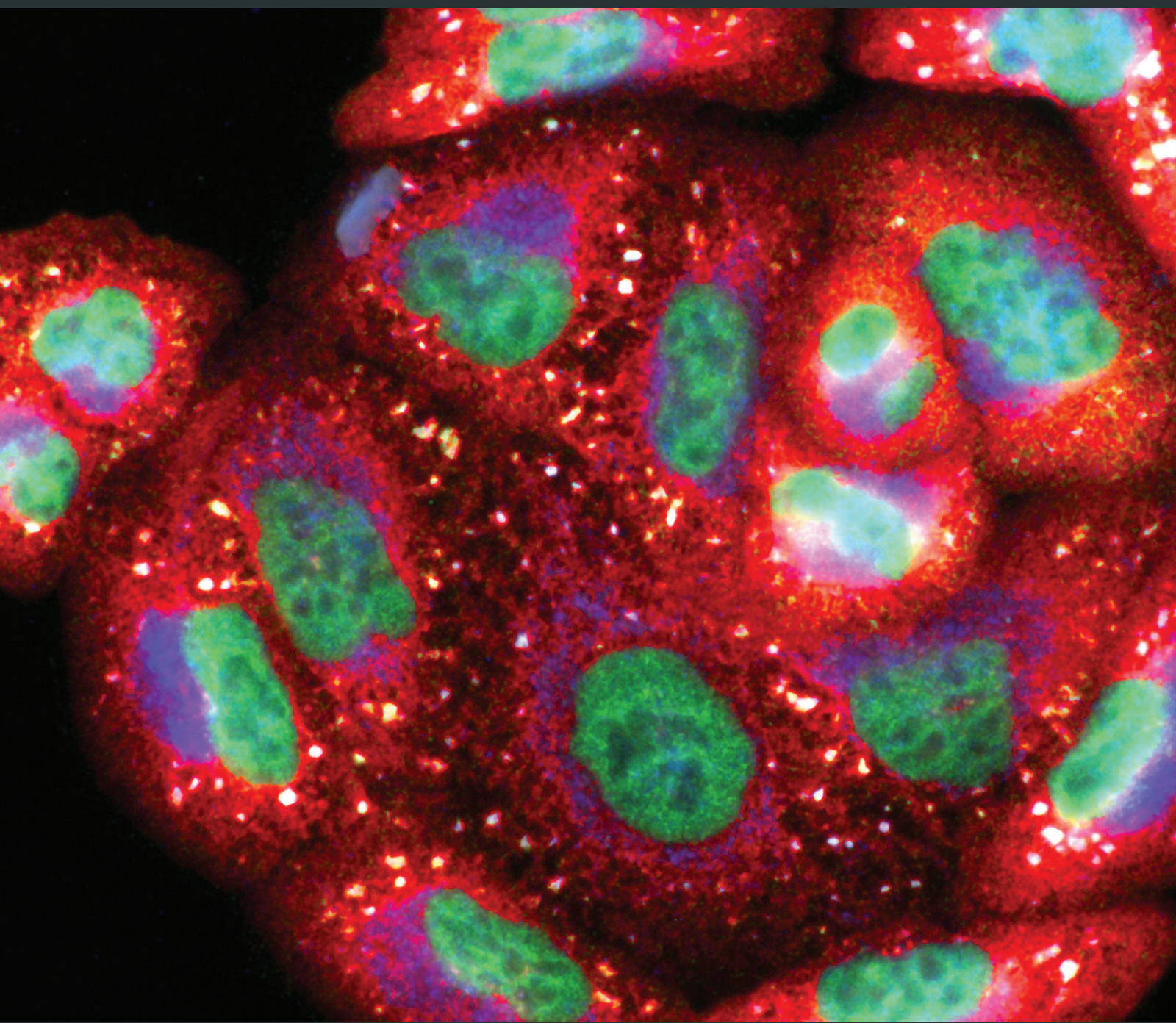
Oxidative Medicine and Cellular Longevity

Medicinal Plants in Therapy: Antioxidant Activities

Guest Editors: Mohamed A. Dkhil, Denis Delic, Hesham A. El Enshasy, and Ahmed E. Abdel Moneim



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Editorial Board

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Editorial

Medicinal Plants in Therapy: Antioxidant Activities

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In view of the oxidative stress implicated in the pathophysiology of a wide variety of different diseases, a therapeutic strategy to elevate the antioxidant defenses of the body may be of assistance in protecting from different diseases and disorders. This special issue includes 19 articles that emphasize the importance of medicinal plants in augmenting oxidative stress in cancer, aging, inflammation, diabetes, and heavy metal toxicity.

Several original and review articles discuss the role of medicinal plants in oxidative stress participating in diseases. T. Esatbeyoglu et al. and H. Qiao et al. open the issue by presenting the antioxidant and anti-inflammatory properties of a stilbenoid-rich root extract of *Vitis vinifera* and polydatin extracted from *Polygonum cuspidatum* in protecting against hydrogen peroxide-induced DNA damage. Q. Wang et al. show the ability of bioactive peptides from *Angelica sinensis* to attenuate aging process in *Caenorhabditis elegans* through antioxidant activities independent of dietary restriction. A. C. F. Salgueiro et al. find that tea prepared from *Bauhinia forficata* can protect from diabetes-induced liver damage and N. Sarega et al. present interesting data on the ability of *Clinacanthus nutans* to attenuate hyperlipidemia-associated oxidative stress in rats. Two other research articles focus on the anticancer effects of caffeic acid phenethyl ester and cocoa beans. D. Bauer et al. discuss the ability of cocoa beans to inhibit cell proliferation, arrest cell cycle in different phases, and increase apoptosis in human lung carcinoma cells. T. K. Motawi et al. show that caffeic acid phenethyl ester augmented tamoxifen cytotoxicity via multiple mechanisms, providing a novel therapeutic approach for breast cancer treatment that can overcome resistance and lower toxicity. In addition, A. Curnow and S. J. Owen examine *Althea officinalis* and *Astragalus membranaceus* extracts against UVA-induced DNA damage in cultured human lung and skin fibroblasts. The authors found that both the plants protected from UVA-induced oxidative stress and DNA damage for a greater period of time than the commercial field-grown roots. H. Khan et al. examined the antiplasmodial effects of 11-O-galloylbergenin isolated from *Mallotus philippensis* and they found that the isolated compound is good enough for its potency and effectiveness against *Plasmodium falciparum*. Furthermore, F. V. S. de Araújo Pinho et al. and J. Coccimiglio et al. studied the potential activities of *Duguetia furfuracea* A. St.-Hil. and *Origanum vulgare*. Finally, H.-R. Choi et al. isolated phlorizin from *Eleutherococcus senticosus* and studied its potential on human keratinocytes and skin equivalents. In this study, the authors found that phlorizin can affect the proliferative potential of epidermal cells in part by microenvironment changes via miR135b downregulation and following increased expression of type IV collagen.

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Research Article

Phytochemical Composition, Antifungal and Antioxidant Activity of *Duguetia furfuracea* A. St.-Hill

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Background. *Duguetia furfuracea* is popular plant used in popular medicine. *Hypothesis/Purpose.* This claim evaluated the phytochemical composition of the hydroethanolic extract (HEDF), fractions of *Duguetia furfuracea*, and antioxidant and antifungal activity. *Methods.* The chemical profile was carried out by HPLC-DAD. The total phenolic contents and flavonoid components were determined by Folin-Ciocalteu and aluminium chloride reaction. The antioxidant activity was measured by scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and ferric reducing ability of plasma (FRAP) methods. The antifungal activity was determined by microdilution assay. Results. HPLC analysis revealed caffeic acid and rutin as major compounds (HEDF), caffeic acid and quercitrin (Mt-OH fraction), and quercitrin and isoquercitrin (Ac-OEt fraction). The highest levels of phenols and total flavonoids were found for Ac-OEt fraction, and the crude extract showed higher *in vitro* antioxidant potential. The antifungal activity showed synergic effect with fluconazole and EHDF against *C. krusei*, fluconazole and Mt-OH against *C. krusei* and *C. tropicalis*, and Ac-OE and fluconazole against *C. albicans*. *Conclusion.* The highest levels of phenols and total flavonoids were marked with antioxidant effect. This is the first report of bioactivity of the synergic effect of HEDF and fractions. More studies would be required to better clarify its mechanism of synergic action.

1. Introduction

The species *Duguetia furfuracea* is a shrub which belongs to the Annonaceae family, being popularly known as "araticum do cerrado," "ata brava." The leaves are popularly used for treatment of rheumatism and renal colic, antihyperlipidemic agent, and anorexic agent [1].

Studies with different structures of *D. furfuracea* have pointed out its cytotoxic, bactericidal, and antitumoral properties. Extracts from leaves and roots of *Duguetia furfuracea* presented larvicide activity against *Aedes aegypti* [2]. Alkaloids from peels of subterranean stem showed antitumor, trypanocide [3], and leishmanicidal activities [4, 5]. Extracts from subterranean parts of the plant displayed toxic effect to *Artemia salina* [4]. Our group demonstrated recently the prooxidant and insecticidal activity of the hydroalcoholic extract from leaves of *D. furfuracea* in *Drosophila melanogaster* [6]. Studies regarding antifungal and antioxidant properties of *Duguetia furfuracea* are scarce; thus the present work contributes to amplifying the knowledge about this species.

The antioxidant potential of the plants has been associated mainly with the presence of phenolic compounds. The chemical structure and reductive properties of these compounds make them active molecules in the scavenging of free radicals and in chelation of transition metals [7]. Secondary metabolites are substances with a generally complex structure playing several roles in the adaptation of plants to the environment; these substances have been attracting interest for their pharmacological and biotechnological properties such as antioxidant [8], anti-inflammatory [9], antimutagenic, anticarcinogenic, gastroprotection [10–12], and antimicrobial [13] ones.

The occurrence of fungal infections is increasing at alarming rates, especially among immunocompromised subjects, such as AIDS patients, transplanted patients, and neonates [14]. Among the pathogens, species of *Candida* are generally associated with these infections, whose incidence is attributed to a variety of factors, either exogenous or endogenous. More than 100 species of *Candida* are known and the frequency of distribution for *Candida spp.* varies in accordance with geographical location [15, 16].

Actually, conventional treatments for fungal infections are not fully effective, since the available drugs lead to secondary effects or to development of resistance [17]. Therefore, the search for new drugs and alternative therapies (including natural products) for the treatment of *Candida* infections has become critical. In this aspect, plants and their derivatives have been contributing to pharmacological research due to their potential as a source for a variety of biologically active ingredients used in drug development. The antimicrobial activity of plants has been identified in some species; however, it should be taken into consideration that besides its beneficial effects, the use of plants may interfere with conventional treatments by interaction with drugs, thus potentiating or minimizing clinical efficacy [18].

Our aim of the present study was to describe the phytochemical characterization, *in vitro* antioxidant potential and to evaluate, for the first time, the antifungal and/or modulatory activity of the hydroalcoholic extract, methanolic and ethyl acetate fractions of *Duguetia furfuracea*.

2. Materials and Methods

2.1. Drugs, Reagents, and Equipment. Sabouraud Dextrose Agar (semisolid) and Sabouraud Dextrose Broth were from Difco Laboratories (Michigan, MI, USA). Dimethyl sulfoxide (DMSO), acetonitrile, and formic, gallic, chlorogenic, ellagic, and caffeic acids were purchased from Merck (Darmstadt, Germany). Antifungal agents fluconazole and nistatina, catechin, quercetin, quercitrin, isoquercitrin, rutin, kaempferol, ethanol, methyl alcohol, hexane, ethyl acetate, Folin-Ciocalteu Reagent, gallic acid, sodium carbonate, aluminium chloride, quercetin, DPPH, ascorbic acid, sodium acetate, TPTZ, ferric chloride, and ferrous sulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with the system of HLPC (Shimadzu, Kyoto, Japan), autosampler prominence (SIL-20A), equipped with high-pressure plunger pumps LC-20AT Shimadzu connected to DGU degasser 20A5 with integrator CBM 20A, UV-VIS detector DAD (diode) SPD-M20A, and software solution LC 1,22 SP1. The absorbance measurements were obtained using EnSpire® multimode plate reader (PerkinElmer, USA). All chemical products were of the highest analytical grade.

2.2. Collection of Plant Material. Leaves of *D. furfuracea* were collected from Barreiro Grande, Crato, Ceará (7°22′2.8″S, 39°28′42.4″W and altitude of 892 m above sea level), Brazil, in September 2011 and identified by Dr. Maria Arlene Pessoa da Silva. A voucher specimen (n. 6703) was deposited in the Herbarium Caririense Dárdano de Andrade Lima (HCDAL) of the Regional University of Cariri (URCA).

2.3. Preparation of the Hydroalcoholic Extract, Methanolic and Acetate Fractions. Leaves (1.050 g) of *D. furfuracea* were washed in running water, crushed, and put into glass flasks containing hydroalcoholic solution of extraction (99,8% of ethanol in distilled water) in the proportion of 1 : 1, for three days. The suspension was filtered, solvent evaporated under reduced pressure, and lyophilized to obtain 261.13 g of crude ethanolic extract. 80 g of this was partitioned with ethyl acetate and methanol to obtain g of 2.28 ethyl acetate fraction (EAF) and 75.6 g of methanolic fraction [19]. The procedure yielded 24.87% for HEDF, 94.5% for methanolic fraction (Mt-OH), and 3.35% for the ethyl acetate (Ac-OEt) fraction.

2.4. Identification and Quantitation of Phenolic Compounds of HEDF by HPLC. Mt-OH and Ac-OEt fractions were submitted to the chromatographic analysis of reversal phase. The chemical composition of the HEDF was previously determined by our group [6] using the same procedure described in this section.

The chromatographic analyses were performed under the same gradient conditions using the column C18 (4.6 mm × 250 mm) charged with particles of 5 μm of diameter. The mobile phase was water containing 1% of formic acid (A), acetonitrile (B) and the composition gradient was 13% of B

until 10 minutes and changed to obtain 20%, 30%, 50%, 60%, 70%, 20%, and 10% of B at 20, 30, 40, 50, 60, 70, and 80 minutes, respectively, according to Boligon et al. [20] with some modifications. Fractions were analyzed at a concentration of 5 mg/mL. The presence of ten antioxidant compounds was investigated: gallic acid, chlorogenic acid, ellagic acid, caffeic acid, catechin, quercetin, quercitrin, isoquercitrin, rutin, and kaempferol. The identification of these compounds was performed by comparing their retention time and the UV radiation absorption spectral to the commercial standards. The flow rate was 0.7 mL/min, injection volume 40 μL, and the wavelength 254 nm to the gallic acid, 280 nm to the catechin, 325 nm to caffeic acid, ellagic acid, and chlorogenic acid, and 365 nm to quercetin, quercitrin, isoquercitrin, rutin, and kaempferol. All the samples and the mobile phase were filtered through a membrane filter of 0.45 μm (Millipore) and, after this, they were degassed with ultrasonic bath before the use. The solutions of the standards of reference were prepared in a mobile phase of HPLC in a range of concentrations from 0.030 to 0.250 mg/mL to kaempferol, quercetin, quercitrin, isoquercitrin, rutin, and catechin and from 0.030 to 0.250 mg/mL to gallic acid, caffeic acid, ellagic acid, and chlorogenic acid. The peaks of chromatography were confirmed by comparing their retention time to the standards of reference and by the spectral of DAD (200 a 400 nm). All the operations of chromatography were performed at room temperature and in triplicate. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated on the basis of the standard deviation of the responses and of the inclination of three analytical independent curves. LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve [20].

2.5. Determination of Total Phenols. The quantification of phenolic compounds was performed using the Folin-Ciocalteu method that involves the reduction of the reagent by phenolic compounds present in the samples forming a blue complex whose intensity increases linearly at 760 nm, as described by Swain and Hillis (1959) [21]. For the assays, 4 μL of samples (HEDF, Mt-OH, and Ac-OEt) at a concentration of 100 μg/mL to an incubation medium consisting of 0.1 N Folin-Ciocalteu reagent and 1.25% (w/v) Na_2CO_3 in a final volume of 284 μL. After two hours of incubation in the dark, at room temperature, the absorbance was measured at 760 nm. The experiments were carried out in triplicate. The index of total phenolic compounds was expressed as equivalents of gallic acid *per* gram of the sample (mg GAEq/g), calculated through a curve of gallic acid, built with concentrations ranging from 50 to 500 μg/mL.

2.6. Determination of Total Flavonoids. The quantification of flavonoids was made according to Quettier-Deleu et al. 2000 [22]. The method is based on the measurement of absorbance, at 415 nm, of the complex formed between flavonoid compounds and aluminum cation in ethanol. For the assays, samples were incubated with $AlCl_3$ (2%) in a 1 : 1 reaction. The final volume of the reaction medium was 300 μL. The experiments were carried out in triplicate. The index of total flavonoids was expressed as equivalents of quercetin *per* gram of the sample (mg QEq/g), calculated through a curve of quercetin, built with concentrations ranging from 0.625 to 25 μg/mL.

2.7. Antioxidant Activity

2.7.1. Scavenging Activity of the DPPH Radical. The antioxidant activity of the extract and fractions was also checked by the DPPH method as described by Brand-Williams et al. (1995) elsewhere [23] with minor changes. This test is based on the reduction of the stable free radical DPPH which presents a deep violet color in solution and turns to a yellowish color when neutralized. The mixture of the reaction was composed of 50 μL of samples (extract and fractions), 50 μL of solvent, and 100 μL of solution and 0.3 mM of the radical DPPH in ethanol. The measurement of the absorbance was at 517 nm after 30 minutes. The samples (HEDF, Mt-OH, and Ac-OEt) were diluted in ethanol and water (1 : 1) and the standard substance, ascorbic acid, was diluted in water. All tests were made in triplicate. Results were expressed as IC_{50} values defined as the concentration of antioxidant required to sequester 50% of the DPPH radicals. IC_{50} was calculated by nonlinear regression.

2.7.2. FRAP (Ferric Reducing Antioxidant Power). The FRAP assay was conducted as described previously by Benzie and Strain (1996) [24]. FRAP solution consisted of 10 mM TPTZ, 20 mM ferric chloride in acetate buffer 0.3. In 96-well microtiter plate, we added 9 μL of the samples (HEDF, Mt-OH, and Ac-OEt); 27 μL of water; and 270 μL of the FRAP solution. After incubation at 37°C for 30 minutes absorbance was taken at 595 nm. Samples readings were compared to a ferrous sulfate II standard curve. Results were expressed as μM ferrous sulfate II ($FeSO_4$) equivalents *per* gram of sample. Experiments were done in triplicate.

2.8. Antifungal Activity

2.8.1. Culture Medium and Inoculums. The antifungal activity was evaluated using the standard fungal strains *C. albicans* (ATCC 40277), *C. krusei* (ATCC 6438), and *C. tropicalis* (ATCC 40042) donated by the Universidade Estadual da Paraíba. In the biological tests, we used the following culture medium: Sabouraud Dextrose Agar (semisolid) and Sabouraud Dextrose Broth (liquid) prepared according to the manufacturer's specifications. Fungal cultures kept at 4°C were transported to the Sabouraud Dextrose Agar medium and incubated at 35°C for 24 hours. As to the preparation of the inoculum, the pricked out strains were transferred to the sterile saline solution (0.9% NaCl), composing of a fungal suspension (inoculum) until obtaining the concentration of 10^5 UFC/mL according to the scale of McFarland [25].

2.8.2. Minimum Inhibitory Concentration Test: CIM and the Modulation of Standard Antifungal Action. The method of microdilution in sauce was used to determine the minimum inhibitory concentration (CIM). The samples (HEDF, Mt-OH, and Ac-OEt) were weighed and solubilized initially in

Table 1: Quantification of phenolic compounds of HEDF. Me-OH and Ac-OEt.

Compounds	HEDF (modified from [6]) mg/g	Me-OH fraction mg/g	Ac-OEt fraction mg/g	LOD μg/mL	LOQ μg/mL
Gallic acid	5.29 ± 0.01	5.47 ± 0.03	9.85 ± 0.01	0.015	0.049
Catechin	5.31 ± 0.01	2.69 ± 0.01	3.16 ± 0.02	0.032	0.105
Chlorogenic acid	16.03 ± 0.02	7.18 ± 0.01	25.78 ± 0.01	0.009	0.029
Caffeic acid	33.17 ± 0.03	32.47 ± 0.03	21.90 ± 0.03	0.024	0.078
Ellagic acid	7.30 ± 0.01	7.25 ± 0.01	11.17 ± 0.01	0.013	0.042
Rutin	20.05 ± 0.01	19.67 ± 0.02	5.49 ± 0.02	0.027	0.090
Isoquercitrin	18.61 ± 0.01	14.83 ± 0.01	31.56 ± 0.01	0.008	0.026
Quercitrin	19.07 ± 0.02	31.96 ± 0.03	32.97 ± 0.037	0.035	0.114
Quercetin	5.87 ± 0.01	6.95 ± 0.01	18.73 ± 0.01	0.019	0.063
Kaempferol	5.36 ± 0.01	6.91 ± 0.02	20.98 ± 0.02	0.026	0.085

Results are expressed as mean ± SE of three determinations. LOD: limit of detection. LOQ: limit of quantification.

dimethyl sulfoxide (DMSO) and diluted at 1024 μg/mL using sterile distilled water (test solution).

We distributed 100 μL of inocula, prepared previously, in each cavity of a 96-well microtiter plate and, thereafter, it was submitted to a serial double dilution using 100 μL of the samples with concentrations that range from 1024 to 0.5 μg/mL. The plates were transported to the incubator for 24 hours at 35°C [26]. The identification of CIM was performed through the visual observation of the turbidity provoked by the fungal growth, with the CIM being defined as the lowest concentration of the sample in which no fungal growth was observed [25].

To observe how these samples could affect the action of the standard antifungal agents against the strains tested, we used the method proposed by [27]. The extract and the fractions were tested using a subinhibitory concentration (MIC/8 = 64 μg/mL). We distributed, in each well, 100 μL of solution containing 1.675 μL of liquid medium (Sabouraud Dextrose Broth) 10%; 200 μL of inoculum (fungal suspension); and 125 μL of the natural product (extract and fractions). After that, 100 μL of the antifungal agents was added to the first cavity and following the serial dilution along the other cavities. The concentrations of the antifungal agents ranged from 1024 to 0.5 μg/mL. The tests were performed in triplicate.

2.9. Statistical Analysis. The results of the tests were done in triplicate and expressed as geometric mean [28]. Statistical differences between samples were tested by analysis of variance ANOVA followed by Tukey's or Dunnett's *post hoc* test when necessary. The differences were considered significant when $P < 0.05$.

3. Results and Discussion

3.1. Identification and Quantification of Phenolic Compounds by HPLC. The chromatographic and spectral profile of Mt-OH and Ac-OEt fractions revealed the presence of gallic acid (t_R = 9.95 min; peak 1), catechin (t_R = 16.08 min; peak 2), chlorogenic acid (t_R = 20.14 min; peak 3), caffeic acid (t_R = 24.63 min; peak 4), ellagic acid (t_R = 37.29 min; peak 5); rutin (t_R = 39.87 min; peak 6); isoquercitrin (t_R = 44.93 min; peak 7); quercitrin (t_R = 48.15 min; peak 8); quercetin (t_R = 51.07 min; peak 9); and kaempferol (t_R = 61.56 min; peak 10).

Table 2: Total phenolic contents and flavonoids present in extract and fractions of *D. furfuracea*.

Samples	Total phenolic contents mg of GEA/g of the sample	Total flavonoids mg of EQ/g of the sample
HEDF	231.26 ± 1.15[a]	76.26 ± 2.73[a]
Mt-OH	289.33 ± 1.22[b]	87.57 ± 2.48[b]
Ac-OEt	657.05 ± 6.33[c]	120.9 ± 2.53[c]

The values were expressed as mean ± SD (n = 3); EAC: equivalent of gallic acid; EQ: equivalent of quercetin. Averages followed by different letters differ by Tukey's test at $P < 0.05$.

The main compounds present in the Mt-OH fraction were caffeic acid (32.47 ± 0.03 mg/g) and quercitrin (31.96 ± 0.03 mg/g) while catechin (2.69 ± 0.01 mg/g) and the gallic acid (5.47 ± 0.03 mg/g) were the least abundant. In the Ac-OEt fraction, major compounds were quercitrin (32.97 ± 0.037 mg/g) and isoquercitrin (31.56 ± 0.01 mg/g) while catechin (3.16 ± 0.02 mg/g) and rutin (5.49 ± 0.02 mg/g) were the least present (Figure 1 and Table 1). The chromatographic profile of HEDF demonstrated the presence of caffeic acid and rutin as major compounds (33.17 ± 0.03 mg/g and 20.05 ± 0.01 mg/g, resp.) while gallic acid (5.29 ± 0.01 mg/g) and catechin (5.31 ± 0.01 mg/g) were the least abundant [6].

The determination of total phenols and flavonoids is showed in Table 2. The content of total phenols and flavonoids was higher in Ac-OEt fraction (657.05 mg/EAG/g and 120.9 mg EQ/g, resp.), followed by Mt-OH fraction (289.33 mg/EAG/g and 76.26 mg EQ/g, resp.) and HEDF (231.26 mg EAG/g and 87.57 mg EQ/g, resp.). It is recognized that flavonoids are preferably extracted by ethyl acetate solvent.

The *in vitro* antioxidant potential of crude extract (HEDF) and fractions of *D. furfuracea* was evaluated by two different methods, FRAP, which measures the ferric reducing antioxidant power of compounds, and ability of sequestering

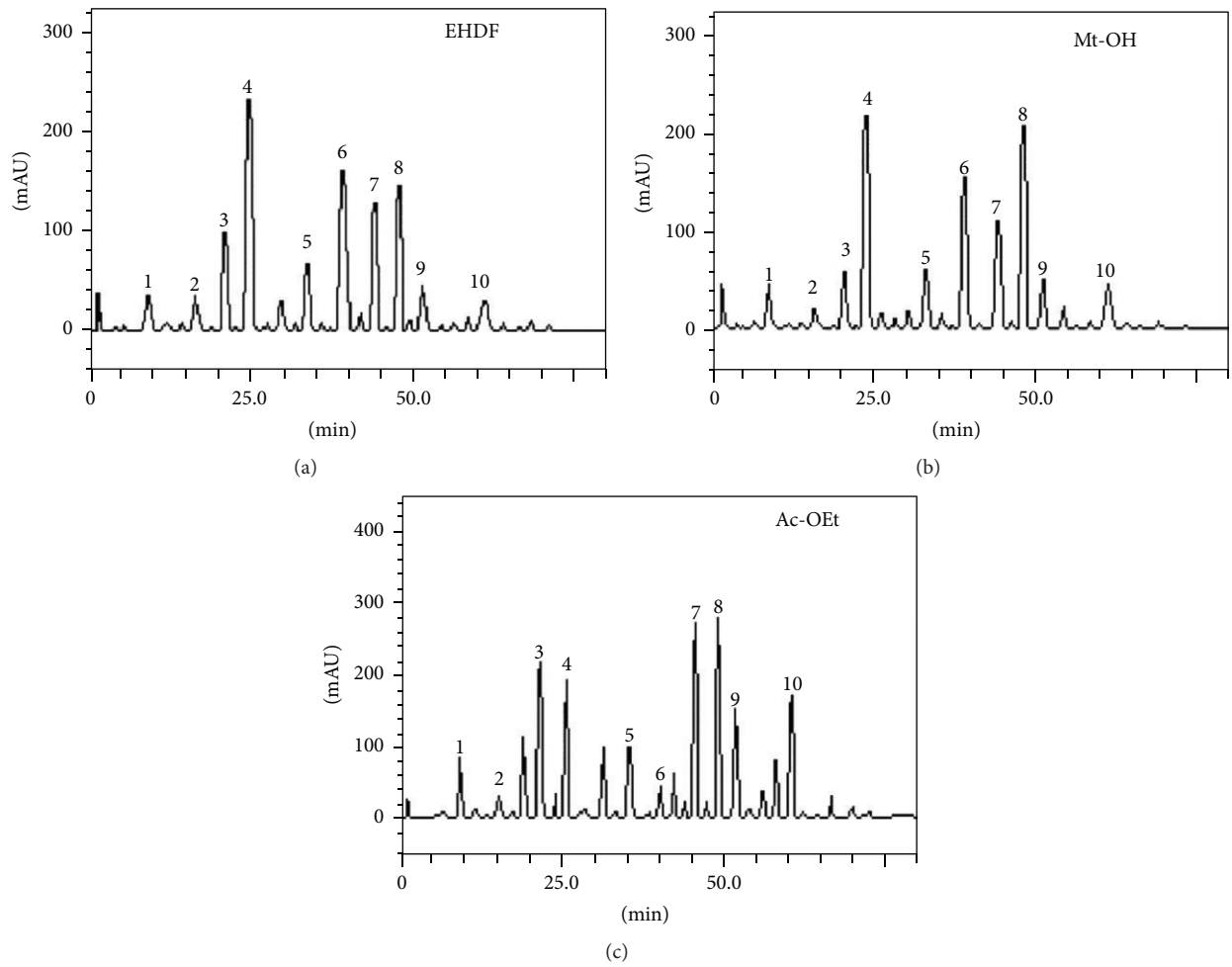

Figure 1: Elution profiles of high performance liquid chromatography analysis: (a) HEDF (modified from [6]), (b) methanolic fraction (Mt-OH) and (c) ethyl acetate (Ac-OEt). Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), rutin (peak 6), isoquercitrin (peak 7), quercitrin (peak 8), quercetin (peak 9), and kaempferol (peak 10). Calibration curve of the gallic acid: $Y = 14286x + 1395.8$ ($r = 0.9996$); catechin: $Y = 15097x + 1189.3$ ($r = 0.9997$); caffeic acid: $Y = 12758x + 1259.7$ ($r = 0.9996$); chlorogenic acid: $Y = 13461x + 1275.3$ ($r = 0.9992$); ellagic acid: $Y = 13576x + 1346.4$ ($r = 0.9999$); rutin: $Y = 12845 + 1305.7$ ($r = 0.9999$); quercetin: $Y = 13560x + 1192.6$ ($r = 0.9991$); isoquercitrin: $Y = 12873x + 1325.6$ ($r = 0.9998$); quercitrin: $Y = 11870x + 1329.8$ ($r = 0.9993$); and kaempferol: $Y = 14253x + 1238.9$ ($r = 0.9997$).

the synthetic radical DPPH. The crude extract (HEDF) presented the best ferric reducing potential ($166.73 \pm 5.13\ \mu$M of Fe^{+2}/g of sample), followed by Mt-OH ($126.43 \pm 4.98\ \mu$M of Fe^{+2}/g of sample) and Ac-OEt fractions ($118.20 \pm 1.08\ \mu$M of Fe^{+2}/g of sample) (Table 3). The potential of scavenging of radical DPPH was expressed as IC_{50} in μg/mL of extract or fractions and compared with the positive control ascorbic acid. HEDF presented the better antioxidant activity in the DPPH test with IC_{50} values of 33.15 μg/mL when compared to Ac-OEt (39.32 μg/mL) and Mt-OH (42.32 μg/mL).

In this study, the antifungal potential of the hydroalcoholic extract of *D. furfuracea* (HEDF) and methanolic (Mt-OH) and ethyl acetate (Ac-OEt) fractions was tested against standard strains of *C. albicans*, *C. tropicalis*, and *C. krusei*. According to the results, both extract and fractions presented minimal inhibitory concentration (CIM) $\geq$1024 μg/mL against all the fungi strains tested. However, the extract and the fractions of *D. furfuracea* presented synergic effect when they were associated with fluconazole, indicating its modulatory action against fungi when associated with clinically relevant drugs. The HEDF and Mt-OH fraction potentialized the effect of the fluconazole when tested against *C. krusei* as observed in Figures 2(a) and 2(b). The methanolic fraction also presented synergism with fluconazole against *C. tropicalis* (Figure 2(b)) and Ac-OEt fraction potentialized the effect of fluconazole against *C. albicans* (Figure 2(c)).

4. Discussion

Phenolic compounds and some of their derivatives are known by their antioxidant properties. The antioxidant activity of some medicinal plants is correlated to the total phenolic and flavonoids indexes [29]. The level of total phenols for *D. furfuracea* extract and fractions is comparable with

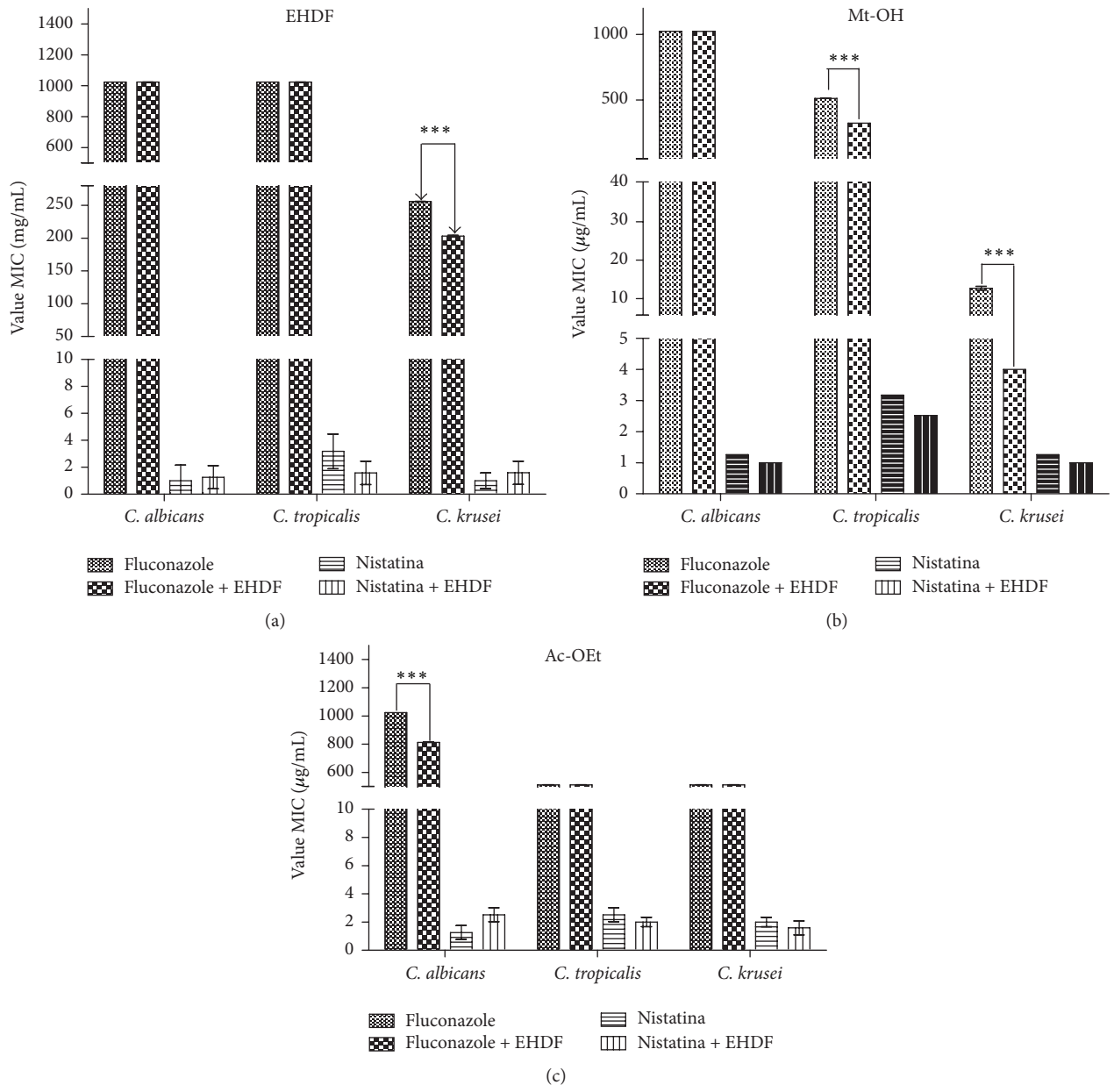

Figure 2: Modulatory activity of (a) HEDF (hydroalcoholic extract of *D. furfuracea*), Mt-OH, and Ac-OET *against the fungi C. albicans*, *C. krusei*, and *C. tropicalis* (concentrations ranging from 1024 μg/mL to 0.5 μg/mL). $P < 0.001$ related to control group; (b) Mt-OH (methanolic extract of *D. furfuracea*) against the fungi *C. albicans*, *C. krusei*, and *C. tropicalis* (concentrations ranging from 1024 μg/mL to 0.5 μg/mL); (c) Ac-OEt (ethyl acetate extract of *D. furfuracea*) against the fungi *C. albicans*, *C. krusei*, and *C. tropicalis* (concentrations ranging from 1024 μg/mL to 0.5 μg/mL). Statistical analysis: one-way ANOVA followed by Student-Newman-Keuls test. $^{***}P < 0.001$ versus fluconazole.

other Brazilian medicinal plants of *Duguetia* genus [30, 31]. Although the total index of phenols and flavonoids has been higher in the fractions than in crude extract, it is possible to connect these compounds with *in vitro* antioxidant activity, as determined by FRAP and DPPH methods. Though the *in vitro* antioxidant activity was higher in the crude extract than the fractions, this analysis suggests that other compounds in the crude extract of *D. furfuracea* contribute to its more effective antioxidant activity [32]. In a previously published study by our group, the crude extract (HEDF) was demonstrated to present alkaloids in its phytochemical constitution [6]. In this work we can see a direct correlation between the concentration of flavonoids and antioxidant FRAP activity ($r = -0.801$). Those results were confirmed with others results present in literature [33–35].

The primary mechanism of fluconazole's action occurs by the inhibition of the fungal enzyme lanosterol 14α-demethylase (CYP51), which is a cytochrome enzyme P-450, involved in the synthesis of the ergosterol, the most important sterol in the fungal cell membrane [36]. It is known that many medicinal plants may modulate the activity of several antimicrobial agents [37, 38]. In a previous study, the aqueous extract of

Table 3: Antioxidant activity of HEDF and fractions of *D. furfuracea*.

Samples	Sequestering of the radical DPPH EC_{50} (μg/mL)	FRAP μM Fe^{+2}/g of the sample
HEDF	33.15[b]	166.73 ± 5.13[a]
Mt-OH	42.32[c]	126.43 ± 4.98[b]
Ac-OEt	39.32[c]	118.20 ± 1.08[b]
Asc. Ac.	17.50[a]	—

The values were expressed as mean ± SD ($n = 3$); EFe^{+2} = equivalent of iron; HEDF (hydroalcoholic extract of *D. furfuracea*); Mt-OH (methanolic fraction); Ac-OEt (ethyl acetate fraction); and Asc. Ac. (ascorbic acid). Results are expressed as mean ± SEM ($n = 3$). Averages followed by different letters differ by Tukey's test at $P < 0.05$.

the leaves and fractions of *D. furfuracea* when combined with aminoglycosides presented synergic activity against *Escherichia coli* and *Staphylococcus aureus* [39].

Phenolic compounds and flavonoids have demonstrated potential therapeutic activities as antifungal, antibacterial, and antioxidant agents [40, 41]. Although the mechanisms underlying antimicrobial pharmacology of the phenolic compounds are rather variable, many of them act by promoting damage to the function of the cell membrane or cell wall [42]. The analysis by HPLC of the extract and fractions of *D. furfuracea*, as described previously, revealed the predominance of the following compounds: caffeic acid, chlorogenic acid, rutin, quercitrin, and isoquercitrin. There is a study that demonstrated that chlorogenic acid presented antifungal activity against the yeast of the gender *Candida* [43]. Six flavonoids that were isolated from plants, among them, rutin, presented antibacterial and antifungal activity [40].

Sun et al. (2004) showed the influence of phenolic compounds in fluconazole antifungal properties. This paper shows that the concentration of fluconazole in *C. albicans* was found to be increased with the increment of the phenolic compounds concentration when they were in combination. This result corroborated synergetic activity present in this work [44].

It is possible to speculate that some of these chemical constituents, especially the flavonoids, are responsible for the pharmacological properties found. However, the isolation and the activity of alkaloids and acetogenines have stood out in studies with plants of the Annonaceae family. The biological activity as antimicrobial capacity and antioxidant activity present in *A. muricata* can be attributed to the presence of acetogenines [37, 45]. Alkaloids as aphorphinoids present the bark of *Annona salzmannii* D. C. was responsible for the antioxidant and antimicrobial capacity [46].

5. Conclusion

We can conclude that the crude extract of *D. furfuracea* (HEDF) and its methanolic (Mt-OH) and ethyl acetate (Ac-OEt) fractions have an important antioxidant activity (*in vitro*) when compared to other natural compounds. The crude extract (HEDF) presented highest antioxidant activity *in vitro* when compared to Mt-OH and Ac-OEt, as determined by DPPH and FRAP methods. However, it was not possible to observe a positive correlation between the antioxidant activity and the total index of phenols and flavonoids identified, indicating that compounds other than phenolics may contribute to the antioxidant potential of the plant extracts. The crude extract and fractions of *D. furfuracea* presented a synergistic activity with fluconazole when tested against strains of *C. albicans, C. tropicalis, and C. krusei*, indicating a potential antifungal activity via modulation of clinically used drugs against fungal infections. More studies are needed to clarify the mechanisms involved in this phenomenon as well as other potential biomedical and biotechnological applications of *D. furfuracea*.

Abbreviations

HEDF: Hydroethanolic extract of *Duguetia furfuracea*
Mt-OH: Methanolic fractions of *Duguetia furfuracea*
Ac-OEt: Acetate fractions of *Duguetia furfuracea*
HPLC DAD: High performance liquid chromatography with diode array
DPPH: 2,2-Diphenyl-1-picrylhydrazyl
FRAP: Ferric reducing antioxidant power.

Competing Interests

All authors wish to confirm that there is no known conflict of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Research Article

Antioxidant Activity and Cytotoxicity Effect of Cocoa Beans Subjected to Different Processing Conditions in Human Lung Carcinoma Cells

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Lung cancer is a common malignancy in men and the second leading cause of cancer-related mortality in men in the western world. Phenolic cocoa ingredients have a strong antioxidative activity and the potential to have a protective effect against cancer. In the present study, we have evaluated the influence of cocoa beans subjected to different processing conditions on cell viability and apoptosis of human lung cancer cells (A549). We measured the viability of lung cells treated with cocoa beans, unroasted slates (US), roasted slates (RS), unroasted well fermented (UWF) cocoa, and roasted well fermented (RWF) cocoa for 24 h. Using an MTT assay, we observed a decrease in the viability of A549 cells after treatment with cocoa bean extracts. Flow cytometer analysis revealed that cocoa beans increased the percentage of cells in sub-G_1 phase and promoted up to twofold increase of apoptotic cells when compared to the control group. Taken together, the present study suggests that cocoa beans may have a protective effect against lung cancer.

1. Introduction

Lung cancer is a major health concern since it is one of the leading causes of death worldwide [1, 2]. It is estimated that the annual incidence rate is nearly 1.23 million. In Brazil, it is the second most frequent type of cancer and it has the highest mortality rate, due to late diagnosis and the aggressiveness of the tumor type. In 80–90% of cases, it is caused by smoking and exposure to pollutants. The existing therapeutic strategies for cell lung cancer include surgery, radiotherapy, chemotherapy, and physical therapy. The survival rate of non-small-cell lung cancer patients is less than 1% [3, 4].

The proposals that cancer might be preventable, and that food and nutrition might influence the risk of cancer, were first made in the 19th and 20th centuries. Throughout recorded history, wise choices of food and drink, and of habitual behavior, have been recommended as a protective measure against cancer [5]. Researchers are still unsure about the role of diet in lung cancer. Bright yellow-orange beta-carotene is one of a number of carotenoids thought to have anticancer activity even greater than vitamin A. Other possible lung protectors are foods high in bioactive compounds such as vitamin C and other antioxidants present in fruits and vegetables. These nutrients may protect lung linings but cannot totally prevent damage [6].

Among the different bioactive compounds, phenolic compounds from fruits and vegetables have gained much attention over the years because of their antioxidant activity that indirectly reflects their potential effects on human health [7, 8]. Some studies reported that phenolic compounds found

in cocoa beans may present different properties such as antioxidant, anticarcinogenic, and antiradical activities [9–11]. Polyphenols are the main antioxidant-active constituents of cocoa. Flavanols and procyanidins have previously been identified as the active antioxidant agents of cocoa [12]. The polyphenol content of cocoa products depends on many factors, especially the cultivated variety and the postharvest handling that includes fermentation, drying, and roasting of the beans and nibs. There is evidence that fermentation and roasting of the beans tend to reduce their flavanol content [13, 14]. The antioxidant activity of the phenolic compounds is primarily due to their redox properties that allow them to act as reducing agents, hydrogen donors, and scavengers of reactive oxygen species (ROS) and metal ions [8, 15–17]. The emergence of natural extracts with antioxidant properties may help reduce the current dependence on synthetic drugs.

Potential mechanisms for cancer prevention of bioactive compounds include prevention of DNA adduct formation enhanced carcinogen elimination, inhibition of inflammatory processes, and a direct cytotoxic effect on tumour cells [18–20]. In line with efforts to balance the conservation of biodiversity and encourage the controlled exploitation of plant resources for economic gain, especially in biopharming, waste of valuable resources should be minimized [17]. The aim of this study was to evaluate and compare the antioxidant activity of cocoa beans classified as slate, roasted, and unroasted with well fermented beans submitted to the same processing and their cytotoxic effects on human lung carcinoma cell line (A549).

2. Methods

2.1. Samples and Extractions. Samples of cocoa beans, unroasted slates (US), roasted slates (RS), unroasted well fermented (UWF) cocoa, and roasted well fermented (RWF) cocoa were harvested and preprocessed (fermented and dried) in the cocoa producing region of Ilhéus (Bahia, Brazil). These samples were classified according to their fermentation status and donated by a company of the cocoa sector from the same region. At least three different lots of each cocoa class were mixed to form the samples used in this study. The roasting as well as the fine grinding of all beans was carried out in the State University of Feira de Santana (BA). All samples were kept at −5°C and sent to the Functional Foods and Biotechnology Laboratory of the Federal University of Rio de Janeiro State (UNIRIO), where all analyses were conducted.

2.2. Extraction of Samples. The samples of cocoa were extracted with 3 different solution extractors: methanol (I), methanol 50% (II), and methanol 50% : acetone 70% (1 : 1) (III). 1.25 g of sample was weighed and suspended in 10 mL of extracting solution for 1 hour under stirring, protected from light. The crude extracts were filtered and completed in 25 mL with distilled water. For cellular analysis, 1.25 g of each sample was weighed and suspended in saline solution (PBS) at 2% dimethyl sulfoxide (DMSO).

2.3. Total Phenolic Assay. Total phenolic content of the extracts was determined according to the Folin-Ciocalteu method as described by Singleton and Rossi [21] with minor modifications. Aliquots of 0.5 mL of the extracts were added to 2.5 mL of Folin-Ciocalteu reagent and 2.0 mL of 4% sodium carbonate solution and the mixture was allowed to rest for 2 hours in the dark. Measurements were performed at 750 nm in triplicates, applying a Turner® 340 spectrophotometer. Gallic acid, in the concentration range of 0–100 mg/mL^{-1}, was used to construct a calibration curve. The concentration of total phenolic compounds in the extract was expressed as gallic acid equivalents, which reflect the phenolic content as the amount of gallic acid in mg/100 g dry weight of the samples.

2.4. Antioxidant Activity Analyses

2.4.1. DPPH Assay. Aliquots of 0.5 mL of the extracts were mixed with 2.5 mL DPPH methanolic solution (0.06 mM) and allowed to react for 1 hour, in the dark. Measurements were performed at 515 nm applying a Turner 340 spectrophotometer. The analysis was performed in triplicates; the decline in the DPPH radical absorbance concentration caused by the extracts was compared to a trolox standard. The results were expressed as μmol trolox equivalents/g dry basis. [22].

2.4.2. Trolox Equivalent Antioxidant Capacity (ABTS/TEAC). The TEAC$^{\bullet+}$ cation was prepared by mixing a TEAC stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was allowed to stand for 16 hours at room temperature until the reaction was completed and the absorbance was stable.

The antioxidant capacity assay was carried out by the improved ABTS/TEAC method as described by Rufino et al. [23]. TEAC solution (2.5 mL) was added to extracts or commercial antioxidant (trolox) and mixed thoroughly. Absorbance was recorded at 734 nm during 6 min. Aliquots of 5, 10, and 20 μL of the extracts were tested and their volume was completed to 0.5 mL with water. Results were expressed as μmol trolox/g dry basis.

2.4.3. Ferric Reducing Ability (FRAP). The extracts were measured for antioxidant activity by FRAP according to Rufino et al. [24]. Aliquots of 2.7 mL of TPTZ reagent (ferric 2,4,6-tripyridyl-s-triazine) were mixed with 0.5 mL of sample extract (aliquots 5, 10, and 20 μL). After 30 min at 37°C temperature, the absorbance was read at 595 nm. The antioxidant capacity (FRAP) was expressed as Fe^{3+} equivalents (μmol Fe^{3+}/g dry basis).

2.4.4. Cell Culture and Treatment Protocol. Cell lines were obtained from the Rio de Janeiro Cell Bank which certified their identity and quality (INMETRO, Rio de Janeiro, RJ, Brazil). Human lung carcinoma cell line (A549) was plated in 25 cm^2 tissue culture flasks (5.0×10^6 cells/flask) and maintained routinely in Dulbecco's Modified Eagle's Medium-high glucose (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin (PS), pH 7.4, under 5% CO_2

atmosphere. Stock flasks were grown to 70% confluence and subcultured routinely. Medium renewal was done 3 times weekly. For each experiment, cells were seeded at 3.5×10^5 cells/cm^2 and 2×10^4 cells/cm^2 densities in 6-well plates and 96-well plates for cell cycle and cell proliferation analyses, respectively. After 24 h, medium was removed and cells were treated with increasing concentrations of cocoa nibs extract (100 to 10000 μg/mL) dissolved in DMEM. The controls, DMEM and DMEM + 2% DMSO, were included on each plate. The cells were then incubated for 48 hours.

