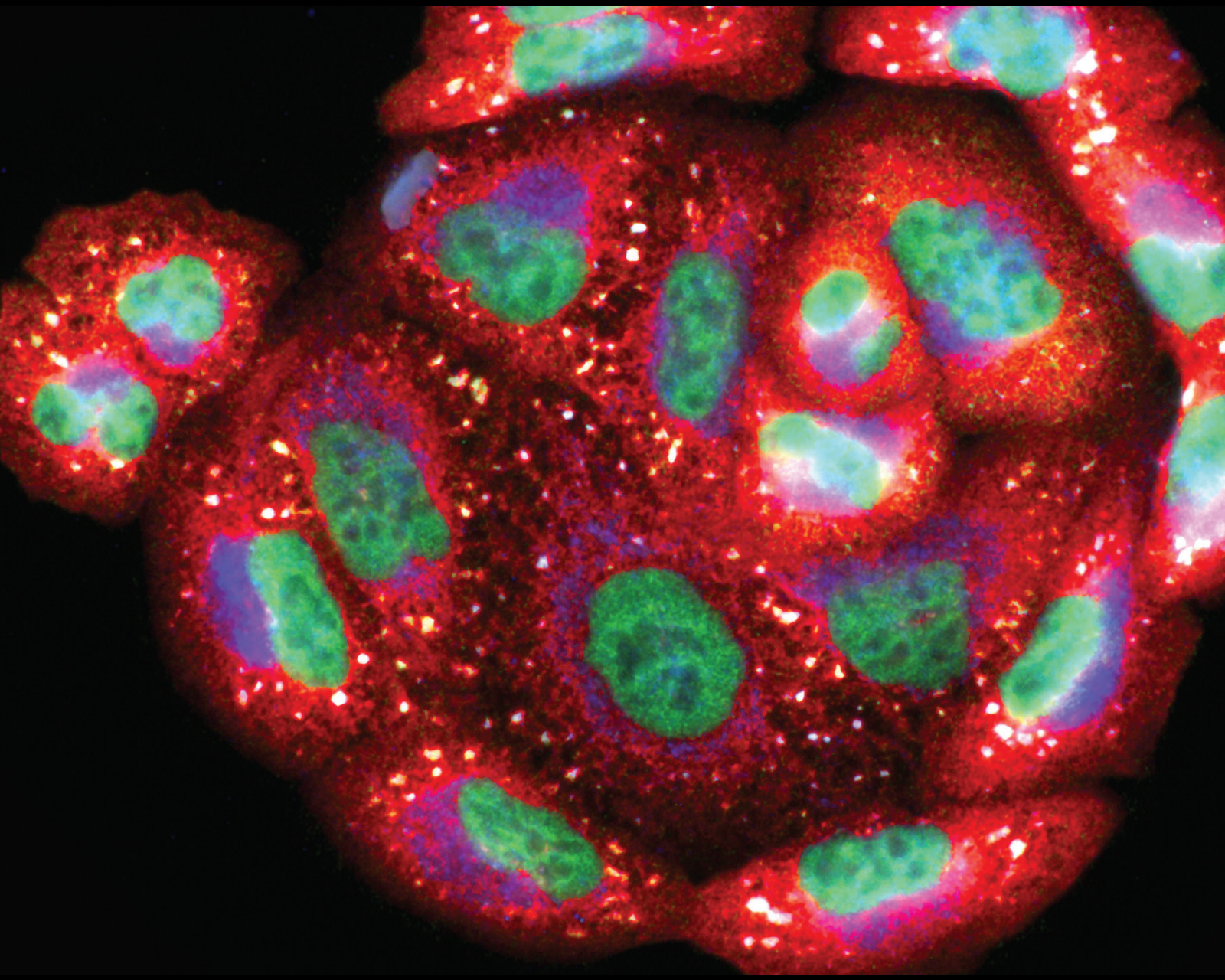
Oxidative Medicine and Cellular Longevity

Bioactive Compounds of Food: Their Role in The Prevention and Treatment of Diseases 2021

Lead Guest Editor: Anderson Junger Teodoro
Guest Editors: German Gil and Felipe Leite de Oliveira



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4,7-Didehydro-neophysalin B Protects Rat Lung Epithelial Cells against Hydrogen Peroxide-Induced Oxidative Damage through Nrf2-Mediated Signaling Pathway

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The administration of 4,7-didehydro-neophysalin B is expected to be a promising strategy for mitigating oxidative stress in respiratory diseases. This study was aimed at investigating the efficacy of 4,7-didehydro-neophysalin B for apoptosis resistance of rat lung epithelial cells (RLE-6TN) to oxidative stress and evaluating its underlying mechanism of action. The RLE-6TN cells treated with hydrogen peroxide (H_2O_2) were divided into five groups, and 4,7-didehydro-neophysalin B was administered into it. To evaluate its mechanism of action, the expression of oxidative stress and apoptotic proteins was investigated. 4,7-Didehydro-neophysalin B significantly inhibited H_2O_2-induced RLE-6TN cell damage. It also activated the Nrf2 signaling pathway which was evident from the increased transcription of antioxidant responsive of KLF9, NQO1, Keap-1, and HO-1. Nrf2 was found to be a potential target of 4,7-didehydro-neophysalin B. The protein levels of Bcl-2 and Bcl-xL were increased while Bax and p53 were decreased significantly. Flow cytometry showed that 4,7-didehydro-neophysalin B protected RLE-6TN cells from apoptosis and has improved the oxidative damage. This study provided a promising evidence that 4,7-didehydro-neophysalin B can be a therapeutic option for oxidative stress in respiratory diseases.

1. Introduction

The respiratory epithelial cell damage is a key indicator of respiratory diseases. Investigation of respiratory epithelial cell injury and its underlying mechanism of action and development of new drugs against it are crucial steps to prevent and treat respiratory diseases. Hydrogen peroxide (H_2O_2) is the main reactive oxygen species (ROS) involved in the regulation of redox reactions in biological activities through specific protein targets [1]. Recently, oxidative stress is considered as a key risk factor for respiratory diseases [2]; evidences showed that the causative factors of diseases including mycoplasma pneumonia are also linked with the oxidative stress [3–6]. Therefore, the active compounds with antioxidative properties tend to be the potential agents for the prevention of respiratory diseases.

In recent years, the use of active natural compounds against pathological conditions has gained considerable recognition. 4,7-Didehydro-neophysalin B and *Physalin* B are biologically active substances extracted from *Physalis alkekengi L. var. franchetii*. It has been confirmed that *Physalin* B has various pharmacological effects such as anti-inflammatory [7], antitumor [8], antibacterial [9], and immune regulation [10] verified by various bioassay both

Table 1: Nrf2 siRNA: a pool of 3 different siRNA duplexes.

siRNA	Primer (5′→3′)
Nrf2 A	Sense: GCAUGCUACGUGAUGAAGAtt Antisense: UCUUCAUCACGUAGCAUGCtt
Nrf2 B	Sense: CUCCUACUGUGAUGUGAAAtt Antisense: UUUCACAUCACAGUAGGAGtt
Nrf2 C	Sense: GUGUCAGUAUGUUGAAUCAtt Antisense: UGAUUCAACAUACUGACACtt

Table 2: Primers used in RT-PCR.

Gene	Primer (5′→3′)
Nrf2	Forward: GAGAGCCCAGTCTTCATTGC Reverse: TTGGCTTCTGGACTTGGAAC
Keap1	Forward: TTCAAGGCCATGTTCACCAA Reverse: TGGATACCCTCAATGGACACC
NQO1	Forward: GGAGAGTTTGCTTACACTTACGC Reverse: AGTGGTGATGGAAAGCACTGCCTTC
HO-1	Forward: CGCCTTCCTGCTCAACATT Reverse: TGTGTTCCTCTGTCAGCATCAC
KLF9	Forward: GGGAAACCTCCGAAAA Reverse: CGTTCACCTGTATGCACTGTA
GAPDH	Forward: GGAGATTACTGCCCTGGCTC Reverse: GACTCATCGTACTCCT

RT-PCR was performed on the ABI PRISM® 7500 real-time PCR analyzer (Applied Biosystems, Foster City, CA, USA) using the SYBR® Premix Ex Taq™ RT-PCR Kit. The relative mRNA expression was calculated by means of $2^{-\Delta\Delta Ct}$ and was normalized to the mean mRNA expressions of GAPDH. The results were calculated with the following formulae: ratio = $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{Sample} - (Ct_{target} - Ct_{GAPDH})_{Control}$.

in vitro and in vivo. 4,7-Didehydro-neophysalin B is a new kind of *Physalin* B lacking two hydrogen atoms. There is evidence that *Physalin* B has efficient antioxidant activity [8]. However, the protective effect and mechanism of 4,7-didehydro-neophysalin B against H_2O_2-induced lung injury remained elusive.

Nuclear factor-erythroid 2-related factor-2 (Nrf2) has been identified as a key regulator of antioxidative, anti-inflammatory, and conjugation/detoxification proteins including NAD(P)H, quinine oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1) [11]. After oxidative stress caused by H_2O_2 or other factors, the Nrf2 pathway is rapidly activated to eliminate intracellular ROS generation, thereby attenuating DNA damage induced by H_2O_2 to reduce risk of subsequent lung apoptosis [12].

Activation of antioxidative defense such as phase II enzyme expression is an effective way to protect cells against oxidative damage [13]. Nrf2 combined with antioxidant response element (ARE) system is one of the most important defensive signaling pathways to regulate the transcription activity of antioxidases [14]. The RLE-6TN cell line has been widely used to evaluate lung function due to their hypersensitivity to H_2O_2 [15, 16]. Although many bioactive compounds have been reported against oxidative stress, still there is no relevant study on the cytoprotective effect of 4,7-didehydro-neophysalin B on RLE-6TN cells.

In this study, H_2O_2 was used to establish the basic oxidative damage model so that we can investigate the effect of 4,7-didehydro-neophysalin B on the oxidative stress, cell apoptosis, and the role of Nrf2 signaling pathway. This study will promote the development of nutraceutical and functional food from *Physalis alkekengi L. var. franchetii* or its extracts for reducing the risk of oxidative stress-induced lung injury.

2. Materials and Methods

2.1. Preparation of 4,7-Didehydro-neophysalin B. Physalin was isolated from the stems of *Physalis alkekengi L. var. franchetii*. The plant was identified by Dr. Qiongming Xu from the College of Pharmaceutical Science, Soochow University. Physalin was purified, and the purity of Physalin is 72.39% by chromatography [17]. Physalin from the above reaction was pulverized, extracted with ethanol, loaded on a silica gel column, eluted, and purified with ethyl acetate. The obtained contents of 4,7-didehydro-neophysalin B were detected by chromatography.

2.2. Cytotoxicity of 4,7-Didehydro-neophysalin B. The RLE-6TN cells (ATCC® CRL-2300™, 4000 cells per well) were subcultured in 96-well plates. After different concentrations of 4,7-didehydro-neophysalin B (50, 25, 10, and 5 μg/mL) treatments, cells were incubated with CCK-8 (CK04, Dojindo, Japan) solution (10%) for 1 h at 37°C. The resulting absorbance was measured at 450 nm by using a microplate reader (Tecan, Switzerland). The maximum nontoxic concentration (MNTC) value was calculated by Prism 6 software (San Diego, CA, USA) [18].

$$\text{Cell proliferation rate} = \frac{A_{450} \text{ of treatment group}}{A_{450} \text{ of blank group}} \times 100\%. \quad (1)$$

2.3. Culture of RLE-6TN Cells. The RLE-6TN cell line was cultured in DMEM containing 15% fetal bovine serum, 2 mmol/L glutamine, 5 mmol/L sodium pyruvate, and 25 mmol/L HEPES, containing 100 UI/mL penicillin and 100 μg/mL streptomycin, placed in an incubator and grown at 37°C, 5% CO_2-saturated humidity. 0.25% trypsin-EDTA digested and passaged, and cells in logarithmic growth phase were used for experiments [19]. RLE-6TN cells were seeded in 96-well cell culture plates and incubated in a 5% CO_2 humidified incubator for 24 h at 37°C. Various concentrations of H_2O_2 prepared in 2% medium (200, 100, 50, and 25 μmol) were added and incubated again for 4, 8, 12, and 24 h. The cell proliferation rate of H_2O_2 was determined using the CCK-8 (CK04, Dojindo, Japan) kit. To develop a cellular oxidative damage model, H_2O_2 (100 μmol) prepared in 2% medium was added to each well except the blank group and incubated for the next 12 h. Different concentrations of 4,7-didehydro-neophysalin B with low-dose group (2.5 μg/mL), medium-dose group (5 μg/mL), and high-dose

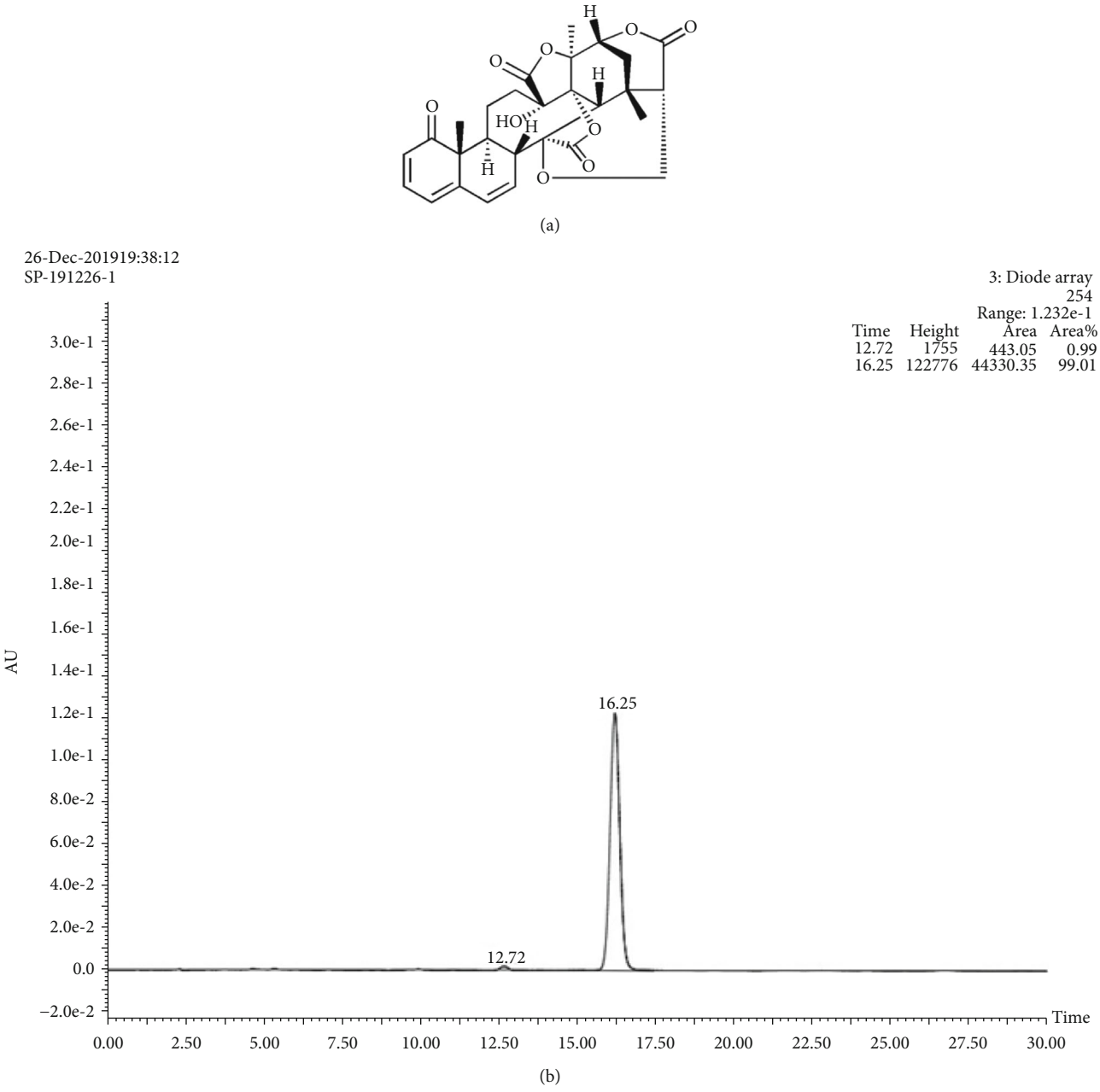

Figure 1: (a) The structure of 4,7-didehydro-neophysalin B. (b) The contents of 4,7-didehydro-neophysalin B.

group (10 μg/mL) were added as treatment and incubated again for 24 h.

$$\text{Cell proliferation rate} = \frac{A_{450} \text{ of treatment group}}{A_{450} \text{ of blank group}} \times 100\%. \quad (2)$$

2.4. Transfection. (1) Culture the RLE-6TN cells for 3 to 5 generations, select the cells with good growth condition, digest the cells with 0.25% trypsin, inoculate 2×10^5 cells/well in 6-well plates, add 10% fetal bovine serum, culture in F12 medium (Gibco, USA) without double antibody for 24 h, and after the cell confluence reached 70%, transfection was carried out. (2) Mix 7.5 μL of Lipofectamine 2000 (Life, USA) with 125 μL of OPTI-MEM (Gibco, USA) medium. Take another EP tube and mix 6 μL of siRNA (0.75 μg) with 125 μL of OPTI-MEM medium. This is the amount for one well in a 6-well plate. (3) Gently mix the two tubes of mixture in step 2, and let them act together at room temperature for 15 min. (4) The liquid after the joint action in step 3 was directly added to the original 6-well plate medium, and after 6 hours in a 37°C, 5% CO_2 incubator, the medium was replaced and the subsequent experiments were carried out. For siRNA transfection, siRNA duplex targeting Nrf2 (sc-37030, Santa Cruz Biotechnology, Santa Cruz, CA, USA, primer shown in Table 1) were used. siRNA (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was selected as a

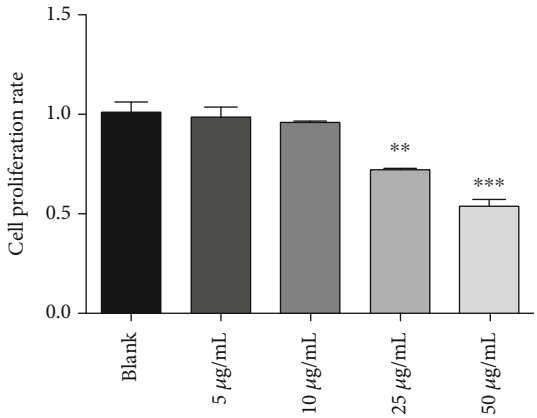

FIGURE 2: Cell viability (CCK-8) assay results of RLE-6TN cells: 4,7-didehydro-neophysalin B-induced cell proliferation rate. Compared with the blank group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

control to determine whether Nrf2's siRNA was successfully transfected.

2.5. Detection of Gene Expression in Tissues by RT-PCR. The total RNA was extracted from cells using TRIzol reagent, and the absorbance was measured at 260 and 280 nm by an ultraviolet spectrophotometer. The RNA content was calculated from the absorbance (A) value at a wavelength of 260 nm while the RNA purity was identified by the ratio of A_{260}/A_{280}. Reverse transcription of total RNA was performed with reverse transcriptase. The PCR primers (shown in Table 2) were designed and synthesized by KeyGEN BioTECH with GAPDH as endogenous control, and the PCR reactions were carried out according to the recommended conditions.

2.6. Flow Cytometry. After treating the cells with/without 100 μmol of H_2O_2 for 12 h, different concentrations of 4,7-didehydro-neophysalin B (0, 2.5, 5, and 10 μg/mL) were added to the rat lung epithelial cells RLE-6TN for 24 h. The cells were collected, washed twice with cold PBS, and centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the cells were made into a single cell suspension. 5 μL of Annexin V-FITC and 5 μL PI were added, mixed, and incubated for 15 min in the dark. Finally, 400 μL of binding buffer was added and cell apoptosis was detected using flow cytometry.

2.7. Western Blot. RLE-6TN cells were homogenized with RIPA lysate (Solarbio, Beijing, China) containing protease inhibitor PMSF and phosphatase inhibitor (Solarbio). The nuclear and cytosolic proteins were extracted by a nuclear protein extraction kit (KeyGEN BioTECH, Jiangsu, China). The protein concentrations were measured by bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). 40 μg equivalent protein samples were separated by SDS-PAGE gel electrophoresis and were transferred into a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with appropriate primary and HRP-conjugated secondary antibodies. Chemiluminescence was visualized with an enhanced chemiluminescence kit (BOSTER, Wuhan, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (Sanying, Wuhan, China) and the TATA-binding protein (TBP) (Sanying, Wuhan, China) were used as loading control. The antibodies for Nrf2, heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide phosphatase:quinone-acceptor 1 (NQO1), Krueppel-like factor 9 (KLF9), Keap-1, p53, Bcl-2-associated X protein (Bax), B cell lymphoma gene 2 (Bcl-2), and B cell lymphoma-extra large (Bcl-xL) were purchased from Abcam (UK). All secondary antibodies were from Sanying (Wuhan, China). Protein expression was analyzed by scanning densitometry using ImageJ software (USA).

To further explain the molecular mechanism by which 4,7-didehydro-neophysalin B treated H_2O_2-induced oxidative damage in RLE-6TN cells, western blot was performed. Nrf2's siRNA was generated to inhibit Nrf2 expression, and the inhibitory efficiency was verified using western blot. Nrf2's siRNA was transfected into RLE-6TN cells with or without H_2O_2 treatment. Then, 10 μg/mL 4,7-didehydro-neophysalin B was administrated for treatment and the expression of Nrf2 was explored using western blotting.

2.8. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance with Tukey's test post hoc comparisons and Student's t-test when comparing between 2 groups using SPSS 19.0 software (USA). The data were presented as the mean ± SEM. Values with $p < 0.05$ was considered statistically significant.

3. Results

3.1. Structure and Content of 4,7-Didehydro-neophysalin B. The structure and contents of 4,7-didehydro-neophysalin B are shown in Figures 1(a) and 1(b). The chemical formula of 4,7-didehydro-neophysalin B is $C_{28}H_{28}O_9$, and its purity is 99.01%.

3.2. The Cytotoxicity of 4,7-Didehydro-neophysalin B. The maximum nontoxic concentration of 4,7-didehydro-neophysalin B in RLE-6TN was investigated. Compared with the blank group, RLE-6TN cells treated with 25 μg/mL 4,7-didehydro-neophysalin B for 24 h showed significant reduction in cell viability (74.87 ± 1.54%, $p = 0.008 < 0.01$) while 10 μg/mL 4,7-didehydro-neophysalin B had negligible effect on cell viability (93.6 ± 1.03%, $p = 0.07 > 0.05$) (Figure 2). Therefore, we choose 10 μg/mL 4,7-didehydro-neophysalin B as the maximum nontoxic concentration.

3.3. The Effect of 4,7-Didehydro-neophysalin B on RLE-6TN Pretreated with H_2O_2. Compared with the blank group, the working concentration for H_2O_2 in RLE-6TN cells treated with different concentrations was determined. Results showed that 100 μmol H_2O_2 treated for 12 h significantly reduced (59.87 ± 1.32%, $p = 0.0004 < 0.001$) while 50 μmol H_2O_2 showed less effect on the cell viability (80.37 ± 1.96% , $p = 0.0007 < 0.001$). The mortality of cells treated with 200 μmol of H_2O_2 was too high (41.28 ± 2.84%, $p = 0.0003$

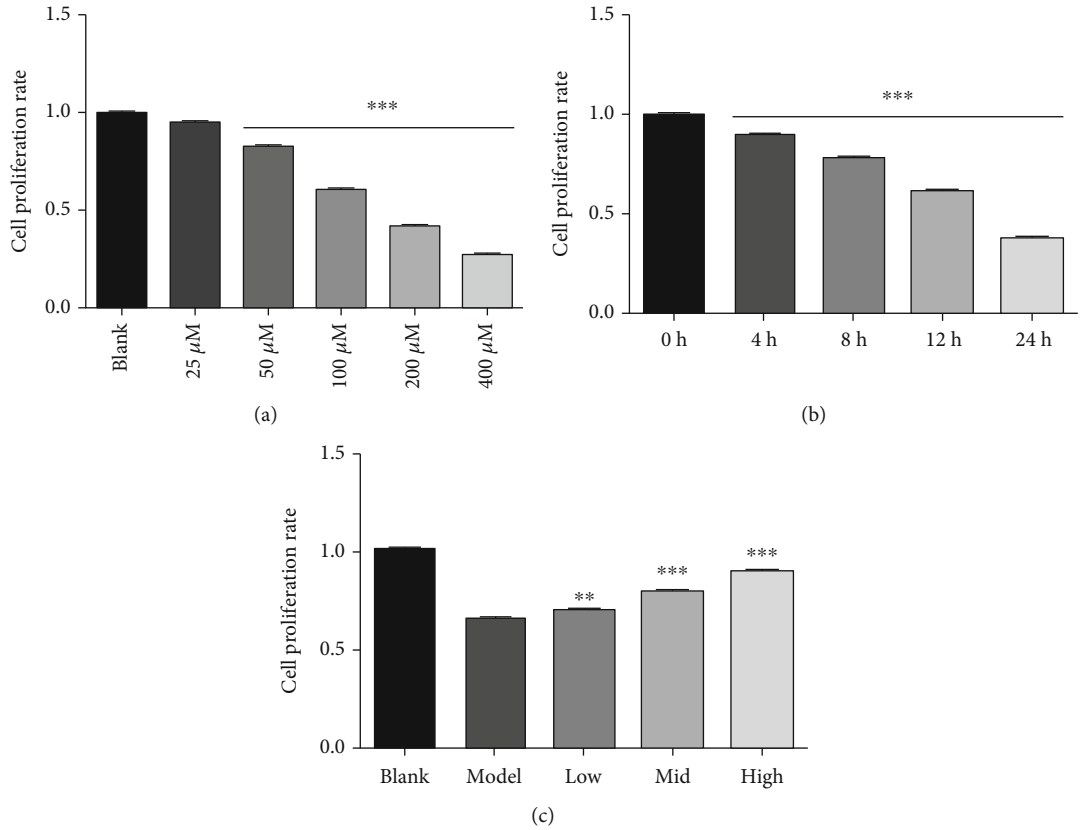

Figure 3: Cell viability (CCK-8) assay results of RLE-6TN cells. (a) H_2O_2-induced cell proliferation rate (concentration-dependent); (b) H_2O_2-induced cell proliferation rate (time-dependent); (c) 4,7-didehydro-neophysalin B treatment of the H_2O_2-challenged cells. Low: 2.5 μg/mL 4,7-didehydro-neophysalin B-treated RLE-6TN cell group; mid: 5 μg/mL 4,7-didehydro-neophysalin B-treated RLE-6TN cell group; high: 10 μg/mL 4,7-didehydro-neophysalin B-treated RLE-6TN cell group; the same as below. Compared with the blank group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Compared with the model group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

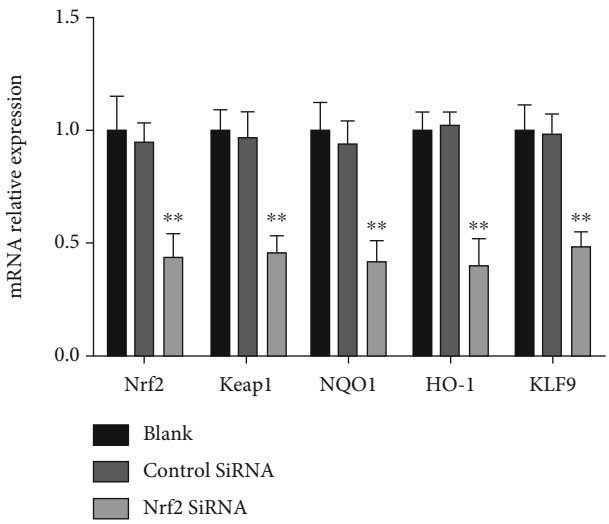

Figure 4: The mRNA expression of Nrf2 and its downstream genes. Compared with the blank group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

< 0.001) as shown in Figure 3(a). Therefore, 100 μmol H_2O_2 was selected as a working concentration.

Compared with the blank group, the working time for 100 μmol H_2O_2 in RLE-6TN cells treated with different times was determined. The results showed that RLE-6TN cells treated with 100 μmol H_2O_2 for 24, 12, 8, and 4 h treatment all had significant effect on cell viability, but the RLE-6TN cells treated with 100 μmol H_2O_2 for 24 ($37.84 \pm 1.62\%$, $p = 0.0005 < 0.001$), 8 ($76.62 \pm 1.45\%$, $p = 0.0006 < 0.001$), and 4 ($87.61 \pm 2.33\%$, $p = 0.0009 < 0.001$) hours were too high or low on cell viability, as shown in Figure 3(b). Therefore, 12 h ($68.88 \pm 0.95\%$, $p = 0.0002 < 0.001$) was selected as a working time.

Conclusively, 100 μmol of H_2O_2 was used to treat RLE-6TN for 12 h, followed by the addition of 4,7-didehydro-neophysalin B to examine the effect on cell viability. As shown in Figure 3(c), 4,7-didehydro-neophysalin B had significantly reversed the cell viability caused by H_2O_2 in a dose-dependent manner in which 10 μg/mL 4,7-didehydro-neophysalin B showed the strongest effect ($84.93 \pm 1.86\%$, $p = 0.0007 < 0.001$).

3.4. Expressions of Nrf2 in RLE-6TN under the Intervention of Nrf2's siRNA. The mRNA expression of Nrf2 and its downstream genes was detected by RT-PCR. The A_{260}/A_{280} ratio of the RNA samples was 1.8-2.0. Nrf2's siRNA treatment significantly reduced the mRNA expression of Nrf2 and its downstream genes compared with the control siRNA

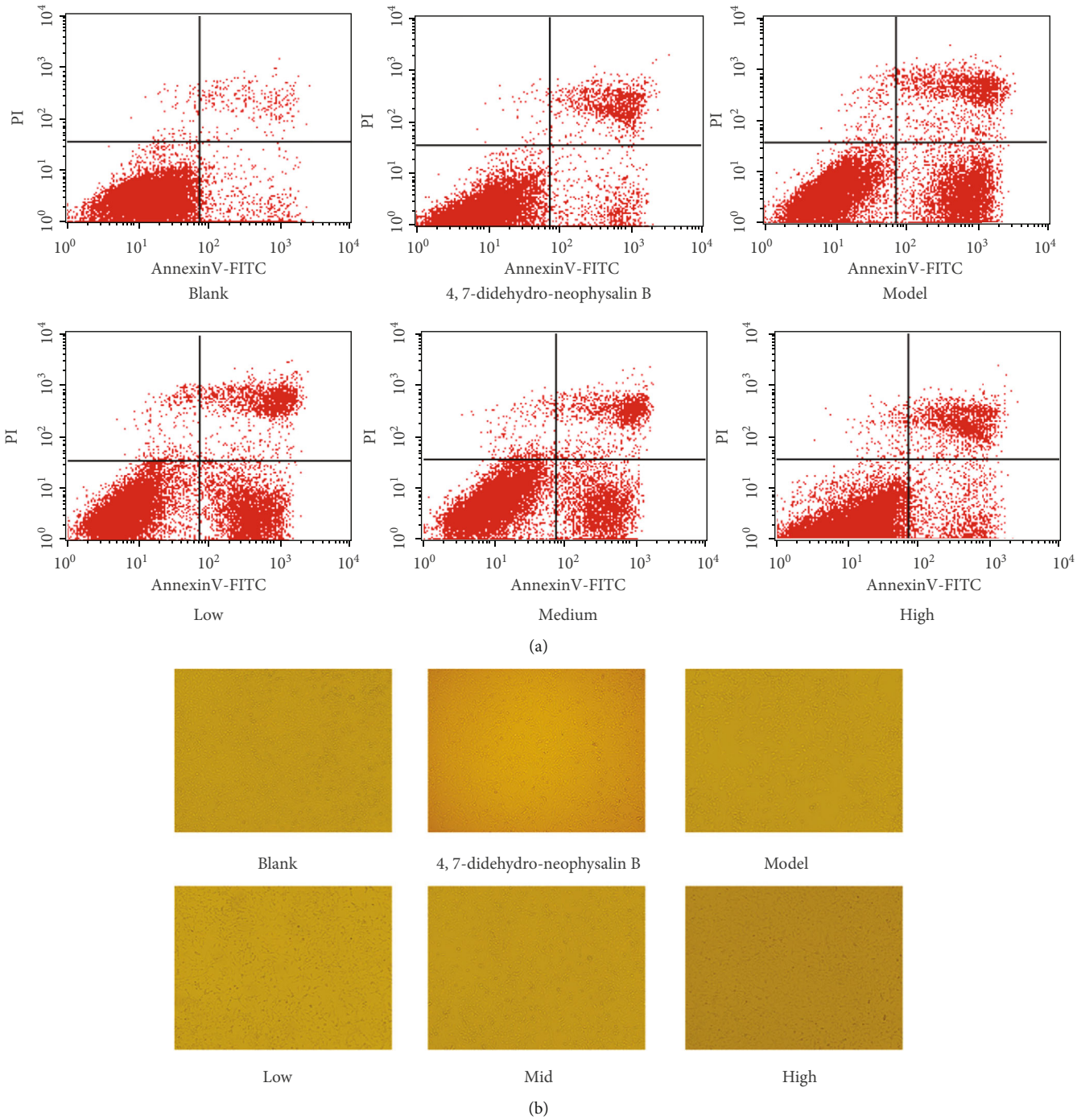

Figure 5: (a) The effect of 4,7-didehydro-neophysalin B on H_2O_2-induced apoptosis in RLE-6TN cells. (b) The microscope images of H_2O_2-induced cells after treatment with 4,7-didehydro-neophysalin B.

which have shown negligible effect on the mRNA expression of Nrf2 and its downstream genes, as shown in Figure 4.

3.5. 4,7-Didehydro-neophysalin B Mitigates H_2O_2-Induced Apoptosis in RLE-6TN. We investigated the effect of 4,7-didehydro-neophysalin B on H_2O_2-induced cell apoptosis by flow cytometry. Compared with the blank group, H_2O_2 caused a significant increase in RLE-6TN cell apoptosis while only 4,7-didehydro-neophysalin B treatment showed no significant effect on cell viability. Similarly, 4,7-didehydro-neophysalin B treatment dramatically mitigated H_2O_2-induced apoptosis. These results elucidated that 4,7-didehydro-neophysalin B had only a significant effect on H_2O_2-induced early apoptosis but had no significant effect on late apoptosis (early apoptosis: 4,7-didehydro-neophysalin B group $7.98 \pm 1.15\%$, $p = 0.07 > 0.05$; model group $37.48 \pm 3.84\%$, $p = 0.0005 < 0.001$; low-dose group $29.83 \pm 3.68\%$, $p = 0.0007 < 0.001$; medium-dose group $21.44 \pm 2.99\%$, $p =$

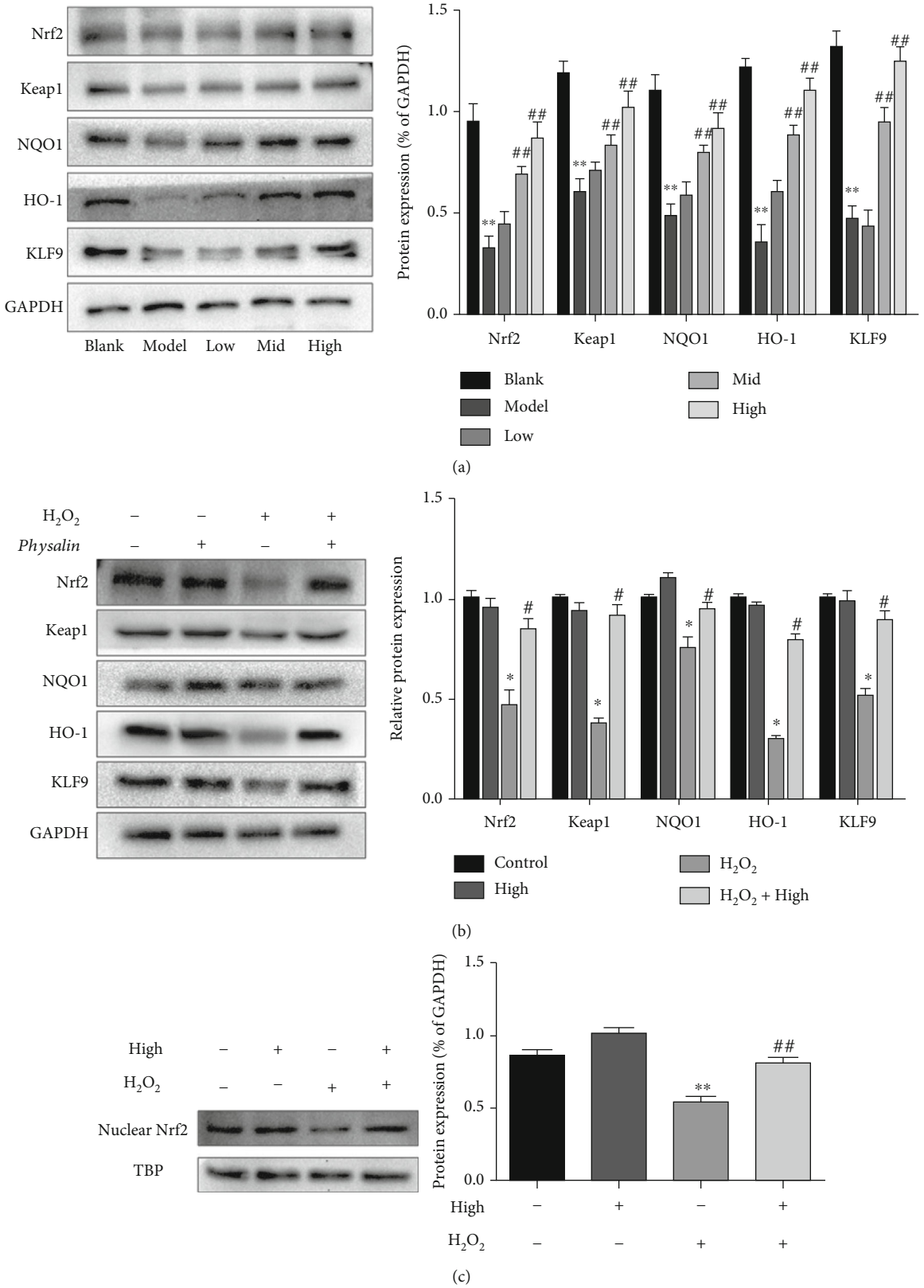

Figure 6: Western blot analysis of the Nrf2 signaling pathway. (a) After being added with different concentrations of 4,7-didehydro-neophysalin B, Nrf2 pathway-related protein expression in RLE-6TN cells and its quantitative band intensity analysis. (b) Effect of 4,7-didehydro-neophysalin B on the expression of Nrf2 pathway-related proteins and the quantitative band intensity analysis. (c) Expression of Nrf2 protein in the nucleus and its quantitative band intensity analysis. Compared with the blank group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Compared with the H_2O_2+0% 4,7-didehydro-neophysalin B group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$.

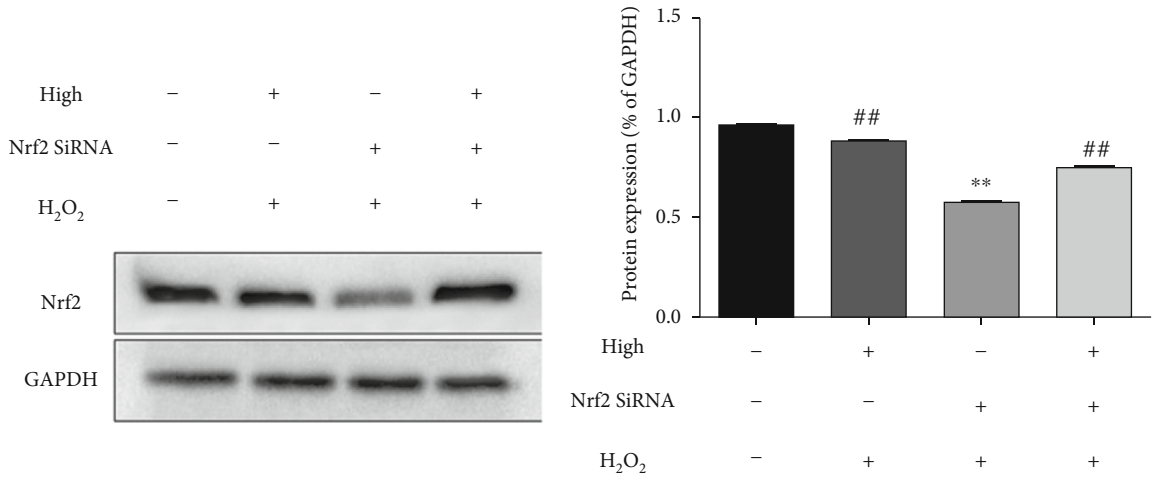

Figure 7: After using Nrf2 siRNA to knock down the expression of Nrf2 protein, effect of 4,7-didehydro-neophysalin B on the expression of Nrf2 pathway-related proteins and their quantitative band intensity analysis. Compared with the blank group, $^{*}p < 0.05$ and $^{**}p < 0.01$. Compared with the H_2O_2+0% 4,7-didehydro-neophysalin B group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

$0.0008 < 0.001$; high-dose group $15.35 \pm 1.68\%$, $p = 0.03 < 0.05$, compared with the blank group) (late apoptosis: 4,7-didehydro-neophysalin B group $13.32 \pm 2.47\%$, $p = 0.008 < 0.01$; model group $24.49 \pm 2.96\%$, $p = 0.006 < 0.01$; low-dose group $17.92 \pm 2.86\%$, $p = 0.007 < 0.01$; medium-dose group $15.58 \pm 3.15\%$, $p = 0.008 < 0.01$; high-dose group $19.44 \pm 3.57\%$, $p = 0.006 < 0.01$, compared with the blank group), as shown in Figure 5(a). The microscopic images of cells after treatment are shown in Figure 5(b).

3.6. The Effect of 4,7-Didehydro-neophysalin B on Nrf2-Dependent Signaling Pathway in H_2O_2-Induced Oxidative Damage. Nrf2 signaling pathway proteins were detected by western blotting. In vitro treatment of H_2O_2 significantly reduced the Nrf2, NQO1, Keap1, HO-1, and KLF9 protein expressions. However, 4,7-didehydro-neophysalin B treatment has significantly reversed these effects induced by H_2O_2 with 10 μg/mL 4,7-didehydro-neophysalin B showing the strongest effect as shown in Figure 6(a). The addition of 4,7-didehydro-neophysalin B alone showed no effect on the protein expression of Nrf2, NQO1, Keap1, HO-1, and KLF9 in RLE-6TN as shown in Figure 6(b). H_2O_2 had markedly reduced the expression of nuclear Nrf2 which was significantly reversed by 4,7-didehydro-neophysalin B as shown in Figure 6(c).

3.7. Knockdown of Nrf2 Declines the Treatment Effect of Physalin B. The Nrf2's siRNA treatment significantly reduced the expression of Nrf2 as shown in Figure 7. Compared with the model group, the treatment effect of 4,7-didehydro-neophysalin B in the Nrf2's siRNA treatment group was significantly reduced.

3.8. Effects of 4,7-Didehydro-neophysalin B on Bcl-2 Family and p53 Proteins. The molecular basis of a protective effect of 4,7-didehydro-neophysalin B against H_2O_2-induced cell apoptosis was investigated. H_2O_2 treatment decreased the expression of antiapoptotic Bcl-2 and Bcl-xL proteins in RLE-6TN cells. In H_2O_2-treated cells, 4,7-didehydro-neophysalin B administration significantly induced the expression of Bcl-2 and Bcl-xL. The expression of proapoptotic protein Bax was significantly increased post H_2O_2 treatment which was significantly reduced by 4,7-didehydro-neophysalin B treatment. Compared with the control group, p53 expression was also increased post H_2O_2 treatment. 4,7-Didehydro-neophysalin B attenuated H_2O_2-induced p53 expression with the strongest effect of 10 μg/mL 4,7-didehydro-neophysalin B as shown in Figure 8(a) (Bcl-xL expression: blank 1.0 ± 0.14, control 0.37 ± 0.05, $p = 0.004 < 0.01$ compared with the blank group; low 0.28 ± 0.07, mid 0.59 ± 0.09, $p = 0.03 < 0.05$ compared with the control group; high group 0.98 ± 0.05, $p = 0.11$ compared with the control group; Bcl-2 expression: blank 1.0 ± 0.16, control 0.48 ± 0.35, $p = 0.005 < 0.01$ compared with the model group; low 0.53 ± 0.005, mid 0.59 ± 0.77, $p = 0.04 < 0.05$ compared with the control group; high group 0.98 ± 0.05, $p = 0.11 < 0.01$ compared with the control group). The addition of 4,7-didehydro-neophysalin B alone showed no effect on the protein expression of Bcl-2, Bcl-xL, Bax, and p53 in RLE-6TN as shown in Figure 8(b).

4. Discussion

A variety of chemicals and environmental factors causes oxidative stress in the human body which damages and induces a variety of diseases. H_2O_2-induced RLE-6TN cell damage is a classical model of oxidative stress injury. Studies have shown that the damage caused by oxidative stress is mediated by reactive oxygen species which plays an important role in neurodegenerative diseases [20, 21]. H_2O_2 produced in the body during metabolism is equivalent to reactive oxygen species and can aggravate the body oxidative stress injury [22, 23]. The results of this study showed that H_2O_2 induced oxidative damage and apoptosis in RLE-6TN cells which was reduced by 4,7-didehydro-neophysalin B treatment as shown in Figure 2(c). This study demonstrated that

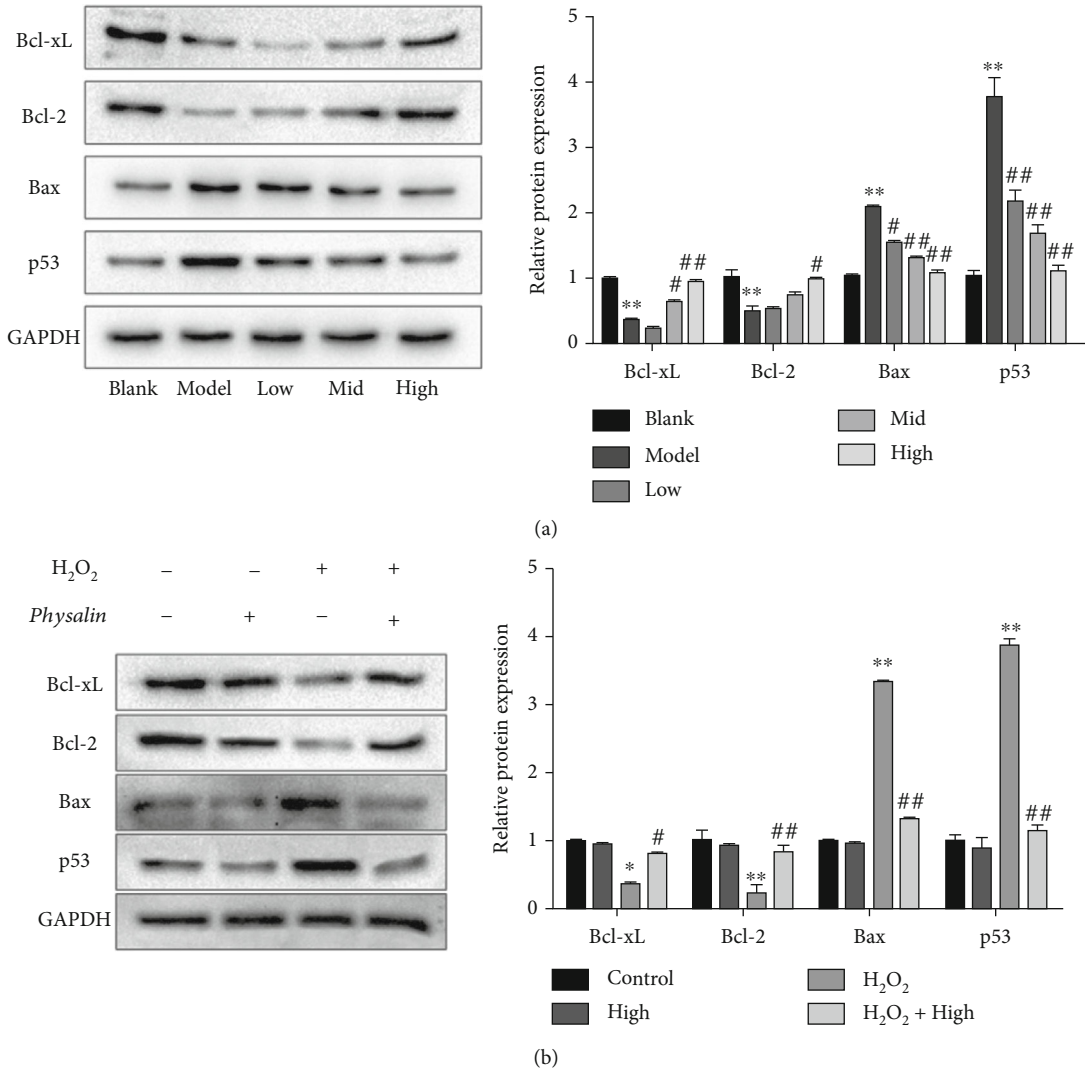

Figure 8: Western blot analysis of p53 pathway protein. (a) Post addition of different 4,7-didehydro-neophysalin B concentrations, p53 pathway-related protein expression in RLE-6TN cells and their quantitative band intensity analysis. (b) Effect of 4,7-didehydro-neophysalin B on the expression of p53 pathway-related proteins and the quantitative band intensity analysis. Compared with the blank group, $^{*}p < 0.05$ and $^{**}p < 0.01$, and with the H_2O_2+0% 4,7-didehydro-neophysalin B group, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

4,7-didehydro-neophysalin B possessed therapeutic effects in the form of antioxidant activities against H_2O_2-induced oxidative stress injury.

When the cellular level of ROS exceeds the body's antioxidant capacity, it induces oxidative stress leaving the cells in a redox state by producing peroxides and free radicals [24–26]. Mitochondrial dysfunction and DNA damage are caused by the accumulation of reactive oxygen species which mediate and accelerate apoptosis [27–30]. DNA damage causes activation of p53which serves as a major mediator of cellular stress [31]. Our results (Figure 7) indicated p53-mediated apoptosis in H_2O_2-induced lung injury. p53 along with the members of Bcl-2 family proteins regulates protein–protein interactions and causes activation of Bax which promotes mitochondrial membrane permeability and hence induces apoptosis [32]. In this study, 4,7-didehydro-neophysalin B suppressed p53, decreased the level of proapoptotic member Bax, and increased the level of prosurvival members Bcl-2 and Bcl-xL. Moreover, the results of apoptotic index (Figure 2) and cell viability (Figure 4) of RLE-6TN were consistent with the observed effects on Bcl-2 family proteins. These findings suggest that 4,7-didehydro-neophysalin B has a pivotal role in regulating apoptosis and inhibiting oxidative stress-induced cell death. It is also noteworthy that some studies showed that NQO1 stabilizes the tumor suppressor p53 [33]. HO-1 upregulates Bcl-2 and Bcl-xL expressions [34] which are downstream proteins of Nrf2. A possible explanation is that 4,7-didehydro-neophysalin B protects lung injury induced by H_2O_2 via activating the Nrf2 pathway.

The transcription factor Nrf2 plays an important role in protection against oxidative damage [35, 36]. Nrf2 senses the presence of oxidative stress and regulates transcription of genes encoding cytoprotective enzymes and other proteins crucial for maintaining cellular homeostasis. Under physiological conditions, the Nrf2 inhibitor, Keap-1, which is a

negative regulator of Nrf2 binds to Nrf2 and retains Nrf2 in the cytoplasm [37]. During oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus, and activates ARE-dependent gene expression including transcription of target genes NQO-1 and HO-1 [38], thereby improving the antioxidant capacity in the body [39, 40]. Accumulation of Nrf2 in the nucleus is a necessary condition which is closely related to the induction of cellular defense genes [41]. The increase in cell death caused by H_2O_2 might be attributed to the insufficient ROS removal caused by the failure of Nrf2 activation. However, Figure 5 shows that 4,7-didehydro-neophysalin B activates the antioxidant pathway affecting both Nrf2 gene levels and expression of its target proteins. Therefore, this study suggested that Nrf2 activation was required for protection of the lungs by 4,7-didehydro-neophysalin B from H_2O_2-mediated cell death.

5. Conclusions

In conclusion, our study demonstrated that *Physalin* B attenuated H_2O_2-induced lung injury by regulating the Nrf2/P53 signaling pathway. *Physalin* B with great therapeutical potential can be used as an antioxidant agent. This study provided beneficial evidences for the application of *Physalin* B supplementation as an alternative treatment strategy for lung injury.

Abbreviations

H_2O_2: Hydrogen peroxide
ROS: Reactive oxygen species
Nrf2: Nuclear translocation of erythroid-2-related factor 2
PVDF: Polyvinylidene fluoride
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
ARE: Antioxidant response element
TBP: TATA-binding protein
HO-1: Heme oxygenase-1
NQO1: Nicotinamide adenine dinucleotide phosphatase:quinone-acceptor 1
KLF9: Krueppel-like factor 9
Keap-1: Kelch-like ECH-associated protein
Bax: Bcl-2-associated X protein
Bcl-2: B cell lymphoma gene 2
Bcl-xL: B cell lymphoma-extra large
SEM: Standard errors of the mean.

Data Availability

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Conceptualization and methodology were done by Qiu Zhong and Yao-gui Sun; investigation, data curation, and writing—original draft preparation were done by Qiu Zhong; validation was done by Qiu Zhong and Yao-gui Sun; writing—review and editing were done by Ajab Khan, Jianhua Guo, Zhirui Wang, and Na Sun; funding acquisition was done by Yaogui Sun and Hongquan Li; project administration was done by Hongquan Li. All authors have read and agreed to the reviewed version of the manuscript.

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Review Article

Flavonols and Flavones as Potential anti-Inflammatory, Antioxidant, and Antibacterial Compounds

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Plant preparations have been used to treat various diseases and discussed for centuries. Research has advanced to discover and identify the plant components with beneficial effects and reveal their underlying mechanisms. Flavonoids are phytoconstituents with anti-inflammatory, antimutagenic, anticarcinogenic, and antimicrobial properties. Herein, we listed and contextualized various aspects of the protective effects of the flavonols quercetin, isoquercetin, kaempferol, and myricetin and the flavones luteolin, apigenin, 3′,4′-dihydroxyflavone, baicalein, scutellarein, lucenin-2, vicenin-2, diosmetin, nobiletin, tangeretin, and 5-O-methyl-scutellarein. We presented their structural characteristics and subclasses, importance, occurrence, and food sources. The bioactive compounds present in our diet, such as fruits and vegetables, may affect the health and disease state. Therefore, we discussed the role of these compounds in inflammation, oxidative mechanisms, and bacterial metabolism; moreover, we discussed their synergism with antibiotics for better disease outcomes. Indiscriminate use of antibiotics allows the emergence of multidrug-resistant bacterial strains; thus, bioactive compounds may be used for adjuvant treatment of infectious diseases caused by resistant and opportunistic bacteria *via* direct and indirect mechanisms. We also focused on the reported mechanisms and intracellular targets of flavonols and flavones, which support their therapeutic role in inflammatory and infectious diseases.

1. Introduction

Flavonoids are an essential and diversified class of secondary plant metabolites present in different concentrations in the leaves, flowers, roots, and fruits. Flavonoid concentrations are influenced by natural factors such as temperature, ultraviolet (UV) radiation, season, pollutant, drought, and salinity stress owing to their effect on plant metabolism (Li [1]).

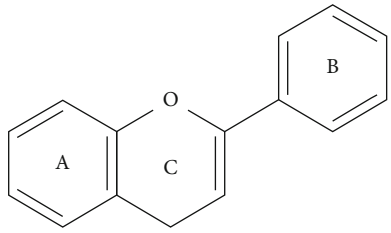

Figure 1: The basic skeleton of flavonoids (C6-C3-C6).

The preparations containing flavonoids has been used to treat various human diseases. Flavonoids have anti-inflammatory, antimutagenic, anticarcinogenic, and antimicrobial properties [2–4]. They modulate the activities of several enzymes, including xanthine oxidase (XO), cyclooxygenase (COX), lipoxygenase (LOX), and phosphoinositide 3-kinase [5]) involved in pathological processes.

Inflammation is a complex biological response to noxious stimuli. It protects organisms against pathogens, cell damage, and irritating chemicals. Moreover, it initiates the healing process, which involves different cells and chemical mediators [6, 7]. Nevertheless, exacerbated and uncontrolled inflammation can also cause deleterious tissue damage. Flavonoids can alter cellular responses and production of chemical mediators related to inflammatory processes [8] caused by many agents, including natural toxins, pharmaceuticals, heavy metals, and environmental chemicals [9].

Oxidative stress is induced by mitochondrial dysfunction and inflammation [10, 11]. Continuous oxidative stress generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing lipid peroxidation, protein and nucleic acid oxidation, and even cell death [12]. These effects are dependent on redox balance because they act as damaging agents and physiological regulators of cell functions [13]. Plant extracts are valuable sources of bioactive compounds with antioxidant properties. For example, honey shows antioxidant and anti-inflammatory properties owing to its high flavonoid content [14–16]. Moreover, honey intake led to reduction in malondialdehyde and ROS levels generated by high physical activity in athletes and murine models [17–20]. A recent review discussed the role of consuming plant foods rich in antioxidants in protection against cognitive decline [12].

Similar to all secondary plant metabolites, plant defense mechanisms include flavonoid production; for example, a plant under attack by microorganisms may trigger the production of flavonoids having antimicrobial effects [21]. Herein, we discussed many aspects of the protective effects of flavonols and flavones in detail.

2. Flavonoids: Structural Characteristics and Subclasses

Flavonoids present a C6-C3-C6 carbon skeleton structure that consists of at least two aromatic rings, called A and B, linked by the three carbons chain that can form a heterocyclic ring containing oxygen (ring C) with ring A (Figure 1) [175]. Flavonoids are divided into flavones, flavonols, flavanones, flavanols, isoflavones, leucoanthocyanidins, anthocyanidins, and chalcones [5] (Figure 2). The subclasses are determined by minor structural variations, including the absence of ring C, position of the bond between ring B and ring C, degree of unsaturation, and ring C oxidation [22, 23]. In addition, substitutions at these rings can lead to differences in hydrogenation, hydroxylation, methylation, malonylation, sulfation, and glycosylation, which determine different pharmacological activities [5]. Currently, there have been reported more than 9000 different flavonoids [24].

We focused on two groups: (a) flavones that have a double bond between C2 and C3 in the flavonoid skeleton, without substitution at C3 and oxidized at C4, and (b) flavonols that differ from flavones by a hydroxyl group at C3 (Figure 2).

2.1. Flavonols and Flavones: Importance, Occurrence, and Food Sources. Almost all natural flavonoids exist in plants in O-glycosides or C-glycosides forms. C-glycosides are sugar moieties combined directly to flavonoid backbone as C–C covalent bonds. O-glycosides are formed by attaching sugar to hydroxyl oxygen [25]. The most common sugars attached to these flavonoids are D-glucose and L-rhamnose, but at least eight monosaccharides or combinations thereof may bind to different hydroxyl groups of flavonoids, resulting in a large number of known glycosides [26, 27]. They are widely present in plants and are potential metabolites involved in plant signaling and defense mechanism. Furthermore, they are vital ingredients in human diet and have significant health benefits [28–30]. Flavonols, which are primary flavonoids in nature, are found in various fruits and vegetables, such as apples, berries, grapes, tomatoes, and onions, and play a key role in attracting pollinators and seed disseminators [27, 31].

Flavones and flavonols have antioxidant effects and are essential for protecting plants from UV radiation [32, 33]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is one of the most representative flavonoids, widely distributed in nature, and an abundant dietary flavonol, found in food, such as onions, peas, and apples [22, 26]. Quercetin-3-O-rutinoside, known as rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) and also called vitamin P due to contribute to reducing the permeability of blood vessels, was introduced in the market and has been explored for important pharmacological effects [34]. Luteolin (*3′,4′,5,7*-tetrahydroxyflavone) and apigenin (*4′,5,7*-trihydroxyflavone) are the main flavones found in food, such as onions, celery, red pepper, and grapes, and have a wide range of effects in biological systems, including anti-inflammatory and anticarcinogenic [35]. Luteolin is found in cereals and herbs, and glycosylated luteolin in vegetables such as carrots and broccoli [31, 36]. Luteolin inhibits cancer in head and neck squamous cell carcinoma (HNSCC) xenograft in a mouse model by inhibiting p300 lysine acetyltransferase activity *via* acting at multiple levels of gene expression, miRNA expression, and miRNA processing [37]. Luteolin effectively prevented pulmonary and hepatic fibrosis [38, 39] and reduced cancer cell proliferation [40]. Both effects are due to the reduction in oxidative

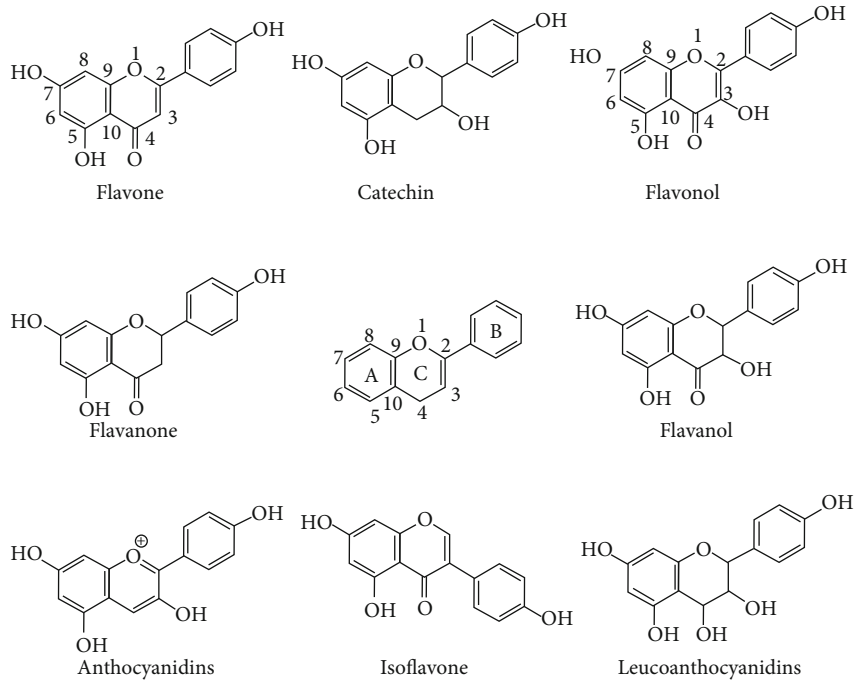

Figure 2: Chemical structures of some representative flavonoids and the basic skeleton of the flavonoids in the middle.

stress and inflammatory responses [41]. Interestingly, luteolin can also play a protective role in inflammation and cancer by modulating the microbiota ([42]. The flavonoid hesperidin possesses antioxidant and anti-inflammatory properties and plays a role in inhibition of tumor cell metastasis and angiogenesis [43]. Table 1 shows some flavones and flavonols aglycones and its natural sources and content (mg/100 g or mg/100 ml).

2.2. Inflammation. Inflammation is a complex response initiated by an insult by chemical, physical, or biological agents, which leads to resolution and promotes tissue repair [54, 55]. During the inflammatory process, immune system cells, such as neutrophils, monocytes, eosinophils, and lymphocytes, migrate to the lesion site [56, 57].

The lipid mediators eicosanoids and cytokines produced during the inflammatory response cause vascular and cellular alterations such as vasodilation, an increase in vascular permeability and activation, adhesion, and cellular transmigration [54]. The increase in the concentrations of inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, is crucial for leukocyte migration to the lesion [58–60]. The production of mediators and increased vascular permeability generate an exudate rich in plasma proteins [61]. The production of inflammatory mediators leads to endothelial increased permeability and leukocyte activation and migration to the injury site, cellular, and tissue damage and triggers the characteristic symptoms of inflammation, known by the five cardinal signs: pain, redness, heat, edema, and loss of function [54, 62]. When leukocytes reach the inflammation site, they are activated and respond to eliminate noxious stimuli, thus releasing the content of their toxic granules, which includes ROS, reactive nitrogen species (RNS), proteinase, cathepsin G, and elastase [61, 63].

The inflammatory process involves the production of numerous cytokines that bind to cellular receptors triggering intracellular signaling, such as JAK kinase, phosphatidylinositol-3-kinase (PI3K), V-Akt murine thymoma viral oncogene homolog 1 (AKT1), inhibitory kappa B kinase (IKK), and mitogen-activated protein kinases (MAPKs), which are responsible for the activation and nuclear translocation of transcriptional factors (signal transducer and activator of transcription [STAT], nuclear factor kappa B [NF-κB], and activator protein 1[AP-1]) [64–67].

Inflammatory responses must be fine-tuned because if exacerbated, it will damage tissues and, if insufficient, will not cause complete elimination of pathogens. Exacerbation of immune response with uncontrolled inflammation results in poor outcomes [68]. Drugs or natural compounds with strong anti-inflammatory effects allow microbes to grow and decrease survival [69]. However, adequate anti-inflammatory effects may prevent organ damage and improve host survival [69, 70].

2.3. ROS and Oxidative Stress. ROS are presented by one or more unpaired electrons produced in cells during mitochondrial oxidative metabolism and in cellular response to xenobiotics, cytokines, bacterial invasion, UV radiation, and pollutants, collectively known as oxidizing agents [71]; they promote cascade reactions and cause oxidative damage [71, 72].

Normal metabolism generates reactive species that play a prominent role in physiological functions, including gene expression, cell signaling, and immune responses. Antioxidants neutralize reactive species in excess [71, 72]. An

Table 1: Natural sources and content (mg/100 g or mg/100 ml) of some flavonols and flavones.

Substance	Food source	Estimated content (mg/100 g fresh weight)	Reference
Quercetin	Tomatoes	7-55	[26]
	Lettuce	2-911	
	Onions	10-1359	
	Peas	98-145	
	Apple	2-26	[22]
	Apricot	2-5	
	Broccoli	0-3	
	Chives	10-30	
	Cranberry	149	
	Grape, red	1-3	
	Cranberry	25	[44]
	Oregano	42	
	Dill	79	
	Lovage	170	[45]
	Radish leaves	70	
	Watercress	29	
Kaempferol	Kale	47	[44]
	Spinach	55	
	Chives	12	
	Dill	40	
	Cabbage	20-25	[45]
	Watercress	23	
Luteolin	Celery	6-40	[26]
	Mexican oregano	901-1137	[46]
	Spices, celery seed	811	[47]
Apigenin	Celery	17-191	[26]
	Spinach	1	
	Green pepper	0-1	[48]
	Welsh onion	8-11	
	Parsley	4503	
	Chamomile	300-500	[49]
	Vine-spinach	62	
Baicalein	Wine, tea, citrus fruits, dark chocolate, and herbs	n.d.	[50]
	Roots of *Scutellaria baicalensis (traditional Chinese herbal medicine)*	n.d.	[51]
	Welsh onion	9	[48]
	Spinach	19	
Scutellarein	Mexican oregano	24-48	[47]
Tangeretin	Citrus fruits, Orange juice (pure)	8*	[52]
Nobiletin	Grape fruits, teas (*Camellia sinensis*), herbal tea, and black tea	n.d.	[53]

n.d.: not described; *mg/100 ml.

antioxidant is a molecule able to donate an electron to a rampaging free radical and neutralize it [73]. Some antioxidants can interact with other antioxidants regenerating their original properties called the "antioxidant network" [74]. They neutralize ROS by different mechanisms including directly scavenging free radicals, directly scavenging free radicals, and inhibiting lipid peroxidation between others [75]. Antioxidant system can be enzymatic or nonenzymatic. The nonenzymatic system blocks the initiation of oxidation. The enzymatic system comprises antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). In the nonenzymatic antioxidant system, glutathione (GSH); ascorbate (AsA); numerous simple

phenolic acids such as hydroxybenzoic acids, hydroxycinnamic acids, and different flavonoid compounds; carotenoids such as xanthophylls, beta-carotene, and α-carotene; vitamin E; and pigments such as chlorophylls, betaxanthins, betalains, and betacyanins such as amaranthine, isoamaranthine, betanidine, and isobetadine are the most noticeable components ([76]). Antioxidants act by stabilizing ROS. An imbalance in ROS production and antioxidant activity in biological systems causes oxidative stress. As a result of this imbalance, cellular dysfunction may occur *via* modulating gene expression, protein stability, and membrane fluidity, leading to cell damage and death [72]. An increase in reactive species levels occurs with aging and the development of diseases, including cancer, Parkinson's disease, diabetes, and cardiovascular diseases [77]. ROS increase is linked to different diseases, reviewed by [78]. For instance, excessive ROS accumulation leads to glutathionylation modification and proteasomal degradation of MAP kinase phosphatase-1, which prevents insulin signaling. Also, ROS in excess inactivates AKT, inhibiting glucose uptake, which eventually causes type 2 diabetes. In addition, mitochondria-derived ROS elicit the production of NLRP3 and IL-1β, accounting for an inflammatory phenotype in atherosclerosis pathogenesis. Furthermore, phosphorylated tau protein and Aβ are risk factors in Alzheimer's disease, which originate from the downregulated Nrf2 and increased ROS burden. Finally, extracellular and intracellular ROS activate TGF-β signaling triggering the epithelial-mesenchymal transition of epithelial cells and survival of circulating tumor cells during cancer metastasis [78].

The search for new antioxidant compounds as therapeutic agents for diseases associated with imbalance in reactive species production and clearance is gaining attraction of researchers. Natural antioxidants, such as phenolic compounds (flavonoids and phenolic acids), have emerged as primary therapeutic agents as nonenzymatic antioxidants because they can sequester ROS [79]. They also act as anti-inflammatory agents.

2.4. Limitations Associated with the Use of Flavonoids. We consume fruits and vegetables that containing flavonoids, such as apples, berries, onions, and broccoli, on a daily basis [80, 81]. However, their bioavailability can vary. A trial conducted with 97 humans showed that the plasma concentrations of total polyphenolic metabolites was 0–4 μmol/L after an intake of 50 mg aglycone equivalents, and the urinary excretion was 0.3–43% of the ingested dose [82]. Flavonoids are absorbed in the small intestine. Flavonoids that cannot be absorbed in the small intestine are degraded in the colon by microorganisms to phenolic acids [83].

The absorption of flavonoids from food was long considered to be very low, as they are present in foods linked to sugars such as β-glycosides (except catechins). Only aglycones forms were considered able to pass the gut wall, and no enzymes that can split these predominantly β-glycosidic bonds are secreted into the gut or present in the intestinal wall [84]. Unlike terpenes with high bioavailability, flavonoids can have low bioavailability due to the extensive first-pass effect and glucuronidation [85]. However, research on the mechanisms for aglycone transfer across the gut wall is lacking. In a study on ileostomized humans, the absorption of orally administered quercetin was 24%. These data show that humans absorb appreciable amounts of quercetin and that absorption of glycosides in the small intestine is possible. Humans consume, on average, between 0.45 and 1.17 mg of apigenin daily [86]. Apigenin when taken orally (whose bioavailability is about 30%) is systemically absorbed and recirculated by enterohepatic and local intestinal pathways [87].

Despite these concerns about bioavailability, which seems to be influenced by their chemical nature, flavonoids effectively prevent or improve diseases. There are three possible explanations for this: (1) act in the lumen or intestinal epithelium, (2) act in the peripheral tissues even at very low circulating levels, or (3) the microbial metabolites of flavonoids are also active in the peripheral tissues [88]. Flavonoid amount consumed through food is generally nontoxic. However, when consumed in large amount, they can show toxic effects. Administration of high doses ($\geq$200 mg/kg) and prolonged use of luteolin may increase hepatic enzyme activity; however, no significant liver damage was observed, whereas low doses ($\leq$50 mg/kg) are hepatoprotective [89].

The purification or production of bioactive compounds on a large scale is challenging. Although a specific bioactive compound has been identified and proven to be beneficial in treating inflammatory or infectious diseases, its purification or bioengineering remains a challenge. Moreover, where bioactive flavonols have been identified in natural food, the among ingested is difficult to determine because it varies from place to place depending on the environment in which the plant grows. Another crucial challenge is the development of optimum delivery formulations. Nevertheless, the flavonoid properties in biological systems are indisputable because they are bioactive compounds with the potential to treat or prevent diseases; therefore, future studies based on better delivery systems, such as nanoformulations, should be encouraged [90, 91].

2.5. Flavonols and Inflammation. The current strategies for treating inflammatory and infectious diseases often present side effects that markedly affect the quality of life of patients. In addition, the use of immunosuppressants may be essential to control the immune response in infectious diseases; however, it can increase the risk of spread of the infection and should be used with caution. Flavonols, such as quercetin, kaempferol, and myricetin, are the most common plant flavonoids. We discussed recent studies on anti-inflammatory effects of flavonols and listed them in Table 1.

2.6. Anti-Inflammatory Effects of Quercetin, Isoquercetin, Kaempferol, and Myricetin. First isolated in 1857 from oak (*Quercus*), quercetin is probably the most investigated flavonoid. It has strong and prolonged anti-inflammatory effects [75, 92, 93]. Quercetin acts through several mechanisms such as by inhibiting lipopolysaccharide (LPS)-induced TNF-α production in macrophages [94], inhibiting LPS-induced IL-8 in A549 lung carcinoma epithelial cells [95], and inhibiting the production of inflammatory mediator-

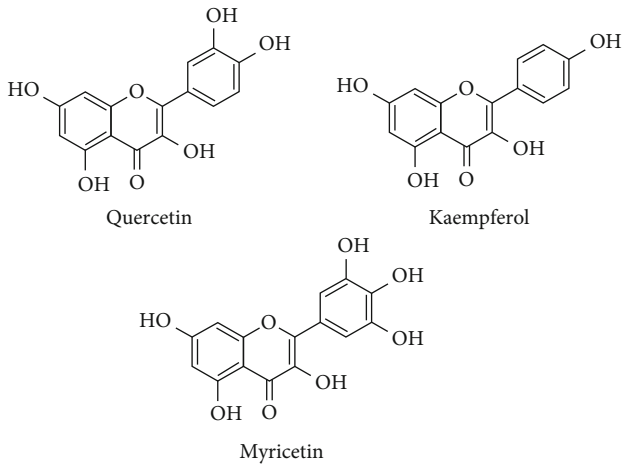

Figure 3: Chemical structures of quercetin, myricetin, and kaempferol.

producing enzymes such as COX and LOX [96, 97]. Quercetin showed cardioprotective effect against isoproterenol-induced myocardial infarction in rats [98] (Figure 3). Additionally, it reduced levels of biomarkers of ischemic induction, such as creatine kinase myocardial band (CK-MB) and cardiac troponin I (cTnl), inhibited inflammatory cell infiltration into the myocardium, reduced the expression of TNF-α and IL-6, and inhibited upregulation of calpain genes 1 and 2 that protect the myocardium [98].

Treatment with quercetin improved LPS-induced inflammatory damage in pulmonary fibroblasts by increasing cell viability, Treatment with quercetin improvedaverting apoptosis, and reducing pro-inflammatory cytokine production (TNF-α and IL-6) [95]. In an experimental model of acetic acid-induced gastric ulcers in rats, quercetin, isolated from *Madhuca indica*, inhibited cytokines (IL-1β and TNF-α), NO, and prostaglandin production by downregulating COX-2 expression [99]. Similarly, quercetin treatment reduced IL-1β and TNF-α levels [100]. Macrophage inflammatory protein 1α/chemokine (C-C motif) ligand 3 (MIP-1α/CCL3) is a key to monocytes/macrophage recruitment, and the release from adipocytes and macrophages tissue causes tissue inflammation [101]. Quercetin suppressed MIP-1α effects by downregulation of CCR1/CCR5 and inhibition of activation of c-Jun N-terminal kinase (JNK), p38 mitogen-activated-protein kinase (MAPK), and IKK and IκBα degradation [3].

Oral administration of quercetin for the treatment of arthritis induced by inactive *Mycobacterium butyricum* in a rat model showed that quercetin could diminish inflammatory markers, such as IL-1β, C-reactive protein, and MPC-1 and restore the plasma antioxidant capacity. In addition, it inhibited 12/15-LOX activity in the lungs and liver and increased heme oxygenase-1 (HO-1) expression in joints and lungs [102]. Quercetin treatment decreased inflammation and attenuated pathological alterations in the pancreas and ileum of animals with acute necrotizing pancreatitis. This suggests that the flavonoid acts by partial inhibition of TLR4/MyD88/P38 and MAPK ERK, thus reducing inflammation and interrupting intestinal damage [103]. In a model of monohydrate crystal–induced arthritis fatty acids activates TLR2 inducing interleukin-1β production via the ASC/caspase 1 pathway [104]. In addition, quercetin inhibited the release of ROS, IL-1β, and IL-18, resulting in the protection of mitochondrial integrity in Caco-2 cells stimulated with *Escherichia coli* [105]. It also blocked the inflammasome NLRP3 activation in the rat kidneys [106, 107].

Moreover, quercetin suppressed the CXCR4 upregulation in otitis media induced by non-typificable gram-negative bacillus *Haemophilus influenzae* (NTHi), both *in vitro* and *in vivo* [102]. It downregulated the TLR3/Myd88 signaling by blocking NF-κB activation [108] and IKKα and p38 phosphorylation in mice inoculated with NTHi [102]. A recent study showed that isoquercetin treatment reversed weight loss, decreased glucose levels, and increased insulin production in streptozotocin-induced diabetic rats. Furthermore, isoquercetin reduced lipid peroxidation by increasing SOD and CAT activity, which improved the lipid profile in the diabetic group. In addition, this flavonol suppressed inflammatory genes that code for NF-κB, IL-1, IL-6, TLR, TNF-α, and COX-2 [109].

Kaempferol (from genus *Kaempferia*) is a flavonol abundantly present in various species and plant-derived foods; it has been shown to inhibit adipogenesis by blocking 3 T3-L1 differentiation and lipid accumulation in mature adipocytes. It modulated adipogenic gene expression and decreased lipid accumulation in mature adipocytes (CEBPA) *via* upregulating mRNA expression of Pnpla2 and Lipe [110]. Similarly, kaempferol treatment reduced lipid and protein accumulation along with the expression of the adipogenic markers PPARγ and C/EBPα in 3 T3-L1 cells [108]. These changes in adipocyte lipid metabolism affect the response of adipocytes to the production of inflammatory mediators.

First isolated from the bark of *Myrica nagi*, myricetin is a common flavonol occurring also in both free or glycosylated forms, with anti-inflammatory activity and cardioprotective effect, which prevents myocarditis by reducing apoptosis [111]. It markedly reduced cleaved caspase-3 and Bcl-2 levels and increased Bax levels in H9c2 cells. The flavonol decreased MCP-1 and IκBα phosphorylation levels by blocking the NF-κB activation in LPS-challenged H9c2 cells. Treatment with semisynthetic flavone derived from myricetin (the M10 compound) prevented chronic inflammation and ulcerative colitis induced by azoxymethane/dextran in colorectal tumorigenesis [24].

2.7. Flavones and Inflammation. Flavones are characterized by the absence of a hydroxyl group at C3 when compared to flavonol that also exhibit structural variations of hydroxylation, O-methylation, C-methylation, or prenylation. This wide range of structural combinations results in several types of flavones, making them as an abundant subclass [112]. Herein, we discussed the most frequent flavones present in human diet, luteolin, and apigenin. Luteolin modulates the immune response more efficiently than other naturally derived compounds such as quercetin, genistein, and hesperidin [113] (Table 2). Besides, apigenin is also known for its anti-inflammatory and antioxidant effects by inhibiting cytokine secretion and downregulating enzymes such as inducible nitric oxide synthase (iNOS) and COX-2 [114].

Table 2: Anti-inflammatory effect of flavonols.

Flavonoid	Experimental Model	Treatment/dose	Mechanism of action or main results	Author/ year
Quercetin	***In vivo*** Acetic acid induced ulcer gastric in male Wistar rats	2.5, 5 and 10 mg/ kg	(i)Reduction of expression of the pro-inflammatory cytokines TNF-α and IL-6 (ii)Inhibition of COX-2	[99]
	In vivo Isoproterenol-induced myocardial infarction in Wistar rats	50 mg/kg	(i)Protection against heart damage (ii)Attenuation of oxidative stress (iii)Protection of cardiac architecture (iv)Modulation of calpain expression (v)Reduction of expression of the pro-inflammatory cytokines TNF-α and IL-6	[98]
	In vitro Human colonic epithelial cell line Caco-2 cell	200 μM	(i)Inhibition of NLRP3 activation (ii)Reduced ROS production (iii)Reduction in IL-1 and IL-18 levels	[105]
	In vivo Acute necrotizing pancreatitis (ANP) model in rats	50 mg/kg	(i)Mitigation of damage to the pancreas, ileum, and intestine (ii)Suppression of TLR4/MyD88/p38 and inhibition of endoplasmic reticulum stress	[103]
	In vitro Human middle ear epithelial cells (HMEECs), renal epithelial cells (HK2), and mouse lung epithelial cells (MLE-12), human liver epithelial cells (L02), and human lung epithelial cells (BEAS-2B)	40, 80 and 120 μM	(i)Suppressed NTHi-induced CXCR4 expression levels *in vitro* and *in vivo* (ii)Blocked CXCR4 activation through direct IKKβ phosphorylation inhibition	[102]
	In vivo Otitis NTHi-induced in mice	20, 40 and 80 mg/ kg		
Isoquercetin	***In vivo*** Streptozotocin-induced diabetes in male Wistar rats	40 mg/kg	(i)Reduction in inflammation marker levels (IL-1β, C-reactive protein, and MPC-1) (ii)Restoration of plasma antioxidant capacity (iii)Inhibition of 12/15-LOX activity in the lungs and liver (iv)Upregulation of HO-1 expression in the joints and lungs (v)Reversal of weight loss, reduction in glucose levels, and increase in insulin production (vi)Increased SOD and CAT activity (vii)Suppression of the inflammatory genes NF-κB, IL-1, IL-6, TLR, TNF-α, and COX-2	[109]
Kaempferol	***In vitro*** Anti-adipogenic effects on 3 T3-L1 cells	7.5, 15, and 30 μM	(i) Blockage of 3 T3-L1 differentiation and lipid accumulation in mature adipocytes	[108]
	In vivo Wild-type zebrafish	5, 10, and 20 μM	(ii)Reduction in expression of the adipogenic markers PPARγ and C/EBPα in 3 T3-L1 cells	
	In vitro Anti-adipogenic effects on 3 T3-L1 cells	20, 40, 60, 80, 100, 120, 150, and 170 μM	(i)Modulation of adipogenic gene expression in mature adipocytes (ii)Decreased accumulation of lipids in mature adipocytes (iii)Upregulation of Pnpla2 and Lipe mRNA expression	[110]
Myricetin	LPS stimulated H9c2 cells	H0, 10, 30, and 50 μM	(i)Reduction in cleaved caspase-3 and Bcl-2 levels	[111]

Table 2: Continued.

Flavonoid	Experimental Model	Treatment/dose	Mechanism of action or main results	Author/ year
			(ii)Increase in Bax levels in H9c2 cells (iii)Reduction in MCP-1/CCL-2 and IκBα phosphorylation levels	

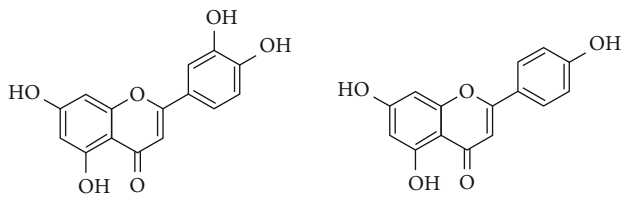

Figure 4: Chemical structures of luteolin and apigenin.

2.8. Luteolin, Apigenin, and Inflammation. Luteolin and apigenin are the most abundant flavones occurring in nature. They differ structurally by the presence of a hydroxyl group at C3` (Figure 4).

Luteolin was first isolated in 1829 as the main yellow-colored compound extracted from *Reseda luteola* and structure confirmed by synthesis in 1900 [115]. Luteolin has antimicrobial, anti-inflammatory, chemopreventive, chemotherapeutic, cardioprotective, antidiabetic, neuroprotective, and antiallergic properties [116–119]. Moreover, consumption of luteolin-rich food reduces the risk of developing chronic diseases [120].

Luteolin exhibited its anti-inflammatory effect by reducing the production of pro-inflammatory agents, such as NO, prostaglandin E2 (PGE2), TNF-α, MMP-2, MMP-8, and MMP-9, and by downregulating COX-2, iNOS, MMP-3, and MMP-13 expression in both *in vitro* and *in vivo* arthritis models [1, 113]. Luteolin also showed effects in human neutrophils by inhibiting NET formation, elastase release, CD11 expression, chemotaxis, and the RAF1-MEK-1-Erk signaling pathway, resulting in decreased neutrophil infiltration and activation, thereby attenuating the inflammatory response [1]. It also blocked ROS, TNF-α, IL-6, and nitric oxide (NO) production in human and mouse cells [121].

In addition, luteolin showed a cardioprotective effect in cardiomyocyte cultures and experimental type 1 diabetes. This flavone reduced inflammation by inhibiting the NF-κB signaling, cytokine production, and oxidative stress by blocking the activation of the nuclear factor 2 (NRF2) pathway. It has also been shown to prevent cardiac fibrosis, hypertrophy, and dysfunction in diabetic rats [122]. Moreover, luteolin inhibited the production of IL-8 and COX-2 and reduced the expression of iNOS and NO in colon HT-29 cells [123]. Hence, it has emerged as a potential candidate for the treatment of bowel disease because of its ability to modulate intracellular inflammatory signaling cascade. Inhibition of the JAK/STAT signaling pathway seems to be a key mechanism for anti-inflammatory action of luteolin in bowel diseases [124]. These results indicate that luteolin negatively modulates the main signaling pathways involved in the intestinal inflammation.

Luteolin inhibited the expression of TNF-α, IL-1β, and IL-6; suppressed IκBα and NF-κB p65 phosphorylation levels; and downregulated the expression of MMP-2 and MMP-9 in *S. aureus*-induced mastitis [125], showing its potential in reducing tissue damage and inflammation caused by *S. aureus*-induced mastitis. Moreover, luteolin showed effects in arthritis models through the involvement of metalloproteinase [113]. Luteolin plays a critical role as an anti-inflammatory agent by reducing the production of inflammatory mediators, inhibiting crucial inflammatory intracellular pathways, and blocking the translocation and activation of inflammatory transcription factors. Therefore, it is a potential agent for the treatment and prevention of inflammatory, infectious, and metabolic diseases.

Apigenin, a constituent from celery (*Apium graveolens*) also common in other food plants, such as parsley and chamomile [49], has therapeutic potential for various diseases (diabetes, cancer, and neurological and cardiovascular disorders) [126], considering its anti-inflammatory, antioxidant, and anticancer effects. The mechanism of action of apigenin involved the suppression of p65 phosphorylation in monocytes and macrophages and reduction in the expression of COX-2 and NO in rat macrophages [8]. Zhang et al. have reported that apigenin inhibited CD38, an inflammatory biomarker, reduced pro-inflammatory cytokines, and led to reduction in adipose tissue mass in a metabolic syndrome model [127]. Treatment with apigenin prevented pulmonary fibrosis and bleomycin-induced inflammation by decreasing TNF-α and TGF-β1 levels and attenuating the reduction in SOD activity [128]. An *in vivo* experiment on concanavalin A-stimulated rat splenocytes showed that apigenin prevented iNOS induction, downregulated COX-2 expression, and reduced pro-inflammatory cytokine production (TNF-α, IFN-γ, and IL-2). The flavone has also demonstrated an antiproliferative effect on lymphocytes [114].

2.9. Effect of Other Flavones on Inflammation. Other flavones with anti-inflammatory effects, such as 3′,4′-dihydroxyflavone, baicalein, scutellarein, lucenin-2, vicenin-2, diosmetin, nobiletin, tangeretin, and 5-O-methyl-scutellarein, have been described (Figure 5).

3′,4′-Dihydroxyflavone suppressed the MAPK and NF-κB signaling pathways, in addition to the inhibition of NO, PGE2, and pro-inflammatory cytokine production in microglial cells and mice [129]. Baicalein, one of the main constituents of *Scutellaria baicalensis* Georgi [127] improved motor deficit and attenuated brain injury in rats chronically exposed to rotenone. Baicalein inhibited IL-6, TNF-α, and NO production, blocked IκBα phosphorylation and NF-κB translocation, and negatively modulated TLR4 in BV2 microglial cells. Similarly, scutellarein (*Scutellaria barbata*)

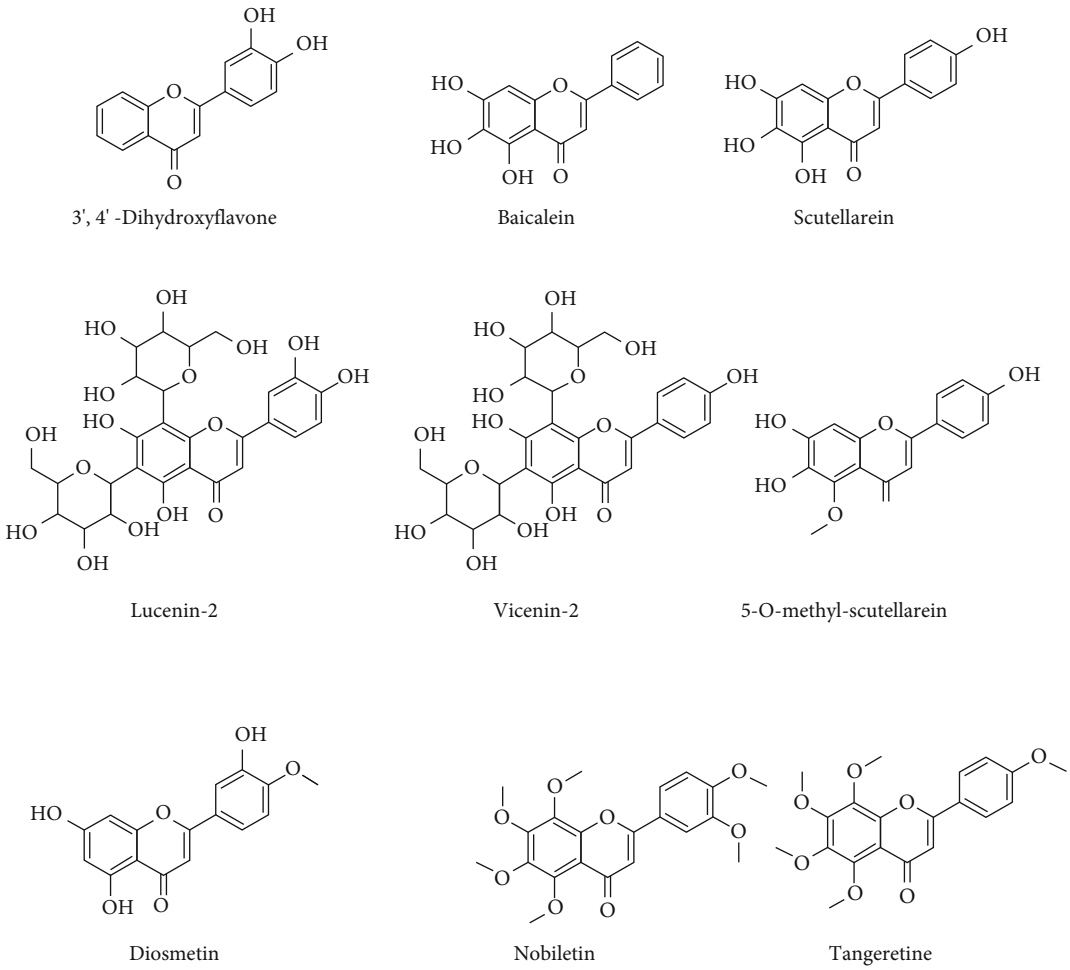

Figure 5: Chemical structures of 3′,4′-dihydroxyflavone, baicalein, scutellarein, lucenin-2, vicenin-2, diosmetin, nobiletin, tangeretin, and 5-O-methyl-scutellarein.

improved LPS-induced cognitive deficit in rats and decreased the levels of hippocampal malondialdehyde (MDA) and antioxidant defense elements such as SOD, catalase, and GSH [130]. Thus, the authors suggest possible mechanisms underlying anti-inflammatory activity as inhibition of the levels of NF-κB, TNF-α, IL-6, and NRF2, in addition to the inhibition of autophagy markers and decreased expression of matrix metalloproteinase.

Diosmetin (3′,5,7-trihydroxy-4′-methoxyflavone) is a flavone common in *Citrus* species, which exerts anti-inflammatory and other effects [131]. Diosmetin-7-O-rutinoside, known as diosmin (3′,5,7-trihydroxy-4′-methoxyflavone 7-rhamnosylglucoside), is well stablished to treat vessel permeability and inflammatory diseases. A recent study was showed that diosmetin attenuates experimental ulcerative colitis in rats via suppression of NF-κB, TNF-α, and IL-6 signaling pathways correlated with downregulation of apoptotic events [132].

Glycosylated flavones, lucenin-2, vicenin-2, and scutellarein-2, obtained from *Korthalsella japonica*, possess anti-inflammatory activity [133]. Both the extract and isolated flavones inhibited the production of NO and reduced the expression of iNOS and COX-2 in LPS-stimulated RAW 264 cells (M. K. [133]). Vicenin-2 negatively regulated the secretion of cytokines (TNF-α and IL-1β) and increased the expression of IL-10 and IL-1Ra in LPS-induced mononuclear cells [134]. Furthermore, vicenin-2 inhibited the reporter activity of NF-κB and increased homodimerization of P50 subunit of transcription factor by heterodimerization of P50/P65 [135].

Nobiletin, a polymethoxyflavone (3′,4′,5,6,7,8-hexamethoxyflavone), reduced the myeloperoxidase activity in the pancreas, cellular damage, and phosphorylation of p38 and AKT in a model of acute peritonitis. It also reduced the levels of TNF-α, IL-1β, IL-6, and IL-10, attributing to its anti-inflammatory activity [136].

Tangeretin, a flavone isolated from dried fruit shells of *Citrus tachibana*, attenuated colitis parameters such as colon shortening; activation of myeloperoxidase, NF-κB, and MAPKs; production of IFN-γ, TNF-α, and IL-17; and expression of COX-2 and iNOS. Therefore, it has shown significant anti-inflammatory effect [137]. Hydroethanolic extract of *Arrabidaea chica* leaves and its isolated flavone 4′,6,7-tri-hydroxy-5-methoxyflavone (5-O-methyl-scutellarein) dose-dependently inhibited inflammatory cell recruitment and TNF-α, IL-1β, and IL-10 production [138].

A recent study reviewed preclinical and clinical data on anti-inflammatory and analgesic properties of flavonoids; most data demonstrated the potential therapeutic role and efficacy of flavonoids in cardiovascular diseases, osteoarthritis, Parkinson's disease, colitis, cancer pain, arthritis, and neuropathic pain. Nevertheless, the mechanisms of action of flavonoids have yet to be fully elucidated. Considering the pharmacological activities of flavonoids, it would be promising to further develop delivery formulations containing flavonoids to treat inflammatory diseases and pain [139].

Different approaches to evaluate the anti-inflammatory activity of flavones described above, both *in vitro* and *in vivo*, showed a reduction in the production of pro-inflammatory mediator, and inhibition of activation of the signaling pathway and nuclear factor. Regardless of the chosen model of arthritis, colitis, or gastric ulcer, flavones inhibited different signaling pathways, production of mediators, transcription factor activation, and ROS production.

2.10. Antioxidant Effects of Flavonols and Flavones. The balance between ROS production and antioxidant cell mechanisms regulates oxidative stress in cells and can lead to cell damage when unregulated. Antioxidant enzymes, SOD and CAT, can directly detoxify the generated ROS or facilitate antioxidant activity using GSH as a reducing agent [143]. CAT is responsible for peroxide decomposition in a cell [144].

The antioxidant activity of quercetin can be explained by its direct effect on GSH, signal transduction pathways, enzymatic activity, and ROS, which maintain oxidative balance. Quercetin regulates the levels of GSH, a hydrogen donor in decomposition of H_2O_2 to H_2O, by inducing its synthesis. By influencing the signal transduction pathways, the compound can inhibit the enzymatic activity of numerous enzymes associated with oxidative mechanisms, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), and enhance the expression levels of antioxidant enzymes such as SOD, CAT, and GSH peroxidase. Finally, quercetin can remove ROS through various mechanisms involved in regulation of ROS production and modulation of antioxidant-related gene expression. Clinically, quercetin exhibits antitumor effect and is used for cancer and metastasis prevention [145]. It has also been shown to have therapeutic effects in cardiovascular diseases and significant antidepressant outcome [146]. Quercetin isolated from *Flaveria bidentis* (L.) inhibited ROS production in human mononuclear cells and polymorphic leukocytes more efficiently than that of vitamin C [147]. Quercetin reversed the oxidative damage induced by $FeSO_4$ ferrous ions in bovine sperm cells [148]. The flavonol also induced the expression of HO-1 and the subsequent end product, suppressing oxidative stress derived from NADPH oxidase [142]. Furthermore, quercetin decreased MDA levels in the myocardial tissue and activated SOD and GPx enzymes [98, 142], demonstrating the antioxidant activity of quercetin in a rat model of myocardial infarction. In a similar study, treatment with quercetin decreased MDA levels in the myocardial tissue and further stimulated SOD and GPx enzymes [149].

Luteolin attenuated oxidative damage and reduced the generation of intracellular ROS by inducing the expression of the antioxidant enzyme HO-1, thus counteracting the damage induced by H_2O_2 in rat cortical primary cell cultures [141]. Treatment with the flavone normalized AST and ALT levels. The protective role of luteolin against ROS and oxidative damage occurs by normal GSH level restoration and lipoperoxidation suppression in the plasma and liver tissues [140]. Moreover, the flavone blocked superoxide anion and ROS production in human neutrophils [150], strengthening the role of luteolin as an antioxidant agent. Chanput et al. evaluated the antioxidant activity of three classes of flavonoids: (i) flavonol: quercetin and myricetin; (ii) flavanone: eriodictyol and naringenin; and (iii) flavone: luteolin and apigenin [151]. The flavonol group (myricetin and quercetin) showed the best antioxidant activity among the three groups, which might be due to the presence of more hydroxyl groups in their structure and the presence of double bond between C2 and C3 of the aromatic ring that improve their antioxidant properties by the electron distribution pattern [152] compared to the other groups.

2.11. Antibacterial Activity of Flavones and Flavonols. Several studies have supported the use of medicinal plants for their antimicrobial activities. Flavonoids act on different microorganisms. Herein, we discuss their effects on resistant and opportunistic bacteria. Antibiotic abuse may lead to the select bacterial resistance to current drugs. Medicinal plant extracts contain a variety of phytochemical substances that can act synergistically to inhibit the bacterial growth and prevent the development of drug resistance. Table 3 shows the flavonoids used for antibacterial purposes and their mechanisms of action.

Methanolic fraction of the extract from *Justicia wynaadensis* contains 3,3,4-trihydroxyflavone that exhibits antimicrobial activity against multiresistant and opportunistic organisms. Flavones can effectively kill bacteria present in diabetic wounds such as *Enterococcus faecalis*, *Streptococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* [153]. The methanolic extract of *J. wynaadensis* also showed antibacterial activity against *K. pneumoniae* (Ponnamma SU2015). The methanolic extract of *Tragia involucrata* was effective against *Enterobacter aerogenes*, *P. aeruginosa*, *S. aureus*, *Proteus vulgaris*, and *E. coli* associated with diabetic foot ulcer and ICU [154].

The methanolic root extract from *Garcinia macrophylla* is an important medicinal herb used to treat diverse diseases and disorders in Traditional Chinese Medicine and showed antimicrobial activity against bacterial strains of *Micrococcus luteus*, *E. faecalis*, *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus uberis*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* isolated from burn wounds, with the lowest minimum inhibitory concentration against *S. epidermidis* and the highest MIC value against *E. coli* [150]. The authors observed equivalent values for minimum bactericidal concentration (MBC). The antibacterial activity of *Gentiana kurroo* methanolic extract overcame the results presented by other comparable studies [155].

Table 3: Mechanisms underlying anti-inflammatory action of flavones.

Flavonoid	Experimental Model	Treatment	Mechanism of action or main results	Author/ year
Luteolin	***In vitro*** Rat chondrocytes	25, 50, and 100 μM	(i)Reduction of the levels of NO, PGE2, TNF-α, MMP-2, MMP-8, and MMP-9 induced by IL-1β (ii)Reduction of the expression of COX-2, iNOS, MMP-1, MMP-3, and MMP-13 (iii)Reversal of IL-1β-induced collagen II degradation (iv)Inhibition of IL-1β-induced phosphorylation of NF-κB	[113]
	In vivo *Osteoarthritis in Wistar rats*	10 mg/kg	(i)Prevention of cartilage destruction and increased collagen II expression in rats	
	In vitro Cardiomyocytes	5 e 10 μg/kg	(i)Reduction of inflammatory mediator production (ii)Inhibition of oxidative stress (iii)Inhibition of the NF-κB pathway (iv)Activation of the NRF2 pathway	[122]
	In vivo Mice C57BL/6 with diabetes type I induced by streptozotocin	20 mg/kg	(i)Inhibition of fibrosis, hypertrophy, and cardiac dysfunction in streptozotocin-induced diabetic mice	
	In vitro Human neutrophils	3, 10, and 30 μM	(i)Inhibition of superoxide anion production (ii)Inhibition of NO production and NET formation in neutrophils (iii)Reduction in elastase production and inhibition of CD11b expression and chemotaxis (iv)Inhibition of ERK and kinase-1 activated protein (MEK-1)	[1]
	In vivo Male mice (C57BL/6) Paw edema CFA-induced	50 mg/kg	(i)Reduction of neutrophil infiltration (ii)Improvement in adjuvant-induced edema (iii)Reduction in ROS production	
	In vivo Swiss albino mice Paw edema carrageenan-induced	10, 25, and 50 mg/kg	(i)Reduction in the number of abdominal constrictions and licking induced by acetic acid and glutamate (ii)Inhibition of nociceptive response in both phases of formalin test (iii)Inhibition of carrageenan-induced paw edema (iv)Reduction in the levels of TNF-α, IL-1β, and IL-6	[117]
	In vivo BALB/c mice Model of mastitis induced by *S. aureus*	25, 50, and 100 mg/kg	(i)Protection of tissue destruction of mammary glands (ii)Inhibition of inflammatory cell infiltration (iii)Inhibition of TNF-α, IL-1β, and IL-6 expression (iv)Inhibition of IκBα and NF-κB p65 phosphorylation levels (v)Downregulation of MMP-2 and MMP-9 expression (vi)Increase in the levels of tissue metalloproteinase inhibitors (TIMP)-1 and TIMP-2	[125]
	In vivo Male BALB/c mice model of hepatic cancer chemically induced	1, 5, 10, 20 and 50 μg/kg	(i)Modification in the levels of α-fetoprotein and antioxidant enzymes (SOD and CAT) (ii)Modification of enzymatic markers such as AST and ALT (iii)Reduction in the levels of glutathione and inflammatory cytokines such as IL-2 and IFN-γ in the plasma and liver	**[140]**
	In vitro Colon tumor cell HT-29	50 μM and 100 μM	(i)Inhibition of IL-8 production, and COX-2 and iNOS expression (ii)Downregulation of the JAK/STAT signaling pathway	[123]
	In vitro Primary cultures of rat cortical cells subjected to oxidative stress	3–30 μM	(i)Attenuation of oxidative damage (ii)Inhibition of ROS generation (iii)Increase in bad phosphorylation in serine-112 (iv)Attenuation of induced caspase-3 activation (v)Upregulation of HO-1 expression (vi)Increase in the levels of ERK, p38, JNK, and AKT phosphorylation	[141]

Table 3: Continued.

Flavonoid	Experimental Model	Treatment	Mechanism of action or main results	Author/ year
Apigenin	***In vivo*** Model of bleomycin-induced lung fibrosis in Wistar rats	10, 15, and 20 mg/kg/ day	(i)Prevention of pulmonary fibrosis (ii)Prevention of bleomycin-induced inflammation by decreasing TNF-α and TGF-β1 levels (iii)Attenuation of reduced SOD activity (iv)Inhibition of leukocyte infiltration	[128]
3′,4′ -dihydroxyflavone	***In vitro*** Murine BV2 microglial cells	0-10 μM	(i)Inhibition of the production of NO and PGE2 chemokines (ii)Inhibition of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (iii)Inhibition of MAPK phosphorylation (iv)Inhibition of the NF-κB pathway	[129]
	In vivo Male C57BL/6 mice	5 Mg/kg	(i)Inhibition of LPS-mediated iNOS and COX-2 expression (ii)Reduces LPS-induced neuroinflammation in the mouse brain	
Baicalin	***In vivo*** Rotenone-induced brain injury in rats Sprague-Dawley (SD) rats	200 mg/kg	(i)Mitigation of motor deficit (ii)Attenuation of brain injury (iii)Suppression of the production of TNF-α and IL-6 (iv)Modulation of activation of astrocytes and microglia (v)Blockage of NF-κB activation (vi)Inhibition of the MAPK pathway	[142]
Scutellarin	***In vitro*** (LPS)-induced cognitive deficit in male albino Wistar rats	5, 25, or 50 mg/kg/day	(i)Dose-dependently improved spatial deficit and recognition memory (ii)Decreased expression of NF-κB, TNF-α, and IL-6	[130]
Lucenin-2 Vicenin-2	***In vitro*** Murine monocyte/macrophage cell line	0, 1, 10, and 100 mg/mL	(i)Inhibition of NO production and reduction of iNOS and COX-2 expression	[133]
Vicenin-2	***In vitro*** LPS stimulated inflammatory activities in PMA-differentiated THP-1 cells and human primary mononuclear cells	1.6-160 nM	(i)Stimulated IL-18 decrease (ii)Inhibition of the NF-κB pathway—increase in P50 transcriptional subunit homodimerization	[135]
Nobiletin	***In vivo*** AP induced with L-arginine in male C57BL/6 mice	50 mg/kg	(i)Reduction in plasma amylase levels (ii)Reduction in myeloperoxidase activity in the pancreas (iii)Inhibition of inflammatory factors (iv)Inhibition of phosphorylated p38 and AKT	[136]
Tangeretin	***In vitro*** LPS-stimulated dendritic cells	5, 10, or 20 μM	(i)Inhibition of TNF-α, IL-12, and IL-23 expression (ii)Inhibition of the NF-κB and MAPK pathways (iii)Activation of myeloperoxidase activity (iv)Inhibition of Th1 and Th17 cell differentiation (v)Inhibition of T-bet and RORγt expression (vi)Inhibition of IFN-γ, IL-12, and IL-17	[137]
	In vivo Colitis TNBS-induced in male mice C57BL/6	10 or 20 mg/kg	(i)Tangeretin may attenuate colitis by inhibiting IL-12 and TNF-α expression	[137]
5-O-methyl-scutellarein	***In vitro*** LPS stimulations in murine macrophages RAW 264.7 cells	2, 10, and 50 μg/mL	(i)Dose-dependent inhibition of total cell recruitment (ii)Inhibition of neutrophil infiltration (iii)Reduction in the levels of TNF-α, IL-1β, and IL-10	[138]
	In vivo Lipopolysaccharide (LPS) -induced peritonitis model in mice Swiss Webster	4 and 20 mg/kg	(i)Decreased leukocyte migration to the peritoneal cavity and a reduction in the concentrations of pro-inflammatory cytokines (TNFα and IL-1β)	
Diosmetin	***In vivo*** Colitis TNBS-induced		(i)Showed protective effect by suppression of TNF-α, IL-6 and NF-κB	[132]

Table 3: Continued.

Flavonoid	Experimental Model	Treatment	Mechanism of action or main results	Author/ year
		50, 100, and 200 mg/kg	(ii)Elevation of SOD and CAT levels in the colonic mucosa and depletion of MDA which further confirms the antioxidant activity of diosmetin in ulcerative colitis	

Table 4: Antibacterial actions of flavonoids.

Flavonoid	Experimental Model	MIC	Mechanism of action or main results	**Author/ year**
3′,3′,4-trihydroxyflavone	***In vitro*** Pathogens: *E. faecalis, S. aureus*, *E. coli*, *E. aerogenes*, *S. epidermidis*, *K. pneumoniae S. haemolyticus*, and *P. aeruginosa*	32 μg/mL (*E. faecalis* and *S. aureus*) 64 and 128 μg/mL (*K. pneumoniae*, *E. aerogenes*, and *E. coli*)	(i)Killing bacteria from diabetic wounds	[153]
3′,4′,7-trihydroxyflavone	***In vitro*** Pathogens: *E. coli*, *E. aerogenes*, *K. pneumoniae*, *P. stuartii*, and *P. aeruginosa*	MIC values ranging from 4 to 128 μg/mL	(i)Antibacterial and antibiotic sensitizing activity against MDR gram-negative enteric bacteria Substrates for efflux pump	[166]
Isoquercitrin	***In vitro*** Pathogen: *E. coli*	MIC 4.64 μg/mL MBC 18.56 μg/mL	Membrane alterations, a rapid increase in DNA fragmentation, and caspase activation	[167]
Baicalin	***In vitro*** Broth-microdilution method	Sub-MIC 256 μg/mL	(i)Anti-QS activity against *P. aeruginosa* (ii)Marked reduction in inflammation indicated by reduced accumulation of cellular infiltration in peritoneal tissue Significant decrease in IL-4 in the peritoneal flushing fluid Significant increase in INF-γ production	[160]
	In vivo Mouse intraperitoneal foreign-body biofilm infection model	100 mg/kg subcutaneous		
Rutin	***In vitro*** AI-2 bioluminescence assay APEC-O78 strain (CVCC141)	12.5, 25 and 50 μg/mL	(i)Inhibition of QS gene expression in *P. aeruginosa* (ii)Decreased production of the signaling molecule AHL by *P. aeruginosa* (iii)Reduction in CFU count of *P. aeruginosa*	[162]

The methanolic extract of *B. purpurascens* leaves, a medicinal herb containing diverse flavonoids (bergenin, catechin, naringenin, and myricetin), is used to treat wounds and bacterial infections. The extract exhibited strong antibacterial properties, particularly against *S. aureus* and *Streptococcus* spp. These results corroborate those of previous studies demonstrating the inhibition of growth of different bacteria by *Bergenia* spp. [156, 157] and that silver-plated nanoparticles from *Bergenia cilita* crude extract had antimicrobial activity against several bacterial strains. Moreover, *B. purpurascens* extract reduced the mortality caused by infection of mice with *S. aureus in vivo* [158, 159].

Microorganisms' growth and survival are associated with biofilm formation. Quorum sensing (QS) is an intercellular signaling or cell-cell communication process that is necessary for coordinating biofilm formation. The QS system is related to opportunism and resistance mechanisms developed by many bacterial strains. Blocking QS in bacteria is a far-reaching strategy for attenuating their virulence and making them more susceptible to elimination by the host immune system at lower doses of antibiotics. The modulatory effect of kaempferol-loaded chitosan nanoparticles on autoinducer (AI)-mediated anti-QS activity by *Chromobacterium violaceum* CV026 was used to evaluate the effect of this flavonol. Changes in bacterial cell density led to AI production *via* signaling molecules involved in communication among bacteria, composing QS, and managing growth. The results of anti-QS evaluation using the disk diffusion method showed that kaempferol produces a remarkable anti-QS effect; thus, its use against resistant bacteria is promising [158, 159].

Baicalin, a flavonol extracted from the Chinese medicinal plant *Scutellaria baicalensis*, has also demonstrated *in vitro* and *in vivo* anti-QS activities [160]. Baicalin concentrations below the MIC (sub-MIC) inhibited Pseudomonas biofilm formation, which increased the bactericidal effects of various conventional antibiotics. MIC and broth microdilution methods were used to evaluate the MIC and MBC of baicalin and antibiotics, biofilm inhibition assay, biofilm dispersion evaluation, and combined antibiotic

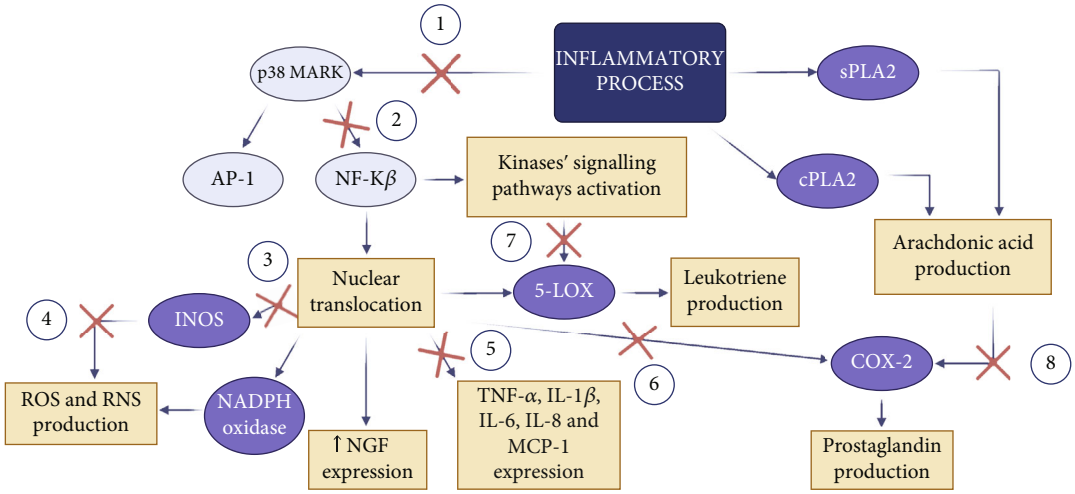

Figure 6: Summary of the effects of flavonols and flavones. The figure presents a range of signaling pathways of the inflammatory process and the stages where the compounds block the signaling cascade. The number represents the pathways inhibited by different compounds. For example, pathway 1 is blocked by the following compounds: quercetin, luteolin, 3′,4′-dihydroxyflavone, baicalein, nobiletin, and tangeretin. Scutellarin, vicenin-2, apigenin, 5-O-methyl-scutellarin, and the substances are able to block pathway 1 and inhibit pathway 2. Only luteolin and lucenin-2 inhibit pathway 3. Furthermore, pathway 4 can only be inhibited by luteolin. Most of the compounds previously mentioned in this review block pathway 5, except nobiletin, tangeretin, vicenin-2, and lucenin-2. Furthermore, luteolin, scutellarin, vicenin-2, and lucenin-2 inhibited pathway 6. Only isoquercetin blocks pathway 7, and luteolin blocks pathway 8.

use. Treatment with different concentrations of baicalin inhibited *P. aeruginosa* biofilm formation in a dose-dependent manner. The lower number of viable cells and lower biomass indicated a synergistic effect compared to treatment with flavonol alone and antibiotics [161], demonstrating that baicalin co-treatment increased the antimicrobial activities of three different antibiotics.

Treatment with sub-MIC of baicalein reduced QS. It decreased most virulence factors, corroborating previously reported data and demonstrating that baicalin exerts anti-QS activity against *P. aeruginosa* PAO1 by inhibiting the QS gene expression and production of signaling molecules such as acyl-homoserine lactone (AHL). Treatment with a sub-MIC of baicalin also considerably increased the survival rate of *C. elegans*. Moreover, baicalin inhibited the motility and production of the QS molecule pyocyanin and significantly reduced the number of colony-forming units (CFUs) of *P. aeruginosa* in implants [160]. Previous studies have also demonstrated reduced bacterial growth in implants treated with baicalin and antibiotics, particularly levofloxacin, ampicillin/clavulanic acid, and ceftazidime [162]). *In vivo* experiments have shown a higher survival rate of worms in the baicalin-treated group, which indicates that treatment with baicalin reduced the pathogenicity of *P. aeruginosa* in *C. elegans*. In addition, baicalin treatment increased the clearance of *P. aeruginosa* from the implants of infected animals in a peritoneal implant infection model.

Quercetin, both free and chitosan-associated, and baicalein have shown a potential inhibitory effect on biofilm formation, QS formation, and cytotoxicity in free mammalian cells treated with chitosan-based nanocapsules [163]. A transformed *E. coli* Top 10 biosensor strain showed QS formation. These results are in line with the data of a previous study [164] that suggested that these phytochemicals may inhibit biofilm formation [163]. Indeed, baicalin inhibited *P. aeruginosa* PAO1 biofilm formation, whereas quercetin showed the highest anti-QS effect in PAO1 strain [165].

The flavonol rutin inhibited QS, biofilm formation, and virulence genes in pathogenic *E. coli* APEC-O78 *in vitro*. Bioluminescence assay demonstrated that AI-2 secretion and activity were significantly decreased after rutin treatment, resulting in marked inhibition of biofilm formation. The expression of APEC virulence genes (csgA, csgB, Flic, FyuA, IucD, LsrB, LsrK, Rpos, H-NS, Luxs, and Pf) decreased after treatment with rutin. These results highlight the role of rutin in controlling *E. coli* pathogenicity. Furthermore, flavonols reduced cellular damage in type II pneumocytes infected with pathogenic *E. coli* by interfering with the QS, thereby inducing a decrease in AI-2 production, biofilm formation, and virulence gene expression [162].

The efflux pump proteins encoded by genes involved in bacterial cellular functions may cause antibiotic resistance. Crude extracts and 3′,4′,7-trihydroxyflavone compound extracted from *Myristica fragrans* Houtt. (MSF) have shown *in vitro* antibacterial and antibiotic sensitizing activity against *E. coli* AG102, which is a multidrug-resistant (MDR) gram-negative enteric bacterium, and against *P. stuartii* strains. This study observed a synergistic effect of these substances with a PAβN efflux pump inhibitor. The antibacterial potential was improved by 73.37% with crude MFS against the tested MDR, whereas it was improved by 100% with 3′,4′,7-trihydroxyflavone. Therefore, both substances are considered substrates for efflux pumps because they have intracellular targets [166]. There was a synergistic action between the extracts and at least one of the six antibiotics tested against 50% of MDR bacterial strains. The compound 3′,4′,7-trihydroxyflavone improved the activity of 70% of the antibiotics tested. These results suggest that this

compound may be considered as a potential efflux pump inhibitor because of its antibacterial effect and antibiotic-modifying activity [166].

The effects of the hydroalcoholic extract of *Glycyrrhiza glabra* and its flavonoids were evaluated in an *in vivo* model of *P. aeruginosa*-induced pulmonary infection in rats. The study elucidated the mechanism of action, inhibition of biofilm formation, pathogenesis, and efflux activity of one of its pure glycyrrhizic acid components [167]. The findings were validated using molecular docking analysis using ExoS segmentation, which confirmed the potential of the herb *G. glabra* [153]. Isolated *G. glabra* glycosides altered membrane permeability and inhibited efflux activity and biofilm formation by *P. aeruginosa* [166]. These flavonoids isolated from licorice showed inhibitory effects on acute lipopolysaccharide-induced pneumonia [167]. These studies provide evidence of preclinical efficacy of *G. glabra* in *P. aeruginosa* pneumonia.

In addition to interfering with the QS and efflux pump, antimicrobials act by direct killing of microbial cells. Antibacterial activity was evaluated by MIC in DNA fragmentation test, caspase activity, lipid peroxidation, inner and external membrane permeabilization, membrane fluidity disturbance, and membrane damage. Isoquercitrin isolated from the perennial herb *Aster yomena*, generally found in Korea and Japan, inhibited *E. coli* growth or completely destroyed bacterial cells. Sub-MIC concentrations of isoquercitrin led to a rapid increase in DNA fragmentation and caspase activation, which are characteristics of apoptosis. The antibacterial activity of isoquercitrin in *E. coli* is attributed to membrane alterations, most likely caused by the induction of oxidative stress and apoptosis [167]. A double-blind, placebo-controlled clinical trial showed that propolis oral spray could be used to improve and immediately resolve the symptoms of uncomplicated upper respiratory tract bacterial infections, without any pharmacological treatment [168].

The antibacterial actions of flavonoids and flavonols range from QS disturbance and alterations in the efflux pump to direct induction of apoptosis (Table 4). Unfortunately, there are few studies detailing the mechanisms involved, but their microbicidal and synergistic effects with antibiotics should be further explored in the future.

3. Concluding Remarks

Conclusively, flavonoids are bioactive components found in fruits and vegetables being recognized as highly versatile substances with therapeutic applications, representing a vast source of pharmacological potential. Herein, we discuss the pivotal role of some flavonoids against inflammation and bacterial infection, which are responsible for millions of casualties every year and severely affect hospitalized and susceptible individuals. Currently available drugs used to treat of these individuals will probably no longer be effective shortly. Bioactive compounds derived from plants play a critical role in treating or preventing diseases either individually or synergistically with antibiotics. Furthermore, flavonoids play a critical role in modulating oxidative stress and treating inflammatory and infectious diseases, particularly those caused by resistant and opportunistic bacteria, through diverse mechanisms of action. Despite their low bioavailability, a proper delivery system such as nanoformulation may aid in their clinical applications. Nanotechnology offers multiple benefits in treating chronic human diseases by site-specific and target-oriented delivery of precise medicines, improving the efficacy of drugs and natural products [169, 170]. For instance, phytochemical cyclodextrin complexes have the potential for transformation into drug delivery systems [171], and nanotechnology-based drug delivery for therapeutics are bioactive compounds from plant sources [172]. Besides, plant phenolic compounds have potential use as a natural feed additive [173], enhancing antioxidant status, immune function, and growth performance of farm animals [174]. We focused on the reported mechanisms and intracellular targets of flavonols and flavones (Figure 6) to boost research on discovering bioactive compounds and their mechanisms of action, anticipating that they can be used as standard treatment for inflammatory and infectious diseases in the future.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Adriana R. Silva and Cassiano F. Gonçalves-de-Albuquerque contributed equally to this work.

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Review Article

Diosgenin: An Updated Pharmacological Review and Therapeutic Perspectives

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Plants including *Rhizoma polgonati*, *Smilax china*, and *Trigonella foenum-graecum* contain a lot of diosgenin, a steroidal sapogenin. This bioactive phytochemical has shown high potential and interest in the treatment of various disorders such as cancer, diabetes, arthritis, asthma, and cardiovascular disease, in addition to being an important starting material for the preparation of several steroidal drugs in the pharmaceutical industry. This review aims to provide an overview of the in vitro, in vivo, and clinical studies reporting the diosgenin's pharmacological effects and to discuss the safety issues. Preclinical studies have shown promising effects on cancer, neuroprotection, atherosclerosis, asthma, bone health, and other pathologies. Clinical investigations have demonstrated diosgenin's nontoxic nature and promising benefits on cognitive function and menopause. However, further well-designed clinical trials are needed to address the other effects seen in preclinical studies, as well as a better knowledge of the diosgenin's safety profile.

1. Introduction

Currently, innovative approaches are being adopted to combat the onset and progression of human health problems [1–3]. One strategy involves using compounds derived from edible dietary plants, seeds and traditional medicinal herbs that have an impact on not only treating but also preventing cancer, either alone or along with existing treatment approaches [4–6]. Natural medicines which are derived from medicinal herbs or different vegetables or plants and fruits with promising health benefits are proved to be effective in treating and also preventing a wide range of human ailments, including obesity, neurological disorders, cancer, metabolic syndromes, cardiovascular disease, and diabetes [7–10]. A recent review by Newman and Cragg [11] reported the various natural products as a source of safe drugs for the treatment of various ailments in humans [11]. Over the decades, natural compounds derived from Chinese herbs have been used to a large extent in traditional practices of medicine. There are natural compounds that can be taken from plants, such as the anticancer medicine paclitaxel from *Taxus brevifolia* quercetin found in numerous vegetables and fruits [12, 13] and the antimalarial medication artemisinin from *Artemisia apiacea*, which have a wide spectrum of pharmacological effects [14].

Sapogenins are a group of chemicals that are found in a variety of natural products in glycoside form and enhance overall health. The most effective bioactive chemicals derived from natural product sources are steroidal sapogenins (otherwise known as spirostans) [15, 16]. The majority of steroidal sapogenins show pharmacological action in vitro and in preclinical animal models [15, 17]. Sapogenins have been the subject of several clinical investigations, which are either finished or ongoing [18, 19]. Diosgenin is a steroidal sapogenin that can be found in the *Rhamnaceae*, *Liliaceae*, *Scrophulariaceae*, *Dioscoreaceae*, *Amaryllidaceae*, *Solanaceae*, *Leguminosae*, and *Agavaceae* families [20–23]. It is plentiful in *Rhizoma polgonati*, *Smilax china, Dioscorea villosa*, *Trigonella foenum-graecum*, and Dioscorea rhizome, among other plants with medicinal values. In the pharmaceutical sector, diosgenin is considered to be a fundamental material for starting the steroidal medicines to synthesize. One of the leading causes of death in the whole world is considered to be chronic diseases, and despite recent advancements in the procedures of treatment, synthetic medications still exhibit grating side effects along with chemoresistance, restricting the usage of these medications.

Phytochemicals have gained popularity due to their low risk of negative effects [8, 24]. Diosgenin is a phytochemical that has acquired prominence due to its usefulness in the treatment of lethal diseases such as nervous system disorders, diabetes, cancer, arthritis, asthma, cardiovascular disease, and others [25]. Scientists have used it in the treatment of inflammation, malignancies, hyperlipidemia, and infections as it has a wide spectrum of therapeutic attributes and pharmacological actions [26]. Diosgenin has been investigated extensively for the management and treatment of cancer [17], skin illnesses [27], cardiovascular illnesses [28], diabetes mellitus [29], atherosclerosis [30], and osteoporosis [31].

Diosgenin has been largely talked of and written about for its huge potential in pharmacological studies, as well as the intriguing rudimentary means of action, validating and expanding the comprehensive knowledge gained through its conventional use. Several mechanistic and preclinical studies have been conducted in this context, primarily during the last two decades, to better apprehend the true advantages and significance of diosgenin against manifold illnesses [32, 33]. Overall, the findings of multiple investigations suggest that diosgenin could be used as a novel multi-target-based therapeutic or chemopreventive drug for a variety of chronic diseases. As a result, it has become an agenda of great interest to develop effective ways to derive diosgenin from various natural resources, furthermore to establish medication dosage forms to grant its administration [34, 35]. Recently, the role of diosgenin in diabetic and cardiac diseases has been reviewed, but a holistic review of various biological activities based on preclinical and clinical studies is still missing in the literature to the best of our literature review [36, 37]. The fundamental objective of this review is to summarize the in-depth pharmacological activity moving from preclinical to clinical evidence and safety issues, to upcoming strategies to overcome present limitations.

2. Review Methodology

To conduct this comprehensive study, the most relevant studies on the pharmacological properties of diosgenin were analyzed. We searched for scientific publications published in journals electronic databases such as PubMed/MEDLINE, DOAJ, Scopus, Web of Science, and SciFinder, using the next MeSH terms: "Diosgenin/analogs & derivatives," "Diosgenin/pharmacology," "Diosgenin/therapeutic use," "Humans," Anti-Asthmatic Agents/pharmacology" "Chronic Disease/prevention & control," "Cardiovascular Diseases/drug therapy," "Inflammation/drug therapy," "Neoplasms/drug therapy," "Nervous System Diseases/drug therapy" "Saponins/pharmacology," "Saponins/therapeutic use," "Saponins/toxicity," "steroids," and "Signal Transduction/drug effects." Studies that explained the molecular mechanisms, signaling pathways, molecular targets, and well-defined doses of pharmacological experiments were included. Duplicates, experimental pharmacological studies involving other test substances, and homeopathic preparations were excluded. The most relevant information was summarized in tables and images. The taxonomy of plants has been validated according to The Plant List [38, 39] and the chemical formulas verified with the PubChem database [40].

3. Sources

The compound diosgenin was discovered by Fuji and Matsukawa in 1936 [41], and its application in the synthesis of cortisone and other drugs was reported by Marker and coworkers [42]. The *Dioscorea* genus is the main source of diosgenin, and ~137 species of this genus contain diosgenin

Table 1: Botanical sources of diosgenin.

SN	Botanical name	Family	References
1	*Trigonella foenum-graecum*	Fabaceae	Arya and Kumar [49]
2	*Costus speciosus*	Costaceae	Selim and Al Jaouni [50]
3	*Tribulus terrestris* L	Zygophyllaceae	Wang et al., [51]
4	*Smilax china* L.	Smilacaceae	Yin et al. [52]
5	*Rhizoma polgonation*	Asparagaceae	Chen et al. [53]
6	*Helicteres isora* L.	Malvaceae	Deshpande and Bhalsing [21]
7	*Paris polyphylla*	Melanthiaceae	Gupta et al. [54]

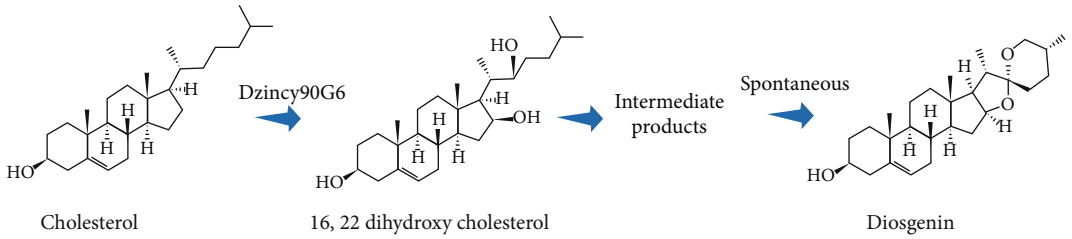

Figure 1: The biosynthetic pathways of diosgenin. *Dioscorea zingiberensis*, for diosgenin biosynthesis uses two P450 genes, DzinCYP94D144 and DzinCYP90G6, the orthologs of PpCYP94D108 and PpCYP90G4, which, from cholesterol, catalyze the formation of diosgenin.

[26] including *Dioscorea nipponica* [43], *Dioscorea zingiberensis* [44], *Dioscorea composita* [45], and *Dioscorea deltoidea* [46, 47]. It is also isolated from other botanicals including *Trigonella foenum-graecum*, *Costus speciosus*, *Tribulus terrestris* L., *Rhizoma polgonati*, and *Paris polyphylla* (Table 1). The compound diosgenin is mostly produced from the hydrolysis process of steroidal saponins in the presence of strong acid, base, or enzyme catalyst, while microbial transformation technics are gaining much attention due to their highly specific nature, low cost, and environmentally friendly conditions [48].

4. Biosynthesis

Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) methods were used for a better yield of diosgenin by using different solvent systems including acetone, ethanol, hexane, and petroleum ether with different concentrations (40, 60, 80, and 100%) and treatment time (MAE: 1.5, 3.0, 4.5, and 6.0 min; UAE: 30, 40, 50, and 60 min). The result of this study indicates that different parameters such as solvent type, concentration, treatment time, and extraction method have a significant impact on diosgenin extraction (yield). The UAE method (21.48%, 40.37 mg/100 g) showed better yield and diosgenin content compared to the MAE method (7.38%, 35.50 mg/100 g) at 80% ethanol concentration at 6 and 60 min, respectively [49]. In the industry of pharmaceutical sector, most hormonal medicines have diosgenin as their precursor. This makes diosgenin a classic precursor which is generated mostly by *Dioscorea* species. The mechanisms underlying the emergence and evolution or progression of biosynthesis of diosgenin in plants are unknown. The geneses of the diosgenin biosynthesis pathway, as well as its evolution, in yam, were validated in recent experimental research. The authors used a variety of diosgenin biosynthesis routes in this study [55] (Figure 1).

Metabolic engineering has also been used to produce diosgenin from yeast. Because cholesterol biosynthesis is more efficient in animal cells than in plant cells, researchers have created a strain with yeast chassis that produces cholesterol by integrating animal genes [56]. As a common precursor for triterpenoid biosynthesis, 2,3-oxidosqualene could be produced in significant amounts from it; *Saccharomyces cerevisiae* strain BY-T3 which was previously modified was chosen as the starting strain [57]. First, Cheng and coauthors discovered that positive selection-driven P450 gene neofunctionalization and duplication were significant in the diosgenin biosynthesis pathway formation. They discovered that CpG islands, which developed to maintain the balance of carbon flux between the production of diosgenin and starch and modulate or control gene expression in the diosgenin pathway, were responsible for diosgenin enrichment in the yam lineage. Finally, we heterologously produced diosgenin to 10 mg/L in yeast that was genetically modified by combining genes from plants, mammals, and yeast [55].

5. Pharmacological Properties of Diosgenin: Underlying Molecular Mechanisms and Signaling Pathways

5.1. Neuroprotective. The oxidative stress caused by the reactive oxygen stress leads to various ailments such as neurological disorders, cardiovascular diseases, and cancer [58–60]. Health benefits of diosgenin administration are most commonly determined as a neuroprotective agent in terms of

Table 2: Pharmacological studies regarding diosgenin's neuroprotective effect with mechanism of action.

Tested compounds	Model	Potential mechanisms	References
Diosgenin	C6 rat glioma cells in vitro	↓ the dosage regimen of TMZ, ↑ MMP-2, ↑ apoptosis	Rajesh et al. [82]
Diosgenin	5XFAD mice in vivo	↓amyloid plaques, ↓neurofibrillary tangles in the cerebral cortex and hippocampus, ↑$1,25D_3$-MARRS	Tohda et al. [83]
Diosgenin	ddY mice in vivo	Memory enhancement effects mediated by $1,25D_3$-MARRS-triggered axonal growth, ↑ $1,25D_3$-MARRS	Tohda et al. [84]
Diosgenin	Transgenic 2576 mice in vivo	↓AChE, ↓Bax/Bcl-2, ↓amyloid plaques production in the granule cells, ↑ NGF, ↑ SOD, p^{75} (NTR)	Koh et al. [85]
Diosgenin	Senescent mice induced via D-galactose in vivo	↑learning and memory ability, ↑ SOD, ↑ GSH-Px, ↓ MDA level, ↑ endogenous antioxidant enzymatic activities	Chiu et al. [61]
Diosgenin-rich extract	Senescent mice induced via D-galactose in vivo	↑learning and memory ability, ↑ SOD, ↑ GSH-Px, ↓ MDA level, ↑ endogenous antioxidant enzymatic activities	Chiu et al. [86]
Diosgenin	Sprague-Dawley rats in vivo	↓ neuronal death rate, ↓pro-inflammatory cytokines, ↑impaired neurological functions at 100 and 200 mg/kg, ↑ IkBα, ↓ p65, ↓NF-κB	Zhang et al. [87]
Diosgenin + curcumin (bivalent)	MC65 neuroblastoma cells in vitro	Antioxidant, anti-oligomerization ↓ amyloid-β oligomer formation	Chojnacki et al. [88]
Diosgenin	Sprague-Dawley rats with lipopolysaccharide in vivo	↓TLR/NF-κB ↓ TLR2, ↓TLR4, ↓NF-κB	Li et al. [89]
Diosgenin carbamate derivatives	D-galactose aging mice in vivo	Anti-inflammatory, anti-oxidant, β- amyloid, ↓ NO ↓ IL-1β, IL-6, TNF-α	Yang et al. [90]
Arginyl–diosgenin	Neuroinflammation model using BV2 cells induced by LPS C57BL/6 mice in vitro/in vivo	↓activation of microglia, microphages, ↓$CD4^+$ T cell proliferation, ↓Th1/Th17 cell differentiation, ↓ NO, ↓ iNOS, ↓ COX-2, ↓PGD2, ↓IL-6, ↓IL-1β, ↓ TNF-α	Cai et al. [65]
Diosgenin	Primary murine microglial cell line BV-2 in vitro	↓pro-inflammatory M1 markers via activation of microglia and without affecting M2 makers, ↓IκB-α, ↓ERK, ↓ MAPK, ↓p38	Wang et al. [51]
Diosgenin	SH-SY5Y cell line H9c2 cell line in vitro	Protective effects against SH-SY5Y cells, ↓angiogenesis at high concentration, ↓apoptosis	Cai et al. [91]
Diosgenin	Trimethyltin-injected transgenic 2576 mice in vivo	Neuroprotective effects against different brain damages via NGF biosynthesis stimulation, ↓ AChE, ↓ Bax/Bcl-2, ↑ NGF, ↑ SOD	Koh et al. [85]

Symbols: ↑ = increased; ↓ = decreased.

cognitive effects [61–63], neuroinflammation [51, 64–67], multiple sclerosis [68, 69], spinal cord injury [70, 71], stroke and thrombosis [72], and neuropathic pain [43, 73–75]. Few recent investigations based on diosgenin and related aspects have been studied through in silico, in vitro, and in vivo approaches and discussed here. Regarding this, a new structure of the neuronal network has been developed to calculate the solubility of the diosgenin compound. The ordinary neural network modeling was used to improve the calculation accuracy and estimate the solubility of diosgenin in the n-alkanols with more carbon atoms due to its rational design concept [76].

In another study, diosgenin was evaluated against cerebral ischemia-reperfusion injury through in silico (proteome dynamic approach), in vitro, and in vivo methods. The authors investigated 5043 regulatory proteins from the brain samples and different signaling pathways [77]. Screening of small molecules for targeting the fungal virulence factors without any effect on viability was conducted by Aaron and the group [78]. They demonstrated that *Cryptococcus neoformans* (*Cn*) was prohibited from crossing the blood-brain barrier via suppressing the proteolytic activity of Mpr 1 with maintaining *Cn* viability. In another word, out of 240 compounds, diosgenin with two other compounds significantly inhibited Mpr 1 proteolytic activity (IC_{50}: <10 μM) without any cell toxicity and blocked *Cn* crossing the BBB [78].

Cheng et al. evaluated the antiapoptotic effects of diosgenin in D-galactose-induced ageing brain (cerebral cortical apoptosis) [79]. A total of 36 male (12-week-old) Wistar rats were recruited and divided into four groups (control: 1 mg/kg/day of saline, i.p.; DD0: 150 mg/kg/day of D-galactose, i.

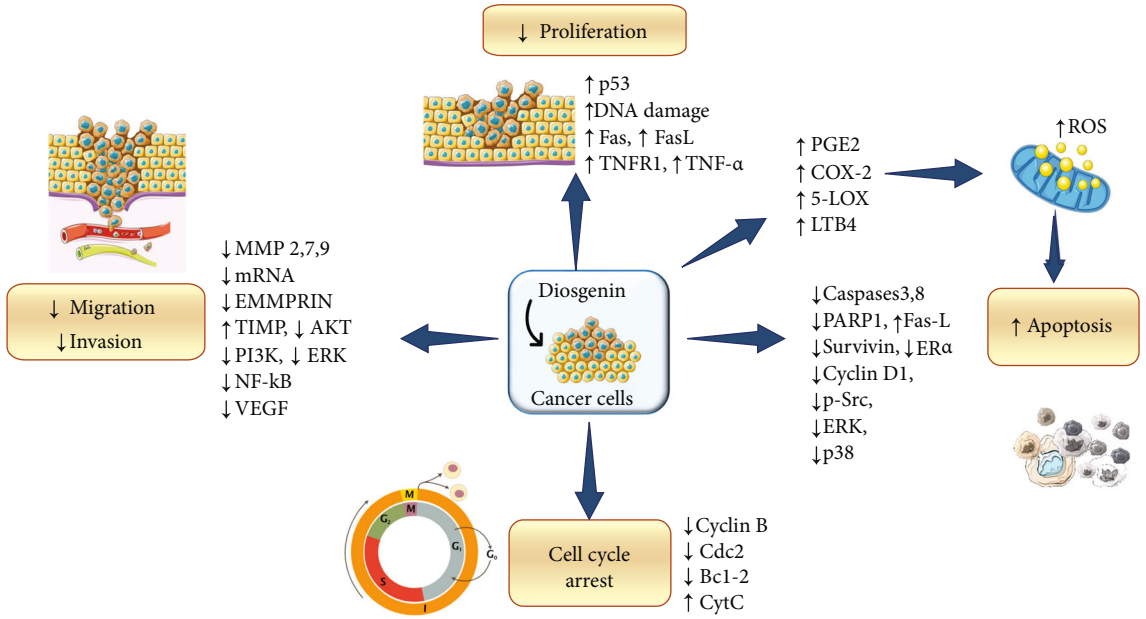

Figure 2: Diagram with cellular targets and molecular mechanisms involved in anticancer effect of diosgenin. Abbreviations and symbols: ↑: increased; ↓: decreased; MMPs: matrix metalloproteinases; abbreviated mRNA: messenger RNA; TIMP: metallopeptidase inhibitor 1; AKT: serine/threonine kinase 1; PI3K: phosphatidylinositol-3-kinase; ERK: extracellular signal-regulated kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; VEGF: vascular endothelial growth factor; DNA: deoxyribonucleic acid; TNFR1: tumor necrosis factor receptor 1; TNFα: tumor necrosis factor; PGE2: prostaglandin E_2; COX: cyclooxygenase; LOX: 5-lipoxygenase; LTB4: leukotriene B4; PARP-1: poly(ADP-ribose) polymerase 1; ERα: estrogen receptor alpha; Src: proto-oncogene tyrosine-protein kinase; Cyt C: cytochrome C.

p.; DD10: D-galactose +10 of diosgenin; and DD50: D-galactose+50 mg/kg/day of diosgenin orally) and treated up to 8 weeks. The overall results of this study showed the suppressing (D-galactose-induced neuronal Fas-dependent and mitochondria-dependent apoptotic pathways) and enhancing (Bcl-2 family-associated prosurvival and IGF-1-PI3K-AKT survival pathways) effects of diosgenin, which may trigger neuroprotective effects against D-galactose-induced ageing brain [79].

The neuroprotective effects of diosgenin in the diabetic mice model were also evaluated by Leng and the group [80]. All mice (male C57) were fed for up to 8 weeks (high fat diet) and intraperitoneally injected with streptozotocin (dose: 100 mg/kg for 2 days). Eligible mice were dived into four groups including control ($n = 6$), diabetic group ($n = 6$), low-dose diosgenin group ($n = 6$, 50 mg/kg), and high-dose diosgenin group ($n = 6$, 100 mg/kg). The main outcome of this study indicates that diosgenin significantly reduced the level of blood glucose and increased the body weight of diabetic mice. Diosgenin attenuated the level of MDA (in dose-dependent manner) but increased the activity of antioxidant enzymes (superoxide dismutase (SOD) and glutathione peroxidase (GPx)) and expression of signaling pathways (nuclear factor-erythroid factor 2-related factor (Nrf2), heme oxygenase (HO), and NAD(P)H dehydrogenase [quinone] (NQO)-1) in diabetic mice. These signaling pathways were involved in its neuroprotective activities [80].

Oyelaja-Akinsipo et al. investigated the neuroprotective and glucose-lowering ability of compound diosgenin in hyperglycemia-mediated cerebral ischemic brain injury using the zebra-fish model of type II diabetes mellitus [81]. Diabetes was developed in an experimental model by using streptozotocin (20 mg/kg b.w.) for 28 days, and two different doses (20 and 40 mg/kg b.w) of diosgenin were used. The results of this study indicated that diosgenin significantly decreased the concentration of glucose from 175.87 to 105.68 mg/d/L and 82.06 mg/d/L in both doses. The compound treatment increased the body weight and growth in diabetic zebra fishes ($p < 0.05$ and $p < 0.001$) and also enhanced the catalytic activity. Its activity also protects the brain from the possibility of hyperglycemic-mediated brain injury and apoptotic brain cell death [81].

The most representative studies on neuroprotective activities of diosgenin have been presented in Table 2.

5.2. Anticancer. Cancer is the most dangerous disease that affects people around the globe, with one in every six deaths due to cancer [92–94]. Different therapeutic tactics, including radiotherapy, chemotherapy, and laser-based therapy, are currently under practice [59, 95, 96]. A series of experiments on cytotoxicity aspects of diosgenin was conducted by several researchers worldwide. In a recent study, cytotoxic effects of standardized extracts, fraction and their compounds (*Trigonella foenum-graecum*) were evaluated against human cancer cells (SKOV-3, HeLa, and MOLT-4 cells).

Table 3: Anticancer properties of diosgenin and their derivatives in different types of cancer.

Model/cancer cell lines/ IC_{50}	Mechanism	Pharmacological action	References
MCF-7 Hs578T $IC_{50} = 0 - 40\ \mu M$	G2/M phase arrest, ↓cyclin B, ↓ Cdc2, ↓ Bcl-2 ↑caspase 3	Regulation of the level of proteins which triggered cell cycle blockade at the G2/M phase	Liao et al. [103]
MDA-MB-231 $IC_{50} = 5\ \mu M$	↓ Cdc42 ↓ Vav2	↓ cancer cell migration ↓actin polymerization ↓Vav2 phosphorylation ↓Cdc42 activation	He et al. [104]
MCF-7 $IC_{50} = 10 - 30\ \mu M$	↓caspases-3,8, ↓PARP1, ↑Fas-L, ↓Survivin, ↓ERα, ↓cyclin D1, ↓c-Myc, ↓p-Src, ↓ ERK½, ↓p38	↓expression of ER-α ↑apoptosis via extrinsic pathway	Chun et al. [105]
MCF-7 MDA-MB-231 $IC_{50} = 1.15 - 5.76\ \mu M$	↑ GATA3, ↑ DNMT3A, ↑ ZFPM2, ↑ E-cadherin, ↑ TET2, ↑ TET3, ↓ TET1, ↓ vimentin, ↓ MMP9	Diosgenin mediated pathways modulate the GATA3 expression at transcription and translation	Aumsuwan et al. [106]
MDA-MB-231 MDA-MB-453 T47D $IC_{50} = 0 - 8\ \mu g/mL$	↓ Bcl-2 ↓ cIAP-1 ↓ McI-1	↑ apoptosis via downregulation of proteins related with inhibition ↑apoptotic process	Kim et al. [107]
MCF-7 MCF-10 MDA-231 $IC_{50} = 20 - 30\ \mu M$	↓ pAKT (Ser473), ↓ AKT kinase activity, ↓ p-GSK3β, ↓ Raf, ↓ p-MEKs 1/2, ↓ p-MEKs 3/6, ↓ MEK-1, ↓ MEK-4, ↑ pElk-1, ↑ p21, ↓ XIAP, ↓ Bcl-2, ↓ Cdk-2, ↓ Survivin, ↓ cyclin D1, ↓ NF-κBp65, ↓ p65, ↑ IκB-α, ↓ pElk-1, ↑ Bax, ↓ NF-κB, ↑ caspase-3	↑ G1 cell cycle arrest apoptosis in MCF-7 and MDA-231 cells while did not cause in MCF-10A cells	Srinivasan et al. [108]
HuCCT1 QBC939 HuH28 SK-ChA1 RBE Mz-ChA-1 $IC_{50} = 0 - 40\ \mu M$	↑Bax/Bcl-2, ↑p21, ↑caspase-3, ↑ pARP-1,↓CyclinB1 ↑Cyt c, ↑GSK3β-PY216 ↓ GSK3β-PS9	G2/M phase arrest ↑apoptosis The compound suppressed cholangiocarcinoma cells and triggered	Mao et al. [109]
HT-29 HCT-116 $IC_{50} = 40\ \mu M$	↑ PGE2, ↑ COX-2, ↑ 5-LOX, ↑ LTB4	↑ apoptosis in both cancer cell lines	Lepage et al. [110]
HCT-116	↑ ROS, ↑ Ca2+, ↑ NO, ↑ iNOS, ↑ DNA damage, ↑ Gna11, ↑ATP6V0C, ↑Ppp2r5e, ↑COX6C ↑mRNA	The compound triggered mitochondrial damage and G2/M cell cycle arrest	Chen et al. [111]
HeLa CaSki	↑ caspases-3, 8, 9	Diosgenin and its glycoside derivatives showed strong anticancer activity with low necrotic activity and selective action	Hernández-Vázquez et al. [112]
HeLa SiHa $IC_{50} = 1.25 - 5.0\ \mu g/mL$	↑ ROS, ↑ Ca^{2+}, DNA damage, ↑ Bid, Bcl-2, ↓ Bcl-xL, ↑ caspases-3, 9, ↑ Bax, ↑ Bak, ↑ p53	↑ apoptosis ↓ cell proliferation, ↑DNA damage in both cell lines via modulation of protein level	Zhao et al. [113]
HeLa	↑ apoptosis, ↑ caspase-3 and -9 activity, ↓ Bcl-2	The compound significantly induced apoptosis in a dose and time-dependent manner	Cai et al. [114]
HeLa $IC_{50} = 0 - 40\ \mu M$	G2/M phase, ↑ apoptosis, ↑ ROS	The compound significantly inhibits cell proliferation, transformed cell morphology, arrests the cell cycle, and regulates apoptosis via death receptor and mitochondrial pathways.	Ma et al. [115]
KYSE510 $IC_{50} = 0.5 - 20\ \mu M$	G1/S arrest, ↑ apoptosis, ↑ cleaved caspase-9, ↑ Bax, ↑ Cyt c, ↑ ROS, ↓ Bcl-2	Peroxiredoxins 1 and 6 play an important role in compound induced apoptosis	Zhiyu et al. [116]

Table 3: Continued.

Model/cancer cell lines/ IC_{50}	Mechanism	Pharmacological action	References
NOZ SGC996 $IC_{50} = 0 - 8\ \mu M$	↓ ROS-mediated PI3K/AKT	↑ apoptosis via inhibition of reactive oxygen species-mediated PI3K/AKT signaling	Song et al. [117]
MGC-803 MKN-45 $IC_{50} = 1.25 - 5.0\ \mu g/mL$	↑ ROS, ↑ Ca^{2+}, ↑ RBM-3, ↑ GALR-2, ↓ CliC-3, ↓ Bcl-2, ↑ Bax, ↑ caspase-3, 9,↑ MAPKs, ↓ CAP-1, ↓ Tribbles-2	Anticancer effects against human gastric cancer via inducing cell apoptosis, DNA damage, etc.	Zhao et al. [118]
SGC-7901 $IC_{50} = 0.65 - 2.6\ \mu g/mL$	↑ Fas, ↑ FasL, ↑ TNFR1, ↑ TNF-α, ↓ Bcl-2, ↑ Bax, ↑ Bak, ↓ bid, ↓ Bcl-xL, ↑ p53 mRNA ↑ caspase-3, 8	Anticancer activity	Hu et al. [119]
HGC-27 MGC-803 SGC-7901 $IC_{50} = 0 - 60\ \mu mol$	↓proliferation ↓ HOTAIR ↓Hox	↓proliferation of gastric cancer cells	Ma et al. [120]
C6 allograft $IC_{50} = 1.25 - 5.0\ \mu g/mL$	↑ROS, ↑Ca^{2+}, ↑MDA, ↑NO, ↑GSSG, ↓GSH, ↓Bcl-2, Bcl-xL, ↑Bak, ↑Bax, ↑caspase-3, 9	Anticancer activity	Lv et al. [121]
HepG2 $IC_{50} = 0 - 100\ \mu M$	↓TAZ ↓β-catenin	↓ cell growth, ↑apoptosis, ↑ apoptosis, ↑G2/M phase arrest	Chen et al. [122]
HepG2 SMMC-7721 $IC_{50} = 0 - 100\ \mu M$	G2/M phase arrest, ↑ DDX3, ↓mRNA, ↓ cyclin D1, ↑p21, ↑E-cadherin, ↓ Notch-1, ↓β-catenin	↓ cell growth ↑apoptosis via upregulation of DDX3	Yu et al. [123]
HepG2 $IC_{50} = 0 - 40\ \mu M$	↑ caspase-3, 8, 9 ↑ Bax, ↓Bcl-2, ↓Bid, ↑ROS, ↑ASK1	↓ cell growth ↑apoptosis in HepG2 cells via Bcl-2 protein-mediated pathways	Kim et al. [124]
Bel-7402 $IC_{50} = 0.25 - 63\ \mu mol/L$	↑ TP53, ↑ Bax, ↓ Bcl-2 ↑ caspase-3	↓cell growth ↑apoptosis via modulation of protein expression	Zhang et al. [125]
HepG2 $IC_{50} = 0 - 20\ \mu M$	↑ apoptosis, G2/M phase arrest, ↓ cyclin B1, ↑ Bax, ↑ Bcl-2	↓cell growth ↑apoptosis via modulation of protein expression	Wang et al. [126]
DU145 $IC_{50} = 0.2 - 100\ \mu g/mL$	↑ LC3-II, ↑ caspase-9, ↓ PI3K, ↓AKT, ↓mTOR, ↑ Beclin-1, ↓ Bcl-2	↓cell growth ↑apoptosis, ↑autophagy due to inhibition of mitochondrial pathways	Nie et al. [127]
DU145 $IC_{50} = 33\ ng/mL$	↓vimentin, ↓ Mdm2, ↓ c-Met, ↓ ERK ↓AKT, ↓mTOR	↑ apoptosis ↓ HGF induced ↑ Mdm2, ↑vimentin ↓phosphorylation of Akt, mTOR	Chang et al. [128]
PC-3 $IC_{50} = 0 - 30\ \mu M$	↓ MMP 2,7,9, ↓mRNA, ↓ EMMPRIN, ↑ TIMP, ↓ AKT, ↓ PI3K, ↓ ERK, ↓c-JNK, ↓NF-κB, ↓VEGF	↓ cancer cell growth ↑apoptosis via modulation of signaling pathways	Chen et al. [129]
PC-3 $IC_{50} = 0 - 100\ \mu M$	G2/M phase arrest, ↓ NEDD4, ↓ p73, ↑ LATS1, ↓ p-AKT, ↓ TAZ	↓cell growth, ↑apoptosis cell cycle arrest	Zhang et al. [130]
PC3 $IC_{50} = 250 - 1000\ \mu M$	↑ [Ca^{2+}]i, ↑ Mn^{2+}	Significant anticancer activity	Sun et al. [131]

The steroid saponins fraction (C) showed strongest cytotoxic activity on cancer cells (IC_{50}: 3.94 (HaCaT), 3.91 (HeLa), 3.97 (SKOV-3), and 7.75 (MOLT-4)). The fraction significantly increased reactive oxygen species production and caspases activity in the cells [97].

A total of 28 diosgenin amino acid ester derivatives (3a-3g and 7a-7g) were designed and synthesized by Ma et al. and evaluated for their cytotoxicity against six human cancer cells including K562, T24, MNK45, HepG2, A549, and MCF-7 [98]. The majority of derivatives displayed cytotoxic potential against these six tumor cells. Out of 28 derivatives, compound 7g exhibited significant cytotoxicity against the K562 cells (IC_{50}: 4.41 μM) compared to diosgenin (IC_{50}: 30.04 μM). Compound 7 also triggered K562 cells apoptosis through mitochondria-related pathways. The cytotoxicity of two different extracts of *Paris polyphylla* rhizomes (ethanol extract and diosgenin rich extract) were evaluated against human breast cancer cells (MCF-7 and MDA-MB-231), cervical cancer cells (HeLa), and Hep-2 cell lines. The diosgenin-rich extract significantly reduced the proliferation of all cancerous cells, and the maximum activity was observed in MCF-7 cells. Diosgenin-rich extract triggered

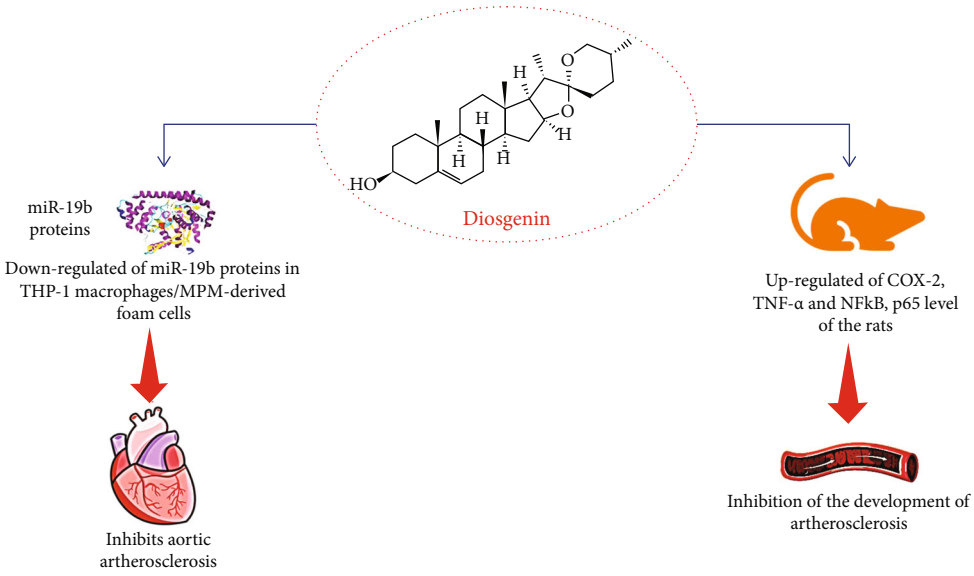

Figure 3: Schematic diagram showing the beneficial effect in atherosclerosis, thus preventing cardiovascular and neurodegenerative diseases. Abbreviations: Cox-2: cyclooxygenase-2; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

upregulation of Bax and downregulation of Bcl-2 and BIRC5 pre-mRNA transcripts of genes [54].

Yin and coworkers developed and synthesized thirty-two new diosgenin derivatives and evaluated their cytotoxic activity against three human cancer cells (A549, MCF-7, and HepG2). Among them, compounds 8, 18, 26, and 30 were more potent compared to diosgenin. Compound 8 showed strong and low cytotoxic activity against HepG2 cells (IC_{50}: 1.9 μM) and low L02 cells (IC_{50}: 18.6 μM), respectively. Additionally, compound 8 induces G_0/G_1 cell cycle arrest and apoptosis in HepG2 cells. A molecular docking study also suggested that p38α-MAPK is a suitable target for compound 8 and fits well its active site [99]. Two new azasteroids were synthesized from diosgenin through the modification in the A and B rings and evaluated for their antiproliferative activity [100].

Diosgenin significantly inhibited the cell viability and motility of breast cancer cells and stimulate apoptosis via suppression of S-phase kinase-associated protein Skp-2 in breast cancer cells [101]. Although NF-κB promotes cancer initiation and development, but some reports demonstrate its role in tumor suppression [102]. Part of this several studies has been conducted to investigate the role of diosgenin, and its derivatives against different types of cancers are presented in Figure 2 and Table 3.

5.3. Antiatherosclerosis. Atherosclerosis is a disease of the middle and large arteries characterized by the formation in the inner tunic and middle deposits of atheromatous plaques, which contain accumulations of LDL-cholesterol, lipophage, and sometimes calcifications on former lesions that prevent normal blood flow through the vessel. It is a disease in which plaque accumulates inside the arteries and can trigger serious problems including heart failure, stroke, or death [132–134]. The pathogenic mechanisms are complex, involving lipid peroxidation, oxidative stress, inflammation, or altered immune response, causing aging and degenerative brain damage [135–137].

The antiatherosclerosis potential of diosgenin and its derivatives were studied by few researchers. In this context, the curative effects of diosgenin on macrophage cholesterol metabolism and its mechanism were investigated by Lv and the group [30]. The diosgenin treatment significantly enhanced the expression of ATP-binding cassette transporter A1 (ABCA1) protein without any effect on liver X receptor α levels. Additionally, diosgenin treatment also inhibits aortic atherosclerosis progression via downregulation of miR-19b proteins in THP-1 macrophages/MPM-derived foam cells (Figure 3). The in vivo study of the diosgenin compound and its impact on Wistar rats treated with an atherogenic diet were performed by Binesh and coworkers. In this study, the atherogenic diet triggered the inflammatory mediators in the heart, liver, and brain via upregulation of COX-2, TNF-α, and NFkBp65 levels of the rats, while diosgenin treatment downregulated the level of these inflammatory markers and inhibit the development of atherosclerosis (Figure 3) [64]. The same research group reported the downregulation of NF-κB expression and polarization of macrophages by diosgenin treatment [138]. In another study, compound dioscin was evaluated for its inhibitory activity against atherosclerosis and postmenopausal atherosclerosis in ovariectomized LDLR-/-mice [139, 140].

5.4. Antiasthmatic. Asthma is a long-term condition which results in inflammation of the lower respiratory tract and affects children and adults. Junchao and coworkers evaluated the molecular mechanism of anti-trachea inflammatory effects produced by diosgenin via interactions with glucocorticoid receptor alpha [141]. They used ovalbumin-induced asthmatic mice and primary tracheal epithelial cells as

Table 4: Different biological activities of diosgenin and their derivatives with the mechanism of action.

Disease	Experimental model	Mechanism	Pharmacological action	References
Cardiac fibrosis	Rat cardiac fibrosis cells in vitro $IC_{50} = 0 - 10\,\mu M$	↓ α-SMA ↓ TGF-β1 ↓ p-Smad3	↓ proliferation of Ang II-induced cardiac fibrosis, ECM synthesis of rat cardiac fibrosis and expression of TGF-beta 1 and Smad3 phosphorylation in cardiac fibrosis cells	Zhou et al., [149]
Pulmonary hypertension	ICR mice in vivo Dose = 0.1 – 10 mg/kg	↓ NF-κB ↓p50/p65 ↓ MAPK/p38 ↓ iNOS	Pretreatment with diosgenin significantly suppresses the LPS-induced NF-κB, MAPK/p38 activation protective effects against acute lung injury or sepsis	Gao et al., [150]
Graves disease	BALB/c mice in vivo Dose = 20 – 100 mg/kg/day	↓ mRNA, ↓IGF-1, ↓NF-κB, ↓ cyclin D1 ↓PCNA	Diosgenin treatment significantly reduces the TT4 level and thyroid size without affecting TRAb in graves' disease mice	Cai et al., [151]
Diabetes	Albino rats in vivo Dose =5-10 mg/kg/day	↓serum glucose, ↓ MDA, ↑GSH, ↑ SOD, ↑ GPx, ↓ protein carbonyl ↑ catalase	↓ blood glucose, ↓LDL cholesterol ↓cardiovascular risk	Kalailingam et al. [28]
	Wistar rats in vivo Dose = 15 – 60 mg/kg/day	↓ serum glucose	Diosgenin significantly reduced the blood glucose, increase the insulin blood level	Saravanan et al. [152]
	Swiss mice in vivo Dose = 1 mg/mL	↓ α-amylase ↓ α-glucosidase	Diosgenin demonstrated significant antidiabetic activity	Ghosh et al., [153]
Osteoporosis	Ovariectomized rats	↓ RANKL ↑ OPG	Diosgenin demonstrated significant antiosteoporotic activity compared to OVX control	Zhang et al. [154]
Arthritis	C57BL/6 mice in vivo	↓ p-JAK2, ↓ p-STAT3, ↓ SDH, ↓COX, ↓ SOD, ↑ Bax	Diosgenin treatment significantly inhibited the apoptosis and upregulated the mitochondrial oxidative stress capacity of chondrocytes in experimental mice with osteoarthritis	Liu et al. [155]
Cardiotoxicity	Male Balb/c mice in vivo Dose = 130 mg/kg/day	↓TBARS, ↓ ROS, ↓ caspase-3, ↓ NF-κB, ↑ cGMP, ↑ cAMP, ↓ PDE5	Cardioprotective Diosgenin displayed antioxidant, anti-apoptotic, cGMP modulation activities	Chen et al. [156]

experimental models. The results demonstrated that diosgenin significantly reduces the secretion of different inflammatory factors including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 via upregulation of glucocorticoid receptors, secretory leukocyte protease inhibitor, glucocorticoid-induced leucine zipper, mitogen-activated protein kinases (MAPK) phosphatase 1, and downregulation of heat shock proteins (HSP70).

5.5. Hepatoprotective. Liver disease can have genetic causes or it can be caused by a variety of factors that affect the liver, such as viruses or alcohol consumption [142–144]. Obesity is also associated with liver disease. Over time, liver damage results in scarring (cirrhosis), which can lead to liver failure, a life-threatening condition [145, 146]. Under this topic, Xie et al. [147] reported on the impact of diosgenin on transforming growth factor (TGF)-β1-induced hepatic stellate cells and its mechanism of action for antifibrotic effects. The results of this study showed that diosgenin significantly inhibited the proliferation of TGF-β1-induced hepatic stellate cells and reduced the expression of collagen I and alpha-smooth muscle action as well as the expression of TGF-β receptors I and II. Additionally, diosgenin also downregulated the expression of TGF-β1-induced phosphorylation of Smad3 in hepatic stellate cells and displayed potential effects to treat liver fibrosis. In another study, Zhang and coworkers described the dioscin and its effect on alcoholic liver fibrosis (in vitro and in vivo). The results demonstrated that dioscin improved the condition of alcoholic liver fibrosis via modulation of toll-like receptor 4/myeloid differentiation primary response 88/NF-κB signaling pathway [148]. Other important studies related to diosgenin and its derivatives have been presented in Table 4 and shown in Figure 4.

6. Clinical Studies

A clinical pilot study was conducted to investigate the efficacy and safety profile of diallyl thiosulfinate associated with nuciferine and diosgenin in the cure of primary and secondary erectile dysfunction. A total of 143 candidates (age 18-39 Y) were selected and treated orally with

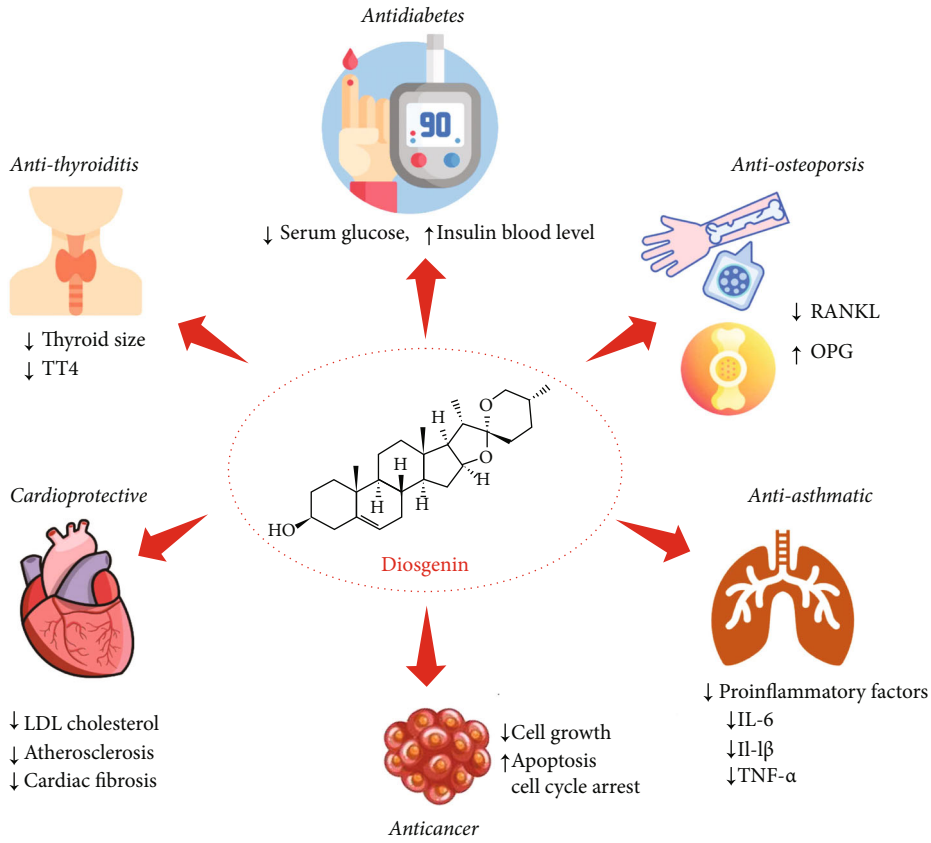

Figure 4: Summarized scheme showing the most representative biological activities of the diosgenin. Abbreviations and symbols: ↑: increase; ↓: decrease; T4: thyroxine; LDL: low-density lipoprotein; OPG: osteoprotegerin; RANKL: receptor activator of nuclear factor kappa beta; IL: interleukin; TNF-α: tumor necrosis factor-alpha.

nuciferine and diosgenin for up to three months (single dose, alternative days). After three months of treatment, each candidate was screened in terms of the international index of erectile-5, premature ejaculation diagnostic tool, and male sexual health questionnaire. A significant enhancement was observed in terms of the international index of erectile-5 (8.7 vs. 14.01; $p < 0.001$) when compared with baseline and follow-up visits. The overall observation of the study indicates that these three compounds are capable to enhance the control of ejaculation in candidates suffering from premature and erectile dysfunction without any side effects [157].

In another study, Tohda et al. evaluated the impact of diosgenin-rich yam extract on synaptic loss and memory dysfunction using a transgenic mouse model of Alzheimer's. A placebo-controlled, randomized, double-blind, cross-over study was performed on 28 healthy volunteers (age: 20–81 years) and randomly treated with yam extract or placebo (12-weeks intake and 6-week washout period). The Japanese version of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) test and the adverse effects were evaluated. The diosgenin-rich yam extract consumption (12 weeks) significantly increased the synaptic fluency or enhance cognitive function without any side effects [158].

The standardized multinutrient supplement including folic acid, selenium, vitamin E, catechins, glycyrrhizin, diosgenin, damiana, and omega-3-fatty acids showed beneficial effects on in vitro fertilization/intracytoplasmic sperm injection in terms of embryo quality [159]. The curative effects of *Dioscorea villosa* extract on menopausal systems, lipids, and sex hormones were evaluated [160].

A placebo-controlled, randomized, double-blind, cross-over study was conducted on 23 healthy women suffering from symptoms of menopause. All candidates were treated with wild yam cream or placebo for up to three months, and no significant adverse effects were observed in both the treatments. Additionally, no changes were also recorded in weight, systolic or diastolic blood pressure, total serum cholesterol, triglyceride, high-density lipoprotein cholesterol, glucose, estradiol, or serum [160].

7. Toxicological Profile: Safety and Side Effects

Only a few articles have acknowledged diosgenin's toxicity, even though there has been substantial research on its part and maneuver in the treatment along with prevention of cancer and further different chronic disorders. A study was published in 2009 on breast cancer cells by Srinivasan and coworkers where it was shown that diosgenin modulates AKT to regulate breast cancer cell survival and that this drug has no effect on normal breast epithelial cells (MCF-10A) except for its selective toxicity to cancer cells [108].

Steroidal saponins of different doses from *D. zingiberensis* were given to mice that were experimental in another in vitro investigation. Diosgenin was the metabolite and the main component of these saponins. There were no harmful effects up to a level of 562.5 mg/kg, according to the findings. However, steroidal saponins, which include diosgenin, displayed deleterious consequences and even death in a dose-dependent manner at dosages of 1125 mg/kg and higher. Interestingly, the steroidal saponins dosage, which is traditional, is 510 mg/kg/day, implying that steroidal saponins, together with diosgenin, have no significant toxicity at this dosage [161]. A recent study also discovered that diosgenin derivatives had antithrombotic properties. In vivo experiments revealed that they appeared to be protective and comparable to aspirin, with a decreased risk of bleeding and less stomach mucosal injury [162]. Furthermore, investigations have shown that diosgenin has a modest inhibitory impact on cytochrome P450 enzymes (CYPs), suggesting diosgenin fused with any other medicine would be safe to consider that they would have no toxicity [163]. These investigations demonstrated that diosgenin and its derivatives are nontoxic and have underlined their utility in the medicaments of chronic disorders including cancer.

8. Therapeutic Perspectives and Limitations

Diosgenin is a natural compound mostly found in *Dioscorea* species and starting material for the commercial synthesis of different steroids including cortisone, pregnenolone, and progesterone. The natural antioxidant compound diosgenin possesses different biological activities such as anticancer [164, 165], antidiabetic [28, 36, 152, 166–169], multiple sclerosis [68, 69], and spinal cord injury [70, 71] and helps in the management of these diseases. Therefore, diosgenin could be functional and helpful in the treatment and prevention of a variety of disorders. The identification of diosgenin-specific targets, on the other hand, is critical for further validating its use in the treatment and elimination of diseases.

The main therapeutic limitations of diosgenin are represented by its low bioavailability; therefore, a special emphasis is being paid to the production of nanoformulations or conjugate complexes to improve the compound's bioavailability and pharmacokinetic features to develop it into a possible medicine. The potential of this chemical, its analogs, or combinations of this molecule with others has previously been demonstrated; however, carrier systems such as nanoparticles must be developed to govern diosgenin to the location where it works, boosting effectiveness and lowering adverse impacts. For example, encapsulated diosgenin PCL [poly(caprolactone)]-pluronic nanoparticles (PCL-F68-D-NPs) were developed by the nanoprecipitation method to improve performance in brain cancer therapy [170]. The developed nanoparticles (PCL-F68-D-NPs) displayed significant cytotoxicity against U87-MG cells compared to free diosgenin. Additionally, developed nanoparticles demonstrated suitable properties in terms of size distribution, stability, morphology, chemical and mechanical properties, encapsulation, and loading efficiency [170].

9. Overall Conclusions

A steroidal saponin, diosgenin, and its chemical and structural variants are useful in the treatment of a great variety of chronic conditions, including cardiovascular disease, several forms of lethal malignancies, nervous system problems, and autoimmune diseases. They have gotten a lot of interest from researchers all across the world. Diosgenin and its derivatives have been shown to have pharmacological benefits against cancer, diabetes, osteoporosis, Alzheimer's disease, and stroke in several investigations. Diosgenin has been shown to act on several molecular targets that are essential players in the occurrence and incidence of many serious disorders. Its multitargeting capability allows it to influence multiple molecular targets and signaling pathways at the same time. Diosgenin has an edge over the most commercial medicinal medicines available today because of this property. Furthermore, investigations demonstrating its nontoxic nature significantly promote the inclusion of this medicine in additional clinical studies or trials in the forthcoming days. Overall, diosgenin demonstrated great promise in the treatment and prevention of a variety of chronic diseases; nevertheless, additional clinical research is needed in the nearing days to come to confirm the preclinical findings and demonstrate the effective and secure usage of these purely natural compounds.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization and design were performed by J.S.-R., P. S., W.C.C., and A.R.; validation, investigation, data curation, and writing were performed by P.S., S.P., T.A.-I., A.R., A.S., M.K., J.S.-R., M.M.A., R.D., Y.T., S.M., S.D.D., T.B.E., and W.C.C.; review and editing were performed by J.S.-R., D. C., P.S., W.C.C., and A.R. and contributed to the final revision and major edits on the manuscript. All the authors read and approved the final manuscript.

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Review Article

Neurobiological Promises of the Bitter Diterpene Lactone Andrographolide

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Andrographolide (ANDRO), a bitter diterpene lactone found in *Andrographis paniculata* (Burm.f.) Nees, possesses several biological effects such as antioxidant, anti-inflammatory, and organo-protective effects. Scientific reports suggest that it also has neuroprotective capacity in various test systems. The purpose of this review was to synthesize the neuropharmacological properties of ANDRO and highlight the molecular mechanisms of action that highlight these activities. A careful search was

done in PubMed and Google Scholar databases using specific keywords. Findings suggest that ANDRO possess neuroprotective, analgesic, and antifatigue effects. Prominent effects were stated on neuro-inflammation, cerebral ischemia, Alzheimer's and Parkinson's diseases, multiple sclerosis, and brain cancer in mice and rats. Furthermore, ANDRO and its derivatives can enhance memory and learning capacity in experimental animals (rats) without causing any toxicity in the brain. Thus, ANDRO may be one of the most promising plant-based psychopharmacological lead compounds for new drug development.

1. Introduction

Neurological disorders are very common and important health issues in elderly people [1, 2]. The inflammation plays a crucial role in several neurodegenerative diseases, including Alzheimer's (AD) disease and Parkinson's (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS). Oxidative stress is an important factor in the pathophysiology of many chronic diseases such as diseases of the central system or cancers [3, 4]. Therefore, much attention has been given to the discovery and development of neurological drugs from various origins [5, 6].

Current evidence suggests that plants are a promising source of phytochemicals for the treatment of various diseases [7–9], and thus, phytochemicals are considered one of the most popular tools for sound health maintenance throughout the world [10–13]. Diterpenes are evident in their many promising biological activities, including neuroprotective capacity [14, 15].

Andrographolide (ANDRO, $C_{20}H_{30}O_5$; Figure 1), a bicyclic diterpene lactone isolated from *Andrographis paniculata* (Burm.f.) Nees (Family: *Acanthaceae*), has shown diverse biological activities [15–18].

ANDRO has neurobiological properties, such as antidepressant [19], anti-Alzheimer [20, 21], anti-Parkinsonism [22], neuroprotective [23], antioxidant [24], anticancer [25], and cognitive improvement [26] effects. It also shows neuroprotective effects by inhibiting proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) production and microglial activation [27, 28]. In addition, ANDRO (0.25–8 mg/kg) is known to exert antifatigue activity in experimental animals [29]. Moreover, solid lipid nanoparticles prepared by ANDRO have been found to improve the transport facility through the BBB in healthy rats [30]. No serious toxic effects in mice were observed with two ANDRO derivatives, 3,19-isopropylidenyl- and 3,19-dipalmitoyl, up to 100 mg/kg [31].

In this sense, the present review is aimed at focusing on ANDRO neurobiological effects.

2. Search Strategy

A search was done in the following databases: PubMed/Medline and Google Scholar using the next MeSH terms: "Diterpenes", "Neuroprotective Agents", "Andrographolide", "Animals", "Central Nervous System/drug effects", "Diterpenes/pharmacology", "Diterpenes/therapeutic use", "Humans", "Neuroprotective Agents/pharmacology", and "Neuroprotective Agents/therapeutic use".

The research was done according to the consensus statement of researchers active in ethnopharmacology and with particular input by the ConSEFS Advisory group [32], and the name of the plant was verified according to the PlantList [33]. No language restrictions were imposed, and manuscripts were evaluated for dose/concentration, administration route, test systems, results, discussion, conclusion, and proposed mechanisms of action.

The following inclusion criteria were considered:

(1) Studies developed *in vitro*, *ex vivo*, or *in vivo*, and humans and their derived tissues and cells

(2) Studies with ANDRO and its derivatives

(3) ANDRO or its derivative joint effects with other chemical compounds

(4) Studies with or without proposing mechanisms of action

After careful search and strict analysis, 38 reports (PubMed: 28; Google Scholar: 10) were included. The most important mechanism of ANDRO' effects on AD, PD, MS, cerebral ischemia, intracerebral haemorrhage, neuropathic pain, brain tumour, and depression is summarized in Table 1 and Figure 2.

3. The Neurobiological Role of Andrographolide: Molecular Mechanisms and Pathways

3.1. Andrographolide and Neurodegenerative Diseases. Neurodegeneration occurs in the CNS and involves the loss of neuronal structure and function, triggered by several factors including CNS inflammation [34, 35]. CNS degeneration involves the progressive chronic loss of neural structure and function, resulting in functional and mental neurological deficiencies [7, 13].

3.1.1. Alzheimer's Disease. Alzheimer's disease (AD) is a chronic neurodegenerative disease, which usually has a slow progression and gradually worsens over time. It is the cause of 60–70% of cases of dementia [36, 37].

Current treatment protocols are not sufficient for effectively preventing AD signs and symptoms [38, 39]. Many natural resources have been discovered in recent decades which can be used as adjuvant therapies in the treatment of AD. [8, 40, 41].

In some recent studies, ANDRO administration (2 and 4 mg/kg i.p.) suppressed the spatial learning and memory function impairment in *Octodon degus* [42], and the proposed mechanisms of ANDRO were as follows: (1) recovery of learning performance and spatial memory, (2) synaptic basal transmission recovery, (3) protection of synaptic

proteins, and (4) lowering of amyloid-beta (Aβ) aggregate maturation and phosphorylated tau protein [42].

In another study, ANDRO (2 mg/kg) was found to improve learning/memory by activating Wnt signaling. Wnt is a signaling pathway that enhances glucose metabolism via gene expression and/or activity enhancement of hexokinase, phosphofructokinase, and AMP-activated protein kinase (AMPK) [20, 43].

ANDRO also reduced the Aβ levels and tau phosphorylation and changed amyloid plaques in AβPPswe/PS-1 double transgenic male mice [26]. Hence, ANDRO recovered synaptic proteins, increases β-catenin levels, reduces active glycogen synthase kinase- (GSK-) 3β levels, increases synaptic transmission, and protects long-term potentiation (LTP) at the same time that suppresses postsynaptic-density-protein 95 (PSD-95), GluA2, GluN2B, and Shank decrease in the hippocampus [26]. Besides, the activation of nuclear factor erythroid 2-related factor 2- (Nrf2-) mediated heme oxygenase (HO)-1 expression, ANDRO (1–10 μM) also inhibited Aβ42-overexpression in microglial BV-2 cells [44].

In human microglia cells, ANDRO also inhibited nuclear factor- (NF-) κB translocation via IκB phosphorylation modulation and attenuated Aβ- (1-42-) induced Jun N-terminal kinase- (JNK-) mitogen-activated protein kinase (MAPK) overactivation [45]. ANDRO sulfonate (2.5 and 5 mg/kg, 5 months) was found to inhibit AD *via* mitochondria protection in APPswe/PSENΔ9 double transgenic mice having AD [46].

Other studies narrate that ANDRO treatment during chronic cerebral hypoperfusion suppressed astrocyte activation supported by decreased expression of the glial fibrillary acid protein (GFAP), enhanced brain-derived neurotrophic factor (BDNF) and tyrosine kinase receptor B (TrkB) expression, and reversed upregulated expression of TNF-α, IL-1β, and caspase-3. Thus, in the rat model of chronic cerebral hypoperfusion, ANDRO improved impaired spatial learning and memory [47].

3.1.2. Parkinson Disease. Parkinson's disease (PD) is described as a gradual loss of midbrain *substantia nigra* dopaminergic neurons [48, 49].

Some researchers have shown that pretreatment with ANDRO (0.5–5 μM) abolished lipopolysaccharide- (LPS-) induced decrease in dopamine (DA) uptake but failed to affect 1-methyl-4-phenyl-pyridine- (MPP-) induced decrease in DA uptake. Thereby, ANDRO (1–5 M) reduced the tyrosine hydroxylase- (TH-) immunoreactive neuron loss and shortened TH-immunoreactive dendrites [22]. Microglia-derived toxic factors include proinflammatory mediators such as reactive oxygen species (ROS), prostaglandin E2 (PGE2), TNF-α, inducible nitric oxide synthetase (iNOS), nitric oxide (NO), and cyclooxygenase 2 (COX2) [50, 51].

Anxiety and depressive disorders are frequently comorbid with PD [52]. Thus, a forced swimming test was performed in mice to measure depressive symptoms. After ANDRO (5 mg/kg) treatment, the swimming time was noticeably enhanced showing an improvement of depressive symptoms [53]. Geng et al. [54] provided evidence that

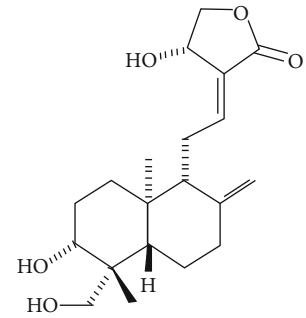

Figure 1: Andrographolide chemical structure.

ANDRO attenuated DA neuron loss, oxidative stress, and preserved mitochondrial morphology and also reduced mitochondrial malfunctions, reduced cell death, and inhibited GTPase activity in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) induced PD. Apoptosis is also an important factor that triggers PD.

ANDRO reduced Ca^{2+} influx [55], intracellular ROS production [56], and lipid peroxidation. In addition, ANDRO regulated Bcl-2, Bid, Bax, and apoptosis-inducing factor levels. ANDRO also inhibited the phosphorylation of mitogen-activated protein kinases (p38, extracellular signal-regulated kinase (ERK), and *c*-JNK) [55].

ANDRO (0.1–10 μM) was found to decrease apoptosis and inhibited IL-2 [57] and maybe an antagonist of phorbol-12-myristate-13-acetate (PMA) which stimulated remarkable ROS production [56].

3.1.3. Multiple Sclerosis. Multiple sclerosis (MS) is a well-known immune-mediated disorder, in which insulating covers of nerve cells in the spinal cord and brain are damaged in the CNS [58]. From the MS pathogenesis, it was found that $CD4^+$ T-cell-mediated autoimmunity is crucial in MS pathogenesis, mainly for early disease initiation [59, 60]. T-helper type 1 (Th1) cells, characterized by interferon- (IFN-) γ production, mediate the MS pathogenesis [61, 62], but IL-17-expressing T-helper cells (Th17) are also involved. $CD8^+$, as well as $CD4^+$ T cells, was equally immune-stained for IL-17 and IL-17 production inactive areas of MS lesions [63].

ANDRO inhibits the dendritic cells ability and generates peptide-major histocompatibility complexes required for T cell activation. In LPS-treated dendritic cells, ANDRO attenuated the upregulation of the maturation markers $I\text{-}A^b$, CD40, and CD86 (B7.2) [16]. Besides, ANDRO also suppressed T cell function, IFN-γ, and IL-2 production [57]. These effects may contribute to ANDRO's therapeutic potential, ameliorating MS symptoms in autoimmune encephalomyelitis mice through inhibition of T-cell activation and antibody responses directed to the myelin sheath [16].

3.2. Andrographolide and Stroke

3.2.1. Ischemic Stroke. Cerebral ischemia occurs when the metabolic demand of brain is not satisfied due to an insufficient blood flow [48, 64]. This involves cerebral hypoxia

Table 1: ANDRO neuropharmacological activities and possible mechanism of action.

Neurological/psychiatric disorders	Results/possible mechanisms	References
Alzheimer disease	Neuroprotective ↓ Aβ protein ↓ Caspase-3 expression ↓ Apoptosis ↓ Neuronal cell death	([20], [44], [47], [26], [46])
Parkinson's disease	Neuroprotective ↓ ROS, ↓ NO ↓ TNF-α ↓ IL-1β ↓ Lipid peroxidation ↓ Apoptosis ↓ Cell death	([22], [94], [95], [52], [50], [51], [55], [56], [57], [54])
Multiple sclerosis	Neuroprotective ↓ T-cell activation ↓ IFN-γ ↓ TNF-α ↓ IL-1β, ↓ IL-2 ↓ Apoptosis	([16], [62], [61], [63], [57], [96])
Cerebral ischemia	Neuroprotective ↓ β-Catenin ↓ Caspase-3 ↓ NF-κB ↓ Apoptosis ↓ Neuroinflammation	([24], [27], [68], [69], [97], [70])
Intracerebral hemorrhage	↓ Brain injury ↓ Catenin-1 ↓ TNF-α ↓ IL-1β ↓ IL-6	([15], [23], [98], [71], [72])
Neuropathic pain	↓ TNF-α ↓ IL-1β ↓ Neuronal excitability ↓ Central and peripheral pain sensitization	([31], [75], [76], [77])
Brain tumor	↑ p53 and ERK phosphorylation ↑ Caspase-7 ↑ Apoptosis	([83], [81], [82], [84])
Depression	↓ Inflammation ↓ NO, ↓ iNOS ↓ COX-2 ↓ TNF-α ↓ IL-1β, ↓ IL-6 ↑ β-Catenin ↑ BDNF ↑ pSer9	([19], [93], [92], [54], [90], [91], [89])

↑: increase; ↓: decrease; Aβ: amyloid beta; COX-2: cyclooxygenase 2; ERK: extracellular signal-regulated kinase; IFN: interferon; IL: interleukin; NF-κB: nuclear factor κB; NO: nitric oxide; ROS: reactive oxygen species.

leading to the death of brain tissues [65, 66]. Cerebral ischemia is one of the serious causes of morbimortality worldwide. The treatment options against cerebral ischemia/stroke are limited [67].

Some studies have shown that ANDRO (0.1 and 1 mg/kg i.p) lowered the infarct volume and neurological deficits and drastically reduced microglia cells in permanent middle cerebral artery occlusion- (pMCAO-) induced rat model [27]. On the other hand, ANDRO showed a neuroprotective effect by reducing inflammation in the MCAO-induced rat model. ANDRO abolished neuroinflammatory markers, such as IL-1β and TNF-α. ANDRO also suppressed NF-κB activation [27].

Another study showed that ROS production and protein nitrosylation, iNOS, gp91phox/NADPH oxidase 2 (NOX2), IL-1β, and HIF-1α levels were decreased by ANDRO (5 and 10 μg/kg, i.v.) in cerebral ischemia in rats [68]. Furthermore, NOX2 and iNOS expression were reduced by impairing PI3K/protein kinase B- (AKT-) dependent NF-κB and HIF-1α activation in cerebral ischemia in mice [24].

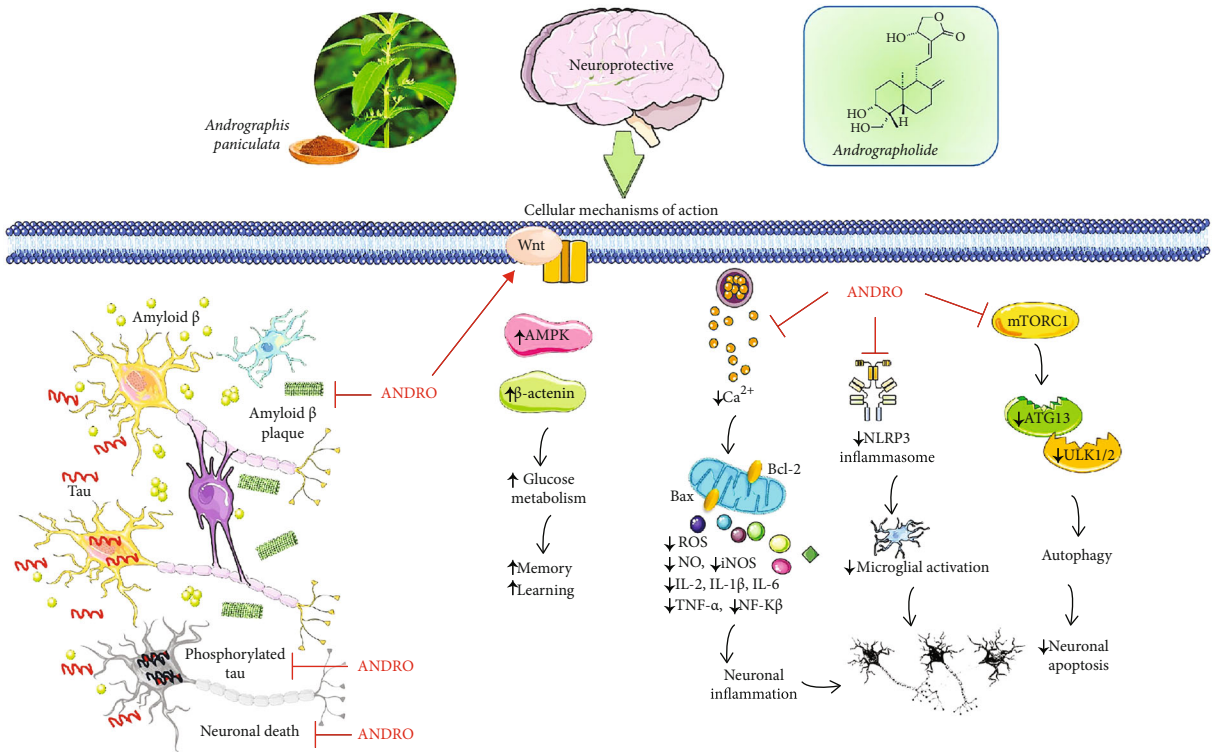

Figure 2: Schematic representation of the most important molecular mechanisms and signaling pathways of andrographolide in central nervous system disorders. ↑: increase; ↓: decrease; ANDRO: andrographolide; ROS: reactive oxidative species; NO: nitric oxide; iNOS: inducible nitric oxide; IL: interleukins; TNF: tumor necrosis alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells. ATG 13 autophagy-related protein 13, ULK ½ Unc-51 like autophagy activating kinase.

ANDRO, in rat hippocampal cultures, also inhibited GSK-3β in a non-ATP-competitive, substrate-competitive way [69].

In a recent study, ANDRO (5–100 μg/kg, i.p.) increased Wnt/β-catenin signaling as evidenced by the enhanced nuclear β-catenin expression and inhibited GSK-3β (pSer9) [70].

3.2.2. Hemorrhagic Stroke. Intracerebral hemorrhage (ICH) occurs within brain tissue or ventricles [71] and is a prime CNS health problem all over the world with high morbimortality rates. So far, no effective strategies exist to treat this disorder, and nearly 20% of patients achieve therapeutic outcomes [72]. There are several causes of, including neuroinflammation and microglial activation. Therefore, prevention and secondary treatment of brain injury are important for patients with ICH [23, 73].

In some studies, ANDRO (1 and 2 mg/kg) was able to reduce neurobehavioral damage, the water content in the brain, alleviate neuronal cell death, and degeneration in ICH-induced SBI rats [23]. Further experimental analysis shows that ANDRO has inhibitory effects on CD11b + and CD16+ microglia cells and attenuated TNF-α and IL-6 level by deactivating the NF-κB signaling pathway through reverse phosphorylation of IκBα and p65 in ICH rats [23]. Furthermore, in the ICH brain, ANDRO reduced caspase-1-caspase-recruitment domain (ASC) and NLRP3-ASC interaction, thereby inhibiting caspase-1/gasdermin D cleavage and IL-1β production [23]. In addition, ANDRO suppresses NF-κB and NLPR3 inflammasome activation via p65 translocation assembly inhibition of NLRP3/ASC/caspase-1 complex [23] and can reduce SBI after ICH [15].

3.3. Andrographolide and Neuropathic Pain. Pain sensation occurs when tissue injury is detected by nociceptors [74]. ANDRO (25, 50, 100 mg/kg) is evident to exert analgesic effects by reducing writhing reflex [75]. ANDRO and its derivatives 14-deoxy-11,12-didehydroandrographolide, 14-acetyl-3,19-isopropylidenyl-, and 3,19-dipalmitoyl-derivatives (4 mg/kg) exerted analgesic effects in hot plate and writhing test in mice [31].

ANDRO is also evident to exert analgesic effects in Charles Foster male albino rats [76]. ANDRO (25 mg/20 mL) also attenuated mechanical and thermal hyperalgesia and downregulated the expression of the P2X7 receptor. Besides this, ANDRO decreased TNF-α and IL-1β expression, increased IL-10 expression, inhibited ERK signaling pathways activation, and also decreased the coexpression of GFAP and P2X7 receptors [77], thus reducing neuropathic pain in the HIV rat model.

3.4. Andrographolide and Brain Tumors. Glioma is a tumor occurring in the glial cells of the brain or spine [78] and comprises about 30% of all brain tumors and 80% of all malignant brain tumors in CNS [79]. Targeted therapies for cancer are a rapidly advancing field for treating tumor, and natural products have become the best choice for researchers ([80], Sharifi-Rad et al., 2021b).

ANDRO showed anticancer potential in several cancer cell types [25, 81]. ANDRO (15 μM) induced cell death of glioblastoma (C6) cells by inducing apoptosis through ROS-extracellular receptor kinase- (ERK-) p53-caspase 7- and PARP-pathways in mouse glioblastoma (C6) cells. ANDRO could increase apoptosis through both phosphorylations of p53 and p53 activation. Because ANDRO increased p53 levels in neural cells [55], findings suggest that ANDRO can promote p53 protein activation, which activates the downstream caspase 7-PARP cascade [82] and is regulated by ERK [83]. In a recent study, ANDRO accelerated RSC96 cell proliferation [84].

3.5. Andrographolide and Depression. One of the most common neuropsychiatric disorders in the world is depression [85–87]. Characterized by a variety of signs and symptoms, antidepressants have been increasingly used for depression treatment in daily life, but their multiple side effects and high rates of failure have triggered the researchers' interest to find more effective and safer therapeutic strategies [88]. ANDRO (5 mg/kg) administration can improve depressive-like behaviour, as well as to attenuate the expression of pro-inflammatory mediators and cytokines, including NO, iNOS, COX-2, IL-1β, IL-6 and TNF-α, NF-κB signaling (p-p65, p-IκBα), and NLRP3 inflammasome assembly (NLRP3, ASC, and caspase-1) in the prefrontal cortex. Besides, ANDRO (5 mg/kg) increased Beclin1 expression and abrogated phosphorylated mTORC1 (p-mTOR), revealing autophagic activity in the prefrontal cortex of chronic unpredictable mild stress mice [54]. Beclin1 stimulates the initial stages of autophagy, and p-mTOR inhibits autophagy through ATG13 and ULK1/2 phosphorylation [89]. ANDRO-generated autophagy can attenuate depressive-like symptoms, inhibits inflammation, and shows antidepressive effects [54].

In another study performed in mice, ANDRO (20 and 50 mg/kg) activated hippocampal BDNF system, thus showing antidepressant effects [19, 90]. Also, ANDRO inhibited the long-term depression in a concentration-dependent way, showing β-catenin accumulation and reducing the GSK-3β active state [91–93].

4. Conclusions

The antioxidant and anti-inflammatory effects of ANDRO and some of its derivatives are well-known. It is also widely recognized that substances with these kinds of properties are cytoprotective and can protect animal organs. This review gives insights on the neuro-pharmacological effects of ANDRO and its derivatives in several test systems. In light of these data, ANDRO can be considered one of the most important neuro-protective phytochemicals that can be considered as an adjuvant treatment in neurodegenerative diseases such as AD, PD, and MS. Further studies are needed to find solutions such as nanocarriers, to increase the bioavailability of ANDRO in order to cross the blood-brain barrier by incorporating in pharmaceutical formulations such as nanoparticles.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, interpretation, or in all these areas that is revising or critically reviewing the article, giving final approval of the version to be published, agreeing on the journal to which the article has been submitted, and confirming to be accountable for all aspects of the work.

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Review Article

Nutraceutical Profiling, Bioactive Composition, and Biological Applications of *Lepidium sativum* L.

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The roots, leaves, and seeds of *Lepidium sativum* L., popularly known as Garden cress in different regions, have high economic importance; although, the crop is particularly cultivated for the seeds. In traditional medicine, this plant has been reported to possess various biological activities. This review is aimed at providing updated and critical scientific information about the traditional, nutritional, phytochemical, and biological activities of *L. sativum*. In addition, the geographic distribution is also reviewed. The comprehensive literature search was carried out with the help of different search engines PubMed, Web of Science, and Science Direct. This review highlighted the importance of *L. sativum* as an edible herb that possesses a wide range of therapeutic properties along with high nutritional values. Preclinical studies (in vitro and in vivo) displayed anticancer, hepatoprotective, antidiabetic, hypoglycemic, antioxidant, antimicrobial, gastrointestinal, and fracture/bone healing activities of *L. sativum* and support the clinical importance of plant-derived bioactive compounds for the treatment of different diseases. Screening of literature revealed that *L. sativum* species and their bioactive compounds may be a significant source for new drug compounds and also could be used against malnutrition. Further clinical trials are needed to effectively assess the actual potential of the species and its bioactive compounds.

1. Introduction

A large number of people or community from developed and developing countries depend on medicinal plants for treatments, skin care, cultural progress, and economic growth [1, 2]. The World Health Organization (WHO) projected that 80% of the world's population relies on traditional medicines, and around 19.4 billion global revenue were recorded for herbal remedies in 2010 [3, 4]. The market demand for medicinal plants is increasing continuously and according to WHO the demand will be more than the US $ 5 trillion in 2050 [5].

Lepidium sativum L. popularly known as garden cress in different regions of the world is an edible annual and fast-growing herb belongs to the family Brassicaceae and genus *Lepidium* [6]. The genus consists of more than 175 species around the world; among them, several species are known for their nutritional and therapeutic properties [7, 8].

From prehistoric time, *L. sativum* has been consumed by ancient Egyptians and Romans for several health-promoting benefits [9]. Traditionally, *L. sativum* is used for the treatment of various diseases like asthma, tumors of the uterus, ulcers, hemorrhoidal haemorrhage, coughing, wounds, dermatomycosis, dysmenorrhea, sciatica, and nasal polyps. The seeds of this species have been utilized as a galactagogues and abortive agent and are also used to treat sore throat, headache, cough, asthma, malaria, syphilis, and impotence [10]. A seed paste prepared in water is used for skin problems and sunburns while the mucus of seeds is used against diarrhoea and irritation of the intestines in dysentery, and germinating seeds are used for constipation [11, 12]. The leaves of *L. sativum* are diuretic, mildly stimulant, and also used in liver problems and scorbutic diseases [13].

People consume it in the form of salad, sprouts, and spicy seasoning, and the oil extracted from their seed is used for seasoning [14, 15]. Different parts such as roots, leaves, and seeds of this plant species have immense economic importance; although, the crop is particularly cultivated for the seeds [16].

L. sativum has been reported to possess various biological activities such as antimicrobial, bronchodilator, hypotensive, allopathic, hypoglycemic, hepatoprotective, antioxidant, and against hiccup [17–21]. Its mucilage possesses various characteristics such as gelling, binding, and disintegrating, which help in the development of desirable pharmaceutical dosage forms [16]. The phytochemical profiling of *L. sativum* showed the presence of flavonoids, phenols, cardiotonic glycosides, cardiac glycosides, alkaloids, coumarins, proteins, and amino acids [10].

The purpose of the present review is to provide updated and quantified scientific information about the traditional, nutritional, phytochemical, and biological activities of *L. sativum*.

2. Methodology

For this review, we collected literature published in English from scientific databases such as PubMed, Web of Science, and ScienceDirect, before July 2021 on phytochemistry, nutritional profile, and pharmacology of Lepidium sativum species. The following MESH terms were used for searching: "Lepidium sativum/chemistry," Seeds/chemistry, Oxidative Stress/drug effects, "Plant Extracts/chemistry," "Plant Extracts/pharmacology," "Antineoplastic Agents," "Antioxidants/chemistry," "Antioxidants/pharmacology," "Apoptosis/drug effects," "Cell Line, Tumor," "DNA Damage/drug effects," "Flavonoids/chemistry," "Flavonoids/pharmacology," "Blood Glucose," "Diabetes Mellitus," "Experimental/drug therapy," "Hyperglycemia/drug therapy," "Hypoglycemic Agents/pharmacology," "Animals," and "Humans."

All selected papers were analyzed and summarized to prepare this comprehensive review.

The plant taxonomy was verified by the database "The PlantList," and the chemical formulas were validated with Chemspider [22, 23].

3. Bioactive Compounds

Active compounds or secondary metabolites are produced in plants as a byproduct of various metabolic reactions; although they do not play a primary role in plant reactions, they are important in many plant defence mechanisms and are also known for their biological or therapeutic activities [24–26]. The most important class of secondary metabolites are phenols, flavonoids, terpenoids, alkaloids, saponins, and glycosides [27–29].

Phytochemically, the seeds, leaves, roots, and seed oil of *L. sativum* are a rich source of alkaloids, glucosinolates, saponins, terpenes, saturated, and essential fatty acids [13, 30–33].

Glucosinolates are a wide group of secondary metabolites consisting of sulphur and nitrogen molecules and are mainly known for their nutritional effects and other therapeutic properties like antimicrobial, antioxidant, anticancer, and anti-inflammatory [34, 35].

Total phenolic and flavonoid content of *L. sativum* leaves of two cultivars (Dadas and Izmir from Turkey) was measured to be 0.573 mg gallic acid equivalent (GAE)/g fresh weight (FW) and 6.332 mg GAE/g DW for Dadas cultivar and 0.774 mg GAE/g FW and 7.401 mg GAE/g DW for Izmir cultivar, respectively [36]. The ascorbic acid content for *L. sativum* leaves was measured to be 54 mg/100 g FW and 74 mg/100 g FW for Dadas and Izmir cultivars [36]. However, the methanolic extract of seeds showed the presence of 0.5% and 0.375% of phenolic and flavonoid content [32].

Malar et al. [37] reported the ascorbic acid content in stem (11.74 ± 0.83 mg), leaves (7.4 ± 0.38 mg), whole plant (12.5 ± 0.60 mg), and seeds (9.68 ± 0.72 mg) of *L.* sativum.

Chatoui et al. [38] showed the presence of tannin in the ethanolic and methanolic seed extract of *L. sativum* collected from different regions of Morocco. The maximum tannin acid (31.50 ± 0.11 mg catechin/g extract) was observed in methanolic seed extract of *L. sativum* of Ben-Ahmed region, Morocco, whereas the minimum (8.33 ± 0.11 mg catechin/g extract) amount of tannin was measured in the ethanolic extract of *L. sativum* of Rommani region, Morocco [38]. Other studies from different regions also showed that *L. sativum* has a significant amount of phenolic and flavonoid content (Table 1).

Table 1: Total phenolic and flavonoid content in *Lepidium sativum*.

Country	Plant part and solvents	Total phenolic content (mg gallic acid equivalent/g extract)	Total flavonoid content (mg quercetin equivalent/g extract)	Ref.
India	Ethanolic seed extract	4.46 ± 0.14	3.57 ± 1.2	[39]
	Ethanolic seed extract	11.03 ± 0.75	4.79 ± 0.24	[40]
Pakistan	Methanolic seed extract	120.26 ± 1.52*	—	[41]
Egypt	Aqueous seed extract	126.24	007.21	[42]
	Ethanolic seed extract	88.08	00.65	[42]
	Ethanolic seed extract	46.00 ± 0.86	82.00 ± 0.93	[43]
	Aqueous seed extract	34.00 ± 0.67	53.00 ± 0.58	[43]
Turkey	Methanolic extract of aerial part	184.14 ± 2.5**	12.63 ± 1.5***	[44]
Morocco				
Tafraout region	Methanolic seed extract	94.48 ± 1.82	37.63 ± 2.14	[38]
	Ethanolic seed extract	86.48 ± 0.22	32.51 ± 0.81	[38]
El-Haouz region	Methanolic seed extract	83.36 ± 0.98	33.58 ± 0.33	[38]
	Ethanolic seed extract	80.28 ± 0.28	29.24 ± 0.47	[38]
Ben-Ahmed region	Methanolic seed extract	69.46 ± 0.09	24.85 ± 0.48	[38]
	Ethanolic seed extract	65.15 ± 1.07	23.92 ± 0.64	[38]
Rommani region	Methanolic seed extract	59.40 ± 0.62	21.09 ± 0.21	[38]
	Ethanolic seed extract	52.79 ± 0.30	20.04 ± 0.04	[38]

*mg catechin equivalent/g extract; ** μg gallic acid equivalent/mg extract; *** μg quercetin equivalent/mg extract.

Regarding the essential oil composition, Afsharypuor and Hadi [45] identified the presence of 1,8-cineole, benzyl isothiocyanate, α-pinene, and phenyl acetonitrile in seeds, benzyl isothiocyanate, α-pinene, palmitic acid, and linoleic acid in roots, and benzyl isothiocyanate, α-pinene, palmitic acid, phenyl acetonitrile, sabinene, and limonene, β-thujone in the aerial part of *L. sativum* by gas chromatography-mass spectrometry (GC-MS) analysis [45].

The seeds of *L. sativum* are comprised of 24% oil which contains linoleic acid and α-linoleic acid. It is reactively more stable due to the presence of phytosterols and antioxidant content [46, 47].

Singh et al. [48] reported the presence of 2-pentanoic acid, penta-decadienoic acid, pentanoic acid, succinic acid, butyric acid, acetic acid, oxalic acid, carbonic acid, propanoic acid, and cyclohexane carboxylic acid in the seed oil of *L. sativum*. The chemical structures of bioactive compounds present in the essential oil of the species are shown in Figures 1(a) and 1(b) while a detail description of essential oil composition has been presented in Table 2.

The analyses of the chemical composition of *L. sativum* extract revealed the presence of five glucosinolates in seeds (glucotropaeolin and 2-phenyl ethyl glucosinolate) and fresh herb (glucotropaeolin, methyl glucosinolate, 2-ethyl butyl glucosinolate, and butyl glucosinolate) [56]. Williams et al. (2009) reported the presence of glucotropaeolin as a principal glucosinolate and gluconasturtiin in the seeds of *L. sativum* [57]. Hussain et al. (2011) [58] reported the presence of 19 phytochemicals in the methanolic leaves to extract *L. sativum* including campesterol, cis-vaccenic acid, 2-naphthalenol, 1-nitro-2-propanol,1-deoxy-d-mannitol, allyl isothiocyanate, and paromomycin, among others.

Maier et al. [59] identified the imidazole alkaloid lepidine along with five new dimeric (lepidines B, C, D, E, and F) and two monomeric (semilepidinosides A and B) imidazole alkaloids in seeds of *L. sativum* [59], while the presence of 10 major compounds includes benzyl nitrile, 2,3,4-trimethoxycinna-mic acid, 5-hydroxy-methyl furfural, and furfural was reported by El-Gendy [60].

A complete screening of phytochemicals present in *L. sativum* seeds was evaluated by ultrahigh-performance liquid chromatography (UHPLC)/photodiode array detection (PDA)/electrospray ionization-mass spectroscopy (ESI-MS) method as well as head space solid-phase microextraction (SPME)-GC/MS methods [61]. A total of 32 metabolites from flavonoid, glucosinolate, phenolic acid, sugar, coumarin, lignan, glycoalkaloid, steroid, and fatty acid classes were identified via UHPLC/PDA/ESI-MS, and 66 metabolites from alcohol, acid, ester, aromatic, ketone, aldehyde, monoterpene hydrocarbon, and among other classes were identified by (SPME)-GC/MS [61]. All the above studies are reported from different regions including Saudi Arabia, India, Egypt, and Iraq, which indicates that the leaves, seed, or seed oil of *L. sativum* could be a valuable source of important active compounds with significant biological activity.

The chemical structure of bioactive compounds present in the extracts of *L. sativum* has been displayed in Figure 2 while a detailed description of bioactive compounds present in different parts of the species has been presented in Table 3.

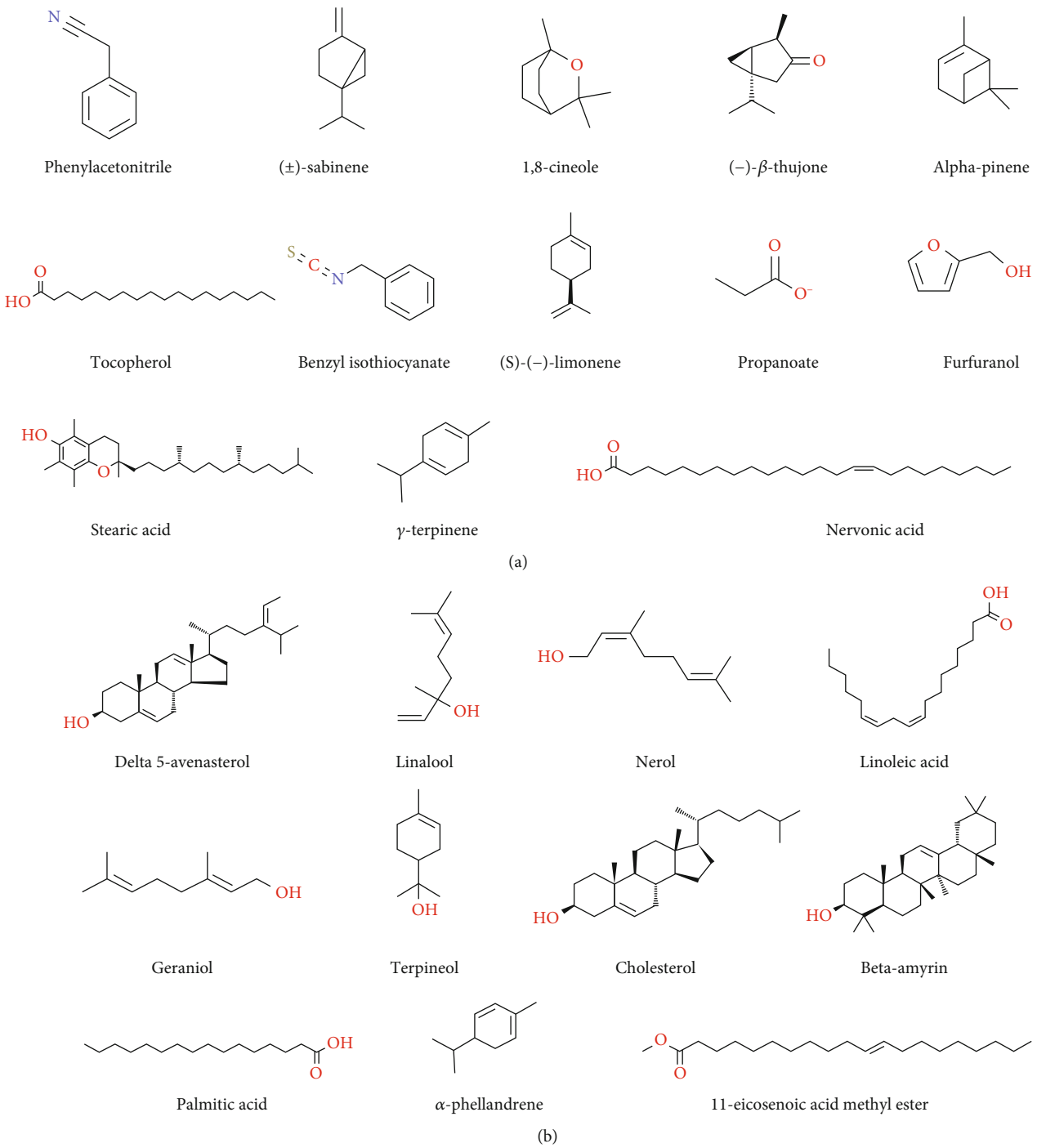

Figure 1: (a) Chemical structure of several bioactive compounds present in essential oil of *L. sativum*. (b) Chemical structure of several bioactive compounds present in the essential oil of *L. sativum*.

4. Nutritional Profile

L. sativum is considered a valuable source of nutrition with significant therapeutic properties. In the last few years, several researchers from different regions have investigated the nutritional profiling of the leaves, seed, and seed oil of *L. sativum* (Tables 4 and 5).

Hassan et al. [68] evaluated that in *L. sativum* leaves, the highest amount of mineral value was observed for potassium (1850.00 ± 43.30 mg/100 g dry weight (DW)) followed by calcium (829.13 ± 20.70 mg/100 g DW), and the minimum value was observed for chromium (0.36 ± 0.27 mg/100 g DW); however, the maximum amino acid content in leaves was calculated for glutamic acid (9.36 ± 0.06 g/100 g protein DW), and minimum value was shown by cysteine (0.42 ± 0.20 g/100 g protein DW) [58].

In three studies from different regions (Nigeria, Saudi Arabia, and Pakistan), the highest mineral value of *L.*

Table 2: The chemical composition of essential oils isolated from different parts of *L. sativum*.

Plant part used	Bioactive compounds	Regions/ country	References
Aerial part	Alpha-pinene; sabinene; limonene; 1,8-cineole; β-thujone; phenylacetonitrile; benzyl isothiocyanate; hexadecanoic acid; linoleic acid.	Iran	[45]
Seeds	Alpha-pinene; 1,8-cineole; phenylacetonitrile; benzyl isothiocyanate.	Iran	[45]
	Alpha-pinene; sabinene; alpha-phellandrene; eucalyptol; gamma-terpinene; linanool; terpinen-4-ol; alpha-terpineol; propanoate; alpha-terpinyl acetate; E-nerolidol.	Greece	[49]
	Docosatrienoic acid; linoleic acid; eicosenoic acid; palmitic acid; arachidonoic acid; eruic acid; stearic acid; arachidic acid.	Ethiopia	[50]
	Alpha-tocopherol; gamma-tocopherol; cholesterol; campesterol; stigmasterol; sitosterol; avenasterol.	USA	[47]
	7,10-Hexadecadienoic acid, methyl ester; 11-octadecenoic acid, methyl ester; behenic acid, methyl ester; 7,10,13-hexadecatrienoic acid, methyl ester; stearic acid, methyl ester; hexadecanoic acid, 15-methyl-, methyl ester; 15-tetracosenoic acid, methyl ester; 10-octadecenoic acid, methyl ester; heneicosanoic acid, methyl ester.	Saudi Arabia	[51]
	Myristic acid; palmitic acid; palmitoleic acid; stearic acid; oleic acid; linoleic acid; linolenic acid; arachidic acid; gadoleic acid; cholesterol acid; stigmasterol; campesterol; beta-sitosterol; 5-avenasterol; alpha-tocopherol; beta-tocopherol; gamma-tocopherol.	Morocco	[38]
	Toluene, octane, (E,Z)-1,3,5-octatriene, ethylbenzene, 2-furanmethanol, styrene, methional, benzaldehyde, benzyl alcohol, benzaldehyde, benzyl alcohol, 1-isocyano-2-methylbenzene, benzyl isothiocyanate, benzylurea, 1-benzyl-2(1 H)-pyridone, (Z)-8-heptadecene, palmitic acid, cyclic octaatomic sulfur, oleic acid, linoleic acid, nonadecanamide, arachidic acid, etc.	India	[20]
	Linolenic acid, oleic acid, arachidic acid, palmitic acid, stearic acid.	India	[52]
	Myristic acid; palmitoleic acid; palmitic acid; alpha-linolenic acid; octadecenoic acid; stearic acid; 9-octadecen-12-ynoic acid; paullinic acid; arachidic acid; erucic acid; behenic acid; nervonic acid; lignoceric acid.	Saudi Arabia	[53]
	Alpha-linolenic acid; oleic acid; linoleic acid; eicosanoic acid; palmitic acid; erucic acid; arachidic acid; stearic acids.	India	[46]
	Beta-amyrin; 9,12,15-octadecatrienoic acid methyl ester; 9-octadecenoic acid methyl ester; alpha-amyrin; 11-eicosenoic acid methyl ester; 9,12-octadecadienoic acid; hexadecanoic acid methyl ester.	Saudi Arabia	[54]
	Geraniol; citronellol; nerol; triacontane; palmitic acid; 1,6-octadien-3-ol, 3,7-dimethyl.	Egypt	[55]
Roots	Alpha-pinene; benzyl isothiocyanate; hexadecanoic acid; linoleic acid.	Iran	[45]

sativum seed was calculated for potassium (1193.95 ± 10.51; 785.0 ± 7.51; 1236.5 ± 1.67 mg/100 g) followed by phosphorus (514.59 ± 10.67; 616.50 ± 9.67; 608.63 ± 1.39 mg/100 g) [41, 69, 70], and the minimum mineral value was observed for molybdenum (0.43 ± 0.08 mg/100 g) [70].

The amino acid analyses showed different results in terms of the maximum and minimum amino acid value, and it was recorded for glutamic acid (19.33 ± 0.19 g/100 g protein) and methionine (0.97 ± 0.02 g/100 g protein) [70]; however, in another study, the highest amino acid value was measured for leucine (9.03 ± 0.007 g/100 g protein), and lowest amino acid values were measured for cysteine (0.80 ± 0.00 g/100 g protein) [69].

The estimation of fatty acid was done for three seed oil extracts of *L. sativum* prepared from the cold press extraction method, Soxhlet extraction method, and supercritical carbon dioxide extraction method. The study findings showed that in all the seed oil extracts, the maximum fatty acid content was measured for linoleic acid (~34-35%), and the minimum was observed in oleic acid (~2.8%) [46].

The nutritional profiling showed that the leaves, seeds, and seed oil of *L. sativum* possess appropriate nutritional content which can help in combating anemia, malnutrition, and several micronutrient deficiencies (Figure 3).

5. Pharmacological Properties

The major role of food is to fulfil the requirement of necessary nutrients in the body and to satisfy hunger; however, nowadays, food from edible plants also plays a significant role in preventing and curing several diseases and disorders due to the presence of different bioactive compounds [75]. The species comprise a variety of bioactive compounds along with strong nutraceutical potential and showed several biological activities [76]. In this section, we discussed different biological applications of the species including anticancer, hepatoprotective, antidiabetic and hypoglycemic, antioxidant, antimicrobial, gastrointestinal, and fracture/bone healing activities.

The most relevant pharmacological properties and their mechanisms of action are summarized in Figure 4.

5.1. Anticancer. Globally, cancer is the second leading cause of death and modern drugs and techniques used to treat

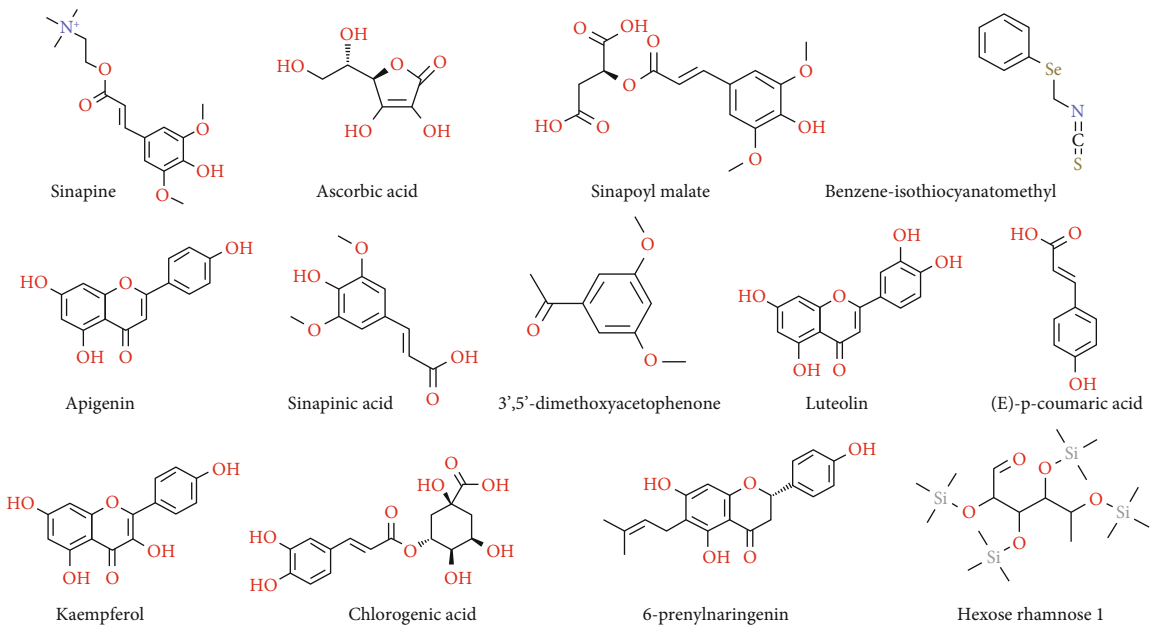

Figure 2: Chemical structure of bioactive compounds present in *L. sativum* extracts.

cancer possess several toxicities and side effects [77–79]. Easily available traditional medicines and natural remedies for cancer have less or no side effects relative to modern drugs [80, 81]. Many plant extracts and plant-derived secondary metabolites are presently used to treat cancer and to eliminate the side effects of chemotherapy [82].

In the anticancer activity of *L. sativum* leave extract (aqueous) against CAL-27, a human tongue squamous carcinoma was evaluated a dose-dependent manner (70, 100, and 150 μg/mL). The best result was shown at 100 and 150 μg/mL of concentrations where the aqueous leaves extract of *L. sativum* caused significant damage to DNA and increase the apoptosis up to 30% and 60%. The results also showed the increase in reactive oxygen species (ROS) level in the mitochondria of CAL-27 [83]. The hydroalcoholic leave extract of *L. sativum* showed optimum antiproliferative and apoptotic activity against cervical cancer cell lines (HeLa) cell lines at 100 μg/mL [84].

The combination of shoots stems and leave hydroalcoholic extracts before and after flowering was tested for cytotoxic effect against leukemia cell line (K562) at different concentrations ranging between 12.5 and 100 μg/mL [85]. The hydroalcoholic extracts before and after flowering exhibit cytotoxic effect against K562 cell lines and the best results are shown at 25 μg/mL of concentration.

The methanolic extract of *L. sativum* shows cytotoxic effect against lymphocyte cells and colon and endometrium cancer cell lines (DLD-1 and ECC-1) through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [44]. The necrotic effect, apoptotic activity, and genotoxic activity of plant extract were also investigated by lactate dehydrogenase (LDH), DNA ladder fragmentation, enzyme-linked immunosorbent assay (ELISA), ethidium bromide staining, and comet assay. The extract showed cytotoxic activity in a concentration-dependent manner against colon and endometrium cancer cells; however, the maximum apoptotic and genotoxic activity was seen at 200 μg/mL of a concentration [44].

Kassie et al. [86] reported the chemoprotective effect of *L. sativum* seed extract and their compounds (glucotropaeolin and benzylisothiocyanate) on 2-amino-3-methyl-imidazo [4,5-f] quinoline- (IQ-) induced genotoxic effects and colonic preneoplastic lesions in male Fischer 344 rats. The pretreatment of the experimental model with *L. sativum* extracts (0.8 mL) and their compounds (GT: 150 mg/kg, BITC: 70 mg/kg) up to three days (consecutive) triggered a significant reduction in IQ-induced DNA damage in colon and liver cells ranging between 75 and 92% [86]. The aqueous seed extract of *L. sativum* showed cytotoxic effect against breast cancer cell lines (MCF-7) by sulforhodamine B and tryphan blue dye exclusion assay at concentration and time-dependent manner (25%, 50%, and 75%) [87].

The cytotoxic activity of seed extracts (chloroform, n-hexane, methanol, and ethyl acetate) of *L. sativum* was tested against human neuroblastoma (IMR-32), colon cancer (HT-15 and 29), and lung cancer (A-549) cell lines [88]. The study showed that all the extracts exhibited significant in vitro cytotoxicity against all the cell lines; however, methanolic seed extract shows the highest per cent of growth inhibition activity (90 ± 0.88, 95 ± 0.24, 91 ± 0.20, and 87 ± 0.65) for all the cell lines (IMR-32, HT-15, HT-29, and A-549) [88].

The aqueous seed extract of *L. sativum* with the lowest and highest concentration of 200 and 400 mg/kg was evaluated for anticancer activity against dextran sulfate sodium/azoxymethane-induced colon cancer in the albino mice model [89]. The result showed that at 400 mg/kg of concentration, the seed extract exhibits higher apoptosis and higher anticancer activity against colon cancer with a decrease in colon tumor/polyp size and incidence and tissue disorder [89]. The seed extract of *L. sativum* alone and with silver nanoparticles showed significant anticancer activity against

Table 3: The chemical composition of *Lepidium sativum* extracts.

Plant part used	Bioactive compounds	Regions/country	References
Leaves	Benzyl nitrile n,n-Dimethylaminoethanol 2-Hydroxy-1-(1′-pyrrolidiyl)-1-buten-3-one d-Proline Butyrolactone	Iraq	[62]
	Apigenin Quercetin Kaempferol Luteolin 7-Hydroxy-4′,5,6-trimethoxyisoflavone; Sinapic acid Chlorogenic acid p-coumaric acid Ascorbic acid α-Tocopherol 6-prenylnaringenin.	Egypt	[63]
Seeds	Glucotropaeolin; sinapine K di-hexose rhamnose Sinapoyl di-glucose; sinapoyl malate K hexose rhamnose 1 K rhamnose (benzo) di-hexose 1	Algeria	[64]
	Benzyl nitrile Benzene-isothiocyanatomethyl 3′,5′-dimethoxyacetophenone Hexadecanoic acid methyl ester cis-Vaccenic acid cs-11-Eicosenoic acid-methyl ester 7,8-Epoxylanostan-11-ol, 3-acetoxyeergosta-14,22-dien-3-ol- acetate -3 beta-5 alpha	India	[65]
	Benzyl cyanide Benzyl thiocyanate Benzyl isothiocyanate Benzaldehyde Benzonitrile Benzyl thiocyanate Benzyl isothiocyanate	Poland	[66]
Aerial part	Stigmast-5-en-3 β27-Diol 27-benzoate	India	[67]

HT-29 colon cancer cell lines by inducing apoptosis and mitotic cell arrest. They also increase the p53 expression and prevent cell division of HT-29 colon cancer cells [90].

Ait-Yahia et al. [91] studied the cytotoxic effect of aglycones (flavones/flavonoids), *C*-glycosides, and *O*-glycosides, isolated from the seed and leave extract of *L. sativum* against human laryngeal carcinoma cells (HEp2). The findings showed that all the compounds possess cytotoxic activity, whereas the highest cytotoxic effect was observed for the *O*-glycosylate rich acetate ethyl extract at 57 μg/mL of concentration [91].

5.2. Hepatoprotective. The liver is a crucial part of the body that play a fundamental role in different physiological processes and functions including secretion, metabolism, and storage [92]. Numerous studies proved its important role in the detoxification and excretion of endogenous waste metabolites and exogenous toxic compounds from the body [93, 94].

The liver is also involved in various biochemical processes of nutrient and energy supply, growth, etc. Additionally, it helps in carbohydrate and fat metabolism, bile secretion, and vitamin storage [95, 96]. However, biological factors, genetic factors, environmental factors, autoimmune diseases, toxic compounds, and chemicals result in damage of the cell, structure, tissues, and functioning of the liver and cause hepatic diseases. Modern drugs can also cause an adverse effect on liver as they possess numerous side effects [97]. Thus, there is a need to identify the alternative treatment of hepatic diseases to discover more effective and less toxic natural agents [98–100].

Hepatoprotective activity of the seed and herb extracts (petroleum ether and alcohol) of *L. sativum* was evaluated against carbon tetrachloride- (CCl_4-) induced toxicity in

Table 4: Nutritional composition of leaves of *Lepidium sativum*.

(a)

Component	Proximate composition		
	Nigeria (g/100 g DW ± SD) [68]	Bangladesh (g/100 g DW ± SD) [71]	Nigeria (%) [72]
Moisture	91.05 ± 1.41	87.13 ± 0.088	81.85
Ash	15.38 ± 0.21	1.80 ± 0.015	3.25
Crude fiber	9.31 ± 0.13	2.38 ± 0.015	8.69
Crude protein	18.25 ± 0.1	2.53 ± 0.041	1.01
Total carbohydrate	55.34 ± 0.20	5.47 ± 0.025	5.82
Total lipid	1.72 ± 0.18	—	8.08
Total fat	—	0.70 ± 0.029	—

(b)

Principal component	Minerals Nigeria (mg/100 g DW ± SD) [68]
Potassium	1850.00 ± 43.30
Phosphorus	4.10 ± 0.44
Magnesium	160.60 ± 6.56
Calcium	829.13 ± 20.70
Iron	63.47 ± 5.27
Sodium	141.13 ± 38.19
Copper	0.39 ± 0.02
Chromium	0.36 ± 0.27
Zinc	2.28 ± 0.07
Manganese	5.74 ± 0.11

(c)

Principal component	Aminoacids Nigeria (g/100 g protein DW ± SD) [68]
Isoleucine (Ile)*	3.26 ± 1.05
Leucine (Leu)*	6.84 ± 1.02
Lysine (Lys)*	3.5 ± 0.21
Methionine (Met)*	1.11 ± 0.1
Cysteine (Cys)	0.42 ± 0.21
Phenylalanine (Phe)*	4.77 ± 2.02
Tyrosine (Tyr)	2.59 ± 1.20
Threonine (Thr)*	2.61 ± 1.04
Valine (Val)*	3.85 ± 0.25
Alanine (Ala)	4.31 ± 0.90
Arginine (Arg)*	4.32 ± 1.78
Aspartic acid (Asp)	7.73 ± 2.77
Glutamic acid (Glu)	9.36 ± 0.06
Glycine (Gly)	1.24 ± 0.24
Histidine (His)*	2.09 ± 1.00
Proline (Pro)	2.16 ± 0.16
Serine (Ser)	2.31 ± 0.01

Table 4: Continued.

*Essential amino acids. DW: dry weight; SD: standard deviation.

hepatocytes at different concentrations, and the results showed that both the extracts of seed and herb at a minimum concentration of 50 μg/mL possess a hepatoprotective effect on the hepatocytes against CCl_4 cytotoxicity; however, the concentration that prevents the growth of half of the cells was 150 μg/mL and 200 μg/mL, respectively [56]. The results also showed that the alcoholic extract is safer than petroleum ether extract [56].

L. sativum seed show in vivo hepatoprotective activity for the prevention of CCl_4-induced liver damage in Wistar albino rats at different concentrations ranging from 100 mg/kg to 400 mg/kg body weight [53, 101, 102]. The total alkaloid fraction of seeds of *L. sativum* was screened for the hepatoprotective activity against CCl_4 at 50, 150, and 250 mg/kg (i.p.) of concentrations, and the finding showed that in all concentrations, the extract showed hepatoprotective activity, and the maximum activity was observed at 250 mg/kg [103].

Sakran et al. [104] reported in vivo hepatoprotective activity of a new isoflavonoid (5,6-dimethoxy-2′,3′-methylenedioxy-7-*C*-β-D-gluco-pyranosyl isoflavone) isolated from the seeds of *L. sativum* against paracetamol-induced hepatotoxicity in Sprague Dawley male rats at 100 mg/kg of dose. Al-Sheddi et al. [105] reported the hepatoprotective effect of chloroform extract of seed of *L. sativum* at 5, 10, and 25 mg/mL of concentrations against hepatotoxicity induced by hydrogen peroxide in HepG2 cell lines [105].

Hepatoprotective activity of *L. sativum* seed extract (ethanolic) was evaluated at 150 and 300 mg/kg of doses against D-galactosamine/lipopolysaccharide-induced hepatotoxicity in the Wistar rat model. The result revealed the hepatoprotective activity of the *L. sativum* seed ethanolic extract and showed that the pretreatment of the extract upregulates Bcl-2 protein expression and downregulated caspase-3 in mice [21].

5.3. Antidiabetic. In the last few decades, the global prevalence of diabetes has risen faster not in developed but also in developing countries. Diabetes also causes dysfunction, damage, and failure of a various organ systems which can lead to premature death. Existing synthetic antidiabetic drugs show several limitations and therefore, the search for new antidiabetic agents from natural resources continues [106].

The hypoglycemic activity of aqueous seed extract of *L. sativum* was evaluated in vivo in streptozotocin-induced diabetic Wistar rats at 20 mg/kg of concentration [19, 107]. The result showed significant hypoglycemic activity in the rat

Table 5: Nutritional composition of seed of *Lepidium sativum*.

(a)

Component	Proximate content				
	India (g/100 g) [70]	Indian (g/100 g DW) [73]	Saudi Arabia (%) [69]	Pakistan (%) [41]	Egypt (%) [74]
Moisture	4.14 ± 0.05	4.82 ± 0.09	4.89 ± 0.050	3.92 ± 1.06	7.05 ± 0.45
Ash	4.65 ± 0.09	4.95 ± 0.00	5.83 ± 0.389	4.25 ± 0.13	4.8 ± 0.88
Crude fiber	7.01 ± 0.08	9.72 ± 0.32	6.80 ± 0.080	6.75 ± 1.02	18.79 ± 0.79
Crude protein	22.47 ± 0.78	26.31 ± 0.03	19.82 ± 0.205	24.18 ± 1.5	19.73 ± 1.03
Total carbohydrate	34.24 ± 0.92	29.25 ± 0.27	34.24 ± 0.092	32.87 ± 0.29	35.45 ± 1.65
Total lipid	—	—	—	28.03 ± 1.05	—
Total fat	27.48 ± 0.14	24.96 ± 0.02	—	—	14.18 ± 0.94

(b)

Principal component	Mineral composition		
	India (mg/100 g ± SD) [70]	Saudi Arabia (mg/100 g ± SD) [69]	Pakistan (mg/100 g of seed ± SD) [41]
Potassium	1193.95 ± 10.51	785.0 ± 7.51	1236.51 ± 1.67
Phosphorus	514.59 ± 10.67	616.50 ± 9.67	608.63 ± 1.39
Magnesium	315.25 ± 3.63	—	339.23 ± 2.13
Calcium	296.60 ± 1.04	253.0 ± 1.04	266.35 ± 1.44
Iron	7.62 ± 0.04	53.81 ± 0.04	8.31 ± 0.36
Sodium	24.64 ± 0.02	—	19.65 ± 0.98
Copper	5.53 ± 0.09	1.90 ± 0.09	5.73 ± 2.11
Zinc	5.05 ± 0.07	4.10 ± 0.07	6.99 ± 0.54
Manganese	2.57 ± 0.04	—	2.00 ± 1.08
Sulphur	293.02 ± 14.27	—	—
Aluminum	2.82 ± 0.13	—	—
Boron	1.41 ± 0.03	—	—
Molybdenum	0.43 ± 0.08	—	—

(c)

Fatty acid	Fatty acid profile		
	India (%) [70]	Saudi Arabia (%) [69]	Pakistan (%) (g/100 g of *L.sativum*) ± SD [41]
Palmitic acid	8.7	8.80	10.30 ± 0.12
Oleic acid	19.9	23.49	30.50 ± 0.16
Palmitoleic acid	—	—	0.70 ± 0.30
Stearic acid	3.2	3.49	1.90 ± 0.19
Myristic acid	1.9	1.50	—
Linolenic acid	12.1	30.07	—
Linoleic acid	30.2	11.35	8.60 ± 0.38
Eicosenoic acid	10.3	12.60	—
Erucic acid	—	4.64	—
Arachidic acid	3.2	4.06	—

(d)

Principal component	Amino acid composition Saudi Arabia (g/100 g protein ± SD) [69]	Pakistan (g/100 g protein ± SD) [70]
Isoleucine (Ile)*	5.21 ± 0.014	5.11 ± 0.03
Leucine (Leu)*	9.03 ± 0.007	8.21 ± 0.01
Lysine (Lys)*	2.26 ± 0.390	6.26 ± 0.39
Methionine (Met)*	1.86 ± 0.000	0.97 ± 0.02
Cysteine (Cys)	0.80 ± 0.000	—
Phenylalanine (Phe)*	5.80 ± 0.004	5.65 ± 0.03
Tyrosine (Tyr)	3.82 ± 0.000	2.69 ± 0.09
Threonine (Thr)*	5.39 ± 0.019	2.66 ± 0.09
Valine (Val)*	6.24 ± 0.007	8.04 ± 0.03
Alanine (Ala)	—	4.83 ± 0.02
Arginine (Arg)*	—	4.51 ± 0.03
Aspartic acid (Asp)	—	9.76 ± 0.03
Glutamic acid (Glu)	—	19.33 ± 0.19
Glycine (Gly)	—	5.51 ± 0.07
Histidine (His)*	3.51 ± 0.007	3.87 ± 0.14
Proline (Pro)	—	5.84 ± 0.38
Serine (Ser)	—	4.96 ± 0.09
Phenylalanine + tyrosine (Phe + Tyr)	9.62 ± 0.000	—
Methionine + cysteine (Met + Cys)	1.86 ± 0.000	—

*Essential amino acids. DW: dry weight; SD: standard deviation.

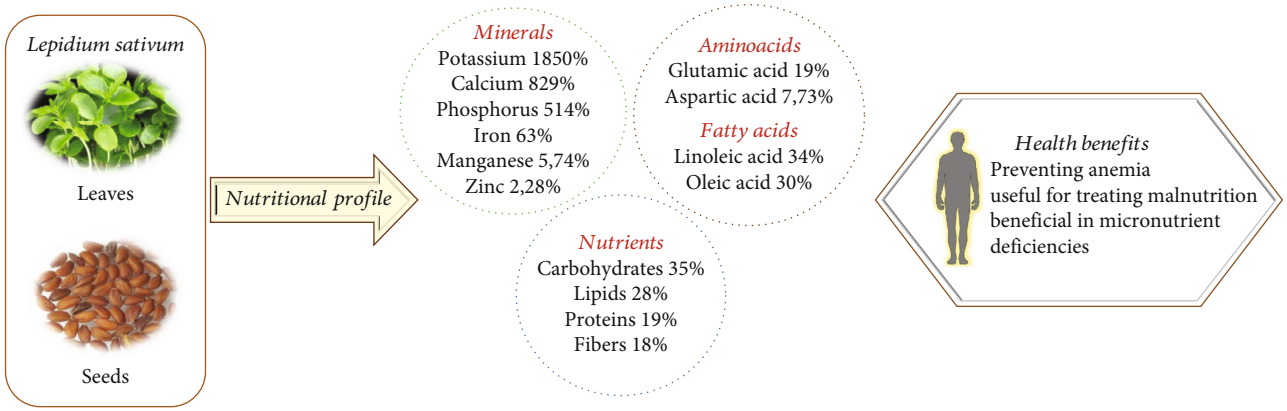

Figure 3: The most representative nutritional compounds of *Lepidium sativum* and the correlation with their beneficial effects for human health.

model without showing any effect in basal plasma insulin concentration [19, 107].

Mishra et al. [108] also investigated the hypoglycemic activity of seeds of *L. sativum* on streptozotocin-induced diabetic Wistar rat and showed the reduction in glucose, alkaline phosphate, and creatinine levels at 20 mg/kg of dose [108]. The total alkaloid fraction of *L. sativum* seed was investigated for antidiabetic activity in alloxan-induced diabetic Wistar rat model at different (50, 150, and 250 mg/kg, i.p.) concentrations [109].

Kamani et al. [110] reported that the methanolic seed extract of *L. sativum* at 200 and 400 mg/kg of doses showed antidiabetic activity against streptozotocin-induced diabetic in albino rats. The fraction suppresses blood glucose, cholesterol, triglyceride, and urea level and showed the best antidiabetic results at 250 mg/kg of concentration [110]. The

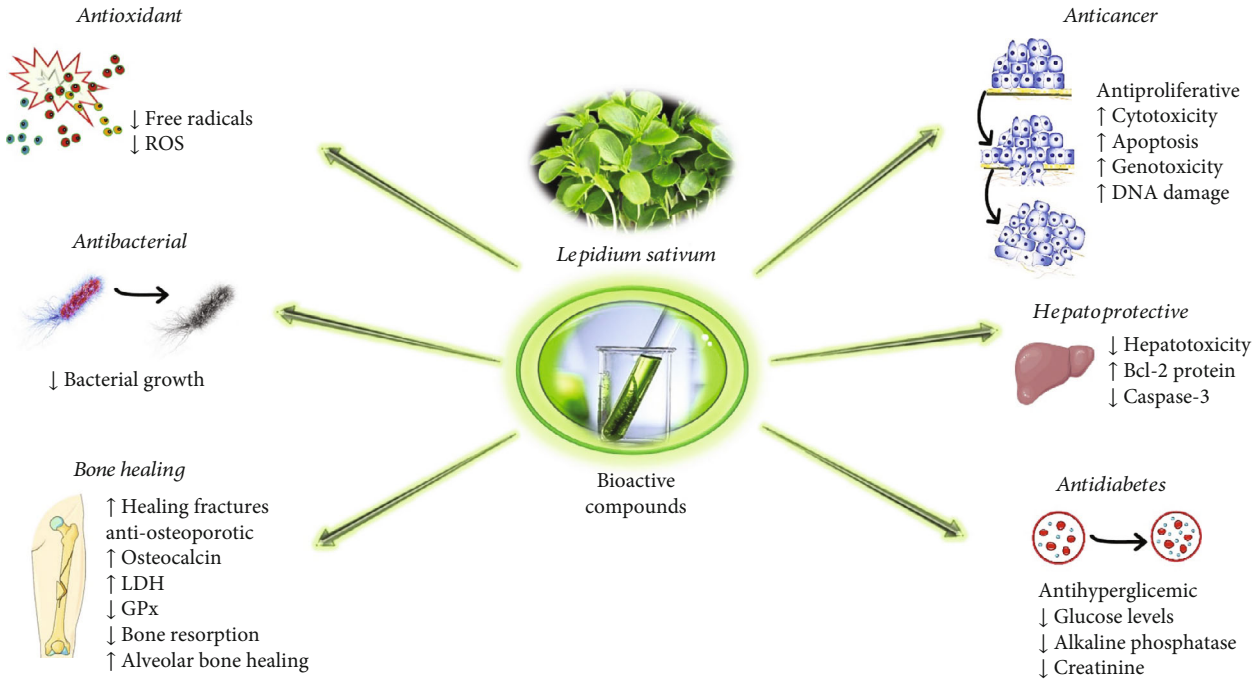

FIGURE 4: Summarized diagram with pharmacological properties of *Lepidium sativum* and its potential mechanism of actions. Abbreviations and symbols: ↑: increase; ↓: decrease; Bcl-2: B-cell lymphoma 2; GPx: glutathione peroxidase; LDH: lactate dehydrogenase; ROS: reactive oxygen species.

methanolic seed extract of *L. sativum* also showed the highest antidiabetic activity against alloxan-induced albino rat at 300 mg/kg of dose [111].

5.4. Antioxidant. Plants are the major source of natural antioxidants, which function as free radical scavengers and reducing agents against reactive oxygen species and free radicals [112, 113].

The antioxidants present in the plant are found in the form of vitamins, phenols, terpenoids, flavonoids, coumarins, alkaloids, etc.

Researchers reported the antioxidant potential of *L. sativum* using different important antioxidants like gallic acid, coumarin acid, caffeic acid, quercetin, tocopherol (α, β, γ, δ), and among others [40, 41]. The ethanolic extract of stem, leaves, whole plant, and seeds of *L. sativum* was tested for antioxidant activity by several methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, reduced glutathione assay, reducing power assay, and ascorbic acid content determination [37]. The result from the study suggests that all the parts (stem, leaves, whole plant, seeds) of *L. sativum* possess scavenging activity; however, the maximum per cent (12.19% ± 0.2) was noted for the whole plant, and the minimum per cent (2.69% ± 0.5) was noted for stem part. In reduced glutathione assay, all the extracts showed enhanced antioxidant activity; however, the highest value was measured in ethanolic leaf extract, i.e., 9 μg/mL. Reducing power or Fe^{3+}-Fe^{2+} transformation ability assay showed that all the plant parts possess the significant reducing ability [37].

Sat, Yildirim, Turan, and Demirbas [36] reported the antioxidant potential of species using DPPH assay in terms of EC_{50} value (EC_{50}: 330.99 μg/mL (Dadas, Turkey) and 346.65 μg/mL (Dadas, Turkey) for FW and 128.08 and 85.97 μg/mL for DW). However, Al-Saad and Al-Saadi [62] reported the IC_{50} value of 149.541 μg/mL for the leaves of *L. sativum* by DPPH assay.

The DPPH, ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric [Fe(III)] ion–reducing antioxidant power), and β-carotene bleaching assays were performed to investigate the antioxidant activity of ethanolic and aqueous seed extract of *L. sativum*. The results showed 31.15% and 18.07% of DPPH activity and 24.61% and 21.14% ABTS activity for ethanolic and aqueous seed extracts, respectively. The FRAP and β-carotene/linoleic bleaching assay also confirm the antioxidant potential of the ethanolic and aqueous extract of *L. sativum* [42].

Kadam, Palamthodi, and Lele [40] also determined that the ethanolic seed extract of *L. sativum* possesses significant antioxidant activity using DPPH (IC_{50}: 162.4 ± 2.3 μg/mL), ABTS (IC_{50}: 35.29 ± 1.02 μg/mL), superoxide scavenging activity (IC_{50}: 187.12 ± 3.4 μg/mL), and metal chelating property (IC_{50}: 119.32 ± 1.5 μg/mL) assays [40].

Chatoui, Harhar, El Kamli, and Tabyaoui [38] evaluated the methanolic and ethanolic seed extracts from Tafraout, Rommani, Ben-Ahmed, and El-Haouz regions in Morocco for the estimation of antioxidant activity. The results showed that the highest DPPH (IC_{50}: 119.3 μg/mL), ABTS (IC_{50}: 187.8 μg/mL), and FRAP (EC_{50}: 777.0 μg/mL) activities in the methanolic seed extract of *L. sativum* are collected from Tafraout region [38]. Nitric oxide assay, total antioxidant capacity assay, reducing power assay, and hydrogen peroxide scavenging assay of aqueous and ethanolic seed extract

of *L. sativum* showed the presence of significant antioxidant activity [43]. Few more studies from different regions confirm that the seed extract of *L. sativum* possesses significant amount of antioxidants and antioxidant activity [32, 39, 44, 74, 114, 115].

5.5. Antimicrobial. Presently, due to several environmental, biological, physical, chemical, and anthropogenic factors, the incidences of pathogenic microorganisms are increasing constantly, and this became a major concern among several scientific communities [116, 117]. The plant serves as a source of secondary metabolites which possess low or no side effects with other nutritional benefits. The antimicrobial activity of numerous medicinal plants has been studied against a range of microorganisms including bacteria, yeast, fungi, and virus, and many research groups are working continuously to discover novel antimicrobial compounds.

Hussain, Khattak, Muhammad, Khan, Khan, Ullah, and Haider [58] studied the antimicrobial activity of aqueous and chloroform plant extracts of *L. sativum* against a few bacterial strains including *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus, Escherichia coli*, and two fungal strains, *Aspergillus niger* and *Candida albicans* by the agar well diffusion method [58]. The result showed that both the extracts possess antimicrobial activity against all the bacterial and fungal strains; however, the maximum and minimum zone of inhibition (ZI) for chloroform extract was shown by *P. vulgaris* (13 mm) and *S. typhi* (1 mm) and for aqueous extract, the maximum and minimum ZI was observed in *P. vulgaris* (16 mm) and *E. coli* (2 mm) [58].

The root, stem, and leaves were extracted with methanol, ethyl acetate, chloroform, and aqueous solvents and evaluated for antimicrobial activity. All extracts showed antimicrobial activity against bacterial strains (*E. coli*, *S. aureus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*) and fungal strains (*Aspergillus flavus*, *Aspergillus fumigatus*, *A. niger*, and *Fusarium solani*) [118]. Plant extract of *L. sativum* (ethanolic and aqueous) also showed antimicrobial activity against *Proteus* spp., *S. aureus*, and *Streptococcus* mutants by well diffusion method, whereas by minimum inhibitory concentrations (MIC), all the strains (*K. pneumoniae*, *Proteus spp.*, *S. mutans*, *P. aeruginosa*, and *Staphylococcus aureus*) found to be sensitive to all concentrations (12.5%, 25%, 50%, 75%, and 100%) of the aqueous and ethanolic extracts of *L. sativum* [119].

The sprouts (dried and freeze dried) extract of the plant (*L. sativum*) is also examined for their antimicrobial activity against *K. pneumoniae*, *Proteus mirabilis*, *S. aureus*, *Salmonella infantis*, *S. epidermidis*, *E.coli*, and *P. aeruginosa* through well diffusion method [66]. Among dried and freeze-dried sprout extracts, the best result was observed in freeze-dried sprout extract showing maximum activity for *S. aureus* (21.5 mm), no activity was seen against *K. pneumoniae* and *E. coli*, and the MIC value for freeze-dried extract ranges between 0.5 and 1 mg/mL [66].

Ibrahim and Kebede [120] evaluated the antibacterial activities of aqueous and methanolic extracts of leaves of *L. sativum* against human pathogenic bacteria (*S. aureus*, *S. typhi*, *Streptococcus agalactiae*, *Shigella boydii*) [120]. Along with leaves, different seed extracts of *L. sativum* showed potential antimicrobial activity against a series of microbial strains (Table 6).

Gacemi et al. [121] reported the antifungal activity of lepidines B and E and compounds present in seeds of *L. sativum* against of *C. albicans*. The seed oil of *L. sativum* possesses antifungal and antibacterial activity against *S. aureus*, *B. subtilis*, *P.aeruginosa*, *E. coli*, *Salmonella enterica*, and *C. albicans*. The essential oil extracted by clevenger type apparatus from seeds of *L. sativum* showed the best activity at 1 mg/mL of concentration against *S. aureus* (15.57 ± 0.46 mm ZI), *B. cereus* (13.12 ± 1.16 mm ZI), *E. coli* (9.78 ± 065 mm ZI), and *K. pneumoniae* (8.17 ± 0.32 mm ZI) by disc diffusion assay [121].

5.6. Gastroprotective. Gastrointestinal infections are one of the most common problems in tropical countries. They involve various parts of the gastrointestinal tract and organs like the pancreas, liver and gallbladder [122]. They are responsible for causing problems like diarrhoea, abdominal distention, intestinal obstruction, abdominal pain, and gastrointestinal bleeding [123]. Gastrointestinal diseases directly or indirectly have an economic impact and also alter the quality of life Natural active compounds possess preventive and healing activity against gastrointestinal diseases [122, 124].

The methanolic extract of seed of *L. sativum* at 50, 100, and 200 mg/kg p.o. concentration was investigated for antidiarrheal activity against castor oil-induced diarrhoea in Swiss albino and Wistar rat models [125]. The highest antidiarrheal activity was observed in 200 mg/kg of concentration.

Rehman et al. [126] investigated the antidiarrheal and antispasmodic activities of seed extract of *L. sativum* against castor oil-induced diarrhoea in Sprague Dawley rat model at 100-300 mg/kg of doses [126]. The crude extract of seed was found to possess significant antidiarrheal and antispasmodic activity.

Another study by Mehmood et al. [127] investigated the aqueous-methanolic seed extract of *L. sativum* for indigestion and constipation at 30 and 100 mg/kg of doses in BALB/c mice, guinea pigs, and rabbits. The study showed the laxative and prokinetic effects of *L. sativum* seeds in the mice model [127].

5.7. Fracture/Bone Healing. Fracture healing or bone healing is a complicated physiological process that requires the participation of hematopoietic and immune cells in the bone marrow. Medicinal plants have important properties to reduce inflammation and pain of fractures and also help in fracture fast recovery [133, 134].

The impact of *L. sativum* seeds on fracture induced bone healing in rabbit (*Oryctolagus cuniculus*) model was evaluated. The test group had a statistically significant increase in the healing of fractures compared with the control group. The results showed the significant effect of *L. sativum* seeds in fracture induced bone healing [135]. Yadav et al. [136]

Table 6: Antimicrobial activities of different extracts of *L. sativum*.

Extract/concentration	Control drug used	Microorganisms	Agar well diffusion method/agar disc diffusion method ZI (mm); control drug (ZI)	MIC/MBC (mg/mL)	Regions	References
Chloroform extract (100 mg/mL)	Gentamicin	*Escherichia coli*	NZ; 22	NT	India	[128]
		Salmonella typhi	NZ; 24			
		Pseudomonas aeruginosa	NZ; 21			
		Staphylococcus aureus	10; 25			
		Bacillus cereus	NZ; 28			
		Micrococcus luteus	11; 27			
Ethyl acetate extract (100 mg/mL)	Gentamicin	*Escherichia coli*	14; 22	NT		
		Salmonella typhi	NZ; 24			
		Pseudomonas aeruginosa	NZ; 21			
		Staphylococcus aureus	18; 25			
		Bacillus cereus	NZ; 28			
		Micrococcus luteus	16; 27			
Methanol extract (100 mg/mL)	Gentamicin	*Escherichia coli*	14; 22	6.25/25		
		Salmonella typhi	13; 24	25/ND		
		Pseudomonas aeruginosa	14; 21	6.25/25		
		Staphylococcus aureus	22; 25	1.56/6.25		
		Bacillus cereus	16; 28	6.25/25.0		
		Micrococcus luteus	16; 27	12.5/ND		
Dichloromethane extract (100 mg/mL)	Gentamicin	*Escherichia coli*	NZ; 22	NT		
		Salmonella typhi	NZ; 24			
		Pseudomonas aeruginosa	NZ; 21			
		Staphylococcus aureus	NZ; 25			
		Bacillus cereus	NZ; 28			
		Micrococcus luteus	10; 27			
Petroleum ether extracts (2.5%)	Gentamicin/ Ketoconzol	*Staphylococcus aureus*	25; 32			
		Escherichia coli	25; 32			
		Klebsiella pneumoniae	26; 35			
		Proteus vulgaris	21; 34			
		Pseudomonas aeruginosa	18; 32			
		Candida albicans	32; 33			
Methanolic extract (2.5%)	Gentamicin/ Ketoconzol	*Staphylococcus aureus*	15; 32	NT	Sudan	[129]
		Escherichia coli	17; 32			
		Klebsiella pneumoniae	16; 35			
		Proteus vulgaris	18; 34			
		Pseudomonas aeruginosa	17; 32			
		Candida albicans	9; 33			
Aqueous extract (10%)	Gentamicin/ Ketoconzol	*Staphylococcus aureus*	NZ; 32			
		Escherichia coli	19; 32			
		Klebsiella pneumoniae	17; 35			
		Proteus vulgaris	19; 34			
		Pseudomonas aeruginosa	16; 32			
		Candida albicans	21; 33			
Ethanolic extract (1 mg/mL)	Not mentioned	*Staphylococcus aureus*	10*	NT	Egypt	[54]
		Listeria monocytogenes	10*			
		Salmonella Enteritidis	20*			
		Escherichia coli	12*			
		Serratia marcescens	7			
Methanol extract (1 mg/mL)	Not mentioned	*Salmonella Enteritidis*	15			
		Serratia marcescens	9			
Methanol extract (100 mg/mL)	Gentamicin/ nystatin	*Escherichia coli*	14; 32	NT	Sudan	[130]
		Staphylococcus aureus	13; 35			
		Bacillus subtilis	13; 29			
		Candida albicans	14; 17			
		Aspergillus niger	20; 20			

Table 6: Continued.

Extract/concentration	Control drug used	Microorganisms	Agar well diffusion method/agar disc diffusion method ZI (mm); control drug (ZI)	MIC/MBC (mg/mL)	Regions	References
Ethanol extract (50 mg/mL)	Vancomycin	*Escherichia coli* *Pseudomonas aeruginosa*	22.63; 18 10; NA			
Methanol extract (50 mg/mL)	Vancomycin	*Escherichia coli* *Pseudomonas aeruginosa*	22.37; 18 9; NA	NT	Ethiopia	[131]
Chloroform extract (50 mg/mL)	Vancomycin	*Escherichia coli* *Pseudomonas aeruginosa*	10.67; 18 11.33; NA			
Ethyl acetate extract	Erythromycin	*Rhodococcus equi*	15.5; 23			
Methanolic extract	Chlorophénicol	*Rhodococcus equi*	13.15; 34	NT	Morocco	[18]
Petroleum ether extract	Ciprofloxacine	*Rhodococcus equi*	10.35; 30			
Ethanol extract	Not mentioned	*Pseudomonas aeruginosa* *Klebsiella pneumonia* *Escherichia coli* *Staphylococcus aureus* *Streptococcus pyogenes* MβL, *P. aeruginosa* ESβL, *E. coli* MRSA MDR *S. pyogenes*		12.5/25 6.25/12.5 3.13/3.13 6.25/6.25 50/50 25/25 12.5/12.5 12.5/25 100/50		
Acetone extract	Not mentioned	*Pseudomonas aeruginosa* *Klebsiella pneumonia* *Escherichia coli* *Staphylococcus aureus* MβL, *P. aeruginosa* ESβL, *Klebsiella pneumonia* ESβL, *E. coli* MRSA	NT	25/25 12.5/25 6.25/12.5 3.13/6.25 25/50 12.5/12.5 12.5/25 12.5/25	Egypt	[132]
Aqueous extract	Not mentioned	*Pseudomonas aeruginosa* *Escherichia coli* *Staphylococcus aureus* *E. coli* MRSA		25/25 6.25/6.25 6.25/12.5 12.5/25		
n-Butanol extract	Not mentioned	*Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus* (methicillin-sen.) *Staphylococcus aureus* (methicillin-res.) *Enterococcus faecalis*	NT	5 4 4 4 3.5	Algeria	[64]

Abbreviations: NZ: no zone of inhibition; NA: not applicable; NT: not tested; MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration.

reported the effect of ethanol seed extract (400 mg/kg p.o.) of *L. sativum* on fracture healing in the Wistar rat model.

The osteoprotective effect of *L. sativum* seeds (doses: 50-100 mg/kg) was studied in an ovary ectomized Wistar rat model [61]. Results revealed the antiosteoporotic actions of *L. sativum* with improved perpendicular and longitudinal femur compression strength.

Extract also enhanced the osteocalcin levels, and serum bone formation biomarkers lactate dehydrogenase (LDH) activity and inhibit the glutathione peroxidase (GPx) activity and deposition of lipid peroxides in bone tissues [61].

L. sativum showed a promising protective effect with no side effects against glucocorticoid-induced bone resorption in guinea pigs [137] and accelerates the alveolar bone healing and improves the formation of bone in periodontal diseases [138]. Alharbi et al. [139] investigated the in vivo effect of *L. sativum* seeds in osteogenic enhancement in bone fractures induced in *O. cuniculus* and concluded that the seeds can be used in the treatment of bone fractures [139].

6. The Challenge of Standardizing Extract, Toxicity, and Bioavailability of the Extract

Medicinal plants have shown immense pharmacological activities like fungicidal, bactericidal, virucidal, analgesic, anticancer, anti-inflammatory, neuroprotective, sedative,

and antioxidant, due to the presence of significant phytochemicals or active compounds including flavonoids, phenols, terpenoids alkaloids, tannins, saponins, and glycosides [140–143].

Presently, excessive use of synthetic drugs and antibiotics has developed serious side effects, toxicity, and resistance against pathogenic microorganisms, which has limited their use in many countries; therefore, researchers are now paying more attention to traditional herbal medicines and their active compounds to fight against diseases and disorders [144–146].

One of the main clinical challenge is the reduced bioavailability and absorption of bioactive compounds from plants. As a result, their inclusion in nanoformulations with increased absorption, bioavailability, and transport to the target was the optimal therapeutic solution.

Bloukh et al. (2021) evaluate the antimicrobial potential of *Lepidium sativum* silver nanoparticles against a series of microbes by using agar well and disk diffusion assays. Pure extract and *Lepidium sativum* silver nanoparticle formulations displayed a significant antimicrobial activity (very good to intermediate) against 10 microbial strains (*S. pneumoniae, S. aureus, S. pyogenes, E. faecalis, B. subtilis, P. mirabilis, P. aeruginosa, E. coli, K. pneumoniae, C. albicans*) at the concentrations of 1.08 μg/mL, 0.54 μg/mL, and 0.27 μg/mL [147].

Yasin et al. [148] evaluated the cytotoxicity of nanocapsulated lectin isolated from *L. sativum* against hepatocellular carcinoma cells (HepG2). The methanolic seed extract of *L. sativum* showed anticancer activity against in vivo Ehrlich ascite carcinoma (EAC) cell lines in Swiss albino mice at 500 mg/kg body weight of concentration [149].

L. sativum seed acetone extract and its combination with biogenic silver nanoparticles were found to be nontoxic to splenic cells [90].

7. Concluding Remarks

The current review discussed the traditional uses, nutritional values, chemical composition, and biological activity of *L. sativum*. Under this study, we summarized the presence of important minerals (potassium, calcium, phosphorus, iron, etc), amino acids (glutamic acid, leucine, etc.), fatty acid and essential oils (oleic acid, linoleic acid, linolenic acid, alpha-pinene, gamma-terpinene, alpha-terpineol, sabinene, alpha-phellandrene, etc.), and other secondary metabolites like campesterol, glucosinolates, napthalenol, furfural, coumarin, flavonoid, and phenolic acid in different extracts of *L. sativum*. The study also shows that it is an important edible herb that possesses wide range of therapeutic properties and high nutraceutical potential and can be used against malnutrition. However, most of the studies are restricted to in vitro studies and very few in vivo. Therefore, further research is needed to develop new phytopharmaceuticals based on *L. sativum*, and well-designed clinical studies are necessary to validate the biological activities reported in preclinical models mentioned in this review. Other than these scientific perspectives, people participation is needed regarding the planting, conservation, and sustainable use of *L. sativum* as a source of nutritionally rich food. Based on the scientific evidence, it can be concluded that *L. sativum* is a rich source of nutritional components along with bioactive compounds and could be used as a functional food.

Data Availability

The data supporting this review are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Pretreatment with 6-Gingerol Ameliorates Sepsis-Induced Immune Dysfunction by Regulating the Cytokine Balance and Reducing Lymphocyte Apoptosis

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Sepsis is characterized by an initial net hyperinflammatory response, followed by a period of immunosuppression, termed immunoparalysis. During this immunosuppressive phase, patients may have difficulty eradicating invading pathogens and are susceptible to life-threatening secondary hospital-acquired infections. Due to progress in antimicrobial treatment and supportive care, most patients survive early sepsis. Mortality is more frequently attributed to subsequent secondary nosocomial infections and multiorgan system failure. 6-Gingerol is the major pharmacologically active component of ginger. Although it is known to exhibit a variety of biological activities, including anti-inflammation and antioxidation, the role of 6-gingerol in sepsis-induced immune dysfunction remains elusive. Thus, we investigated whether 6-gingerol improves septic host response to infections during sepsis. 6-Gingerol-treated mice showed significantly lower mortality in polymicrobial sepsis induced by cecal ligation and puncture LPS via enhanced bacterial clearance in the peritoneum, blood, and organs (liver, spleen, and kidney) and inhibited the production of TNF-α and IL-6 in TLR2 and/or TLR4-stimulated macrophages. In addition, we demonstrated that survival improvement of secondary infection following septic insult was associated with an initial response of enhanced neutrophil numbers and function at the infection site, reduced apoptosis of immune cells, and a shift from a T helper cell type 2 (Th2) to a T helper cell type 1 (Th1) cytokine balance in the hypoinflammation phase. Our overall findings suggest that 6-gingerol potentially restores sepsis-induced immune dysfunction by shifting the balance of Th1/Th2 and by regulating apoptosis of immune cells.

1. Introduction

The immune response in sepsis can be characterized by a proinflammatory cytokine-mediated hyperinflammatory phase and subsequent hypoinflammatory phase with significant immunosuppression [1–3]. Patients fail to eradicate invading pathogens and are susceptible to opportunistic organisms, particularly bacterial infection of the lungs in the hypoinflammatory phase [4, 5]. Over 70% of sepsis-related deaths occur during this immunosuppressive phase, typically due to secondary (opportunistic) infections such as bacterial pneumonia [4–6]. Increasing incidence of nosocomial infections and the emergence of multidrug-resistant bacteria require the study of immunomodulators in the prevention and treatment of sepsis.

Despite the development of powerful antibiotics and specialized treatment available in intensive care units, sepsis remains a serious disorder with high rates of morbidity and mortality. Sepsis, septic shock, and the ensuing multiple organ failure continue to be the most common causes of mortality in surgical intensive care units [7]. Thus, several laboratories have sought an increased understanding of sepsis and septic shock to develop new therapeutic agents that can either reduce or prevent this ever-increasing problem

in surgical critical care. The cecal ligation and puncture (CLP) rodent model has been developed as a suitable animal model for polymicrobial sepsis in humans since it can produce symptoms (i.e., appendicitis and peritonitis) similar to those of patients with sepsis [7]. The CLP model produces a hyperdynamic, hypermetabolic state that can lead to a hypodynamic, hypometabolic stage and eventual death. The early hyperdynamic stage is characterized by enhanced cardiac output, tissue perfusion, and reduced vascular resistance. The hallmark of this early stage is the proinflammatory state mediated primarily by neutrophils and macrophages, stimulated by bacteria and/or their toxins. The late hypodynamic stage includes reduced tissue and microvascular blood flow, decreased cardiac function, and enhanced indices of organ injury and dysfunction. The CLP model has been used extensively to investigate clinical manifestations of sepsis and septic shock [8, 9].

6-Gingerol (1-[4′-hydroxy-3′-methoxyphenyl]-5-hydroxy-3-decanone) is the major pungent compound in ginger (*Zingiber officinale*), which has many reported pharmacological effects, including inflammation, indigestion, and hypertension [9–11]. Several studies have demonstrated that 6-gingerol treatment reduced the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1 from LPS-stimulated macrophages [12–14]. In this study, we investigated the effect of 6-gingerol in a CLP-induced polymicrobial sepsis model, which involves various Gram-negative and Gram-positive bacteria. We found that 6-gingerol prevented polymicrobial sepsis induced by CLP through increased bacterial clearance and inhibited inflammatory cytokine production. In addition, we demonstrated that 6-gingerol improved survival of secondary infection following septic insult using the second-hit pneumonia model of sepsis. The well-accepted and widely used CLP model was used as the first hit, and *Pseudomonas aeruginosa* was used to induce pneumonia in mice after CLP as a second hit [15]. CLP induces an immunosuppressive state that leaves the host susceptible to a secondary infection [15–17]. *P. aeruginosa* has been shown to cause little to no mortality when administered alone but was shown to induce a high level of mortality when delivered as a secondary injury in a mouse model of primary peritonitis [15–17]. Therefore, we investigated the protective effect of 6-gingerol from infection, as well as its immune regulation for Th1/Th2 cytokine balance in sepsis, which helps to provide scientific insight for the development of this compound as a drug for the regulation of immune cells in sepsis. Our results demonstrate the value of developing this compound as a drug for regulating immune cells in sepsis.

2. Materials and Methods

2.1. Reagents. 6-Gingerol (Sigma-Aldrich, St Louis, MO) was dissolved to 3.5 μg/ml of 50% ethanol and diluted with drinking water, as required (the final ethanol concentration was less than 0.01%). The following antibodies were purchased from BD Pharmingen, San Diego, CA: phycoerythrin- (PE-) conjugated anti-Ly6G, fluorescein isothiocyanate- (FITC-) conjugated anti-CD11b, PE-conjugated anti-CXCR2, FITC-conjugated anti-CD4, FITC-conjugated anti-CD8, PE-conjugated anti-IFN-γ, PE-conjugated anti-CD4, PE-conjugated anti-CD8, and FITC-conjugated antiannexin V antibodies. *P. aeruginosa* (ATCC27853) was obtained from the American Type Culture Collection (ATCC). For the M1 and M2 macrophage analysis, cells were separately stained with monoclonal antibodies specific for F4/80-PE from eBioscience; F4/80-FITC and CD206-PerCP/Cy5.5 from Bio-Legend, San Diego, CA; and CD80-FITC and iNOS-FITC from BD Transduction Laboratories, San Diego, CA. Bacteria were grown in brain heart infusion (BHI) media (Difco) at 37°C for 18 h, and aliquots were frozen at −80°C.

2.2. Model of Polymicrobial Sepsis. BALB/c (7~8 weeks of age, males, average weight: 20 g) mice (Orient Bio-Charles River, Seoul, Republic of Korea) were maintained under specific pathogen-free conditions at 22°C. All animal experiments were conducted according to the regulations of the Animal Committee of the University of Ulsan. For cecal ligation and puncture (CLP), mice were anesthetized via intraperitoneal injection of a mixture of Zoletil 50 (30 mg/kg; Virbac Carros, France) and Rompun (10 mg/kg; Bayer Korea Ltd., Seoul, Korea). Under sterile conditions, a 1-2 cm incision was made in the lower left abdomen, and the cecum was exposed. The distal portion of the cecum was ligated with a 4-0 silk suture, punctured once with a 26- or 21-gauge (G) needle to induce moderate or severe sepsis, respectively [18]. For the second-hit pneumonia model of sepsis, to create a long-term infection with a relatively low mortality (<10%), we used cecal ligation and punctured once with a 29-gauge (G) needle [19]. The abdomen was closed in two layers, and 1 ml of 0.9% saline was administered subcutaneously (s.c.). Sham-operated mice were subjected to similar laparotomy without CLP. For the LPS or *Escherichia coli* (*E. coli*) model, LPS (150 μg/mouse) or *E. coli* (2×10^7 cells/mouse) was injected i.p., respectively. Survival was recorded every 12 h for 7 days. Mice were injected intraperitoneally with 6-gingerol (50 μg/mouse) or control (0.01% ethanol/mouse) at 2 days before CLP.

Mice were fed drinking water containing 3.5 μg/ml of 6-gingerol every day for 5 days before CLP, while control mice were fed drinking water containing 0.01% ethanol until the end of the experiment. Freshly made 6-gingerol-containing drinking water was provided every 2 days.

2.3. Analysis of Blood and Cells in the Peritoneal Cavity. At 4 h and 24 h after surgery, two punctures with a 26-gauge needle were made for CLP; then, mice ($n \geq 5$ mice/group) were killed and blood was collected and stored for analysis of bacteria and cytokines. The peritoneal cavities were washed with 3 ml of sterile phosphate-buffered saline (PBS), and the lavage fluids were collected. After a 10 μl aliquot of the lavage fluid was removed for assessment of bacterial colony-forming units (CFUs), the remaining fluid was centrifuged at $400 \times g$ for 5 min at 4°C, and the supernatants were collected and stored at -80°C to determine cytokine concentrations. Cell pellets were treated with red blood cell lysing buffer, washed twice, and resuspended in PBS containing 3 mM EDTA. For flow cytometry, cells were

incubated with 1 μg/ml of antimouse Fcγ MAb (clone 2.4G2) for 15 min to block nonspecific binding of mAbs and then stained with 1 μg/ml of PE-conjugated anti-Ly6G, F4/80, B220, or FITC-conjugated anti-CD11b, CD3, and annexin V antibodies and analyzed with FACS. $CD11b^+$ $Ly6G^+$ cells were considered as neutrophils and $CD11b^+$ $F4/80^+$ cells as macrophages.

2.4. Induction of Secondary Bacterial Pneumonia. To create a relevant model of secondary bacterial pneumonia, on Day 4 post-CLP, the surviving mice were anesthetized, as mentioned above. *P. aeruginosa* (ATCC 27853) suspension was used as the secondary injury [19]. Using a pipette, 20 μl of a *P. aeruginosa* suspension (8×10^7 CFU/mouse) was slowly injected intranasally (divided equally between the nares), observed to be aspirated on inhalation, and held in position for 1 min. Mice were fed drinking water containing 3.5 μg/ml of 6-gingerol, while control mice were fed drinking water containing 0.01% ethanol from Day 5 pre-CLP until the end of the secondary bacterial pneumonia experiment. Freshly made 6-gingerol-containing drinking water was provided every 2 days. Survival was recorded for 14 days following pneumonia. Animals were then sacrificed 4 days post-CLP or 4 h and 24 h after secondary injury.

2.5. Bronchoalveolar Lavage. Mice were anesthetized as mentioned previously. For bronchoalveolar lavage (BAL), animals were placed supine and the trachea was exposed by dissection. A 24-gauge angiocatheter was inserted into the trachea, and 1 ml of sterile saline was injected and immediately withdrawn. BAL fluid was placed in sterile vials and analyzed for cytokine levels, bacteria count, and immune cells.

2.6. CFU Assay. Lungs were collected in sterile phosphate-buffered saline (PBS), and extracted lobes were homogenized and serially diluted. BAL fluid or lung tissue homogenate was spread onto *BHI agar* (Difco) plates. Following overnight culture in a 37°C incubator, colonies were counted and the bacteria number was calculated. Bacteria numbers in BAL and lung were indicated as CFU/ml BAL and CFU/ml lung tissue, respectively.

2.7. Isolation of Cells and Culture. Peritoneal macrophages were isolated as previously described [20]. Peritoneal exudated cells were harvested by lavaging the peritoneal cavity with 10 ml sterile ice-cold PBS. Contaminating erythrocytes were lysed by washing cells in red blood cell lysing buffer. Cells were washed three times and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were then cultured in plastic tissue culture flasks at 37°C in a CO_2 incubator for 2 hours. The cultures were then washed three times to remove all nonadherent cells; 95% of the adherent cell population was macrophages as determined by flow cytometry. Mouse neutrophils were isolated from the bone marrow by MACS using the Anti-Ly6G MicroBead Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocols. Isolated neutrophils were 98% pure, as assessed by flow cytometry.

Macrophages and neutrophils (5×10^5 cells/well) in RPMI-1640 medium containing 10% FBS were cultured with control, 6-gingerol (1 μg/ml), Pam3 (500 ng/ml), or LPS (500 ng/ml).

2.8. Cell Depletion In Vivo. For macrophage depletion, mice were injected i.p. with clodronate liposomes (Clodrolip) (2 mg/20 g) on day 1.5 before polymicrobial sepsis induced by CLP (Day 0). Empty liposomes were used as the control. Clodrolip contains approximately 18 ± 2 mg of clodronate per milliliter (0.5 micromoles of clodronic acid disodium salt tetrahydrate, $CH_2Cl_2Na_2O_6P_2{\cdot}4H_2O$). After Clodrolip injection, liposomes were phagocytosed and digested by macrophages, followed by intracellular release and accumulation of clodronate, causing >90% depletion of macrophages within 24–36 h [21].

2.9. Cytokine Analysis. Cytokines in BAL were quantified using a cytometric bead array (CBA) kit mouse inflammation (BD Biosciences, San Diego, CA), according to the manufacturer's protocol. It was analyzed on a FACSCanto™ II (BD Biosciences, San Diego, CA) with FACSDiva software. IL-17 production was assessed according to the manufacturer's protocol (mouse IL-17 DuoSet ELISA, R&D Systems).

2.10. Assessment of Apoptosis. Apoptosis was evaluated by a FACScan flow cytometer (BD Biosciences, San Diego, CA) using the Annexin V-FITC kit (BD Biosciences).

2.11. Determination of Intracellular Cytokines. Splenocytes were cultured for 6 h with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml), and cytokine release was prevented by treatment with Golgi-stop (BD Pharmingen, San Diego, CA). Following surface staining for $CD4^+$ or $CD8^+$, cells were fixed in Cytofix/Cytoperm solution (BD Pharmingen) and stained with PE-conjugated anti-mouse IFN-γ (BD Pharmingen). Finally, cells were analyzed on a FACSCanto™ II (BD Biosciences) with FACSDiva software.

2.12. Bacterial Killing Assays. The killing of bacteria was measured as described previously [22]. Briefly, macrophages were mixed with opsonized *E. coli* at an MOI of 40 : 1 and incubated at 37°C for 10 min with continuous rotation. Non-ingested bacteria were discarded by centrifugation, and cells were cultured with 6-gingerol for the next 60 min with slow rotation. Killing was stopped by spinning the cells onto ice after the addition of 1 ml distilled water containing 0.01% bovine serum albumin, and the number of viable bacteria was determined by plating tenfold serial dilutions. The percent killing was calculated as $[1 - (\text{number of viable bacteria at 60 min/number of viable bacteria at 0 min})] \times 100$.

2.13. Measurements of ROS Generation. Purified macrophages were stained with 2 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, MO) for 20 min at 37°C in the dark. After incubation, the macrophages were washed twice with PBS, incubated with control or 6-gingerol (1 μg/ml) and *E. coli* in an MOI of 20 : 1 at 37°C for 1 h, and analyzed by FACS. Reactive oxygen species

(ROS) production was expressed as mean fluorescence intensity (MFI). To prevent ROS production, macrophages were preincubated with 10 μM of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase inhibitor diphenyleneiodonium chloride (DPI; Sigma) for 1 h at 37°C before the addition of 6-gingerol.

2.14. Histology. For histological examination, lung tissue was fixed with 10% neutral buffered formaldehyde and embedded in paraffin. The section was deparaffinized and stained with hematoxylin and eosin (H&E) solution.

2.15. Statistical Analysis. All data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Data obtained from two and three groups were analyzed by *t*-test and one-way ANOVA with post hoc Tukey's test, respectively. Survival curves were compared by using the log-rank test.

3. Results

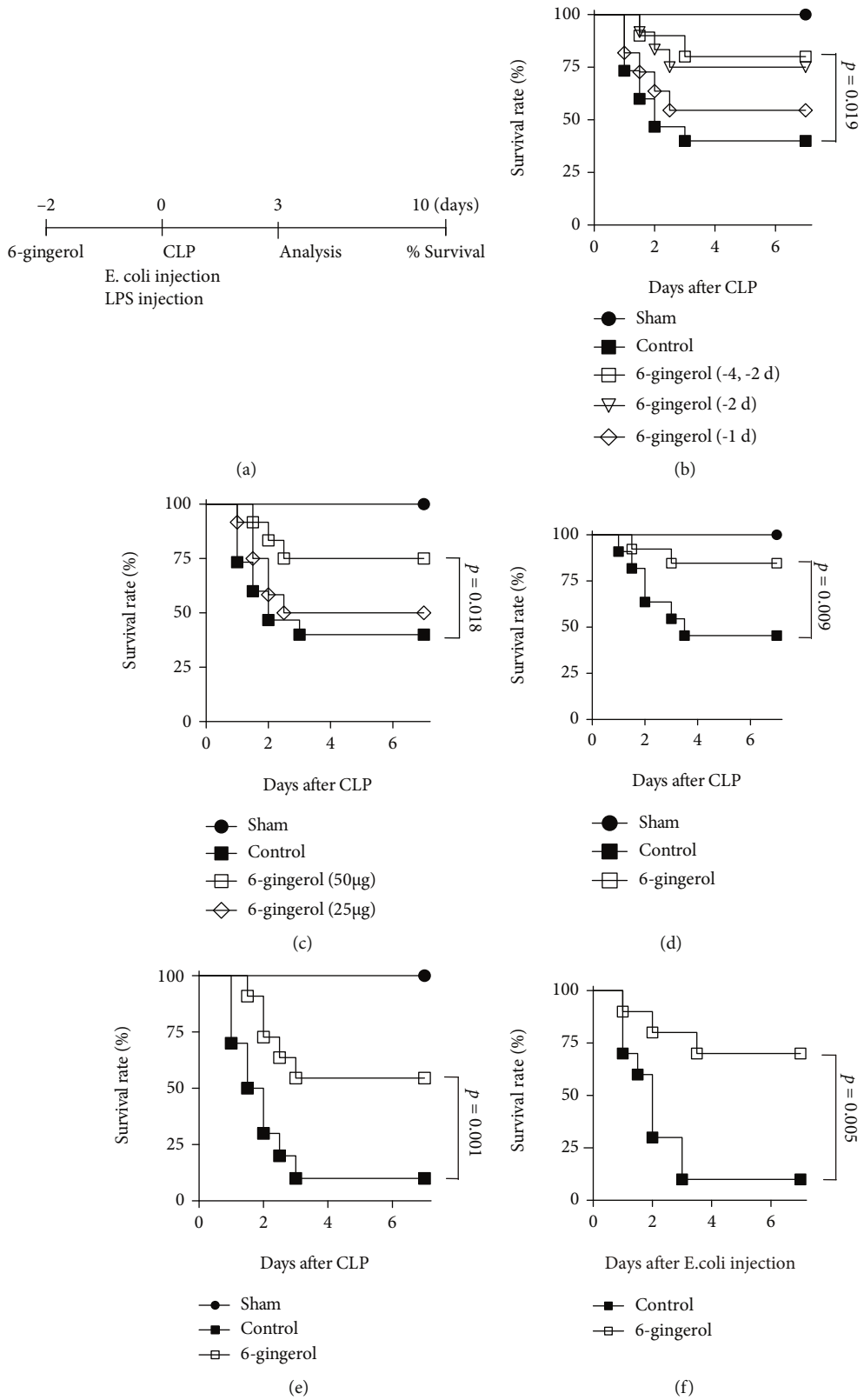
3.1. Administration of 6-Gingerol Protected against Sepsis-Induced Mortality. To investigate whether 6-gingerol has a protective effect on polymicrobial sepsis, CLP was performed on BALB/c mice, and their survival was monitored for up to 7 days. In the moderate CLP experiment, mice were subjected to CLP using 26-gauge needles and two punctures. To examine the effect of 6-gingerol treatment, mice were i.p. injected with 6-gingerol (50 μg/mouse) or control (0.01% ethanol) before CLP. As shown in Figure 1(b), treatment with 6-gingerol 2 days before CLP significantly increased mouse survival, compared with the control. By Day 7 post-CLP, 75% of the 6-gingerol-treated mice were still alive (9 of 12 mice), compared to 40% of the control-treated mice (6 of 15 mice). When 6-gingerol was injected 2 and 4 days before CLP, the protective effect was similar to that observed following injection of 6-gingerol 2 days before CLP. Figure 1(c) shows that treatment with 6-gingerol significantly increased mice survival in a dose-dependent manner. On Day 7 post-CLP, the survival rate for mice treated with 50 μg/mouse 6-gingerol was 75% (9 of 12 mice), while that for mice treated with 25 μg/mouse 6-gingerol was 50% (6 of 12 mice). For mice fed drinking water containing 3.5 μg/ml of 6-gingerol every day for 10 days before CLP, survival also increased, compared with the control (Figure 1(d)). Only 45% of the control-treated mice (5 of 11 mice) survived, compared to 85% of the 6-gingerol-treated mice (11 of 13 mice). In the severe sepsis experiment (using 21 G needles and two punctures), the 6-gingerol-treated mice were also more resistant to sepsis than control-treated mice (Figure 1(e)). On Day 7 post-CLP, 55% of the 6-gingerol-treated mice (6 of 11 mice) were still alive, compared to 10% of the control-treated mice (1 of 10 mice).

Sham surgery did not cause any mortality, and the survival of 6-gingerol-treated mice with a C57BL/6 background with CLP-induced sepsis was also higher than that of control-treated C57BL/6 mice with CLP-induced sepsis, indicating the 6-gingerol effects were not mouse strain-specific (data not shown). *E. coli* is the most common cause of Gram-negative bacteria-induced sepsis. Similarly, LPS-induced endotoxemia is marked by the activation of inflammatory responses, which can lead to shock, multiple organ damage, and even death. Therefore, *E. coli* and LPS were used to evaluate the protective effects of 6-gingerol in other sepsis mouse models. Figure 1(f) showed that treatment with 6-gingerol increased the survival of *E. coli*-infected mice. On Day 7 post-*E. coli* infection, the survival rate for mice treated with 6-gingerol was 70% (7 of 10 mice), while that for control mice was only 10% (1 of 10 mice). Moreover, 6-gingerol treatment also reduced the mortality of LPS-injected mice (Figure 1(g)). The survival rate for 6-gingerol-treated mice on Day 7 was 66% (8 of 12 mice) in septic shock induced by LPS, while survival in control-treated mice was only 23% (3 of 13 mice).

3.2. Administration of 6-Gingerol Increased Immune Cell Infiltration and Bacteria Clearance and Reduced Cell Apoptosis. We investigated whether the survival of 6-gingerol-treated mice was higher than that of the control-treated mice, and whether survival was associated with enhanced bacterial clearance. As shown in Figure 2(a), bacterial clearance in the organs of 6-gingerol-treated mice in the CLP model was greater than that in the organs of control-treated mice. At 24 h post-CLP, CFUs of bacteria in the blood, peritoneum, and organs (liver, spleen, and kidney) of 6-gingerol-treated mice were 66 to 88 times lower than those in the control-treated mice.

In the CLP-induced sepsis model, neutrophils and macrophages are the first recruited into the peritoneum and play an important role in host defense [23–25]. As shown in Figure 2(b), at 24 h post-CLP, 6-gingerol-treated mice had a higher number of infiltrated peritoneal cells, including neutrophils and macrophages, than in control-treated mice. T- and B cell numbers were similar between 6-gingerol-treated mice and control-treated mice.

Apoptosis of neutrophils and macrophages in sepsis induces defects in immunity and has been considered a critical factor in determining sepsis-induced mortality. As shown in Figure 2(c), CLP-induced sepsis caused neutrophil and macrophage apoptosis in the peritoneum; however, this effect was inhibited by 6-gingerol treatment. The 6-gingerol-treated mice had a lower level of annexin V expression on neutrophils and macrophages than in control-treated mice. To determine whether macrophages were required for resistance to CLP-induced sepsis in 6-gingerol-treated mice, we depleted macrophages by *in vivo* injection of clodronate liposomes. Following macrophage depletion, the bacterial load in the peritoneum or liver did not change in comparison to the control-treated mice (Figure 2(d)). In addition, macrophages ingest microorganisms via phagocytosis, and the ingested bacteria were killed by ROS derived from superoxide, which is produced by an activated, phagosome-bound NADH oxidase [25–27]. We evaluated whether the enhanced bacterial clearance in 6-gingerol-treated macrophages was involved in ROS generation. Cells were preincubated with ROS inhibitor, DPI before incubation with 6-gingerol, and *E. coli*, and antibacterial activities were determined. As shown in Figure 2(e), 6-gingerol enhanced bacterial killing activity



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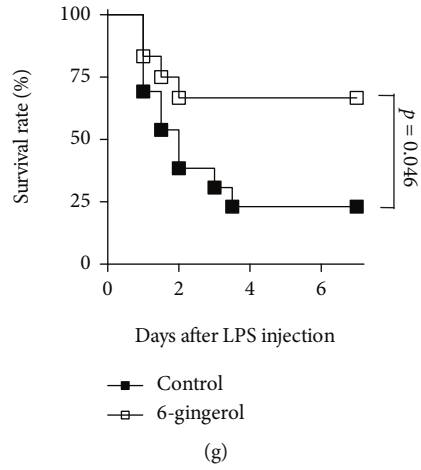


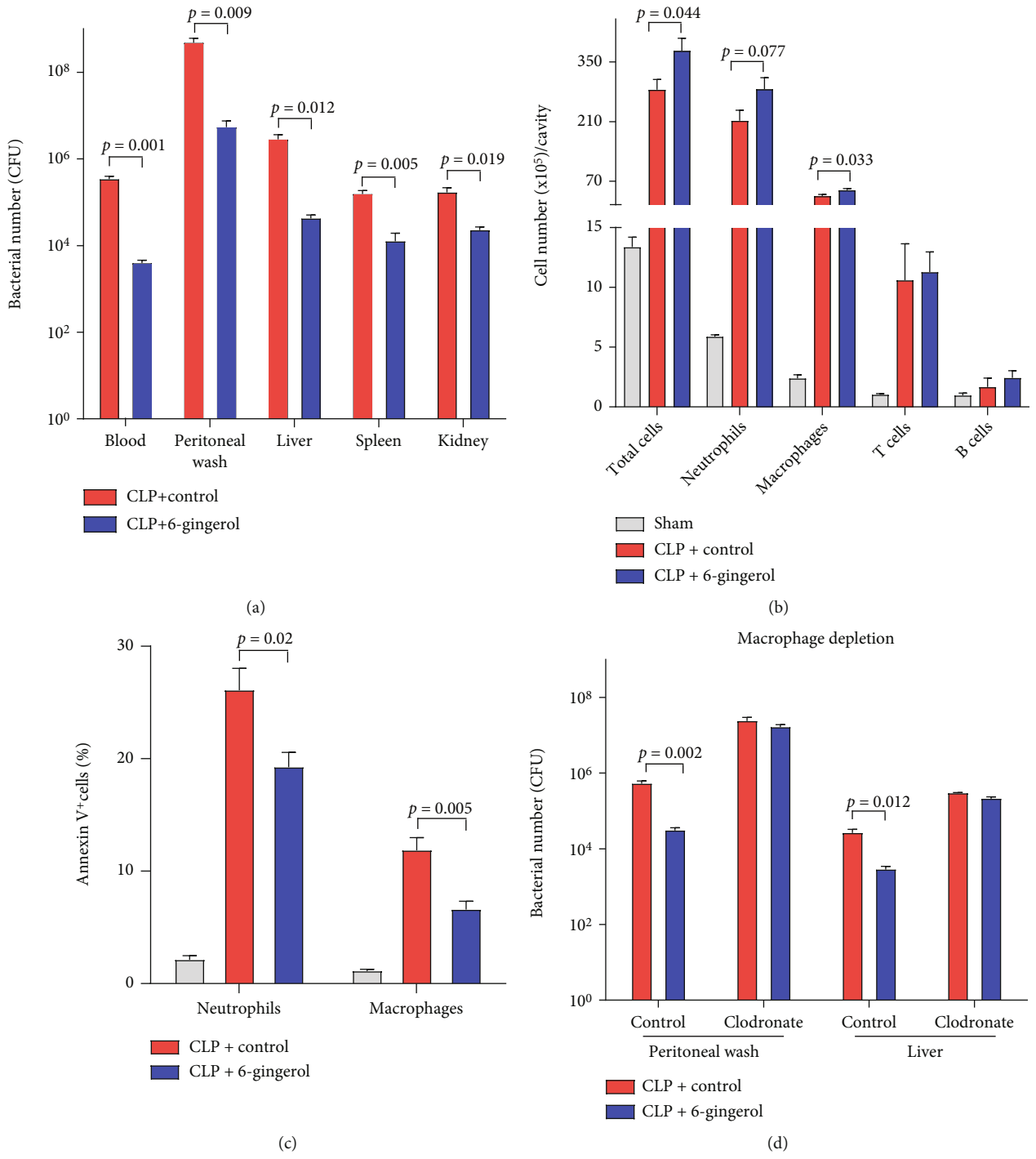
Figure 1: Administration of 6-gingerol protected mice from septic death. (a) Schematic diagram for the model of polymicrobial sepsis. (b–d) Moderate sepsis in mice was induced using 26-gauge needles with two punctures on Day 0. (b) 6-Gingerol (50 μg) was treated at indicated times before CLP. (c) 6-Gingerol was i.p. injected into mice at indicated dose before CLP. (d) Mice were fed drinking water containing 3.5 μg/ml of 6-gingerol, while control mice were fed drinking water containing 0.01% ethanol every day for 7 days before CLP. (e) Mice were i.p. injected with control or 6-gingerol (50 μg). After 2 days, mice were subjected to severe CLP using 21-gauge needles with two punctures. (f, g) Sham mice were subjected to only laparotomy without CLP. Control or 6-gingerol (50 μg) treatment was i.p. injected into mice Day 2 before peritoneal injection with *E. coli* (2×10^7 cells/mouse) (f) or with LPS (150 μg/mouse) (g). Mouse survival was monitored every 12 h for 7 days. Each group contained 10 to 15 mice, and results of two or three experiments were pooled. The curve comparison with the log-rank (Mantel-Cox) test revealed statistically significant differences as shown on the graph.

and ROS production by up to 68% or 65% in macrophages, respectively. However, DPI treatment inhibited 6-gingerol-induced effects. This indicates that 6-gingerol-induced antibacterial activities are, in part, regulated by the production of ROS in macrophages. Our results thus support the notion that macrophages may play a critical role in the resistance to CLP-induced sepsis in 6-gingerol-treated mice.

3.3. 6-Gingerol Inhibited Production of Inflammatory Cytokines. Sepsis is associated with high cytokine levels and sustained infection, which contribute to multisystem organ failure, the direct cause of mortality [28, 29]. Studies have shown that the production of inflammatory cytokines and chemokines are augmented during CLP-induced sepsis and are associated with mortality when they are expressed at high levels [30, 31]. Therefore, we determined the effect of 6-gingerol treatment on inflammatory cytokine/chemokine levels after CLP. Cytokine profiles of sham, control, and 6-gingerol-treated mice were measured at 4 or 24 h after surgery (Figure 3(a)). Sham surgery did not cause a significant rise in cytokine production. Inflammatory cytokine/chemokine (TNF-α, IL-6, MCP-1, and IL-10) levels at 24 h were higher than those at 4 h post-CLP. Inflammatory cytokine/chemokine levels in the peritoneum (Figure 3(a), top panels) and serum (Figure 3(a), bottom panels) of 6-gingerol-treated mice were significantly lower than the levels in the peritoneum and serum of control-treated mice at 24 h post-CLP. Only IL-6 levels in septic 6-gingerol-treated mice were significantly lower than those in control-treated mice at 4 h post-CLP. These results indicate that the inflammatory response to sepsis in 6-gingerol-treated mice was less vigorous than that in control-treated mice, which may be one of the reasons for less severe organ failure and decline in mortality in septic 6-gingerol-treated mice.

Polymicrobial sepsis was induced by CLP, which involves various Gram-negative (G-) and Gram-positive (G+) bacteria. Of all identified TLRs, it is known that TLR2 and TLR4 differentially recognize G+ and G- bacteria. TLR4 recognizes LPS, the most potent immunostimulant of Gram-negative bacteria; whereas, TLR2 plays a major role in detecting G+ bacteria by recognizing lipoproteins and lipoteichoic acid [24]. In order to evaluate the effect of 6-gingerol on inflammatory cytokine production by macrophages and neutrophils, purified cells were incubated with combinations of 6-gingerol, Pam3, and LPS. Cytokines produced in culture supernatants were then determined. The release of TNF-α and IL-6 from macrophages (Figure 3(b)) or the release of TNF-α and IL-10 from neutrophils (Figure 3(c)) by Pam3 and LPS was inhibited by 6-gingerol treatment. Based on changes to the cytokine profile after 6-gingerol treatment, we investigated the effects of 6-gingerol on M1 and M2 macrophage polarization. Flow cytometry data indicated that 6-gingerol could decrease the ratio of $CD80^+/F4/80^+$ or $iNOS^+/F4/80^+$ and significantly increase the expression of $CD206^+/F4/80^+$ than in the control group (Figures 3(d) and 3(e)). The results suggest that 6-gingerol inhibits sepsis-induced hyperpolarization of M1 and promotes M2 phenotypic alteration in macrophages.

3.4. Administration of 6-Gingerol Improved Survival and Bacterial Clearance to Intranasal P. aeruginosa Infection in Postseptic Mice. To investigate the role of 6-gingerol in host defense against secondary *P. aeruginosa* pneumonia after sepsis, we first established that these polymicrobial sepsis-induced mice had survival rates of >90% following CLP. Mice were subjected to CLP, via one puncture with a 29-gauge needle. This level of injury was utilized in order to create a prolonged infection with relatively low mortality



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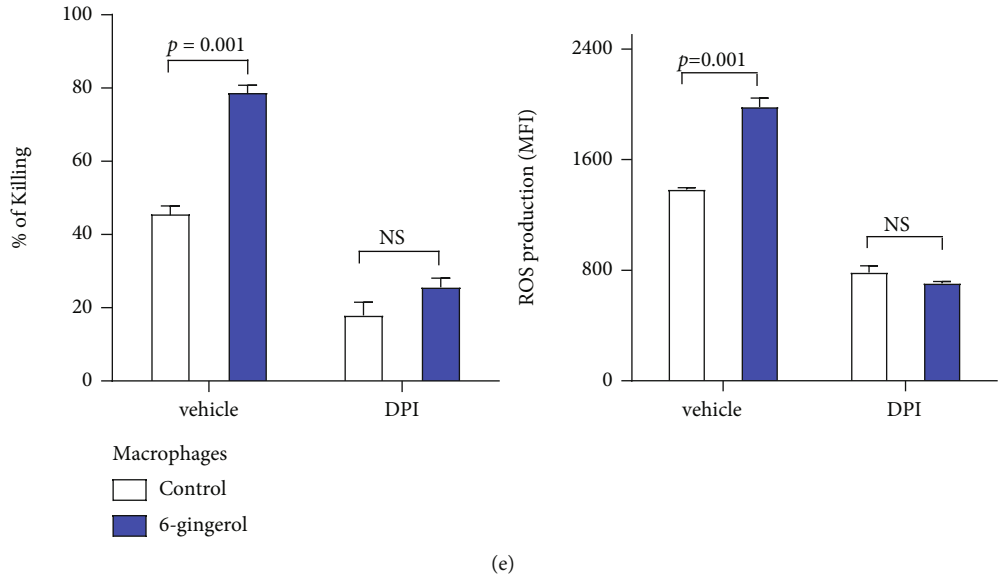


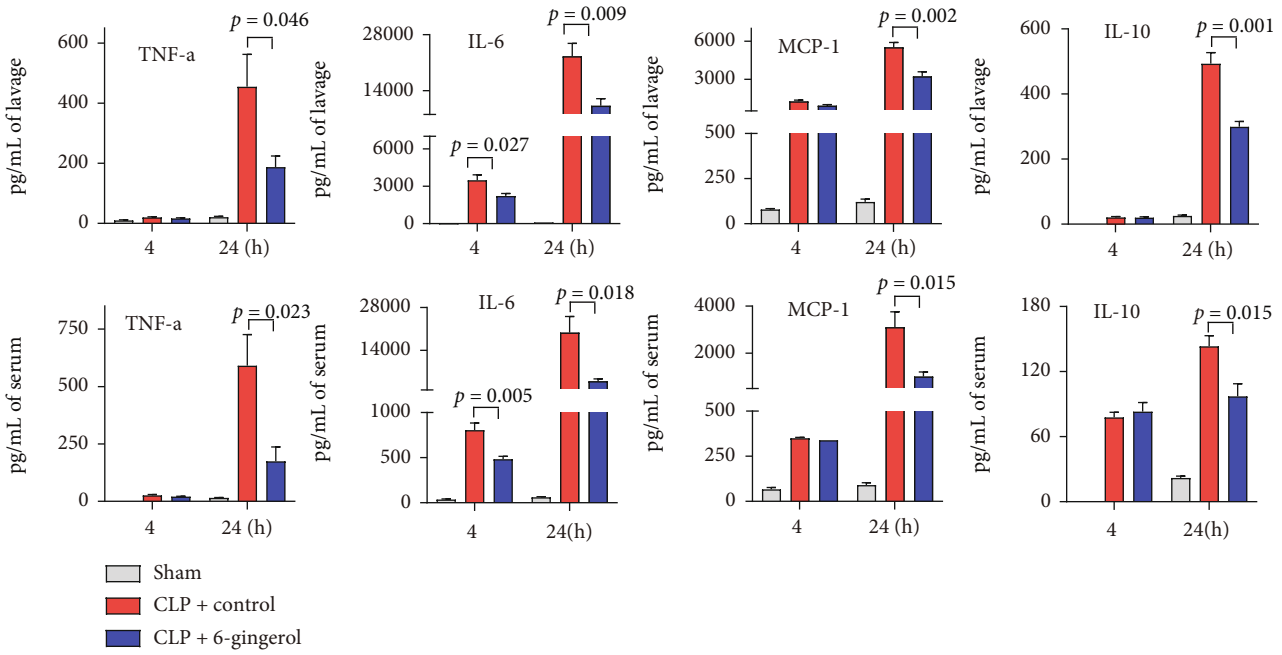
(e)

Figure 2: Administration of 6-gingerol increased immune cell infiltration and bacteria clearance and reduced cell apoptosis. Mice were i.p. injected with control or 6-gingerol (50 μg). After 2 days, mice were subjected to CLP using 26-gauge needles with two punctures. (a–c) Bacterial load (a), cell recruitment to peritoneum (b), and cell apoptosis (c) were determined at 24 h after CLP, as described in Materials and Methods. (d) Mice were i.p. injected with control or 6-gingerol (50 μg) on Day 2 before CLP and then injected with clodronate liposomes to deplete macrophages. After depletion, mice were subjected to CLP using 26-gauge needles with single punctures on Day 0. Twenty hours after CLP, bacterial load was evaluated by colony plating assay. (e) Macrophages were preincubated with ROS inhibitor DPI (10 μM) for 1 h before 6-gingerol (1 μg/ml) treatment. Bacterial killing activities (left panel) and ROS production (right panel) by macrophages against *E. coli* were determined. Data are the means and standard errors of means obtained for individual mice. Data are representative of at least three independent experiments in which each group contained four to six mice. Statistically significant differences are shown on the graph. NS: not significant.

(<10%). Mice were administrated 3.5 μg/ml of 6-gingerol contained in drinking water daily. Because each mouse used in our experiments weighed approximately 20 g and consumed 3-4 ml of drinking water per day, dosages of 6-gingerol were 525-700 μg/kg body weight/day. Control mice were fed water containing 0.01% ethanol. Survivors on day 4 after CLP were then subjected to intranasal instillation of a *P. aeruginosa* bacterial load of 1.6×10^8 CFU. Postseptic control mice displayed high susceptibility to secondary pneumonia (mortality rate, 50%). In contrast, 6-gingerol-treated mice displayed resistance to secondary pulmonary infection, with survival rates of 90% (Figure 4(b)).

To understand how 6-gingerol treatment confers protection against secondary *P. aeruginosa* pneumonia, we assessed lung clearance of *P. aeruginosa* through quantitative bacterial cultures of BAL fluids and lung homogenates collected 24 hours after intranasal instillation. Bacterial clearance significantly improved in 6-gingerol-treated mice compared to control mice (Figures 4(c) and 4(d)). In addition, microscopic analysis of lung sections stained with hematoxylin-eosin showed that 6-gingerol treatment attenuated lung damage in secondary *P. aeruginosa* pneumonia. Lung sections of 6-gingerol-treated mice showed significant decreases in extensive foci of consolidation with loss of pulmonary structure (Figure 4(e)). These observations indicate that 6-gingerol enhances the bacterial clearance of intranasal *P. aeruginosa* infection in postseptic mice compared to the control group, which reduced lung tissue damage and was associated with an increased survival rate.

3.5. Administration of 6-Gingerol Increased Neutrophil Infiltration into BAL Fluid following Secondary Challenge by Intranasal Instillation of P. aeruginosa. To observe the effects of 6-gingerol in host defense against secondary *P. aeruginosa* pneumonia, we collected and analyzed immune cells in BAL fluid 4 hours after secondary *P. aeruginosa* infection. We observed a significant increase in the total number of immune cells and neutrophils in BAL fluid of 6-gingerol-treated mice when compared to control mice. The total number of white blood cells of 6-gingerol-treated mice in BAL fluid was 3.3 times that of control mice (Figure 5(a)). In BAL fluid, most infiltrated cells were neutrophils, and 6-gingerol-treated mice showed an increased percentage and number of neutrophils compared to control mice (Figures 5(b) and 5(c)). Neutrophil numbers in BAL fluid from 6-gingerol-treated mice were 3.9 times greater than those from control mice (Figure 5(c)). Because 6-gingerol-treated mice showed increased neutrophils in BAL fluid compared to control mice, we investigated whether 6-gingerol has antiapoptotic effects *in vivo*. As shown in Figure 5(d), neutrophils harvested from BAL fluid of 6-gingerol-treated mice showed significantly decreased neutrophil apoptosis than those found in control-treated mice. In addition, the migration and microbicidal activity of



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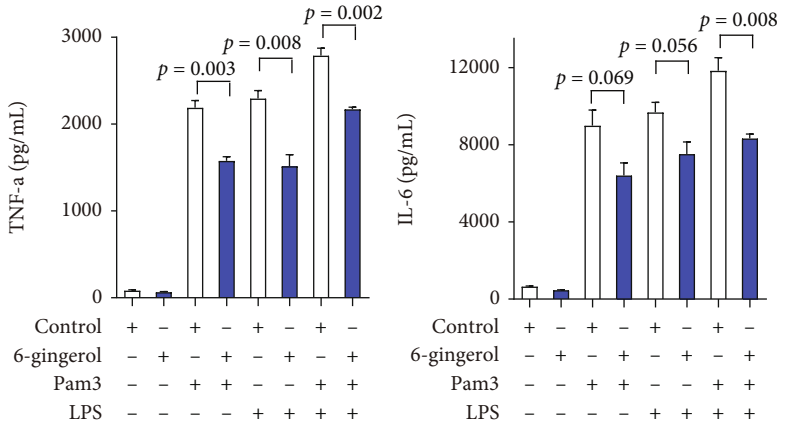

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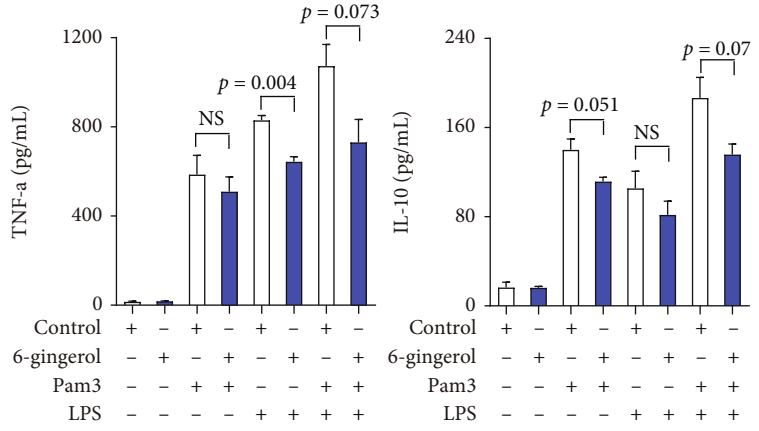

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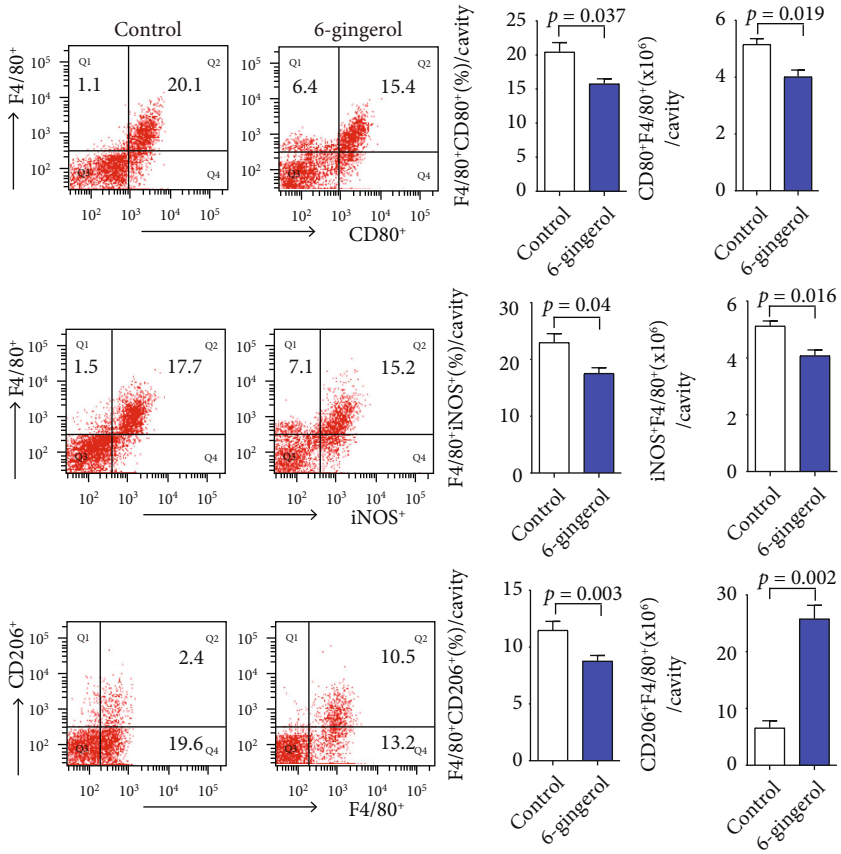

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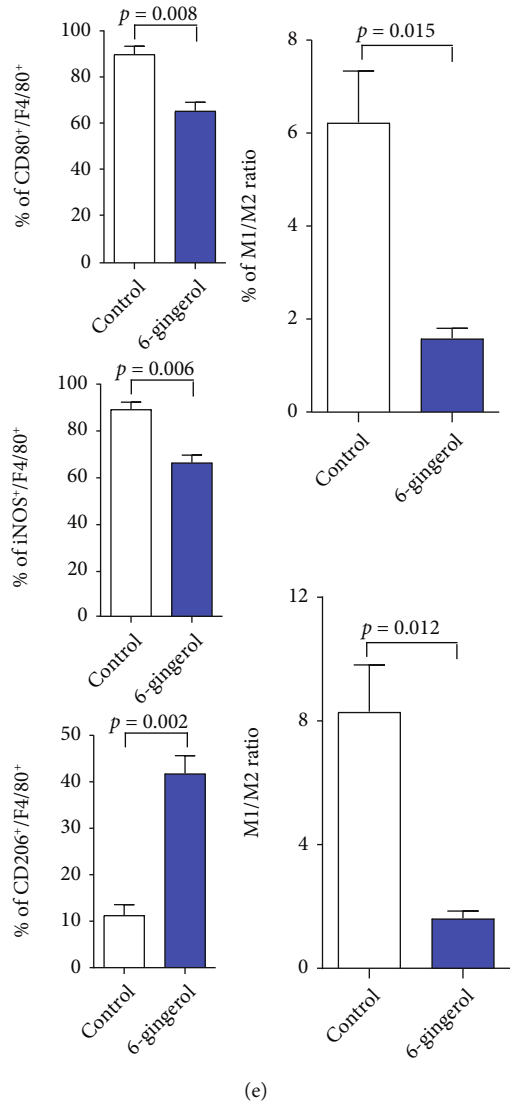


Figure 3: 6-Gingerol inhibited the production of inflammatory cytokines. (a) Mice were i.p. injected with control or 6-gingerol (50 μg). After 2 days, mice were subjected to CLP using 26-gauge needles with two punctures. Blood was collected at 4 or 24 h after CLP, and their peritonea were washed with 3 ml of PBS. After centrifugation at 400 × g, 5 min, cytokine concentrations in the peritoneum (top panels) and serum (bottom panels) were determined using a CBA kit; ND: not detected. Purified macrophages (b) and neutrophils (c) were cultured with combinations of control, 6-gingerol (1 μg/ml), Pam3 (500 ng/ml), and LPS (500 ng/ml), as indicated. After 24 h, supernatants were harvested, and cytokine levels were measured with the CBA kit. Data are presented as the means of triplicate cultures. (d, e) Mice were i.p. injected with control or 6-gingerol (50 μg). After 2 days, mice were subjected to CLP using 26-gauge needles with two punctures. Peritoneal cells were harvested and analyzed. (d) Representative flow plots (left panels), percentages (middle panels), and numbers (right panels) presented M1 ($CD80^+$ $F4/80^+$, $iNOS^+$ $F4/80^+$) or M2 ($CD206^+$ $F4/80^+$) macrophages in peritoneal cavity and (e) percentages of $CD80^+$, $iNOS^+$, or $CD206^+$ cells in macrophages ($F4/80^+$) (left panels). The M1/M2 ratio was calculated based on the percentages (top panel) and cell numbers (bottom panel) of $iNOS^+$ $F4/80^+$ cells and $CD206^+$ $F4/80^+$ cells in peritoneal cavity cells, respectively. Error bars indicated the mean ± SD for three separate experiments, $n = 3$ for each group. Statistically significant differences are shown on the graph. NS: not significant.

neutrophils in sepsis is regulated by IL-17 [32, 33]. A decrease in IL-17 is correlated with the risk of bacteremia [34]. Thus, we investigated whether 6-gingerol augments IL-17 levels, resulting in the migration of neutrophils. The 6-gingerol treatment augmented the IL-17 levels in BAL fluid (Figure 5(e)). Besides IL-17, CXCR2 plays a crucial role in the migration of neutrophils to infected sites during sepsis [35–37]. We observed that 6-gingerol increased CXCR2 expression in neutrophils (Figure 5(f)). Therefore, 6-gingerol promotes neutrophil infiltration to the BAL fluid through an increase in IL-17 and CXCR2 levels.

3.6. 6-Gingerol Modulated Cytokine Balance toward a Proinflammatory Pattern in Secondary P. aeruginosa-Infected Mice. To further investigate the role of 6-gingerol in the immune response to secondary *P. aeruginosa* pneumonia, we assessed pulmonary levels of cytokines involved in antibacterial defense. IL-6 and TNF-α levels in BAL fluid

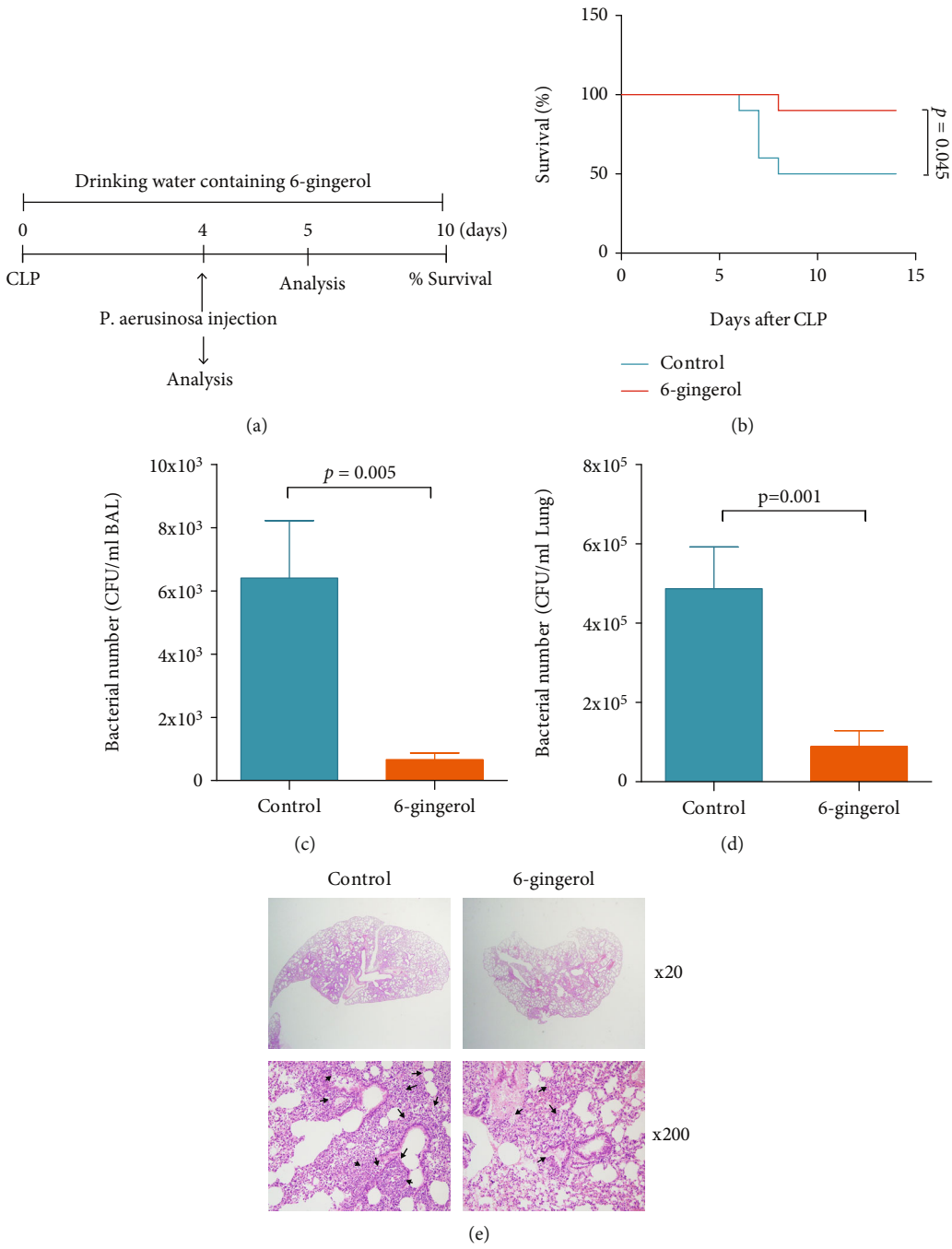


Figure 4: Administration of 6-gingerol improved survival and bacterial clearance to intranasal *P. aeruginosa* infection in postseptic mice. (a) Schematic diagram for induction of secondary bacterial pneumonia. (b) Mice were subjected to CLP using 29-gauge needles and punctured once. From 5 days before CLP to the end of the experiment, mice were fed drinking water containing 3.5 μg/ml of 6-gingerol every day, while control mice were fed drinking water containing 0.01% ethanol. On Day 4 post-CLP, mice were challenged with an intranasal administration of 8×10^7 CFU of *P. aeruginosa*. Mouse survival was monitored every 24 h for 14 days. The curve comparison with the log-rank (Mantel-Cox) test revealed statistically significant differences as shown on the graph ($n = 10$). After CLP, survivors on Day 4 were secondarily challenged by intranasal instillation of *P. aeruginosa* (8×10^7 CFU). (c, d) Bacterial load was determined 24 h after instillation, by quantitative cultures of BAL fluid (c) and lung homogenates (d). (e) Lung damage was assessed by microscopic examinations of hematoxylin and eosin-stained lung sections from control and 6-gingerol-treated mice 24 hours after intranasal inoculation. Data represent the mean ± SEM. Data are representative of at least three independent experiments in which each group contained six mice. Statistically significant differences are shown on the graph.