2.4.5. Cell Viability. Cell viability was monitored by MTT assay (Amresco, Solon, OH). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a pale yellow substrate that is reduced by living cells to yield a dark blue formazan product. This requires active mitochondria, and even recently dead cells do not reduce significant amounts of MTT. Exponentially growing cells were adjusted to 2.0×10^4/cm^2 with DMEM, plated in 96-well plates (Corning, Tewksbury, MA) at 200 μL/well and incubated for 24 h according to the routine procedure. The cells were then incubated with cocoa nibs unroasted slates (US), roasted slates (RS), unroasted well fermented (UWF) cocoa, and roasted well fermented (RWF) cocoa (5–10 mg/mL) for 48 h (6 wells for each sample). Each well was also incubated with MTT (10 μL/well; 5 g/mL) for 4 h. After 85 μL/well the liquid was removed and 50 μL/well sodium dodecyl sulfate was added to dissolve the solid residue. Finally, the absorbance was measured using a microplate reader (POLARIS, CELER®) at 570 nm. The cell proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 − average value of experimental group/average value of control group) × 100%.

2.4.6. Cell Cycle Analysis. Cells were rinsed briefly with calcium and magnesium-free phosphate-buffered saline and detached with trypsin at room temperature. After centrifugation, the cells were washed twice with phosphate-buffered saline; cells were resuspended in 500 μL of ice-cold Vindelov solution [25] containing 0.1% Triton X-100, 0.1% citrate buffer, 0.1 mg/mL RNase, and 50 mg/mL propidium iodide (Sigma Chemical Co., St. Louis, MO). After 15 min of incubation, cell suspension was analysed for DNA content by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The relative proportions of cells with DNA content indicative of apoptosis (<2n), G_0/G_1 diploid (2n), S (phase >2n but <4n), and G_2/M phase (4n) were obtained and analyzed using the CellQuest WinMDI 2.9. The percentage of cell population at a particular phase was estimated with FlowJo software. Cell dissociation procedure does not affect fluorescence under the experimental conditions that were used in this study or in any other studies of which we are aware. Nuclei of viable cells were gated according to FL-2W × FL2-A relation.

2.4.7. Apoptosis Assay. To measure the rate of apoptosis, the cells were subjected to staining with Annexin V conjugated to FITC (BD Pharmingen, San Diego, CA). The nonadherent cells were collected, and adherent cells were quickly washed with buffered saline solution (BSS) calcium/magnesium-free and were detached with trypsin/EDTA 0.125% (Sigma chemical Co., St. Louis, USA) at room temperature. Subsequently, apoptotic and necrotic cells were stained with Annexin VFITC/propidium iodide (PI) (BD Pharmingen, New Jersey, USA) according to the manufacturer's instructions, quantified by flow cytometer (FACSCalibur, BD Bioscience, New Jersey, USA), and analyzed using two specific programs, Cell Quest and FlowJo software.

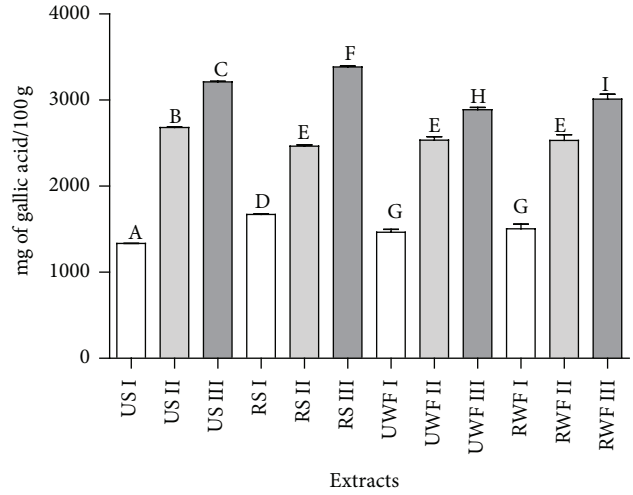

Figure 1: Total phenolic compounds of cocoa nibs unroasted slates (US), roasted slates (RS), unroasted well fermented (UWF) cocoa, and roasted well fermented (RWF) cocoa. Extracting solutions: I, methanol; II, methanol 50%; III, 1/2 50% methanol : 1/2 70% acetone. Means with different letters differ significantly ($p < 0.05$, Tukey's test).

2.5. Statistical Analysis. Results are presented as mean with the corresponding standard deviation of 3 independent experiments done in triplicates ($n = 9$). Data were analysed with the statistical software GraphPad Prism (version 5.04, GraphPad Software, San Diego, CA) and Statistica (version 7.0, StatSoft Inc., Tulsa, OK). One-way analysis of variance (ANOVA) test with the posttest of Tukey at a confidence level of 95% was used to test cell viability, cell cycle, and apoptosis.

3. Results and Discussion

3.1. Phenolic Compounds and Antioxidant Activity of Cocoa Beans Samples. The method that yielded higher phenolic extracts was the one using acetone, in all samples tested, as can be observed in Figure 1. Dreosti [26] reported that 60% of the total phenolics in raw cocoa beans are flavanol monomers (epicatechin and catechin) and procyanidin oligomers (dimer to decamer) [27]. Benayad et al. [28], Cheng et al. [29], and Boulekbache-Makhlouf et al. [30] have shown that the use of acetone, when compared to the use of other polar organic compounds, potentiated the extraction of flavonoids and flavonols from different plant materials. In addition, extraction of procyanidins from cocoa with acetone solutions has been successfully accomplished at least since 1999.

Table 1: Effect of extracts of cocoa, cocoa nibs roasted slates (RS), and unroasted well fermented (UWF) cocoa (5–10 mg/mL) on cell cycle progression in human lung cancer cell line after 48 hours.

	Cell cycle phase	CT	5 mg/mL	10 mg/mL
RS	sub-G_1	6.65 ± 1.00	55.65 ± 0.49	77.10 ± 2.26
	G_0/G_1	75.75 ± 4.45	30.65 ± 0.92*	20.63 ± 0.90*
	S	5.40 ± 0.77	4.89 ± 0.16	0.74 ± 0.40*
	G_2/M	7.68 ± 1.77	6.73 ± 0.38	0.70 ± 0.64*
UWF	sub-G_1	1.37 ± 0.29	13.55 ± 2.62*	50.28 ± 7.09**
	G_0/G_1	75.80 ± 6.02	25.65 ± 7.57*	21.78 ± 5.35*
	S	5.61 ± 1.17	2.64 ± 1.58*	2.20 ± 1.01*
	G_2/M	11.58 ± 3.54	33.95 ± 3.04**	16.08 ± 4.01*

Results are expressed as the percentage of total cells. The data represent mean ± SD values of triplicate experiments. Tukey's test; * $p < 0.05$; ** $p < 0.01$.

The present work found amounts of phenolic substances (Table 1) compatible with the results displayed by Kadow et al. [31] for both raw and "fermentation-like" treated samples. Unprocessed cocoa beans usually present a high phenolic content of about 12–18% (dry weight) [32]. The processing of raw cocoa includes a number of stages and each stage in the processing alters cocoa's chemistry and composition [11]. When extractor III was considered, there were significant differences among all samples tested and slates showed higher phenolic content when compared to well fermented samples. Generally, it is expected that well fermented beans show lower phenolic contents, because low mass phenols are related to astringency and anthocyanins are related to the purple color of unfermented beans, both considered undesirable characteristics in chocolate [33, 34].

Figures 2(a)–2(c) clearly show that the overall higher values were obtained for the DPPH method, followed by TEAC and FRAP, respectively. The slate samples showed higher antioxidant activity compared to well fermented beans or nibs. All these findings were in agreement with previous results as it was expected to obtain higher antioxidant activity in extracts with higher concentrations of phenolic compounds [14, 35–37].

The roasted slate (RS) samples showed a decrease in antioxidant activity after fermentation. Roasting is considered one of the processing steps of the cocoa nibs that leads to the loss of phenolic compounds and should cause a decrease in the antioxidant activity, as may be seen when comparing UWF and RWF samples (Figure 2). It is possible that, during roasting, while phenolic compounds are degraded, other antioxidant potential compounds are formed through the Maillard reaction, especially reductones and melanoidins [38, 39]. The reason why this formation may have been significant in slates but not in well fermented beans is probably related to the different compositions of these two samples before the roasting process.

FRAP assay showed a decrease in antioxidant activity after roasting. According to Apak et al. [40], FRAP methodology is not capable of measuring the antioxidant activity of compounds in complex matrix, since it takes more time to perform the analysis of antioxidant function, and not all antioxidants have the specific ability to reduce iron [41].

Recovery of antioxidant compounds from plant materials is typically accomplished through different extraction techniques, taking into account their chemistry and uneven distribution in the plant matrix. These compounds tend to present different polarities as well as other variable characteristics. Thus, the solubility in a particular solvent is a unique feature of the phytochemicals to be taken into account. Methanol and solutions of 50% methanol in water are commonly applied solvents that efficiently extract phenolic compounds. Methanol and acetone are also suitable solvents for anthocyanin extraction from various raw materials [42–44], and acetone-water mixtures have been suggested to grant better extraction results of procyanidins and phenols when compared with other extractors [45].

It is now recognized that diet and nutrients play an import role in cancer development and progress, with many dietary components found to be associated with cancer risk. However, almost all the clinical intervention trials with isolated nutrients, such as vitamin A, vitamin E, vitamin C, and phenolic compounds supplements, failed to demonstrate their protective effects against cancer. Due to the complexity of cocoa matrix, it is very difficult to characterize all components and even say which major component is responsible for the cytotoxic effect, due to the synergistic and antagonistic effects.

3.2. Cells Results

3.2.1. Effect of Cocoa Extracts on Cell Viability. The slate nibs were the supplement which caused the largest decrease in viability compared to control (34.45%, Figure 3), while cells exposed to US had the highest percentage of viability, 78.07%, at a concentration of 10 mg/mL (Figure 3(b)). However, surprisingly, crisp sample obtained higher potential reduction in cell viability (Figure 3(c)), with cell viability of 77.15% (5000 μg/mL) and 63.55% (10.000 μg/mL).

Well fermented cocoa bean extract decreased the number of viable A549 cells within 48 hours. In UWF sample, cell viability decreased from the concentration of 100 μg/mL by 45% compared with the control group ($p < 0.05$) (Figures 4(a) and 4(b)). The concentrations that caused the largest decrease in cell viability were 5000 and 10000 μg/mL, reduced by 58.77% and 72.35%, respectively (Figure 3(b)). For RWF sample, the reduction was smaller (Figure 3(c)), with effect only at concentrations of 5000 (83.07% viable cells) and 10000 μg/mL (72.20% viable cells).

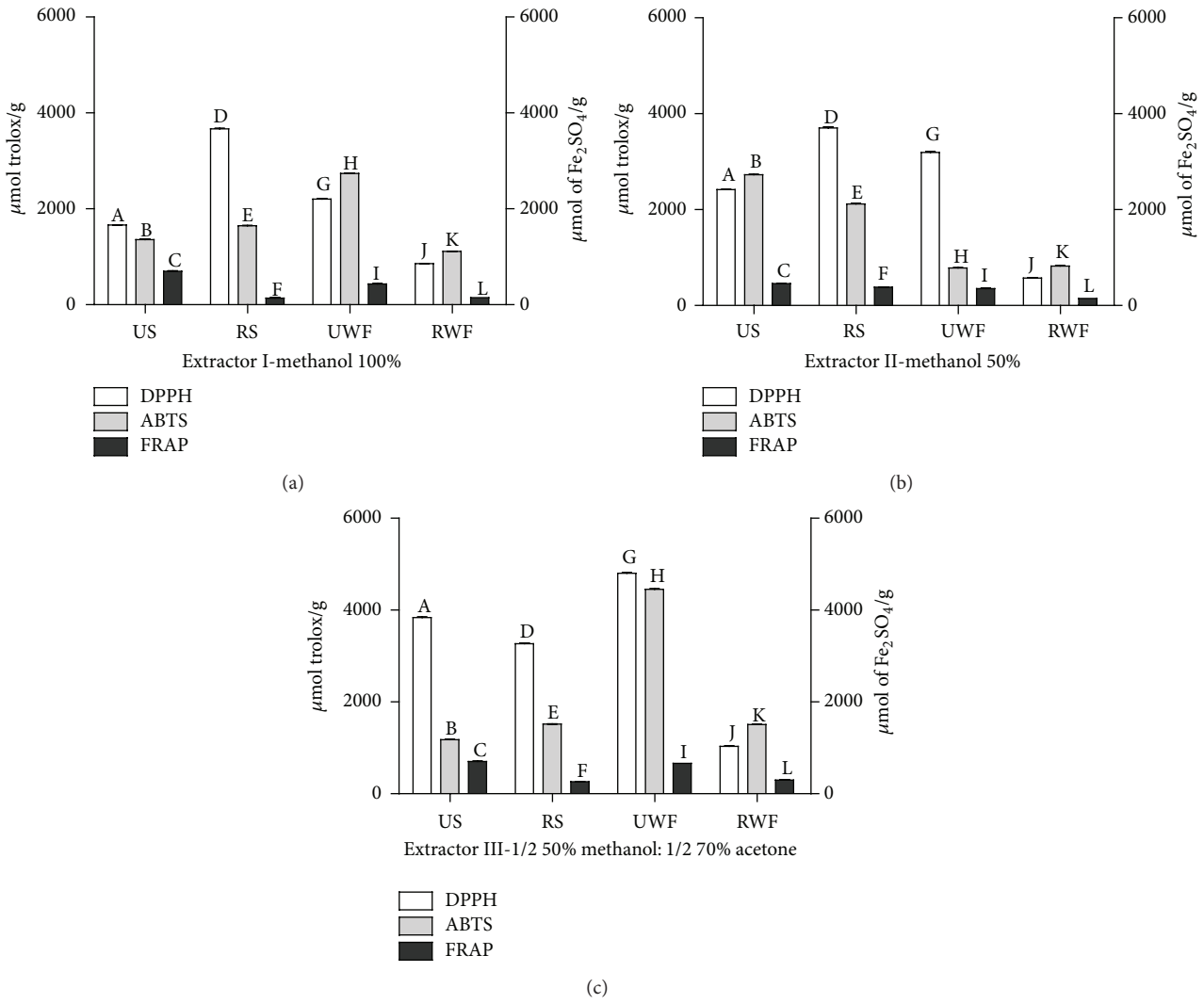

Figure 2: Antioxidant activity of cocoa nibs unroasted slates (US), roasted slates (RS), unroasted well fermented (UWF) cocoa, and roasted well fermented (RWF) cocoa for DPPH, TEAC, and FRAP assays by different extracting solutions (I–III). Means with different letters differ significantly ($p < 0.05$, Tukey's test).

Cell culture studies constitute a useful tool to elucidate the molecular mechanisms of action of cocoa extracts and their polyphenolic compounds in different cancer cell lines. It has been shown that cocoa components induced a time-dependent regulation of survival/proliferation pathways in HepG2 liver cells [46]. Moreover, a cocoa procyanidin fraction inhibited TPA-induced neoplastic transformation of JB6P+ mouse epidermal cells, COX-2 expression, and phosphorylation of MEK and p90 ribosomal s6 kinase and attenuated activator protein-1 (AP-1) and NF-κB stimulations [47].

The physiological impact of polyphenols depends on their absorption. However, it is important to bear in mind that the most common polyphenols in diet are not necessarily the most bioavailable, since their structure plays an important role. Most native polyphenols in foods are in glycoside form (flavonols, flavones, flavanones, isoflavones, and anthocyanins), together with the less frequent oligomers (proanthocyanidins), which cannot be absorbed in the intestinal mucosa [48]. Through this, we can observe that the sample that most reduced the viability of human lung carcinoma cells was again the sample which had the parent compounds of the modified crude cocoa, such as well fermented sample, and also the sample which has not been roasted, since many bioactive compounds are lost during this processing.

3.2.2. Effect of Cocoa Extracts on Cell Cycle Progression. Uncontrolled cell proliferation is a characteristic of cancer [49], and extracts of cocoa beans have been shown to inhibit the proliferation of A549 cells. Previous trials of antioxidant activity and MTT have determined which samples have the greatest potential for use in the analysis of cell cycle and apoptosis. To probe inhibition of cell growth mediated by RS and UWF extracts, we examined the cell cycle by flow cytometry. The effects of the RS and UWF extracts on cell cycle progression in A549 cells are shown in Table 1.

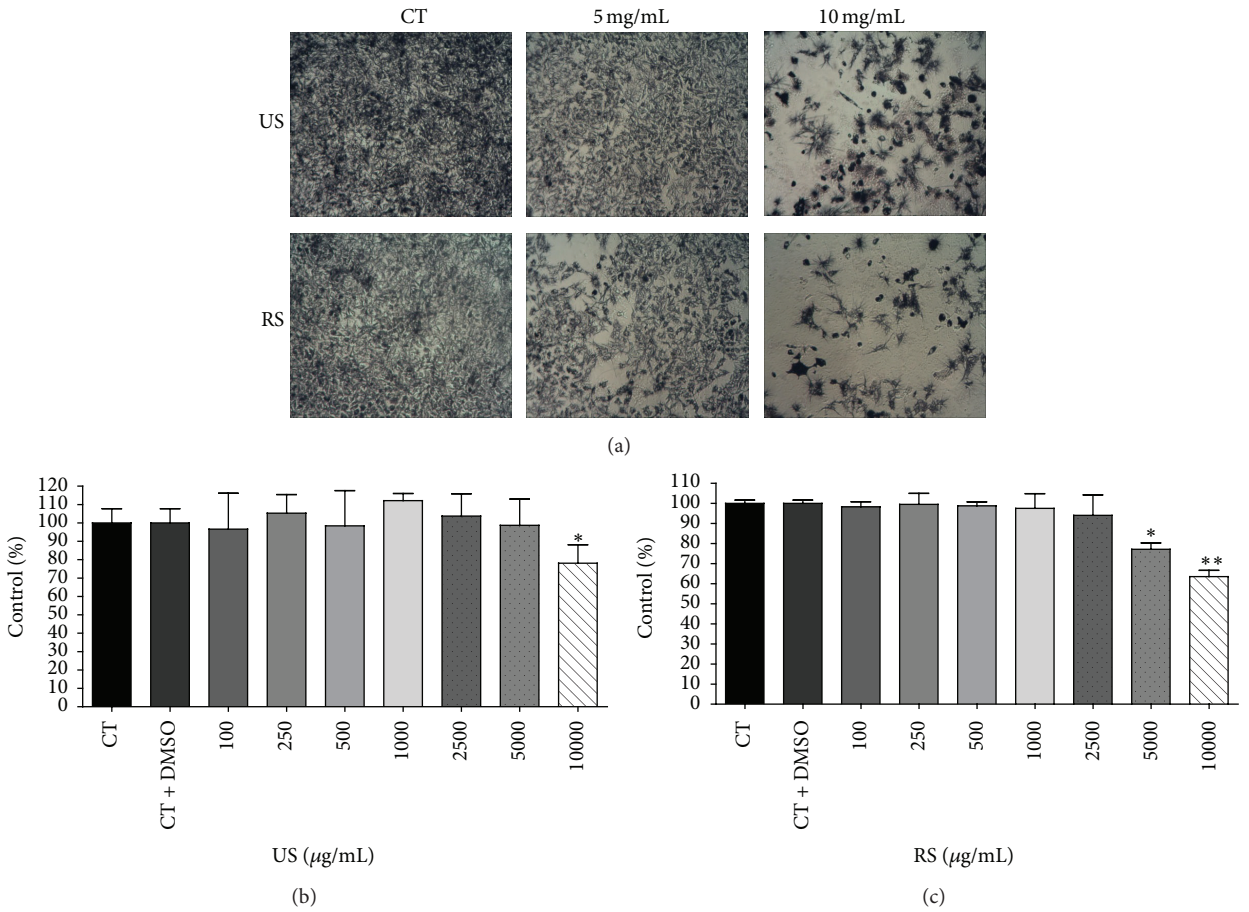

Figure 3: The effect of unroasted slates (US) and roasted slates (RS) nibs extract in cell control (a). Cocoa nibs US (b) and cocoa nibs RS (c), after forty-eight hours on viability A549 cells after exposure using MTT assays. The results are expressed as mean ± standard error and significant differences between cells treated with US and RS nibs extract (100–10000 μg/mL) were compared using Tukey's test ($^{*}p < 0.05$; $^{**}p < 0.01$).

After 48 h of growth, the population control in the sub-G_1 G_0/G_1 phase reached values between 6.65 and 1.37% and between 75.75% and 75.80%, respectively. Meanwhile, cells treated in the sub-G_1 population increased those treated in G_0/G_1 phase decreased in a dose-dependent manner in both treatments. The loss of the ability to regulate the cell cycle is characteristic of cancer cells and results in uncontrollable proliferation. The cell progression through the first gap phase of the cell cycle (G_1) is a step which is frequently disordered in cancer [50].

Treatments with RS resulted in the highest percentage of cells in sub-G_1 (77.01%, 10 mg/mL) and in G_0/G_1 (30.65%, 5 mg/mL) phases. As the G_0/G_1 phase population increased the G_2/M phase population of cells decreased, whereas the population of phase S cells showed no difference within 48 h. Treatment with UWF cocoa at a concentration of 10 mg/mL showed the highest value for sub-G_1 (50.27%) reduction within phases G_0/G_1 and S and an increase in the G_2/M phase. Treatment with 5 mg/mL of UWF cocoa showed the smallest number of cells in sub-G_1 and the G_0/G_1 phases and therefore a greater cell population in the G_2/M phase (33.95%).

Cocoa-derived pentameric procyanidin (pentamer) caused a G_0/G_1 cell cycle arrest in human breast cancer MDA MB-231, MDA MB-436, MDA MB-468, SKBR-3, and MCF-7 cells and in benzo(a)pyrene-immortalized 184A1N4 and 184B5 cells, whereas normal human mammary epithelial cells in primary culture and spontaneously immortalized MCF-10A cells were resistant [51]. Similarly, procyanidin-enriched extracts from cocoa caused growth inhibition with blockade of the cell cycle at G_2/M phase in human colonic Caco-2 cells [52], and EC induced S phase arrest in the cell cycle progression in LoVo colon cancer cells [53].

Our results indicate that cocoa nibs extracts were able to modify cell cycle. The deregulation in cell cycle control is a fundamental aspect in the development of cancer. Faults in the cell cycle regulation process can cause a greater proliferation of cancer cells. However, the reversal of this process leads to a delay in growth and induces cell death [54].

3.2.3. Apoptosis. We examined the effect of RS and UWF cocoa on apoptotic death in A549 cells. Table 2 shows the percentages of viable, early apoptotic, late apoptotic, and nonapoptotic cells treated with 5 mg/mL and 10 mg/mL

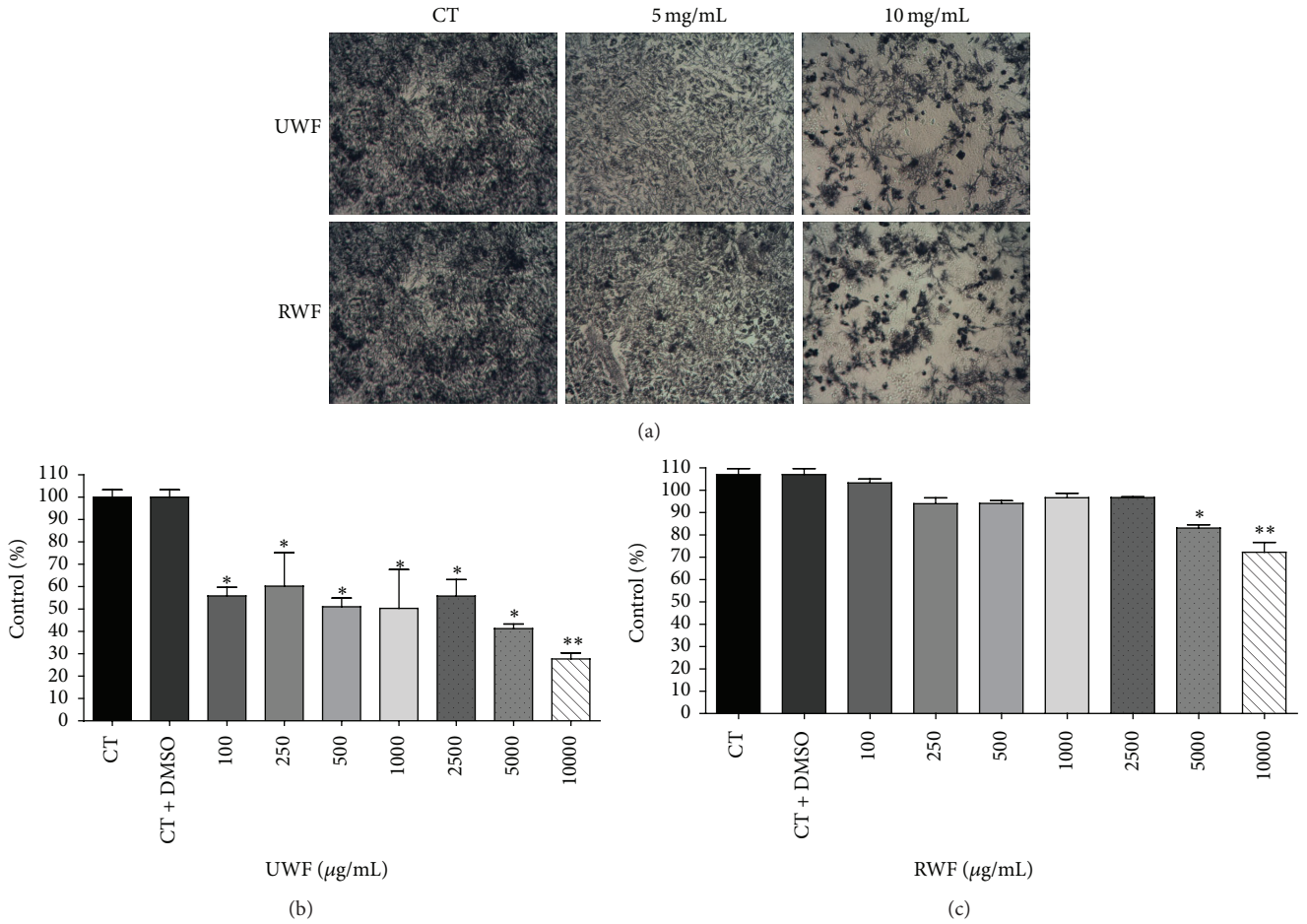

Figure 4: The effect of unroasted well fermented (UWF) nibs and roasted well fermented (RWF) nibs extracts in cell control (a). UWF cocoa nibs (b) and RWF cocoa nibs (c), after forty-eight hours on viability A549 cells after exposure using MTT assays. The experiment is expressed as mean ± standard error and significant differences between cells treated with UWF and RWF nibs extract (100–10000 μg/mL) were compared using Tukey's test ($^{*}p < 0.05$; $^{**}p < 0.01$).

Table 2: Effect of extracts of cocoa nibs roasted slates and unroasted well fermented cocoa (5–10 mg/mL) on programmed cell death in human lung cancer cell line after 48 hours.

Stages of cell death	CT	RS (mg/mL)		UWF (mg/mL)	
		5	10	5	10
Viable cells (Annexin V−/PI−)	95.80 ± 0.57	89.10 ± 2.26	86.73 ± 1.96	91.37 ± 1.33	72.03 ± 4.67*
Early apoptosis (Annexin V+/PI−)	2.16 ± 1.03	1.80 ± 0.83	8.22 ± 2.48**	3.87 ± 1.22	4.85 ± 2.05
Late apoptosis (Annexin V+/PI+)	0.90 ± 0.25	2.56 ± 1.30	4.92 ± 0.95*	2.23 ± 0.67	8.13 ± 0.06**
Nonapoptotic cells (Annexin V−/PI+)	1.12 ± 0.69	6.54 ± 0.13*	0.14 ± 0.69	2.52 ± 0.68	14.93 ± 0.42**

Results are expressed as percentage of total cells. The experiment is expressed as mean ± standard deviation; significant differences between untreated cells (CT) and cells treated with lycopene (5–10 μM) were compared by one-way ANOVA with the posttest of Tukey ($^{*}p < 0.05$; $^{**}p < 0.01$).

RS and UWF cocoa. Figure 4 shows the influence of the extracts of cocoa nibs on the rate of apoptosis. Cells treated with 5 mg/mL and 10 mg/mL RS and UWF cocoa for 48 hours resulted in a significant increase in the percentage of apoptotic cells compared with untreated cells (control). The concentrations used in this cellular model promoted a change in cell cycle and induction of apoptosis by lower doses than isolated compounds or cocoa products used in studies with human models. [55–57]. This has been demonstrated in a study by Ottaviani et al. [57], where adult males were given

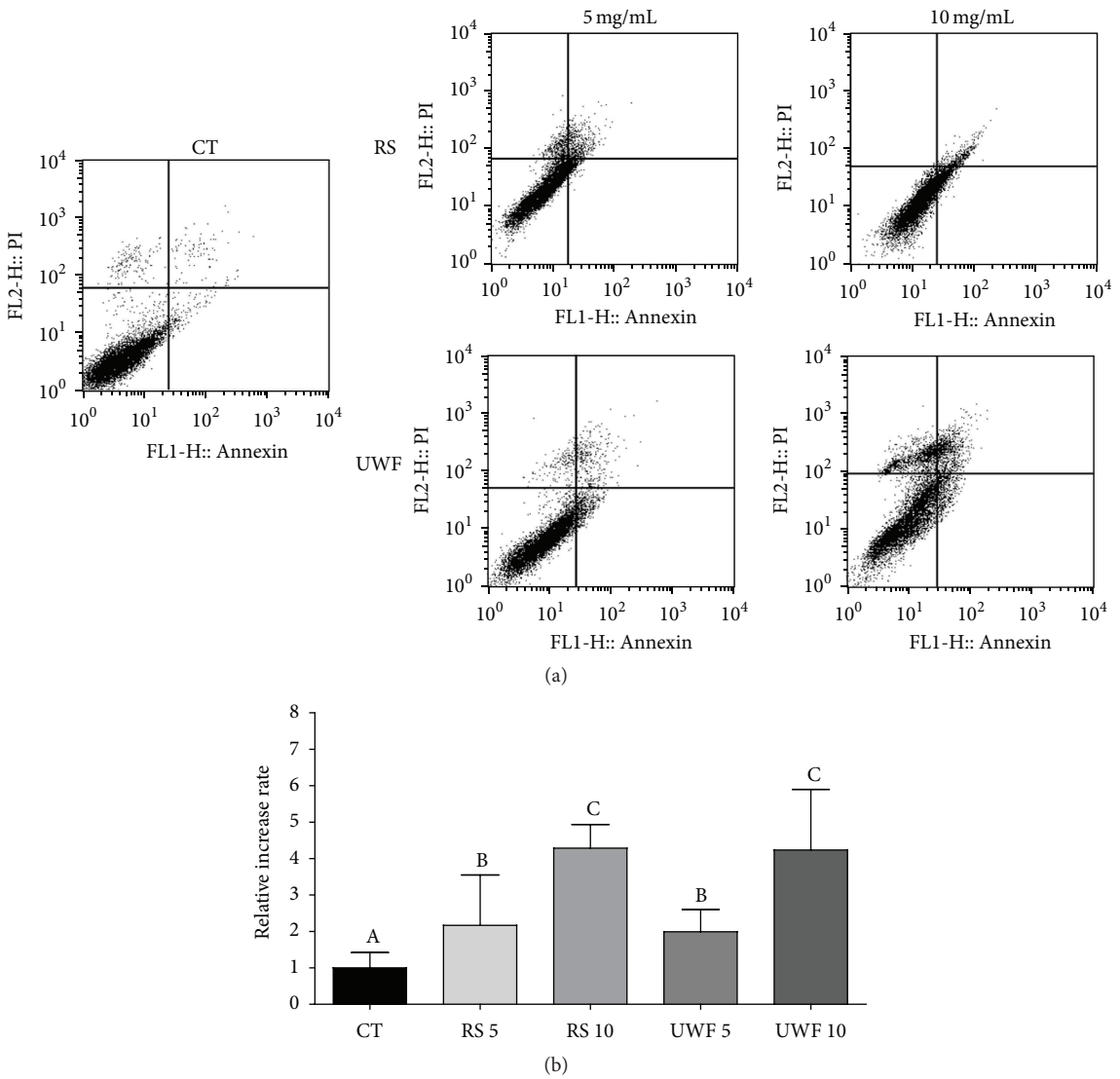

Figure 5: The effect of unroasted well fermented cocoa and roasted slates on the process of programmed death in A549 cells after treatment for 48 h. (a) Flow cytometry analysis of UWF cocoa and RS according to the exposure time and concentration of the compounds. (b) Quantitative effects of UWF cocoa and RS at 5 mg/mL and 10 mg/mL on A549 cells after exposure for 48 h. The results are expressed as mean ± SD, with significant differences between untreated cells (CT) and cells treated with UWF cocoa and RS (5–10 mg/mL) compared by 1-way ANOVA followed by Tukey's multiple comparison post hoc test. $^{*}p < 0.05$. $^{**}p < 0.01$.

1.5 mg/kg of a concentrated cocoa solution, and two hours after ingestion it was noted that there were many metabolites of flavonols in cocoa in the blood of the volunteers.

Increase of early apoptosis was observed in A549 cells treated with 10 mg/mL RS, whereas increase in late apoptosis was observed in cells treated with 10 mg/mL UWF cocoa for 48 h. A549 cells incubated with 5 mg/mL and 10 mg/mL RS UQG for 48 hours showed a decrease in the population of viable cells and an increase of up to 4.3 times in the percentage of apoptotic cells compared with control, in a dose-dependent manner (Table 2 and Figure 5).

Apoptosis is characterized by a series of distinct changes in cell morphology, loss of cell attachment, cytoplasmic contraction, DNA fragmentation, and other biochemical changes, including the activation of caspases through extrinsic and/or intrinsic mitochondrial pathways [58]. Increased resistance to apoptosis is a hallmark of many tumor cells. The functional inhibition of specific antiapoptotic factors may provide a rational basis for the development of novel therapeutic strategies. Therefore, apoptotic deficiency is considered to be a major cause of therapeutic resistance of tumors, since many chemotherapeutic and radiotherapeutic agents act through the induction of apoptosis [59]. However, the apoptotic effect of the active ingredient of cocoa bean samples on A549 cells has not yet been studied in detail.

4. Conclusion

In conclusion, cocoa extract presented itself as a potent antioxidant agent, with antioxidant capability influenced by the processing method and extraction. Slate cocoa beans extract inhibited cell proliferation, arrested cell cycle in different phases, and increased apoptosis in human lung carcinoma cells, in a time-dependent and dose-dependent manner. Our study has far reaching health relevance as cocoa could be projected as functional foods which, in addition to providing nutrition, would provide preventive therapeutic value against the development of cancer.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Antioxidant, Antibacterial, and Cytotoxic Activities of the Ethanolic *Origanum vulgare* Extract and Its Major Constituents

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Oregano is a perennial shrub that grows in the mountains of the Mediterranean and Euro/Irano-Siberian regions. This study was conducted to identify the major constituents of the ethanolic *Origanum vulgare* extract and examine the cytotoxic, antioxidant, and antibacterial properties of the extract but more importantly the contribution of its specific major constituent(s) or their combination to the overall extract biological activity. Gas chromatography/mass spectroscopy analysis showed that the extract contained monoterpene hydrocarbons and phenolic compounds, the major ones being carvacrol and thymol and to a lesser extent p-cymene, 1-octacosanol, creosol, and phytol. A549 epithelial cells challenged with the extract showed a concentration-dependent increase in cytotoxicity. A combination of thymol and carvacrol at equimolar concentrations to those present in the extract was less cytotoxic. The A549 cells pretreated with nonlethal extract concentrations protected against hydrogen-peroxide-induced cytotoxicity, an antioxidant effect more effective than the combination of equimolar concentrations of thymol/carvacrol. Inclusion of p-cymene and/or 1-octacosanol did not alter the synergistic antioxidant effects of the carvacrol/thymol mixture. The extract also exhibited antimicrobial properties against Gram-positive and Gram-negative bacterial strains including clinical isolates. In conclusion, the oregano extract has cytotoxic, antioxidant, and antibacterial activities mostly attributed to carvacrol and thymol.

1. Introduction

Herbs and spices contain a wide array of phytochemicals with strong biological and pharmacological properties [1]. One of them, oregano, is a perennial shrub native to the dry, rocky calcareous soils in the mountainous area of the Mediterranean and Euro/Irano-Siberian regions but it is also cultivated for its uses as a herb and therapeutic properties. Studies examining the antioxidant activities of different forms of oregano (fresh, dry, and ready-to-use herb blend pastes) showed that oregano retains its strong antioxidant capacity in both fresh and dry form [2]. The leaves and dried herb of oregano as well as its essential oil are traditionally used for respiratory disorders, indigestion, and rheumatoid arthritis [3–6].

The antibacterial and antioxidant properties of oregano have been attributed mainly to carvacrol and thymol, which are the major components of its essential oil [7]. Antibacterial effects have been reported for oregano against *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [8–10]. Studies comparing the antioxidant properties of Mediterranean food spices and common food additives have shown that extracts from oregano were more effective than butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in inhibiting lipid peroxidation [11]. The use of synthetic antioxidants to prevent free radical damage

can involve questionable nutritional value and toxic side effects while natural antioxidants present in many plants reduce oxidative damage and help in preventing mutagenesis, carcinogenesis, and aging due to their radical scavenging activities [12]. The role of free radicals has been implicated in several pathological conditions, including cancer, cardiovascular diseases, neurodegenerative disorders, and drug toxicity [13–15].

This study was carried out to identify the main components of *Origanum vulgare* from Mt. Parnon, Southern Greece, assess the cytotoxic, antioxidant, and antimicrobial properties of the oregano extract, and delineate the contribution of the extract's major components towards such effects.

2. Materials and Methods

2.1. Herb Material and Chemicals. The wild-grown herb *Origanum vulgare* was collected and authenticated by Dr. Z. Suntres from the southern slope of Mt. Parnon (37°19′N 22°39′E) in Kynouria Peloponnese, during the early summer of 2009 and voucher specimens are stored in the Department of Chemistry, Lakehead University, Dr. C. Gottardo's laboratory. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and all other chemicals (i.e., thymol, carvacrol, *p*-cymene, and 1-octacosanol) were purchased from Sigma-Aldrich (Oakville, ON, Canada). The chemicals used were of analytical reagent grade.

2.2. Preparation of Extracts. Ground leaves from *Origanum vulgare* (5.5 g) were extracted in 100 mL of ethanol at room temperature for three times, each for 24 h. The combined ethanol solution (300 mL) was concentrated using a rotary evaporator *in vacuo* (at 28°C), resulting in an organic oil (1.04 g, 19% from the dried sample).

2.3. Gas Chromatography/Mass Spectrometry (GC-MS). Two hundred (200) μg of dried extract was dissolved in a 1.1 mL ethanol. The sample and standards (1 μL) were loaded into the Varian 300 GC-MS. The samples were analyzed by a Varian model-450 gas chromatograph coupled with a Varian model 300-MS quadruple GC-MS mass spectrometer. This was attached with a factor four capillary column (VF-5 ms, 30 mm × 0.25 mm ID, DF = 0.25 μm). Helium was used as the carrier gas with a flow rate of 1.0 mL/min. Samples were introduced through a split mode method. This involved a one in ten split by a Varian 450 autosampler, with a high temperature injection port (280°C). The oven temperature was initially 50°C for 1 min. This was increased to a final temperature of 280°C at a rate of 10°C/min. The final 280°C temperature was held for 6 min. Electron ionization conditions were used with ionization energy of 70 eV. The scan range was from 70 to 600 amu. Lastly, the GC-MS interface temperature was set to 270°C.

The components were identified based on the comparison of their RI (retention indices) and mass spectra (NIST08 Mass Spectrum Library) of the GC-MS system. Synthetic components (Sigma-Aldrich Co., Oakville, ON, Canada) were used as references for retention time calculations. Major peaks with respective Mass Spectrum Library matches are shown in Table 1. All determinations were performed in triplicate.

2.4. Cell Culture. Human alveolar type II-like epithelial A549 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Corning Costar 0.2 μm vent cap cell culture flasks (Corning, NY, USA) with standard Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham supplemented with 10% iron-fortified bovine calf serum (SAFC Biosciences, Lenexa, KS, USA), 2 mM L-glutamine, and antibiotic/antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B) from Gibco (Carlsbad, CA, USA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and subcultured when they were 80% confluent. Prior to plating, cell counts and viabilities were assessed using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, Canada).

2.5. Cytotoxic and Antioxidant Properties of Oregano Extracts and Major Extract Components. The A549 cells were seeded into sterile flat-bottom 96-well plates at 10,000 cells/well and grown to 80% confluence overnight before treatments began. To determine the cytotoxicity of ethanolic extracts and their individual components, cells were treated with the different treatments (thymol, carvacrol, p-cymene, and/or 1-octacosanol dissolved in 1% ethanol [the vehicle solution had no effect on the antioxidant and cytotoxic activities of the extract and/or its constituents]) in media. To determine the antioxidant effects of the oregano extracts and their individual components (thymol carvacrol, p-cymene, and/or 1-octacosanol dissolved in 1% ethanol), A549 cells were first pretreated for 24 h with the different treatments in media, followed by treatment with 500 μM hydrogen peroxide. The MTT assay, a commonly used measure of cell viability, was used to assess cell survival as described by the manufacturer (Sigma-Aldrich Co., Oakville, ON, Canada). Viabilities of challenged cells were assessed relatively to control cells.

2.6. Bacterial Strains. *P. aeruginosa* (ATCC 25619 and clinical isolates), *Bordetella bronchiseptica* (ATCC 4617, ATCC 10580), *Escherichia coli* (ATCC 25922, ATCC 700973), *Burkholderia cenocepacia* (ATCC 25608 and clinical isolates), *Acinetobacter lwoffii* (ATCC 17925), *Acinetobacter baumannii* (ATCC 19606), *Moraxella catarrhalis* (ATCC 8176), *Bacillus subtilis* (ATCC 6633), and *S. aureus* (ATCC 29213 and clinical isolates) were purchased from Cedarlane (Burlington, ON, Canada) or obtained from the Clinical Microbiology Laboratory of Memorial Hospital (Sudbury, ON, Canada). For experimentation, the strains were inoculated onto Mueller Hinton II agar plates and incubated for 24 h at 37°C.

2.7. The Minimum Inhibitory Concentrations (MIC) of Oregano Extract against Gram-Positive and Gram-Negative Strains. The inhibitory concentrations were determined by the agar dilution method as previously described [16]. Bacterial inocula were prepared from an overnight culture in Mueller Hinton II agar and adjusted to contain an equivalence

Table 1: Composition of *Origanum vulgare* assessed by GC-MS analysis.

Peak	Retention time (min)	% area	Compound
1	8.959	6.900	1-Methyl-4-(1-methylethyl) benzene-(p-cumene)
2	9.664	1.904	1-Methyl-4-(1-methylethyl)-1,4-cyclohexadiene (γ-terpinene)
3	13.113	2.110	1-Methoxy-4-methyl-2-(1-methylethyl) benzene (creosol)
4	14.072	25.008	2-(1-Methylethyl)-5-methylphenol (thymol)
5	14.263	59.468	2-Methyl-5-(1-methylethyl)-phenol (carvacrol)
6	19.327	0.560	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (phytol)
7	25.600	4.050	1-Octacosanol

of a 0.5 McFarland standard with phosphate-buffered saline. One (1) μL of the adjusted inocula was then delivered onto Mueller Hinton II agar plates containing twofold serial dilutions of oregano from 25000 μg/mL to 3100 μg/mL, using the Replianalyzer system (Oxoid Inc., Nepean, ON, Canada). The lowest concentration of oregano that prevented the appearance of a visible growth within the inoculation area after 24 h at 36°C was defined as the MIC.

2.8. Carvacrol and Thymol Cellular Uptake. The cellular uptake of carvacrol and thymol was examined following exposure of A549 cells (3×10^6 cells) to 56 μM carvacrol or 23.3 μM thymol (i.e., equimolar concentrations to those found in the alcoholic extract) or a combination of the two components. Treated cells were removed at 1, 4, 8, 12, and 24 h, washed twice with PBS, and lysed with NP-40 lysis buffer. Cytosolic and membrane fractions were separated via centrifugation at 10,000 rpm for 12 minutes. Membrane and cytosolic fractions were dissolved in diethyl ether, evacuated with nitrogen gas, capped, and crimped. The samples were analyzed by gas chromatography/mass spectrometry (GC-MS) as previously described.

2.9. Statistical Analysis. All data were expressed as means ± SD of triplicate measurements. Data were evaluated by one way analysis of variance (ANOVA). If the F values were significant, Student's t-test was used to compare all groups. The level of significance was accepted at $p < 0.05$.

3. Results

3.1. GC-MS Analysis. In the present study, the GC-MS analysis of the ethanol extract of wild-growing herb *Origanum vulgare* obtained from Southern Greece revealed an abundance of monoterpene hydrocarbons and phenolic compounds with the main constituents being carvacrol (2-methyl-5-(1-methylethyl)phenol) (59.46%), followed by thymol (5-methyl-2-(1-methylethyl)phenol) (25.00%), p-cymene (6.90%), and 1-octacosanol (4.05%) (Table 1).

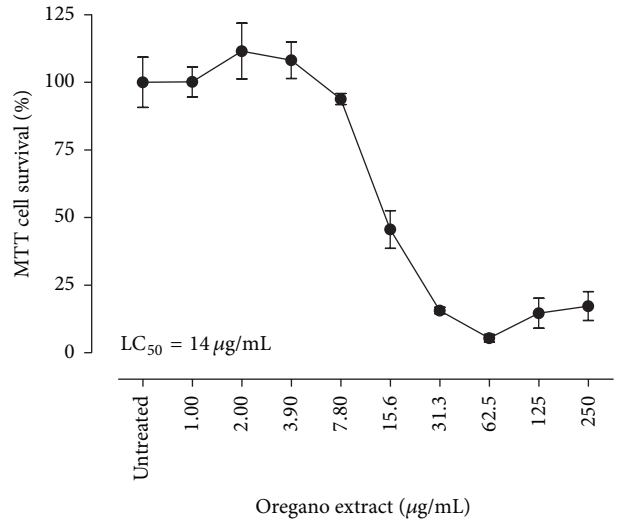

Figure 1: The cytotoxic properties of the ethanolic *O. vulgare* extract in A549 cells. All experiments were repeated three times in two replicates as outlined in Section 2.

3.2. Chemotherapeutic Properties of the Oregano Components. In order to examine the chemotherapeutic properties of the oregano extracts, A549 human lung adenocarcinoma epithelial cells were treated with increasing concentrations of oregano ethanolic extracts (0–250 μg/mL final concentration) and cell viability was assessed 24 h after treatment. Treatment of A549 cells with oregano extract resulted in a concentration-dependent decrease in cell viability with a calculated LC_{50} = 14 μg/mL (Figure 1).

To assess the contribution of the major components isolated from the oregano extract on cytotoxicity, the cell viability of A549 cells challenged with thymol, carvacrol, p-cymene, 1-octacosanol, or a mixture containing all four major constituents was examined. As shown in Figure 2(a), challenge of A549 cells with increasing concentrations of thymol, carvacrol, p-cymene, or 1-octacosanol alone resulted in a concentration-dependent decrease in cell viability, with

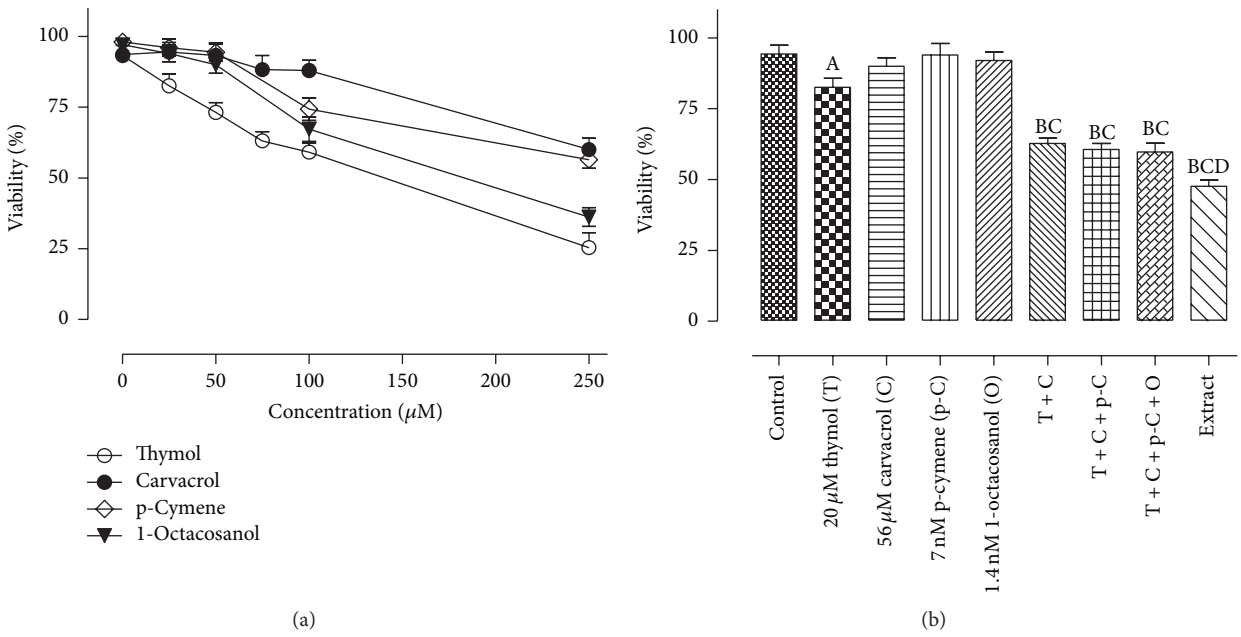

Figure 2: The cytotoxic effects of carvacrol, thymol, p-cymene, and 1-octacosanol in A549 cells. All experiments were repeated three times in two replicates as outlined in Section 2. The thymol and carvacrol concentrations are equimolar to those present in the ethanolic extract. (A) Significantly different from control, $p < 0.05$; (B) significantly different from thymol-treated group, $p < 0.05$; (C) significantly different from carvacrol-treated group, $p < 0.05$; (D) significantly different from carvacrol + thymol + p-cymene + 1-octacosanol-treated group, $p < 0.05$.

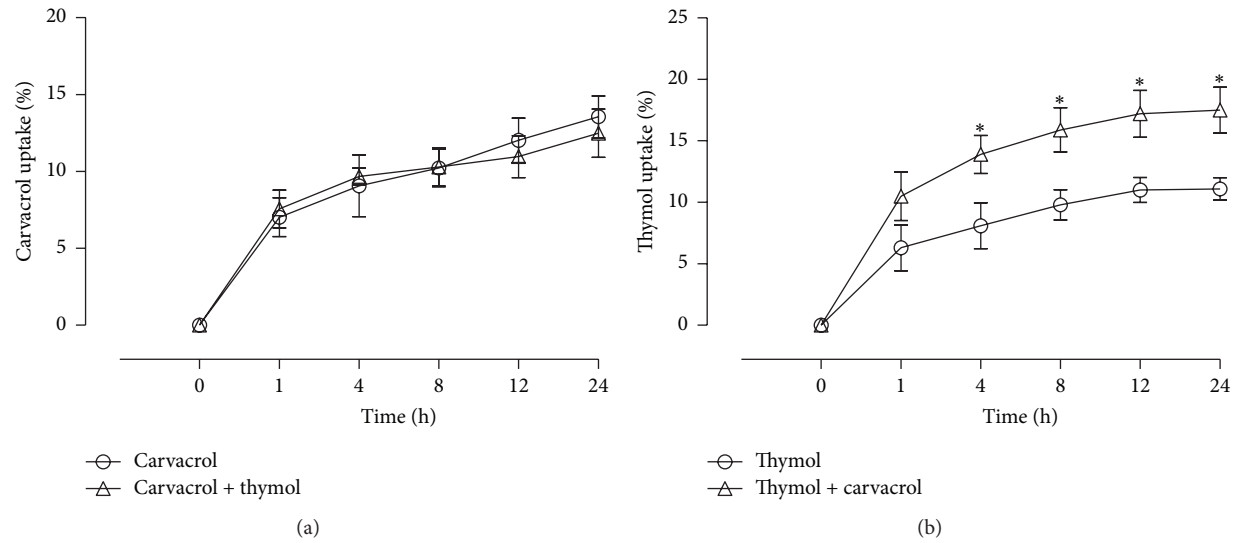

Figure 3: Uptake of carvacrol and thymol in A549 cells. All experiments were repeated three times in two replicates as outlined in Section 2. ∗ denotes significant difference from thymol-treated group, $p < 0.05$.

thymol being the most cytotoxic. Challenge of A549 cells with a combination of thymol, carvacrol, *p*-cymene, and 1-octacosanol at equimolar concentrations to the extract (56 μM carvacrol, 23.3 μM thymol, 7.19 nM *p*-cymene, and 1.38 nM 1-octacosanol) was less cytotoxic than the extract itself (% viability of mixture versus oregano extract: 62.65 ± 1.97 versus 49.59 ± 2.26). It should be noted that the contribution of *p*-cymene and 1-octacosanol to the cytotoxicity of the mixture was negligible (Figure 2(b)).

3.3. Carvacrol and Thymol Cellular Uptake. To investigate the synergistic effect of carvacrol and thymol cytotoxicity, the cellular uptake of carvacrol and thymol was examined (note: 56 μM carvacrol resulted in cell viability of 94.20 ± 2.56% while 23.3 μM thymol resulted in 82.60 ± 2.12% and their combination resulted in 62.65 ± 1.97% viability). Incubation of cells with carvacrol or thymol alone resulted in a time-dependent increase in their uptake (Figures 3(a) and 3(b)). Coincubation of cells with a mixture of carvacrol

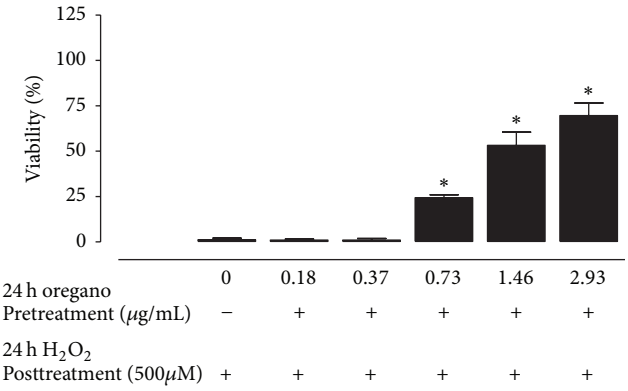

FIGURE 4: The antioxidant effect of the ethanolic *O. vulgare* extract against H_2O_2-induced cytotoxicity in A549 cells. All experiments were repeated three times in two replicates as outlined in Section 2. * denotes significance from the control group treated with H_2O_2 only, $p < 0.05$.

and thymol did not have any effect on the uptake of carvacrol (Figure 3(a)); a small but significant increase in the uptake of thymol was observed (Figure 3(b)).

3.4. Antioxidant Protective Effects of the Oregano Extract. To examine the antioxidant effects of the oregano extract, noncytotoxic concentrations of extract that corresponded to greater than 95% cell viability were used. As shown in Figure 4, pretreatment of A549 cells with the oregano extract at concentrations ranging from 0 to 2.93 μg/mL resulted in a concentration-dependent protective effect against H_2O_2. Preincubation of A549 cells with nontoxic concentrations of carvacrol or thymol showed that both phenolic isomers protected against hydrogen-peroxide induced cytotoxicity, with carvacrol producing a better protective effect than thymol (Figure 5(a)). Preincubation of cells with a combination of both isomers at equimolar concentrations (12 μM carvacrol and 5 μM thymol) to those present in the oregano extract (at the 2.93 μg/mL concentration) resulted in a less protective effect than that produced by the extract (% viability of carvacrol + thymol mixture versus oregano extract: 37.14 ± 0.77 versus 69.60 ± 5.00) (Figure 5). Inclusion of p-cymene and/or 1-octacosanol to the carvacrol and thymol mixture did not contribute to the antioxidant effects of the mixture (data not shown); however, the inclusion of the other oregano extracts to the carvacrol/thymol mixture was not examined.

3.5. Antimicrobial Properties of the Oregano Extract. The antibacterial properties of the ethanolic oregano extract were measured using an agar dilution method. The oregano extract inhibited the growth of reference ATCC Gram-negative and Gram-positive bacterial strains with varying degree, but no trends were realised (Table 2). The inhibitory concentrations were 2- to 4-fold lower against nonmucoid than mucoid clinical isolates of *P. aeruginosa* and exhibited greater effects against clinical isolates of *B. cenocepacia* compared to the ATCC reference strain (Table 2).

4. Discussion

Results from the GC-MS analysis of the ethanol extract of wild-growing herb *Origanum vulgare* obtained from Southern Greece revealed an abundance of monoterpene hydrocarbons and phenolic compounds with the main constituents being carvacrol and thymol (Table 1). This finding is consistent with that reported by other investigators who found that in spite of the high variability of individual compounds in the essential oil of *O. vulgare* from 23 localities scattered all over Greece, the sum of carvacrol, thymol, *p*-cymene, and γ-terpinene was consistent, amounting to >80% [10, 17, 18]. Plants collected from the northern region of Greece were rich in thymol (30.3–42.8% of total oil), whereas those from the southern part of the country were rich in carvacrol (57.4–69.6% of total oil) [17]. *p*-Cymene (1-methyl-4-(1-methylethyl)-benzene) and γ-terpinene (1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene) are the precursors of carvacrol and thymol in species of *Origanum* [19].

Studies examining the effect of oregano extract on cancer prevention and cytotoxicity are limited [20]. In the present study, challenge of A549 human lung adenocarcinoma epithelial cells with oregano ethanolic extracts (0–250 μg/mL final concentration) resulted in a concentration-dependent decrease in cell viability with a calculated LC_{50} = 14 μg/mL (Figure 1). It is not known whether the cytotoxicity of oregano extract is attributed to a specific component or combination of components. Results shown in Figure 2 demonstrated that challenge of A549 cells with thymol, carvacrol, p-cymene, or 1-octacosanol alone resulted in a concentration-dependent decrease in cell viability, with thymol being more cytotoxic than the other three compounds. Challenge of A549 cells with a combination of the four compounds at equimolar concentrations (56 μM carvacrol, 23.3 μM thymol) to those present in the oregano extract was less cytotoxic than the extract itself. The cytotoxicity of the oregano extract is mostly attributed to presence of carvacrol and thymol. *p*-cymene and 1-octacosanol, although cytotoxic at high concentrations, did not contribute to the cytotoxicity of the mixture, most likely attributed to their low potency and very low availability in the mixture (nM range). It appears that other unmeasured ethanolic constituents at lower concentrations than those determined in this study might possess higher potencies and play a role in the overall effects of the oregano extracts.

The mechanisms by which thymol and carvacrol cause cell death in mammalian cell lines have not been thoroughly investigated. Results from an *in vitro* study showed that carvacrol is very potent inhibitor of cell growth in A549 cell line as evidenced by the concentration-dependent decreases in cell number, degeneration of cell morphology, and a decrease in total protein amount [21]. Thymol induces cell death in human osteosarcoma and astrocytes and may involve apoptosis via mitochondrial pathways [22, 23]. Whether the synergistic effects of carvacrol and thymol regarding cell viability are related to a combination of the purported mechanisms is under investigation. It is evident from the results of this study that coincubation of carvacrol and thymol increased the uptake of the more cytotoxic thymol and enhanced the cytotoxicity of the mixture. Carvacrol is

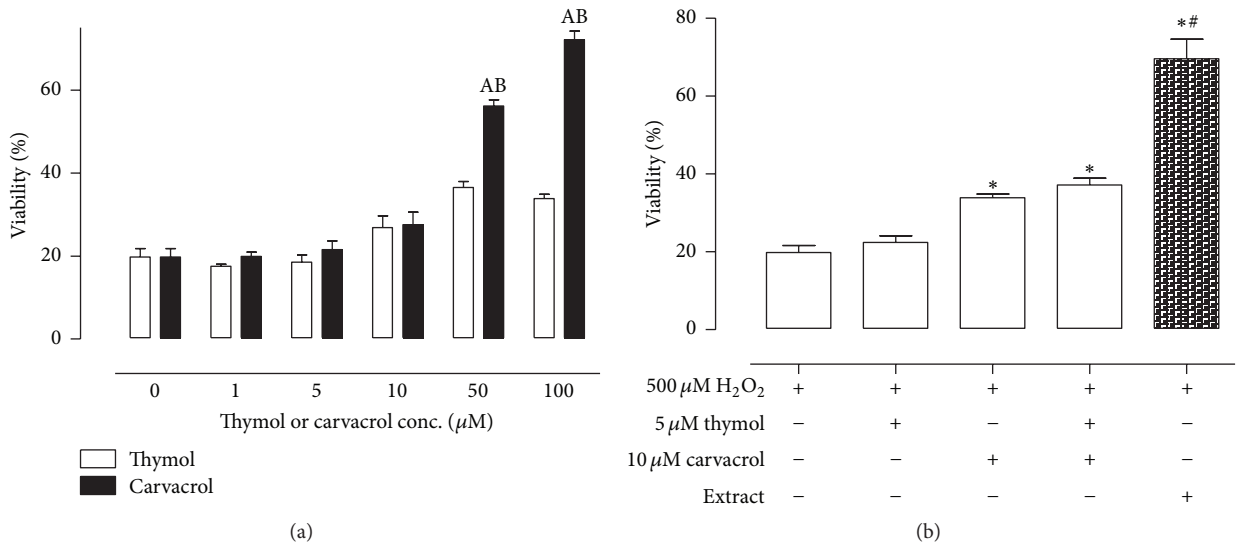

Figure 5: The antioxidant effect of (a) carvacrol and thymol and (b) thymol and/or carvacrol. Equimolar concentration to carvacrol and thymol is present in the extract at the 2.93 μg/mL concentration, as well as in ethanolic extract against H_2O_2-induced cytotoxicity in A549 cells. All experiments were repeated three times in two replicates as outlined in Section 2. (A) denotes significance from the control group treated with H_2O_2 only, $p < 0.05$; (B) denotes significance from the thymol pretreated group challenged with H_2O_2, $p < 0.05$; $*$ denotes significance from the control group treated with H_2O_2 only, $p < 0.05$; # denotes significance from the control group pretreated with thymol and carvacrol mixture and challenged with H_2O_2, $p < 0.05$.

slightly more lipophilic than thymol with partition coefficient in octanol/water ($P_{o/w}$) of 3.64 and 3.30, respectively [24–26], suggesting that carvacrol is partitioned deeper in the cytoplasmic membrane, thereby causing an expansion of the membrane [24] altering its permeability [27].

Oxidative stress describes the outcome of an increased reactive oxygen species (ROS) production and/or a decrease in their elimination. ROS (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, and lipid peroxides) can be formed as normal products of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions. ROS attack biological macromolecules such as membrane lipids, nucleic acids, carbohydrates, and proteins resulting in damage [13–15, 28]. Pretreatment of A549 cells with the oregano extract at nontoxic concentrations ranging from 0 to 2.93 μg/mL resulted in a concentration-dependent protective effect against H_2O_2. H_2O_2 has been used as a model of oxidative stress because it can be generated *in vivo* by the spontaneous and/or enzymatic dismutation of the superoxide anion radical. The antioxidant effectiveness of oregano extracts *in vitro* is perhaps due to their ability to act as reducing agents and free radical scavengers, as quenchers of singlet O_2 formation and to complex with prooxidant metal ions [29, 30]. Phenols (thymol, carvacrol) monocyclic hydrocarbons (terpinolene, R-terpinene, and γ-terpinene) belong to the most active natural antioxidants found in the essential oils [29, 30]. Indeed, in our study nontoxic concentrations of both phenolic isomers protected against hydrogen-peroxide induced cytotoxicity, with carvacrol producing a better protective effect than thymol. However, a combination of both isomers at equimolar concentrations (12 μM carvacrol and 5 μM thymol) to those present in the oregano extract (at the 2.93 μg/mL concentration) resulted in a less protective effect than that produced by the extract (Figure 4). Although inclusion of *p*-cymene and/or 1-octacosanol to the carvacrol and thymol mixture did not contribute to the antioxidant effects of the mixture, other components present in the extract at lower levels might possess higher antioxidant potencies and play a role in the overall effects of the oregano extracts. For example, β-caryophyllene, a constituent in oregano at very low levels, has a higher inhibitory capacity on lipid peroxidation than probucol, α-humulene, and α-tocopherol [31, 32].

Gram-negative pathogens like *P. aeruginosa* and *B. cenocepacia* are commonly found opportunistic bacilli in our environment that establish infections in patients suffering from pulmonary diseases like cystic fibrosis [33, 34]. The results of this study showed that the ethanolic extracts of oregano exhibited antibacterial properties as indicated by their ability to inhibit nonmucoid and mucoid clinical isolates of *P. aeruginosa*, and clinical isolates of *B. cenocepacia*. It is important to note that the extracts were less effective against the mucoid clinical isolates of *P. aeruginosa* possibly due to the secretion of negatively charged alginate-rich matrix to the surroundings, which inhibit antibiotic penetration. Earlier work by Lambert et al. [8] credits the activity of oregano essential oil and its active constituents (carvacrol and thymol) to interference with the pH gradient and membrane permeability. Burt et al. [35] have previously described that carvacrol may also be involved in inhibiting *E. coli* flagellin. A study examining the separate and combined antibacterial activities of the main chemical constituents of oregano and

Table 2: Minimum Inhibitory Concentrations (MIC) of bacterial strains.

Bacterial strains	Source	Ethanolic oregano extract (μg/mL)
Pseudomonas aeruginosa		
25619	ATCC	25
Mucoid clinical isolate 1	Cystic fibrosis isolate	25
Mucoid clinical isolate 2	Cystic fibrosis isolate	25
Mucoid clinical isolate 3	Cystic fibrosis isolate	25
Mucoid clinical isolate 4	Cystic fibrosis isolate	25
Mucoid clinical isolate 5	Cystic fibrosis isolate	12.5
Nonmucoid clinical isolate 1	Cystic fibrosis isolate	6.3
Nonmucoid clinical isolate 2	Cystic fibrosis isolate	6.3
Nonmucoid clinical isolate 3	Cystic fibrosis isolate	25
Nonmucoid clinical isolate 4	Cystic fibrosis isolate	12.5
Bordetella bronchiseptica		
10580	ATCC	12.5
4617	ATCC	12.5
Escherichia coli		
25922	ATCC	25
700973	ATCC	12.5
Burkholderia cenocepacia		
25608	ATCC	25
Clinical isolate 1	Cystic fibrosis isolate	12.5
Clinical isolate 2	Cystic fibrosis isolate	6.3
Clinical isolate 3	Cystic fibrosis isolate	12.5
Clinical isolate 4	Cystic fibrosis isolate	12.5
Acinetobacter lwoffii 17925	ATCC	12.5
Acinetobacter baumannii 19606	ATCC	12.5
Moraxella catarrhalis 8176	ATCC	12.5
Bacillus subtilis 6633	ATCC	6.3
Staphylococcus aureus		
29213	ATCC	25
Clinical isolate 1	Cystic fibrosis isolate	12.5
Clinical isolate 2	Cystic fibrosis isolate	25

other spices (namely, eugenol, cinnamaldehyde, thymol, and carvacrol) showed that each component possessed antibacterial properties and the components acted synergistically with each other [8, 36]. The mechanism by which the oregano extract and individual components produce the antimicrobial effect on mucoid and nonmucoid bacteria is currently under investigation.

In conclusion, the results of this study showed that the ethanolic extract of *Origanum vulgare* possesses strong cytotoxic, antioxidant, and antibacterial activities which are attributed mostly to the presence of the isomeric phenolic constituents, carvacrol, and thymol.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Research Article

Phlorizin, an Active Ingredient of *Eleutherococcus senticosus*, Increases Proliferative Potential of Keratinocytes with Inhibition of MiR135b and Increased Expression of Type IV Collagen

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E. senticosus extract (ESE), known as antioxidant, has diverse pharmacologic effects. It is also used as an antiaging agent for the skin and phlorizin (PZ) is identified as a main ingredient. In this study, the effects of PZ on epidermal stem cells were investigated. Cultured normal human keratinocytes and skin equivalents are used to test whether PZ affects proliferative potential of keratinocytes and how it regulates these effects. Skin equivalents (SEs) were treated with ESE and the results showed that the epidermis became slightly thickened on addition of 0.002% ESE. The staining intensity of p63 as well as proliferating cell nuclear antigen (PCNA) is increased, and integrin $\alpha 6$ was upregulated. Analysis of ESE confirmed that PZ is the main ingredient. When SEs were treated with PZ, similar findings were observed. In particular, the expression of integrin $\alpha 6$, integrin $\beta 1$, and type IV collagen was increased. Levels of mRNA for type IV collagen were increased and levels of miR135b were downregulated. All these findings suggested that PZ can affect the proliferative potential of epidermal cells in part by microenvironment changes via miR135b downregulation and following increased expression of type IV collagen.

1. Introduction

Eleutherococcus senticosus (Acanthopanax senticosus), known as Siberian ginseng or ciwujia, is a kind of small woody shrub which belongs to the family Araliaceae. It has diverse pharmacological effects, including antifatigue, learning improvement, anti-inflammatory, immune-enhancing, and antidepressive effects [1]. It has protective effect against oxidative damage, and it has been used as a skin antiaging agent [2].

Skin is maintained by epidermal stem cells that self-renew, proliferate, and differentiate [3, 4]. Therefore, skin aging is induced by impaired stem cell mobilization or by a reduction in the number of stem cells that can respond to proliferative signals [5]. Self-renewal of stem cells is controlled intrinsically by gene expression and it is modulated through interaction with extrinsic cues from the environment [6]. Thus, modulation of the stem-cell niche is important for the self-renewal and multipotency of stem cells [7]. It is reported that low oxygen tensions (hypoxia) are necessary to maintain undifferentiated stem cell phenotypes and also influence proliferation and stem cell fate [8]. We also reported that redox status is critical for stem cell activities [9]. It is

well known that ESE has antioxidant activity [2] and PZ is found to be the main ingredient and it has antioxidant activity [10]. It has also been reported that PZ protects skin against UVB-induced skin damage by decreasing ROS overproduction [11]. These findings suggested that PZ may have beneficial effects on skin stem cells. In the current study, we investigated the effects of an extract of *E. senticosus* (ESE) and its active ingredient, phlorizin (PZ) {3,5-dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenyl β-D-glucopyranoside}, on human epidermal cells. Results showed that PZ recovered proliferative potential of epidermal cells by affecting basement membrane which constitute niche of epidermal basal cells. Recently, we reported that type IV collagen is a target of miR135b and that miR135b suppression may improve the microenvironment and also increase the proliferative potential of epidermal basal cells [12]. In this study, the effect of PZ was investigated in terms of miR135b and type IV collagen expression.

2. Materials and Methods

2.1. Reagents. Unless otherwise specified, reagents including phlorizin (Phlorizin, P3449) were obtained from Sigma-Aldrich. The antibodies that we used in this study were as follows: p63 antibody (#sc-8431, Santa Cruz Biotechnology, Santa Cruz, CA), proliferating cell nuclear antigen antibody (PCNA, #M0879, DAKO, Glostrup, Denmark), integrin α6 antibody (#sc-6597, Santa Cruz Biotechnology), integrin β1 antibody (#sc-9970, Santa Cruz Biotechnology), and type IV collagen antibody (239M-16, Cell Marque, Rocklin, CA).

2.2. Preparation of E. senticosus Extracts and Identification of Phlorizin. Air-dried powdered roots (100 g) of *E. senticosus* were extracted with 80% aqueous methanol (1000 mL) at room temperature. The 80% aqueous methanol extract was concentrated under reduced pressure and then lyophilized to yield 21 g of dry extract, which was stored at −20°C. Concentrated extract was suspended in water. The extract was then fractionated successively with equal volumes of hexane, chloroform, ethyl acetate, and butan-1-ol. The ethyl acetate fraction was further purified by reversed-phase chromatography (LiChroprep RP-C18, Merck). The EtOAc fraction was dissolved in 10% MeOH (100 mL) and subjected to open-column chromatography. Elution was carried out with aqueous MeOH increasing the MeOH content in 10% increment from 10% to 100% (100 mL each). The 30% aqueous MeOH fraction was concentrated in vacuo. The active compound was then separated by preparative reversed-phase HPLC [Luna C18 (2) column, 21.2 × 250 mm, 5 μm, Phenomenex]. The mobile phase was a 45-min linear methanol-water gradient (5 : 95 to 95 : 5) flowing at a rate of 24 mL/min. To identify the active ingredient of ESE, we examined the effects of the various HPLC fractions by means of MTT assay. The active ingredient was found to be present in HPLC fractions 32–36. HPLC fractions 32–36 were combined and concentrated in vacuo (13 mg), and the chemical structure of the isolated product was determined by examining its 1H- and 13C-NMR spectra (Bruker Avance-500, 500 MHz).

2.3. DPPH Radical Scavenging Assay. A sample of each stock solution (2 μL, 10 mg/mL) was added to 80 μL of 0.25 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 118 μL of 70% ethanol to give a final DPPH concentration of 0.1 mM. Next, the mixture was vigorously shaken and then left to stand for 10 min in the dark. The absorbance at 517 nm was then measured using ELISA reader (TECAN, Salzburg, Austria) [13].

2.4. Cell Culture and Toxicity Assay. Normal human fibroblasts and keratinocytes were isolated from human foreskins obtained during circumcision following our previous protocol [9]. All samples were obtained with informed consent. For culture of keratinocytes or fibroblast, keratinocyte growth medium (KGM, Clonetics, San Diego, CA) or Dulbecco's modified Eagle's medium (DMEM) (LM001-05, WelGENE, Daegu, South Korea) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific HyClone, Logan, UT) was used. The medium was changed every two or three days. For keratinocytes, 4×10^4 cells suspended in KGM were seeded into each well of a 24-well plate. After 24 h, the medium was replaced with keratinocyte basal medium (KBM, Clonetics) supplemented with 0.1% bovine serum albumin (BSA). For fibroblasts, cells were also incubated for 24 h in FBS-free DMEM before treatment. ESE or PZ was added and the cells were incubated for further 24 h at 37°C. A 5 mg/mL solution of 2-(4,5-dimethyl-1,3-thiazol-2-yl)-3,5-diphenyl-2*H*-tetrazol-3-ium bromide (MTT) in DPBS (100 μL) was mixed and the plates were incubated for 4 h. After removing the supernatant, the formazan crystals were dissolved in 1 mL of dimethyl sulfoxide. The optical density was then determined at 540 nm by using ELISA reader (Tecan Austria GmbH, Grödig, Austria).

2.5. Construction of Skin Equivalents. Skin equivalents (SEs) were prepared by our method [14]. Type I collagen was extracted from the tendons of rat tails. Collagen substitutes were prepared as follows: eight volumes of type I collagen with one volume of 10x concentrated DMEM and one volume of neutralization buffer (0.05 M NaOH, 0.26 mM $NaHCO_3$, and 200 mM 2-[1-(2-hydroxyethyl)piperazin-1-ium-4-yl]ethanesulfonate) (HEPES) and then adding 5×10^5 fibroblasts. After gelling (3.0 μm Millicell, Millipore, Bedford, MA), keratinocytes (1×10^6 cells) were then seeded onto the surface of collagen substitute. After 1 day in a submerged state, they were maintained at the air-liquid interface for additional 12 days. The growth medium consists of DMEM and Ham's nutrient mixture F12 in a ratio of 3 : 1, supplemented with 5% FBS, 0.4 μg/mL hydrocortisone, 1 μM isoproterenol, 25 μg/mL ascorbic acid, and 5 μg/mL insulin. Every week, the medium was changed for 3 times. All experiments were repeated at least twice under the same conditions. Samples were treated with the medium for 7 days before harvesting the SEs.

2.6. Histology and Immunohistochemistry. Finally, the SEs were fixed with Carnoy's fixative and embedded in paraffin. To stain for type IV collagen, antigen retrieval was performed by using proteinase K (Roche Applied Science, Penzberg,

Germany). For other antibodies, thermal antigen retrieval was performed by using Trilogy solution (Cell Marque) and a pressure cooker. Immunohistochemical staining was performed by using the avidin-biotin-peroxidase complex technique (DAKO). All experiments were repeated twice. For image analysis, same condition was applied for staining and taking pictures. After taking pictures, expression of type IV collagen and integrins was measured by stained areas in three representative areas. In case of PCNA (proliferating cell nuclear antigen) and p63, expression was measured by OD (optical density: intensity/stained area) in three representative areas. Images were analyzed quantitatively using MetaMorph Offline version 7.7.0.0 image analysis software (Molecular Devices, Downingtown, PA). To see the effects of phlorizin on fibroblasts, SEs were stained with Hoechst 33342 (1 μg/mL, 40047, Biotium Inc., Hayward, CA, USA) for 10 min at RT. Then number of dermal cells in 3 representative areas was compared.

2.7. RT-PCR Analysis. One hundred thousand of keratinocytes were seeded in well of 6-well plate. The cells were cultured in phlorizin (0, 50, 100, and 200 μM) containing media for 72 hrs. Total RNA was isolated using AllPrep DNA/RNA/Protein Mini Kit (80004, Qiagen, Valencia, CA). Quality and amount of the RNA were assessed using Experion RNA StdSens analysis kit (700-7104, Bio-Rad, Hercules, CA) by Experion automated electrophoresis system (700-7001, Bio-Rad). For the qRT-PCR of human COL4A3, ITGA6, and ITGB1, one microgram of total RNA was reverse transcribed into cDNA (ImProm-II Reverse Transcription System, A3800, Promega, Madison, WI) with Oligo(dT) 15 Primer following manufacturer's instructions. Real-time PCR was performed with TaqMan Gene Expression Master Mix (4369016, Applied Biosystems, Foster City, CA) and target gene primers: COL4A3 (4331182, Hs01022542_m1, Applied Biosystems), ITGA6 (4331182, Hs01041011_m1, Applied Biosystems), and ITGB1 (4331182, Hs00559595_m1, Applied Biosystems). The housekeeping gene, GAPDH (43352934E, Applied Biosystems), was used as an endogenous control. For qRT-PCR of has-miR135b, 10 ng of total RNA was reverse transcribed into cDNA using TaqMan MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems). Real-time PCR was performed with TaqMan Universal PCR Master Mix II (4440038, Applied Biosystems, Foster City, CA) and TaqMan MicroRNA Assays (4427975, 002261, Applied Biosystems) specific for has-miR135b on ViiA 7 Real-Time PCR System (Applied Biosystems). RNU6B (4427975, 001093, Applied Biosystems) was used as an endogenous control.

2.8. Statistical Analysis. Data were compared by using a Mann-Whitney test (PASW Statistics 18, PASW, Chicago, IL).

3. Results

3.1. The Effects of ESE on LSE. Cells were treated with ESE. The results showed that ESE was slightly toxic at a concentration of 0.005% (S-Figure 1a, 1b, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3859721). To confirm these effects, different fraction was tested and similar results were obtained (S-Figure 1c, 1d). We therefore chose concentrations of 0.001% and 0.002% for LSE construction. In all the SE models, well stratified epidermis was observed and the epidermis became slightly thickened on treatment with a higher concentration (0.002%) of ESE (S-Figures 2 and 3). Integrin α6, extracellular adhesion receptors, is located along the dermal epidermal junction and it means the presence of hemidesmosomes [15]. Intense and linear staining of α6 integrin was observed along the basement membrane in ESE models (S-Figures 2 and 3). p63 is known as a stem cell marker that belongs to the p53 family of genes and it is expressed in basal and suprabasal layers of the epidermis [16]. ESE-treated SEs showed increased intensity of p63 compared with control SEs (S-Figures 2 and 3). Expression of PCNA [17] is observed in the basal layer and there was increased intensity in PCNA staining in the ESE-treated models (S-Figures 2 and 3). All experiments were repeated twice.

3.2. Identification of Phlorizin. To identify the active ingredient of ESE, we examined the effect of various fractions from an aqueous methanolic extract of ESE. For proliferation assay of keratinocytes, full medium was used by means of MTT assay (data not shown). The chemical structure of the purified compound from *E. senticosus* was determined. The 1H NMR spectrum showed the presence of two mutually coupled doublets (δ = 5.95 and 6.15, J = 1.9 Hz) that are characteristic of an unsymmetrically substituted phloroglucinol ring, a quartet (δ = 6.65 and 7.01, J = 8.4 Hz) corresponding to protons of the *para*-disubstituted benzene ring and a multiplet (δ = 3.41) and a triplet (δ = 2.85, J = 7.6 Hz) attributable to methylene protons adjacent to a ketone structure. In addition, the 1H NMR spectrum also exhibited signals arising from a β-glucopyranosyl unit (δ = 5.01, J = 7.0 Hz) and a sugar moiety (δ = 3.08–3.71). The 13C NMR spectrum showed signals for 21 carbon atoms. Analysis of COSY, HMQC, and HMBC data showed that these NMR features were consistent with the structure of phlorizin {3,5-dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenyl β-D-glucopyranoside} (S-Figure 4).

1H NMR (500 MHz, CD3OD): δ = 2.85 (t, J = 7.6 Hz, 2H, H-9), 3.08 (m, 1H, H-4″), 3.10 (m, 1H, H-5″), 3.15 (m, 1H, H-2″), 3.17 (m, 1H, H-3″), 3.41 (m, 2H, H-8), 3.50 (dd, J = 5.1 and 11.9 Hz, 1H, H-6″), 3.71 (d, J = 11.9 Hz, 1H, H-6″), 5.01 (d, J = 7.0 Hz, 1H, H-1″), 5.95 (d, J = 1.9 Hz, 1H, H-5), 6.15 (d, J = 1.9 Hz, 1H, H-3), 6.65 (d, J = 8.4 Hz, 2H, H-2′ and H-6′), 7.01 (d, J = 8.4 Hz, 2H, H-3′ and H-5′). 13C NMR (125 MHz, CD3OD): δ = 30.8 (t, C-9), 46.9 (t, C-8), 62.2 (t, C-6″), 71.3 (d, C-4″), 74.6 (d, C-2″), 78.1 (d, C-5″), 78.3 (d, C-3″), 95.2 (d, C-5), 98.3 (d, C-3), 102.1 (d, C-1″), 106.8 (s, C-1), 115.8 (d, C-2′ and C-6′), 130.2 (d, C-3′ and C-5′), 133.6 (s, C-1′), 56.3 (s, C-4′), 162.2 (s, C-4), 165.6 (s, C-6), 167.5 (s, C-2), 206.5 (s, C-7).

4. Antioxidant Activity of PZ

Vitamin C was chosen as a control. Scavenging activity was tested by DPPH radical scavenging assay. Compared to vitamin C, PZ showed relatively weak antioxidant activity

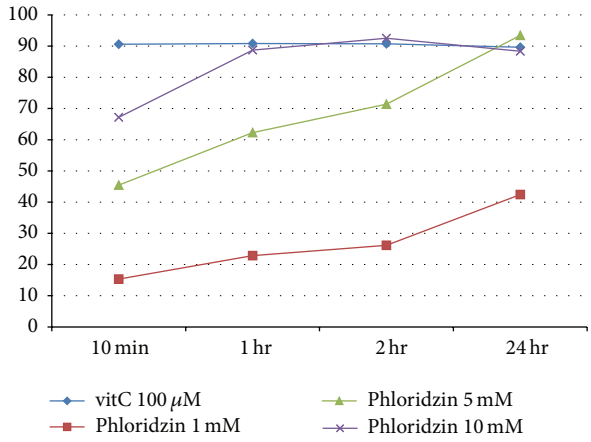

Figure 1: DPPH assay of vitamin C and phlorizin.

at low concentrations but good antioxidant activities at high concentrations (10 mM) (Figure 1).

4.1. Cytotoxicity of PZ. The cytotoxicity of PZ was analyzed by MTT assay, in which normal human keratinocytes and fibroblasts were treated with PZ. PZ was not toxic at any of the concentrations tested up to 200 μM (S-Figure 5). To test the proliferative effects on keratinocytes, normal human keratinocytes were treated for 72 hr and results showed that PZ is not stimulatory at any concentrations (S-Figure 6). The effects of PZ were then tested at concentrations of 50 μM to 1 mM in LSE.

4.2. Histology of PZ-Treated SEs. The presence of a stratified epidermis was observed in all the models. PZ increased the thickness of the epidermis (Figures 2 and 3). To see the effects on fibroblast, Hoechst 33342 stained cells were counted in 3 different parts of dermal portion in SEs and there was no significant difference according to the concentration of PZ (S-Figure 7).

4.3. Immunohistochemical Staining of PZ-Treated SEs. Integrin α6 is a marker of extracellular adhesion receptors [15] and integrin β1, expressed throughout the basal cell membrane, is important in cell-matrix and cell-cell interactions [18]. Integrins α6 and β1 were observed along the basement membrane, and the staining of both integrins α6 and β1 was significantly increased at dose-dependent manner (Figures 2 and 3). In addition, type IV collagen, a major component of basement membrane [19], was stained. Linear staining of type IV collagen increased in a dose-dependent manner in the PZ-treated models (Figures 2 and 3). p63 and PCNA expression were also increased in a dose-dependent manner (Figures 2 and 3). Experiments were repeated at least twice and the results are representative data from repeated experiments.

4.4. RT-PCR Analysis for miR135b and mRNA of Integrins and Type IV Collagen. Results showed that levels of miR135b were decreased and expression of type IV collagen mRNA was increased. But mRNA of integrin α6 and integrin β1 was not significantly changed (Figure 4).

5. Discussion

E. senticosus has similar herbal properties to *Panax* ginseng. We found that PZ is the main ingredient of ESE and the effect of PZ on human epidermal cells was tested.

Redox balance is important determinant of stem cell fate [9] and ESE as well as PZ is known to have antioxidant activity [2]. Thus, the effects of PZ were tested in terms of stem cell fate in the skin.

Epidermis is the outermost layer of the skin and needs to self-renew and regenerate continuously. This ability is dependent on keratinocyte which can proliferate and respond to differentiation cues [20]. ESE definitely increased the intensity of p63 staining compared with control SEs and it means that ESE increases the proliferative potential of epidermal basal cells. The PCNA staining also showed increased intensity in ESE-treated SEs (S-Figures 2 and 3). Extraction and chromatography showed that PZ is an active ingredient of ESE. Then, the effects of PZ were tested by similar pattern of experiments. PZ increased the thickness of the epidermis (Figures 2 and 3). Furthermore, PZ is not stimulatory to keratinocytes proliferation at any concentrations tested (S-Figure 6) in monolayer culture. This suggests that PZ may increase the proliferative potential of epidermal cells by indirect mechanism. Epidermal homeostasis depends on a balance between renewal and differentiation of stem cells and it is regulated by extrinsic signals from the extracellular matrix (ECM) [21]. The status of the basement membrane was stained. Staining of integrin α6, integrin β1, and type IV collagen increased along the basement membrane, and the staining intensity increased dose dependently (Figures 2 and 3). We then stained for p63 and PCNA. The results clearly showed that the intensity of p63 and PCNA staining are also increased in a dose-dependent manner (Figures 2 and 3). These findings suggest that PZ can affect the stem cell characteristics and proliferative potential of epidermal cells. As far as we know, this is the first report of the fact that PZ, a key component of ESE, might affect the stem cell characteristics of keratinocytes by regulating extracellular matrix proteins or transmembrane protein such as type IV collagen and integrins. Because PZ also may affect epidermal cells through fibroblasts, the number of dermal cells was compared in SEs. But there were no significant differences according to the concentration of PZ (S-Figure 6). These finding suggested that PZ may not affect keratinocytes through fibroblasts.

It is described that the behavior of all stem cells is controlled by the interplay between intrinsic transcriptional programs and extrinsic signals [21]. Recently we also reported that oligosaccharides of hyaluronic acid and various antioxidants can affect the stem cell character and proliferative potential of epidermal basal cells by providing more favorable microenvironment [9, 22]. PZ inhibits polycystic ovary disease progression by targeting sodium-glucose cotransporter [23]. Furthermore, the calcium-dependent desmosome formation was affected by the sodium-regulated keratinization in frog skin cultures [24]. In addition, human keratinocytes

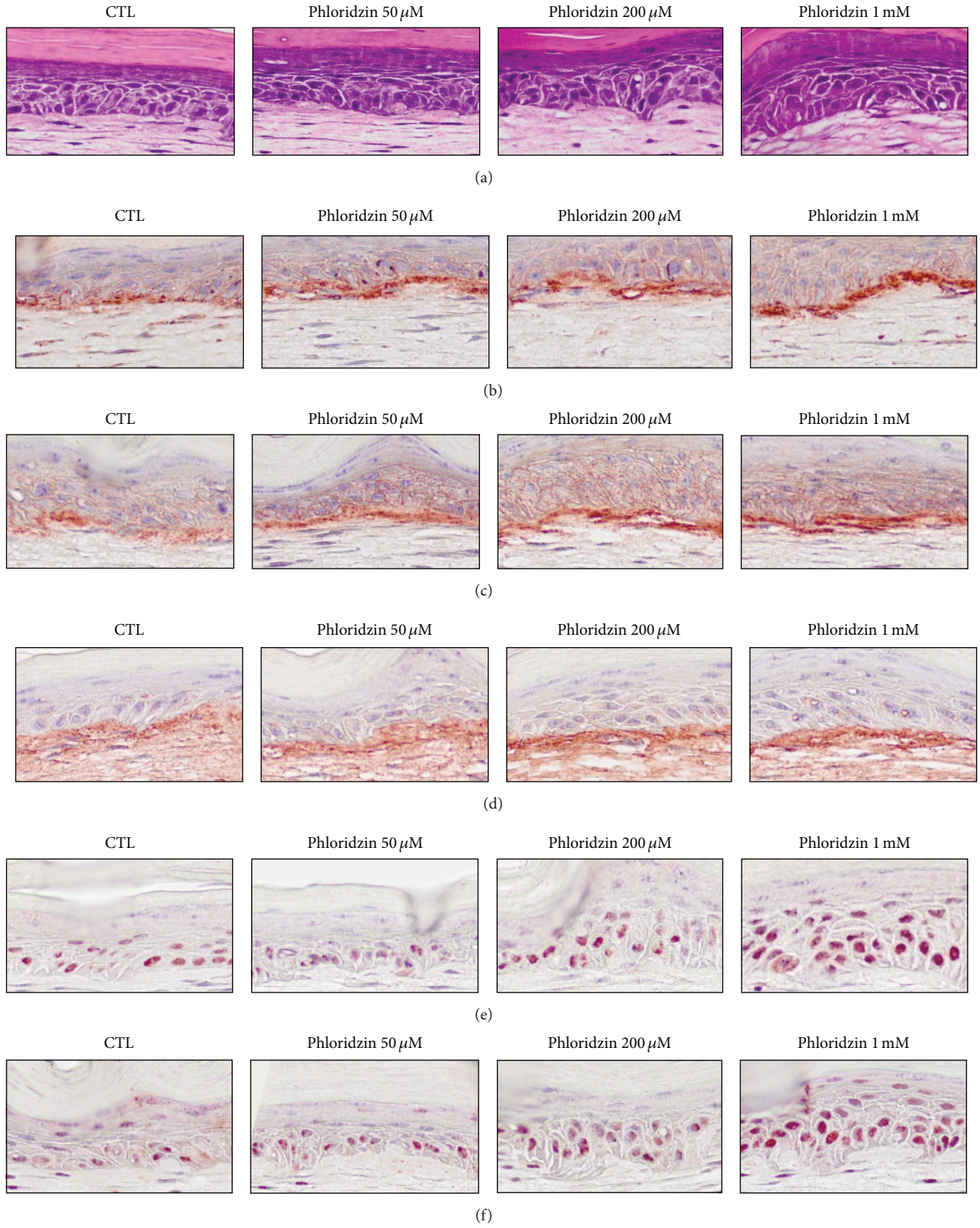

Figure 2: Histologic findings for PZ-treated SEs. SEs were constructed and then incubated in the presence of PZ (0, 50, and 200 μM or I mM). Sections of SEs were stained with hematoxylin and eosin and analyzed by immunohistochemical staining ((a): H&E staining, (b): integrin α6, (c): integrin β1, (d): type IV collagen, (e): p63, and (f): PCNA).

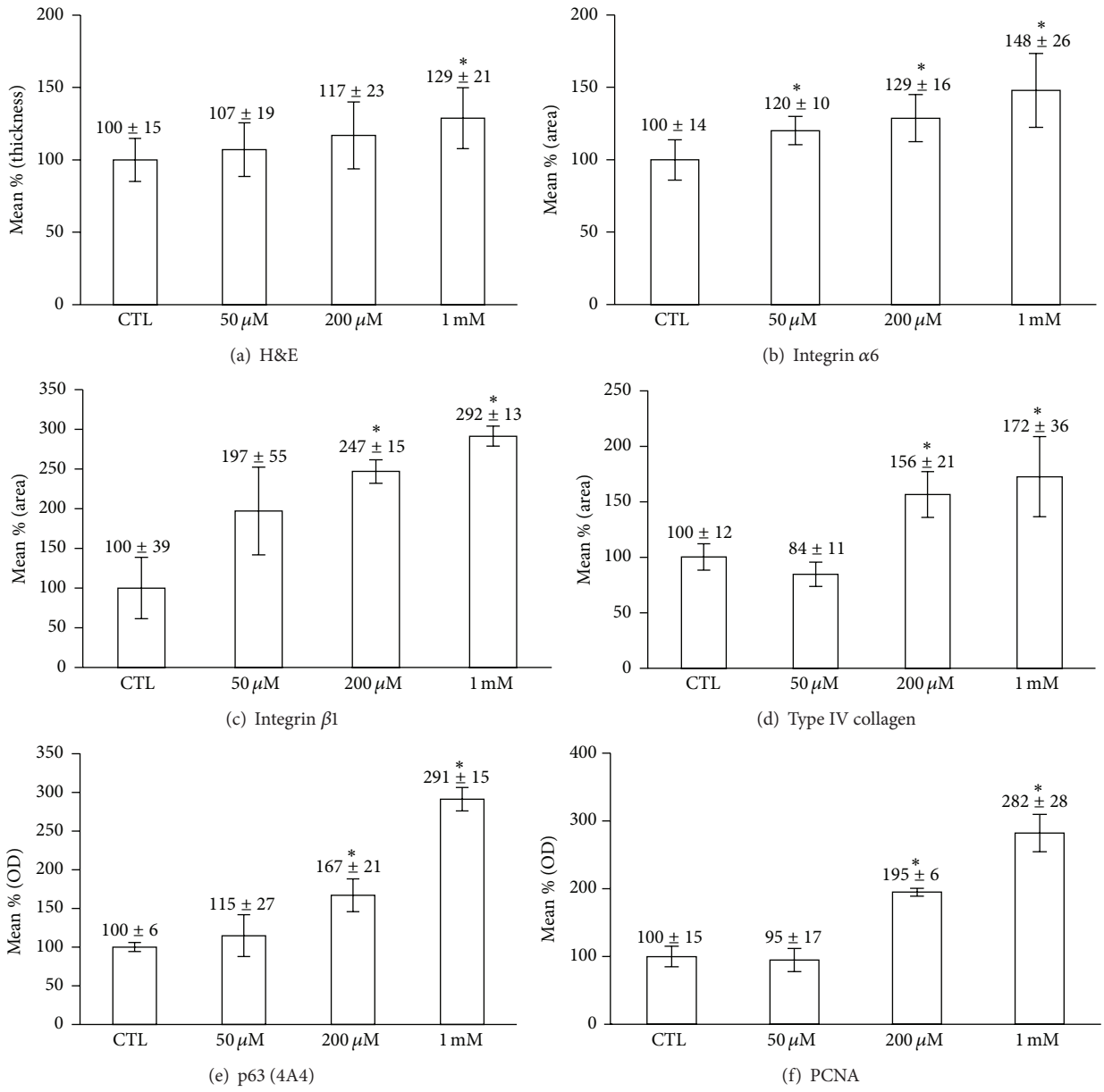

Figure 3: Comparison of epidermal thickness, staining intensity, and the numbers of p63- and PCNA-positive cells. Immunohistochemical staining was analyzed quantitatively by using Image J software (National Institute of Health, Bethesda, Maryland, USA). The positivity of p63 and PCNA was measured as described in Section 2. $^{*}P < 0.05$ compared to contro.

stem cells survive for months in sodium chloride [25]. Thus, PZ may regulate keratinocytes by targeting sodium-glucose transport. Interestingly, it is also reported that there is a relationship between sodium chloride concentration and free radical scavenging activity [26]. Cell fate is regulated by the expression of specific genes [27] and microRNAs (miRNAs) are regulators of gene expression. Recently, we found that type IV collagen is a target of miR135b and that miR135b suppression improved the microenvironment and increase the proliferative potential of basal cells [12]. It disclosed a role of miR135b in epidermal keratinocytes and may provide a way to control stem cell fate in the skin. All these findings showed that topical application of ESE or PZ may have antiaging effects on the skin through miR135b-type IV collagen pathway. Thus, miR and extracellular matrix mediated pathway can be an important antiaging strategy.

In this study, PZ increased the staining intensity of $\alpha 6$ integrin, $\beta 1$ integrin, and type IV collagen which is associated with increased number of p63 and PCNA positive cells. RT-PCR also showed that type IV collagen mRNA was increased but the expression levels of both integrins $\alpha 6$ and $\beta 1$ mRNA was not changed. The reason of these differences may be derived from different models but needs further extensive study. Furthermore, level of miRNA135b was decreased by PZ treatment. Type IV collagen is a main scaffold of basement membrane. Thus, all these findings suggested that PZ can

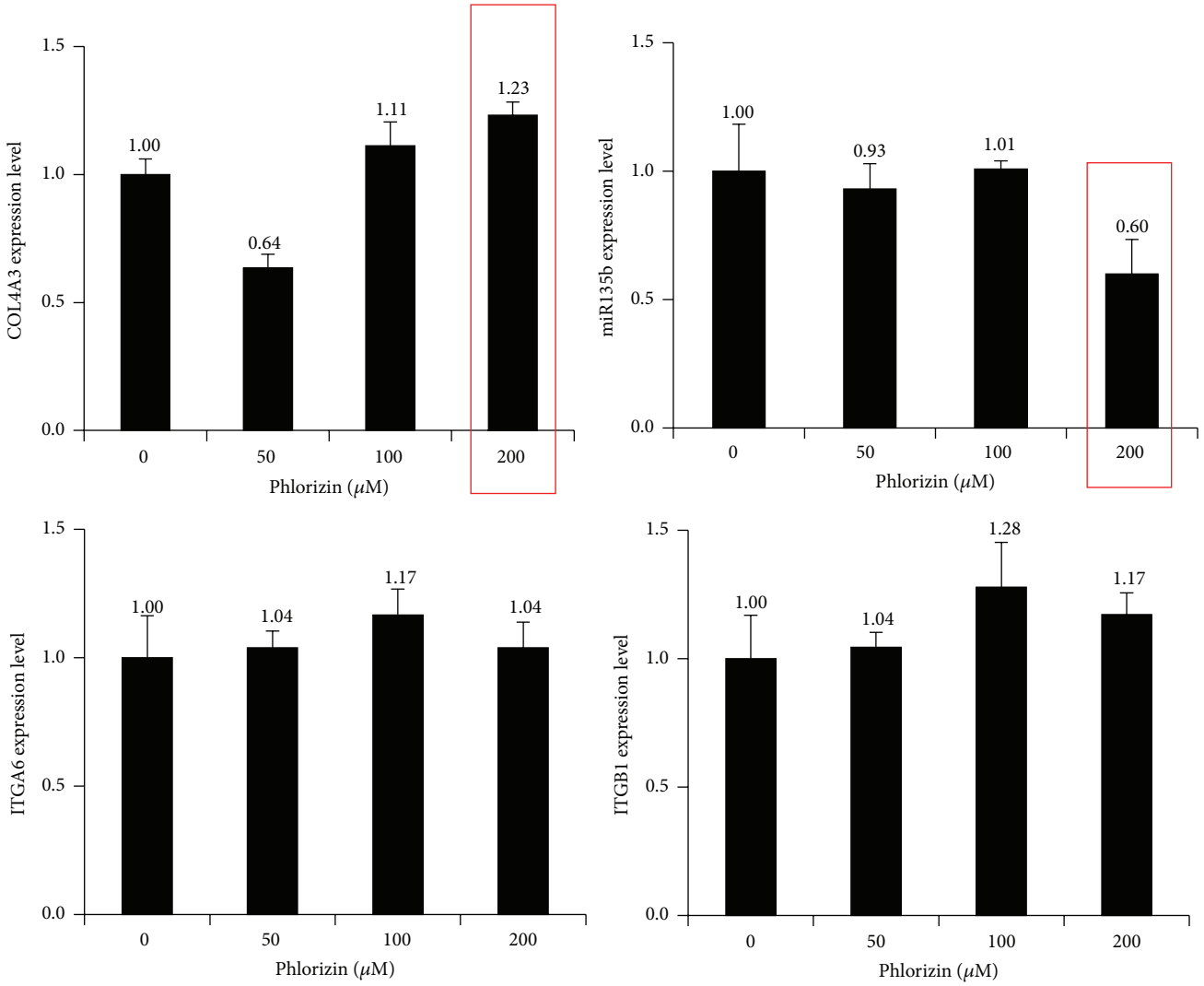

Figure 4: RT-PCR analysis of miR135b and mRNA of integrins and type IV collagen. Keratinocytes were treated with increasing doses of PZ and total RNA was extracted and reverse transcribed into cDNA. Real-time PCR was performed as described in Section 2.

affect the niche condition by increased expression of type IV collagen.