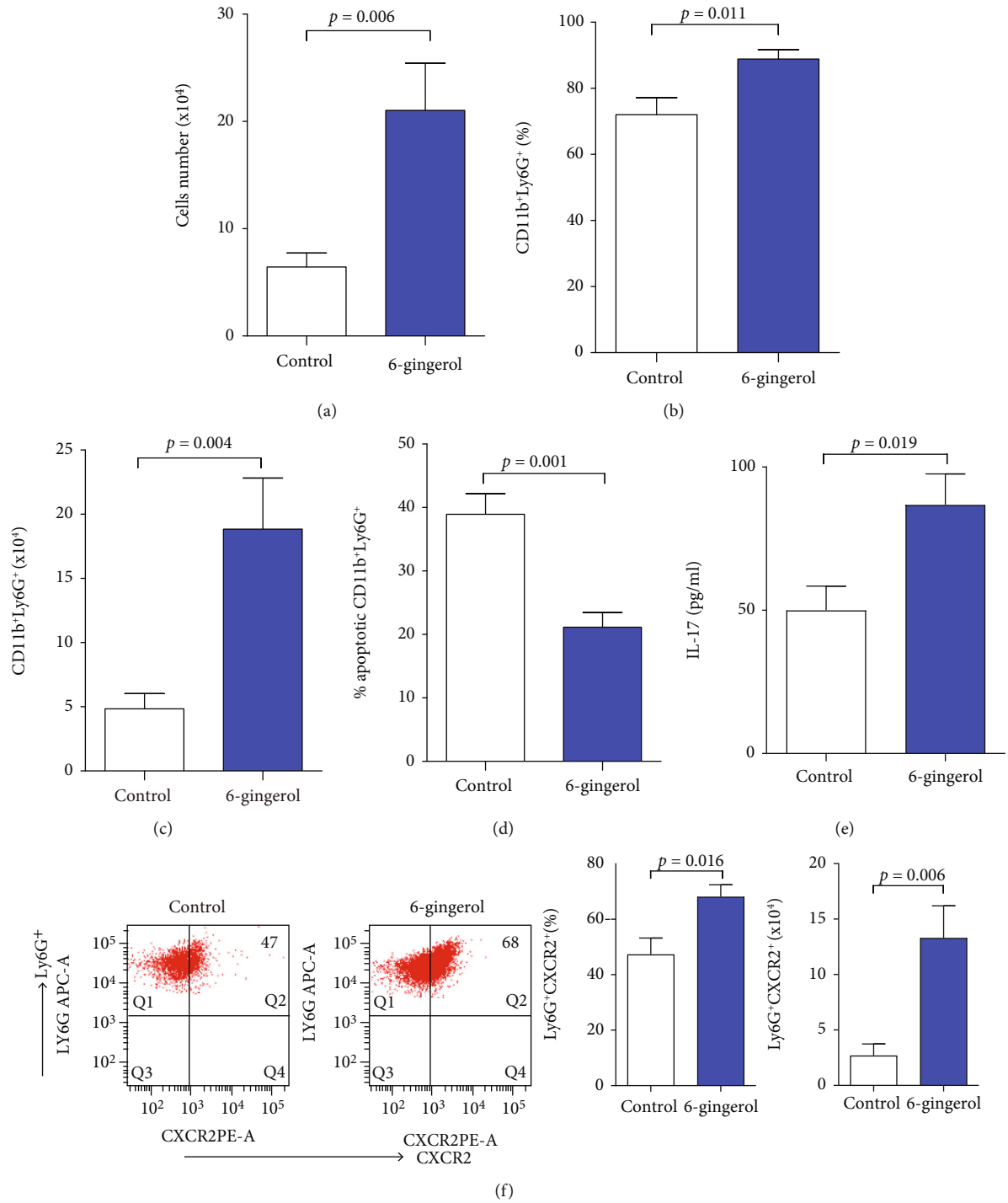
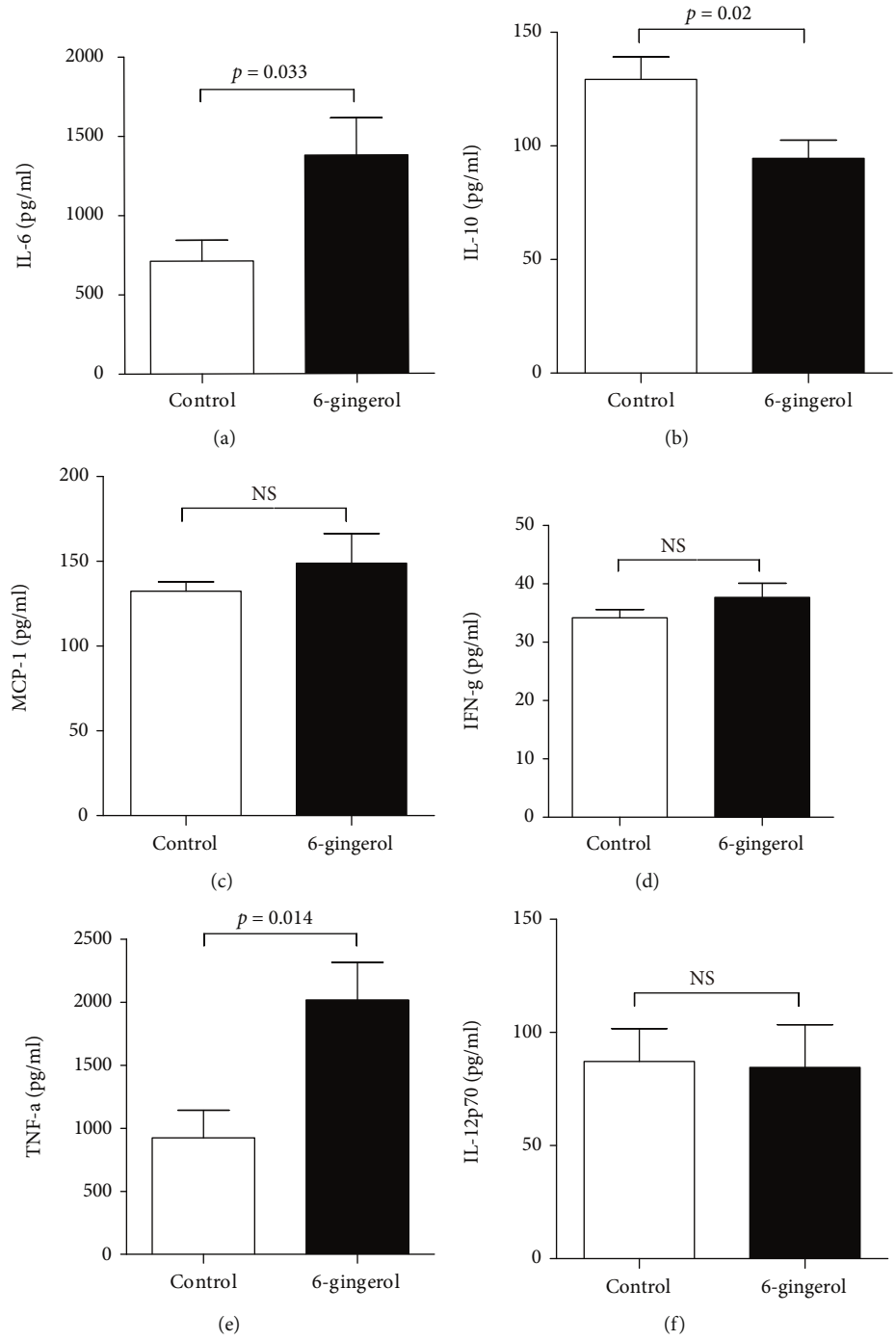


Figure 5: Administration of 6-gingerol increased neutrophil infiltration into BAL fluid and decreased neutrophil apoptosis. Mice were subjected to CLP and fed 6-gingerol as mentioned in the Materials and Methods. Survivors on Day 4 were secondarily challenged by intranasal instillation of *P. aeruginosa* (8×10^7 CFU). (a) White blood cell numbers were assessed in BAL fluid 4 hours after instillation. (b–d) Neutrophil percent (b) and numbers (c), percent of apoptosis, and (d) IL-17 of BAL fluid sup (e) representative flow plots of $CXCR2^+$ expression on neutrophils (*left panels*), percentage (*middle panel*), and numbers (*right panel*) (f) in BAL fluid were determined as described in Materials and Methods. Results are representative of three independent experiments and expressed as the mean ± SD. Statistically significant differences are shown on the graph.

from 6-gingerol-treated mice were significantly higher than levels in BAL fluid from controls. In contrast, IL-10 levels in 6-gingerol-treated mice were significantly lower than those in control-treated mice at 24 hours after intranasal instillation (Figures 6(a)–6(f)). As an index of the overall balance of pro- and anti-inflammatory cytokines, ratios of IL-6 to IL-10 and TNF-α to IL-10 in BAL fluid were calculated. 6-Gingerol-treated mice showed a shift from a Th2



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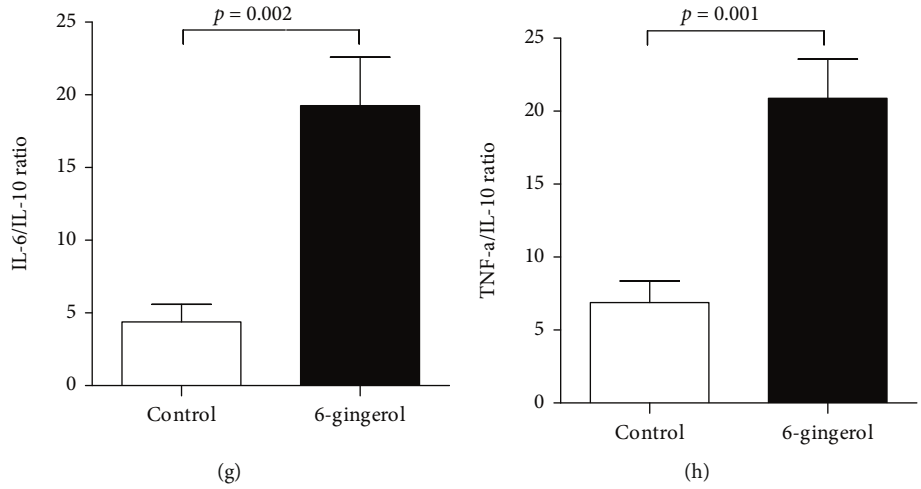


Figure 6: 6-Gingerol modulated cytokine balance toward a proinflammatory pattern in secondary *P. aeruginosa*-infected mice. Mice were subjected to CLP and fed 6-gingerol. Survivors were challenged on Day 4 by intranasal instillation of *P. aeruginosa* (8×10^7 CFU). (a–f) Following a second septic insult, evaluation of BAL cytokines, including IL-6 (a), IL-10 (b), MCP-1 (c), IFN-γ (d), TNF-α (e), and IL-12p70 (f), were measured 24 hours after instillation with the CBA kit. They were analyzed on a FACSCanto™ II (BD Biosciences) with FACSDiva software. (g, h) As an index of overall balance of pro- and anti-inflammatory cytokines, the rate of IL-6 to IL-10 (g) and the rate of TNF-α to IL-10 (h) in BAL were calculated. Data are presented as the means of triplicate cultures. Results are representative of three independent experiments and expressed as the mean ± SD. Statistically significant differences are shown on the graph. NS: not significant.

to a Th1 cytokine balance in the hypoinflammation phase (Figures 6(g) and 6(h)). These results indicate that a secondary *P. aeruginosa* infection in control mice resulted in a shift in the pro- and anti-inflammatory cytokine balance favoring predominantly IL-10 response, while 6-gingerol modulated a cytokine balance toward a proinflammatory pattern.

3.7. Splenocytes from 6-Gingerol-Treated Mice Showed Significant Increases in IFN-γ Production and Decreased Apoptosis Compared to Control 4 Days Post-CLP. To determine whether enhanced survival of 6-gingerol-treated mice with a secondary infection of *P. aeruginosa* was associated with the immune status of mice undergoing CLP, on Day 4, prior to secondary injury, we analyzed the spleens of 6-gingerol-treated and control mice. Lymphocyte loss is thought to be partially responsible for the profound immunosuppression seen in sepsis [2, 38, 39]. To determine whether 6-gingerol treatment was associated with decreased lymphocyte apoptosis, we evaluated spleen cell numbers on Day 4 after CLP. Splenocyte numbers in 6-gingerol-treated mice were significantly higher than those in control mice (Figure 7(a)). In addition, 6-gingerol-treated mice had a significantly lower level of annexin V expression on $CD4^+$ and $CD8^+$ T-cells, compared to control mice (Figure 7(b)). Next, we evaluated T-cell production of IFN-γ to determine the host's ability to mount an effective inflammatory response prior to secondary infection. Splenocytes harvested 4 days after CLP were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml), and IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells were analyzed by FACS. Splenocytes from 6-gingerol-treated mice demonstrated significantly enhanced percentages and numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells compared to control-treated CLP mice (Figures 7(c) and 7(d)). These results indicate a better systemic immune response in the 6-gingerol-treated mice subjected to CLP surgery compared to control mice.

4. Discussion

Sepsis is characterized by sustained infection and uncontrolled systemic inflammatory response, which results in tissue damage and, ultimately, multisystem organ failure, the clinical hallmark of sepsis and direct cause of mortality [40]. The first host response against an invading pathogen involves the recruitment of leukocytes such as neutrophils and macrophages, to infectious foci and their activation which allows these cells to successfully localize, kill, and clear pathogens. In sepsis, bacterial signals recognized by blood neutrophils and macrophages induce the production or release of inflammatory cytokines that increase blood flow to infected tissues, enhance the permeability of local blood vessels, and recruit inflammatory cells to the site of infection [41]. In the present study, we found that 6-gingerol-treated mice exhibited reduced bacterial load in organs, had higher numbers of infiltrated peritoneal neutrophils and macrophages, and had lower levels of cell apoptosis in the CLP model when compared to control-treated mice (Figures 2(a)–2(c)).

Cytokines and chemokines play a critical role in the recruitment of leukocytes to inflamed tissues, yet they are often described as "double-edged swords." An appropriate concentration of cytokines is necessary for the recruitment and activation of immune cells in response to foreign antigens, however, when excess cytokines are produced, they damage the host. A successful host defense response during sepsis requires a fine balance of anti- and proinflammatory

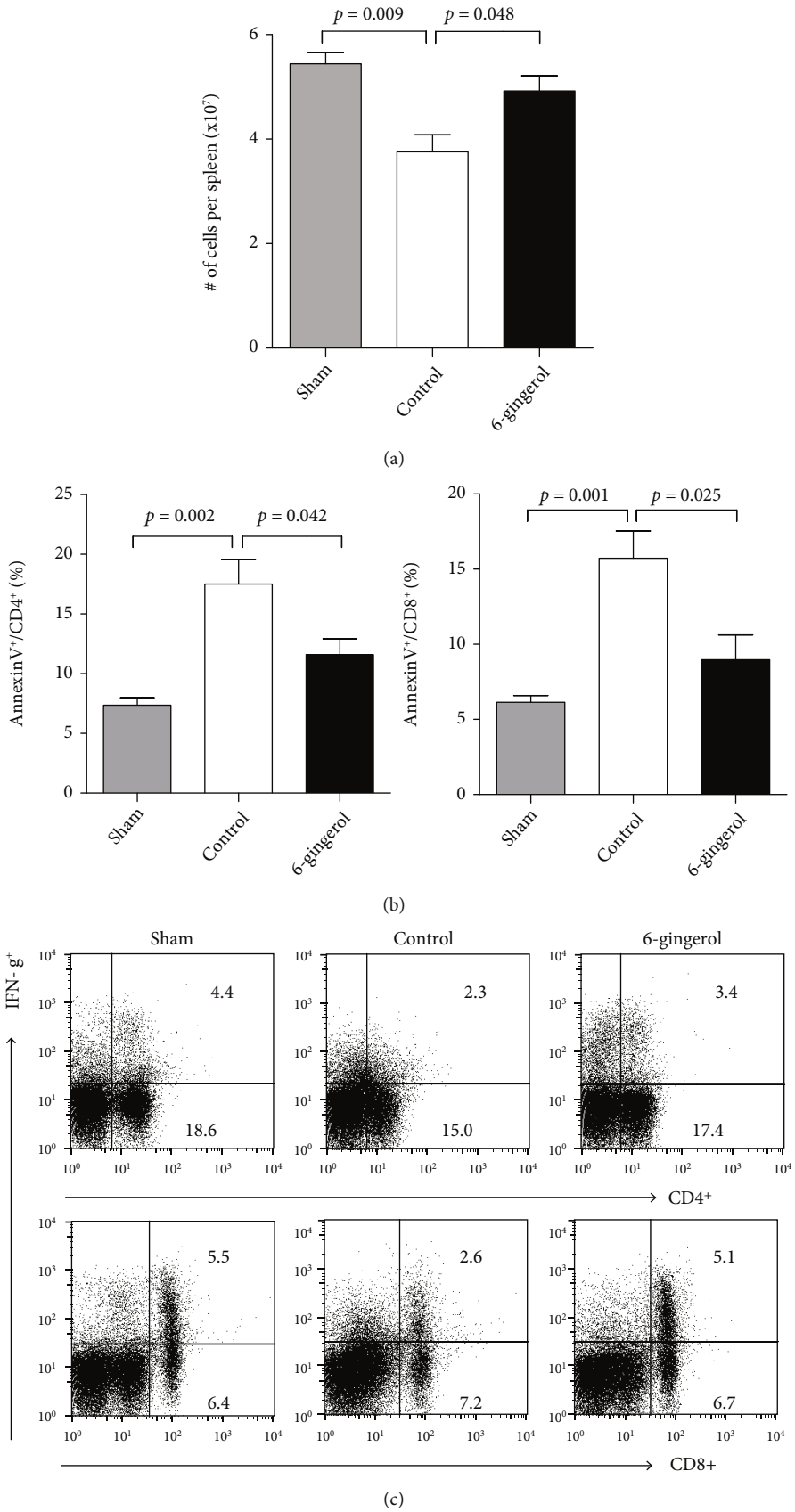
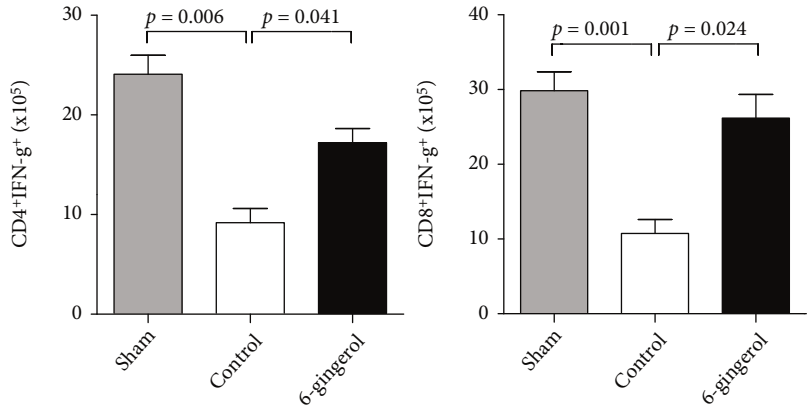


Figure 7: Continued.



(d)

Figure 7: T-cells from 6-gingerol-treated mice showed significant increases in IFN-γ production compared to control 4 days post-CLP. Mice were subjected to CLP and fed 6-gingerol. (a) Spleen cell numbers on Day 4 after CLP. (b) Annexin V expression on $CD4^+$ and $CD8^+$ T-cells was analyzed by FACS. (c) Splenocytes harvested 4 days after CLP (prior to secondary injury) were evaluated by intracellular staining after ex vivo stimulation of splenocytes with PMA plus ionomycin as mentioned in the Materials and Methods. Data were analyzed on a FACSCanto™ II (BD Biosciences) with FACSDiva software. Representative flow plots showing the percentage of IFN-γ-producing $CD4^+$ and $CD8^+$ T-cells among splenocytes in sham, control, or 6-gingerol-treated mice. Panel values indicate percentage of each cell population among total cells. (d) Number of $CD4^+IFN\text{-}\gamma^+$ (left panel) and $CD8^+IFN\text{-}\gamma^+$ (right panel) cells among splenocytes in sham, control, or 6-gingerol-treated mice. Results are representative of three independent experiments. Statistically significant differences are shown on the graph.

cytokines [23, 42]. Several reports have demonstrated that TNF-α, IL-6, and IL-10 can serve as both makers and mediators of sepsis severity and that elevated cytokine levels predict mortality following CLP [43–45]. Macrophages may be excessively activated during the early phase of sepsis and produce excessive proinflammatory cytokines [46], which may be a major cause of the high mortality rate during this early stage [47]. Several studies have demonstrated that 6-gingerol inhibited proinflammatory cytokines production from LPS-stimulated macrophages [12, 13]. In our study, we found that 6-gingerol inhibited the production of TNF-α and IL-6 from TLR2, 4 ligand-activated macrophages as well as TNF-α and IL-10 from TLR2, 4 ligand-activated neutrophils (Figures 3(b) and 3(c)). Moreover, it has been documented that the plasma levels of TNF-α and IL-6 increased significantly in patients with sepsis and in animal models [48, 49]. In *in vivo* studies, 6-gingerol-treated mice showed a less severe inflammatory response and reduced bacterial burden in CLP-induced sepsis (Figures 2(a) and 3), indicating that the balance between efficient pathogen clearance and a harmful overactive inflammatory response was affected by 6-gingerol treatment, which may be one of the reasons for less severe organ failure and decreased mortality in these septic mice. To overcome the excessive inflammation, macrophages undergo apoptosis or polarize to an M2 phenotype to protect the host from excessive injury [50]. Given that 6-gingerol inhibited inflammatory cytokines, such as TNF-α and IL-6, involved in M1 macrophage polarization (Figures 3(a)–3(c)), 6-gingerol promoted M2 macrophage polarization (Figure 3(d)). As expected, 6-gingerol decreased the M1/M2 ratio (Figure 3(e)). Macrophage depletion abrogated the improved bacterial clearance in 6-gingerol-treated mice (Figure 2(d)). Therefore, our results indicate that 6-gingerol effectively prevents the development of severe sepsis after microbial infection via enhanced bacterial clearance, inhibited inflammatory cytokine production, and increased ROS production. In addition, we investigated the immunomodulatory effects of 6-gingerol on intranasal *P. aeruginosa* infection in postseptic mice. Results showed that administration of 6-gingerol improved the survival of intranasal *P. aeruginosa* infection. To investigate the role of 6-gingerol in host defense against secondary *P. aeruginosa* pneumonia, we analyzed bacteria clearance, immune cell population, apoptosis, and cytokine balance. The immunoparalysis caused by sepsis is associated with a decreased ability of the host to clear bacteria [19]. Given this, we evaluated the ability of the host to clear *P. aeruginosa* from BAL fluid and lungs following double injury. As shown in Figures 4(c) and 4(d), 6-gingerol-treated mice significantly decreased the bacterial concentration in BAL fluid and lungs 24 h after intranasal instillation. Because of this, we analyzed immune cell numbers at a much earlier time point after intranasal instillation. In BAL fluid, most infiltrated cells were neutrophils and 6-gingerol-treated mice showed significantly increased neutrophil numbers when compared to control mice. Consistent with increased neutrophil numbers, neutrophil apoptosis in 6-gingerol-treated mice was significantly reduced compared to control mice (Figure 5(d)). In addition, increased IL-17 and CXCR2 levels in 6-gingerol-treated mice compared to controls in BAL fluid suggest that 6-gingerol is also associated with neutrophil infiltration (Figures 5(e) and 5(f)). The respiratory system is continuously exposed to a variety of bacteria. To combat these intruders, one of the most important components of the initial innate immune response against bacterial infection is the vigorous recruitment of neutrophils. Neutrophils play a primary and unambiguous role in *P. aeruginosa* clearance during acute pulmonary infection, which is clearly

demonstrated by the extreme susceptibility of neutropenic mice to this pathogen [51–53]. Our data indicate that 6-gingerol contributed to the effective clearance of bacteria at the site of infection due to the promotion of neutrophil recruitment and reduction in apoptosis in the hypoinflammatory state of postseptic mice.

Cytokine balance is another key mechanism related to survival. A balanced and regulated production of pro- and anti-inflammatory cytokines is essential to eradicate pathogens without tissue damage. Sepsis-induced immunosuppression is characterized by an imbalance in the expression of proinflammatory and anti-inflammatory cytokines, especially a shift towards an anti-inflammatory cytokine pattern, which predisposes the host to secondary pulmonary infections, particularly the development of nosocomial pneumonia [3, 54]. Early death in sepsis is usually a result of a hyperinflammatory response driven by proinflammatory cytokines. The early hyperinflammatory state evolves to a subsequent hypoinflammatory state with significant immunosuppression [1–3]. Researchers highlighted the importance of IL-10 as a classic anti-inflammatory cytokine [55]. During the immunosuppressive phase of sepsis, IL-10 leads to suppression of macrophage cytokine secretion and decreased activity of macrophage and neutrophil functions [55, 56]. This anti-inflammatory reaction is further characterized by low levels of circulating lymphocytes, increased lymphocyte apoptosis, and a shift from Th1 to Th2 subpopulations [57]. Previous research reported that higher plasma IL-10 concentrations contributed to higher mortality of sepsis [58, 59]. On the contrary, the importance of TNF-α production in response to infection has been demonstrated by multiple research groups. Lukacs et al. [60] reported that TNF-α mediates the recruitment of neutrophils during airway inflammation. As shown in Figure 6, 6-gingerol modulates the cytokine balance toward a proinflammatory pattern in mice with a secondary infection of *P. aeruginosa*. These data suggest that 6-gingerol treatment improves host defense by removing bacteria. Several studies have demonstrated that 6-gingerol inhibited the production of proinflammatory cytokines from LPS-stimulated macrophages [12, 13]. In our model, 6-gingerol treatment reduced hyperinflammation in CLP mice, thereby preventing them from moving towards the immunosuppressive phase.

Multiple research studies using animal models and patients dying from sepsis have demonstrated a profound loss of immune effector cells by apoptosis [2, 38, 61]. In order to evaluate the immune status of mice undergoing CLP, on Day 4, prior to secondary injury, we analyzed the spleens of 6-gingerol-treated and control mice. As shown in Figures 7(a) and 7(b), splenocyte numbers in 6-gingerol-treated mice were significantly higher, while the levels of T-cell apoptosis were lower than in control mice. In addition, splenocytes from 6-gingerol-treated mice exhibited enhanced T-cell production of IFN-γ compared to control mice (Figure 7(c)). IFN-γ treatment has been shown to improve the clinical course of sepsis in humans [62]. Hotchkiss and Karl [2], in adoptive transfer experiments, showed that increased animal survival was due to IFN-γ upregulation. Therefore, the reduction in T-cell apoptosis and improvement in IFN-γ production ability in 6-gingerol-treated mice indicate a better systemic immune response than those in control mice.

5. Conclusion

In conclusion, our results indicate that 6-gingerol effectively prevents the development of severe sepsis after microbial infection via enhanced bacterial clearance and inhibited inflammatory cytokine production. In addition, we found that 6-gingerol improves survival rates from infection in a murine model of sepsis-induced immune dysfunction. Survival improvement was associated with enhanced neutrophil numbers and function early on the infection site, a shift from a Th2 to a Th1 cytokine balance in the hypoinflammation phase, and decreased lymphocyte apoptosis.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Authors' Contributions

Seong-A Ju and Quang-Tam Nguyen contributed equally to this work (co-first authors).

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Research Article

Effects of Different Green Tea Extracts on Chronic Alcohol Induced-Fatty Liver Disease by Ameliorating Oxidative Stress and Inflammation in Mice

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Alcoholic fatty liver disease (AFLD) is a common chronic liver disease and has become a critical global public health problem. Green tea is a popular drink worldwide and contains several bioactive compounds. Different green teas could contain diverse compounds and possess distinct bioactivities. In the present study, the effects of 10 green teas on chronic alcohol induced-fatty liver disease in mice were explored and compared. The results showed that several green teas significantly reduced triacylglycerol levels in serum and liver as well as the aminotransferase activities in mice at a dose of 200 mg/kg, suggesting that they possess hepatoprotective effects. Moreover, several green teas remarkably decreased the expression of cytochrome P450 2E1, the levels of malondialdehyde and 4-hydroxynonenoic acid, and the contents of proinflammatory cytokines, indicating that they could alleviate oxidation damage and inflammation induced by chronic alcohol exposure. In addition, Seven Star Matcha Tea and Selenium-Enriched Matcha Tea could increase glutathione level. Furthermore, the main phytochemical components in green teas were determined and quantified by high-performance liquid chromatography, and the correlation analysis showed that gallic acid, gallocatechin, catechin, chlorogenic acid, and epigallocatechin gallate might at least partially contribute to protective effects on AFLD. In conclusion, Selenium-Enriched Chaoqing Green Tea, Xihu Longjing Tea, Taiping Houkui Tea, and Selenium-Enriched Matcha Tea showed the strongest preventive effects on AFLD. This research also provides the public with new insights about the effects of different green teas on AFLD.

1. Introduction

Alcoholic liver disease (ALD) exerts significant morbidity and mortality worldwide [1]. Alcoholic fatty liver disease (AFLD) is the earliest response and primary consequence of chronic excessive alcohol consumption and could develop into more severe pathological stages of ALD, such as alcoholic fibrosis, cirrhosis, and hepatocellular carcinoma [2, 3]. AFLD is characterized by a series of changes, such as hepatic oxidative stress, inflammation, and steatosis, which together contribute to hepatocytes damage [1, 4, 5]. Accordingly, the key to prevent and manage liver injury induced by chronic alcohol exposure is to prevent oxidative stress, inflammation, and steatosis.

Reactive oxygen species (ROS) are required in many important physiological functions, but excessive ROS will react with biological macromolecules in cells, such as lipids, proteins, and nucleic acids and induce various chronic diseases [6, 7]. Oxidative stress is stimulated when the balance of ROS production and antioxidant defense capability is disrupted, which is implicated in certain chronic diseases, such as liver diseases, cardiovascular diseases, and cancers [8–10]. Additionally, several lines of evidence have indicated that the production of excess ROS can be induced by alcohol

Table 1: The detailed information of 10 green teas from China.

Number	Name	Production place
GT1	Dianqing Tea	Kunming, Yunnan
GT2	Liping Xiang Tea	Liping, Guizhou
GT3	Selenium-enriched Chaoqing Green Tea	Enshi, Hubei
GT4	Xihu Longjing Tea	Hangzhou, Zhejiang
GT5	Chaoqing Green Tea	Yichang, Hubei
GT6	Taiping Houkui Tea	Huangshan, Anhui
GT7	Jieyang Chaoqing Tea	Jieyang, Guangdong
GT8	Fenggang Zinc-Selenium-Enriched Tea	Guiyang, Guizhou
GT9	Seven Star Matcha Tea	Shaoxing, Zhejiang
GT10	Selenium-enriched Matcha Tea	Enshi, Hubei

exposure and metabolism [11]. In fact, the liver is the main organ responsible for metabolizing alcohol [12]. Generally, alcohol is firstly converted into acetaldehyde mainly by alcohol dehydrogenase (ADH), and then aldehyde dehydrogenase (ALDH) metabolizes acetaldehyde to acetic acid [13]. However, during chronic alcohol exposure, cytochrome P450 2E1 (CYP2E1) is induced to replace ADH, playing the major role in alcohol metabolism, which can produce a significant amount of ROS, such as hydrogen peroxide and superoxide anion radicals, resulting in severe oxidative stress [14]. Besides, accumulating studies have revealed that overwhelming oxidative stress induced by alcohol can further lead to the production of lipid peroxides, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) [15]. Consequently, it has been found that oxidative stress plays a prominent role in the occurrence and progression of AFLD [2]. On the other hand, mounting studies have convinced that inflammation plays an essential role in the initiation and development of AFLD [16, 17]. Furthermore, considerable research has reported that long-term alcohol consumption could influence multiple signaling pathways for lipid metabolism, which increase lipogenesis, inhibit β-oxidation of fatty acids, and cause hepatocytes steatosis [18, 19]. Although immediate alcohol abstinence is the most effective therapeutic treatment for AFLD, it is very difficult to carry out for persons with alcohol dependence [20]. Besides, there are no effective drugs for the treatment of AFLD currently. In recent years, early intervention of dietary natural products with strong antioxidant and anti-inflammatory properties has received increasing attention in the prevention and management of AFLD, and more relevant research is urgently needed [3, 21] .

Tea (*Camellia sinensis*) is a widely consumed beverage around the world due to its multiple health benefits [22]. Several lines of studies have proven that green tea imparts numerous biological functions, such as antioxidant and anti-inflammatory activities [23]. In addition, mounting studies have revealed that the various bioactivities of green tea are greatly ascribable to the several bioactive ingredients, such as polyphenols [24]. Moreover, catechins and phenolic acids are the main polyphenols in green tea [25, 26]. Accumulating in vitro evidences have suggested that the catechins in green tea are mainly composed of epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC), which have been considered as the major contributor of potent antioxidant property [25, 27]. Several studies have reported the effect of a green tea extract as well as catechins or caffeine from green tea on liver injury induced by alcohol exposure [28–30]. However, different green teas could have distinct effects on AFLD. Thus, in the current study, our goal is to explore and compare the effects of different green teas on AFLD in chronic ethanol-exposed mice, and the main components in green teas are determined and quantified by high-performance liquid chromatography (HPLC). The results could serve the public to select the tea possessing the strongest biological activity on AFLD. Several teas could be also developed into functional foods for the prevention and management of AFLD.

2. Materials and Methods

2.1. Preparation of Green Tea Extracts. For the preparation of green tea extracts, we depended on the procedures provided in our previous report, including extraction, concentration, and freeze-drying [31]. Firstly, 10 g of green tea was extracted 3 times by using boiling deionized water (100 mL) in water bath (98°C) for 10 min each time, and all extracted solutions were filtered. Secondly, the filtered solution was concentrate, and then completely freeze-dried into a powder for further experiment. Table 1 displays the detailed information of 10 types of green teas obtained from China.

2.2. Measurement of Main Components in Green Teas. The HPLC method was used to determine and quantify the main components in 10 green tea extracts based on our previous report [31], among which the standard compounds were gain from Derick Biotechnology Co., Ltd. (Chengdu, China).

2.3. Animals and Experimental Design. Male C57BL/6 J mice (8 weeks old) were obtained from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). All the mice were kept in a specific-pathogen-free (SPF) experimental environment with 12-hour light/dark cycle at 22 ± 0.5°C and had free access to chow diet and water. In addition, all experimental protocols on animals were performed with approve by the "Principles of Care and Use of Laboratory

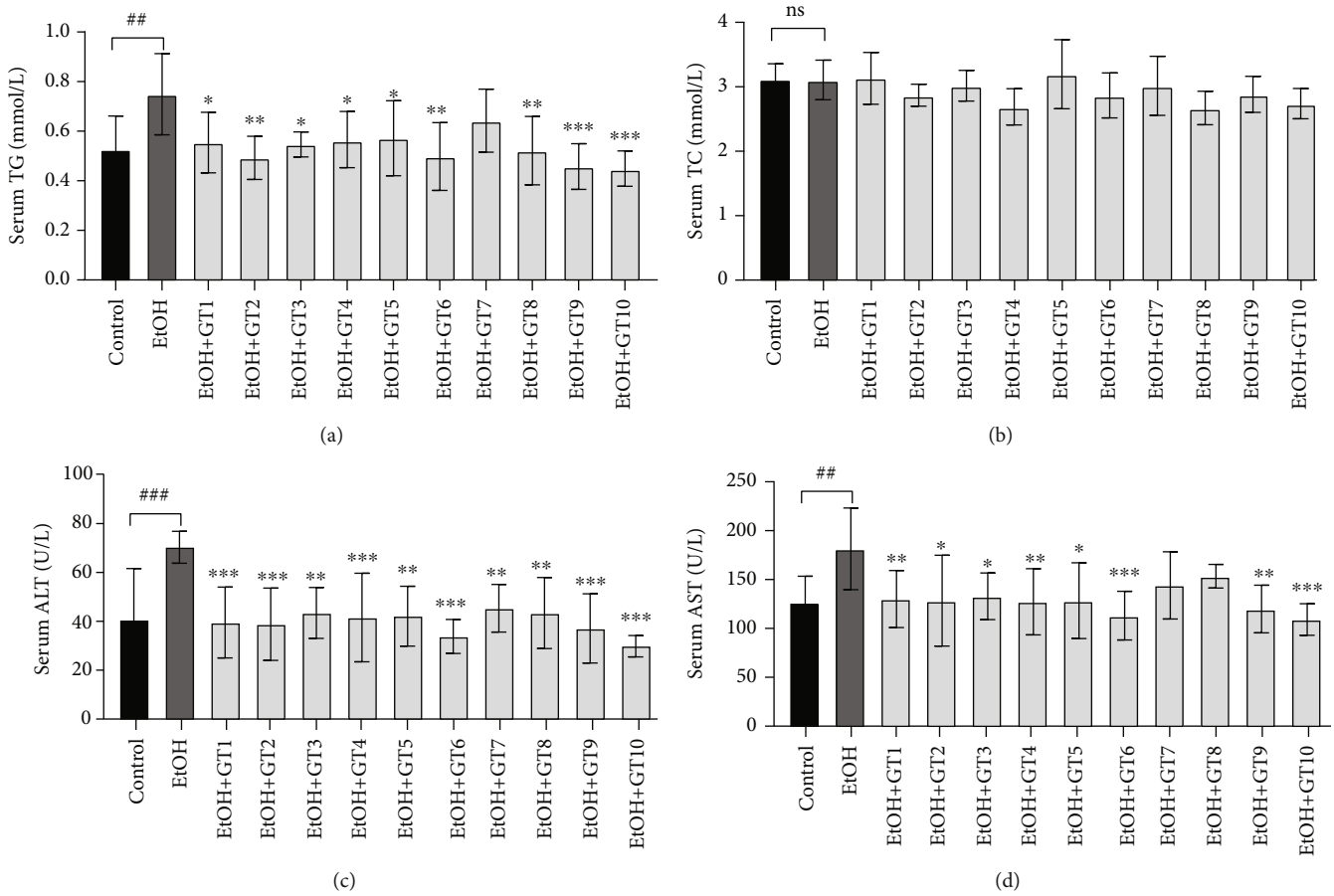


Figure 1: The effects of green teas on serum biomarkers in chronic alcohol-induced fatty liver mice. (a) Serum TG: triacylglycerol. (b) Serum TC: total cholesterol. (c) Serum ALT: alanine aminotransferase. (d) Serum AST: aspartate transaminase. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ## $p < 0.01$; ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

Animals" from the School of Public Health, Sun Yat-Sen University (No. 2019-002; 28 February 2019).

In this study, the Lieber–DeCarli liquid diet purchased from TROPHIC Animal Feed High-tech Co., Ltd. (Nantong, China) was used to establish the AFLD mouse model based on a previous report [32]. This diet contained 4% ethanol (*w*/*v*) and the ethanol supplied 28% of total calories. In briefly, after one week of acclimatization, all mice were fed Lieber-DeCarli control liquid diet for 5 days to adapt to the liquid diet. After that, according to body weight, the mice were randomly classified into a control group (9 mice) and ethanol-fed groups, which received alcohol adaptation for 6 days. Subsequently, the mice in ethanol-fed groups were further classified into eleven groups (9 mice in each group) including 10 green tea extract supplementary groups and a model group, which were fed with the Lieber-DeCarli ethanol liquid diet. However, the control mice were fed a Lieber-DeCarli control liquid diet during the entire experiment. On the 12th day, mice in all green tea extract supplementary groups were administered with 200 mg/kg.b.w green tea extracts for 4 weeks [33, 34]. Equivalently, about 3.0 g, the green tea was drunk for a person with 60 kg body weight. For another thing, the control and model groups were treated with sterile distilled water (10 mL/kg) for 4 weeks.

At the end of experiment, all mice fasted for 9 hours were anesthetized. The blood samples were obtained from the ophthalmic venous plexus and were centrifuged at the condition of 4,000 × g for 10 min to obtain the serum for biochemical analysis. In addition, the mice were sacrificed, and their liver tissue was collected for further experiments.

2.4. Determination of Serum Biochemical Indicators. The liver function biomarkers of alanine transaminase (ALT) and aspartate transaminase (AST) as well as the serum lipid profile of triglyceride (TG) and total cholesterol (TC) were determined with the corresponding kits by using an automated biochemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

2.5. Hepatic Histopathological Evaluation by Hematoxylin-Eosin (H&E) Staining. After the mice were sacrificed, the liver samples were immediately fixed in 4% formalin and

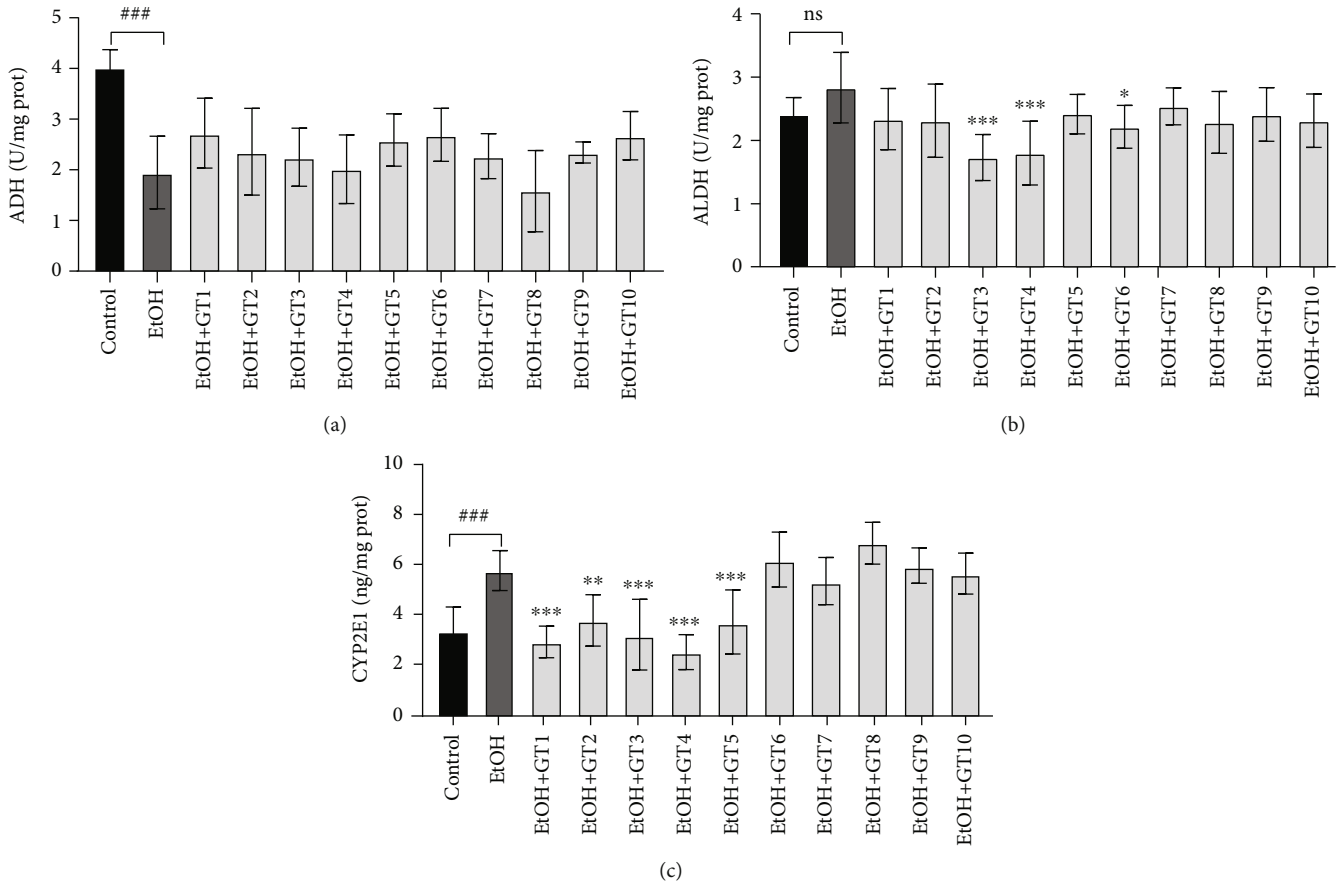


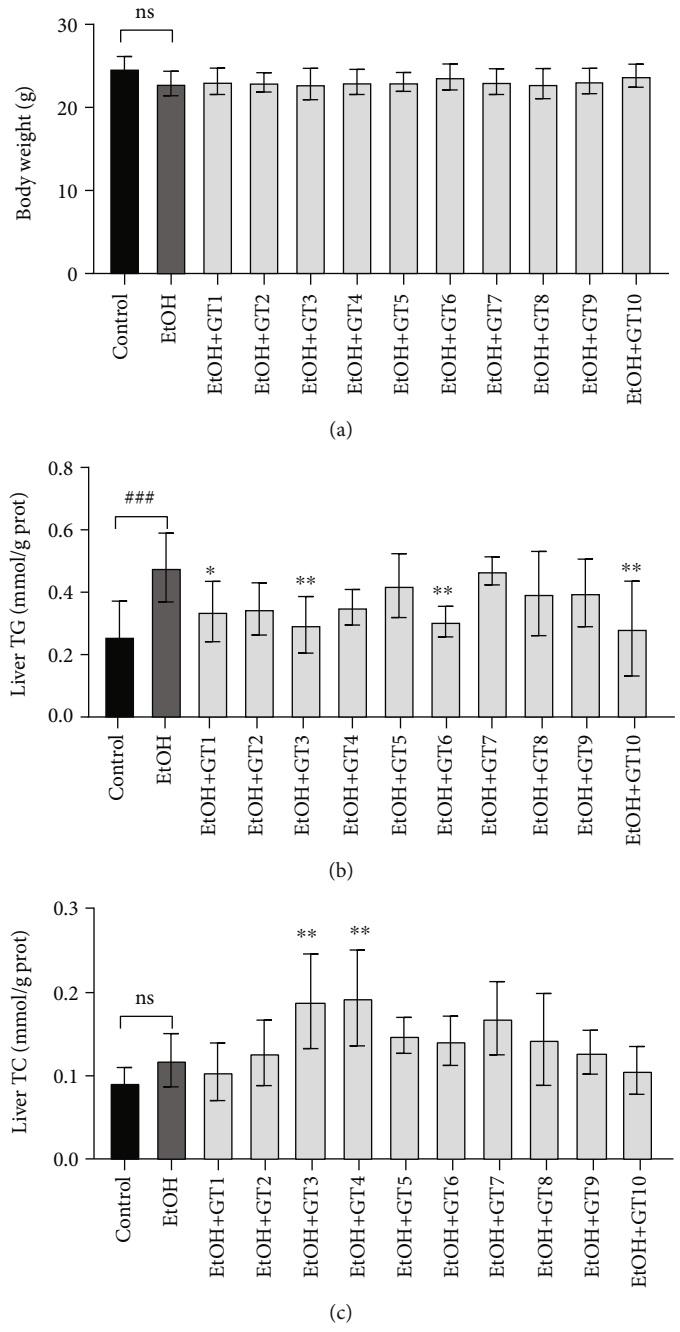
Figure 2: The effects of green teas on alcohol metabolism in chronic alcohol-induced fatty liver mice. (a) ADH: alcohol dehydrogenase; (b) ALDH: aldehyde dehydrogenase; (c) CYP2E1: cytochrome P450 2E1. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

then embedded in paraffin as well as sectioned. Subsequently, the processed liver tissues were stained using hematoxylin and eosin to assess liver injury induced by chronic alcohol exposure, such as infiltration of inflammatory cells, hepatocyte rearrangement, and lipid accumulation. Finally, a microscope (Leica, Solms, Germany) was used to visualize the histological images. Moreover, using Image-Pro Plus 6.0 analysis software (Media Cybemetics, U.S.A), the vacuolar pixel area within the hepatocytes in each image and tissue pixel area was measured separately (the vacuolar area percentage = vacuolar pixel area/tissue pixel area × 100%).

2.6. Analysis of Hepatic Alcohol Metabolism Enzymes. The hepatic samples from all experimental groups were weighed and homogenized in ice-cold 0.9% NaCl to prepare a 10% (w/v) liver tissue homogenate. The activities of ADH and ALDH in liver tissue were determined according to the protocol provided by the kit manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [35, 36].

2.7. Analysis of Hepatic Biochemical Indicators. The 200 mg of liver tissue was mixed with 1.8 mL of 0.9% NaCl, and then the mixture was homogenized. Afterward, the liver homogenate was centrifuged (2500 × g, 4°C, and 10 min) to obtain the supernatant, which was used to detect superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) activities, and glutathione (GSH) content. The levels of SOD, CAT, GSH-Px, and GSH were measured as previously described by using detection kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) [37]. On the other hand, the concentrations of TG, MDA, and total protein were determined by detection kits gained from Apply-gen Technologies Inc. (Beijing, China) [38].

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). The levels of hepatic 4-HNE, CYP2E1, and inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured by enzyme linked immunosorbent assay (ELISA) based on the instructions (Meimian, Jiangsu, China) [39, 40]. The 0.2 g liver tissue of each mouse was weighed and homogenized thoroughly with 1.8 mL of ice-cold phosphate buffer saline (PBS) and then centrifuged at 3,000 × g for 20 min at 4°C to obtain the supernatant for the assay.



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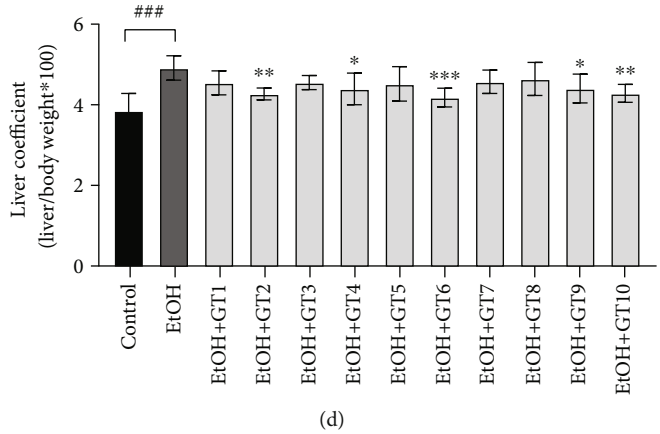


(d)

Figure 3: The effects of green teas on liver lipid profiles in chronic alcohol-induced fatty liver mice. (a) Body weight. (b) Liver TG: triacylglycerol. (c) Liver TC: total cholesterol. (d) liver coefficient. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

2.9. Statistical Analysis. All experimental data were analyzed with the software SPSS 20.0 (IBM SPSS Statistics, IBM Corp, Somers, NY, USA), and the results were expressed as mean ± standard deviation. In addition, a one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) tests was performed to determine statistical difference between experimental groups. When the p value <0.05, the result was considered statistically significant. Moreover, graphs were drawn using the software GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

3.1. Effects of Green Tea Extracts on Serum Biomarkers. Increasing evidence has revealed that chronic alcohol consumption could result in lipid metabolism disorders [41, 42]. Additionally, it is well known that the liver is an essential place for modulating lipid metabolism, and the blood is an important transport medium for these metabolites [43]. Thus, the effects of green tea extracts on serum TG and TC levels in chronic alcohol-induced fatty liver mice were studied as shown in Figures 1(a) and 1(b). The serum TG level in the model group was significantly elevated in comparison with the control group ($p < 0.01$). Moreover, the increased serum TG level induced by chronic alcohol exposure was effectively restored by the supplementation of most green tea extracts, except for Jieyang Chaoqing Tea (GT7). On the other hand, there was no marked difference in serum TC level among all groups ($p > 0.05$).

It has been demonstrated that increased serum ALT and AST levels are reliable indicator of liver injury [44, 45]. Seen from the Figures 1(c) and 1(d), the model group exhibited a significant elevation in serum ALT ($p < 0.001$) and AST ($p < 0.01$) activities compared with the control group. Consistent with our results, previous reports have revealed that an increase in serum AST and ALT activities was observed in mice and rats exposed to alcohol [46, 47]. In the present study, treatment with most green tea extracts effectively ameliorated liver injury by inhibiting the elevated ALT and AST activities induced by alcohol consumption, while supplementation with Jieyang Chaoqing Tea (GT7) and Fenggang Zinc-Selenium-enriched Tea (GT8) only reduced AST activity. The results indicated that most green tea extracts possessed preventive effects on AFLD induced by chronic alcohol exposure. It has been reported that some green tea extracts, such as Liping Xiang Tea and Seven Star Matcha Tea, could protect acute alcohol consumption-induced liver injury by reducing the activities of AST and/or ALT [31]. Furthermore, in agreement to our finding, a previous animal study showed that an increased ALT activity caused by alcohol was inhibited by the treatment of green tea extracts in rats [28].

3.2. Effects of Green Tea Extracts on Alcohol Metabolism. The effects of green teas on alcohol metabolism in chronic alcohol-induced fatty liver mice are presented in Figure 2. In the present study, we found that the activity of ADH was significantly inhibited, and the CYP2E1 enzymes were remarkably induced by chronic alcohol exposure. Although an increased tendency in ALDH activity was shown in the model group compared with the control group, there was no significant difference. Under normal conditions, alcohol metabolism consists of two steps, where ethanol is initially converted to acetaldehyde via ADH, and then acetaldehyde is further oxidized to acetic acid by ALDH [48, 49]. Accumulating evidence has demonstrated that ADH would be replaced by CYP2E1 enzymes in the liver under long-term alcohol exposure, which could result in severe oxidative stress [2, 50].

As shown in Figure 2(c), the elevated CYP2E1 enzymes induced by chronic alcohol exposure were effectively restored by supplementation with Dianqing Tea (GT1), Liping Xiang Tea (GT2), Selenium-Enriched Chaoqing Green Tea (GT3), Xihu Longjing Tea (GT4), and Chaoqing Green

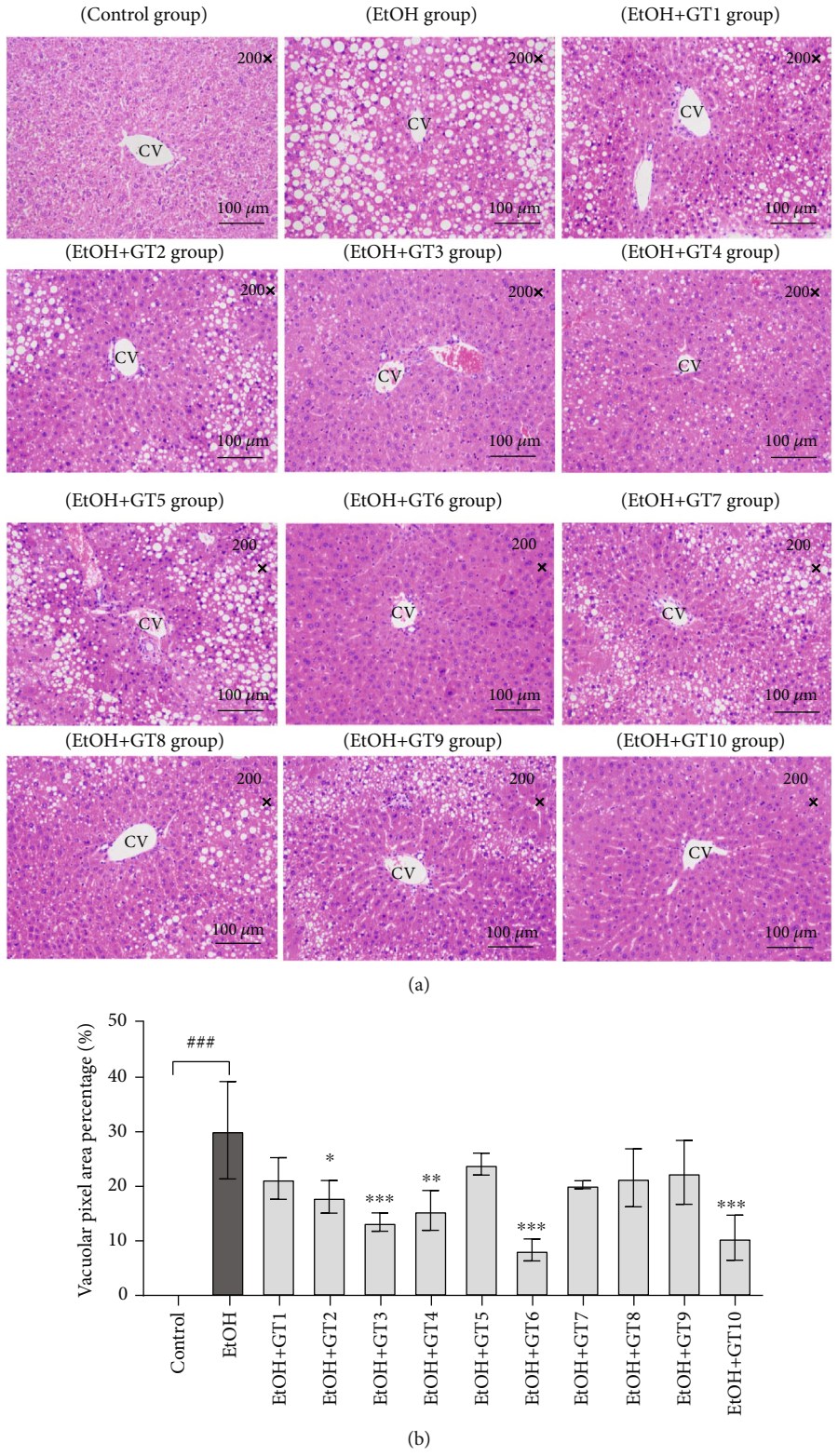


Figure 4: The histopathological evaluation for all experiment groups. (a) The photomicrographs of hematoxylin and eosin (H&E) stained for liver sections (magnification: 200; scale bar: 100 μm; CV: central venous). (b) The vacuolar pixel area percentage. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

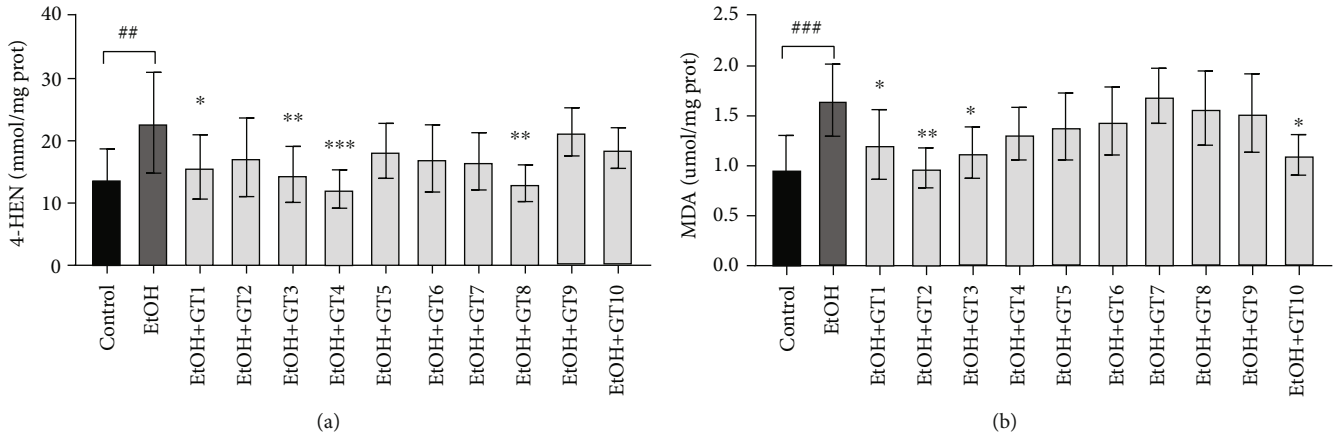


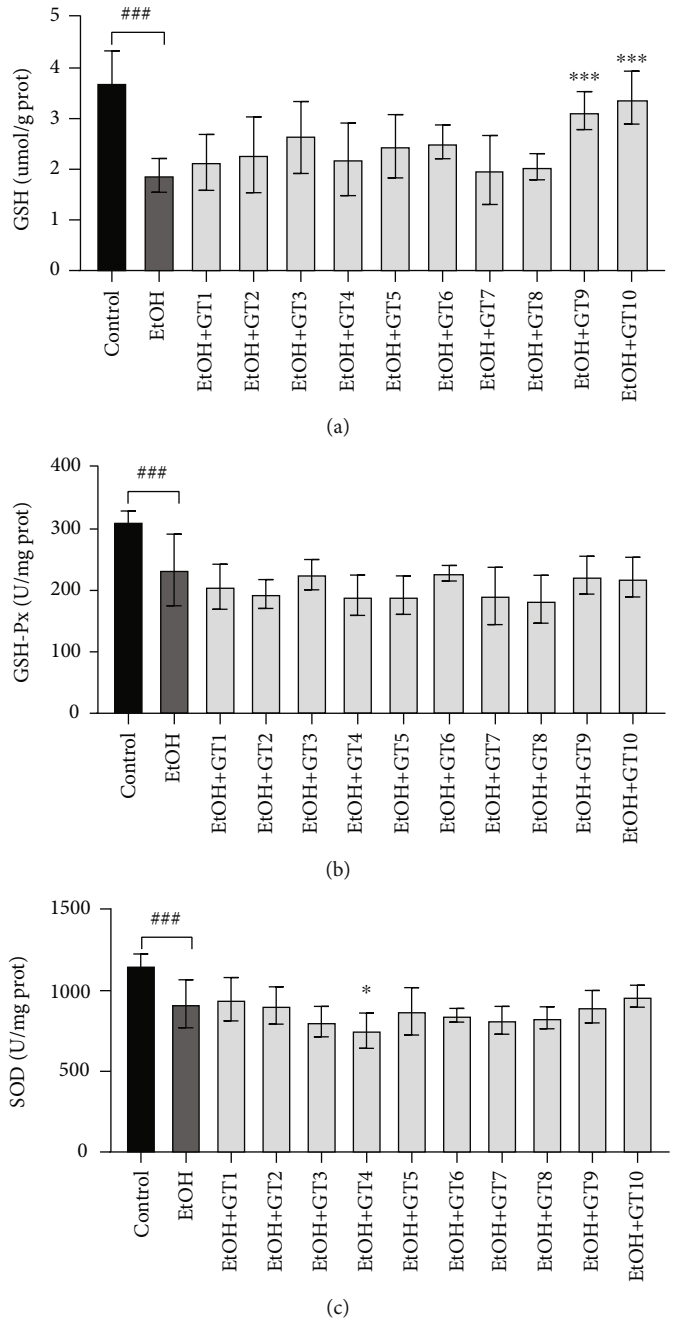
FIGURE 5: The effects of green teas on oxidative stress damage in chronic alcohol-induced fatty liver mice. (a) 4-HNE: 4-hydroxynonenoic acid; (b) MDA: malondialdehyde. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ## $p < 0.01$; ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

Tea (GT5). The results suggested that these teas could ameliorate AFLD caused by alcohol through inhibiting CYP2E1 enzymes. Consistent with our results, a previous in vitro study revealed that intervention with catechin and caffeine from green tea extract could obviously restrain the overexpression of CYP2E1 in the HepG2 cell model [51]. In addition, a previous animal experiment reported that catechins derived from green tea extracts decreased the expression of oxidative stress-derived enzymes, such as CYP2E1 [28]. According to the results, all green tea extracts insignificantly influenced the activity of ADH compared with the model group, while the ALDH activity was markedly inhibited by the supplementation of Selenium-Enriched Chaoqing Green Tea (GT3), Xihu Longjing Tea (GT4) ,and Taiping Houkui Tea (GT6). It illustrated that these green teas inhibited the conversion of acetaldehyde to acetic acid followed by an increase in the acetaldehyde concentration, which would aggravate health damage [52, 53]. Moreover, the results indicated the side effects of these green teas on the alcohol drinking.

3.3. Effects of Green Tea Extracts on Body Weight and Liver Lipid Profiles. In the current study, the liquid feed intake volume of the mice in control and all green tea extract supplementary groups were determined based on that of the model group in the previous day to control the energy intake to be equal. Thus, it was observed that there was no significant difference in body weight among mice of all groups at the end of experiment (Figure 3(a)).

As displayed in Figures 3(b)–3(d), compared with the control group, hepatic TG concentration and liver coefficient were remarkably elevated in the model group, but there was no significant change in the hepatic TC content. The results were agreement with previous studies in which reported long-term excessive consumption of alcohol could result in abnormal metabolism of hepatic TG and liver steatosis [54, 55]. Seen from our results, the increased hepatic TG level induced by chronic alcohol exposure was effectively reduced by the supplementation of Dianqing Tea (GT1), Selenium-Enriched Chaoqing Green Tea (GT3), Taiping Houkui Tea (GT6), and Selenium-Enriched Matcha Tea (GT10), indicating that these green teas could modulate abnormal hepatic TG accumulation in chronic alcohol-induced mice. Meanwhile, the abnormity of liver coefficient caused by alcohol was obviously restored by the treatment of Liping Xiang Tea (GT2), Xihu Longjing Tea (GT4), Taiping Houkui Tea (GT6), Seven Star Matcha Tea (GT9), and Selenium-Enriched Matcha Tea (GT10). In addition, the supplementation of Selenium-Enriched Chaoqing Green Tea (GT3) and Xihu Longjing Tea (GT4) could obviously increase hepatic TC content, which is different from the results of serum. This could be because the liver is the main organ for the synthesis and storage of total cholesterol.

3.4. Histopathological Evaluation. To further confirm the preventive effect of green teas on AFLD induced by chronic ethanol exposure, H&E staining was used to observe the histopathological morphologies of the liver sections. As displayed in Figure 4, compared with the normal group mice, the liver of chronic alcohol-treated mice showed remarkable deposition with large numbers of medium and small lipid droplets in the parenchyma cells. However, supplementation with certain green teas reduced lipid droplets in hepatic cells and attenuated the liver injury induced by ethanol, especially Selenium-Enriched Chaoqing Green Tea (GT3), Xihu Longjing Tea (GT4), Taiping Houkui Tea (GT6), and Selenium-Enriched Matcha Tea (GT10). In addition, it should also be point out that the degree of inflammatory damage in ethanol-fed mice was less severe in this study, and the inflammatory damage of ethanol-fed mice was not observed by hepatic histopathological evaluation; although, the release of proinflammatory cytokines was obvious (see latter section).



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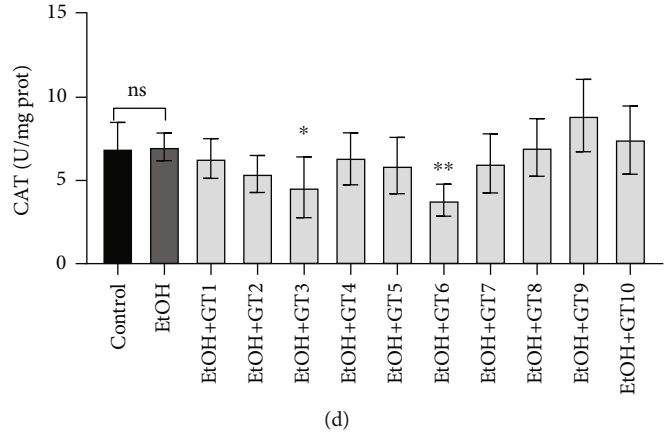

Figure 6: The effects of green teas on oxidant ability in chronic alcohol-induced fatty liver mice. (a) GSH: glutathione. (b) GSH-Px: glutathione peroxidase. (c) SOD: superoxide dismutase. (d) CAT: catalase. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

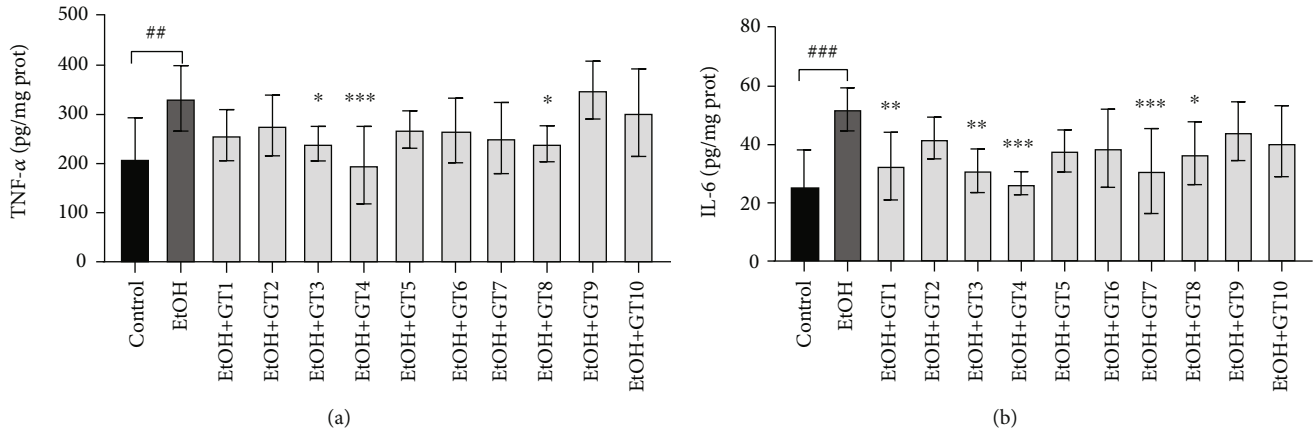

Figure 7: The effects of green teas on inflammation in chronic alcohol-induced fatty liver mice. (a) TNF-α: tumor necrosis factor-α. (b) IL-6: interleukin-6. Control: the control group; EtOH: the model group; GT1: Dianqing Tea; GT2: Liping Xiang Tea; GT3: Selenium-Enriched Chaoqing Green Tea; GT4: Xihu Longjing Tea; GT5: Chaoqing Green Tea; GT6: Taiping Houkui Tea; GT7: Jieyang Chaoqing Tea; GT8: Fenggang Zinc-Selenium-enriched Tea; GT9: Seven Star Matcha Tea; GT10: Selenium-Enriched Matcha Tea. ## $p < 0.01$; ### $p < 0.001$, the model group compared with the control group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, the green tea extract supplementary groups compared with the model group.

3.5. Effects of Green Tea Extracts on Oxidative Damage. Recent work has revealed that CYP2E1 enzymes induced by chronic alcohol exposure can result in producing excessive ROS, which are correlated with lipid peroxidation [56]. Seen from the Figure 5, we observed that the levels of 4-HNE and MDA were significantly increased by chronic alcohol exposure compared with the control group. A previous study revealed that oxidative stress in transgenic mice overexpressing CYP2E1 was enhanced [57]. However, it has been reported that alcohol-medicated lipid peroxidation was significantly blocked by CYP2E1 deletion, and the liver damage was relieved [58]. In the present work, Dianqing Tea (GT1), Selenium-Enriched Chaoqing Green Tea (GT3) and Xihu Longjing Tea (GT4), and Fenggang Zinc-Selenium-enriched Tea (GT8) effectively alleviated liver lipid peroxidation by decreasing the 4-HNE level. The results were consistent with the fact that several teas could significantly decrease chronic alcohol-induced CYP2E1 enzymes level. In addition, the level of MDA was obviously reduced by Dianqing Tea (GT1), Liping Xiang Tea (GT2), Selenium-Enriched Chaoqing Green Tea (GT3), and Selenium-Enriched Matcha Tea (GT10). The results indicated that these teas possessed preventive effects on lipid peroxidation of liver caused by ethanol.

3.6. Effects of Green Tea Extracts on Antioxidant Capacity. It has been identified that alcohol exposure will significantly decrease in vivo antioxidant capacity [59]. In the present

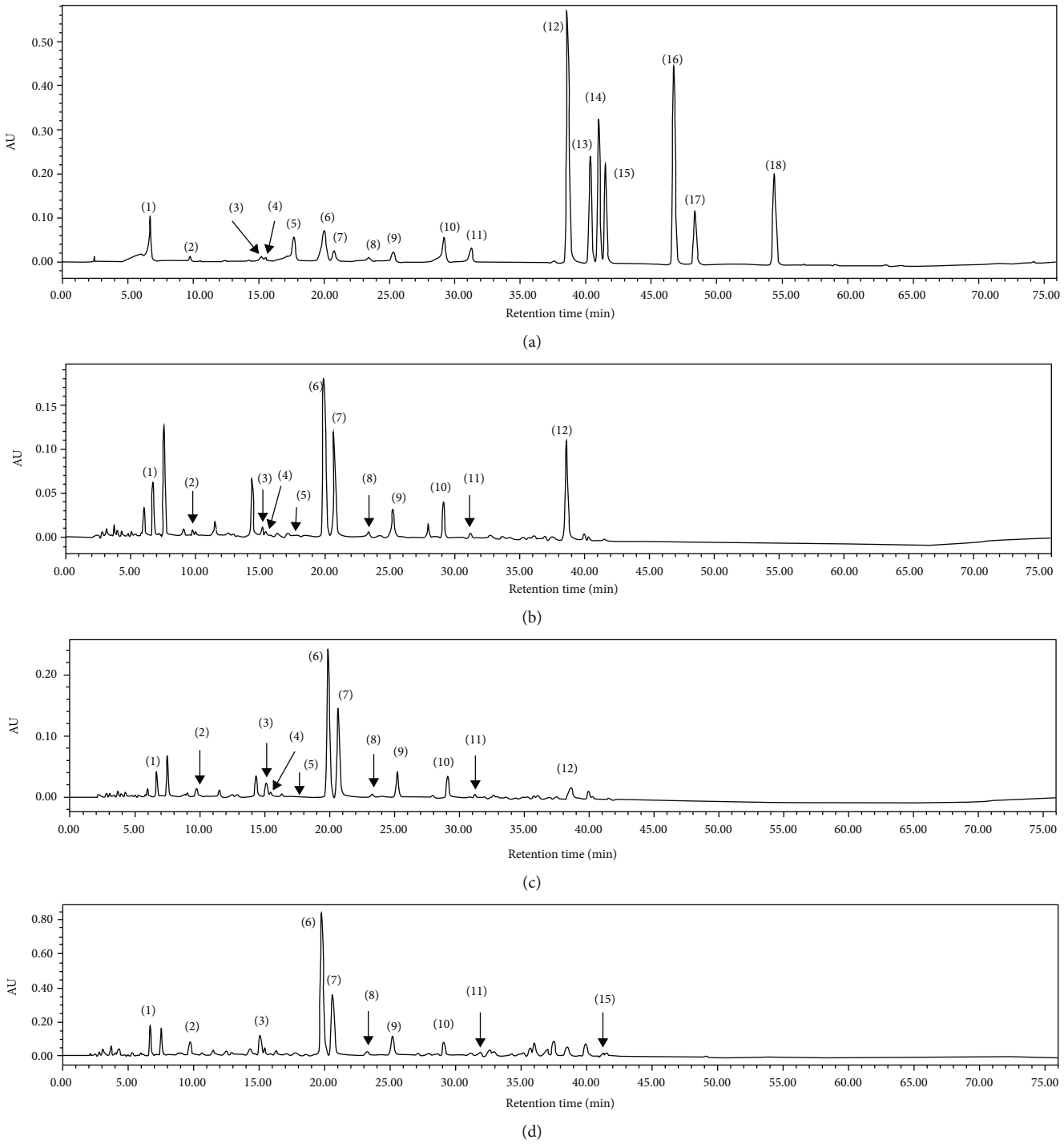

Figure 8: The HPLC chromatograms of the standard compounds. (a) Xihu Longjing Tea. (b) Taiping Houkui Tea. (c) Selenium-Enriched Matcha Tea. (d) Under 254 nm. 1, gallic acid; 2, gallocatechin; 3, epigallocatechin; 4, catechin; 5, chlorogenic acid; 6, caffeine; 7, epigallocatechin gallate; 8, epicatechin; 9, gallocatechin gallate; 10, epicatechin gallate; 11, catechin gallate; 12, ellagic acid; 13, myricetin; 14, quercitrin; 15, astragalin; 16, quercetin; 17, theaflavin; 18, kaempferol.

study (Figure 6), chronic alcohol consumption resulted in a remarkable reduction in hepatic GSH content as well as the activities of GSH-Px and SOD compared with the control group, which indicated a significant inhibition in the antioxidant capacity of the liver. A previous animal research reported that the activities of enzymatic antioxidants (GSH-Px and SOD) were inhibited, and the content of nonenzymatic antioxidants (GSH) was reduced in acute alcohol-induced liver damage mice [60]. In addition, compared with the control group, there was no statistical difference in the change in CAT activity induced by chronic alcohol exposure.

Table 2: The contents (mg/g DW) of main phytochemicals in 10 green teas.

(a)

Main phytochemicals	Dianqing Tea	Liping Xiang Tea	Selenium-Enriched Chaoqing Green Tea	Xihu Longjing Tea	Chaoqing Green Tea
Gallic acid	1.698 ± 0.027	1.588 ± 0.029	1.575 ± 0.035	1.657 ± 0.064	0.683 ± 0.086
Gallocatechin	4.577 ± 0.257	7.901 ± 0.120	6.411 ± 0.416	8.050 ± 0.060	4.941 ± 0.180
Epigallocatechin	13.573 ± 0.253	23.390 ± 0.029	8.654 ± 0.397	19.028 ± 0.040	9.339 ± 0.281
Catechin	4.985 ± 0.197	2.313 ± 0.003	0.690 ± 0.014	9.533 ± 0178	6.010 ± 0.062
Chlorogenic acid	1.230 ± 0.003	—	0.830 ± 0.001	0.804 ± 0.000	—
Caffeine	33.925 ± 0.172	27.664 ± 0.174	31.137 ± 0.228	39.859 ± 0.234	32.766 ± 0.327
Epigallocatechin gallate	53.368 ± 0.463	37.705 ± 0.193	43.304 ± 0.889	34.741 ± 0.284	40.110 ± 1.120
Epicatechin	8.901 ± 0.153	2.665 ± 0.056	5.517 ± 0.076	5.295 ± 0.104	5.249 ± 0.180
Gallocatechin gallate	16.895 ± 0.344	6.222 ± 0.119	—	15.134 ± 1.029	13.950 ± 1.056
Epicatechin gallate	17.649 ± 0.537	3.329 ± 0.020	6.340 ± 0.158	17.188 ± 0.052	17.683 ± 0.030
Catechin gallate	3.475 ± 0.039	0.391 ± 0.009	—	1.747 ± 0.031	2.244 ± 0.041
Ellagic acid	0.778 ± 0.023	—	—	0.950 ± 0.004	—
Myricetin	—	—	—	—	—
Quercitrin	—	—	—	—	—
Astragalin	0.616 ± 0.007	2.702 ± 0.039	—	—	1.053 ± 0.006
Quercetin	—	—	—	—	—
Theaflavin	—	—	—	—	—
Kaempferol	—	—	—	—	—

(b)

Main phytochemicals	Taiping Houkui Tea	Jieyang Chaoqing Tea	Fenggang Zinc-Selenium-Enriched Tea	Seven Star Matcha Tea	Selenium-Enriched Matcha Tea
Gallic acid	0.929 ± 0.043	1.84 ± 0.023	2.335 ± 0.053	1.298 ± 0.031	1.411 ± 0.027
Gallocatechin	9.721 ± 0.076	6.109 ± 0.030	8.667 ± 1.164	5.736 ± 0.075	7.409 ± 0.091
Epigallocatechin	17.590 ± 0.201	9.151 ± 0.219	13.039 ± 0.074	20.865 ± 0.073	9.425 ± 0.050
Catechin	6.407 ± 0.447	6.635 ± 0.143	8.362 ± 0.214	—	—
Chlorogenic acid	0.862 ± 0.005	1.095 ± 0.005	0.819 ± 0.000	—	—
Caffeine	46.218 ± 0.377	25.973 ± 0.054	37.421 ± 0.445	28.715 ± 0.158	29.340 ± 0.270
Epigallocatechin gallate	38.205 ± 0.873	38.687 ± 0.198	62.164 ± 0.298	13.462 ± 0.206	24.264 ± 0.743
Epicatechin	5.268 ± 0.112	6.307 ± 0.114	7.125 ± 0.060	7.421 ± 0.133	3.379 ± 0.052
Gallocatechin gallate	16.140 ± 0.574	9.724 ± 0.147	16.612 ± 0.153	—	9.030 ± 0.069
Epicatechin gallate	16.259 ± 0.180	8.073 ± 0.025	11.411 ± 0.158	8.727 ± 0.079	9.378 ± 0.038
Catechin gallate	1.577 ± 0.024	1.584 ± 0.109	3.056 ± 0.090	0.375 ± 0.001	4.844 ± 0.109
Ellagic acid	0.893 ± 0.044	—	2.069 ± 0.019	—	0.241 ± 0.043
Myricetin	—	—	—	—	—
Quercitrin	—	—	—	—	—
Astragalin	—	2.839 ± 0.024	—	1.823 ± 0.094	—
Quercetin	—	—	—	—	—
Theaflavin	—	—	—	—	—
Kaempferol	—	—	—	—	—

Note. -: not determined; DW: dry weight of tea.

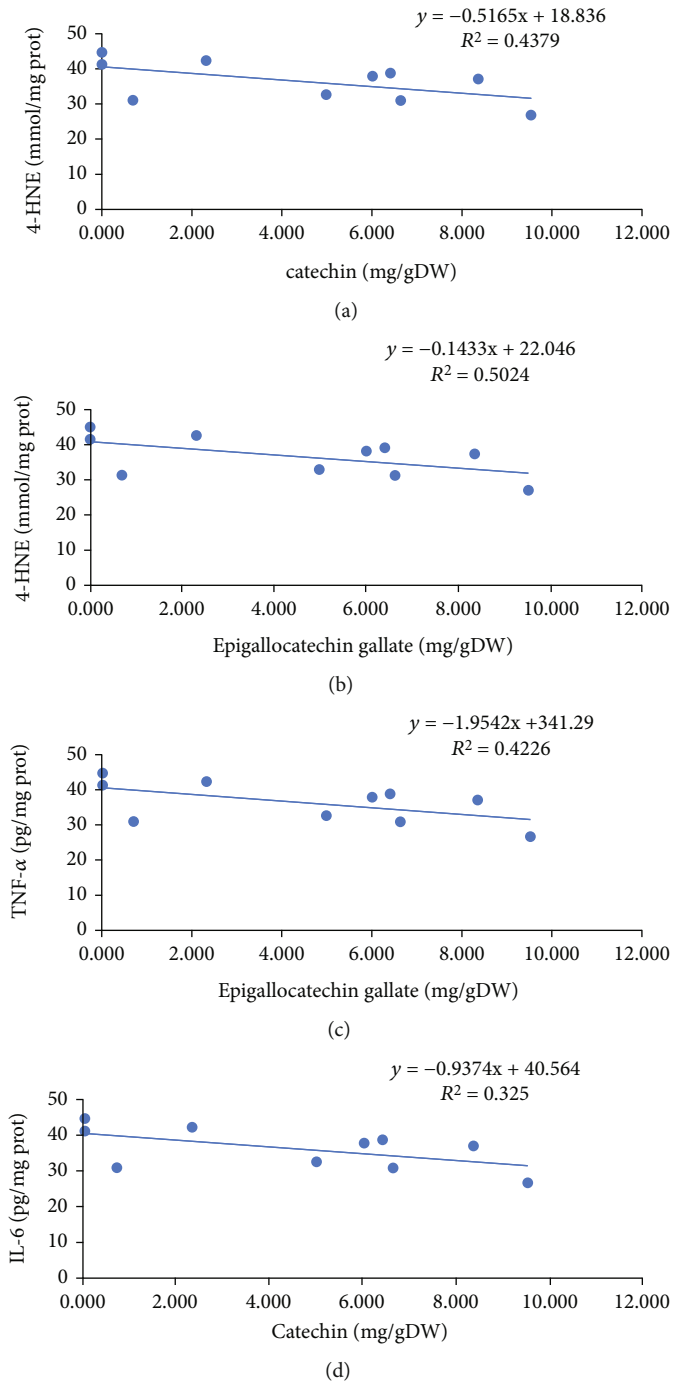

Figure 9: The relationships between biochemical indicators and phytochemical components of green tea. (a) Correlation between the level of 4-HNE and catechin. (b) Correlation between the level of 4-HNE and epigallocatechin gallate. (c) Correlation between the concentration of TNF-α in the liver and epigallocatechin gallate. (d) Correlation between the concentration of IL-6 in serum and catechin. 4-HNE: 4-hydroxynonenoic acid; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6.

Accumulating findings have demonstrated that green tea contained abundant phenolic acids and catechins, especially EGCG, which were responsible for the strong antioxidant properties of green tea [25, 61]. In addition, it has been reported that green tea had strong in vitro antioxidant ability [62]. In this study, we found that only Seven Star Matcha Tea (GT9) and Selenium-Enriched Matcha Tea (GT10) significantly elevated the GSH content, while the intervention of most green tea extracts did not influence the activities of SOD, CAT, and GSH-Px (Figure 6). Moreover, Xihu Longjing Tea (GT4) obviously inhibited the SOD activity and Selenium-Enriched Chaoqing Green Tea (GT3) and Taiping Houkui Tea (GT6) remarkably decreased CAT activity, which illustrated that these teas could reduce the in vivo antioxidant ability in chronic alcohol-induced fatty liver mice.

3.7. Effects of Green Tea Extracts on Inflammation Levels. The available evidence revealed that alcohol exposure results in inflammation [63]. In this study, we observed that chronic alcohol consumption induced a significant increase in the levels of IL-6 and TNF-α in liver compared to the control group. As shown in Figure 7, Selenium-Enriched Chaoqing Green Tea (GT3), Xihu Longjing Tea (GT4), and Fenggang Zinc-Selenium-enriched Tea (GT8) effectively ameliorated inflammation induced by alcohol through decreasing IL-6 and TNF-α levels. Moreover, the IL-6 level was reduced by Dianqing Tea (GT1) and Jieyang Chaoqing Tea (GT7). Consistent with our results, a previous study reported that catechins from tea could effectively inhibited proinflammatory signal pathways and relieve inflammation [28].

3.8. Relation of Biochemical Indicators and Components of Green Teas. In the current study, the main components in 10 green tea extracts were detected and quantified by HPLC. The chromatograms of standard components, Xihu Longjing Tea (GT4), Taiping Houkui Tea (GT6), and Selenium-Enriched Matcha Tea (GT10) under 254 nm wavelength, are displayed in Figure 8. Additionally, the results of main components in 10 green teas are shown in Table 2.

Based on our results, 13 components in 10 green tea extracts have been determined and quantified, including 8 kinds of catechins and 5 other active ingredients (gallic acid, chlorogenic acid, caffeine, ellagic acid, and astragalin). The results showed that catechins are the most abundant phytochemicals in these green tea extracts, but the content of catechins in theses green teas varied greatly. We observed that EGCG was the richest catechin with a range from 13.462 ± 0.206 to 62.164 ± 0.298 mg/g DW (dry weight of tea). In addition, all green teas were found with high contents of caffeine, with a range of 25.973 ± 0.054 to 46.218 ± 0.377 mg/g DW. However, myricetin, quercitrin, quercetin, theaflavin, and kaempferol have not been identified.

The relationship between the main components in green teas and the biochemical indicators was analyzed in the present study. According to the results, some phytochemicals in green tea were associated with serum TC and TG concentrations as well as serum aminotransferase activity (such as AST). For example, a middle negative ($p < 0.05$) correlation has been found between the content of gallocatechin and the serum TC level, and the R^2 value was 0.6018. However, the content of catechin was positively ($p < 0.05$) related to serum TG level, and the R^2 value was 0.3618. In addition, we observed that the contents of gallic acid and EGCG were positively ($p < 0.05$) associated with the activity of AST, and the R^2 values were 0.4972 and 0.4967, respectively. Moreover, the results showed that the content of gallic acid was negatively ($p < 0.05$) related to the activity of ADH, and the R^2 value was 0.5194.

The contents of gallic acid, catechin, chlorogenic acid, and EGCG were all negatively ($p < 0.05$) related to the level of 4-HNE, and the R^2 values were 0.3433, 0.4379, 0.4317, and 0.5024, respectively (Figures 9(a) and 9(b)). The results indicate that gallic acid, catechin, chlorogenic acid, and EGCG in green tea extracts might be closely related to ameliorating chronic alcohol-induced lipid peroxidation damage. In addition, the concentration of GSH showed a significant negative ($p < 0.05$) correlation with the contents of catechin, chlorogenic acid, and EGCG, and the R^2 values were 0.6031, 0.4225, and 0.5726, respectively. Moreover, a weak negative ($p < 0.05$) correlation was observed between the activity of GSH-Px and the content of catechin, and the R^2 value was 0.4541. Besides, the content of catechin was negatively ($p < 0.05$) associated with the activity of SOD, and the R^2 value was 0.367. The results suggest that catechin, chlorogenic acid, and EGCG might at least partially inhibit the antioxidant activity of green tea in vivo.

The correlations between these main components and the inflammatory cytokines (TNF-α and IL-6) were also analyzed (Figures 9(c) and 9(d)). The results showed that the concentrations of catechin, chlorogenic acid, and EGCG were negatively ($p < 0.05$) correlated with the level of TNF-α, and the R^2 values were 0.5438, 0.4167, and 0.4226, respectively. Furthermore, the level of IL-6 showed an obvious negative ($p < 0.05$) correlation with the contents of catechin and chlorogenic acid, and the R^2 values were 0.325 and 0.5724, respectively. These results indicate that catechin, chlorogenic acid, and EGCG in green tea might be related to anti-inflammatory property.

4. Conclusions

The effects of 10 green teas on mice with AFLD caused by chronic alcohol exposure and the correlation between their main components and AFLD were studied and compared. The results revealed that most green teas significantly reduced the levels of TG and the aminotransferase activities (such as AST and ALT), indicating that they possess hepatoprotective effects. In addition, certain green teas remarkably decreased the expression of CYP2E1, the levels of MDA and 4-HNE, and the contents of TNF-α and IL-6, suggesting that they could relieve oxidation damage and inflammation induced by chronic ethanol consumption. Moreover, Seven Star Matcha Tea and Selenium-Enriched Matcha Tea could increase GSH content. On the other hand, the most abundant ingredients are catechins and caffeine among the 10 green teas. Moreover, the correlation analysis showed that gallic acid, gallocatechin, catechin, chlorogenic acid, and EGCG might at least partially contribute to preventive effects on AFLD. On the other hand, some green teas could increase hepatic TC content and decrease SOD, CAT, and ALDH activities, which could result in some adverse effects. In previous review paper [62], the adverse effects of various teas have been summarized in detail, most studies have focused on nonalcoholic fatty liver disease and normal persons, and no report about green teas against ALD could be found. In the future, a special attention should be paid to the adverse effects of teas (including green tea) and their components against ALD. In total, four teas, including Selenium-Enriched Chaoqing Green Tea, Xihu Longjing Tea, Taiping Houkui Tea, and Selenium-Enriched Matcha Tea, showed the stronger preventive effects on AFLD than other six teas, but the difference was very less among four teas. These four green teas could be developed into

functional foods for the prevention and management of AFLD. Furthermore, the results provide the public and nutritionists with new insights about the effects of green teas on AFLD.

Data Availability

The data is kept in School of Public Health, Sun Yat-Sen University.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization was contributed by B.-Y.L., R.-Y.G., and H.-B.L. Investigation was contributed by B.-Y.L., H.-Y.L. D.-D.Z., S.-Y.H., M.L., Q.-Q.M., A.S., and A.S. Resources were contributed by B.-Y.L. Data curation was contributed by B.-Y.L. Writing—original draft preparation was contributed by B.-Y.L. Writing–review and editing was contributed byR.-Y.G. and H.-B.L.

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Review Article

Lasia spinosa Chemical Composition and Therapeutic Potential: A Literature-Based Review

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Lasia spinosa (L.) is used ethnobotanically for the treatment of various diseases, including rheumatoid arthritis, inflammation of the lungs, bleeding cough, hemorrhoids, intestinal diseases, stomach pain, and uterine cancer. This review is aimed at summarizing phytochemistry and pharmacological data with their molecular mechanisms of action. A search was performed in databases such as PubMed, Science Direct, and Google Scholar using the keywords: "*Lasia spinosa*," then combined with "ethnopharmacological use," "phytochemistry," and "pharmacological activity." This updated review included studies with in vitro, ex vivo, and in vivo experiments with compounds of known concentration and highlighted pharmacological

mechanisms. The research results showed that *L. spinosa* contains many important nutritional and phytochemical components such as alkanes, aldehydes, alkaloids, carotenoids, flavonoids, fatty acids, ketones, lignans, phenolics, terpenoids, steroids, and volatile oil with excellent bioactivity. The importance of this review lies in the fact that scientific pharmacological evidence supports the fact that the plant has antioxidant, anti-inflammatory, antimicrobial, cytotoxic, antidiarrheal, antihelminthic, antidiabetic, antihyperlipidemic, and antinociceptive effects, while protecting the gastrointestinal system and reproductive. Regarding future toxicological and safety data, more research is needed, including studies on human subjects. In light of these data, *L. spinosa* can be considered a medicinal plant with effective bioactives for the adjuvant treatment of various diseases in humans.

1. Introduction

Traditional, herbal, and Ayurvedic medicine comprise an important and prestigious form of treatment for various diseases and conditions in different locations all over the world from the beginning of human civilization on Earth [1]. Several plants and their corresponding preparations have been used for various therapeutic purposes for a long time ago. The history of traditional, herbal, and Ayurvedic medicine is the eldest to establish a treatment pattern [1].

Lasia spinosa (L.) Thwaites, often known as Chengmora/Sibru in Assames, Kata-kachu in Bengali, Janum-Saru in Manipuri, Kohila/Mahakohila/Engilikohila in Sri Lanka, Zawangzang in Mizoram, and Laksmana in Sanskrit [2–4], belongs to the *Araceae* family [5]. It is found in Asia-Bangladesh, China, the Indian subcontinent, Myanmar, Thailand, Indo-China, Indonesia, and Papua New Guinea [6].

Briefly, *L. spinosa* is an aquatic or terrestrial plant, short-stemmed spiny heirs with underground rhizome that usually occurs in wet forests, open marshes, wetlands, or in permanently standing water [7]. *L. spinosa* is a large marsh plant with the stem stout 1 m high and the leaves broadly arrow-shaped in outlines, 20-30 cm long deeply divided into 4-6 pairs of narrow side lobes. The petiole is 30-40 cm long, veins beneath the petiole and peduncle prickly [8]. Morpho-anatomical feature of *L. spinosa* has been recently reported by Lakshmi et al. [9]. The plant is harvested from the wild for its edible leaves and various medicinal uses. Sometimes, it is also cultivated as a vegetable crop along ponds margins [10]. Recently, it has been reported that *Fusarium fujikuroi* caused leaf spot on *L. spinosa* in China [11].

With regards to their biological effects, the tender leaves and rhizomes of this plant, used as a vegetable and in indigenous medicine, have been recommended for a variety of conditions [12–15].

Given the multiple potentialities of this plant, this review provides up-to-date data on *L. spinosa* chemical composition and biological effects based on the scientific reports found in the databases.

2. Review Methodology

In this study, the literature on botanical classification of *L. spinosa*, ethnomedicinal applications, secondary metabolites, biological properties were compiled, reviewed, and summarized. For the compilation of all written papers on this species, scientific search engines such as PubMed, ScienceDirect, SpringerLink, Web of Science, Scopus, Wiley Online, Scifnder, and Google Scholar have been used. In this study, the literature on botanical classification of Lasia spinosa, ethnomedicinal applications, secondary metabolites, and biological properties were compiled, reviewed, and summarized. For searching, the next MeSH terms were used: "Phytotherapy", "Plants", "Medicinal", "Plant Extracts/administration & dosage", "Plant Extracts/isolation & purification", "Plant/chemistry", "Structure-Activity Relationship", "Disease Models", "Animal", and "Plant Extracts/toxicity". Using the Chemsketch version 12.01 program, chemical structures were drawn. The scientific names of the plants have been verified according to the PlantList [16, 17].

Inclusion criteria: works published in English on Lasia spinosa that highlighted the following data: chemical compounds isolated from each genus, preclinical pharmacological research highlighting molecular mechanisms, and *in vitro*/*in vivo* pharmacological studies that contained the concentration and dose at which the chemical compounds studied were pharmacologically active and toxicological data. The most important results obtained were summarized in the tables.

Exclusion criteria: abstracts, letters to the editor, papers in languages other than English, studies that did not have dose-effect correlations, and studies that did not have proven molecular mechanisms which underline the pharmacology.

3. Ethnopharmacology

L. spinosa is a medicinally important plant, traditionally used by different ethnic communities all over the world. There are various reports on *L. spinosa* medicinal and economical properties. Often used for treating colic, tuberculosis of lymph nodes, swollen lymph nodes, rheumatism/rheumatoid arthritis, injuries, snake bites, and insect bites, this plant is also recommended as effective for the treatment of sore throat, constipation, to purify the blood, on lung inflammation, bleeding cough, and uterine cancer [14, 18–20]. Rhizomes (roots) are most often used as a remedy for hemorrhoids in Sri Lanka and Malays and to confer protection for some of the above conditions, because of their high fibre content and antioxidant compounds [15].

Besides, leaves and stalks have demonstrated profound antihelminthic, anticestode, and antinematode efficacy [12, 15, 18, 19, 21, 22]. The root decoction is also useful in gastrointestinal diseases and stomachache [4], while also stimulating liver function [22]. Paste from tender leaves is externally used in burns [4].

4. Phytochemical Profile

L. spinosa whole plant contains several essential phytochemicals, including alkaloids, flavonoids, tannin, saponins, steroids, terpenoids, and varying amounts of micronutrients, like zinc (Zn), magnesium (Mg), calcium (Ca), iron (Fe), copper (Cu), manganese (Mn), and molybdenum (Mo) (Table 1).

Nutritional analysis of *L. spinosa* showed that it contains proteins (17.6 kcal/100 g), fats (1.16 kcal/100 g), and carbohydrates (35.7 kcal/100 g), with a nutritive value of 224 kcal/100 g [2, 23]. In another study, the protein, fats, and carbohydrate content on a dry weight basis were 17.9, 3.8, and 45.5 g/100 g edible portion for protein, fats, and carbohydrate, respectively, for *L. spinosa* leaf, with a nutritive value of 288.5 kcal/100 g [24]. *L. spinosa* roots/rhizome contains dietary fibre, Ca, and provitamin A carotenoids [18, 25]. *L. spinosa* leaf contains 15.4 g of fibre, 250 mg of Ca, 19.2 mg of Fe, and 455 mg of vitamin C for 100 g edible portion on a dry weight basis [24, 26].

In fresh weight, other studies reported content of proteins, fats, and carbohydrates of 3.68 ± 0.28, 0.44 ± 0.03, and 4.78 ± 0.38 g/100 g, respectively, the mineral content of 158.08 ± 3.98, 321.73 ± 7.00, 73.17 ± 2.37, 53.86 ± 3.86, and 0.92 ± 0.08 mg/100 g for Ca, K, P, Mg, and Fe, and 2.99 ± 0.11 and 0.28 ± 0.01 mg/100 g of vitamins C and E, respectively [27].

When specifically addressing the different extracts prepared from *L. spinosa*, hexane extracts leaves and root contains the alkaloid berberine [28], lignan (e.g., lyoniresinol, meridional, secoisolariciresinol; 5,5′-dimethoxysecoiso-lariciresinol; 2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)-1,2-butanediol; (7′S,8S,8R)-4,4′-dihydroxy-3,3′5,5′-tetra methoxy-7′,9-eproxylignan-9′-ol-7-one; 5,5′-dimethoxy-lariciresinol; 5′-methyoxlariciresinol, dihydrodehydrodiconifery alcohol; syringaresinol) [27–29], aldehyde (e.g., p-hydroxy benzaldehyde) [30], phenolic (e.g., procyanidin A1) [31] and other compounds (e.g., 4-hydroxybenzoic acid, 2-(4′- methoxyphenyl)-ethanol, 4-methoxyphenyl alcohol, 1-tetracosane) [30], from stem carotenoids (e.g., α-carotene, β-carotene, β-carotene-5,6, 5′, 6′-diepoxide; 5, 6, 5′, 6′-diepoxy-5, 8, 5′,8′-tetrahydro-β, cis-neoxanthin, and unidentified carotenoids I, II, III, and IV) are isolated [24, 32, 33].

The aerial parts of *L. spinosa* contain terpenoids (e.g., limonene, aqualene, caryophyllene), volatile oil (e.g., methyl octadec-6,9-dien-12-ynoate, α-glyceryl-linolenate α-pinene, α-selinene, camphene, δ-3-carene, camphor) [21, 34], phenolic compounds (e.g., 4-hydroxybenzoic acid, morin, cinnamic acid, syringic acid, gentisic aid) [21, 28, 34], fatty acids (e.g., methyl ester of oleic acid, palmitic acid, stearic acid, epoxyoleic acid) [34], steroids (e.g., spinasterone, β-sitosterol, γ-sitosterol, stigmasterol, campesterol, crinosterol) [21, 34], alkane (e.g., hexatriacontane and heptacosane) [35].

The whole plant contains phenolics (e.g., gentisic acid, isovanilic acid, syringic acid, chlorogenic acid, p-hydroxy benzoic acid, (+)-catechin) [28], flavonoids including flavonoid glycosides and flavonoid aglycones (e.g., vitexin, vitexin-2”-O-β-D-glucopyranoside; isorhamnetin 3-O-rutinoside, morin, apigenin, 3′-methyl-quercetin-3-O-α-L-rhamnopyranosyl-(1/6) β-D-glucopyranoside; triglochinin) [28, 32, 35], and ketone (e.g., hexahydrofarnesyl acetone) (21).

Table 1: Amounts of micronutrients of *Lasia spinosa* in ppm (parts per million) [2].

Elements	Amounts (ppm)
Zn	7.442 ± 0.01
Mg	6.228 ± 0.11
Fe	17.06 ± 0.87
Cu	0.316 ± 0.02
Mn	1.334 ± 0.08
Mo	1.180 ± 0.06

The chemical structures of such compounds are shown in Table 2 and Figure 1.

5. Pharmacological Properties: Mechanisms and Targeted Molecular Pathways

5.1. Antioxidant. Oxidative stress is the basis of premature ageing of the body, the basis of disease, and is triggered by free radicals [29] more precisely occurs as a result of the imbalance between the amount of reactive oxygen produced in the body and its ability to eliminate it [30, 31]. Oxidative stress can be alleviated by approaching a balanced lifestyle that includes a healthy diet and sports [32]. Physical exercise reduces cellular oxidation by deep oxygenating tissues, eliminating stress, and relaxing the body [33]. On the other hand, the diet has a very important role, and the best treatment against oxidative stress is antioxidants [34]. They are found in many herbs and can kill free radicals [35, 37]. Medicinal plants usually contain a high level of antioxidants that can counteract the oxidative stress process linked to a disease [38, 39].

The free radical scavenging activity of *L. spinosa* leaves extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) had been assessed and showed significant antioxidant activities [40]. The ethyl acetate fraction showed the highest free radical scavenging activity ($IC_{50} = 16.42\,\mu$g/mL) when compared to the positive control-butylated hydroxytoluene (BHT). At the same time, the aqueous fraction also exhibited moderate antioxidant potential ($IC_{50} = 73.20\,\mu$g/mL) [40]. In DPPH and ABTS assay, ethanol extract (leaves) showed antioxidant activity ($SC_{50} = 17.25\,\mu$g/mL and 16.47 μg/mL, respectively). Antioxidant activity is due to the presence of high levels of polyphenolic compounds [38]. In a study performed with different extracts of *L. spinosa* aerial parts, the highest free radical scavenging activity (DPPH) was stated to the methanol extract ($IC_{50} = 0.48 \pm 0.04\,\mu$g/mL), whereas in the metal chelating activity of ferrous ions (Fe^{2+}) assay, the highest activity was observed for hexane extract ($IC_{50} = 0.55 \pm 0.08\,\mu$g/mL) [23]. In another study, the antiradical activity ($1/EC_{50}$) of *L. spinosa* leaf determined by the DPPH method was 0.1 [24]. The same

Table 2: Phytochemical profile of *Lasia spinosa*.

Plant parts	Phytochemical class	Compounds	Ref.
Leave	Alkaloids	Berberine	[25]
Leaf and root/ rhizome	Aldehyde	*p*-Hydroxy benzaldehyde	[26]
	Other compounds	4-Hydroxybenzoic acid, 2-(4′-methoxyphenyl)-ethanol, 4-methoxyphenethyl alcohol, 1-tetracosane	
Stem	Carotenoid	α-Carotene, β-carotene, β-carotene-5,6, 5′, 6′-diepoxide; 5, 6, 5′, 6′-diepoxy-5, 8, 5′,8′-tetrahydro-β, cis-neoxanthin	[24, 31] Priyadarshani and Jansz, [25]
Aerial parts	Terpinoid	Limonene, β-elemene, squalene, caryophyllene	[21]; Rahman et al., [23]
	Volatile oil	Methyl octadec-6,9-dien-12-ynoate, α-glyceryl-linolenate α-pinene, α-selinene, camphene, δ-3-carene, camphor	[21]; Rahman et al., [23]
	Phenolics	4-Hydroxybenzoic acid, morin, cinnamic acid, syringic acid, gentisic acid	Rahman et al., [23]; [21, 28]
	Fatty acids	Methyl ester of oleic acid, palmitic acid, stearic acid, epoxyoleic acid	Rahman et al., [23]
	Steroid	Spinasterone, β-sitosterol, γ-sitosterol, stigmasterol, campesterol, crinosterol,	Rahman et al., [23]; [21]
	Alkane	Hexatriacontane, heptacosane	Rahman et al., [23]
Root/ rhizome	Lignan	Lyoniresinol, meridinol, secoisolariciresinol; 5,5′-dimethoxysecoisolariciresinol; 2-(4-hydroxy-3,5-dimethoxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)-1,2-butanediol; (7′S,8S,8R)-4,4′-dihydroxy-3,3′5,5′-tetramethoxy-7′,9-eproxylignan-9′-ol-7-one; 5,5′-dimethoxy-lariciresinol; 5′-methyoxlariciresinol, dihydrodehydrodiconifery alcohol; syringaresinol	Alam et al., [36]; [28, 29, 32]
	Phenolic	Procyanidin A1	[30]
Whole plant	Phenolic	Gentisic acid, isovanilic acid, syringic acid, chlorogenic acid, p-hydroxy benzoic acid, (+)-catechin	[28]
	Flavonoids (glycosides and aglycones)	Vitexin, vitexin 2”-O-β-D-glucopyranoside; isorhamnetin 3-O-rutinoside, morin, apigenin, 3′-methyl quercetin-3-O-α-L-rhamnopyranosyl-(1/6) β-D-glucopyranoside; triglochinin	[27, 28, 31]
	Ketone	Hexahydrofarnesyl acetone	[21]

study reported a total phenolic content of 6.4 mg gallic equivalents/g and total flavonoid content of 4.4 retinol equivalent in *L. spinosa* leaf [24]. In other studies, *L. spinosa* showed a total phenolic content of 2.1 mg gallic equivalents/g and low antioxidant activity, through ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays, in comparison to indigenous vegetables from Southern Thailand, such as young cashew leaves (*Anacardium occidentale* L.) and Mon-pu (*Glochidion zeylanicum* (Gaertn.) A.Juss.) [27].

5.2. Anti-Inflammatory. Inflammation is part of the complex biological response of body tissues to harmful stimuli such as pathogens, damaged cells, or irritants [41] and a protective response involving immune system cells, molecular mediators, among others [42–44].

In lipopolysaccharide-induced RAW 264.7 macrophages, the anti-inflammatory activity of *L. spinosa* leaf extract was addressed [45], is stated that it can activate the nuclear factor- (NF-) κappa B, and nuclear factor erythroid 2-related factor 2/heme-oxygenase-1 (Nrf2/HO-1) pathways and to suppress mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathways. Furthermore, *L. spinosa* leaf extract suppresses the upregulation enzyme iNOS (NOS2), COX2, and proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and increases cytokines (IL-10) which produced anti-inflammatory effect [40].

In another study, the anti-inflammatory activity of *L. spinosa* hydroalcoholic extract in xylene-induced ear oedema model mice was assessed, being stated a significant inhibitory effect on oedema formation 17.1% at 250 mg/kg and 27.9% at 500 mg/kg. An inhibitory potential was also stated in a carrageenan-induced paw oedema model rat, and it was highest at 3 h, with 26.72% inhibition at 250 mg/kg and 38.70% at 500 mg/kg, when compared to the standard drugs (diclofenac sodium (10 mg/kg): 29.52%, and phenylbutazone (100 mg/kg): 40.47%) (Figure 2) [46].

5.3. Antimicrobial. An antimicrobial agent is that able to kill or stop microorganisms' growth. For that, antibacterial and antifungals are used to fight bacterial and fungal infections, respectively [47–49]. Specifically addressing antibacterial, their prolonged use is closely related to a marked decrease

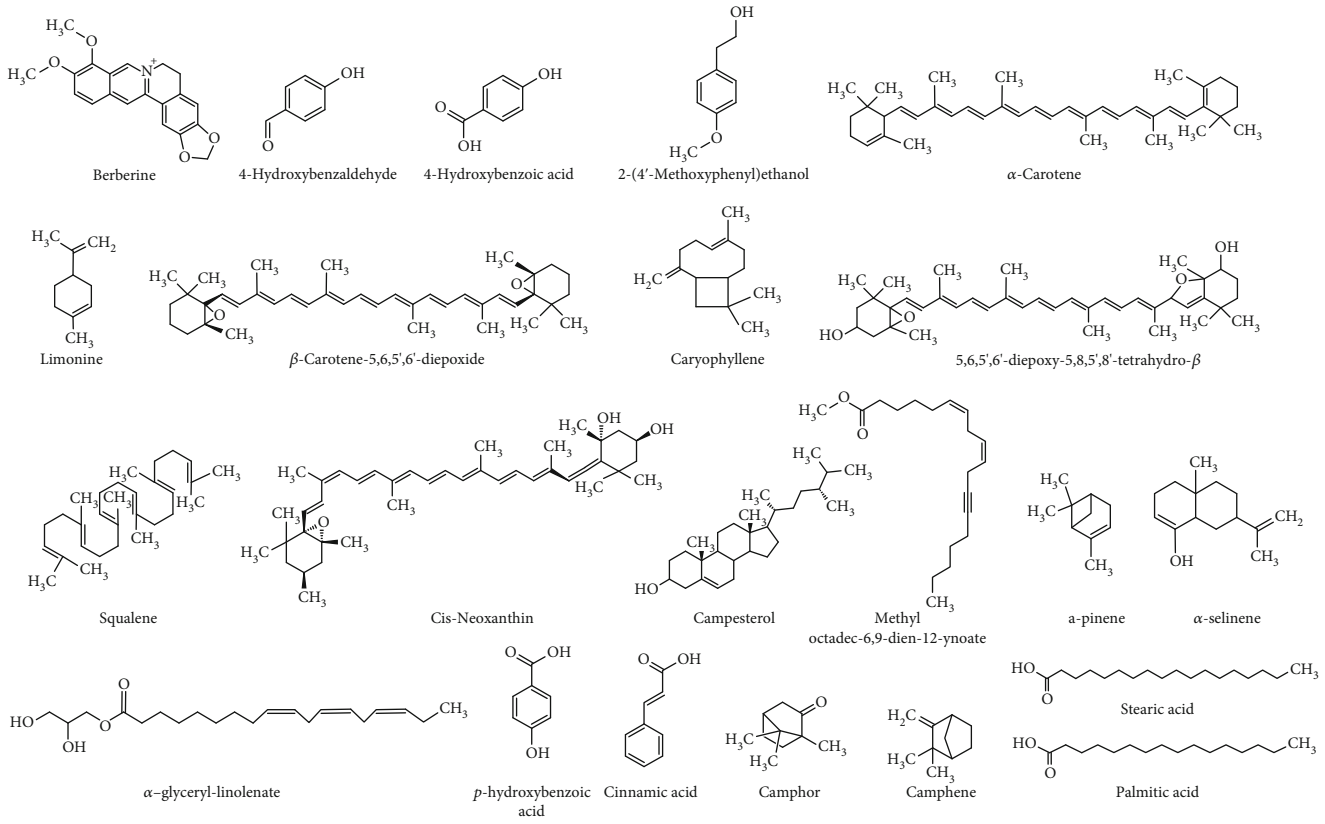

Figure 1: Chemical structures of the most important phytochemicals found in different parts of *Lasia spinosa*.

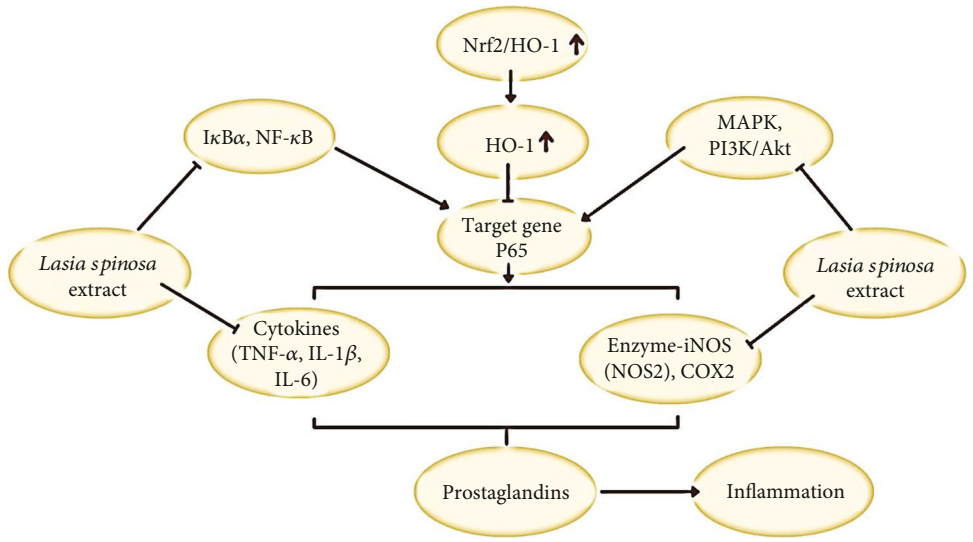

Figure 2: Diagram with molecular mechanisms of anti-inflammatory effect of *Lasia spinosa*.

in the number of enteric bacteria, thus, having a major negative impact on health and wellbeing [50, 51]. In this sense, the consumption of probiotics and a prebiotics-rich diet may help to replace the destroyed gut microbiota [52]. Stool transplants may also be proposed for patients with difficulty in recovering from prolonged antibiotic treatment, as for recurrent *Clostridium difficile* infections [53, 54].

The organic extracts (hexane, chloroform, ethyl acetate; 300 μg/disc) and essential oil of *L. spinosa* aerial parts showed potent antibacterial activity against *Escherichia coli*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* in comparison of standard antibiotics (tetracycline 30 μg/disc, streptomycin 30 μg/disc, and erythromycin 15 μg/disc),

except for methanol extract [23]. In another study, *L. spinosa* leaves methanol extract showed moderate antimicrobial properties against *Bacillus subtilis*, *E. coli*, *Bacillus cereus*, *S. aureus*, *Candida albicans*, *Aspergillus niger*, and *Vibrio para hemolyticus* at 400 μg/disc by disc diffusion assay [36]. In other studies, the methanol extract of *L. spinosa* edible parts did not show antimicrobial activity against *C. albicans* [46].

5.4. Cytotoxic. Cytotoxicity destroys cancer cells or prevents them from multiplying [55–57]. This cytotoxicity is done in different ways: some bioactive compounds can affect the genetic material of cells, and others act by blocking the access of malignant cells to the nutrients needed for division and multiplication [58, 59].

The cytotoxic potential of *L. spinosa* extracts has also been assessed. Brine shrimp lethality bioassay technique was applied to determine the cytotoxic potential of crude extracts. The aqueous extract from *L. spinosa* leaves showed moderate cytotoxicity (LC_{50} = 98.66 μg/mL) in brine shrimp lethality bioassay [40], while the methanol extract from the whole plant led to significant cytotoxic effects (IC_{50} = 13.49 μg/mL) on brine shrimp [36].

5.5. Antidiarrheal. In the castor oil-induced diarrheal mice model, both standard antimotility drug loperamide and hydroalcoholic extract from *L. spinosa* root significantly reduced the number of stools in a dose-dependent manner compared to the negative control group. The mean number of stools found was 11.6 for 250 mg/kg and 8.2 for 500 mg/kg b.w., whereas for the standard drug (5 mg/kg) was of 5.6.*L. spinosa* root extract at 250 mg/kg b.w, causing a little increase in the latent period, but at 500 mg/kg b.w. led to a significant increase [46]. *L. spinosa* root extract exhibited a potent antidiarrheal activity, supporting their traditional use for diarrhea.

5.6. Antihelminthic. Helminthic infections continue to be the major people's health hazard, especially in those living in tropical developing countries [60]. *L. spinosa* leaves methanol extract significantly exhibited paralysis and triggered worms' death, especially at high doses (100 mg/mL) against *Pheritimaposthuma* [61], in a hymenolepisdiminuta–rat animal model [12] and infected mice with *Trichinellaspiralis* (800 mg/kg; *p.o.*) [19].

5.7. Antidiabetic. Diabetes mellitus is a chronic metabolic disease with numerous complications, like retinopathy, neuropathy, and peripheral vascular insufficiency. Several synthetic agents are available for diabetes treatment, but several side effects have been reported [62]. Plant-based medicinal products have been used since ancient times to manage diabetes in traditional medicine in many countries all over the world. *L. spinosa* stem hydroalcoholic extract have revealed antidiabetic activity at 200 and 400 mg/kg (p.o.) in dexamethasone (10 mg/kg s.c.)-induced diabetic albino rats by preventing serum glucose levels rise triggered by dexamethasone [63] and significantly reducing the triglycerides levels [64]. This extract also ameliorated hyperglycemia, and it likely has greater therapeutic potential as they may also exert beneficial effects on the clinical course of noninsulin-dependent diabetes mellitus (NIDDM), hypertension, and coronary artery disease [63]. A study developed by Shafie et al. [65] showed inhibitory effects of different parts (leaves, stems, and roots) of *L. spinosa* extracts (aqueous hot/cold, ethanol) against pancreatic lipase, α-amylase, and α-glucosidase.

5.8. Antihyperlipidemic. L. spinosa leaves have also the potential to prevent hyperlipidemia-induced pancreatitis in rats at concentrations of 400 and 800 mg/kg (p.o.) while exerting cardioprotective effects by significantly increasing serum high-density lipoprotein-cholesterol (HDL-c) at 100 mg/kg, p.o., and Triton-X 100 at 480 mg/kg, i.p. in an induced hyperlipidemic animal model [3].

5.9. Antinociceptive. Antinociception is the action or process of blocking the detection of a painful or injurious stimulus by sensory neurons, and antinociceptives are agents that block painful stimulus [66, 67]. The acetic acid-induced writhing test is used for detecting both central and peripheral analgesia, whereas the hot plate is most sensitive to centrally acting analgesics [68]. In acetic acid-induced writhing and hot plate-induced pain in mice the hydroalcoholic extract of *L. spinosa* roots revealed antinociceptive activity in mice, being stated 37% and 50% writhing inhibition at 250 mg/kg and 500 mg/kg b.w., respectively, while increased pain threshold [46]. On the other hand, the methanol extract from *L. spinosa* leaves at 400 mg/kg led to a significant decrease in the number of writhes and elongated the reaction time in the acetic acid writhing method and radiant heat tail flicking method, respectively [69].

5.10. Gastroprotective. A study revealed that *L. spinosa* leaves ethanol extract has gastroprotective effects. In albino rats with indomethacin (5 mg/kg, p.o.) and cold restrain stress-induced ulcers, 3 doses (100, 200, and 400 mg/kg, p.o.) of *L. spinosa* extract were tested, with gastroprotective effects being mainly conferred by the extractability to create a defensive layer in stomach, through scavenging free radicals and inhibiting lipid peroxidation [70]. In gastric secretion studies, *L. spinosa* significantly evidenced a tendency to decrease gastric juice, free acidity, and total acidity [70]. Thus, after isolation of the individual compounds present in the extract, those responsible for the observed effect can be used both to treat ulcers and to reduce their severity.

5.11. Effect on Reproductive Activity. Testosterone plays an important role in Sertoli and Leydig cell proliferation and hyperplasia that can increase the testis size [71]. Testosterone is also involved in spermatogenesis and the growth and development of testis and male accessory reproductive glands [64]. The hydroalcoholic extract of *L. spinosa* rhizomes was revealed to be able to increase the serum testosterone levels and sperm count in male rats at 40 g/kg b.w. These data were confirmed afterward by an increase in testicular weight and sperm count, with the increase in the absolute weight of testis being attributed to the elevation of androgen biosynthesis leading to an increase in serum testosterone levels [64].

The most important pharmacological properties are summarized in Table 3 and Figure 3.

Table 3: Pharmacological activities of *Lasia spinosa*.

Activity	Sources	Test system	Dose tested	Positive value	Results	Ref.
Antioxidant	Ethyl acetate extract (leaves)	DPPH	IC_{50} = 73.20 μg/mL	BHT IC_{50} = 23.19 μg/mL	Moderate potential	[40]
	Ethanol extract (leaves)	DPPH ABTS	DPPH, IC_{50} = 17, 25 μg/mL; ABTS SC_{50} = 16.47 μg/mL	Vitamin C Dose = 5.38 μg/mL Trolox Dose = 3.17 μg/mL	↑ antioxidant activity due to the presence of high levels of polyphenolic compounds	[45]
	Hexane, chloroform, ethyl acetate, and methanol extracts (aerial parts)	DPPH Chelating activity of ferrous ions (Fe^{2+})	IC_{50} = 0.48 ± 0.04 μg/mL (methanolic) IC_{50} = 0.55 ± 0.08 μg/mL (hexane)	Not studied	↑ antioxidant activity	[23]
Anti-inflammatory	Ethanol extract (leaves)	Lipopolysaccharide-induced RAW 264.7 macrophages *in vitro*	Dose = 50, 100, 200, 400 μg/mL	L-NAME Dose = 100 μM	↑ NF-κB, ↑ Nrf2/HO-1 ↓MAPK, ↓ PI3K/Akt	[45]
	Hydroalcoholic extract (roots)	Carrageenan-induced paw edema model rats and xylene-induced ear edema mice *in vivo*	Dose = 250, 500 mg/kg bw, i.p. n = 6	Nalbuphine Dose = 10 mg/kg	↓oedema formation	[46]
Antimicrobial	Methanol extract (leaves)	Disc diffusion assay *in vitro*	Dose = 400 μg/disc	Kanamycin Dose = 30 μg/disc	Moderate antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger*, and *Vibrio parahemolyticus*	[36]
	Hexane, chloroform, ethyl acetate, and methanol extracts (aerial parts) and essential oil	Disc diffusion assay *in vitro*	Dose = 300 μg/disc	Tetracycline Dose = 30 μg/disc Streptomycin Dose = 30 μg/disc Erythromycine Dose = 15 μg/disc	Potent antibacterial activity against *Escherichia coli*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*, with the exception of methanolic extract	[23]
Cytotoxicity	Hydromethanolic extract (leaves)	Brine shrimp lethality bioassay *in vitro*	LC_{50} = 98.663 μg/mL	Vincristine sulphate LC_{50} = 0.544 μg/ml	Moderate cytotoxic effect	[40]
	Methanol extract (whole plant)	Triton-X 100 (480 mg/kg, i.p.) induced hyperlipidemic rat model *in vivo*	IC_{50} = 13.49 μg/mL	Vincristine sulphate $LC_{50=}$20 μg/ml	↑cytotoxicity	[36]
			Dose = 200, 400, 800 mg/kg, p.o. n = 6	Triton-X 100 Dose = 480 mg/kg	↓triglycerides, ↓LDL-C ↓VLDL-C	
Antidiarrheal	Hydroalcoholic extract (roots)	Castor oil-induced diarrhea mice model *in vivo*	250 and 500 mg/kg b.w., i.p. n = 6	Loperamide Dose = 5 mg/kg	↓number of stools ↑latent period of diarrhea	[46]
Anthelmintic						[61]

Table 3: Continued.

Activity	Sources	Test system	Dose tested	Positive value	Results	Ref.
	Methanol extract (leaves)	*Pheritima posthuman* *in vivo*	Dose = 25, 50, 100 mg/mL, $n = 6$	Albendazole Dose = 10 mg/ml	↑paralysis, ↑worm death, especially at 100 mg/ml	
	Extract (leaves)	*Hymenolepis diminuta* rat model *in vivo*	Dose = 200, 400, 800, 1600 mg/kg, p.o., $n = 6$	Praziquantel Dose = 5 – 10 mg/kg	↓eggs per gram of feces ↓worm recovery rates	[12]
Antidiabetic	Hydroalcoholic extract (stem)	Dexamethasone 10 mg/kg s.c. induced diabetes rats *in vivo*	Dose = 200, 400 mg/kg, p.o. $n = 6$	Dexamethasone dose = 10 mg/kg	↑antidiabetic activity	[63]
Antihyperlipidemic	Methanol extract (leaves)	Cholestero,l 100 mg/kg p.o, induced hyperlipidemic rat model *in vivo*	Dose = 200, 400, 800 mg/kg, p.o. $n = 6$	Cholesterol Dose = 100 mg/kg	↓cholesterol	[3]
		Triton-X 100, 480 mg/kg, i.p. induced hyperlipidemic rat model *in vivo*	Dose = 200, 400, 800 mg/kg, p.o. $n = 6$	Triton-X 100 Dose = 480 mg/kg	↓triglycerides ↓LDL-C ↓VLDL-C	
Antinociceptive	Hydroalcoholic extract (roots)	Acetic acid-induced writhing and hot plate-induced pain in mice *in vivo*	Dose = 250, 500 mg/kg b.w., i.p. $n = 6$	Diclofenac sodium Dose = 10 mg/kg	50% writhing inhibition ↑pain threshold	[46]
	Methanol extract (leaves)	Acetic acid writhing method and radiant heat tail flicking method *in vivo*	Dose = 200, 400 mg/kg, p.o. $n = 5$	Diclofenac sodium Dose = 50 mg/kg	↓number of writhes ↑reaction time at dose 400 mg/kg	[69]
Gastroprotective	Ethanol extract (leaves)	Indomethacin, 5 mg/kg b.w., p.o. Cold restraint stress-induced ulcers in rats *in vivo*	Dose = 100, 200, 400 mg/kg, p.o. $n = 5$	Indomethacin dose = 5 mg/kg	Development of a defensive layer; ↑free radical scavenging activity; ↓LPO	[70]
Reproductive activity	Hydroalcoholic extract (rhizomes)	Male rats *in vivo*	Dose = 5, 10, 20, 40 g/kg b.w., p.o. $n = 5$	Distilled water	↑serum testosterone	[64]

Abbreviations: ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; BHT: butylatedhydroxytoluene; DPPH: 1,1-diphenyl-2-pecrylhydrazyl; HO-1: heme-oxygenase-1; L-NAME: *N*-Nitro-L-arginine methyl ester; LDL-C: low-density lipoprotein cholesterol; MAPK: mitogen-activated protein kinase; NF-κB: kappa B; Nrf2: nuclear factor erythroid 2-related factor 2; PI3K/Akt: phosphoinositide-3-kinase/protein kinase B; VLDL-C: very-low-density lipoprotein cholesterol.

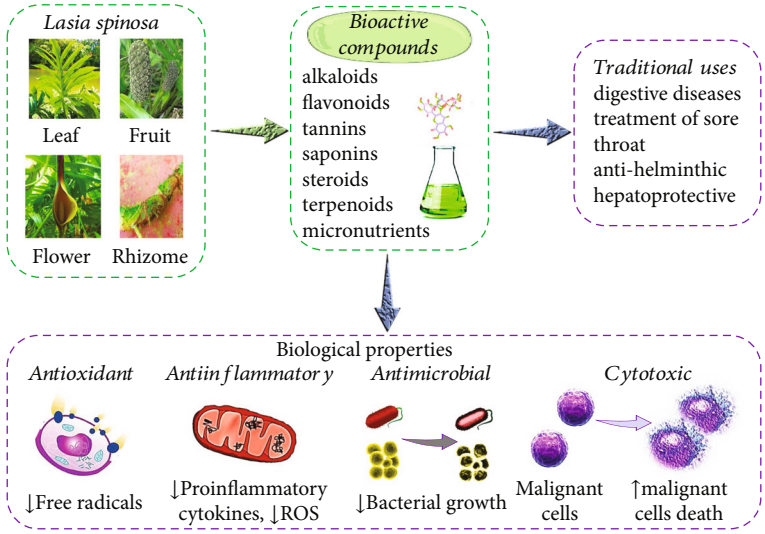

Figure 3: Summarized scheme with traditional uses and the most representative biological properties of *L. spinosa*.

6. Toxicological Data and Clinical Gaps

Oral administration in an acute analysis of 5, 10, 20, and 40 gm/kg of an extract, there were no mortality or physiological changes demonstrated. In the subchronic assay for 28 gm/kg, administration of 5 or 20 gm/kg of extract for 28 gm/kg, no animal deaths were announced that day. No differences in haematological parameters were noticed in either case [52].

Therapeutic limitation of natural bioactive compounds from *Lasia spinosa* results from the relatively reduced bioavailability of bioactive compounds. In addition, numerous interactions with other prescription drugs may occur. Interactions between medicinal plants interfere with the metabolism or elimination of the drug/chemotherapy from the body. Drug metabolism/elimination is mediated by enzymes that metabolize drugs in the cytochrome P450 (CYP) family and drug transport proteins. These interactions can change the concentration of drugs in the body [72].

Interactions between plants and drugs may occur due to inhibition or activation by plant phytochemicals of CYP enzymes or drug transport proteins that metabolize the drug [73]. Some therapeutic pharmacological agents must be activated by CYP to be effective. Once CYPs are inhibited, such drugs that need to be activated will be ineffective. There may be interactions between plants and drugs that lead to increased elimination of drugs due to CYP activation, which could lead to subtherapeutic exposure to drugs and could lead to therapy failure [74]. Some plant-drug interactions due to CYP inhibition may lead to the accumulation of cytotoxic drugs due to delayed clearance and may increase drug toxicity due to high doses of drugs. Cancer patients are already taking several medications at the same time due to other conditions associated with cancer and comorbidities, which present a risk of drug interactions [75]. The use of herbs/herbal products may further increase the risk of these potentially harmful interactions that interfere with the impact of the drug.

7. Overall Conclusions and Future Perspectives

Natural plant sources have contributed to many drug developments. In this study, we comply with the traditional uses, pharmacological properties, and chemical constituents of *L. spinosa*, information that can be useful for further research. Many phytochemicals present in *L. spinosa* may be responsible for its biological effects in various test systems, but more studies are needed to identify and characterize the active compounds responsible for the pharmacological activities of this hopeful medicinal plant. Future directions must be oriented to toxicological studies which are scarce, and there are necessary new reports to ensure the safety of this plant. Besides, clinical studies are required to confirm the preclinical biological effects in humans.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

The Effect of Berberine on Metabolic Profiles in Type 2 Diabetic Patients: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Objective. *Rhizoma Coptidis* is an herb that has been frequently used in many traditional formulas for the treatment of diabetic mellitus (DM) over thousands of years. Berberine, the main active component of *Rhizoma Coptidis*, has been demonstrated to have the potential effect of hypoglycemia. To determine the potential advantages of berberine for diabetic care, we conducted this systematic review and meta-analysis to examine the efficacy and safety of berberine in the treatment of patients with type 2 DM. *Methods*. Eight databases including PubMed, Embase, Web of Science, the Cochrane library, China National Knowledge Infrastructure (CNKI), Chinese Biomedical Database (SinoMed), Wanfang Database, and Chinese VIP Information was searched for randomized controlled trials (RCTs) reporting clinical data regarding the use of berberine for the treatment of DM. Publication qualities were also considered to augment the credibility of the evidence. Glycemic metabolisms were the main factors studied, including glycosylated hemoglobin (HbA1c), fasting plasm glucose (FPG), and 2-hour postprandial blood glucose (2hPG). Insulin resistance was estimated by fasting blood insulin (FINS), homeostasis model assessment-insulin resistance (HOMA-IR), and body mass index (BMI). Lipid profiles were also assessed, including triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), along with inflammation factors such as C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). Serum creatinine (Scr), blood urea nitrogen (BUN), and adverse events were applied to evaluate the safety of berberine. *Results*. Forty-six trials were assessed. Analysis of berberine applied alone or with standard diabetic therapies versus the control group revealed significant reductions in HbA1c (MD = −0.73; 95% CI (−0.97, −0.51)), FPG (MD = −0.86, 95% CI (−1.10, −0.62)), and 2hPG (MD = −1.26, 95% CI (−1.64, −0.89)). Improved insulin resistance was assessed by lowering FINS (MD = −2.05, 95% CI (−2.62, −1.48)), HOMA-IR (MD = −0.71, 95% CI (−1.03, −0.39)), and BMI (MD = −1.07, 95% CI (−1.76, −0.37)). Lipid metabolisms were also ameliorated via the reduction of TG (MD = −0.5, 95% CI (−0.61, −0.39)), TC (MD = 0.64, 95% CI (−0.78, −0.49)), and LDL (MD = 0.86, 95% CI (−1.06, −0.65)) and the upregulation of HDL (MD = 0.17, 95% CI (0.09, 0.25)). Additionally, berberine improved the inflammation factor. *Conclusion*. There is strong evidence supporting the clinical efficacy and safety of berberine in the treatment of DM, especially as an adjunctive therapy. In the future, this may be used to guide targeted clinical use of berberine and the development of medications seeking to treat patients with T2DM and dyslipidemia.

1. Introduction

Diabetes mellitus (DM) is a chronic, noncommunicable disease that has become a worldwide threat to public health. The global prevalence of DM has been remarkably increased to 463 million among adults and has been predicted to surge to about 700 million by the year 2045. In 2019, approximately 4.2 million adults had an estimated cause of death related to diabetes and its complications. This comes out to about one death every eight seconds [1]. Though 10% of the global health expenditure is currently spent on DM, this disease remains in the top 4 causes of noncommunicable disease deaths [2]. Thus, it is essential to explore more effective and safe strategies for the prevention and treatment of DM. China currently has the largest number of type 2 diabetic patients, so this condition has become a leading public health challenge in China [3].

In addition, with the development of society and the change of diet structure, the characteristics of the diabetic population are changing. The classic symptoms, like polydipsia, polyphagia, polyuria, and weight loss, are less often recognized in those with type 2 diabetes mellitus (T2DM). It has been reported that 44% of type 2 diabetic respondents had no classic symptoms in the previous year [4]. Moreover, patients with T2DM are more likely to be overweight or obese, which indicates insulin resistance and dyslipidemia along with hyperglycemia [5, 6]. For T2DM patients, glycemic or lipid values and their variability can significantly predict all-cause mortality and the occurrence of complications, including micro- and macrovascular complications [7, 8]. Growing evidence has indicated that chronic inflammation, hyperglycemia, and dyslipidemia are all involved in insulin resistance, pathogenesis of T2DM, and systematic diabetic complications [9, 10]. In T2DM patients, IL-6 and TNF-α levels are strikingly increased. This is also associated with a downregulation of several drug metabolizing enzymes, which leads to poor drug effects [11].

Huanglian (*Rhizoma Coptidis*) is an herb that has been frequently used in many traditional formulas for the treatment of T2DM. This treatment has been used for thousands of years. Alkaloids from Huanglian have been widely used for the treatment of diabetes and hyperglycemia with inconspicuous toxicities and side effects [12]. Among the different alkaloids, berberine is an important lead compound with a wide spectrum of pharmacological activities, including antitumor, anti-inflammatory, hypoglycemic, hypolipidemic, and antiobesity effects [13]. Additionally, evidence has shown that berberine regulates the gut microbiota as well [14, 15], which is closely associated with systematic inflammation that adds to the progression of T2DM. Systematic reviews have previously reported the efficacy of berberine on the regulation of glycemic and lipid metabolisms [16, 18]. However, its comprehensive effects on glycemic metabolisms, lipid profiles, and inflammation factors of patients with T2DM have not been well evaluated. Since berberine is the main active component of *Rhizoma Coptidis*, we conducted this meta-analysis to comprehensively evaluate the efficacy and safety of berberine and *Rhizoma Coptidis* on T2DM.

2. Methods

2.1. Search Strategy and Study Selection. This study was performed according to the Cochrane Handbook for Systematic Reviews of Interventions and was reported according to Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) [19]. The protocol has been registered on PROSPERO as CRD42020155086.

The literature research was conducted with the use of the following eight databases with no time restriction: PubMed (up to March 29, 2021), Embase (up to March 29, 2021), Web of Science (ended up to March 29, 2021), the Cochrane library (up to March 29, 2021), China National Knowledge Infrastructure (CNKI) (up to April 14, 2021), Chinese Biomedical Database (SinoMed) (up to April 14, 2021), Wanfang Database (up to April 14, 2021), and Chinese VIP Information (up to April 14, 2021). The MeSH and free-text terms were applied based on the characteristics of specific database as follows: berberine, huanglian, *Rhizoma Coptidis*, traditional Chinese medicine, diabetes mellitus, insulin resistance, and randomized controlled trials. No restrictions on publication language or date were set. The terms were searched as "diabetes mellitus" OR "insulin resistance" OR "metabolic syndrome" OR "Xiaoke syndrome" AND "TCM" OR" Traditional Chinese Medicine" OR "herbal medicines" OR "plant medicines" OR "berberine" OR "huanglian" OR " Coptidis " AND "randomized controlled trial" OR "controlled clinical trial" OR "random" OR "double-blind" OR "single-blind." The full electronic search strategy for PubMed was provided in Supplementary Files 1 according to the search history.

The included clinical studies fulfilled the following criteria:

(1) Types of studies: only RCTs were eligible for this review. Single-blinded and open label trials were also considered. The sample size was greater than or equal to 60 participants, and the intervention duration was no less than four weeks

(2) Types of participants: adults aged 18 years or older with T2DM or prediabetes were included

(3) Types of interventions: intervention with berberine or *Rhizoma Coptidis* was considered. The control intervention included placebo, life interventions such as changes in exercise or dietary habits, or any active antihyperglycemia intervention

(4) Types of outcomes: primary outcomes included glycosylated hemoglobin (HbA1c), fasting plasma glucose (FPG), and 2-hour postprandial plasma glucose (2hPG)

Secondary outcomes included insulin resistance and the associated index of fasting plasma insulin, homeostasis model assessment-insulin resistance (HOMA-IR), and body mass index (BMI). Lipid profiles include triglyceride, total cholesterol, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) along with inflammation markers, such as C-reactive protein.

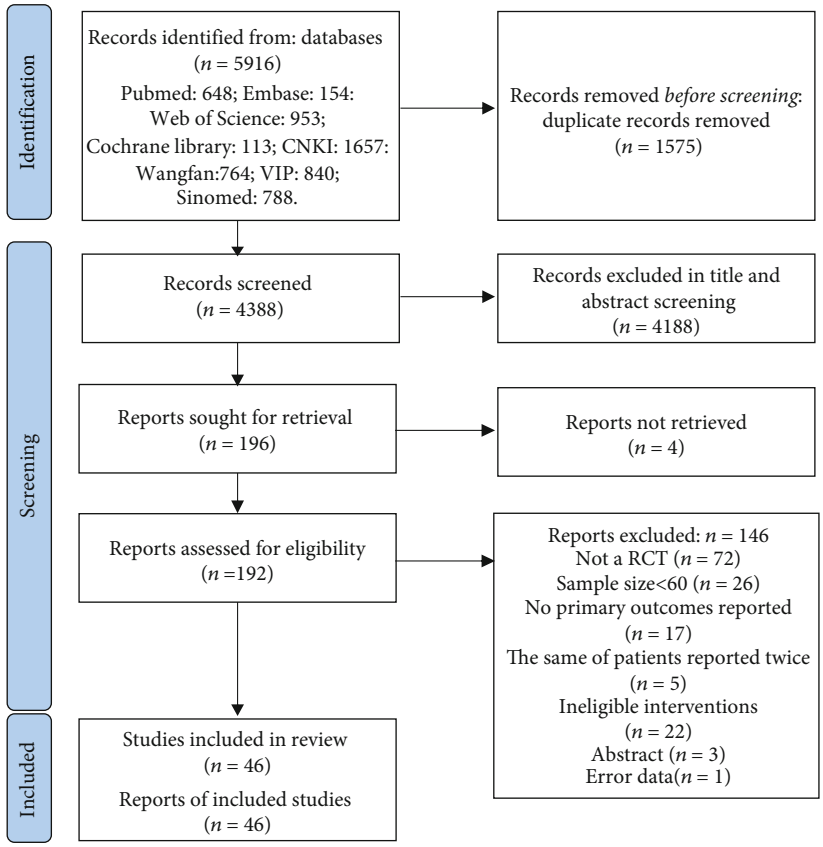


Figure 1: Flow diagram.

Safety outcomes included the incidence of adverse reactions and adverse reactions.

The excluded criteria were as follows:

(1) Other TCM treatments applied in either the treatment or control group

(2) If the publication was a review, an abstract, a protocol, or had no outcomes of interest, it would not be considered

(3) Full texts were not available

2.2. Data Collection and Analysis. Titles and abstracts were screened independently by two investigators. The NoteExpress 3.4 literature management software was used to screen references. It was suggested to reviewers to contact the author for the complete information if this was indicated. If there was a disagreement, another reviewer would help determine a solution. Kappa statistics were employed to analyze the consistency of the results.

Data extraction was carried out independently by two authors using standard data extraction forms that included participant details, the interventions used in both treatment and control group, and the primary and secondary outcomes. Where more than one publication of one study existed, reports were grouped together and the publication with the most complete data was used in the analyses.

2.3. Assessment of Risk of Bias in Included Studies. Two reviewers independently assessed the quality of each article, using the risk of bias assessment tool in the Cochrane. Another reviewer was asked to help decide if there was a disagreement.

2.4. Measures of Treatment Effect. Categorical outcomes were expressed as risk ratio (RR) with 95% confident intervals (CI), while continuous measurement was pooled by the standardized mean difference (SMD) or mean difference (MD). If data were missing or unclear, it was suggested that reviewers contact the authors of studies to request this information.

2.5. Assessment of Heterogeneity. Heterogeneity was analyzed using a Chi^2 test with an alpha of 0.1. The I^2 statistic was used for statistical significance. When I^2 was less than or equal to 50% and $P > 0.1$, the heterogeneity was acceptable. In addition, when I^2 was greater than 50% and $P < 0.1$, the heterogeneity among the trials was significant.

2.6. Data Synthesis and Analysis. Revman 5.4 software was used to conduct the data synthesis and analysis. In the analysis, pooling data was processed as overall RR and/or MD. A random effects model was applied if there was any heterogeneity observed. If pooling was not possible, the data were summarized descriptively.

Table 1: Specific characteristics of included trials.

Study	Participants	No. (T/C)	Age	Sex (M/F)	Course (year)	Intervention	Control	Duration (weeks)	Outcomes
Zhang et al. 2020 [15]	T2DM	98/103	T: 53 (42–61) C: 54 (46–61)	T: 59/39 C: 61/42	Newly diagnosed	Berberine 0.5 g bid	Placebo	12	①②③④⑤⑥⑦⑧⑨⑩
Hu 2020 [28]	T2DM	60/58	T: 43.21 ± 8.14 C: 42.85 ± 6.23	T: 34/26 C: 30/28	T: 2.31 ± 1.06 C: 2.61 ± 0.84	Berberine 0.3 g tid Metformin 0.5 g tid	Placebo Metformin 0.5 g tid	24	①③④⑤⑥⑦⑧⑨⑩
Sanjari et al. 2020 [26]	T2DM	42/38	T: 51.8 ± 9.3 C: 46.5 ± 10	T: 18/24 C: 7/31	T: 4.5 ± 0.25 C: 4.25 ± 0.42	Berberis integerrima root extract 480 mg	Metformin 1 g/d	12	①②③⑥⑦⑧⑨
Li et al. 2021 [20]	Prediabetes	35/35	T: 54.11 ± 8.14 C: 42.85 ± 6.23	T: 22/13 C: 17/18	—	Berberine 0.3 g tid	Life intervention	24	①②③⑥⑦⑧⑨⑩
Tahmasebi et al. 2019 [27]	T2DM	40/40	T: 54.05 ± 8.00 C: 53.07 ± 7.74	T: 17/23 C: 15/25	Less than 10 years	1000 mg dry extract and 157.3 mg berberine	Placebo	6	①②④⑥⑦⑧⑨⑩
Ma 2019 [24]	T2DM	50/50	T: 50.8 ± 3.6 C: 50.2 ± 4.2	T: 31/19 C:28/22	T: 1.77 ± 0.57 C: 1.81 ± 0.53	C+Huanglian 3 g tid	Metformin 0.5 g tid	8	①②③④⑥⑦⑧⑨
Deng et al. 2019 [29]	T2DM	35/35	T: 54.6 ± 2.5 C: 55.3 ± 2.6	T:23/12 C:21/14	T: 6.04 ± 2.43 C: 6.61 ± 2.75	C+berberine 0.3 g tid	Glipizide 5 mg tid	12	①②③④⑥⑦⑧⑨
Chen et al. 2018 [30]	T2DM	40/40	T: 53.27 ± 8.15 C: 52.71 ± 7.89	T: 22/18 C: 24/16	T: 5.59 ± 3.74 C: 5.64 ± 3.58	C+berberine 0.5 g tid	Metformin 0.5 g tid	12	①②③⑩
Wu 2018 [31]	T2DM	63/63	T: 58.61 ± 8.37 C: 58.43 ± 8.26	T: 37/26 C: 38/25	NA	C+berberine 0.2 g tid	Metformin 0.5 g tid	12	①②③⑤⑥⑦⑧⑨⑩
Rashidi et al. 2018 [32]	T2DM	40/41	T: 50.18 ± 4.22 C: 45.12 ± 9.55	T: 14/28 C: 19/22	T: 4.5 ± 1.8 C: 4.3 ± 2.04	Berberine 0.5 g bid	Placebo	4	①②④⑥⑦⑧⑨⑩
Li et al. 2018 [33]	T2DM	57/57	T: 53 ± 15 C: 57 ± 12	T: 22/35 C: 24/33	T: 5.92 (1.92, 8) C:7.25 (2, 10.2)	C+berberine 300 mg tid	Antidiabetic agents	24	①②③⑤⑥⑦⑩
Sha et al. 2018 [34]	T2DM	30/30	T: 58.79 ± 12.27 C: 60.46 ± 11.73	T: 15/13 C: 14/15	T: 7.08 ± 2.68 C: 8.68 ± 4.13	Berberine 500 mg tid	NA	12	①③④⑩
Zhao 2018 [22]	Prediabetes	32/32	T: 54.7 ± 0.8 C: 54.9 ± 0.16	T: 24/8 C: 26/6	—	Berberine 0.3-0.5 g tid	Life intervention	12	①②③⑤⑥⑦
Guan et al. 2017 [35]	T2DM	53/53	T: 65.74 ± 3.85 C: 65.28 ± 3.29	T: 29/24 C: 28/25	T: 6.14 ± 2.44 C: 6.25 ± 2.41	C+berberine 0.5 g tid	Metformin 0.5 g tid	4	①③
Zhang 2017 [36]	T2DM	40/40	T: 58.24 ± 6.15 C: 58.13 ± 6.24	T: 21/19 C: 23/17	T: 5.8 ± 1.9 C: 5.7 ± 1.8	Berberine 3 g tid	Metformin 750 mg/d	12	①②③④
		40/40	T: 58.91 ± 6.58 C: 58.13 ± 6.24	T:22/18 C:23/17	T: 6.0 ± 1.7 C: 5.7 ± 1.8	C+berberine 3 g tid	Metformin 750 mg/d	12	①②③④
Xing 2017 [37]	T2DM	35/35	T: 53.3 ± 6.2 C: 54.1 ± 6.1	T: 18/17 C: 17/18	T: 5.9 ± 2.8 C: 5.8 ± 2.7	Berberine 500 mg tid	Metformin 250 mg tid	12	①③④⑤⑥⑦⑧⑨⑩

Table 1: Continued.

Study	Participants	No. (T/C)	Age	Sex (M/F)	Course (year)	Intervention	Control	Duration (weeks)	Outcomes
Sun 2017 [38]	T2DM	91/91	T: 58.95 ± 10.57 C: 58.34 ± 11.21	T: 53/38 C: 51/40	T: 3.98 ± 1.62 C: 3.64 ± 1.75	C+berberine 30 mg tid	Metformin 0.5 g tid	8	①②③④⑥⑦⑧⑨⑩
Chen et al. 2017 [39]	Prediabetes	50/50	T: 36.5 ± 14.4 C: 35.2 ± 15.1	T: 31/19 C: 30/20	—	Berberine 0.5 g tid	Metformin 0.5 g tid	24	①②⑤
Li et al. 2017 [40]	T2DM	30/30	T: 50.54 ± 3.78 C: 51.24 ± 3.91	T: 18/12 C: 17/13	T: 6.04 ± 2.43 C: 6.61 ± 2.75	C+berberine 0.3 g tid	Sitagliptin 100 mg tid	12	①②③
Li 2016 [41]	T2DM	90/90	T: 56.2 ± 2.9 C: 57.8 ± 2.7	T: 55/35 C: 55/35	T: 3.7 ± 1.9 C: 3.8 ± 1.7	C+berberine 0.3 g tid	Metformin 1.7 g/d	12	①②③④
Lang and Zhu 2016[42]	T2DM	50/50	46.3 ± 4.7	63/37	NA	Berberine 500 mg tid	Placebo	12	①②③
Du 2016 [43]	T2DM	37/36	T: 67.8 ± 4.6 C: 56.5 ± 7.1	T: 21/16 C: 18/18	T: 10.9 ± 6.3 C: 11.6 ± 6.9	C+berberine 4 pills tid	Metformin 0.5 g tid	4	①②③
Li 2016 [44]	T2DM	60/60	T: 65.85 ± 4.78 C: 67.92 ± 4.73	T: 26/24 C: 28/32	T: 5.32 ± 1.08 C: 5.28 ± 1.31	C+berberine 0.5 g tid	Glipizide 5 mg tid	12	③④⑥⑦⑧⑨
Cui 2016 [45]	T2DM	40/40	T: 50.85 ± 7.26 C: 51.59 ± 8.97	T: 28/12 C: 30/10	T: 8.65 ± 3.33 C: 9.07 ± 3.47	C+berberine 0.5 g tid	Metformin 0.5 g tid	16	①②③⑥⑦
Ren et al. 2016 [46]	T2DM	31/30	T: 47.74 ± 7.39 C: 46.87 ± 7.74	T: 21/10 C: 19/11	Newly diagnosed	C+berberine 0.5 g tid	Life interverntion	12	①②③⑥⑦⑧⑨
Wang 2015 [25]	T2DM	39/36	T: 51.6 ± 11.7 C: 53.4 ± 11.3	T: 22/17 C: 21/15	T: 1.7 ± 0.78 C: 1.9 ± 0.71	C+Huanglian 3 g tid	Metformin 0.5 g tid	8	①②③④⑥⑦⑧⑨
Yu 2015 [47]	T2DM	49/48	T: 51.7 ± 4.6 C: 50.9 ± 3.8	T: 23/26 C: 25/23	T: 4.1 ± 0.5 C: 4.3 ± 0.7	C+berberine 0.5 g tid	Metformin 0.5 g qd	12	①③⑥⑦
Zhu et al. 2015 [48]	T2DM	59/59	T: 66.4 ± 7.6 C: 65.6 ± 7.2	T: 35/24 C: 33/26	T: 4.5 ± 1.8 y C: 4.3 ± 2.04	C+berberine 100 mg tid	Gliclazide modified release tablets 30 mg qd	12	①②③⑥⑦⑧⑨
Shu 2014 [49]	T2DM	32/32	T: 62.80 ± 12.20 C: 61.21 ± 13.52	T: 19/13 C: 20/12	T: 6.53 ± 2.61 C: 6.79 ± 2.93	C+berberine 300 mg tid	Metformin 0.5 g tid	24	①④
Zhou 2014 [50]	T2DM	33/33	65.3 ± 2.5	46/20	7.4 ± 1.4	C+berberine 300 mg tid	Metformin 0.5 g bid	12	①②③⑤⑥⑦⑧⑨
Cao 2012 [51]	T2DM	38/40	T: 51.26 ± 14.71 C: 52.53 ± 6.37	T: 22/16 C: 24/16	Newly diagnosed	C+berberine 0.5 g tid	Metformin 0.5 g tid	16	①②③④⑤⑥⑦⑧⑨⑩
Zhou and Huang 2012 [52]	T2DM	45/45	46.67 ± 8.52	48/44	4.81 ± 0.29	C+berberine 0.5 g tid	Metformin 0.5 g tid	12	①②④⑤⑥⑦⑧⑨
Zhang and Yuan 2012 [53]	T2DM	38/38	No difference	T: 21/17 C: 20/18	No difference	C+berberine 0.5 g-0.8 tid	Metformin 0.5 g tid	12	①②③
Xue et al. 2012 [54]	T2DM	44/45	No difference	No difference	No difference	C+berberine 0.5 g tid	Metformin 1.5 g/d SU	12	①②③④

Table 1: Continued.

Study	Participants	No. (T/C)	Age	Sex (M/F)	Course (year)	Intervention	Control	Duration (weeks)	Outcomes
Lou et al. 2011 [55]	T2DM	30/30	36-74	32/28	Newly diagnosed	C+berberine 0.3 g tid	Metformin 1.5 g/d	24	①②③④⑥⑦⑧⑨
Qiu et al. 2011 [56]	T2DM	49/51	T: 42.2 ± 11.4 C: 54 ± 12.5	T: 26/23 C: 28/23	NA	C+berberine 0.3 g tid	Antidiabetic drugs	8	①②③④⑩
Meng et al. [57] 2011	T2DM	30/30	T: 51 ± 13.3 C: 53 ± 13.9	T: 17/13 C: 16/14	Newly diagnosed	C+berberine 0.3 g tid	Insulin injection	12	①②③
Ye 2010 [58]	T2DM	40/40	T: 43•5 ± 9•8 C: 42•5 ± 8•6	T: 24/16 C: 22/18	T: 0.84 ± 0.36 C: 0.8 ± 0.35	C+berberine 0.5 g tid	Glimepiride 0.1 mg bid Metformin 0.5 g tid	12	①②③⑤⑥⑦⑧⑨
Sheng and Xie 2010 [59]	T2DM	30/30	T: 52 ± 11 C: 51 ± 8	T: 18/12 C: 11/19	T: 5 ± 3 C: 6 ± 3	C+berberine 0.5 g tid	Glipizide 5 mg bid Metformin 0.5 g tid	12	①④⑤⑩
Xu et al. 2008 [60]	T2DM	32/32	43.4 ± 2.1	34/30	Newly diagnosed	C+berberine 0.3 g tid	Pioglitazone 30 mg qd	12	①②⑦
Zhang et al. 2008 [61]	T2DM	59/57	T: 51 ± 9 C: 51 ± 10	T: 30/28 C: 31/21	Newly diagnosed	Berberine 0.5 g bid	Placebo	12	①②③④⑤⑥⑦⑧⑨⑩
Li et al. 2008 [62]	T2DM	33/32	T: 47.5 C: 49.2	T: 17/16 C: 16/16	T: 9.01 ± 1.99 C: 8.11 ± 2.24	Berberine 500 mg tid	Metformin 250 mg tid	12	①②③④⑤⑥⑦⑧⑨⑩
Liu and Hu 2008 [63]	T2DM	30/30	T: 52 ± 9.81 C: 53.07 ± 8.51	T: 10/20 C: 14/16	T: 9.01 ± 1.99 C: 8.11 ± 2.24	C+berberine 0.3-0.5 g tid	Metformin 500 mg tid	8	①②③④
Ju et al. 2007 [23]	Prediabetes	46/44	T: 46.7 ± 4.2 C: 45.6 ± 4.4	T: 28/18 C: 26/18	—	Berberine 0.6 g/d	NA	48	①②⑤⑥⑦⑧⑨⑩
Zhang 2005 [64]	T2DM	48/46	43 ± 11	50/44	6-10	SU+berberine 0.3 g tid	Metformin 1.5 g/d + SU	12	①②③④
Cao 2004 [65]	T2DM	30/30	T: 55.3 ± 11.5 C: 55.4 ± 10.7	T: 13/17 C: 12/18	T: 0.8 ± 0.28 C: 0.825 ± 0.275	Berberine 0.5 g tid Life intervention	Life intervention	12	①②③④⑤⑥⑦⑧⑨⑩

Note: ① FBG: fasting blood glucose; ② 2hPBG: 2-hour postprandial blood glucose; ③ HbA1c: glycated hemoglobin; ④ FINS: fasting insulin; ⑤ BMI: body mass index: ⑥ TC: total cholesterol; ⑦ TG: triglyceride; ⑧ HDL-C: high-density lipoprotein cholesterol; ⑨ LDL-L: low-density lipoprotein cholesterol; ⑩HOMA-IR.

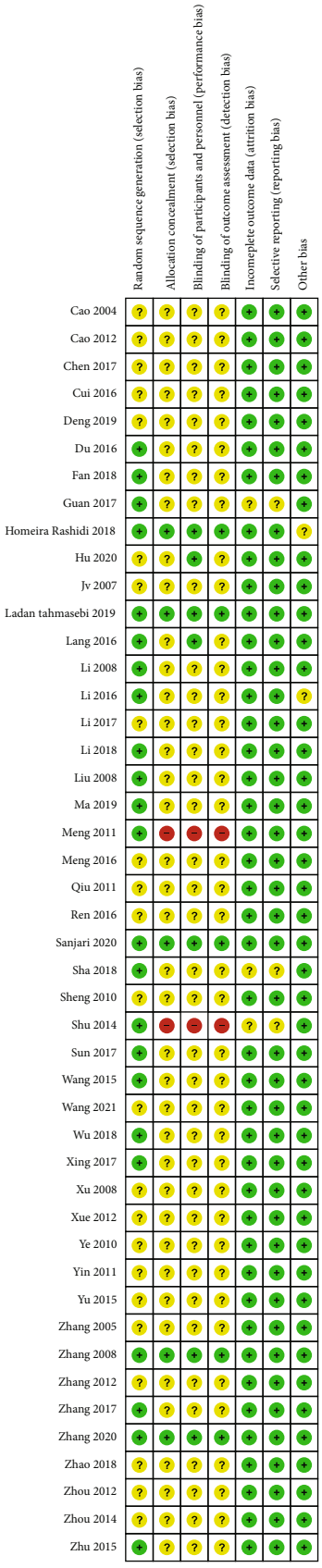


Figure 2: Risk of bias of assessment. The quality of each article independently using the risk of bias assessment tool in the Cochrane.

Subgroup analysis was planned to explore the source of heterogeneity according to the differences of treatment methods among trials. Sensitivity analyses were conducted using a leave-one-out method to identify studies contributing to significant heterogeneity, which had an I^2 value of greater than 50%. Funnel plots were planned to assess publication bias as well.

3. Results

A total of 46 clinical trials were considered and included in the quantitative meta-analysis (Figure 1). As to the literature screening and selection, the result of kappa statistics was $k = 0.785$, indicating a good consistency. Of these, 38 were related to berberine in the treatment of T2DM, and four [20–23] were for prediabetes. Two trials [24, 25] were aimed at evaluating the effectiveness and safety of *Rhizoma Coptidis* in the treatment of T2DM, and two [26, 27] were for root dry extracts including berberine. The publication time of the included studies was from 2004 to 2021.

A total of 4,158 participants were enrolled, including 2,063 participants in the experimental group and 2,095 participants in the control group. The intervention duration ranged from 4 weeks to 6 months. Eleven trials compared berberine with placebo or none, while four trials compared berberine with metformin. Specific characteristics of the included trials are illustrated in Table 1.

All the included studies were RCT designs and twenty-two of them provided information on random sequence generation. Five studies reported information on allocation concealment procedures and the blindness of outcome assessments. Five studies were conducted with blinding of both participants and personnel. No risks of incomplete outcome data or selective reporting were found for any of the recruited studies. The risks of bias assessment across the recruited studies are illustrated in Figure 2. To comprehensively illustrate the effect of berberine for diabetes, glucose metabolism-related index (HbA1c, FPG, and 2hPG), insulin resistance-related index (FINS, HOMA-IR, and BMI), lipid profiles (TC, TG, HDL, and LDL), and inflammation index (CRP, IL-6, and TNF-α) were reported, along with the safety including effect of berberine on serum creatinine (SCR), blood urea nitrogen (BUN), and any associated adverse events.

3.1. Berberine for Glucose Metabolism of T2DM. Forty trials, 45 trials, and 36 trials provided the data of HbA1c, FPG, and 2hPG, respectively. The meta-analyses showed that berberine remarkably decreased the HbA1c level (MD = −0.75, 95% (−1.00, −0.51); $P < 0.05$; $I^2 = 98\%$, 95% CI (0.97, 0.98)), the FPG level (MD = −0.89, 95% CI (−1.13, −0.64), $P < 0.05$, $I^2 = 97$, 95% CI (0.96, 0.97)), and the 2hPG level (MD = −1.31, 95% CI (−1.69, −0.93), $P < 0.05$, $I^2 = 96\%$, 95% CI (0.96, 0.97)).

Subgroup analysis showed that berberine could slightly lower the HbA1c level (MD = −0.38, 95% CI (−0.49, −0.27), $P < 0.05$) and the FPG level (MD = −0.58, 95% CI (−0.81, −0.35), $P < 0.05$) but remarkably reduce the 2hPG

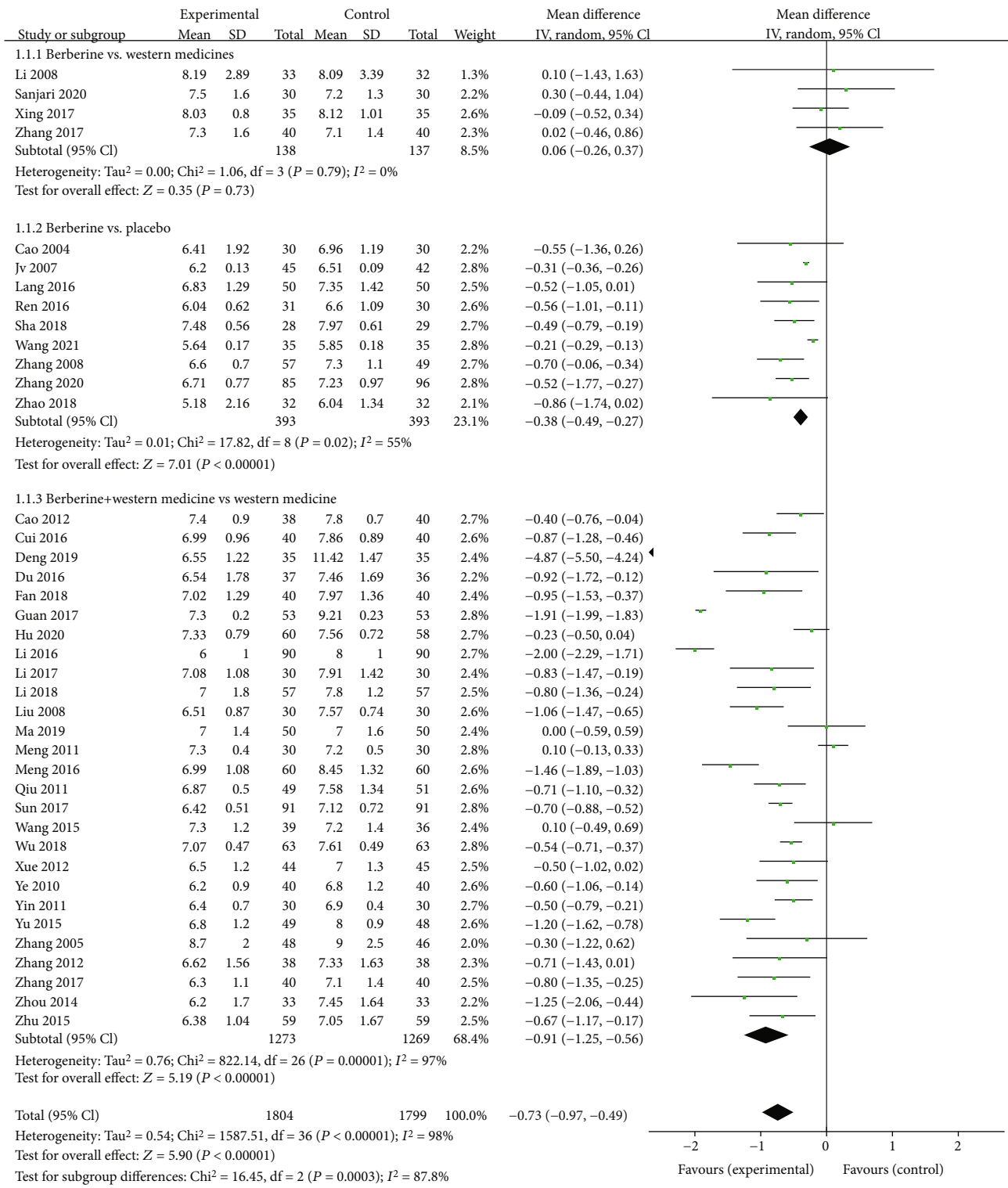


Figure 3: Meta-analysis of the effect of berberine on glycosylated hemoglobin (HbA1c).

level (MD = −1.48, 95% CI (−2.16, −0.79), $P < 0.05$) when it was used alone. When combined with antidiabetic agents, berberine strikingly reduce the HbA1c level (MD = −0.91, 95% CI (−1.25, −0.56), $P < 0.05$), the FPG level (MD = −1.06, 95% CI (−1.34, −0.79), $P < 0.05$), and the 2hPG level (MD = −1.34, 95% CI (−1.73, −0.96), $P < 0.05$). However, compared with Western medicine, there was no significant difference observed in the HbA1c level, the FPG level, or the 2hPG level when berberine was applied alone (Figures 3–5).

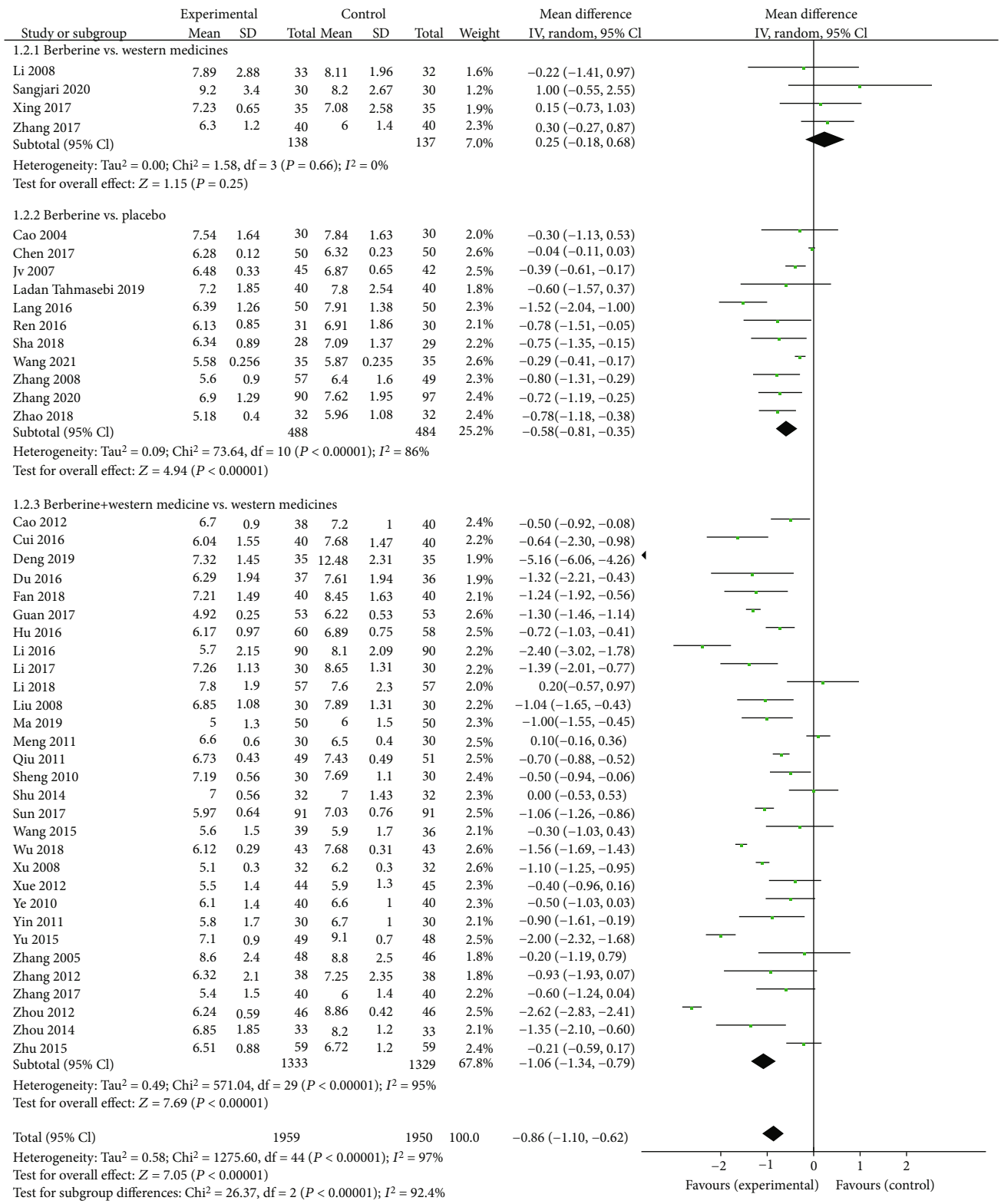


Figure 4: Meta-analysis of the effect of berberine on fasting plasma glucose (FPG).

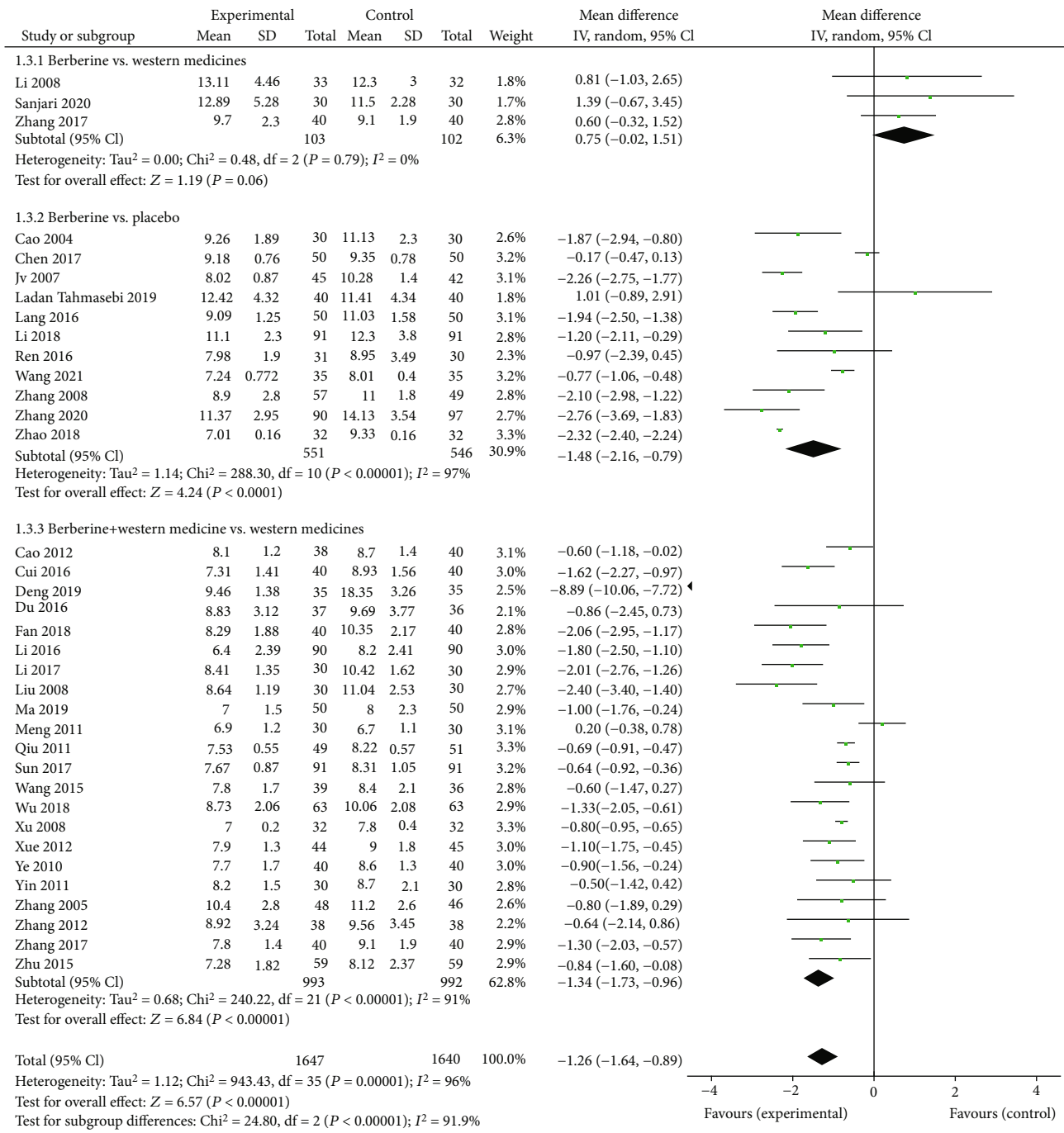


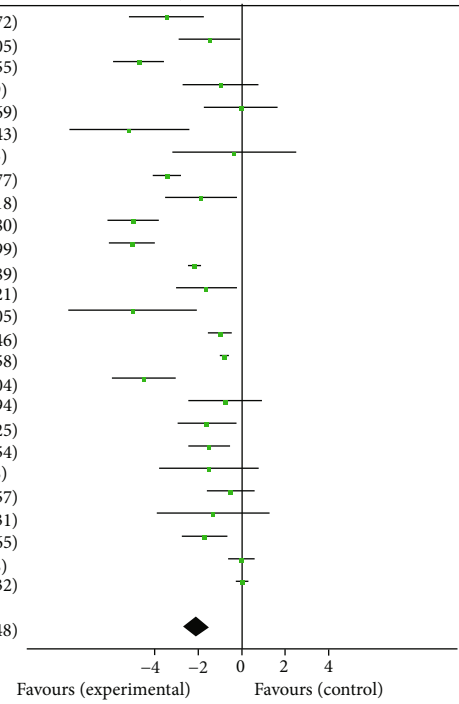
Figure 5: Meta-analysis of the effect of berberine on 2-hour postprandial plasma glucose (2hPG).

3.2. Berberine for Insulin Resistance-Associated Index of T2DM. Figure 6 shows the efficacy of berberine on the FINS level, the HOMA-IR level, and the BMI level of T2DM patients. The FINS concentration of the trial group decreased by 2.05 (95% CI (−2.62, −1.48), $P < 0.05$, $I^2 = 93$ %, 95% CI (0.91, 0.95)), the HOMA-IR level of the berberine group decreased by 0.71 (95% CI (−1.03, −0.39), $P < 0.05$, $I^2 = 96\%$, 95% CI (0.94, 0.97)), and the BMI level of the berberine group decreased by 1.07 (95% CI (−1.76, −0.37), $P < 0.05$, $I^2 = 91\%$, 95% CI (0.87, 0.94)).

3.3. Berberine for Lipid Profiles of T2DM. The pooled results of berberine efficacy in the treatment of T2DM on lipid profiles are shown in Figures 7 and 8. Twenty-four trials were assessed on TG (Figure 7(a)), and the result showed that berberine significantly lowered the TG level in patients with T2DM (MD = −0.5, 95% CI (−0.61, −0.39), $P < 0.05$, $I^2 = 92\%$, 95% CI (0.89, 0.94)). Pooled results of 25 trials showed that the TC concentration of the berberine group decreased by 0.64 (95% CI (−0.78, −0.49), $P < 0.05$, $I^2 = 79\%$, 95% CI (0.69, 0.86)) (Figure 7(b)). There was a

Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI
Cao 2004	21.7	1.65	30	25.15	4.53	30	3.5%	−3.45 (−5.18, −1.72)
Cao 2012	8.08	3.35	38	9.54	2.97	40	3.9%	−1.46 (−2.87, −0.05)
Deng 2019	9.65	2.36	35	14.38	2.67	35	4.2%	−4.73 (−5.91, −3.55)
Hu 2020	13.57	4.95	60	14.53	4.77	58	3.4%	−0.96 (−2.71, 0.79)
Jv 2007	11.06	4.02	45	11.06	4.02	42	3.5%	0.00 (−1.69, 1.69)
Ladan Tahmasebi 2019	9.49	3.84	40	14.67	7.99	40	2.3%	−5.18 (−7.93, −2.43)
Li 2008	9.79	5.98	33	10.12	5.77	32	2.2%	−0.33 (−3.19, 2.53)
Li 2016	11.04	2.21	90	14.45	2.18	90	4.8%	−3.41 (−4.05, −2.77)
Liu 2008	17.63	3.17	30	19.49	3.46	30	3.5%	−1.86 (−3.54, −0.18)
Ma 2019	10	2.7	50	15	3.4	50	4.1%	−5.00 (−6.20, −3.80)
Meng 2016	9.98	2.29	60	15.03	3.52	60	4.3%	−5.05 (−6.11, −3.99)
Qiu 2011	6.26	0.78	49	8.43	0.65	51	5.0%	−2.17 (−2.45, −1.89)
Sha 2018	5.85	2.33	28	7.47	3.07	29	3.9%	−1.62 (−3.03, −0.21)
Sheng 2010	9	2	30	14	8	30	2.1%	−5.00 (−7.95, −2.05)
Shu 2014	2	0.86	32	3	1.29	32	4.9%	−1.00 (−1.54, −0.46)
Sun 2017	7.06	0.65	91	7.85	0.78	91	5.0%	−0.79 (−1.00, −0.58)
Wang 2015	10.6	2.9	39	15.1	3.5	36	3.8%	−4.50 (−5.96, −3.04)
Xing 2017	9.12	3.15	35	9.87	4.03	35	3.5%	−0.75 (−2.44, 0.94)
Xu 2008	8.6	1.7	32	10.2	3.5	32	3.9%	−1.60 (−2.95, −0.25)
Xue 2012	11.7	2.2	44	13.2	2.4	45	4.4%	−1.50 (−2.46, −0.54)
Yin 2011	14.7	5.2	30	16.2	3.7	30	2.8%	−1.50 (−3.78, 0.78)
Zhang 2005	9.5	2.5	48	10	2.8	46	4.3%	−0.50 (−1.57, 0.57)
Zhang 2008	9.8	5.7	57	11.1	7.7	49	2.4%	−1.30 (−3.91, 1.31)
Zhang 2017	11.6	2.3	40	13.3	2.5	40	4.3%	−1.70 (−2.75, −0.65)
Zhang 2020	10.8	1.78	90	10.82	2.36	97	4.8%	−0.02 (−0.62, 0.58)
Zhu 2015	2.89	0.78	59	2.86	0.81	59	5.0%	−0.03 (−0.26, 0.32)
Total (95% CI)			1215			1209	100.0%	−2.05 (−2.62, −1.48)

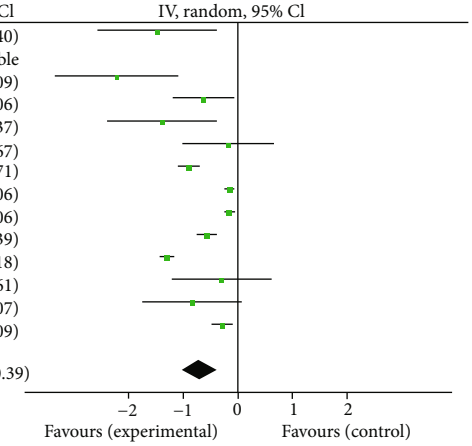
Heterogeneity: $\text{Tau}^2 = 1.66$; $\text{Chi}^2 = 362.58$, df = 25 ($P < 0.00001$); $I^2 = 93\%$
Test for overall effect: $Z = 7.06$ ($P < 0.00001$)



(a)

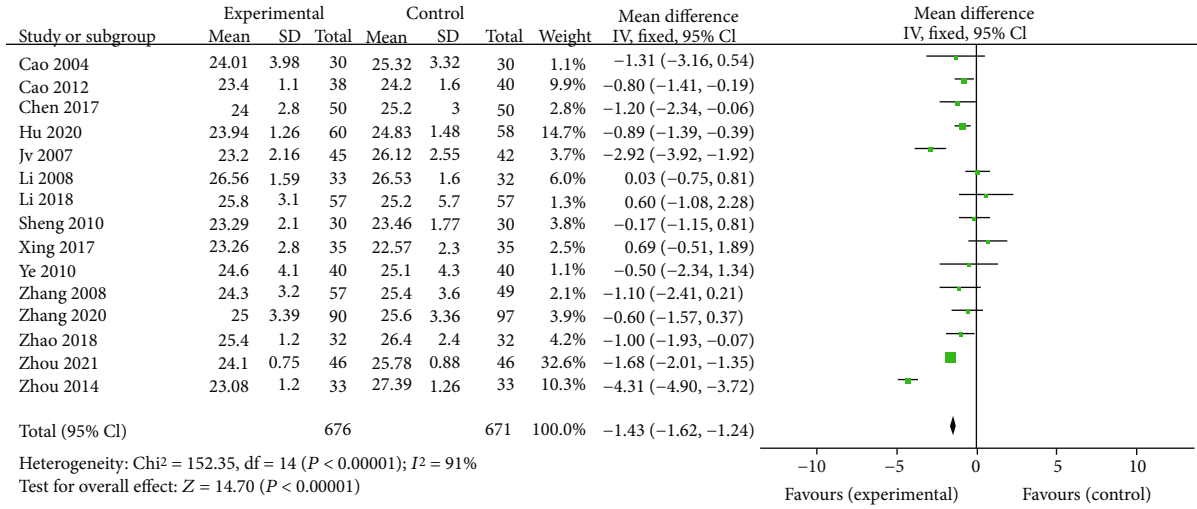
Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV, random, 95% CI
Cao 2004	7.27	2.28	30	8.76	2.03	30	4.7%	−1.49 (−2.58, −0.40)
Cao 2012	0	0	0	0	0	0		Not estimable
Fan 2018	4.37	2.45	40	6.61	2.79	40	4.5%	−2.24 (−3.39, −1.09)
Jv 2007	3.19	1.12	45	3.82	1.55	42	7.8%	−0.63 (−1.20, −0.06)
Ladan Tahmasebi 2019	3.21	1.59	40	4.61	2.92	40	5.0%	−1.40 (−2.43, −0.37)
Liu 2008	5.36	1.56	30	5.54	1.78	30	6.0%	−0.18 (−1.03, 0.67)
Qiu 2011	1.87	0.48	49	2.78	0.56	51	10.0%	−0.91 (−1.11, −0.71)
Sha 2018	0.19	0.16	28	0.34	0.2	29	10.3%	−0.15 (−0.24, −0.06)
Sheng 2010	0.47	0.11	30	0.63	0.26	30	10.3%	−0.16 (−0.26, −0.06)
Sun 2017	1.87	0.91	91	2.45	0.24	91	10.0%	−0.58 (−0.77, −0.39)
Wu 2018	1.61	0.3	63	2.92	0.41	63	10.2%	−1.31 (−1.44, −1.18)
Xing 2017	8.23	1.96	35	8.54	1.98	35	5.6%	−0.31 (−1.23, 0.61)
Zhang 2008	2.44	1.67	57	3.29	2.88	49	5.6%	−0.85 (−1.77, 0.07)
Zhang 2020	3.3	0.58	90	3.59	0.78	97	10.0%	−0.29 (−0.49, −0.09)
Total (95% CI)			628			627	100.0%	−0.71 (−1.03, −0.39)

Heterogeneity: $\text{Tau}^2 = 0.26$; $\text{Chi}^2 = 292.46$, df = 12 ($P < 0.00001$); $I^2 = 96\%$
Test for overall effect: $Z = 4.33$ ($P < 0.00001$)



(b)

Figure 6: Continued.



(c)

Figure 6: Meta-analysis of the effect of berberine on insulin resistance-associated index. (a) Fasting plasma insulin (FINS). (b) Homeostasis model assessment-insulin resistance (HOMA-IR). (c) Body mass index (BMI).

slightly upregulated tendency in HDL (MD = 0.17, 95% CI (0.09, 0.25), $P < 0.05$, $I^2 = 92\%$, 95% CI (0.89, 0.95)) (Figure 8(a)), as compared to the control group and the LDL concentration of the berberine group decreased by 0.86 (95% CI (−1.06, −0.66), $P < 0.05$, $I^2 = 92\%$, 95% CI (0.89, 0.94)) (Figure 8(b)).

3.4. Berberine for Inflammation Factors of T2DM. Pooled results of six clinical trials proved that berberine markedly lowered the CRP levels in patients with T2DM (SMD = − 2.13, 95% CI (−2.98, −1.28), $P < 0.05$, $I^2 = 96\%$, 95% CI (0.94, 0.97)).

Six trials were considered for the analysis of the efficacy of berberine on IL-6 levels. The IL-6 concentration in the trial group decreased by 1.83 (95% CI (−3.05, −0.61), $P = 0.003$; $I^2 = 97\%$, 95% CI (0.95, 0.98)).

Among them, five reported the efficacy of berberine on TNF-α, and the results found that berberine reduced the level of TNF-α in patients with T2DM to some extent (SMD = −1.44, 95% CI (−2.72, −0.16), $P = 0.03$, $I^2 = 97\%$, 95% CI (0.95, 0.98)) (Figure 9).

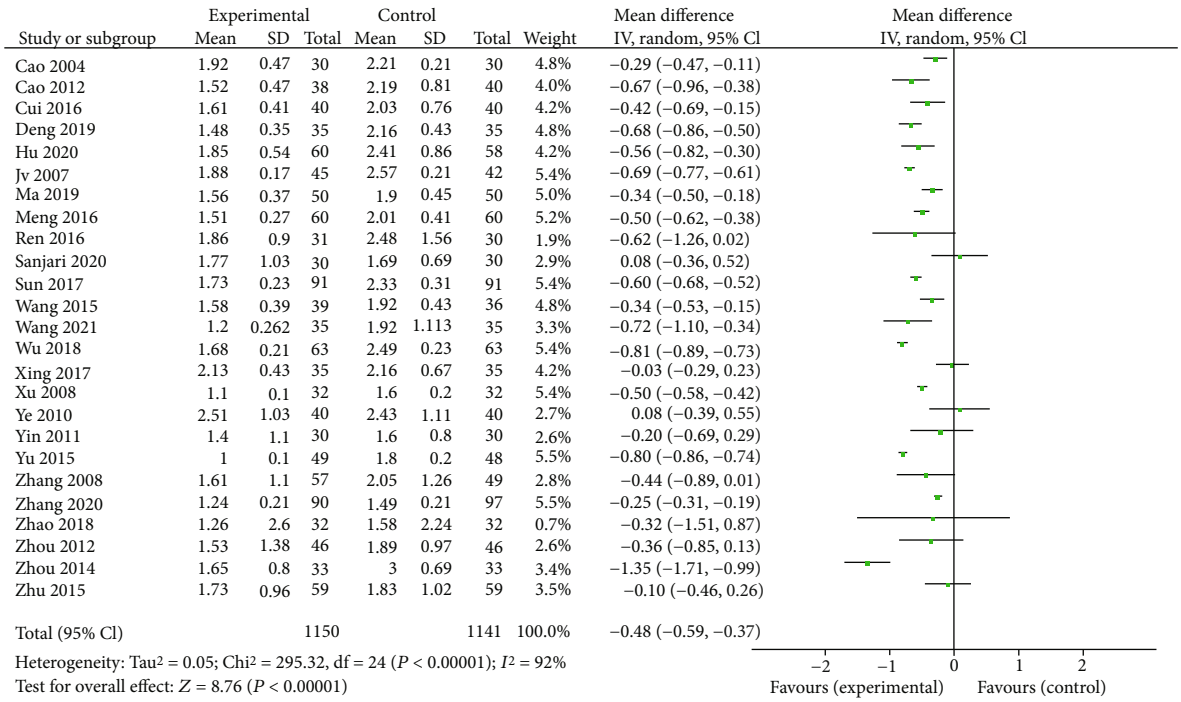
3.5. Safety of Berberine on Patients with T2DM. Figure 10(a) shows the effects of berberine on Scr in patients with T2DM. There were a total of 288 volunteers in the trial group and 294 in the control group. The Scr concentration of the berberine group decreased by 2.02 (95% CI (−3.63, −0.42), $P = 0.01$, $I^2 = 0\%$, 95% CI (0, 0.86)). Berberine was found to have a beneficial effect on Scr.

Figure 10(b) illustrates the effects of berberine on BUN. There were a total of 288 volunteers in the trial group and 294 in the control group. Compared to the control group, berberine had no significant effect on the BUN level (SMD = −0.29, 95% CI (−0.69, −0.11), $P = 0.16$, $I^2 = 97\%$, 95% CI (0.94, 0.98)).

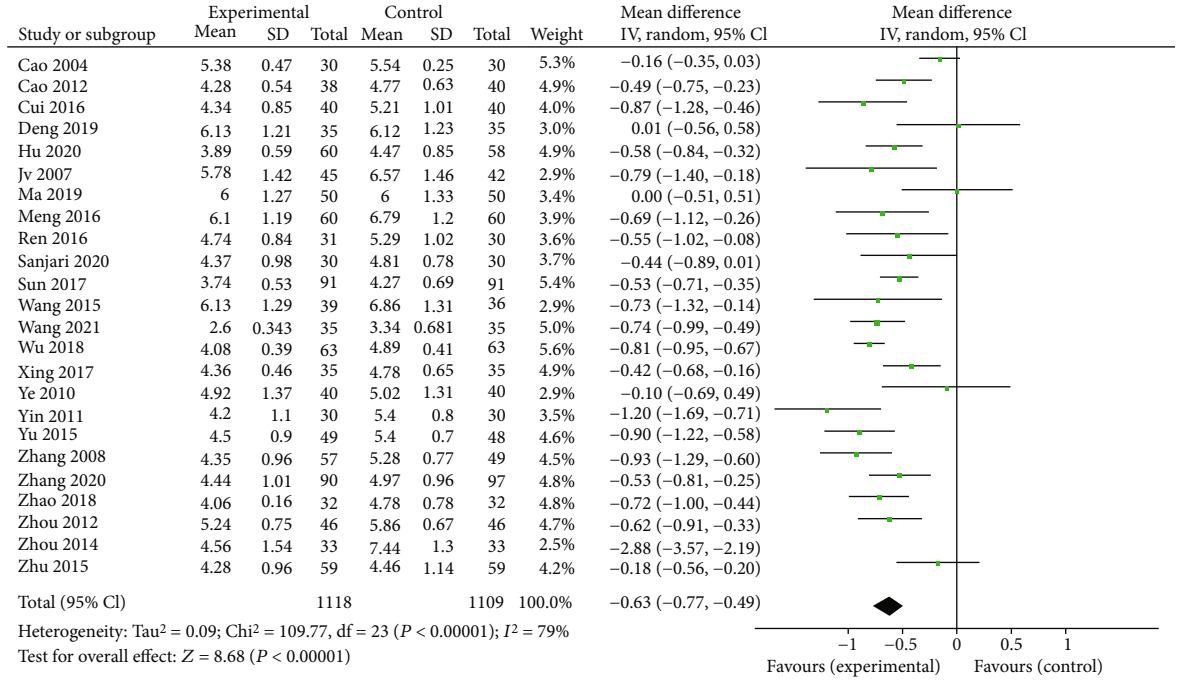
The incidence of adverse events (AEs) was used to assess the safety of berberine in 17 studies, including a total of 858 patients in the berberine group and 856 in the control group. Pooled results of 17 trials showed that berberine applied for the treatment of T2DM appeared to have better safety compared to the control group in the incidence of AEs (RR = 0.70, 95% CI (0.57, 0.87), $P = 0.0009$, $I^2 = 28\%$, 95% CI (0, 0.6)) (Figure 10(c)). The main reported adverse events of berberine treatment were gastrointestinal responses like diarrhea, abdominal distention, or constipation. Among the 17 trials, 15 specifically reported the number of gastrointestinal AEs, which included 732 volunteers in the trial group and 730 in the control group. These results demonstrated that berberine did not have more gastrointestinal AEs as compared to the control group (RR = 0.81, 95% CI (0.46, 1.14), $P = 0.45$, $I^2 = 52\%$, 95% CI (0.13, 0.73)) (Figure 10(d)). The pooled results demonstrated that berberine was generally safe as a complementary or alternative therapy for the treatment of T2DM.

3.6. Publication Bias. A funnel plot was used to evaluate potential publication bias. Comparisons of HbA1c, FPG, and 2hPG were conducted using funnel plots. Approximately symmetrical dispersion points suggested rare publication bias. These results are shown in Figure 11.

3.7. Sensitivity Analysis. According to the different interventions, daily dosage of berberine applied, the intervention duration, and disease courses, we conducted the subgroup analyses for main outcomes including HbA1c, FPG, and 2hPG (Supplementary Files 2). Results found that different interventions were the source of heterogeneity. Mutual conversion between a random-effects model and a fixed-effect model was conducted as a sensitivity analysis for testing the stability of the research. The results showed that the I^2 value did not change in the mutual conversion. The results



(a)



(b)

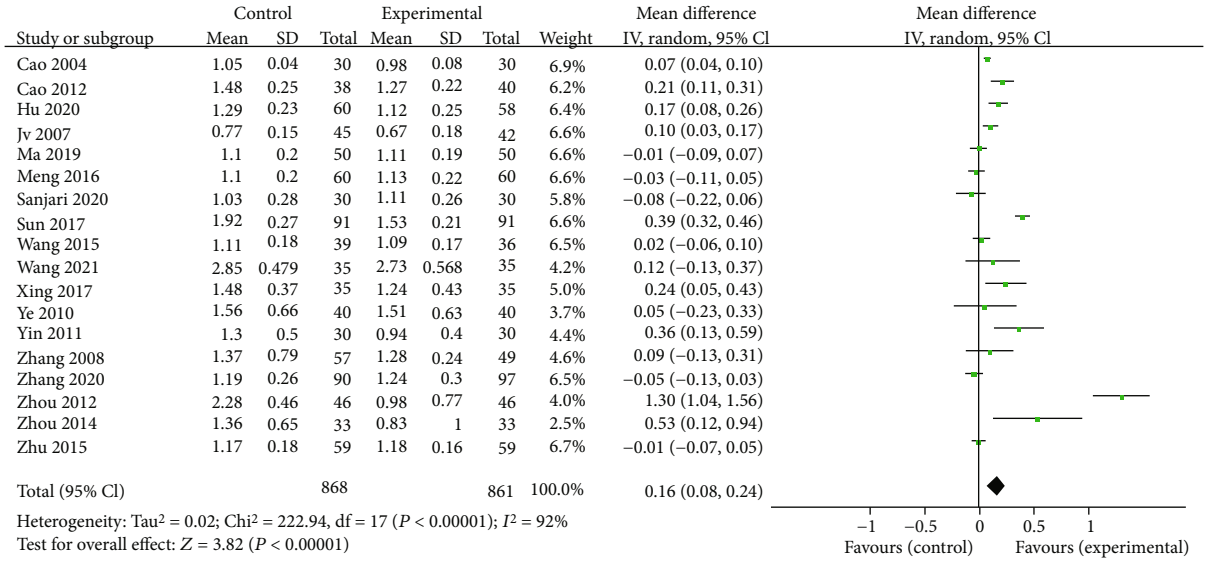
Figure 7: Meta-analysis of the effect of berberine on triglyceride (a) and total cholesterol (b).

of meta-analyses were not changed either. This suggests that our findings were stable.

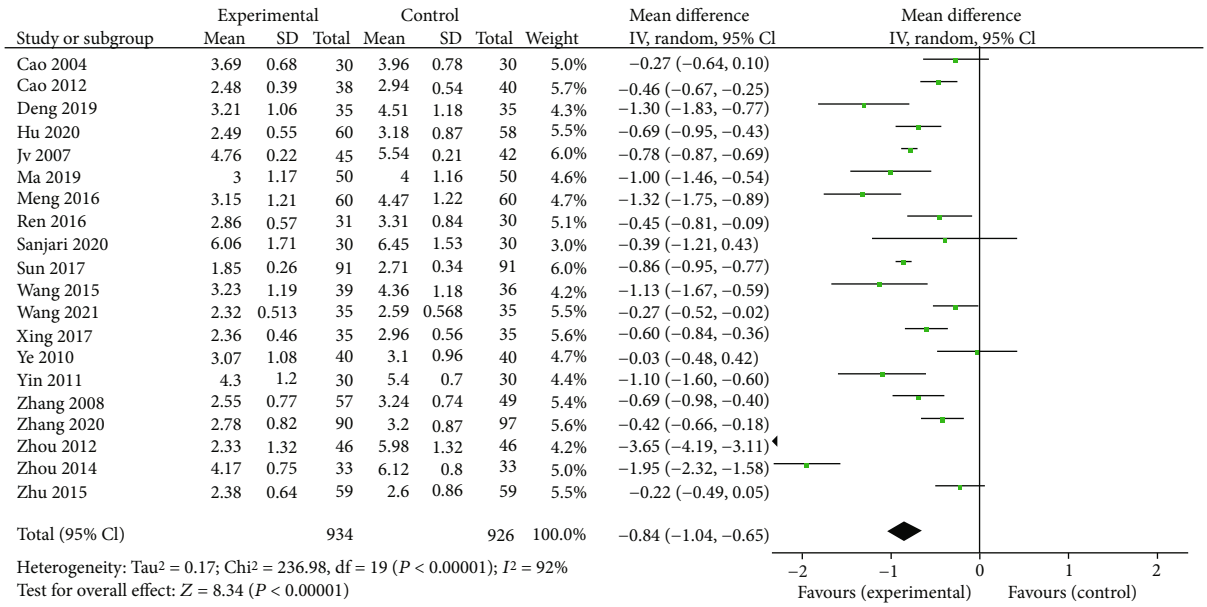
4. Discussion

In the current systematic review, we evaluated the efficacy and safety of berberine for the treatment of T2DM. Our findings suggested that berberine, used along or combined with antidiabetic agents, significantly improved glucose and lipid metabolisms along with inflammation markers.

Berberine showed effectiveness in lowering blood glucose comparable with metformin. As an adjunctive therapy, berberine presented better reduction of HbA1c, FPG, and 2hPG. In recent years, three meta-analyses [17, 66, 67] were



(a)



(b)

Figure 8: Meta-analysis of the effect of berberine on high-density lipoprotein (a) and low-density lipoprotein (b).

conducted to explore the effects of berberine for the treatment of T2DM. The latest study was published in 2019, which first performed subgroup analyses to examine the source of heterogeneity and identify the potential factors which likely determine the effects of berberine. These results showed that berberine seemed to achieve better effects on glucose levels when patients aged less than 60 years were treated with a daily dosage of 1.5–2 g. After adding new research with limited sample size and intervention durations in the included criteria, our results suggested that low-dose berberine (< 1 g/d) achieve promising effects of FPG, especially for those patients with a disease duration of no more than five years. The efficacy of berberine seemed to decrease with an increased treatment course. Low and medium doses of (1–2 g) berberine showed the same effects on reducing HbA1c and 2hPG. Better efficacy on 2hPG was observed in patients with an intervention duration of no less than 12 weeks. For HbA1c, the highest level of efficacy was observed with an intervention duration of no more than eight weeks and less than twelve weeks, especially for those patients with T2DM that have had this condition for 5–10 years. It appeared that the early application of berberine mainly functioned to reduce FPG. With prolonged intervention duration and disease progression, this effect was mainly observed as the reduction of 2hPG.

The regulation of berberine on blood homeostasis is partly due to the improvement of insulin resistance, the hallmark of T2DM, which is given rise to obesity. Systematic reviews showed that berberine improved obesity parameters

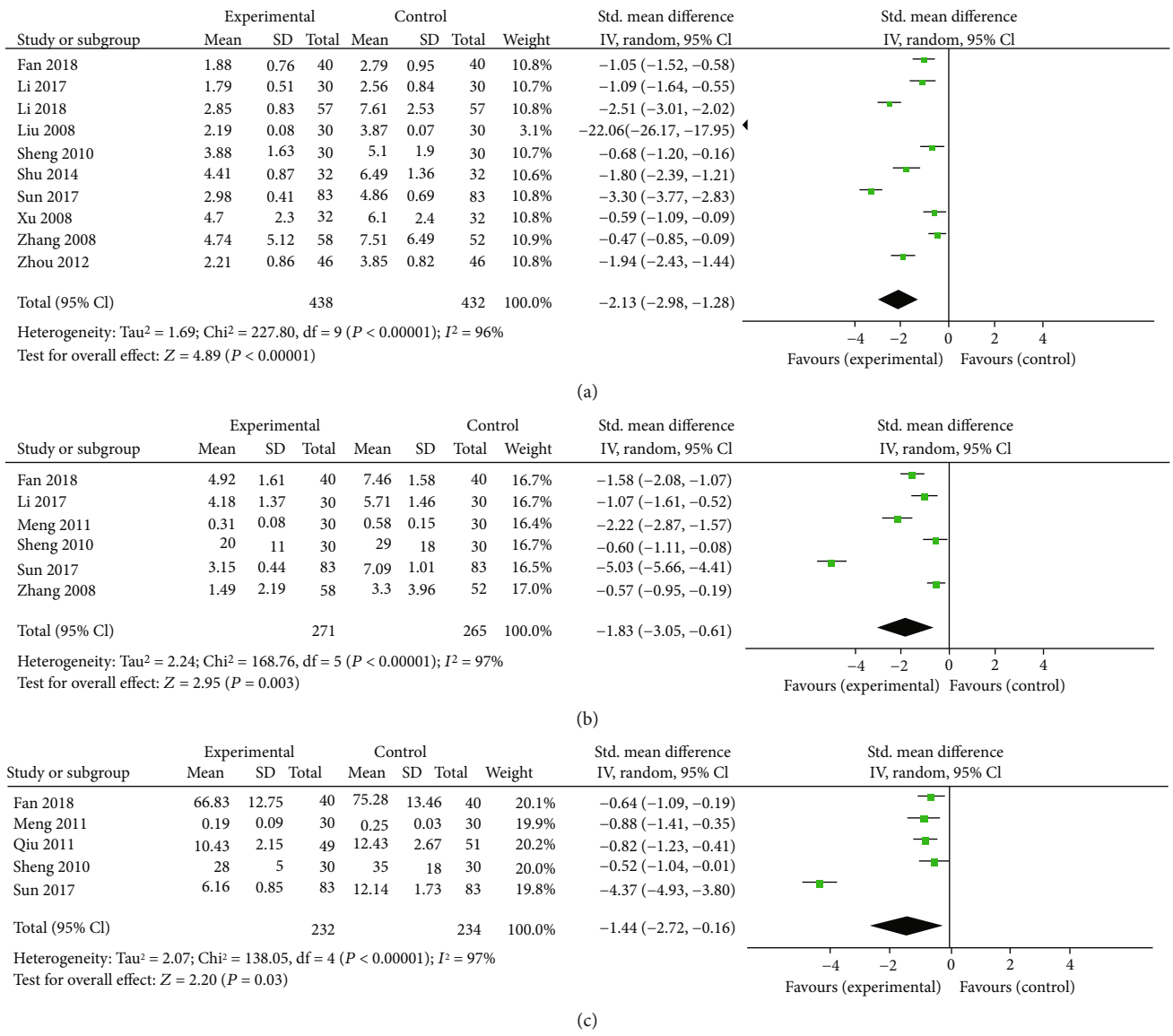
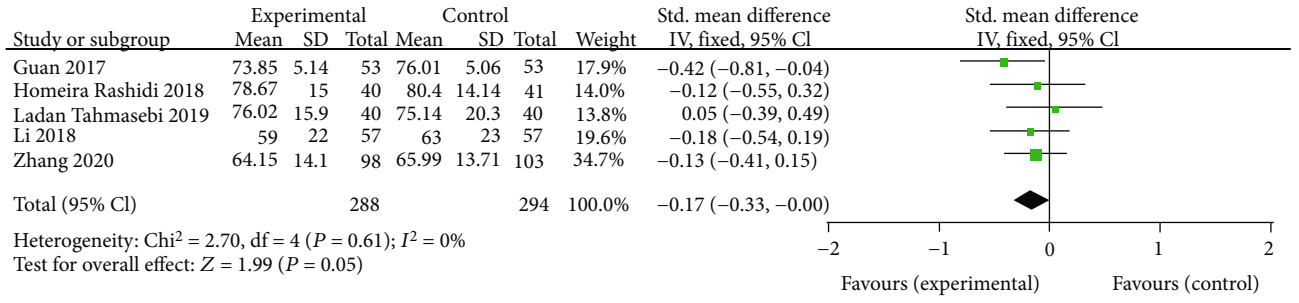


Figure 9: Meta-analysis of the effect of berberine on inflammation factors. (a) C-reaction protein (CRP). (b) Interleukin-6 (IL-6). (c) Tumor necrosis factor-α (TNF-α).

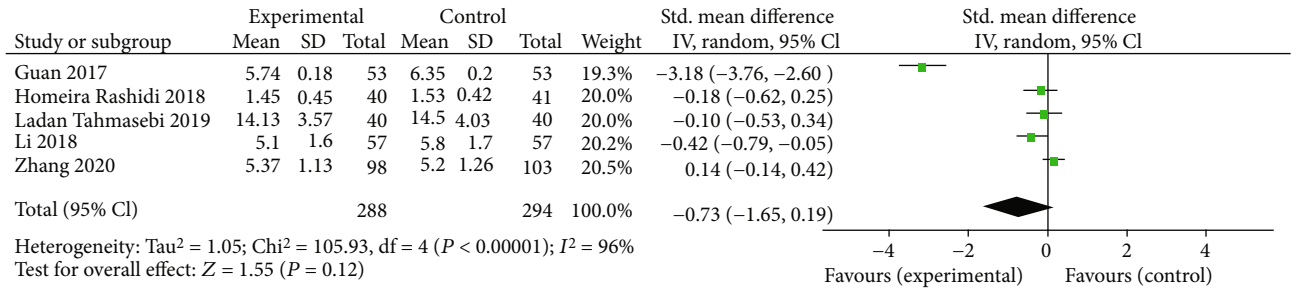
including BMI [68, 69]. Meanwhile, a case-control clinical trial reported that the HOMA-IR level of T2DM decreased by 73% with 500 mg (×3 daily) berberine for 3 months [70]. Our results showed that berberine remarkably lower fasting blood insulin, improve HOMA-IR, and decrease BMI, which demonstrated the advantages of berberine on improving insulin resistance.

Evidence suggests that clinically tested lipid-lowering nutraceuticals including berberine could be safely used to improve lipid levels in patients with mild-to-moderate dyslipidemia [71]. Our work also showed the plasma lipid profiles of diabetic patients were improved by berberine intake. The efficacy of berberine on dyslipidemia has been widely researched. Whether used alone or combined with other therapies, meta-analyses [16, 72] suggested that berberine improve obesity and hyperlipidemia by reducing TG, TC, and LDL and increasing HDL in the setting of several metabolic disorders along with improving glucose metabolism. This is consistent with the current meta-analysis specific to T2DM, which showed a remarkable lowering of TC, TG, and LDL, along with moderate upregulation of HDL.

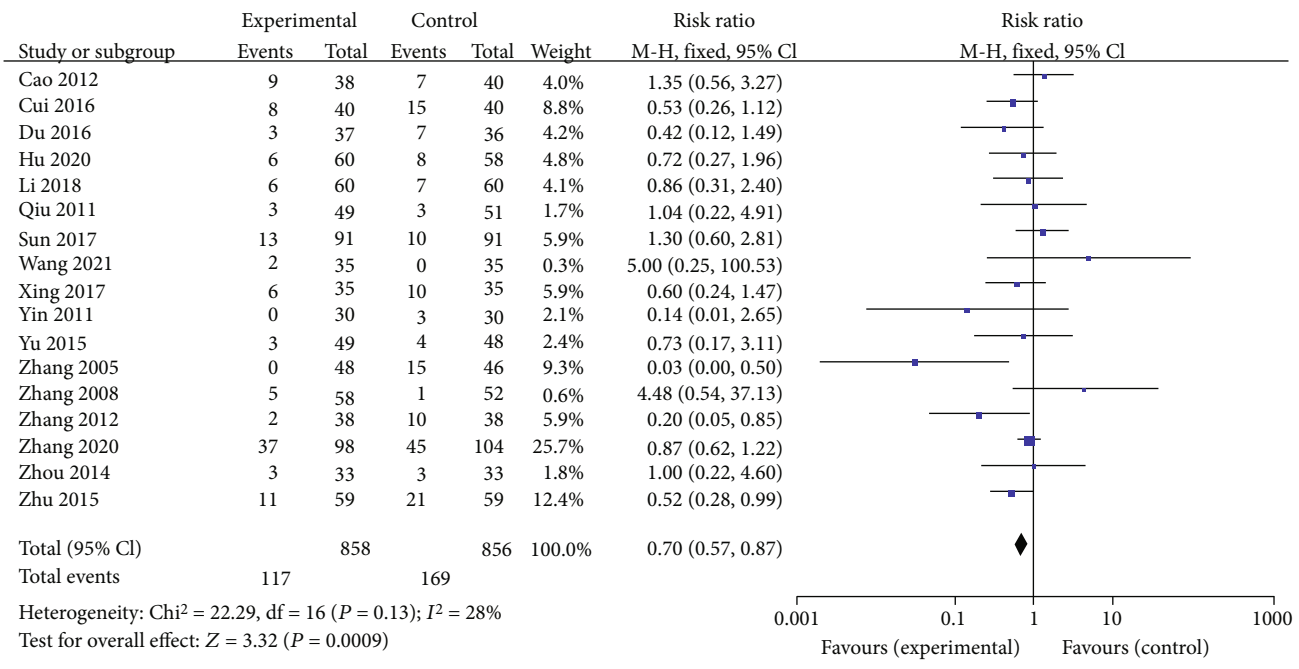
In T2DM, obesity and dyslipidemia bring about low-grade inflammation and factor like IL-6 and TNF-α levels were found to be strikingly increased. This was associated with a downregulation of several drug metabolizing enzymes, which led to poor drug effect. Berberine has been demonstrated as a chronic inflammation regulator as well [73]. A meta-analysis proved that berberine supplementation ameliorates the state of chronic inflammation by lowering the serum level of CRP [74]. Our meta-analysis illustrated that berberine significantly downregulates CRP, IL-6, and TNF-α. This indicates that berberine as an



(a)

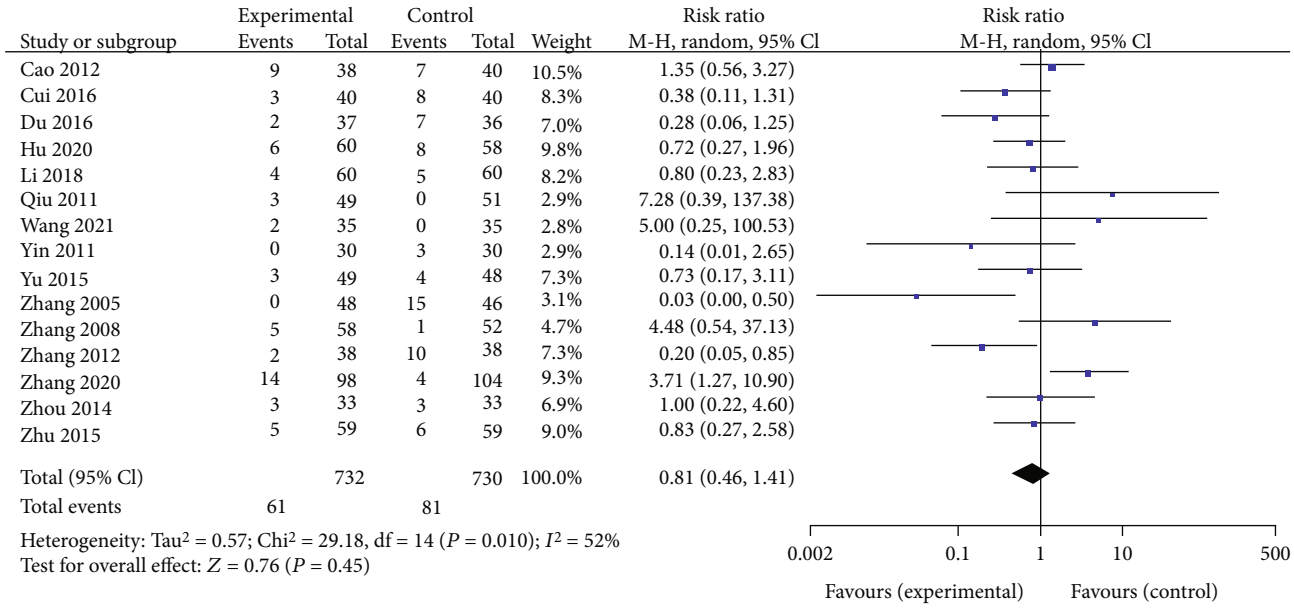


(b)



(c)

Figure 10: Continued.



(d)

Figure 10: Meta-analysis of the safety of berberine in the treatment of diabetes mellitus. (a) Serum creatinine (Scr). (b) Blood urea nitrogen (BUN). (c) Total adverse events. (d) The gastrointestinal adverse events.

additional therapy shows synergistic benefits with hypoglycemic agents. Above all, berberine is suggested to be applied to diabetic patients with insulin resistance and dyslipidemia, especially for those patients newly diagnosed with T2DM accompanied by obesity and dyslipidemia.

Regarding safety, our work demonstrated that berberine had no toxic effects on Scr and BUN. In addition, this treatment did not increase the risk of serious adverse events when the routine dosage ranged from 0.6 g to 1.5 g.

Berberine has been demonstrated to have comparable effects in the treatment of T2DM with antidiabetic drugs like metformin that display multiple targets and pathways. For patients with T2DM, the main function of berberine is as a SIRT1 or AMPK agonist to mimic energy restriction [75–77] and to target on NF-κB [78] to improve insulin resistance and inflammation as well as to alleviate the activation of ox-LDL-induced macrophages [79]. In addition, berberine stabilizes LDL receptor mRNA to increase the clearance rate of plasma LDL [80]. This corresponds to its effects of improving insulin resistance and the regulation of glycemic and lipid metabolisms. As we all know, dyslipidemia and inflammation are risk factors of micro- and macrovascular leisures, our meta-analysis suggest that berberine has potential benefits on diabetes with cardiovascular and chronic kidney disease, which has been reported in preclinical studies [81, 82].

Excessive statistical heterogeneity was induced by several factors in our work. First, different antidiabetic agents used as controls also had a distinct influence on the outcomes. Different dosage levels of berberine and the duration of treatment may have resulted in inconsistent efficacy and different disease courses. To account for these, subgroup analyses were used to assess primary outcomes, including HbA1c, FPG, and 2hPG. The results showed that the different interventions in the control groups seemed to be a potential impact factor of heterogeneity. Meanwhile, the literature qualities may have influenced the results. In the future, the association of each factor with the effect of berberine for the treatment of T2DM patients should be quantified by metaregression analysis.

5. Strengths and Limitations of the Current Study

Compared to previous meta-analyses, the current study included 46 trials and comprehensively showed the efficacy and safety of berberine for the treatment of T2DM. Subgroup analyses were also conducted to clarify how berberine is used. Additionally, GRADE criteria (Supplementary Files 3) were applied to determine the certainty in the estimate of effect for primary outcomes.

There are some limitations of this review. First, most of the trials were conducted among Chinese patients, which limited the widespread application of this data. Second, excessive statistical heterogeneity appeared in some comparisons; however, the primary source of heterogeneity could not be determined. Third, literature qualities were uneven, although the included trials were RCTs. Many of these studies did not report the methods of blinding and allocation concealment. Lastly, more information on the long-term intervention of berberine for the treatment of T2DM is needed to assess the occurrence risk of diabetic complications.

In conclusion, berberine positively regulated glucose metabolism and lipids, improving insulin resistance and

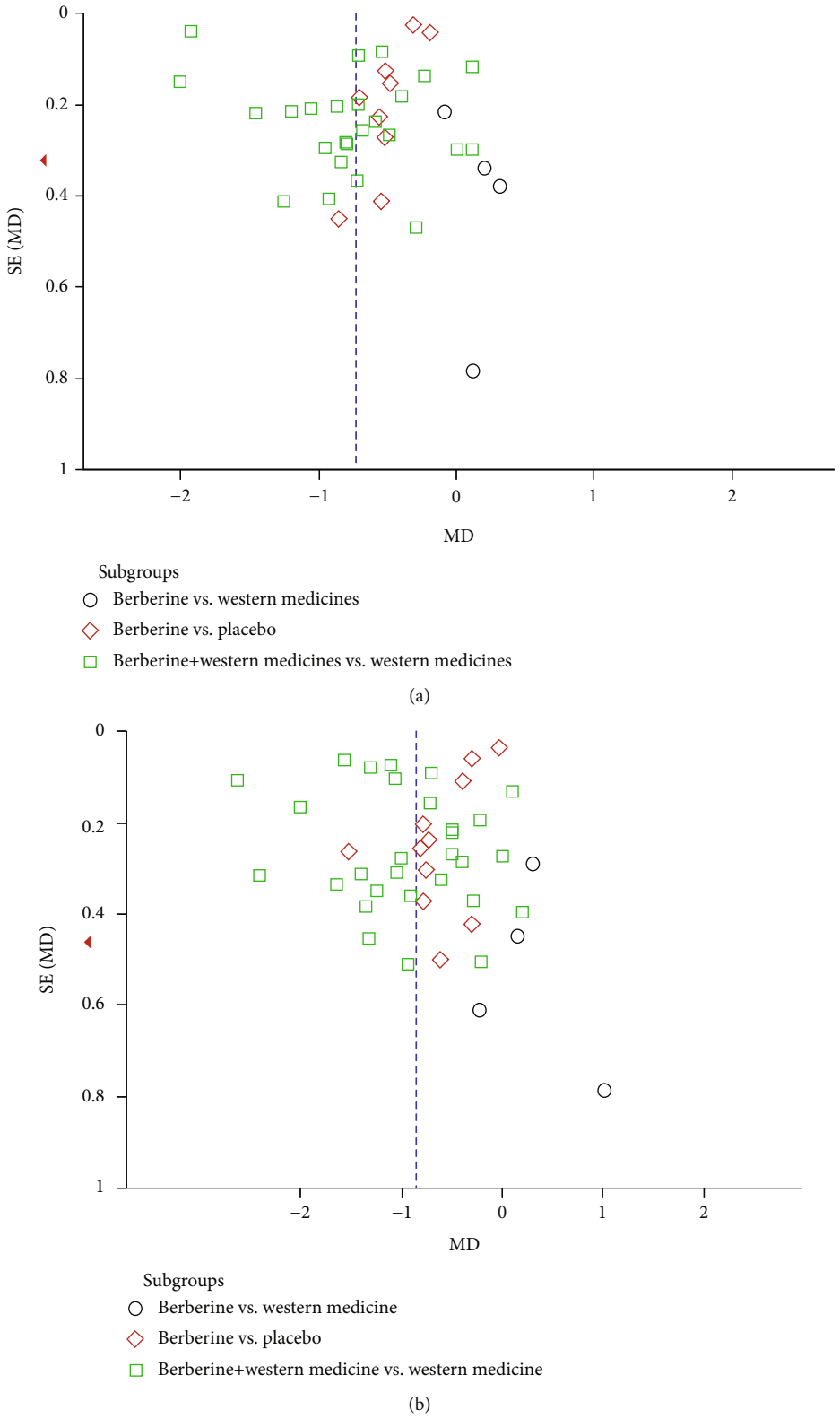


Figure 11: Continued.

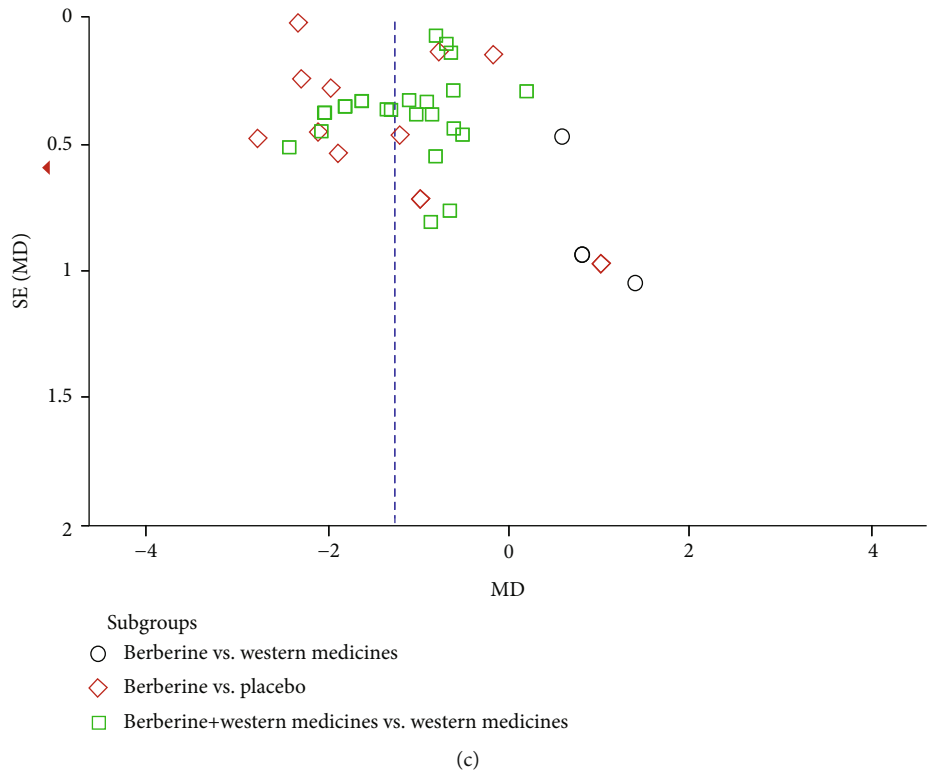


Figure 11: Funnel plots of comparison. A funnel plot was used to evaluate potential publication bias. Comparison in HbA1c, FPG, and 2hPG was conducted by funnel plots.

inflammation in patients with T2DM. Thus, berberine was recommended as an adjunctive therapy for T2DM.

Abbreviations

DM: Diabetes mellitus
RCT: Randomized controlled trials
HbA1c: Glycosylated hemoglobin
FPG: Fasting plasm glucose
2hPG: 2-hour postprandial blood glucose
FINS: Fasting blood insulin
HOMA-IR: Homeostasis model assessment-insulin resistance
BMI: Body mass index
TG: Triglyceride
TC: Total cholesterol
LDL: Low-density lipoprotein
HDL: High-density lipoprotein
CRP: C-reactive protein
IL-6: Interleukin-6
TNF-α: Tumor necrosis factor-α
Scr: Serum creatinine
BUN: Blood urea nitrogen.

Data Availability

The original contributions presented in the study are included in the article material; further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare that they do not have any potential conflict of interest.

Authors' Contributions

This study was designed by W.J. Liu and J.Guo. J. Guo and X.Q. Zhang searched literature. J. Guo and H.D. Chen screened and selected the eligible trials. W.J. Lou, Pingna Zhang, Y.H. Qiu, and C. Z performed the data collection. Analysis of the data was performed by J. Guo and H.D. Chen. J. Guo and X.Q. Zhang wrote the manuscript. Y. X Wang and W.J. Liu checked and revised the manuscript. J. Guo and H.D. Chen revised the manuscript according to reviewers' suggestions. Jing Guo and Hongdong Chen contributed equally to this study.

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Supplementary Materials

Supplementary 1. Supplementary Files 1 shows the full electronic search strategy for PubMed according to the search history.

Supplementary 2. Supplementary Files 2 shows results of subgroup analyses for main outcomes including HbA1c, FPG, and 2hPG.

Supplementary 3. Supplementary Files 3 shows GRADE criteria to determine the certainty in the estimate of effect for primary outcomes including HbA1c, FPG, and 2hPG.

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Review Article

A Literature-Based Update on *Benincasa hispida* (Thunb.) Cogn.: Traditional Uses, Nutraceutical, and Phytopharmacological Profiles

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Benincasa hispida (Thunb.) Cogn. (Cucurbitaceae) is an annual climbing plant, native to Asia with multiple therapeutic uses in traditional medicine. This updated review is aimed at discussing the ethnopharmacological, phytochemical, pharmacological properties, and molecular mechanisms highlighted in preclinical experimental studies and toxicological safety to evaluate the therapeutic potential of this genus. The literature from PubMed, Google Scholar, Elsevier, Springer, Science Direct, and database was analyzed using the basic keyword "*Benincasa hispida*." Other searching strategies, including online resources, books, and journals, were used. The taxonomy of the plant has been made by consulting "The Plant List". The results showed that *B. hispida* has been used in traditional medicine to treat neurological diseases, kidney disease, fever, and cough accompanied by thick mucus and to fight intestinal worms. The main bioactive compounds contained in *Benincasa hispida* have cytotoxic, anti-inflammatory, and anticancer properties. Further safety and efficacy investigations are needed to confirm these beneficial therapeutic effects and also future human clinical studies.

1. Introduction

Food and food products are being used as medicines over centuries worldwide. Many species from the family Cucurbitaceae have been used as medicaments in various diseases in Ayurveda and ancient Chinese medicine. This family is also known as the gourd family. It provides approximately 5 to 6% of the total vegetables in the world. To date, 825 species from under 118 genera have been reported growing in temperate regions of the world [1]. It should be mentioned that the Cucurbit species can grow in diverse climatic conditions, including arid deserts, tropical, subtropical, and temperate regions. These various types of species are included in food systems and Indian traditional medicines. Generally, the gourd family vegetables provide vitamins, essential minerals, antioxidants, and soluble fibres [2].

The word "herb" derived from the Latin word "*herba*" and an old French word "*herbe*" refers to any part of the plant like fruit, seed, stem, bark, flower, leaf, stigma, or a root, as well as a non-woody plant. Many herbs are currently under-using as a source of foods, flavonoids, medicines, or perfumes as well as in certain spiritual activities. Ancient era literature including Unani manuscripts, Chinese writings, and Egyptian papyrus also depicted the use of herbs in various diseases. The Indian Vaids, Unani Hakims, and European and Mediterranean cultures are using herbs for more than 4000 years as medicines. Native people of Iran, Rome, Egypt, Africa, and America used medicinal herbs in healing habits. The Unani, Ayurveda, and Chinese Medicine are using herbal remedies systematically. These all are the potential sources of medicinal plant-based modern medicines. According to World Health Organization (WHO), about 80% of people in the world depend on herbal medicines to fulfil their basic health care needs, and around 21,000 species of plants have been identified as potential medicinal plants. In developed countries, around 25% of the total drugs come from plant origin, while in fast-developing countries as much as 80% [3].

Benincasa hispida (Thunb.) Cogn. (synonym: *Benincasa cerifera* Savi) (Cucurbitaceae) especially in Asian countries is considered as one of the famous crops under the Cucurbitaceae family that grows mainly for its fruits and well renowned for its nutritional and medicinal properties [4, 5]. Scientific reports suggest that *B. hispida* possesses many important nutritious substances, including vitamins, natural sugars, amino acids, organic acids, and mineral elements [4, 6, 7]. This review is aimed at sketching an up-to-date scenario on the indigenous uses, nutraceutical, and phytochemical composition along with the pharmacological activities of *B. hispida* based on database reports.

2. Review Methodology

Using the PubMed database and the search engines Google scholar, Elsevier, Springer, Science Direct, research articles, and reviews related to *B. hispida* were analyzed. Abstracts and papers peer reviewed were selected according to the objectives of the research: the molecular pharmacological mechanisms of action proven by preclinical experimental studies [8] and which scientifically justify the traditional uses of *B. hispida*.

Other sources of "grey literature" information such as Web pages, book chapters, and specialized monographs were also analyzed to obtain maximum updated information on the biological properties of this plant. The keywords used were "*Benincasa hispida*" or "traditional uses" or "phytochemistry" of "pharmacological properties" or "biological activities" or "toxicology" or "safety" or "side effects." The scientific names of the plants were verified according to PlantList, and the chemical formulas were revised by consulting the PubChem database (https://scholar.google.com).

Inclusion criteria: the most relevant articles written in English on taxonomy, ethnopharmacology, phytochemistry, pharmacology, various biological activities, and toxicity of this plant were included and analyzed.

Exclusion criteria: the papers containing homoeopathic preparations, papers written in languages other than English, publications without pharmacological mechanisms of action.

3. Botany and Traditional Uses

3.1. Botany (Plant Profile). B. hispida (Figure 1), also known as *kundur fruit*, *chalkumra*, *wax gourd*, *winter gourd*, *ash gourd*, *winter melon*, *white gourd*, *tallow gourd*, *Chinese preserving melon*, *ash pumpkin*, and *(alu) puhul*, a creeper grown for its very big size fruit, is eaten as a green mature vegetable or greens [9–11].

There is a fine hairs fuzzy coating outer side of the young fruit and has solid thick white flesh of sweet in tastes. The mature fruit sheds its hairs and forms a waxy white coating, giving the name of "*wax gourd*." The gourd wax coating increases the storage facilities of it. It can grow of a length up to 80 cm and also have broad leaves and yellow flowers. The taste is rather bland. *B. hispida* is a native of South and Southeast Asia. However, it is commonly grown all over Asia, including Japan, Burma, Ceylon, Sri Lanka, Java, and Australia [10].

Taxonomy
Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Oder: Cucurbitales
Family: Cucurbitaceae
Genus: Benincasa
Species: *Benincasa hispida* (Thunb.) Cogn.

3.2. Traditional Uses and Ethnopharmacology. In India, *B. hispida* is used as a winter season vegetable for a wide variety of diseases. Its medicinal properties have been also recognized in the Ayurvedic system of medicine, spiritual traditions of India and Yoga. In Vietnam, its soup (cooked with pork short ribs) is traditionally used by breastfeeding mothers. In north India and almost all regions in Bangladesh, it is added with pulses like as moong which usually crushed, along with wax gourd, makes a dish locally called *bori*, which after sun drying is used in curry dishes and eaten with rice or chapati [12]. To make wax gourd soup in China, it is used in stir-fries or added into pork or pork/beef bones, which often served in the scooped-out gourd, carved by

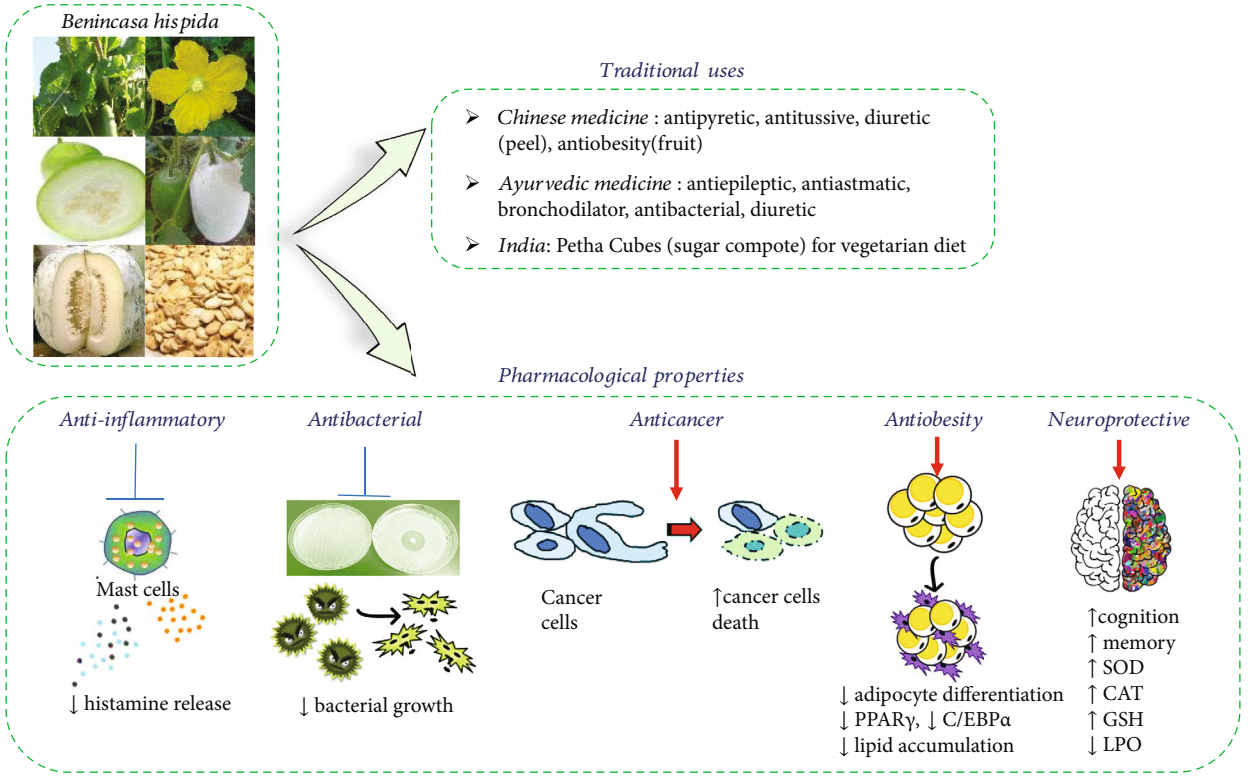

Figure 1: Diagram with different parts (aerial parts, flower, fruit pulp unripe, fruits, fruit pulp mature, and seeds) of *Benincasa hispida* (Thunb.) Cogn, traditional uses, and its most important pharmacological properties. Abbreviations: PPARγ: peroxisome proliferator-activated receptor gamma; C/EBPα: CCAAT enhancer-binding protein alpha; CAT: catalase; SOD: superoxidase dismutase; GSH: reduced glutathione; LPO: lipid peroxidations.

scraping off the waxy coating. It is also cut into pieces, candied and normally eaten during the time of New Year festivals, or used as filling in Sweetheart cake. For the Moon Festival, the Chinese and Taiwanese also used it in moon cakes as a base filling. It is candied by the people of the Philippines and is used as a pastry filling for bakpia. In some savoury soups and stir-fries, it also acts as an ingredient. In Nepal, India, and Bangladesh, the tendrils, shoots, and leaves of the plant are consumed as green vegetables [6].

B. hispida is widely used in Chinese medicine, in the treatment of fever, cough accompanied by thick mucus and urinary disorders, it is used especially in bark with a very good diuretic effect. The fruit is recommended for overweight people who want to follow diets. In Ayurvedic medicine, it is used in the treatment of epilepsy, cough, lung disease, hiccups, asthma, internal bleeding, and urinary retention. In India, a fruit compote called Petha Cubes is made from the pulp of the fruit, which is recommended for vegetarians [13].

The fruit is also used in peptic ulcer, and it is also used in diabetes mellitus, urinary infection, haemorrhages from internal organs, insanity, epilepsy, and other nervous disorders in Ayurveda [14]. The fruit is sweet and traditionally used as a cooling, styptic, antiperiodic, laxative, diuretic, tonic, aphrodisiac, and cardiotonic, and also in jaundice, dyspepsia, urinary calculi, blood disease (e.g., haemorrhages from internal organs), insanity, epilepsy, asthma, diabetes, vitiated conditions of pitta, fever, menstrual disorders, and balancing the body heat [15] (Figure 1).

3.3. Phytochemical Profile

3.3.1. Nutritional Composition. The edible portion of *B. hispida* contains moisture (93.80-96.80/100 g), proteins (0.30-0.70/100 g), carbohydrates (1.10-4.00/100 g), fat (0.02-0.20/100 g), fibre (0.50-2.10/100 g), and ash (0.27-0.70/100 g).

Vitamins present in the edible portion (per 100 g) of this plant are vitamin C (1.35-68.00), thiamin (0.02-0.04), riboflavin (0.02-0.31), niacin (0.20-0.46), and vitamin E. Major minerals in the edible portion (per 100 g) include sodium (0.14-6.00), potassium (77.00-131.00), calcium (5.00-23.32), iron (0.20-0.49), and phosphorus (225.39-234.61) [6, 16, 17]. The fruit contains water-soluble polysaccharides [18], such as arabinogalactans [19].

The fruit pulp contains homogalacturonan, β-(1 → 4)-D-galactan, acidic arabinan [20], and natural sugars (e.g., glucose and fructose) [21]. The mature fruit also contains organic acids such as malic and citric acid.

3.3.2. Chemical Phytoconstituents. The leaf contains alkaloids, flavonoids, steroids [22], and the fruit amino acids, pectic polysaccharides [20], hemicellulose polysaccharides [18], terpenes and terpenoids, flavonoid C–glycosides, sterols [23], proteins [24], phenols, alkaloids, glycosides, tannins, saponins [25], hydroxybenzoic acids, flavonols, hydrocinnamic acids, and triterpenes [9] (Figure 2).

The seeds contain proteins [24], carbohydrates, phenolic compounds, amino acids, flavonoids, sterols [26], glycosides,

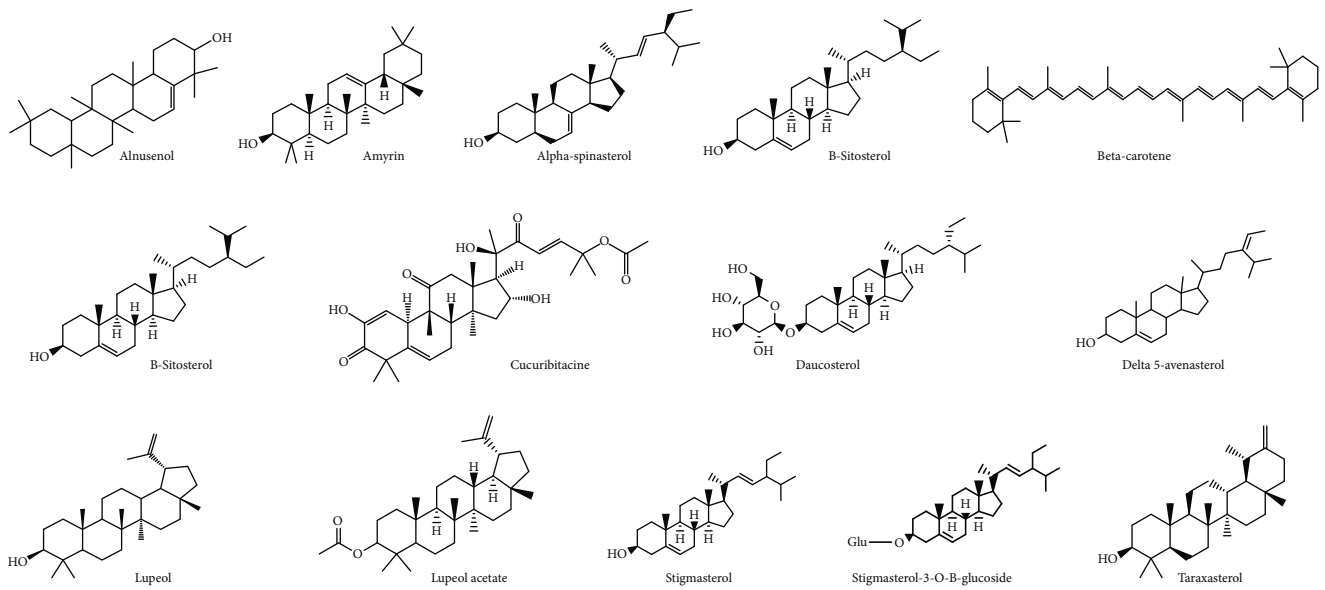

Figure 2: The chemical formulas of most important representative sterols and terpenes from *Benincasa hispida*.

alkaloids, fixed oils and fats, phenolic compounds, steroids [27], and unsaturated fatty acids [28]. The peel contains alkaloids, saponins, steroids, carbohydrates, flavonoids [29], tannins, carotenoids, oxalates, and phytate [17].

The root contains proteins [24]. The fruit contains many volatile compounds, including (*E,E*)-2,4-nonadienal, (*E*)-2-hexenal, n-hexanal, n-hexyl formate, (*E,E*)-2,4-heptadienal, (*Z*)-3-hexenal, (*E*)-2-heptenal, 1-octen-3-ol [30], 2,5-dimethylpyrazine, 2-methyl pyrazine, 2-ethyl-5-methyl pyrazine, and 2,6-dimethylpyrazine, 2,3,5-trimethylpyrazine [30].

B. hispida is rich in phenolic compounds. Several other bioactive compounds present in it are isomultiflorenyl acetate, isovitexin, 1-sinapoylglucose, multiflorenol, 5-gluten-3-β-ylacetate, alnusenol, and benzylalcolcohol-*O*-α-l-arabinopyranosyl-(1-6)-β-d-glucopyranoside [31]. The most representative phytochemicals present in *B. hispida* has been shown in Figures 2–5 and Table 1.

4. Pharmacological Activities

4.1. Antioxidant Effects. Oxidative stress is a term used for free radical diseases [51, 52]. It is defined as the imbalance between free radicals and antioxidants, given that oxidants (free radicals) are more and have a destructive potential on the human body [53, 54].

The methanolic seed extract showed a concentration-dependent (25-200 μg/mL) 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide radical scavenging effects [55]. Another study revealed that the ethanolic seed extract shows better DPPH and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging along with total phenolic content (TPC) than its ethyl acetate and n-hexane extracts [43]. The seed oil (0.1 mg/mL) also showed significant DPPH and ABTS radical scavenging capacity [56]. This study also determined the TPC in seed oil. The aqueous extract of this plant reduced reactive oxygen species (ROS) in human umbilical vein endothelial cells (HUVECs) [57].

Polysaccharides of fruit extract showed DPPH free radicals scavenging activity with an EC_{50} value of 0.98 mg/mL [50]. The seed oil also showed DPPH and ABTS radical scavenging capacity. However, the antioxidant activity was lower than the catechin and BHT at the same concentration (0.1 mg/mL) [44]. Petroleum ether and methanol fruit extracts increased in catalase (CAT) levels in gastric ulcer rats [58]. Hispidalin isolated from this herb also showed DPPH radical scavenging and inhibition of lipid peroxidation capacity [59]. The aqueous fruit extract significantly increased the antioxidant status as well as levels of vitamin C concentration in gastric juice or rats [60].

Antioxidant effects of various parts of *B. hispida* on various test models have been also observed by several authors [17, 28, 29, 56, 61]. Table 2 shows the antioxidant effects of various parts of *B. hispida*.

4.2. Anti-Inflammatory Effect. The methanolic seed extract (100-300 mg/kg, p.o.) showed dose-dependent anti-inflammatory effects on carrageenan-induced paw oedema rat ($n = 6$) model [55]. The fruit peel methanolic extract showed an anti-inflammatory effect on egg albumin-induced inflammation in rats [62]. The petroleum ether and methanolic fruit extract of *B. hispida* (300 mg/kg, p.o.) showed a dose-dependent anti-inflammatory effect on cotton pellet-induced granuloma models in rats, carrageenan-induced paw oedema, and histamine-induced paw oedema [58].

4.3. Antimicrobial, Antihelmintic, and Larvicidal Effects. Due to the excessive use of antibiotics that can lead to the development of antibiotic resistance of various strains of bacteria [63–65], attempts have been made to use natural antibiotic alternatives [66, 67]. Most of these options include plants with antiviral and antibacterial properties that can be

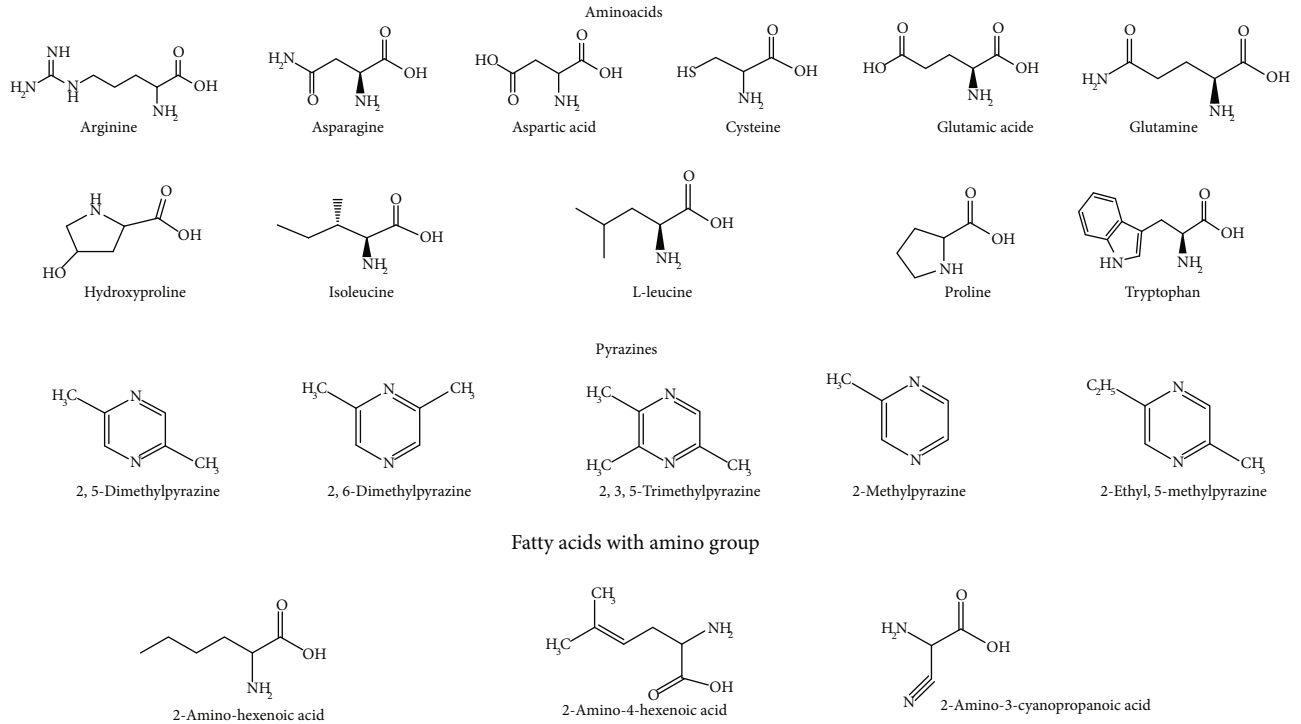

Figure 3: The chemical formulas of most important representative carbohydrates from *Benincasa hispida*.

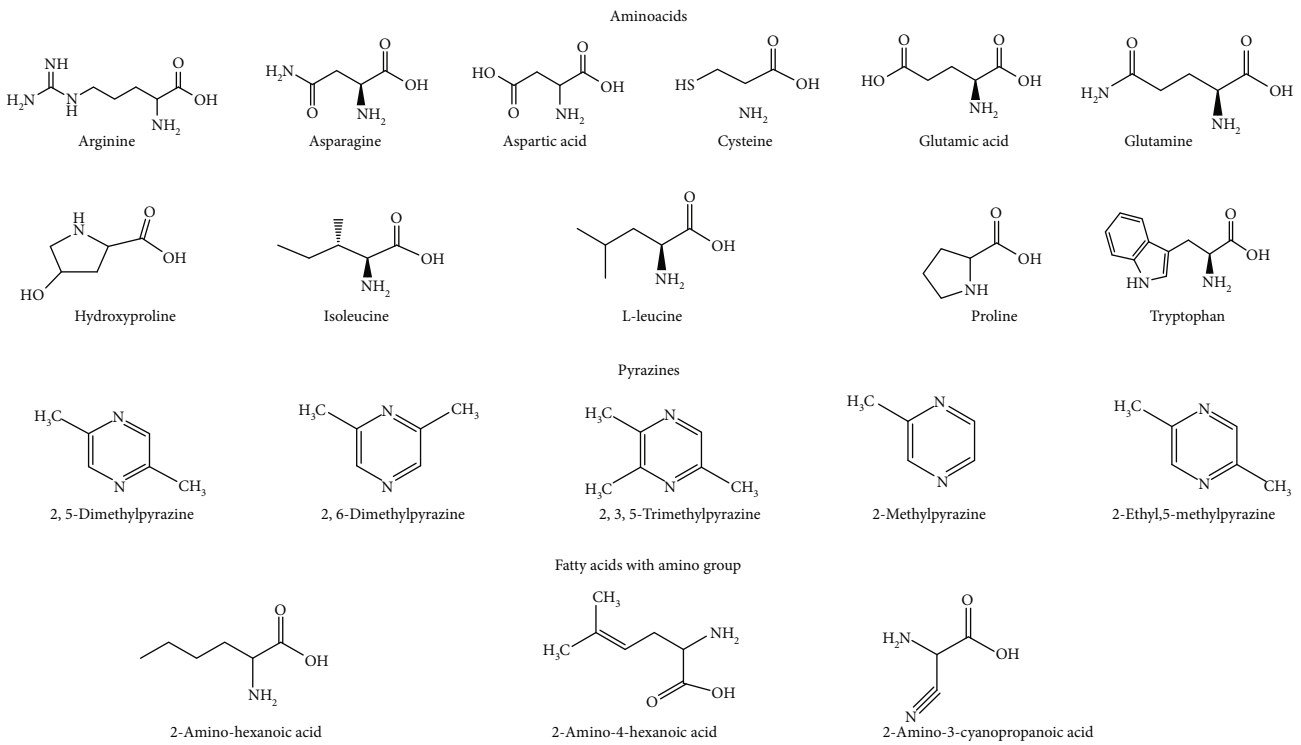

Figure 4: The chemical formulas of most important representative aminoacids, pyrazines, and fatty acids with amino group from *Benincasa hispida*.

effective against gram-negative and gram-negative germs, which are often difficult to eradicate [68, 69]. The methanolic whole plant extract (500 μg/disc) was found to act against *Pseudomonas aeruginosa and Vibrio parahaemolyticus* [70]. In the latter case, the zone of inhibition was 6 mm only. Hispidalin, an isolated compound from this herb, was found to act against several bacteria (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella*

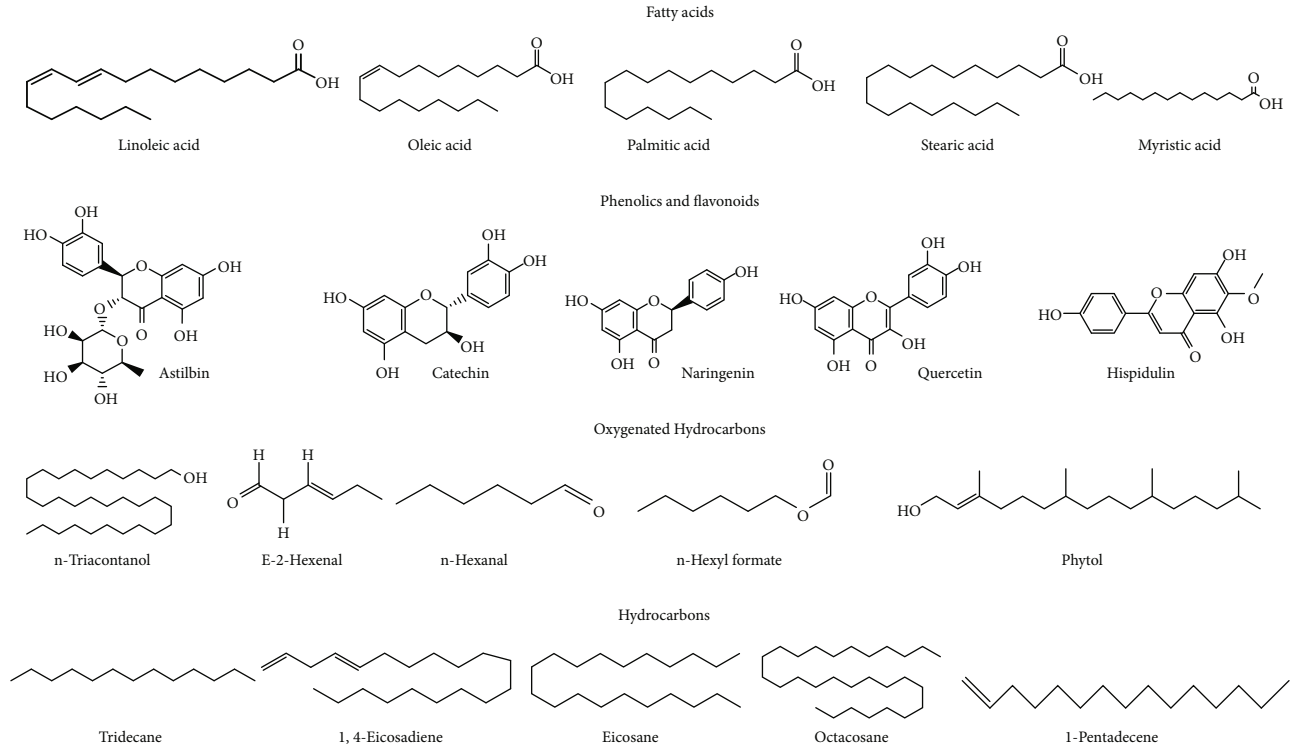

Figure 5: The chemical formulas of most important representative fatty acids, phenolic and flavomoids, oxygenated hydrocarbons, and hydrocarbons from *Benincasa hispida*.

enterica) and fungi (e.g., *Aspergillus flavus*, *Penicillium chrysogenum*, *Fusarium Solani*, and *Colletotrichum gloeosporioides*) *[59]*. In this case, the minimum inhibitory concentrations (MIC) were 30-120 and 100-200 μg/mL for bacterial and fungal strains, respectively. Moreover, the aqueous, methanol, and petroleum extracts of seeds showed significant therapeutic efficacy with methanol extract being the best comparable to the antibiotic ciprofloxacin. In other study, the aqueous peel extract showed strong antibacterial activity against *S. aureus* (MIC = 14.5 μg/mL), *Micrococcus luteus* (MIC = 8.6 μg/mL), *E. coli* (MIC = 6.1 μg/mL), and *Klebsiella pneumoniae* (MIC = 13.4 μg/mL) [11]. The herb shows prebiotic activity [71]. Table 3 shows the antimicrobial effects of various parts of *B. hispida*.

Ethanolic seed extract (20, 40 and 60 mg) showed a dose-dependent anthelmintic in anthelmintic activity on *Pheretima posthuma* [72]. The phloem lectin-like protein from the exudate of the herb exerted an inhibitory effect on the *Samia ricini* larvae [46] (Table 3).

4.4. Cytotoxic and Anticancer Effects. Cancer is a term used to define malignancies in which abnormal cells multiply in an uncontrolled and continuous manner and can invade the surrounding healthy tissues [73, 74]. Abnormal cells come from any tissue in the human body and can occur anywhere in the body [75–77]. Natural anticancer alternatives can have a direct effect on malignant cells, as well as by stimulating the body's immune capacity in the fight against the aggression of carcinogenic factors, internal or external [78, 79]. The favourable effects of some medicinal plants are due to the main biochemical components: flavonoids—-which inhibit the activity of carcinogens and prevent the metastasis of malignant cells; carotenoids—which protect the body against colon cancer; terpenes in essential oils—-block the action of carcinogens, having a strong antioxidant action; β-carotene, a powerful antioxidant with anticancer protection and a recognized inhibitor of malignant cells; antioxidant vitamins C, E, and A, destroy free radicals, prevent cancer, and block the metastasis process [80–83].

The fruit, seed, and root proteins (10-1000 μg/mL) exerted a concentration-dependent cytotoxic effect on *Artemia salina.* The median lethal concentration (LC_{50}) values of fruit, seed, and root extract were 44, 41, and 50 μg/mL, respectively [24]. In this study, the root proteins inhibited the proliferation of HeLa and K-562 cells by 28.50 and 36.60%, respectively. Another study reveals that the whole plant methanolic extract *(5-50* μg/mL) exerted a cytotoxic effect on *A. salina* (LC_{50}: 45.187 μg/mL) [70]. Moreover, the aqueous seed extract (20-800 μg/mL) did not exert cytotoxic effects on HUVECs and normal fibroblast (NIH/3T3) cells. On male C57BL/6 mice, the extract showed a potent inhibitory effect on basic fibroblast growth factor- (bFGF-) induced angiogenesis [84]. The aqueous extract (1–20 μg/mL) also reduced cell adhesion molecules activation by inhibiting monocyte adhesion, ROS, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) on high glucose (25 mM) induced HUVECs cells [57] (Figure 6).

Table 4 shows the cytotoxic and anti-cancer effects of various parts of *B. hispida*, and Figure 1 summarizes the most important anticancer mechanism.

Table 1: Chemical phytoconstituents of *Benincasa hispida* (Thunb.) Cogn.

Compounds	Plant parts	Locality/country	References
E-2-hexenal, n-hexanal and n-hexyl formate; however, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylprazine, 2-methylpyrazine, 2-ethyl-5-methylpyrazine	Fruit	Taipei, Taiwan/China	[30]
Cucumisin-like protease	Sarcocarp	Kagoshima/Japan	[32]
Triterpenes, sterols, flavonoid C-glycoside, benzyl glycoside, alnusenol, multiflorenol	Fruit	Kyoto/Japan	[33]
Osmotin-like protein	Seeds	New York/USA	[34]
Chitinase	Seeds	New York/USA	[35]
Astilbin, catechin, naringenin	Fruit	Hainan/China	[31]
Di-2-ethylhexyl phthalate	Fruit	Hainan/China	[36]
W-sitosterol, V-amyrin, quercetin	Stem	Visakhapatnam/India	[37]
β-Carotene	Fruit	Faisalabad/Pakistan	[16]
Tryptophan	Fruit	Gwalior/India	[38]
Linoleic, palmitic, oleic, and stearic acids	Fruit	Temerloh, Pahang/ Malaysia	[39]
Acetoin, octanal, nonanal	—	Mumbai/India	[40]
α-Tocopherol, δ-tocopherol, linoleic acid, β-sitosterol, campesterol, stigmasterol, Δ^5-avenasterol	Fruit	Serdang, Selangor/ Malaysia	[41]
Galactose, glucose, xylose, sorbose	Peel	Karnataka/India	[42]
Linoleic acid, linolenic acid	Seeds	Serdang, Selangor/ Malaysia	[43]
Myristic acid, palmitoleic acid, oleic acid, linoleic acid, stearic acid, α-linolenic acid, palmitic acid, other saturated and unsaturated fatty acids	Seed oil	Serdang, Selangor/ Malaysia	[44]
3α,29-O-di-trans-cinnamoyl-D:C-friedooleana-7,9(11)-diene, oleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1 $\rightarrow$ 4)]-(1 $\rightarrow$ 3)-α-L-rhamnopyranosyl (1 $\rightarrow$ 2)-α-L-arabinopyranoside, oleanolic acid 28-O-β-D-glucopyranosyl-(1 $\rightarrow$ 3)-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1 $\rightarrow$ 4)]-(1 $\rightarrow$ 3)-α-L-rhamnopyranosyl-(1 $\rightarrow$ 2)-α-L-arabinopyranoside, multiflorenol, isomultiflorenyl acetate, stigmasterol, stigmasterol 3-O-β-D-glucopyranoside, α-spinasterol, α-spinasterol 3-O-β-D-glucopyranoside, β-sitosterol, daucosterol, arbutin, nicotinic acid, (+)-pinonesinol, ethyl β-D-glucopyranoside	Fruit	Jinghong/China	[45]
Phloem lectin-like protein	Exudate	Fukuoka/Japan	[46]
Linoleic acid, palmitic acid, oleic acid, stearic acid	Seeds	Rambagh, Allahabad/ India	[47]
Gallic acid	Fruit	Kota Bharu/Malaysia	[15]
Lupeol	Seeds	Mumbai/India	[27]
Gallic acid, linoleic acid	Seeds	Serdang, Selangor/ Malaysia	[28]
β-Sitosterol	Seeds	Mumbai/India	[48]
Ascorbic acid	Fruit	Kubang Kerian, Kelantan/Malysia	[49]
β-Carotein, ascorbic acid	Peel	Mysore/India	[17]
Polysaccharides	Fruit	Guangzhou/China	[50]
Gallic acid, catechin, epicatechin, rutin, quercetin, quercetin-3-D-galactoside, trans-ferrulic acid, oleanolic acid, ursolic acid	Fruit	Buzau/Romania	[9]

4.5. Gastrointestinal Protective Effects

4.5.1. Gastroprotective Effect. Fresh juice (1-4 mL/animal, p.o.), ethanol (12, 24 and 48 mg/kg, p.o.), and pet ether extract (0.75, 1.5 and 3 mg/kg, p.o.) in swimming stress, aspirin plus restraint, serotonin-induced ulcers, and indomethacin plus histamine displayed a dose-dependent antiulcerogenic effect in rats and mice [13].

The petroleum ether and methanol fruit extracts (300 mg/kg, p.o.) significantly ($P < 0.05$) reduced ulcer index, vascular permeability, and malondialdehyde (MDA) content, while an increase in CAT levels in comparison to

Table 2: Antioxidant properties of different parts or their extracts/fractions of isolated compounds.

Extract/isolated compounds	Test system	Results	References
Crude oil from seeds	DPPH ABTS TPC	DPPH: EC_{50} = 0.1 mg/mL ABTS: EC_{50} = 0.1 mg/mL Significant antioxidant effect Standards: methyl ether, fatty acids	[56]
Seeds extract	DPPH, ABTS, total phenolic content	EC_{50} = 10 − 100 μg/mL Significant antioxidant effect Standards: methyl ether, fatty acids	
Methanolic and aqueous peel extracts	DPPH	EC_{50} = 10 − 100 μg/mL Concentration-dependent radical scavenging activity The methanolic extract exhibited a better antioxidant effect Standard: DPPH	[29]
Aqueous seeds extract	TPC, TFC DPPH, ABTS, H_2O_2, linoleic acid oxidation nitrite scavenging assay	TPC: EC_{50} = 81.3 ± 1.4 μg gallic acid/g TFC: EC_{50} = 486.8 ± 4.1 μg catechin/g dry mass DPPH: EC_{50} = 0.6 − 3 mg/mL Concentration-dependent antioxidant activity Standards: catechin 0.05-0.5 mg/mL, BHT, ascorbic acid 10 mg/mL	[61]
Seed oil	DPPH ABTS radical scavenging assay	DPPH: EC_{50} = 0.1 mg/mL The antioxidant activity of the seed oil was lower than the catechin and BHT at the same concentration. Standard: FAME	[44]
Hispidalin	DPPH Lipid peroxidation assay	DPPH: EC_{50} = 2 − 40 μg/mL EC_{50} = 40 μg/mL Significant DPPH radical scavenging and inhibition of lipid peroxidation capacity Standard: methyl ether	[59]
Methanol, ethanol, aqueous peel extracts	DPPH Reducing power assay	Significant antioxidant effect Standard: acarbose 20, 40, 60, 80, 100 μg.mL^{-1}	[17]
Seed extract	DPPH ABTS	Significant antioxidant effect Standard: FAME	[28]
Polysaccharides of fruit extract	DPPH	EC_{50} = 0.98 mg/mL Significant antioxidant effect Standard: glucose	[50]

Abbreviations: TPC: total phenolic contents; TFC: total flavonoid contents (TFC); ABTS: 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl free radical-scavenging ability; BHT: antioxidant butylated hydroxytoluene; FAME: fatty acid methyl ester; EC_{50}: the half-maximal effective concentration.

the control group in pylorus ligated (PL) gastric ulcers, ethanol-induced gastric mucosal damage, and cold restraint stress- (CRS-) induced gastric ulcer rat models [58]. The fruit extract (1 mL/kg, p.o.) also decreased ulcer index as well as MDA, superoxide dismutase (SOD), and vitamin C levels in indomethacin-induced gastric ulcer in rats [23].

The hydromethanol, ethyl acetate, and aqueous ripe fruit extracts (20 mg/kg, p.o./alternative days) were treated for 14 days in ranitidine (5 mg/kg, p.o.) induced hypochlorhydria in rats. The aqueous extract showed better effects on the test animals. It increased the antioxidant status as well as levels of pepsin, vitamin C, and gastric juice chloride concentration than the other extracts [60]. On the other hand, the extract of fruits with the whole plant of *Fumaria vaillantii* Loisel (1 : 1) (20 mg/kg, p.o.) was administrated in ranitidine (5 mg/kg) induced hypochlorhydria in rats as pre-and cotreatment manners. The extract significantly ($P < 0.05$) enhanced the concentration of pepsin, iron levels in serum, chloride level in gastric juice, and liver along with blood haemoglobin level in experimental animals [85].

A prospective pilot study on dyspeptic patients ($n = 20$) (baseline between 30 days and 45 days) aged between 18 and 45 years with only single dose of 200 mL fruit juice every morning in empty stomach for thirty days suggests that a significant improvement of pain, nausea, belching, retrosternal burning, and bowel habits among the patients [86]. Table 5 shows the gastrointestinal -protective effects of various parts of *B. hispida*.

4.5.2. Antidiarrheal Effect. Diarrhoea is a condition characterized by frequent watery stools, and usually, diarrhoea persists for a few days and is treated with diet [87]. But there are also more serious situations, in which diarrhoea requires drug/complementary treatment and is more difficult to cure [88, 89].

Table 3: Antimicrobial, anthelmintic, and larvicidal effects of different parts or their extracts/fractions or isolated compounds.

Extract/isolated compounds	Dose/concentration model (*in vitro*/*in vivo*)	Results/mechanisms	References
Antimicrobial effects			
Methanolic whole plant extract	*Pseudomonas aeruginosa* *Vibrio parahaemolyticus* *In vitro* Standard: DMSO	IC_{50} = 500 μg/disc Zone of inhibition = 6 mm	[70]
Hispidalin	*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella enterica*; Fungi: *Penicillium chrysogenum*, *Fusarium solani*, *Aspergillus flavus*, *Colletotrichum gloeosporioides* *In vitro* Standard: acetoin (0.01–20 μg/μl)	Antibacterial: MIC = 30 – 120 μg/mL, Antifungal: MIC = 100 – 200 μg/mL	[59]
Aqueous peel extract	*Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Klebsiella pneumoniae* *In vitro* Standard: DMSO 150 μL	Antibacterial: MIC = 6.1 – 14.5 μg/mL	[11]
Anthelmintic effect			
Ethanolic seed extract	*Pheretima posthuman*/*in vitro* Standard: phenytoin sodium	IC_{50} = 20, 40, and 60 mg Dose-dependent anthelmintic effect	[72]
Larvicidal effect			
Phloem lectin-like protein from the exudate	*Samia ricini* larvae/*in vitro* Standards: Precision Plus Protein™, serum albumin	↑ inhibitory activity against the larvae Dose: 70 μg/g	[46]

Abbreviations: IC_{50}: value concentration that inhibits cell growth by 50%; MIC: minimum inhibitory concentration.

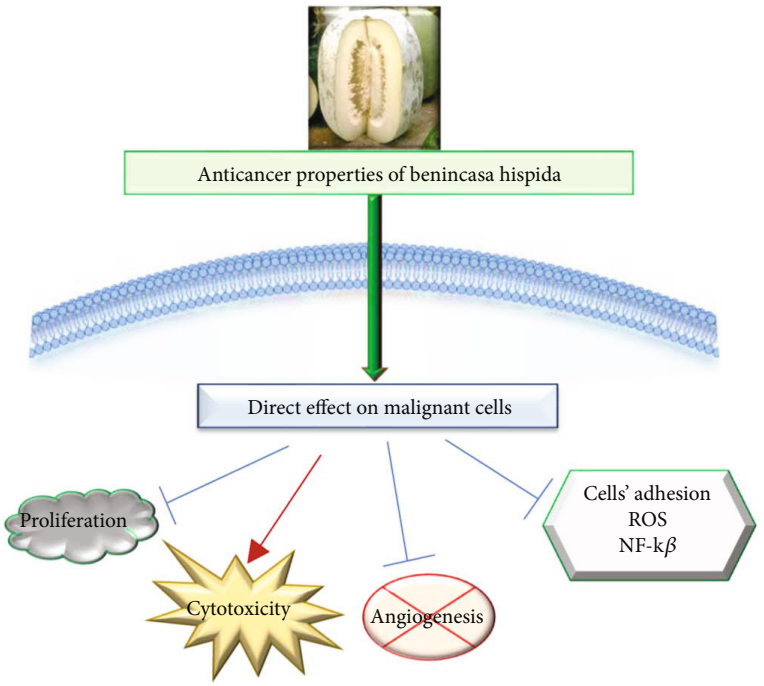

Figure 6: A schematic diagram with anticancer mechanisms of natural compounds from *Benincasa hispida*. Legend: blue arrow: inhibition, reduction; red arrow: increase, stimulation, ROS reactive oxygen species, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).

Table 4: Cytotoxic and anticancer effects of various parts of *B. hispida* extracts/fractions.

Extract/isolated compounds	Model dose/concentration	Results/mechanisms	References
Aqueous seed extract	HUVECs, NIH/3T3 cells/*in vitro* Male C57BL/6 mice/*in vivo* IC_{50} = 20 – 800 μg/mL Standard: NNGH	No cytotoxicity on HUVECs, NIH/3T3 cells decrease bFGF-induced angiogenesis in mice	[84]
Fruit, seed, root proteins	HeLa, K-562 cells/*in vitro* IC_{50} of fruit, seeds root extract = 44, 40-50 μg/mL IC_{50} = 10 – 1000 μg/mL in *Artemia salina* IC_{50} = 10 – 50 μg/mL on HeLa, K-562 cells Standards: lysozime, tyrosine, carbonic anhydrase, ovalbumin, albumin	Decrease cell proliferation by 28.50-36.80%	[24]
Aqueous extract	HUVECs cells/*in vitro* IC_{50} = 1 – 20 μg/mL on high glucose (25 mM) Standards: glucose 25 mM, glucose and ABH 5 μg/ml, 20 μg/ml	Decrease cell adhesion molecules activation, Decrease ROS, NF-κB Decrease inhibiting monocyte adhesion	[57]
Methanolic/whole plant extract	*Artemia salina*/*in vitro* IC_{50} = 45.186 μg/mL Standard: DMSO, vincristine sulphate 0.91 μg/mL	Increase cytotoxic effect concentration-dependent	[70]

Abbreviations: IC_{50}: value concentration that inhibits cell growth by 50%; bFGF: basic fibroblast growth factor; ROS: reactive oxygen species; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NNGH: *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid.

The *B. hispida* fruit methanolic extract displayed potential antidiarrheal activity on the castor oil-induced diarrheal rat model. It was also seen to inhibit induced PGE2, enter pooling, and reduce in the motility of gastro-intestine in charcoal meal rats [90]. The same extract also possessed a significant inhibitory activity against castor oil-induced diarrhoea and induced PGE2, enter pooling and gastrointestinal motility at 200, 400, and 600 mg/kg (p.o.) in castor oil, charcoal meal, and antienter pooling models in rats [90] (Table 5).

4.6. Effects on Metabolic Diseases

4.6.1. Antidiabetic Effects. The methanolic stem extract (50,100, 200 mg/kg, p.o.) dose-dependently lowered the blood glucose level in alloxan-induced diabetic rats [37]. The chloroform fruit extract (250 and 500 mg/kg, p.o.) dose-dependently ameliorated the derangements in lipid metabolism in alloxan-induced diabetic albino rats after 14 days of treatment [91]. The study reveals that the methanol, ethanol, and aqueous peel extracts showed significant α-amylase inhibition activity [17]. The ethanol and ethyl ethanoate leaf extracts lowered the blood glucose level of the diabetic mice in a dose-dependent manner [22]. Antidiabetic effects of various parts of *B. hispida* have been shown in Table 6.

4.6.2. Antiobesity and Lipid-Lowering Effect. Lipids are fatty organic substances that are the largest source of energy for the body. The vast majority of fats are stored in solid form in various organs or skin, and a small part circulates in the blood in liquid form [92, 93]. Imbalances in lipid metabolism lead to pathophysiological changes and the appearance of chronic diseases such as cardiovascular disease, fatty liver, endocrine disorders, and diabetes [94, 95]. Methanolic fruit extract (0.2-1 g/kg, i.p.) reduced food intake, suggesting anorectic activity in mice [96]. Hexane fraction from the aqueous fruit extract inhibited adipocyte differentiation by blocking leptin gene expression, peroxisome proliferator-activated receptor gamma (PPARγ), and CCAAT enhancer-binding protein alpha (C/EBPα), resulting in the reduction of lipid accumulation, increased releasing of glycerol and intracellular triglycerides in 3T3-L1 cells [97].

4.7. Neuroprotective Properties

4.7.1. Anticonvulsant Effects. The fruit methanol extract (0.2-1 g/kg, p.o.) showed a dose-dependent anticonvulsant activity in pentylenetetrazole, strychnine and picrotoxin, and maximal electro seizures model [98]. On the other hand, the fruit peel methanolic extract exerted a dose-dependent (0.25-1.5 g/kg) anticonvulsant effect on pentylenetetrazol-induced convulsion in mouse models [62]. Ethanolic seed extract (250 and 500 mg/kg, p.o.) showed a dose-dependent anticonvulsant effect in anticonvulsant activity in Swiss albino mice [72].

4.7.2. Effects on Alzheimer's Disease. Neurodegenerative diseases such as Alzheimer's disease are characterized by the presence of the central nervous system, protein aggregates, inflammation, and oxidative stress [99, 100]. Several factors are involved in triggering neurodegenerative diseases, including the lifestyle that leads to the gradual deterioration of the health of the nervous system, with serious consequences on the quality of life of the patient with such a disease [101]. Although there are still no treatment solutions to restore nerve function in neurodegenerative diseases, more and more studies insist on several natural formulas that have been shown to have the effect of reducing symptoms and improving the quality of life of patients with neurodegenerative diseases [102, 103].

Table 5: Gastrointestinal protective effects of different parts or their extracts/fractions of *B. hispida*.

Gastroprotective	Model/dose/concentration	Mechanisms	References
Antiulcer effect			
Fresh juice, petroleum ether, alcoholic/fruits extract	Aspirin plus restraint, serotonin-induced ulcers, indomethacin plus histamine Swimming stress Mice/*in vivo* Dose: 1 ml/mouse	↓ulcer index formed by several ulcerogenic	[13]
Fresh juice, ethanol, petroleum ether extracts (5% v/v)	Aspirin plus restraint, swimming stress, indomethacin plus histamine, and serotonin-induced ulcers Rats and mice/*in vivo* Fresh juice (1-4 mL/animal, p.o.), Dose: ethanol extract 12, 24, and 48 mg/kg, p.o. Dose: petroleum ether extract 0.75, 1.5, 3 mg/kg, p.o.	Dose-dependent anti-ulcerogenic effect The fresh juice treatment for 3 months did not change the indices (i.e., WBC, RBC counts HCT, HB, MCV, MCH urea, and sugar) No behavioural changes in experimental animals.	[13]
Petroleum ether, methanol/fruits extract	Pylorus ligated (PL) gastric ulcers, ethanol-induced gastric mucosal damage, cold restraint-stress- (CRS-) induced gastric ulcer Rats/*in vivo* Dose: 300 mg/kg, p.o.	↓ulcer index ↓MDA ↓vascular permeability ↑ CAT	[58]
Fruit extract	Indomethacin-induced gastric ulcer Rats/*in vivo* Dose: 1 mL/kg, p.o.	↓ulcer index, ↓MDA ↓SOD, ↓vitamin C	[23]
Hydromethanol, aqueous ripe fruit, ethyl acetate extracts	Ranitidine (5 mg/kg) induced hypochlorhydria Rats/*in vivo* Dose: 20 mg/kg, p.o./alternative days for 14 days	The aqueous extract showed better effects: ↑antioxidant status, ↑pepsin, ↑vitamin C, ↑chloride in gastric juice	[60]
Fruit extract with the whole plant of *Fumaria vaillantii* Loisel (1 : 1)	Ranitidine (5 mg/kg) induced hypochlorhydria Rats/*in vivo* Dose: 20 mg/kg, p.o.	↑iron levels in serum, ↑pepsin, ↑gastric juice chloride level and liver ↑blood haemoglobin level	[85]
Fruit juice	Prospective pilot study Dyspeptic patients ($n = 20$) (baseline between 30-45 days); age 18-45 years; 200 mL single-dose every morning in empty stomach for thirty days	↓pain ↓belching retrosternal burning ↓nausea ↓bowel habits	[86]
Antidiarrheal effect			
Methanolic fruit extract	Castor oil-induced diarrheal, PGE2-induced, enter pooling and charcoal meal models Rats/*in vivo* Dose: 200, 400, and 600 mg/kg, orally by gavage	Dose-dependent antidiarrheal effect ↓PGE2- induced, enter pooling ↓gastrointestinal motility	[90]
Methanolic fruit extract	Castor oil, charcoal meal, and antienter pooling models in rats/*in vivo* Dose: 200, 400, 600 mg/kg, p.o.	↓ activity against castor oil-induced diarrhoea; ↓PGE2 induced enter pooling ↓gastrointestinal motility	[90]

Abbreviations and symbols:↑(increased); ↓(decreased); WBC: white blood cells; RBC: red blood cells; HCT: hematocrit; HB: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin concentration; MDA: malondialdehyde; CAT: catalase; SOD: superoxide dismutase; PGE2: prostaglandin E2.

The fruit extract at a dose of 400 mg/kg (p.o.) showed a protective effect on colchicine-induced Alzheimer's disease rats, possibly through the presence of both vitamin E and β-carotene protecting rat neurons against oxidative stress. On the other hand, the aqueous fruit pulp extract (100-450 mg/kg, p.o.) dose-dependently increased SOD, CAT, and GSH, while reduced in LPO levels in the colchicine-induced Alzheimer's rat model [16].

4.7.3. Effects on Memory and Cognitive Behaviour. Cognitive disorders are characterized by changes in brain structure and function that affect learning, orientation, judgment, memory, and intellectual abilities [104–106]. The methanolic fruit extract (200, 400, or 600 mg/kg, p.o.) showed a significant dose-dependent anticompulsive effect in marble-burying and motor coordination test models in mice [38]. The petroleum ether, methanolic, and aqueous fruit extracts (100, 200, and 400 mg/kg, p.o.) showed a dose-dependent nootropic activity in the cognitive behaviour mouse model [107]. Kumar and Nirmala [108] also studied the possible nootropic effects of the fruit on experimental animals.

4.7.4. Antidepressant and Anxiolytic Effects. Anxiety is defined as a diffuse fear, without a well-defined cause regarding various events of daily life [109]. Methanolic fruit extract (50, 100, and 200 mg/kg, p.o.) showed a dose-dependent

Table 6: Other pharmacological activities of *Benincasa hispida* (Thunb.) Cogn.

Extract/isolated compounds	Model dose/concentration	Results/potential mechanisms	References
Lipid-lowering effect			
Hexane, chloroform, ethyl acetate/aqueous fruit extract	3T3-L1 cells/*in vitro*	Hexane extract: ↓adipocyte differentiation, ↓PPARγ, ↓C/EBPα, ↓leptin gene expression, ↓lipids accumulation, ↑releasing of glycerol, ↑ triglycerides	[97]
Antidiabetic effect			
Methanolic/stem extract	Alloxan-induced diabetes Rats/*in vivo* Dose: 50,100, 200 mg/kg p.o.	↓blood glucose level dose-dependent	[37]
Chloroform/fruits extract	Alloxan-induced diabetes Rats/*in vivo* Dose: 250, 500 mg/kg p.o.	Dose-dependently ameliorated the disorders in the metabolism of lipids in diabetic mice	[91]
Ethanol, hexane, ethyl ethanoate/leaf extract	STZ-induced diabetes Mice/*in vivo* Dose: 0.2-1 g/kg, i.p	Ethanol, ethyl ethanoate extracts: ↓blood glucose level	[22]
Antiobesity effect			
Methanolic fruit extract	Mice/*in vivo* Dose: 0.2-1 g/kg, i.p.	Anorexic activity ↓food intake	[96]
Antiageing of skin			
Petroleum ether, chloroform, ethyl acetate, methanol/dried fruit pulp extract	Stratum corneum of human skin and dansyl chloride fluorescence models *In vitro*	Cream prepared from the fruit extract showed the significant antiageing effect	[12]
Effects on other diseases			
Fruit methanol extract	Antigen-antibody induced reaction in rats exudate cells/*in vitro*	↓histamine release, anti-inflammatory effect Triterpenes, sterols, multiflorenol, alnusenol exerted better inhibitory effects	[33]
Methanolic fruits extract	Histamine and acetylcholine-induced bronchospasm Guinea pigs/*in vivo* Dose: 50, 200, 400 mg/kg, p.o.	Bronchodilator effect: dose-dependent protection against histamine and acetylcholine-induced bronchospasm	[115]
Petroleum ether, methanolic/fruits extract	Histamine stimulated paw oedema carrageenan- stimulated paw oedema cotton pellet stimulated granuloma Rats/*in vivo* Dose: 300 mg/kg, p.o.	↓histamine release Anti-inflammatory effect	[58]
Juice	Isolated rat aortic ring/*in vitro* Cultured porcine endothelial cells/*in vitro* Rats/*in vivo* Dose: 0.4–1.6 mL/kg, i.v.	Antihypertensive effect dose-dependent ↓blood pressure ↑relaxation, ↓contraction of isolated rat aortic ring ↑NO in cultured porcine aortic endothelial cells	[116]
Methanolic fruit extract	Renal ischemia/reperfusion injury model Rats/*in vivo* Dose: 500 mg/kg/day, p.o. for 5 days	Nephroprotective ↓MDA, ↑SOD, CAT, ↑GSH	[117]
Ethanolic seeds extract	Ethylene glycol induced chronic Hyperoxaluria model Rats/*in vivo* Dose:250, 500 mg/kg, p.o. for 35 days	Nephroprotective ↓ urinary oxalate, ↓endogenous oxalate synthesis; ↓urinary protein excretion, ↓kidney oxalate and calcium; ↓elevated serum levels of sodium, creatinine, calcium, phosphorus	[118]
Neuroprotective effects			
Fruit juice	Morphine addiction model Mice/*in vivo* Dose: 1 mL/mouse, p.o.	The development of morphine addiction prevented along with the suppression of opioid withdrawal symptoms	[113]

Table 6: Continued.

Extract/isolated compounds	Model dose/concentration	Results/potential mechanisms	References
Methanolic fruit extract	Spontaneous motor, muscle relaxant, antihistaminic effect and barbiturate induced hypnosis models Mice, rats, and guinea pigs/*in vivo* Dose: 200-3000 mg/kg, p.o.	↑ barbiturate induced hypnosis ↑ antihistaminic activity	[114]
Fruit methanol extract	Pentylenetetrazole, strychnine, picrotoxin, and maximal electro seizures model Rats/*in vivo* Dose: 0.2-1 g/kg, p.o.	Dose-dependent anticonvulsant activity	[98]
Methanolic fruit extract	Acetic acid-induced writhing and hot plate Model Mice/*in vivo* Dose: 200, 400, 600 mg/kg, p.o.	Dose-dependent analgesic effect	[111]
Aqueous pulp extract	Colchicine-induced Alzheimer's model Rats/*in vivo* Dose: 100-450 mg/kg, p.o.	↑SOD, ↑CAT, ↑GSH, ↓LPO dose-dependent	[16]
Ethanolic seed extract	Rats/*in vivo* Dose: 250, 500 mg/kg, p.o.	Dose-dependent analgesic and antipyretic effects	[26]
Methanolic fruit extract	Marble-burying and motor coordination tests Mice/*in vivo* Dose: 200, 400, 600 mg/kg, p.o.	Significant dose-dependent anticompulsive effect	[38]
Methanolic leaf extract	Acetic acid-induced writhing Mice/*in vivo* Dose: 50, 100, 200, 400 mg/kg, p.o.	Dose-dependent analgesic effect	[112]
Fruit peel methanolic extract	Egg albumin-induced inflammation in rats; acetic acid-induced writhing, formalin-induced pain, hot plate-induced, and pentylenetetrazol-induced convulsions Mice/*in vivo* Dose: 50, 100, 200, 400 mg/kg, p.o.	Dose-dependently (0.25-1.5 g/kg) inhibited acetic acid-induced writhing, formalin-induced pain licking, and hot plate-induced pain in mice. Significantly inhibition of egg albumin-induced inflammation in rats and pentylenetetrazol-induced convulsion in mice	[62]
Ethanolic seed extract	Anticonvulsant activity Mice/*in vivo* Dose: 250, 500 mg/kg, p.o.	Dose-dependent anticonvulsant effects	[72]
Methanolic fruit extract	TST and FST model Mice/*in vivo* Dose: 50, 100, 200 mg/kg, p.o.	Dose-dependent antidepressant effect possibly through GABAergic involvement.	[110]
Petroleum ether, methanolic, aqueous/fruit extracts	Motor coordination, locomotor, cognitive behaviour, anxiolytic, haloperidol-induced catalepsy, and anticonvulsant models Mice/*in vivo* Dose: 100, 200, 400 mg/kg, p.o.	Dose-dependent anxiolytic, analgesic, and nootropic activity	[107]

antidepressant-like effect in TST and FST models possibly through GABAergic involvement in mice in Swiss mice [110].

Petroleum ether, methanolic, and aqueous fruit extracts (100, 200, and 400 mg/kg, p.o.) confirmed a dose-dependent anxiolytic activity in mice [107]. Effects of various parts of *B. hispida* on the nervous system have been shown in Table 6.

4.8. Analgesic and Antipyretic Effects. The methanolic fruit extract (200, 400, and 600 mg/kg, p.o.) showed a dose-dependent analgesic effect in acetic acid-induced writhing and hot plate model in mice [111]. The ethanolic seed extract (250 and 500 mg/kg, p.o.) exerted a dose-dependent analgesic effect in rats [26]. The fruit peel methanolic extract also dose-dependently (0.25-1.5 g/kg) inhibited acetic acid-induced writhing, formalin-induced pain licking, and hot plate-induced pain in mice [62].

In another study, the methanolic seed extract (100-300 mg/kg, p.o.) also showed a dose-dependent analgesic effect on the rats ($n = 6$) model [55]. The methanolic leaf extract (50-400 mg/kg, p.o.) exerted a dose-dependent analgesic effect in an acetic acid-induced writhing mouse model [112]. Petroleum ether, methanolic, and aqueous fruit extracts (100, 200 and 400 mg/kg, p.o.) showed a dose-

dependent analgesic effect in the mouse model [107]. The fruit juice (1 mL, p.o.) prevents morphine addiction development along with the suppression of opioid withdrawal symptoms [113]. In experimental animals such as rats, mice, and guinea pigs, the methanolic fruit extract (200-3000 mg/kg, p.o.) significantly potentiated the barbiturate stimulated hypnosis [114].

Qadrie et al. [26] reported that the ethanolic seed extract (250 and 500 mg/kg, p.o.) displayed a dose-dependent antipyretic effect in rats.

4.9. Other Potential Biological Activities

4.9.1. Bronchodilatator Effect. Fruit methanol extract inhibited histamine release. In this study, two triterpenes, the triterpenes and sterols, multiflorenol and alnusenol exerted better inhibitory effects [33]. The methanolic extract (50, 200, and 400 mg/kg, p.o.) of *B. hispida* exhibited significant protection in guinea pigs against the histamine and acetylcholine-induced bronchospasm [115]. The methanolic fruit extract (200-3000 mg/kg, p.o.) showed significant antihistaminic activity on experimental animals (e.g., rats, mice, and guinea pigs) [114].

4.9.2. Antihypertensive Effect. The ACE inhibitory effect of the plant may show the pharmacological basis in the treatment of high blood pressure for its long time uses in traditional Chinese medicine. The fruit juice (0.4 – 1.6 mL/kg, i.v.) dose-dependently lowered blood pressure, concentration-dependently showed relaxation of isolated rat aortic rings and produced nitric oxide (NO) from the cultured porcine aortic endothelial cells [116]. Polysaccharides of fruit extract showed an antiglycation effect [50].

4.9.3. Nephroprotective Effects. Methanolic fruit extract (500 mg/kg/day, p.o.) for five days reduced the MDA content, while the increase in SOD, CAT, and GSH levels in renal ischemia/reperfusion injury in female Wistar albino rats [117]. The seed ethanolic extract (250 and 500 mg/kg, p.o.) for 35 days significantly lowered the increased urinary oxalate, presenting a regulatory action on endogenous oxalate synthesis; decreased in the urinary excretion and kidney retention levels of protein, oxalate, and calcium; and reduced the increased serum levels of sodium, calcium, phosphorus, and creatinine levels in ethylene glycol induced chronic hyperoxaluria in Wistar albino rat [118].

Effects of various parts of *B. hispida* on the kidney have been shown in Table 6.

4.9.4. Antiageing of Skin. A cream prepared from the dried fruit pulp extract (petroleum ether, chloroform, ethyl acetate, and methanol) showed a significant antiageing effect on the stratum corneum of human skin and dansyl chloride fluorescence models [12].

5. Toxicological Profile: Safety and Adverse Effects

The fresh juice (5% *v*/*v*) treatment for 3 months did not change the total white blood cells (WBC), red blood cells (RBC), haemoglobin (HB), mean corpuscular haemoglobin (MCH), hematocrit (HCT), mean corpuscular volume (MCV), sugar, and urea levels in rats and mice. The treatment also caused no behavioural changes in experimental animals [13]. The methanolic extract of fruit was nontoxic and did not cause the death of mice, rats, and guinea pigs in doses up to 3.0 g/kg [114]. Other studies, performed in female and male rats, concluded that the standardized hydroalcoholic (70% ethanol) extract of the fruit pulp of *B. hispida* administered orally was relatively safe when to female and male rats [119]. Up to oral dose (1000 mg/kg body weight/day) level, no-observe-adverse-effect-level (NOAEL) was obtained for the extract in the 90-days toxicity study. The ethanolic seed extract up to 5000 mg/kg (p.o.) did not exert toxicity in rats [26]. Di-2-Ethylhexyl phthalate (18.3-75.5 mg/kg), isolated from the fruit of this herb, is a popularly used plasticizer and is harmful to human health [36].

6. Conclusions and Future Perspectives

Benincasa hispida (Cucurbitaceae) is an annual plant, originating in Indonesia. The Chinese have been cultivating it for over 2000 years; its medicinal uses first appeared in the medical field of the Tang Dynasty. In Chinese medicine, the crust is used to treat urinary dysfunction, and the fruits are used to treat fever. In Ayurveda, the fruits are also used to treat epilepsy, lung diseases, asthma, cough, and urinary retention. Starting from these traditional uses, the present paper evaluated the latest in vivo and in vitro pharmacological studies that demonstrated the molecular mechanisms which confirmed ethnopharmacological uses. However, a limiting aspect of this paper is the lack of clinical trials in human subjects. In the future, they are needed to complete the pharmacological properties and to pave the way for new pharmaceutical forms based on natural compounds with proven therapeutic effects. Improvements in control standards are also needed for future pharmacological studies that include *B. hispida*. In our work, they are relative, phytochemical compounds being identified only by high-performance liquid chromatography (HPLC). Another limiting aspect is represented by the antioxidant action of this plant which has been researched only in vitro, which does not guarantee the same effect on in vivo experimental models. Also, in future studies, the bioavailability, pharmacokinetics, mechanism of action, and study of the activity relationship of the identified and isolated pure phytochemicals should be analyzed, to better understand the reported biological actions.

Although experimental toxicological studies in animals have not shown any adverse effects, no human clinical trials have been performed to demonstrate pharmacological properties or to systematically assess toxicity and safety in humans. These studies are very important for the evaluation of short- and long-term toxicity as well as clinical therapeutic efficacy. However, the results of the present study support the clinical use of *B. hispida* in modern medicine and can serve as a basis for further studies based on this plant.

Abbreviations

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ALP: Alkaline phosphatase
bFGF: Basic fibroblast growth factor
CAT: Catalase
C/EBPα: CCAAT enhancer-binding protein alpha
DPPH: 2,2-diphenyl-1-picrylhydrazyl
HPLC: High-performance liquid chromatography
HUVECs: Human umbilical vein endothelial cells
GSH: Reduced glutathione
ICAM-1: Intercellular adhesion molecule
IL-6: Interleukin 6
LC_{50}: Median lethal concentration
LPO: Lipid peroxidation
MCV: Mean corpuscular volume
MDA: Malondialdehyde
NIH/3 T3: Normal fibroblast cells
NO: Nitric oxide
PGE2: Prostaglandin E2
PPARγ: Peroxisome proliferator-activated receptor-gamma
ROS: Reactive oxygen species
SGOT: Serum glutamic oxaloacetic transaminase
SGPT: Serum glutamic pyruvic transaminase
SOD: Superoxidase dismutase
STZ: Streptozotocin
TPC: Total phenolic content
TNF-α: Tumour necrosis factor-alpha.

Data Availability

The data supporting this review are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Antiosteoporosis Effect and Possible Mechanisms of the Ingredients of Fructus Psoraleae in Animal Models of Osteoporosis: A Preclinical Systematic Review and Meta-Analysis

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Objective. *Fructus Psoraleae* (*FP*) and its ingredients (IFP) have a variety of biological activities and are widely used to treat osteoporosis (OP). Herein, we conducted a systematic review to evaluate the efficacy of IFP for an animal model of OP from the current literatures. Potential mechanisms of IFP in the treatment of OP were also summarized. *Materials and Methods*. We carried out a search for electronic literature in the PubMed, Chinese National Knowledge Infrastructure, EMBASE, Wanfang, Web of Science, Chinese Biomedical Literature Database, and Cochrane Library, as well as Chinese VIP databases targeting articles published from inception to June 2021. The inclusion criteria were animal studies that assessed the efficacy and safety of IFP for OP, regardless of publication status or language. The exclusion criteria included (1) other types of studies (in vitro studies, case reports, clinical trials, reviews, abstracts, comments, and editorials), (2) combination with other compounds, (3) compared with other traditional Chinese medicine, (4) not osteoporosis or bone loss model, (5) studies with insufficient data, (6) lack of a control group, and (7) duplicate publications. The modified Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Stroke (CAMARADES) 10-item quality checklist was used to evaluate the risk of bias of included studies. We computed the relative risk (RR) and the standard mean difference (SMD) for dichotomous outcomes and continuous outcomes, respectively. When heterogeneity was detected or there was significant statistical heterogeneity ($P < 0.05$ or $I^2 > 50\%$), a random-effects model was employed, followed by further subgroup analysis and metaregression estimations to ascertain the origins of heterogeneity. Otherwise, we used a fixed-effects model ($P \geq 0.05$ or $I^2 \leq 50\%$). The primary outcome measures were bone mineral density (BMD), serum osteocalcin(S-OCN), bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), bone maximum load, and elasticity modulus. The secondary outcome measure was the antiosteoporosis mechanisms of IFP. The STATA 12.0 software was used to analyze the data. *Results*. Overall, 16 studies focusing on 379 animals were enrolled into the study. The risk of bias score of included studies ranged from 4 to 7 with an average score of 5.25. The present study provided the preliminary preclinical evidence that administration of IFP could significantly increase the S-OCN, BMD, BV/TV, and Tb.N while Tb.Th and Tb.Sp were remarkably decreased by IFP in OP model animals ($P < 0.05$). Moreover, IFP could significantly improve the bone biomechanical indicator bone maximum load and elasticity modulus ($P < 0.05$). In terms of the possible mechanisms of treatment of OP, IFP exerts anti-OP effects in animal models probably through osteoprotegerin/receptor activator of the nuclear factor-κB ligand/receptor activator of nuclear factor-κB (OPG/RANKL/RANK), peroxisome proliferator activated receptor γ (PPAR-γ)/Axin2/Wnt, antioxidative stress via forkhead box O3a (FoxO3a)/Axin2/Wnt, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR), estrogen-like effect, and gamma-aminobutyric acid/gamma-aminobutyric acid receptor (GABA/$GABA_BRI$) signaling pathway. *Conclusion*. Taken together, the findings suggest the possibility of developing IFP as a drug or an ingredient in diet for the clinical treatment of OP. We recommend that rigorous, as well as high-quality, trials involving large sample sizes should be conducted to confirm our findings.

1. Introduction

Osteoporosis (OP), is a systemic skeletal disease characterized by loss of bone mass and bone microarchitectural deterioration, resulting in increased bone fragility and a greater risk of fractures, especially in the spine, hip, and wrist [1, 2]. Many risk factors are associated with OP, including age, race, smoking, alcohol, low physical activity, hormone-related factors, drugs such as glucocorticoids, low calcium, and vitamin D levels [3]. Reports have showed that about 9.9 million Americans suffer from OP, with an additional 43.1 million experiencing low bone mineral density [4]. In China, the prevalence of OP was 14.94% before 2008 and increased to 27.96% from 2012 to 2015, with the rate being higher in females relative to males [5]. Approximately, in the United States, 16% of men and 29.9% of women aged more than 50 years have OP on the basis of the diagnostic criteria of the National Bone Health Alliance [6]. With the aggravation of global population aging, OP has become a serious global health problem. OP has been attributed to decreased quality of life and increased risks of death along with an elevated burden on health systems economically [7]. Thus, the management of patients with OP is extremely urgent.

Up to now, calcium and vitamin D supplementation are the standard choices for OP treatment [8]. Pharmacological therapies, including bisphosphonates, denosumab, and teriparatide, are also recommended to reduce the risk of vertebral or hip fractures in patients with OP [9]. Estrogen therapy, menopausal estrogen plus progestogen therapy, or raloxifene is suitable for postmenopausal women [10]. However, despite the availability of numerous anti-OP medications with diverse pharmacological properties, as well as fixed-dose combination therapy, the targeted therapeutic effect is not attained in significant numbers of individuals with OP, and the mitigation of OP fracture has remained suboptimal [11, 12]. Therefore, finding a drug that is effective and safe for osteoporosis is an important challenge for the industry.

Recently, the growing utilization of complementary and alternative medicine consists of herbal medicine in research and in clinical practice medicinal: they usually have few side effects and are easily accessible [13, 14]. Herbal medicines are typically used as a complementary and adjunct therapy for a wide range of diseases such as OP [15]. *Fructus Psoraleae* (FP) is the dried matured fruits of *Psoralea corylifolia* Linn, which has a long history and a wide range of applications in Asia, particularly in China, Japan, and Korea for their effects on OP and bone fracture [16]. Studies have shown that FP has the effects of dilating coronary artery, increasing coronary blood flow, antitumor, protecting liver, estrogen-like, and anti-OP effects [17]. The ingredients of FP (IFP) are shown in Figure 1, which mainly contains furanocoumarins (mainly psoralen and isopsoralen), coumestrol (such as psoralidin), flavonoids (mainly corylifolin, corylifolinin, corylin, and bavachalcone), and phenolic terpenoids (such as bakuchiol). Many IFP have estrogen-like effects [18]. Recent reports have shown that phytoestrogens have anti-OP effects similar to natural estrogen with less estrogen-like side effects [19]. Several studies have demonstrated that multiple IFP may possess anti-OP effects both *in vivo* and *vitro* [20, 21]. Corylifolin could prevent estrogen deficiency-induced bone loss in ovariectomized rats and induced primary human osteoblast differentiation [20]. Psoralen and bakuchiol improved osteoclast differentiation and bone resorption via inhibiting the protein kinase B (AKT) and activator protein-1 (AP-1) pathway [21]. However, the scattered evidence and uncertain mechanisms limited the application of FP and IFP in the clinic. Systematic review and meta-analysis of animal studies are considered to be a valuable tool to provide important insights into the validity of animal studies, improve the precision of estimated effects, and support further generalization to human clinical trials [22]. We speculated that IFP could exert anti-OP effects in animal models of OP. However, it is difficult to translate these beneficial effects of IFP from basic research to clinical application. Besides, the uncertain mechanisms and adverse drug reaction of IFP should also be taken into consideration, which were also the uncertainties and conflicts that underlie the hypotheticals. The evidence of studies was beneficial to find out a potential medication to prevent OP for healthy individuals and to treat OP for OP patients with less adverse drug reaction. Now clinically, FP has not been used to treat OP and there is a lack of clinical evidence, which is the focused clinical question we review and address in our study. Our study might provide a theoretical basis for the application of FP in OP. Therefore, we presented a systematic review and meta-analysis from the preclinical evidence of IFP in animal models of OP to summarize the significant outcomes on efficacy and mechanisms.

2. Methods

We used the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement (Table 1) to perform a systematic review and meta-analysis [23]. There are no protocols preregistered for this review.

2.1. Database and Search Strategies. Electronic searches were performed in eight databases from their respective inception to June 2021: PubMed, EMBASE, Web of Science, Cochrane Library, Chinese National Knowledge Infrastructure, Chinese Biomedical Literature Database, Chinese VIP Database, and Wanfang Database. No language restrictions were applied. The following search terms were used in PubMed and were modified to suit other databases: "Fructus Psoraleae extract" AND "Osteoporosis." In addition, reference lists from the resulting publications and reviews were also searched carefully for the eligible studies.

2.2. Eligibility Criteria. The studies were included according to the following PICOS criteria.

2.2.1. Types of Participants. We included controlled studies assessing the administration of IFP for OP animal models established by different methods, regardless of animal species, age, weight, and gender.

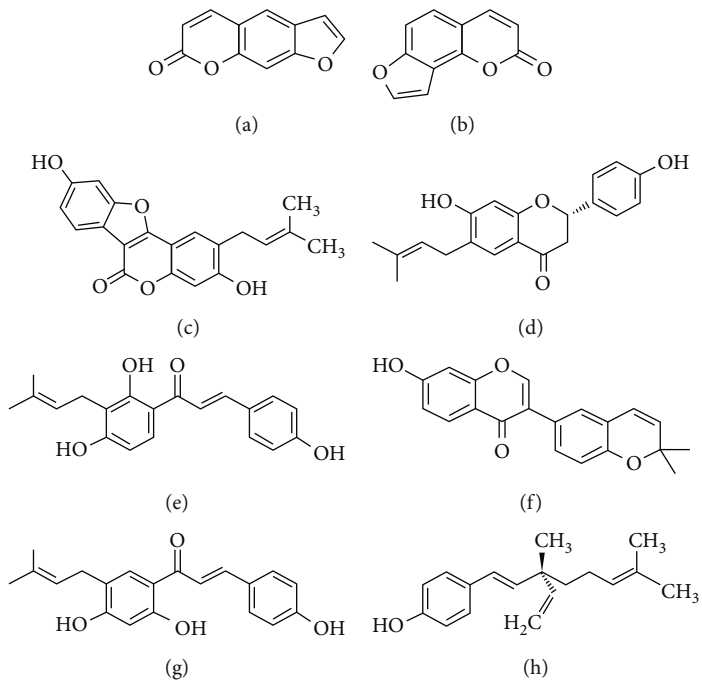


Figure 1: The chemical structure of IFP: (a) psoralen; (b) isopsoralen; (c) psoralidin; (d) corylifolin; (e) corylifolinin; (f) corylin; (g) bavachalcone; (h) bakuchiol.

2.2.2. Types of Interventions. The treatment group received IFP as monotherapy, regardless of dosage, medicament type, route of administration, and time for the medicine application.

2.2.3. Types of Controls. Blank treatment or isometric placebo was received in the control group.

2.2.4. Types of Outcome Measures. The primary outcome measures were the following: (1) bone mineral density (BMD, including BMD-lumbar spine and BMD-femur), (2) serum osteocalcin(S-OCN), (3) bone volume over total volume (BV/TV), (4) trabecular number (Tb.N), (5) trabecular thickness (Tb.Th), (6) trabecular separation (Tb.Sp), (7) bone maximum load, and (8) elasticity modulus. The secondary outcome measure was the antiosteoporosis mechanisms of IFP.

2.2.5. Types of Studies. Only animal studies that assessed the efficacy and safety of IFP for OP were included, regardless of publication status or language.

2.3. Exclusion Criteria. Exclusion criteria are as follows: (1) other types of studies (in vitro studies, case reports, clinical trials, reviews, abstracts, comments, and editorials), (2) combination with other compounds, (3) compared with other traditional Chinese medicines, (4) not osteoporosis or bone loss model, (5) studies with insufficient data, (6) lack of control group, and (7) duplicate publications.

2.4. Selection of Literature. We used the PRISMA flow diagram to select the included studies. The results of literature search were imported into the software Endnote X7. Two authors independently assessed the potentially eligible studies. Firstly, the titles and abstracts were screened to exclude the duplicated and apparently irrelevant ones or those that do not meet our inclusion criteria. After that, the remaining potential studies were full-text downloaded and reviewed. Any disagreement between the two above authors was sent and discussed with the third independent author.

2.5. Data Extraction. Two reviewers independently extracted data, and the third reviewer checked the consistency between them. A standard form was used; the extracted items included the general study information: the author's name(s), publishing date, animal species, age, gender, weight, sample size, OP modeling method, the use of anesthetics in the course of the experiment, the therapeutic regimen of the treatment and control groups, and primary and secondary outcomes and its intergroup differences. For continuous outcomes, we extracted the mean, standard deviation (SD), and participant number. The data in other forms was recalculated when possible to enable pooled analysis. If the study was involved in multiple intervention groups, we extracted data only for the group(s) involving IFP and the control group(s). Disagreements between two researchers were resolved by discussion. Whenever necessary, we contacted the authors of the studies for the missing data and additional information.

2.6. Quality Assessment of Included Studies. Two authors independently performed methodological quality and risk of bias assessment of the included studies using the CAMARADES 10-item quality checklist with minor modification [24]. The modification is listed as follows: D: blinded induction of model (group randomly after modeling), F: use of anesthetic without significant protective and toxic effects

Table 1: PRISMA 2020 checklist.

Section and topic	Item #	Checklist item	Location where item is reported
Title			
Title	1	Identify the report as a systematic review	Page 1
Abstract			
Abstract	2	See the PRISMA 2020 for abstract checklist	Page 2
Introduction			
Rationale	3	Describe the rationale for the review in the context of existing knowledge	Page 4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses	Page 6
Methods			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses	Page 7
Information sources	6	Specify all databases, registers, websites, organisations, reference lists, and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted	Page 7
Search strategy	7	Present the full search strategies for all databases, registers, and websites, including any filters and limits used	Page 7
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and, if applicable, details of automation tools used in the process	Page 8
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and, if applicable, details of automation tools used in the process	Page 9
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g., for all measures, time points, analyses), and if not, the methods used to decide which results to collect	Page 9
	10b	List and define all other variables for which data were sought (e.g., participant and intervention characteristics, and funding sources). Describe any assumptions made about any missing or unclear information	Page 9
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and, if applicable, details of automation tools used in the process	Page 9
Effect measures	12	Specify for each outcome the effect measure(s) (e.g., risk ratio, mean difference) used in the synthesis or presentation of results	Page 10
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g., tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5))	Page 10
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics or data conversions	Page 10
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses	Page 10
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used	Page 10
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g., subgroup analysis, meta-regression)	Page 10
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results	Page 10

Table 1: Continued.

Section and topic	Item #	Checklist item	Location where item is reported
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases)	Page 10
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome	Page 10
Results			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram	Page 10
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded	Page 10
Study characteristics	17	Cite each included study and present its characteristics	Page 11
Risk of bias in studies	18	Present assessments of risk of bias for each included study	Page 12
Results of individual studies	19	For all outcomes, present, for each study, the following: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g., confidence/credible interval), ideally using structured tables or plots	Table1
Results of syntheses	20a	For each synthesis, briefly summarize the characteristics and risk of bias among contributing studies	Pages 13-15
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g., confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect	Pages 13-15
	20c	Present results of all investigations of possible causes of heterogeneity among study results	Pages 15-17
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results	Page 17
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed	Page 17
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed	Page 17
Discussion			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence	Page 18
	23b	Discuss any limitations of the evidence included in the review	Page 19
	23c	Discuss any limitations of the review processes used	Page 19
	23d	Discuss implications of the results for practice, policy, and future research	Pages 20-22
Other information			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered	Page 7
	24b	Indicate where the review protocol can be accessed or state that a protocol was not prepared	Page 7
	24c	Describe and explain any amendments to information provided at registration or in the protocol	None
Support	25	Describe sources of financial or nonfinancial support for the review and the role of the funders or sponsors in the review	Page 25
Competing interests	26	Declare any competing interests of review authors	Page 25
Availability of data, code, and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review	Page 24

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372: n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/.

on bones, and G: appropriate animal model with complications or risk factors (including age, hyperlipidemia, diabetes, or hypertensive).

2.7. Quality Assessment of Evidence. The assessment of evidence quality was evaluated according to the GRADE criteria [25, 26]. The evidence quality of included outcomes

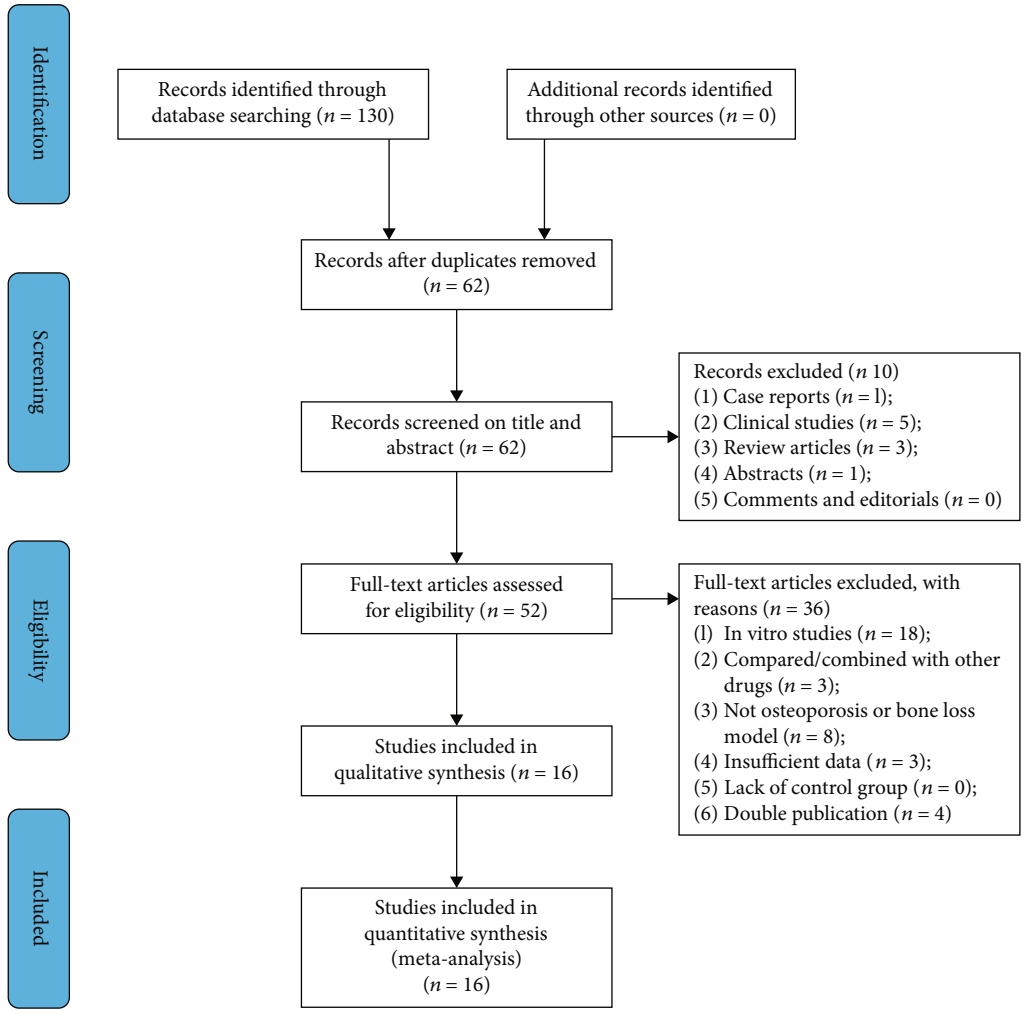


Figure 2: Flowchart of study selection.

was graded as high, moderate, low, or very low. RCTs were initially classified as having high-quality evidence. The quality of each outcome was downgraded for the following five factors: risk of bias, inconsistency, indirectness, imprecision, and publication bias. GRADE pro3.6.1 software was used for the data analysis and synthesis.

2.8. Statistical Analysis. The data was collected and input into the STATA software (version 12.0; StataCorp, College Station, TX) for meta-analysis. A random-effects model was applied when heterogeneity was detected or the statistical heterogeneity was high ($P < 0.05$ or $I^2 > 50\%$), and then, further subgroup study and metaregression analysis (the number of included studies was more than ten) were performed to detect the origin of heterogeneity. Otherwise, a fixed-effects model was used ($P \geq 0.05$ or $I^2 \leq 50\%$). To test the strength and stability of the pooled results, we performed a sensitivity analysis by omitting the individual studies one by one. Moreover, the effect of publication bias was investigated by Egger's test. Standard mean difference (SMD) was calculated for continuous outcomes.

3. Results

3.1. Study Selection. We identified 130 potentially relevant hints from eight databases. Removing duplication of literature, there were 62 articles left. We excluded 10 studies that are not related to this study after reading the titles and abstracts in detail. Through reading the full text of 52 articles, 36 articles were excluded for at least one of the exclusion criteria. Finally, 16 articles [17, 27–41] were included for analysis. Figure 2 presents the detailed flow diagram of the search and selection process.

3.2. General Characteristics of the Included Studies. The characteristics of all the 16 included studies are summarized and shown in Table 2. All of them were published between 2008 and 2021. All studies involved a total of 379 subjects with 191 in the experimental group vs. 188 in the control group. As for animal species, Sprague-Dawley rats were used in ten studies [27–29, 31, 32, 34, 37–39, 41], Wistar rats in three [17, 30, 40], C57BL/6 mice in two [35, 36], and ICR mice in one [33]. The weight of rats varied between 160 g and 290 g, while the weight of mice varied between 16.6 g

Table 2: Characteristics of the included studies.

Study (year)	Species (sex, n = experimental/control group, age)	Weight	Model (method)	Anesthetic	Experimental group	Control group	Outcome index	Intergroup differences
Chai et al. (2018) [21]	Female SD rats (20/20, 6 months old)	NG	By intramuscular injection of DXM (2.5 mg/kg, biw) for 12 weeks	Chloral hydrate	By oral gavage of ISO (25 mg/kg/d, qd) after modeling lasted 12 weeks	By oral gavage of isometric NS after modeling lasted 12 weeks	(1) BMD (F-BMD; L-BMD; P-BMD; total-BMD) (2) Tb.N, BV/TV (3) Serum ALP, TRACP, Ca, P (4) PPAR-γ, Axin2, β-catenin protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$
van Luijk et al. (2013) [22]	Female SD rats (9/9, 7 weeks old)	180-220 g	By oral gavage of tretinoin (70 mg/kg/d, qd) for 2 weeks	NG	By oral gavage of ISO (100 mg/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD; L-BMD) (2) Serum Ca (3) Serum P (4) OCN (5) Runx2, MMP 13 mRNA, and protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P > 0.05$ (4) $P < 0.05$ (5) $P < 0.05$
Moher et al. (2009) [23]	Female SD rats (10/10, NG)	200-280 g	Bilateral oophorectomy was performed on rats	Chloral hydrate	By oral gavage of PSO (88 mg/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD; L-BMD) (2) Serum Ca, PINP, OCN (3) Serum BMP2, VEGF (4) PI3K, AKT, mTOR mRNA, and protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$
Macleod et al. (2004) [24]	Female and male Wistar rats (12/12, NG)	160-200 g	By intramuscular injection of DXM (2.5 mg/kg, biw) for 12 weeks	NG	By oral gavage of PSO (126 mg/kg/d, qd) after modeling and lasted 9 weeks	By oral gavage of isometric NS after modeling and lasted 9 weeks	(1) BMD (F-BMD) (2) Elasticity modulus, maximum load, yield load (3) Content of bone mineral salt (4) RANKL, OPG protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$
Guyatt et al. (2011) [25]	Female SD rats (10/10, 3 months old)	NG	Bilateral oophorectomy was performed on rats	NG	By oral gavage of PSO (1.26 g/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD) (2) BV/TV, Tb.Th, Tb.N, Tb.Sp (3) Elasticity modulus, maximum load (4) Serum PINP, CTX, ALP	(1) $P < 0.05$ (2) $P > 0.05$ (3) $P < 0.05$ (4) $P < 0.05$ (5) $P > 0.05$ (6) $P < 0.05$

Table 2: Continued.

Study (year)	Species (sex, n = experimental/control group, age)	Weight	Model (method)	Anesthetic	Experimental group	Control group	Outcome index	Intergroup differences
Balshem et al. (2011) [26]	Female SD rats (20/20, 8 months old)	200-220 g	By oral gavage of prednisone (0.005 mg/kg/d, qd) for 14 weeks	Chloral hydrate	By oral gavage of ISO (25 mg/kg/d, qd) after modeling and lasted 14 weeks	By oral gavage of isometric NS after modeling and lasted 14 weeks	(5) Serum AST, ALT (6) Runx2, OPG, RANKL, collagen-I protein expression (1) BMD (F-BMD; L-BMD; P-BMD; total-BMD) (2) Serum BAP, OCN, OPG, RANKL, TRACP5b (3) CAT, SOD, MDA (4) Elasticity modulus, bending energy, maximum load, stiffness coefficient (5) FoxO3a, Axin2, β-catenin protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$ (5) $P < 0.05$
Wang et al. (2021) [27]	Female ICR mice (12/11, 6 weeks old)	20-22 g	Bilateral oophorectomy was performed on rats	Chloral hydrate	By oral gavage of bakuchiol (20 mg/kg/d, qd) after modeling and lasted 8 weeks	By oral gavage of isometric NS after modeling and lasted 8 weeks	(1) BV/TV, Tb.Th (2) Tb.N, Tb.Sp, DA (3) Maximum load (4) Serum OCN (5) Serum ALP, TRACP	(1) $P > 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$ (5) $P > 0.05$
Heinrich et al. (2020) [14]	Female and male Wistar rats (12/12, NG)	190-210 g	By oral gavage of tretinoin (70 mg/kg/d, qd) for 2 weeks	Pentobarbital sodium	By oral gavage of PSO (0.9 g/kg/d, qd) after modeling and lasted 8 weeks	By oral gavage of isometric NS after modeling and lasted 8 weeks	(1) Of BV, Conn.D (2) Tb.N, Tb.Sp (3) Tb.Th (4) Serum ACP (5) Serum ALP, ALP/ACP	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P > 0.05$ (4) $P < 0.05$ (5) $P > 0.05$
Shang et al. (2021) [28]	Female SD rats (10/10, 3 months old)	210-230 g	Bilateral oophorectomy was performed on rats	Chloral hydrate	By oral gavage of ISO (25 mg/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD; L-BMD; total-BMD) (2) Elasticity modulus, bending energy, maximum load (3) Serum OCN, TRACP 5b (4) OPG, RANKL protein expression	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$

Table 2: Continued.

Study (year)	Species (sex, n = experimental/control group, age)	Weight	Model (method)	Anesthetic	Experimental group	Control group	Outcome index	Intergroup differences
Chen and Tang (2021) [29]	Male C57BL/6 mice (10/10, 9 weeks old)	17.7 ± 1.1 g	Bilateral oophorectomy was performed on rats	NG	By oral gavage of PSO (20 mg/kg/d, qd) after modeling and lasted 6 weeks	By oral gavage of isometric NS after modeling and lasted 6 weeks	(1) Serum ALP, CTX (2) Elasticity modulus, maximum load	(1) $P < 0.05$ (2) $P < 0.05$
Zhou et al. (2020) [30]	Male C57BL/6 mice (6/6, NG)	NG	Bilateral oophorectomy was performed on rats	NG	By oral gavage of ISO (20 mg/kg/d, qd) after modeling and lasted 8 weeks	By oral gavage of isometric NS after modeling and lasted 8 weeks	(1) BV/TV, Tb.Th, Tb.N, Tb.Sp (2) Runx2, PPAR-γ protein expression	(1) $P < 0.05$ (2) $P < 0.05$
Liu et al. (2020) [31]	Female SD rats (12/12, 4 months old)	250 ± 25 g	Bilateral oophorectomy was performed on rats	Pentobarbital sodium	By oral gavage of PSO (1 g/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD; L-BMD) (2) BV/TV, Tb.Th, Tb.N, Tb.Sp (3) Elasticity modulus, maximum load (4) Serum ALP, IL-1, IL-6	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$ (4) $P < 0.05$
Wang et al. (2019) [32]	Female SD rats (18/17, 3 months old)	180 ± 20 g	Bilateral oophorectomy was performed on rats	Pentobarbital sodium	By oral gavage of bavachin (50 mg/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD) (2) Urinary Ca (3) GABA, $GABA_BRI$	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$
Zhang et al. (2019) [33]	Female SD rats (12/12, 4 months old)	230 g	Bilateral oophorectomy was performed on rats	Pentobarbital sodium	By oral gavage of psoralidin (16 mg/kg/d, qd) after modeling and lasted 13 weeks	By oral gavage of isometric vegetable oil after modeling and lasted 13 weeks	(1) BMD (F-BMD; L-BMD) (2) Serum estradiol, calcitonin (3) Elasticity modulus	(1) $P < 0.05$ (2) $P < 0.05$ (3) $P < 0.05$
Liu et al. (2017) [34]	Female Wistar rats (8/7, 4 months old)	290.9 ± 1.5 g	Bilateral oophorectomy was performed on rats	Ketamine	By oral gavage of PSO (60 mg/kg/d, qd) after modeling and lasted 12 weeks	By oral gavage of isometric NS after modeling and lasted 12 weeks	(1) BMD (F-BMD) (2) Serum estradiol (3) Serum OCN, 1,25-dihydroxyvitamin D3, TNF-α	(1) $P < 0.05$ (2) $P > 0.05$ (3) $P < 0.05$

Table 2: Continued.

Study (year)	Species (sex, n = experimental/control group, age)	Weight	Model (method)	Anesthetic	Experimental group	Control group	Outcome index	Intergroup differences
Huang and Zhou (2017) [35]	Female SD rats (10/10, 5 weeks old)	NG	Bilateral oophorectomy was performed on rats	NG	By subcutaneous injection of bakuchiol (30 mg/kg/d, qd) after modeling and lasted 6 weeks	By oral subcutaneous injection of isometric NS after modeling and lasted 6 weeks	(1) BMD (F-BMD) (2) Serum ALP, P, Ca, estradiol	(1) $P < 0.05$ (2) $P < 0.05$

NG: not given; SD: Sprague-Dawley; DXM: dexamethasone; ISO: isopsoralen; PSO: psoralen; NS: normal saline; BMD: bone mineral density; F-BMD: femoral bone mineral density; L-BMD: lumbar bone mineral density; P-BMD: pelvis bone mineral density: BV/TV: bone volume over total volume; Tb.N: trabecular linear density; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; ALP: alkaline phosphatase; ACP: acid phosphatase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TRACP: tartrate-resistant acid phosphatase; Ca: calcium; P: phosphorus; PPAR-γ: peroxisome proliferator activated receptor γ; OCN: osteocalcin; Runx2: runt-related transcription factor 2; MMP 13: matrix metalloproteinase 13; PINP: N-terminal propeptide of type 1 procollagen; BMP2: bone morphogenetic protein 2; VEGF: vascular endothelial growth factor; PI3K: phosphatidylinositol 3-kinase; AKT: protein kinase B; mTOR: mammalian target of rapamycin; RANKL: receptor activator of nuclear factor-κB ligand; OPG: osteoprotegerin; CTX: C-terminal cross-linked telopeptide of type I collagen; CAT: catalase; SOD: superoxide dismutase; MDA: malondialdehyde; FoxO3a: forkhead box O3a; Conn.D: connectivity density; IL-1: interleukin-1; IL-6: interleukin-6; GABA: gamma-aminobutyric acid; $GABA_BRI$: gamma-aminobutyric acid receptor; qd: once a day; biw: twice a week.

Table 3: Detailed information of IFP in each study.

Study (year)	Chemical composition	Source	Purity (%)	Quality control reported
Chai et al. (2018) [21]	Isopsoralen	Chengdu Ruisifen Biological Technology Co., Ltd., CHN	NG	Batch number: 20180122
van Luijk et al. (2013) [22]	Isopsoralen	Shanghai Chunyou Biotechnology Co., Ltd., CHN	≥98%	HPLC
Moher et al. (2009) [23]	Psoralen	Guangdong Jingxin Biological Technology Co., Ltd., CHN	98%	Batch number: 1202133231
Macleod et al. (2004) [24]	Psoralen	Xinjiang Qikang Harbowei Pharmaceutical Co., Ltd., CHN	≥98%	Batch number: 20151027
Guyatt et al. (2011) [25]	Psoralen	National Institute for Food and Drug Control	NG	HPLC
Balshem et al. (2011) [26]	Isopsoralen	Chengdu Ruisifen Biological Technology Co., Ltd., CHN	NG	Batch number: 20180122
Wang et al. (2021) [27]	Bakuchiol	Tianjin Crescent Lake Technology Co. Ltd., CHN	≥95%	HPLC
Heinrich et al. (2020) [14]	Psoralen	Shenzhen China Resources Sanjiu Pharmaceutical Co., Ltd., CHN	≥98%	Batch number: 20120920
Shang et al. (2021) [28]	Isopsoralen	National Institute for the Control of Pharmaceutical and Biological Products	≥98%	Batch number: 110738-201509
Chen and Tang (2021) [29]	Psoralen	Jiangsu Yongjian Pharmaceutical Co., Ltd., CHN	NG	NG
Zhou et al. (2020) [30]	Isopsoralen	Sigma-Aldrich Corporation, USA	≥99%	HPLC
Liu et al. (2020) [31]	Psoralen	Henan Luoyang Orthopedic Hospital, CHN	≥98%	HPLC
Wang et al. (2019) [32]	Bavachin	NG	NG	NG
Zhang et al. (2019) [33]	Psoralidin	School of Pharmacy of Central South University, CHN	≥98%	HPLC
Liu et al. (2017) [34]	Psoralen	The Second People's Hospital of Shanxi Province, CHN	NG	NG
Huang and Zhou (2017) [35]	Bakuchiol	Korea Research Institute of Chemical Technology	≥98%	HPLC

HPLC: high-performance liquid chromatography; NG: not given.

and 22 g. Eleven studies [29, 31, 33–41] established an OP model by bilateral oophorectomy, two studies [27, 30] by intramuscular injection of DXM (2.5 mg/kg, biw) for 12 weeks, two studies [17, 28] by oral gavage of 7% tretinoin (10 ml/kg/d, qd) for 2 weeks, and 1 study [32] by oral gavage of prednisone (0.005 mg/kg/d, qd) for 14 weeks. Anesthetics were reported in ten studies [17, 27, 29, 32–34, 37–40], of which chloral hydrate was reported in five studies [27, 29, 32–34], pentobarbital sodium in four studies [17, 37–39], and ketamine in one study [40]. Detailed information of IFP in each study is displayed in Table 3. With regard to the specific IFP, isopsoralen was reported in five studies [27, 28, 32, 34, 36], psoralen in seven studies [17, 29–31, 35, 37, 40], bakuchiol in two studies [33, 41], psoralidin in one study [39], and bavachin in one study [38]. Fifteen studies [17, 27–40] used a dose gradient of IFP by oral administration ranging from 16 mg/kg/d to 1.26 g/kg/d, and one study [41] used subcutaneous injection administration by 30 mg/kg/d. In terms of primary outcome, BMD-femur was measured in twelve studies [27–32, 34, 37–41], BMD-lumbar spine in seven studies [27–29, 32, 34, 37, 39], S-OCN in five studies [28–30, 32, 40], BV/TV in five studies [27, 31, 33, 36, 37], Tb.N in six studies [17, 27, 31, 33, 36, 37], Tb.Th in four studies [17, 31, 33, 36], Tb.Sp in five studies [17, 31, 33, 36, 37], bone maximum load in six studies [30–32, 34, 35, 37], and elasticity modulus in six studies [30, 31, 34, 35, 37, 39].

3.3. Study Quality. The CAMARADES 10-item quality checklist was adopted to judge the risk of bias of each study, and the number of criteria met varied from 4/10 to 7/10 with the average of 5.25. Detailed results of methodological quality are presented in Table 4. All the included studies were peer-reviewed publications; three studies [31, 37, 39] did not mention control of temperature. Two [32, 34] of the included studies did not declare randomisation. The ways of blinding induction of model were reported in seven studies [29–31, 33, 35, 36, 41], and all of them reported that the animals were grouped randomly after modeling. No study mentioned the calculation of the sample size, and none used a blinding method during outcome assessment and an appropriate animal model. Ten studies [17, 27, 29, 32–34,

Table 4: Risk of bias of the included studies.

Study	A	B	C	D	E	F	G	H	I	J	Total
Chai et al. (2018) [21]	√	√	√			√			√	√	6
van Luijk et al. (2013) [22]	√	√	√						√	√	5
Moher et al. (2009) [23]	√	√	√	√		√			√	√	7
Macleod et al. (2004) [24]	√	√	√	√					√		5
Guyatt et al. (2011) [25]	√		√	√					√	√	5
Balshem et al. (2011) [26]	√	√				√			√		4
Wang et al. (2021) [27]	√	√	√	√		√				√	6
Heinrich et al. (2020) [14]	√	√	√			√			√	√	6
Shang et al. (2021) [28]	√	√				√			√	√	5
Chen and Tang (2021) [29]	√	√	√	√							4
Zhou et al. (2020) [30]	√	√	√	√					√	√	6
Liu et al. (2020) [31]	√		√			√				√	4
Wang et al. (2019) [32]	√	√	√			√			√	√	6
Zhang et al. (2019) [33]	√		√			√			√	√	5
Liu et al. (2017) [34]	√	√	√			√					4
Huang and Zhou (2017) [35]	√	√	√	√					√	√	6

Note: studies fulfilling the criteria of the following: A: peer-reviewed publication; B: control of temperature; C: random allocation to treatment or control; D: blinded induction of model (group randomly after modeling); E: blinded assessment of outcome; F: use of anesthetic without significant protective and toxic effects on bones; G: appropriate animal model (aged, hyperlipidemia, hypertensive, or diabetes); H: sample size calculation; I: compliance with animal welfare regulations (including three or more of the following points: preoperative anesthesia, postoperative analgesia, nutrition, disinfection, environment temperature, environment humidity, circadian rhythm, and euthanasia); J: statement of potential conflict of interests.

37–40] used the anesthetic without protective and toxic effects on bones. Compliance with animal welfare regulations was not described in four studies [33, 35, 37, 40], and the potential conflict of interests was not mentioned in four studies [30, 32, 35, 40].

3.4. Effectiveness

3.4.1. S-OCN. Five studies [28–30, 32, 40] compared IFP with a control group regarding S-OCN. As shown in Figure 3, the pooled results showed that IFP significantly increased the S-OCN in contrast with control (SMD = 2.825; 95%CI = 2.302 to 3.349; $P < 0.001$; heterogeneity $\chi^2 = 3.66$, df = 4, $I^2 = 0\%$, $P = 0.454$, Figure 3).

3.4.2. BMD

(1) BMD-Femur. Twelve studies [27–32, 34, 37–41] reported IFP with the control group according to BMD at the femur. The pooled results indicated that IFP was significant for lifting BMD at the femur compared to the control group (SMD = 3.424; 95%CI = 2.186 to 4.661; $P < 0.001$, heterogeneity $\chi^2 = 159.09$, df = 11, $I^2 = 93.1\%$, $P < 0.001$, Figure 4). The causes of heterogeneity in the results were explored by metaregression. The metaregression analysis for sample size, intervention time, dosage of IFP, publication year, and age of animals (weeks) was performed to analyze the potential sources of interstudy heterogeneity (Figure 5). Overall, the sample size ($\beta = -0.244$; $P = 0.028$; Adj $R^2 = 35.83\%$) might be one of the sources of heterogeneity. However, intervention time ($\beta = -0.644$; $P = 0.216$; Adj $R^2 = 8.40\%$), publication year ($\beta = 0.209$; $P = 0.122$; Adj $R^2 = 15.95\%$), dosage of IFP ($\beta = 0.001$; $P = 0.595$; Adj $R^2 = -7.13\%$), and age of animals ($\beta = 0.117$; $P = 0.062$; Adj $R^2 = 31.60\%$) were not the major causes of heterogeneity for BMD at the femur.

(2) BMD-Lumbar Spine. Seven studies [27–29, 32, 34, 37, 39] compared IFP with the control group about the BMD at the lumbar spine. As shown in Figure 6, the pooled results showed that IFP was significant for improving BMD at the lumbar spine compared with the control group (SMD = 1.880; 95%CI = 0.754 to 3.005; $P = 0.001$; heterogeneity $\chi^2 = 56.71$, df = 6, $I^2 = 89.4\%$, $P < 0.001$).

3.4.3. BMD-Related Indicator under Micro-CT

(1) BV/TV. Five studies [27, 31, 33, 36, 37] reported IFP versus the control group according to BV/TV. The pooled results indicated that IFP was significant for raising BV/TV compared to the control group (SMD = 3.433; 95%CI = 1.412 to 5.455; $P = 0.001$; heterogeneity $\chi^2 = 47.06$, df = 4, $I^2 = 91.5\%$, $P < 0.001$, Figure 7(a)).

(2) Tb.N. There were six studies [17, 27, 31, 33, 36, 37] comparing IFP with the control group about Tb.N. The pooled results indicated that IFP significantly increased Tb.N compared to the control group (SMD = 2.737; 95%CI = 2.267 to 3.208; $P < 0.001$; heterogeneity $\chi^2 = 6.59$, df = 5, $I^2 = 24.1\%$, $P = 0.253$, Figure 7(b)).

(3) Tb.Th. Four studies [17, 31, 33, 36] compared IFP with the control group regarding the Tb.Th. As shown in

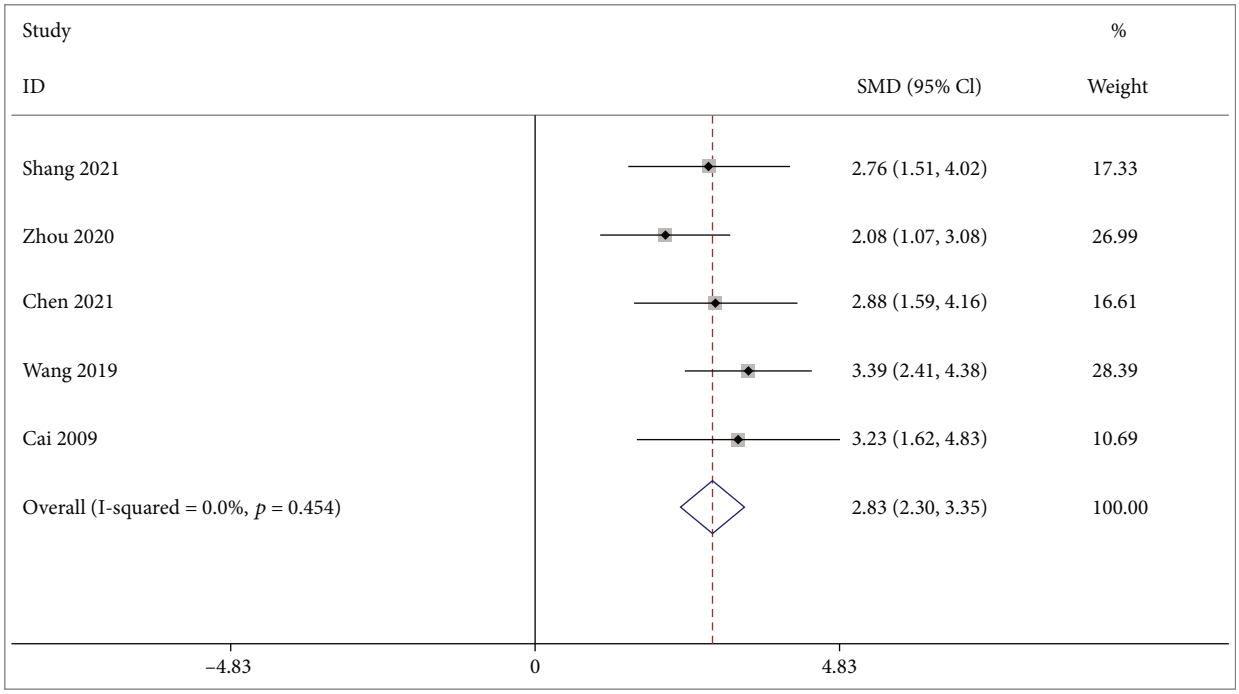


Figure 3: Forest plot of IFP versus control with regard to S-OCN.

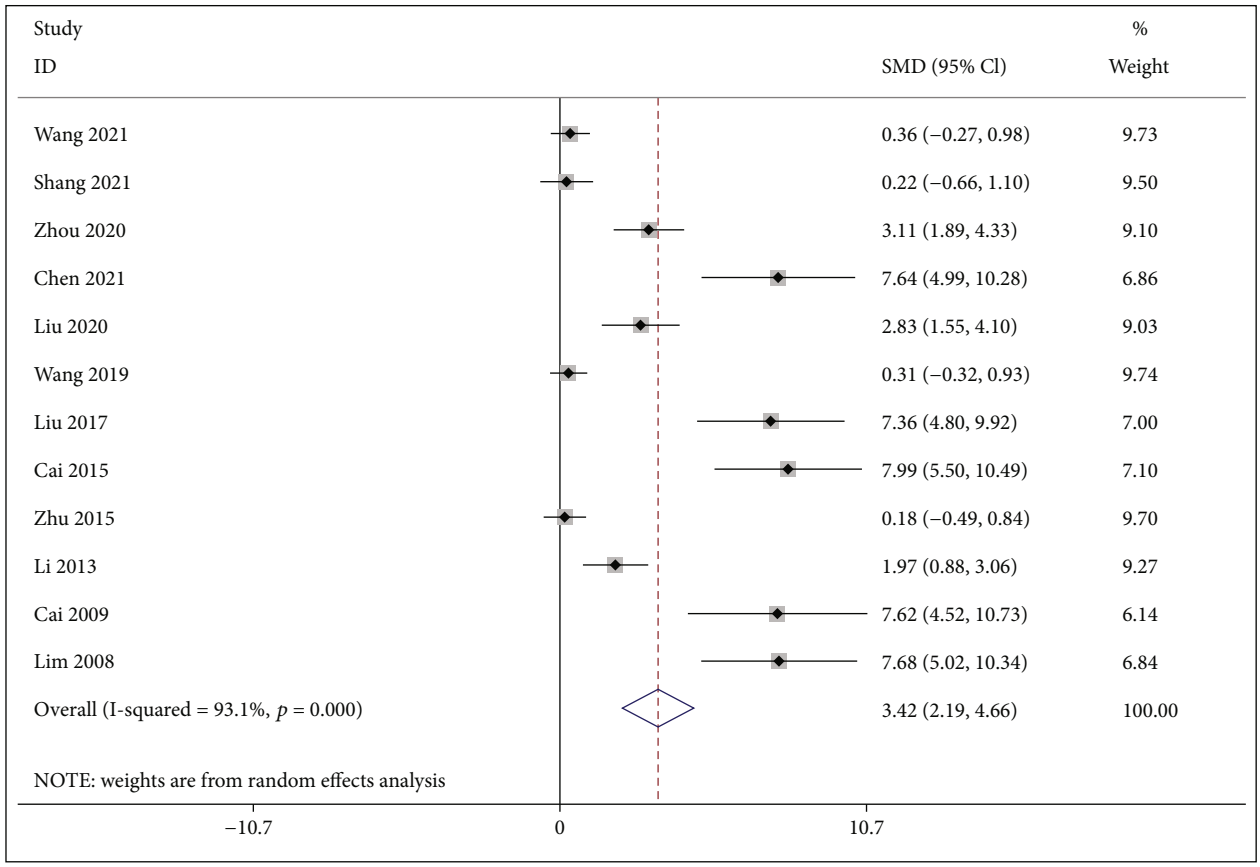


Figure 4: Forest plot of IFP versus control with regard to BMD-femur.

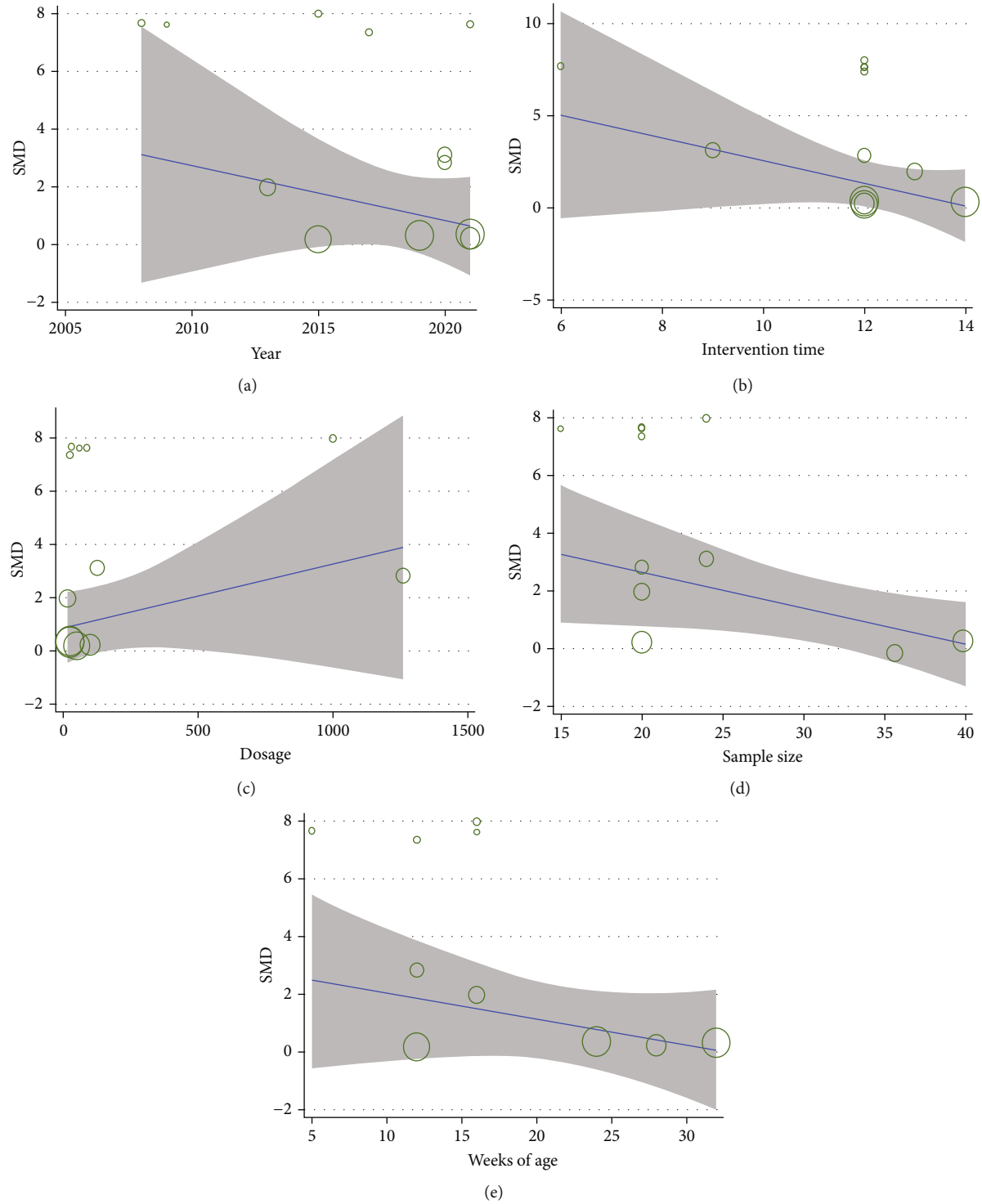


Figure 5: Metaregression analysis of BMD-femur: (a) publication year; (b) intervention time; (c) dosage of IFP; (d) sample size; (e) weeks of age.

Figure 7(c), the pooled results showed that IFP was significant for decreasing Tb.Th compared with the control group (SMD = −0.600; 95%CI = −1.056 to − 0.145; $P = 0.010$; heterogeneity $\chi^2 = 4.09$, df = 3, $I^2 = 26.6\%$, $P = 0.252$).

(4) Tb.Sp. Five studies [17, 31, 33, 36, 37] compared IFP with the control group with regard to Tb.Sp. The available data demonstrated that IFP significantly reduced Tb.Sp in contrast with the control group (SMD = −1.393; 95%CI = –

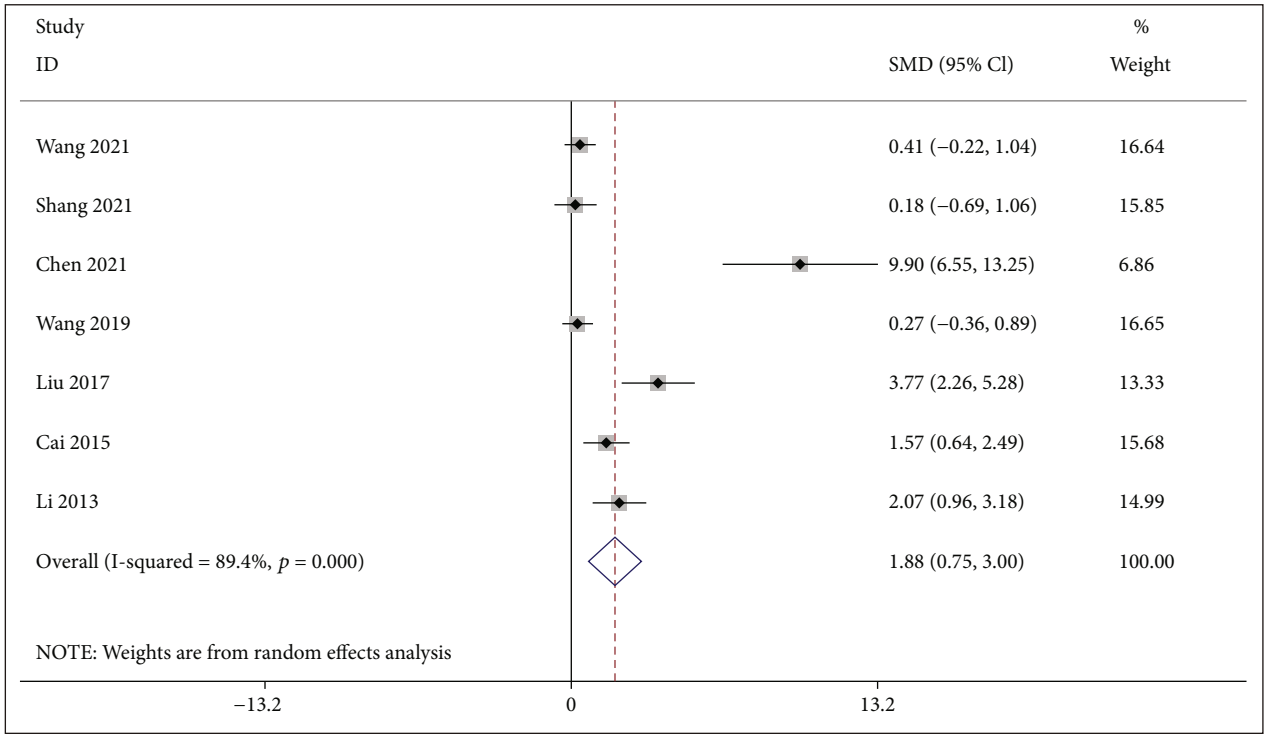


Figure 6: Forest plot of IFP versus control with regard to BMD-lumbar spine.

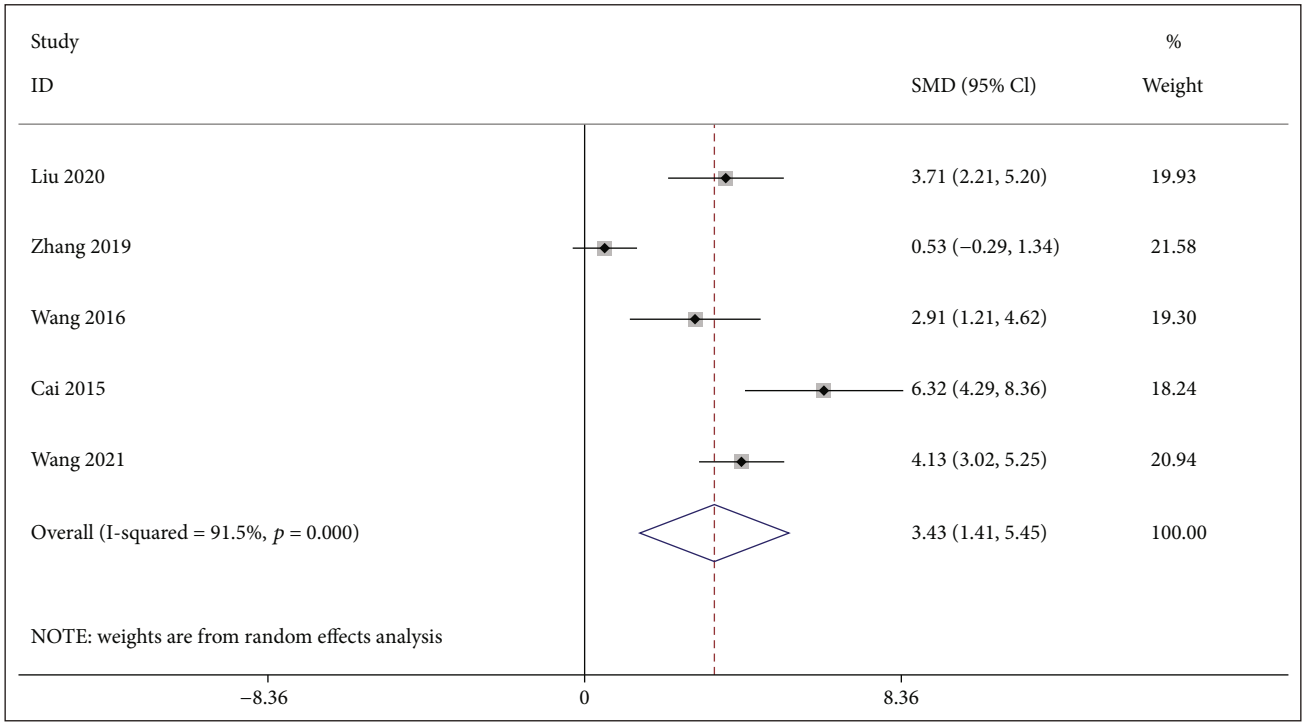
1.833 to − 0.954; $P < 0.001$; heterogeneity $\chi^2 = 5.60$, df = 4, $I^2 = 28.6\%$, $P = 0.231$, Figure 7(d)).

3.4.4. Bone Biomechanical Indicator

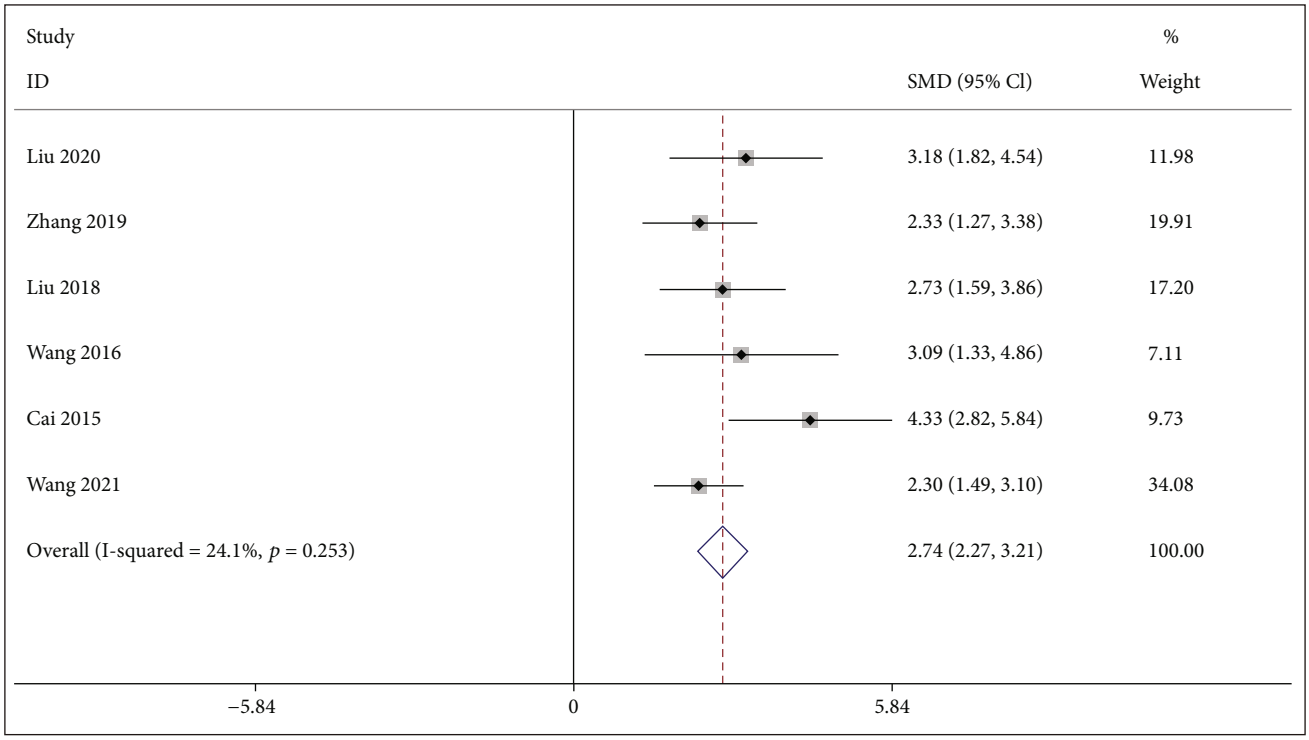
(1) Bone Maximum Load. There were six studies [30–32, 34, 35, 37] comparing IFP with the control group about the bone maximum load. The pooled results indicated that IFP significantly improved bone maximum load compared to the control group (SMD = 2.253; 95%CI = 1.828 to 2.678; $P < 0.001$; heterogeneity $\chi^2 = 7.65$, df = 5, $I^2 = 34.6\%$, $P = 0.177$, Figure 8(a)).

(2) Elasticity Modulus. Six studies [30, 31, 34, 35, 37, 39] reported IFP with the control group according to elasticity modulus. The pooled results indicated that elasticity modulus in the IFP group was significantly larger than that in the control group (SMD = 1.691; 95%CI = 1.274 to 2.107; $P < 0.001$; heterogeneity $\chi^2 = 8.18$, df = 5, $I^2 = 38.8\%$, $P = 0.147$, Figure 8(b)).

3.5. Subgroup Analysis. The potential confounding factors (including animal species, modeling methods, kind of IFP, sample size, and dosages of IFP) that may increase the heterogeneity of outcome measures were explored using subgroup analysis of BMD-femur and BMD-lumbar spine. As for BMD-femur, the subgroup analysis of animal species showed that no difference was seen in the effect size between the SD rat group and the Wistar rat group (SMD = 3.654 ± 3.560 versus SMD = 5.368 ± 3.192, respectively, $P = 0.544$, Figure 9(a)), and heterogeneity of both groups did not decrease obviously. In the subgroup analysis of modeling methods, the ovariectomized model group showed better effect size than the nonovariectomized model group (SMD = 5.409 ± 3.193 versus SMD = 1.000 ± 1.408, respectively, $P = 0.027$, Figure 9(b)) with significantly reduced heterogeneity of both groups. In the subgroup analysis of kind of IFP, significant difference was found between the three groups (SMD = 2.063 ± 3.533 versus SMD = 6.839 ± 2.090 versus SMD = 1.276 ± 0.963, respectively, $P = 0.021$, Figure 9(c)), and the heterogeneity of the three groups decreased substantially. However, no difference was shown between the high-sample group (>20) and the low-sample group (≤20) (SMD = 2.390 ± 3.365 versus SMD = 5.047 ± 3.248, respectively, $P = 0.199$, Figure 9(d)). Besides, the high-dosage IFP group (>25 mg/kg, qd) showed greater effect size than in the low-dosage IFP (≤25 mg/kg, qd) (SMD = 5.535 ± 3.013 versus SMD = 0.750 ± 0.815, $P = 0.0122$, Figure 9(e)), and heterogeneity of two groups reduced substantially. In terms of BMD-lumbar spine, the subgroup analysis of kind of IFP showed that no significant difference was revealed between the isopsoralen group and nonisopsoralen group (SMD = 1.157 ± 1.743 versus SMD = 4.512 ± 4.672, respectively, $P = 0.2499$, Figure 10(a)) without a significant decline in heterogeneity between subgroups. In the subgroup analysis of modeling methods, the ovariectomized model group showed better effect size than the nonovariectomized model group (SMD = 6.326 ± 2.941 versus SMD = 0.2870 ± 0.1149, respectively, $P = 0.0179$, Figure 10(b)) with significantly reduced heterogeneity of both groups. Moreover, the low-sample group (≤20) exhibited better effect size than the high-sample group (>20) (SMD = 5.980 ± 2.690 versus SMD = 0.7480 ± 0.7130, respectively, $P = 0.0254$, Figure 10(c)), and the heterogeneity

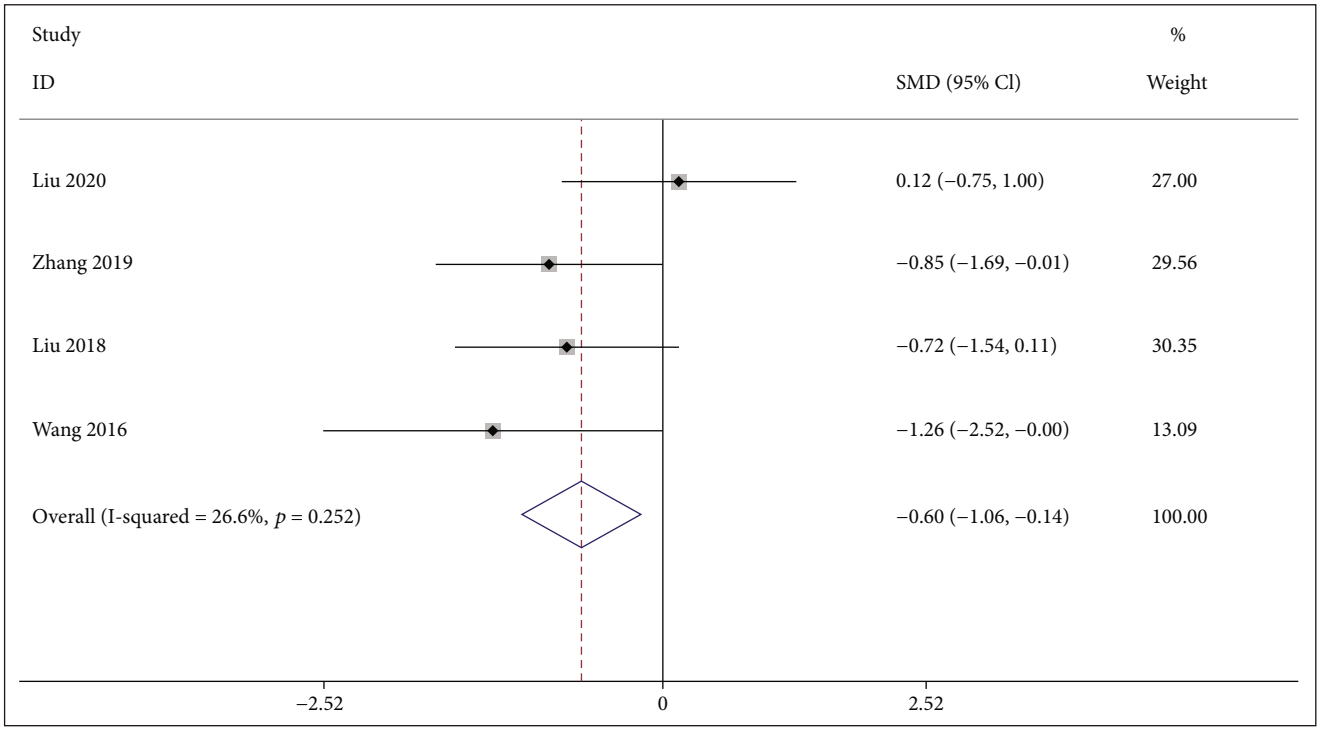


(a)

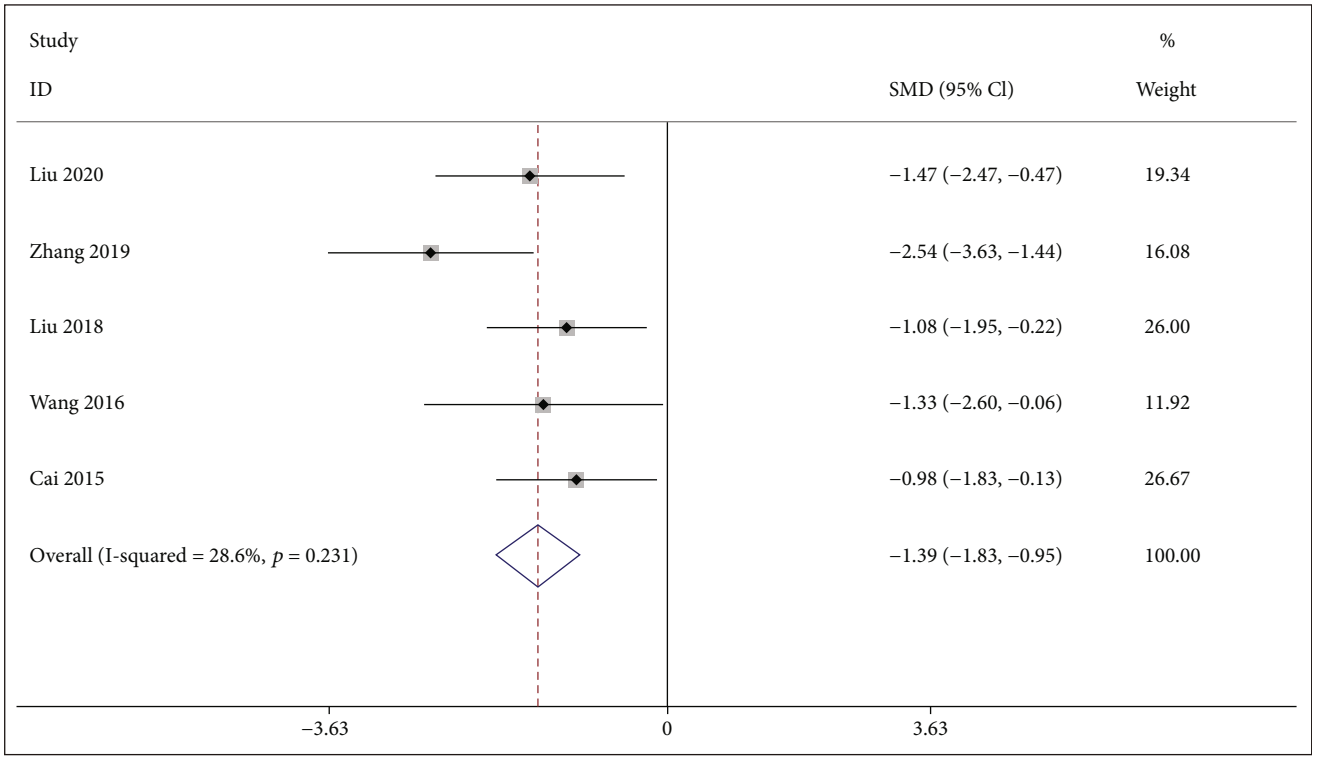


(b)

Figure 7: Continued.



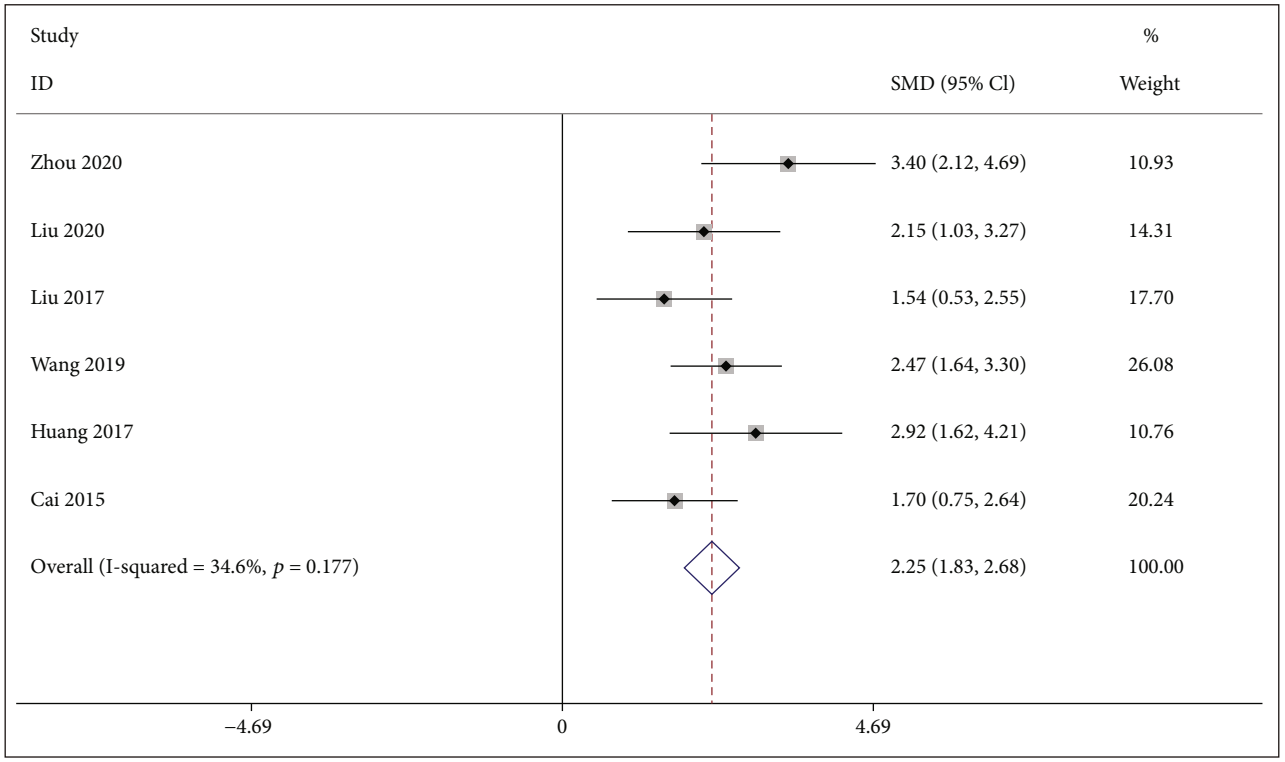
(c)



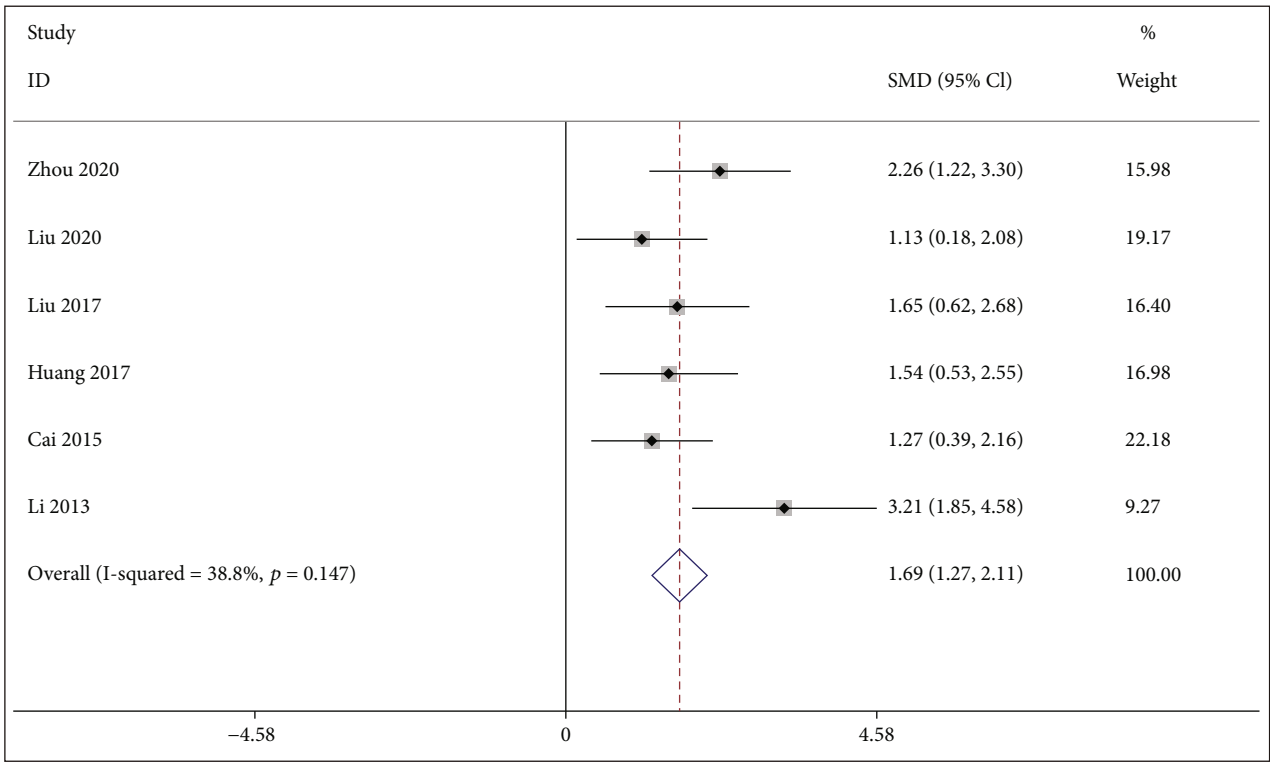
(d)

Figure 7: Forest plot of IFP versus control with regard to BMD-related indicator under micro-CT: (a) BV/TV; (b) Tb.N; (c) Tb.Th; (d) Tb.Sp.

experienced a marked decline in the high-sample group, whereas no difference was found between the high-dosage IFP group (>25 mg/kg, qd) and the low-dosage IFP group (≤25 mg/kg, qd) (SMD = 3.883 ± 5.255 versus SMD = 1.629 ± 1.644, respectively, $P = 0.539$, Figure 10(d)) without a significant decrease in heterogeneity between subgroups.



(a)



(b)

Figure 8: Forest plot of IFP versus control with regard to bone biomechanical indicator: (a) bone maximum load; (b) elasticity modulus.

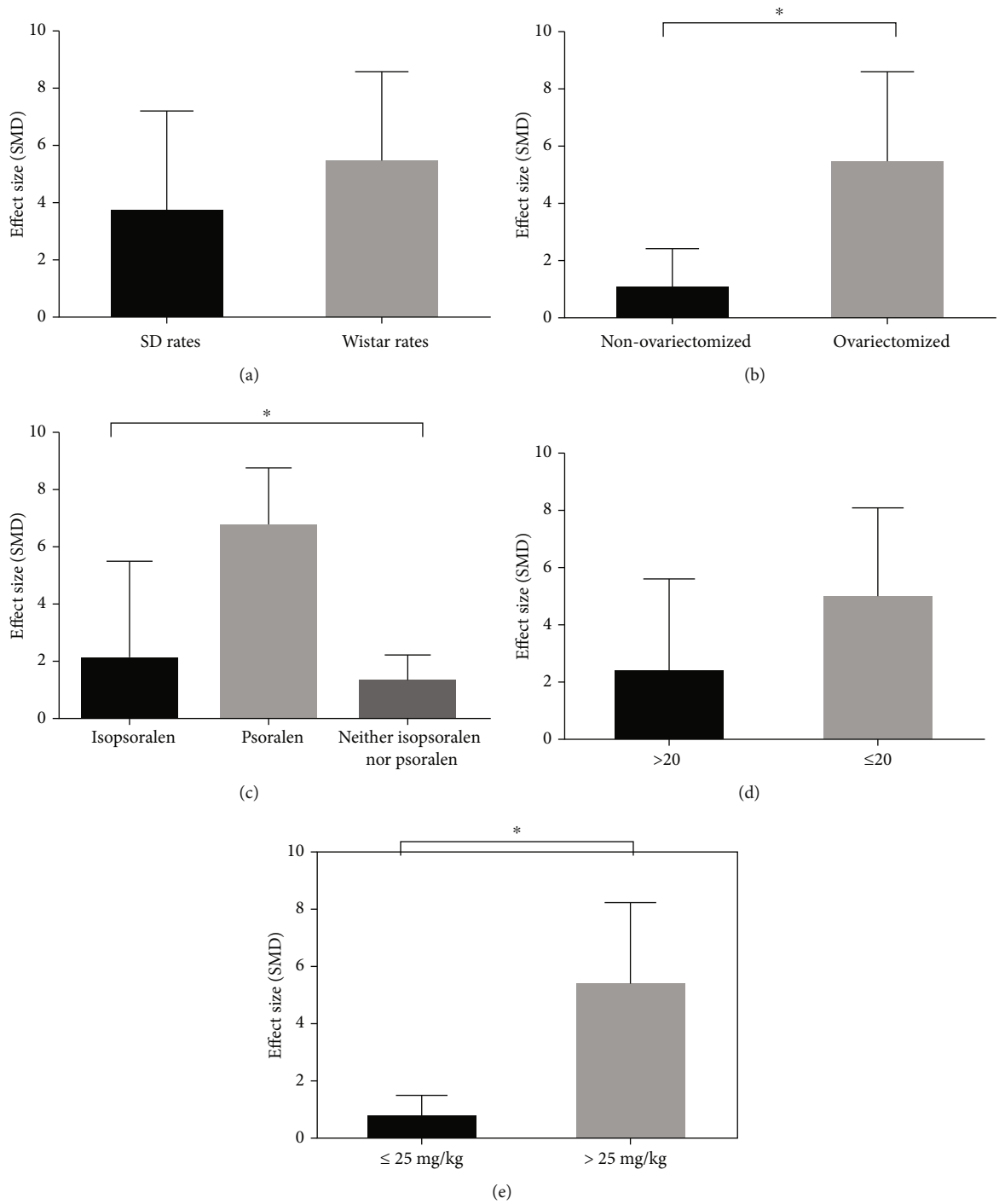


Figure 9: Subgroup analyses of the BMD-femur. (a) The different effect sizes between the SD rat group and the Wistar rat group. (b) The different effect sizes between the ovariectomized model group and the nonovariectomized model group. (c) The different effect sizes between the different kinds of IFP group. (d) The different effect sizes between different sample size groups. (e) The different effect sizes between different dosage groups. $^*P < 0.05$ between subgroups.

3.6. Publication Bias and Sensitivity Analysis. Egger's test (Figure 11) was used to assess the potential publication bias of the BMD-femur in this meta-analysis. The P values from Egger's tests indicated that there was no significant publication bias for BMD-femur ($P = 0.416$).

To determine the influence of each study on the pooled data for BMD-femur, BMD-lumbar spine, and BV/TV to verify the robustness of our results, sensitivity analysis was performed by omitting one study at a time and calculating the pooled data for the remaining studies. The results of the sensitivity analysis indicated that no significant effect was observed after excluding any single study, suggesting that the results of this meta-analysis were relatively robust (Figure 12).

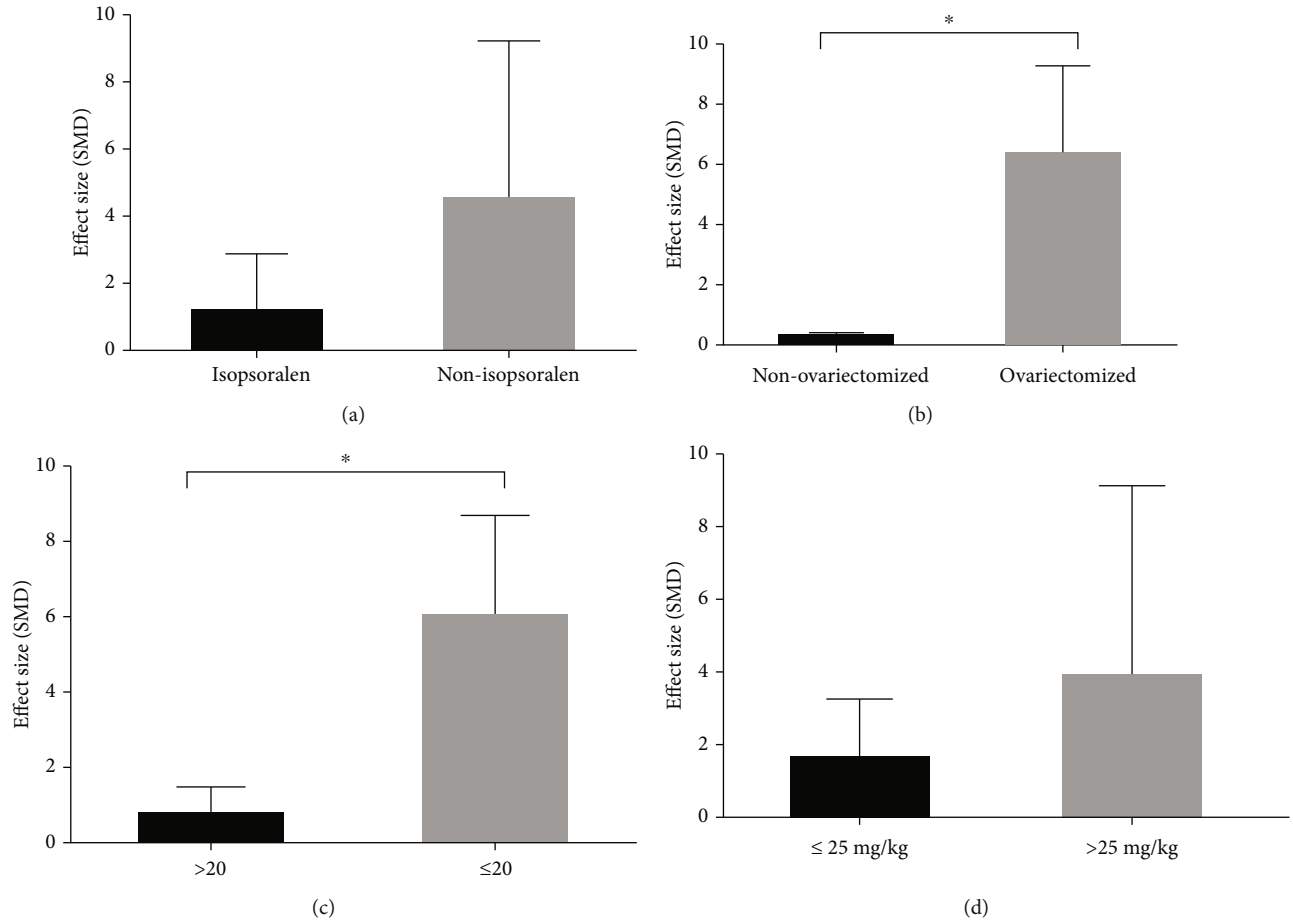


Figure 10: Subgroup analyses of the BMD-lumbar spine. (a) The different effect sizes between the different kinds of IFP groups. (b) The different effect sizes between the ovariectomized model group and the nonovariectomized model group. (c) The different effect sizes between different sample size groups. (d) The different effect sizes between different dosage groups. $^*P < 0.05$ between subgroups.

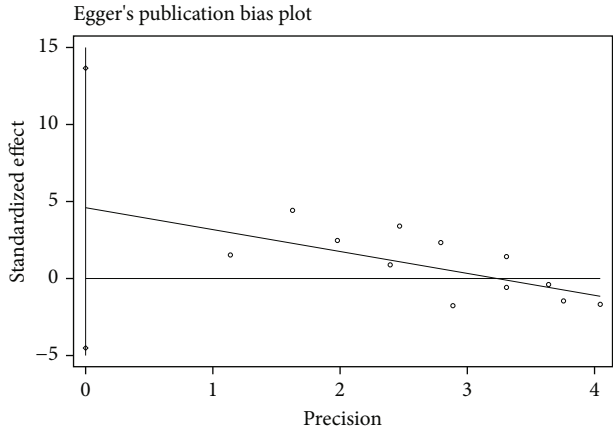


Figure 11: Egger's test of BMD-femur.

3.7. GRADE Assessment. The GRADE system was used to assess the level of evidence for the outcomes, which indicated moderate, low, or very low quality with methodological problems and heterogeneity problem. The GRADE evidence profiles are shown in Table 5.

4. Discussion

4.1. Summary of Evidence. This is the first preclinical systematic review and meta-analysis to estimate the efficacy and possible mechanism of IFP for the OP animal model. Sixteen high-quality studies involving 379 animals with the OP model were enrolled in the analysis. The primary findings of present systematic review illustrated that IFP could significantly increase the S-OCN, BMD, BV/TV, and Tb.N while Tb.Sp and Tb.Th were remarkably decreased by IFP in OP model animals. Moreover, IFP could significantly improve the bone biomechanical indicator bone maximum load and elasticity modulus. Therefore, the findings revealed that FP is a potential anti-OP drug through multiple mechanisms. However, the outcomes BMD-femur, BMD-lumbar spine, and BV/TV represented high heterogeneity in our meta-analysis. According to the results of subgroup analysis and metaregression in our study, the source of heterogeneity was from the sample size, OP modeling methods, kind of IFP, and dosages of IFP. Thus, more high-quality studies involving large sample sizes should be conducted to confirm our findings.

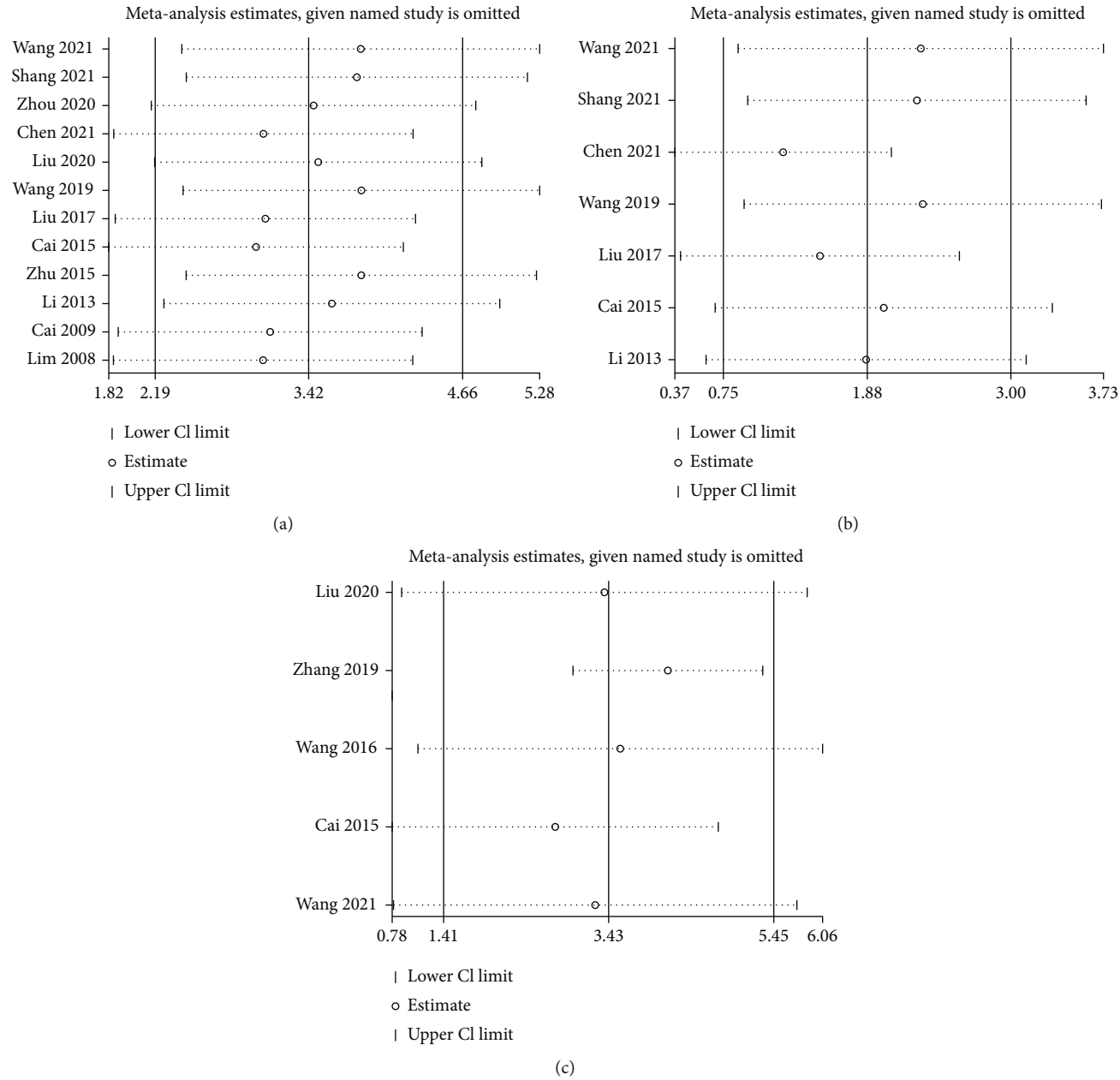


Figure 12: Sensitivity analysis for BMD-femur (a), BMD-lumbar spine (b), and BV/TV (c).

4.2. Strengths. The strengths of this meta-analysis study consisted of a clearly defined research question, which reduced the bias in the choice of the included studies, fidelity, and consistency to a precise research approach that we designed before the meta-analysis, an in-depth search of the literature, the agreement between the two researchers regarding the entry data components, and the quality control appraisal of all the data. The quality of included original studies was relatively moderate, which suggests that the results were relatively reliable. Additionally, the number of trials and the overall sample size was comparatively large (16 trails with 379 animals). We performed subgroup assessments and metaregression evaluation to identify the origin of heterogeneity. Consequently, no publication bias was reported in this meta-analysis, and sensitivity estimation revealed that the findings of this meta-analysis are comparatively robust.

4.3. Limitations. Some limitations that may affect the accuracy of the study should be considered. Firstly, the included primary studies had some intrinsic and methodological shortcomings: (1) Only 14 trials had sufficient information on the generation of random allocation. (2) The blinding procedure and sample size calculation were not reported or remained unclear in some studies, making it a challenge to bias findings unintentionally or intentionally and to help allow the credibility of study conclusions. Secondly, selection bias was unavoidable because only eight frequently used databases were searched for English and Chinese language

Table 5: GRADE evidence profile.

Quality assessment							No. of patients		Effect			
No. of studies	Design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	Ingredients of Fructus Psoraleae	Control	Relative (95% CI)	Absolute	Quality	Importance
S-OCN (better indicated by lower values)												
5	Randomised trials	Serious[1]	No serious inconsistency	No serious indirectness	No serious imprecision	None	60	59	—	SMD 2.73 higher (2.2 to 3.25 higher)	⊕⊕⊕O Moderate	Important
BMD-femur (better indicated by lower values)												
12	Randomised trials	Serious[1]	Serious[2]	No serious indirectness	No serious imprecision	None	150	148	—	SMD 3.22 higher (2.04 to 4.41 higher)	⊕⊕OO Low	Critical
BMD-lumbar spine (better indicated by lower values)												
7	Randomised trials	Serious[1]	Serious[2]	No serious indirectness	No serious imprecision	None	92	92	—	SMD 1.76 higher (0.69 to 2.83 higher)	⊕⊕OO Low	Critical
BV/TV (better indicated by lower values)												
5	Randomised trials	Serious[1]	Serious[2]	No serious indirectness	No serious imprecision	None	60	60	—	SMD 3.29 higher (1.32 to 5.26 higher)	⊕⊕OO Low	Important
Tb.N (better indicated by lower values)												
6	Randomised trials	Very serious[1,3]	No serious inconsistency	No serious indirectness	No serious imprecision	None	72	72	—	SMD 2.64 higher (2.17 to 3.11 higher)	⊕⊕OO Low	Important
Tb.Th (better indicated by lower values)												
4	Randomised trials	Serious[1,4]	No serious inconsistency	No serious indirectness	No serious imprecision	None	40	40	—	SMD 0.57 lower (1.03 to 0.12 lower)	⊕⊕⊕O Moderate	Important
Tb.Sp (better indicated by lower values)												
5	Randomised trials	Serious[1]	No serious inconsistency	No serious indirectness	No serious imprecision	None	52	52	—	SMD 1.34 lower (1.78 to 0.89 lower)	⊕⊕⊕O Moderate	Important
Bone maximum load (better indicated by lower values)												
6	Randomised trials	Very serious[1,4]	No serious inconsistency	No serious indirectness	No serious imprecision	None	74	74	—	SMD 2.18 higher (1.75 to 2.6 higher)	⊕⊕OO Low	Critical
Elasticity modulus (better indicated by lower values)												
6	Randomised trials	Serious[1]	No serious inconsistency	No serious indirectness	No serious imprecision	None	64	64	—	SMD 1.62 higher (1.2 to 2.04 higher)	⊕⊕⊕O Moderate	Critical

GRADE working group grades of evidence: high quality: further research is very unlikely to change our confidence in the estimate of effect; moderate quality: further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate; low quality: further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate; very low quality: we are very uncertain about the estimate. [1]Some of the included studies did not report the implementation of blinding. [2]Heterogeneity ($I^2 > 50\%$, $P < 0.05$) was found. [3]No details of random protocol were reported. [4]Some of the included studies lack allocation concealment.

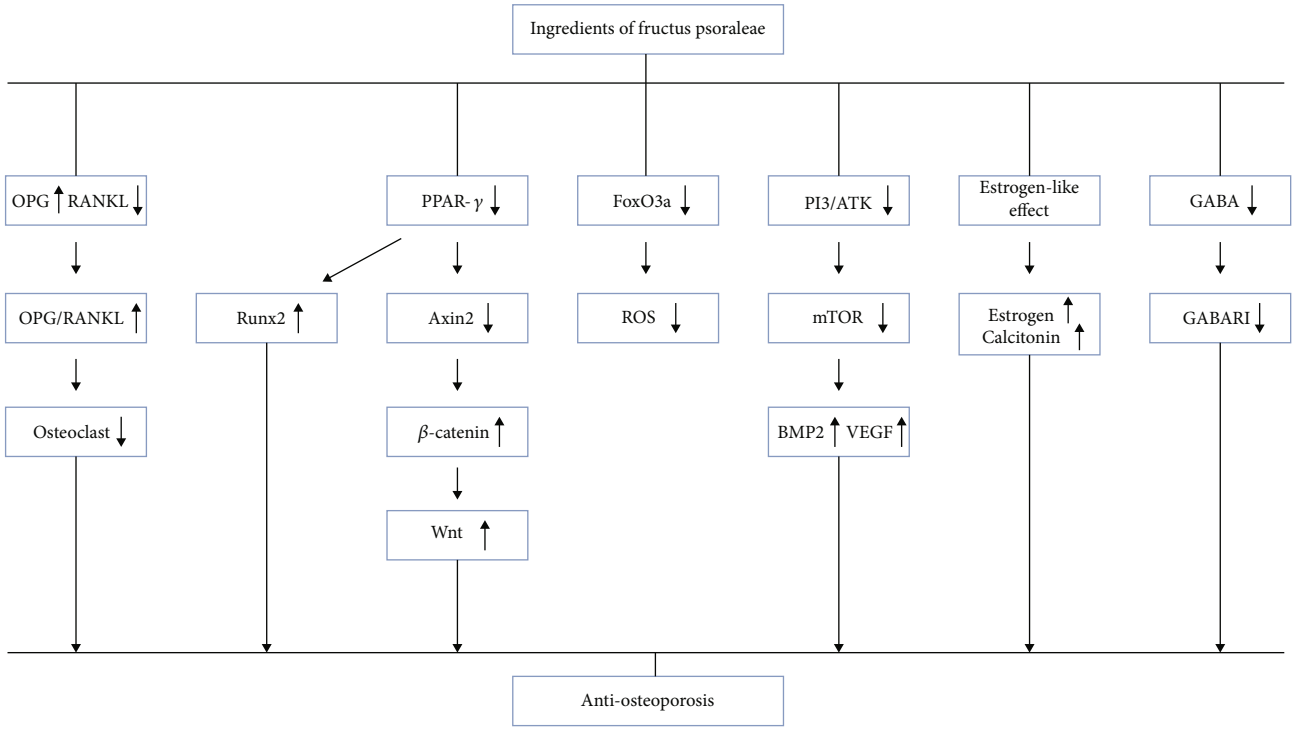


Figure 13: A schematic representation of antiosteoporosis mechanisms of IFP.

studies. Therefore, the potentially relevant studies published in other languages could have been left out. Thirdly, the absence of negative studies might have led to the true effect of IFP being overestimated. Fourthly, though the metaregression and subgroup analysis were done, the high heterogeneity of BMD-femur, BMD-lumbar spine, and BV/TV could not be neglected. The OP modeling methods, the specific kind of IFP, dosage of IFP, administration approaches, and period of IFP treatments differed remarkably in the included studies. This heterogeneousness could compromise the viability of our findings. Fifthly, most of the included studies in the meta-analysis were conducted in China, a potential limitation to the generalizability of our findings. Sixthly, the overall quality of evidence of this study was low (Table 5). Finally, many of the included studies suffer from significant sources of bias; this also will jeopardize the validity of results.

4.4. Implications. High-quality methodologies of studies are the cornerstones of translating animal research into clinical drug treatments for human disease [42]. The score (mean 5.25) by prudent assessment of included studies was better than that of most studies of traditional Chinese medicine [43]. There were limitations in terms of blinding and sample size calculation. The blinding methods in the animal model establishment and outcome assessment were usually seen as technical difficulties for most studies. A sample size calculation could avoid the waste of resources caused by oversize and the imprecision of study result by undersize; the specific steps could be referred from the literature [44]. Besides, the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines are aimed at improving the quality of research reports by guiding complete and transparent reporting of in vivo animal research. Employing of experimental animals with comorbidities such as advanced age, obesity, hyperglycemia, or other risk factors may be more in line with the physiology of OP patients and may be helpful for the clinical translation of experimental results. These should be adopted in the future study management of IFP for OP.

Using different animal models at different research stages of disease is crucial to study the pathophysiology and treatments [45]. Factors that need to be considered include pathogenesis of model, availability of the animals, technical requirements, and cost and ethical considerations [46]. According to the pathogenesis, animal models of OP can be divided into two types: models with increased bone resorption as the dominant mechanism (such as ovariectomized OP model, disused OP model, tretinoin induction OP model, nutritional OP model, and glucocorticoid OP model) and models with reduced bone formation as the dominant mechanism (such as senile OP model) [47]. This study comprehensively includes the ovariectomized OP model and nonovariectomized OP model (tretinoin induction OP model and glucocorticoid OP model) to evaluate the efficacy and mechanisms of IFP for OP. The results of subgroup analysis suggested that the ovariectomized OP model group showed better effect size than the nonovariectomized OP model group in regard to BMD-femur ($SMD = 5.409 \pm 3.193$ versus $SMD = 1.000 \pm 1.408$, respectively, $P = 0.027$, Figure 9(b)), which suggests that the different OP model methods may be the source of high heterogeneity. In clinical practice, postmenopausal OP has a similar pathogenesis with ovariectomized OP model [48].

The ovariectomized OP model has become the most widely used animal model to study OP [49], and it also was the most adopted model in eligible studies. Thus, we suggest an ovariectomized OP model be adopted to assess OP in future studies.

The results of subgroup analysis regarding the specific kind of IFP in BMD-femur demonstrated that the prosalen group gave a higher effect size than the isoprosalen group and neither the isoprosalen nor the prosalen group (SMD = 2.063 ± 3.533 versus SMD = 6.839 ± 2.090 versus SMD = 1.276 ± 0.963, respectively, $P = 0.021$, Figure 9(c)), indicating that different kinds of IFP might be the origin of high heterogeneity. Psoralen is one of the most major ingredients of FP and was also the most used IFP in the included studies (reported in 7 studies) because it has the highest content in FP and is easy to extract from FP. Besides, psoralen has been reported to possess potential anti-OP effects in animal OP models through multiple signal pathways [17, 29, 35]. For example, psoralen could significantly increase BMD of femur and vertebra, serum levels of calcium, osteocalcin, N-terminal propeptide of type 1 procollagen (P1NP), bone morphogenetic protein 2 (BMP2), and vascular endothelial growth factor (VEGF) via phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) in postmenopausal rats [29]. Isoprosalen is another furanocoumarin compound of FP and is derived from psoralen. Analogously, several studies have shown that isoprosalen exerted mighty protective effects of OP in animal OP models [27, 28, 36]. Isopsoralen could increase BMD and improve serological indicators inhibiting Axin2/peroxisome proliferator activated receptor (PPAR-γ) signaling pathway and activating Wnt signaling pathway in rats with glucocorticoid-induced OP [27]. Other IFP including bakuchiol, bavachin, and psoralidin were also reported in our included studies; however, the number of studies was relatively less and their effect size was lower than prosalen and isoprosalen. Therefore, prosalen and isoprosalen may be recommended as potential candidates of anti-OP drugs in the future studies. However, their safety and toxicity should be taken into consideration, which will be the direction of future research.

4.5. Possible Mechanisms. Systemic review of preclinical studies is conducive to understand comprehensively pathological mechanisms of disease and pharmacological effects of drugs [50]. The possible mechanisms of IFP that mediated anti-OP effects in the included studies are summed up as follows: (1) *Osteoprotegerin/receptor activator of nuclear factor-κB ligand/receptor activator of nuclear factor-κB (OPG/RANKL/RANK) signal pathway*: IFP could highly increase OPG secretion and reduce RANKL expression, resulting in an enhancement in OPG/RANKL ratio, which leads to a decrease in the number and activity of osteoclasts [30, 31, 34]. (2) *PPAR-γ/Axin2/Wnt signal pathway*: IFP suppressed the PPAR-γ/Axin2 signaling pathway involved in lipid metabolism, blocked the inhibition of Axin2 on the Wnt signaling pathway, upregulated the expression of β-catenin protein, and made β-catenin form a complex with nuclear transcription factors after entering the nucleus. Eventually, β-catenin activated the Wnt signaling pathway to regulate the lipid metabolism of rat bone marrow, thereby affecting bone metabolism and promoting bone formation [27]. Besides, the inhibition of PPAR-γ resulted in the increase of Runx2 expression, which facilitates osteogenic differentiation of bone mesenchymal stem cells in rats [36]. (3) *Antioxidative stress through the forkhead box O3a (FoxO3a)/Axin2/Wnt signal pathway*: IFP inhibited the activation of the FoxO3a signaling pathway through its antioxidant effect; meanwhile, it upregulated the expression of β-catenin, bound it to FoxO3a, initiated the transcriptional program that regulates bone tissue cell apoptosis and eliminates reactive oxygen species (ROS), which promotes the generation and formation of osteoblasts. Then, the expression of Axin2 was downregulated by removing excessive ROS, the PPAR-γ/Axin2 signaling pathway was inhibited to participate in lipid metabolism and the inhibition of Axin2 on the Wnt signaling pathway was blocked, which ultimately exerts its anti-OP effect [32]. (4) *PI3K/Akt/mTOR signaling pathway*: IFP restrained the expression of PI3K/Akt/mTOR in the rat's femur and increased the levels of BMP2 and VEGF, which improves bone formation and angiogenesis [29]. (5) *Estrogen-like effect*: IFP had the similar effect of phytoestrogen on inhibiting bone resorption by participating in the binding of estrogen receptor. On the one hand, IFP may promote the synthesis and secretion of estrogen outside the ovary [40, 41]. On the other hand, it may enhance the secretion of thyroid calcitonin to exert it anti-OP effect [39]. (6) *Gamma-aminobutyric acid/gamma-aminobutyric acid receptor (GABA/$GABA_BRI$) signaling pathway*: IFP was observed to reduce the production of GABA and $GABA_B$RI to inhibit the activity of osteoblast [38]. The mechanism diagram is summarized in Figure 13.

5. Conclusion

This preclinical systematic review provided preliminary evidence that IFP was capable of partially exerting anti-OP effects in animal models probably through the OPG/RANKL/RANK, PPAR-γ/Axin2/Wnt, antioxidative stress via FoxO3a/Axin2/Wnt, PI3K/Akt/mTOR, estrogen-like effect, and GABA/$GABA_B$RI signaling pathway. Taken together, the findings suggest the possibility of developing IFP as a drug for the clinical treatment of OP.

Abbreviations

OP: Osteoporosis
FP: *Fructus Psoraleae*
IFP: Ingredients of *Fructus Psoraleae*
SD: Sprague-Dawley
DXM: Dexamethasone
ISO: Isopsoralen
PSO: Psoralen
NS: Normal saline
BMD: Bone mineral density
F-BMD: Femoral bone mineral density
L-BMD: Lumbar bone mineral density
BV/TV: Bone volume over total volume

Tb.N: Trabecular linear density
Tb.Th: Trabecular thickness
Tb.Sp: Trabecular separation
PPAR-γ: Peroxisome proliferator activated receptor γ
OCN: Osteocalcin
Runx2: Runt-related transcription factor 2
BMP2: Bone morphogenetic protein 2
VEGF: Vascular endothelial growth factor
PI3K: Phosphatidylinositol 3-kinase
AKT: Protein kinase B
mTOR: Mammalian target of rapamycin
RANKL: Receptor activator of nuclear factor-κ B ligand
OPG: Osteoprotegerin
FoxO3a: Forkhead box O3a
ROS: Reactive oxygen species
GABA: Gamma-aminobutyric acid
$GABA_BRI$: Gamma-aminobutyric acid receptor.

Data Availability

Previously reported data were used to support this study. These prior studies and datasets are cited at relevant places within the text as references.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this study.

Authors' Contributions

Z.L. was responsible for the conceptualization, methodology, and software. S.X.D. was responsible for data curation and writing of the initial draft. J.J.Z. was responsible for visualization and investigation. M.M.C. provided supervision. S.X.D. was responsible for software and validation. J.R.C. was responsible for reviewing and editing.

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Review Article

Quercetin Impact in Pancreatic Cancer: An Overview on Its Therapeutic Effects

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Pancreatic cancer (PC) is a lethal malignancy cancer, and its mortality rates have been increasing worldwide. Diagnosis of this cancer is complicated, as it does not often present symptoms, and most patients present an irremediable tumor having a 5-year survival rate after diagnosis. Regarding treatment, many concerns have also been raised, as most tumors are found at advanced stages. At present, anticancer compounds-rich foods have been utilized to control PC. Among such bioactive molecules,

flavonoid compounds have shown excellent anticancer abilities, such as quercetin, which has been used as an adjunctive or alternative drug to PC treatment by inhibitory or stimulatory biological mechanisms including autophagy, apoptosis, cell growth reduction or inhibition, EMT, oxidative stress, and enhancing sensitivity to chemotherapy agents. The recognition that this natural product has beneficial effects on cancer treatment has boosted the researchers' interest towards more extensive studies to use herbal medicine for anticancer purposes. In addition, due to the expensive cost and high rate of side effects of anticancer drugs, attempts have been made to use quercetin but also other flavonoids for preventing and treating PC. Based on related studies, it has been found that the quercetin compound has significant effect on cancerous cell lines as well as animal models. Therefore, it can be used as a supplementary drug to treat a variety of cancers, particularly pancreatic cancer. This review is aimed at discussing the therapeutic effects of quercetin by targeting the molecular signaling pathway and identifying antigrowth, cell proliferation, antioxidative stress, EMT, induction of apoptotic, and autophagic features.

1. Introduction

Pancreatic cancer (PC) is an increasingly common cancer of the gastrointestinal tract (GIT), with survival rates less than 5% at 5 years after diagnosis, and about 50% of all patients die over 6 months of diagnosis. According to estimations in the United States, PC will become the second most common cause of cancer death in the next twenty to thirty years. However, patients' prognosis with localized and respectable tumors remains poor with only 20% survival rate after surgery [1]. In addition, in line with GLOBOCAN 2018 assessments, PC which accounts for approximately 459,000 new cases and 432,000 deaths is the seventh leading cause of global cancer death [2]. In Europe, it is assumed that PC will quickly exceed breast cancer as the third cause of cancer death after colorectal and lung cancers [3].

PC is characterized as a tumor of the exocrine pancreas and ductal adenocarcinoma; however, a minor subset of patients also represents neuroendocrine tumors. Indeed, pancreatic intraepithelial neoplasia or precursor lesions are the operative factors in the acquisition of genetic shifts which trigger discernible pancreatic ductal adenocarcinoma (PDA) [4]. Unfortunately, PC symptoms do not begin up to the advanced stages of cancer and are usually vague, including nausea, vomiting, severe abdominal pain, and weight loss. Besides, previous studies revealed that type 2 diabetes, family history, obesity, and tobacco usage are the major risk factors for PC [1–5]. Therefore, studies are headed for PC prevention. A broad range of recent studies have explored the anticancer features of phytochemicals and have indicated that polyphenols, flavonoids, and flavones can be occupied against diverse types of cancers [6]. Flavonoids are secondary metabolites of plants with pharmacological activities (Table 1). Hence, fruits or vegetables, such as cocoa and coffee, are valuable sources of flavonoids [7, 8]. Based on chemical structure, oxidation degree, and unsaturation of linking chain, flavonoids are categorized into 6 main classes: isoflavonoids, flavones, flavanols, flavanones, flavonols, and anthocyanidins [9]. Quercetin and kaempferol are some of the most frequently found flavonols [10]. Quercetin ($C_{15}H_{10}O_7$) is called by IUPAC (International Union of Pure and Applied Chemistry) as follows: 3,3,4,5,7-pentahydroxyflavone and 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one [11]. It has been documented that quercetin offers antifungal, antioxidant, cytotoxic, hepatoprotective, and anticancer activities [12]. Specifically, both quercetin and its derivatives can prevent cancer-related diseases by regulating cellular signaling pathways. However, the anti-inflammatory and antioxidant properties of quercetin are the main factors for its activity as cell cycle inhibitors, and the apoptosis-inducing effect of quercetin has a key anticancer role [13, 14]. Noteworthy, quercetin is a general phytochemical in the regular dietary program of people worldwide since it can be widely found in daily foods, like tea, coffee, different vegetables, nuts, and fruits [15]. Quercetin and its derivatives pose biological inhibitory effects on the progression of the cancerous cell cycle; therefore, the metabolic pathways of quercetin are deemed as a significant factor in the plants' adaptive reaction. A number of recent studies have focused on the quercetin content of fruits and vegetables for its therapeutic purposes [16–19]. Additionally, as mentioned by Harwood et al. [20], commercially accessible quercetin can be consumed orally at a dose of 1 g daily, which is absorbed up to 60% and safe enough. In this sense, this review aims to discuss the anticancer properties of quercetin against PC, considering its low cost in comparison to synthetic drugs. In addition, the latest trends on quercetin features and their molecular mechanisms in cancer therapy are also summarized. Therefore, different research studies have analyzed the probable mechanisms through which quercetin exerts its antitumor effects against pancreatic cancer cells. Since there is not any review article on this subject based on our searches, we aimed to discuss the therapeutic effects of quercetin against pancreatic cancer cells for the first time.

2. Pancreatic Cancer (PC)

Currently with an average 5-year survival rate, PC is estimated to be the second cause of cancer-related deaths by 2030 in the United States [21–23]. The possibility of developing PC is about 1.5% in both genders [23], despite it mainly occurs in elderly, between 70 and 80 years, mostly in unlocalized and incurable forms [21, 24]. PC often remains undetectable until it transforms into a metastatic tumor [25]. While the etiology of PC has not been completely understood, several genetic and environmental risk factors are known to increase the risk, including smoking, obesity, diets rich in animal fat, cystic fibrosis, and genetic predispositions [26]. According to Huang et al. [27], the highest incidence and mortality of PC are in countries with very high human development index or age-standardized rates (ASRs) or the countries with a higher prevalence of alcohol drinking, smoking, hypertension, physical inactivity, obesity, and high cholesterol. The highest

Table 1: A number of pharmacological activities of quercetin reported in recent studies.

Dose	Model	Administration	Activity	Ref.
25 μM	MCF-7 and MDA-MB-231	Direct treatment	Apoptosis induction and anticancer effect	[129]
20 μM	Human umbilical vein endothelial cells (HUVECs)	Direct treatment	Autophagy and promoted cell survival	[130]
5.7 μM (DPPH assay)	Erythrocytes	Direct treatment	ROS and free radical scavenging activity	[131]
100 mg kg^{-1}	C57BL/6J mice on ethanol-containing Lieber De Carli liquid diets	Gavage	Suppressed autophagic flux, decreased liver injury by ethanol consumption	[132]
100 mg kg^{-1}	*Streptococcus suis* infected mice	Subcutaneous	Antimicrobial effect against *Streptococcus suis*	[133]
100 mg kg^{-1}	Chronic ethanol feeding C57BL/6J mice	Oral	Decreased fat accumulation in liver (ethanol induced)	[134]
30 mg kg^{-1}	STZ-induced diabetic rats	Intraperitoneal	Higher insulin levels, improved dyslipidemia, reduced serum blood glucose levels, decreased oxidative stress	[135]
100-200 mg kg^{-1}	STZ-induced diabetic Wistar rats	Oral	Controlled insulin resistance, reduced blood sugar, pancreatic cells protection	[136]
5-20 mg kg^{-1}	STZ-induced diabetic rats	Oral	Controlled body weight and blood glucose, performance in the Morris water test	[137]
40 mg kg^{-1}	STZ-induced diabetic mice in the Morris water maze task	Oral	Enhanced the time spent by mice in the target quadrant in the Morris water maze task	[138]

incidence rates of PC were reported in Western Europe (ASR, 8.3), North America (ASR, 7.6), and Central and Eastern Europe (ASR, 7.5). The incidence of PC has men to women ratio of 1.4 : 1.0. More detailed information about the PC incidence and mortality based on the region and sex are presented in the main reference [27].

An earlier diagnosis would be very helpful in the successful treatment of this malignancy, despite the scarce presence of symptoms among individuals. Regarding treatment, surgery, chemotherapy, and radiotherapy are the most common therapeutic strategies applied for PC treatment. Actually, the standard course of treatment is surgery following adjuvant therapy; however, the recurrence of 70-80% of resected tumors ultimately occurs. Patients who are eligible for surgical resection comprise merely almost 10-15% of all patients with advanced PC. With the majority of patients being diagnosed at later stages, chemotherapy remains as only treatment option for PC. 5-Flourouracil (5-FU) and gemcitabine (GMC), alone or in combination with radiation, are the standard chemotherapy regimen for PC's treatment, even though the response rate is usually below 31%. GMC has some advantages over 5-FU, such as the ability to relieve most disease's symptoms and having a modest survival advantage; however, it could not extend the average survival rate much beyond 6 months, like other chemotherapeutics [28, 29]. Hence, with the limited success of current standard therapies, the search for new and effective treatment strategies and agents is urgently needed.

3. Naturally Occurring Phytochemicals for Anticancer Purposes

Various observational and prospective studies have revealed an indirect association between fruit and vegetable consumption with the occurrence of some cancers and the great potential of natural compounds to change the natural history of carcinogenesis [30–32]. Plants with some bioactive nonnutrient compounds isolated, characterized, and identified as phytochemicals have been ever more searched for their ability to treat different diseases, especially cancer [33–41]. It seems that natural products still hold out the best options to find effective novel components in the treatment of human diseases [42]. In addition, the development of scientific technologies such as genome mining, genetic engineering, and using of nanoparticles as carriers [43] improve the discovery of new drugs in cancer therapy [44]. The word 'phytochemical' refers to plant (phyto in Greek) chemicals. Many of these phytochemicals could regulate a wide range of cellular signaling pathways which are involved in oxidative stress, growth, proliferation, differentiation, and death [37, 45–48]. For example, they exhibit antioxidant properties by affecting Nrf2-Keap1 pathway, where upon activation, Nrf2 translocate into the nucleus, binds to ARE (antioxidant response elements) or EpREs (electrophile response elements) and increases the expression of ATP-dependent drug efflux pumps, detoxification enzymes, and endogenous antioxidants [49]. These events eventually lead to the protection of cells against ROS (reactive oxygen species) [50, 51]. Phytochemicals could also suppress tumor progression and induce apoptosis in pre-neoplastic or neoplastic cells by affecting cell cycle, JAK-STAT, NF-κB, and cytochrome C signaling pathways [52, 53]. One of the phytochemicals is garcinol, a Polyisoprenylated Benzophenone that can inhibit STAT-3 pathway by suppressing the upstream kinases (c-Src, JAK1, and JAK2) in HNSCC cells. Garcinol also inhibits NF-κB activation by the suppression of TGF-β and inhibitor of IκB kinase (IKK) activation in HNSCC cells [54]. In addition, Li et al. showed that garcinol prevents the growth of human HNSCC xenograft tumors in male athymic nu/nu mice [54]. It can be concluded that

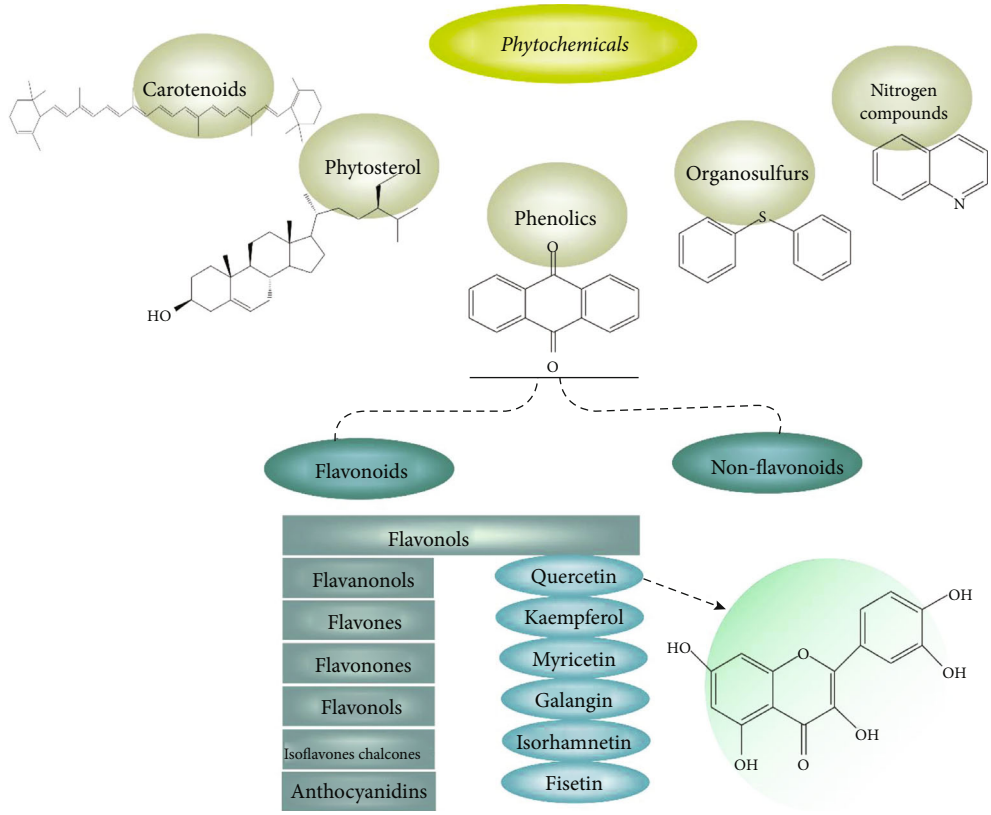

Figure 1: Classified phytochemicals with anticancer potential and their chemical structure.

garcinol has potential antitumor effects in head and neck carcinoma through the suppression of multiple proinflammatory cascades. Activator protein 1 (AP-1) as a key transcription factor in the control of several cellular processes is involved in inflammatory disorders and cancer. Several natural compounds such as kaempferide, resveratrol, apigenin, isorhamnetin, citrifolinoside A, viscolin, curcumin, and quercetin can modulate AP-1-associated signaling pathways for cancer prevention and intervention [55].

Among the plethora of biologically active phytochemicals with anticancer potential, they are chemically categorized into phenolics, carotenoids, phytosterols, organosulfur compounds, and nitrogen-containing compounds [56, 57]. Phenolics are structurally characterized with one (phenolic acids) or more (polyphenols) aromatic rings with one or more hydroxyl (OH) groups [58]. Phenolic compounds can be divided into flavonoids and nonflavonoids [59]. Flavonoids, including glycosides, aglycone, and methylated derivatives, comprise half of phenolic compounds [60]; flavonoids are subgrouped into flavones, flavanones, flavanonols, flavanols, flavonols, isoflavones, chalcones, and anthocyanidins [61, 62]. Nonflavonoids also have several subgroups which include stilbenes, phenolic acids, lignans, coumarins, and tannins [63] (Figure 1).

4. Flavonoids and Anticancer Effects: Key Focus on Quercetin

Belonging to the class of polyphenolic flavonoid compounds and the subclass of flavonols, quercetin is ubiquitous in daily foods, including various plants, vegetables, nuts, seeds, fruits, tea, and red wine [64, 65]. However, fruits and plants are being studied as promising sources of quercetin [17, 66–68]. Quercetin comprises the characteristic structure of flavonoids (backbone C6-C3-C6) in which two benzene rings are bonded by a 3-carbone heterocyclic pyrone [69, 70]. Quercetin has two antioxidant pharmacophores in this structure, which allow to act as a free radical scavenging agent and join to transitional metal ions [69]. The ideal arrangement of the catechol and the OH group at C_3, the position in quercetin structure, also adds to its free radical scavenging ability [69, 71]. The replacement of its various OH groups grants quercetin different biochemical and pharmacological functions [72]. It has been estimated that the average daily intake of quercetin could be about 25 mg [20]. The bioavailability of quercetin relies on its metabolic form in the food [73]. Quercetin may be found as free or aglycone state and conjugated forms, in which it interacts with several molecules, including lipids, carbohydrates, alcohols, and sulfate groups to form its derivatives, including prenylated quercetin, quercetin ethers, quercetin glycoside, and quercetin sulfate [72]. In plants, the form of quercetin is quercetin glucosides (quercetin-glucose conjugates). Quercetin glucosides undergo hydrolysis to form quercetin aglycone following the absorption in the apical membrane of the enterocytes. Then, enterocytic transferases metabolize quercetin aglycone to the glucuronidated, sulfonylated, and methylated forms [73]. These quercetin metabolites when transported to the liver undergo other conjugation processes to generate Que-3-glucuronide and quercetin-3′-sulfate [73–75]. The peak

plasma concentration of quercetin varies from 3.5 to 5.0 μM once being absorbed in the form of glucosides. However, its peak plasma concentration is less than 0.33 μM when absorbed in the unconjugated form, showing less efficient absorption [76].

Quercetin has numerous benefits on human health, including anticancer, antioxidant, antidiabetic, antiulcer, anti-inflammatory, antiviral, antiallergic, antihypertension and anti-infection, cardioprotective, gastroprotective, and immune-modulatory effects [69, 77]. With the specific impact on tumor cells and without any impact on normal and nontransformed ones, quercetin has fascinated many researchers to investigate its potential as an adjuvant to suppress oxidative stress, proliferation, and metastasis [78]. Several studies showed the inhibitory impacts of quercetin against pancreatic, colorectal, prostate, lung, ovarian, nasopharyngeal, breast, and kidney cancers [79–85]. A number of recent clinical studies have scrutinized quercetin's effect on PC. In this regard, Liu et al. [86] have explored the anticancer effects and mechanistic actions of quercetin in GMC-resistant cancer cells. In this survey, BxPC-3, PANC-1 and HepG2, and Huh-7 cell lines were studied. Proliferation assays presented that quercetin had cytotoxic effects on GMC-resistant cell lines including HepG2 and PANC-1, and flow cytometric analysis specified a noteworthy proapoptotic effect on these cell lines. GMC treatment, along with quercetin, caused increased anticancer effects compared with GMC alone, and quercetin led to S phase arrest in resistant cell lines. Hoca et al. [5] investigated the effect of quercetin and resveratrol on epithelial-mesenchymal transition (EMT) of $CD133^+$ and $CD133^-$ pancreatic cancer cells. $CD133^+$ cells were obtained from the PANC-1 cells by the MiniMACS system. $CD133^+$ and $CD133^-$ PANC-1 cells were treated with different concentrations of resveratrol and quercetin. Immunocytochemistry tests using antibodies such as TNF-α, ACTA-2, N-cadherin, IL-1β, and vimentin were applied for assessing the anticancer and antimetastatic properties of resveratrol and quercetin. Results revealed that the immunostaining intensity of $CD133^+$ cells was stronger than that of $CD133^-$ cells. ACTA-2, N-cadherin, and IL-1β immunoreactivities were significantly decreased, whereas vimentin and TNF-α immunoreactivities increased in quercetin treated $CD133^+$ cells. In addition, quercetin was more effective than resveratrol in inhibiting metastasis. Guo et al. [87] have studied the therapeutic potential of quercetin in targeting sonic hedgehog (SHH) signaling of PDA. The effects of quercetin on the apoptosis, migration, and invasion of pancreatic cancer cells (PCCs) were evaluated in PDA xenograft mouse models. According to the results, quercetin inhibited the PCC proliferation by downregulating c-Myc expression and suppressed the EMT by reducing TGF-β1 level, which inhibited the PCC migration and invasion. Quercetin treatment reduced the PDA growth and metastasis in nude mouse models by decreasing SHH activity. Additionally, SHH activated TGF-β1/Smad2/3 signaling and stimulated EMT by inducing the expression of Snail1and Zeb2 that instigated a partial reversal of quercetin-mediated inhibition of PCC migration and invasion.

4.1. Molecular Mechanisms Underlying Quercetin-Mediated Effects in Cancer

4.1.1. Effect in Autophagy and Apoptosis Induction. According to Pang et al. [88], quercetin can affect CD36 and decrease the death rate of PC by facilitating the uptake of fatty acids, improving the cell adhesion, stimulating immune response, and regulating thrombospondin-1. Furthermore, previous trends indicated that quercetin has proapoptotic activity in suppressing Bcl-2 protein and in upregulating the p53 gene; however, inhibition of Bcl-2 transcription could prevent the tumors development [12]. In an illustrative study, Serri et al. [89], have investigated the effect of GMC with biodegradable nanoparticles (NPs) loaded within quercetin on PC cell lines. The manufactured NPs decorated with hyaluronic acid (HA) and loaded with quercetin and GMC presented a developed cytotoxicity on PANC-1 and Mia-PaCa-2 cell lines when compared with the bare drugs and the NPs nondecorated with HA on the surface. The results indicated that, the NPs exposing HA may enhance the anti-inflammatory activity of Que, which led to a reduction of interleukin (IL) expression levels in cell lines and preliminarily increased with lipopolysaccharides (LPS). In another survey, Lan et al. [90] showed that quercetin accelerates cell death and chemosensitivity of human PC cells. The results showed that silencing of a receptor for advanced glycation end products (RAGE) by RAGE-specific siRNA intensified the autophagy and apoptosis through suppressing PI3K/AKT/mTOR axis in MIA Paca-2 and GMC-resistant cells (MIA Paca-2 ^{GMCR} cells). Moreover, quercetin reduced RAGE expression and facilitated the apoptosis, autophagy, and chemosensitivity to GMC in MIA Paca-2 ^{GMCR} cells, which suggests that further cytotoxicity has been achieved by the addition of quercetin in treatment with GMC. Yu et al. [91] showed that quercetin initiated inhibitory activities against PATU-8988 and PANC-1 cells and reduced the release of matrix metalloproteinase (MMP). In this study, they used STAT-3 and IL-6 activation to scrutinize the effects of quercetin treatment on cell malignancy. The MMP secretion and epithelial mesenchymal transition (EMT) stimulated the STAT-3 signaling pathway, while quercetin reversed IL-6-induced EMT and invasion. As main findings, this study showed that quercetin is an effective agent in PC treatment as it blocks the STAT-3 signaling pathway, leading to the suppression of EMT and metastasis. In addition, Nwaeburu et al. [92] have explored the effect of quercetin on miRNA expression in PC cells and concluded that quercetin treatment induced the expression of miR-200b-3p in AsPC1 cell lines, which has a crucial role in the irregular division of PDA cells by notch signaling regulation (Figure 2).

4.1.2. Effect in Proliferation and Cell Growth. Inhibition of PC cell proliferation could signify a distinct mechanism of anticancer effects of quercetin (Table 2). In this way, Pham et al. [93] studied the effect of quercetin on dysregulated epigenetic readers, including bromodomain and extraterminal domain (BET) proteins, in *in vitro* and xenograft models of PC. According to the results, after treatment with BET

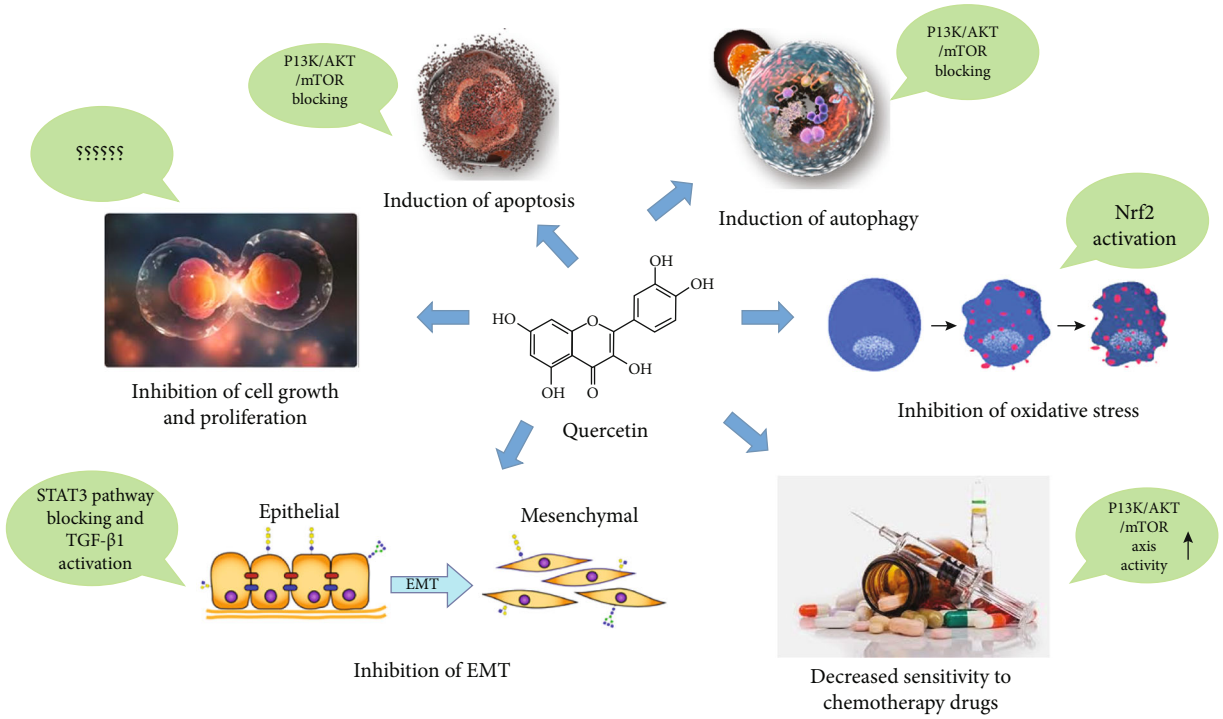

Figure 2: Schematic representation of the molecular structure of quercetin composition and its role in physiological conditions through signaling pathways. The flavonoid quercetin through inhibitory and stimulatory mechanisms performs functions such as inducing autophagy and apoptosis and reducing or inhibiting cell growth and proliferation, EMT, oxidative stress, and sensitivity to chemotherapy drugs.

Table 2: Anticancer effects of quercetin against PC.

Dose	*In vitro*/*in vivo*	Cell line	Effective mechanism	Ref.
100 μM and 75 mg kg^{-1}	*In vivo* and *in vitro*	PANC-1 and Patu8988	EMT suppression by reducing TGF-β1 level, inhibition of growth, invasion, and migration of cells, apoptosis of cancer cells by antagonizing TGF-β/Smad and SHH signaling pathways	[87]
20 μM	*In vitro*	Mia-PaCa-2 and PANC-1	Reduced IL-6 and IL-8 expressions and enhanced cytotoxicity against Mia-PaCa-2 and PANC-1 cell lines	[89]
100 μM	*In vitro*	PANC-1	Reduced immunoreactivities such as ACTA-2, IL-1β, and N-cadherin, increased TNF-α and vimentin, prevention of EMT	[139]
20 μM and 40 mg kg^{-1}	*In vivo* and *in vitro*	PDAC	Improved effects of BET inhibitors at suppressing tumor development and reduced hnRNPA1 *in vivo*	[93]
50-200 μM	*In vitro*	MIA Paca-2, BxPC-3, AsPC-1, HPAC and PANC-1	Quercetin showed a RAGE silencing like effect that attenuate RAGE expression to accelerate apoptosis, autophagy, and chemosensitivity of MIA Paca-2 ^{GEMR} cells	[90]
20-80 μM	*In vitro*	PANC-1 and PATU-8988	Quercetin reversed IL-6-induced EMT by the stimulation of the STAT3 signaling pathway and prevented the migration	[91]
50 μM	*In vivo*	AsPC-1 and PANC-1	Upregulation of miR-200b-3p that promoted the Notch signaling pathway of daughter cells to turn into symmetric	[92]
50 μM	*In vitro*	AsPC-1, CRL-4023, and PANC-1	Notch inhibition by quercetin-induced let-7c and marker progression, upregulation of Numbl, and tumor development reduction	[94]
100 nM	*In vitro*	CFPAC-1 and SNU-213	Suppressed TGF-β- and VEGF-A-induced migratory activity induced at low dosages in CFPAC-1, but not in bFGF-activated SNU-213 cells	[140]

inhibitors and quercetin the proliferation and sphere-forming ability of cancer cells was reduced, and apoptosis stimulated. In addition, quercetin diminished the nuclear protein hnRNPA1 which control mRNA translation and export of antiapoptotic proteins, *in vivo* and increased the BET inhibitors effects at suppressing cells proliferation and tumor growth. In another study, Nwaeburu et al. [94] explored the quercetin influence on PC cells proliferation by the activation of Notch-inhibitor Numbl as let-7c target gene. *In vivo* xenotransplantation of PDA cell and following IV injection of let-7c provoked a noteworthy reduction of tumor mass in the fertilized chick egg model. Immunohistochemistry analysis demonstrated that let-7c upregulated the Numbl and reserved Notch and progression markers. The findings illustrate that Que-induced let-7c declines cancer cell divisions and tumor growth.

4.1.3. Effect in Oxidative Stress. Redox homeostasis is very important for cell function and ROS have an essential role in cell signaling. However, the disturbance in the antioxidant system could lead to excessive intracellular ROS levels, such as hydroxy free radicals and H_2O_2 [95]. The excessive intracellular levels of ROS result in oxidative damages to many biological macromolecules which includes lipids, proteins, and genetic material, giving rise to pathological conditions, like cancer, inflammation, atherosclerosis, angiogenesis, as well as aging [96–100]. Therefore, helping cells to keep redox homeostasis is of great value, which can be provided by consuming natural nutritional components, such as Que.

With the numerous OH groups and conjugated π orbitals allowing it to donate hydrogen or electrons, and thus scavenge superoxide anion ($\bullet O_2-$) and H_2O_2. Quercetin is regarded as outstanding free-radical scavenging antioxidant [101]. Quercetin could generate the semi-quinone radical and H_2O_2 by reaction with $\bullet O_{2-}$, while also decreases H_2O_2 levels in the presence of peroxidases and keeps cells safe against H_2O_2 damages [64]. The semi-quinone is one of potentially harmful reactive oxidation products and undertakes a second oxidation reaction with Que, producing additional quinone (Que-Quinone; QQ) [64]. QQ is held culprit for lipid peroxidation as well as protein and DNA damages with higher affinity to react with lipids, proteins, and DNA [64, 102]. QQ with high reactivity towards thiols could arylate protein thiols, impairing several vital enzymes; however, it generates relatively stable glutathione (GSH)-oxidized adducts including 8-glutathionyl-quercetin (8-GSQ), 6-glutathionyl-quercetin (6-GSQ), and 2′-glutathionyl-quercetin (2′-GSQ) when reduced GSH exists [103, 104]; this reaction is reversible and glutathionyl-quercetin adducts could be constantly disassociated into QQ and GSH [105]. Consequently, high GSH concentrations within cells, oxidized quercetin forms GSQ by reaction with GSH, neutralizing the toxicity of QQ. Yet, oxidized quercetin reacts with protein thiols while lower concentrations of GSH exist within cells, showing the prooxidant effect of quercetin [105, 106]. Therefore, the GSH concentration within cells determines whether the antioxidant effect of quercetin could prevail over its prooxidant effect. Indeed, high levels of GSH limit quercetin cytotoxicity and permit it to show its antioxidant activity but not prooxidant activity [107]. Besides, it has been shown that quercetin induces GSH synthesis [108, 109]. Moreover, quercetin also exerts antioxidant activity by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) as well as its downstream targets, which are vital for maintaining cell redox hemostasis [110, 111].

4.1.4. Effect in Epithelial-to-Mesenchymal Transition (EMT). A physiological process, epithelial-to-mesenchymal transition (EMT), has an key function in mammalian embryonic development and cell and tissue balance; however, it has also an important role in tumorigenesis and tumor progression [112]. During EMT, epithelial cells undergo some changes including losing cellular polarity, disabling junctions between cells and adhesive connections, and obtaining penetration and migration capabilities [113, 114]. EMT can be monitored by protein markers, including E- and N-cadherin, Snail and Vimentin [114, 115]. Furthermore, MMPs closely related to EMT are recently introduced as EMT markers and as a predisposing factor for it, providing an appropriate condition for tumor infiltration and metastasis by degrading the extracellular matrix (ECM) and basement membrane (BM) nearby the tumor exterior [116, 117]. The impact of quercetin on EMT in PC cells have been investigated by a few studies. In one study, it has been shown that quercetin treatment could decrease EMT and MMP secretion in PATU-8688 PC cell line [91]. Quercetin reduced mRNA and protein expressions level of N-cadherin, Slug, Vimentin Zeb1, Twist, and Snail, indicating the potential of quercetin to reverse the EMT process; however, it increased E-cadherin expression [91]. Quercetin also inhibited MMP2 and MMP7 protein expressions [91]. Besides, it has been indicated that quercetin exerted its inhibitory impacts on EMT, invasion, and metastasis in PC cells through suppressing the STAT-3 signaling pathway [91]. Another study showed that quercetin repressed EMT by suppressing SHH and TGF-β/Smad signaling pathways, involved in promoting EMT by the induction of Zeb2 and Snail1 expressions [87]. Quercetin downregulated Vim (encoding vimentin) and Acta2 (encoding α-SMA) gene expressions, and upregulated Cdh1 (encoding E-cadherin) gene expression in PANC-1 and Patu8988 cells; upon quercetin treatment, the protein levels of type I collagen, N-cadherin, α-SMA and vimentin were reduced,; however, the protein level of E-cadherin was increased in cells [87]. Quercetin decreased TGF-β1 expression and that of EMT-TFs (EMT-inducing transcription factors) Snail1 and Zeb2 [87]. EMT-TFs (Snail1 and Zeb2) are the key downstream target of TGF-β1/Smad2/3 signaling pathway suppressing E-cadherin expression [118, 119]. Furthermore, the nuclear translocation and phosphorylation of Smad2 and Smad3 were also suppressed by quercetin [87]. It has been reported that upon activation by TGF-β1 and forming heteromeric complexes with Smad4, Smad2 and Smad3 translocate to the nucleus and induce EMT-TFs' expression [120]. It has also been indicated that quercetin may inhibit EMT in PC stem cells by suppressing the expression of N-cadherin [5]. Quercetin downregulated Twist2 expression, a protein involved in EMT, in PC stem cells [121], suggesting EMT inhibition by quercetin [122].

4.1.5. Effect in Chemo-Sensitivity. By the improved effectiveness in combination with other dietary agents, quercetin has been investigated as a promising adjuvant to increase the effectiveness of numerous chemotherapeutics [122, 123]. Lan, Chen, Kuo, Lu and Yen [90] showed that quercetin may decline cell viability, promote autophagy, and increase apoptosis by suppressing receptors for advanced glycation end products (RAGE) in GMC-resistant PC cells, with a greater impact once accompanied with GMC. The results revealed that RAGE silencing promoted GMC-induced cytotoxicity in MIA Paca-2 and MIA Paca-2 ^{GEMR} cell via the PI3K/AKT/mTOR axis [90]. As RAGE silencing, quercetin reduced the expression of RAGE, which led to cell cycle arrest, apoptosis, autophagy, and promoted GEM efficacy in MIA Paca-2 GEMR cells [90], proposing quercetin quercetin as enhancer of chemotherapy efficacy of drugs against PC. In another study, quercetin promoted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in TRAIL-resistant PC cells [124], and decreased cellular FLICE-like inhibitory protein (cFLIP) expression, while activated c-Jun N-terminal kinase (JNK), leading to the proteasomal degradation of cFLIP and eventually making PC cells more susceptible to TRAIL-induced apoptosis [124]. It has also been reported that quercetin decreased the viability of PC cell lines including PANC-1, MiaPaCa-2, and BxPC-3 [125, 126]. Once combined with other chemotherapeutics, such as GMC or 5-FU, quercetin could affect chemotherapy efficacy depending on cell lines applied, either to suppress proliferation of or have no impact on cancer cells [125, 126]. Borska et al. [127] indicated that quercetin induced apoptosis and suppressed cell proliferation in both daunorubicin sensitive EPP85-181P and resistant EPP85-181RDB PC cell lines. Quercetin had synergistic effects with daunorubicin in both sensitive and resistant cells [127]. They also showed that quercetin treatment could decrease P-glycoprotein expression [128].

5. Conclusion

Food consumption combined with therapeutic agents has been considered a key for the successful treatment of several diseases, including cancer. Conventional therapies like natural components besides other therapeutic methods due to their lower cost and side effects have been increasingly considered by researchers. Specifically, quercetin exerts an anticancer effect against PC cancer cells by mediating apoptosis, but recent studies have also indicated that quercetin affects various signal transduction pathways to reduce cancer progression. Quercetin suppresses the expression of N-cadherin, MMP-9, STAT-3 signaling pathways and potentially inhibits EMT, invasion, and metastasis. Quercetin enhances gemcitabine chemosensitivity in pancreatic cancer cells through the inhibitory effect on RAGE expression. Meanwhile, it has wide accessibility, efficacy and low toxicity comparing with other studied compounds, make it an appealing agent in cancer treatment. More recently, quercetin has been introduced and applied as a promising drug in the treatment of various cancers alone or in combination with other chemotherapeutic agents. Future well-designed clinical studies are needed to help the scientists to evaluate the safety and potential of quercetin against PC.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no competing interests.

Authors' Contributions

Parina Asgharian and Abbas Pirpour Tazehkand contributed equally to this work.

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Review Article

Bioactive Compounds from Kefir and Their Potential Benefits on Health: A Systematic Review and Meta-Analysis

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Despite evidence of health benefits from kefir administration, a systematic review with meta-analysis on bioactive compounds associated with these benefits is still absent in the literature. Kefir is fermented milk resulting from the metabolism of a complex microbiota in symbiosis. Recent researches have investigated the bioactive compounds responsible for the preventive and therapeutic effects attributed to kefir. However, differences in functional potential between industrial and artisanal kefir are still controversial. Firstly, we identified differences in the microbial composition among both types of kefir. Available evidence concerning the action of different bioactive compounds from kefir on health, both from *in vitro* and *in vivo* studies, was subsequently summarized to draw a primary conclusion of the dose and the intervention time for effect, the producer microorganisms, the precursor in the milk, and the action mechanism. Meta-analysis was performed to investigate the statistically significant differences ($P < 0.05$) between intervention and control and between both types of kefir for each health effect studied. In summary, the bioactive compounds more commonly reported were exopolysaccharides, including kefiran, bioactive peptides, and organic acids, especially lactic acid. Kefir bioactive compounds presented antimicrobial, anticancer, and immune-modulatory activities corroborated by the meta-analysis. However, clinical evidence is urgently needed to strengthen the practical applicability of these bioactive compounds. The mechanisms of their action were diverse, indicating that they can act by different signaling pathways. Still, industrial and artisanal kefir may differ regarding functional potential—OR of 8.56 (95% CI: 2.27–32.21, $P \leq .001$)—according to the observed health effect, which can be associated with differences in the microbial composition between both types of kefir.

1. Introduction

Fermentation of a matrix produces kefir. Milk is the matrix generally used, resulting in a beverage acidic, slightly alcoholic, and with a creamy consistency [1]. It results from milk fermentation by microorganisms that live in symbiosis in kefir grains. Kefir differs from other fermented milk because it is a metabolic result of a diversity of microorganisms. Lactose fermenting and nonfermenting yeast species (*Kluyveromyces*, *Pichia*, and *Saccharomyces*), with a predominance of lactic

acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*), besides acetic acid bacteria [2] make up the grain's microbiota.

In recent years, there has been an increase in scientific research on kefir motivated by the association of beverage consumption with therapeutic effects [3]. Regular consumption of kefir has been associated to the reduction of severity of inflammatory bowel disease [4], antihypertensive effect [5], anticarcinogenic effect [6], increased insulin sensitivity [7], improved lipid profile [8], therapeutic effects on osteoporosis [9], and neurodegenerative disease [10]. The positive health effects have been related to the antioxidant capacity [11] and modulation of the intestinal microbiota [12] by the kefir drink. Bioactive compounds present in kefir, produced by microorganisms during fermentation and storage of beverage, have been attributed to these benefits; kefiran, exopolysaccharides, bioactive peptides, and organic acids are the bioactive compounds commonly implicated with the therapeutic potential of kefir [13–17]. However, there is still a need for a deeper discussion about the bioactive compounds present in the kefir drink [13] to distinguish them according to their therapeutic potential for each disease.

In addition, the possible difference in the functional potential between artisanal and industrial kefir is controversial in the literature. The use of kefir grains results in artisanal kefir [18], while previously selected starter culture of bacteria and yeast species leads to commercial or industrial kefir [19]. Some studies have reported artisanal kefir with greater therapeutic potential due to its greater microbiological diversity [20, 21]. In contrast, other studies have described industrial kefir as promising in treating diseases [22], while Ebner et al. [23] have found no significant difference between both. In this context, a meta-analysis could be helpful to elucidate the inconsistencies observed between studies.

Thus, there have been some reports on bioactive compounds from kefir and health benefits in recent years. However, there is still a lack of an overview and in-depth approach in this research field, so a systematic review with meta-analysis will be relevant for this purpose. Therefore, it is necessary to summarize the bioactive compounds from kefir produced by different microorganisms that make up its microbiota, their beneficial effects, action mechanisms, and their precursors in the milk. Based on this knowledge, it will be possible to provide a theoretical basis for developing functional formulations by the food industry to prevent specific diseases. In addition, the pharmaceutical industry may prepare formulations with therapeutic potential from bioactive compounds isolated from kefir. In this scenario, this review summarized the antimicrobial, antioxidant, immune-modulatory, gut microbiota-modulatory, anticancer, antiosteoporosis, antihypertensive, antidiabetic, and lipid profile-modulatory role of the bioactive compounds from milk's kefir. Prevention and treatment of neurodegenerative diseases also were covered. The functional differences between both types of kefir finished this review.

2. Methods

2.1. Focus Questions. The development of the question was according to the population, intervention, comparison, and outcome (PICO) method. The questions formulated were as follows: What are the bioactive compounds in milk kefir and their producing microorganisms? What are the precursors of these bioactive compounds in milk? What are the mechanisms of action of the main bioactive compounds in kefir? Do bioactive compounds differ in terms of concentration and intervention time to obtain the same effect on health? Is artisanal kefir drink more functional than the one produced industrially?

2.2. Data Collection Process and Eligibility Criteria. Two authors (C.P.V and A.P.A.C) independently conducted the preliminary selection of identified abstracts and titles of research articles published in English; we removed the time filter not to limit the number of manuscript resulting. Thus, the initial screening publications covered the period from 1986 to 2021. Abstracts were then removed in this initial screening if the papers did not investigate any of the following health aspects: antimicrobial activity, antioxidant activity, effect on cancer, neurodegenerative diseases, lipid profile, blood pressure, plasma glucose, gut microbiota modulation, inflammation, and osteoporosis both *in vitro*, in situ, *in vivo* animal, and human clinical trials. The criterion used to choose these specific health benefits was that they had been the most investigated in the scientific literature in the last two decades [24–26]. Papers about nonmilk kefir were excluded, including editorials, letters, reviews, commentaries, monographs, preprints, and Ph.D. thesis. Based on the entire reading of the paper, all studies included in the present work were controlled experiments and with a quantitative approach for data analysis. Only studies in which it was possible to determine the bioactive compounds responsible for the observed health effect were included. Studies addressing the microbial composition of kefir grains or starter culture of milk kefir were also included. Some studies considered essential to compose the present work such as those that address conventional therapies, recent reviews on the effect of kefir, and pathology of the diseases studied that was not included in any of the research bases were added to compose our introduction and discussion.

Finally, we summarized information about the type of kefir (artisanal or industrial) from which the bioactive compound was derived, the study model, the definition of the bioactive compound, and its effects compared to the control treatment. The results were reported in agreement with the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) statement.

2.3. Information Sources. Our search protocol strategy used search strings constructed and adapted for five electronic databases: Science Direct, Pubmed, Embase, Web of Science, and Scopus. The initial screening process was conducted from February 2021 to March 2021. Also, directed searching was carried out by checking the reference lists of relevant articles. The research questions were used to summarize the search strings through which the manuscripts were recovered. The string was based on predetermined groups of keywords related to microbial composition of artisanal

and industrial milk kefir, bioactive compounds in the milk kefir, and their effect on health, as follows:

(i) *Search Component 1*. microorganisms OR "microbial composition" OR "kefir grains" OR "starter culture" AND "milk kefir"

(ii) *Search Component 2*. "bioactive compounds" OR "functional compounds" AND "milk kefir"

(iii) *Search Component 3*. "health benefits" OR "health effects" OR "functional effects" AND "milk kefir"

2.4. Risk of Bias Assessment. Possible sources of bias include eligibility criteria, the impact of missing data, missing primary results, chosen database, chosen language (English), and article type selected for our review.

2.5. Meta-Analysis. Two authors (A.I.L.S.R and D.K.A.R) to conduct the meta-analysis extracted data from the included articles. Any inconsistency in retrieved data was solved by discussion. *In vitro*, *in situ*, and *in vivo* papers were pooled for examination. From every publication, each different outcome point was extracted as an independent study. Then, we investigated a statistically significant difference between intervention and control. However, only health effects with an appropriate number of studies were included in the meta-analysis: antimicrobial (including eight groups of microorganisms, totalizing 182 *in vitro* and *in situ* studies from 15 papers), antioxidant (149 *in vitro* and *in situ* studies included in 7 articles), anticancer (44 *in vitro* studies comprising five publications), immune modulation (271 *in vitro* and *in vivo* studies from 11 papers), and microbiota modulation (40 *in vitro* and *in vivo* studies retrieved from 5 publications) effects.

The analyses were conducted evaluating the presence or absence of health benefits for the different outcomes. The definitions of presence/lack of action varied across publications, as different methodologies were used on the selected studies. In this case, the resolution was directed by three authors based on the specific outcome. For example, considering antimicrobial research, if a result was expressed in log UFC, decreasing log UFC by kefir treatment was deemed "presence of action." Similarly, if a study result was defined as an inhibition zone, reducing zone size (mm) by kefir treatment was considered a "lack of action".

In addition, to measure the heterogeneity among studies, I^2 test was used. The I^2 assumes the null hypothesis that all the studies are homogeneous or that each study is measuring an identical effect so that a P value tests this hypothesis. In this scenario, the I^2 statistic describes the percentage of variation across studies due to inconsistency (heterogeneity) rather than sampling error (chance). A significance level of 0.05 was used herein. Studies with $I^2 \leq 50\%$ were considered homogenous. The I^2 was quantified as follows [27]:

$$I^2\,(\%) = \left(\frac{Q - d_f}{Q}\right).100, \tag{1}$$

where I^2 is the inconsistency across studies, Q is the Cochran's heterogeneity statistic, and d_f is the degrees of freedom.

Subsequently, functional differences between artisanal and industrial kefir were investigated for health effects. Both types of kefir were tested: antimicrobial (182 *in vitro* and *in situ* studies) and antioxidant activity (149 *in vitro* and *in situ* studies). Then, studies were analyzed to compare the health effects of consuming artisanal kefir drinks rather than the industrial variety, evaluated by odds ratio (OR) and corresponding 95% confidence intervals (CIs). All analyses were performed using Review Manager 5.4 (Cochrane Collaboration, London, UK).

3. Results

3.1. Literature Search. There were 451 articles identified at Web of Science, 242 at Embase, 180 at Scopus, 107 at Pubmed, and 37 at Science Direct. Still, 8 articles were manually identified by other sources for addition of effect on neurodegenerative disease. Of these, 333 were duplicates or triplicates and were excluded, with 692 papers remaining. After reading the titles and abstracts, 263 papers were selected for the full read, but only 88 met the eligibility criteria (Figure 1). Among the articles read in full, the main reasons for exclusion were as follows: nonidentification of the bioactive compound ($n = 112$) and uncontrolled experiments ($n = 23$). This highlights the need for experiments with a more elaborate experimental design, as well as that identify the bioactive compound responsible for the health effects observed due to the use of kefir.

3.2. Meta-Analysis: Study Selection and Characteristics. Data extraction for meta-analysis consisted of 45 papers. Studies were conducted in Argentina, Brazil, Canada, China, Egypt, Iran, Italy, Japan, Malaysia, South Korea, Thailand, Taiwan, Turkey, the United Kingdom, and the United States of America. The publication year of studies ranged from 2005 to 2021. The concentration of kefir used in the studies varied between 0.01 and 1000 mg/mL. The interventions lasted between 0.5 and 1344 hours.

3.3. Structure and Microorganisms of the Kefir Grains. Kefir is a beverage commonly produced from milk and involves a complex fermentative process from the microbiological diversity in "kefir grains" [28]. Typically, kefir grains are inoculated into milk in proportions of 5% to 10% [29], which gives characteristics such as a creamy fermented milk with a slightly acidic taste depending on the starter culture and mixture of the inoculated microorganisms [1].

The diversity of the "kefir grains" embraces numerous symbiotic interactions between lactic acid bacteria (LAB), acetic acid bacteria, mycelial fungi, and yeasts [2], resulting in an acidic and alcoholic fermentation [30]. These microorganisms present in kefir are interspersed in a matrix composed of proteins and polysaccharides [31]. Macroscopically, this matrix can be characterized as solid, cauliflower-like grains viscous and firm consistency, with a color that varies from white to yellowish [32, 33].

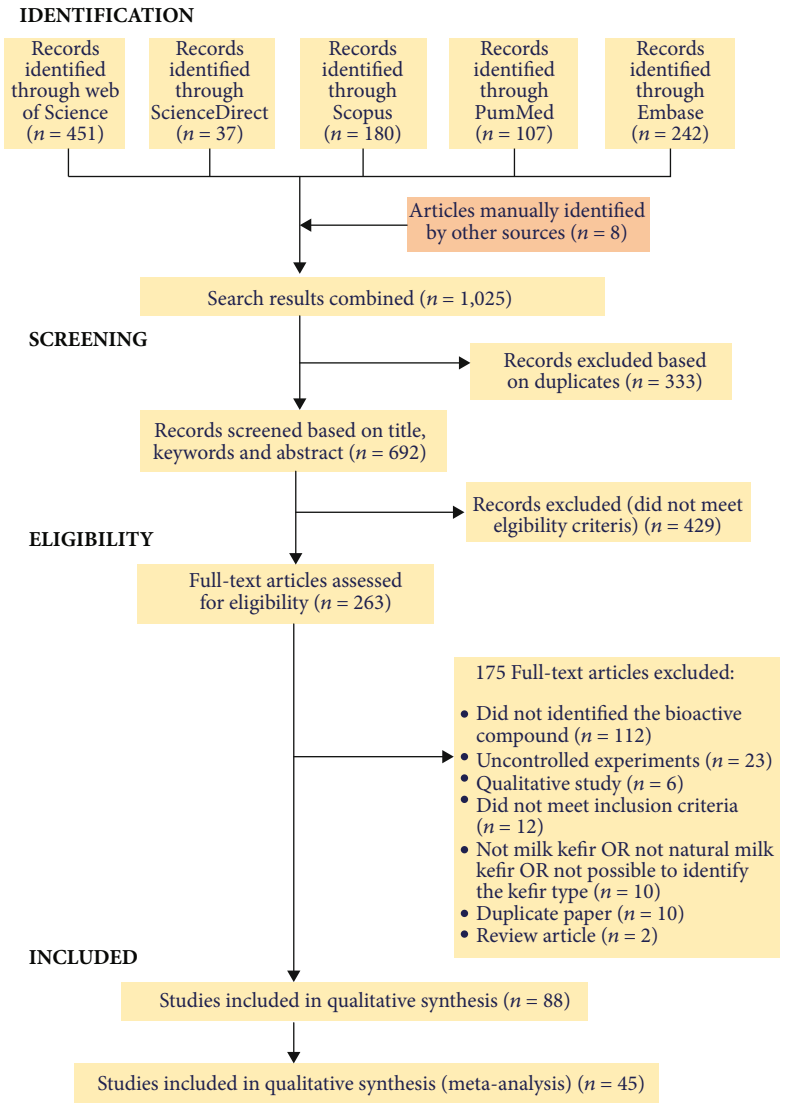

Figure 1: PRISMA flow diagram with results of the systematic search.

According to Khokhlacheva et al. [34], the consortium between microorganisms that evolves an adaptive capacity and enzymatic activity is the key mechanism for their development and survival in milk kefir. In kefir grains, the lactic acid bacteria (LAB) usually present greater levels when compared with acetic acid bacteria. This composition presents itself dynamically and generally changes according to the fermentation time, where the dominance of *Lactobacillus kefiranofaciens* can be observed in the early stages of fermentation, giving rise to the most prominent growth of *Leuconostoc mesenteroides* in the final stages of the process [35]. Several studies around the world are aimed at determining the composition of kefir microbiota (Table 1) since it varies according to geographic, climatic, and cultural factors [33, 36].

The inoculated kefir grains ferment the artisanal kefir [18], while a previously selected starter culture of bacteria and yeast species results in commercial or industrial kefir [19]. According to Table 1, although the *Lactobacillus* and *Lactococcus* genera predominate in both types of kefir, *Enterococcus* was not reported in industrial kefir. Another important aspect can be pointed out in industrial kefir by commercial species not commonly found in artisanal kefir: *Saccharomyces boulardii*, *Lactobacillus lactis*, *Bifidobacterium lactis*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Lactobacillus reuteri*. Korsak et al. [29] corroborated these findings, reporting that industrial kefir is typically composed of selected cultures and conventionally used in dairy products.

Concerning fungal composition, although both types of kefir have presented common species, such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Kazachstania unispora*, the fungal diversity in artisanal kefir was dramatically greater. In addition, *Kazachstania exígua*, *Kazachstania turicensis*, and *Saccharomyces florentinus* were reported only in industrial kefir (Table 1). Other differences between both kefirs include differences in efficiency under conditions of nutrient competition [41], location and adhesion of microorganisms in the structure of kefir grains, or even different abilities of species to grow in milk [18].

However, more microbiota characterization from artisanal and industrial kefir is necessary to define the microbial

Table 1: Microbiological diversity in artisanal and industrial milk kefir.

Type of kefir	Microbial diversity	Source of kefir culture	References
Industrial	*A. syzygii* K03D05, *Lb. plantarum* K03D08	Chile	Dinamarca et al., 2021
	Lb. plantarum CIDCA 83114, *Kl. marxianus* CIDCA 8154, *Streptococcus thermophilus* CIDCA 321	Argentina	Kakisu et al., 2011
	Kz. unispora, *Kodamaea ohmeri*, *Sc. boulardii*, *Sc. cerevisiae*	Malaysia	Azhar et al., 2019
	A. fabarum, *A. orientalis*, *D. anomalus*, *Kl. marxianus*, *Kz. exígua*, *Kz. turicensis*, *Kz. unispora*, *Lb. kefiranofaciens ssp. kefiranofaciens*, *Lb. kefiranofaciens ssp. kefirgranum*, *Lb. kefiri*, *Lb. helveticus*, *Lb. paracasei*, *Lb. parakefiri*, *Lb. reutrei*, *Lc. lactis ssp. cremoris*, *Lc. lactis ssp. lactis*, *Ln. mesenteroides*, *Sc. cerevisiae*	Germany	Nejati et al., 2020
	Lb. lactis, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. casei*, *Sc. florentinus*, *Ln. mesenteroides* subsp. *cremoris*, *Bif. lactis*, *Bif. longum*, *Bif. Breve*, *Lb. acidophilus*, *Lb. reuteri*, *Streptococcus diacetylactis*	Canada	Bourrie et al., 2021
Artisanal	*Aspergillus amstelodam*, *Cordyceps bassiana*, *Fusarium solani*, *Lb. casei*, *Lb. kefiranofaciens*, *Lb. kefiri*, *Lb. mali*, *Lb. paracasei*, *Lb. satsumensis*, *Lc. lactis*, *Lc. lactis ssp. cremoris*, *Lc. lactis ssp. lactis*, *Ln. mesenteroides*	Brazil	Brasiel et al., 2021 [37]; Leite et al., 2015 [32]; Vieira et al., 2017 [38]; Zanirati et al., 2015 [36]
	Enterococcus durans, *Lb. kefiri*, *Lc. lactis*, *Ln. mesenteroides* subsp. *dextranicum*	Taiwan	Chang-Liao et al., 2020
	Lactobacillus sp., *Lb. delbrueckii*, *Lb. kefiri*, *Lb. paracasei*, *Lb. plantarum*, *Lb. sakei*, *Lc. lactis*, *Ln. gelidum*, *Ln. mesenteroides*, *Pediococcus pentosaceus*	Russia	Khokhlacheva et al. 2015 [34]; Mantzourani et al., 2019 [39]
	Cryptococcus sp. *vega*, *Cyberlindnera jadinii*, *Davidiella tassiana*, *Dekkera bruxellensis*, *Dioszegia hungarica*, *Eurotium amstelodami*, *Ganoderma lucidum*, *Heterobasidion annosum*, *Kz. barnettii*, *Kz. Unispora*, *Kl. marxianus*, *Malassezia pachydermatis*, *Microdochium nivale*, *Naumovozyma Castelli*, *Penicillium* sp. *vega*, *Peziza campestres*, *Pichia fermentans*, *Pichia kudriavzevii*, *Sc. cerevisiae*, *Teratosphaeria knoxdaviesii*, *Wallemia sebi*, *Zygosaccharomyces lentus*	Ireland, United Kingdom, United States, Spain, France, Italy, Canada, Germany	Marsh et al., 2013 [33]
	A. fabarum, *A. okinawensis*, *A. orientalis*, *Enterococcus durans*, *Kz. unispora*, *Kl. marxianus*, *Lb. diolivorans*, *Lb. kefiri*, *Lb. kefirofaciens*, *Lb. otakiensis*, *Lb. paracasei*, *Lc. lactis*, *Sc. cerevisiae*	Turkey	Purutoglu et al., 2020
	A. orleanensis, *A. pasteurianus*, *Acidocella aluminiidurans*, *Gluconobacter morbifer*, *Lb. acidophilus*, *Lb. apis*, *Lb. casei*, *Lb. crispatus*, *Lb. delbrueckii*, *Lb. gigeriorum*, *Lb. helveticus*, *Lb. kefiranofaciens*, *Lb. paracasei*, *Lb. rhamnosus*, *Lb. ultunensis*, *Lc. lactis*, *Lent. Kefiri*, *Ln. mesenteroides*, *Streptococcus thermophilus*	South Korea, Ireland, Lithuania, Britain, the Caucuses	Sindi et al., 2020 [40]
	Lactobacillus helveticus	Indonesia	Raras et al. 2019

Table 1: Continued.

Type of kefir	Microbial diversity	Source of kefir culture	References
Artisanal and industrial	*A. syzygii*, *Alternaria tenuissima*, *Bacillus sporothermodurans*, *Cladosporium cladosporioides*, *Didymella negriana*, *Filobasidium magnus*, *Hanseniaspora thailandica*, *Kl. Marxianus*, *Kz. unispora*, *Lb. Kefiranofaciens*, *Lb. parakefiri*, *Lb. plantarum Lc. lactis*, *Ln. pseudomesenteroides*, *Sc cerevisiae*, *Pichia manshurica*, *Pichia orientalis*, *Pichia fermentans*, *Torulaspora delbrueckii Wickerhamiella pararugosa*	Bosnia and Herzegovina	Garofalo et al., 2020 [18]
	A. lovaniensis, *A. orientalis*, *Enterobacter amnigenus*, *Gluconobacter frateurii*, *Gluconobacter cerinus*, *Kz. khefir*, *Kl. marxianus*, *Lb. kefiranofaciens*, *Lb. parakefiri*, *Lb. kefiri*, *Lc. lactis*, *Ln. mesenteroids*, *Naumovozyma* sp.	Belgium	Korsak et al., 2015 [29]

A.: Acetobacter; *Bif.: Bifidobacterium*; *Kl.: Kluyveromyces*; *Kz.: Kazachstania*; *Lb.: Lactobacillus*; *Lc.: Lactococcus*; *Ln.: Leuconostoc*; *Sc.: Saccharomyces.*

similarities and particularities between these two types of milk kefir. This characterization is highly relevant since the beverage's bioactive compound profile is closely related to the producing microorganisms present [42, 43]. The use of kefir grains in the artisanal beverage is correlated to higher counts in the final product and remarkable survival during passage through the gastrointestinal tract, proving significant probiotic properties compared to industrial cultures [42]. Thus, the analysis proposed here may justify possible functional differences between both types of kefir.

Kefir grain's microorganisms can present the ability to produce bioactive compounds during the fermentation and storage of kefir beverages. Consistently, from 48 strains isolated from Russian kefir grains, ten species of *Lactobacillus* sp. were recognized with probiotic potential [39]. Some yeast strains, such as *Saccharomyces cerevisiae* KU200284, present double importance: a starter culture and a probiotic [44]. In Korean kefir, the acetic acid bacterial strain *Acetobacter fabarum* DH1801 had viability as a functional starter with food preservative mechanisms and the potential as a probiotic agent [45]. In addition, the species of LAB has a fundamental role in the formation of exopolysaccharide (EPS), which is a significant bioactive compound in kefir [37]. In this scenario, the *Lactobacillus kefiranofaciens* is considered the main piece in the formation of kefir grains [18] since its genes demonstrate a great capacity to produce exopolysaccharides (such as kefiran) which make up the structure of the kefir grain [43]. Similarly, *Lactococcus lactis* ssp. *cremoris* MRS 47 does found for Vieira et al. [38] to be capable of producing conjugated linoleic acid (CLA), a bioactive compound, from milk fat.

3.4. Bioactive Compounds from Kefir and Their Effects on Health

3.4.1. Antimicrobial Activity. The antimicrobial activity of kefir was mainly attributed to exopolysaccharides (EPSs), specially kefiran, and organic acids, especially lactic acid. However, bioactive peptides with antimicrobial activity have also been identified (Table 2).

Regarding the producing microorganisms, kefiran and EPS were produced by *Lactobacillus kefiranofaciens*, while bioactive peptides were synthesized by *Lactobacillus paracasei*, species belonging to the *Lactococcus* genus and yeast. Biofilms and S-layer proteins, turn on, were produced by *Lactobacillus plantarum* and *Lactobacillus kefir*, respectively (Table 2). It demonstrates that microorganisms of the *Lactobacillus* genus are relevant for the production of bioactive compounds with antimicrobial activity in kefir. In addition, among the 18 articles selected from the systematic review addressing the antimicrobial effect of the compounds, only 1 of them investigated the precursor of the compound in milk; thus, the investigation of the precursors of antimicrobial compounds in kefir is currently necessary. *In vitro* antimicrobial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus* was attributed to bioactive peptides produced from β-casein, k-casein, αs1-casein, and αs2-casein present in the milk [64].

Kefiran has demonstrated an antimicrobial effect for *Streptococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus faecalis*, and *Fusarium graminearum* [61, 69, 71]. In addition, kefiran also had action on fungi (*Aspergillus flavus* AH3) producing aflatoxins; a decrease from 100% to 33.3% of aflatoxin B1 production accompanied a decline in the mycelial dry weights [71]. Still, kefiran presented a more dramatic antimicrobial effect on *E. coli* than *S. aureus*, which can be attributed to the peptidoglycan in the cell wall of the latter. Peptidoglycan, in turn, hinders the diffusion of the antimicrobial through the cell. In addition, reports suggest that kefiran can reduce the concentration of antibiotics needed to obtain the antimicrobial effect, as illustrated by its synergism with ciprofloxacin [69]. It is interesting since extended administration of ciprofloxacin results in gastric and intestinal side effects [69].

Still, given the promising results of kefiran as a natural antimicrobial potential, its extraction methods from kefir have also been studied to maximize its antimicrobial effects.

Table 2: Effect and conditions of intervention with kefir bioactive compounds for different health benefits.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
Anticarcinogenic	*In vitro*: MTT assay of MCF7 human breast cancer cell line	Kefiran	-	500 to 4000 μg/mL	48 h	Cell viability		-	[46]
						500 to 2000 μg/mL	↓up to 45%		
						4000 μg/mL	Without effect		
					72 h	500 μg/mL	Without effect		
						1000 to 4000 μg/mL	↓up to 15.6%		
	In vitro: MTT assay of HT-29 human colon cancer cell line	Exopolysaccharide MSR101	*Lactobacillus kefiri* MSR101	50 to 400 μg/mL	24 h	Cell viability	↓up to 55.9%	Upregulates the expression of apoptosis-related genes (Cyto-c, BAX, BAD, caspase 3, caspase 8, and caspase 9) in HT-29 cells	[47]
	In vitro: MTT assay of HepG2 human hepatocellular carcinoma cell line and HeLa human cervical carcinoma cell line	Kefiran	*Lactobacillus kefiranofaciens*	15.6 to 1000 μg/mL	24 h	Cell viability HeLa cells	↓up to 72%	Adversely affected the morphological characteristics of HeLa and HepG2 carcinoma cell lines	[48]
						HepG2 cells			
						250 to 1000 μg/mL	↓up to 82%		
						Below 250 μg/mL	Without effect		
	In vitro: fecal water-induced DNA damage assay in HT-29 human colon cancer cell line	Lactic and acetic acids	-	20 to 200 μL/mL	30 min (preincubation)	DNA damage		Antioxidant activity	[49]
						200 μL/mL	↓ 20%		
						Below 200 μL/mL	Without effect		
	In vitro: cell nuclei counting assay of MCF7-E3 human breast cancer estrogen-sensitive cells	Bioactive peptides	-	0.31 to 10% (v/v)	6 days	Cell number	↓up to 88%	-	[15]
Anti-inflammatory	*In vivo*: DSS-induced acute colitis model in male wild-type C57BL/6 mice; chronic colitis model aggravated by Piroxicam in IL10$^{-/-}$ C57BL/6 mice	Extracellular vesicles (PRCC-1301 EVs)	*Lactobacillus kefirgranum* PRCC-1301	0.03 and 3 mg/kg bw/day	Acute colitis: from 2 days before administration of DSS to 5 days	Acute colitis		Promoting the intestinal barrier integrity through the expression of occlusion proteins (ZO-1, claudin-1, and occludin) in colon epithelial cells and inhibition of the NF-κB signaling pathway in the distal and proximal colon in an acute colitis model	[50]
						Body weight	↑16%		
						Colon length (3 mg/kg bw)	↑29.6%		
						Histological score	↓up to 63%		
					Chronic colitis: from 14th to 28th day (Piroxicam from day 0 to day 14)	Chronic colitis			
						Body weight	Without effect		
						Colon length (3 mg/kg bw)	↑14.3%		
						Histological score	↓up to 85.3%		
	In vitro: RAW264.7 cell line (murine macrophages)	Exopolysaccharide R-17-EPS	*Lactobacillus pentosus* LZ-R-17	50 to 400 μg/mL	24 h	Macrophage cell viability	↑up to 38%	-	[51]
						Phagocytosis index	↑up to 44%		
						Acid phosphatase activity	↑up to 78%		
						NO production	↑up to 97.2%		
						TNF-α			
						50 and 400 μg/mL	Without effect		

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
						100 to 200 μg/mL	↑up to 10.2%		
						IL-6			
						50 to 200 μg/mL	↑up to 16.7%		
						400 μg/mL	↓ 9.5%		
						IL-1β			
						50 and 400 μg/mL	Without effect		
						100 to 200 μg/mL	↑up to 14%		
						IL-10			
						100 to 400 μg/mL	↑up to 6.4%		
						50 μg/mL	↓ 6.4%		
	In vitro: DSS-induced acute colitis model in Caco-2 cell line cultures (human intestinal epithelial cells)	Extracellular vesicles	*Lactobacillus kefirgranum* PRCC-1301	100 μg/mL	6 h	Cytokine gene expression:		-	Kang et al. (2020) [50]
						IL-2	↓58.3%		
						IL-8	↓64.3%		
						TNF-α	↓67%		
						Macrophage cell viability	↑up to 19%		
						Phagocytosis index	↑up to 35.9%		
						Acid phosphatase activity	↑up to 44%		
						NO production	↑up to 44.4%		
						TNF-α			
						50 μg/mL	Without effect		
	In vitro: RAW264.7 cell line (murine macrophages)	Exopolysaccharide (R-5-EPS)	*Lactobacillus helveticus* LZ-R-5 from Tibetan kefir	50 to 400 μg/mL	24 h	100 to 200 μg/mL	↑up to 25.5%	-	[52]
						400 μg/mL	↓27.5%		
						IL-6	↑up to 54.3%		
						IL-1β			
						100 and 200 μg/mL	↑up to 20.5%		
						50 and 400 μg/mL	↓up to 13.6%		
						IL-10			
						50 to 200 μg/mL	↑up to 18.2%;		
						400 μg/mL	Without effect		
	In vitro: PBMC culture isolated from human total peripheral blood	Kefiran	-	1000 and 5000 μg/mL	48 h	IL-6	Control: not detected Treatment:	-	Jenab et al. (2020) [46]
						1000 μg/mL	220 ng/L		
						5000 μg/mL	270 ng/L		
				500 to 4000 μg/mL	24 h, 48 h, 72 h, and 96 h	PBMC viability: 24 h			

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
						500 to 1000 μg/mL	Without effect		
						2000 to 4000 μg/mL	↑up to 200%		
						48 h to 96 h	Without effect		
	In vitro: TNF-α-induced intestinal inflammation model in Caco-2 cell line	Extracellular vesicles (80 to 400 nm)	*Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum*	1×10^9 extracellular vesicles/mL (A strains mix ratios: 1 : 1 : 1)	24 h	IL-8 mRNA level	↓up to 65.6%	Reducing the phosphorylation of p65, a subunit of NF-κB	[53]
						IL-8 Secretion	↓up to 96.8%		
	In vitro: a cell-free system containing sodium nitroprusside (10 mM)	Kefiran	-	5000 to 10.000 μg/mL	2.5 h	NO radical production	↓up to 40.91%	Nitric oxide radical scavenging capacity of kefiran	[54]
	In vitro: FliC-induced intestinal inflammation model in Caco-2 cell line	Exopolysaccharide	*Lactobacillus paracasei* CIDCA 8339, CIDCA 83123, and CIDCA 83124 strains	*L. paracasei* suspensions (OD_{590} 0.25)	1 h (preincubation)	Promoter induction: CCL20	↓up to 55%	-	[13]
						Body weight	↑up to 12.5%		
	In vivo: TNBS-induced inflammatory bowel disease in Balb/c mice	Extracellular vesicles	*Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, and *Lactobacillus kefirgranum*	3×10^8 and 3×10^{10} extracellular vesicles/head/day (A strain mix ratios: 1 : 1 : 1)	10 days	Rectal bleeding severity	↓up to 75%	Blocking MPO activation in mouse serum	Seo et al. (2018) [53]
						Diarrheal conditions	↓up to 91.4%		
						Histopathological damage	↓up to 85%		
	In vitro: cell-free system	Polysaccharide extract	-	5000 μg/mL	72 h	Hyaluronidase inhibition	↓up to 35%	-	[55]
	In vitro: intestinal inflammation model in Caco-2 cell line induced by IL-1β, TNF-α, or FliC	Lactate	-	100 mM	30 min (preincubation)	Promoter inhibition CCL20		Inhibition of the NF-κB signaling pathway	[56]
						FliC-induced	↓78%		
						IL-1β-induced	↓80%		
						TNF-α-induced	↓42%		
	In vitro: peritoneal macrophages isolated from six-week Balb/c female mice	Protein (*molecular mass* > 30 kDa)	*Lactobacillus kefiranofaciens* M1	5 μL of kefir supernatant/mL	24 h	Secretion		-	[57]
						TNF-α	↑1000%		
						IL-1β	↑700%		
						IL-6	↑1300%		
						IL-12	↑3000%		
	In vivo: Six- to 8-week-old BALB/c female mice	Kefiran	*Lactobacillus kefiranofaciens*	100 mg/kg bw/day	2, 5, or 7 days	Small intestine Mucosa		-	[58]
						IgA			
						IL-10	↑up to 50%		
						IL-6	↑up to 22%		
						IL-12	↑up to 33%		
						Fluid			

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
						IL-4	↑up to 164%		
						IL-12	↑up to 67.5%		
						Large intestine			
						IgA	↑up to 43%		
						IgG	↑up to 41.7%		
						IL-4	↑up to 44.4%		
						IL-10	↑up to 47.2%		
						IL-6	↑up to 30%		
						IFN	↑up to 21.2%		
						TNF	↑up to 20%		
						Serum:			
						IL-4	↑ up to 209%		
						IL-6	↑ up to 254%		
						IL-10	↑ up to 74.5%		
						IFN	↑ up to 170%		
	In vivo: cotton-induced granuloma in Wistar rats	Kefiran	-	1 mL kefir suspension/day	7 days	Granuloma weight	↓44%	-	[16]
[59]	*In vitro*: *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Bacillus cereus* (BC), *Staphylococcus aureus* (SA), *Staphylococcus epidermidis* (SE), *Escherichia coli* (EC), clinical isolates *Proteus mirabilis* (PM), and *Listeria monocytogenes* (LM)	Lactic acid	Cow milk kefir: mesophilic aerobic bacteria, yeast, *Lactobacillus*, and *Lactococcus* Donkey milk kefir: mesophilic aerobic bacteria, yeast, *Lactobacillus*, *Lactococcus*, and *Leukonostoc*	Cow milk kefir: 0.90% (w/w) Donkey milk kefir: 0.80% (w/w)	24 h	Microorganism growth Cow's milk kefir BC KP SA SE LM PA EC PM Donkey milk kefir BC KP SA SE LM PA EC PM	 ↓132.33% ↓72.05% ↓33.33% ↓31.89% ↓16.66% Without effect Without effect Without effect ↓183.33% ↓17.39% ↓4.34% ↓16.89% ↓58.33% Without effect ↓33.33% ↓58.33%	-	[60]
	In vitro: *Escherichia coli* (EC), *Salmonella* Typhimurium (ST), and *Staphylococcus aureus* (SA)	Lactic, acetic, and pyruvic acids	*Acetobacter orientalis*, *Lactococcus lactis*, *Lactobacillus gallinarum*,	25, 50, 75, and 100% (v/v)	48 h	Microorganism growth EC	 ↓100%	-	[14]

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
			Kazachstania unispora, *Pichia kudriavzevii*, *Galactomyces candidum*, *Geotrichum bryndzae*, *Lactobacillus kefiri*, and *Saccharomyces cerevisiae*			ST	↓100%		
						SA	↓98.6% to 100%		
						Zone of inhibition Kefiran extracted by hot water			
						EC	↓41.6%		
						PA	↓51.3%		
						SF	↓60.2%		
						AS	↓61.6%		
	In vitro: *Staphylococcus aureus* (SA), *Streptococcus faecalis* (SF), *Pseudomonas aeruginosa* (PA), and *Escherichia coli* (EC)	Kefiran	-	1% (w/v)	24 h	Kefiran extracted by ultrasound			[61]
						EC	↓33.7%		
						PA	↓43.0%		
						SF	↓50.4%		
						AS	↓51.4%		
						Kefiran (hot water +ultrasound)			
						EC	↓23.6%		
						PA	↓32.8%		
						SF	↓42.9%		
						AS	↓43.4%		
	In vitro: *Pseudomonas aeruginosa* (PA) and methicillin-resistant *Staphylococcus aureus* (MRSA)	FK-1000 (composed of sugars and amino acids)	*Lactobacilacea*, *Acetobacteraceae*, *Pseudomonadacea*, *Streptococcaceae*, *Leuconostocaceae*, *Enterobacteriaceae*, *Alphaproteobacteria*, *Aeromonadaceae*, and *Pseudomonadales*	200 μL/well of Muller Hinton	18 h	Microorganism growth pH 5		-	[62]
						MRSA	↓100%,		
						PA	↓100%		
						pH 7			
						MRSA	↓83%		
						PA	Without effect		
	In vitro: *Pseudomonas putida* extracted from spoiled chicken	Fraction with *MM* < 6000 in the supernatant	*Lactobacillus paracasei FX-6*	0.078%, 0.156%, 0.3125%, 0.625%, 1.25%, 2.5%, 5.0%, and 10% (w/v)	21 h	Microorganism growth *Pseudomonas putida* 1.25% (w/v)	↓13.5% to 98%	Performance on the plasma membrane, DNA and proteins of pathogenic microorganisms	[63]
	In vitro: *Escherichia coli* (EC), *Lactobacillus plantarum* (LP), *Micrococcus luteus* (ML), *Listeria monocytogenes* (LM), *Salmonella enteritidis* (SE), *Staphylococcus*	Bacteriocin	-	100, 150, 200, and 250 μL/wells with a diameter of 7 mm	24 h	Zone of inhibition LP	↑up to 15 times	-	[40]
						ML	↑up to 20 times		

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
	aureus (AS), *and Bacillus cereus* (BC)					BC LM	↑up to 7 times ↑up to 14 times		
						SA	↑up to 8 times		
						SE	↑up to 10 times		
						EC	Without effect		
						Antimicrobial activity			
						SA	↑up to 100%		
						EC	↑80% to 100%		
	In vitro: *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA), *Enterococcus faecalis* (EF), *Bacillus cereus* (BC), *Bacillus subtilis* (BS), *and Staphylococcus aureus* (SA)	Bioactive peptides	*Lactobacillus*, *Lactococcus*, and yeast	2.5% (w/v)	24 h	KP	↑up to 99.98%	-	[64]
						EF	↑94.5%to100%		
						BS	↑up to 75.2%;		
						PA	↑34.7% to 51%		
						BC	↑up to 12.2%		
						Microorganism growth 0.3% (w/v)			
	In vitro: *Listeria monocytogenes* (LM) and *Salmonella* enteritidis (SE)	Exopolysaccharide DN1	*Lactobacillus kefiranofaciens* DN1	0.35, 1% and 2.5% (w/v)	24 h	LM	↓56%	-	[65]
						SE 1% and 2.5% (w/v)	↓5.45%		
						LM	↓100%		
						SE	↓100%		
						Microorganism growth 50-100% (v/v)			
	In vitro: *Escherichia coli* (EC), *Bacillus cereus* (BC), and *Salmonella* enteritidis (SE)	Lactic Acid	-	25, 50, 75, and 100% (v/v)	24 h	EC	↓31% to 99%	-	[66]
						SE	↓up to 98.7%		
						BC 25% (v/v)	↓up to 87%		
						SE	↑6%		
						BC	↑24%		
	In vitro: methicillin-resistant *Staphylococcus aureus* (MRSA) (S547)	Biofilms	*Lactobacillus plantarum*	10^6 CFU/mL	12, 24, 36, 48, 60, 72, and 84 h	Microorganism growth MRSA	↓1.4% to 30%	Inhibition of biofilm production by MRSA	[67]
	In vitro: *Escherichia coli* (EC)	F1 bioactive peptide	*Lactobacillus paracasei* subsp. *Tolerans FX-6*	0.00625% (w/v)	20 h	Microorganism growth EC	↓33% to 57%	Damage to the outer and inner cell membrane with extravasation of potassium ions and cytoplasmic β-galactosidase; binding to the bacterial DNA	[68]
	In vitro: *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP),	Kefiran	*Lactobacillus kefiranofaciens*	1% (w/v)	13 h	Microorganism growth		-	[69]

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
	Pseudomonas aeruginosa (PA), *Salmonella* Typhymurium (ST), and *Staphylococcus aureus* (SA)					EC	↓up to 25.2%		
						PA	↓up to 19%		
						KP	↓up to 9.2%		
						ST	↓up to 3.3%		
						AS	↓up to 2%		
	In situ (whey fermented with kefir grains): *Aspergillus flavus* (AFL), *Penicillium crustosum* (PC), *Aspergillus terreus* (AT), *Aspergillus Fumigatus* (AF), *Trichoderma longibrachiatum* (TL), *Rhizopus sp.*, and *Aspergillus parasiticus* (AP)	Lactic and acetic acids	-	95% (v/v)	24 h	Microorganism germination		-	[70]
						Rhizopus sp.	↓70%		
						AP	↓67		
						TL	↓61%		
						AF	↓60%		
						AFL	↓34%		
						PC	Without effect		
						AT	Without effect		
	In vitro: spent culture supernatant (SCS) obtained from *Clostridium difficile*	*Thermolabile fraction* > 10 kDa in the supernatant	-	OD_{550} = 1	1 h	Biological activity of *C. difficile* SCS on Vero cells	↓80%	-	
						Supernatant from *Lactococcus lactis* subps. *lactis* or from a mixture of all microorganisms of kefir			
						Supernatant from *Lactobacillus kefir*, *Lb. plantarum*, *Saccharomyces cerevisiae*, and *Kluyveromyces marxianus*	Without effect		
	In vitro: *Streptococcus faecalis* (SF), *Pseudomonas aeruginosa* (PA), *Salmonella* Typhi (STP), *Bacillus subtilis* (BS), *Bacillus cereus* (BC), *Escherichia coli* (EC), *Klebsiella pneumoniae* (KP), *Staphylococcus aureus* (SA), and *Fusarium graminearum* (FG) *Aspergillus flavus* AH3 (mycelial dry weights and aflatoxin B1)	Kefiran	-	0.1 mL/5 mm diameter paper disks 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10% (v/v)	24 h for bacteria and yeasts and 7 days for fungi 10 days	Zone of inhibition		-	[71]
						STP	↑75%		
						SF	↑66.6%		
						BC	↑62.5%		
						PA	↑50%		
						BS	↑44.4%		
						EC	↑37.5%		
						SA	↑16.6%		
						FG	↑15.4%		
						KP	↑9.09%		
						Aflatoxin B1	↓33.3% to 100%		

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
Antioxidant	*In vitro*: *Salmonella Typhimurium* (ST), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), and *Staphylococcus aureus* (SA)	Lactic acid (partial effect)	-	0.9% (w/w)	24 h	Mycelial dry weights	↓14.9% to 100%	-	[17] – Industrial kefir
						Zone of inhibition			
						ST	↓60%		
						SA	↓56.6%		
						EC	↓56.5%		
						PA	Without effect		
					48 h	ST	↓41.4%		
						SA	↓42.1%		
						EC	Without effect		
						PA	Without effect		
					7 days	ST	↓1.67%		
						SA	Without effect		
						EC	Without effect		
						PA	Without effect		
	In vitro: *Salmonella* enteritidis (SE)	S-layer proteins	*Lactobacillus kefir* strains CIDCA 8344 and CIDCA 8348	2×10^8 CFU/mL	4 h	Microorganism growth S-layer proteins from *L. kefir* 8348		-	[72]
						Salmonella enteritidis	↓up to 99.96%		
						S-layer proteins from *L. kefir* 8344			
						Salmonella enteritidis	↓50% to 99.93%		
	In situ (kefir-fermented milk): spores and vegetative cells de *Bacillus cereus* and toxin production by *B. cereus*	Organic acids	-	1% and 5% (w/v)	24 h	1% (w/v)		Reduction of the biological activity of the pathogenic microorganism	[31]
						Vegetative cells	↓70% to 98%		
						Number of spores	↓up to 80 times		
						5% (w/v)			
						Vegetative cells	↓70% to 99.8%		
						Number of spores	↓from 80 to 50,000 times		
	In vitro: 2,2-diphenyl-1-picrylhydrazyl—DPPH; reduce Fe^{3+} to Fe^{2+} (FRAP)	Kefiran	-	0.08%, 0.04%, 0.02%, 0.01%, and 0.005% (w/v)	30 min	DPPH	↓25 to 85%	-	[61]
						FRAP	↓37 to 84%		
	In situ (cow milk kefir): 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid—ABTS; oxygen radical absorbance capacity—ORAC	Bioactive peptides	-	-	-	ABTS	↑35.8%	The antioxidant effect cannot be attributed to the ion chelating ability	[14]
			-	20 μL/well (v/v)	80 min	ORAC	↑111.6%		
		Kefiran	-	1% and 0.5% (w/v)	20 min				[54]

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
	In vitro: ascorbic acid equivalent reducing capacity (AAEC)					Ascorbic acid equivalent reducing capacity	4.44 μg/mL (hyaluronic acid) Control 0.5% (w/v) 1% (w/v) Without effect Treatment	Metal chelating activity and sequestering activity of hydroxyl and superoxide radical	
	8.47 μg/mL								
	In situ (cow and ewe milk kefirs)					Ewe milk kefir			
	2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid—ABTS	Total phenolic compounds	-	5% (v/v)	3 min	ABTS	↓4.6% to 46%	-	[73, 74] – Industrial kefir
						DPPH	↓20% to 50%		
				8.3% (v/v)	30 min	FRAP	↑24% to 134%		
	2,2-Diphenyl-1-picrylhydrazyl—DPPH			8.3% (v/v)	30 min	Cow milk kefir			
						ABTS	↓52% to 70%		
	Reduce Fe^{3+} to Fe^{2+} (FRAP)					DPPH	↑70% to 220%		
						FRAP	↑13% to 120%		
				0.625% (w/v)	6, 30, 60, 90, 150, and 180 min	ABTS	↑2% to 25.6%		
						DPPH			
	In vitro 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid–ABTS 2,2-Diphenyl-1-picrylhydrazyl–DPPH	Bioactive peptides	-	2.5%, 1.25%, 0.62, and 0.31% (w/v)	120 min	2.5% peptides 1.25% peptides	↓2.64% to 17.7%	-	[64]
						7 days	↑2.99%		
						14 days	↓0.3% to 6.32%		
						0.62% peptides			
						7 days	↓1.15% to 11.03%		
						From 14 days	↑13.22% to 18.85%		
						0.31% peptides	↓17.0% to 30.0%		
	In vitro: 2,2-diphenyl-1-picrylhydrazyl—DPPH	Exopolysaccharides	*Bacillus amyloliquefaciens*, *Uncultured Bacillus* sp. *clone*, *Bacillus subtilis*, *Bacillus subtilis*, *Bacillus tequilensis*, and ↓*Bacillus siamensis*	1 mL (10^9 CFU/mL)/3 mL	30 min	Antioxidant activity	↑10% to 20%	Resistance to hydrogen peroxide	[73]
	In vitro: 2,20-azobis(2-methypropionamidine) dihydrochloride)—APPH—in bovine serum albumin (BSA)	Exopolysaccharides	*Acetobacter okinawensis*, *Leuconostoc pseudomesenteroides*, and *Kazachstania unispora*	0.05%, 0.1%, 0.15%, 0.20%, and 0.25% (w/v)	360 min	AAPH-oxidized BSA protein	↓31% to 96%	-	[28]
	In situ (cow milk kefir): 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid—ABTS	Exopolysaccharides	-	0.00113% (w/v)	6 min	Antioxidant activity	↑8.43%	-	[74]

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
ORAC	*In situ* (goat milk kefir): 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid—ABTS; oxygen radical absorbance capacity—ORAC	Phenolic compounds	-	38.93, 61.60, 26.62, and 123.23% (w/v)	-	ABTS ↑10% to 40%	↑62% to 120%	-	[75]
Lipid accumulation in adipocytes								exopolysaccharides isolated from their producer microorganisms	
0.01 mg/dL									
LMDH4				*Lactobacillus kefiri* (LKDH1, LKDH3, and LKDH5 strains) and *Leuconostoc mesenteroides* (LMDH4, LMDH6, LMDH7, LMDH8, and LMDH9 strains)	0.01 mg/mL, 0.1 mg/mL, and 0.2 mg/mL of	↓12%			
Other strains						Without effect			
0.1 mg/dL									
LMDH4		*In-vitro*: exopolysaccharides (EPSs) in DMEM medium containing 3T3-L1 adipocytes	Exopolysaccharides			↓22%	-	[76]	6 days
LMDH7						↓24%			
LKDH5						↓14%			
Other strains						Without effect			
0.2 mg/dL									
LMDH7	Cholesterol					↓28%			
LDMH4						↓22%			
LKDH3						↓22%			
LKDH5						↓19%			
LMDH6						↓10%			
Other strains		Without effect							
In vivo: C57BL/6J high-fat and high-fructose diet-induced obese mice and with oral administration of heat-killed lactic acid bacteria (HLAB) from kefir		Exopolysaccharides	*Leuconostoc mesenteroides* LMDH4 and *Lactobacillus kefiri* LKDH5	10 mL/kg bw of a mixture *Leuconostoc mesenteroides* LMDH4 (1×10^{10} CFU/mL) and *Lactobacillus kefiri* LKDH5 (1×10^{9} CFU/mL)	8 weeks	Total cholesterol HDL LDL Triglyceride Adipose tissue weight	Without effect Without effect Without effect Without effect ↓36%	Reduction in the proinflammatory genes expression (*Hp* and *Wfdc21*) and genes (*Fabp4* and *Fasn*) related to the synthesis of fatty acids in the adipose tissues	[76]
In vitro: *Lactococcus lactis WH-C1* from kefir in GM17 broth supplemented with cholesterol (30 mg/100 mL)	Exopolysaccharides	*Lactococcus lactis* WH-C1	4% (v/v) inoculum *Lactococcus lactis WH-C1* from Tibet kefir grains	24 h	Cholesterol in the medium	↓up to 31.23%	-	[77]	

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
Blood pressure	*In situ* (cow milk kefir): assay of inhibitory activity on angiotensin-converting enzyme (ACE)	Bioactive peptides	-	11.2 mg/100 mL	24 h	ACE activity	↓98.4%	-	[14]
Glucose	*In vivo*: C57BL/6J high-fat and high-fructose diet-induced obese mice and with oral administration of heat-killed lactic acid bacteria (HLAB) from kefir	Exopolysaccharides	*Leuconostoc mesenteroides* LMDH4 and *Lactobacillus kefiri* LKDH5	10 mL/kg bw of a mixture *Leuconostoc mesenteroides* LMDH4 (1×10^{10} CFU/mL) and *Lactobacillus kefiri* LKDH5 (1×10^{9} CFU/mL)	8 weeks	Plasma glucose	Without effect	-	[76]
	In vivo: Streptozotocin-nicotinamide- (STZ-NA-) induced diabetic rats/male rats of 8-12 weeks old	Alcohol and phenolic compounds	-	5 to 20 mL kefir with black rice extract (1 : 1)/kg bw	4 weeks	Number of Langerhans islet	↑up to 199%	Proton-radical scavenging activity	[78]
						Insulin-positive β-cells	↑up to 2330%		
Intestinal microbiota modulation	*In vitro*: fecal samples from healthy children aged between 8 months and 3 years old	Exopolysaccharides 8339 and 83124	*L. paracasei* CIDCA 8339 and CIDCA 83124	0.3% (w/v)	72 h	*L. paracasei* CIDCA 8339		-	[79]
						Lentisphaerae	↑32%		
						Firmicutes	↑12%		
						Victivallis	↑33%		
						Acidaminococcu	↑15%		
						Comamonas	↑6%		
						Proteobacteria	↓31%		
						Bacteroidetes	↓11%		
						Actinobacteria	↓1.5%		
						L. paracasei CIDCA 83124			
						Proteobacteria	↑15%		
						Comamonas	↑52%		
						Firmicutes	↓17%		
						Bacteroidetes	↓10%		
						Actinobacteria	↓1.3%		
						Escherichia	↓28%		
						Bacteroides	↓11% to 12%		
						Klebsiella	↓6%		
	In vivo: thirty 16-week-old female C57BL/6J ovariectomized mice	Bioactive peptides	-	100 mg of bioactive peptides/kg bw	56 days	*Firmicutes*/ *Bacteroidetes*	↑17%	-	[80]
						Alloprevotella	↑326%		
						Romboustsia	↓85%		
						Anaerostipes	↓66%		

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
						Ruminococcus	↓55%		
						Parasutterella	↓46%		
						Streptococcus	↓39%		
						Firmicutes/ Bacteroidetes	↑0.04% to 1.8%		
						Lactobacillaceae	↑14.59%		
	In vivo: male Balb/c mice aged 16 weeks	Exopolysaccharide	*Lactobacillus kefiranofaciens* XL10	0.4 mL of an XL10 suspension (10^8 CFU/mL)	21 days	*Ruminococcaceae* (day 7)	↑15.12%	-	[81]
						Bifidobacteriaceae	↑0.2% to 0.59%		
						Rikenellaceae	↓2.63% to 2.74%		
						Abundance in the gender	↓ up to 39.19%		
	BALB/c female mice (6 to 8 weeks old)	Kefiran	-	Daily intake 0.75 to 1 mg kefiran per day	21 days	*Bifidobacteria*	↑ up to 17%	-	[82]
	In vitro: *Bifidobacterium bifidum* PRL2010	Kefiran	-	0.3% (w/v)	72 h	*Bifidobacterium bifidum* PRL2010	Control (MRSc without carbon source) Without growth Treatment 5.8×10^8 CFU/mL	Enhanced transcription of genes that act as effector molecules in the microbe-host interaction, such as pili; transcription of genes involved in the metabolism of diet glucans	[41]
	In vitro: MTT assay of PC12 cell line (from rat adrenal gland -phaeochromocytoma)	Kefiran	-	5 and 10%	1 to 6 days	Cell viability		-	[46]
						1 day	↓up to 26.7%		
						2 days	Without effect		
						4 days (10%)	↓15.4%		
						6 days (10%)	↓21.2%		
Nervous system	*In vivo*: aging mouse model induced with D-galactose (oxidative stress)	Exopolysaccharide	*Lactobacillus plantarum* YW11	Low dose: 20 mL/ kg bw/day of 1 mg/ mL EPS solution High dose: 20 mL/ kg bw/day of 2.5 mg/mL EPS solution	12 weeks	Low dose		Modulation of gut microbiota and reduction of oxidative stress of the intestinal tract (decrease of NOx fecal content and increase of content of short-chain fatty acids—acetic and butyric)	[83]
						T-AOC	↑27.7%		
						MDA	Without effect		
						GSH-Px	Without effect		
						SOD	Without effect		
						CAT	Without effect		
						High dose			
						T-AOC	↑38.18%		
						MDA	↓49.6%		
						GSH-Px	↑21.55%		

Table 2: Continued.

Health effect	Model/participants	Bioactive compound	MO[1]	Concentration tested	Intervention time	Effect tested/ condition/ bioactive compound concentration	Effect compared to control	Action mechanism	Reference
Osteoporosis	*In vivo*: thirty 16-week-old female C57BL/6J ovariectomized mice	Bioactive peptides (KPS)	-	100 mg of KPs/kg bw	56 days	SOD	↑33.14%	-	[80]
						CAT	↑61.09%		
						Trabecular bone volume	↑264%		
						Trabecular number	↑235%		
						Bone mineral density	↑41%		
						Mechanical properties	↑42%		
						Hardness of the bones	↑36%		
						Trabecular separation areas	↓36.5%		
						Nanoindentation areas	↓33%		

[1]Producer microorganism. ↑: increase; ↓: decrease; EPS: exopolysaccharide; IL: interleukin; TNF-α: tumor necrosis factor-α; IFN: interferon; ZO-1: zonula occludens-1; NO: nitric oxide; PBMC: peripheral blood mononuclear cell; DSS: dextran sulfate sodium; TNBS: 2,4,6-trinitrobenzene sulfonic acid; FliC: flagellin; DO: optical density; CCL20: chemokine-ligand-20; bw: body weight; MPO: myeloperoxidase; MDA: malondialdehyde; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; T-AOC: total antioxidant capacity; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl); Cyto-c: cytochrome c; BAX: BCL2-associated X; BAD: BCL2-associated agonist of cell death.

The ultrasound combined with hot water in the kefiran extraction process showed synergistic results about the antimicrobial activity compared to the kefiran extracted by the isolated methods [61]. *Lactobacillus kefiranofaciens* DN1 produced an EPS—composed of mannose, arabinose, glucose, galactose, and rhamnose—which at 0.3% demonstrated a bacteriostatic effect against *Listeria monocytogenes* and *Salmonella enteritidis*. In higher concentrations (1% and 2.5%), the bactericidal effect was obtained by completely inhibiting the growth of both microorganisms, being considered a new bioactive compound that can be used as a natural antimicrobial [65].

Organic acids are other antimicrobial compounds produced by kefir microorganisms. In this context, lactic acid was a bioactive compound associated with the antimicrobial effect of cow milk kefir and donkey milk kefir. Donkey milk kefir reduced *Klebsiella pneumoniae*, *Bacillus cereus*, and *Proteus mirabilis* by 8%, 37%, and 58% compared to the kanamycin antibiotic. In contrast, cow milk kefir decreased *B. cereus* by 12.9% while had the same impact on *Proteus mirabilis* compared to kanamycin, demonstrating the tremendous antimicrobial potential associated with lactic acid [60, 66]. However, there was no antimicrobial effect against *Pseudomonas aeruginosa* [17, 60]. Other organic acids present in kefir, such as acetic and pyruvic acid, also demonstrated an antimicrobial effect when administered together with lactic acid [14, 31, 70]. Kakisu et al. [31] identified that organic acids act against the germination of *B. cereus* spores in a dose-dependent manner. Using a higher concentration of kefir grains (5%) for the fermentation of milk reduced the pH value more significantly and more quickly, inhibiting the germination of the spores. This more significant lowering of the pH may be associated with more outstanding organic acid production in the medium and, consequently, a more significant antimicrobial effect. Moreover, kefir grains at 5% reduced in a range of 33.3% to 61.8% the output of NheA toxin by *B. cereus* [31].

Bacteriocin, a bioactive protein produced by microorganisms in kefir, demonstrated antimicrobial effects against several microorganisms. In this context, *Lactobacillus plantarum*, *Micrococcus luteus*, *Listeria monocytogenes*, *Salmonella enterica* serovar *Enteritidis*, *Staphylococcus aureus*, and *Bacillus cereus* were sensible when compared to the negative control (sterile deionized water). In contrast, *E. coli* proved resistant to the antimicrobial action of the bacteriocin. However, it is essential to note that *E. coli* was also resistant to the positive controls, nisin, and polylysine, which are natural antimicrobial peptides commonly employed as preservatives by the food industry. For *S. enterica*, antimicrobial effects similar to nisin were observed [40].

Some microorganisms in kefir produced fractions with antimicrobial activity against *Clostridium difficile*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* [59, 62, 63]. FK-1000 at 25 mg/mL inhibited the growth of *Pseudomonas aeruginosa* by 91%. However, even when in a lower concentration (0.25 mg/mL), FK-1000 presented a synergistic effect with streptomycin, potentiating five times the outcome of this antibiotic. It demonstrates the potential of FK-1000 for use in combination therapy. Another important aspect is that a 50 mg/mL concentration of FK-1000 was not toxic to human epithelial cells, increasing the relevance of this compound's use as a treatment [62].

In addition, thermolabile fraction greater than 10 kDa produced by *Lactococcus lactis* subsp. *lactis* CIDCA 8221 showed to inhibit the toxigenic effect of *C. difficile*; the fraction inhibited the interruption of the actin network and displacement of Vero cells caused by *C. difficile*. Also, a reduction in the formation of TcdA and TcdB toxins by *Clostridium* has been observed [59].

The proteolysis in the milk by kefir's microorganisms during fermentation leads to bioactive peptides with antimicrobial activity. F1 bioactive peptides reduced *E. coli* growth in a range of 33% to 57%. Consistently, a mixture of bioactive peptides from kefir had antimicrobial activity against several microorganisms such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, and *Staphylococcus aureus* [64, 68].

However, it is essential to note that the bioactive compounds from kefir appear to have different antimicrobial potencies. In general, when comparing interventions with similar target microorganisms (*E. coli*, *S. typhimurium*, and *S. aureus*), the concentration and time of intervention with organic acids were substantially higher (40 and 2 times, respectively) than for interventions with kefiran or bioactive peptides (Table 2). Therefore, kefiran and bioactive peptides may have more potent effects than organic acids, requiring lower concentrations for similar antimicrobial results. However, this premise needs further investigation.

Lactobacillus plantarum, a microorganism present in kefir grains, produces a biofilm that acts as an antimicrobial, inhibiting the growth of methicillin-resistant *Staphylococcus aureus* in the range of 1.4 to 30%. Medium's time, temperature, and aeration influenced the biofilm production by the kefir's microorganism, which may be related to cell maturation, enzymatic reactions, and activation expression of specific genes. Thus, external factors and factors related to the strain used can influence the antimicrobial activity associated with the production of biofilms by *Lactobacillus plantarum* [67].

Finally, the inhibitory effect of some microorganisms present in the kefir about pathogens may be associated with the presence of S-layer proteins on the outer surface of their cell membrane. Thus, the preincubation of *Lactobacillus kefir* with *S. enteritidis* allowed direct interaction between them through the S-layer proteins of *L. kefir*, reducing then the sites binding to enterocytes on the pathogen's cell membrane. In addition, S-layer proteins from *Lactobacillus kefir* strains also decreased the viability of *Salmonella enteritidis*. Interestingly, even S-layer proteins obtained from noncoaggregation strains, which do not interact with *Salmonella*, could interact with *S. enteritidis*. The conformation and the active groups present in the S-layer may differ between the protein isolated in the solution and the one present on the bacterium's surface (*Lactobacillus kefir*). Therefore, S-layer proteins have the potential to be used as a natural antimicrobial [72].

The meta-analysis for the antimicrobial category corroborates with the systematic review findings herein, as results

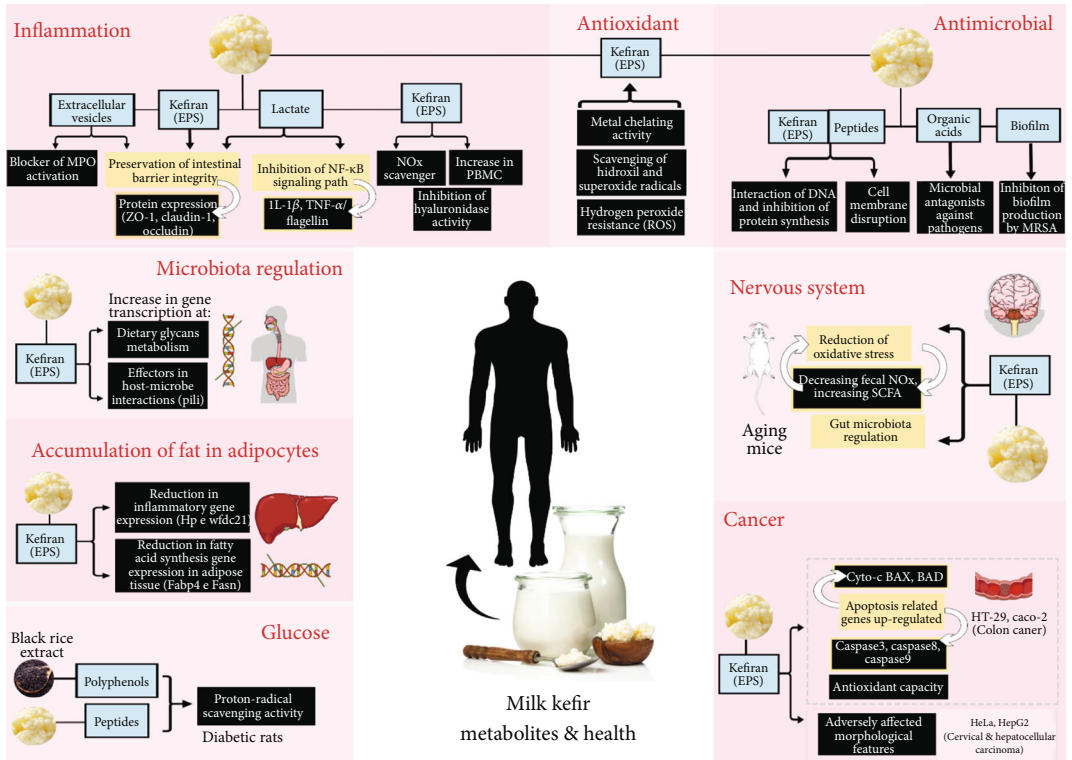

Figure 2: Mechanisms of action of bioactive compounds in milk kefir. EPS: exopolysaccharide; MPO: myeloperoxidase; ZO-1: zonula occludens-1; IL: interleukin; TNFα: tumor necrosis factor-α; NO: nitric oxide; PBMC: peripheral blood mononuclear cell; ROS: reactive oxygen species; MRSA: methicillin-resistant *Staphylococcus aureus*; Hp: haptoglobin-2; Wfdc21: Wfdc21 protein; Fabp4: fatty acid-binding protein 4; Fasn: fatty acid synthase; SCFA: short-chain fatty acid; Cyto-c: cytochrome c; BAX: BCL2-ssociated X protein; BAD: BCL2-associated agonist of cell death.

show a statistically significant overall effect of kefir bioactive compounds against bacteria and fungi (Supplementary Figure S1). As the distinct outcomes' measurements differed between trials, we used the standardized mean differences (SMDs) to estimate the effects, whereas a negative SMD value indicates microbial reduction. Therefore, the standard mean differences (SMDs) were estimated for the overall antimicrobial effect (SMD (-1.35) (95% CI: -1.79–-0.91, $P \leq .001$)) and separately for fungi (SMD (-7.18) (95% CI: -9.08–-5.28, $P \leq .001$)), *Bacillus cereus* (SMD (-0.25) (95% CI: -1.06–0.56, $P = 0.54$)), *Escherichia coli* (SMD (-0.67) (95% CI: -2.26–0.92, $P = 0.41$)), *Klebsiella pneumoniae* (SMD (-1.79), (95% CI: -4.22–0.63, $P = 0.15$)), *Pseudomonas* spp. (SMD (-1.98), (95% CI: -3.48–-0.49, $P = 0.009$)), *Salmonella* spp. (SMD (-0.53), (95% CI: -1.12–0.06, $P = 0.08$)), and *Staphylococcus* spp. (SMD (-0.88), (95% CI: -1.81–0.06, $P = 0.07$)) as showed in the Supplementary Figure S1. In this context, kefir demonstrated a statistical significance concerning the overall antimicrobial effect, although the bioactive compounds from kefir only had significant effects against fungi and *Pseudomonas* spp. However, we can see a tendency to favor kefir treatments for all the studied microorganisms, although not statistically significant.

To assess the heterogeneity of the data, I^2 tests for all nine analyses showed statistically significant considerable heterogeneity for fungi ($I^2 = 83\%$, $P \leq .001$) and statistically significant moderate heterogeneity for overall antimicrobial effect ($I^2 = 72\%$, $P \leq .001$), *Bacillus cereus* ($I^2 = 63\%$, $P \leq .001$), *Escherichia coli* ($I^2 = 67\%$, $P \leq .001$), *Klebsiella pneumoniae* ($I^2 = 64\%$, $P = 0.004$), *Pseudomonas* spp. ($I^2 = 61\%$, $P = 0.0003$), and *Salmonella* spp. ($I^2 = 68\%$, $P \leq .00001$).

Despite the scarcity of studies investigating antimicrobials' mechanisms, these mechanisms appear diverse, presenting singularity for each type of bioactive compound. For bioactive peptides, the action occurs on the cell membrane and the pathogen's DNA, while organic acids reduce biological activity. Biofilms, in turn, inhibit the production of biofilm by the pathogenic microorganism (Figure 2). In this context, bioactive compounds form pores in the pathogen's membrane, which damages the integrity of the plasma membrane, increasing its permeability with consequent efflux of potassium ions, leakage of proteins, and nucleic acids. These compounds also were described bind to the genomic DNA of the pathogen [64, 68].

It is essential to highlight that although the *in vitro* and *in situ* studies imply an antimicrobial role of bioactive compounds from kefir, the absence of controlled *in vivo* studies is a gap in the literature that limits the assessment of the extent of this effect in physiological systems.

3.4.2. Antioxidant Activity. The intervention with antioxidant molecules is crucial since they interact with free

radicals, ending the chain chemical reaction and reducing the attack on proteins [28] and DNA [49] that would cause cell damage [84].

The main bioactive compounds identified with antioxidant activity from kefir were the EPS, including kefiran, although bioactive peptides and phenolic compounds were also described (Table 2). In addition, there have been few studies identifying the producing microorganisms of the compounds with antioxidant properties. However, the *Acetobacter*, *Leuconostoc*, *Bacillus*, and *Kazachstania* genera strains were reported (Table 2). Concerning the precursors in milk, there was an evident scarcity of their investigation; only one study identified the precursors of bioactive peptides as being β-casein, k-casein, αs1-casein, and αs2-casein [64].

The antioxidant activity of kefiran *in vitro* was 8.47 μg/mL and 4.44 μg/mL, for 1% and 0.5% concentrations, respectively, as measured by reducing power activity in ascorbic acid equivalent capacity (AAEC). Interestingly, the hyaluronic acid used in gold standard viscosupplementation treatment did not demonstrate reducing power at similar concentrations to those employed for kefiran. It shows the antioxidant potential of the compound isolated from kefir [54]. The scavenging of free radicals and ferric ion reduction represented 25% to 85% and 37% to 84%, respectively, of vitamin C activity, which is considered a positive control for these activities. The relatively low concentrations (0.005% to 0.08%) tested of kefiran can justify its inferior effect compared to vitamin C [61].

The antitoxic activity of the EPS produced by microorganisms in the kefir promoted the protection of biological molecules from oxidation. Inline, low concentrations of EPS (0.05% to 0.25%) could protect BSA protein from oxidation induced by APPH (2,20-azobis(2-methypropionamidine) dihydrochloride). The protein oxidation-decrease ranged from 31% to 96%. Interestingly, this protection was higher than observed in the negative control (protein without induced oxidation) [28]. In addition, the antioxidant capacity *in situ* related to EPS was higher 8.43% after 24 hours of fermentation [74]. The ability to eliminate the DPPH (1,1-diphenyl-2-picrilhidrazil) free radical, turn in, increased from 10% to 20%. This fact can be attributed to the metabolic activity of kefir microorganisms during fermentation, leading to the production and accumulation of EPS. Thus, studies have found that the EPS present in kefir demonstrates potential as an antioxidant agent [73]. The EPS also confers resistance to hydrogen peroxide, completely reversing the detrimental effect of this compound on the cell growth of microorganisms present in the kefir. Consistent, cells without EPS significantly lose resistance to hydrogen peroxide [73].

Smaller fractions present in kefir, such as bioactive peptides, also demonstrated an antioxidant effect. An enhancement in scavenging ability of oxidized 2,2′-zino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) ranged from 2% to 25.6% for sheep's milk kefir and 35.8% for cow's milk kefir both compared to control [14, 64]. A significant improvement (111.6%) in oxygen radical absorbance capacity (ORAC) for cow's milk kefir confirmed the antioxidant activity of bioactive peptides [14]. We believe that the observed difference between the antioxidant activity of sheep's milk kefir and cow's milk kefir may be associated with the difference in composition between milk, especially about the protein content and its design which are precursors of bioactive peptides. This finding highlights the relevance of identifying the precursors of bioactive compounds in milk to increase its functional potential.

The fermentative process of milk by kefir grains showed to elevate its antioxidant activity by synthesizing phenolic compounds. In artisanal kefir, the antioxidant capacity was increased up to 120% when measured by ABTS and up to 40% by ORAC [75]. In contrast, for commercial kefir, the antioxidant capacity by ABTS reduced, while that by FRAP—ferric antioxidant power—increased [85]. Thus, phenolic compounds produced by starter culture appear to exert antioxidant action through their reducing power but are ineffective as to the scavenging ability of oxidized compounds. Still, the DPPH scavenging varied according to the type of kefir, reducing in ewe kefir and increasing in cow kefir compared to control [85]. Therefore, according to the matrix, the profile of phenolic compounds can influence the *in situ* radical scavenging ability. Indeed, Satir and Guzel-Seydim [75] observed increased antioxidant activity in the presence of gallic acid, catechin, epicatechin, caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid, and photocatechuic acid. Phenolic compounds resulting from the metabolic activity of kefir microorganisms can improve up to 120% of the *in situ* antioxidant capacity during fermentation [85].

The intervention times were more remarkable for bioactive peptides than EPS and phenolic compounds for similar methodologies (Table 2). This finding suggests that the peptides need a longer time to exert significant antioxidant activity. On the other hand, phenolic compounds required concentrations up to 3 times higher than other bioactive compounds (Table 2).

Consistent with the described findings, the meta-analysis showed an antioxidant tendency of the kefir bioactive compounds. However, it was not considered statistically significant compared to control treatments, even though we observed a narrow confidence interval. It resulted in a standard mean difference (SMD) of (SMD (-0.83) (95% CI: -1.65–0.00, $P = 0.05$)) (Supplementary Figure S2). Besides, the outcomes of the studies showed a significant substantial heterogeneity ($I^2 = 84\%$, $P \leq .001$), which may have contributed to the lack of significance.

Although there is a scarcity of description of the mechanisms of action in the literature, EPS seems to exercise effect through metal chelating activity and sequestering activity of hydroxyl and superoxide radicals, with consequent resistance to the hydrogen peroxide. In contrast, the antioxidant activity of bioactive peptides cannot be attributed to the ion chelating ability [14].

As reported previously for antimicrobial effect, the absence of controlled *in vivo* experiments evaluating the antioxidant potential of bioactive compounds from kefir is a gap in the literature that limits assessing the extent of this effect in physiological systems.

3.4.3. Modulation of the Intestinal Microbiota. Maintaining the intestinal microbiota in symbiosis with the host is essential for human health since it favors the integrity of the intestinal barrier, the balance of the immune system, and controlling inflammatory processes. Therefore, the search for probiotics or bioactive compounds which favor the modulation of the intestinal microbiota has been extensively studied in the recent literature.

Exopolysaccharides (EPSs), including kefiran, were the predominant described bioactive compounds capable of modulating the intestinal microbiota *in vitro* and *in vivo* models (Table 2). However, there is a lack of studies identifying the precursors of these bioactive compounds. In addition, the scarcity of studies determining the microorganisms that produce them in milk highlights a gap in the literature. Interestingly, the concentration and intervention time using bioactive peptides was considerably higher than those with EPS for *in vivo* animal models; the concentration employed of peptides was up to 2.5-fold greater. The intervention time was up to 2.6-fold longer compared to EPS use (Table 2). It suggests that EPS can be a bioactive compound more potent for gut modulation. However, this premise needs further investigation.

EPS produced by *L. paracasei* CIDCA 8339 and CIDCA 83124 in kefir demonstrated modifying the microbiota present in infant fecal samples and, consequently, changing the short-chain fatty acid profile (SCFA). The butyric and propionic acids produced are compounds with biological activity associated with health benefits. Among these benefits stand out are as follows: strengthening the intestinal epithelial barrier and inhibiting the cholesterol synthesis at the liver. Still, the expression of leptin, YY polypeptide (PYY), and glucagon-like peptide 1 (GLP-1) promoted by them regulate the lipogenesis in adipose tissue and the appetite [79].

Considering the phylum level, the use of EPS and bioactive peptides commonly improved *Firmicutes* to the detriment of *Bacteroidetes*, as demonstrated in Table 2. Regarding the genus level, a reduction in microorganisms associated with pathogenicities, such as *Klebsiella* and *Escherichia,* has been reported, demonstrating the potential of EPS in contributing to a healthy intestinal microbiota [79]. The concomitant decrease in *Rikenellaceae* is also a promoter of the health of colon epithelial tissue [81]. In contrast, EPS favored *Victivallis*, *Acidaminococcus*, *Comamonas*, and the *Ruminococcaceae* family [79, 81]. Selecting certain species of the *Acidaminococcus* genus may be responsible for increasing organic acids such as propionate and butyrate, which are beneficial in intestinal levels.

On the other hand, the modulation of other genera also considered beneficial to health, such as *Lactobacillus* and *Bifidobacterium*, depended on the bioactive compound used during the intervention (Table 2). Thus, *Lactobacillus* and *Bifidobacterium* were not favored by EPSs 8339 and 83124 in Bengoa et al. [79]. In contrast, in the study of Xing et al. [81], using adult male mice, EPS produced by *Lactobacillus kefiranofaciens* XL10 provided growth of 14.59% for *Lactobacillaceae* and up to 0.59% for *Bifidobacteriaceae*. Growth of up to 17% in *Bifidobacterium* in adult female mice was also observed when using kefiran, although no change was observed in the *Lactobacillus* population [82]. *In vitro*, 0.3% kefiran elevated a *Bifidobacterium bifidum* PRL2010 population up to 5.8×10^8 CFU/mL. In contrast, there was not *Bifidobacteria* population growth in the control (medium without carbon source). This fact indicates that the target microorganisms should be considered in deciding the most appropriate bioactive compound for intestinal modulation in each intervention.

Interestingly, EPS was more potent in promoting intestinal microbiota diversity than inulin, a prebiotic commonly used by the food industry. Still, EPS led to a different short-chain fatty acid profile, increasing the butyrate content and benefiting the gut microbial population more than inulin [79]. Kefiran also proved to be a better source of carbon than glucose for the growth of *Bifidobacterium bifidum* PRL2010; kefiran increased the development of the strain by 20 to 700% compared to the use of glucose [41].

Bioactive peptides, other bioactive compounds present in kefir, were also related to changes in the intestinal microbiota. The oral administration of peptides to female mice partially reversed the detrimental effect on the intestinal microbiota caused by oophorectomy; the peptides from kefir reduced 46% of *Parasutterella* and 39% of *Streptococcus*. Bacteria potentially pathogenic belonging to genera *Klebsiella* and *Escherichia* were also decreased. *Romboutsia*, together with *Streptococcus*, has been linked to obesity and presented an 86% reduction (Table 2). Bioactive peptides from kefir elevated the *Alloprevotella* population by more than 30% by reducing oophorectomy-induced renal fat accumulation. *Ruminococcus*-1, SCFA producer bacteria, increased in ovariectomized mice, subsequently decreasing with bioactive peptides' administration. It is essential to highlight that the growth of butyrate-producing bacteria after estrogen deficiency in ovariectomized mice can negatively lead to a detrimental accumulation of SCFAs in the intestine. In contrast, bioactive peptides have not been able to restore the reduction in the *Deferribacteres* phylum caused by oophorectomy; the decrease of *Deferribacteres* is related to the detriment of vitamins and amino acid metabolism [80].

Thus, bioactive peptides can enrich beneficial bacteria and decreasing potentially harmful pathogens in the gut of ovariectomized females [80]. Still, these findings reveal that further studies, analyzing the relationship between intestinal microbiota and estrogen deficiency and the role of the bioactive peptides from kefir in this relationship, are necessary for a better understanding.

Concerning the richness and diversity of microbiota, interestingly, it is not affected by bioactive peptides in ovariectomized mice. However, peptides significantly improve intestinal microbiota diversity compared to control without ovariectomized procedure [80].

Regarding meta-analysis, treatments with bioactive compounds did not present statistically significant effects on gut microbiota modulation compared to control treatments (SMD (-0.39) (95% CI: -1.32–0.54, $P = 0.41$)) (Supplementary Figure S3). However, we can notice a tendency that favors bioactive efficacy, consistent with our findings. This analysis presented a statistically significant moderate heterogeneity ($I^2 = 58\%$, $P < .001$), which may have contributed at least partially to the lack of significance.

Finally, most studies failed to evaluate the mechanisms of action of the bioactive compounds on gut microbiota modulation, representing a gap in the literature. However, enhanced transcription of genes involved in the microbe-host interaction was proposed for the kefiran action.

3.4.4. Immune Response Modulation. Bioactive compounds from kefir exerted an anti- or proinflammatory impact depending on the model's presence or absence of inflammatory insult. Thus, they exerted an inhibitory effect in inflammatory diseases' models, while they had an immunostimulatory effect for models without inflammatory insult (Table 2).

The predominantly studied inflammatory disease model was that of acute colitis, both *in vitro* and *in vivo*. For colitis, mainly EPS and extracellular vesicles, but also lactate, they have been described to have an anti-inflammatory role against a variety of acute inflammatory insults: DSS (dextran sulfate sodium), TNFα, FliC (flagellin), IL-1β, and TNBS (2,4,6-trinitrobenzene sulfonic acid). For chronic colitis, extracellular vesicles presented an anti-inflammatory effect against piroxicam. Kefiran, in turn, had an inhibitory effect on cotton-induced granuloma in rats (Table 2). The *L. kefirgranum*, *L. kefir*, *L. kefiranofaciens*, and *L. paracasei* species were responsible for producing these bioactive compounds. Therefore, the *Lactobacillus* genus seems relevant for making anti-inflammatory compounds in the kefir [13, 50, 53]. Galactose and glucose, and to a lesser extent, mannose, arabinose, and rhamnose, were the significant precursors of the polysaccharide component of the bioactive compounds [55].

Extracellular vesicles at 100 μg/mL from *L. kefirgranum* reduced the gene expression of IL-2, IL-8, and TNFα proinflammatory cytokines by 58, 64, and 67%, respectively, in Caco-2 cells for DSS-induced acute colitis model [50]. Extracellular vesicles (1×10^9 particles/mL) also inhibited TNFα-induced colitis. They reduced the expression and secretion of IL-8 by 65 and 96%, respectively, in the Caco-2 cell line. Interestingly, the extracellular vesicles were just as effective as budesonide [53], a glucocorticoid steroid commonly used to treat Crohn's disease (inflammatory bowel disease) [86]. Still, treatment of Caco-2 cells with extracellular vesicles showed a longer intervention time than treatments with EPS or lactate. In addition, the effect observed with preincubation of cells with EPS or lactate (Table 2) indicates the potential of these compounds as preventive agents of intestinal inflammation. However, further studies exploring pre- and postincubation concerning the inflammatory insult must be conducted to elucidate these bioactive agents' preventive and therapeutic potentials.

In mice, oral administration of extracellular vesicles could mitigate acute and chronic colitis, corroborating the previous findings *in vitro*. For DSS-induced acute colitis, both high and low dosages of vesicles prevented weight loss in mice by up to 16% and reduced damage to colon tissue by up to 63%. However, only the highest dosage (3 mg/kg bw) reduced colon atrophy by 29.6%. Similarly, only the highest dosage mitigated colon atrophy by 14.3% for chronic colitis aggravated by piroxicam. Nevertheless, both dosages reduced colon histological damage by up to 85%.

In contrast to acute colitis, ingestion of extracellular vesicles was ineffective in preventing weight loss in chronic colitis [50]. Therefore, due to the broader effects obtained for 3 mg/kg bw, the high dosage seemed more effective for chronic and acute colitis treatment. Vesicles' administration against TNBS-induced acute colitis also effectively prevented the mouse weight loss by up to 12.5% at 3×10^8 and 3×10^{10} vesicles/-head. Moreover, the administration reduced rectal bleeding and diarrheal condition severity by 75 and 91%, respectively. Damage to colon tissue, in turn, was decreased by up to 85% [53]. Therapy with vesicles from *Lactobacillus* of kefir was more effective than the prednisolone drug (2 mg/kg) in preventing weight loss, the severity of rectal bleeding, and diarrheal conditions well as in mitigating colon histological damage [53]. Prednisolone is an anti-inflammatory steroid used to treat inflammation in colitis and Crohn's disease; however, it does not prevent recurrence of the disease, in addition to having several side effects [87, 88]. Therefore, treatment with bioactive compounds from kefir would be promising both for effectiveness and reduced side effects.

Suspensions of EPS-producer *Lactobacillus paracasei* ($OD_{590} = 0.25$) inhibited by up to 55% the induction of the CCL20 proinflammatory promoter in Caco-2 cells for flagellin-induced acute colitis model. *Lactobacillus paracasei* CIDCA 8339 strain showed more dramatic anti-inflammatory potential than the other tested strains [13], which indicates that the functional potential of the EPS is strain-dependent. Similarly, lactate at 100 mM inhibited by 78, 80, and 42% the CCL20 promoter induction by flagellin, IL-1β, and TNFα, respectively, in Caco-2 cells. Inline, human intestinal epithelial cells express the lactate receptor. Still, lactate solution and supernatant from kefir with corresponding lactate concentration showed similar inhibitory effects on Caco-2 cells [56], indicating that the kefir matrix does not reduce the impact of this bioactive compound. *In vivo*, oral administration of kefiran-rich kefir supernatant (1 mL/day) was responsible for reducing the weight of cotton-induced abdominal granulomas by 44% in rats. Kefir was as effective as dexamethasone (0.2 mg/kg) in reducing these granulomas [16]. Dexamethasone is a corticosteroid medication used as a primary option in the treatment of granulomas [89]. This evidence reinforces the anti-inflammatory potential of bioactive compounds from kefir.

Therefore, in general, bioactive agents inhibited the expression of proinflammatory cytokines and the activation of the CCL20 promoter for *in vitro* inflammatory models with Caco-2 cells. In *in vivo* colitis models, bioactive compounds reduced weight loss, atrophy, and colon histological damage.

The compounds displayed anti-inflammatory action mechanisms by inhibiting the NF-κB pathway in the Caco-2 cells and the colon mucosa due to the expression of the IκBα inhibitor [50, 53, 56]. In addition, bioactive agents promoted the integrity of the intestinal barrier, increasing the expression of occludin, ZO-1, and claudin-1 occlusion proteins [50]. Additional anti-inflammatory mechanisms proposed for EPS were nitric oxide radical scavenging ability [54] and inhibition of hyaluronidase activity in cell-free *in vitro* systems [55]. Hydrolysis of the extracellular matrix by hyaluronidase releases compounds, like hyaluronan,

throughout inflammatory pathologies [90]. For extracellular vesicles, blocking myeloperoxidase activation in the mouse plasma has also been reported [53]. Oxidative stress in inflammatory bowel disease activates inflammatory cells, such as neutrophils, whose myeloperoxidase catalyzes the production of reactive oxygen species [91]. In this scenario, extracellular vesicles were as effective as the prednisolone drug in inhibiting myeloperoxidase [53]. Thus, the evidence suggests that bioactive compounds from kefir may play a decisive anti-inflammatory role.

However, EPS, including kefiran, can also have the opposite effect, acting as immunostimulants, in cases where there is no inflammatory insult (Table 2); this role was also corroborated in an *in vivo* model [58]. Their precursors in milk were glucose and galactose [51, 52]. In a minority way, bioactive peptides have also been reported as immunostimulants [57]. The *Lactobacillus* genus was relevant to produce these immunostimulants, especially the *L. helveticus*, *L. pentosus*, and *L. kefiranofaciens* species.

For EPS, although the intervention time has been similar for both a pro- and anti-inflammatory effect assay, the EPS concentration employed was dramatically higher; *in vitro*, the concentration for immunostimulating varied from 50 to 5000 μg/mL [4, 46, 51], while for inhibition, it reached the maximum of 100 μg/mL [50]. *In vivo*, 100 mg/kg bw was administered orally for immunostimulating [58], while to inhibit the immune response, the concentration ranged from 0.03 to 3 mg/kg bw [50]. This fact suggests that the concentration of EPS is a significant factor in determining the role that it will play on the immune system. In line, EPS has been reported to stimulate or inhibit the secretion of TNFα, IL-10, and IL-6 by *in vitro* murine macrophages, depending on the concentration tested [51, 52]. Thus, it appears that EPS can act by different cell signaling pathways, according to its concentration. However, the immunostimulatory mechanisms of action still need to be studied.

In vitro, the immunostimulatory role of EPS and bioactive peptides has been demonstrated in the macrophage's cell line and primary culture, in addition to human peripheral blood mononuclear cells (PBMCs) (Table 2). R-5-EPS and R-17-EPS at 50-400 μg/mL stimulated proliferation, phagocytosis, phosphatase activity, IL-6 secretion, and NO production by RAW264.7 murine macrophage cells. Still, in the concentration range of 100 to 200 μg/mL, they stimulated the secretion of TNFα, IL-1β, and IL-10. EPSs were as efficient as lipopolysaccharides in promoting cell proliferation, phagocytosis, and cytokine secretion by macrophages [51, 52]. Kefiran at 1000 to 5000 μg/mL increased IL-6 secretion, and concentrations from 2000 to 4000 μg/mL stimulated cell proliferation of human PBMC culture by up to 200% after 24 h [46]. Bioactive peptide from *L. kefiranofaciens*, turn on, improved secretion of TNFα, IL-1β, IL-6, and IL-12 by 1000, 700, 1300, and 3000% by murine peritoneal macrophage culture. However, peptides from different microbial strains showed differences in immunostimulatory capacity [57], suggesting that the functional potential of the peptide is strain-dependent. In addition, bioactive peptide acted via the TLR2 receptor [57]; Toll-like receptor 2 enables macrophages to recognize microbial ligands, thereby promoting inflammation [92].

Consistently, oral administration of kefiran (100 mg/kg bw) to healthy mice for up to 7 days enhanced IgA, IL-10, IL-6, and IL-12 in the mucosa of the small intestine, as well as IL-4 and IL-12 in the fluid of the small intestine. In serum, kefiran increased IL-4, IL-6, IL-10, and IFN. However, broader immunostimulation occurred in the large intestine, increasing IgA, IgG, IL-4, IL-10, IL-6, INF, and TNF content. The most evident stimulatory activity in the large intestine has been attributed to the kefiran fermentation by intestinal microbiota [58]. Therefore, it appears that bioactivity may vary according to the biochemical transformations that these molecules undergo throughout the digestive process.

Finally, it is essential to highlight that immune stimulation can be interesting for a better prognosis of infectious conditions [93] and stimulating immunoglobulin production after vaccination [94]. Thus, the concentration and environmental context (presence or absence of inflammatory insult) are relevant factors to be considered according to the purpose of administering the bioactive compound.

Meta-analysis results corroborated the benefits of kefir bioactive compounds on immune modulation since the findings indicated that treatments had significant immune-modulatory activity compared to control (Supplementary Figure S4). The estimated (SMD) was (SMD (-1.17) (95% CI: -1.47–-0.87, and $P \leq .001$)). Heterogeneity was statistically significant moderate for this analysis ($I^2 = 67\%$, $P \leq .001$).

3.4.5. Anticancer Effect. Cancer is a term that encompasses more than 100 different types of malignancies that have in typical disordered cell growth, which can metastasize [95]. However, although human *in vitro* models have reported anticancer effects of bioactive compounds from kefir on breast, colon, cervical and hepatocellular cancers, the lack of *in vivo* studies limits the understanding of the extent of their anticancer effect in organisms. Among the bioactive ones, EPS, including kefiran, had a broader impact, covering different types of cancer: breast, colon, cervical, and hepatocellular cancers (Table 2). The main precursors of EPS in the milk were glucose and galactose [47]. The *Lactobacillus* genus seemed relevant for EPS production with an anticancer effect on kefir (Table 2).

Kefiran reduced up to 45% of MCF7 breast cancer cell viability after 48 h of intervention, losing effect under the highest tested concentration of 4 mg/mL. However, efficiency decreased to 15.6% after 72 h, without effect at the lowest tested concentration of 0.5 mg/mL [46]. For HepG2 hepatocellular carcinoma cells, kefiran reduced their viability by up to 82% from the minimum concentration of 250 μg/mL. In contrast, for HeLa cervical carcinoma cells, kefiran reduced the viability by up to 72% in a dose-dependent manner [48]. Similarly, EPS MRS101 presented a dose-dependent effect on the viability of HT-29 colon cancer cells, reducing the number of viable cells by up to 55.9% after 24 h [47]. Therefore, the EPS concentration and the intervention time seem to be decisive for obtaining or not the anticarcinogenic effect. However, it is essential to note that developmental toxicity was observed in zebrafish embryos for kefiran concentrations above 100 μg/mL so that kefiran at 1000 μg/mL reaches 80% mortality [48].

Inline, 10^{-3} to 3 μg/mL of EPS showed no toxicity on typical Vero cell culture [55]. Therefore, EPS can affect the growth of normal tissues depending on the concentration used, so this factor should be considered in future clinical applications.

The proposed mechanism of action for EPS was the upregulation of the apoptotic genes Cyto-c (cytochrome c), BAD (BCL2-associated agonist of cell death), BAX (BCL2-associated X protein), and caspases 3, 8, and 9; the increase in the expression of these genes ranged from 15 to 120%. In contrast, EPS downregulated by 70% the BCL2 gene, which is involved with the oxidative stability of the mitochondria [47]. Such changes in gene expression may be related to the adverse effects on the morphology of cancer cells. Loss of adherence capacity and formation of intracellular vacuoles were the more common changes resulting in cell death [48].

On the other hand, bioactive peptides have been reported only in an estrogen-sensitive breast cancer model. In addition, they showed a longer intervention time than other bioactive compounds described (Table 2). However, peptides appear to have lower toxicity as an advantage to EPS since peptides from kefir did not exert an antiproliferative effect on normal human mammary epithelial cells at doses of 0.31 to 10% (v/v). On the other hand, on those exact dosages, peptides reduced dose-dependent manner by up to 88% the MCF7-E3 human breast cancer estrogen-sensitive cell number [15], which indicates the specificity of this bioactive compound. Still, yogurt extracts showed dramatically lower efficiency than kefir extracts, in addition to presenting toxicity on normal human epithelial cells from a 5% (v/v) concentration. Nonfermented milk, unlike, had a negative effect, stimulating the proliferation of MCF7-E3 cancer cells [15]. These findings corroborate the anticarcinogenic specificity of peptides from kefir.

Although the anticancer effect of organic acids has not been directly tested, lactic and acetic acids present in kefir reduced fecal water-induced-DNA damage in HT-29 cells. Cultivation with fecal water for 30 min increased by 36% the DNA damage in HT-29 cells, being this effect more toxic than that caused by hydrogen peroxide [49]. However, fecal water-induced-DNA damage was inhibited with kefir supernatant by 20%. Thus, organic acids can have a preventive effect against colon cancer, attributed to their antioxidant activity. Inline, kefir presented antioxidant capacity (Trolox equivalent) 78.6% greater than unfermented milk [49], corroborating the role of organic acids produced during fermentation in protecting DNA.

Finally, the HT-29 colon cancer cell line [47, 49] appeared to be more sensitive to EPS than the MCF7 breast cancer cell line [46]. For the latter, the concentration reported for the assay was ten times greater (Table 2). Thus, sensitivity to the bioactive compounds from kefir appears to be dependent on cell type.

Validating our findings, treatments with bioactive compounds presented a significant anticarcinogenic effect, as the estimated (SMD) for overall anticancer effect was (SMD (-2.44) (95% CI: -3.41–-1.47, $P \leq .001$)) (Supplementary Figure S5). The data were homogeneous ($I^2 = 46\%$, $P \leq 0.0006$), which means good consistency among studies.

3.4.6. Plasma Glucose. Diabetes mellitus is a chronic glucose metabolism disorder with severe clinical consequences, such as retinopathy, nephropathy, and stroke [96]. Type 2 diabetes mellitus is a metabolic disorder marked by the rise in blood glucose due to a decrease in insulin secretion by pancreatic β-cells and insulin resistance. Additionally, the increase in the prevalence of diabetes mellitus worldwide in recent decades is associated with the rise in the prevalence of obesity in the population [96].

In this scenario, bioactive compounds in kefir were tested on glucose metabolism in obesity and diabetes murine models *in vivo* (Table 2). Oral administration (10 mL/kg bw) of a mixture of EPS-producer *Leuconostoc mesenteroides* LMDH4 and *Lactobacillus kefiri* LKDH5 during eight weeks did not affect the plasma glucose level in diet-induced obese mice. However, this treatment effectively reduced adipocyte tissue weight by 36%, in addition to downregulating the pro-inflammatory and fatty acid synthesis gene expression in the adipocytes [76]. This fact indicates that the regulation of fatty acid metabolism in adipose tissue is not a determining factor in promoting a significant change in plasma glucose.

In contrast, administration of a mixture of 5 to 20 mL of kefir with black rice extract—1 : 1/kg bw—during four weeks enhanced by up to 199% and 2330% of the Langerhans islet in the pancreas and insulin-positive β-cells, respectively, in diabetic rats. The concentration from 10 mL/kg bw completely reversed the pancreatic damage induced by STZ-NA (Streptozotocin-nicotinamide), achieving a similar effect to the glibenclamide [78]. It is an antidiabetic drug of the second-generation sulfonylureas class that reduces blood glucose by increasing insulin secretion from pancreatic β-cells [97]. The effect was attributed to the proton-radical scavenging activity from alcohol and phenolic compounds present in the beverage [78]. This finding demonstrates that the antioxidative capacity must be a significant factor in promoting the homeostasis of insulin production by the pancreas. However, as the study did not test a formulation without adding black rice extract, it is impossible to determine how much of this positive effect on β-cells can be attributed to the kefir alone. Indeed, the addition of black rice extract increased by 56.8% the antioxidant capacity of the beverage compared to the kefir alone, as measured by DPPH [78].

Therefore, the current findings in the literature on the effect of bioactive compounds of kefir on glucose metabolism are inconclusive, so further preclinical studies with pure kefir drink or with bioactive compounds isolated from it are urgently needed.

3.4.7. Effect on Serum Cholesterol and Accumulation of Fat in the Adipose Tissue. Cardiovascular disease (CVD) is one of the major leading causes of morbidity and mortality worldwide. However, deaths by CVD are attributable to manageable risk factors, the main ones being high total serum cholesterol, high blood pressure, and smoking [98]. In addition, hypercholesterolemia is one of the risk conditions related to obesity [99]. In this scenario, reducing total serum cholesterol and fat accumulation in adipocytes is one of the strategies used to prevent CVD. However, pharmacological

drugs to treat obesity reportedly cause several side effects related to blood pressure, hepatic failure, pancreatitis, and headaches [100]. Therefore, research on natural compounds for the treatment of obesity has been encouraged.

Among the bioactive compounds from kefir, only EPS has been associated with lowering adipocyte fat and reducing *in vitro* cholesterol. *Lactococcus lactis* WH-C1, *Lactobacillus kefiri*, and *Leuconostoc mesenteroides* were the microorganisms identified as producers of these EPSs in the kefir [77]. However, the lack of investigation of their precursors in the milk shows a gap in the literature (Table 2).

EPS-producer *Lactococcus lactis* WH-C1 (4%, v/v) isolated from Tiber kefir grain could remove up to 31.23% of cholesterol from culture medium, demonstrating a potential hypocholesterolemic property *in vitro* [77]. In contrast, EPS was ineffective on an *in vivo* experimental model; supplementation for eight weeks with a mixture of EPS-producer heat-killed lactic acid bacteria (HLAB) isolated from kefir did not change the lipid profile in obese mice. Thus, serum HDL, LDL, total cholesterol, and triglycerides were not affected in diet-induced obese mice [76]. The lack of effect on serum cholesterol indicates that the concentration of EPS-producing microorganisms employed (1.1×10^{10} CFU/mL) may have been insufficient to achieve significant changes in the cholesterol level *in vivo*. It must be taken into account that microorganisms became unable to continue the EPS production when they died from heating. Indeed, the greater the growth of the EPS-producing microorganism, the greater the cholesterol removal rate from the medium was demonstrated *in vitro* [77].

Similarly, kefir supplementation, which did not lead to a modulation of gut microbiota, is associated with low propionic acid production in the colon, which is insufficient to promote changes in the serum cholesterol level [101]. Also, the diet's inclusion of extract rich in polyphenol (wine grape seed flour -2.5%, v/v) did not alter the serum cholesterol levels [76]. Thus, antioxidative capacity may not be a significant factor in reducing blood cholesterol levels.

On the other hand, the EPS ability to reduce the fat accumulation in the adipocytes was confirmed *in vitro* and *in vivo* animal models (Table 2). EPS isolated from strains of *Lactobacillus kefiri* and *Leuconostoc mesenteroides* showed inhibition (up to 28%) of lipid accumulation in 3T3-L1 adipocytes *in vitro*; this inhibition, in general, occurred in a dose-dependent manner [76]. In addition, the minimum concentration of EPS to obtain the effect appeared to be strain-dependent since only EPS from *L. mesenteroides* LMDH4 presented an effect at 0.01 mg/mL. At the same time, those from *L. kefiri* LKDH3 and *L. mesenteroides* LMDH6 affected the fat accumulation only at the highest concentration tested (0.2 mg/mL). In contrast, EPS from *L. mesenteroides* LMDH8 and LMDH9 did not act even at the highest concentration tried (Table 2). This finding implies that the functional potential of EPS on fat accumulation varies according to its microbial origin.

About *in vivo* animal experiment, a diet supplemented with EPS-producer HLAB during eight weeks reduced by 36% the adipose tissue weight in C57BL/6J mice fed a high-fat and high-fructose diet [76]. This way, it suggests that EPS from kefir's microorganisms can be a functional ingredient capable of being used in obesity cases. EPS performance occurred through action on adipose tissue; expression of *Wdfc21* and *Hp* proinflammatory genes was lowered by 56% and 57%, respectively, in the adipose tissue compared to the control group. Also, the expression of genes related to the synthesis of fatty acids (*Fabp4* and *Fsan*) was downregulated by 55% and 43%, respectively, in the adipose tissue of the HLAB group. *Fabp4* encodes fatty acid-binding protein 4 (Fabp4), while *Fsan* encodes fatty acid synthase. It demonstrates that EPS has roles in anti-inflammation and inhibiting the fatty acid synthesis in adipocytes. However, these effects on the *Wdfc21*, *Hp*, *Fabp4*, and *Fsan* genes did not result in serum cholesterol changes, as its level has not been altered by treatment with HLAB.

Interestingly, supplementation of the diet with HLAB in conjunction with wine grape seed flour (2.5%), which is rich in polyphenol, potentiated the effect of reducing fat accumulation by 25% [76]. Therefore, antioxidant activity may be a relevant factor for reducing fat accumulation and can enhance the impact of EPS on adipocytes. Consequently, the synergistic treatment was the only one capable of significantly reducing the serum triglyceride.

3.4.8. Antihypertensive Effect. Hypertension is a chronic disease that leads to an increase in blood pressure [102]. Angiotensin-converting enzyme (ACE) is a key component of the renin-angiotensin system as part of the homeostatic mechanism to maintain adequate blood pressure levels in mammals. Conversion of ACE 1 to ACE 2 (an enzyme with a vasoconstrictor effect) is a common mechanism to regulate blood pressure [103]. But in abnormal conditions, this constriction can cause high blood pressure and increase the work required for the heart to pump blood into the body's main arteries [104]. In this way, inhibition in the conversion of ACE 1 to ACE 2 plays a role in managing hypertension.

As the systematic review selected only one article, it was the major limitation to a consistent understanding of the effect of bioactive compounds from kefir on blood pressure. Still, this study did not investigate the microorganism that produces the bioactive compound, its mechanism of action, nor the precursor of this bioactive compound in milk. In addition, the model studied was *in situ*. The absence of an investigation *in vivo* limits the understanding of the functional potential of bioactive compounds from kefir on blood pressure in physiological systems (Table 2).

In situ, the ACE activity was reduced by 98.4% in the milk after fermentation with kefir grains for 24 h. The effect observed was attributed to bioactive peptides released by kefir's microorganisms during the fermentation [14]. It suggests that bioactive peptides from kefir can have the potential to be used in the treatment of hypertension; however, further investigations are urgently needed to reach more consistent conclusions in the future.

3.4.9. Antioxidant Effect in Aging Models Related to Neurodegenerative Diseases. Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis are neurodegenerative diseases characterized by progressive loss of neuron cells, detriment of motor or cognitive functions, and accumulation of abnormal protein aggregates [105].

Aging is the primary risk factor for most neurodegenerative diseases, including Alzheimer's and Parkinson's disease. One in 10 individuals aged ≥65 has Alzheimer's disease, and the prevalence of the disease continues to increase as age advances [106]. Mitochondrial dysfunction and consequent oxidative stress during aging are well-established factors that significantly influence the progress of neurodegenerative diseases [106]. The reactive oxygen species (ROS) production in aged mitochondria increases, the membrane potential becomes smaller, the ATP synthesis reduces, and the activity of respiratory enzyme complexes declines.

Consequently, oxidation and aggregation of proteins occur, in addition to the oxidation of mitochondrial DNA, which starts to present mutations and deletions [105]. Few or no effective treatments for age-related neurodegenerative diseases are available, so they tend to progress irreversibly. In this context, stem cell therapy is one of the alternative methods studied for the treatment of neurodegenerative diseases to regenerate the neuronal population [107].

The present systematic review selected only two properly controlled studies testing bioactive compounds from kefir as a therapeutic strategy in neurodegenerative diseases. In both, the potential of EPS was assessed (Table 2). The effect of kefiran on the proliferation of neural stem cell culture (PC12 cell culture) was investigated by Jenab et al. [46] for 5 and 10% kefiran concentrations associated with pure poly-acrylonitrile (PAN) to form nanofibers. When comparing with the control (PAN alone), however, both kefiran concentrations tested reduced up to 26.7% of the PC12 viability after one-day incubation, showing no significant effect after two days. On the fourth and sixth days, 10% kefiran reduced by 15.4% and 21.2%, respectively, the viability of the PC12 cell line. Thus, kefiran was not a suitable compound for promoting neuronal regeneration; unlike, it showed toxicity from 10% concentration.

On the other hand, oral administration of EPS (20 mL EPS solution/kg bw) during 12 weeks had a beneficial effect on the aging mouse model induced with D-galactose since EPS could mitigate the resulting oxidative stress. At a low dose (1 mg/mL), EPS enhanced by 27.7% the total antioxidant capacity in the serum. However, in addition to increasing the total antioxidant capacity, EPS increased serum glutathione peroxidase, superoxide dismutase, and catalase by 21.55, 33.14, and 61.09%, respectively. A 49.6% reduction in serum malondialdehyde accompanied the increased activity of the antioxidant enzymes reported [83]. Malondialdehyde, in turn, has been proposed as a biological marker of the progression of neurodegenerative diseases, including Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease. It is because the elevation of peripheral malondialdehyde levels occurs in these diseases [108]. Thus, the reduction of serum malondialdehyde suggests that EPS can mitigate the progression of neurodegenerative diseases.

Lactobacillus plantarum YW11 was identified as the EPS-producing microorganism with antioxidant activity (Table 2). The antioxidant effect of EPS at the peripheral level was attributed to its ability to modulate intestinal microbiota positively. This modulatory effect was associated with a reduction of oxidative stress in the intestinal tract; the decrease in NOx with a simultaneous increase in acetic and butyric acids in feces illustrates the potential of EPS in improving the oxidative conditions of the host's intestinal tract (Figure 2). Interestingly, EPS was more efficient than ascorbic acid in reducing the level of NOx and just as efficient in raising the content of short-chain fatty acids in the intestine. The antioxidant capacity of EPS can be attributed to its scavenging ability on hydroxyl radicals, DPPH radicals, and superoxide anion, in addition to Fe^{2+} chelating ability [83].

Therefore, EPSs from kefir seem more promising in terms of preventive effects than for therapeutic purposes. Indeed, the aging mouse model exhibits similar characteristics to those presented in the early stage of neurodegenerative diseases, such as oxidative stress. Therefore, this model is well-established for studying the earliest neurodegenerative changes associated with these diseases [109]. However, human clinical trials and more preclinical assays must be performed to corroborate this hypothesis.

3.4.10. Effect on Osteoporosis. The systematic review here selected only 1 study on the effect of the bioactive compounds on osteoporosis [80]. However, the *in vivo* animal study did not investigate the microorganisms that produce the bioactive compound in kefir, nor its precursor in milk. The mechanisms of action have also not been described. Therefore, further studies are urgently needed better to understand the effect of kefir bioactive compounds on osteoporosis.

The ovariectomized procedure in female mice resulted in osteoporosis associated with the fall of the estrogen hormone due to the removal of the ovaries. These are the main glands that produce estrogen, which caused an imbalance between osteoblastic and osteoclastic activities in the bone. Consequently, there was a reduction of approximately 16%, 70%, and 70% of the bone mineral density, trabecular bone volume, and trabecular number in mice. However, the supplementation with bioactive peptides from kefir (100 mg/kg body weight) to ovariectomized mice could inhibit bone loss. Thus, the peptides increased the bone mineral density, trabecular bone volume, trabecular number, and the mechanical properties of the elastic modulus and hardness of the bones by 41%, 264%, 235%, 42%, and 36%, respectively. Consequently, a reduction of 36.5% and 33% in the trabecular separation and nanoindentation areas on the femur was obtained. It is important to note that peptide supplementation could ultimately reverse the condition of osteoporosis since the bone mineral density, trabecular number, trabecular separation, elastic modulus, hardness, and nanoindentation areas were similar to those of the negative control (nonovariectomized female).

Administration of calcium carbonate, standard procedure for osteoporosis treatment, also could enhance the trabecular bone volume and trabecular number. However, these values were about 0.7 times lower than those obtained by supplementation with bioactive peptides, demonstrating the potential of the peptides from kefir as a therapeutic agent in osteoporosis. Finally, no significant difference in the effects of calcium and bioactive peptides' combined action concerning isolated bioactive peptides revealed no synergism between these two treatments [80].

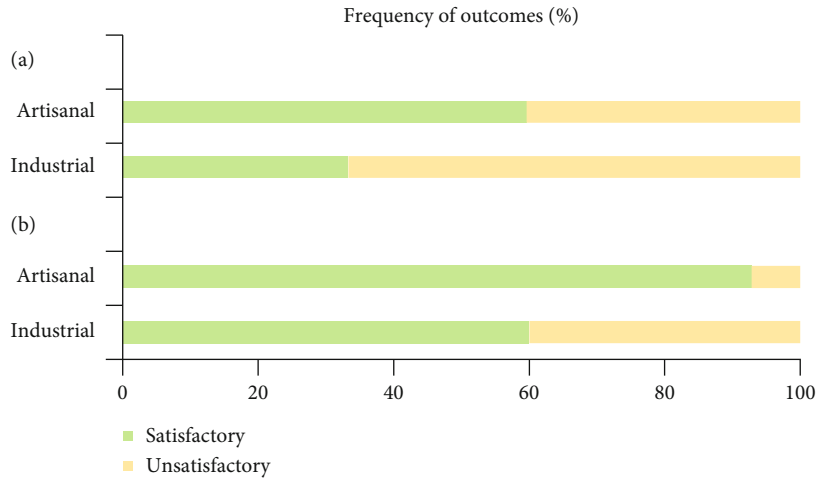

Figure 3: Frequency of significant satisfactory and unsatisfactory outcomes from artisanal and industrial kefir trials included in the meta-analysis. (a) Antimicrobial potential outcomes. (b) Antioxidant potential outcomes.

3.5. Do Bioactive Compounds from Artisanal and Industrial Kefir Differ in Terms of Their Functional Potential? Two groups were assessed for comparing artisanal (kefir grain) and industrial (starter culture) kefir trials regarding their functional properties (Figure 3). First, we target effects related to antibacterial/antifungal potential resulting in an estimated OR of 2.96 (95% CI: 0.87–10.05, $P = 0.07$). Therefore, the type of kefir was not significantly associated with its antimicrobial potential. This result agrees with our findings (Table 2), which reported the organic acids (especially lactic acid) as one of the main responsible bioactive compounds for the antimicrobial effect in both types of kefir.

After aiming to evaluate artisanal and industrial kefir outcomes concerning antioxidant potential, another group analysis was carried out, resulting in an estimated OR of 8.56 (95% CI: 2.27–32.21, $P \leq .001$). Therefore, the utilization of artisanal kefir may provide 756% higher chances of having antioxidant effects than kefir from the industrial process. As stated before, phenolic compounds were the bioactive compounds responsible for industrial kefir antioxidant activity. In contrast, artisanal kefir presented a greater diversity of bioactive antioxidant compounds; in addition to phenolic compounds, artisanal kefir also contained bioactive peptides and EPS, including kefiran (artisanal kefir-exclusive structure) (Table 2). Although the phenolic compounds in industrial kefir have reducing power, they were ineffective in terms of the radical scavenging ability [85], unlike the phenolics present in artisanal kefir [75]. These findings suggest a different profile of phenolic compounds between both kefirs that can affect the antioxidant potential. In addition, the greater diversity of bioactive compounds in artisanal kefir may have contributed to potentiate the antioxidant capacity of this type of kefir (Table 2).

The difference in the antioxidant potential may be related to the different microbial profiles between the types of kefir since the microorganisms reported as producers of the antioxidant compounds present in artisanal kefir [28, 73] are not commonly found in industrial kefir (Table 1). Indeed, artisanal kefir's more diverse microbial profile has been suggested as responsible for granting better functional benefits than industrial kefir [20]. Consistently, as discussed earlier, we observed a dramatically greater fungal diversity in artisanal kefir. In addition, the bacterial composition between both kefirs presented particularities (Table 1). Therefore, the microbial profile of artisanal kefir seems to be related to producing a greater diversity of bioactive compounds with antioxidant potential.

However, it is essential to report that because artisanal kefir is often primarily selected for kefir studies, only a few studies reporting industrial kefir findings were included in the meta-analysis, which was a limiting factor for comparison between kefir types.

4. Conclusion and Prospect

This review highlights the potential of bioactive compounds from kefir as preventive and therapeutic agents. There is an abundance of studies addressing the health benefits of kefir administration. However, a literature limitation is uncontrolled experiments or not identifying the bioactive compounds responsible for the observed health benefits. In addition, the relative scarcity of studies investigating the bioactive compounds-producer microorganisms and their substrates in milk constitutes a gap in the literature. The meta-analysis corroborated the antimicrobial, anticancer, and immune-modulatory activities. Kefiran and lactic acid were the main components with global antimicrobial action. However, kefiran presented a concentration threshold from which it exerted a bactericidal rather than a bacteriostatic effect. At the same time, lactic acid inhibited the germination of bacterial spores in a dose-dependent manner. Still, EPSs, including kefiran, were primarily responsible for the activity against colon and breast cancer. Nevertheless, the EPS concentration and intervention time were decisive for obtaining or not the anticancer effect, with EPS presenting a dose-dependent inhibitory effect for cervical carcinoma and colon cancer.

Regarding the anti-inflammatory effect, EPS, extracellular vesicles, and lactate were the main bioactive agents involved. However, EPS can also have an immunostimulatory effect, depending on its concentration and environmental conditions

(absence or presence of inflammatory insult). To immunostimulate, the EPS concentration had to be dramatically higher, despite the intervention time similar to that used for the anti-inflammatory effect. A high dosage of extracellular vesicles seemed more effective against colitis than low dosages. Therefore, these factors must be carefully considered in future clinical applications. It is noteworthy that the studies were limited to *in vitro* and *in vivo* experiments, so clinical evidence is urgently needed to help advance the practical application of bioactive compounds from kefir. The mechanisms of action of bioactive compounds were diverse, indicating that they can act by different signaling pathways. Although antioxidant activity and gut modulation have shown a trend that favors kefir efficacy, they did not reach statistical significance, possibly due to the high heterogeneity of the data. Thus, standardization of the methodologies used to evaluate these effects will help compare different studies in the future. Bioactive compounds on plasma glucose level, neurodegenerative disease, lipid profile, blood pressure, and osteoporosis were inconclusive, and further studies are needed for consistent conclusions. Finally, artisanal and industrial kefir can have different functional potential depending on the health effect, which can be associated with differences in the microbial composition between both types of kefir.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: forest plot of the standardized mean difference (SMD) and 95% confidence intervals for studies concerning the presence or lack of antimicrobial effect of kefir bioactive compounds compared to control treatments; Figure S2: forest plot of the standardized mean difference (SMD) and 95% confidence intervals for studies concerning the presence or lack of antioxidant effect of kefir bioactive compounds compared to control treatments; Figure S3: forest plot of the standardized mean difference (SMD) and 95% confidence intervals for studies concerning the presence or lack of gut microbiota modulation effect of kefir bioactive compounds compared to control treatments; Figure S4: forest plot of the standardized mean difference (SMD) and 95% confidence intervals for studies concerning the presence or lack of immune modulation effect of kefir bioactive compounds compared to control treatments; Figure S5: forest plot of the standardized mean difference (SMD) and 95% confidence intervals for studies concerning the presence or lack of anticancer effect of kefir bioactive compounds compared to control treatments. *(Supplementary Materials)*

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Research Article

Total Sesquiterpene Glycosides from Loquat Leaves Ameliorate HFD-Induced Insulin Resistance by Modulating IRS-1/GLUT4, TRPV1, and SIRT6/Nrf2 Signaling Pathways

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Loquat (*Eriobotrya japonica* Lindl.), a subtropical fruit tree native to Asia, is not only known to be nutritive but also beneficial for the treatment of diabetes in the south of China. To expand its development, this study was undertaken concerning the potential therapeutic role of total sesquiterpene glycosides (TSGs) from loquat leaves in insulin resistance (IR), the major causative factor of type 2 diabetes mellitus (T2DM). Male C57BL/6 mice were fed on high-fat diet (HFD) to induce IR and then were given TSG by oral administration at 25 and 100 mg/kg/day, respectively. TSG notably improved metabolic parameters including body weight, serum glucose, and insulin levels and prevented hepatic injury. Moreover, inflammatory response and oxidative stress were found to be remarkably alleviated in IR mice with TSG supplement. Further research in liver of IR mice demonstrated that TSG repaired the signalings of insulin receptor substrate-1 (IRS-1)/glucose transporter member 4 (GLUT4) and AMP-activated protein kinase (AMPK), which improved glucose and lipid metabolism and prevented lipid accumulation in liver. It was also observed that TSG suppressed the expression of transient receptor potential vanilloid 1 (TRPV1), whereas the signaling pathway of sirtuin-6 (SIRT6)/nuclear factor erythroid 2-related factor 2 (Nrf2) was significantly promoted. Based on the results, the current study demonstrated that TSG from loquat leaves potentially ameliorated IR *in vivo* by enhancing IRS-1/GLUT4 signaling and AMPK activation and modulating TRPV1 and SIRT6/Nrf2 signaling pathways.

1. Introduction

Owing to the popularity of fast food and takeout, dietary habits in the form of high fat are so common that metabolic disorders are prevalently increasing worldwide. Insulin resistance (IR) is regarded as a main pathological feature of metabolic disorders, such as obesity, type 2 diabetes mellitus (T2DM), and nonalcoholic fatty liver disease (NAFLD). IR is a complex pathological condition, which refers to the inadequate response of insulin target tissues during insulin stimulation. The molecular mechanism of IR is not fully understood, but it is confirmed that imbalanced glucose and lipid metabolism, inflammation, and oxidative stress are major factors associated with the development of IR [1–3].

In normal state, insulin binds to the insulin receptor, and then, insulin receptor substrate-1 (IRS-1) is phosphorylated. It activates its downstream targets including glucose transporter member 4 (GLUT4) and regulates blood glucose balance [4]. AMP-activated protein kinase (AMPK) is a vital regulator in the metabolism of glucose and lipid [5]. AMPK has been believed to be a therapeutic target for many metabolic disorders including IR [6, 7]. Moreover, a few earlier studies have revealed that the activated AMPK could positively regulate the expression of GLUT4 [8, 9].

The suppression of AMPK and IRS-1/GLUT4 has been proven to be associated with dysregulated glucose and lipid metabolism in IR-related models [9–11]. Conversely, molecules that could upregulate IRS-1/GLUT4 signaling or activate AMPK always display great effects on IR [10, 12, 13].

Transient receptor potential vanilloid 1 (TRPV1) is a nonselective cation channel, which is associated with inflammation, nociception, and thermosensation [14]. TRPV1 can be activated by heat, pH, and chemicals. As a member of chemicals, natural products were also found to interact with TRPV1, including vanilloids, flavonoids, terpenoids, and cannabinoids [15]. TRPV1 is not only localized to sensory neurons but also present in nonneuronal tissues. Hence, it plays pivotal roles in many disease progressions, such as inflammation in chronic obstructive pulmonary disease, oxidant stress-induced pain, and neuronal injury [16–18]. Recently, TRPV1 has drawn rising attention with its role in metabolic disorders [19–21]. TRPV1 deletion displayed anti-inflammatory effect in murine models involving metabolism disorder [22]. Accordingly, targeting TRPV1 may be a useful therapy for IR.

Sirtuin-6 (SIRT6) is a nuclear protein expressed in all of tissues, including liver, heart, and adipose [23–25]. Since its discovery, SIRT6 has been substantiated that it positively involved in numerous physiological processes. Furthermore, it has been regarded as a therapeutic agent for IR [26, 27]. SIRT6 deficiency could make diet-induced IR heavier [28]. SIRT6 has been well established to promote nuclear factor erythroid 2-related factor 2 (Nrf2) binding the antioxidant response elements (AREs) and to protect cells from oxidative stress [29]. Additionally, robust expression of SIRT6 has been shown an inhibitory effect on TRPV1-regulated inflammation [30, 31]. Thus, promoting the SIRT6/Nrf2 signaling will be a distinctive strategy to curb IR.

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical fruit tree that is widely distributed in China. Its leaf is a new source of food approved by the Ministry of Health of the People's Republic of China in 2014. Besides, loquat leaf has been found to possess beneficial properties such as anti-inflammatory, antioxidant effects, and improvement of metabolic diseases [16, 32]. Mounting evidence has shown that different extracts from loquat leaves were helpful in controlling IR. The triterpenoid acids from loquat leaves have been demonstrated to act on signalings of AMP-activated protein kinase (AMPK), insulin receptor substrate-1 (IRS-1), and Nrf2, which corrected abnormity of glucose and lipid metabolism in high-fat diet (HFD) mice and contributed to alleviating IR [33, 34]. As previously reported, the total sesquiterpene glycosides isolated from loquat leaves were considered to be the possible secondary metabolites responsible for its antidiabetic activity [35]. However, it is elusive that how these total sesquiterpene glycosides (TSGs) affect the glucose and lipid metabolism, inflammation, and oxidative stress *in vivo* under IR condition.

To expand their development, this study was undertaken concerning the potential therapeutic role of TSG from loquat leaves in IR, the major causative factor of T2DM. The present study confirmed the effects of TSG on HFD-induced IR *in vivo* and explored its underlying mechanism.

2. Materials and Methods

2.1. Preparation and Analysis of TSG from Loquat Leaves. Total sesquiterpene glycosides from loquat leaves were prepared in our lab following the method that we reported earlier [36, 37]. In brief, the powder of dried loquat leaves was percolated with 80% ethanol solution twice. The combined extracts were concentrated and centrifuged. Next, the supernatant was evaporated and subjected to column chromatography on macroporous resin XAD16, using solvent gradient system from H_2O to EtOH. The 60% and 70% EtOH eluted fractions were further column chromatographed over polyamide eluting with solvent gradient system from H_2O to MeOH. The H_2O eluted fraction was further column chromatographed by RP-C18 with different mixtures of H_2O and MeOH to obtain TSG.

HPLC was performed on Dionex Ultimate 3000 HPLC systems (Thermo Fisher Scientific Inc., Germany), equipped with a quaternary solvent delivery pump, an autosampler, DAD detector, and a Chromeleon Workstation. The analytical column Inertsil ODS-SP (4.6 mm × 250 mm, 5 μm) was used, and the temperature of column was kept at 35°C. The mobile phase was composed of methanol (A) and 0.1% formic acid-water (B) with gradient elution (0-10 min, 57% A; 12-20 min, 58% A; 20-35 min, 60% A; and 35-50 min, 65% A). The flow rate was 0.5 mL/min, and the elute was monitored at 210 nm. For quantification, nerolidol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (SG1), nerolidol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (SG2), nerolidol-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (SG3), and nerolidol-3-O-α-L-arabinopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside (SG4) were purified as standard compounds, and the same conditions were applied to HPLC. The chemical structure of SG1-4 is shown in Figures 1(b) and 1(c) and was consistent with the reported literatures [35, 37–39].

2.2. Reagents and Antibodies. The assay kits of glucose, triglycerides (TGs), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), superoxide dismutase (SOD), malondialdehyde (MDA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and Oil Red O staining were obtained from Nanjing Jiancheng Bioengineering Institute, China. Enzyme-linked immuno-sorbent assay (ELISA) kits of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were obtained from Lianke Biotechnology, Hangzhou, China. ELISA kit of insulin was purchased from Elabscience Biotechnology, Wuhan, China. The kit for HE staining was purchased from Solarbio, Beijing, China. The BCA assay kit was obtained from Biosharp, China.

Primary antibodies for TRPV1 (1 : 1000) and SOD1 (1 : 200) were from Santa Cruz Biotechnology, USA. Antibodies for SIRT6 (1 : 1000), glucose transporter type 4 (GLUT4, 1 : 1000), SOD2 (1 : 2000), and GAPDH (1 : 20000) were obtained from ProteinTech, China. Antibodies for Nrf2

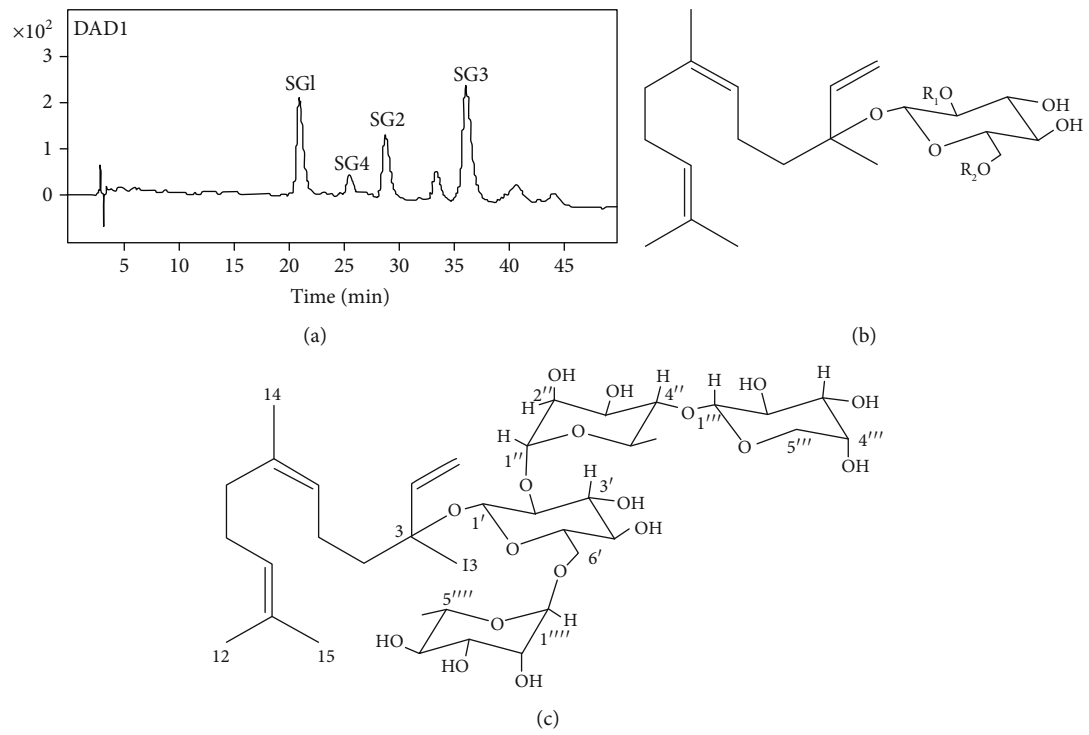

Figure 1: Compositional analysis of TSG from loquat leaves. (a) Chromatograms of the constituents through HPLC. (b) Chemical structure of SG1-SG3, SG1: R_1 = Rha (1 ⟶ 4) Rha, R_2 = Rha; SG2: R_1 = Rha (1 ⟶ 4) Rha, R_2 = H; and SG3: R_1 = Rha, R_2 = Rha. (c) Chemical structure of SG4.

(1 : 1000), phosphor-IRS-1 (Tyr895) (1 : 1000), and IRS-1 (1 : 1000) were purchased from Cell Signaling Technology, USA. Anti-AMPK (1 : 1000) and anti-phosphor-AMPK (1 : 1000) were purchased from Bioworld Technology, China. Second antibodies for rabbit-source and mouse-source were obtained from Abmart, Shanghai, China.

2.3. Animals and Treatment. Six-week-old healthy male C57BL/6 mice weighting 17-20 g were obtained from Shanghai Sino-British SIPPR/BK Lab Animal Co., Ltd., China. All the mice studied were maintained on a 12 h light/dark cycle at 23 ± 2°C with free access to water and food throughout the experiment. Mice were separated into four random groups with eight mice in each group as follows: regular diet (CON), HFD (HFD), low-dose TSG (25 mg/kg) with HFD (HFD+TSG-L), and high-dose TSG (100 mg/kg) with HFD (HFD+TSG-H). After a week of acclimation, the CON group was treated with regular diet for 8 weeks, which contained 20% (weight/weight) flour, 10% rice flour, 20% corn, 26% drum head, 20% bean, 2% fish powder, and 2% bone powder (XieTong Organism Inc., China). Simultaneously, to induce a mouse model with basic pathophysiology of IR, the other groups were provided with HFD for 8 weeks, which were made in our lab (18% lard (w/w), 5% egg powder, 1% cholesterol, 20% sucrose, 0.1% bile salt, and 55.9% regular diet). During the last 4 weeks of induction, groups of HFD+TSG-L and HFD+TSG-H were administered with oral gavages of 25 and 100 mg/kg TSG, respectively. Correspondingly, the groups of CON and HFD received equal volumes of saline by intragastric administration during the last 4 weeks. Mouse body weights were monitored weekly. All the experimental procedures and animal treatments were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

2.4. Sample Collection. After 8 weeks of treatment, mice were fasted for 12 h before sacrificed for blood and liver samples. The blood samples were collected by cardiac puncture and centrifuged at 2500 × g for 15 min at 4°C to obtain serum and then stored at -80°C for further experiment. The liver tissues were dissected, photographed, weighed, frozen in liquid nitrogen, and stored at -80°C until analysis.

2.5. Biochemical Analysis. The levels in serum of glucose, TG, TC, LDL-C, HDL-C, MDA, SOD, ALT, and AST were measured with commercial kits and analyzed by a Molecular Devices Spectra Max Plus automatic plate reader (Molecular Device, Sunnyvale, CA, USA). The levels of TNF-α, IL-1β, IL-6, and insulin in serum were tested with ELISA kits following the manufacturer's protocol.

2.6. Hematoxylin-Eosin Staining and Oil Red O Staining. For hematoxylin-eosin (HE) staining, liver samples were fixed in formalin buffer, then embedded in paraffin blocks, and sectioned. Sliced sections were stained with hematoxylin and eosin. Pathological changes were observed using a light microscope (Olympus, Japan). For Oil Red O staining, the frozen liver sections were fixed in 10% formaldehyde for 10 min, then washed by isopropanol, stained in Oil Red O, and processed for hematoxylin counter staining. Photomicrographs of tissue sections were taken using a light microscope.

2.7. Western Blotting. Liver tissues were first lysed on ice using RIPA lysis buffer and then centrifuged. Protein concentrations were determined using a protein assay kit, with BSA as standards. Equal amount of proteins was electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a 0.45 μm PVDF membrane. Blocked for 2 h with TBST containing skimmed milk powder at room temperature, the membranes were incubated with primary antibodies separately overnight at 4°C. Then, membranes were washed with TBST for 3 times and incubated with secondary antibodies at room temperature for 1 h. Signals were detected by chemiluminescence using the ECL detection reagent. The bands were quantified and analyzed by the Image J software (National Institutes of Health, USA).

2.8. Statistics Analysis. Data were presented as mean ± SEM. with GraphPad Prism (GraphPad Software Inc., USA). One-way ANOVA with Tukey's multiple comparison test was used to analyze intergroup significance. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Compositional Analysis of TSG. Four known sesquiterpene glycosides were detected in the TSG sample (Figure 1(a)). The content of sesquiterpene glycosides in TSG was 84.2% ± 2.8%.

3.2. TSG Ameliorated Weight Gain and Hepatic Damage Induced by HFD in IR Mice. After diet intervention, mice in HFD group were evidently fatter than mice in the CON group (Figures 2(a) and 2(c)). The liver tissues were dark brown both in CON and TSG groups while those were yellowish and significantly heavier in HFD mice (Figures 2(b) and 2(d)). Oral administration of TSG efficaciously reduced body and liver weights of HFD mice, despite low or high doses ($P < 0.001$, Figures 2(c) and 2(d)). Liver is an insulin-sensitive organ with a vital role in maintaining metabolism. When IR develops, abnormal lipid accumulation usually occurs in liver. Hence, HE and Oil Red O staining were performed to evaluate the hepatic pathology and morphology, assessing the effect of TSG on mouse liver. There were dramatical increase of vacuoles and lipid droplets in liver of the HFD group. TSG administration enormously lessened vacuoles and lipid droplets in liver tissues (Figures 2(e) and 2(f)). As important supplements to the histological changes of liver, the activities of ALT and AST were tested to monitor liver function. Activities of ALT and AST were dramatically elevated in the HFD mice, which were significantly decreased after TSG treatment ($P < 0.01$, Figures 3(a) and 3(b)).

3.3. TSG Relieved Hyperglycemia and Hyperlipidemia in IR Mice. Hyperglycemia and hyperlipidemia are common in IR patients. Compared with the mice on regular diet, excessive serum insulin and glucose were detected in mice on HFD. The HFD-driven increases of serum insulin and glucose were both reduced in mice treated with TSG at 25 and 100 mg/kg ($P < 0.001$, Figures 3(c) and 3(d)). Next, we assayed the concentration of TC, TG, LDL-C, and HDL-C in serum to confirm the hypolipidemic function of TSG in IR mice. The levels of TC and TG were highly stimulated by HFD, and similar trend was found in serum LDL-C level ($P < 0.001$, Figures 3(e)–3(g)). In the meantime, lower serum HDL-C level was detected in the HFD group ($P < 0.01$, Figure 3(h)). We found that TSG treatment achieved hypolipidemic effect, as indicated by the reduction of TC, TG, and LDL-C and elevation of HDL-C in serum.

3.4. Anti-Inflammatory and Antioxidant Effects of TSG in IR Mice. Systemic inflammation in mice was estimated by the content of inflammatory cytokines using ELISA kits. Compared with regular-diet mice, each concentration of TNF-α, IL-1β, and IL-6 in serum was dramatically higher in HFD mice. These inflammatory cytokines were enormously reduced in TSG-treatment groups (Figures 4(a)–4(c)).

MDA, a landmark product of lipid peroxidation, was overproduced in mice by continuous HFD treatment. In addition, HFD consumption suppressed the activity of SOD which was identified as one of antioxidant defense system. Supplementation of TSG induced significant reduction in serum MDA content while remarkable increase in SOD activity ($P < 0.001$, Figures 4(d) and 4(e)).

3.5. Effect of TSG on Hepatic IRS-1/GLUT4 and AMPK Signaling Pathways. HFD-induced IR markedly impaired insulin signaling, including the decrease of IRS-1 tyrosyl phosphorylation and GLUT4 expression. TSG treatment revealed an apparent improvement in p-IRS-1 and GLUT4 expression compared with the HFD group (Figures 5(a)–5(d)). Meanwhile, TSG significantly reversed HFD-induced suppression of AMPK phosphorylation in IR mice (Figures 5(e) and 5(f)).

3.6. Effect of TSG on Regulating Hepatic TRPV1. Based on the role of TRPV1 in inflammation and metabolism, we continued to analyze whether TSG prevented the IR-caused damage through TRPV1 on protein level. TRPV1 expression of the HFD group was greatly raised in comparison with the CON group. Hepatic TRPV1 expression was effectively reduced in mice treated with TSG, especially with the high dose of TSG (Figures 6(a) and 6(b)).

3.7. Effect of TSG on SITR6/Nrf2 Signaling. Considering the former reports suggested that SIRT6 promoted Nrf2 signaling in respond to oxidative stress, we also verified whether TSG had a protective function in HFD-induced IR by regulating SIRT6/Nrf2 pathway. Protein expressions of both SIRT6 and Nrf2 were greatly inhibited in livers of HFD mice. In accordance with Nrf2, protein levels of SOD1 and SOD2 were lower than those in the CON group. After TSG treatment, all the decreased trends were distinctly reversed (Figures 6(c)–6(g)).

4. Discussion

Loquat (*Eriobotrya japonica* Lindl.) leaf is used as not only a delicious tea but also a kind of diet-therapy food with a rich resource in China. As traditional Chinese medicine, loquat leaf now is clinically used to treat cough and has been recorded in history for hundreds of years. Besides, it has

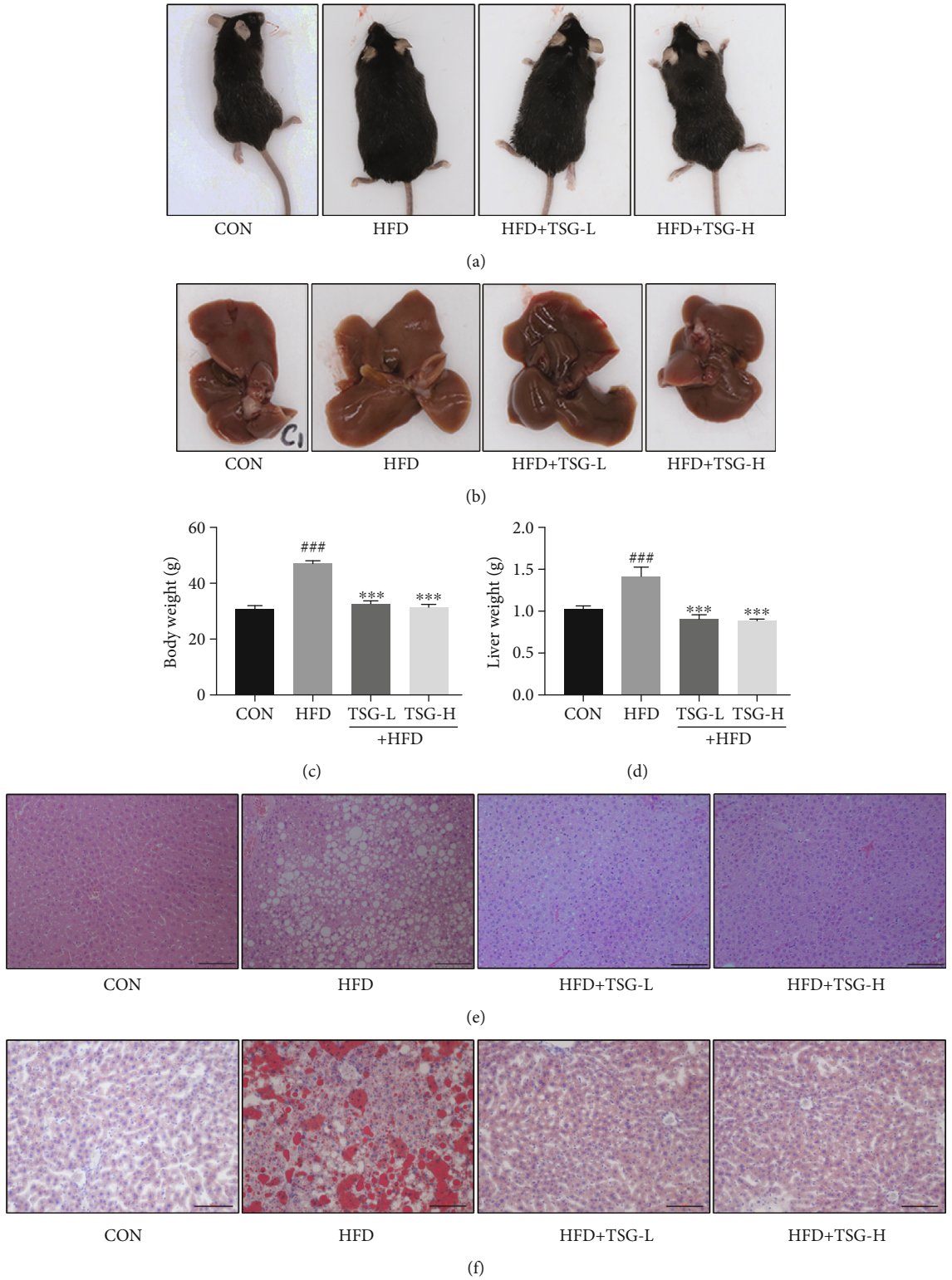

FIGURE 2: Effects of TSG on body weight, liver weight, and hepatic pathological changes. (a, b) Pictures of mice's bodies and liver tissues. (c, d) Weights of body and liver. (e, f) Liver tissues stained with HE and Oil O Red. The size bar in photomicrograph was 100 μm. Data were presented as mean ± SEM. $^{###}P < 0.001$ vs. mice in the CON group. $^{***}P < 0.001$ vs. mice in the HFD group.

therapeutic potential for T2DM and obesity. The bioactivities of loquat leaf extracts or compounds have been authenticated scientifically and methodically by researchers, including anti-inflammation and antioxidation [16, 40–42]. According to earlier studies, extracts of loquat leaves reduced TC and promoted glucose uptake in HFD-induced IR condition, which was instrumental in treating IR [43, 44]. Our previous investigation indicated that sesquiterpene

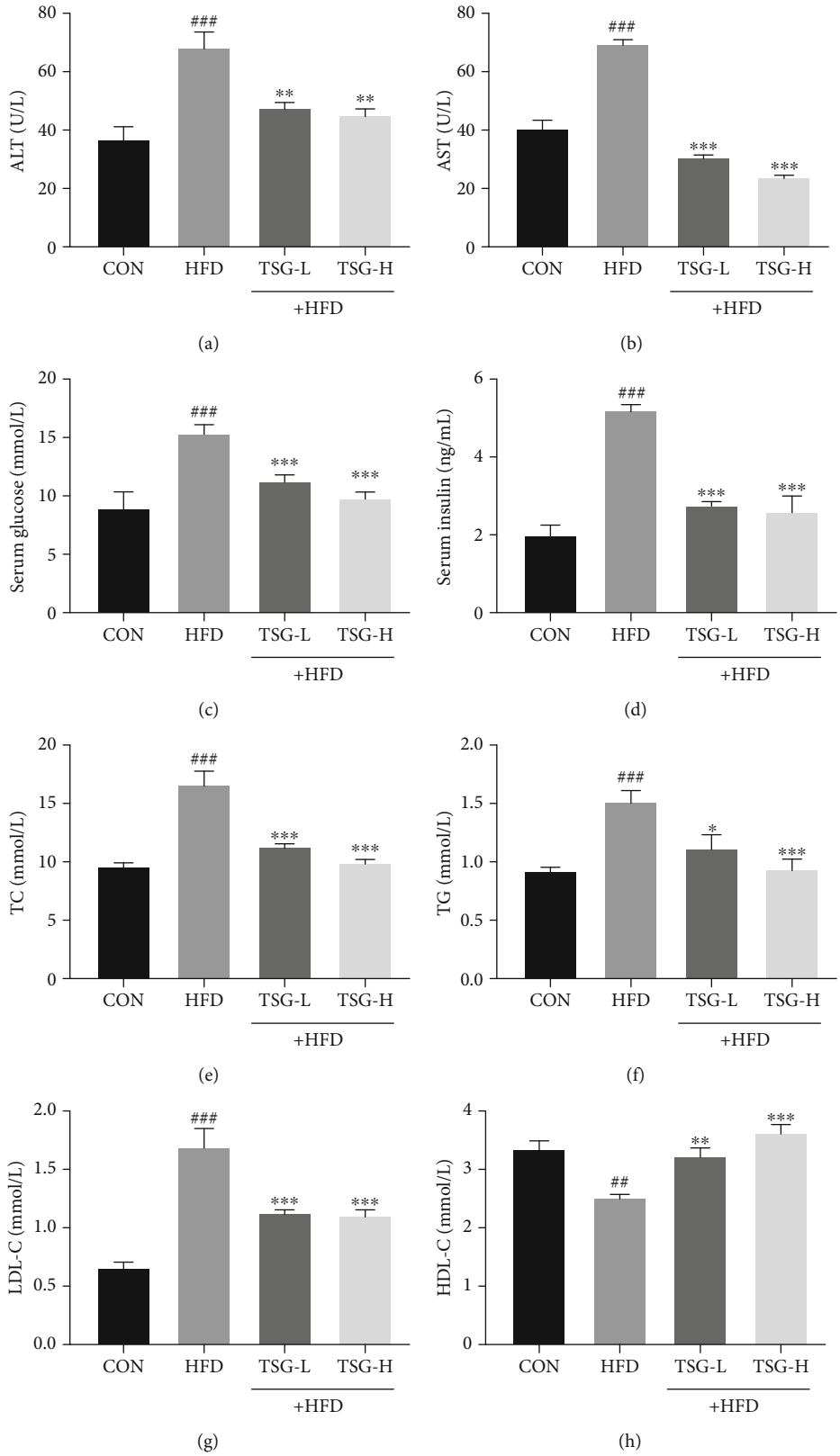

Figure 3: Effects of TSG on metabolic parameters and aminotransferase levels. Activities of (a) ALT and (b) AST. Quantitation of (c) glucose and (b) insulin in serum. Quantitation of (e) TC, (f) TG, (g) LDL-C, and (h) HDL-C. All data were presented as mean ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. mice in the CON group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. mice in the HFD group.

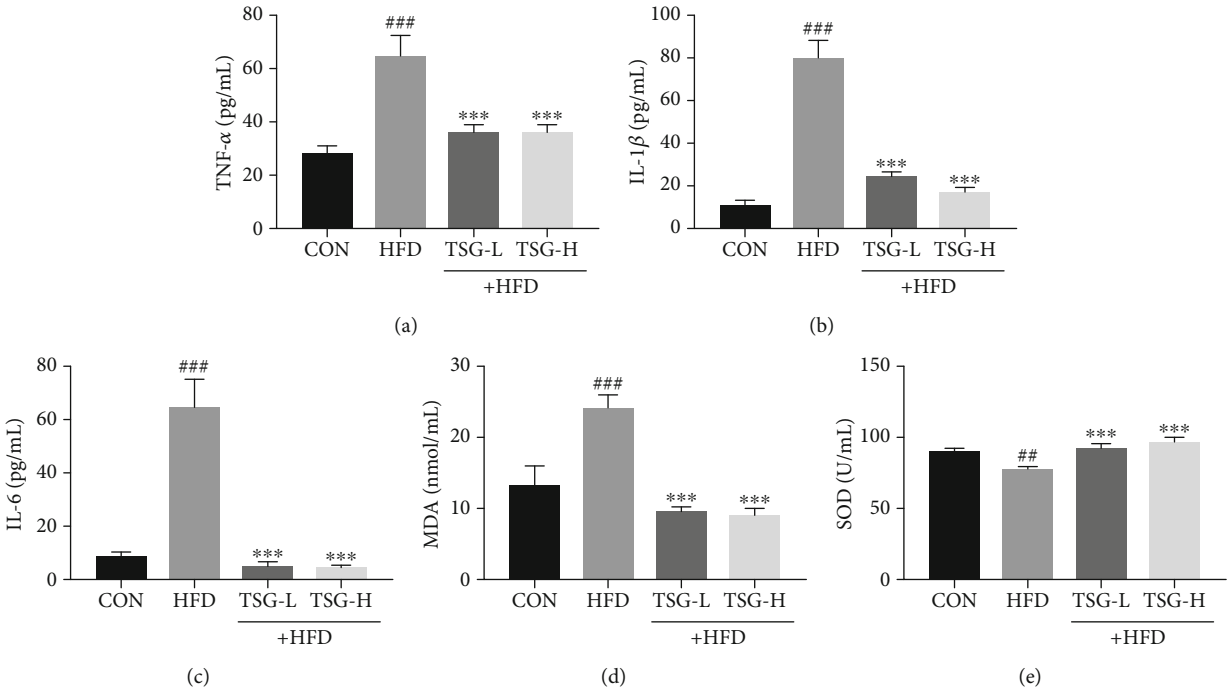

Figure 4: Anti-inflammatory and antioxidant effects of TSG. Quantitation of (a) TNF-α, (b) IL-1β, and (c) IL-6 in serum. (d) The content of MDA in serum. (e) Activity of SOD in serum. All data were presented as mean ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. mice in the CON group. $^{***}P < 0.001$ vs. mice in the HFD group.

glycosides from loquat leaves exhibited the bioactivity of reducing lipid accumulation and enhancing glucose uptake in insulin-resistant HepG2 cells via AMPK pathway [37].

Insulin resistance is defined as failure of insulin-target tissues to respond to insulin stimulation. IR induced by HFD disrupts normal regulation mechanism of glucose and lipid, leading to hyperglycemia and abnormal lipid accumulation in nonadipose tissues [45]. In this study, TSG extracted from loquat leaves effectively prevented HFD-induced obesity and irregular glucose and lipid metabolism, showing protective effect against IR. And the protection of TSG was also considered to be associated with anti-inflammation and antioxidation. The modulation of IRS-1/GLUT4, AMPK, TRPV1, and SIRT6/Nrf2 signal pathways might be involved in the potential molecular mechanism (Figure 7).

Liver is a unique organ in maintaining glucose and lipid homeostasis. IR is usually accompanied by lipid accumulation in liver and even steatosis. Our data confirmed that TSG had hypolipidemic effect and prevented abnormal lipid accumulation in liver. Activities of ALT and AST were notably decreased by TSG treatment, and histological examination further presented the protective effect of TSG on livers in IR condition. Taken together, TSG protected liver from damage caused by abnormal lipid deposition, conducing to controlling IR.

In physiological state, insulin binding to insulin receptor leads to IRS-1 phosphorylation. It initiates insulin signal transduction and then promotes GLUT4 and glucose transport. During IR occurrence, the signaling of IRS-1/GLUT4 is inhibited. It has been well proved that AMPK serves as an important regulator in glucose and lipid metabolism [5, 46]. AMPK has been recommended as a target in the treatment of numerous diseases [7, 47]. Activation of AMPK has been shown to improve insulin sensitivity, attenuate gluconeogenesis, and ameliorate abnormal lipid metabolism [47–49]. Moreover, there were evidences showing that the expression of GLUT4 could be positively regulated by activated AMPK [8, 9]. As our results showed (Figure 5), the signaling pathways of IRS-1/GLUT4 and AMPK were suppressed in IR, bringing out disordered metabolism of glucose and lipid. TSG was found to repair the glucose metabolism and insulin signaling by promoting expression of IRS-1 and GLUT4. In the meantime, TSG noticeably reversed the suppression of AMPK activation, which attenuated gluconeogenesis and profited hepatic lipid metabolism and further prevented blood glucose from increasing and hepatic steatosis.

In the past decade, studies on insulin resistance have gradually revealed a relation between low-grade systematic inflammation and pathological insulin sensitivity, which was evidenced by changes in biochemical markers of inflammation [50, 51]. We observed the increased levels of TNF-α, IL-1β, and IL-6 in mice under IR condition, supporting the correlation between systemic inflammatory state and IR. These results were consistent with previous studies [52, 53]. TSG greatly reduced inflammatory cytokines, indicating that inflammation induced by HFD was alleviated due to TSG treatment.

Of wide knowledge, TRPV1 is a wide-expression calcium channel. TRPV1 can be activated by physical and chemical stimulation, mediating harmful reactions under pathological conditions [54]. Raised expression of TRPV1 on mRNA and protein levels has been reported in various inflammatory

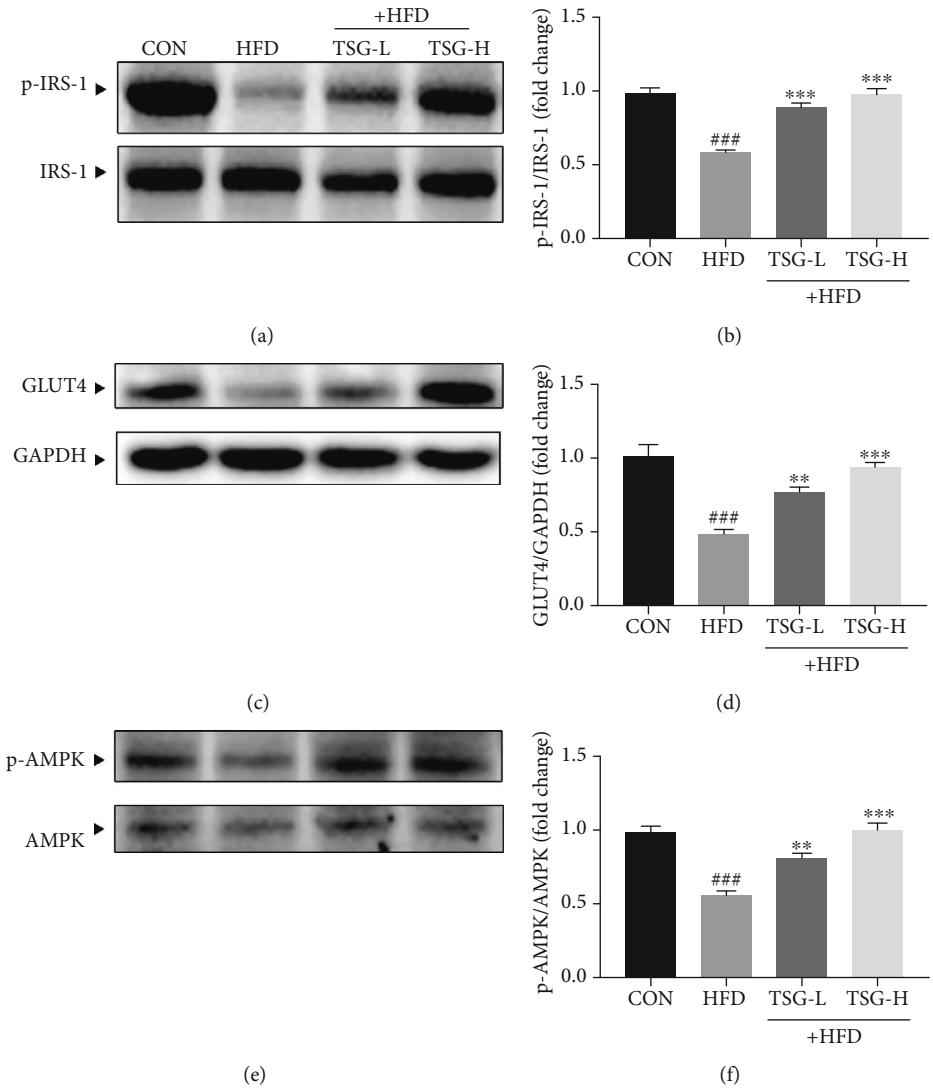

Figure 5: Enhanced signaling of IRS-1/GLUT4 and AMPK following TSG administration. (a, b) The phosphorylated and total forms of IRS-1 in liver. (c, d) GLUT4 expression in liver tissue. (e, f) The phosphorylated and total forms of AMPK in liver. All data were presented as mean ± SEM. $^{###}P < 0.001$ vs. mice in the CON group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. mice in the HFD group.

models [55, 56]. There was evidence providing that the level of TRPV1 expression was increased with exposure to TNF-α [57, 58]. TRPV1 can be stimulated by reactive oxygen species (ROS) as well and mediates generation of ROS and inflammatory mediators, contributing to inflammation and tissue damage [18, 59–61]. Furthermore, a range of studies demonstrated that application of TRPV1 deletion or TRPV1 antagonists could alleviate metabolism-related low-grade inflammation *in vivo*. HFD raised the levels of IL-1β and IL-6 in wild-type mice but had not affects in TRPV1-null mice, and lack of TRPV1 improved glucose tolerance though did no significant differ on weight gain [22]. Similarly, application of TRPV1 antagonists made obvious decrease in levels of serum proinflammatory markers (TNF-α, IL-6, and NF-κB) and alleviated IR as well [62]. Our results supported a harmful function of increased TRPV1 expression in IR mice. Based on above findings, we suggested that TSG decreased the levels of proinflammatory cytokines, relieving chronic inflammation in IR mice, via suppressing TRPV1 expression.

On the other hand, oxidative stress has been proven to be another key even in the onset and progress of IR [63, 64]. Excessive fat intake makes lipid accumulation and peroxidation in body, producing superfluous ROS [65, 66]. And when the antioxidant system cannot overcome the generation of ROS, oxidative stress eventually occurs [67]. In the present study, the generation of MDA was increased whereas the activity of SOD was lowered in IR mice, implying the imbalance between oxidation and antioxidant capacity. We confirmed that TSG reversed the abnormal accumulation of MDA in serum and compromised activity of SOD, thus exhibited antioxidant effect.

SIRT6, a kind of deacetylases, has been reported to regulate a myriad of biologic processes [68–70]. SIRT6 inhibition has been established in clinical and animal models of IR [29, 71]. Mounting studies have believed that SIRT6 protected

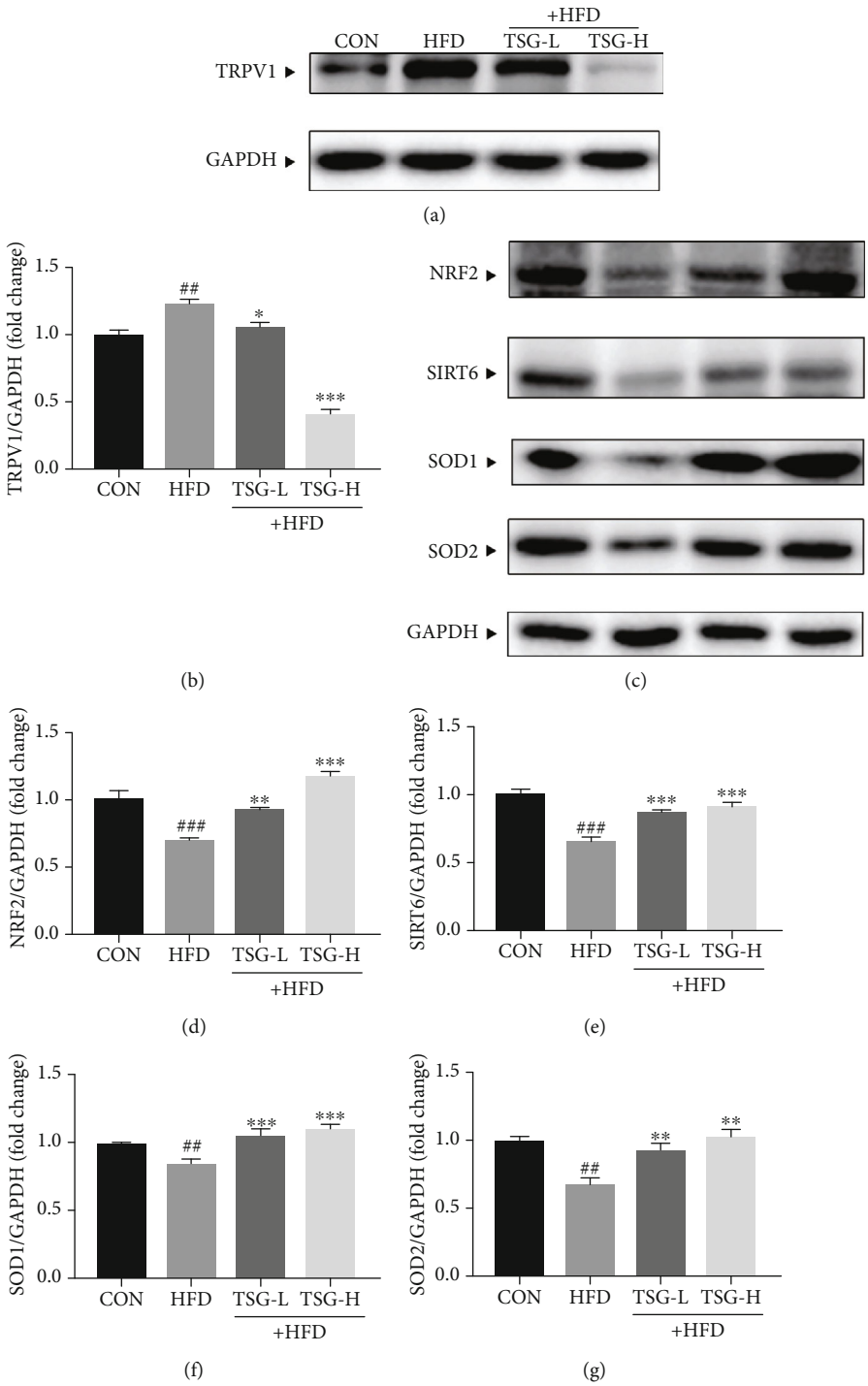

Figure 6: Effects of TSG on the TRPV1 and SIRT6/Nrf2 signaling pathways. (a, b) The protein expression of TRPV1 in livers. (c–g) The protein expression of Nrf2, SIRT6, SOD1, and SOD2 in liver tissues. Data were presented as mean ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. mice in the CON group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. mice in the HFD group.

cells in many diseases including NAFLD, T2DM, and obesity based on IR [25, 72–74]. Overexpression of SIRT6 has been proven to reduce blood glucose levels in mice with both standard chow and high-calorie diet and enhance insulin sensitivity in mouse liver [75]. In recent years, SIRT6 has been identified as a coactivator of Nrf2, promoting activation of Nrf2 and its downstream enzymes against oxidative stress [23, 76]. It was found that diet-induced hepatic IR exacerbated in hepatocyte-specific SIRT6-knockout mice due to downregulation of Nrf2 as well as upregulation of Nrf2 functional repressor [29]. In the present study, we observed that TSG greatly upregulated hepatic protein expression of SIRT6, Nrf2, SOD1, and SOD2 in IR mice. Our results indicated that TSG from loquat leaves attenuated

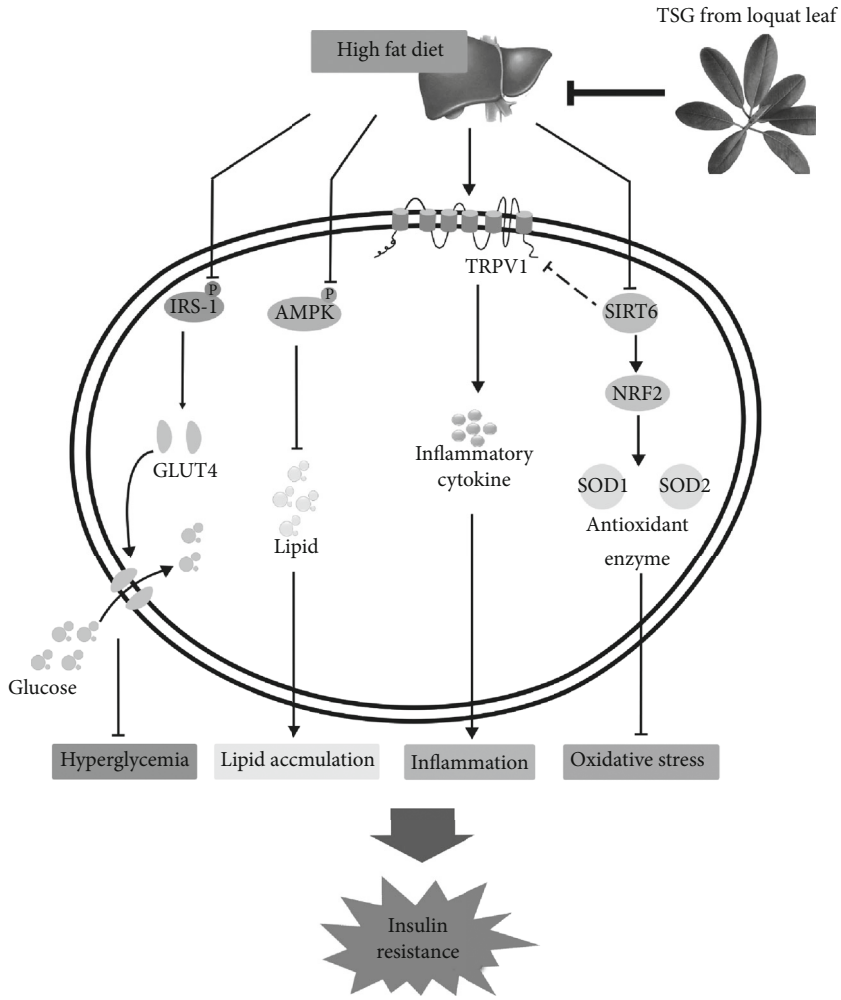

Figure 7: A schematic illustration of major points of conclusion.

hepatic oxidative stress in IR mice by potentiating SIRT6/Nrf2 signaling and its downstream enzymes. Furthermore, it is worth noting that SIRT6 has been suggested to take a part in TRPV1-mediated inflammation. In the inflammatory state, the level of SIRT6 was decreased while TRPV1 expression was upregulated [30, 77]. Overexpression of SIRT6 could suppress TRPV1 and result in significant downregulation ROS and NF-κB production, achieving anti-inflammatory effect [31]. It proved a support for the negative regulatory role of SIRT6 in TRPV1-mediated inflammation. Our results showed the low level of SIRT6 and high level of TRPV1 in IR that was accompanied with inflammation while TSG reversed the trend. From this point of view, further studies are required to explore the deep mechanism of TSG on anti-inflammation by TRPV1 and SIRT6.

5. Conclusion

Our study demonstrated that TSG from loquat leaves prevented HFD-induced IR by reducing abnormal lipid accumulation, inflammation, and oxidative stress. These effects were attributed to enhanced signaling of IRS-1/GLUT4 and activated AMPK as well as the modulation of TRPV1 and SIRT6/Nrf2 signaling pathways. Given the potential of IR amelioration by TSG in this study, TSG can be used to treat T2DM. And further studies can be performed with every monomer in TSG to evaluate the potential of each individual sesquiterpene glycosides on IR reduction *in vivo*.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ruoyun Wu and Tunyu Jian contributed equally to this work.

Acknowledgments

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Research Article

Bavachin Induces Ferroptosis through the STAT3/P53/SLC7A11 Axis in Osteosarcoma Cells

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Ferroptosis is a new form of regulated cell death, which is mediated by intracellular iron. Although it is reported that bavachin has antitumour effects on several tumour cells and prompts the reactive oxygen species (ROS) generation, it is unclear whether ferroptosis can be induced by bavachin in osteosarcoma (OS) cells. In this study, we found that bavachin inhibits the viability of MG63 and HOS OS cell lines along with an increase in the ferrous iron level, ROS accumulation, malondialdehyde overexpression, and glutathione depletion. Moreover, iron chelators (deferoxamine), antioxidants (Vit E), and ferroptosis inhibitors (ferrostatin-1 and liproxstatin-1) reverse bavachin-induced cell death. Bavachin also altered the mitochondrial morphology of OS cells, leading to smaller mitochondria, higher density of the mitochondrial membrane, and reduced mitochondrial cristae. Further investigation showed that bavachin upregulated the expression of transferrin receptor, divalent metal transporter-1, and P53, along with downregulating the expression of ferritin light chain, ferritin heavy chain, p-STAT3 (705), SLC7A11, and glutathione peroxidase-4 in OS cells. More importantly, STAT3 overexpression, SLC7A11 overexpression, and pretreatment with pifithrin-α (P53 inhibitor) rescued OS cell ferroptosis induced by bavachin. The results show that bavachin induces ferroptosis via the STAT3/P53/SLC7A11 axis in OS cells.

1. Introduction

Osteosarcoma (OS) is the most frequent bone tumour in children and adolescents, in addition to being the most common aggressive malignancy originating from mesenchymal cells [1, 2]. Although neoadjuvant chemotherapy combined with surgery and postoperative chemotherapy has been used to treat OS, the five-year survival rate of OS is not satisfactory on account of development of early metastases and chemotherapy resistance [3, 4]. Thus, it is imperative to find new targets and pharmaceuticals to improve the prognosis.

Ferroptosis is a new form of regulated cell death (RCD) that is mediated by intracellular iron and is quite different from other forms of cell death, such as apoptosis, autophagy, or necrosis [5]. Intracellular iron reacts with H_2O_2 through Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + OH^-$) to generate many reactive oxygen species (ROS) and trigger lipid peroxidation (LP) to induce ferroptosis [6]. Aberrant iron metabolism, ROS generation, and abnormal LP are the hallmarks of ferroptosis [5, 7]. Morphologically, ferroptotic cells demonstrate shrunken mitochondria, higher density of the mitochondrial membrane, and reduced or diminished mitochondrial cristae [8]. Additionally, ferroptosis can be inhibited by iron chelators or antioxidants and activated by some small compounds (erastin), or by inhibiting glutathione peroxidase-4 (GPX4) [9]. The cystine/glutamate antiporter system Xc^-, which is composed of two subunits, SLC7A11 (light chain) and SLC3A2 (heavy chain), is closely linked to ferroptosis [10]. SLC7A11, which transports cystine into the cells, enhances glutathione (GSH) synthesis, further promoting the inhibition of ferroptosis by GPX4. P53, as an upstream mediator of SLC7A11, mediates the repression of

SLC7A11 to initiate ferroptosis in tumour cells [11]. In addition to breast cancer, colorectal cancer, and anaplastic thyroid cancer [12–14], ferroptosis also exerts anticancer effects in OS [15, 16]. Some clinical drugs including sulfasalazine and sorafenib have proven to induce ferroptosis to trigger anticancer effects [17], via inhibiting the system Xc^- [18, 19]. Therefore, activating P53 to downregulate SLC7A11/GPX4 to trigger ferroptosis could be a potential method to inhibit progression of OS cells.

Signal transducer and activator of transcription 3 (STAT3) belongs to the STAT family and is a vital transcription factor involved in inflammation and tumour progression [20]. Classical STAT3 activation involves the phosphorylation of STAT3 at Tyr705 (p-STAT3 (705)), which interacts with and inhibits P53 [21]. Wang et al. discovered that high expression of STAT3 is relevant to increased malignancy and poor prognosis [22]. As described above, it is critical to inactivate STAT3 to halt tumour progression.

Flavonoids have multiple biological functions, especially anticancer effects induced by the prooxidant activity [23]. Amentoflavone inhibits glioma cells via inducing ROS accumulation and ferroptosis [24]. Robustaflavone also induces ferroptosis by increasing ROS and lipid peroxidation in MCF-7 cells [25]. Bavachin belongs to flavonoids and is a bioactive compound extracted from the fruit of *Psoralea corylifolia* and displays various functions including anti-inflammatory, lipid-lowering, and cholesterol-reducing effects [26]. A previous study showed that bavachin stimulates osteoblast differentiation by activating the Wnt pathway and is considered an estrogen supplement [27]. Recently, it has been shown that bavachin also exerts antitumour effects. Bavachin has been demonstrated to inhibit human hepatocellular carcinoma cells by inducing apoptosis, accompanied by ROS accumulation [28]. It has been reported that bavachin inhibits melanoma cells by downregulating the MAPK signaling pathway [29]. Notably, bavachin was shown to trigger the apoptosis of multiple myeloma cells by inhibiting p-STAT3 (705) and increasing P53 [30]. Although it has been reported that bavachin can exacerbate ROS accumulation and suppress several tumour cells, it is unclear whether ferroptosis could be induced by bavachin, and if so, what is the underlying mechanism?

We hypothesized that bavachin increases intracellular ferrous iron, ROS, and malondialdehyde (MDA) levels and induces ferroptosis by the downregulation of SLC7A11 through inactivating STAT3 to upregulate P53. We hope that our research will provide promising pharmaceutical targets for the treatment of OS.

2. Materials and Methods

2.1. Reagents. Bavachin was purchased from MCE (China), and pifithrin-α (PFT-α), deferoxamine (DFO), ferrostatin-1 (Fer-1), and liproxstatin-1 (Lip-1) were purchased from Topscience (China). Vitamin E was purchased from Beyotime (Shanghai, China). Rabbit polyclonal anti-transferrin receptor antibody (TFRC, AF5343, 1 : 1000), rabbit polyclonal anti-divalent metal transporter-1 antibody (DMT1, DF12740. 1 : 1000), rabbit polyclonal anti-ferritin light chain antibody (FTL, DF6604, 1 : 1000), rabbit polyclonal anti-ferritin heavy chain antibody (FTH, DF6278, 1 : 1000), rabbit polyclonal anti-SLC7A11 antibody (DF12509, 1 : 1000), rabbit polyclonal anti-P53 antibody (AF0879, 1 : 1000), rabbit polyclonal anti-STAT3 antibody (AF6294, 1 : 1000), rabbit polyclonal anti-p-STAT3 (705) antibody (AF3293, 1 : 1000), and rabbit polyclonal anti-glutathione peroxidase-4 antibody (GPX4, DF6701, 1 : 1000) were purchased from Affinity Bioscience (China). HRP goat anti-rabbit IgG was purchased from Earthox (USA).

2.2. Cell Culture. The human OS cell lines MG63 and HOS were purchased from Procell (China). The cells were cultured in a humidified CO_2 incubator at 37°C and 5% CO_2 and grown in DMEM/high-glucose (Gibco, USA) supplemented with 10% foetal bovine serum (MRC, China).

2.3. Cell Viability. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) (APExBIO, China). According to the protocol, the absorbance values of the samples were read at 450 nm using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). Subsequently, the cell death ratio was calculated using the formula: $\text{cell death ratio}\,(\%) = (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100$.

2.4. Transmission Electron Microscope (TEM). MG63 and HOS cells were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at room temperature for 24 h. Then, the cells were fixed in 2% osmium tetroxide in PBS at room temperature for 1 h. Next, the samples were dehydrated with a graded series of ethanol solutions (50% to 100%). Subsequently, the samples were embedded in epoxy resin and sectioned into ultrathin sections (60 nm). Finally, the ultrathin sections were steeped with uranyl acetate (1%) and lead citrate (0.1%) and observed and photographed using an H-7500 TEM (Hitachi, Japan).

2.5. Mitochondrial Membrane Potential (MMP) Detection. MMP was observed using JC-1 assay kit (Beyotime, China). According to the protocol, the cells were stained with the JC-1 probe and observed under a fluorescence microscope (DMIL4000, Leica, China). Green fluorescence indicates a low MMP, whereas red fluorescence indicates a high MMP.

2.6. Ferrous Iron Assay. Cellular ferrous iron levels were detected using FerroOrange (Dojindo, China). According to the protocol, the cells were incubated with FerroOrange for 0.5 h. Fluorescence intensity (Ex: 543 nm, Em: 580 nm) was assessed using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). The ferrous iron levels were finally expressed as a ratio to the fluorescence intensity value of the control.

2.7. ROS Assay. Cellular ROS levels were measured using a commercial ROS assay kit (Beyotime, China). According to the protocol, the cells were loaded with a 2,7-dichlorodihydrofluorescein diacetate probe (10 μM). The cells were then incubated at 37°C for 20 min. Finally, the fluorescence intensity (Ex: 488 nm, Em: 525 nm) was assessed using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China).

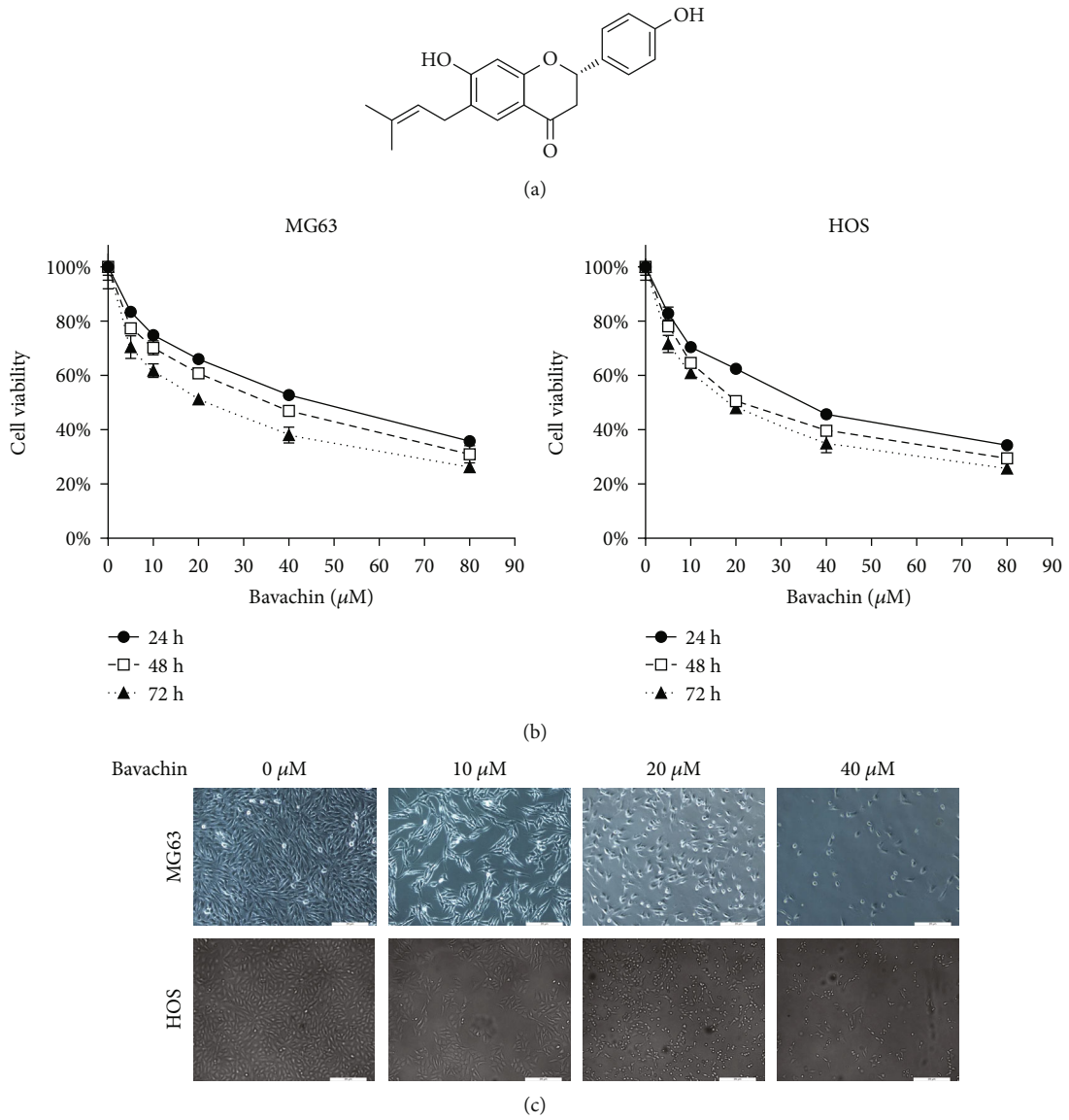


Figure 1: Bavachin inhibits osteosarcoma (OS) cell viability. (a) Chemical structure of bavachin. (b) CCK8 assay shows bavachin inhibits OS cells in a concentration- (5 μM, 10 μM, 20 μM, 40 μM, and 80 μM) and time- (24 h, 48 h, and 72 h) dependent manner. (c) Morphologic features of OS cells on microscopy. Cells became round and showed shrunk after bavachin (10 μM, 20 μM, and 40 μM) treatment for 24 h.

2.8. GSH Assay. GSH levels were assayed using the GSH assay kit (Nanjing Jiancheng, Nanjing, China). The harvested cells were crushed by sonication to obtain the supernatant used for measuring the GSH levels in a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). The levels of GSH were expressed as a ratio to the absorbance value of the control cells assessed at 405 nm.

2.9. Malondialdehyde (MDA) Assay. MDA levels were assayed using the MDA Assay Kit (Beyotime, China). First, the cells were lysed using the RIPA Lysis Buffer (Servicebio, China) with a protease inhibitor and centrifugated at 100,00 g for 10 min at 4°C to obtain the supernatant. The protein concentration of the samples was measured using the BCA Protein Assay Kit (Beyotime, China). Subsequently, the collected supernatant (0.1 ml) was added to thiobarbituric acid (0.2 ml) and incubated at 100°C for 15 min. Finally, the samples were read at 532 nm using a fluorescence microplate reader (Varioskan&LUX, Thermo Fisher, China). The MDA levels were expressed as a ratio to the absorbance value of the control.

2.10. Western Blotting Analysis. The cells were lysed and homogenized using RIPA Lysis Buffer (Servicebio, China) with a protease inhibitor. The protein concentration of the samples was measured using the BCA Protein Assay Kit (Beyotime, China). The proteins were added to the loading buffer (Beyotime, China) and denatured in boiling water

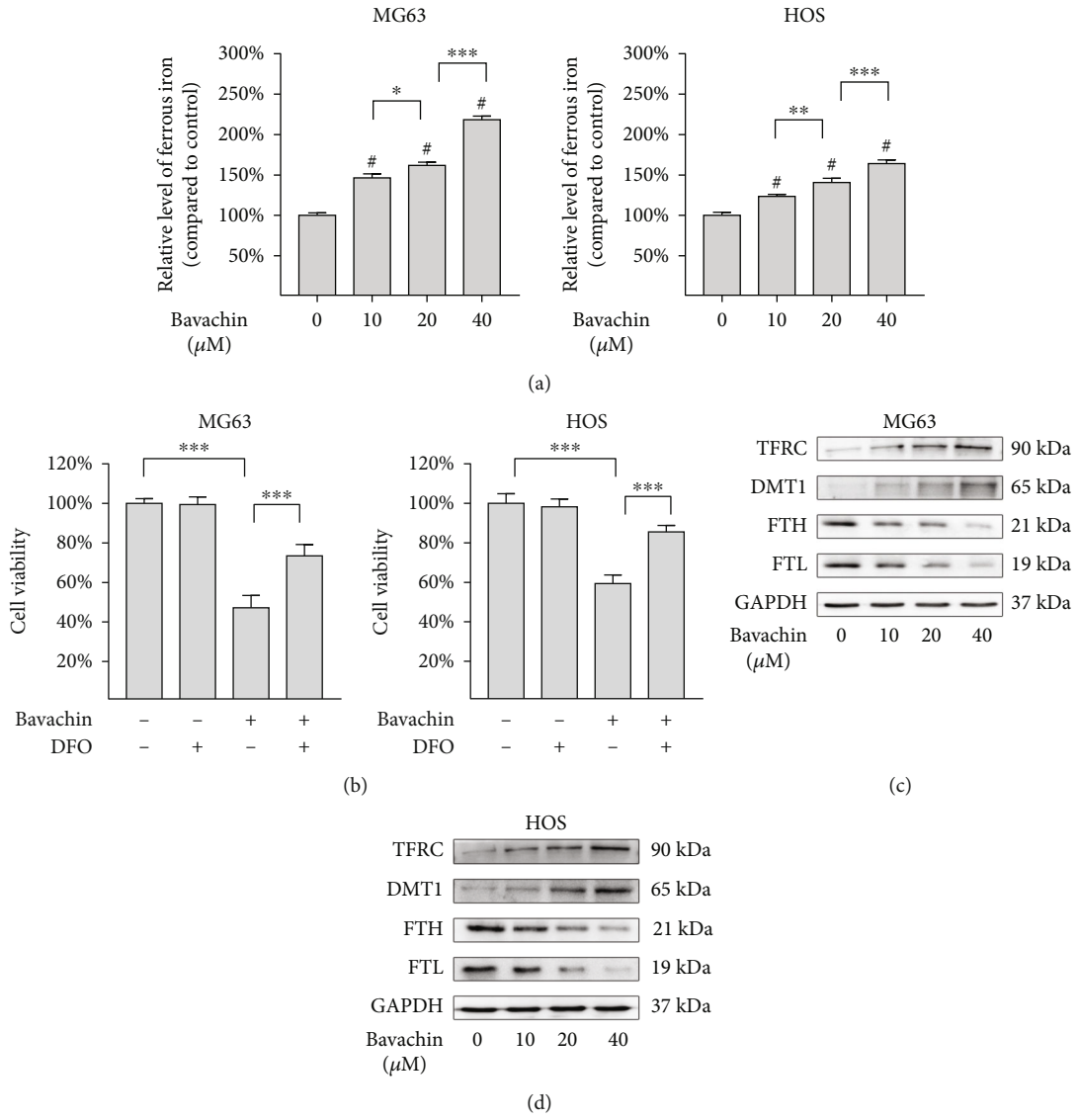


Figure 2: Bavachin-induced ferrous iron accumulation initiates death of osteosarcoma (OS) cells. (a) Bavachin (24 h) induced ferrous iron accumulation in OS cells occurs in a concentration-dependent manner. (b) Deferoxamine (DFO) rescues cells from bavachin-induced cell death. (c, d) Western blotting analysis shows the protein levels of transferrin receptor (TFRC), divalent metal transporter-1 (DMT1), ferritin light chain (FTH), and ferritin heavy chain (FTL) in MG63 and HOS cells treated with bavachin (10 μM, 20 μM, and 40 μM) for 24 h. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{\#}p < 0.001$ vs. the control group.

for 5 min. Each protein sample (40 μg) was loaded onto an SDS-PAGE (10%–12%) gel and transferred to PVDF membranes. The membranes were blocked with NcmBlot Blocking Buffer (NCM Biotech, China) and incubated with the primary antibodies at 4°C overnight. The membranes were subsequently incubated with the secondary antibody at room temperature for 1.5 h. Finally, the bands were developed using a HyperSignal ECL kit (4A Biotech, China).

2.11. Cell Transfection. For evaluating the overexpression of STAT3 and SLC7A11, an overexpressing plasmid along with a negative control (NC) plasmid was purchased from Vigene Biosciences (China). Briefly, cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen, China). After 48 h, the cells were subjected to subsequent experiments.

2.12. Statistical Analysis. Data are presented as means ± SD ($n = 3$). One-way ANOVA and Tukey's multiple comparisons test were used to analyze the data. All statistical analyses were performed using GraphPad Prism 7 (https://www.graphpad.com/). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Bavachin Inhibits OS Cell Viability. To evaluate the effects of bavachin on OS cells, MG63 and HOS cells were treated with bavachin (5 μM, 10 μM, 20 μM, 40 μM, and

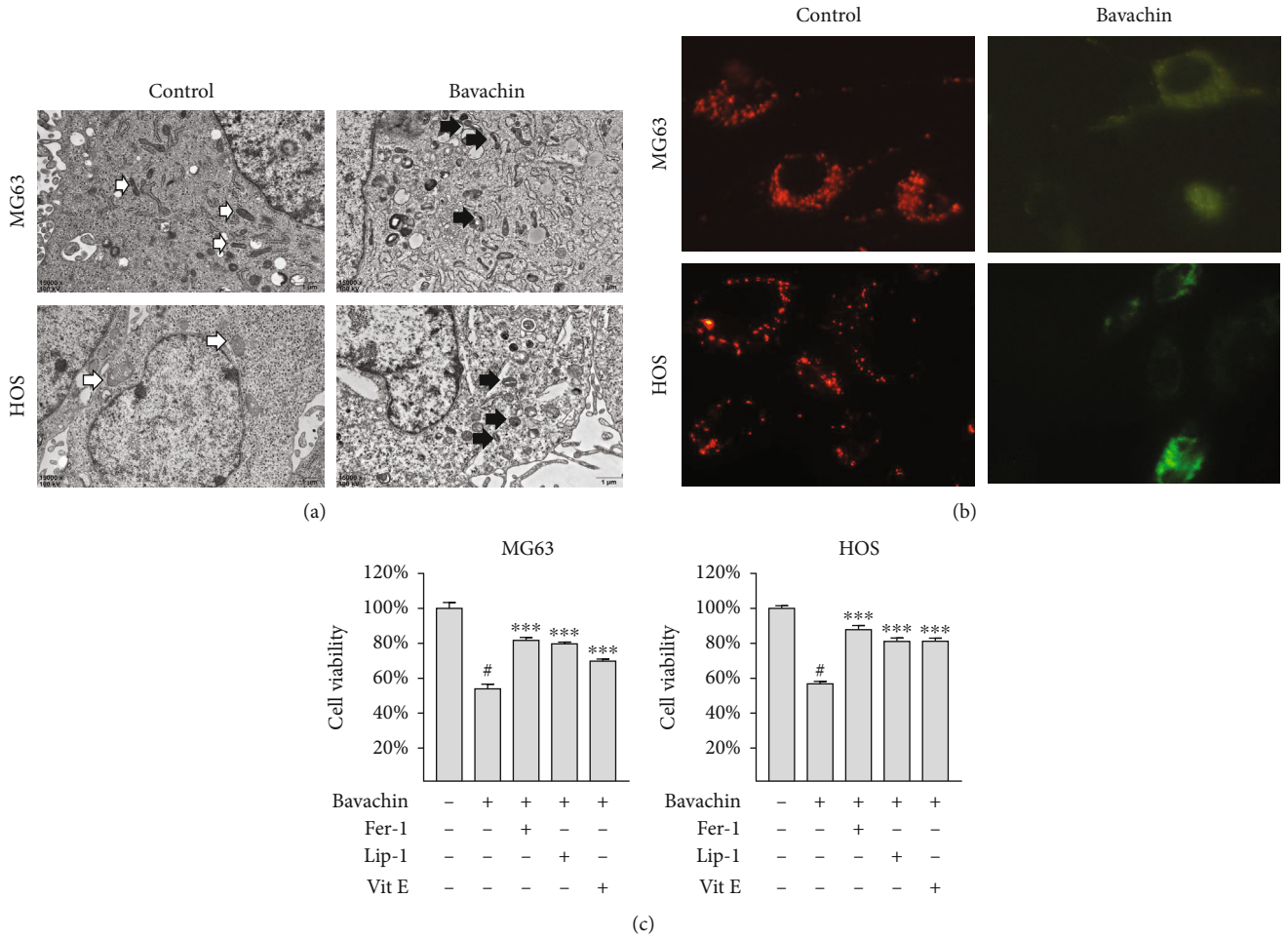


Figure 3: Bavachin induces ferroptosis in osteosarcoma (OS) cells. (a) The ultrastructure of MG63 and HOS cells was observed using transmission electronic microscopy. Cells in the bavachin (40 μM, 24 h) group display shrinking mitochondria, higher density of the mitochondrial membrane, and reduction or disappearance of the mitochondrial cristae. (b) Merged images of JC-1 staining demonstrate the change in the mitochondrial membrane potential (MMP) (red: aggregates and high MMP; green: monomers and low MMP). (c) CCK8 assay shows that deferoxamine (DFO), ferrostatin-1 (Fer-1), and liproxstatin-1 (Lip-1) rescue bavachin-induced cell death in OS cells. $^{***}p < 0.001$ vs. the bavachin group. $^{\#}p < 0.001$ vs. the control group.

80 μM) for 24 h, 48 h, and 72 h, and then cell viability was measured using the CCK8 assay. As shown in Figure 1(b), bavachin inhibited the viability of both MG63 and HOS cells in a concentration- and time-dependent manner. According to CCK8 assay, the half-maximal inhibitory concentration (IC_{50}) of bavachin at 24 h was determined. The IC_{50} values for MG63 and HOS cells were 42.32 μM and 34.2 μM, respectively. Therefore, 40 μM of bavachin concentration was selected for the following experiments. Morphological changes in MG63 and HOS cells following bavachin treatment were observed. Light microscopy images showed that the shape of the cells became round, and there was an increase in cell shrinkage and cell death with increasing concentration of bavachin (Figure 1(c)).

3.2. Bavachin Induces Ferrous Iron Accumulation to Initiate OS Cell Death. To evaluate whether bavachin affects ferrous iron accumulation, we assessed the expression of ferrous iron in OS cells treated with bavachin (10 μM, 20 μM, and 40 μM) for 24 h. Compared to the control group, bavachin increased the ferrous iron concentration in a concentration-dependent manner (Figure 2(a)). As illustrated in Supplementary Figures1(a) and 1(b), ferrous iron levels reached maximum at 24 h of 40 μM bavachin stimulation. Furthermore, OS cells pretreated with 100 μM of DFO (iron chelator) for 1 h recovered from bavachin-induced (40 μM, 24 h) cell death (Figure 2(b)). Finally, western blotting was used to explore the mechanism of ferrous iron accumulation induced by bavachin. As shown in Figures 3(c) and (d), bavachin treatment increased TFRC and DMT1 expressions and decreased FTH and FTL expression in a concentration-dependent manner (10 μM, 20 μM, and 40 μM).

3.3. Bavachin Induces Ferroptosis in OS Cells. To demonstrate bavachin-induced ferroptosis in OS cells, we looked into the morphology of the treated cells. TEM showed that

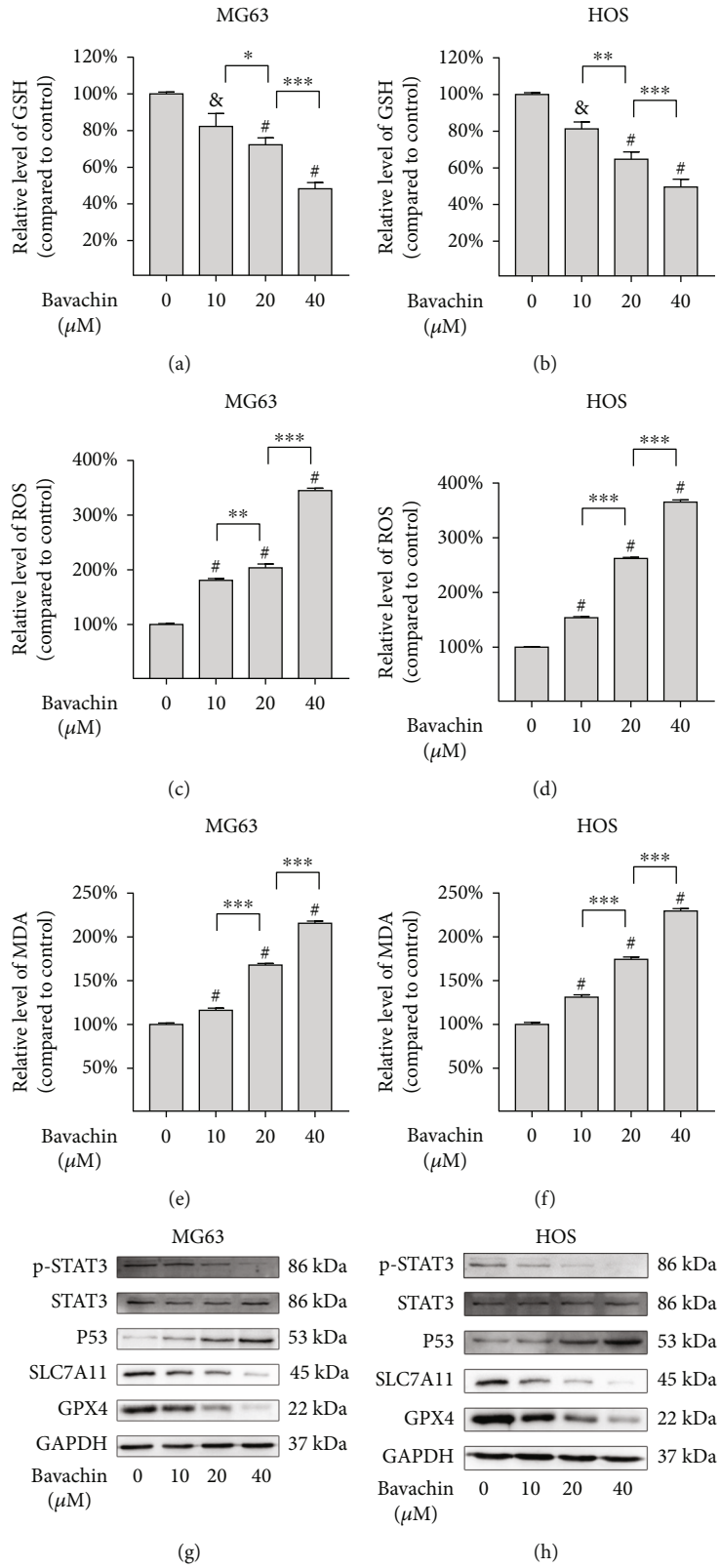
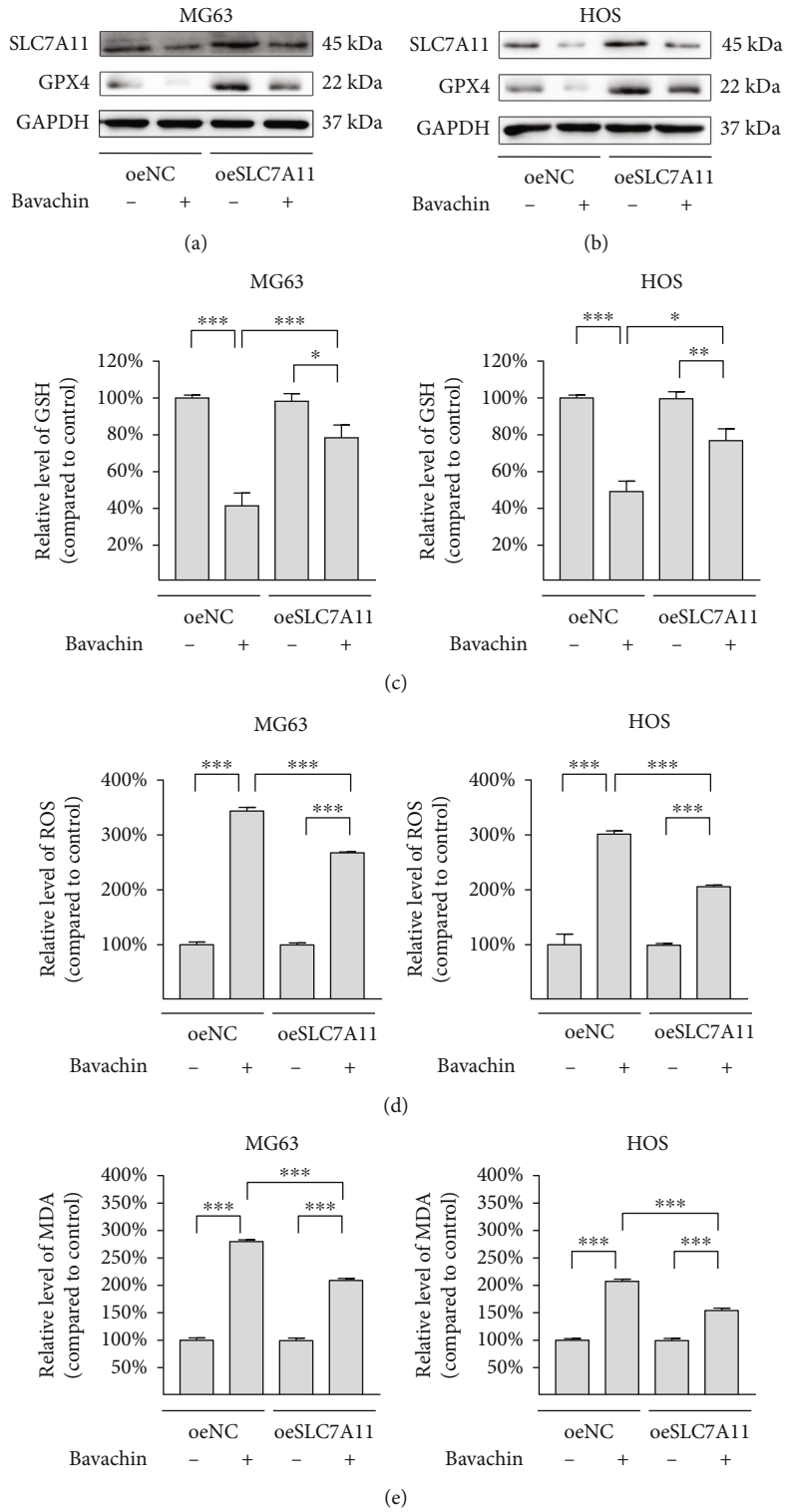


Figure 4: Bavachin induces GSH depletion and lipid peroxidation accumulation in osteosarcoma (OS) cells. (a, b) Bavachin induces GSH depletion in a dose-related manner over 24 h. (c, d) Bavachin greatly induces ROS generation in OS cells over 24 h. (e, f) Bavachin increases MDA accumulation in a dose-related manner. (g, h) Western blotting analysis shows the expression of p-STAT3, STAT3, P53, SLC7A11, and GPX4 in OS cells treated with bavachin (10 μM, 20 μM, and 40 μM) for 24 h. $^{\&}p < 0.01$ vs. the control group, $^{\#}p < 0.001$ vs. the control group, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.