6. Conclusions

It can be concluded that PZ, which has antioxidant activity, can affect stem cell fate in the skin via inhibition of miR135b and following synthesis of type IV collagen of basement membrane.

Conflict of Interests

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

Authors' Contribution

Hye-Ryung Choi, Kyung-Mi Nam, and Seung-Hye Yang contributed equally to this work.

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Review Article

Ageing-Associated Oxidative Stress and Inflammation Are Alleviated by Products from Grapes

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Advanced age is associated with increased incidence of a variety of chronic disease states which share oxidative stress and inflammation as causative role players. Furthermore, data point to a role for both cumulative oxidative stress and low grade inflammation in the normal ageing process, independently of disease. Therefore, arguably the best route with which to address premature ageing, as well as age-associated diseases such as diabetes, cardiovascular disease, and dementia, is preventative medicine aimed at modulation of these two responses, which are intricately interlinked. In this review, we provide a detailed account of the literature on the communication of these systems in the context of ageing, but with inclusion of relevant data obtained in other models. In doing so, we attempted to more clearly elucidate or identify the most probable cellular or molecular targets for preventative intervention. In addition, given the absence of a clear pharmaceutical solution in this context, together with the ever-increasing consumer bias for natural medicine, we provide an overview of the literature on grape (*Vitis vinifera*) derived products, for which beneficial effects are consistently reported in the context of both oxidative stress and inflammation.

1. Introduction

With ageing, the capacity of the body to function optimally declines. There is a combination of genetic and lifestyle factors which may either accelerate or slow down the ageing process. A number of chronic diseases are associated with advanced age: these include cardiovascular disease (CVD), diabetes, metabolic syndrome, and Alzheimer's disease. This results in an exponential increase in the disease burden on modern society, relative to a few decades ago, due to longer life expectancy. The World Health Organisation states that from 2015 to 2050, the proportion of the world's population over 60 years will increase from 12% to 22% [1]. Taking into consideration that predominant research in the field of age-associated diseases considers the sixth decade of life to be a risk factor for the rapid progression and onset of age-associated diseases, the disease burden will almost double in the next 35 years. It is thus vital not only to elucidate the causes and progression of these chronic conditions, but also to actively search and investigate potential preventative therapies that may slow the processes contributing to physiological ageing.

Although the ageing-associated chronic disease states are each uniquely complex in terms of their aetiology, development, and progression, they do share common aetiologies which stem from two main entities, namely, cumulative oxidative stress and chronic inflammation. Briefly, oxidants are produced by normal cell metabolism and various physiological responses. However, when the production of oxidants outweighs the capacity of endogenous antioxidant systems, oxidative stress is incurred. Furthermore, while inflammation is crucial for repair of tissue injury and primary defence against invading pathogens and chemicals, it also results in unintended detriment to previously uninjured cells. Although these are necessary systems in the body, both oxidative stress and the inflammatory response, if unchecked, can have detrimental consequences which have been linked to accelerated ageing and the progression of

age-associated disease. We postulated that the effects of the inflammatory immune system and oxidative stress on allostatic load are interlinked. This has led us to investigate the potential of antioxidants as treatment options to attenuate the cumulative effects of both oxidative stress and chronic low grade inflammation. Given the modern consumer bias for natural medicines, we focused on a group of plant medicines which are consistently associated with beneficial effects on these processes in the literature, grape-derived polyphenols. In this review, we will provide a more in-depth review of interconnected molecular mechanisms of oxidative stress and inflammation in the physiological ageing process, before moving our focus to a discussion of the merits of these plant medicines as potential preventative therapy in this context.

2. Contribution of Oxidative Stress to Premature Ageing

In the context of ageing, reactive oxygen and nitrogen species (RONS) are generally the major molecules which contribute significantly to oxidative stress. The most frequently studied free radicals are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($^\bullet OH$), peroxynitrite (ONO_2^-), and nitric oxide (NO). The generation of these free radicals is necessary as they are essential for host defence: phagocytic cells use ROS to digest invading pathogens and debris. Furthermore, they act as signalling molecules, regulating cell growth and apoptosis, adhesion, and differentiation. More specifically, RONS are formed during processes such as the mitochondrial electron transport chain as well as enzyme systems such as cytochrome P450, lipoxygenase and cyclooxygenase, the NADPH-oxidase complex, xanthine oxidase, and peroxisomes [2]. In contrast to these roles in growth, repair, and immune functions which are all beneficial to the host, these molecules also have the ability to oxidise signalling molecules, DNA, macromolecules, and cell structures such as lipid membranes of healthy host cells, all of which are to the detriment of these cells. Usually, each cell has defence mechanisms to counteract the occurrence of oxidative stress. These endogenous enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase, catalases, glutathione/TrxR, and peroxiredoxins. An appropriately nutritious diet is vital to maintain these systems, as these natural antioxidants are supplemented or replenished by antioxidant constituents of various fruits and vegetables. It is only when the capacity of the body's antioxidant defences is outweighed by the rate of production of free radicals that oxidative stress is incurred by unquenched free radicals which can alter surrounding cell structures and environment.

With advancing age, various factors, among these is a natural progressive decline in endogenous antioxidant capacity, cause disruption in the balance between pro- and antioxidant mechanisms and RONS accumulate beyond the normal endogenous antioxidant system's quenching capacity, resulting in cumulative oxidative stress. Eventually this causes cell damage which cannot be repaired by internal mechanisms, leading to loss of organ mass and functionality, which ultimately culminates in system dysfunction [3]. Indeed, long-term oxidative stress states have been linked to various diseases associated with advanced age, most notably diabetes, chronic obstructive pulmonary disease (COPD), cardiovascular disease, cancer, diabetes, and asthma [4]. Many of these diseases are not only associated with ageing anymore, but also with the high-obesity, high-stress, and sedentary modern lifestyle (albeit perhaps in milder form) [5]. It is thus vital to address the prevention of these detrimental long-term outcomes, as they are afflicting not only the aged, but also relatively young populations, such as university students [6, 7].

The aetiological mechanism(s) of the various chronic diseases mentioned are each different, as each disease has its own complexities. However, chronic cumulative oxidative stress is a common factor in these diseases, highlighting this system as a vital therapeutic and/or preventative medicine target [8].

Even in the absence of age-associated disease, various theories have linked oxidative stress directly to the normal ageing process. We briefly mention two here. Firstly, the Free Radial Theory of Ageing proposes that the presence of free radicals and their effects on cells are one of the causes of cell ageing and subsequent cell death, which in turn lead to loss of organ mass and other features of whole organism ageing. This theory was first suggested by Harman in 1956 [9], who hypothesized that irradiation of cellular components resulting from the liberation of $^\bullet OH$ and $^\bullet HO_2$ radicals would lead to dysfunction and mutations which cannot be reversed and thus result in ageing. This theory was later supported and expanded as research began elucidating the details of his proposed theory, for example, by describing the role of SOD [10]. Phenomenally, a very recent meta-analysis [11] confirmed the sustained validity of this theory, 60 years later. Of course, given the technological advances made over this period, it is no surprise that subsequent research has added more detail on mechanisms in support of this theory, although many unresolved questions remain [12].

Secondly, the Replicative Senescence Theory is based on the hypothesis that oxidative stress induces cell death and/or senescence, which necessitates an increase in the rate of cell replication. This in turn accelerates the detrimental effects associated with repeated cycles of mitosis. Telomere length and its accelerated shortening due to reparative replication is the basis of this theory, which was first described by Hayflick in 1965, in response to the observation of a decline in functionality of cell cultures (fibroblasts) which had undergone numerous cell divisions [13]. This phenomenon was subsequently confirmed by experiments on primary peripheral blood mononuclear cells (PBMCs) and fibroblasts from a largely aged population with increased risk for vascular dementia [14]. In this report, decreased telomere length in both fibroblasts and PBMCs was correlated to risk for dementia induced by stroke. In addition, telomere shortening rate was reported to decrease with increasing antioxidant capacity in fibroblasts of the same population.

This theory, together with the subsequent data, presents evidence that, firstly, oxidative stress is responsible for the accelerated shortening of telomeres brought about by more frequent reparative replication and, secondly, that an increase in antioxidant defence capacity could slow down the ageing process. Note at this point that we do not infer that telomere shortening results only from oxidative stress: other mechanisms such as chromosomal instability have indeed been linked to both ageing and pathological conditions such as cancer [15]. However, to remain focused on the main topic of this review, we have limited this discussion to *accelerated* telomere shortening in the context of cumulative oxidative stress.

Holistic interpretation of the two theories introduced above implicates oxidative stress as major causative role player in the damage to cell constituents by oxidising membranes, molecules, and DNA. This initiates a cascade of events leading to the need for either cell growth and replication for repair, or death. This implicates ROS as rate determining factor of cell lifespan due to the direct damage it inflicts. Furthermore, oxidative stress is implicated in causing irreversible damage to the mechanism of replication through accelerated telomere shortening and thus ultimately decreases the capacity of the cell to replicate optimally, or at all. With both repair and replication affected, cell senescence is encouraged.

In keeping with the idea of a holistic approach, consideration of oxidative stress in isolation is insufficient. A basic but practical example of the interplay between the oxidative stress system and another system implicated in ageing, inflammation [16], is data available on detrimental effects of cigarette smoking. In this context, both acute smoking and long-term smoking were shown to overwhelm the glutathione antioxidant defence system within the lungs, which was associated with significant infiltration of inflammatory immune cells in the lungs [17]. This study clearly shows system interaction. Furthermore, in the same study, the severity of this maladaptation increased with duration of habitual smoking (in years) and was exacerbated by natural decreases in antioxidant capacity as experienced with ageing, resulting in increased oxidative stress, illustrating the significance of cumulative damage.

However, before considering these interactions in more detail, the literature providing proof of a role for chronic inflammation in the process of ageing will be briefly reviewed.

3. Chronic Low Grade Inflammation Facilitates Premature Ageing

As is the case for oxidative stress, the inflammatory response is a system essential for normal body function [18]. As component of the innate immune response, inflammation is a major first-line defence against infection and injury [18]. Apart from this largely independent, nonspecific role in immunity, inflammation is also vital for many specific immune responses to run its course [19]. However, in the process of repair and restoration after insult, the inflammatory response inadvertently disrupts cellular homeostasis of previously unharmed or unaffected cells [20, 21]. The injury-repair cycle which inflammation regulates is an efficient system during youth, when optimal sensitivity and response to signalling molecules (such as cytokines, growth factors, prostaglandins, and peptides) maintain the general health of circulating immune cells and the tissue microenvironment, with minimum secondary damage. However, during natural chronological ageing, long-term, repeated stimulus-response cycles change the receptor expression levels and thus sensitivity to these molecular stimuli [22]. This may necessitate relatively increased concentrations of any particular stimulus to maintain the required effect. Commonly reported characteristics of the natural ageing process include inflammation or oxidative stress-associated symptoms such as directionally inaccurate chemotaxis, premature or suboptimal respiratory burst, and increased proinflammatory signalling from immune cells, all of which form the basis of immunosenescence [23].

Immunosenescence is the term used to describe ageing of immune cells and the functioning of the immune system as a whole. This occurs naturally with advancing chronological age or as result of lifestyle factors, as already mentioned. There is more than one way in which the immune system is compromised upon ageing. Firstly, immunocompetent cells are derived from hematopoietic stem cells. With ageing, a natural bias develops for stem cells to commit to expansion of the myeloid lineage at the cost of the lymphoid lineage [24]. This results in a shift in the balance of immune cells available to enter the circulation. Secondly, the chronic low grade inflammation associated with ageing recruits larger numbers of cells into circulation from the hematopoietic tissue. However, despite the higher circulating cell counts, phagocyte Toll-like receptor expression and phagocytic capacity are decreased in the aged [22], leaving the immune system with a lower capacity for becoming activated and for responding to a more acute insult, such as viral infection. These maladaptations, which together further predispose the individual to proinflammatory responses, are postulated to stem at least in part from alterations in HPA-axis signalling.

The process of immunosenescence may be accelerated by unhealthy lifestyle, such as psychological stress and obesity. As mentioned before, lifestyle-associated diseases share clinical symptoms associated with normal ageing. Indeed rutin, a potent antioxidant, has been shown to protect against ageing-related metabolic dysfunction [25]. Thus, studies focused on those conditions may provide much insight in terms of ageing and the supplementation options to limit its progress.

For example, in the context of obesity and/or inactivity, cytokines release from adipose tissue macrophages has been demonstrated by many researchers [26–29]. Tumour necrosis factor-α (TNF-α), interleukin- (IL-) 1β, and IL-6 are among some of the cytokines shown to be released from resident macrophages in adipose tissue, resulting in a proinflammatory microenvironment [30]. Furthermore,

chronic stress, and in particular psychological stress, is a generally accepted cause of chronic low grade inflammation. For example, in 38 medical students, psychological stress was associated with increased proinflammatory cytokine (TNF-α, IL-6, IL-1Ra, and IFN-γ) levels, as well as decreased anti-inflammatory regulators (IL-10 and IL-4) [31]. Interestingly, these effects were exacerbated by high anxiety proneness as trait, again suggesting that a cumulative stimulus (in this case lifelong anxiety) further exacerbates the undesirable adaptation.

Also in posttraumatic stress disorder (as extreme form of chronic psychological stress), an initial glucocorticoid hyperresponse is followed by glucocorticoid hyporesponsiveness, which is associated by a relatively proinflammatory state. In this condition of continuous proinflammatory signalling, the feedback systems, which usually downregulate inflammation, adjust overtime and result in maladaptations such as chronic but low grade upregulation of proinflammatory mediators (e.g., IL-6, TNF-α, IL-1β, and prostaglandin E_2) [32]. Incidentally, the secretion of the first three is mediated by the NF-κB pathway, which is activated in response to cellular stress [33].

In addition to increased proinflammatory signalling, other more mechanical cellular mechanisms also seem to be compromised over time. For example, inappropriate and/or insufficient neutrophil responses result from its decreased phagocytic capacity, increased basal levels of intracellular calcium, and the resulting reduction in capacity for chemotaxis [34]. The result is a chronic low grade inflammatory status in relative absence of a specific threat which can persist for extended periods of time and cause harm and inefficiency of the system. Thus, although varied in specific causative mechanism, the outcome of all of these suboptimal life events or lifestyle habits, for example, chronic stress leading to glucocorticoid resistance and/or cardiovascular disease and high-calorie diets and inactivity (obesity) leading to insulin resistance and diabetes, is that of a chronic inflammatory state [16].

Of particular interest in the context of ageing is the fact that, apart from the now notorious low grade inflammation as primary culprit, this maladaptation results in a compromised capacity to mount an efficient inflammatory response to acute insults. As overviewed in the review by Weinberger and colleagues [35], several reports from clinical literature support the notion that, in the aged individual, that there is a significant increase in the convalescence period required for recovery from injury and pathogen clearance, as well as a decrease in the quality of repair, thus favouring disease progression and morbidity due to injury. Very recently, Baëhl and colleagues (2015) demonstrated, in a longitudinal study of elderly patients, that the acute stress of a hip fracture had a negative effect on neutrophil function immediately after injury. While some neutrophil functions (chemotaxis, phagocytosis) were recovered over time, several others (superoxide production, complement C5A and CD11b receptor level, and cytokine secretory profile) were still impaired even 6 months after injury. From this, the authors concluded that the acute stress had a long-term negative effect on neutrophil responses, which negatively influenced clinical outcomes, such as the resolution of long-term inflammation, recovery, and susceptibility to opportunistic infections [36].

It is thus clear that ageing is an inflammatory-mediated process. However, from the inflammation/ageing literature, it is clear that inflammation and oxidative stress cannot be separated as causative factors in this context. For example, the ageing-related shift in balance between the glucocorticoid and inflammatory systems has been linked to increased ROS production, which in turn exacerbates low grade immune activation [37].

4. Links between Inflammation and Oxidative Stress in the Ageing Process: Identifying Therapeutic Targets

From the above sections it is evident that, in ageing, oxidative stress and inflammation are interdependent mechanisms. We postulate that unravelling and understanding of these intricate links between the two responses hold the answer to identification of the major contributor(s) to allostatic load and maladaptation associated with ageing and age-related pathology.

Generally, repeated exposure to reactive oxygen and nitrogen species causes cell damage and thus a proinflammatory signalling response. For example, in aged mice, unquenched reactive oxygen and nitrogen species act as proinflammatory signalling molecules and mediators of inflammation within the cell itself [38]. More specifically, oxidative damage to cells prompts the release of TNF-α from these damaged cells [21]. Binding of TNF-α to cell surface TNF-α receptors activates the NF-κB inflammasome, which results in the further production of other proinflammatory cytokines, most notably IL-1β. Incidentally, TNF-α specifically has also been implicated in ROS-mediated upregulation of adhesion molecules which facilitate the infiltration of immune cells into tissue [39], with more on this later. Upregulation of inflammation via the NF-κB inflammasome is probably the main aetiological mechanism for age-related chronic conditions with an inflammatory component. Indeed, TNF-α upregulation, which is a direct result of increased flux through the inflammasome, has been implicated as causative factor in cardiovascular disease [40]. Of particular relevance to the topic of ageing, the NF-κB inflammasome has regulatory roles in cell growth, survival, and proliferation. However, as recently reviewed [41], ROS production may have either inhibitory or stimulatory roles in the NF-κB pathway, suggesting a dose-dependency of the effects of ROS. This unfortunately also means that development of an intervention strategy/product to modulate this target mechanism is no simple feat and will have to be approached in a very tightly controlled "modification range."

From the literature consulted, it is clear that bidirectional communication is in place. For example, both neutrophils and macrophages are producers of oxidants *via* the NADPH-oxidase system [42]. The NADPH-oxidase (NOX) proteins

aid the transport of electrons across biological membranes and are found in all cells [43]. They are also one of the major generators of ROS in all cells. Particularly, these proteins are the predominant ROS producers in phagocytic cells, a process required for the normal respiratory burst that phagocytes use to kill pathogens and digest cell debris.

Furthermore, activated neutrophils release myeloperoxidase (MPO), which contributes to the formation of hypochlorous acid (HOCL) by acting as a catalyst when reacting with hydrogen peroxide (H_2O_2). This directly increases the production of ROS [44]. The oxidative burst of neutrophils in itself releases oxidants such as H_2O_2, which are harmful to healthy cells and tissue [45]. Besides directly increasing the production of reactive oxygen species itself which causes damage to surrounding cells, MPO specifically has been implicated as a risk factor for coronary artery disease, due to its capacity for oxidation of lipid membranes [46]. The increased oxidant concentration due to immune cell functions, as well as the resultant cell damage, results in increased metabolism in surrounding healthy tissue.

Also at gene level, role players have been elucidated in the context of a ROS-inflammation link. For example, sirtuins are Class III histone deacetylases which are responsible for the deacetylation at N-epsilon lysine residues, a reaction which consumes NAD+. SIRT1 specifically is a sirtuin commonly associated with antioxidant function [47]. Its regulation of oxidative stress is threefold: firstly, it stimulates the expression of antioxidants *via* the fork-head box protein O (FOXO) pathway. Secondly, it is involved in inhibiting the NF-κB signalling pathway. In contrast, however, excessive ROS can inhibit SIRT1 activity by oxidatively modifying its cysteine residues and thereby releasing its inhibition of the NF-κB pathway [47]. It is thus theoretically possible for cumulative stress to downregulate SIRT1 activity in the ageing process. Thirdly, SIRT1 has also been implicated in regulation of apoptosis by deacetylating p53 to inhibit p53-dependent transcription in models of cellular stress [48]. This tripartite role defines SIRT1 as another important molecular target in the context of both normal and accelerated ageing.

Apart from these targets related to cellular signalling, cell functional capacity should also be a focus. A striking example of the oxidative stress, inflammation link in ageing, is the decreased capacity for neutrophil chemokinesis reported in the elderly both in terms of motility and accuracy [49]. Normally, immune cells are attracted by chemotactic signals from injured tissue, to migrate to sites of injury or pathogen invasion. During this chemokinetic response, immune cells, typically neutrophils and classically activated macrophages, migrate through tissue toward the site of injury. This movement is facilitated largely *via* adhesion molecules such as the beta-integrins and I-CAM1 in the case of neutrophils [21]. However, as mentioned earlier, expression of adhesion molecules on neutrophils increases with ageing [39], so that their movement is slowed. In addition, due to yet unclear mechanisms, but most probably due to adaptation of cellular "homing" molecules, directional accuracy of neutrophil migration is also compromised in the elderly. Sapey et al. showed that inaccurate neutrophil migration was causally associated with increased constitutive phosphoinositide 3-kinase (PI3K) signalling [49]. This results not only in inefficient inflammation due to prolonged response time, but the directional inaccuracy of movement also results in mechanical and oxidative damage to relatively more cells in the path of the migrating inflammatory cell [49].

It is clear that both oxidative stress and inflammation are able to induce and exacerbate one another (both indirectly and directly). Furthermore, regardless of the primary signal or which pathway was activated first, these interrelated processes form a vicious cycle which is difficult to target therapeutically because of its complexity. However, it is also this interrelated nature of the two systems that has led us to investigate the possibilities of antioxidants as treatment options to attenuate the cumulative effects of oxidative stress and in turn low grade chronic inflammation.

5. Are Grapes the Answer to Prevention of Ageing?

Despite the huge range of nonsteroidal and natural product anti-inflammatories on the market today, the scientific literature shows a conspicuous lack of consistent support for any specific medication. This is perhaps at least in part due to the fact that researches investigating these products cannot keep up with the rate at which new ones are pushed onto the market. Hopefully new legislation on the control of these substances will affect this trend to the benefit of the consumer, by allowing for (or demanding) appropriate testing of these products.

Nevertheless, antioxidants are being used almost routinely by many individuals who wish to supplement for enhancement of general health or as adjuvant therapy in conjunction with more mainstream, pharmaceutical medication. Although they are generally not regarded as a primary therapeutic option, antioxidants may hold particular potential in the realm of preventative medicine. The potential benefits of appropriate antioxidant supplementation are vast, especially when considering the connection between oxidative stress and inflammation. An antioxidant with the capacity to modulate inflammatory status can thus be beneficial to both normal ageing individuals and those suffering from lifestyle-associated diseases.

A comprehensive search of the scientific literature revealed that grape-derived antioxidants are consistently reported to have high benefit and low risk in the context of both oxidative stress and inflammation. These positive results are further strengthened by the fact that these consistent findings were reported across many different models and using a variety of different preparations, ranging from relatively crude extracts to highly purified ones. For example, in terms of purified polyphenols, resveratrol, one of the best-known polyphenols present in grapes as well as

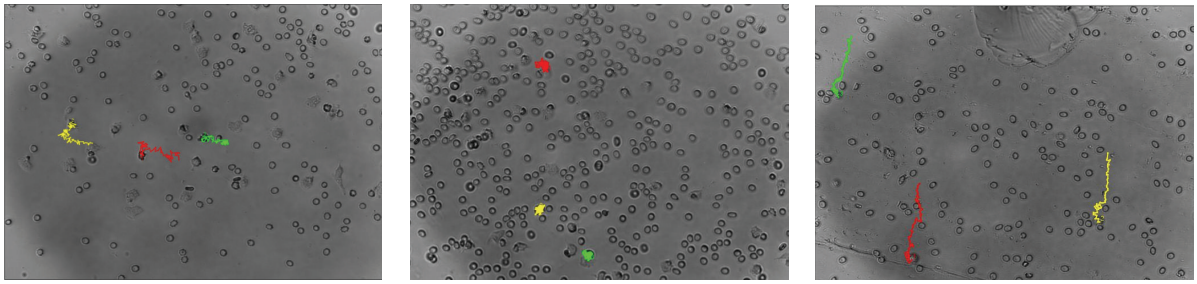
other plants, is commonly linked to anti-inflammatory [50], antioxidant [51, 52], and thus by implication antiageing effects in the scientific literature, as well as anecdotally. Indeed, a recent paper [53] elucidated a role for resveratrol to protect against inflammatory damage via SIRT1 inhibition of the NF-κB pathway (a mechanism discussed above in the context of ageing). In addition, more advanced studies have been undertaken on this polyphenol to better understand the relationship between the chemical structure of resveratrol and its biological activity, especially in terms of oxidant scavenging [54]. Also, pharmaceutical groups have been working on optimisation of delivery systems for resveratrol [55]. Such information may further advance the popularity of this very promising natural product with the pharmaceutical industry, to the benefit of consumers. The phenomenal frequency at which new papers on resveratrol appear, all providing evidence of positive effects in this context, suggests that this particular polyphenol should be investigated in the context of ageing as a matter of urgency.

Even more promising than the many positive effects described for resveratrol above is the fact that resveratrol is only one from a range of equally beneficial substances contained in grapes. The flavonoids quercetin and dihydroquercetin (DHQ), as well as proanthocyanidins and anthocyanins, all of which are present in grapes and a variety of other plant sources, have similarly been linked to both antioxidant and anti-inflammatory effects [50, 52]. To date, despite appearance of a few very promising reports in this context, ageing specifically has not been the focus of many studies investigating these substances. Therefore, for the purpose of this review, we provide a comprehensive overview of the few existing ageing-related studies in this context that were available to us. Results from relevant papers that did not have ageing as a focus were also included, where those results contribute to our understanding of the role of grape-derived polyphenols in the oxidative stress, inflammation link in the context of prevention or deceleration of the ageing process.

When considering *in vivo* studies on ageing as a starting point, resveratrol (0.1 μM to 2.5 μM) exhibited a clear dose-dependent effect on longevity in fish with known short lifespan: resveratrol supplemented fish almost doubled their expected 13-week lifespan and continued to produce healthy offspring long after all control fish had died. Even though resveratrol supplementation was only started in adulthood in this study (i.e., it compares to when humans might start to consider supplementation), it effectively delayed age-dependent compromise of locomotor and cognitive performance and reduced expression of neurofibrillary degeneration in the brain [56]. This result of improved neural morphology was recently further substantiated in an aged rat model, where chronic resveratrol treatment prevented detrimental changes in dendritic morphology which is linked not only to ageing, but also to Alzheimer's disease [57]. Similarly, 2 months of ingesting polyphenols in the form of 10% grape juice resulted in enhanced potassium-evoked dopamine release and cognitive performance in aged rats [58].

A more recent review [59] provides more insight into the potential mechanisms by which age-related cognitive disorders may be curbed by grape polyphenols. Some of these mechanisms at first seem unrelated to the scope of this review, for example, preventing of amyloid-beta deposition associated with Alzheimer's dementia [60]. However, recent research suggests a role for inflammation in the development of the disease [61], while natural antioxidants have been linked to prevention of amyloid-beta deposition [62]. Together, these data suggest that even these seemingly unrelated mechanisms may be interconnected to either inflammation, or oxidative stress, or both. However, more clearly in context of this review, resveratrol was reported to increase NO production, resulting in vasodilation [59, 63], which may play a role in the maintenance of central circulation and thus perhaps slower degenerative central processes, as has indeed been reported for resveratrol, as mentioned earlier. However, the role of NO in the context of antioxidant status is much more complex, so that this effect of grape polyphenols should probably receive more attention before it can be interpreted fully in terms of mechanism(s) involved. Furthermore, recently in a coculture simulation of the human blood-brain barrier, another grape polyphenol, proanthocyanidin, was associated with significant inhibition of monocyte infiltration and proinflammatory cytokine secretion in HIV-associated neuroinflammation [64]. Such inhibition of neuroinflammation is associated with a better prognosis in terms of HIV-related neurodegeneration and dementia, further confirming the neuroprotective potential of grape-derived antioxidants. Taken together, these studies suggest that the neuroprotective effects of grape polyphenols involve both antioxidant and anti-inflammatory mechanisms, with the latter including not only modulated cytokine signalling, but also modulation of both motility and functional capacity of leukocytes, as previously illustrated by our group [64, 65].

One may argue that both these results may be the result of decreased cell activation, perhaps as a result of the known altered cytokine environment. However, an age-associated lack of neutrophil chemokinetic accuracy in response to the chemotaxin fMLP has been reported [49], which suggests that the mechanism is probably related to age-induced compromise of specific cellular mechanisms, rather than activation. In addition, in an ongoing study by our group, we have been able to illustrate by using Dunn chamber chemokinetic assays that grape polyphenols (specifically proanthocyanidin) are able to correct this age-associated anomaly (unpublished data). From Figure 1, which depicts typical digital images obtained for the path of individual neutrophils, it is clear that a more purposeful, directionally accurate movement was achieved in proanthocyanidin-treated neutrophils. This will ensure a more optimal inflammatory response (i.e., the response will be effective, result in relatively insignificant secondary damage, and be resolved in the minimum amount of time). In contrast the rather "aimless wander" of untreated neutrophils from aged individuals will result in not only ineffective immune cell infiltration to sites where they are required, but also relatively more secondary tissue damage and thus prolonged and exacerbated inflammation.



(a) (b) (c)

Figure 1: Typical neutrophil chemotaxis pathways for (a) a young participant (<25 yr), (b) an aged participant (>65 yr), and (c) an aged participant after acute *in vitro* treatment with grape-deed derived proanthocyanidin. The Olympus Cell® system IX-81 inverted fluorescent microscope system with an F-view cooled CCD camera (Soft Imaging Systems) at 20x magnification was used to capture these images, which was analysed using Image J (Java software).

Turning attention to inflammation and oxidative stress again, the most probable targets for therapeutic intervention in the context of ageing have been described in Section 4. Since the ageing literature is relatively lacking in terms of papers on polyphenol intervention, we have tabulated effects of grape polyphenols reported for these identified targets in Table 1, citing relevant data that was mostly obtained in models other than ageing. The aim with this table was not to present all studies on grape-derived products; rather, it is an attempt to show the many models, species, and disease systems in which beneficial effects on oxidative stress and inflammatory status have consistently been reported. Also, although we have included doses used for *in vivo* studies for general comparison and again to illustrate the large variations in doses, these doses are product/extract specific and cannot be extrapolated across board.

From Table 1, which is by no means a complete list of all studies reporting on grape-derived substances, grape-derived products are undeniably beneficial in limiting the magnitude of the inflammatory response as well as to increase antioxidant activity and seem to have multiple targets.

Of course, when considering potential antiageing modalities, it is also of interest to include evaluation of changes in quality of life. Since ageing is associated with natural muscle wasting or sarcopenia [83], it is important to note that, in this context, supplementation with grape polyphenols (50 mg/kg/day) for 4 weeks mitigated skeletal muscle atrophy in a mouse model of chronic inflammation [84]. This was achieved via modulation of two distinct pathways: one directly linked to inflammation (decreased NF-κB activation) and the other due to antioxidant function (limited ROS-associated mitochondrial damage and caspase-3 and -9 activation). Since caspase-3 activation is also a known proapoptotic signal [85], reduced activation and thus apoptosis may result in fewer mitotic cycles. This, in the context of the telomere hypothesis, may point to deceleration of ageing by the polyphenols. Very recently, grape proanthocyanidin treatment in rats was reported to have an antiapoptotic effect which reduced damage after ischemia/reperfusion of the liver [86], which further substantiates this theory.

Interestingly, a study in mice fed a high-fat diet indicated that grape polyphenols may modify gut microbial community structure to result in lower intestinal and systemic inflammation [87]. This extraordinary result serves to remind us of the potential complexity of plant medicines and the requirement for comprehensive investigation of mechanisms of actions and interactions of any potential product *via* the traditional clinical trial process followed for new pharmaceutical drugs.

6. Conclusion

Ageing and accelerated ageing are not new concepts, but rather the norm in modern society. With a population that is growing relatively older due to advances in medicine, we are however pressed for answers on how to alleviate the symptoms or slow the progression of this inevitable phenomenon. From the literature consulted, no negative effects of grape-derived products became evident, while beneficial effects in the context of oxidative stress and inflammation were consistently reported in the context of numerous cellular targets. Huge variation in product content and prescribed dosage complicates interpretation of the fast growing body of literature on this topic. A recommendation for future studies is the inclusion of more parameters per study, so that a more comprehensive interpretation of specific mechanisms becomes possible. Measurement of only basic indicators of either antioxidant status or inflammatory status, while providing proof of efficacy of the product, does not contribute much information on its mechanism of action. Despite this shortcoming, the literature clearly indicates that grape-derived products are undeniably a therapeutic force to be reckoned in the combat of ageing and age-associated conditions.

Conflict of Interests

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

Table 1: Representative reports on antioxidant and anti-inflammatory effects of grape-derived crude extracts and purified products.

Model	Treatment	Outcomes: inflammation	Outcomes: oxidative status	References
In vitro:				
Glucose and LPS-induced inflammation in HUVEC cells	Red grape polyphenols	↓IL-6, IL-8, and NF-κB at protein and mRNA levels ↓PECAM and ICAM-1 levels	↓ROS formation in dose-dependent manner	[66]
Primary human chondrocytes challenged with *E. coli* LPS (arthritis model)	Grape extract containing resveratrol, hopeaphenol, and viniferin	↓PGE_2 production	↑scavenging of DPPH radicals	[67]
Osteoblast-like cells (MC3T3-Ei), treated with TGF-β to induce VEGF synthesis	Resveratrol	↓VEGF and VEGF mRNA, but no effect on p38 or SAPK/JNK, suggesting SIRT1 activation	n.a.	[68]
Yeast models of sirtuin activation (*c. elegans*, *D. melanogaster)*	Resveratrol	↑sirtuin (SIRT1) activation	n.a.	[69, 70]
Human adipose derived stem cells (hASCs)	Red grape (muscarine) grape seed oil, in comparison to rice bran and olive oils	↓adipogenetic factors (PPARγ and aP2) ↓IL-6 and IL-8 response to LPS ↓proinflammatory gene expression in adipocytes	Shown to be source of γ-tocopherol	[71]
High-glucose induced oxidative stress in porcine proximal tubule cells (LLC-PK_1)	Grape seed polyphenols	↓NF-κB pathway	↓intracellular ROS	[72]
In vivo animal:				
Rats exposed to localised bowel irradiation	Grape polyphenols OR pure quercetin 3-*O*-β-glucoside (10 mg/mL, 7.14 mL/kg body mass) by oral gavage for 5 days prior to irradiation	↓MPO activity ↓CINC-1 levels	↓SOD activity No change in glutathione peroxidase (GSHPx) activity No change in plasma malondialdehyde (MDA) concentration	[73]
Rats subjected to *E. coli*-induced septic shock	75 and 200 mg/kg/day grape seed procyanidin, by ip. injection for 15 days pre-*E. coli* challenge	↓IL-6 gene expression	↓NO in liver, spleen, plasma, and RBCs ↓iNos gene expression ↓GSSG: total glutathione ratio	[74]
Rat model of osteoarthritis	500 mg/kg body mass of grape extract daily for 28 days	Prevented joint deterioration	n.a.	[67]

Table 1: Continued.

Model	Treatment	Outcomes: inflammation	Outcomes: oxidative status	References
Rat model of skeletal muscle contusion injury	Acute OR 2-week supplementation, proanthocyanidins	↓proinflammatory cytokine signalling (TNF-α; IL-6) ↓neutrophil migration capacity Earlier macrophage switch from pro- to anti-inflammatory phenotype	↑plasma and skeletal muscle ORAC	[50, 75]
Rat model of ageing	Drinking water supplemented with 15 g/L grape powder for 3 weeks	↓age-associated increase in corticosterone	↓plasma 8-isoprostane	[76]
Rat model of obesity	Grape procyanidin B2	↓IL-1β and NLRP3 levels in pancreas	n.a.	[77]
Middle-aged mice on high-calorie diet	Diet supplemented with 0.04% resveratrol	↓IGF-1	↑AMPK and PGC-1α activity ↑mitochondrial number	[78]
Mouse model of pulmonary fibrosis	7-day oral resveratrol (50 mg/kg/day) OR quercetin OR dihydroquercetin (both 10 mg/kg/day)	↓neutrophil infiltration into lung tissue ↓inflammatory cells in bronchoalveolar lavage fluid ↓COX-2 ↓NF-κBp65 translocation	↓iNOS ↓oxidative lung damage (↓nitrotyrosine and poly-ADP-ribose polymerase levels)	[79]
Rabbit model of acute (*E. coli*) inflammatory arthritis	500 mg/kg body mass of extract acutely prior to *E. coli* challenge	↓PGE_2 production	n.a.	[67]
In vivo human:				
Nondiabetic haemodialysis patients	Grape powder (500 mg polyphenols/day) for 5 weeks	Prevented increase in plasma CRP levels	↑glutathione peroxidase activity	[80]
Humans at risk for metabolic syndrome, aged 30–65	16 weeks of 20 g wine grape pomace powder (822 mg polyphenols) per day	n.a.	↑ γ- and δ-tocopherol	[81]
Hypertensive, T2DM males, aged ≈55–65	8 mg grape extract daily for 1 year	↓alkaline phosphatase ↓TNF-α, IL-1β, IL-6, and CCL3 levels ↑transcriptional repressor LRRFIP-1 in PBMCs Modulation of expression of group of miRNAs known to regulate inflammatory response	n.a.	[82]

ADP, adenosine diphosphate; AMPK, adenosine monophosphate-activated protein kinase; CCL3, chemokine(C-C motif) ligand 3; CINC-1, cytokine-induced neutrophil chemoattractant-1; COX-2, cyclooxygenase-2; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide; ICAM-1, intercellular adhesion molecule-1; IGF-1, insulin growth factor-1; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; LRRFIP-1, leucine-rich repeat in flii-interacting protein-1; NO, nitric oxide; NF-κB, nuclear factor-kappa beta; ORAC, oxygen radical absorbance capacity; PECAM, platelet endothelial adhesion molecule; PGC-1α, peroxisome proliferator activated receptor gamma coactivator 1-alpha; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; SAPK, stress activated protein kinase; VEGF, vascular endothelial growth factor.

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Research Article

Antioxidant and Antiplasmodial Activities of Bergenin and 11-*O*-Galloylbergenin Isolated from *Mallotus philippensis*

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Two important biologically active compounds were isolated from *Mallotus philippensis*. The isolated compounds were characterized using spectroanalytical techniques and found to be bergenin (**1**) and 11-*O*-galloylbergenin (**2**). The *in vitro* antioxidant and antiplasmodial activities of the isolated compounds were determined. For the antioxidant potential, three standard analytical protocols, namely, DPPH radical scavenging activity (RSA), reducing power assay (RPA), and total antioxidant capacity (TAC) assay, were adopted. The results showed that compound **2** was found to be more potent antioxidant as compared to **1**. Fascinatingly, compound **2** displayed better EC_{50} results as compared to α-tocopherol while being comparable with ascorbic acid. The antiplasmodial assay data showed that both the compound exhibited good activity against chloroquine sensitive strain of *Plasmodium falciparum* (D10) and IC_{50} values were found to be less than 8 μM. The *in silico* molecular docking analyses were also performed for the determination of binding affinity of the isolated compounds using *P. falciparum* proteins PfLDH and Pfg27. The results showed that compound **2** has high docking score and binding affinity to both protein receptors as compared to compound **1**. The demonstrated biological potentials declared that compound **2** could be the better natural antioxidant and antiplasmodial candidate.

1. Introduction

Medicinal plants, their extracts, or the isolated purified constituents have been used extensively as remedy for treatment of several diseases. The flora of Pakistan is quite rich with the naturally gifted medicinal plants. However, very little attention was given to explore the medicinal potentials of such worthy materia medica [1–3]. Various ailments such as Parkinson's, Alzheimer's, cancer, inflammation, neurodegeneration, aging, injury to blood vessel membranes, heart, and brain, and a number of other diseases may be caused by the free radicals present in the body. Antioxidants are free radical scavengers that may prevent, protect, or reduce the extension of such damage [4, 5]. A number of chemical species including both synthetic and natural products may act as antioxidants. Plants are considered to be the best source of natural antioxidants [1–3, 6]. Similarly, the various antimalarial and antiparasitic drugs such as quinine and artemisinin were also reported from the medicinal plants [7].

Bergenin and 11-*O*-galloylbergenin are the two natural products. The biological and pharmacological activities of bergenin are well documented in the literature [8–21]. However, very little work has been reported on the biological potentials of 11-*O*-galloylbergenin [22, 23] which need to be more explored. In the present study, the abovementioned natural products were isolated from *Mallotus philippensis* and their antioxidant and antiplasmodial activities were investigated. For confirmation of the experimental results, the computational study was also performed using *in silico* molecular docking.

2. Materials and Methods

2.1. General Experimental Procedures. ^{1}H-NMR and ^{13}C-NMR were recorded at 400 MHz for ^{1}H and at 100 MHz for ^{13}C using TMS as internal standard with Bruker DPX-400 instrument in deuterated solutions. Mass spectra were recorded on Agilent 5973N instrument using EI mode. IR spectra were determined using a Jasco A-302 spectrophotometer. UV and UV-visible spectra were recorded using U-3200 (Hitachi, Japan) and SP-3000 PLUS (Optima, Japan) spectrophotometers. For TLC and column chromatography, aluminum sheets precoated with silica-gel 60 F254 (20 × 20 cm, 0.2 mm thick; E. Merck, Germany) and silica gel (200–300 mesh), respectively, were used. The commercial solvents were used for extraction purpose and were redistilled. For the antioxidant and antiplasmodial activities, analytical grade reagents and chemicals were used.

2.2. Plant Material. The stem wood of *M. philippensis* (Euphorbiaceae) was collected from district Bunner located in the north of Pakistan in July 2006 and identified via Voucher Number 1013 (pup) by Professor Dr. Abdur Rashid, Department of Botany, University of Peshawar, Peshawar, Pakistan.

2.3. Extraction and Isolation. Air dried plant material was chopped, grinded, and extracted three times with commercial ethanol for 72 h which afforded 4.32% of the crude extract. From the crude extract, *n*-hexane soluble fraction was removed by solvent extraction with water. The aqueous fraction was then dried under vacuum which was further processed for the isolation of compounds **1** and **2** as depicted in Scheme 1. The isolated compounds were characterized using various spectroanalytical techniques.

2.4. Antioxidant Potential. The antioxidant potential of the isolated compounds was determined using DPPH radical scavenging activity (RSA) [1–3, 24], reducing power assay (RPA) [24, 25] and total antioxidant capacity (TAC) assay [26].

2.5. Antiplasmodial Activity. For antiplasmodial activity, the isolated compounds were tested against chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using the reported method [27]. For the quantitative assessment of antiplasmodial activity, parasite lactate dehydrogenase assay was adopted [28]. The IC_{50} values were obtained using a nonlinear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

2.6. Molecular Docking. For the *in silico* molecular docking study, the crystal structures of receptor proteins were downloaded from the protein data bank, code number PfLDH (*P. falciparum* lactate dehydrogenase) and PFG27 (gametocyte protein) of *P. falciparum*. The water molecules were removed and 3D protonation of the receptor molecules was carried out. The energies of the retrieved receptors were minimized using the default parameters of MOE energy minimization algorithm (gradient: 0.05, force field: MMFF94X). For the molecular docking of the isolated compounds, default parameters of MOE-dock program were used. To find the correct conformations of the ligands and to obtain minimum energy structure, ligands were allowed to be flexible. At the end of docking, the best conformations of the ligand were analyzed for their binding interactions.

3. Results and Discussion

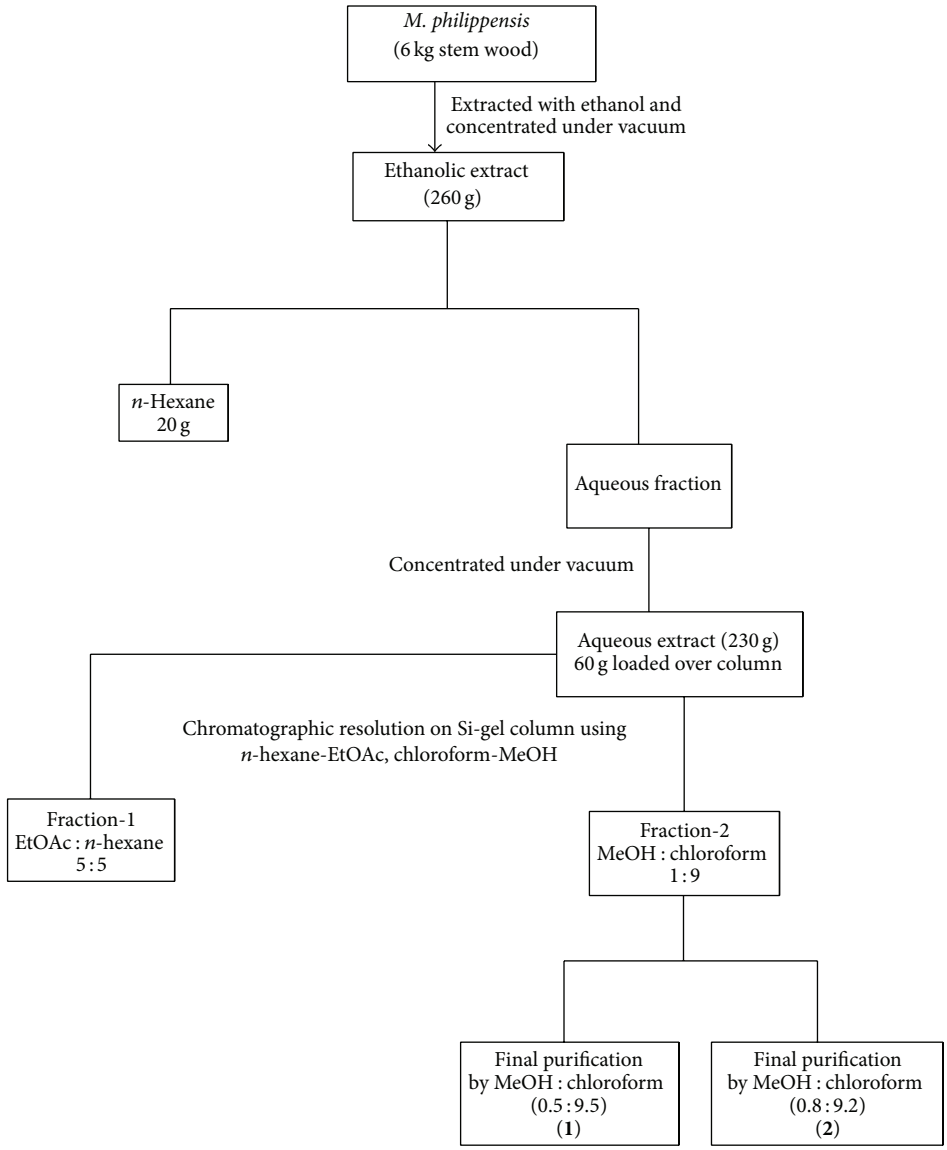
3.1. Characterization of the Isolated Compounds. The spectral analyses of the two isolated compounds are summarized as follows.

*Compound **1**.* White needles; mp = 237°C; UV λ_{max} (log ε) = 279(4.28); IR (KBr) λ_{max} = 3310, 3350, 1712, 1632, 1609, 1510, 1230 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 6.97 (1H, s, H-7), 5.67 (1H, d, H-10b), 4.98 (1H, dd, H-4a), 3.89 (1H, dd, H-4), 3.81 (2H, d, H-11), 3.75 (3H, s, H-12), 3.62 (1H, m, H-2), 3.48 (1H, dd, H-3); ^{13}C-NMR (DMSO- d_6, 100 MHz): δ 60.0 (C-12), 61.1 (C-11), 70.7 (C-3), 72.1 (C-10b), 73.7 (C-4), 79.8 (C-4a), 81.7 (C-2), 109.4 (C-7), 116.0 (C-10a), 118.1 (C-6a), 140.6 (C-9), 148.1 (C-10), 151.0 (C-8), 163.5 (C-6); EIMS *m/z* (rel. int.): 328 (34), 208 (100), 237 (7), 170 (30).

Compound **1** was obtained as white crystals. The mass spectral data of compound **1** gave a molecular ion peak at *m/z* 328 which leads to a molecular formula of C_{14} $H_{16}O_9$. The melting point of 237°C is consistent with the published melting point of bergenin, that is, 238°C. The ^{1}H-NMR spectral analysis showed a signal for one aromatic proton at δ 6.97 (1H, s) and a signal for methoxy protons at δ 3.8 (3H, s). In the ^{13}C-NMR spectrum, the signals were observed at δ 163.5 and 60.0 for carbonyl and methoxy groups, respectively. Compound **1** was characterized as bergenin (Figure 1) by comparing its physical and spectral data with previous literature [23, 29].