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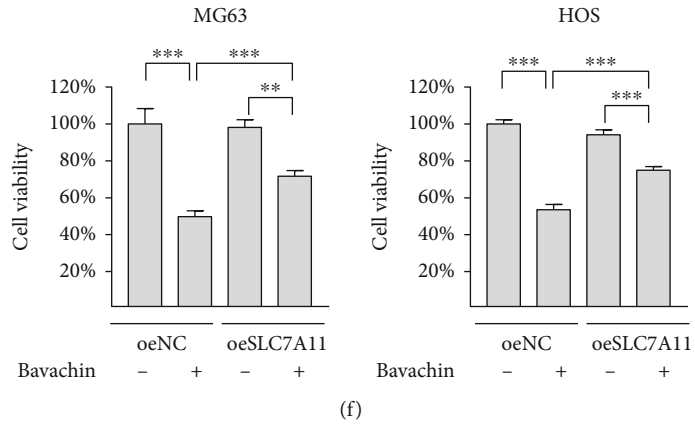


Figure 5: SLC7A11 overexpression alleviates bavachin-induced ferroptosis in osteosarcoma (OS) cells. OS cells were transfected with a SLC7A11 overexpressing plasmid and treated with bavachin (40 μM, 24 h). (a, b) Western blotting demonstrates the relative levels of SLC7A11 and GPX4 protein expression. (c) Overexpression of SLC7A11 restores bavachin-induced GSH depletion in OS cells. (d, e) Overexpression of SLC7A11 reduces bavachin-induced ROS and MDA accumulation. (f) Overexpression of SLC7A11 recovers bavachin-induced ferroptosis in OS cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

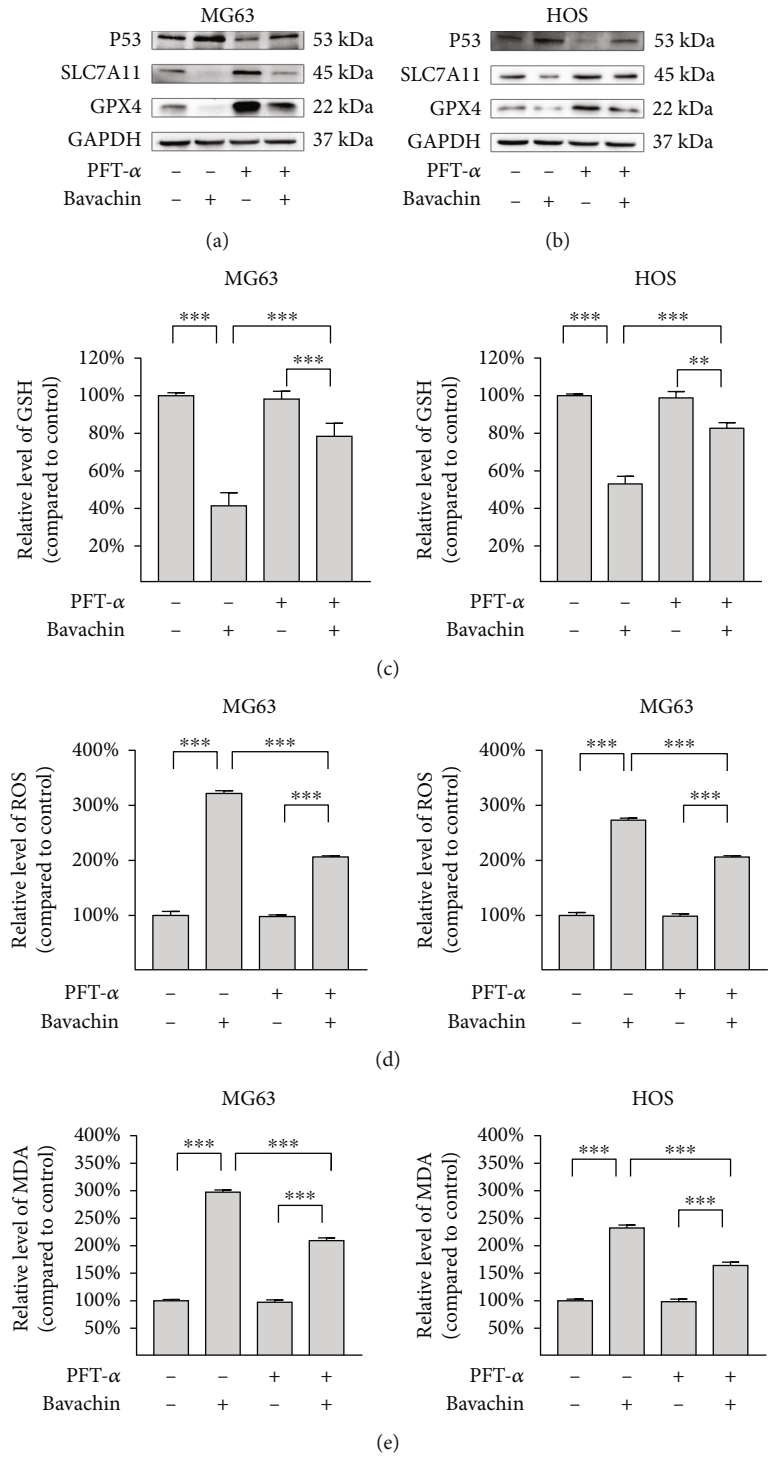
MG63 and HOS cells treated with 40 μM bavachin for 24 h demonstrated shrinking mitochondria, higher density of the mitochondrial membrane, and reduction or disappearance of mitochondrial cristae, which are typical morphological features of ferroptosis (Figure 3(a)). Moreover, in fluorescence images of JC-1 staining, green fluorescence was observed in the bavachin (40 μM for 24 h) group and red fluorescence in the control group (Figure 3(b)). Bavachin treatment reduced the MMP of OS cells compared with that of the control group. Finally, to clarify whether bavachin induced ferroptosis, OS cells were pretreated with ferroptosis inhibitors Fer-1 (5 μM), Lip-1 (2 μM), and Vit E (150 μM) for 1 h. As shown in Figure 3(c), the results of the CCK8 assay showed that Fer-1, Lip-1, and Vit E rescued bavachin-induced OS cell death.

3.4. Bavachin Induces GSH Depletion and LP Accumulation in OS Cells. The levels of GSH, ROS, and MDA were measured to determine whether bavachin induces oxidative stress in OS cells. It was found that the level of GSH declined gradually as bavachin concentration (10 μM, 20 μM, and 40 μM) increased over 24 h (Figures 4(a) and 4(b)). However, ROS levels were remarkably upregulated by bavachin stimulation (Figures 4(c) and 4(d)). Moreover, ROS levels reached their highest values at 24 h of 40 μM bavachin stimulation (Supplementary Figure 1(c) and 1(d)). As illustrated in Figures 4(e) and 4(f), bavachin markedly elevates the MDA level, which is a marker of LP, in a concentration-dependent manner for 24 h. To further analyze the molecular mechanism of bavachin-induced LP in OS cells, western blotting was performed to detect the expression of related proteins. The results demonstrated that bavachin inhibited GPX4, SLC7A11, and p-STAT3 expressions and increases P53 expressions in OS cells (Figures 4(g) and 4(h)).

3.5. SLC7A11 Overexpression Alleviates Bavachin-Induced Ferroptosis in OS Cells. OS cells were transfected with the SLC7A1 overexpressing plasmid to examine whether SLC7A1 overexpression could rescue bavachin-induced ferroptosis. As indicated in Figures 5(a)–5(c), SLC7A11 upregulation increases the decline in GPX4 expression and GSH content induced by bavachin (40 μM, 24 h). SLC7A11 overexpression attenuates the bavachin-induced accumulation of ROS and MDA (Figures 5(d) and 5(e)). Importantly, SLC7A11 overexpression rescues bavachin-induced OS cell death (Figure 5(f)). Overall, SLC7A1 overexpression inhibits bavachin-induced ferroptosis in OS cells.

3.6. P53 Inactivation Upregulates SLC7A11 and Alleviates Bavachin-Induced Ferroptosis in OS Cells. Our previous experiments (Figure 4) showed that bavachin upregulated P53 and downregulated SLC7A11-induced ferroptosis in OS cells. To investigate the regulatory relationship between P53 and SLC7A11, OS cells were pretreated with 5 μM PFT-α, a P53 inhibitor, for 0.5 h followed by treatment with 40 μM of bavachin for 24 h. As shown in Figures 6(a) and 6(b), PFT-α inactivates P53 to upregulate the expression of SLC7A11 and GPX4 induced by bavachin. PFT-α also restores bavachin-induced GSH consumption (Figure 6(c)). Meanwhile, PFT-α significantly reduced bavachin-induced ROS and MDA accumulation (Figures 6(d) and 6(e)). Subsequently, PFT-α rescued bavachin-induced ferroptosis in OS cells (Figure 6(f)). In summary, these results show that P53 negatively regulates SLC7A11 and plays a promoting role in bavachin-induced ferroptosis in OS cells.

3.7. P-STAT3 Activation Downregulates P53 Expression to Rescue Bavachin-Induced Ferroptosis in OS Cells. To evaluate whether STAT3 inhibits P53 expression to alleviate bavachin-induced ferroptosis in OS cells, OS cells were transfected with a STAT3 overexpressing plasmid, followed by treatment with 40 μM bavachin for 24 h. As shown in Figures 7(a) and 7(b), compared with that in the NC group, p-STAT3 was activated, although the p-STAT3/STAT3 ratio was unchanged. p-STAT3 activation inhibited the upregulation of P53 expression induced by bavachin, while increased GPX4 expression suppressed by bavachin. p-STAT3 activation recovered GSH depletion (Figure 7(c)) and decreased



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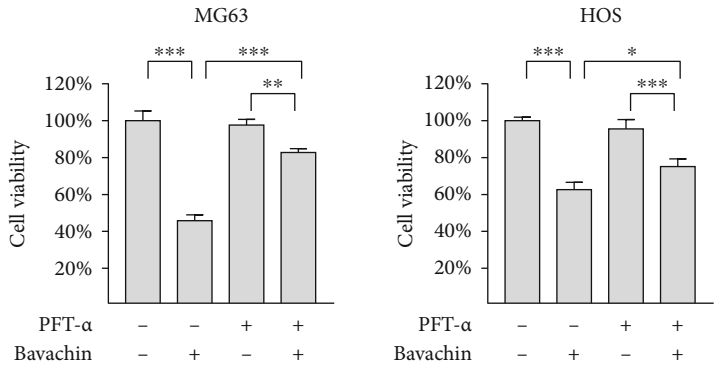


Figure 6: P53 inactivation upregulates SLC7A11 to alleviate bavachin-induced ferroptosis in osteosarcoma (OS) cells. OS cells were pretreated with pifithrin-α (PFT-α, 5 μM) followed by treatment with bavachin (40 μM, 24 h). (a, b) Western blotting shows the relative levels of P53, SLC7A11, and GPX4 protein expression. (c) PFT-α (5 μM) restores bavachin-induced GSH depletion. (d, e) PFT-α reduces bavachin-induced ROS and MDA accumulation. (f) PFT-α recovers bavachin-induced ferroptosis in OS cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

the accumulation of ROS and MDA, which is stimulated by bavachin (Figures 7(d) and 7(e)). Moreover, p-STAT3 upregulation rescued bavachin-induced ferroptosis in OS cells (Figure 7(f)). As described above, p-STAT3 activation inhibits P53 expression and rescues bavachin-induced ferroptosis in OS cells.

4. Discussion

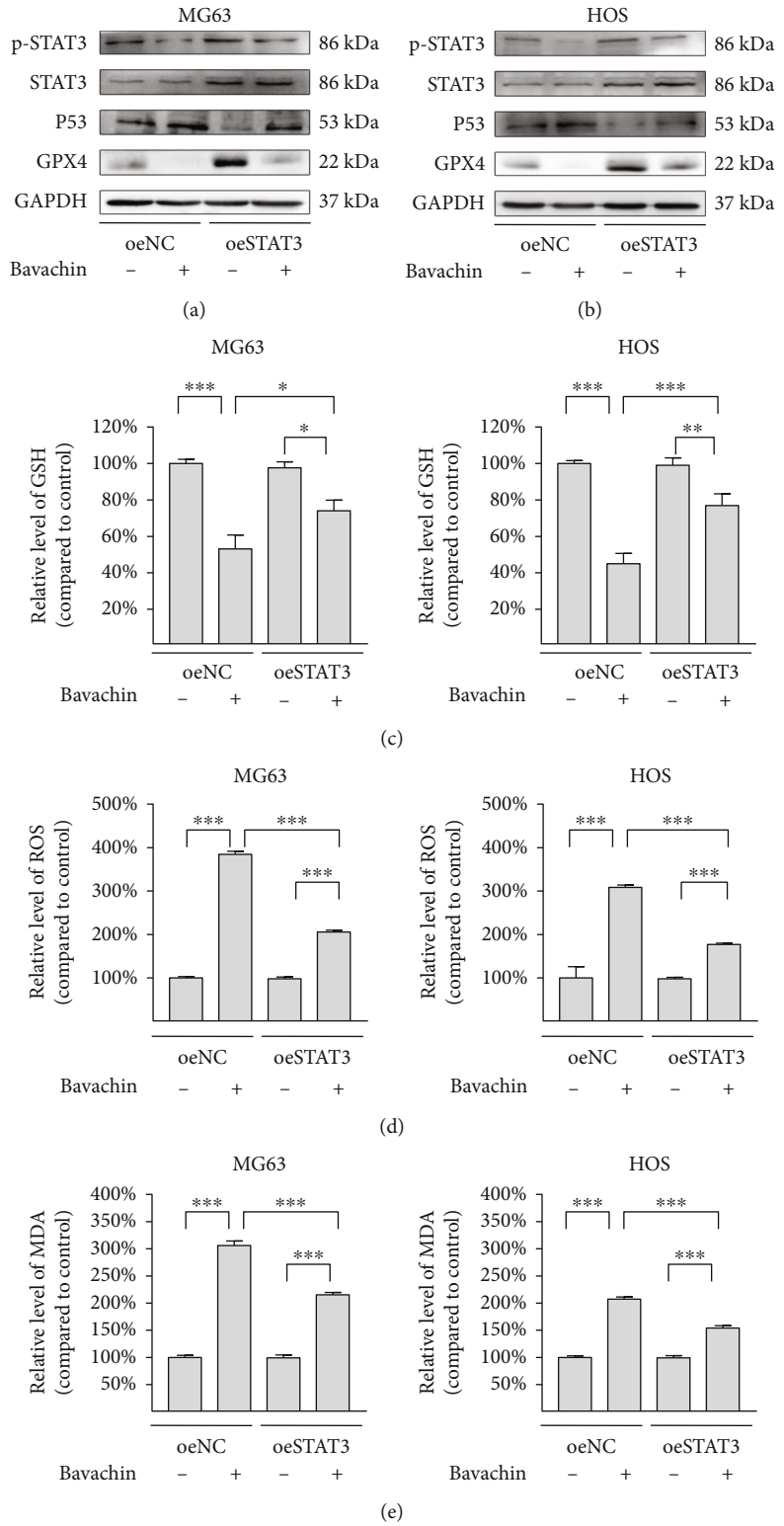
Our study revealed that bavachin could induce OS cell death, which was reversed by iron chelator (DFO) and ferroptosis inhibitors (Fer-1, Lip-1, and Vit E). Moreover, bavachin reduced the MMP and led to mitochondrial shrinkage, increased mitochondrial membrane density, and reduced mitochondrial cristae in OS cells. Furthermore, bavachin elevated intracellular ferrous iron levels by increasing TFRC and DMT1 expression and decreasing FTH and FTL expressions. Bavachin also reduced SLC7A11 and GPX4 expression and promoted ROS and MDA accumulation by downregulating p-STAT3 to upregulate P53 expression (Figure 8).

As a new form of RCD, ferroptosis significantly differs from other types of cell death, which is due to characteristics of iron-dependence, GPX4 inhibition, and abnormal LP [5]. Moreover, ferroptosis is rescued by iron chelators and antioxidants [31, 32]. Our study consistently showed that bavachin increases ferrous iron levels and induces OS cell death, which can be rescued by Fer-1, Lip-1, Vit E, and DFO. After treatment with bavachin, morphological changes in OS cells are consistent with the classical ferroptosis morphology, which is characterised by a smaller mitochondria, higher mitochondrial membrane density, and reduced mitochondrial cristae [8]. These findings indicate that bavachin induces ferroptosis in OS cells.

Aberrant intracellular iron metabolism, especially ferrous iron overloading, is an initiating factor for ferroptosis. TFRC and DMT1 mediate ferrous iron formation and transport [33]. Previous studies suggest that downregulation of TFRC expression inhibits ferroptosis [34], and overexpression of TFRC could increase iron levels to increase ferroptosis sensitivity [35]. Similar to TFRC, DMT1 is also a positive regulator of iron accumulation, leading to ferroptosis [36]. Consistent with previous studies, our findings show that TFRC and DMT1 expressions are positively related to ferroptosis induced by bavachin. Additionally, ferritin, composed of FTL and FTH, also serves as a critical mediator of iron storage and a regulator of iron homeostasis. Previously published studies have revealed that ferritin has a two-sided effect on ferroptosis. For example, overexpression of ferritin inhibits ferroptosis in PC-12 cells via ferritinophagy [37], whereas ferroptosis occurs in glioma cells by upregulating FTL and FTH expression [38]. Although the expression of FTH and FTL in ferroptosis is diverse, our results demonstrate that bavachin downregulates FTL and FTH expressions to promote ferroptosis, which can probably be ascribed to ferritinophagy-degrading ferritin to release Fe^{2+} that is involved in ferroptosis progression [9].

Triggering LP, which is described as a process by which ROS attacks polyunsaturated fatty acids to cause dehydrogenation, is an essential event that drives ferroptosis [39]. Both ROS, generated from iron in the Fenton reaction, and MDA, the final product of LP, are contributing factors for ferroptosis activation [40]. Bavachin has been demonstrated to promote ROS generation via suppressing the MMP to trigger HepG2 cell apoptosis [28]. However, whether bavachin induces MDA has not been reported in the literature. Similarly, our results suggest that bavachin elevates the level of ROS in a dose-dependent manner and suppresses the MMP. Moreover, we found that bavachin distinctly increased the MDA content to promote ferroptosis.

The SLC7A11/GPX4 axis efficiently protects cells from ferroptoss [41]. SLC7A11, a key subunit of the system Xc-, is responsible for transporting cystine, which is reduced to cysteine for synthesis of GSH. As a vital nonenzymatic antioxidant, GSH not only scavenges free radicals but also acts as a



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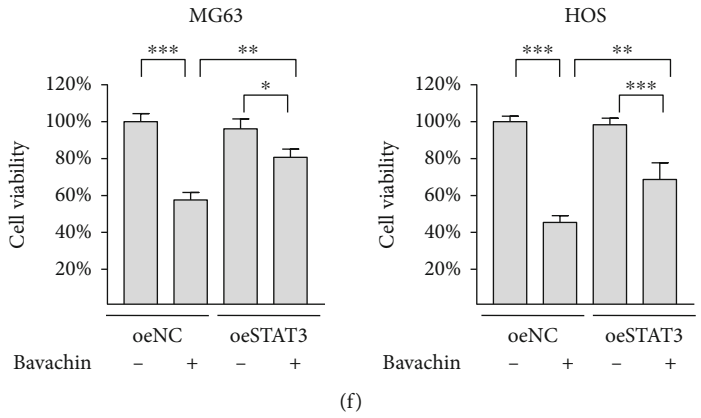

Figure 7: p-STAT3 activation downregulates P53 expression to rescue bavachin-induced ferroptosis in osteosarcoma (OS) cells. (a, b) Western blotting shows the relative levels of p-STAT3, STAT3, P53, and GPX4 protein expression. (c) p-STAT3 activation restores bavachin-induced GSH depletion. (d, e) p-STAT3 activation reduces bavachin-induced ROS and MDA accumulation. (f) p-STAT3 activation rescues bavachin-induced ferroptosis in OS cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

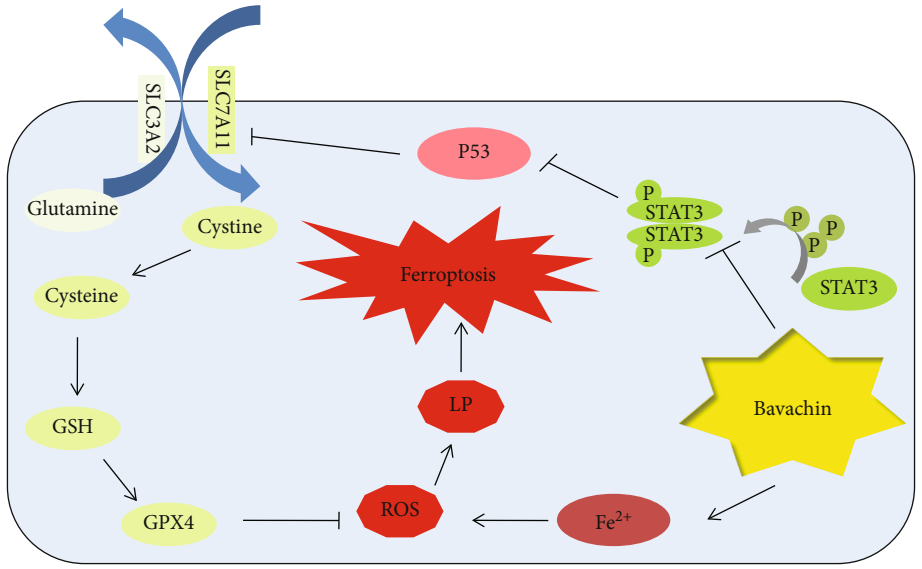

Figure 8: Schematic diagram displays potential mechanism of bavachin-induced ferroptosis in osteosarcoma cells. LP: lipid peroxidation.

cofactor of the GPX4-mediated reduction reaction of lipid hydroperoxides (LOOH). Inhibition of SLC7A11/GPX4, which is the most important antioxidant enzyme in the body, can trigger ferroptosis, while activation of SLC7A11/GPX4 facilitates the suppression of ferroptosis [42]. Shi et al. reported that tirapazamine induced ferroptosis in OS cells via downregulation of SLC7A11 and GPX4 expression, whereas the phenotype of cells induced by tirapazamine was reversed after overexpression of SLC7A11 [43]. Lin et al. also found that EF24, a synthetic drug, triggered ferroptosis in OS cells via inhibiting GPX4 [16]. Similarly, our results showed that bavachin led to ferroptosis in OS cells with accumulation of ROS and MDA, GSH depletion, and downregulation of GPX4 expression, while overexpression of SLC7A11 rescued OS cells from ferroptosis caused by bavachin. Therefore, we believe that bavachin triggers OS cell ferroptosis via the SLC7A11/GPX4 axis.

P53 is known as a tumour suppressor protein and can inhibit tumour cells by triggering ferroptosis [44]. Jiang et al. discovered that P53 induced ferroptosis by negatively regulating SLC7A11 to inhibit cystine transport [11]. However, a few studies have reported a contrasting view about the function of P53 in ferroptosis. In colorectal cancer cells, P53 inhibited LP and ferroptosis by binding to DPP4, while the loss of P53 led to the recovery of DPP4 to increase cell ferroptosis [45]. Tarangelo et al. found that P21, a downstream effector of P53, elevates intracellular GSH levels and GPX4 expression, leading to P53-delayed ferroptosis in

some cells [46, 47]. Therefore, the two-sided functions and mechanisms of P53 in ferroptosis need to be further explored in diverse extracellular environments and cell lines. Our study showed that bavachin increased P53 expression and downregulated SLC7A11 expression to induce ferroptosis in OS cells. However, in OS cells pretreated with PFT-α, a P53 inhibitor, these effects were reversed. These findings suggest that bavachin triggers ferroptosis in OS cells by activating P53 to inactivate SLC7A11.

STAT3 inactivation is also associated with ferroptosis. It has been reported that STAT3 inhibits P53 to facilitate tumour progression by binding to the promoter of p53, which is an important regulator of ferroptoss [21, 48]. Qiang et al. found that increasing the expression of p-STAT3 could alleviate ferroptosis via SLC7A11 in MLE12 cells by regulating SLC7A11 [49]. In OS cells, overexpression of STAT3 aggravated ROS accumulation and ferroptosis via the Nrf2/GPX4 axis [50]. According to these studies, STAT3 appears to be a potential pharmacological target. Although bavachin was verified to inactivate p-STAT3 and increase P53 expression in multiple myeloma cells, the regulatory relationship between STAT3 and P53 has not yet been explored [30]. Our study displayed that bavachin triggers ferroptosis by inhibiting p-STAT3 but not STAT3 and increases P53 expression; however, after OS cells were transfected with the STAT3 overexpression plasmid, which simultaneously increased STAT3 and p-STAT3 expressions, bavachin no longer induced ferroptosis. It has been suggested that bavachin enables ferroptosis by inhibiting STAT3 to upregulate P53 in OS cells.

Taken together, our study displayed that bavachin triggers ferroptosis in OS cells by increasing intracellular ferrous iron levels and inhibiting the STAT3/P53/SLC7A11 axis. It suggests that bavachin could be a promising pharmaceutical for the treatment of OS.

Data Availability

The data of this study is included within the article. The data is available from the first author (royalman1984@sina.cn) upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Zhenming Hu and Junming Feng contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: bavachin induces ferrous iron accumulation and ROS generation in a time-dependent manner in osteosarcoma (OS) cells. *(Supplementary Materials)*

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Review Article

Paclitaxel: Application in Modern Oncology and Nanomedicine-Based Cancer Therapy

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Paclitaxel is a broad-spectrum anticancer compound, which was derived mainly from a medicinal plant, in particular, from the bark of the yew tree *Taxus brevifolia* Nutt. It is a representative of a class of diterpene taxanes, which are nowadays used as the most common chemotherapeutic agent against many forms of cancer. It possesses scientifically proven anticancer activity against, e.g., ovarian, lung, and breast cancers. The application of this compound is difficult because of limited solubility, recrystalization upon dilution, and cosolvent-induced toxicity. In these cases, nanotechnology and nanoparticles provide certain advantages such as increased drug half-life, lowered toxicity, and specific and selective delivery over free drugs. Nanodrugs possess the capability to buildup in the tissue which might be linked to enhanced permeability and retention as well as enhanced antitumour influence possessing minimal toxicity in normal tissues. This article presents information about paclitaxel, its chemical structure, formulations, mechanism of action, and toxicity. Attention is drawn on nanotechnology, the usefulness of nanoparticles containing paclitaxel, its opportunities, and also future perspective. This review article is aimed at summarizing the current state of continuous pharmaceutical development and employment of nanotechnology in the enhancement of the pharmacokinetic and pharmacodynamic features of paclitaxel as a chemotherapeutic agent.

1. Introduction

As a result of the nonavailability of adequate treatment, cancer is nowadays becoming a major and life-threatening disease worldwide. Recent studies indicated that the number of cancer deaths globally exceeded the mortality rate of cardiac diseases [1]. It represents one of the greatest challenges of medicine today with the presumed increase in newly diagnosed cases in the near future and powerful impact at any stage of sociosanitary care [2]. General actions in prevention, early diagnosis, screening, and treatment of cancer have been questioned by its complex nature and mutability [3]. The occurrence of different tumour types may be attributable to several lifestyles and environmental factors such as radiation, infections, low physical activity, use of immunosuppressants, smoking, overweight, quality of diet, and alcohol consumption [1].

Over the past years, different cancer therapies have been developed. Fundamental treatments in cancer are surgery and radiation therapy, followed by various forms of specific treatments such as chemotherapy, immunotherapy, hormonal therapy, radiotherapy, and targeted therapy [1]. Even though therapies such as radiation and the application of standard chemotherapeutics possess significant tumouricidal potential, they usually kill normal cells besides cancer cells inducing that way serious hematologic toxicity and tissue damage in affected individuals [4]. Furthermore, chemotherapy shows narrow therapeutic benefits as a result of the occurrence of the intrinsic or acquired multiple drug resistance [5].

Compared to conventional therapies, targeted therapies with a multi-advantageous approach are much desirable [6]. They specifically stop the growth and expansion of cancer cells [7]. Their selective targeting profile is reflected in the fact that they achieve controlled release of the specific agent and its site-specific delivery and accumulation in tumour sites. As more efficient, targeted therapies reduce the demand for high doses of chemotherapeutics, that way, they generate less systemic adverse reactions [1].

The application of nanomedicine, which represents the integration of nanotechnology and medicine, in diagnosing, monitoring, and treating diseases has considerably enhanced the result of treatment of complicated and lethal diseases by retaining the therapeutic dose at the target site [8]. It includes the manipulation of the drug substance at the nanoscale which ultimately affects its physicochemical properties. Formulation approaches employing nanomedicines implicate the usage of sophisticated nanocarriers of natural and synthetic origin which shows immense potential to liberate therapeutics inside the body [1]. Numerous factors such as solubility, stability, biocompatibility, and biodegradability contribute significantly to the selection of nanomaterials for drug delivery demands [9]. Nanotechnology and nanoparticles provide certain advantages such as increased drug half-life, lowered toxicity, and specific and selective delivery over free drugs [10].

Nanomedicines that are presently used in clinical practice have been moderately effective in tackling the problems associated with drug development such as low solubility and inadequate circulation time [11]. Paclitaxel is a broad-spectrum anticancer drug from the group of the most commonly applied chemotherapeutic agents primarily for various solid tumours [12]. However, its application is significantly restricted as a result of limited solubility, recrystalization upon dilution, and cosolvent-induced toxicity [13]. The application of proper pharmaceutical carriers could overcome these limitations [14].

So far, paclitaxel nanoformulations have been efficiently utilized in clinical practice for the treatment of ovarian cancer, lung cancer, and breast cancer [15]. This narrative review article summarized the current state of the continuous pharmaceutical development and employment of nanotechnology in the enhancement of the pharmacokinetic and pharmacodynamic features of paclitaxel as a chemotherapeutic agent. As a novelty, this review highlights the therapeutic importance of paclitaxel nanoparticles in the targeted therapy of cancer cells in different types of tumours as well as data on their toxicity and safety.

2. Key Aspects of Targeted Therapy for Effective Cancer Management

Chemotherapy, lacking in specificity, is fundamentally based on the assumption that cancer cells have mitosis more

frequently than normal cells, and for this reason, they can be more affected than healthy tissues [16]. However, this is not true for all cell types, thus, deriving the nonspecific toxicity of chemotherapeutic drugs for healthy tissues, characterized by cells with rapid proliferation rates, such as hair follicles, bone marrow, and gastrointestinal tract cells, generating the characteristic side effects of chemotherapy [17]. We can state that all chemotherapeutic drugs have two faces: pharmaceutical drugs at the right (for a specific case) concentration and toxic substances at higher concentrations. Nonspecific toxicity toward normal cells, narrow therapeutic windows, and the onset of multidrug resistance mechanisms [18, 19] have highlighted the limits of conventional cancer treatment with chemotherapeutics and the need to explore new ways to deal with the disease. Therefore, many researchers have been stimulated to develop new therapeutic approaches allowing a more targeted drug delivery to the cancer cell.

The idea to target cancer cells in a specific manner started from the hypothesis and studies of the Nobel Prize laureate Paul Ehrlich (in 1900), who postulated the "magic bullet concept": an ideal drug that can go straight to its intended target on the cell surface [20]. Inspired by this concept, one of the most popular research approaches is based on the linking of a pharmacologically active molecule to carriers for selective delivery to target cells. These drugs could specifically target cancer cells to a larger extent with only a minimum impact on healthy cells.

Many different strategies have been exploited to carry toxic moieties. Different nanoparticles can facilitate drug delivery by both passive and active targeting mechanisms and highlight the potential advantages of utilizing nanotechnology within the field of cancer therapy [21]. Passive targeting is defined as the preferential accumulation of the drug in the tumour. Nanoparticles can be made from various materials including lipids [22]; inorganic materials such as gold, carbon, and iron oxide [23]; proteins [24]; and polymeric systems [25]. The accumulation and delivery of the drug are determined by the capability of the drug delivery system to overcome biological barriers and the inherent characteristics of the drug itself (e.g., size, material, and charge). In this strategy, drugs are enveloped in nanoparticles that can protect the therapeutic payload from blood degradation, with an improvement of their therapeutic index. However, due to the absence of specificity of these nanoparticles, it is possible to obtain only a limited reduction of some side effects. The size and the material of nanoparticles can affect their distribution and their intended goals.

Active chemotherapeutic drug targeting is aimed at blocking one or more specific hallmarks of cancer [26]: proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [27]. This strategy is aimed at blocking specific pathways or cancer proteins, i.e., receptors, growth factors, and proteins related to apoptosis and angiogenesis, that are expressed in normal tissues but resulted in being overexpressed or mutated in cancer [28]. An essential requirement for this approach is that the target molecule is possibly confined to the cell population to be destroyed, or at least that it is not present on stem cells or other cell types essential for the organism's survival.

A type of active nanoparticles can be considered antibodies. They are the most utilized nanocarriers, due to their stability in blood and their avidity and affinity for the target antigen. To obtain these hybrid antibody-drug conjugates, the two molecules must be linked together. The linker should be stable in blood but cleavable within the target cells by specific enzymatic or chemical degradation, thus, releasing the drug in an active form that can kill the cancer cell [29]. In conclusion, although more than a century has passed from Erlich's initial idea, the concept of a "magic bullet" for cancer therapy has not yet faded.

3. Paclitaxel: Pharmacotherapeutic Synopsis

Paclitaxel was discovered as a consequence of a joint input from the US Department of Agriculture (USDA) and the National Cancer Institute (NCI) through the application of a plant-screening program for effective discovery of a novel drug that could be applied as an effective drug for the management of cancer diseases [30]. The program was conducted by the National Cancer Institute Plant directed by Jonathan Hartwell who is a chemist who deals with natural products in collaboration with a botanist from the US Department of Agriculture named Robert Perdue. They evaluated almost 15,000 medicinal plants globally as well as evaluated their crude extracts numbering approximately 115,000 to validate their effectiveness as an anticancer drug against cancer diseases for a duration that varies from 1960 to 1981. Moreover, the program involved in the selection of the active compound that was performed by NCI in 1981 showed that paclitaxel was the only active biological ingredient that fits into this category and met the standard that could be utilized for effective management of cancer diseases most especially from the clinical trials [31, 32].

Paclitaxel was derived from a medicinal plant, specifically from the bark of the yew tree *Taxus brevifolia* Nutt. which belong to the *Taxaceae* family. The medicinal plant was later transported from Gifford Pinchot National Forest to their headquarters at the US Department of Agriculture in Maryland in August 1962. This was performed by the US Department of Agriculture botanist Arthur S. Barclay [30, 31, 33, 34]. Also, the extract derived from the bark of this plant was established to possess a cytotoxic action that could affect the *in vitro* development of 9KB cell cultures responsible for the development of human oral epidermoid carcinoma [35, 36]. In the year 1990, a motion was raised to include the severely depleted *T. brevifolia* as among the number of endangered species in the Pacific Yew Act being passed that was signed into law that year to prevent the intellectual properties of the tree in the year 1992 [37]. The most active component was eventually identified through the help of Monroe Wall and a second person, Mansukh Wani, who worked in collaboration and with the help of the National Cancer Institute at the Research Triangle Institute to perform this action. The active constituent was named paclitaxel in the year 1967 [30].

Paclitaxel was shown to possess a great range of antitumour action after several *in vivo* screens carried out on tumours implanted in laboratory mice [31, 38]. The modes of action of paclitaxel were further established by Dr. Susan Horwitz. This experiment was performed at the Albert Einstein College of Medicine. Moreover, another milestone on paclitaxel that was documented establishes the capability of the plant to inhibit the development of tumour development most especially in the mammary tumour xenograft [30]. Also, Hartwell discovered that only half a gram of purified paclitaxel extract from the crude extract which contains 13 kilograms of dried bark contains the active component while other components are invaluable. This constitutes a factor that affected the investigation of paclitaxel on numerous cancers which mushroomed when carried out at clinical trials.

Furthermore, a great challenge that was encountered on the discovery of paclitaxel as an effective anticancer drug was that it has a high level of insolubility in an aqueous solution, but this problem was resolved through an enhanced formulation that entails Cremophor EL and ethanol. Cremophor EL has been identified as polyethoxylated castor oil that could be applied as a carrier for solubilization of hydrophobic drugs such as paclitaxel [39]. Moreover, one of the side effects of paclitaxel was its hypersensitivity reactions which limit their application using Cremophor EL as the drug vehicle, and this consequence affected any additional clinical trials due to the high number of deaths recorded. An extended and dawdling infusion was established to require 24 hours to minimize this severe adverse influence [40]. The application of paclitaxel was documented as a treatment option agent for the management of one of the most dangerous and common diseases called ovarian cancer. The scientist also affirms the efficacy of paclitaxel for the management of advanced breast cancer.

3.1. Paclitaxel: Chemical Characteristics. Paclitaxel has been recognized as a group of taxane drugs that could be referred to as diterpenoid pseudoalkaloid possessing an empirical formula of $C_{47}H_{51}NO_{14}$, while 853.9 g/mol represents its molecular weight. There is the presence of 2 molecules having a homochiral ester side chain at C13 as well as the presence of a taxane ring having a four-membered oxetane side ring around C4 and C5 together with a homochiral ester side chain at C13. Moreover, the side chain present at C13 has been affirmed to perform an important function which represents the most effective portion that could join to the microtubules which play an important action in the stabilization of the tubulin bundles and triggers the disorganization of the microtubules available in a guanosine triphosphate- (GTP-) independent manner. This led to the prevention of cell penetration by inhibiting the cell cycle most especially at the metaphase/anaphase borderline. It has been established that an ester side chain and an intact taxane ring are very crucial for cytotoxic action [41, 42].

3.2. Paclitaxel: Mechanism of Action. It has been stated that the application of paclitaxel as an anticancer drug possesses the ability to attack microtubules. Microtubules possess a cylindrical hollow body shape in nature with a diameter that varies from 25 to 30 nm, which is made up of numerous polymers of tubulin which are in dynamic equilibrium together with tubulin heterodimers which entails beta- and alpha-constituents of the protein subunits [43, 44].

The fabrication of tubulin and the collation of microtubules happened at the prophase of mitosis and the G2 phase. It has been stated that microtubules exist in the state of dynamic steadiness most especially with the subunit tubulins such as β and α placed in a head-to-tail arrangement. The plus ends tend to be faster when compared to the minus ends which is slower at the other end. Moreover, it has been documented that the length of the microtubule remains unaltered under steady-state conditions.

Furthermore, it has been stated that the minus part that constitutes part of the microtubules are usually joined to the centrosome [45], but the plus ends use the cytoplasm and relate with the cellular structures [46, 47]. Dr. Horwitz established that comparison of paclitaxel to vinca alkaloids showed that they can hinder cell separation by enhancing the joining together of stable microtubules particularly from the ?-tubulin heterodimers as well as the prevention of the process of depolymerisation which plays a crucial function toward the inhibition of the G2/M phase of the cell cycle, death of the cell, and prevention of cell replication [48, 49].

Paclitaxel also possesses the capability to join precisely in a rescindable manner to the N-terminal 31 amino acids available at the beta-tubulin subunit present in the microtubules relatively to tubulin dimers [49, 50]. Moreover, paclitaxel can enhance microtubule formation *in vitro* when subjected to cold temperatures (4°C) without the presence of GTP [48]. Furthermore, investigation on the proper knowledge of the molecular modes of action of the microtubule development has established that paclitaxel possesses the capability that could enable the cell to avoid drug toxicity, which might lead to the development of a more resistant drug which might affect its application as a chemotherapy drug [47], and the investigation has discovered that the microtubule dynamics does not have any relationship to the cell division.

It was discovered that the application of treatment to the following mutant cell lines which entails Tax 18 and Tax 11-6 when tested at concentrations of 50 to 100 nmol/L decreases the microtubule dynamics instead of restoring behaviour to normal. Under normal conditions, there should be an enhancement of microtubule assembly to additional normal levels which enables the cell to grow into a normal cell [47]. It has also been discovered that the presence of a lower concentration of paclitaxel reduced dynamics, which did not show any effect on the degree of microtubule separation. The presence of a greater therapeutic concentration stimulates cell division which greatly prevented the disinterestedness in the mutant cell lines which enable them to be geared back to their normal level. This could be linked to the potential of paclitaxel to prevent microtubule separation instead of its capability to inhibit or prevent microtubule changing aspects, which could be established through the help of live cell imaging which shows that microtubule detachment that occurs at the centrosomes could be a crucial

factor that constitutes the development of microtubule fragments, a process that could be overturned by paclitaxel [47].

Also, it has been established that the presence of paclitaxel, whenever it is present in a lower concentration, most especially when available in nanomolar concentrations, could prevent the process involved in the depolymerisation of microtubules, but their presence at a very high concentration could enhance the mass of microtubules and their number. Therefore, improving the steadiness of microtubules could prevent their nonfunctional action through the inhibition of the process involved in the removal of the microtubule minus ends, most especially from the centrosomes, when compared to the plus end [47, 51].

Also, based on the apoptotic influence of paclitaxel performed on the modes of action carried out during a weekly route experiment, it was shown that paclitaxel possesses a capability to stimulate the apoptotic modulator genes which shows that paclitaxel is independent of microtubule maintenance. This might be linked to the regulation of the process of the transcription of numerous genes which includes inflammation, DNA-damage response proteins, apoptosis, and proteins or cytokines that play a crucial role in the regulation of cellular proliferation. The rate of cell apoptotic influence of paclitaxel has been established to depend on the dose and the time of exposure. For example, the application of a concentration containing 10 nM which has been exposed for 12 hours may trigger cell death through the induction of the S phase without mitotic arrest. It has been established that the application of a dose containing ≥9 nM paclitaxel could stimulate Raf-1 activation which regulates cell death control. Also, the dose containing ≥9 nM showed the nonappearance of Raf-1 kinase participation, but the cell death induction is regulated through the action of p21 and p53 [52, 53]. Moreover, it was affirmed that the presence of the same dose when applied at 24 hours could lead to cell death and generate an irretrievable mitotic arrest [54]. The presence of p53 as a significant tumour suppressor plays a crucial role in the prevention of cell death and cell proliferation while mutation could happen in more than 50% of human cancers; however, in a normal cell, it has been stated that the damage of DNA in p53 could stimulate the G1 cell cycle arrested by p21 to enhance the process involved in the repair mechanisms or cell death which naturally prevent the production of genetically transmuted cell clones.

It has been established that a well-designed p53 signalling route is paramount to the stimulation of cancer cells and all the chemical therapeutic techniques involved in its prevention. It has been shown that paclitaxel action does not depend on p53 status which may be mutated or silent as well as constitute a major factor that decreases its chemoresistant action [55–58].

Also, it has been demonstrated that paclitaxel possesses the capability to stimulate the action of many signal-transduction routes that are related to pre-cell death signalling. The route related to paclitaxel entails the following which was P38 mitogen-activated protein, stimulator of transcription factor route, TLR-4-dependent pathway, Janus kinase signal transducer, c-Jun N-terminal kinase, and nuclear factor kappa B [55–58].

Furthermore, the stimulation of cell death has been documented through a mitogen-activated protein kinase route leading to phosphorylation of the pre-cell death protein Bax and Bad, Bcl2 decreased cell death, phosphorylation of Bcl2, and Bad and Bax enhanced cell death, all of which constitute a regulatory protein that plays a crucial role in programmed cell death. It has been shown that the stimulation of proinflammatory proteins and cytokines could lead to the production of the immunomodulatory influence of paclitaxel when applied to a lower dose and the stimulation of apoptosis when applied to a higher concentration. The alteration that exists in these pathways is accountable for the high level of resistance to paclitaxel [59–62].

Also, it has been established that paclitaxel possesses the capability to exhibit a greater angiogenic inhibitory action when performed weekly [63, 64], while the application of paclitaxel in a murine evaluation shows that it portends the tendency to decrease the new vessel formation when applied at a lower, noncytotoxic concentration containing 0.3 and 6 $mg\,kg^{-1}\,day^{-1}$ in mice by decreasing the manifestation of vascular endothelial growth factor [65, 66]. Also, other modes of action through which paclitaxel exhibits an effect involves the postponement of the time of administration and may play a crucial role in patients suffering from cancers becoming more resistant to drugs when administered on a 3-weekly conventional schedule [67, 68].

The application of paclitaxel as an anticancer has been affirmed that this drug could stimulate the presence of hydroperoxide through the triggering of the action of the nicotinamide adenine dinucleotide phosphate oxidase [69, 70]. Also, the synergetic effect of paclitaxel together with inhibitors of glucose such as 2DG and 2-deoxy-D-glucose as well as substances that contain hydroperoxide metabolisms such as L-buthionine-S,R-sulfoximine have been established to prevent the cancer cell inhibition of the breast through the action of the H_2O_2-stimulated metabolic-induced oxidant in the management of breast cancers [70].

4. Paclitaxel: Nanomedicine-Based Cancer Therapy—Recent Advances and Challenges

Nanomedicine could be stated as the utilization of nanotechnology most especially in the medical field for the management and diagnosis of life-threatening diseases such as cancer [71]. It has been observed that the application of nanomedicine gives a more precise and accurate detection and encourages the effectiveness of the drugs applied by minimizing the adverse influence related to standard therapeutics [72]. The application of nanomedicine has been observed in the treatment of cancer such as trajectory delivery of nanodrugs, nanopharmaceuticals, and nanoanalytical disparity chemicals in a research laboratory and animal prototypical research [73].

Also, the discovery of numerous new nanomedical products during the clinical trials and their eventual distribution into the commercial market has grown tremendously over the years, most especially their application for the treatment of cancer and their application in the diagnosis of cancer diseases [74].

This enhances the therapeutic effects of some active compounds through several novel techniques that have been evaluated that could lead to enhanced retention of drugs most especially when carried out *in vitro* studies. Typical examples of such involve the incorporation of high molecular substance agents such as hydrogels and nanoparticles [75–80].

The utilization of nanotechnology has been recognized as an effective technique that could deliver nanodrugs [81, 82]. It has been established that nanodrugs possess the capability to buildup in the tissue which might be linked to enhanced permeability and retention as well as enhanced antitumour influence possessing minimal toxicity in normal tissues [83]. The enhanced permeability and retention influence could be linked to certain features of solid tumours such as immature lymphatic drainage, hyperpermeability of tumour vessel walls, and partial vascular architecture [84]. Leveraging on all these concepts, numerous types of nanodrugs have been fabricated for the management of several types of cancer diseases [85].

It has been observed that repetitive intraperitoneal introduction of paclitaxel together with simultaneous systemic chemotherapy has been established to show a high level of effectiveness for the treatment of peritoneal metastases obtained from gastric cancer. However, the process involved in the biochemical alterations has been established to influence the bioavailability of intraperitoneally administered paclitaxel.

In 2017, Kitayama et al. [86] utilized a xenograft repetitive intraperitoneal model utilizing human gastric cancer cells. Fluorescein-conjugated paclitaxel was repetitively administered intraperitoneally, and the intratumour circulation of paclitaxel was assessed utilizing fluorescein microscopy. The result obtained indicates that paclitaxel was observed to unswervingly penetrate up to numerous hundreds of micrometers most especially superficially through the repetitive intraperitoneal administration immediately after intraperitoneal inoculation. The coadministration of 5% nonanimal stabilized hyaluronic acid enhances paclitaxel infiltration and inhibited the growth of peritoneal metastases when compared to paclitaxel alone. Moreover, it was discovered that the solubilization of paclitaxel along with an amphiphilic polymer possessing 2-methacryloyloxyethyl phosphorylcholine and n-butyl methacrylate led to the development of a micellar formation which has a diameter that ranges from 50 to 100 nm in diameter. Additionally, it was discovered that the administration of nanomicellar paclitaxel (paclitaxel-30W) improved tumour penetration and prevented the development of peritoneal metastases when compared with paclitaxel solubilized with cremophor-ethanol (paclitaxel-Cre). Furthermore, repetitive intraperitoneal injection of NK105, which represent another nanomicellar paclitaxel, prevents the development of subcutaneous tumours, such as together with peritoneal metastases, when compared to the unadventurous paclitaxel-Cre in the same murine model. Their study showed that the repetitive intraperitoneal paclitaxel injection could enhance the penetrability potential of peritoneal metastases which could serve as techniques for the management of peritoneal metastases. The alteration of drug-using nanotechnology could improve the level of infiltration of peritoneal metastases leading to enhanced clinical effectiveness.

It has been shown recently that the prevention of liver metastases derived mainly from lung and breast cancers have exhibited little effectiveness. This might be linked to the fact that liver metastases are not properly vascularized, which prevented the capability of transport therapeutics mainly through the systemic circulation to lesions.

In 2016, Tanei et al. [87] utilized a system that could introduce nanoparticle albumin-bound paclitaxel (nAb-paclitaxel) into a nanoporous solid multistage nanovector material that improves the movement of the therapeutic agent into the tumour microenvironment. This might also trigger their relationship with the liver macrophages. Also, it was shown that nanoporous solid multistage nanovector enablement could enhance nAb-paclitaxel effectiveness and persistence in mouse models of lung liver and breast metastasis. Nanoporous solid multistage nanovector-nAb-PTX also amplified the buildup of paclitaxel and the level of nanoporous solid multistage nanovector available in the liver, most especially the macrophages, but the level of the paclitaxel available in the blood remained unaltered after injecting nAb-paclitaxel and nanovector-nAb-paclitaxel.

The *in vitro* study carried out showed that the macrophages administered with nanoporous solid multistage nanovector-nAb-paclitaxel maintained their viability and internalized, maintained, and discharged meaningfully advanced amounts of paclitaxel when compared to the treatment with nAb-paclitaxel. The cytotoxic effects of the liberated paclitaxel were also affirmed in the tumour cells cultured together with the supernatants of macrophages applied with the nanoporous solid multistage nanovector-nAB-paclitaxel. Their study established that conveying nAb-paclitaxel to liver macrophages most especially when it occurs within the tumour microenvironment could produce an enhanced therapeutic reaction most especially in patients having metastatic liver cancer without any sign of enhancing systemic side influence.

The effectiveness of chemotherapy which is derived from radiation methods has been discovered to often undergo swift clearance and off-target toxicity. The application of a drug delivery system and the control of therapeutic drugs in a controlled release manner have been observed to enhance the effectiveness of the therapeutic efficiency of drugs most especially those that contain small molecules.

In 2018, Yang et al. [88] synthesized 2 new nanodrugs using oxidative and reductive techniques for the fabrication of paclitaxel prodrugs which can quickly conjugate with albumin in an *in vivo* study. It was discovered that albumin could serve as a natural means of transporting the nanodrugs to the tumours which might be linked to the increase in the retention and permeation influence. It was observed that paclitaxel could induce the liberation of reactive oxygen species/glutathione cancer cell microenvironment. Their experiment indicated that the bioresponsive prodrug approach shows the effectiveness of chemotherapeutic effectiveness when tested in an *in vivo* assay which may affect the management of cancer diseases.

It has been stated that multifunctional nanoparticles could be applied to enhance the management of index and minimize the side influence of antitumour drugs.

In 2019, Lei et al. [89] synthesized a highly biocompatible drug which is a multifunctional nanoparticle incorporated with gemcitabine, paclitaxel, and folic acid using self-assembly toward targeted and affected cancer cells. The result obtained from the transmission electron microscopy established that the fabricated nanoparticles which are multifunctional FA targeting nanoparticles (MF-FA NPs) are spherical with encouraging enhanced structural stability most especially when present in an aqueous solution. Furthermore, it was observed that the MF NPs and the MF-FA NPs showed proliferation inhibition when applied to the breast cancer cell 4T1 when compared to pure drugs. The result of the *in vivo* antitumour experiment carried out established that the nanoparticles could enhance the anticancer effects in comparison with the synthetic drug. Moreover, MF-FA NPs showed an enhanced greater tumour growth inhibition when compared to the MF NPs which might be linked to the presence of some specific influence directing FA to cancer cells. Therefore, the innovative MF-FA NPs might be utilized as an important nanodrug for the management of breast cancer.

Paclitaxel has been recognized as one of the major active and potent chemotherapeutic drugs that have been discovered to show higher inhibitory activities against different types of cancer such as ovarian, breast, and lung cancers. It has been discovered that paclitaxel has a lower solubility in water; therefore, paclitaxel is normally formulated in a mixture containing a combination of dehydrated ethanol and cremophor EL (50 : 50, *v*/*v*) which constitutes a combination referred to as taxol. Moreover, several side effects as a result of utilizing ethanol and cremophor EL have been reported. Therefore, there is a need to develop an alternative taxol formulation [90, 91].

Therefore, the application of encapsulation techniques that utilize paclitaxel which is a biodegradable and nontoxic nanodelivery system will go a long way toward the prevention of the drugs from dilapidation most especially during the process of circulation and thereby prevent the body from any available toxic or adverse effect. Moreover, it will also show a more enhanced patient compliance, minimize its toxicity, enhance its circulation half-life, and enhance pharmacokinetic profiles. Moreover, it has been affirmed that the nanoparticle-based delivery systems have several merits such as improved permeability and retention influence most especially for the targeting of passive tumours which shows that they are promising carriers that could increase the therapeutic index and reduce of the total side effect of paclitaxel [12]. In 2017, Wang et al. [92] wrote a comprehensive review on the expansion of paclitaxel drug delivery systems and evaluate the design values supporting each delivery stratagem.

The application of paclitaxel albumin-bound nanoparticles (Abraxane®) has been approved by the Food and Drug Administration for the management of breast and lung cancer. Moreover, numerous new paclitaxel nanoparticle formulations have been tested in clinical trials. Therefore, in 2013, Ma and Mumper [12] wrote a comprehensive review on the application of paclitaxel nanodelivery systems such as nanoparticles, polymeric nanoparticles, cyclodextrin, lipid-based formulations, nanocrystals, polymer conjugates, carbon nanotubes, and inorganic nanoparticles.

In 2016, Paciotti et al. [93] carried out the fabrication of different types of thiolated paclitaxel analogues which has been identified as a new nanomedicine program that was formulated toward effective development of nanomedicine involving paclitaxel and gold nanoparticles, most especially for tumour targeted drug delivery. The initial assessment of the novel nanomedicine entails 27 nm gold nanoparticles, thiolated polyethene glycol (PEG-thiol), numerous thiolated paclitaxel analogues, and tumour necrosis factor-alpha (TNF-α). There is a higher demand for an effective and more effective drug without any side effects. This has led to the discovery of innovative paclitaxel formulations, which have been subjected to clinical trials.

In Table 1, the type of nanoformulation, anticancer activity, and mode of delivery of paclitaxel are shown.

5. Cancer and Targeted Therapy

5.1. Cancer and Its Triggering Factors. Cancer is a specific disease where cells are formed that cannot divide without control, are immune to death, and can attack other tissues [113]. It is considered one of the leading causes of death in the world [114]. According to previous research, in 15-20% of cancer cases, autoimmunity in the same tissue or organ site, chronic inflammation, or infection was also diagnosed [115, 116]. In such cases, inflammation may appear earlier than tumour formation. The most prominent examples include inflammatory bowel disease (IBD), chronic hepatitis, helicobacter-induced gastritis, or schistosoma-induced bladder inflammation that increases the risk of colorectal cancer (CRC), liver cancer, stomach cancer, or bladder cancer, respectively [117].

Factors that can predispose to cancer include environmental factors that can cause some or all of the chronic inflammation, sometimes of low intensity [118]. In these cases, the inflammation that is present may develop with or before the tumour. Concerning the host, these factors might be systemic or site and organ specific. The development of lung cancer and mesothelioma may be caused by the formation of pneumonia and the respiratory tract by inhaling tobacco smoke and asbestos [119, 120]. Inflammation caused by obesity, hyperglycemia, and excessive lipid accumulation is usually systemic and may increase the risk of various types of cancer such as liver, pancreatic, colon, breast, and other malignancies [121, 122].

Type II diabetes is classified as one of the independent cancer risk factors. It is believed to lead to cancer formation through obesity-induced inflammation and obesity-related tissue injury. Obesity is an increasingly common problem in westernized countries, so it is important to determine the mechanism by which obesity and inflammation associated with it affect the growth of cancer. This is to alleviate the consequences of metabolic diseases common in society. In the late stages of the tumour, systemic inflammation can also take place, for instance, due to tobacco smoke, obesity, and bacterial-product-induced inflammation. Neutrophils are activated, and their extracellular trap formation

Table 1: The effect of paclitaxel nanoformulation against different types of cancer.

Paclitaxel nanoformulation	Target tissue/ type of cancer	Mode of delivery	Reference
Nanoformulated paclitaxel and AZD9291	Lung cancer	AZD9291-loaded disulfide cross-linking micelles (DCMs)	[94]
Low-dose nanoparticle albumin-bound paclitaxel (nab-PTX)	HER2-negative metastatic breast cancer	Albumin nanoparticles	[95, 96]
Nab-paclitaxel–gemcitabine combination	Pancreatic cancer	Albumin nanoparticles	[97]
Milk-derived exosomal formulation of PAC (ExoPAC)	Lung cancer	Exosomes	[98]
PTX-GemC12-LNC formulation	Brain tumour (glioblastoma)	Liquid nanoparticle	[99]
Paclitaxel-loaded PCL-TPGS nanoparticles: PTX-loaded poly(caprolactone)–alpha-tocopheryl polyethylene glycol 1000 succinate (PCL-TPGS) NPs	Breast cancer	PCL-TPGS copolymer	[100]
Paclitaxel-loaded poly(glycolide-*co*-ε-caprolactone)-*b*-D-α-tocopheryl polyethylene glycol 2000 succinate nanoparticles (PTX-loaded (PGA-*co*-PCL)-*b*-TPGS2k	Lung cancer	Amphiphilic copolymer (PGA-*co*-PCL)-*b*-TPGS2k	[101]
Paclitaxel and superparamagnetic iron oxide-loaded PEGylated poly(lactic-*co*-glycolic acid) (PLGA)-based nanoparticles (PTX/SPIO-NPs)	Glioblastoma	Superparamagnetic iron oxide-PEGylated poly(lactic-*co*-glycolic acid)-based nanoparticles	[102]
PTX micelles	Pulmonary carcinoma	Poly(ethylene glycol)-poly(3-caprolactone) copolymers (MPEG-PCL micelles)	[103]
Paclitaxel-loaded vitamin E-TPGS nanoparticles	Lung cancer Breast cancer Colorectal cancer Brain cancer Prostate cancer	TPGS nanoparticles	[75, 100, 104–107]
PTX nanomicelles using poly(styrene-*co*-maleic acid) (SMA); SMA-PTX micelles	Murine orthotopic colon cancer model	Poly(styrene-*co*-maleic acid)	[108]
Lumbrokinase/paclitaxel/poly(ethylene glycol)-*b*-(poly(ethylenediamine l-glutamate)-*g*-poly(ε-benzyoxycarbonyl-l-lysine)-*r*-poly(l-lysine)) LK/PTX/PEG-*b*-(PELG-*g*-(PZLL-*r*-PLL))	Bladder cancer	Poly(ethylene glycol)-*b*-(poly(ethylenediamine l-glutamate)-*g*-poly(ε-benzyoxycarbonyl-l-lysine)-*r*-poly(l-lysine))	[109]
Paclitaxel dimer-methoxypoly(ethylene glycol)*2K*-block-poly(D,L-lactide)*2K* (PTX dimer)/PEG-PDLLA formulations	Cervical cancer	Methoxypoly(ethylene glycol)*2K*-block-poly(D,L-lactide)*2K* (PEG-PDLLA) micelles	[110]
PTX-loaded redox-sensitive HSV nanoparticles	Lung cancer	Hyaluronic acid-disulfide-vitamin E succinate (HA-SS-VES, HSV) conjugate	[111]
Thermostable RNA-PTX nanoparticles (4WJ-X-24 PTX nanoparticles)	Breast cancer	RNA nanoparticles	[112]

functions to promote breast cancer metastasis into the lungs [123].

5.1.1. Inflammation and Oncogenesis. Tumour initiation occurs as a result of two independent events:

(1) One leads to the accumulation of mutations and/or epigenetic alterations of genes and signalling pathways involved in tumour suppression (inactivation) and oncogenic pathways (activation). They were usually associated mainly with environmental factors (e.g., UV, carcinogens, and/or variable radiation), as well as with errors in DNA repair and replication (Figure 1). It is worth noting that inflammatory reactions also involve mechanisms contributing to the accumulation of mutation and various epigenetic changes in adjacent epithelial cells.

Chronic intestinal inflammation leads to the accumulation of mutations in Tp53 and other cancer-related genes in intestinal epithelial cells [124–127] and can trigger tumour formation even without additional extrinsic mutagens being present [128].

Interestingly, evolutionary inflammation has the potential to induce mutation and DNA damage, an example being IL-22, which induces DNA damage response (DDR) gene

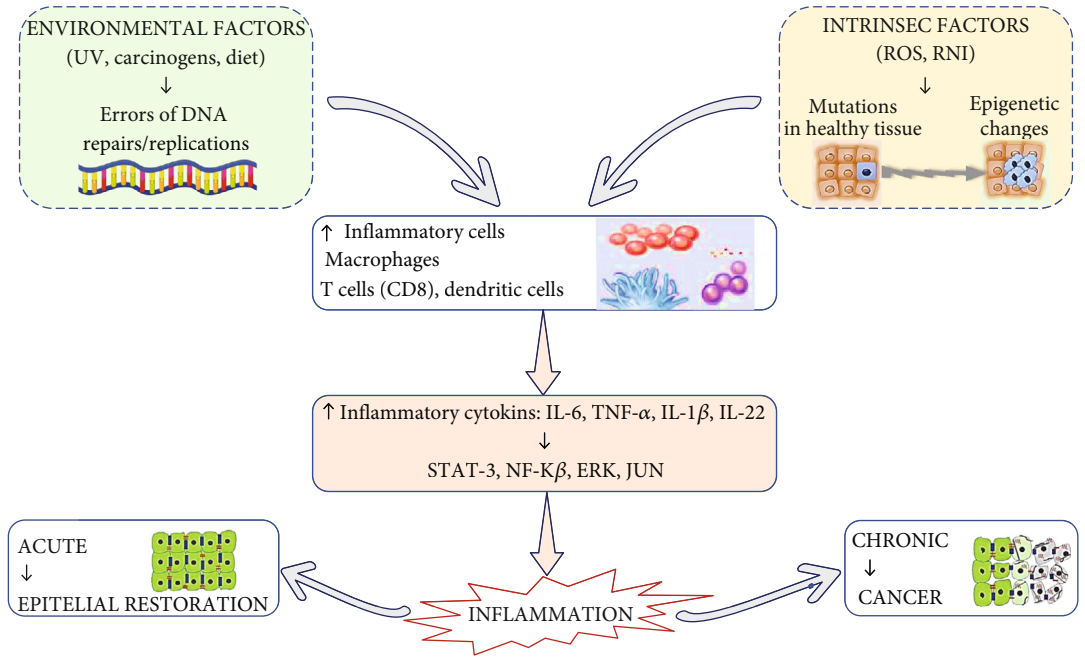

Figure 1: A diagram regarding the correlation between different factors (environmental, intrinsic), inflammation processes, and the effects on tissues. *Abbreviations*: ROS—reactive oxygen species; RNI—reactive nitrogen species; IL-6—interleukin 6; TNF-α—tumour necrosis alfa; IL-1β—interleukin 1β; IL-22—interleukin-22; STAT3—signal transducer and activator of transcription 3; NF-κB—nuclear factor kappa-light-chain-enhancer of activated B cells; ERK—extracellular signal-related kinase.

expression to counter possible inflammation-induced genotoxic injury [129]. Besides, cytokine signalling (i.e., IL-6, TNF-α, and IL-1b) produced by inflammatory cells activates epigenetic machinery in epithelial cells, including DNA and histone modification components (Dnmt1, Dnmt3, and telomeric silencing disruptor 1 (DOTL1)), microRNA- (miRNA-) and long noncoding RNA- (lncRNA-) modulating oncogene expression levels, and tumour suppressors [115].

The net outcome of such epigenetic changes is proposed to be the same as inactivating mutations in tumour suppressors and activating mutations in oncogenes and can potentially be achieved in a "bi-allelic" manner at the same time. There are many cases where stem cells are considered to be "cells of origin" for cancer, and therefore, inflammatory processes can cause dedifferentiation of postmitotic epithelia into tumour-initiating stem-like cells [130].

Tissue damage that may result from chronic inflammation leads to the weakening of the barrier, and thus cells may be exposed to carcinogens, e.g., environmental carcinogens, or leads to the presence of stem cells close to active inflammatory cells producing genotoxic compounds. In another case, cancer occurring in, e.g., the colon, is rich in microbes and inflammation may be amplified thereby causing changes in qualitative characteristics of epithelial-adhesive microbiota, enriching the content of species harbouring genotoxic gene products, such as colibactin in some strains of *E.coli*, which may be capable of inflicting mutations in host cells [8, 131] which may be capable of inflicting mutations in host cells.

(2) The development of transformed and/or malignant clones, however, should be accompanied by their growth into a frank tumour, a cycle to which inflammatory pathways can make a major contribution. Inflammation is induced by reactive oxygen (ROS) and nitrogen (RNI) species which are produced by macrophages and neutrophils. This contributes to the development of mutagenesis and the accumulation of mutations in healthy tissue (Figure 1).

For example, cytokine receptor signalling in mutated cells can converge in the activation of prosurvival pathways, especially mediated by NF-κB, STAT3, and other types of signalling [132, 133] thereby increasing the probability of survival of transformed clones or allowing proliferation. Specifically, early inflammatory signalling may be needed in tumour initiation. Some of the cancer cells may have not yet evolved a full-scale, auxiliary TME that needed growth factors to support tumours. There is also an inflammation-driven cell survival problem regarding cancer immune surveillance and tumour removal process of mutated and stressed cells [134, 135].

Signals activating STAT3 shield epithelial cells from CD8 T cytotoxic cell attack [136, 137] and IFN signalling, while normally anticytokine, enhances the expression of T cell fatigue, causing molecular programmed death-ligand 1 (PD-L1) in the transformed epithelium, which is recognized by T cells. Inflammatory signs may be used to the same goal, to improve fitness and decrease the expression of "stress ligands" on cancer cells needed for appropriate identification [138–140], and inflammation and injury cause cell turnover in tissues, providing space for the development of malignant clones [141].

As per the study, the tendency of inflammation to induce tumourigenicity leads to tumour progression. As per the translational standpoint, tumour growth can be driven by

inflammation, and inhibition of inflammation can inhibit tumour growth and result in further progression [142]. The insight information widens the opportunity for early detection of cancers and shedding light on metastatic seeds spreading out even if distantly established. Multiple preclinical animal models have been instrumental to reveal the significant mechanisms associated with inflammation and cancer. Conceptually, there are many interdependent molecular and cellular mechanisms at play. First, inflammatory agents similar to tumour initiation may function as direct growth factors for rising tumours. Moreover, inflammatory factors in the tumour microenvironment (TME) is important in the formation of cell plasticity, as a result of which it influences tumour growth in three separate mechanisms [143].

Studies of NF-κB inactivation in myeloid cells have demonstrated for the first time that inflammation may play a role in tumour promotion. Ablation of the nuclear factor-κB (IKKb) kinase inhibitor resulted in a reduction in tumour enlargement in a colitis-associated cancer model [144]. In reality, NF-κB regulates cytokine expressed particularly in immune cells which promotes the survival and expression of chemokines by acting on epithelial and tumour cells which is necessary for the recruitment of the tumour microenvironment (TME).

Genetic and pharmacological inactivation of NF-κB-dependent cytokines reduces tumour growth due to activating other monogenic pathways in epithelial and cancer cells, including STAT3, extracellular signal-regulated kinase (ERK), JUN, and Tyr kinase receptor (TKR) [133, 145–148].

5.2. Cancer and the Need for Anticancer Drug Use. Cancer cells may be targeted at DNA, RNA, or protein at the molecular level; at the organelle or nucleus at the cell level; and at the endothelium and extracellular matrix at the tissue level according to the action mechanism of anticancer drugs [149]. Most traditional chemotherapeutic agents interact with cancer cell DNA, while monoclonal antibodies are directed against proteins or the endothelium and extracellular matrix [150]. Chemotherapy by traditional drug administration is the most common cancer treatment, but it involves several issues, including low drug solubility, low precision, high toxicity, and low therapy index [113, 151].

The platinum-based family of anticancer medication is composed of cisplatin (CDDP), carboplatin, oxaliplatin, and nedaplatin. These are antineoplastic drugs by blocking DNA replication and are used to treat a range of cancers including testicular cancer, cancer of the ovaries, cancer of the breast, and cancer of the bladder and lung [152]. Trabectedin (Yondelis®, ET-743), a semisynthetic tetrahydroisoquinoline alkaloid originally extracted from the marine tunicate *Ecteinascidia turbinata*, was the first marine anticancer agent to be approved for soft tissue sarcoma patients in the European Union [153, 154].

Among the clinically approved polyketides, ribulin mesylate (E7389) can be distinguished, its primary source of origin being the marine sponge *Halichondria okadai*. This compound is an analogue of halichondrin B that acts as a non-taxane microtubule dynamics inhibitor. Since 2010, compound E7389 has been approved by the FDA for the treatment of liposarcoma and breast cancer [155]. Bryostatin 1 is sourced from sponges and tunicates; it is a polyketide isolated from *Bryozoan bugula neritin*. This compound is active against many carcinomas [156]. Cytarabine is another compound (Ara-C) which is synthetically derived from spongothymidine and isolated from the marine sponge *Cryptotheca crypt*. This compound is a nucleoside that has already been licensed and marketed and has been used primarily in leukaemia [157]. Gemcitabine is a nucleoside analogue (specifically, a fluorinated cytarabine derivative) that could be used in cancer drug development [158, 159] and is used in numerous carcinomas, such as non-small-cell lung cancer, pancreatic cancer, prostate cancer, and breast cancer [157]. Doxorubicin is a metabolite of *Streptomyces petitcetius*. DOX is used as an antineoplastic agent in the treatment of fluid and solid cancers including breast cancer leukaemia. Unfortunately, DOX usage is correlated with toxicity that may result in extravasation, diarrhoea, vomiting, haematopoietic repression, alopecia, and cardiotoxicity [160].

Paclitaxel (PTX) in the taxane family of chemotherapeutics and herbal diterpenoid pseudoalkaloid is a white crystalline powder with a lipophilic nature. It has an empiric form $C_{47}H_{51}NO_{14}$ and a molecular weight of 853.9 g/mol [161]. It demonstrates low solubility in water and melts at a temperature of 217 to 2°C [162].

PTX consists of two molecules, that is, a taxane ring with a four-piece oxetane side ring at positions C-4 and C-5, and a homochiral ester aspect chain at position C-13. The aspect chain at C-13 plays a crucial role as an active element that attaches to the microtubules, which stabilizes the tubulin bundles and activates the disassembly of microtubules in the guanosine triphosphate residue in an independent environment. As a result, cellular proliferation is disrupted by halting the phone loop at the periphery of metaphase/anaphase and by developing nonaccomplished chromosomes, which eventually contributes to the stability of the microtubule dynamics. A comprehensive lookup has shown that a broken taxane ring and an ester side chain are necessary for the development of cytotoxic action [41, 42].

PTX is an antitumour drug that activates microtubules. Microtubules contain cylindrical hole bodies that are 25–30 nm in diameter and include dynamic structured tubulin polymers along with heterodimers of tubulin molecules (comprising 5-007 as well as β protein subunits) [163]. The cytotoxic activity of PTX has been correlated with the stabilization of microtubules [164]. Microtubules play an important role in the formation of mitotic spindles at some stage in the process of mobile division. It is important to preserve the cell structure, motility, and cytoplasmic motion within the cells. The development of tubulin as well as the assembly of microtubules takes place at some point in the G2 segment and the mitosis prophase. Microtubules are in complex equilibrium with their tubulin subunits 5-007 and β, arranged in a head-to-tail manner with preferential faster growth (plus ends) at one end and slower boom (minus ends) at the other hand. Tubulin is used as a target factor for the cytotoxic activity of PTX, of which tumour cells in the mitosis segment have been arrested to cause tubulin affiliation to microtubules [48, 165].

5.3. Modifiable and Nonmodifiable Factors in Cancer Therapy. Cancer risk factors are those factors that increase a person's risk of developing cancer due to certain reasons. In most cases, the root cause of cancer development cannot be given, which causes one person to get sick and the other not. However, certain risk factors may increase a person's chances of developing cancer. The risk factor may be categorised as follows: modifiable cancer risk factors including the lifestyle and health behaviour of a person (e.g., consumption of tobacco products, alcohol, and food items that increases obesity); nonmodifiable cancer risk factors including the genetics of the family, their age, gender, race, and ethnicity; and environmental risk factors including exposure to chemical substances, radiation, infectious diseases, or any other agents which may cause cancer (Figure 1) [118, 166, 167].

It is also understood that for a particular type of cancer, different risk factors may apply. That means a complex interaction of multiple factors is playing together for a person's risk of developing cancer.

5.3.1. Nonmodifiable Factors of Host

(1) Age. Age is a great risk factor for the development of cancer. It is being said that cancer cells are present in the human system. When and how it will express is a matter of concern and seems to be related to age. Cancer risk factors are often in age-old people as compared to the younger generation. For example, in the US, pancreatic cancer usually occurs between the ages of 40 and 80 years [168]. In India, the condition is slightly different. This incident of pancreatic cancer starts rising in the 5th decade and is prominent in the 6th decade [169].

(2) Gender. The incidence and mortality rate of various cancers are often related to sex disparities. Sex differences are one of the most significant findings in cancer epidemiology [170]. In most cases of cancer development, men are more susceptible than women, e.g., especially in haematological malignancies. Similar gender differences can be observed in nonmalignant diseases including autoimmunity, attributed due to hormonal (like estrogen, progesterone) or behavioural differences. The mortality of cancer is reported more in men than in women, especially for lung, colorectal, and stomach cancers [171, 172]. Female-specific cancers such as breast, ovarian, and uterine corpus and male-specific cancers such as prostate cancer are showing a high mortality rate. Men have a 34% higher risk of death than women in the case of melanoma cancer [173]. Antitumour effects of paclitaxel [174] that can interfere with cell division by depolymerizing cytoskeletal microtubules was reported in a female patient showing 20% lower elimination of paclitaxel than in a male patient [175].

(3) Ethnicity. Several risk factors can increase the chance of developing cancer in human beings. One of them is race as a risk factor for developing cancer. Race is a recognized risk factor for most cancer progression. Statistics analysis reveals that certain racial groups are more prone to some types of cancer than other racial groups around the globe. For instance, in the US, African-Americans are more susceptible to pancreatic cancer than Caucasians, and the lowest incidence was visible in Asian-American and Pacific Islanders [176]. The higher incidence in African-Americans is attributed and related to modifiable risk factors such as diet, vitamin D insufficiency, and alcohol consumption [177]. Asian patients appear to have less vulnerability and a better survival rate to cancer risk than Americans [178].

(4) Blood Group. The blood group of human beings can be discussed for cancer risk. ABO blood type is associated with the risk of all cancer types. However, the incidence of cancer risk varies according to the type of cancer. As compared with blood type A, blood type B is less susceptible to cancer risk factors [179]. Blood type B and AB have a lower risk of gastrointestinal cancer and colorectal cancer. Blood type B is likely to have a lower risk of stomach cancer and bladder cancer while blood type AB is more susceptible to liver cancer [179].

5.3.2. Modifiable Risk Factors

(1) Dietary Factors. Lifestyle and dietary consumption play a significant role in risking cancer development. Nearly 30-40% of all cancers can be prevented by changing the diet and lifestyle [180]. The consumption of low-fibre food, concentrated sugars, refined flour products, and red meat and an imbalanced intake of omega 3 and omega 6 fatty acids puts one at more risk to develop cancer [181]. Intake of vegetables and fruits and other antitumour seeds will lower the risk of cancer development. Vegetables like cruciferous, allium, and broccoli contain sulforaphane, and these are beneficial in protecting and preventing against cancer. Some other diets which are useful to protect and prevent against cancer development may include selenium, folic acid, vitamin B-12, vitamin D, chlorophyll, and antioxidants. When a good guideline diet is properly taken, it is likely to reduce 60-70% of breast cancer, colorectal cancer, and prostate cancer, and even 40-50% in the case of lung cancer, and other similar reductions in cancer development [180].

(2) Alcohol. Alcohol is the common term for ethanol or ethyl alcohol. It is usually derived from rice, wheat, maize, grapes, etc., through the process of fermentation by yeast. It is being utilized as an important component in some medicines, mouthwashes, and other household products. There is a strong consensus from scientific reports that the consumption of alcohol causes different types of cancers, and it is one of the high risks for cancer development [182]. Consumption of alcohol is a known human carcinogen. From much evidence, it is indicated that consumption of alcohol regularly causes a higher risk of alcohol-associated cancer in a later phase of one's life [183]. A total of 3.5% of cancer deaths in the US are due to alcohol consumption [184]. Numerous studies have examined whether consumption of alcohol is a risk factor prominently causing different cancers which include head and neck cancer [185], esophageal cancer [186], liver cancer [187], and colorectal cancer [188].

(3) Smoking. Smoking is dangerous to health. Smoking will lead to tobacco-related cancer (TRC). According to WHO, it is estimated that one out of two young people who becomes a chain smoker will lead to TRC [189]. In India, the risk of developing cancer in between the age groups of 35 and 70 years due to the consumption of tobacco-related products was high in males (4.75%) as compared to females (2.16%). The total cancer risks due to tobacco consumption are around 45% in males and 20% in females [190]. The relation between smoking and lung cancer was first identified by a US surgeon in 1964. Subsequently, different other cancers like acute myeloid leukaemia (AML), cervix cancer, oesophagus cancer, kidney cancer, larynx cancer, trachea cancer, oral cancer, and pancreas cancer were also developed from smoking.

(4) Infections. After dietary factors and tobacco-related factors, infectious disease factors represent the third cause for cancer development worldwide. Several chronic viral papillomaviruses (several HPV types), herpesviruses (EBV and KSHV), polyomaviruses (SV40, MCV, BK, and JCV), and hepadnaviruses (HBV); RNA viruses, such as flaviviruses (HCV), defective viruses (HDV), and retroviruses (HTLV-I, HTLV-II, HIV-1, HIV-2, HERV-K, and XMRV); bacterial (*H. pylori*, *S. typhi*, *S. bovis*, *Bartonella*, and *C. pneumoniae*) and protozoan infections (*P. falciparum*); nematodes (*S. haematobium*, *S. japonicum*, *S. mansoni*, *O. viverrini*, *O. felineus*, and *C. sinensis*); and other microorganisms have been reportedly linked with human cancer development affecting different anatomical sites of the body. Infectious disease and parasitic disease claimed 10% of the population of cancer patients in the US [191], 10-20% in the UK, 3.6% in France, and 29.4% in China [192]. In the world, cancer that developed due to infectious diseases is estimated to be 15.6% in 1990 [193], 17.8% in 2002 [194], and 16.1% in 2008 [195]. Most of the viruses linked with the risk of cancer infestation can be passed from one person to another through the blood or other body fluids. One can lower the risk of cancer development by getting vaccinated.

5.4. Cancer-Targeted Therapy and Personalized Medicine. Traditional chemotherapy is based on the fact that the drug kills normal and cancerous cells. It is not only affecting the cancerous cells but also affects other surrounding cells. The targeted therapies are also termed precision medicine or personalized medicine which works differently from traditional therapies [196].

The idea of precision medicine is not new, but the recent advancement in technologies, materials, drugs, and medicine have helped to boost this area of research. Targeted therapies are applied only to that target cancer cells without hampering other normal cells in the body. The targeted therapies are emerging and most wanted technologies acquired by an oncologist for better diagnosis and treatment of cancer patients by looking to the current trends of increasing cases [197]. They are designed to interfere with the cellular activities of targeted cells by stopping the function of the gene. In some cases, the drug will attach to the molecules directly and stopping their metabolic activities. By doing so, the cancer cell will stop performing its normal metabolic activities thereby decreasing the growth and spread of the cancer cells. It is being called personalized medicine because of the patient' tumour cells. Every cancer patient has a unique molecular profile and different mutational mechanisms. The mutation of the cancer cell may vary according to the patient and the type of mutation. This means that certain types of drugs that works for one patient with lung cancer may not work for another. For instance, immune-oncology is a kind of immunotherapy that uses the patient's immune system to fight against cancer cells. Sometimes, this kind of therapy allows the doctor to selectively treat the patients based on their genetic history. This system of therapy is affected by three components which include biological, psychological, and social factors. Targeted therapies can be given in the form of pills or infusions in the bloodstream as per the requirement. The recent advancement in nanomedicine accumulation with curcumin is an effective therapy for targeted delivery in cancer cells [198].

5.4.1. Antibodies as Targeted Therapies. Antibody exploitation in cancer therapy has become a major strategy for most of the oncologists to treat the patient due to the ability of antibodies binding to the specific primary and metastatic cancer cells. They have a high affinity toward targeted cells and produced antitumour effects through the following:

(a) Complement-mediated cytolysis and antibody dependent

(b) Cell-mediated cytotoxicity (naked antibodies)

(c) Focused delivery of radiation

(d) Cellular toxins (conjugated antibodies) [199–201]

In the US, the FDA has approved the use of 8 different anticancer therapeutic antibodies to increase the availability of drugs to the general public, and the details were provided by Ross et al. in 2004 [202].

Studies have shown that the use of the mouse monoclonal antibodies does not bring the expected results due to low overall potency of naked mouse antibodies as anticancer drugs, poor tumour cell penetration of antibodies, small radioisotope and toxin conjugate production, and the development of human antimouse antibodies (HAMAs) [203]. There are also partially humanized antibodies known to reduce HAMA responses and other related problems. Later on, human antibodies were developed based on murine sources and recombinant DNA technologies [204]. Modern antibody therapy is capable of reducing cytotoxicity of the drugs and other protein toxins that may create the problem.

6. Paclitaxel and Other Drugs: Emphasis on Synergistic and Potentiation Reactions

Paclitaxel is a representative of a class of diterpenes taxanes, which nowadays are widely used as a chemotherapeutic agent against various types of cancer. Another name of paclitaxel in use is taxol [205]. The source of paclitaxel is the

Pacific yew tree (*Taxus brevifolia*). Initially, the class of taxanes was isolated from natural sources and later obtained by artificial synthesis [206]. The common hazel plant *Corylus avellana* shells and leaves have been reported to be a source of paclitaxel and other taxanes [207].

Taxanes are difficult to synthesize because of their numerous chiral centres—paclitaxel (or taxol) has 11 of these. Paclitaxel (Taxol) and docetaxel (Taxotere) are known to be used as chemotherapy agents [208]. Docetaxel is an efficient alternative in the treatment of patients with metastatic breast cancer for whom previous treatment has not worked [209]. The effect of docetaxel monotherapy, which consists of a 1-hour infusion every 3 weeks is comparable or more effective than that of doxorubicin, paclitaxel, and fluorouracil plus vinorelbine [210]. Cabazitaxel is a compound that has been classified by the FDA to treat hormone-refractory prostate cancer [211].

Due to the limited solubility in the water of taxanes, drug formulation is the difficult therapeutic potential of the taxanes paclitaxel (Taxol) and docetaxel (Taxotere), which can be limited by encumbrances faced by anticancer drugs such as toxicities, acquired multidrug resistance, and *de novo* refraction [205]. Due to their toxicity, both paclitaxel and docetaxel can predispose to and/or cause drug-induced lupus erythematosus, which most commonly manifests as subacute cutaneous lupus erythematosus [212–214]. To decrease the toxicity, some chemotherapeutic agents used the potential of synergistic reactions. Synergy is a mechanism by which the actions of certain substances together bring greater benefits and effects than the sum of their monoeffects.

The synergistic effects can be obtained between herbal products and conventional drugs or biochemical compounds [215]. In the in vitro model of non-small-cell lung cancer, it was claimed that there was synergism between paclitaxel and naturally occurring diet-based flavonoid fisetin. Studies have shown the ability to stimulate mitotic catastrophe and autophagic cell death [216]. The experimental studies of synergistic antitumour effect of α-pinene and β-pinene with paclitaxel against non-small-cell lung carcinoma showed morphological changes characteristic of apoptosis-like chromatin condensation and fragmentation of the nucleus [49].

Discodermolide is a polypropionate-based specific secondary metabolite isolated from the sea sponge. Discodermia dissolute in the past years was found to stabilize microtubules [217], and its synergistic potential for paclitaxel (Taxol) efficacy has been studied. The experimental studies for evaluation of drug combination of discodermolide and taxol indicated drug-induced aneuploidy enhancing effect rather than mitotic arrest in human ovarian cancer cells and in an in vivo model of ovarian carcinoma. Furthermore, in an animal model of ovarian carcinoma, the studied discodermolide and paclitaxel combination may suppress angiogenesis and induce regression of tumours [218]. Another secondary metabolite which belongs to the terpenes class was found to potentiate the lethality of paclitaxel via ROS-mediated mechanisms, and it was described that gelomulide K, which is a caspase-independent cell death-inducing agent, synergizes with paclitaxel on breast cancer cells and also has low toxicity to healthy cell treatment with gelomulide K-activated PARP-1 hyperactivation, AIF nuclear translocation, and cytoprotective autophagy, which relate to the intensification of ROS production [219].

The synergistic interaction of the conventional drug gemcitabine (brand name Gemzar) and paclitaxel by modulating acetylation and polymerization of tubulin in non-small-cell lung cancer cell lines have been shown and that the combination allows gemcitabine with paclitaxel to synergistically suppress tumour growth [220].

The other known conventional antifungal drug rapamycin (sirolimus) is able to potentiate the effects of paclitaxel in endometrial cancer cells through induction of apoptosis, inhibition of cell proliferation, acetylation of tubulin, and potentially increasing polymerization [221].

The antiparasite conventional drug praziquantel (brand name Biltricide) could greatly enhance the anticancer efficacy of paclitaxel resulting in a more pronounced inhibition of tumour growth compared with either drug alone in a mouse xenograft model [222].

7. A Brief Overview of Clinical Trials

Paclitaxel was produced as a natural material derived from the plant into a commercially viable semisynthetic drug that was developed to meet the increase in the demand for this drug [36]. Rapid advances in biomedical research have stimulated the development of nanomedicine and over the last 15 years, the number of studies employed to address its application in cancer therapy significantly increased [9, 223]. Due to the complexity and heterogeneity of the tumour environment, the distribution of nanomedicine and its interaction within the tumour area has shown considerable challenges [224].

The positive outcome of nanoparticles in clinical trials is conditioned with various factors:

(i) Stability and time spent by the particles in the circulation

(ii) The capacity of the particles to pass physiological barriers and reach affected anatomic sites

(iii) Bioavailability of particles at the affected area

(iv) The safety profile of particles

Upgrading these features should help to achieve greater efficacy of nanomedicines, subsequently facilitating their promotion into standard cancer treatment [225].

Nowadays, there are numerous immunoconjugates under clinical trials. The great interest in the field of institutional investigators and pharmaceutical companies is also demonstrated by the increasing number of patented immunoconjugates, and many researchers agree that immunoconjugates will likely become important actors in cancer therapy in the foreseeable future. This research is today particularly interesting due to the availability of a new generation of mAbs, such as chimeric or humanized molecules, that are already at the clinical stage [226]. The development of

combination therapies may result in new effective options for the treatment of cancer, and the knowledge of the mechanism of action of the substances is mandatory to design proper effective protocols.

Paclitaxel anticancer activity was established in a clinical trial after 22 years after it was discovered in the year 1984. Also, the first clinical trial was reported in 1987 by Wiernik et al. [40]. Paclitaxel was documented to have cytotoxic action most especially when carried out at the clinical trial during a trial that was performed on ovarian cancer. It was finalized that only 30% of ill people suffering from platinum-resistant ovarian cancer diseases showed a positive response to the treatment of paclitaxel whether partially or completely [227].

Some other clinical trials performed in the year 1994 showed that the drug exhibited a more inhibitory action against cancer diseases. The Food and Drug Administration finally approved this effective paclitaxel for utilization for the cure of breast cancer diseases. Paclitaxel is now applied when combined with other drugs such as carboplatin and cisplatin and as a single drug for the management of several cancer diseases [228]. The trial of the paclitaxel in patients with neck and head cancer, advanced ovarian cancer, and metastatic melanoma led to stability in these diseases [229–231].

The efficacy and clinical safety of albumin-bound paclitaxel (Abraxane®) as nanoparticles has been demonstrated in numerous clinical trials in various types of cancers. Thus, data available from 106 patients included in two open-label, single-arm studies and from 454 patients treated in a randomized phase III comparative study supported the efficacy of Abraxane in metastatic breast cancer [96, 232]. Another multicenter, multinational, randomized, open-label study was conducted, in which 861 patients compare the combination of Abraxane/gemcitabine with monotherapy with gemcitabine as first-line treatment in patients with metastatic pancreatic adenocarcinoma. There was a statistically significant improvement in progression-free survival in patients treated with Abraxane/gemcitabine compared to gemcitabine monotherapy, with a mean increase in survival of 1.8 months. A multicenter, randomized, open-label study was performed in 1052 patients with non-small-cell lung cancer in Stage IIIb/IV, in which no chemotherapy was administered. The study compared Abraxane in combination with carboplatin with the solvent-based paclitaxel in combination with carboplatin for first-line treatment in patients with non-advanced small-cell lung cancer. Patients in the Abraxane/carboplatin group had a significantly higher overall response rate larger compared to patients in the control group [233, 234].

8. Toxicity and Safety Aspects

Chemotherapeutic drugs for cancer management are accompanied by undesired adverse effects especially causing damage to other tissues of the body. Therefore, the application of nanotechnology for targeted drug delivery and biocompatibility and biodegradability offers an amazing opportunity to enhance the efficacy of cancer drugs while diminishing the toxic effects at the same time.

Paclitaxel (PTX) is a broadly used anticancer drug for a variety of cancers including breast, ovarian, gastric, and lung cancer [161]. The use of PTX clinically is reduced because of the concerns of toxicity such as low solubility, poor cancer cell selectivity, and speedy removal from the body. Likewise, PTX is associated with unwanted adverse effects such as hair loss, neurotoxicity, genotoxicity, hepatotoxicity, hypersensitivity, cardiac toxicity, allergic reaction, and repression of bone marrow [235].

The cancer patient's essence and worth of life is negatively affected because of the PTX-associated toxicity. Hence, this reduces the actual dose necessary for the beneficial effects of the chemodrug. The improvement of the mode of administration or delivery of PTX alongside exceptional anticancer potential with a good safety profile is necessary [236].

The application of nanotechnology in the field of biomedicine especially for the targeted drug delivery to a specific tissue of interest has gained research attention and clinical use, particularly for cancer treatment. Nanotechnology has been used to reduce toxicity and improve the safety and potency of PTX through the application of nanomaterials for efficient and effective delivery of the nanoformulated PTX drugs to the carcinoma cells and tissues [237]. Many biocompatible and biodegradable polymer-based nanoparticles have been used for drug delivery purposes [238].

8.1. Nanoparticle Albumin-Bound Paclitaxel (Nab-PTX). The nab-PTX chemo-drug has been documented to possess limited safety issues compared to paclitaxel. However, nab-PTX is associated with neuronal toxicity (Figure 2). Furthermore, nab-paclitaxel is beneficial more than Crepaclitaxel, since it has a tremendous diminished possibility of hypersensitivity and neutropenia, and quick improvement of peripheral neuronal toxicity upon cessation of treatment [239]. Zong et al. have also reported the toxicity associated with nab-PTX chemotherapy to include haematological toxicity such as low neutrophil and leukocyte counts as well as elevated alanine aminotransferase and aspartate aminotransferase activities which were corresponding but higher in number than what was observed in the paclitaxel-treated group. Furthermore, nausea, vomiting, fatigue, and diarrhoea were also seen in the nab-PTX group [96]. Interestingly nab-PTX was reported to be cheap for the management of breast cancer when compared to other traditional taxanes because it reduces the toxic side effects and cost of management of any ensuing hospitalization [240, 241].

8.2. Bovine Milk-Derived Exosomal Formulation of Paclitaxel (ExoPTX). Exosomes derived from milk is a feasible option because of their simplicity thereby enabling good mass production, quite comparable distribution in the biological system when administered through the oral route, and well-suited to the intracellular environment. Milk-derived exosomes possess innate defensive capability because of their antacid characteristics. Hence, they can serve as a great oral drug delivery system for cancer drugs such as PTX [98]. One of the most important functions of milk-derived exosomes is that they enhance the ease of drug usage. Milk-derived

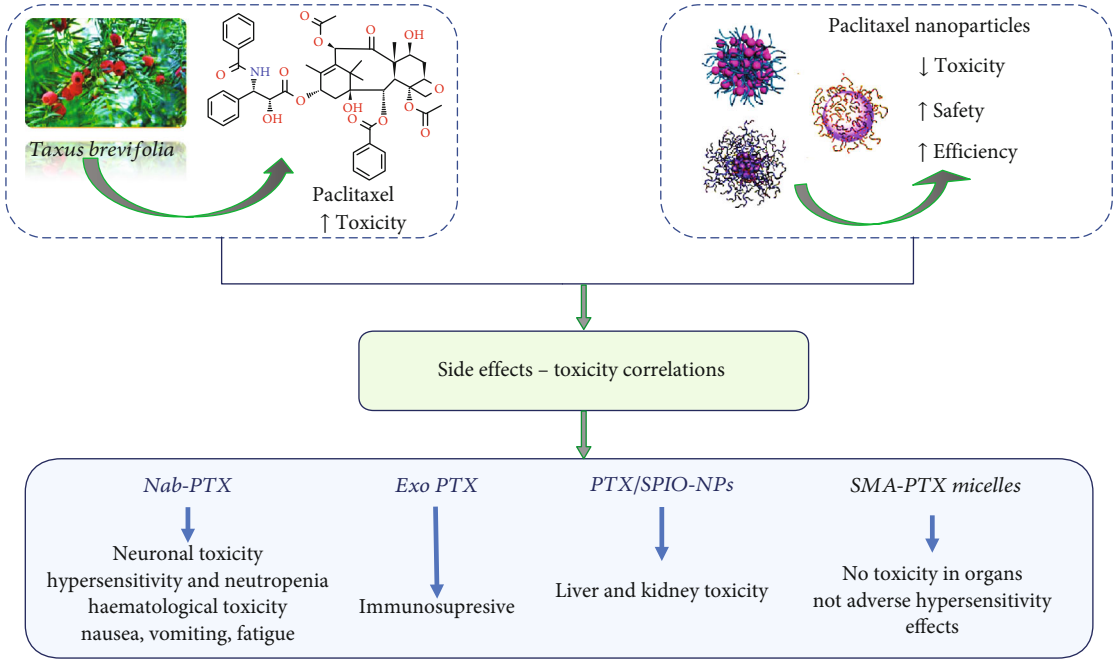

Figure 2: Summarized scheme regarding side effects of PTX nanoformulations.

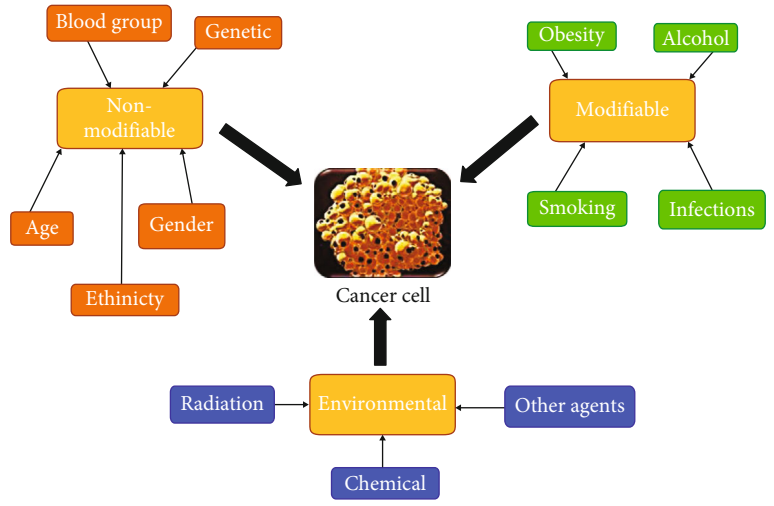

Figure 3: Risk factors for cancer development.

exosomes were shown to be immune-competent without any significant alterations in the spleen size, haematological parameters, and bone marrow. Therefore, ExoPTX did not have adverse effects on the immunological system of the body, unlike PTX which causes alteration in the responses of the immune system through the reduction in white blood cells. The toxic immunosuppressive effect associated with intravenous PTX administration has been completely taken cared of by the ExoPTX oral administration (Figure 2). The improvement offered by ExoPTX will enhance patient's experience and minimize the cost of cancer care [98].

8.3. Paclitaxel and Superparamagnetic Iron Oxide-Loaded PEGylated Poly(lactic-co-glycolic Acid)- (PLGA-) Based Nanoparticles (PTX/SPIO-NPs). The understanding of the polymeric nanoparticles used for diagnosis and therapies is very crucial especially when it comes to the elucidation of their toxicity [242]. Since PTX/SPIO-loaded PLGA nanoparticles store up in the hepatic tissue, then it is quite important to ensure that they do not have any adverse effects. PTX/SPIO-NPs have been demonstrated to be safe for the liver because there was a histomorphological alteration in the hepatocytes of the PTX/SPIO-NP-treated mice. Furthermore, PTX/SPIO-NPs did not result in any elevation in levels of blood urea nitrogen, aspartate aminotransaminase, or alanine aminotransferase when compared with the control (saline-treated group), signifying that the nanoparticle does not have toxic effects on the liver and kidneys. Furthermore, there was no significant alteration in the body weight and creatine myocardial band (CK-MB) of the PTX/SPIO-

NP-treated mice and the control showing the safety of the NPs in the heart. Therefore, PTX/SPIO-NPs will offer a good anticancer treatment [102].

8.4. Toxicity Analysis of SMA-PTX Micelles in the Orthotopic Colon Cancer Model. Treatment with SMA-PTX micelles did not alter the bodyweight signifying enhanced broad health of the mice. Furthermore, the SMA-PTX micelle-treated mice also had similar organ weights when compared with the control showing that there is no toxicity in the organs. Likewise, the SMA-PTX micelle group did not have any alterations in the alkaline phosphatase activity and creatinine levels indicating that there was no renal and hepatic tissue toxicity as a result of the treatment (AHC, 2015). Furthermore, SMA-PTX micelle nanoformulation is soluble in water and possesses no adverse hypersensitivity effects which are associated with PTX treatment. Interestingly, SMA-PTX micelle nanoformulation did not lead to any alteration in the anticancer effects of PTX. Therefore, the SMA-PTX micelle nanoformulation will offer an efficacious and nontoxic oral drug delivery. SMA-PTX micelle nanoformulation led to improved anticancer activity against colorectal cancer when compared with PTX [108] (Figure 3).

9. Overall Conclusions and Future Perspectives

Our work proves the usefulness of nanomedicine in cancer therapy. A special example is paclitaxel, which is a broad-spectrum anticancer drug. Scientific studies have confirmed its action against ovarian, lung, and breast cancer.

The review focused on the chemical structure of paclitaxel, its difficulties in application, and various nanoparticles containing paclitaxel—its usefulness and prospective toxic effect. It has been confirmed that the importance of nanomedicine is increasing in cancer therapy due to the reduced toxicity and improved safety and potency of such compounds as paclitaxel through the effective delivery to the carcinoma cells and tissues

To summarize, nanotechnology gives more opportunities to use the drug with proven anticancer effects, but its application is significantly restricted by limited solubility, recrystalization upon dilution, and cosolvent-induced toxicity.

Conflicts of Interest

The authors report no declarations of interest.

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Research Article

Tackling of Renal Carcinogenesis in Wistar Rats by *Silybum marianum* Total Extract, Silymarin, and Silibinin *via* Modulation of Oxidative Stress, Apoptosis, Nrf2, PPARγ, NF-κB, and PI3K/Akt Signaling Pathways

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The present work was designed to assess the efficacy of *Silybum marianum* total extract (STE), silymarin (Sm), and silibinin (Sb) against experimentally induced renal carcinogenesis in male Wistar rats and their roles in regulating oxidative stress, inflammation, apoptosis, and carcinogenesis. The diethylnitrosamine (DEN)/2-acetylaminofluorene (AAF)/carbon tetrachloride (CCl_4)-administered rats were orally treated with STE (200 mg/kg b.w.), Sm (150 mg/kg b.w.), and Sb (5 mg/kg b.w.) every other day either from the 1st week or from the 16th week of carcinogen administration to the end of 25th week. The treatments with STE, Sm, and Sb attenuated markers of toxicity in serum, decreased kidney lipid peroxidation (LPO), and significantly reinforced the renal antioxidant armory. The biochemical results were further confirmed by the histopathological alterations. The treatments also led to suppression of proinflammatory mediators such as NF-$\kappa\beta$, p65, I$\kappa\beta\alpha$, and IL-6 in association with inhibition of the PI3K/Akt pathway. Furthermore, they activated the expressions of PPARs, Nrf2, and IL-4 in addition to downregulation of apoptotic proteins p53 and caspase-3 and upregulation of antiapoptotic mediator Bcl-2. The obtained data supply potent proof for the efficacy of STE, Sm, and Sb to counteract renal carcinogenesis *via* alteration of varied molecular pathways.

1. Introduction

Kidney cancer is one of the most common malignant tumors of the genitourinary system, and it is among the cancer types that have the highest growth rate in all age and racial groups worldwide [1]. It has been stated that it is the most resistant malignancy, responding to conventional therapies either very little or not at all [2]. Despite the discovery of many novel chemotherapeutic drugs in the last decade as well as advances in the understanding of molecular pathways implicated in the development of kidney carcinogenesis, the disease remains incurable and fatal [3]. Several risk factors are claimed to predispose to kidney cancer involving chronic renal disease, hereditary syndrome, hypertension, obesity, cigarette smoking, long-term dialysis, genetic susceptibility, and maybe the result of exposure to different environmental toxicants [4].

Oxidative stress is defined as an imbalance between the generation of reactive oxygen species (ROS) and the biological system's ability to counteract the effects of reactive free

radicals or repair oxidative damage [5]. High amounts of ROS can also compromise the antioxidant defense system resulting in DNA, protein, and lipid damage [6]. The diminished effectiveness of the antioxidant defense system may aggravate oxidative damage [7]. ROS such as superoxides, peroxides, and hydroxyl radicals can be produced by mitochondria or extramitochondrial NAD (P)H oxidase (Nox) system [8, 9]. Oxidative stress is a major cause of various renal diseases that stimulates progression from acute to chronic damage and the development of kidney cancer [10].

Accumulating evidence indicates that tumorigenesis, rather than occurring spontaneously, appears to be linked to chronic inflammation and immune dysfunction [11, 12]. It has been proved that inflammation causes chromosomal instability, a proliferation of cancer cells, and stimulation of angiogenesis and tissue remodeling [13, 14]. Oxidative stress triggers the activation of nuclear factor-kappa B (NF-κB), exacerbating inflammation *via* inhibition of peroxisome-activated receptors (PPARs) and nuclear factor erythroid 2-related factor 2 (Nrf2) and anti-inflammatory interleukins [15–18].

Nrf2 controls the transcription of several antioxidant genes that maintain detoxification genes and cellular homeostasis in check, allowing toxins and carcinogens to be removed before they cause damage [19]. Nrf2 is known to be expressed in all cell types; however, it translocates into the nucleus during elevated cellular stress and stimulates the transcription of target genes encoding proteins related to xenobiotic efflux, redox regulation, iron metabolism, protein homeostasis, resistance to apoptosis, and repairing DNA [20]. Consequently, Nrf2 is thought to promote several anti-inflammatory effects involving inhibition of NF-κB activity, reduction of the expressions of several inflammatory mediators involving chemokines, cytokines, adhesion molecules, MMP-9, iNOS, and COX-2 [21–23], and interfering with interleukin production [24] and directly enhances PPARγ expression [25]. A previous study has examined the strategy of utilizing Nrf2 promoting compounds to mitigate ROS to inhibit the progression of renal diseases [26].

The PI3K/Akt signaling pathway is the most commonly activated pathway implicated in the oncogenesis of several cancers [27]. Activation of this pathway causes phosphorylation of additional proteins that regulate cell cycle entry, cell proliferation, and carcinogenesis [28, 29]. ROS can act directly on Akt or its downstream targets such as p53 and FOXO or indirectly through modulators such as GSK3, PTEN, Src, and others [30]. Genetic alterations in the PI3K/Akt pathway are common in kidney cancer [31, 32]. Therefore, PI3K/Akt signaling represents a wide window for developing targeted therapy.

Earlier studies investigated the roles of various dietary and natural antioxidants against damage caused by oxidative stress affecting glomerular and tubular functions [33, 34]. Silymarin (Sm) and silibinin (Sb) are investigated to exert strong antineoplastic effects in both *in vitro* and *in vivo* models of cancer, including cancers of the prostate, bladder, skin, colon, lung, breast, and kidney [35–41]. During Sm and Sb treatment, several mechanisms have been applied, such as DNA repair, cell cycle arrest, inhibition of growth and proliferation in addition to antiangiogenic effects, and blockage of invasion and metastasis [42, 43].

In conductance with the previous literature, the purpose of this study was to evaluate the potential preventive and therapeutic effects of STE, Sm, and Sb on diethylnitrosamine (DEN)/2-acetylaminofluorene (AAF)/carbon tetrachloride (CCl_4)-mediated renal carcinogenesis in addition to assessing their preventive mechanisms on apoptosis, Nrf2, PPARγ, NF-κB, and PI3K/Akt signaling pathways.

2. Materials and Methods

2.1. Chemicals. DEN, AAF, CCl_4, and Sb were obtained from Sigma chemicals company (St. Louis, MO, USA). Assay kits for urea, creatinine, and uric acid were purchased from Biosystem (Spain). Antibodies for p-PI3K and β-actin were obtained from Santa Cruz Biotechnology (USA). t-PI3K, t-Akt, p-Akt, t-p65, p-p65, t-IκBα, and p-IκBα antibodies were purchased from Cell Signaling Technology, Inc. (USA). Cleaved-caspase-3 antibody was obtained from Merck Millipore (Germany). Other analytical grade reagents and chemicals were purchased from local suppliers.

2.2. Preparation of Silymarin and Silybum marianum Total Extract. The fruits of *Silybum marianum* were obtained from a local vendor and used to extract Sm and STE using a Soxhlet apparatus. The extraction was performed in two steps: first, the fruits were defatted for 6 hrs using petroleum ether, and then, these defatted fruits were soaked in methanol for 5 hrs to obtain pure Sm. Each extract was evaporated under reduced pressure at a temperature not exceeding 40°C. Finally, two extracts were obtained: the first extract is about a pure Sm and the second extract is STE which is about Sm and the petroleum ether extract. Quantitative nuclear magnetic resonance (NMR) was used for quantitative estimation of flavonolignans in the extracts used in assessing biological activities. This technique is a nondestructive process, and the samples need only a few steps to be prepared. It is easily automated and supplies both quantitative and qualitative analyte measurements without the use of chromatographic separation or extra analytical instrumentation. Sb was used as a reference standard compound. The calibration curve was done by plotting integration values versus the molar concentration. A linear relationship of the standard curve was used to obtain the regression equation $[y = 0.5699x - 0.733\ (R^2 = 0.9987)]$ [44].

2.3. Animals. Eighty adult male Wistar rats (100–120 g body weight (b.w.)) were obtained from the Egyptian Organization for Biological Products and Vaccines (Helwan Station, Cairo, Egypt). Rats were kept under pathogen-free conditions. All procedures were carried out according to the rules of the animal care and use guidelines stated by the Experimental Animal Ethics Committee of Faculty of Science, Beni-Suef University, Egypt (Ethical Approval Number: BSU/FS/2017/6).

2.4. Experimental Design. After one week of adaptation, the rats were divided into eight groups each with 10 rats. Group I acted as a normal control group. Rats of the other seven groups were intraperitoneally injected with DEN (100 mg/kg b.w.)

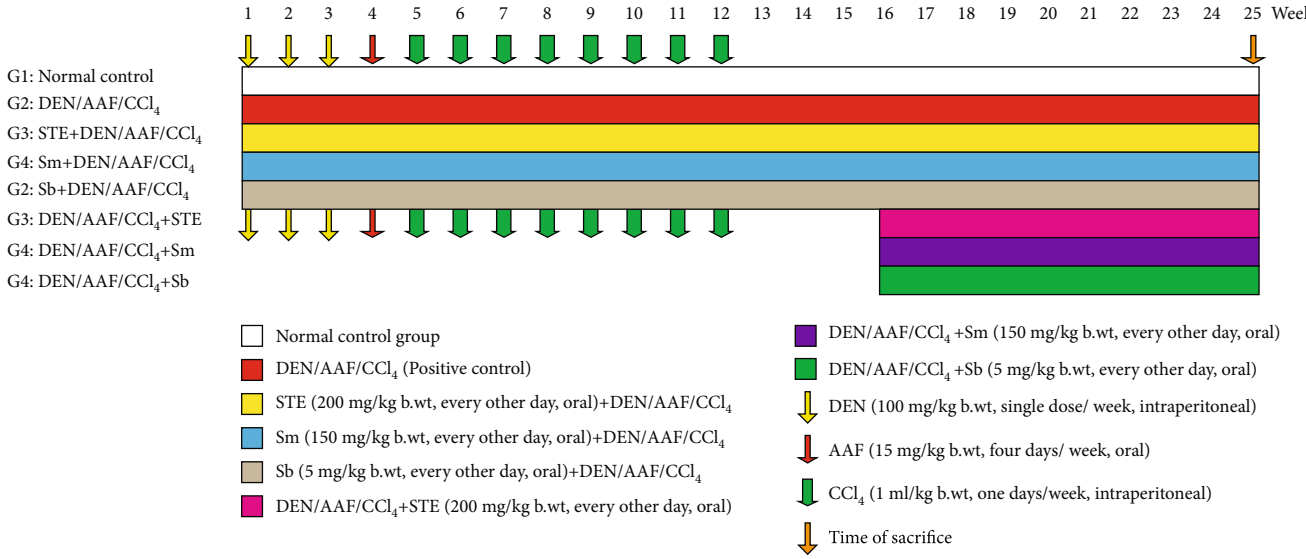


Figure 1: Experimental design.

once a week for three successive weeks, followed by oral administration of AAF (15 mg/kg b.w.) four times a week for one week only. Starting from the 5th week, all rats were injected with CCl_4 (1 mL/kg b.w.) intraperitoneally until the 12th week of the experiment [45]. Group II served as the DEN/AAF/CCl_4 control group. Groups III, IV, and V received DEN/AAF/CCl_4 like group II and were orally concurrently treated with STE (200 mg/kg b.w.) [46], Sm (150 mg/kg b.w.) [47], and Sb (5 mg/kg b.w.) [48], every other day for 25 weeks. Groups VI, VII, and VIII received DEN/AAF/CCl_4 like group II, and after 16 weeks, they were treated with STE (200 mg/kg b.w.) [46], Sm (150 mg/kg b.w.) [47], and Sb (5 mg/kg b.w.) [48], every other day till the end of the 25th week of the experiment (Figure 1).

2.5. Blood and Kidney Sampling. At the end of the experiment, the experimental rats were anesthetized and then sacrificed for obtaining the blood samples to be analyzed. To determine both the kidney LPO and the content of kidney total thiol, glutathione, and antioxidant enzymes, kidney samples were homogenized (0.1 M) in phosphate-buffered saline (cold). Other parts from the kidney tissues were collected and put in 10% neutral buffered formalin for histological examination. Other parts of the kidney were stored at -70°C for western blotting, RNA extraction, and quantitative RT-PCR analysis.

2.6. Detection of the Concentrations of Serum Urea, Creatinine, and Uric Acid. The renal function parameters including urea, creatinine, and uric acid levels were estimated according to the methods of Tabacco et al. [49], Fabiny and Ertingshausen [50], and Fossati et al. [51].

2.7. Histological Investigation. For histological investigation, a part from the kidney of each rat was postfixed in neutral buffered formalin (10%) for twenty-four hours. After complete fixation, specimens (3-4 mm^3) were cleared in xylene and embedded in paraffin wax, then sectioned using a microtome at a thickness of 4 μm, and stained with hematoxylin and eosin (H&E) for light microscopy investigation [52].

2.8. Evaluation of the Kidney Oxidative Stress and Antioxidant Defense Markers. Kidney lipid peroxidation (LPO) and reduced glutathione (GSH) content were assessed using the methods of Preuss et al. [53] and Beutler et al. [54], respectively. Kidney total thiol, glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx), and superoxide dismutase (SOD) were estimated using the reported methods according to Koster et al. [55], Goldberg [56], Mannervik and Gutenberg [57], Matkovics et al. [58], and Marklund and Marklund [59], respectively, with minor modifications.

2.9. Isolation of RNA and RT-PCR Gene Expression Analysis. The total amount of RNA from frozen kidney tissues was extracted using a Qiagen tissue extraction kit (USA) and quantified. According to the manufacturer's procedure, the concentration was estimated spectrophotometrically at A260. Two μg of total RNAs were used for the reverse transcription system (Fermentas, USA). A qRT-PCR was carried out using SYBR Green mix (Thermo Fisher Scientific, USA) with PCR condition. The sequences of the primer pairs are given in Table 1. The expression of genes was determined using the $2^{-\Delta\Delta Ct}$ method. The β-actin gene was amplified as an internal control, and samples were run in triplicate.

2.10. Western Blot Analysis. Proteins were extracted from kidney tissue samples by RIPA buffer containing a cocktail of protease inhibitors (Bio-Rad Inc.). Protein was quantified using a Bradford assay kit (SK3041; Bio Basic Inc., Markham Ontario L3R 8T4 Canada), then separated by 10% SDS-PAGE (Bio-Rad Laboratories Inc. Cat#161-0181), and transferred to PDVF membranes. Membranes were immunoblotted with indicated primary antibodies prepared at

Table 1: Primer sequences used in qRT-PCR analysis.

Gene	GenBank accession number	Sequence (5′–3′)
Nrf2	NM_031789.2	F: TTGTAGATGACCATGAGTCGC R: TGTCCTGCTGTATGCTGCTT
NF-κB	NM_001276711.1	F: TACCATGCTGTTTTGGTTAC R: TCAAGCTACCAATGACTTTC
IL-6	NM_012589.2	F: AGTTGCCTTCTTGGGACTGA R: ACTGGTCTGTTGTGGGTGGT
IL-4	NM_201270.1	F: GGAACACCACGGAGAACG R: GCACGGAGGTACATCACG
PPARγ	NM_001145367.1	F: GGACGCTGAAGAAGAGACCTG R: CCGGGTCCTGTCTGAGTATG
p53	NM_030989.3	F: CAGCGTGATGATGGTAAGGA R: CAGCGTGATGATGGTAAGGA
Bcl-2	NM_016993.1	F: GGGATGCCTTTGTGGAACTA R: CTCACTTGTGGCCCAGGTAT
β-Actin	NM_031144.3	F: AAGGATTCCTATGTGGGCGACG R: GCCTGGATAGCAACGTACATGG

Table 2: Effects of STE, Sm, and Sb on serum levels of urea, creatinine, and uric acid of DEN/AAF/CCl_4-administered rats.

Groups	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
Normal control	53.17 ± 3.72	0.70 ± 0.06	1.45 ± 0.10
DEN control	$98.50 \pm 6.87^{***}$	$1.12 \pm 0.05^{***}$	$5.12 \pm 0.35^{***}$
STE+DEN/AAF/CCl_4	$56.50 \pm 2.68^{\#\#\#}$	$0.77 \pm 0.05^{\#\#\#}$	$1.87 \pm 0.16^{\#\#\#}$
Sm+DEN/AAF/CCl_4	$59.83 \pm 3.56^{\#\#\#}$	$0.82 \pm 0.03^{\#\#\#}$	$2.93 \pm 0.30^{***\#\#\#}$
Sb+DEN/AAF/CCl_4	$59.00 \pm 3.42^{\#\#\#}$	$0.80 \pm 0.06^{\#\#\#}$	$2.64 \pm 0.19^{**\#\#\#}$
DEN/AAF/CCl_4+STE	$63.00 \pm 1.90^{\#\#\#}$	$0.78 \pm 0.03^{\#\#\#}$	$2.43 \pm 0.12^{*\#\#\#}$
DEN/AAF/CCl_4+Sm	$45.00 \pm 1.92^{\#\#\#}$	$0.80 \pm 0.03^{\#\#\#}$	$2.50 \pm 0.16^{*\#\#\#}$
DEN/AAF/CCl_4+Sb	$69.17 \pm 2.59^{\#\#\#}$	$0.78 \pm 0.04^{\#\#\#}$	$2.79 \pm 0.10^{***\#\#\#}$

Data are presented as mean values ± SEM. The number of detected rats is six. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

5% blocking buffer (1 : 1000) and were cultured overnight at 4°C. After extensive washing in TBS buffer, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies (Goat anti-rabbit IgG-HRP-1 mg Goat mab-Novus Biologicals) and developed using the chemiluminescent substrate (ClarityTM Western ECL substrate Bio-Rad Cat#170-5060). β-Actin was used as a loading control. Image analysis software was assessed by normalization procedure of each phosphorylated active target protein versus corresponding control sample total protein on the ChemiDoc MP imager.

Primary antibodies contained antibody t-PI3K (Cat#3358), p-PI3K (sc-1637), t-Akt (Cat#9272), p-Akt (Cat#9271), t-p65 (Cat#3034), p-p65 (Cat#3033,), t-NF-κB inhibitor α (IκBα, Cat#9242), p-IκBα (Cat#2859), cleaved-caspase-3 (Cat#AB3623), and β-actin (sc-8432).

2.11. Statistical Analysis. The results (mean ± SEM) obtained were performed using a one-way ANOVA test followed by Tukey's test post hoc analysis with the help of GraphPad prism 5 (GraphPad Software, CA, USA). The values of $P > 0.05$were nonsignificant while those of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were presumed to be significant, highly significant, and very highly significant, respectively.

3. Results

3.1. Effects of STE, Sm, and Sb on Nephrotoxicity Biomarkers in Serum. A significant difference was observed in the renal function markers when compared in all rat groups (Table 2). The concentrations of urea, creatinine, and uric acid were significantly ($P < 0.001$) increased in the serum of DEN/AAF/CCl_4-administered rats comparing to the normal control group. The groups supplemented with STE, Sm, and Sb from either at the 1st week or the 16th week of carcinogen administration revealed a significant decrease ($P < 0.001$) in the serum levels of urea, creatinine,

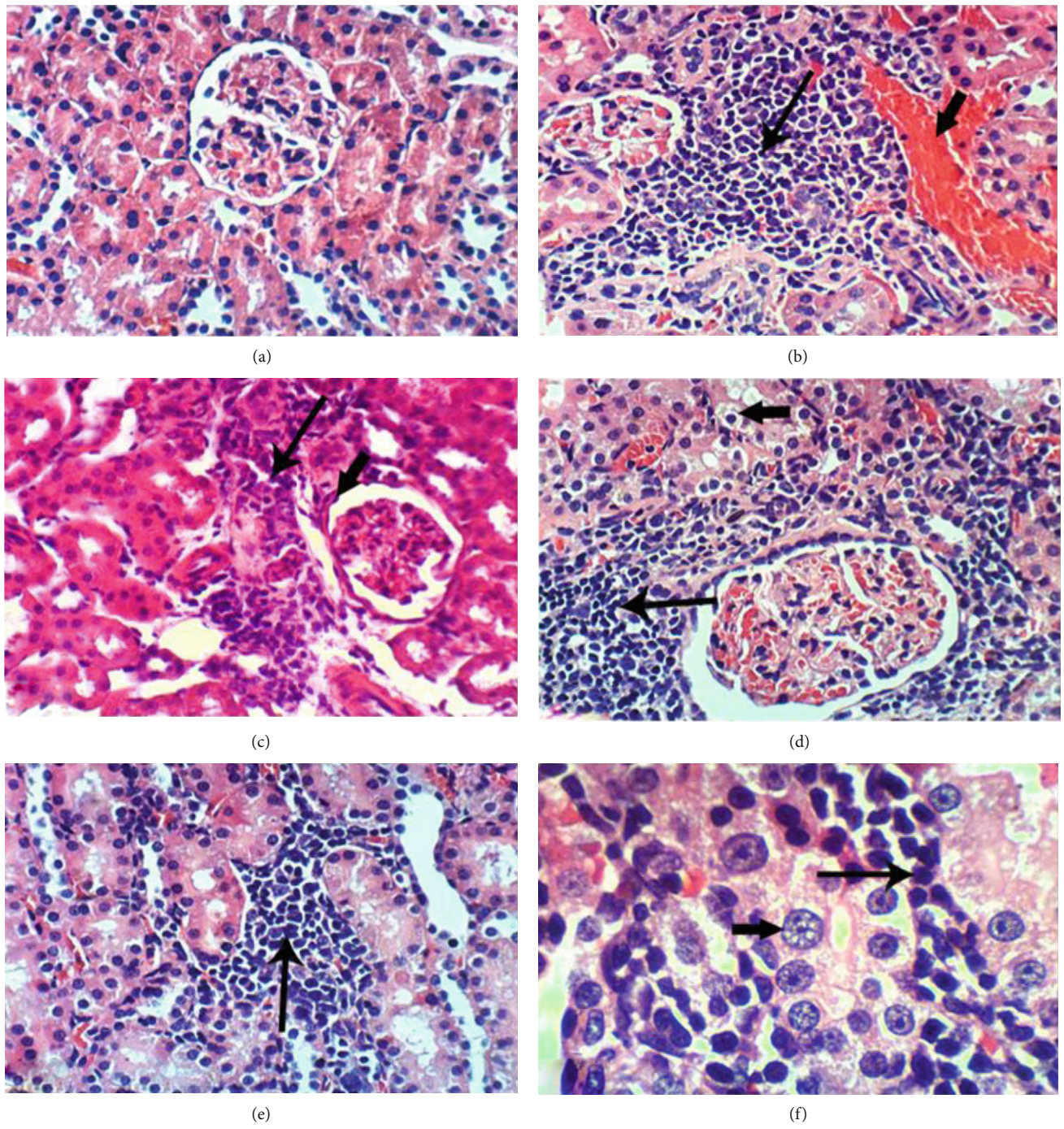


Figure 2: H&E-stained kidney sections photomicrographs of normal control and DEN/AAF/CCl_4-administered rats. There were no histological changes that have been observed in normal control rats ((a); 400x). On contrary, the DEN/AAF/CCl_4-administered rats exhibited congestion of renal blood vessel (short arrow) ((b); 400x), focal necrosis of renal tubules associated with inflammatory cell infiltration (long arrow) (b, c), thickening of the parietal layer of Bowman's capsule (short arrow) ((c); 400x), vacuolation of the renal tubular epithelium (short arrow), periglomerular inflammatory cell infiltration (long arrow) and congestion of glomerular tuft ((d); 400c), interstitial nephritis (long arrow) (e, f), and dysplastic renal tubules with karyomegalic nuclei (short arrow) ((f); 1000x).

and uric acid in comparison to the DEN/AAF/CCl_4-administered group.

3.2. Effects of STE, Sm, and Sb on Kidney Histological Changes. According to H&E staining (Figures 2–4), the cells in kidney tissues of DEN/AAF/CCl_4-administered rats showed tubular interstitial necrosis, periglomerular inflammatory cell infiltration, glomerular tuft congestion, vacuolation of epithelial lining renal tubules, interstitial nephritis, thickening of the parietal layer of Bowman's capsule, and karyomegaly of the nuclei with more than one nucleolus (Figures 2(b)–2(f)) in comparison with the intact

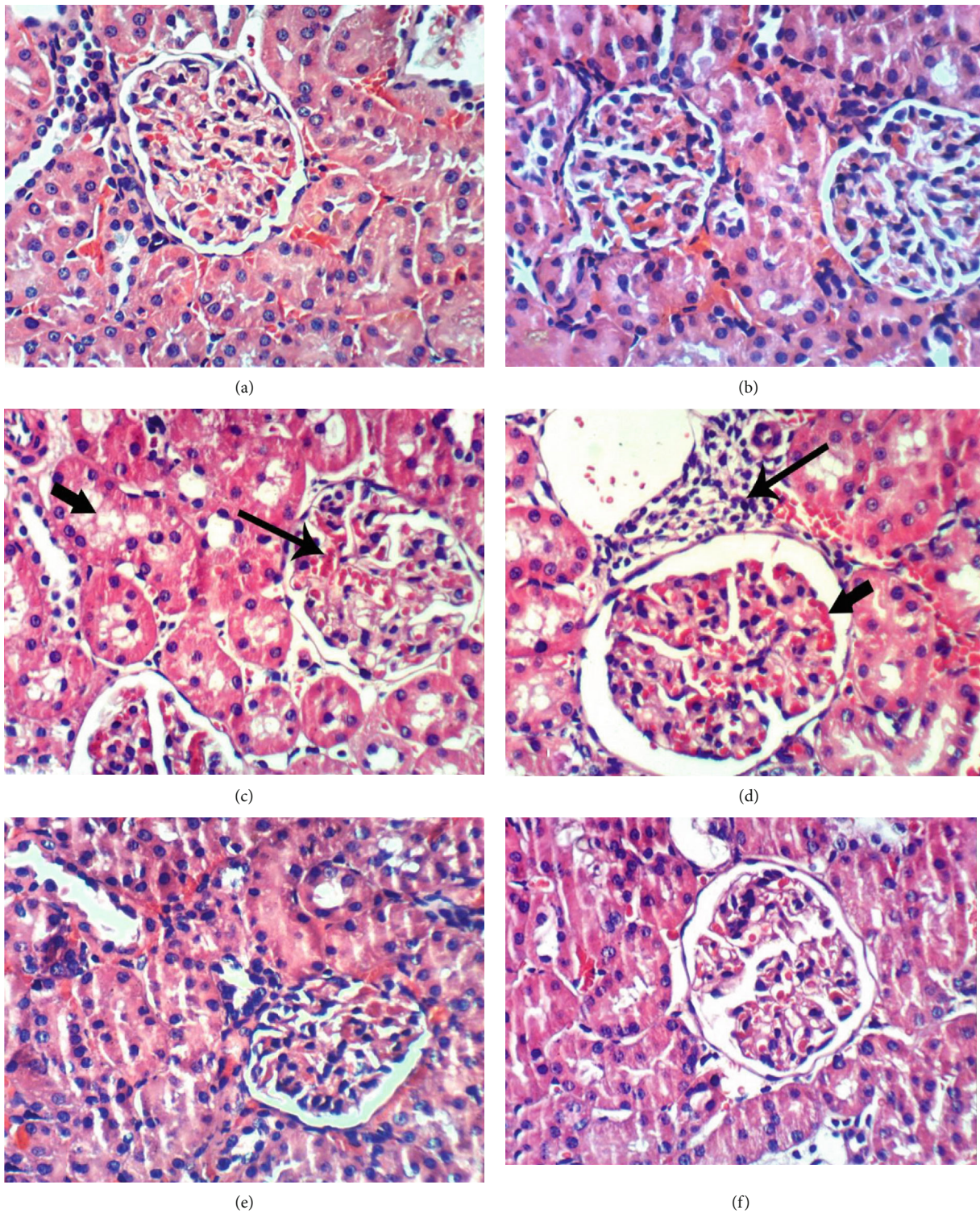


Figure 3: Photomicrographs (H&E; 400x) of kidney sections of DEN/AAF/CCl_4-administered rats treated with STE, Sm, and Sb from the 1st week of carcinogens-induction till the end of the experiment showing no histological changes in group-administered STE (a, b) as well as groups supplemented with Sb (e, f) in addition to vacuolation of the renal tubular epithelium (short arrow), congestion of glomerular tuft (long arrow) (c), and focal mononuclear cell infiltration (long arrow) (d) in rats treated with Sm.

architecture of kidney tissue of the normal control rats (Figure 2(a)), whereas the treatment with STE, Sm, and Sb from the 1st week of DEN/AAF/CCl_4 administration showed normalized kidney structure in both the STE-treated group (Figures 3(a) and 3(b)) and the Sb-treated group (Figures 3(e) and 3(f)). Furthermore, renal tubular epithelium vacuolation, glomerular tuft congestion, and focal mononuclear cell infiltration were detected in the Sm-

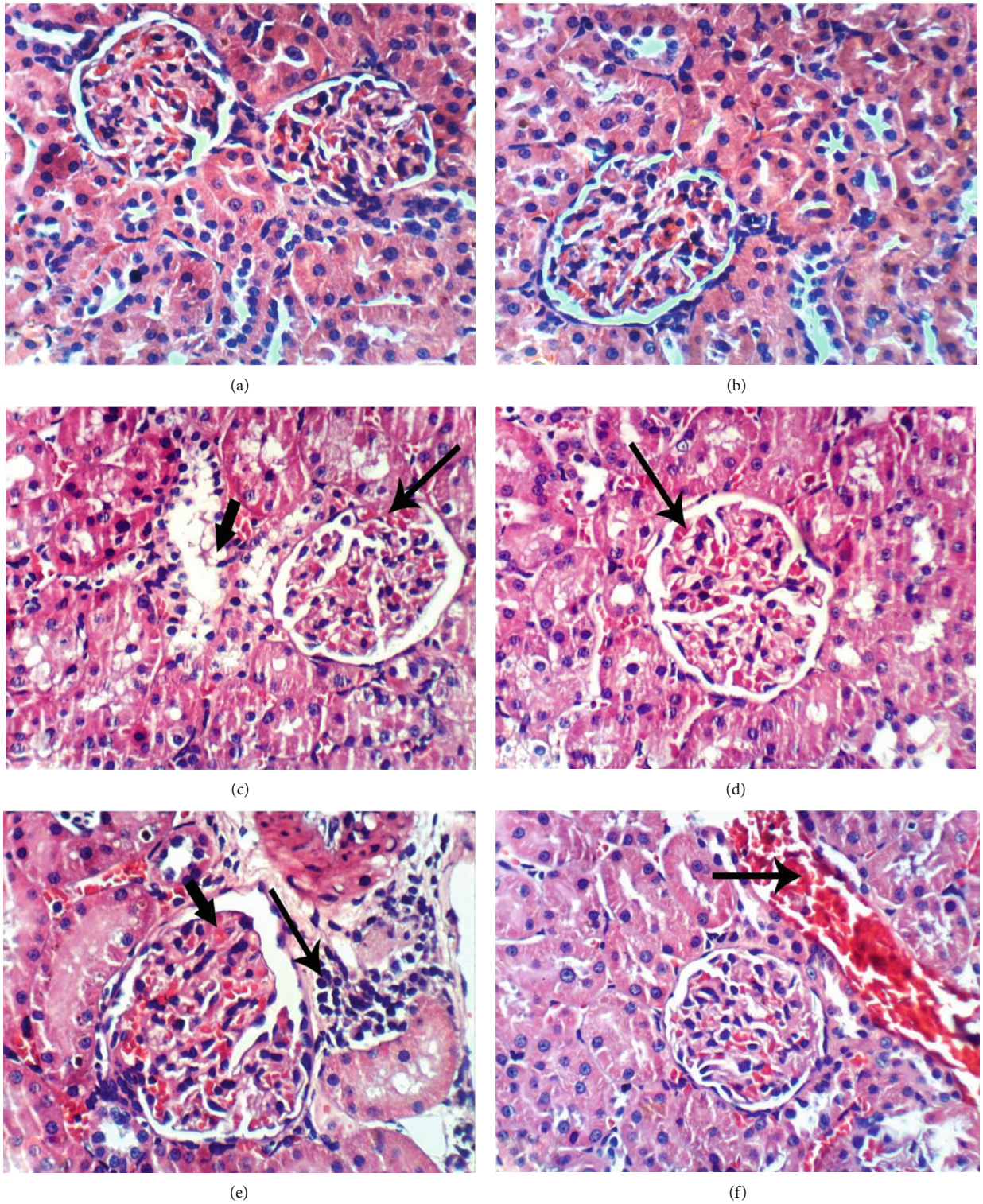


Figure 4: Photomicrographs of kidney sections (H&E; 400x) of DEN/AAF/CCl_4-administered rats treated with STE, Sm, and Sb from the 16^{th} week of DEN injection till the end of the experiment. The DEN/AAF/CCl_4-administered rats treated with STE showed no histological changes (a, b). Slight vacuolation of the renal tubular epithelium (short arrow) and congestion of glomerular tuft (long arrow) (c, d) were detected in kidney sections of rats treated with Sm. Similarly, the treatment with Sb produced a marked improvement of the kidney histological changes compared to those rats administered DEN/AAF/CCl_4 alone; there was congestion of glomerular tuft (short arrow), focal mononuclear cell infiltration (long arrow) (e), and congestion of renal blood vessel (f).

Table 3: Effects of STE, Sm, and Sb on kidney LPO, SOD, and GPx activities in DEN/AAF/CCl_4-administered rats.

Groups	LPO (nmol MDA/100mg tissue/hour)	SOD (U/g tissue)	GPx (mU/100mg tissue)
Normal control	13.78 ± 1.09	114.30 ± 4.70	165.30 ± 2.39
DEN/AAF/CCl_4	$22.53 \pm 0.99^{***}$	$70.99 \pm 2.80^{***}$	$133.10 \pm 1.91^{***}$
STE+DEN/AAF/CCl_4	$14.47 \pm 0.63^{\#\#\#}$	$112.30 \pm 7.31^{\#\#\#}$	$152.90 \pm 2.13^{**\#\#\#}$
Sm+DEN/AAF/CCl_4	$13.00 \pm 0.34^{\#\#\#}$	$110.60 \pm 5.27^{\#\#\#}$	$153.60 \pm 1.85^{**\#\#\#}$
Sb+DEN/AAF/CCl_4	$11.70 \pm 0.33^{\#\#\#}$	$115.10 \pm 7.07^{\#\#\#}$	$153.80 \pm 1.84^{**\#\#\#}$
DEN/AAF/CCl_4+STE	$11.82 \pm 1.14^{\#\#\#}$	$120.70 \pm 2.97^{\#\#\#}$	$155.10 \pm 2.03^{*\#\#\#}$
DEN/AAF/CCl_4+Sm	$13.46 \pm 0.42^{\#\#\#}$	$113.40 \pm 6.02^{\#\#\#}$	$150.60 \pm 1.54^{***\#\#\#}$
DEN/AAF/CCl_4+Sb	$15.45 \pm 0.40^{\#\#\#}$	$116.00 \pm 1.52^{\#\#\#}$	$155.50 \pm 1.48^{*\#\#\#}$

Data are presented as mean values ± SEM. The number of detected rats is six. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

Table 4: Effects of STE, Sm, and Sb on kidney total thiol and GSH contents as well as GR and GST activities in DEN/AAF/CCl_4-administered rats.

Groups	Total thiol (nmole/100mg tissue)	GSH (nmole/100mg tissue)	GR (mU/100mg tissue)	GST (U/100mg tissue)
Normal control	217.40 ± 11.43	62.51 ± 4.55	307.60 ± 9.07	455.00 ± 10.83
Control DEN	$116.40 \pm 5.04^{***}$	$32.20 \pm 3.34^{***}$	$75.44 \pm 5.00^{***}$	$218.90 \pm 18.28^{***}$
STE+DEN/AAF/CCl_4	$214.60 \pm 11.14^{\#\#\#}$	$57.28 \pm 3.86^{\#\#\#}$	$300.50 \pm 13.70^{\#\#\#}$	$431.40 \pm 31.49^{\#\#\#}$
Sm+DEN/AAF/CCl_4	$195.90 \pm 12.85^{\#\#\#}$	$51.41 \pm 4.01^{\#}$	$269.30 \pm 13.76^{\#\#\#}$	$421.30 \pm 8.14^{\#\#\#}$
Sb+DEN/AAF/CCl_4	$200.60 \pm 14.33^{\#\#\#}$	$52.83 \pm 4.57^{\#\#}$	$299.90 \pm 7.83^{\#\#\#}$	$427.90 \pm 19.24^{\#\#\#}$
DEN/AAF/CCl_4+STE	$218.10 \pm 15.27^{\#\#\#}$	$54.51 \pm 2.69^{\#\#}$	$286.70 \pm 17.29^{\#\#\#}$	$424.50 \pm 30.16^{\#\#\#}$
DEN/AAF/CCl_4+Sm	$199.40 \pm 13.81^{\#\#\#}$	$52.50 \pm 4.01^{\#\#}$	$284.00 \pm 16.62^{\#\#\#}$	$395.90 \pm 15.24^{\#\#\#}$
DEN/AAF/CCl_4+Sb	$204.60 \pm 9.57^{\#\#\#}$	$54.77 \pm 2.42^{\#\#}$	$295.20 \pm 10.80^{\#\#\#}$	$390.10 \pm 18.94^{\#\#\#}$

Data are presented as mean values ± SEM. The number of detected rats is six. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

supplemented group (Figures 3(c) and 3(d)). Similarly, the treatment with STE, Sm, and Sb from the 16th week of DEN/AAF/CCl_4 administration revealed no histological changes in STE-treated rats (Figures 4(a) and 4(b)), slight vacuolation of renal tubular epithelium and congestion of glomerular tuft in Sm-treated rats (Figures 4(c) and 4(d)), and congestion of glomerular tuft, focal mononuclear cell infiltration, and congestion of renal blood vessel in Sb-treated rats (Figures 4(e) and 4(f)).

3.3. Effects of STE, Sm, and Sb on Kidney Oxidative Stress and Antioxidant Defense Markers. As shown in Tables 3 and 4, DEN/AAF/CCl_4 administration resulted in a significant increase ($P < 0.001$) in kidney LPO and a significant decrease in the GSH kidney content, in addition to a significant decline ($P < 0.001$) in total thiol content and SOD, GPx, GR, and GST activities as compared to normal control rats. Administration of STE, Sm, and Sb, either from the 1st week or from the 16th week of DEN administration, significantly reduced kidney LPO ($P < 0.001$) in comparison with the DEN/AAF/CCl_4-administered group. In contrast, these treatments significantly restored ($P < 0.001$) the kidney total thiol content as well as SOD, GPx, GR, and GST activities when compared with the DEN control group. Besides, the content of kidney GSH was markedly improved either in groups administered DEN/AAF/CCl_4 and treated with STE ($P < 0.001$), Sm ($P < 0.05$), and Sb ($P < 0.01$) from the 1st week of DEN injection or in groups treated with STE ($P < 0.01$), Sm ($P < 0.01$), and Sb ($P < 0.01$) from 16th week of DEN injection to the end of the experiment.

3.4. Effects of STE, Sm, and Sb on the Expression of the NF-κB Pathway. The mRNA expression of NF-κB was strongly induced in the DEN/AAF/CCl_4-administered group when compared with the normal control group. The treatment with STE, Sm, and Sb either from the 1st week or from the 16th week of DEN administration reduced the NF-κB expression ($P < 0.001$) to a great extent (Figure 5).

The administration of DEN/AAF/CCl_4 activated the protein levels of p/t-p65 (Figures 6(a) and 6(b); $P < 0.001$) and p/t-IκBα (Figure 6(c); $P < 0.001$) comparing to normal control rats, whereas the groups treated with STE, Sm, and Sb either from the 1st week or from the 16th week of carcinogen administration showed a significantly declined ratio of

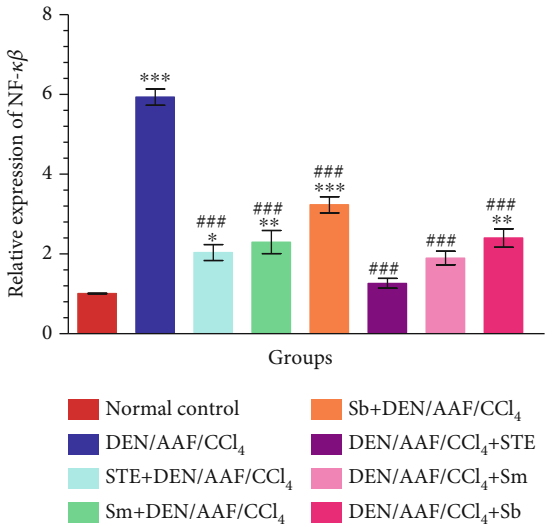


Figure 5: The effect of STE, Sm, and Sb on mRNA expression of NF-κB of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

p/t-p65 (Figures 6(a) and 6(b); $P < 0.001$). Similarly, the rats administered DEN/AAF/CCl_4 then treated with STE, Sm, and sb showed a significant decline in the level of p/t-IκBα (Figures 6(a) and 6(c); $P < 0.001$) manifesting that the STE, Sm, and Sb restrained NF-κB signaling pathways.

As illustrated in Figure 7, the administration of DEN/AAF/CCl_4 significantly ($P < 0.001$) increased the mRNA expression of IL-6 compared to normal control rats. On contrary, the treatment with STE, Sm, and Sb either from the 1st week or from the 16th week of carcinogen administration suppressed the expression ($P < 0.001$) of IL-6 when compared with the DEN/AAF/CCl_4-administered group.

3.5. Effect of STE, Sm, and Sb on the Expression of Nrf2 in Rats Administered DEN/AAF/CCl_4. As shown in Figure 8, DEN/AAF/CCl_4 induction resulted in a marked decline in the mRNA expression of Nrf2 ($P < 0.001$) when compared with normal control rats. On contrary, in comparison with the DEN/AAF/CCl_4-administered group, STE, Sm, and Sb supplementation either from the 1st week or from the 16th week of DEN administration activated this defense pathway to a great extent.

3.6. Effects of STE, Sm, and Sb on the mRNA Expressions of PPARγ and IL-4. DEN/AAF/CCl_4 administration significantly ($P < 0.001$) decreased the expression of PPARγ comparing to the normal control group, whereas supplementation of STE, Sm, and Sb either from the 1st week or from the 16th week of DEN administration significantly ($P < 0.001$) ameliorated this effect by increasing the mRNA expression of PPARγ (Figure 9).

As depicted in Figure 10, a significant decline in the mRNA expression of IL-4 in rats administered DEN/AAF/CCl_4 alone was detected ($P < 0.001$). On contrary, STE, Sm, and Sb supplementation either from the 1st week or from the 16th week of DEN administration significantly ($P < 0.001$) activated the expression of IL-4 when compared with the DEN/AAF/CCl_4-administered group.

3.7. STE, Sm, and Sb Inhibit Kidney Carcinogenesis through Inhibition of the PI3K/Akt Pathway. Expressions of PI3K/Akt in kidney tissues of Wistar rats in the current study were assessed by western blot. As shown, the levels of p/t-PI3K (Figures 11(a) and 11(b); $P < 0.001$) and p/t-Akt (Figures 11(a) and 11(c); $P < 0.001$) were significantly increased in rats administered DEN/AAF/CCl_4, whereas the rats treated with STE, Sm, and Sb from the 1st week or the 16th week of carcinogen administration showed a significant ($P < 0.001$) reduction in the protein levels of p/t-PI3K (Figures 11(a) and 11(b); $P < 0.001$). Furthermore, the protein level of p/t-Akt was significantly (Figures 11(a) and 11(c); $P < 0.001$) decreased in rats treated with STE, Sm, and Sb from either the 1st week or the 16th week of DEN injection.

3.8. STE, Sm, and Sb Ameliorate Nephrocyte Apoptosis Induced by DEN/AAF/CCl_4. As shown in the current study, a significant ($P < 0.001$) increased mRNA expression of p53 as well as decreased expression ($P < 0.001$) of Bcl-2 in the renal tissue of rats-administered DEN/AAF/CCl_4 was detected when compared with normal control rats. These changes were significantly ($P < 0.001$) alleviated by the coadministration of STE, Sm, and Sb from either the 1st week or the 16th week of DEN administration by producing low expression of p53 and strong expression of Bcl-2 comparing to DEN/AAF/CCl_4-administered rats (Figures 12 and 13).

Comparing with the normal control rats, the rats administered DEN/AAF/CCl_4 showed a significant increment in the protein level of cleaved-caspase-3 (Figures 14(a) and 14(b); $P < 0.001$), whereas the groups treated with STE, Sm, and Sb from the 1st week of carcinogen administration showed a significant ($P < 0.001$) decrease in the protein level of cleaved-caspase-3. Similarly, the level of caspase-3 was found to be reduced in rats treated with STE ($P < 0.001$), Sm ($P < 0.01$), and Sb ($P < 0.001$) starting from the 16th week of DEN induction (Figures 14(a) and 14(b)).

4. Discussion

The kidney plays an important role in the elimination of different xenobiotics; thereby, it is more exposed to damage because of its high sensitivity to numerous drugs and toxins [60]. DEN is a well-known carcinogenic N-nitroso compound that causes preneoplastic lesions in a variety of organs in experimental animals [61, 62]. It is found mainly in agriculture products, cosmetics, alcoholic drinks, cigarette smoke, groundwater, occupational settings, dried and salted fish, foods such as cheese, and soybean [63]. Additionally, as a result of the absorption of various drugs into the body,

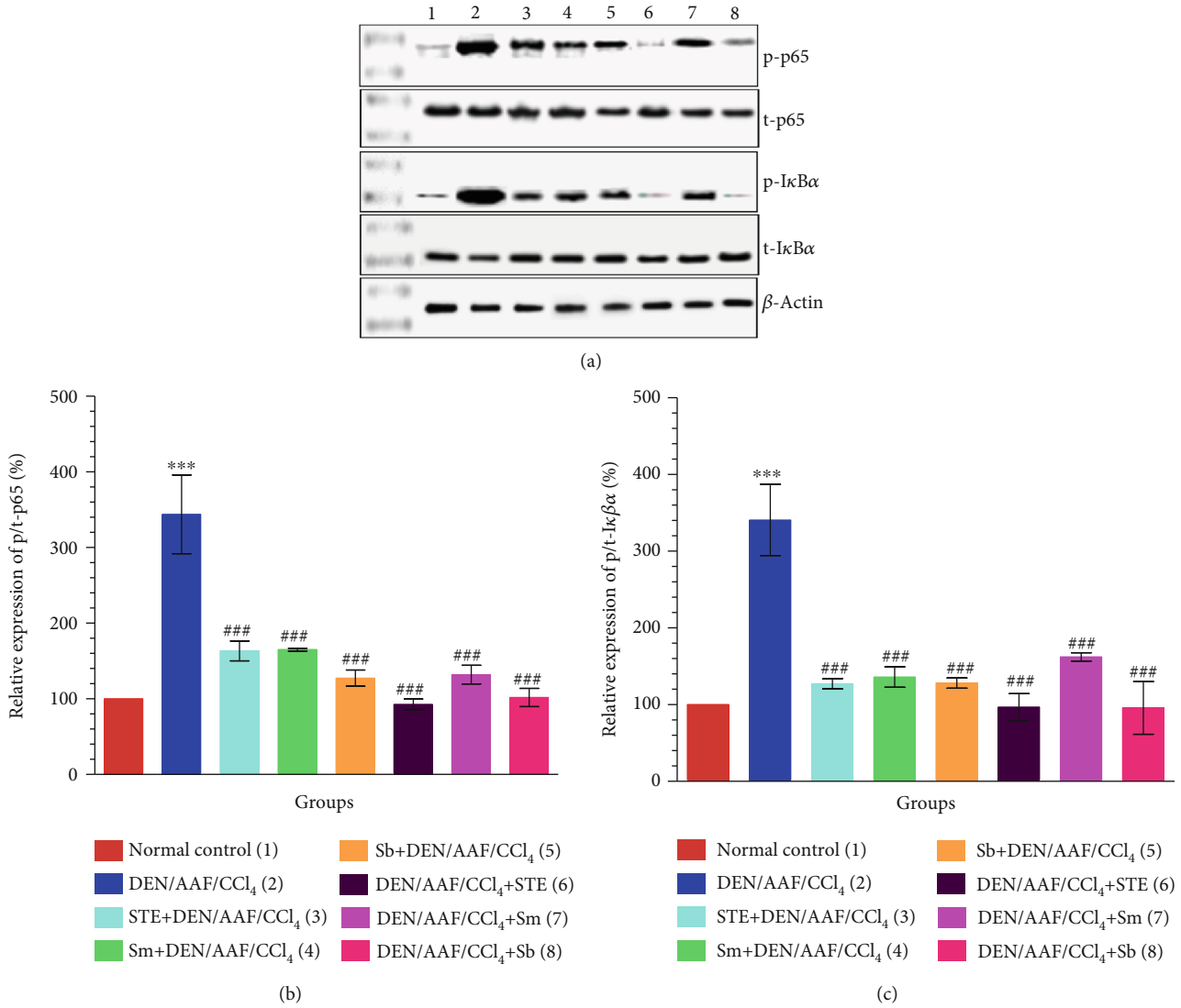


Figure 6: Effect of STE, Sm, and Sb on the protein levels of p65 and IκBα in kidney tissues of rats given DEN/AAF/CCl_4. Western blot assay was used to measure the expressions of p/t-p65 (b) and p/t-IκBα (c) proteins in kidney tissues of the experimental groups. Representative immunoblots for quantification of p/t-p65 and p/t-IκBα proteins were depicted in (a). Data are presented as mean values ± SEM with results from 3 independent biological repeats. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE+DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

DEN is produced in one form or repeated forms [64]. Accumulating evidence indicates that the administration of DEN in combination with AAF or CCl_4 promotes carcinogenesis [65, 66]. When DEN is metabolized in the body by cytochrome P450, it generates highly reactive free radicals which trigger the lipid peroxidation process, oxidative damage to proteins, and DNA in other organelles of the cell [67, 68]. As a result, we investigated the preventive role of STE, Sm, and Sb against DEN-induced kidney carcinogenesis through their antioxidant property which is retained by the chemical constituents of STE, Sm, and Sb.

Urea, creatinine, and uric acid are the main breakdown products of the body processes which must be filtered and excreted by the kidneys [69]. If the kidney malfunction, these substances are not easily eliminated by them. As a result, levels of blood urea, creatinine, and uric acid will accumulate in the serum. Similarly, the administration of DEN/AAF/CCl_4 to Wistar rats showed a significant increase in serum levels of urea, creatinine, and uric acid. Upgradation in serum creatinine levels is a key indicator of renal failure, and this range reflects the glomerular function [69]. The rise in the blood level of uric acid causes hyperuricemia, and it is a prognostic marker of inflammation that occurs at different parts of the body [70]. The administration of STE, Sm, and Sb showed a successful improvement in renal functions involving the serum levels of urea, creatinine, and uric acid in comparison with the rats administered DEN/AAF/CCl_4 alone. Similar results were reported [34, 71], in which, Sm

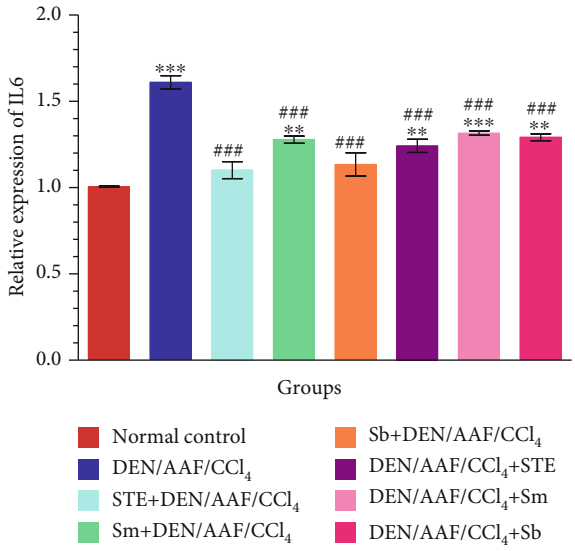


Figure 7: The effect of STE, Sm, and Sb on mRNA expression of IL-6 of rats given DEN/AAF/CC_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE+DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

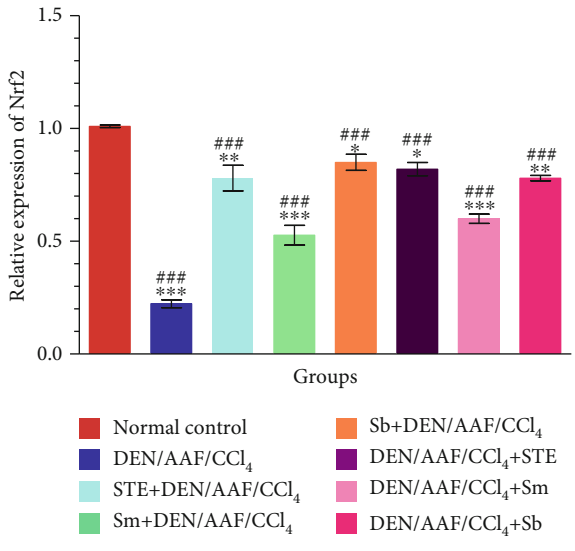


Figure 8: The effect of STE, Sm, and Sb on mRNA expression of Nrf2 of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE+DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

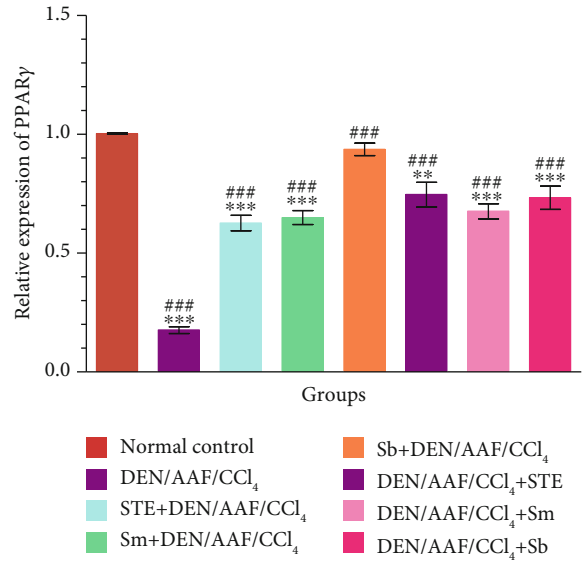


Figure 9: The effect of STE, Sm, and Sb on mRNA expression of PPARγ of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

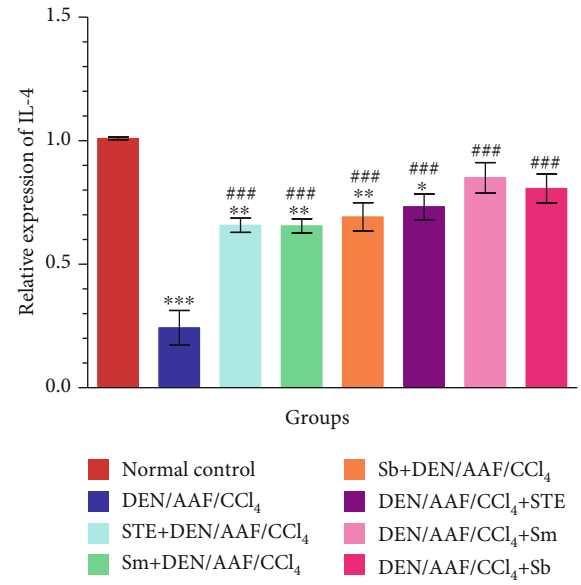


Figure 10: The effect of STE, Sm, and Sb on mRNA expression of IL-4 of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

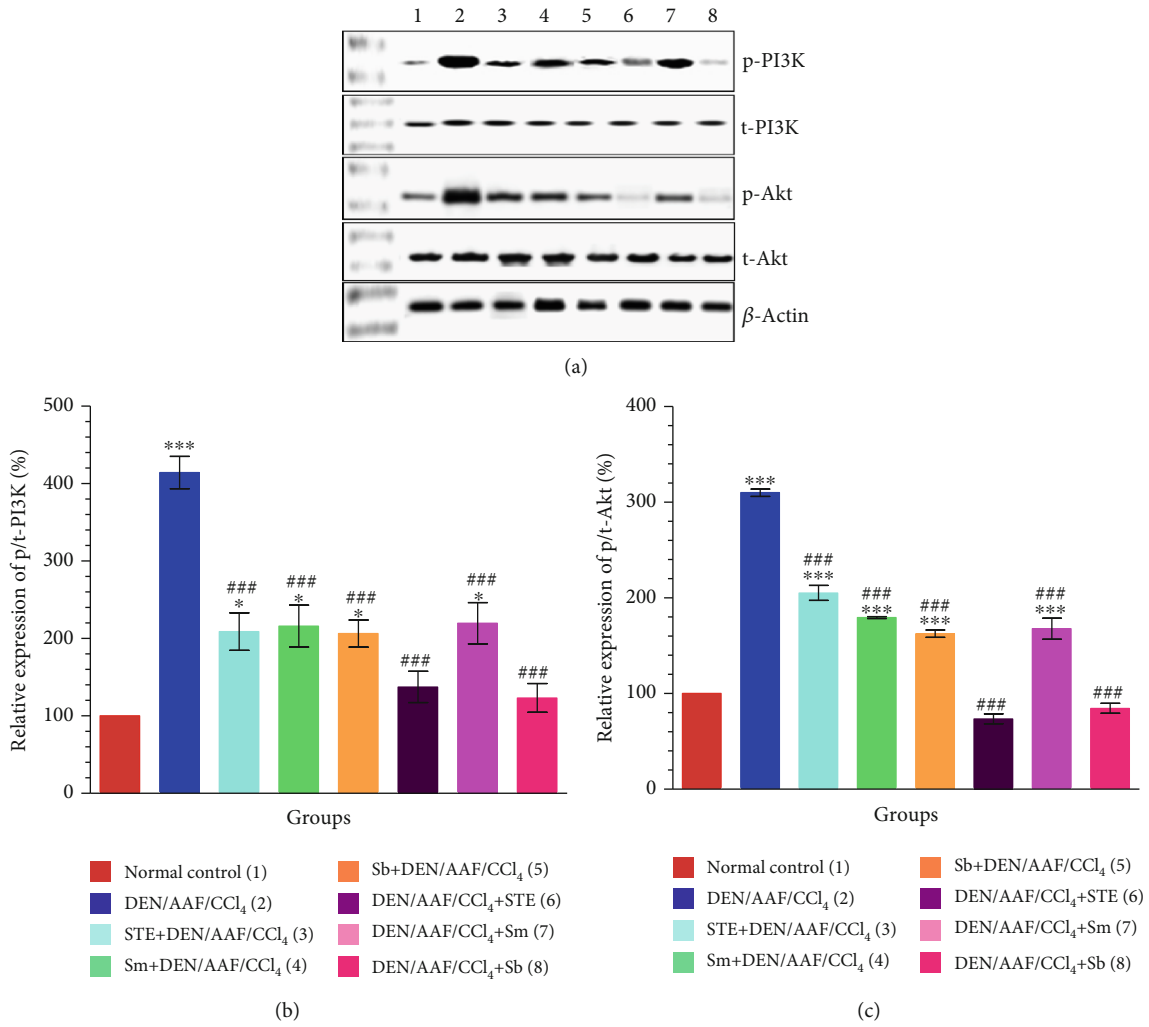


Figure 11: Effect of STE, Sm, and Sb on PI3K/Akt signaling pathway in kidney tissues of rats given DEN/AAF/CCl_4. Western blot assay was used to measure the expressions of p/t-PI3K (b) and p/t-Akt (c) proteins in kidney tissues of the experimental groups. Representative immunoblots for quantification of p/t-PI3K and p/t-Akt proteins were depicted in (a). Data are presented as mean values ± SEM with results from 3 independent biological repeats. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

and Sb showed an improvement in renal functions by reducing the serum levels of creatinine, urea, and uric acid.

These biochemical changes were supported by the histopathological analysis of the renal tissue exposed to DEN/AAF/CCl_4 showing dysplastic renal tubules and karyomegaly of the nuclei with more than one nucleolus as precancerous lesions. However, these pathological alterations were restored by STE, Sm, and Sb administration.

The presence of the long-chain polyunsaturated fatty acids in lipid composition makes the kidney vulnerable to damage that occurs by free radicals. The nitrosamine breakdown has been suggested as a way to generate ROS. Increased amounts of ROS induce somatic mutations in addition to neoplastic transformations [72]. A variety of processes in tumor cells including genetic instability and mutation progression, cellular proliferation, alterations in anticancer drug susceptibility, angiogenesis, and metastasis can be affected by intrinsic oxidative stress [73, 74]. The administration of DEN/AAF/CCl_4 resulted in a significant elevation of MDA activity with a decrease in the glutathione (free thiols), and its oxidized glutathione disulfide, implying a substantial redox reaction within the cell. GSH is a nonenzymatic antioxidant that reacts directly with ROS or acts as a coenzyme or cofactor in the detoxification mechanism [75]. In comparison to the normal control group, the drop in kidney content of GSH may be attributed to the direct conjugation with DEN and its metabolites with free or protein bound-SH groups and a significant reduction in the GPx activity was observed. It was reported that the administration of DEN induced inflammation and disturbed the redox cycle in the kidney [76]. Moreover, DEN/AAF/CCl_4 administration also decreased other antioxidant enzyme activities

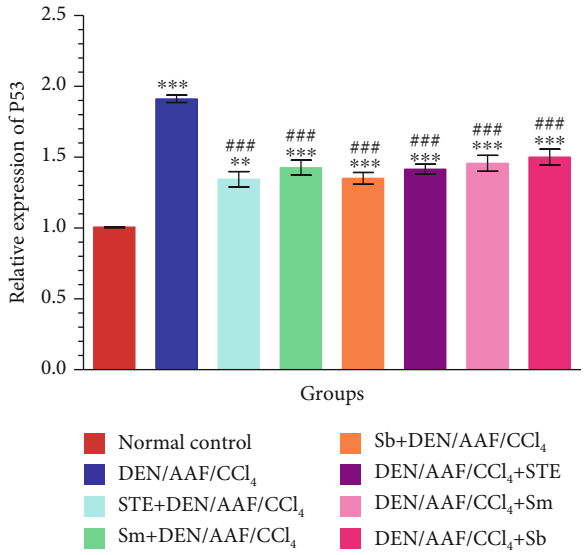


Figure 12: The effect of STE, Sm, and Sb on mRNA expression of p53 of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

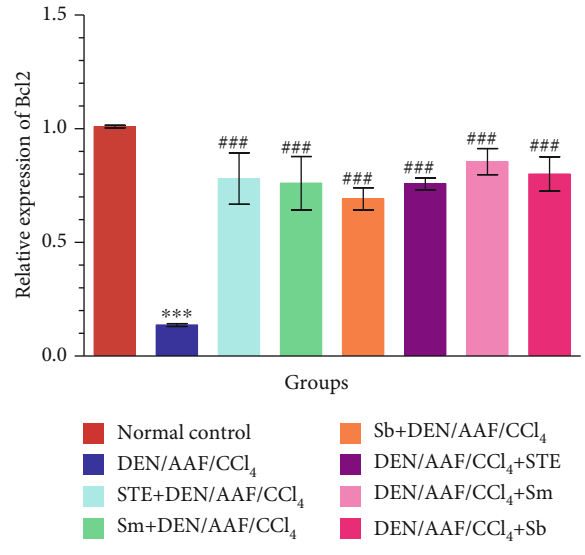


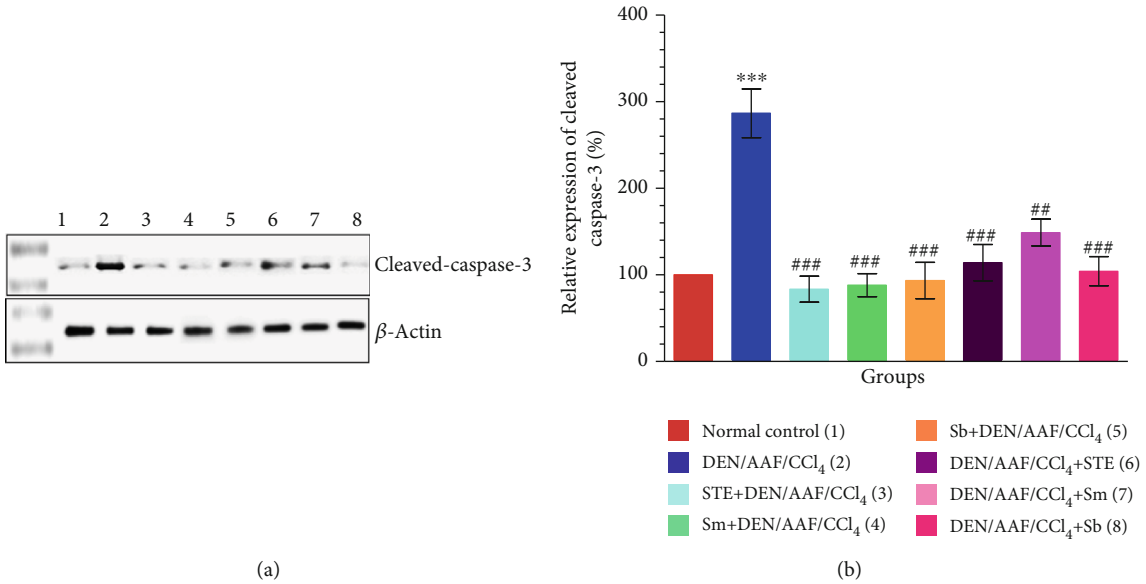
Figure 13: The effect of STE, Sm, and Sb on mRNA expression of Bcl-2 of rats given DEN/AAF/CCl_4. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE +DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

such as GST and GR. These results were strongly supported by previous studies which reported that DEN induction increased the level of MDA and reduced the kidney GSH content and the antioxidant enzymes such as SOD, GPx, GR, and GST [77, 78]. In contrast, STE, Sm, and Sb treatment significantly attenuated the elevated MDA level demonstrating that these treatments are effective in quenching free radicals. Furthermore, supplementation with STE, Sm, and Sb upregulated the activities of all renal antioxidant enzymes implying the nephroprotective effects of those treatments against DEN/AAF/CCl_4-induced oxidative stress.

The redox imbalance, induced by excess production of ROS, triggers the transfer of Nrf2 from the cytoplasm to the nucleus, where it regulates the expression of its downstream proteins, involving HO-1 and SOD-1/-2 [79]. Accordingly, the administration of DEN/AAF/CCl_4 resulted in a reduction in mRNA abundance of Nrf2 in the rat kidney tissues. On the other hand, the treatment with STE, Sm, and Sb succeeded in the activation of the production of Nrf2 which attains both activations of antioxidant machinery and inhibition of NF-κB mediated proinflammatory pathways.

The administration of DEN/AAF/CCl_4 activated the expression of the proinflammatory cytokine, IL-6, which may be under the direct transcriptional directive of NF-κB. This cytokine is important for inflammation, vascular permeability, and cell proliferation. Because of numerous proinflammatory cytokines, oxidative stress is also a major factor in the modulation of inflammation [80]. It also has a critical role in the development of kidney carcinoma. The redox system also plays an important role in the regulation of NF-κB and various genes involved in cell transformation, proliferation, and invasion. ROS and NF-κB have a complicated relationship in between. The blockage of the NF-κB pathway is a good approach to control carcinogenesis and cancer progression [81]. Normally, NF-κB is found in an inactive form in the cytoplasm, attached to one of a number of inhibitory molecules (IκBs), the most prevalent of which is IκBα. IκBα interacts with p50/p65 in the cytoplasm, forming an inactive complex. IκB kinase is activated by oxidative stress which phosphorylates IκB [82]. The phosphorylated IκBα is responsible for the transfer of the active NF-κB to the nucleus and interacts with κB sites on target genes to promote the oncogene transcription which controls multidrug resistance, apoptosis, invasion, and metastasis [83]. It has been proved that NF-κB competes with Nrf2 for transcription coactivator CREB binding protein (CBP) and can directly suppress the transcriptional level of Nrf2 [84]. Additionally, the N-terminal region of the p65 subunit of NF-κB physically associates with Keap1 and inhibits the Nrf2 pathway [85]. DEN/AAF/CCl_4 administration activated NF-κB which is very delicate to ROS. STE, Sm, and Sb supplementation reduced IL-6 levels and protected against DEN/AAF/CCl_4-induced renal carcinogenesis by inhibiting p65 and IκBα phosphorylation, indicating further inhibition of NF-κB by them (Figure 15).

Furthermore, comparing to the DEN/AAF/CCl_4-administered group, the supplementation of STE, Sm, and Sb increased the level of IL-4. The anti-inflammatory effects of



(a) (b)

FIGURE 14: Effect of STE, Sm, and Sb on cleaved-caspase-3 in kidney tissues of rats given DEN/AAF/CCl_4. Western blot assay was used to measure the expressions of cleaved-caspase-3 (b) protein in kidney tissues of the experimental groups. Representative immunoblots for quantification of cleaved-caspase-3 protein were depicted in (a). Data are presented as mean values ± SEM with results from 3 independent biological repeats. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were significant as compared with normal control rats, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were significant as compared with the DEN/AAF/CCl_4-administered group. STE+DEN/AAF/CCl_4, Sm+DEN/AAF/CCl_4, and Sb+DEN/AAF/CCl_4: groups orally treated with STE, Sm, and Sb, respectively, from the 1st week of DEN injection; DEN/AAF/CCl_4+STE, DEN/AAF/CCl_4+Sm, and DEN/AAF/CCl_4+Sb: groups orally treated with STE, Sm, and Sb, respectively, from the 16th week of DEN injection.

Sm and Sb have already been demonstrated in various reports against various inflammatory animal models [86, 87] which may be based on the Sm content of lipopolysaccharide, phorbol ester, ceramide, and okadaic acid [88].

The ligand-activated transcription factors, PPARs, have been revealed to play a major role in the transcription of many genes involved in various kidney physiological functions such as lipid metabolism, glucose homeostasis, and renal mineral control [89]. PPARγ plays a significant role in the control of inflammation *via* suppression of the activities of many transcription factors, including NF-κB and signal transducer and activator of transcription (STATs) [90, 91] involved in inflammation. PPARγ is considered a tumor suppressor by controlling cell differentiation, regulation of inflammation, and its ligands may inhibit angiogenesis by downregulating the vascular endothelial growth factor (VEGF) [92]. Moreover, the interaction between Nrf2 and PPARγ was investigated by various studies [25, 93] which have been proposed to suppress NF-κB [94]. The current work indicated that targeting the PPARγ pathway may be one of the prospective therapeutic strategies that may help in the treatment of renal cell carcinogenesis induced by DEN/AAF/CCl_4. The present data demonstrated that the treatment of STE, Sm, and Sb significantly activated the expression of PPARγ which may stop the production of downstream inflammatory mediators in the renal tissues of rat-administered DEN/AAF/CCl_4 (Figure 15).

The PI3K-Akt signaling is permanently active in different human malignancies, and the genetic alterations of the PI3K/Akt pathway are commonly found in kidney cancer where it plays a crucial role in tumor development and therapy resistance [31, 32, 95, 96]. Our study showed that DEN/AAF/CCl_4 exposure activated the PI3K/Akt signaling pathway. When PI3K is activated, it generates phosphoinositol triphosphate (PIP3) and other related lipid second messengers, which triggers Akt/protein kinase B (PKB). Activated Akt phosphorylates a number of cytoplasmic proteins and controls a number of important cellular functions. The mTOR protein subfamily is one of Akt's most significant downstream effectors [97]. Once mTOR is activated, it enhances proliferation and inactivates BAD which inhibits apoptosis and promotes cell survival [98]. Normally, this pathway is negatively regulated by the phosphatase and tumor suppressor phosphatase on chromosome 10 (phosphatase and tensin homolog (PTEN)) which dephosphorylates PIP3 and prevents Akt activation and functions as a negative regulator of PI3K/Akt signaling pathway [98] (Figure 15).

Taken together, there is a strong link between the PI3K/Akt signaling system and carcinogenesis, suggesting that inhibiting this pathway might be an effective kidney cancer therapy strategy. Therefore, the current study targeted this pathway as one of the most promising targets to explore safer and effective therapeutic strategies against kidney cancer. As shown above, the treatment with STE, Sm, and Sb either from the 1st week or from the 16th weeks of carcinogen induction significantly blocked the PI3K/Akt signaling pathway *via* inhibition of the protein levels of PI3K and Akt in DEN/AAF/CCl_4-administered rats (Figure 15).

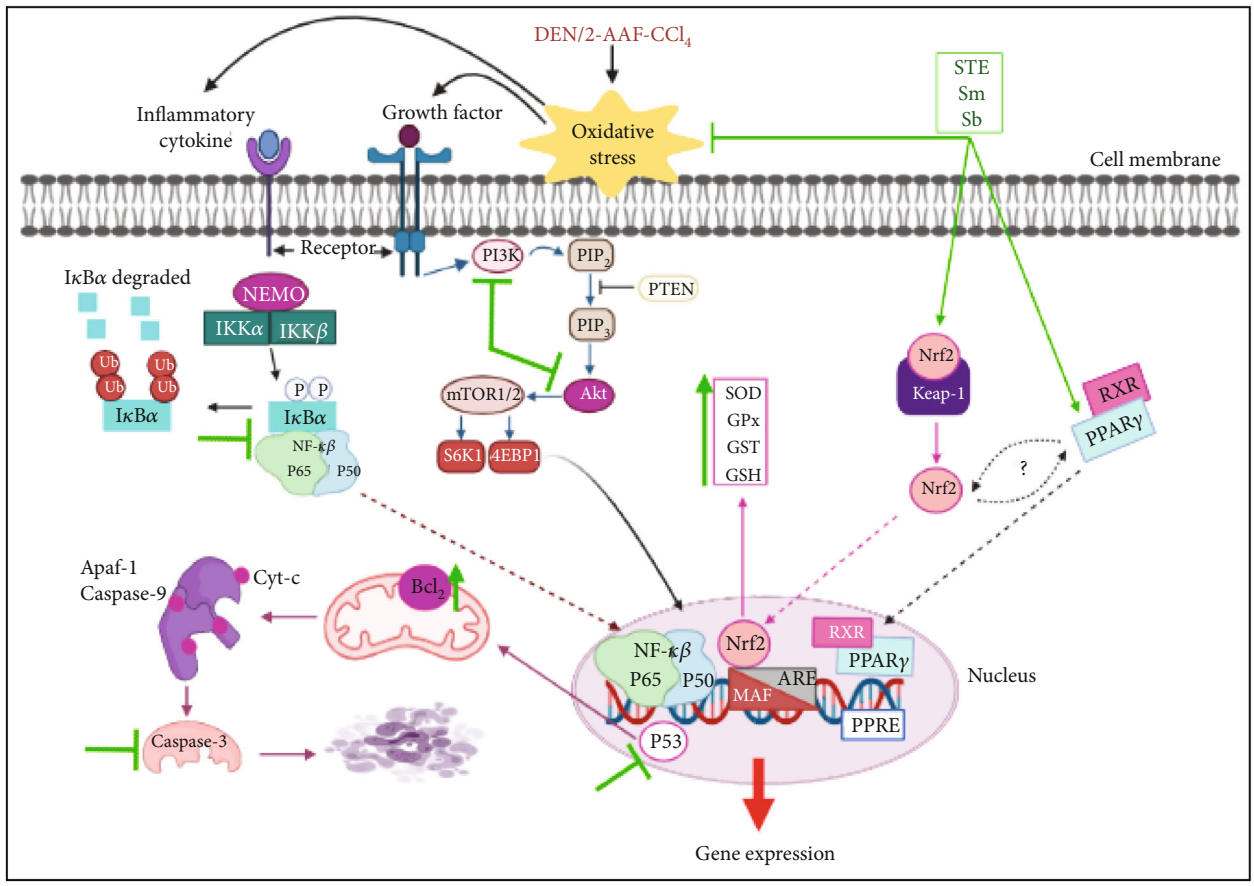


Figure 15: A proposed schematic diagram of the effective role of STE, Sm, and Sb on targeting NF-κB, Nrf2, PPARγ, PI3K/Akt, and apoptotic pathways of renal carcinogenesis.

High ROS exposure increases mitochondrial permeability transition and alters transmembrane potential, resulting in mitochondrial oxidative damage and increased ROS release into the cytoplasm [99]. In conjugation with mitochondrial apoptosis, proapoptotic molecules produced from mitochondria, involving cytochrome c, trigger the release of caspase-3 and caspase-9, which cleave particular substrate proteins, such as PARP, to activate damage of DNA [100]. Meanwhile, DEN/AAF/CCl_4 administration resulted in an imbalance in the expression of pro- and antiapoptotic Bcl-2 family members. ROS may be generated due to this imbalance and consequently activates caspase-8 and caspase-9 [101]. In a feedback loop, once caspase-8 is activated, it not only activates caspase-3 but also leads to mitochondrial apoptosis through cleaved Bid [102]. On the other hand, the treatment with STE, Sm, and Sb increased the expression of Bcl-2 while reduced the levels of p53 and cleaved-caspase-3, confirming that STE, Sm, and Sb may have an anticarcinogenic mechanism. This might be due to the modulation of oxidative stress-induced apoptosis.

According to the previous findings, STE is the most potent antikidney carcinogenic agent rather than Sm and Sb. This can be explained by the added oily part in STE which contains a higher amount of tocopherols particularly αtocopherol [103]. Tocopherols are one of the important antioxidants in seed oils that can regulate signal transduction, gene expressions, and cell function modification and prevent the risk of certain cancers [104].

5. Conclusions

Based on the above data, it can be reached to many suggestions and conclusions. First, STE, Sm, and Sb reduced oxidative stress and limited the release of free radicals through multiple mechanisms in addition to stabilization of the kidney function markers. Second, STE, Sm, and Sb regulated various transcription factors such as inhibition of proinflammatory cytokines, inhibition of PI3K/Akt signaling, activation of Nrf2 and PPARγ expressions, and suppression of apoptosis. Given these findings together, the present study proposed that STE, Sm, and Sb can exert potent antirenal carcinogenic effects. To discover the accurate mechanisms of these treatments in renal carcinogenesis, more molecular and cellular investigations are recommended.

Data Availability

All datasets analyzed and described during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Supplementary figure: original western blot for three repeats. *(Supplementary Materials)*

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Research Article

One-Two Punch Therapy for the Treatment of T-Cell Malignancies Involving p53-Dependent Cellular Senescence

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T-cell malignancies are still difficult to treat due to a paucity of plans that target critical dependencies. Drug-induced cellular senescence provides a permanent cell cycle arrest during tumorigenesis and cancer development, particularly when combined with senolytics to promote apoptosis of senescent cells, which is an innovation for cancer therapy. Here, our research found that wogonin, a well-known natural flavonoid compound, not only had a potential to inhibit cell growth and proliferation but also induced cellular senescence in T-cell malignancies with nonlethal concentration. Transcription activity of senescence-suppression human telomerase reverse transcriptase (*hTERT*) and oncogenic *C-MYC* was suppressed in wogonin-induced senescent cells, resulting in the inhibition of telomerase activity. We also substantiated the occurrence of DNA damage during the wogonin-induced aging process. Results showed that wogonin increased the activity of senescence-associated β-galactosidase (SA-β-Gal) and activated the DNA damage response pathway mediated by p53. In addition, we found the upregulated expression of BCL-2 in senescent T-cell malignancies because of the antiapoptotic properties of senescent cells. Following up this result, we identified a BCL-2 inhibitor Navitoclax (ABT-263), which was highly effective in decreasing cell viability and inducing apoptotic cell death in wogonin-induced senescent cells. Thus, the "one-two punch" approach increased the sensibility of T-cell malignancies with low expression of BCL-2 to Navitoclax. In conclusion, our research revealed that wogonin possesses potential antitumor effects based on senescence induction, offering a better insight into the development of novel therapeutic methods for T-cell malignancies.

1. Introduction

T-cell malignancies are a highly heterogeneous group of disorders [1, 2], which have been widely acknowledged to be typically aggressive and resistant to chemotherapy and have poor prognosis [3–5]. T-cell malignancies commonly consist of two categories, T-cell acute lymphoblastic leukemia (T-ALL) and T-cell non-Hodgkin lymphoma (T-NHL), both of which have similar clinical therapies and poor prognosis [6]. Due to the varied morphology of subtypes and being highly heterogeneous, the diagnosis and treatment of this disease have become a great challenge [7]. Although the standard first-line treatment of T-cell malignancies is a CHOP regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone), which is still the representative chemotherapy [8], most patients still have a poor prognosis with an ORR of 65.8% because of rapid relapse [8]. Relapse and cytotoxicity always become an unavoidable problem for

several patients. And just a few of patients who achieve remission are suitable for hematopoietic stem cell transplantation [9, 10]. Thus, it is urgent to find more effective therapy options for patients who fail to respond to chemotherapy or relapse after chemotherapy.

Cellular senescence is a complex stress response accompanied by permanent cell cycle arrest and inhibition of proliferation, which is induced by DNA damage, telomere shortening, ionizing radiation, or oncogene [11, 12]. The typical characteristics of senescent cells are large and flat morphology and positive expression of senescence-associated β-galactosidase. Moreover, senescence-associated heterochromatin foci (SAHF) are also a biomarker of senescent cells, which are formed by nuclei as the heterochromatin structures [13]. In hematologic malignancies, cells abnormally overexpress human telomerase reverse transcriptase and decrease the expression of senescence-promoting genes, which contribute to immortalization of cancer cells [14]. Previous reports have shown that *hTERT* also exerts antisenescent effect through telomerase- and telomere length-independent mechanisms [15]. As a novel therapeutic concept, the use of drugs which can accumulate DNA damage and trigger cellular senescence in cancer cells can provide a new insight into treating hematologic malignancies. However, induced cellular senescence alone cannot achieve the ideal therapeutic effect. Many chemotherapy drugs can induce cellular senescence and alert the tumor microenvironment, which is a double-edged sword for cancer therapy [11, 16]. Although senescent cells induced by agents had vulnerabilities such as persistent growth inhibition, persisting existence of senescent cells can promote cancer progression and invasion in B-cell lymphoma and T-cell acute lymphoblastic leukemia [17]. Thus, exploiting vulnerabilities and removing senescent cells are important for the second step of senescence-focused cancer therapies [18–20]. As the characteristic of resistance to apoptosis in senescent cells, senolytics are identified to eliminate aging cells including quercetin/dasatinib, pan-BCL inhibitors through the inhibition of the BCL-2 family of proteins (BCL-2, BCL-xL, and BCL-w) [21, 22]. Therefore, a one-two punch cancer therapy may be regarded as a potentially therapeutic strategy [20].

Nowadays, DNA-damaging drugs are widely used in cancer therapy, which not only induce cell death but also activate cellular senescence [23]. DNA damage response (DDR) plays a core role in senescence. The biomarker of DDR is the activation of H2A.X, which depends on the phosphorylation at Ser139 (γ-H2AX). As a main factor of DDR, p53 acts as a protagonist during the process of aging and tumor suppression [24]. A previous study reported that in adult acute lymphoblastic leukemia, the alterations of *TP53*, the most important tumor suppressor gene, including deletions, sequence mutations, and defect in its expression, result in an adverse prognosis [25]. As the activation of DNA damage, p53 is activated through phosphorylation on serine-15 and serine-20 by ataxia-telangiectasia mutated (ATM) or ataxia-telangiectasia Rad3-related (ATR) and the checkpoint kinase 2 (CHK2), respectively [26, 27]. And then, the interaction of MDM2 and p53 is impaired, promoting the accumulation and stabilization of p53 in response to DNA damage activation [28]. After activation of p53, the downstream effectors like p21 regulate different cell responses such as cell cycle arrest and senescence.

Wogonin (5,7-dihydroxy-8-methoxy-2-phenyl-4h-1-benzopyran-4-one) is a type of flavonoid extracted from *S. baicalensis* Georgi, which has prominent biological activities including anti-inflammatory, antiviral, and anticancer activities [29, 30]. However, cytostatic concentrations of wogonin have other characteristics such as inducing cellular senescence, which can combine with senolytics as a new therapeutic regimen. Here, we first investigated wogonin-induced cellular senescence and found the suppression of telomerase activity in wogonin-treated senescent cells. Moreover, DNA damage response mediated by p53 was involved in cellular senescence induced by wogonin. Finally, we also clarified that the combination sequential treatment of wogonin and Navitoclax, a BCL-2 inhibitor, could obtain better effect and become a novel strategy in T-cell malignancies.

2. Materials and Methods

2.1. Compounds and Reagents. Wogonin (purity ≥ 99%) is isolated from *S. baicalensis* Georgi according to previously reported protocols [31]. Wogonin was dissolved in dimethyl sulfoxide (DMSO) as a stock solution (100 mM) and stored at -80°C. The solution was freshly diluted with basal medium to designated concentrations, and the final concentration of DMSO will not be over 0.1%. Cells treated with the highest concentration of DMSO were used as the control in corresponding experiments. Navitoclax (CSN12932) was purchased from CSNpharm (Chicago, USA). Navitoclax powder was dissolved with DMSO to 10 mM and stored at -20°C. Primary antibodies against β-actin, GAPDH, trimethyl-histone H3-K9, BCL-2, hTERT, c-Myc, caspase-3, active caspase-3, p27, Bax, CHK2, cyclin D1, cyclin E1, CDK4, CDK6, HRP goat anti-mouse IgG (H+L), and HRP goat anti-rabbit IgG (H+L) were obtained from ABclonal Technology (Wuhan, China). Phospho-CHK2 (T68), phospho-p53 (Ser15), p21, and phospho-histone H2A.X (γ-H2A.X) were obtained from Cell Signaling Technology (Danvers, MA, USA). p53, p16, BCL-xL, Bim, and PARP-1 were obtained from Proteintech (CA, USA).

2.2. Cell Culture. The human T-ALL cell line (Jurkat cells), human T-cell lymphoma cell lines (Hut-102 and Hut-78 cells), and HEK293T cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry & Cell Biology. All the cells were expanded and stored in liquid nitrogen upon receipt, and each aliquot was passaged for fewer than 25-30 times in our laboratory. Jurkat, Hut-102, and Hut-78 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, USA). HEK293T cells were cultured in DMEM medium (Gibco, Carlsbad, USA). Medium was supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA), 100 U/mL of penicillin, and 100 U/mL of streptomycin in a humidified environment (Thermo Fisher Scientific) with 5% CO_2 at 37°C.

2.3. Cell Viability Assay. Cell proliferation of cells was detected using Cell Counting Kit-8 (KeyGen Biotech, Nanjing, China) according to the manufacturer's instruction. Briefly, cells (1×10^4 cells/well) were seeded into a 96-well plate in 100 μL medium and treated with wogonin at different concentrations for 24 h. Each group consisted of six parallel wells. Then, 10 μL of CCK8 solution was added to each well, and cells were incubated at 37°C for 1–4 h. The absorbance was measured at 450 nm using an Automated Microplate Reader ELx800 (BioTek). The cell survival rate (%) was calculated using the following equation: survival rate (%) = $(OD_{treated} - OD_{blank})/(OD_{standard} - OD_{blank}) \times 100$ %.Inhibition rate (%) = 100% − survival rate (%).

2.4. Quantitative Real-Time RT-qPCR. RT-qPCR was performed according to the manufacturer's instructions [32].

The primer sequences were as follows:

GAPDH

Forward 5′-GCAGGGGGGAGCCAAAAGGG-3′

Reverse 5′-TGCCAGCCCCAGCGTCAAAG-3′

P16

Forward 5′-GATCCAGGTGGGTAGAAGGTC-3′

Reverse 5′-CCCCTGCAAACTTCGTCCT-3′

P21

Forward 5′-TGTCCGTCAGAACCCATGC-3′

Reverse 5′-AAAGTCGAAGTTCCATCGCTC-3′

P27

Forward 5′-AACGTGCGAGTGTCTAACGG-3′

Reverse 5′-CCCTCTAGGGGTTTGTGATTCT-3′

C-MYC

Forward 5′-GGCTCCTGGCAAAAGGTCA-3′

Reverse 5′-CTGCGTAGTTGTGCTGATGT-3′

hTERT

Forward 5′-TATGTCACGGAGACCACGTT-3′

Reverse 5′-GTGCTGTCTGATTCCAATGC-3′

2.5. Flow Cytometric Analysis of Apoptosis. Apoptosis was evaluated using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's instruction. Cellular fluorescence was performed by flow cytometric analysis using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Our data was analyzed by using FlowJo software.

2.6. Cell Cycle Analysis. Cells were collected and washed with PBS and then fixed with 70% ethanol for 2 h at 4°C. After fixation, the cells were washed with PBS buffer and stained with PI for 30 min. The fluorescence was detected by flow cytometry. The cell cycle was analyzed and quantified using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle was determined by the PI fluorescence signal peak versus the integral which was analyzed by using ModFit LT 4.0 software (Becton Dickinson, San Jose, CA, USA).

2.7. CFSE Assay. CFSE-labeled cells (CellTrace CFSE Proliferation Kit, Invitrogen) were cultured with wogonin for the indicated time, cells were harvested, and cell proliferation capacity was determined by using a flow cytometer.

2.8. Cell Staining of Senescence-Associated β-Galactosidase (SA-β-Gal). SA-β-Gal activity was measured by using a β-Galactosidase Staining Kit (Yeasen, Shanghai, China) according to the standard protocol. The stained cells were observed and photographed under the microscope (Carl Zeiss, Germany). Cells were considered positive when the cytoplasm was stained with SA-β-Gal. Total numbers of cells and SA-β-Gal-positive cells were counted for five fields per well. The ratios of SA-β-Gal-positive cells were calculated compared to the total cell numbers per area.

2.9. Flow Cytometric Detection of SA-β-Gal. For flow cytometry-based detection of senescence, the fluorogenic substrate 5-dodecanoylaminofluorescein di-beta-d-galactopyranoside (C_{12}FDG) was used as described previously [33]. According to the manufacturer's instructions, cells were incubated with 33 μM C_{12}FDG (ImaGene Green C_{12}FDG lacZ Gene Expression Kit) at 37°C for 30 min. Then, cells were washed with PBS and measured immediately with a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Data were analyzed using FlowJo software. SA-β-Gal activity was estimated using the relative mean fluorescence intensity of the cell population.

2.10. Western Blotting Analysis. Whole cell proteins were collected after the treatment with wogonin and were extracted by using RIPA buffer containing protease/phosphatase inhibitors on ice for 50 min. Then, cell lysates were clarified by centrifugation at 14,000 rpm (5430R; Eppendorf, Hamburg, Germany) for 25 min at 4°C. Concentrations of proteins were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Protein extracts were equally loaded on 8%-12% SDS-polyacrylamide gels and then transferred onto the PVDF membranes (Millipore, Boston, MA, USA). Membranes were blocked with 3% BSA in PBS at room temperature for 1 h and then incubated with primary antibodies overnight at 4°C. After that, the membrane was incubated with an HRP goat anti-rabbit IgG (H+L) or HRP goat anti-mouse IgG (H+L) secondary antibody for 1 h at room temperature. Chemiluminescence detection was performed by using ECL reagents (Thermo Fisher Scientific, USA) upon using the AI600 imaging system (GE Healthcare, Pittsburgh, PA, USA).

2.11. Immunofluorescence. Cells were collected and seeded onto glass coverslips. Then, the cells were fixed in the ice-cold methanol for 10 min and incubated with 0.15% (*v*/*v*) Triton X-100 for 20 min. After the cells were blocked with 3% BSA at room temperature for 1 h, the cells were incubated with primary antibodies overnight at 4°C. Following this, the cells were stained with an Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Becton Dickinson) for 1 h and DAPI (Beyotime Biotechnology, Shanghai, China) for 5 min. Cells were observed under a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan).

2.12. RNA Interference. For RNA interference by lentiviral vectors, *P53* shRNA constructs and a negative control

construct created in the same vector system (PLV3ltr-puro-U6) were purchased from Corues Biotechnology. A *P53* shRNA construct and a negative control were transfected into HEK293T cells together with the Lentiviral Mix and HG Transgene™ Reagent according to the manufacturer's instruction of the Lentiviral Packaging Kit (Yeasen, 41102ES20) for 48 h. After this, the virus supernatant was harvested. Then, the virus supernatant infected target cells. 2 μg/mL puromycin was added to screen for expressing shRNA construct cells.

2.13. Statistical Analysis. All results were expressed as the mean ± standard error of the mean (SEM). The data shown were obtained from triplicate independent parallel experiments. Statistical analyses were performed by using one-way analysis of variance (ANOVA), post hoc intergroup comparisons, and Tukey's test. Two-tailed Student's *t*-test was used to determine differences between groups. $^{*}p < 0.05$ was considered statistically significant, $^{**}p < 0.01$ very significant, and $^{***}p < 0.001$ highly significant. Statistical analyses were performed using GraphPad Prism 6.0 Software (GraphPad, San Diego, CA).

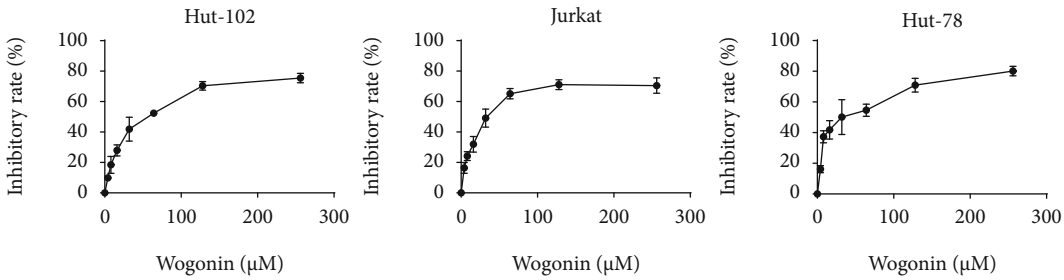
3. Results

3.1. Wogonin Induced Growth Inhibition but Not Apoptosis in Hut-102 and Jurkat Cells. In order to investigate the growth suppression effects of wogonin on T-cell malignancy cells, the growth inhibition rates of cells were detected by the CCK8 assay firstly. We performed our studies using T-NHL cell lines (Hut-102 and Hut-78) and the T-ALL cell line (Jurkat). Cells were treated with various concentrations of wogonin (0–256 μM) for 24 h. As shown in Figure 1(a), wogonin inhibited cell proliferation in a concentration-dependent manner. To investigate whether low concentration of wogonin still has proliferation inhibition on T-cell malignancies for a long-time exposure, we chose 20 μM as the treatment concentration. 20 μM wogonin inhibited cell proliferation in T-cell malignancies notably, as the decreased fluorescence intensity in CFSE-labeled cells was prevented by wogonin (Figures 1(b) and 1(c)). However, 20 μM wogonin did not induce significant apoptosis in Hut-102 and Jurkat cells after treatment for 1, 3, 5, and 7 d, but in Hut-78 cells, apoptosis rates showed a slight increase (Figure 1(d)). It is concluded that wogonin suppressed growth and proliferation of human T-cell malignancy cells.

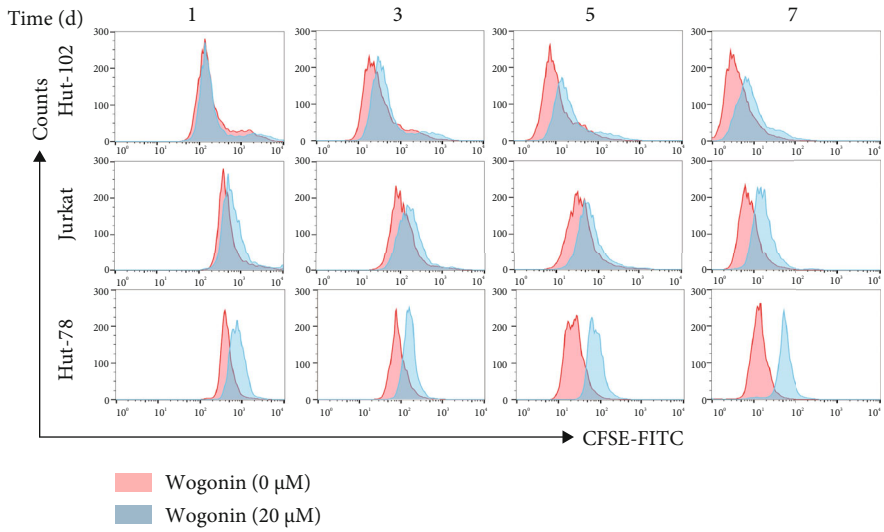
3.2. Wogonin Induced Cellular Senescence in Hut-102 and Jurkat Cells but Not Hut-78 Cells. A previous study has confirmed that small-molecule compounds can induce the cellular senescence in a few of cases [34]. It is a viable therapeutic option named one-two punch therapy to induce senescence of cancer cells and then remove them [19]. In order to validate the one-two punch therapy for the treatment of T-cell malignancies, firstly we identified whether wogonin could induce cellular senescence in these T-cell malignancy cells. C_{12}FDG and senescence-associated β-galactosidase staining test were adopted to assess the cellular senescence in Hut-102, Jurkat, and Hut-78 cells treated with 20 μM wogonin for 5 d. Higher SA-β-Gal activity measured by C_{12}FDG using flow cytometry was observed in wogonin-treated Hut-102 and Jurkat cells (Figures 2(a) and 2(b)). Moreover, more positive β-galactosidase-stained cells were found in wogonin-treated Hut-102 and Jurkat cells compared with control cells (Figures 2(c) and 2(d)). Morphologically, the senescent cells were enlarged and flattened compared with control cells (Figures 2(c) and 2(d)). Interestingly, cellular senescence was not observed in Hut-78 cells after wogonin treatment for 5 d (Figures 2(a) – 2(d)). Overall, these results showed that low concentration of wogonin (20 μM) could induce cellular senescence in Hut-102 and Jurkat cells.

3.3. Wogonin-Induced Cell Cycle Arrest Was Accompanied by Cellular Senescence. Cell cycle blockage is a well-known feature of cellular senescence, which contributes to permanent growth arrest in senescent cells [35, 36]. Thus, the effects of wogonin on cell cycle progression were measured in Hut-102 and Jurkat cells treated with 20 μM wogonin for 5 d. Results showed that the proportion of cells increased in the G_1 phase and decreased in the S phase compared to the untreated cells (Figures 3(a) and 3(b)). The CDK4/6-cyclin D1 complex plays a key role in promoting transition of the G_1 to S phrase, and the CDK2-cyclin E1 complex, the downstream of the CDK4/6-cyclin D1 complex, promotes cells to enter the S phrase through the restriction point [37]. Western blots indicated that wogonin decreased the expression of cyclin D1, CDK4, and CDK6, while the cyclin E1 expression was stable (Figures 3(c) and 3(d)). Furthermore, the increased expression of p16,p21, and p27 may contribute to cell cycle blockage in senescent cells [38, 39]. RT-PCR results showed that wogonin increased the mRNA level of *P21* by over 20-fold in senescent Hut-102 and Jurkat cells, while the mRNA level of *P27* did not change in those two cell lines (Figure 3(e)). The mRNA level of *P16* was slightly increased in Jurkat cells but not in Hut-102 cells (Figure 3(e)). All these data demonstrated that wogonin arrested the cell cycle at the checkpoint of G_0/G_1, which was correlated with cellular senescence in T-cell malignancy cell lines including Hut-102 and Jurkat.

3.4. Wogonin Suppressed Telomerase Activity by Inhibition of hTERT and c-Myc in Wogonin-Induced Senescent Cells. Telomere length affects cell life, and when the telomere shortens to a certain extent, cell senescence occurs [40]. Previous studies have shown that telomerase activity affects telomere length [41]. As a main component of telomerase, human telomerase reverse transcriptase is overexpressed in many malignant tumors including hematologic malignancies [42, 43]. c-Myc, a transcription factor, binds to the promoter region of *hTERT* and drives overexpression of *hTERT* in cancer cells, resulting in the activation of telomerase [44]. In order to verify whether wogonin induced cellular senescence involved in inhibiting telomerase activity, we first detect the mRNA levels of *C-MYC* and *hTERT*. Results showed that the mRNA expression of *C-MYC* and *hTERT* were decreased in Hut-102 and Jurkat cells after treatment with 20 μM wogonin for 5 d (Figure 4(a)). By the way, we also investigate the protein expression of c-Myc and hTERT using western blot,



(a)



(b)

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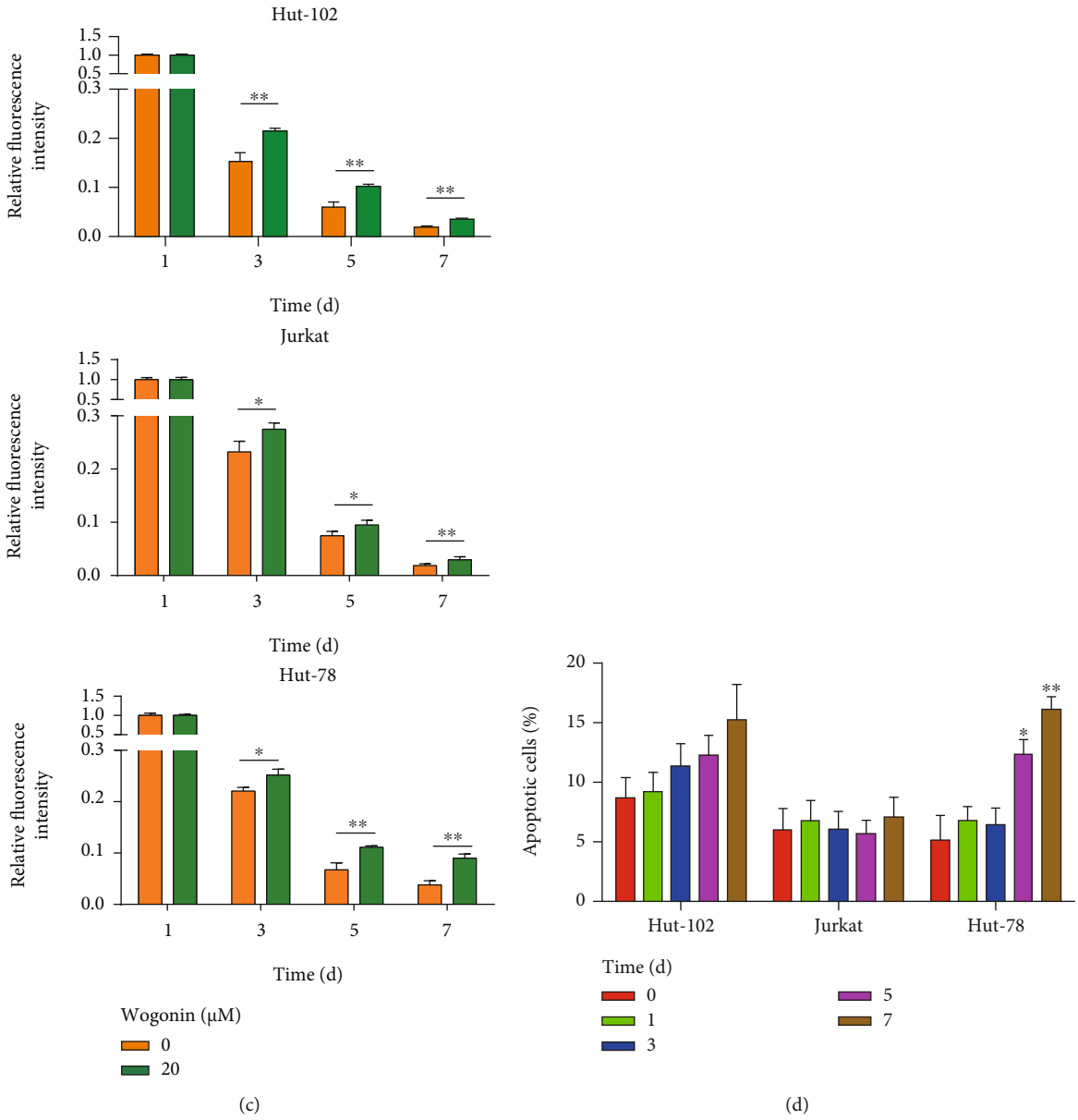


Figure 1: Wogonin induced growth inhibition but not apoptosis in Hut-102 and Jurkat cells. (a) Hut-102, Jurkat, and Hut-78 cells were exposed to wogonin at indicated concentrations (0-256 μM), respectively. The inhibitory rate of cell viability was measured by the CCK8 assay after 24 h. (b) Hut-102, Jurkat, and Hut-78 cells were treated with or without 20 μM wogonin; then, the cells were labeled with CFSE for 1, 3, 5, and 7 d, respectively. Results of CFSE expression were analyzed by flow cytometry. (c) Quantification of CFSE expression. Ordinate represents relative changes of fluorescence intensity (GEOmean). Significant difference represents the fluorescent intensity between the control group and wogonin group in the same day. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (d) Hut-102, Jurkat, and Hut-78 cells were treated with 20 μM wogonin for the indicated times (0, 1, 3, 5, and 7 d). Then, the cell apoptosis rates were analyzed via Annexin V/PI staining by flow cytometry. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group.

and results showed that wogonin decreased the expression of c-Myc and hTERT in Hut-102 and Jurkat cells after treatment for 5 d (Figures 4(b) – 4(e)). Thus, we concluded that wogonin could inhibit telomerase activity in senescent cells.

3.5. Wogonin Induced Senescence-Associated Heterochromatin Foci in Senescent Cells. Cell senescence is accompanied by the change of chromosome structure, especially the phenomenon of heterochromatin foci, which is the biological marker of cell senescence [13, 45]. In order to determine whether wogonin induced SAHF in senescent cells, Hut-102 and Jurkat cells were exposed to 20 μM wogonin for 5 d and formation of DNA agglutination points appeared (Figure 5(a)). The ratios of agglutination point-positive cells were 38.00% ± 5.10% and 44.22 ± 11.09% in wogonin-treated Hut-102 and Jurkat cells, respectively (Figure 5(b)). Next, we verified the molecular marker of SAHF named H3K9me3 by western blots and under a laser scanning confocal microscope. Results of

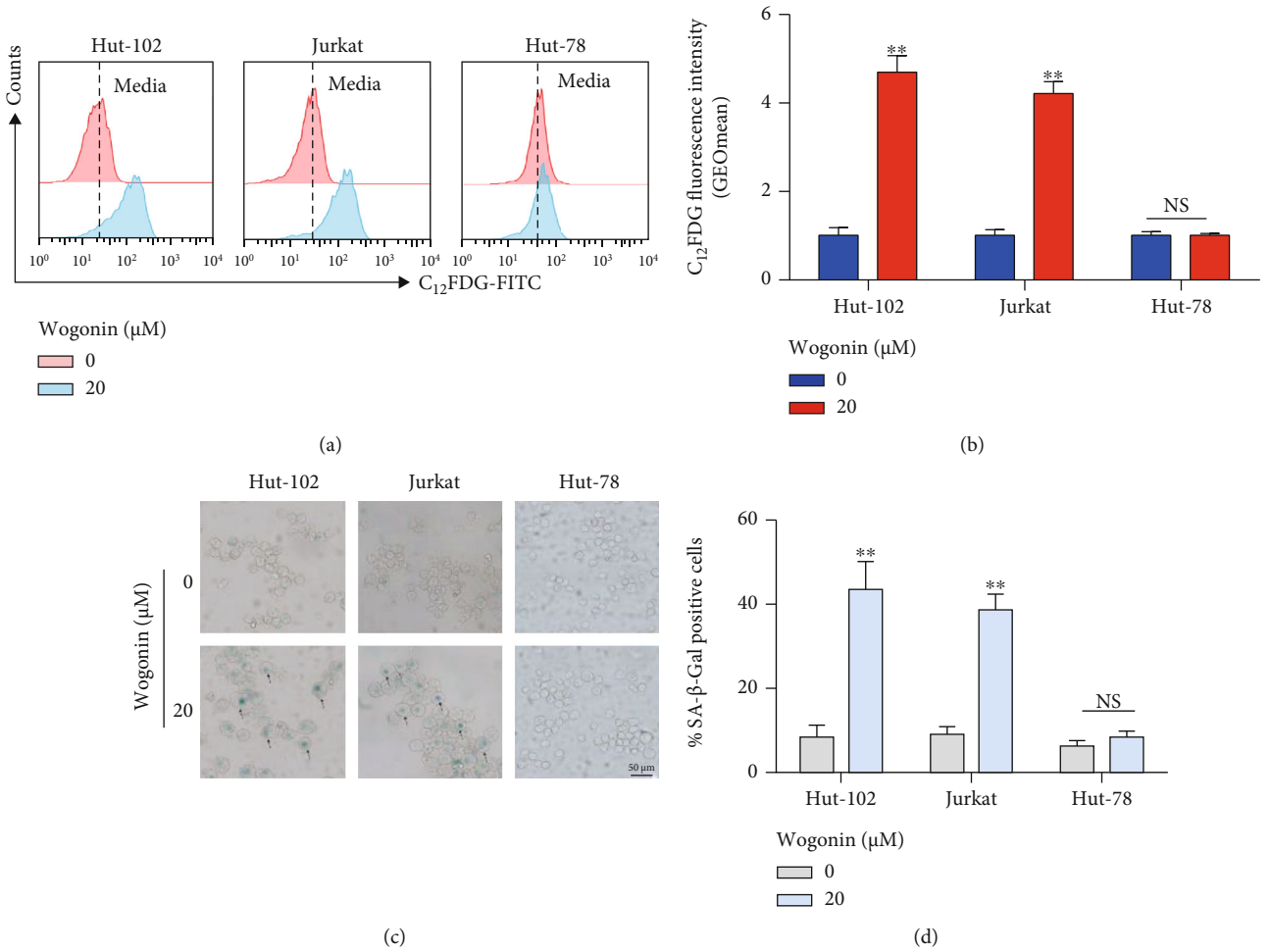


Figure 2: Wogonin induced cellular senescence in Hut-102 and Jurkat cells but not Hut-78 cells. (a) Cells (Hut-102, Jurkat, and Hut-78 cells) were treated with or without 20 μM wogonin for 5 d. SA-β-Gal activity was measured by C_{12}FDG fluorogenic substrate of SA-β-galactosidase using flow cytometry. Representative peak graphs from one of the three independent experiments, illustrating C_{12}FDG fluorescence. (b) Quantification of SA-β-Gal activity measured by C_{12}FDG fluorescence. The dark blue histogram represents control cells, and the red histogram represents cells that were cultured in the presence of 20 μM wogonin. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (c) SA-β-Gal activity was measured by SA-β-Gal staining in cells (Hut-102, Jurkat, and Hut-78 cells) treated with or without 20 μM wogonin for 5 d. Representative images from one of the three independent experiments. Representative pictures are shown (magnification, ×200). (d) Quantification of SA-β-Gal activity measured by cell staining. The grey histogram represents control cells, and the blue histogram represents cells that were cultured in the presence of 20 μM wogonin. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group.

western blots showed that the expression of H3K9me3 was upregulated after the treatment with wogonin for 3, 5, and 7 d in Hut-102 cells; meanwhile, H3K9me3 was also upregulated after the treatment with wogonin for 5 and 7 d in Jurkat cells (Figures 5(c) and 5(d)). Immunofluorescence analysis showed that the fluorescence intensity of H3K9me3 increased and colocated with the agglutination point in Hut-102 and Jurkat cells (Figure 5(e)).

3.6. Wogonin Induced Cellular Senescence through p53-Mediated DNA Damage Response. Many studies have shown that anticancer agent-induced cellular senescence is mediated by the DNA damage pathway [24, 46]. To figure out the mechanism of cellular senescence induced by wogonin, we first performed western blot experiments to detect the protein expression of the DNA damage marker protein γ-H2AX in Hut-102 and Jurkat cells. We found that the γ-H2AX expression increased obviously after treatment with 20 μM wogonin for 5 d and 7 d (Figure 6(a)), while in Hut-78 cells, the level of γ-H2AX was increased slightly with the treatment with 20 μM wogonin for 7 d (Figure S1a). In line with that, results of the immunofluorescence experiment showed that the fluorescence intensity of γ-H2AX was enhanced and colocalized with the nucleus after treatment with 20 μM wogonin in three T-cell malignancies (Figure 6(b), Figure S1b). The enhanced expression of γ-H2AX proved the wogonin-induced DNA damage response. We then analyzed the activation level of the CHK2/p53/p21 axis, which is involved in DNA damage response [47]. Western blot indicated that the level of p-

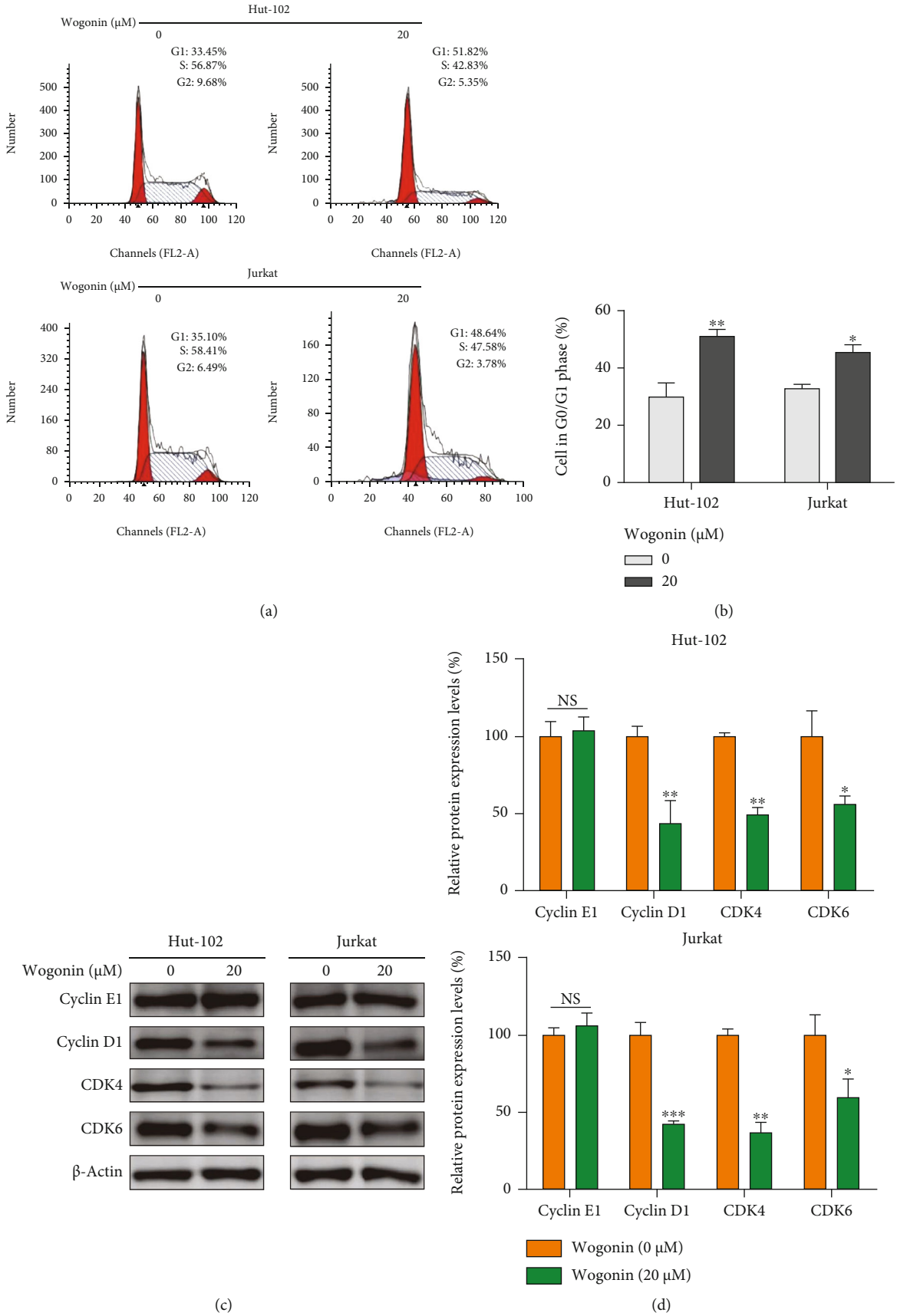


Figure 3: Continued.

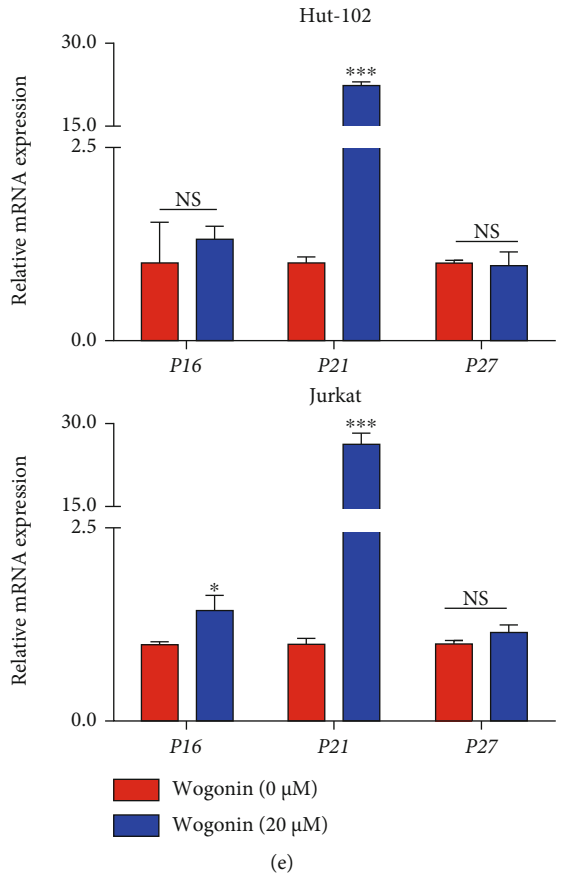


Figure 3: Wogonin-induced cell cycle arrest was accompanied by cellular senescence. (a) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. Representative cell cycle was performed by PI staining and analyzed by flow cytometry. (b) The percentages of cells in the G_0/G_1 phases of the cell cycle following 20 μM wogonin treatment for 5 d are shown. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the control group. (c) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. Western blot analyses of cell cycle regulatory proteins cyclin E1, cyclin D1, CDK4, and CDK6. β-Actin was used as loading controls. (d) Relative protein expression levels of cyclin D1, cyclin E1, CDK4, and CDK6 were determined. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the control group. (e) The relative mRNA levels of *P16*, *P21*, and *P27* in control and wogonin-treated Hut-102 and Jurkat cells. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with the control group.

CHK2 (Thr68) was gradually upregulated in wogonin-treated Hut-102 and Jurkat cells in a time-dependent manner, as well as p-p53 (Ser15) and p21. However, the expression of CHK2, p53 and p16, and p27 was stable in Hut-102 and Jurkat cells after treatment with wogonin (Figures 6(c) and 6(d)). But in Hut-78 cells, a cell line lacking p53 expression resulted from a homozygous point mutation at codon 196 (CGA-TGA) [48] and the level of CHK2 and p-CHK2 (Thr68) was increased after treatment with wogonin for 7 d; meanwhile, p21 was slightly increased and p16 and p27 expression was not changed (Figure S1c, d). However, the change degree of p21 in Hut-78 cells is generally slighter than that in Hut-102 and Jurkat cells (Figure 6(d), Figure S1d).

To further investigate whether wogonin-induced senescence depended on the functionally intact p53, we performed our studies using lentiviral-transfected *P53* shRNA and negative control (NC) in wt-p53 Hut-102 cells. Transfected efficiency of *P53* shRNA is shown in Figures 6(e) and 6(f). Flow cytometric analysis showed that the activity of SA-β-Gal alleviated in cells transfected with *P53* shRNA compared with the negative control group after wogonin treatment (Figures 6(g) and 6(h)). Moreover, western blot showed that wogonin-induced expression of p-p53 (Ser15) and p21 was inhibited by *P53* knockdown (Figures 6(e) and 6(f)). As a result, DNA damage response is involved in wogonin-induced cellular senescence, and the participation of p53 is critical for the cellular senescence induced by wogonin.

3.7. Wogonin-Induced Cellular Senescence Sensitized Navitoclax to Kill T-Cell malignancy cells. Recent research suggested that the expression of prosurvival networks increased in senescent cells including BCL-2, resulting in resistance to apoptosis [19]. We used senolytic drugs to target senescent cells, which is the second step of one-two punch therapy specific to Achilles' heel of senescent cells [19, 49]. Navitoclax, a senolytic BH3 mimetic drug, can selectively kill senescent cells [21, 50]. Navitoclax binds to BCL-2, BCL-xL, and BCL-w *in vitro*, while BCL-2 plays a key role *in vivo* and high expression of BCL-2 exhibited sensitivity to Navitoclax

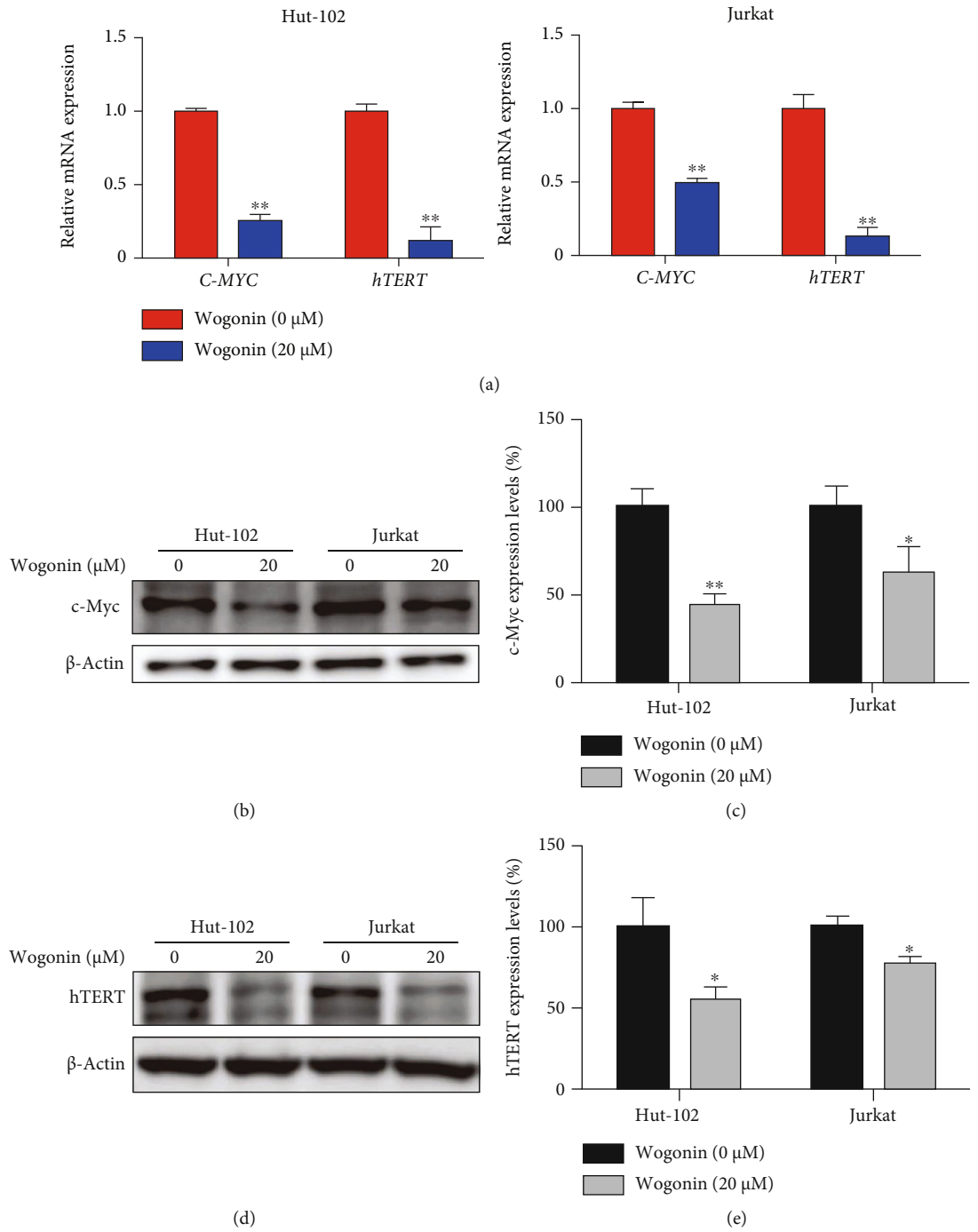


Figure 4: Wogonin suppressed telomerase activity by inhibition of hTERT and c-Myc in wogonin-induced senescent cells. (a) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. The relative mRNA level of *hTERT* and *C-MYC* was measured by RT-PCR. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (b) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. The expression of c-Myc protein was performed by western blot. β-Actin was used as loading controls. (c) Relative protein expression level of c-Myc. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (d) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. The expression of hTERT protein was performed by western blot. β-Actin was used as loading controls. (e) Relative protein expression level of hTERT. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group.

in human non-Hodgkin lymphomas instead of BCL-xL or BCL-w [51]. To investigate whether wogonin-induced senescence promoted the expression of prosurvival protein BCL-2 and BCL-xL, western blot was used and results showed that the expression of BCL-2 was upregulated but the expression of BCL-xL was not changed after treatment with 20 μM

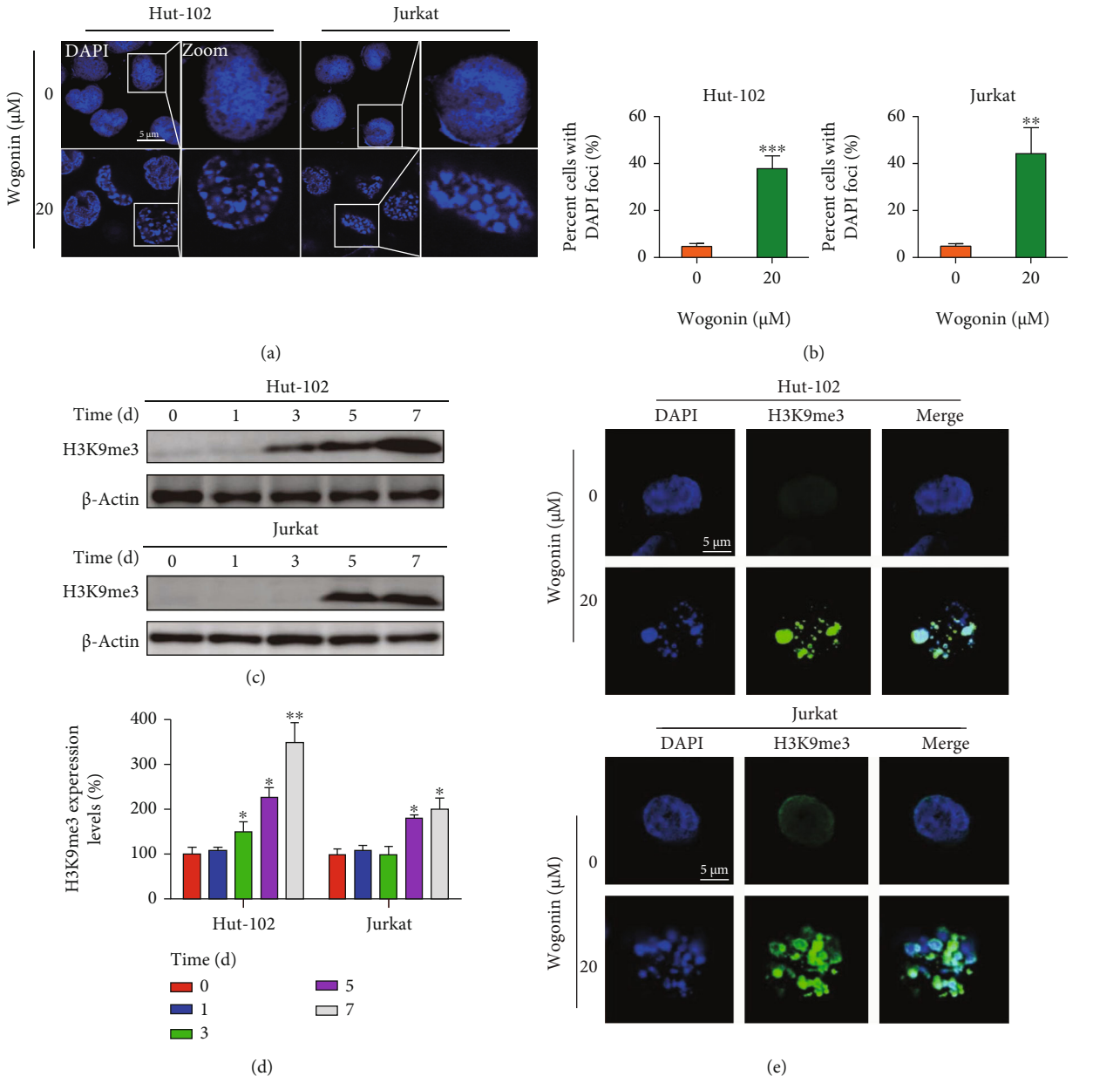
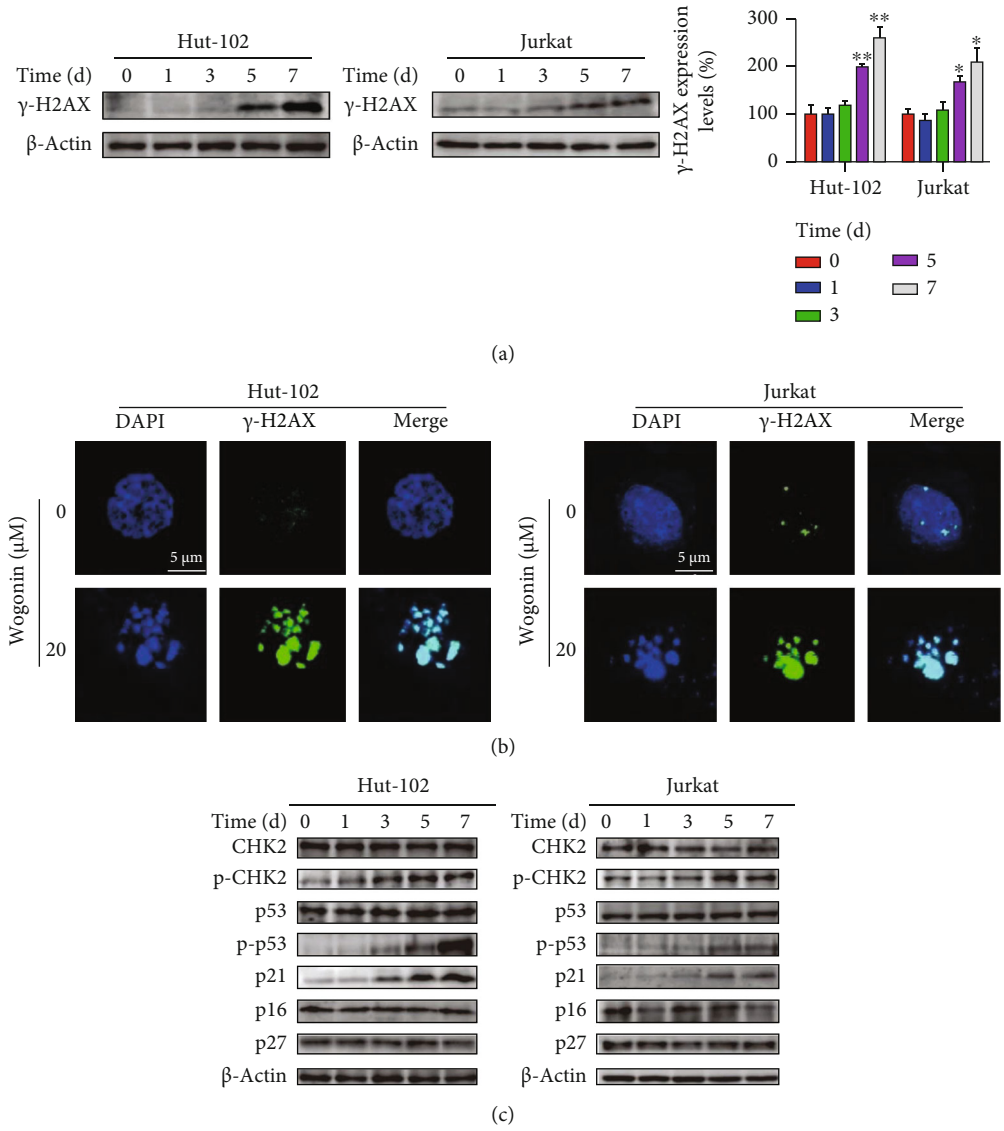


Figure 5: Wogonin induced senescence-associated heterochromatin foci in senescent cells. (a) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d; then, the cells were fixed and permeabilized, and nuclei were stained with DAPI (blue). SAHF formed in wogonin-treated Hut-102 and Jurkat cells. Representative confocal images for wogonin-induced SAHF formation in cells from confocal laser scanning microscopy are shown (original magnification ×1000; immersion objective ×100/×100 with immersion oil type). Images are representative of three independent experiments. (b) The percentage of positive cells with the DNA agglutination points (DAPI foci) was counted (with 20 cells counted per field). Columns represent the mean from three parallel experiments (mean ± SEM). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group. (c) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d. The expression of the SAHF marker protein H3K9me3 in control and wogonin-treated Hut-102 and Jurkat cells was performed by western blot. β-Actin was used as loading controls. (d) Relative protein expression level of H3K9me3 was determined. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (e) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d; then, the cells were fixed, permeabilized, and stained with an antibody against H3K9me3 (green), while nuclei were stained with DAPI (blue). Immunofluorescent images showed the distribution of H3K9me3 in Hut-102 and Jurkat cells (original magnification ×1000; immersion objective ×100/×100 with immersion oil type). Images are representative of three independent experiments.

wogonin for 5 d in Hut-102 and Jurkat cells (Figures 7(a) and 7(b)). We chose Jurkat cells to assess cell viability affected by Navitoclax. The CCK8 assay indicated that wogonin-induced senescent cells exhibited more significant sensitivity to Navitoclax than control cells (Figure 7(c)). Meanwhile, cell apoptosis analyses showed that Navitoclax could induce more apoptosis in wogonin-treated cells than in untreated cells (Figure 7(d)). Moreover, western blot results showed that



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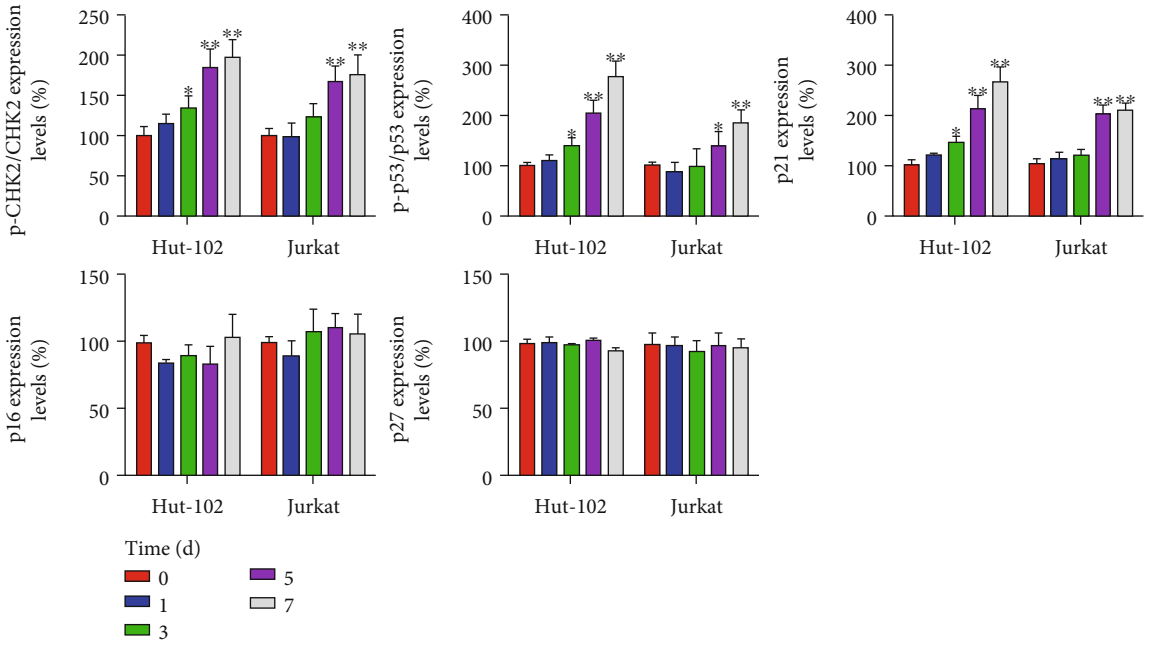
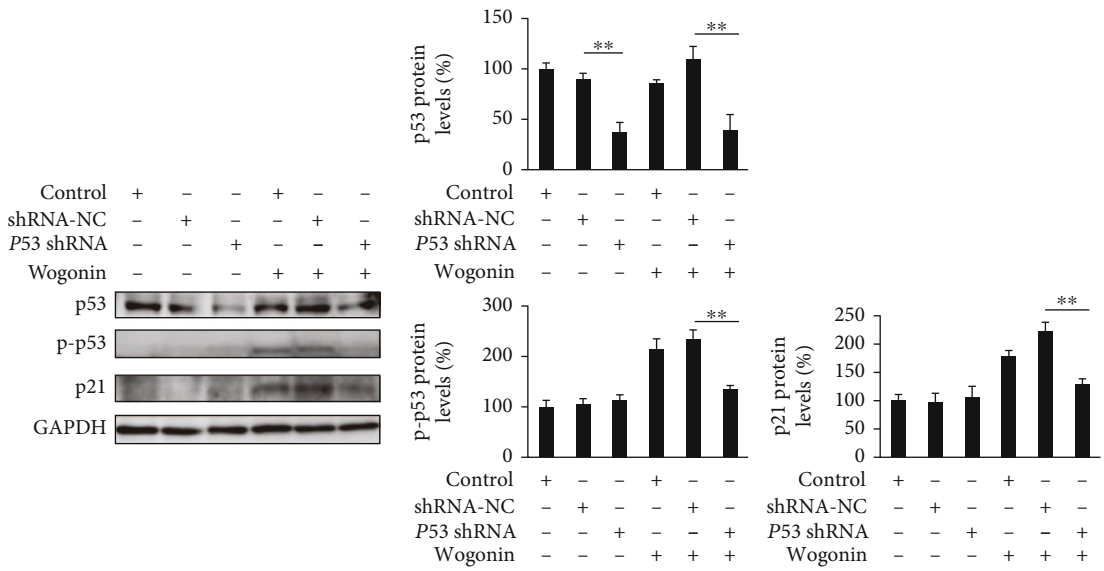


(d)



(e) (f)

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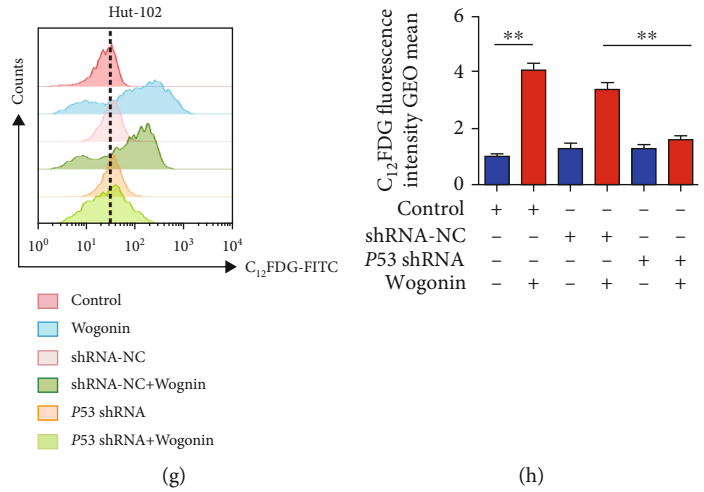

Figure 6: Wogonin induced cellular senescence through p53-mediated DNA damage response. (a) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for the indicated time periods, and western blot was used to detect the expression change of the DNA damage marker protein γ-H2AX. β-Actin was used as loading controls. Relative protein expression level of γ-H2AX was determined. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the control group. (b) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d; then, the cells were fixed, permeabilized, and stained with an antibody against γ-H2AX (green), while nuclei were stained with DAPI (blue). Immunofluorescent images showed the distribution of γ-H2AX in Hut-102 and Jurkat cells (original magnification ×1000; immersion objective ×100/×100 with immersion oil type). Images are representative of three independent experiments. (c) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for the indicated time periods (0, 1, 3, 5, and 7 d), and western blot was performed to detect the expression changes of CHK2, p-CHK2 (T68), p53, p-p53 (Ser15), p21, p16, and p27. β-Actin was used as loading controls. (d) Relative protein expression levels of p-CHK2/CHK2, p-p53/p53, p21, p16, and p27 were determined. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the control group. (e) The protein expression levels of p53, p-p53 (Ser15), and p21 were detected by western blot. *P53* shRNA-transfected Hut-102, shRNA-NC, and control Hut-102 cells were treated with or without 20 μM wogonin for 5 d. GAPDH was used as loading controls. (f) Relative protein expression levels of p53, p-p53 (Ser15), and p21. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the control group. (g) Hut-102 cells were transfected with *P53* shRNA and shRNA-NC by a lentiviral vector. *P53* shRNA-transfected Hut-102, shRNA-NC, and control Hut-102 cells were treated with or without 20 μM wogonin for 5 d. The activity of SA-β-Gal was measured by flow cytometry (cells were stained with C_{12}FDG). (h) Relative SA-β-Gal activity was assessed by using the GEOmean of groups, which represented C_{12}FDG fluorescence intensity. Columns represent the mean from three parallel experiments (mean ± SEM). $^*p < 0.05$, $^{**}p < 0.01$, compared with the NC group.

the expression of apoptosis protein levels like cleaved caspase-3/caspase-3, cleaved PARP-1/PARP-1, Bax, and Bim_L was increased in Navitoclax-treated senescent cells (Figures 7(e) and 7(f)). In conclusion, wogonin-induced senescent cells were more sensitive to Navitoclax cytotoxicity and were effectively killed.

4. Discussion

T-cell malignancies remain to have poor prognosis and are difficult to have specific treatments [3, 52]. Clinically, chemotherapy based on the ABVD and CHOP regimen is still the preferred treatment for T-cell malignancies, and toxic effects and drug resistance brought by traditional chemotherapy regimens are unavoidable [53–55]. Cellular senescence makes senescent cells as a druggable target to repress tumor progression [22, 56]. Thus, inducing cellular senescence makes senescent cells display an induced vulnerability to senolytics drugs, which could be a novel one-two punch therapy for the treatment of T-cell malignancies [19, 50, 57].

TP53 mutations in hematologic malignancies are less frequent (5%-15%) but closely associated with poor prognosis [58]. Different sites, forms, and frequencies of *TP53* mutation lead to different disease states [58–60]. Because of the central role of p53 in regulating cells' normal life activity, drug discovery concentrates on manipulating p53 and eradicating cancer cells. Wogonin is a well-known flavonoid and has a wide range of anticancer effects especially producing a better effect in hematologic malignancies [61–64]. However, although moderate and high concentrations of wogonin (over 40 μM) had a better therapeutic effect, its insoluble characteristic and cytotoxicity in high doses were inescapable and limited its use in clinic. We found that low concentration of wogonin (20 μM) could induce cellular senescence via DNA damage response in T-cell malignancies and verified the application of one-two punch therapy involving cellular senescence. Targeting senescent cells may benefit to increase therapeutic efficacy and reduce risk of recurrence [19]. Moreover, a previous study also reported that wogonin could target the Warburg Effect which depends on wild-type p53 to produce anticancer effects [65]. Except in the field of metabolism, p53 also plays an important role in regulating cellular senescence [66]. Our results showed that wogonin induced cellular senescence in p53 functional cell lines (Hut-102, Jurkat) but not the p53-deficient Hut-78 cell line. And we also found senescence-related SAHF phenomenon in Hut-102

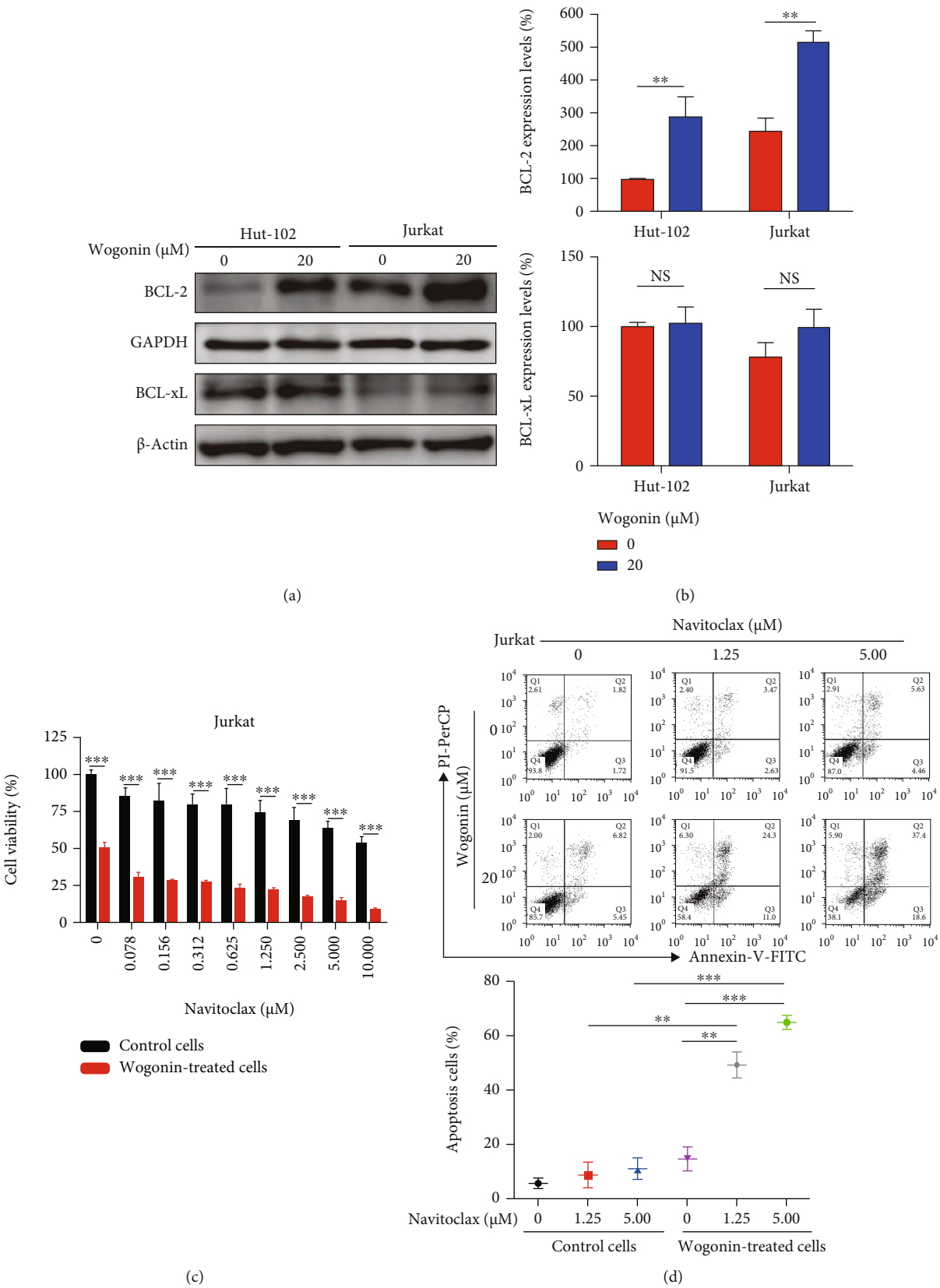

Figure 7: Continued.

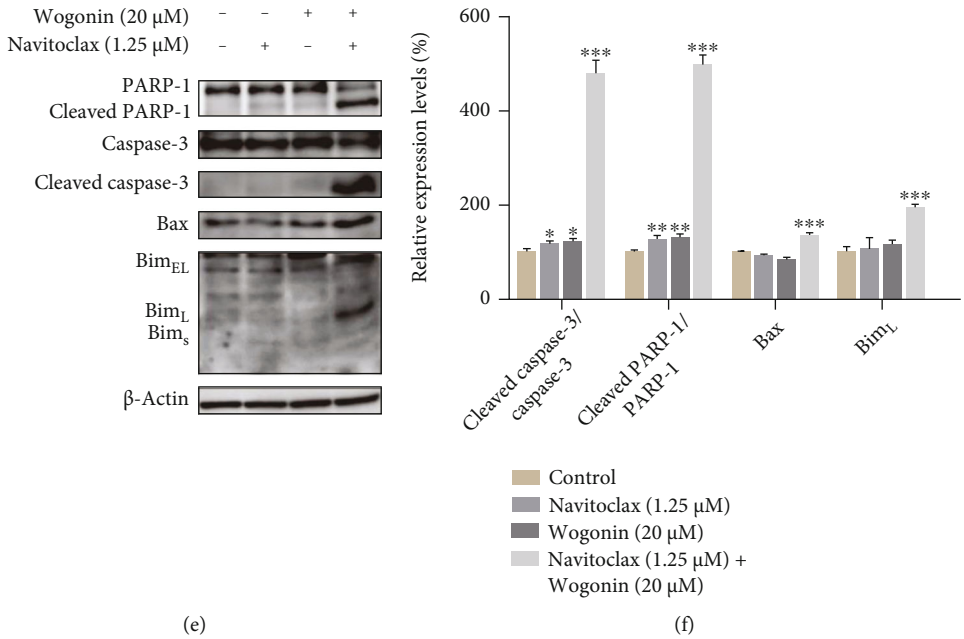

Figure 7: Wogonin-induced cellular senescence sensitized Navitoclax to kill T-cell malignancy cells. (a) Hut-102 and Jurkat cells were treated with or without 20 μM wogonin for 5 d, and western blot was used to detect the expression change of BCL-2 and BCL-xL. GAPDH and β-actin were used as loading controls. (b) Relative protein expression levels of BCL-2 and BCL-xL. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the control group. (c) Jurkat cells were exposed to 20 μM wogonin for 5 d, and then, control or wogonin-treated Jurkat cells were treated with the indicated concentrations of Navitoclax (0-10 μM) for 48 h. Cell viability was assayed by CCK8. The data represent the mean ± SEM of three different experiments. $^{***}p < 0.001$, compared with the control group. (d) Jurkat cells were exposed to 20 μM wogonin for 5 d, and then, control or wogonin-treated Jurkat cells were then treated with the indicated concentrations of Navitoclax (1.25, 5 μM) for 48 h. Cell apoptosis was assayed by flow cytometry after Annexin-V/PI staining. Data represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with the control group. (e) Jurkat cells were exposed to 20 μM wogonin for 5 d, and then, control or wogonin-treated Jurkat cells were then treated with the indicated concentrations of Navitoclax (1.25 μM) for 48 h. The protein expression levels of PARP-1, cleaved PARP-1, caspase-3, cleaved caspase-3, Bax, and Bim were analyzed by western blot. β-Actin were used as loading controls. (f) Relative protein expression levels of cleaved caspase-3/caspase-3, cleaved PARP-1/PARP-1, Bax, and Bim$_L$. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with the control group.

and Jurkat cells but not in Hut-78 cells, suggesting that p53 may be involved in wogonin-induced senescence. Meanwhile, the phosphorylation of p53 (Ser15) was increased in senescent cells after the treatment with wogonin for 5 d. The knocking down assay confirmed that wogonin-induced senescence depended on the expression of p53. It is indicated that p53 may be a core factor in mechanisms of wogonin-treated T-cell malignancies.

According to our study, wogonin induced DNA damage response and increased the expression of γ-H2AX in Hut-102 and Jurkat cells. As the consequences of DNA damage response, phosphorylation of CHK2 (Thr68) was activated after the treatment with wogonin in senescent cells. The mRNA and protein expression of p21 playing a crucial role was obviously increased by wogonin treatment to render the cell cycle arrest irreversible and induce the senescence phenotype. The mRNA level of p27 did not change in Hut-102 and Jurkat cells, and the mRNA level of p16 had slightly changed in Jurkat cells but not in Hut-102 cells. It is also worth noting that the protein expression of p16 did not change in Hut-102 and Jurkat cells, and the results prompted us that the p16/Rb pathway may not mainly participate in wogonin-induced cellular senescence in T-cell malignancies. In a word, the results suggested that the CHK2/p53/p21 pathway may be involved in wogonin-induced cellular senescence.

DNA damage is only the mediator of cellular senescence, but the change of telomere function is one of the real causes to trigger DNA damage response resulting in cellular senescence [67]. Telomere dysfunction leads to aging, which is a proliferation disorder dependent on p53 and pRb, and hinders the continuous accumulation of mutations, thus resulting in blocking the development of cancer. In contrast, experimental inactivation of p53 and pRb enables fibroblasts to bypass aging and continue to divide [68, 69]. As a mild senescence inducer, nonlethal concentration of wogonin showed a unique function to T-cell malignancies. 20 μM wogonin obviously suppressed the transcription of *C-MYC* and its downstream gene *hTERT*. Furthermore, wogonin decreased the protein expression of c-Myc and hTERT. *C-MYC* is a well-known oncogene and regulates the occurrence and progression of cancer. The promoter region of *hTERT* has oncoprotein c-Myc binding site-E-boxes, which induce the transcriptional activity of *hTERT* and activate telomerase

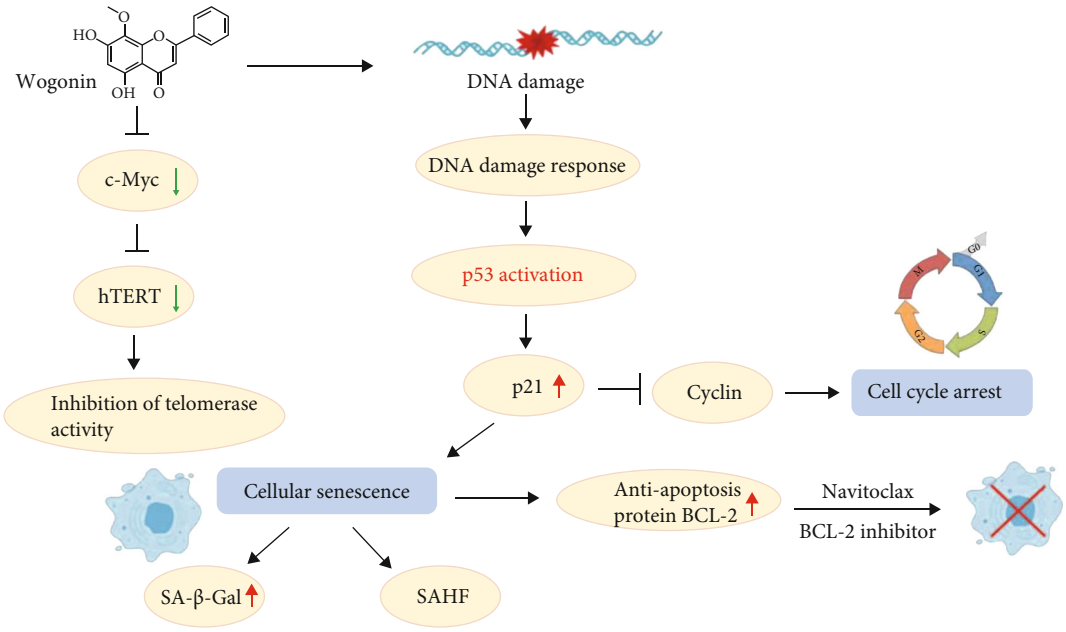

Figure 8: Graphical abstract on the mechanisms of one-two punch therapy involved in wogonin-induced cellular senescence in T-cell malignancies.

[70]. We can infer that wogonin induced cellular senescence partly through the inhibition of telomere function. The underlying mechanisms are still being investigated.

In this paper, we used Hut-78 cells as the p53 deficiency model to verify the mechanism of wogonin. We did not observe obvious cellular senescence in Hut-78 cells after wogonin treatment. It is worth mentioning that we found that wogonin increased the level of γ-H2AX in Hut-78 cells (Figure S1a, b). Following the results, we detected the expression of CHK2, p-CHK2 (T68), p21, p16, and p27. The results showed that the expression of CHK2 and p-CHK2 (T68) were all increased after wogonin treatment for 7 d. The expression of p21 had slightly increased on day 7, but p16 and p27 had no change (Figure S1c, d). The upregulated folds of p21 in Hut-102 and Jurkat cells were more than those in Hut-78 cells. The expression of p21 was slightly increased in Hut-78 cells after treatment with wogonin slighter (Figure 6(d) and Figure S1d). However, the lack of p53 and slightly increased level of p21 may not be enough give rise to senescence in Hut-78 cells. CFSE experiments suggested that the decreased fluorescence intensity in CFSE-labeled cells was prevented by wogonin in Hut-78 cells. Thus, we supposed that there could be other signaling pathways to account for the mechanism which regulates cell proliferation induced by wogonin in Hut-78 cells. More follow-up studies are warranted to unveil the true nature of wogonin in Hut-78 cells.

Some studies have shown that overexpression of BCL-2 or BCL-xL is commonly observed in acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) [71, 72]. Navitoclax is a specific BH3-mimetic drug especially used in hematologic malignancies [73]. However, BCL-2 was verified as playing a key role in lymphoid cells, and overexpression of BCL-2 could increase the sensibility to Navitoclax [51]. But lymphoid cells with low expression of BCL-2 lack sensibility to Navitoclax and lose opportunity for optimal cell killing. The expression of BCL-2 was upregulated, contributing to the resistance of senescent cells to death. In our study, the increased expression of BCL-2 in senescent cells induced by wogonin could be detected by western blot. Then, the CCK8 assay showed that senescent cells were more sensitive to Navitoclax compared to untreated cells. Thus, the sequential combination of the BCL-2 inhibitor Navitoclax could promote the death of tumor cells, so there was no doubt that adopting this treatment method was of positive significance.

In summary, our findings suggest that wogonin not only inhibited telomere function but also induced p53-dependent cellular senescence, which was correlated with DDR. Cellular senescence promoted the expression of BCL-2 and consequently increased the sensibility to Navitoclax and removed senescent cells. Our work indicates prosenescence therapy induced by wogonin, sequentially combined with Navitoclax, providing a novel clinical approach for T-cell malignancy treatment (Figure 8).

5. Conclusion

In conclusion, our study revealed that wogonin inhibited telomerase activity via inhibition of c-Myc regulating the expression of hTERT. On this basis, we also found that wogonin induced p53-dependent cellular senescence mediated by DDR in T-cell malignancies. Moreover, wogonin-induced senescent cells increased the sensibility of Navitoclax resulting in killing T-cell malignancy cells effectively. cells. This may be considered a promising aspect of clinical cancer treatment.

Data Availability

The authors declare that the data supporting the findings of this study are available from the corresponding author upon reasonable request.

Additional Points

Additional Information. The online version of this article contains supplementary material, which is available to authorized users.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YJQ and HL designed and performed the research, analyzed the data, and wrote the paper. YZZ and PH performed the research and analyzed the data. XYW and XXY performed the research. MYZ and HZW collected the data and performed statistical analysis. ZYW collected and analyzed the data, and QLG and HH conceptualized the project and directed the experimental design data analysis. Yingjie Qing and Hui Li contributed equally to this work.

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Supplementary Materials

Supplemental Figure. Effect of 20 μM wogonin on Hut-78 cell line. (a) Hut-78 cells were treated with or without 20 μM wogonin for the indicated time periods (0, 1, 3, 5, 7 d), and western blot was used to detect the changes in the expression of DNA damage marker protein γ-H2AX. β-actin was used as loading controls. The right panel: relative protein expression level of γ-H2AX. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group. (b) Hut-78 cells were treated with or without 20 μM wogonin for 7 d, then the cells were fixed, permeabilized and stained with antibody against γ-H2AX (Green), while nuclei were stained with DAPI (Blue). Immunofluorescent images showed the distribution of γ-H2AX in Hut-78 cells (original magnification ×1000; immersion objective ×100/×100 with immersion oil type). Images are representative of three independent experiments. (c) Hut-78 cells were treated with or without 20 μM wogonin for the indicated time periods (0, 1, 3, 5, 7 d), and western blot was performed to detect the changes in the expression of CHK2, p-CHK2 (T68), p21, p16 and p27. β-actin was used as loading controls. (d) Relative protein levels of p-CHK2/CHK2, p16, p21 and p27 were determined. Columns represent the mean from three parallel experiments (mean ± SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group. *(Supplementary Materials)*

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Review Article

A Decade of Research on Coffee as an Anticarcinogenic Beverage

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Coffee consumption has been investigated as a protective factor against cancer. Coffee is a complex beverage that contains more than 1000 described phytochemicals, which are responsible for its pleasant taste, aroma, and health-promoting properties. Many of these compounds have a potential therapeutic effect due to their antioxidant, anti-inflammatory, antifibrotic, and anticancer properties. The roasting process affects the phytochemical content, and undesirable compounds may be formed. In recent years, there have been contradictory publications regarding the effect of coffee drinking and cancer. Therefore, this study is aimed at evaluating the association of coffee consumption with the development of cancer. In PubMed, until July 2021, the terms "Coffee and cancer" resulted in about 2150 publications, and almost 50% of them have been published in the last 10 years. In general, studies published in recent years have shown negative associations between coffee consumption and the risk or development of different types of cancer, including breast, prostate, oral, oral and pharyngeal, melanoma, skin and skin nonmelanoma, kidney, gastric, colorectal, endometrial, liver, leukemic and hepatocellular carcinoma, brain, and thyroid cancer, among others. In contrast, only a few publications demonstrated a double association between coffee consumption and bladder, pancreatic, and lung cancer. In this review, we summarize the *in vitro* and *in vivo* studies that accumulate epidemiological evidence showing a consistent inverse association between coffee consumption and cancer.

1. Introduction

Drinking coffee has often been discouraged, due to its association with the description of negative effects, mainly attributed to caffeine. Consumers' beliefs about the effects of coffee are strongly influenced by this idea: only 25%, a relevant minority of consumers, think that drinking coffee could have beneficial effects on health [1]. A survey performed by the World Cancer Research Fund (WCRF) revealed that as many as 36% of health professionals thought drinking coffee increases the risk of cancer [2]. Despite the "bad reputation" that has been conferred on coffee for many years, it has been increasingly demonstrated that it offers numerous health benefits, ranging from reduced risk of several cancers to reduced risk of developing depression.

Coffee is widely consumed, ranking as one of the four most commonly consumed beverages in the world [3]. The generic name coffee covers about one hundred species of plants, cultivated in multiple countries with different types of weather conditions: Hawaii, Colombia, Jamaica, Ethiopia, Kenya, Brazil, Vietnam, and some African countries [4]. The coffee tree appertains to the Rubiaceae family, and there are more than 80 different species of the genus *Coffea L.* Globally, only two species are traded: *Coffea arabica*, accounting for 63% of the world production, and *Coffea canephora*, accounting for the rest of the world production [5]. As de Mejia et al. highlight, the species and the origin are only two of the factors that determine that we never drink two cups of coffee with the same chemical composition. Additionally, the final composition of coffee will depend on cultivation practices (traditional or organic), postharvest techniques (wet or dry), duration and conditions of storage, roasting degree (light, medium, or dark), kind of roasting process (standard or torrefaction), type of commercial coffee (roasted ground or instant), and grinding and brewing method (boiled, filtered, or espresso) [6].

The consumption of coffee may have a substantial effect on public health. In 2017, two large reviews based on meta-analyzes were published, which established that coffee consumption was more often associated with benefit than harm, a probable decrease in the risk of breast, colorectal, colon, endometrial, and prostate cancer; cardiovascular disease and mortality; Parkinson's disease; and type 2 diabetes [7, 8]. Then, it is no surprise that coffee motivates the interest of researchers and clinicians [9]. In PubMed, until April 2021, the search for the term "coffee" resulted in around 17,200 publications, including 1,430 reviews, 800 clinical trials, and 260 meta-analyses. However, the impact of coffee intake on chronic diseases, including cancer, has been a matter of debate in the last two decades. Accordingly, the search performed by combining both terms, "coffee and cancer", resulted in about 2,150 publications, and almost 50% of them have been published in the last 10 years.

In this work, we present an update on the last decade of research related to coffee and its anticancer activity. For this purpose, a broad approach was used, as follows: (1) analysis of the main coffee compounds and their modifications by the roasting process, (2) review of the bioactivity of coffee extracts by *in vitro* assays, (3) analysis of the antioxidant activity of coffee and its components, and (4) data collection of large observational human studies.

1.1. Search Strategy. We searched on the PubMed database for basic and meta-analysis articles published from 2010 to June 2021. The search included the following relevant terms: "coffee" paired with "cancer," "roasting," "green," "dark," "bioactive," "composition," "*in vitro*," "cell line," "antioxidant," "breast," "leukemic," "oral," "oral and pharyngeal," "gastric," "non-melanoma skin," "melanoma," "endometrial," "hepatocellular," "prostate," "esophageal," "ovarian," "colorectal," "liver," "brain," "glioma," "thyroid," "bladder," "pancreatic," "laryngeal," "lung," "caffeine," "polyphenol," "Trigonelline," "chlorogenic acid," "cafestol," "kahweol," "caffeic acid," "melanoidin," "nicotinic acid," "flavonoid," and "N-methylpyridinium."

No language restrictions were imposed. When available, priority was given to the conclusions of meta-analyses and systematic reviews. Within these studies, we selected those that reported statistically significant associations in their analyses, whether positive or negative.

2. The Main Bioactive Compounds of Coffee and Their Modifications by the Roasting Process

The traditional recommendation to reduce the consumption of coffee as a beverage or not to drink it at all because of a global risk profile has progressively given rise to a less negative view due to its better-known phytochemistry [10]. The knowledge that coffee and caffeine are not equivalent has increased the interest in discovering whether other components of coffee might contribute to the protective action in the human body [9]. Coffee is a complex beverage containing more than 1000 described phytochemicals responsible for its pleasant flavor, aroma, and health promoters [1, 6, 10]. Many of these compounds have therapeutic potential antioxidant, anti-inflammatory, antifibrotic, and anticancer effects.

As a consequence of roasting, profound changes occur in the chemical composition, which leads to the transformation of natural substances present in green beans into compounds derived from the Maillard reaction [4]. Table 1 shows the main compounds of green coffee, i.e., coffee beans before roasting, and the appearance of or increase in specific components in black coffee beans. This well-known roasting process involves the caramelization of carbohydrates and the pyrolysis of organic compounds. Here, we detail the main phytochemicals belonging to groups of sugars and sugar metabolites, protein and amino acids, fatty acids, chlorogenic acids (CGAs), organic acids, and other compounds.

"Bioactive compounds" are extra nutritional constituents that typically occur in small quantities in foods and have a positive effect on human health. These are also referred to as nutraceuticals, a term that reflects their existence in the human diet and their biological activity. They consist of a wide range of chemical compounds with different structures, physiological activities, and molecular mass between 200 and 1000 Da [11]. Green coffee beans have been shown to have high levels of bioactive compounds, but even after the roasting process and exposure to hot water, dark coffee maintains and develops numerous new phytochemicals that are beneficial to consumers' health [12].

As highlighted in Table 1, during the roasting process, the concentration of some nutraceutical compounds, such as 5-CQA, 3,4-diCQA, 3,5-diCQA, phenolic acid, trigonelline, polyphenolic, cafestol, and kahweol, decreases while others are formed, such as melanoidins, chlorogenic lactones, acid gallic, acid nicotinic, caffeic acid, flavonoids, and N-methylpyridinium (all of them marked in bold). This delicate balance between the formation and decomposition of bioactive compounds allows the biological effects of dark roasted coffee to be found in experimental models. Sometimes, these effects are higher in dark than lighter roasted coffee, despite the antioxidant content in dark coffee is lower [1]. In fact, Priftis et al. reported that in eight out of 13 coffee varieties, toasted coffee increased the free radical scavenging activity [13].

The five most abundant bioactive constituents in green coffee are *(I) caffeine*, which has been positioned by numerous studies as a protective agent for cell membranes against oxidative damage, with anticancer activity [4, 14, 15] and anti-inflammatory effects [16]; *(II) polyphenols*, which can cause a variety of important bioactivities with beneficial effects on human health [4]; *(III) trigonelline*, which has hypoglycemic, neuroprotective, antitumor (anti-invasive), antibacterial, and antiviral activities [3, 4, 17]; *(IV) chlorogenic acids* (CGAs), the most important class of polyphenols, which can be grouped into caffeoylquinic acids (CQA), feruloylquinic acids (FQA), and di-caffeoylquinic acids (diCQA), all of which are known to have powerful antioxidant, anticancer, anti-inflammatory, antibacterial, antipyretic, hepatoprotective, and neuroprotective effects and can help prevent retinal degeneration, obesity, and hypertension [4, 12, 18, 19]; and *(V) cafestol* and *kahweol*, main coffee

Table 1: Compounds that increase or decrease during the coffee bean roasting process. The left column shows the most abundant compounds in green coffee that are lost during the roasting process, while the right column shows those that appear during the roasting process. The known bioactive compounds are marked in bold.

Coffee bean roasting process		
Polysaccharide sugars and sugar metabolites	Sucrose [3, 19, 63–66], fructose [4, 19, 66], arabinos, galactose, glucose [4, 66], mannose, manitol, xylose, ribose [4]	Hydroxymethyl-furfural [3, 63, 64]
Proteins and amino acids	Asparagine [4, 19, 64], proline, glutamine, histidine, leucine, phenylalanine [19], alanine [4, 64]	Protein [66]
Lipids Fatty acids	Palmitic, stearic [4, 33], arachidic, linolenic, oleic, arachidic [4, 33]	(PUFA) n-6, MUFA+PUFA [33]
Polyphenols Chlorogenic acid (CGA)	1-FQA [66], 4-CoQA [66], 4-FQA [66, 67], 3-CQA, **5-CQA**, 4-CQA 4, [64, 66, 67], 5-FQA [66, 67], 4,5-diCQA [67], **3,4-diCQA**, **3,5-diCQA** [66, 67]	3-CoQA, 3-FQA, 5-CoQA [66], **chlorogenic lactones** [4]
Organic acids	**Citric**, GABA, malic [64], quinic [4, 64], **phenolic** [4, 14, 19]	**Gallic** [12, 63], **caffeic** [4, 12, 63], acetic [63], formic, lactic [63, 64], syllo-quinic [64], **nicotinic** (**Niacin** and **Vitamin B3)** [3, 4, 64]
Other compounds	**Caffeine** [3, 4, 19, 64, 66], **trigonelline** [3, 4, 64], **polyphenolic content** [13, 48], choline [3, 64], **cafestol**, **kahweol** [68], fluoride [42]	γ-Quinide [4, 64], **flavonoids** [69], **N-methylpyridinium** [3], alkyl esters, shikimoyl esters [4], **melanoidins** [3, 4, 13]

diterpenes that have demonstrated anti-inflammatory, hepatoprotective, anticancer (tumor cell-inducing apoptosis and antiangiogenesis), antidiabetic, and antiosteoclastogenesis activities [20, 21].

In contrast, the main bioactive molecules in roasted coffee are *(I) caffeic acid*, with biological activities such as antitumor, antioxidation, anti-inflammatory, and immune regulation properties [4, 12, 17]; *(II) melanoidins*, whose nutritional antiradical, antioxidant, chelating, antimicrobial, antimutagenic, anticariogenic, antihypertensive, anti-inflammatory, and antiglycative properties have been described [22, 23]; (*III) nicotinic acid* (Niacin or Vitamin B3), which plays a role in DNA repair (interacting with PARP), has tumor suppressive effects, and inhibits cancer cell invasion (blocking epithelial-mesenchymal transition) [24, 25]; *(IV) flavonoids*, which exhibit a great diversity of biological activities, such as antioxidant, antiaging, anti-inflammatory, immunomodulation, cardioprotective, antibacterial, antiviral, antiparasitic, antihypertensive, antiulcerogenic, antidiabetic, and hepatoprotective properties, as well as prevention against cancer (including carcinogen inactivation, antiproliferation, cell-cycle arrest, induction of apoptosis, inhibition of angiogenesis, antioxidation, and reversal of multidrug resistance or a combination of these mechanisms) [26, 27]; and *(V) N-methylpyridinium*, identified as inducers of antioxidant response element pathway [28].

In summary, although *in vitro* and *in vivo* studies using individual components of coffee revealed multiple biological activities, the physiological properties of whole coffee will likely differ because coffee is a complex, nonstandardized beverage. Therefore, it is a variable mixture of hundreds of compounds, and its bioactivity may be influenced by possible matrix, synergistic, and/or antagonist effects. Additionally, only a small percentage of the ingested compounds may enter the circulatory system and reach the tissues, and very little of the absorbed material may retain the original structure present in the beverage [4]. For these reasons, it is worth pointing out that the prevention of various diseases derived from coffee consumption is usually the joint action of multiple components, and sometimes the synergistic effect of various types of compounds is much better than the activity of single compounds [29].

3. Bioactivity of Coffee Extracts, Evidence Provided by *In Vitro* Studies

Despite all the bioactivities described above for individual compounds isolated from coffee, in this work, we highlight

Table 2: *In vitro* studies in cell lines. Effects of different types of coffee on a broad spectrum of human cancer cell lines.

Author, date (year)	Cell lines	Cancer type	Coffee variety	Coffee type	IC_{50}	Effects
Pounis, 2017 [32]	PC-3 DU145	Prostate	Uninformed	Roasted	Uninformed	Antiproliferative
Montenegro, 2021 [5, 31]	PC-3 DU145	Prostate	Arabica Brazil	Roasted Green	1-5 mg/mL	Cell-cycle arrest Antiproliferative, apoptosis, high antioxidant capacity
Palmioli, 2017 [70]	MDA-MB-231	Breast	Arabica (Brazil, Burundi and Colombia) Robusta (Uganda, Vietnam, and Tanzania)	Roasted Green	121 ± 10 ng/μl 72 ± 7 -315 ± 17 ng/μl	Antiproliferative
Amigo-Benavent, 2017 [18]	OE-33 T24 A549	Esophageal Urinary bladder Lung	*Coffea arabica L.* (Colombia)	Green	01-1 mg/mL	Antiproliferative
Bauer, 2018 [19]	DU-145	Prostate	*Coffea canephora* var. Robusta (Brazil)	Dark, medium, light, green	Uninformed	Antiproliferative, induced apoptosis
Mojica, 2018 [12]	HT-29 SCC-25	Colon Oral	Columbia Supremo	Geen, cinnamon, city, full city, full city plus	Uninformed	Antiproliferative, antioxidant activity
Funakoshi-Tago 2020 [71]	MCF-7	Breast	Columbia Arabica Japan	Roasted coffee	2.5, 5 v/v%	Cell-cycle arrest, apoptosis, enhances tamoxifen proapoptotic activity.
Makino 2021 [35]	ACHN Caki-1	Kidney	Arabica or Robusta Coffee Japan	Roasted	Uninformed	Antiproliferative, antimigratory, apoptosis
Oleaga, 2012 [36]	HT29 MCF-7	Colon Breast	Instant caffeinated coffee		Uninformed	Cyclin D1, STAT5B, and ATF-2 downregulated
Nigra, 2021 [30]	MDAMB-231 MCF-7 HCT116 U2OS T98G	Breast Colon Osteosarcoma Glioblastoma	Coffea canephora var. Robusta Brazil	Roasted green	1 mg/mL	Antiproliferative, cell-cycle arrest, induced apoptosis, mitochondrial dysfunction

the studies that consider whole -/coffee, because it has been shown that after a person drinks 2-3 cups of coffee, many components are metabolized (thus changing their original structures) or only reach transient and very low plasma concentrations [4, 12]. Additionally, it has not been possible to attribute the diverse and extensive bioactive capabilities of coffee to any pure compound.

In the last decade, numerous research groups have focused on the effect of coffee on tumors by conducting *in vitro* studies, to understand the bioactivities of this beverage in greater depth. Table 2 presents many of these *in vitro* studies, which have addressed a great diversity of cancer types, coffee varieties, degrees of roasting [5, 19, 30, 31], and diverse effects including cell-cycle arrest, antiproliferative effects, and antiapoptotic and high antioxidant activities.

Remarkably, most of the investigations report a wide range of coffee effects on cancer cells. For example, studies on breast cancer cells focused on antiproliferative and antioxidant effects, cell-cycle arrest, induced apoptosis, regulation of gene expression, modulation of enzyme activity, and enhanced efficiency of cancer treatments [30, 32–34]. Studies on prostate cancer cells reported antiproliferative effects, cell-cycle arrest, apoptosis, and high antioxidant capacity [5, 19, 31, 32]. Research on kidney cancer cells found antiproliferative and antimigratory effects, EMT downregulation, and gene downregulation [35]. Furthermore, antiproliferative effects were reported in esophageal, urinary, bladder, lung, colon, oral, osteosarcoma, and glioblastoma cancer cells [12, 18, 30, 36].

Additionally, as Samoggia and Riedel and Montenegro et al. pointed out, intake of green coffee-based beverages has become popular in recent years due to the belief in their beneficial antioxidant properties [1, 31]. Some researchers have observed that green coffee treatments have antiproliferative activity, though not as powerful as that of roasted coffee treatments [12]. However, further cell experiments

should be conducted to evaluate the deep molecular mechanisms and pharmacokinetics involved in the effects observed. The bioavailability and bioaccessibility of extracts should be investigated to determine the quantity of coffee required to achieve such effects, due to possible losses during digestion, absorption, and metabolization by the gut microbiota. Toxicity assays should be performed to ensure safety. In addition, *in vivo* and clinical tests would be required to recommend the consumption of different coffee types to help protect against cancer.

4. Antioxidant Properties of Coffee

As highlighted in the previous sections, among the main biological activities of the beverage and its components, the antioxidant activity was made evident in numerous investigations, both in chemical tests and different tumor cell cultures.

When, in aerobic life and our metabolism, the electron flow becomes uncoupled (transfer of unpaired single electrons), free radicals are continuously produced by the body's normal use of oxygen such as respiration. Oxygen-centered free radicals or ROS radicals are superoxide, hydroxyl, peroxyl, alkoxyl, hydroxyl radical, nitric oxide, and lipid hydroperoxides, while ROS nonradicals are singlet oxygen, hydrogen peroxide, and hypochlorous acid. Therefore, these intermediates, also called oxidants or prooxidants, can easily initiate the peroxidation of membrane lipids [37]. The imbalance towards high ROS concentration could result in oxidative damage to critical cellular biopolymers (proteins, lipids, and nucleic acids), especially if the free radicals are produced and accumulate unchecked for a prolonged period. The sustained oxidative damage of these biological macromolecules is linked to the development of chronic diseases like cancer.[38]. The reason why antioxidants are thought to be beneficial to our health is that ROS have been shown to be involved in many disease processes, including cancer [39].

In terms of food, an antioxidant has been defined as any substance that, when present at lower concentrations than those of an oxidizable substrate, significantly delays or inhibits the oxidation of the substrate. In recent years, there has been great interest in identifying alternative natural and safe sources of food antioxidants, especially those of plant origin. Nowadays, there is a growing interest in substances exhibiting antioxidant properties, which are supplied to human organisms as food components or as specific preventive pharmaceuticals [37]. The possibility of complementing the body's natural antioxidant defense system with exogenous antioxidants has continued to receive significant research attention as a result of their potential for wide applications [38].

In this sense, the antioxidant activity of coffee and of many of its components has been demonstrated [29, 40–43]. Additionally, recent studies have shown that coffee components can trigger tissue antioxidant gene expression and protect against gastrointestinal oxidative stress [44].

Recent studies conclude that coffee infusions significantly extend the chronological lifespan of the Saccharomyces cerevisiae yeast cells by protecting cells against reactive oxygen species, double DNA-strand break, and the decrease in metabolic activity [45]. However, the bibliography currently gives contradictory information as to whether slightly roasted or dark coffee generates greater antioxidant activity. Water extracts from green coffee are characterized by significant antioxidant properties and a high capacity to reduce transition metal ions because it contains many polyphenolic compounds that oxidize in the potential range tested [19, 46]. Jung et al. found that the cellular antioxidant activity of coffee extracts (in AML-12 and RAW 264.7 cells) has physiological antioxidant and anti-inflammatory activities, and that these effects are negatively correlated with roasting levels in the cell models [47]. Bobková et al. also support this theory, since they established that total antioxidant capacity reached the highest values in light roasted coffee, and the roasting process affected both the oxidative activity and the polyphenolic content [48]. Schouten et al. confirmed that acrylamide levels and antioxidant activity reached a maximum in the first coffee roasting degrees and then decreased as the heating process continued, both in Arabica and Robusta samples [49]. Wolska et al. showed that the method of brewing Arabica coffee and green coffee (simple infusion, espresso maker, French press, overflow espresso, or Turkish coffee) significantly affected the antioxidant potential of infusions [42]. In other research, antioxidant activity significantly rose with the degree of roasting, where strongly roasted coffee had higher activity than lightly roasted coffee [50].

As mentioned above, many coffee micronutrients are considered bioactive due to their high antioxidant capacity. Investigations have revealed that crude caffeine possesses hydrophilic antioxidant activity (145 ?mol Trolox equivalent (TE)/g) and lipophilic antioxidant activity (66 ?mol TE/g) [51], and its administration has led to the inhibition of the cyclooxygenase-2 enzyme [40]. The antioxidant activities of CGA exhibited protection against oxidative damage of macromolecules such as DNA, lipids, and proteins. When administered to mice under scopolamine-induced amnesia, CGA showed a neuroprotective function via the inhibition of acetylcholinesterase [40]. Coffee melanoidins, compounds formed during coffee roasting, have also been demonstrated as potential food ingredients due to their antioxidant properties. The antioxidant activity of these compounds, evaluated *in vitro*, was 725-750 μmol Trolox/g, and it was tripled by the addition of sugar during coffee roasting, namely, torrefaction, known to increase the content of melanoidins [41]. Czachor et al. provide strong evidence that coffee flavonoids are responsible for scavenging free radicals and leading to longevity in yeast lacking Sod1, Sod2, and Rad52 proteins [45].

5. Have We Been Drinking a Medicine Daily for Hundreds of Years without Knowing It?

In recent years, the number of consistent epidemiological evidence of coffee and cancer has accumulated but due to similar methodological limitations, it is sometimes unsatisfactory. Therefore, interest has focused on meta-analyses or

Table 3: Coffee consumption and cancer risk. 78 meta-analyses, cohort, or prospective studies, published between 2010 and 2021, which reported some type of significant association between coffee consumption and different cancer types, were analyzed. Italic rows: studies that report a negative association between coffee and cancer risk; bold rows: studies that report a positive association between coffee and cancer risk. Abbreviations: Pt: participants; Cn: control; Cn: cancer cases; c/d: cups/day.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Yu, 2011 [72]	Breast	40 prospective cohort studies (2,179,126 Pt and 34,177 Cc)	March 2010	Meta-analysis showed coffee drinking had an inverse association with cancer.
Li, 2013 [73]		16 cohort and 10 case-control studies (49,497 Cc)	July 2012	An inverse association was observed in ER^- negative subgroup.
Jiang, 2013 [74]		37 articles (966,263 Pt and 59,018 Cc)	December 2012	A strong and significant association with cancer risk was found for BRCA1 mutation carriers. The risk of breast cancer decreased by 2% for every 2 days.
Lowcock, 2013 [52]		1 cohort study (3,427 Cn and 3,062 Cc)	2002-2003	High coffee consumption, but not total caffeine, may be associated with reduced risk of ER^- and postmenopausal cancers.
Simonsson, 2013 [75]		1 preoperative study (634 Pt)	2002-2008	Tamoxifen-treated patients with ER^+ tumors who consumed 2 or more c/d had significantly decreased risk for early events.
Rosendahl, 2015 [76]		1 cohort (1,090 Pt with invasive primary cancer)	2002-2012	A moderate (2–4 c/d) to high (≥5 c/d) coffee intake was associated with smaller invasive primary tumors and a lower proportion of ER^+ tumors.
Lafranconi, 2018 [77]		21 prospective studies	March 2017	Coffee intake was associated with a 10% reduction in postmenopausal cancer risk.
Sánchez-Quesada, 2020 [78]		1 cohort study (10,812 Pt)	Uninformed	Among postmenopausal women, more than 1 c/d of coffee was associated with a lower incidence of cancer.
Yu, 2011 [72]	Leukemic	40 prospective cohort studies (2,179,126 Pt and 34,177 Cc)	March 2010	It confirmed that coffee consumption is associated with a reduced risk of cancer.
Zhang, 2015 [79]	Oral	12 studies (1,872,231 Pt and 4,037 Cc)	March 2015	Higher consumption might reduce the risk of cancer, especially in Europe.
Li, 2016 [80]		11 case-control and 4 cohort studies (2,832,706 Cn and 5,021 Cc)	2015	A protective benefit in oral cancer
He, 2020 [81]		14 case-control and 5 cohort studies (6456 Cc)	September 2018	High and intermediate versus low coffee intake was associated with a reduced risk of cancer. Coffee intake might have protective effects against cancer.
Farvid, 2021 [82]		2 preoperative studies (8900 Cc)	1980-2010 1991-2011	>3 c/d of coffee was associated with a 25% lower risk of cancer. Among cancer survivors, higher postdiagnostic coffee

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
				consumption was associated with better cancer and overall survival.
Turati, 2011 [83]	Oral and pharyngeal	1 cohort and 8 case-control studies (2,633 Cc)	October 2009	Coffee drinking is inversely related to oral pharyngeal cancer risk.
Miranda, 2017 [84]		13 case-control and 4 cohort studies	August 2016	An inverse association between high consumption and the risk of both cancer types
Hildebrand, 2013 [85]		A prospective US cohort study (967,564 Cn and 868 Cc)	1982-2008	Intake of >4 c/d was associated with a 49% lower risk of cancer. Caffeinated coffee intake was inversely associated with oral/pharyngeal cancer mortality.
Vaseghi, 2016 [86]	Nonmelanoma skin	6 independent studies (320,370 Pt and 104,770 Cc)	January 2016	Caffeinated coffee might have chemopreventive effects dose-dependent effects against basal cell carcinoma
Caini, 2017 [87]		13 articles (37,627 Cc)	February 2016	A moderate protective effect against basal cell cancer development
Wang, 2016 [88]	Cutaneous melanoma	23 studies (2,268,338 Pt)	August 2015	The risk of cancer decreased by 3% and 4% for 1 c/d increment of total coffee and caffeinated coffee consumption, respectively.
Liu, 2016 [89]	Melanoma	2 case-control (846 Cc and 843 Cn) and 5 cohort studies (844,246 Pt and 5,737 Cc)	November 2015	Caffeinated coffee might have chemopreventive effects against cancer.
Yew, 2016 [90]		9 studies (927,173 Pt and 3,787 Cc)	September 2015	Beneficial effects of regular coffee consumption on cancer.
Micek, 2018 [91]		7 studies (1,418,779 Pt and 9,211 Cc)	March 2017	An increase in consumption of one c/d was associated with a 3% reduction in cancer risk. Coffee intake may be inversely associated with the incidence of melanoma.
Je, 2012 [92]	Endometrial	10 case-control and 6 cohort studies (6,628 Cc)	October 2011	Increased intake is associated with a reduced risk of cancer.
Zhou, 2015 [53]		13 articles (1,534,039 Pt)	May 2015	Risk decreased by 5% for every 1 c/d intake, 7% for every 1 c/d of caffeinated coffee intake, 4% for every 1 c/d of decaffeinated coffee intake, and 4% for every 100 mg of caffeine intake/d
Lafranconi, 2017 [93]		12 studies	March 2017	Increasing consumption by 4 c/d was associated with a 20% reduction in risk and a 24% reduction in postmenopausal cancer risk.

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Lukic, 2018 [94]		12 cohort and 8 case-control studies (11,663 Pt and 2,746 Cc)	August 2016	Protective effect
Bravi, 2013 [95]		8 cohort and 8 control studies (3,153 Cc)	September 2012	The risk of cancer is reduced by 40% for any coffee consumption vs. no consumption regardless of the subjects' sex.
Bai, 2016 [96]		11 studies (340,749 Cn and 2,795 Cc)	August 2015	An inverse association between coffee consumption and cancer risk was observed, with quantitative evidence.
Bravi, 2017 [97]	Hepatocellular carcinoma	12 studies (3,414 Cc)	Uninformed	The meta-analysis provides a precise quantification of the inverse relation between coffee consumption and the risk of cancer.
Kennedy, 2017 [54]		18 cohorts (2,272,642 Pt and 2,905 Cc) and 8 case-control studies, (4,652 Cn and 1,825 Cc)	Uninformed	An extra 2 cups of caffeinated and decaffeinated coffee were associated with reductions of 27 and 14% in the risk of cancer. Increased consumption is associated with a reduced risk of cancer, including preexisting liver disease.
Discacciati, 2014 [98]		3 case-control and 5 cohort studies	July 2013	Inversely associated with the risk of fatal cancer
Lu, 2014 [99]		12 case-control (9,461 Cn and 7,909 Cc) and 9 cohort studies (455,123 Pt)	June 2013	High (highest ≥4 or 5 c/d) consumption may not only be associated with a reduced risk of cancer but also inversely associated with fatal and high-grade cancer.
Cao, 2014 [100]		10 cohort studies (206,096 and Pt8,973 Cc)	June 2013	Coffee consumption may decrease the risk of cancer.
Zhong, 2014 [101]	Prostate	12 case-control and 12 cohort studies (42,179 Cc)	July 2013	An increase of 2 c/d was associated with a 7% decreased risk of cancer. A significant inverse relationship was also found for fatal cases and high-grade cancers.
Huang, 2014 [102]		13 cohort studies	August 2013	A significant reverse association was found between highest versus none/lowest consumption and risk of cancer.
Liu, 2015 [103]		13 cohort studies (539,577 Pt and 34,105 Cc)	Uninformed	Coffee consumption may be associated with a reduced risk of cancer, and it also has an inverse association with nonadvanced cancer.
Xia, 2017 [104]		14 case-control and 14 cohort studies (42,399 Pt)	July 2016	An effect on reducing the localized cancer risk

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Pounis, 2017 [32]		1 cohort study (6,989 Pt and 100 Cc)	March 2005-April 2010	Reduction of 53% lower cancer risk by Italian-style coffee consumption
Chen, 2021 [105]		16 cohort studies (1.081.586 Pt and 57,732 Cc)	September 2020	Higher coffee consumption was significantly associated with a lower risk of cancer.
Zheng, 2013 [106]	Esophageal	24 case-control and cohort studies (7,376 Cc)	October 2011	Borderline significantly inverse association of highest versus non/lowest consumption against risk (protective effects)
Zhang, 2018 [107]		11 studies (457,010 Pt and 2,628 Cc)	January 2017	An inverse association between coffee consumption and incidence of cancer was found in East Asian participants.
Shafiei, 2019 [55]	Ovarian	22 case–control and 20 studies (40,140 Pt)	April 2018	Inverse association between decaffeinated coffee consumption and risk of cancer
Galeone, 2010 [108]	Colorectal	24 studies (14,846 Cc)	May 2010	A moderate favorable effect on cancer risk
Li, 2013 [109]		25 case-control (15,522 Cc) and 16 cohort studies (10 443 Cc)	May 2011	Coffee consumption can significantly decrease the risks of colorectal and colon cancer, especially in Europe and for females.
Tian, 2013 [110]		21 studies case-control and 12 cohort studies	Uninformed	A significant association was found between consumption and decreased risk of colorectal and colon cancer among subjects consuming ≥4 c/d.
Gan, 2017 [60]		19 cohort studies (2,046,575 Pt and 22,629 Cc)	August 2015	Coffee consumption was significantly associated with a decreased risk of cancer at ≥5 c/d.
Nakagawa-Senda, 2017 [111]		2 case-control studies (13,480 Cn and 2,696 Cc)	1988 – 2000 2001-2005	The study found a significant inverse linear trend between consumption and distal colon cancer and a tendency toward a lower risk of rectal cancer.
Micek, 2019 [43]		14 prospective studies (1,381,085 Pt and 28,404 Cc)	August 2018	Restriction to decaffeinated coffee revealed a 15% lower risk of cancer for the highest category consumption. Coffee consumption was related with a decreased risk of cancer in a subgroup of never-smokers and in Asian countries.
Sartini, 2019 [56]		26 prospective studies	Uninformed	Regarding colorectal cancer, a protective effect emerged in US subjects. Concerning colon cancer, a significant protective effect was noted only in European men and only in Asian women. Decaffeinated coffee exhibited a protective effect against colorectal cancer in men and women combined.

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Mackintosh, 2020 [57]		1 prospective observational cohort study (1171 Pt)	2005-2018	Increased consumption of coffee was associated with decreased risk of cancer progression. Significant associations were noted for both caffeinated and decaffeinated coffee.
Um, 2020 [58]		1 prospective cohort study (107,061 Pt and 1,829 Cc)	1999-2015	A higher intake of decaffeinated coffee was associated with a lower risk of colorectal, colon, and rectal cancer.
Sang, 2013 [112]	Liver	9 case-control and 7 cohort studies	May 2012	An inverse association was observed between coffee consumption and cancer.
Yu, 2016 [113]		20 cohort studies from 10 publications	Jan 2016	A significant linear dose-response relationship was found between consumption and cancer risk.
Godos, 2017 [114]		13 studies	March 2017	An inverse correlation was noted between consumption and cancer. Increasing consumption by 1 c/d was associated with 15% reduction in cancer risk.
Tamura, 2019 [115]		6 cohort studies from 5 publications	Uninformed	Consumption among Japanese people has a significant role in preventing cancer.
Tanaka, 2019 [116]		4 cohort and 4 case-control studies	September 2018	Coffee drinking decreases the risk of primary cancer among the Japanese population.
Bhurwal, 2020 [117]		20 prospective studies	June 2019	Higher doses of coffee consumption were associated with a significant decrease in the risk of developing cancer.
Song, 2019 [118]	Brain	11 articles	November 2018	A statistically significant protective effect of consumption and cancer risk was reported.
Creed, 2020 [119]	Glioma	1 prospective study (379,259 Pt and 487 Cc)	2006-2010	A suggestive inverse association was observed with greater consumption of coffee.
Pranata, 2021 [61]		12 studies (1,960,731 Pt and 2,987 Cc).	October 2020	Dose-response meta-analysis showed that every 1 c/d of coffee decreases the risk of glioma by 3%.
Shao, 2019 [120]	Thyroid	10 studies (379,825 Pt and 1,254 Cc)	February 2019	Inversely associated with cancer occurrence in a linear dose-response manner. The occurrence of cancer was reduced by 5% with each 1 c/d increment of coffee consumption.
Sugiyama, 2017 [121]	Bladder	2 cohort studies (73,346 Pt and 274 Cc)	Uninformed	A significant inverse association was observed between coffee consumption and the risk of cancer.

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Wu, 2015 [122]		34 case-control and 6 cohort studies	Uninformed	An increased risk between coffee consumption and cancer was found.
Yu, 2020 [123]		12 cohort studies (2601 Cc and 501,604 Pt)	Uninformed	Positive associations are suggested between coffee consumption and cancer among male smokers but not among never-smokers and females.
Dong, 2011 [124]	Pancreatic	14 studies (669,584 Pt and 1,496 Cc)	August 2010	An inverse relationship was found between coffee drinking and the risk of cancer.
Ran, 2016 [125]		20 cohort studies	June 2015	High coffee consumption is associated with reduced risk.
Nie, 2016 [126]		20 articles	November 2015	Every 1-cup increase was associated with a 1% increase in risk. Coffee consumption may weakly increase the risk of cancer.
Li, 2019 [127]		13 cohort studies (959,992 Pt and 3,831 Cc)	February 2018	Coffee consumption is related to increased risk of cancer in a dose-response manner.
Xie, 2016 [128]	Gastric	9 cohort and 13 case-control studies (1,019,693 Cn and 7,631 Cc)	July 2014	An increase in consumption was associated with a decreased risk of cancer.
Shen, 2015 [129]		8 studies (311,564 Pt and 1,429 Cc)	October 2013	Coffee consumption is associated with the development of cancer. More coffee drinking could result in an increased risk of cancer.
Zeng, 2015 [130]		9 studies, 15 independent prospective cohorts (1,289,314 Pt and 2,019 Cc)	February 2015	High coffee consumption (>6.5 c/d) might increase the risk of cancer in the US population.
Deng, 2016 [131]		13 cohort studies (1,324,559 Pt and 3,484 Cc)	September 2014	High coffee consumption is a risk factor for cancer.
Chen, 2014 [132]	Laryngeal	10 studies (503,234 Cn and 2,803 Cc)	October 2013	Coffee consumption would increase cancer risk.
Kudwongsa, 2020 [133]	Lung	1 prospective cohort study (12,668 Pt and 138 Cc)	1990-2016	Coffee consumption was associated with a reduced risk of cancer. Consumption may be a protective factor for cancer among this cohort.
Tang, 2010 [134]		5 prospective and 8 case-control studies (104,911 Pt and 5347 Cc)	January 2009	Highest consumption was significantly associated with an increased risk of cancer.
Wang, 2012 [135]		9 publications (3,008 Cc)	2005	A significantly positive association was found between coffee consumption and the risk of cancer.

Table 3: Continued.

First author, date	Type of cancer	Study group	Date of completion of included data	Observed effect on coffee consumption
Xie, 2016 [136]		5 cohort and 12 case-control studies (102,516 Cn and 12,276 Cc)	March 2015	Cancer risk is significantly increased by 47% in the population with the highest category intake of coffee compared with that with the lowest category intake.
Zhu, 2020 [137]		17 prospective cohort studies (1.1 million Pt and 20,280 Cc)	Uninformed	Higher consumption of coffee is associated with increased cancer risk.

pooled analyses to bypass the shortage of individual studies [10].

The database of human meta-analysis studies is more abundant today than ever before. Among them, there is a great diversity and due to contradictions between them or the limited scope of their conclusions, they are often not considered, or their interpretation is difficult. For this reason, in this review, we endeavored to make an exhaustive summary of meta-analysis studies (78 out of 115 published in PubMed until April 2021), which found a significant association (positive or negative) between coffee consumption and different types of cancer development, treatment, or advancement.

Table 3 shows meta-analyses, cohort, or prospective studies published between 2010 and 2021. Importantly, the large number of patients involved in these studies provides evidence on the effect of years of coffee consumption on humans. We collected and analyzed more than 75 studies (as shown in Table 3), the vast majority of which (63) reported beneficial effects of coffee consumption, such as less development, metastasis, or mortality from cancer of different origins.

Most publications showed a negative association between coffee consumption and the risk or development of different types of cancer (Table 3, italic rows); these include breast, oral, oral, and pharyngeal, melanoma, skin and skin nonmelanoma, prostate, colorectal, endometrial, liver, leukemic and hepatocellular carcinoma, brain, and thyroid cancer among others (18 cancer types). Instead, a dual association was observed in bladder, gastric, pancreatic, and lung cancer, although only a few publications demonstrated this association. Surprisingly, we found that only 12 publications showed positive associations between cancer and coffee consumption (bold rows), as observed in a few types of cancer, including lung, bladder, pancreatic, laryngeal, and gastric cancer.

Although caffeine has been shown to have anticancer activity both *in vivo* and *in vitro*, many epidemiological studies have published evidence indicating that the risk of cancer decreased even in people who ingested decaffeinated coffee [43, 52–58]. In this sense, Hall et al. discovered that the amounts of many of the bioactive components, such as caffeic acid, chlorogenic acid, ferulic acid, pyrogallic acid, and trigonelline, did not change when decaffeinated coffee was compared to caffeinated coffee [59].

This update reveals a growing body of statistically significant evidence from epidemiological studies, suggesting that coffee drinking in most people (including different sexes, ethnicity, and ages) is beneficial and inversely associated with cancer risk [6, 60]. The fact that we found few publications on the positive association of coffee consumption and cancer does not imply that it is less important but rather suggests that more research should be carried out, as this would make it possible to formulate a clearer hypothesis.

Undoubtedly, moderate coffee consumption of up to 4 cups/day can be enjoyed as part of a healthy, balanced diet and an active lifestyle [9], since health claims associated with its consumption are broad enough to recommend it as a protective beverage. Probing for the detrimental effects of coffee should be focused on further drawing a conclusive approach for end-users to eliminate the ambiguities [29].

Higher doses of coffee have higher benefits in terms of risk reduction. However, further biological and epidemiological studies are required to determine the exact mechanism and analyze the specific subgroups [61]. In addition, it is important to recommend caution to avoid the concomitant use of coffee with drugs that have a significant interaction with coffee. There should be an adequate time interval between the intake of drugs and coffee based on the properties of the drugs. Pharmacists and physicians must be aware of the potential risks of drug-coffee interaction and counsel patients appropriately. Further *in vitro* and *in vivo* studies should be performed on frequently prescribed drugs to obtain robust evidence of the pharmacokinetic interaction with coffee [62].

6. Conclusions

In the last decade of research, the overall view on the impact of coffee on health has shifted from mostly detrimental outcomes towards a beneficial profile. In fact, the data on cancer disease are mostly balanced towards beneficial effects. The subsequent production of more clinical data with a higher number of cases, together with a better understanding of the components of coffee, has contributed to changing this perspective. It can be concluded according to the current knowledge that the labeling of coffee as a mostly unhealthy beverage lacks scientific support.

Throughout this review, we have detailed the main components of coffee, their variation in the roasting process, and the bioactivity of many of them. We provide a wealth of up-

to-date information on the existing experimental evidence for the anticarcinogenic effects of this beverage and a comprehensive review of the important significant evidence for these effects on humans. We have summarized the accumulating epidemiological evidence pointing towards a consistently inverse association between coffee consumption and the risk of breast, oral, pharyngeal, melanoma, endometrial, hepatocellular, prostate, colorectal, liver, and brain cancers, among others.

The prospects for new bioactive compounds and the development of new drugs are focused on natural products. Considering the wide range and depth of evidence already collected, coffee, a beverage that has been widely included in the human diet for years, could be used not only for its well-known taste and stimulant, pleasurable effects, but also for social, pharmaceutical, and clinical purposes, as it provides health benefits and significant effects on cancer treatments.

More research is needed to find the right dosage and balance between the beneficial health effects of coffee (such as anticancer and antioxidant activities) and those traditionally considered negative. However, many of the "harmful" effects noted in antiquity can be attributed to excessive consumption or specific compounds (such as caffeine), which can be regulated or even eliminated from the beverage, thus minimizing the associated risks, without necessarily affecting the important beneficial effects described by the numerous research studies included in this review.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

Authors' Contributions

All authors contributed equally to this work. Ayelén D. Nigra performed the conceptualization and acquisition of data, methodology, validation, formal analysis, investigation, and writing of the original draft, writing and editing of the review, and final approval of the manuscript. Anderson Junger Teodoro performed the conceptualization and interpretation of data, investigation, methodology, resources, writing and editing of the review, supervision, project administration, funding acquisition, and final approval of the manuscript. German A. Gil performed the conceptualization and design of the study, formal analysis and interpretation of data, investigation, methodology, resources, writing of the original draft, writing and editing of the review, supervision, project administration, funding acquisition, and final approval of the manuscript.

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Review Article

Nano-Derived Therapeutic Formulations with Curcumin in Inflammation-Related Diseases

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Due to its vast therapeutic potential, the plant-derived polyphenol curcumin is utilized in an ever-growing number of health-related applications. Here, we report the extraction methodologies, therapeutic properties, advantages and disadvantages linked to curcumin employment, and the new strategies addressed to improve its effectiveness by employing advanced nanocarriers. The emerging nanotechnology applications used to enhance CUR bioavailability and its targeted delivery in specific pathological conditions are collected and discussed. In particular, new aspects concerning the main strategic nanocarriers

employed for treating inflammation and oxidative stress-related diseases are reported and discussed, with specific emphasis on those topically employed in conditions such as wounds, arthritis, or psoriasis and others used in pathologies such as bowel (colitis), neurodegenerative (Alzheimer's or dementia), cardiovascular (atherosclerosis), and lung (asthma and chronic obstructive pulmonary disease) diseases. A brief overview of the relevant clinical trials is also included. We believe the review can provide the readers with an overview of the nanostrategies currently employed to improve CUR therapeutic applications in the highlighted pathological conditions.

1. Introduction

The incidence of chronic diseases, including cardiovascular (CV), cerebrovascular (CeV), neurodegenerative, metabolic, pulmonary, autoimmune, endocrine, and osteoarticular, is alarming growing worldwide [1–6]. In this regard, the widely recognized crosstalk between inflammation, oxidative stress, and excessive proinflammatory cytokine production results in one of the main triggering factors in promoting the onset and progression of the aforementioned chronic conditions [7–9].

Increasing evidence indicates a critical interplay between oxidative stress and inflammation in disease pathogenesis. Reactive oxygen species (ROS) released from inflammatory cells lead to oxidative stress, which is widely recognized as the direct link between the inflammatory process and disease onset and progression [10–12]. Indeed, both ROS and reactive nitrogen species (RNS) boost cell signaling pathways linked to increased proinflammatory gene expression, despite inflammation is regarded as a self-defense response of the human body to hazards, including injuries or allergens [4]. In this regard, synthetic medicines have been widely used for controlling and suppressing inflammation, although they are often associated with a plethora of undesirable side effects. On the other hand, natural antioxidants and anti-inflammatory (AIF) agents have shown instead the ability to achieve the necessary pharmacological impact with the lowest side effects compared to commonly used synthetic drugs [4, 13–15].

Naturally occurred bioactive products have been a source of new therapeutic medications for decades [15–19]. In this regard, curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; CUR) is a bioactive molecule isolated from *Curcuma longa* L. rhizomes with a plethora of therapeutic applications [20]. Synonymously known as diferuloylmethane, CUR is the major polyphenolic compound of *Curcuma* spp. [10, 21] with the ability to target various cell signaling pathways and modulate a wide range of biological activities [22]. Being CUR a natural and virtual nontoxic compound is the object of an intense number of investigations. Among others, CUR's AIF effects result from its ability to interact with multiple molecules and modulate the activity of several intracellular signaling pathways. Indeed, CUR has been reported to interact with the cellular redox status and modulates the activity of several protein kinases. CUR can downregulate inflammatory reaction-related transcription factors, cytokines, and enzymes that promote inflammation, besides to be able to activate the cellular apoptotic process via receptor- and mitochondrial-mediated pathways in a caspase-dependent fashion [23, 24]. Nonetheless, despite its vast therapeutic potentialities, as most of the natural bioactive compounds [25], CUR suffer of low bioavailability, partially due to its poor stability and solubility in the digestive tract, which ultimately restricts its therapeutic uses. Some catalyst compound-based approaches have been used to improve CUR bioavailability, including novel liquid and solid oral delivery systems, which have been tested to counteract both low CUR absorption and faster excretion from the human body [26]. In addition, nanotechnology-based CUR formulations have also been designed and tested for treating various diseases [27–29]. In this context, several types of nanoparticles, including micelles, polymeric nanoparticles, liposomes, solid lipids, nanogels, dendrimers, niosomes, silvers, and cyclodextrins, have been found suitable for CUR loading or encapsulation to improve its effectiveness as therapeutic agent in various diseases [30, 31].

First, extraction methodologies, therapeutic properties, and advantages and disadvantages of CUR employment are briefly addressed in this review. Then, new emerging nanoformulations and nanodelivery systems aimed at improving CUR efficacy against selected oxidative stress- and inflammatory-associated diseases such as wounds, arthritis, psoriasis, colitis, Alzheimer atherosclerosis, asthma, and chronic obstructive pulmonary diseases are collected, analyzed, and discussed.

2. Origin and General Bioactivity of Curcumin

CUR is the dominant polyphenol found in turmeric (*C. longa*) rhizomes and less often in other *Curcuma* species [1, 10, 32, 33]. Turmeric is a perennial herbaceous plant widely grown and cultivated in tropical and subtropical regions of South Western and Southern Asia [32–34]. Turmeric rhizomes contain essential oil (4.2–14%), fatty oil (8.76–12.7%), CUR (up to 5%), and other phytoconstituents [2]. The major turmeric essential oil components are α-turmerone (42.6%), β-turmerone (16.0%), and ar-turmerone (12.9%), which are responsible for its potent antioxidant, antifungal, and antibacterial effects [35, 36].

CUR was formerly isolated from turmeric about two centuries ago, whereas its structure was elucidated around one century ago (in 1910) [3]. CUR is considered the most important turmeric secondary metabolite [4, 37], although two other dominant curcuminoids are also present, namely, demethoxycurcumin and bis-demethoxycurcumin [37]. Column chromatography has been the majorly used technique for CUR separation from the curcuminoid mixture [33]. CUR content in crude curcuminoids' powder is around 76.8%, whereas in recrystallized powder, its purity can be as higher as 99.45% [38].

CUR has a bright yellow-orange color and exists in two tautomeric forms associated with different properties and activities. The keto-enol form is present in neutral or acid solutions, and the enol-form in alkalis solutions (Figure 1). CUR exists in its enolic form in ethanol or other organic solvents, while its keto form predominately presents in water [39]. Over time, and with the increasing number of studies performed, researchers have recognized the methoxy groups on CUR phenyl rings responsible for its therapeutic effects [10].

Turmeric is a key ingredient in both traditional Chinese medicine and Indian holistic systems. China and India population have been using CUR for centuries to treat infectious, skin disorders, depression, and stress [34, 40]. Turmeric is also used as a spice ingredient in curry and a coloring food additive compound, E100 [41]. It is also widely used as an herbal supplement for food and beverage flavoring and coloring [10]. Based on the recent findings suggesting monotargeted therapies less effective than multitargeted ones, turmeric can be considered the ideal "Spice for Life" [3]. Indeed, numerous pharmacological effects have been ascribed to CUR, the most abundant turmeric bioactive compound, including antioxidant, AIF, antitumor, antimicrobial, hypolipidemic, antidiabetic, neuroprotective, and hepatoprotective activities (Figure 2) [1, 37, 40]. But, worth noting is that most CUR's health benefits are mainly ascribed to its AIF and antioxidant properties [3].

CUR oral administration has no toxic effect on animals, and human studies have shown that its intake (up to 6 g/day) caused no toxicity even though prolonged for several weeks [24]. Moreover, based on the Food and Drug Administration (FDA), CUR consumption at a dose as high as 8 g per day is considered safe [10]. Thus, given all these properties, CUR can be regarded as an excellent potential candidate for nutraceuticals and pharmaceuticals formulation. Nonetheless, CUR presents some usage limitations, such as low bioavailability and low water solubility, which markedly limits its uses and therapeutic efficacy [10]. In this sense, as referred above, various strategies have been developed to overcome such obstacles in order to obtain effective CUR formulations [40, 42], with a progressively higher bioavailability and improved applicability [43]. For instance, other natural compounds, such as piperine, well-known for its CUR bioavailability enhancers action, have also been included CUR formulation. In this regard, data obtained so far reveal that piperine inclusion can raise CUR bioavailability by 2000% [10]. Also, more recently, chemistry computations have highlighted that CUR solubility improves in natural deep eutectic solvents [44].

3. General Overview of Curcumin Inflammation Modulatory Properties

C. longa has a very long history of use in the Indian system of medicine, known as Ayurveda, and in Chinese traditional medicine [45, 46]. Epidemiological observations suggest that turmeric consumption exerts protective effects in humans, decreasing the risk of several diseases, especially those associated with chronic inflammation and oxidative stress [34]. These properties are considered paramount in people's modern society modus vivendi characterized by lack of sleep, exaggerate consumption of junk food, alcohol, cigarette smoke, environmental pollutants, and stress (chemical, physical, mechanical, or psychological) which are the most common triggering factors of several inflammatory chronic diseases. Indeed, these factors have been now recognized as the main ones responsible for excessive free radical production and activation of proinflammatory factors, such as TNF and NF-κB [47], which boost neurodegenerative, cardiovascular, pulmonary, metabolic, and autoimmune diseases [3].

Different synthetic drugs have been tested and successfully used to treat these diseases; however, many of them are linked to numerous side effects, which often reduce their effectiveness or are even responsible for therapy failure. [48, 49]. In this light, CUR has appeared as a promising bioactive capable of preventing or controlling oxidative stress and chronic inflammation, either used as a supplement or adjuvant in different pathologies to promote the beneficial effects or reduce the toxicity of synthetic drugs [50–54].

CUR antioxidant and AIF activities are regarded as the key components underpinning this naturally occurring compound's plethora of health benefits [1, 10, 55]. Indeed, CUR's antioxidant activity has been tightly connected to its ability to trigger several intracellular signaling pathways and modulates multiple cell functions. Various preclinical (*in vitro* and *in vivo*) and clinical studies have shown that CUR is a beneficial compound for inflammatory disease treatment and prevention [23, 56]. CUR can indeed promote its antioxidant activity by scavenging ROS and RNS, as well as by regulating the activity of key antioxidant enzyme systems such as superoxide dismutase, catalase, and glutathione reductase [5, 43]. Furthermore, CUR lipophilic properties also make it an excellent peroxyl radicals' absorber with chain-breaking antioxidant properties [10, 11]. On the other hand, this polyphenol is a well-documented AIF agent [23, 57], being able to counteract not only oxidative stress but also inflammation [1]. In this regard, molecular docking studies have underlined that CUR and its analogs act as effective cyclooxygenase- (COX-) 2 inhibitors [58]. Moreover, they also inhibit the secretion of several inflammatory cytokines, such as chemokines, interleukins (ILs), and other inflammatory enzymes, including nitric oxide synthase, thereby attenuating the overall cytokine-associated proinflammatory environment and inhibiting the chronic ROS production [2, 6, 56, 57]. In addition, it has been stated that CUR exerts AIF actions through the suppression of nuclear factor-kappa B (NF-κB) activity and stimulation of peroxisome proliferator-activated receptor-gamma pathway [59] and at the same time inhibiting a number of kinases, including protein kinase C [58]. In this regard, CUR can counteract inflammatory and oxidative processes by inhibiting NF-κB activation (inhibiting IkBα kinase and AKT), which in turn suppresses the actions of all mediators connected with cell apoptosis, proliferation, invasion, and angiogenesis [60, 61]. CUR anti-inflammatory properties result from its modulatory action on several intracellular pathways, as highlighted by its ability to bind both COX-2 and 5-LOX and inhibiting their activity. [62]. Another important target

Figure 1: Turmeric (a) as a source of CUR; tautomeric forms of CUR (b): keto-form (B1) and enol-form (B2).

Figure 2: Biological activities of curcumin.

of CUR's effectiveness is the Toll-like receptor- (TLR-) 4, which engagement activates important signals involved in the immune response modulation, as well as in cytokines and inflammatory chemokine production. INdde, scientific outcomes have shown that CUR mitigates inflammatory response through the direct action on TLR-4 or on its downstream route [42].

To cite some examples, in an *in vivo* study using the carrageenan-induced inflammatory test, cotreatment of *C. longa* and *Allium hookeri* Thwaites extracts resulted in

effective suppression of inflammatory cytokine production and fast recovery of skin morphological changes in rats [5]. It has also been demonstrated that CUR has a crucial role in decreasing endometriosis progression by blocking oxidative stress, inflammation, and angiogenesis [63]. Similarly, turmeric extract supplementation inhibits inflammation and muscle damage in athletes [64], while nano-CUR supplementation exerts inflammation decreasing effects in females with metabolic syndrome [65]. CUR is also a potent inhibitor of the TLR-4-mediated action, which plays a significant role in the immune response regulation by improving inflammatory cytokine production [42]. CUR intraperitoneal injection (100 mg/kg) alleviates acute neuroinflammatory injury in mice via TLR4-mediated mechanism [66]. This polyphenol also modulates essential T-lymphocytes functions [11], indicating to be helpful in the treatment of T-helper-mediated inflammatory and autoimmune diseases [67]. CUR application (25 μM) in glioma cells also triggered the reduction of critical inflammatory mediators such as the activator protein 1 (AP-1) and NF-κB [68]. CUR can also downregulate the secretion of several proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12, thus modulating their target cell functions [68]. Via NF-κB inhibition, CUR can also block the TNF-α-stimulated T-cell attachment to endothelial cells by reducing the expression of vascular cell adhesion molecules (VCAM)-1, intracellular adhesion molecules (ICAM)-1, and endothelial leukocyte adhesion molecules (ELAM)-1 [68].

Taken together, the reported data indicated CUR as a naturally occurring molecule that can effectively alleviate inflammation-associated diseases and their clinical manifestations compared to commonly used drugs [4, 69]. In a clinical study with anterior uveitis-baring patients, 2 weeks of CUR administration triggered a significant disease remission [4]. Also, it was proven that tolfenamic acid and CUR coadministration enhances tolfenamic acid AIF effects while reducing its toxic effects on the stomach and liver [70]. Moreover, CUR has been proposed to alleviate chronic inflammation after chemotherapy or radiotherapy [57]. A recent study revealed that CUR, vitamin C, and glycyrrhizic acid coadministration helps regulate immune response and fight the severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) outcome by preventing the cytokine storm and inhibiting the plethoric inflammatory level [71]. In cases of ulcerative colitis-associated chronic colon inflammation, CUR has also been revealed to promote disease remission by acting in an NF-κB-dependent fashion. Besides ameliorating inflammatory bowel disease, clinical trials have also proven CUR effectiveness in patients with gastric ulcers after 12 weeks of oral administration [4]. Dietary CUR consumption has been reported to attenuate myeloperoxidase (MPO) activity and leucocyte infiltration, simultaneously downregulating the levels of proinflammatory cytokines in intestinal diseases connected with oxidative stress and chronic inflammatory processes [72, 73]. In addition, CUR treatment has been also linked to reduced NO and O_2^- levels along with the inhibition of NF-κB activation in the colonic mucosa, confirming thus its beneficial effects in experimental colitis and valuable application in inflammatory bowel disease treatment.

Following CUR treatment, interesting achievements have been reported in rheumatoid arthritis (RA) [74]. RA is a chronic proinflammatory disease featured by uncontrolled synovial fibroblast growth where smoking and stress have been identified as leading causes [75, 76]. Most RA treatments so far in use are focused on pain and disability reduction, delay in disease progression, and improvement in patients' quality of life. Although AIF and antirheumatic drugs are the best choices for RA treatment [77], they are linked to undesired side effects, significantly reducing patients' compliance. In this sense, CUR has proven to be a valid adjuvant in treating arthritis [78]. Important immunomodulatory properties have been attributed to CUR [79]; among them is the ability to suppress the TNF-α expression in primary chondrocytes reducing cartilage breakdown [80] and inhibit inflammatory processes associated with arthritis [81].

By counteracting the release of essential inflammation mediators [82], CUR may also exert beneficial effects in several skin diseases including psoriasis [83]. In addition, CUR is able to inhibit keratinocyte proliferation, corroborating its effectiveness in psoriasis treatment [84, 85]. Psoriasis is a highly diffuse and painful disorder involving NF-κB, signal transducer, and activator of transcription (STAT)-3 and TNF, which is usually treated with corticosteroids [86]. Nonetheless, as in other diseases, many of the most promising therapies are also linked to undesired side effects and often therapy failure; therefore, CUR can be a valuable and safe alternative. Moreover, CUR has shown significant wound healing properties [87], facilitating tissue remodeling, granulation tissue formation, and collagen deposition [88].