*Compound **2**.* White amorphous powder; mp = 180°C; $[\alpha]_D^{15}$ = +37.6° (EtOH; c 1.2), UV λ_{max} (log ε) = 277(4.22); IR (KBr) λ_{max} = 3310, 1712, 1632, 1609, 1510, 1230 cm^{-1}; ^{1}H-NMR (90 MHz, Me_2CO-d): δ 7.21(2H, s, gall-H), 7.10 (IH, s, H-7), 3.90 (3H, s, OMe); ^{13}C-NMR (90 MHz, CD_3OD): δ 81.2 (C-2), 72.2 (C-3), 75.7 (C-4), 81.1 (C-4a), 166.5(C-6), 120.0, (C-6a), 112.0 (C-7), 153.1 (C-8), 143.3 (C-9), 150.0 (C-10), 117.7 (C-10a), 74.7 (C-10b), 65.1 (C-11), 61.3 (C-12), 121.8 (C-1′), 111.1 (C-2′, C-6′), 147.4 (C-3′, C-5′), 140.0 (C-4′), 169.2 (C-7′); EIMS *m/z* (rel. int.): 480 (32), 328 (34), 208 (100), 237 (7), 170 (30).

Compound **2** was interpreted and analyzed as $C_{21}H_{20}O_{13}$ to be monogalloyl ester of bergenin by its ^{1}H-NMR, ^{13}C-NMR, and EIMS spectral data and also by acid hydrolysis which gave bergenin and gallic acid. The position of the galloyl group was established by ^{13}C-NMR spectrum and 2D techniques. The carbon signals other than that of C-11 in the glucose moiety of bergenin were assigned as given below. The carbon carrying free hydroxyl group (C-3, C-4, and C-11) was unequivocally distinguished from the others (C-2, C-4a, and C-10b) by the deuterium induced differential isotope shift



Scheme 1: Schematic representation of extraction and isolation of compounds **1** and **2** from *Mallotus philippensis* stem wood.

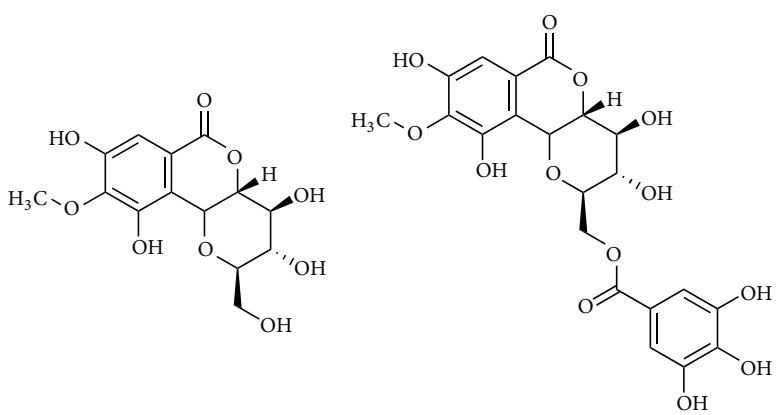


Figure 1: Chemical structure of isolated compounds **1** (bergenin) and **2** (11-*O*-galloylbergenin).

Table 1: Antioxidant activity of the isolated compounds and standards.

Tested compounds	% radical scavenging activity (RSA)	Reducing power assay (RPA)	Total antioxidant capacity*
Bergenin (**1**)	6.858 ± 0.329	0.055 ± 0.002	49.159 ± 3.136
11-*O*-Galloylbergenin (**2**)	87.26 ± 1.671	1.315 ± 0.027	951.50 ± 109.64
Ascorbic acid	97.85 ± 0.623	3.351 ± 0.034	2478.36 ± 173.81
Gallic acid	98.12 ± 0.931	1.435 ± 0.031	2201.05 ± 152.33
Quercetin	98.35 ± 0.871	1.772 ± 0.041	2030.29 ± 134.51
α-Tocopherol	92.26 ± 0.547	22.026 ± 0.074	565.17 ± 25.32

Each reading is mean ($n = 3$) ± SD (standard deviation). For RSA and RPA, 100 and 25 μg/mL, respectively, were used. * As ascorbic acid equivalent (μmol/mg).

Table 2: EC_{50} values of the isolated compounds and standards.

Tested compounds	Radical scavenging assay (EC_{50}) (μg/mL)[a]	Reducing power assay (EC_{50}) (μg/mL)[b]
Bergenin (**1**)	99.807 ± 3.120	24.915 ± 1.326
11-*O*-Galloylbergenin (**2**)	7.276 ± 0.058	5.208 ± 0.095
Ascorbic acid	6.571 ± 0.303	3.551 ± 0.073
Gallic acid	4.732 ± 0.187	1.542 ± 0.062
Quercetin	4.355 ± 0.099	2.073 ± 0.065
α-Tocopherol	33.675 ± 2.019	22.152 ± 1.153

Each reading is mean ($n = 3$) ± SD (standard deviation). [a]EC_{50}: effective concentration at which 50% of DPPH radicals are scavenged and [b]EC_{50}: effective concentration at which the absorbance is 0.4.

(DIS) measurement. Compound **2** was characterized as 11-*O*-galloylbergenin (Figure 1) [23, 30].

3.2. Antioxidant Activity. In DPPH radical scavenging assay, the isolated compounds **1** and **2** showed 6.858 ± 0.329 and 87.26 ± 1.671% RSA, respectively when compared with the selected standards whose %RSA were in the range from 92.26 ± 0.547 to 98.35 ± 0.871 (Table 1). The demonstrated %RSA of compound **2** clearly indicates that it is the high potency toward DPPH free radical. Similarly, in the RPA, the reducing power capacity of compound **2** was found to be much higher as compared to compound **1** while being comparable with gallic acid and quercetin as depicted in Table 1. The TAC of the isolated compounds and standards was determined as ascorbic acid equivalent as shown in Table 1. As can be seen from the results, again compound **2** displayed better activity as compared to compound **1** and even α-tocopherol. From the above discussion, compound **2** could be declared as the better antioxidant candidate. The antioxidant properties of various plants extract or their purified constituents are well documented in the literature [1–3, 6, 22, 24, 31–34].

Table 2 shows a comparative analysis of EC_{50} values of the isolated compounds and standards using DPPH radical scavenging and reducing power assays. The EC_{50} values showed more prominent performance of compound **2** as compared to compound **1**. For the studied assays data, the EC_{50} vales for compound **2** showed better results as compared to α-tocopherol while being comparable with the ascorbic acid (Table 2). In a previous study, the antioxidant activity of various compounds isolated from the methanolic extract of the aerial parts of *Vitex agnus-castus* Linn. plant was evaluated using a DPPH radical scavenging assay and the results obtained were in the range from no activity to strong activity. However, the IC_{50} value was not reported [6]. The results obtained in the present study are comparable with the reported data [22].

Table 3: The *in vitro* antiplasmodial activity of the isolated compounds and standard.

Tested compounds	Antiplasmodial activity (IC_{50} in μM)
Bergenin (**1**)	6.92 ± 0.43
11-*O*-Galloylbergenin (**2**)	7.85 ± 0.61
Chloroquine	0.031 ± 0.002

Each reading is mean ($n = 3$) ± SD (standard deviation).

3.3. Antiplasmodial Activity. Compounds **1** and **2** were also tested for the *in vitro* antiplasmodial activity against the CQS D10 strain of *P. falciparum* and the results obtained are presented in Table 3. As can be seen, both the tested compounds had displayed good activity even at low concentration with IC_{50} values of 6.92 ± 0.43 and 7.85 ± 0.61 μM for compounds **1** and **2**, respectively, while IC_{50} value of 0.031 ± 0.002 μM was recorded for chloroquine (Table 3). The analogous results were also reported previously for mentioned compounds isolated from the roots of *Bergenia ligulata*.

3.4. Molecular Docking. The binding interaction of the isolated compounds and *P. falciparum* proteins (PfLDH and Pfg27) was also investigated using *in silico* molecular docking. The selected proteins are very important because PfLDH has a role in glycolysis for energy production during asexual cycle, while Pfg27 is vital protein for the gametocyte production during sexual phase of the parasite; thus both proteinsare potential molecular targets for antimalarial drugs. The results of molecular docking with compound **1** and

Table 4: The *in silico* docking score of the isolated compounds against *P. falciparum* proteins (PfLDH and Pfg27).

Isolated compounds	Docking result Moldock score			
	PfLDH		PfG27	
	Moldock score	Binding affinity (pKi)	Moldock score	Binding affinity (pKi)
1	−12.13	10.20	−10.01	8.78
2	−16.22	12.43	−11.84	9.29

PfLDH showed that compound was bound in the binding pocket of the enzyme, making interactions with the residues Lys198, Arg109, Asn108, and Asn197 (basic, side chain donors and backbone donor). Lys98 interacts with oxygen of one hydroxyl group of ring and Arg109 with other hydroxyl group oxygen while oxygen of third hydroxyl group established interaction with the Asn197 whereas the oxygen atom of the ring interacts with Asn108 (Figure S2 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1051925). Compound **2** was completely docked in cavity of the enzyme PfLDH and established large number of interactions with the residues Arg185, Ser170, Glu256, Lys173, Val166, Gly165, Thr169, Ala253, and Ala249. In the above docking process, the residue Arg185 formed three interactions, that is, one with oxygen of one hydroxyl group of benzene ring, the second with oxygen of carboxylic group of compound **2,** and the third (arene-arene interaction) with benzene ring. The residue Ser170 established two interactions (backbone donor and acceptor) with hydrogen of hydroxyl group of two cyclic rings and one with oxygen of hydroxyl group of benzene ring of compound **2**. The residue Glu256 showed interaction with the hydrogen atom of benzene hydroxyl group (side chain acceptor) and Lys173 has two interactions (side chain donor) with the two oxygen atoms of two hydroxyl groups of benzene ring. The residue Gly165 formed one interaction with hydrogen of hydroxyl group of benzene ring and residue Thr169 (side chain acceptor) showed one interaction with hydrogen of one hydroxyl group. Val166 (backbone acceptor) formed two interactions with the two hydrogen atoms of one hydroxyl group of one benzene ring and with the other hydroxyl group of another benzene ring. Ala253 and Ala249 (backbone donor) both expressed interactions with oxygen of hydroxyl group and carboxyl group (Figure S3).

The results of molecular docking of compound **1** and Pfg27 protein binding showed that **1** was bound into the binding cavity of protein (Pfg27) making interactions with the residues Arg131 (basic, side chain donor) and Asp40. Arg131 interacts with oxygen (carboxyl group) to one side of benzene ring while Asp40 was found in polar interaction with H (hydroxyl group) of compound **1** (Figure S4). Similarly, Arg36 residue also showed prominent interaction with oxygen of hydroxyl group as shown in Figure S5. In case of compound **2**, Arg131 residue established arene-cation interaction with one of the benzene rings of compound and the residue Glu134 formed three-side interaction, that is, two sides with hydrogen of two hydroxyl groups and one side with one oxygen group of carboxyl group (Figure S6). Further, compound **2** also showed arene-arene interaction the residue His28, while Arg36 and Gln130 formed interaction with hydrogen of hydroxyl groups (Figures S7 and S8). From the MOE-docking studies, it was observed that, for both the proteins, compound **2** has good agreement of docking score and binding affinity to protein receptors as compared to compound **1** as shown in Table 4. The results demonstrated that the isolated compounds are good enough for their potency and effectiveness against *P. falciparum*.

4. Conclusions

The current study deals with the isolation and characterization of two biologically active compounds from *M. philippensis*. The natural products were found to be bergenin (**1**) and 11-*O*-galloylbergenin (**2**). The isolated constituents were evaluated for their antioxidant and antiplasmodial potentials and from the results it was evident that compound **2** was found to be a potent and effective antioxidant as compared to compound **1** and its synthetic derivatives [8, 30]. The isolated compounds also offered good antiplasmodial activity against the tested *P. falciparum* strain which was further confirmed using *in silico* molecular docking. It is therefore concluded that the demonstrated medicinal properties of the isolated compounds could be used as scaffolds for the generation of advanced natural products and may play a vital role in drug development and design.

Conflict of Interests

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

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Research Article

Screening SIRT1 Activators from Medicinal Plants as Bioactive Compounds against Oxidative Damage in Mitochondrial Function

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Sirtuin type 1 (SIRT1) belongs to the family of NAD^+ dependent histone deacetylases and plays a critical role in cellular metabolism and response to oxidative stress. Traditional Chinese medicines (TCMs), as an important part of natural products, have been reported to exert protective effect against oxidative stress in mitochondria. In this study, we screened SIRT1 activators from TCMs and investigated their activities against mitochondrial damage. 19 activators were found in total by *in vitro* SIRT1 activity assay. Among those active compounds, four compounds, ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A, were further studied to validate the SIRT1-activation effects by liquid chromatography-mass spectrometry and confirm their activities against oxidative damage in H9c2 cardiomyocytes exposed to tert-butyl hydroperoxide (t-BHP). The results showed that those compounds enhanced the deacetylated activity of SIRT1, increased ATP content, and inhibited intracellular ROS formation as well as regulating the activity of Mn-SOD. These SIRT1 activators also showed moderate protective effects on mitochondrial function in t-BHP cells by recovering oxygen consumption and increasing mitochondrial DNA content. Our results suggested that those compounds from TCMs attenuated oxidative stress-induced mitochondrial damage in cardiomyocytes through activation of SIRT1.

1. Introduction

Sirtuin type 1 (SIRT1) belongs to the family of class III histone deacetylases (HDAC) that consume NAD^+ during deacylation cycle. It has been reported that, in mammals, SIRT1 plays a critical function in cellular metabolism and response to oxidative stress [1–4].

Recently, researchers have found that SIRT1 activators can protect mitochondrial function from oxidative-induced mitochondrial damage in various types of cell through regulating PGC-1α and multiple transcription factors [5–9], which are tightly related to mitochondrial biogenesis and metastasis [10, 11]. It is also reported that activators of SIRT1, such as resveratrol [12], can extend lifespan and regulate metabolic disorders [13–15]. Therefore, SIRT1 activators exhibit unique pharmacological potentials for treating mitochondrial dysfunction related diseases. Meanwhile, several clinical trials of SIRT1 activators such as SRT1720 for type 2 diabetes and obesity are under way [16].

Natural products have historically been regarded as an important resource of therapeutic agents in pharmaceutical discovery over the past century [17]. Traditional Chinese medicines (TCMs), as an important part of natural products, are mainly governed by empirical experience and fundamental theories such as the Yin and Yang concept [18]. TCMs with Qi Tonification effects including *Astragalus membranaceus* [19, 20], *Panax ginseng* [21, 22], and *Panax notoginseng* [23, 24] have been reported to exert protective effect against oxidative stress in mitochondria. Several compounds isolated from TCMs are reported to regulate SIRT1 activity [25]. However, a comprehensive screening of SIRT1 activators from TCMs has not yet been performed to investigate their protective effects on mitochondrial function against oxidative stress.

The aim of present study is to discover SIRT1 activators from TCMs and validate their activities against mitochondrial damage. A sensitive *in vitro* assay to screen SIRT1 activators was performed to discover bioactive compounds from TCMs, and the lead compounds were validated by liquid chromatography-mass spectrometry (LC-MS) analysis. Effects of identified SIRT1 activators on mitochondrial function were further investigated in cardiomyocytes exposed to tert-butyl hydroperoxide (t-BHP). ATP content, intracellular ROS formation, and activity of Mn-SOD were measured. Moreover, oxygen consumption and mitochondrial DNA content of cardiomyocytes were used to evaluate the effects of those SIRT1 activators on mitochondrial function.

2. Materials and Methods

2.1. Supplies and Chemicals. SIRT1 protein (human recombinant) and lysyl endopeptidase were purchased from Cayman Chemical (USA). Ginsenoside F1, ginsenoside Rc, and schisandrin A were purchased from Shanghai Winherb Medical Technology Company (China). Ginsenoside Rb_2 was purchased from National Institute for Food and Drug control (Beijing, China).

2.2. Cell Culture. H9c2 (from Cell Bank of Chinese Science Academy, Shanghai, China) were cultured in DMEM (Corning, USA) containing 10% fetal bovine serum (Sigma, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). All the cells were grown in 5% humidified CO_2 atmosphere at 37°C.

2.3. Fluorescent Probe Based Assay for SIRT1 Modulation Effects of Compounds. The measurement of SIRT1 activity and effects of compounds on SIRT1 activation were performed by a previously reported fluorescent method [26]. Briefly, SIRT1 was incubated with a TPE-GK(Ac)YDD probe (20 μM) in the presence of the tested compound (50 μM). Fluorescence intensity was recorded by a TECAN infinite F200 microplate reader with excitation wavelength 320 nm and emission wavelength 465 nm.

A total of 195 constituents of TCMs were screened by the assay to evaluate their regulatory effects on SIRT1 activity. The detailed information related to the chemicals and their CAS number were illustrated in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4206392. The inhibition or activation of SIRT1 was calculated by the following equation:

$$\text{Activation rate}\,(\%) = \left(\frac{\left(I_s - I_{s0}\right)/I_{s0}}{\left(I_c - I_0\right)/I_0} - 1 \right). \quad (1)$$

I_s and I_c represented the fluorescence intensity of tested sample group with various test compounds and control group without test compounds. I_{s0} and I_0 represented the fluorescence intensity of tested sample and control when incubated without SIRT1 protein. To explore the dose-related effects of these compounds, several active compounds, including ginsenoside Rb_2, ginsenoside Rc, and schisandrin A, were further tested with the concentrations of 1, 10, 25, and 50 μM.

2.4. Validation of SIRT1 Activators by LC-MS Analysis. In order to validate activation of SIRT1, SIRT1 (10 μg/mL) was added to TPE-GK(Ac)YDD (20 μM) and NAD^+ (Sigma, 3 mM) for 1 h incubation at 37°C in the presence or absence of ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A (50 μM), respectively. Samples were boiled at 100°C for 10 min to degenerate SIRT1 and terminate reaction.

Samples were analyzed by Agilent 1100 LC system (Agilent Technologies, USA) and Finnigan LCQ Deca XP^{plus} ion trap mass spectrometer with an ESI source (Thermo, USA). The acquisition parameters for LC/ESI-MS were as follows: nebulizing gas, high-purity nitrogen (N_2); collision gas, high-purity helium (He); capillary voltage: −15 V; capillary temperature: 350°C; ion spray voltage: −3 kV; tube lens offset voltage: −30 V; mass range: m/z 100–1500. Chromatographic separation was performed by a reversed-phase ZORBAX SB-C_{18} analytical column. The mobile phase included water containing 0.1% (v/v) formic acid (A) and acetonitrile (B). The flow rate was 0.6 mL/min. A gradient program was carried out as the following profile: 0 min, 50% B; 5 min, 50% B; 30 min, 95% B; 40 min, 95% B.

2.5. Measurement of ATP Content and Intracellular ROS. H9c2 cells were seeded in 96 wells at the density of 4,000/well. The cells were preincubated with ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A (20 μM) for 24 h before being exposed to t-BHP (300 μM) for 1 h. Intracellular ATP content was measured by CellTiter-Glo® Luminescent Assay kit (Promega) according to the instruction of manufacture. Intracellular ROS content was measured by DCFH-DA probe (5 μM) whilst fluorescence intensity was recorded by a TECAN infinite F200 Multifunction microplate with excitation wavelength 485 nm and emission wavelength 535 nm. The changes of ATP content and ROS accumulation were calculated by comparing the luminescent or fluorescent signal of the treated cells with that of untreated H9c2 cells.

2.6. Detection of Mn-SOD Activity. Manganese superoxide dismutase (Mn-SOD) was an antioxidative enzyme, which protected against ROS-induced damage [27]. To measure Mn-SOD activity, H9c2 cells were seeded in 6-well plate in the density of 4×10^5/mL. H9c2 cells were preprotected for 24 h by ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A (20 μM) before being exposed to t-BHP (300 μM) for 1 h. The cells were lysed and the concentration of total protein was measured by BCA assay kit. Mn-SOD activity in total protein was measured by Mn-SOD assay kit (Beyotime, China).

2.7. Oxygen Consumption Assay. 2×10^6 H9c2 cells were seeded in 100 mm culture plate. After being grown to stable attachment, cells were preincubated with ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A at final concentrations (20 μM) for 18 h before being exposed to t-BHP (100 μM) for 1 h. Cells were washed with PBS twice, subsequently collected by trypsinization followed by centrifugation, and resuspended in fresh medium. Respiratory activity was measured with a Clark-type oxygen electrode

(Oxytherm, Hansatech Instruments, UK). An aliquot (1 mL) of suspended cells (2×10^6 cells/mL) was placed in the air-tight liquid-phase oxygen electrode chamber. The system was maintained at 37°C. After equilibration, the slope of oxygen consumption in H9c2 cells was measured. Every 1×10^6 cells oxygen consumption was calculated as the basic respiration rate of each group. The experiment was also performed in the presence of SIRT1 inhibitor, EX527 at final concentration of 20 μM to investigate whether the effect of ginsenoside Rb_2 can be prevented by SIRT1 inhibitor.

2.8. Measurement of Mitochondrial DNA Content. Real-time PCR was used to determine relative quantities of mitochondrial DNA content in H9c2 cells exposed to t-BHP and cells incubated with SIRT1 activators. Cells were preincubated for 24 h with ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A at final concentrations (20 μM) before being exposed to t-BHP (300 μM) for 1 h. Cells in normal condition were used as control group. Total DNA was extracted using Mammalian Genomic DNA Miniprep Kits (Sigma, USA). DNA was quantified by measuring A_{260} values, and 50 ng of total DNA was used for PCRs by GenElute™ QuantiFast SYBR Green PCR Kit (QIAGEN, Germany). Primers specific to the mitochondrial-encoded Atp6 gene (Fw: 5′-ATT ACG GCT CCT GCT CAT A-3′; Rev: 5′-TGG CTC AAC CAA CCT TCT A-3′) were used to assess mitochondrial DNA copy numbers. Primers designed against the nuclear-encoded Rpl13 gene (Fw: 5′-CAC AAG AAA ATG GCA CGC AC-3′; Rev: 5′-GAG CAG AAG GCT TCC TGG G-3′) were used for normalization. C_T values were obtained automatically. The number of mitochondrial genes was calculated by $2^{-\Delta\Delta C_T}$ method.

2.9. Statistical Analysis. All values were expressed as the means ± SD. One way ANOVA was used to analyze differences among groups. Statistical analysis was performed using GraphPad Prism. p values of less than 0.05 were considered statistically significant.

3. Results

3.1. Screening of SIRT1 Activators from Compound Library of TCM. To screen SIRT1 activators, we chose 195 compounds from TCMs with different efficacy, which were defined according to the TCMs theory. For example, *Panax ginseng* and *Ophiopogon japonicas* were regarded as Tonifying herbs, whilst *Schisandra chinensis* was Astringent herb and *Sophora flavescens* came from heat-clearing medicinal. Figure 1 exhibited a heatmap of activation or inhibition rate of each compound. The corresponding values were listed in Supplementary Table. A total of 19 SIRT1 activators were found, including 20(S)-ginsenoside Rg_3 (60%), ginsenoside Rb_3 (28%), ginsenoside F1 (22%), and ginsenoside F2 (45%) from *Panax ginseng*, gypenoside XVII (43%) and notoginsenoside Ft1 (40%) from *Panax notoginseng*, polyphyllin I (24%), polyphyllin III (32%), polyphyllin VI (31%), and polyphyllin VII (32%) from *Paris polyphylla*, liriopesides B (65%) and *Liriope muscari* baily saponins C (57%) from *Liriope muscari*, ophiopogonin D′ (54%) from *Ophiopogon japonicas*, saikosaponin A (25%) from *Bupleurum chinense*, schisandrin B (30%) *from Schisandra chinensis*, and anisodine hydrobromide (60%) from *Anisodus tanguticus*. It was obvious that most of identified activators belonged to Tonifying herb. Moreover, potential inhibitors of SIRT1 almost belonged to herbs with heat-clearing efficacy.

Representative compounds were chosen to validate their activation effects on SIRT1 activity. Ginsenoside Rb_2 showed 8% to 152% activation with the concentration range of 1~50 μM. Ginsenoside Rc exerted 88% activation at the concentration of 50 μM. Schisandrin A showed 28% activation at the concentration of 50 μM.

3.2. Validation of SIRT1 Activators by LC-MS Analysis. To validate the SIRT1-activation effect of the compounds, liquid chromatography-mass spectrometry (LC-MS) analysis was employed. The specific substrate of SIRT1, TPE-GK(Ac)YDD (Figure 2(a)), was incubated with SIRT1 (10 μg/mL) and NAD^+ (3 mM) for 1 h. The reaction product was identified by LC-MS based on the molecular weight. As shown in Figure 2(b), the deacetylated peptide, TPE-GKYDD, was detected with a loss of Ac (43 Da). When analyzing the deacetylated reaction in the presence of SIRT1 activators, including ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A, the peaks of deacetylated product were significantly elevated (Figure 2(c)). Our findings indicated that these compounds activated SIRT1 in enzymatic reaction.

3.3. Effects of SIRT1 Activators on Cardiomyocytes Oxygen Consumption. The effects of SIRT1 activators on mitochondrial function were further investigated by measuring cellular respiration in H9c2 cells. As shown in Figure 3(a), basal respiration of t-BHP treated cardiomyocytes was significantly dropped comparing with control group. Preincubation with ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A attenuated the decrease of oxygen consumption. Figure 3(b) showed the representative oxygen consumption slope of normal cells (1.65 ± 0.31 nmol O_2/mL/min), t-BHP treated cells (1.10 ± 0.25 nmol O_2/mL/min), and ginsenoside Rb_2 treated cells (1.43 ± 0.25 nmol O_2/mL/min). The results suggested that these SIRT1 activators recovered the oxygen consumption rate in t-BHP injured cardiomyocytes. In the presence of SIRT1 inhibitor EX527 [28, 29], the protective effect of ginsenoside Rb_2 was blocked, which indicated that the effect of ginsenoside Rb_2 to recover the oxygen consumption rate was SIRT1 dependence (Figure 3(c)).

3.4. Effects of SIRT1 Activators on ATP Content and ROS Accumulation. As a specific parameter of mitochondrial function, intracellular ATP contents in cardiomyocytes exposed to oxidative stress were measured. After cells were exposed to t-BHP for 1 h, the content of ATP was significantly decreased. As shown in Figure 4, preprotection of cells by ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A led to the recovery of ATP content, suggesting that those SIRT1 activators reversed the decreased mitochondrial energy metabolism induced by t-BHP.

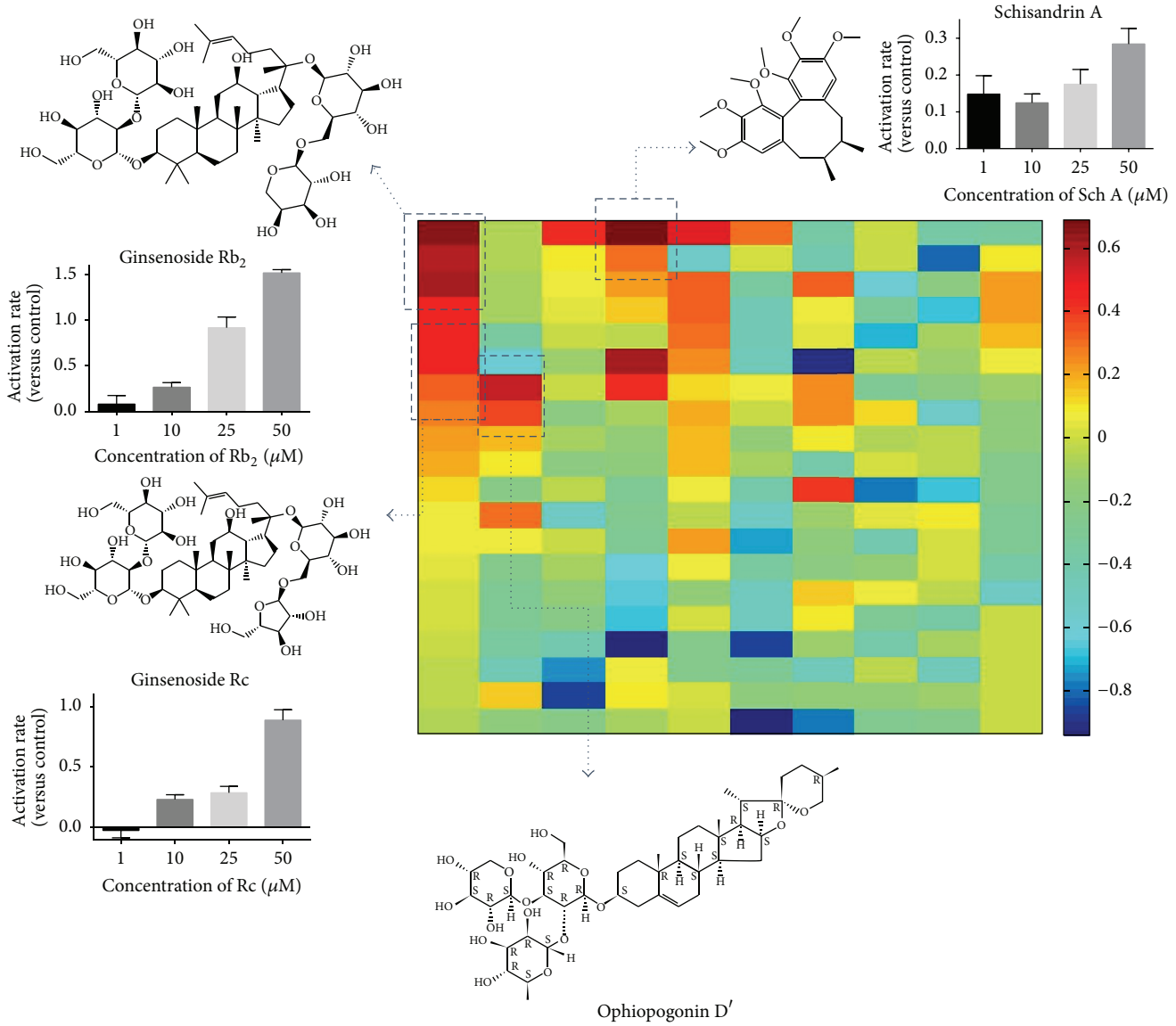

Figure 1: Screening results of 195 compounds from TCMs and dose dependent activation of representative activators.

Mitochondrial oxidative stress was often caused by increased intracellular ROS formation. Figure 5 showed that the intracellular ROS was significantly increased after t-BHP treatment. Pretreatments of SIRT1 activators, ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A, kept intracellular ROS levels on the normal condition.

3.5. Effects of SIRT1 Activators on Mn-SOD Activity. Mn-SOD was one of the antioxidative enzymes in mitochondria that assured mitochondrial oxidative stress resistance. As shown in Figure 6, when cells were exposed to t-BHP (300 μM) for 1 h, activity of Mn-SOD was decreased. Preprotection of cells by ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A enhanced the activity of Mn-SOD compared with t-BHP group.

3.6. Effects of SIRT1 Activators on Mitochondrial DNA Content. To verify the effect of ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A of mitochondrial protection or biogenesis, cells were injured by t-BHP after compounds preprotection, and then mitochondrial DNA content was analyzed. As shown in Figure 7, t-BHP treatment reduced mitochondrial DNA content compared to control group. Pretreatments of ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, or schisandrin A significantly elevated the mitochondrial DNA content. Our findings suggested that these natural SIRT1 activators facilitated mitochondrial biogenesis.

4. Discussion

In the present study, we screened and identified 19 SIRT1 activators, such as ginsenoside Rg_3, ginsenoside Rb_2, ginsenoside

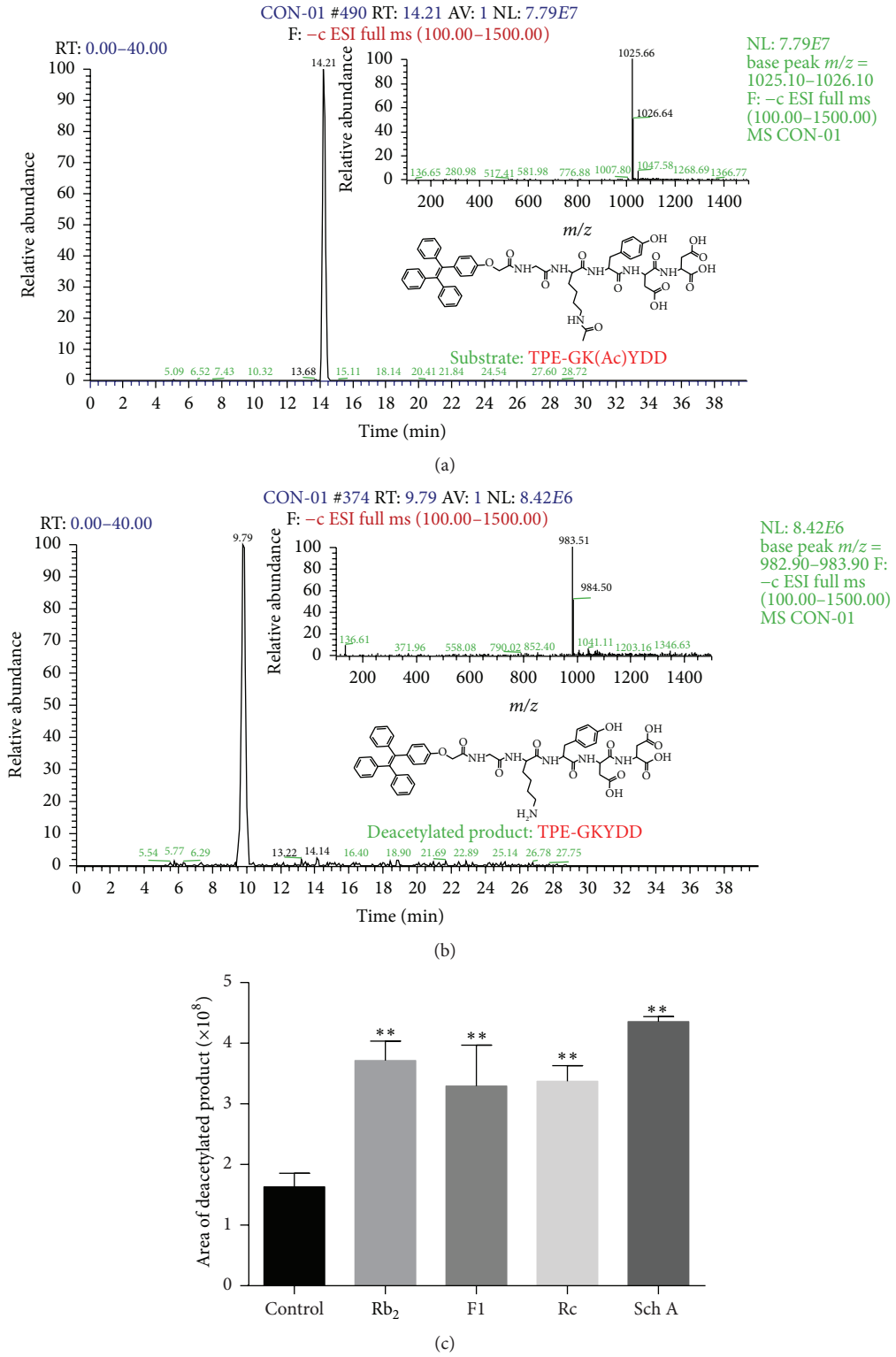

Figure 2: LC-MS results of SIRT1 activation. LC-MS chromatograms in negative ion mode and structures of (a) substrate TPE-GK(Ac)YDD and (b) deacetylated product TPE-GKYDD. (c) Area of deacetylated product in LC-MS chromatogram. SIRT1 was incubated with or without ginsenoside Rb_2 (Rb_2), ginsenoside F1 (F1), ginsenoside Rc (Rc), and schisandrin A (Sch A). Each bar represented the mean ± SD of triplicate experiments. Compared with control group, $^{**}P < 0.01$.

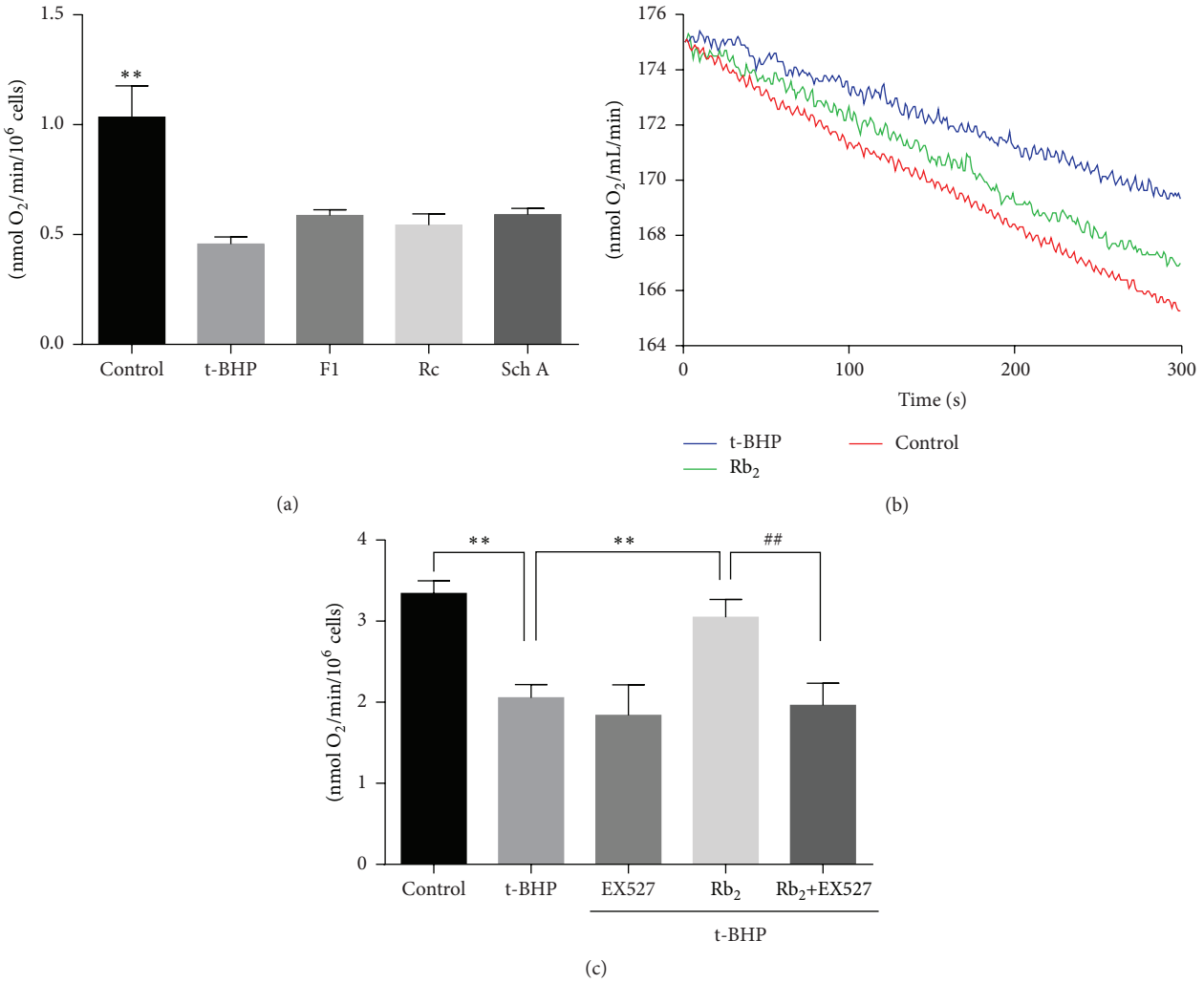

Figure 3: Effects of SIRT1 activators on mitochondrial oxygen consumption. (a) Respiration rates of H9c2 cells, t-BHP injured cells, and t-BHP injured cells preincubated with F1, Rc, and Sch A. (b) Representative curves of oxygen consumption recorded by the Clark-type oxygen electrode, and t-BHP injured cells were preincubated with Rb_2. (c) In the presence of EX527, the effect of ginsenoside $Rb_2$was blocked. Each bar represented the mean ± SD of triplicate experiments. Compared with t-BHP group, $^{**}p < 0.01$. Compared with t-BHP + Rb_2 + EX527 group, $^{\#\#}p < 0.01$.

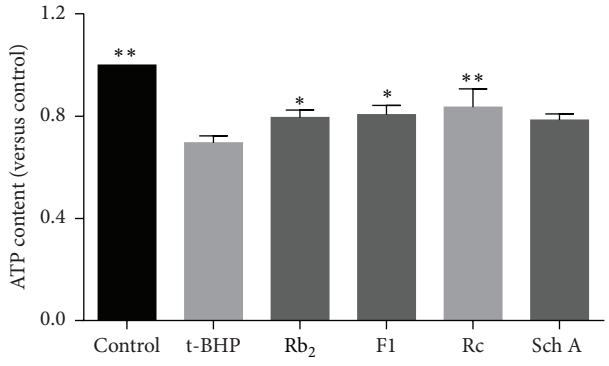

Figure 4: Effects of SIRT1 activators on the ATP content in t-BHP treated H9c2 cells. Each bar represented the mean ± SD of triplicate experiments. Compared with t-BHP group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

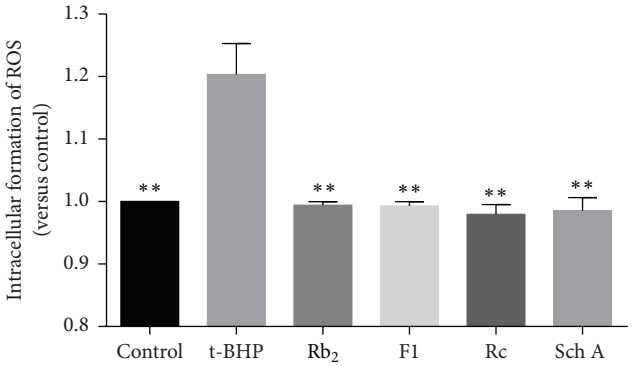

Figure 5: Effects of SIRT1 activators on the intracellular ROS level. Each bar represented the mean ± SD of triplicate experiments. Compared with t-BHP group, $^{**}p < 0.01$.

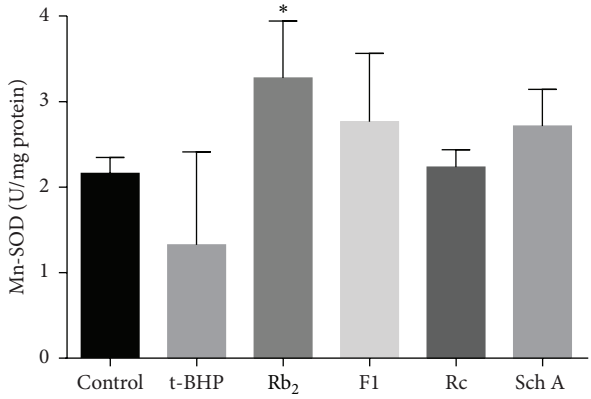

Figure 6: Effects of SIRT1 activators on Mn-SOD activity. Each bar represented the mean ± SD of triplicate experiments. Compared with t-BHP group, $^{*}p < 0.05$.

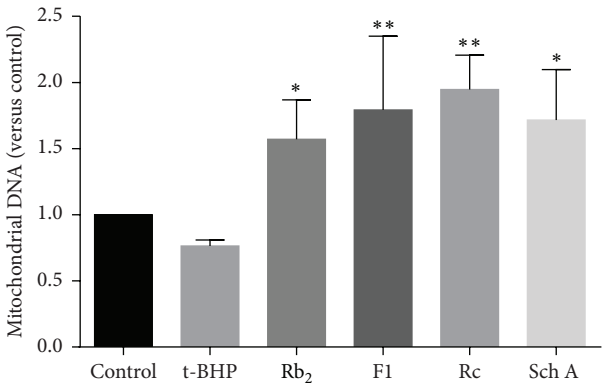

Figure 7: Effects of SIRT1 activators on mitochondrial DNA content. Each bar represented the mean ± SD of triplicate experiments. Compared with t-BHP group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Rb_3, ginsenoside F1, and ginsenoside Rc from *Panax ginseng*, ophiopogonin D′ from *Ophiopogon japonicas,* and schisandrin A and schisandrin B from *Schisandra chinensis*. Interestingly, those herbs consisted of a traditional Chinese formula named Shengmai San, which have been clinically used for the treatment of coronary heart diseases [30, 31] and heart failure [32, 33]. Four SIRT1 activators from Shengmai San, including ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A, were validated by LC-MS analysis and we found their effects against mitochondrial oxidative damage in further study. Our findings were in accordance with previous reports on other cell lines. Ginsenoside Rc was reported to suppress oxidative stress in HEK293T cells [34], whilst schisandrin A inhibited apoptosis induced by H_2O_2 in intestinal epithelial cells [35].

Mitochondrial dysfunction has been one of mechanisms in organ injuries and diseases due to its influence on ATP formation, metabolism, and apoptosis [36]. Our findings indicated that those SIRT1 activators elevated ATP content, prevented ROS formation, and increased the activity of Mn-SOD. Mitochondrial DNA content and oxygen consumption were also moderated by SIRT1 activators. Those results indicated that SIRT1 activators protected mitochondrial function by improving mitochondrial DNA content, which led to promotion of ATP content, mitochondrial oxygen consumption, and reduction of ROS formation.

5. Conclusion

In summary, we identified 19 SIRT1 activators from TCMs. Four active compounds, ginsenoside Rb_2, ginsenoside F1, ginsenoside Rc, and schisandrin A, exerted significant activities against t-BHP induced oxidative damage in cardiomyocytes. Our findings provided useful evidence to illustrate the cardioprotective effects of TCMs with Tonification effects and led to a new insight into the scientific illustration of TCMs theory.

Abbreviations

TCMs: Traditional Chinese medicines
AIE: Aggregation induced emission
LC-MS: Liquid chromatography-mass spectrometry
t-BHP: tert-Butyl hydroperoxide
ROS: Reactive oxygen species
F1: Ginsenoside F1
Rc: Ginsenoside Rc
Sch A: Schisandrin A
Rb_2: Ginsenoside Rb_2.

Conflict of Interests

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

Acknowledgments

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Review Article

Role of Viral miRNAs and Epigenetic Modifications in Epstein-Barr Virus-Associated Gastric Carcinogenesis

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MicroRNAs are short (21–23 nucleotides), noncoding RNAs that typically silence posttranscriptional gene expression through interaction with target messenger RNAs. Currently, miRNAs have been identified in almost all studied multicellular eukaryotes in the plant and animal kingdoms. Additionally, recent studies reported that miRNAs can also be encoded by certain single-cell eukaryotes and by viruses. The vast majority of viral miRNAs are encoded by the herpesviruses family. These DNA viruses including Epstein-Barr virus encode their own miRNAs and/or manipulate the expression of cellular miRNAs to facilitate respective infection cycles. Modulation of the control pathways of miRNAs expression is often involved in the promotion of tumorigenesis through a specific cascade of transduction signals. Notably, latent infection with Epstein-Barr virus is considered liable of causing several types of malignancies, including the majority of gastric carcinoma cases detected worldwide. In this review, we describe the role of the Epstein-Barr virus in gastric carcinogenesis, summarizing the functions of the Epstein-Barr virus-encoded viral proteins and related epigenetic alterations as well as the roles of Epstein-Barr virus-encoded and virally modulated cellular miRNAs.

1. Introduction

The Epstein-Barr virus (EBV) was the first discovered human tumor-causing virus considered as the etiologic agent of Burkitt's lymphoma (BL), an unusual African pediatric lymphoma [1].