By interacting with redox-regulated copper/iron-bound proteins, CUR can inhibit oxidative stress preventing and counteracting the development of neurological disorders, such as AD and PD, and thus preventing cognitive impairment [89–91]. Furthermore, CUR also counteracts Alzheimer's disease- (AD-) associated damaging plaques and restore injured neurites in a mice model AD [92]. In addition, by suppressing the expression of TLR-4, high-mobility group box 1 protein, and receptor for advanced glycation, CUR can effectively inhibit microglial neuroinflammation in AD patients [93]. Through its antioxidant and AIF properties, CUR has also been shown to counteract atherosclerosis development, reduce myocardial ischemia and/or infarction damages, and prevent chemotherapy-induced cardiotoxicity in cancer patients [94–96].

Despite its numerous therapeutic benefits, physicochemical property-derived CUR poor bioavailability results in its poor absorption and rapid metabolism [97]. Moreover, its high liver metabolism and fast elimination also reduce CUR therapeutic effectiveness, especially in oral-administered formulations [98]. In this context, nanocarriers have appeared as a promising strategy to improve the CUR bioavailability and, consequently, its therapeutic effects. Indeed, thanks to their nanometric size and physicochemical property easy tuning; nanoparticles [99], liposomes

[100–102], micelles, and phospholipid vesicles [103] are capable of potentiating CUR effectiveness at the desired level. In addition, as observed for several naturally occurring compounds, it is worthy of note that CUR antioxidant and AIF effects can be related to its metabolites, tetrahydro-CUR and octahydro-CUR [15, 104, 105]. In this regard, following tetrahydro-CUR and octahydro-CUR administration, a dose-dependent inflammation inhibition was observed in experimental mice models of acute inflammation. Noteworthy, tetrahydro-CUR and octahydro-CUR effect resulted more satisfying than CUR, especially in terms of COX-2 and NF-κB pathway suppression [104].

4. Nanotechnology for Curcumin-Enhanced Efficacy

The achievement of controlled and targeted drug delivery has been studied for many years and appeared as a new challenge for pharmaceutical research, with nanomedicine emerging as a promising tool [106]. Nanotechnology has significantly changed the therapeutic perspective of several drugs, giving new and effective alternatives, especially for treating chronic diseases and many types of cancer [106]. Regardless of their structure and composition, and due to their small size and high surface area, nanosystems can modify the pharmacokinetic features of bioactive molecules, especially those characterized by low bioavailability rates. Nanosystems include nanoscale formulations or nanocarriers, which may ensure passive or active drug targeting, and improve drug circulation time and biodistribution, simultaneously protecting the incorporated drug from exogenous (i.e., light and heat) and endogenous (i.e., acid media, enzymes, and first-pass effect) degradation insults [107]. Moreover, nanotechnological carriers' research attention increased significantly in recent years as emerging nanosystems can coload therapeutic mediators and coordinate the delivery to specific target cells [108].

The promising findings obtained with nanocarrier-based drug delivery systems have raised researchers' interest in improving the efficacy of natural molecules, which are broadly conceived as safer and less expensive than synthetic drugs and effective for treating various diseases [109]. Combining folk medicine-derived biological molecules with new pharmaceutical nanotechnologies has emerged as a significant advance in developing new and safe therapeutic systems [110].

Given its excellent bioactive and therapeutic effects, CUR has aroused the scientific community's attention [22]. However, CUR yield extraction depending on the plant content in bioactive, besides being strongly affected by the extraction methodologies and solvents used [111]. In this regard, although several extraction methods have been reported, many of them are not suitable for industrial applications since they are energy-dissipative or involve toxic extraction solvents.

4.1. Curcumin Extraction Methods. CUR can be separated from *C. longa* rhizomes using different methods. Turmeric grinding and powder extraction has been performed since ancient times. Recently, additional and improved extraction methods have been explored and tested [33, 112, 113]. Among all, solvent extractions followed by column chromatography and Soxhlet, and ultrasonic and microwave extractions have become the most used because of the high reproducibility and extraction [114–116]. Also, a particular focus has been devoted to developing suitable and scalable extraction methods given the CUR rising demands from pharma, food, and cosmetic industries [33]. In this light, less expensive green extraction techniques capable of providing good yield have been chosen and accurately modified by researchers in order to be easily transferred at the industrial level [117]. As a result, pulse ultrasonic and microwave-assisted extraction methods, especially when high temperatures are employed, have been recently tested and chosen as innovative methods to improve CUR yield extraction [114, 118].

4.2. Curcumin-Loaded Nanocarriers. Pharmaceutical nanotechnology embraces intelligent and innovative systems or carriers characterized by nanometric size, which can be obtained using different materials, including polymers (either natural or synthetic), lipids, oils, surfactants, and other additives [119]. Main challenges that must be considered during nanocarrier formulation are payload physicochemical properties and biological barriers and defense mechanisms activated by the human body [120]. Among the different carriers tested for CUR delivery, phospholipid vesicles have largely been used and demonstrated to be the most effective in improving its stability and bioavailability irrespective of the chosen administration route [121–123]. The most used phospholipid vesicles are liposomes, mainly composed of phospholipids and water [124]. Due to their composition, they are highly biocompatible and can be appropriately modified with other additives such as water cosolvents, surfactants, lipids, polymers, and fibers to improve their delivery performance.

5. Curcumin Nanoformulations in Inflammatory Diseases

5.1. Curcumin-Loaded Nanocarriers in Pulmonary Ailments. It has been reported that CUR-loaded phospholipid vesicles can significantly improve CUR's anti-inflammatory properties, enhancing its overall therapeutic efficacy [99, 125]. In this light, new CUR-loaded phospholipid vesicle formulations and studies testing their potential in treating pulmonary disorders, such as asthma or chronic obstructive pulmonary diseases, have progressively increased. For instance, Manca et al. [126] formulated and used glycerosomes, which are vesicles containing high amounts of glycerol employed for CUR lung delivery through aerosol therapy. In this study, glycerosome formulation was improved by adding sodium hyaluronate or trimethyl chitosan chloride to ameliorate vesicle stability and performances during aerosolization process. The improved polymer-glycerosomes could deliver CUR in the last stages of the next-generation impinger to a better extent than regular glycerosomes. Moreover, glycerosomes in general and polymer-

glycerosomes in particular, significantly improved CUR effectiveness by (i) inhibiting proinflammatory cytokine production (IL-6 and IL-8) and protects oxidatively stressed A549 cells in vitro and (ii) increasing CUR deposition in the deeper respiratory tract *vivo*. Similarly, Manconi et al. [127] formulated chitosan- and hyaluronan-coated liposomes for CUR pulmonary delivery and addressed carriers' influence on its effectiveness against oxidative stress. CUR incorporation in liposomes or polymer-coated liposomes significantly promoted CUR lung deposition and improved its antioxidant power, a phenomenon likely due to vesicles' ability to interact with cells and release CUR in the cytoplasm. CUR-loaded liposomes were also tested as an antiasthmatic system [128], leading to a significant reduction of inflammatory markers, such as IL-6, IL-8, IL-1β, and TNF-α compared to positive control. In this regard, the lower CUR-tested dosage (1 μg/mL) reduced the inflammatory markers release to a better extent than higher doses, which is not surprising considering that natural compound-beneficial effects are now recognized to be influenced by several factors, including dose and redox environment [129–135]. Other studies also demonstrated that CUR liposomal formulations effectively reduced the expression of proinflammatory markers (IL-6, IL-8, and TNF-α) in human synovial fibroblasts and mouse macrophages (RAW264.7) stressed with LPS [136, 137]. Cytokine storm, which refers to the increased secretion of cytokines such as IL-1β, IL-6, TNF-α, and IL-18 is a characteristic of COVID-19 patients with lung damage [138, 139]. In this regard, a clinical trial performed on COVID-19 patients indicated the ability of a nanomicellar form of CUR to significantly decrease the mRNA expression and cytokine secretion levels of IL-6 and IL-1β [140], which may ameliorate disease's clinical manifestation and promote overall recovery.

5.2. Curcumin-Loaded Nanocarriers in Skin Ailments. Curcumin has also been incorporated in phospholipid vesicles tailored for skin applications. Hyalurosomes, a new class of phospholipid vesicles immobilized with sodium hyaluronate, have been specifically formulated to treat skin wound-associated inflammatory and oxidative processes [102]. Thanks to their peculiar structure and viscosity, hyalurosomes vesicles could incorporate a high CUR amount and retain it over 3 months of storage [102]. CUR-loaded hyalurosomes significantly improved CUR antioxidant activity being able to effectively protect keratinocytes from oxidative stress and even promoting cell proliferation [102]. Hyalurosomes also promoted CUR accumulation in different skin strata and wound healing *in vivo* in a mouse model of 12-O-tetradecanoylphorbol-13-acetate- (TPA-) induced lesions by inhibiting edema and MPO activity [102]. CUR-loaded phospholipid vesicles have also been used to reduce psoriasis-associated inflammatory and oxidative processes. In this regard, vesicles facilitated lipophilic payload penetration in different skin layers, ensuring its delivery in the damaged site [141, 142]. Recently, Zhang et al. [143] formulated hyaluronic acid-enriched ethosomes as topical systems for the treatment of psoriasis. In this work, hyaluronic acid was added to vesicle surface as it can interact with CD44 protein, which is overexpressed in inflammation- and oxidative stress-associated diseases and can be considered a potential targeting system capable of increasing both CUR skin retention and efficacy [144]. As expected, the CUR cumulative amount detected in the skin following hyaluronic acid-modified ethosome application was very high. This result may be due to the ethosomal bilayer's high flexibility that may overcome the stratum corneum barrier and reach the deepest skin strata, especially in the dermis, where psoriatic skin lesion-associated inflammatory cytokines, such as IL-17 and -22, are mainly located. *In vitro* results were confirmed by confocal observation of CUR accumulation in the skin, which was more evident in the deeper skin strata when hyaluronic acid-associated liposomes were used [144]. In particular, the CUR-associated fluorescence was preferentially located in the epidermis, where CD44 is highly expressed in psoriasis-like skin, thus promoting improved CUR accumulation at the inflammation site. Improved skin CUR accumulation is also linked to a keratinocyte's CUR significant uptake mainly because its incorporation into these flexible polymer-associated liposomes promotes "vesicle-cell" interaction and CUR internalization [144]. CUR-loaded chitosan nanoparticles linked with epidermal growth factor (EGF) were synthesized to develop an EGF-modified spray solution (EGF@CCN) for treating skin wounds [145] Such nanoformulation tested in a Wistar rats' model of full-thickness dermal defect shows the ability to promote an almost complete wound healing after 12 days postoperation [145].

CUR-alginate-based nanomicelle (C-A-NM) also show colonic wound healing properties in rats as evidenced by both histopathology/colonoscopy evaluation and increased protein and collagen synthesis in damaged sites [146]. C-A-NM also increased TGFβ1 expression while decreasing that of NFκB, a phenome that may explain the observed healing effect [146]. A randomized clinical trial performed with different CUR nanoformulation showed that CUR-loaded nanostructured lipid carriers (NLC) are able to provide a better drug delivery and physiological skin parameters ameliorations as compared to nanosized emulsions based on monoacyl-phosphatidylcholine (MAPL) [147]. Further clinical studies are needed to better understand the optimal nanoformulation able to provide the best therapeutic results in the different pathological conditions.

5.3. Curcumin-Loaded Nanocarriers in Rheumatoid and Osteoarthritis. CUR-loaded hyalurosomes were also tested as topical carriers for the treatment of RA [148]. Briefly, RA is a chronic inflammation due to an uncontrolled proliferation of fibroblast-like synovial cells responsible for the release of proinflammatory cytokines [149]. Treatment of fibroblast-like synovial cells with CUR-loaded hyalurosomes significantly inhibited IL-15 and IL-6 production, key molecules in RA pathogenesis. Moreover, CUR-loaded hyalurosomes stimulated the production of IL-10, which is considered the most important AIF cytokine. CUR-loaded hyalurosomes also suppressed NF-κB release and ROS production, confirming the double effect of this CUR

formulation in reduction of joint damage by inhibition of TNF-α and ROS generation [148].

CUR-loaded liposomes enriched with cholesterol have been formulated for the treatment of osteoarthritis. Data indicated that this new CUR nanosystem formulation provided improved 7F2 osteoblastic cell survival and bioactive accumulations into cells [150]. They were also capable of inhibiting NO production in stimulated RAW264.7 macrophages and preventing osteoclast differentiation by downregulating cathepsin K and tartrate-resistant acid phosphatase (TRAP) expression. Moreover, reduced levels of IL-1β-induced COX-2 and MMP-3 were also detected in 7F2 osteoblasts exposed to these CUR-loaded cholesterol-enriched liposomes. Therefore, CUR-loaded liposomes may have a promising effect against subchondral bone turnover slowing the osteoarthritis progression [150]. In this regard, a recent clinical trial reports that a nanomicellar formulation of CUR significantly improves the symptoms of osteoarthritis patients [151]. Indeed, an overall improvement of pain, stiffness, and physical activity subscales of the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire were found in treated patients compared with the placebo group [151].

5.4. Curcumin-Loaded Nanocarriers in Neurodegenerative Disorders. The antioxidant and AIF potential of CUR-loaded nanosystems has also been proven in the treatment of neurodegenerative disorders [152]. Wheat germ agglutinin-conjugated liposomes incorporated with cardiolipin reduced SK-N-MC cell neurodegeneration and amyloid-β plaque deposition providing neuronal protection in a rodent model of AD [153]. In another study, Sokolik [154] demonstrated that CUR-loaded liposomes reduced angiotensin-converting enzyme activity in brain-targeted regions potentiating memory recovery in rats with AD. A set of clinical trial provided evidence regarding the ability of ω-3 fatty acids and nanocurcumin combination to significantly reduce migraine attack frequency [155–158]. The drug combination was able to reduce the serum levels of VCAM and C-reactive protein (CRP) along with proinflammatory cytokines IL-1β and IL-6, which may be the mechanism at the basis of the reported therapeutic effect [155–158].

5.5. Curcumin-Loaded Nanocarriers in Cardiovascular Diseases. CUR-loaded phospholipid vesicles have also been proven to be effective in the treatment of CV diseases. It is now widely accepted that CV diseases such as hypertension and atherosclerosis result from endothelial cell dysfunction induced by inflammatory factors and plasma lipid deposition at the damaged sites [159, 160]. Liposomes specifically designed to codeliver atorvastatin calcium and CUR for atherosclerosis treatment have been recently proposed [161]. Atorvastatin calcium is currently used as an antiatherosclerotic drug, and CUR is a safe adjuvant capable of promoting antiatherosclerotic effects and reduces atorvastatin calcium cytotoxicity. Liposomes were surface modified with specific ligands capable of targeting vesicles at the desired level to improve payload antiatherosclerotic and AIF effects. Indeed, the combination of the two active substances effectively reduced atherosclerotic areas and proinflammatory factor levels [161]. Moreover, the vesicle surface functionalization further improved both drugs' effectiveness suggesting their possible use for preventing or treating endothelial cell disfunction-associated CV diseases. Consonant with the above-reported findings, the AIF, antioxidant, and CV protective effects of CUR-loaded liposomes have been previously reported [29, 125]. A recent clinical trial reports that CUR nanomicelle (80 mg/day) ameliorates lipid profile and oxidative and inflammatory markers in patients undergoing coronary angioplasty [162]. The same trial indicates that the CUR nanoformulation provides significantly better result in lowering triacylglycerol (TG), total cholesterol (TC), malondialdehyde (MDA), superoxide dismutase (SOD), and tumor necrosis factor-alpha (TNF-α) levels as compared to CUR alone (500 mg/day) [162]. Another trial performed in hemodialysis patients shows that nanocurcumin (120 mg/day) decreases the serum levels of CRP, along with VCAM-1 and ICAM-1, two proinflammatory adhesion molecules involved in endothelial dysfunction [163]. CUR nanomicelle also significantly improve the levels of TG in patients with metabolic syndrome, while failed to ameliorate other biochemical parameters such as TC, LDL-C, HDL-C, fasting blood sugar (FBS), hemoglobin A1c (HbA1c), homeostatic model assessment (HOMA) for insulin resistance (HOMA-IR), and pancreatic β cell function (HOMA-β) [164]. The various protective effects of CUR-loaded nanocarriers on different human body ailments are illustrated in Figure 3.

6. Role of Nanocarriers in Curcumin Bioavailability and Stability

Due to its low absorption and degradation at gastric and liver levels, the lowest bioavailability of CUR is generally obtained following oral administration [165]. However, since oral administration is the patients' preferred route of administration, many efforts have been carried out to find effective oral formulations of CUR, especially using nanocarriers. For this purpose, CUR has been loaded in phospholipid vesicles enriched with fibers and polymers called nutriosomes [166]. Nutriosomes resulted stable in acidic and neutral environments mimicking the gastrointestinal tract and improved the protective activity of CUR against hydrogen peroxide-stressed CaCo2 cells. These findings appear mainly connected with an enhanced "nutriosome-cell" interaction confirmed by the improved vesicle internalization detected by confocal microscopy. Nutriosomes increased the *in vivo* CUR biodistribution and reduced the 2,4,6-trinitrobenzene sulfonic acid- (TNBS-) induced intestinal inflammation in rats [167].

In a similar study, CUR was incorporated into eudragit-hyaluronan multicompartment liposomes aiming at improving its intestinal bioavailability [100]. The long-term vesicle stability was ensured by their lyophilization. However, they can be easily rehydrated in liquid or semisolid foods to obtain an extemporaneous CUR-enriched food with beneficial properties for human health [100]. Due to their multicompartmented structure, these vesicles have shown

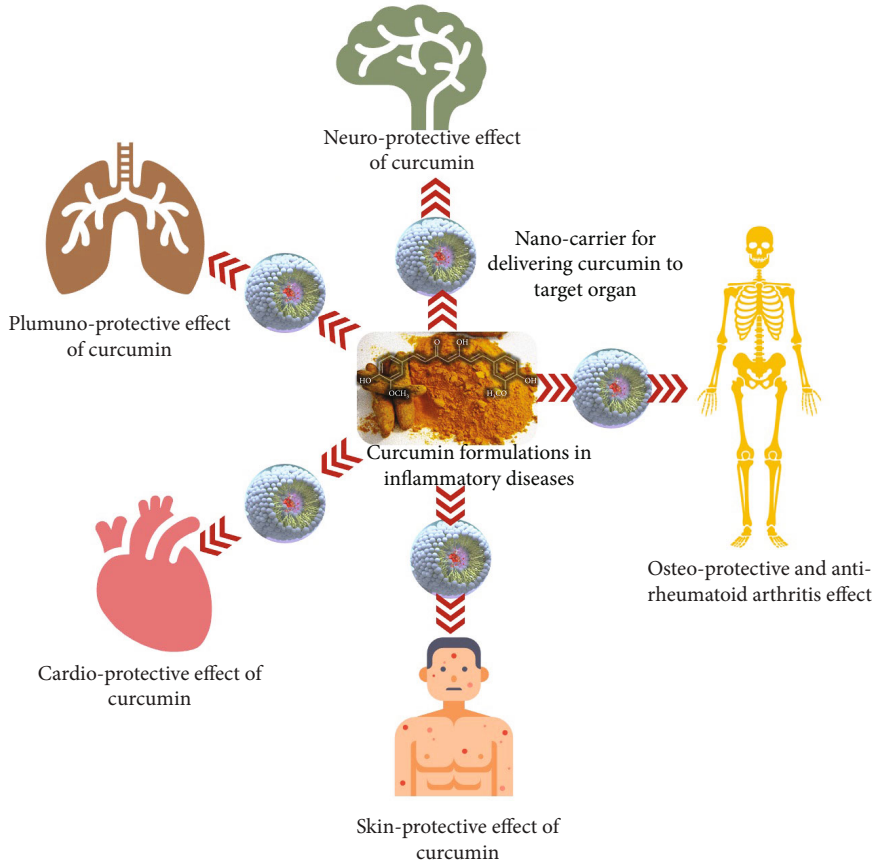

Figure 3: Protective effect of curcumin-loaded nanocarriers on various ailments in human body.

remarkable stability under the gastrointestinal tract harsh conditions, such as high ionic strength and pH variations, and ensured a more significant CUR deposition in the intestines in comparison with CUR dispersion. Indeed, CUR-containing vesicles could reach intact intestines to exert the potential therapeutic effect by releasing their payload [100]. The use of polymers as a phospholipid vesicle stabilizer seems promising for formulating delivery systems specifically designed for oral administration. In this regard, De Leo et al. [168] prepared Eudragit S100-coated liposomes with the intent to improve vesicle stability. The coating process allowed a polymer layer formation, which efficiently protected both the liposomes and the incorporated CUR. Indeed, CUR released experiments have proved the polymeric layer high stability and ability to protect CUR against degradation after treatment of coated liposomes with bile salts capable of destroying liposomes [168]. Moreover, eudragit-coated liposomes also preserved CUR antioxidant property under the gastrointestinal tract's harsh conditions as its antioxidant activity was detected only when the layer was dissolved in basic pH condition mimicking the intestinal environment.

7. Conclusions and Future Perspective

This review's finding highlights the CUR potential as a natural and safe bioactive for treating different inflammation- and oxidative stress-related diseases. Nonetheless, numerous studies highlighted the CUR limitations when employed as a free drug because of its low bioavailability and regardless of the administration route chosen. In this light, studies developing and investigating CUR nanoformulations as a way to improve its bioavailability and effectiveness are exponentially increasing. In this regard, liposomes and derived phospholipid vesicles disclosed promising performance in CUR delivery to the lungs, gut, and skin. Overall, data obtained in the last years confirmed the potential of phospholipid vesicles as ideal carriers for CUR, especially when they are ad hoc formulated for a specific route of administration and a particular disorder by using appropriate additives or ligands. Noteworthy, most of the available literature reports in vitro or animal models data while in vivo clinical studies are so far scarce. Nonetheless, some of the available clinical trials provide the first evidences that CUR nanoformulations may wield better result in terms of therapeutic effect as compared to CUR alone. Moreover, nanocombinations of CUR with other natural compounds or currently used drugs appear another promising way to improve the overall therapeutic effect of the employed compounds. In this regard, more research effort should be directed at understanding whether CUR nanoformulations may be routinely included into the standardized therapeutic treatments to reduce the amount of the main drugs with the intent to obtain improved therapeutic effect with less toxicity. Moreover,

simple, cost-effective and scalable technologies to produce effective CUR nanocarrier should also be developed in order to make the "nanodrug sector" competitive in the pharmaceutical industry towards the classic drugs. As evidenced by this review, to better maximize CUR efficacy, different and specific nanoformulations are required, and larger and deeper clinical studies should be carried out to understand its pharmacokinetic behavior and *in vivo* therapeutic effects.

Data Availability

The data supporting this review are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

N.C.-M., W.C.C., and J.S.-R. are responsible for the conceptualization and design; C.Q., N.C.-M., M.L.M., M.M., O.S., N.H., M.S., G.P., M.K., M.M., Y.T., W.C.C., and J.S.-R. for the validation investigation, resources, data curation, and writing; N.C.-M., M.K., G.P., W.C.C., and J.S.-R for the review and editing. All the authors read and approved the final manuscript.

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Review Article

Therapeutic Potential of Isoflavones with an Emphasis on Daidzein

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Daidzein is a phytoestrogen isoflavone found in soybeans and other legumes. The chemical composition of daidzein is analogous to mammalian estrogens, and it could be useful with a dual-directional purpose by substituting/hindering with

estrogen and estrogen receptor (ER) complex. Hence, daidzein puts forth shielding effects against a great number of diseases, especially those associated with the control of estrogen, such as breast cancer, diabetes, osteoporosis, and cardiovascular disease. However, daidzein also has other ER-independent biological activities, such as oxidative damage reduction acting as an antioxidant, immune regulator as an anti-inflammatory agent, and apoptosis regulation, directly linked to its potential anticancer effects. In this sense, the present review is aimed at providing a deepen analysis of daidzein pharmacodynamics and its implications in human health, from its best-known effects alleviating postmenopausal symptoms to its potential anticancer and antiaging properties.

1. Introduction

Nutraceuticals contain selective combinations of specific plant-derived bioactive components with renowned medicinal, disease-preventing, and/or health-enhancing properties. Such compounds include polyphenols, carotenoids, flavonoids, isoflavonoids, terpenoids, glucosinolates, phytoestrogens, and phytosterols. Studies on these phytochemicals have also shown positive pharmacological activities in human health [1].

Regarding phytochemical-rich plant sources, and to what concerns to isoflavonoid sources, soybeans and other leguminous plants are the main sources of active isoflavones genistein and daidzein [2]. Daidzein [7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one] (Figure 1) is a naturally occurring phytoestrogen fitting into the category of nonsteroidal estrogens [3], with numerous pharmacological activities, such as antihemolytic, antioxidant, and anti-inflammatory activities [4, 5]. Daidzein can be found in soy-derived food products such as soy-based infant formulas, soy flour, textured soy protein, soy protein isolates, tofu, tempeh, and miso. In addition, soy flour is used for the fortification of other flours, including wheat, rice, and corn. The daidzein content of these products is quite variable, i.e., the daidzein amount is 22 mg in a half cup of miso, 15 mg in 3 ounces of tempeh, 8 mg in 3 ounces of tofu, and 7 mg in one cup of soy milk [6].

The chemical structure of daidzein is analogous to mammalian estrogens, making it a promising candidate for a dual purpose by substituting/hindering such hormones and their corresponding receptors. Hence, daidzein could be a therapeutic strategy for estrogen-dependent health conditions, such as breast [7] and prostate [8] cancer, diabetes, osteoporosis, and cardiovascular disease (CVD) [9]. However, daidzein also has other estrogen receptor- (ER-) independent biological activities, for instance, the ability to reduce oxidative damage, regulate the immune reaction [10], and induce apoptosis, directly linked to their anticancer effects [11]. Thus, such activities, along with minimum toxicity features, make daidzein a promissory compound for drug design. In this sense, the present review is aimed at providing an in-depth overview of daidzein's potential use to prevent or treat some burdening human health conditions. First, we focus on daidzein's pharmacodynamics and current limitations for its use. Then, we briefly describe some proposed mechanisms of action, and finally, we review its implications in human health showing the latest research in the field, namely, focusing on its ability to alleviate postmenopausal symptoms, and its potential anticancer and antiaging properties.

2. Daidzein Pharmacodynamics

Daidzein is predominantly found in soy and many unfermented foods not only in the form of daidzin, a glycoside conjugate [12, 13], but also as acetylglycoside and aglycone [14]. Daidzin is not directly absorbed in the gut and instead needs to be hydrolyzed into the aglycone form daidzein [15] by β-glucosidases in the small intestine [16]. The aglycone form is either absorbed or metabolized to different types of metabolites by human gut bacteria, including dihydrodaidzein [15], equol, and *O*-desmethylangolensin (*O*-DMA, a metabolite with no estrogenic activity) (Figure 2) [17]. This intestinal biotransformation is accomplished through several reactions, such as reduction, methylation and demethylation, hydroxylation, and C-ring cleavage [18]. The absorbed aglycone is metabolized mainly to glucuronidated derivatives and, to a lesser extent, into sulfated conjugates by phase I and II enzymes [19–21]. Then, these metabolites can be further metabolized in the liver or might be secreted into the bile and recycled [22]. Finally, both the unabsorbed daidzein and biliary derivatives that reach the colon undergo deconjugation by bacterial enzymes and are then reabsorbed or metabolized [18, 22–25].

Studies on daidzein absorption, bioavailability, distribution, and excretion are still limited [15, 26, 27], with data obtained so far revealing the appearance of a small peak in plasma around 1 h after ingestion, with daidzein being absorbed in the small intestine [28]. A larger peak appears after 5-8 h, from the conjugates recycling and colon absorption. Interestingly, daidzein can be found in plasma mostly in its conjugated form and a small proportion in the form of aglycone [29]. A clinical study showed that daidzein ingestion in the form of glucoside results in higher bioavailability than consuming the aglycone form [30], while a previous study showed contrary data [31]. These controversial results could be explained by differences in the type of glycosides or the influence of other isoflavones on their metabolism [32]. Regardless of these studies, it seems that daidzein reaches the maximum concentration in plasma approximately 7 h after ingestion [33], which appears to be directly linked to its complex absorption process. Finally, a study by Setchell et al. [33] suggested that almost all daidzein is rapidly absorbed and metabolized, as the excretion in feces and urine was minimal, although up to 30% of daidzein intake can be recovered in urine.

Regarding daidzein's biological activity, as well as of other isoflavones, it is highly dependent on their biotransformation, and huge differences have been stated in daidzein metabolism between humans, rats, and mice, which suggest that not all research studies regarding daidzein and its effects

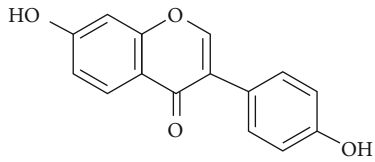

Figure 1: Chemical structure of daidzein.

can be extrapolated to humans. In humans, glucuronides are the major plasma phase II metabolites and the proportion of plasma daidzein and other aglycones (0.5-1.3%) is significantly low compared to other animals [21].

Several factors, such as age, gender, or diet, have been described to influence the bioavailability of isoflavones in humans. For instance, the main source of isoflavones among the Asian population is fermented soy products, which contain isoflavones in the form of aglycones and can be directly absorbed. On the other hand, in the Western diet, the principal source is cooked soybeans, soy milk, and vegetable proteins, which contain the glucoside form [34]. Interestingly, an increased daidzein intake or its prolonged consumption appears not to change its bioavailability or pharmacokinetics (Setchell, Faughnan, Avades, Zimmer-Nechemias, Brown, Wolfe, Brashear, Desai, Oldfield, Botting and [3]).

Another important factor that determines daidzein bioavailability is the different food matrices used [26, 35]. Cassidy et al. [36] showed that daidzein absorption is faster when consuming soy milk, with glucoside conjugates, than solid soy food, with a significant difference of 2 h. Another study found that insoluble fiber, such as inulin, might increase the daidzein absorption [37, 38], partly due to bacterial growth stimulation [9]. However, there is a key aspect of daidzein metabolism that needs to be considered when studying its potential benefits. Plasma levels do not correlate well with the concentration that may effectively reach the different tissues. In fact, the quantification of isoflavones and their derivatives in human tissues is not usually determined and can vary in a large extent [24]. For instance, in humans, equol levels range between 22 and 36 nmol/kg in breast adipose tissue and 456-559 nmol/kg in glandular tissue [23, 39].

These complex daidzein pharmacokinetic features, along with its insolubility in water and oil, have blocked their use as a highly common compound in medicine or as a nutraceutical. Thus, several strategies have been developed to enhance daidzein's bioavailability, including emulsifying formulations or encapsulation with cyclodextrins [9]. For example, Peng et al. [40] designed fat-soluble derivatives by sulfonic acid esterification and stated that these are able to improve both daidzein cell uptake and its biological activities. Several techniques for modification of natural compounds are being developed and are discussed in other reviews [41, 42].

Equol (4′,7-isoflavandiol) is the daidzein metabolite that shows the strongest biological activity. Only a small percentage of the world population can metabolize daidzein to equol by gut bacteria [43]. The equol nonproducers, which have a prevalence between 80 and 90% in human subjects, convert a large part of daidzein into *O*-DMA [18]. Equol and *O*-DMA are likely produced by different bacterial taxa. Lu and Anderson [44] documented that only 30% of their study population presented equol conjugates in urine following soy administration, and no differences were reported regarding the type of diet. Furthermore, a prolonged soy intake led to the ability to produce equol in a small fraction of equol nonproducer women. In this regard, some known factors that limit the ability to produce equol are ethnicity and dietary habits [18]. For instance, up to 50-70% of the Asian population are equol producers, compared to only 20-30% of Western individuals [45]. Brown et al. [46] suggested that the ability to produce equol is developed during the first years of life, and it appears to be related to the diet composition in the early years, as they observed that breastfed infants showed the lowest percentage of equol producers. Some other studies have tried to improve equol production by dietary habit modification. For example, Kruger et al. [47] analyzed the effects of a supplement of isoflavones with kiwifruit, expecting to see an improvement in equol production. Surprisingly, supplementation with kiwifruit had no effect on equol production and, in fact, attenuated the effects of isoflavone supplementation on reducing high-density lipoprotein (HDL) levels in postmenopausal women. Fructo-oligosaccharide supplementation also failed to increase equol production in postmenopausal Japanese women [48].

So far, most equol-producing bacterial strains belong to the family of *Coriobacteriaceae*, and they include *Adlercreutzia equolifaciens*, *Asaccharobacter celatus*, *Enterorhabdus mucosicola*, and *Slackia isoflavoniconvertens* and *Slackia equolifaciens*. Other equol-producing strains have also been identified, namely, *Bifidobacterium*, *Lactobacilllus*, *Lactococcus*, *Pediococcus*, and *Proteus* species [18]. The involvement of gut microbiota in daidzein metabolism highlights the importance of analyzing how diet and, specifically, how soy products can affect the balance of such microorganisms and understand the triggers for individual differences [43]. For instance, a recent study showed that isoflavone administration did not change the copy number of *Coriobacteriaceae* species in feces regardless of diet [18]. Iino et al. [49] reported that daidzein intake increased with age, as also the ability to produce equol. Interestingly, both equol producers and nonproducers held equol-producing bacteria, although the relative abundance of 2 species, namely, *A. celatus* and *S. isoflavoniconvertens*, was significantly higher in equol producers.

3. Daidzein Pharmacological Activities: Emphasis on Clinical Evidence

Epidemiological data suggest that isoflavone consumption may have health benefits and lower the risk of some age-related diseases, including osteoporosis, CVD, and several types of cancer, as well as reduce menopause-associated symptoms [18]. Table 1 resumes the different human studies reporting the effects of daidzein or isoflavones in several disorders.

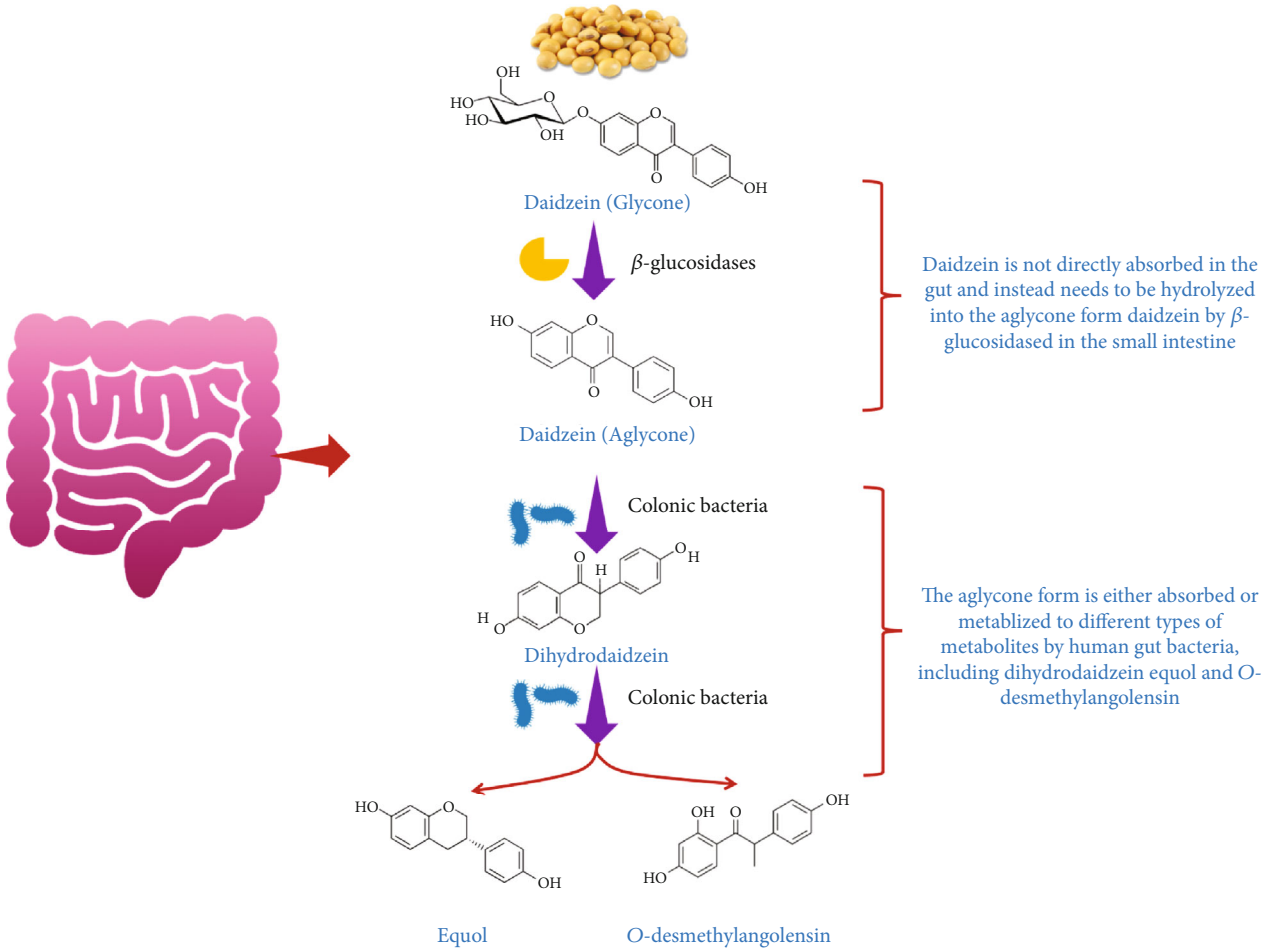

Figure 2: Biotransformation of daidzein in human gut.

In the Asian population, with a predominance of soy products in their diet, isoflavone intake can be up to 50 mg/day, while in Western countries, it is less than 2 mg, although it may be higher in menopausal women [109]. As a phytoestrogen, daidzein may induce its effects through the interaction with ERs, as it possesses a strong similarity with 17-β-estradiol (E2), the main female sex hormone. Two ER subtypes, namely, ERα and ERβ, have been described with different tissue distribution and ligand-binding affinities. ERα is mainly found in breast and uterine tissues and has been associated with higher cell proliferation. On the other hand, ERβ is the predominant isoform in the brain, bones, and blood vessels and is related to cell differentiation. Thus, to assess the overall effects of daidzein or any other phytoestrogen, the ERα/ERβ ratio needs to be considered, as the cell response may considerably differ from one tissue to another [110, 111].

Both daidzein and equol are ERα and ERβ agonists, with higher affinity for the latter, and can interfere with their signaling pathway. However, other ER-independent signaling mechanisms have been described, including protein kinase regulation, enzymatic inhibition, growth factor modulation, antioxidant activity, or epigenetic changes [111].

3.1. Daidzein and Allergies. Although estrogens are known to regulate the immune response, epidemiologic studies assessing the association between dietary isoflavones and allergic disorders are still limited. Miyake et al. [50] suggested that soy consumption and daidzein may reduce allergic rhinitis in Japanese women, although there was no dose-response effect. On the other hand, other products, such as tofu or fermented soybeans, showed no differences in allergic rhinitis prevalence. Nonetheless, it must be taken into account that soy is a strong food allergen, thus its consumption may be counterproductive when it comes to allergic disorders. Smith et al. [51] evaluated the soy isoflavone supplementation in poorly controlled asthmatic patients and found no differences in lung function between control patients and patients with isoflavone supplementation.

3.2. Effects of Daidzein on Osteoporosis and Menopausal Symptoms. Osteoporosis has a high incidence among menopausal women as estrogens regulate bone metabolism and ultimately prevent bone loss. Thus, the reduction in estrogens is associated with a higher risk of osteoporosis, and hormone replacement therapy has been proposed as a solution to reduce such risk [112]. In this regard, soy isoflavones have also been studied to prevent osteoporosis. Indeed, isoflavone supplementation for 4 and 6 months in

Table 1: Pharmacological activities of daidzein reported in human studies.

Pharmacological activity	Study type	Dose/type of treatment	Results	References
Antiallergic	Cross-sectional study	Regular soy products consumption	Possible reduction in allergic rhinitis incidence	[50]
	RCT	100 mg isoflavones/day, 24 weeks	No effects	[51]
Antiosteoporotic	CT	59.6 mg genistein + 15.6 mg daidzein/day, 6 months	Increase in bone mineral density in lumbar spine and decrease bone resorption biomarkers	[52]
	CT	70 mg isoflavones/day, 12 weeks	Increase in bone formation markers	[53]
	CT	60 mg genistein + 60 mg daidzein + 16.6 mg glycitein, 5 days/week, 2 years	No direct effects on serum calcium levels	[54]
	RCT	105.23 mg isoflavones/day, 50 days	Increase in calcium retention	[55]
	CT	136.6 mg isoflavones, 5 days/week, 2 years	Decrease in mineral bone density with low calcium levels	[56]
	SR	Different isoflavone extracts, 7 weeks up to 3 years	Bone health improvement and blocked mineral density loss	[57]
Menopausal symptoms reduction	RCT	10, 20, or 40 mg equol/day, 8 weeks	Reduced hot flash frequency and improved muscle and joint pain	[58]
	RCT	10 mg equol, 1 or 3 times/day, 12 weeks	Anxiety scores reduction	[59]
	CT	10 mg equol/day, 12 weeks	Reduced hot flash frequency and severity, improved sweating and muscle stiffness	[60]
	RCT	135 mg isoflavones/day, 1 week	Improved hot flashes, sweating and fatigue	[61]
	CT	100-200 mg isoflavones/day, 12 weeks	Improved hot flash intensity	[62]
	CT	63 mg daidzein/day, 6 months	Improved renal function	[63]
	RCT	150 mg isoflavones/day, 16 weeks	No effects on menopausal symptoms	[64]
	SR	Different isoflavones extracts, 3-12 months	Possible reduction in hot flash frequency	[65]
	SR	Different isoflavones extracts, 12 weeks to 4 years	Reduced hot flash frequency, improved vascular health, no effects on urogenital symptoms	[66]
Anticancer	Cohort study	Regular soy products consumption, 23 months	Reduced recurrence in tamoxifen-treated breast cancer patients	[67]
	Cohort study	Regular soy products consumption, 3.9 years	Reduced mortality and recurrence of breast cancer	[68]
	Meta-analysis	Regular isoflavones consumption	Decreased risk of breast cancer	[69]
	Review with meta-analysis	Regular phytoestrogen consumption	Possible role in survival from breast cancer	[70]
	CT	51.6 g soy protein/day (1.2 mg genistein/g protein, and 0.8 mg daidzein/g protein), 7-30 days	Increased markers of cell proliferation in breast cancer patients	[71]
	RCT	154 mg isoflavone soy protein/day, 3 years	No effects on endometrial cancer	[72]
	Cohort multicenter study	25 g isoflavone/day, 5 years	No effects on endometrial cancer	[73]
	RCT	80 mg isoflavones/day, 6 weeks	No changes in PSA in prostate cancer patients	[74]
	CT	47 mg isoflavones 3 times/day, 12 months	Reduced PSA levels in prostate cancer patients	[75]
	CT	60 mg isoflavones/day, 12 months	Reduced prostate cancer incidence in 65 years or older patients	[76]
		Regular soy products consumption	Reduced endometrial cancer risk	

Table 1: Continued.

Pharmacological activity	Study type	Dose/type of treatment	Results	References
	Meta-analysis			([77], [78])
	Meta-analysis and CT	Regular soy products consumption	Reduced ovarian cancer risk	([79], [80])
	Meta-analysis	Regular soy products consumption	Reduced mortality and recurrence of breast cancer	[81]
	Review	Regular soy products consumption	Reduced prostate cancer risk in equol producers	[82]
	Review	Regular soy products consumption	Reduced prostate cancer risk, no differences in PSA	[83]
	Review	Regular isoflavones consumption	Reduced prostate cancer risk for daidzein, not for equol	[84]
	Meta-analysis	Regular soy products consumption	Reduced colorectal cancer risk in women	[85]
	Review	Regular isoflavones consumption	No effect on stomach and colorectal cancer	[86]
	Review	Regular phytoestrogen consumption	Possible reduction in colorectal cancer risk	[86]
Cardioprotective	CT	Regular soy products consumption	Reduced CVD risk	[87]
	CT	Regular soy products consumption	Reduced myocardial infarction risk	[88]
	CT	Regular soy products consumption	No changes in CVD mortality	[89]
	CT	Regular phytoestrogen consumption	No changes in CVD risk	[90]
	CT	Regular isoflavones consumption	Reduced CVD risk	[91]
	CT	63 mg daidzein/day, 6 months	Improved renal function in postmenopausal women with prehypertension	[63]
	RCT	40-80 mg daidzein/day, 6 months	Reduced TG and uric acid	[43]
	Review	Regular soy products consumption	Reduced platelet aggregation and atherosclerosis	[92]
	Meta-analysis	Regular soy products consumption	Reduced TC, LDL-C and TG	[93]
	Review	Regular soy products consumption	Improved arterial stiffness and antiatherogenic effect	[45]
	Review	Different phytoestrogen supplementation	Improved body composition in postmenopausal women	[94]
Antiaging and cognitive promotion	RCT	27 g soy protein/day, 12 weeks	Decreased gain in muscle strength in older adults	[95]
	RCT	25 g soy/day, 16 weeks	Increase in muscle strength in postmenopausal women	[96]
	RCT	60 mg isoflavones/day, 6 months	Enhanced cognitive performance	[97]
	RCT	80 mg isoflavones/day, 4 months	Enhanced cognitive performance and memory	[98]
	CT	Regular phytoestrogen consumption	Improved verbal memory	[99]
	RCT	52 mg genistein + 36 mg daidzein + 3 mg glycitein daily, 2.5 years	Possibly improved visual memory	[100]
	RCT	100 mg isoflavones/day, 6 months	No beneficial effects for Alzheimer's disease patients	[101]
	CT	50 mg phytoSERM/day, 12 weeks	Possible enhancement of cognitive activity	[102]
Thyroid function	CT	80-120 mg isoflavones/day, 2 years	No changes in thyroid function	[103]
	CT	80 mg isoflavones/day, 3 months	No changes in thyroid function	[104]
Antidiabetic	CT	Regular isoflavones consumption	Decreased type 2 diabetes risk	[105]
	RCT	40 g soy protein/day, 57 days		[106]

Table 1: Continued.

Pharmacological activity	Study type	Dose/type of treatment	Results	References
			No beneficial effects for glycemic control in diabetic patients	
	RCT	50 mg daidzein/day, 12, 24 weeks	No beneficial effects for glycemic control or insulin sensitivity in diabetes patients	[107]
	RCT	10 mg equol/day, 12 weeks	Possibly improved glycemia control in overweight patients	[108]

CT: clinical trial; LDL: low density lipoprotein; RCT: randomized controlled trial; SR: systematic review; TC: total cholesterol; TG: triglycerides.

postmenopausal women resulted in an increased bone density and improvement in bone resorption and formation biomarkers [52, 53]. Abdi et al. [57] reported in their systematic review that isoflavones may improve bone health and prevent mineral density loss in menopausal women.

Estrogens also exert direct effects on calcium homeostasis through ER-independent mechanisms. In fact, a correlation between estradiol and calcium levels has been described and inversely correlated with osteoporosis-associated fractures in humans [113]. Recently, Lu et al. [54] reported no changes in serum calcium levels with isoflavone pills uptake, containing 60 mg genistein and daidzein, 5 days/week for 2 years. However, a potential association was proposed between daidzein urinary excretion and serum calcium and chloride levels.

Pawlowski et al. [55] showed that a treatment with 105.23 mg total isoflavones/day, including genistein, daidzein, and glycitein, led to an increase in calcium retention in bones, although no differences were reported when equol producers and nonproducers were compared. On the other hand, Nayeem et al. [56] found a correlation between urinary isoflavone levels and decreased mineral density in women with low calcium levels.

Several studies have analyzed the effect of daidzein and equol in menopausal symptom reduction in women, like hot flashes and muscle and joint pain [2, 58]. Supplementation with 10 mg of equol 3 times/day reduced symptoms like anxiety, depression, and fatigue in postmenopausal women [59]. Other studies have also shown an improvement in some symptoms, including hot flash frequency, muscle stiffness, sweating, and renal function [58, 60, 63]. Interestingly, in several studies, equol-producing women showed a decrease in anxiety [59] and hot flash scores, as well as in sweating and fatigue [61], and hot flashes intensity [62] compared to equol nonproducing women. However, other studies have reported no benefits of daidzein or isoflavone supplementation in menopausal symptom reduction [64].

To address such controversy, some meta-analyses have been performed. Chen et al. [65] reported no evidence of improvement in the Kupperman Index, a questionnaire on menopausal symptoms, for women under a phytoestrogen treatment. However, based on the data obtained, the authors revealed that phytoestrogens seem to reduce hot flash frequency without having any marked side effects. Another meta-analysis reported such reduction in hot flashes with isoflavones, as well as other beneficial effects on vascular health, although they were not able to improve the urogenital symptoms [66]. Thus, taken together, such controversial results on the potential effects of daidzein and other isoflavones are presumably due to a lack of standardized protocol treatments, since different doses, study periods, supplement composition, and methods to determine the outcomes are used. Another proposed reason for this discrepancy in results is that most studies fail to distinguish equol producers from nonproducers and to determine the levels of free, unconjugated equol, which is presumably the main effector [114].

3.3. Daidzein and Cancer. Incidence and mortality rates of hormone-dependent tumors, such as breast, prostate, and ovarian cancer are considerably lower in Asia when compared to Western countries. This fact has been attributed to the higher soy isoflavone consumption in the Asian population, which has increased the interest in soy isoflavones for both prevention and treatment of such types of cancers [115]. However, some issues have yet to be resolved, such as the bioavailability of these compounds in the target tissue. Most studies show a dual effect of isoflavones on cancer depending on their concentration. Thus, tissue distribution and concentration must be determined to understand whether daidzein or other compounds may have beneficial or harmful effects in cancer [116]. For instance, Bolca et al. [23] analyzed the concentration of isoflavones in normal breast tissue following a dietary intervention increasing isoflavone intake, and they found that isoflavones may reach significant levels in breast to elicit a beneficial effect.

Several *in vitro* studies have described an anticancer effect for daidzein in different types of tumors [117–121]. Among the mechanisms described, daidzein was reported to induce apoptosis and cell cycle arrest in the SKOV3 ovarian cancer cell line [122] or induce epigenetic changes *in vivo* [123]. Furthermore, daidzein could modulate long noncoding RNA (lncRNA) expression in some cancer types, as several isoflavones have been reported to target these molecules [124].

The impact of soy on breast carcinogenesis has been widely evaluated. A meta-analysis conducted by Chi et al. [81] revealed that soy isoflavones may be associated with a lower incidence of breast cancer and that ER-negative breast cancer patients could benefit from an isoflavone supplementation. A decrease in breast cancer recurrence has been described for both soy consumption [67] and daidzein supplementation [68] in postmenopausal women. Interestingly, soy consumption has also been associated with a decreased

expression of HER2/neu and PCNA in tumors, directly related to a more proliferative, malignant tumor phenotype [125]. On the other hand, Shike et al. [71] described a gene signature associated with higher cell proliferation in women with breast cancer with a supplement of soy protein, warning on the possible counterproductive effects of soy supplementation for breast cancer patients. Nevertheless, the American Association for Cancer Research recommends soy consumption in women, including those diagnosed with breast cancer [2]. In a meta-analysis, isoflavones showed a nonsignificative association with a decreased risk of breast cancer, as well as with either individual compounds as genistein, daidzein, and glycitein [69]. Soy isoflavone consumption has also been associated with a reduced risk of endometrial [77, 78] and ovarian cancer [79, 80]. However, other studies have found no effects of soy administration regarding endometrial health and cancer [72, 73]. In a recent review with meta-analysis, the authors suggested that phytoestrogens may play a role in breast cancer disease, although in other cancers, the evidence is too limited to draw this conclusion [70].

Prostate cancer incidence and mortality are significantly higher among North American and European men compared to Asian men. This difference has been attributed, in part, to the ability to produce equol, which is substantially higher among the Asian population [82]. Some studies have described a lower prostate cancer risk with soy isoflavone intake, although no changes in prostate-specific antigen (PSA) levels were observed under short-term treatments [2, 8, 74–76, 83, 126–128]. Zhang et al. [84] reported that while total isoflavones and equol were not correlated with prostate cancer risk, daidzein and other isoflavones could reduce the risk of developing this type of cancer.

Epidemiological data suggest that soy intake may have benefits for other types of cancer. For instance, phytoestrogen administration may be associated with a lower risk of colorectal cancer [85, 86]. Phytoestrogens have been described to increase the expression of ERβ in normal colonic mucosa in humans [129], which could explain the protection against this type of cancer. However, since soy consumption is usually associated with healthier diet choices, this risk reduction may not be entirely due to daidzein and other soy components. Interestingly, Jiang et al. [34] detected that only in case-control and not in cohort studies the risk of colorectal cancer seemed to be reduced with isoflavone administration.

3.4. Daidzein and Cardiovascular Diseases. In animal models, daidzein was able to reduce platelet aggregation and nitric oxide production, suggesting a cardioprotective effect [130]. In this regard, daidzein has been reported to interfere with the inducible nitric oxide synthase (iNOS) expression pathway resulting in the downregulation of this enzyme (Figure 3) [131].

The first reports on the beneficial effects of soy products on human CV health were made more than two decades ago, with a meta-analysis showing that soy protein intake decreased total cholesterol (TC) and low-density lipoprotein- (LDL-) cholesterol levels [93]. Isoflavones have been found to enhance endothelial function and limit atherosclerosis progression [92], as well as lower blood pressure, improve lipid profile, and reduce oxidative stress and inflammation [132]. Daidzein administration only lowered serum triglycerides (TG) and uric acid, while the rest of the lipid profile and glucose remained unchanged. Interestingly, participants with a specific ER genotype were those who most benefit from this intervention [43]. Furthermore, equol has shown potential as an antiatherogenic agent and could prevent coronary heart disease [45].

Controversial results have been described in epidemiological studies analyzing the effects of isoflavones on coronary heart disease. The Shanghai Women's Health Study [87] and a Japanese cohort study [88] reported an inverse correlation between heart disease and dietary soy intake, while the Singapore Chinese Health Study [89] and the European Prospective study Into Cancer and Nutrition [90] showed no association. Zhang et al. [91] described a significant inverse correlation between coronary heart diseases and equol, but no effects were stated to soy isoflavones or their metabolites. On the other hand, another report suggested that the benefit for cardiovascular health is only seen in equol producers after 6 months of soy supplementation but not with the use of purified daidzein [63].

Finally, a meta-analysis performed by Glisic et al. [94] analyzed the effect of phytoestrogen on body weight and body composition in postmenopausal women. Phytoestrogen administration did not produce any changes in these parameters, although those participants with preexisting conditions such as diabetes or hyperlipidemia suffered an increase in body weight. Furthermore, daidzein could be associated with nonbeneficial effects in body composition. Miller et al. [133] suggested that gut microbiota could influence the incidence of obesity, as they reported that both peri- and postmenopausal women who did not produce *O*-DMA metabolite showed higher rates of overweight and obesity.

3.5. Effects of Daidzein on Aging and Cognitive Activities. Aging is usually associated with a decline in muscle mass and strength. Thomson et al. [95] analyzed the effects of soy intake on training resistance in older adults. Interestingly, they reported that those participants with soy protein supplementation did not gain as much muscle strength when compared to adults with regular protein or dairy protein intake. On the other hand, Orsatti et al. [96] reported a significant increase in muscle strength after 16 weeks of training resistance and soy supplementation in postmenopausal women.

Another hallmark of aging is a mild cognitive decline regarding learning, memory, and perception. The incidence of neurodegenerative diseases and dementia is also rapidly growing among the elderly population. Some studies have proposed estrogen therapy as a treatment for improving memory and preventing Alzheimer's disease in postmenopausal women [134]. Likewise, isoflavone administration may also improve cognitive functions and memory [97–100]. However, although a protective effect against Alzheimer's disease has been described in mice [135], following

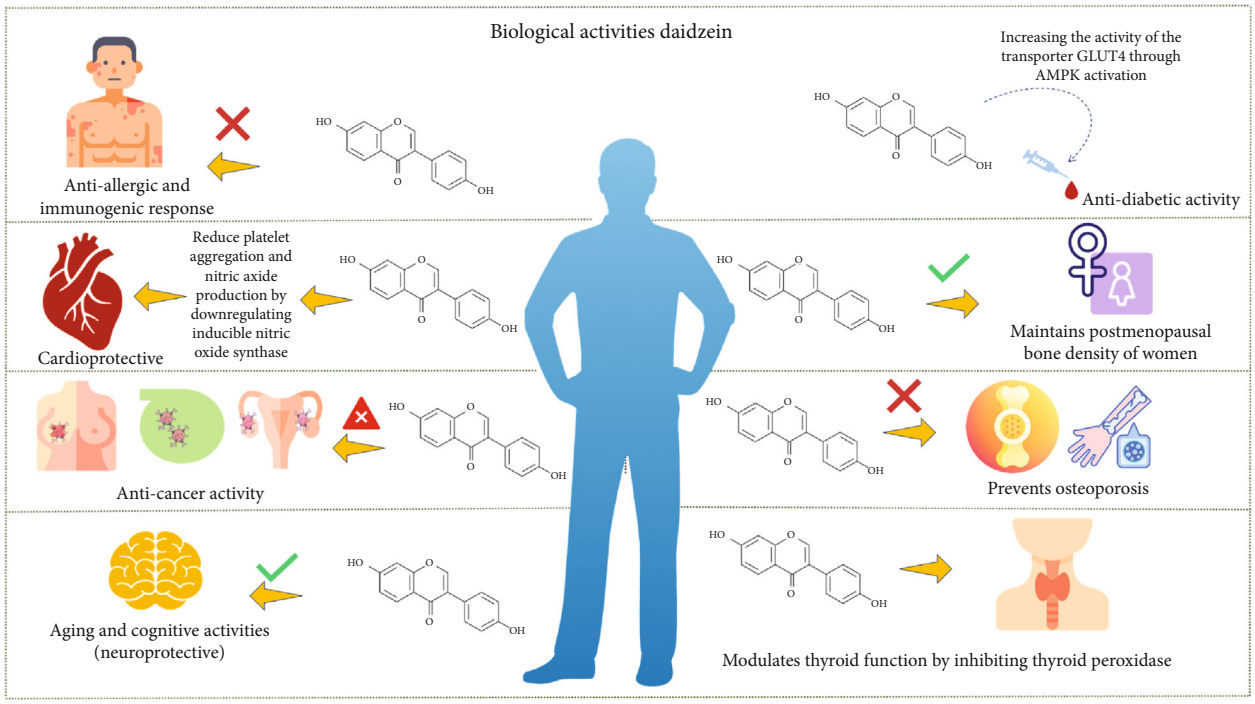

Figure 3: Various biological activities of the daidzein.

analysis of isoflavone supplementation effects in Alzheimer's disease patients, Gleason et al. [101] concluded that there were no significant benefits. Lately, Hernandez et al. [136] and Schneider et al. [102] tested PhytoSERM for 12 weeks in perimenopausal women, a mixture composed of genistein, daidzein, and equol. With a daily dose of 50 mg, the participants declared a reduction in menopausal symptoms and a better cognitive function, with no associated side effects. In this regard, more studies increasing the number of participants and analyzing the effects of PhytoSERM on cognitive decline are still undergoing.

3.6. Effects of Daidzein on Thyroid Function. Daidzein and other isoflavones are known enzymatic inhibitors and, theoretically, they may interfere with thyroid function as they inhibit thyroid peroxidase. However, several studies were measured thyroid function, and no special impact of isoflavones was found [103, 137]. Sosvorová et al. [104] confirmed that both genistein and daidzein are targets of thyroid peroxidase by detection of iodinated derivates of these isoflavones in human urine, although no effects were described in free thyroid hormones levels. Thus, there is no evidence that daidzein consumption could be harmful to thyroid gland disorders.

3.7. Daidzein and Diabetes. Isoflavones have also been studied for the treatment of diabetes. Interestingly, these compounds have the ability to modulate gut microbiota, which is altered in diabetes, and their potential use to prevent and manage this disease is currently being analyzed [138]. Some studies suggest that daidzein could enhance glucose and lipid metabolism, regulating glycemia and TC levels in animal models [139, 140] and increasing the activity of the transporter GLUT4 through AMPK activation [141]. Furthermore, the detection of equol in urine has been associated with a reduction in type 2 diabetes incidence among the Chinese population [105]. However, Gobert et al. [106] reported that isoflavones had no significant effect on glycemic control in patients with type 2 diabetes, and Ye et al. [107] found that daidzein improved neither insulin sensitivity nor glycemia following 6 months of treatment. Nevertheless, body weight control can be helpful for diabetes management. In this regard, isoflavones have shown potential to reduce fat accumulation and improve insulin resistance in animals [2, 142, 143]. Similarly, isoflavones could help in weight loss in humans [2, 108, 144], as these compounds have shown higher lipolytic potential [145]. Various biological activities of daidzein are shown in Figure 3.

3.8. Soy and Soy-Derived Metabolites in Children. Phytoestrogens may theoretically interfere with ER signaling in the developing brain of children or produce gut dysbiosis, although these results are controversial [146]. Soy-based formulas are often used for infants under certain circumstances, such as allergy and intolerance to milk, lactose intolerance, or galactosemia. Vandenplas et al. [147] evaluated the safety of these formulas and found that, although the levels of genistein and daidzein were higher in formula-fed infants, no harmful effects regarding anthropometric growth, immunity, cognition, or endocrine functions were found.

4. Conclusions and Future Perspectives

As mentioned before, daidzein has potent antioxidant and estrogenic activities, which has led to a wide interest in

developing a functional food containing this compound. In adults, daidzein and other phytoestrogens are well-tolerated and have low levels of toxicity, while in infants, there are reports concerning their harmful effects. In the last years, there has been an increase in the consumption of soy products. For a better understanding of the properties of such soy products, it would be necessary to indicate, in addition to the quantity, the type of isoflavones that these products contain. Food processing technologies could affect both retention and distribution of different isoflavone isomers present in soy products. Both transformation and/or loss of some isoflavones, especially genistein and daidzein, may affect the nutraceutical characteristics of these soy products.

Although some of the benefits of isoflavones such as daidzein have been demonstrated, the side effects (for example the potential fertility problems among male humans) of long-lasting high consumption of these soy products need to be studied in a more in-depth way. In fact, clinical trial data are conflicting, showing both negative and positive effects of daidzein on human health. That is why a correct standardization and documentation of these clinical trials is essential to advance in the study of daidzein's beneficial effects on human health. Despite being possible to control all the independent variables in clinical trials, the ability of each individual to metabolize daidzein closely depends on individual's microbiota composition, the ability of this microbiota to assimilate the dose administered, and the different bioavailability of daidzein that could influence the data heterogeneity.

In the future, the use of genetic screening techniques could represent a great advance in personalized medicine. One of the uses of such techniques could be the assessment of the genetic predisposition of an individual to metabolize daidzein, which could initially help to select comparable groups for clinical trials and then filter the possible recipients of a treatment with daidzein, depending on individual's ability to metabolize this phytoestrogen. Moreover, the consumption of soy-rich products should be monitored by physicians, especially in cases of diseases for which daidzein is known to play an essential role, such as breast cancer [148].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Cyperus spp.: A Review on Phytochemical Composition, Biological Activity, and Health-Promoting Effects

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Cyperaceae are a plant family of grass-like monocots, comprising 5600 species with a cosmopolitan distribution in temperate and tropical regions. Phytochemically, *Cyperus* is one of the most promising health supplementing genera of the Cyperaceae family, housing ≈950 species, with *Cyperus rotundus* L. being the most reported species in pharmacological studies. The traditional uses of *Cyperus* spp. have been reported against various diseases, *viz.*, gastrointestinal and respiratory affections, blood disorders, menstrual irregularities, and inflammatory diseases. *Cyperus* spp. are known to contain a plethora of bioactive compounds such as *α*-cyperone, *α*-corymbolol, *α*-pinene, caryophyllene oxide, cyperotundone, germacrene D, mustakone, and zierone, which impart pharmacological properties to its extract. Therefore, *Cyperus* sp. extracts were preclinically studied and reported to possess antioxidant, anti-inflammatory, antimicrobial, anticancer, neuroprotective, antidepressive, antiarthritic, antiobesity, vasodilator, spasmolytic, bronchodilator, and estrogenic biofunctionalities. Nonetheless, conclusive evidence is still sparse regarding its clinical applications on human diseases. Further studies focused on toxicity data and risk assessment are needed to elucidate its safe and effective application. Moreover, detailed structure-activity studies also need time to explore the candidature of *Cyperus*-derived phytochemicals as upcoming drugs in pharmaceuticals.

1. Introduction

Immemorially, human societies have been using herbs and their products as sources of medicine, nutrition, and industrial applications [1]. As an example of the role of plant species in human life, in ancient Egypt, the first paper was made from papyrus (*Cyperus papyrus* L.), a species of the Cyperaceae family [2]. Cyperaceae includes grass-like monocots, comprising around 5600 species and 100 genera, and the family is widespread on all continents with the exception of Antarctica. The second largest genus in this family is *Cyperus*, with ~950 species [3]. *Cyperus* spp. are most commonly known as weeds, despite some cultures using them for medicinal purposes and as a source of food [4]. *Cyperus* spp. predominantly exist in the wetlands throughout the globe in the tropical regions and act as source of primary productivity. The tubers, shoots, and fruits of this species are produced in larger quantities and act as a source of food for amphibians and aquatic animals [5].

The traditional use of *Cyperus* plants has been reported from all over the world as a remedy against various human ailments [6] including treatment of stomach and bowel disorders, as diuretic, digestant, and lactodepurant purposes. The plant extracts also act as a selective drug for the treatment of bronchitis, blood disorders, menstrual irregularities, amenorrhea, diarrhea, dysentery, and inflammatory diseases [7]. Interestingly and despite *Cyperus* including more than 950 species, the three most commonly reported species are purple nutsedge (*Cyperus rotundus* L.), yellow nutsedge (*Cyperus esculentus* L.), and *C. papyrus*. *Cyperus rotundus* is the most well-known species of *Cyperus* in South Asia, a perennial weed that grows best in high-moisture soil and reproduces easily through rhizomes and tubers [7]. This species is indigenous to the tropical and subtropical parts of the Old World, and despite the fact that it can be found detrimental in cultivated fields, it has several beneficial uses as medicine since ancient times [8]. *Cyperus rotundus* rhizomes and tubers are mentioned in Oriental traditional medicine to treat fever, digestive disorders, and menstrual irregularities in several countries including China, India, Iran, and Japan [9, 10].

Cyperus esculentus L. is an edible perennial grass-like plant native to the Old World. This species exists widely throughout tropics and subtropics of North America [11]. The earliest records of its use dated back to predynastic times about 6000 y ago in North America and Egypt; however, its different varieties are mostly found in Southern Europe, South-Middle East, and Africa [12]. It has been also considered as a foodstuff since ancient times, especially in ancient Egypt. It is a crop of early domestication and was regarded important with the other crops of the Nile Valley. Its dry tubers have been found in tombs from predynastic times about 6000 y ago. *Cyperus esculentus* tubers were roasted and used as a sweetmeat in Egypt during the ancient times [12]. *Cyperus esculentus* is widely cultivated for its edible tubers, called earth almonds or tigernuts [13], which are consumed as a popular snack in Africa and for making a sweet milk-like beverage, horchata de chufa, commonly consumed in Spain and other European and Latin-American countries [14]. Tigernut is a rich source of protein and minerals making the beverage highly nutritious (phosphorus and potassium) [12].

Cyperus papyrus L. is an aquatic sedge mostly known for its use in the preparation of the paper by the traditional Egypt, Greek, and Roman civilizations. Paper made from dried, pressed, and woven strips of culm pith had been used since 3500 BC by ancient civilizations in the Egypt and the Mediterranean Basin. It was the only widespread recording medium until the 8th century in Europe [15]. Other species of the *Cyperus* family include *Cyperus compressus* L., *Cyperus javanicus* Houtt., and *Cyperus monocephalus* Roxb. (*Cyperus cephalotes*). For instance, *C. compressus* is a grass-like plant and is widely distributed across the tropical and subtropical regions of the world. In India, the powdered roots of *C. compressus* have long been used in traditional medicine by the Santhal tribes to treat intestinal helminthic infections [16]. Examples of folk medicinal and edible uses of *Cyperus* spp. reported from different parts of the world are briefly shown in Table 1.

Taken together, the multiple potentialities reported so far for the most widely exploited *Cyperus* spp. were considered. This review is the first of its kind that gives a comprehensive discussion on the recent findings related to chemical composition, biological activities, and pharmacological effects of such promissory naturally occurring matrices. The safety and toxicity effects of the *Cyperus* sp. extracts are also considered in the scope of the manuscript. The diagram showing various components discussed in the review are presented in Figure 1.

2. Chemical Composition

The Cyperaceae family is one of the largest flowering plant families and is ranked the third largest monocot family after Orchidaceae and Poaceae [46]. A rising number of studies have highlighted that the multiple potentialities of the species of this family as medicines are attributed to the presence of several bioactive constituents. For example, the cypriol, isolated from *Cyperus scariosus* R.Br. essential oil, is present in various perfumes and medicines. In fact, cypriol's ambery, balsamic, spicy, warm, and woody features make it highly demanded in perfume industry [47]. In addition, the essential oil is also present in various other species of *Cyperus*, such as *C. articulatus* L., *C. rotundus*, and *Cyperus maculatus* Boeckeler [48]. Summarization of phytochemicals present in the major six species of the Cyperus genus is summarized in Table 2, and in the next subsections, a brief description of the most abundant phytochemicals in the recently investigated *Cyperus* spp. is also presented.

2.1. Cyperus articulatus L. Cyperus articulatus is a perennial herb with underground perennial rhizomes having scales which grade into culm leaves. They have exceptionally high photosynthesizing function compared to other plants and are also regarded as herbal switch plants as they are a reservoir of potentially useful drugs for the treatment metabolic disorders [49]. Various specific compounds isolated from *C.*

Table 1: Examples of folk medicinal uses of a selection of *Cyperus* species.

Plant species	Country/region	Plant part (s)	Traditional use	Instruction	Reference
Cyperus rotundus L.	North-West Himalaya/India	Roots	Skin diseases	Decoction prepared by burning and adding the ash of fresh leaves of *A. baccifera* (10 g) and *C. rotundus* roots (10 g) and fresh ginger (5 g) in sesame oil.	[7]
	Pakistan/India	Tubers	Diabetes	10–12 g of dry tuber powder administered daily twice for 2–3 months.	[17]
	India	Whole plant	Menstruation problem	Juice of the *Citrus maxima* fruit (100 ml) and 30 g dried powder of *C. rotundus* is taken once daily for a week.	[18]
	Tamil Nadu/India	Tubers	Snake bite	Paste of leaf and root bark of *Albizia amara*, root bark of *Jasminum angustifolium*, and tubers of *C. rotundus* is heated with oil and applied externally on affected places for 10 days.	[19]
	India	Roots/tubers	Urinary trouble-stone removal	Decoction of the plant is used.	[20, 21]
	India	Whole plant	Epilepsy	Plant decoction (10 ml) with 5 ml of honey is orally administered to treat epilepsy.	[22]
	India	Roots	Cholera	Roots are boiled with equal quantity of mint and given for cholera.	[23]
	India	Roots	Pimples	Roots along with turmeric and curd are made into a paste which is applied on the face for pimples and beautification	[23]
	India	Roots	Increase lactation	Paste of the roots is applied on breasts to increase lactation.	[23]
	North-West Himalaya	Roots	Intermittent fevers	The decoction prepared from 10 g of *C. rotundus* roots and 5 g of fresh ginger is used.	[24] Das, & Misra [25]
	India	Tubers	Dermatitis	The decoction prepared from tuberous roots of *C. rotundus* and leaf of *Trichosanthes anguina* is taken orally to cure dermatitis.	[25]
	India	Tubers	Dysentery	The tuberous root of *C. rotundus* with other plants which are orally used to treat dysentery is taken in three doses to cure dysentery.	[25]
	India	Tubers	Indigestion disorders, stomachache	A powder was prepared from 10 g of tuber of *C. rotundus*, 10 g stem bark of *Holarrhena antidysenterica*, and 10 g of *Zingiber officinalis* after being sun dried. 30 g powder is given internally along with 250 ml of buttermilk twice daily till cure.	[26]
	India	Tubers	Vaginal discharge	Tubers crushed with *Abutilon indicum* leaves and sufficient quantity of *Cuminum cyminum* seeds; extract administered daily twice for three days.	[27] Jahan et al. [28]
	India	Whole plant	Loss of libido in men	Leaves of *Psidium guajava*, leaves of *Punica granatum*, and whole plants of *C. rotundus* are mixed, warmed, and macerated to obtain juice. 1/2 cup of the juice is taken with 10–15 drops of honey twice daily for 3 days.	[28]
	India	Tubers	Constipation	1/2 cup of juice obtained from macerated tubers is taken three times daily.	[28]
	India	Whole plant	Bone fracture	Whole plant of *C. rotundus* and 7 slices of ginger are crushed and made into a paste. The paste is warmed and applied to fractures.	[29]

Table 1: Continued.

Plant species	Country/region	Plant part (s)	Traditional use	Instruction	Reference
	India	Tubers	Jaundice	Fresh rhizome with tuberous root of C. *rotundus* and fruits of *Phyllanthus emblica* are taken in equal quantities and ground. 2 spoonfuls of paste mixed in a glass of water are administered daily once for 8 days.	[30]
	India	Bark	Malaria	The decoction is prepared from a mixture of 200 g of rhizome of *Costus speciosus*, 200 g bark of *C. rotundus*, and 200 g bark of *Azadirachta indica*. 2–4 spoons of decoction were prescribed after meal for 15 days.	[31]
	India	Tubers	Bronchitis	Tubers of *C. rotundus* and leaves of *Tinospora cordifolia* with fruits of *Pergularia daemia* are ground. 2 spoons of paste with honey are orally administered twice daily for 30 days.	[32]
	China	—	Coughs	—	[33]
	Rarotonga	Tubers	Sore throat	Twenty to thirty tubers of *C. rotundus* and a handful of *Pandanus tectorius* bark which is crushed into the water of four green coconuts. Half the mixture is drunk hot, and the remainder cold. The treatment lasts for three days.	[33]
Cyperus javanicus Houtt.	Rarotonga	Leaves	Fractures/sprains	Leaves without flowers are pounded and squeezed into a small basin of water. The treatment lasts for three days.	[33]
	Rarotonga	Leaves	Irregular menstrual	Leaves with those of several other herbs.	[33]
Cyperus brevifolius (Rottb.) Hassk.	Malaysia	Tubers	Sore legs	—	[33]
Cyperus kyllingia Endl.	Rarotonga	Tubers	Oral thrush	Tubers of *C. kyllingia*, 4 *Aleurites moluccana* inside nuts, and a handful of the aerial roots of *Ficus prolixa* are pounded then squeezed through a cloth into a liter of water.	[33]
Cyperus monocephalus Roxb.	Philippines	Tubers	Dermatosis	Decoction is prepared from tuberous root.	[33]
	Tami Islands	Tubers	Ringworm	Decoction of tubers prepared by adding lime.	[33]
Cyperus compressus L.	India	Roots	Helminthiasis	Powdered roots orally administered.	[16]
Cyperus articulatus L.	Central Africa Republic	Tubers	Headache, migraine	Decoction is prepared from tuberous root.	[34]
Cyperus pedunculatus(R.Br.) J.Kern	West Africa	Stem and leaves	Diarrhea, kidney disease, fever, pain, and inflammations	Extract is made from the whole plant	[35]
Cyperus nitidus Lam.	South Africa	Rhizomes	Respiratory and digestive disorders	Extract is made from the rhizomes	[36]
Cyperus sexangularis Nees	South Africa	—	Asthma, fatigue, fever, pneumonia, and TB	—	[37]
Cyperus sexangularis Nees	South Africa	Roots	Antimicrobial, emollient, diuretic, stimulant, anthelmintic, and analgesic treatment	Extract is made from the roots	[38]
	East Africa	Roots	Various animal diseases	Extract is made from the roots	[39]

Table 1: Continued.

Plant species	Country/region	Plant part (s)	Traditional use	Instruction	Reference
Cyperus kilimandscharicus Kük.					
Cyperus latifolius Poir.	East Africa	Roots	Tuberculosis and related ailments	Extract is made from the roots	[40]
Cyperus maculatus Boeck.	West Africa	Tubers	Cattle worms	—	[41]
Cyperus natalensis Hochst.	South Africa	Roots	Treatment of gynaecology and obstetric complaints	Decoction is prepared from the roots	[42]
Cyperus erectus (Schumach.) Mattf. & Kük.	South Africa	—	Reduces foot swelling	Ground plant is used for the medicinal purposes	[43]
Cyperus mundii (Nees) Kunth	Madagascar	—	Treatment of evacuation of the placenta, tuberculosis, and paludism	Whole plant extract	[44]
Cyperus esculentus L.	Oaxaca, Santa María Tecomavaca	Roots	Depression	Root extracts	[45]
Cyperus flavescens L.	Oaxaca, Santa María Tecomavaca	Roots	Depression	Root extracts	[45]

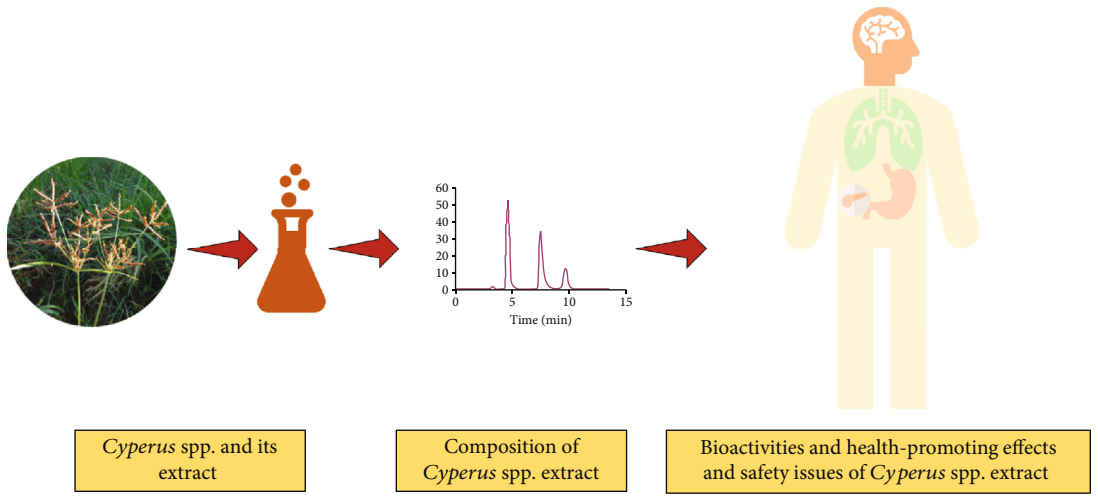

Figure 1: Diagram showing various components discussed in the review.

articulatus include α-cyperone, α-corymbolol, α-pinene, caryophyllene oxide, cyperotundone, and mustakone. Researchers also identified articulone, myrtenal, and myrtenol from volatile oil of Nigerian *C. articulatus* [55]. Cameroonian *C. articulatus* hexane extracts displayed the presence of isopatchoul-4(5)en-3-one, mandassidione, mustakone, and almost all sesquiterpene diketones [56]. Similar compounds were also identified in the Brazilian rhizome volatile oil with mustakone (14%), caryophyllene oxide (10.2%), and α-pinene (6.4%) [57]. Volatile oil from *C. articulatus* rhizomes showed the presence of α-pinene (3.5–25.2%), β-pinene (2.3–12.6%), *trans*-pinocarolol (2.2–5.5%), myrtenal + myrtenol (2.3–5.6%), α-copaene (1.3–2.6%), cyperene (0.7–1.6%), β-selinene (0.8–2.4%), lithol (0.9–5.1%), caryophyllene oxide (3.1–8.3%), mustakone (3.4–9.9%), cyperotundone (2.6–4.1%), and α-cyperone (3.2–8.8%) [58]. These reports suggest a qualitative and quantitative difference in the volatile oil composition. The difference was attributed to various factors like air pollution, altitude, harvesting time, developmental stage, luminosity, seasonality, temperature, water availability, nutrients, UV radiation, and pathogens [59, 60].

Table 2: Phytochemicals present in different *Cyperus* species.

Cyperus species	Chemical constituents	Plant part	References
Cyperus articulatus L.	α-Campholenal, α-corymbolol, α-cyperone, α-pinene, cyperol, cyclocolorenone, β-copaen-4-α-ol, p-cymene, caryophyllene oxide, corybolane, cyperotundone, limonene, thuja-2,4(10)-diene, *trans*-pinocarveol, p-mentha-1,5-dien-8-ol, myrtenal, mustakone	Thick rhizomes	[49, 50]
Cyperus conglomeratus Rottb.	Saponins, steroids, tannins, triterpenes	Whole plant powder	[51]
Cyperus distans L.f.	Artemisia ketone, α-cyperone, cyperene, α-pinene, 1,8-cineole, caryophyllene oxide, endesma-2,4,11-triene, humulene epoxide II, germacrene D, pinocarveol, myrtenol, nor-copernone, zierone	Rhizomes	Lawal et al. [52]
Cyperus esculentus L.	β-Pinene, cymene, cyperene, coumaran, cyperotundone, p-vinylguaiacol, vanillin, cyprotundone	Rhizomes	Gugsa & Yaya [46]
Cyperus longus L.	α-Caryophyllene oxide, β-himachalene, β-caryophyllene oxide, aristolone, humulene oxide, irisone, longiverbenone, viridiflorol	Whole plant powder	Memariani et al. [53]
Cyperus rotundus L.	Isobutyl lactate, thiazol-4(5H)-one.5-(4-nitrobenzylidenol)-2-phenyl, *cis*-pinen-3-ol, *trans*-p-mentha-2,8-dienol, pyranone, *cis*-10-nonadecenoic acid, β-santalol, α-copaen-11-ol, β-vatirenene, elema-1,3-dien6a-ol, β-nootkatol, *cis*-13,16-docasadienoic acid, 25,26-dihydroxy-vitamin D3	—	El-Wakil et al. [54]

2.2. Cyperus conglomeratus Rottb. Cyperus conglomeratus is a perennial monocot with coarse rhizomes up to 12–16 cm long and 0.2–0.3 cm in width. It is native to India but also grows in temperate, tropical, and subtropical regions [61]. It is a perennial weedy herb commonly found with slim and sheathing leaf base. Phytochemical analysis of several plant extracts revealed the existence of different types of constituents at different amounts with medicinal activities. For example, the crude powder is rich in steroids, while the solvent extract is rich in triterpenes [62]. The therapeutic efficacy was not limited to a specific plant part; every plant part displays a pharmacological activity. Henceforth, the pharmacognostic studies of different plant parts have been performed for different plant organs [63], such as aerial parts, flower, fruit, leaf root, and stem [64–71]. The fatty acid profile of *C. conglomeratus* showed the presence of palmitic, linoleic, heptadecanoic, oleic, myristic, arachidonic, lignoceric, and stearic acid. The unsaponifiable fractions of *C. conglomeratus* constituted two other bioactive compounds (β-sitosterol and α-amyrin).

2.3. Cyperus distans L.f. Cyperus distans is an annual herb of about 0.6–1.5 m tall, mostly found in humid areas along roadsides and rivers and as weeds [72]. The phytochemical study of *C. distans* revealed the presence of scabequinone with antifeeding effects [73]. From 80% of its oil composition, almost 22 compounds were isolated, being zierone (33.8%) the main component. Other noteworthy compounds were caryophyllene oxide (14.1%), α-cyperone (9.1%), humulene epoxide II (3.8%), cyperene (3.2%), endesma-2,4,11-triene (2.9%), nor-copernone (2.9%), and germacrene D (2.8%) [74–77].

2.4. Cyperus esculentus L. Cyperus esculentus, also known as yellow nutsedge, is a perennial herb growing in tropical and temperate regions of the world. Naturally, it is found as a weed in farming areas and in wastelands [78]. The taste of tubers is sweet and reported to have health and nutritional benefits [79]. The common names of *C. esculentus* are chufas, earth almond, nutsedge, and rush nut [80]. Cyprotundone, the volatile component p-vinylguaiacol (2-methoxy-4-vinylphenol) [81], and vanillin (4-hydroxy-3-methoxy benzaldehyde) have been identified [82], with interesting bioactive effects that been reported to these biomolecules. As a matter of fact, these molecules are helpful in tracing components in cosmetics, dairy products, drug preparations, and pastry products [83].

2.5. Cyperus longus L. Cyperus longus is an Egyptian plant, distributed throughout Africa and Europe and to Indian subcontinent and is used as herbal tonic and diuretic [84]. Compounds isolated from this plant are flavonoids, stilbenes, and terpenoids [85–88]. Other compounds from *C. longus* essential oil were also identified such as β-himachalene (46.6%), α-humulene (16.7%), and γ-himachalene (10.1) as main components [86]. In another study, 32 components were identified consisting 83.50% of essential oil using gas chromatography-mass spectroscopy (GC-MS) analysis [53].

2.6. Cyperus rotundus L. Cyperus rotundus is popularly known as Nagarmotha or purple nutsedge or nut grass [89]. This is a perennial herb with creeping rhizomes 1–3 cm long having a bulbous base. The stems of this herb can attain as the size of about 140 cm, and leaves are grooved on the upper surface. The ethanolic extracts of *C. rotundus* were determined using HPLC, and it was reported to contain two bioactive phenolics, i.e., quercetin and chlorogenic acid [90]. Structures of important members of bioactive compounds from *Cyperus* spp. are shown in Figure 2.

3. Bioactive Effects: Preclinical Evidence

3.1. Antioxidant Activity. Antioxidants are substances which remove reactive species or free radicals from cells and play a

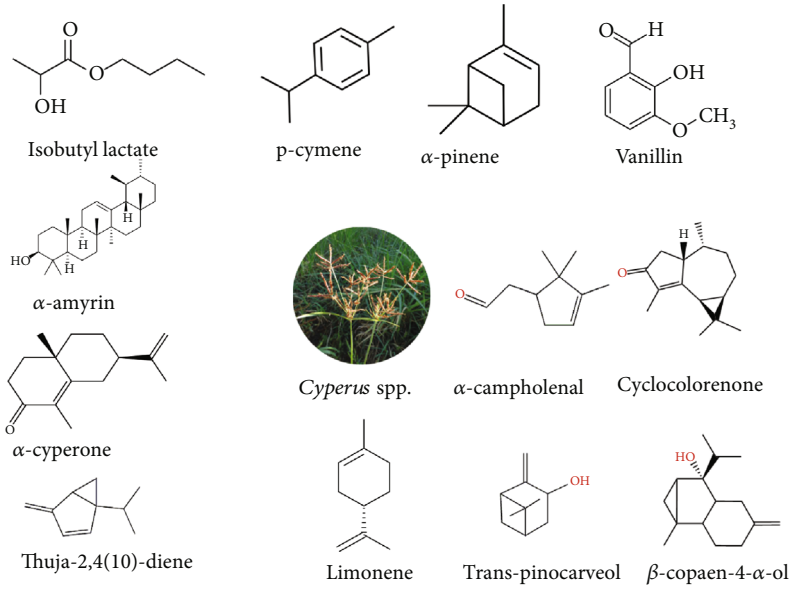

Figure 2: Structure of important members of bioactive compounds from *Cyperus* spp.

crucial role in maintaining the health and by preventing the diseases. The antioxidant capacity of *Cyperus* spp. is attributed to the plethora of phytochemicals present. Phenolic compounds, specifically flavonoids, tannins, and coumarins, are present in this species. The presence of these phytochemicals is directly correlated with antioxidant effects [91]. For example, a study assessed the nutritional value, mineral composition, secondary metabolites, and antioxidant activity of 5 wild geophytes: 2 from the Cyperaceae family (*Cyperus capitatus* Vand. and *C. conglomeratus* Rottb.) and 3 from the Poaceae family (*Elymus farctus* (Viv.) Runemark ex Melderis, *Lasiurus scindicus* Henrard, and *Panicum turgidum* Forssk.) collected from the Egyptian coastal desert (Mediterranean coast of the Delta) and the interior desert (Wadi Hagoul). Strong radical scavenging activity with $EC_{50} < 1$ mg/ml assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was reported from the extracts of *C. conglomeratus* and *C. capitatus* [92]. An experiment assessing the antioxidant activity of the extracts of *Cyperus tegetum* Roxb. demonstrated significant DPPH radical, superoxide anion, and hydrogen peroxide scavenging activities compared to the standards, viz., hydroxybutylanisol, butylhydroxytoluene, and ascorbic acid, respectively [93]. In addition, the milk extracted from *C. esculentus* tubers, commonly known as tigernut, was utilized in a study on rats to assess its effect on preventing acetaminophen-induced liver damage (APAP). Its presence led to an increased activity of antioxidant enzyme superoxide dismutase (SOD), while malondialdehyde concentrations were lower than the control group, thus demonstrating a good antioxidant activity [94]. The improvement in the levels of antioxidant enzymes resulted in the decreased reactive species and free radical in acetaminophen-treated liver, thus, managing the situation of liver damage. Similarly, the polyphenolic content of aquatic extracts from *C. rotundus* tubers was found to have protective effects on liver and kidney function caused by exposure to heavy metals (cadmium chloride) in rats, through scavenging of free radicals [95]. Also, components of the essential oils of *C. articulatus* rhizome encapsulated in chitosan nanoparticles revealed a high potential to eliminate free radicals [96]. The encapsulation improves the stability and also the efficiency of extracts from Cyperus spp. resulting in decreasing the stress imposed by free radicals.

Cyperus plant extracts have proven to have a neuroprotective effect caused due to reactive oxygen species (ROS). The deposition of beta-amyloid in the hippocampus promotes oxidative stress, reactive ROS formation, reduction of the antioxidant enzymes activity, and consequently, neuronal death. Previous studies have shown that flavonoids can modulate the function of immune cells, exerting a direct effect against inflammation and oxidative stress [97]. Thus, the antioxidant activity showed by the flavonoids present in *C. rotundus* extracts explains the increase in hippocampal neurogenesis of beta-amyloid in rat models and consequently improves the memory [98]. Orientin, a flavonoid found in *C. esculentus*, decreased oxidative stress generating a neuroprotective effect against cerebral ischemia/reperfusion injury in Sprague-Dawley rats through the middle cerebral artery occlusion method [99].

3.2. Anti-Inflammatory Activity. Numerous studies have proven the potent anti-inflammatory activity of extracts obtained from various plant parts of the *Cyperus* genus [100]. The anti-inflammatory action of the extract from *C. rotundus* rhizome was first described in 1971 [101], and since then, investigations have been done to confirm and understand the anti-inflammatory effect of the different plant parts or active constituents of *C. rotundus*. The compound α-cyperone, one of the main phytochemicals found in *C. rotundus* oil, was found to inhibit lipopolysaccharide- (LPS-) stimulated inflammatory response in a murine BV-2 microglial cell line, by activating Akt (protein kinase B)/nuclear

factor-E2-related factor (Nrf)-2/heme oxygenase- (HO-) 1 and suppressing the nuclear factor kappa light chain enhancer of the activated B cell (NF-κB) pathway [102]. A study concluded that α-cyperone exerts a neuroprotective activity by attenuating the production of inflammatory cytokines in BV-2 cells through activating Akt/Nrf2/HO-1 and suppressing the NF-κB pathway.

Another study using methanol extracts from *C. rotundus* rhizomes revealed that cyperalin A has high anti-inflammatory activity through inhibition of prostaglandin E2 (PGE-2), cyclooxygenase-2 (COX-2), and arachidonate 5-lipoxygenase (LOX-5) and that sugetriol triacetate, another compound of biological interest in *C. rotundus*, presented a similar effect on PGE-2, COX-2, and LOX-5 enzymes in peripheral blood mononuclear cell (PBMC) lines [103]. α-Cyperone revealed to suppress the inflammatory response in lipopolysaccharide- (LPS-) induced acute lung injury in mice, through inhibiting the growth of inflammatory cells along with cytokines and downregulating the NF-κB and NLR family pyrin domain containing 3 (NLRP3) signalling pathways [104]. Moreover, recent evidence has shown that the topical application of C. *rotundus* rhizome extract in a rat model with chronic and acute dermatitis leads to a reduction in ear oedema and inflammatory cell infiltration generated by exposure to 12-*O*-tetradecanoylforbol-acetate (TPA). This ultimately suggested that the extract could be a potential new therapeutic tool for the treatment of inflammatory skin disorders [90].

3.3. Antimicrobial Activity. The antimicrobial activity of *C. rotundus* extract has been shown in numerous studies [7, 100, 105–109]. In general, it was documented that Gram-positive bacteria were more sensitive to *Cyperus* extracts than Gram-negative bacteria. However, direct comparison of different studies was difficult due to variety of microbiological tests, microbial genera and species, presence of saccharides, herb cultivation conditions, extraction methods, and so on [110]. For example, a study carried out with *C. articulatus* essential oils revealed interesting inhibitory effects on *Staphylococcus aureus* and *Escherichia coli* [96]. Traditional medicine practitioners make use of water primarily as a solvent, but studies have shown that alcohol extracts of plants are much potent and efficacious [111]. The effects of aqueous and alcohol extracts with essential oils from *C. rotundus* tubers on cultures of *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans* were investigated. Alcoholic extracts displayed marked inhibition of *S. mutans* and *A. actinomycetemcomitans* growth, making these extracts future candidates for both treatment and prevention of periodontitis and oral cavity affections [105].

Another study used chloroform extracts of *C. conglomerates*, orally administered to mice, aiming to determine the degree of consumption toxicity. There was no damage to the liver or kidney at the doses used, and a negative impact on the growth of *C. albicans*, *C. dubliniensis*, *C. famata*, *C. glabrata* and *C. inconspicua* was listed. β-Sitosterol and α-amyrin were the most abundant components identified and were the chemicals responsible for the significant effect on the growth of *C. famata* and *C. albicans*, respectively [106]. *C. conglomeratus* chloroform extracts were also assessed for their antibacterial activity [112]. Extracts demonstrated powerful activity against Gram-positive and Gram-negative bacterial strains, such as *S. aureus*, *Enterococcus aureus*, *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa*, while butanol and ethyl acetate extracts showed a moderate activity. The antimicrobial activity of *Cyperus* sp. extracts is mainly contributed by the essential oil components. The essential oil components from *Cyperus* spp. easily penetrate inside and create pores in bacterial cells which results in leakage of intracellular components resulting to cell death.

The antimycobacterial activity of *C. rotundus* extracts, evaluated on multidrug-resistant strains of *Mycobacterium tuberculosis*, also revealed to be prominent [113]. In addition, such extracts were used to assess their antibacterial activity and the mode of action against ampicillin-resistant *S. aureus*. The extract revealed a synergistic activity when in combination with ampicillin at the lowest inhibitory concentration. Using electron microscopy, it was observed that the combined treatment induced damages to the peptidoglycans and cell membrane, generating an increase in membrane permeability and revealing an inhibitory activity against β-lactamase [114]. Another study found that the fermented extracts of *C. rotundus* inhibited the growth of *P. aeruginosa*, *B. subtilis*, and *E. coli* [115]. For such reasons, these extracts may be conceived as a natural remedy against infections caused by pathogenic bacteria. More recently, copper oxide nanostructures synthesized using *C. rotundus* extracts revealed an excellent antibacterial activity against *Klebsiella pneumoniae* strains, and the observed inhibitory effects seemed to be associated with factors such as mechanical damage, oxidative damage, and genetic toxicity [116].

3.4. Anticancer Activity. The anticancer activity of *C. rotundus* extracts has also been assessed; the mechanism of action also elucidated the influence on genetic expression. For example, human cervical cancer (HeLa) cell lines exposed to different doses of *C. rotundus* extracts revealed morphological modifications and changes in the degree of chromatin condensation. Microarray analysis also showed that the extract led to the upregulation of 449 genes and downregulation of 484 genes, classified into different interaction pathways, with gene expression induction being associated with apoptosis and cell cycle arrest [117]. The main mechanism of anticancer activity of plant extracts is by inhibiting the cell proliferation or by inducing apoptosis in the cancerous cells. Both of these mechanisms are impaired in the cancerous cells. Plant extracts either halt the cell division or induce apoptosis of cancerous cells by activating the apoptotic factors. *C. rotundus* ethanol extracts were used to evaluate its effects on triple-negative breast cancer cells (TNBC) (negative for estrogen, progesterone receptors, and human epidermal growth factor receptor 2 (HER2) protein overexpression). As main findings, the authors stated that the chemical components present in the extracts inhibited the TNBC cell proliferation, which might be related to cell cycle arrest at the G_0/G_1 phase, thus inducing apoptosis by promoting Bcl-2-associated X protein (Bax) expression and inhibiting B cell lymphoma (Bcl) expression. The n-hexane extract from *C.*

rotundus rhizomes also exhibited an anticancer activity on Michigan Cancer Foundation-7 (MCF-7) breast cancer cell lines, by inducing apoptosis and halting them in G_0-G_1 stages of the cell cycle [118].

The cytotoxic effects of benzoquinones isolated from *Cyperus* sp. roots and tubers were also studied in adenocarcinoma gastric (AGS) and human gastric cancer cell lines. As main achievements, the authors stated that benzoquinones exerted their toxic effect by activating stress in the endoplasmic reticulum, increasing expression of C/EBP homologous protein (CHOP) (mRNA and protein levels), intracellular ROS, changes in calcium dynamics, and caspase-4 activation. The proteasome inhibition caused by hydroxyl cyperaquinone (causing cell death) was first described by the inositol-requiring enzyme 1α- (IRE1α-) independent/(PKR-like ER kinase) PERK-dependent pathway in stomach cancer cells [119]. Recently, the anticancer activity was also tested using silver nanoparticles in combination with *C. conglomeratus* extracts. The cytotoxic effect was assessed in MCF-7 breast cancer cells and normal fibroblasts using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and a selective cytotoxicity against MCF-7 was stated, while in fibroblasts, no toxic effect was reported. Furthermore, the apoptotic effects were confirmed using annexin V-fluorescein isothiocyanate-propidium iodide (FITC-PI) and real-time PCR for apoptotic genes [120]. Various biological activities of the extracts from *Cyperus* spp. are shown in Figure 3.

3.5. Other Biological Activities. Six sesquiterpenes isolated from *C. rotundus* rhizome methanol extracts were analyzed as an alternative to hormone replacement therapy (HRT). Its estrogenic activity was evaluated in MCF-7 cell lines through its competitive binding to estrogen receptor- (ER-) α and ER-β. These isolated compounds revealed to be useful as an alternative to HTR [121]. Tongbi-san (TBS), the name attributed to a set of 3 herbs: *C. rotundus*, *Citrus unshiu*, and *Poria cocos*, has been used in traditional Korean medicine for dysuria. In a study, a significant reduction in body weight and a decrease in weight of epididymal and visceral white adipose tissue were found following oral administration of TBS to male mice (C57BL/6N) for 11 weeks. TBS enhanced the expression of AMP-activated protein kinase (AMPK) and inhibited the expression of transcription factors, such as CCAAT-enhancer-binding proteins (C/EBPs), sterol regulatory element-binding transcription factor 1 (SREBP1), and peroxisome proliferator-activated receptors (PPARγ) in the liver and white adipose tissue of the epididymis [122].

Cyperus eragrostis seed extracts have also demonstrated vasodilatory properties and ability to inhibit the mammalian arginase enzyme in both an ex vivo experiment on rat aortic rings and an *in vitro* assay with purified bovine liver arginase [123]. Through an *in vivo* study in rats, mice, and chicks and *in vitro* study using isolated tissues of the jejunum and ileum of rabbits and rats, the antispasmodic, antidiarrheal, and antiemetic effects of *Cyperus niveus* Retz. were assessed. The presence of flavonoids, phenols, alkaloids, tannins, saponins, and glycosides in the extracts was described to be responsible for the significant inhibition of diarrhea in rats and for the marked decrease in the intestinal motility of mice [124].

Cyperus articulatus ethanolic extracts revealed to prevent pentylenetetrazol-induced seizures (PTZ) and to increase the gamma aminobutyric acid (GABA) levels in mice with PTZ [125]. Other preclinical (*in vivo* and *in vitro*) were experiments performed with *C. rotundus* to evaluate its effect on gastrointestinal, bronchial, and vascular disorders, as well as pain, emesis, pyrexia, and bacterial infections. A study revealed that the crude extract from *C. rotundus* has remarkable spasmolytic, bronchodilator, and vasodilatory effects, possibly through blockade of calcium channels [126]. A recent study evaluated the antiulcer potential of methanol and ethyl acetate extracts of *Cyperus alternifolius* L. rhizomes and aerial structures in fasted rats with orally administered indomethacin (30 mg/kg). The extracts led to a significant reduction in the number of ulcers and TNF-α content in the stomach. Histopathological examination revealed an improvement in damaged mucosa, with the effect generated by tubers being more effective than that of the control ranitidine [127].

4. Health-Promoting Effects: Data from Clinical Findings

As previously referred (Section 1), *Cyperus* spp. have been used over the years in folk medicine around the world to prevent and even treat different medical affections. *C. rotundus* is the most widely used and exploited species from this genus, and the metabolites present in this species are currently well elucidated [128, 129], so as their bioactive effects, analyzed through a number of investigations [130]. For example, the combined effects of *C. rotundus* with other medicinal plants taken as capsules of dehydrated plants or in the form of decoction were found effective against overweight, obesity, Alzheimer's disease (AD), depression, and rheumatoid arthritis. However, no clinical trials on *C. rotundus* used alone could be found.

An Ayurvedic polyherbal formulation, called Trimad, composed of *C. rotundus* tubers, *Embelia ribes* Burm.f. fruits and *Plumbago zeylanica* L. roots traditionally used for the management of overweight and obesity, was investigated by Salunke et al. [131] in 20 overweight and obese individuals. In this clinical study, the authors aimed to assess the effect of the aqueous extract of triphala (formulation made from *Emblica officinalis* L., *Terminalia bellirica* (Gaertn.) Roxb., and *Terminalia chebula* Retz.) (two tablets of 500 mg, twice a day after meals), Trimad, and placebo (dextrin) for the management of obesity and overweight, over 90 days. Significant differences were stated in visceral and subcutaneous fat, as well as other benefits, like positive bowel regulation and a decrease in fatigue in patients who received triphala [131]. Similarly, an Ayurvedic polyherbal mixture (no common name was mentioned) proved to be effective in the management of type 2 diabetes, with findings being compared to metformin effects in a 6-month treatment. The capsules, consisting of *Berberis aristata*, *C. rotundus*, *Cedrus deodara*, *Emblica officinalis*, *Terminalia chebula*, and *Terminalia bellirica*, following HPLC analysis revealed the presence of

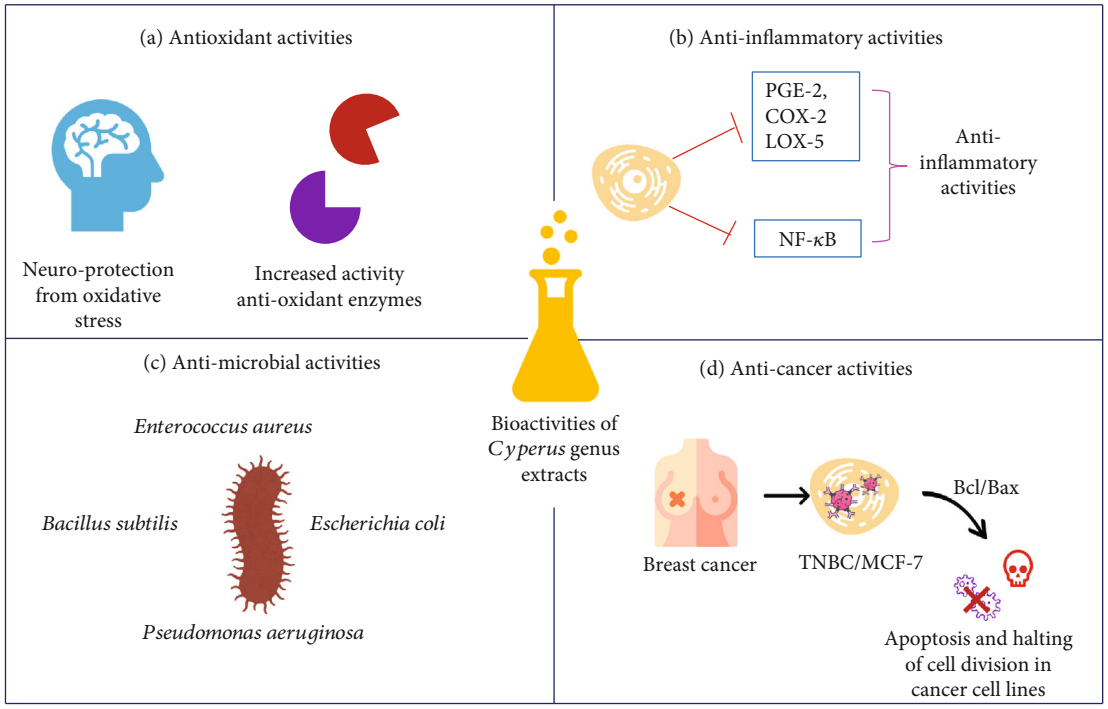

Figure 3: Biological activities of the extracts from *Cyperus* spp.

berberine (1.27%), quercetin (0.01%), and gallic acid (3.09%). The randomized study included 93 participants, and 48 of them received 3 g of the herbal treatment for 6 months. The study concluded that the formulation had a positive effect on blood glucose levels, on glycosylated hemoglobin, and on the lipid profile of the patients without showing any adverse effects [132]. Finally, another traditional Ayurvedic formulation with *C. rotundus* [133] was found to be useful for rheumatoid arthritis. The herbomineral formulation (5 g of each plant), consisting of *Vitex negundo*, *C. rotundus*, *Nyctanthes arbor-tristis*, *Smilax glabra*, *Delphinium denudatum*, and *Withania somnifera*, was combined with Maha yogaraj Guggulu Vaiswanar churna and Simhanada Guggulu. No information on its safety or chemical composition was provided in the article, despite the fact that patients (39%) who receive the preparation twice a day before meals for 1 year exhibited a good response to the treatment.

The possible use of *C. rotundus* against neurocognitive disorders has also been reported. The combination of *C. rotundus* with *Crocus sativus* and *Astragalus membranaceus* honey was assessed for its ability to treat a major neurocognitive disorder. A double-blind clinical trial on 60 patients previously diagnosed was performed. The intervention group took two daily capsules of 500 mg each one of the combinations for three months. The results indicated that the combination could be useful to improve the cognitive and depression score. However, the extract used was not characterized, and the preparation method was not indicated [134]. The traditional Iranian herbal medicine, Davaie Loban, composed of *C. rotundus*, *Zingiber officinale*, *Acorus calamus*, *Piper nigrum*, and *Boswellia carterii* has also been tested against AD. In the study, 24 patients with mild to moderate AD took 500 mg of the capsules 3 times daily for 12 weeks. Results showed that the herbal mixture might have an improvement in the memory of patients. However, despite the safety of the administered mixture which was tested, the preparation method was not detailed [135]. In traditional Chinese medicine, the Guilpi decoction, an herbal mixture, is used given its ability to regulate blood pressure and as an antidepressant [136]. So, Zhuang et al. [137] tested the effect of Guilpi decoction in 120 elderly patients affected with hypertension and depression. The decoction was prepared as follows: 15 g of a mixture formed by *Codonopsis pilosula*, *Atractylodes*, *Angelica sinensis*, membranous milkvetch root, *Polygala tenuifolia*, and *Arillus longan*; 20 g of *Poria cocos* and *magnesium*; 30 g of calcined Os Draconis, calcined oyster shell, light wheat, and concha haliotidis; and 10 g of elecampane, fresh ginger, Chinese date, colla corii asini, mint, *Albizia julibrissin*, *C. rotundus*, jasmine, and *Coptis chinensis* as well as spina date seed of 40 g. The mixture was decocted, and patients received 400 ml of extract for a month. The control group was treated with sertraline. Results concluded that the mixture exerted a curative effect and improved the patients' quality of life by alleviating depression symptoms, although no significant differences were reported in blood pressure.

Some other clinical trials have been performed using the extracts of *Cyperus* spp., but the species investigated were not specified in a large amount of cases. These are the cases of mixtures made with *Cyperus* for sexual dysfunction [138], as anti-inflammatory and analgesic [139] agents and even as lactation inducers [140]. Another critical limitation present in many clinical trials is that in most cases, as previously listed, the herbal mixture was not characterized, and when a chemical characterization was reported, only some polyphenolic compounds were analyzed. In a case, the herbal mixture

was consumed as an aqueous extract, but the polyphenol analysis by HPLC was done in a methanol/water extract. Therefore, the active metabolites responsible for the therapeutic action remain unknown, and the possible synergistic and antagonistic interactions among the plant constituents were not addressed neither described. In addition and also worth noting is that few researches have done a microbiological or safety study on the dehydrated plants when consumed in the form of capsules of dried plants. However, the presence of pathogens, spores, heavy metals, and even aflatoxins was not described in the preparations, which otherwise would have been a potential risk for patients. Moreover, such clinical trials were done with a few patients, and in most of them, both treatment monitoring and fidelity were not precise. Therefore, there is an urgent need to design more robust and reproducible clinical trials to prove the potential health benefits of *Cyperus* spp., which have been widely described via a plethora of *in vitro* and *in vivo* tests.

5. Safety and Adverse Effects

Due to its richness in chemical constituents, *Cyperus* plants have been widely used in folk medicine for multiple affections [100, 141, 142]. Thorough screening of literature available on *Cyperus* plants as a popular remedy among various ethnic groups, researchers have increasingly explored their therapeutic potential [143]. However, it is also of extreme interest to evaluate the toxicological aspects of botanical drugs and products for their reliable and safe usage among consumers.

Toxicological data from *C. rotundus* extracts have been reported by several investigators [144]. Most of them reported the use of *Cyperus* extracts as safe [100, 109], with no side effects or even only minor side effects being reported. For example, *C. rotundus* extracts were studied *in vivo* for toxicity and biochemical activities in mice and rats. The lethal dose LD_{50} of *C. rotundus* root extract, when administered intraperitoneally, was 90 g/kg [145, 146]. Ethanolic extract of dried roots of this plant administered to mice of both sexes showed $LD_{50} > 0.5$ mg/kg [147]. Aqua-ethanolic (1 : 1) extracts of rhizome administered to mice of both sexes produced LD_{50} of 681.0 mg/kg [148]. In turn, the LD_{50} of *Cyperus* essential oils was 5000 mg/kg in rats [149]. Other studies reported that a single oral administration of *C. rotundus* ethanolic extract at 5000 mg/kg did not produce signs of toxicity, behavioral changes, mortality, or differences on gross appearance of internal organs in the animals. In subacute toxicity, all rats received a repeated oral dose of 1000 mg/kg of the ethanolic extract for 14 days. The parallel group received the ethanolic extract in the same period but was kept for further 14 days. Application did not produce any effects or reversibility of toxic effects. Thus, it was concluded that the extract did not cause changes in terms of general behaviors, mortality, weight gain, and hematological and clinical blood chemistry parameters in comparison to the control group [150]. Another research also confirmed the safety of *Cyperus* extracts. *C. rotundus* methanolic extracts in mice at doses of 250 and 500 mg/kg body weight showed no toxic effects [151]. On the other side, Lemaure and coworkers [152] documented that the administration of 45 or 220 mg/kg/day of *C. rotundus* tubers' hexane extracts for 60 days in rats stimulated a significant reduction in weight gain but without toxic effects. Raut and Gaikwad [153] also observed no toxicity symptoms following the administration of *C. rotundus* crude extract at different concentrations and oral doses. Jebasingh et al. [154] performed acute toxicological studies with *C. rotundus* extract and found no mortality or morbidity up to 2000 mg/kg body weight in rats. Toxicity studies also revealed no changes in food, water consumption, and body weight of animals with an increase in white blood cell count and hemoglobin level as well as improvement in kidney and liver function. Krisanapun et al. [155] carried out the acute toxicity test of *C. rotundus* water extracts in rats and reported the single oral $LD_{50} > 5$ g/kg body weight. The *C. rotundus* extract used at three doses, 10, 100, and 1000 mg/kg, did not exhibit any sign of toxicity. However, it was observed that at 1000 mg/kg, motor activity was slightly decreased. The effects of *C. rotundus* extract were also assessed on different biochemical parameters (glucose, lipid profile, cardiac enzymes, liver enzymes, and kidney function test). Liver enzymes were found normal, and a nonsignificant increase in serum bilirubin, gamma-glutamyl transferase (GGT), and serum glutamic-pyruvic transaminase (SGPT) was recorded. Also, hematological studies did not show any significant toxic changes, and histopathological examination confirmed that the tested extract was safe and nontoxic [156]. Finally and also worth noting is the use of *Cyperus* spp. for biotechnological purposes, namely, regarding its use as a functional food additive. For example, Carvalho Barros and coworkers [157] evaluated the replacement of beef fat in beef burgers using a tigernut (*C. esculentus*) oil emulsion, to reduce total fat and saturated fatty acids in the studied samples. As main findings, the authors stated that total replacement of beef fat using tigernut oil emulsions in beef burger resulted in a well-accepted and healthier meat product with reduced total and saturated fat contents, as well as increased unsaturated fatty acids [157]. However, further studies are needed to further explore other *Cyperus* sp. agroindustrial and biotechnological potentialities.

6. Conclusions and Perspectives

Across the diverse traditional systems of medicine, *Cyperus* sp. is popularly employed as a potent ethnomedicine owing to its plethora of pharmacological attributes, *viz.*, antioxidant, anti-inflammatory, neuroprotective, antidepressive, antiarthritis, antiobesity, antimicrobial, anticancer, vasodilator, spasmolytic, bronchodilator, and estrogenic properties. This wide array of biological activities is closely linked to the presence of phytochemicals such as α-cyperone, α-corymbolol, α-pinene, caryophyllene oxide, cyperotundone, germacrene D, mustakone, and zierone. However, its wide-ranging use in folk medicine and expansive pharmacological properties are not corroborated with incontrovertible evidences employing animal models, where despite the bioactive phytochemicals of *Cyperus* spp., they have been well-deciphered. Comprehensive investigations on the pharmacological efficacies of isolated compounds are still inadequate.

Moreover, structure-activity analyses on the obtained phytoconstituents have also uncovered the perception of the underlying molecular insights of action of its active extracts and/or phytochemicals. On the other side and although toxicological data have indicated the use of *Cyperus* spp. as safe and effective, conclusive studies encompassing its clinical, toxicological, and safety features are still sparse. In addition and given that reported clinical trials lack herbal mixtures' characterization containing *Cyperus* spp., further studies are needed to explore the precise active constituents. Moreover, the subchronic toxicity as well as their interactions with commonly used conventional drugs to assure a safe and long-term consumption by human subjects also needs further attention of the researchers. Lastly and not least interesting to underline is that, considering the extensive pharmacological potential of *Cyperus* spp., more in-depth research is needed to attain a greater clarity of its mechanism of action.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

All authors contributed equally to the manuscript. Conceptualization was performed by J.S.-R., N.C.-M., M.K., W.C.C., and A.D.; validation, investigation, data curation, and writing were performed by all authors; review and editing were performed by J.S.-R., N.C.-M., M.K., W.C.C., and A.D. All the authors read and approved the final manuscript.

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Review Article

Diplazium esculentum (Retz.) Sw.: Ethnomedicinal, Phytochemical, and Pharmacological Overview of the Himalayan Ferns

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The genus *Diplazium* (family: Athyriaceae) comprises approximately 350 species of pteridophytes. *Diplazium esculentum* (Retz.) Sw. is an important member of this genus and commonly known as a wild vegetable in the Himalayan and sub-Himalayan communities. According to the literature analysis, *D. esculentum* was traditionally used for the prevention or treatment of several diseases such as diabetes, smallpox, asthma, diarrhea, rheumatism, dysentery, headache, fever, wounds, pain, measles,

hypertension, constipation, oligospermia, bone fracture, and glandular swellings. Various extracts of *D. esculentum* were evaluated to elucidate their phytochemical and pharmacological activities. A wide array of pharmacological properties such as antioxidant, antimicrobial, antidiabetic, immunomodulatory, CNS stimulant, and antianaphylactic activities have been recognized in different parts of *D. esculentum*. The review covers a systematic examination of pharmacognosy, phytochemistry, and pharmacological applications of *D. esculentum*, but scientifically, it is not fully assessed regarding complete therapeutic effects, toxicity, and safety in the human body. The published literature on *D. esculentum* and its therapeutic properties were collected from different search engines including Wiley online, PubMed, Springer Link, Scopus, Science Direct, Web of Science, Google Scholar, and ACS publications by using specific terms such as "*Diplazium esculentum,* bioactive compounds, biological activities and health benefits" from 1984 to 2021 (March). Therefore, further studies are required to identify the detailed action mechanism of *D. esculentum in vitro*/*in vivo*, and also, more studies should focus on conservation, cultivation, and sustainable utilization of the species.

1. Introduction

The Himalayan botanicals are well known to produce wide variety of secondary metabolites due to critical climatic conditions [1–4]. These botanicals, including wild plants, have a significant role in food security and socio-economic development of the region [5, 6]. Moreover, these botanicals are locally utilized for food resources, medicines, and other purposes due to the presence of numerous bioactive compounds and high nutritional value [7, 8]. With recent developments in science and technology, the importance of wild plants has been identified as a possible source of nutraceuticals and/or functional foods [9].

Among several high valued functional foods, *Diplazium esculentum* is one of the important species of wild ferns, which is frequently consumed by people living in the hilly areas; it is not growing on much higher altitude. *D. esculentum* ($n = 41$ chromosomes, grade of polyploidy = diploid) is utilized as a traditional vegetable in the Himalayan communities [10]. It is an important member of the genus *Diplazium* which comprises around ~350 species of pteridophytes, mainly distributed in Asia and Oceania [11].

Specifically, *D. esculentum* is distributed through different parts of the globe including Cambodia, China, India, Indonesia, Japan, Malaysia, Papua New Guinea, Pakistan, Philippines, Singapore, Taiwan, Thailand, Vietnam, and Bangladesh. It grows on the banks of rivers and streams, canals, marshy areas, and hills with an altitudinal range up to 2,300 meters [12, 13].

It is locally known by different names such as English: vegetable fern; India: dung-kek, kari-welli-panna-maravara, kasrot, kukari-sag, mairungshai, para-panna-maravara, linguda, kathura; Japan: Kuware-shida; Malay: paku, pakutanjong; Nepali: paninyuro, piraunli; Papua New Guines: sigogo; Philippines: Pako; Thai: kut-kin; and Bangladesh: Dheki Shak [14].

Traditionally, *D. esculentum* is being used in the treatment of various ailments (as shown in Figure 1) such as diabetes, smallpox, asthma, diarrhea, rheumatism, dysentery, headache, fever, wounds, pain, measles, high blood pressure, constipation, oligospermia, bone fracture, glandular swellings, and skin-related diseases by the different communities in India and other countries [15–21].

Recently, a few researchers have validated its nutraceutical and pharmacological properties by using *in vitro* and *in vivo* models/methods. For the development of evidence-based medicine, a critical investigation of current knowledge is required regarding ethnopharmacology, chemical composition, biological activities, and possible side effects of the species. Additionally, *D. esculentum* belongs to the least concern category under International Union for Conservation of Nature (IUCN) 2021-1 (https://www.iucnredlist.org/species/194150/8883499) and needs more attention. Therefore, in this manuscript, we reviewed and discuss the recent scientific information conducted so far on *D. esculentum*, which includes its pharmacognosy, phytochemistry, and pharmacology.

2. Pharmacognosy

2.1. Traditional Uses. Traditionally, *D. esculentum* is one of the most popular vegetables consumed in different parts of the globe, namely, India, Philippines, Nepal, China, Thailand, Indonesia, etc. The literature has revealed that *D. esculentum* is still being used by different communities for the treatment of several diseases including diabetes, smallpox, asthma, diarrhea, rheumatism, dysentery, headache, fever, wounds, pain, measles, and high blood pressure. The detailed information on the traditional uses of the species is summarized in Table 1. Additionally, this species is collected in large amounts and marketed by the rural and tribal communities of India for their livelihood enhancement [22]. The Mishing community of Assam (State of the Indian republic) used it essentially in the religious ceremony of the dead person [23].

2.2. Proximate and Mineral Composition. The nutritional value of any food substance can be analyzed by its proximate and mineral composition [24]. Literature-based screening of the proximate composition of *D. esculentum* revealed the presence of lipids, proteins, carbohydrates, vitamins, fiber, etc., while mineral composition possesses the presence of essential micro and macro compounds [25–35]. The comparative analyses of proximate and mineral composition of *D. esculentum* are presented in Tables 2 and 3.

3. Bioactive Compounds

Traditionally, botanicals are being widely used to cure various ailments due to the presence of high-valued bioactive compounds [61, 62]. Literature-based screening for bioactive compounds of *D. esculentum* revealed the presence of

Figure 1: Traditional uses of *Diplazium esculentum*.

alkaloids, flavonoids, glycosides, phenolic, tannins, terpenoids, steroids, carbohydrates, fats, and oils in different solvent systems [2, 35, 46, 50, 60, 63–69].

In the study of Essien and coworkers [16], the chemical composition of essential oil isolated from *D. esculentum* leaves and the major volatile compounds were identified as β-pinene (17.2%), α-pinene (10.5%), caryophyllene oxide (7.5%), sabinene (6.1%), and 1,8-cineole (5.8%) (Figure 2). The essential oil of this species was composed of monoterpene hydrocarbons, oxygenated sesquiterpenoids, sesquiterpene hydrocarbons, oxygenated monoterpenoids, and nonterpene derivatives.

Few compounds such as ascorbic acid [70], eriodictyol 5-*O*-methyl ether 7-*O*-β-D-xylosyigalactoside [71], tannins and phytates [72], α-tocopherol [73], quercetin [74, 75], pterosin [75], ptaquiloside [76], terpene, hopan-triterpene lactone [51], and lutein [77] were also isolated from *D. esculentum*. Additionally, four phenolic compounds ((2R)-3-(4′-hydroxyphenyl) lactic acid, trans-cinnamic acid, protocatechuic acid, and rutin) and three ecdysteroids (amarasterone A1, makisterone C, and ponasterone A) were isolated from young fronds of *D. esculentum* collected from Japan [78] while 26 bioactive compounds were identified in the methanolic extracts of young fronds of *D. esculentum* collected from Indian Himalaya [60]. The major compounds present in the species were identified as pentadecanoic acid, β-sitosterol, neophytadiene, α-linolenic acid, methyl palmitate, diisobutyl phthalate, phytol, and 10,12 hexadecadien-1-ol [60]. These all major compounds are shown in Figures 3(a) and 3(b), respectively.

4. Biological Applications

Among the functional properties of *D. esculentum*, the antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and immune-modulatory activities can be considered as potentially contributing to the preventive and pharmacological values of this plant species (Figure 4). The following sections reviewed the abovementioned functional biological activities of different *D. esculentum* extracts.

4.1. Antioxidant Activities. The botanicals can be considered as safe and cost-effective natural antioxidants capturing free radicals and may help in the prevention and the treatment of different diseases [79, 80]. Recently, a research group from Indonesia reported that the methanolic extract of *D. esculentum* showed a good antioxidant activity with an IC_{50} value of 123.95 ppm according to 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay [2].

In an *in vitro* study, the nutritional properties and antioxidant capacity of *D. esculentum* were evaluated on the ethanol extract of the edible parts. The phytochemical analysis indicated that the ethanolic extract possesses significant concentrations of flavonoids (90.6–144.5 mg QE/gm) and tannins (26.8–57.2 mg GAE/gm). Considerable antioxidant activities of *D. esculentum* were revealed using different antioxidant assays including DPPH radical scavenging ($IC_{50=}$146.51 μg/mL), superoxide radical scavenging ($IC_{50=}$111.17 μg/mL), hydroxyl radical scavenging ($IC_{50=}$43.45 μg/mL), and reducing power ($IC_{50=}$76.36 μg/mL) assays.

In another study, the antioxidant activity of *D. esculentum*, extracted by using pressurized hot water extraction (PHWE) method, was reported [81]. The results demonstrated that the optimum condition for the best antioxidant activity of PHWE was at 175°C, 21 min extraction time (2 g dried powder in 50 mL water) in Box-Behnken design. The plant extract showed moderate DPPH scavenging activity (EC_{50} = 1241.14 μg/mL). The hydro-alcoholic extract of *D. esculentum* leaf was evaluated for antioxidant activity using the DPPH and nitric oxide assays [82]. The IC_{50} value of the plant extract for DPPH and NO inhibition activity was found to be 138.8 and 151.9 mg/mL, respectively.

The methanolic extract of *D. esculentum* fronds showed promising antioxidant activity using different assays (DPPH, ABTS, NO, metal chelating, and superoxide scavenging activity) [64]. The IC_{50} values of the plant extract was recorded as 3.8, 4.6, 0.59, and 2.24 mg/mL for DPPH, ABTS, metal chelating, and superoxide scavenging activity, respectively, while nitric oxide, hydroxyl ion, and FRAP assays were recorded as 100-10000 μg/mL, 100-10000 μg/mL, and 0.095-0.121 mM Fe^{2+} equivalent. Table 4 includes detailed information about previous antioxidant activities.

4.2. Antimicrobial Activities. Recently, several pathogenic microorganisms have developed antibiotic resistance, and these antibiotics can have undesirable side effects [92]. Thus, researchers are focusing on botanicals for the development of herbal-based antibiotic substitutes [93]. Table 5 includes antimicrobial studies performed with *D. esculentum*. Antimicrobial activity was considered good (minimum inhibitory concentration (MIC) less than 100 μg/mL), moderate (MIC from 100 to 500 μg/mL), weak (MIC from 500 to 1000 μg/mL), or inactive (MIC over 1000 μg/mL). Inactive results of antimicrobial activities of *D. esculentum* did not included in this study [46, 94].

The areal parts of *D. esculentum* were extracted with ethanol to evaluate the antimicrobial properties by using the disk diffusion method. The crude extract showed considerable antimicrobial activity in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) value. The MIC value was recorded from a range of 200-800 μg/mL (200 μg/mL (*Bacillus cereus*), 400 μg/mL

Table 1: Traditional uses of *D. esculentum* in different regions.

Plant part	Ethno-pharmacological uses	Country	References
Fronds/leaves/areal part/whole plant	Cooked and eaten as a vegetable and in soups to maintain good health.	India, Bangladesh, Thailand	[23, 36–38]
	Tender leaves are cooked with fruit of *Dillenia indica* and fish and taken as vegetable.	Malaysia, India	[39, 40]
	Hairs are removed, boiled with salt and water until water is evaporated then fried and eaten as vegetable.	India	[41–45]
	Used in headache, pain, fever, wounds, dysentery, glandular swellings, diarrhea, measles, toothache, high blood pressure, and various skin infections. Fronds used by pregnant women as protection against difficult childbirth. Leaf paste is used in the wounded place externally for the cure of bone fracture. Used as a laxative. Used as insecticides.	Bangladesh, Nigeria, Indonesia, Nigeria, India, Philippines	[15–17, 19, 46–48]
	The tender frond is cooked without salt and is consumed with rice for 5–10 days for the treatment of diabetes.	India	[20]
	Eaten as highly preferred Koche Sag, Neuro/Niuro vegetable.	Nepal	[49]
	Used as vegetable and pickle.	India, Vietnam, Japan, Indonesia, Philippines	[30, 31, 50–53]
Root	About 20 g of fresh root is boiled in 1 liter of water and reduced to one-fourth of its volume. 3 mL of this decoction along with 2 mL of honey is taken orally on an empty stomach twice a day for 15 days to cure spermatorrhea.	India	[54]
	About 50 g juice obtained from macerated root is fed three times for human dysentery. Macerated root extract is also useful for the cattle dysentery.	Bangladesh	[55]
	About 2-3 spoonsful of root juice are taken for 1/2 days, or 1/½ cup of boiling extract of whole plant is taken thrice daily to treat infections and used as an antidote. The root paste is used externally for the treatment of rheumatism and smallpox. Two pills of pulverized root and honey are taken thrice daily for 2 weeks for the treatment of oligospermia.	Bangladesh	[17]
Rhizome	Decoction of rhizome used as a tonic and also used for the cure of hemoptysis and cough.	India	[39, 56]

Table 2: Proximate composition of *Diplazium esculentum* from different regions.

Parameters	Bangladesh (mg/100 g) [34]	Indonesia (%) [29]	India (%) [26]	India (%) [57]	Philippines (%) [33]	Nepal (%) [58]	Indonesia (%) [27]	India (%) [59]	India (%) [60]
Moisture (%)	8.8	—	89.34	92.4	91.82	93.25	90.84	93.1	90.4
Lipid	2.16	—	—	—	—	—	—	—	—
Protein	8.73	6.20-8.30	3.84	31.2	0.87-10.67	0.99	2.23	2.6	8.87
Ash	5.09	1.90-2.11	1.33	16.2	1.42-17.39	1.10	1.38	1.3	—
Total carbohydrate	59.62	—	—	44.3	—	—	—	1.0	18.8
Fiber	15.59	—	5.05	4.6	0.72-9.06	0.99	4.82	—	3.1
Fat	—	0.51-0.68	0.25	8.3	0.28-3.40	0.15	0.04	2.0	2.5
Water level	—	2.70-3.08	—	—	—	—	—	—	—
Vitamin C (mg/100 g)	—	—	21.38	21	—	6.20	—	—	—

(*Escherichia coli* and *Aspergillus ochraceus*), and 800 μg/mL (*Bacillus megaterium*)) while MBC from a range of 800 to >800 μg/mL (800 μg/mL (*B. cereus*, *A. ochraceus*) and >800 μg/mL (*B. megaterium*, *E. coli*)), respectively [95].

Different parts (leaves, rhizomes, and roots) of the *D. esculentum* were extracted with aqueous and alcoholic solvents to evaluate the antibacterial activity by using the disk diffusion method. Four bacterial strains, namely, *E. coli*, *Salmonella arizonae*, *Salmonella typhi*, and *Staphylococcus aureus*, were used in this study. The rhizome and root extracts inhibited the growth of microorganisms while leaf extract did not show any inhibition. Additionally, extracts

Table 3: Mineral composition of *Diplazium esculentum* from different regions.

Parameters	Bangladesh (mg/100 g) [32]	Bangladesh (mg/g) [34]	India (mg/100 g) [26]	Indonesia (μg/g) [31]	Malaysia (mg/kg) [28]	India (mg/100 g) [57]	India (mg/g) [25]	Nepal (mg/100 g) [58]	Indonesia (mg/kg) [27]	India (mg/100 g) [59]
N	—	13.97	—	—	—	—		—	—	—
P	48	1.58	—	—	—	—		117	0.09	—
K	—	7.93	—	—	—	914.4		—	0.24	927.4
Ca	9	—	0.66	—	—	192.7		—	0.39	200.5
Mg	11	—	9.56	—	—	0.36	10-12.11	—	0.14	—
Fe	—	—	14.38	15.7	—	11.2	20.2-23.4	1.03	44.6	—
Mn	—	—	11.91	7.03	3.24-22.5	—	0.04-0.38	—	—	—
Na	54	20.21	0.50	—	—	9.5		—	—	8.1
Cu	—	—	13.37	3.99	3.24-24.3	0.32	1.03-1.28	—	4.24	—
Al	—	—	58.5	18.3	—	—	0.10-0.73	—	—	—
As	—	—	14.6	—	—	—	—	—	—	—
Cd	—	—	0.4	—	—	—	—	—	—	—
Hg	—	—	0.07	—	—	—	—	—	—	—
Li	—	—	2.1	—	—	—	—	—	—	—
Ni	—	—	24.5	—	—	—	—	—	—	—
Pb	—	—	0.8	2.46	0.31-3.26	—	—	—	—	—
Cr	—	—	—	0.05	1.19-3.03	—	—	—	—	—

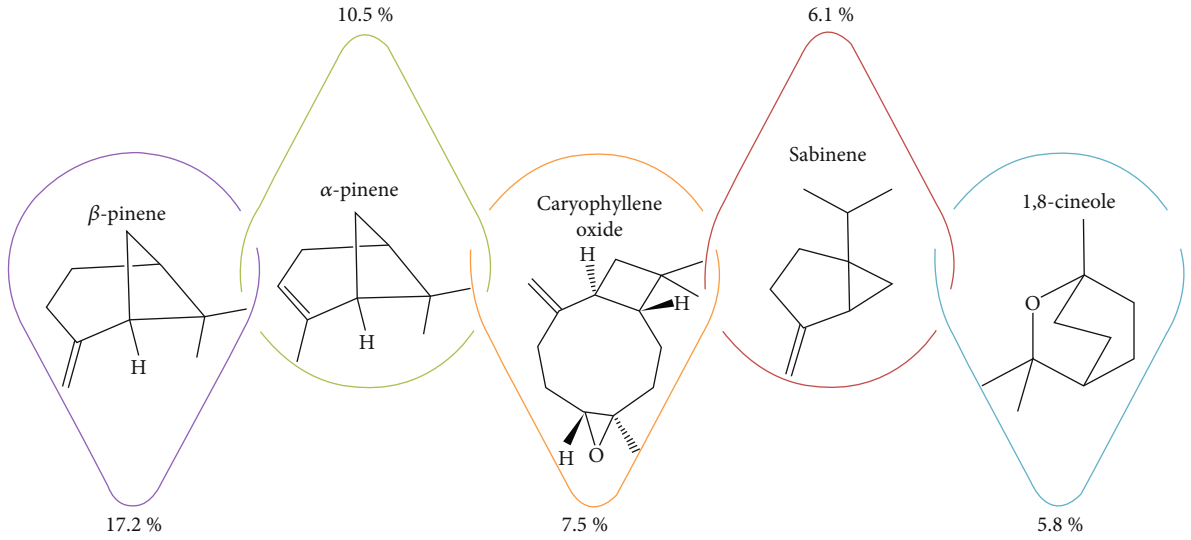

Figure 2: Main essential oil components of *Diplazium esculentum* [16].

combined with the antibiotic (tetracycline in equal amount) were more potent against bacterial strains than the antibiotic alone [96].

The aerial parts of *D. esculentum* extracts were evaluated for antimicrobial activity by using a colorimetric broth microdilution method. A total six different solvent extracts (hexane, chloroform, ethyl acetate, ethanol, methanol, and distilled water) were used against a series of microbial strains including *S. aureus*, *B. cereus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, *Acinetobacter baumannii*, *Candida albicans*, *Candida parapsilosis*, *Issatchenkia orientalis*, *Cryptococcus neoformans*, *Aspergillus brasiliensis*, and

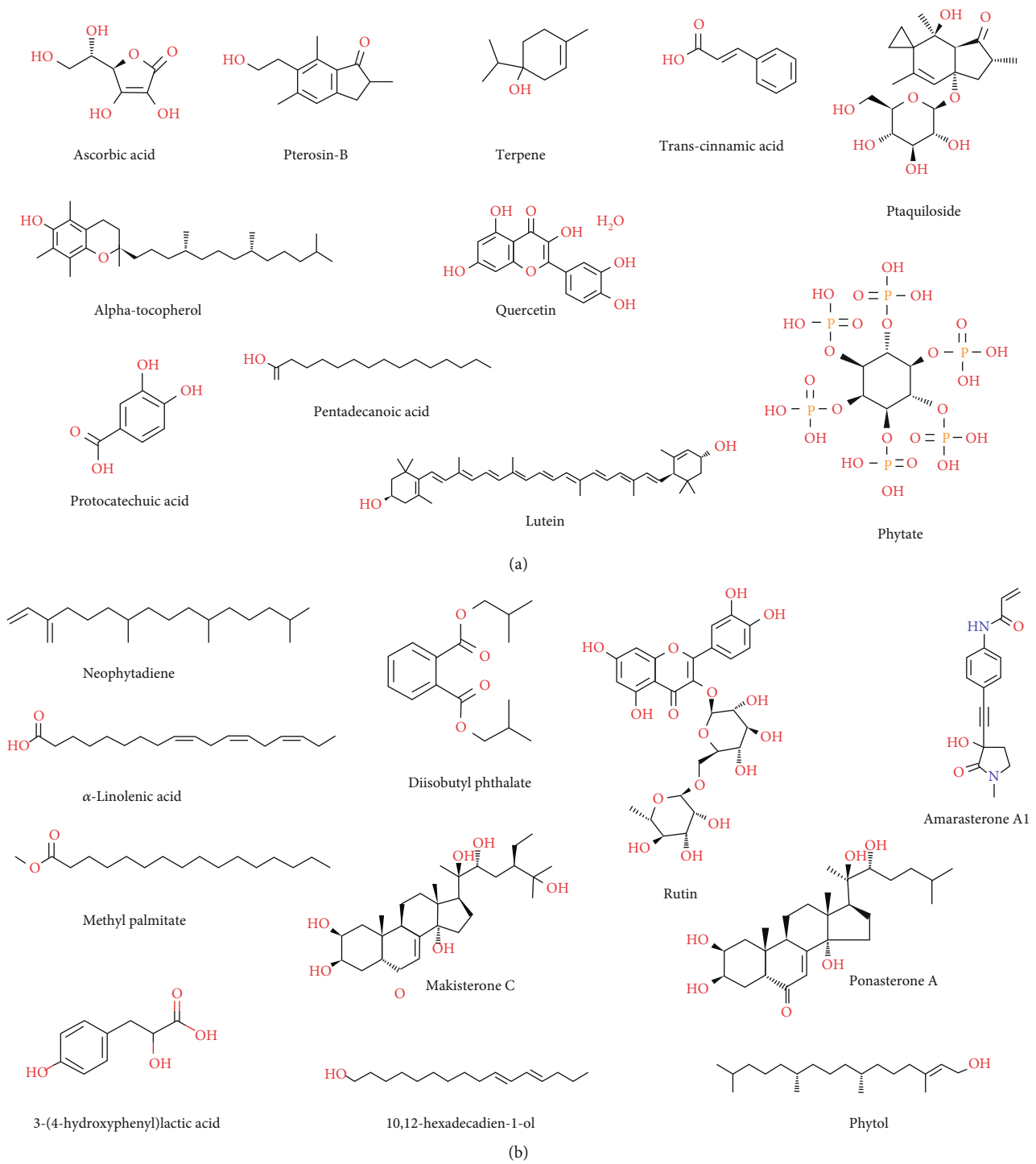

Figure 3: (a) Main nonoil bioactive components of *D. esculentum*. (b) Main nonoil bioactive components of *D. esculentum*.

Trichophyton mentagrophytes. The plant extract only showed a good-moderate antimicrobial activity against *I. orientalis* [97].

The methanolic extract of *D. esculentum* leaves has been evaluated for antibacterial activity by using the disc diffusion method [98]. The plant extract showed slight antibacterial activity (6-10 mm zone of inhibition) against *Salmonella paratyphi*, *Vibrio parahaemolyticus*, *E. coli*, *B. megaterium*, *Shigella dysenteriae*, and *Shigella boydii* among 12 bacterial strains.

The chloroform and methanolic extracts of *D. esculentum* leaves were evaluated for antimicrobial activity by using the disk diffusion method [46]. The plant extracts showed inactive antimicrobial activity against all the microbial strains tested, namely, *K. pneumoniae*, *S. aureus*, *E. coli*, *Salmonella typhimurium*, *Vibrio cholerae*, *Sarcina lutea*,

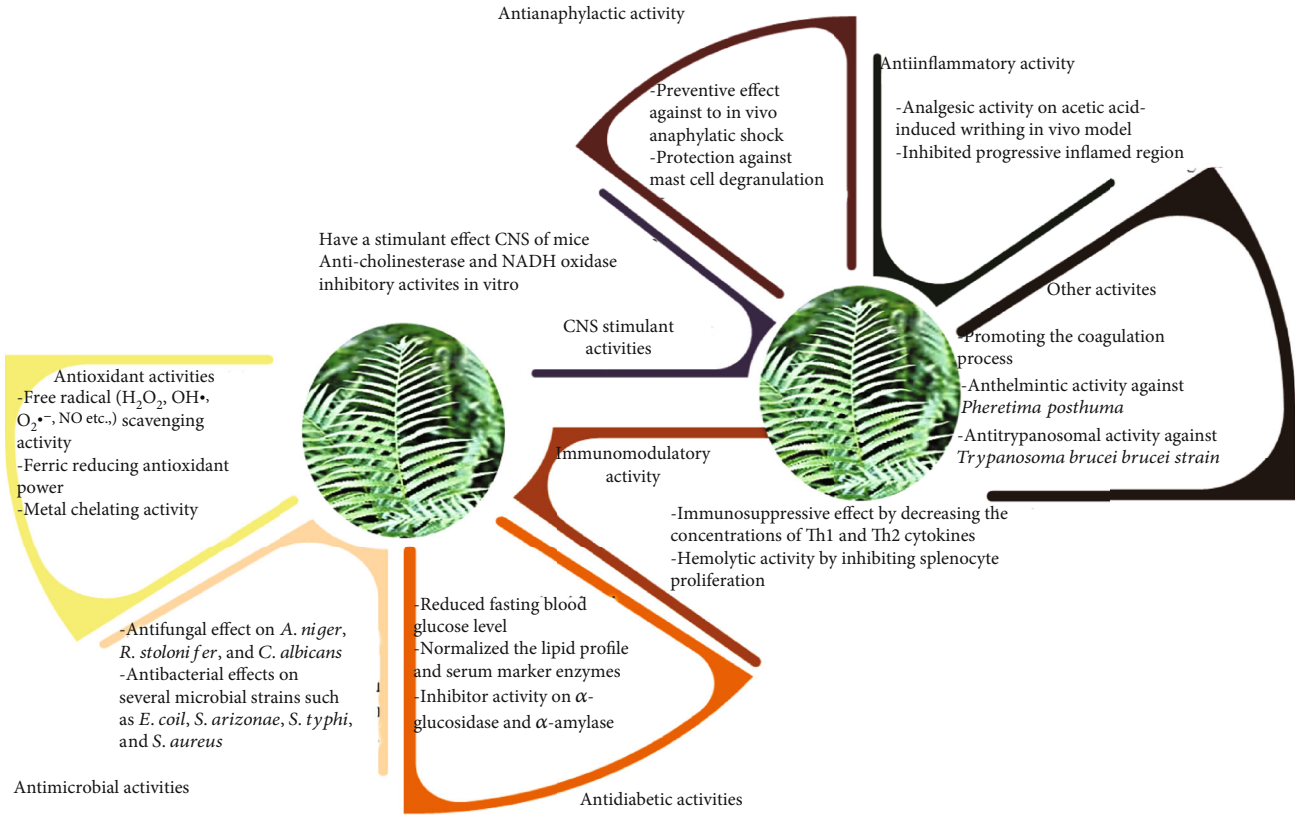

Figure 4: Summary of the proposed biological activities of *Diplazium esculentum*.

Bacillus subtilis, and *Shigella boydii* in terms of MIC (1.6-12.5 mg/mL) value.

The antifungal activity of *D. esculentum* leaves against three fungal strains using the agar diffusion method has been reported [94]. The methanolic extract showed inactive antifungal activity against *Aspergillus niger*, *Rhizopus stolonifer*, and *C. albicans* in terms of MIC (50-100 mg/mL) and minimum fungal inhibition concentration (100-200 mg/mL).

4.3. Antidiabetic Activities. Diabetes mellitus is a chronic carbohydrate, fat, and protein metabolism disorder characterized by the increase in blood glucose level due to defect of insulin secretion [99]. The inhibition of α-glucosidase and α-amylase enzymes, involved in the digestion of carbohydrates, can significantly reduce the postprandial increase of blood glucose and therefore can be an important strategy in the management of blood glucose level in type 2 diabetic and borderline patients. The antidiabetic activity of *D. esculentum* through inhibition of α-glucosidase and α-amylase enzymes has been reported [69]. The results demonstrated that *D. esculentum* extract exhibited the highest α-amylase (92.09%) and α-glucosidase (70.01%) inhibitory activities.

The protective effect of a hydro-alcoholic extract of *D. esculentum* on streptozocin- (STZ-) induced diabetes was evaluated [82]. In this study, a total of 30 rats were used and treated with plant extract up to 21 days. After the treatment, it was observed that the plant extract (500 mg/kg) reduced (50.2%) the blood glucose level in STZ-induced diabetic rats. Additionally, a significant reduction was recorded in plant extract-treated rats for lipid profiling ($p < 0.01$), serum marker enzyme activity ($p < 0.001$), necrosis, and regeneration of beta cells. The plant extract showed dose-dependent activity in all the experiments.

4.4. Immunomodulatory Activity. The immunosuppressive and hemolytic activities of *D. esculentum* extracts in mouse models have been evaluated [100]. A total of 120 Swiss albino mice (6-8 weeks age) were treated with plant extracts up to 180 days. After this treatment, the plant extract showed significant dose-dependent decreases in body weight, relative spleen weight, number of plaques (formation of antibody secreting cells) formed, hemagglutination antibody titer value, the number of peritoneal macrophages, and the number of cultured splenocytes. The *in vitro* analysis showed significant dose-dependent increases in the percentage inhibition of splenocyte proliferation as well as the percentage of hemolysis. In other words, the treatment with *D. esculentum* may act as an immunosuppressive agent.

The impact of boiled *D. esculentum* on Th1 and Th2 cytokine levels of Swiss albino mice that were treated with different doses of plant extract, daily up to 180 days, has been reported [101]. The outcome of the study demonstrated that the plant extract significantly decreases the concentration of Th1 and Th2 cytokines when compared with controls. In other words, boiled *D. esculentum* extract may affect some of the innate and cell-mediated immune responses by modulating the level of Th1 and Th2 cytokines.

Table 4: Previous antioxidant studies in *Diplazium esculentum*.

Plant part used and solvent system	Name of assay	Key results		Antioxidant activity*	References
		Plant extracts	Positive control		
Whole plant, (chloroform, n-butanol, aqueous)	Free radical scavenging (DPPH)	IC_{50} = 287 -404 μg/mL	IC_{50} = 17.45 μg/mL	Moderate	[77]
	Radical cation scavenging activity ($ABTS^+$)	IC_{50} = 191 -273 μg/mL	IC_{50} = 08.44 μg/mL	Moderate	
	Ferric reducing antioxidant power (FRAP)	0.44-0.55 mg/g	—		
Leaves (methanol)	Free radical scavenging (DPPH)	31.35-57.95% inhibition	91.99-97.03% inhibition	Moderate	[57]
Leaves (methanol)	Free radical scavenging (DPPH)	IC_{50} = 402.88 μg/mL	IC_{50} = 324.86 μg/mL	Weak	[83]
Leaves (protein)	Free radical scavenging (DPPH)	IC_{50} = 10.23 mg/mL	—	—	[84]
	Free radical scavenging (DMPD·+)	IC_{50} = 14.67 mg/mL	—	—	
	Radical cation scavenging activity ($ABTS^+$)	IC_{50} = 07.95 mg/mL	—	—	
Leaves (not reported)	Free radical scavenging (DPPH)	336-3359 ORAC unit^2/g	—	—	[50]
Leaves (ethanol, vinegar, acetic acid, aqueous)	Free radical scavenging activity (DPPH)	258-303 μmol TE/100 g	—	—	[85]
Leaves (chloroform, methanol)	Total antioxidant capacities (TAC)	181.94-207.41 mg/g	—	—	[46]
	Free radical scavenging (DPPH)	IC_{50} = 5907 -95669 μg/mL	IC_{50} = 13.76 μg/mL	Weak	
Leaves (methanol)	Free radical scavenging activity (DPPH)	IC_{50} = 1.73 mg/mL	—	—	[86]
	Metal chelating activity	52.07 mg/mL	—	—	
	Ferric reducing antioxidant power (FRAP)	2.12 μg/mg	—	—	
	Radical cation scavenging activity ($ABTS^+$)	IC_{50} = 0.03 mg/mL	—	—	
Fronds (aqueous, ethanol)	Radical cation scavenging activity ($ABTS^+$)	09.60-57.84% inhibition	—	—	[65]
	Hydrogen peroxide scavenging (H_2O_2)	15-40% inhibition	50% inhibition	Strong	
Leaves (methanol)	Hydroxyl radical scavenging (OH·)	IC_{50} = 811.00 μg/mL	IC_{50} = 571.00 μg/mL	Weak	[87]
	Superoxide anion scavenging ($O_2\cdot^-$)	IC_{50} = 90.39 μg/mL	IC_{50} = 42.06 μg/mL	Strong	
	Nitric oxide radical scavenging (NO)	IC_{50} = 204.28 μg/mL	IC_{50} = 90.82 μg/mL	Moderate	
	Hydrogen peroxide scavenging (H_2O_2)	IC_{50} = 4.17 mg/mL	IC_{50} = 3.24 μg/mL	Strong	
	Peroxynitrite scavenging ($ONOO^-$)	IC_{50} = 3.35 mg/mL	IC_{50} = 0.87 μg/mL	Strong	
	Singlet oxygen scavenging ($^1O_2$)	IC_{50} = 278.88 μg/mL	IC_{50} = 46.15 μg/mL	Moderate	
	Hypochlorous acid scavenging (HOCl)	IC_{50} = 338.96 μg/mL	IC_{50} = 235.95 μg/mL	Moderate	

Table 4: Continued.

Plant part used and solvent system	Name of assay	Key results			References
		Plant extracts	Positive control	Antioxidant activity*	
	Iron chelating	IC_{50} = 1.33 mg/mL	IC_{50} = 0.001 μg/mL	Strong	
	Lipid peroxidation inhibition	IC_{50} = 141.67 μg/mL	IC_{50} = 6.76 μg/mL	Moderate	
Leaves (petroleum ether, chloroform, acetone, methanol, aqueous)	Ferric reducing antioxidant power (FRAP)	0.22-7.6 mM/dry weight	—	—	[88]
Leaves (aqueous-methanol, acetone)	Free radical scavenging (DPPH)	IC_{50} = 0.92 -3.60 mg dry wt.	—	—	[89]
	Ferric reducing antioxidant power (FRAP)	4.99-8.78 mg/g	—	—	
Leaves (methanol)	Free radical scavenging (DPPH)	EC_{50} = 3353.2 μg/mg	EC_{50} = 322.4 μg/mg	Weak	[90]
Leaves (aqueous)	Free radical scavenging (DPPH)	50 μmol/g	—	—	[91]
	Ferric reducing antioxidant power (FRAP)	100 mol/g	—	—	
	Cupric ions chelation assay	80% inhibition	—	—	

(-): not mentioned in the reference papers; (*): antioxidant activity was considered strong (less than 100 μg/mL), moderate (100 to 500 μg/mL), weak (500 to 1000 μg/mL), or inactive (over 1000 μg/mL) compared with control.

4.5. CNS Stimulant Activities. The impact of "Ulam" (a fresh Malaysian vegetable, *D. esculentum*) on cognitive status has been evaluated [102]. In this cross-sectional study, a total of 132 adults were recruited. Socio-demographic information, anthropometric measurements, dietary history, food frequency, and cognitive function were assessed. The average ulam intake by the participants was 15.1 ± 8.2 g/day. The outcome of the study indicated that "pucukpaku" showed protective effects (62.9%) against cognitive decline.

The anticholinesterase and NADH oxidase inhibitory activities of a methanolic extract of *D. esculentum* have been evaluated [83]. Recently, most of the studies reported that the inhibition of anticholinesterase has been shown to be a strategy for the treatment of neurodegenerative disorders. The results of the study demonstrated that the methanolic extract of *D. esculentum* inhibited acetyl-cholinesterase and NADH oxidase in a dose-dependent manner, with IC_{50} values of 272.97 and 265.81 μg/mL.

The CNS stimulant effect of *D. esculentum* in a mouse model using digital acto-photometer has been reported [88]. The plant water extract showed statistically significant ($p < 0.0001$) and dose-dependent activity when compared with control and standard caffeine.

4.6. Toxicity Studies (In Vitro and In Vivo). The methanolic and chloroform extracts of *D. esculentum* were evaluated for their toxicity using brine shrimp lethality bioassay. Both extracts produced dose-dependent increment in percent mortality of brine shrimp nauplii which indicates the presence of toxic compounds in the extracts. The LC_{50} values were recorded as 1.87 μg/mL (chloroform), 1.62 μg/mL (methanol), and 0.66 μg/mL (vincristine sulphate as standard drug) [46]. In another study, the toxicity of methanolic extract of *D. esculentum* using brine shrimp lethality bioassay was reported as significant (LC_{50} = 18.6 μg/mL). [98]. In other study, the cytotoxicity of ethanolic extract of *D. esculentum* was evaluated in different cell lines including breast cancer (MDA-MB-231 and MCF-7), colon cancer (Caco-2), liver cancer (HepG2), and normal liver (Chang liver), and no cytotoxic effect was observed [103].

The systemic toxicity and several pathological effects of *D. esculentum* were evaluated on rabbits and guinea pigs [104]. The study indicated that the plant extract decreased all the pathological functions including growth, body weight, forced motor activity, alterations of blood glucose values, erythrocyte sedimentation rate, mean corpuscular volume, mean corpuscular hemoglobin, total leukocyte count, neutrophil, lymphocyte, and monocyte count, while increased blood SGOT level in both rats and guinea pigs. In other words, the plant extract indicated toxic effects in guinea pigs and rabbits, while rats showed a little adverse effects. Junejo and coworkers reported the nontoxic effects of *D. esculentum* extract on experimental models and recommended as a potential functional food [67].

The toxicological impact of *D. esculentum* on male reproductive functions of Swiss albino mice has been reported [105]. A total of 120 male Swiss albino mice of 6-8 weeks of age were fed orally with 80, 160, and 320 mg/kg b.w. of plant material and treated up to 180 days. After this successful treatment, the boiled plant extract showed significant dose and time-dependent decreases in body weight, absolute and relative testis

Table 5: Antimicrobial activities of *Diplazium esculentum*.

Plant part used and solvent system	Microorganism	Antimicrobial activity	MIC (μg/mL)	MBC or MFC (μg/mL)	Reference
Aerial parts (ethanol)	*Bacillus cereus*	Moderate	200	800	[95]
	Escherichia coli	Moderate	400	>800	
	Aspergillus ochraceus	Moderate	400	800	
	Bacillus megaterium	Weak	800	>800	
Aerial parts (ethanol)	*Staphylococcus aureus*	Moderate-weak	310-630	NA	[97]
	Bacillus cereus	Moderate-weak	310-630	1250	
	Klebsiella pneumoniae	Moderate-weak	310-630	NA	
	Pseudomonas aeruginosa	Weak	630	NA	
	Candida albicans	Weak-inactive	630-1250	2500	
	Candida parapsilosis	Weak-inactive	1250-2500	NA	
	Cryptococcus neoformans	Moderate	310	310	
	Issatchenkia orientalis	Good-moderate	80-160	160	
Aerial parts (hexane)	*Cryptococcus neoformans*	Moderate	310	310	[97]
	Issatchenkia orientalis	Good	80	160	
Aerial parts (chloroform)	*Staphylococcus aureus*	Moderate-weak	310-630	NA	[97]
	Bacillus cereus	Moderate	310	630	
	Klebsiella pneumoniae	Moderate-weak	310-630	NA	
	Pseudomonas aeruginosa	Weak-inactive	630-1250	NA	
	Trichophyton mentagrophytes	Weak-inactive	630-1250	1250	
	Candida albicans	Weak-inactive	630-1250	NA	
	Cryptococcus neoformans	Moderate	310	310	
	Issatchenkia orientalis	Good	80	160	
Aerial parts (ethyl acetate)	*Pseudomonas aeruginosa*	Weak	630	NA	[97]
	Cryptococcus neoformans	Moderate	310	310	
	Issatchenkia orientalis	Moderate	160	310	
Aerial parts (methanol)	*Staphylococcus aureus*	Weak-inactive	630-1250	NA	[97]
	Bacillus cereus	Weak-inactive	630-1250	2500	
	Klebsiella pneumoniae	Weak-inactive	630-1250	NA	
	Pseudomonas aeruginosa	Weak-inactive	630-1250	NA	
	Cryptococcus neoformans	Moderate	310	310	
	Issatchenkia orientalis	Moderate	160	310	
Aerial parts (aqueous)	*Pseudomonas aeruginosa*	Weak-inactive	630-1250	NA	[97]
	Cryptococcus neoformans	Moderate	160-310	310	
	Issatchenkia orientalis	Moderate	160	310	
Leaves, rhizomes, and roots (aqueous and alcoholic)	*Escherichia coli*		-*		[96]
	Salmonella arizonae		-*		
	Salmonella typhi		-*		
	Staphylococcus aureus		-*		
Leaves (methanol)	*Salmonella paratyphi*		-*		[98]
	Vibrio parahemolyticus		-*		
	Escherichia coli		-*		
	Bacillus megaterium		-*		
	Shigella dysenteriae		-*		
	Shigella boydii		-*		

MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration; MFC: minimum fungal concentration; NA: no activity; "-": not tested. Antimicrobial activity was considered good (MIC less than 100 μg/mL), moderate (MIC from 100 to 500 μg/mL), and weak (MIC from 500 to 1000 μg/mL). * Only zone inhibition test was performed.

weight, the relative weight of other organs and their biochemical parameters, percentage of live spermatozoa, fertility, and fecundity in plant extract fed mice. In other words, the main outcome of this study is boiled extracts of *D. esculentum* possess toxic properties that can be slow down the male reproductive functions and may induce infertility.

4.7. Antianaphylactic and Mast Cells Stabilizing Activity. D. esculentum were extracted with aqueous and ethanolic

solvents and evaluated for mast cell stability and antianaphylactic activity. In this study, Swiss albino mice (18-20 g) and Wistar rats (150-170 g) were used. A significant reduction was observed in the number of degranulated mast cells of the plant extracts-treated models ($p < 0.001$). After the administration of both extracts at 250 and 500 mg/kg doses, it showed 72.83%, 76.67%, 69%, and 71.67% intact mast cells. Plant extract demonstrates protective activity against mast cell degranulation. The 500 mg/kg dose of both extracts showed maximum inhibition of the release of myeloperoxidase from lung tissue. Additionally, the plant extract had stabilized the mast cell membrane and decreased the level of nitric oxide in serum and peritoneal fluid [106].

4.8. Anti-inflammatory Activity. The ethanolic extract of *D. esculentum* was evaluated for anti-inflammatory activity [107]. A total of 25 male mice were recruited in this experiment and divided into 5 groups. The ethanolic extract indicated anti-inflammatory activity on hind paw oedema in terms of inflamed inhibition percent of 125 mg/kg b.w. (71.72%), 250 mg/kg b.w. (81.49%), and 250 mg/kg b.w. (92.60%) in the treated group. In another study, a considerable analgesic activity of *D. esculentum* was recorded using the acetic acid-induced writhing method in mice [63].

4.9. Other Biological Activities. The aqueous and powder extract of *D. esculentum* leaves was evaluated for coagulant activity [108]. The plant extracts combined with polyaluminium chloride showed a synergistic effect for all the measured parameters in Kuala Sepetang Landfill Site (KSLS) leachates. The combination was identified as a high molecular weight polymer, and it acted as an anionic coagulant and was also capable of promoting the coagulation process.

Extracts of the rhizome of *D. esculentum* extracts were evaluated for their anthelmintic activity against *Pheretima posthuma*. The study included three solvents (ethanol, aqueous, and petroleum ether) and three concentrations (10, 25, and 50 mg/mL), and all the extracts demonstrated significant anthelmintic activity in terms of the time of paralysis and time of death. Ethanolic extract showed the highest activity compared to other solvents, and the activity was recorded in dose-dependent patterns [109].

Silver nanoparticles (10-45 nm) were synthesized using the leaf powder of *D. esculentum* [110]. The synthesized nanoparticles were evaluated as a catalyst in the degradation of methylene blue and rhodamine B and also evaluated for the anticoagulation activity. The synthesized Ag NPs showed considerable anticoagulation activity. Besides, prominent photocatalytic activity in the degradation of methylene blue and rhodamine B was also recorded.

The antitrypanosomal activity of *D. esculentum* leaves was evaluated against *Trypanosoma brucei brucei* strain BS221 [111]. In this study, the ethanolic extract was used with seven different concentrations (0.01 to 12.5 μg/mL), and the extract showed significant antitrypanosomal activity with IC_{50} value 4.32 μg/mL and a selectivity index (SI) value > 23 in mammalian cell line (Vero, $IC_{50} > 100\,\mu$g/mL) when compared with the positive control (pentamidine, I $C_{50} = 4.51$ ng/mL).

5. Concluding Remark and Future Prospective

The present manuscript reports traditional uses, nutraceuticals, pharmacognosy, phytochemistry, and pharmacological studies in *D. esculentum*. The literature survey revealed that *D. esculentum* is one of the most important and popular wild species of ferns in the Himalaya. It is a widely used species in different traditional systems, but the complete chemical composition and active compounds need to be further elucidated and authenticated by bioassay-guided isolation. However, very limited studies are available for this species, not only in terms of chemical characterization but also in terms of pharmacological evaluation as well. Most of the studies are limited to the *in vitro* screening and a few for *in vivo*. Clinical trial studies should be performed to evaluate the safety profile of wild ferns in the human body in terms of antimicrobial activity, antidiabetic activity, anti-inflammatory activity, and immunomodulatory aspects. Apart from this, educating the local people regarding the cultivation, conservation, and sustainable utilization of this plant will help for improving the population size of the species.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declared that they have no conflict of interest regarding this manuscript.

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Review Article

Natural Coumarins: Exploring the Pharmacological Complexity and Underlying Molecular Mechanisms

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Coumarins belong to the benzopyrone family commonly found in many medicinal plants. Natural coumarins demonstrated a wide spectrum of pharmacological activities, including anti-inflammatory, anticoagulant, anticancer, antibacterial, antimalarial, casein kinase-2 (CK2) inhibitory, antifungal, antiviral, Alzheimer's disease inhibition, neuroprotective, anticonvulsant, phytoalexins, ulcerogenic, and antihypertensive. There are very few studies on the bioavailability of coumarins; therefore, further investigations are necessitated to study the bioavailability of different coumarins which already showed good biological activities in previous studies. On the evidence of varied pharmacological properties, the present work presents an overall review of the derivation, availability, and biological capacities of coumarins with further consideration of the essential mode of their therapeutic actions. In conclusion, a wide variety of coumarins are available, and their pharmacological activities are of current interest thanks to their synthetic accessibility and riches in medicinal plants. Coumarins perform the valuable function as therapeutic agents in a range of medical fields.

1. Introduction

Plants synthesize a large collection of natural products called secondary metabolites [1, 2]. Secondary metabolites have important ecological functions, supporting plant protection against microbes and herbivores and participate in the attraction of pollinators etc. Humans use secondary metabolites as a source of drugs, aromatization, flavouring agents, and for a wide spectrum of other use [3]. Natural compound coumarins have been exposed to considerable phytochemical and pharmacological exploration in the last few decades. It is shown that over the last three years, over 400 coumarins have been described in scientific publications [4].

Coumarins are present naturally in a large number of plants, considerably in high concentration in *Coumarouna odorata* (tonka bean) (Fabaceae/Leguminosae) [5]. It is also present in high content in vanilla grass (*Anthoxanthum odoratum*) [6], sweet clover (genus *Melilotus*) [7], cassia cinnamon (*Cinnamomum cassia*) [8], in extracts of *Justicia pectoralis* [9], and a large number of cherry blossom trees [10]. Some species from the Apiaceae family (*Prangos* Lindl., *Ferula* L., *Heracleum* L., *Pachypleurum* Hoff., *Conioselinum* Fisch., *Libanotis* L., and *Seseli* L.) were rich with coumarin contents [11]. Many plants contain different concentrations of coumarin. Tonka beans, liquorice, and cassia cinnamon have a high concentration of natural coumarins. Some cherry blossom strawberries and apricots contain coumarin in smaller quantities. Despite its sweet smell, animals tend to avoid plants that contain coumarins due to their bitter taste [12]. Coumarins are natural bioactive compounds recognized for their anti-inflammatory, anticoagulant, antibacterial, antifungal, antiviral, anticancer, antihypertensive, antituberculous, anticonvulsant, antiadipogenic, and antihyperglycemic pharmacological activities, as well as its antioxidant and neuroprotective actions [13]. Over 1300 coumarins have been identified from plant sources. Tonka beans can contain, on average, 1% to 3% coumarin [14]. Animal studies have shown that these grains can be toxic, even in small amounts, causing serious liver damage in just a few weeks [15].

Coumarins belong to the benzopyrone family commonly found in many medicinal plants (Figure 1). Their structure consists of two six-membered *rings* with lactone carbonyl groups. Most coumarin compounds are thermally stable and have notable optical activity [16]. The coumarin biosynthesis takes part in involving multiple P450 enzymes [17]. *Ortho*-hydroxylation is a key point for the biosynthesis of natural coumarins in plants [18].

Naturally occurring coumarins have been reported for their wide pharmacological activities, including antidiabetic [19], antioxidant, anabolic and hepatoprotective activities [19, 20], antifungal, and anti-inflammatory effects [21–23] (Figure 1). Calanolides, a natural coumarin, isolated from the *Calophyllum* genus have demonstrated strong anti-HIV activity [24] (Figure 2).

Accordance to evidence of varied pharmacological properties, the current paper presents an overall perspective of the derivation, availability, and biological capacities of coumarins with further consideration of the essential mode of their therapeutic actions.

2. Review Methodology

For this updated review, useful data were collected from the scientific databases PubMed, Science Direct, Scopus, Web of Science, and Elsevier, on the molecular mechanisms and pharmacological studies of natural coumarins and their derivatives. The following MeSH terms were used to search for them: "coumarins," "chemistry," "structure-activity relationship," "pharmacology," "drug therapy," "humans," "clinical trials," "diabetes," "anti –Inflammatory," "neuroprotective," "antibacterial," " antifungal," and "antiviral." The scientific names of the plants were verified using Plant-List (http://www.theplantlist.org/), and the chemical formulas were verified with ChemSpider.

Inclusion criteria are as follows: preclinical pharmacological studies that highlighted the molecular mechanisms of action and signaling pathways and experimental *in vitro* and *in vivo* pharmacological studies.

Exclusion criteria are as follows: studies without obvious mechanisms and doses, works that were not written in English, and studies that included homeopathic preparations.

3. Bioavailability of Coumarins

Bioavailability is described as "the extent and rate to which the active drug ingredient or active moiety from the drug product is absorbed and becomes available at the site of drug action." Usually, bioavailability mentions to the absorption of a compound from the gastrointestinal tract following oral administration of a dosage form [25, 26]. Therefore, it is not enough to know the dosage of taken compound(s); the more important issue is how much of that present is bioavailable. However, compound concentrations regularly cannot be determined directly at the site of action. Therefore, many bioavailability examinations concern to determine the concentration of the active compound in the blood or urine. Thus, bioavailability is involved with how fast and how much of a compound appears in the blood after a specific dose is administered.

A pharmacokinetic study was carried out by Ritschel and Hoffmann [27] who have compared between prolonged-release of coumarin containing tablets with intravenous and peroral administration of coumarin solution in humans, where a correlation was established between the percent of drugs released *in vitro* and the area under the curve (AUC). Xie and colleagues' [28] study showed that coumarin-based prodrug system produced a fourfold growth in oral bioavailability over the parent drug meptazinol in rats. In another kinetic study, Abraham et al. [29] presented that the absorption of coumarin from cinnamon powder is slightly lower than that of isolated coumarin.

In another research, the exchange of chloride anion by coumarin-3-carboxylate in sertraline, an antidepressant drug, enhances the pharmacological activity of the native drug, binding to bovine serum albumin with higher effect

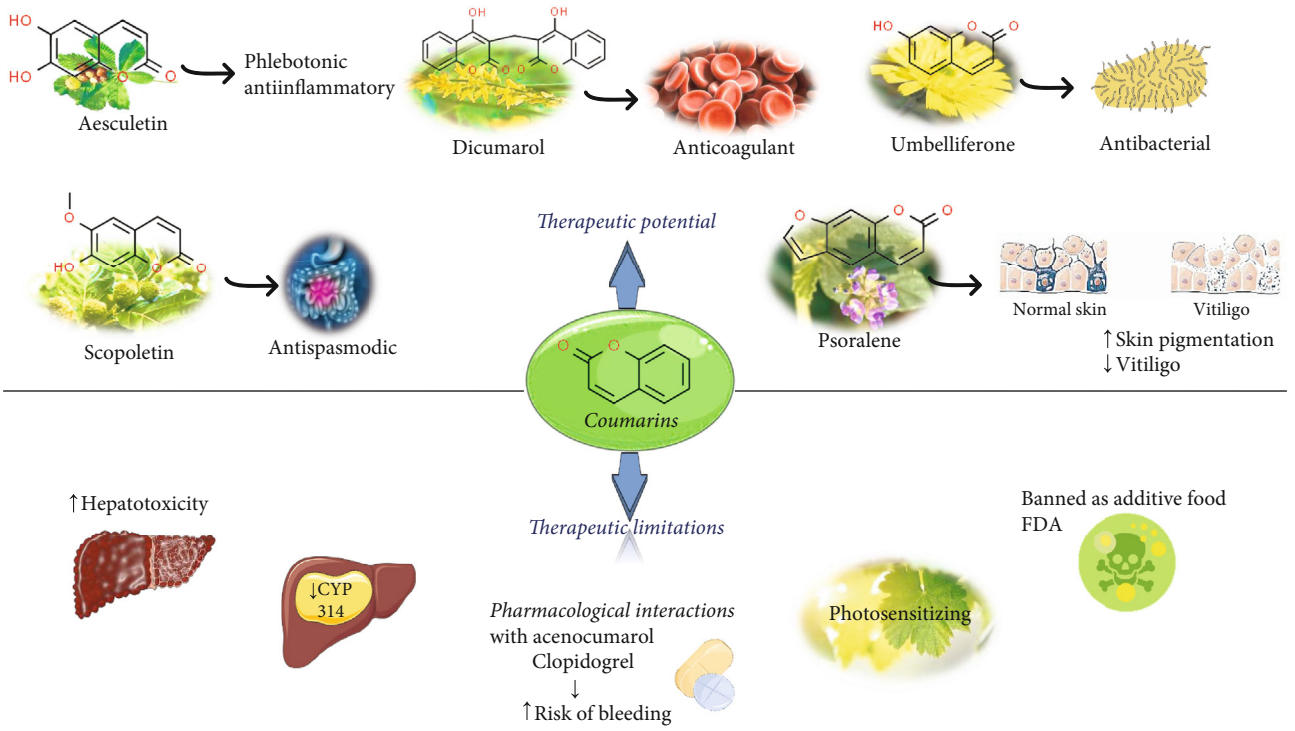


Figure 1: Diagram with the most relevant pharmacological properties related to chemical structures of natural coumarins and their derivatives.

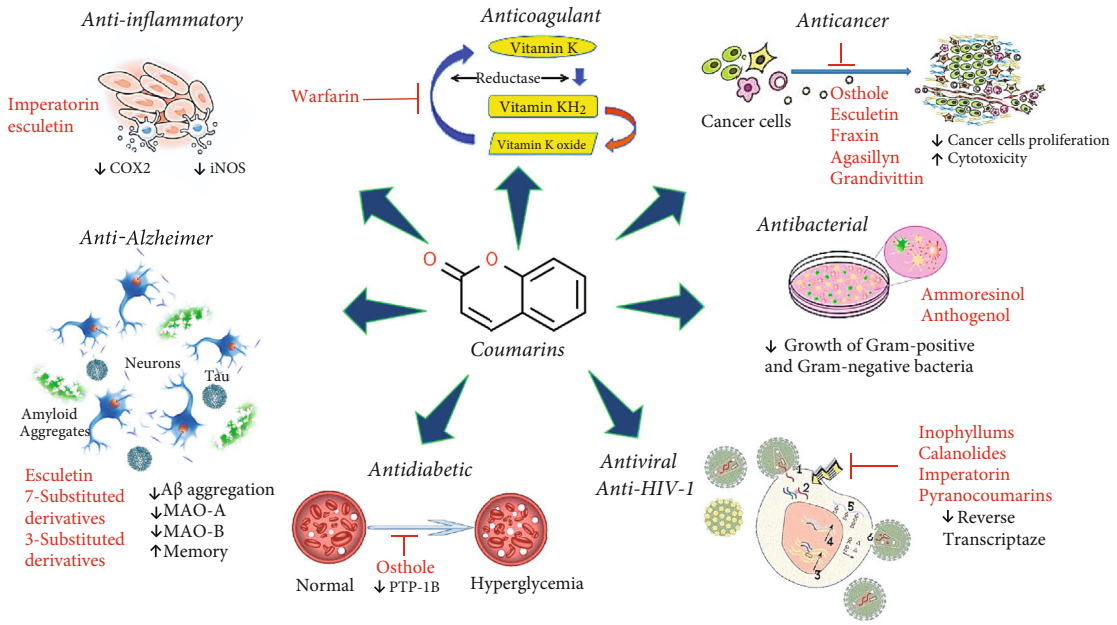


Figure 2: Schematic representation of molecular mechanisms and signaling pathways of the most representative coumarins and their derivatives. Abbreviations: COX-2: cyclooxygenase 2; iNOS: inducible nitric oxide synthase, PTP-1B: protein tyrosine phosphatase; MAO: monoamine oxidase.

and saving the antimicrobial properties of the antidepressant molecule [30]. Newly designed coumarins can be oppressed for suppression of P-gp-mediated efflux to promote bioavailability of paclitaxel and can suppress breast cancer stem cell development which is crucial for planning potent anticancer drugs [31].

There are very few studies on the bioavailability of coumarins; therefore, future pharmacological studies are needed

to study the bioavailability of naturally pharmacologically active coumarins.

4. Natural Coumarins and the Link between Chemical Structures and Pharmacological Effects

Coumarin molecules are made of combined benzene and α-pyrone rings and are chemically 2H-1-benzopyran-2-one which involves a large group of phenolic constituents in plants. The term coumarin derives from Coumarona odorata, a South American vegetable from which the molecule was first isolated in the 1820s. Coumarins were originally isolated in *Dipteryx odorata* Wild (tonka bean) [32].

They have been observed in over 150 various plants which belong to almost 30 diverse families, of which some important ones are Apiaceae, Caprifoliaceae, Clusiaceae, Guttiferae, Nyctaginaceae, Oleaceae, Rutaceae, and Umbelliferae. Naturally occurring coumarins are chiefly grouped into six types. In Figure 1 and Table 1, different types of coumarin are listed with their reported pharmacological activities.

At the end of the 19th century, farmers in North America introduced sweet clover (*Melilotus officinalis*) to animal feed and soon a hemorrhagic epidemic arose, which later proved to be correlated with the consumption of sweet clover. During the drying of the clover, coumarin undergoes a series of chemical transformations—partly spontaneous and partly mediated by Aspergillus fungi resulting in dicumarol [56]. Dicumarol interferes with the blood clotting process, blocking the synthesis of vitamin K-dependent clotting factors. One of its chemical derivatives, warfarin, is currently used as an oral anticoagulant in the treatment of deep vein thrombosis, prophylaxis of pulmonary embolism, myocardial infarction in patients with atrial fibrillation, or valve prostheses. Another chemical derivative of dicumarol, acenocoumarol, the active ingredient in sintrom drug, has similar therapeutic indications [57].

Scopoletin from the species Viburnum prunifolium and Angelica (essential oil extracted from the roots) has hypotensive and spasmolytic properties—able to inhibit the spastic contraction of smooth gastrointestinal and genitourinary muscle [58]. Khelline and visnagine, two coumarins of the species *Ammi visnaga*, have spasmolytic action on the smooth muscle of the coronary vessels with an antianginal effect [59]. Umbelliferone, a coumarin present in the grassy aerial parts of *Pilosella* and the resins of many Umbelliferae species, is used as a sunscreen and has antibiotic properties against brucellosis [60].

Esculetin, a coumarin present in chestnut leaves (*Aesculus hippocastanum*) due to the aglycone esculetin decreases the permeability of capillaries and increases their resistance, has anti-inflammatory and phlebotonic effects, improving venous tone. The same coumarin, from the flowers of the sweet clover (Melilotus officinalis), demonstrated antiedematous properties in vivo using animal models. Due to these properties, esculetin in combination with flavonoids is recommended as a phlebotonic adjuvant in the treatment and prophylaxis of chronic venous insufficiency [61]. Esculetin has also bacteriostatic and antifungal properties, while dafnoretin and 3-phenylcoumarins have antiviral properties against hepatitis B virus and anti-HIV. Esculetin also inhibits the synthesis of prostaglandins, thromboxanes and leukotrienes, and proinflammatory molecules involved in asthmatic, allergic, and inflammatory reactions [62].

Phytoalexins are plant-derived coumarins with general protective properties to fungal infection, physical injury, chemical damage, or a pathogenic progression. The main function of phytoalexins is to constrain or destroy the attacking agents like bacteria, viruses, and insects. Ayapin (6,7-methylenedioxycoumarin) is phytoalexin which was initially isolated from *Eupatorium ayapana*, a member of Asteraceae. Later on it was also isolated from several plants such as *Artemisia apiacea* [63], *Helianthus annuus*, *Pterocaulon polystachyum* [64], and *Pterocaulon virgatum* [65].

In plants, coumarin can be found in both free and glycosidic form, bound in the form of an aglycone to a glucose molecule. The high structural heterogeneity of coumarins justifies the great pharmacological variability with benefits for human health.

5. The Mechanisms behind the Pharmacological Activities of Natural Coumarins

5.1. Anti-Inflammatory Activity. Inflammation starts with five major signs: pain, swelling, redness, heat, and loss of function which ended up in vascular permeability, leukocytes invasion, production of pain, local oedema, and necrosis [66, 67]. Inflammation can be a denotation of multiple diseases like osteoarthritis, Alzheimer, and atherosclerosis [3]. Moreover, many types of cancers are related to chronic inflammation. Therefore, it is important to deal with inflammation before it turns up into a severe disease [68, 69].

Coumarin possessed anti-inflammatory characteristics, and its mechanism of action involves phagocytosis, production of the enzyme, and proteolysis to eradicate protein and oedema fluid from wounded tissue. One of its derivatives imperatorin also reported for its anti-inflammatory property in lipopolysaccharide-stimulated mouse macrophage (RAW264.7) *in vitro* as well as in carrageenan-induced mouse paw oedema model *in vivo*. It stops the protein expression of inducible cyclooxygenase-2 and NO synthase in lipopolysaccharide-stimulated RAW264.7 [70], whereas another derivative esculetin showed its anti-inflammatory potential in rat colitis induced by trinitrobenzene sulfonic acid [34, 71] This coumarin was extracted from *Bougainvillea spectabilis* and *Cichorium intybus*.

Coumarins possess antioxidant properties that may contribute to their anti-inflammatory effects [72]. The coumarin 1,2-benzopyrone has long been known to be effective in slowly reducing shoulder lymphedema. The underlying mode of action is unclear, but macrophage-induced proteolysis of the oedema proteins may be implicated.

5.2. Anticoagulant Activity. A complex system controls the blood fluidity in the human body [73]. Blood should remain inside the vasculature and also must clot quickly on exposure to injury [74]. Vitamin K is denoted as a blood anticoagulant,

Table 1: Summarized biological activities of different type of coumarins.

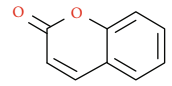
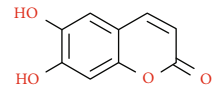
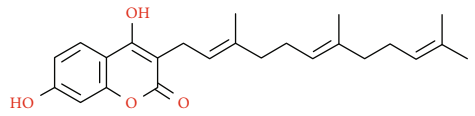
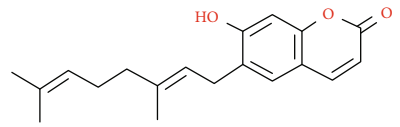
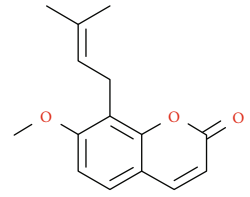
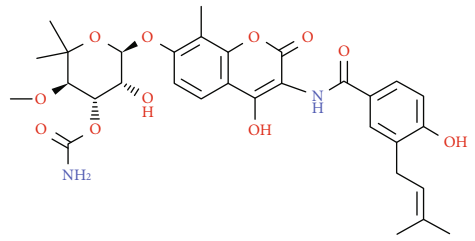
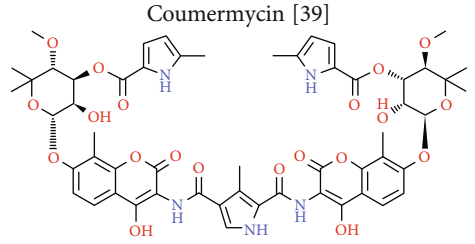
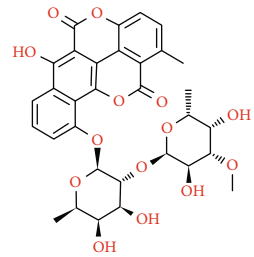
Coumarin type	Example	Pharmacological activities
Simple coumarins	Coumarin [33] 	Anti-inflammatory
	Esculetin [34] 	Antioxidant Anti-inflammatory Anticancer Neuroprotective
	Ammoresinol [35] 	Antibacterial
	Ostruthin [35] 	Antimicrobial
	Osthole [36, 37] 	Antioxidant Antimicrobial Antitumor Anticonvulsant Antidiabetic
	Novobiocin [38] 	Antibacterial
	Coumermycin [39] 	Antibacterial
	Chartreusin [40] 	Antibacterial Anticancer

Table 1: Continued.

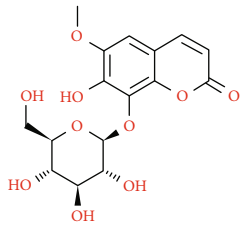
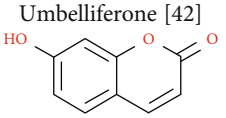
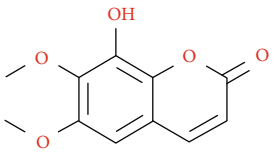
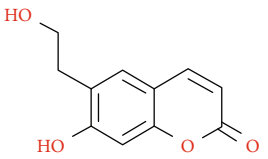
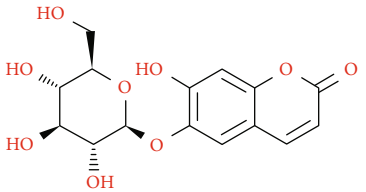
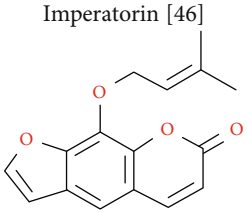
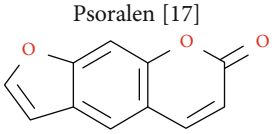
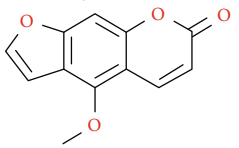
Coumarin type	Example	Pharmacological activities
	Fraxin [41] 	Antioxidant Anticancer Antiadipogenic
	Umbelliferone [42] 	Antitubercular
	Fraxidin [43] 	Antiadipogenic Antihyperglycemic
	Phellodenol A [44] 	Antitubercular
	Esculin [45] 	Antiadipogenic
Furano coumarins	Imperatorin [46] 	Anti-inflammatory Antimicrobial Anticancer Anticonvulsant
	Psoralen [17] 	Antifungal Antituberculosis
	Bergapten [45] 	Antituberculosis

Table 1: Continued.

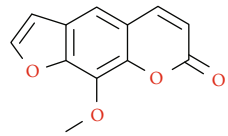
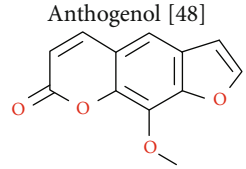
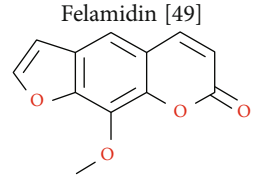
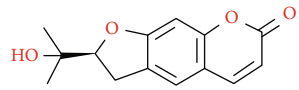
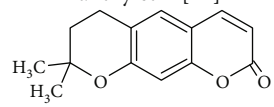
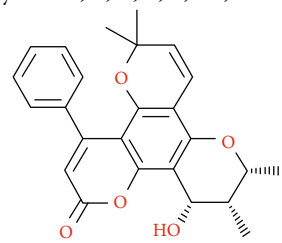
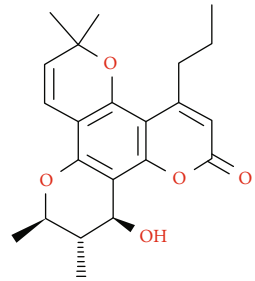
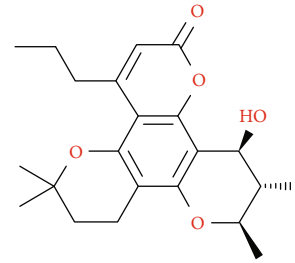
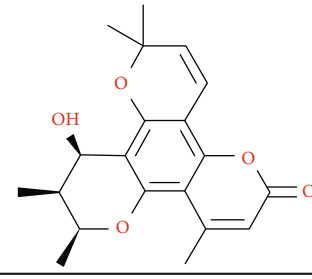
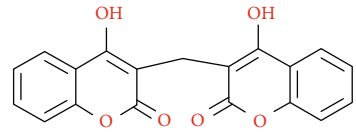
Coumarin type	Example	Pharmacological activities
	Methoxsalen [47] 	Cytochrome P450 inhibitor
Dihydrofurano coumarins	Anthogenol [48]  Felamidin [49]  Marmesin [42] 	Antibacterial Antibacterial Antituberculosis
Linear type	Agasyllin [50] Aegelinol benzoate [50] Xanthyletin [42] 	Antibacterial Antibacterial Antituberculosis
Angular type	Inophyllum A, B, C, E, P, G1, and G2 [51]  Calanolide A, B, and F [52] 	Antiviral Antiviral Antiviral Antiviral

Table 1: Continued.

Coumarin type	Example	Pharmacological activities
	(+)-Dihydrocalanolide A and B [53] 	
	Pseudocordatolide C [54] 	
Bicoumarins	Dicumarol [55] 	Anticoagulant

and the structure of warfarin is quite similar to this vitamin. Thus, warfarin is regarded as a vitamin K antagonist because of this structural and functional similarity [75].

Initially, warfarin was used as a poison for rat extermination during laboratory experiments; however, for the past 60 years, it was regarded as an anticoagulant. By mechanism, it inhibits the production of vitamin K-dependent coagulation factors (II, VII, IX, and X) in the liver microsomal. Vitamin K possibly induces the vitamin K-dependent coagulation factors (II, VII, IX, and X), N-terminal glutamate remains to form γ-carboxyglutamates. The reduced vitamin K (Vit KH2) contributes to the γ-carboxylation, and warfarin constrains the epoxide reductase to hinder the Vit KH2 development [75] (Figure 2).

5.3. Anticancer Activity. Cancers are characterized by an uncontrolled division of a group of cells, with the ability to invade other tissues in the body, either by a direct growth in those tissues or by the migration of cells to more distant places in the body (metastasis) [76]. In most cases of cancer, no specific cause of the disease can be identified [77]. Risk factors for cancer include exposure to anthropogens, chemicals, eating habits, and lifestyle [78].

In a recent study, coumarin was reported as a protective agent for mucosa and salivary glands in those patients who are undergoing head and neck radiotherapy [79]. Coumarin also can directly deal with cancer as osthole is active in controlling the migration and attack of breast cancer cells by healing the wound or transwell assays. Luciferase and zymography methods have shown that osthole efficiently hinders matrix metalloproteinase-s promoter and enzyme action, which could be one of the reasons that tend the relocation inhibition and attack by osthole [80], whereas esculetin is reported for its antitumor effects [81] and saves cultured primary neurons from N-methyl-D-aspartate toxicity [82].

Fraxin's defensive properties have been observed in the protection of cytotoxicity prompted by hydrogen peroxide in endothelial cells of the human umbilical vein [41]. Maximum of the coumarins grandivittin, agasyllin, aegelinol benzoate, and osthole displayed marginally cytotoxic effect across the A549 lung cancer cell, and they are usually extracted from *Ferulago campestris* [49].

5.4. Antibacterial Activity. It had been observed that pathogenic resistance against pharmaceutical and natural antimicrobial agents has been increased in recent times [83, 84]. Therefore, new prototype combinations are required to address this condition [85, 86]. In this regard, plant-based natural antimicrobial agents combined with antibiotics had shown a good potential [87, 88] Coumarins had been reported for combating against both Gram-positive and Gram-negative type of bacteria [89]. Long-chain derivatives of coumarin like ammoresinol and ostruthin showed more efficacy against *Bacillus megaterium*, *Micrococcus luteus*, *Micrococcus lysodeikticus*, and *Staphylococcus aureus.* However, another type of coumarin, i.e., anthogenol (extracted from *Aegle marmelos*) [90], displays a potent effect against *Enterococcus*. Moreover, a furanocoumarin compound imperatorin, which is extracted

from *Angelica dahurica* and *A. archangelica* (Umbelliferae) [91], controls the growth and spread of *Shigella dysenteriae* [92]. A substantial antibacterial effect against clinically extracted Gram-positive and Gram-negative bacterial strains (such as *Staphylococcus aureus*, *Salmonella typhi*, *Enterobacter cloacae*, and *E. aerogenes*) was reported in *aegelinol* and *agasyllin*.

5.5. Antifungal Activity. An antifungal coumarin derivative, i.e., osthole, was extracted from *Angelica pubescens*, *Cnidium monnieri* [93], and *Peucedanum ostruthium* [94]. It showed a broad range of fungicidal property against microorganisms like *Botrytis cinerea*, *Fusarium graminearum*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* [36]. After multiple antifungal experimentations, three most efficient have been reported, i.e., psoralen, imperatorin, and ostruthin [17].

5.6. Antiviral Activity. Coumarin is one of the herbal products which possess anti-HIV capabilities [95]. Inophyllums and calanolides (coumarin derivatives) presented new HIV inhibitory potential. Inophyllums A, B, C, E, P, G1, and G2 were extracted from *Achatina fulica*. Inophyllums B and P are reported to stop the activity of HIV reverse transcriptase with IC_{50} values of 38 and 130 nM, respectively. Moreover, both were also found effective against HIV-1 in cell culture (IC_{50} of 1.4 and 1.6 μM) [51].

Calanolides A and B were extracted from the foliar parts of *Calophyllum lanigerum*. Studies reported both calanolides A and B as totally protective against replication of HIV-1 [52]. The (+)-calanolide A is a nonnucleoside reverse transcriptase suppressant with the potential efficacy against HIV-1. However, (−)-calanolide B and (−)-dihydrocalanolide B showed antiviral activities comparable to (+)-calanolide A [53, 96]. Formerly inophyllum A and (−)-calanolide B were derived from the seed oil of *Calophyllum inophyllum* Linn and *C. cerasiferum* Vesque, correspondingly. Both of them are members of the family Clusiaceae and known for effective HIV-1 reverse transcriptase blockers [97]. Pyranocoumarins (pseudocordatolide C and calanolide F) were extracted from *Calophyllum lanigerum* var. *austrocoriaceum* and *C. teysmannii* var. *inophylloide* (King) P. F. Stevens (Clusiaceae). Both of these compounds demonstrated anti-HIV effect properties [54], whereas imperatorin also reported inhibiting both vesicular stomatitis and gp160-enveloped recombinant HIV-1 infection in numerous T-cell and HeLa cell lines [98].

Coumarin-linked benzoxazole-5-carboxylic acids showed the inhibitory activity against hepatitis virus RNA polymerase. Inhibiting effect against hepatitis virus RNA polymerase can be demonstrated by coumarin-linked benzoxazole-5-carboxylic acids. Currently, it has been discovered that benzoxazole is conjugated with a coumarin with this methylene linker which displayed strong inhibitory activities on the hepatitis virus [99].

The coumarins have been found to exert antiviral activities including an interesting potential activity against the hepatitis C virus [100]. Likewise, different coumarins such as novobiocin and its analogues have shown antimicrobial activity. Novobiocin, extracted from *Streptomyces niveus*, is principally effective against Gram-positive bacteria. This drug is little used, however, due to the high incidence of associated adverse reactions and the frequent appearance of resistant strains.

5.7. Neuroprotective Effect. Neurodegenerative diseases are characterized by progressive dysfunction and neuronal loss, due in most cases to the accumulation of proteins with altered physicochemical properties [101]. This process causes the death of neurons in which it accumulates and the alteration of neural connections, influencing movement, speech, memory, intelligence, and other brain functions, following the areas where changes occur in the central nervous system [102].

Alzheimer disease is the degenerative disorder of the CNS (central nervous system) which is distinguished by weakening in memory, intellectual poor operation, personality alteration, and language obstruction [103, 104]. The prevalence of this disease is increasing day by day, and it is most common in old aged people [105]. Researches in recent times showed that coumarin and its derivatives can inhibit this disease [106].

A researcher group of Matos synthesized a sequence of 3-substituted coumarin derivatives, which work as an active inhibitor of monoamine oxidase A and B (MAO-A and MAO-B) isoforms and acetylcholinesterase (AChE) [107].

Esculetin showed neuroprotective action on cerebral ischemia damage in a middle cerebral artery-blocking model in mice at 20 μg/mL [108].

Coriandrum sativum (coriander) is an essential medicinal plant containing coumarin as an effective component with neuroprotective effects that improve memory. A series of coumarin 7-substituted derivatives have been developed to demonstrate the inhibitory properties of choline esterase and monoamine oxidase B (MAO-B) [109]. In this regard, it has been reported that various coumarin derivatives selectively inhibit the enzyme MAO-B, implicated in Alzheimer's disease, and prevent Aβ1-42 aggregation, with low toxicity in the studied cells lines. These results suggest possible applications in the management of Alzheimer's disease.

5.8. Antidiabetic Properties. There is a clear association between diabetes mellitus and cardiovascular complications, and in this regard, the coumarins would be even more valuable if they were found to be effective against diabetes. In theory, these drugs would not only lower blood glucose but also improve the outcomes in terms of the cardiovascular complications of diabetic patients. In this regard, different studies have found plant extracts containing coumarins to exhibit antidiabetic activity [110]. Osthole containing volatile oil from roots of *Prangos pabularia* significantly inhibited protein tyrosine phosphatase 1B (PTP-1B) with an IC_{50} value of 0.06 ± 0.01 μg/mL [37].

6. Coumarins in Clinical Trials

6.1. Role of Coumarin in Cardiovascular Diseases. On reviewing the ClinicalTrials.gov database, we found only 7 clinical studies registered using the keywords "coumarin" or "coumarins" (Table 2). However, on adding the term "antivitamin

Table 2: Registered coumarin clinical trials on the Clinicaltrials.gov database.

Title	Participants	Type of trial	Period	Outcomes
Effectiveness of a Multidisciplinary Medication Review with Follow-up for Patients Treated with Coumarin Anticoagulants in Primary Care *NCT03154489*	204	Randomized	May 2017-May 2018	Effectiveness of medication review with follow-up. Principally studying INR control of these patients during 6 months
Efficacy and Safety of Coumarin and Troxerutin in the symptomatic Treatment of Chronic Venous Insufficiency *NCT01848210*	829	Randomized	May 2013-September 2015	Mean Change (Reduction) from Baseline in Volume of Reference Le g at Week 16 Change in the partial volume of legs will be measured using a water plethysmometer.
Efficacy and Safety Study of BERIPLEX® P/N (Kcentra) Compared with Plasma in Patients with Acute Major Bleeding Caused by Anticoagulant Therapy *NCT00708435*	216	Randomized	-	Percentage of participants achieving hemostatic efficacy of stopping an ongoing major bleed: at 1 and 4 hours after the end of infusion Percentage of participants who had a rapid decrease of the international normalized ratio (INR) time frame: 30 minutes after the end of infusion
Anticoagulation in Liver Fibrosis *NCT00180674*	12	Nonrandomized	August 2005-October 2006	-
An Open-label, Randomized, Multicenter Phase IIIb Study to Assess the Efficacy, Safety and Tolerance of BERIPLEX® P/N (Kcentra) Compared with Plasma for Rapid Reversal of Coagulopathy Induced by Vitamin K Antagonists in Subjects Requiring an Urgent Surgical Procedure (BE1116_3003) *NCT00803101*	176	Randomized	February 2009-February 2013	Percentage of participants achieving hemostatic efficacy during surgery the start of infusion until the end of surgery Percentage of participants who had a rapid decrease of the INR: 30 minutes after the end of infusion
Therapeutic Equivalence between Branded and Generic WARFArin Tablets in Brazil (WARFA) *NCT02017197*	100	Randomized	August 2014-August 2016	Difference between Delta INR at the fourth week of each period
Rivaroxaban Compared to Vitamin K Antagonist upon Development of Cardiovascular Calcification *NCT02066662*	192	Randomized	July 2013-active	Progression of coronary and aortic valve calcification (Agatston, volume & mass score as assessed by cardiac CT)

K," we identified 10 publications, and the addition of "warfarin" yielded 375 registered studies.

In the last few years, modern direct oral anticoagulants (DOACs) have been generated. Instead of acting upon some factors within the coagulation cascade (like the VKAs), these drugs are targeted to a specific component of the cascade. Furthermore, DOACs show scant interaction with other medications or foods and can be administered at fixed doses without having to monitor the patient coagulation status. This allows simplification of long-term anticoagulation therapy [111].

Randomized studies have evaluated the role of oral anticoagulation with VKAs in the primary prevention of thromboembolic events in patients with nonvalvular atrial fibrillation [112–118]. One of the first of these studies was the Copenhagen AFASAK trial [113], which showed the superiority of anticoagulation with VKAs as thromboembolic prophylaxis among patients with atrial fibrillation. The research found warfarin to be more effective than aspirin or placebo in this context, with an annual stroke incidence of 2.0% (95% confidence interval [95% CI] 0.6-4.8) in the warfarin group versus 5.5% (95% CI 2.9-9.4) in the aspirin and placebo groups. The drug also reduced overall vascular mortality.

The results of the SPAF trial became known one year later [112]. This was a work involving a larger sample size and a follow-up period of 16 months. It recorded thromboembolic episodes, bleeding, and mortality and reported an annual incidence of thromboembolic events of 2.3% in the warfarin group versus 7.4% in the placebo group (relative risk [RR] = 0.67 in favour of warfarin [95% CI 0.27-0.85]; $p = 0.01$). In turn, the bleeding risk was 1.2% annually in the warfarin group and proved much lower than in the Copenhagen AFASAK trial [113], probably because of the use of less intense anticoagulation (INR 2.0-4.5). Likewise, in 1991, the CAFA (Canadian Atrial Fibrillation Anticoagulation) trial [115] compared warfarin (INR 2-3) in 187 patients versus placebo in 191 patients, with a follow-up of 16 months. The study endpoints were thromboembolic events and bleeding, and here again, the findings favoured warfarin both in terms of the number of such events and the incidence of bleeding.

Lastly, in 1992 ,the SPINAF (Stroke Prevention in Nonrheumatic Atrial Fibrillation) examination [116] contrasted low-dose warfarin (INR 1.4-2.8) versus placebo. A total of 571 males (525 in the context of primary prevention and 46 with previous CVA) were distributed to either warfarin ($n = 281$) or placebo ($n = 290$), with a follow-up period of 22-month years. The study endpoints were thromboembolic events, bleeding, and mortality. Besides the patients with no history of CVA, 19 of those administered placebos suffered thromboembolic events (annual rate 4.3%) versus only four of those administered warfarin (annual rate 0.9%) (RR = 0.79 in favour of warfarin [95% CI 0.52-0.90]; $p = 0.001$)—this observation causing early termination of the trial.

In 2016, Cannon et al. (ClinicalTrials.gov NCT 02164864) [111] studied a series of patients with atrial fibrillation subjected to coronary stent placement and anticoagulation for the prevention of CVA and stent thrombosis. The patients with atrial fibrillation subjected to stent placement were seen to have a lesser risk of bleeding when dual therapy was provided in the form of dabigatran and a P2Y12 inhibitor than when triple therapy was administered with warfarin, a P2Y12 inhibitor, and aspirin. Dual healing was not inferior to the triple-drug strategy in terms of thromboembolic risk.

About deep venous thrombosis, the EINSTEIN-DVT trial (ClinicalTrials.gov NCT00440193) [119] was an open-label study in which patients were randomized to rivaroxaban or enoxaparin, followed by a VKA agent that could be warfarin or acenocoumarol, during 3, 6, or 12 months. The sufficiency results were found to be independent of the type of VKA administered, thus suggesting that the efficacy of the two VKAs may be similar.

The coagulation system plays a crucial role in tumor development and metastasis. This has led to increasing interest in the potential benefits of anticoagulation in this field. Concerning cancer patients, several researchers [120–122] have reported a substantial risk of recurrent thrombosis in such individuals despite oral anticoagulation therapy. On comparing low molecular weight heparin (LMWH) versus coumarin, dalteparin was seen to be more active than an oral anticoagulant in risk reduction of recurrent thromboembolism, apart from incrementing bleeding risk.

Ntaios et al. [123] conducted a meta-analysis of 28 observational surveys matching the application of DOACs versus VKAs in patients with atrial fibrillation. All of the evaluated DOACs were supported to a clear and marked decrease in intracranial haemorrhage. In the latest research published by Esteve-Pastor et al. [124], non-VKA anticoagulants have been proposed as an alternative for patients with atrial fibrillation. Direct oral anticoagulants are an interesting therapeutic option, but further investigations are required before their widespread use can be recommended.

6.2. Studies on the Efficacy of Coumarins in Cancer Patients. Grotz et al. [125] studied the validity of combination coumarin/troxerutin therapy in protecting the salivary glands and mucous membranes of patients subjected to head and neck radiotherapy. In turn, Thornes et al. reported the efficacy of coumarin (warfarin) as adjuvant therapy in melanoma [126, 127] (Table 3).

The antitumor effect of coumarin in patients with renal cell carcinoma has been studied by administering the drug at an oral dose of 100 mg/day with the addition of cimetidine (four 300 mg doses a day from day 15 of treatment), with interesting results and practically no side effects [128, 129].

Concerning prostate cancer, patients have been treated with coumarin (3 g/day), and although the results indicated partial tumor response, the tumor burden was seen to decrease in all cases [130].

Warfarin has proved notably positive achievements in the handling of small-cell lung carcinoma. Mousa [131] found that anticoagulation with typically used drugs like nonfractionated heparin and warfarin (Coumadin®) prevents cancer development by limiting tumor cell capacity. The recent finding indicates that anticoagulant drugs and

Table 3: Clinical studies with warfarin in treating cancers.

Cancer [Ref.]	Intervention	Study design	Observation
Colorectal cancer [138]	Warfarin for 24 months vs. control	RCTa, unblinded	No significant difference (72.2% in the warfarin group vs. 69.5% in the control group)
Breast cancer [139]	Warfarin during chemotherapy vs. placebo	RCTa, double-blind	Insignificant variation for 225-day survival (57% in warfarin group vs. 63% in the placebo group)
Lung cancer [140]	Warfarin life-long vs. control	RCTa, unblinded	Insignificant variation for median survival (9.7 months in the warfarin group vs. 8.9 months in the control)
[132]	Warfarin life-long vs. control	RCTa, unblinded	Significant increase in median survival (49.5 weeks in warfarin group vs. 23 weeks in control)
[141]	Warfarin life-long vs. control	RCTa, unblinded	No significant difference in median survival
Melanoma stage Ib and II melanoma [126]	Warfarin for 24 months after surgery vs. placebo	RCTa, double-blind	Significant decrease in recurrence (4/13 in warfarin group vs. 10/14 in the placebo group)
Advanced head and neck cancer [133]	Warfarin life-long vs. control	RCTa, unblinded	No significant difference in median survival (18.0 weeks in the warfarin group vs. 20.7 weeks in the control group)

treatment with cimetidine in malignant disease can enhance patient survival and inhibit metastatic action [131].

Zacharski et al., in a phase III trial (study 75), evaluated patients with small cell and non-small-cell lung carcinoma, colon cancer, head and neck malignancies, and pancreatic cancer. The patients received chemotherapy together with warfarin or chemotherapy alone. The authors found warfarin to be associated with improved survival among the patients with small-cell lung carcinoma subjected to chemotherapy versus the patients with non-small-cell lung carcinoma ($p = 0.018$). No considerable variation was noted in the rest of the malignancies [132–134]. A randomized, prospective study of advanced small-cell lung carcinoma reported benefits in terms of tumor response and survival, though at the cost of more frequent bleeding [135]. A Norwegian population study published in JAMA Internal Medicine [136] provided data on the influence of warfarin on cancer risk. Among the subjects administered warfarin, 9.4% were diagnosed with cancer, versus 10.6% of the population not treated with warfarin. This lesser presence of malignancy in the warfarin group was particularly manifested in patients with lung cancer and atrial fibrillation, where the incidence was lower than among the rest (incidence rate ratio [IRR]: 0.39). However, in the case of colon cancer, warfarin showed no effects in terms of incidence in this population.

Lastly, a recent Cochrane review (2017) on the effects of oral VKAs in cancer patients [137] has concluded that these drugs offer no benefits in terms of survival in patients not diagnosed with thrombosis (i.e., without anticoagulation indications). Concerning the antitumor potential of VKAs (warfarin), the high associated bleeding rates have limited their use in this field. Furthermore, the negative data of the Cochrane review and the great variability of the blood levels of these drugs when administered with cytostatic agents indicate that they afford suboptimal therapeutic outcomes.

Further clinical trials are needed, with patient groups that are homogeneous in terms of the variety of cancer involved, the phase of the disease, and lifetime.

7. Discussion

The pharmacological activities and therapeutic applications of the simple coumarins are dependent upon their chemical substitution profile [23]. Anticancer drugs are designed to defect the abnormally dividing cell by disturbing the cell division action [142, 143]. Chemicals like DNA intercalating and cross-linking agents, topoisomerase inhibitors, cytoskeleton disrupting agents, and antimetabolites are in practice as anticancer drugs [144]. However, apart from the effectiveness of these drugs, multiple side effects have been seen (e.g., haematopoietic system). Occasionally, collective therapies are more preferably used to treat the disease which allows the least side effects during treatment. At present, surgery, chemotherapy, and radiotherapy in combination offer the best treatment for patients. Coumarin is not only effective in cancer treatment but also helps to reduce undesirable outcomes resulted in radiotherapy [65, 145].

High doses of bergapten, a coumarin found in bergamot and citrus essential oil in general, are mutagenic and carcinogenic [146]; in addition, they appear to be responsible for the inhibitory activity of the CYP3A4 isoform of cytochrome P450, characteristic of grapefruit juice (which therefore reduces the metabolism of many drugs, increasing therapeutic activity with the risk of adverse reactions from overdose) [146].

Other studies have shown that coumarin lowers glutamate levels in the hippocampus and stimulates the release of endogenous amino acids such as glutamate, taurine, and glycine in the prefrontal cortex, thus justifying the efficacy of coumarin in the treatment of neurodegenerative diseases, which are directly correlated with central nervous system imbalance. Due to its inhibitory effect on MAO, coumarins may have antidepressant properties.

Although in this updated review, some potential therapeutic effects beneficial to human health have been highlighted; there are still some limitations and clinical pitfalls that must be considered before recommending them as complementary/adjuvant treatments.

Coumarin chromen-2-one is a poisonous chemical compound found in many plants such as cinnamon, mint, green

tea, and lavender [147]. In addition, this compound is the precursor of several anticoagulant drugs such as warfarin.

Coumarin derivatives such as warfarin and acenocoumarol, both of which are vitamin K antagonists (VKAs)—are widely prescribed throughout the world as oral anticoagulants to prevent and treat thromboembolic disorders. The efficacy and safety of acenocoumarol have been evaluated in atrial fibrillation, deep venous thrombosis (DVT), and heart valve replacement surgery, following acute myocardial infarction, in the postoperative period of major surgery and also in cases of prolonged hospital stay. Acenocoumarol is effective and safe in all age groups. In comparison with warfarin, it affords greater stability of the anticoagulant effect. However, treatment with coumarin derivatives remains a challenge, since the response to a given dose is characterized by great inter- and intraindividual variability. This is because certain CYP2C9 and VKORC1 polymorphisms are related to lesser dose prescription and greater bleeding risk. Acenocoumarol is inexpensive and may be an adequate oral coagulant for prolonged use. The VKAs are well tolerated and have an excellent cost-efficacy ratio. Moreover, the experience gained with the use of these drugs is very extensive, and the anticoagulation control afforded in the real-life setting is reasonable (with excellent control in the presence of patient self-control). On the other hand, the VKAs have a universally available antidote (vitamin K) that is very easy to use and inexpensive. In general, VKAs pose a slightly increased risk of intracranial haemorrhage, though a probably lesser risk of gastrointestinal bleeding compared with other drugs [148–151]. Optimum anticoagulation should seek a proportion among the prohibition of cerebrovascular accidents (CVAs) and the appearance of bleeding complications. In this respect, standardized VKA therapy based on an international normalized ratio (INR) of 2-3 is effective.

On the other hand, VKAs have well-known limitations, including a narrow therapeutic window, easy access and disappearance of effect, numerous pharmacological and food interactions, unpredictable pharmacokinetics (great inter- and intraindividual variability in response to a given drug dose, as commented above), and the need for periodic laboratory test monitoring with frequent dose adjustments that complicate treatment and interfere with patient quality of life [23, 148–151].

Natural coumarins have also a casein kinase-2 (CK2) inhibitory activity. CK2 is perhaps the best-known pleiotropic protein kinases which have greater than the three hundred recognizable protein substrate, and this could be the probable reason for its fermentative action. The two enzymatic subunits are effective in either presence or absence of regulatory subunits. However, the active CK2 is universal, crucial, and associated in a diverse range of imperative cell functions, but evidence suggested that its enzymatic subunits can also act as oncogenes. It is observed that they show an antiapoptotic influence in prostatic carcinoma cell lines. Coumarin-linked piperazine is considered the most promising inhibitor of CK2 [152]. The experiment showed that this inhibitor crystalized in complex with CK2, and the trial binding mode has been used to derive the linear interaction energy (LIE) model. In the past several years, multiple testing programs have been done by employing both *in silico* and conventional methods to discover novel powerful and electively CK2 blocking agents.

Particular attention should be paid to the use of dried plants containing coumarin, due to the already mentioned capacity to produce dicumarol in certain situations. These natural compounds are contraindicated in patients undergoing treatment with coumarin anticoagulants such as sintrom or antiplatelet agents (aspirin and clopidogrel).

Although the existing clinical trials are limited, their findings suggest a positive impact of anticoagulation in cancer patients. Nevertheless, this advantage is not the same in all cases, since some histological types of tumor appear to be extra responsive to the action of anticoagulation therapy than others in the early stages of neoplastic development [120–122].

Due to their photosensitizing properties, 6, 7-furanocoumarins are contraindicated in case of prolonged exposure to sunlight, due to the risk of photodermatitis, burns, and melanomas [153]. Doses of coumarin in a human's daily diet were calculated at 0.02 mg/bw, and the concentration of coumarin varies from 1 m/bw in celery to 7000 mg/bw in Ceylon cinnamon and over 87000 mg/bw in Cassia cinnamon. Therefore, the association of nutritional supplements containing coumarins with these plants should be avoided [14]. Due to the proven hepatotoxicity in experimental studies in rats, FDA has banned the use of coumarin as a food additive [154]. However, adverse events in humans after natural coumarin administration were rare.

8. Concluding Remarks and Perspectives

Coumarins belong to the benzopyrone family widespread in nature. Natural coumarins demonstrated a wide spectrum of pharmacological activities, including anti-inflammatory, anticoagulant, anticancer, antibacterial, antimalarial, casein kinase-2 (CK2) inhibitory, antifungal, antiviral, Alzheimer's disease inhibition, neuroprotective, anticonvulsant, phytoalexins, ulcerogenic, and antihypertensive. There are very few studies on the bioavailability of coumarins; therefore, extra investigations are required to study the bioavailability of different coumarins which already showed good biological activities in previous studies.

Based on the wide range of pharmacological effects, this review presents a common analysis of the origin, availability, and biological capacities of coumarins with a further discussion on the most crucial mode of their therapeutic actions. The coumarins play an important task in different pharmacological pathways and make a significant contribution to the development of new therapeutic targets. In conclusion, a broad variety of coumarins are available, and their pharmacological activities are of current interest thanks to their synthetic accessibility and richness in plants and other natural objects. Coumarins play an essential role as therapeutic agents in a range of medical fields.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

J.S-R., N.-C.M., W.C.C., and D.C. conceived and designed the paper; P.L.J., E.P.F.L, N.H., B.Y., A.B., O.S., S.S., F.S., Y.T., and A.O.D. were responsible for the collection of literatures and draft the manuscript; J.S.-R., A.O.D., W.C.C., and D.C. contributed to reviewing the manuscript; J.S.-R. and D.C. edited and amended the final draft of the manuscript. All authors approved this version of the manuscript.

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Review Article

Paving Plant-Food-Derived Bioactives as Effective Therapeutic Agents in Autism Spectrum Disorder

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Autism spectrum disorder (ASD) is a neurodevelopmental disorder, where social and communication deficits and repetitive behaviors are present. Plant-derived bioactives have shown promising results in the treatment of autism. In this sense, this review is aimed at providing a careful view on the use of plant-derived bioactive molecules for the treatment of autism. Among the plethora of bioactives, curcumin, luteolin, and resveratrol have revealed excellent neuroprotective effects and can be effectively used in the treatment of neuropsychological disorders. However, the number of clinical trials is limited, and none of them have been approved for the treatment of autism or autism-related disorder. Further clinical studies are needed to effectively assess the real potential of such bioactive molecules.

1. Introduction

Autism spectrum disorder (ASD) is a set of behavioral and neurodevelopmental diseases featuring social and communication deficits accompanied by increased repetitive and/or restrictive behaviors [1]. The most commonly registered behaviors under ASD are presented in Figure 1. In the last years, the prevalence of ASD has dramatically risen [2], and as per the World Health Organization (WHO), it is estimated that 1 in 160 children in the world has ASD [3]. Although the etiology and pathogenesis of this disorder are not fully understood, several environmental and genetic factors have been proposed as mediators, thus, limiting key molecular mediators' identification as well as possible neurological and biochemical mechanisms. However, recent evidences are pointing to alterations in hormones, amino acids, and several biochemical markers as possible mediators in autistic individuals [4, 5]. Furthermore, unifying etiology involving immune dysfunction, abnormal lipid metabolism, glutamatergic dysfunction, and raised susceptibility to oxidative stress has been proposed for ASD [6], with studies increasingly linking mitochondrial dysfunction to ASD [1, 7, 8]. Indeed, mitochondrial dysfunction and oxidative metabolism defects are characteristic of many chronic illnesses, such as bipolar disorder, multiple sclerosis, Parkinson's disease (PD), schizophrenia, depression, ASD, and chronic fatigue syndrome [9]. Since mitochondria is an integral part of many cell processes, it becomes susceptible to many insults that could affect its integrity. Furthermore, evidences are pointing at immune dysregulation in ASD etiology [9]. Several studies have revealed the presence of immune abnormalities in those suffering from autism [10, 11], with markers of autoimmunity, abnormal cell immunity, aberrant expression of cytokines, and other soluble immunity mediators being evident in ASD children [10–13].

Nonetheless, faced by data scarcity on ASD etiology and underlying causes, the ability to develop and mobilize effective treatments is still limited. Hence, efforts have been done on alleviating only comorbid manifestations of the disorder [1]. However, as a result of the limited treatment options available to improve ASD symptoms, financial challenges, and drugs side effects, dietary and nutritional approaches are becoming popular components of ASD management [14, 15]. In this sense, the present review is aimed at providing a brief overview on ADS and related pathophysiology, as well as on the promissory therapeutic abilities evidenced by plant-food bioactives.

2. Autism Spectrum Disease: A Brief Overview

ASD is an increasingly frequent disorder found among children. To date, several risk factors (prenatal, perinatal, and postnatal) that are either genetic or environmental have been stated [16]. Approximately, 15% cases of ASD are linked to genetic disorders (fragile X syndrome, neurofibromatosis, tuberous sclerosis, and Rett syndrome) [17], and various genetic alterations, such as de novo mutations in coding regions, copy number variations, and chromosomal alterations, are the most frequent variants that have been associated with ASD development [18, 19]. The significance of genetic factors in ASD development could be easily explained in twin studies, with results from these studies indicating that the ASD concordance rates could be around 30–99% in monozygotic twins, 0–65% in dizygotic twins, and 3–30% in siblings, respectively [20–23], while a recent study showed a lower ASD concordance rate in monozygotic twins (50%) [24].

However, some evidences have indicated controversial results in terms of environmental factors and ASD development. These studies suggest that environmental factors, such as vaccination, advanced parental age, maternal smoking, pregnancy and birth complications, thimerosal exposure, deficiency of vitamin D, and reproductive technologies have strongly correlated with ASD [16, 24, 25], but few studies showed no relation with ASD risk [24]. On the other hand, few studies also suggested that both genetic and environmental factors act in a synergistic manner, and epigenetic modifications could be mediators in the gene/environment interface [26, 27]. Various mechanisms have been proposed in such way, including DNA methylation, MECP2 mutation, folate-methionine pathway enzymes, histone acetylation, and chromosome remodeling for tracking of epigenetic changes [28].

Additionally, while few research groups reported that oxidative stress has been involved in ASD [29], also genetic abnormalities in glutathione (GSH) metabolism may be related to altered mitochondrial function [30]. Indeed, GSH, the key intramitochondrial reactive oxygen species (ROS) neutralizer, is decreased in ASD [31, 32]. Also, changes in glutamate metabolism have been implicated in ASD, with glutamate being postulated as a likely cause of mitochondrial dysfunction and selective Purkinje neuron degeneration stated in ASD [8].

3. From the Historical Perspective to Current Clinical Practice

Autism was first identified by Kanner [33] as an "inborn autistic disturbance of affective contact." Then, many researchers tried to identify the definition and diagnostic criteria of autism [34]. The criteria for the diagnosis of childhood autism was established in 1993 [35]. Lobotomy was one of the initial ASD treatments [36, 37]. Psychotherapy

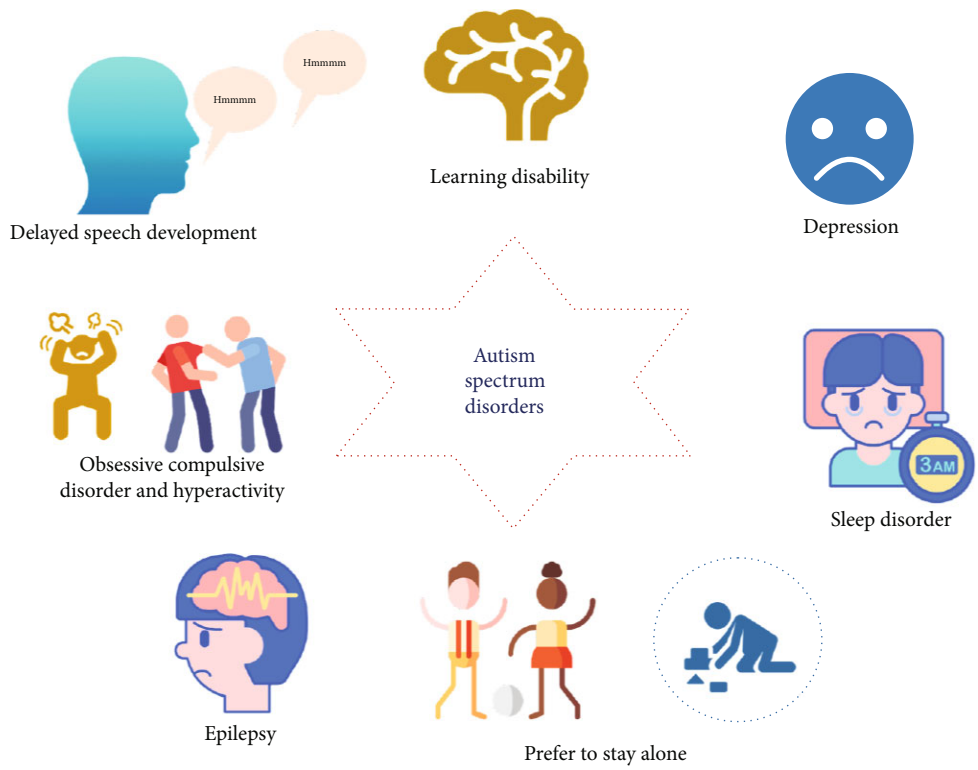


Figure 1: Illustration showing autism spectrum diseases (ASD).

[38] and holding therapy [39] were also practiced. The first medical drug that was approved to be used in autism was risperidone [40].

During the last decade, the frequency rate of the disease has been progressively rising [41]. Neurological dysfunctions such as autism substitute for a high impact in societies via the world. Though the indications resulting from those illnesses are popular, the mechanisms and reasons are composite and rely on multiple parameters [42]. ASD contains multiple dysfunctions with different stages of verbal skills, logical functioning, and several genetic etiologies [43].

Neurobiological systems being important for social functioning are debatably the most hopeful signaling pathways for ASD biomarker and therapeutic target detection. Today, two such nominees are the arginine vasopressin (AVP) and oxytocin (OXT) signaling pathways [44].

Apart from the earlier clinical studies, traditional medicine has been commonly used in ASD treatment [45]. Nevertheless, the negative effects of these medications or their adverse interaction between other medications are questionable, and the parents of the autistic children receiving treatment should be given information about the risks [46].

Japanese traditional herbal medicine, Kami-shoyo-san and Yokukansan, can be helpful in improving behavioral problems in autism [47, 48]. Moreover, Ayurvedic, Siddha, and homeopathic treatments are commonly used in India [49]. For instance, Panchagavya gritha was suggested as an effective Ayurvedic drug for autism [50]. In addition, acupuncture is also widely used in China [51, 52]. Positive effects of electroacupuncture on children with ASD were reported [53, 54].

It was suggested in several studies that ASD can be associated with nutritional and gastrointestinal problems, such as food selectivity, nutritional deficiencies, food allergies, intolerances, diarrhea, and constipation [55–59]. Thus, dietary approaches, including ketogenic diets, gluten-free and casein-free diets, high-fat diets, probiotic use, food additives, camel milk consumption, and other diets, have been frequently investigated [57]. Multivitamin/mineral supplementations are also frequently prescribed [60]. Some food components seem to cause both behavioral and gastrointestinal symptoms in children having ASD [61]. Autoimmunity may rise out of enhanced immune response to potential cross reactivity and dietary proteins to proteins in the brain or gut. Remarkably, children on specific protein-restricted diets display lower stages of activated underlying lamina propria lymphocytes (CD3+TNF-α+) known to be rich in immune and colonic intraepithelial lymphocytes compared to children on unrestricted diets [62].

When it comes to digestive capacity, if it is impaired in a subgroup of ASD children, another treatment option is the use of digestive enzyme supplementation, precisely with a full panel of protease enzymes, despite the limited number of researches carried out to assess the effectiveness of digestive enzyme supplementation on behavioral and gastrointestinal conditions [63].

On the one hand, although probiotic therapy has been advised in several reviews as a potential cure for children with ASD and gastrointestinal indications [64], it has been

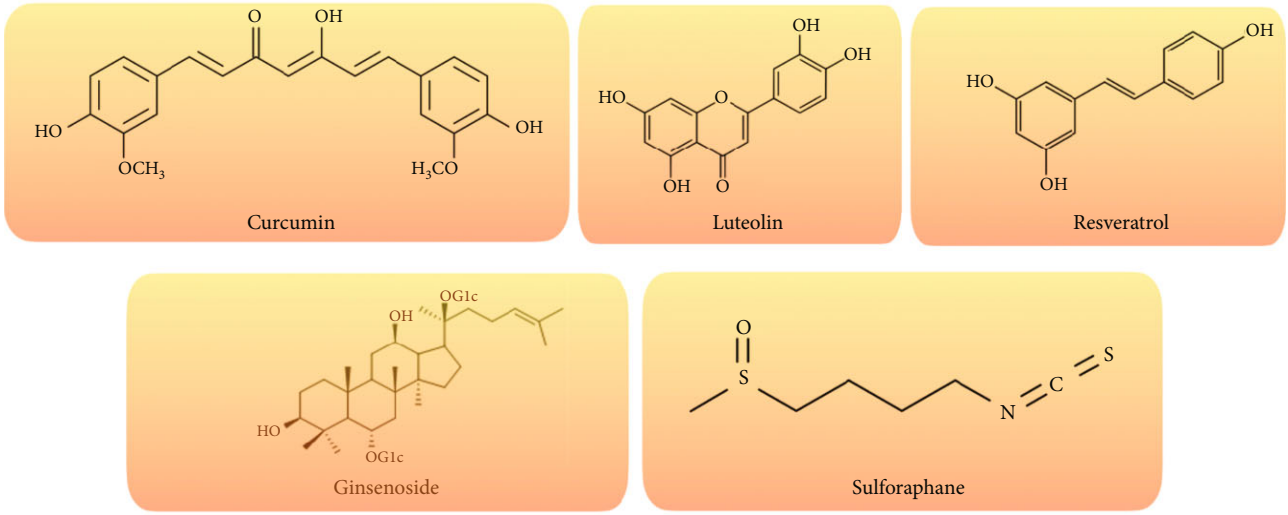


Figure 2: The chemical structure of some potential phytochemicals for ASD treatment.

explained that as of 2009, only one-fifth of physicians were inspired to use probiotics in children with ASD [65]. One research showed that *L. acidophilus* management (5×109 CFU/day, twice a day for 2 months) expressively decreased a marker of offensive pathogenic candidiasis in children with ASD and gastrointestinal indications [66], although half of the children in the researches were on limited diets and the research design did not have a control group [66]. In 2014, Rossignol and Frye [67] systematically reviewed a large number of studies on Alzheimer's medications in ASD including donepezil, galantamine, rivastigmine, tacrine, and memantine. The results of some medications showed encouraging evidence for effectiveness against treating core and associated ASD symptoms, but clinical trials are also needed to confirm their efficacy for treating ASD individuals.

4. Bioactive Molecules: An Upcoming Key in Autism Spectrum Disease

Even though ASD is a lifelong neurodevelopmental disorder with no cure, there are some pharmacological treatments available to suppress symptoms, like irritability and suppression, and to treat other psychiatric problems that accompany ASD, such as depression, bipolar disorder, and anxiety [68]. Several researchers have reported that ASD is associated with nutritional disorders. Thus, the importance of diet and food consumption is huge in ASD treatment [69, 70]. Certain bioactive molecules have been suggested to support the treatment of autism. The effects of plant-based bioactive materials on autism are summarized in the following subsections.

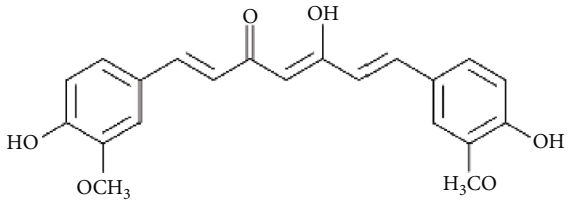
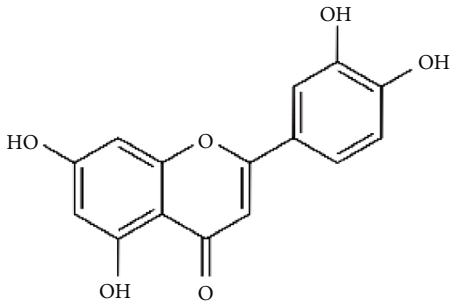
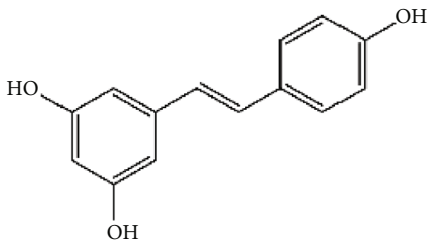
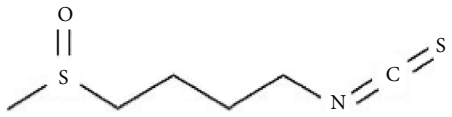
4.1. The Role of Bioactive Molecules in Autism Spectrum Disease. Bioactive molecules that have neuroprotective effects can be used as natural agents in the treatment of neuropsychological disorders, such as ASD, depression, and bipolar disorder [42, 71, 72]. Curcumin, luteolin, and resveratrol are the frequently investigated plant-based active components (Figure 2).

Curcumin, which is a bioactive compound of turmeric (*Curcuma longa* L.), has the potential to be used in the treatment of neuropsychiatric disorders including autism [71]. Even though clinical trials are yet to come, there are a couple of promising studies on rodents. Bhandari and Kuhad reported that after curcumin treatment at a daily dose of 50, 100, and 200 mg/kg for 4 weeks in rats with propionic acid- (PPA-) induced autism, curcumin restored the core and associated symptoms of autistic phenotype by suppressing oxidative-nitrosative stress, mitochondrial dysfunction, and TNF-α and MMP-9 in PPA-induced autism in rats [73]. Curcumin could be developed as a potential pharmacotherapeutic adjuvant for ASD. In addition, Al-Askar et al. [74] reported the postnatal therapeutic role of curcumin in improving most of the impaired parameters in valproic acid- (VPA-) induced rodent models with persistent autistic features.

It was reported that luteolin can reduce maternal immune activation-induced neural abnormalities, such as ASD [75]. In addition, Asadi and Theoharides [56] reported that it can be utilized in ASD treatment, as luteolin inhibited mast-cell activation due to allergy augmentation and mitochondria stimulation. Moreover, dietary luteolin supplementation resulted in the reduction of serum interleukin-6 and tumor necrosis factor levels in children with ASD [76]. Dietary supplementation (1 capsule per 10 kg body weight) containing luteolin (100 mg/capsule), quercetin (70 mg/capsule), and rutin (30 mg/capsule) in olive kernel oil for 26 weeks enhanced adaptive functioning and behavioral disorders in children with ASD [77]. Moreover, Theoharides et al. [78] reported that a dietary supplement, NeuroProtek®, that contains luteolin, quercetin, and rutin, improved symptoms related to gut and brain inflammation in children (aged 4-12 y) with ASD.

Resveratrol has shown therapeutic and anti-inflammatory effects on neurological disorders [79]. For instance, prenatal resveratrol treatment of rats that were also given VPA during

Table 1: Protective action of bioactive compounds from plants in the management of ASD.

Phytocompounds	Dose	Treatment of ASD behavior	References
 Curcumin	Curcumin: 50, 100, and 200 mg/kg for 4 weeks	Neurological, behavioral, biochemical and molecular changes lead to management of ASD	[71, 73, 74]
 Luteolin	Luteolin: 100 mg/capsule Quercetin: 70 mg/capsule Rutin: 30 mg/capsule For 26 weeks	Improved adaptive functioning and behavioral disorders for managing ASD	[56, 75–78]
 Resveratrol	Resveratrol: 3.6 mg/kg for 13 days	Improved neurological behavioral, sensorial, biochemical, and molecular changes leading to the management of ASD	[79–81]
 Sulforaphane	Sulforaphane: 50 μmol/capsule	Successful management of ASD	[72, 86]

the prenatal stage to induce autism, with daily 3.6 mg/kg injections for 13 days, resulted in enhancement in behavioral changes with low interaction between resveratrol and VPA [80]. In another study, the restoration of the autism-related dysfunctions, such as neurological, behavioral, sensorial, biochemical, and molecular changes, were reported in rats with PPA-induced autism after daily oral resveratrol treatment of 5, 10, and 15 mg/kg for 4 weeks [81].

Korean red ginseng supplementation improved social interaction of mice with autism-like disorder [82]. Gonzales et al. [83] reported an improvement in autism-related behavioral problems with no effects on motor coordination ability due to a daily Korean red ginseng oral dose of 100 or 200 mg/kg in mice with VPA-induced autism (prenatal exposure).

Niederhofer [84] suggested that *Ginkgo biloba* can be added to the treatment of autism patients, as they observed improvement on the "aberrant behavior" and symptom checklist after *Ginkgo biloba* treatment of three patients. However, *Ginkgo biloba* extract was used in adjunction to standard autism medication, risperidone, for treatment of autistic children, and no significant effect was observed [85].

Isothiocyanates such as sulforaphane that is found in high amounts in broccoli sprouts has been found to ameliorate ADS symptoms [72]. Moreover, capsules of sulforaphane-rich broccoli sprout extracts (50 μmol/capsule) were given to male patients with ASD (aged 13-30 y) in doses according to body weight (one capsule for <100 lb, two capsules for 101–199 lb, and three capsules for >150 lb), and improvement in ASD-related problems, such as oxidative stress, weakened GSH synthesis, lower mitochondrial function, decreased oxidative phosphorylation, lipid peroxidation, and neuroinflammation, were reported [86].

All these studies show that some natural plant-bioactive compounds can be promising tools in the control of ASD (Table 1).

Other formulations of bioactives can also be used in the treatment of autistic disorders. For instance, folinic acid

(800 μg oral powder in juice, twice a day), betaine (1000 mg oral powder in juice twice a day), and methylcobalamin (75 μg/kg injectable liquid twice a week) improved the metabolic imbalance in autistic children [87]. Moreover, improvement in glutathione redox status of ADS children was observed after methylcobalamin (75 μg/kg injected every third day) and folinic acid (400 μg twice per day) treatment [88]. In another study, it was reported that ubiquinol improved ASD-related problems, such as behavior, eating, and sleep disorders in children (aged 3-6 y) [89].

Methylcobalamin and folinic acid have also been studied in combination with sapropterin, a synthetic form of tetrahydrobiopterin (BH4) [90]. These supplementations have shown therapeutic potential by affecting the folate, methylation, and glutathione pathways. There are also trials where supplementation with only BH4 has been investigated, where the treatment dose is 20 mg/kg/day of BH4. A trial with ASD children has demonstrated the significant improvements over placebo in social awareness, autistic gestures, hyperactivity, and inappropriate speech [91]. Other assays have shown that BH4 is involved in multiple metabolic pathways. It has been reported that this substance is involved in the improvement of the redox state, pterin, biogenic amines, nitric oxide, or inflammation [92–95].

4.2. Drifting from Natural to Synthetic Drugs. Plant-bioactive molecules demonstrated promising results in ASD treatment. However, the number of clinical trials is limited, and none of them have been approved for ASD or ASD-related disorder treatment. Thus, bioactive compounds and vitamin/mineral supplements are used as an adjunct to the synthetic drugs used to treat autism. The medications of autism generally target behavioral problems, developmental disorders, physiological destructions, and other cooccurring psychiatric and medical conditions, such as sleep and gastrointestinal problems, hypertension, and diabetes [96, 97].

Antipsychotic agents, such as risperidone and aripiprazole, are commonly used for treatment of ADS. DeVane et al. [98] reported that risperidone or aripiprazole treatments improved the Aberrant Behavior, Checklist-Irritability scores, behavior, and weight gain of patients with autistic disorder. The positive effects of risperidone on several problems associated with autism, such as Aberrant Behavior Checklist scores, and behavioral problems (e.g., aggression, explosivity, hyperactivity, irritability, repetition, self-injury, and social withdrawal) with a side effect of weight gain, were reported in several studies [99–103]. Aripiprazole also gave similar results in the treatment of ASD patients, such as improvements in behavioral disorders (hyperactivity, irritability, sleep problems, etc.) often with side effects, such as weight gain, and higher risk for sedation and tremor [104–107].

Although risperidone or aripiprazole are the only medications approved by the US Food and Drug Administration for ASD treatment [98, 108, 109], there have been several studies that some drugs developed to treat other mental disorders can be used in the treatment of ASD [67]. For instance, donepezil [110, 111], galantamine [112], and memantine [113], which were approved to treat Alzheimer's disease, were reported to improve autistic disorders, such as behavioral, communication, and social impairments. For instance, guanfacine developed for hypertension treatment was found effective in improving disorders in people with pervasive developmental disorders (PDD), such as ASD [114]. However, there have been several studies showing that metformin can be helpful in controlling weight gain in autistic patients [115–118].

Several other drugs are being investigated and new drugs are also being developed by scientists to treat autism. For instance, a single dose of suramin (20 mg/kg) improved behavioral problems due to autism and schizophrenia in mice models [119]. In addition, Naviaux et al. [120] reported that 20 mg/kg intravenous infusion (one dose) of suramin resulted in improvements in autism syndromes such as behavior and language in autistic children. Ong et al. [121] reviewed the use of phospholipase A2 inhibitors in the treatment of neurological disorders including autism.

Methylphenidate [122, 123], sertraline [124, 125], atomoxetine [126–128], and alpha agonists [129] were also reported to have potential to be used in autism treatment, even though they may have some side effects, such as mood change, irritability, and gastrointestinal disturbance.

Apart from central drugs such as the antidepressants or antipsychotics mentioned above, drugs such as propranolol, memantine, d-cycloserine, and oxytocin can be used [130]. One of the most important is propranolol, a beta-adrenergic antagonist that inhibits anger in higher doses improving various neuropsychiatric disorders [131, 132]. Trials have shown that propranolol improves altered emotional states that occur with anxiety, aggressiveness, self-harm, and hypersexuality [133].

Furthermore, a dysregulation in the hypothalamic-pituitary-adrenal axis and consequently in cortisol levels has been observed among people with autism spectrum disorder (ASD) [134]. Therefore, the regulation of steroid signaling has been suggested as a potential therapeutic route for the treatment of ASD and other disorders of the central nervous system [135].

Food selectivity and picky eating habits in patients with ASD are common, and the patient may also suffer from nutrient deficiencies even though the deficiency itself elevates the symptoms. Thus, food supplements are generally viewed as essential elements of ASD treatment. As vitamin and mineral deficiencies are common in ASD patients [55, 136–139], vitamin-mineral supplements are frequently investigated [140, 141]. Improvements in several ASD symptoms due to vitamin and mineral supplementations were reported [60, 142–145]. On the other hand, Stewart et al. [146] suggested that dietary supplementation, including vitamin and mineral supplements, should be done carefully, as they may not provide sufficient amounts of the deficient nutrient or may cause excessive intake. On the other hand, ASD patients are often treated with several drugs and food supplements at the same time, and the interactions between them are not well known and can be undesired [147]. In addition, further comprehensive investigations on the side effects of these medications are still required.

5. Plant-Food-Derived Bioactive Studies in Autism Spectrum Disease

5.1. In Vitro Studies. Mitochondrial function is critical to CNS as evident in its role in brain energy turn-over [148] and maintenance of ionic gradients critical to neurotransmission and plasticity [149], and its involvement in neural stem cell proliferation, differentiation, and maturation as well as formation of dendritic processes, developmental and synaptic plasticity, and cell survival and death [1, 150, 151] reinforces the importance of mitochondrial dysfunction in ASD etiology. Several phytochemicals have been revealed to protect or restore mitochondrial function and chief among them are the polyphenols with potent antioxidant properties.

Quercetin, a polyphenol broadly distributed in many plants and vegetables, exhibits strong antioxidant properties and could prevent oxidative stress. Research has shown that quercetin and epigallocatechin-3-gallate are bioaccumulated in the mitochondria in its active form, and this could be responsible for their mitochondria protective effect via ROS-scavenging mechanism in vivo [152, 153]. Studies have revealed the modulatory effect of polyphenols such as resveratrol, quercetin, and hydroxytyrosol on the mitochondrial biogenesis process via the stimulation of several coactivators and transcription factors such as the proliferator-activated receptor coactivator-1α (PGC-1α) [153]. Furthermore, several polyphenols have been shown to activate silent information regulation 2 homolog 1 (SIRT1) in vitro; thus, they have been increasingly searched as potential inducers of mitochondrial biogenesis through PGC-1α deacetylation-mediated activation [154]. Recently, resveratrol has been reported to stimulate the SIRT1/PGC-1α-dependent effect on mitochondrial biogenesis in cultured endothelial cells [155]. Furthermore, resveratrol has been revealed to positively influence mitochondrial performance in C2C12 cells via evoking AMP-dependent protein (AMPK) activation and increased mitochondria biogenesis [156]. Sulforaphane (isothiocyanate) preserved mitochondrial functions in ischemia- or toxin-induced damages in normal noncancerous cells [157] and also stimulated mitochondrial biogenesis [158]. Quercetin, rutin, and resveratrol have been shown to prevent the ATP drop and indomethacin-induced alteration in mitochondrial membrane potential in Caco-2 cells [159].

Glutamate dysregulation and toxicity have been observed in ASD, and there is ample evidence that phytochemicals could counteract the effect of altered glutamate metabolism. Polyphenols from green tea have been shown to attenuate glutamate excitotoxicity via antioxidative and antiapoptotic pathways in cultured cortical neurons [160]. In 2003, Lee et al. revealed the protective effect of polyphenols baicalin, baicalein, and wogonin isolated from *Scutellaria baicalensis* Georgi against glutamate/glucose-induced neurotoxicity in primary cultured rat central neurons via an increase in the cell viability, attenuation of increased intracellular calcium ions and nitric oxide production with baicalein being the most effective [161]. Furthermore, phlorofucofuroeckol isolated from brown algae species improves glutamate-induced neurotoxicity through modulation of oxidative stress-mediated mitochondrial dysfunction in PC12 cells [162].

All these in vitro studies with plant-food-derived bioactives in autism spectrum disease are summarized in Table 2.

5.2. In Vivo Studies. Given the rapidly growing disabilities triggered by ASD, characterized by social deficits, communication impairment, and cognitive flexibility deficits, McKinnell et al. evaluated in 2021 the changes in rodent behavior (social and anxiety) and cognitive flexibility in the VPA model of autism and control. The results indicated that VPA rats showed loss in performing the set-shifting task. In other words, females with ASD displayed unique behavioral profiling compared to males with ASD [163]. In 2021, Rebolledo-Solleiro et al. examined the effect of bisphenol A (BPA) on behavior, neurodevelopment, and neurodegeneration through a systematic review. As their main findings, the authors underlined that BPA modulates the normal functioning of the reproductive system, metabolism, and brain functions, while triggering the development of few neurodevelopmental disorders including ASD [164].

Similarly, an increasing amount of naturally occurring bioactive compounds have been used for ASD management. Among them, *Camellia sinensis* (green tea) is an important dietary source of polyphenols, specifically flavonoids, with catechins, such as epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, and epicatechin being the dominant ones. However, the presence of gallic, chlorogenic, and caffeic acids and flavonol derivatives, like kaempferol, myricetin, and quercetin have been stated [165, 166]. Significant improvement in behavioral assessments and neuroprotection and lowered oxidative stress were observed in autistic mice treated with flavonoid extract from green tea [166]. Furthermore, histological findings revealed the presence of a distinct Purkinje layer and cells after treatment with green tea, thus suggesting its neuroprotective effect [166]. The positive effect of plant extracts (green tea and black pepper) in the management of autistic behavior in rat models is illustrated in Figure 3.

Also, piperine, the major alkaloid found in *Piper longum* L. and *Piper nigrum* L. (black pepper), has been reported to possess antioxidant, neuroprotective, anxiolytic, and cognition-enhancing effects [167–169]. A previous study has revealed ameliorative effects of piperine on behavioral alterations and oxidative stress markers in ASD murine model as evident by improved/restored motor deficits and decreased reorientation time, due to its capability to mitigate sodium valproate-induced cerebellar damage [170]. Piperine treatment also brought on cerebellum integrity restoration via a decrease in Purkinje cell number, which is connected with the cerebral cortex and the limbic system [170]. For these properties and anti-inflammatory, antioxidant, and neuroprotective effects, resveratrol could also be relevant in ASD treatment [171, 172].

A study in an experimental murine model revealed that resveratrol improves social skills in valproic acid-induced autistic rats. It regulates and activates sirtuins, members of the class-III histone deacetylases, and also exerts neuroimmunomodulatory effects by regulating transcription factor signaling, decreasing proinflammatory molecules (IL-6 and TNF-α) on dopaminergic neurons, inhibiting NF-κB

Table 2: Summary of in vitro studies.

Phytocompounds	In vitro model	Results	References
Quercetin	Jurkat cells	Antioxidant properties Mitochondria protective effect	[152, 153]
Resveratrol	Endothelial cells	Stimulate SIRT1/PGC-1α and increase of mitochondria biogenesis	[155]
Resveratrol	C2C12 cells	AMPK activation and increase of mitochondria biogenesis	[156]
Sulforaphane	Ischemia induced in normal noncancerous cells	Preserve mitochondrial functions and increase of its biogenesis	[157, 158]
Quercetin, rutin, and resveratrol	Indomethacin-induced Caco-2 cells	Prevent the ATP	[159]
Green tea polyphenols	Cortical neurons	Antioxidative and antiapoptotic properties	[160]
Baicalin, baicalein, and wogonin isolated from *S. baicalensis*	Primary culture rat central neurons	Antioxidative properties, increased cell viability, reduced intracellular calcium ions and nitric oxide production	[161]
Phlorofucofuroeckol	Glutamate-induced toxicity in PC12 cells	Improvement of mitochondrial dysfunction	[162]

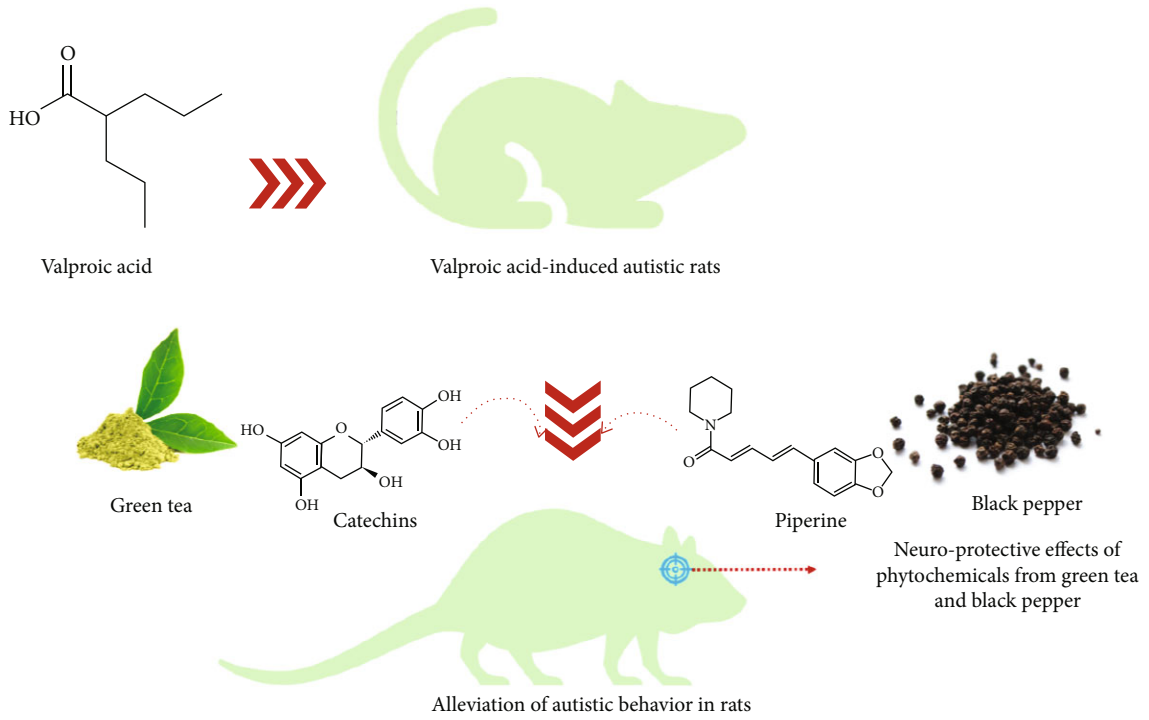


Figure 3: Positive effect of plant extracts (green tea and black pepper) in the management of autistic behavior in rat model.

activation, and suppressing T cells [80, 173]. Another study reported that resveratrol suppresses neuroinflammation, mitochondrial dysfunction, oxidative/nitrosative stress, and TNF-α expression in propanoic acid-induced autistic rats [81], thus making it a potential therapeutic agent to ameliorate ASD's neurobehavioral, and biochemical changes [81].

The impact of turmeric on neurodegenerative diseases and neuropsychiatric disorders has also been documented. Curcumin (diferuloyl methane), the major curcuminoid present in turmeric, a relatively nontoxic and permeable compound to the blood-brain barrier [174, 175], has been reported to have positive effects on the treatment of autistic rats as it targets several cell signaling pathways. For instance, it increases intracellular GSH levels, reduces inflammatory components, and mitigates mitochondrial dysfunction and oxidative/nitrosative stress as well as protein aggregation [73, 176]. This study has also shown that curcumin can alleviate autistic phenotype-associated symptoms via suppressing oxidative-nitrosative stress and mitochondrial dysfunction in propanoic acid-induced autistic rats [73]. In addition, curcumin is able to ameliorate delayed brain maturation and brain toxicity in an autistic animal model via restoration of altered neurological, behavioral, biochemical, and molecular changes related to ASD phenotype [73, 74].

Bacosides are medicinal substances widely used by Indian tribes and are the main bioactive compounds extracted from

Table 3: Summary of in vivo studies.

Phytocompounds	In vivo model	Results	References
Bisphenol A	Different ASD animal models	Modulates the function of the reproductive system, metabolism, and brain functions	[164]
Green tea	ASD mice model	Neuroprotective and antioxidant properties, and improvement of behavior	[166]
Piperine	ASD murine model	Antioxidant, neuroprotective, anxiolytic, and cognition-enhancing effects	[167–169]
Resveratrol	VPA-induced ASD rats	Activates sirtuins and decreases IL-6, TNF-α, NF-κB, and T cells	[80, 173]
Resveratrol	Propanoic acid-induced ASD rats	Reduces neuroinflammation, mitochondrial dysfunction, and oxidative/nitrosative stress	[81]
Curcumin	ASD rats	Increases GSH levels and reduces inflammation, mitochondrial dysfunction, and oxidative/nitrosative stress	[73, 176]
B. monnieri	ASD murine model	Improvement of behavior and antioxidant, anxiolytic, and analgesic properties	[181]
Ginsenoside-rich extract	ASD mice model	Improvement of behavior and locomotor activity	[83]
Co-ultra-PEA-LUT	ASD murine model	Reduces proimflammatory markers (nitrotyrosine and NF-κB), improves neuroplasticity and neurogenesis, and modulates apoptosis	[186]
PEA	BTBR T+tf/J mice	Reduces mitochondrial dysfunction and inflammatory effects Improvement of behavior, PPAR-α activation Microbiota-gut-brain axis regulation	[187]

Bacopa monnieri (L.) Wettst [177]. This plant is traditionally known for its intellect- and cognition-improving properties as well as being a nerve tonic [178, 179]. The pharmacological properties of *B. monnieri* have been attributed to its constituent alkaloids, saponins, and sterols [180]. *B. monnieri* has significantly improved behavioral alterations, decreased oxidative stress markers, reduced pain threshold, and normalized locomotor deficiencies as well as anxiety in a murine model of autism. The improvement in locomotive activity was attributed to the antianxiety properties of *B. monnieri* and its ability to decrease accumulated glutamate and restore cerebellum architecture [181].

Long-time, daily oral administration of ginsenoside-rich extract has been found to improve social interaction, repetitive behaviors, locomotor activity, and other ASD-related behaviors in mice models [83]. Thus, ginsenoside may be viewed as a possible drug candidate for ASD-associated phenotypes and symptom management as well as neurobehavioral deficits. Ginsenosides are Korean red ginseng (*Ginkgo biloba* L.) phytoconstituents known for their therapeutic properties, such as improvement in cerebral blood flow, stimulation of neuronal plasticity, learning, memory, and cognition improvement as well as CNS-associated disease treatment [182–184]. In addition, this plant has been reported to possess antistress, neuroprotective, anti-inflammatory, and antioxidant properties [85, 185].

Furthermore, the effect of the ultramicronized lipid molecule, N-palmitoylethanolamide (PEA) with luteolin (co-ultra-PEA-LUT), in an ASD murine model revealed a reduction in proinflammatory molecules, such as nitrotyrosine and nuclear factor kappa B (NF-κB), an improvement in neuroplasticity and neurogenesis, and the modulation of the apoptotic mechanism in several brain regions (cerebellum and hippocampus) following treatment with co-ultra-PEA-LUT [186]. Another study evaluated PEA on the autistic behavior of BTBR T+tf/J mice and shed light on the contributing mechanisms [187]. PEA improved the behavioral phenotype of BTBR mice dependent on PPAR-α activation. PEA restored the hippocampal BDNF signaling pathway and mitochondrial dysfunction and reduced the general inflammatory status of the mice, reducing the expression of proinflammatory cytokines at the hippocampal, serum, and colonic levels. It also improved intestinal permeability by increasing the expression of the tight junctions of the colon and the composition of the intestinal microbiota.

All these in vivo studies with plant-food-derived bioactives in ASD are summarized in Table 3.

PEA: N-palmitoylethanolamide; co-ultra-PEA-LUT: ultramicronized N-palmitoylethanolamide with luteolin.

5.3. Clinical Studies. Given the huge impact of ASD on the health, wellbeing, and quality of life of patients and facing the increasing evidences of the role of diet in both prevention and management of several diseases, Hartman and Patel reviewed a large number of reports on food approaches to the ASD management and their relation with gastrointestinal, behavioral, neurological, and immune functions through food supplements (fatty acid and pro- and prebiotics, vitamins, minerals, phytochemicals, and hormones) [188]. As their main statements, gastrointestinal issues were correlated with a number of behavioral and neurological deficits, with food approaches being able to improve the lives of patients with ASD [188]. In another study, Gogou and Kolios

Table 4: Summary of clinical studies.

Phytocompounds	Subjects	Results	Reference
Food supplements including choline, folic acid, and multivitamins	Pregnant patient	Reduced expression of ASD-related genes	[189]
Omega-3 and vitamin D	Case study of ASD patient	Reduced ASD symptom	[190]
Metformin	ASD adult patients	Stabilized BMI *z*-score	[118]
Cannabis and cannabidiol	ASD adult patients	Reduced ASD symptom	[193]
Luteolin and quercetin	Children with ASD	Improvement in gastrointestinal features, eye contact, and attention social interactions	[194]
Luteolin, quercetin, and rutin	Children with ASD	Improvement in adaptive functioning and in overall behavior	[77]
Co-ultra-PEA-LUT	ASD adult patients	Reduced motor stereotypes, anxiety, and worsening in social skills	[186]
PEA	Children with ASD	Improvement of cognitive aspects, expressive language, and sociability Reduced general severity of autism	[195]
Flavonoid component extracted from *G. biloba*	ASD adult patients	Slight improvements in irritability, hyperactivity, eye contact, and speech	[84]

reported on the impact of food administration during pregnancy on the risk of ASD offspring. In this study, authors included both clinical and experimental studies, and food supplement (i.e., folic acid, iron, vitamins, choline, vitamin D, and docosahexaenoic acid) studies were also included [189]. Food supplements including choline, folic acid, and multivitamins had a significant impact on the expression of ASD-related genes, while iron had no effect. Thus, a suitable amount of multivitamins, vitamin D, and docosahexaenoic acid can help in reducing the risk of ASD in offspring [189]. Infante et al. reported a case study of a 23-year-old young adult male with ASD. Omega-3 and vitamin D combination therapy showed beneficial effects to the patient [190]. Also, Sivamaruthi et al. assessed the role of the microbiome, food supplements, and probiotics in the development of ASD and underlined that the maternal diet and lifestyle are greatly associated with the development of ASD [191].

In 2017, Wink et al. described the long-term impact of metformin on antipsychotic-associated weight gain in youth with and without ASD using brief ASD mealtime behavior inventory (BAMBI) and the behavior pediatric feeding assessment scale [118]. The authors stated that metformin treatment stabilized BMI *z*-score, and BAMBI demonstrated good consistency, test-retest reliability, and criterion-related reliability. In 2001, Ahearn et al. reported on the feeding behavior in children (ages 3–14 years; exposed to 12 food items in 6 sessions) with ASD and pervasive developmental disorder-not otherwise specified (PDD-NOS) [192]. Different parameters, including food acceptance, expulsion, and disruptive behavior were recorded on a trial-by-trial basis, with data obtained clearly indicating that some children were sensitive to food texture or category, while others were indifferent.

In addition, and when looking at clinical evidence reporting the use of plant-derived bioactive molecules on ASD, the anti-inflammatory, antioxidant, antiallergy, and neuroprotective effects of different biomolecules have been well documented. Mostafavi and Gaitanis analyzed a data set including preclinical and clinical data regarding the use of cannabis and cannabidiol in the treatment of ASD [193]. The results of the analysis suggested that both compounds revealed promising therapeutic benefits in some persons with ASD.

In the case of specific compounds, luteolin and quercetin extracted from *Chamomile* sp. and *Sophora* sp. leaves were used in children with ASD, and marked improvements in ASD symptoms were stated. Most of the patients (75%) reported a significant improvement in gastrointestinal features, such as in stool shape, smell, form, and color. In about 50% of children, habits were improved within a period of 2 to 3 weeks and "allergic-like" skin symptoms were also markedly reduced. Eye contact and attention also improved in about 50% of patients. In addition, 30%–50% of patients showed learned tasks and social interactions and about 10% of children started speaking words or short sentences. However, no improvements were recorded for hyperactivity or aggressiveness [194]. In 2013, Taliou et al. reported an improvement in adaptive functioning and in overall behavior of about 26.6%–34.8% of the autistic children when treated with luteolin, quercetin, and rutin extracted from *Chamomile* sp. and *Sophora* sp. leaves [77]. In another clinical study, co-ultra-PEA-LUT led to an improvement in ASD-related symptoms and reduced motor stereotypes [186]. Another study with PEA showed beneficial effects of this treatment in two child patients with ASD [195]. These subjects showed significantly improved cognitive aspects, expressive language, and sociability, as well as the general severity of autism. Furthermore, slight improvements were observed in irritability, hyperactivity, eye contact, and speech following treatment with a flavonoid component extracted from *G. biloba* leaves [84].

All these clinical studies with plant-food-derived bioactives in ASD are summarized in Table 4.

6. Conclusions and Upcoming Perspectives

Plant-bioactive molecules demonstrated promising results in ASD treatment. However, there is a scarce number of clinical trials available so far, and none of them have been approved for the treatment of ASD or ASD-related disorder. Thus, further preclinical and clinical studies are needed for a more in-depth understanding of plant-derived bioactives as drug discovery candidates on ASD treatment.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

All authors contributed to the manuscript. J.S.-R., N.C.-M., and W.C.C. were involved in conceptualization. All authors were involved in validation, investigation, resource acquisition, data curation, and writing of the manuscript. C.Q., I.-C.B., M.B., F.S., N.C.-M., J.S.R., V.L., M.M.A., W.C.C., and F.L. were involved in reviewing and editing. All authors have read and agreed to publish this version of the manuscript.

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Review Article

Phenolic Bioactives as Antiplatelet Aggregation Factors: The Pivotal Ingredients in Maintaining Cardiovascular Health

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Cardiovascular diseases (CVD) are one of the main causes of mortality in the world. The development of these diseases has a specific factor—alteration in blood platelet activation. It has been shown that phenolic compounds have antiplatelet aggregation abilities and a positive impact in the management of CVD, exerting prominent antioxidant, anti-inflammatory, antitumor, cardioprotective, antihyperglycemic, and antimicrobial effects. Thus, this review is intended to address the antiplatelet activity of phenolic compounds with special emphasis in preventing CVD, along with the mechanisms of action through which they are able to prevent and treat CVD. *In vitro* and *in vivo* studies have shown beneficial effects of phenolic compound-rich plant extracts and isolated compounds against CVD, despite that the scientific literature available on the antiplatelet aggregation ability of phenolic compounds *in vivo* is scarce. Thus, despite the current advances, further studies are needed to confirm the cardioprotective potential of phenolic compounds towards their use alone or in combination with conventional drugs for effective therapeutic interventions.

1. Introduction

Cardiovascular diseases (CVDs) are a major cause of human mortality and morbidity in Western countries, and they are considered a huge problem for todays' health care system [1]. Parallel to the improvement of life expectancy, deaths caused by CVDs have been increasing. Currently, smoking, obesity, poor nutrition, and sedentary lifestyles comprise the main contributors [2]. Scientists estimate that by 2030, in low-income countries, the number of individuals dying from CVD will be significantly higher in comparison to other infectious diseases and nutritional disorders combined [3].

Changes in blood platelet activation are considered a specific key triggering factor for the development of CVD among others [4]. In healthy individuals, hemostatic plugs are formed as the blood clots at the bleeding site. In such situations, they are beneficial since they prevent both blood and plasma from escaping into surrounding tissue. On the other hand, thrombus expansion in the unruptured blood vessels can be harmful [5]. As a result of blood vessel injury and/or atherosclerotic plaque erosion, the endothelium liberates protein factors, enabling that way the platelets' adhesion to the subendothelium and initiating their activation. Activated platelets release biologically active ligands, including thromboxane A2 (TXA2), adenosine diphosphate, and serotonin, which further increase platelet activation, aggregation, and thrombus formation [6]. Thus, the proper control of platelet function is mandatory for the prevention of thrombotic events [7]. Indeed, platelet hyperactivity has been increasingly linked to the development and complication of certain CVDs (Figure 1), including atherosclerosis, thrombosis, peripheral artery disease, myocardial infarction, and ischemic stroke [3].

On the other side, several side effects have been reported with the currently used synthetic antiplatelet agents (aspirin, clopidogrel, and dipyridamole). For example, the chronic consumption of aspirin implies an increased risk of developing intestinal ulcers and major bleeding [6]. Moreover, these drugs are also not completely effective in suppressing platelet aggregation besides increasing the risk of drug resistance and bleeding. As a consequence, these limitations have prompted researchers to launch the quest for natural alternatives as new, effective, and safer antiplatelet drugs. In a recent review, the use of natural products as therapeutic agents has been highlighted considering the data of the last four decades (Newman and Cragg [8]). The review highlighted the use of natural products as anticancer, antidiabetic, and multiple sclerosis agents. The polyphenolic compounds present in plant-based products have been found to play the central part of many scientific studies due to their potential health benefits, with special attention being given to their positive impact on CVD [9–11]. In fact, polyphenol-rich diets have revealed a great impact on the vascular system, improving both platelet and endothelial functions [12]. Moreover, platelet activation pathways involve arachidonic acid (AA), adenosine diphosphate, serotonin, and nitric oxide (NO) pathways. Numerous medicinal plants have also demonstrated their bioactivities in reducing platelet aggregation via these mechanisms [13]. Phenolic compounds may be better for treating CVD than synthetic antiplatelet agents, addressing the problems of drug resistance and bleeding that the synthetic antiplatelet agents cannot solve. A number of reviews which focused on the role of phenolic compounds in improving the cardiovascular health have been published recently [14, 15]. These reviews are more focused on the effect of the phenolic extracts on cardiovascular health, but limited aspects of antiplatelet aggregation activity of phenolic bioactive compounds in maintaining cardiovascular health were discussed. In this sense, considering the importance of the phenolic bioactive compounds in the management of CVDs by their antiplatelet activity, the aim of this review is to collate and summarize the finding of the researchers on polyphenolic compounds with antiplatelet activity, with emphasis on the possible molecular mechanisms responsible for their action against CVDs.

2. Basic Structure and Physicochemical Properties of Phenolic Compounds

Phenolic compounds account for about 40% of organic carbon circulating in the biosphere and are by definition any

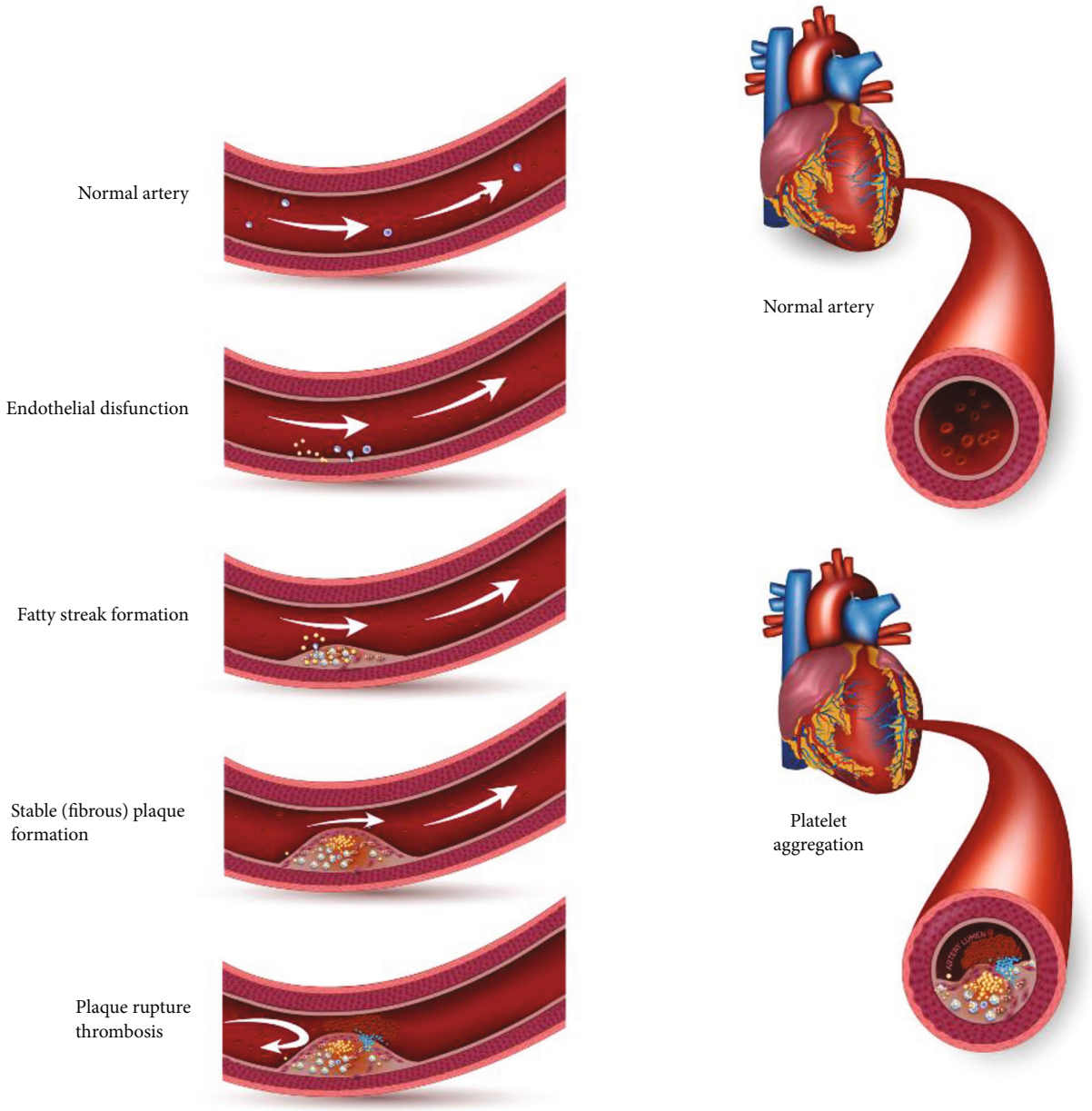

Figure 1: Process of platelet aggregation and formation of thrombosis during CVDs.

compound with a hydroxylated benzene ring. A polyphenolic compound has more than one phenol group, or more than one hydroxyl on a single benzene ring. Phenolic compounds come from simple, low-molecular-weight phenolic molecules to highly polymerized, high-molecular-weight, complex polyphenolic compounds [16]. They are synthesized either as soluble or cell-wall-bound compounds, generally appearing as esters and glycosides rather than free compounds. They are classified based on the number of phenolic rings that they contain and the radicals that bind the rings to one another [16]. The hydroxyl group of phenolics is influenced by the presence of the aromatic ring which makes the hydroxyl group's hydrogen labile and then makes them weak acids [16].

Water solubility increases with the number of hydroxyl groups. For analytical purposes, methanol, ethanol, water, and alcohol-water mixtures have been the most commonly used solvents to dissolve and extract phenolic compounds. The intense absorption in the spectrum of the UV region is exhibited by all phenolic compounds, and the colored ones also absorb strongly in the visible region [16].

Polyphenolic flavonoids constitute the largest group of low-molecular-weight phenolic compounds, characterized by a phenyl-benzo-pyran chemical structure consisting of two aromatic C6 rings (A and B) and a heterocyclic benzopyran ring (C) with one oxygen atom [16]. They are crystalline compounds; some are colored, while others are colorless. They occur as aglycones, glycosides, and methylated derivatives, with differences in glycosides being attributed to the number of positions for glycosylation, types and numbers of sugars involved, and level of glycosylation. Briefly, glycosides can

either be O- or C-linked in positions 3 or 7 to L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose [16].

Phenolic acids (phenolcarboxylic acids) are members of the phenolic compounds' group containing a phenolic ring and at least one organic carboxylic acid function [16]. Tannins are water-soluble polyphenols, characterized by their relatively high molecular weight, being often found in complexes with alkaloids, polysaccharides, and proteins. Stilbenes exhibit a C6-C2-C6 structure derived from the same biosynthetic pathway as flavonoids and are featured by the presence of a 1,2-diphenylethylene nucleus with hydroxyl groups substituted on the aromatic rings [16].

2.1. Main Sources of Phenolic Compounds. According to Lattanzio [17], plant phenolic content depends on several factors, such as growing conditions, cultivation techniques, cultivar, and ripening process. In addition, while they are natural antioxidants and health promoting phytochemicals, phenolic compounds are among the health promoting phytochemicals found abundantly in plant-based foods, including fruits, vegetables, spices, and whole grains, especially cereals [17]. The subclass of flavonoids is widely addressed for their protective effects on CVDs by inhibiting platelet aggregation. Plant-based food groups are known to be richer in such compounds than others. Below, some few examples of plant-based food groups which are rich in these flavonoid compounds are presented. The concentrations of a particular flavonoid are given in mg/100 g edible portion.

(i) Fruits: berries (blueberries (flavones: 7.5–19.7), raspberries (flavonols: 1.11–1.114), strawberries (flavonols: 1.3–1.65), and cranberries (flavones: 0.03)), cherry (flavonols: 2.43–28.6), apple (flavones: 0.01–0.12; flavonols: 0.42–3.87; flavan-3-ols: 6.64–12.3; and anthocyanidins: 0–5), grape (flavonols: 1.05–2.39), lemon (flavonols: 1.67; flavanones: 49.8), lime (flavonols: 0.40), banana (flavonols: 0.18), orange (flavonols: 0.22–0.73; flavanones: 29.0–42.6), pears (flavan-3-ols: 1.88–4.81; anthocyanidins: 2.06), plums (flavonols: 0.90–12.5; anthocyanidins: 0.30–558.2), peaches (flavonols: 0.45–0.88; anthocyanidins: 0.97–1.92; and flavan-3-ols: 1.87–16.3), and apricot (flavan-3-ols: 8.41)

(ii) Cereals and legumes: soybean (flavan-3-ols: 37.4), cowpea (flavonols: 21.9), sorghum (flavones: 2.99–6.47; flavanones: 1.96), broad beans (flavanols: 0.9), beans (anthocyanidins: 2.74–44.5; flavan-3-ols: 0.10–324.2), purple wheat (anthocyanidins: 25.9), and peanuts (flavan-3-ols: 0.66)

(iii) Vegetables: eggplants (flavones: 0.03), onions (flavonols: 3.63–46.7; flavon-3-ols: 0–0.08; and anthocyanidins: 9.56), broccoli (flavonols: 1.05–11.2), carrots (flavonols: 0.49), cabbage (flavonols: 0.05–22.5), lettuce (flavonols: 1.63–7.63), and tomatoes (flavonols: 0.03–0.8)

(iv) Herbs and spices: chili, coriander (flavonols: 52.9), garlic (flavonols: 3.61), ginger (flavonols: 0.19–33.6), turmeric (flavonols: 6.96), and thyme (flavones: 47.75)

(v) Beverages: red wine (flavones: 0.04–0.17), tea (flavan-3-ols: 9.8–324.2), coffee (flavan-3-ols: 0.08; flavonols: 0.10), and cocoa (flavan-3-ols: 1.33–52.7)

Therefore, there is a chance of increased consumption by human on a daily basis, since they are widespread in plant-based foods and are easily absorbed due to their simplicity.

3. Phenolic Compounds and Health Promotion

Phenolic compounds are highly abundant secondary metabolites in the plant kingdom, possessing aromatic rings with one or more hydroxyl substituents or some functional derivatives, such as esters, methyl ethers, and glycosides [18]. Both taste and color, as well as some features of vegetables and fruits are strongly associated with the presence of such compounds, widely recognized for their wide range of activities, making them extremely beneficial to human health.

The physiological functions of phenolic compounds are vast because of their beneficial effects, not only for health promotion and maintenance but also for therapeutic purposes, given their wide range of pharmacological activities; thus, taken together, these aspects have made phenolic compounds highly important secondary metabolites and the target of an intense investigation in current times [19]. Taking a look at their versatile health benefits, they are effective antioxidant, antitumor, antimicrobial, antihyperglycemic, immunomodulatory, cardioprotective, vasodilatory, antithrombotic, anti-inflammatory, and UV radiation skin protective agents, and therefore interesting candidates for pharmaceutical and medicinal applications [20–23].

Due to the medicinal plant's abundance in phenolic compounds, interesting biological activities have been reported, which are extremely useful in the prevention of the onset of age-related diseases and closely related to high oxidative stress levels. With regard to the broad-spectrum activities, there is a shift towards natural product industrialization, especially in pharmaceutical and cosmetic industries. For example, flavonoids regulate key proteins involved in inflammation and signal transduction pathways [24]. Both the absorption and metabolism of these dietary phenolic compounds determine the extent of their health benefits, which in turn are determined by their structure (conjugation with other phenolics, degree of glycosylation/acylation), solubility, and molecular size. Their effectiveness decreases with the substitution of the hydroxyl groups in their structure for sugars; thus, aglycones exhibit more potent activities than their corresponding glycosides.

4. Phenolic Compounds That Inhibit Platelet Aggregation via Affecting Vascular Environment

4.1. Phenolic Compounds and Cardiovascular Activity: Emphasis on Molecular Ways. The regular consumption of diets rich in fruits, vegetables, olive oil, and wine, composed

of a wealth of phenolic compounds, exert a wide range of beneficial properties, such as antioxidant and anti-inflammatory effects as well as metabolic modulatory activities to hinder not only the disease onset but also its progression [25–28].

4.1.1. Cardioprotective Activity of Phenolics. CVD pathogenesis has been increasingly linked to oxidative stress. Typically, featured by an inflammatory condition, CVD is recognized as one of the major causes of death worldwide, with a rise in prevalence in developed countries [29]. As polyphenols are known for their antioxidant, immunomodulatory, and vasodilatory properties, an increased intake of such dietary antioxidants contributes to CV risk reduction [9] and inhibits oxidation of human low-density lipoproteins (LDL), ultimately preventing atherosclerotic plaque formation. The mechanisms involved in the cardioprotective effects of phenolic compounds have been studied and reported in preclinical (*in vitro* and *in vivo*) and clinical studies, acting via inhibition of ROS production, mitochondrial dysfunction, apoptosis, nuclear factor kappa B (NF-κB), p53, and DNA damage. For example, resveratrol as a case study, is a phenolic compound (stilbene) widely abundant in grapes and red wine that confers extraordinary cardioprotective effects by acutely improving endothelial function in coronary heart disease (CHD) patients [30].

Regarding CVD risk factors, hypertension is the most prominent one, contributing to one third of global mortality. Recently, naturally occurring phytochemicals have been employed not only to reduce but also to manage hypertension risk [31]. The renin-angiotensin-aldosterone (RAAS) system has been identified as an important target in the treatment/management of hypertension, myocardial infraction, stroke, and kidney diseases [32], given its role in the maintenance of vascular tone/tension. Briefly, the RAAS system mediates vascular tension via a sequential conversion of angiotensinogen to angiotensin II by a series of enzymatic cleavages. The angiotensinogen is cleaved by renin to produce angiotensin I, which is further cleaved to produce angiotensin II by the angiotensin-converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor, and its presence also promotes aldosterone production, thus contributing to hypertension [32]. Hence, inhibitors of renin and ACE have shown to be beneficial in the treatment of vascular tension and as antifibrotics [33]. Recently, phenolic compounds have revealed promising effects both as ACE and renin inhibitors, with phenolic-rich foods also revealing good antihypertensive abilities in experimental models (Figure 2) [34, 35].

Experimental and clinical studies have suggested that flavonoids and flavanol-rich foods could reduce blood pressure and CVD risk in humans [36]. For instance, caffeic acid along with its 19 novel derivatives, chlorogenic acid, quercetin, and captopril showed prominent effects as inhibitors of ACE and renin, as well as modulators of aldosterone secretion [31]. Other evidences have also shown that phenolic compounds, particularly flavonoids, are able to reverse vascular endothelial dysfunction [37].

Indeed, phenolic supplementation has been shown to boost endothelial function by stimulating endothelium-derived NO bioactivity, and this may explain some of the favorable effects of high phenolic intake seen in epidemiological studies [37]. For example, a randomized controlled trial performed in pre- and hypertensive patients, revealed that olive oil enriched with its own phenolic compounds show more benefits on endothelial function than standard virgin olive oil [38].

4.2. Role of Phenolic Compounds as Antiplatelet Agent via Redox Modulation. As the largest phytochemical molecules grouped under phenolic compounds, phenolic acid and flavonoids are commonly known to have great antioxidant properties and prove to be more effective than vitamins C and E and carotenoids [39]. These antioxidant abilities are mediated through several mechanisms, such as scavenging free radicals, suppressing ROS formation, inhibiting some enzymes, chelating trace metals, and upregulating or protecting antioxidant defense to ensure a proper redox balance [40]. The hydrogen-donating specificity and interaction of their hydroxyl groups (acting as the antioxidant) with ROS is a termination reaction which breaks the cycle of a new radical generation. The main factor on antioxidant/reduction activity of phenolic compounds is the number and position of its hydroxyl groups which are strengthened by steric hindrance; thus, flavonoids possess more hydroxyl groups and higher antioxidant activity than the other molecules of the group [41, 42].

ROS are generated intracellularly and exogenously as a byproduct of normal metabolism or due to cells' exposure to some environmental triggers [43]. The imbalance between ROS generation and defense mechanism is known as oxidative stress, which is pivotal in CVD development [44, 45]. Oxidized LDL promotes vasoconstriction and progression of platelet aggregation by promoting the smooth muscle cells' proliferation and inhibiting the endothelial nitric oxide synthase (eNOS) [46].

Phenolic compounds exert an antioxidant activity via their free radical scavenging properties and inhibiting ROS-generating enzymes (e.g., iNOS), as well as boosting antioxidant enzyme activity, like hemeoxygenase-1, glutathione peroxidase, and glutathione-S-transferase in cardiac and aortic smooth muscle cells [44, 45]. They are also able to regulate vascular reactivity by inducing nuclear Nrf2 accumulation and targeting eNOS, thereby enhancing NO bioavailability [47, 48]. Polyphenols also contribute to the antioxidant defense of endothelial cells by reducing NADPH oxidase expression [49]. Several *in vitro* studies have reported that phenolic compounds could effectively reduce oxidized LDL and increase the level of high-density lipoproteins (HDL), ultimately improving endothelial function [50, 51]. Moreover, numerous phenolic compounds exert cardioprotective effects at a localized or systemic level by inducing antiplatelet effects [46], with such effects being majorly attributed to the *O*-dihydroxyl group in the A and/or B ring (Figure 3) [52].

4.3. Role of Phenolic Compounds as Antiplatelet Agent via Inflammatory Process. Production of proinflammatory mediators during inflammation is common in some cells, mainly in macrophages, with interleukins (IL), tumor necrosis factor- (TNF-) α, ROS, NO, and prostaglandins (PGs) being

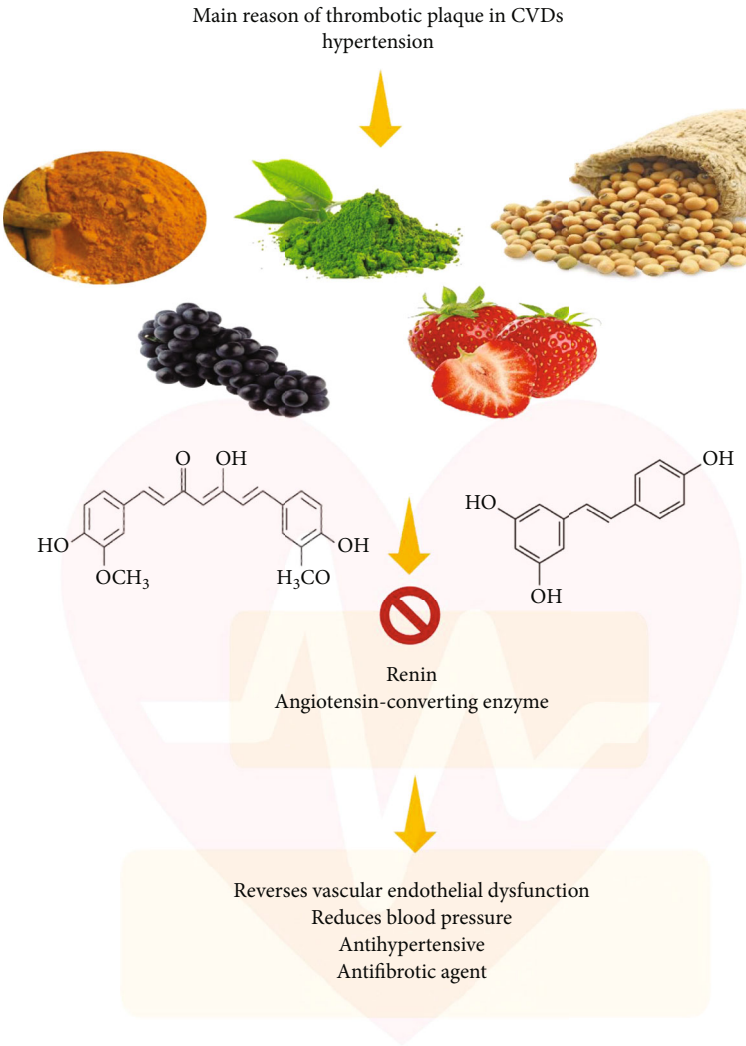

Figure 2: Antithrombotic and antihypertensive properties of the phenolic compounds.

the most commonly produced inflammation mediators [53]. Also, the association of phenolic compound structures and their anti-inflammatory activity with different targets of inflammation have been established [54]. Indeed, they are able to modulate transcriptional factors, like downregulating NF-κB or upregulating Nrf-2 [55, 56]. NF-κB regulates the expression of several proinflammatory cytokines, such as IL-1β, TNF-α, and enzymes, like iNOS and cyclooxygenase (COX-2). Nrf-2 regulates the expression of anti-inflammatory enzymes by possessing an antioxidant responsive element (ARE) able to activate several antioxidant enzymes needed for redox balance. Thus, they inhibit the gene expression and the activity of pro-inflammatory mediators at the same time that they activate the expression and activity of anti-inflammatory mediators that are targets of transcription factors.

The inhibition of inflammatory mediators, such as ROS, NO, and PGE2, and proinflammatory mediators, like cytokines, TNF-α, and COX-2, is one of the major targets for CVD treatment (Figure 3). The overexpression of TNF-α and IL is linked to NF-κB activation, which regulates the release of inflammatory mediators [57]. Additionally, phosphorylation of p38 mitogen-activated protein kinases (MAPK) plays an important role in chronic inflammation by activating NF-κB as well as regulating the NO and proinflammatory gene production from macrophages [58, 59].

Procyanidins were found to reduce the protein expression of iNOS, COX-2, lipoxygenase- (LOX-) 15 and some proinflammatory cytokines, such as IL-1β, TNF-α, and monocyte chemoattractant protein- (MCP-) 1 [58, 60, 61]. These effects might be due to suppression of NF-κB activity via downregulation of p38 and MAPK pathways [44, 58]. Rius et al. [62] proved that resveratrol supplementation may partially protect against CVD especially during the early atherosclerotic phase. This effect could be due to a decrease in the overexpression of intercellular and vascular cell adhesion molecules by inhibiting the NF-κB pathway in TNF-α-activated endothelial cells [63], and other reports have shown that it is related to a reduction of circulating levels of MCP-1 and MIP-1α [62]. Polyphenols extracted from roasted cocoa beans suppressed inflammation via oxidative pathways, which lead to an increase in oxygen consumption by mitochondria and ATP production via oxidative phosphorylation [64].

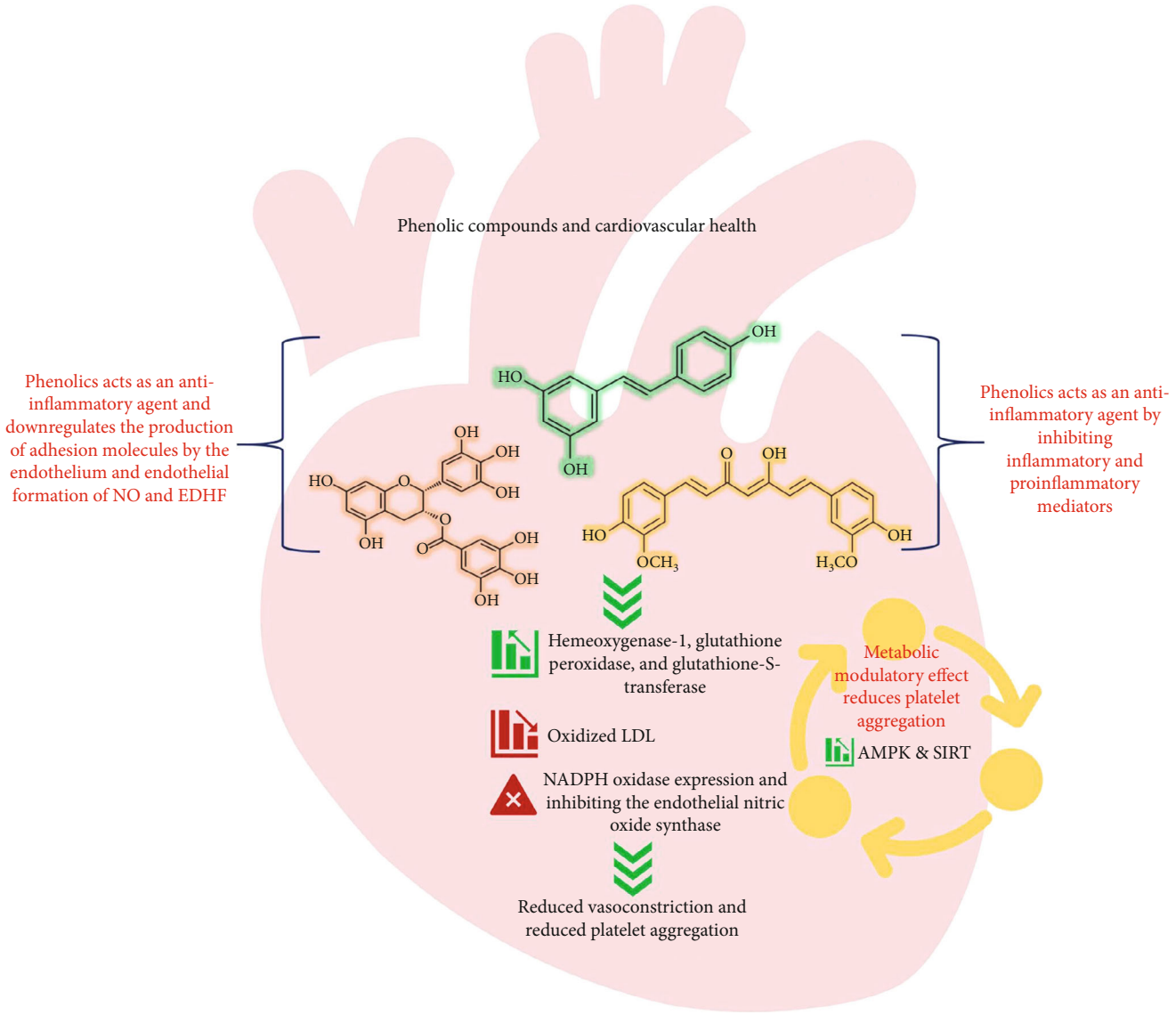

Figure 3: Role of phenolics as antiplatelet factors via redox modulation, anti-inflammatory responses, and metabolic modulatory effect.

As stated above, phenolic compounds present in extravirgin olive oil have also been stated to be potent anti-inflammatory agents by preventing the expression of iNOS, COX-2, LOX, and phospholipase A2 and thus blocking the production of eicosanoids (PGI2, leukotriene B4) [65]. They can also reduce platelet aggregation by decreasing the production of thromboxane B2 (TXB2) and 27-hydroxyleicosatetraenoate [66]. The same findings have also been said about resveratrol, which forms stable complexes in platelet COX-1 channels [67]. Moreover, the supplementation with curcumin or cinnamon bark extract was able to reduce the levels of C-reactive protein (CRP), an acute phase protein that plays a key role in CVD progression [68, 69]. The potential therapeutic and health-promoting roles of curcumin are also highlighted in the recent review by Moballegh Nasery et al. [70].

On the other hand, endothelial dysfunction results in the formation of vasoconstrictive factors, such as endothelin-1 in the arterial wall, implicated in CVD development [71]. Dietary polyphenols have also been shown to downregulate the production of adhesion molecules by the endothelium and to modify the endothelial formation of NO and endothelium-derived hyperpolarizing factor (EDHF), which improve endothelial function [9].

4.4. Role of Phenolic Compounds as Antiplatelet Agents via Metabolism Modulation. CVDs are related to alterations in metabolism. Dasgupta and Milbrandt in 2007 found that resveratrol can target and activate AMP-activated protein kinase (AMPK), having an important role in reducing fat accumulation, cholesterol synthesis, and inflammatory cytokines [72]. Resveratrol could also stimulate sirtuin 1 (SIRT1) at an amplitude of ~10-fold, which is a NAD-dependent lysine deacetylase that plays a vital role in energy metabolism (Figure 3) [73]. SIRT1 is known to regulate a variety of cell functions, mainly mitochondrial function, by activating the transcriptional activity of peroxisome proliferator-activated

receptor-gamma coactivator- (PGC-) 1α and triggering the activation of AMPK [74, 75]. Stimulation of SIRT1 and AMPK boosts the eNOS activity in human coronary arterial endothelial cells and increases NO production and mitochondrial biogenesis, which triggers vasodilation and decreases atherosclerosis [76]. Recent data have shown that polyphenols interact directly with the activator site of both estrogen receptor- (ER-) α and ER-β, leading to eNOS activation and stimulation of NO production and endothelium-dependent vasorelaxation [77, 78].

5. Phenolic Compounds with *In Vitro* Antiplatelet Aggregation Activity

The inhibition of platelet aggregation and thrombus formation comprises a vital target in preventing atherosclerotic events [79]. This concept has motivated researches to find therapeutic strategies targeting to reduce platelet aggregation. In fact, diets rich in phenolic compounds could represent a natural alternative for inhibiting platelet aggregation in a dose-dependent manner and helping reduce the individual risk of developing CVD [80]. From a mechanistic point of view, the inhibitory activity of phenolic compounds greatly depends on the phenolic class and is mainly due to their anti-inflammatory and antioxidant capacities, with an IC_{50} in the range of μM [81]. Pignatelli et al. [82] have reported that a combination of 25 μM catechin/L and 5 μM quercetin/L can synergistically inhibit platelet aggregation by blunting hydrogen peroxide production. Wang et al. [83] reported that kaempferol inhibits NADPH oxidase, thus reducing the ROS production. Similarly, Meshkini and Tahmasbi reported in 2017 that the antiplatelet activity of walnut hull extract is linked to its capacity to inhibit the rise in ROS levels induced by thrombin in platelets [7]. Cocoa polyphenols reduced platelet NADPH oxidase activation and the platelet formation of ROS and eicosanoids [84].

The main reported mechanisms of action for phenolic compounds, other than the antioxidant effects, include the suppression of cytoplasmic Ca^{2+} increase and inhibition of thromboxane formation and AA pathway [81, 85]. Quercetin was found to completely inhibit AA-induced platelet aggregation at 200 μM [86] and to inhibit platelet aggregation induced by thrombin by impairing Ca^{2+} mobilization and serotonin secretion [87]. Resveratrol could also inhibit the arachidonate-dependent synthesis of inflammatory agents, such as TXB2, hydroxyheptadecatrienoate, and 12-hydroxyeicosatetraenoate [67]. Apart from the antiplatelet activity of resveratrol, it also modulates the expression of noncoding RNAs in ovarian cancer cells (Vallino et al., 2020). Another study also showed the anticancer activity of resveratrol by inhibiting the STAT3 signaling pathway (Baek et al., 2016). Similarly, resveratrol might improve cardiovascular health by affecting the gene expression of platelet aggregating factors. Son et al. [88] reported that green tea catechins exert antithrombotic effects through the inhibition of TXA2 formation by modulating AA liberation and TXA2 synthase. Rutin and α-naphthoflavone have also been shown to inhibit phosphoinositide breakdown and several other steps, such as Ca^{2+} mobilization, protein kinase C (PKC) activation, and TXA2 formation in collagen-activated platelets [89, 90].

Flavonoids also inhibit the platelets' stimulation through phosphoinositide 3-kinase (PI3K)/PKB (AKT) and by extracellular signal-regulated kinase (ERK) 1/2, p38, and cJun N-terminal kinase (JNK) 1/2 pathways [91]. Several researches proved that quercetin at 50-60 μM completely inhibits all PI3k isoforms [92]. Moreover, some flavonoids have been shown to inhibit phospholipase C, platelet-activating factor, or collagen-receptor antagonism and glycoprotein IIb-IIIa activation [93]. It has also been revealed that quercetin and catechin are able to downregulate the expression of GPIIb/IIIa in platelets by increasing the NO production [94]. Hydroxytyrosol acetate and hydroxytyrosol were also found to synergistically inhibit collagen-induced platelet aggregation [95]. The inhibition of phospholipases, tyrosine kinases, phosphodiesterases, LOX, and COX are other mechanisms involved [96]. *In silico* docking studies showed that resveratrol could form stable complexes in platelet COX-1 channels [67], while Hubbard et al. [97] concluded that quercetin inhibits collagen-induced phosphorylation mainly due to its tyrosine-kinase inhibitory activity.

From the point of view of a structure-activity relationship (Figure 4), the hydroxyflavones were more effective than their corresponding methoxyflavones, considering that the hydroxyl group position also influences platelet function [98]. This is explained by the fact that methylation changes the electrical charge of the flavonoid and so decreases its affinity for TXA2 receptors. Glycosylation also decreases the antiplatelet activity of flavonoids by enlarging their size, thus complicating binding to the receptor [85]. The double bond in C2–C3 and/or 4-C=O in the C-ring of flavonoids has also a key importance for the antiplatelet activities [99]. It was also observed that not only does the phenyl group of a B ring play a critical role in antiplatelet activity, but the heteroatoms of the B ring also largely influence this activity [98].

Data have also shown that apigenin, genistein, and luteolin have high affinity to the TXA2 receptor due to their structural characteristic conjugation, with the presence of a lactone structure [85]. Indeed, epigallocatechin gallate, catechin gallate, and epicatechin gallate are catechins containing a galloyl group in the 3′ position, all inhibiting thrombin-induced aggregation and phosphorylation of p38 MAPK and ERK1/2. Catechins without a galloyl group (catechin, epicatechin) or with a galloyl group in the 2′ position (epigallocatechin) did not inhibit platelet aggregation [100].

6. Phenolic Compounds with *In Vivo* Antiplatelet Aggregation Activity

In vitro evidence cannot be fully translated to the *in vivo* condition in animals because parent molecule administration is followed by the presence of conjugated metabolites in the plasma with lower biological effects [101]. Additionally, experimental data related to the antiplatelet aggregation activity of dietary polyphenols *in vivo* are scarce, and results are often conflicting. For example, Ostertag et al. [102] demonstrated in an ex vivo study that phenolic compounds affect

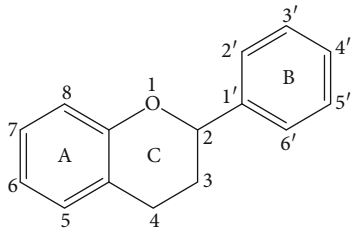

Figure 4: Basic structure of flavonoids.

the collagen-induced platelet aggregation and thrombin receptor-activating peptide-induced P-selectin expression but only at very high nonphysiologically attainable concentrations. In streptozotocin-induced diabetic rats, Schmatz et al. [103] also proved in an *ex vivo* study that moderate consumption of grape juice and red wine modulates the hydrolysis of the adenine nucleotides and decreases platelet aggregation.

Schumacher et al. [104] studied the effect of 300 mL chicory coffee rich in caffeic acid given daily to 27 healthy volunteers for 1 week. They found that the whole blood and plasma viscosity were both significantly decreased. In another study, 20 healthy subjects daily consumed 7 mL/kg of both red wine and purple grape juice for 14 days [104]. Platelet aggregation was inhibited and platelet-derived NO production increased, whereas the superoxide release decreased significantly [105]. In an intervention trial that lasts for 8 weeks, berry consumption resulted in favorable changes in platelet function, and antiplatelet activity was induced by both ADP and collagen [106]. Similar findings were also stated when consuming 2 or 3 kiwi fruits per day for 28 days [107]. The daily consumption of 50 mL of pomegranate juice (1.5 mmol total polyphenols) for 2 weeks was found to reduce LDL susceptibility to aggregation and retention and to increase the activity of serum paraoxonase by 20% [108]. Data from several studies shows that the consumption of 100 mg flavanols (equivalent to 11 g dark chocolate, 52 g milk chocolate, or 50 to 100 mL cocoa drink) also inhibits the collagen–epinephrine- and collagen–ADP-induced closure [109]. Hamed et al. [110] proved that the intake of dark chocolate (700 mg flavonoids/day) for a week significantly reduced ADP- and AA-induced platelet activation in addition to activated glycoprotein IIb/IIIa. Similar findings were reported by Rull et al. [111] who proved that dark chocolate (with high and low flavanol levels) supplementation for 6 weeks lowered platelet responsiveness to ADP and to thrombin receptor activator peptide. Furthermore, Wright et al. [112] found that both methylated and sulphated flavonoid metabolites have higher platelet inhibitory effects than the glucoronidated metabolites.

Conversely, in a study conducted on 20 subjects, a polyphenol-rich meal every lunchtime for 5 days had no ex vivo effect on platelet aggregation although the total plasma flavonoids significantly increased [113]. In the same way, a daily supplementation with 200 mg of flavonoids from grape seeds had no effect on platelet aggregation in a double-blind randomized study performed in male smokers [114]. Platelet aggregation did not evidence significant differences in 21 postmenopausal women supplemented with wine polyphenols [115]. Additionally, the daily intake of 2 g of cocoa flavonols in healthy volunteers during 12 weeks did not exert significant differences in platelet aggregation [116].

Taken together, these findings underline that further *in vivo* studies are absolutely required to confirm the potentiality of phenolic compounds as antiplatelet aggregation agents. This potentiality renders polyphenols an important dietary element in the prevention of CVD and possibly an alternative to pharmacological treatments of platelet aggregation.

7. From Effects of Phenolic Compounds to Absorption and Bioavailability

The bioavailability of polyphenols and related metabolites following oral intake has been viewed as a hot research topic in the last decades. Indeed, results obtained so far have indicated that the bioavailability of phenolic compounds is related to the cleavage and release of the aglycone by digestive enzymes and microbial fermentation, as shown in Figure 5 [117]. The identification of phenolic compounds in biological samples to check its bioavailability, metabolism, and intestinal absorption is very crucial. These metabolomic analyses are frequently done using nuclear magnetic resonance and mass spectrometry ([118]).

Lactase phlorizin hydrolase and cytosolic β-glucosidase were found to be the main digestive enzymes that hydrolyze phenolic glycosides and release the aglycones which enter the epithelial cells [119, 120]. Before entering into the systemic circulation, polyphenolic aglycones undergo some degrees of phase II metabolism through the action of sulfotransferases, uridine-5′-diphosphate glucuronosyl transferases, or catechol-*O*-methyltransferases [121]. On the other side, compounds with a high degree of polymerization are exposed to microbial catabolism in the colon before reaching the liver, where they can also be subjected to conjugation [122]. Also worthy of note is that the different compositions of the colonic microflora between individuals lead to variations in the released metabolites [123]. In contrast, some polyphenols show specific pharmacokinetic features, as their glycosides are considered to be more bioavailable than their aglycones. As an example, some anthocyanins, glycosides, and isoflavone-glycosides could be efficiently absorbed across the gastrointestinal mucosa [124, 125]. For instance, Kay et al. [126] revealed that the absorption rate for quercetin glycosides is higher than that of the aglycones.

8. Phenolic Compounds: Looking at Adverse Effects, Drug Interaction, and Related Toxicity

The beneficial effects of phenolic compounds could be diminished by aspects as lifestyle, gender, age, genetic factors, underlying diseases, and interactions with conventional drugs, the latter being of great importance with regard to safety concerns. In fact, although the risk of adverse effects due to interactions between herbal medicinal products and conventional drugs is still an underexploited problem, some

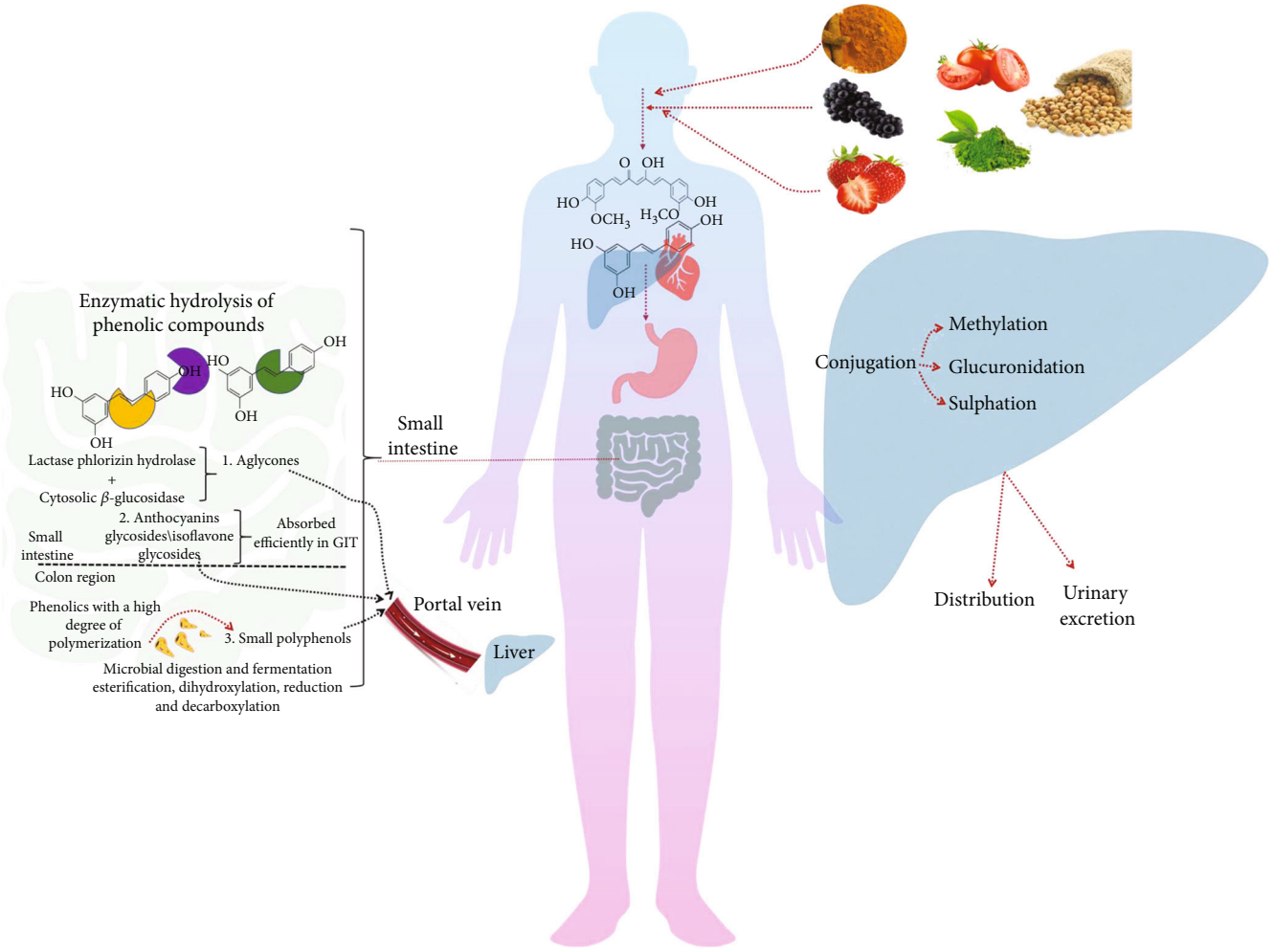

FIGURE 5: Absorption and bioavailability of phenolics as an antiplatelet agent through the liver distribution system.

studies highlighting concerns regarding antithrombotic agents have been reported [96, 127, 128]. Overall, it is clear that the majority of these works do not address a single/specific drug and, on the contrary, preferentially consider anticoagulant (e.g., warfarin) and antiplatelet (e.g., aspirin, clopidogrel, or ticlopidine) drugs [129] as the whole focus of the study (Table 1).

The most referred mechanisms in such interactions include inhibition of thromboxane synthesis and/or COX activity and interference in drug metabolism, which globally results in synergistic effects with drugs, and ultimately, promoting platelet inhibition and increasing the risk of bleeding in some patients [127, 129]. Yet, in most cases, the mechanism of action is not deeply determined, and a great number of aspects on herbal-antiplatelet interactions remain unexplored. In addition, most of them lack the identification of the active compounds' class in such interactions, just referring to the name of the herbal product [130, 131]. Nevertheless, phenolic compounds of some herbal plants, such as *Camellia sinensis* (L.) Kuntze, *Citrus paradisi* Macfad., *Crataegus* spp., *Curcuma longa* L., *Crocus sativus* L., *Foeniculum vulgare* Mill. *Ginkgo biloba* L., *Matricaria recutita* L., and *Vaccinium myrtillus* L. have been pointed out as probable plant components capable of interacting with antithrombotic drugs.

9. Paving the Way for Effective Clinical Applications

Phenolic compounds are gaining a huge importance in the promotion, prevention, and maintenance of good health, as well as in the treatment of multiple diseases, because of their remarkable bioactive effects, with special emphasis on their antioxidant, antitumor, antihyperglycemic, cardioprotective, antithrombotic, vasodilatory, and anti-inflammatory activities. This wide range of activities makes such biomolecules of hot potential for both pharmaceutical and medical applications.

As stated, phenolic compounds are able to modulate some transcriptional factors (e.g., NF-κB) to regulate the expression of some proinflammatory cytokines (e.g., IL-1β and TNF-α) and even enzymes (e.g., iNOS and COX-2) that are present in inflammatory processes. NF-κB is associated with the Nrf-2 regulation that regulates the expression of anti-inflammatory enzymes [55, 56]. Considering such mechanisms and their ability to inhibit ROS, the

Table 1: Botanical species identified in literature acting as antithrombotic agents.

Species/herbal product Common name	Conventional drugs	Mechanism of action	Negative effect	Observations	References
Allium sativum Garlic	Anticoagulants and antiplatelets (aspirin, warfarin)	↔ thromboxane synthesis ↑ INR ↓ platelet aggregation	↔ with platelet ↓ blood clotting time Spontaneous spinal epidural hematoma causing paraplegia secondary	Ajoene, allicin, aliin, and thiocyanates are potentially responsible for the interaction Need more clinical assays	([128]; [131]; [132]; [133]; [134])
Borago officinalis Borage seed oil	Anticoagulants and antiplatelets	Potentiate the effects of warfarin	↑ the risk of bleeding		[131]
Camellia sinensis Green tea	Anticoagulants and antiplatelets	⦸ platelet aggregation ↔ coagulation mechanisms (e.g., antagonized the effects of warfarin)		Catechins, tannins, and others (caffeine, vitamin k) are potentially responsible for the interaction *In vitro* studies Clinical significance unknown	([127]; [135])
Carthamus tinctorius Safflower	Anticoagulants and antiplatelets	Synergism	↑ the effects of blood thinning agents		[131]
Cnicus benedictus Blessed thistle	Anticoagulant and antiplatelets	Synergism	↑ bleeding risk	Theoretical interaction	[127]
Commifora mukul Guggul	Anticoagulant and antiplatelets	Synergism	↑ bleeding risk	Theoretical interaction	[127]
Citrus paradise Grapefruit, juice	Cilostazol, clopidogrel, and ticagrelor	Synergism (cilostazol, ticagrelor) ⦸ CYP2C19 and CYP3A4 (clopidogrel) ⦸ CYP3A4-mediated first pass metabolism (ticagrelor)	↑ antiplatelet effects (cilostazol, ticagrelor) ⦸ effect (clopidogrel) Purpura (cilostazol)	Coumarins and flavonoids are potentially responsible for the interaction Need more clinical assays	([131]; [136]; [137]; [138])
Crataegus monogyna, *Crataegus laevigata*, *Crataegus* spp., *Crataegus oxyacantha* Hawthorn	Anticoagulants and antiplatelets	⦸ biosynthesis of thromboxane A2	Potentially ↑ risk of bleeding	Procyanidins, phenolic acids, and flavonoids are potential responsible for the interaction Need more clinical assays	([127]; [134]; [139])
Curcuma longa Turmeric	Antiplatelets	Synergism	↑ risk of bleeding	Curcumin and *ar*-turmerone are potentially responsible for the interaction	([127]; [131]; [140])
Cassia senna Senna	Anticoagulant and antiplatelets	Synergism	↑ risk of bleeding		[127]
Crocus sativus Saffron		Synergism ↓ platelet aggregation		4-*trans*-Crocin, picrocrocin, safranal, and flavonoids are potentially responsible for the interaction	[127]

Table 1: Continued.

Species/herbal product Common name	Conventional drugs	Mechanism of action	Negative effect	Observations	References
Eugenia aromatic Clove	Anticoagulants and antiplatelets	Synergism ↓platelet aggregation *in vivo* and in clinical studies ↑ INR	↑ risk of bleeding		[131]
Foeniculum vulgare Fennel	Antiplatelets (aspirin)	Synergism	↑ risk of bleeding	Anetol, fenchone, isorhamnetin, luteolin, and naringenin are potentially responsible for the interaction	[141]
Ganoderma lucidum Reishi mushroom	Anticoagulant and antiplatelets	Synergism	↑ risk of bleeding	Theoretical interaction	[127]
Ginkgo biloba Ginkgo	Anticoagulants (warfarin and others), antiplatelets (aspirin, clopidogrel), NSAIDs	⦸ platelet aggregation ⦸ platelet-activating factor and altering bleeding times ↑ anticoagulant effect of warfarin and aspirin Platelet-activating factor receptor antagonists	↑ risk of bleeding Severe spontaneous bleeding Subdural haematomas	Ginkgolides (ginkgolide B), bilobalides, flavonoids, terpenic lactones, amentoflavone, and ginkgolic acids are potentially responsible for interaction Need more clinical assays	[127, 128] ([132]; [133]; [134]; [135]; [142])
Glycyrrhiza glabra Licorice	Anticoagulants and antiplatelets	⦸ thrombin ⦸ platelet aggregation			[134]
Harpagophytum procumbens Devil's claw	Anticoagulants (e.g., warfarin) and antiplatelets	Synergism	Purpura (warfarin)	Need clinical assays	([127]; [131]; [143])
Lavandula angustifolia Miller lavender	Anticoagulant and antiplatelets	Synergism			[127]
Larrea tridentate, *Larrea divaricate* Chaparral	Aspirin	↓ platelet aggregation	Delayed its onset	Nordihydroguaiaretic acid (NDGA) are potentially responsible for the interaction	([127]; [131])
Leonurus cardiaca Motherwort		↓ platelet aggregation ↓ fibrinogen levels			[134]
Matricaria recutita Chamomile	Anticoagulants and antiplatelets	Synergism		Coumaric compounds are potentially responsible for the interaction Need clinical assays	[144]
Monascus purpureus Red yeast rice	Anticoagulants (warfarin) and antiplatelets	↓ serum thromboxane concentration		Inconclusive conclusions	[131]
Piper methysticum Kava	Antiplatelets	⦸ thromboxane synthesis ⦸ cyclooxygenase		Isolated use is associated to reduced platelet size	[127]
Policosanol	Anticoagulants and antiplatelets	⦸ platelet aggregation *in vitro*			[131]

Table 1: Continued.

Species/herbal product Common name	Conventional drugs	Mechanism of action	Negative effect	Observations	References
Pinus pinaster spp. *Atlantica* Pycnogenol®	Aspirin	↓ coagulation	↓ efficacy of acetylsalicylic acid		[131]
Panax ginseng Ginseng	Anticoagulants (warfarin), antiplatelets (aspirin) and NSAIDs	⦸ platelet aggregation ↓ platelet adhesiveness ↓ INR	↑ action of NSAIDs		([127]; [131]; [141])
Pelargonium sidoides Umckaloabo	Anticoagulant and antiplatelets	Synergism	↑ risk of bleeding	Theoretical interaction	[127]
Rosmarinus officinalis Rosemary	Antiplatelets	Synergism ⦸ platelets aggregation ↓ fibronectin ↓ fibrin			[127]
Salvia hispanica Chia	Anticoagulant and antiplatelets	Synergism	↑ risk of bleeding		[127]
Salvia miltiorrhiza Danshen	Anticoagulant and antiplatelets	↓ elimination of warfarin ⦸ cyclic adenosine monophosphate phosphodiesterase ↑ prothrombin time	↑ INR Over anticoagulation and antiplatelet effects: increased bleeding		([134]; [141])
Serenoa repens Saw palmetto	Anticoagulant and antiplatelets (aspirin)	Synergism ⦸ lipoxygenase ⦸ cyclooxygenase	↑ risk of bleeding		([127]; [131]; [141])
Tanacetum parhenium Feverfew	Anticoagulant (warfarin) and antiplatelets (aspirin)	Synergism	Additive platelet inhibition		([132]; [141]; [142])
Trigonella foenum-graecum Fenugreek	Anticoagulant and antiplatelets	⦸ effect	↑ INR		[127]
Trifolium pratense Red clover	Anticoagulant and antiplatelets	Synergism	Bleeding disorders		[127]
Vaccinium myrtillus Bilberry	Antiplatelets	Synergism		Anthocyanidins are potentially responsible for the interaction Theoretical interaction	[135]
Zingiber officinale/herbal products containing ginger Ginger	Anticoagulant (e.g., warfarin) and antiplatelets	NSAID-like activity (?) LOX ⦸ COX ↔ platelet		Gingerol, zingerone, and ginkgolide B are potentially responsible for the interaction Need more clinical assays	([128]; [135]; [144])

↑: increase; ↓: decrease; ⦸: inhibition; ↔: interference; INR: international normalized ratio; NSAIDs: nonsteroidal anti-inflammatory drugs.

cardioprotective effects of such molecules are due to its ability to improve endothelial dysfunction in CHD [37], since it also increases the bioactivity of endothelial NO. Moreover, evidence has stated that flavonoids reverse endothelial dysfunction in addition to lowering blood pressure [36].

To name a few mechanisms, there is increasing *in vitro* evidence that shows that polyphenols reduce platelet NADPH oxidase activation [83, 84], ROS formation, thromboxane formation, AA pathway [84, 85], and platelet stimulation through PI3K/AKT [91]. Nonetheless, there is still little *in vivo* evidence showing that polyphenols can inhibit platelet aggregation, increase NO production [105], inhibit ADP-induced platelet activity [106], and reduce AA-induced platelet activation [110]. So, there is no doubt that the *in vitro* data cannot be translated into the *in vivo* condition, because the molecules undergo biotransformation reactions that alter their bioavailability. As a consequence, this would lead to discrete and contradictory biological effects.

There is increasing evidence showing that the effectiveness of selective phenolic compounds is supported by their important role in whole foods, where when used in combination, such as through the intake of whole plant foods with a mixture of polyphenols, better antiplatelet effects are reached when compared to isolated compounds. For these reasons, further studies are needed to confirm its potential.

10. Conclusions and Upcoming Perspectives

Phenolic compounds have revealed several positive effects in CVD models *in vitro* and *in vivo*. However, for *in vivo* models, the evidence is scarce. However, with the described evidence, it can be affirmed that polyphenols are a key element in CVD prevention. Regarding the effectiveness of phenolic compounds, little has been studied about their interactions with medications, like anticoagulants and antiplatelets. Such interactions, that include mechanism of inhibition of the synthesis of thromboxane or COX activity, may increase the risk of bleeding in such patients, despite that there is a wide range of herbal products that interact with antithrombotic drugs in a synergistic manner.

Taken together, data presented here clearly underline the need for more *in vivo* studies and clinical trials to evaluate the phenolic compounds' potential and to guarantee their efficacy. Also, more investigations are desired on isolated or mixed phenolic compounds and their relationship with CVDs in order to elucidate their potential and success for both prevention and treatment. Finally, and not least important, as it is poorly described, is the design of more clinical trials, because most of the currently performed clinical studies with these herbal products lack identification and quantification of phenolic compounds.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

J.S.-R., M.B., M.M., N.C.-M., and W.C.C. contributed to the study conception and design, acquisition of data, and the analysis and interpretation of data. C.Q., W.Z., M.K., S.M.C., O.R.P., A.O.A., O.A., A.C.M., J.Ž., F.N., S.A.A., F.K., M.F., I.C.B., R.V.B., M.M.A, literature review. J.S.-R., M.M., W.C.C., M.B., and N.C.-M. contributed to drafting the manuscript and critically revising the manuscript. All authors prepared the manuscript, contributed equally, and read and approved the final manuscript.

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Research Article

Naringin and Hesperidin Counteract Diclofenac-Induced Hepatotoxicity in Male Wistar Rats *via* Their Antioxidant, Anti-Inflammatory, and Antiapoptotic Activities

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This study is aimed at evaluating the preventive effect and at suggesting the mode of actions of naringin and hesperidin and their combination in diclofenac-induced hepatotoxicity. Male Wistar rats, intraperitoneally injected with diclofenac sodium (3 mg/kg b.wt/day), were orally treated with naringin (20 mg/kg b.wt/day) and hesperidin (20 mg/kg b.wt/day) and their combination for 4 weeks. The administrations of naringin and hesperidin to diclofenac-injected rats led to a significant decrease in the elevated serum ALT, AST, LDH, ALP, GGT, total bilirubin, TNF-α, and IL-17 levels as well as liver lipid peroxidation and liver p53 and caspase-3 mRNA expressions. In contrast, serum IL-4 level, liver GSH content, and liver GPx and SOD activities increased. In association, diclofenac-induced deleterious histological alterations including hydropic degeneration, cytoplasmic vacuolization, apoptosis, and focal hepatic necrosis of hepatocytes associated with inflammatory cells' infiltration were remarkably improved by treatments with naringin and hesperidin. In conclusion, naringin, hesperidin, and their combination, which was the most potent, counteract diclofenac-induced liver injury *via* antioxidant, anti-inflammatory, and antiapoptotic actions. Thus, this study recommends the use of naringin and hesperidin or their combination to resolve the side effects of drugs like diclofenac on the liver.

1. Introduction

Nonsteroidal anti-inflammatory drugs)NSAIDs) have various side effects [1]. Diclofenac, as one of this category of drugs, is a phenylacetic acid derivative that has long been used as analgesic and an anti-inflammatory drug to treat certain conditions [2]. In its mechanism of action, diclofenac inhibits cyclooxygenase- (COX-) 2 enzymes with greater potency than it acts on COX-1 [3]. Although diclofenac has potent therapeutic effects, its sustained use is associated with serious dose-dependent adverse effects [3]. Increase in the oxidative stress and suppression of antioxidant defense system were reported by various publications that they were implicated in the induction of toxicity and side effects of the drug [4, 5]. The use of antioxidant such as citrus flavonoid in combination with the diclofenac may counteract the diclofenac-induced oxidative stress and thereby may prevent drug side effects and toxicity.

Naringin responsible for the sour flavor and bitter taste of the fruits of *Citrus species* have broad-spectrum pharmacological and therapeutic properties as lipid lowering, anti-inflammatory, free radical-scavenging, antioxidative, antihyperlipidemic,

antiapoptotic, and antiatherogenic properties [6, 7]. Hesperidin is another flavonoid isolated from *Citrus species* that had several biological properties, particularly antioxidant and anti-inflammatory [8]. It has been reported that hesperidin improved the cytochrome-induced liver tissue injury observed by histopathological examination [9]. It was also revealed that hesperidin was effective in suppressing inflammation and allergic symptoms and in the treatment of allergic rhinitis [10].

Therefore, this study was conducted to assess the possible preventive effects of naringin and hesperidin on diclofenac-induced liver toxicity and to explore the roles of modulation of inflammation, oxidative stress, and apoptosis in the preventive action.

2. Materials and Methods

2.1. Chemicals and Kits. Diclofenac sodium was obtained from El-Nasr Pharmaceutical Chemical Company, Egypt. Naringin and hesperidin were produced by Sigma-Aldrich Company (St. Louis, MO, USA). All other used chemicals are of analytical grade and were obtained from local chemical companies. Alanine transaminase (ALT) and aspartate transaminase (AST) kits were obtained from Spinreact, Ctra Sta Coloma, 7. 17176 St., Esteve de Bas, Girona, Spain. Lactate dehydrogenase (LDH) and γ-glutamyltransferase (GGT) was obtained from Spectrum, Egyptian Company for Biotechnology, Obour City Industrial Area, Cairo, Egypt. Alkaline phosphatase (ALP) kit was obtained from Biodiagnostic, 29 Tahrir St., Dokki, Giza, Egypt. Albumin and bilirubin kits were obtained from Diamond, 24 El Montazah St., Heliopolis, Cairo, Egypt.

2.2. Experimental Animals. Adult male rats of Wistar strain weighing 100-130 g (9-11 weeks) were used as experimental animals in the present work. They were obtained from Animal House of Ophthalmology Research Institute, Giza, Egypt. The animals were housed in good aerated cages at 12 hours daily of light-dark cycles and temperature between 20 and 25°C. Rats were supplemented daily with standard pelleted diet and water *ad libitum*. The animals were maintained for two weeks under observation before starting the experiment for adaptation and to exclude any intercurrent infection. All animal methodologies are in accordance with the guidelines and instructions of the Experimental Animals Ethics Committee of the Faculty of Science for Care and Use of the Animals, Beni Suef University, Egypt. The ethical approval number is BSU/FS/2015/15. All efforts were done to reduce the suffering and distress of animals.

2.3. Animal Grouping. Thirty male Wistar rats were used in this experiment and were allocated into five groups (Figure 1). The rats of group 1 (normal) received the equivalent volume of saline (the vehicle in which diclofenac was dissolved) by intraperitoneal injection and also given the equivalent volume of 1% carboxymethyl cellulose (CMC; the vehicle in which naringin and hesperidin was dissolved) by oral gavage daily for 4 weeks. Group 2 (diclofenac-injected group) was injected with diclofenac (dissolved in saline) by intraperitoneal administration at a dose of 3 mg/kilogram body weight (kg b. wt)/day [11] and was also given the equivalent volume of 1% CMC by oral gavage daily for 4 weeks. Group 3 (diclofenac-injected group treated with naringin) was given diclofenac as in group 2 and was orally treated with naringin (dissolved in 1% CMC) at a dose level of 20 mg/kg b. wt/day by oral gavage [12] for 4 weeks. Group 4 (diclofenac-injected group treated with hesperidin) was given diclofenac as in group 2 and was orally treated with hesperidin (dissolved in 1% CMC) at a dose of 20 mg/kg b. wt/day [13] for 4 weeks. Group 5 (diclofenac-injected group treated with naringin and hesperidin) was supplemented with diclofenac as in group 2 and was orally treated with naringin and hesperidin (dissolved in 1% CMC) at a dose of 20 mg/kg b. wt/day for 4 weeks.

2.4. Blood and Liver Sampling. At the end of the experiment, rats deprived of food and blood samples were collected from jugular vein. The blood samples were left to coagulate at the room temperature and then centrifuged at 3000 rpm for 15 minutes. The serum of each blood sample was fractioned into three vials that were kept at -30°C until used for biochemical analysis.

After taking blood samples from jugular vein, the rats were euthanized and dissected. The liver from each rat was rapidly excised and washed in sterile saline (0.9% NaCl). Pieces of the liver were fixed in 10% neutral buffered formalin for histological investigation. Other parts were kept in deep freezer at -30°C pending homogenization in sterile isotonic saline (0.9% NaCl) for determination of oxidative stress and antioxidant defense markers. Third parts of the liver were kept at -30°C in sterile Eppendorf tubes for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

2.5. Detection of Liver Function Parameters in Serum. ALT and AST activities were estimated using the nicotinamide adenine dinucleotide hydrogen (NADH) oxidation method according to methods of Murray [14] and Murray [15], respectively, using reagent kits purchased from Spinreact, Spain. In brief, ALT catalyzes the conversion of alanine to pyruvate, which is reduced to lactate by LDH, and NADH is oxidized to nicotinamide adenine dinucleotide (NAD). The rate of decrease in concentration of NADH, measured photometrically at 340 nm, is proportional to the catalytic concentration of ALT present in the serum sample. On the other hand, AST catalyzes the conversion of aspartate to oxaloacetate, which is reduced to malate by malate dehydrogenase (MDH), and NADH is oxidized to NAD. The rate of decrease in concentration of NADH, measured photometrically at 340 nm, is proportional to the catalytic concentration of AST present in the serum sample. Serum LDH activity was detected according to method of Van der Heiden et al. [16]. In this method, LDH catalyzes the reaction between pyruvate and NADH to produce NAD^+ and L-lactate. The rate of decrease in concentration of NADH, measured photometrically at 340 nm, is proportional to the catalytic concentration of LDH present in the sample. Serum ALP activity was determined according to method of Belfield and Goldberg [17] using kits obtained from Biodiagnostic, Egypt. By adding serum sample to phenyl phosphate (5 mmol/L) at pH 10,

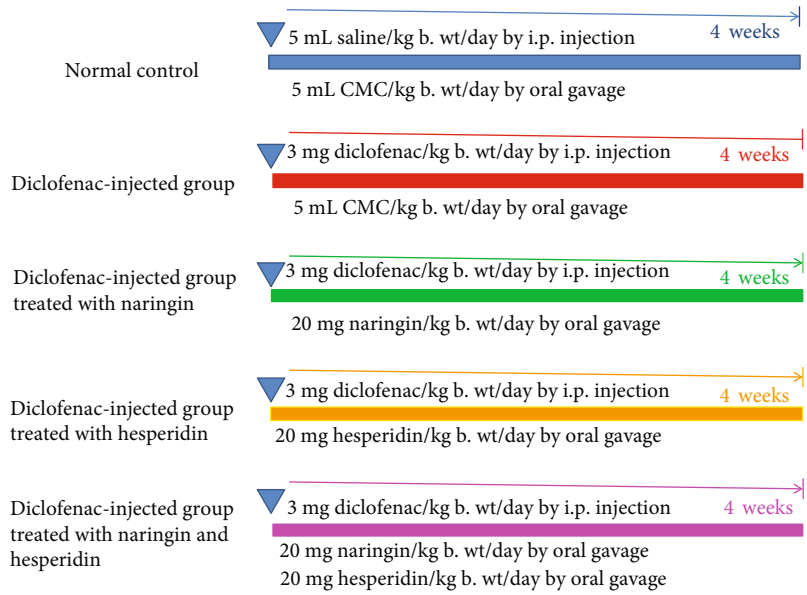

Figure 1: Experimental design and animal grouping. i.p.: intraperitoneal.

ALP leads to formation of phenol and phosphate. The liberated phenol is measured colorimetrically at 510 nm in the presence of 4-aminophenazone and potassium ferricyanide. GGT activity was determined according to method of Szasz and Persijn [18] using kits obtained from Spectrum Diagnostic, Egyptian Company for Biotechnology, Egypt. GGT transfers the γ-glutamyl group from the substrate L-γ-glutamyl-p-nitroanilide, liberating the chromogen p-nitroanilide (pNA) which is measured at 418 nm and its formation is proportional to the GGT activity. Serum albumin level was determined according to the method of Doumas et al. [19] using reagent kits purchased from Diamond, Egypt. The method in brief was performed by adding 10 μL serum to 2 mL bromocresol green (0.12 mmol/L) in citrate buffer pH 3.8. The albumin/bromocresol green complex, formed by the reaction, was measured at 630 nm. Serum bilirubin level was assayed according to methods of Malloy and Evelyn [20] using reagent kits purchased from Diamond, Egypt. This method for bilirubin estimation is based on van den Bergh reaction. In this reaction, bilirubin reacts with diazotized sulfanilic acid to produce azobilirubin which is purple in color which was measured photometrically at 578 nm. Intensity of color is directly proportional to the amount of bilirubin in the serum.

2.6. Evaluation of Liver Oxidative Stress and Antioxidant Defense System Parameters. Liver lipid peroxidation (LPO) was determined according to method of Preuss et al. [21]. In brief, the protein was precipitated by adding 0.15 mL 76% trichloroacetic acid (TCA) to 1 mL liver homogenate. Then, 0.35 mL of thiobarbituric acid (TBA) was added, as a color-developing agent, to the separated supernatant. The developed faint pink color was measured at 532 nm after incubation in water bath at 80°C for 30 minutes. MDA (malondialdehyde or 1,1,3,3-tetramethoxypropane) was used as standard. Reduced glutathione (GSH) content in the liver was determined by adding 0.5 mL 5,5′-dithiobis (2-nitrobenzoic acid), Ellman's reagent (as a color-developing agent), and phosphate buffer solution (pH 7) to homogenate supernatant after protein precipitation based on the procedure of Beutler et al. [22]. The developed yellow colors in samples and GSH standard were measured at 412 nm against blank. Liver GPx activity was measured according to the procedure of Matkovics et al. [23] with some modifications. This method is based on the detection of the GSH that is converted to oxidized glutathione (GSSG) by the enzyme through detection of the residual GSH and subtracting it from the total. Briefly, 50 μL homogenate supernatant was added to a Wasserman tube containing 350 μL Tris buffer (pH 7.6), 50 μL GSH solution (2 mM), and 50 μL H_2O_2 (3.38 mM). Then, after 10 minutes of incubation, the residual GSH content was measured by the previously described method for GSH determination at 430 nm. Standard test was prepared by adding 50 μL distilled water instead of 50 μL sample and blank test was prepared by adding 100 μL distilled water instead of 50 μL sample and 50 μL GSH solution. After detection of residual GSH content in the sample, the GSH converted to oxidized form (GSSG) and the enzyme activity can be calculated. Liver superoxide dismutase (SOD) activity was detected according to the method of Marklund and Marklund [24]. The reaction is based on the inhibition of autooxidation of pyrogallol by SOD. The process is dependent on the presence of superoxide ions. The amount of the enzyme that causes a 50% inhibition in the extinction changes in 1 minute compared to the control is regarded as one unit of the enzyme.

2.7. Determination of Serum TNF-α, IL-17, and IL-4 Levels. Serum TNF-α, IL-17, and IL-4 levels were estimated according to specific enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. Rat TNF-alpha ELISA kit (Catalog No.: MBS355371), IL-17 ELISA

kit (Catalog No.: MBS5203506), and IL-4 ELISA kit (Catalog No.: MBS494192) were obtained from MyBioSource, Inc., San Diego, CA, USA. These ELISA kits use sandwich ELISA. In brief, the micro ELISA plates provided in these kits have been precoated with an antibody specific to the concerned cytokine. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibodies. Then, a biotinylated detection antibody specific for the concerned cytokine and avidin-horseradish peroxidase (HRP) conjugate is added to each microplate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain cytokine, biotinylated detection antibody, and avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of the tested cytokine and its concentration in the samples can be determined by comparing the OD of the samples to the standard curve.

2.8. Detection of mRNA Expression of p53

2.8.1. RNA Isolation. Total RNA was isolated from the liver tissue according to the method of Chomzynski and Sacchi [25] and Boom et al. [26] using Thermo Scientific GeneJET RNA purification kit obtained from Thermo Fisher Scientific Inc., Rochester, New York, USA. Briefly, liver samples are lysed and homogenized in lysis buffer, which contains guanidine thiocyanate as a chaotropic salt and β-mercaptoethanol capable of protecting RNA from endogenous RNases. The lysate is then mixed with ethanol and loaded on a purification column. The chaotropic salt and ethanol cause RNA to bind to the silica membrane while the lysate is spun through the column. Subsequently, impurities are effectively removed from the membrane by washing the column with wash buffers. Pure RNA is then eluted under low ionic strength conditions with nuclease-free water. The levels of isolated RNA were determined and quantified using ultraviolet (UV) spectrophotometer and taking the absorbance at optical densities (OD) at 260 nm and 280 nm. RNA was quantified and qualified based on Sambrook and Russell [27] formula
(RNA (μg/μL) = OD at 260 nm × dilution × 40 μg/mL/1000). For each extracted RNA sample, the ratio between OD at 260 nm and OD at 280 nm and the ratio ranged between 1.7 and 2.0 ensure high purity of extracted RNA.

2.8.2. RT-PCR Assay. RT-PCR assay was applied using Thermo Scientific Verso 1-Step RT-PCR Reddy Mix Kit (Thermo Fisher Scientific Inc., Rochester, New York, USA) for detection of the mRNA expression of liver p53. In brief, in one step reaction, RNA was reverse transcribed into cDNA and the produced cDNA was amplified by Techne 32 Thermocycler. The reaction mixture consists of Veso Enzyme Mix (1 μL), 2X One-Step PCR ReadyMix (25 μL), RT enhancer (25 μL), forward primer (10 μM; 1 μL), reverse primer (10 μM; 1 μL), template RNA (1 ng; 1.5 μL), and water nuclease free to complete total volume to 50 μL. The thermal cycling program includes cDNA synthesis (temp. 50°C, 15 minutes, 1 cycle), Verso inactivation (temp. 95°C, 2 minutes, 1 cycle), denaturation (temp. 95°C, 20 seconds), annealing (temp. 55°C, 30 seconds), extension (temp. 72°C, 1 minute), and final extension (temp. 72°C, 5 minutes, 1 cycle). The number of cycles of denaturation followed by annealing and extension is 35 cycles. After reverse transcription and amplification, 10 μL of PCR products was analyzed on a 1.5% agarose gel stained with ethidium bromide in 1× Tris Borate EDTA buffer (TBE) pH 8.3–8.5. The electrophoretic picture was visualized and analyzed by gel documentation system (GelDocu Advanced), and values were normalized to the quantity of β-actin. The primer pair sequences p53 were 5'd CAGCGTGATGATGGTAAGGA 3' (forward) and 5'd GCGTTGCTCTGATGGTGA 3' (reverse) according to Asiri [28].

2.9. Histopathological Investigations. After sacrifice and dissection, the liver from each rat was rapidly excised and then perfused in saline solution. Pieces from the liver of each rat (3 mm^3) were taken and fixed in 10% neutral buffered formalin for twenty-four hours. The fixed livers were sent to Pathology Department, Faculty of Veterinary Medicine, Beni Suef University, for further processing, blocking in wax, sectioning, and staining with haematoxylin and eosin (H&E) [29]. The stained liver sections were examined for detection of histopathological score. In three randomly selected fields of each section (×100), lesions or injuries were graded as absent (0), mild (I), moderate (II), and severe (III) for changes, 0%, less than 30%, 30-50%, and more than 50%, respectively [30]. The graded lesions included hydropic and vacuolar degeneration, inflammatory cell infiltration, necrosis, apoptosis, Kupffer cell proliferation, fatty changes and steatosis, congested blood vessels and sinusoids, and hyperplasia of epithelial lining of the bile duct.

2.10. Immunohistochemical Detection of Caspase-3. The paraffin-embedded liver samples were transferred to the Department of Pathology, National Cancer Institute, for sectioning into 5 μm thick sections that were mounted on positive-charged slides (Fisher Scientific, Pittsburgh, PA). Caspase-3 reactivity was processed according to the methods of Galaly et al. [31] and Ahmed and Ahmed [32]. In brief, after antigen retrieval, diluted primary antibody for caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated with liver sections for 1 hour. Diluted biotinylated secondary antibody (DakoCytomation Kit) was added, and incubation was done for 15 minutes at 37°C. Thereafter, horseradish peroxidase conjugated with streptavidin (DakoCytomation Kit) was applied for further 15 minutes incubation. Bound antibody complex was visualized by the reaction of 3,3′-diaminobenzidine (DAB) substrate and counter staining with haematoxylin. All liver sections were incubated under the same conditions with the same dilutions of antibodies and at the same period, so the immunostaining was comparable among the different study groups. For each preparation, a negative control was performed (a slide without primary antibody). The sections were

visualized under a light microscope, and the extent of cell immunopositivity was assessed. Images of sections of the liver were captured using a digital camera (Leica, DM2500M Leica, Wetzlar, Germany). Examination and analysis of labeling were performed using free software version ImageJ (1.51d) [33]. The ImageJ software was used to measure the integrated intensities (in pixels) of positive reaction of caspase-3.

2.11. Statistical Analysis. Results were represented in tables and graphs as the mean ± standard error (SE). The data were analyzed by LSD analysis to discern the main effects and to compare various groups with each other using PC-STAT, University of Georgia (USA) [34]. Values of $p > 0.05$ were considered statistically nonsignificantly different, while values of $p < 0.05$ were significantly and highly significantly different.

3. Results

3.1. Effect on Serum Parameters Related to Liver Function. The daily intraperitoneal injection of diclofenac for 4 weeks induced a significant elevation ($p < 0.05$) in serum ALT, AST, LDH, ALP, and GGT activities as well as serum total bilirubin level. The cotreatment of diclofenac-injected animals with naringin and/or hesperidin induced a decrease in these elevated levels. While the cotreatment of diclofenac-injected rats with naringin induced a significant decrease ($p < 0.05$) in the elevated serum AST, LDH, ALP, GGT, and total bilirubin levels, it produced a nonsignificant effect ($p > 0.05$) on the ALT activity. The cotreatment of diclofenac-injected animals with hesperidin induced a significant effect ($p < 0.05$) on the elevated ALT, AST, LDH, GGT, and total bilirubin levels while it failed to produce a significant effect in serum ALP activity ($p < 0.05$). The cotreatment with naringin and hesperidin in combination successfully caused a significant decrease ($p < 0.05$) in all elevated parameters related to liver function including ALT, AST, LDH, ALP, GGT, and total bilirubin levels. Moreover, the treatment with the combination of naringin and hesperidin appeared to be the most effective in improving the elevated AST, ALP, and GGT activities (Table 1). Serum albumin level, on the other hand, exhibited a nonsignificant change ($p > 0.05$) in all groups.

3.2. Effect on Liver Oxidative Stress and Antioxidant Defense System. The daily intraperitoneal injection of diclofenac for 4 weeks induced a significant elevation ($p < 0.05$) in liver LPO and a significant decrease ($p < 0.05$) in liver GSH content and GPx and SOD activities. The treatment of diclofenac-injected animals with naringin and/or hesperidin induced a significant decrease ($p < 0.05$) in liver LPO. Naringin and its combination with hesperidin were more potent than hesperidin alone in decreasing the elevated liver LPO product. While the treatment with naringin and/or hesperidin produced a significant increase ($p < 0.05$) in liver GPx and SOD activities, only naringin and hesperidin in combination induced a significant increase ($p < 0.05$) in liver GSH content (Table 2).

3.3. Effect on Liver p53 mRNA Expression. As indicated in Figure 2, the proapoptotic protein p53 mRNA expression significantly increased ($p < 0.05$) in the liver of diclofenac-injected rats. On the other hand, the treatment with naringin, hesperidin, and their combination produced a significant ($p < 0.05$) decrease in p53 mRNA expression in comparison with diclofenac-injected control. There is no significant difference between the three treatments when compared with each other.

3.4. Effect on Serum TNF-α, IL-17, and IL-4 Levels. In Table 3, the daily intraperitoneal injection of diclofenac for 4 weeks induced a significant increase ($p < 0.05$) in serum TNF-α and IL-17 levels and a significant decrease in serum IL-4 level. The cotreatment of diclofenac-injected rats with naringin and/or hesperidin induced a significant improvement ($p < 0.05$) of altered serum TNF-α, IL-17, and IL-4 levels. The treatment with the combination of both flavonoids was the most potent in increasing the lowered IL-4 level; the effect of the combination was more significantly potent ($p < 0.05$) as compared with the effect of either naringin or hesperidin.

3.5. Liver Histopathological Findings. Figure 3 depicts the effect of naringin and/or hesperidin on liver histological changes of diclofenac-injected rats. Figure 3(a) shows the liver with normal histological structure of hepatic lobule. On the other hand, the liver of diclofenac-injected rats exhibited marked deleterious histological changes like hydropic degeneration of hepatocytes, apoptotic cells, and portal infiltration with mononuclear cells (Figure 3(b)) in addition to cytoplasmic vacuolization of hepatocytes and focal hepatic necrosis associated with focal inflammatory cells infiltration (Figure 3(c)). These alterations were amended to some extent in the diclofenac-injected rats treated with naringin showing only fatty change of sporadic hepatocytes (Figure 3(d)). The treatment of diclofenac-injected rats with hesperidin produced an improvement of the liver histological changes as compared with the diclofenac-injected control but still exhibited fatty change of sporadic hepatocytes, slight activation of Kupffer cells, and presence of few apoptotic cells (Figure 3(e)). Similarly, the treatment of diclofenac-injected rats with a combination of naringin and hesperidin improved the liver histological changes as compared with diclofenac-injected control. However, the liver of diclofenac-injected rats with a combination of both flavonoids exhibited slight Kupffer cell proliferation and slight cytoplasmic vacuolization (Figure 3(f)). It seemed that the combinatory effect of naringin and hesperidin was the most potent in improving the liver histological architecture.

Histopathological change scores of all groups are recorded in Table 4. The liver section of normal control showed zero score for all histological lesions. The liver diclofenac-injected rats exhibited various grades of histological lesion scores ranging from grade III to grade 0. The administration of hesperidin and naringin to diclofenac-injected rats resulted in remarkable improvements in histological lesions including hydropic and vacuolar degeneration, inflammatory cells infiltration, necrosis, apoptosis, Kupffer cell proliferation, fatty changes and steatosis, congested

Table 1: Effect of naringin, hesperidin, and their combination on serum biochemical parameters related to liver function in diclofenac-administered rats.

Parameter	Normal control	Diclofenac	Diclofenac+naringin	Diclofenac+hesperidin	Diclofenac+naringin+hesperidin
ALT (U/L)	48.0 ± 1.34^{c}	62.00 ± 0.89^{a}	58.83 ± 0.94^{ab}	51.00 ± 0.63^{c}	55.50 ± 2.4^{b}
AST (U/L)	138.20 ± 5.11^{b}	244.00 ± 30.44^{a}	163.83 ± 16.31^{b}	141.00 ± 6.76^{b}	136.67 ± 8.46^{b}
LDH (U/L)	366.00 ± 51.42^{c}	786.00 ± 18.33^{a}	615.66 ± 65.19^{b}	580.66 ± 14.53^{b}	669.00 ± 1.79^{b}
ALP (U/L)	193.66 ± 18.92^{c}	329.33 ± 33.80^{a}	259.83 ± 9.10^{b}	270.17 ± 21.05^{ab}	226.33 ± 10.83^{bc}
GGT (U/L)	5.30 ± 1.10^{b}	17.0 ± 0.80^{a}	5.00 ± 1.00^{b}	7.00 ± 1.00^{b}	4.50 ± 0.80^{b}
Albumin (g/dL)	3.0 ± 0.04^{a}	3.03 ± 0.02^{a}	2.95 ± 0.09^{a}	3.02 ± 0.05^{a}	2.90 ± 0.04^{a}
Bilirubin (mg/dL)	0.30 ± 0.04^{b}	0.50 ± 0.04^{a}	0.33 ± 0.02^{b}	0.30 ± 0.04^{b}	0.35 ± 0.05^{b}

Data are expressed as the mean ± SE. Number of animals in each group is six. Means, which share different superscript symbols in the same row, are significantly different at $p < 0.05$.

Table 2: Effect of naringin, hesperidin, and their combination on liver LPO and antioxidant defense markers in diclofenac-administered rats.

Parameter	Normal control	Diclofenac	Diclofenac+naringin	Diclofenac+hesperidin	Diclofenac+naringin +hesperidin
LPO (MDA nmol/g tissue)	181.75 ± 5.92^{c}	386.90 ± 14.08^{a}	174.63 ± 14.83^{c}	265.03 ± 29.66^{b}	194.35 ± 19.22^{c}
GSH (nmol/100 mg tissue)	30.50 ± 2.47^{a}	19.73 ± 1.0^{c}	20.80 ± 0.79^{bc}	22.68 ± 0.92^{bc}	23.73 ± 0.77^{b}
GPx (mU/100 mg tissue)	175.00 ± 4.08^{a}	46.33 ± 8.34^{c}	151.17 ± 13.19^{ab}	140.00 ± 11.90^{b}	135.00 ± 11.47^{b}
SOD (U/g tissue)	18.43 ± 0.37^{a}	12.28 ± 0.31^{c}	15.98 ± 0.75^{b}	15.65 ± 0.54^{b}	16.56 ± 0.68^{b}

Data are expressed as the mean ± SE. Number of animals in each group is six. Means, which share different superscript symbols in the same row, are significantly different at $p < 0.05$.

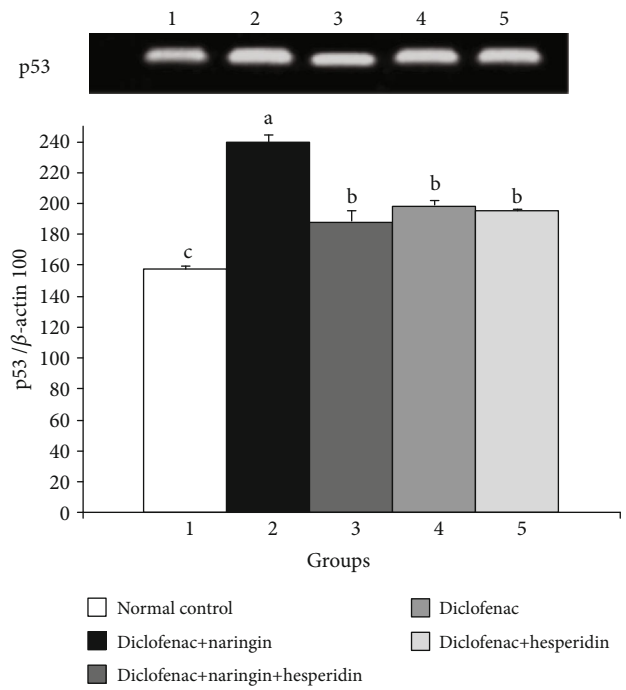

Figure 2: Effect of naringin, hesperidin, and their combination on p53 mRNA expression in the liver of diclofenac-administered rats. Means, which share different symbols, are significantly different at $p < 0.05$.

blood vessels and sinusoids, and hyperplasia of epithelial lining of the bile duct; thereby, the liver exhibited lower grades of histopathological change scores in the diclofenac-injected groups treated with naringin and/or hesperidin in comparison with diclofenac-injected control rats. The combinatory effect of naringin and hesperidin was the most potent in decreasing the histopathological lesion scores.

3.6. Immunohistochemical Assay. The immunohistochemically stained liver sections depicted in Figure 4 and the results of image analysis are demonstrated in Figure 5.

The photomicrographs in Figure 4 and data in Figure 5 revealed that caspase-3 expression was significantly increased in diclofenac-injected rats. The treatment of diclofenac-injected rats with naringin, hesperidin, and their combination resulted in a significant decrease in the elevated caspase-3 expression in the liver. The combinatory effect of naringin and hesperidin was the most potent in decreasing the elevated caspase-3 content. The treatment with the combination normalized the liver caspase-3 expression.

4. Discussion

Diclofenac sodium is a well-known representative of drugs (NSAIDs) and it is widely used to control pain and inflammation of rheumatic and nonrheumatic origin [35]. It had been linked to serious side effects including gastric ulcers and liver, renal, and heart injuries [36–38]. Citrus fruit is one of the mostly consumed fruits worldwide, and numerous studies had revealed its remarkable health-promoting activities, such as antioxidant, anticancer, anti-inflammatory, and cardiovascular protection activities [39]. Such activities largely depend on the diverse chemical constituents of citrus fruits, including vitamins, minerals, terpenoids, and flavonoids which had attracted growing interest due to their distinct beneficial effects on human health. The daily ingested fruits and vegetables are rich sources of both nutrients such as carbohydrate, vitamins, and minerals, and nonnutritive

Table 3: The effect of naringin, hesperidin, and their combination on serum TNF-α, IL-17, and IL-4 levels in diclofenac-administered rats.

Serum parameter	Normal control	Diclofenac	Diclofenac+naringin	Diclofenac+hesperidin	Diclofenac+naringin+hesperidin
TNF-α (pg/mL)	33.07 ± 0.51^{c}	154.12 ± 11.63^{a}	102.95 ± 3.44^{b}	88.08 ± 2.37^{b}	91.50 ± 4.67^{b}
IL-17 (pg/mL)	40.05 ± 3.16^{c}	151.48 ± 3.60^{a}	83.30 ± 1.27^{b}	77.60 ± 4.26^{b}	77.60 ± 3.99^{b}
IL-4 (pg/mL)	130.00 ± 3.77^{a}	70.37 ± 3.12^{d}	100.53 ± 0.60^{c}	106.43 ± 2.65^{c}	116.88 ± 2.16^{b}

Data are expressed as the mean ± SE. Number of animals in each group is six. Means, which share different superscript symbols in the same row, are significantly different at $p < 0.05$.

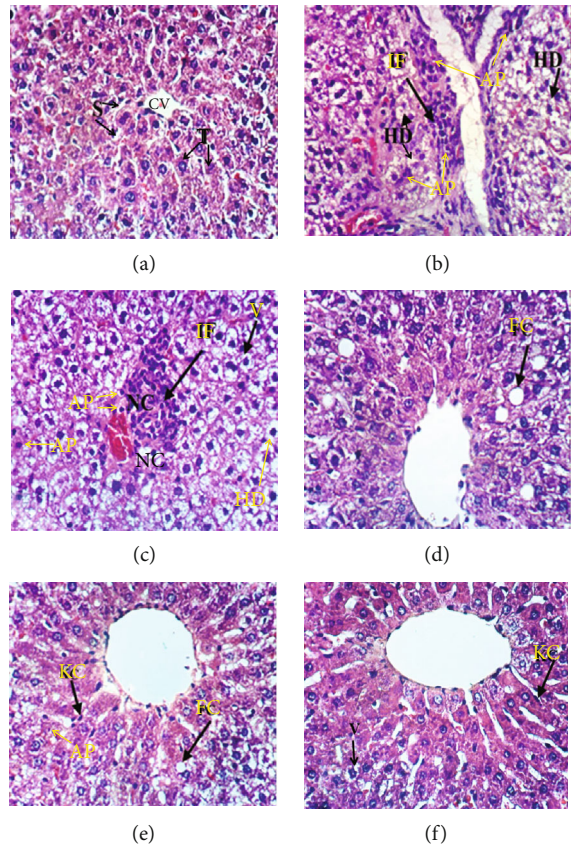

Figure 3: Photomicrographs of liver sections of the normal (a), diclofenac-injected group (b, c), and diclofenac-injected groups treated with naringin (d), hesperidin (e), and their combination (f). T: trabeculae; S: sinusoids; CV: central vein; IF: inflammatory cell infiltration; HD: hydropic degeneration; AP: apoptosis; NC: necrosis; FC: fatty changes; V: vacuolization; KC: Kupffer cells (H&E; ×400).

constituents, particularly polyphenols including flavonoids and phenolic acids [40]. Flavonoids especially flavanones, which contain hesperidin and naringin, are well known for their antioxidant properties and as health-promoting molecules with multifunctional biological activities; they had been shown to attenuate inflammation, to quench active oxygen species [41] and to prevent liver, kidney, and heart toxicities as well as several forms of cancer [42–44].

The present study was conducted to evaluate the preventive effects of the naringin, hesperidin, and their combination against diclofenac-induced liver injury in rats and to elucidate the mode of actions by assessing the effects on oxidative stress, antioxidant defense system, inflammation, and apoptosis.

The present study showed that diclofenac administration profoundly produced a significant increase in serum enzyme (ALT, AST, LDH, ALP, and GGT) activities, bilirubin level, and proinflammatory cytokine (TNF-α and IL-17) levels as well as liver LPO, caspase-3, and p53 mRNA expression but it induced a significant decrease in serum anti-inflammatory cytokine (IL-4) level and liver GSH content as well as SOD and GPx activities. These alterations reflect hepatocyte damage and necrosis, biliary liver dysfunction, increase in oxidative stress, suppression of antioxidant defense system, and augmentation of inflammation and apoptosis. The present study is in agreement with many previous publications [45–51]. The present biochemical and pathophysiological alterations in diclofenac-injected rats are associated with liver histological alterations including hydropic and vacuolar degeneration of hepatocytes, inflammatory cells' infiltration, focal hepatic necrosis, apoptosis, congestion of blood vessels and sinusoids, fatty changes, Kupffer cells' proliferation, and

Table 4: Histopathological scores of liver lesions in the normal control, diclofenac-injected group, and diclofenac-injected groups treated with naringin, hesperidin, and their combination.

Histopathological changes	Score	Normal control	Diclofenac	Diclofenac +naringin	Diclofenac +hesperidin	Diclofenac+naringin +hesperidin
Hydropic and vacuolar degeneration	0	6 (100%)	1 (16.7%)	3 (50.0%)	2 (33.3%)	4 (66.7%)
	I	—	1 (16.7%)	2 (33.3%)	2 (33.3%)	2 (33.3%)
	II	—	2 (33.3%)	1 (16.7%)	2 (33.3%)	—
	III	—	2 (33.3%)	—	—	—
Inflammation (inflammatory cell infiltration)	0	6 (100%)	—	3 (50.0%)	3 (50.0%)	5 (83.3%)
	I	—	1 (16.7%)	1 (16.7%)	2 (33.3%)	1 (16.7%)
	II	—	2 (33.3%)	2 (33.3%)	1 (16.7%)	—
	III	—	3 (50.0%)	—	—	—
Necrosis	0	6 (100%)	—	1 (16.7%)	2 (33.3%)	4 (66.7%)
	I	—	2 (33.3%)	2 (33.3%)	3 (50.0%)	2 (33.3%)
	II	—	2 (33.3%)	3 (50.0%)	1 (16.7%)	—
	III	—	2 (33.3%)	—	—	—
Apoptosis	0	6 (100%)	—	3 (50.0%)	3 (50.0%)	4 (66.7%)
	I	—	1 (16.7%)	3 (50.0%)	2 (33.3%)	1 (16.7%)
	II	—	2 (33.3%)	—	1 (16.7%)-	1 (16.7%)
	III	—	3 (50.0%)	—	—	—
Kupffer cell proliferation	0	6 (100%)	—	3 (50.0%)	3 (50.0%)	4 (66.7%)
	I	—	1 (16.7%)	1 (16.7%)	2 (33.3%)	1 (16.7%)
	II	—	3 (50.0%)	2 (33.3%)	1 (16.7%)	1 (16.7%)
	III	—	2 (33.3%)	—	—	—
Fatty changes and steatosis	0	6 (100%)	4 (66.7%)	4 (66.7%)	4 (66.7%)	6 (100%)
	I	—	1 (16.7%)	1 (16.7%)	—	—
	II	—	1 (16.7%)	1 (16.7%)	2 (33.3%)	—
	III	—	—	—	—	—
Congested blood vessels and sinusoids	0	6 (100%)	—	3 (50.0%)	4 (66.7%)	4 (66.7%)
	I	—	2 (33.3%)	—	1 (16.7%)	2 (33.3%)
	II	—	3 (50.0%)	2 (33.3%)	1 (16.7%)	—
	III	—	1 (16.7%)	1 (16.7%)	—	—
Hyperplasia of epithelial lining of the bile duct	0	6 (100%)	4 (66.7%)	5 (83.3%)	6 (100%)	6 (100%)
	I	—	—	—	—	—
	II	—	—	1 (16.7%)	—	—
	III	—	2 (33.3%)	—	—	—

0: absence of lesion; I: mild; II: moderate; III: severe. Number of animals in each group is 6. Numbers 1, 2, 3, 4, 5, and 6 represent the number of animals for each grade. The % in parentheses is the percent of animals in each grade.

hyperplasia of the epithelial lining the bile canaliculi. The histological scores of these lesions were much lower in diclofenac-injected rats treated with naringin and/or hesperidin than those in diclofenac-injected control; the effect of combination was the most potent in decreasing the lesions' scores. The previous histological lesions in diclofenac-injected rats may be attributed to the increase in the oxidative stress and excessive release of free radicals and ROS. This exacerbated production of ROS administration activates apoptosis through intrinsic pathway, aggravates cell necrosis through peroxidation of membrane lipids, and stimulates DNA breakages and mutations by oxidative damage [43, 52] (Figure 6). In addition to its necrotic effects, the elevation of TNF-α may augment hepatocyte apoptosis through linking to TNF receptor (TNFR) and death receptors leading to activation of extrinsic pathway of apoptosis [43, 52, 53] (Figure 6). In turn, the improvement effects of naringin and hesperidin on liver histological lesions as well as ameliorations of serum biochemical parameters may be due to the suppressive effects of these two flavonoids on oxidative stress and enhancement of the antioxidant defense system in addition to the suppressive effects on inflammatory mediators such as TNF-α and IL-17 (Figure 6). In agreement with our results, GökÇimen et al. [54], Tan et al. [55], and El-Kordy and Makhlouf [56] revealed hepatocyte necrosis and interstitial and periportal inflammation, which indicate acute hepatitis in diclofenac-administered animals. Also, Alqasoumi [49] reported that the diclofenac-treated rats revealed early bridging necrosis and lymphocytic infiltrate.

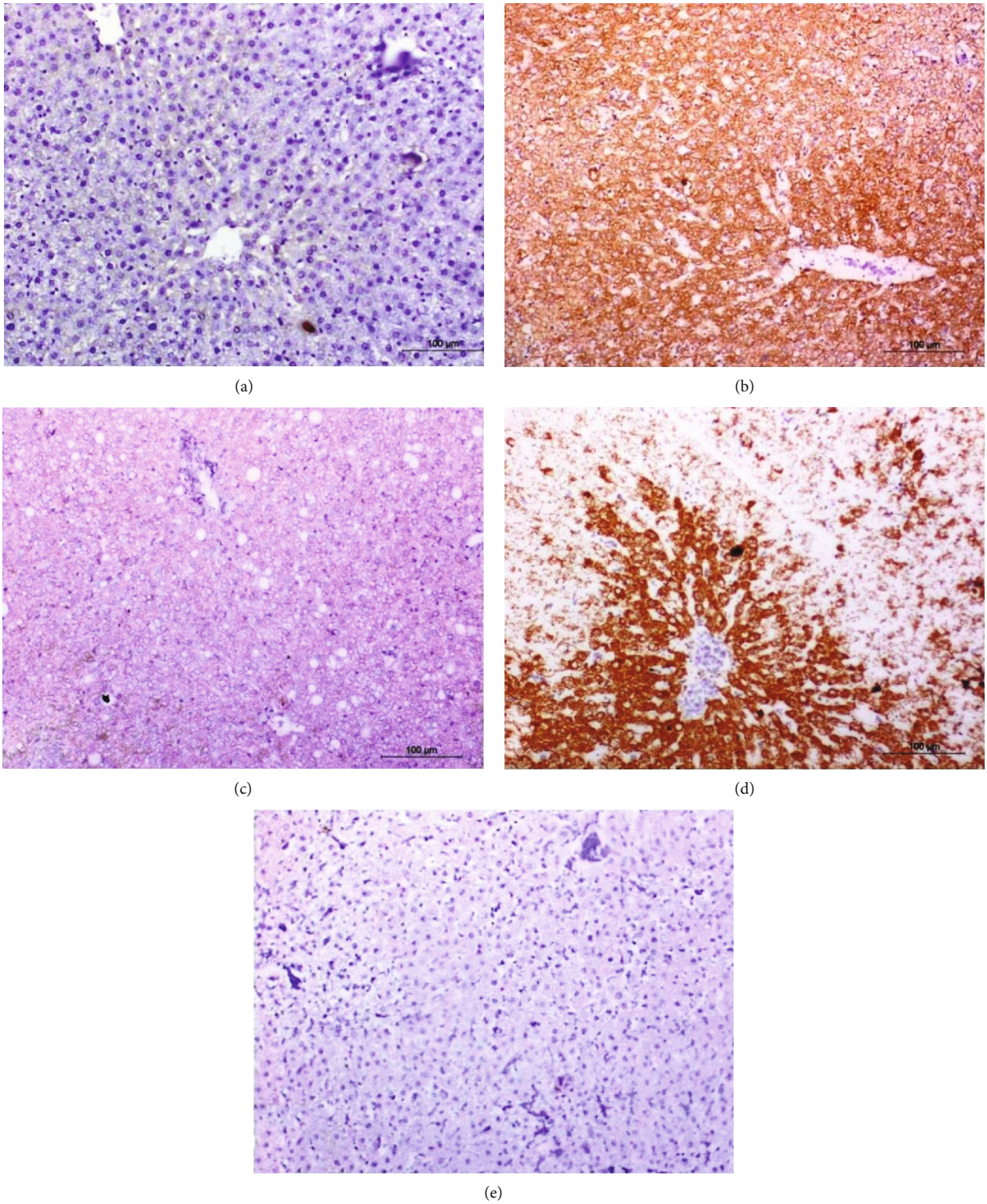

Figure 4: Immunohistochemical stained liver sections showing caspase-3 expression in the liver of the normal (a), diclofenac-injected group (b), and diclofenac-injected groups treated with naringin (c), hesperidin (d), and their combination (e) (×200).

In the current study, the serum albumin levels were not significantly altered in all groups. The serum albumin reflects the synthetic capacity of liver cells as albumin is formed by liver cells (hepatocytes). The nonsignificant change in serum albumin level in the diclofenac group in spite of the significant increase in serum enzymes, mostly or partially leaked from the liver to plasma, led us to suggest that the stressed and survived hepatocytes may have increased capability to synthesize more albumin as a compensatory mechanism.

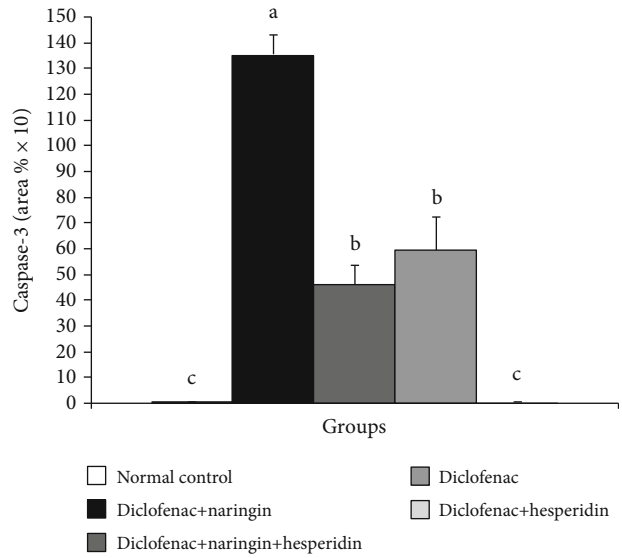

Figure 5: Immunohistochemical staining intensity of caspase-3 in the liver of the normal control, diclofenac control group, and diclofenac groups treated with naringin and/or hesperidin. Means, which share different symbols, are significantly different at $p < 0.05$.

In the present study, the inflammatory cell infiltration in the liver of diclofenac-injected rats was associated with an elevation in serum levels of inflammatory cytokines (TNF-α and IL-17) and a decrease in serum level of anti-inflammatory cytokine, IL-4. These findings are in agreement with many previous publications. Hussien et al. [50] reported that oral administration of diclofenac sodium led to a significant increase in serum TNF-α level. Deng et al. [57], Schmidt-Weber et al. [58], and Ahmed et al. [59] showed that overproduction of TNF-α and IL-17 had been implicated in the pathogenesis of several inflammatory conditions by inducing and maintaining inflammation. Moreover, IL-17 stimulates inflammatory responses *via* NF-κB, which plays the important role as the key regulator of transcriptional responses to TNF-α [60, 61]. On the other hand, IL-4 is the complicated cytokine whose role varies between anti- and proinflammation in autoimmunity. IL-4 had an immunoregulatory role in immune-mediated drug-induced liver injury; this represents a unique condition where hepatitis can be initiated by drug haptens or self-proteins [62]. In the current study, the diclofenac has a suppressive effect on IL-4 production in Wistar rats.

The elevation in the TNF-α level in diclofenac-injected rats may activate extrinsic apoptotic pathway through TNF receptor (TNFR) and death receptor. On the other hand, the increased mRNA expression of p53 in association with the increase in oxidative stress may reflect the stimulation of intrinsic pathway. Furthermore, caspase-3, which serves as a convergence point for both intrinsic and extrinsic pathways, is significantly elevated in diclofenac-injected rats. Thus, it can be suggested that diclofenac may activate apoptosis in Wistar rats *via* stimulation of both apoptotic pathways (Figure 6).

The oral administration of naringin and/or hesperidin to diclofenac-injected rats produced a marked decrease in the elevated serum ALT, AST, LDH, ALP, and GGT activities and serum total bilirubin level reflecting improvement of liver function and integrity; the combinatory effect seemed to be the most potent on AST, ALP, and GGT activities. These improvements in biochemical parameters related to liver function were associated with alleviations in liver histological architecture; the treatment with the combination was the most effective. Such decrease in the elevated serum ALT, AST, LDH, ALP, and GGT activities and serum total bilirubin level as well as alleviated liver histological architecture and integrity were accompanied with a decrease in the elevated liver LPO, serum Th1 cytokine (TNF-α), serum Th17 cytokine (IL-17), and liver proapoptotic mediators (p53 and caspase-3) expression. On the other hand, serum Th2 cytokine (IL-4) level, liver GSH content, and antioxidant enzyme (GPx and SOD) activities were enhanced by treatment of diclofenac-injected rats with naringin and/or hesperidin. These results go parallel with Ahmed et al. [43] who found that the administration of naringin to acetaminophen-supplemented rats improved the deteriorated changes in serum ALT, AST, LDH, ALP, GGT, TNF-α, and IL-4 levels as well as liver LPO and antioxidant defense system. With regard to apoptosis, Ahmed et al. [43] reported decrease in apoptotic proteins p53 and caspase-3 as result of treatment of acetaminophen-injected rats with naringin and naringenin. The present results are also in concurrence with Omar et al. [63] who found that hesperidin significantly reduced cisplatin-induced elevations in serum ALT and AST activities as well as liver LPO, NO content, and NF-κB. The NF-κB has the important role as the key regulator of transcriptional responses to TNF-α expression (Figure 6). In the same regard, Çetin et al. [64] stated that hesperidin caused an increase in liver GSH, catalase, and SOD levels and induced a decrease in LPO in the liver of CCl4-injected rats. Thus, based on the results of the present study and of past publications, it can be suggested that the ameliorative effects of naringin and hesperidin, singly or in combination, on liver function and structural integrity may be attributed to their suppressive effects on the inflammation, oxidative stress, and apoptosis and to their stimulatory effects on anti-inflammatory effects on antioxidant defense system (Figure 6). This suggestion was supported by previous publications which stated the antioxidant and anti-inflammatory effects of naringin and hesperidin in different diseased conditions and drug toxicities [65–75].

Naringin and hesperidin may decrease apoptosis in diclofenac-injected rats through extrinsic pathway and/or intrinsic pathway. In the present study, naringin and hesperidin treatment of diclofenac-injected rats resulted in a decrease in TNF-α which in turn activates extrinsic apoptotic pathway through TNFR and death receptor. Naringin and hesperidin treatment of diclofenac-injected rats also caused a decrease in mRNA expression of p53 which is mediator in intrinsic pathway. In addition, naringin and hesperidin induced a decrease in liver caspase-3, which is a convergence point for both intrinsic and extrinsic pathways (Figure 6).

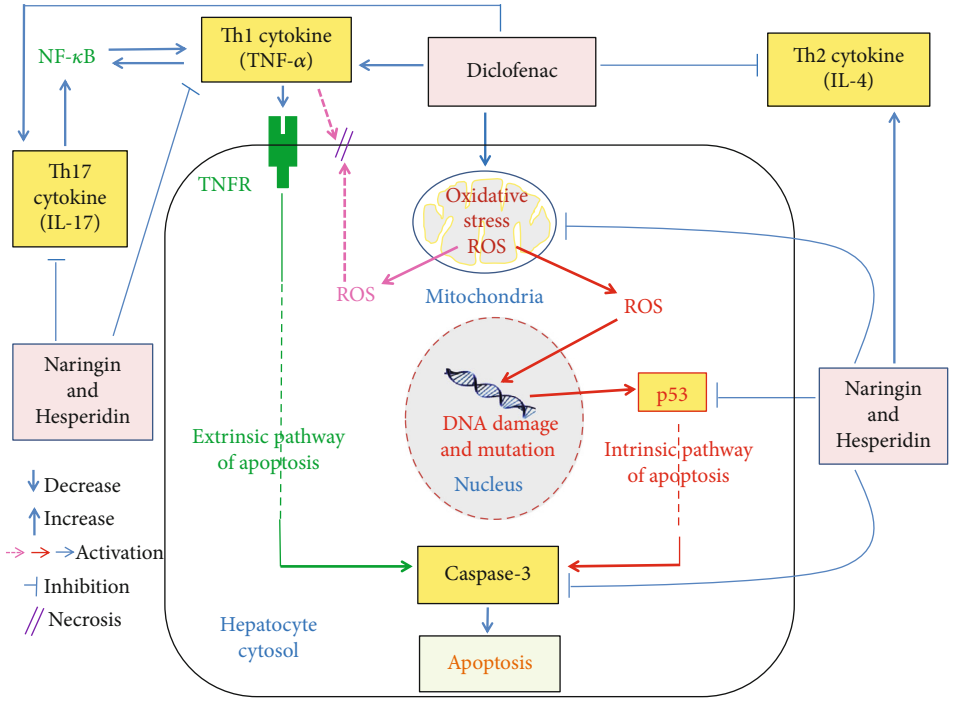

Figure 6: Schematic diagram showing the effects of naringin and hesperidin to suppress oxidative stress, inflammation, and apoptosis in diclofenac-injected rats.

Overall, it can be concluded that multiple long-term diclofenac administration induces liver toxicity. Administration of hesperidin, naringin, and their combination, which was the most potent, potentially counteracts diclofenac-induced liver injury and toxicity *via* enhancement of antioxidant defense system and anti-inflammatory effect as well as suppression of oxidative stress and apoptosis. However, further clinical investigations are required to assess the efficacy and safety of hesperidin and naringin in human beings.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that no conflict of interest exists in the submission of this manuscript.

Authors' Contributions

The manuscript is approved by all authors for publication.

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Research Article

Mining Natural Products with Anticancer Biological Activity through a Systems Biology Approach

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Natural products, like turmeric, are considered powerful antioxidants which exhibit tumor-inhibiting activity and chemoradioprotective properties. Nowadays, there is a great demand for developing novel, affordable, efficacious, and effective anticancer drugs from natural resources. In the present study, we have employed a stringent in silico methodology to mine and finally propose a number of natural products, retrieved from the biomedical literature. Our main target was the systematic search of anticancer products as anticancer agents compatible to the human organism for future use. In this case and due to the great plethora of such products, we have followed stringent bioinformatics methodologies. Our results taken together suggest that natural products of a great diverse may exert cytotoxic effects in a maximum of the studied cancer cell lines. These natural compounds and active ingredients could possibly be combined to exert potential chemopreventive effects. Furthermore, in order to substantiate our findings and their application potency at a systems biology level, we have developed a representative, user-friendly, publicly accessible biodatabase, NaturaProDB, containing the retrieved natural resources, their active ingredients/fractional mixtures, the types of cancers that they affect, and the corresponding experimentally verified target genes.

1. Introduction

Cancer represents one of the leading causes of death globally. Despite the availability of anticancer therapeutics, cancer incidence is increasing gradually. According to the World Cancer Report of the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO), the global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018 (GLOBOCAN 2018 database: https://gco.iarc.fr/). Nowadays, research efforts are directed towards the discovery of naturally derived chemical compounds with anticancer potential [1]. A great number of highly potent bioactive compounds, derived from plants, have been found to possess anticancer properties, and this number is increasing exponentially [2, 3]. For example, there are natural products with antioxidant and anti-inflammatory capacities [4, 5], thereby preventing oxidative stress and inflammation, which can cause damage to DNA, eventually leading to genomic instability and eventually to carcinogenesis [6, 7].)

Natural products derived from plants and natural product-based anticancer drugs have been associated with

reduced cancer mortality and risk. Fruits and vegetables contain vitamins, minerals, folate, plant sterols, carotenoids, and various phytochemicals such as flavonoid and polyphenols, which are suggested to have cancer chemopreventive potential [8–11].

Another category of plants that possess antitumor, antioxidant, and anti-inflammatory properties is spices and herbs [12]. They contain tannins, alkaloids, phenolic diterpenes, vitamins, flavonoids, and polyphenols. Spices and herbs such as curcumin, clove, rosemary, sage, oregano, and cinnamon are excellent sources of antioxidants due to the high content of phenolic compounds [8, 13, 14]. The antioxidants in edible and medicinal plant extracts have been shown to counteract ROS-mediated damage in diverse human cancers [15].

Furthermore, nuts are enriched in nutrients of high biological value. Nuts contain high amounts of vegetable protein [16, 17] and fat, mostly unsaturated fatty acids [17, 18]. They are also rich in a variety of other nutrients, as well as dietary fiber [19], vitamins (e.g., folic acid, niacin, tocopherols, and vitamin B6), minerals (e.g., calcium, magnesium, and potassium), and many other bioactive constituents such as phytosterols [17, 20] and phenolic compounds [17, 21]. Among the dietary plants, nuts, like peanuts and walnuts, contain the highest total content of antioxidants [21]. Nuts are associated with cancer prevention and they exert their potential chemopreventive effect through documented anti-antioxidant, anti-inflammatory, proapoptotic, antiproliferation, and antimetastatic activities [22].

Notably, natural compounds with cancer chemopreventive potential have also been derived from nonplant resources. For example, many cyclic peptides and analogues derived from marine organisms have been shown to possess anticancer, antimicrobial, anti-inflammatory, antiproliferative, and antihypertensive properties [23–25]. In addition, lactoferrin, a multifunctional protein found in bovine and camel milk, possesses anticancer, antimicrobial, and immune system boosting effects [26–29].

In the present work, taking into account the numerous studies on the biological (chemopreventive) activities of natural products, we have employed an in silico methodology to determine an effective combination of such products, retrieved from the biomedical literature, affecting diverse types of cancers and cancer-related pathways. Furthermore, we have developed a representative database, NaturaProDB, containing the retrieved data, which is maintained by the National Technical University of Athens, Greece.

2. Materials and Methods

2.1. Data Acquisition and Compilation. To obtain a broad spectrum of naturally occurring products with demonstrated anticancer activity, the bibliographic database MEDLINE/-PubMed (https://www.ncbi.nlm.nih.gov/pubmed) was searched manually for full-text articles (from January 2003 up to 20 October 2020) using relevant keywords, including "natural products" or "naturally occurring compounds" or "natural ingredients" or "natural agents" or "natural substances" or "natural extracts" or "superfoods" and "cancer" or "malignancy." The criteria applied to assess the anticancer potential of the natural products/fractional extracts were based on their ability to (i) suppress the growth of cancer cell lines and (ii) alter the expression of target genes, either oncogenes or tumor suppressor genes. Genus and species names (binomial nomenclature) were assigned to the source organism of the natural products according to the NCBI Taxonomy database [30]. The different types of cancers were classified according to NCBI's MeSH [31]. The official HUGO Gene Nomenclature Committee (HGNC) [32] symbols were used for the human genes.

The adverse effects of the natural compounds/extracts on human health were also assessed via extensive literature mining using the keywords "adverse effect" or "toxic∗" or "side effect" and "natural product." The articles retrieved from MEDLINE/PubMed were carefully examined for any association between the active compounds/extracts and toxicity in humans.

2.2. Pathway Enrichment Analysis. The retrieved target genes were provided as input to WebGestalt (WEB-based GEne SeT AnaLysis Toolkit) [33], an online tool for functional annotation enrichment analysis, to identify statistically significant overrepresented cancer-associated WikiPathways. The threshold for the FDR-adjusted p value was set at 10^{-3}, and hypergeometric distribution was used.

2.3. Functional Association Network. The associations among the genes/proteins under study were investigated and visualized with the usage of STRING (Search Tool for Retrieval of Interacting Genes/Proteins) v11.0 [34, 35], a database of either known or predicted, direct or indirect, association data among genes or proteins. These data are derived from diverse resources, including text mining of the scientific literature, biological and biochemical pathways, gene coexpression, high-throughput experiments, and gene fusion. The confidence score for displaying interactions was set to 0.9.

The software platform Cytoscape (http://www.cytoscape.org/) [36] was used for network analysis, processing, and visualization.

2.4. Statistical Analyses. All statistical analyses were performed with the R package "Stats" and Microsoft Excel Macros.

To identify the minimum number of superfoods that target all cancer types, as well as the maximum number of target genes, we utilized the R package "RcppGreedySetCover" for resolving set cover problems.

2.5. Differential Gene Expression Analysis. RNA sequencing (RNA-seq) gene expression data for 27 tumor and corresponding normal tissue samples (Table S1), from the TCGA and GTEx databases, respectively, were downloaded from the GEPIA2 (Gene Expression Profiling Interactive Analysis) online web server [37] (http://gepia2.cancer-pku.cn/). The differentially expressed genes (DEGs) between tumor and normal samples were identified using one-way analysis of variance (ANOVA), by setting the cutoff value for absolute log fold change $|\log_2 FC| \geq 2$ and FDR-adjusted p value ≤ 0.05.

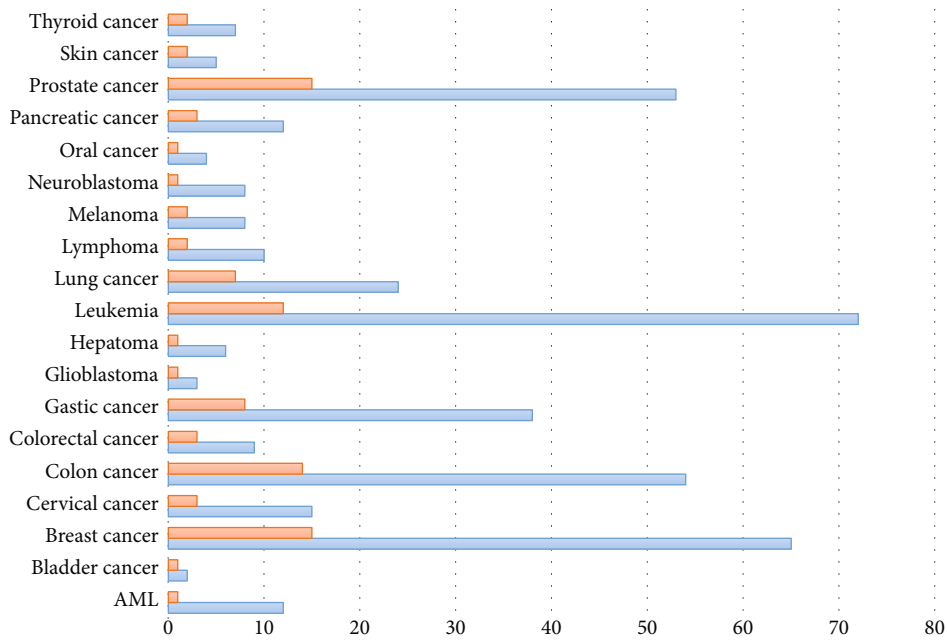


Figure 1: Bar graph depicting the distribution of natural products (orange) and genes (blue) with respect to cancer types.

2.6. Database Design

2.6.1. Database Storage. "Cloud Firestore," a NoSQL cloud database, was used to store and sync data for the client- and server-side web development. Cloud Firestore real-time data read-write feature was used for automatic data synchronization, thereby providing the user with the most updated data (https://firebase.google.com/docs/database).

2.6.2. Website Design. Google's "Firebase Hosting" was utilized to host NaturaProDB's static assets (HTML and JavaScript); the popular front-end framework VueJS was offered through officially maintained supporting packages for creating the website user interface. The generated networks were processed and analyzed by Cytoscape (http://www.cytoscape.org/); Cytoscape.js library (https://js.cytoscape.org/) was used for network implementation, visualization, and interaction in the designed user interface.

3. Results

3.1. Data Retrieval and Assembly. A total of 562 relevant articles were selected after thorough review; of those, 86 articles were included in our study according to the eligibility criteria. Data collected from those studies regarding the scientific name of the source organism or food, natural product or fractional extract mixture, target cancer type, cell lines used for assessing anticancer activity in *in vitro* experimental studies, and target gene symbol along with its expression status (up- or down-regulated) were merged and recorded in a table (Table S1). The natural products were divided into eight major groups: vegetables/fruits, herbs, spices, nuts, dairy products, cereals, marine organisms, and oil. The vast majority of natural products originated from plants; however, natural compounds were also extracted from fungi, bacteria, marine organisms, and dairy products. Collectively, 87 source organisms/foods, 19 types of cancers, 105 target genes, and 66 cell lines were retrieved from the eligible studies and the relevant data were recorded in an Excel worksheet. The distribution pattern of natural products or target genes with respect to types of cancers is shown in Figure 1. The greatest percentages of natural products and target genes are distributed within the solid tumor breast and colon cancer, as well as the blood cancer leukemia.

A total of 81 pivotal target genes were found to participate in cancer-relevant, interdependent pathways. These 81 genes and their corresponding proteins appear to be highly interconnected within the functional network shown in Figure 2. The generated network is quite dense, with an average node degree of 12.4 (Figure 2), suggesting tight associations, either physical or functional, among molecules so as to exert their antineoplastic effect. The distribution of genes across pathways is depicted in Figure 3. Among the overrepresented pathways are those associated with genomic instability, prosurvival pathways, TP53-mediated signaling, apoptosis, and cell cycle.

We found a total of 22 natural foods targeting 57 genes (Table 1), most of them plants, except the fungus hazel mushroom. The active substances/functional extracts of the corresponding natural foods, as well as the effective concentration needed for cell growth inhibition, are listed in the second and third columns of Table 1, respectively. Based on literature research, no adverse health effects of the natural compounds/functional extracts were reported for the same dose range as in the one in the third columns in Table 1. In addition, a bipartite network was generated (Figure 4) based on the identified DEGs in diverse cancer tissue samples

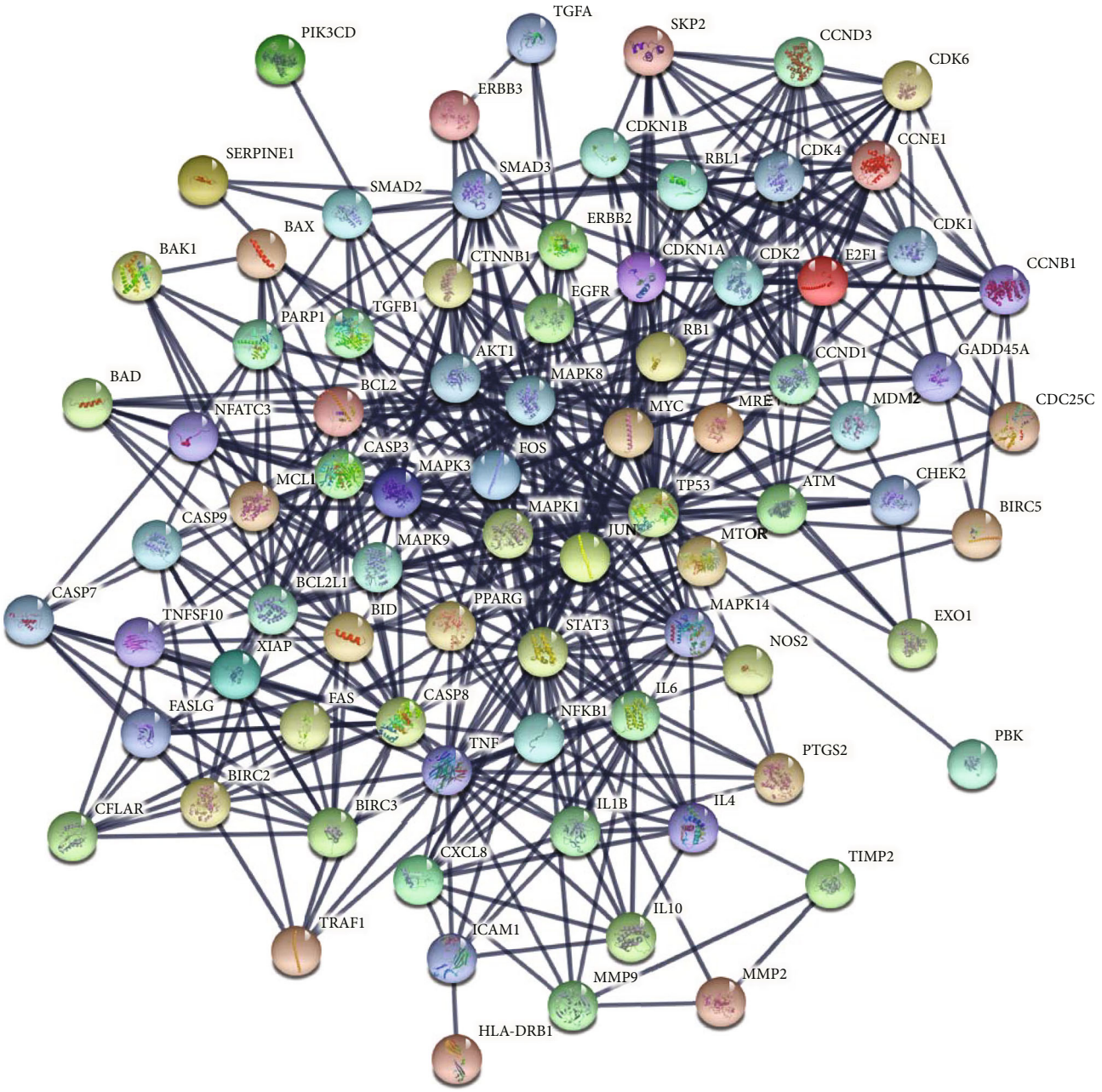


Figure 2: STRING output functional network of the 81 genes/gene products. The nodes represent molecules and the connecting lines (edges) indicate an interaction confidence score above 0.9.

(Table 2). Collectively, 45 out of the 57 target genes appear to be highly connected due to their differential coexpression in 27 types of cancers (Figure 4).

Among the targeted genes are the key proapoptotic BAX (BCL2-associated X protein) [38], the caspases CASP3/8/9, and the cardinal player TP53 which are consistently upregulated in diverse cancer cell lines following treatment with natural compounds (Table 1). Furthermore, the antiapoptotic genes BCL2 and XIAP (X-linked inhibitor of apoptosis) [39, 40] are downregulated by natural products and upregulated in several cancer tissues (Tables 1 and 2). BCL2 can also suppress apoptosis by inhibiting the activity of caspases indispensable for apoptosis, such as CASP3 [41, 42]. The DNA damage response-associated gene PARP1 (poly(ADP-ribose) polymerase 1), pharmacological inhibitors of which are used in anticancer treatment [43], and CDKN1A (cyclin dependent kinase inhibitor 1A), a universal inhibitor of CDK/cyclin complexes [44, 45], are involved in DNA damage detection and DNA damage-induced cell cycle arrest [44, 46], respectively (Table 1). In addition, several natural products can potentially exert their antineoplastic effect on the oncogene *AKT1* (*AKT serine/threonine kinase 1*) [47]. Among the genes that are targeted by the 22 superfoods is *NFKB1*, which plays a protagonistic role in inflammatory responses [48, 49] (Table 1). NFKB1 also plays a dual role in apoptosis, either as inducer or as inhibitor of apoptosis

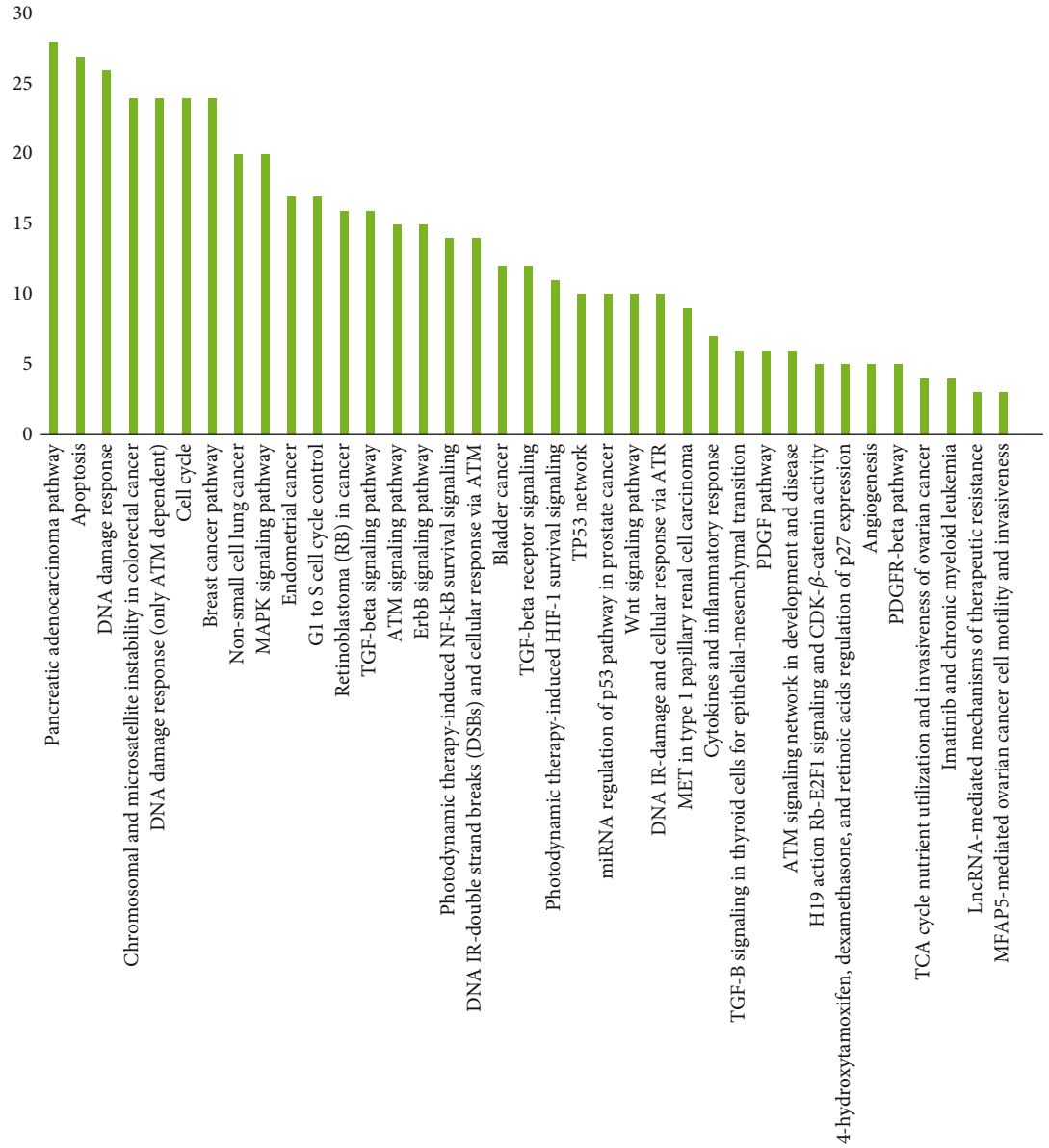


Figure 3: Bar plot depicting the overrepresented cancer-related pathways across target genes.

[50]; thus, *NFKB1* was found to be both up- or downregulated by natural compounds in different cancers, as well as the same type of cancer (Table S1).

3.2. NaturaProDB. The data presented in Table S1 were deposited in a repository, referred to as the National Technical University of Athens Anticancer Products Database (NaturaProDB; https://naturaprodb.web.app/). This database has a user friendly interface and can be queried using different options, that is, by (a) source, (b) natural product, (c) target cancer, (d) target gene, and (e) expression status of target gene, as well as the combination of the above options (Figure 5(a)). The results appear in a new window, in a tabulated format. Each entry contains the (i) general class of natural sources, (ii) food or organism, (iii) constituent compound/functional extract, (iv) target cancer, (v) target gene, (vi) gene status (up- or downregulated), (vii) cell line tested, and (viii) a hyperlink to the corresponding PubMed webpage (Figure 5(b)). The search output is provided in a JSON or TSV format. By clicking on the "Networks" tab, two interactive networks are displayed: the "TCGA Network" shown in Figure 4 and the "Natural Products Network" which includes food-target cancer-target gene associations (listed in Table S1). The latter network is highly interconnected, suggesting that natural products target multiple and diverse cancer cells and corresponding genes (Figure 5(c)).

4. Discussion

There is an ongoing need for alternative, effective, economical resources for drug development. Natural compounds with antineoplastic potential, as compared to synthetic compounds, are considered to be more efficacious and

Table 1: List of the selected organisms/foods, active compounds/extracts, effective inhibitory concentration, affected cancer type, and target genes.

Source	Active substance/extract	Effective concentration	Cancer type	Target gene
Actinidia callosa var. callosa	Ethyl acetate fraction of *Actinidia callosa* var. callosa (EAAC)	IC_{50}: 73.3 and 92.7 μg/ml for 24 h treatment	Hepatoma	AKT1, MAPK1, MMP2, MMP9, PIK3CD, TIMP2
Aegle marmelos	Beta-caryophyllene and caryophyllene oxide fractions of *Aegle marmelos* extract	Maximum inhibition: 50 μg/ml for 24 h treatment	Lymphoma; neuroblastoma	ATM, BAK1, BAX, BCL2, CASP8, CASP9, MDM2, PTGS2
Allium sativum (garlic)	Allicin	Maximum inhibition: 90 μg/ml for 24 h treatment	Glioblastoma	BAX, BCL2
Allium sativum (garlic)	N-Benzyl-N-methyldecan-1-amine (NBNMA)	Maximum inhibition: 50 μg/ml for 24 h treatment	Leukemia	BAD, BAX, BCL2, BCL2L1, BIRC2, CASP3, CASP8, CASP9, CDK2, CDKN1A, XIAP
Aloe vera (Barbados aloe)	Aloin (AL)	IC_{50}: 10 μmol/l for 72 h treatment	Colorectal cancer	BCL2L1, MYC, STAT3
Anacardium occidentale (cashews)	Cardanol monoene (CM) extracted from cashew nut shell liquid (CNSL)	IC_{50}: 23.15 ± 2.42 μM (24 h); 12.30 ± 1.67 μM (48 h)	Melanoma	BAX, BCL2, TP53, CASP3, PARP1
Annona muricata	Ethyl acetate extract of *Annona muricata* leaves (EEAM)	IC_{50}: 11.43 ± 1.87 μg/ml (HT-29); 8.98 ± 1.24 μg/ml (HCT-116) for 24 h treatment	Colon cancer	CASP3, CASP7, CASP8, CASP9, BAX, BCL2
Arachis hypogaea (peanuts)	Resveratrol	IC_{50}: 46.81 ± 1.26 μM (MCF-7); 53.02 ± 2.42 μM (HeLa) for 72 h treatment	Breast cancer; cervical cancer	CDK4, CDKN1A, MAPK3, TP53
Arachis hypogaea (peanuts)	Peanut skin procyanidins (PSP)	IC_{50}: 48.57 μg/ml for 24 h treatment	Prostate cancer	BAX, BCL2, CASP3, TP53
Basella rubra (spinach)	Natural antioxidants (NAOs) from spinach extract	MIC: 3.2 mg/ml for 24 h treatment	Prostate cancer	CDK2, CDKN1A, E2F1, RB1, RBL1
Black pepper	Piperine	IC_{50}: 54 ± 5 μM to 126 ± 3 μM for 72 h treatment	Colon cancer	AKT1, BIRC5, CCND1, CCND3, CDKN1A, CDKN1B, MAPK8
Black soybean	Black soybean extract	IC_{50}: 3.69 mg/ml for 72 h treatment	Gastric cancer	BAX, BCL2, CASP3
Brassica spp. vegetables (cabbage, cauliflower, and brussels sprouts)	Indole-3-carbinol (I3C)	Maximum inhibition: 50 μM for 24 h treatment	Acute myeloid leukemia (AML); leukemia	AKT1, BCL2, BIRC2, BIRC5, CCND1, MMP9, NFKB1, NOS2, PTGS2, TNF, TRAF1, XIAP
Castanea (chestnut)	Ethanol extracts of raw chestnut (RCE)	Maximum inhibition: 200 μg/ml for 24 h treatment	Gastric cancer	ASP3, CASP7, CASP8, FASLG, HLA-DRB1,PARP1, TNFSF10, XIAP
Citrus aurantium	Flavonoids	IC_{50}: 99 μg/ml for 24 h treatment	Gastric cancer	CASP3, CCNB1, CDK1, PARP1
Curcuma longa (turmeric)	Curcumin	Maximum inhibition: 25 μmol/l for 24 h treatment	Bladder cancer	NFKB1, PTGS2
Curcuma longa (turmeric)	Curcumin	IC_{50}: 11 to 46 μM (72 h)	Pancreatic cancer	CXCL8, NFKB1, PTGS2
Curcuma longa (turmeric)	Curcumin	Maximum inhibition: 50 μM for 24 h treatment	Thyroid cancer	SMAD2, SMAD3, TGFB1
Daphne genkwa	Yuanhuadine	Maximum inhibition: 32 nM for 72 h treatment	Lung cancer	AKT1, CDK2, CDK4, CDKN1A, MYC
Ipomoea batatas (sweet potato)	Sporamin	Maximum inhibition: 100 μM for 72 h treatment	Pancreatic cancer	BAX, BCL2, BCL2L1, NFKB1
	Juglanin		Breast cancer	

Table 1: Continued.

Source	Active substance/extract	Effective concentration	Cancer type	Target gene
Juglans mandshruica (Manchurian walnut)		IC_{50}: 20.07 to 29.13 μM for 24 h treatment		BAD, BAX, BCL2, CASP3, CASP8, CASP9, CDC25C, CDK1, CDKN1B, CHEK2
Juglans mandshruica (Manchurian walnut)	Juglone	IC_{50}: 8 μM for 24 h treatment	Leukemia	AKT1, CASP3, MTOR, PIK3CD
Lebanese *Daucus carota* (wild carrot)	Daucus carota oil extract (DCOE)	IC_{50}: 10.2 ± 0.90 to 19.1 ± 0.98 μM for 48 h treatment	Skin Cancer	AKT1, BCl2, BAX
Naematoloma sublateritium (hazel mushroom)	Hexane fraction of N. sublateritium extract (HFNS)	IC_{50}: 200 μg/ml for 24 h treatment	Breast Cancer	JUN, MAPK14, MAPK8, MAPK9, MMP9, NFKB1, SERPINE1, TIMP2
Pulsatilla koreana	*Pulsatilla koreana* extract (PKE)	IC_{50}: 120 to 140 μg/ml for 24 h treatment	Thyroid cancer	BAX, BCL2, CASP3, PARP1
Red sorghum bran	3-Deoxyanthocyanidins	IC_{50}: 300 μg/ml for 24 h treatment	Breast Cancer	BCL2, TP53
Sanguinaria canadensis (bloodroot)	Sanguinarine	IC_{50}: 1 μM; maximum inhibition: 2 μM for 24 h treatment	Oral cancer	AKT1, CASP3, CASP9, PIK3CD

MIC: minimum inhibitory concentration. IC50: half-maximal effective inhibitory concentration.

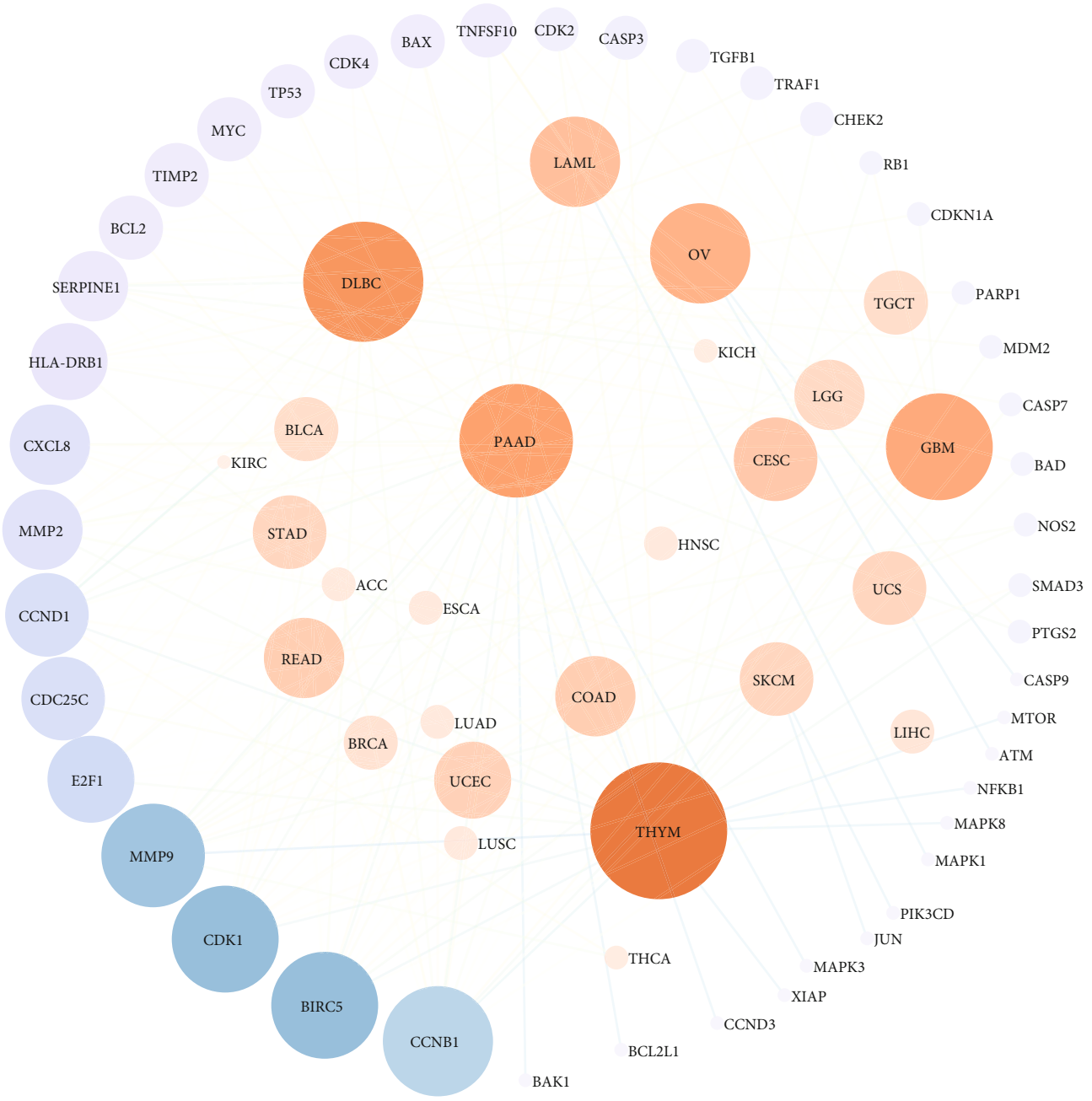


FIGURE 4: Bipartite network displaying TCGA-derived cancer-gene associations. The interactions are presented in a circular mode; the cancer types are shown at the center and the genes at the periphery. The size of the nodes is proportional to their connectivity degree.

bioavailable and cost effective, with less toxic adverse effects [1]. The constant demand for natural antioncogenic medicines is also reflected in the number of databases dedicated to natural products with anticancer activity, such as CancerHSP [51], CHMIS-C [52], InPACdb [53], NPACT [54], and NCARE [55].

Another parameter that must be taken into consideration is the cellular, genetic, and metabolic heterogeneity of cancers and the complex tumor microenvironment. Designing of broad spectrum therapeutic strategies represents an intriguing solution to this problem. The so-called "dirty" drugs have multiple, instead of single, molecular targets. Within this context, the anticancer activity of several natural compounds could be safely combined in such a way as to maximize their inherent additive and synergistic effects and avoid any side effects, towards the design of potent dirty drugs. In our study, we suggest a combination of 22 natural foods which, based on in vitro experimental studies, could potentially target all 19 types of cancers and 57 key cancer-relevant genes under investigation. These genes are implicated in interconnected pathways (Figure 2), including those related to genomic integrity and cell cycle control. In particular, chromosomal instability and DNA damage response and repair (DDR/R) pathways are known to contribute largely to carcinogenesis. One of the characteristics of cancer is the presence of genomic lesions, caused either directly or

Table 2: Differentially expressed genes in diverse TCGA-derived cancers.

Gene	TCGA cancer type	Status
ATM	OV	Down
BAD	DLBC,THYM	Up
BAK1	PAAD	Up
BAX	DLBC,GBM,PAAD,TGCT,THYM	Up
BCL2	CESC,OV,UCS	Down
BCL2	DLBC,LAML,THYM	Up
BCL2L1	PAAD	Up
BIRC5	LAML	Down
BIRC5	BLCA,BRCA,CESC,COAD,DLBC,GBM,LIHC,LUAD,LUSC,OV,PAAD,READ,SKCM,STAD,THYM,UCEC,UCS	Up
CASP3	DLBC,GBM,PAAD,THYM	Up
CASP7	DLBC,THYM	Up
CASP9	OV	Down
CCNB1	LAML	Down
CCNB1	ACC,BLCA,BRCA,CESC,COAD,DLBC,GBM,LIHC,LUAD,LUSC,OV,PAAD,READ,SKCM,STAD,THYM,UCEC,UCS	Up
CCND1	LAML	Down
CCND1	COAD,DLBC,KIRC,LGG,OV,PAAD,READ,STAD,THCA,THYM	Up
CCND3	PAAD	Up
CDC25C	LAML	Down
CDC25C	CESC,COAD,DLBC,GBM,OV,READ,STAD,THYM,UCEC,UCS	Up
CDK1	LAML	Down
CDK1	ACC,BLCA,BRCA,CESC,COAD,DLBC,GBM,LIHC,LUAD,LUSC,OV,PAAD,READ,STAD,THYM,UCEC,UCS	Up
CDK2	DLBC,GBM,SKCM,THYM	Up
CDK4	DLBC,GBM,LGG,SARC,TGCT,THYM	Up
CDKN1A	OV	Down
CDKN1A	GBM	Up
CHEK2	DLBC,TGCT,THYM	Up
CXCL8	CESC,COAD,ESCA,GBM,HNSC,PAAD,READ,SKCM,STAD,UCEC	Up
E2F1	BLCA,BRCA,CESC,COAD,DLBC,LIHC,OV,PAAD,READ,THYM,UCEC,UCS	Up
HLA-DRB1	DLBC,GBM,LAML,LGG,OV,PAAD,STAD,TGCT,THYM	Up
JUN	SKCM	Down
MAPK1	LAML	Down
MAPK3	PAAD	Up
MAPK8	THYM	Up
MDM2	DLBC,SARC,THYM	Up
MMP2	ACC,CESC,OV,SKCM	Down
MMP2	DLBC,GBM,LAML,LGG,PAAD,THYM	Up
MMP9	THYM	Down
MMP9	BLCA,BRCA,CESC,COAD,ESCA,GBM,HNSC,OV,PAAD,READ,SKCM,STAD,TGCT,THCA,UCEC,UCS	Up
MTOR	THYM	Up
MYC	COAD,DLBC,GBM,LGG,READ,THYM	Up
NFKB1	THYM	Up
NOS2	COAD,READ	Up
PARP1	DLBC,THYM	Up
PIK3CD	SKCM	Up
PTGS2	LAML,PAAD	Up
RB1	GBM,THYM	Up
SERPINE1	KICH,OV	Down
SERPINE1	DLBC,ESCA,GBM,HNSC,PAAD	Up

Table 2: Continued.

Gene	TCGA cancer type	Status
SMAD3	UCEC	Down
SMAD3	THYM	Up
TGFB1	GBM,LGG,PAAD	Up
TIMP2	BLCA,CESC,OV,UCEC	Down
TIMP2	LAML,PAAD	Up
TNFSF10	KICH	Down
TNFSF10	CESC,LAML,PAAD,TGCT	Up
TP53	DLBC,GBM,LAML,LGG,THYM	Up
TRAF1	OV,UCS	Down
TRAF1	DLBC	Up
XIAP	THYM	Up

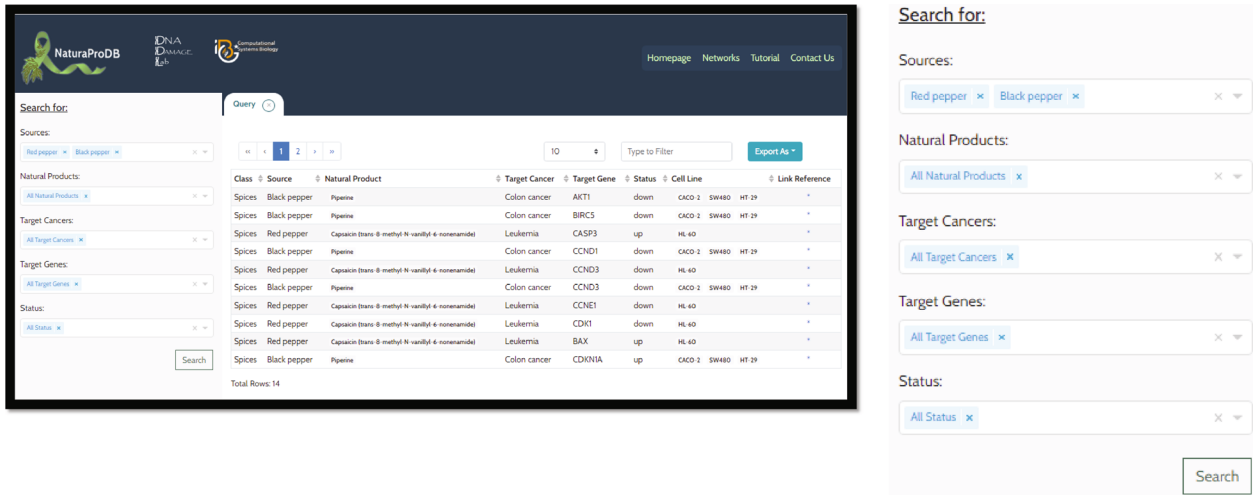
indirectly, through the generation of DNA-damaging intermediates, like reactive oxygen species (ROS) and in general free radicals (e.g., hydrogen ion and hydroxide) [56, 57]. These genomic lesions, if not properly processed, could lead to genomic instability and eventually to carcinogenesis [58]. ATM (Table 1) plays a protagonistic role in the initial stage of DDR/R, that is, DNA damage detection and stress-response signaling [59, 60]. ATM signaling is activated by a wide variety of DNA lesions and DNA replication stress [61, 62]. Cyclin D1 (CCND1) (Table 1) was demonstrated to induce post-DNA damage cell cycle arrest and apoptosis in different types of cancers [63, 64].

Moreover, several cell prosurvival pathways (Figure 2) were found to be enriched in the gene set. For example, the transforming growth factor beta (TGFB), MAPK, ERBB, HIF-1, WNT, PDGF/PDGFRB, and NFKB signaling pathways are implicated in several aspects of cancer initiation, promotion, and progression [65–71], by mediating survival of cancer cells. The apoptotic pathway is also overrepresented. Cancer cells have developed the ability to evade apoptosis, mainly due to deregulation of key proapoptotic molecules like TP53, BAX, BCL2, BIRC5, and XIAP (Table 1).

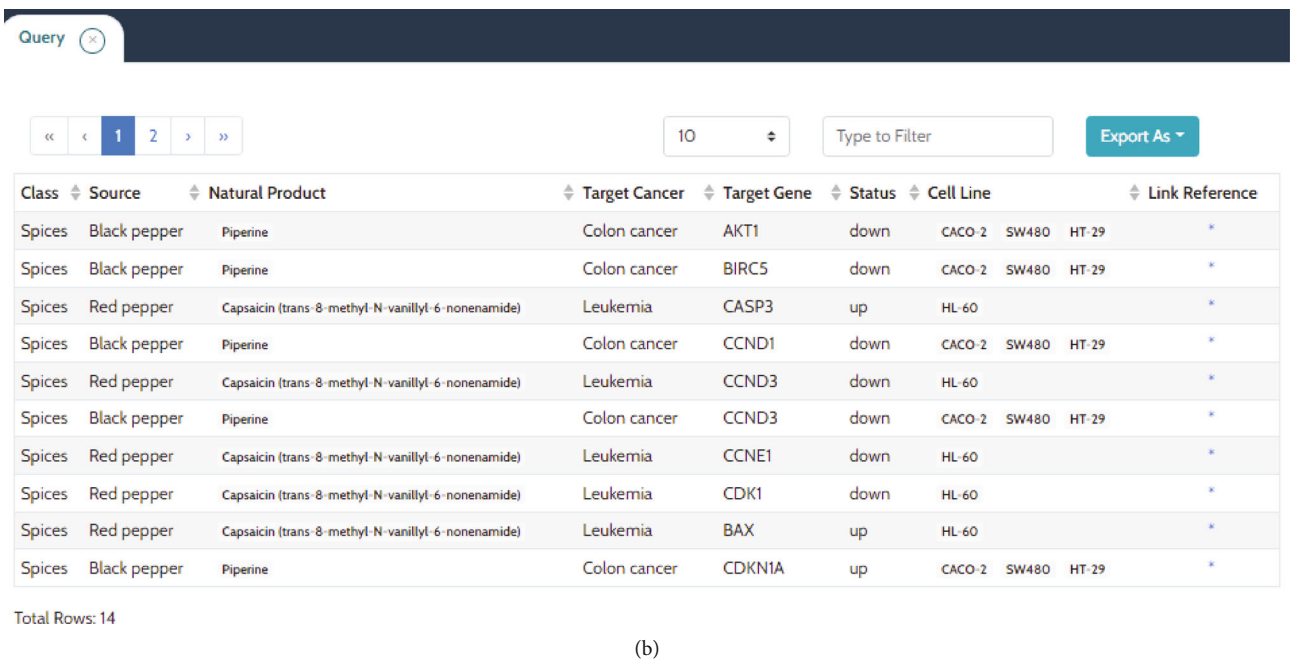
Several of the plants (e.g., garlic, peanuts, spinach, and black soybean) listed in Table 1 contain more than one active compound or phytochemicals with anticancer activity (Table S1), suggesting that they exert their anticancer effect in an additive or synergistic manner. One of the garlic clove ingredients, the phenylamine NBNMA (Table 1), was shown to induce cell cycle arrest, via downregulation of the cell cycle M-phase inducer cyclin dependent kinase 2 (CDK2) and overexpression of CDKN1A, as well as apoptosis, by activation of the proapoptotic factors CASP3, -8, -9, BAX, and BAD and, conversely, inactivation of the antiapoptotic BCL2, BCL2L1, BIRC2, and XIAP in leukemic cells [72]. As it is shown in Table 2, BCL2 is overexpressed in acute myeloid leukemia. Another garlic compound, allicin was shown to sensitize hepatocellular carcinoma cells to anticancer agents via a ROS-dependent signaling pathway [73]. Allicin, also, increases the sensitivity of colorectal cancer cells to radiation through the inhibition of a NFKB1-mediated pathway [74]. Furthermore, allicin potentiates apoptosis in human glioblastoma cells, by elevating the expression of BAX and downregulating BCL2 (Table 1) [75]. Peanuts, which contain procyanidins in their skin, can inhibit the proliferation of prostate cancer cells and promote apoptotic cell death by downregulating BCL2 and upregulating the proapoptotic factors BAX, CASP3, and the TP53 [76]. The phenolic compounds, which comprise the largest group of phytochemicals, are known to exert their antineoplastic effect by contributing to cell proliferation, apoptosis, angiogenesis, metastasis, and inflammation under oxidative stress [77–81]. The phenolic antioxidant resveratrol in peanuts (Table 1) was shown to exhibit antiproliferative activity in cervical and breast cancer cells, by decreasing the expression of the DNA damage-induced prosurvival protein kinases MAPK3 and CDK4 and, conversely, elevating TP53 and CDKN1A [82].

Juglone, a phenolic compound in the Manchurian walnut, was shown to inhibit the proliferation of human leukemia cells and enhance apoptosis [83]. In particular, Juglone (Table 1) markedly inhibited the phosphorylation of PI3K/AKT/mTOR, a major antiapoptotic and prosurvival signaling pathway which is overactivated in multiple cancers [84], and induced the cleavage and activation of the proapoptotic procaspase-3. Moreover, juglanin was shown to inhibit the proliferation of breast cancer cell proliferation through the differential regulation of cell cycle-associated proteins (i.e., CDC25C, CDK1, CDKN1B, and CHEK2), the activation of the proapoptotic factors BAD, BAX, CASP3, -8 and -9, and, conversely, the suppression of the antiapoptotic protein BCL2 [85]. Of note, CDK1 (cyclin-dependent kinase 1), which is downregulated by the flavonoid compound juglanin (Table 1), was found to be overexpressed in invasive breast cancer (Table 2); Izadi et al. suggest that CDK1 is the best CDK target for breast cancer therapy [86].

The cancer-preventive properties of the cruciferous vegetables (belonging to the family *Brassicaceae*) have been acknowledged for a long time. The active ingredient indole-3-carbinol (I3C) has been documented to play a role in the prevention of several cancers [87]. For example, I3C was found to inhibit the growth of prostate cancer cells and induce G1 cell cycle arrest and apoptotic cell death [88]. Furthermore, it was demonstrated by Takada and colleagues [89]

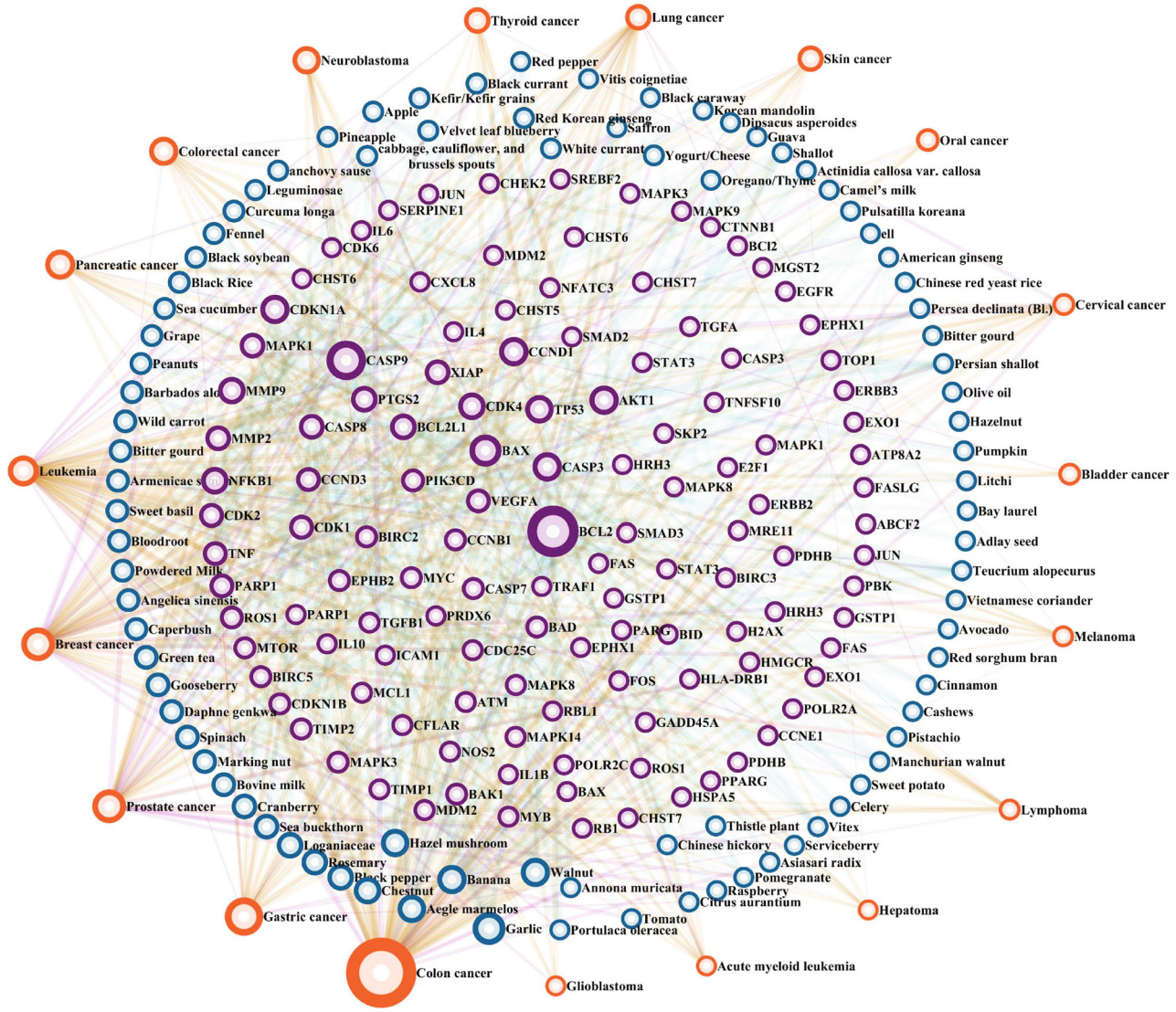


(a)



(b)

Figure 5: Continued.



(c)

Figure 5: NaturaProDB workflow showing the (a) example input query, (b) example results page, and (c) "Natural Products Network," where the node size is proportional to their connectivity degree.

that I3C (Table 1) blocks the expression of NFKB1-regulated prometastatic, proproliferation, and antiapoptotic gene products (i.e., AKT1, BCL2, BIRC2, BIRC5, CCND1, MMP9, PTGS2, TNF, and XIAP) in myeloid and leukemia cells. Of those genes, *BCL2* and *PTGS2* were shown to be upregulated in acute myeloid leukemia (Table 2). In the same study, the gene encoding NOS2 (nitric oxide synthase 2), which catalyzes the production of the reactive free radical nitric oxide, was downregulated, leading to the suggestion that I3C might exert antioxidant activity.

Flavonoids present in citrus fruits have been documented to exhibit cancer-preventive potential by participating in cell cycle inhibition, suppression of metastasis, and angiogenesis, as well as anti-inflammatory signal transduction pathways [90]. In particular, flavonoids isolated from *Citrus aurantium* were shown to inhibit the growth of human gastric cancer cells, by suppressing the proteins CCNB1 (cyclin B1) and CDK1 (Table 1) which control cell cycle progression; the corresponding genes *CCNB1* and *CDK1* were found to be upregulated in stomach cancer (Table 2). In addition, activation of CASP3 together with the inactivation of PARP1, which is involved in DNA damage repair, potentiated apoptosis [91].

The spice *Curcuma longa*, commonly known as turmeric or "Indian saffron," has been used in the folk medicine of India for thousands of years. Curcumin, the major active ingredient of turmeric, is a powerful antioxidant, with well-documented anti-inflammatory and anticancer potential [92]. Curcumin was shown to suppress growth of bladder and pancreatic cancer cell lines through the inhibition of NFKB1-regulated proinflammatory and proproliferative gene products PTGS2 [93] and CXCL8 [94] (Table 1); both CXCL8 and PTGS2 were also found to be downregulated in pancreatic cancer (Table 2). Moreover, curcumin was demonstrated to inhibit metastasis in human papillary thyroid

carcinoma cells by downregulating components of the prometastatic signaling pathway TGFB1/SMAD2/SMAD3 [95] (Table 1).

Other phytocompounds, like sporamin in sweet potato [96–98], piperine in black pepper [99], sanguinarine in bloodroot [100–102], 3-deoxyanthocyanins in red sorghum bran [103], and aloin in *Aloe vera* [104], have been documented to exhibit anticancer effects in several cancers. Of importance, these agents possess antioxidant properties [105–108]. Aloin [104] and piperine [109] can inhibit proliferation of (colo)rectal cancer cells; the genes *MYC* and *BIRC5*, the expression of which is decreased by the two compounds (Table 1), are otherwise overexpressed in colon adenocarcinoma (Table 2). Similarly, the antiapoptotic *BCL2L1* gene, which was found downregulated in sporamin-induced apoptotic pancreatic cancer cells [97] (Table 1), is overexpressed in pancreatic adenocarcinoma (Table 2). Furthermore, β-caryophyllene and β-caryophyllene oxide extracted from *Aegle marmelos* are suggested to possess anti-inflammatory potential and were demonstrated to induce apoptosis in cancer cells of diverse tissue origin, that is, lymphoma (i.e., haematological cancer) and neuroblastoma (i.e., nerve tissue neoplasm), through overexpression of the proapoptotic (*ATM*, *BAK1*, *BAX*, and *CASP8/9*) and underexpression of the antiapoptotic (*BCL2*, *MDM2 proto-oncogene*, and *PTGS2*) genes [110] (Table 1). The genes *BCL2* and *MDM2* are also upregulated in lymphoid neoplasm diffuse large B-cell lymphoma (Table 2).

5. Conclusions

Nowadays, there is a great need for drugs derived from nature that can be 100% compatible if possible to the human organism, with a potential applicability in the fight against human diseases. In this study, we have targeted human cancer. Based on systematic searches and use of current bioinformatics methodologies, we have designed a biodatabase exceeding the existing standards of natural product databases, i.e., at a systems biology level. We have found that diverse natural products, with no observed adverse health effects, can target dissimilar cancer types through the significant alteration of the expression of multiple, common genes, which are involved in shared interconnected cancer-relevant pathways. Therefore, the efficient and safe combination of bioactive compounds derived from natural resources can be potentially applied to exert cytotoxic effects on diverse types of cancer cells and regulate the expression of numerous target genes which play a central role in cancer pathways. Further studies in animal models should be directed towards the investigation of the chemopreventive actions and safety of those compounds. The aforementioned findings should be taken into consideration in the rational design of drugs with broad spectrum activity, such as "dirty" drugs. Lastly, NaturaProDB was developed to facilitate the retrieval of relevant information. In conclusion, in this study, we provide a novel and effective systems biology approach to investigate the potential value of the combined activity of natural products in cancer chemoprevention, which can be exploited in the development of anticancer multitargeted therapies.

Abbreviations

ACC: Adrenocortical carcinoma
BLCA: Bladder urothelial carcinoma
BRCA: Breast-invasive carcinoma
CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma
COAD: Colon adenocarcinoma
DLBC: Lymphoid neoplasm diffuse large B-cell lymphoma
ESCA: Esophageal carcinoma
GBM: Glioblastoma multiforme
HNSC: Head and neck squamous cell carcinoma
KICH: Kidney chromophobe
KIRC: Kidney renal clear cell carcinoma
LAML: Acute myeloid leukemia
LGG: Brain lower-grade glioma
LIHC: Liver hepatocellular carcinoma
LUAD: Lung adenocarcinoma
LUSC: Lung squamous cell carcinoma
OV: Ovarian serous cystadenocarcinoma
PAAD: Pancreatic adenocarcinoma
READ: Rectum adenocarcinoma
SARC: Sarcoma
SKCM: Skin cutaneous melanoma
STAD: Stomach adenocarcinoma
TGCT: Testicular germ cell tumors
THCA: Thyroid carcinoma
THYM: Thymoma
UCEC: Uterine corpus endometrial carcinoma
UCS: Uterine carcinosarcoma.

Data Availability

The data used to support the findings of this study are available from the corresponding authors upon logical request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Conceptualization was done by A.G.G., D.T., and A.P.; methodology was done by D.T., I.T., G.K., and A.P.; software was acquired by D.T., I.T., G.M.B., and V.Z.; validation was done by D.T., I.T., V.Z., and A.P.; formal analysis was done by D.T., I.T., G.K., and A.P.; investigation was done D.T.; data curation was done by D.T., I.T., G.M.B., and A.P.; writing and original draft preparation were done by A.G.G., D.T., I.T., and A.P.; writing and review and editing were done by all authors.; visualization was done by I.T., G.M.B., G.K., and A.P.; supervision was done by A.G.G. and A.P.; project administration was done by A.G.G. and A.P.; all authors have read and agreed to the current version of the manuscript. Dionysia Theofylaktou and Işıl Takan contributed equally to this work.

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Supplementary Materials

Table S1: the class, natural source, active ingredient, the affected cancer type(s), target gene(s), gene expression status, cell line used in the experimental study, and corresponding PubMed link are listed. *(Supplementary Materials)*

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Review Article

Quercetin as a Novel Therapeutic Approach for Lymphoma

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Lymphoma is a name for malignant diseases of the lymphatic system including Hodgkin's lymphoma and non-Hodgkin's lymphoma. Although several approaches are used for the treatment of these diseases, some of them are not successful and have serious adverse effects. Therefore, other effective treatment methods might be interesting. Studies have indicated that plant ingredients play a key role in treating several diseases. Some plants have already shown a potential therapeutic effect on many malignant diseases. Quercetin is a flavonoid found in different plants and could be useful in the treatment of different malignant diseases. Quercetin has its antimalignant effects through targeting main survival pathways activated in tumor cells. *In vitro*/*in vivo* experimental studies have demonstrated that quercetin possesses a cytotoxic effect on lymphoid cancer cells. Regardless of the optimum results that have been obtained from both *in vitro*/*in vivo* studies, few clinical studies have analyzed the antitumor effects of quercetin in lymphoid cancers. Thus, it seems that more clinical studies should introduce quercetin as a therapeutic, alone or in combination with other chemotherapy agents. Here, in this study, we reviewed the anticancer effects of quercetin and highlighted the potential therapeutic effects of quercetin in various types of lymphoma.

1. Introduction

The lymphatic system has important functions such as maintaining tissue fluid homeostasis, initiating humoral and cellular immune responses, and transporting intestinal lipids to the blood [1]. According to the classical theory of metastasis, malignant cells that reach the sentinel lymph nodes may spread further into the distal lymph nodes, and subsequently form organ metastases [2]. Traditionally, four treatment methods including surgery, radiotherapy, chemotherapy, and immunotherapy are used alone or in combination to treat patients' malignant diseases [3, 4]. Chemotherapy is used as a common approach for the treatment of these patients; however, drug resistance is the key reason for

decreased effectiveness of chemotherapy and occurs due to adaptation of cancer cells to chemotherapy agents [5]. The development of analytical tools, genome extraction, engineering strategies, and microbial science have opened a new window on the sciences of pharmacotherapy and cancer therapy. Given this development, in relation to cancer and some infectious diseases, their natural products and structural analogues are of great help to researchers in the field of pharmacology [6]. Producing substances which are capable of killing malignant cells without being toxic is important and has attracted increasing interest. In recent years, it has been shown that some substances derived from plants can have beneficial effects on malignant cells [7–10]. Flavonoids have been the most studied. They interact in specific stages of the carcinogenesis process to prevent cell proliferation and consequently cause apoptosis [11–15]. Quercetin (3,3′, 4′,5,7″ penta-hydroxyl flavones) is a polyphenolic flavonoid found in apples, red grapes, onions, raspberries, honey, cherries, citrus fruits, and green leafy vegetables [16].

In recent years, several studies have demonstrated that quercetin has different biological effects [17], including antiviral, cell-cycle modulation, inhibitory effects on antioxidant angiogenesis, anticancer, apoptosis-inducing induction, and C-blocking protein kinase [18–20]. It was suggested that chronic daily intake of quercetin might prevent some types of malignant diseases as well as lymphoid cancers. Several pathways have been identified that are affected by quercetin in various malignancies [21, 22]. Here, for the first time, we reviewed the anticancer and therapeutic effects of quercetin in different types of lymphoid cancers.

2. Lymphomas and Possible Causes of Increased Lymphoma

The lymphatic system is essential for immune response, maintaining tissue fluid balance, transporting antigen and antigen-presenting cells (APCs) to the lymph nodes, and transporting lipids absorbed from the intestine into the blood. Therefore, disorders of the lymphatic system can cause lymphedema, compromise local immune response, and cause intestinal malabsorption [23]. The lymphatic system includes the lymph nodes, spleen, thymus, and bone marrow. Lymphomas can affect all of them as well as other organs. Lymphoma is a malignant disease of the lymphatic system. There are several types of lymphoma: Hodgkin's lymphoma (formerly called Hodgkin's disease) and non-Hodgkin's lymphoma [24]. Causes and risk factors that increase the susceptibility to lymphoma include age (people over 60), gender (more prevalent in women than men), obesity, ethnicity (e.g., high percentage within white Americans), exposure to chemicals or radiation (such as pesticides and nuclear radiation), infections (such as HTLV-1 virus, *H. pylori*, hepatitis C, and EBV), autoimmune diseases (such as RA and celiac disease), immune deficiencies (such as AIDS), or receiving immunosuppressive drugs [25–27]. The role of the lymphatic system in the progression of metastasis of malignancies has attracted an increased interest in the last 15 years [28]. The lymphatic system also plays a critical role in cancer progression, because the metastatic cancer cells can spread through the lymph vessels to the lymph nodes [23]. The most common sites of solid tumor metastases are lymph nodes. Their presence indicates a poor prognosis and usually suggests the need for systemic treatment in patients with malignant disease. On the other hand, the presence of cancer cells in the lymph nodes may reflect the ability of the primary tumor to metastasize; however, their actual presence in the lymph nodes may be inconclusive [29, 30]. This reasoning indicates that lymph node metastases need to be treated for prevention of distant metastasis and ultimately eradication of the disease [31–33]. The metastases in lymph nodes might indicate whether there is a possibility of further expansion of the malignancy in lymph nodes or whether distal metastases have already occurred [34]. Lymph node metastasis is thought to be regulated in two stages; the first one being the uptake of malignant cells into the lymph vessels and the second one a successful penetration of these cells into lymph nodes [35]. Because of the lack of functional lymph vessels in tumor cells, interstitial fluid pressure increases and the lymph flow to the lymph nodes changes, which causes the tumors to drain [36]. Tumor-induced VEGF-C and VEGF-D increase the proximal contraction of lymphatic vessels [37]. The expansion of malignant cells to the lymph nodes is decreased when tumor-induced lymph vessel regeneration is prevented [36, 38–40].

3. Quercetin and Its Antitumor Effects

Despite great advances in the treatment of malignant diseases, they are still recognized as life-threatening diseases. However, the effects of chemotherapy which is used as a common treatment for many malignant diseases are limited because of resistance to this type of therapy and serious adverse effects. Today, natural compounds such as quercetin are recognized as an important factor in malignant disease prevention and treatment because of their effects, high therapeutic potential, and low toxicity [41]. Quercetin, as a lipophilic compound, can cross cell membranes and initiate several intracellular signaling pathways. Quercetin's unique characteristic includes its dual function as a peroxidant or antioxidant [42]. It seems that quercetin can act as a potential antiproliferation and antimalignant as well as apoptosis inducer (Figure 1) [41, 43]. The mitochondrial-mediated pathway has been suggested as the main mechanism by quercetin to induce apoptosis [44–46]. It also induces apoptosis by increasing proapoptotic molecules such as Bax, caspase-3, caspase-9, p53, or decreasing of antiapoptotic substances [47]. Quercetin prevents the cell cycle by reducing D1/Cdk4 and E/Cdk2 and regulating p21, which ultimately stops the G1 cell cycle [48]. Quercetin binds directly to tubulin and eliminate the polymerization of cell microtubules, which causes stopping of the cell cycle [49]. It seems that quercetin interferes with a large variety of molecules involved in the cell cycle. The complex of PI3K/AKT/PKB is a pathway involved in different processes such as regulation of cell survival, cell cycle, growth progression, and carcinogenesis [50]. This pathway can be targeted with quercetin in different types of malignancies and cause induction of apoptosis, inhibition

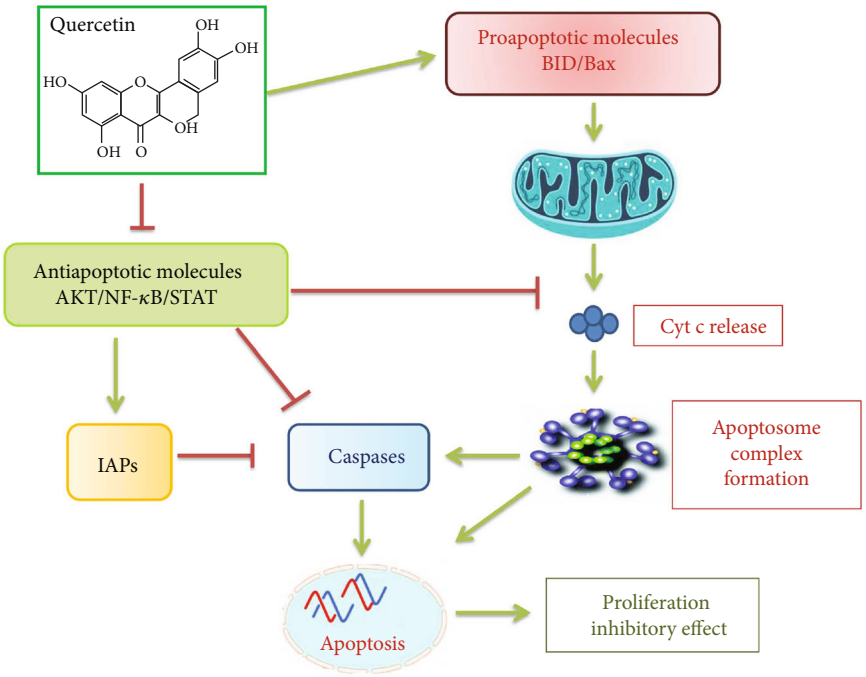

Figure 1: Suggested model for quercetin effects on lymphoma cells. Quercetin inhibits cell growth by apoptosis induction via inhibitory effects on antiapoptotic signaling molecules and proapoptotic protein induction which cause activation of mitochondrial-mediated caspase activation and apoptosis [43, 51, 59, 60].

of cancerogenesis, and development of malignancy [51]. Numerous studies have reported that quercetin causes phosphorylation and stabilization of p53 levels. p53 is considered to be a key molecule in the proapoptotic and antimalignant effects of quercetin [52]. p53 has an antioxidant effect through regulating several genes, including aldehyde dehydrogenase ALDH4A1, Mn-SOD2, microsomal GSH homologous transferase PIG12, Gpx1, and catalase [53–55]. A decrease in p53 sensitizes cells to H_2O_2 damage, which causes decreased viability and increased apoptosis and DNA oxidation. The function of P53 in malignant cells is significantly blocked by poor regulation of its genes [56]. Increased ROS as oxidative stress causes DNA damage and mutations. Quercetin can reduce ROS by electron donation that leads to reduce ROS-mediated DNA damage [57, 58]. According to the results of related studies, it could be concluded that quercetin has an important role to treat and control malignancies. Regulation of cell-cycle-related proteins, antioxidant effects, induction of apoptosis, and induction of cell-cycle arrest are some of the mechanisms by which quercetin has its effects in the treatment and inhibition of malignancies.

4. Molecular Targets of Quercetin in Malignant Cells

Malignant diseases have an abnormal activation of various survival signaling pathways, e.g., a different antiapoptotic protein expression [61]. Aberrant activation of signaling pathways such as serine-threonine kinase, activator protein 1 (AP-1), AKT kinase/protein kinase B (AKT/PKB), nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), androgen receptor, Raf/Ras pathways, and estrogen receptors are the key mechanisms that can cause tumorigenesis [62, 63].

Tyrosine kinases like Jak and Src kinases are activated in malignant cells [64]. These signaling transductions are essential for normal cell proliferation and physiological function. In malignant cells, deregulated expression of these pathways results in abnormal cell growth and apoptosis resistance. Several studies have been conducted to explore the strategies of targeting the overactivated molecules involved in survival pathways to induce apoptosis in malignant cells [16]. It has been shown that quercetin inhibits cell growth by cell-cycle arrest and induces apoptosis in different malignant cells by targeting different molecular pathways such as activation of NF-κB and signal transducers/activators of transcription STAT3 (Figure 2) [65–68]. It can also downregulate the expression NF-κB, Bcl-2, cyclooxygenase 2 (COX-2), matrix metalloprotease 9 (MMP-9), tumor necrosis factor (TNF), cyclin D1, and adhesion molecules [69–73]. NF-κB is one of the important transcription factors identified in the nucleus of B lymphocytes and plays a critical role in regulating the function of the human immune system. This transcription factor controls the expression of many genes and has been linked to inflammatory, neurological, and cancer diseases [74]. Numerous studies have shown that NF-κB activation is associated with some lymph node diseases, including large cell lymphoma, Burkitt's lymphoma, diffuse large B-cell lymphoma, fibrosarcoma, Hodgkin's lymphoma, mammary carcinoma, and mantle cell lymphoma melanoma [75].

Several research indicated that the chemopreventive/-chemotherapeutic effects of quercetin are based on its

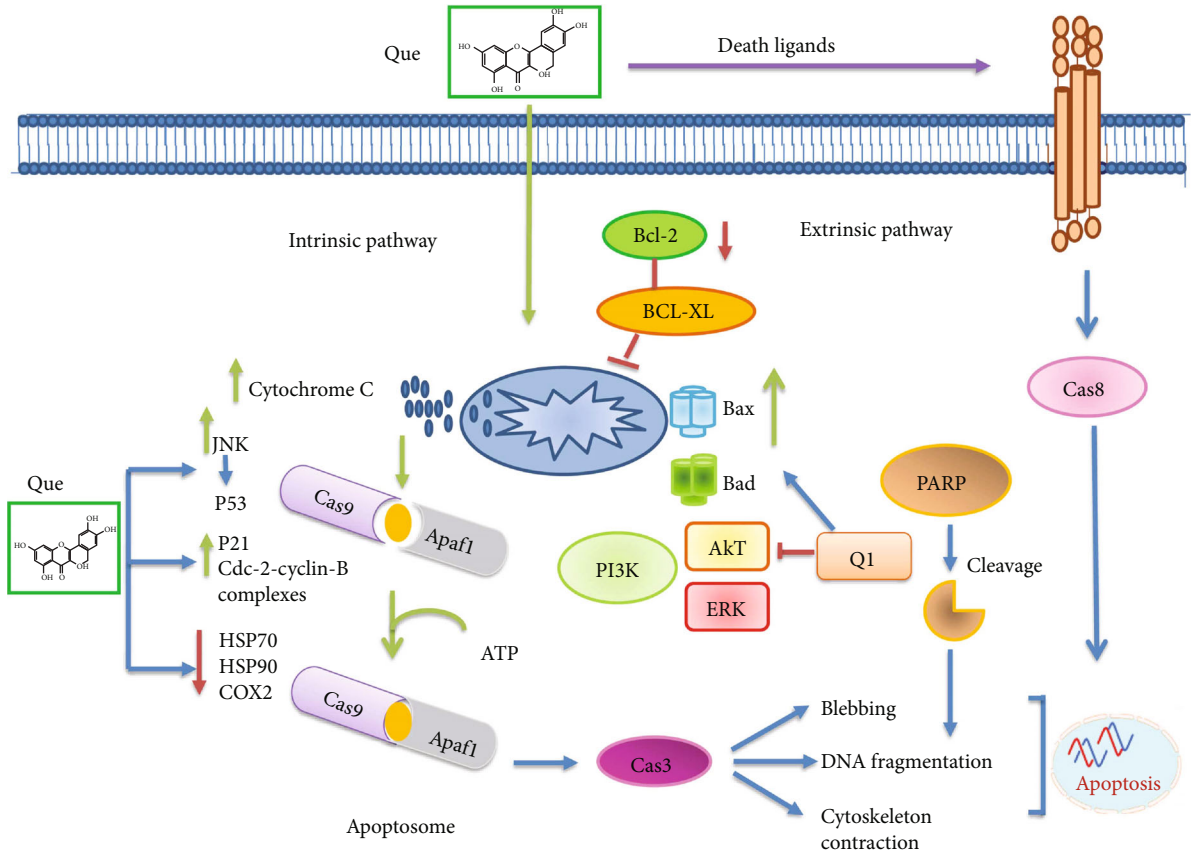

Figure 2: A view of the effect of quercetin on the expression level of genes involved in apoptosis pathway [65–68].

concentration. At low doses, quercetin acts as an antioxidant. However, at high doses, quercetin has effects of a prooxidant and prompts chemotherapeutic effects. The antitumor effects of quercetin are related to its potential in reducing tumor growth, inducing apoptosis, promoting cell-cycle arrest, and suppressing mitotic processes, which are performed by modulating cyclin, proapoptotic, PI3K/AKT, and mitogen-activated protein kinase (MAPK) molecular pathways (Table 1) [76]. Quercetin also downregulates p-ERK1/p-ERK 2 in the multiple myeloma (MM) cell lines which results in inhibition of MAPK pathway activation and suppression MM cell proliferation.

5. Quercetin and Lymphomas

5.1. Multiple Myeloma and Quercetin. Multiple myeloma is characterized by accumulation of plasma cells in the bone marrow and an increased monoclonal protein concentration in blood and/or urine [89]. The effects of quercetin on multiple myeloma cells have been reported in several studies [85, 86, 90]. The deregulation of apoptosis in plasma cells plays a key role in the pathogenesis and chemoresistance of multiple myeloma. Quercetin inhibits IQ motif-containing GTPase activating protein 1 (IQGAP1) expression in multiple myeloma cells. IQGAP1 activates growth factor-mediated activation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK)/ERK kinase (MEK). The RAS/MEK/ERK signaling pathway plays a key role in MM pathogenesis [86]. Xu et al. demonstrated that quercetin activates the apoptosis-related proteins and induces cell-cycle arrest in MM cell lines. It also upregulates the apoptosis-related proteins including caspase-3, 8, and 9; upregulates PARP; and inhibits Bcl-2 expression. Quercetin induces the expression of P53, P21, and P27 and reduces the phosphorylation levels of p-ERK and p-A KT in MM cell lines [85].

5.2. Quercetin and T-Cell Lymphoma. T-cell lymphomas are a group of diseases characterized by clonal expansion and dysfunction of T-cells. T-cell lymphomas have been classified into two groups: cutaneous TCL (CTCL) and peripheral TCL (PTCL) [91, 92]. The PI3K/AKT pathway is the main target in antimalignant therapy and PI3K as a lipid kinase plays a key role in malignant cell transformation. PI3K is involved in regulating cell growth, survival, apoptosis, and angiogenesis. It also activates AKT which has a key function in both physiological and pathological signaling pathways. The antimalignant effect of PI3K is based on inhibition in hematological malignancies via the blocking of survival signaling pathways within malignant and nonmalignant cells along with controlling cytokine secretion and activating the immune response. Maurya et al. indicated that in human leukemic cell lines, quercetin can inhibit upregulation of AKT1/2 and tensin homolog (PTEN) pathways by reducing PI3K/AKT activity and suppressing the proliferation in human leukemic cell lines. AKT induces cell survival by

Table 1: The effects of quercetin in treatment of lymphomas.

	Dose	In vitro/ in vivo	Cell line	Effective molecular mechanism	Molecular targets of quercetin	Ref.
Large B-lymphomas	10 or 20 μM	*In vitro*	U937	Arrests cell cycle in the G2/M phase, increases cyclin B protein levels, and decreases cyclin D and E and E2F1 and E2F2 levels	Cyclin B, cyclin D, and cyclin E	[77]
	50 μM	*In vitro*	Daudi and TMD-8	Decreases expression of Notch1 protein	Notch1 protein	[78]
	—	*In vitro*	Namalawa	Arrest of cells in the G2/M phase	Caspase-3	[79]
	20 μM	*In vitro*	VAL, RL, and SUDHL-4 cell lines	Reduces expression of survivin and Mcl-1 and reduces restoration of the TRAIL pathway	TRAIL, Mcl-1	[80]
	20 μM	*In vitro*	DLBCL	Inhibits p-STAT3 and decreases expression of Mcl-1, survivin, and Bcl-x1 genes	STAT3 protein	[81]
	50 μM	*In vitro*	BC1, BC3, and BCBL1	Increases the number of G1 events; increases the inhibition of PI3K and mTOR kinase; reduces the Wnt activation pathway; decreases expression of c-FLIP, cyclin D1, c-Myc, and p65 NF-κB; reduces the release of IL-6 and IL-10; increases expression of HLA II (HLA-DR); decreases expression of HLA I	c-FLIP, cyclin D1, c-Myc, IL-6, IL-10, KSHV proteins, HLA-DR, and calreticulin	[59]
	50 mg	*In vivo*	—	Increases and decreases expression of the *HIGD2A* gene	Higd2a protein	[82]
	2.5, 10, and 25	*In vivo*	—	Inhibits primary viral antigens	Ornithine decarboxylase (ODC)	[83]
Burkitt's lymphoma	30 μM	*In vitro*	Raji cell	Inhibits PI3Kδ; decreases expression of cIAP-1, cIAP-2, and anti-/proapoptotic molecules; inhibits the mTOR pathway; increases expression of the γH2A.X protein	PI3K and mTOR	[84]
	50 and 100 μM	*In vitro*	Raji, Akata, 2A8, Ramos, and BL-41	Inhibits c-Myc expression and the PI3K/AKT/mTOR pathway, reduces expression of HSP-70, and stimulates autophagy	c-Myc protein, PI3K, AKT, and mTOR	[60]
Multiple myeloma	100 and 200 μmol/L	*In vitro*	NCI-H929	Inhibits CDK4 expression; downregulates p-ERK and p-AKT; activates apoptosis-related proteins caspase-3, caspase-8, caspase-9, and PARP	Caspase-3, caspase-8, caspase-9, PARP, Bcl-2, P53, P21, P27, CDK4, p-ERK, and p-AKT	[85]
	40, 80, 160, and 320 μmol/L	*In vitro*	U266, KM3, RPMI8226, and MM	Downregulates IQGAP1 expression and p-ERK1/2 and inhibits the MAPK pathway in MM cells	IQGAP1 protein and ERK1/2	[86]
T-cell lymphoma	—	*In vivo*	—	Downregulates p-AKT, p-PDK1, p-BAD, p-GSK-3β, p-mTOR, p-IkBα, VEGF-A COX-2, iNOS, and NO level. Upregulates p-PTEN	AKT, PDK1, BAD, GSK-3β, mTOR, IkBα, VEGF-A, COX-2, iNOS, and PTEN	[87]
	300 μmol/L	*In vitro*	DLA	Downregulates p-AKT 1, p-PDK1, p-BAD, TNFR1, PKCα, and upregulates PTEN	AKT, PDK1, BAD, TNFR1, and PTEN	[88]

phosphorylating/inhibiting Bcl-2-associated agonist of cell death (BAD) which promotes cell death and apoptosis by its dephosphorylation. Therefore, BAD phosphorylation stimulates cell survival. Quercetin attenuates the PI3K/AKT pathway and prevents survival signals in lymphoma growth [88].

5.3. Quercetin and Large B Lymphomas (Hodgkin and Non-Hodgkin). Another type of lymphoma is the B-cell lymphoma, which is classified into Hodgkin's and non-Hodgkin's lymphomas. According to the World Health Organization (WHO), B-cell lymphomas are classified into five major categories: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, marginal zone B-cell lymphoma (MZL) or mucosa-associated lymphatic tissue lymphoma (MALT), small lymphocytic lymphoma (SLL), and mantle cell lymphoma (MCL) [93]. Aggressive lymphomas require serious treatment. On the other hand, this type of malignancy therapy depends on the type of lymphoma and the stage and grade of the disease. For example, slow-growing lymphomas can be treated with radiation therapy, but the aggressive type has to be treated with chemotherapy in addition to radiation therapy [94, 95]. Chemotherapy and radiation therapy have many adverse effects. Therefore, some researchers have tried to use therapies with fewer side effects, such as herbal compounds. One of these plant substances is quercetin, which is rich in polyphenols and flavonoids and has been shown to have anti-inflammatory, antimalignant, and antioxidant (free radical scavenger) effects [96–98]. Fil'chenkov et al. investigated the antiapoptotic effects of quercetin and resveratrol in vitro on B-cell lymphoma cell lines (Namalawa). Using flow cytometry and 1H NMR spectroscopy, they found that these two substances activated caspase-3 apoptotic cycles and increased hypodiploid cells as well as the number of motile lipid domains. Thus, the combination with quercetin causes time-dependent arrest of cells in the G2/M phase [79]. Lee et al. studied the U937 lymphoma cell line and found that the main mechanism of inhibition of lymphoma cell growth is related to cell-cycle arrest in the G2/M phase and induction of the caspase-mediated apoptotic process. They found that quercetin stabilized U937 cells in the G2/M phase, which in turn increased cyclin B protein levels and decreased cyclin D, E, E2F1, and E2F2 levels, and vice versa. Quercetin stimulates cell death by modulating Bcl-2 and Bcl-xl without altering the expression of Bcl-2 and Bcl-xl proteins, and this action is mediated by caspase-3. Finally, quercetin makes time-dependent accumulation of U937 cells in the G2/M phase in which arrest occurs and increases the time-dependent cell population in the sub-G1 phase [77]. Frankenfeld et al. studied the association of dietary flavonoid intake with a reduced risk of developing non-Hodgkin's lymphoma (NHL). They found that consuming more flavonols such as quercetin, epicatechins, anthocyanidins, and proanthocyanidins reduces the risk by 47% in the highest quarter compared to the lowest (95% CI: 31%, 73%), and that the more flavonols were consumed, the lower was the risk of non-Hodgkin lymphoma [99]. Kawahara et al. studied lymphoma and leukemia cell lines, especially Daudi (Burkitt's lymphoma) and TMD-8 (diffuse large B-cell lymphoma), and found that cyclopamine and quercetin inhibited cell line growth and stimulated apoptosis. Cyclopamine targets the Hh signaling protein called Gli1 and reduces its expression. Quercetin reduces the expression of Notch1 protein and its active component in the DND-41 lymphocytic T leukemia cell line. They concluded that these natural substances could be used as targeted treatment for chemotherapy-resistant lymphoma and leukemia [78].

Jacquemin et al. found that quercetin combined with TRAIL therapy could be beneficial for malignant diseases such as non-Hodgkin's lymphoma (NHL). They found that pretreatment of malignant cells with 20 μM quercetin for 24 hours overcomes the resistance of lymphoma cell lines to tumor necrosis factor-related apoptosis-inducing ligand-(TRAIL-) stimulated cell death. This compound completely activates caspase-3, and then it activates caspase-8 and caspase-9. Using flow cytometric analysis, they have shown that quercetin pretreatment prevents the expression of TRAIL receptors in lymphoma cell lines such as RL and VAL. In these cells, quercetin increases the expression of caspase-10 following TRAIL stimulation, but it is not needed for the reversal of apoptosis. Quercetin sensitization is independent of caspase-10 and the regulation of TRAIL-RISC complex formation. Quercetin reactivates the caspase-independent mitochondrial pathway by reducing the expression of survivin and Mcl-1, except p53 [80]. Rituximab is a chimeric monoclonal antibody against the CD20 protein on the surface of B cells and causes cell death after binding of the ligand to the receptor. This drug is used in the treatment of non-Hodgkin's lymphoma (NHL), chronic myelocytic leukemia (CLL), autoimmune diseases such as rheumatoid arthritis (RA), and skin lesions caused by the EBV virus. Li et al. studied the effects of monoclonal antibody in combination with quercetin on DLBCL lymphoma cell lines (WUS-DLCL-2 and SUDHL-4). They found that by using the cell viability method, these cell lines are sensitive to 20 μM quercetin; furthermore, by using flow cytometry, quercetin induces apoptosis in these cell lines. The amount of 20 μM quercetin together with 5 μg/ml of antibody increased the sensitivity of the cell lines to the rituximab antibody. They also found that antibody-induced apoptosis was amplified by quercetin on the cell lines, and the quercetin-antibody complex inhibited the p-STAT3 pathway (involved in the regulation of Mcl-1, survivin and Bcl-x1 genes) because the expression of these genes was significantly reduced [81].

In addition to the STAT3 signaling pathway, the PI3K/AKT/mTOR and Wnt/β-catenin pathways can be inhibited by natural products such as quercetin. These pathways cause highly aggressive B-cell lymphomas, such as primary effusion lymphoma (PEL). The Granato research team found that quercetin induces the death of the BC1, BC3, and BCBL1 cell lines in a dose-dependent manner by increasing the number of G1 events and causing cell death by PARP cleavage. They also found that quercetin inhibited PI3K and mTOR kinase and reduced the Wnt activation pathway, which eventually caused cell death to occur by inhibiting PI3K/AKT/mTOR signaling. mTOR was less phosphorylated, and quercetin reduced AKT phosphorylation at the Ser473 position. Quercetin also decreased prosurvival molecules downstream of these pathways, such as c-FLIP,

cyclin D1, and c-Myc, and it reduced nuclear localization of p65 NF-κB. They showed that protein phosphatase inhibitors neutralized dephosphorylation of tyrosine STAT3 by quercetin in the BC3 cell line, and since it has two-way communication between these pathways, it seems that quercetin reduces the release of the IL-6 and IL-10 cytokines. According to them, quercetin regulates the autophagy process by inhibiting the mTOR pathway and the STAT3 pathway and reducing the expression of FADD-like IL-1β-converting enzyme- (FLICE -) inhibitory protein (c-FLIP) and viral FLICE-Inhibitory Proteins (v-FLIPs) in the cell lines. In addition, the survival of BC3 and body-cavity-based lymphoma (BCBL1) cells is reduced by the quercetin-bortezomib complex and quercetin increases the activity of this proteasome inhibitor. Following the activation of T_h $CD4^+$ cells, the expression of HLA II molecules (HLA-DR) in cells increases and the expression of HLA I decreases, and quercetin induces exposure to calreticulin on the surface of malignant cells [59].

Salazar et al. studied the *HIGD2A* gene (involved in cell survival under hypoxia) and its role in diffuse large B-cell lymphoma (DLBCL) by modulating the expression of this gene in mice. Using RT-qPCR analysis, they found that exposure to 50 mg/ml quercetin caused the expression of this gene in the bone marrow tissue (reduced hypoxia) of lymphoma mice. In addition, it increased in the liver and spleen tissue (increased hypoxia) due to the rapid proliferation of tissue, while in healthy mice, the expression of this gene in the bone marrow and spleen increased but it decreased in the liver. Finally, they found that *HIGD2A* gene expression increased in DLBCL cells and decreased in nodal marginal lymphoma (NMZL) [82].

5.4. Quercetin and Burkitt's Lymphoma. Another malignant disease of the lymphatic system is Burkitt's lymphoma. This type of malignancy is most often associated with B lymphocytes in the germinal regions [100]. Clinically, this malignancy is classified into three categories including the endemic or African variant in which the EBV virus infects children, the sporadic or non-African variant hosted by children, and the immune system defect-related variants that are often associated with HIV infection or in people who are immunocompromised [101–103]. These variants are often associated with mutations in c-Myc gene which is a regulator and protooncogene [104]. Risk factors for the disease include HIV/AIDS infection, posttransplantation immunosuppression, and malaria infection [105]. Treatment of this type of malignancy depends on the type of the disease. Nevertheless, the first line of treatment, depending on when the disease is diagnosed and whether the tumor is slow or fast growing, is the use of chemotherapy. Drugs which are used to treat Burkitt's lymphoma are cyclophosphamide, doxorubicin, methotrexate, rituximab, etc. In addition to chemotherapy, immunotherapy, bone marrow transplantation, stem cell transplantation, radiotherapy, etc., are also used for treatment [100]. Recently, herbal products with active ingredients have been used to treat this type of malignancy because they have less adverse effects. Based on their research on Raji cells (Burkitt's lymphoma cells), Okamoto et al. found that quercetin inhibited the stimulation of primary antigens (EA) by the EBV virus which was induced by 12-O-tetradcanoyl phorbol-13-acetate (TPA). They also found that doses of 25, 10, and 2.5 μg/ml of quercetin inhibited 82, 74, and 32% of primary viral antigens, respectively. Finally, they concluded that natural inhibitory substances such as quercetin may also be beneficial in increasing lymphoma inhibition by TPA [106].

Inadequate functioning of the PI3K pathway is involved in the growth and development of a variety of malignancies. As mentioned earlier, quercetin inhibits the PI3K pathway. Lim et al. studied a subset of a quercetin called fisetin. They found that because PI3K has several isoforms and is expressed in the Raji cell line (gamma and delta isoforms are expressed more on these cells than alpha and beta), tPI3Kδ can be targeted by fisetin followed by induction of apoptosis. They also showed that fisetin reduced the expression of anti-/proapoptotic molecules. Therefore, using the immune-blotting technique, 30 μM concentration of fisetin reduced cIAP-1 and cIAP-2 protein expression. It also inhibited the mTOR pathway by inhibiting phosphorylation of the mTOR pathway (S2448) and its downstream targets, p70S6K and 4E-BP1. Following this process, apoptosis increased in Raji cells. 30 μM of fisetin also increased the expression of γH2A.X protein (a marker of cell DNA damage), which in turn indicated the proapoptotic activity of this substance on Raji cells [84].

As mentioned earlier, one of the causes of Burkitt's lymphoma is overexpression of the c-Myc gene and its translocation. Granato et al. demonstrated that quercetin generally reduced and inhibited the c-Myc expression and the PI3K/AKT/mTOR pathway and induced cytotoxicity against Raji, Akata, 2A8, Ramos, and BL-41 as Burkitt's lymphoma cells. The found that quercetin reduced tumorigenesis in BL-41 cells because 50 and 100 μM concentrations of this substance decreased c-Myc expression in BL-41, Raji, Akata, and Ramos cells. By phosphorylating 70S6K and 4E-BP1, the mTOR pathway was inhibited. The effect of quercetin-mediated toxicity on BL-41 and Ramos cells was greater than on Raji and Akata cells. Western blot analysis showed that quercetin was involved in PARP fragment cleavage in apoptotic cell death. The decreased expression of the c-Myc protooncogene and inhibition of the PI3K/AKT/mTOR pathway leads to BL-41 cell death. It seems that quercetin reduces the expression of heat shock protein (HSP-70) followed by a decreased expression of c-Myc protein in BL-41 cells. Quercetin also affects HSP-90 expression and decreases HSP-70 expression in Raji and Akata cell lines. Quercetin stimulates the autophagy process in BL-41 cells because the lipidation of cells (autophagy markers) treated with this substance is increased and the induction of the autophagy process helps to reduce c-Myc expression in some cell lines [60].

5.5. Antimalignant Effect of Quercetin (Molecular Mechanisms). Since it seems that quercetin has chemopreventive and chemotherapeutic effects in vivo and in vitro, it has been used as an antitumor drug. This natural product has antioxidant effects in small amounts and prooxidant effects in large amounts; therefore, it can be used as a chemotherapy drug by reducing malignant cell proliferation, apoptosis,

cell-cycle arrest, etc., through various molecular pathways that have become more and more interesting [107–109].

5.6. Role in Cell Proliferation. One of the main roles of quercetin is to inhibit the growth and proliferation of many types of malignant cells, including lymphoma, prostate, cervix, lung, breast, and colon. Quercetin can cause cell-cycle arrest in the G2/M phase or G1 phase, depending on the type of cancer cell [77, 110, 111]. Cyclins as well as kinase-dependent cyclins (cdks) play a major regulatory role in the cell cycle [112, 113]. The degree of cell inhibition depends on the concentration of quercetin and has often been studied in vitro [114]. Quercetin-mediated cell-cycle regulation can be interceded by p21, p27, p53, and Chk2 proteins. Regulation of reduced expression of Cdk1 and cyclin B1 as well as pRb phosphorylation can be shown on many types of malignant cell lines [115, 116]. As mentioned before, Granato et al. have investigated cell biology, growth, and proliferation of B lymphoma cells (including BC1, BC3, and BCBL1 cell lines) at different concentrations of quercetin [59].

5.7. Role in Oxidative Stress. Oxidative stress is caused by an imbalance between oxidants (such as ROS) and antioxidants (such as detoxification systems) [117]. Quercetin increases the activity of antioxidants by increasing the glutathione (GSH) level. GSH as a donor is involved in the conversion of H_2O_2 to H_2O by the enzyme superoxide dismutase (SOD). Quercetin stimulates GSH production [118]. The high activity of signaling pathways involved in cell survival, proliferation, and migration is directly related to oxidative stress. For example, ROS can activate the PI3K/AKT pathway, which in turn plays a crucial role in the growth and development of various malignancies [119]. According to Maurya et al., 300 μM of quercetin inhibited the increase of ROS production, which in turn phosphorylated the AKT, PDK1, and Bcl-2 promoters, followed by downregulation. In Dalton lymphoma ascite (DLA) cells treated with H_2O_2, quercetin increased the level of PTEN protein [88].

5.8. Role in Autophagy. One of the natural cell mechanisms to remove unnecessary cell components is the process of autophagy, and due to this, the cell components are decomposed and recycled. This process helps to stimulate malignant growth by increasing the survival of malignant cells [120–122]. Several studies have shown that quercetin causes instability and does not induce autophagy in malignant cells. It also diminishes autophagy loss by reducing the stabilization of β-catenin and HIF-1α and inhibiting the phosphorylation of AKT, mTOR, and ERK [76]. The STAT pathway (signal transducer and transcription activator) is associated with JAK family proteins and is also involved in mediating cellular responses to cytokines. In general, STAT3/5 is involved in the inflammatory and carcinogenic processes, and conversely STAT1 is involved in the inflammatory process [123]. Granato et al. found that quercetin induced autophagy in PEL cells by inhibiting the STAT3 and PI3K/AKT/mTOR signaling pathways. They also indicated that quercetin induced prosurvival autophagy in PEL cells [59].

5.9. Role in Apoptosis. In cellular organisms, one of the main causes of changes in cell morphology and eventually cell death is apoptosis or programmed cell death. This process generally occurs through two paths, intrinsic and extrinsic, both of which cause cell death by caspase proteins [124]. During apoptosis, changes occur on the cell surface, including bulging of the cell surface, cell shrinkage, fragmentation of the cell nucleus, chromatin condensation, and mRNA loss [112, 125]. Quercetin stimulates apoptosis through the mitochondrial pathway such as activation of caspase-3 and caspase-9 proteins, the release of cytochrome c, and cleavage of the PARP fragment (poly-ADP-ribose polymerase) [126, 127]. In a study on lymphoma cell lines (U937), quercetin and shHSP27 reduced the expression of proteins involved in the Notch/AKT/mTOR signaling pathway [128]. In another study on the Burji lymphoma cell line (Raji cell line), it was shown that 120 μM of quercetin increased the rate of cell apoptosis [16].

5.10. Therapeutic Potential of Quercetin for Lymphoma. Quercetin as an important polyphenolic bioflavonoid that exists in most fruits and vegetables [129] has antimalignant effects besides its antioxidant effects. Recently, some studies indicated that quercetin could suppress the proliferation of cancer cells in vitro [130–132]. Moreover, quercetin has different antimalignant activities against non-Hodgkin's lymphoma including a modulatory effect on the protein kinase (PKC) signaling pathway and apoptosis induction by inhibition of reactive oxygen species and tumor necrosis factor (TNF) receptor, suppression of the STAT3 pathway, and downregulation of Mcl-1, survivin, and p53 [81, 133, 134].

As mentioned above, it has been suggested that the mitochondria are the main targets for quercetin. The treatment with quercetin increased the release following TRAIL stimulation. Quercetin had synergic effects with TRAIL; however, the underlying molecular mechanism has not been identified [135]. The TRAIL-R2 stabilized in quercetin-mediated sensitization to TRAIL. Subsequently the expression of TRAIL-R2 at the cell surface upregulated and increased TRAIL DISC formation [136–138]. Since the mitochondrial apoptotic pathway is suppressed in resistant B-lymphoma cell lines, quercetin could restore the TRAIL apoptotic mechanism [80]. Quercetin-induced restoration of the mitochondrial apoptotic mechanism was correlated with Mcl-1 and survivin dysregulation. Survivin suppresses the mitochondrial Smac release, and it stabilizes XIAP which causes inhibition of caspase-9/3 activation at the postmitochondrial level [139]. Various transcription factors, e.g., signaling pathways such as p53 and AKT, negatively regulate the expression of survivin. In VAL and RL cells, the expression of survivin was inhibited following quercetin stimulation. Further studies are needed to clarify how the expression of survivin was inhibited by quercetin stimulation. Quercetin restores TRAIL-induced apoptosis in aggressive B-lymphoma cell lines via Mcl-1-induced proteasomal degradation. Mcl-1 preserves the cells from TRAIL-induced apoptosis by suppressing Bak, and Bid affects Bax activation. Moreover, quercetin-induced Mcl-1 proteasomal degradation was correlated with Mcl-1 ubiquitination and upregulation. Quercetin at higher concentrations

could induce apoptosis via downregulating Mcl-1 and activating Bax [140]. The advantages of quercetin including its low cost, potential beneficial effects, and its pharmacological safety suggest that quercetin could be a promising therapeutic approach for lymphoma.

5.11. Limitation of Quercetin Therapy. As mentioned earlier, the plant flavonoid quercetin has frequent biological and pharmacological functions. Numerous studies have shown that quercetin has anti-inflammatory, antioxidant, anticancer, antitoxic, and immunomodulatory properties. In addition to the beneficial and potential properties of quercetin in the treatment of diseases, their use has limitations that researchers need to pay close attention to. These limitations include poor solubility, low bioavailability, hydrophobic nature, and poor permeability. Many researchers are trying to overcome these limitations to use quercetin as a drug with minimal side effects and pharmacological limitations. For example, Nathiya et al. used polymer capsules to increase bioavailability and solubility by placing quercetin inside them [141]. Examining the pros and cons of quercetin in the brain and their diseases, Dajas et al. found that quercetin oxidation leads to the formation of quinones that are not reduced by antioxidants such as tocopherol (vitamin E) and ascorbate (vitamin C) and cause more damage to neurons (neurotoxicity). They concluded that modulating kinases could restore the redox equilibrium and was the only way to reduce quercetin restriction by preventing quinone formation [142]. In addition to these limitations, instability in physiological environments such as the stomach and intestines, a short half-life, and high metabolism in the liver before reaching the bloodstream reduces oral bioavailability. These factors limit the use of quercetin; to overcome these limitations, researchers have proposed efficient drug delivery systems that prevent drug damage to the gastrointestinal tract and reduce its instability when it enters the colon [143–145].

5.12. Clinical Studies with Quercetin. Previous studies have shown that quercetin has beneficial effects on many types of diseases. From recent years until now, the clinical (in vivo) use of quercetin in the treatment of various types of cancerous tumors has attracted the attention of many researchers. Several studies have shown that quercetin can exert its anticancer properties through different molecular mechanisms. It has been found that this compound with properties such as cell-cycle arrest, increased apoptosis, inhibition of angiogenesis and metastasis in vivo can help prevent the proliferation of tumor cells and reduce tumor size [146]. For example, in xenograft models of cancers such as leukemia and breast cancer, different doses of quercetin inhibit the cell cycle and increase programmed cell death by inhibiting the AKT/mTOR signaling pathway [147, 148]. In a study on prostate cancer, quercetin inhibited the growth of mouse prostate tumor cells in vivo by increasing the expression of Thrombospondin-1, an antiangiogenic factor [149]. The effect of quercetin on pulmonary-colorectal metastasis was also investigated, and it was found that a dose of 50 mg/kg of this compound reduces tumor metastasis [150]. Another study in BALB/c mice found that a dose of 150 mg/kg quercetin increased the apoptosis of liver tumor cells [151]. In a Phase I clinical trial, Ferry et al. examined the antiproliferative effect of quercetin on cisplatin-resistant ovarian cancer. Following treatment with 420 mg/m^2, in one patient the level of CA 125 decreased from 295 to 55 units/m^2; in another patient, the level of serum alpha phytoprotein decreased. As a result, they found that i.v. quercetin inhibits tyrosine kinase activity in lymphocytes and ultimately has an antitumor effect [152]. As a result, due to the fact that quercetin has limited side effects and is well tolerated in humans, it can be used in vivo (clinical trial) to treat a variety of cancers. Unfortunately, clinical studies have not been used to treat lymphoma, so due to the high activity of researchers, it is hoped that this combination can be used to treat lymphoma.

6. Conclusion

Quercetin possesses antitumor effects on malignant cells and induces its anticancer effect against cancer cells via modulating various signaling pathways involved in cancer development and progression. Quercetin promotes apoptosis and autophagy by activating caspase-3 and inhibiting AKT, mTOR, and ERK phosphorylation in cancer cells. In addition, quercetin inhibits metastasis by downregulating VEGF and MMP. Quercetin targets mitochondria in cancer cells, decking the bioenergetics and triggering the intrinsic pathway of apoptosis. Quercetin could be helpful as a supplement in cancer prevention and as a low-toxicity therapeutic molecule for cancer treatment. It seems that quercetin can strengthen the effects of other chemotherapeutic drugs. However, more investigations are required to completely elucidate its fully exact mechanisms of action against lymphoid cancer. Taken together, quercetin may be a promising candidate for lymphoid cancer treatment especially in combination with other chemopreventive drugs due to its potential synergistic effects.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

The conceptualization of this study came from S.R.S., V.T., P.A., W.C.C., and J.S.-R. They also coordinated the other authors and analyzed the collected papers. K.H., H.F., T.G., V.T., P.A., and J.S.-R. wrote the manuscript in collaboration with Ž.R. and W.C.C. Final editing was done by V.T. and J.S.-R. All authors have read and approved the manuscript. S.R.S. and K.H. contributed equally to this work.

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Research Article

Saccharomyces boulardii Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice by Regulating NF-κB and Nrf2 Signaling Pathways

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Saccharomyces boulardii (*S. boulardii*) is a probiotic yeast that is widely used to treat gastrointestinal disorders. The present study is aimed to explore the therapeutic effects of *S. boulardii* on dextran sulfate sodium- (DSS-) induced murine ulcerative colitis (UC) and illustrate the mechanisms of action. C57BL/6 mice were administered *S. boulardii* (10^5 and 10^7 CFU/ml, *p.o.*) for 3 weeks and then given DSS [2.5% (*w*/*v*)] for one week. Administration of *S. boulardii* prevented DSS-induced reduction in body weight, diarrhea, bloody feces, decreased colon length, and loss of histological structure. Moreover, *S. boulardii* protected the intestinal barrier by increasing the levels of tight junction proteins zona occludens-1 and Occludin and exerted immunomodulatory effects in DSS-induced mice. Furthermore, *S. boulardii* suppressed the colonic inflammation by reducing the levels of Interleukin-1β, Interleukin-6, and Tumor necrosis factor alpha and restored myeloperoxidase activity in mice exposed to DSS. *S. boulardii* also mitigated colonic oxidative damage by increasing the levels of antioxidant enzymes (superoxide dismutase, catalase, and heme oxygenase 1) and glutathione and decreasing malondialdehyde accumulation. Further studies identified that *S. boulardii* suppressed the nuclear translocation of nuclear factor kappa B (NF-κB) p65 subunit by decreasing IκKα/β levels, while promoted the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) in DSS-exposed mice. Collectively, *S. boulardii* possessed an appreciable therapeutic effect against the experimental mice model of UC. The protective mechanism of *S. boulardii* may involve inhibition of NF-κB-mediated proinflammatory signaling and activation of Nrf2-modulated antioxidant defense in addition to intestinal barrier protective and immunomodulatory effects.

1. Introduction

Ulcerative colitis (UC), one of the inflammatory bowel diseases, is characterized by persistent progression or relapsing inflammation that mainly involves the colonic mucosa and submucosa [1]. The incidence and prevalence of UC have been increasing worldwide in recent years with accelerating circadian rhythm and actuating pressure [2]. The relapse-remission cycles of patients by UC can progress to severe and even face total colectomy and develop colon cancer [3]. Up to now, the specific pathogenesis of UC is still unclear; thus, it is difficult to treat UC in clinic practice. Current

treatments (either biological applications or chemical drugs) are not able to obtain a satisfactory curative effect. Severe adverse responses like lymphoma and infection, as well as low efficacy, are always observed [4, 5]. Accordingly, alternative therapies or drugs with low side effects are urgently needed in UC management.

The etiology of UC is complicated, in which genetic susceptibility, hyperreactive immune system, mucosal barrier dysfunction, and alteration of intestinal flora are crucial contributory factors [6, 7]. During the development of colitis, activated immunocytes secrete multiple proinflammatory mediators such as reactive oxygen species (ROS), neutrophil infiltration, and cytokines, which stimulate an inflammatory cascade, resulting in the breakdown of the intestinal epithelial barrier and cell apoptosis, perpetuating chronic intestinal inflammation [8, 9]. Remarkably, nuclear transcription factor kappa B (NF-κB), a key regulatory component in inflammatory process, has been found to be observably activated in UC patients and strongly affects the progression of mucosal inflammation [10]. Moreover, as one of the pivotal transcription factors against inflammation and oxidative stress, nuclear factor erythroid 2-related factor 2 (Nrf2) is reported to be implicated in the suppression of proinflammatory signaling and inflammation-associated pathogenesis [11, 12]. In this regard, it is likely that NF-κB and Nrf2 signaling pathways are promising targets for the molecular therapy of UC.

Saccharomyces boulardii (*S. boulardii*) is a probiotic yeast that is resistant to low pH and is highly tolerant to bile acids [13]. It has been received more and more attention in the clinical application in recent years due to its beneficial properties including intestinal immune regulation and intestinal barrier protection [14]. For its few side effects, *S. boulardii* is widely used to treat gastrointestinal disorders, such as traveler's diarrhea, antibiotic-associated diarrhea, tube feeding-associated diarrhea, and acute diarrhea in children [15]. Experimental studies have shown that *S. boulardii* has specific probiotic properties involving impacts on enteric pathogen adhesion, mucosal immune factors, host cell signaling, and mediators of the inflammatory process [15, 16]. *S. boulardii* is able to block NF-κB activation and NF-κB-mediated inflammatory genes expression through producing a low molecular weight soluble factor in intestinal epithelial cells and monocytes [17]. Moreover, a recent study has shown that *S. boulardii* is capable of reducing ROS accumulation via activating Nrf2 pathway, thereby counteracting oxidative stress [18]. However, whether *S. boulardii* has the protective effect on UC and whether this benefit involves modulating oxidative stress and inflammatory reaction are yet poorly known.

Hence, the current study was aimed to examine the therapeutic effect of *S. boulardii* on a well-established mice model of colitis induced by dextran sodium sulfate (DSS) that mimics many clinical symptoms of human UC and, further, investigate the potential mechanisms involved.

2. Experimental Section

2.1. Chemicals. The lyophilized preparation of *S. boulardii* was obtained from Angel Nutritech Co., Ltd (Hubei, China. 2017081701). Commercial DSS was purchased from MP Biomedicals (Irvine, CA, USA). In addition, other chemicals mentioned in this paper were also laboratory specific.

2.2. Animals and Treatment. The animal experiments involved in this article were approved by the Animal Care and Use Committee (ACUC) of Tongji Medical College, Huazhong University of Science and Technology approval (s2135).

Female C57BL/6 mice (8 weeks old, 20-24 g) were from Beijing HuaFuKang Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were kept in a specific pathogen-free (SPF) environment with suitable temperature ($25 \pm 2^{\circ}$C) and humidity (55%), and the room followed a 12/12-hour light and dark cycle. These mice were provided with adequate amounts of food and water. Every fifth mouse was placed in a plastic cage. After one week of adaptive feeding, the mice were randomly divided into four groups, namely, the control group (noncolitis), the DSS group (colitis), the *Sb* − L group (10^5 CFU/ml) + DSS group, and the *Sb* − H group (10^7 CFU/ml) + DSS group ($N = 20$ per group). Mice were treated with distilled water or *S. boulardii* suspension by oral gavage for 21 consecutive days. On the 22nd day, the mice received 2.5% (wt/vol) DSS for 7 days to induce ulcerative colitis (Figure 1(a)). The doses of suspension were allocated by 10^5 and 10^7 CFU *S. boulardii* in 300 μL double-distilled water per day, which were proximately 0.1 and 10 folds of clinical doses. The weight changes, gross blood in the feces, and stool consistency were monitored daily for each mouse in a blind manner.

Once the mice were sacrificed, the blood was collected. The colon was removed aseptically and weighed and measured its length as well. The colonic tissue and fecal samples were frozen at -80°C for future use.

2.3. Disease Activity Index (DAI). To assess Disease Activity Index (DAI), the changes in the weight and feces of the mice were observed and recorded every other day. To quantify the score, the Cooper method was slightly modified [19]. The DAI assesses the severity of colitis in mice from three aspects: body weight loss, diarrhea, and blood (Table S1). Weight loss is defined as the difference between the initial weight at the beginning of the experiment and a given day. The four groups were scored for the DAI from the 21st to the 30th day.

2.4. Histologic Analysis. After obtaining the mice's distal colon tissues (1-2 cm from the anal verge), placed them in 4% paraformaldehyde solution and fixed them on a shaker for 24 hours. Then, the tissues were dehydrated, embedded, and sliced. To observe the histopathological changes between different groups, the colon tissues were stained with hematoxylin and eosin (HE) and observed and photographed under a microscope (Olympus, Japan).

Colonic histological damage was scored based on the combination of the severity of inflammation, mucosal and crypt damage, and ulceration [20]. The rules of scoring were shown detailly in Table S2. Six random fields were taken from each colon.

2.5. Biochemical Determinations in Colonic Tissue. Colonic tissues were lavaged, weighed, and put into normal saline.

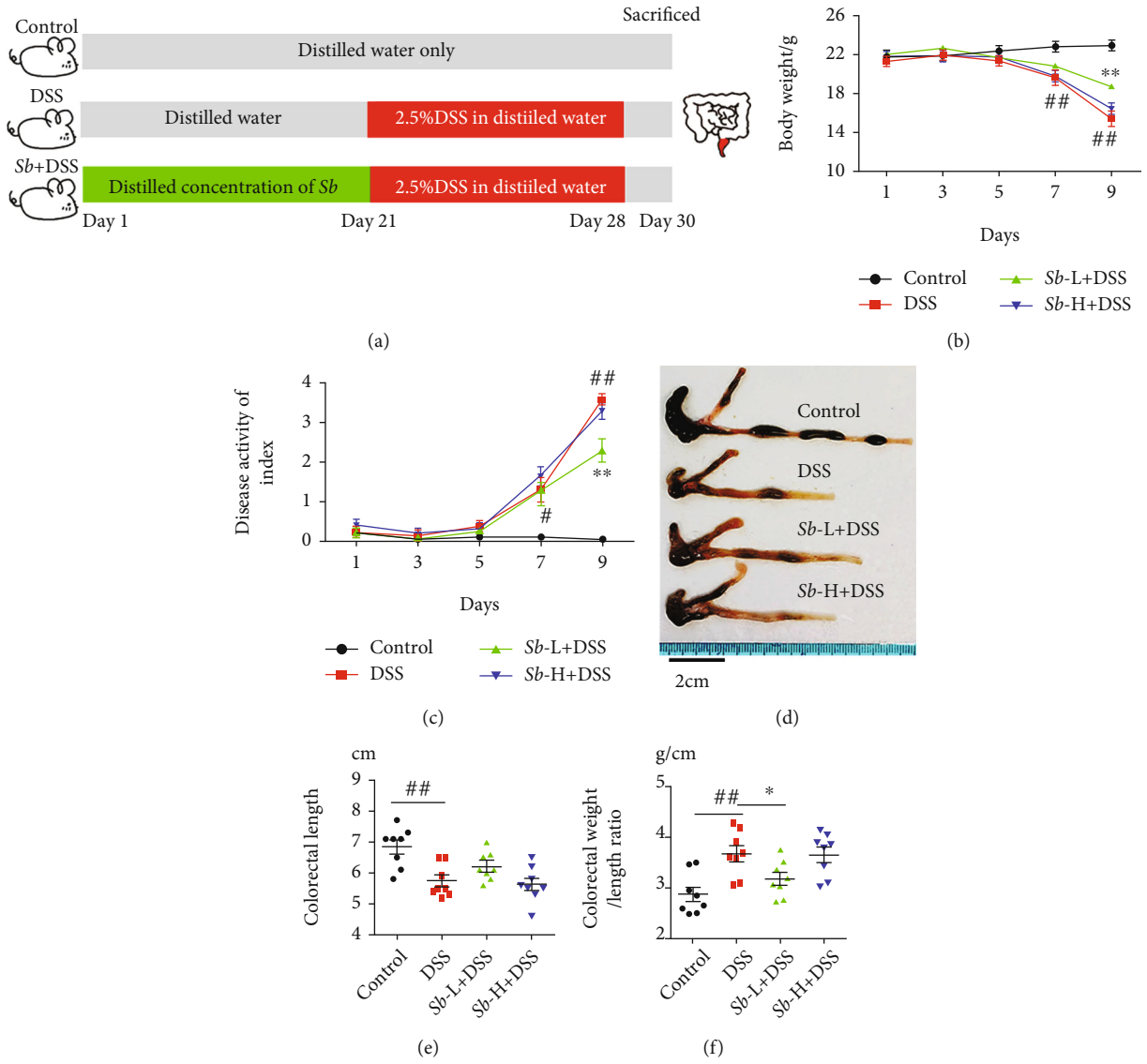


FIGURE 1: *S. boulardii* alleviated the symptoms in mice with ulcerative colitis. Mice were continuously administrated with 2.5% DSS for 7 days with or without *Sb* (10^5 and 10^7 CFU/ml) pretreatment for 21 days. (a) Scheme of the experiment. (b) Weight changes in mice. (c) DAI was recorded every other day. (d) The representative pictures of colon. (e) Average colorectal lengths. (f) Colorectal weight/length ratio in each group. $N = 8$. Values are presented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the control group. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the other groups. *Sb*: *S. boulardii*.

After tissue samples were homogenized, centrifuge at 4000 r/min for 10 min. The supernatant was collected for further detection of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and malondialdehyde (MDA) (Beyotime Biotechnology, Nantong, China). Kits for myeloperoxidase (MPO) analysis were from Jiancheng Bioengineering Institute (Nanjing, China). The detection is carried out according to the instructions of the commercial kits.

2.6. Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick-End Labeling (TUNEL) Assay. To assess apoptosis in colonocytes, the fluorescein (FITC) Tunel Cell Apoptosis Detection Kit (Servicebio, China) was used to perform TUNEL assay. Specifically, the paraffin-embedded colons were cut into 4 μm thick sample sections. After the sample sections were deparaffinized and rehydrated, incubated them with TUNEL reaction mixture in a 37°C incubator for 60 min. After that, incubated the samples with 4,6-diamidino-2-phenylindole (DAPI) (Roche, Switzerland) for 10 min. The results of TUNEL assay could be observed under a microscope (Olympus, Japan). Cells that were positive for apoptosis showed bright green nuclear staining. In each sample, six randomly optical areas were taken to quantify the staining.

2.7. Immunofluorescence Staining. The tissue sample sections were heated in EDTA/citrate antigen retrieval solution. The sections were deparaffinized with xylene, hydrated with ethanol of different concentration gradients; blocked with serum at room temperature for 30 minutes; then incubated with

Table 1: Sequences of primers used for Q-PCR.

Gene		Gene sequence
Il1b	Forward primer	GTGGCTGTGGAGAAGCTGTG
	Reverse primer	GAAGGTCCACGGGAAAGACAC
Il6	Forward primer	GAGGATACCACTCCCAACAGACC
	Reverse primer	AAGTGCATCATCGTTGTTCATACA
Tnf	Forward primer	CTGAACTTCGGGGTGATCGG
	Reverse primer	GGCTTGTCACTCGAATTTTGAGA
Tgfb1	Forward primer	CAACAATTCCTGGCGTTACCTT
	Reverse primer	TCGAAAGCCCTGTATTCCGTCT
Tjp1	Forward primer	GAGCGGGCTACCTTACTGAAC
	Reverse primer	GTCATCTCTTTCCGAGGCATTAG
Ocln	Forward primer	TGAAAGTCCACCTCCTTACAGA
	Reverse primer	CCGGATAAAAAGAGTACGCTGG
Gapdh	Forward primer	TGACCTCAACTACATGGTCTACA
	Reverse primer	CTTCCCATTCTCGGCCTTG

specific primary antibodies such as ZO-1 (1 : 100), Occludin (1 : 100), F4/80 (1 : 100), Ly6G (1 : 100), NF-κB p65 (1 : 100), and Nrf2 (1 : 100) overnight at 4°C; and followed by fluorescently labeled secondary antibodies in 37°C incubator for 60 min. After counterstaining with DAPI, the colon samples were observed and photographed by a microscope (Olympus, Japan). In each sample, six randomly optical areas were taken to analyze.

2.8. RNA Preparation and Q-PCR. Trizol reagent (Invitrogen, USA) was used to extract total RNA from colon tissues according to the instructions of the manufacturer. Single-stranded cDNA was reverse transcribed from RNA by a reverse transcription kit (Takara, Japan). Quantitative PCR was performed on the Roche light 480II. The mRNA levels of Interleukin-1β (*Il1b*), Interleukin-6 (*Il6*), Tumor necrosis factor alpha (*Tnf*), Transforming growth factor-β1 (*Tgfb1*), Tight junction proteins zona occludens-1 (*Tjp1*), and Occludin (*Ocln*) were measured by Q-PCR. The mRNA relative expression levels were normalized to the expressions of *Gapdh*. The primers used are listed in Table 1.

2.9. Western Blot. Colon tissues were homogenized in the protein lysis mix. The same amount of proteins was loaded on sodium dodecylsulfatepolyacrylamide gel and transferred to PVDF membranes (Millipore, USA). After blocking with skim milk for 60 minutes at room temperature, the PVDF membranes were incubated with corresponding primary antibodies at 4°C overnight. The following primary antibodies were used: rabbit anti-Occludin (1 : 1000, Abcam, UK), rabbit anti-ZO-1, anti-Nrf2, anti-heme oxygenase 1 (HO-1) (1 : 1000, ABclonal, China), rabbit anti-NF-κB p65, anti-IκKα, anti-IκKβ, anti-Phospho-NF-κB p65 (Ser536) (1 : 1000, Cell Signaling Technology, USA), and mouse anti-GAPDH (1 : 5000, Abbkine, China). HRP-conjugated secondary antibodies (1 : 4000, ABclonal, China) were used to incubate the blots, and the blots were observed by enhanced chemiluminescence (Bio-Rad, USA). The relative intensity of the target blot was quantified by Image J (NIH, USA). Four independent experiments were performed.

2.10. Flow Cytometry and Cell Sorting. The colon was digested into a single-cell suspension. Cells were incubated with PE/Cy7-conjugated anti-mouse CD45 (eBoscience, USA), FITC-conjugated anti-mouse CD3, BV421-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, APC-conjugated anti-mouse/human CD11b, and PE-conjugated anti-mouse F4/80 (BioLegend, USA) at room temperature in dark for 30 min for fluorescence-activated cell sorting (FACS) flow cytometry.

2.11. Statistical Analysis. All results are showed as mean ± SEM, and the differences among the means were determined by one-way analysis of variance (ANOVA) using SPSS 21.0 (IBM SPSS, Inc., USA). $P < 0.05$ was considered statistical significance.

3. Results

3.1. S. boulardii Alleviates the Clinical Symptoms of DSS-Induced Colitis. Mice exposed to DSS showed significant weight loss from the seventh day (Figure 1(b)), and correspondingly, a notable increase in DAI (Figure 1(c)) evidenced by apparent diarrhea and rectal bleeding when compared with the control animals. Moreover, DSS-treated mice displayed a significant reduction in colon length (Figures 1(d) and 1(e)), while a marked increase in colon weight/length ratio (Figure 1(f)) in comparison to controls. However, administration of *S. boulardii* at low dosage significantly prevented the weight loss, improved the DAI, and decreased the colon weight/length ratio in DSS-exposed mice (Figures 1(b)–1(f)). Collectively, these results suggest that *S. boulardii* alleviates the clinical symptoms of colitis in DSS-exposed mice.

3.2. S. boulardii Attenuates Histopathological Changes in DSS-Induced Colitis. Colon tissues from control mice were free of histological lesions. In contrast, mice with ulcerative colitis exhibited severe damage in intestinal segments, including the loss of histological architecture, decreased in crypt numbers, and infiltration of granulocytes and monocytes in the mucosa and submucosa (Figure 2(a)). Additionally, the severity of DSS-induced colitis was evaluated, and the histopathological damage score was higher in DSS-exposed mice than in control animals (Figure 2(b)). However, *S. boulardii* effectively alleviated these histological lesions and decreased the damage score in DSS-treated mice (Figures 2(a) and 2(b)).

To further confirm the degree of neutrophil infiltration, MPO activity, which positively reflects the number of neutrophil granulocytes, was determined. As shown in Figure 2(c), MPO activity was markedly increased in DSS-treated mice versus the normal controls ($P < 0.01$), which was significantly alleviated by administration of *S. boulardii* (10^5 CFU/ml) ($P < 0.01$).

To investigate the role of apoptosis in DSS-induced colitis and the antiapoptotic effect of *S. boulardii*, TUNEL assay was

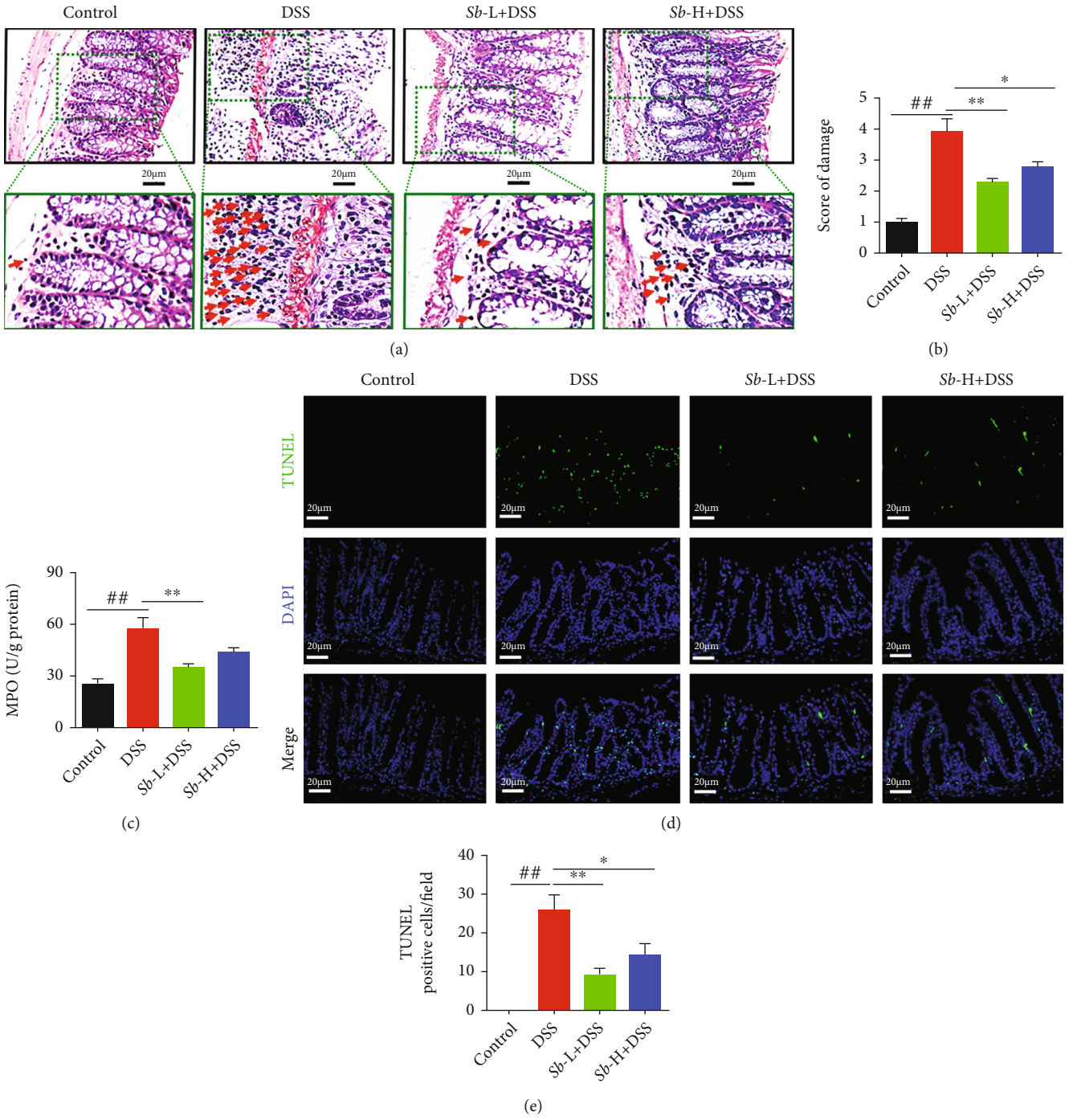
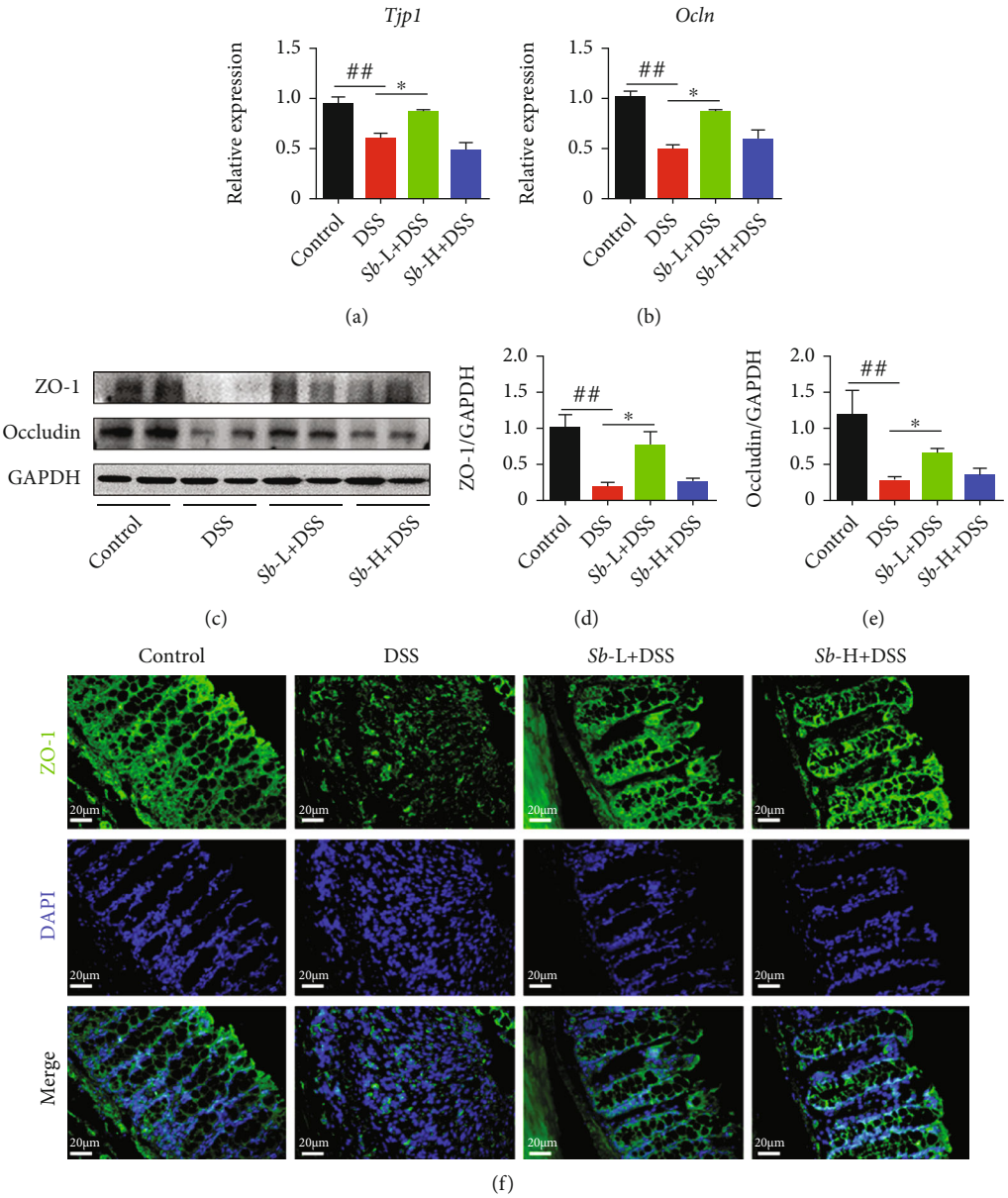


Figure 2: *S. boulardii* alleviated pathological alterations in ulcerative colitis. (a) Representative images of colon injury shown by hematoxylin and eosin (HE) staining (Magnification: ×400; Scale bars: 20 μm). Arrows indicated the infiltration of neutrophils and monocytes in mucosa. (b) Histopathological score on colons. (c) Colonic MPO level. (d) Representative colon images of colon cell apoptosis. (e) Quantification of TUNEL positive cell number/field. $N = 8$. Values are presented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the control group. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the other groups. *Sb*: *S. boulardii*.

performed. As depicted in Figure 2(d), the colonic epithelial cells of control animals were rarely TUNEL positive, while DSS-treated mice showed a dramatic elevation in TUNEL-positive cells ($P < 0.01$). Nevertheless, treatment with *S. boulardii* (10^5 and 10^7 CFU/ml) significantly prevented the increase in colonic epithelial cell apoptosis in DSS-exposed mice (Figure 2(e)).

3.3. S. boulardii Protects against Intestinal Barrier Dysfunction in DSS-Induced Colitis. To explore the effect of *S. boulardii* on intestinal barrier dysfunction in mice exposed to DSS, the levels of ZO-1 and Occludin, two key proteins within tight junctions and essential to the integrity of the epithelial and endothelial barriers, were determined. As shown in Figures 3(a) and 3(b), the mRNA levels of *Tjp1* and *Ocln*



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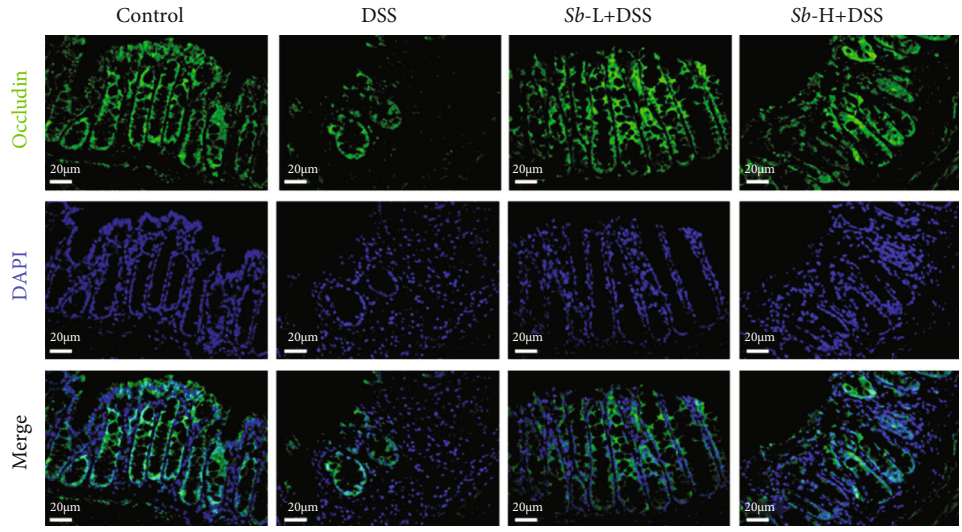


(g)

Figure 3: *S. boulardii* alleviated pathological alterations in colitis induced by DSS. Relative expression of mRNA in the colon (a) *Tjp1* and (b) *Ocln*. Representative western blot pictures (c) and quantification of (d) ZO-1 and (e) Occludin. (f, g) Representative immunofluorescence images of ZO-1 (f, green), Occludin (g, green) in distal ileum (Magnification: ×400; Scale bar: 20 μm). DAPI was used for nuclear counterstaining (blue). $N = 8$. Values are presented as mean ± SEM. $^{\#\#}P < 0.01$ compared with the control group. $^{*}P < 0.05$ versus the other groups. *Sb*: *S. boulardii*.

were significantly reduced in DSS-exposed mice as compared with the control animals. However, treatment with low-dosage *S. boulardii* uniformly elevated *Tjp1* and *Ocln* levels in DSS-exposed mice (both $P < 0.05$). At the same time, the protein levels of ZO-1 and Occludin were obviously decreased in the colons from DSS-exposed mice as compared to the controls (both $P < 0.01$, Figures 3(c)–3(e)). Nevertheless, administration of *S. boulardii* (10^5 CFU/ml) significantly suppressed these alterations. Consistently, immunofluorescence of tight junctions in the distal ileum demonstrated that *S. boulardii* administration ameliorated the changes in ZO-1 and Occludin levels induced by DSS (Figures 3(f) and 3(g)). These results suggest that *S. boulardii* attenuates the DSS-induced intestinal barrier dysfunction through promoting ZO-1 and Occludin expression.

3.4. S. boulardii Displays Immunomodulatory Effects in DSS-Induced Colitis. Next, the immunomodulatory properties of *S. boulardii* were characterized in colitis mice, involving in macrophages, neutrophils, and the intestinal immune response. First, the level of the F4/80, a unique marker of murine macrophage, was assessed. As exhibited in Figures 4(a) and 4(b), the intestinal macrophages expressing F4/80 were notably accumulated in DSS-exposed mice compared to those in controls ($P < 0.01$). Interestingly, administration of *S. boulardii* at both concentrations (10^5 and 10^7 CFU/ml) resulted in a significant reduction of F4/80$^+$ macrophages in DSS-treated mice (both $P < 0.05$).

And then, the neutrophil surface antigen Ly6G, which regulates leukocyte migration and neutrophil recruitment, was examined. As exhibited in Figures 4(c) and 4(d), an obvious neutrophil infiltration in colon evidenced by Ly6G staining was seen in DSS-treated mice when compared to control animals (Figures 4(c) and 4(d)). Of note, the inhibition of Ly6G$^+$ granulocytes recruitment was achieved only by 10^5 CFU/ml *S. boulardii* treatment ($P < 0.05$).

Finally, the percentages of CD4$^+$ and CD8$^+$ T cells, which are very important for immune defense, were determined. As presented in Figures 4(e)–4(g), the percentage of CD4$^+$ T cells was dramatically increased, while CD8$^+$ T cells were obviously decreased in DSS-exposed mice compared with the controls (both $P < 0.05$). However, *S. boulardii* treatment at both dosages (10^5 and 10^7 CFU/ml) reversed these changes in DSS-treated mice (both $P < 0.05$). These data indicate that the administration of *S. boulardii* regulates the immune response in colitis mice.

3.5. S. boulardii Modulates Inflammation by Suppressing NF-κB Signaling in Colitis Mice. To identify further the inflammation modulatory effect of *S. boulardii* on DSS-elicited colitis, the levels of several inflammatory cytokines mRNA (*Il1b*, *Il6*, *Tnf*, and *Tgfb1*) were measured by Q-PCR. As depicted in Figures 5(a)–5(d), the levels of *Il1b*, *Il6*, and *Tnf* were significantly increased in the colon of DSS-exposed mice in comparison with the control animals (all $P < 0.01$). However, administration of *S. boulardii*, especially at the low dosage, suppressed the upregulation of those cytokines in the colons of mice exposed to DSS (all $P < 0.05$). In addition, *Tgfb1* level was profoundly decreased in the inflamed colonic tissue of DSS-exposed mice while reversed by *S. boulardii* treatment ($P < 0.05$).

Since the NF-κB signaling pathway has a vital role in the inflammatory process, the key components of this pathway were assessed by western blot. As compared to the controls, the levels of p-NF-κB p65, NF-κB p65, and inhibitor of kappa B (IκB) kinase α/β (IκKα/β) were obviously increased in the colons from DSS-exposed mice (all $P < 0.01$, Figures 5(e)–5(i)), indicative of NF-κB signaling activation. Nevertheless,

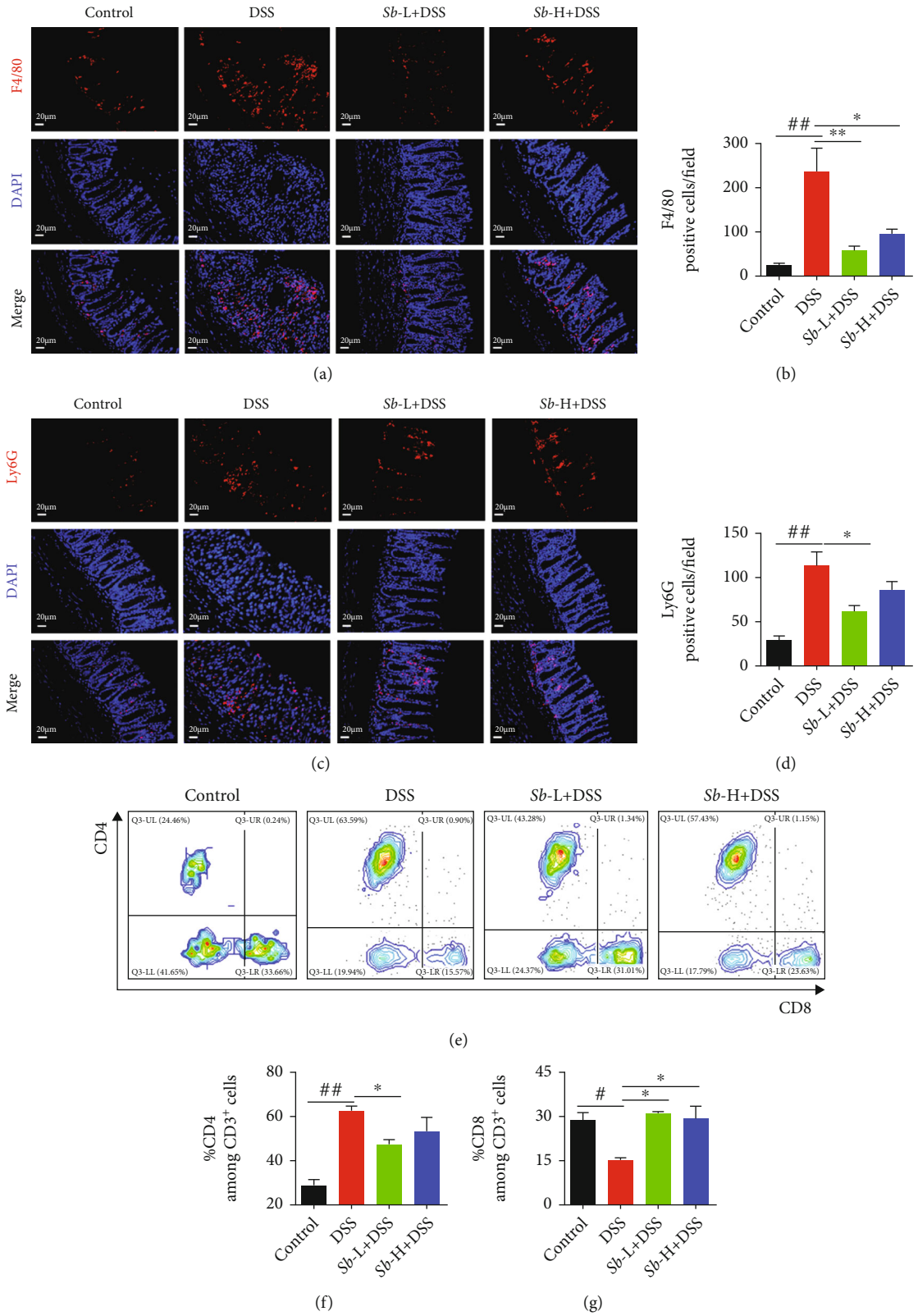
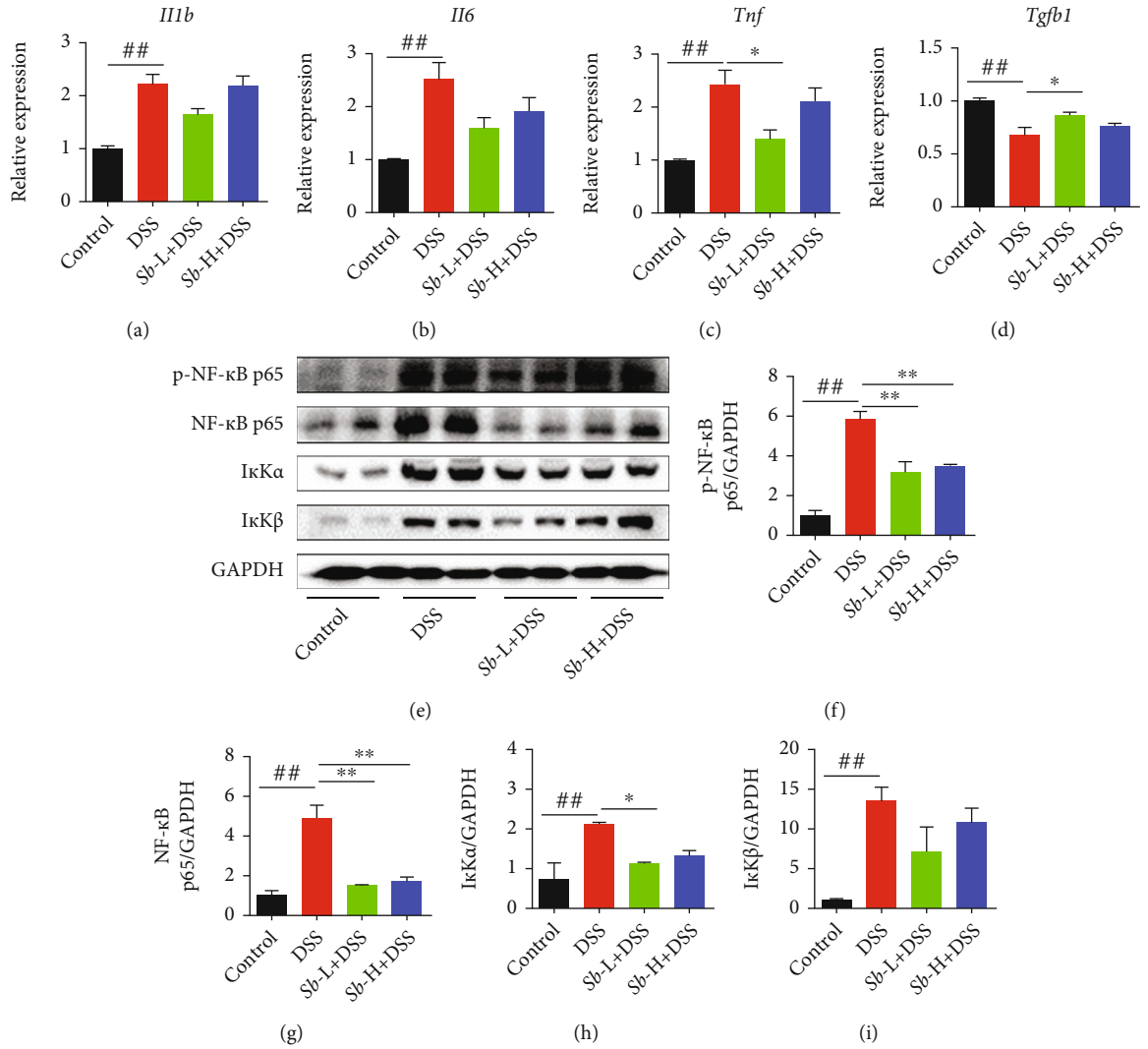


Figure 4: The immunomodulatory effects of *Sb* (10^5 and 10^7 CFU/ml) on colons of DSS-induced colitis mice. (a) Immunofluorescence staining analysis of F4/80-positive macrophages (red) in colon mucosa. (Magnification: ×200; Scale bar: 20 μm). DAPI was used for nuclear counterstaining (blue). (b) Statistics of F4/80. $N = 8$. (c) Immunofluorescence analysis of Ly6G positive neutrophils (red) in colon mucosa. (Magnification: ×200; Scale bar: 20 μm). DAPI was used for nuclear counterstaining (blue). (d) Statistics of Ly6G. $N = 8$. (e–g) The $CD4^+$ and $CD8^+$ T cell percentages were detected by flow cytometry. $N = 4$. Values are presented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with control group. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the other groups. *Sb*: *S. boulardii*.



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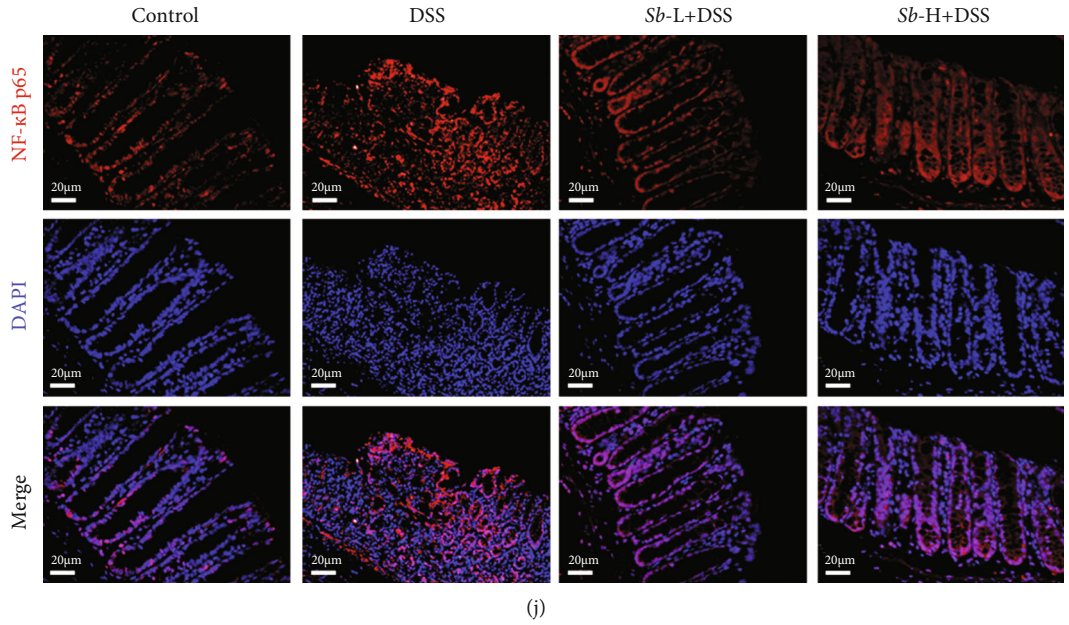

(j)

Figure 5: Inhibitory effect of *S. boulardii* on NF-κB signaling pathway in ulcerative colitis induced by DSS in mice. Relative expression of mRNA in the colon (a) *Il1b*, (b) *Il6*, (c) *Tnf*, and (d) *Tgfb1*. Representative western blot images (e) and quantification of (f) p-NF-κB p65, (g) NF-κB p65, (h) IκKα, and (i) IκKβ. (j) Immunofluorescence analysis of NF-κB p65 (red) in colon (Magnification: ×400; Scale bars: 20 μm). DAPI was used for nuclear counterstaining (blue). $N = 6$. Values are presented as mean ± SEM. $^{\#}P < 0.05$ versus control group. $^{*}P < 0.05$, and $^{**}P < 0.01$ compared with the other groups. *Sb*: *S. boulardii*.

administration of *S. boulardii* (10^5 and 10^7 CFU/ml) significantly suppressed these alterations. Of note, the degradation of IκKα but not IκKβ was only observed in mice treated with 10^5 CFU/ml *S. boulardii* ($P < 0.05$).

To further confirm NF-κB activation, the subcellular localization of the active subunit NF-κB p65 was evaluated. Immunofluorescence results demonstrated that the NF-κB p65 subunit was low intensity and mainly located in the cytoplasm in the control colons. Nonetheless, it has a much higher intensity than in the controls and is mainly located in the nucleus in the colons of DSS-exposed mice. As expected, administration of *S. boulardii* (10^5 and 10^7 CFU/ml) markedly reduced NF-κB p65 staining in the colons of DSS-exposed mice (Figure 5(j)). Taken together, these findings suggest that *S. boulardii* modulates inflammation by suppressing NF-κB signaling in colitis mice.

3.6. S. boulardii Activates Nrf2 Signaling to Reduce Oxidative Stress in Colitis Mice. In inflammatory diseases such as UC, inflammatory cells and epithelial cells generated a large amount of ROS, causing direct damage to colon epithelial cells and contributing to UC development [21, 22]. To test the antioxidant capacity of *S. Boulardii* in UC, the levels of oxidative stress markers were examined. As showed in Figure 6, the levels of SOD, CAT, and GSH in colons of mice exposed to DSS were notably reduced, while the level of MDA was obviously increased when compared to the control animals (all $P < 0.05$), suggestive of oxidative stress induced by DSS. However, treatment with *S. Boulardii* significantly relieved the oxidative stress by enhancing colonic SOD, CAT, and GSH levels and reducing colonic MDA level in DSS-exposed mice (all $P < 0.05$).

Since antioxidant enzyme gene expression is regulated by Nrf2 signaling pathway, the underlying mechanism implicated in the antioxidative effect of *S. boulardii* on DSS-induced colitis was explored by evaluating the expression and distribution of Nrf2 proteins. As shown in Figures 6(e)–6(g), the level of Nrf2 and HO-1 was significantly reduced in mice exposed to DSS compare to the controls ($P < 0.01$). However, *S. boulardii* significantly enhanced its level in DSS-treated mice ($P < 0.01$). Moreover, the effect of *S. boulardii* on Nrf2 activation was confirmed by immunofluorescence analysis of Nrf2 in colon mucosa cells (Figure 5(h)). In addition to activating the transcription of various antioxidant enzyme genes, Nrf2 is also able to activate the genes of transcription of phase II detoxification enzyme such as HO-1. As expected, *S. boulardii* notably reversed the DSS-induced decrease in HO-1 level in colons of treated mice. Collectively, these results suggest that *S. boulardii* mitigates oxidative stress by activating Nrf2 signaling in colitis mice.

4. Discussion

In this study, mice exposed to DSS demonstrated an elevated DAI with body weight loss, diarrhea, bloody feces, and decreased colon length. These were accompanied by severe histological alterations including the loss of morphological structure, reduction in number of crypts, and inflammatory cell infiltration. Those clinical signs resemble and are comparable to the symptoms usually observed in human UC [23, 24], suggestive of successful establishment of DSS-induced colitis mice model in this research. The present findings are consistent with previous studies [24–26]. Moreover, DSS

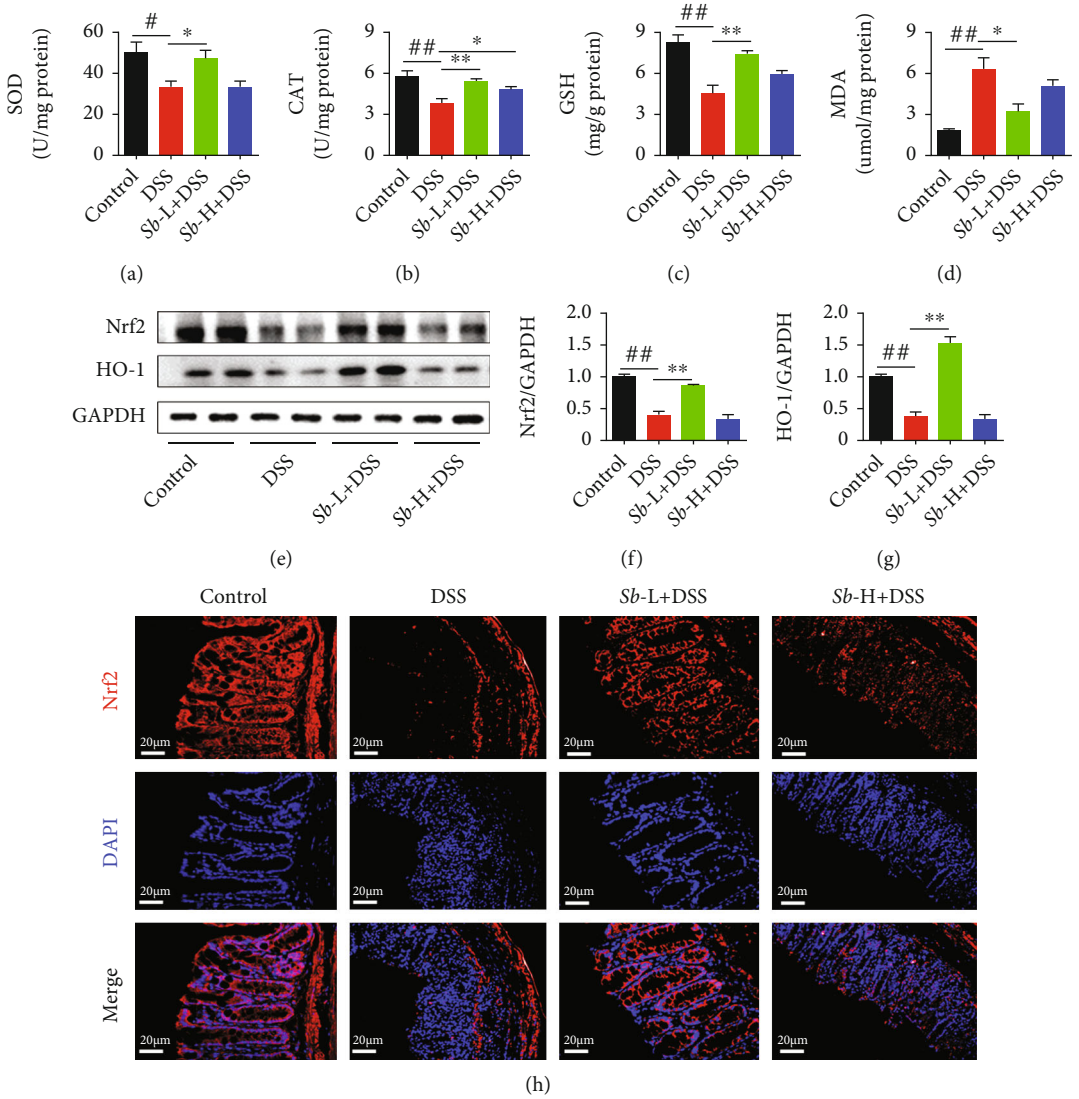

Figure 6: Detraction effect of *S. boulardii* on Nrf2 signaling pathway in DSS-induced colitis mice. Colon oxidative stress-related indicators (a) SOD, (b) CAT, (c) GSH, and (d) MDA. Representative western blot images (e) and quantification of (f) Nrf2 and (g) HO-1. (h) Immunofluorescence staining of Nrf2 (red) in colon (magnification: ×400; scale bar: 20 μm). DAPI was used for nuclear counterstaining (blue). $N = 6$. Values are presented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the DSS groups. *Sb*: *S. boulardii*.

induced excessive cell apoptosis in colonic tissue, which may play a crucial role in these observed morphological alterations. Additionally, DSS exposure displayed epithelial barrier dysfunction, evidenced by the decreased levels of tight junction proteins ZO-1 and Occludin in colons of mice. However, administration of *S. boulardii* reestablished the gut health and exerted a significant effect on structural and functional maintenance of colons in DSS-treated mice, demonstrating the promising preventive potential in UC.

Although the underlying specific etiologic causes and mechanisms of UC are not completely clear yet, the immune response abnormality has attracted more and more attention [27]. In the present study, DSS exposure induced a significant elevation in $F4/80^{+}$ macrophages, $Ly6G^{+}$ granulocytes, and $CD4^{+}$ T cells in mice colons, indicating immune response disorder. However, administration of *S. boulardii* exerted its immunomodulatory effects by reversing these alterations in DSS-treated mice. In addition, inflammatory response has also been implicated in the pathogenesis of UC [1]. Increasing evidence has shown that the proinflammatory cytokines result in intestinal tissue damage by amplifying the inflammatory cascade in DSS-induced UC [28, 29]. In agreement with this, the current work demonstrated a notable increase in the levels of *Il1b*, *Il6*, and *Tnf* in mice exposed to DSS. These observations are in line with previous findings from patients with UC [30] and in experimental colitis [31]. The levels of proinflammatory cytokines are affected by inflammatory cells including neutrophils, macrophages, and leukocytes. Infiltration of inflammatory cells in the colon has been suggested to contribute to the mucosal tissue impairment,

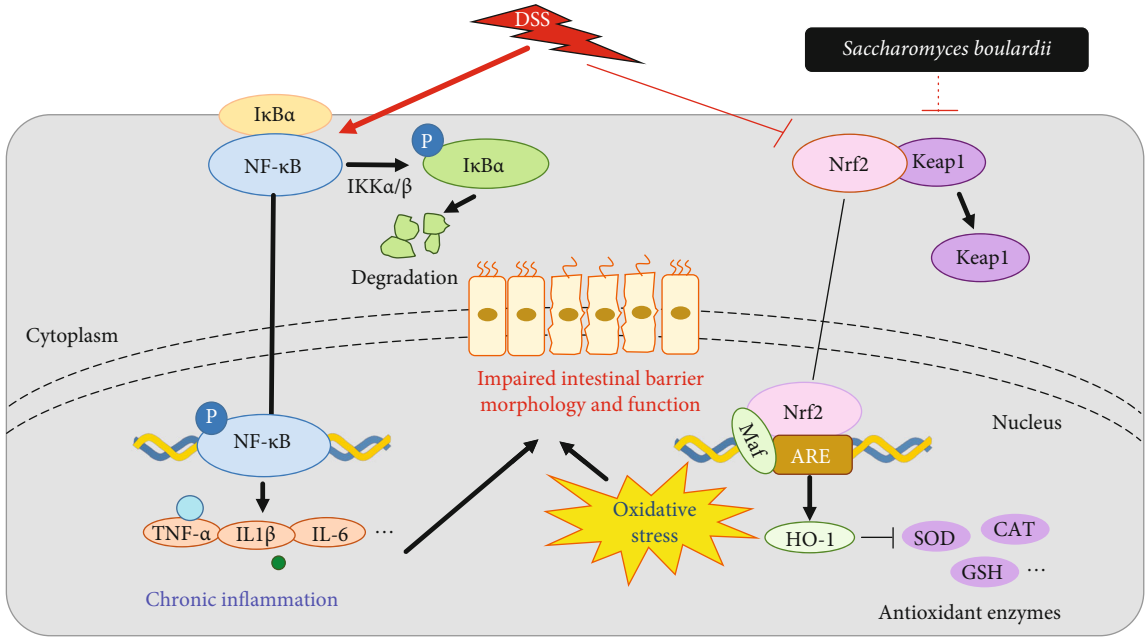

Figure 7: A proposed model for the mechanism of *S. boulardii* against DSS-induced UC.

mucosal barrier disruption, and subsequent inflammatory response [32]. This is further supported by the present result on elevated MPO activity, which is an index of colon mucosa neutrophil infiltration, a marker of acute inflammation [33]. Interestingly, administration of *S. boulardii* greatly reduced the activity of MPO and inhibited the expression of those cytokines in DSS-induced colitis mice, indicating that the protective effect of *S. boulardii* against colonic damage is associated with the regulation of inflammatory cytokines. Together, these observations indicate the ability of *S. boulardii* to modulate immune response and prevent inflammatory cells infiltration and inflammatory response in the colonic tissues of DSS-exposed mice.

The present study also provided insights into the molecular mechanism through which *S. boulardii* exerted its anti-inflammatory effect in DSS-induced colitis. It is known that the transcription of inflammatory cytokines relies on NF-κB activation, which is triggered by a sequential cascade including degradation of its inhibitory subunit IκBα by IκB kinases IκKα/β, as well as nuclear translocation of the cytosolic p65 subunit of NF-κB [34]. The current work showed that the levels of p-NF-κB p65 and IκKα/β in colons of DSS-exposed mice were significantly increased compared with control animals. Moreover, an intensive staining of NF-κB p65 subunit in nucleus was also observed in mice exposed to DSS, further confirming that the NF-κB signaling pathway was activated by DSS. Nonetheless, administration of *S. boulardii* downregulated the levels of p65 NF-κB, IκKα, and IκKβ, as well as the downstream inflammatory cytokines. Hence, it is likely that *S. boulardii* prevented IκBα degradation by decreasing IκKα/β levels, suppressing nuclear translocation of NF-κB, therefore inhibiting activation of NF-κB signaling, finally mitigating DSS-induced colitis. An earlier study by Sougioultzis et al. demonstrated that *S. boulardii* inhibits NF-κB activation and related proinflammatory signaling in host cells by producing a soluble anti-inflammatory factor [17], supporting the present findings. It is worth noting that *S. boulardii* increased *Tgfb1* mRNA expression in the current study. Since TGF-β1 induction has been shown to be effective for the recovery of inflammation-related colitis [35], further studies are needed to clarify the regulatory mechanism of inflammatory response by *S. boulardii* when treating colitis and other inflammatory diseases.

It has been shown that inflammation and oxidative stress create a vicious cycle contributing to the pathogenesis of many inflammatory diseases including UC [36]. During colitis, proinflammatory factors activate phagocytes, which are recruited to the mucosa and result in ROS generation [9], causing oxidative stress. Consistent with this, in the present work, DSS exposure induced accumulation of MDA level in the colonic tissues, suggestive of lipid peroxidation and increased production of ROS. The antioxidant defense system comprises the enzymatic and nonenzymatic antioxidants such as SOD, CAT, and GSH, which are responsible for the removal of free radicals and counteracting oxidative stress. The significant reduction in colonic SOD, CAT, and GSH levels was parallel with the marked elevation in MDA level in DSS-exposed mice in the current investigation. These findings suggest the inactivation of antioxidant defense system in the colonic tissues of DSS-exposed mice. Importantly, administration of *S. boulardii* notably improved the DSS-induced reduction in these antioxidants and accumulation of MDA in the exposed mice. Therefore, these observations imply that *S. boulardii* restored colitis-linked depletion of antioxidant system via, at least in part, boosting the levels of enzymatic and nonenzymatic antioxidants in the colons of DSS-exposed mice.

The Nrf2 signaling pathway plays a crucial part in cellular antioxidant defense [37–39]. Under physiological conditions, cytoplasmic protein chaperone Keap1 interacts with Nrf2 to retain the quiescent state. Upon oxidative stress, Nrf2 is able to escape from Keap1 and translocate to the nucleus, where it induces the transcription of a series of genes for antioxidant enzymes (including SOD, CAT, and GSH) as well as genes for phase II detoxification enzymes (such as HO-1) [40]. In the present study, *S. boulardii* significantly increased Nrf2 level and its nuclear localization in colons of DSS-exposed mice. Moreover, these were accompanied by restoration of DSS-induced decline in levels of SOD, CAT, GSH, and HO-1, as well as accumulation of MDA. Taken together, the above results indicate that *S. boulardii* promotes Nrf2 signaling activation to decrease the DSS-induced oxidative injury in colons.

5. Conclusion

The current results indicate that the administration of *S. boulardii* is effective for the prevention of DSS-induced mice UC. More importantly, *S. boulardii* exerts attractive intestinal barrier protective and immunomodulatory effects and, particularly, protects against UC via inhibition of NF-κB-mediated proinflammatory signaling and activation of Nrf2-modulated cytoprotective antioxidant defense mechanism (Figure 7). Thus, *S. boulardii* might be a new and attractive therapeutic option for UC.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Authors' Contributions

T.Y. and Y.Y. conceived and designed the experiments; H.G., Y.L., J.S., and H.X. performed the experiments; M.W., N.W., Y.G., Z.C., P.Z., and X.L. analyzed the data; X.Z., Q.F., T.Y., and Y.Y. contributed to the reagents/materials tools; H.G. and Y.L. wrote the paper. Hui Gao and Yinzheng Li contributed equally to this work. All authors read and approved the final manuscript.

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Supplementary Materials

Tables S1-S2 were listed in the Supplementary Material. *(Supplementary Materials)*

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Research Article

Grape Seed Proanthocyanidins Inhibit Migration and Invasion of Bladder Cancer Cells by Reversing EMT through Suppression of TGF-β Signaling Pathway

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Bladder cancer (BC) is the most common cancer of the urinary system. Despite advances in diagnosis and therapy, the prognosis is still poor because of recurrence and metastasis. Epithelial-mesenchymal transition (EMT) is considered to play an important role in the invasion and metastasis of BC. Grape seed proanthocyanidins (GSPs) exhibit chemopreventive and chemotherapeutic activities against several types of cancer. However, their effects and underlying mechanisms on the invasive potential of BC remain unclear. In this study, we found that GSPs inhibited migration, invasion, and MMP-2/-9 secretion of both T24 and 5637 bladder cancer cells at noncytotoxic concentrations. We also discovered that 5637 cells were more suitable than T24 cells for the EMT study. Further study showed that GSPs inhibited EMT by reversing the TGF-β-induced morphological change and upregulation of mesenchymal markers N-cadherin, vimentin, and Slug as well as downregulation of epithelial markers E-cadherin and ZO-1 in 5637 cells. GSPs also inhibited TGF-β-induced phosphorylation of Smad2/3, Akt, Erk, and p38 in 5637 cells without affecting the expression of total Smad2/3, Akt, Erk, and p38. Taken together, the results of the present study demonstrate that GSPs effectively inhibit the migration and invasion of BC cells by reversing EMT through suppression of the TGF-β signaling pathway, which indicates that GSPs could be developed as a potential chemopreventive and therapeutic agent against bladder cancer.

1. Introduction

Bladder cancer (BC) is the most common cancer of the urinary system. It ranks tenth among 36 cancers worldwide, with an estimated 549,000 new cases and about 200,000 deaths during 2018. Especially in men, it is the sixth most common cancer and the ninth leading cause of cancer death [1]. In China, BC is the 13th most common cancer and the 12th leading cause of cancer death. It is estimated that there were 78,100 new cases and 32,100 cancer death in 2014 [2]. Furthermore, according to Cai et al., both incidence and mortality of BC will continue to increase among some countries and regions in the next 15 years [3]. Despite significant progresses in combined therapies including surgery, chemotherapy, and radiotherapy, BC is still characterized by a poor prognosis and a low survival rate during the past decades, particularly as a result of recurrence and metastasis [4]. Therefore, it is necessary to identify mechanisms underlying BC development as well as develop novel therapeutic reagents for its treatment.

Epithelial-mesenchymal transition (EMT) is a process in which polarized epithelial cells undergo a phenotypic

transition that enables them to obtain mesenchymal cells traits. Hallmarks of EMT include the loss of expression of epithelial markers, such as E-cadherin and occludins, and the concomitant increase of mesenchymal markers including N-cadherin and vimentin [5–7]. EMT is considered a pathological process that promotes cancer progression, particularly invasion and metastasis. Through this process, cancer cells detach from the primary tumor and acquire migratory and invasive capabilities as well as resistance to therapy [8–10]. Therefore, inhibition of EMT would be an effective measure to block cancer invasion and metastasis. The most well-characterized factor responsible for the induction of EMT by far is TGF-β. TGF-β has a dual role in tumor progression. In the early stage, TGF-β is considered as a tumor suppressor by inducing cell differentiation and apoptosis. In the late stage, TGF-β contributes to cancer progression by promoting tumor invasion and metastasis, which is related to its induction of EMT [11, 12]. In bladder cancer, studies have shown that cancer-associated fibroblasts induce bladder cancer cell EMT and invasiveness through secreting TGF-β [13]. Therefore, inhibition or reversion of the TGF-β signaling pathway-mediated EMT might be an effective measure for bladder cancer treatment.

It is reported that many polyphenolic compounds have anti-EMT activity [14, 15]. Proanthocyanidins are dimers, trimers, and other oligomers of flavan-3-ols. As a kind of natural polyphenolic compounds, proanthocyanidins have significant antioxidant activities and are rich in grapes, especially in grape seeds [16]. Grape seed proanthocyanidins (GSPs) have been reported to possess chemopreventive and chemotherapeutic potential against several types of cancers. The underlying mechanisms are related to inhibition of proliferation, induction of apoptosis, arrest of cell cycle, inhibition of angiogenesis, and suppression of metastasis [17–19]. GSPs have also been shown to have the ability to reverse the process of EMT [20–22]. However, the effects and underlying mechanisms of GSPs on the invasive potential of BC remain unclear.

In the present study, we investigated the antimetastic activity and associated mechanisms of GSPs against human BC cells *in vitro*. Our results show that GSPs inhibit the migration, invasion, and MMP-2/9 secretion of both T24 and 5637 bladder cancer cells. GSPs also inhibit TGF-β-induced EMT of 5637 cells, which are related to the blocking of the TGF-β signaling pathway. Hence, the present study provides scientific evidences supporting GSPs as chemopreventive and chemotherapeutic agents against BC.

2. Materials and Methods

2.1. Chemicals and Reagents. Grape seed proanthocyanidins (GSPs) containing ≥95% proanthocyanidins, ≥1.8% proanthocyanidins B2, and ≥60% oligomers were obtained from Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). RPMI-1640 culture medium was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from National HyClone (Lanzhou) Bio-engineering Co., Ltd. (Lanzhou, China). Sulforhodamine B (SRB) and gelatin from porcine skin were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Millicell Cell Culture Inserts were obtained from EMD Millipore (Billerica, MA, USA). Matrigel and transforming growth factor-β (TGF-β) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). RIPA lysis buffer (protease and phosphatase inhibitors added) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies directed against N-cadherin, vimentin, E-cadherin, and β-actin were purchased from Abcam (Cambridge, MA, USA). Primary antibodies directed against ZO-1, Slug, Smad 2/3, p-Smad2 (Ser465/467)/Smad3 (Ser423/425), Akt, p-Akt, p44/42 MAPK (Erk1/2), p-p44/42 MAPK (Erk1/2), p38 MAPK, p-p38 MAPK, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). SuperSignal™ West Pico PLUS Chemiluminescent Substrate was purchased from Thermo Scientific (Waltham, MA, USA). GSPs were dissolved in DMSO as stock and diluted to appropriate concentrations with RPMI 1640 medium before use. The maximum final concentration of DMSO in cultures was less than 0.5%.

2.2. Cell Culture. Human bladder cancer cell lines T24 and 5637 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO_2.

2.3. Cell Viability Assay. The viability of cells was determined by the SRB assay as previously described [23] with certain modifications. Cells were plated into 96-well plates at a density of $4 - 6 \times 10^3$ cells/well in 100 μl 1640 medium supplemented with 10% FBS. Following incubation at 37°C overnight, cells were treated with GSPs at varying concentrations (0-200 μg/ml) for 24, 48, or 72 h. Subsequently, the cultures were fixed with cold 10% trichloroacetic acid at 4°C for 1 h and washed with water. Next, the plates were air-dried and the fixed cells were stained with 0.4% SRB at room temperature for 10 min and washed repeatedly with 0.1% acetic acid to remove the unbound dye. The bound SRB was dissolved in 1% Tris (pH 10.5). The optical density was measured at 515 nm using a microplate reader.

2.4. Cell Migration Assay. Cell migration was assayed in Millicell Cell Culture Inserts using a polycarbonate filter with a pore size of 8 μm as previously described [24]. Cells were trypsinized and suspended in a serum-free 1640 medium. A total of 8×10^4 cells in 0.4 ml medium were seeded into the upper chamber of cell culture inserts with various concentrations of GSPs (0-50 μg/ml). The culture inserts were then placed into 24-well plates filled with 0.6 ml 1640 medium supplemented with 20% FBS as a chemoattractant. Following incubation at 37°C for 24 h, the nonmigrated cells on the upper surface of the membrane were wiped off with a cotton swab. Cells that had crossed over the inserts were fixed with methanol for 30 min and then stained with 0.1% crystal violet for 10 min at room temperature. Images were captured using light microscopy (magnification, ×100). The number of cells that had migrated was counted using the Image J software

(version 1.52v; National Institutes of Health, Bethesda, MD, USA).

2.5. Cell Invasion Assay. A cell invasion assay was performed as previously described [25]. Briefly, the upper chamber of Millicell Cell Culture Inserts using a polycarbonate filter with a pore size of 8 μm was coated with 50 μl Matrigel diluted 1 : 8 with PBS. Subsequently, 4×10^5 cells in 0.4 ml serum-free 1640 medium with or without GSPs (0-50 μg/ml) were added to the upper chamber. The culture inserts were then placed into 24-well plates. Subsequently, the lower chamber was filled with 0.6 ml 1640 medium supplemented with 20% FBS as a chemoattractant to induce invasion. Following incubation at 37°C for 24 h, the culture inserts were removed, and the noninvasive cells on the upper surface of culture inserts were scraped away with a cotton swab. The cells that invaded through the Matrigel were fixed with methanol for 30 min and then stained with 0.1% crystal violet for 10 min at room temperature. Images were captured by light microscopy (magnification, ×100), and the number of cells was counted using the Image J software.

2.6. Gelatin Zymography. The enzymatic activities of MMP-2 and MMP-9 were examined by gelatin zymography as described previously [24]. Subconfluent cells were treated with GSPs (0-50 μg/ml) for 24 h in a serum-free 1640 medium. Following treatment, the conditioned medium was collected and centrifuged at 300 × g for 10 min at 4°C to remove cellular debris. The supernatants were incubated with sample buffer (without β-mercaptoethanol) for 0.5 h at 37°C and then subjected to 7.5% SDS-PAGE in a gel containing 1% gelatin. Following electrophoresis, the gels were washed twice with washing buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 2.5% Triton X-100] for 1 h and incubated in a reaction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 10 mM $CaCl_2$] at 37°C for 36 h. Subsequently, the gels were stained with 0.25% Coomassie Blue R250 for 30 min and destained for 10 min with 25% methanol and 7.5% acetic acid at room temperature. Enzyme activity was visualized as a bright band on a blue background. The band intensities were measured using the Adobe Photoshop CS5 software.

2.7. Western Blot Analysis. After treatment with TGF-β or TGF-β with or without GSPs for 24 h, cells were lysed using RIPA buffer. Total protein was collected and then quantified using the Bradford assay. Protein was separated via 10% SDS-PAGE (40 μg protein/lane). Following electrophoresis, the separated proteins were transferred to PVDF membranes. Next, the membranes were probed with the primary antibodies at 4°C overnight. Following this, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 4°C for 2 h. The desired proteins were detected using SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's protocol. Bands were quantified using Adobe Photoshop CS5.

2.8. Statistical Analysis. Data were expressed as the mean ± SD. Statistical significance was determined by Student's t -test and ANOVA using the SPSS software (version 20; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. GSPs Decrease the Viability of BC Cells. The effects of GSPs on the viability of BC cells were examined using the SRB assay. As shown in Figure 1, treatment with different concentrations of GSPs in different times inhibited the viability of both T24 and 5637 cells in a dose-dependent manner. The half-maximal inhibitory concentration values (IC_{50}) to T24 cells were 206.8, 86.9, and 56.2 μg/ml for 24, 48, and 72 h. Accordingly, the IC_{50} values to 5637 cells for 24, 48, and 72 h were 288.3, 98.0, and 52.6 μg/ml, respectively. As treatment with 6.25-50 μg/ml GSPs for 24 h had no significant inhibitory effects on the viability of both T24 and 5637 cells, we carry out subsequent experiments under these conditions.

3.2. GSPs Inhibit Migration of BC Cells. Cancer cell migration and invasion are the initial and critical steps in the process of cancer metastasis. We then investigated the effects of GSPs on the migration of BC cells using a transwell migration assay. After stimulated T24 cells with 20% FBS for 24 h, a large number of cells migrated to the lower side of the chamber. Treatment with different concentrations of GSPs inhibited the FBS-induced migration of T24 cells in a dose-dependent manner. The inhibition rates of 12.5, 25, 50 μg/ml GSPs were 20.7%, 42.6%, and 67.5%, respectively (Figure 2(a)). The similar inhibitory effects of GSPs were also observed on 5637 cells. As shown in Figure 2(b), GSPs treatments inhibited migration of 5637 cells dose-dependently with the inhibition rates of 3.1%, 38.2%, and 67.9% for 12.5, 25, and 50 μg/ml GSPs, respectively.

3.3. GSPs Inhibit Invasion of BC Cells. The effects of GSPs on the invasive capacity of BC cells were also investigated. As shown in Figure 3(a), the addition of 20% FBS in the lower chamber significantly induced the invasion of T24 cells. GSPs significantly inhibited FBS-induced invasion of T24 cells dose-dependently. The inhibition rates of GSPs at 12.5, 25, and 50 μg/ml were 19.1%, 56.0%, and 80.0%, respectively. Similarly, GSPs treatment for 24 h also inhibited the invasion of 5637 cells. The inhibition rates of GSPs at 12.5, 25, and 50 μg/ml were 19.8%, 28.2%, and 45.1%, respectively (Figure 3(b)).

3.4. GSPs Inhibit the Secretion of MMP-2 and MMP-9 by BC Cells. The secretion of MMPs for extracellular matrix degradation is a key event in the process of tumor metastasis. So we further examined the effects of GSPs on the secretion of MMP-2 and MMP-9 by BC cells. Gelatin zymography showed that MMP-2 and MMP-9 activities in the conditioned medium collected from both T24 and 5637 cells were markedly reduced by GSPs dose-dependently (Figure 4).

3.5. Establishment of TGF-β-Induced EMT Model in BC Cells. EMT plays an important role in promoting tumor invasion and metastasis. TGF-β is one important inducer of EMT. To investigate whether GSPs decreased cancer migration

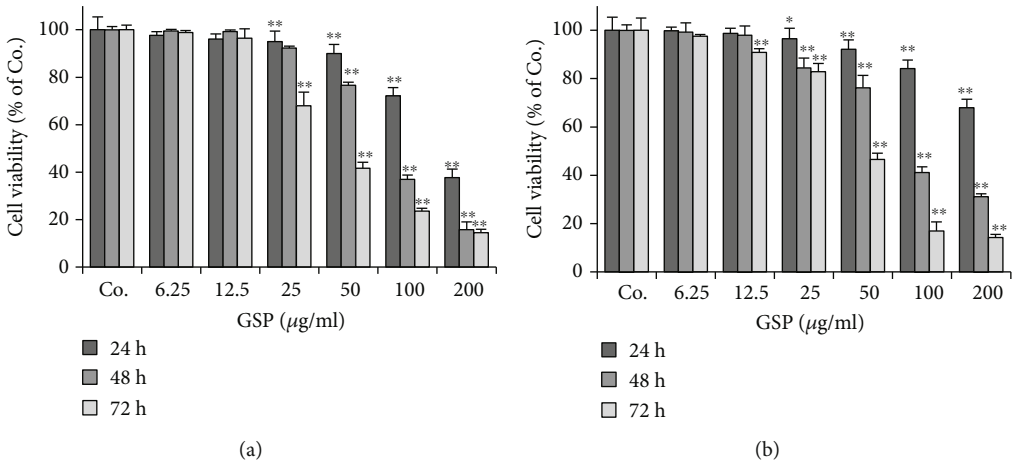

Figure 1: GSPs decrease cell viability of T24 (a) and 5637 (b) cells. After being treated with GSPs for the indicated time, the cell viability was measured with the SRB assay. The data were presented as mean ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

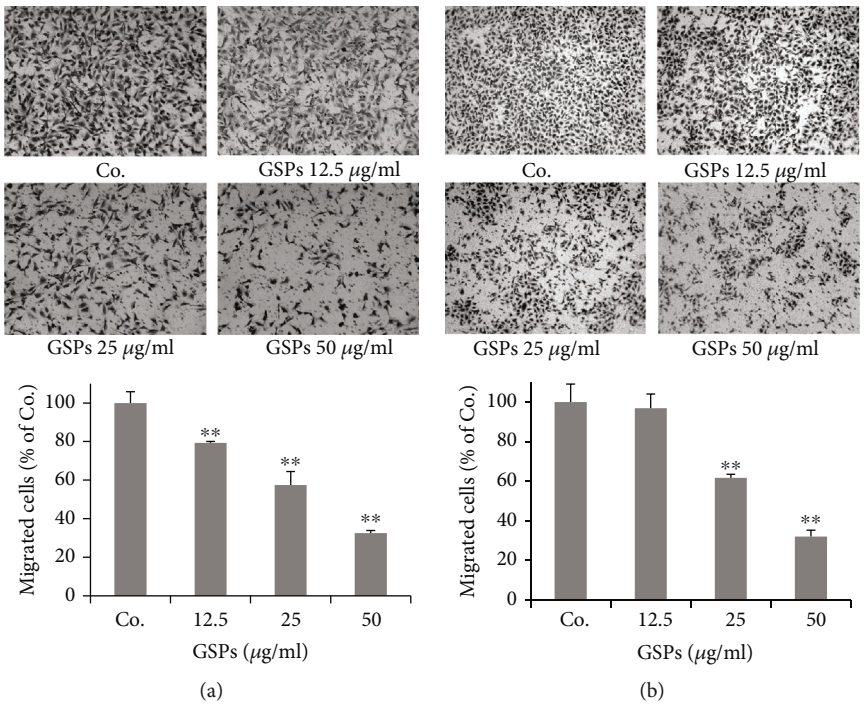

Figure 2: GSPs inhibit migration of T24 (a) and 5637 (b) cells. After incubated with GSPs for 24 h in the upper chamber of Millicells, cells passed through the membrane were fixed, stained, and counted. Representative images were shown. The data were presented as mean ± SD ($n = 4$). $^{**}P < 0.01$ vs. control group.

and invasion through inhibiting EMT, we established the TGF-β-induced EMT model with T24 and 5637 cells. For this purpose, both T24 and 5637 cells were treated with different concentrations of TGF-β for 24 h and then cell morphology and EMT markers were detected. As shown in Figure 5, treatment with TGF-β for 24 h did not induce obvious morphologic changes of T24 cells (Figure 5(a)). Western blotting results showed that TGF-β treatment for 24 h increased the expression of mesenchymal biomarker vimentin in T24 cells. However, only 5 ng/ml TGF-β enhanced the expression of another mesenchymal biomarker N-cadherin (Figure 5(b)). We did not detect the expression of epithelial biomarker E-cadherin (data not shown) in T24 cells. In 5637 cells, treatment with different concentrations of TGF-β for 24 h resulted in a morphological change from the initial polygonal epithelial appearance to an elongated mesenchymal morphology (Figure 5(c)). The expression of the mesenchymal marker N-cadherin was increased, while the epithelial marker E-cadherin was decreased by TGF-β treatment (Figure 5(d)). These results indicated that 5637 cells were more suitable than T24 cells for the EMT study. Therefore, we selected 5637 cells and 5 ng/ml

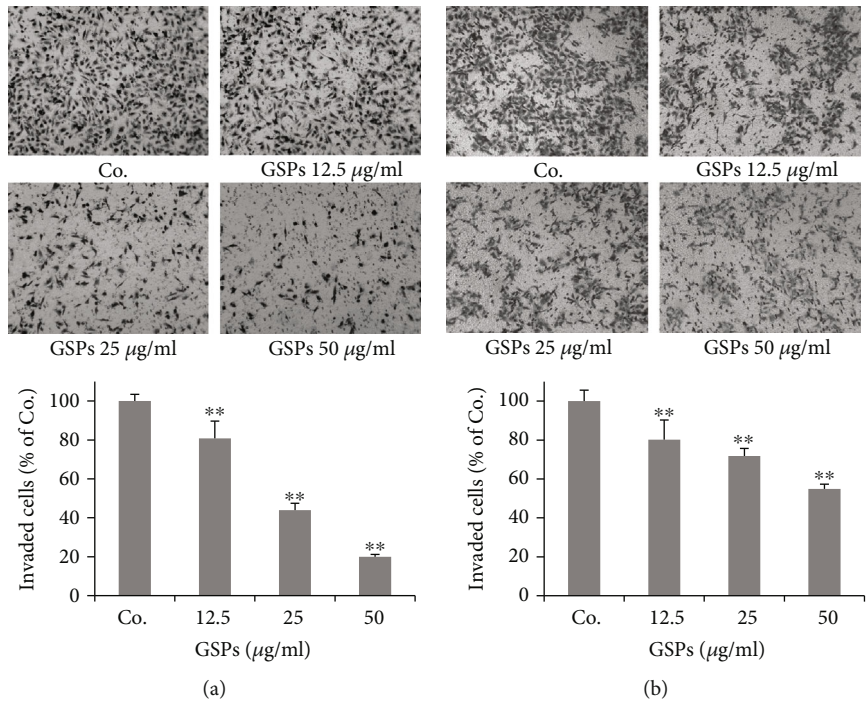

Figure 3: GSPs inhibit invasion of T24 (a) and 5637 (b) cells. After incubated with GSPs for 24 h in the upper chamber of Millicells, cells passed through the membrane coated with Matrigel were fixed, stained, and counted. Representative images were shown. The data were presented as mean ± SD ($n = 4$). $^{**}P < 0.01$ vs. control group.

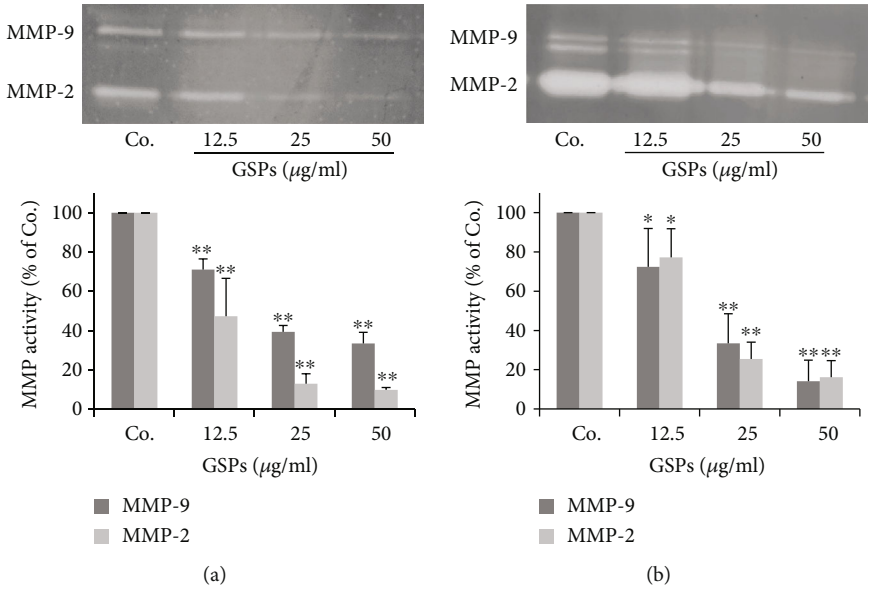

Figure 4: GSPs inhibit the secretion of MMP-2/9 of T24 (a) and 5637 (b) cells. Subconfluent cells were treated with different concentrations of GSPs in a serum-free medium for 24 h. The conditioned medium was collected. The activities of MMP-2 and MMP-9 in conditioned medium were detected by gelatin zymography and the results were quantified. The data were presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

TGF-β treatment to establish the EMT model induced by TGF-β.

3.6. GSPs Inhibit TGF-β-Induced EMT in 5637 Cells. Consistent with results of Figure 5, treatment with TGF-β at the concentration of 5 ng/ml for 24 h led to a morphological change from an epithelial morphology to a mesenchymal phenotype in 5637 cells. However, GSPs reversed this morphological change induced by TGF-β (Figure 6(a)). Results from western blotting also showed that treatment with TGF-β at the concentration of 5 ng/ml for 24 h upregulated the expression of mesenchymal markers N-cadherin and

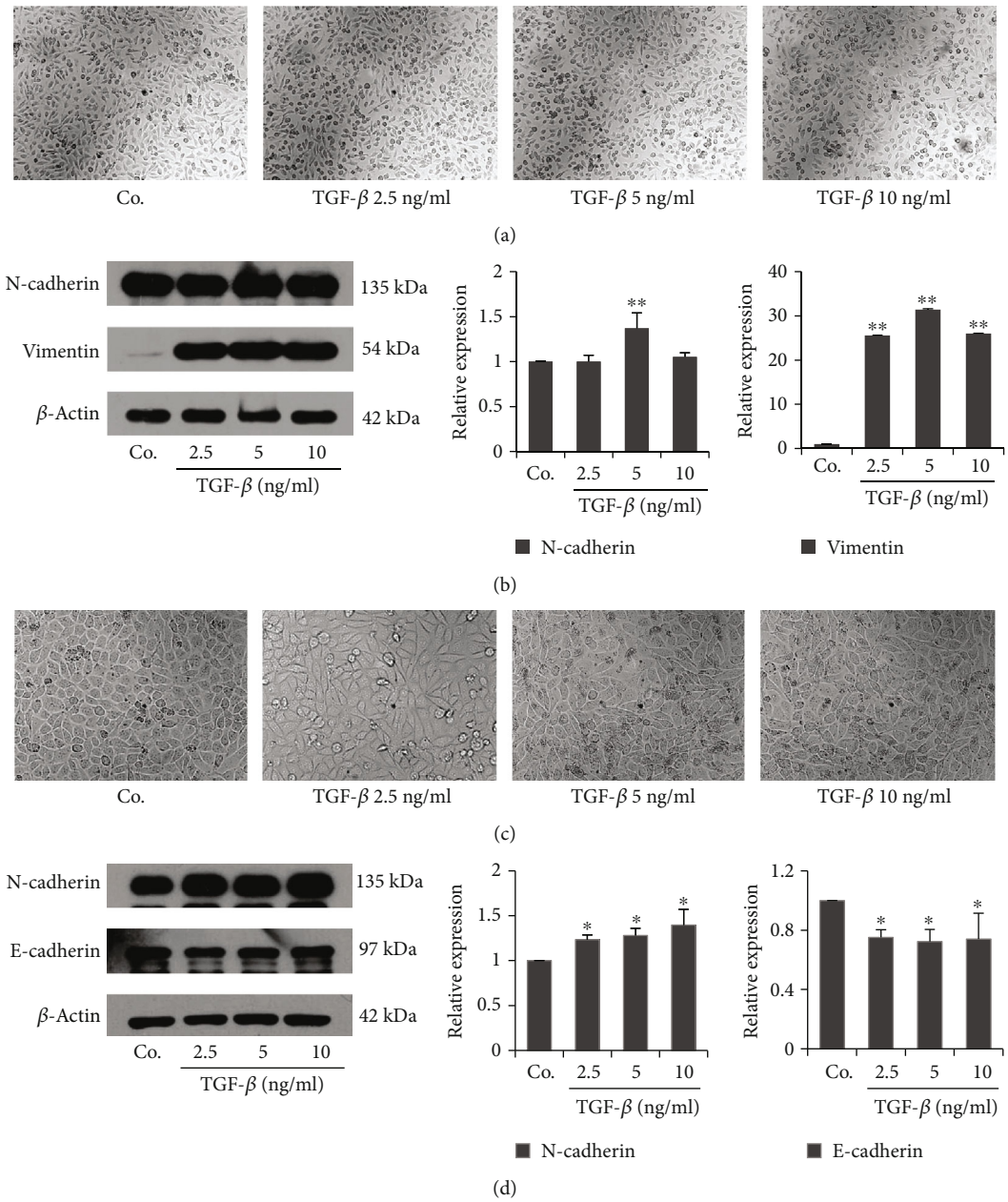

Figure 5: Establishment of TGF-β-induced EMT model of BC cells. T24 (a, b) and 5637 (c, d) were treated with TGF-β for 24 h. The morphological changes were observed under a phase-contrast microscope and photographed (100x). The level of EMT markers was examined by western blotting and the results were quantified. The data were presented as mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

vimentin as well as EMT-related transcription factors Slug but downregulated the expression of epithelial marker E-cadherin and ZO-1 in 5637 cells. GSPs treatment reversed the TGF-β-induced upregulation of N-cadherin, vimentin, and Slug as well as downregulation of E-cadherin and ZO-1 dose-dependently (Figure 6(b)). These results suggested that GSPs were able to inhibit TGF-β-induced EMT.

3.7. GSPs Inhibit TGF-β Signal Pathway. To elucidate potential molecular mechanisms of GSPs on EMT in 5637 cells, we examined the effects of GSPs on the activation of downstream signal molecules of TGF-β. As shown in Figure 7, treatment with TGF-β (5 ng/ml) enhanced the phosphorylation of Smad 2/3, Akt, Erk, and p38. GSPs treatment blocked the TGF-β-induced phosphorylation of Smad 2/3, Akt, Erk, and p38 without affecting the total SMAD2/3, Akt, Erk, and p-38 expression level.

4. Discussion

Increasing evidences demonstrated that many natural polyphenolic compounds including resveratrol and curcumin possessed a wide spectrum of pharmacological properties, including antimetastasis activities. The mechanisms of this

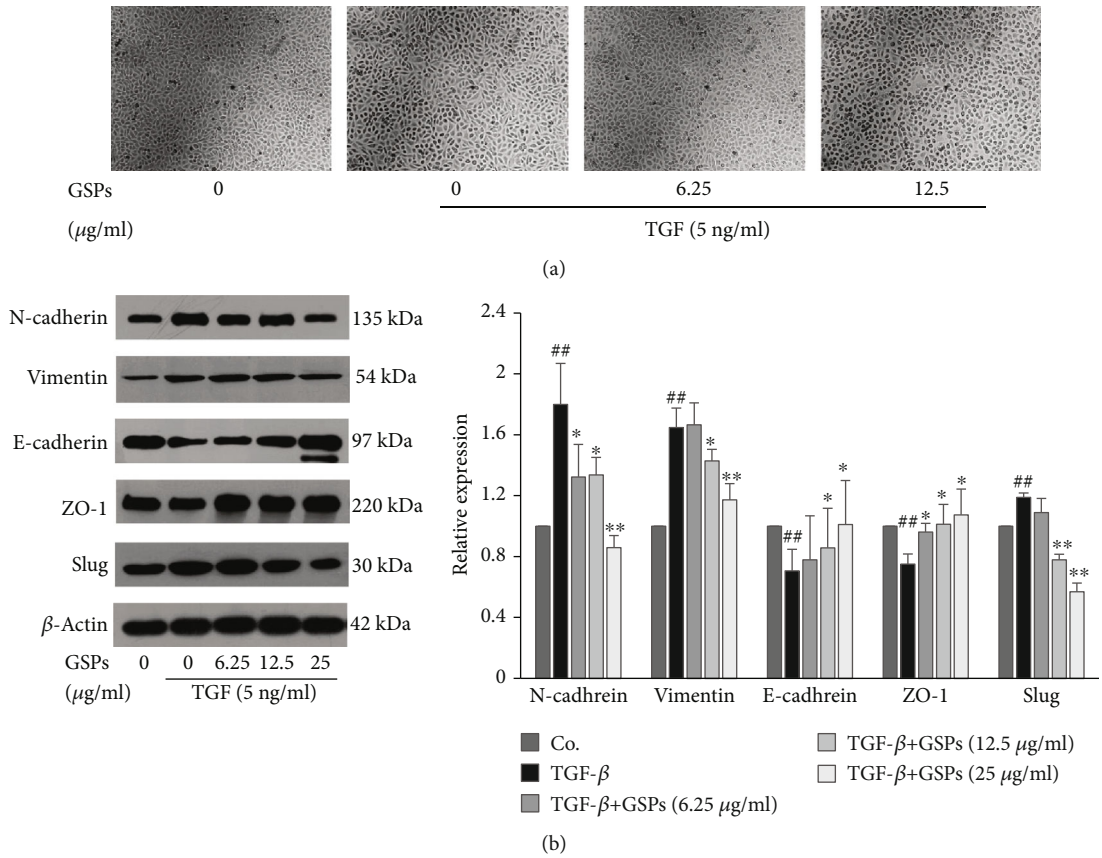

Figure 6: GSPs inhibit TGF-β-induced EMT in 5637 cells. 5637 cells were treated with GSPs in the presence or absence of 5 ng/ml of TGF-β for 24 h. (a) The morphological changes were observed under a phase-contrast microscope and photographed (100x). (b) The expression of EMT markers was detected by western blotting and the results were quantified. The data were presented as mean ± SD ($n = 3$). $^{\#\#}P < 0.01$ vs. control group and $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TGF-β treatment alone.

action were mainly related to the inhibition of EMT phenotype through suppression of multiple signaling pathways, such as PI-3K/Akt, NF-κB, FAK, and TGF-β1/SMADs pathway [26–29]. GSPs have also been shown to inhibit the metastasis of various types of cancer including tongue squamous cell carcinoma [30], nonsmall cell lung cancer [31], pancreatic cancer [21], and lung cancer [32]. The molecular targets of GSPs include NF-κB, PI3K/Akt, and MAPK signaling pathway. The goal of this study was to investigate the antimetastatic activity and associated mechanisms of GSPs against human BC cells *in vitro*. We found that GSPs inhibited the migration, invasion, and secretion of MMP-2/9 of T24 and 5637 cells at noncytotoxic concentrations.

EMT has an important role in promoting the invasion and metastasis of cancer cells. This process is regulated by multiple signaling molecules. Among them, TGF-β is a potent stimulator of EMT [33]. Therefore, we select TGF-β to establish the EMT model of bladder cancer cells. Although Both T24 and 5637 cells are derived from bladder cancer, we found that morphology change induced by TGF-β was more obvious in 5637 cells than in T24 cells. Moreover, in T24 cells, TGF-β treatment only increased the expression of the mesenchymal marker N-cadherin and vimentin. No expression of epithelial marker E-cadherin was detected. Unlike T24 cells, treatment of 5637 cells with TGF-β resulted in the increased expression of N-cadherin, at the same time, the reduced expression of E-cadherin. The results were consistent with previous studies [34, 35]. Further studies showed that treatment 5637 cells with TGF-β for 24 h led to the increased expression of N-cadherin, vimentin, and Slug and decreased of E-cadherin and ZO-1, indicating the occurrence of EMT. All the results indicated that 5637 cells were better suited for EMT study than T24 cells. Therefore, we selected 5637 to establish the TGF-β-induced EMT model.

It has been reported that GSPs inhibit EMT in head and neck cutaneous squamous cell carcinoma cells and pancreatic cancer cells [20, 21]. However, whether GSPs can also inhibit EMT in BC cells remains to be explored. In the present study, we found that treatment with GSPs reversed the TGF-β-induced morphological changes as well as the expression of EMT markers. These suggest that GSPs have the ability to inhibit EMT in 5637 cells and that this may be one of the possible mechanisms through which GSPs inhibit the migration and invasion of BC cells.

It is reported that TGF-β induces EMT through a Smad-dependent canonical pathway and the Smad-independent noncanonical pathway. In the Smad-dependent pathway, the binding of TGF-β to cell surface receptor results the phosphorylation and activation of Smad2 and/or Smad3. The receptor-activated Smad2 and/or Smad3 then form

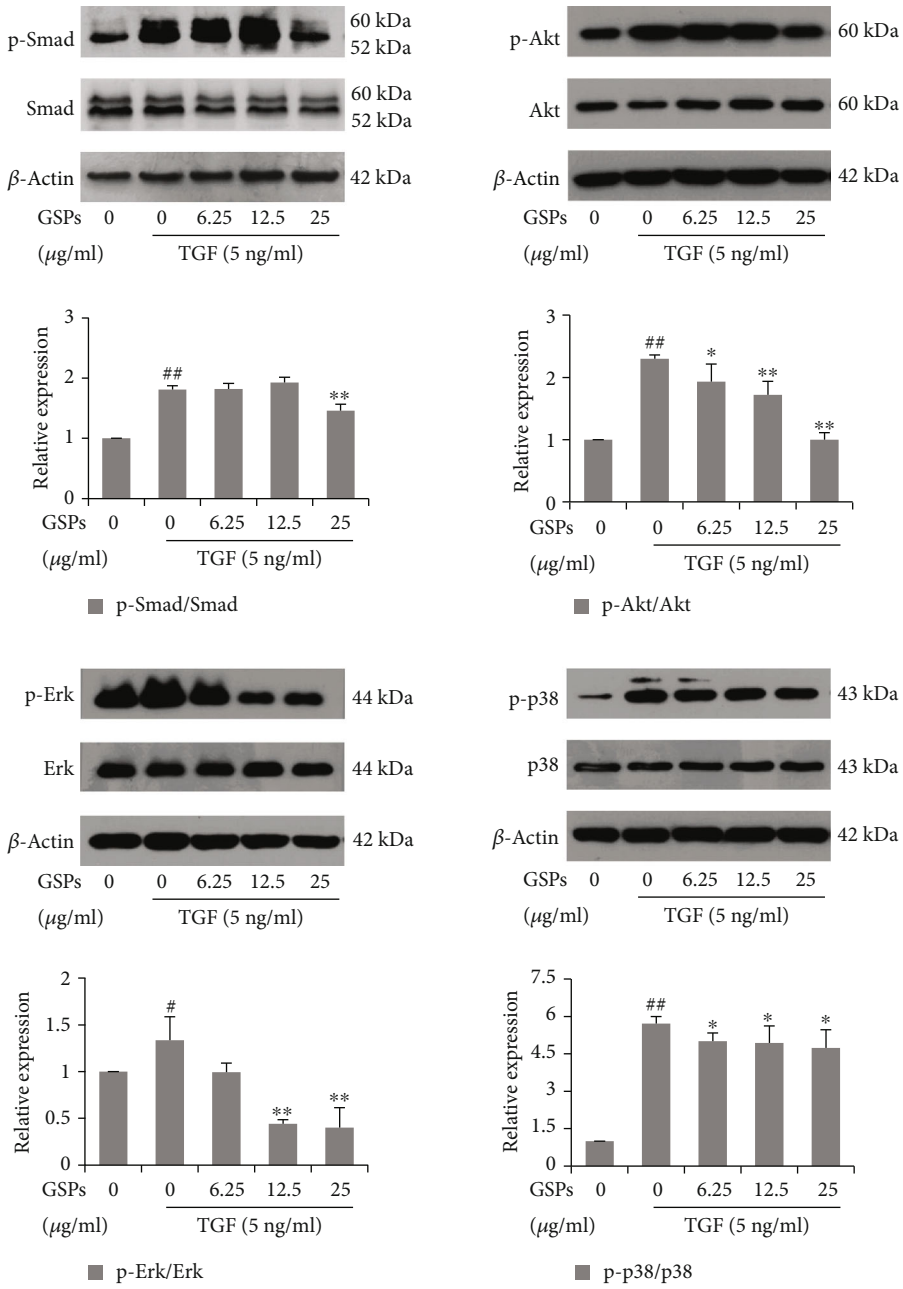

FIGURE 7: GSPs inhibit the TGF-β signal pathway. 5637 cells were treated with GSPs in the presence or absence of 5 ng/ml of TGF-β for 24 h. The expression of indicated signaling molecules was detected by western blotting and the results were quantified. The data were presented as mean ± SD ($n = 3$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group and $^{*}P < 0.05$, $^{**}P < 0.01$ vs. TGF-β treatment alone.

Smad complexes with Smad4 and translocate into the nucleus to regulate transcription of target genes that mediate EMT. In addition to the Smad signaling pathways, TGF-β also induces EMT in certain types of cells through Smad-independent, noncanonical pathway, which involves the activation of other signaling pathways such as RHO-like GTPases, PI3K-Akt, and MAPK (including ERK, p38, and JNK) [36–38]. Therefore, many natural compounds were reported to inhibit EMT through targeting TGF-β signaling. Lee et al. reported that Farnesol, a fragrant component of essential oils, inhibited TGF-β-induced EMT through blocking the PI3K/Akt/mTOR signaling pathway in lung cancer cells [39]. Bergamottin, a naturally occurring furanocoumarin, suppressed the metastasis of lung cancer cells through inhibiting the TGF-mediated EMT process by blocking the activation of PI3K/Akt/mTOR signaling [40]. In the present study, treatment with TGF-β induced the phosphorylation of Smad2/3, Akt, Erk, and p38 in 5637 cells, suggesting that both Smad-dependent and non-Smad-dependent signaling pathways are related to TGF-β induced EMT. GSPs blocked the TGF-β-induced phosphorylation of Smad2/3, Akt, Erk, and p38 in 5637 cells but did not affect the total expression

levels of these signal molecules, indicating that the reverse of TGF-β-induced EMT by GSPs is associated with the suppression of the TGF-β signaling pathway.

However, it is important to note that although our results have demonstrated that GSPs inhibit EMT through suppression of the TGF-β signaling pathway. It would make the results more convincing to further explore the mechanisms of GSPs by blocking or overexpressing the expression of downstream effectors of TGF-β signaling, such as Smad, Akt, p38 MAPK, and Erk, using siRNAs. Moreover, many studies have shown that in addition to TGF-β, EMT can be initiated by a variety of signaling molecules, including microRNA [41, 42]. It is necessary to establish EMT models induced by other signaling molecules to explore the effects and mechanisms of GSPs. In addition, this is a preliminary study on cell culture. Other BC-derived cell lines and *in vivo* studies will be needed in the future to further confirm the findings.

5. Conclusions

In conclusion, the results of our study indicate that GSPs inhibit migration and invasion of bladder cancer cells by reversing EMT through suppression of the TGF-β signaling pathway. Our data suggest that GSPs may be developed as a potential chemopreventive and therapeutic agent against bladder cancer.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Ninggang Yang and Jing Gao contributed equally to this work.

Acknowledgments

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Review Article

Glycyrrhiza Genus: Enlightening Phytochemical Components for Pharmacological and Health-Promoting Abilities

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The *Glycyrrhiza* genus, generally well-known as licorice, is broadly used for food and medicinal purposes around the globe. The genus encompasses a rich pool of bioactive molecules including triterpene saponins (e.g., glycyrrhizin) and flavonoids (e.g., liquiritigenin, liquiritin). This genus is being increasingly exploited for its biological effects such as antioxidant, antibacterial, antifungal, anti-inflammatory, antiproliferative, and cytotoxic activities. The species *Glycyrrhiza glabra* L. and the compound glycyrrhizin (glycyrrhizic acid) have been studied immensely for their effect on humans. The efficacy of the compound has been reported to be significantly higher on viral hepatitis and immune deficiency syndrome. This review provides up-to-date data on the most widely investigated *Glycyrrhiza* species for food and medicinal purposes, with special emphasis on secondary metabolites' composition and bioactive effects.

1. Introduction

The *Glycyrrhiza* genus belongs to the Fabaceae family and is generally well-known as licorice in English, *Bois doux* in French, and *Regalizia* in Spanish [1]. *Glycyrrhiza* is derived from the old Greek terms *glykos* which means "sweet" and *rhiza* which means "root" [2]. Composed of ~30 species, this genus is distributed worldwide [3], and is extensively spread in the Mediterranean basin of Africa, Europe, and Asia, extending to Australia, North America, and temperate regions of South America. *Glycyrrhiza glabra* L. is commercially the most important species under this genus, native to Afghanistan, Syria, Persia, Southern Europe, and North Africa [4, 5]. This species consist of 3 varieties, viz., *Glycyrrhiza glabra* var. *violacea*, known as Persian and Turkish licorice; *Glycyrrhiza glabra* var. *gladulifera*, known as Russian licorice; and *Glycyrrhiza glabra* var. *typica* L., known as Spanish and Italian licorice [6]. The second most commercially important species of this genus is *Glycyrrhiza uralensis* Fisch. which extends from Western to Eastern Siberia, as well as from Northern China to Mongolia [7]. *Glycyrrhiza echinata* L. is common in the Balkans and in Russia [8]. *Glycyrrhiza pallidiflora* Maxim. is native to the far East and China, *Glycyrrhiza asperrima* L.f. is commonly found in Russia and in Central Asia, *Glycyrrhiza astragalina* Hook. & Arn. is found Chile, and *Glycyrrhiza bucharica* Regel is found in Central Asia [8]. In Pakistan, the genus is represented by only 3 species, viz., *Glycyrrhiza glabra* L., *Glycyrrhiza triphylla* Fisch. & C.A.Mey., and *Glycyrrhiza uralensis* Fisch. [1, 9]. *Glycyrrhiza áspera* Pall., *Glycyrrhiza echinata* L., *Glycyrrhiza glabra* var. *glabra*, *Glycyrrhiza glabra* var. *glandulifera*, and the endemic species *Glycyrrhiza asymmetrica* Hub.-Mor., *Glycyrrhiza icónica* Hub.-Mor, and *Glycyrrhiza flavescens* subsp. *flavescens* grow among the Turkish flora [10–14]. Nonetheless, and looking at large-scale use, some taxa of the genus are now commercially cultivated in Spain, Sicily, England, North America, and Northern India [15–18]. The world map showing the countries which widely cultivate licorice can be seen in Figure 1.

Licorice has certain bioactive components such as flavonoids and glycyrrhizin, which have many pharmacological properties such as antioxidant, antiviral, anti-infective, and anti-inflammatory properties [19, 20]. The spreading cultivation of the *Glycyrrhiza* spp. is due to its high market demands pertaining to their pharmaceutical properties. Traditionally, licorice is widely used as an antidote to reduce the toxicity caused by drug therapies. In Ayurveda, licorice is considered as "Rasayana," which means to have nourishing, rejuvenating, and strengthening effects. Its rhizomes and roots are used to treat renal, hepatic, gastric, and respiratory disorders. Recent findings established its role in numerous biological activities in the human body such as having an anti-inflammatory and antioxidant role and having a protective effect on various organs [21]. It is evident that licorice has a various health-promoting activities proved using preclinical and clinical trials; however, a comprehensive compilation of these research advances is lacking. In this sense, the present work is aimed at providing an in-depth revision on the most widely used and investigated *Glycyrrhiza* spp. for food and medicinal purposes. A special emphasis is given to the botanical aspects, traditional uses, and secondary metabolite composition of widely studied species of *Glycyrrhiza* (*Glycyrrhiza glabra* L. and *Glycyrrhiza uralensis* Fisch.) and their biological effects. The basic components discussed in the current review are presented in Figure 2.

2. Botanical Features

The *Glycyrrhiza* genus is a subshrub, erect, and attaining heights of up to 2.5 m tall. It has highly developed stoloniferous roots. Roots are thick and branched with yellowish to red color [22]. Leaves are alternate, petiolate, and imparipinnate, with small, free, deciduous stipules; leaflets are elliptical to lanceolate, 4–7 pairs; apex acute to obtuse, margin entire, smooth [23, 24]. The inflorescence has an axillary spike, and bracts are very small, conspicuously present or absent, and caduceus. Flowers are stipitate, narrow, and zygomorphic. The calyx is short, persistent, and bilabiate. The corolla is typically papilionaceous and blue, lavender to purple, or violet in color; the banner petal (vexillum) is narrow or oblanceolate; the wing petals (alae) are narrow, oblanceolate to oblong; and the keel petal is bipartite, acute, and straight. Stamens are diadelphous, filaments are glabrous, and anthers are simple and rounded. Style is filiform, and stigma is blunt. Legume or pod is the fruit of *Glycyrrhiza* genus which is compressed, oblong, unilocular, up to 1.5 cm long, sometimes falcate, erect, glabrous or covered with brownish echinate glandular hairs (*Glycyrrhiza uralensis* Fisch.), and

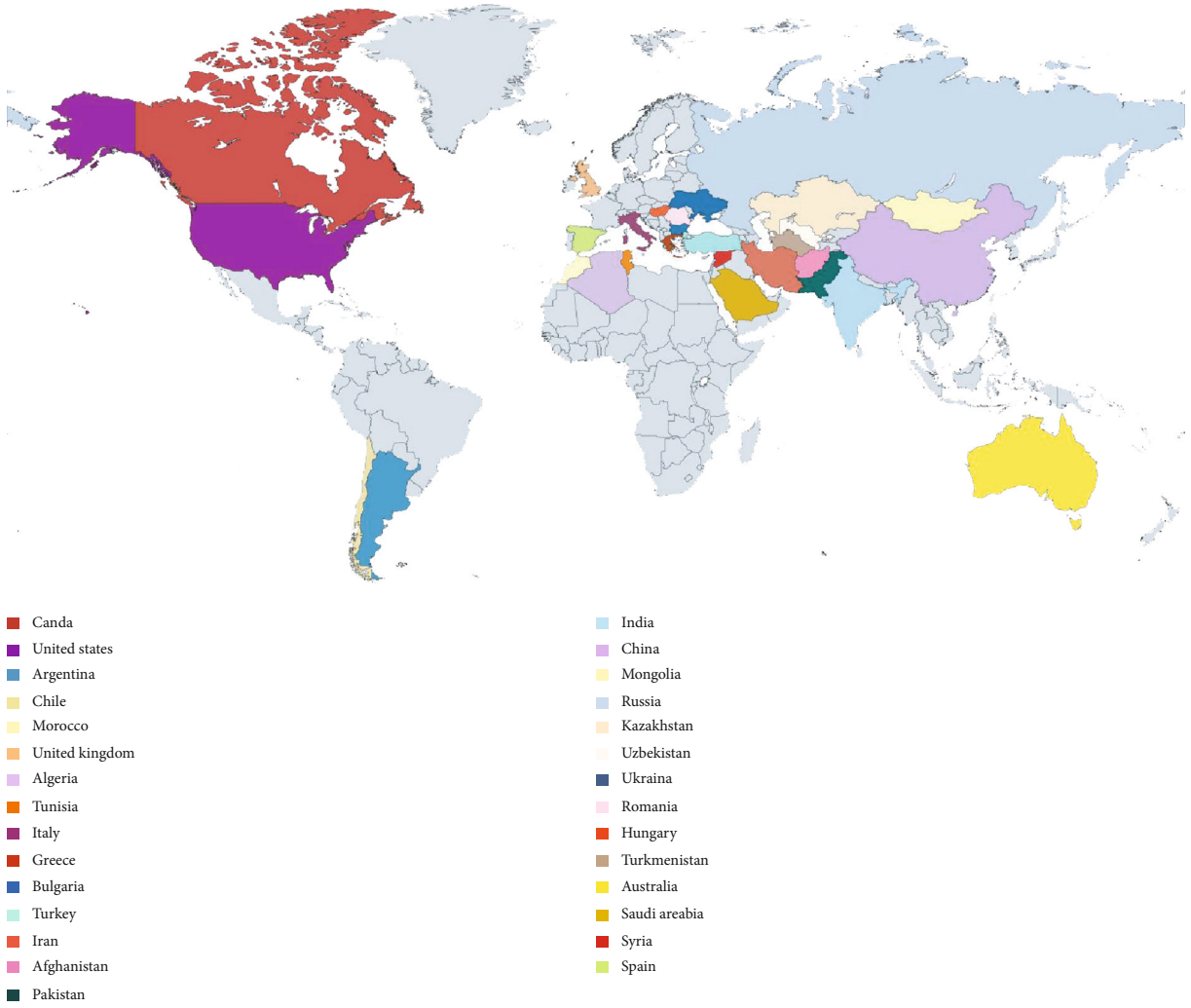

Figure 1: World map showing the major countries producing *Glycyrrhiza* spp.

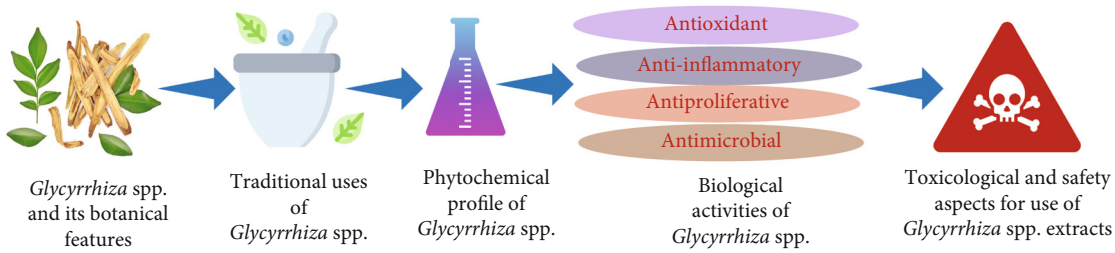

Figure 2: Diagram showing various components discussed in the review.

weakly dehiscent or indehiscent. Seeds are reniform; smooth; small; and brown, black, or deep grey [10, 22, 24, 25].

3. Traditional Uses

The *Glycyrrhiza* genus is used around the world for food and medicinal purposes [26]. The Generally Recognized as Safe (GRAS) status of licorice allows its application in a plethora of foods at typical concentrations. Also, the sweet flavor of licorice makes it suitable for various applications in foods, like confectionary and condiments, where the most commonly used plant part are the rhizomes and roots. For example, both London drops and Red Vines® are flavored with licorice. In condiments, licorice powder is generally used in sweet chili sauce and soy sauce to add a unique flavor. In traditional medicine and clinical practice of different cultures, *Glycyrrhiza* has been applied for treating various types of ailments [2]. Although there are more than 20 species identified belonging to this genus, just a few of these species are traditionally used to treat diseases.

The roots of *Glycyrrhiza glabra* L. are traditionally most commonly used in Albania and Italy against sore throat and as an antitussive [27]. In Brazil, *Glycyrrhiza glabra* L. is used as an emollient and diuretic, and it is also used for inflammatory diseases [28]. In the countries of the Commonwealth of Independent States, *Glycyrrhiza glabra* L. has been used as an expectorant, emollient, anti-inflammatory, antispasmodic, antacid, antiallergic, antihistamine, laxative, diaphoretic, analgesic, diuretic, wound-healing medicine, tonic, potency enhancer, detoxificant, sedative, antiviral, antiulcer, hypotensive medicine, capillary-strengthening medicine, antibacterial, and antioxidant [29, 30]. The *Glycyrrhiza glabra* L. rhizome is traditionally used in the Northern Navarra, Iberian Peninsula, for digestive disorders [31]. In India, *Glycyrrhiza glabra* L. barks are used by local traditional practitioners in the Thiruvarur district for gonorrhea, while the whole plant is used for hepatitis B virus (HBV) infection [32]. The whole ripe fruit and rhizome are used to increase the sperm count and to treat colds and cough [33]. Fruit and stem bark of this species are also used for paralysis [34]. Wild grown roots and leaves are used in different parts of Iran for gastralgia, gastric ulcer, hemorrhoids, liver disorders, muscle spasm, bone pains, and dyspnea [35, 36]. In the Kerman province of Iran, the roots and stems are used by local people for colds, stomach pain, ulcers, acidification, joint pain (back and leg), and bone fractures [37, 38]. In the Qaysari Market, Erbil, Iraq, *Glycyrrhiza glabra* L. radix is applied for pneumonia, sour eructation, and duodenal inflammation [39]. *Glycyrrhiza glabra* L. is traditionally used to treat sore throat and lung infections in Mauritius [40]. In South Africa, local people often use *Glycyrrhiza glabra* L. rhizomes for chest ailments, rheumatism, arthritis, and ulcers [41, 42]. *Glycyrrhiza glabra* L. rhizomes are used for common colds in the Granada province of Southern Spain [43]. The roots of this plant are used for the treatment of heart diseases by Turkish communities in Germany [44]. Also, roots are used by the local people in Turkey for respiratory tract diseases, flu, bronchitis, pain, epilepsy, cancer, gastrointestinal diseases, and high cholesterol levels [45–47]. Besides, roots are used for cardiac disorders, diabetes, and hemorrhoids [48, 49].

Among other *Glycyrrhiza* spp., *Glycyrrhiza echinata* L. is used in West Azerbaijan (Iran) in cases of cough, bronchitis, ulcer, and pharynx [50]. Also, the underground parts of *Glycyrrhiza uralensis* Fisch. are used in Kazakhstan as an anticoagulant, antifibrinolytic, diuretic, anti-inflammatory, and detoxificant, as well as for handling diabetes mellitus and hypertension [51]. The use of the licorice extracts is also mentioned in traditional Chinese medicine. It was recommended for relieving the situation of dyspnea, spasms, cough, phlegm, pain, and toxicity as traditional Chinese medicine. Kampo, which is a traditional Japanese medicine also named as "yokukansan," shows a neuroprotective effect, whereas in South Korea it has been used in the treatment of cold and cough. It is conspicuous that rhizomes and roots from licorice are used widely in many countries as traditional medicine, and it may act as an important ingredient in the formulation of functional foods.

The medicinal properties of this plant are mainly conferred by the phytoconstituents, and it is crucial to know the exact profile. The next section will highlight the phytochemical profile of licorice.

4. Secondary Metabolite Composition

As licorice represents a historical background of use, and various investigations have been performed to find the active principles responsible for the extreme health potentialities. Glycyrrhizin is the principal substance present in licorice roots (Figure 3), along with the flavonoid liquiritin apioside [52, 53]. Four compounds were isolated including two new flavonoids from the licorice of Sinkiang, China. The known compounds were licochalcones A and B. A new compound, glycyrrhisoflavanone, was obtained as colorless needles. The electron ionization mass spectrometry (EIMS) of both compounds showed the molecular ion peaks at 368 and 354, which correspond to the molecular formulas $C_{21}H_{20}O_6$ and $C_{20}H_{18}O_6$, respectively [54].

Phenolic compounds, such as liquiritigenin, isoliquiritigenin, 4′-O-methylglabridin, isoprenyl chalcone, formononetin, glabridin, and hispaglabridins A and B are also present at greater amounts and possess numerous bioactivities [55–59]. Stilbene derivatives have also been isolated from licorice leaves [60, 61]. Moreover, 49 phenolic compounds and 15 different saponins have been identified from licorice roots [62]. Saponins (licorices A3, G2, and J2), chalcones (isoliquiritin, licochalcone B, and neolicuroside) [63], coumarins (glycycoumarin) [64], and flavonoids (glychionides A and B, glabrene, glabrone, glabraisoflavanones A and B, isoviolanthin, 5,7-dihydroxyflavanone, and rhamnoliquiritin) [24, 65–67] have also been reported in licorice. Glucoliquiritin apioside, prenyl licoflavone A, shinflavanone, shinpterocarpin, and 1-methoxy phaseolin are also present in licorice roots [68]. The optimization of the extraction of glycyrrhizic acid and glabridin from Chinese licorice was investigated [69]. In another study, it was found that the main constituents of *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch., and *Glycyrrhiza inflata* Batalin were glycyrrhizin, liquiritin and its apioside, and liquiritigenin [53]. A brief description of characteristic compounds present in some of the important species of licorice is also presented below.

Regarding *Glycyrrhiza* spp. essential oils, a remarkable difference has been found in volatile constitution. Volatile compounds, such as linalool oxides A and B, terpinen-4-ol, α-terpineol, and geraniol, have been identified from roots [70].

4.1. Glycyrrhiza glabra L. The methanol based-root extract of *Glycyrrhiza glabra* L. was partitioned between water and ethyl acetate to obtain water and ethyl acetate soluble fractions. These fractions were then separated using column chromatography and yielded 3 known compounds, viz., kanzonol Y, licochalcone C, licoagrochalcone B, and one new compound, named glycyglabrone [71]. Another group of researchers found the presence of 15 sugars in the ethanol extract of *Glycyrrhiza glabra* L., with ribitol, saccharose, glucofuranose, sorbose, fructose, mannitol, galactofuranose, mannopyranose, hydroxyethylglucose, glucopyranose, and mannopyranosyl-D-glucitol being the most common [72]. The same authors separated the components of *Glycyrrhiza glabra* L. roots based on acid-base properties. Glabridin, hispaglabridin B, and 4-O-methylglabridin were the major compounds of the fraction identified by gas chromatography-mass spectrometry (GC-MS) [73].

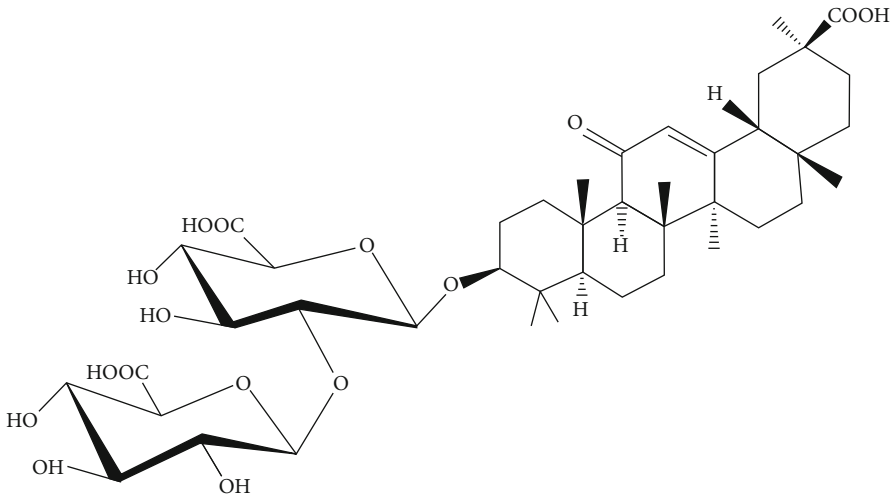

Figure 3: Glycyrrhizin (glycyrrhizinic acid) structure.

Two new compounds, viz., 1,2 dihydroparatocarpin A and neolignan lipid esters, along with 7 known phenolic compounds, viz., paratocarpin B, formononetin, isoliquiritigenin, glabridin, 4-O-methylglabridin, hemileiocarpin, and hispaglabridin B were discovered from the chloroform extract of *Glycyrrhiza glabra* L. roots and stolons [55]. In addition, three new compounds, namely, glabroisoflavanones A and B and glabrocoumarin were isolated from the dichloromethane extract of *Glycyrrhiza glabra* L. roots [74]. Ten monodesmosidic saponins were isolated, namely, 20-α-rhaoglycyrrhizin, 20-α-galacturonoylglycyrrhizin, 11-deoxo-20-α-glycyrrhizin, rhaogalactoglycyrrhizin, rhaoglucoglycyrrhizin, 11-deoxorhaoglycyrrhizin, rhaoglycyrrhizin, 24-hydroxyglucoglycyrrhizin, glycyrrhizin 20-methanoate, and 30-hydroxyglycyrrhizin were isolated for the first time using the aquamethanolic extract of *Glycyrrhiza glabra* L. roots [75]. The main components found in *Glycyrrhiza glabra* L. are represented in Figure 4.

4.2. Glycyrrhiza uralensis Fisch. Flavonoid glycosides (liquiritin, isoliquiritin, neoisoliquiritin, ononin, liquiritin apioside, isoliquiritin apioside, and licraside) and triterpene glycosides (glycyrrhizin; araboglycyrrhizin; 18 α-glycyrrhizin; apioglycyrrhizin; and licorice saponins A3, E2, G2, H2, and L3) were extracted from the *Glycyrrhiza uralensis* Fisch. roots [76]. Echinatin, licoflavone A, licochalcone A, liquiritin, formononetin, glabrono, licochalcone B, isoliquiritin, 4,7-dihydroxy-flavone, liquiritigenin, and medicarpin 3-O-β-D-glucopyranoside were also identified in *Glycyrrhiza uralensis* Fisch. [77]. Other researchers also found licochalcone B in the ethanolic extract from *Glycyrrhiza uralensis* Fisch. roots [78]. Isoliquiritigenin, its glycoside isoliquiritin, and isoliquiritin apioside forms were also isolated from *Glycyrrhiza uralensis* Fisch. aqueous extract [79]. Hayashi et al. [80] also isolated a triterpene saponin, glucoglycyrrhizin, from *Glycyrrhiza uralensis* Fisch.

Three novel dihydrostilbenes, particularly glycydipytilbene and glycypytilbenes A and B with 12 known compounds, were isolated from *Glycyrrhiza uralensis* Fisch. leaves. The compounds were identified as isoglycyrol, ononin, glycycoumarin, glycyrrhizic acid, licuraside, liquiritin, isoliquiritin apioside, liquorice saponin A3, licorice glycosides A and B, liquiritin apioside, licorice saponin G2, and isolicoflavonol [81]. In another research, thirty-four known compounds and 2 new compounds were identified from the ethanolic extract of *Glycyrrhiza uralensis* Fisch. roots [82]. In another work, glycyrin, glycyrol, glycycoumarin, and liquiritigenin were also obtained from the dried powder of *Glycyrrhiza uralensis* Fisch. roots [83]. The main components found in *Glycyrrhiza uralensis* Fisch. are represented in Figure 5.

4.3. Other Glycyrrhiza Species. Three pure compounds, namely, glepidotins A, B, and D were identified from *Glycyrrhiza lepidota* Pursh leaves. These compounds were extracted by a solvent system containing H_2O, and CH_2Cl_2 : MeOH in a 1 : 1 ratio. The identification was done using bioassay-guided fractionation and high-performance liquid chromatography (HPLC) [84].

A new retrochalcone and three known compounds were isolated from the roots of *Glycyrrhiza inflata* Batalin. The structure of retrochalcone and licochalcone E were elucidated through spectroscopic analysis. Isoliquiritigenin, licochalcone A, and licochalcone C were also identified by analysis of the mass spectrometry (MS), nuclear magnetic resonance (NMR), and infrared spectroscopy (IR) spectra of each compound and by comparing with those found in the literature [85].

It is evident that 4 species under the *Glycyrrhiza* genus, namely, *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza lepidota* Pursh, and *Glycyrrhiza inflata* Batalin, are widely studied for the investigation of bioactive compounds, and these compounds may reveal important use as health-promoting agents.

5. Biological Activities

The biological effects of the *Glycyrrhiza* genus have been mainly assessed through *in vivo* and *in vitro* experiments. *Glycyrrhiza* plant extracts have been majorly assessed for its

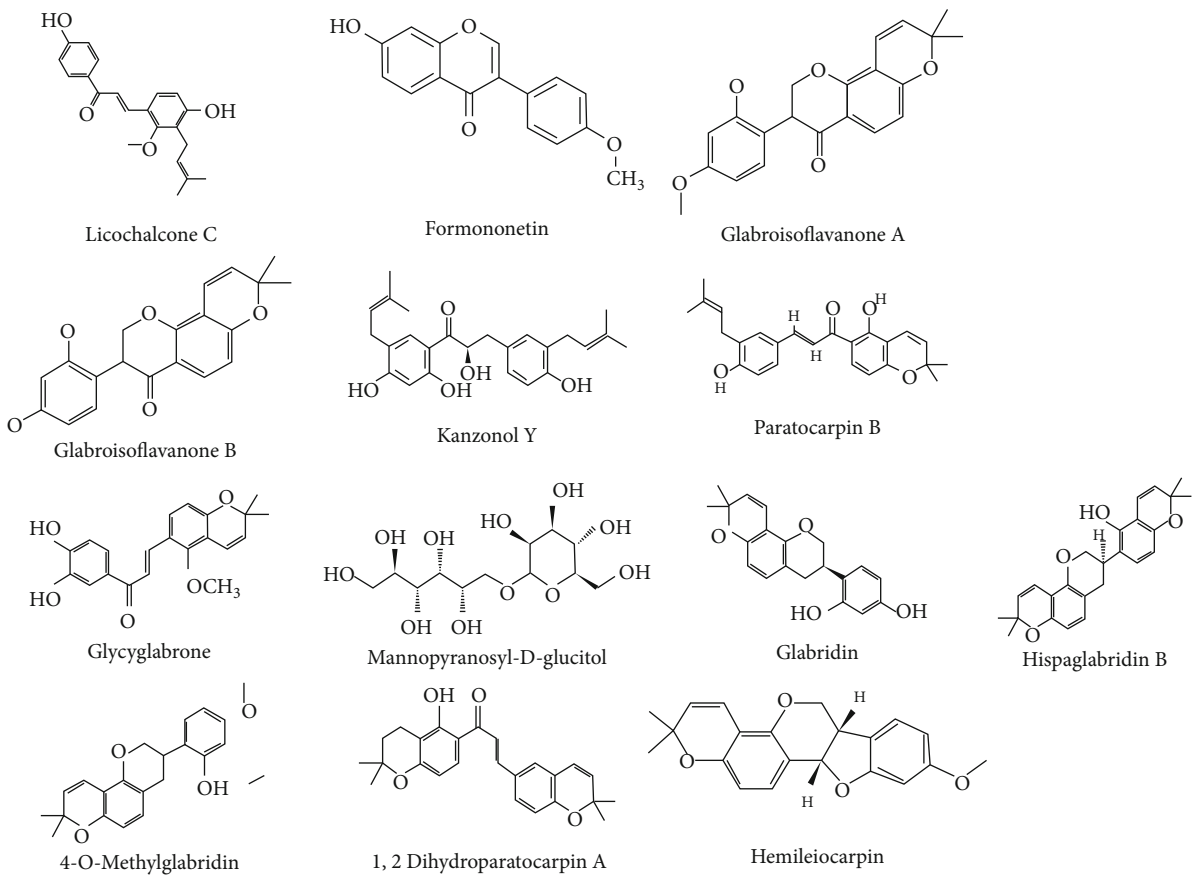

Figure 4: Chemical structures of the main components reported in *Glycyrrhiza glabra* L.

antioxidant, antimicrobial, anti-inflammatory, antiproliferative, and cytotoxic activities. Nevertheless, the observed biological activities of *Glycyrrhiza* can vary according to the extraction method [86], geographical origin [87, 88], drying method [89], and harvesting time [90].

5.1. Antioxidant Activity. The antioxidant potential of *Glycyrrhiza* spp. has been reported by several studies [91–98] as assessed through its *in vitro* radical scavenging potential, phosphomolybdenum, cupric-reducing antioxidant capacity (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, hydrogen peroxide scavenging capability, and β-carotene/linoleic acid bleaching assays. Shakeri et al. [99] assessed the biological activity of *Glycyrrhiza triphylla* Fisch. essential oils and found an appropriate antioxidant activity with an IC_{50} of 110.4 μg/mL using the DPPH assay. Polysaccharide fractions (GUPs-1, GUPs-2, and GUPs-3) extracted from *Glycyrrhiza uralensis* Fisch. were reported to have antioxidant effects [100]. Among all the fractions, GUPs-1 demonstrated the highest scavenging activity (70%) at a concentration of 4 mg/mL followed by GUPs-2 (60%) and GUPs-3 (30%). Licorice phenolic extract at a concentration of 0.54 μM was revealed to have the highest oxidative stress protection with 72% of cell viability in Caco-2 cells [95]. Haraguchi et al. [101] reported that isoflavans extracted from *Glycyrrhiza glabra* L. inhibited oxidative stress in liver mitochondria. The most potent inhibitor of NADH-dependent lipid peroxidation was 3′-hydroxy-4′-O-methylglabridin with an IC_{50} of 0.1 μM. The high *in vitro* antioxidant capacity and inhibitory effect of *Glycyrrhiza glabra* L. extracts on peroxidation of lipids in mice liver was also reported by Saeed et al. [102]. Furthermore, the oral intake of *Glycyrrhiza glabra* L. polysaccharides led to an improvement in immune system activity and decreased oxidative stress in high-fat mice by enhancing the activity of the antioxidant enzymes [103]. A dose of 300 mg/kg of *Glycyrrhiza glabra* polysaccharides resulted in the highest activity of superoxide dismutase (150 U/mL), catalase (3.33 U/mL), and glutathione peroxidase (20.67 U/mL). On the other hand, dehydroglyasperins C and D and isoangustone A separated from *Glycyrrhiza uralensis* Fisch. inhibited lipid peroxidation with IC_{50} values varying between 0.205 and 0.418 mM in rat tissue. Further, 10 μM licorice extracts also inhibited H_2O_2-induced reactive oxygen species (ROS) production by 53–85% in human hepatoma (HepG2) cells [104]. The level of ROS induced by *Glycyrrhiza glabra* L. root infusions was also reported to have a good mineral content and *in vitro* antioxidant activity with 52% of OH scavenging ability. The extracts did not cause degenerative effects up to 50 mg/kg b.w. in rat hepatocytes (Salawu, Ibukun, and Esan, 2019 [105]). Furthermore, Liu et al. [106] isolated eight new triterpenoid saponins from *Glycyrrhiza uralensis* Fisch. and observed that two compounds, glyuralsaponins B and H, displayed lipid peroxidation inhibition activity against Fe^{2+}/cysteine-induced liver

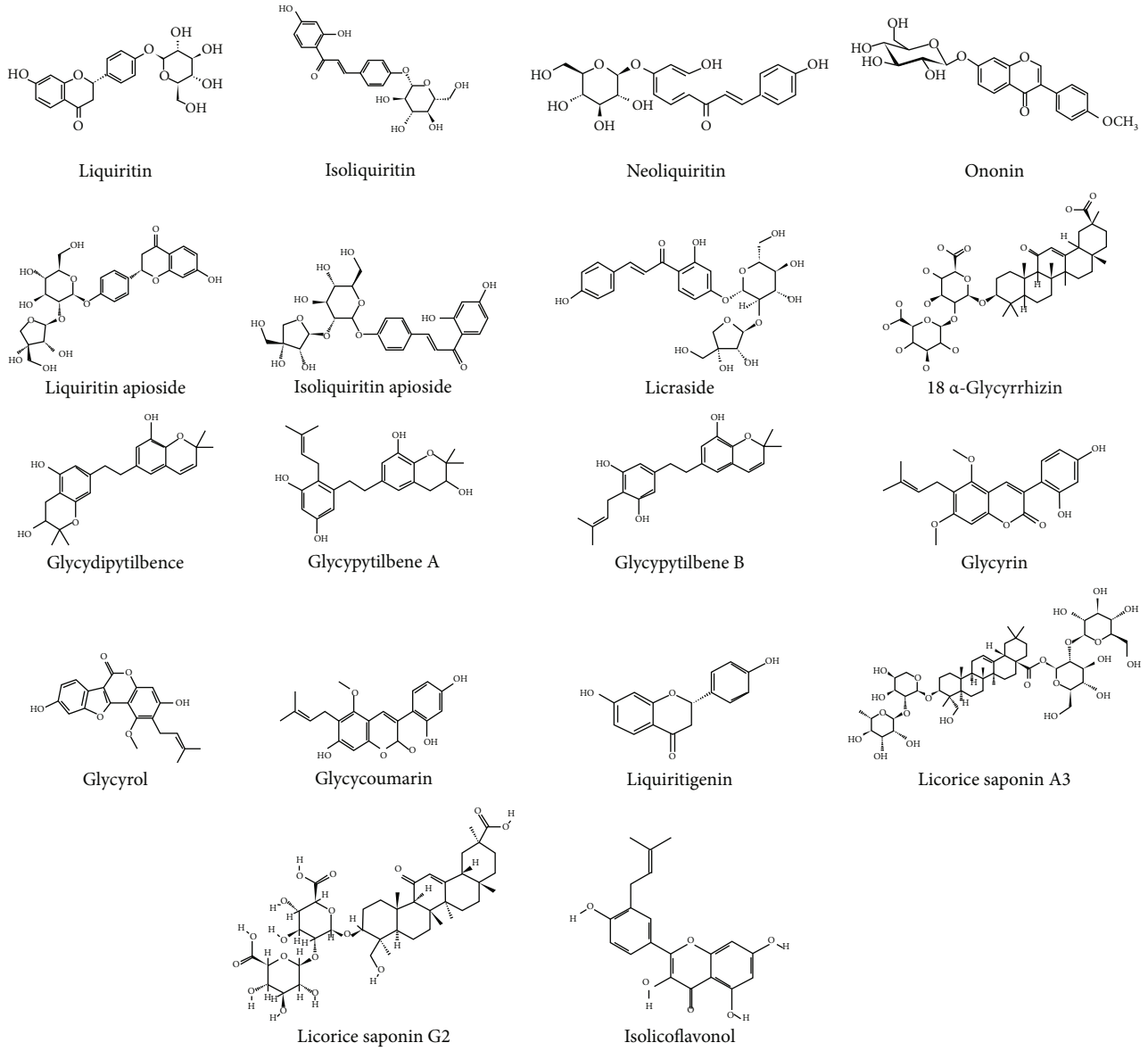

Figure 5: Chemical structures of the main components reported in *Glycyrrhiza uralensis* Fisch.

microsomal enzyme system at a concentration of 0.1 μM with inhibition values of 79 and 91%, respectively.

Besides these aspects, licorice has also revealed a good ability to be used as a natural antioxidant in food products. For instance, licorice extract was effective in controlling rancidity in precooked pork [56]. The value of thiobarbituric acid-reactive substances in the control sample of pork patties was found to be 9.4 mg/kg after 14 days of storage, whereas it was only 4.4 mg/kg in the case of licorice extract-treated pork patties. Furthermore, its antioxidant activity in fish oil was also assessed by various methods, as reported by Ucak [107]. On the other hand, Zhang et al. [108] recommended licorice extract to be fed to sheep as a bioadditive to enhance the antioxidant effect of their meats. Compared to the control samples, the supplementation of 3000–4000 mg/kg feed led to increased DPPH (39%) free radical scavenging activity.

5.2. Anti-Inflammatory Activity. The methanol extract from leaves of *Glycyrrhiza glabra* L. and *Glycyrrhiza uralensis* Fisch. demonstrated anti-inflammatory activity on lipopolysaccharide- (LPS-) induced RAW264.7 cells [109–112]. Moreover, Frattaruolo et al. [113] reported that the M2 fraction (licoflavanone) from *Glycyrrhiza glabra* L. leaves at an IC_{50} value of 60.49 μM exhibited anti-inflammatory activity by reducing the NF-κB translocation as confirmed by using immune-fluorescence monitoring and reducing the nitrite levels by ~100-fold while reducing the proinflammatory cytokines and cyclooxygenase 2/inducible nitric oxide synthase expression. The anti-inflammatory activity of *Glycyrrhiza uralensis* Fisch. was also reported [114]. The authors reported that acetone licorice fractions can act as a strong anti-inflammatory agent and caused 77.9% inhibition at 62 μg/mL.

Regarding the *in vivo* findings, licorice flavonoids at a concentration of 30 mg/kg revealed anti-inflammatory effects by reducing the expression of TNFα and IL-1β mRNA expression on mice with acute pulmonary inflammation and licochalcone A at a concentration of 20 μM also revealed anti-inflammatory effects on mice with acute lung injury, as demonstrated by Me et al. [115] and Chu et al. [116], respectively. Liu et al. [117] also reported that licochalcone A of licorice roots has anti-inflammatory activity in mice, while Khattab et al. [118] reported that 10 mg/kg of licorice extracts demonstrated anti-inflammatory activities in mice with ovalbumin-induced bronchial asthma by reducing the levels of interleukin- (IL-) 5 and 13 and IgE.

5.3. Antiproliferative and Cytotoxic Activity. *Glycyrrhiza glabra* L. methanolic extract demonstrated interesting antiproliferative effects in a skin cancer melanoma cell line (WM1316A) at an IC_{50} value of 35.2 μg/mL [119]. Vlaisavljević et al. [120] evaluated the chemical composition of licorice root extracts and reported that fresh root extracts had antiproliferative activity against human cancer cell lines of gynecological origin containing four breast lines (T47D, MCF7, MDA-MB-231, and MDA-MB-361), two cervical cancer cell lines (HeLa and SiHa), and one ovarian cancer cell line (A2780). The authors reported that 30 μg/mL fresh root extracts of licorice demonstrated >50% growth inhibition in all the cell lines except HeLa. In addition, Jo et al. [121] also concluded that *Glycyrrhiza glabra* L. roots exhibited preventive activity against breast cancer. *Glycyrrhiza glabra* L. extract also demonstrated cytotoxicity against hepatocellular, breast, and colorectal cancer cell lines with IC_{50} values in the range of 5.6 to 33.6 μg/mL [122]. It was also revealed that the cytotoxic activity of the methanol extract of licorice roots against immortal human keratinocyte, lung adenocarcinoma, and liver carcinoma cell lines varied depending on geographical origin [123].

Rasul and Ma [124] reported the cytotoxic activity of *Glycyrrhiza uralensis* Fisch. against the gastric adenocarcinoma (SGC-7901) cell line with IC_{50} values in the range of 8.7 to 64.9 μg/mL for different compounds. Moreover, Fan et al. [125] identified the biologically active components of *Glycyrrhiza uralensis* Fisch. leaves and noted that most of these compounds exhibited an antiproliferative effect on human hepatic stellate cells with IC_{50} values in the range of 43 to more than 90 μg/mL for different compounds. *Glycyrrhiza pallidiflora* Maxim. also showed cytotoxicity on human T-cell leukemia (MT-4), human monocyte (U-937), and lymphoblastoid leukemia (CEM-13) cells [126]. The authors reported that isoflavonoid calycosin demonstrated the best results against human T-cell leukaemia MT-4 cells (cell toxicity dose $(CTD)_{50}$, 2.9 μM). Furthermore, the protein extract of licorice roots at concentrations of 50 and 100 μg/mL revealed inhibitory effects and induced apoptosis of colon cancer cells [127]. In another study, it was claimed that glycyrrhetic acid induced apoptosis in non-small-cell lung cancer cell lines [128].

Regarding the *in vivo* findings, licorice extract has shown antitumor activity in mice with colon cancer [129]. Moreover, Liu et al. [117] reported that licochalcone A of licorice root has cytoprotective activity in mice.

5.4. Antimicrobial Effects. Several studies have reported the antimicrobial capacity of *Glycyrrhiza* spp. against several pathogens. *Glycyrrhiza glabra* L. extract showed interesting antimicrobial effects against several microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *Candida albicans*, *C. glabrata*, and *Aspergillus niger* [97, 130–134]. Martins et al. [135] also evaluated the antimicrobial potential using the disc diffusion method and the antibiofilm activity of *Glycyrrhiza glabra* L. against *Candida* strains. The authors concluded that *Candida tropicalis* was most susceptible with a disc diffusion diameter of 10-13 mm. Furthermore, the antimicrobial capacity of licorice roots against *Helicobacter pylori* was reported by Nariman et al. [136]. The minimum inhibitory concentration (MIC) of the licorice root extract ranged between 15.6 and 250 μg/mL.

Similarly, the *Glycyrrhiza uralensis* Fisch. ethanolic extract was also able to inhibit *E. coli*, *B. cereus*, and *S. aureus* with a diagram of zones of inhibition of 18, 14, and 12.4 mm, respectively [137]. *Glycyrrhiza uralensis* Fisch. hexane fraction also demonstrated a good antimicrobial effect with an MIC of 0.25 mg/mL against methicillin-resistant *S. aureus*, whereas the chloroform fraction had a 2.5-fold higher antimicrobial activity [138]. Chouitah et al. [139] also reported interesting antibacterial effects of the *Glycyrrhiza glabra* L. essential oil against *E. coli*, *S. typhi*, and *S. aureus* with an MIC of 4.2, 14.5, and 14.5 μg/mL, respectively. It was reported that iconisoflavan, (3S)-licoricidin, licorisoflavan A, and topazolin exhibited antibacterial activity against *Salmonella typhimurium* [140]. Moreover, glycyrrhizin isolated from *Glycyrrhiza glabra* L. exerted antimicrobial activity against *S. sciuri*, *E. coli*, *S. typhi*, *S. aureus*, *Rhizopus* ssp., and *Aspergillus awamorii* with an inhibition diameter of 25, 28, 20, 25, 14, and 12 mm, respectively [98]. In addition, Chidambaram and Swaminathan [141] claimed that *Glycyrrhiza glabra* L. and glycyrrhizin exhibit antimicrobial activity against *Mycobacterium tuberculosis* with an MIC value of 500 μg/mL. Similarly, the *Glycyrrhiza glabra* L. extract was revealed to be effective against *M. tuberculosis* in U937 human macrophage cell lines with an MIC varying from 0.97 to 1.95 μg/mL [142]. Saponins from *Glycyrrhiza glabra* L. showed good antimicrobial potential on *E. coli*, *S. aureus*, and *P. aeruginosa* with an MIC of 3.12 mg/mL, whereas the MIC was 25 mg/mL for *Proteus mirabilis* strains [143]. An isolated compound from *Glycyrrhiza glabra* L., 18-β-glycyrrhetinic acid, was also effective against *C. albicans* isolated from patients with vulvovaginal candidiasis [144]. The authors reported that a concentration of 6.2 μg/mL of 18-β-glycyrrhetinic acid can inhibit the growth of *C. albicans*. In another *in vivo* investigation, the *Glycyrrhiza glabra* L. extract (80 mg/kg dose) was significantly active for *P. aeruginosa* in mice with lung infection [145]. The authors reported that bacterial load increased constantly in control animals but was reduced by 3 $\log_{10}$ CFU/mL after 7 days of treatment with licorice extract. Moreover, Bawankule et al. [146] reported that CIM-Candy, prepared with *Ocimum sanctum* L., *Phyllanthus emblica* L., and *Glycyrrhiza glabra* L. according to Ayurvedic techniques, demonstrated humoral immune response in a mouse model.

Phytochemical constituents particularly licoricidin, glycyrin, and glycycoumarin were isolated from *Glycyrrhiza uralensis* Fisch., and licoricidin demonstrated the highest antibacterial activity against respiratory infection-associated bacteria, specifically *S. pyogenes*, *Haemophilus influenza*, and *Moraxella catarrhalis* with an MIC of 12.5 μg/mL [147]. In addition, 1-methoxyficifolinol, licorisoflavan A, and 6,8-diprenylgenistein extracted from *Glycyrrhiza uralensis* Fisch. extract was also effective in preventing *S. mutans* biofilm formation [148]. On the other hand, Rajput et al. [149] reported the antimicrobial potential of the *Glycyrrhiza uralensis* Fisch. extract against the plant pathogens *Rhizoctonia solani* and *Pythium aphanidermatum*.

Licochalcones A-E isolated from *Glycyrrhiza inflata* Batalin demonstrated antibacterial activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci* with the MIC ranging from 10 to 20 μg/mL [150]. In addition, Tsukiyama et al. [151] reported a good antimicrobial activity to licochalcone A isolated from *Glycyrrhiza inflata* Batalin against several spore-forming bacteria. It was also reported that phenolic compounds isolated from licorice, such as licoarylcoumarin, glycycoumarin, and gancaonin I, showed potent or moderate antimicrobial activity against vancomycin-resistant *Enterococcus* with the MIC varying from 8 to 16 μg/mL [152]. In another study, Celik and Duran [153] claimed that glycyrrhizinic acid exhibited antimicrobial activity against *H. pylori* strains. At the same time, Lv et al. [154] stated that *Glycyrrhiza* spp. could be used as an antimicrobial in cotton fabric.

It is undisputable from the bioactivities (antioxidative, antimicrobial, antiproliferative, and anti-inflammatory) (Figure 6) of the extracts that licorice is certainly a potential candidate for wider application in functional foods and in the pharmaceutical industry for improving the health standards in humans. These health-promoting effects of the licorice extracts are also evident from the clinical studies presented in the next section.

6. Health-Promoting Effects: From Preclinical to Clinical Evidence

The pharmacological effects of various *Glycyrrhiza* spp.-derived bioactivities have been subject to several clinical studies in humans. Neo Miniphogen-C and glycyrrhizin are the most widely experimented derivatives in humans, and the efficacy has been reported to be significantly high in viral hepatitis and immune deficiency syndrome.

In 1993, Acharya et al. studied the activity of the Stronger Neo Minophagen-C (SNMC) interferon stimulator derived from *Glycyrrhiza glabra* L. at a dose of 40 or 100 mL daily for 8 weeks in 18 hepatitis patients, and a survival rate of 72.2% was obtained ($p < 0.01$). Moreover, SNMC demonstrated an anti-inflammatory activity in patients with liver disease [155]. In another study, an improvement in liver histology was reported by using SNMC, containing 40 mg glycyrrhizin, injected to patients with chronic viral hepatitis [156]. Glycyrrhizin also induced a decrease of 1.5-fold in serum liver enzymes and improved liver histology when intravenously administered [156, 157]. A similar finding on glycyrrhizin has also suggested that it may enhance liver function with occasional complete recovery [158–160].

A retrospective research was carried out to assess the ability of SNMC to prevent chronic hepatitis C (CHC) development, and it was found that long-term SNMC administration has proven to be effective [161]. In addition, SNMC could inhibit liver necrosis and inflammation in CHC patients [162]. Other authors also showed that glycyrrhizin notably reduces the hepatocarcinogenesis rate [163].

Glycyrrhizin derived from *Glycyrrhiza* spp. was tested intravenously at 400–1600 mg/daily for a period of more than one month to three hemophiliacs with acquired immune deficiency syndrome (AIDS), with glycyrrhizin being able to inhibit the human immunodeficiency virus-(HIV-) 1 replication *in vivo* [164, 165]. CD4 lymphocyte count and cluster of differentiation (CD)4/CD8 ratio increased by drip infusion of SNMC (5 mg glycyrrhizin/kg) in AIDS patients [166]. In another research, glycyrrhizin administration to HIV-positive hemophilia patients showed preventive effects by raising the CD4-positive T-lymphocyte number [167]. Also, the application of glycyrrhizinic acid for 12 months produced a positive effect with a 30–40% rate of success in patients with chronic viral hepatitis B [168].

Compared to other species of *Glycyrrhiza*, the *Glycyrrhiza glabra* L. aqueous extract was found to be active in reducing oral mucositis in cancer patients [169]. Another clinical trial suggested that an ImmunoGuard® drug—a standardized fixed herbal combination of *Glycyrrhiza glabra* L. with *Andrographis paniculata* (Burm.f.) Nees, *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim., and *Schisandra chinensis* (Turcz.) Baill. extracts—was safe and effective in Familial Mediterranean Fever (FMF) patient management [170].

In another clinical trial conducted with 236 patients (randomized and double-blind study), licorice-based gargles were found effective in postoperative sore throat and postextubation coughing as compared to sugar water-based gargles. The incidence of sore throat in the case of licorice-based gargles was 19% after a half-hour and only 10% after one hour of surgery, whereas incidence was, respectively, 36 and 35% in the case of sugar water-based gargles [171]. Ghalayani et al. [172] compared a licorice mucoadhesive film with a triamcinolone acetonide film on radiotherapy-induced oral mucositis in a randomized double-blind trial with 60 patients ($n = 30$ for the licorice group and $n = 30$ for the triamcinolone group). The study concluded that both licorice and triamcinolone mucoadhesive-based films are effective in reducing oral discomfort in oral mucositis during radiotherapy. Licorice whole extract was also tested for improving the neurological emergency situation in 75 patients suffering from ischemic stroke [173]. Licorice extract was prescribed at the rate of 450 mg or 900 mg capsules for 7 days with a daily frequency of 3 times. In addition, a decline in the National Institute of Health Stroke Scale and also the modified Rankin Scale scores suggest the beneficial effect of licorice extract in improving the neurological condition in acute ischemic stroke patients. In another double-blind clinical trial, licorice roots were evaluated for their ability to relieve hot flashes and their reoccurrence in 90 menopausal women [173]. Consumption of 3 capsules daily containing

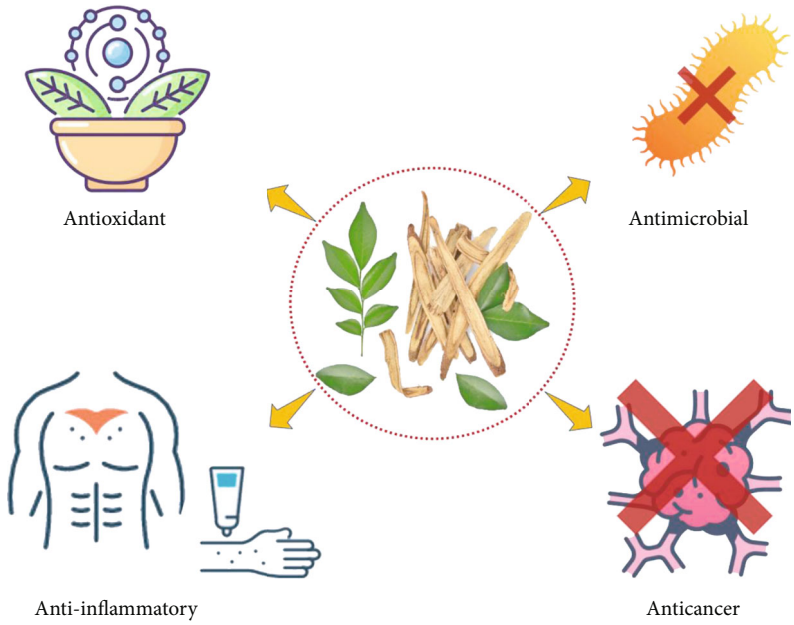

Figure 6: Various biological activities of the *Glycyrrhiza* species extract discussed in this study are presented in the diagram.

330 mg of licorice root extract resulted in a decreased severity and frequency of hot flashes. Taken together, data presented here underline that *Glycyrrhiza* spp. has shown to be effective in reducing the discomfort in cancer, ischemic, neurological, and a few other complications in humans. But, the small number of patients in clinical trials and the very limited trials remain a concern, so that there is a need to pay specific attention in the future to establish *Glycyrrhiza* plant extracts as potential pharma ingredients.

7. Toxicological Data, Safety, and Bioavailability Features

Published literature data showed that *Glycyrrhiza glabra* L. is capable of inducing serious adverse effects [174]. Historically, glycyrrhizin is the term used to describe the crude acid extract of licorice. A component of glycyrrhizin is glycyrrhizinic acid that occurs as calcium, potassium, or ammonium salts. Glycyrrhizinic acid consists of 18β-glycyrrhetinic acid and 2 glucuronic acid molecules [175].

Generally, the assessment of the pharmacological effects of *Glycyrrhiza glabra* L. are mostly related to its principal components, glycyrrhizin and glycyrrhetinic acid (GA), with both demonstrating positive biological activities, as described in the previous sections [176]. Glycyrrhizinic acid and its salts are hydrolysed by intestinal bacteria and absorbed from the gastrointestinal (GI) tract, with glycyrrhizin hydrolysis being related to specialized β-glucuronidase activity [177]. In earlier studies, it was reported that prior to absorption, the microbial hydrolysis of glycyrrhizin leads to the formation of 3β-monoglucuronyl-18β-glycyrrhetic acid [178], and then GA [179]. Gut microbiota acts differently on glycyrrhizin; some microbial glucuronidases, particularly from *Eubacterium* and *Bacteroides* spp., can fully D-glucuronidate glycyrrhizin, whereas some others from *Streptococcus* spp. strains only remove one glucuronide moiety [180]. For instance, after application of 800 or 1600 mg ammonium salt or licorice extract in healthy adult volunteers, the time to reach maximum plasma concentration of 18β-glycyrrhetinic acid was 10 and 12 h, respectively [181]. The interindividual variation in glycyrrhizin response, metabolism, and kinetics is also present, being at least partially caused by the gut microbiota. GA becomes more apparent when the administration occurs on a daily basis, and its complete elimination takes several days [182].

GA is notably absorbed by tissues; however, it greatly adheres to serum albumin [175, 177], with its elimination being directly related to serum protein binding saturation. GA uptake at doses < 10 mg/kg b.w. results in a rapid elimination *via* bile [175]. The plasma clearance of 18β-glycyrrhetinic acid is essentially reduced (<38–64 mL/h/kg) in patients with CHC and liver cirrhosis [177]. In special situations, during premenstrual syndrome, the *Glycyrrhiza glabra* L. application may cause water retention and bloating [183].

Regarding glycyrrhizin consumption, it is commonly well-tolerated at low amounts, while at high doses, it may become toxic [184, 185]. In addition, the interaction of glycyrrhizin with other components present in licorice extracts during intestinal intake may lead to modified bioavailability and serious adverse effects [181]. In this regard, a synergistic suppression of NO production and inducible NO synthase expression was observed while using licorice extracts, while the glycyrrhizin treatment alone did not show these effects [186]. Nonetheless, regarding glycyrrhizin, the most important issue seems to be related to the fact that glycyrrhizin suppresses the 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD2) enzyme that leads to high cortisol concentrations in tissues, resulting in inappropriate mineralocorticoid activity with sodium and water retention and resulting in loss of potassium. Thus, because of the long half-life of 18β-glycyrrhetinic acid, large doses of KCl supplementation are

necessary for weeks [180]. Worthy of note is that this phenomenon has been noted to occur in both humans and animals [187]. Nonetheless, in general, the main adverse effects of *Glycyrrhiza glabra* L. are related to hypertension and various hypokalemic-induced secondary complications (e.g., compensatory reduction in plasma aldosterone and renin concentrations, and hypokalemia) [188]. The summary of the side effects of licorice is presented in Table 1.

Licorice-induced hypokalemia was first described by Revers in 1946 [189]. GA induces pseudohyperaldosteronism by inhibiting 11-β-HSD2 [201]. In addition, hypokalemia can result in hypokalemic paralysis, proximal myopathy, rhabdomyolysis, and flaccid quadriparesis [175]. Other reports have also demonstrated other interactions of licorice [202]. Hypokalemia is well-known to aggravate glucose intolerance. Therefore, licorice ingestion may affect blood glucose levels and interfere with hypoglycemic therapy. The licorice component, isoliquiritigenin, prohibits aldose reductase, which reduces glucose to sorbitol, and inhibits sorbitol accumulation in tissues and cells. Nonetheless, patients with apparent mineralocorticoid excess (AME), a rare form of hypertension caused by mutations in the 11-β-HSD2 gene, may be responsive to both licorice and its components. It is worth noting that untreated AME may prompt damage to different organs (kidney, colon, salivary glands, and placenta) [203]. On the other hand, individuals may exhibit digoxin toxicity because of hypokalemia induced by licorice absorption [204]. The potentiation of warfarin effects due to the licorice inhibitory effect on the hepatic microsomal enzyme system was also reported [205]. Bilateral median neuropathy in a patient possibly related to licorice-induced water retention was also reported [193]. Other complications include hypersensitivity to glycyrrhizin [206], occupational asthma [207], myoclonus [191], or contact dermatitis [208]. In addition, a number of researches have reported the occurrence of ocular complications related to licorice ingestion [209–212]. Also, individuals with reduced liver function may be extremely vulnerable to an extra use of licorice, as an excessive consumption may cause thrombocytopenia [201]. However, some case statements have clinically proven licorice interactions with drugs. It interacts with different kinds of drugs, namely, steroids that may result in a quick metabolism of the coadministered drugs, *via* induction of different enzymes [213]. For example, the extracts from *Glycyrrhiza glabra* L., *Glycyrrhiza inflata* Batalin, and *Glycyrrhiza uralensis* Fisch. inhibit several drug-metabolizing cytochrome P (CYP) 450 enzymes. In addition, some licorice components, like isoliquiritigenin, licoricidin, licochalcone A, 18β-glycyrrhetinic acid, and glycycoumarin, inhibit one or more members of the CYP2C family. CYP1A2, CYP2B6, and CYP3A4 are also inhibited by glycycoumarin and licochalcone A [214].

Although the glycyrrhizin responsiveness is affected by the health status, some patients may show manifestations of toxicity with administration of small doses [215]. Of course, these very sensitive subgroups are comprised of people with decreased 11-β-HSD2 activity, or even with a prolonged GI transit [187]. In one of the studies, it was reported that glycyrrhizin and GA bind to nucleic acids. Spectroscopic data have indicated that glycyrrhizin binds to DNA *via* the A-T and PO2 group, with the affinity of ligand-DNA binding of glycyrrhizin being higher than that of GA [216, 217]. In turn, RNA is binding *via* the G-C and A-U base pairs, with the affinity of ligand-RNA binding being in the same order [218].

Glycyrrhiza glabra L. crude extracts also have estrogenic effects [219]. The obtained data have demonstrated that *Glycyrrhiza* spp. have different zones of estrogenic action, and this underlined the need for precise labeling of herbal supplements. The application of licorice was demonstrated by a rise in blood pressure of pregnant women [220]. Moreover, in another study, the administration of ammonium glycyrrhizinate to rats from 7 to 17 days of pregnancy led to a rise in the prevalence of external hemorrhages and hematomas [221]. Also, high glycyrrhizin doses did not notably affect the birth weight or maternal blood pressure [222], despite that high glycyrrhizin exposure was correlated with shorter gestational period, raising more than two times the risk of preterm (<37 weeks) delivery [223]. Other authors also stated that when children age reaches 8 years, they have poorer cognitive completion, externalizing symptoms and attention issues after high glycyrrhizin exposure [224]. At the age of 12 years, girls, but not boys, were taller, heavier, and had higher body mass index for age [225]. Moreover, children scored lower on tests of intelligence quotient, had faulty memory, and had higher odds of attention deficit/hyperactivity disorder. The outcome in these investigations indicated the potential adverse action during pregnancy. Based on some findings, the negative health effects of glycyrrhizin administration in mothers or their fetus or child were found at doses $\geq$ 500 mg/week [222–226]. Nonetheless, due to the various adverse effects reported, complex well-designed human trials of pregnant women with accurately estimated exposure are needed [227, 228].

The basic problem with licorice dosing lies in its availability in diverse forms and at distinct doses. The combination of a long persistence and enterohepatic circulation along with the unreliable bioavailability has made difficulty in establishing a clear dose-response correlation for glycyrrhizinate substances. Another issue found is the high interindividual variation in sensitivity to glycyrrhizin and glycyrrhizic acid. Many possible reasons for this fact exist; however, the main reason seems to be related to differences in the gut microbiota ability to hydrolyze glycyrrhizic acid to glycyrrhetic acid [177]. Rare causes of apparent mineralocorticoid excess include the genetic deficiency of 11-β-HSD2, sometimes present in adulthood, which although usually appears in childhood, it is often asymptomatic and may stay undiagnosed until adulthood [229]. An *in vitro* evaluation demonstrated that glycyrrhetinic acid in humans at relevant concentrations may disturb cell adhesion, effect anoikis-like cell death, cause morphologic changes, and disturb cytoskeletal proteins [230].

The Food and Drug Administration (FDA) declared licorice root extracts and ammoniated glycyrrhizin as generally recognized as safe (GRAS). However, glycyrrhizinic acid is used for the production of many additive agents in the USA at the following maximum permitted levels: baked foods = 500 mg/kg; alcoholic beverages = 1000 mg/kg; nonalcoholic beverages = 1500 mg/kg; chewing gum = 11000 mg/kg; hard

Table 1: Typical examples of *Glycyrrhiza glabra* L. and glycyrrhizin salt-related side effects in humans.

Gender	Age (in years)	Consumption	Symptoms/complications	Reference
Man	70	Licorice candies, 60–100 g/daily for 4–5 years	Hypertension, hypokalemia, rhabdomyolysis	[190]
Woman	90	Antacid with licorice	Hypertension, hypokalemia, myoclonus, metabolic alkalosis	[191]
Woman	55	High amount of licorice, daily, for 4 years	Hypertension, hypokalemia	[192]
Woman	44	Chewing licorice sticks, 3 days	Edema, bilateral nocturnal hand pain, paresthesias in fingers, bilateral carpal tunnel syndrome	[193]
Woman	46	Herbal tea with licorice, 1-2 cups/daily for 7 years	Hypertension, hypokalemia, reduced plasma aldosterone, and renin	[194]
Woman	39	Herbal medicine with licorice/daily for 8 weeks	Hypertension, hypokalemia, muscle weakness, acute renal failure	[195]
Woman	38	Licorice root tea, 3 times per day for 2 months	Polymorphic, ventricular tachycardia	[196]
Man	52	Licorice, 1.5 g/daily for 2 months	Hypertension, severe asthenia, muscle cramps	[197]
Man	49	Herbal formulation containing licorice, for 1.5 years	Hypokalemia, somnolence, rhabdomyolysis, acute renal failure	[198]
Man	57	Licorice, 900 g/weekly for 3–4 months	Hypertension, acute visual impairment, hypokalemia	[199]
Man	72	Licorice, 2 oz/weekly for 1 month	Hypertension, metabolic alkalosis, severe hypokalemia, increase in rhabdomyolysis indexes myoglobinuria	[200]

candy = 160000 mg/kg; herbs and seasonings = 1500 mg/kg; plant protein products = 1500 mg/kg; soft candy = 31000 mg/kg; and vitamin or mineral dietary supplements = 5000 mg/kg. The maximum levels of glycyrrhizin allowed in food products are 16% for hard candy and 3.1% for soft candy. The maximum allowable levels in other foods ranged from 0.05 to 0.15% [231].