Specifically, EBV is ubiquitous member of the human gamma-herpesvirus family that causes mononucleosis during acute and lytic infection and also establishes a persistent and latent infection in more than 90% of the human population. EBV latent infection has been demonstrated to be involved in multiple types of cancer that primarily develop in lymphocytes and epithelial cells. These include malignant tumors that develop in the immunocompromised conditions such as AIDS-associated lymphomas and posttransplant lymphoproliferative disease [2, 3] and also several human cancers that develop in the immunocompetent patients such as BL, Hodgkin's lymphoma, B-cell and T-cell lymphomas, epithelial nasopharyngeal carcinoma (NPC), and some forms of gastric carcinomas [4–6]. Gastric cancer is the fourth

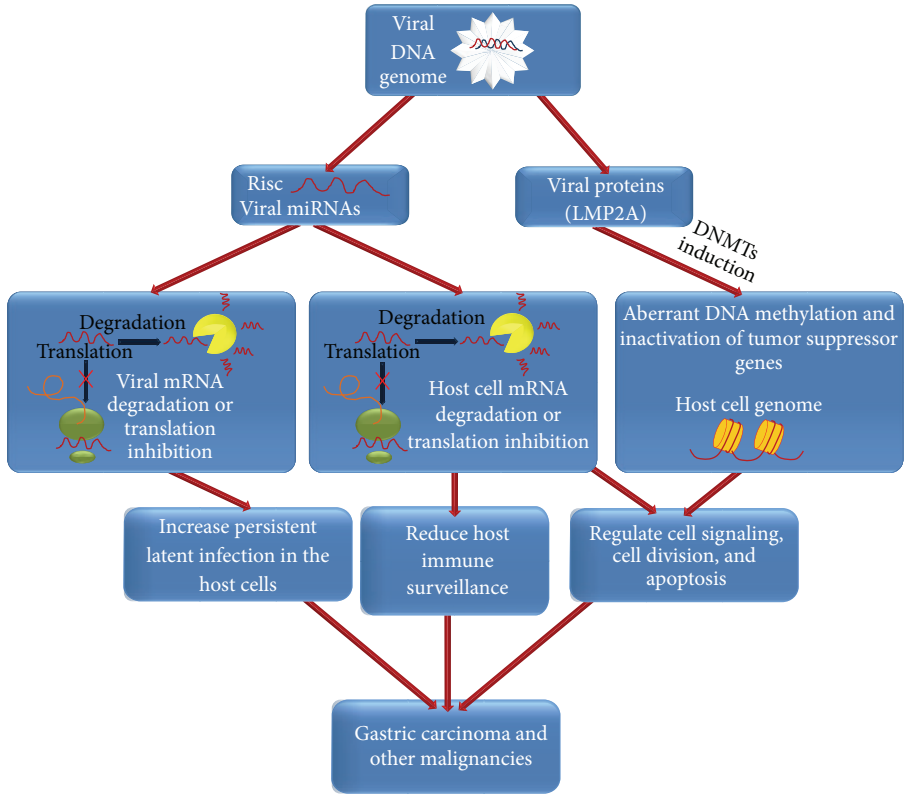

Figure 1: Graphical representation of possible mechanisms by which viral miRNAs and viral proteins might contribute to EBV-associated gastric carcinogenesis. This model indicates that EBV-encoded miRNAs (e.g., BART miRNAs and BHRF-1 miRNAs) target viral genes to mediate immune evasion or maintenance of latency, whereas some viral proteins (e.g., LMP2A) promote aberrant host DNA methylation and subsequent inactivation of tumor suppressor genes via DNA methyltransferases (DNMTs) induction. In addition, these viral miRNAs incorporated into RISC complex can also interact directly with specific host genes involved in immune surveillance, cell proliferation, and apoptosis, playing a crucial role in the aetiology of diverse diseases including EBVaGC.

most common cancer in the world and the second leading cause of cancer-related death. Globally, gastric cancer poses a significant public health burden, both economically and socially [7, 8]. Risk factors of gastric cancer are multifactorial; hence genes, diet, age, and chronic inflammation need to be evaluated in connection with infectious agents (EBV, *Helicobacter pylori*) and environmental factors (e.g., alcohol and smoking) [8]. Notably, EBV-associated gastric carcinoma (EBVaGC) represents almost 10% of all gastric carcinoma cases and expresses restricted EBV latent genes (Latency I) [4, 9]. In recent years, it has become increasingly evident that EBV may contribute to gastric carcinogenesis through the expression of viral proteins and microRNAs (miRNAs) [10] (Figure 1). A growing body of scientific evidence also suggests that, in addition to genetic alterations, epigenetic alterations, including aberrant DNA methylation of CpG islands and posttranslational modifications of histones, are involved in the development and progression of EBVaGC [10, 11]. This review briefly summarizes remarkable advancements in our understanding of the functions and mechanisms of action of herpesviral miRNAs in gastric carcinoma in recent years. In particular it discusses how the expression of viral proteins and epigenetic alterations contribute to EBVaGC and the roles of EBV-encoded and virally modulated cellular miRNAs in the respective viral life cycles and in EBV-associated gastric carcinoma.

2. Biogenesis of EBV-Encoded miRNAs

The generation of viral miRNA and selection of targets are totally dependent on the host molecular miRNA apparatus involved in the maturation and silencing. Most viruses utilize a strategy similar to that of the host cell to produce the viral pri-miRNAs by using the host RNA polymerase II (RNAP II) [12, 13]. Exceptionally, certain viral miRNAs (such as those from mouse γ-herpesvirus 68, MHV68) are produced from tRNA-like genes transcribed by host RNA polymerase III (RNAP III) and processed Drosha independently by host tRNase Z [14]. A minority of viruses from the herpesvirus and retrovirus families also utilize noncanonical pathways other than "tRNA-like" to generate pre-miRNA molecules [15–22]. For instance, herpesvirus saimiri (HVS), an oncogenic γ-herpesvirus that infects New World monkeys, expresses small nuclear RNAs (snRNAs) of the Sm-class called HSURs which are processed by the integrator complex to produce viral pre-miRNAs. Subsequently, both the MHV68 and HVS pre-miRNAs are processed by Dicer to generate miRNAs [23]. Additionally, some retroviruses such as foamy viruses (FVs)

and bovine leukemia virus (BLV) are able to express pri-miRNAs via RNAP III [18, 19, 21]. While some retroviral pri-miRNAs are processed by Drosha in the frame of RNAP III transcripts, some others may be directly processed by Dicer bypassing Drosha processing. Production of retroviral miRNAs without having recourse to Drosha-mediated cleavage of the RNA genome intermediate has been found to represent an important biogenesis strategy that may eventually take an active part in reducing overall viral fitness [18, 19, 21]. In brief, viral miRNA biogenesis initiates in the nucleus, where after transcription by host RNA polymerase II the microprocessor complex which contains the host RNase III endonuclease Drosha and its interaction partner DGCR8 (also known as Pasha in Drosophila) [24] cleaves pri-miRNA hairpin structure to pre-miRNAs. The vast majority of pri-miRNAs contain approximately 80 nucleotide hairpin secondary structures that can be intronic or exonic. Approximately 60 pre-miRNA nucleotides are liberated and rapidly exported from the nucleus to the cytoplasm by exportin-5/Ran GTPase pathway. Once in the cytoplasm, they are further processed by a second host RNase III endonuclease, Dicer, into a short double-stranded (ds) RNA or RNA duplex. The strand guide of these mature miRNAs of approximately 22 nucleotides is then incorporated into a protein complex known as the RNA-induced silencing complex (RISC), while the other strand called the passenger is rapidly degraded [13, 25]. RISC complex can incorporate both strands of miRNAs generated in EBV-infected cells. The major component of RISC is the Argonaute (Ago) protein. In mammalian cells there are four Ago proteins which are all incorporated into RISC; however, only Ago2 shows endonuclease activity. Binding of Ago-loaded miRNAs (miRISC) to a mRNA bearing extensive sequence complementary to miRNA generally results in mRNA cleavage and degradation, whereas binding to mRNAs bearing partial complementarity to miRNA results mainly in translational arrest [12, 13, 26]. There is no proof that any animal virus encodes additional miRNA-processing factors or RISC components. Therefore, viral miRNAs biogenesis largely relies on host-derived enzymes or proteins. Interestingly, EBV miRNAs can be subdivided into two groups, Bam HI fragment H rightward open reading frame 1 microRNAs (BHRF1 miRNAs) and Bam HI-A rightward transcripts microRNAs (BART miRNAs), based on their locations [27, 28]. Specifically, BHRF1 miRNAs are located within introns of the BHRF1 gene and generated from the long EBNA transcripts; whereas BART miRNAs are instead located in introns included within the BART transcripts [27, 28]. Nevertheless, how EBV miRNA expression is finely regulated remains largely unknown. Various studies suggest that BART transcripts/miRNAs are transcribed from P(1) and P(2) promoters in both B-cells and epithelial cells [29]. The BART promoter region contains putative binding sites for diverse transcription factors. Among transcription factors, C/EBPβ was reported to positively modulate the expression of BART [30]. Notably, expression of the EBV miRNAs was detected in several human cancer cell lines; in fact, expression of the EBV miRNAs was observed in EBVaGC [31] and peripheral T-cell lymphoma [32] besides being shown in B-cell lines and nasopharyngeal carcinoma EBV-infected cells [27, 28, 33]. Most plant miRNAs as well as some rare viral miRNAs bind to their targets with perfect complementarity even though this is generally uncommon in higher order animals. Once bound to the target, miRNAs behave similar to siRNAs, inducing specific and irreversible endonucleolytic cleavage event in the target transcripts [34]. The question why this mode of miRNA-mediated action is so rarely used by animal host miRNAs is puzzling [26].

3. Mechanism of EBV Infection, Viral Proteins Expression Profile during Latency, and EBV-Associated Gastric Carcinogenesis

Mounting scientific evidence describes herpesviruses having two distinct life cycles: lytic replication and latency. Notably, EBV may infect host gastric epithelial cells through direct and indirect mechanisms. In the direct infection, viral glycoproteins attach to cellular receptors and drive viral proteins conformational changes that promote fusion of the viral envelope with the epithelial cell membrane [35]. In the indirect infection, instead, EBV initially infects B lymphocytes. Subsequently, EBV-infected B-cells, through direct cell-to-cell contact, infect epithelial cells via the capped adhesion molecules [36]. Following primary infection, EBV, after an early replication phase, establishes persistent latent infection in the host cells. During latency, viral genomes exist as extrachromosomal episomes in the nucleus and, being largely silenced by host-driven methylation of CpG island motifs, are only able to express a small subset of genes including latent proteins with oncogenic potential and viral miRNAs [36].

As a result, the latency enables the virus to efficiently evade the host immune response causing persistent infections over a lifetime. This is a feature common to all herpesviruses [4, 37]. Considering the subset of viral genes expressed, herpesviruses-associated tumors have been subdivided into four types: latency Ia, latency Ib, latency II, and latency III. EBVaGC belongs to latency type I, where the viral genes EBV nuclear antigen 1 (EBNA1), latent membrane protein 2A (LMP2A), Bam HI-A rightward transcripts (BARTs), and EBV-encoded small RNA (EBER1/2) may be expressed [10, 38, 39]. In particular, the expression of latency genes fulfils a relevant task in the initiation and neoplastic progression of EBV-associated epithelial cancers including EBVaGC. For instance, the viral protein EBNA1 is absolutely required in maintaining and replicating the viral episomal genome by the host cell DNA polymerase machinery in all EBV-associated malignancies. EBNA1 may contribute to cell survival after DNA damage and to genetic instability in EBV-infected epithelial cells [40].

Specifically, in both NPC and EBVaGC, EBNA1 can promote the decline of the promyelocytic leukemia (PML) nuclear bodies, including many regulatory proteins, by binding and modulating casein kinase 2 (CK2) or ubiquitin-specific protease 7, which in turn degrades p53 [41]. Therefore, EBNA1 may increase the genetic instability of the EBV-infected epithelial cells and facilitate the oncogenesis mainly by decreasing p53 levels following DNA damage. Moreover,

EBNA1 may also participate in tumor development by suppressing transforming growth factor-beta (TGF-β) [42] and improving the nuclear factor-kappa B (NF-κB) signaling [43]. In addition to EBNA1, half of all EBVaGCs also express LMP2A, and therefore EBV latency configurations should be classified into Ia or Ib based on the presence or absence of LMP2A [38, 44]. LMP2A viral protein, therefore, plays a paramount role in the transformation of epithelial cells by the induction and maintenance of tumor phenotypes. These activities range from resistance to apoptosis to cell proliferation, invasion, motility, and angiogenesis [45]. LMP2A also inhibits apoptosis by upregulation of the cellular survivin gene through the NF-κB pathway [46]. In epithelial cells, LMP2A may activate not only the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) pathway and phosphorylation of glycogen synthase kinase-3 but also the β-catenin signaling pathway. These molecular mechanisms induce several remarkable phenotypic changes including anchorage-independent growth and inhibition of differentiation [47–49]. These data herein reviewed collectively suggest that LMP2A viral protein not only has an impact on the NF-κB pathway which upregulates survivin gene, therefore inhibiting apoptosis, but also promotes the induction of cancer stem cells in EBV-associated epithelial cancers.

4. Role of Epigenetic Alterations in EBV-Associated Gastric Carcinogenesis

The term epigenetics currently refers to the inheritable changes in gene expression which occur without an alteration of the genome at the level of nucleotide sequences. Epigenetic mechanisms are necessary to support the physiological organ growth and development and moreover to insure normal gene expression in different tissues [50–52]. However, nowadays, gastric carcinogenesis processes can be explained not only by genetic modifications [53–56] but also by epigenetic alterations such as DNA methylation, histone modifications, and noncoding RNAs [51, 57]. These modifications are part of the "Epigenetic code" and are essential to regulate the normal development and maintenance of tissue-specific gene expression in different mammalian cell types [51, 52]. Increasing evidence suggests that some environmental factors such as aging, diet, physical activity, chronic inflammation, and microbial infection can also affect gene methylation in gastric epithelia and promote the development of gastric cancer [58, 59]. Specifically, EBV infection was reported as a cause of increased methylation and repression of tumor suppressor genes in MKN7, a low methylation GC cell line [11]. Further studies also confirmed that abnormal DNA methylation in the promoter regions of the gene, which provides inactivation of tumor suppressor and other cancer-related genes, is the most well-defined epigenetic characteristic in EBVaGC but not in EBV nonassociated GC (EBVnGC) [11, 60–66]. Specifically, hypermethylation of tumor suppressor genes such as E-cadherin (CDH1), p14, p15, p16, p73, adenomatous polyposis coli (APC), and phosphatase and tensin homolog (PTEN) were observed in EBVaGC but not in EBVnGC [67, 68]. In addition, Hino et al. [46] also demonstrated that LMP2A induced the expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), which stimulated the upregulation of DNA methyltransferases (DNMT) DNMT1 and DNMT2 in EBV-infected GC cells [69]. Recent studies by Zhao et al. [60] also reported that hundreds of genes implicated in cancer signaling pathways such as mitogen-activated protein kinase signaling, wnt signaling pathway, and cell adhesion molecules were hypermethylated following EBV infection [60]. The same authors also suggested that EBV infection induced aberrant CpG hypermethylation of several genes by upregulation of DNMT3b through LMP2A in EBV-positive AGS cells compared to EBV-negative AGS cells. Another important cellular alteration in EBVaGC is its resistance to programmed cell death (apoptosis). The frequency of apoptosis is markedly reduced in EBVaGC compared to EBVnGC [70]. Some studies hypothesize that both genes, somatostatin receptor 1 (SSTR1) and glutathione S-transferase P1 (GSTP1), are frequently hypermethylated in GC infected EBV tissues and modulate cell migration, proliferation, and apoptosis [67, 68, 71, 72]. However, the molecular mechanism of the aberrant DNA methylation following EBV infection is still unclear. As mentioned above, one possible mechanism is EBV induction of LMP2A overexpression which promotes STAT3 phosphorylation, further inducing DNMTs expression [60, 69]. Therefore, LMP2A may play an important role in cellular epigenetic dysregulation involved in the development and maintenance of EBV-associated cancer by increasing DNMTs expression.

5. Role of EBV-Encoded miRNAs in Gastric Carcinogenesis

The identification of two miRNAs, miR-15a and miR-16a, located in a deletion region of 30 kb on chromosome 13q14, identified in 50% of chronic lymphocytic leukemia (CLL) [73], was one of the first indications of possible involvement of miRNAs in human tumorigenesis. After this first experimental evidence, the correlation between the genomic locations of a large number of miRNAs and cancer-associated genomic regions has been better identified [74].

From the functional and evolutive point of view, miRNAs represent for viruses an element of fundamental regulation not only of their genes but also of the host genes. Compared to genes coding for proteins, the genes coding for miRNAs are small; this characteristic is ideal for space-constrained restricted viral genomes. Moreover, their small size could facilitate rapid adaptation to new targets through small changes in the level of their nucleotide composition. In fact, a miRNA can have multiple targets and inhibit the expression of different genes simultaneously. These characteristics make miRNAs ideal candidates for control of host-pathogen interactions [26]. Increasing evidence suggests that EBV is a DNA tumor virus belonging to the human gamma-herpesvirus family, which is capable of establishing a latent infection mainly in human B lymphocytes and epithelial cells, and is associated with several human lymphoid and epithelial cell malignancies including EBVaGC [1–6]. Probably, the

Table 1: List of main EBV-associated gastric carcinogenesis studies discussed in this review.

First author and year of publication	Study design	Paper number in references section	Summary of findings
Yau et al., 2014	Review	[10]	EBV infection contributes to gastric carcinogenesis through the expression of viral proteins and microRNAs as well as aberrant DNA methylation and histone modification and relative therapeutic implications.
Shinozaki-Ushiku et al., 2015	Review	[57]	In the present review latest findings on EBVaGC from clinicopathological and molecular perspectives were discussed to provide a better understanding of EBV involvement in gastric carcinogenesis. In addition to genetic and epigenetic changes, posttranscriptional gene expression regulation by cellular and/or EBV-derived microRNAs was also considered.
Shinozaki-Ushiku et al., 2015	Experimental research	[90]	Comprehensive profile of the expression of 44 known EBV miRNAs from EBV-associated gastric carcinoma patients was presented. Of several highly expressed EBV miRNAs, EBV-miR-BART4-5p plays a partial role in suppressing proapoptotic protein Bid, leading to reduced apoptosis. The present work provides novel insights into the roles played by EBV miRNAs in gastric carcinogenesis and identifies future potential therapeutic targets.
Kim et al., 2015	Experimental research	[91]	miR-BART20-5p contributes to the tumorigenesis initiation and/or maintenance of EBVaGC by directly targeting 3′-UTR of Bcl-2-associated death promoter (BAD) involved in cell proliferation and apoptosis. Inhibition of miR-BART20-5p can exert a therapeutic effect for this neoplasia.
Kanda et al., 2015	Experimental research	[92]	A causative relationship between BART miRNA expression and epithelial carcinogenesis in vivo was demonstrated. In particular, it was shown that NDRG1 protein, which is a putative target of BART miRNAs, can be used as an epithelial differentiation marker and a suppressor of metastasis.
Fu et al., 2013	Review	[94]	Potential mechanisms by which EBV contributes to its own latency and the formation tumors including EBVaGC were considered. Particularly, this review describes the interactions of EBV gene products including viral miRNAs and the Bcl-2 family members involved in cell death (apoptosis) and survival pathways. A better understanding of this complicated network of interactions could be of great importance for creating novel therapeutic strategies for EBV-associated diseases.
Tokunaga et al., 1993	Epidemiological research	[104]	EBV infection contributed significantly to gastric carcinogenesis in Japan. It occurred predominantly in males, especially in cancers of the upper and middle parts of the stomach, with greater cell type variation in men, suggesting that novel factors may play important causal roles in EBV-associated gastric carcinogenesis.

induction of tumors is not the main advantage of this virus but rather an accidental need to alter the cell cycle, preventing cell death, and evade the immune response [75]. In EBV-associated epithelial malignancies (e.g., NPC and EBVaGC) the gene products encoded by EBV play a crucial role at the beginning of the carcinogenesis process; therefore only few additional acquired genetic changes are required for the neoplastic transformation [10, 40, 41]. Specifically, in EBV-associated epithelial cancers, the latent genes (EBNA1, EBERs, and miR-BARTs) are intensely expressed in all cancer cells. Unlike EBNA1 and EBERs, miR-BARTs are expressed at high levels only in EBV-infected epithelial cancers, but not in EBV-transformed lymphocytes [56, 76, 77], pointing out their involvement in EBV-associated epithelial cancers. Interestingly, the expression of latency genes plays an important role in the initiation and neoplastic progression of gastric cancer by inducing strong antiapoptotic signals. For instance, EBER1 can confer an apoptotic-resistant phenotype by increasing the expression of insulin growth factor-1 (IGF-1), an autocrine growth factor which potentiates cell proliferation in EBVaGC [78]. During EBV infection of epithelial cells, these EBV-encoded viral regulatory RNAs may also modulate the host innate immune responses [79]. A recent study by Banerjee et al. [80] also suggested that EBERs could increase IL-6 expression and activate its downstream regulator STAT3, which was responsible for downregulation of the cell cycle inhibitors p21

and p27 in gastric carcinoma cells and associated cancer cell resistance. The same authors also demonstrated that EBERs could downregulate antimetastatic molecules, RhoGD1 and KAI-1, and activate the prometastatic molecules, pFAK and pPAK1, which induced cell migration. In addition, many EBV miRNAs also repress apoptosis by targeting the proapoptotic proteins p53-upregulated modulator of apoptosis (PUMA), Bcl-2 interacting mediator of cell death (BIM), and translocase of outer mitochondrial membrane 22 homolog (TOMM22), respectively [81–83]. Specifically, Choy and colleagues [81] have demonstrated that EBV miR-BART5 inhibits production of the proapoptotic protein PUMA by targeting mRNA which encodes the cellular factor PUMA. PUMA is one of the six members of the BH3-only group in the Bcl-2 family. The BH3-only proteins, the essential initiators of apoptosis, are responsible for controlling the release of cytochrome C from the mitochondrial intermembrane [84]. In EBV-infected carcinoma cells miR-BART5 depletion induces high levels of PUMA-mediated apoptosis, suggesting a crucial role of miR-BART5 in the inhibition of apoptosis both in EBV-infected epithelial cells and in EBV-transformed cells. Therefore, miR-BART5 may contribute to the survival of infected cells during the natural viral infection and influence the cell survival in virus-associated cancers. Another BH3-only group proapoptotic protein, Bim, has been reported to be a target of both EBV miR-BART4 and miR-BART15 [82, 85]. It is possible that this activity is linked to the observed inhibition of apoptosis by miRNAs of the BART cluster in the human gastric carcinoma cell line AGS. Interestingly, Bim's 3′-UTR is not responsive to any of the individual miR-BARTs in stable transfectants indicating possible cooperation of miR-BARTs in cluster 1 for Bim expression. Another potential target of miR-BART-16 is TOMM22, a protein forming part of a mitochondrial pore complex that stands as a receptor for the proapoptotic protein, Bcl-2-associated X (Bax) [83]. EBV can gain benefit from the repression of TOMM22 since siRNA-mediated knockdown of TOMM22 has been shown to inhibit the association of Bax protein to mitochondria, therefore preventing Bax-dependent apoptosis [86]. Other EBV miRNAs of the BART cluster variably expressed in EBVaGC tissue samples and cell lines are EBV-miR-BART1-3p, 5-5p, 7-3p, 15-3p, 19-3p, and 22-3p [87–89]. Recently, Shinozaki-Ushiku et al. [90] also reported that EBV-miR-BART4-5p exerts a crucial role in gastric tumorigenesis through modulation of apoptosis. Specifically, these authors demonstrated that reduction of apoptosis in clinical samples from EBVaGC patients was attributable to the expression of EBV-miR-BART4-5p which plays a partial role in suppressing proapoptotic protein Bid, also termed as the BH3 interacting domain death agonist. Another important finding has been recently reported by Kim et al. [91]. These authors suggested that miR-BART20-5p reduced Bcl-2-associated death promoter (BAD) expression in EBV-infected GC cells as opposed to EBV-negative GC cells by directly targeting 3′-UTR of BAD in order to promote host cell survival. Recently, Kanda et al. [92] also demonstrated that multiple EBV-encoded miRNAs contribute to EBVaGC by targeting N-myc downstream regulated gene 1 (NDRG1). This oncosuppressor protein was highly expressed in primary epithelial cells and significantly reduced in the BART(+) virus-infected epithelial cells, playing an important role in carcinogenesis and preventing the metastasis and invasion of gastric cancer cells. These data also implicate that NDRG1 could be utilized as a prognostic and/or diagnostic marker as well as a potential therapeutic target against gastric cancer [92, 93] (Table 1). Additional studies by Fu et al. [94] also demonstrated that EBV BHRF1 miRNAs can promote cell survival by interacting with several proapoptotic proteins such as Bcl-2 homologous antagonist/killer (Bak), Bcl-2-related ovarian death gene (Bod), Bcl-2-related ovarian killer (Bok), Bim, and Bcl-2-interacting killer (Bik) in various cell lines including gastric carcinoma cell lines. The same authors also indicate that BARF1 (Bam HI-A fragment rightward reading frame 1) might promote cell transformation by activating antiapoptotic protein Bcl-2 [94]. In addition to these effects, EBV infection may also affect host cell miRNA expression and exert effects on immune responses and oncogenesis. For instance, studies by Shinozaki et al. [95] have demonstrated downregulation of the cellular miR-200 family (e.g., miR-200a and miR-200b) in EBV-associated gastric carcinoma by repressing transcription of pri-miRNAs and by posttranscriptional dysregulation of the miRNA in EBVaGC compared to EBVnGC and adjacent mucosa. Specifically, these authors indicate that all the latency type I genes such as Bam HI-A rightward reading frame (BARF0), EBERs, EBNA1, and LMP2A have a synergetic effect on these processes and contribute to the downregulation of the mature miR-200 family and the subsequent upregulation of the E-cadherin transcription repressors, zinc finger E-box binding factor 1 (ZEB1) and ZEB2, resulting in the inhibition of E-cadherin expression and induction of the epithelial-to-mesenchymal transition (EMT) [95]. This is a pivotal stage in the process of carcinogenesis of EBV-associated gastric carcinoma. Another report recently published by Du and colleagues [96] also revealed that miR-141, a member of the miR-200 family, was downregulated in 80% of primary gastric cancer tissues showing its inhibitory effect on cell proliferation. A recent study by Marquitz et al. [97] suggests that, by infecting EBV-negative AGS gastric carcinoma cell lines with a recombinant EBV, a clear hallmark of transformation becomes apparent with an anchorage independent phenotype. Specifically, the authors showed that the cells have levels of BART miRNAs similar to EBV-positive gastric cancer. They also reported that EBNA1 overexpression in AGS gastric carcinoma cell lines significantly reduced miR-143 expression, which acts as tumor suppressor in several types of cancer [98–100]. More recently, Marquitz et al. [101] also indicate that EBV infection induces downregulation of host tumor suppressor miRNAs including the Let-7 family and the miR-200 family through a mechanism that is independent of latent protein expression. It seems that EBV miR-BART6 can repress the expression level of miRNA processing human DICER1 enzyme in EBV-infected cells compared to noninfected cells, playing an important role in the progression of EBV-associated tumors [102]. Therefore, EBV-encoded miRNAs may be an aetiological factor in cancer development in immunocompetent individuals that could bypass the requirement for viral protein expression

and the consequential recognition by the immune system. Surprisingly, not all miR-BARTs inhibit apoptosis or promote cell growth. For example, Choi et al. [103] reported the tumor suppressive effect of miR-BART15 in gastric carcinogenesis. The authors demonstrated that miR-BART15 inhibited cell growth and induced early apoptosis in AGS gastric carcinoma cell in part by targeting the BRUCE gene which encodes the BRUCE protein, an inhibitor of apoptosis proteins (IAPs). In addition, miR-BART15 might also indirectly contribute to cancer development by inducing its proapoptotic activity in the adjacent immune cells by targeting BRUCE [103]. These results collectively suggest that some EBV-encoded miRNAs may regulate the expression of several key cancer-related proteins, including those involved in latency maintenance, immune suppression, and tumor promotion. Additional data would warrant a more solid conclusion on this issue.

6. Conclusions

Accumulating evidence suggests that miRNAs can be encoded not only by eukaryotes but also by certain viruses. The vast majority of viral miRNAs are encoded by the herpesviruses family including pathogens such as, EBV, herpes simplex viruses (HSVs), human cytomegalovirus (HCMV), Kaposi's sarcoma herpes virus (KSHV), and MHV 68. Specifically, EBV expresses multiple noncoding RNAs during all types of latency, including two clusters of miRNAs: BART miRNAs and BHRF1 miRNAs. EBV-transformed cells encode at least 44 mature viral miRNAs that target viral and cellular genes. Some viral miRNAs, including those which are analogs to host miRNAs and those which are virus-specific, seem to exert an important role in the establishment of a latent viral infection by suppression of an effective host immune response or through blockade of apoptotic processes in the infected cells [105, 106]. In all EBV-associated cancers, the viral infection also promotes important changes in the cellular miRNAs profile. It has been shown that many cell miRNAs in cell lines or in tumor tissues are deregulated upon EBV infection. The presently available information suggests that viral miRNAs also contribute to induction or maintenance of the transformed phenotype. In fact, latent infection with EBV is considered responsible for several malignancies, including a big amount of all gastric carcinomas. EBVaGC is characterized by unique clinical and pathologic features including male predominance, the presence of EBV genomes and EBV-encoded small RNA (EBER) in gastric carcinoma cell lines, and monoclonal proliferation of EBV-infected carcinoma cells [4, 9, 57, 104]. In addition, it also presents elevated levels of serum antibodies against EBV early antigen and EBV capsid antigen as well as a lymphoepithelioma-like histology and a relatively favourable prognosis [4, 9, 57, 104]. EBVaGC also shows abnormal hypermethylation of several tumor suppressor gene promoter regions, causing downregulation of their expression. In addition, EBVaGC is distinctive owing to the limited number of EBV latent genes expressed in cancer cells [96]. It is classified as latency type I because the expressed latent genes are restricted to BARF0, EBERs, EBNA1, and LMP2A, excluding EBNA2 or LMP1 which are essential for its transforming ability [107–109]. All four of these genes play an important role in the downregulation of mature miR-200 and the subsequent upregulation of transcription repressors ZEB1/ZEB2, resulting in the inhibition of E-cadherin expression and induction of the EMT, which is a crucial step in the carcinogenesis of EBVaGC. Notably, downregulation of mature miR-200 may be mediated by aberrant DNA methylation due to overexpression of DNMTs following EBV infection. EBER1 may also increase the expression of IGF-1, an autocrine growth factor which accelerates cell proliferation in EBVaGC [78]. Another important aspect underlined by Marquitz et al. [101] is that in several EBV-infected AGS gastric carcinoma cell lines a significant fraction of the changes in cellular expression likely reflect(s) the expression of the viral noncoding RNAs such as the BART RNAs and not the latent protein expression. These changes comprise a decrease in host tumor suppressor miRNAs levels and increased expression of viral miRNAs with putative oncogenic potential. However, the precise role of EBV in the multifactorial aetiology of gastric carcinoma is still not well known. In particular, it will be necessary to identify more targets of viral and deregulated cellular miRNAs. A general problem in EBV-encoded miRNAs research is the lack of an animal model. The advancement of humanized mice should facilitate the eventual development of EBV infection mouse models. Such models will ultimately be necessary to evaluate viral miRNAs as potential therapeutic cancer targets. They might be also used to evaluate different chemopreventive agents. In this respect, scientific evidence suggests that natural bioactive agents such as green tea catechins could be useful for the modulation of the epigenome [110] and the subsequent inhibition of viral infections and virus-associated malignancies [111–114]. Furthermore, green tea catechins may also inhibit chronic inflammation involved in viral oncogenesis by regulating the Nrf2 and NF-κB signaling pathways [115–117].

Conflict of Interests

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

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Research Article

Protective Effect of Parsley Juice (*Petroselinum crispum*, Apiaceae) against Cadmium Deleterious Changes in the Developed Albino Mice Newborns (*Mus musculus*) Brain

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Parsley was used as a probe of the current experiment to prevent the behavioral, morphological and biochemical changes in the newborn brain following the administration of cadmium (Cd) to the pregnant mice. The nonanesthetized pregnant mice were given daily parsley juice (*Petroselinum crispum*) at doses of 20 mg/kg and 10 mg/kg. Pregnant mothers were given Cd at a dose of 30 mg/kg divided into 3 equal times. The newborns have been divided into 6 groups: Group A, mothers did not take treatment; Groups B and C, mothers were treated with low and high dose of parsley, respectively; Group D, mothers were treated only with Cd (perinatal intoxication); Groups E and F, mothers were treated with Cd doses and protected by low and high doses of parsley, respectively. Light microscopy showed that Cd-induced neuronal degeneration by chromatolysis and pyknosis in the brain regions. The low dose of parsley 10 g/kg/day exhibited significant effects in neutralizing and reducing the deleterious changes due to Cd exposure during pregnancy on the behavioral activities, neurotransmitters, oxidative stress, and brain neurons morphology of the mice newborns.

1. Background

Cadmium (Cd) exposure produces severe toxicity in multiple organ tissues because it produces oxidative stress, disrupts aquaporins, and interferes with functions of essential cations such as zinc and magnesium [1]. Cd causes high risks to the young, as exposures early in life during development [2]. It is classified as one of the most toxic and carcinogenic metals [3]. It was reported as a serious industrial and environmental pollutant and may cause serious health hazards to humans and animals [4]. Many sources of Cd exposure for humans and animals could be from several industries (such as petroleum mining, metal plating, pigments, plastics, batteries, toys, and alloy), cigarette smoking, or dietary consumption [5]. Exposure to Cd may cause lesions in many organs such as central nervous system (CNS), liver, kidney, and testis [1, 3, 6].

The long-term changes in neurobehaviors such as alterations in attention and memory as well as in the psychomotor and vasomotor functioning and speed in workers are due to Cd exposure [7]. Moreover, rat studies have observed increased aggressive and anxiety-like behaviors, impaired learning and memory processes, and changes in the development of the visual system [8]. Some studies on Cd toxicity have found an association with behavioral disturbances and cholinergic neurotransmission since an increase or a decrease in the acetylcholine esterase activity was verified in both animal models and humans that showed behavioral impairments after exposure to Cd [9]. This enzyme hydrolyses the neurotransmitter acetylcholine in the synaptic cleft of cholinergic synapses and neuromuscular junctions [10]. Alterations in the acetylcholine activity in various diseases and poisonings suggest that this enzyme could be an important physiological

and pathological parameter [11]. In addition, maternal Cd exposure during pregnancy induced fetal growth restriction [12]. Nevertheless, the molecular mechanism for Cd-induced development toxicity remains obscure.

Cd has well-documented teratogen and embryotoxic effects in a large variety of species, including man [13]. Cd is more toxic to newborns and young rats than to adult [14]. This metal accumulates in the brain of developing and adult rats [11] leading to brain intracellular accumulation, cellular dysfunction, and cerebral edema. Also, it can affect the degree and balance of excitation-inhibition in synaptic neurotransmission as well as the antioxidant levels in animal brain [15]. Cd embryotoxicity is partly due to oxidative DNA damage associated with increased producing of oxygen reactive species (ROS) and decreased antioxidant enzyme levels, and the interaction of Cd with the enzymes that repair damaged DNA [16]. Many culinary herbs (e.g., parsley) have been shown to function as natural antioxidants [17].

Parsley (*Petroselinum crispum*, Apiaceae) is an annual herb which is important dietary source of vitamins and essential metals. Its supplementation at sufficient levels can promote the levels of the vitamins and essential metals in the human body, which in turn can decrease the risks of Cd toxicity [18]. Phytochemical screening of parsley has revealed the presence of some compounds such as flavonoids [19], carotenoids [20], ascorbic acid [21], and tocopherol [22]. These components of fresh parsley leaf scavenge superoxide anion in vitro and hydroxyl radical in addition to protecting against ascorbic acid-induced membrane oxidation [19], where lipid oxidation is a major cause of food quality deterioration. Supplementation of diets with fresh parsley leaf can increase antioxidant capacity of rat plasma and decrease oxidative stress in humans [23]. Similarly, aqueous and ethanol extracts of fresh parsley leaf strongly inhibit linoleic acid oxidation and lipid oxidation [24].

Parsley is reported as a good source of antioxidant which may prevent Cd toxicity and teratogenicity. Also, it is one of the most used medicinal plants to treat arterial hypertension [25], diabetes, cardiac [25], and renal diseases [26]. Moreover, in experimental studies, it has been reported that this herb has strong diuretic [27], antihyperglycemic [28], antihyperlipidemic, anticoagulant [29], antioxidant [30], antimicrobial [31], and laxative activities [32]. Alcoholic extract of parsley has a protective effect against toxicity induced by sodium valproate (SVP) in male rats [33]. Parsley leaf was used for treatment of constipation, jaundice, colic, flatulence edema, and rheumatism. It was used as an aphrodisiac, improved productive performance in broiler, antimicrobial, antianemia, hemorrhagic, anticoagulant, antihyperlipidemic, antihepatotoxic, and laxative [32, 33]. It was used to treat eczema, knee, ache, impotence, and bleed [34]. However, according to our knowledge, no investigations have been reported in the literature on the protective effect of parsley against Cd teratogenicity.

In the present work we investigated the hypothesis of the protective effect of parsley juice against Cd intoxication during pregnancy and lactation periods in albino mice newborns. Behavioral and motor performances of newborns have been investigated. Also, we have evaluated the effects of this metal on oxidative stress, neurotransmitter activities, and brain structures of the newborns.

2. Methods

2.1. Chemicals. The current cadmium chloride ($CdCL_2$) and other chemicals have been purchased from Sigma Company (St. Louis, MO, USA).

2.2. Parsley Juice Preparation. *Petroselinum crispum* (Mill.) Nym. ex A.W. Hill from the family Apiaceae (alt. Umbelliferae) is commonly known as parsley. The origin of parsley is from Mediterranean region, but today it is cultivated wherever of the world. Botanic identification was performed by taxonomist in the Department of Botany and Microbiology, Collage of Sciences, King Saud University, Riyadh, Saudi Arabia. The plain leaf of parsley type was daily collected from vegetable market in Riyadh (Saudi Arabia) and was carefully washed under tap water. The fresh parsley juice was prepared daily using a vegetable juices machine. Two concentrations of the juice were prepared: the first is 10% juice, that is, 10 g parsley squash in 100 mL drinking water. The second is 5% juice, that is, 5 g parsley squash in 100 mL drinking water. The prepared juice has been filtered using a filter paper after preparation and before drinking by the animals to remove fibers and other insoluble materials.

2.3. Ethics Statement. All the experimental protocols and investigations were approved and complied with the Guide of Laboratory Animals Use and Care which have been published by the United States of America National Institutes of Health (NIH Publication number 85–23, revised 1996) and have been approved by the Animal Experimentation Ethics Committee at the King Saud University (Permit number: PT 983).

2.4. Animals and Dosing Schedule. The current study used a total of 45 albino mice (*Mus musculus*), 15 mature males and 30 mature virgin females (weighing 30–35 g), collected from the animal house in the College of Pharmacy, King Saud University. The animals were housed in mouse cages (1 animal/cage) under pathogen-free and healthy conditions. Males and females lived at 22–25°C on a light/dark cycle (12 : 12 h), in addition to provided water and food *ad libitum*. Mating was done between proesterous females and males overnight by housing of one female with one male in special cages used for mating (stainless steel wire cages). Vaginal plug deposition at morning determined the day zero of gestation. Parsley juice was orally administered daily to nonanesthetized parsley treated groups by gastric intubation at a dose of 20 g/kg/day and 10 g/kg/day from day zero of gestation till postnatal day 30.

Totally, 30 mg/kg of $CdCl_2$ was dissolved in 30 mL saline and intraperitoneally injected to partially anesthetized Cd treated groups on three stages: at day 7 of gestation, postnatal day 1, and postnatal day 15 (10 mg/kg every time).

The mothers were labeled and divided into 6 groups as follows:

Group A: pregnant mice were given tap water orally and saline intraperitoneally (did not take treatment as control group).

Group B: pregnant mice were given 5% parsley juice orally and saline intraperitoneally (*5% parsley group*).

Group C: pregnant mice were given 10% parsley juice orally and saline intraperitoneally (*10% parsley group*).

Group D: pregnant mice were given tab water orally and Cd doses intraperitoneally (*Cd intoxicated group*).

Group E: pregnant mice were given 5% parsley juice orally and Cd doses intraperitoneally (*5% parsley-Cd group*).

Group F: pregnant mice were given 10% parsley juice orally and Cd doses intraperitoneally (*10% parsley-Cd group*).

2.5. Newborns Body Weights Assessment during Lactation Period. A physical developmental landmark like body weight is a useful indicator of the development through the entire lactation period. Thus, the newborns were weighed every day from postnatal day 1 until day 28.

2.6. Cd Estimation Assay. The analytical determination of Cd in the brain was carried out according to Shah et al. [35] using Inductively Coupled Plasma Mass Spectrometer (ICP-MS) under operation conditions of the instruments shown in Table (S1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2016/2646840).

2.7. Behavioral Assays. Ten meal mice pups from each group (first generation) were investigated in the current study at postnatal day 30 (postweaning period). Each newborn was used only in one test. For tests conduction, the pups were brought into investigation room of dim red light used for this purpose (25°C). The tests were done blindly by one experimenter according to Ajarem and Ahmad [36].

2.7.1. Cage Activity Assay. The Ugo Basile 47420-Activity Cage was used to record spontaneous coordinate activity in mice and variation of this activity in time either horizontal or vertical movements. This test was performed for 3 min/animal.

2.7.2. Grip-Strength Meter Assay. The Ugo Basile 47200 Grip-Strength Meter suitable for mice automatically measures Grip Strength (i.e., peak force and time resistance) of the forelimbs in mice. The aim was to assess forelimbs muscle strength. Each animal was tested three times and the peak force of each mouse was recorded. The mean of three values of each mouse was recorded.

2.7.3. Rota-Rod Assay. The Ugo Basile rota-rod instrument has been used to estimate the balance ability in the investigated animals. The newborns were placed on a horizontally oriented rod which mechanically rotates at 10 ×g. The newborns innately will try to stay on the rota-rod (rotating rod) and will try to avoid falling down on the instrument sensor. The length of time that the tested newborns stay on the rota-rod was used as a measure of their coordination, physical condition, balance, and motor activity.

2.8. Biochemical Assays. Six newborns from each group were anesthetized by light ether and sacrificed by decapitation at postnatal day 30. The brain was dissected and 0.5 g tissue was homogenized in 5 mL of cold 0.1 M $HClO_4$ containing 0.05% EDTA. The homogenate was centrifuged at 1000 ×g for 10 min at 4°C and the clear supernatant was collected in a microfuge tube (0.5 mL each) and was stored at −40°C until used.

2.8.1. Dopamine and Serotonin Determination Assay. The levels of neurotransmitters serotonin or dopamine were estimated in the brain using the modified method of Eghwrudjakpor et al. [37] as mentioned by Abu-Taweel et al. [38].

2.8.2. Acetylcholine Determination Assay. The estimation of acetylcholine was done according to the method of Ichikawa et al. [39].

2.8.3. Lipid Peroxidation Assay. Lipid peroxidation was estimated by assessment of thiobarbituric acid-reactive substances (TBARS) using the method of Preuss et al. [40].

2.8.4. Glutathione (GSH) Assay. The reduced glutathione content was estimated using the modified method of Beutler et al. [41] as reported in Allam et al. [42].

2.8.5. Peroxidase Activity Determination Assay. Peroxidase enzyme activity was estimated using the procedure of Kar and Mishra [43].

2.9. Histological Studies Using Light Microscopy. Four pups of each group have been anesthetized using light ether and decapitated at postnatal day 30 followed by dissection. Histological preparations of the left loop of cerebellum, cerebral cortex, and medulla oblongata were done as mentioned by Allam et al. [42]. The use of Haematoxylin and Eosin stains for the paraffin sections was done according to the method of Mallory [44].

2.10. Statistical Analysis Assays. The current study data has been analyzed using the software program Statistical Package for the Social Sciences (SPSS for Windows version 11.0; SPSS Inc., Chicago). The comparative analyses were conducted by using the general linear models procedure (SPSS, Inc.). The data has been tested by one-way and two-way analysis of variance (ANOVA) followed by LSD computations to compare various groups with each other. Results were expressed as mean ± SD. The level of significance was expressed as very

highly significant at $^{***}P < 0.001$, highly significant at $^{**}P < 0.01$, and significant at $^{*}P < 0.05$.

3. Results

3.1. General Observations. The signals of toxicity due to Cd exposure were noticed postnatally in group D mothers represented by weakness and ataxia of hindlimb muscles causing alteration in maternal behavior, so their newborns suffered from bad lactation and consequently malnutrition. These signals of toxicity were reduced in groups E and F and especially group E due to parsley supplementation. The insignificant difference in letters size was observed in groups B, C, and E while highly significant and very highly significant reduction were detected in groups F and D (Figure 1(a)) compared to group A. The body weights of the pups of the present experimental groups were varied from postnatal day 1 to day 28 (Figure 1(b)). The pups of groups D, E, and F suffered from a noticeable decrease in the body weight gain especially group D. The pups of group E showed marked increase in their body weight gain by the third week of age to become near the values of normal pups.

The Cd concentration in the brain tissues was estimated in the brain tissues of the newborns. Figure 1(c) showed the Cd bioaccumulation in the brain of groups B and C was highly significantly reduced while in group D it was very highly significantly elevated and in groups E and F it was highly significantly elevated compared to group A. The current results showed that parsley significantly reduces the Cd concentration in pup's brain of parsley treated group.

3.2. Behavioral Investigations. In the activity cage and in comparison with the newborns of group A, the newborns of group D showed a significant elevation in the vertical and horizontal movement in comparison with group A. Contrary to the vertical movement, the newborns of groups B and C showed significant increase in the value of horizontal movement. Despite Cd and parsley treatment, the newborns of groups E and F displayed vertical and horizontal movement values near the values of normal newborns (Figures 2(a) and 2(b)).

The forelimb muscles of the newborns of groups A, B, and C recorded relatively similar beaks in the grip strength examination scores. While the recorded beaks of group D newborns appeared significantly small, the beaks achieved by the newborns of groups E and F showed significant improvements (Figure 2(c)).

In rotator test and in comparison with the newborns of group A, the time of group D pups on the rod was significantly small. The staying times of the newborns of groups B, C, E, and F on the rod were less than the time of group A but the difference was not significant (Figure 2(d)).

3.3. Biochemical Studies

3.3.1. Neurotransmitters. In groups A, B, and C, the dopamine concentration in the brain appeared nearly similar with little elevation in group C. In comparison with group A, very highly significant depletion of dopamine concentrations has been detected in group D ($P < 0.001$) and highly significant reduction in groups E and F ($P < 0.01$) as in Figure 3(a).

No significant differences in the brain-serotonin concentration were detected between groups A, B, C, and F while highly significant reduction ($P < 0.01$) and significant decrease ($P < 0.05$) have been detected in groups D and E, respectively (Figure 3(b)).

The present acetylcholine concentrations showed similar results to dopamine. In groups A, B, and C, its concentration did not have any insignificant differences while it displayed very highly significant ($P < 0.001$), highly significant ($P < 0.01$), and significant ($P < 0.05$) depletion in groups D, E, and F, respectively (Figure 3(c)).

3.3.2. Oxidative Stress. In comparison with control newborns, the brain lipid peroxidation was insignificantly reduced in group B while insignificant increase was observed in group C (Figure 4(a)). The elevation of TBARS was highly significant ($P < 0.01$) in group D and insignificant ($P > 0.05$) in groups E and F (Figure 4(a)).

Cd exposure produced noticeable reduction in GSH content ($P < 0.001$) in group D (~50%). Improvement was observed in groups E and F although the difference between them and control group was still significant ($P < 0.05$) according to Figure 4(b). Insignificant difference ($P > 0.05$) was observed between groups A, B, and C (Figure 4(b)).

Peroxidase activity showed fluctuations in the experiment groups. No significant difference was observed between groups A and C while an insignificant reduction was noticed in group B. Cd exposed groups showed significant increase of peroxidase activity in both groups D and F while the increase was insignificant ($P > 0.05$) in group E (Figure 4(c)). The maximal elevation of peroxidase activity was observed in group D ($P < 0.01$).

3.4. Brain Histoarchitecture Changes. At postnatal day 30, the normal pyramidal neurons exhibited their general characteristic shape. The nuclei of these cells were rounded, large, and centrally located (Figure 5). The normal cerebral cortex cells had pyramidal or spherical perikaryon with large nuclei where the cells were arranged in a uniform pattern (Figures 5(a), 5(b), and 5(c)). These neurons seemed more developed if we move toward the white matter. The cerebral investigation of many sections in the Cd exposed groups showed some pathological cases. In group D, chromatolysis and pyknosis have been observed in the pyramidal neurons. In groups E and F, parsley juice showed significant neuronal protection through reducing the rate of chromatolysis and pyknosis (Figures 5(d), 5(e), and 5(f)).

In the cerebellum, the fold layers (molecular, Purkinje cells, and internal granular) have become completely mature and the external granular layer disappeared completely (Figure 6). The neuronal density in the molecular layer of normal and parsley treated groups was the highest compared to Cd treated groups. The normal Purkinje cells had a pear-shaped perikaryon and large nucleus and were arranged in a single row. The lateral processes disappeared and the apical processes formed the permanent dendritic tree (Figure 6). In

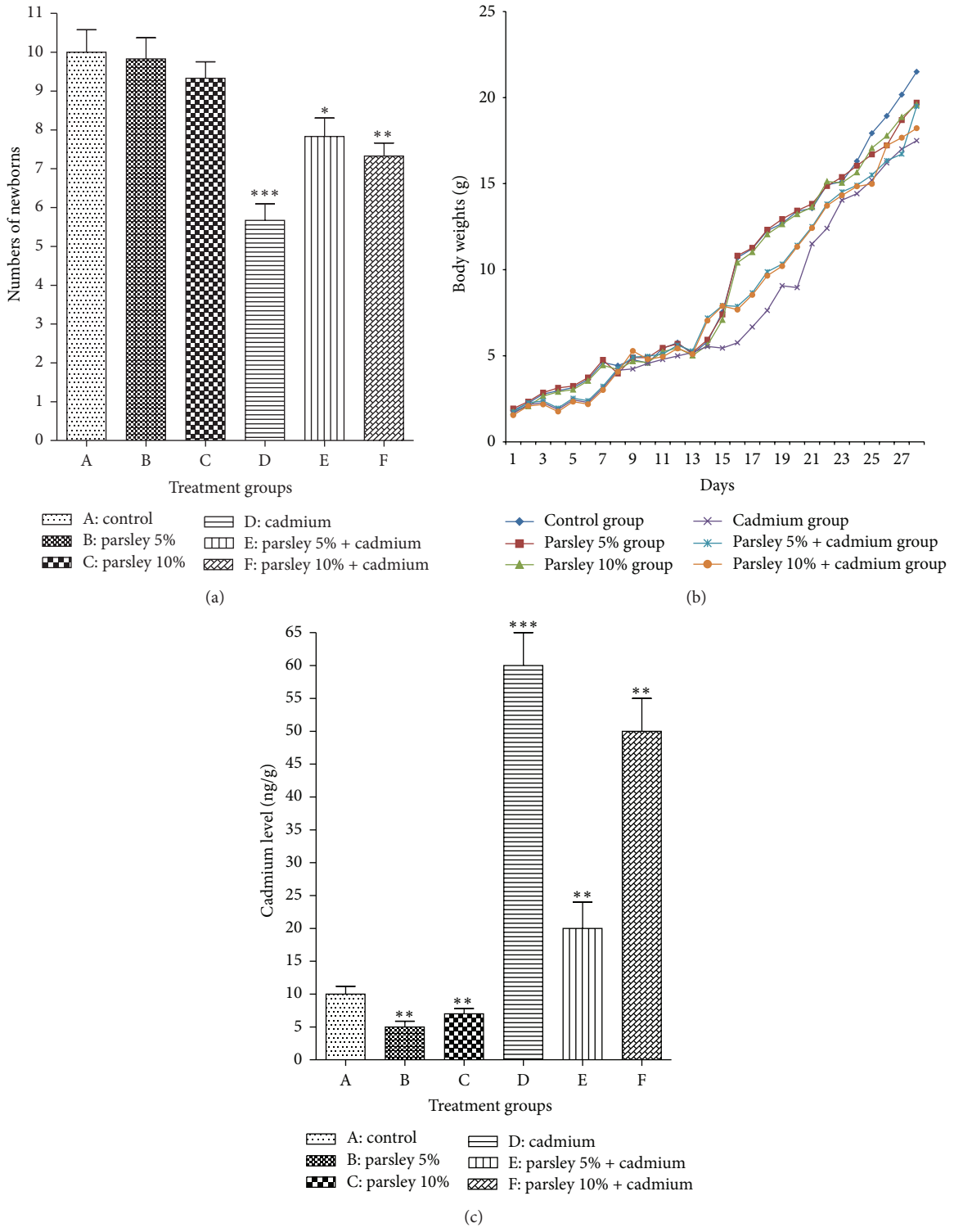

FIGURE 1: Postnatal developmental observations. (a) The number of alive delivered newborns (litter size) of each group at postnatal day 1. (b) The newborns body weights of each group from postnatal day 1 to day 28. (c) The Cd concentration in the brain tissues of the newborns of each group at postnatal day 30. Data are expressed as mean ± SE ($N = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, significantly different from the control group).

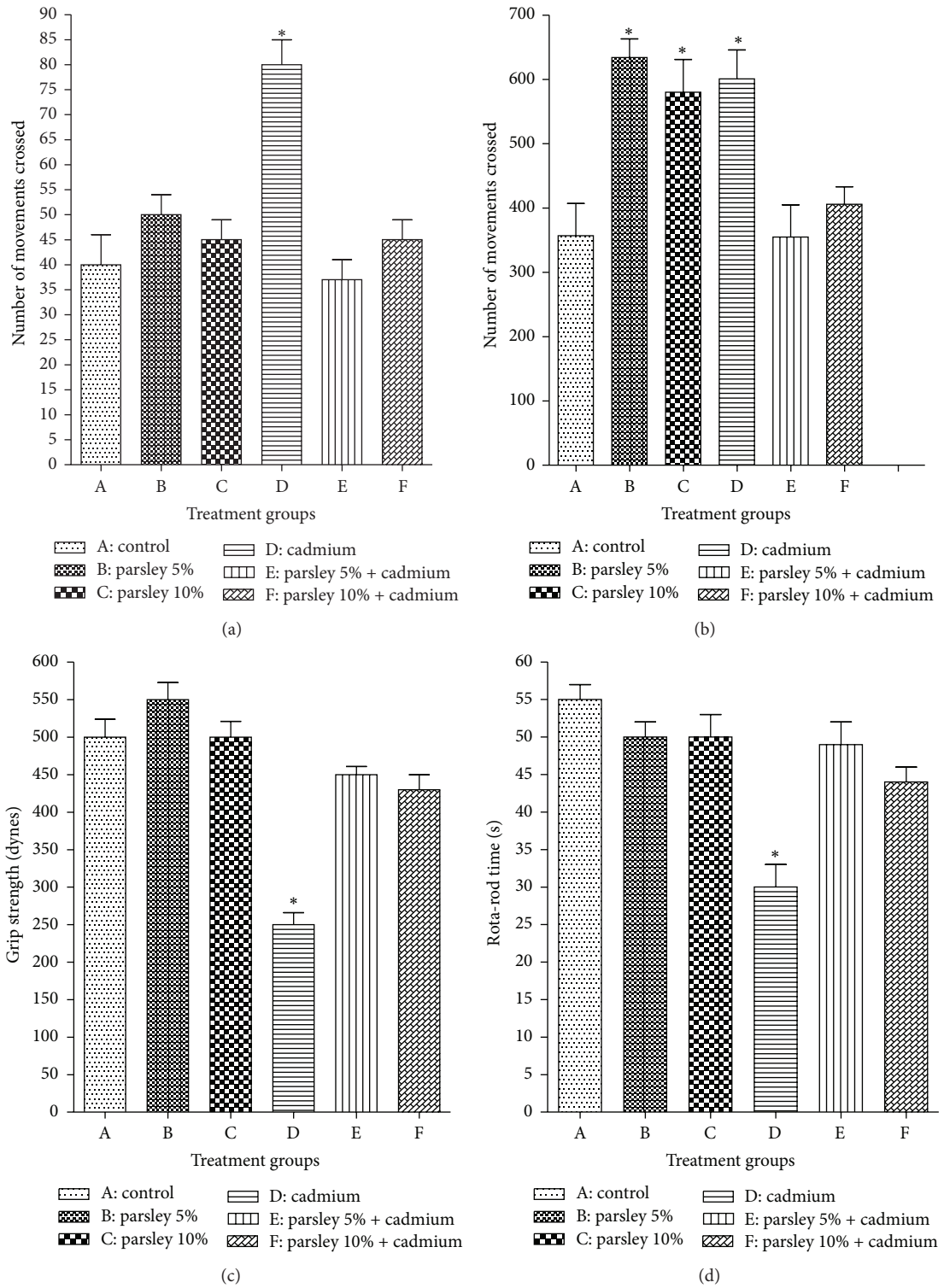

Figure 2: Behavioral investigations of the newborns of each group at D 30. (a) Vertical movements, (b) horizontal movements, (c) grip strength records for the forelimb, and (d) rota-rod records. Data are expressed as mean ± SE ($N = 6$; $^*P < 0.05$, significantly different from the control group).

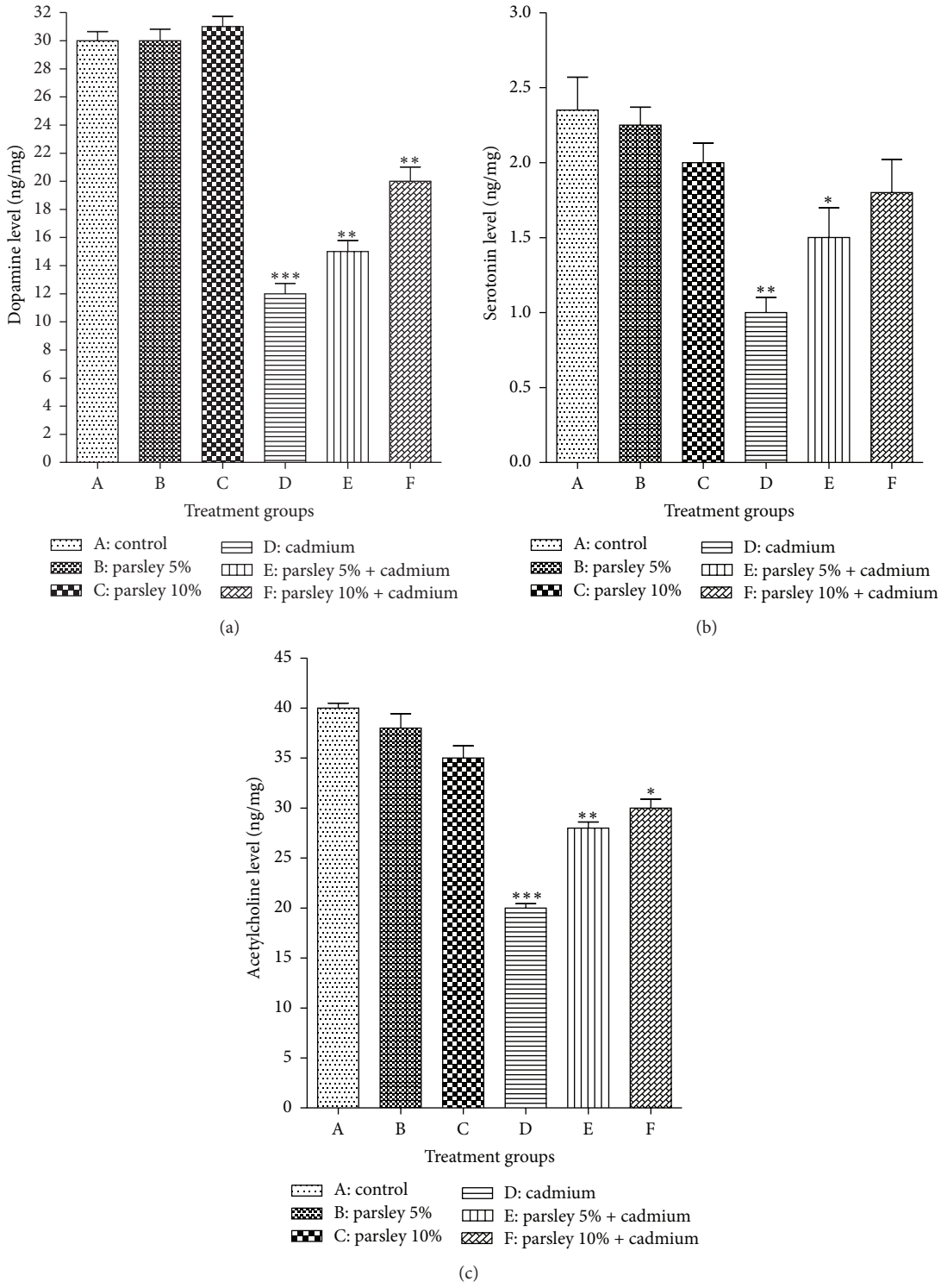

Figure 3: Extracellular neurotransmitters concentration in the newborns brain tissues at postnatal day 30. (a) Dopamine, (b) serotonin, and (c) acetylcholine. Data are expressed as mean ± SE ($N = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, significantly different from the control group).

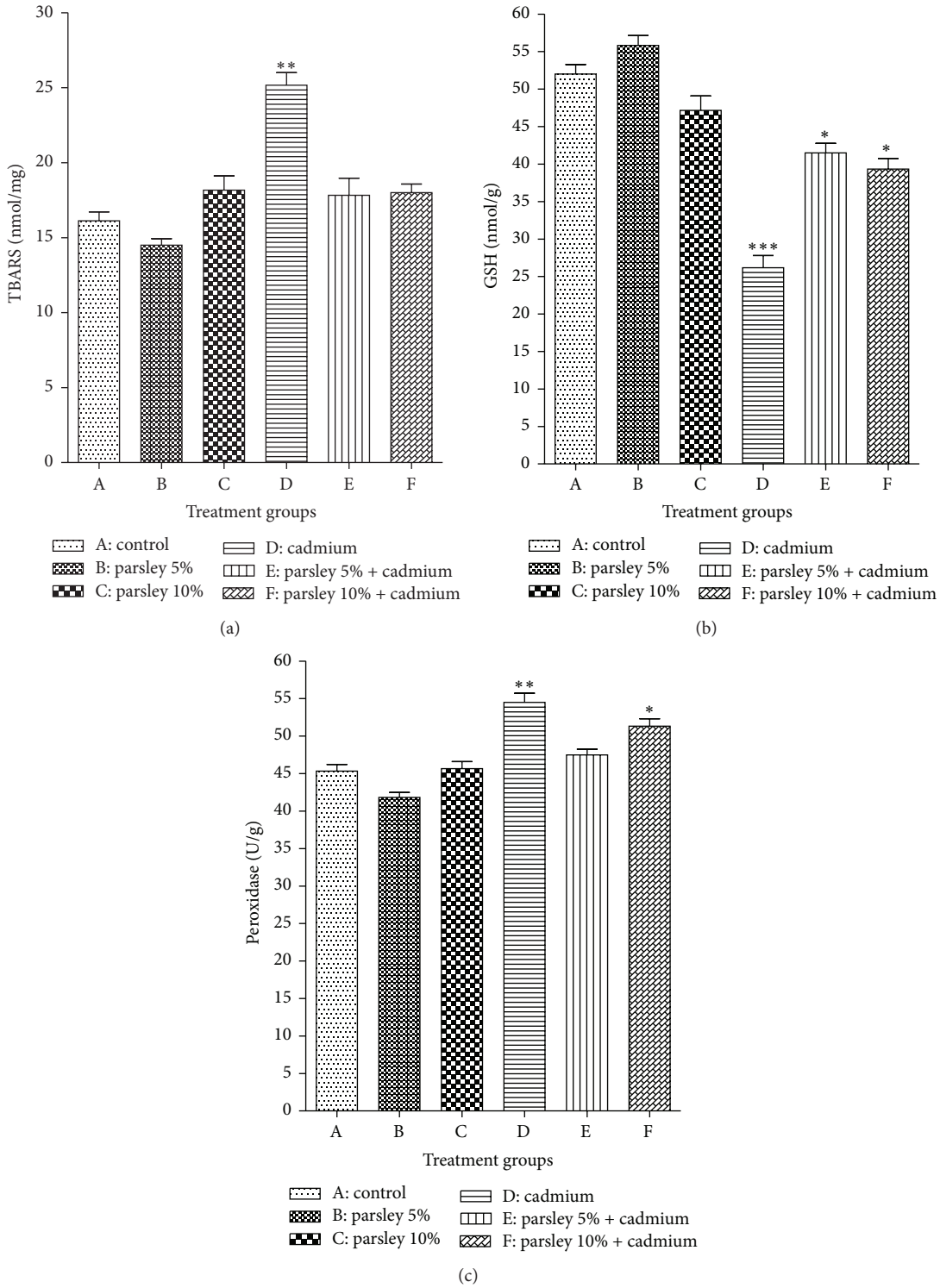

Figure 4: Oxidative stress parameters in the brain tissues of the newborns of each group at postnatal day 30. (a) TBARS, (b) GSH, and (c) peroxidase. Data are expressed as mean ± SE ($N = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, significantly different from the control group).

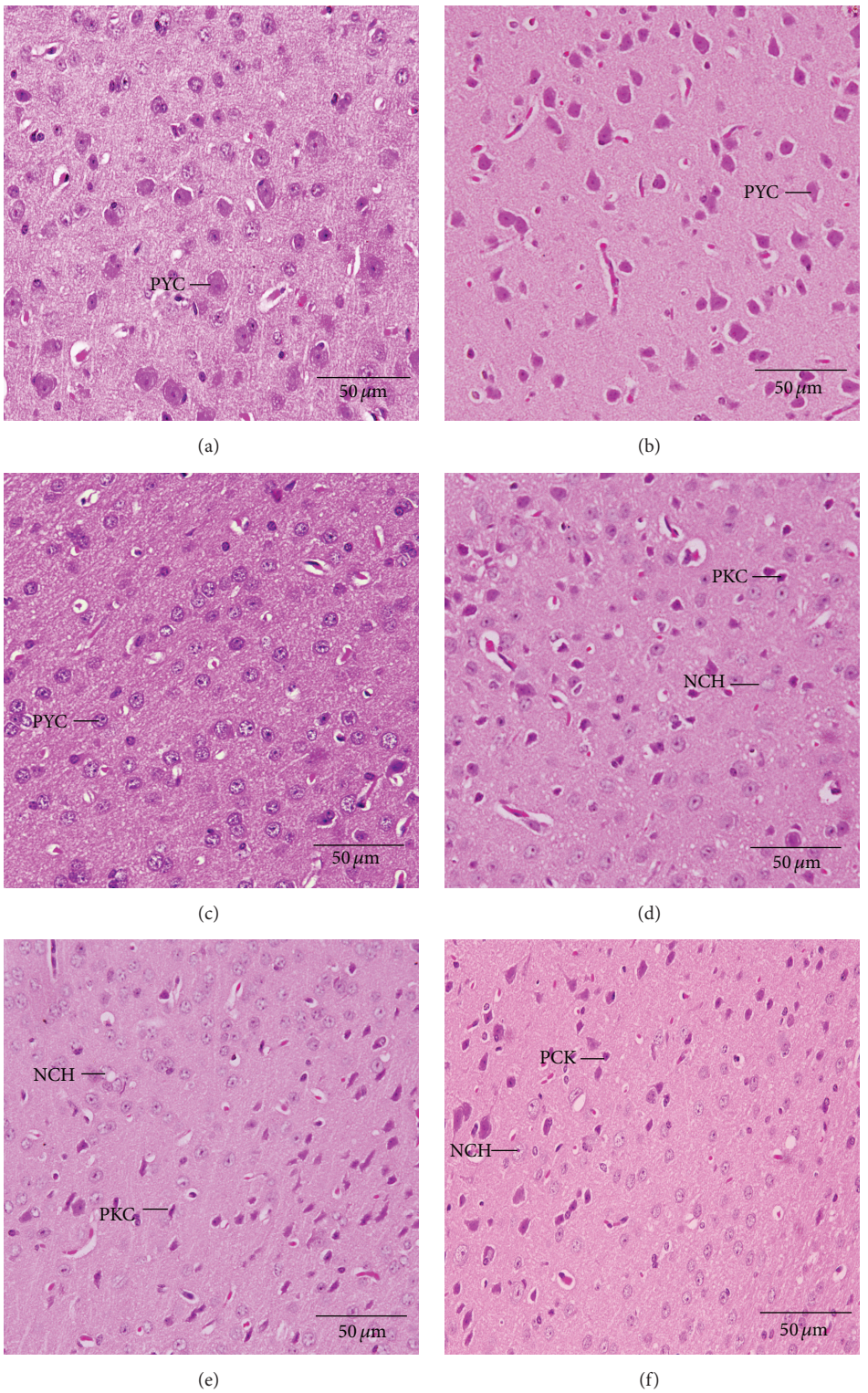

Figure 5: Histological changes in the cerebral cortex of the newborns at postnatal day 30 showing pyramidal neurons (PYC), degenerated pyramidal cells (PKC), and neurocyte chromatolysis (NCH). (a) Control group, (b) parsley 5% group, (c) parsley 10%, (d) cadmium inoculated group, (e) cadmium inoculated group + parsley 5%, and (f) cadmium inoculated group + parsley 5% (H&E stain).

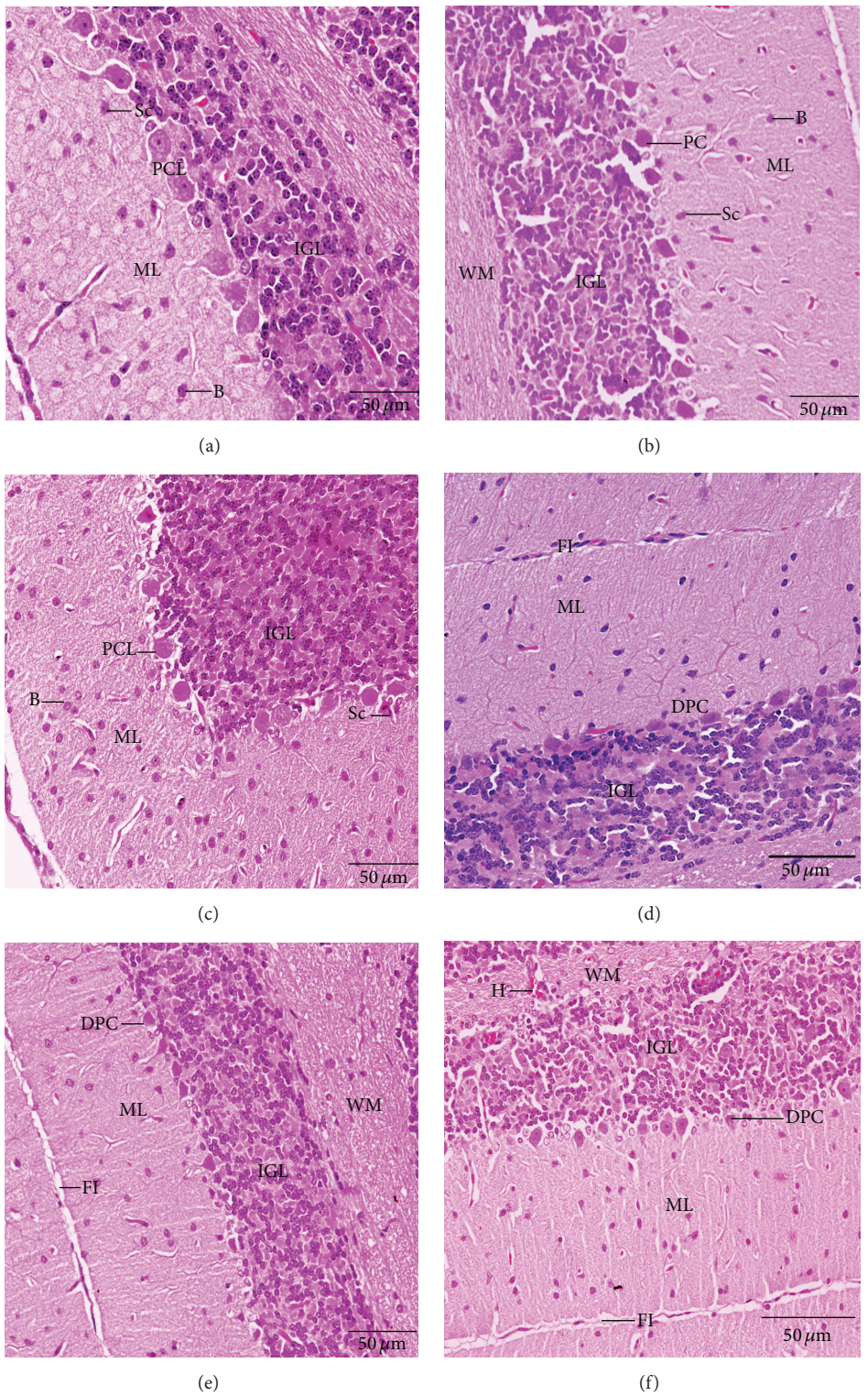

Figure 6: Histological changes in the cerebellar cortex of the newborns at postnatal day 30 showing Purkinje cell (PC), Purkinje cell layer (PCL), degenerated Purkinje cell (DPC), fissure (FI), hemorrhage (H), internal granular layer (IGL), molecular layer (ML), and white matter (WM). (a) Control group, (b) parsley 5% group, (c) parsley 10%, (d) cadmium inoculated group, (e) cadmium inoculated group + parsley 5%, and (f) cadmium inoculated group + parsley 5% (H&E stain).

Cd treated groups, some pyknotic and degenerated Purkinje cells were observed and some were more spindle-shaped and small. These numbers of degenerated Purkinje neurons reduced in groups E and F (Figures 6(d), 6(e), and 6(f)). Variations have been observed in the folds size of the groups where small folds appeared in group D.

The normal medulla neurons appeared to be large in size, polygonal, and varied in shape and had round nuclei (Figures 7(a), 7(b), and 7(c)). In Cd treated group, most of medulla neurons appeared small and pyknotic (Figure 6(d)). Cd-parsley treated groups medulla neurons showed improvement (Figures 6(e) and 6(f)).

4. Discussion

The current study has been designed to investigate the protective role of parsley juice against Cd teratogenicity in albino mouse newborns. These results proposed that the intake of parsley may improve the malformations due to exposure of female pregnant mice to Cd. The effect of daily supplementation of the two different doses of parsley on the deleterious changes of Cd in litter size, pups body weight, behavioral activities, brain neurotransmitters, brain oxidative stress, brain Cd concentration, and brain histoarchitecture was discussed in the current study. The small litter size of the nonprotected Cd treated group shows the hazardous effect of Cd on pregnancy. Antonio et al. [14] recorded that prenatal Cd exposures induce fetal resorption and abortion. Cd passes through placenta and is dispensed in the embryos tissues during pregnancy [11]. The high accumulation of Cd in brain tissues of Cd treated groups due to prenatal and postnatal passing through placenta and mother's milk to its newborns as reported early by Shaheed et al. [45]. The low concentration of Cd in the pup's brain tissue in groups E and F may be explained by the ability of the mothers of these two groups to get off the administrated Cd due to the ingested parsley.

The current results reported that the Cd exposed groups showed reeducation in the pup's body weights as reported in earlier studies. The reasons this weight loss may be prenatal due to intrauterine Cd exposures that produce growth deficiency for the developed fetus or postnatal as maternal Cd exposure causes bad lactation due to maternal bad behaviors caused also by Cd and consequently leads to postnatal malnutrition fort the neonates [45]. In addition, Friedman et al. [46] confirmed the main reason for the postnatal pup's weight loss because they culminate from maternal behaviors alterations in Cd exposed groups as well as a decrease in the lactation index. Pups body weights are the most sensitive indicator of developmental toxicity [47]. The results of groups E and F showed that parsley juice has an observable protective effect against Cd accumulation and prenatal effects, especially the low dose. These may be due to the significant effect of parsley in the excretion of heavy metals such as Cd from mother's bodies so the complications of Cd toxicity reduced and disappeared in the pups of parsley ingested groups [27].

The present levels of the neurotransmitters were depleted significantly by Cd treatment in the brain tissue of the Cd exposed groups. The inhibition of dopamine, serotonin, and acetylcholine occurs in depressing the hyperexcitability of brain neurons as what appeared by the poor performance of the treated pups in the current behavioral examinations [3]. Recently, a growing body of research has focused on the participation of serotonin in the neurochemical mechanisms of cognition and especially of learning and memory. Potential toxic mechanisms of action for Cd may include disruption in serotonergic neurotransmission through disturbed levels of neurotransmitters in the brain [3]. It was mentioned before that Cd toxicity disrupts the action of the acetylcholine esterase activity, so the sensorimotor performances of the newborns will be affected [9]. The disturbances in the levels of current neurotransmitters levels in the toxicity exposed groups may be due to the impact of Cd on the neurons functions [3], while the improvements in the parsley juice ingested groups may be because the ability of this component to limit the Cd impacts the neurons.

Although groups D, E, and F pups were exposed to Cd perinatally (during gestation and lactation), group D displayed a marked elevation in the oxidative stress and a depletion of the antioxidants, compared to groups E and F. One aspect of the oxidative stress elevation was the significant increase of the lipid peroxidation in group D. Méndez-Armenta and Ríos [15] reported that the lipid peroxidation and TBARS increased after acute Cd toxicity. Glutathione is one of the most important compounds for the preserve of cell integrity because of its reducing ability and participation in the cell metabolism as well as a well sensitive reflector of the oxidative stress state in laboratory animals and human [48].

It was observed in many oxidative stress states that the reduction of GSH induces the elevation of lipid peroxidation. Therefore, GSH is an important indicator and biomarker of oxidative stress [49, 50]. Almost the level of GSH is regulated by glutathione reductase (GR) which is a NADPH dependent enzyme. Therefore the limitation in active GR and NADPH may adversely affect GSH levels [50]. Furthermore, interaction of electrophilic xenobiotics such as heavy Cd and its metabolites products with GSH forms glutathione-S conjugates which deplete GSH concentrations in the tissue cells [40, 51]. Abu-Taweel et al. [3] reported that Cd exposure depleted GSH content and elevates peroxidase enzymes activities in adult mice. Increasing of peroxidase activity may be due to the presence of generated free radicals [52]. Peroxidases are considered as one of the antioxidant enzymes which constitute a mutually supportive defense team against free radicals such as reactive oxygen species (ROS) as mentioned by Allam et al. [50].

Several mechanisms may explain the reasons of oxidative stress increasing due to heavy metals toxicity, especially the chronic toxicity. For example, the chronic Cd inhalation leads to Cd accumulation which elevates ROS and reactive nitrogen species (RNS) production by the mitochondrial respiratory system. This will exhaust the antioxidant enzymes and an imbalance of glutathione redox status will be produced [7]. At altitude concentration, ROS/RNS will damage the components of the cell including nucleic acids, proteins, amino acids, and lipids [53]. Such oxidative modifications affect several cell metabolic reactions, functions, and gene

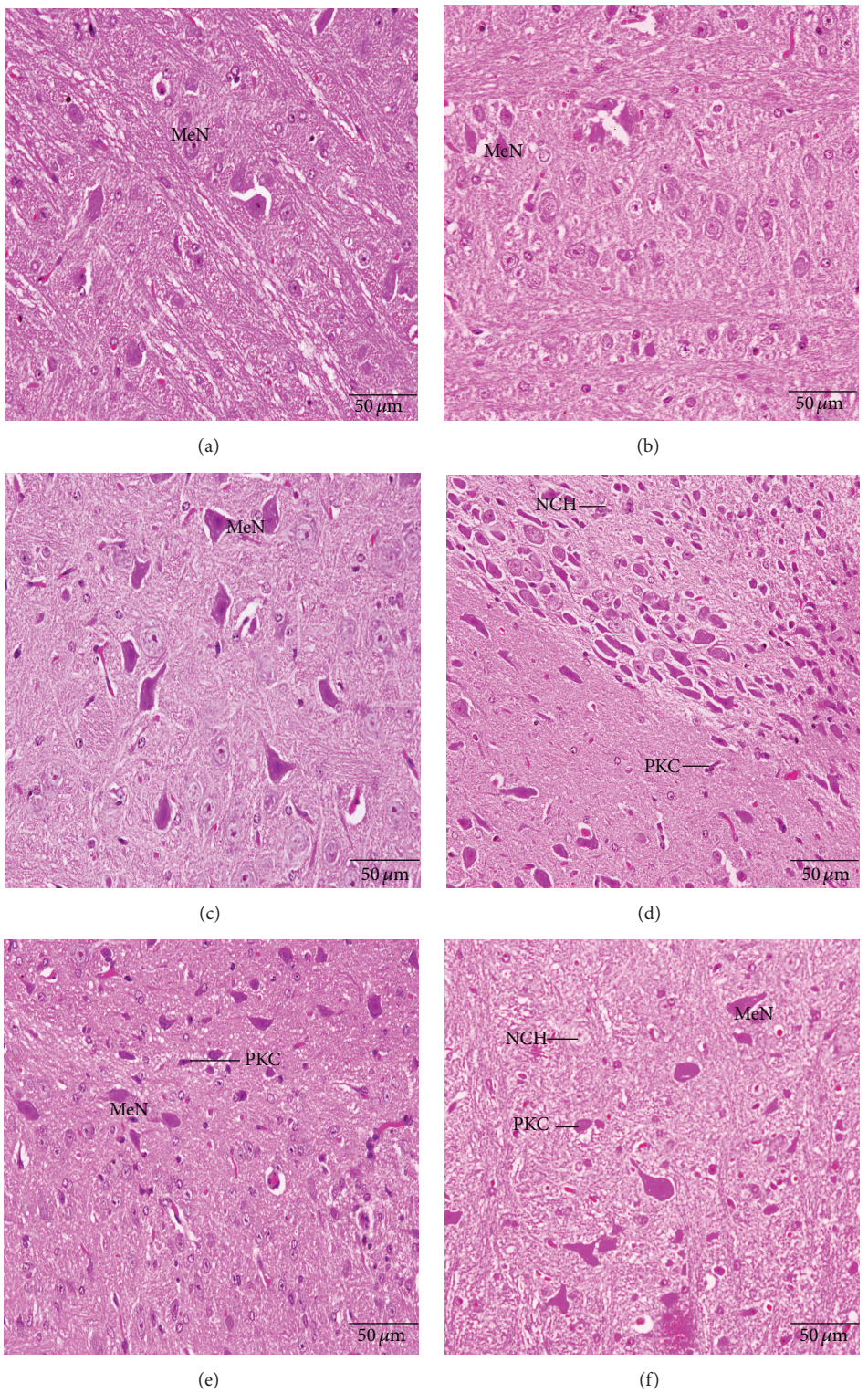

Figure 7: Histological changes in the medulla oblongata of the newborns at postnatal day 30 showing medulla neurons (MeN), degenerated medullary cells (PKC), and neurocyte chromatolysis (NCH). (a) Control group, (b) parsley 5% group, (c) parsley 10%, (d) cadmium inoculated group, (e) cadmium inoculated group + parsley 5%, and (f) cadmium inoculated group + parsley 5% (H&E stain).

expression which in turn can cause other pathological conditions [54]. The oxidative stress leads to neuronal damage in several brain regions [40, 49, 55]. For example, neuronal loss in cerebrum impairs animal's memory [38], neuronal loss in cerebellum can have effect on balance and coordination [40], and neuronal loss in medulla oblongata and spinal cord can affect physical activity of mice [56].

Phytochemical screening of parsley has revealed the presence of some compounds such as flavonoids [19], carotenoids [20], ascorbic acid [21], and tocopherol [22]. These components of fresh parsley leaf scavenge superoxide anion in vitro and hydroxyl radical in addition to protecting against ascorbic acid-induced membrane oxidation [19]. Supplementation with parsley juice for 50 days prevent to somewhat the Cd toxicity showed significant improvement in the physical balance, coordination, motor activities, muscles strength, and brain neurotransmitters levels in Cd treated pups of groups E and F. Parsley supplement also restored GSH balance and decreased lipid peroxidation and peroxidase activity. Overall, this study demonstrated that low dose of parsley supplementation significantly improved pathological alterations in mice as reported by Zhang et al. [30]. Parsley juice components were found to be significant suppressors to H_2O_2 and ROS levels in brain and other tissues in mice by stimulating production of glutathione synthesis and thereby boosting cellular antioxidant defense [30]. Therefore, we suggest that parsley may be an important therapeutic tool to combat oxidative stress-associated diseases. We propose that diluted parsley juice may ameliorate Cd neurotoxicity complications in mice by its ability to neutralize free radicals and thereby prevent neuronal damage caused by oxidative stress.

5. Conclusion

Parsley has a protective effect against Cd neurotoxicity and teratogenicity in albino mice. Parsley juice supplementation improves the behavior of perinatally Cd intoxicated mice newborns and reduces neuronal aberrations in the brain caused by oxidative stress.

Conflict of Interests

The authors declare that they have no competing or conflict of interests.

Acknowledgment

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Research Article

An Evaluation of Root Phytochemicals Derived from *Althea officinalis* (Marshmallow) and *Astragalus membranaceus* as Potential Natural Components of UV Protecting Dermatological Formulations

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As lifetime exposure to ultraviolet (UV) radiation has risen, the deleterious effects have also become more apparent. Numerous sunscreen and skincare products have therefore been developed to help reduce the occurrence of sunburn, photoageing, and skin carcinogenesis. This has stimulated research into identifying new natural sources of effective skin protecting compounds. Alkaline single-cell gel electrophoresis (comet assay) was employed to assess aqueous extracts derived from soil or hydroponically glasshouse-grown roots of *Althea officinalis* (Marshmallow) and *Astragalus membranaceus*, compared with commercial, field-grown roots. Hydroponically grown root extracts from both plant species were found to significantly reduce UVA-induced DNA damage in cultured human lung and skin fibroblasts, although initial *Astragalus* experimentation detected some genotoxic effects, indicating that *Althea* root extracts may be better suited as potential constituents of dermatological formulations. Glasshouse-grown soil and hydroponic *Althea* root extracts afforded lung fibroblasts with statistically significant protection against UVA irradiation for a greater period of time than the commercial field-grown roots. No significant reduction in DNA damage was observed when total ultraviolet irradiation (including UVB) was employed (data not shown), indicating that the extracted phytochemicals predominantly protected against indirect UVA-induced oxidative stress. *Althea* phytochemical root extracts may therefore be useful components in dermatological formulations.

1. Introduction

Skin cancer incidence is known to have increased significantly in the last 20 years, despite nonmelanoma skin cancer (NMSC) being greatly under reported [1–4]. As lifetime exposure to ultraviolet radiation (UV) has risen, the health effects have also become more apparent particularly within older populations (60 years of age plus) [5]. More than 70% of all skin cancer cases presenting in this age group are NMSC, which are primarily thought to be caused by excess UV exposure accumulated over time [6]. Although NMSC is rarely fatal, its morbidity is significant and treatment often places a significant burden on healthcare provision. Exposure to sunlight is not entirely avoidable or indeed desirable however, as it is also necessary for essential biological functions to occur (i.e., vitamin D metabolism) [7].

UV radiation consists of three wavelength ranges UVA (315–400 nm), UVB (280–315 nm), and UVC (<280 nm). Of these, mainly UVA and UVB are of physiological importance as UVC is absorbed by oxygen and ozone in Earth's atmosphere [8, 9]. Acute effects of overexposure of the skin to UV manifest as erythema (sunburn), whereas chronic effects can develop into skin cancer or lead to premature photoageing [10]. The involvement of UV as the major causal factor in the aetiology of skin cancer is very persuasive and has arisen from extensive animal studies and the effect of solar radiation on genetic mutation [6, 11]. UVB radiation has sufficient energy to directly damage DNA by inducing

base modifications such as cyclobutane pyrimidine dimers (CPDs), 6-4 photoproducts (6-4PPs), and thymidine glycols [11–16]. CPDs are generally the more abundant lesion type leading to cytotoxicity, with 6-4PPs being less represented but potentially more mutagenic. Lower energy UVA can penetrate deeper into the skin than UVB and causes indirect DNA damage through the activation of reactive oxygen species (ROS). These genotoxic reactions induce single strand breaks (SSBs) in DNA, DNA-protein cross-linking, or oxidisation of bases [17]. There is also an increasing evidence from both animal and human *in vitro* studies that UVA irradiation has a more significant role in skin carcinogenesis than previously thought [18–24]. Historically, UVA-induced carcinogenesis has been attributed to oxidative DNA base modification such as 7,8-dihydro-8-oxoguanine (8-OHG) [25, 26]. More recent studies have indicated that, along with 8-OHG, pyrimidine dimers are a major contributor in UVA mutagenesis particularly CPDs at cytosine-dipyrimidine sites [27–29]. It is speculated that a weak activation of p53 following UVA exposure may be more mutagenic than UVB exposure as there is increased chance of cell survival with nonrepaired DNA damage, potentially leading to the induction of skin carcinogenesis [24]. This is particularly significant when many modern tanning devices employ the UVA spectrum rather than the UVB [30] and sunscreens predominantly provide protection against the latter with less protection against the DNA damage induced by UVA irradiation being incorporated [31].

The cells of the body, including the skin, have very effective defence mechanisms in place however to protect UV-absorbing nucleic acids and proteins, in particular cellular DNA, from damage [17]. The availability and abundance of these mechanisms (be they physically absorbing or reflecting UV irradiation, scavenging free radicals, or repairing cellular damage) are essential to minimize the potential mutagenic and carcinogenic effects of UVA and/or UVB within the cellular environment [32]. It is impossible however for these defence systems to completely inhibit UV-induced damage and the resulting impact can lead to cell death, senescence, or carcinogenesis [33]. Therefore, there has been a significant effort in recent years to stem the rising incidence of UV-related skin cancer through education programmes [34]. The development of sunscreen products and skincare formulations containing UV protection factors for a range of skin types has also become a prominent feature in the cosmetic industry [31]. Such products are marketed heavily on their ability to prevent sunburn while still allowing the skin to tan, permitting the length of sun exposure time to be increased whilst also suggesting a reduction in the likelihood of developing skin cancer and photoageing. Traditionally, sunscreens were designed to prevent sunburn (UVB-induced erythema), the sun protection factor (SPF) indicating the number of minimal erythema doses (MED) an individual can tolerate before developing erythema. To date, there is no validated measure regarding the protection proffered by sunscreens to indirect skin damage caused by UVA although several methods have been proposed [35–39].

Increasing public demand for dermatological products containing components derived from nature has also increased the desire to identify novel naturally occurring UV protecting compounds that can be employed in such formulations [21, 33, 40–44]. Phytochemicals have been used in herbal medicine and traditional remedies for many years and can have beneficial or detrimental effects depending on their use [44]. *Aloe vera* and vitamin E are two of the most prominent naturally derived plant chemicals employed in skin care products. In addition to negating the effects of erythema and inflammation in the skin, phytochemicals may also provide important antioxidant and UV-absorbing properties, which could reduce or prevent the UV-induced DNA damage [45] that may potentially initiate skin carcinogenesis.

An initial general review of the literature indicated that *Althea officinalis* (AL; Marshmallow) and *Astragalus membranaceus* (AS; also known as Huang Qi in traditional Chinese medicine) are just two of the many plant species that have been used in alternative medical therapies for many years (e.g., Chinese medicine) to treat a variety of ailments including digestive disorders, compromised immunity, colds, wound healing, and inflammation. Unlike in other plants, however, in both these particular species the roots are of particular interest as these are thought to contain skin protecting polysaccharides and/or UV-absorbing compounds which may have potential in protecting against solar-induced skin damage. Root extracts of each of these species (AL and AS) have therefore been investigated here to see if they could protect the DNA of cultured human cells from the DNA damage known to be induced by UV irradiation. Additionally, as there is currently a move away from using organic solvents due to their potential toxicity and/or environmental concerns relating to their disposal, the root extracts investigated were prepared by aqueous extraction using supercritical water to increase the hydrophobicity above that of water at normal atmospheric pressure. The alkaline comet assay (single-cell gel electrophoresis) as described by Singh et al. [46] can assess DNA damage in the form of single strand breaks, double strand breaks, and alkali labile sites and so has been employed to assess the effect of these plant-derived compounds (phytochemicals) on protecting the integrity of cellular DNA *in vitro*.

2. Materials and Methods

2.1. Phytochemical Sample Preparation. In order to be used in a commercial capacity, a pure and consistent source of the phytochemicals was essential. The extracted root material supplied for this study was therefore produced by cultivating each plant species, *Althea officinalis* (AL; Marshmallow) and *Astragalus membranaceus* (AS) separately using carefully maintained hydroponic or soil growing conditions in a glasshouse (by ADAS UK Ltd., UK) as follows.

Seeds of AL and AS were utilised from a single stock supplied by Horizon Herbs LLC (Williams, OR 97544, USA). Plants were raised initially in rock wool plugs and later transplanted to the hydroponic or soil based system. Hydroponic production employed a nutrient film technique (NFT), and plants were placed in net pots at densities of 40 plants/m^2 for AL and 80 plants/m^2 for AS. A standard nutrient regime with 124 mg NO_3 L^{-1} was used for both

species, and electrical conductivity and pH were monitored and controlled automatically. Soil-grown plants were grown in peat based compost in growbags in the glasshouse alongside the NFT units, such that both treatments experienced the same light and temperature regimes. Plant density in the growbags mirrored that in the adjacent hydroponic channels. Roots were cut off when the NFT channels became full and the plants were then allowed to re-grow. The soil-grown plants were harvested once at the end of the season, as roots could not be harvested continuously as in the NFT system. Soil-grown roots were washed in a commercial carrot washer to remove compost prior to drying.

The materials grown in the glasshouse were compared to samples of field-grown roots sourced from commercial suppliers [AL; G. Baldwin and Co. (Walworth Rd, London, UK) and/or Base Formula Co. (North Street, Melton Mowbray, UK); AS; G. Baldwin and Co].

The root crop subsequently harvested was dried gently at 40°C in a forced air flow oven until reaching constant temperature before processing using a novel extraction method using superheated water (University of Leeds/Critical Processes Ltd., UK) to optimize the production and extraction of UV protecting molecules and polysaccharides [47]. Ten and a half grams of root material was extracted at 150°C for 120 minutes, collecting 240 mL in 3 continuous 80 mL portions. These samples were labelled extracts 1, 2, and 3, respectively. This was done to see if different phytochemical components were eluted at different time points from the extraction process and if these different fractions possessed different biological activity on subsequent analysis. An equal portion of each of these three timed extract samples was then reserved to form three "whole" extract samples (labelled "W1," "W2," and "W3," resp.) which contained a representation of all the phytochemicals extracted from the plant root over each subsequent period of elution. Further equal portions of extracts 1, 2, and 3 were then subjected to ultrafiltration using disposable in-line membrane filters designed for the use in a laboratory centrifuge. Membranes were chosen with a cut-off of 30 kDa to separate polysaccharides from low molecular weight species. This produced six more samples for analysis, a retentate (labelled "R") and filtrate (labelled "F") of each of these three timed extracts.

These processes produced consistent, pure samples of the roots of each plant species ready for testing (or use in a sunscreen product). All samples for comet assay analysis were supplied in phosphate buffered saline (PBS) at various concentrations (mg/mL) and were confirmed to be sterile solutions via microbiological analysis.

2.2. Human Fibroblast Cell Culture and Phytochemical Exposure. Initial experiments were conducted using human fetal lung fibroblasts (MRC-5) (ECACC Number 84108101, UK) derived from the normal lung tissue of a 14-week-old male. Human skin fibroblasts (84BR) (ECACC No 90011805, UK) derived from a biopsy of a radiosensitive female were used for subsequent experimentation. Fibroblasts were cultured at 37°C with 5% CO_2 in Eagles modified medium (EMEM) supplemented with 10 or 15% fetal calf serum (FCS), respectively, 200 mM L-glutamine and 2% penicillin/streptomycin solution (1000 iu penicillin and 1 g streptomycin). All reagents were supplied by Sigma (UK) unless otherwise stated. Monolayers of cells were grown aseptically in 25 cm^2 vented tissue culture flasks until they were 70% confluent and were then washed twice with PBS to remove the spent medium. Fresh medium was added (10 mL) and supplemented with extract (which had been passed through a 0.22 μm filter to maintain sterility) at a dilution factor of 1 : 100 (as determined from an initial dose escalation experiment conducted over the concentration range of 1 : 10 to 1 : 1000 with MRC-5 lung fibroblasts; data not shown) and incubated for one hour before harvesting (the time selected from previous studies [48]). Cells were detached from the bottom of the culture flasks using 0.25% trypsin/EDTA and centrifuged for 3 minutes at 1500 rpm before suspension in PBS. Cell viability was assessed using trypan blue dye exclusion (>95%) and suspensions diluted to provide 600,000 cells/mL for comet assay analysis.

2.3. Alkaline Single-Cell Gel Electrophoresis (Comet Assay). Alkaline single-cell gel electrophoresis (comet assay), described by Singh et al. [46], can assess DNA damage in the form of single strand breaks, double strand breaks, and alkali labile sites. To assess the efficacy of phytochemicals in the extracts of plant root material, the comet assay was used to determine the level of DNA damage induced by a controlled light insult in the cells incubated in the presence or absence of the test substances. All samples were tested in quadruplicate (60 comets scored per area, 240 comets per sample). The alkaline comet assay was carried out as described in detail by Morley et al. [48]. Briefly, 50 μL of cell suspension was mixed with 500 μL premolten (43°C) 0.5% low melting point agarose (LMP; LMAgarose, AMS, Trevigen Inc., USA). Aliquots (75 μL) of this cell/LMAgarose mixture were then transferred to each of the two circular sample areas of CometSlide™ glass microscope slides (AMS, Trevigen Inc., USA). Slides were left to set at 4°C for 15 minutes before irradiation. Following irradiation (described below), slides were immediately immersed in lysis solution (AMS, Trevigen Inc., USA) to prevent cellular repair and kept at 4°C for 1 hour. The DNA was allowed to unwind in an alkaline solution (pH > 13) (200 mM EDTA, NaOH) for 1 hour at room temperature before carrying out electrophoresis at (20 V, 275 mA) for 24 minutes. Following electrophoresis, the slides were rinsed with ethanol and then left to dry at room temperature before analysis. The DNA was stained using ethidium bromide (10 μg/mL) and DNA migration (% tail DNA) analysed using a fluorescence microscope connected to specialist image analysis software (comet assay II, Perceptive Instruments, UK).

2.4. Light Source and Irradiation. Irradiation was administered using a 200 W xenon-mercury UV light source (Lightningcure L5, Hamamatsu Photonics Ltd., UK) with a four-furcated liquid light guide directed towards test areas on four separate comet slides simultaneously. Test areas were exposed to uniform, stable light intensity within the same wavelength range as that of terrestrial solar radiation, with

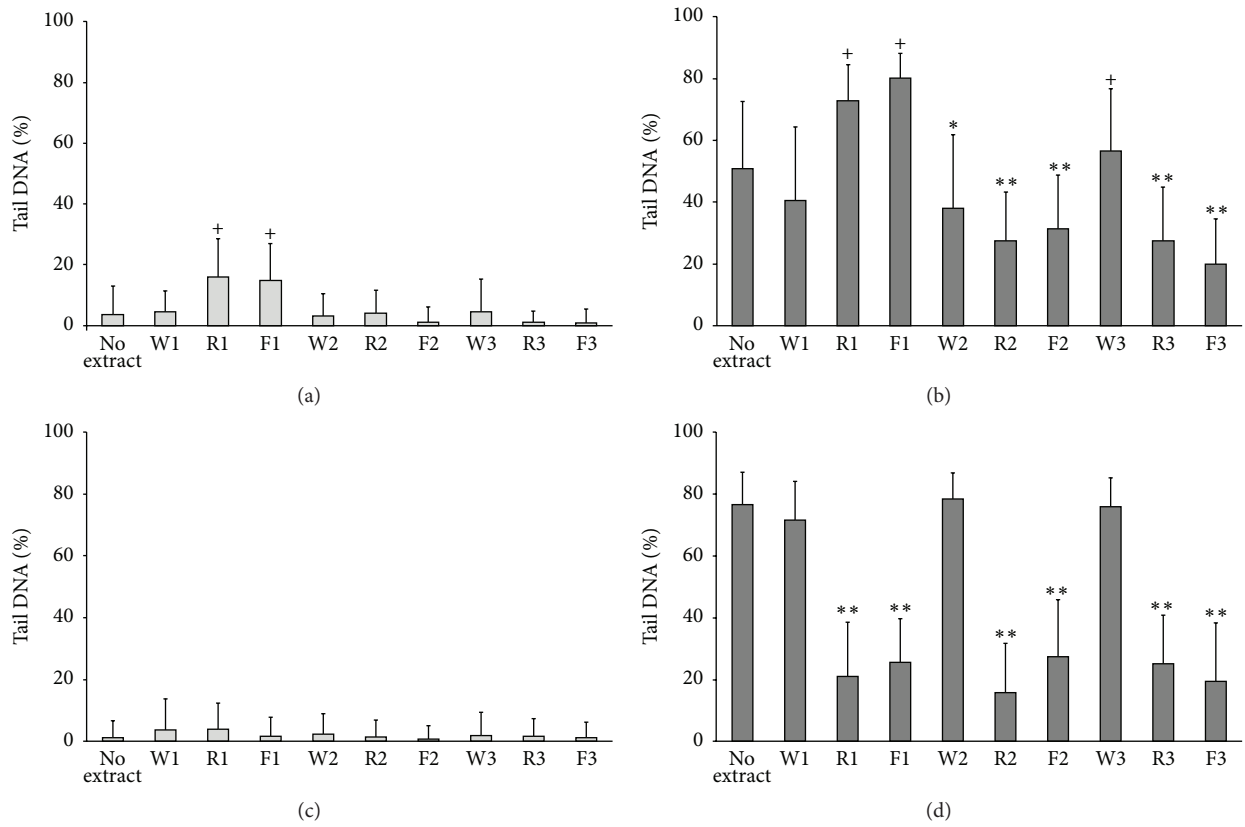

Figure 1: Median percentage DNA damage in the tail of comets derived from cultured human lung fibroblasts exposed to (a) *Astragalus* extracts without irradiation, (b) *Astragalus* extracts with 16 minutes of filtered xenon-mercury irradiation (UVA + visible light), (c) *Althea* extracts without irradiation, and (d) *Althea* extracts with 16 minutes of filtered xenon-mercury irradiation (UVA + visible light). W = whole extract, R = retentate, and F = filtrate collected from elution periods 1, 2, and 3. Bars indicate the 75 percentile of the data set. + indicates a statistically significant increase in damage ($p < 0.001$) when compared with the corresponding control group without extract exposure. $*$ and $**$ indicate a statistically significant decrease in damage ($p < 0.05$ and $p < 0.001$, resp.) when compared with the no extract control group.