The Flavor and Extract Manufacturers Association (FEMA) has noted the following maximum levels of glycyrrhizic acid ammonium salt: 51 mg/kg for nonalcoholic beverages; 59 mg/kg for alcoholic beverages; 61 mg/kg for baked goods; 79 mg/kg for gelatin/puddings; 91 mg/kg for frozen dairy products; 625 mg/kg for frosting confectionery; 676 mg/kg for hard candies; 1511 mg/kg for soft candies; and 2278 mg/kg for chewing gum [177]. The safe levels of glycyrrhizic acid and its salt are given in Table 2.

However, according to the FAO/WHO Committee, the administration of 80–100 mg glycyrrhizic acid daily was referred to as being capable of provoking hypertension, so that the safety evaluation of glycyrrhizinic acid should be based on human data [175]. In Europe, the European Medicines Agency (EMA) concluded that there are no clinical data in the research-based report to support a "well-established medicinal use." Although the short-term application (≤4–6 weeks) of licorice preparations was judged as safe, there is insufficient data to support licorice root safety during pregnancy and lactation, and in children and adolescents (<18 years). So, the use is not recommended for these groups [213]. The European Food Safety Authority (EFSA) panel decided that licorice extract as a food additive is safe for the main human adult population up to 100 mg daily [232]. Taken together, all data presented above indicate that licorice is safe to consume for maintaining human health, but the data also reinforced the need of a precise labeling of botanical supplements containing licorice compounds.

Table 2: Summary of suggested safe levels of glycyrrhizic acid and its salts.

Recommended safe levels	Source
GRAS	[231]
100 mg daily	[233]
100 mg daily	[175, 234]
Not possible to establish	[213]
100 mg daily	[232]

8. Conclusions

Glycyrrhiza spp. has been used for food and medicinal purposes around the world from ancient times. It is conspicuous that licorice rhizomes and roots have been widely used in many countries, with its medicinal properties being mostly conferred by phytoconstituents. Glycyrrhizin along with liquiritigenin, isoliquiritigenin, 4′-O-methylglabridin, isoprenyl chalcone, formononetin, glabridin, and hispaglabridins A and B are the main phytochemicals present in *Glycyrrhiza* spp., although many new compounds have been progressively discovered. Both the isolated compounds and extracts from *Glycyrrhiza* spp. have shown key antioxidant, antimicrobial, anti-inflammatory, antiproliferative, and cytotoxic effects through *in vivo* and *in vitro* experiments. The biological activities of glycyrrhizin (glycyrrhizic acid) and *Glycyrrhiza glabra* L. are the most evaluated compound and plant species. Clinical trials based on *Glycyrrhiza* spp. have established that its extracts are useful in reducing the issues of discomfort in cancer, ischemic, and neurological patients. For continuing work on licorice, more in-depth studies are needed to clarify some gaps related to its safety and toxicological features along with clinical trials that are needed for establishing *Glycyrrhiza* plant extracts as potential pharma and food ingredients.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

All authors contributed equally to the manuscript. C.Q., J.H.-B., L.H.B., R.K., D.K., Y.U., A.B., B.Y., C.K., S.S., K.A., M.K., U.S., R.M.K., and H.A.R.S. contributed in conceptualization. All authors contributed in the validation, investigation, and data curation as well as in writing the manuscript. B.Ö., N.C.-M., F.S., A.F.A.R., and J.S.-R contributed in reviewing and editing the manuscript. All the authors read and approved the final manuscript.

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Research Article

Polydatin Induces Differentiation and Radiation Sensitivity in Human Osteosarcoma Cells and Parallel Secretion through Lipid Metabolite Secretion

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Osteosarcoma is a bone cancer characterized by the production of osteoid tissue and immature bone from mesenchymal cells. Osteosarcoma mainly affects long bones (femur is most frequently site) and occur in children and young adults with greater incidence. Here, we investigated the role accomplished by polydatin, a natural antioxidative compound, in promoting osteogenic differentiation alone or after radiation therapy on osteosarcoma cells. In vitro, polydatin significantly induced cell cycle arrest in S-phase and enhanced bone alkaline phosphatase activity. Moreover, the differentiation process was paralleled by the activation of Wnt-β-catenin pathway. In combination with radiotherapy, the pretreatment with polydatin promoted a radiosensitizing effect on osteosarcoma cancer cells as demonstrated by the upregulation of osteogenic markers and reduced clonogenic survival of tumor cells. Additionally, we analyzed, by mass spectrometry, the secretion of sphingolipid, ceramides, and their metabolites in osteosarcoma cells treated with polydatin. Overall, our results demonstrate that polydatin, through the secretion of sphingolipids and ceramide, induced osteogenic differentiation, alone and in the presence of ionizing therapy. Future investigations are needed to validate the use of polydatin in clinical practice as a potentiating agent of radiotherapy-induced anticancer effects.

1. Introduction

Osteosarcoma (OS) is the most common primary tumor of the bone in children [1]. The incidence rate is about 3.4 cases per million people per year with a higher percentage in males than in females (5.4 vs. 4.0 cases) [2, 3]. Osteosarcoma can affect all bone districts, but in 90% of cases, it concerns long bones and in 50% of cases the knee. Some studies define osteosarcoma as a pathology associated to differentiation, in which genetic and epigenetic changes interrupt the normal differentiation process starting from mesenchymal stem cells. Tumorigenesis may involve a deregulation of proliferative capacity of stem cancer cells that give rise to an aberrant differentiated osteoblastic progeny [4]. Although most osteosarcomas are sporadic, the main risk factors associated with their incidence include the exposure to radiation or chemotherapy, especially alkylating or anthracycline agents, used to treat other cancers. When OS is diagnosed, approximately 15-20% of patients already have macroscopic evidence of metastases, most commonly in the lungs (85-90%), less frequently in the bones (8-10%), and occasionally in the lymph nodes [5]. OS treatment consists of surgical resection, chemotherapy, and radiotherapy [6]. Osteosarcoma is generally considered a radiation resistant tumor; however, radiotherapy

is indicated as primary treatment in unresectable or surgically inaccessible lesions, with 60-70 (Gray) Gy total dose [7]. Radiation therapy can also be used as adjuvant treatment in R1 or R2 margins lesions, alone or with chemotherapy, with total doses of 64-68 Gy at high-risk sites, or as palliative treatment on primary site or on metastases in case of pain or high risk of pathological fracture (8-45 Gy total dose) [8]. Polydatin (PD), (trans-piceid 3,5,4′-trihydroxystilbene-3-O-β-D-glucopyranoside) is a natural precursor of resveratrol extracted from *Polygonum cuspidatum* plant. PD has a glycosidic ring bound in position C-3 that replaces the hydroxyl group of resveratrol. This modification leads to changes in biological properties of PD that shows better oral absorption and metabolic stability than resveratrol [9]. Moreover, it is absorbed into the cell, using a secondary active transport mechanism, through sodium-dependent glucose transporters (SGLT1) mainly expressed in the stomach and intestines. Previous studies demonstrated a variety of biological functions of PD, including antioxidant properties and protection against ischemia/reperfusion injury, congestive heart failure, endometriosis, and hemorrhagic shock [10–15]. Recent studies have shown that PD is able to improve the survival of mesenchymal stem cells derived from the bone marrow, protecting them from oxidative damage [16]. In addition, PD inhibits the growth of lung cancer and osteosarcoma cells and promotes differentiation of the human colorectal Caco-2 cells [17–19]. Recent studies by Mele et al. demonstrated that PD exerts a significant cytotoxic effect on cancer cells by G6PD inhibition, a rate-limiting enzyme in the pentose phosphate pathway altered in different malignant tumors [20, 21]. The present study is aimed at investigating the role played by PD on the osteosarcoma cells and its effects on osteogenic differentiation process alone or together with radiation therapy.

2. Materials and Methods

2.1. Drugs and Reagents. Cell culture plastics were purchased from Becton Dickinson (Lincoln Park, NJ). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), L-glutamine, trypsin, and antibiotics were purchased from Gibco (Life Technologies, Carlsbad, CA). Transpolydatin with a purity grade higher than 99% has been provided by Glures S.r.l., Academic Spin-Off of Ca' Foscari University of Venice (Venice, Italy).

2.2. Cell Culture. Human osteosarcoma cell lines MG-63 and Saos-2 were available within our research network. MG-63 cells were cultured in RPMI-1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Saos-2 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Both media were enriched with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 1% di L-glutamine, and antibiotics (1.0×10^4 μg/mL penicillin and 1.0×10^4 μg/mL streptomycin). All cell lines were grown at 37°C in a 5% CO_2-humidified atmosphere.

2.3. Cell Viability Assay. Saos-2 and MG-63 were seeded in a 96-well plate at appropriate density (3.0×10^3 and 1.6×10^3 cells/well, respectively). After 24 h, cells were exposed to polydatin at increasing concentrations ranging from 0 to 150 μM. Cell viability was evaluated after 24, 48, and 72 hours by MTT assay as previously described [22]. Each experiment was performed at least three times, and the data were expressed as the mean ± SD.

2.4. Alkaline Phosphatase (ALP) Activity. ALP activity was used as a marker of osteogenic differentiation in human Saos-2 cells. To discriminate different ALP isoenzymes, heat fraction was performed warming the samples for 15 minutes at 56°C; bone ALP (bALP) is heat-labile, and liver ALP is stable. ALP activity was determined using colorimetric alkaline phosphatase assay kit colorimetric (Abcam ab83369). Each experiment was conducted three times, and the data were expressed as the mean ± SD.

2.5. Cell Cycle Analysis. The percentage of cells in G0/G1, S, and G2/M phases was determined by flow cytometry. Briefly, Saos-2 cells were seeded in a 6-well plate at density of 2.0×10^5 cells/plate and after 24 h exposed to 48 μM of polydatin. Then, after 72, 96 h of treatment the cells was detached and washed in PBS 1x, centrifuged, and directly stained in a propidium iodide (PI) solution (50 μg/mL PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) for 60 min at 4°C in the dark. Flow cytometric analysis was performed using a BD Accuri™ C6 instrument (Becton Dickinson, San Jose, CA, USA). To evaluate cell cycle, PI fluorescence was collected as FL2. Analysis was carried out in triplicate in three separate experiments, and the data were expressed as the mean ± SD.

2.6. Immunostaining and Laser Scanning Confocal Microscopy. Saos-2 cells were fixed for 20 min with a 3% (*w*/*v*) paraformaldehyde solution and permeabilized with 0.1% (*w*/*v*) Triton X-100 in PBS at room temperature. To prevent nonspecific interactions of antibodies, cells were treated for 2 h in 5% (*w*/*v*) BSA in phosphate-buffered saline and then were incubated with a specific rabbit Ab raised against anti-β-catenin (Alexa Fluor®, BD Pharmingen™) for 2 h at 37°C. After several washes, the slides were mounted using ProLong™ Diamond Antifade Mountant with DAPI. The analyses were performed with a Zeiss LSM 510 microscope equipped with a plan-apochromat objective X 63 (NA 1.4) in oil immersion.

2.7. Wound Healing Assay. Saos-2 cells were seeded in 6-well plates under normal culture conditions at the density of 5.0×10^5 cells/well. Once it reached ~90% confluence, the medium was removed and the cell monolayer was scratched across the center using a sterile pipet tip (200 μL) to produce a uniform wound area. Through gently washing with sterile PBS1X, cellular debris was removed from the well and the cells incubated with 48 μM of polydatin. Cell migration was monitored by microscopy using an inverted light microscope (Eclipse TE 300 Nikon, New York, USA), and images were captured (10x magnification) at different time points after the scratch (0–48 h). The images acquired were analyzed quantitatively, using a specific wound healing tool of ImageJ software (Maryland, USA). The percentage of relative wound closure was assessed according to the following equation [23]:

$$Wound\ closure\% = \frac{W_0 - W_n}{W_0} \times 100, \quad (1)$$

in which W_n is the width of the cut after the treatment time and W_0 is the width of the scratch before treatment.

2.8. Colony Formation Assay. Clonogenic assay was used to evaluate the effect of polydatin on the radiation-resistant osteosarcoma cells. Saos-2 cells were seeded in a 6-well plate at density of 1.0×10^4 cells/well and exposed to polydatin (48 μM) for 96 h. Then, cells were irradiated with 0, 2, 4, and 6 Gy doses of X-rays (Siemens Mevatron LINAC). After 10 days, colonies were fixed with 70% ethanol and stained with 0.25% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The area of colonies was estimated using ImageJ software. Each sample was processed in triplicate, and the data were expressed as the mean ± SD.

2.9. Real-Time PCR. Total RNA was extracted using mirVana PARIS kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality and quantity were measured by NanoDrop ND-1000 microspectrophotometry (Thermo Fisher Scientific, Wilmington, NC, USA). Reverse transcription was performed according to High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) instructions. qPCR results were obtained from the mean of triplicate measurements for osteopontin, Notch1, and Notch2 using ViiA 7 real-time PCR system (Applied Biosystems, CA, USA), normalized for the mRNA levels of the housekeeping reference gene (HPRT1, also amplified in triplicate).

2.10. LC-MS/MS Lipid Analysis. Lipid were extracted from samples in chloroform : methanol (2 : 1) [24]. MS analysis was performed by direct injection of lipid extracts using an automatic infusion syringe coupled with a nano-ESI-linear ion trap (LIT) Thermo XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were injected (Thomas Scientific, NJ, USA) at a flow rate of 5 μL/min. MS data were scanned in both positive and negative ion modes from 200 to 2000 m/z mass range. Data-dependent MS/MS spectra were collected from the upon fragmentation using CID activation with 35.0% normalized collision energy, activation Q of 0.25, and activation time of 30 ms. Spectra were processed using the Xcalibur Software 3.1 version (Thermo Fisher Scientific, Waltham, MA, USA).

2.11. Statistical Analysis. Statistical analysis was performed using GraphPad Prism (version 6.01, GraphPad Software Inc., San Diego, CA), and values of $p \leq 0.05$ were considered significant. Differences between the treatment and control groups were calculated using Student's t-test.

3. Results

3.1. Antiviability Effects of Polydatin on Osteosarcoma Cell Lines. We evaluated the effect of polydatin on cell viability, *in vitro*, of both MG-63 and Saos-2 human osteosarcoma cell lines. Osteosarcoma cells were treated for 24, 48, and 72 hours with increasing concentrations of polydatin (0-150 μM) and

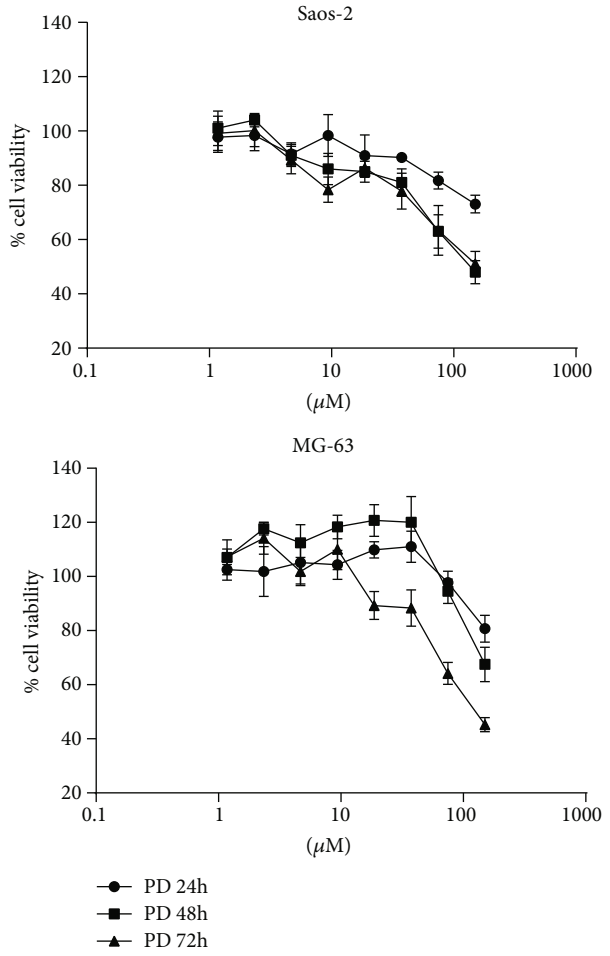

Figure 1: PD effects on osteosarcoma cell line growth. Cell viability was evaluated by MTT assay, after 24, 48, and 72 h of treatment with polydatin in MG-63 and Saos-2 osteosarcoma cell lines.

Table 1: The IC25 (μM) values of PD on osteosarcoma cells.

	Saos-2	MG-63
24 h	122	Not determined
48 h	48	125
72 h	43	55

Table 2: Effect of polydatin on the percentage of cells in different stages of cell cycle Saos-2 cells as measured by flow cytometric analysis ($^*p < 0.05$; $^{****}p < 0.0001$).

	G0/G1 (% of total)	S (% of total)	G2 (% of total)
72 h			
Saos-2	30.6 ± 6.96	12.7 ± 3.45	54.9 ± 9.91
Saos-2 +PD	28.9 ± 7.88	****26.9 ± 5.22	*43.5 ± 10.0
96 h			
Saos-2	30.6 ± 7.52	8.3 ± 2.38	59.9 ± 10.1
Saos-2 +PD	30.0 ± 7.02	****22.5 ± 6.70	*47.6 ± 10.50

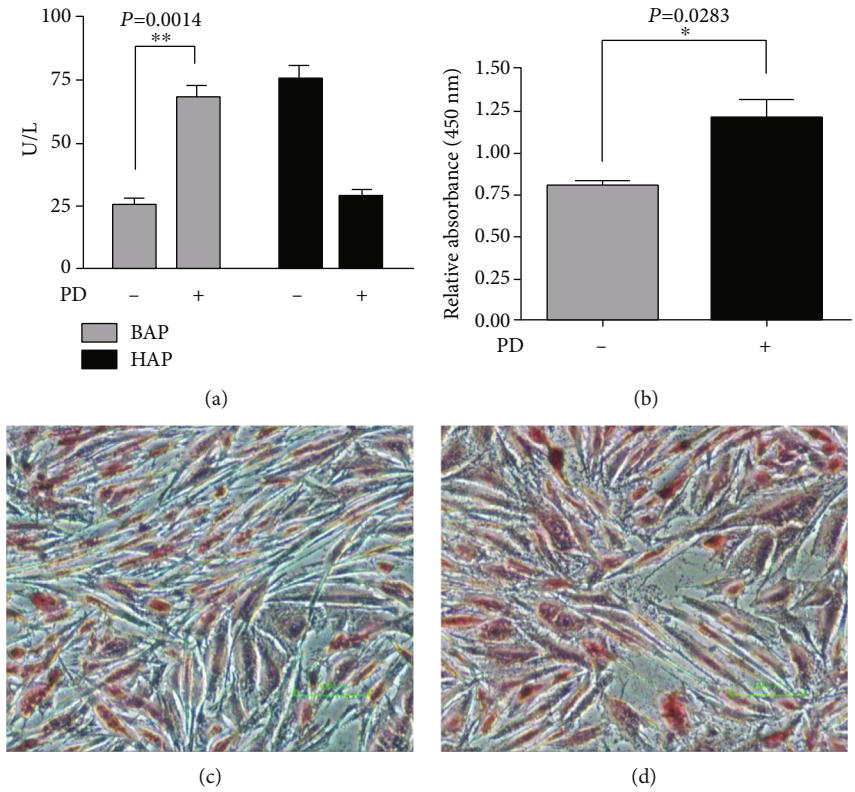

Figure 2: PD increases BAP activity and mineralization: (a) effect of PD on both bone (BAP) and hepatic alkaline phosphatase (HAP) activities in Saos-2 cells. Cells were treated with 48 μM of polydatin and BAP and HAP activities measured using a colorimetric assay. The values are expressed as the means ± SD of three independent experiments; (b) quantitative measurement of the mineralization process after Alizarin Red staining at 405 nm absorbance; images of Alizarin Red staining of (c) untreated Saos-2 cells and (d) cells treated with polydatin.

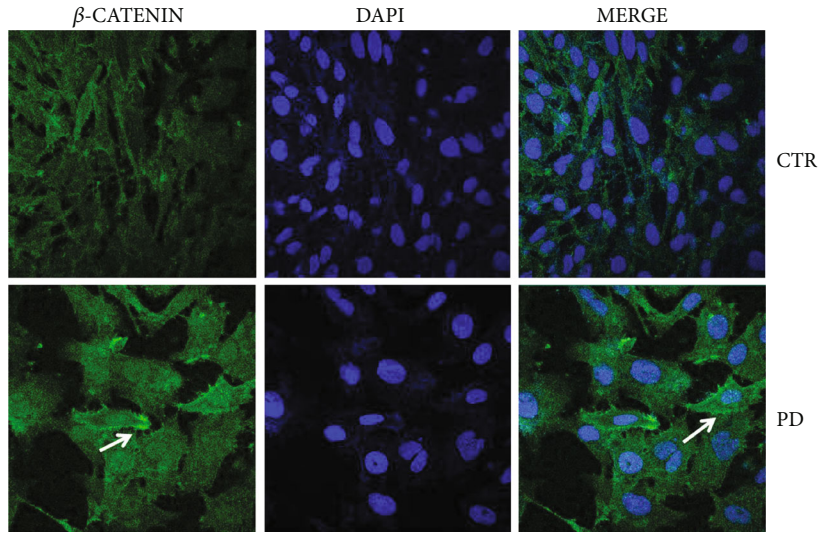

Figure 3: Confocal microscopy images of untreated Saos-2 cells (CTR) and treated with polydatin (PD) at 48 μM for 96 h. The merged images are on the right side: β-catenin (green) and DAPI (blue).

cell viability assessed with MTT assay as reported in Materials and Methods (Figure 1). Polydatin (PD), after 48 hours of treatment, induced an about 25% cell growth inhibition (IC25), in MG-63 and Saos-2 cells at 125 and 48 μM, respectively (Table 1).

Between these two cell lines, Saos-2 cells resemble human osteoblasts because of low proliferation rate, high matrix mineralization ability, and elevated levels of alkaline phosphatase (ALP) activity [25]. In our previous paper, we have demonstrated that polydatin induced a transition from

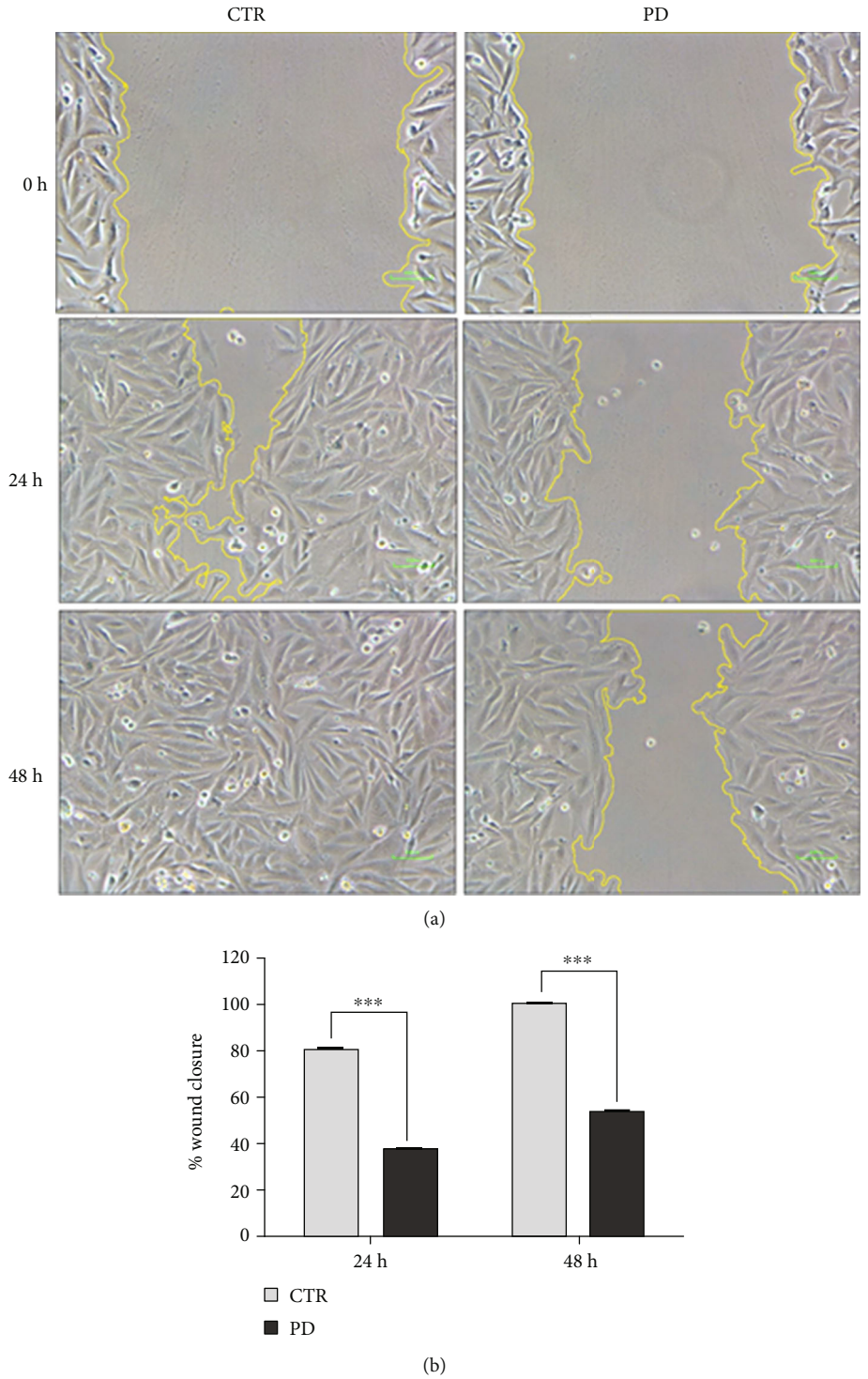

Figure 4: Wound healing assay to determine cell migration. The wound-healing assays were performed at 0, 24, and 48 h in Saos-2 cells treated with polydatin (PD) and untreated cells (CTR). (a) Representative phase contrast microscope images showing the area covered by the cells at 0, 24, and 48 h after wounding (10x magnification). (b) The percentage of relative wound closure was assessed using a specific wound healing tool of ImageJ software ($^{***}p < 0.001$).

a proliferative morphology to cell-specific differentiated structures in human colon cancer Caco-2 cells [19]. Additionally, ALP in Saos-2 cells can be additionally stimulated by molecules such as dexamethasone that induces an osteoblast-like differentiated phenotype [26]. Therefore, the cellular model chosen appeared to be the most suitable in demonstrating the ability of PD to induce differentiation and sensitize osteosarcoma cells to radiotherapy. All subsequent experiments were performed using Saos-2 cells exposed to polydatin at 48 μM in order to evaluate molecular

mechanisms induced by PD alone or in combination with radiation exposure.

3.2. Polydatin Induces Cell Cycle Arrest and Enhances Bone Alkaline Phosphatase Activity. In order to elucidate whether PD was involved in cell cycle regulation, we analyzed its effects on cell cycle distribution by flow cytometry. Saos-2 cells were treated for 72 and 96 hours with PD and subsequently labeled with propidium iodide for 1 h, and the percentage of cells in each phase of the cell cycle (G0/G1-S-G2/M) was determined. The treatment with PD induced a significant increase in S phase with a concomitant reduction in G2/M phase if compared to the control cells (Table 2). In PD-treated cells, the percentage in S phase increased from 8.3% to 22.5% and in G2/M phase decreased from 59.8% to 47.6%, while the G0/G1 phase remained unaltered if compared to control cells. The cell cycle arrest in S phase is one of the prerequisites for cell differentiation [27]. Therefore, we determined the effect of PD on osteogenic differentiation assessing the bone alkaline phosphatase (BAP) activity. As shown in Figure 2, a significant increase of BAP activity was detected after 96 hours in PD-treated Saos-2 cells compared to untreated cells. On the contrary, the level of hepatic alkaline phosphatase activity (HAP) decreased in PD-treated cells if compared to control cells (Figure 2(a)). In addition, we examined the effects of PD on osteoblast-mediated mineralization by ARed Assay (Figures 2(b)–2(d)).

We found that PD promoted osteoblast-mediated mineralization over the control cells. These findings indicate that PD promoted osteoblast differentiation and mineralization in Saos-2 cells. In addition, after 96 hours, the PD-treatment of Saos-2 cells induced an increase of approximately 20% of extracellular nitric oxide production, compared to the untreated cells (data not shown). Our findings suggest that the reduction in cell viability in Saos-2 cells elicited by PD was associated to the increased level of free NO that might be responsible of cellular differentiation and structural changes. Polydatin redistributes β-catenin protein and attenuates migration of osteosarcoma cancer cells.

Cancer-cell migration is a critical process to create distant metastases. The development and progression of cell motility depend on cytoskeletal modifications, changes in cell-substrate adhesive properties, and alterations in the extracellular matrix [28]. In order to study the impact of PD on the metastatic potential of osteosarcoma cells, we assessed the morphological changes triggered by a sublethal concentrations of PD. We have observed, through confocal microscopy, in PD-treated Saos-2 cells a higher cytoplasm/nucleus ratio and an irregular shape if compared to untreated cells (CTR). Moreover, PD induced, after 96 h, a more compact layer of cells that were adherent to neighboring cells together with plasmatic membrane localization of β-catenin (Figure 3), suggesting a less-invasive phenotype.

To analyze the capacity of polydatin in inducing the impairment of cancer cell migration, a wound healing assay experiment was performed. A cell-free area was created in a confluent monolayer by removing Saos-2 cells from the area through mechanical damage, and cells were exposed to the

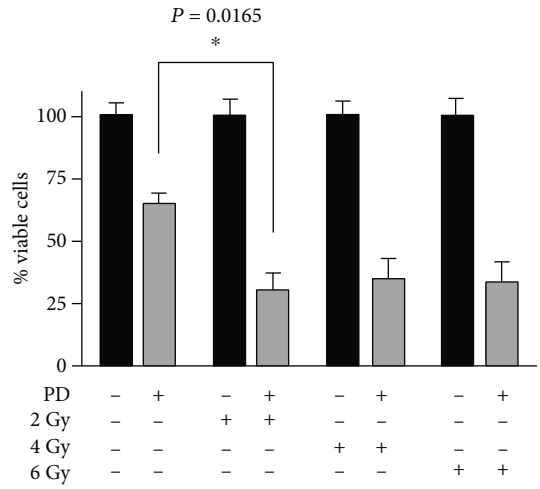

Figure 5: PD effect on cell viability of irradiated Saos-2 cells. Saos-2 cells were pretreated for 96 h with 48 μM polydatin and then exposed to 0, 2, 4, and 6 Gy. The effect of irradiation on cell growth was assessed by Trypan Blue assay after 24 h. The percentage of cell viability of the PD-pretreated cells exposed to 2 Gy was significantly lower than those of the PD-pretreated cells (0 Gy) ($^*p < 0.05$).

polydatin for 24 and 48 hours. As shown in Figures 4(a) and 4(b) at 48 h, control cells had substantially closed the wound, whereas the migration of PD-treated cells was impaired, and the percentage of open wound area was equal to 53.6. Our data clearly show that treatment with PD caused a significant inhibition of cell migration at sublethal concentration in OS cells.

3.3. Polydatin Sensitizes Saos-2 Cells to Ionizing Radiation. It was previously reported that osteosarcoma is associated with poor prognosis due to its high incidence of metastases and chemo and radioresistance. Generally, combination treatment strategies have the aim to potentiate the anticancer activity and/or reduce the therapeutic doses of the anticancer drugs in order to decrease detrimental side effects. To investigate if PD pretreatment could increase the effectiveness of radiotherapy, both cell viability (Figure 5) and colony formation assays (Figure 6) were performed. Saos-2 cells were pretreated with 48 μM of PD for 96 hours and then irradiated with 2, 4, and 6 Gy. The results showed that Saos-2 cells were not sensitive to radiation. In fact, the percentage of viable cells was unchanged compared to the control cell, while PD pretreatment for 96 hours significantly increased the sensitivity of the cells to low radiation dosages (2 Gy) inducing a decrease in viability of about 4- and 2-fold if compared to either untreated or PD-pretreated cells, respectively (Figure 5).

Thereafter, we performed a clonogenic survival assay in order to understand whether the effects of PD on Saos-2 cells may be associated to increased cell radiosensitivity. Saos-2 cells were pretreated for 96 h with 48 μM of PD and then exposed to 0, 2, 4, and 6 Gy radiation, respectively. The cells were cultured for 10 days and colonies stained with crystal violet (Figure 6).

Saos-2 cells treated with PD showed a significant decrease of cell survival when compared to the control cells

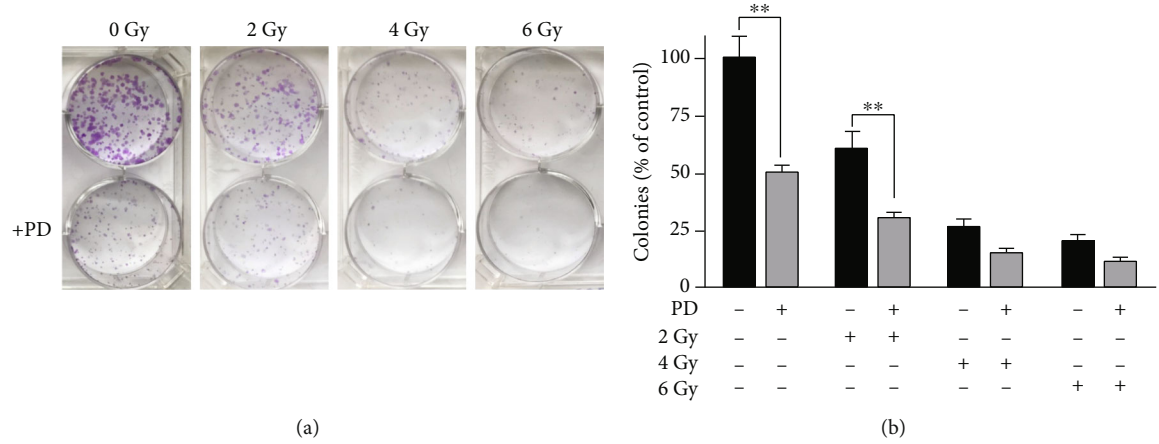

Figure 6: PD effects on colony formation of irradiated Saos-2 cells. PD-pretreated Saos-2 cells were exposed to 0, 2, 4, and 6 Gy of X-irradiation, and crystal violet staining was performed on day 10 after irradiation. (a) Images of clonogenic assay performed in six-well plates, with clones produced by Saos-2 osteosarcoma cells. (b) The area of colonies was estimated using ImageJ software. Data were expressed as the mean ± SD of 3 wells ($^{**}p < 0.01$).

($p = 0.0018$). After exposure to radiation, the PD-pretreated Saos-2 cells showed a decreased ($p = 0.0059$) cell survival when compared to Saos-2 cells exposed to radiation alone. Zhang et al. reported that differentiated cancer cells, after radiotherapy, may reacquire stem cell traits, increased proliferation, and cellular migration [29]. Moreover, in literature, the involvement of Notch pathway during osteogenic differentiation in human osteosarcoma cell line was reported. Activation of Notch2, inducing osteoblast differentiation, not the depression of Notch1, maintained an undifferentiated status [24]. Considering that OS could be induced by disruptions of differentiation of osteoblasts, we have analyzed the mRNA expression level of osteopontin (OPN), a late marker of mesenchymal stem cell differentiation, and Notch1 and Notch2 after the PD and radiation treatment of Saos-2 cells. The results showed a significant increase of OPN and Notch2 mRNA expression level in Saos-2 cells treated with PD in combination with 2 Gy radiation (Figure 7), when compared to the untreated PD cells. Interestingly, in PD-pretreated cells exposed to 2, 4, and 6 Gy radiation, we found a Notch1 decreased expression level compared to the Saos-2 cells submitted at the same radiation doses without PD-pretreatment. The transformation of poorly differentiated Saos-2 cells into highly differentiated cells induced by the PD in combination with radiation could be helpful to induce terminal differentiation of OS cells [30].

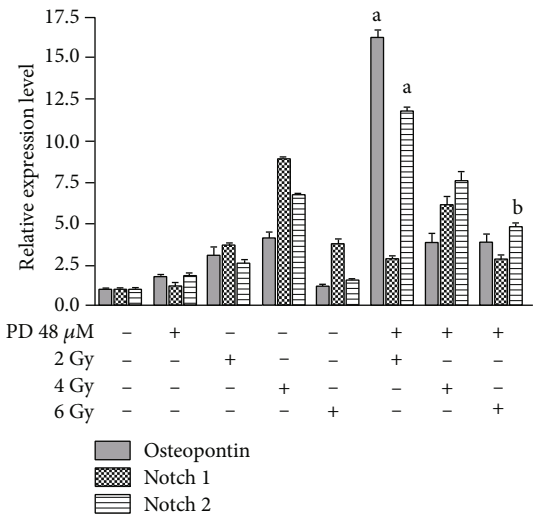

Figure 7: PD effect on relative expression of osteopontin (OPN), Notch1, and Notch2 of irradiated Saos-2 cells. The expression profiles of OPN, Notch1, and Notch2 were determined in the Saos-2 cells without and with PD-pretreatment and subjected to 0, 2, 4, and 6 Gy using a real-time qPCR method $^{a}p < 0.0001$ and $^{b}p < 0.0003$, vs. control represented from the marker values evaluated in cells treated only with 2,4, and 6 Gy.

3.4. Polydatin Modulates the Synthesis and Secretion of Sphingolipids, Ceramides, and Their Metabolites. The different lipid species released in the medium of the Saos-2 cells exposed or not to PD, 2 Gy, and PD+2Gy were detected in a positive ionization mode in the range 200–2000 of *m/z* (Figure 8). In all mass spectra, even if with different intensities, we detected the molecular peak $[M+H]^+$ at 288 *m/z*, 316 *m/z*, 409 *m/z* 906 *m/z*, 950 *m/z*, and 978 *m/z* identified as C17-Sphinganine, dehydrophytosphingosine, LPA (16 : 1), PI (18 : 1 (9Z)/21 : 0), PI-Cer (d20 : 0/26 : 0), and PI (22 : 0/22 : 1), respectively, while the molecular peak $[M+H]^+$ at 381 *m/z*, 437 *m/z*, 619 *m/z*, 685 *m/z*, 1043 *m/z*, and 1347 *m/z* identified as Sphinganine-1-P, PE (P-16 : 0), CerP (d18 : 1/16 : 0), Cer (18 : 0/24 : 0 (2OH)), PIM1 (18 : 1/18 : 1), and PIM3 (18 : 2/18 : 1), respectively, were only present in Saos-2 cells treated with PD alone or in combination with 2 Gy. Phospholipids are the primary components of the plasma membrane. Some of them can act as secondary messengers including phosphoinositides, which are derived from phosphoinositols. Phosphoinositols are in turn involved in intracellular signaling cascades following the activation of specific phospholipases, such as phospholipase C (PLC) in mammalian cells [31].

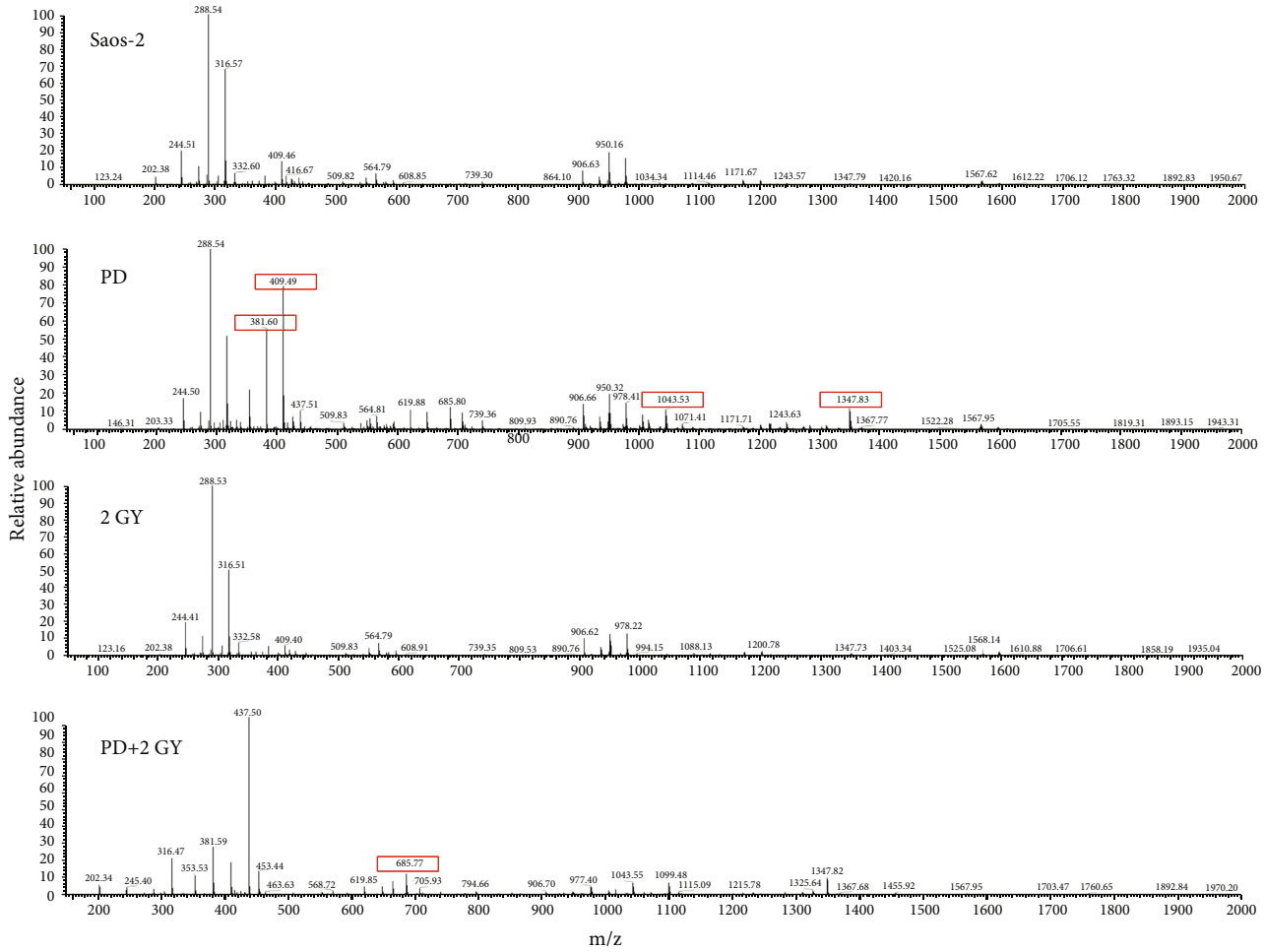

Figure 8: Effect of PD on lipid extracted from media of irradiated Saos-2 cells. Positive ion electrospray mass spectra of lipid molecular species in lipid extracts from the medium of the Saos-2, treated with PD before and after 2 Gy irradiation. Aliquots of chloroform extracts were analyzed directly by electrospray as described in Materials and Methods. Selected peaks are indicated by their m/z values.

4. Discussion

Osteosarcomas are bone tumors occurred principally in children or adolescents [32, 33]. Unfortunately, the presence of mesenchymal component at high-rate proliferation can induce metastasis and relapse and novel therapies need to be urgently discovered [34, 35]. Clinical treatment with polydatin is available for the therapy of patients with liver diseases in China but the knowledge of pathway trigged by its action inside the cells is still unclear. Polydatin has shown potential therapeutic applications due to its antiosteoporotic and anti-inflammatory activities; furthermore, it promotes the osteogenesis of human bone marrow stromal cells via the BMP2-Wnt/β-catenin pathway. In the present study, we have demonstrated that the PD is able to affect the osteosarcoma cell proliferation by inducing cell cycle arrest and a different localization of β-catenin. Moreover, at 48 μM the PD showed no antiproliferative effect on mesenchymal stem cells (data not shown). The effect of PD on OS cell morphology suggests that the migration may also be negatively affected, likely involving EMT through the Wnt/β-catenin pathway.

We also showed that pretreatment with polydatin sensitizes Saos-2 cells to ionizing radiation. As reported by Schwarz et al., radiotherapy is an important option as a local treatment of unresectable tumors or to avoid metastases. The probability of long-term survival, however, is low. In fact, the high radiation doses required to sterilize unresectable osteosarcomas are difficult to achieve with fractionated radiation therapy techniques [36]. By approaching OS as a disease treatable with a combination therapy using polydatin and radiation, we suggest developing novel therapies that can target OS differentiation and subsequently improve the effect of radiotherapy. Recent investigations show how osteogenic differentiation has a role in the pathogenesis of OS considering that OS tumors deregulate the signaling pathways associated with osteogenic differentiation by arresting the cells as undifferentiated precursors [37]. In this context, the expression of osteopontin (OPN) appears to be critical for the status of osteoblasts. OPN is necessary for modulating osteoblast differentiation through integrin $\alpha v\beta 3$-mediated cell signaling [38]. Although OPN overexpression has been associated with worse prognosis in many cancers, in osteosarcoma, the inhibition of OPN expression reduces the differentiation of

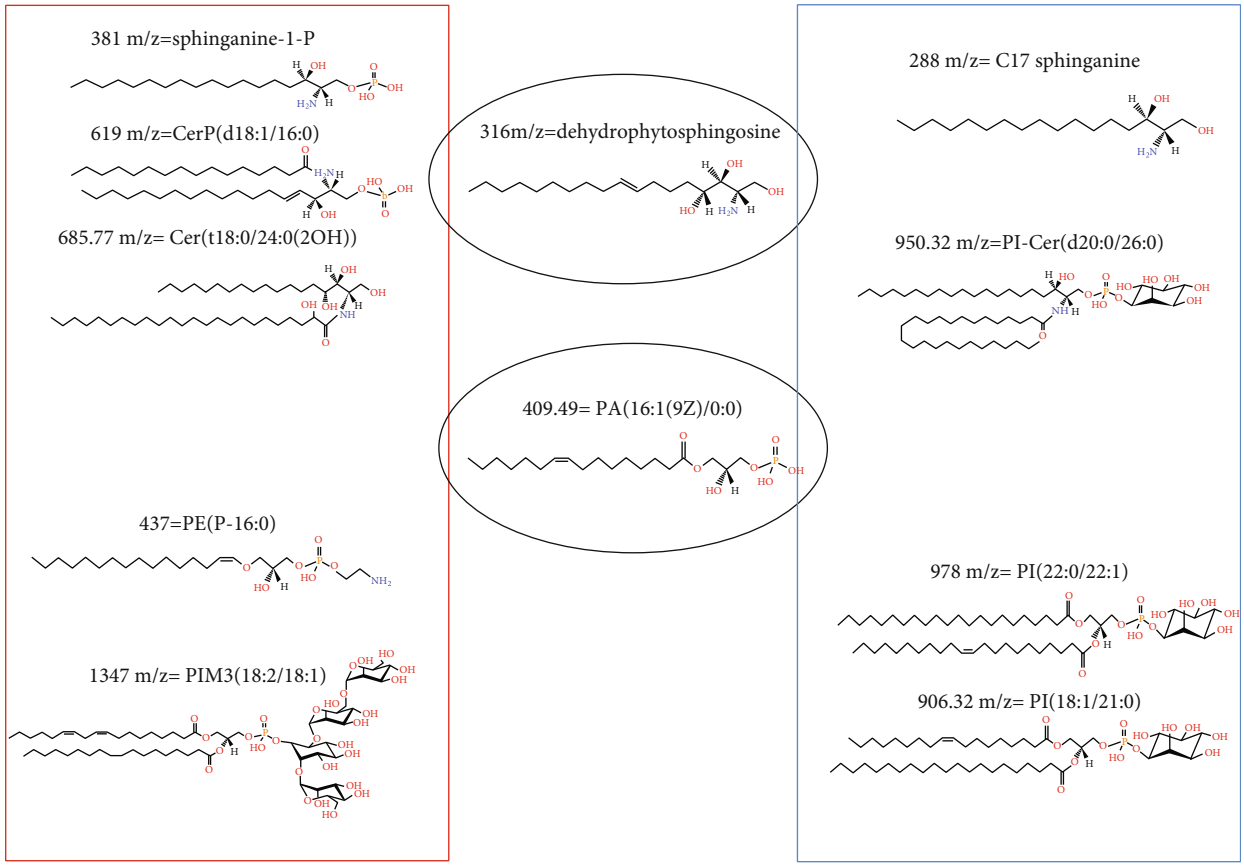

Figure 9: Sphingolipids secreted in the medium of PD-treated (red square) and PD-untreated Saos-2 cells (blue square). The sphingolipids indicated with black circles were commonly expressed in all assessed experimental conditions.

mesenchymal stem cells or immature osteoblasts into mature osteoblasts thus leading to immature osteoblastic-like cells [30, 39]. Along with OPN, Notch signaling was important in osteogenic differentiation of adult stem cells and enhanced human periodontal ligament cells mineralization [40]. Our results proved that polydatin is a natural agent able to promote osteoblast-mediated mineralization and enhance the activity of bone phosphatase in osteosarcoma cells. Marrelli et al. [41] reported a case of adenomatoid odontogenic tumor (AOT) developed in an 18-year-old woman with familial adenomatous polyposis (aFAP). After surgical eradication and through the use of adjuvants of bone regeneration, a reconstruction of eroded bone tissue was performed using biotechnological products. Moreover, it was also reported that lymphoproliferative disorders can affect the oral cavity with possible involvement of the bone [42]. In these cases, the possible use of osteodifferentiating agents such as polydatin could be useful as adjuvant strategies for the bone reconstruction. In past years, evidence has accumulated that $2'$-hydroxy ceramide/sphingolipids have distinct biological functions to regulate various cellular processes [43]. They also play a role in physical properties of cell membranes and regulate cell differentiation by binding to specific target proteins. Moreover, Sphingosine-1-phosphate (SP1) was reported to inhibit the osteoclast formation and the mineralization, respectively [22]. Finally, it was also reported that SP1 and its analogue FTY720 increase radiation sensitivity of human breast cancer cells *in vitro* [23]. Therefore, it can not be excluded that also in our cellular model the increase of SP1 levels induced by PD may act as a potentiating agent of anticancer effects of radiation therapy. In Figure 9, we have reported the scheme of the sphingolipid structures identified by mass spectrometry in the medium of PD-treated and PD-untreated Saos-2 cells. The production of the ceramides ($619\ m/z = \mathrm{Cer\,P(d18:1/16:0)}$; $685.77\ m/z = Cer(t18:0/24:0(2OH))$ secreted in the medium of the PD-treated Saos-2 cells may be induced by de novo synthesis starting from serine and palmitoyl-CoA or by hydrolysis of sphingomyelin or glycosphingolipids (GSL). Therefore, on the basis of the identified structures, we hypothesize that PD induced a sphingolipid rheostat dynamic process suggesting new strategies for therapy of osteosarcoma based upon concomitant treatment of OS with radiotherapy and PD administration on a biological rationale.

5. Conclusions

OS is a tumor of bone which frequently develops metastases following treatments and is partially sensitive to radiotherapy. In the present study, we demonstrated that PD was able to induce cell cycle arrest in S phase, redistributed β-catenin protein, and enhanced osteoblast differentiation markers including BAP, osteopontin, and Notch2. We also found that PD sensitized Saos-2 cells to ionizing radiation, and this

effect was paralleled by increased expression and secretion of ceramides and sphingolipids. The use of polydatin in combination with radiotherapy can consolidate the response to therapy of OS cells and can be used as an adjuvant added to radiotherapy for the local control of the disease and for the prevention of the local recurrence. Overall, our preliminary results suggest that PD may be considered a candidate for improving the efficacy of radiotherapy in patients with osteosarcoma and it is a starting point for additional studies on the mechanisms of OS radio-sensitization and PD activity.

Data Availability

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

AL, MC, and PS are responsible for the conceptualization. AL and PS curated data. PCM and PS did the formal analysis. AL, SL, CC, and PF did the investigation. AI, AS, and SC are responsible for the resources. MC and PS are involved in the supervision. AL, PCM, and PS worked on the visualization. AL, SL, PCM, AI, AS, CC, MC, and PS wrote the original draft. AL, PF, SC, MC, and PS wrote the review and did the editing. All authors have read and agreed to the published version of the manuscript.

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Review Article

Genus *Viburnum*: Therapeutic Potentialities and Agro-Food-Pharma Applications

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The genus *Viburnum* (Adoxaceae, Dipsacales) is of scientific interest due to the chemical components and diverse biological activities found across species of the genus, which includes more than 230 species of evergreen, semievergreen, or deciduous shrubs and small trees. Although frequently used as an ornament, the *Viburnum* species show biological properties with health-promoting effects. Fruits, flowers, and barks of certain species are used for pharmaceutical purposes or as cooking ingredients, hence containing biochemical compounds with health-promoting activity such are carotenoids, polyphenols, and flavonoids. However, its taxonomical determination is difficult, due to its wide distribution and frequent hybridizations; therefore, an objective classification would allow us to understand its biological activity based on its phytochemical components. More than

sixty phytochemical compounds have been reported, where vibsanin-type diterpenes and their derivatives are the most prevalent. Leaves and twigs of *V. dilatatum* contain the largest number of phytochemicals among the genus. Through preclinical evidence, this study provides insight regarding antioxidant, antibacterial, anti-inflammatory, cytotoxic, and anticancer activities of genus *Viburnum*.

1. Introduction

The genus *Viburnum* (Adoxaceae, Dipsacales) is comprised of more than 230 species of evergreen, semievergreen, or deciduous shrubs and small trees distributed primarily within the temperate forest regions of the northern hemisphere but also in the mountains of Central and South America, Southeast Asia (Philippines, Malaysia), and southeastern Australia and Tasmania.

Although the species of the genus are generally well adapted to mesic forest environments, particular species have been reported to inhabit both, cold boreal forests, and tropical rainforests, as is the case of *Viburnum edule* and *Viburnum amplificatum,* respectively. The regions of highest species diversity are eastern Asia and Latin America [1–4]. *Viburnum* was established by Linnaeus, classified in the Caprifoliaceae family [5] and soon after reclassified in its own family Viburnaceae [6]. Based on the Dipsacales phylogeny research, *Viburnum* was subsequently listed in Adoxaceae, together with *Adoxa*, *Sinoadoxa*, *Tetradoxa*, and *Sambucus* [7] This classification is widely accepted today [1, 4, 8]. Nevertheless, certain plant classification systems, e.g., the one from Takhtajan [9], still recognize *Viburnum* as a member of the Viburnaceae family.

Leaves of the *Viburnum* species are simple, petiolate, and opposite and rarely whorled. The small hermaphroditic flowers form paniculate or umbellate inflorescences of 15-500 flowers each. Marginal flowers are sometimes sterile, and their purpose is to attract pollinators. The plants are self-incompatible. The androecium consists of five stamens. Filament bases are attached to corolla petals. The gynoecium is of three carpels. Two of the three inferior ovaries are aborted. The fruit is a red to purple black single-seeded drupe. Floral nectaries located at the gynoecium apex additionally differentiate viburnums from another Adoxaceae [10–14].

Taxonomical determination of the genus *Viburnum* is difficult, due to its wide distribution and frequent hybridizations, both natural and horticultural [15, 16]. Traditionally accepted classification is based on plant morphology (inflorescences, flowers, extrafloral nectaries, trichomes, and pollen grain exine) and recognizes the sections *Solenotinus*, *Viburnum*, *Pseudotinus*, *Tomentosa*, *Tinus*, *Megalotinus*, *Lentago*, *Oreinotinus*, *Odontotinus*, and *Opulus Opulus* ([17, 18]); however, several mostly regional-oriented section level revisions were made ([3] and references therein). Besides, plant morphology, taxonomical values of the phytochemical amentoflavone isolated from *Viburnum* leaves and branchlets [19], as well as anatomical characteristics of fruits, cork, assimilating parenchyma, and leaf epidermal cells [20, 21], were also assessed. More recently, assessments of *Viburnum* diversification have been made at a molecular level [3, 4, 8, 22, 23]. Clement et al. [24] proposed a *Viburnum* phylogenetic classification and provided formal phylogenetic definitions for 30 clades.

Many viburnums are of high ornamental value. Fruits, flowers, and barks of certain species are used for pharmaceutical purposes or as cooking ingredients, hence containing biochemical compounds with health-promoting activity such as carotenoids, polyphenols, and flavonoids. Many viburnums are of high ornamental value. Fruits, flowers, and barks of certain species are used for pharmaceutical purposes or as cooking ingredients, hence containing biochemical compounds with health-promoting activity such are carotenoids, polyphenols, and flavonoids. These biochemical components are responsible for the main biological activities of Viburnum plants, and although the antioxidant, antibacterial, anti-inflammatory, and cytotoxic activity have been well documented, some current research also links to the chemical components found in Viburnum exerting protection and treatment against diseases. Chronic diseases including diabetes [25], cancer [26], Alzheimer's [27], and all diseases that can be caused by a clot [28].

Substantial research has been carried out in relation to the mechanisms responsible for the synthesis, location, and accumulation of bioactive compounds. The most widely researched species are *V. opulus*, *tinus*, *lantana*, and *orientale*; although, various biochemical constituents have also been isolated from *V. arboricolum*, *awabuki*, *ayavacense*, *betulifolium*, *cylindricum*, *davidii*, *dilatatum*, *erosum*, *furcatum*, *grandifolium*, *japonicum*, *jucundum*, *luzonicum*, *odoratissimum*, *phlebotrichum*, *pichinchense*, *prunifolium*, *rhytidophyllum*, *suspensum*, *urceolatum*, and *wrightii* [21, 29–31]. Studies undertaken to assess within-species variability in terms of bioactive compounds profile and content [32–34] facilitate breeding and subsequent growing of viburnums as pharmaceutical crops.

2. Traditional Uses

Plants and humans are in a forever codependent relationship. Plants are considered the lungs of the earth but also provide food, shelter, timber, medicines, etc. for humans. Medicinal plants represent a rich reservoir of bioactive chemicals of therapeutic potential [35]. A review of the scientific literature reveals multiple applications from medicinal plants of the genus *Viburnum* (Table 1), implying the need for further research and documentation.

3. Phytoconstituents

Although *Viburnum* initially diversified in East Asia, other regions such as eastern North America, the mountainous regions of Mexico, Central America, and northern South America are also viewed as centers of high diversity. Most of the American *Viburnum* species seem to have derived

Table 1: Ethnomedical use of genus Viburnum.

S. no.	Species name	Part used	Medicinal applications	References
1	*Viburnum grandiflorum*	Wood, leaf, flower, bark	Digestive problems, purgative, abdominal pain, diuretic, antimalarial, respiratory diseases, toothaches, yphoid, whooping cough, anesthetic	([35]; [36]; [37]; [38]; [39])
2	*Viburnum opulus*	Fruit	Gall bladder, liver disease, diuretic; bleeding, heart disease, blood pressure, coughs, cold, neurosis, diabetes	([40]; [41]; [42]; [43]; [44]; [45]; [34])
3	*Viburnum cylindricum*	Leaves	Cough, diarrhea, rheumatoid arthritis, and tumefaction Cough, diarrhea, rheumatoid arthritis, and tumefaction Cough, diarrhea, rheumatoid arthritis, tumefaction, insecticide	([46]; [30]; [47])
4	*Viburnum dilatatum*	Fruits	Spice, pickles	[48]
5	*Viburnum cotinifolium*	Bark	Hepatic and digestive problems	[47]
6	*Viburnum erubescens*	Roots, stem, leaves	Cough, insecticide	([49]; [47])
7	*Viburnum lantana*	Bark	Rubefiant, analgesic	[41]
8	*Viburnum foetens*	Whole plant, bark	Purgative, sedative, cleaning teeth "miswak"	[50]
9	*Viburnum punctatum*	—	Fever, stomach disorders	[51]
10	*Viburnum prunifolium*	Roots	Dysmenorrhea, menstrual irregularities, convulsions, hysteria, fever, palpitation, heart diseases, hysterical fits, arthritis, heart tonic, improves blood circulation	([52]; [51])
11	*Viburnum prunifolium*	—	Sedatives, muscle relaxants, cardiotonics	[53]
12	*Viburnum nervosum*	Leaf, bark, root	Purification of blood, carminative, hemorrhage, uterine disorders, asthma, furunculosis, menorrhagia	([54]; [55]; [52])
13	*Viburnum coriaceum*	Root and bark	Antispasmodic, uterine relaxant	([56]; [52])
14	*Viburnum foetidum*	Leaves, aerial parts	Menorrhagia, hypothermic, cardiovascular; uterine disorders, skin disease, emmenetic	([52]; [57])
15	*Viburnum jucundum*	—	Cancer, gastrointestinal diseases	([52]; [58])

from Asian clades, while the species in Latin America appear to be directly related to those in the eastern United States [59]. In this way, the genus represents a classic pattern of disjunct distribution between the Old and the New World.

Roughly 200 species have been reported for the genus *Viburnum*, among shrubs and trees, distributed mainly on the Asian side, used primarily in traditional medicine for the treatment of diseases such as rheumatoid arthritis, cough, diarrhea, tumefaction, swelling, kidney cramps [60], antitumor, antimicrobial, antioxidant, antihyperglycemic, anti-inflammatory, and neuroprotective activities [61]. Leaves, flowers, and fruits are currently being used in Turkish folk medicine [62].

The study of *Viburnum* is of scientific interest due to the chemical components and diverse biological activities found across species of the genus. Although the highest number of chemical compounds has been found in leaves, the presence of phytochemicals has also been reported in fruits, roots, and seeds (Table 2). *V. awabuki*, *V. dilatatum*, *V. fordiae Hance*, *V. odoratissimum*, and *V. opulus* have the highest amount of extracted phytochemical constituents (diterpenes, triterpenes, iridioides, monoterpenes, sesquiterpenes, flavonoids, lignans, etc.) and are dependent on the specific part of the plant from which the extract is obtained, as well as the type of solvent used.

In the case of *V. awabuki*, few studies have been found regarding the description of its phytochemical constituents (Table 2). The number of chemical compounds reported amounts to more than sixty, where vibsanin-type diterpenes and their derivatives are the most prevalent (Figure 1). These phytochemicals can also be present in species such as *V. odoratissimum* and *V. suspensum*. These diterpenoids can chemically be eleven-membered ring, seven-membered ring, and rearranged types, represented by vibsanine B, vibsanine C, and neovibsanine A, respectively. Some vibsane-type diterpenoids have exhibited increasing biological activities, and their challenging structures combined with attractive neurotrophs have drawn synthetic attention [29]. Table 2 reports the compounds found in *V. awabuki* corresponding to methanolic extracts of leaves and twigs of the plant

Table 2: Main phytochemicals found in the species of the genus *Viburnum*.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
V. arboricolum	Viburolide	—	Leaves and twigs	Antitumor effects	[31]
V. ayavacense	7,10,2′,3′-Tetraacetylsuspensolide F, 7,10,2′,3′-tetraacetylisosuspensolide F, 7,10,2′,6′-tetraacetylisosuspensolide F, 2′,3′-diacetylvalerosidate, 2′,3′-diacetylisovalerosidate, isoviburtinoside II, isoviburtinoside III, isosuspensolide E, isosuspensolide F.	—	Leaves and twigs	Antitumor effects	[31]
V. awabuki	6-O-methyl-6,7-dihydroxyvibsanin B, 4-hydroxyvibsanin A, 14(R*),15-epoxyneovibsanin B, 14(S*),15-epoxyneovibsanin B, (8Z)-neovibsanin B, 18-O-methylvibsanin C, (8Z)-vibsanin E.	—	Leaves	—	[29]
	Vibsanin G, vibsanin H, vibsanin K, vibsanin O, vibsanin P, vibsanin Q, vibsanin R, vibsanin S, vibsanin T, vibsanin U, vibsanin V, vibsanin W, furanovibsanin A, furanovibsanin B, furanovibsanin C, furanovibsanin D, furanovibsanin E, furanovibsanin F, furanovibsanin G, neovibsanin A, neovibsanin B, neovibsanin C, neovibsanin D, neovibsanin G, neovibsanin H, neovibsanin I, spirovibsanin A, 7-epineovibsanin D, 3-*O*-methylfuranovibsanin A, 7-epifuranovibsanin B, 15,18-di-*O*-methylvibsanin H, 18-*O*-methylvibsanin K, cyclovibsanin A, 15-*O*-methylcyclovibsanin A, 15-*O*-methylcyclovibsanin B, 3-hydroxy-15-*O*-methylcyclovibsanin A, 15-*O*-methylneovibsanin F, 15-*O*-methyl-14-epineovibsanin F, 15-*O*-methyl-18-oxoneovibsanin F, 2-*O*-methylneovibsanin H, 2-*O*-methylneovibsanin I, 14-epineovibsanin G, 5-epivibsanin C, 5-epivibsanin E, 5-epivibsanin H, 5-epivibsanin K, 18-*O*-methyl-5-epivibsanin K, 3-hydroxyvibsanin E, 3b,28-dihydroxyolean-12-en-1-one, 3b,28-dihydroxyolean-12-en-11-one, 13,28-epoxyolean-11-en-3-one, 6a-hydroxy-3-oxolup-20(29)-en-28-oic acid, Ψ-taraxasterol acetate, 6b-hydroxy-3,20-dioxo-30-norlupan-28-oic acid, 4,20-dihydroxy-3,4-secolupane 3,28-dioic acid 3-methyl ester, awabukinol, 3-hydroperoxyawabukinol, 4-hydroperoxyawabukinol, epicatechin, catechin, 7R-dihydrodehydrodiconferyl alcohol 4-*O*-β-D-glucopyranoside, 8R-dihydrodehydrodiconferyl alcohol 4-*O*-β-D-glucopyranoside, vibsanol, 9′-*O*-methylvibsanol, dihydrodehydrodiconiferyl alcohol, 3′,6′′-*O*-diacetylscopolin, 2′-*O*-acetylscopolin, 6′-*O*-acetylscopolin	—	Leaves and twigs	—	[31]
V. betulifolium	Viburnalloside, decapetaloside	—	Leaves and twigs	Antitumor effects	[31]
V. chinshanence	Lignan, chinshanol A.	—	Roots	—	[63]
V. coriaceum	Phytosterols, triterpenoids, phenolics, phenolic glycosides	—	Roots	—	[64]
V. cotinifolium	Biflavonoid	Petrol, benzene and methanol	Leaves	—	[65]
V. cylindricum	3-*O*-Caffeoylquinic acid methyl ester, 4-*O*-caffeoylquinic acid methyl ester, 5-*O*-caffeoylquinic acid methyl ester	—	Leaves and twigs	Antitumor effects	[31]
	2′-O-Acetylhenryoside, 2',3'-di-O-acetylhenryoside, 2′,6′-di-O-acetylhenryoside, 2′,3′,6′-tri-O-acetylhenryoside, 2′,3′,4′,6′-tetra-O-acetylhenryoside, 2-[(2,3-di-O-acetyl-beta-D-glucopyranosyl)oxy]-6-hydroxybenzoic acid, 6-hydroxy-2-[(2,3,4,6-tetra-*O*-acetyl-beta-D-glucopyranosyl)oxy]benzoic acid	Methanolic extract	Leaves and stems	—	[66]

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
V. dilatatum	Viburnol A, viburnol B, viburnol C, viburnol D, viburnol E, viburnol F, viburnol G, viburnol H, viburnol I, viburnol J, viburnol K, viburnudienone B1 methyl ester, viburnudienone B2 methyl ester, viburnenone B1 methyl ester, viburnenone B2 methyl ester, viburnudienone H1, viburnudienone H2, 2,3,4-trihydroxybutyl 6-*O*-(E)-caffeoyl- *β*-D-glucopyranoside, 2,3,4,5-tetrahydroxyhexyl 6-*O*-(E)-caffeoyl-*β*-D-glucopyranoside, arbutin, furcatin, 4-allyl-2-methoxyphenyl 6-*O*-*β*-D-apiofuranosyl(1 → 6)-*O*-*β*-D-glucopyranoside, *p*-hydroxyphenyl 6-*O*-(E)-caffeoyl- *β*-D-glucopyranoside, *p*-hydroxyphenyl 6-*O*-(E)-caffeoyl-*β*-D -allopyranoside, salidroside, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, dilaspirolactone, kuromanin	—	Leaves and twigs	—	[31]
	Jiamizioside E, jiamizioside A, jiamizioside B, jiamizioside C, jiamizioside D	—	Fruits	—	([67]; [68])
	Cyanidin 3-sambubioside, 5-caffeoyl quinic acid	—	Fruits	—	[69]
	Cyanidin 3-sambubioside, cyanidin 3-glucoside, quercetin, 5-O-caffeoyl-4-methoxyl quinic acid, chlorogenic acid.	—	Fruits	—	[3]
	Cyanidin 3-sambubioside, cyanidin 3-glucoside, 4-methoxy chlorogenic acid, chlorogenic acid, quercetin	—	Fruits	—	[70]
	3Z-Hexenol, l-linalool	—	Flower	—	[71]
	2-(-Glucopyranosyloxy)-benzyl 3-(-glucopyranosyloxy)-benzoate	—	Roots	—	[72]
	7-*O*-Tigloylsecologanolic acid, 7-ketologanin, 7-*O*-benzoylsecologanolic acid, 7-ketologanin	Methanolic, ethyl acetate, n-butanol, water	Stems	—	[73]
V. erosum	Vibruresinol, (70 R,8S,80 S)-3,50-dimethoxy-30,4,80,90 -tetrahydroxy70,9-epoxy-8,80–lignan, (+)-syringaresinol, (+)-pinoresinol, (+)-pinoresinol-4-*O*-*β*-D-glucopyranoside, herpetol, vibsanol, (-)-dehydrodiconiferyl alcohol, icariside E4, (-)-dihydrodehydrodiconiferyl alcohol	Methanolic extract	Stems	Neuroprotective activity on glutamate-induced cell death in HT22 cells	[74]
	Loganic acid, sweroside, 7-O-tigloylsecologanol, 3,7-dihydroxy-8-methyl-cyclopenta[c] pyran-4-carboxylic acid, rel-(1S,5R,9S)-9-ethenyl-1-(beta-D-glucopyrinosyloxy)-5,9-dihydro-5-{2-[(2-methylbut-2-enoyl)oxy]ethyl}-1H-pyran-4-carboxylic acid, viburnin, epi-7-O-tigloylsecologanolic acid.	Methanolic extract	Stems	—	[75]
V. erubescens	Phytosterols, triterpenoids, and phenolic compounds and their glycosides	—	Roots	—	[64]
	Phytosterols, triterpenoids, glycosides (saponins), phenolic compounds (flavonoids and procyanidins)	Soxhlet method	Leaves and stems	—	[76]
V. fordiae Hance	(7S,8R)-4-Hydroxy-3,3′,5′-trimethoxy-8′,9′-dinor-8,4′-oxyneolignan-7,7′,9-triol, (7R,8R)-4-hydroxy-3,3',5'-trimethoxy-8′,9′-dinor-8,4′-oxyneolignan-7,7′,9-triol, (7R,8R)-4-hydroxy-3,3′,5′-trimethoxy-8,4′-oxyneolignan-7,9,9′-triol-7′-one, *γ*-lactone, 3-(3,4-dihydroxyphenyl)-4-pentanolide, uvaol, 28-nor-urs-12-ene-3b,17b-diol, 2,3-O-isopropylidenyl-2a,3a,19a-trihydroxyurs-12- en-28-oic acid, erythrodiol, oleanolic acid, lupeol, megastigmadien-3,9-dione, loliolide, dehydrololiolide, 2a-hydroxycineole, (+)-isolariciresinol, umbelliferone, 3-(4-	—	Aerial parts	—	([77]; [78])

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
	hydroxy-3-methoxyphenyl)propane-1,2-diol, 1-(4-hydroxy-3-methoxyphenyl)-1-methoxypropan-2-ol, coniferyl aldehyde, p-hydroxylcinnamaldehyde, (+)-2-hydroxy-1-(4-hydroxy-3- methoxypheny) propan-1-one, syringaldehyde, protocatechuate, 3,4-dihydroxybenzoic acid methyl ester, vanillin, p-hydroxybenzaldehyde, salicylic acid, benzyl alcohol, hydroquinone 7,8-bis-*O*-Isopropylidene-dihydroeugenol	—	Air-dried plants	—	[79]
	Fordioside, alangilignoside D, salicin, rhapontigenin	—	Leaves	—	[67]
	C-13-Norisoprenoid, alangionoside C, pisumionoside, koaburaside, 3,5-dimethoxy-benzyl alcohol 4-O-β-D-glucopyranoside, 3,4,5-trimethoxybenzyl-β-D -glucopyranoside, arbutin, salidroside, (3R,9R)-3-hydroxy-7,8-didehydro-β-ionyl 9-O-α-D-arabinopyranosyl-(1 → 6)-β-D-glucopyranoside, 2-(4-O-β-D-glucopyranosyl) syringylpropane-1,3-diol	—	Stems	—	[80]
	Norneolignan glycoside, 7-noraryl-4′,7-epoxy-8,5′-neolignan glycoside, (7R,8R)-guaiacylglycerol 4-O-β-D -(6-O-vanilloyl) glucopyranoside, (7S,8S)-guaiacylglycerol 4-O- β-D-(6-O-vanilloyl) glucopyranoside, (7S,8R)-guaiacylglycerol 4-O-β-D-(6-O-vanilloyl) glucopyranoside, coniferyl alcohol 4-O-[6-O- (4-O-β-D-glucopyranosyl)vanilloyl]-β-D-glucopyranoside	—	Stems	—	([77]; [78])
	Viburfordoside A, viburfordoside B, viburfordoside C, viburfordoside D, viburfordoside E, viburfordoside F, viburfordoside G, viburfordoside H, viburfordoside I.	—	Fruits	—	[81]
	Fordiane A, fordiane B				
V. formosanum	Dioxatricyclodecane	Methanolic extract, ethyl acetate	Leaves	—	[82]
V. furcatum	Furcatoside A, furcatoside B, furcatoside C, isoquercitroside, kaempferol 3-*O*-b-d-glucopyranosyl-7-O-a-l-rhamnoside, furcatin,	—	Leaves and twigs	Antitumor effects	[31]
V. grandifolium	Luteolin, 3′-*O*-b-d-xylopyranosyl (1 → 2)-*O*-b-d-glucopyranoside	—	Leaves and twigs	Antitumor effects	[31]
V. japonicum	2′,3′-*O*-Diacetylfurcatoside C, chavicol	—	Leaves and twigs	Antitumor effects	[31]
	2′-Acetyldihydropenstemide, 2′-acetylpatrinoside, 3′-acetylpatrinoside, lantanoside, dihydropenstemide, betulalbuside A.	—	Leaves and twigs	Antitumor effects	[31]
V. lantana	2-Heptanone, n-heptanal, benzaldehyde, 1-octen-3-ol, 6-methyl-5-hepten-2-one, 2-pentylfuran, 2, 4 heptadienal, n-octanal, limonene, n-octanol, cis-linalool oxide, L-linalool, n-nonanal, α-terpineol, methyl salicylate, n-decanal, 2E, 4E-nonadienal, 2E-decanal, cinnamaldehyde, 2E, 4Z-decadienal, 2E, 4E-decadienal, α-cubebene, α-copaene, β-bourbonene, E-caryophyllene, β-copaene, geranyl acetone, γ-murolene, α-amorphene, germacrene D, β-ionone, α-muurolene, γ-cadinene, Δ-cadinene, trans-cadina-1, 4-diene, α-calacorene, occidentalol, E-nerolidol, spathulenol, caryophyllene oxide, salvial-4(14)-en-1-one, γ-eudesmol, α-muurolol, β-eudesmol, α-cadinol, occidenol, eudesma-4(15,7-dien-1-β-ol, pentadecanal, 6, 10, 14-trimethyl-2-pentadecanone, nonadecane, n-heneicosane, n-docosane, n-tricosane	Hydrodistillation	Air-dried whole plants	Antimicrobial activity	[83]

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
V. luzonicum	Luzonial A, luzonial B, luzonoside A, luzonoside B, luzonidial A, luzonidial B, luzonoside C, luzonoside D, luzonoid A, luzonoid B, luzonoid C, luzonoid D, luzonoid E, luzonoid F, luzonoid G,	—	Leaves and twigs	Antitumor effects	[31]
V. macrocephalum	Methyl (2-*α*-L-rhamnopyranosyloxy)acetate, methyl (2R-3-*α*-L-rhamnopyranosyloxy)glycerate, methyl (3R-4-*α*-L-rhamnopyranosyloxy-3-hydroxy)butanoate, bridelionoside B (4), (6S,7E,9R)-roseoside, linarionoside A, 3,7,11-trimethyl-1,6-dodecadien-3,10,11-triol, (+)-8-hydroxylinalool, beta-sitosterol and daucosterol	Ethanolic extract	Branch	Insecticidal and antimicrobial activities	[61]
	Apigenin-7-*O*-[6-*O*-(5-methoxy-3-hydroxy-3-methyl-5-oxovaleryl)]-beta-D-glucopyranoside, kaempferol-3-*O*-(6″-*O*-acetyl)- *β*-D-glucopyranoside, kaempferol-3-*O*-(6″-*O*-crotonyl)- *β*-D-glucopyranoside, kaempferol 4′-*O*-*α*-L-rhamnopyranoside, (+)-naringenin-7-*O*-*β*-D-glucopyranoside, (−)-naringenin-7-*O*-*β*-D-glucopyranoside, afzelin, apigenin-7-*O*-*β*-D-glucopyranoside	Ethanolic extract	Branch	—	[84]
	(7R,8S)-Guaiacylglycerol4-*O*-*β*-D-(6-*O*-vanilloyl) glucopyranoside.	Ethanolic extract	Fruits	Intestinal alpha-glucosidase inhibitory activity	[85]
V. melanocarpum	(−)-(7R,7′R,8S,8′S)-Pinoresinol 4′-*O*-*β*-dglucopyranosyl-4-*O*-(6-*O*-vanilloyl)-*β*-d-glucopyranoside, (7′E,7S,8R)-7,9,9′-trihydroxy-3,3′,5′-trimethoxy-8-O-4′-neolignan-4-*O*-[6-*O*-(4-*O*-*β*-D-glucopyranosyl)vanilloyl]-*β*-d-glucopyranoside, pinoresinol 4,4′-*O*-*β*-dglucopyranoside, pinoresinol 4′-*O*-*β*-d-glucoside, syringaresinol 4′-*O*-*β*-d-glucopyranoside, pinoresinol 4-*O*-*β*-d-glucopyranosyl-(1→6)-*β*-d-glucopyranoside	Ethanolic extract	Stems	Inhibitory activity against alpha-glucosidase	[86]
	(7R,8S)-Syringylglycerol 8-O–D-allopyranoside, (7S,8S)-syringylglycerol 8-O–D-allopyranoside.	Ethanolic extract	Stems	Radical scavenging and glucosidase inhibitory activities	[87]
	Vibsane, vibsanol I, 15-hydroperoxyvibsanol A, 14-hydroperoxyvibsanol B, 15-O-methylvibsanin U, 5,6-dihydrovibsanin B, 14,18-O-diacetyl-15-O-methylvibsanin U, vibsanin K	—	Leaves	—	[88]
	Vibsane, vibsanin B, vibsanin F, neovibsanin B, neovibsanin	—	—	—	[89]
	Vibsanin I, vibsanin L, 14-hydroxyvibsanin F, 14R*,15-epoxyvibsanin C, 14S*,15-epoxyvibsanin C	—	Leaves	—	[90]
	Vibsanol C, vibsanol D, vibsanol E, vibsanol F, vibsanol G, vibsanol H, vibsanin X	—	Leaves and twigs	—	[91]
V. odoratissimum	Vibsanin A, vibsanin B, vibsanin C, vibsanin D, vibsanin E, vibsanin F, vibsanin I, vibsanin L, vibsanin M, aldovibsanin A, aldovibsanin B, aldovibsanin C, 7-epialdovibsanin A, 5-epivibsanin G, 18-O-methylvibsanin G, 14-hydroxyvibsanin F, (14R*)-14,15-epoxyvibsanin C, (14S*)-14,15-epoxyvibsanin C, vibsanol A, vibsanol B, 6*β*-hydroxy-3-oxolup-20(29)-ene-27,28-dioic acid, 6*α*-hydroxy-3-oxolup-20(29)-ene-27,28-dioic acid, quercetine.	—	Leaves and twigs	—	[31]
	Vibsanin C, vibsanin H, dehydrovibsanin G, vibsanol, 9-aldehydevibsanol, (+)-9′-O-senecioyllariciresinol, (8Z)-10-epi-vibsanin C, (+)-9′-O-isovaleryllariciresinol	—	Leaves and branch	—	[92]

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
	5-epi-Vibsanin G, 18-O-methylvibsanin G, vibsanin M, aldovibsanin C	—	Leaves and flowers	—	[93]
	Vibsanin B, vibsanin E, vibsanol A, vibsanol B, 6β-hydroxylup-20(29)-en-3-oxo-27,28-dioic acid, 6α-hydroxylup-20-(29)-en-3-oxo-27,28-dioic acid, 6α-hydroxylup-20(29)-en-3-oxo-28-oic acid	—	Leaves and flowers	—	[94]
	Benzaldehyde, exo-2-methylnorbornane, cis-linalool oxide (furanoid), linalool, nonanal, isophorone, 4-oxoisophorone, trans-linalool oxide (pyranoid), methyl salicylate, decanal, methyl nonanoate, eucarvone, 1-[2-(1-hydroxy-1-methylethyl)cyclopropyl]-ethanone, nonanoic acid, methyl geranate, methyl o-anisate, α-ionone, geranyl acetone, pentadecane, hexadecane, β-eudesmol, heptadecane, methyl eudesmate, octadecane, hexahydrofarnesyl acetone, phthalic acid, decyl isobutyl ester, methyl palmitate, methyl linoleate, methyl linolenate, heneicosane, docosane	—	Flowers	—	[95]
	β-Amyrin, α-amyrin, stigmasta-4-en-3-one, ergosta-4,6,8(14),22-tetraen-3-one, Olean-12-en-3-one, lupeol, 3-hydroxyolean-12-en-1-one, 3-acetoxyolean-12-en-28-ol, 3-acetoxyolean-12-en-28-oic acid, 3,28-dihydroxyolean-12-ene, 3,28-dihydroxyurs-12-ene, 28-hydroxyolean-12-en-3-one, trans-phytol, betulin	—	Roots	—	[96]
	Ascorbic acid, total phenolics, total anthocyanin	—	Leaves	—	[97]
	Methyl pentanoate, 3Z -Hexen-1-ol, n-heptanal, 2-pentylfuran, phenyl acetaldehyde, linalool oxide, terpinolene, L-linalool, n-nonanal, 2E, 6Z-nonadienal, 4-terpineol, α-terpineol, methyl salicylate, myrtenol, n-decanal, trans-carveol, geraniol, 2E-decanal, 2E, 4Z–decadienal, 2E, 4E-decadienal, α-copaene, rans-β-damascenone, trans-α-ambrinol, α-amorphene, germacrene D, β-ionone, γ-cadinene, Δ-cadinene, trans-cadina-1, 4-diene, α-calacorene, ledol, tetradecanal, α-muurolol, α-cadinol, pentadecanal, manool, n-heneicosane, phytol, n-docosane, n-tricosane	—	Air-dried whole plants	—	[83]
	Chlorogenic acid	—	Fruits	—	[98]
V. opulus	Gallic acid, procyanidin B1, (+)-catechin, procyanidin B2, (−)-epicatechin, neochlorogenic acid, chlorogenic acid, rutin, isorhamnetin, isorhamnetin 3-O-rutinoside, quercetin, anthocyanins, cyanidin-3-O-sambubioside, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside	—	Fruits		[99]
	Coumaroyl-quinic acid, chlorogenic acid dimer, procyanidin B2, catechin, procyanidin trimer epicatechin, proanthocyanidin dimer monoglycoside, quercetin-hexose + pentose, rutin, quercetin-hexose, quercetin-deoxyhexose	—	Fruits	—	[100]
	Quinic acid, catechin dimer, catechin, chlorogenic acid (3-O-caffeoylquinic acid), procyanidin C1, epicatechin, neochlorogenic acid (5-O-caffeoylquinic acid)	—	Fruits	—	[101]
	Ethyl alcohol, 1-propano, 2-butanone, acetic acid, ethyl acetate, isobutanol, 2-pentanone, 3-methyl-1-butano, 2-methyl-1-butanol, 1-pentanol, 2-hexanone, 2-hexanol, hexanal, 3-methyl-butanoic acid, 2-methyl-butanoic acid, 3-hexen-1-ol (Z), 1-hexanol, 2-heptanone, 2-heptanol, heptanal, 3-methyl-pentanoic acid, 1-heptanol, 1-octen-3-ol, 6-methyl-5-hepten-2-one, 2-octanone, ethyl hexanoate, 2-octano, octanal, hexyl acetate, a-terpinene, p-cymene, limonene, 1,8-cineole, trans-	—	Fruits	—	[101]

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
	linalool oxide (furanoid), 2-nonanone, linalool L, nonanal, dill ether, α-terpineol, ethyl decanoate, β-caryophyllene (E)				
	Gallic acid, ascorbic acid, vitamin C	—	Fruits	—	[102]
	L-Malic acid, L-ascorbic acid, oxalic acid	—	Fruits	—	[103]
	Chlorogenic acid, (+)-catechin, (-)-epicatechin, cyanidin-3-glucoside, cyanidin-3-rutinoside and six different glucosides of quercetin.	—	Fruits	—	[104]
	Chlorogenic acid, oxalic acid, citric acid, tartaric acid, malic acid, quinic acid, succinic acid, fumaric acid, procyanidin B2, (−)-epicatechin, p-coumaric acid, isorhamnetin 3-O-rutinoside, isorhamnetin 3-O-glucoside, quercetin 3-O-glucoside.	—	Fruits, flowers, and bark	—	[34]
	β-Sitosterol, stigmasterol, colesterol, α-amyrin-urs-12-en-3-β-ol, β-amyrin-Olean-12-en-3-β-ol, lupeol, 3-keto-urs-12-ene, 3-keto-Olean-12-ene, A:D-neoolean-12,14-diene, A-neoolean-5,12-diene	—	Seeds	—	[105]
V. orientale	Chlorogenic acid.	Methanolic extract and aqueous	Fruit, leaf, and branch	Enzyme inhibitory and antioxidant effect	[106]
	Betulalbuside A, anatolioside E, betulalbuside B, anatolioside, anatolioside A, anatolioside B, anatolioside C, anatolioside D,	—	Leaves and twigs	Antitumor effects	[31]
V. phlebotrichum	Phlebotrichin, p-hydroquinone, arbutin,	—	Leaves and twigs	Antitumor effects	[31]
	Dideoxyplicatumoside A, erythro-syringylglycerol-β-O-4′-(+)-isoeucommin A 4″-O-β-D-glucopyranoside,	—	Leaves	—	[107]
	Plicatumoside A, (+)-neomedioresinol 4,4′-di-O-β-D-glucopyranoside, (+)-neomedioresinol 4,40-O-di-β-D-glucopyranoside	—	Leaves	—	[108]
V. plicatum	7-O-Tigloylsecologanol, 7-O-tigloylsecologanolic anolic acid, 3′-O-[(2S)-2-methylbutanoyl]henryoside, (4R)-α-terpineol O-β-D-glucopyranoside2), (7S,8R)-dihydrodehydrodiconiferyl alcohol 9-O-β-d-glucopyranoside2), (7R,8S)-dihydrodehydrodiconiferyl alcohol 9-O-β-d-glucopyranoside2), quercetin 3-O-robinobioside2), quercetin 3-O-rutinoside2), kaempferol 3-O-robinobioside2), kaempferol 3-orutinoside2).	Methanolic extract, chloroform, ethyl acetate, butanol, water	Leaves	—	[109]
V. propinquum	(3,4,2′,4′-tetrahydroxy-trans-chalcone), (3,4,2′,4′-tetrahydroxy-trans-chalcone-2′-O-β-D-glucoside), quercetin, (+)-dihydroquercetin, eriodictyol, taraxerol, β-sitosterol, stigmasterol, 3β,28-dihydroxy-12-ursene, ursolic acid, daucosterol, 4,2′,4′-trihydroxy-dihydrochalcone, 4,2′,4′-trihydroxy-dihydrochalcone-2′-O-β-D-glucoside.	—	Leaves and stems	Antioxidant activity	[110]
	Scopoletin	—	Haw	Antispasmodics	[111]
V. prunifolium	2′-Acetyldihydropenstemide, 2′-acetylpatrinoside, patrinoside, 2′-(E)-p-coumaroyldihydropenstemide	—	Leaves and twigs	Antitumor effects	[31]
V. punctatum	Phytosterols, triterpenoids, and phenolic compounds and their glycosides	—	Roots	—	[64]

Table 2: Continued.

Viburnum spp.	Phytoconstituents	Type of extract	Part of plant	Biological activities	References
V. rhytidophyllum	Ursolic acid, 7,10,2′-triacetylpatrinoside, 7-*p*-coumaroylpatrinoside, 10-acetylpatrinoside, catechin, arbutin, henryoside, salicin, viburnine	—	Leaves and twigs	Antitumor effects	[31]
V. sargentii	(-)-Epicatechin, 5,7,4-trihydroxy-flavonoid-8-C–d-glucopyranoside, 1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3–l-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propane-diol (erythro), 1-(4-hydroxy-3-methoxyphenyl)-2-[4-(3–l-rhamnopyranoxypropyl)-2-methoxyphenoxy]-1,3-propanediol (threo), (R)-4-hydroxylphenol *O*-(6-*O*-oleuropeoyl)–d-glucopyranoside, (R)-3-methoxy-4-hydroxylphenolO-(6-*O*-oleuropeoyl)–d-glucopyranoside, quercetin-3-*O*-rutinoside.	Ethanolic extract and aqueous	Fruits	Antioxidant activity	[112]
	Neovibsanin, vibsanins B, vibsanins F, vibsanin F, neovibsanin B.	—	—	—	[89]
V. suspensum	Neovibsanin F, gomojoside A, gomojoside B, gomojoside C, gomojoside D, gomojoside E, gomojoside F, gomojoside G, gomojoside H, gomojoside I, gomojoside J, gomojoside K, gomojoside L, gomojoside M, gomojoside N, gomojoside O, gomojoside P, gomojoside Q, 3-oxooleana-11,13(18)-dien-28-oic acid, 24-hydroxy-3-oxooleana-11,13(18)-dien-28-oic acid, 6β-hydroxy-3-oxooleana-11,13(18)-dien-28-oic acid, 2′,6′-*O*-diacetylscopolin.	—	Leaves and twigs	Antitumor effects	[31]
V. tinus	3-*O*-β-D-Galactopyranosyl-(1 → 2)-*O*-β-D-glucuronopyranosideoleanolic acid 28-*O*-β-D-glucopyranosyl ester, 3-*O*-(β-D-glucuronopyranosyl)oleanolic acid, 28-*O*-β-D-glucopyranosyl ester, oleanolic acid, viburtinoside A, viburtinoside B, viburtinoside I, viburtinoside II, viburtinoside III, viburtinoside IV, viburtinoside V, suspensolide F, suspensolide A, isoquercitroside, kaempferol 3-*O*-β-D-galactopyranoside, quercetine, nobiletin, rutin, afzelin, scopoletin 7-*O*-sophoroside, 2,6-Di-C-methylnicotinic acid 3,5-diethyl ester	—	Leaves and twigs	Antitumor effects	[31]
V. urceolatum	α-Amyrin palmitate, lupeol palmitate, β-amyrin acetate, ursolic acid, urceolatoside A, urceolatoside B, urceolatoside C, urceolatoside D, urceolide,	—	Leaves and twigs	Antitumor effects	[31]
V. wrightii	α-Amyrin palmitate, ursolic acid, astragalin, kaempferol 3-*O*-β-D-galactopyranoside, kaempferol 3-*O*-rutinoside, apigenin 7-O-β-D-glucoside, arbutin, *p*-hydroxypheny β-D-allopyranoside, 6-*O*-acetylarbutin, 4′-hydroxycinnamic acid, viburnolides A, viburnolides B, viburnolides C	—	Leaves and twigs	Antitumor effects	[31]

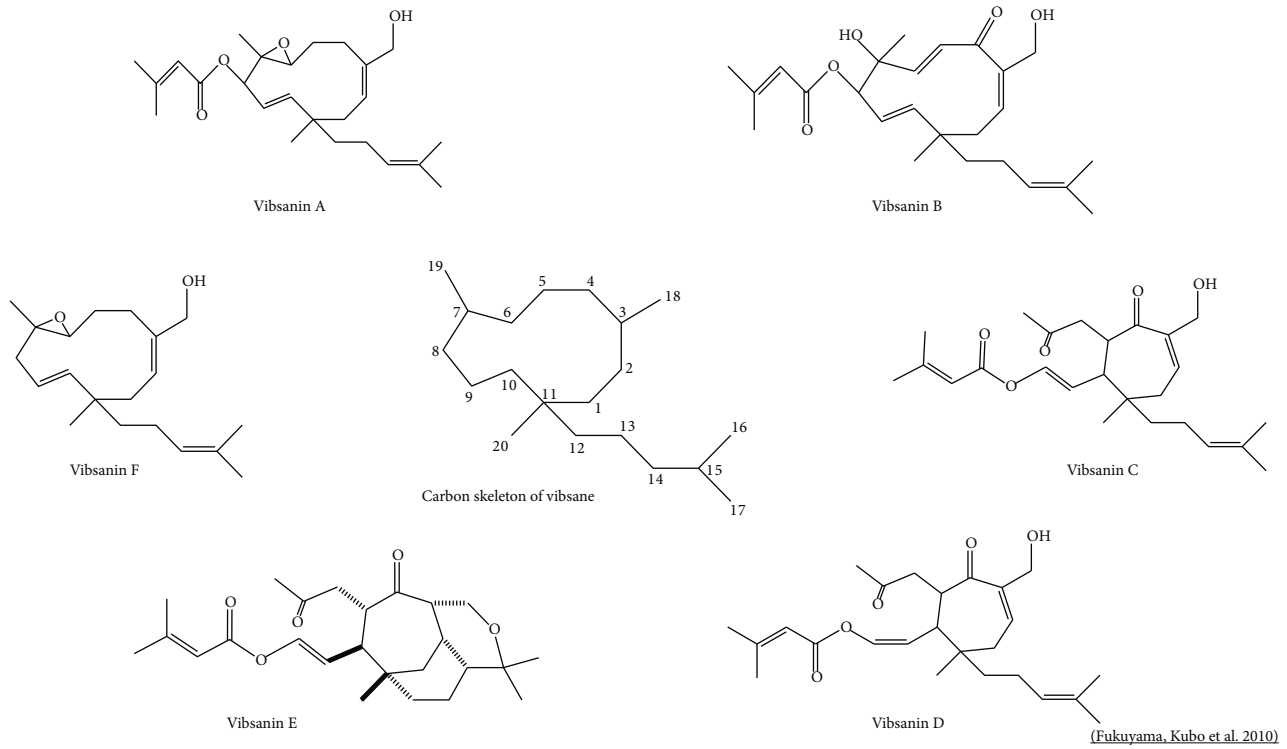

Figure 1: Chemical structure of vibsane-type diterpenoids components reported on the genus *Viburnum*.

[29, 31]. Other compounds such as triterpenoid derivatives, sesquiterpenes (such as awabukinol), specific flavonoids of the catechin type, coumarin derivatives, and lignans have also been observed.

Leaves and twigs of *V. dilatatum* contain the largest number of phytochemical constituents within the genus (Table 2). The triterpenoids viburnols (Viburnol A, B, C, D, E, F, G, H, I, J, and K), viburnudienone, and viburnenone are present in leaves, as well as, flavonoids, phenolic, and lactone type compounds [31]. The main compounds in the essential oils of *V. dilatatum* flowers are phenethyl alcohol, 3Z-hexenol, and l-linalool [71]. Glycosylated phenolic compounds of the jiamizioside type (A, B, C, and D) and anthocyanidins and quercetin flavonoids have also been found in methanolic and squeezed juice extracts of fruits. Compounds derived from phenylpropanoids such as 5-O-caffeoyl-4-methoxyl quinic acid and polyphenolic esters (chlorogenic acid) were also reported in the fruits, along with cyanidin 3-sambubioside, 5-caffeoyl quinic acid [3, 69, 70], cyanidin 3-glucoside, 4-methoxy chlorogenic acid, chlorogenic acid, and quercetin [3, 70]. These extracts show a significant antioxidant activity related to the described compounds [113]. Only one study reports the presence of the compound, 2-(-glucopyranosyloxy)-benzyl 3-(-glucopyranosyloxy)-benzoate, in the methanolic extract of roots [72].

This field of research is relatively novel and phytochemicals in *V. fordiae* have been found in leaves, branches, and fruits (Table 2) [61, 79]. First reports of the phytochemical compounds in *V. fordiae* were made in methanolic extracts of leaves. Compounds such us glycosylated phenolic type (fordioside), lignan glucoside (alangilignoside D), alcoholic β-glucoside (salicin), sand tilbenoid (rhapontigenin) were reported [67]. Recent studies describe essential oils [114], terpenoids, neolignans [81], and 52 phenolics [67] in stems, leaves, and roots. These compounds have shown to exhibit weak or moderate antioxidant, anti-inflammatory, and α-glucosidase inhibition properties [78, 81]. Neolignan glycosides, viburfordosides A–I, neolignans, fordianes A and B (Figure 2), and analogues present in the ethanolic fruit extract have been described to serve as functional foods and for the prevention and treatment of type 2 diabetes (T2D) [81].

Studies have described a wide range of phytochemical constituents for members of the genus *Viburnum*, such as diterpene, triterpene, and flavonoid type for *V. odoratissimum*. vibsanin, vibsane [89], and vibsanol, and their derivates present in leaves (Figure 2) [88, 90, 93, 94], twigs [91], and branches [92], have been found using methanol and ethanol as primary extraction solvents (Table 2). Triterpenes 6β-hydroxy-3-oxolup-20(29)-ene-27, 28-dioic acid, and 6α-hydroxy-3-oxolup-20(29)-ene-27, 28-dioic acid [31, 94] have been reported in leaves. Volatiles in *V. odoratissimum* flowers consist of esters, alkanes, ketones, alcohols, aldehydes, and acids. The main compounds in flowers were methyl o-anisate, heneicosane, methyl salicylate, 1-[2-(1-hydroxy-1-methylethyl) cyclopropyl] ethanone, linalool, nonanal, and methyl palmitate [95]. Compounds such as triterpenes and trans-phytol fatty alcohol have been detected in the root [96].

Studies carried out on fruit juice [100, 103, 104], aqueous methanolic extracts [98], acidic mixtures of water/methanol [102], and mixtures of methanol/acetone/water [99] have been assessed in *V. opulus* (Table 2). It is one of the few

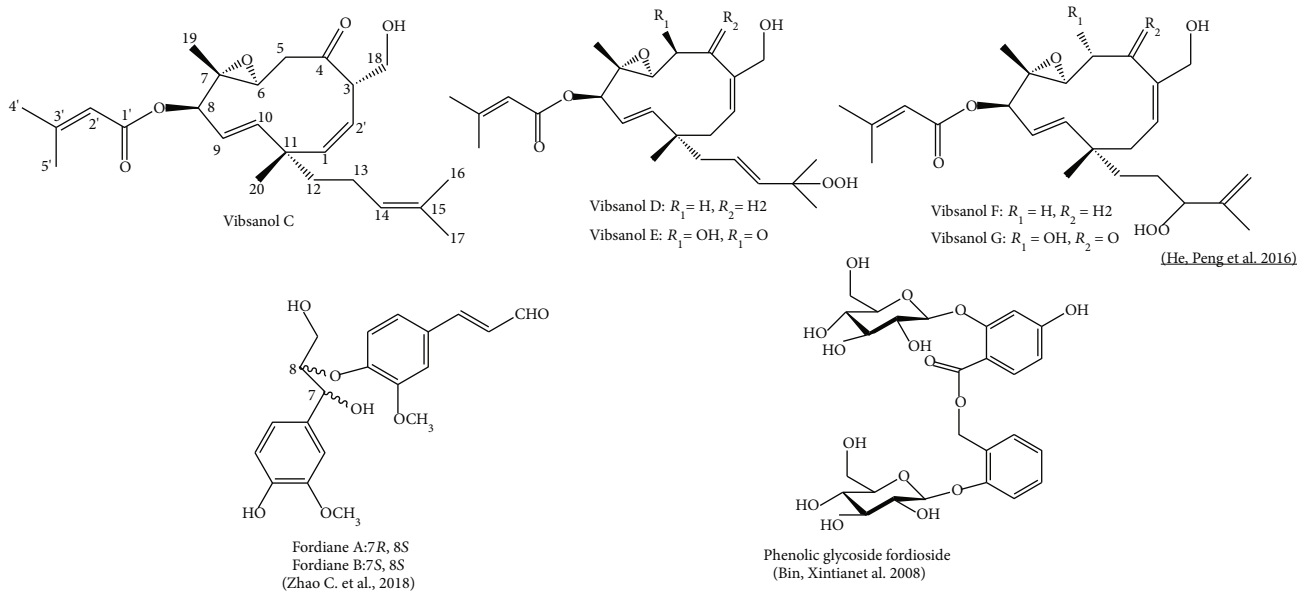

Figure 2: Chemical structure of the visanin components reported on the genus *Viburnum*.

species where the volatile compounds of its fruits are described [115]. Mass spectrometry analysis has identified nine components in *V. opulus* juice, using ultra high-performance liquid chromatography (UPLC) coupled to quadruple time-of-flight mass spectrometers (QTOF-MS) [100]. *Viburnum* fruits have been reported to contain lipids, pectins, proteins, lipid compounds (carotenoids, essential oils, steroids, and saponins), tannins, flavonoids, and anthocyanins-type polyphenols. *V. opulus* fruits have a higher content of carotenoids, polyphenols, flavonoids, steroids, and pectins than *V. lantana*; the latter species being characterized by a higher content of proteins, saponins, and essential oils. Metabolites in *V. opulus* fruits have been found in different layers of the pericarp with greater amounts in the skin [21]. Phenolic compounds have been reported in fruit juice via hydrochloric acid analysis [101] while triterpenic compounds have been obtained from seeds after triterpene esters hydrolysis (terpenes of the sterols-I type, triterpenyl alcohols and their derivatives-II) [105]. In a review published in 2010, it was reported that only 3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid were isolated from the *V. opulus* [31]. The presence of phenolic compounds, anthocyanins, and others constituents (Table 2) have been reported in leaves and hydrodistillation extracts of dried *V. opulus* plants [83, 97].

In this context, the genus *Viburnum* contains chemical compounds grouped in diterpenes, triterpenes, iridoids, monoterpenes, sesquiterhytopenes, flavonoids, lignans, phenols, coumarins, lactones, and alkaloids. Among the chemical contents of *Viburnum*, vibsane-type diterpenoids are characteristic of the genus, as have not been found in other higher plants. Compounds of the type vibsanin A-F, vibsanol (C-F), phenolic glycoside fordioside, fordiane A and B, and their derivatives have also been highlighted (Figure 2). The base structure of the visanin (A-F) compound group corresponds to a vibsane carbon skeleton, with a 20-carbon structure (Figure 2).

Another key aspect is the type of solvent used for the phytochemical extraction, where water, methanol, ethanol, and their mixture are used in higher proportion. In some cases, less polar solvents such as ethyl acetate or n-butanol are used, from branches and leaves. Chloroform has been used in leaves, while benzene or petroleum ether for the extraction of phytochemicals from branches.

Table 2 shows the main phytochemicals found in the genus *Viburnum*, as well as the type of extract obtained, the part of the plant used and the biological activity.

4. Biological Activities

4.1. Antioxidant Activities of Viburnum Plants (In Vitro Studies/In Vivo Studies). Many traditionally used medicinal herbs exert a beneficial impact on human health thanks to their antioxidant potential. Phenolic compounds, commonly found in plants, are the largest group of natural antioxidants. Plants produce them to protect their cells from oxidative damage caused by oxygen radicals and molecular excitation [116, 117]. *Viburnum* plant species have been extensively studied *in vitro* and *in vivo* assays. Most antioxidant studies relate to *Viburnum* fruits and juices, being *V. opulus* the most investigated plant species. According to the numerous published results, it can be said that *Viburnum* species and their products are exceptional antioxidants finding their place as naturally safe agents.

4.1.1. In Vitro Studies. The fruit juice of *V. opulus* (from the Eastern Black Sea Region, Turkey) had a prominent activity in the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) system in comparison to methanol, acetonitrile, and aqueous extracts of fruits skin and seeds, where the seed extract contained a higher number and quantity of antioxidant compounds. Coumaroyl-quinic acid, chlorogenic acid, procyanidin

B2, and procyanidin trimer were dominant in the juice [100]. Turkish *V. opulus* fruit, flesh, and seeds were analysed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Fruits and seeds expressed a more potent effect with EC_{50} of 2.35 mg/mg DPPH, as compared to EC_{50} of 24.56 mg/mg DPPH found in the flesh [103]. Antiradical activity tests in DPPH and ABTS, and antilipoperoxidation in the β-carotene/linoleic acid bleaching, were applied in aqueous and methanol extracts of the same species. Methanol extract of dried fruits had a greater performance in the DPPH test with IC_{50} of 0.104 mg/ml, while that of fresh fruits scavenged more ABTS radicals. All extracts inhibited the linoleic acid peroxidation, thus protecting the loss of β-carotene. The methanol extract reduced the ferric (III) to the ferro (II) form more effectively than the aqueous extract [118]. Sagdic et al. [119] also tested the fruits' methanol extract of Turkish *V. opulus* using the phosphomolybdenum complex method and found a value of 315.50 ± 8.2 mg/g in relation to the ascorbic acid. The antioxidant capacities of aqueous extracts of commercially available fruits, bark, and flowers of *V. opulus* from Poland were assessed by ABTS, hydroxyl radical scavenging, and peroxyl radical scavenging (ORAC) and FRAP techniques. The bark aqueous extract displayed the highest antioxidant capacity, followed by flowers and fruits. Strong correlations were found between total phenolic, flavanol, and proanthocyanidin contents with these assays [34]. The same authors investigated the antioxidant capacities of *V. opulus* fruits, flowers, and bark ethanol extracts by measuring of ABTS-, hydroxyl- (HORS), peroxyl- (ORAC), and superoxide- (SORS) free radicals scavenging and reducing power (FRAP). The antioxidant activity of different parts of the herb was in the following order for HORS, SORS, and ORAC tests: bark>flowers>fruits, and for ABTS and FRAP tests: bark>-fruits>flowers. The dominant compound in *V. opulus* bark was (+)-catechin, while chlorogenic acid was dominant in flowers and fruits [34].

Andreeva et al. [120] determined the antioxidant potential of Russian *V. opulus* bark extracts using the cathode voltammetry method measuring the relative decrease in the oxygen electroreduction. Results showed that the ethyl acetate fraction of the 70% ethanol extract expressed a higher antioxidant potential than chloroform and aqueous fractions, 70% and 30% ethanol extract, respectively. Bubulica et al. [121] conducted an antioxidant effects screening across several extracts of *in vitro* assays of Romanian plants. The authors found that the *V. opulus* methanol bark extract produced an antiradical effect against DPPH radicals with IC_{50} of 0.918 ± 0.46 mg/ml, as well as ferrous ion chelating effects with IC_{50} of 1.865 ± 0.05 mg/ml. Additionally, the antioxidant activity of the ethanol extract of the fruits was determined by the ABTS test. After 24 h of refrigerating, a 16% decrease in the antioxidant effect was recorded, followed by a 22% increase in the next 24 h, showing no correlation between the total the antioxidant potential and the phenol content in the extract during storage [122]. Paşayeva et al. [123] suggests that Turkish *V. opulus* fruits could be used as a neuroprotective agent. Antioxidant properties of both the decoction ethanol extract and fruit juice were used against hydrogen peroxide-induced oxidative stress in human SH-SY5Y neuronal cells. The Polish *V. opulus* fresh juice and extracts acted as scavenging potential agents toward ABTS and peroxyl radical cations estimated by ABTS and ORAC assays, expressed using Trolox equivalents (TE). The phenolic-rich fraction from fresh juice was compared to the methanol-acetone extract from pomace. The former was the most active in all identified phenol compounds (flavanols, falvonols, hydroxycinnamic acids, and anthocyanins) with values within the range of 2619.59 ± 123.1 and 7810.29 ± 342.3 μg TE/g. Also, the *V. opulus* extracts had powerful chemopreventive effects against oxidative stress in Caco-2 cells induced by tert-butylhydroperoxide and against DNA damage through the repair induction after cell exposure to hydrogen peroxide and methylnitronitrosoguanidine [99].

Studies have compared the antioxidant effects of different *V. opulus* genotypes and cultivars. Kraujalytė et al. [101] reported that *V. opulus* var. *sargentii* had the highest radical scavenging capacity (77.5%), followed by *V. opulus* var. *americanum*, and *V. opulus* P3. The strongest activity in a FRAP test system was observed in *V. opulus* var. *sargentii* juice while the weakest was expressed by the 'Shukshinskaya' cultivar. The *V. opulus* var. *sargentii* juice was most effective in the ORAC method with the highest TEAC (Trolox equivalent antioxidant capacity) value, while 'Shukshinskaya' was powerless as observed in the previous test. Moskalets et al. [124] assessed the antioxidant capacity of Ukrainian *V. opulus* fruit varieties using a Blisar A analyser. The antioxidant activity was expressed through gallic acid in a range between 387 and 540 mg%. The cultivar with the maximal tested effect was EF 3-10-2010. Furthermore, approximately 10 genotypes of Turkish *V. opulus* fruits showed antioxidant potential, marked SIV 1-10 by a FRAP test system. SIV genotypes in the forms of acetone extracts displayed effects in a narrow range from 21.02 ± 2.6 to 34.90 ± 4.5 μmol TE/g, where SIV-10 had the greatest capacity, suggesting the higher synthesis or accumulations of phenolics and others antioxidant compounds in fruits [33]. Scavenging effects of fruit porridge (25%) of *V. opulus* var. *edule* cultivars Leningradskaya otbornaya, Souzga, and Taezny rubiny on nitric oxide, superoxide anion, hydroxyl radical, and lipid peroxidation inhibition, were moderate from 11.20 to 28.50%. Fruits' total antioxidant activity was determined by the ABTS and DPPH methods, where Taezny rubiny was most prominent. A high correlation between total phenolics and ascorbic acid content, and antioxidant activity was reported [102]. *V. opulus* (Latvia) fruits and pomace were subjected to supercritical carbon dioxide extraction (SFE-CO2) with different conditions to obtain the optimal lipophilic fraction. The antioxidant potential of *V. opulus* SFE-CO2 extracts was assessed by the ORAC method. The antioxidant capacity values of the washed pomace, unwashed pomace, and dried berry extracts, at the highest yield (optimal SFE-CO2 conditions), were 65.3 ± 1.8, 74.3 ± 2.2, and 142.4 ± 3.6 μmol TE/g, respectively. It can be concluded that the dried berry extracts were twice as higher in the antioxidant activity expression in comparison to the pomace extracts [125].

Çanga and Dudak [126] implemented cellulose acetate/-gum Arabic fibers loaded with a *V. opulus* (Turkey) fruit extract. Within the examination of the materials, they tested

the antioxidant activity of the loaded fibers and observed high values of DPPH radical inhibitions ranging 56-59% and 55–58% at 4°C and 25°C, respectively.

These free radical inhibitions were more effective than the free *V. opulus* fruit extract (40% and 34%, respectively). Barak et al. [98] studied the difference in the antioxidant potential of Turkish *V. opulus* methanol, and water fruit extracts before and after *in vitro* gastrointestinal human digestion. Antioxidant effects of the extracts were assessed in the following phases: nondigested, postgastric, colon available, and serum available, by different methods (N,N-dimethyl-p-phenylendiamine-DMPD, cupric reducing antioxidant capacity (CUPRAC), DPPH, FRAP, and total antioxidant capacity). The methanol extract was superior in antioxidant expression than the aqueous one, and the antioxidant effect decreased during the *in vitro* digestion. An interesting investigation was conducted with *V. opulus* (Turkey) fruit pomace in wheat flower cakes at concentrations of 0, 5, 10, and 15%. Cakes' total phenolic content and antiradical activity (DPPH) increased in correlation to the level of *V. opulus* fruit pomace addition (from 10.26 ± 1.5 to $76.83 \pm 4.5\%$) [127]. The *V. opulus* (guelder rose) fruit concentrate (65%) decreased the thiobarbituric acid reactive substance (TBARS) levels in turkey meat samples during storage at concentrations of 5% and 10%, particularly after 10, 15, and 30 days. The addition of 10% concentrate to the meat significantly reduced the TBARS in comparison to control and butylated hydroxytoluene ($p < 0.05$) both under aerobic and anaerobic conditions [128].

Erdogan-Orhan et al. [129] observed the antioxidant effects of Turkish *V. opulus* and *V. lantana* ethyl acetate, methanol, and aqueous extracts from branches, leaves, and fruits. The most powerful antioxidant agents in the ferrous ion chelating capacity test were the *V. opulus* ethyl acetate leaf extract ($44.62 \pm 0.02\%$ of inhibition; 2000 μg/ml) and the *V. lantana* ethyl acetate fruit extract ($58.72 \pm 1.00\%$ of inhibition; 2000 μg/ml). *V. opulus* aqueous extracts from branches and *V. lantana* methanol leaf extracts exhibited the highest effects (3.396 ± 0.01 and 3.401 ± 0.02; 2000 μg/ml, respectively) using the FRAP method with chlorogenic acid as a reference. In the β-carotene bleaching assay, *V. opulus* ethyl acetate fruit extracts and *V. lantana* methanol leaf extracts were significant antioxidant agents with coefficients of 60.5 ± 1.36 and 79.50 ± 1.76, respectively, at 2000 μg/ml. The authors noticed that a higher total phenolic content in the tested extracts usually indicates greater antioxidant effects [129]. Both *Viburnum* species were also studied by Altun et al. [41]. The antioxidant effects of different water extracts (branches, fruits, and leaves) were assessed using the DPPH and superoxide anion scavenging methods. Branch extracts successfully reduced the effects of the superoxide anion ($IC_{50} = 3.7$ and 3.1 mg/ml, respectively). On the contrary, the extracts produced a positive antiradical effect on the DPPH radical with various inhibition values, especially the *V. opulus* branch extract with IC_{50} values at 0.014 mg/ml [41].

Interestingly, during autumn migration birds select quality fruits rich in anthocyanins, phenolic profile, and strong antioxidant activity. Bolser et al. [130] revealed that birds preferred *V. recognitum* and *V. dentatum* fruits which have the highest total antioxidant content. Serteser et al. [131] investigated the antioxidant properties of selected wild-growing plants in Turkey. Among them, the *V. lantana* methanol fruit extract proved to be a moderate antioxidant agent in the DPPH method ($EC_{50} = 1.523$ mg/mg DPPH), the Fe^{+2} chelating assays ($39.43 \pm 2.69\%$), and through H_2O_2 inhibition ($43.37 \pm 2.86\%$). *V. lanata* buds, macerated in a glycerin-ethanol solution (as part of a gemmotherapy preparation for respiratory diseases), showed the weakest antiradical effect in the DPPH test (30.08 ± 2.14 μg/ml) in comparison to other herbs (buds of *Betula pubescens*, *Ribes nigrum*, *Carpinus betulus*, and offshoots of *Rosa canina*). *V. lanata* buds were the poorest in total phenolic and flavonoid, caffeic, and chlorogenic acid contents [132]. Iranian *V. lantana* methanol leaf extract was evaluated by the DPPH method and IC_{50} value of 52 μg/ml, demonstrating a great antioxidant capacity. This extract contained two chalcone glycosides (trans-3-ethoxy-4-O-(glucopyranoside)-2′, 3′, 4′, 5′, 6′-pentahydroxy chalcone and trans-3-methoxy-4-O-(glucopyranoside)-2′, 3′, 4′, 5′, 6′-pentahydroxy chalcone) isolated for the first time from *V. lantana* leaves [133].

The GC-MS data for the methanol soluble fractions of *V. sargentii* extract (originating from the Republic of Korea) highlights the presence of guanosine, levoglucosan, vitamin E, stigmast-5-en-3-ol, and stigmata-5,24(28)-dien-3-ol. Patil et al. [134] showed a significant antioxidant potential of the extract in a concentration-dependent manner for all three methods. The extract produced a strong antiradical effect in the DPPH assay with an IC_{50} value of 15.33 ± 0.58 μg/ml. For both remaining methods, the extract had an electron-donating capacity which reflected its reducing power to change the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) form and Mo (VI) to Mo (V), respectively [134]. *V. nervosum* roots, essential oil, and extracts showed strong antiradical effects in DPPH system. Methanol and ethanol extracts (100% and 80%) were more potent with $IC_{50} = 22.97 \pm 0.38$-25.65 ± 1.02 μg/ml than essential oil ($IC_{50} = 33.32 \pm 0.67$ μg/ml). Additionally, the inhibition capacity of the extracts was greater in a linoleic acid system, where lipoperoxidation inhibition ranged from 47.67 ± 0.87 to $69.25 \pm 1.67\%$ in comparison to oil $44.03 \pm 0.96\%$ where the dominant compounds were α-eudesmol, caryophyllene oxide, linalool, spathulenol, and ledene Awan et al. [135]. Fu et al. [136] conducted an antioxidant potential screening of edible fruits from South China. They reported that *V. sempervirens* (in particular *V. fordiae*) fruits produced great total antioxidant effects estimated by FRAP and TEAC. However, it is important to note that the *V. sempervirens* nonpolar fruit fraction showed greater effects as compared to *V. fordiae*, which polar fruit fraction was more active. The authors also noticed strong correlations of antioxidant expression with total phenols [136]. Leiner et al. [137] evaluated the antioxidant capacity of Alaskan berries by the ORAC method and revealed an excellent effect of *V. edule* fruits (117 mmol of TE/g), after *Vaccinium vitis-idaea*. Antioxidant potential was also confirmed for *V. dilatatum* leaves and fruits [138, 139]. Iwai et al. [138] from Japan examined *V. dilatatum* juice (fruit squeezing solution) claiming its strong antioxidant activity.

The *in vitro* antioxidant potential of the *V. dilatatum* sample was measured by the XYZ-dish and electron spin resonance (ESR) method. In the XYZ-dish technique, the tested sample expressed effective activity against OH· (10.163 ± 2.376 units/ml), as opposed to its antiperoxide effect (H_2O_2) (0.529 ± 0.127 units/ml). The activity of the *V. dilatatum* juice to scavenge OH· radicals, measured by the ESR method and represented as DMSO equivalent concentration, was also strong (0.937 ± 0.176 mM DMSO eq/ml) [138]. Three Indian *Viburnum* species and their methanol leaf extracts were investigated in a DPPH test, a nitric oxide (NO) scavenging test, and an assay of reduced glutathione and ferrous sulphate-induced lipid peroxidation. Ponnudurai et al. [140] concluded that these extracts could be effective antioxidants in the following order: *V. erubescens*>*V. coriaceum*>*V. punctatum*. *V. awabuki* (originating from China) and its extracts exhibited exceptional activities. The ethyl acetate-soluble fraction of the crude methanol-chloroform extract was dominant in antioxidant effects in DPPH radical inhibition (1000 μg/ml = 61.88 ± 0.23%) and reducing power activity (1000 μg/ml = absorbance of 0.287 ± 0.006), while the basic crude extract was prominent in the hydroxyl radical-scavenging activity test (100 μg/ml = 71.26 ± 0.38%). On the contrary, other *V. awabuki* extracts: petroleum ether-soluble fraction, n-butanol-soluble fraction, and aqueous residue, were significantly weaker [141]. Abbasi [142] revealed that *V. foetens* fruit from the Himalayan region-Pakistan represented a rich source of natural antioxidants possessing significant antioxidant effects. Compared with the water extract, the acetone extract showed higher levels of the total antioxidant capacity (84.67 ± 0.48 μM AAE/100 g), DPPH antiradical capacity (84.62 ± 0.63%), and hydroxyl radical scavenging capacity (75.53 ± 0.95%). They also found significant correlations between the ascorbic acid, phenols, and metal contents with free radical scavenging activity [142]. Nine *V. tinus* extracts from Turkey were screened in detail for their antioxidant potential. Antioxidant capacities of the ethyl acetate, methanol, and water extracts of leaves, branches, and fruits were tested against DPPH, DMPD, superoxide, and NO radicals. The methanol extracts of leaves, branches, and fruits and leaves' aqueous extract exhibited a remarkable DPPH antiradical activity, over 89%. The fruits' methanol extract was prominent against the DMPD radicals (67.1 ± 0.33%), the branches' aqueous extract in NO radical quenching (near 80%), and the fruits' ethyl acetate extract was the only active in the superoxide radical scavenging test (38.4 ± 1.01%). The fruits' methanol extract displayed the strongest activity in the FRAP and phosphomolybdenum-reducing antioxidant power (PRAP) tests. In the metal-chelation capacity test, the aqueous extracts were predominant with over 60% of the activity [143]. *V. tinus* from Tunisia and its leaves' acetone extract also produced strong antiradical effects in a DPPH test with high total phenolic, flavonoid, and tannin content [144]. The Indian *V. punctatum* leaf ethanol extract was tested for its scavenging effects in a DPPH and ABTS system and total antioxidant capacity (reducing power activity) in a phosphomolybdenum assay. The extract proved to be an efficient scavenging agent with IC_{50} values of 83.29 μg/ml and 92.04 μg/ml, respectively, with excellent reduction effects of 65.67 ± 0.15% with the maximal concentration of 100 μg/ml [145]. *V. grandiflorum*, among other wild berries of the Northwestern Himalayan region, demonstrated promising antioxidant properties. Namely, the methanol fruit and leaf extract produced an antiradical effect in a DPPH system with IC_{50} values of 294.5 and 125.82 μg/ml, respectively, as well as effective chelating and reducing power [146]. Fruit, leaf, and branch extracts from *V. orientale* (traditionally used in Turkey -Anatolia) were studied for their antioxidant and neurobiological effects. The fruit and branch methanol extracts (characterized by the highest total phenolic contents) showed the highest inhibition capacity against DPPH radicals and in FRAP and phosphomolibdenum reducing antioxidant power assays. The leaf aqueous extract showed the greatest NO scavenging (75.00 ± 1.22%), DMPD scavenging (33.70 ± 1.13%), and metal-chelating (54.66 ± 3.56 %) at the concentration of 2500 μg/ml [106]. The aqueous extract of the aerial parts of *V. punctatum* from India showed a significant antiradical effect in a DPPH test system. The extract inhibited from 44.33 ± 0.21% to 93.65 ± 0.56% of free radicals at the concentrations from 20 to 100 μg/ml Susmitha et al. [147]. The antioxidant effects of *V. odoratissimum* seed extract, from China, were analysed in superoxide radical scavenging, reducing power, and lipid peroxidation inhibition assays. The butanol-soluble fraction of the methanol-chloroform crude extract was dominant in the first two tests, which was in accordance with the total phenol and flavonoid contents. The authors observed that the petrol ether-soluble fraction had the highest antilipoperoxidant activity and that the high temperature was not effective for the lipid peroxidation inhibition [148]. *V. mullaha* (India) acetone fruit extracts showed prominent antioxidant activities *in vitro* assays: ABTS, DPPH, superoxide anion, and linoleate peroxyl radicals scavenging and ferric reducing except ferrous metal chelating activity. The HR-LC-MS analysis detected 15 phenolic compounds: chlorogenic acid, acetyl salicylic acid, dihydroquercetin, dihydrorobinetin, dihydromyricetin, 2-isoprenylemodin, rutin, cosmosiin hexaacetate, pectolinarin, eriodictyol, iriginol hexaacetate, theaflavin, epicatechin pentaacetate, lomatin, and peucenin [149]. Methanol, ethyl acetate, and water extracts made of *V. grandiflorum* (Pakistan) stem exhibited antiradical activity in a DPPH assay. The water extract had the highest potential (IC_{50} = 255 μg/ml) followed by ethyl acetate (IC_{50} = 322 μg/ml) and methanol (IC_{50} = 742 μg/ml) extracts [39].

4.1.2. In Vivo Studies. *In vivo* studies of the antioxidant effects of *Viburnum* species are not as extensive as *in vitro* studies. The literature refers to several experimental studies which mainly included *V. opulus* and *V. dilatatum*.

The *V. opulus* (Turkey) fruit methanol extract showed protective effects against ischemia/reperfusion- (I/R-) induced oxidative stress during lung transplantation in rats, presumably due to its antioxidant effects and ability to neutralize free radicals. Namely, the treatment with the extract (200 mg/kg, intraperitoneally) significantly increased the levels of the antioxidant system (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and total glutathione)

and repaired the total antioxidant plasma status of rats ($69.59 \pm 8.9\,\mu$mol Trolox eq/mg protein) compared to the untreated group ($43.02 \pm 4.75\,\mu$mol Trolox eq/mg protein). In addition, the extract reduced the malonyldialdehyde (MDA) and protein carbonyl levels. It is considered that malic, caffeic, quinic, coumaroyl-quinic, and chlorogenic acid, as well as particular caffeic acid, derivatives are responsible for the antioxidant effects previously described [150]. The impact of *V. opulus* water fruit extract (also originating from Turkey) on testicular and epididymal rats tissue treated by i.p. injection of taxane-based chemotherapeutics was investigated by measuring the lipid peroxidation level and antioxidant activities. Docetaxel and paclitaxel imbalanced an oxidant/antioxidant system, which was repaired with an oral dose of 100 mg/kg of the extract. MDA levels were significantly lower in rats' testis and epididymis while the levels of superoxide dismutase, glutathione peroxidase, and catalase were increased. The authors identified several compounds in the extract by GC-MS (α- and β-pinene, butanoic acid, DL-limonene, α-terpineol, and germacrene D) [151]. Furthermore, the lyophilized *V. opulus* juice and the commercial lyophilized *V. opulus* juice showed a significant antiurolithiatic activity in rats (100 mg/kg) compared to the Cystone standard. Ilhan et al. [152] attributed this effect to their antioxidant and diuretic activity and the inhibitory effects on the oxalate levels. The antioxidant action was estimated by measuring TBARS, total thiols, and glutathione in kidney tissues. TBARS levels were significantly reduced, after administration of *V. opulus* juices, with increased levels of total thiols and glutathione [152]. *V. opulus* proanthocyanidins produced gastroduodenoprotective effects against water immersion and restraint stress in rats, improving the levels of antioxidant enzymes' superoxide dismutase, catalase, and gluthatione peroxidase and decreasing the MDA content. Proanthocyanidins, as *V. opulus* extract, were intragastrically administered in three doses at 25, 50, and 75 mg/kg body weight [40].

V. dilatatum juice showed an inhibitory effect on gastric ulcer formation and oxidative damage caused by water immersion restraint stress in rats. The concentrations of lipid peroxides, assessed by TBARS, in the plasma, liver, and stomach were significantly lower compared with the group treated with water [138]. The same Japanese researchers tested the *V. dilatatum* crude fruit extract, proving ineffective for preventing oxidative injury induced by water immersion restraint stress. The extract improved the lipid peroxidation in the plasma, liver, and stomach but had weak effects on the enzymatic activities (superoxide dismutase, catalase, and gluthatione peroxidase). However, ferrous ascorbate-induced oxidation in hepatic homogenate of rats was inhibited. According to the results, *V. dilatatum* fruits cannot induce antioxidant enzymes and its absorbed antioxidant components have a direct effect on the oxidative injury in the body [153]. In addition, the *V. dilatatum* crude fruit extract was subjected to the experiment with streptozotocin-induced diabetic rats. The animals received *V. dilatatum* extract solution (16.8 mg/ml) for 10 weeks. TBARS levels in the plasma, erythrocytes, liver, kidney, and pancreas were significantly reduced together with plasma glucose levels. The extract contained two cyanidin glycosides, two chlorogenic acids, and quercetin. It is suggested that cyanidin 3-sambubioside is crucial for the physiological effects of *V. dilatatum* fruit, given the strong antioxidant nature of the compound [70, 154].

The leaf methanol extract of *V. tinus* from Egypt produced a significant effect on serum lipid peroxides (measuring of thiobarbituric acid-reactive substance) and nitric oxide levels (Griess reaction) with the dose of 50 mg/kg i.p. on CCl4-induced hepatotoxicity in rats, although not at lower doses of 25 mg/kg [155].

4.2. Antimicrobial Activities of Viburnum Plants (In Vitro Studies). During the last two decades, antimicrobial activity of plant species of the genus *Viburnum* L. has been extensively studied. Antimicrobial activity of the essential oils from the air-dried whole plants of *V. opulus*, *V. lantana*, and *V. orientala* were tested against the bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus cereus*, and the fungus *Candida tropicalis*. The oils were at a maximum concentration of 250, 500, and 1000 μg/ml in hexane, respectively. The activity was tested using the agar dilution MIC assay. The oils of *V. lantana* and *V. opulus* showed no activity against the microorganisms tested. The essential oil of *V. orientale* showed a weak antibacterial activity against Gram-positive bacteria such as *E. faecalis*, *S. aureus*, and *B. cereus* [83]. In another study, the essential oil of *V. betulifolium* was analysed and tested for antimicrobial activity using microdilution assay of human pathogenic bacteria and yeast. *V. betulifolium* is an evergreen shrub widely distributed throughout the Yunnan Province and southwestern parts of China. The main essential oil constituents of the species are phytol (9.8%), trans-b-damascenone (5.9%), α-cadinol (5.7%), γ-cadinene (5.6%), Δ-cadinene (5.3%), methyl pentanoate (4.6%), and tetradecanal (3.8%). The oil showed strong antimicrobial activity against both Gram-positive and Gram-negative bacteria and the yeast, the effect being more significant against Gram-positive than Gram-negative bacteria. Positive inhibitory activity was shown by *Pseudomonas aeruginosa* (MIC 125 μg ml^{-1}) and *Candida albicans* (MIC 62.5 μg ml^{-1}) [156]. Awan et al. [135] investigated antimicrobial activities of *Viburnum nervosum* root essential oil and several methanolic and ethanolic root extracts. *V. nervosum* is a large deciduous precocious shrub, 2-3 m tall with stiff stout branches. In Kashmir's traditional medicine, it is used as an astringent and emmenagogue, as well as for the treatment of acute furunculosis. Bergenin, a trihydroxybenzoic acid glycoside, was isolated from its roots. This glycoside is also useful in the treatment of hypercholestraemia, kidney stones, fever, diarrhea, and pulmonary infection. The essential oil of the root possesses carminative, insecticidal, antiseptic, and disinfectant properties. The main compounds of the root oil are eudesmol (30.3%), caryophyllene oxide (17.0%), spathulenol (10.7%), and linalool (12.65%). The essential oil and root extracts of *V. nervosum* were tested against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli* and *Pasteurella multocida*), and pathogenic fungi (*Aspergillus niger*, *Aspergillus flavus*, *Fusarium solani*, and *Rhizopus solani*) with the

disc diffusion method. The oil showed maximum activity against *B. subtils*, *A. niger*, and *R. solani*. However, no activity was observed by the extracts Awan et al. [135]. Nonetheless, the chemical composition and antimicrobial activity of *Viburnum* species essential oils were examined, neither were the antimicrobial activities of the dominant compounds investigated.

Bibi et al. [157] tested the antibacterial activity of the methanolic dry extract of *Viburnum foetens* (20 mg/ml), with an agar well diffusion method, against *Bacillus subtilis*, *Micrococcus leuteus*, *Salmonella setubal*, *Salmonella aureus*, and *Pseudomonas picketii*. All bacteria tested were sensitive to the extract. *S. setubal* was the most sensitive bacteria. The extracts of *V. foetens* were subject of another study. Awan et al. [158] examined four different extracts of *V. nervosum* and *V. foetens* leaves for antibacterial activities against eight different bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Klebsiela pneumoniae*, *Proteus vulgaris*, *Citrobacter freundii*, and *Streptococcus pneumoniae*) using the disc diffusion method. The study concluded that the ethanolic extract was the most effective, contrary to that of the petroleum ether extract. The ethanolic and methanolic extracts showed prominent activities against all tested bacteria, in comparison to the chloroform extract which had moderate activity. Turker et al. [159] used the same antibacterial activity evaluation method by analysing the antimicrobial activity of the *V. lantana* extracts obtained from dry and fresh fruits (water and ethanol) against Gram-positive bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*). Gram-positive bacteria were more susceptible to the inhibitory effects of the plant extracts than the Gram-negative bacteria. The extracts of *V. lantana* fresh fruits exhibited antibacterial activities. The inhibition capacity of the hot ethanolic extract was greater than the cold ethanolic extract against *S. aureus*, *S. epidermidis*, and *S. pyogenes.* Both *S. marcescens* and *P. aeruginosa* were resistant to all examined fruit extracts.

Eryilmaz et al. [160] also studied the antimicrobial activity of *V. lantana*, together with *Viburnum opulus* L., *V. orientale* Pallas, and *V. tinus* L. against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Candida albicans*. The disc diffusion and tube dilution techniques were used to determine the activities of the extracts. Ethanolic and water plant extracts of leaf, stem, and fruit were used in the experiment. Ethanolic extracts from all analysed species showed antimicrobial activity against all the tested microbes. Water extracts were either weak or not effective against tested microorganisms. The antimicrobial activity of the dried fruit methanolic extract of *V. opulus* was also analysed by another study with an agar diffusion method [119]. The study analysed ten microorganisms (*Aeromonas hydrophila*, *Bacillus cereus*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Yersinia enterocolitica*). A 15% concentration extract completely inhibited the growth of all analysed bacteria. The same plant species have shown the capacity to reduce the potential of *Staphylococcus aureus* and *S. epidermidis* to colonize inert substratum and form biofilms [121]. Similarly, fruit juices and ethanolic extracts of *V. opulus* genotypes were tested against ten Gram-positive and Gram-negative bacterial cultures and nine yeast strains. The fruit juices showed greater antibacterial activity compared to the ethanol extracts. The most effective antibacterial activity was exhibited by the juices against *Salmonella typhimurium*, *Salmonella agona*, and *Listeria monocytogenes*. The fruit juices and ethanol extracts showed weak or no activity on the yeast strains [161]. Antimicrobial activities of fruit juices of six *V. opulus* genotypes were evaluated by Česoniene et al., [42], using the agar well diffusion method against ten Gram-positive and Gram-negative bacteria and seven strains of yeast. The juices strongly inhibited the growth of Gram-negative (*S. typhimurium* and *S. agona)* and *Gram-positive (S. aureus*, *L. monocytogenes*, and *Enterococcus faecalis*) bacteria. As previously reported, the effect of the juices on the yeast was low or lacking [42]. Up to Česonienė's studies, most *Viburnum* plant extracts had presented the highest effect on Gram-positive bacteria strains and some yeasts. However, antimicrobial analysis of the juices has shown that they can be used to fight Gram-negative microorganisms. Differences in antimicrobial activity are most likely due to the chemical composition of the extracts and juices. Therefore, further research of *Viburnum* species should connect the chemical composition with the antimicrobial activity.

Paulauskas et al. [162] went one step further. They analysed the antimicrobial activity of unripe mashed berries and ripe berry juice of *V. trilobum* Marshall, *V. sargentii* Koehne, and *V. opulus* cultivar "Leningradskaya Otbornaja." The unripe berry mass and ripe berry juices both significantly influenced the bacteria. The unripe berry mass manifested greater antibacterial activity, similarly, on both the Gram-positive and Gram-negative bacteria. *Micrococcus* sp. and *S. aureus* were the most sensitive bacteria to the mashed berries and all analysed juices. *Viburnum* juice impacted the microscopic fungi the least.

Methanolic extract of *V. cotinifolium* leaves from Pakistan was tested against four Gram-positive bacteria, five Gram-negative bacteria, and ten fungal strains with the agar diffusion method. The extract demonstrated maximum activity against *Aspergilus flavus* and *A. fumigates*. The extract also showed positive antimicrobial activity against *A. niger*. The *V. cotinifolium* extract showed the most effective activity against *Enterococcus faecalis* and *Enterobacter coccus*. As can be observed, the extract of *Viburnum* species was also effective against Gram-negative bacteria [163]. This indicates that *V. cotinifolium* leaf methanolic extract has great potential as a natural antimicrobial agent. Hence, chemical analyses of the extract should be carried out and associated with antimicrobial activity. This connection is supported by the research carried out by Roy [164], which proves that the methanolic extract (and fraction) from the whole plant of *V. foetidum* exhibit a significant antimicrobial activity against Gram-positive and Gram-negative bacteria

strains, as well as significant antifungal activity. The ethyl acetate (EA) fraction from the methanolic extract exhibited the highest antimicrobial potential. Agrobacterium species were most susceptible to the EA fraction of the extract, and clearly, the EA fraction differs in its composition from its counterparts. Unfortunately, detailed chemical analyses of the extracts and/or fractions have currently not been carried out.

4.3. Anti-Inflammatory Activities of Plant Species of the Genus Viburnum L. (In Vitro Studies/In Vivo Studies). Inflammatory diseases are usually treated by steroid drugs, nonsteroidal anti-inflammatory drugs, and immunosuppressant. Although the effects of these drugs have been proven, their side effects are not negligible. The usage of these drugs is often associated with bleeding gastrointestinal and peptic ulcers [165]. In search for new harmless drugs, scientists are once again turning to medicinal plants. Among these plants, the species of the genus *Viburnum* are of interest. For that purpose, anti-inflammatory activities of *V. lantana*, *V. trilobum*, *V. pichinchense*, *V. sargentii*, *V. fordiae*, and *V. opulus* were investigated [78, 166–170].

The bark of this species has been used in Turkish traditional medicine as a rubefacient and analgesic [41]. *Viburnum lantana* L. leaf water extract was investigated for anti-inflammatory activity, in rats with a carrageenan-induced rat paw edema test. The anti-inflammatory activity of the extract at doses of 100 and 200 mg/kg has been low as compared to indomethacin [169].

Viburnum trilobum Marshall (American highbush cranberry) is widely used in traditional medicine as it displays an anti-inflammatory and antidiabetic effect, sometimes used to improve lipid metabolism. The bark can act as a sedative and pain reliever. Due to the high content of ursolic acid, which expresses anti-inflammatory properties, the bark acts as an anti-inflammatory agent. This effect is proven in a RAW 264.7 macrophage cell system. All fractions of the *V. trilobum* ethanolic extract significantly inhibited the levels of IL-1β, IL-6, and TNFα [168].

Viburnum pichinchense Benth also displays anti-inflammatory properties. The anti-inflammatory effects of the methanol extract were demonstrated using LPS-stimulated macrophages and HCl/EtOH-induced gastritis model mice. The extract expresses anti-inflammatory activity by targeting NF-κB and caspase-11 noncanonical inflammasome pathways in macrophage-mediated inflammatory responses [167].

Leaves, stems, and fruits of the plants have been used in traditional folk medicines as therapeutic agents, as styptics and analgesics, to treat boils, rheumatoid arthritis, traumatic injuries, ringworm, skin itching, and coughs [170]. In the last two decades analgesic, anti-inflammatory, and hepatoprotective activities of its methanol extract were confirmed. The butanol fraction of the methanolic extract showed the highest activity on inflammatory reactions [170].

Viburnum fordiae Hance, a small tree widely distributed in the south of China, has been used in traditional Chinese medicine for centuries to treat rheumatic arthralgia and allergic dermatitis. Recent studies have reported a new, unusual ɣ-lactone, obtained from the aerial parts of these plants, capable of expressing an *in vitro* anti-inflammatory effect *vitro* [78].

V. opulus is well known as a medicinal and horticultural plant with a dietary value. Its fruits have been used in traditional medicine to cure pulmonary, stomach, cardiovascular, and kidney diseases, as well as for the treatment of cramps, diabetes, bleeding, coughs, and colds. Arginase activity and arterial vasodilation of the plant extract have also been proven [100, 101, 104, 171–173]. The anti-inflammatory activity of *V. opulus* water leaf extract was conducted in rats by a carrageenan-induced rat paw edema, test at doses of 50, 100, and 200 mg/kg, i.p., proving the extract had no anti-inflammatory effect at these doses [166].

4.4. Cytotoxic Activities of Viburnum Plants (In Vitro Studies). For centuries, herbs and plants have had a role in the treatment of various forms of tumors as have also shown to reduce the risk of cancer development or serve as a treatment for different types of cancer [174]. *Viburnum* species and their products have been extensively studied for their cytotoxic properties, being promising anticancer agents. To date, the most studied species in this regard is *V. opulus*.

Sauter and Wolfensberger [175] were the first to report the cytotoxic activity of *Viburnum* extracts. Aqueous fruit extracts of *V. opulus* and *V. lantana*, from Switzerland, showed no cytotoxic activity on BT 20 breast cancer cells within 72 h of incubation. Similarly, further research revealed no cytotoxicity of *V. opulus* extracts. An Indian aqueous bark extract was tested for its cytotoxic effect using a simple bioassay, brine shrimp lethality test, but no remarkable effect was observed [3]. The same findings were reported for *V. opulus* seed extracts by Cantrell et al. [176]. Russian *V. opulus* fruits and its ethanol extract exhibited low cytotoxicity, suppressing cell growth at concentrations above 200 μg/ml [3].

On the contrary, some authors consider *V. opulus* as an effective cytotoxic agent. Laux et al. [177] investigated the aldehyde fraction of the *V. opulus* chloroform-methanol fruit extract (Canada) for the cytotoxic effect on human gastric carcinoma cells. The fraction containing (E) 2-hexenal, (Z) 2-decenal, 2,4–decenal, (E) 2-octenal, and 2-undecenal produced a direct antiproliferative effect on the growth of the carcinoma cells with death at a concentration of 27 μM [177]. The methanol and acetone extract, juice, and juice after extraction to the solid phase of *V. opulus* from Poland showed cytotoxic activity against human breast (MCF-7) and cervical (HeLa) cancer cell lines. The strongest toxic agent towards both cell lines was observed for the juice obtained after purification with IC_{50} values of 63.541 and 19.380 μg/ml for HeLa and MCF cell lines, respectively [178]. According to IC_{50} values (250-450 μg/ml), the cytotoxicity of the same origin *V. opulus* extracts against Caco-2 cells, measured with the PrestoBlue assay, were in the following order: phenoli- rich fraction from fresh juice> methanol-acetone extract from pomace>acetone extract from pomace>fresh juice. This effect could be attributed to the highest content of phenolic compounds (flavanols, falvonols, hydroxycinnamic acids, and anthocyanins) [99]. The commercial *V. opulus* juice from Turkey produced a

cytotoxic effect against Caco-2 (human colon adenocarcinoma) and HeLa cells but was not active in a test using A549 (human type II lung epithelium) cells over a 72-hour period with the concentrations of 10-80 μg/ml. Futhermore, *V. opulus* juice caused no significant decrease in the viability of MDCK (Madin Darby Canine Kidney) and HUVEC (Human umbilical vein endothelial cells) normal cell lines [179].

Antitumor activities of water and ethanol extracts prepared using the hot and cold procedures of fresh and dried *V. opulus* and *V. lantana* fruits (Turkey) were tested with the potato disc tumor induction method. *V. opulus* was more active with the inhibition of 61.9-100%. Both water extracts from dried fruits were the most effective in the assay. Among V. lantana extracts, hot water and ethanol extracts were the most effective, 90.5% and 95.2%, respectively [159]. The same technique was used to assess the antitumor activity of aqueous, ethanol, and methanol *V. lantana* (Turkey) leaf and fruit extracts. The methanol extract was the most effective with 100% of tumor inhibition, followed by the ethanol (90.9%) and the aqueous one (86.4%) [180].

The researchers from Pakistan, Shah et al. [37], conducted an identical assay for cytotoxicity with *V. grandiflorum* methanol extract and its n-hexane, chloroform, ethyl acetate, and n-butanol fractions. The chloroform extract was more active, expressing a strong ability to kill brine shrimp (EC_{50} = 107.45 μg/ml). The methanol extract of *V. grandiflorum* from China exerted a strong impact on lung cancer cells H1650, HCC827, and H1299 by decreasing their viability in a concentration- and time-dependent manner. The viability of the H1650, HCC827, and H1299 cells decreased to 34%, 31%, and 29%, respectively, after three days of treatment with the extract. A detailed analysis found that the viability of cells was inhibited by the apoptosis activation through a caspase-dependent pathway [181].

V. punctatum from India was tested in several studies. A *V. punctatum* methanol extract made from aerial parts displayed cytotoxic activity against human liver cancer cells (HepG2) with CTC50 (cytotoxicity 50%) values of 205.8 ± 1.92 μg/ml by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and against human laryngeal epithelial carcinoma (Hep2) with CTC50 value 197.3 ± 2.89 μg/ml [182]. *In vitro* anticancer activity of aerial parts of *V. punctatum* was tested using chloroform and methanol extracts and the HCT 15 cell line (human colon carcinoma). Maximal concentrations of the extracts (400 μg/ml) inhibited 63.93 ± 2.76% and 80.16 ± 2.13% of cell viability, respectively [183]. *V. punctatum* chloroform and methanol extracts expressed a hepatoprotective activity *in vitro* protecting the Chang liver cells against CCl4-induced toxicity in the MTT test. The cell viability ranged from 62% to 84% at concentrations of 200-400 μg/ml, with the methanol extract being more effective [184]. The ethanol extract of *V. punctatum* leaves showed anticancer activity against MCF-7 in MTT test with IC_{50} of 56.73 μg/ml [185].

The *V. foetens* (Pakistan) methanol crude extract and fractions showed a significant anticancer effect against the breast cancer cell line MCF-7. The crude extract was active 90.5% at 200 μg/ml, while the fractions were less effective at the same concentrations. The highest MCF-7 cell line inhibition percentage was reported for the methanol (83%), followed by the chloroform 55.5%, the hexane 25.11%, and water (2%) fractions [157]. The crude methanol extract and fractions of *V. foetens* (Pakistan) were evaluated against MCF-7, MDA-MB-468, and Caco-2 cancer cell lines by the MTT test and NRU (neutral red uptake) assay. The crude extract inhibited the cancerous cell growth in a dose-dependent manner. The ethyl acetate fraction significantly reduced Caco-2 cells (93.44%) growth in the MTT test. The methanol and ethyl acetate fractions decreased 99% and 96% cell growth of MCF-7 and Caco-2 cell lines, respectively, in the NRU assay. Also, ethyl acetate fraction of the *V. foetens* extract exhibited a considerable inhibition of MDA-MB-468 cells in both used assays. Other fractions (chloroform, hexane, and aqueous) produced a weaker effect on cancer cell proliferation [186]. Methanol leaf extract of *V. dilatatum* (Korea) produced a cytotoxic effect on MCF-7 human breast cancer cells with IC_{50} of 139 ± 16 μg/ml. The authors reported that this effect could not be considered strong, according to the screening program of the National Cancer Institute, USA, which recommends IC_{50} under 20 μg/ml to be the effective cytotoxic agent [139]. Roy [164] reported significant lethality in a brine shrimp cytotoxicity assay for the *V. foetidum* crude methanol extract and its petroleum ether and n-hexan fractions with LC50 of 39.81, 25, and 25 μg/ml, respectively, while LC50 for standard vincrinstine sulphate was 10.44 μg/ml. The Colombian *V. cornifolium* leaf dichlormetan extract showed high cytoxicity tested on the V79 cell line (Chinese hamster lung fibroblasts) with IC_{50} of 25 μg/ml [187]. Ponnudurai et al. [188] (India) tested methanol leaf and chloroform root extracts of *V. coriaceum* and *V. erubescens* for their bacterial strain-based cytotoxicity (*E. coli* AB 1157 strain), using the MTT method (MCF-7 breast cancer cell lines and HeLa cervical cell lines). All extracts, except the chloroform root extract of *V. erubescens*, showed an effect on the bacterial strain-based carcinogenicity. IC_{50} values in the MTT test, only determined for the *V. coriaceum* extracts, indicated a moderate anticancer activity (over 500 μg/ml for MCF-7 cells and 300 μg/ml HeLa cells) [188]. Calderón-Montaño et al. [189] reported cytotoxic effects for the Spanish V. tinus water fruit and leaf extracts in the MTT test. The fruit extract was more potent in the inhibition of proliferation of A549-human lung adenocarcinoma cells and MRC-5-human lung fibroblastic cells with IC_{50} of 26.6 ± 6.5 and 65.4 ± 8.6 μg/ml, respectively. Methanol extracts of Chinese *V. odoratissimum* wood and bark successfully inhibited melanin biosynthesis and cell proliferation of B16 melanoma cells at 100 and 50 μg/ml, respectively [190].

4.5. Anticancer Effects of Viburnum Plants In Vivo. The anticancer effect of *V. opulus* juice was previously reported on Ehrlich ascites carcinoma cells [26]. However, Ceylan et al. [191] from Turkey investigated *V. opulus* juice for its antitumor potential in an in vivo experiment with experimental Balb/c mice. To implement tumors to mice, they applied Ehrlich ascite carcinoma (EAC) 1×10^6 cells i.p. and lyophilized *V. opulus* juice at a dose of 1000, 2000, and 4000 mg/kg. The tumor weight significantly decreased in mouse groups

treated with the juice compared to the control group. The survival rate of Ehrlich ascites tumor cells was reported to be 88.72%, 69.02%, and 51.87%, respectively. The results of the *in vitro* assay indicated the cytotoxicity of the juice with the IC_{50} value of 199.58 μg/ml [191]. Also, the same authors reported that gilaburu fractions below and above 50 kDa can stop the cell cycle at the G0/G1 stage and slow the cell division of the Ehrlich ascites tumor [123].

Ulger et al. [192] (Turkey) experimented with Balb-c male mice to study the effect of *V. opulus* juice on colon tumorogenesis induced with 1,2-dimethylhydrazine (DMH). All groups treated with DMH developed colon tumors as observed by histogenesis. However, mice that received the juice showed a reduced number of tumor lesions, as well as the incidence of invasive carcinoma, as compared to untreated mice. The authors concluded that *V. opulus* juice could be useful at the initiation stage and prevention of colon cancer.

4.6. Other Health-Promoting Effects. A 1000 mg *Viburnum opulus* dose and diclofenac *on-demand* were administered, orally, to 53 patients with urethral stones < 10 mm, in comparison to 50 patients receiving only diclofenac *on-demand*. It was observed that the expulsion of the stones was greater, and the passage time was faster in the treatment with *V. opulus*. The demand for diclofenac was lower compared to patients who received only diclofenac [193]. There are no clinical trial reports for the premenstrual syndrome [111], only the folk medicine use, as antispasmodic, in menstrual cramps, dysmenorrhea, and miscarriage prevention [194]. No side effects or reports regarding safety were found [194, 195].

5. Conclusions

The genus *Viburnum* includes about 200 species, distributed mainly on the Asian side Fruits, flowers, and barks of certain species are used in traditional medicine for the treatment of diseases, such as rheumatoid arthritis, cough, diarrhea. They contain a plethora of biochemical compounds with health-promoting activity, including carotenoids, polyphenols, and flavonoids, which can explain the high antioxidant activity as shown by in vitro studies. Preclinical evidence supports antibacterial, anti-inflammatory, cytotoxic, and anticancer properties of certain species, such as *V. opulus*.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Effects and Mechanisms of Resveratrol on Aging and Age-Related Diseases

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The aging of population has become an issue of great concern because of its rapid increase. Aging is an important risk factor of many chronic diseases. Resveratrol could be found in many foods, such as grapes, red wine, peanuts, and blueberries. Many studies reported that resveratrol possessed various bioactivities, such as antioxidant, anti-inflammatory, cardiovascular protection, anticancer, antidiabetes mellitus, antiobesity, neuroprotection, and antiaging effects. The antiaging mechanisms of resveratrol were mainly ameliorating oxidative stress, relieving inflammatory reaction, improving mitochondrial function, and regulating apoptosis. Resveratrol could be an effective and safe compound for the prevention and treatment of aging and age-related diseases. In this review, we summarize the effects of resveratrol on aging, life extension, and several age-related diseases, with special attention paid to the mechanisms of antiaging action.

1. Introduction

The expansion in the aged population has emerged as a matter of great concern. Over the past 60 years, the proportion of the world population aged 60 and over had experienced only a slight increase, from 8% to 10%. However, in the next 40 years, this group is estimated to soar to 22% of the total population, jumping from 0.8 billion to 2 billion [1]. In the progress of aging, living organisms experienced a series of progressively degenerative changes and became more sensitive to the internal and external stimuli, leading to aggravation of oxidative stress, accumulation of inflammation, apoptosis of cells, and damage to structures and functions of cells and organs [2–4]. Hence, as age is growing, the risks of susceptibility to various diseases (such as neurodegenerative diseases, sarcopenia, cardiovascular diseases, diabetes mellitus, obesity, and malignant tumors) and vulnerability to death are increased [5–7]. In this respect, a challenge facing the aging population is to decrease the impact of aging on health and avoid the occurrence and development of age-related diseases. The plethora of literature has supported that bioactive compounds from traditional Chinese medicine or natural products could be an effective and safe strategy to protect against aging and age-related diseases [8–12].

Polyphenols are bioactive compounds that extensively existed in plant foods and have many health-promoting effects by the different mechanisms, such as antioxidation, anti-inflammation, immunomodulation, and modulating gut microbiota [11, 13]. Resveratrol is a natural phenolic compound and has been found in many foods, such as grapes, peanuts, blueberries, and red wine. Resveratrol has shown many bioactivities, such as antioxidant, anti-inflammatory, immunomodulatory, hypotensive, and hypolipidemic actions, as well as efficacies in the prevention and

management of cancer, cardiovascular diseases, neurodegenerative diseases, and obesity [14, 15]. In addition, many studies highlighted its importance in aging treatment through suppressing oxidative stress, inhibiting inflammatory response, improving mitochondrial function, and modulating apoptosis [16, 17]. Moreover, the interaction between resveratrol and gut microbiota in the protection of health has attracted increasing attention in recent years [18]. In this review, we summarize the effects of resveratrol on antiaging, lifespan, and several age-related diseases (neurodegenerative diseases, sarcopenia, cardiovascular diseases, and malignant tumors), and the mechanisms of antiaging are paid special attention.

2. Effect of Resveratrol on Life Extension

Longevity is a vital target of antiaging research and an important index reflecting the antiaging efficacy [19]. Accumulating studies revealed that resveratrol could extend lifespan (Table 1). For example, a meta-analysis indicated that resveratrol acted as a life-extending agent, concluding from 19 published papers with six species (yeast, nematodes, mice, fruit flies, Mexican fruit flies, and turquoise killifish) [20]. Autophagy could promote the clearance of impaired cellular organelles (mitochondria, endoplasmic reticulum, etc.) and dysfunctional proteins, which contributes to the life extension and antiaging effect [21, 22]. A study demonstrated that resveratrol could induce autophagy in human cells *in vitro* and *Caenorhabditis elegans in vivo* and prolong lifespan in *Caenorhabditis elegans*, while this effect was prevented under the condition of knockdown or knockout of sirtuin (Sirt)1. The results suggested that resveratrol promoted longevity through the Sirt1-dependent induction of autophagy [23]. Another study compared the effect of thymol and resveratrol on the life span of honey bees and found that the bees fed with resveratrol syrup lived longer (25 days) than those fed with thymol or control syrup (23 and 20 days, respectively) [24]. Moreover, a study evaluated the difference in adult longevity of short-lived *Drosophila melanogaster* populations fed with different concentrations of resveratrol. Resveratrol supplementation prolonged adult longevity in both the male and female flies in a nonlinearly dose-dependent manner by scavenging reactive oxygen species (ROS) and neuroprotection, without affecting reproduction [25]. In a study, a transgenic resveratrol rice DJ526, which had 180 times more resveratrol than the conventional grain, remarkably prolonged the median longevity of *Drosophila melanogaster* by up to 50% in contrast to the control. It also improved age-related symptoms, including locomotive deterioration, body weight gain, eye degeneration, and neurodegeneration [26]. Another study showed that the supplement of resveratrol extended the lifespan of *Nothobranchius guentheri* (a wild type of annual fish) but had no effect on their body size. Resveratrol-treated fish exhibited better cognitive and locomotor activities than the fish in the control group, and resveratrol retarded the aging-related histological markers, including the expression of senescence-associated β-galactosidase activity and lipofuscin formation [27]. In addition, a study used HtrA2 knockout mice to evaluate the effect of resveratrol on lifespan, which usually developed neuromuscular abnormalities around postnatal day 23 and died around day 28. The results showed that oral administration of resveratrol increased the median survival of the mice by 10 days (from 32 days to 42 days) [28]. However, inconsistent evidence existed. For example, a study pointed out that red wine and equivalent oral pharmacological doses of resveratrol could delay vascular aging but had no effect on lifespan extension in rats [29].

To sum up, resveratrol could extend lifespan in many animal models mainly by inducing autophagy, reducing oxidative stress and neuroprotection. In addition, some fruits and vegetables, such as grape, peanut, blueberry, cucumber, tomato, red cabbage and spinach, have relatively high contents of resveratrol, and their effects on lifespan should be evaluated in the future.

3. Effect of Resveratrol on Age-Related Diseases

Lowering the risk of age-related diseases is another crucial goal of antiaging research. Here, we summarized the effects of resveratrol on several age-related diseases (Table 2 and Figure 1).

3.1. Effect of Resveratrol on Neurodegenerative Diseases. Aging is reported to be the primary risk factor of neurodegenerative diseases. Aging is accompanied by neuroinflammation, autophagy dysregulation, neuronal apoptosis, and elevated oxidative status, which leads to progressive memory loss and motor impairment. It eventually increases the risk of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and dementia [30, 31]. The plethora of literature showed the protection of resveratrol against neurodegenerative diseases. For example, a study revealed the protection of resveratrol treatment on hippocampal plasticity and memory performance in female Balb/C mice. Resveratrol induced neuronal differentiation in adult hippocampal precursor cells without affecting the proliferation *in vitro*. Also, resveratrol intervention improved behavioral performance, increased the production of new neurons, elevated the population of doublecortin-expressing intermediate cells, and promoted hippocampal neurogenesis *in vivo*. The resveratrol-treated mice showed an increase in the levels of phosphoprotein kinase B (Akt) and phosphoprotein kinase C, indicating the involvement of these signaling pathways [32]. Furthermore, after intraventricular injection of resveratrol for 7 days, the long-term memory formation and the long-time potentiation induction from hippocampus CA1 were improved in C57BL/6J mice, while these effects were blocked in Sirt1 mutant mice [33]. In another study, aged male F344 rats were treated with resveratrol or vehicle for 4 weeks. The results found that resveratrol-treated animals showed the improved learning, memory, and mood functions. Resveratrol also increased net neurogenesis and microvasculature, decreased astrocyte hypertrophy, and microglial activation in the hippocampus [34]. Moreover, a study evaluated the effects of chronic administration of resveratrol on aged male Wistar rats by testing tryptophan hydroxylase (TPH) and tyrosine hydroxylase (TH) activities

Table 1: Effect of resveratrol on life extension.

Study type	Subjects	Administration methods	Dose & duration	Main effects	Ref.
In vitro, *in vivo*	Human cells, *Caenorhabditis elegans*	Culture	100 μM	Induced autophagy in human cells and *Caenorhabditis elegans*, prolonged lifespan in *Caenorhabditis elegans* through the Sirt1-dependent induction of autophagy	[23]
In vivo	Honey bees	Administered in candy and syrup	10 ppm	Inhibited *Nosema ceranae* infection and prolonged lifespan of honey bees	[24]
In vivo	*Drosophila melanogaster*	Diet supplement	25-800 μM	Extended adult longevity in both the male and female flies by scavenging ROS and neuroprotection	[25]
In vivo	Rats	Diet supplement	NA	Delayed vascular aging, but did not extend lifespan	[29]
In vivo	*Nothobranchius guentheri* (fish)	Diet supplement	200 μg/g food	Prolonged longevity, improved cognitive ability and aging-related histological markers	[27]
In vivo	*Drosophila melanogaster*	Culture	NA	Prolonged longevity, ameliorated age-related symptoms	[26]
In vivo	HtrA2 knockout mice	Oral administration	25 mg/kg BW	Prolonged longevity, delayed worsening of the motor phenotype	[28]

Note: NA: not available; ROS: reactive oxygen species; BW: body weight.

(mediating central monoaminergic neurotransmitter synthesis) and hippocampal-dependent working memory. The results reported that resveratrol reversed an age-dependent decline in cognitive functions through enhancing the secretion of neurotransmitters, including serotonin, noradrenaline, and dopamine. These changes were largely due to the increased activities of TPH and TH [35]. Additionally, another study compared the difference between the young and old rats treated with or without resveratrol. The results found that old rats showed emotional and spatial learning memory damage compared to young rats, while resveratrol could reverse the cognitive impairment via inhibiting the production of inflammatory cytokines [36].

In short, resveratrol showed protective effects against neurodegenerative diseases by enhancing the secretion of neurotransmitters, increasing the production of new neurons, decreasing neuroinflammation and oxidative stress, and promoting hippocampal neurogenesis.

3.2. Effect of Resveratrol on Cardiovascular Diseases. Cardiovascular disease is a leading cause of death in the world. Aging is associated with impaired vascular function due to endothelial dysfunction and altered redox balance, thus increasing the risk of cardiovascular diseases [37, 38]. Resveratrol could decrease atherosclerosis and improve cardiovascular health. For instance, an *in vitro* study found that senescent endothelial cells and aortas derived from old Wistar Kyoto rats demonstrated higher levels of superoxide production and oxidative stress as well as a lower level of bioactive nitric oxide (NO) than those from their young controls. Resveratrol could reduce superoxide generation, enhance NO level, and improve oxidative stress, thus protecting against aging-associated vascular diseases [39]. Another *in vitro* study carried on human umbilical vein endothelial cells showed that pretreatment with resveratrol could enhance the cell viability and superoxide dismutase (SOD) levels, reverse the elevated levels of senescence-associated β-galactosidase and intracellular ROS induced by H_2O_2 treatment, and upregulate autophagy, thus delaying the aging process of human umbilical vein endothelial cells [40]. Furthermore, methylglyoxal incubation significantly inhibited endothelium-dependent vasodilatation and decreased the expression of endothelial nitric oxide synthase (eNOS) in thoracic aorta of aged Wistar rats *in vitro*, while resveratrol treatment could improve methylglyoxal-induced endothelial dysfunction by increasing eNOS expression and activity [41]. In another study, cardiotoxicity was induced by doxorubicin in senescence-accelerated mice and led to a disturbance of ubiquitin-specific protease 7-related catabolic/proapoptotic signaling in hearts. The administration of resveratrol attenuated these adverse changes in aged hearts with the absence of Sirt1 inhibitors, like sirtinol, indicating that resveratrol exerted a cardioprotective effect through restoring the activity of Sirt1 [42]. In addition, resveratrol treatment significantly lowered aorta media thickness, inflammation, fibrosis, and oxidative stress in aged male C57BL/6 mice compared to the control group, protecting against arterial aging through modulating the activity of the renin-angiotensin system [43]. Moreover, a study reported that mitochondrial aldehyde dehydrogenase 2 (ALDH2) could exacerbate aging-induced cardiac remodeling and contractile dysfunction, which were mitigated by resveratrol treatment [44].

In conclusion, resveratrol exerted a cardioprotective effect mainly through enhancing the production of NO, modulating the activity of the renin-angiotensin system, ameliorating oxidative stress, and restoring the activity of Sirt1.

3.3. Effect of Resveratrol on Sarcopenia. Sarcopenia is an age-related syndrome characterized by the progressive loss of muscle mass and function [45, 46]. Many studies showed that resveratrol effectively improved the mass and function of skeletal muscle and decreased the happening of sarcopenia. In a study, aged male Fischer 344 x Brown Norway rats received different interventions (resveratrol, calorie restriction, or resveratrol combined with calorie restriction) to evaluate their efficacy of protecting against sarcopenia. The

Table 2: Effect of resveratrol on age-related diseases.

Study type	Subjects	Administration methods	Dose & duration	Effects and mechanisms	Ref.
Neurodegenerative diseases					
In vivo	C57BL/6J mice	Intraventricular injection	5 μg/μL for 1 week	Improved learning and memory functions in a Sirt1-dependent way	[33]
In vivo	Aged C57BL/6 mice	Oral administration	200 mg/kg for 10 days	Rescued cortical neurovascular coupling responses to improve neuronal activity and function by restoring cerebromicrovascular endothelial function and decreasing ROS production	[65]
In vivo	C57BL/6 mice	Intraperitoneal injection	100 mg/kg for 7 days	Alleviated the hippocampus-dependent cognitive impairment via anti-inflammation and antiapoptosis actions	[66]
In vivo	Male F344 rats	Intraperitoneal administration	40 mg/kg for 4 weeks	Improved memory and mood functions, increased hippocampal neurogenesis and microvasculature, and reduced glial activation	[34]
In vivo	Old male rats	Chronic administration	20 mg/kg for 4 weeks	Improved cognitive impairment by enhancing the secretion of neurotransmitters (serotonin, noradrenaline, and dopamine), which is largely due to the increased activities of TPH and TH	[35]
In vitro, *in vivo*	Adult hippocampal precursor cells; female Balb/C mice	Cell culture; injection	40 mg/kg for 2 weeks	Induced neuronal differentiation in adult hippocampal precursor cells without effects on proliferation *in vitro*, improved behavioral performance, increased production of new neurons, elevated population of doublecortin-expressing intermediate cells, and promoted hippocampal neurogenesis *in vivo* through the phospho-Akt and phosphoprotein kinase C signaling pathways	[32]
In vivo	Aged Sprague-Dawley rats	Intraperitoneal injection	100 mg/kg for 7 days	Inhibited neuronal apoptosis and improved behavioral performance via Sirt1-p53 signaling pathway	[67]
In vivo	Male Wistar rats	Oral administration	50 mg/kg for 12 weeks	Improve aging-induced emotional and spatial learning memory impairment via inhibiting inflammation	[36]
In vitro, *in vivo*	SH-SY5Y neuroblastoma cells; male C57BL/6 mice	Cell culture; feeding	1 or 5 μM; 120 mg/kg	Alleviated age-related motor decline and exerted neuroprotection via the promotion of dopamine neuronal survival and activation of the ERK1/2 pathways	[68]
Cardiovascular disease					
In vitro	Aging endothelial cells; aortas of old WKY rats	Cell and tissue culture	10 μmol/L	Inhibited S6K1 signaling, reduced superoxide generation, and enhanced NO levels	[39]
In vivo	Senescence-accelerated mice prone 8 (SAMP8)	Intraperitoneal injection	20 mg/kg for 3 days	Attenuated doxorubicin-induced cardiotoxicity through restoring the activity of Sirt1	[42]
In vivo, *in vitro*	Aged male C57BL/6 mice; vascular smooth muscle cells	Dietary supplementation	40 mg/kg for 6 months	Lowered aorta media thickness, inflammation, fibrosis, and oxidative stress and protected against arterial aging through modulating the activity of the renin-angiotensin system	[43]
In vivo	Wistar albino rats	Drinking water	0.05 mg/mL for 6 weeks	Altered vessel responsiveness and biomarkers related to vascular functions	[69]
In vitro	Human umbilical vein endothelial cells	Cell culture	10 μM	Enhanced the cell viability and SOD levels, inhibited the increased levels of senescence-associated β-galactosidase and intracellular ROS induced by H_2O_2 treatment, and upregulated autophagy	[40]
In vitro	Thoracic aorta of aged Wistar rats	Organ culture	30 μM for 4 h or 24 h	Improved methylglyoxal-induced endothelial dysfunction by increasing eNOS expression and activity	[41]
Sarcopenia					
In vivo	Fischer 344 x Brown Norway rats	Dietary supplementation	50 mg/kg for 6 weeks	Protected against aging-induced muscle loss via modestly altered key mitochondrial regulatory and apoptotic signaling pathways in glycolytic muscle	[47]
In vivo	Fischer 344 x Brown Norway rats	Oral gavage	125 mg/kg	Improved muscle mass, increased the fiber cross-sectional area of type IIA and IIB fibers during reloading after hindlimb suspension due to decreases in the abundance of proapoptotic proteins	[48]
In vivo	Aged C57BL/6J mice	Oral gavage	25 mg/kg BW for 4 weeks	Resveratrol combined with exercise training showed stronger muscle strength and endurance performance of aged mice than the resveratrol or exercise training alone	[49]
In vivo	Aged male Sprague-Dawley rats	Dietary supplementation	150 mg/kg for 6 weeks	Increased the relative grip strength and muscle mass and reduced the increment in sarcomere length, I-band, and H-zone via antiapoptotic signaling pathways through the activation of AMPK/Sirt1	[51]
In vivo	C57/BL6 mice	Dietary supplementation	0.04% for 6 months	Inhibited tubular aggregates and showed better resistance to fatigue	[70]
In vivo	C57BL/6J male mice			Showed a better fatigue resistance	[71]

Table 2: Continued.

Study type	Subjects	Administration methods	Dose & duration	Effects and mechanisms	Ref.
		Dietary supplementation	0.04% for 6 months		
Cancers					
In vitro	Ovarian cancer cells	Cell culture	25-800 μM	Suppressed proliferation and evoked apoptosis via inhibiting glycolysis and targeting AMPK/mTOR signaling pathway	[53]
In vitro	Gastric cancer cell	Cell culture	0, 25, 50, and 100 μM for 96 h	Inhibited cell proliferation and survival through inhibition of PIM-1 kinase activity	[54]
In vitro	Human prostate cancer cell lines	Cell culture	50 μM for 48 h	Inhibited cell proliferation and migration through the NF-κB pathway	[55]
In vitro, in vivo	Colon cancer cells; nude mice	Cell culture; intraperitoneal injection	0-240 μmol/L; 150 mg/kg BW	Inhibited invasion and metastasis through the reversal of EMT via the Akt/GSK-3 β/Snail signaling pathway	[56]
In vitro	Human colon cancer cell lines	Cell culture	0-50 μM	Inhibited cell viability, induced apoptosis, and decreased expression of cyclooxygenase-2 and prostaglandin receptor	[57]
In vitro	Prostate cancer cells	Cell culture	25-100 μM	Inhibited proliferation and promoted apoptosis	[58]
Other diseases					
In vitro	Mouse oocytes	Cell culture	1 μM	Improved the quality of postovulatory aging oocytes via maintaining mitochondrion distribution and the normal morphology of spindle, alleviating oxidative stress, ameliorating apoptosis, and decreasing the loss of sperm binding	[60]
In vivo	Female C57BL/6 mice	Drinking water	30 mg/L for 6 or 12 months	Reserved the ability of reproduction and showed a larger follicle pool, improved the number and quality of oocytes, telomerase activity, telomere length, and age-related gene expression in ovaries	[61]
In vivo	Aged male Wistar rats	Drinking water	10 mg/kg for 10 weeks	Increased bone volume, bone trabecular number, and cortical thickness and reduced spacing between trabeculae	[63]
In vivo	C57BL/6 mice	Dietary supplementation	300 mg/kg for 10 weeks	Accelerated osteoblast activity and bone growth, and promoted bone formation in a Sirt1-dependant way	[64]

Note: Sirt1: sirtuin1; ROS: reactive oxygen species; TPH: tryptophan hydroxylase; TH: tyrosine hydroxylase; Akt: protein kinase B; ERK1/2: extracellular-regulated kinases 1 and 2; S6K1: ribosomal protein S6 kinase, polypeptide1; NO: nitric oxide; SOD: superoxide dismutase; eNOS: endothelial nitric oxide synthase; AMPK: 5′-monophosphate-activated protein kinase; mTOR: mammalian target of rapamycin; PIM-1 kinase: proviral integration site for Moloney murine leukemia virus-1 kinase; BW: body weight; NF-κB: nuclear-factor kappa B; EMT: epithelial-mesenchymal transition; Akt/GSK-3 β/Snail: protein kinase B/glycogen synthase kinase-3 β/Snail signaling.

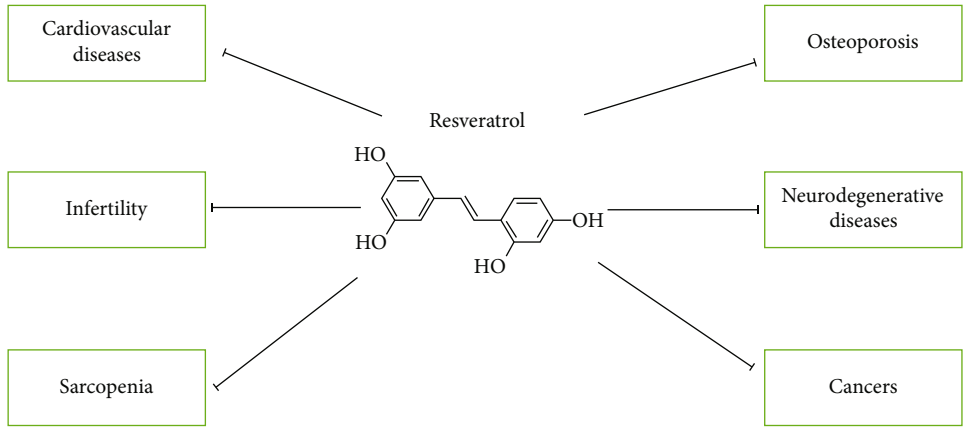

Figure 1: The effects of resveratrol on age-related diseases. Resveratrol could protect against age-related diseases, such as neurodegenerative diseases, cardiovascular diseases, sarcopenia, infertility, osteoporosis, and cancers.

results indicated that short-term moderate resveratrol, calorie restriction, or resveratrol combined with calorie restriction could modestly alter key mitochondrial regulatory and apoptotic signaling pathways in the glycolytic muscle, which protected against aging-induced muscle loss [47]. Moreover, another study found that resveratrol treatment did not attenuate the decrease in the plantaris muscle wet weight during hindlimb suspension (creating disuse condition) in aged rats,

but it could improve muscle mass during reloading after hindlimb suspension. Resveratrol enhanced the fiber cross-sectional area of type IIA and IIB fibers and promoted myogenic precursor cell proliferation in response to reloading after hindlimb suspension [48].

There was evidence that resveratrol in combination with exercise had a better effect on sarcopenia. A study found that aged mice treated with resveratrol combined with exercise training showed stronger muscle strength and endurance performance than with resveratrol or exercise training alone [49]. Another study showed that resveratrol supplementation or exercise training improved physical endurance in aged mice through increasing mitochondrial biogenesis and function, and their combination displayed better results [50]. Furthermore, resveratrol, exercise, or their combination significantly increased the relative grip strength and muscle mass in aged rats and reduced the increment in sarcomere length, I-band, and H-zone via antiapoptotic signaling pathways through activation of adenosine $5'$-monophosphate-activated protein kinase (AMPK)/Sirt1 [51].

In brief, resveratrol could protect against sarcopenia, and its combination with exercise had a better effect. The effect might be mediated by the inhibition of apoptosis, the promotion of mitochondrial biogenesis and function, and the activation of Sirt1.

3.4. Effect of Resveratrol on Cancers. Age is reported to be one of the most important risk factors in the occurrence and development of cancers [52]. Considerable researches elucidated that resveratrol treatment could suppress the formation of cancer via inhibiting cell proliferation. An *in vitro* study showed that resveratrol significantly inhibited the proliferation, migration, and invasion of ovarian cancer cells, meanwhile damaging glycolysis, evoking apoptosis in these cells via increasing the expression and activation of AMPK and caspase 3, and decreasing the expression and activation of AMPK downstream kinase mammalian target of rapamycin (mTOR). In addition, an *in vivo* study manifested that resveratrol suppressed ovarian cancer growth and liver metastasis in the xenograft mouse model [53]. In another study, resveratrol could inhibit cell growth and proliferation in human gastric cancer SNU-601 cells by suppressing the activity of proviral integration site for Moloney murine leukemia virus-1 (PIM-1) kinase [54].

Several studies found that resveratrol could inhibit tumor progression by suppressing cell metastasis. In a study, prostate cancer cells cultured with resveratrol showed a decreased level of epithelial-mesenchymal transition- (EMT-) related proteins and inhibited cell migration and cell growth through the nuclear-factor kappa B (NF-κB) pathway [55]. In another study, the effects of resveratrol on cell invasion and metastasis as well as changes in the expression of EMT markers and Akt/glycogen synthase kinase-3 β (GSK-3 β)/Snail signaling pathway were investigated in colon cancer cells. Furthermore, an *in vivo* lung metastasis model of colon cancer was developed in mice to investigate the effects of resveratrol on lung metastasis in colon cancer. *In vitro* and *in vivo* results indicated that resveratrol significantly inhibited cell migration and invasion in colon cancer through the reversal of EMT via the Akt/GSK-3 β/Snail signaling pathway [56].

The induction of apoptosis also contributed to the anticancer capacity of resveratrol. In a study, results showed that resveratrol significantly inhibited cell viability, induced apoptosis, and decreased cyclooxygenase-2 and prostaglandin receptor expression in human colon cancer cell lines [57]. Moreover, another *in vitro* study reported that resveratrol induced apoptosis in prostate cancer cells in a dose-dependent manner. At the molecular level, resveratrol suppressed the expression of androgen receptor protein as well as Akt phosphorylation [58].

To sum up, many studies showed that resveratrol was effective in the prevention and treatment of cancers mainly through inhibition of cell proliferation, induction of cell apoptosis, and suppression of cell migration.

3.5. Effects of Resveratrol on Other Diseases. Except for the above mentioned, some diseases were also closely associated with aging, like infertility and osteoporosis, and resveratrol was able to ameliorate these age-related diseases.

Aging leads to the loss of oocytes and follicles and impairs the quality of oocytes, which promotes age-associated ovarian aging and infertility [59]. In an *in vitro* study, the effects and mechanisms of resveratrol on postovulatory aging mouse oocytes were investigated. The results showed that resveratrol treatment significantly improved the quality of the oocytes and increased the ratios of fertilization and blastocyst via maintaining mitochondrion distribution and the normal morphology of spindle, alleviating oxidative stress, ameliorating apoptosis, and decreasing the loss of sperm binding [60]. In another study, after long-term (12 months) administration of resveratrol, female C57BL/6 mice reserved the ability of reproduction and showed a larger follicle pool than age-matched controls. Resveratrol significantly improved the number and quality of oocytes, telomerase activity, telomere length, and age-related gene expression in ovaries of mice [61].

Osteoporosis is a common disease of old age, characterized by the decrease of bone density and mass, the destruction of bone microstructure, and the increase of bone brittleness [62]. A study showed that resveratrol increased bone volume, bone trabecular number, and cortical thickness and reduced bone trabecular spacing in aged male Wistar rats [63]. Moreover, another study revealed that resveratrol accelerated osteoblast activity and bone growth and promoted the bone formation of C57Bl/6 mice in a Sirt1-dependant way [64].

Collectively, resveratrol showed abilities in the prevention and treatment of several age-related diseases, such as neurodegenerative diseases, cardiovascular diseases, sarcopenia, cancers, infertility, and osteoporosis.

4. Mechanisms of Resveratrol on Aging

Aging is the result of many factors on the body. The accumulation of oxidative stress, low-grade inflammation, and cell apoptosis is the prominent characteristic in the aging progress [72, 73]. Additionally, increasing evidence shows that mitochondrial dysfunction and gut microbiota imbalance

contribute to the aging progress [74, 75]. Hence, we discuss the mechanisms of the antiaging effect of resveratrol as below (Table 3 and Figure 2).

4.1. The Suppression of Oxidative Stress. Oxidative stress is a crucial contributor to the aging process as well as the occurrence and development of age-related diseases. Lipid peroxidation, protein peroxidation, and an impaired defense system induced by excessive ROS gradually compromise cell structures and functions, eventually giving rise to cell senescence and accelerating the aging process [76, 77]. Many studies showed that resveratrol could inhibit the formation of oxidative stress, thus exerting an antiaging effect. For example, an *in vitro* study carried out on erythrocytes from healthy humans demonstrated that resveratrol treatment could activate the plasma membrane redox system and ascorbate free radical reductase in a dose-dependent manner, protect against lipid peroxidation and protein carbonylation, and recover the cellular redox homeostasis during aging [78]. An *in vitro* study also found that resveratrol treatment reduced the generation of ROS in immortalized lymphocytes from Alzheimer's disease patients or healthy controls, as well as upregulated the gene expression of antioxidant enzymes, like catalase (CAT), SOD, and antiaging factors, like Sirt1 and Sirt3 [79]. In addition, a study found that markers of oxidative damage to DNA, lipid oxidation, and protein oxidation age-dependently accumulated in the majority of mouse tissues, but chronic resveratrol treatment (6 or 12 months) could reverse the damage caused by oxidative stress. However, it also found that a 12-month resveratrol intake caused a significant increase in these oxidative damage markers in the kidney, suggesting that chronic resveratrol treatment might result in nephrotoxicity; hence, attention should be paid to the safe dosage and duration of resveratrol consumption [80].

The grey mouse lemurs were assigned to different groups to receive the standard-fed control diet, 30% fewer calories than the standard-fed control diet, and the standard-fed control diet supplemented with 200 mg/kg resveratrol for 3, 9, 15, and 21 months, to compare the effects of resveratrol and calorie restriction on oxidative stress. The results showed that oxidative stress age-dependently accumulated in grey mouse lemur, while both resveratrol and calorie restriction effectively ameliorated oxidative stress [81]. Another study evaluated the effects of resveratrol and exercise on endogenous antioxidant activities in livers of different-age mice. Aging induced the accumulation of oxidative damage in the liver, particularly impairing the glutathione-dependent system. Both resveratrol and exercise reversed the impact of aging on antioxidant capacity and sustained high activities of glutathione (GSH), glutathione peroxidase (GPx), and GSH transferase in old mice [82]. Moreover, oral administration of 10 mg/kg body weight resveratrol decreased the level of NO and retarded the lipid peroxidation in the cardiac tissue of male Wistar rats during the aging process, but the activities of CAT and SOD in the resveratrol intervention group had no significant difference from the controls, indicating that the antioxidant ability of resveratrol in the cardiac tissue might not result from the activation of CAT and SOD [83].

4.2. The Inhibition of Inflammation. A chronic low-grade inflammation level commonly exists in the aging process, which is known as inflammaging. Increasing evidence has highlighted the importance of inflammaging in the progress of aging and age-related metabolic diseases, and it is an extraordinary crucial risk factor for morbidity and mortality in older people [84, 85]. There are findings that suggest that resveratrol exerted an antiaging effect though improving inflammatory response. An *in vitro* study reported that treatment with resveratrol reversed most of the age-related changes in the secretory phenotype of vascular smooth muscle cells derived from aged Macaca mulatta. Resveratrol notably reduced the secretion of proinflammatory cytokines, such as interleukin- (IL-) 1β, IL-8, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) in aged vascular smooth muscle cells. It also decreased the production of $O_2^{\cdot-}$ in mitochondria and upregulated the transcriptional activity of nuclear factor erythroid-2 related factor 2 (Nrf2) [86]. Another *in vitro* study developed a model for cultured hippocampal astrocytes from newborn, adult, and aged Wistar rats and found that with increasing age, the levels of proinflammatory cytokines increased, while antioxidant defenses decreased in hippocampal astrocytes. Resveratrol treatment significantly lowered the level of proinflammatory cytokines TNF-α and IL-1β and improved antioxidant defenses [87].

In a study, neuroinflammatory response and cognitive deficits were induced by intraperitoneal injection of lipopolysaccharide in adult (3-6 months) and aged (22-24 months) mice. Aged mice showed being more prone to peripheral immune stimulation than adult mice. The dietary supplementation of resveratrol mitigated inflammatory response and cognitive deficits caused by lipopolysaccharides in aged mice and reduced the increase of IL-1β in plasma and the hippocampus [88]. In another study, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) knockout and wild-type mice were randomly assigned to different groups intervened with or without resveratrol/exercise for 12 months, to analyze whether exercise and resveratrol inhibit age-associated low-grade inflammation in a PGC-1α-dependent manner. The results showed that long-term exercise training prevented an age-associated increase in the inflammatory response in a PGC-1α-dependent manner, while resveratrol supplementation reduced age-associated inflammation independently of PGC-1α [89]. Furthermore, metabolic stress was induced by a high-fat diet, leading to inflammation and cognitive disturbances in aged C56/BL6 mice (24 months). Resveratrol treatment reversed the increased levels of some proinflammatory cytokines, like TNF-α, IL-1, and IL-6, as well as other adverse changes in aged mice [90].

4.3. The Improvement of Mitochondrial Function. As a key factor in the metabolism of aerobic organisms, mitochondria are essential not only for obtaining ATP from glucose and fatty acids but also in many other essential functions in cells, including amino acid metabolism, pyridine synthesis, phospholipid modifications, and calcium regulation [91]. It is widely acknowledged that mitochondrial dysfunction and

Table 3: Effects and mechanisms of resveratrol on aging.

Study type	Subjects	Administration methods	Dose & duration	Effects and mechanisms	Ref.
The suppression of oxidative stress					
In vitro	Human erythrocytes	Cell culture	0.1–100 μM	Activated the plasma membrane redox system and ascorbate-free radical reductase, protected against lipid peroxidation and protein carbonylation, and restored the cellular redox homeostasis	[78]
In vitro	Immortalized lymphocytes	Cell culture	10 and 50 μM	Reduced the generation of ROS, upregulated the gene expression of antioxidants and antiaging factors	[79]
In vivo	F2 four-way cross-hybrid mice	Drinking water	14.09 mg/L for 6 or 12 months	Reversed oxidative damage but might result in nephrotoxicity	[80]
In vivo	Male grey mouse lemur	Diet supplement	200 mg/kg for 3-21 months	Ameliorated oxidative stress with age increase	[81]
In vivo	Male C57BL/6J mice	Drinking water	500 μg/animal for 6 months	Retarded the impact of aging and sustained high activities of GSH, GPx, and GSH transferase activities	[82]
In vivo	Male Wistar rats	Oral administration	10 mg/kg for 2-8 months	Decreased the level of NO and retarded the lipoperoxidation in the cardiac tissue	[83]
In vivo	C57BL/6 mice	Diet supplement	0.05% for 10 days	Blunted the exercise-induced increase in xanthine oxidase activity in muscles, lower H_2O_2, and Nox4 protein levels, increased the ratio of reduced GSH to oxidized GSH, prevented the increase in lipid oxidation, increased CAT and SOD activities	[112]
In vivo	Young and aged rats	Perfusion	NA	Ameliorated H_2O_2-induced oxidative stimulus in both young and aged rat brains and ameliorated basal oxidative stress in aged rat brains	[113]
In vivo	Aged C57BL/6 mice	Oral administration	30 mg/kg	Ameliorating renal oxidative stress via the Sirt1-mediated klotho expression	[114]
The inhibition of inflammation					
In vitro	Vascular smooth muscle cells	Cell culture	1 μM	Reduced the secretion of IL-1β, IL-8, TNF-α, and MCP-1, decreased the production of $O_2^{\cdot-}$ in mitochondria, and upregulated the transcriptional activity of Nrf2	[86]
In vitro	Hippocampal astrocyte	Cell culture	10 μM	Decreased proinflammatory cytokines IL-1β and TNF-α and increased antioxidant defenses	[87]
In vivo	Male BALB/c mice	Diet supplement	0.4% for 4 weeks	Mitigated inflammatory response and cognitive deficits and reduced the increase of IL-1β in plasma and the IL-1β mRNA in the hippocampus	[88]
In vivo	Female C57BL/6 mice	Diet supplement	4 g/kg for 12 months	Reduced age-associated inflammation independently of PGC-1α	[89]
In vivo	Aged female mice	Oral gavage	0.1 mg/kg for 10 days	Attenuated peripheral and brain inflammation and ischemic brain injury	[115]
In vivo	Male C57BL/6J mice	Diet supplement	1 g/kg, *W*/*W*	Reduced the inflammation and cognitive disturbances induced by metabolic stress	[90]
The improvement of mitochondrial function					
In vitro	Oocytes and granulosa cells	Cell culture	20 μM	Affected both oocytes and granulosa and improved the quality of oocytes through upregulation of mitochondrial biogenesis and degradation	[94]
In vivo	Aged mice	Oral gavage	15 mg/kg for 4 weeks	Improved physical endurance and oxidative stress via the regulation of mitochondrial biogenesis and function	[50]
In vivo	Female ICR mice		50 mg/kg BW		[95]

Table 3: Continued.

Study type	Subjects	Administration methods	Dose & duration	Effects and mechanisms	Ref.
		Intraperitoneal injection		Improved mitochondrial function, alleviated oxidative stress, and prevented apoptosis	
In vivo	Aged zebrafish	Administration	20 mg/L	Promoted mitochondrial function and downregulated Akt/mTOR pathway activity	[17]
The regulation of apoptosis					
In vivo	Aged Sprague-Dawley rats	Intraperitoneal injection	100 mg/kg for 7 days	Modified the performance of learning and memory, suppressed neuronal apoptosis	[67]
In vivo	Aged senescence-accelerated mice	Drinking water	5 mg/kg	Modulated the inflammatory, oxidative, and apoptotic status related to aging	[16]
In vivo, in vitro	Male albino Wistar rats; human diploid fibroblast strain	Oral administration	50, 100 mg/kg	Displayed antiaging activities by inhibiting senescence and apoptosis and recovering cognitive impairment and oxidative damage	[98]
In vitro	Mouse neuronal N2a cells	Cell culture	1.5 to 25 μM	Counteracted apoptosis, autophagy, and oxidative stress, associated with mitochondrial and peroxisomal dysfunction induced by 7-Ketocholesterol	[116]
In vivo	Sprague-Dawley rats	Oral gavage	80 mg/kg	Decreased apoptotic index, improved mitochondrial function, and inhibited oxidative stress	[99]
In vivo	Senescence-accelerated mice	Diet supplement	4.9 mg/kg for 8 months	Improved exercise capacity and voluntary motor behavior, increased the protein expression of antiapoptotic Bcl2	[100]
In vivo	Male senescence-accelerated mice	Intraperitoneal injection	20 mg/kg/day for 3 days	Attenuated the doxorubicin-induced elevations of apoptotic and catabolic markers measured as Bax, caspase 3 activity, apoptotic DNA fragmentation, ubiquitinated proteins, and proteasomal activity in aged muscles	[117]

Note: ROS: reactive oxygen species; GSH: glutathione; GPx: glutathione peroxidase; NO: nitric oxide; Nox4: NADPH oxidase 4; CAT: catalase; SOD: superoxide dismutase; Sirt1: sirtuin1; IL: interleukin; TNF-α: tumor necrosis factor-α; MCP-1: monocyte chemoattractant protein-1; Nrf2: nuclear factor erythroid-2 related factor 2; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α; Bcl2: B-cell lymphoma-2; Bax: BCL2-associated X.

decreased mitochondrial content are hallmarks of aging and play an important role in promoting aging [92, 93]. Increasing literature reported that resveratrol regulated the mitochondrial function, thus delaying the aging process. An *in vitro* study showed that resveratrol treatment increased the expression of Sirt1 and evoked autophagy in both oocytes and granulosa cells derived from aged cows. Resveratrol also increased the mitochondrial DNA (mtDNA) copy numbers and the ATP content in oocytes and promoted oocytes to develop into the blastocyst stage, thus improving the quality of oocytes. All of these effects were associated with the regulation of mitochondrial biogenesis and degradation [94]. In addition, an *in vivo* study showed that aged mice receiving resveratrol and/or exercise-training for 4 weeks showed a markedly longer time to exhaustion with lower blood lactate and free fatty acids levels and an improved oxidative status with decreased gastrocnemius muscle lipid peroxidation and increased activities of antioxidant enzymes (like CAT and SOD). The improvement of physical endurance and oxidative stress was associated with the regulation of mitochondrial biogenesis and function [50]. Furthermore, an *in vivo* study demonstrated that short-term administration of resveratrol significantly improved mitochondria function and alleviated oxidative stress-induced damage in postovulatory aging oocytes of middle-aged mice. Resveratrol treatment also slowed down the aging-induced oocyte deterioration, upregulated the expression of the antiaging molecule Sirt1, decreased the level of ROS, and prevented apoptosis, showing a multifactor effect on aging [95]. Another *in vivo* study showed that mtDNA integrity, mtDNA copy number, mitochondrial fusion regulators, mitophagy, and the expression of antioxidant-related genes were all decreased in zebrafish retinas upon aging, whereas Akt/mTOR activity and inflammation were increased. Resveratrol treatment could improve mitochondrial quality and function, as well as the downregulated Akt/mTOR pathway in zebrafish retinas to reverse the age-dependent changes [17].

4.4. The Regulation of Apoptosis. As a consequence of intracellular or extracellular damage, apoptosis is activated as an adaptive response for the maintenance of homeostasis. On the one hand, apoptosis contributed to the disappearance of nonfunctional and damaged cells, such as cancer cells. On the other hand, its dysregulation played an essential role in the development of age-related pathologies. For example, excessive neuronal apoptosis promoted the development of neurodegenerative diseases [96, 97]. Many studies showed that resveratrol could regulate apoptosis to protect against aging and age-related diseases. In an *in vivo* study, impairment of learning and memory ability in aged rats was

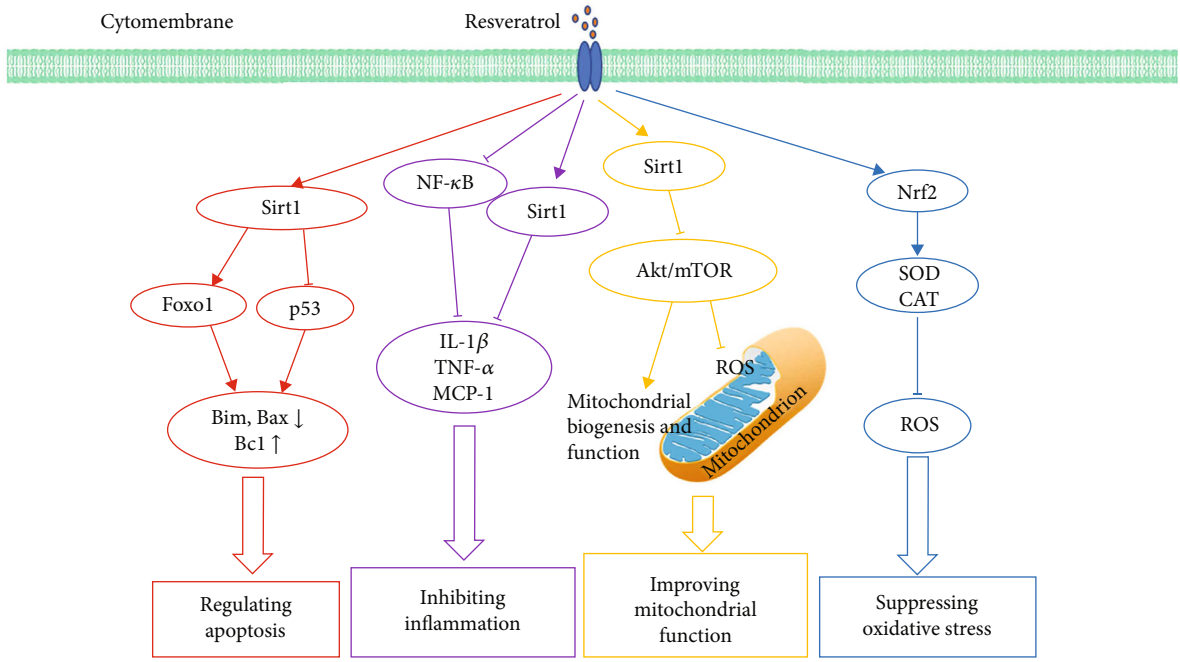

Figure 2: The mechanisms of resveratrol against aging. Resveratrol could stimulate the activity of nuclear factor erythroid-2 related factor 2 (Nrf2) and promote the activities of antioxidant enzymes, like superoxide dismutase (SOD) and catalase (CAT) to inhibit the production of reactive oxygen species (ROS), thus suppressing oxidative stress. Resveratrol could activate antiaging factor sirtuin1 (Sirt1) and downregulate the Akt/mTOR pathway to inhibit ROS in mitochondria and increase mitochondrial biogenesis and function, which could improve mitochondrial function. Resveratrol could promote the activities of nuclear-factor kappa B (NF-κB) and Sirt1 to decrease the levels of inflammatory markers, like interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) to inhibit inflammation. Resveratrol could upregulate Sirt1, subsequently promote forkhead box protein O1 (Foxo1), and inhibit p53, thereby modulating the levels of apoptotic proteins Bim and Bax and antiapoptotic protein Bcl, which could regulate apoptosis.

induced by sevoflurane and nitrous oxide, accompanied with neuronal apoptosis, but pretreatment with resveratrol modified the performance of learning and memory and suppressed neuronal apoptosis by upregulating the expression of Sirt1 in aged rats [67]. Moreover, aged senescence-accelerated mice showed the decreased activities of antiapoptosis and antioxidants as well as mRNA expressions of Sirt1 with the aging process and the increased levels of inflammatory response and NF-κB protein expression. Resveratrol administration could improve apoptotic, proinflammatory, and prooxidant statuses and increase Sirt1 mRNA expression, as well as decrease NF-κB expression, thus exerting an antiaging effect [16]. Additionally, a study compared the antiaging effects of resveratrol and caloric restriction. The results showed that both resveratrol and caloric restriction displayed antiaging activities by inhibiting senescence and apoptosis and recovering cognitive impairment and oxidative damage. The 10 μM resveratrol *in vitro* and the high-dose group (100 mg/kg body weight) *in vivo* showed more powerful effects on antiaging and stimulating the Sirt1 level than caloric restriction [98]. In a study, Sprague-Dawley rats were randomly distributed to the sham control group, aging model group, and aging rats with different treatment groups (resveratrol, soy isoflavones, resveratrol combined with soy isoflavones, and estrogen replacement therapy). The results showed that the increased apoptotic index, elevated mitochondrial swelling and vacuolation, accelerated oxidative stress, and decreased mitochondrial integrity were observed in the aging model group. All of the treatment groups could markedly decrease the apoptotic index, improve mitochondrial function, and reduce oxidative stress. The combination of resveratrol and soy isoflavones exerted a stronger effect than that in the alone administration [99]. In another study, aging significantly impaired exercise capacity and voluntary motor behavior and elevated the contents of p53, proapoptotic protein, and apoptotic DNA fragmentation in aged rats, while long-term resveratrol treatment could improve physiological performance and increase the expression of the antiapoptotic protein in aged rats via the activation of Sirt1 deacetylase activity [100].

4.5. The Modulation of Gut Microbiota. In recent years, the effects of gut microbiota on health have attracted increasing attention [101]. Accumulating studies have shown that natural products could play an important role in modulating the homeostasis of gut microbiota, thus protecting against many diseases [102–105]. As shown in some studies, resveratrol treatment markedly regulated the composition and metabolic function of gut microbiota in high-fat diet-induced obese mice, exerting an antiobesity ability [106–108]. Some studies also showed that resveratrol could protect against diabetic nephropathy, hepatic steatosis, and hypertension through the regulation of gut microbiota [109–111]. However, there is little evidence about the effect of resveratrol on antiaging via targeting with gut microbiota; hence, it is of great significance to investigate whether resveratrol could

stave off the process of aging via an intestinal flora regulatory way, which represents a novel target for interventions.

In conclusion, resveratrol exerted an antiaging action through the suppression of oxidative stress, inhibition of inflammatory response, modulation of mitochondrial function, and regulation of apoptosis. Considering the importance of gut microbiota in the maintenance of health, the antiaging effect of resveratrol targeting the regulation of gut microbiota should be studied in the future.

5. Clinical Trials

Several clinical trials studied the antiaging effect of resveratrol. For example, 200 mg/d resveratrol intake enhanced memory performance accompanied with improved glucose metabolism and hippocampal functional connectivity in 23 healthy overweight older adults, exerting beneficial effects on brain aging [118]. In addition, the effect of a cream containing trans-resveratrol on human volunteers was evaluated by measuring different skin parameters, and all patients showed a visible improvement of clinical conditions with a significant decrease of aging signs [119]. However, a clinical trial showed that resveratrol in combination with exercise could abolish the positive effects of exercise on cardiovascular health in aged men, and the negative effects of resveratrol supplementation on training-induced benefits were partly related to the antioxidant properties of this compound [120]. In the future, more clinical trials are necessary to confirm the effectiveness of resveratrol and observe its possible risks, such as nephrotoxicity [80].

6. Conclusions

Many studies show that resveratrol has tremendous potential in the prevention and treatment of aging. Accumulating studies revealed that resveratrol could markedly extend lifespan and protect against a series of age-related diseases, such as neurodegenerative diseases, cardiovascular diseases, sarcopenia, cancers, infertility, and osteoporosis. Some clinical trials also showed the antiaging effect of resveratrol. Several mechanisms contributed to the antiaging effect of resveratrol, mainly including the suppression of oxidative stress, the inhibition of inflammation, the regulation of mitochondrial function, and the regulation of apoptosis. In the future, studies about whether resveratrol could protect against aging through the modulation of gut microbiota should be carried out. In addition, the synergetic effects of resveratrol with other natural compounds should be studied to guide future product development and reduce the cost of new formulations. Moreover, effects of some fruits and vegetables on lifespan should be evaluated, such as grape, peanut, blueberry, cucumber, tomato, red cabbage, and spinach, because they have relatively high contents of resveratrol. Furthermore, more clinical trials are necessary to confirm the effectiveness of resveratrol, and attention should also be paid to the safe dosage and duration of resveratrol consumption since chronic resveratrol treatment might result in nephrotoxicity.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Conceptualization was handled by D.-D.Z., R.-Y.G., and H.-B.L. The original draft preparation was handled by D.-D.Z., M.L., S.-Y.H., A.S., and A.S. The review and editing were handled by R.-Y.G. and H.-B.L. Supervision was handled by R.-Y.G. and H.-B.L. Funding acquisition was handled by R.-Y.G. and H.-B.L.

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Review Article

Targeting Mitochondrial Biogenesis with Polyphenol Compounds

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Appropriate mitochondrial physiology is an essential for health and survival. Cells have developed unique mechanisms to adapt to stress circumstances and changes in metabolic demands, by meditating mitochondrial function and number. In this context, sufficient mitochondrial biogenesis is necessary for efficient cell function and haemostasis, which is dependent on the regulation of ATP generation and maintenance of mitochondrial DNA (mtDNA). These procedures play a primary role in the processes of inflammation, aging, cancer, metabolic diseases, and neurodegeneration. Polyphenols have been considered as the main components of plants, fruits, and natural extracts with proven therapeutic effects during the time. These components regulate the intracellular pathways of mitochondrial biogenesis. Therefore, the current review is aimed at representing an updated review which determines the effects of different natural polyphenol compounds from various plant kingdoms on modulating signaling pathways of mitochondrial biogenesis that could be a promising alternative for the treatment of several disorders.

1. Introduction

Healthy physiology of mitochondria is essential for human health, and their dysfunction is connected with the pathogenesis of different diseases such as type 2 diabetes, cancer, neurodegenerative, and cardiovascular diseases [1–5]. For this reason, the removal of damaged mitochondria (mitophagy) and the generation of new ones are required to preserve the cellular and mitochondrial homeostasis.

Emerging evidence indicates that the biogenesis and clearance of mitochondria are tightly controlled by a multistep process involving fusion, fission, and mitophagy events [6]. The biogenesis of mitochondria is extremely plastic in response to environmental stimuli, developmental signals, and cellular energy demand [7] and requires mitochondrial DNA (mtDNA) replication, synchronous production of mitochondrial and nuclear proteins, and localization of nuclear-encoded proteins and phospholipids in different subcompartments of mitochondria [8, 9].

Recent data highlight the impact of the peroxisome proliferator-activated receptor-γ coactivator- (PGC-) 1α, a master controller of mitochondrial biogenesis. PGC-1α, through activation of different transcription factors, mainly Nrf-1 (nuclear respiratory factor 1) and Nrf-2 triggering mitochondrial transcription factor A (TFAM), promotes mitochondrial biogenesis [8, 10]. Nrf-2 also induces mtDNA replication and transcription. The activation of the PGC-1α/Nrf/TFAM pathway results in mtDNA and protein synthesis and generation of new mitochondria [11, 12]. Different key factors including ROS production, nitric oxide,

calcineurin, AMPK (AMP-activated protein kinase), energy sensor, and sirtuins can induce PGC-1α and mitochondrial biogenesis [8, 13].

Given the essential role of mitochondrial impairment in the pathogenesis of different diseases, the knowledge of the molecular mechanisms, signals, and actors regulating the biogenesis and function of mitochondria is thus of high prominence which can be used to develop novel mitochondrial-based therapies. Numerous natural extracts such as polyphenols, flavonoids, and saponins are reported to antioxidant and anti-inflammatory activities [14, 15] and stimulate the biogenesis of mitochondria. In this review, we will highlight an updated report of implying the effects of polyphenol compounds on mitochondrial biogenesis and function by modulating intracellular signaling pathways in different models focusing on biogenesis processes as the main mechanism involved in the modulation of energy expenditure rather than their well-known antioxidant properties.

2. Mitochondrial Biogenesis

The membrane-enclosed organelles recognized as "power generators" of eukaryotic cells are called mitochondria. Moreover, various metabolic pathways (Krebs cycle, OXPHOS (oxidative phosphorylation), pyruvate oxidation, fatty acid beta-oxidation, etc.) are carried out through their molecular cascades [5]. Mitochondria's advantages are not only for their contribution to cell metabolism but also are required to regulate energy-producing pathways. Their commitment to steering stress responses and cell death has gained significant attention lately [16–18]. Additionally, inside the cells, mitochondria serve as the ROS's prime source [19]. Mitochondria's biomedical growing concerns are due to their diverse executions covering multiple genetic diseases (through inheriting mitochondrial DNA (mtDNA) mutations), contributions to neurodegeneration, aging, inflammation, and carcinogenesis [20].

The biogenesis of organelles is connected with their inheritance through cellular division. Mitochondrial biogenesis is linked with the proliferation and division of prior organelles, a process which is coordinated by cell cycle cascades [21]. However, different stimuli such as exercise, oxidative stimulus, elevated energy demand, hormones, and physiological development as well as certain diseases also trigger mitochondrial biogenesis [22]. The individual mitochondrial mass increases during mitochondrial biogenesis. Mitochondrial biogenesis has recently been proposed as a potential target of intervention in different diseases which have not an effective cure up to date. This is due to the crucial roles of mitochondria in numerous cellular events [23]. Thus, pharmacological manipulations are plausible in order to induce mitochondrial biogenesis.

Besides proceeding mitochondrial biogenesis among healthy cells, the importance of mitochondrial biogenesis and oxidative phosphorylation in damaged cells has been reported [24]. Stimulation of PGC-1α (through deacetylation or phosphorylation) enrols the pathway, which is later followed by stimulation of nuclear transcription series factors such as estrogen-related receptor-α (ERR-α), NRF-1, and NRF-2, and succeeding with a rise in TFAM's expression ultimately leads to mtDNA transcription and replication [22, 25, 26]. The translation of mtDNA encoded genes is facilitated by particular translation factors, including the initiation factor 2 (mtIF2) and mtIF3, the elongation factors Tu (mtEFTu), mtEFT, and mtEFG1, and translational release factor 1-like (mtRF1L) and recycling factors 1 (mtRRF1) and mtRRF2. Besides, the binding of mitochondrial RNA (mRNA) to the translational activator of cytochrome c oxidase 1, aka TACO1, controls the mitochondrial proteins' level.

Triggering of silent information regulator-1 (SIRT1) initiates transcription of nuclear and mitochondrial genes encoding proteins mediated by PGC-1α, which occurs during the proliferation of mitochondria. The amplified production of proteins contributed to the oxidation of fatty acid, oxidative phosphorylation, and tricarboxylic acid cycle in handled by SIRT3. Additionally, in parallel to that, AMPK and PGC-1α are affected collaterally. C1q/tumor necrosis factor-related protein-3 contributes to the AMPK/PGC-1α pathway that advances biogenesis. Additionally, the phosphorylation and activation of histone acetyltransferase 1 by AMPK lead to a far less constricted chromatin-DNA structure that ultimately activates transcription. Besides, phosphorylation of DNA methyltransferase 1 as an epigenetic factor, aka DNMT1 by AMPK, restricts the access of transcription factors to promoters. [27]. Figure 1 represents the signaling pathways involved in mitochondrial biogenesis.

Synthesis, importing, and assembling of nDNA-encoded mitochondrial proteins are important orchestrated steps. Cytosolic preproteins are the source of such mitochondrial proteins and compromise targeting signals at amino-terminals. Translocase TIM23 transmits the messages of these preproteins to the mitochondrial matrix. Within there, they are assembled and assorted to the matrix of the inner mitochondrial membrane (IMM). This pathway's energy supply is catered through oxidative phosphorylation (ATP) and the mitochondrial membrane potential [28, 29]. Conducted studies of outer mitochondrial membrane (OMM)'s biogenesis have been done on yeast, Saccharomyces cerevisiae, and other unicellular organisms. The OMM plays an essential role in communication with other intracellular organelles and is crucial for fission, fusion, and other processes contributing to mitochondrial dynamic changes. [30]. The escalation of mtDNA:nDNA percentage, the expression level of the mitochondrial gene, and mtDNA quantity indicate the level of mitochondrial biogenesis [31]. Although cancerous cells have declined mitochondria number, some extracted data present increments in the expression of TFAM, PGC-1α, and NRF1 genes [32–34]. However, a late report raises the uncertainty of the TFAM level's indicated as a biogenesis marker, due to its failure in accommodation to mtDNA quantity and the expression of mtDNA-encoded polypeptides [35]. The consequences of mitochondrial biogenesis comprise of boosting of mitochondrial-associated dysfunction, declining of pathologic oxidative stress, and oxidative phosphorylation efficiency. [25]. mtDNA synthesis and mitochondrial membrane

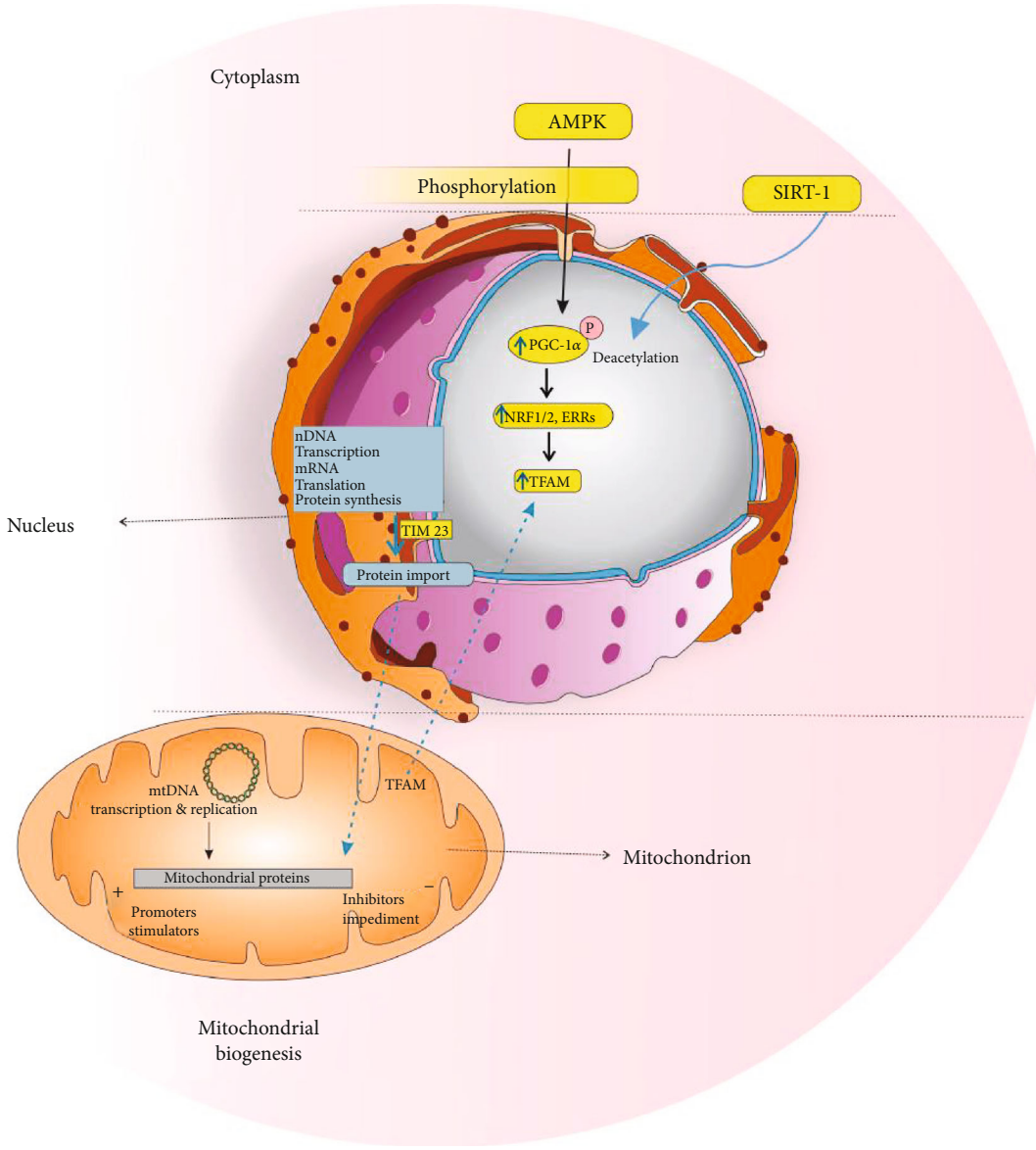

Figure 1: The role of different signaling pathways in mitochondrial biogenesis.

phospholipids are encompassed with certain strategies for measuring mitochondrial biogenesis. Notably, variation in mitochondria's number do not represent a definite sign of biogenesis (because number changes are not limited to synthesis) [36]. Moreover, remarkably, mitochondrial biogenesis could lead to detrimental effects, including reactions of unfolded protein's silencing to the endoplasmic reticulum and misfolded proteins' organelle importation [37].

3. Natural Agents Modifying Mitochondrial Biogenesis

Within the physiological circumstances, cells react to diminished energy by affecting transcription factors through up-/downregulation, which contributes to mitochondrial biogenesis enhancement and/or restraining (Table 1). Pathological disturbances to mitochondrial biogenesis lead to two conditions: (a) hampering, this requires stimulating of the process, and (b) abnormal intensifying. Enhancing mitochondrial biogenesis is up to transcription factors' activation that affects stimulating local translation of mitochondrial proteins and mitochondrial genes. Polyphenols have been considered as the main natural compounds interfering with mitochondrial biogenesis. Thus, the present review focuses on the role of these components in the regulation of mitochondrial biogenesis.

4. Polyphenols

Activating mitochondrial biogenesis in vitro has been indicated by various polyphenols. Hence, they are under inspection as potential inducers through initiating PGC-1α activation by deacetylation as a mediator [10].

Table 1: Summary of effects of the different natural agents on mitochondrial biogenesis.

Substance	Model (cell or animals)	Result	References
Resveratrol	C57BL/6NIA mice	Resveratrol increased insulin sensitivity, AMP-activated protein kinase (AMPK), and peroxisome proliferator-activated receptor coactivator 1α (PGC-1α) receptor activity and improved mitochondrial number. Moreover, resveratrol improved motor function and reduced insulin-like growth factor-1 (IGF-1). These changes led to the improvement of health and survival of high-calorie diet mice. Therefore, resveratrol could be considered as a promising substance for the treatment of obesity-related disorders and diseases of aging.	[38]
Resveratrol	C57Bl/6J mice	The administration of resveratrol protected mice against diet-induced obesity and insulin resistance by improving mitochondrial function and activating SIRT1 and PGC-1α.	[39]
Resveratrol	Endothelial cells	The mitochondrial biogenesis of endothelial cells was increased in presence of resveratrol by activating the PGC-1α/SIRT1 cascade.	[40]
Resveratrol	Transgenic rats	The administration of resveratrol for transgenic rats enhanced mitochondrial biogenesis and ameliorated Ang II-induced cardiac remodeling.	[41]
Resveratrol	C57BL/6J mice	Resveratrol activated AMPK and increased NAD+ in a SIRT1 dependent manner. This procedure led to the improvement of mitochondrial function.	[46]
Resveratrol	SIRT1 KO mice	Moderate doses of resveratrol improved mitochondrial function by SIRT1, which is required for AMPK activation.	[50]
Resveratrol	HepG2 cells	Resveratrol banded to the subunits of nicotinamide adenine dinucleotide (NADH) dehydrogenase to activate the mitochondrial complex I.	[51]
Resveratrol	C57BL/6J mice	Brain mitochondria of young mice were affected by resveratrol. This substance simulated complex I activity while the respiration rate was not improved.	[52]
Resveratrol	Rat	The results demonstrated that resveratrol inhibited the function of complexes I to III of mitochondrial respiratory chain competing with coenzyme Q.	[53]
Resveratrol	C2C12 myoblasts, C3 cancer cells, and mouse embryonic fibroblasts	Resveratrol treatment improved cellular metabolism and growth and mitochondrial fusion. Moreover, cellular respiratory capacity and the activity of complexes I to IV have shown a surge after resveratrol treatment.	[54]
Resveratrol	Rat	Resveratrol had regulatory effects on the synthesis of ATP and complex V activity.	[55]
Resveratrol	Sprague-Dawley rats	Resveratrol demonstrated inhibitory effects on the enzymatic activity of both rat brain and liver F0F1-ATPase/ATP synthase.	[56]
Resveratrol	Human fibroblasts	The resveratrol treatment excessed primary oxygen intake rates and ATP formation on human fibroblasts derived from the skin of patients.	[57]
Resveratrol	Human skin fibroblasts	Resveratrol improved mitochondrial biogenesis using SIRT1- and AMPK-independent pathways. This improvement involved the estrogen receptor (ER) and estrogen-related receptor alpha (ERRα) signaling pathway.	[58]
Resveratrol	Complex I-deficient human fibroblasts	Resveratrol treatment reduced oxidative stress in mitochondrial complex I deficiency using SIRT3. The growth in SIRT3 creativity led to dramatic decreases in ROS level and enhancement of SOD2.	[60]
Resveratrol	Bhas 42 cells	Resveratrol enhanced mitochondrial content by protecting against benzo [a] pyrene-induced bioenergetic dysfunction and ROS generation in neoplastic transformation model.	[61]
Quercetin	ICR mice	The administration of quercetin elevated mitochondrial biogenesis and exercise tolerance in the brain and muscle.	[63]

Table 1: Continued.

Substance	Model (cell or animals)	Result	References
Quercetin	Young adult male	The administration of quercetin increases mtDNA numbers and improved mitochondrial biogenesis that led to enhanced physical performance.	[64]
Quercetin	HepG2 cells	Administration of quercetin increased the mitochondrial DNA content and biogenesis by activating HO-1 in HepG2 cells.	[65]
Quercetin	Obese mice	Administration of quercetin had protective effects on traumatic brain injury of obese mice by regulation of the NRF-2/HO-1/PGC-1α signaling pathway.	[67]
Quercetin	Rat	After induction of hypobaric hypoxia in the rat hippocampus, the administration of quercetin led to increased levels of mitochondrial DNA, TFAM, PGC-1α, and NRF-1.	[68]
Quercetin	Rodent	The functions of complexes II, IV, and V were elected after the administration of quercetin. Moreover, this substance improved ATP levels, which could affect the activity of OXPHOS. The provocation of mitochondrial biogenesis in presence of quercetin was occurred due to the activation of the PGC-1α/NRF-1-NRF-2-TFAM cascade.	[69]
Quercetin	Male C57BL/6 mice	Quercetin enhanced hepatic mitochondrial oxidative metabolism by inducing heme oxygenase-1 via the Nrf-2 pathway.	[70]
Quercetin	Neuronal cell	Quercetin reduced ischemic neuronal cell death by preserving mitochondrial spare respiratory capacity. Moreover, this substance completely blocked neuroprotection by oxide synthase.	[71]
Quercetin	Wistar rats	The combination of oral quercetin supplementation and exercise prevents brain mitochondrial biogenesis.	[75]
Quercetin	Male C57BL/6 mice	Exercise, but not quercetin, ameliorates inflammation, mitochondrial biogenesis, and lipid metabolism in skeletal muscle after strenuous exercise by high-fat diet mice.	[76]
Quercetin	C57BL/6J mice	Quercetin increased skeletal muscle mitochondrial number and function.	[77]
Quercetin	OA rat model	The administration of quercetin in OA rats reduced ROS levels and ameliorated mitochondrial damages which led to the preservation of the integrity of the extracellular matrix of joint cartilage. This procedure might involve the regulation of the AMPK/SIRT1 signaling pathway.	[79]
Hydroxytyrosol	Retinal pigment epithelial cells	As shown in the retinal pigment epithelial cells, hydroxytyrosol deacetylated through SIRT1 and activated PGC-1α, which promoted mitochondrial biogenesis.	[81]
Hydroxytyrosol	Rat	The administration of hydroxytyrosol regulated the expression of mitochondrial complexes I and II in skeletal muscle by the PGC-1α signaling pathway.	[82]
Hydroxytyrosol	Murine 3T3-L1 adipocytes	The administration of hydroxytyrosol improved protein expression and function of mitochondrial complexes I, II, III, and V.	[83]
Hydroxytyrosol	Human fibroblasts	Hydroxytyrosol increased the phosphorylation of PKA and CREB, which regulated the biogenesis of OXPHOS.	[84]
Hydroxytyrosol	Endothelial cells	Hydroxytyrosol stimulated PGC-1α expression, which led to NRF-1 and TFAM stimulation, the elevation of mitochondrial DNA content, and ATP synthesis.	[85]
Isoflavones (daidzein, genistein, and formononetin)	Rabbits' proximal renal tubular cells	Rabbit's proximal renal tubular cells in exposer to daidzein, genistein, and formononetin had shown elevated mitochondrial biogenesis through the PGC-1α/SIRT1 pathway.	[86]
Flavones (wogonin and baicalein)	Rats' L6 skeletal muscle cells	Antimycin A-induced mitochondrial dysfunction of rat L6 cells was ameliorated by Scutellaria baicalensis extracts.	[87]
Flavan-3-ol	Skin fibroblasts from Down's syndrome patients	Epigallocatechin-3-gallate prevents oxidative phosphorylation deficit and promotes mitochondrial biogenesis in human cells from subjects with Down's syndrome.	[88]

Table 1: Continued.

Substance	Model (cell or animals)	Result	References
Green tea's polyphenols	Rats	Green tea elevated mtDNA contact, mRNA, and proteins of TFAM, PGC-1α, and complex IV in mitochondria.	[89]
Epicatechin-rich cocoa	Patients with type 2 diabetes	The expressions of SIRT1 and PGC-1α were enhanced in T2D human patients after administration of epicatechin-rich cocoa. This enhancement led to the improvement of mitochondrial biogenesis in skeletal muscle.	[90]
Curcumin	Mice	The mitochondrial membrane potential (MMP) and ATP contents in the brain of fast-aging augmented senescence-8 mice were increased due to the enhancement of PGC-1α protein expression in presence of curcumin.	[92]
Curcumin	Mice	The administration of curcumin supplementation elevated the levels of PGC-1α, TFAM, ATP, and levels of mitochondrial respiratory complexes in the APO3-mutant mice's brain.	[93]
Yerba mate	Obese mice	C2C12 cells were showed increased mitochondrial respiratory capacity and DNA content in presence of yerba mate. Moreover, in the obese mice, this substance increased mtDNA levels in brown adipose tissue and skeletal muscle. These effects were related to the AMPK/SIRT1/PGC-1α-mediated cascade in presence of yerba mate.	[94]
Curcumin	Rat skeletal muscle	The increasing of cAMP levels, mtDNA amounts, SIRT1 expression, PGC-1α deacetylation, AMPK phosphorylation, and NAD^+/NADH ratio was observed due to the curcumin treatment on skeletal muscle.	[95]
Epigallocatechin-3-gallate (EGCG)	Obese mice	EGCG modulated the biogenesis of mitochondrial and brown adipose tissue thermogenesis through AMPK triggering in brown adipose tissue and stimulating the mitochondria DNA replication.	[96]
Epigallocatechin gallate (EGCG)	Hepa1-6 cells	The amounts of the cytochrome C, oxygen consumption, ATP synthesis, and NAD^+/NADH ratio were increased by modified EGCG derivatives in Hepa1-6 cells.	[98]
Procyanidins	Mice	The expression of the PGC-1α gene and copy number of DNA were increased after oral administration of apple procyanidin in OA models of mice.	[99]
Digitoflavone	PC12 cells	Mitochondrial biogenesis improved in presence of digitoflavone by regulation of AMPK and increasing of antioxidant capacity of cells.	[100]
Anthocyanins	3T3-L1 preadipocytes	Anthocyanin inhibited adipocyte differentiation through activation of the AMPK signaling pathway.	[101]
Cyanidin-3-glucoside	3T3-L1 preadipocytes	The intracellular levels of CAMP were signification increased in preadipocytes after Cy36 exposure.	[102]
Anthocyanins	3T3-L1 preadipocytes	The lipogenesis stage during adipocyte differentiation of 3T3-L1 preadipocytes was inhibited by anthocyanins. This substance regulated BAT's function through AKT and ERK signaling pathways.	[103]
Mulberry anthocyanins, cyanidin 3-glucoside, and cyanidin 3-rutinoside,	BAT-cMyc cell	Mulberry anthocyanins, cyanidin 3-glucoside, and cyanidin 3-rutinoside increase the number of mitochondria during brown adipogenesis.	[104]
Mulberry and mulberry wine	$C_3H_{10}T_{1/2}$ mesenchymal stem cell	The number and function of mitochondria were increased during brown adipogenesis by exposure to the mulberry and mulberry wine.	[105]
Rutin	Mice	The administration of rutin decreased the blood levels of lactic acid in the forced swimming mouse model. Moreover, in these animals, the levels of malondialdehyde (MDA) were decreased in the muscle and brain. In these tissues, regulation of SOD and GPx increased PGC-1α and sirtuin 1 (SIRT1). The antioxidative effects of this flavonoid were also observed in the brain of mice by regulation of TPI, GDI, and CB1.	[107]

Table 1: Continued.

Substance	Model (cell or animals)	Result	References
Glycyrrhizic acid	Mice	Glycyrrhizic acid (GA) had neuroprotective effects and increased memory and antioxidant-related enzymes.	[108]
Glycyrrhizic acid	PC12 cells	Mitochondrial function and biogenesis were enhanced against aluminum toxicity of PC12 cells by glycyrrhizic acid treatment.	[109]
Glycyrrhizic acid	Renal tubular epithelial cell	The high glucose-related renal tubular epithelial cell injury was ameliorated by glycyrrhizic acid treatment.	[110]
Glycyrrhizic acid	Human coronary artery endothelial cell	Mitochondria regulation due to the glycyrrhizic acid protective effects ameliorated hypoxia/reoxygenation-induced human coronary artery endothelial cell damage.	[111]
Citrus tangeretin	Kunming mice and C2C12 myoblasts	Mitochondrial biogenesis in skeletal muscle was improved by activation of the AMPK-PGC1-α pathway in presence of citrus.	[112]
Cyanidin-3-glucoside	Human hepatocyte cell	The treatment of hepatocyte cell line by cyanidin-3-glucoside showed the upregulation of PGC-1α and SIRT 1 expression in a dose- and time-dependent manner. Moreover, the expression of NRF1 and TFAM was increased which led to improvement of function and biogenesis of mitochondria.	[113]
Isorhamnetin	3T3-L1 cells	The expression of mitochondrial genes, activating AMPK, and replicating of mtDNA in presence of isorhamnetin led to antiobesity effects through the regulation of mitochondrial biogenesis.	[114]
Nobiletin	C57BL/6 mice	The activation of SIRT-1/FOXO3a-medicated autophagy and mitochondrial biogenesis in presence of nobiletin ameliorated hepatic ischemia and reperfusion.	[115]
Eriocitrin	HepG2 cells	The oral administration of eriocitrin upregulated the levels of cytochrome c, oxidase subunit 4, TFAM, NRF1, and ATP synthase, which improved liver function in contribution to the mitochondrial biogenesis.	[120]
Polymethoxy flavonoids (black ginger extract)	C2C12 myoblasts	The black ginger's flavonoids including 5-hydroxy-7-methoxyflavone, 5-hydroxy-3,7,40-trimethoxyflavone, and 5,7-dimethoxyflavone enhanced ATP production on C2C12 myoblasts through regulation of PGC-1α and AMPK pathway.	[121]
Polymethoxylated flavone (sudachitin)	C57BL/6J mice	Sudachitin improved the SIRT 1 and PGC-1α, which caused the amelioration of metabolic disorders and dyslipidemia through improving mitochondrial biogenesis.	[122]
Chikusetsu saponin	Neuroblastoma cells	Chikusetsu saponin has plummeted the oxidative hazard in neuroblastoma cells that had been exposed to H2O2 through the elevation of PGC1a and SIRT-1 activation.	[123]
Platycodon grandiflorum	Brown adipose cells	The ethanolic extract of Platycodon grandiflorum upregulated the mitochondrial genes such as PGC1α, SIRT3, and Nrf, which has exhibited beneficial effects in brown adipose.	[124]
Amla	C2C12 myotubes	The protective effects of amla in myotubes subjected to tBHP have been observed in the context of mitochondrial function and biogenesis. The activation of AMPK and Nrf signaling in C2C12 myotubes cells has been attributed to these protective effects.	[125]

4.1. Phenolic Compounds

4.1.1. Resveratrol. At the point, resveratrol appears to stimulate PGC-1α activity through boosting deacetylation with SIRT1's mediation and enhancing transcriptional activity in the liver and muscles tissues of mice [38, 39]. Additionally, resveratrol promotes the motor function and survival rate of high fat diet-received mice [38, 39]. In a recently published paper, the effects of resveratrol on the activation of PGC-1α/SIRT1 cascade have shown an upward trend in endothelial cells *in vitro* [40]. Besides, similar effects were observed in the aorta of type 2 diabetes (T2D) animals and the cardiac tissues of human renin angiotensin genes expressing transgenic rats [41]. Despite these beneficial effects, the exact molecular mechanisms of resveratrol-induced mitochondrial biogenesis remain unclear. Resveratrol's effects on mediating mitochondrial biogenesis through initiating AMP protein kinase (AMPK) have been presented by various authors [42]. Indeed, evidence illuminating the blockade of oxidative phosphorylation (OXPHOS) complex III, phosphodiesterase, or ATP synthase by resveratrol is mainly contributed to the activation of AMPK [43–46]. Moreover, extending the

levels of intracellular NAD+ due to triggering of SIRT1 as a collateral outcome of resveratrol's contribution to AMPK has been suggested [47]. Other researches have proposed that resveratrol activates SIRT1, which in turn terminates in liver kinase B1 deacetylation and AMPK activation accompanied by enhancement (in vivo and vitro) of mitochondrial function [47–50]. Besides, the direct involvement of resveratrol in OXPHOS system has been detected. Resveratrol influences the activity of mitochondrial complex I via binding to the subunits of nicotinamide adenine dinucleotide (NADH) dehydrogenase, which contributes to an antagonistic characteristic on OXPHOS, in which low dose resveratrol stimulates complex I activity and high dose results in its prohibition in liver cells [51]. Resveratrol substantially enhances the function of complex I in young mice, while old animals are not influenced [52]. Throughout another experiment, resveratrol was shown to inhibit the function of complex III through competing with coenzyme Q *in vitro* [53]. Conversely, cellular respiratory capacity and the activity of complexes I to IV have shown a surge in a recent *in vitro* study subsequent to resveratrol treatment [54]. It has been revealed that resveratrol shows regulatory effects on the synthesis of ATP and complex V, while in lower doses stimulates the complex V activity [55] and derives an inhibiting role on the generation of ATP [56]. Recently, the role of resveratrol in the maintenance of cellular mitochondrial DNA replication was assessed. Among human fibroblasts derived from (skin of) patients carrying homoplasmic mutations of the mitochondria-encoded ATP Synthase Membrane Subunit 6 (MT-ATP6), mitochondria-encoded TRNA Leucine 1 (UUA/G) (MT-TL1), and mitochondrial-encoded TRNA Lysine (MT-TK) gene, resveratrol excessed primary oxygen intake rates and ATP formation [57]. Besides, resveratrol provokes fusion in mitochondria, leading to extremely expanded mitochondrial network divisions. Additionally, in patients suffering from Complex I and IV inadequacy, resveratrol has demonstrated growth in fibroblasts' biogenesis of mitochondria [58]. Correspondingly, in isolated cells from patients suffering from complex I inadequacy consuming resveratrol, the ratios of lactate to pyruvate became normal. Additionally, it regained the ratios of NADH to NAD+. Serious lactic acidosis develops among patients with a deficiency of OXPHOS [59]. This reassures the previous finding [44], and resveratrol consumption can have advantageous gains in such conditions. Resveratrol treating was promising among fibroblasts of patients suffering from complex inadequacy. The authors discovered a considerable ROS level plunge and increasing in SOD2 mediated by growth in SIRT3 activity [60]. Therefore, current evidence indicates the beneficial effects of resveratrol in OXPHOS-related diseases.

The effects of resveratrol on mitochondrial biogenesis has been investigated and compared with the flavonoids quercetin, catechin, berberine (a quaternary alkaloid and O-methyl polyphenol impacting mitochondria), cyanidin (various forms of 3′,4′-dihydroxyflavonoids), and cyanidin-3-glucoside (an intactly absorbed representative glycoside). After exposure (46% after 24 hours and 30% after 72 hours) to benzo[a]pyrene (B[a]P), the membrane potential of mitochondria (MMP) and cellular ATP was declined, and production of cellular ROS and superoxide in mitochondria was elevated. Reduced mitochondrial content could be preserved by polyphenol content after 72 h by 75%. The 24 h mRNA measurements of the activities of SIRT1 and other related proteins in mitochondria proposed interference with the biogenesis of mitochondria by the impact of this polyphenols. Furthermore, combined treatment with polyphenols mitigated mitochondrial deficiencies due to benzo[a]pyrene (primarily resveratrol) and developed the production of ROS and mitochondrial superoxide. Remarkably, 24 hours polyphenol pretreatment severely suppressed further B[a]P-stimulated increases in the production of ROS and mitochondrial superoxide, in which resveratrol is the most effective agent. Furthermore, the evidence promotes resveratrol as a chemoprophylaxis for enhancing mitochondrial biogenesis [61]. Resveratrol has also shown beneficial effects in several in vitro and in vivo models of neuropathies such as Alzheimer's, Down syndrome, cognition impairments, and aging via modulation of SIRT1-AMPK-PGC1-α pathway [62].

4.1.2. Quercetin. Quercetin as another polyphenol has been excessively investigated and has displayed significant efficacy in inducing mitochondrial biogenesis. In the brain and skeletal muscle cells of mice, reports demonstrate induction in cytochrome C content, triggering SIRT1 and PGC-1α, and the mitochondrial DNA [63]. A concurrent physical endurance development in quercetin-administered animals has been reported. Similar practical impact engaged in enhancing the PGC-1α and SIRT1 expression [63]. A study on young adult male individuals (untrained) assessed the impact of quercetin on the biogenesis of mitochondria along with endurance exercise. Investigations showed that quercetin improved mtDNA number besides the notable increase in physical performance [64]. In line, in vitro findings proposed that administration of quercetin elevated the mitochondrial DNA content [65] and in a dose-dependent manner affected the expression levels of TFAM, NRF-1, and PGC-1α. Moreover, the OXPHOS complex IV function was also enhanced with quercetin. Hence, the collected data from experimental investigations suggest that quercetin, by assisting PGC-1α/NRF-1 and TFAM signaling, stimulates biogenesis of mitochondria [66]. Furthermore, the involvement of quercetin in the upregulation of Nrf2 expression and cytochrome c affects the mitochondrial function in traumatic brain injury in male mice (Figure 2) [67]. Quercetin also has elevated mitochondrial DNA levels and contents of TFAM, PGC-1α, and NRF-1 in a dose-depending way in the rat hippocampus under hypobaric hypoxia [67, 68]. Additionally, quercetin improves complexes II, IV, and V functions, besides ATP level's growth, therefore influencing the activity of OXPHOS [68]. Similar consequences were derived from the striatum and hippocampus exposed to aluminum of the rodent. Here, through the PGC-1α/NRF-1-NRF-2-TFAM cascade, quercetin provokes the biogenesis of mitochondria and maintains the mitochondrial shape and number [69].

Rayamajhi et al. perceived that 15 μM quercetin provoked mtDNA quantity after 1 hour in a cultured human hepatocellular carcinoma cell line (HepG2 cells) [65]. Quercetin simultaneously leads to the upregulation of TFAM,

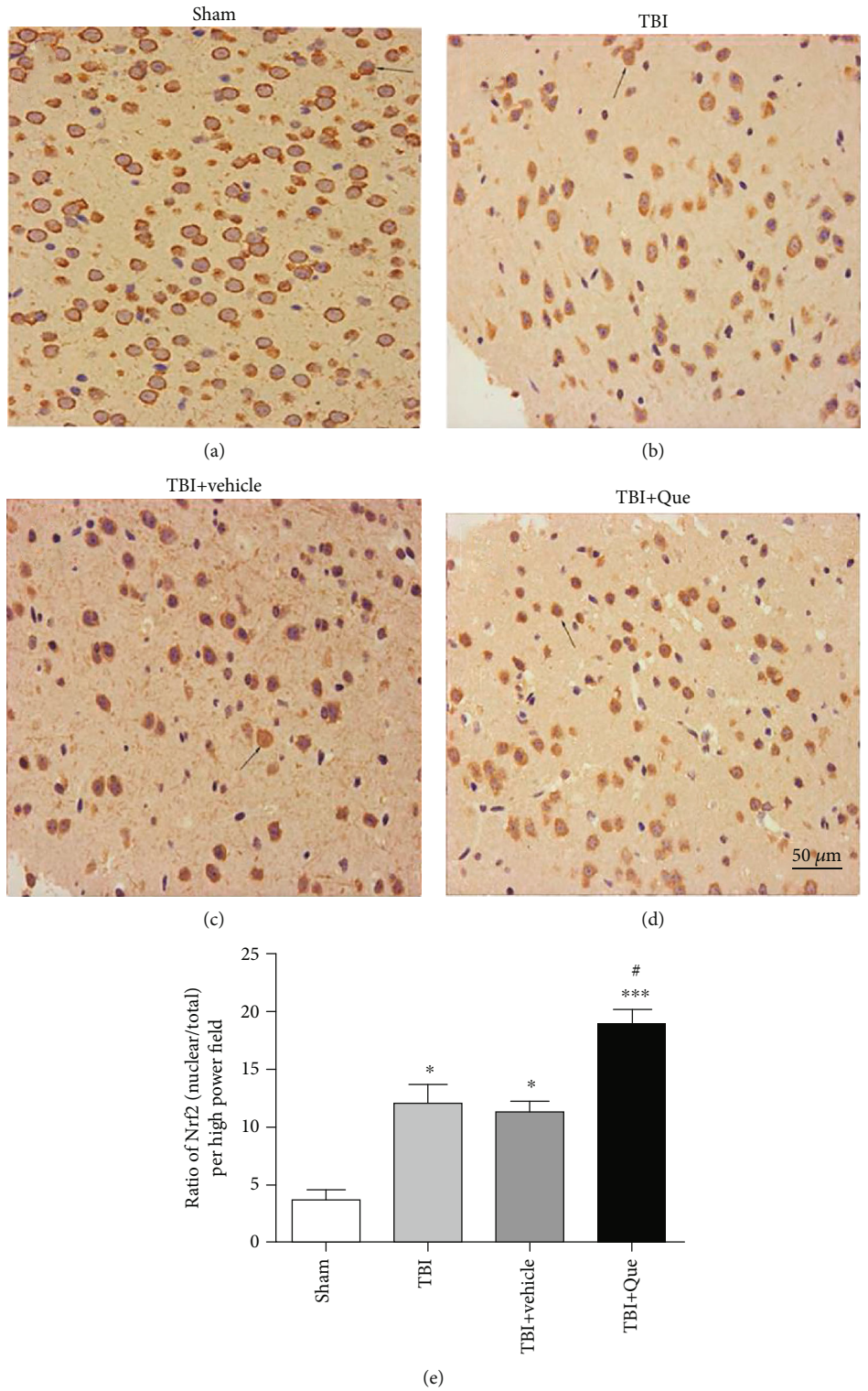

Figure 2: Immunohistochemistry and ratio of Nrf2 in mouse brain 24 h after traumatic brain injury in different groups ((a) sham, (b) traumatic brain injury, (c) traumatic brain injury with vehicle, (d) effect of quercetin on the concentration of Nrf2 in the nucleus, and (e) significant increase in the ratio of Nrf2 in the quercetin group in compared with the traumatic brain injury/vehicle group). Data represent the mean ± SEM ($n = 5$ per group). Scale (20 μm). Reproduced under the terms and conditions of the Creative Commons Attribution 4.0 International License. Copyright 2016, PLOS [67].

PGC-1α, and NRF-1 in a dose- and time-dependent manner. Furthermore, the authors indicated that quercetin surged the mETC constituent of complex IV in the respiratory chain in immunohistochemistry studies. The data derived from research concerning the process of quercetin eliciting impacts manifest amplified genes associated with the biogenesis of mitochondrial genes in a heme oxygenase-1- (HO-1-) dependent way. Quercetin augmented the mRNA of heme oxygenase-1 and immune-ocontent across 0.5–3 hours. Additionally, quercetin impacts on complex IV, NRF-1, mitochondrial DNA, TFAM, and PGC-1α were restrained by combined treatment with an HO-1 suppressor, tin protoporphyrin IX (SnPP). Moreover, exposure of quercetin-treated HepG2 cells to hemoglobin (CO scavenger) terminated in resembled outcomes, including dropped mtDNA and the quantity of complex IV, and plummeted expression of molecules attributed to mitochondrial biogenesis, when contrasted to HepG2 cells having SnPP treatment. These researchers further discovered (in vivo) stimulated mouse liver mitochondrial biogenesis through HO-1/CO/PGC-1α/NRF-1-TFAM-dependent fashion occur when quercetin (50 mg/kg) supplementations are added for 7 days. Correspondingly, Kim et al. [70] lately inspected the expression of PPARα and PGC-1α through an Nrf-2/HO-1-dependent process in primary hepatocytes of animals when receiving 10 μM quercetin for 24 hours. Hence, carbon monoxide uses especial suppressors to afflict the impact of quercetin on the biogenesis of mitochondria. Therefore, quercetin appears to have an essential effect on the enhancement of mitochondrial biogenesis. However, in stimulating mitochondrial biogenesis in primary cortical neurons facing diminished oxygen and glucose, quercetin (0.1 or 1 μM for 24 h) fails to show significant effects [71]. Furthermore, various quercetin concentrations could result in varying terminates considering mitochondrial biogenesis. Davis et al. [63] proposed that applying quercetin at concentrations 12.5 or 25 mg/kg for seven days by gavage sets off mitochondrial biogenesis in mouse muscle and brain by involving the upregulation of cytochrome c, PGC-1α, and NAD + -dependent protein deacetylase SIRT1. Additionally, examining 25 mg/kg quercetin (assumed as high dose) stimulates mtDNA levels in brain and muscle tissues. Potentially, modulation of PGC-1α by SIRT1 occurs through deacetylation of this coactivator function, amplifying and generating mitochondrial biogenesis by triggering engaged transcription factors in the mechanism [66]. Kim et al. [70] reported an alternative mechanism, whereby quercetin probably stimulated mitochondrial biogenesis. It presented upregulating of Nrf-2/HO-1/PGC-1α axis in the liver. The animals were held below a high-fat diet supplied with 0.05%–0.1% quercetin for nine weeks. These authors examined the administrating of 0.01% quercetin for nine weeks, which stimulated the complex IV expression in mice liver supplemented with a high-fat diet. Liu et al. [68] manifested that in rats hippocampus exposed to hypobaric hypoxia; a surge of DNA content, number, length, and mitochondrial area occurs when quercetin (50, 75, or 100 mg/kg/day) is administrated orally for seven days. Consuming 50–100 mg/kg daily quercetin leads to a rise of TFAM, NRF-1, SIRT1, and PGC-1α in the rat hippocampus experiencing hypobaric hypoxia in a dose-dependent fashion. On the other hand, the group taking 100 mg/kg quercetin demonstrated a function upregulation of complexes II, IV, and V. Besides, elevated ATP in the hypobaric hypoxia exposed rat hippocampus was observed. The authors further manifested that 50–100 mg/kg quercetin plunged the expression of dynamin-related protein 1 (Drp1), and 75 or 100 mg/kg quercetin plunged fission one homolog protein (Fis1)'s expression. Moreover, 100 mg/kg quercetin upregulated the expression of mitofusin 1 and 2 proteins (Mfn1 and 2). Drp1 and Fis1 are engaged in the regulation of mitochondrial fission [72, 73], and Mfn1 and Mfn2 modulated mitochondrial fusion. Elevated Fis1 results in autophagy [74]. Therefore, quercetin incompletely hampered cellular mitophagy challenging with hypoxia. Overall, similar data propose that quercetin plunges mitochondrial degradation through reducing fission and stimulating fusion. Lately, Sharma et al. [69] investigated that acquiring a daily quercetin regimen for 12 weeks by gavage stimulated the biogenesis of mitochondria in a PGC-1α/NRF-1-NRF-2-TFAM-related process in the aluminum-exposed corpus striatum and hippocampus of rats. Moreover, quercetin maintained the mitochondrial shape and elevated mitochondria quantitatively in the brains of aluminum exposed animals. Mitochondrial biogenesis stimulator functions of quercetin were analysed in some investigation models such as exercise in which 25 mg/kg is consumed by gavage during six weeks [75] and conducted high-fat diet studies in which 10 mg/kg is consumed by gavage during five days per week for eight weeks [76] in lab animals. Surprisingly, another investigation [77] discovered that a high-fat diet mouse acquired with 17 mg/kg quercetin for nine weeks generated elevated mitochondrial DNA in skeletal muscles (quadriceps and gastrocnemius) but declined expression of cytochrome c oxidase and NADH dehydrogenase (complex I) subunits. The authors did not analyse if the modulation impacted mitochondrial function as a whole, or these modulations caused the uncoupling of mitochondria and further ATP generation deterioration. Additionally, quercetin failed to spark the biogenesis of mitochondria in the human skeletal muscle [64]. Hence, the role of quercetin as a stimulator agent of mitochondrial biogenesis is vague. Further information demands to comprehend the task, as an example, carbon monoxide and SIRT1 in similar biological events. Moreover, more analyses assist in confirming more certainly whether there is an alternative role in signaling pathways terminating in the enhancement of mitochondrial biogenesis mediated by quercetin. For example, AMPK/protein kinase B, following quercetin's potential generation in declining cellular energy, possibly leads to an elevation in AMP and consequently triggers AMPK/SIRT/PGC-1α signaling pathway. Notably, the debates about quercetin and its initiating potential on the biogenesis of mitochondria require to be more illuminated. Therefore, similar biological parameters demonstrate substantial results on human health in disease treatment and prophylaxis in connection with mitochondrial deterioration [78]. In spite of the severity of osteoarthritis (OA), ongoing medical approaches focus on managing symptoms and easing pain, and currently, there is no medication with high

treatment efficacy. To discover the therapeutic impact of quercetin on osteoarthritis remission, an OA rat model was experimented through analysing the ECM (extracellular matrix) integrity, ROS quantity, and mitochondria activity. Quercetin could hinder ROS production and upregulate the expression of glutathione and glutathione peroxidase (GSH and GPx) in OA rats. Besides, it showed pivotal effects on oxygen utilization, mitochondrial ATP amount, stimulating mitochondrial membrane potential, and also elevating mitochondrial number. Additionally, quercetin could cease the interleukin-1β-induced condensed NO matrix metalloproteinase 3 and 13 (MMP-3 and -13). Thus, the potential therapeutic effect of quercetin on OA was possibly in connection with the AMPK/SIRT1 signaling pathway. In conclusion, quercetin promotes OA treatment by lowering the ROS quantity, conversing with the impairment of mitochondria, and maintaining the extracellular matrix of joint cartilage's integrity, which possibly engage in adjusting of AMPK/SIRT1 signaling pathway [79, 80].

4.1.3. Hydroxytyrosol. Hydroxytyrosol as a polyphenol that has strong ties with mitochondrial biogenesis appears in olives and extra virgin olive oils. Conducted *in vitro* studies manifest the potential of hydroxytyrosol in activating PGC-1α by deacetylation through SIRT1, which promotes the biogenesis of mitochondria within retinal pigment epithelial cells, ARPE-19 [81]. In the former group's later studies, it has been shown that rats receiving hydroxytyrosol regulate the expression of mitochondrial complexes I and II in skeletal muscle submitted to ergometric exercise and also their PGC-1α function. Furthermore, among the trained group, the potential growth of endurance exercise was also determined [82]. Furthermore, although between moderate exercise experimental groups, the enhancement of mitochondrial biogenesis was demonstrated, and extreme exercise reduced the PGC-1α. This phenomenon was completely reversed by administration of hydroxytyrosol. Another study manifested the consequences of this polyphenol in improving the function of mitochondria in murine 3T3-L1 adipocytes (Figure 3). It comprised an enhanced function and protein expression of mitochondrial complexes I, II, III, and V, including extended oxygen utilization and declined free fatty acid content [83].

Moreover, collected data from human fibroblasts (*in vitro*) presented the impacts of hydroxytyrosol on the PGC-1α cascade function. The stated response is related to surges in phosphorylation of PKA and CREB, engaging processes in coordinating OXPHOS [84]. Hydroxytyrosol, besides stimulating PGC-1α expression also within endothelial cells, presents NRF-1 and TFAM stimulation and increases mitochondrial DNA content and ATP synthesis [85].

4.1.4. Other Polyphenols. As well as hydroxytyrosol, resveratrol, and quercetin, there are other polyphenols with stimulating comparable outcomes on mitochondria. The activating of mitochondrial biogenesis through the PGC-1α/SIRT1 pathway was determined in rabbit's proximal renal tubular cells when exposed to isoflavones (daidzein, genistein, and formononetin) [86], flavones (wogonin and baicalein) in L6 skeletal muscle cells [87], and flavan-3-ol, in skin fibroblasts from Down's syndrome patients [88]. *In vivo* assessment of green tea's efficacy on the biogenesis of mitochondria in rats indicated a rise in the mtDNA content, including mRNA and proteins of TFAM, PGC-1α, and complex IV [89]. Administration of epicatechin-rich cocoa in an experiment of T2D human patients suffering from heart failure enhanced the expressions of SIRT1 and PGC-1α and consequently enhanced the biogenesis of mitochondria among skeletal muscle. Besides, it elevated mitochondrial complex I, mitochondrial complex V, and porin [90]. The positive efficacy of long-term acquired treatment of curcumin, a turmeric stemmed polyphenols, and in coordinating the biogenesis of mitochondria has been presented [15, 91]. Curcumin affects the brain of fast-aging augmented senescence-8 mice, develops the PGC-1α protein expression, and stimulates mitochondrial membrane potential (MMP) and ATP, along with maintaining the mitochondrial fusion process, pointing its preserving role in diseases resulting from mitochondrial impairment [92]. Following prior investigations, a later study presents that the administration of curcumin elevated the levels of mitochondrial respiratory complexes (exclusively complex IV), PGC-1α, and TFAM, which also increased ATP in the APO3-mutant mice's brain [93]. One of South America's most consumed plants is Yerba mate (Ilex paraguariensis). It has abundant bioactive composites as well as polyphenols. It has been shown that increment in mitochondrial spare respiratory capacity and mitochondrial DNA content was observed in cultured muscle C2C12 cells due to yerba mate consumption. In obese mice with high-fat diet, yerba mate enhances mtDNA levels as well as energy outflow in brown adipose tissue and skeletal muscle. Notably, the stated impacts had close bounded to bodyweight decline. Hence, yerba mate could relatively hinder diet-related obesity through escalating ATP consumption and induction of the biogenesis of mitochondria by AMPK/SIRT1/PGC-1α-mediated cascade [94]. The green tea's efficacy on the biogenesis of mitochondria was investigated *in vivo*. Epicatechin-rich cocoa administration throughout a study in T2D human patients suffering from heart failure expressed an increase in the SIRT1 and PGC-1α expression that promoted mitochondrial biogenesis in skeletal muscle. Furthermore, the amounts of mitochondrial complex I and mitochondrial complex V and porin have also escalated [90]. The favorable impacts of chronic administration of curcumin in mediating mitochondrial biogenesis have also been reported. Curcumin enhanced the PGC-1α protein expression in the brain of fast-aging accelerated senescence-8 mice and increased the MMP and ATP contents. Moreover, it replenished the mitochondrial fusion phenomenon, supporting the involvement of mitochondrial dysfunction in disease progression [92]. Another experiment indicated the role of curcumin supplementation in the elevation of TFAM and PGC-1α and respiratory chain complexes in vivo in brain tissues [93].

One study has covered the compound of citrate synthase (CS) function in rat skeletal muscle, curcumin therapy on the expression of OXPHOS subunits, AMPK, mtDNA copy number, SIRT1, PGC-1α, and endurance training (eTR).

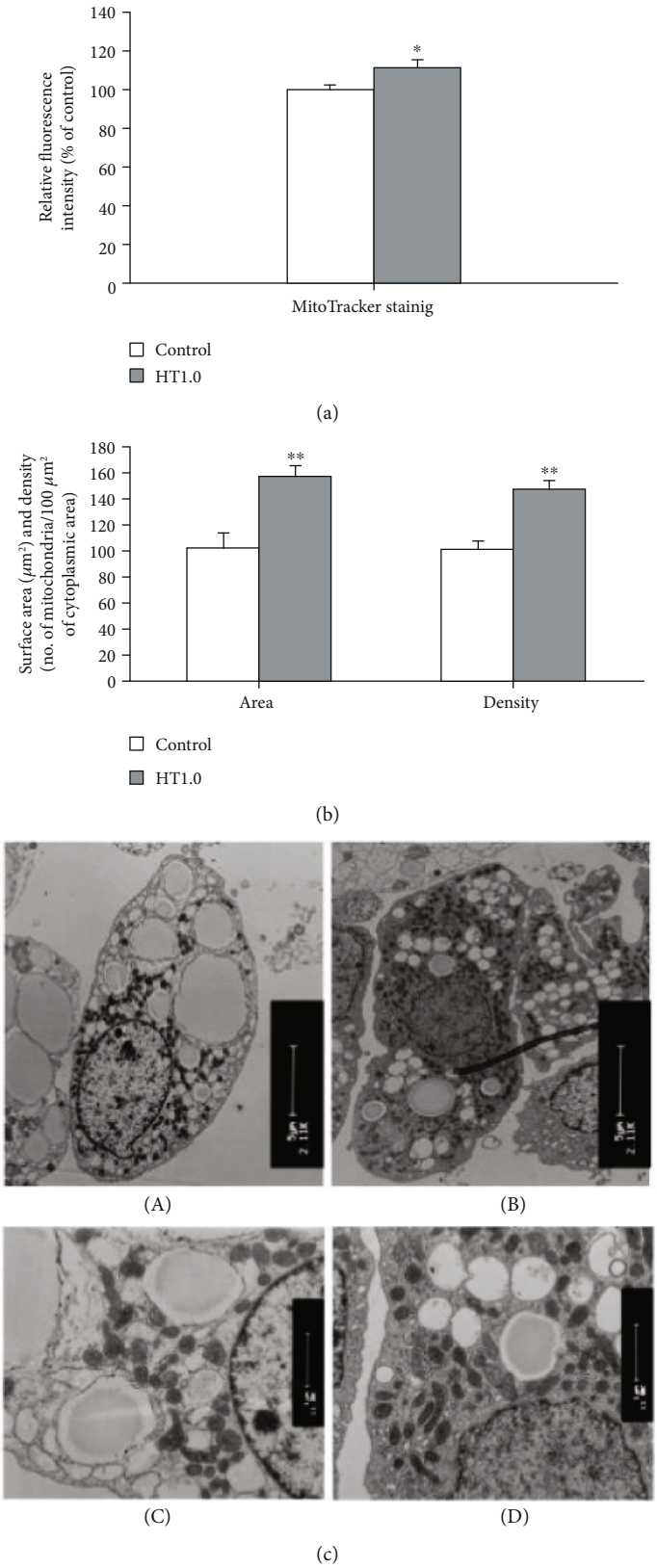

Figure 3: Alteration in ultrastructural and mass of adipocyte mitochondria induced by hydroxytyrosol treatments (1.0 μM for 48 h). (a) Upsurge in the fluorescence intensity in MitoTracker staining. (b) Significant change ($p < 0.01$) in mitochondrial surface area and density in morphometric analysis using electron microscopy. (c) Electron microscope comparison of mitochondrial profiles (A, B) (magnification ×2110) and (C, D) (magnification ×11,000). Reproduced with permission of Elsevier [83].

Before administering 50 mg/kg Bw/day as low doses of curcumin or 100 mg/kg Bw/day as a high dose of it intraperitoneally through injection in all animals diluting it in dimethyl sulfoxide is a prerequisite. This evaluates curcumin's effect alone and the incorporation effects of curcumin eTR. To determine the proteins' presence, Western blotting and immunoprecipitation were executed. The outcome signified that combined treatment of curcumin with eTR enhanced the expression of CS function and mtDNA copy quantity in the gastrocnemius and soleus muscles as well as elevated SIRT1 expression, PGC-1α deacetylation, AMPK phosphorylation, and the ratio of NAD+ to NADH [95]. Epigallocatechin-3-gallate (EGCG) as a mitochondria-aiming molecule stimulates the biogenesis of mitochondria through coordinating chief modulators of mitochondrial metabolism and prohibits mitochondrial dysfunction. Presumably, dietary EGCG declined liver lipid, plasma, and body weight. Moreover, in diet provoking obese mice improved fecal lipid excretion. Such EGCG contributions were moderately mediated through the gene regulation associated with biogenesis of mitochondria and thermogenesis. EGCG could have direct bonds on development of AMPK, triggered in brown adipose tissue and mitochondrial DNA replication. Therefore, EGCG could prohibit obesity (as a moderately linked factor with gene expression regulators of various genes throughout the biogenesis of mitochondria and thermogenesis), AMPK triggering in brown adipose tissue (BAT) of diet-induced obese mice, and stimulate mDNA replication. Stated exhibits propose that EGCG probably performs undeniable tasks in modulating the biogenesis of mitochondria and brown adipose tissue thermogenesis for obesity improvement [96].

Peracetylated (-)-epigallocatechin-3-gallate (AcEGCG) and its 4″-O-alkyl congeners catered by formerly exhibited mechanisms were assessed biologically. Notably, EGCG and AcEGCG had trivial efficacy in enhancing the biogenesis of mitochondria. EGCG is recognized to be a mitochondrion-targeting medicinal compound, mediating mitochondrial metabolism, including mitochondrial biogenesis [97]. However, AcEGCG congeners carrying an alkyl group at the 4″-O position alteration exhibited a considerable elevation in biological functions when set on their root composite. Among the congeners, 3f with methyl-branched carbonate chain at the 4″-O position presented the most stimulation on enhancing mitochondrial biogenesis. 3f treatment received Hepal-6 cells protested increasing 1.5 times in mitochondrial mass and 1.5 times in corresponding mitochondrial DNA content with nDNA. Besides, as a mitochondrial biogenesis stimulator, it elevated mitochondrial activity modulators' expression, comprising 4.2-fold in SIRT1, 1.6-fold in NRF-1, 4-fold in PGC-1α, 1.7-fold in NRF-2, 1.6-fold in mtTFA, 1.8-fold in ERRα, and 2.5-fold in p-AMPK. An examination's results in the accompany of 3f on oxidative phosphorylation of mitochondria exhibited that 3f enhanced the ratio of NAD+ to NADH, cytochrome c number, ATP synthesis, and oxygen utilization in Hepa1-6 cells by 2.2-, 1.4-, 1.5-, and 2.1-fold, accordingly. Altogether, outcomes proclaim and extent the structure-function association study for EGCG isolation to achieve new stimulators for the biogenesis of mitochondria [98].

A class of polyphenols known as procyanidins is attainable in apples that possess expressed efficacy on the circulatory system and skeletal organs. OA as a locomotive syndrome through a histology perspective is characterized by cartilage diminishment linked to deficiency of proteoglycan homeostasis in chondrocytes. Yet, there is still no effective therapy to preserve diminishing cartilage. The beneficial effects of apple polyphenols (procyanidins) or cartilage homeostasis have been examined. According to the assay's manifestations (in vitro), apple polyphenols enhance the function of mitochondrial dehydrogenases related to escalating mitochondrial DNA copy number and gene expression of PGC-1α. Stimulation of proteoglycan biosynthesis through primary chondrocyte upregulation of aggrecan occurs because of procyanidins found in apples. Notice, articular cartilage degradation in OA models in mice responded promising to oral treatment with apple procyanidins and was escalated through chondrocytes' mitochondrial impairment. According to their research, the authors proposed that apple procyanidins are reassuring OA development restrains through stimulating proteoglycan homeostasis and the biogenesis of mitochondria in chondrocytes [99].

4.2. Flavonoids. Phosphorylation of AMPK leads to increased levels of mitochondrial biogenesis factors that could be decreased by H2O2. Digitoflavone could have positive effects in this procedure by elevating mitochondrial biogenesis factors besides the regulation of AMPK [100]. *In vitro* studies showed that anthocyanins elevate the expression of mitochondrial TFAM, PGC-1α, and NRF-2. These increased levels could affect the phosphorylation of AMPK and mitochondrial biogenesis [101, 102]. Moreover, during brown adipogenesis, anthocyanin decreases oxidative stress and elevates thermogenin (UCP1) expressed in brown fat cells [103, 104].

The function of brown adipocytes depends on the expression of different genes such as protein kinase B (AKT), extracellular signal-related kinases, mitogen-activated protein kinase (ERK-MAPK), NRF-1, and NRF-2. Briefly, elevated mitochondrial amounts of UCP1, cytochrome c (Cytc), PGC-1α, and PR16 domain (PRDM16) participate in the lipid metabolism of the body through initializing of AKT and ERK-MAPK [105, 106].

Rutin is a flavonoid that is derived from Saussurea involucrate with proven antifatigue properties. Su et al. showed that the oral administration of rutin to the mice improved the different problems, which related to physical fatigue. The administration of this flavonoid decreased lactic acid levels in the blood and malondialdehyde (MDA) levels in the muscle and brain. Moreover, the administration of rutin regulated SOD and GPx and increased PGC-1α and sirtuin 1 (SIRT1) in the muscle and brain that caused elevated levels of maximal endurance capacity. The evaluation of antianxiety effects of rutin on the brain of mice showed regulation of TPI, GDI, and CB1, which are known as anxiety-related proteins [107].

Glycyrrhizic acid (GA), as a major component of *Glycyrrhiza glabra* roots, belongs to flavonoids, which exhibits antioxidant, anti-inflammatory, and antiexcitotoxic properties [108]. GA substantially increases the expression rate and

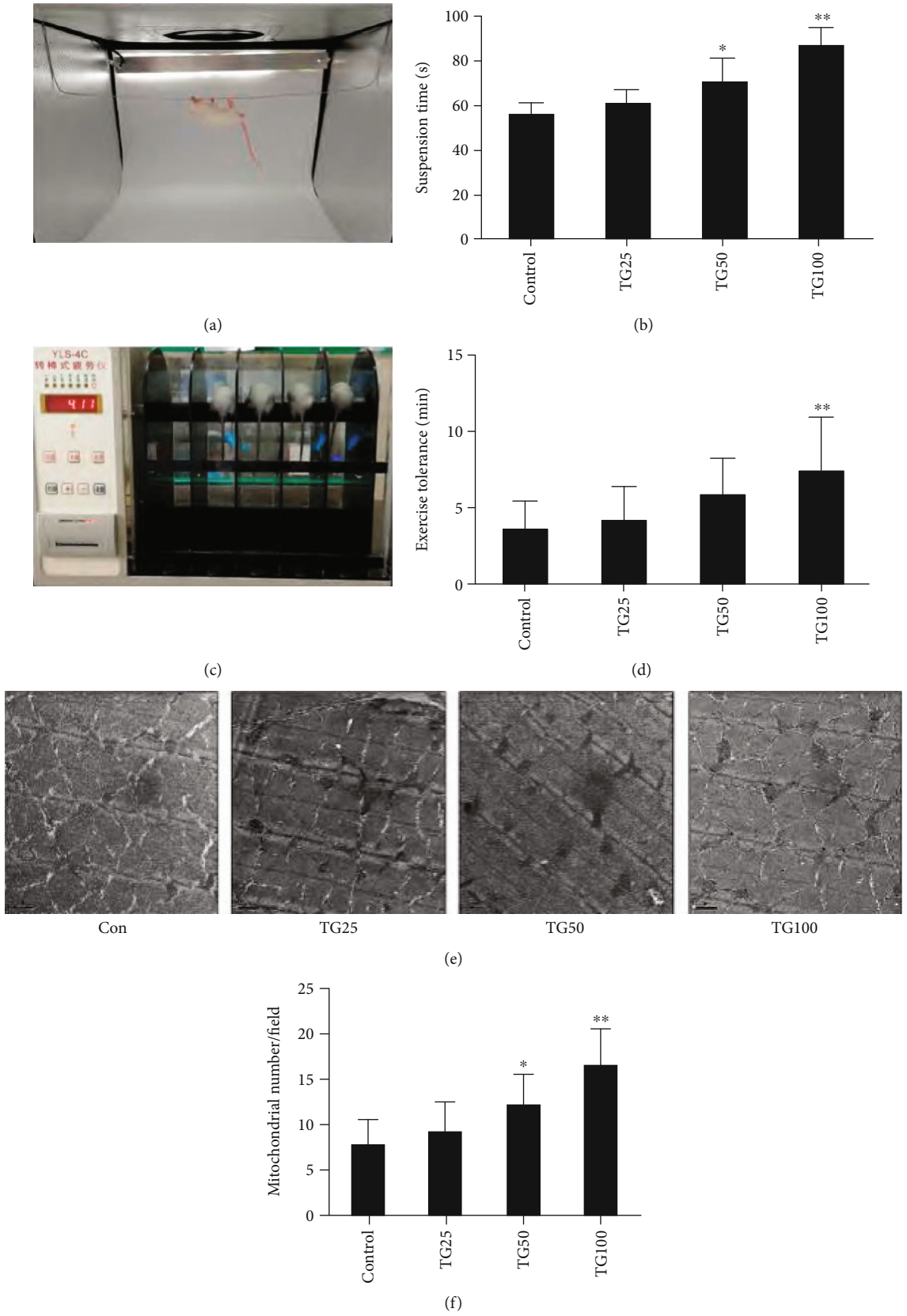

Figure 4: Improvement of exercise performance and skeletal muscle mitochondrial biogenesis of mice using tangeretin. Hanging wire test (a, b), exercise tolerance test (c, d), transmission electron microscope micrographs (e), and mitochondrial numbers (f). Con (control), TG25 (25 mg/kg tangeretin group), TG50 (50 mg/kg tangeretin group), and TG100 (100 mg/kg tangeretin group) (mean ± SD), $^{*}p < 0.05$, $^{**}p < 0.01$ in comparison with the control group. Scale bars = 1 μm. Reprinted (adapted) with permission from [112]. Copyright (2018) American Chemical Society.

mass of mitochondrial genes subsequent to aluminum toxicity [109]. The protective role of GA against aluminum-induced toxicity was proved to be in tight connection with the improvement of mitochondrial function and biogenesis [109]. Since aluminum toxicity is a good model of neurodegenerative disease, GA could be considered as a supplementary natural product in this context. Moreover, GA has significantly increased the expression of SIRT1 in high glucose-exposed renal tubular epithelial cells [110]. Mitochondrial DNA copy number and mitochondrial transcriptional activity have shown an upward trend in hypoxia/reoxygenation-induced human coronary artery endothelial cell injury after GA treatment [111].

Tangeretin is another flavonoid with positive effects on mitochondrial biogenesis. This substance is extracted from mandarin fruits. The study conducted on Kunming mice, and C2C12 myoblasts for evaluation of mitochondrial biogenesis revealed the significant activation of the signaling pathway of AMPK-PGC1-α in C2C12 myoblasts and remarkable improvement on the physical fatigue of mice. Based on these results, it could be suggested that this flavonoid might be a new potential mitochondrial regulator with sufficient results (Figure 4) [112].

Cyanidin-3-glucoside (Cy3g) is another flavonoid, which is founded in different fruits. The effects of this component on mitochondrial biogenesis showed increased levels of intracellular mitochondrial reductase, mitochondrial membrane potential, and ATP production. This substance also upregulates the expression of PGC-1α and SIRT1 in a dose- and time-dependent manner. Moreover, the expression of NRF1 and TFAM was increased in the presence of Cy3g. The upregulated amounts of the mentioned genes indicate the improvement of mitochondrial function and biogenesis in presence of Cy3g [113].

Isorhamnetin (ISOR), 3-O-methylquercetin, is a quercetin-derived flavonoid with antiobesity effects. It seems that the antiobesity effects of this metabolite relate to the regulation of mitochondrial biogenesis in adipocytes through the expression of mitochondrial genes, activating AMPK, and replicating of mtDNA [114].

Nobiletin is a natural flavonoid, which is known for anti-inflammatory, anticancer, and antioxidative properties. Dusabimana et al. evaluated the effects of nobiletin (5 mg/kg) on C57BL/6 mice with hepatic ischemia, which was caused by mitochondrial dysfunction and impaired autophagy.

Nobiletin decreases inflammation, oxidative damage, and necrosis by improving mitochondrial biogenesis and functions besides controlling autophagy. The positive effects of nobiletin on mitochondrial biogenesis are related to the regulation of SIRT-1/FOXO3a and PGC-1α and AKT pathways as well as other flavonoids [115]. SIRT1 is an important factor for the deacetylation of PGC-1α, which is considered as a transcription factor that plays an important role as a regulator in mitochondrial biogenesis and metabolism [113]. Activation of this sensor consequent to cellular stress and caloric restriction leads to the regulation of genes that associate with mitochondrial biogenesis and function. Moreover, reduced levels of FOXO3a due to the deacetylation of this component occurred after the activation of SIRT1 [116–118]. This important metabolic sensor is one of the main targets for most of the flavonoids for regulating metabolic activities and mitochondrial biogenesis [119].

Eriocitrin (eriodictyol 7-rutinoside) is a flavonoid that is found on Citrus limon with notable antiobesity effects. This bioactive substance, while administered orally, enhanced liver function through upregulation of genes that contribute to mitochondrial biogenesis including cytochrome c oxidase subunit 4, TFAM, NRF1, and ATP synthase. Moreover, in HepG2 cells, mitochondrial size and mtDNA amounts were increased in the presence of eriocitrin that could lead to the producing of ATP. Therefore, this could be concluded that intake of eriocitrin in the daily diet activates mitochondrial biogenesis, which improves diet-induced hepatic steatosis [120]. 5-Hydroxy-7-methoxyflavone, 5-hydroxy-3,7,40-trimethoxyflavone, and 5,7-dimethoxyflavone are common flavonoids that are founded in black ginger extract (KPE). KPE showed notable positive results in the treatment of obesity-associated problems and lipid metabolism through activation of brown adipocytes and lipolysis. The effects of these flavonoids on mitochondrial biogenesis and lipid metabolism on C2C12 myoblasts indicate that mitochondrial biogenesis and ATP production improved through regulation of PGC-1α and AMPK pathways [121].

Sudachitin is extracted from citrus sudachi fruit. This flavonoid ameliorates metabolic disorders and dyslipidemia through improving mitochondrial biogenesis and triglyceride reduction. This substance elevates the expression levels of SIRT1 and PGC-1α as well as nobiletin and other flavonoids, which leads to better mitochondrial biogenesis and metabolic function [122].

5. Other Compounds

Saponins, such as tomatine and digitonin, have also been found to be effective compounds in mediating mitochondrial biogenesis. It has been reported that chikusetsu saponin has plummeted the oxidative hazard in neuroblastoma cells which had been exposed to H2O2 through the elevation of PGC1a and SIRT-1 activation. The rhizome of Panax japonicus, which has been shown to possess anti-inflammatory and antioxidant effects, contains chikusetsu saponin [123]. The ethanolic extract of Platycodon grandiflorum has exhibited beneficial effects in brown adipose cells in combating obesity via the upregulation of mitochondrial genes such as PGC1α, SIRT3, and Nrf [124]. Amla is another traditional plant with antioxidant and antiaging effects. The protective effects of amla in myotubes subjected to tBHP have been observed in the context of mitochondrial function and biogenesis. The activation of AMPK and Nrf signaling in cells has been attributed to these protective effects [125].

6. Conclusion

The efficiency of homeostasis in healthy eukaryotic cells incorporated with mitochondrial biogenesis and function. Physiologically, cells react to diminished energy by either up- or downregulating the transcription factors which stimulate and/or inhibit mitochondrial biogenesis. Any

dysfunction in this intracellular pathway could cause metabolic syndrome, neurodegenerative diseases, and cancer. Polyphenols are wide-ranged therapeutic agents that founder in the plant kingdom and have been known for their antioxidant, anticancer, and antiaging properties. As described in the current study, these properties were related to the ability of these functional components to modulate the intracellular pathways of mitochondrial biogenesis. This information suggests that polyphenol components of plants, fruits, and natural extracts could be a promising alternative for preventing or treatment of metabolic and aging-related disorders such as type 2 diabetes, cancer, and neurodegenerative diseases. Also, other natural compounds such as flavonoids and saponins can exert therapeutical effects via interfering with mitochondrial biogenesis-related pathways.

The significance of mitochondria in both healthy and pathologic situations highlights the importance of better understanding the element required in mitochondrial biogenesis and turnover. Data suggest that pharmacological interventions in this context might significantly increase mitochondrial utility. Although there is incipient information on the use of polyphenols as mitochondrial biogenesis inducer, they might serve as fundamental opportunities in developing novel drug classes in mitochondrial involved pathologies.

Despite the aforementioned advantages of polyphenols in this context, more human studies are required to prove their beneficial effects on expanding mitochondrial volume and/or density in order to overcome different diseases. The current knowledge has well demonstrated that protective antioxidant role of polyphenols is at least partially due to their capacity to stimulate mitochondrial biogenesis and improve their function, which elevates mitochondrial efficiency resulting in diminished ROS production. Interestingly, mitochondrial produced ROS are vital for mitochondrial quality control. However, contra-indicatory results are obtained from the outputs of human studies directing at finding an ergogenic effect of polyphenols. In addition, polyphenol compounds possess weak pharmacokinetics properties such as low bioavailability and solubility which demands novel technologies to enhance polyphenol delivery to the target tissue.

Abbreviations

ROS: Reactive oxygen species
PGC-1α: Proliferator-activated receptor-γ coactivator
Nrf-1: Nuclear respiratory factor 1
TFAM: Mitochondrial transcription factor A
AMPK: AMP-activated protein kinase
ERR-α: Estrogen-related receptor-α
mtIF: Initiation factor
mtEFTu: Elongation factors Tu
mtRF1L: Translational release factor 1-like
mtRRF1: Recycling factors 1
SIRT1: Silent information regulator-1
IMM: Inner mitochondrial membrane
OA: Osteoarthritis
GSH and GPx: Glutathione and glutathione peroxidase
MMP: Mitochondrial membrane potential
EGCG: Epigallocatechin-3-gallate
AcEGCG: Peracetylated (-)-epigallocatechin-3-gallate.

Data Availability

This being a review article, no data was generated during the preparation of this manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Synergistic Antioxidant Effects of Araloside A and L-Ascorbic Acid on H_2O_2-Induced HEK293 Cells: Regulation of Cellular Antioxidant Status

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Araloside A is a pentacyclic triterpenoid saponin, and L-ascorbic acid is a globally recognized antioxidant. In this study, coadministered araloside A and L-ascorbic acid were found to have a strong synergistic antioxidant effect, and correlations between cellular antioxidant indexes and free radical scavenging ability were found. Individual and combined pretreatment with araloside A and L-ascorbic acid increased both cell viability and antioxidant enzyme activity and inhibited the release of lactate dehydrogenase (LDH); the accumulation of malondialdehyde (MDA), lipid peroxidation (LPO) products, and H_2O_2; and the production of intracellular reactive oxygen species (ROS), protein carbonyls, and 8-hydroxy-2-deoxy guanosine (8-OHdG). Free radical scavenging ability was positively correlated with superoxide dismutase (SOD) and catalase (CAT) activity, the glutathione (GSH)/oxidized glutathione (GSSG) ratio, and total antioxidant capacity (T-AOC). Our study is the first investigation of araloside A and L-ascorbic acid coadministration for the treatment of diseases caused by oxidative stress. The synergistic antioxidant effects of araloside A and L-ascorbic acid support their potential as functional food ingredients for the elimination of oxidative stress-induced adverse reactions.

1. Introduction

Selye first proposed the concept of oxidative stress in 1936 [1]. Oxidative stress is essentially caused by an imbalance between excessive free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS), and weakened antioxidant defence systems [2, 3]. Low-level oxidative stress is a normal process for maintaining life, but excessive oxidative stress causes damage to biomolecules, which leads to the development of certain diseases [4]. H_2O_2 not only transmits redox signals under physiological conditions (1-10 nM) but also causes oxidative damage to cells when its concentration exceeds 100 nM [5]. Exogenous H_2O_2 can penetrate the cell membrane to induce the production and accumulation of ROS; react with metal ions in the cell; and cause DNA damage, mitochondrial dysfunction, and even H_2O_2-mediated apoptosis. The H_2O_2-induced cellular oxidative stress model is commonly used to study the functions of various biologically active materials [6, 7]. Therefore, the antioxidant capacities of araloside A, L-ascorbic acid, and their combination were investigated in a human embryonic kidney cell line (HEK293 cells) using H_2O_2-induced oxidative stress, which has been utilized in a large number of previous studies. Traditional chemical methods can rapidly detect the antioxidant capacity of active substances but do not reflect the biological behaviour of active substances and downstream molecular mechanisms in living cells. Therefore, the present study is the first investigation of the in vitro and intracellular antioxidant activities of araloside A and L-ascorbic acid and the correlation between these compounds.

HEK293 cells are immortalized cells characterized by normal kidney function with apical zona occludens and relatively inconspicuous brush borders. The metabolic conditions and physiological characteristics of HEK293 cells are closer to those of normal cells than those of cancer cells. These characteristics are more reflective of the true state of

oxidative stress, and many researchers have also used HEK293 cells as a cell model of oxidative stress [8, 9].

Triterpenoid saponins, an important class of bioactive products, are primarily divided into tetracyclic triterpenoid saponins and pentacyclic triterpenoid saponins, among which pentacyclic triterpenes are common. Oleanane-type pentacyclic triterpenoid saponins are broadly distributed and have been widely studied [10, 11]. Araloside A (Figure 1), an oleanolic acid-type pentacyclic triterpenoid saponin, is extensively distributed in many Aralia plants, such as *Aralia elata* [12], *Aralia taibaiensis* [13], *Aralia armata* [14], *Aralia chinensis L.* [15], and *Aralia spinifolia* [16]. The mechanism of araloside A absorption in the human body involves multiple transport pathways and mechanisms, such as passive diffusion, paracellular pathways, and mechanisms involving the participation of efflux transporters [17]. Araloside A at doses of 50 and 100 mg/kg was shown to significantly alleviate HCl·ethanol-induced gastric injury and aspirin-induced gastric ulcer [12]. Ding et al. [18] reported that araloside A effectively alleviated inflammation in rheumatoid arthritis fibroblast-like synoviocytes via inhibition of the nuclear factor kappa B pathway. L-Ascorbic acid is a water-soluble vitamin and the essential micronutrient in the human body. At normal physiological concentrations, L-ascorbic acid plays an important role in protecting cells from oxidative damage at normal physiological concentrations, and at higher pharmacological concentrations, it prevents cancer [19]. Araloside A and L-ascorbic acid are widespread in common foods, including fruits, vegetables, herbs, and other plant-based foods. These active ingredients in the daily diet exert antioxidant interactions in the body when ingested simultaneously. These antioxidant interactions may be synergistic, antagonistic, or additive depending on the ratio at which these ingredients are mixed [20]. The antioxidant effects of different active ingredients have been a research focus for many scientists in recent years. However, few scholars have studied the synergistic effects of araloside A and L-ascorbic acid on antioxidant activity.

The objective of this study was to investigate the independent and combined effects of araloside A and L-ascorbic acid on oxidative stress injury in HEK293 cells by monitoring cell viability, antioxidant enzyme capacity, ROS production, lactate dehydrogenase (LDH) release, H_2O_2 levels, the extent of lipid peroxidation (LPO), malondialdehyde (MDA) levels, protein carbonyl levels, and 8-hydroxy-2-deoxy guanosine (8-OHdG) levels. In addition, the chemical antioxidant activities of araloside A and L-ascorbic acid were evaluated, and correlations between parameters related to cell antioxidant status and chemical antioxidant activity were analysed.

2. Materials and Methods

2.1. Materials and Reagents. Araloside A and L-ascorbic acid at a purity ≥98% were purchased from Shanghai Yuanye Biotechnology Co. Ltd. (Shanghai, China). H_2O_2 solution (30%) was purchased from Tianli Chemical Reagent Co. Ltd. (Tianjin, China). Minimal essential medium (MEM), penicillin-streptomycin (PSS) solution, foetal bovine serum (FBS), trypsin, RIPA lysis buffer, fluorescence probe (DCFH-DA), phosphate-buffered saline (PBS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). GoTaq Green Master Mix and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) were purchased from Promega Biotechnology Co. (Madison, WI, USA). Kits used to assay LDH, MDA, LPO, H_2O_2, protein carbonyl levels; superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities; total antioxidant capacity (T-AOC); and the glutathione/oxidized glutathione (GSH/GSSG) ratio were purchased from Nanjing Jiancheng Bioengineering Co. Ltd. (Nanjing, Jiangsu, China). ELISA kit was purchased from AmyJet Scientific Co. Ltd. (Wuhan, Hubei, China). Xanthine oxidase, β-nicotinamide adenine dinucleotide, and lactate dehydrogenase were obtained from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China).

2.2. Cell Culture and Treatment. HEK293 cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in MEM supplemented with a 1% PSS and 10% FBS at 37°C in an incubator with 5% CO_2. The HEK293 cells ($5 - 6 \times 10^3$ cells/mL) were seeded into flat-bottomed plates and cultured for 24 h in an incubator with CO_2. When the cells reached approximately 70-80% confluence, the medium was removed, and the cells were pretreated with araloside A (1, 10, or 20 μM), L-ascorbic acid (1, 10, or 20 μM), or both (combined ratio of 1 : 1) for 24 h. Then, the medium was gently removed, and the cells were exposed to H_2O_2 for 4 h.

2.3. Establishment of the H_2O_2-Induced Oxidative Stress Model. HEK293 cells were treated with H_2O_2 at different concentrations for 4 h to establish an oxidative stress model. The appropriate amount of H_2O_2 was added to medium deprived of serum and antibiotics, and the final concentration of H_2O_2 was maintained at 100, 200, 300, 400, 500, 600, 700, or 800 μM. Complete medium without cells served as a blank group, and HEK293 cells without H_2O_2 were used as a control group. Cell viability was measured using the MTS assay. MTS (20 μL) was added to the cells, which were then incubated at 37°C for 4 h. The 96-well plate was placed in a multimode microplate reader (Tecan, Infinite M200 Pro) to measure the absorbance at 490 nm. Six parallel experiments with cells in each treatment group were performed, and the results are expressed as average values. All the assays were repeated three times. The concentration of H_2O_2 (producing a cell viability of approximately 50%) used to establish the oxidative stress model was selected for subsequent experiments. Cell viability was calculated using the following equation:

$$\text{Cell viability}(\%) = \frac{\text{OD}_{\text{injury group}} - \text{OD}_{\text{blank group}}}{\text{OD}_{\text{control group}} - \text{OD}_{\text{blank group}}}. \quad (1)$$

2.4. Assessment of the Effects of Araloside A and L-Ascorbic Acid on Cytotoxicity/Proliferation. Normal cells without treatment were regarded as the control group. The cells in the experimental groups were treated with araloside A

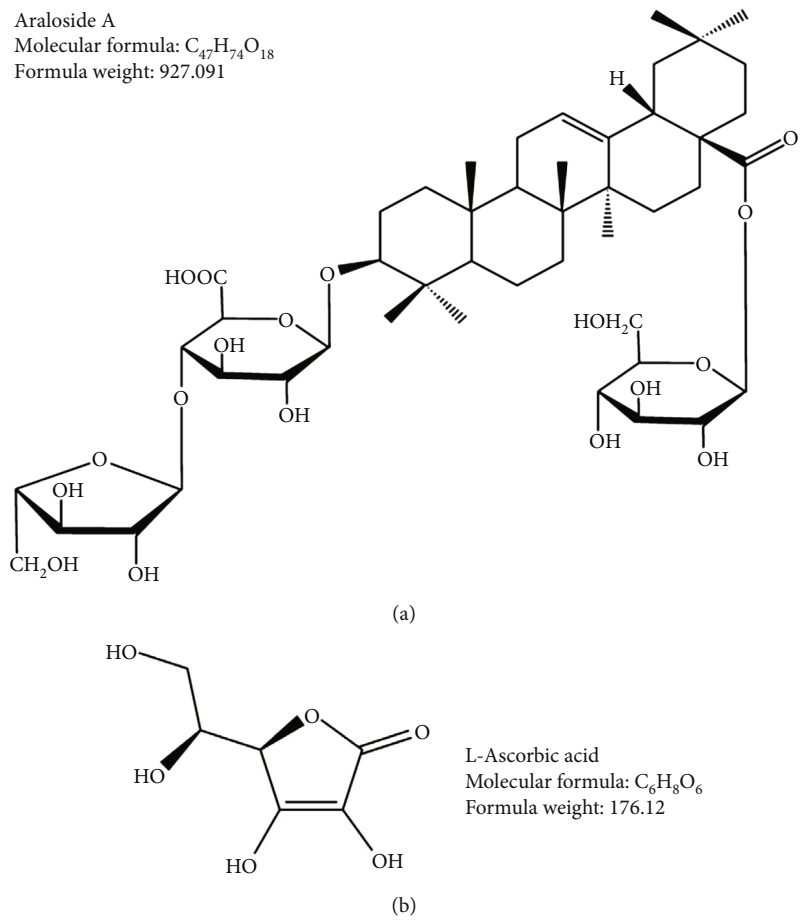


Figure 1: Chemical structure of araloside A (a) and L-ascorbic acid (b).

(1~100 μM) and L-ascorbic acid (1~100 μM) for 24 h at 37°C with 5% CO_2. Cell viability was measured using the MTS assay. Six parallel experiments with cells in each treatment group were performed, and all assays were repeated at least three times.

2.5. Effects of Araloside A and L-Ascorbic Acid at Different Ratios on Cell Viability. Normal cells without treatment were used as the control group, and H_2O_2-treated cells served as the injury group. Cells treated with araloside A and L-ascorbic acid at different ratios (1 : 1, 1 : 2, 2 : 1, 1 : 3, or 3 : 1) constituted the synergy groups. HEK293 cells were treated with araloside A and L-ascorbic acid at different ratios for 24 h. The medium was then gently removed, and the cells were exposed to H_2O_2 (400 μM) for an additional 4 h. Cell viability was measured using the MTS assay. Six parallel experiments with cells in each treatment group were performed, and all assays were repeated at least three times.

2.6. Protective Effects of Araloside A, L-Ascorbic Acid, and the Combination on Cell Viability. Normal cells without treatment were used as the control group, and H_2O_2-treated cells constituted the injury group. Cells in the experimental groups were treated with araloside A (1, 10, or 20 μM), L-ascorbic acid (1, 10, or 20 μM), or the combination (ratio of 1 : 1) for 24 h at 37°C in the presence of 5% CO_2. The cells were treated with H_2O_2 (400 μM) for an additional 4 h, and cell viability was measured using the MTS assay. Six parallel experiments with cells in each treatment group were performed, and all assays were repeated at least three times.

2.7. Measurement of Intracellular ROS Production. A DCFH-DA fluorescent probe assay was used to determine intracellular ROS production. Incomplete medium containing DCFH-DA (10 μM) was added to the cells, which were then incubated at 37°C for 20 min. The fluorescent probe (DCFH-DA) was removed, and the cells were washed three times with PBS. The green fluorescence from DCF in the HEK293 cells was determined by fluorescence microscopy (BX43 upright microscope, Olympus, Long Island, New York, USA). Fluorescence intensity was immediately detected using a multimode microplate reader with excitation wavelength of 485 nm and an emission wavelength of 525 nm. All assays were repeated at least three times.

2.8. Measurement of Relevant Indicators of Cell Antioxidant Status. Cells digested with trypsin were washed twice with PBS. The cell pellet remaining after centrifugation was lysed in ice-cold RIPA lysis buffer containing 1 mM PMSF for 40 min. The relevant indicators were determined according to the instructions provided with the corresponding assay kits, and the principles of each biochemical methods were as

follows. SOD activity (superoxide dismutase assay kit, A001-3) was determined via colorimetric analysis of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium formazan at 450 nm. GSH-Px activity (glutathione peroxidase assay kit, A005-1) was determined by colorimetric analysis via measurement of the consumption of GSH at 412 nm. CAT activity (catalase assay kit, A007-1) was determined by measuring the decomposition of H_2O_2 via absorbance at 405 nm. T-AOC was estimated by measuring the absorbance of $ABTS^+$ at 415 nm. The cyclic reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) was used to determine the total glutathione and GSSG levels (total glutathione/oxidized glutathione assay kit, A061-1). The GSH content was equal to the total glutathione content minus 2 times the GSSG content. The MDA level (cell malondialdehyde assay kit, A003-4) was analysed colorimetrically by measuring condensation of thiobarbituric acid at 523 nm. The H_2O_2 level (hydrogen peroxide assay kit, A064-1) was evaluated by measuring the amount of the complex formed by the reaction between H_2O_2 and molybdic acid at 405 nm. The LPO content (lipid peroxidation assay kit, A106-1) was analysed by measuring the absorbance following the reaction between LPO and the developer at 586 nm. The protein carbonyl content (protein carbonyl assay kit, A087-1) was determined by measuring the absorbance of 2,4-dinitrophenylhydrazone at 370 nm. The medium was collected, and LDH release (lactate dehydrogenase assay kit, A020-2) was determined by measuring the change in colour of pyruvate-dinitrophenylhydrazone at 450 nm. The amount of 8-OHdG was determined using a standard ELISA kit following the manufacturer's instructions. All assays were repeated at least three times.

2.9. Determination of In Vitro Antioxidant Activity. The DPPH assay was performed according to the method described by Fattouch et al. with slight modifications [21]. Samples (2 mL) and 2 mL of a $DPPH^·$ solution were reacted at room temperature for 30 min in the dark, and the absorbance at a wavelength of 517 nm was measured and recorded as A_i. The sample was replaced with 70% methanol, and the absorbance was measured and recorded as A_c. The $DPPH^·$ solution was replaced with 70% methanol, and the absorbance was measured and recorded as A_t. The $DPPH^·$ scavenging ratio was calculated as follows:

$$DPPH^· \text{ scavenging ratio}(\%) = \left(1 - \frac{A_i - A_t}{A_c}\right) \times 100\%. \quad (2)$$

The ABTS assay was performed as described by Re et al. [22]. Three millilitres of $ABTS^+$ solution and 100 μL of sample were mixed at 30°C for 6 min in the dark, and the absorbance at 743 nm was measured and recorded as A_i. The sample was replaced with 70% methanol, and the absorbance was measured and recorded as A_c. The $ABTS^+$ working solution was replaced with 70% methanol, and the absorbance was measured and recorded as A_t. The calculations were performed as described above.

Hydroxyl radical scavenging capacity was determined according to Denev et al. with some modifications [23]. The sample (1 mL) was added to 1 mL of an $FeSO_4$ solution (10 mmol/L), 1 mL of a salicylic acid solution (10 mmol/L), and 1 mL of H_2O_2 (6 mmol/L). The mixture was cooled rapidly, and the absorbance at 510 nm was measured and recorded as A_i. The sample was replaced with 70% methanol, and the absorbance was measured and recorded as A_c. The $FeSO_4$ solution was replaced with 70% methanol, and the absorbance was measured and recorded as A_t. The calculations were performed as described above.

Superoxide anion radical scavenging capacity was determined according to Chan and Bielski with some modifications [24]. The sample (200 mL) was added to 1 mL of sodium phosphate buffer (pH 7.0), 500 μL of β-nicotinamide adenine dinucleotide, and 100 μL of lactate dehydrogenase and allowed to react at 25°C for 1 min. Xanthine oxidase (10 mL) was then added, and the absorbance at 340 nm was measured and recorded as A_i. The absorbance of an equal volume of distilled water (instead of the sample) was measured and recorded as A_c, and the absorbance of an equal volume of distilled water instead of the pyrogallol solution was measured and recorded as A_t. The calculations were performed as described above. All assays were repeated at least three times.

2.10. Assessing the Synergistic Effect of Araloside A and L-Ascorbic Acid in Combination by the Isobologram Method. The antioxidant interaction of araloside A and L-ascorbic acid was evaluated by the isobologram method according to Jiang et al. [25]. The synergistic rate (SR) was calculated by the equation: SR = EV/TV, where EV is the experimental value of the combination group and TV is the theoretical value of the combination group. TV was calculated as follows: $TV = a * R_A + b * R_B$, where a and b are the combined ratios of substance A and substance B, and R_A and R_B are the experimental results obtained when substance A and substance B are used separately. SR > 1 indicated synergistic effects, SR < 1 indicated the antagonistic effects, and SR = 1 indicated additive effects. In this study, the SRs based on LDH release, ROS generation, GSSG content, MDA content, H_2O_2 level, LPO content, protein carbonyl level, and 8-OHdG level were less than 1, indicating synergistic effects.

2.11. Statistical Analysis. All assays were repeated at least three times, and the data are expressed as the mean ± standard deviation. The normality of the distribution was checked using the KURT and SKEW functions in Microsoft Office Excel 2019. Homogeneity of variance was tested with the homogeneity of variance test using SPSS 20.0 (IBM Corp., Armonk, NY, USA). A correction for multiple comparisons was calculated. Correlations between data were assessed with bivariate and Pearson correlation coefficients using SPSS 20.0. The data were subjected to one-way analysis of variance (ANOVA) in SPSS 20.0 to analyse the statistical significance. Significance was established at a level of $p < 0.05$.

3. Results

3.1. Cytotoxic Effects of H_2O_2 in HEK293 Cells. HEK293 cells were treated with 100-800 μM H_2O_2 for 4 h, and cell viability was measured using the MTS method. As shown in

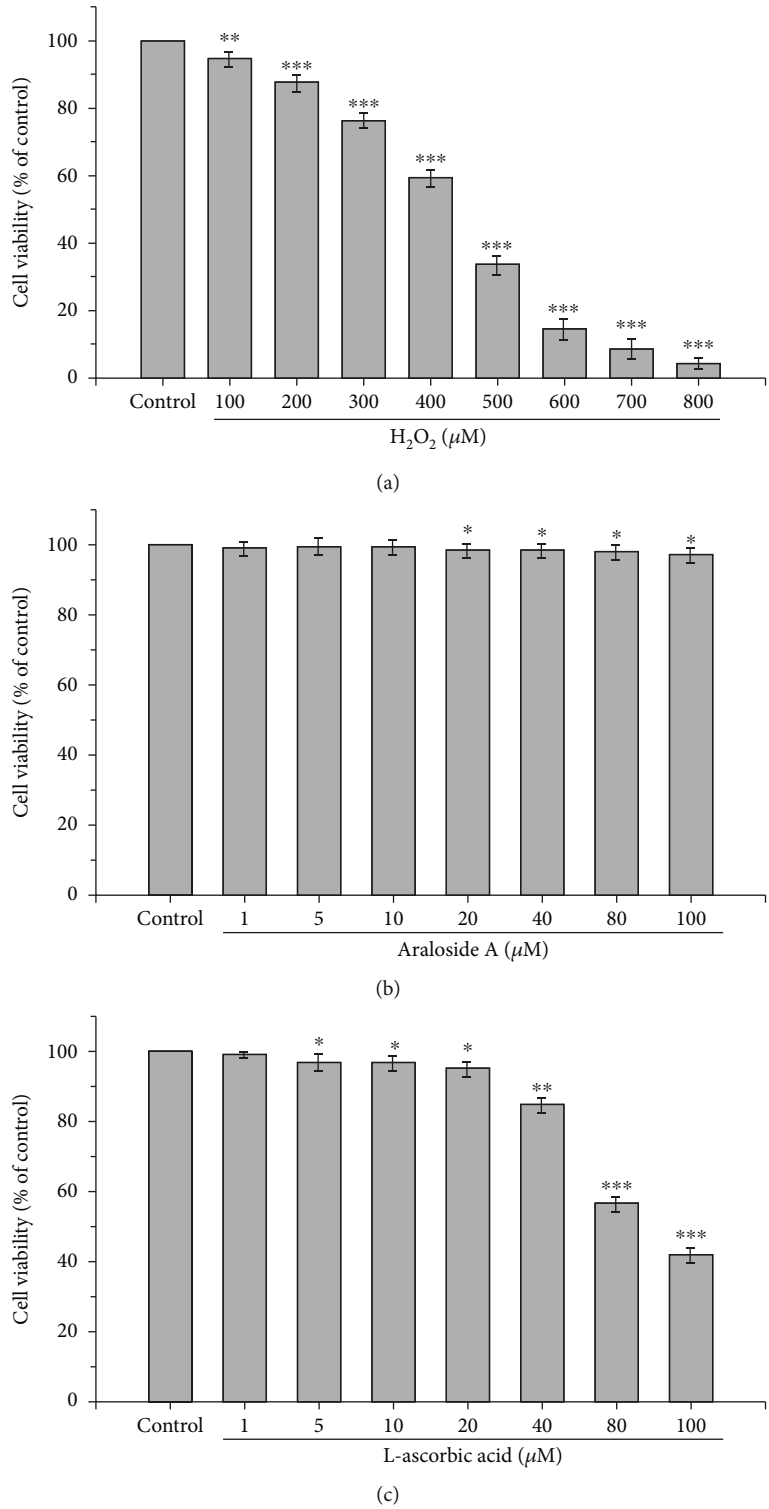


FIGURE 2: Cytotoxicity effects of H_2O_2 (a), araloside A (b), and L-ascorbic acid (c) on the viability of HEK239 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the control group.

Figure 2(a), cell viability underwent a concentration-dependent decrease with increasing H_2O_2 concentration. The cell viability obtained with the minimum treatment concentration (100 μM) and the maximum treatment concentration (800 μM) was 94.45% and 3.91%, respectively. Treatment with 400 μM H_2O_2 reduced cell viability to approximately 41% of that of the control group. Therefore, 400 μM H_2O_2 was selected as the optimal concentration to establish the oxidative stress model in HEK293 cells in the subsequent experiments.

3.2. Cytotoxic Effects of Araloside A and L-Ascorbic Acid. In this study, a cell viability between 95% and 105% indicated no cytotoxicity or proliferation of HEK293 cells. Araloside A did not significantly enhance or inhibit the viability of HEK293 cells when its concentration was below 100 μM (Figure 2(b)). L-Ascorbic acid did not significantly enhance or inhibit the viability of HEK293 cells when its concentration was below 20 μM (Figure 2(c)). Moreover, the plasma concentrations of L-ascorbic acid following food intake do not exceed 100 μM [26]. Considering that the actual plasma concentration of araloside A is relatively low and that physiological araloside A concentrations in previous studies did not exceed 20 μM [18], L-ascorbic acid and araloside A concentrations were maintained under 20 μM for further experiments.

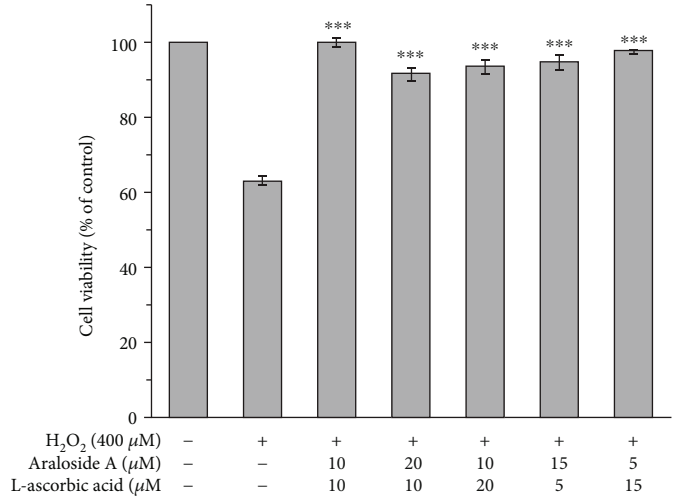
3.3. Effects of Araloside A and L-Ascorbic Acid at Various Ratios on Cell Viability. The inhibitory effects of araloside A and L-ascorbic acid in combination at various ratios on the induction of oxidative stress-related damage were evaluated using the MTS assay. After the coadministration of araloside A and L-ascorbic acid at a ratio of 1 : 1, the cell viability reached 101.01 ± 1.10%, which was 39.10% higher than that obtained in the injured group and 9.5%, 8.44%, 7.49%, and 3.40% higher than that obtained after their coadministration at ratios of 1 : 2, 1 : 3, 2 : 1, and 3 : 1, respectively (Figure 3(a)). Thus, araloside A and L-ascorbic acid at a ratio of 1 : 1 exerted the best inhibitory effects on H_2O_2-induced oxidative stress-related damage.

3.4. Individual and Combined Protective Effects of Araloside A and L-Ascorbic Acid against H_2O_2-Induced Oxidative Stress. As shown in Figure 3(b), the viability of the injured group was 59.59 ± 1.88%, which was significantly lower than that of the control group. Cell viability after pretreatment with araloside A, L-ascorbic acid, or their combination increased from 71.16 ± 1.05% to 95.44 ± 1.55% compared with that of the injury group. Pretreatment araloside A and/or L-ascorbic acid at higher concentrations increased cell viability. The cell viability obtained with combined araloside A and L-ascorbic acid pretreatment was significantly higher than that obtained upon pretreatment with the individual compounds. According to the isobologram method, the SR at all three concentrations (1, 10, or 20 μM) was greater than 1 (Table 1). The viability of the cells under combination pretreatment with 1/2 araloside A and 1/2 L-ascorbic acid was significantly improved compared with that upon pretreatment with individual araloside A or L-ascorbic acid treatment at the same concentration. These results indicate that the combination of araloside A and L-ascorbic acid significantly enhanced the inhibition of H_2O_2-induced oxidative stress damage. Therefore, the combination of araloside A and L-ascorbic acid improved the cytoprotective effect against oxidative damage in HEK293 cells via synergy.

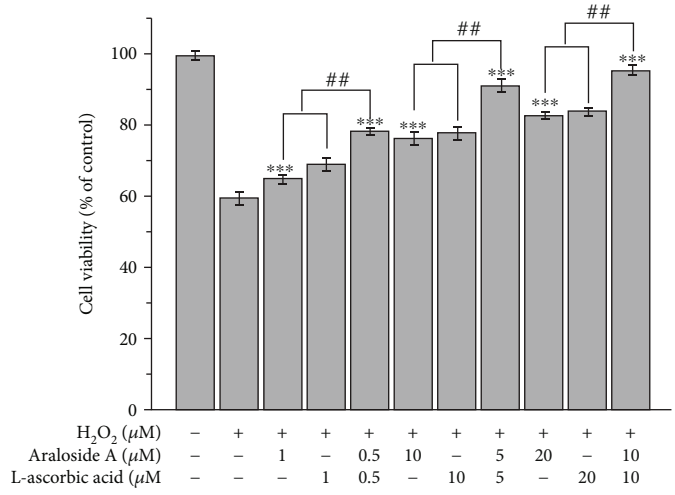
3.5. Individual and Combined Effects of Araloside A and L-Ascorbic Acid on LDH Release. The individual and combined protective effects of araloside A and L-ascorbic acid on plasma membrane integrity were evaluated by measuring the release of LDH. As shown in Figure 3(c), LDH release in the control group was significantly lower than that in the injury group. The damage to HEK293 cells induced by H_2O_2 resulted in destruction of the plasma membrane and thus a sharp increase in the level of LDH release compared with that of the control group. However, LDH release after pretreatment with araloside A or L-ascorbic acid was significantly reduced compared with that in the injury group, and the combination of araloside A and L-ascorbic acid induced a significantly larger reduction in LDH release compared with that upon pretreatment with either individual compound. The amount of LDH released by the combination group (10 μM araloside A + 10 μM L − ascorbic acid) was 42.13 and 55.04 U/g protein lower than those of the groups treated with 20 μM araloside A and 20 μM L-ascorbic acid alone, respectively. The SR of the group treated with araloside A+L-ascorbic acid (10 μM + 10 μM) was 0.70 ± 0.01, showing the significant synergistic effects of the two compounds (Table 1). These results confirm that araloside A and L-ascorbic acid synergistically stabilize the release of LDH and improve the protective capacity of the cell plasma membrane.

3.6. Individual and Combined Effects of Araloside A and L-Ascorbic Acid on H_2O_2-Induced ROS Generation. Intracellular DCF fluorescence images showed that the control group exhibited a weak green fluorescence intensity, which indicated that the production and elimination of ROS in HEK293 cells were in equilibrium (Figure 4(a)). The green fluorescence intensity of the injury group was significantly enhanced, implying the rapid generation and accumulation of ROS in the HEK293 cells. However, the green fluorescence intensity after pretreatment with araloside A or L-ascorbic acid was obviously weaker than that of the injury group, and the combination of araloside A and L-ascorbic acid attenuated the green fluorescence intensity to a greater extent than pretreatment with the individual compounds.

The level of ROS generated in the control group was designated 1.0 and used to express relative ROS production in the other groups. The data showed that relative ROS production in the injury group was approximately 2.3-fold higher than that in the control group (Figure 4(b)). Relative ROS production after pretreatment with araloside A, L-ascorbic acid, or their combination was significantly lower than that in the injury group, and ROS production after pretreatments gradually approached the baseline obtained from the control group in a concentration-dependent manner. Notably, the combination of araloside A and L-ascorbic acid significantly decreased relative ROS production compared with that of the individual pretreatment groups. The isobologram method showed that the combination of 5 μM araloside A and 5 μM L-ascorbic acid produced a greater effect than 10 μM araloside A or L-ascorbic acid, which indicated that araloside A and L-ascorbic acid had a synergistic effect (SR = 0.73 ± 0.05, Table 1). These data demonstrated that araloside A and L-ascorbic acid synergistically protected HEK293 cells against H_2O_2-induced oxidative stress and that these effects might be due to improvement in the ability to scavenge or inhibit ROS.



(a)



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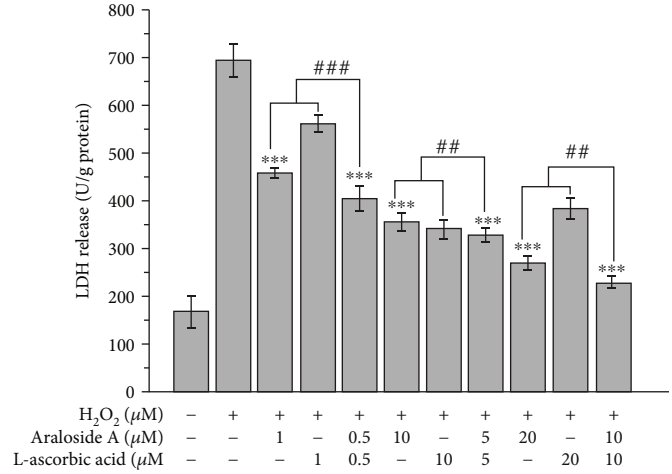


(c)

Figure 3: Individual and combined effect of araloside A and L-ascorbic acid on HEK293 cells. (a) The effects of araloside A and L-ascorbic acid with variously combined ratio on H_2O_2-induced oxidative damage in HEK293 cells. (b) Protective effect of araloside A and L-ascorbic acid on H_2O_2-induced oxidative damage in HEK293 cells. (c) Individual and combined effect of araloside A and L-ascorbic acid on lactate dehydrogenase (LDH) release. Vertical bars indicate the mean value ± SD. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the araloside A+L-ascorbic acid group. $^{***}p < 0.001$ versus the injury group.

Table 1: Synergistic rate (SR) of the combination pretreatment of araloside A and L-ascorbic acid in H_2O_2-induced HEK-293 cells.

	Concentrations (araloside A+L-ascorbic acid)								
	EV			TV			SR		
	1	2	3	1	2	3	1	2	3
Cell viability (%)	78.37 ± 0.96	91.29 ± 1.94	95.44 ± 1.55	67.13 ± 1.17	77.08 ± 1.71	83.32 ± 1.98	1.07 ± 0.03^{Cde}	1.18 ± 0.06^{Ab}	1.14 ± 0.03^{Be}
LDH (U/g pro)	405.29 ± 21.93	329.61 ± 11.23	230.39 ± 12.32	484.01 ± 11.35	349.36 ± 14.19	328.97 ± 16.07	0.84 ± 0.01^{Bghi}	0.94 ± 0.02^{Ae}	0.70 ± 0.01^{Ck}
ROS	2.34 ± 0.06	1.79 ± 0.07	1.50 ± 0.02	2.79 ± 0.19	2.42 ± 0.07	1.91 ± 0.03	0.84 ± 0.02^{Ahi}	0.73 ± 0.05^{Cj}	0.78 ± 0.07^{Bi}
CAT (U/mg pro)	10.18 ± 1.06	14.78 ± 0.64	17.50 ± 0.34	8.22 ± 0.26	9.98 ± 0.92	12.03 ± 0.68	1.24 ± 0.06^{Ca}	1.48 ± 0.05^{Aa}	1.45 ± 0.03^{Bb}
SOD (U/mg pro)	16.02 ± 0.39	17.04 ± 0.81	18.54 ± 0.59	14.33 ± 0.71	14.39 ± 0.43	15.66 ± 0.61	1.12 ± 0.02^{Cc}	1.18 ± 0.03^{Bb}	1.88 ± 0.04^{Aa}
GSH-Px (U/mg pro)	54.19 ± 1.82	65.01 ± 0.28	74.51 ± 0.58	50.74 ± 1.56	60.88 ± 0.98	70.22 ± 0.57	1.07 ± 0.03^{Ae}	1.06 ± 0.04^{Bd}	1.06 ± 0.05^{Bf}
T-AOC (mM TE/g FM)	0.80 ± 0.36	0.82 ± 0.01	0.97 ± 0.03	0.67 ± 0.04	0.73 ± 0.02	0.83 ± 0.02	1.19 ± 0.02^{Ab}	1.12 ± 0.01^{Cc}	1.17 ± 0.03^{Bd}
GSSG (μmol/L)	32.79 ± 0.94	27.19 ± 1.04	20.12 ± 1.22	36.90 ± 1.67	33.11 ± 2.14	24.66 ± 1.14	0.88 ± 0.04^{Ag}	0.82 ± 0.04^{Bg}	0.81 ± 0.07^{Ch}
GSH/GSSG	0.57 ± 0.01	0.78 ± 0.03	0.87 ± 0.04	0.52 ± 0.01	0.66 ± 0.02	0.71 ± 0.02	1.10 ± 0.02^{Ce}	1.18 ± 0.02^{Bb}	1.22 ± 0.03^{Ac}
MDA (nmol/mg pro)	9.11 ± 0.23	8.55 ± 0.16	5.71 ± 0.15	10.69 ± 0.27	9.00 ± 0.06	7.06 ± 0.16	0.85 ± 0.03^{Bgh}	0.95 ± 0.04^{Ae}	0.80 ± 0.02^{Ch}
H_2O_2 (mmol/g pro)	50.29 ± 0.34	37.20 ± 1.43	23.39 ± 0.31	54.16 ± 0.49	48.32 ± 1.05	30.30 ± 1.39	0.92 ± 0.06^{Af}	0.76 ± 0.06^{Ci}	0.77 ± 0.04^{Bij}
LPO (μmol/g pro)	16.13 ± 0.17	14.55 ± 0.13	10.71 ± 0.17	18.70 ± 0.35	17.03 ± 0.16	14.06 ± 0.25	0.86 ± 0.03^{Agh}	0.85 ± 0.01^{Bf}	0.76 ± 0.06^{Cj}
Protein carbonyl (nmol/mg pro)	363.93 ± 5.39	213.39 ± 3.35	170.23 ± 6.88	383.30 ± 4.13	275.86 ± 5.81	197.28 ± 2.74	0.95 ± 0.01^{Af}	0.77 ± 0.04^{Bi}	0.86 ± 0.02^{Cg}
8-OHdG	3.01 ± 0.03	2.83 ± 0.04	2.54 ± 0.02	3.73 ± 0.16	3.52 ± 0.05	2.94 ± 0.06	0.80 ± 0.03^{Bi}	0.80 ± 0.01^{Bh}	0.86 ± 0.02^{Ag}

1: 0.5 μM araloside A + 0.5 μM L − ascorbic acid; 2: 5 μM araloside A + 5 μM L − ascorbic acid; 3: 10 μM araloside A + 10 μM L − ascorbic acid. The synergistic rate (SR) was calculated by the equation: SR = EV/TV. EV was the experimental value of the combination group. TV was the theoretical value of the combination group: $TV = a * R_A + b * R_B$. Among them, a and b were the combined ratios of substance A and substance B, and R_A and R_B were the experimental results obtained when substance A and substance B are used separately. SR > 1 indicated the synergistic effects; SR < 1 indicated the antagonistic effects; SR = 1 indicated the addictive effects. In this table, SRs based on LDH release, ROS generation, GSSG content, MDA content, H_2O_2 level, LPO content, protein carbonyl level, and 8-OHdG level were less than 1, which represented the synergistic effects. LDH: the release of lactate dehydrogenase; ROS: the production of reactive oxygen species; CAT: the activity of catalase; SOD: the activity of superoxide dismutase; GSH-Px: the activity of glutathione peroxidase; T-AOC: the total antioxidant capacity; GSSG: oxidized glutathione; GSH/GSSG: the glutathione/oxidized glutathione ratio; MDA: the malondialdehyde levels; H_2O_2: the H_2O_2 levels; LPO: the lipid peroxidation contents; 8-OHdG: the 8-hydroxy-2-deoxy guanosine levels. Different uppercase letters indicate significant differences between SR values in the same row ($p < 0.05$); different lowercase letters indicate significant differences between SR values in the same column ($p < 0.05$).

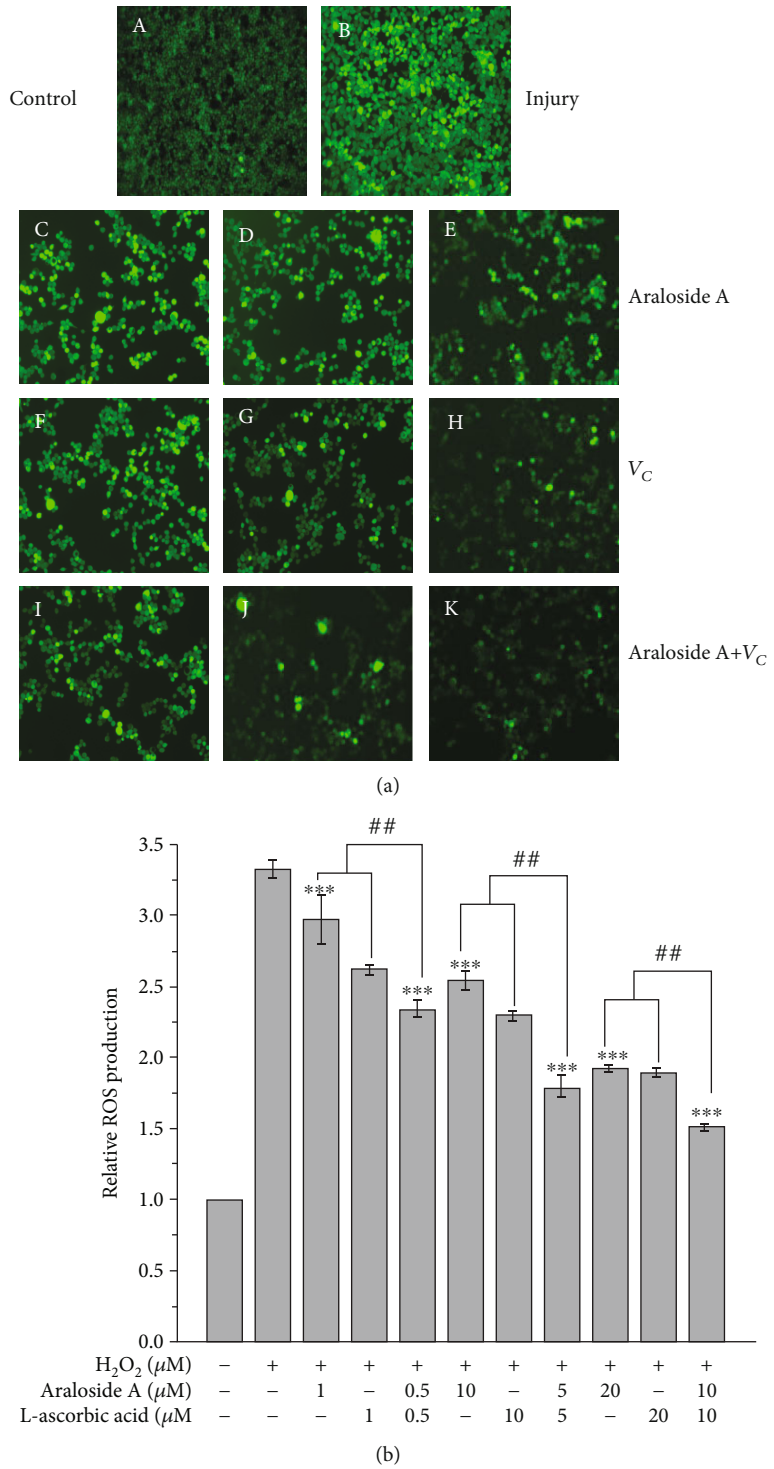


FIGURE 4: Individual and combined effect of araloside A and L-ascorbic acid (V_C) on reactive oxygen species (ROS) generation. (a) Fluorescence images in HEK293 cells were collected by laser scanning confocal microscopy (LSCM) 200x. (A) Control group; (B) injury group; (C) 1 μM araloside A + 400 μM H_2O_2; (D) 10 μM araloside A + 400 μM H_2O_2; (E) 20 μM araloside A + 400 μM H_2O_2; (F) 1 μM L – ascorbic acid + 400 μM H_2O_2; (G) 10 μM L – ascorbic acid + 400 μM H_2O_2; (H) 20 μM L – ascorbic acid + 400 μM H_2O_2; (I) 0.5 μM araloside A + 0.5 μM L – ascorbic acid + 400 μM H_2O_2; (J) 5 μM araloside A + 5 μM L – ascorbic acid + 400 μM H_2O_2; (K) 10 μM araloside A + 10 μM L – ascorbic acid + 400 μM H_2O_2. (b) Production of intracellular ROS was measured using the fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Vertical bars indicate the mean values ± SD. $^{\#\#}p < 0.01$ versus the araloside A+L-ascorbic acid group. $^{***}p < 0.001$ versus the injury group.

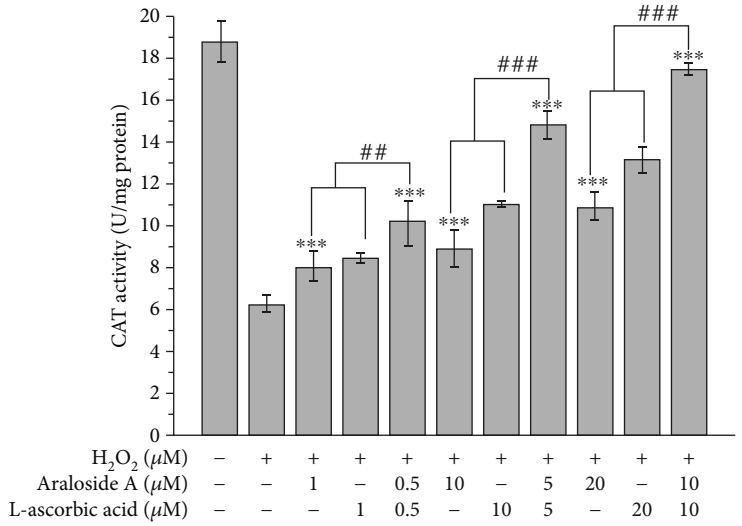
3.7. Individual and Combined Regulatory Effects of Araloside A and L-Ascorbic Acid on the Antioxidant Status of H_2O_2-Exposed HEK293 Cells. H_2O_2 exposure induces oxidative stress-related damage in HEK293 cells by reducing the activity of antioxidant enzymes and destroying the antioxidant defence system. The individual and combined effects of araloside A and L-ascorbic acid on the activities of antioxidant enzymes (CAT, SOD, and GSH-Px) are shown in Figure 5. CAT, SOD, and GSH-Px activities were significantly decreased in the H_2O_2-treated group, but pretreatment with araloside A, L-ascorbic acid, or their combination effectively prevented the H_2O_2-induced reduction in antioxidant enzyme activity. However, combined pretreatment with araloside A and L-ascorbic acid inhibited the reduction in antioxidant enzyme activity more effectively than individual araloside A or L-ascorbic acid pretreatment. For example, the SR of SOD was 1.88 ± 0.04 (SR > 1) in the araloside A-L-ascorbic acid group (10 μM), indicating synergistic effects (Table 1). H_2O_2 treatment also decreases the T-AOC of HEK293 cells. T-AOC activity decreased by 0.38 mM TE/g FM in the H_2O_2-treated group compared with that of the control group. However, the T-AOC after treatment with the combination of araloside A and L-ascorbic acid (10 μM) increased by 0.35 mM TE/g, which was markedly higher than the increases obtained with individual pretreatment. HEK293 cells exposed to H_2O_2 exhibited a decreased GSH/GSSG ratio and an increased level of GSSG (Figures 5(e) and 5(f)). Compared with individual pretreatment, combined pretreatment with araloside A and L-ascorbic acid significantly reduced the GSSG content and increased the GSH/GSSG ratio in a concentration-dependent manner. Araloside A and L-ascorbic acid inhibited H_2O_2-induced oxidative stress-related damage by increasing antioxidant enzyme activity and maintaining the antioxidant defence system. Table 1 shows that the SRs of antioxidant enzymes, T-AOC, and the GSH/GSSG ratio were all greater than 1. These results further support the presence of a synergistic effect between araloside A and L-ascorbic acid.

3.8. Individual and Combined Effects of Araloside A and L-Ascorbic Acid on the MDA Content, LPO Content, and H_2O_2 Level. To further evaluate the individual and combined effects of araloside A and L-ascorbic acid on H_2O_2-induced oxidative stress, the MDA level, LPO content, and H_2O_2 level of each treatment group were measured using kits (Figure 6). The MDA level of the control group was 12.26 nmol/mg protein, and H_2O_2 exposure significantly increased the MDA level to approximately 4-fold that of the control group. However, the MDA level obtained after pretreatment with araloside A or L-ascorbic acid was significantly lower than that of the injury group, which indicated that pretreatment with araloside A or L-ascorbic acid effectively inhibited the H_2O_2-induced production of MDA. In addition, the MDA level in HEK293 cells treated with araloside A combined with L-ascorbic acid was lower than those after individual pretreatment, and this effect was concentration-dependent. Furthermore, the LPO content in the combined pretreatment groups (0.5 μM + 0.5 μM: 16.13 μmol/g protein; 5 μM + 5 μM: 14.55 μmol/g protein; 10 μM + 10 μM: 10.71 μmol/g protein) was also lower than that after individual pretreatment (1, 10, and 20 μM araloside A: 19.17, 17.11, and 14.49 μmol/g protein, respectively; 1, 10, and 20 μM L-ascorbic acid: 18.22, 16.95, and 13.63 μmol/g protein, respectively). Measurement of the H_2O_2 levels revealed the same trend after comparison in the same way. This experiment indicated that araloside A and L-ascorbic acid inhibited H_2O_2-induced oxidative stress-related damage in HEK293 cells by reducing the degree of lipid oxidation and levels of free radicals in a synergistic manner (SR < 1).

3.9. Individual and Combined Protective Effects of Araloside A and L-Ascorbic Acid against Protein and DNA Damage. H_2O_2-induced oxidative stress caused varying degrees of oxidative damage to proteins and DNA (Figures 6(d) and 6(e)). Figure 6 shows that the protein carbonyl and 8-OHdG levels in HEK293 cells exposed to H_2O_2 were significantly higher than those of the control group. However, the protein carbonyl and 8-OHdG levels after pretreatment with araloside A, L-ascorbic acid, or their combination were significantly reduced compared with those of the injury group. Surprisingly, the reduction in the combined pretreatment group (araloside A+L-ascorbic acid) was more significant than those after individual pretreatment. For example, the SRs for protein carbonyl and 8-OHdG levels in the araloside A-L-ascorbic acid group (10 μM + 10 μM) were 0.77 ± 0.04 and 0.80 ± 0.01 (SR < 1), respectively, indicating an obvious synergistic effect (Table 1). These results demonstrated that araloside A and L-ascorbic acid synergistically protect proteins and DNA from H_2O_2-induced damage.

3.10. Combined Effects of Araloside A and L-Ascorbic Acid on In Vitro Free Radical Scavenging Capacity. The combined chemical antioxidant potential of araloside A and L-ascorbic acid was evaluated by measuring the capacity to scavenge $DPPH^{\cdot}$ and $ABTS^{+}$ free radicals, the hydroxyl radical, and the superoxide anion (Figure 7). The results showed that araloside A and L-ascorbic acid induced concentration-dependent increases in antioxidant activity against $DPPH^{\cdot}$ radicals. The $DPPH^{\cdot}$ radical scavenging rate upon combination treatment with 10 μM araloside A and 10 μM L-ascorbic acid was 54.36%, which was significantly higher than that upon treatment of the combination of 0.5 μM araloside A and 0.5 μM L-ascorbic acid. The potential of araloside A and L-ascorbic acid to scavenge $ABTS^{+}$ radicals also increased in a concentration-dependent manner. The effects of combination treatment with araloside A and L-ascorbic acid on the hydroxyl radical and superoxide anion radical scavenging rates followed a trend similar to that shown by the $ABTS^{+}$ radical scavenging rate, and the findings showed that the scavenging rates gradually increased with increasing the pretreatment concentration.

3.11. Correlation between Cell Antioxidant Status and Chemical Antioxidant Capacity. The correlation between chemical antioxidant capacity (the capacity to scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion radicals) and the antioxidant status of HEK293 cells after combined treatment is shown in Figure 8. CAT activity in H_2O_2-injured HEK293 cells after the coadministration of araloside A and L-



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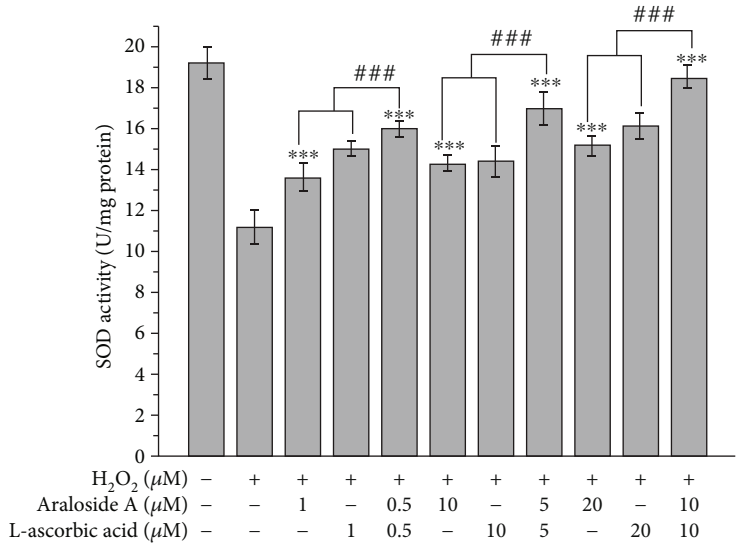

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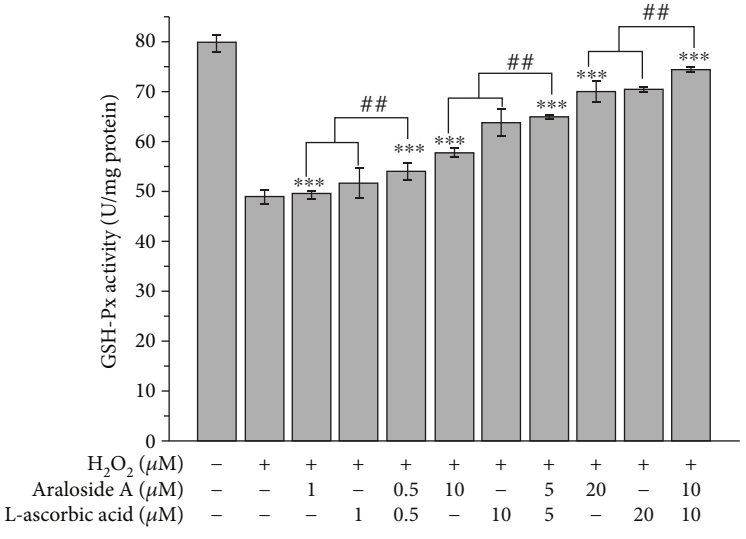

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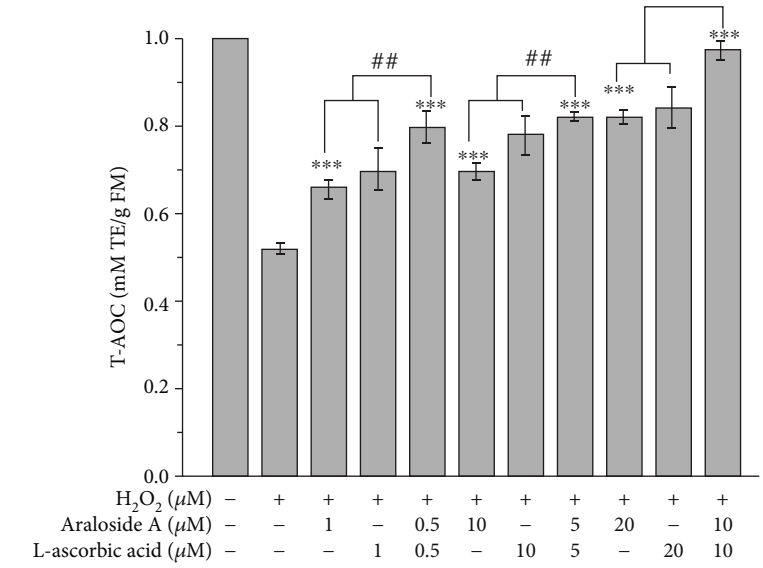

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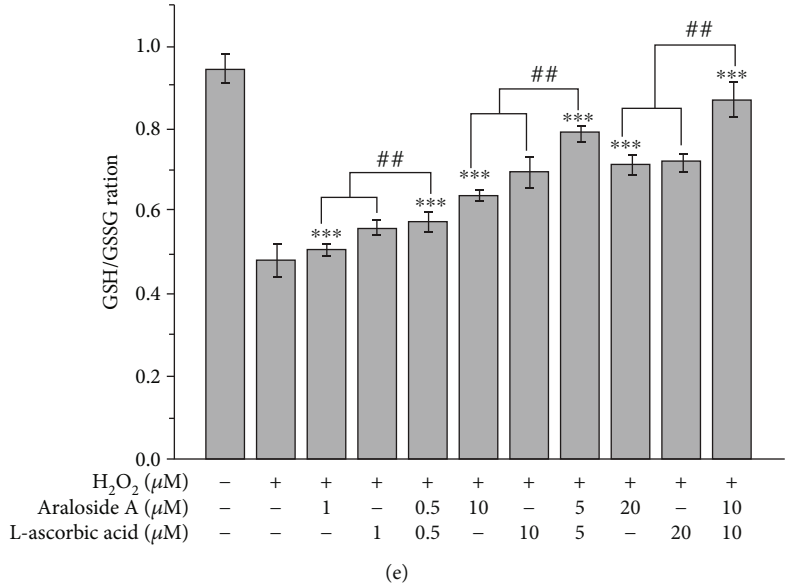

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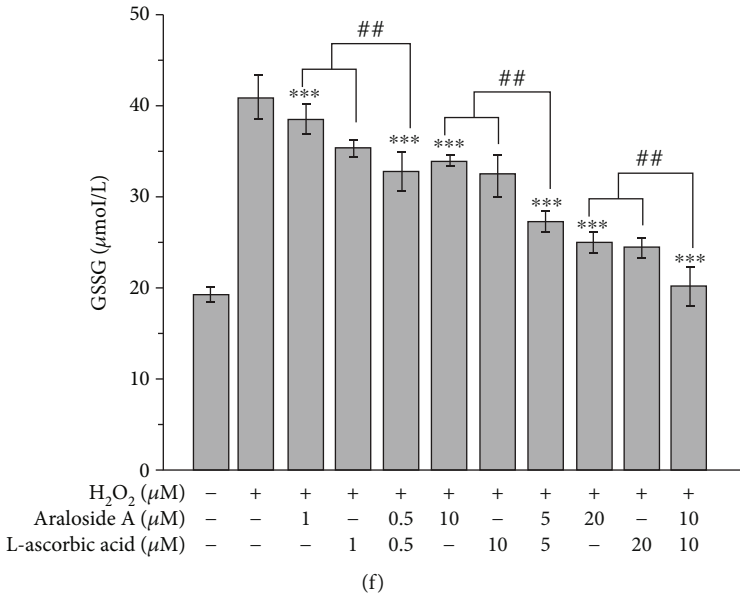


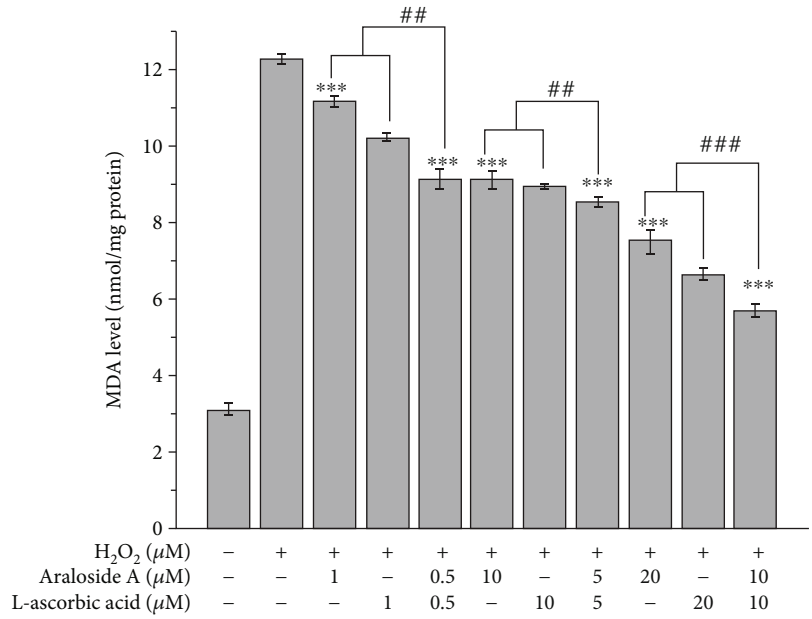
Figure 5: Individual and combined effect of araloside A and L-ascorbic acid on antioxidant status of H_2O_2-exposed HEK293 cells. (a) Catalase (CAT) activity. (b) Superoxide dismutase (SOD) activity. (c) Glutathione peroxidase (GSH-Px) activity. (d) Total antioxidant capacity (T-AOC) activity. (e) Glutathione/oxidized glutathione (GSH/GSSG) ratio. (f) Oxidized glutathione (GSSG) levels. Vertical bars indicate the mean values ± SD. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the araloside A+L-ascorbic acid group. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the injury group.

ascorbic acid was significantly and positively correlated with the capacity to scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion radicals, with correlation coefficients of 0.750 ($p = 0.020$, <0.05), 0.901 ($p = 0.001$, <0.01), 0.740 ($p = 0.023$, <0.05), and 0.717 ($p = 0.030$, <0.05), respectively. A significant positive correlation between SOD activity and chemical antioxidant capacity was found, with correlation coefficients of 0.849 ($p = 0.004$, <0.01, DPPH), 0.779 ($p = 0.013$, <0.05, ABTS), 0.709 ($p = 0.032$, <0.05, hydroxyl), and 0.681 ($p = 0.043$, <0.05, superoxide anion). No significant correlation between GSH-Px activity and the viability or radical scavenging ability of HEK293 cells was found. In addition, the data showed a positive correlation between T-AOC and $DPPH^{\cdot}$ radical scavenging capacity ($r = 0.723$, $p = 0.028$, <0.05). A significant negative correlation ($r = -0.724$, $p = 0.027$, <0.05) was observed between GSSG content and $DPPH^{\cdot}$ radical scavenging capacity, and a positive correlation ($r = 0.722$, $p = 0.028$, <0.05) was found between the GSSG/GSH ratio and $DPPH^{\cdot}$ radical scavenging capacity. The study of oxidation products revealed significant negative correlations ($r = -0.709$, $p = 0.032$, <0.05, MDA; $r = -0.943$, $p = 0.001$, <0.01, LPO) between the MDA level and LPO content with the $ABTS^{+}$ radical scavenging capacity. Moreover, a negative correlation ($r = -0.693$, $p = 0.038$, <0.05) between LDH release and $DPPH^{\cdot}$ radical scavenging capacity was also found. In addition, free radical levels were negatively correlated with protein and DNA damage (protein carbonyl and 8-OHdG levels). Additionally, the capacity to scavenge $DPPH^{\cdot}$ ($r = -0.780$, $p = 0.013$, <0.05, ROS; $r = -0.784$, $p = 0.012$, <0.05, H_2O_2) and $ABTS^{+}$ ($r = -0.698$, $p = 0.036$, <0.05, ROS; $r = -0.768$, $p = 0.016$, <0.05, H_2O_2) was negatively correlated with relative ROS production and the H_2O_2 level.

4. Discussion

Synergistic or antagonistic effects may occur between ingested foods and drugs. Ginsenoside Rg1 and heat shock protein 70 were shown to synergistically inhibit tert-butyl hydroperoxide-induced damage in neural stem cells [27], and synergistic antioxidant effects among vitamin E, epicatechin, and catechin ester acids have been found [28]. In addition, flavanols, pigments (beets, lycopene, and β-carotene), and common anthocyanins in foods exhibit synergistic effects on antioxidant activity by synergistically inhibiting free hydrogen peroxide radicals [29]. Relatively few studies have investigated whether araloside A exerts synergistic or antagonistic effects with certain ingredients in food and medicine. In this study, the synergistic antioxidant effects of araloside A and L-ascorbic acid were evaluated using the isobologram method. The results showed that araloside A and L-ascorbic acid significantly inhibited H_2O_2-induced oxidative stress in HEK293 cells and exerted synergistic effects to reduce intracellular ROS production, the MDA content, the LPO content, LDH release, H_2O_2 levels, the protein carbonyl content, and 8-OHdG levels and increase the activity of related antioxidant enzymes. Moreover, the combination of araloside A and L-ascorbic acid exerted significant effects in the scavenging of $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion radicals. In addition, the inhibitory effects of araloside A and L-ascorbic acid on H_2O_2-induced oxidative stress in HEK293 cells were found to be correlated with their ability to scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion free radicals.

Araloside A can scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion radicals, proving its potential to act as a



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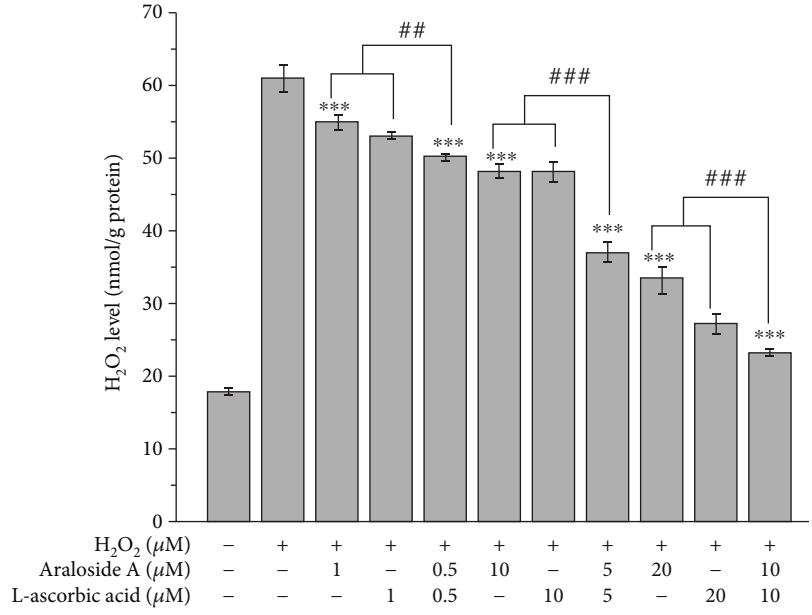

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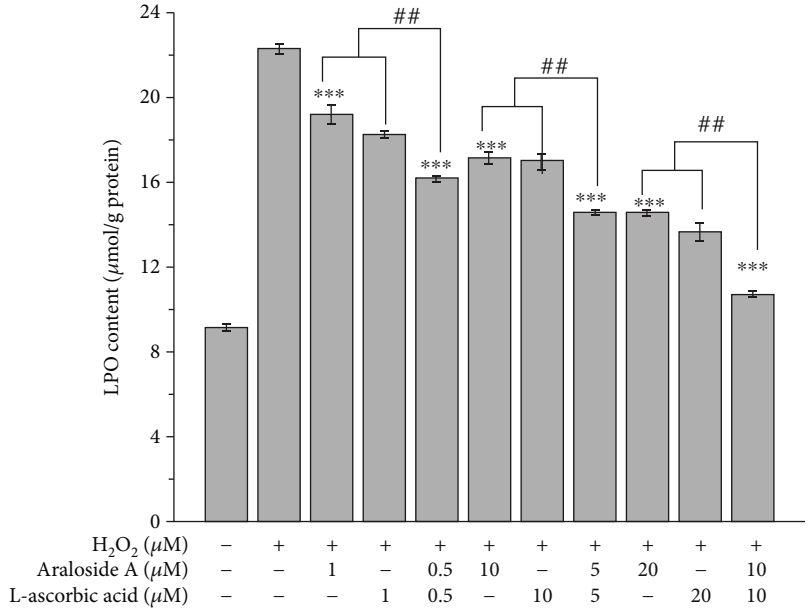

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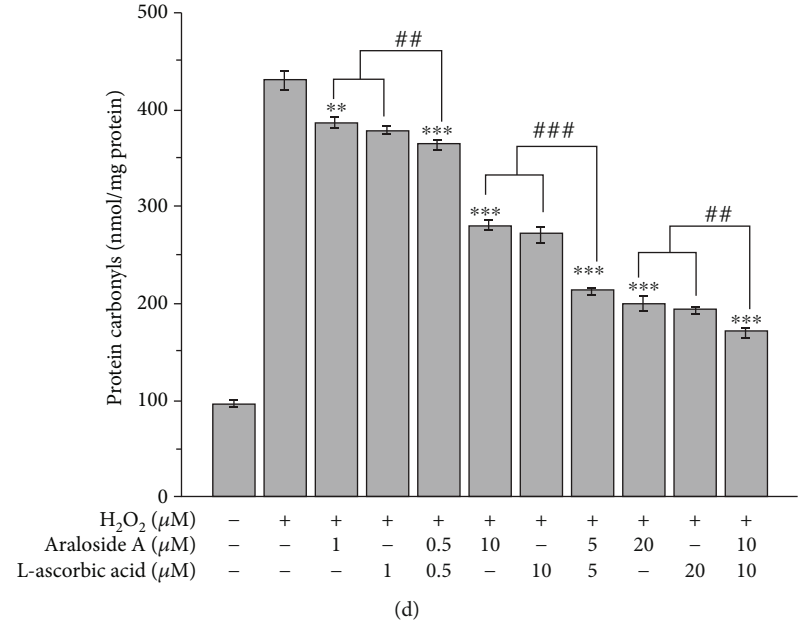

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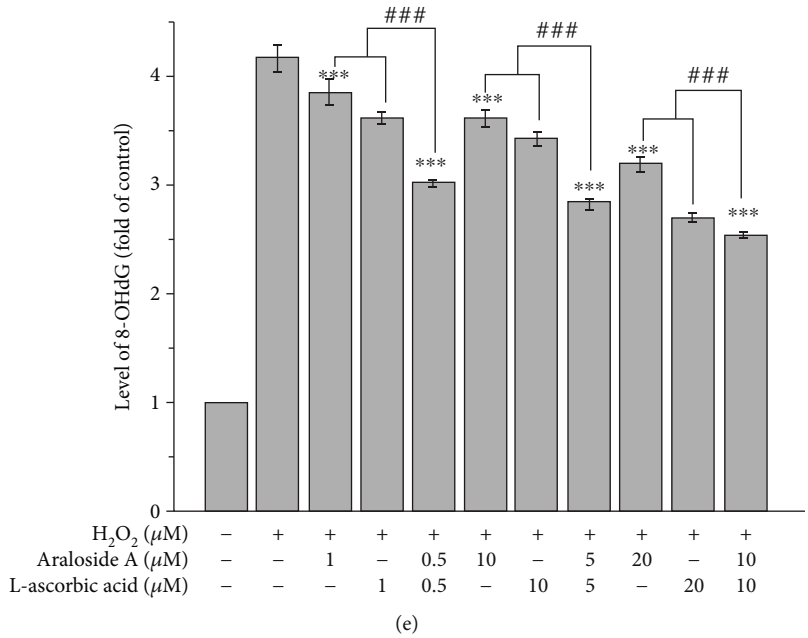

Figure 6: Individual and combined effect of araloside A and L-ascorbic acid on lipid, protein, and DNA damage. (a) Malondialdehyde (MDA) level. (b) H_2O_2 level. (c) Lipid peroxidation (LPO) contents. (d) Protein carbonyl levels. (e) 8-Hydroxy-2-deoxy guanosine (8-OHdG) levels. Vertical bars indicate the mean values ± SD. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the araloside A+L-ascorbic acid group. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the injury group.

prooxidant under certain conditions. Prooxidants at low or moderate concentrations are beneficial for antioxidant defence systems, but at higher concentrations, they act as toxic agents that cause oxidative damage. When the concentration of araloside A exceeded 16 μM or 108 μM, the survival rates of human rheumatoid arthritis fibroblast-like synoviocytes and Caco-2 cells were less than 50% [17, 18]. However, the role of araloside A as a prooxidant should attract attention in cancer therapy. Previous studies have shown that araloside A is an oleanolic acid trioside with glucose, arabinose, and glucuronic acid residues and sugar residues attached to the C-3 and C-28 sites of its mother nucleus (Figure 1(a)) [30]. Yoshikawa et al. suggested that the basic active structure of several oleanosides, such as araloside A, contains a monosaccharide chain at the C-3 position of the mother nucleus [31]. Data from our current study showed that araloside A alleviated H_2O_2-induced oxidative stress in HEK293 cells. This result indicated that araloside A might have lost its glucose residues through the hydrolysis of glycosidic bonds and thus achieved partial antioxidant activity. Among a dozen globally recognized vitamins, L-ascorbic acid has the simplest molecular structure (Figure 1(b)). The antioxidant capacity of L-ascorbic acid, an acidic 3-keto-L-furanogluonic acid lactone with an enol-like structure containing six carbon atoms, primarily manifests through its rapid reaction with free radicals, such as $O_2^{\cdot-}$, $HO^{\cdot}$, and $RO^{\cdot}$. This reaction not only produces semidehydroascorbic acid but also scavenges singlet oxygen and reduces free sulphur radicals [32]. This antioxidant capacity depends on the reversible dehydrogenation reaction [33]. Araloside A and L-ascorbic acid exerted synergistic antioxidant effects, probably due to the combination of the glycosylation activity of araloside A and the dehydrogenation reaction of ascorbic acid.

H_2O_2, a stable source of free radicals, is widely used in the construction of in vitro oxidative stress models. However, its effects depend on the cell type and may differ from those of other prooxidant agents. HepG2 cells were damaged by 10 mM H_2O_2, resulting in a cell viability of approximately 50%. H_2O_2 challenged the antioxidant defence system of HepG2 cells, causing ROS accumulation and protein and lipid damage in this oxidative stress model [34]. However, treatment of SH-SY5Y cells with 250 μM H_2O_2 resulted in a cell death rate of approximately 50% [35]. Wang et al. used HTR8/SVneo cells treated with 200 μM H_2O_2 for 12 h to investigate the protective effects of resveratrol against oxidative stress injury [36]. In contrast, the maximum oxidative damage was achieved when Chinese hamster cells were incubated with high-dose H_2O_2 for 5 min [37]. The optimal conditions for use in our study were 400 μM H_2O_2 and 4 h (Figure 2), which agrees with the experimental results reported by Liu et al. [38]. The individual and combined protective effects of araloside A and L-ascorbic acid against H_2O_2-induced oxidative damage were evaluated by measuring cell viability and LDH release, and the SRs were screened. The results showed that araloside A, L-ascorbic acid, and their combination all increased the viability of H_2O_2-exposed HEK293 cells and decreased the release of LDH (Figure 3). These results reveal the potential synergistic effects of araloside A and L-ascorbic acid and demonstrate that araloside A in combination with L-ascorbic acid alleviates oxidative damage and maintains the integrity of the plasma membrane.

ROS and free radicals are by-products of normal cell metabolism and do not damage cells at low concentrations but contribute to interactions between cells [39]. However, at a too-high intracellular concentration, ROS and free

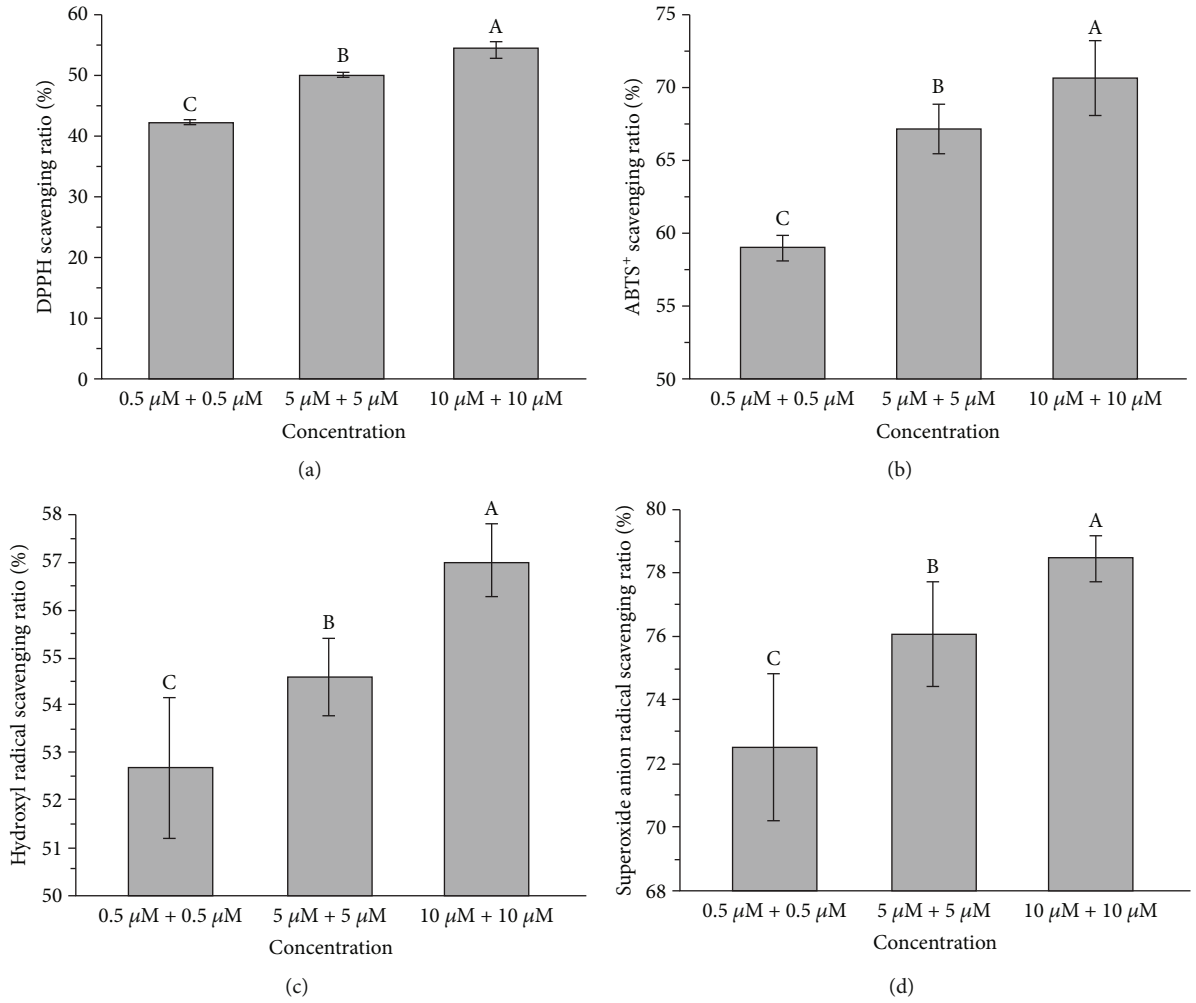

Figure 7: Combined effect of araloside A and L-ascorbic acid on chemical antioxidant capacity. (a) $DPPH^{\cdot}$ free radical scavenging capacity. (b) $ABTS^{+}$ free radical scavenging capacity. (c) Hydroxyl radical scavenging capacity. (d) Superoxide anion radical scavenging capacity. Vertical bars indicate the mean values ± SD. Different letters within each column indicate significant differences at $p < 0.05$.

radicals severely damage cells and tissues, which decreases the mitochondrial membrane potential and induces protein damage and DNA fragmentation [40]. In our experiments, we used the DCFH-DA fluorescent probe assay to evaluate the production and elimination of ROS in HEK293 cells. DCFH-DA is a nonfluorescent substance that can pass through the cell membrane; after entering a cell, this probe is delipidized into DCFH and further oxidized by ROS in the cell to form the highly fluorescent compound DCF [38]. The fluorescence intensity and density of DCF obtained by our experimental measurements reflected the effects of araloside A, L-ascorbic acid, and their combination on the production of intracellular ROS. The results showed that combined pretreatment with araloside A and L-ascorbic acid was more effective in inhibiting H_2O_2-induced intracellular ROS production than individual pretreatment (Figure 4). The protein carbonyl and 8-OHdG levels were used to represent the damaging effect of H_2O_2 on protein and DNA, respectively (Figure 6). Interestingly, compared to individual pretreatment, combined pretreatment more significantly reduced the protein carbonyl and 8-OHdG content. This result suggests that the combination of araloside A and L-ascorbic acid could protect HEK293 cells from H_2O_2-induced oxidative stress-related damage and that this effect might be partially due to the accelerated scavenging of intracellular ROS and reductions in protein and DNA damage.

Antioxidant defence systems include enzymes, including SOD, GSH-Px, and CAT, and nonenzymatic defences, including GSH, alpha tocopherol, and other antioxidants [41]. Antioxidant enzyme activity (SOD, GSH-Px, and CAT), T-AOC, and the GSH/GSSG ratio in HEK293 cells treated with H_2O_2 were decreased compared with those of the untreated cells. However, pretreatment with araloside A, L-ascorbic acid, and their combination significantly increased SOD, GSH-Px, and CAT activities, T-AOC, and the GSH/GSSG ratio (Figure 5). These results demonstrate that araloside A and/or L-ascorbic acid can be used as an exogenous antioxidant to increase the antioxidant defence capacity of cells and that their combination exerts significant synergistic effects. Pal et al. also reported that L-ascorbic acid can increase GSH-Px, SOD, and CAT activity and T-AOC [42], which is consistent with our results. MDA and LPO

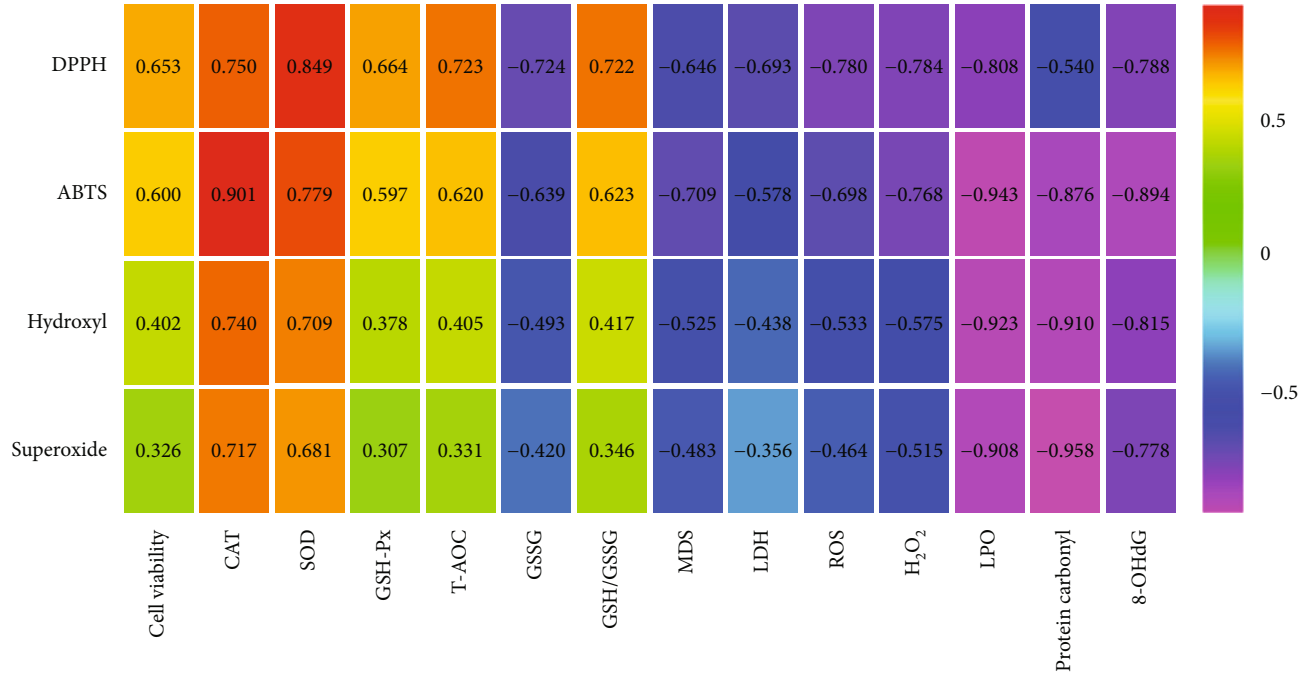

Figure 8: Correlation coefficients of cell antioxidant status and $DPPH^{\cdot}$ radicals scavenging capacity, $ABTS^{+}$ radicals scavenging capacity, hydroxyl radicals scavenging capacity, and superoxide anion radicals scavenging capacity of combination of araloside A and L-ascorbic acid. CAT: the activity of catalase; SOD: the activity of superoxide dismutase; GSH-Px: the activity of glutathione peroxidase; T-AOC: the total antioxidant capacity; GSSG: oxidized glutathione; GSH/GSSG: the glutathione/oxidized glutathione ratio; MDA: the malondialdehyde levels; LDH: the release of lactate dehydrogenase; ROS: the production of reactive oxygen species; H_2O_2: the H_2O_2 levels; LPO: the lipid peroxidation contents; 8-OHdG: the 8-hydroxy-2-deoxy guanosine levels.

are important products of lipid peroxidation often used as biomarkers of oxidative stress levels. L-Ascorbic acid exerts protective effects against cellular oxidative stress-related damage by reducing the cellular MDA content [43]. The data from our study showed that araloside A effectively inhibited the increases in MDA and LPO contents and that combined pretreatment with araloside A and L-ascorbic acid was more effective in inhibiting these increases in the MDA and LPO contents than individual pretreatment with either compound (Figure 6). These results provided evidence demonstrating that araloside A and L-ascorbic acid synergistically inhibit cell lipid peroxidation.

The measurement of $DPPH^{\cdot}$, a stable source of nitrogen free radicals, is one of the most reliable and accurate methods to determine antioxidant capacity and is often used to study the antioxidant activity of active components in plants and animals [44]. Hydroxyl radicals are the most destructive ROS in foods and biological systems [45]. In our study, the capacity to scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anions was measured in vitro to evaluate the combined antioxidant potential of araloside A and L-ascorbic acid. The results also suggested that araloside A and L-ascorbic acid exhibit strong antioxidant capacity at lower concentrations.

Detection of the antioxidant capacity of active ingredients of plants requires determining antioxidant capacity both in vitro and in cells or animals, and whether a correlation between these systems exists must be studied. Ait Lahcen et al. evaluated the antioxidant capacity of a Moroccan *Cistus creticus* leaf extract by measuring $DPPH^{\cdot}$ and FRAP free radical scavenging abilities [46]. Santos et al. used assays to measure oxygen radical absorbance capacity, ferric reducing antioxidant power, and DPPH radical scavenging capacity to evaluate the antioxidant capacity of *Spathodea campanulata* [47]. Wang et al. evaluated the protective effects of araloside C against H_2O_2-induced oxidative stress by assessing the release of LDH, mitochondrial function, and bioenergetics in H9c2 cardiomyocytes [48]. The antioxidant activity of araloside A and L-ascorbic acid was studied in vitro, and their correlation in cellular pathways was analysed. The antioxidant status of HEK293 cells showed a significant and direct correlation with the ability of araloside A and L-ascorbic acid to scavenge $DPPH^{\cdot}$, $ABTS^{+}$, hydroxyl, and superoxide anion radicals (Figure 8). The free radical scavenging ability was found to be positively correlated with SOD and CAT activity, the GSH/GSSG ratio, and T-AOC but negatively correlated with GSSG, MDA, LPO, LDH, protein carbonyl, 8-OHdG, H_2O_2, and ROS levels. Furthermore, the radical scavenging ability showed no correlation with cell viability or GSH-Px activity. In vitro antioxidant activity, particularly the $DPPH^{\cdot}$ free radical scavenging ability, might predict the effects of araloside A and L-ascorbic acid on the antioxidant status of cells.

In general, our manuscript still has some limitations. First, we evaluated only the synergistic antioxidant effects of araloside A and L-ascorbic acid, but determining whether these agents have synergistic anti-inflammatory and anticancer effects requires further discussion. Second, the indicators

used to assess the protective effect of araloside A and L-ascorbic acid against protein and lipid damage are insufficient. The next step is to determine the signalling pathways that regulate their synergistic antioxidant mechanism, such as the Nrf2-Keap1 system.

5. Conclusions

The synergistic antioxidant activity of araloside A and L-ascorbic acid indicates that their combination has the potential to alleviate oxidative damage to food and biological systems. Studying the synergistic effects of natural antioxidants is important for the treatment of diseases related to oxidative stress. Our research results provide new ideas regarding the interaction between different plant active ingredients and the feasibility of the use of various dietary combinations involved in multiple pathways to regulate oxidative stress-induced damage and diseases.

Data Availability

Data will be available on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Probiotics Fermentation Technology, a Novel Kefir Product, Ameliorates Cognitive Impairment in Streptozotocin-Induced Sporadic Alzheimer's Disease in Mice

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Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive impairment. Gut microbiota dysfunction (dysbiosis) is implicated in the pathology of AD and is associated with several detrimental consequences, including neurotransmitter depletion, oxidative stress, inflammation, apoptosis, and insulin resistance, which all contribute to the onset of AD. The objective of this study was to assess the effectiveness of Probiotics Fermentation Technology (PFT), a kefir product, in alleviating AD symptoms via regulation of the gut microbiota using a streptozotocin- (STZ-) induced AD mouse model and to compare its activity with simvastatin, which has been proven to effectively treat AD. Mice received one intracerebroventricular injection of STZ (3 mg/kg). PFT (100, 300, 600 mg/kg) and simvastatin (20 mg/kg) were administered orally for 3 weeks. PFT supplementation mitigated STZ-induced neuronal degeneration in the cortex and hippocampus, restored hippocampal acetylcholine levels, and improved cognition in a dose-dependent manner. These effects were accompanied by reductions in oxidative damage, proinflammatory cytokine expression, apoptosis, and tau hyperphosphorylation. Moreover, PFT hindered amyloid plaque accumulation via the enhancement of insulin-degrading enzyme. These beneficial effects were comparable to those produced by simvastatin. The results suggest that PFT can alleviate AD symptoms by regulating the gut microbiota and by inhibiting AD-related pathological events.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder [1]. It is a devastating disease that is characterized by progressive cognitive impairment and memory loss [2]. The fundamental pathological hallmarks of AD are amyloid plaques (extracellular accumulations of abnormally folded amyloid beta protein (Aβ)) and neurofibrillary tangles that are composed of hyperphosphorylated tau protein [3]. The pathophysiological aspects of AD have not yet been fully investigated; however, synaptic dysfunction, membrane permeabilization, oxidative stress, inflammation, apoptosis, and a reduction in cerebral glucose utilization have been identified as risk factors of AD progression [4, 5]. Interestingly, it has recently been proposed that gut microbiota dysfunction (dysbiosis) correlates with the onset of AD [6, 7]. This hypothesis is based on many lines of evidence. First, the gut microbiota composition is influenced by aging, the main risk factor for AD [8]. Second, alterations in the microbiota lead to the release of significant quantities of amyloids and lipopolysaccharides, which modulate signaling pathways, increase the permeability of the intestine and the blood-brain barrier, and produce proinflammatory cytokines, which are all related to AD pathogenesis [7, 9, 10]. Third, dysbiosis generates oxidative stress, which is also associated with AD [11]. Furthermore, disturbance of the gut microbiome confers insulin resistance, which has also been linked with AD [8]. Therefore, AD is intricately interrelated with gut microbiota imbalance and may initiate from the gut. To this end, the modulation of

the gut microbiota has become of increasing interest in the quest for new AD therapeutic agents.

Probiotic fermentation technology (PFT), a kefir grain product, is extracted from kefir (fermented milk) [12, 13]. Kefir is a health-endorsing probiotic drink that is formed by the fermentation of milk with kefir grains and is composed of mainly *Lactobacillus kefiri*. Earlier studies have demonstrated the numerous health benefits of kefir-derived *Lactobacillus kefiri*: it improves gut health by preserving the probiotic bacteria balance and reduces oxidative stress, inflammation, and insulin resistance [12, 14–17]. Interestingly, PFT has recently been examined for its ability to exert anticancer effects in animals with Ehrlich ascites carcinoma and in human gastric cancer cells in vitro [18, 19]. Since the majority of AD patients are elderly, the development of safe, well-tolerated drugs is important. Our recent study showed the effectiveness of PFT supplementation in modulating age-associated oxidative stress, suggesting that PFT may be a valuable therapeutic intervention for AD [20]. In addition, PFT has been shown to be a safe, nontoxic agent with no side effects [18].

In this study, we explore the potential effects of PFT in the treatment of AD, as well as comparing its activity with simvastatin, which has been proven to treat dementia, reduce cognitive decline in AD patients, and diminish the prevalence of AD [21, 22].

2. Materials and Methods

2.1. Animals. Adult male albino mice (25–30 g) were obtained from the animal facility of the Faculty of Pharmacy, Cairo University, Egypt. Mice were housed at a constant temperature (25°C ± 2°C) and humidity level (60% ± 10%) under a 12/12 h light/dark cycle and were allowed a standard chow diet and water ad libitum. The study was approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) (Permit Number: CU-III-F-35-20) and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

2.2. Chemicals and Drugs. PFT kefir grain product is a mixture that primarily (~90%) consists of a heat-killed freeze-dried form of *L. kefiri* P-IF, whose characteristics have been reported previously [12, 13]. PFT also contains ~2%–3% of the following: one bacterial strain (*L. kefiri* P-B1) and the yeast strains *Kazachstania turicensis*, *Kazachstania unispora*, and *Kluyveromyces marxianus*. Positron-emission tomography scans showed 99.6% homology with regular kefirs. The yeast strains are not intentionally added but are present in large amounts when the product is obtained from the Caucasus mountains and are filtered out in order to maximize the *Lactobacillus kefiri* levels. PFT was provided by Paitos Co. Ltd. (Yokohama, Kanagawa, Japan).

Streptozotocin (STZ) and simvastatin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals and reagents, unless otherwise specified, were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Induction of AD. Intracerebroventricular (ICV) injection of STZ, first described by Pelleymounter et al. and modified by Warnock, was used in the current study for the avoidance of cerebral vein penetration [23–25]. Mice were anesthetized with thiopental (5 mg/kg, i.p.), then the mouse's head was stabilized using downward pressure above the ears, and the needle was inserted directly through the skin and skull into the lateral ventricle, which was targeted by visualizing an equilateral triangle between the eyes and the center of the skull to locate the bregma, allowing the needle to be inserted at the following coordinates from the bregma: 1 mm mediolateral, −0.1 mm anteroposterior, and−3 mm dorsoventral. Mice behaved normally approximately 1 min following the injection. The accuracy of the injection technique was established by injecting methylene blue dye that was detected in the lateral ventricles [26].

2.4. Experimental Design. Mice were randomly allocated into six groups, with 12 mice in each group. The study was conducted for a total of 21 days. Group I, the sham control group, received one ICV injection of 0.9% saline, in addition to oral administration of Tween 80 with 0.9% saline, daily for 21 consecutive days. Group II, the AD-model group, received one ICV injection of STZ (3 mg/kg) dissolved in 0.9% saline [27]. Groups III to VI received one ICV STZ injection (3 mg/kg), and group III was treated with simvastatin (20 mg/kg, p.o.) suspended in Tween 80 with 0.9% saline 5 h after STZ injection and then daily for a total of 21 doses [28]. Groups IV to VI were treated with PFT (100 mg, 300 mg, and 600 mg/kg, p.o., respectively) suspended in Tween 80 with 0.9% saline 5 h after STZ injection and then daily for a total of 21 doses (Figure 1) [29].

2.5. Behavioral Assessment. Twenty-four hours after the last drug doses, behavioral tests were conducted. These tests were arranged sequentially from least stressful to most stressful and were carried out during the light cycle under top lighting in order to lessen potential circadian variability.

2.5.1. Novel Object Recognition Test. The novel object recognition (NOR) test, used to assess learning and memory, is based on the innate preference for novelty that mice exhibit, where mice that remember the familiar object will take more time exploring a novel object. This test was accomplished by following the procedure described by Antunes and Biala [30]. The test took place over three consecutive days: habituation day, training day, and test day (days 20, 21, and 22, respectively). On habituation day, mice were placed individually into a wooden box (40 cm × 40 cm × 40 cm) and were allowed to explore the environment (without objects) for 10 min. On training day, mice were placed individually in the middle of the box and given 10 min to visually explore two identical objects placed along the diagonal of the box (one in the northwest corner and one in the southeast corner). On testing day, one of the previously explored objects was replaced by a novel object (the diagonal used on the training day was the same as that used on the testing day), and each mouse was given 3 min to explore the objects in the box. Objects that are simply discriminated by mice with

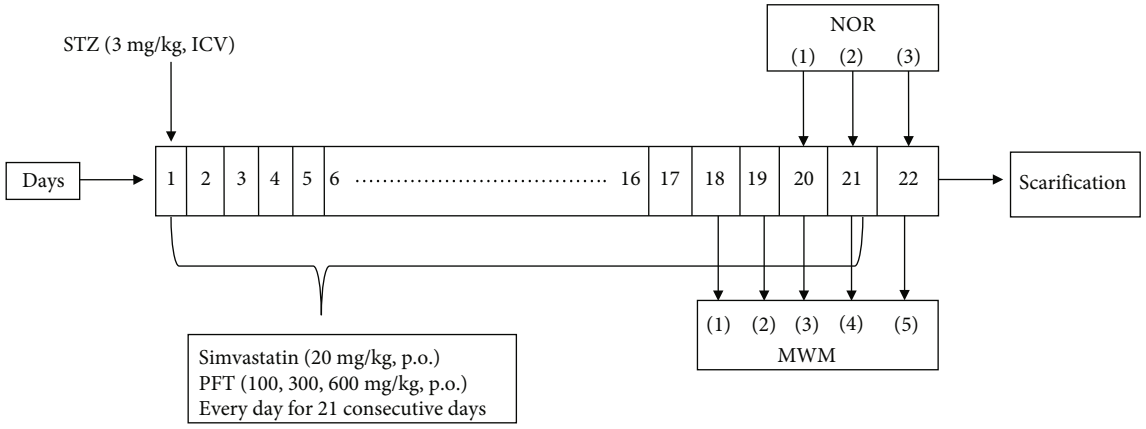

Figure 1: Experimental design.

the presence of protrusions/intrusions on the surface and have a comparable degree of complexity (texture, shape, color patterning, and brightness, etc.) were selected in order to diminish bias due to object preference [31]. Moreover, objects that are mouse-sized or only slightly larger to encourage exploration were used, and they were made of nonbreakable material, to avoid object damage during experimentation and hence interfere with the continuing testing and to avoid animals' injury. Great attention was directed to the object odors; thus, multiple copies of the sample objects were available and each copy was used once only for the same animal and all objects were cleaned with 70% ethanol after each test to eliminate possible bias due to odors left by previous animals [32]. Exploration was defined as directing the nose to the object at a distance of less than 2 cm, while sitting on the object was not considered exploration. The animals' behavior was video recorded, and the following parameters were calculated:

(1) Discrimination Index. Difference in time exploring the novel and familiar objects divided by the total time spent exploring both objects (this value varies between −1 and +1, with a negative score indicating that the mouse spends more time exploring the familiar object, a zero-score indicating that the mouse has no preference, and a positive score indicating that the mouse spends more time exploring the novel object).

(2) Recognition Index. Time spent by the animal exploring the novel object as a percentage of the total exploration time.

2.5.2. Morris Water Maze Test. The Morris water maze (MWM) test, used to investigate spatial memory and learning, was performed as described by D'Hooge and De Deyn [33]. A stainless-steel circular tank (150 cm in diameter and 60 cm in height) was filled with water (25°C ± 2°C) to a depth of 35 cm and divided into four quadrants. A black platform (10 cm width, 28 cm height) was positioned inside the target quadrant, and the tank was filled to 2 cm below the top of the platform. The platform was maintained in the same location during the training and testing procedures. During testing, the platform was made invisible to the mice by making the water opaque with a purple-colored nontoxic tempera paint. It is important to ensure that animals can swim adequately before performing the training and testing procedures [34]. This was accomplished by allowing each mouse to swim in the pool to reach a platform that is maintained above the water level. The animal that can swim directly to the visible platform without difficulty was used to perform training and testing procedures. Memory acquisition trials (120 s/trial) were performed over four consecutive days (days 18, 19, 20, and 21), twice a day, with at least 15 min between trials. During training, animals were left to locate the hidden platform in the target quadrant. If the mouse found the hidden platform within 120 s, it was kept there for an additional 20 s before being removed, while if it failed to find the hidden platform during the designated time, the mouse was gently guided to the platform and kept there for 20 s. The mean escape latency was calculated as the time taken by each mouse to find the hidden platform and was used as an index of acquisition or learning. On the fifth day (day 22), mice underwent a probe trial session, in which the platform was removed, and each mouse was given 60 s to explore the pool. The time each mouse spent in the target quadrant in which the hidden platform was previously placed was determined and used as an indicator of retrieval or memory.

2.6. Brain Processing. After evaluation of cognitive performance, the mice in each group were divided into three sets and were euthanized by cervical dislocation under light anesthesia. Subsequently, the brains were rapidly dissected and washed with ice-cold saline. In the first set ($n = 3$ per group), brains were fixed in 10% (v/v) formalin for 24 h to perform histopathological staining with hematoxylin and eosin (HE), Congo red, and Nissl stain. In the other sets, the hippocampal tissues were excised from each brain on an ice-cold glass plate. In the second set ($n = 6$ per group), the hippocampi were homogenized in ice-cold physiological saline (10% w/v), then the hippocampal homogenates were used for determination of the levels of acetylcholine (Ach), amyloid $\beta_{1\text{-}42}$, oxidative stress parameters (malondialdehyde, MDA; nuclear factor erythroid 2-related factor 2, Nrf-2; heme oxygenase-1, HO-1; and glutathione, GSH), inflammatory markers (nuclear factor kappa beta, NF-κB; NLRP3;

Table 1: The primer sequences.

Gene	Forward primer	Reverse primer
β-Actin	5′-TATCCTGGCCTCACTGTCCA-3′	5′-AACGCAGCTCAGTAACAGTC-3′
ERK1/2	5′-TCAAGCCTTCCAACCTC-3′	5′-GCAGCCCACAGACCAAA-3′
p38-MAPK	5′-AGGGCGATGTGACGTTT-3′	5′-CTGGCAGGGTGAAGTTGG-3′

interleukin 1β, IL-1β; and tumor necrosis factor-alpha, TNF-α), caspase-3, and insulin-degrading enzyme (IDE). In the third set ($n = 3$ per group), hippocampi were used for the assessment of protein expression of phosphorylated tau, glycogen synthase kinase 3β (GSK-3β), and mammalian target of rapamycin (mTOR), as well as the determination of extracellular signal-regulated protein kinase (ERK1/2) and p38 mitogen-activated protein kinase (p38-MAPK) gene expression.

2.7. Measured Parameters

2.7.1. Enzyme-Linked Immunosorbent Assay of Levels of Ach, Amyloid $\beta_{1\text{-}42}$, MDA, Nrf-2, HO-1, NF-κB, NLRP3, IL-1β, TNF-α, Caspase-3, and IDE in the Hippocampus. Mouse ELISA kits for Ach (Cat. # MBS733116), MDA (Cat. # MBS269473), Nrf-2 (Cat. # MBS2516218), HO-1 (Cat. # MBS760394), NF-κB (Cat. # MBS2023542), NLRP3 (Cat. # MBS920134), caspase-3 (Cat. # MBS733100), and IDE (Cat. # MBS2019335) were obtained from Mybiosource (San Diego, CA, USA). Mouse ELISA kits for amyloid $\beta_{1\text{-}42}$ (Cat. # KMB3441), IL-1β (Cat. # BMS6002), and TNF-α (Cat. # BMS607-3) were purchased from Invitrogen (Carlsbad, CA, USA). All procedures were performed according to the manufacturer's instructions. The results were expressed as ng/mg protein for Ach, Nrf-2, NLRP3, and caspase-3, as pg/mg protein for HO-1, amyloid $\beta_{1\text{-}42}$, NF-κB, IL-1β, TNF-α, and IDE, and as nmol/mg protein for MDA.

2.7.2. Colorimetric Determination of GSH. GSH, an antioxidant peptide, was assessed using a colorimetric kit (Biodiagnostics, Cairo, Egypt). Briefly, 0.1 ml of the hippocampal homogenate was mixed with 0.5 ml of trichloroacetic acid (500 nmol/L) and left for 5 min for precipitation of protein SH-groups. Then, the solutions were centrifuged at 3000 rpm for 15 min. After centrifugation, 0.5 ml of a protein-free supernatant was added to 1 ml of phosphate buffer solution (100 mmol/L) and 0.1 ml of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) (1 mmol/L), which is reduced by GSH to form a stable yellow product (5-mercapto-2-nitrobenzoic acid) that is directly proportional to the amount of GSH in the sample. The absorbance was measured at 405 nm after 5-10 min against blank, which was similarly prepared using 0.5 ml of distilled water instead of the sample, using a double beam computerized spectrophotometer (Thermo Electron Corporation, evolution 100, Altrincham, England). GSH concentration was calculated from the following equation and was expressed as mmol/mg protein.

GSH concentration = absorbance of the sample × 2.22/mg tissue used.

2.7.3. Western Blot Analysis of Phosphorylated Tau, GSK-3β, and mTOR Protein Expression in the Hippocampus. Protein solutions were extracted from hippocampal tissues; then, equal amounts of protein were attached onto a sodium dodecyl sulfate-polyacrylamide gel to be separated by electrophoresis according to their molecular weight. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Bioscience, Piscataway, NJ, USA) using semidry transfer apparatus (Bio-Rad, Hercules, CA, USA). The membranes' nonspecific binding sites were then blocked by soaking in 5% skimmed milk. Next, the membranes were incubated at 4°C overnight on a roller shaker with solutions containing antiphosphorylated tau (1 : 10000, Cat. # ab109390), anti-GSK-3β (1 : 5000, Cat. # ab32391), and anti-mTOR (1 : 10000, Cat. # ab134903), which were obtained from Abcam (Cambridge, MA, USA). The membranes were then washed and incubated with the horseradish peroxidase-conjugated secondary antibody solution. Finally, the blots were developed with enhanced chemiluminescence detection reagents (Amersham Biosciences, Arlington Heights, IL, USA). Scanning laser densitometry (GS-800 system, Bio-Rad, Hercules, CA, USA) was used to determine the quantities of the target proteins. The results were normalized with β-actin protein expression and expressed as arbitrary units.

2.7.4. Quantitative Real-Time Polymerase Chain Reaction Analysis of ERK1/2 and p38-MAPK Gene Expression in the Hippocampus. RNA was extracted; then, 1 μg was reverse-transcribed into complementary DNA using an RT-PCR kit (Stratagene, Cat. # 600188, La Jolla, CA, USA) according to the manufacturer's instructions. Quantitative RT-PCR was accomplished using SYBR Green JumpStart Taq ReadyMix (Sigma–Aldrich, Cat. # S5193, St. Louis, MO, USA), where 5 μl of complementary DNA was added to 12.5 μl of SYBR Green, 5.5 μl of RNAse free water, and 2 μl of each primer (5 pmol/μl). The primer sequences are shown in Table 1. The PCR reactions comprised 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The $2^{-\Delta\Delta CT}$ formula was applied to determine the relative expression of target genes, using β-actin as a control.

2.7.5. Determination of Protein Content. The protein content was quantified according to the method described by Lowry et al. [35].

2.8. Histopathological Examination. Brains were carefully separated, washed with ice-cold saline, and immediately fixed in 10% formalin for 24 h. The brains were then washed,

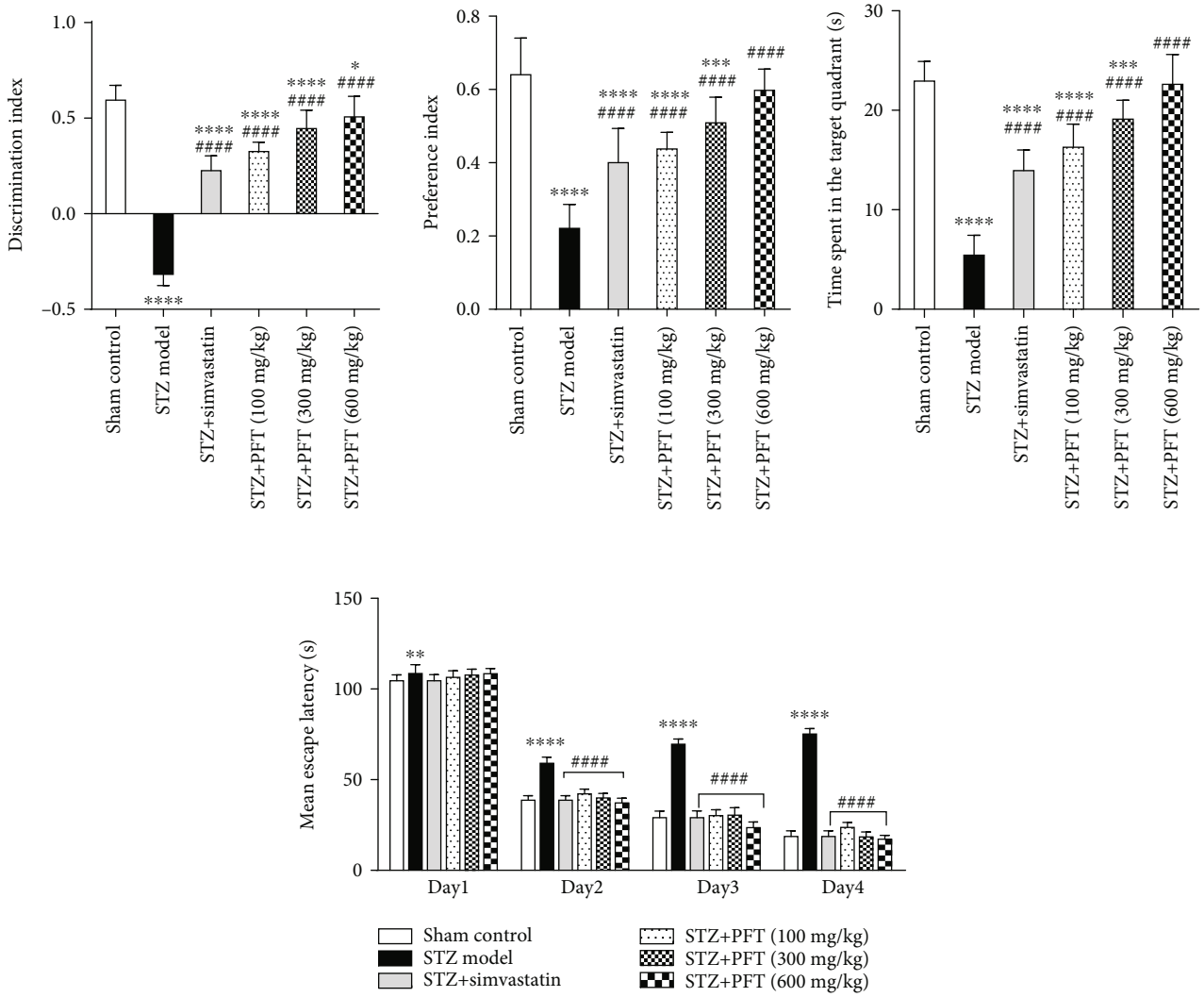

Figure 2: PFT ameliorated STZ-induced cognitive impairment. Each bar with vertical line represents the mean ± SD of 12 mice per group; ∗ significantly different from the sham control group at $p < 0.05$, ∗∗significantly different from the sham control group at $p < 0.01$, ∗∗∗ significantly different from the sham control group at $p < 0.001$, ∗∗∗∗significantly different from the control group at $p < 0.0001$, $^{\#\#\#\#}$significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test for discrimination index, preference index and the time spent in the target quadrant, while using two-way ANOVA for the mean escape latency.

dehydrated in alcohol, and embedded in paraffin blocks. Tissue sections of 4 μm were stained with H & E for preliminary histopathological examination. Congo red stain was applied to detect amyloid plaques, and Nissl stain was used to assess neurodegeneration in the cerebral cortex and the hippocampal regions: the cornu ammonis (CA3&4) and the dentate gyrus (DG), according to the method described by Nobakht et al. [36].

2.9. Statistical Analysis. All data were checked for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Datasets that fulfilled the assumptions for parametric analysis were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test and were expressed as mean ± SD. The amyloid plaque number was analyzed using Kruskal–Wallis nonparametric test followed by Dunn's multiple comparisons test and was expressed as median and range. The mean escape latency in the MWM was analyzed by two-way ANOVA. A probability level of less than 0.05 was accepted as being significant in all statistical tests. Statistical analysis was performed using GraphPad Prism software version 8 (San Diego, CA, USA).

3. Results

3.1. PFT Ameliorated STZ-Induced Cognitive Impairment. In the NOR test, STZ significantly deteriorated memory in mice as compared with the sham control group ($p < 0.0001$) (Figure 2). PFT administration (100, 300, and 600 mg/kg) improved cognitive performance in a dose-dependent manner, which was revealed by a significant increase in the discrimination (2.5%, 39.1%, and 57.9%, respectively) and preference (95.5%, 127.2%, and 166.1%, respectively) indices as compared with the STZ group ($F(5, 66) = 281.6$ and 58.5, respectively, $p < 0.0001$). The effect of PFT was comparable to that of simvastatin, which significantly increased the

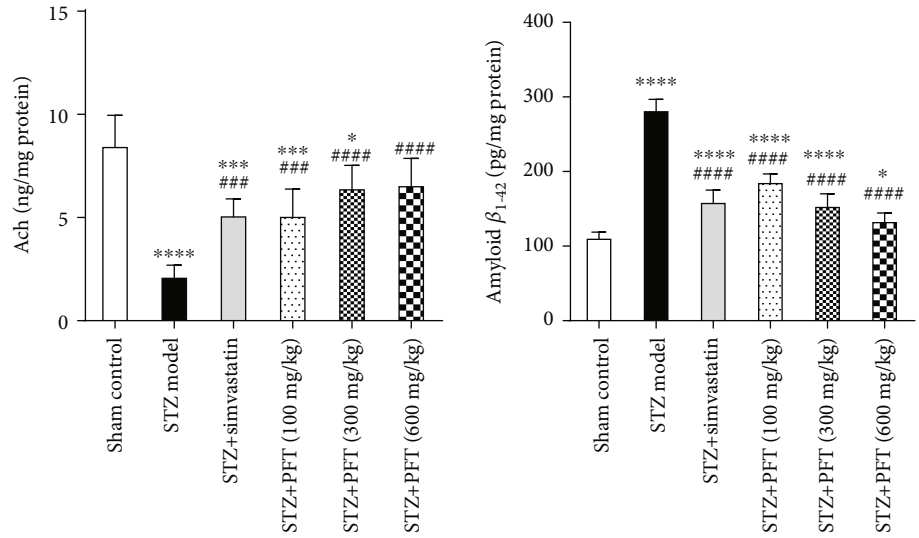

Figure 3: PFT reversed STZ-induced alterations in Ach and amyloid $\beta_{1\text{-}42}$ levels in the hippocampus. Each bar with vertical line represents the mean ± SD of 6 mice per group; ∗significantly different from the sham control group at $p < 0.05$, ∗∗∗significantly different from the sham control group at $p < 0.001$, ∗∗∗∗significantly different from the sham control group at $p < 0.0001$, $^{\#\#\#}$significantly different from STZ group at $p < 0.001$, $^{\#\#\#\#}$significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

discrimination (by 27.9%) and preference indices (by 79.4%) as compared with the STZ group.

On the first day of training in the MWM, there was no significant difference in the mean escape latency between the STZ and treated groups. From the second day until the fourth day, the mice in the treatment groups took a shorter period of time to reach the platform as compared with the STZ group. On the test day, animals treated with PFT (100, 300, and 600 mg/kg) displayed a substantial increase in the time spent in the target quadrant in which the platform was previously located (2.9-, 3.4-, and 4-fold, respectively) as compared with the STZ group ($F(5, 66) = 125$, $p < 0.0001$). The effect of PFT was in line with that of simvastatin (2.5-fold increase as compared with the STZ group) (Figure 2).

3.2. PFT Reversed STZ-Induced Alterations in Ach and Amyloid $\beta_{1\text{-}42}$ Levels in the Hippocampus. Mice that received STZ showed a large decline in Ach levels and an increase in amyloid $\beta_{1\text{-}42}$ level in the hippocampus as compared to their sham control counterparts ($F(5, 30) = 21.9$ and 146, respectively, $p < 0.0001$) (Figure 3). Improvements in cognition by PFT (100, 300, and 600 mg/kg) were reflected by augmentation of hippocampal Ach by 2.4-, 2.9-, and 3-fold, respectively, and suppression of amyloid $\beta_{1\text{-}42}$ by 34.1%, 45.3%, and 52.5%, respectively, as compared with the STZ group ($p < 0.001$ and $p < 0.0001$). These results reflected those exhibited by simvastatin (increase in Ach by 2.4-fold and decrease in amyloid $\beta_{1\text{-}42}$ by 43.4% as compared with STZ group).

3.3. PFT Attenuated STZ-Induced Oxidative Stress. Mice that received STZ exhibited a significant elevation in MDA level ($F(5, 30) = 52.9$) and a reduction in Nrf-2 level, HO-1 level, and GSH activity in the hippocampus as compared with the sham control group ($F(5, 30) = 37.3$, 174.4, and 274.2, respectively) ($p < 0.0001$) (Figure 4). PFT (100, 300, and 600 mg/kg) suppressed the MDA level by 35.2%, 56.2%, and 68.8%, respectively, and restored Nrf-2 level by 2.2-, 2.8-, and 3.8-fold, respectively, as well as HO-1 level by 3.3-, 4.2-, and 5.3-fold, respectively, and GSH activity by 2.1-, 2.5-, and 2.8-fold, respectively, as compared to STZ-exposed mice ($p < 0.0001$). The effect of PFT on MDA level (57.1%) as well as Nrf-2 (2.7-fold), HO-1 (4.7-fold), and GSH activity (2-fold) was similar to that of simvastatin in STZ animals.

3.4. PFT Mitigated STZ-Induced Inflammatory Changes. STZ ICV injection caused an obvious increase in different inflammatory markers, including NF-κB, NLRP3, IL-1β, and TNF-α, in the hippocampus as compared with the sham control group ($F(5, 30) = 21.7$, 68.7, 262.7, and 82.5, respectively, $p < 0.0001$) (Figure 5). PFT treatment (100, 300, and 600 mg/kg) significantly decreased the levels of NF-κB by 37%, 41.8%, and 50.1%, respectively, as well as NLRP3 (20.1%, 48.4%, and 50.3%, respectively), IL-1β (43.8%, 50.1%, and 50.6%, respectively), and TNF-α (39.2%, 43.6%, and 59.7, respectively) as compared with the STZ group ($p < 0.001$ and $p < 0.0001$). The effects of PFT on these markers were similar to those of simvastatin, which decreased NF-κB by 49.1%, NLRP3 by 33.1%, IL-1β by 48.2%, and TNF-α by 50.6%.

3.5. PFT Alleviated STZ-Induced Alterations in Caspase-3 Level in the Hippocampus. In mice that received STZ, the hippocampal caspase-3 level was significantly higher in comparison to the sham control group ($F(5, 30) = 37.8$, $p < 0.0001$) (Figure 6), an effect that was ameliorated by PFT treatment (100, 300, and 600 mg/kg), which decreased the caspase-3 level by 53%, 59.2%, and 63.9%, respectively, as compared with the STZ group ($p < 0.0001$). The activity of PFT was

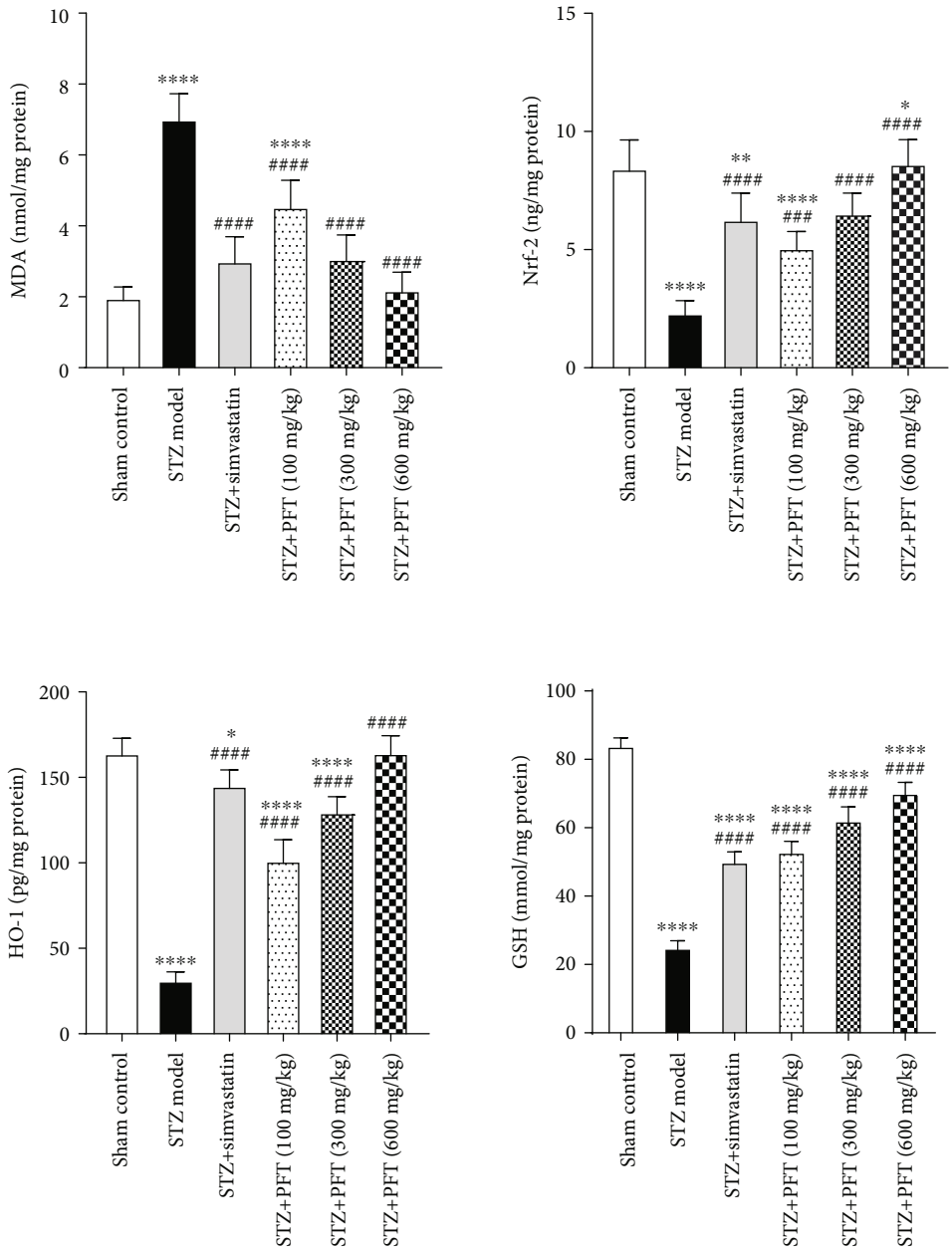

Figure 4: PFT attenuated STZ-induced oxidative stress. Each bar with vertical line represents the mean ± SD of 6 mice per group; ** significantly different from the sham control group at $p < 0.05$, **significantly different from the sham control group at $p < 0.01$, **** significantly different from the sham control group at $p < 0.0001$, ####significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

comparable to that of simvastatin, which hampered caspase-3 activity by 59.2%.

3.6. PFT Inverted STZ-Induced Alterations in the IDE Level in the Hippocampus. IDE, a key Aβ degrading enzyme, was also examined. STZ injection caused an obvious decline in IDE level in the hippocampus as compared with the sham control group ($F(5, 30) = 183.8$, $p < 0.0001$) (Figure 7). Supplementation with PFT (100, 300, and 600 mg/kg) significantly raised the level of IDE 1.5-, 1.6-, and 1.8-fold, respectively, as compared with the STZ group ($p < 0.0001$), an effect that was comparable to that of simvastatin in STZ mice (1.3-fold).

3.7. PFT Amended the Changes Induced by STZ in Phosphorylated Tau, ERK1/2, p38-MAPK, GSK-3β, and mTOR Expression in the Hippocampus. STZ significantly elevated the expression of phosphorylated tau ($F(5, 12) = 130.2$), ERK1/2 ($F(5, 12) = 104.2$), p38-MAPK ($F(5, 12) = 121.7$), GSK-3β ($F(5, 12) = 216.6$), and mTOR ($F(5, 12) = 132.1$) in the hippocampus as compared with the sham control group ($p < 0.0001$) (Figure 8). PFT administration (100, 300, and 600 mg/kg) significantly ameliorated the expression of phosphorylated tau (47%, 51.8%, and 54.3%, respectively), ERK1/2 (51.3%, 53%, and 70.4%, respectively), p38-MAPK (55%, 55%, and 71.8%, respectively), GSK-3β (52.3%, 60.4%,

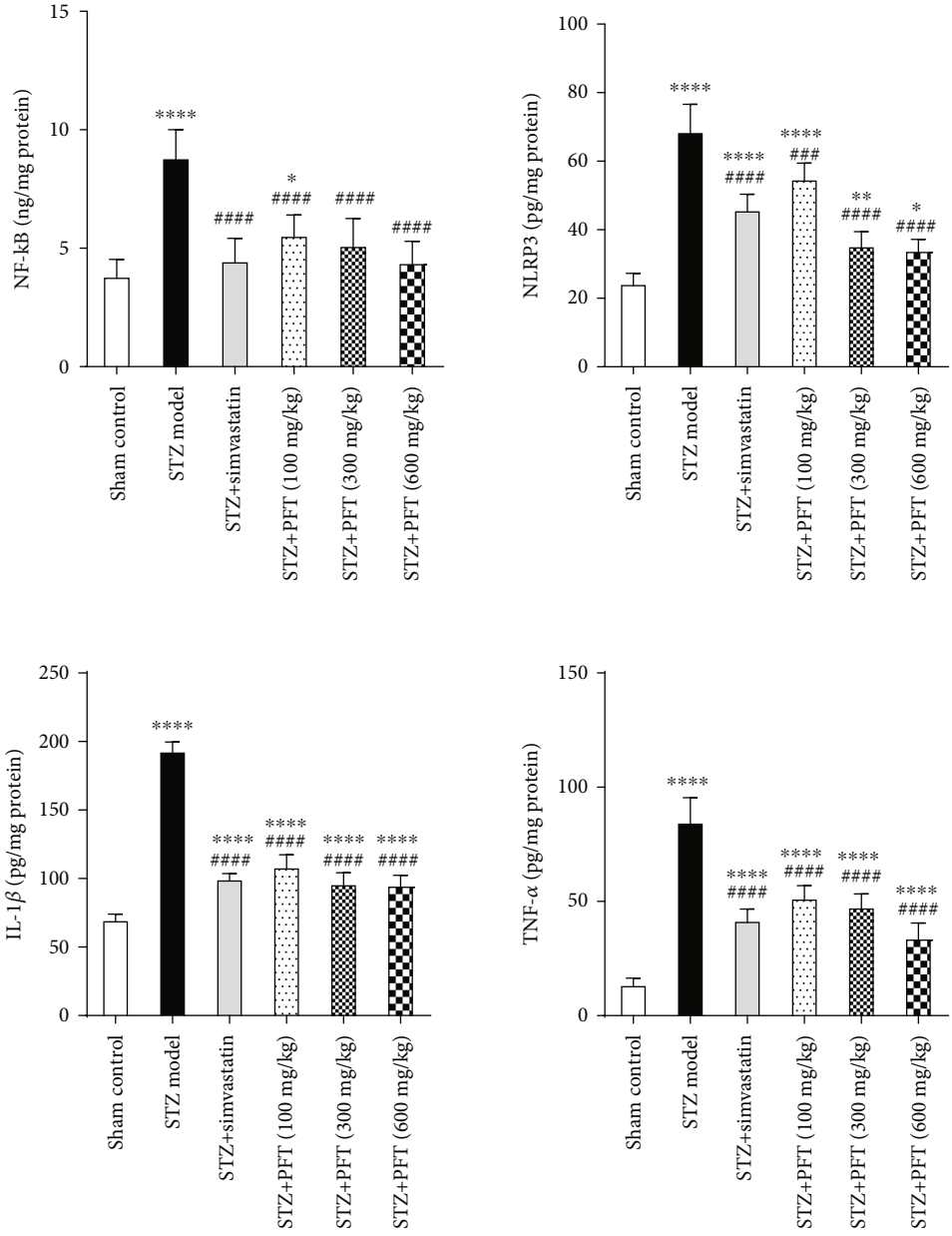

Figure 5: PFT mitigated STZ-induced inflammatory changes. Each bar with vertical line represents the mean ± SD of 6 mice per group; * significantly different from the sham control group at $p < 0.05$, **significantly different from the sham control group at $p < 0.01$, **** significantly different from the sham control group at $p < 0.0001$, ###significantly different from STZ group at $p < 0.001$, ####significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

and 66.7%, respectively), and mTOR (41%, 38.5%, and 37.2, respectively) in the hippocampus as compared with the STZ group ($p < 0.0001$). The favorable outcomes of PFT treatment were comparable to those of simvastatin, which diminished the expression of phosphorylated tau by 45.9%, ERK1/2 by 69.1%, p38-MAPK by 68.8%, GSK-3β by 69.2%, and mTOR by 43.4% in AD mice.

3.8. PFT Rescued STZ-Induced Histological Alterations. Histopathological examination after H & E staining of the sham control group showed normal histological structures in the cerebral cortexes and hippocampi. Meanwhile, the STZ group showed widespread neuronal degeneration and neuronophagia along with activated microglia cells, inflammatory cell infiltration, and diffuse hemorrhages. Simvastatin-treated mice brains had apparently normal neurons with some scattered degenerated neurons and a small number of lymphocyte aggregations. PFT-treated (100 mg/kg) mice brains showed some degenerated neurons that were associated with multifocal areas of hemorrhages. However, mice treated with 300 mg/kg of PFT showed apparently normal structures, except for severely congested blood vessels and some degenerated neurons in the CA3 and CA4 regions. The high dose of PFT (600 mg/kg) conferred the best protection and closely reflected the effects of simvastatin treatment, where the cerebral cortex and hippocampus showed an

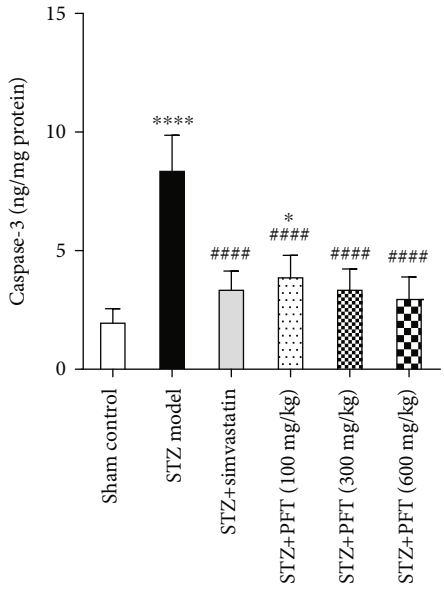

Figure 6: PFT alleviated STZ-induced alterations in caspase-3 level in the hippocampus. Each bar with vertical line represents the mean ± SD of 6 mice per group; ∗significantly different from the sham control group at $p < 0.05$, ∗∗∗∗significantly different from the sham control group at $p < 0.0001$, ####significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

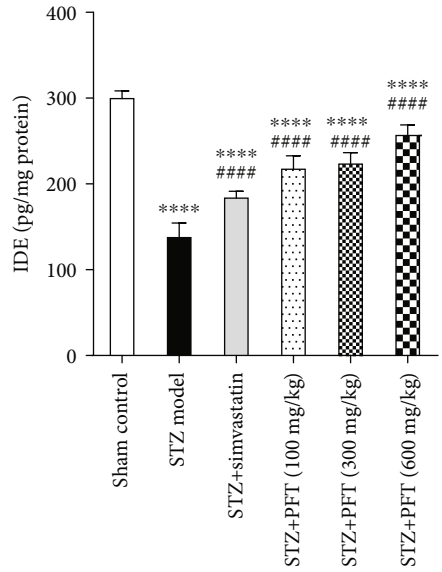

Figure 7: PFT inverted STZ-induced alterations in the IDE level in the hippocampus. Each bar with vertical line represents the mean ± SD of 6 mice per group; ∗∗∗∗significantly different from the sham control group at $p < 0.0001$, ####significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

apparently normal structure except for some individual degenerated cells in the CA3 region (Figure 9).

Cerebral cortex and hippocampus tissues were also examined for amyloid plaques, which were stained with Congo red. The results (Figure 10) showed that the sham control group had no amyloid deposition in the examined tissue sections. In contrast, the model group had a significantly high number of plaques in the cerebral cortex and hippocampus as compared with the sham control group (test statistic = 41.1, $p < 0.001$). However, PFT (100, 300, and 600 mg/kg) and simvastatin treatment conferred a marked reduction in the number of amyloid plaques as compared with STZ. The data regarding the amyloid plaques in the cerebral cortex and hippocampus are shown in Table 2.

Neurodegeneration was examined in the brain sections that were treated with Nissl stain, and the percentage of intact neurons was calculated as the survival rate. The results are summarized in Table 2. STZ resulted in a severe loss of neurons in the cerebral cortex and hippocampal regions CA3, CA4, and DG as compared with the sham control group ($F(5, 12) = 360.7$, 288.8, 696.6, and 592.6, respectively, $p < 0.0001$). Treatment with PFT significantly conserved neurons in the cerebral cortex (1.6-, 1.8-, and 1.9-fold, respectively) and the hippocampus (CA3: 2.8-, 3-, and 3.1-fold, respectively; CA4: 2-, 2.2-, and 2.5-fold, respectively; DG: 2-, 2-, and 2.23-fold, respectively) as compared with the STZ group (Figure 11). The effects of PFT corresponded to those of simvastatin, which preserved neurons in the cerebral cortex 2-fold and in CA3, CA4, and DG 3-, 2.3-, and 2.3-fold, respectively.

4. Discussion

The purpose of the current study was to address the role of gut microbiome modulation through the administration of the natural dietary product, PFT, in the prevention or improvement of AD symptoms in an STZ-induced AD mouse model. This was inspired by recent approaches that focus on the investigation of natural products present in diet as vital bioactive molecules against neurodegenerative diseases [37]. It was revealed that PFT could significantly improve cognitive impairment and dementia, prevent neuronal degeneration in the cortex and hippocampus, restore hippocampal Ach levels, and decrease the presence of amyloid plaques and disease biomarkers in a dose-dependent manner. Our experimental evidence suggests that PFT supplementation attenuates cognitive dysfunction by targeting oxidative stress and inflammatory and apoptotic pathways. Moreover, PFT enhanced the hippocampal level of IDE, a key Aβ-degrading enzyme, with a subsequent decrease in Aβ level in the hippocampus and an improvement in memory deficiency in the STZ-induced AD animal model. Furthermore, PFT significantly reduced tau hyperphosphorylation via suppression of ERK1/2, p38-MAPK, GSK-3β, and mTOR, the chief kinases that regulate tau's hyperphosphorylation. Our results demonstrate that the remarkable effects of PFT in attenuating AD symptoms were comparable to those of simvastatin, a drug whose therapeutic effects have been empirically proven for AD. Notably, simvastatin has been shown in several studies to improve cognitive performance, reduce the levels of Aβ peptides, and prevent neuronal loss in AD through its ability to reduce oxidative stress, inflammation, and apoptosis along with the promotion of IDE secretion and modulation of the PI3K/Akt and MAPK/ERK1/2 pathways [38–41].

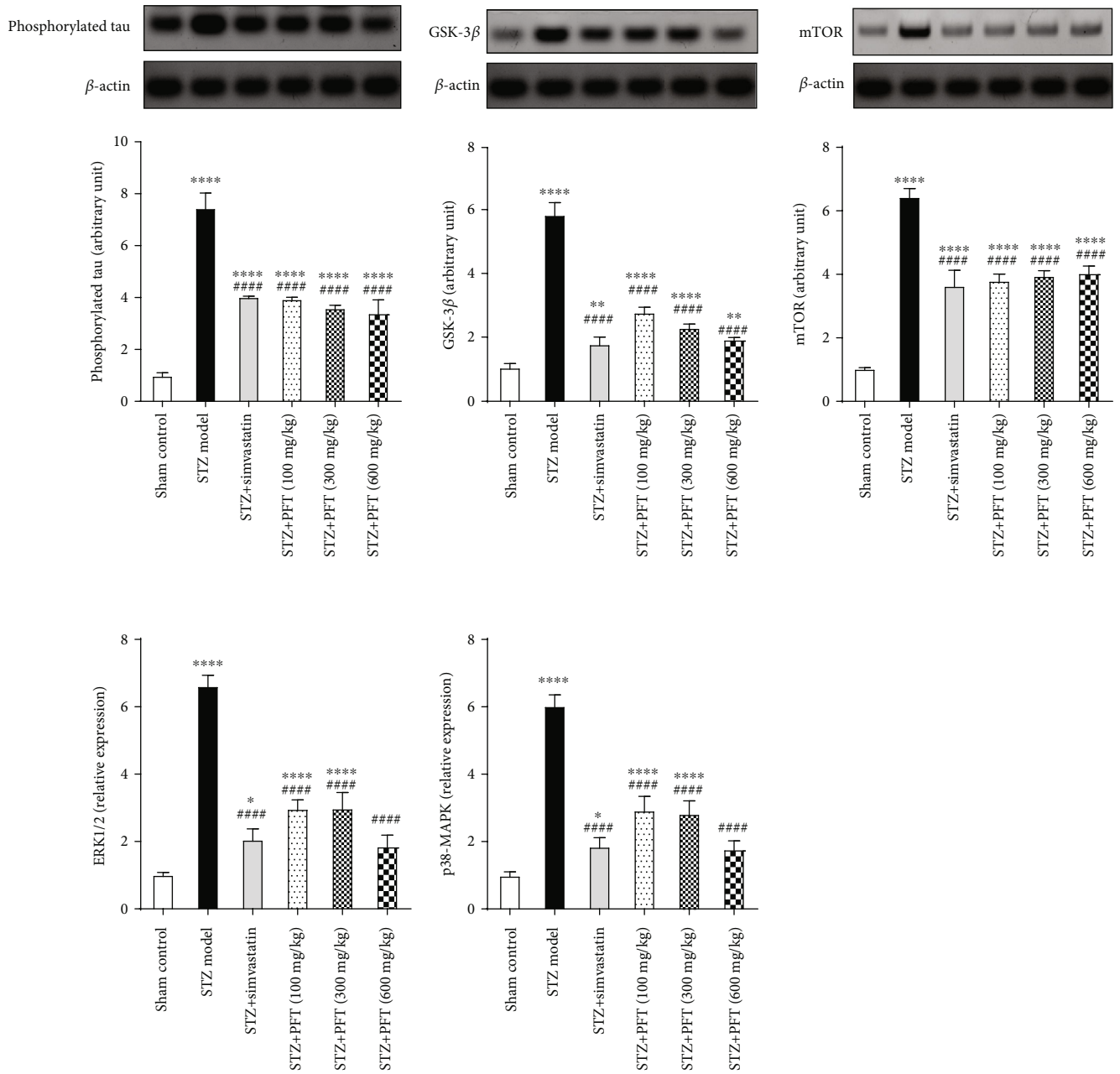

Figure 8: PFT amended the changes induced by STZ in phosphorylated tau, ERK1/2, p38-MAPK, GSK-3β, and mTOR expression in the hippocampus. Each bar with vertical line represents the mean ± SD of 3 mice per group; ∗significantly different from the sham control group at $p < 0.05$, ∗∗ significantly different from the sham control group at $p < 0.01$, ∗∗∗∗significantly different from the sham control group at $p < 0.0001$, $^{\#\#\#\#}$significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test.

The ICV-STZ-induced AD model is considered to sufficiently mimic the progressive pathology of AD in the human brain [42]. In this context, STZ conferred most of the features of AD, with progressive deficits in learning, memory, and cognitive behavior, along with aggregation of Aβ and neuron loss in the cortex and hippocampus, and a massive reduction in hippocampal Ach level, in line with former studies [26, 43, 44].

Aβ accumulation and plaque development are the major biomarkers for the detection of AD, where $A\beta_{1\text{-}42}$ is the most abundant form of Aβ protein and is deposited early as plaques [45]. The molecular mechanisms that trigger the aggregation of Aβ in AD are not fully understood; however, dysbiosis is implicated [8]. Under conditions of dysbiosis, bacteria that inhabit the microbiome release mixtures of lipopolysaccharides, amyloids, and other microbial exudates into their proximal environment. These exudates may leak from the gastrointestinal tract due to increased gut permeability induced by dysbiosis and then accumulate in the brain [8, 9]. It is worth mentioning that aging makes the involvement of gut microbiota in amyloid development and propagation more significant, since both the gut and the blood-brain

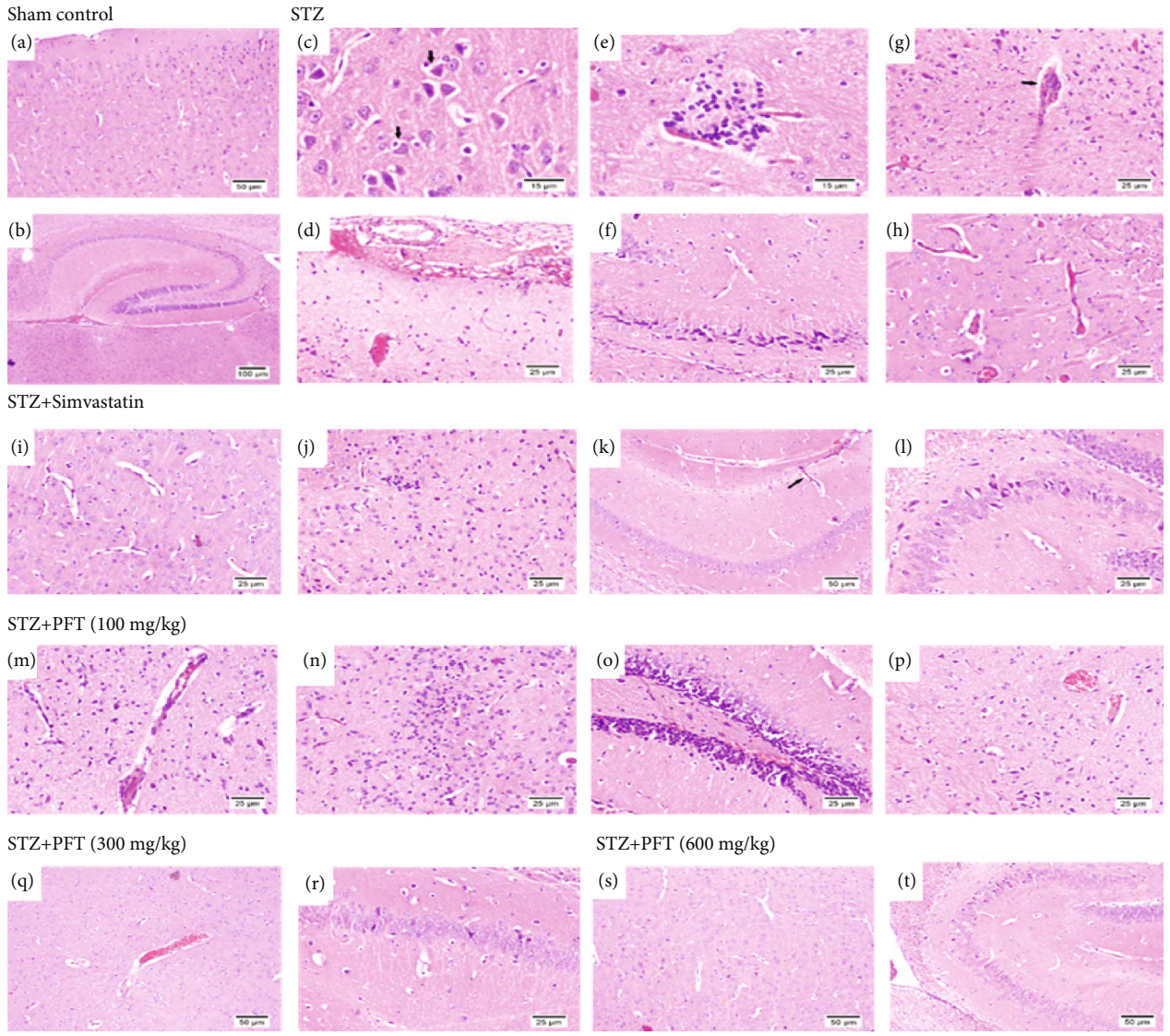

Figure 9: Histological sections of H & E staining in the cerebral cortex and hippocampus of the experimental groups; where sham control mouse showed normal histological structure of the cerebral cortex (a) (magnification ×10) and hippocampus (b) (magnification ×4). STZ model group brain showed neuronal degeneration and neuronophagia (arrows) (c) (magnification ×40), perivascular lymphocytic cuffing in the cerebral cortex (d) (magnification ×20), thickened blood vessel wall (arrow) (e) (magnification ×40), expanded meninges by extensive hemorrhage and inflammatory exudates (f) (magnification ×20), dark shrunken degenerated neurons in CA3 and CA4 of the hippocampus (g), and endothelial capillary proliferation in the striatum (h) (magnification ×20). Mice receiving simvastatin showed apparently normal structure of the cerebral cortex (i) (magnification ×20), small aggregation of lymphocytes (j) (magnification ×20), endothelial capillary proliferation in the hippocampus (arrow) (k) (magnification ×10), and apparently normal neurons in the different CA regions (l) (magnification ×20). PFT (100 mg/kg) brain revealed thickening of blood vessels wall with diffuse gliosis (m), diffuse astrogliosis with lymphocytic infiltration in the cerebral cortex (n), vascular congestion in the cerebral cortex (o), and vascular congestion in the striatum layer (p) (magnification ×20). Brain of PFT (300 mg/kg) showed vascular congestion in the cerebral cortex (q) (magnification ×10), and apparently normal neurons and few scattered degenerated neurons in the CA1 of the hippocampus (r) (magnification ×20). Brain of PFT (600 mg/kg) displayed apparently normal cerebral cortex (s) and individual degenerated neurons in the CA3 of the hippocampus (t) (magnification ×20).

barrier become more permeable to small molecules during aging [46]. In this study, investigation of the $A\beta_{1\text{-}42}$ isoform revealed steep elevation of $A\beta_{1\text{-}42}$ in the hippocampi of STZ-treated mice, an effect that was reversed by modulation of the gut microbiota through supplementation with PFT. Additionally, dysbiosis is associated with alterations in the levels of certain neurotransmitters, including Ach [6]. Cognitive function was revealed to be regulated by Ach, which employs its effect on the striatum, hippocampus, and amygdala [47]. The hippocampus is the core brain region involved in memory and learning processes [48]. Thus, declined cholinergic function in the hippocampus causes cognitive impairments together with learning and memory insufficiencies [44]. In our results, we found that STZ treatment resulted

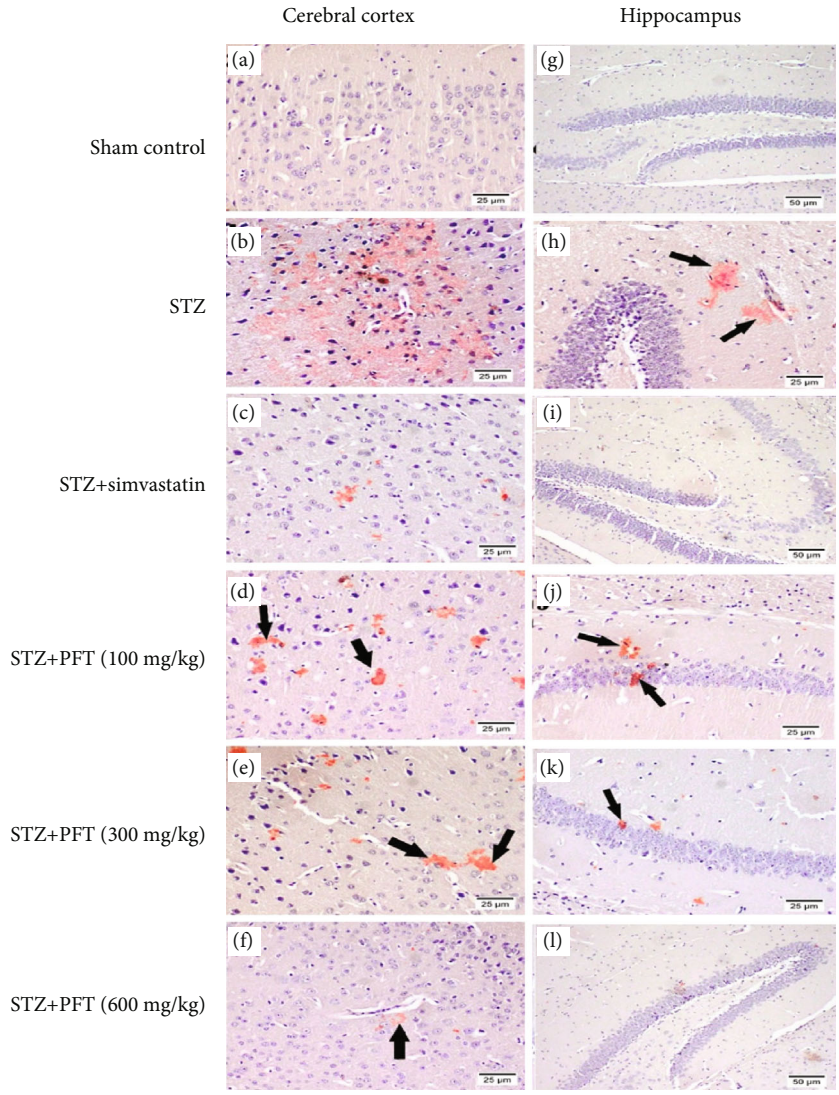

Figure 10: Congo red stained brain sections of mice for amyloid plaques visualization. Sham control group (a, g) showed no deposition of amyloid plaques (magnification ×20 and ×10, respectively). STZ group (b, h) showed diffuse deposition in the cerebral cortex and multifocal deposition in the hippocampus (arrows), respectively (magnification ×20). STZ + simvastatin (c, i) displayed minute deposition of amyloid plaques (magnification ×20 and ×10, respectively). PFT (100 mg/kg) (d, j) revealed multifocal scattered plaques (arrows). PFT (300 mg/kg) (e, k) presented multifocal area in the cerebral cortex and few depositions in the hippocampus (magnification ×20). PFT (600 mg/kg) (f, l) exhibited minute deposition of amyloid plaques (magnification ×20 and ×10, respectively).

in an obvious decrease in hippocampal Ach level, which was accompanied by spatial and short-term memory impairment. Conversely, the administration of PFT in mice with STZ-induced cognitive deficiency prevented this decrease in Ach concentration, with a subsequent improvement in memory and learning, as exemplified by the significant elevation in discrimination and preference indices in the NOR test and the increase in the time spent by mice in the target quadrant during the MWM test probe trial. This valuable effect could be attributed to the restoration of gut microbiota along with the ability of Lactobacillus, the main component of PFT, to yield Ach and to hinder acetylcholinesterase enzyme [49, 50]. The potential effect of PFT treatment was similar to that of simvastatin treatment in STZ-treated mice. The positive effect of simvastatin on cognition is attributed to the restoration of cholesterol homeostasis, since elevated cholesterol levels may result in Aβ formation and cognitive impairment [38]. In addition, the protective effects of simvastatin may also be related to its ability to diminish brain ischemia, prevent cholinergic neuronal loss, modulate brain-derived neurotrophic factor expression, and promote nitric oxide synthesis [28].

Oxidative stress is a chief contributor to aging and age-related diseases including AD [51]. According to hormesis (a dose-response phenomenon, characterized by low-dose stimulation and high-dose inhibition), temporary exposure of neurons to low levels of reactive oxygen species (ROS) has a protective effect, due to the activation of transcriptional regulators called vitagenes that endorse cell adaptive mechanisms to maintain homeostasis and to protect against more severe oxidative stress; nevertheless, chronic exposure to oxidative stress with massive generation of ROS leads to

Table 2: The survival rate percent of intact neurons in the cerebral cortex, different hippocampal regions and amyloid plaques recorded in the brain of the mice.

Groups	Survival rate percent of intact neurons				Amyloid plaques
	Cerebral cortex	CA3	CA4	DG	Cerebral cortex + hippocampus
Sham control	95.3 ± 1.5	92.3 ± 1.5	89.0 ± 1.0	92.3 ± 1.5	—
STZ model	44.7 ±2.1****	27.3 ± 1.5****	31.0 ± 1.0****	36.0 ± 1.0****	12 (10-18)****
STZ + simvastatin	89.0 ± 1.0$^{**\#\#\#\#}$	82.3 ± 1.5$^{**\#\#\#\#}$	71.0 ± 1.0$^{****\#\#\#\#}$	81.0 ± 1.0$^{****\#\#\#\#}$	2.5 (0-5)$^{*\#\#}$
STZ+ PFT (100 mg/kg)	73.3 ± 1.5$^{****\#\#\#\#}$	77.7 ±2.1$^{****\#\#\#\#}$	60.7 ± 1.5$^{****\#\#\#\#}$	71.7 ± 1.5$^{****\#\#\#\#}$	3 (2-6)$^{**\#}$
STZ+ PFT (300 mg/kg)	83.0 ±2.0$^{****\#\#\#\#}$	81.0 ± 1.0$^{***\#\#\#\#}$	66.3 ± 1.5$^{****\#\#\#\#}$	71.7 ± 1.5$^{****\#\#\#\#}$	3 (1-5)$^{**\#}$
STZ+ PFT (600 mg/kg)	85.7 ± 1.5$^{***\#\#\#\#}$	85.7 ± 4.7$^{*\#\#\#\#}$	77.3 ± 1.5$^{****\#\#\#\#}$	80.7 ± 1.5$^{****\#\#\#\#}$	2.5 (0-4)$^{\#\#\#}$

Data of survival rate percent of intact neurons represents the mean ± SD of 3 mice per group and data of amyloid plaques represents the median and range of 3 mice per group; ∗ significantly different from sham control group at $p < 0.05$, ∗∗ significantly different from sham control group at $p < 0.01$, ∗∗∗ significantly different from sham control group at $p < 0.001$, ∗∗∗∗ significantly different from sham control group at $p < 0.0001$, #significantly different from STZ group at $p < 0.05$, ## significantly different from STZ group at $p < 0.01$, ### significantly different from STZ group at $p < 0.001$, #### significantly different from STZ group at $p < 0.0001$ using one-way ANOVA followed by Tukey's multiple comparisons test for statistical analysis of survival rate, and using Kruskal–Wallis one-way ANOVA followed by Dunn's multiple comparisons test for statistical analysis of amyloid plaque number.

significant damage to cellular functions, which eventually results in the initiation of AD [52–54]. The absence of gut microbes is associated with oxidative stress. Aging has also been associated with an increase in gut permeability. Under normal circumstances, the gut microbiota produces antioxidants. However, during dysbiosis, lipopolysaccharides and amyloid dissemination cause an increase in ROS along with an induction of oxidative stress [6, 8]. This, in turn, leads to cognitive impairment since the hippocampus, which regulates cognitive function, is highly vulnerable to oxidative stress [55]. In the present study, STZ was associated with marked oxidative stress, which could possibly be attributed to its ability to induce ROS. This reduction in antioxidant capacity is associated with neuronal damage and consequential cognitive deterioration which was observed in STZ-treated mice. These findings are in agreement with a recent study [44]. Remarkably, PFT acted as a potent antioxidant, which was evidenced by a reduction in MDA content (indicator of lipid peroxidation) and activation of Nrf-2 transcription factor with subsequent induction of its target genes HO-1 and GSH in the hippocampi of STZ-treated mice. Notably, Nrf-2 plays a vital role in suppressing oxidative stress and inflammation through regulation of vitagene transcription, such as HO-1 and glutamate-cysteine synthetase that synthesize GSH, which display antioxidant activity and abate various forms of stress, thus maintaining redox balance and homeostasis [37, 56]. The antioxidant property of PFT was further confirmed by the protection of the brain from Aβ neurotoxicity and the restoration of memory. Numerous studies have suggested that the Lactobacillus in PFT produces exopolysaccharide, which displays high scavenging activity against ROS, increases the activity of antioxidant enzymes, and enhances the overall antioxidant capacity [57, 58]. The antioxidant activity displayed by PFT was equivalent to that exerted by simvastatin. In addition to its direct antioxidant effect, simvastatin has been proven to reduce circulating oxidized low-density lipoproteins and circulating markers of oxidation (such as nitrotyrosine), inhibit oxidant enzymes, and upregulate antioxidant enzyme activity [59].

Besides oxidative stress, inflammation also plays a fundamental role in the pathogenesis of AD [60]. Microglial activation and high levels of proinflammatory cytokines, such as interleukins and TNF-α, have been detected in the serum of AD patients [61]. Dysbiosis contributes to the pathogenesis of AD partly through the generation of a neuroinflammatory state [62]. It has been suggested that an increase in gut permeability following dysbiosis may lead to neuroinflammation and subsequent hippocampal damage [63]. After leaking from the gut, bacteria-derived polysaccharides and amyloids activate NF-κB signaling. NF-κB in turn stimulates proinflammatory microRNA-34a release, which reduces the expression of TREM2 (triggering receptor expressed on myeloid cells 2), resulting in phagocytosis dysfunction and accumulation of Aβ [64]. Furthermore, lipopolysaccharides and amyloids can endorse gut leakiness and trigger ROS in the brain with subsequent microglial activation that enhances proinflammatory cytokine release [65, 66]. It is worth mentioning that ROS and microglial activation along with dysregulation of redox homeostasis by suppression of Nrf-2 transcription results in stimulation of NF-κB signaling, which triggers elevation of TNF-α and upregulation of NLRP3 [37, 67, 68]. NLRP3 inflammasome activation contributes to the inflammatory events in AD pathogenesis, since it enhances the secretion of IL-1β, which further promotes microglial activation and accumulation of inflammatory and neurotoxic factors resulting in a vicious cycle that exacerbates neurodegeneration [67]. To this end, modulating the gut microbiome with probiotics may represent an effective strategy to diminish the level of chronic inflammation and Aβ associated with AD. In this context, STZ treatment resulted in marked neuroinflammation, which was efficiently inhibited by PFT supplementation as verified by the substantial decrease in the expression of hippocampal levels of NF-κB, NLRP3, IL-1β, and TNF-α in STZ-treated mice, with consequent amelioration of Aβ burden in the hippocampus. The anti-inflammatory effect of PFT corresponded to that of simvastatin, which has been shown to inhibit inflammation by reducing neutrophil infiltration, nitrotyrosine formation, NF-κB activation, and inducible nitric oxide synthase expression [69].

Immense neuronal loss is among the pathological features of AD. Apoptosis, a vital aspect of AD pathogenesis,

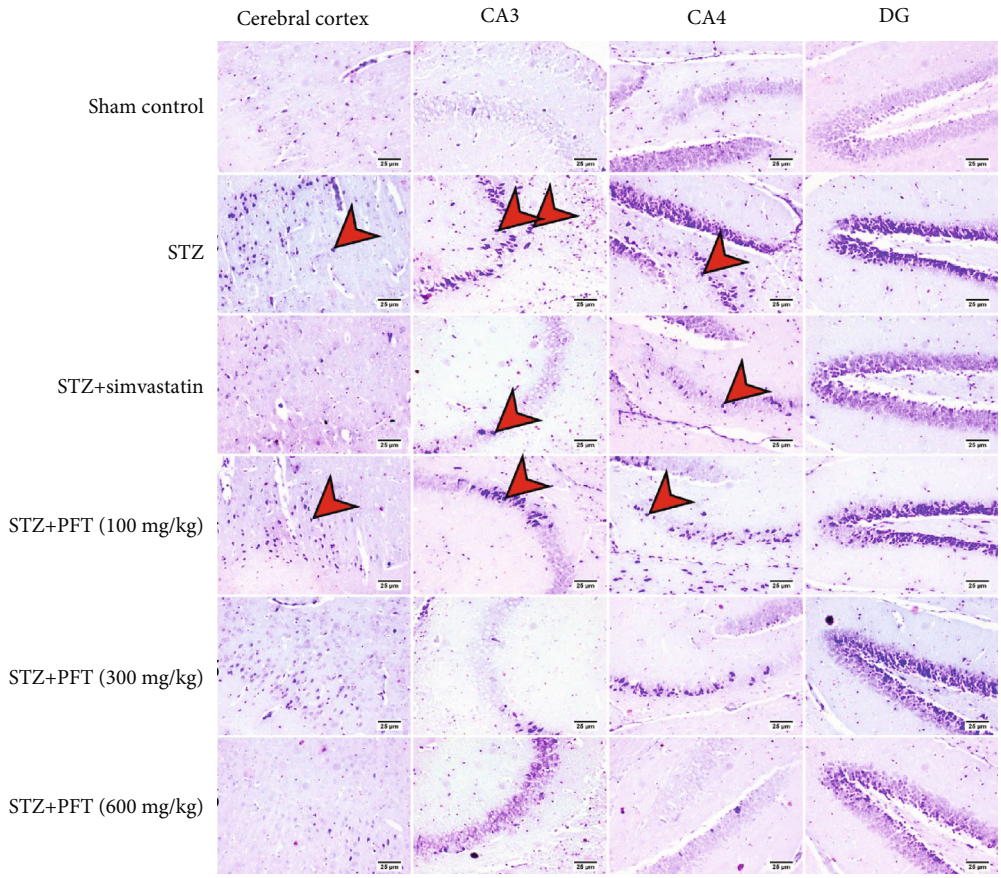

Figure 11: Sections of the cerebral cortex and the hippocampal regions (CA3, CA4, and DG) stained with Nissl stain. The sham control group showed normal intact neurons in all brain regions. An increased number of dark and shrunken neurons with pyknotic nuclei (arrows) was detected in the STZ group, and fewer numbers were recorded in other treated groups (magnification ×20).

is responsible for AD-associated neuronal death [70]. This phenomenon has been assumed to be due to the presence of apoptotic factors in AD brain tissue, in addition to the fact that the brain is highly vulnerable to apoptotic damage [71]. Caspase-3, the major source of apoptotic cell death in neurodegenerative diseases, is directly involved in AD apoptosis [72]. Remarkably, the microbiota can regulate apoptosis via gut-brain axis signaling [73]. In dysbiosis, harmful signals are propagated, leading to increased ROS production and inflammation with consequent mitochondrial dysfunction, which endorses cytochrome c release, thus triggering caspase-3 and inducing apoptosis and neuronal death [74]. In the present study, administration of PFT effectively attenuated the activity of caspase-3 in AD mice, an effect that was associated with neuronal preservation in the hippocampal structures and relevant restoration of attentional performance. PFT's antiapoptotic activity was comparable to simvastatin's effects in STZ-treated mice. Simvastatin has been shown to attenuate the pathology of AD due to its antiapoptotic effects in hippocampal cells [75].

Insulin has also been linked with AD pathology [76]. In this context, elevated insulin levels have been observed in AD brains, resulting in exaggerated inflammatory responses and accumulation of Aβ in the brain [77]. Besides stimulating Aβ secretion, insulin inhibits Aβ degradation by competing for IDE, the key regulator of Aβ in neurons [78]. Insulin not only exerts a direct effect on Aβ metabolism but also promotes mitochondrial dysfunction, oxidative stress, and apoptosis, all of which contribute to the development of AD [79]. In particular, the absence of gut microbes may lead to the development of insulin resistance and the suppression of IDE, which are both found in AD [80]. In this study, restoration of the gut microbiota via PFT supplementation enhanced IDE and significantly decreased Aβ levels in STZ-treated rats. In AD mice, PFT mediated IDE secretion as effectively as simvastatin, which has been reported to affect IDE via an unconventional autophagy-based secretory pathway [40].

Tau hyperphosphorylation is greatly implicated in AD pathogenesis [81]. Tau is a microtubule-binding protein that is responsible for the assemblage and stabilization of microtubules [82]. Pathological tau protein is abnormally hyperphosphorylated and accumulated forming neurofibrillary tangles, a hallmark of AD [82]. Tau phosphorylation is regulated by multiple kinases and phosphatases. ERK1/2, p38-MAPK, GSK-3β, and mTOR are the main kinases that are involved in tau hyperphosphorylation, while protein phosphatase 2A (PP2A) is the chief phosphatase that dephosphorylates tau [83, 84]. It has been demonstrated that the aforementioned kinases are highly activated in AD brains along with the declined activity of PP2A [81, 84]. Of note, Aβ and oxidative stress have been shown to stimulate MAPK

kinase (MEK), which activates ERK1/2 and p38-MAPK through phosphorylation, thereby resulting in tau hyperphosphorylation and prominent neurofibrillary tangle formation [85, 86]. Moreover, Aβ and insulin resistance suppress phosphatidylinositol-4,5-bisphosphate 3-kinase- (PI3K-) Akt signaling leading to activation of GSK-3β and mTOR, which in turn causes PP2A inhibition and subsequent tau hyperphosphorylation [77]. Importantly, there is evidence that activation of mTOR promotes Aβ deposition through the inhibition of autophagy and disposes insulin resistance, thus leading to further tau phosphorylation, resulting in a vicious cycle that aggravates AD [87]. It has been proposed that gut microbiota alteration is associated with tau hyperphosphorylation, based on the fact that factors that enhance tau hyperphosphorylation, including Aβ, oxidative stress, and insulin resistance, are precipitated by dysbiosis [88, 89]. In addition, dysbiosis can induce leucine metabolism disorder, which enhances mTOR activity [90, 91]. Consequently, gut microbiome restoration could dampen tau hyperphosphorylation. Our study shows that PFT decreased tau phosphorylation along with downregulation of ERK1/2, p38-MAPK, GSK-3β, and mTOR expression in the hippocampi of STZ-treated mice, and this favorable effect was accompanied by suppression of Aβ accumulation and enhancement of cognitive function. The action of PFT was parallel to that of simvastatin, which has been demonstrated to modulate the PI3K/Akt and MAPK/ERK1/2 pathways and halt neurodegeneration [39, 92].

5. Conclusions

This study reveals that the gut microbiota affects learning and memory via the microbiota-gut-brain axis and that AD is highly related to alterations in the gut microbiota composition. The delivery of Lactobacillus via PFT, a kefir product, displayed several benefits in STZ-induced AD mice. We have demonstrated that PFT not only improves cognitive function along with amelioration of histopathological markers but also attenuates oxidative stress, suppresses neuroinflammation, reduces apoptosis, and enhances IDE. Thus, PFT ameliorates multiple factors that underlie AD pathology. As a safe, nontoxic agent, PFT represents a useful potential therapy for AD.

Data Availability

The data of the present study including the figures and western blot analysis used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare there are no conflicts of interest.

Authors' Contributions

N El Sayed, E. Kandil, and M Ghoneum planned the study and wrote the manuscript. N El Sayed designed and performed the experiments. All authors revised and approved the manuscript.

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Review Article

Peganum spp.: A Comprehensive Review on Bioactivities and Health-Enhancing Effects and Their Potential for the Formulation of Functional Foods and Pharmaceutical Drugs

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The genus *Peganum* includes four species widely distributed in warm temperate to subtropical regions from the Mediterranean to Mongolia as well as certain regions in America. Among these species, *Peganum harmala* L., distributed from the Mediterranean region to Central Asia, has been studied and its phytochemical profile, traditional folk use, and application in pharmacological and clinical trials are well known. The review is aimed at presenting an insight into the botanical features and geographical distribution of *Peganum* spp. along with traditional folk uses. This manuscript also reviews the phytochemical profile of *Peganum* spp. and its correlation with biological activities evidenced by the *in vitro* and *in vivo* investigations. Moreover, this review gives us an understanding of the bioactive compounds from *Peganum* as health promoters followed by the safety and adverse effects on human health. In relation to their multipurpose therapeutic properties, various parts of this plant such as seeds, bark, and roots present bioactive compounds promoting health benefits. An updated search (until December 2020) was carried out in databases such as PubMed and ScienceDirect. Chemical studies have presented beta-carboline alkaloids as the most active constituents, with harmalol, harmaline, and harmine being the latest and most studied among these naturally occurring alkaloids. The *Peganum* spp. extracts have shown neuroprotective, anticancer, antimicrobial, and antiviral effects. The extracts are also found effective in improving respiratory disorders (asthma and cough conditions), dermatoses, and knee osteoarthritis. Bioactivities and health-enhancing effects of *Peganum* spp. make it a potential candidate for the formulation of functional foods and pharmaceutical drugs. Nevertheless, adverse effects of this plant have also been described, and therefore new bioproducts need to be studied in depth. In fact, the design of new formulations and nanoformulations to control the release of active compounds will be necessary to achieve successful pharmacological and therapeutic treatments.

1. Introduction

The genus *Peganum* is a perennial, herbaceous, glabrous to pubescent, and wild flowering plant with short creeping roots and belongs to the family Zygophyllaceae [1–3]. The genus has four main species (*Peganum harmala* L., *Peganum mexicanum* Gray, *Peganum nigellastrum* Bunge, and *Peganum multisectum* (Maxim.) Bobrov) having significance in health promotion and various biological activities in the human body. These species are widely distributed in warm temperate to subtropical regions from the Mediterranean to Mongolia of the Old World and from Texas to Mexico in the New World [4–10] (Table 1). Among the different species of *Peganum*, *P. harmala* has been studied well in relation to its application as a traditional folk medicine to modern pharmacological usage. *Peganum* spp. has been employed in the treatment of diabetes, rheumatism, Parkinson's disease, hypertension, jaundice, and asthma. The plant is also widely used in traditional Chinese medicine for the treatment of apoplexy and lumbago and also as a stimulant for improving the function of the central nervous system [11]. The benefits of these species are associated with its phytochemical profile. The seeds, roots, leaves, fruits, stems, and flowers of *Peganum* spp. have been widely studied for their phytoconstituents. Bioactive alkaloids (quinazoline alkaloids and β-carboline), essential oil components, and other phenolic compounds mainly contribute to the health-promoting effects. Harmaline, harmine, harmol, harmalol, vasicine, vasicinone, deoxyvasicine, and deoxyvasicinone are few examples of bioactive alkaloids present in *Peganum* spp. Bioactive compounds are important for their numerous biological functions, viz., anticancer, antidiabetic, antimicrobial, anti-inflammatory, antiviral, antidepressant, and antioxidant [12]. These biological activities have been well documented via *in vivo* and *in vitro* investigations.

This current review is aimed at critically discussing the botanical features and geographical distribution of *Peganum* spp. along with traditional folk uses. It highlights the phytochemical profile of the *Peganum* genus along with the biological activities proven by *in vivo* and *in vitro* trials. Finally, the health-promoting effects of *Peganum* spp. plant extracts are well established in this review. The content discussed in this manuscript has been summarized in Figure 1.

2. Geographical Distribution of *Peganum* spp. and Botanical Features

P. harmala is among the most studied species. Commonly known as "Harmal" or "Suryin Rue", it is distributed from the Mediterranean region to Central Asia. It is a drought-tolerant plant, and in Central Asia, this species is found in Mongolia, Kashgaria, Tsaidam, Dzungaria, and Tibet [13]. This species is native to arid and semiarid regions, is widely distributed in North Africa, and is also found in the Middle East, Turkey, Pakistan, India, Iran, Kazakhstan, Mexico, South America, and many other countries [6, 14–21]. *P. nigellastrum* and *P. multisectum* are two other species which are gaining importance due to their health-promoting effects. These species are grown in northwestern China generally in arid and semiarid regions, including Xinjiang Province, Mongolia, and Russia and are vital components of desert vegetation [8, 22–25]. *P. mexicanum* is commonly found growing in the United States and Mexico of North America [23]. A distribution pattern of the *Peganum* spp. throughout the globe is presented in Figure 2.

As already mentioned, the genus *Peganum* is a perennial, herbaceous, glabrous to pubescent, and wild flowering plant with short, creeping roots. Leaves alternate, entire or multifide to palmatisected; flowers 1–3, on subterminal leaf opposed peduncles, white; sepals 4–5, entire to lobed; petals 4–5, whitish to yellow, imbricate, oblong; stamens 8–15, anther bicelled, filamentous inserted at the base of the disc; ovary 2–4 locular, globular; ovules many in each chamber; fruits capsule, globular in shape, splitting by 3 valves or indeniscent fleshy; seeds many, blunt-top and sharp-top shape,

Table 1: Distinctive morphological characteristics of *Peganum* species.

Morphological characteristics	*P. harmala*	*P. multisectum*	*P. nigellestrum*	References
Plant height	40-45 cm	20-65 cm	10-25 cm	[13, 24, 27, 28]
Stem and leaf surface	Glabrous	Sparse setose	Dense setose	
Stem branching	Branched into 5-13 stems	Branched into 70-100 stem	Branched or without branched	
Leaves	Palmatisected into 3-5 linear lobes; lobes 1.5-3.0 mm wide	Bi- or tripalmatisected into linear lobes; lobes 0.4-1.0 mm in width	Bi- or tripalmatisected into linear lobes; lobes 1.2-1.8 mm in width	
Calyx leaves	Calyx leaves entire or slightly incised	Calyx leaves incised into 3 linear lobes	Calyx leaves incised into 5-7 linear lobes	
Seed	Depressed surface	Convex surface	Smooth surface	

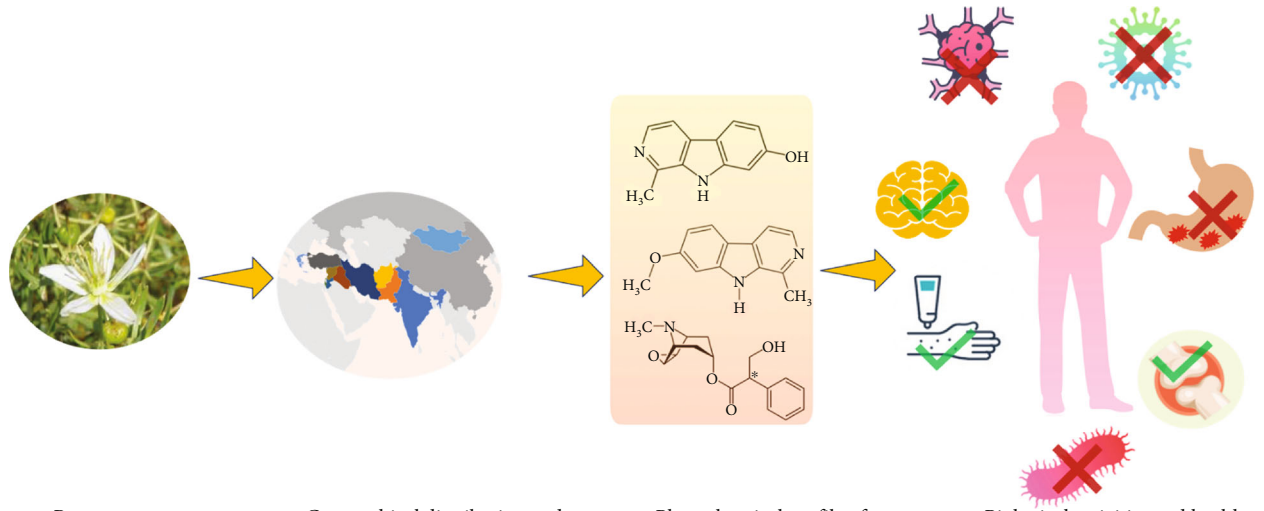

Figure 1: Various components discussed in the review article.

testa rough, spongy; curved embryo [26]. Morphological distinctive features of the main three species *P. harmala*, *P. multisectum*, and *P. nigellestrum* are presented in Table 1.

3. Traditional Uses

P. harmala has been used in various therapeutic, superstitious, and even ritual applications in different cultures and ethnicities. One of the superstitious uses of *Peganum* is found in Morocco where the plant is set on fire for the protection against ghosts and goblins. In Iran, Turkey, China, and other Arab countries in Africa and Asia, it is used to protect a person or condition from the evil eye [29]. One the rituals around the world related to *Peganum* that should be mentioned is a special ceremony in Zoroastrianism, where a prayer is recited and the *Peganum* is set on fire; the other is where *Peganum* is burned in a wedding ceremony in India, so that the bride and groom can avoid a life of darkness [30]. However, the medical aspects of *Peganum* are more prominent as they have long been used in the treatment of diabetes, asthma, arthritis, hypertension, and many other diseases in different cultures. A detailed list of the various traditional uses of the *Peganum* spp. is displayed in Table 2.

4. Essential Oils and Phytochemical Composition of *Peganum* Genus

4.1. Essential Oil and Its Components. Essential oils are volatile, complex, and natural compounds produced by aromatic plants as secondary metabolites [38]. They are used in different industries including aromatherapy, fragrance and flavor industry, biotechnology and pharmaceutical industry, cosmetic and soap industry, and agriculture industry. The chemical composition of essential oils from the *Peganum* genus has been reported by a limited number of authors. Only a few studies are available in the literature about the essential oil composition of *P. harmala* from Morocco [37, 39], Algeria [37], Iran [40], Saudi Arabia [41], Tunisia, Libya, and Egypt [37]. The exhaustive profile of essential oil components from *Peganum* spp. is displayed in Table 3. Oxygenated monoterpenes, sesquiterpenes, eugenol, and oxygenated sesquiterpenes are the principal components in the essential oils from the populations of Morocco, Algeria, Tunisia, Libya, and Egypt, respectively. Figure 3 summarizes some of the most important phytochemical compounds isolated from *Peganum* spp. from different regions.

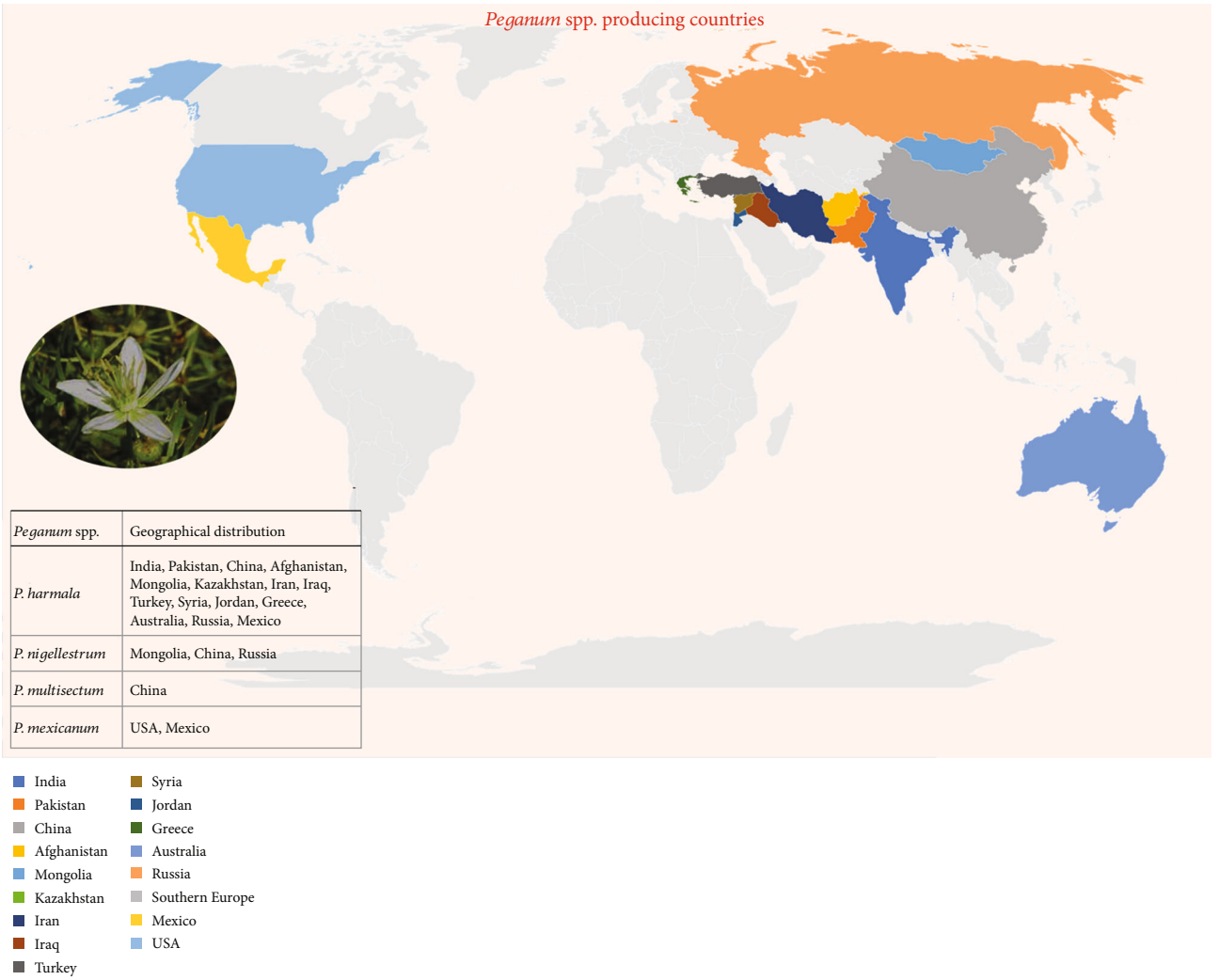

Figure 2: Map presenting the distribution pattern of *Peganum* spp. throughout the globe.

4.2. Other Biologically Active Phytochemicals. The chemical diversity and biologically active compounds in different parts of *P. harmala* and other species of the genus *Peganum* has been reported by the several authors around the world (Table 4).

5. Biological Activities

5.1. In Vitro Studies

5.1.1. Anticancer Activity. P. harmala has been demonstrated to inhibit the effect on the progress of some type of cancers by reducing the growth rate of cancerous cells. The use of Annexin-V-fluos for apoptosis analysis of MDA-MB-231 breast cancer cells showed that the seed extract of *P. harmala* led to declining growth rates depending on the concentration and time that the cells were exposed. A concentration of 30 μg/mL not only reduced the growth rate but also subjected 50% of the cells to apoptosis in nearly 24 h. Furthermore, it was also noticed that the *P. harmala* seed extract caused an extrinsic pathway-induced apoptosis of the cells [88]. Deoxypeganine, harmine, and peganine compounds were tested as a multidrug resistance inhibitor of cancer cells, but they were not successful against the MDR1 gene-transfected mouse lymphoma cell line, even when the highest dose of 40 μg/mL was used [89]. The alkaloid fraction of *P. harmala* seeds was tested for cell toxicity against UCP-Med and Med-mek carcinoma and UCP-Med sarcoma (applied at concentration levels of 20, 40, 60, 80, and 100 μg/mL). Application of the alkaloid fraction of *P. harmala* to UCP-Med carcinoma resulted in extremely slow proliferation of the cells in the first 24 h, followed by cell lysis that started between incubation of 24 and 72 h as per the variation in the treatment. Against the UCP-Med sarcoma, only 20 μg/mL of treatment was enough to slow down the proliferation in the first 24 h. After 24 h exposure of 60–100 μg/mL, the cell death rate was 100%. When alkaloids were applied to Med-Mek carcinoma, a slower growth was observed compared to the other cell lines. Moreover, the inhibition of growth was instantly detected upon exposure to alkaloids, specifically at 80 and 100 μg/mL concentrations. Additionally, the alkaloid fraction of *P. harmala* created cytotoxic effects on the tumoral cell lines, directly proportional with the time and concentration of exposure [90].

Table 2: Traditional uses of *Peganum* species.

Peganum spp.	Part of plant	Country/region	Common name	Effect	Reference
P. harmala	Seeds	Morocco	Madjouna	Diabetes, asthma, rheumatic pain, antihypertensive, anthelmintic/antimicrobial	[29, 31, 32]
P. harmala	Seeds, fruit	Iran/Uzbekistan	Asbatan, fašars ī n, isfanj, ispand, sifand, sipand, isirik	Air purifier, pain relief, anti-Parkinson's	[3, 29, 32]
P. harmala	Seeds, fruit	Turkey	Ilezik, uzarih, üzerlik	To treat intestinal pain, as an antibacterial, necklaces (sometimes also a branch of the plant is hung in the house)	[29, 33]
P. harmala	Seeds	Arabian (Jordan, Saudi Arabia, Syria)	Álqat al-dib, harjal, harmal, hre-milan, huraymilan, legherma, mogannanna, sadab-sami, sadab-bari	Common cold, as a dye, healing ulcers, back pain, blood purifier, psychoactive	[29, 32]
P. harmala	Whole plant, seeds,	Greece, Spain, Italy	Alfarma, armalá, gamarza, harma, hármaga, harmala, African rue, harmal, harmal peganum, harmal shrub, harmel, harmel peganum, Isband, ozallaik, peganum, rue, ruin weed, Syrian rue, wild rue, alfarma, alharma, amargaza, armalá, gamarza, harma, hármaga	Hypotensive, anti-Parkinson's, antidiabetic, leishmaniasis	[29, 34, 35]
P. harmala *P. nigellastrum* *P. multisectum*	Seeds	China	Luo tuo peng, luo tuo hao	Hypertension, diabetes, jaundice	[3, 29, 32]
P. harmala	Seeds	India	Hurmul	Asthma	[30, 32]
P. harmala *P. multisectum*	Seeds	Mexico, southern North America	Isband, ozallaik, peganum, rue, ruin weed, Syrian rue, wild rue	Nervosity	[29, 36]
P. harmala	Seeds	North of Africa	African rue, huraymilan, legherma, mogannanna, sadab-sami, sadab-bari	Emmenagogue	[29, 37]

Cytotoxic effects of raw alkaloid extracts of *P. harmala*'s fruits (TAFr), seeds (TASe), roots [70], and aerial parts (TAAp) were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and quantitative video microscopy analysis of cancer cells (Hs683 and B16F10; the resistant ones to proapoptotic stimuli A549, U373, MCF7, SKMEL-28). Cytotoxic effects were observed on all cancer cells after 72 h of treatment with an IC_{50} range between 1 and 52 μg/mL. Extracts of *P. harmala* presented a clear inhibition of all cancer cell lines, but SKMEL-28 had 38% of survivability after treatment with TAAp at the highest tested dose (100 μg/mL). Analysis of A549 lung cancer cells exhibited only TAAp showing cytotoxic effects instead of cytostatic effects. However, TAFr, TASe, and TAR showed cytostatic effects. While the global growth rate values of TAAp, TASe, and TAFr were 0.19, 0.3, and 0.26, that of TAR was 0.62. Based on cell death rates, TAFr, TASe, and TAR inhibited cancer growth, but not induced cell death. In spite of that, TAAp caused cell death in the same cells by 0.29 [91].

5.1.2. Neuroprotective Activity. Acetylcholinesterase inhibitors (AChEIs) are used for the treatment of Alzheimer's disease. A study which used thin-layer chromatography (TLC, bioautographic assay) showed that methanol extracts of the *P. nigellastrum* seed had an important inhibitory effect. Only 0.01 μg of harmine, harmaline, harmol, and galathamine (which are known as AChE inhibitors) inhibited AChE. Also, 0.1 μg concentrations of vasicine and harmol were enough to inhibit the AChE activity. Not only that, vasicinone, deoxyvasicinone, deoxyvasicine, and nigellastrines I and II exhibited low AChEI effect (1 μg). Therefore, *P. nigellastrum* alkaloids presented a high potential to be used as an AChE inhibitor [66]. The harmane alkaloid from *Peganum* proved to have the most powerful AChE inhibitor activity although butyrylcholinesterase (BChE) inhibitor activity was lower. Similarly, harmine and harmaline showed an important inhibition on AChE and weaker BChE inhibitor activity. On the other hand, harmalol and harmol showed more potent BChE inhibitor activity but mediocre AChE inhibitor activity. Also, both nigellastrine I and nigellastrine II gave more solid BChE inhibitor activity but poorer AChE inhibitor activity results. Unlike all, vasicine was influential in inhibiting both AChE and BChE [92]. A new type of alkaloid (2-aldehyde-tetrahydroharmine) was obtained by the use of an activity-guided fraction. It exhibited an AChE and BChE inhibition effect (IC_{50} values with 12.35 ± 0.24 and 5.51 ± 0.33 μM, alternately). On the other hand, vasicine and deoxyvasicine indicated the most powerful BChE inhibition (IC_{50} values of 0.04 ± 0.01 and 0.1 ± 0.01 μM) [55]. A study that used the piezoelectric spraying method showed that the extract of *P. harmala* seed had an AChE inhibitory effect. The study also indicated that the AChE inhibitory effect was two times more

Table 3: Essential oil composition (%) of *Peganum harmala* L. from different regions.

Compound name	Algeria [37]	Egypt [37]	Libya [37]	Morocco [37]	Tunisia [37]	Iran [40]	S. Arabia [41]	Morocco [39]
p-Cymene	—	—	—	—	0.1	0.8-1.7	—	—
Limonene	0.3	—	—	—	—	1.1-6.4	—	—
2-Acetyl-thiazole	—	1.3	—	—	—	—	—	—
Santolina alcohol	—	2	—	—	—	—	—	—
cis-Dihydro-rose oxide	—	1.8	—	—	—	—	—	—
1-Octen-ol	—	0.7	—	—	—	—	—	—
trans-Dihydro-rose oxide	—	0.4	—	—	—	—	—	—
n-Octanol	—	1.1	—	—	—	—	—	—
Linalool	1.7	—	—	—	—	0.4	—	—
trans-Thujone	—	—	—	—	0.1	—	—	—
Methyl butanoate, 3-methyl-3-butenyl	—	2.9	—	—	—	—	—	—
3-Decanone	—	1.1	—	—	—	—	—	—
Camphor	—	2.7	—	—	—	—	—	—
Benzene acetonitrile	—	1.3	—	—	2.6	—	—	—
Isoborneol	0.9	—	—	—	—	—	—	—
Terpinen-4-ol	0.4	—	—	—	—	—	—	—
Naphthalene	0.2	—	—	—	—	—	—	—
α-Terpineol	0.7	—	—	—	—	—	—	—
1-Dodecene	—	0.7	—	—	—	—	—	—
[2]-Isocitral	0.7	—	—	—	—	—	—	—
Methyl chavicol	1.1	—	—	—	0.1	—	—	—
Isoquinoline	0.5	—	—	—	—	—	—	1.4-6.4
Carvone	0.7	—	—	—	—	—	—	—
Pulegone	0.5	—	—	—	—	—	—	—
Cumin aldehyde	—	—	—	—	0.1	—	—	—
[2]-Anethol	3.7	—	—	—	6.9	—	—	—
α-Terpinen-7-al	—	2.2	—	1	0.1	—	—	—
Thymol	7	—	5.1	5	1.8	—	—	2
Dihydrocarveol acetate	6.2	1	3	0.7	0.3	—	—	—
Terpinyl acetate	—	0.9	—	—	—	—	—	—
4-Methoxyacetophenone	1	—	—	—	—	—	—	—
Eugenol	17.5	17.2	17.8	13.2	69.2	—	5.2	—
Cicloysosativene	2.3	—	—	—	—	—	—	—
α-Ylangene	1.1	—	—	—	—	—	—	—
Decanoic acid	—	1	—	—	—	—	—	—
Thujico acid	—	—	—	—	<0.1	—	—	—
β-Cubebene	—	—	—	0.4	—	—	—	—
n-Undecanol	—	2.3	—	—	—	—	—	—
6,8-Nonadien-2-one,6-methyl-5-(1-methyletildene)	0.3	—	—	—	—	—	—	—
iso-Italicene	1.1	—	—	—	—	—	—	—
β-Longipinene	0.5	—	—	—	0.1	—	—	—
Methyleugenol	—	0.3	—	—	—	—	—	—
β-Ionol	—	—	—	0.7		—	—	—
(Z)-Caryophyllene	2.3	—	0.3	—	0.8	—	—	—
[2]-α-Ionone	0.6	—	—	0.2	—	—	—	—
Nerol acetate	—	—	3.7	—	—	—	—	—
α-Isomethyl-[2]-ionol	7	—	—	—	—	—	—	—
Aromadendrene	—	—	—	—	0.3	—	—	—

Table 3: Continued.

Compound name	Algeria [37]	Egypt [37]	Libya [37]	Morocco [37]	Tunisia [37]	Iran [40]	S. Arabia [41]	Morocco [39]
Bakerol	—	—	—	7.5	—	—	—	—
trans-Cadina-16,4-diene	—	—	—	0.3	—	—	—	—
9-*epi*-[2]-Caryophyllene	—	—	—	—	0.2	—	—	—
α-Acoradiene	0.5	—	—	—	—	—	—	—
γ-Gurjunene	—	—	—	0.6	—	—	—	—
γ-Muurolene	0.3	—	—	—	—	—	—	—
[2]-*β*-Ionone	—	—	—	0.6	—	—	—	8.1
[2]-Methyl isoeugenol	—	0.6	—	—	0.2	—	—	—
10,11-Epoxy-calamenene	—	—	—	0.3	—	—	—	—
α-Zingiberene	0.3	—	—	—	—	—	—	—
γ-Amorphene	1.3	—	—	—	—	—	—	—
Methyl *p*-*tert*-buthylphenil acetate	0.3	0.8	—	2	0.2	—	—	—
10-Undecenol acetate	2	—	—	3.6	0.1	—	—	—
β-Curcumene	—	1.9	—	—	—	—	—	—
7-epi-*α*-Selinene	1.1	—	—	—	—	—	—	—
2E,4E-Dodecandienal	1.2	2.1	2	—	—	—	—	—
α-Thujaplicinol	—	—	—	4.8	—	—	—	—
Eugenol acetate	—	—	—	—	9	—	—	—
α-Bulnesene	—	—	—	0.4	—	—	—	—
(Z)-Nerolidol	0.5	1.6	2.5	1.3	0.1	—	—	—
α-Calacorene	0.6	—	—	0.6	—	—	—	—
Germacrene B	—	—	2.7	—	0.1	—	—	—
β-Calacorene	—	—	—	1.8	—	—	—	—
Dodecanoic acid	—	5.9	—	—	—	—	—	—
Spathulenol	2	2.3	4.2	4	0.2	—	—	—
Caryophyllene oxide	1.7	—	3.8	1.7	0.8	—	—	—
1-Esadecene	—	1.6	—	0.9	0.2	—	—	—
Ledol	—	—	—	0.4	—	—	—	—
β-Oplopenone	—	—	—	—	0.1	—	—	—
n-Exadecane	2.6	—	2.8	2.9	0.1	—	—	—
Isolongifolan-7-*α*-ol	—	—	3.8	2.1	0.1	—	—	—
Cubenol	—	2	—	—	<0.1	—	—	—
Caryophylla-4(12),8(13)-dien-5*α*-ol	—	—	—	—	0.1	—	—	—
epi-*α*-Cadinol	5.3	—	—	0.9	—	—	—	—
β-Acorenol	1.5	2.9	7.4	2.3	0.1	—	—	—
epi-*α*-Muurolol	—	—	—	—	0.1	—	—	—
cis-Guai-3,9-dien-11-ol	0.7	—	—	—	—	—	—	—
allo-Aromadendrene epoxide	—	—	—	1.5	—	—	—	—
Vulgarone B	1.4	—	—	3.7	—	—	—	—
Cedr-8,15-en-10-olo	—	4.1	6	—	0.1	—	—	—
α-Eudesmol	—	1.6	—	—	—	—	—	—
14-Hydroxy-(Z)-caryophyellene	—	—	2.1	—	0.2	—	—	—
n-Tetradecanol	4.8	12.3	11.3	11.1	0.3	—	—	—
Elemol acetate	0.7	—	—	—	—	—	—	—
epi-*α*-Bisabololo	—	3	6.6	—	—	—	—	—
Cyperotundone	1.6	3	4.6	4.1	0.1	—	—	—
Heptadecane	—	1.2	3.8	1.2	0.1	—	—	—
Calamenen-10-one	—	0.5	1.9	—	—	—	—	—

Table 3: Continued.

Compound name	Algeria [37]	Egypt [37]	Libya [37]	Morocco [37]	Tunisia [37]	Iran [40]	S. Arabia [41]	Morocco [39]
Longifolol	—	2.7	—	—	—	—	—	—
Farnesol	—	—	—	0.4	—	—	—	—
3-Otadecine	0.3	—	—	—	—	—	—	—
Farnesale	—	1	—	—	—	—	—	—
Santalol	—	—	—	1	—	—	—	—
[2]-Nerolidil acetate	—	—	—	0.8	—	—	—	—
Amorpha-4,9-diene	—	0.3	—	1.6	—	—	—	—
Lanceol	—	—	—	1.5	—	—	—	—
14-Oxy-α-muurolene	—	—	—	0.3	—	—	—	—
α-Pinene	—	—	—	—	—	60.4-72.6	—	—
Camphene	—	—	—	—	—	1.8-1.1	—	—
Verbenene	—	—	—	—	—	0.9	—	—
Sabinene	—	—	—	—	—	2.6	—	—
β-Pinene	—	—	—	—	—	0.7-2.5	—	—
α-Campholene-aldehyde	—	—	—	—	—	1.7	—	—
trans-Pinocarveol	—	—	—	—	—	2.3	—	—
trans-Verbenole	—	—	—	—	—	3.9	—	—
cis-Verbenole	—	—	—	—	—	1.2	—	—
Karahanaenone	—	—	—	—	—	0.7	—	—
Pinocamphone	—	—	—	—	—	0.5	—	—
Pinocarvone	—	—	—	—	—	0.7	—	—
p-Mentha-1,5-dien 8-ol	—	—	—	—	—	1.7	—	—
Terpineol-4	—	—	—	—	—	0.5	—	—
Myrtenal	—	—	—	—	—	1	—	—
Verbenone	—	—	—	—	—	1.8	—	—
Bornyl acetate	—	—	—	—	—	0.5	—	—
Monoterpene hydrocarbons	—	—	—	—	—	72.9-81.6	—	—
Oxygen-containing monoterpenes	—	—	—	—	—	14.5	—	—
Oxygen-containing sesquiterpenes	—	—	—	—	—	2.4	—	—
2-Octene	—	—	—	—	—	2.3	—	—
3-Octaodiene	—	—	—	—	—	0.7	—	—
2-Methyl-phenol	—	—	—	—	—	0.8	—	—
Methyl benzene	—	—	—	—	—	2.1	—	—
Dimethylbenzene	—	—	—	—	—	2	—	—
Styrene	—	—	—	—	—	4.2	—	—
Nonane	—	—	—	—	—	1.7	—	—
Propyl benzene	—	—	—	—	—	1.9	—	—
1-Hexyl-2-nitrocyclohexane	—	—	—	—	—	—	9	—
(Z)-2-Octadecen-1-ol	—	—	—	—	—	—	8.1	—
3,5,24-Trimethyltetracontane	—	—	—	—	—	—	7.8	—
2-Octadecyl-1,3-propane-diol	—	—	—	—	—	—	6.1	—
[2]-2-Tetradecen-1-ol	—	—	—	—	—	—	5.8	—
11,14-Eicosadienoic acid methyl ester	—	—	—	—	—	—	5.7	—
2,6,10,15-Tetramethyl-heptadecane	—	—	—	—	—	—	4.2	—
11-Tricosene	—	—	—	—	—	—	4	—
2-Piperidinone, n-[4-bromo-n-butyl]	—	—	—	—	—	—	3.4	—
14-Heptadecenal	—	—	—	—	—	—	2.8	—
[2]-9-Tetradecenoic acid	—	—	—	—	—	—	2.7	—

Table 3: Continued.

Compound name	Algeria [37]	Egypt [37]	Libya [37]	Morocco [37]	Tunisia [37]	Iran [40]	S. Arabia [41]	Morocco [39]
Propylic acid	—	—	—	—	—	—	—	2.8-46.3
Butanol	—	—	—	—	—	—	—	3.5
Pent-3-en-2-one	—	—	—	—	—	—	—	3.2-4.9
Butyric acid	—	—	—	—	—	—	—	5.0-7.5
[2]-3-hexenol	—	—	—	—	—	—	—	2.2
Methy-4-methyl valerate	—	—	—	—	—	—	—	0.9-2.3
Tiglic acid	—	—	—	—	—	—	—	13.6

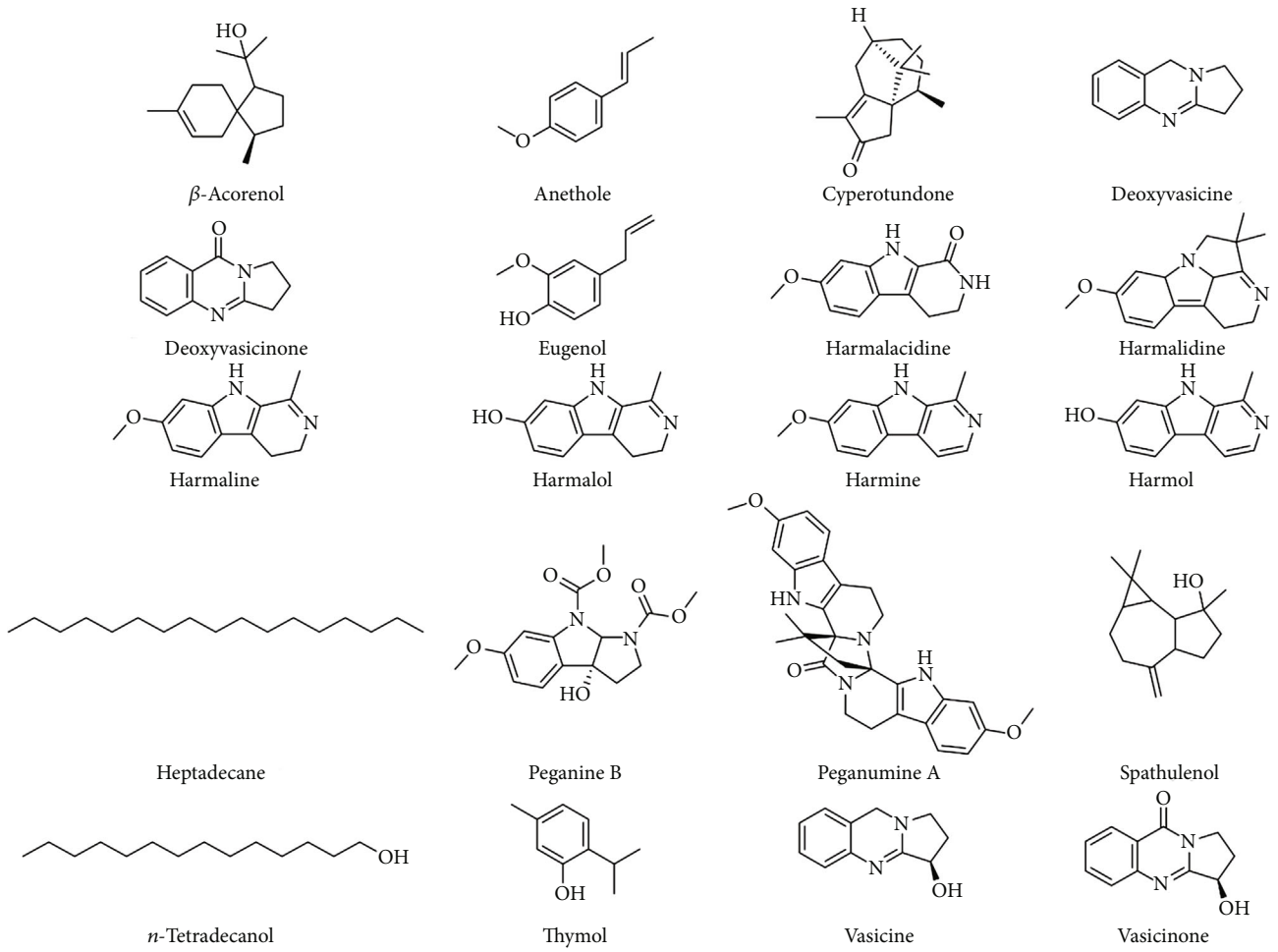

Figure 3: Phytochemical compounds isolated from different *Peganum* spp.

potent than the BChE inhibitory effect [93]. Another study used rat liver microsome incubation (RLMs) to show metabolic profiling of deoxyvasicine. Several metabolites were detected. In total, 6 major metabolites were identified. M1, M2-1, and M2-2 were produced for phase I metabolism, and M10-2, M10-3, and M10-4 were produced for phase II metabolism. Also, the inhibitory effect of deoxyvasicine generally varies as per the dosages [94].

5.1.3. Antiviral Activity. The use of nucleoside analogues such as acyclovir is common for the treatment of herpes simplex virus (HSV) infection (genital herpes transmitted by sexual contact) caused by HSV-2. Nevertheless, overuse leads to the growth of resistant strains. None of the extracts from hexane, dichloromethane, and ethyl acetate (except for the methanol extract) of *P. harmala* seeds reached complete inhibition of virus replication with direct contact during and after virus penetration, but there was no activity when this extract was used for the pretreatment of cells. Additionally, the methanol extract on Vero cells did not show a noteworthy cytotoxicity. The combination of harmine and acyclovir compounds were marked to have a synergetic effect against

Table 4: Phytochemicals present in different parts (seeds, roots, stems, fruits, flowers, leaves, testa, and whole plant) of *Peganum harmala* L. and overall profile of *Peganum multisectum* (Maxim.) Bobrov and *Peganum nigellastrum* Bunge.

Plant parts	Phytoconstituents	References
Peganum harmala L.		
Seeds	Hexadecanoic acid; methyl linoleate; methyl oleate; 9-octadecenoic acid; ethyl linoleate; ethyl oleate; harmaline; harmine; delta-tocopherol	[42]
	Harmine	[43]
	Harmaline; harmine; asparagine; sucrose; choline; phosphorylcholine	[44]
	Harmine; vasicinone; pegamine; H3-hydroxylated; pegamine dimer; ruine; deoxyvasicinone; tetrahydroharmin; peganine; harmaline; harmalol; pegaline; dexoypeganine	[45]
	2-Oxoindole alkaloids; (±)-peganumalines A-E; peganumaline	[46]
	p-Coumaric acid; rutin; catechin; hesperetin; chloregenic acid	[47]
	Peganine A; peganine B; peganumal A; peganumal B; pegaharmine F; pegaharmine G; pegaharmine H; pegaharmine I; pegaharmine J; pegaharmine K	[48]
	Peganine B; peganumal A; peganumal B; pegaharmine F; pegaharmine G; pegaharmine H; pegaharmine I	[49]
	3α-Acetoxy-27-hydroxyolean-12-en-28-oic acid methyl ester; syringin; [5]-caffeyl alcohol 4-O-β-D-glucopyranoside; coniferin; syringinoside; N-acetyl-9-syringinoside	[50]
	Pegaharmine A; pegaharmine B; pegaharmine C; pegaharmine D; pegaharmine E	[51]
	Peganone I (3,6-dihydroxy-8-methoxy-2-methylanthraquinone); peganone II (8-hydroxy-7-methoxy-2-methylanthraquinone)	[52]
	Harmine; harmaline; harmol; harmalol; harmane; norharmane	[53]
	Indole; quinoline,2,3,4-trimethyl-; tetrahydroharman; oleanitrile; tetrahydroharmine; harmaline; harmine; 9-octadecenamide, (z)-; 6-methoxytetrahydro-1-norharmanone; 4-amino-2-ethyl-3 methylquinoline	[54]
	2-Carboxyl-3,4-dihydroquinazoline; 3-hydroxylated harmine; 1-hydroxy-7-methoxy-β-carboline; acetylnorharmine; harmic acid methyl ester; 2-aldehyde-tetrahydroharmine; harmalanine; harmine *N*-oxide; 2-carboxyl-3,4-dihydroquinazoline; 6-methoxyindoline; 1-O-β-D-xylopyranose sinapyl alcohol	[55]
	[5]-Vasicinone-Glu; [5]-vasicinone-Glu	[56]
	3-Hydroxy-3-(N-acetyl-2-aminoethyl)-6-methoxyindol-2-one; 11-methyoxyl-rutaecarpine	[57]
	Harmic acid; 4,5-dihydroblumenol	[58]
	Oleic acid; linoleic acid ((Z,Z)-9,12-octadecatrienoic acid); linolenic acid; palmitic acid (hexadecanoic acid); arachidic acid	[59]
	Peganumine A	[60]
	Peganumine A; peganumine B	[61]
	Harmalidine	[62]
	Harmalacidine	[63]
	Diglycoside vasicine; vasicine; vasicinone; harmalol; ruine; harmol; 8-hydroxy-harmine; tetrahydroharmine; harmaline; harmine	[64]
	Norharmane	[65]
	Harmane; harmol; harmine	[66]
	Tetrahydroharmine	[67]
	Harmine; harmaline; vasicine; vasicinone	[68]
	Quercetin-3-*O*-gentiobioside; quercetin-3-*O*-rutinoside; kaempferol-3-methyoxyl-5-*O*-rutinoside; kaempferol-3,5-dimethyxyl-7-*O*-glucoside; stearic acid; kaempferol-3-methyoxyl-7-*O*-glucoside; isorhamnetin-7-*O*-glucoside; isorhamnetin-3-*O*-rutinoside; anthraquinone glucoside; 9,14-dihydroxyoctadecanoic acid	[69]
	Dodecane; tetradecane; methyl dodecanoate; hexadecane; 2-octanol benzoate; heptadecane; methyl tetradecanoate; 2,6,10,14-tetramethyl pentadecane; octadecane; 2,6,10,14-tetramethyl	[70]

Table 4: Continued.

Plant parts	Phytoconstituents	References
	hexadecane; nonadecane; methyl hexadecanoate; dibutyl phthalate; eicosane; methyl oleate; henicosane; docosane; harmine; tricosane	
	Acacetin 7-0-rhamnoside; acacetin 7-0-[6″-0-glucosyl-2″-O-(3‴-acetylrhamnosyl)] glucoside; acacetin 7-O-(2‴-O-rhamnosyl-2″-O-glucosyl)glucoside; 2‴-0-rhamnosyl-2″-0-gluco-sylcytisoside	[71]
	Ruine; dihydroharmane; dihydroruine; tetrahydroharmol; harmalicine	[72]
Roots	Threonine; acetic acid; lysine; proline; phosphorylcholine; sucrose; β-glucose; formic acid; asparagine; 4-hydroxyisoleucine	[44]
	Harmol	[66]
	Luotonin A; luotonin B	[73]
	Luotonin E; luotonin F	[74]
	Vasicol	[75]
	Harmine; vasicine; vasicinone; harmaline	[76]
Flowers	Harmine; peganine	[77]
	Proline; lysine; asparagine; 4-hydroxyisoleucine; sucrose; vasicine; choline; phosphorylcholine	[44]
Whole plant	1-Octadecene; 6,10,14-trimethyl-2-pentadecanone; [5]-15-heptadecenal; oxacyclohexadecan-2-one; 1,2,2,6,8-pentamethyl-7-oxabicyclo[4.3.1]dec-8-en-10-one; hexadecane-1,2-diol; eicosan-3-ol; tetradecanoic acid; 12-methyl tetradecanoic acid; pentadecanoic acid; 5,9,13-trimethyl tetradecanoic acid; tridecanoic acid; 2-methyl-octadecanoic acid; heptadecanoic acid; [5]-9-dodecenoic acid; (Z)-9-hexadecenoic acid; (Z,Z,Z)-9,12,15-octadecatrienoic acid	[78]
	Harmine; harmaline	[77]
	Deoxyvasicine; deoxyvasicinone	[72]
Leaves	Trigonelline; formic acid; vasicinone; vasicine; harmaline; harmine; maleic acid; sucrose; α-glucose; β-glucose; malic acid; proline; asparagine; betaine; choline; phosphorylcholine; succinic acid; 4-hydroxyisoleucine; acetic acid; alanine; threonine; valine; isoleucine; malic acid; lysine	[79]
	Isoleucine; valine; alanine; betaine; maleic acid; proline; lysine; asparagine; 4-hydroxyisoleucine; acetic acid; sucrose; vasicine; choline; phosphorylcholine	[44]
	Aspartate; threonine; serine; glutamate; glycine; alanine; valine; methionine; isoleucine; leucine; tyrosine; phenylalanine; lysine; histidine; arginine; proline; cystine	[73]
Stem	Lysine; succinic acid; malic acid; vasicinone; proline; asparagine; 4-hydroxyisoleucine; asparagine; sucrose; vasicine; choline; phosphorylcholine	[44]
Areal part	10-Vasicinol; 11-vasicinol; 4-vasicinol; 6-vasicinol; vasicine-glu; vasicine-2glu; vasicine-2glu; methylation-acetylation-vasicine; methylation-acetylation-vasicine; vasicinol-glu; vasicinone-glu; vasicinone-2glu; vasicinone-2glu; pegaline; diosmetin+3glu+rha+ac; acacetin+glu+rha; diosmetin+glu+rha	[75]
	2-Ethoxylpropane; 3-hydroxy-3-methyl-2-butanol; methylcyclopentane; 2-methylhexane; 3-methylhexane; 1-ethoxy-2-methylpropane; heptane; methylcyclohexane; 2,4-dimethylhexane; 1-octen-3-ol; 2-methylheptane; 3-methylheptane; hexanol; *cis*-1,3-dimethylcyclohexane; 2-octanol; octane; *cis*-1,4-dimethylcyclohexane; [5]-2-hexanol; 1,3-dimethylbenzene; benzaldehyde; 6-methyl-5-heptane-2-one; [8]-[5]-5-ethyl-2(5H)-furanone; 3,5,5-trimethyl-2-cyclohexene-1-one; 12-heptadecyn-1-ol; benzenamine; N-phenyl-formamide; geranylacetone; 1,2,3,3,4-pentamethylcyclopenene; 5,6,7,7α-tetratydro-4,4,7α-trimethyl-2(4H)-benzenofranone; 6,10,14-trimethyl-2-pentadecanone; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; dodecanoic acid; hexadecanoic acid	[80]
	Liriodendrin; *trans*-ferulic acid β-D-glucopyranoside; (*6S,7E,9R*)-6,9-dihydroxymegastigma-4,7-dien-3-one-9-*O*-β-D-glucopyranoside (or roseoside); (*3S,5R,6R,7E,9S*)-megastigman-7-ene-3,5,6,9-tetrol-3-O-β-D-glucopyranoside	[81]
	Deoxypeganidine; peganidine; peganol; quinoline; pegamine; hemicellulose; gentisate-2,5-diglucoside	[72]
Fruits	α-Glucose; choline; proline; lysine; asparagine; 4-hydroxyisoleucine; acetic acid; sucrose; vasicine; choline; phosphorylcholine	[44]
	Harmine; peganine; harmaline	[77]

Table 4: Continued.

Plant parts	Phytoconstituents	References
Testa	4-Hydroxyisoleucine; asparagine; proline; vasicine	[44]
Floral nectar	Harmalol; harmine; fructose; glucose; sucrose; aspartic acid; glutamic acid; serine; glutamine; glycine; histidine; alanine; proline; tyrosine; valine; phenylalanine	[82]
Peganum multisectum (Maxim.) Bobrov	2-Methylquinoline; 9-amino-2,3,5,6,7,8-hexahydro-1H-cyclopenta [b] quinoline; vasicinone; harmine; peganine; deoxypeganine; deoxyvasicinone; harmane	[83]
	Vasicine	[84]
	(*S*/*R*)-Vasicinone	[10]
	Harmol; harmane; harmine; harmaline; harmalol	[66]
	Peganetin; deacetylpeganetin; 7,4′-dihydroxy-3′-methoxy-5-O-rutinoside	[85]
	Aspartate; threonine; serine; glutamate; glycine; alanine; valine; methionine; isoleucine; leucine; tyrosine; phenylalanine; lysine; histidine; arginine; proline; cystine	[73]
	2-Ethoxylpropane; 3-hydroxy-3-methyl-2-butanol; methylcyclopentane; 2-methylhexane; 3-methylhexane; 1-ethoxy-2-methylpropane; heptane; methylcyclohexane; 2,4-dimethylhexane; 1-octen-3-ol; 2-methylheptane; 3-methylheptane; hexanol; *cis*-1,3-dimethylcyclohexane; 2-octanol; octane; *cis*-1,4-dimethylcyclohexane; [5]-2-hexanol; 1,3-dimethylbenzene; benzaldehyde; 6-methyl-5-heptane-2-one; [8]-[5]-5-ethyl-2(5H)-furanone; 3,5,5-trimethyl-2-cyclohexene-1-one; 12-heptadecyn-1-ol; benzenamine; N-phenyl-formamide; geranylacetone; 1,2,3,3,4-pentamethylcyclopenene; 5,6,7,7α-tetrahydro-4,4,7α-trimethyl-2(4H)-benzenofranone; 6,10,14-trimethyl-2-pentadecanone; 3,7,11,15-tetramethyl-2-hexadecen-1-ol	[80]
	Deoxyvasicine; deoxyvasicinone	[72]
Peganum nigellastrum Bunge	Vasicine	[64]
	(*S*/*R*)-Vasicinone	[10]
	Diosmetin 7-O-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-[α-L-rhamnopyranosyl (1→6)]-β-D-glucopyranoside	[86]
	Nigellastrine I; nigellastrine II; harmol; harmane; harmine; harmaline; harmalol	[66]
	Dihydrosinapyl ferulate; dihydroconiferyl ferulate	[36]
	3α,27-Dihydroxylup-20(29)-en-28-oic acid methyl ester; 3α-acetoxyolean-12-ene-27,28-dioic acid 28-methyl ester; 3-oxotirucalla-7,24-dien-21-oic acid; 3α-acetoxy-27-hydroxylup-20(29)-en-28-oic acid methyl ester; betulinic acid; 3-O-acetylbetulinic acid; 3-epibetulinc acid; 3-O-acetylepibetulinic acid	[87]
	Acacetin; peganetin; deacetylpeganetin	[85]
	Aspartate; threonine; serine; glutamate; glycine; alanine; valine; methionine; isoleucine; leucine; tyrosine; phenylalanine; lysine; histidine; arginine; proline; cystine	[73]
	2-Ethoxylpropane; 3-hydroxy-3-methyl-2-butanol; methylcyclopentane; 2-methylhexane; 3-methylhexane; 1-ethoxy-2-methylpropane; heptane; methylcyclohexane; 2,4-dimethylhexane; 1-octen-3-ol; 2-methylheptane; 3-methylheptane; hexanol; *cis*-1,3-dimethylcyclohexane; 2-octanol; octane; *cis*-1,4-dimethylcyclohexane; [5]-2-hexanol; 1,3-dimethylbenzene; benzaldehyde; 6-methyl-5-heptane-2-one; [8]-[5]-5-ethyl-2(5H)-furanone; 3,5,5-trimethyl-2-cyclohexene-1-one; 12-heptadecyn-1-ol; benzenamine; N-phenyl-formamide; geranylacetone; 1,2,3,3,4-pentamethylcyclopenene; 5,6,7,7α-tetrahydro-4,4,7α-trimethyl-2(4H)-benzenofranone; 6,10,14-trimethyl-2-pentadecanone; 3,7,11,15-tetramethyl-2-hexadecen-1-ol;	[80]
	Deoxyvasicine; deoxyvasicinone	[72]

HSV-2 replication [43]. Another study indicated that the *P. harmala* seed extract is also effective against influenza A virus considering the concentration and death rates of Madin-Darby canine kidney (MDCK) cells. The IC_{50} value of the *P. harmala* seed extract on influenza A (H1N1) was 15.7 μg/mL, which is clearly under the recommended IC_{50} value (less than 100 μg/mL) of herbal extracts against infectious diseases [95].

5.1.4. Antioxidant and Antimicrobial Agent. To evaluate the antioxidant value of purified protein (132 kDa) from *P. harmala* seeds, several concentrations of the protein were added to the methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution. The result was that the antioxidant activity of the protein was similar to the antioxidant activity of vitamin C reported [96]. The seed extract of *P. harmala* showed strong monoamine oxidase (MAO-A) inhibitory activity with an IC_{50} value of 27 μg/L based upon harmaline and harmine, while the root extracts' IC_{50} value was 159 μg/L based on just harmine. On the other hand, they exhibited weak MAO-B inhibitory activity [97]. To evaluate the MAO-A and B inhibitors in human liver, ultraperformance liquid chromatography-electrospray tandem mass spectrometry

(UPLC-ESI-MS/MS) was used. According to the findings, harmaline, harmine, harmalol, harmane, and tetrahydroharmine indicated important MAO-A inhibitory effects. In contrast, they did not show an MAO-B inhibitory effect [98]. Outcomes from another study, which used a cocktail experiment on human liver enzymes showed that IC_{50} values of harmaline and harmine on MAO-A were $0.1 \pm 0.08\,\mu$M and $0.38 \pm 0.21\,\mu$M, respectively. These results showed that harmaline and harmine selectively performed on MAO. In addition, harmaline and harmine were shown to have more potent MAO-A inhibitory activity than AChE [99].

Urinary tract infectious pathogens (*Proteus mirabilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were isolated and studied to determine the antibacterial effect of the seed and leaf extracts of *P. harmala* on them. Antibacterial activity was detected by agar diffusion. Methanolic extracts of *P. harmala* leaves were more robust in inhibiting the microorganisms than the aqueous extracts. On the other hand, *P. harmala* seed extracts were not inhibitory against the growth of *P. mirabilis* and barely inhibitive against the growth of other microorganisms. The applications of the flavonoid extracts of *P. harmala* to *P. aeruginosa* and *S. aureus* were an exception in that regard. Also, this study exhibited that the Gram-positive strain of *S. aureus* was less resilient to flavonoid extracts of seeds and leaves. However, *E. coli* was found to be resistant to the seed and sensitive to the leaf extracts [100].

Another study on the antibacterial and antifungal activities of beta-carboline from *P. harmala* seeds indicated that alkaloids have positive inhibitory interaction when they are applied as binary mixtures. For example, harmine showed the strongest inhibition zone against *Proteus vulgaris* (24.7 mm), *Bacillus subtilus* (21.2 mm), and *Candida albicans* (22.2 mm), while *E. coli* and *Aspergillus niger* were found to be most sensitive to harman with inhibition zones of up to 20.8 mm. Additionally, stronger inhibition zones against *P. vulgaris* (22.6 mm) and *C. albicans* (21.3 mm) were detected when harmaline was applied. However, the application of harmoline presented mediocre activities. On the other hand, when harman and harmaline were used as a binary mixture, *P. vulgaricus* and *C. albicans* inhibition zones reached to 28.9 and 29.0 mm, respectively. A study also showed that the most effective mixture was the whole β-carboline fraction with inhibition zones varying from 23.7 to 31.5 mm. The results of this study reported that *E. coli*, *P. vulgaris*, *C. albicans*, and *A. niger* were more sensitive against applied alkaloids, while *S. aureus* was less sensitive. The lowest value for minimal inhibition concentration (MIC) was performed by total (crude) harmala alkaloids (0.333 mg/mL) and binary mixtures of harman with either harmine or harmaline [101]. By the use of the piezoelectric spraying procedure, it was presented in a study that the extract of *P. harmala* seeds had the most antibacterial effect on *Aliivibrio fischeri* [93].

5.2. In Vivo Studies

5.2.1. Treatment of Respiratory Disorders. The traditional application of *P. harmala* was for treating some respiratory diseases such as cough and asthma. Use of aerial parts of *P. harmala* (APP) in a study showed that when mice with ammonia-induced cough were treated with the total extract of APP (EXT), cough frequency decreased in 2 minutes at all concentrations of 183.3, 550, and 1650 mg/kg, respectively. Also, the alkaloid fraction of EXT (ALK) showed the same inhibitive effect on the cough model at all concentrations of 10, 30, and 90 mg/kg, respectively [63]. However, the flavonoid fraction of EXT did not present a significant reduction in cough frequency [102]. According to the findings of this cough model, EXT and ALK have stronger inhibitory effect on mice at medium and high concentrations than codeine phosphate which was used on the control mice at 30 mg/kg. In addition, EXT and ALK prolonged the latent period of cough at each concentration and were more efficient at medium and high concentrations than codeine phosphate. When ammonia was replaced with capsaicin, EXT and ALK had an obviously stronger effect compared to codeine phosphate on cough frequency at high concentrations. No significant inhibition was detected with the flavonoid fraction (FLA). Moreover, EXT and ALK were a more potent inhibitor for reducing cough frequency in 5 minutes at high concentrations than codeine phosphate when they were applied on mice with citric acid-induced cough. In the meantime, they also prolonged the latent period of cough. High dosages of EXT and ALK from APP were better as an expectorant than ammonium chloride. All of EXT, ALK, and FLA had bronchodilating effects on guinea pigs by prolonging the preconvulsive time [103]. It was indicated by another study that vasicine, vasicinone, and deoxyvasicine from AAP were inhibitive against cough frequency and prolonged the latent period of cough on mice and guinea pigs at a high dosage of 45 mg/kg, which was similar to 30 mg/kg of codeine phosphate. Vasicine, vasicinone, and deoxyvasicine were proven to be strong expectorants on mice. Moreover, vasicinone was more successful at 15 and 45 mg/kg than ammonium chloride. All alkaloids showed obvious bronchodilation effects on guinea pigs depending on their dosages [104].

5.2.2. As Antiulcer Agent. Peganine hydrochloride from *P. harmala* seeds were applied against cold-resistant, aspirin-, alcohol- [6], and pyloric ligation-induced gastric ulcer models in rats to find out its antiulcer activities. According to this research, the antiulcer activity of peganine hydrochloride was significant in rats. Also, peganine hydrochloride was found to be protective in alcohol, potentially cytoprotective in aspirin, and palliative for free and total acidity in pyloric ligation [105].

5.2.3. Improving Brain Function. Two different dosages of harmine were tested on rats with a delayed-match-sample test and water maze task. Harmine showed noteworthy improvement on short-term working and recent memory. On the other hand, dosages of 1 and 5 mg harmine exhibited no influence on spatial reference memory and the administration of high dosage caused motor deficits on many of these rats [106]. Another study showed that harmine was tested on scopolamine- (1 mg/kg) induced Alzheimer's disease mice and in APP/PS1 transgenic mice with Morris Water Maze Test. Application of harmine (20 mg/kg) for 2 weeks substantially enhanced the spatial learning of mice (scopolamine

induced). In 10 weeks, harmine exhibited little benefit on APP/PS1 mice. Also, this study demonstrated that harmine could penetrate through the blood-brain barrier and activate Egr-1, c-Fos, and c-Jun [107]. According to real time PCR, Western blot analysis on RBE4 cells (*in vitro*), and alternation of cholinergic neurotransmitters, harmaline and harmine selectively performed on AChE [99].

To evaluate the antidepressive-like effect of acute harmine application, rats were acutely administered with harmine at 5, 10, and 15 mg/kg and imipramine at 10, 20, and 30 mg/kg, respectively, and rats' performances were tested in forced swimming and open-field tests. Treatment of harmine with 10 and 15 mg/kg and imipramine with 20 and 30 mg/kg showed reduction in the period of immobility, while climbing and swimming time increased and did not disturb the locomotor activity. In addition, brain-derived neurotrophic factor (BDNF) protein levels were enhanced in rat hippocampus by acute treatment of harmine at greater dosages [108]. To determine the behavioral and physiological influences of harmine in the chronic mild stress (CMS) rat model, the CMS method was carried out. After 40 days of the CMS method, harmine (15 mg/kg/day) was given to rats for a week. Consequently, anhedonic behavior and hypertrophy of adrenal gland were reversed after harmine treatment. Also, harmine regularized adrenocorticotropic hormone circulating levels and BDNF protein levels [109]. Another study showed that harmine and imipramine had similar behavioral outcomes, while on the contrary, they had dissimilar molecular outcomes [110].

Extract and alkaloid fractions isolated from APP were examined for their antiamnesic effects on mice. Extract and alkaloid fractions proved to have positive effects on learning and memory processes of mice for scopolamine-induced cases. Furthermore, alkaloid fractions were confirmed as major effective components of APP [111].

5.2.4. Antiviral Effect. Another research presented that the extract of *P. harmala* seeds (PHS) were therapeutic against PR8 (H1N1) influenza virus. The study indicated that the PHS extract had an antiviral effect similar to oseltamivir (20 mg/kg). Oral application of 200 mg/kg PHS reduced the loss of body weight and enhanced the rate of survival [95].

A comprehensive illustration presenting various bioactivities and health-promoting effects can be seen in Figure 4.

6. Health-Promoting Effects Evidenced by Clinical Trials

Despite various experimental studies in both cell-based and animal models, the amount of published clinical trials regarding *Peganum* spp. is scarce. The main findings are mentioned in the following subsections.

6.1. Knee Osteoarthritis. A randomized, double-blind, placebo-controlled trial (RCT) evaluated the efficacy of a standardized preparation of *P. harmala* oil in pain management, stiffness, tenderness, and function after four weeks of treatment [102]. There was a significant pain reduction in the intervention group (52.56% vs. 17%, $P < 0.05$) according to the Visual Analogue Scale (VAS) and Western Ontario and McMaster Universities Arthritis Index (37.89% vs. 16.41%, $P < 0.001$). Regarding function, there was an 80% decrease in pain on motion factor in patients using the oil, which was significant when compared to the placebo (22.2%, $P < 0.01$). Similarly, tenderness improved by 65% compared to the control group (14.8%, $P < 0.001$). There was no significant improvement in stiffness. These results, while promising, require further examination due to their limited external validity, considering the small sample size ($n = 54$), and only 3 of the patients recruited were males.

6.2. Dermatoses. A clinical trial performed in Egypt evaluated the antibacterial and antiprotozoal activity of *P. harmala* in multiple skin conditions [112]. One hundred and eleven patients with multiple or bilateral lesions were randomly selected and followed for four weeks. All patients received the intervention, an ointment with alkaloids extracted from the plant in question and a placebo. They used the ointment with the active ingredient on one side while they used the placebo on the other. All patients with impetigo ($n = 20$) showed significant improvements in symptoms and laboratory results. Of all patients with pityriasis alba ($n = 18$), 72% had clinically significant improvement. Fifty percent of patients with tinea circinata ($n = 20$) improved considerably, albeit fungi were detected in cultures of 10% of them. Concerning leishmaniasis, the response was favorable in 5 of the seven patients with this disease; however, the lesions were not cleared completely. Patients with itching dermatoses (i.e., Lichen planus, Lichen simplex chronicus) showed a notable change in their symptoms. Psoriasis did not improve at all; in fact, treatment was discontinued during the third week. Placebo did not produce any improvement. These results show a promising use of *P. harmala* as an antibacterial, antipruritic, and antifungal agent. Nevertheless, it is necessary to evaluate the efficacy of the intervention on a grander scale, explicitly focused on each of the dermatoses with a clear description of the inclusion and exclusion criteria.

6.3. Gastroesophageal Cancer. Three studies evaluated the therapeutic and adverse events of Spinal-Z, with *P. harmala* and *Dracocephalum kotschyi* Boiss as its main components, in patients with gastroesophageal cancer. It is used as palliative therapy in combination with standard treatment. The initial study evaluated the response to Spinal-Z in seven patients with cancer in the upper gastrointestinal tract, during a nine-month follow-up [113]. Of these seven patients, one showed a reduction in the growth of metastases. Common side effects included dizziness and vomiting. One patient died of drug-induced hepatitis. Although causality with Spinal-Z was not proven, it should be evaluated in further studies. The remaining studies [114, 115] presented larger sample sizes; one was focused on gastroesophageal cancers in general ($n = 61$), while the other was focused on patients with metastatic gastroesophageal adenocarcinoma ($n = 76$). Both studies showed meaningful improvement in gastrointestinal symptoms such as dysphagia, heartburn, poor appetite, nausea, and constipation ($P < 0.05$). There

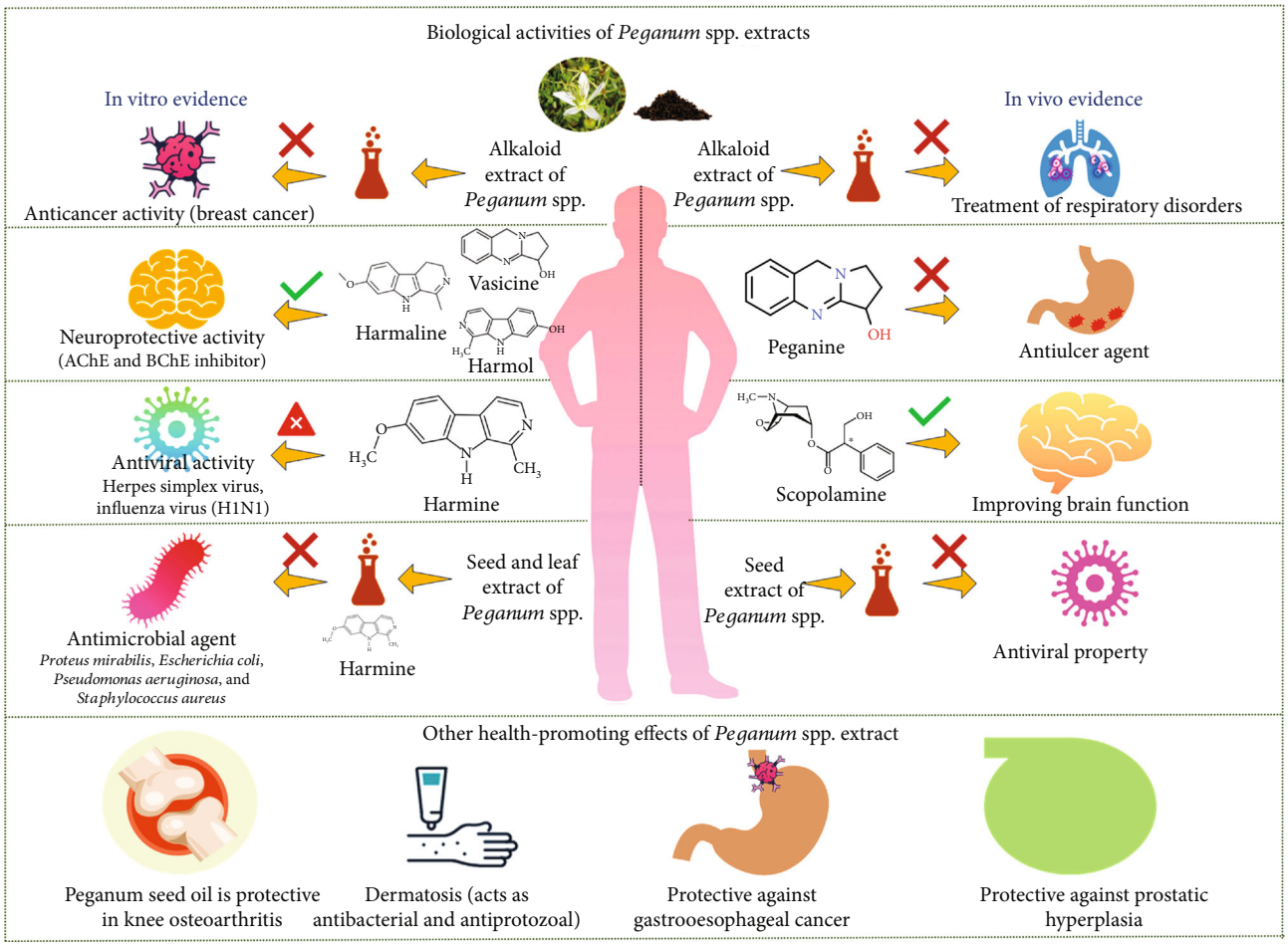

Figure 4: Biological activities and health-promoting effects of *Peganum* spp. extracts.

were no significant changes in laboratory findings ($P > 0.05$) or drug-related adverse symptoms, except for muscle weakness in one of the studies ($P < 0.05$).

6.4. Benign Prostatic Hyperplasia. In a clinical trial study, 90 patients with benign prostatic hyperplasia with lower urinary tract symptoms were randomized into three groups: patients receiving an oral capsule of *P. harmala*, a second group receiving tamsulosin, and a third group receiving combined therapy (*P. harmala* plus tamsulosin) [116]. Efficacy was evaluated based on the International Prostate Standard Survey (IPSS) that evaluates seven symptoms: incomplete discharge, urinary frequency, intermittency, urgent need to urinate, weak flow of urine, straining to urinate, and nocturia. All groups had a statistically significant change in mean IPSS scores after four weeks of treatment ($P < 0.001$). However, there was no significant difference between groups after treatment, with the exception of urinary frequency, intermittency, and nocturia. The combined therapy group seemed to have more effect on alleviating symptoms, although the study does not report whether these results are significant or not. Also, there is no report on adverse events on neither of the groups. Finally, blinding methods are not clearly explained. In the summary section, the article indicates that it is a single-blind study; yet it indicates that it is a double-blinded study on the methods sections. Either way, there is no clear indication of how blinding was performed.

7. Safety and Adverse Effects

As mentioned throughout this manuscript, for *P. harmala*, the whole plant and various parts of this plant, including its seeds, bark, and roots, have been used as traditional medicine in Iran along with many other countries. Recent researchers have shown different pharmacological and therapeutic effects of *P. harmala* and its active alkaloids, especially harmine and harmaline [29].

This plant has also been studied in relation to its bactericidal activity, as a natural alternative where the total alkaloid extract of *P. harmala* seeds was tested *in vitro* on four phytopathogenic bacteria, namely, *Ralstonia solanacearum* Phylotype II, *Erwinia amylovora*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Burkholderia gladioli*, the causal agents of potato brown rot, pear fire blight, potato soft rot, and onion slippery skin diseases, respectively [117]. Hence, the MIC and the minimum bactericidal concentration (MBC) were evaluated *in vitro*, obtaining the MBC of 150 μg/mL for *R. solanacearum*, followed by *B. gladioli* (MBC 200 μg/mL). The extract exhibited a marked inhibitory effect *in vitro* on the pathogen *R. solanacearum* at concentrations

ranging from 4 to 300 μg/mL, but this effect on the other pathogens required higher concentrations (50–300 μg/mL). However, it was observed that *R. solanacearum* cells exposed to 4 μg/mL presented severe cell damage and genome coagulation, as well as a disorganized cytoplasm and a thickened cell wall compared to the control. In general, this study revealed the antibacterial efficacy of the total alkaloid extract of *P. harmala* on phytopathogenic bacteria that could be used as an alternative for chemical antibacterials [117]. Nevertheless, in order to revise the cytotoxic response in bacteria, it would be necessary to deepen the adverse effects in humans.

On the other hand, in a study guided in this sense, the Algerian seeds of *P. harmala* were evaluated for use in therapeutic and clinical trials as mentioned above. In parallel with these trials, there have been some studies on adverse effects and safety that are important to review in this section. Among them, an oral administration of *P. harmala* extract was carried out in mice at a dose of 0–12 g/kg to evaluate the acute toxicity causing adverse effects at an LD_{50} of 2.86 g/kg [118]. In a study to evaluate the subacute effects carried out by the daily oral administration of an aqueous extract for 28 consecutive days, no mortality was obtained, although a significant difference was observed in the weight of the organs between the control animals and those that received the treatment. At a biochemical level, significant differences were also observed in bilirubin, uric acid, and alkaline phosphatase as well as in the hematological analysis, where differences were also observed in the leukocyte count and the estimation of hemoglobin. Only the urinalysis was negative for all parameters except glucose. In addition, from the pathological point of view, some serious abnormalities and histological changes were observed in liver and kidney tissues [118]. These results give us an idea of the high activity of the alkaloids contained in this plant and therefore of the need to deepen the knowledge of the adverse effects that they could present in humans.

8. Conclusions

Future perspectives of new bioactive compounds and development of new drugs have been focusing on natural products. As it has been presented throughout the manuscript, harmine and other alkaloids found in the genus *Peganum* could be used for pharmaceutical and clinical purposes since they provide health benefits and significant effects on treatments of diseases.

These plants have the advantage of functioning as bactericides and virucides and have therapeutic effects showing wide health benefits on humans. Nevertheless, despite all the therapeutic potential, at higher doses and long periods of exposure, extracts from *Peganum* can cause several cytotoxic effects such as several hepatic and nephritic toxicities. In this respect, all new drugs need to be studied in depth, even natural ones, since the adverse effects of these plants also need to be described. Therefore, nanotechnology, development of new drugs, and designing new formulations where release of active compounds may be controlled are necessary to achieve successfully pharmacological and therapeutically treatments.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J.S.-R., W.C.C., C.Q., P.S., S.P., B.Ö., F.E.H., S.S., S.S., K.A., M.A., C.M.S.C., M.D.L, M.S., M.M., H.A.R.S., J.H.-B., and T.G. contributed to the study conception and design, acquisition of data, the analysis and interpretation of data. J.S.-R., M.M., W.C.C., and M.K. contributed to drafting the manuscript and critically revising the manuscript. All authors prepared the manuscript, contributed equally, and read and approved the final manuscript.

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Review Article

Role of Dietary Antioxidants in p53-Mediated Cancer Chemoprevention and Tumor Suppression

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Cancer arises through a complex interplay between genetic, behavioral, metabolic, and environmental factors that combined trigger cellular changes that over time promote malignancy. In terms of cancer prevention, behavioral interventions such as diet can promote genetic programs that may facilitate tumor suppression; and one of the key tumor suppressors responsible for initiating such programs is p53. The p53 protein is activated by various cellular events such as DNA damage, hypoxia, heat shock, and overexpression of oncogenes. Due to its role in cell fate decisions after DNA damage, regulatory pathways controlled by p53 help to maintain genome stability and thus "guard the genome" against mutations that cause cancer. Dietary intake of flavonoids, a C_{15} group of polyphenols, is known to inhibit cancer progression and assist DNA repair through p53-mediated mechanisms in human cells via their antioxidant activities. For example, quercetin arrests human cervical cancer cell growth by blocking the G_2/M phase cell cycle and inducing mitochondrial apoptosis through a p53-dependent mechanism. Other polyphenols such as resveratrol upregulate p53 expression in several cancer cell lines by promoting p53 stability, which in colon cancer cells results in the activation of p53-mediated apoptosis. Finally, among vitamins, folic acid seems to play an important role in the chemoprevention of gastric carcinogenesis by enhancing gastric epithelial apoptosis in patients with premalignant lesions by significantly increased expression of p53. In this review, we discuss the role of these and other dietary antioxidants in p53-mediated cell signaling in relation to cancer chemoprevention and tumor suppression in normal and cancer cells.

1. Introduction

Cancer is a leading cause of mortality in the world, reporting 18.1 million new cases and 9.6 million deaths in 2018 [1]. National Institutes of Health of the US estimated 1.8 million new cancer patients in 2020, with 606,520 deaths in the US [2]. Conventional therapies for cancer include surgery, radiotherapy, chemotherapy, endocrine therapy, and targeted therapy [3, 4]. However, currently available therapies have serious limitations, including severe side effects and dose-limiting toxicities [5]. The common adverse effect of cancer treatments includes nausea, vomiting, peripheral neuropathy, pain, and insomnia, which reduce the quality of life [6]. Hence, the search for alternative therapeutic agents with better patient outcomes is necessary. Currently, epidemiological studies have revealed a strong association between the risk of developing cancer and the consumption of fruit and vegetables [7, 8].

Plants produce various defensive secondary metabolites such as polyphenols, isoprenoids, alkaloids, sulforaphanes, and certain vitamins and their precursors [9–11]. Most of these phytochemicals exert hormetic effects: at low concentrations, they act as antioxidants to scavenge free radicals, including reactive oxygen species (ROS), thus protecting cells from oxidative damage to proteins, lipids, DNA, and RNA [12]. Many studies have suggested that the regular intake of fruits, vegetables, and their products containing phytochemicals is effective in the prevention of various types of cancers [13–16]. Phytochemicals also exhibit anticancer activity through various signaling pathways, including the death receptor or extrinsic pathway, mitochondrial or intrinsic pathway, and perforin/granzyme apoptotic pathway [8, 17].

Polyphenols use the p53 signaling pathway to produce anticancer activity through apoptosis in various types of cancers [18, 19]. p53 regulates various biological processes, including metabolic pathways, aging, development, reprogramming, and reproduction [20, 21]. In this review, we are focusing on p53 regulation by various dietary antioxidants in relation to various cancers.

2. A Brief Overview of p53

In 1979, p53 protein was identified. SDS-PAGE analysis indicated that the protein has 53 kDa molecular mass, thus named p53 [22, 23]. In humans, the p53 gene is located on the short arm of chromosome 17 (17p13.1) [24]. p53 is well known as "the guardian of the genome" and is a powerful tumor suppressor, which comprises 393 amino acid residues and three main domains, namely, transcriptional activation domain (TAD), DNA-binding domain (DBD), and tetramerization (TET) domain. TAD recruits RNA polymerase and activates the DNA-reading machinery. DBD is rich in arginine/lysine residues, most prone to mutations, and binds to the specific regulatory sites present on the DNA response elements. The TET domain assembles the chains of other p53 monomers for oligomerization [25, 26].

3. Significance of p53

The main role of p53 is to control cell cycle progression, DNA repair, and apoptosis. In vertebrates, p53 has the ability to temporarily block the cell cycle and promote DNA repair. Under certain conditions, p53 can also induce senescence or promote apoptosis, thus providing mechanisms against the accumulation of potentially malignant or defective cells [27].

3.1. Apoptosis. Apoptosis is the cellular terminal process in the prevention of the proliferation of cells with anomalous DNA. In mammalian cells, p53 mediates apoptosis through transcriptional activation of proapoptotic target genes by two major apoptotic pathways: extrinsic pathway, mediated by death domain proteins belonging to the tumor necrosis factor receptor (TNF-R), and intrinsic pathway that monitors the B-cell lymphoma 2 (Bcl-2) family of proteins, which lead to cell death through the expression of caspases [28, 29]. The extrinsic apoptotic pathway is initiated by apoptotic genes such as Fas cell surface death receptor (Fas), death receptor 5 (DR5), and p53 apoptosis effector related to PMP22 (PERP) [30, 31]. Overexpression of p53 may increase the level of Fas at the cell surface by promoting the trafficking of the Fas receptor from the Golgi, which allows p53 to rapidly sensitize cells to Fas-induced apoptosis before the transcription-dependent effect operates [32]. Fas is activated by binding of its ligand FasL, which is expressed by T-cells [30]. DR5 is initiated by tumor necrosis factor- (TNF-) related apoptosis-inducing ligand (TRAIL), which promotes apoptosis through the caspase-8 pathway [33]. The intrinsic pathway involves the induction of the proapoptotic Bcl-2 family members Bcl-2-associated X protein (Bax), p53 upregulated modulator of apoptosis (Puma), and damage protein, a proapoptotic BH3-containing protein (Noxa) [33, 34]. The Bcl-2 family is activated by Bcl-2 homology-3 (BH3) domain-only death agonist protein (BID) to form channels in the mitochondrial membrane, allowing efflux of cytochrome c (Cyt c). BID exists in the cytoplasm as an inactive state. After exposure to TNF-R, BID is cleaved by caspase-8 and translocates to the mitochondrial membrane and activates Bax for apoptosome formation. BID is a link between the extrinsic and intrinsic pathways [30, 35]. p53 is also involved in the activation of the apoptosome via induction of Cyt c, apoptotic protease-activating factor 1 (Apaf-1) from mitochondria, and their formation of a complex with procaspase-9, which activates caspase-9 [33, 36]. Caspase-9 responds to changes in the mitochondrial perspective, whereas caspase-8 senses activation of death receptors. These initiator caspases cleave the proenzyme forms of the effector caspases to caspase-3, caspase-6, and caspase-7, which allows for the digestion of essential targets that affect cell viability (Figure 1) [37].

3.2. Senescence. The irreversible cell cycle arrest will lead to cellular senescence that can limit the expansion of mutations in cells [38]. Therefore, senescence is a way to prevent cancer. Apart from this, the depletion of p53 compromises the oncogene-induced senescence, and although molecular mechanisms are still elusive, several microRNAs and p53-targeted genes have been linked to senescence and depletion of p53 [39, 40]. The p53 protein acts both the prooxidant and antioxidant functions in relation to the intensity of the stress, which can contribute to tumor suppression [41]. p53 acts as a prooxidant in permitting the cells to repair damage and survive if stress is mild and also functions as an antioxidant to subsidize the overall action of apoptosis when stress is at an extreme level [19, 42].

3.3. Cell Cycle Arrest. Cyclins and cyclin-dependent kinases (CDKs) are the two major proteins that control cell cycle progression. The cell cycle is arrested at the G_2/M phase during DNA damage, which prevents the propagation of mutagenic lesions to the daughter cells, and these transitions are monitored by p53 downregulatory p21 during the DNA damage [43]. The first evidence of participation of p53 in the cell cycle was confirmed by Kastan and colleagues in 1991, and they found the expression of GADD45, which was dependent on the presence of wild-type p53 by ionizing radiation [44]. DNA damage is also done by proteins such as GADD45 induced by p53. p53 plays an important role in mitotic cell division by regulating the function of centrosomes by blocking the mitotic spindle assembly using nocodazole, which leads to the activation of p53 by ataxia telangiectasia-mutated protein kinase (ATM), the key initiator of the DNA damage repair (DDR) pathway [33, 45].

3.4. DNA Repair. The endpoint of the DDR cascade is DNA repair. Various internal factors that cause DNA damage are ROS, free radicals, certain cellular metabolites, and various external factors such as UV light, certain drugs, and heavy metals. All these factors lead to the moderation of nucleotide bases of DNA, resulting in DNA strand breakage [33, 46]. p53 is active in regulating DNA damage, and its key function

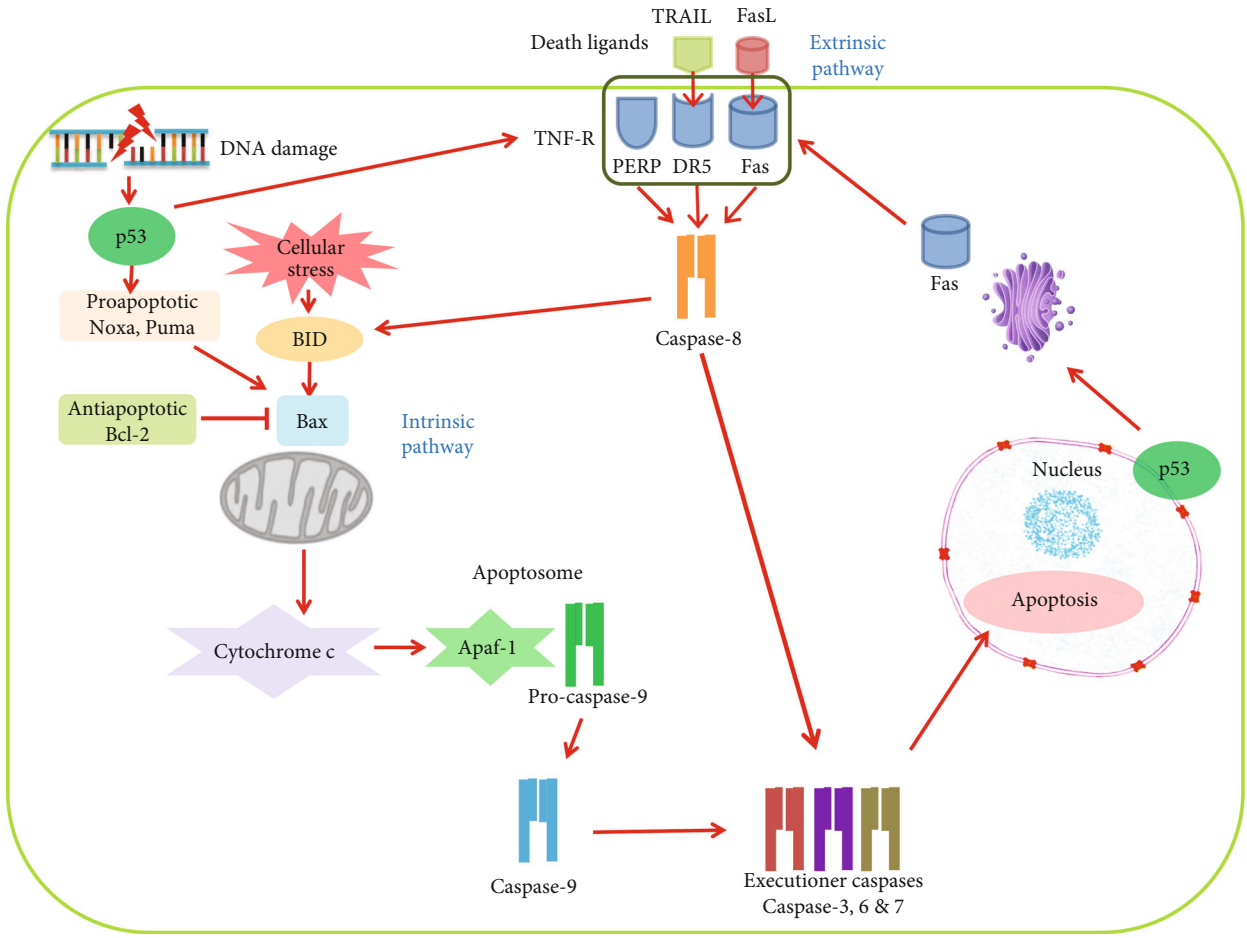

Figure 1: A schematic of the p53-mediated apoptotic pathways in a mammalian cell. The apoptotic pathways involve intrinsic and extrinsic pathways. p53 activates the extrinsic pathway through the induction of the tumor necrosis factor receptor (TNF-R) superfamily that contains apoptotic death domains such as Fas, DR5, and PERP. Overexpression of p53 enhances levels of Fas at the cell surface by promoting the trafficking of the Fas receptor from the Golgi. Fas is activated by its ligand FasL, and DR5 is initiated by the ligand TRAIL. TNF-R is induced by p53 in response to DNA damage, which also promotes cell death through the caspase-9-mediated intrinsic pathway. The intrinsic pathway is dominated by the Bcl-2 family, including Noxa, Puma, and Bax. Bax is activated by BID to increase the mitochondrial outer membrane permeability, allowing efflux of cytochrome c to cytosol. BID is activated by cellular ROS and also cleaved by caspase-8. BID is a crossregulator between the extrinsic and intrinsic pathways. The released cytochrome c and Apaf-1 form a complex with pro-caspase-9 named apoptosome in which caspase-9 is activated. Caspase-8 is activated by death domains of the extrinsic pathway and cleaves proenzyme to form caspase-3, caspase-6, and caspase-7, which affect cell viability and overall apoptosis induced by p53. *Abbreviations*: Apaf-1: apoptotic protease-activating factor 1; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; BID: BH3 domain-only death agonist protein; DR5: death receptor 5; Fas: Fas cell surface death receptor; FasL: Fas ligand; Noxa: damage protein, a proapoptotic BH3-containing protein; p53: tumor suppressor; PERP: p53 apoptosis effector related to PMP22; Puma: p53 upregulated modulator of apoptosis; TNF-R: tumor necrosis factor receptor; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand.

is to promote nonhomologous end joining (NHEJ) and suppress homologous recombination (HR). The two major pathways involved in DNA repair are NHEJ and HR. Certainly, the mechanism of these two processes of DNA repair may differ. HR is an error-free process, while NHEJ is very fast in DNA repair. p53 phosphorylates a series of effector molecules such as rap1-interacting factor 1 (RIF1) and pax transactivation domain-interacting protein (PTIP), prevents resection of DNA 5′ ends, and promotes NHEJ [47–49]. DNA damage results in DNA repair by growth arrest and the DNA damage-inducible gene (GADD45) family that is composed of GADD45α, GADD45β, and GADD45γ nuclear proteins, G_1 arrest by p21, and also results in apoptosis by apoptotic genes. p53 can also induce GADD45, enhancing nucleotide excision repair (NER) to reduce DNA damage and UV photoproducts [50]. p53 also exerts its regulation of DNA function by base excision repair (BER). BER is used to remove the chemically modified bases on the basic (apurinic or apyrimidinic (AP)) nucleotide. The basic nucleotide site is removed by pyramidic endonuclease, resulting in a breach filled by DNA polymerase and a ligase [51]. p53 also activates more DNA damage repair genes to maintain genome stability and regulates the centrosome via duplication and replication. The lack of p53 can be associated with an increased level of genomic agitation, which may be the reason for causing oncogenic progression [52–54].

4. Regulation of p53

Excessive activity of p53 is highly harmful to cell survival and normal growth conditions; thus, the p53 protein level is regulated by ubiquitination and degradation, which is mediated mainly by the E3 ubiquitin ligase mouse double minute 2 homolog (MDM2) [55]. Cells are exposed to various stress signals, including oxidative stress, oncogene activation, telomere shortening, and hypoxia, which leads to p53 activation by inducing MDM2 [56]. The regulation of MDM2 and its close homolog—double minute X human homolog (MDMX)—is achieved by negative and positive regulators, which creates a feedback mechanism. The primary mechanism for suppressing p53 is its negative regulator, MDM2 [33, 57]. MDM2 is induced by p53, and p53 triggers are self-destruction through a negative feedback loop. It has been found that an increased level of MDM2 inactivates the functions of p53, such as apoptosis and cell cycle arrest. MDM2 and p53 form the autoregulatory feedback loop to control cellular activity. MDM2 acts as an E3 ubiquitin ligase and is responsible for the ubiquitination and degradation of p53 [33, 58]. Several reviews broadly discuss the roles of the p53-MDM2 pathway in the initiation, progression, and metastasis of human cancers [59, 60]. Therefore, p53 and MDM2 are critical for controlling their expression levels in both the normal and cancer cells [61, 62].

4.1. Normal Cells. The p53 protein is very low in normal cells due to continuous ubiquitination by MDM2 and MDMX [63]. p53 is controlled by MDM2 and MDMX in normal cells, and this control is necessary for the cells to turn on p53 promptly and effectively utilize its remarkable antioncogenic power to maintain a cancer-free environment [56, 64]. p53 accumulated in normal cells activates the expression of MDM2 in the downstream signaling pathway. MDM2 binds with the transcriptional activation domain of p53 to form the p53-MDM2 complex, which inhibits the transcriptional activity of p53 and creates negative feedback to regulate the pathway [24, 65]. The DNA damage signaling pathway can activate p53 by phosphorylating MDM2 and MDMX and blocks the feedback regulations on this protein through the stress-induced kinase cascade, such as ATM, ATM-Rad3-related protein kinase (ATR), checkpoint kinase 1 (Chk1), and checkpoint kinase 2 (Chk2) [56, 66–68]. p53 protein is activated primarily through posttranslational modifications, which leads to the increase of p53 protein half-life; therefore, p53 protein is accumulated in cells [69]. There are more than 100 target genes of the p53 protein, which have been identified and involved in the regulation of apoptosis by apoptotic genes, such as Bax, Fax, Noxa, DR5, PERP, and Puma, and in cell cycle arrest by genes including p21, GADD45, Yippee-like 3 (Ypel3), and promyelocytic leukemia protein (Pml) [70]. These genes are associated with the regulation of p53, including cellular signaling, regulation of the extracellular matrix, and cellular structure [71]. The activation of p53 is regulated by several modifications, such as acetylation, methylation, ubiquitination, and phosphorylation [26].

4.2. Cancer Cells. The p53 gene is classified as a wild type and mutant type. It has been suggested that more than 50% of all malignant tumors have mutations in the p53 gene [65]. The mutated p53 gene can induce and promote tumorigenesis than the wild-type p53; tumor suppressor genes can transactivate and perform broad-spectrum tumor suppression [72]. Loss of p53 function is strongly associated with cancer development across a wide range of organs [73, 74]. Animals bearing mutant p53 have severely compromised transcriptional activity, making them prone to tumor development [75]. Broad mutation search suggested that more than half of the human cancers possess mutations of p53 [76]. About 95% of p53 mutations were noticeable within the genomic region, encoding the DNA-binding domain [77]. About 30% of mutations occur in six amino acid residues, which are most frequently mutated in human cancers, including Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282 [78]. These mutations are initiated within the DNA-binding domain of p53 and subsequently disrupt the native conformation of the protein. Therefore, the mutant p53 is defective in the sequence-specific transcriptional activation dependent on the wild-type p53-binding consensus element. Moreover, the mutant p53 shows a dominant-negative behavior towards the wild-type p53 through the formation of heterotetramer with wild-type p53 and has the oncogenic potential [79, 80].

Mutant p53 protein interacts with several transcription factors, such as nuclear factor Y (NF-Y), E2F transcription factors (E2Fs), nuclear factor NF-kappa B p65 (NF-κBp65), nuclear factor NF-kappa B p50 (NF-κBp50), sterol regulatory element-binding protein (SREBP), Yes-associated protein (YAP), vitamin D receptor (VDR), and nuclear factor erythroid 2-related factor 2 (Nrf2), which modulates the expression of diverse sets of genes. Mutant p53 proteins lose their ability to activate p53 target genes, and some mutants exert transdominant repression over the wild type [81–84]. The gathering of mutant p53 protein plays an important role in oncogenic activity. The mutant p53 protein is often stabilized in cancer cells, whereas wild-type p53 has a short half-life in both normal tissues and cancer cells [85]. Similar to wild-type p53, mutant p53 degradation is also proteasome-mediated and promoted by the E3 ubiquitin ligases MDM2 and chromatin immunoprecipitation (ChIP) [86]. Still, the mutant p53 becomes stabilized and activated in response to tumor-related stress conditions, similar to wild-type p53 [87]. The accumulating evidence revealed that certain cancer-derived mutant forms of p53 transactivate various target genes, including multiple drug resistance gene 1 (MDR1), c-myc, proliferating cell nuclear antigen (PCNA), interleukin-6 (IL-6), insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), epidermal growth factor receptor (EGFR), asparagine synthetase (ASNS), and telomerase reverse transcriptase (TERT) [76, 88, 89]. Therefore, cancer-derived mutant p53 promotes adaptive responses to growth-promoting and oncogenic genes, thereby leading to the progression of aggressive cancers [90].

5. Regulatory Mechanism of the p53 Signaling Pathway

In response to a broad range of stresses, p53 upregulates and enhances its expression to perform a suitable cellular

response. Oncogenic signaling produces the alternate reading frame (ARF), tumor suppressor, to inhibit the expression of MDM2 and MDMX [91, 92]. Recent research has also been associated with the p53 via the MDM2-ARF pathway leading to cell cycle arrest and apoptosis [19, 93]. DNA damage activates p53 through ATM and ATR protein kinase-dependent pathways [94]. Once ATM and ATR are activated in response to DNA damage, p53 is phosphorylated, resulting in p53 uncoupling from the E3 ubiquitin ligase MDM2 proto-oncogene (MDM2) and negative regulator MDMX, leading to p53 stability and activation. [95]. ATM and ATR are large kinases with sequence similarity to lipid kinases of the phosphatidylinositol 3-kinase (PI3K) family that phosphorylate protein substrates [96]. Chk1 and Chk2 kinases are selectively phosphorylated and activated by ATR and ATM regulators, respectively [97]. The p53 pathways respond to different aberrant DNA structures. ATM is activated primarily at DNA double-strand breaks (DSBs) with the MRE11-RAD50-NBS1 (MRN) complex [98], and ATR is activated at single-strand DNA (ssDNA) with ATR-interacting protein (ATRIP) [99] (Figure 2).

ATM monomers are then recruited to DSBs with the MRN sensor complex stimulating the activation on multiple substrates at the site of damage [100]. Substrates include the variant histone H2AX, forming the DNA damage-associated γH2AX histone mark in the MRN complex itself, and the downstream effector kinase Chk2 [101]. ATM phosphorylates Chk2, leading to transient homodimerization, intermolecular activation loop autophosphorylation, and full activation [102, 103]. Chk2 dissociates from the site of damage and disperses a monomer to act on multiple substrates involved in cell cycle progression, apoptosis, and gene transcription [104]. Chk2 substrates include p53 [105] and its regulator MDMX [106]. Other substrates targeted by ATM at sites of damage include NBS1, BRCA1, MDC1, and p53BP1 [107]. ATM plays an essential role in the activation of the p53 response to DNA damage by phosphorylating p53 itself and the stability regulators such as MDM2 and MDMX [108].

ATR-Chk1 signaling is activated strongly when DNA replication is impeded. DNA polymerases become uncoupled from the replicative helicase, generating tracts of ssDNA that rapidly become coated with the trimeric ssDNA-binding protein complex replication protein A (RPA) [109]. ATR is activated in association with its partner protein ATRIP which interacts with ssDNA complexed with RPA via the 70 kDa RPA1 subunit [110]. ATR activation and Chk1 phosphorylation depend on the action of two mediator proteins, TopBP1 and Claspin. TopBP1, which is recruited to ssDNA-RPA via the PCNA-like RAD9:RAD1:HUS1 checkpoint clamp, contains a domain that stimulates ATR activity [111, 112]. Claspin likely relates to active replication forks when normal replication is subjected to ATR-dependent phosphorylation, which modified and bound Chk1 to serve as a platform for ATR-mediated phosphorylation and activation [113].

ATR-Chk1 and ATM-Chk2 signaling are known for the stabilization and phosphorylation of p53. Chk1 and Chk2 activities are required for p53 phosphorylation [114]. p53 exerts antioxidant activity depending on the extent of stress signals. p53 induces a group of antioxidant genes, including sestrin 1/2, TP53-inducible glycolysis and apoptosis regulator (TIGAR), glutathione peroxidase-1 (GPx1), aldehyde dehydrogenases (ALDH4), glutaminase 2 (GLS2), and Parkin, especially under nonstress or low-stress conditions, to lower ROS levels and to repair the DNA successfully. This antioxidant activity protects cells from oxidative stress-induced DNA damage and mutations and promotes cell survival [115]. Growth arrest and DNA damage-inducible proteins such as GADD45, p21, Ypel3, and Pml are induced by p53 and lead to cell cycle arrest. p53-mediated apoptosis induced by the apoptotic genes, including DR5, Fas, PERP, Bcl-2, Bax, Noxa, and Puma, which takes place in intrinsic and extrinsic pathways, leads to apoptosis in case of failure to repair DNA [30, 116]. If the p53 gene is damaged, tumor suppression is severely reduced. Mutant p53 transactivates several genes, including MDR1, c-myc, PCNA, IL-6, IGF-1, FGF, EGFR, ASNS, and TERT. Mutant p53 promotes oncogenic genes, which lead to the progression of aggressive cancers [90].

5.1. Regulation of p53 by Posttranslational Modifications. p53 is modulated by a few fundamental processes: acetylation, phosphorylation, ubiquitination, sumoylation, neddylation, and methylation [116]. p53 undergoes a sequence of post-translational modifications, mainly due to DNA damage, oncogene activation, and hypoxia. The posttranslational modification in p53 occurs by acetylation and phosphorylation, which leads to tetramerization to reach functional stability and reverse the effects of cellular and genotoxic insults [26, 116].

5.1.1. Phosphorylation. Phosphorylation responses to DNA damage and other stresses are controlled by regulators, subcellular localization, stability, and transcriptional activation of target genes, which prompts the biological effects [116, 117]. Several kinases target specific serine and threonine residues within p53 for phosphorylation [118]. ATM, ATR, and DNA-dependent protein kinase (DNA-PK) are activated by DNA damage and directly phosphorylate Ser15, Ser20, and Ser46 of p53 [76]. Ser15 is targeted by ATR, DNA-PK, and Chk1, whereas Ser20 of p53 is a target in Chk2 kinase [119–121]. Chk1 and Chk2 can also phosphorylate more p53 residues, such as Thr387 by Chk1, Thr18, and Ser366 by Chk2, and also 4 residues, namely, Ser313, Ser314, Thr377, and Ser378, in the C-terminus by both kinases [122]. Ser46 is the major phosphorylation of p53 by several kinases required for apoptosis in response to DNA damage, and increased Ser46 phosphorylation of p53 induces apoptotic target gene transcription [123]. Changes in phosphorylation of p53 either facilitate or inhibit the binding of specific interactors to p53 that can block or promote acetylation [116].

5.1.2. Acetylation. Acetylation of p53 is another mechanism to stabilize the p53 protein that reduces ubiquitination and degradation. SIRT1 plays an important role by deacetylating p53 protein [124]. Acetylation of p53 blocks the ubiquitin sites and prevents ubiquitination by MDM2 and subsequent degradation [125]. Acetylation also recruits p300/CBP/PCAF

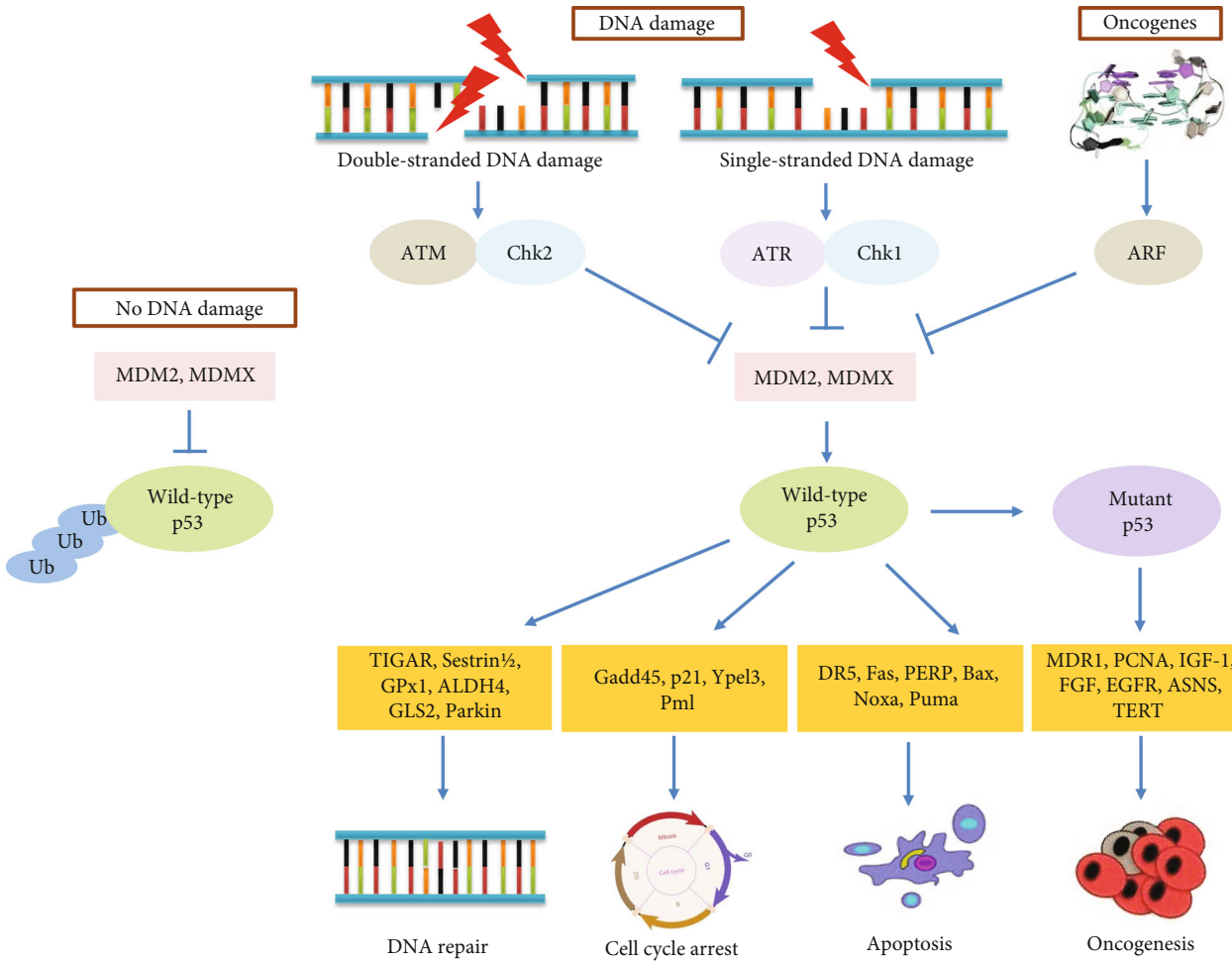

Figure 2: DNA damage and oncogenic signaling active the p53 tumor suppressor to maintain genomic stability and prevent transformation. Both the double-stranded DNA break and the single-stranded DNA break trigger the activation of p53 through ATM and ATR, respectively. Chk1 and Chk2 are selectively phosphorylated and activated by ATR and ATM, respectively. Oncogenic signaling produces the ARF tumor suppressor to inhibit the expression of MDM2 and MDMX. MDM2 and MDMX are the two negative regulators that function as an E3 ubiquitin ligase, which recognizes the N-terminal transcriptional activation domain (TAD) of p53, and as an inhibitor of p53 transcriptional activation. Therefore, under no stress or no DNA damage, p53 is kept inactive by the negative regulation by MDM2 and MDMX. Once activated, p53 induces antioxidant genes such as TIGAR, sestrin 1/2, GPx1, ALDH4, GLS2, and Parkin to repair the DNA. Growth arrest and DNA damage-inducible proteins such as GADD45, p21, Ypel3, and Pml are induced by p53 to cell cycle arrest and apoptotic genes, including DR5, Fas, PERP, Bcl-2, Bax, Noxa, and Puma for p53-mediated apoptosis. If the p53 gene is damaged, tumor suppression is severely reduced. Mutant p53 transactivates several genes, including MDR1, c-myc, PCNA, IL-6, IGF-1, FGF, EGFR, ASNS, and TERT. Mutant p53 promotes oncogenic genes, which lead to the progression of aggressive cancers. *Abbreviations*: ALDH4: aldehyde dehydrogenases; ARF: alternate reading frame; ASNS: asparagine synthetase; ATM: ataxia telangiectasia-mutated protein kinase; ATR: ATM-Rad3-related protein kinase; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Chk1: checkpoint kinase 1; Chk2: checkpoint kinase 2; DR5: death receptor 5; EGFR: epidermal growth factor receptor; Fas: Fas cell surface death receptor; FGF: fibroblast growth factor; GADD45: growth arrest and DNA damage-inducible protein; GLS2: glutaminase 2; GPx1: glutathione peroxidase-1; IGF-1: insulin-like growth factor 1; MDM2: mouse double minute 2 homolog; MDMX: double minute X human homolog; MDR1: multiple drug resistance gene 1; Noxa: damage protein, a proapoptotic BH3-containing protein; p21: cyclin-dependent kinase inhibitor; p53: tumor suppressor; PCNA: proliferating cell nuclear antigen; PERP: p53 apoptosis effector related to PMP22; Pml: promyelocytic leukemia protein; Puma: p53 upregulated modulator of apoptosis; TERT: telomerase reverse transcriptase; TIGAR: TP53-inducible glycolysis and apoptosis regulator; Ypel3: Yippee-like 3.

to the promoter regions for activation of p53-targeted genes, such as p21, MDM2, and Puma [126]. Conversely, loss of eight major acetylation sites in human p53, namely, Lys164, Lys305, Lys320, Lys372, Lys373, Lys381, Lys382, and Lys386, renders p53 transcriptionally and prevents its induction of cell cycle arrest and apoptosis [127]. Six C-terminal residues such as Lys305, Lys372, Lys373, Lys381, Lys382, and Lys386 and one DBD residue Lys164 are acetylated by CBP/p300; however, Lys320 is acetylated by PCAF [126–128]. Histone deacetylase (HDAC) inhibitors are also reported, which induce acetylation in nonhistone proteins [129]. Inhibition of HDAC eliminates HDAC1 and SIRT1

from p53, which increases p53 acetylation and p53-dependent activation of apoptosis and senescence [71].

5.2. Regulation of Autophagy by p53. Autophagy is the mechanism of cells for eliminating unnecessary or dysfunctional cellular components. p53 signaling regulates autophagy, and p53 is an autophagy target protein. Under nutrient starvation and various stressful conditions, such as hypoxia, both the wild-type and mutant p53 can be degraded through autophagy [130–132]. As a result, autophagy is often found in tumor cells that have been subjected to chemotherapy or radiation [133]. In tumor cell proliferation, cell cycle control, apoptosis, senescence, and autophagy, p53 plays a regulatory role. The subcellular localization of p53 is linked to its regulatory function. When p53 is localized in the nucleus, it encourages autophagy in stressed cells, while cytosolic p53 prevents autophagy in nonstressed cells [134]. p53 induces autophagy in the nucleus by transcriptionally controlling the mammalian target of rapamycin (mTOR) pathway as well as transcriptional regulation of key autophagy-related genes (ATGs) [135]. The phosphatase and tensin homolog (PTEN), tuberous sclerosis-2 (TSC2), and AMP-activated protein kinase (AMPK) are some of the p53-targeted genes that have been shown to inhibit mTOR, facilitating autophagy initiation. Furthermore, the tumor suppressor protein p14 ADP-ribosylation factor (ARF) signaling pathway has been linked to p53 and autophagy [136]. Activation of oncogenes during tumorigenesis increases the transcription of ARF, which binds to and inhibits the expression of MDM2, stabilizing p53 [137]. By disrupting the Bcl-xl/Beclin 1 complex and releasing Beclin 1 to induce autophagy, ARF promotes autophagy [138]. Autophagy inhibition with chemical inhibitors or downregulation of the basic autophagy genes ATG1/ULK1, Beclin 1, or ATG5 results in the stabilization of mutant p53 under basal growth conditions [139, 140].

p53 can also mediate functional responses between apoptosis and autophagy when it is subcellularly localized. p53 can localize to mitochondria, where it interacts with antiapoptotic B-cell lymphoma (Bcl) family proteins, allowing proapoptotic factors like Bcl-2-associated X protein (Bax) and Bcl-2-antagonist/killer (BAK) to oligomerize, facilitating mitochondrial outer membrane permeabilization and driving activation of the intrinsic apoptotic cell death pathway [141, 142]. Beclin 1 and ATG7, autophagy-related proteins, avoid mutant p53 degradation caused by turmeric or curcumin [143, 144]. In another study, MDA-MB-231, MDA-MB-468, T47D, and SKBr3 breast cancer cell lines expressing different mutant p53 failed to form complexes with Bcl-2 when compared to human acute myeloblastic leukemia ML-1 cells [145]. On the other hand, overexpression of Beclin 1 or ATG1/ULK1 causes mutant p53 depletion. In MDA-MB-231 and DLD1 cancer cell lines, gambogic acid, a molecule that stimulates the degradation of mutant p53, can induce the degradation of mutant p53 proteins through autophagy [146]. Autophagy inhibition with bafilomycin A1 or chloroquine prevents gambogic acid from degrading mutant p53. Glucose restriction causes mutant p53 deacetylation, which directs it to autophagy for degradation [147]. Depletion of p53 causes autophagy activation and cell death, while the expression of a degradation-defective mutant p53 prevents autophagy and allows survival in the face of glucose restriction. A novel curcumin-based zinc compound has been found to cause mutant p53 protein degradation via autophagy [148].

6. p53 Signaling Pathway Modulated by Dietary Antioxidants

Numerous *in vitro* and *in vivo* studies have demonstrated that dietary biologically active (bioactive) compounds can modulate immunity to delay cancer development and progression through decreased cell proliferation, inactivation of carcinogens, inhibition of angiogenesis, induction of cell cycle arrest, apoptosis, and regulation of various signaling pathways [19, 149–152]. Chemopreventive and anticancer effects of dietary antioxidants have been reported from several normal cell lines, cancer cell lines, and animal studies by the p53 signaling pathway (Table 1).

6.1. Vitamins. In vitro experiments have shown that vitamin C (ascorbic acid) can reduce cell proliferation and induce apoptosis through upregulation of p53, p21, and Bax and downregulation of Bcl-2 in T-cell colonies [153]. Furthermore, Harakeh and colleagues demonstrated that the administration of nontoxic doses of ascorbic acid increased the expression of p53 [153]. Vitamin C increases the ability of the anticancer drug bleomycin to produce DSBs, which makes cancer cells more dependent on functional DNA repair for survival [99]. Vitamin B_6 activates the p53 pathway, which is responsible for controlling p21 mRNA transcription in HT29, Caco2, LoVo, HEK293T, and HepG2 cancer cells. p21 mRNA levels were higher in the colon of mice fed a diet with adequate vitamin B_6 than those fed a vitamin B_6-deficient diet, and this may help to understand the antitumor effect of vitamin B_6 via the activation of p53 and elevation of p21 mRNA [154]. A previous study suggested that 1,25-dihydroxyvitamin D increased oxidative stress through inhibiting transcription of Nrf2, enhancing DNA damage and activation of p16/Rb and p53/p21 signaling in a 1α(OH)ase−/− mouse model [155]. Folic acid (vitamin B_9) might play an important role in the chemoprevention of gastric carcinogenesis. In humans, the tumor suppressor p53 expression in the gastric mucosa was significantly increased, while the expression of Bcl-2 oncogene protein decreased after folic acid supplementation [156]. Furthermore, N-acetylcysteine (NAC) inhibits PDK1 expression through PPARα-mediated induction of p53 and reduction of p65 protein expression and unveils a novel mechanism by which NAC in combination with the PPARα ligand inhibits the growth of non-small-cell lung carcinoma (NSCLC) cells [157]. Water-soluble vitamin E (Trolox) supplementation promoted breast cancer growth by reducing ROS and p53 expression in mice [158]. β-Carotene, ascorbic acid, and vitamin E (α-tocopherol) protect against oxidative stress but reveal no direct influence on p53 expression in rats subjected to stress [159]. In contrast, β-carotene exacerbates DNA oxidative damage and modifies p53-related pathways

Table 1: Dietary antioxidants show the expression of p53 in experimental models.

Antioxidants	Experimental model	Mechanism	Expression of p53	References
(i) Cancer cells				
Vitamin C	T-lymphocyte cell lines (HuT-102, C91-PL, CEM, and Jurkat)	p53 ↑, p21 ↑, Bax ↑, Bcl-2 ↓	Protein	[153]
	Human lung carcinoma cell lines (H460 and A549)	p53 ↑, caspase-3 ↑, caspase-7 ↑	Protein, mRNA	[99]
Vitamin B_6	HT29, Caco2, LoVo, HEK293T, and HepG2 cell lines and ICR mice	p53 ↑, p21 ↑	Protein, mRNA	[154]
Vitamin D	Mice (1α(OH)ase−/−)	p53 ↑, p21 ↓, p16 ↑	Protein, mRNA	[155]
Folic acid	Human	p53 ↑, Bcl-2 ↓	Protein	[156]
N-Acetylcysteine	Non-small-cell lung carcinoma (NSCLC) cell line	p53 ↑, p65 ↓	Protein, mRNA	[157]
Vitamin E	Human breast cancer cell line (MCF-7)	p53 ↓	Protein, mRNA	[158]
β-Carotene	RAT-1 murine fibroblast cell line	p53 ↑, p21 ↑, Bax ↑, Bcl-2 ↓	Protein	[160]
Quercetin	Human cervical cancer cell line (HeLa)	p53 ↑, caspase-3 ↑, caspase-9 ↑, Bax ↑, Bcl-2 ↓	Protein, mRNA	[164]
Apigenin	Human colon cancer cell lines (HCT116 and HT29)	p53 ↑, p21 ↑, NAG-1 ↑	Protein, mRNA	[165]
Acacetin	Human liver cancer cell line (HepG2)	p53 ↑, Bax ↑	Protein, mRNA	[167]
Fisetin	Human colon cancer cell line (HCT116)	p53 ↑, caspase-3 ↑	Protein, mRNA	[168]
Luteolin	Human lung cancer cell lines (A549 and H460)	p53 ↑, p21 ↑, MDM4 ↓	Protein, mRNA	[170]
Theaflavin	Human prostate cancer cell line (LNCaP)	p53 ↑, Bcl-2 ↓, Bax ↑	Protein	[171]
Epigallocatechin-3-gallate (EGCG)	Human prostate cancer cell line (LNCaP)	p53 ↑, p21 ↑, Bcl-2 ↓, Bax ↑, caspase-3 ↑, caspase-8 ↑, caspase-9 ↑	Protein	[93]
Caffeic acid	Human cervical cancer cell line (HeLa)	p53 ↑, Bcl-2 ↓	Protein	[174]
Caffeic acid phenethyl ester	C6 glioma cell line	p53 ↑, Bcl-2 ↓, Bax ↓	Protein	[175]
Ellagic acid	Human cervical cancer cell line (CaSki)	p53 ↑, p21 ↑, GAPDH ↑	Protein, mRNA	[176]
Curcumin	Human cervical cancer cell lines (HeLa, SiHa, and CaSki)	p53 ↑	Protein	[177]
	Multiple myeloma cell line (U266B1)	p53 ↑, p21 ↑	Protein	[178]
Resveratrol	Human breast cancer cell line (MCF-7)	p53 ↑, p21 ↑, p16 ↑	Protein	[180]
	Human colon cancer cell lines (HCT116, CO-115, and SW48)	p53 ↑, PARP ↓, caspase-3 ↓	Protein	[181]
Red wine	Human lung cancer cell line (A549)	p53 ↑, p53 (Ser15) ↑	Protein	[182]
Caffeine	Human T-lymphocyte leukemia MOLT-4 cell line	p53 ↑, p21 ↑, Mcl-1 ↓	Protein	[183]
Harmine	Human breast cancer cell line (MCF-7)	p53 ↑	mRNA	[185]
Colchicine	Human cervical cancer cell lines (CaSki and HeLa)	p53 ↑, Bcl-2 ↓, Bax ↑	Protein, mRNA	[186]

Table 1: Continued.

Antioxidants	Experimental model	Mechanism	Expression of p53	References
(ii) Normal cells				
α-Tocopherol, ascorbic acid, & β-carotene	Rat	p53 ↓, MDM2 ↓	Protein	[159]
Kaempferol	Human umbilical vein endothelial cell line (HUVEC)	p53 ↑, ATM ↑, Fas ↑, DR5 ↑, caspase-3 ↑, caspase-8 ↑, caspase-9 ↑	Protein	[166]
Fisetin	Wistar rat	p53 ↓, p21 ↓, Bax ↓, Bcl-2 ↑	Protein	[169]
Apple flavonoids	Normal human bronchial epithelial cell line (BEAS-2B)	p53 ↓, Chk1 ↓, ATR ↓	Protein	[172]
Cyanidin-3-*O*-glucoside (C3G)	Kunming mice (KM)	p53 ↓, Bax ↓, caspase-3 ↓, caspase-9 ↓, Bcl-2 ↑	Protein	[173]
Curcumin	Wistar rat	p53 ↓, p21 ↓	Protein	[179]
Caffeine	Normal human fibroblast cell line (NHF1)	p53 ↓, p21 ↓	Protein	[184]

of cell proliferation and apoptosis in cultured RAT-1 fibroblasts exposed to tobacco smoke condensate (tar) [160].

6.2. Flavonoids. Flavonoids are suggested to reduce p53-mediated chronic inflammation leading to cancer progression [161]. Quercetin can induce phosphorylation of ATM and phosphorylation of histone H2AX; therefore, p53 accumulation and phosphorylation occurred in ATM-deficient cells, indicating that ATM is not required for quercetin-induced p53 phosphorylation [162]. DNA lesions are produced by quercetin, and the mechanisms to detect this damage must be subtly different from the ability of quercetin to induce ATM-independent p53 phosphorylation [162]. Quercetin increased the phosphorylation of p53 protein and induced apoptosis of the human leukemia cell line in a dose-dependent manner [163]. A recent study revealed that quercetin inhibits HeLa cell proliferation through cell cycle arrest at the G_2/M phase and apoptosis induction through the disruption of mitochondrial membrane potential and activation of the intrinsic apoptotic pathway through p53 induction [164]. Further, apigenin can induce p21, p53, and nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) proteins in kinase pathways, including protein kinase C delta (PKCd) and ATM, which plays an important role in activating these proteins in colorectal cancer cell growth arrest [165]. Further, kaempferol warrants as an anti-angiogenetic agent, which reduced human umbilical vein endothelial cell viability-induced DNA damage and DNA fragmentation through activating the levels of caspase-3, caspase-8, and caspase-9 signaling, which were upregulated by ROS-mediated p53/ATM molecules following stimulations of p53 downstream protein levels of Fas/CD95, death receptor 4 (DR4), and DR5 [166]. Another study revealed acacetin, an O-methylated flavone, which can strongly inhibit tumor growth and induce tumor shrinkage in mice, which is closely correlated with its increasing p53 expression accompanied by decreased retinoic acid receptor gamma (RARγ) and reduced AKT activity in liver cancer cell lines [167]. It was further reported that securin and p53 play an important role in determining the sensitivity of human colon cancer cells to fisetin. Depletion of securin enhances fisetin-induced apoptosis and decreases the resistance of p53-deficient cells to fisetin and might be an attractive strategy for the treatment of human colon cancers [168]. The inhibitory effect of fisetin against bladder cancer by activation of p53 and downregulation of the nuclear factor-kappa B (NF-κB) pathway in a rat bladder carcinogenesis model has been documented, which is a safe and efficacious agent and promising therapeutic approach for bladder cancer [169]. Furthermore, luteolin treatment increases the expression of p53 and p21 proteins and decreases the expression of MDM4 protein in both NSCLC cells and tumor tissues [170]. Theaflavins induced G_2/M arrest by modulating the expression of various proteins, which are involved in signaling. Moreover, theaflavins via p53 signaling inhibited Bcl-2 and interfered phagocytes via modulation of I-κB/NF-κB, as well as the expression of VEGF, and the phosphorylation of VEGFR was reduced in LNCaP cells [171]. Furthermore, epigallocatechin-3-gallate activates p53-dependent downstream targets p21/WAF1 and Bax and downregulates NF-κB-dependent Bcl-2 that results in growth arrest and apoptosis in LNCaP cells [93]. Our previous study revealed that effector proteins like Chk1, Chk2, and p53 were found to be phosphorylated in NNK acetate-treated BEAS-2B cells, and pretreatment with apple flavonoids showed a significant reduction in the levels of phosphorylation of ATR, Chk1, and p53 in NNK acetate-treated cells [172]. Apple flavonoids protect BEAS-2B cells challenged against various carcinogens by assisting DNA repair mechanisms [172]. Further, targeted drug delivery of encapsulated C3G chitosan nanoparticles can downregulate p53-mediated apoptosis in Kunming mice, which provides the powerful photochemopreventive effect [173].

6.3. Phenolic Acids. Caffeic acid increases p53 protein expression in a concentration-dependent manner and induces apoptosis by inhibiting Bcl-2 activity, leading to the release of Cyt c and subsequent activation of caspase-3 in HeLa cells [174]. Pretreatment of caffeic acid with a p38 MAPK specific inhibitor significantly inhibited p53 phosphorylation at serine 15. However, other MAPK-mediated p53 phosphorylation

sites might be activated by CAPE, and the effect of CAPE activated ERKs in C6 glioma cells [175]. A study provided evidence that ellagic acid inhibited cell proliferation via p21-mediated G_1 arrest and cell death, leading to inhibition of tumor cell proliferation and induction of cell death by ellagic acid, which supports as a chemopreventive agent [176].

6.4. Other Polyphenols. Curcumin increases levels of p53, which provides an appropriate cellular environment for p53 and NAD(P)H quinone oxidoreductase (NQO1) to interact in HeLa, SiHa, and CaSki tumor-derived cell lines. At the same time, this interaction promotes the loss of interaction of p53 with its negative regulator E6AP ubiquitin-protein ligase (E6AP) when human papillomavirus E6 (HPV E6) oncoproteins are present [177]. Another study suggested that upregulation of both p53 and p21 in U266 cells exposed to curcumin is involved in cell cycle arrest [178]. Furthermore, curcumin decreased the expression of p53 and p21 in Wistar rats [179]. Resveratrol metabolites can promote a moderate cellular senescence induction in breast cancer cells, which involves inhibiting cell growth by G_2/M phase arrest through the p53/p21Cip1/WAF1 pathway [180]. A previous study has revealed that SET7/9 functions as a mediator of resveratrol-dependent p53 activation, and the results confirm that resveratrol modulates p53 through its monomethylation at K372 [181]. Another study investigated the effect of red wine on anticancer and chemopreventive properties. Polyphenol-rich red wine extracted from grapes could increase the active phosphorylated p53 in human lung cancer cells [182].

6.5. Alkaloids. Caffeine suppresses the ATM/p53 signaling pathway in human T-lymphocyte leukemia MOLT-4 cells. Caffeine suppressed p21 upregulation and inhibited p53 phosphorylation on Ser15 and Ser392, which is ATM-independent, suggesting that caffeine might have another cellular target [183]. Caffeine inhibits γ and UV radiation-induced phosphorylation of p53 and Ser15, which may be modified directly mediated by ATM [183]. Furthermore, Kaufmann et al. [184], in their work on human fibroblasts, described the ATM-dependent phosphorylation of p53 after γ irradiation as resistant to caffeine. Evidence indicated that harmine treatment induced apoptosis in MCF-7 cells through the upregulation of p53 expression and reported that harmine is able to upregulate p53 gene expression in breast cancer cell lines. Furthermore, harmine induced apoptosis in breast cancer cells in a time-dependent manner and real-time PCR outcomes on breast cancer cells showed that apoptosis induction in MCF-7 cells by harmine requires the activation of p53 gene expression [185]. Colchicine exhibits *in vitro* antiproliferative activity against HPV 16/18-positive human cervical cancer cell lines, which was achieved by HPV E6/E7 inhibition and subsequently p53-dependent intrinsic apoptosis [186].

7. Dietary Antioxidants as Anticancer Agents

Dietary antioxidants, including polyphenols and vitamins, have been investigated for their prospective benefits such as antioxidant, antiproliferative, antiangiogenic, anticancer, immunomodulatory, and hypoglycemic properties, which increases their applications in the pharmaceutical, functional food, and cosmetic industries [10, 187, 188]. The anticancer potential of dietary antioxidants was well addressed due to their wide distribution in nature, which includes a large number of dietary sources extracted from plants as well as isolated as pure compounds [189–192]. Moreover, several therapeutic modifications have been employed to get better outputs, such as the application of nanotechnologies to increase the solubility and bioavailability of polyphenols [193, 194]. However, there are several pieces of evidence suggesting that p53 mediated apoptosis by dietary antioxidants, which leads to cancer growth inhibition and prevention. When p53 is mutated or absent, the dietary antioxidants exert an antitumor property, and the cells are resistant to current therapies [19]. Numerous researchers have demonstrated that anticarcinogenic effects of polyphenols are assigned to several mechanisms, including the induction of apoptosis, regulation of various cell signaling pathways, regulation of the cell cycle, DNA repair, and activation of the receptors [195, 196]. Flavonoids are known to activate the Keap1/Nrf2/ARE pathway and contribute to cytoprotection in normal cells [197]; however, some flavonoids such as flavones, luteolin, apigenin, and chrysin are shown to inhibit Keap1/Nrf2/ARE and are proapoptotic in cancer cells [198].

8. Clinical Studies

Anticancer effects of dietary antioxidants targeting the p53 signaling pathway have been identified using several cell-based and experimental animal models [19]. In humans, folic acid supplementation significantly increased p53 expression in the gastric mucosa [156]. Epigallocatechin-3-gallate (EGCG) consumption causes apoptosis in human prostate carcinoma cells by phosphorylating essential serine residues on p53, resulting in upregulation of its transcriptional function [93]. Naringenin has been shown to inhibit cell proliferation and cause apoptotic cell death in human hepatocellular carcinoma, making it a potential candidate for liver cancer therapy [199]. In human hepatocellular carcinoma cells, naringenin treatment causes G_0/G_1 and G_2/M phase arrests, which may be due to rapid p53 accumulation [200]. Quercetin has also been shown to prevent human breast cancer cells from entering the S phase by increasing the levels of the proteins of p53 [201]. A preclinical animal model of skin cancer has demonstrated the synergistic effects of resveratrol and black tea polyphenols in suppressing the development of skin tumors. Treatment with this combination of dietary antioxidants causes a decrease in the expression of phosphorylated mitogen-activated protein kinase (MAPK) family proteins, extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38, as well as an increase in total p53 and p-p53, which shows promise in suppressing the growth of mouse skin tumors [202]. Using *in vitro* and *in vivo* models of research, the synergistic effects of the green tea extract EGCG and tisane herbal infusion in the eradication of malignant human prostate tumors were investigated. Apoptotic genes such as p53, p73, p21, and caspase-3 were all upregulated as a result of the antioxidant combination [203]. Intravesical fisetin has been shown to inhibit bladder cancer in a

rat bladder carcinogenesis model by activating p53 and downregulating the NF-κB pathway. It has been proposed that intravesical fisetin is a novel and promising therapeutic solution for bladder cancer because it is a safe and effective therapeutic agent [169].

9. Conclusion and Future Direction

Cancer is one of the most common causes of disease worldwide. Currently, chemotherapy and radiotherapy are two major cancer treatments, although both are associated with prominent side effects. It is necessary to identify efficacious alternative pharmaceuticals with selective cytotoxicity to cancer cells with the aim of reducing the side effects. Also, novel approaches need to be investigated to reduce the risk of various cancers. Diets rich in polyphenols are implicated in protecting against oxidative stress and inflammation-mediated chronic diseases such as cardiovascular diseases, neurodegenerative disorders, and certain cancers. The cancer chemopreventive effects of dietary polyphenols and other antioxidants are well documented. Dietary polyphenols are able to modulate multiple cellular pathways, which are involved in the initiation and progression of cancer. Polyphenols found in fruits, vegetables, herbs, wine, and teas are safe for consumption. Thus, dietary polyphenols offer multitargeted therapeutic effects than a single drug. The chemopreventive effects of polyphenols have been shown through the modulation of various signaling pathways, including the p53 pathway. p53 signaling activates transcription factors in response to oxidative stresses. In this review, we have shown that certain polyphenols can stabilize p53 protein via phosphorylation and acetylation, contributing to oxidative homeostasis. p53 binds within the DNA and regulates transcriptional genes involved in DNA repair, metabolism, senescence, apoptosis, autophagy, and angiogenesis. Future studies need to be aimed at investigating the efficacy of specific dietary polyphenols and other natural antioxidants and their mixtures against various carcinogen-induced DNA damage through the activation of p53 in healthy cells using *in vitro* models, preclinical experimental animal models, and human studies.

Abbreviations

ATM: Ataxia telangiectasia-mutated protein kinase
ATR: ATM-Rad3-related protein kinase
Bax: Bcl-2-associated X protein
BID: BH3 domain-only death agonist protein
BRCA1: Breast cancer gene 1
Chk1: Checkpoint kinase 1
Chk2: Checkpoint kinase 2
Cyt c: Cytochrome c
DDR: DNA damage response
DNA: Deoxyribonucleic acid
DR5: Death receptor 5
DSBs: Double-strand breaks
Fas: Fas cell surface death receptor
MDM2: Mouse double minute 2 homolog
MDMX: Double minute X human homolog
MRN: MRE11-RAD50-NBS1
p21: Cyclin-dependent kinase inhibitor
p53: Tumor suppressor
PERP: p53 apoptosis effector related to PMP22
ROS: Reactive oxygen species
RPA: Replication protein A
ssDNA: Single-strand DNA.

Conflicts of Interest

GD is a senior scientist, and HPVR is an associate member of the Beatrice Hunter Cancer Research Institute (BHCRI). There is no conflict of interest to declare in this review article.

Authors' Contributions

JPJM wrote the initial draft of the manuscript. HPVR, GD, and KM have reviewed and edited the manuscript.

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Research Article

Bioactive Compounds of Raspberry Oil Emulsions Induced Oxidative Stress via Stimulating the Accumulation of Reactive Oxygen Species and NO in Cancer Cells

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There are growing interests in the complex combinations of natural compounds that may advance the therapy of cancer. Such combinations already exist in foods, and a good representative is seed oils. Two raspberry oils: cold pressed (ROCOP) and one extracted by supercritical CO_2 (ROSCO2) were evaluated for their chemical characteristics and oil emulsions for cell suppression potential against colon adenocarcinoma (LoVo), doxorubicin-resistant colon adenocarcinoma (LoVo/DX), breast cancer (MCF7), doxorubicin-resistant breast cancer (MCF7/DX), and lung cancer (A549) cell lines. The cytotoxicity was also assessed on normal human dermal fibroblasts (NHDFs). With increasing concentration of raspberry oil emulsions (0.5–10%), increasing inhibition of cancer cell viability and proliferation in all of the lines was observed, with different degrees of potency between cancer types and oil tested. ROSCO2 strongly induced free radical production and DNA strand damage in LoVo and MCF7 cells especially doxorubicin-resistant lines. This suggests that ROSCO2 engages and effectively targets the vulnerabilities of the cancer cell. Generally, both ROSCO2 and ROCOP could be a nontoxic support in therapy of selected human cancers.

1. Introduction

Cancer is the leading cause of death in the world. And yet, a growing problem in clinically effective chemotherapeutic treatment is the increased resistance to commonly used cytostatics. Treatment of patients with chemotherapy becomes ineffective and often only results in the side effects of cytostatics and disease progression. Interests of many researches have had focused recently on compounds that are capable of stimulating reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation, which may collectively be responsible for damaging cells, including cancer [1–3]. These compounds are often grouped in complex combinations, acting synergistically, and may modestly advance the therapy of cancer. Such combinations already exist in foods, and a good representative is seed oils. In recent years, oils recovered from various seeds have been an unconventional lipid source occupying an important position in the nutrition field and extensively evaluated regarding their effects on health. Vegetable seed oils are a nutrition source of

polyunsaturated fatty acids, sterols, and tocopherols, but also consist of other bioactive compounds presented in smaller quantities: carotenoids and phenolics. Raspberry seed oil is an excellent source of polyunsaturated fatty acids (PUFAs) exceeding 80% of the total lipid content and various micronutrients of high bioactivity [4–7].

Pharmacological properties of raspberry seed oils have been scrutinized, with anti-inflammatory, antioxidative, and hypolipidemic effects in animal studies [8, 9]. Antioxidant properties of raspberry oil may also suggest their chemopreventive activities. The primary fatty acids are linoleic acid (LA) and α-linolenic acid (ALA), which are present in a ratio of less than two to one. Oleic acid in another significant fatty acid and in raspberry oil is present at 10-12%. Several mechanisms seem to be involved in the antitumor PUFA activity, including alterations in cell signalling, chromatin remodeling, and DNA methylation [10–12]. In addition to PUFA and monounsaturated fatty acids (MUFAs), raspberry seed oils contain high levels of phytosterols (up to 5384 mg/kg), tocopherols (up to 4000 mg/kg), and carotenoids (ca. 25 mg/kg), higher than many other berry species [4, 5, 7]. Among the bioactivities of minor components of raspberry seed oil, the important role of tocopherols in the protection of cell membranes from oxidation, gene regulation, and signal transduction has been well documented [13, 14]. *In vitro* studies have shown the effects of γ- and α-tocopherols in reducing the activity of colon cancer and breast cancer cells, respectively [14]. Previous studies have identified specific antioxidant properties of carotenoids. Both nonoxygenated carotenoids and the oxygenated derivatives xanthophylls, singly and in combination, may suppress cancer development and improve the immune response [15–17]. Sterols present in raspberry oil have shown anti-inflammatory, anticarcinogenic, and wound-healing properties [18]. However, the biological activity of raspberry seed oil with a view to different oil recovery techniques has not been extensively investigated.

Raspberry seeds are a decent oil source; 10-25% of the oil can be recovered. The yield and composition of oil and consequently its biological properties are influenced by the method of oil extraction. Among oil extraction technologies, cold pressing is a widely adopted practice in manufacturing high-quality and environmentally friendly oil. The limitation of cold pressing is low yield and exposing oil rich in PUFA to oxidation. Supercritical CO_2 (SCO2) oil extraction has attracted researchers' attention due to better oil recuperation from residues, the absence of oxygen, and operating at low temperature that can minimize oxidative oil deterioration. Another important asset of supercritical CO_2 is the high penetration and solvating power. In the literature, the authors have highlighted that SCO2 extraction is selective towards nonpolar and low molecular weight nonpolar and slightly polar components such as phytosterols, tocopherols, and phenols [19–21], not to mention the full CO_2 retrievability, which makes the oil extraction environmentally friendly [19].

Taking together all the facts quoted beforehand, this study is primarily aimed at characterizing raspberry oils obtained by supercritical CO_2 extraction and cold pressing and justifying their cell suppression potential against a panel of human normal and cancer cell lines. These findings would provide the basis for further *in vivo* studies as well as for clinical trials.

2. Materials and Methods

2.1. Raw Material. Seeds provided by the Polish manufacturer Oleowita (Milicz, Poland) were purified from any undesirable remains and then freeze-dried (Figure 1). Both methods of oil recovery seeds came from the same harvest.

2.2. Seed Oils. Raspberry oil obtained by supercritical carbon dioxide extraction (ROSCO2) was obtained from seeds crushed in a mill crusher under nitrogen to particle sizes of approximately 0.4–0.6 mm immediately before supercritical carbon dioxide extraction. The supercritical carbon dioxide extraction was carried out on an extraction apparatus pilot plant (ELAB) designed and made by engineers from the Łukasiewicz Research Network–New Chemical Synthesis Institute in Pulawy (Poland). 1660 g of ground seeds was loaded into the extractor basket. The extraction procedure has been described in detail elsewhere [21]. Briefly, the extraction with supercritical carbon dioxide started at a pressure of 340 bar and temperature of 40°C. The flow rate and CO_2 consumption were 89.7 kg CO_2/h. and 62.0 kg CO_2/kg, respectively. The extraction yield was 10.65 wt.%.

Cold-pressed raspberry oil (ROCOP) was obtained by cold pressing in a hydraulic press under nitrogen atmosphere by the Polish manufacturer Oleowita and immediately (within 24 hours) delivered to the laboratory. The extraction yield was 9 wt.%.

Both oils were bottled up under nitrogen atmosphere and stored at -26°C until analysis.

2.3. Oils' Chemical Characteristics

2.3.1. Fatty Acid Compositions. The procedure of fatty acid determination was described previously by Prescha et al. [22]. The chromatographic separation was performed on a gas chromatograph 6890 N (Agilent Technologies, USA) equipped with an FID detector and a Supelco SPTM-2560 fused silica capillary column 100 m × 0.25 mm × 0.2 μm (Bellefonte, USA). Hydrogen at a flow rate of 1.5 ml/min was used as a carrier gas, and the initial temperature of chromatographic separation was 165°C held for 10 min; then, the temperature was increased at the rate of 2°C/min to the final temperature of 220°C held for 10 min. The identification of fatty acids was accomplished using external fatty acid methyl ester (FAME) standards.

2.3.2. Phytosterol and Squalene Content. The analysis of sterols and squalene in oil samples was performed according to the protocol described by Shukla et al. [23]. Volatile components were separated on a DB-5MS capillary column 30 m × 0.25 mm × 0.1 μm (J & W Scientific, USA) using a 7890A gas chromatograph equipped with an electron ionization source and the autosampler system PAL3 RSI (CTC Analytics AG, Switzerland) coupled to a 5975C mass spectrometer (Agilent Technology, USA). Helium at a flow rate of 1.1 ml/min was used as the carrier gas. Source temperature

Figure 1: Raspberry seeds used in cold pressing and supercritical extraction came from the same batch.

was 230°C, and temperature of the transfer line was 290°C; the separation started at 120°C held for 1 minute; then, the temperature was increased at the rate of 5°C/min to a final temperature of 290°C held for 15 minutes. Identification of individual phytosterols and squalene was carried out using mass spectra and retention times of external standards.

2.3.3. Tocopherol and Carotenoid Composition and Content. The procedures of extraction of tocopherols and carotenoids from oil samples and analysis were based on the method already described by Fromm et al. [24]. Then, the unsaponifiable fraction was separated on a C18 UPLC column (Acquity, 100 mm × 2.1 mm × 1.7 μm, Waters, USA) connected to an Acquity UPLC system equipped with a PDA detector (Waters, USA). Two eluents were used as follows: (a) 30% methanol, 50% acetonitrile, and 20% water; (b) 50% methanol and 50% acetonitrile (v/v) at a flow rate of 0.5 ml/min in a timed gradient program: 100% A held for 1 min, linear gradient up to 100% B for 4 min, from 5 min to 10 min 100% B.

2.3.4. Phenolic Compounds' Compositions and Content. The procedure of phenolic compounds' extraction was acquired from the protocol previously published [25] using solid-phase C8 cartridges. Then, the hydrophilic compounds were separated on a C18 UPLC column (BEH Acquity, 100 mm × 2.1 mm × 1.7 μm, Waters, USA) connected to an Acquity UPLC system equipped with PDA 200–500 nm and mass spectrometer Xevo-Q-TOF (Waters, Milford, CT, USA). The mobile phase was a mixture of A—deionized waterformic acid (99.5 : 0.5), and B—acetonitrileformic acid (99.5 : 0.5), at a flow rate of 0.5 ml/min. The electrospray ionization source of the mass spectrometer operated at a capillary voltage of ±2.80 kV in the positive and negative ion mode, respectively, a sampling cone of 66 kV, and an extraction cone of 4.0 kV. Collision energy was set at 0, 20, 20–30, 30, and 30–50 kV. Data were processed using MassLynx 2.0 (Waters, Milford, CT, USA). Single components were identified by comparison of experimental mass, UV absorption spectra, and retention time with standards and literature data.

2.3.5. Statistical Analysis. The statistical significance of differences in the content of oil components was determined using the t-test and Mann-Whiney U test for normally and not normally distributed variables, respectively. The normality of the variable distribution was assessed with the Shapiro-Wilk test.

2.4. In Vitro Studies

2.4.1. Preparation of Oil Emulsions. For *in vitro* studies, the raspberry oil emulsions were formulated in accordance with a protocol developed in a study by Skorkowska-Telichowska et al. [26]. One gram of soybean lecithin, 0.5 ml of Tween 80, and 2.5 ml of oil were mixed gradually and completed with a 5 ml of an aqueous phase containing glycerol 25% v/v. Raspberry oil emulsions were prepared at 0.5%, 1%, 2%, 5%, and 10% at room temperature (RT) and in sterile conditions.

2.4.2. Cell Lines and Culture Conditions. All cell lines were maintained under sterile conditions at 37°C in a humidified atmosphere of 5% CO_2 in the complete culture medium. Normal human dermal fibroblasts (NHDFs; Lonza: CC-2511) and five cancer lines—colon adenocarcinoma cell line (LoVo; ECACC: 870601011-1VL), doxorubicin-resistant colon adenocarcinoma cell line (LoVo/DX), breast cancer (MCF7; ECACC: 86012803-1VL), doxorubicin-resistant breast cancer (MCF7/DX), and lung cancer (A549; ECACC: 86012804-1VL)—were cultured in an appropriate medium supplemented with penicillin (10000 U/ml), streptomycin (10 mg/ml), L-glutamine (200 mM), and 10% fetal bovine serum (FBS). NHDF was purchased from Lonza (Verviers, Belgium; cat. no. CC-2511) and cultured in Dulbecco's Modified Eagle Medium (DMEM) without phenol red. The colon adenocarcinoma cell line (LoVo) was obtained from EATCC (cat. no. 870601011-1VL), and doxorubicin-resistant breast cancer (LoVo/DX) was derived from them as a result of incubation in the presence of low concentrations of doxorubicin for a period of three months [2]. Both types of cell line were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium. The breast cancer line (MCF7) was purchased from the EATCC collection (cat. no. 86012803-1VL), and the doxorubicin-resistant breast cancer line (MCF7/DX) was derived from it as a result of incubation in the presence of low concentrations of doxorubicin for a period of three months [27]. The lung cancer line (A549) was purchased from EATCC (cat. no. 86012804-1VL). Cells of both breast cancer lines and lung cancer were cultured in Minimal Essential Medium (MEM). The cells were evaluated at least twice a week under a microscope and then passaged using TrypLE solution or the appropriate medium was changed.

When the cell confluence was above 70%, the cells were separated from culture flasks using TrypLE. The cell suspension was collected into centrifuge tubes, and an appropriate medium for the cell line was added to inactivate TrypLE activity. The tubes were then centrifuged at 1000 × g for 5

Table 1: Chemical characteristics of raspberry oil obtained by supercritical carbon dioxide extraction (ROSCO2) and cold pressed (ROCOP); statistically significant differences between oils ($p < 0.05$) are marked in bold.

	ROSCO2	ROCOP	p
Fatty acid composition (%)			
Palmitic acid	3.98 ± 0.18	5.37 ± 0.17	**0.00331**
Stearic acid	1.37 ± 0.15	2.58 ± 0.05	**0.00085**
Oleic acid (OA)	15.55 ± 0.85	26.38 ± 0.20	**0.00016**
Linoleic acid (LA)	50.78 ± 0.14	53.25 ± 0.24	**0.00108**
α-Linolenic acid (ALA)	27.22 ± 1.01	11.05 ± 0.11	**0.00008**
Others	1.10 ± 0.04	0.61 ± 0.03	**0.00047**
$\sum$ SFA	6.24 ± 0.30	8.42 ± 0.22	**0.00229**
$\sum$ MUFA	15.76 ± 0.86	27.28 ± 0.18	**0.00015**
$\sum$ PUFA	78.00 ± 1.15	64.29 ± 0.23	**0.00021**
n-6 : n-3	1.87 ± 0.14	4.82 ± 0.06	**0.00001**
Phytosterols (mg/kg)			
Campesterol	616.38 ± 465.99	274.29 ± 46.35	**0.01773**
Stigmasterol	128.48 ± 7.74	147.63 ± 3.70	**0.09433**
β-Sitosterol	15020.17 ± 693.51	4082.44 ± 476.42	**0.00295**
Δ5-Avenasterol	2266.62 ± 161.28	595.91 ± 31.29	**0.00480**
Cycloartenol	445.99 ± 5.46	220.96 ± 12.34	**0.00179**
Δ^7-Avenasterol	117.69 ± 1.25	54.82 ± 7.25	**0.00480**
24-Methylene-cycloartenol	1272.06 ± 42.71	432.04 ± 13.73	**0.00142**
Citrostadienol	221.73 ± 19.25	260.80 ± 60.45	0.47480
β-Amyrin (mg/kg)	181.14 ± 45.77	319.08 ± 35.04	0.16469
Total	20562.28 ± 919.47	6631.60 ± 251.00	**0.00325**
Squalene (mg/kg)	125.16 ± 0.62	110.68 ± 0.86	0.35420
Tocopherols (mg/kg)			
α-Tocopherol	513.37 ± 22.16	321.24 ± 15.02	**0.00128**
γ-Tocopherol	1979.98 ± 120.17	550.16 ± 42.21	**0.00026**
δ-Tocopherol	824.15 ± 11.10	234.06 ± 0.29	**0.00000**
Total	3317.50 ± 109.11	1105.26 ± 7.43	**0.00005**
Carotenoids (mg/kg)			
All-transneoxanthin	-	5.79 ± 1.02	-
All-transzeaxanthin	1329.05 ± 183.59	97.87 ± 11.06	**0.01017**
All-translutein	82.48 ± 5.90	51.55 ± 4.13	0.17910
All-trans-α-cryptoxanthin	255.32 ± 15.49	48.14 ± 1.42	**0.00014**
All-trans-β-cryptoxanthin	205.27 ± 26.38	10.75 ± 0.17	**0.00907**
Fucoxanthin	70.40 ± 11.19	-	-
All-trans-α-carotene	373.87 ± 19.70	27.30 ± 0.39	**0.00161**
All-trans-β-carotene	159.44 ± 12.48	12.49 ± 1.89	**0.00367**
Total	2475.85 ± 443.02	235.41 ± 26.26	**0.01906**
Phenolic compounds (mg/kg)			
4-Hydroxybenzoic acid	4.57 ± 0.038	0.05 ± 0.03	**0.00007**
Vanillic acid	2.88 ± 0.37	0.80 ± 0.21	**0.01382**
4-Hydroxybenzaldehyde	11.57 ± 1.53	3.37 ± 0.61	**0.03244**

Table 1: Continued.

	ROSCO2	ROCOP	*p*
Vanillin	16.32 ± 0.95	1.69 ± 0.10	**0.00184**
Sinapaldehyde	3.07 ± 0.15	0.22 ± 0.02	**0.00224**
t-Ferulic acid	1.06 ± 0.036	0.59 ± 0.02	**0.00676**
*Iso*ferulic acid	2.05 ± 0.09	-	-
Total	41.52 ± 2.98	6.76 ± 0.48	**0.00404**

min. At this time, the number of cells was counted in a Bürker chamber.

A negative control was used in this study as a reference, which was a cell culture incubated in the primary medium without tested oils. All assays were performed in five independent replicates.

2.4.3. Cell Viability Assay. The methylthiazol tetrazolium (MTT) assay, based on the conversation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide to MTT formazan, was used to evaluate the effect of oils' emulsions on cell viability according to the ISO 10.9993 standard, part V. For the assays' purpose, cells were seeded on 96-well plates at a density 1×10^4 cells/ml per well and subjected to 24-hour incubation under 5% CO_2 in air. Then, the cells were treated with increasing concentrations of oil emulsion. At the end of the incubation time, the supernatant was removed, then the cells were washed with PBS, and 50 μl of 1 mg/ml MTT solution in MEM without phenol red was added to the culture plates and left in a CO_2 incubator at 37°C for 2 hours. The supernatant was then removed, and colored formazan was added to the medium, after being dissolved in 100 μl of isopropanol for 30 min while shaking. The percentage of viable cells was calculated by measuring the absorbance of the colored formazan reaction product at 570 nm using a Varioskan LUX microplate reader (Thermo Scientific, USA).

2.4.4. Cell Proliferation Assay. Cell proliferation was determined by the sulforhodamine B assay (SRB). Cells were seeded on 96-well plates at a density of 1×10^4 cells/ml per well. Before the addition of the oil emulsions, one plate from each cell line was fixed with cold 50% trichloroacetic acid (TCA) at 4-8°C (these plates were the control). After 48-hour incubation with increasing concentrations of oil emulsion, the culture plates were also fixed with 50% TCA for 1 h at 4-8°C. The plates were then washed five times under running water, the plates were air-dried, and then, sulforhodaminen B dye was added for another 30 minutes to stain cell proteins. Unbound dye was removed five times by washing with 1% acetic acid and air-dried again. The SRB dye was dissolved in Trizma solution, and the absorbance was measured at 570 nm, using a Varioskan LUX microplate reader (Thermo Scientific, USA).

2.4.5. Scavenging Activity against Reactive Oxygen Species (ROS). The spectrofluorometric method using 2′,7′-dichlorofluorescein-diacetate (DCF-DA) for quantifying the intracellular levels of reactive oxygen species (ROS) produced in five cultured cancer cell lines was utilized. Cells were seeded on 96-well plates at a density 1×10^4 cells/ml per well. After 24-hour incubation with oil emulsion, the supernatant was transferred to new plates, and the DCF-DA solution was added to the culture for the next 1 hour of incubation. After that time, the supernatant was removed, cells were washed twice with phosphate buffered saline (PBS), and then, 100 μl of H_2O_2 was added for the next 30 min. Finally, the fluorescence was measured at $\lambda_{ex.}$=485 nm and $\lambda_{em.}$=535 nm using a Varioskan LUX microplate reader (Thermo Scientific, USA) [28].

2.4.6. Scavenging Activity against Nitric Oxide. For the evaluation of intracellular concentration of nitric oxide, Griess reagent (1 : 1 mixture (v/v) of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride) was added to the supernatant plates. The plates were left for 20 minutes in the dark at RT. Nitrite level was measured at 548 nm using a Varioskan LUX microplate reader (Thermo Scientific, USA) [29].

2.4.7. Fast Halo Assay. The fast halo assay (FHA) was conducted to evaluate the effect of the tested oils on double-strand breaks (DSBs). Cells were seeded on 24-well plates at a density 2.5×10^4 cells/ml per well. After incubating with the tested oils, the supernatant was transferred to centrifuge tubes, which were previously properly signed and prepared, and the culture was washed with PBS, which was also collected into tubes. The cells were separated from the surface of the plate with TrypLE solution up to 3 min at 37°C in a CO_2 incubator, and the cell suspensions were transferred into tubes, which were then centrifuged at 1000 × g for 5 min. After removal of the supernatant, the cell pellet was washed in PBS and again centrifuged at 1000 × g for 5 min. The cells were resuspended in PBS with Ca^{2+} and Mg^{2+} at a density of 1000 cells/μl; the cells in tubes were placed in a water bath. Then, 120 μl of 1.25% low melting agarose in PBS was added to the cells, and the mixture was immediately squeezed between a slide coated with agarose (high melting point) and a coverslip. The slides were placed on a cooling block for 10 min for gel formation. The coverslips were then removed, and the slides were placed in the lysis buffer overnight. The slides were then transferred into alkaline solution (pH = 13.0) for 30 min and then washed twice for 5 min in neutralizing buffer. The preparations were stained using 5

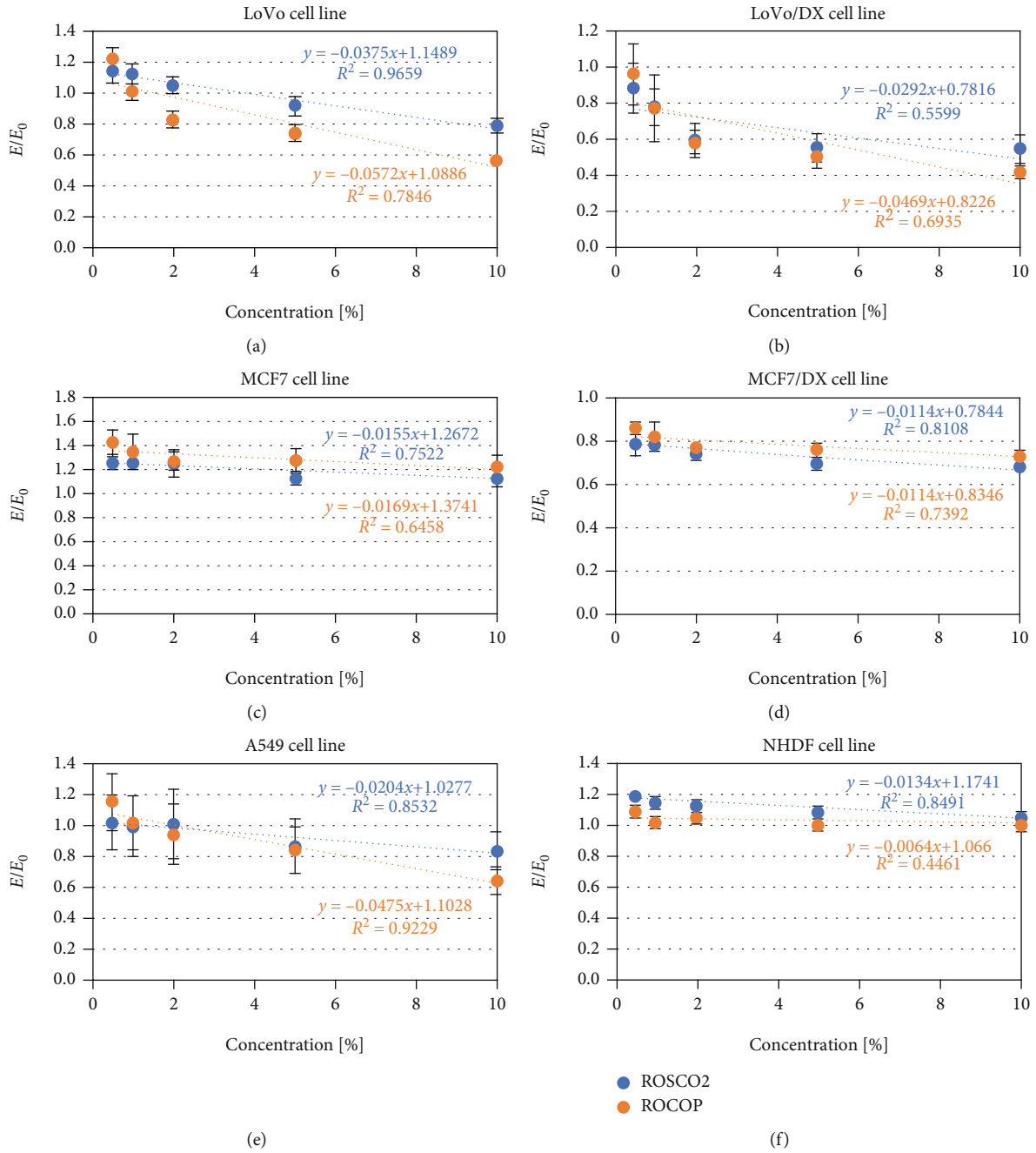

Figure 2: The effect of oil emulsion from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP) at various concentrations—0.5%, 1%, 2%, 5%, and 10%—on the viability of colon adenocarcinoma cell lines (LoVo), doxorubicin-resistant colon adenocarcinoma cell line (LoVo/DX), breast cancer cell line (MCF7), doxorubicin-resistant breast cancer cell line (MCF7/DX), lung cancer cell line (A549), and normal human dermal fibroblast (NHDF). The results were compared to control and expressed as E/E_0—the ratio of spectrometrically measured viability of cells with tested oils (E) to a negative control culture (E_0—cells without tested oils). Linear regression models and coefficients of determination were calculated.

μM DAPI for 20 min and examined immediately under a fluorescence microscope. Photographs were taken, and then, the ratio of cell nucleus diameter to halo diameter (which is a measure of DNA damage) was analyzed [29].

2.4.8. Statistical Analysis. The results obtained in the MTT and SRB assays are presented as IC_{50} values, which indicate the concentrations of the oil emulsions at which 50% inhibition of succinate dehydrogenase activity (MTT assay) or total cellular protein synthesis (SRB assay) was observed (depending on the assay performed) compared to the control. The normality of the data and the equality of variance were checked using the Shapiro-Wilk and Levene'a tests, respectively. Due to the possibility of adopting the hypothesis of

Table 2: IC_{50} values (concentration that causes 50% decrease in cell growth) of oil emulsions from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP) against different cell lines in inhibition of metabolic activity (MTT) assay.

Cell line	IC_{50} values (%)		p value
	ROSCO2	ROCOP	
LoVo	17.3 (3.4)	10.3 (5.2)	0.03
LoVo/DX	9.6 (6.3)	6.9 (5.4)	0.48
MCF7	49.5 (8.4)	51.7 (10.1)	0.72
MCF7/DX	24.9 (2.8)	29.4 (3.1)	0.04
A549	25.9 (17.6)	12.7 (15.6)	0.25
NHDF	51.9 (3.5)	88.4 (3.7)	0.0001

MCF7: breast cancer cell line; MCF7/DX: doxorubicin-resistant breast cancer cell line; A549: lung cancer cell line; NHDF: normal human dermal fibroblast. The results are presented as mean (SD).

normal distribution and equal variance, parametric tests were used for statistical analysis. Other results are shown as mean ± SEM (standard error of the mean) relative to the control (E/E_0). E represents the result obtained for the culture with the test oils after subtraction of the values obtained for the solvent alone, and E_0 is the result for negative control.

The two-way ANOVA was used (with Tukey post hoc test) for the comparison of data obtained for cell lines. In all assays, $p < 0.05$ was used as the significance level. To compare the activity of the tested oils, multiple-criteria decision analysis (MCDA) was carried out using a weighted sum model (WSM). The weights were chosen according to the meaning of each biological assay. Pearson's correlation coefficients were calculated to show the relationship between double-stranded DNA breaks and free radical levels.

3. Results

To compare the impact of bioactive compounds of oils' emulsions on cell treatment, several chemical characteristics were measured.

3.1. Oils' Chemical Characteristics. Raspberry oil extracted by supercritical CO_2 (ROSCO2) contained about 78% PUFA, and in comparison with ROCOP—raspberry oil cold-pressed, this was about 18% more. In both raspberry oils, LA represented slightly more than 50% of total fatty acids, and ALA represented around 27% of ROSCO2. However, in ROCOP, 2.5 times less ALA was recorded than in ROSCO2. Oleic acid (OA) is another significant fatty acid present at 15% in ROSCO2 and 26% in ROCOP.

In addition to high levels of PUFA, raspberry oil contained considerable amounts of phytosterols, reaching over 20 g/kg in ROSCO2, while in ROCOP, the total phytosterol content was only ca. 30% of that determined in ROSCO2 (Table 1). The primary component of the phytosterol fraction in both oils was β-sitosterol, which accounted for 75% in ROSCO2 and 67% in ROCOP, and other significant phytosterols present in smaller amounts were Δ^5-avenasterol and 24-methylenecycloartenol (10-11% and 6-7%, respectively).

Squalene content in both oils was low, and in ROSCO2, it was higher than in ROCOP by ca. 12%. β-Amyrin content was recorded as 1.8-fold higher in ROCOP that in ROSCO2.

The total amount of tocopherols was 1105 mg/kg in ROCOP, and it was 3 times less than the total amount of tocopherols obtained in this study for ROSCO2. In both oils, γ-tocopherol was the dominant one but in ROSCO2, its contribution to the total tocopherols was about 10% higher than that recorded for ROCOP.

Raspberry oil obtained by SCO2 extraction stood out in terms of carotenoid content. The total carotenoid content in ROSCO2 was almost ten times higher than that in ROCOP. Moreover, SCO2 extraction seemed to recover better xanthophylls, especially all-transzeaxanthin, and carotenes.

Phenolic compound recovery in raspberry oils was again higher in ROSCO2 than ROCOP, but still was not so pronounced as it was in the case of carotenoid content or composition. The difference in total phenolic content was 6 times greater. ROSCO2 was characterized by a pronounced presence of vanillin, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid.

3.2. In Vitro Studies

3.2.1. Cytotoxicity. In the entire range of concentrations tested for both oils, no significant reduction in metabolic activity (MTT assay) in normal cells was observed—IC_{50} values were high (51.9% and 88.4%, respectively, for ROSCO2 and ROCOP oils) (Figure 2 and Table 2).

In LoVo cancer cells, ROCOP oil had a stronger effect, while in MCF7/DX cells, ROSCO2 oil had slightly but significantly more affected cells' metabolic activity. Generally, for both oils, a greater reduction of metabolic activity was observed in doxorubicin-resistant cell lines, both colorectal and breast cancer. Based on the calculated IC_{50} values, it can be concluded that in the lung cancer line, ROCOP oil inhibited metabolic activity approximately twice as strongly as ROSCO2.

3.2.2. Cell Proliferation. Raspberry oil (ROSCO2 and ROCOP) emulsions were examined for their antiproliferative activity against a panel of human cancer (5) and normal (1) cell lines. ROSCO2 emulsions exhibited a noncytotoxic effect on human normal fibroblast cells (NHDFs) at all studied concentrations. In contrast to ROSCO2, the ROCOP emulsion showed an antiproliferative effect at 57.0% (IC_{50}) (Figure 3 and Table 3). However, the different cancer cell lines exhibited different sensitivity to raspberry oil emulsions, evident from the different IC_{50} values (Table 3). In the case of colorectal cancer cells (LoVo), ROSCO2 oil was more cytotoxic than ROCOP, but at the same time, the effect was slightly weaker for the doxorubicin-resistant line (LoVo/DX). In contrast to the doxorubicin-sensitive breast cancer cell line (MCF7), where stronger antiproliferative activity was demonstrated for ROCOP oil, in the doxorubicin-resistant MCF7 cells, the effect was similar for

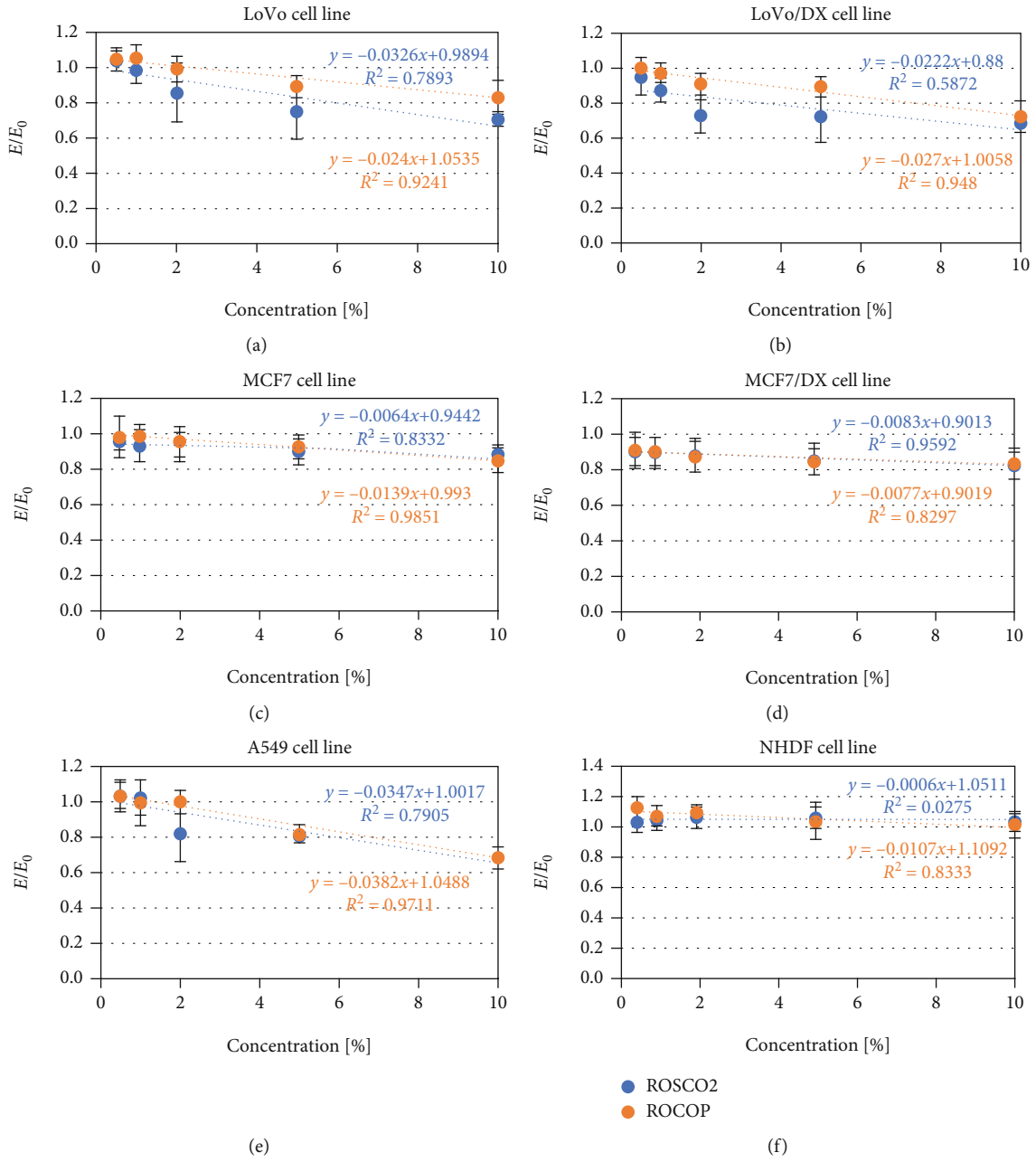

Figure 3: *In vitro* antiproliferative activity of oil emulsion from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP) against human cancer and normal cell lines. Antiproliferative effect of oil emulsions in 0.5%, 1%, 2%, 5%, and 10% concentrations on colon adenocarcinoma cell line (LoVo), doxorubicin-resistant colon adenocarcinoma cell line (LoVo/DX), breast cancer cell line (MCF7), doxorubicin-resistant breast cancer cell line (MCF7/DX), lung cancer cell line (A549), and normal human dermal fibroblast (NHDF). Cell growth percentage was analyzed with sulforhodamine B (SRB) assay. The results were compared to control and expressed as E/E_0. E—cells with tested oils; E_0—a negative control culture (cells without tested oils). Linear regression model and coefficients of determination were calculated.

both oils. For the lung cancer line (A549), the IC_{50} values determined for both oils were almost identical.

3.2.3. Intracellular Levels of Free Oxygen Radicals and Nitric Oxide. To assess the effect of the tested oils on the level of free oxygen radicals and nitric oxide (NO), DCF-DA and Griess assays were performed, respectively. Some cytostatics increase the level of free radicals in cancer cells, which in turn causes DNA damage and subsequent cancer cell death. At the same time, the observed ROS levels are lower in resting cytostatic resistant cell lines compared to the corresponding sensitive cell lines [2].

This study showed significant higher ROS levels in both doxorubicin-resistant cancer cell lines (colorectal and breast)

Table 3: IC_{50} values (concentration that causes 50% decrease in cell growth) of oil emulsions from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP) against different cell lines determined with the SRB assay. The results are presented as mean (SD).

Cell line	IC_{50} values (%)		p value
	ROSCO2	ROCOP	
LoVo	15.0 (2.5)	23.1 (3.4)	0.0026
LoVo/DX	17.1 (2.2)	18.7 (3.0)	0.36
MCF7	69.4 (7.3)	35.5 (8.0)	0.0001
MCF7/DX	48.3 (2.2)	52.2 (2.8)	0.04
A549	14.5 (2.8)	14.4 (2.7)	0.96
NHDF	Nontoxic	57.0 (2.4)	N/A

LoVo: colon adenocarcinoma cell line; LoVo/DX: doxorubicin-resistant colon adenocarcinoma cell line; MCF7: breast cancer cell line; MCF7/DX: doxorubicin-resistant breast cancer cell line; A549: lung cancer cell line; NHDF: normal human dermal fibroblast; N/A: not applicable.

compared to the corresponding sensitive cell lines after incubation with tested raspberry oils, especially ROSCO2 (the increase was 20-80% in colorectal cancer cell and 30-60% in breast cancer cells) (Figure 4). A statistically significantly higher level of free oxygen radicals in resistant lines of colorectal and breast cancer after the use of ROSCO2 oil was observed (40-100% for the colon cancer line and 30-70% for breast cancer) when compared to ROCOP oil. However, the impact of the tested oils on the ROS level in doxorubicin-sensitive lines was independent of the method of oil extraction. In turn, after incubation of lung cancer cells with the examined oils in the concentration range of 0.5-5%, a statistically significant reduction in the level of ROS was observed. At the lowest concentration (0.5%), stronger scavenging of free oxygen radicals was observed after treatment of this cell line with ROSCO2 emulsion in comparison to ROCOP. In the case of A549 cells, incubation with a 10% concentration of ROSCO2 oil statistically significantly increased the level of free oxygen radicals.

In contrast, nitric oxide levels were higher in the doxorubicin-sensitive LoVo and MCF7 cells than in their resistant counterparts (Figure 5). After incubation with each tested oil, a concentration-dependent increase in NO level in the concentration range of 1-10% was observed in these cell cultures. In the doxorubicin-resistant colorectal cancer line, a statistically significant increase in NO level was observed in the entire tested concentration range after incubation with ROSCO2 emulsion and in the concentration range of 2-10% of ROCOP. The level of nitric oxide in LoVo/DX after administration of ROSCO2 at the concentrations of 0.5 and 1.0% was significantly higher than in the case of ROCOP. In the doxorubicin-resistant cell line of breast cancer, after incubation with oils at concentrations of 0.5-2.0%, a decrease in nitric oxide levels was observed compared to the culture incubated only in medium. In the case of the lung tumor cell line (A549), there was a concentration-dependent increase in NO level after treatment with the tested oils. What is more, in the concentrations of 0.5-2.0%, this increase was statistically significantly higher after using supercritical extracted oil compared to cold-pressed oil.

3.2.4. DNA Double-Strand Damage Assessment. To investigate the amount of DNA strand damage after application of the tested oils, a fast halo assay (FHA) was performed. In all cancer lines tested, a concentration-dependent increase in DNA strand breaks was observed regardless of the oil used (Figure 6). At the same time, the amount of DNA damage was always greater after the treatment of cell cultures with ROSCO2 than ROCOP, and these differences were statistically significant for some oil concentrations in doxorubicin-resistant cell lines, both LoVo/DX and MCF7/DX.

Correlation coefficients between DNA damage and ROS or NO levels were assessed. In all cases, after using each tested oil (except for ROSCO2 oil in A549 cells), a strong positive and statistically significant correlation between NO level and DNA damage was found (Table 4). A strong positive correlation was also found between the ROS level and DNA strand damage in the LoVo and MCF7/DX lines after incubation with both tested oils and in the MCF7 line after using ROSCO2 oil.

3.2.5. Multiple-Criteria Decision Analysis. Multiple-criteria decision analysis (MCDA) was performed based on the results obtained from all assays. The MCDA results showed that at concentrations where the oil effect was clearly pronounced (5 and 10%), the activity of the supercritical extracted oil was stronger compared to the cold-pressed oil (Figure 7). At a concentration of 2%, the effect of the oils was very similar. On the other hand, low concentrations showed the opposite activity than expected, and it was stronger in the case of ROCOP oil. Generally, it can be concluded that in the concentration range of 2-10%, the tested oils showed a concentration-dependent suppressive impact on cancer cells.

4. Discussion

The overall objective of this study was to determine whether raspberry oils obtained by different extraction techniques have any diverse and selective effects against the panel of human normal and cancer cell lines. From the current results, it is clear that the two raspberry oils—extracted by supercritical CO_2 and cold pressed—significantly differ in phytochemical characteristics. In comparison to cold-pressed raspberry oil (ROCOP), SCO2-extracted raspberry oil stood out in terms of composition of fatty acids (particularly ALA), phytosterols (substantially β-sitosterol, Δ5-avenasterol, and 24-methylene-cycloartenol), tocopherols, carotenoids, and phenolic compounds. In both raspberry oils, the fatty acid composition was close to the data published so far [5–7, 30], except for ALA and OA in ROCOP, the levels of which were 2- to 3-fold lower than the results previously published [5, 30].

From the proceeding results, it is clear that both raspberry oils exert multiple *in vitro* suppressive effects on the human colon adenocarcinoma cell line (LoVo), doxorubicin-resistant colon adenocarcinoma cell line

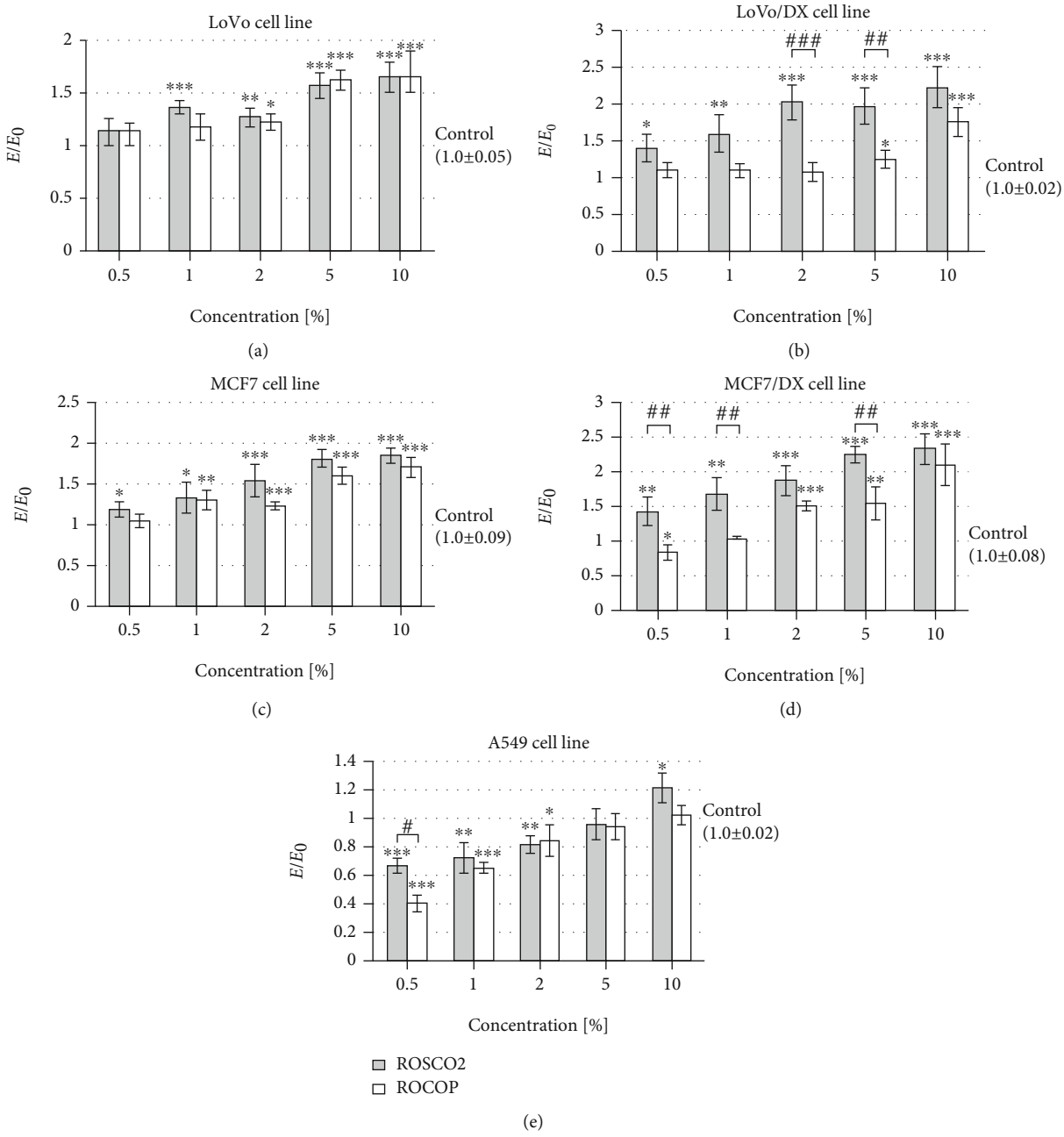

Figure 4: Level of free oxygen radicals after incubation with oil emulsion from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$—significant difference compared to control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$—significant difference between tested oils. The results were compared to control and expressed as E/E_0—the ratio of spectrofluorometrically measured level of free oxygen radicals of cells with tested oils (E) to a negative control culture (E_0—cells without tested oils). Cell lines: colon adenocarcinoma (LoVo), doxorubicin-resistant colon adenocarcinoma (LoVo/DX), breast cancer (MCF7), doxorubicin-resistant breast cancer (MCF7/DX), and lung cancer (A549).

(LoVo/DX), breast cancer cell line (MCF7), doxorubicin-resistant breast cancer cell line (MCF7/DX), and lung cancer cell line (A549), but its cancer cell suppression activity was equally complex. However, the differences in the content of bioactive components of both oils reflected in a distinct cancer-suppressive potential.

The studied oils differ in terms of polyunsaturated fatty acid (PUFA) composition and the ratio of n-6/n-3 PUFA (mainly LA/ALA), which in turn could considerably affect tumor cells. The cancer cell suppression activity of LA and ALA is not definite as yet [31]. It has been previously shown that the action of LA on cancer growth depends on its concentration and the type of cancer cell tested. Lu et al. [32] observed cancer cell suppression at high LA concentration in LoVo by MTT assay. This could explain the stronger inhibition of mitochondrial metabolite activity by ROCOP,

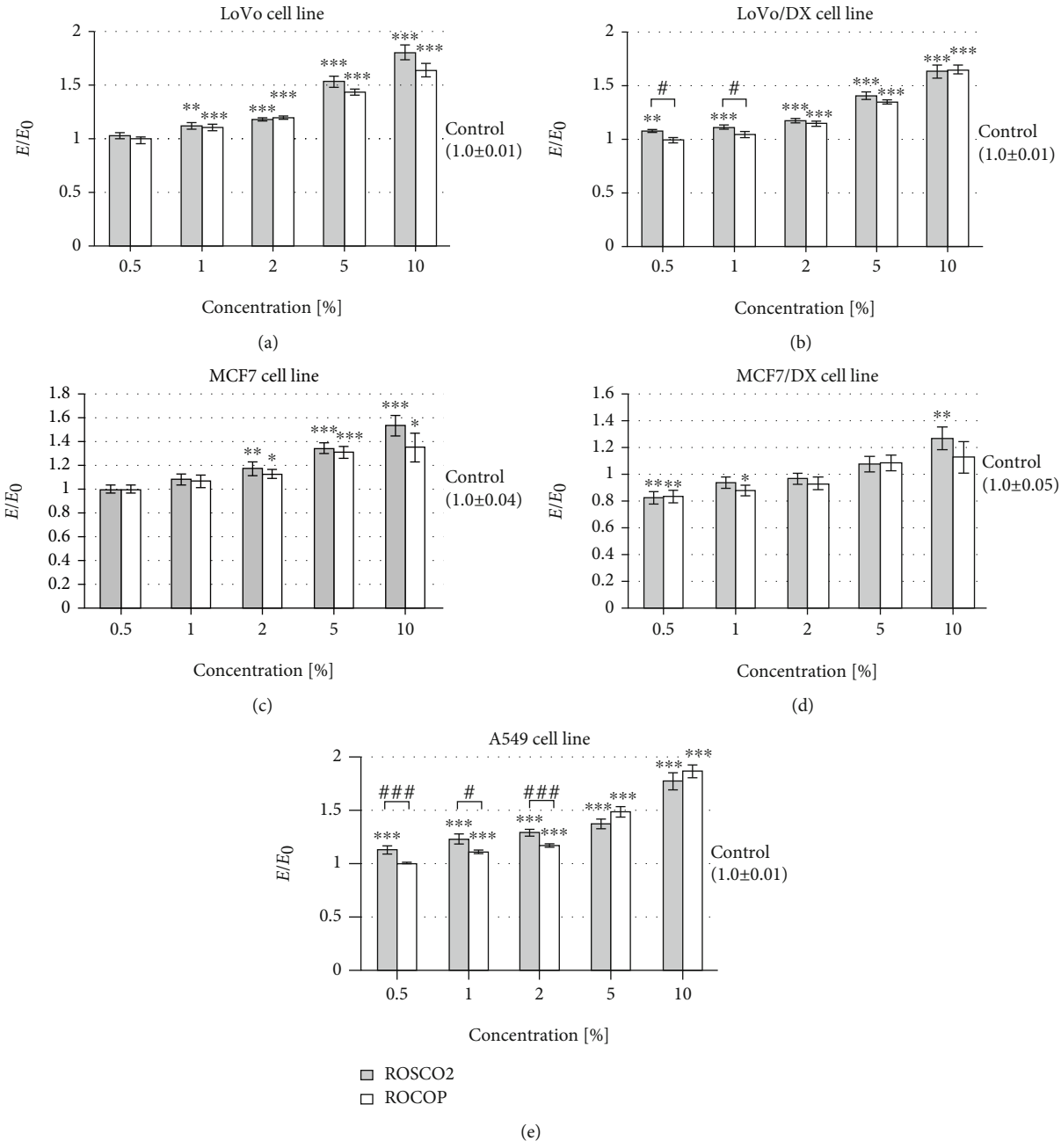

Figure 5: Level of nitric oxide after incubation with oil emulsion from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$—significant difference compared to control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$—significant difference between tested oils. The results were compared to control and expressed as E/E_0—the ratio of spectrometrically measured level of NO of cells with tested oils (E) to a negative control culture (E_0—cells without tested oils). Cell lines: colon adenocarcinoma (LoVo), doxorubicin-resistant colon adenocarcinoma (LoVo/DX), breast cancer (MCF7), doxorubicin-resistant breast cancer (MCF7/DX), and lung cancer (A549).

which is rich in LA, in comparison to ROSCO2. As the reactive oxygen species formation in LoVo cell lines was again more pronounced in ROCOP than ROSCO2, especially in the highest range of concentrations, this may indicate that LA-induced cell death was primed by the mitochondrial apoptotic pathway [33]. Thus, the mechanisms underlying ROCOP apoptotic activity may enhance the cellular oxidant status including mitochondrial dysfunction [32]. Abundant in ALA, ROSCO2 also affected considerably cell viability in LoVo and LoVo/DX cell lines in dose-dependent manners and moreover was more effective than ROCOP in proliferation inhibition of LoVo cells. Apoptotic cell activation via induction of caspase activity, both extrinsic and intrinsic pathways, was previously observed for ALA [10, 11]. These

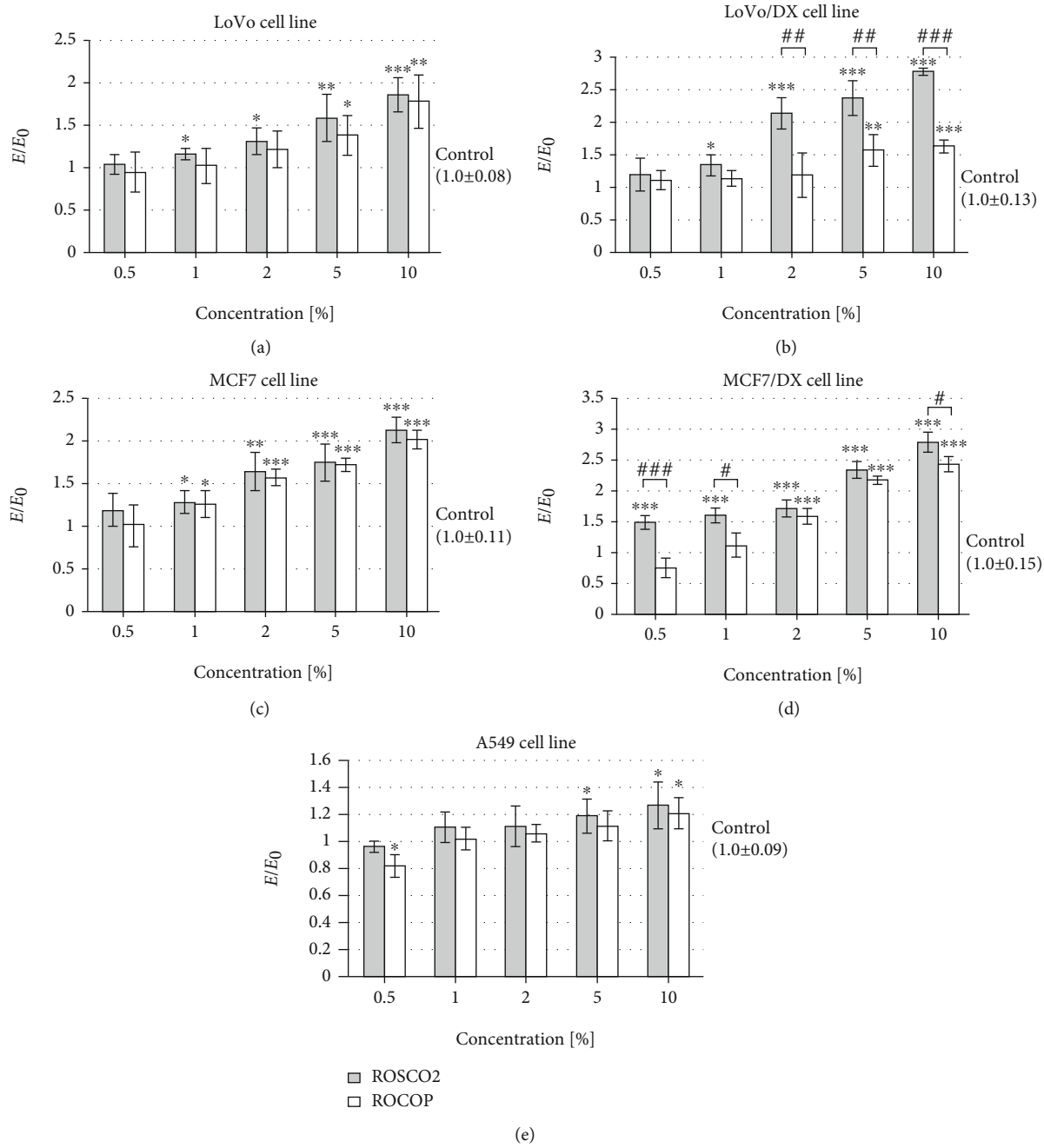

Figure 6: Amount of DNA strand damage after application of the oil emulsion from raspberry oils extracted by supercritical CO_2 (ROSCO2) and cold pressed (ROCOP); $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$—significant difference compared to control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$—significant difference between tested oils. The results were compared to control and expressed as E/E_0. E—cells with tested oils; E_0—a negative control culture (cells without tested oils). Cell lines: colon adenocarcinoma (LoVo), doxorubicin-resistant colon adenocarcinoma (LoVo/DX), breast cancer (MCF7), doxorubicin-resistant breast cancer (MCF7/DX), and lung cancer (A549).

findings also enhance the understanding of the n-6/n-3 ratio in chemoprevention of colon and breast cancers. It seems that smaller ratios could be more favorable in doxorubicin-resistant breast cancer than colon cancer. The observed correlation is in line with previous research where flaxseed oil rich in ALA reduced growth and increased apoptosis in several breast cancer lines, dynamically affecting gene expression and modifying signalling pathways [34].

Polyunsaturated fatty acids may influence cancer cell membrane integrity, facilitating the penetration of other potentially cancer toxic molecules. This means that PUFAs are not solely or even primarily responsible for the apoptosis of cancer cell lines observed in raspberry oil emulsions. The raspberry oils contained a pronounced amount of phytosterols and an interesting compound derivative from 4,4-dimethylphytosterols. 4,4-desmethylphytosterols exhibit

Table 4: Statistically significant correlation coefficients between DNA damage and levels of reactive oxygen species (ROS) and nitric oxide (NO).

Cell line	Oil type	ROS level vs. DNA damage	NO level vs. DNA damage
LoVo	ROSCO2	0.52	0.87
	ROCOP	0.89	0.7
LoVo/DX	ROSCO2	-0.34	0.76
	ROCOP	0.32	0.7
MCF7	ROSCO2	0.5	0.61
	ROCOP	-0.29	0.52
MCF7/DX	ROSCO2	0.64	0.72
	ROCOP	0.56	0.71
A549	ROSCO2	-	-0.36
	ROCOP	0.64	0.76

LoVo: colon adenocarcinoma cell line; LoVo/DX: doxorubicin-resistant colon adenocarcinoma cell line; MCF7: breast cancer cell line; MCF7/DX: doxorubicin-resistant breast cancer cell line; A549: lung cancer cell line; ROSCO2: raspberry oil extracted by supercritical CO2; ROCOP: raspberry oil cold-pressed.

anticancer effects in many cancers including skin cancer, bladder cancer, and colon cancer [18, 35]. The substantially higher amount of β-sitosterol and 4,4-desmethylphytosterols may contribute to the strong antiproliferative effect of ROSCO2 against LoVo and LoVo/DX cells. Tocopherols were also presented in greater concentrations in ROSCO2, especially γ-tocopherol, a strong apoptotic natural compound [14]. An even stronger proapoptotic effect than γ-tocopherol is exhibited by δ-tocopherol, presented in greater concentration in ROSCO2 than ROCOP [36]. Both of these isoforms activate peroxisome proliferator-activated receptor γ (PPARγ) expression, which is known to control inflammation by inducing apoptosis and inhibiting cancer cell proliferation [37, 38]. The γ isoform shows a similar cytotoxic effect as n-3 PUFA. In contrast, α-tocopherol is a weaker proapoptotic inducer. Some studies have revealed that this homolog may attenuate the cell cycle block induction as well as the cytotoxic and proapoptotic effects of n-3 fatty acids on cancer cells [39]. α-Tocopherol has also been shown to compromise the cytotoxic and cytostatic action of chemotherapeutic agents acting as protein kinase inhibitors. In prostate and colon cancer cells, intracellular concentrations increased as the concentration of tocopherols added to the media was increased. This effect was more pronounced in cells treated with γ-tocopherol than those treated with α-tocopherol [14]. Interestingly, the ratio of $\alpha{:}\gamma{:}\delta$ tocopherols found in this study for ROSCO2 is close to that of α-tocopherol mix rich in γ homolog, which shows a broad anticancer activity, including reducing estrogen receptor expression and increasing PPARγ signalling in breast cancer cells [40]. Tocopherols are of course also powerful antioxidants and induce an antioxidant response by upregulating Nrf2 and transcription of its target genes [41]. The lack of influence of the tested oils on the production of ROS and NO in A549 at low concentrations of the emulsions reveals the advantage of the antioxidant effect of oils (rich in tocopherols and other antioxidants). Notwithstanding, in each line at the highest concentrations of oils, the oxidative stress and NO production were strongly expressed, especially under the influence of ROSCO2. The carotenoids possessing provitamin A activity and present in raspberry oils, greatly pronounced in the oil extracted by SCO2, e.g., α-carotene, β-carotene, and β-cryptoxanthin, may be potential agents in biological interference in cancer. Gloria et al. [17] observed inhibition of proliferation of breast cancer cell line MCF7 after treatment with β-carotene at 2.5 and 5 μM for 48 h and found that β-carotene induced programmed cell death. From studies conducted on different cell lines (gastric cancer cell lines and B-cell lymphoma), it has been observed that treatment with β-carotene at a concentration exceeding physiological levels (100 μM) increased the expression of p53 (proapoptotic protein) and decreased antiapoptotic BCL-2 [16]. Overall, the present studies demonstrate complex combinations of compounds that may modestly advance the therapy of cancer. Raspberry oil is an interesting example of such combination already existing in nature, and the way of its recovery may justify its chemopreventive potential.

A growing problem in cancer treatment is the increased resistance to commonly used cytostatics, including doxorubicin. Treatment of patients with chemotherapy becomes ineffective and often only results in the side effects of cytostatics and disease progression [42]. Significantly lower levels of free oxygen radicals (ROSs) are observed in cytostatic-resistant tumors [2]. Accordingly, damage to the DNA strand, and hence the death of cancer cells due to an increase in ROS or RNS, is significantly reduced [43]. As we found in our research, the raspberry oil emulsions were shown to possess good ability to propagate the generation of free oxygen radicals and NO. And especially ROSCO2 stimulates the generation of radicals in cultures of cells resistant to doxorubicin. The observed effects encourage further study to assess the synergistic effect of ROSCO2 with cytostatic.

The results of the calculated correlations may suggest that raspberry oil emulsions affect the NO-dependent mechanism of DNA strand damage in LoVo and MCF7 cells for both doxorubicin-resistant and sensitive lines. This is especially important when the exocytosis of drugs is reduced either by lysosomal or P-glycoprotein action [44, 45]. In the next stage of the research, it is planned to investigate the effect of the studied oils in combination with doxorubicin on cell cultures—evaluating the impact of reducing drug exocytosis and increasing the effectiveness of chemotherapy. Of course, an important aspect is the appropriate preparation of a therapy containing plant compounds that can undergo the first-pass metabolism. Therefore, an important aspect is the development of targeted therapies in the treatment of cancer.

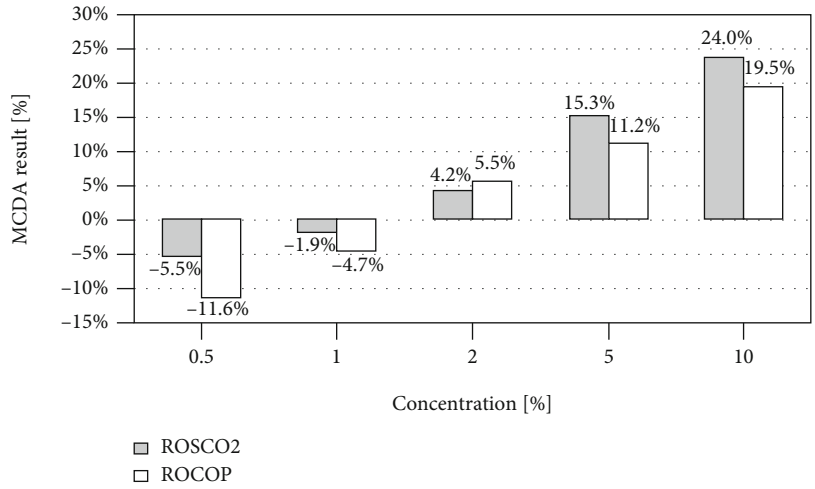

Figure 7: Results of multiple-criteria decision analysis of the collected results of all assays carried out, expressed as the percentage value corresponding to the strength of the positive oil effect; MCDA results greater than zero indicate the expected positive effect on the cancer cell lines.

5. Conclusions

Our study revealed many differences in the response of cell lines to the treatment of raspberry oils obtained by two different methods: extraction by supercritical CO_2 and cold pressed. These data suggest that the way of oil manufacturing is important in terms of chemical characteristics, but what is also essential, may represent a cell suppression potential which may differ regarding the type of tumor. It is also important to conduct further investigations to determine the limitations for some other types of cancer. This study also showed that raspberry oil obtained by SCO2, due to its rich composition, may act in favor of chemoprevention of colon and breast cancers. We also observed that the metabolic activity of colon and breast cancer cell lines may be sensitive to the n-6/n-3 ratio.

Overall, the present study demonstrates the suppressive activity of raspberry oil, and it is the first study to suggest a role of raspberry oil in cancer prevention and/or therapy. The ability to increase the formation of radicals (NO and ROS), especially in doxorubicin cell lines, indicates possible adjunctive therapeutic applications, but this hypothesis needs further clarification.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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