(UVA + visible irradiation) or without (UVB + UVA + visible irradiation) the presence of a 320 nm cut-on filter (CG-WG-320; Elliot Scientific Ltd., UK) to remove wavelengths below 320 nm. UVA + visible light exposure was carried out for a minimum of 12 minutes, UVA + UVB + visible light exposure for 60 seconds (data not shown). These irradiation levels were determined as those required to reliably initiate sufficient DNA damage (circa 50%) in this test system and observe any significant changes (positive or negative) attributable to the presence of phytochemicals in the extracts being tested.

The potential effects of the presence of antioxidants on cellular DNA in this test system were initially assessed using N-acetylcysteine (NAC; Sigma, UK), a low molecular weight antioxidant linked to free radical scavenging and singlet oxygen quenching as a positive test control substance (data not shown).

2.5. Statistical Analysis. Statistical analysis was conducted on median values utilising the nonparametric Mann-Whitney U test. Box-whisker plots were produced using SigmaPlot 11.0 to indicate the median (solid line), ±25% of the data (box), and 10% to 90% spread of the data (whisker).

3. Results and Discussion

3.1. Effects of Hydroponically Grown AL and AS Root Extracts on Human Fibroblasts ± UV Irradiation. MRC-5 cells (human lung fibroblasts) were incubated for one hour with or without timed extracts from AL or AS and then they are exposed to either 16 minutes of filtered xenon-mercury irradiation (UVA + visible light) or no irradiation (dark control) (Figure 1). No increase in DNA damage was observed in nonirradiated cells exposed to any of the AL extracts (Figure 1(c)). Only nonirradiated cells exposed to AS-derived extracts R1 and F1 (Figure 1(a)) showed increase in DNA damage ($p < 0.001$). These extracts were also found to increase levels of DNA damage on exposure to UVA irradiation ($p < 0.001$) (Figure 1(b)). These results suggest that there was a genotoxic effect produced in the cells, by the phytochemical compounds contained within the AS extract, prior to irradiation commencing.

With irradiation (Figure 1(b)), AS whole extract W2 ($p < 0.05$) and ultrafiltrated extracts R2, F2, R3, and F3 ($p < 0.001$) significantly reduced UVA-induced DNA damage. The change in DNA damage in cells following UVA irradiation

incubated with AS whole extract W1 was not found to be of statistical significance and extract W3 was found to statistically increase the level of DNA damage observed ($p < 0.001$). With AL phytochemical exposure followed by irradiation (Figure 1(d)), all ultrafiltrated extracts (R and F) from each sample collection period considered reduced the UVA-induced DNA damage normally induced by the light insult ($p < 0.001$). So, although it would appear that extracts from the AS species could potentially be effective against UVA exposure, their capacity to induce UVA damage cannot be ignored, particularly if considering its use in emollients in future human studies. Future studies therefore concentrated on *Althea*, whose retained and filtered extracts all significantly reduced the UVA-induced genotoxicity produced in this cell type (Figure 1(d)).

Clear and significant reductions in UVA-induced DNA damage were also apparent in the human skin fibroblasts (84BR) when using extracts W2 from both plant species (Figure 2). This was encouraging as these dermatological cells were (as anticipated) less sensitive to the effects of UVA than the lung-derived MRC-5 cells and a more relevant cell type when investigating potential sunscreen/skincare constituents.

Figure 2 demonstrates that with the increasing periods of filtered xenon-mercury irradiation (UVA + visible light) without the presence of any extracts, the % tail DNA damage observed increased ($p < 0.001$) when compared to the dark control. This genotoxic damage was reduced in the presence of whole extract W2 from either AS or AL ($p < 0.001$), although the responses observed with each species at 15 and 18 minutes of exposure were not significantly different from one another ($p > 0.05$). Although these results cannot be directly extrapolated to those of an *in vivo* skin system which has vasculature and immunological factors to consider, there does appear to be target compound or compounds present in the extracts, which is combating the oxidative stress-induced genotoxic damage being produced by UVA radiation. Furthermore, as these positive results were obtained using "whole" root extracts, it appears that the extra processing step of ultrafiltration was not essential for efficacy.

Experimentation was also conducted to consider the effect of UVB on the more robust and UV-sensitive human lung fibroblasts (MRC-5). None of the whole or ultrafiltrated extracts of AL or AS were found to reduce the levels of DNA damage observed using UVB + UVA + visible light with the experimental conditions employed (60 seconds of unfiltered xenon-mercury irradiation; data not shown). This was not unexpected as UVB is able to damage DNA directly and would suggest that either the components of the extracts were not able to absorb the UVB or there was insufficient amount of effective material present. This also indicated that the protection observed against UVA + visible irradiation in Figures 1 and 2 was most likely derived through the prevention of indirectly-induced light-mediated genotoxic damage. In addition, the system was validated prior to investigation of AL and AS extracts using N-acetylcysteine (NAC) (data not shown). So, experimental conditions capable of detecting protection by a known antioxidant compound were employed throughout.

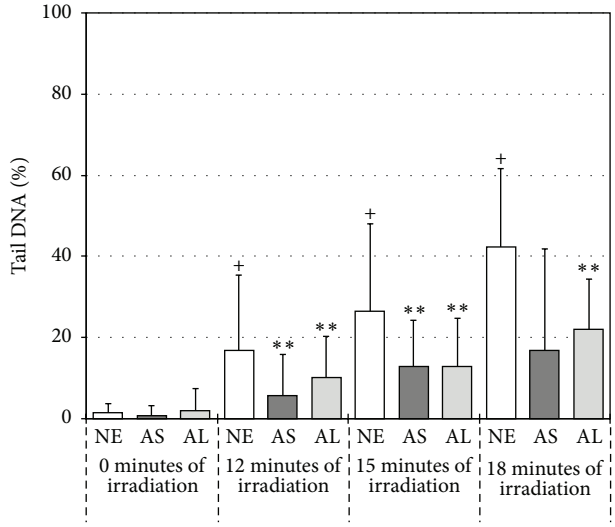

Figure 2: Median percentage DNA damage in the tail of comets derived from cultured human skin fibroblasts incubated with no extract (NE), *Astragalus* extract W2 (AS), or *Althea* extract W2 (AL) for one hour followed by 0, 12, 15, or 18 minutes of filtered xenon-mercury irradiation (UVA + visible light). Bars indicate the 75 percentile of the data set. + indicates a statistically significant increase in damage ($p < 0.001$) when compared with the NE dark control. ** indicates a statistically significant decrease in damage ($p < 0.001$) when compared with the NE control at the same irradiation period.

Due to the novel extraction system used, the exact constituents of the test extracts employed and their concentration were unknown. Chemical analysis (conducted by Royal Botanic Gardens, Kew, UK) established that AS extracts contained simple phenolics (caffeic, p-coumaric acids), various flavonoids, isoflavones, and saponins (astragalosides). AL extracts were found to predominantly contain carbohydrates and simple phenolics as well as 8-hydroxyflavones including luteolin (the latter found more so in extract W2). Several unidentified flavonoid-like compounds were also detected.

3.2. Effects of Hydroponic, Commercial, or Soil Derived AL Root Extracts on Lung Fibroblasts ± UVA Irradiation. Investigation of the efficacy of different AL preparations was carried out to determine if there were differences in the level of UVA protection afforded when the roots were obtained from *Althea* plants that had experienced different growing conditions. Due to the potential genotoxicity of extracts from AS (Figures 1(a) and 1(b)), only AL was deemed suitable for further analysis as a potential candidate for use in a topical dermatological product. Figure 3 indicates how similar the preparations of commercially sourced field-grown (Figure 3(a)), hydroponically glasshouse-grown (Figure 3(b)), or glasshouse soil-grown (Figure 3(c)) derived AL extracts were in their effect on the levels of UVA-induced DNA damage in human lung fibroblasts. Cells were exposed to 0 (dark control), 12 or 15 minutes of filtered xenon-mercury irradiation (UVA + visible light).

Increasing periods of irradiation without extract incubation resulted in increased DNA damage ($p < 0.001$).

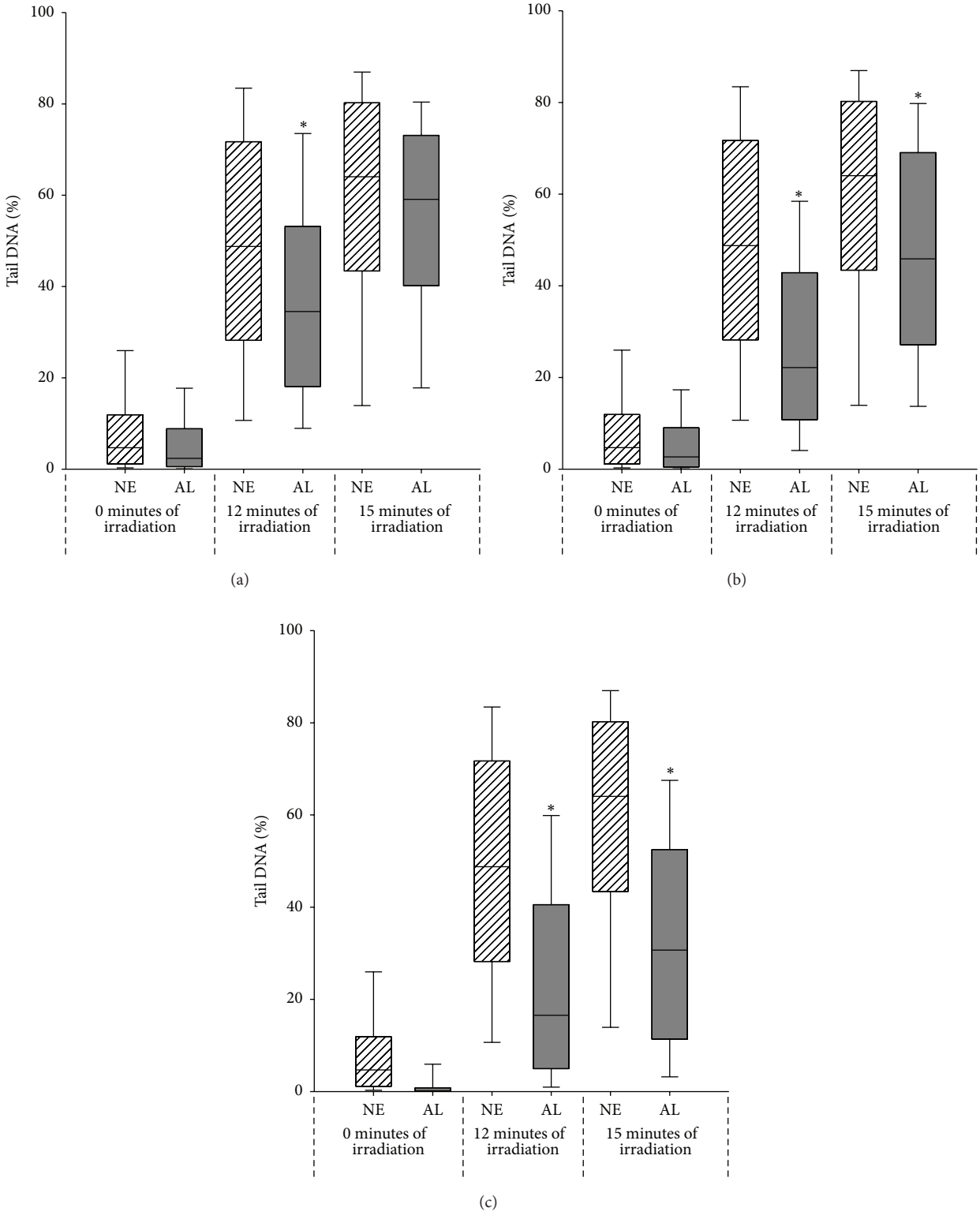

Figure 3: Median percentage DNA damage in the tail of comets derived from MRC-5 cells incubated with no extract (NE) or whole extracts of (a) commercially sourced field-grown, (b) hydroponically glasshouse-grown, or (c) glasshouse soil-grown *Althea* (AL) roots for one hour followed by 0, 12, or 15 minutes of filtered xenon-mercury irradiation (UVA + visible light). Solid bar indicates median, box indicates ± 25% of the data, and the whisker indicates the 10–90% spread of the data. ∗ indicates a statistically significant decrease in damage ($p < 0.001$) when compared with the control group irradiated for the same time period without extract exposure.

All AL extracts (independent of source) significantly reduced the effect of UVA-induced DNA damage with 12 minutes of UVA + visible light ($p < 0.001$) (Figure 3). Hydroponically derived extract (Figure 3(b)) continued to significantly reduce DNA damage up to 15 minutes ($p < 0.001$) as did the glasshouse soil-grown root extract (Figure 3(c)) ($p < 0.001$), although in each case the protection afforded diminished with the continued light exposure. The extract from the commercially derived *Althea* roots offered the least period of protection (Figure 3(a)). These results appear to indicate the presence of similar components in each of the extracts with the most potent being in that of the glasshouse-grown, soil derived AL sample. This is quite possible as different growing conditions may affect the levels of particular phytochemicals and thus the potency of the extracts. The lower activity of the commercially derived field-grown AL extract could also be due to the effects of processing during manufacture, reducing the potency or concentration of the effective compound. Additionally, the glasshouse-grown materials were cultivated from seeds of a particular genetic stock, whereas this was an unknown quantity with the commercially sourced material.

4. Conclusions

Hydroponically grown root extracts from both plant species investigated were found to significantly reduce UVA-induced DNA damage in cultured human lung and skin fibroblasts, although initial AS experimentation detected some genotoxic effects, indicating that AL root extracts may be better suited as potential constituents of dermatological formulations. Glasshouse-grown soil and hydroponic AL root extracts also afforded cultured human cells with statistically significant protection against UVA irradiation for a greater period of time than the commercial field-grown roots, indicating that these systems of cultivation may convey beneficial effects (for instance in terms of antioxidant content) over and above that achieved via more traditional growing methods. No significant reduction in DNA damage was observed when total ultraviolet irradiation (including UVB) was employed, indicating that it is most likely that the extracted phytochemicals predominantly protected against indirectly produced UVA-induced oxidative stress. This factor could be considered in more detail within future experimentation employing the enzyme formamidopyrimidine DNA glycosylase (FPG) to modify the comet assay protocol to reveal oxidised bases.

From the point of view of preventing photoageing and/or potential skin carcinogenesis, the inclusion of such compounds in formulations designed to protect the skin may with further investigation prove to be beneficial. The conclusions that can be drawn from the data presented here in this particular respect, however, are somewhat limited. This is because the alkaline comet assay is only detecting genotoxic DNA damage and does not indicate where in the genome the damage is occurring or whether this damage may potentially be mutagenic or carcinogenic. It cannot therefore be concluded definitively that by preventing this genotoxic damage with phytochemical containing root extracts this would prevent or reduce cancer development, although this may be feasible. Additionally the cells were lysed immediately following light irradiation and so they were given no opportunity to repair the light-induced damage sustained or alternatively to trigger apoptotic cell death, and future investigations should consider these aspects.

So, in conclusion, this investigation has demonstrated that phytochemical containing root extracts do have the potential to be useful natural components in dermatological formulations where a reduction in oxidative stress-induced damage is desired, with the glasshouse-grown soil derived AL roots producing the greatest level of protection against UVA-induced DNA damage observed. Additionally, more extensive chemical analysis of the extracts may be able to identify the individual phytochemical effector(s) involved in the protection afforded by these plants and further research may indicate whether these compounds do indeed have the potential to prevent some of the carcinogenetic processes known to be induced by sunlight.

Conflict of Interests

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

Acknowledgments

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Research Article

Bioactive Peptides from *Angelica sinensis* Protein Hydrolyzate Delay Senescence in *Caenorhabditis elegans* through Antioxidant Activities

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Since excessive reactive oxygen species (ROS) is known to be associated with aging and age-related diseases, strategies modulating ROS level and antioxidant defense systems may contribute to the delay of senescence. Here we show that the protein hydrolyzate from *Angelica sinensis* was capable of increasing oxidative survival of the model animal *Caenorhabditis elegans* intoxicated by paraquat. The hydrolyzate was then fractionated by ultrafiltration, and the antioxidant fraction (<3 kDa) was purified by gel filtration to obtain the antioxidant *A. sinensis* peptides (AsiPeps), which were mostly composed of peptides with <20 amino acid residues. Further studies demonstrate that AsiPeps were able to reduce the endogenous ROS level, increase the activities of the antioxidant enzymes superoxide dismutase and catalase, and decrease the content of the lipid peroxidation product malondialdehyde in nematodes treated with paraquat or undergoing senescence. AsiPeps were also shown to reduce age pigments accumulation and extend lifespan but did not affect the food-intake behavior of the nematodes. Taken together, our results demonstrate that *A. sinensis* peptides (AsiPeps) are able to delay aging process in *C. elegans* through antioxidant activities independent of dietary restriction.

1. Introduction

Reactive oxygen species (ROS) are natural by-products of cellular metabolism and play a role in cell signaling and cellular homeostasis. Excessive accumulation of ROS, however, causes damage to proteins, lipids, and nucleic acids and leads to cell injury and death, which is recognized to contribute to aging process and age-related diseases [1, 2]. Under normal conditions, an organism keeps a dynamic balance between ROS production and scavenging, in which the redundant ROS is largely antagonized by intricate antioxidation systems, including the antioxidant enzymatic scavengers superoxide dismutase (SOD) and catalase (CAT) [1]. Nonetheless, certain external as well as internal factors such as heat shock, toxins, and oxidants can increase ROS level sharply and make the antioxidation defense systems in a weak position, leading to disruption of the balance between generation and elimination of ROS and consequently progression of diseases and even eventual death [3, 4]. Therefore, antioxidants capable of scavenging excessive ROS may help maintain oxidative homeostasis and prevent related damages.

A number of natural products such as resveratrol [5], epigallocatechin gallate [6], salidroside [7], and *Ginko biloba* extract EGb 761 [8] are found to have antioxidant effect.

In recent years, antioxidant peptides derived from food and herbal sources have, as natural and nontoxic products, drawn more and more attention. These bioactive peptides include specific protein fragments of 2–20 amino acid residues, which are concealed in the parental proteins with their biological activities emerged after release by enzymatic hydrolysis [9, 10]. Medicinal plants, especially edible herbs, are shown to be an important source of bioactive peptides with potent antioxidant activities [11–13].

Angelica sinensis (Oliv.) Diels, a popular traditional Chinese medicine first noted in Shen Nong's Herbal Classics (~110 BC), has been widely used for tonifying blood to promote blood circulation and regulate menstruation. It has also been used as health food for women's care in Europe and America [14]. Recent studies have shown that polysaccharides, among other products, from *A. sinensis* have antioxidant and neuroprotective functions [15], but similar activities have not been addressed on peptides from *A. sinensis*. Using the nematode *Caenorhabditis elegans*, a powerful model organism for aging and neurobiology studies, we investigated the antioxidant and anti-aging activities of peptide preparations derived from *A. sinensis* roots, including their effect on oxidative survival, ROS levels, antioxidant enzyme activities, lipid peroxidation product content, age pigments content, and lifespan.

2. Materials and Methods

2.1. Chemicals and Materials. Trypsin from bovine pancreas (≥2,500 IU/mg) and methyl viologen dichloride were purchased from Aladdin Co. (Shanghai, China). Papain (6,000 USP U/mg) was purchased from Solarbio Co. (Beijing, China). 2,7-Dichlorofluorescein diacetate (DCFH-DA) and 5-fluoro-2′-deoxyuridine (FUdR) were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). Sephadex G-25 (medium) was obtained from GE Healthcare Co. (Uppsala, Sweden). Ammonium sulfate (analytical grade) was obtained from Guangzhou Chemical Reagent Factory (Guangzhou, China). SOD, CAT, and malondialdehyde (MDA) assay kits were purchased from Beyotime (Haimen, China). BCA Protein Assay Kit was bought from Pierce Chemical Co. (Rockford, IL, USA).

2.2. Preparation of Angelica sinensis Protein Isolates. Sliced *A. sinensis* roots were purchased from Beijing Tongrentang Yinpian Co., Ltd. (Bozhou, China) and ground into powder. The protein isolates were prepared essentially as described [16]. Briefly, the powder was soaked in 20 mM Tris-HCl buffer at pH 7.4 for 12 h and the suspension was centrifuged at 5,000 ×g for 30 min. The supernatant was collected and then replenished with solid ammonium sulfate at 4°C to reach 80% of saturation. After 4 h, the solution was centrifuged at 5,000 ×g for 30 min at 4°C, and the pellet was collected, resuspended in water, and dialyzed (molecular weight cut-off 3,500 Da) to remove salt and small molecule impurities. The liquid was then freeze-dried as *A. sinensis* protein isolates and stored at 4°C.

2.3. Enzymatic Hydrolysis. The *A. sinensis* protein isolates were hydrolyzed by trypsin and papain as follows. Approximately 10 g of the above protein isolates was dissolved in 200 mL of deionized water and the pH was adjusted to 8.0 with 0.5 M NaOH. After adding 2.4 mL of trypsin solution (6,250 IU/mL), the enzymatic hydrolysis was performed at 45°C in a water bath for 4 h. The solution was then heated to 60°C and the pH was adjusted to 6.0 with 12 M HCl to inactivate the enzyme. After adding 0.5 mL of papain (30,000 USP U/mL), the solution was incubated at 60°C for 4 h. The hydrolysis was terminated by heating in boiling water for 10 min. The solution was then freeze-dried as *A. sinensis* protein hydrolyzate (AsPH).

2.4. Isolation and Purification of Peptides. AsPH (20 mg/mL) was fractionated by centrifugal ultrafiltration (Vivaspin 20, Sartorius) using molecular weight (MW) cut-off of 5 kDa and 3 kDa, respectively, and 3 fractions were obtained: AsPH-F1 (MW > 5 kDa), AsPH-F2 (3 kDa < MW < 5 kDa), and AsPH-F3 (MW < 3 kDa). After bioactivity test (see below), the bioactive AsPH-F3 (20 mg/mL) was further separated on a Sephadex G-25 gel filtration chromatography column (1.2 cm × 150 cm), which was eluted with deionized water at a flow rate of 1 mL/min and monitored at 280 nm with a 785A UV/VIS detector (Perkin Elmer Co., Norwalk, CT, USA). The purified fraction was collected and freeze-dried as AsiPeps.

2.5. Identification of Peptides by LC-MS/MS Analysis. To identify peptide sequence, the peptide samples were separated by reverse phase nanoflow HPLC and analyzed by tandem mass spectrometry. Briefly, 2 μg of peptides was redissolved in solvent A (water/acetonitrile/formic acid, 98 : 2 : 0.1, v/v/v) and loaded on ChromXP C18 (3 μm, 120 Å) nanoLC trap column. The online chromatography separation was employed on the Eksigent nanoLC-Ultra™ 2D System (AB SCIEX, Concord, Ontario, Canada). Desalting procedure was carried out at 2 μL/min for 10 min with 100% solvent A. Then, a linear gradient of 5–35% solvent B (water/acetonitrile/formic acid, 2 : 98 : 0.1, v/v/v) over 50 min was used on an analytical column (75 μm × 15 cm C18, 3 μm, 120 Å, ChromXP Eksigent). LC-MS/MS analysis was performed with a TripleTOF 5,600 System (AB SCIEX, Concord, Ontario, Canada) fitted with a Nanospray III source (AB SCIEX, Concord, Ontario, Canada). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 5 PSI, and an interface heater temperature of 150°C. The MS was operated with TOF-MS scans. For the information dependant acquisition, survey scans were acquired in 250 ms and as many as 25 product ion scans (80 ms) were collected if exceeding a threshold of 150 counts/s and with a +2 to +5 charge-state. A rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (~12 s). All raw data files (*.wiff) were collectively searched with ProteinPilot Software v. 4.5 (AB SCIEX, Foster City, California, USA) against a dicotyledonous plant protein database. The detected protein threshold (unused ProtScore) was set to 1.3 (95% confidence). Peptides were filtered at 1% false discovery rate.

2.6. Strains and Maintenance. Both *Caenorhabditis elegans* (wild-type N2) and *Escherichia coli* (OP50 and NA22) strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, USA). All experiments were performed at 20°C unless otherwise stated.

2.7. Paraquat Survival Assay. The oxidative survival assay was performed as described previously using paraquat [7]. Synchronized L1 larvae were incubated for 42 h, and then 75 μg/mL FUdR was added. After further incubation for 24 h, the nematodes reached young adulthood and were transferred to 96-well plates (~20 nematodes/well; >100 nematodes for each treatment) containing *E. coli* NA22 ($OD_{570\,nm}$ = 0.5) and peptide samples. After incubation for another 24 h, the nematodes were exposed to 70 mM paraquat. The numbers of live and dead nematodes were scored microscopically every 12 h based on their movement and shape; before counting, the plate was gently vibrated to stimulate movement of the animals.

2.8. Determination of ROS Levels. To assess ROS levels in paraquat-stressed nematodes, synchronized young adults were incubated with or without the peptide samples for 24 h before paraquat was added at 2 mM final concentration [17]. After treatment with paraquat for 2 days, the nematodes were harvested and washed for 3 times with M9 buffer to remove remaining paraquat and bacteria. The determination of ROS levels was performed as described previously [18] with modifications. The nematodes were dispensed into black 96-well plates by a COPAS Biosort instrument (Union Biometrica, Inc., Holliston, MA, USA), which is a flow cytometer capable of sorting and dispensing individual nematodes as well as measuring their physical and optical parameters [19]. After the dispense (100 animals per well; 1,000 animals for each treatment), the fluorescent probe DCFH-DA was added at a 50 μM final concentration and the plates were incubated at 20°C for 14 h. Then ROS-related 2,7-dichlorofluorescein (DCF) fluorescence was measured at room temperature by a Fluoroskan Ascent FL plate reader (Thermo Electron Co., Waltham, MA, USA) at excitation 485 nm and emission 520 nm. For determination of ROS levels in senescent nematodes, synchronized young adults with 100 μg/mL of ampicillin (but without paraquat) were incubated with or without the peptides to Day 10 of adulthood, and ROS was determined as above.

2.9. Determination of Antioxidant Enzyme Activity and MDA Content. Antioxidant indexes were determined as previously described [7] with minor modifications. Briefly, about 4,000 nematodes were washed three times, transferred into an Eppendorf tube, and suspended with 600 μL of lysis buffer. The samples were sonicated in ice bath to obtain homogenate. After centrifugation (13,000 ×g, 5 min, 4°C), the supernatant was collected for measurement of SOD activity, CAT activity, MDA content, and protein content using commercial chemical assay kits, respectively. The values of enzyme activities and MDA content were normalized by protein content.

2.10. Age Pigments Accumulation and Lifespan Analyses. The accumulation of age pigments was assessed as described [20]. Briefly, Day-10 adult nematodes with or without peptide treatment were dispensed to black 96-well plates (100 nematodes per well) by COPAS Biosort, and the fluorescence of age pigments was determined by Fluoroskan Ascent FL microplate reader with 355 nm excitation and 460 nm emission. The lifespan assay was performed in 96-well plates using liquid culture as previously described [21]. The number of live nematodes was scored microscopically every 2 days based on their mobility, shape, and pharyngeal pumping until all the nematodes died [22].

2.11. Determination of Extinction, Time of Flight, and Pharyngeal Pumping Rate. Day-3 adult nematodes with or without peptide treatment were used for extinction and time of flight (TOF) and pharyngeal pumping rate determination. Extinction and TOF were determined by COPAS Biosort as previously described [23] using approximately 200 nematodes in each group. The pharyngeal pumping rate was counted manually under a dissecting microscope in one minute [24]. Over 20 animals were randomly selected in each group to score for the pumping rates.

2.12. Acquisition of Visual Images. All images were captured by ImageXpress Micro System (Molecular Devices, Sunnyvale, CA, USA) with a ×10 objective. Specifically, the fluorescent images of DCF were taken with a FITC excitation and emission filter setup, while the bright-field images were taken with 20% transmitted light filter setup.

2.13. Statistical Analysis. GraphPad Prism version 5.01 for Microsoft Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis and one-way analysis of variance (ANOVA) was performed in multiple group comparisons. *C. elegans* survival and lifespan curves were analyzed by Kaplan-Meier method and log-rank test. Probability values of $p < 0.05$ were considered as statistically significant. All experiments were performed at least three times.

3. Results and Discussion

3.1. Increase of Oxidative Survival by A. sinensis Protein Hydrolyzate in C. elegans. Excessive generation of intracellular ROS will damage cellular structure and function, leading to aging and diseases of organisms [25–27]. Most studies on antioxidant peptides, however, are focused on *in vitro* scavenging activities against hydroxyl, superoxide, and other radicals [9–12], despite the fact that discrepancy exists between *in vitro* and *in vivo* antioxidant capacities of compounds [28–30]. Paraquat, a superoxide generator, induces an acute oxidative stress and causes rapid death of *C. elegans* at high doses [31]. Therefore, we performed *in vivo* oxidative survival assay to screen for antioxidant peptides using *C. elegans* models intoxicated by 70 mM paraquat. Preliminarily, we prepared protein hydrolyzates from >40 different medicinal plants and marine organisms. As an example, the effect

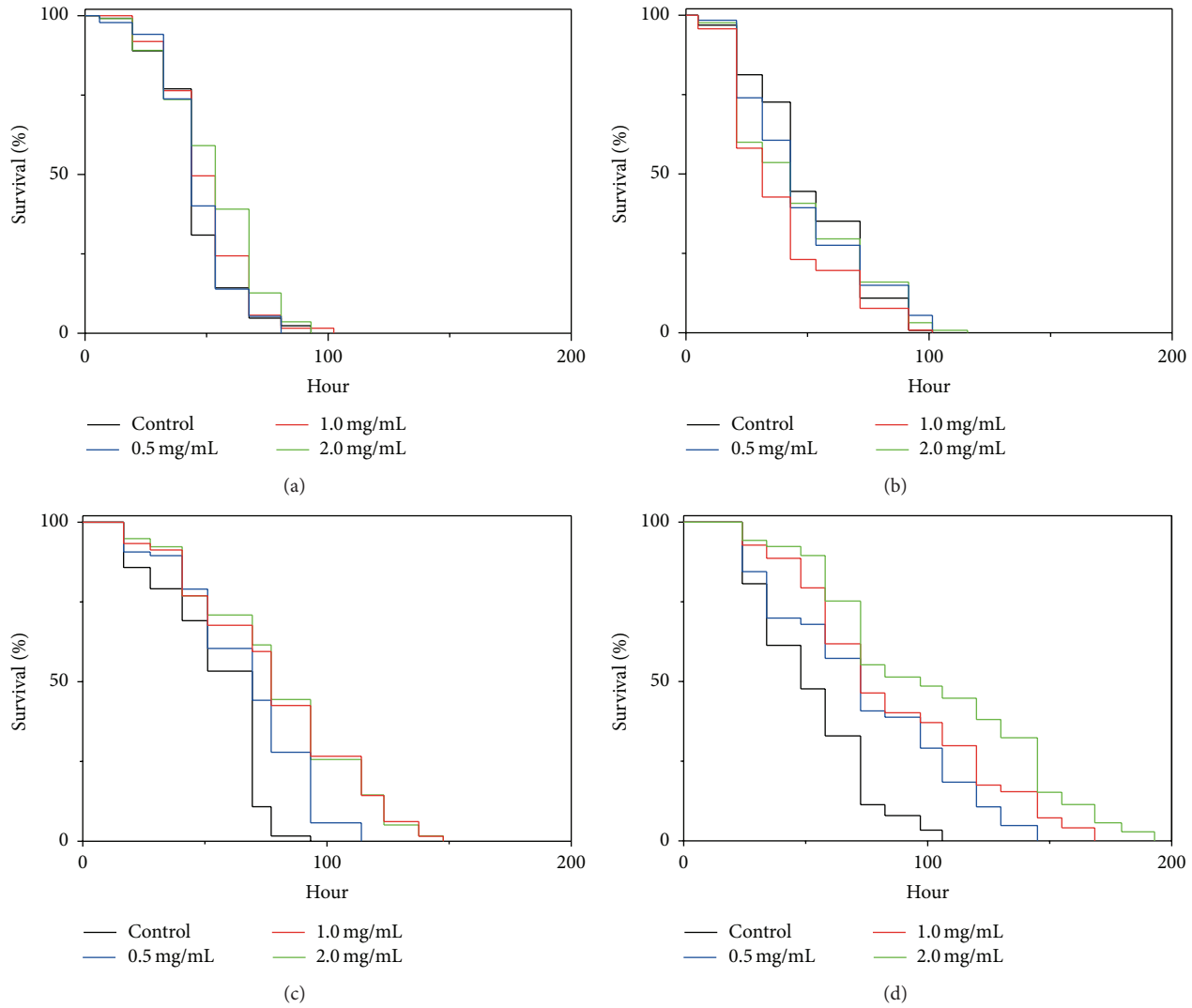

Figure 1: Effect of protein hydrolyzates on survival rates of paraquat-treated *C. elegans*. Young adult nematodes were pretreated with protein hydrolyzates at indicated concentrations at 20°C for 24 h prior to exposure to 70 mM paraquat, and the survival rates were scored every 12 h. (a)–(d) Representative Kaplan-Meier survival curves of nematodes treated with protein hydrolyzates from *Panax ginseng*, *Fallopia multiflora*, *Astragalus membranaceus*, and *Angelica sinensis*, respectively.

of protein hydrolyzates from herbal plants *Panax ginseng*, *Fallopia multiflora*, *Astragalus membranaceus*, and *Angelica sinensis* on the survival rate of paraquat-treated nematodes is presented in Figure 1, in which *A. sinensis* protein hydrolyzate (AsPH) showed noteworthy capacity of improving survival of the nematodes under increased oxidative stress. To further determine the effective doses of AsPH, the nematodes were preincubated with a series of AsPH concentrations and then treated with 70 mM paraquat. As shown in Figure 2, AsPH increased the oxidative survival of nematodes in a dose-dependent manner with concentrations of >2 mg/mL being more potent.

3.2. Isolation and Identification of Antioxidant A. sinensis Peptides. Ultrafiltration, which is an effective approach to fractionate and concentrate proteins and peptides, was used to fractionate AsPH based on molecular weight, and 3 fractions of protein hydrolyzate were obtained, that is, AsPH-F1 (MW > 5 kDa), AsPH-F2 (3 kDa < MW < 5 kDa), and AsPH-F3 (MW < 3 kDa). The antioxidant capacities of the fractions were tested using the above paraquat resistance assay in *C. elegans*, which showed that AsPH-F3 had higher antioxidant activity than the other 2 fractions (data not shown). AsPH-F3 was, therefore, further purified by gel filtration chromatography on a Sephadex G-25 column, and the eluate was collected and freeze-dried as *A. sinensis* peptides (AsiPeps), which was subjected to paraquat resistance assay as above and showed strong antioxidant capacity at 2.0 and 4.0 mg/mL (Figure 3). Using LC-MS/MS, 27 peptides, consisting of 6–19 amino acid residues, were identified from AsiPeps; another peptide was composed of 26 amino acids (Table 1). This is in agreement with previous studies on natural peptides showing that most antioxidant peptides are <20 amino acid

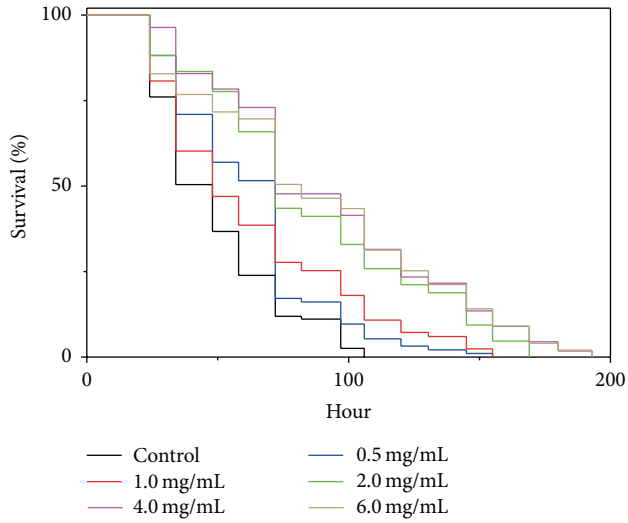

Figure 2: Effect of *A. sinensis* protein hydrolyzate concentration on survival rates of paraquat-treated *C. elegans*. The nematodes were treated with *A. sinensis* protein hydrolyzate and paraquat as in Figure 1. Representative Kaplan-Meier survival curves are shown for the nematodes treated with a series of concentrations of *A. sinensis* protein hydrolyzate.

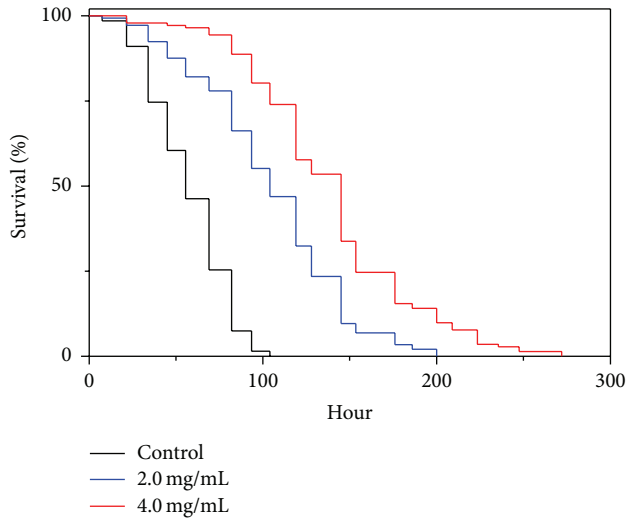

Figure 3: Effect of *A. sinensis* peptides on survival rates of paraquat-treated *C. elegans*. The nematodes were treated with *A. sinensis* peptides (AsiPeps) and paraquat as in Figure 1 and representative Kaplan-Meier survival curves are shown from three independent experiments.

residues [10, 32–34]. For example, a 13-amino-acid peptide (DNYDNSAGKWWVT) from hydrolyzed cocoa by-product is shown to protect *C. elegans* against oxidative stress [35].

3.3. Decrease of ROS Level by A. sinensis Peptides in C. elegans under Oxidative Stress. Since the *A. sinensis* peptides (AsiPeps) showed strong resistant capacity against high dose of paraquat (70 mM), we further tested its effect against paraquat toxicity at a low dose (2 mM), which generated a gentle oxidative stress but did not affect the survival rate of *C. elegans* during the test. The nematodes were preincubated with AsiPeps and then exposed to 2 mM paraquat before ROS determination. After incubation with the fluorescent probe DCFH-DA, the DCF fluorescence in the nematodes, as revealed by ImageXpress Micro System, was reduced after treatment with both 2.0 and 4.0 mg/mL of AsiPeps as compared to the controls, suggesting decreased ROS levels (Figure 4(a)). Further quantification by DCF fluorescence assay using microplate reader also demonstrates that the ROS levels of paraquat-exposed nematodes were reduced for >20% after AsiPeps treatments (Figure 4(b)). These data suggest that the capacity of AsiPeps to decrease endogenous ROS accumulation contributes to their protective effect against oxidative damage.

3.4. Increase of Antioxidant Enzyme Activities and Reduction of MDA Content by A. sinensis Peptides in C. elegans under Oxidative Stress. The antioxidant enzyme system is one of the most important lines of defense to confront oxidative stress and scavenge intracellular ROS in organisms. Accordingly, regulation of the antioxidant enzyme activities may contribute to increased survival rate of *C. elegans* under oxidative stress [36, 37]. On the other hand, malondialdehyde, one of the end-products of lipid peroxidation, is an important biomarker for oxidative stress [38]. Therefore, we investigated the effect of AsiPeps on the activity of antioxidant enzymes SOD and CAT and the content of MDA. As shown in Table 2, the SOD and CAT activities were increased, while the MDA content was decreased in the nematodes pretreated with 2.0 and 4.0 mg/mL AsiPeps, as compared to the control nematodes treated only with 2 mM paraquat. These data demonstrate that AsiPeps are able to improve the antioxidant defense system of nematodes through regulation of antioxidant enzyme activities and lipid peroxidation.

3.5. Effect of A. sinensis Peptides on ROS Level, Antioxidant Enzyme Activities, and Malondialdehyde Content in C. elegans Undergoing Senescence. Aging process is widely recognized to be associated with the degree of oxidation. Therefore, we determined the effect of *A. sinensis* peptides on ROS level, SOD and CAT activities, and MDA content in senescent nematodes. As shown in Figure 4(c), at Day 10 after adulthood, the ROS level was decreased >20% in nematodes treated with 2.0 and 4.0 mg/mL of AsiPeps as compared to the control nematodes without peptide treatment. The SOD and CAT activities were also increased, while the MDA content was reduced in the Day-10 nematodes treated with 4.0 mg/mL AsiPeps as compared to the controls (Table 3). These data demonstrate that the *A. sinensis* peptides are able to increase the antioxidant capacity of nematodes undergoing senescence.

3.6. Reduction of Age Pigments Accumulation and Extension of Lifespan by A. sinensis Peptides in C. elegans. As shown above, the *A. sinensis* peptides (AsiPeps) can improve the antioxidant capability of *C. elegans* both under oxidative stress and at senescent stage, suggesting that AsiPeps are

Table 1: Amino acid sequences of *A. sinensis* peptides identified by LC-MS/MS.

ID	Sequence	Number of residues	Molecular weight (Da)
1	DLTDFL	6	722.79
2	PSIVGRP	7	724.86
3	LTADILPR	8	898.07
4	QTVAVGVIK	9	914.11
5	AGLQFPVGR	9	944.10
6	AMPVEVVQF	9	1019.23
7	ESTAKQVIR	9	1031.18
8	VETGVIKPGM	10	1030.25
9	VVVNIPPTLK	10	1079.35
10	YGVSGYPTLK	10	1084.24
11	TTAGILLPEK	10	1042.24
12	AGFAGDDAPR	10	976.01
13	IGGIGTVPVGR	11	1025.22
14	GTIAGGGVIPH	11	978.12
15	GGVLPNINPVL	11	1092.30
16	AVFPSIVGRPR	11	1198.43
17	VLVSGSIHYPR	11	1227.43
18	TKMDEGVVTKK	11	1235.47
19	AAPFPGQKSLQR	12	1299.50
20	IKGTIAGGGVIPH	13	1219.45
21	LASSGIDHEGRLPR	14	1507.67
22	MIAFNKEQDTDLQSK	15	1767.98
23	IIGATNPAESAPGTIR	16	1567.76
24	EGGDGGYGGGGGGSRW	16	1425.39
25	EGGGGGYGGGGGGYGGR	17	1371.35
26	REGGGGGYGGGGGGYGGR	18	1527.53
27	GGGGYGGGGGGYGGGGGGY	19	1420.37
28	KKVGYNPDKIPFVPISGFEGDNMIER	26	2951.40

Table 2: The effect of *A. sinensis* peptides on the antioxidant enzyme activities and malondialdehyde content in *C. elegans* under oxidative stress. The nematodes were incubated with or without the peptides for 24 h prior to treatment with 2 mM paraquat.

Treatment	Antioxidant enzyme activity[a]		MDA content[b]
	SOD	CAT	
Control	31.44 ± 0.91	1.03 ± 0.01	42.54 ± 0.78
AsiPeps			
2.0 mg/mL	40.87 ± 0.75[c]	1.21 ± 0.02[c]	30.45 ± 1.49[c]
4.0 mg/mL	64.90 ± 1.24[c]	1.33 ± 0.01[c]	34.87 ± 1.34[c]

[a]SOD, U/mg protein; CAT, U/μg protein; [b]MDA, nmol/mg protein; [c]$p < 0.05$.

Table 3: The effect of *A. sinensis* peptides on the antioxidant enzyme activities and malondialdehyde content in *C. elegans* undergoing senescence. The data were determined at Day 10 after adulthood.

Treatment	Antioxidant enzyme activity[a]		MDA content[b]
	SOD	CAT	
Control	30.78 ± 0.53	2.51 ± 0.01	20.33 ± 1.69
AsiPeps			
2.0 mg/mL	32.54 ± 0.26[c]	2.57 ± 0.01	17.50 ± 1.05
4.0 mg/mL	47.13 ± 0.72[c]	3.00 ± 0.01[c]	15.82 ± 1.14[c]

[a]SOD, U/mg protein; CAT, U/μg protein; [b]MDA, nmol/mg protein; [c]$p < 0.05$.

likely to play a role in the delay of aging process. Age-related autofluorescent age pigments, including lipofuscin and advanced glycation end-products, are widely regarded as biomarkers of aging, and their accumulation is shown to be inversely correlated with longevity [39]. In *C. elegans*, the age pigments are accumulated in the intestine over time and increase significantly from the 10th to 15th days after hatching at 20°C and can be quantified using fiber optic-coupled spectrofluorimetry [19]. Therefore, we measured the relative level of age pigments in adult nematodes at Day 10 after adulthood (about 13th day after hatching) to reflect the degree of physiological aging. As shown in Figure 5(a), the relative fluorescent intensity of age pigments in Day-10 nematodes was reduced by 23.2% and 42.7% after treatment with 2.0 and 4.0 mg/mL AsiPeps, respectively, as compared with the nematodes without peptide treatment, demonstrating that AsiPeps were capable of decreasing the accumulation of age pigments in aging nematodes. Interestingly, the wheat gluten hydrolyzate, a peptide-rich product, is recently shown to

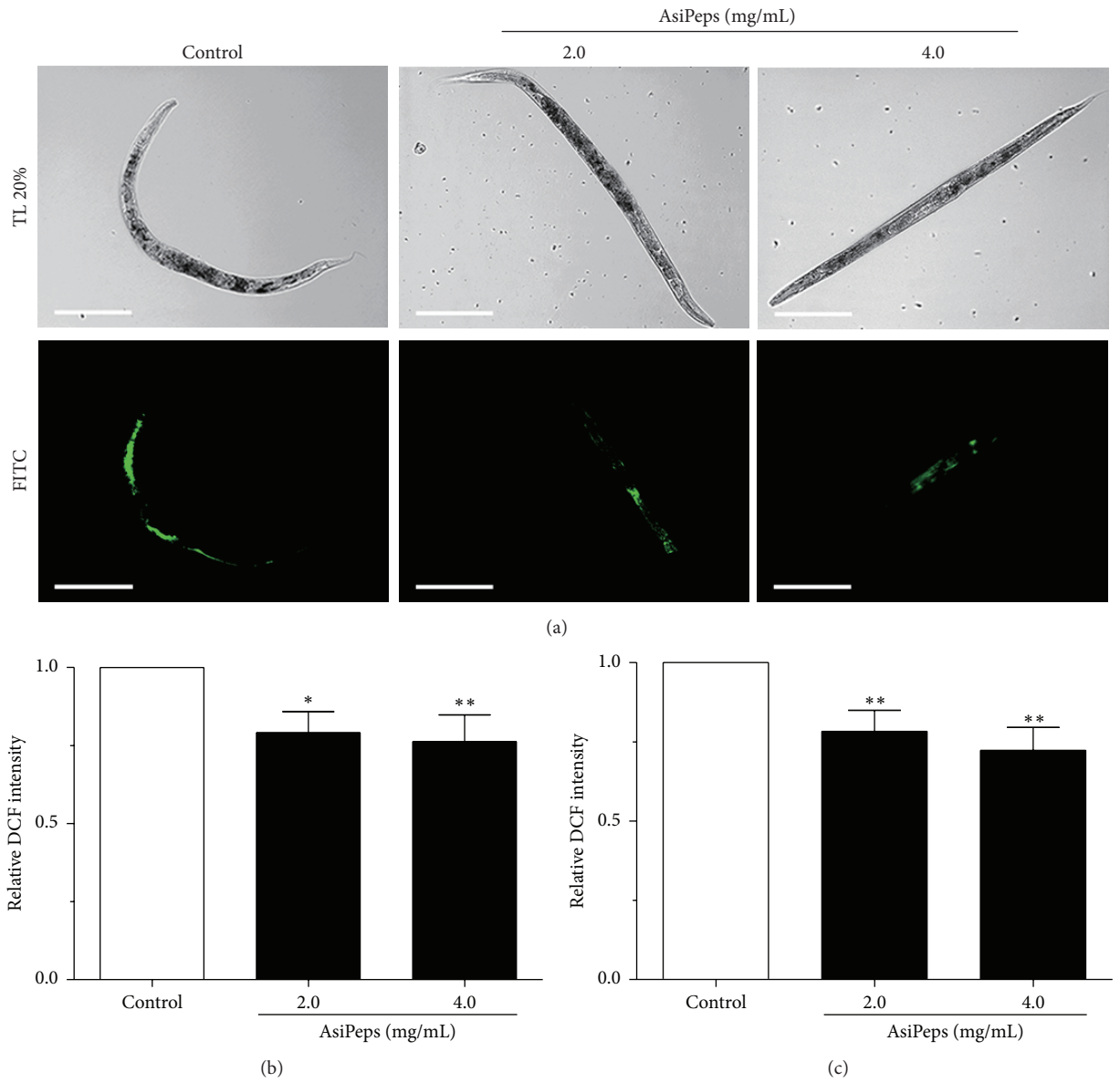

Figure 4: Effect of *A. sinensis* peptides on ROS levels of *C. elegans*. (a) Representative bright-field and corresponding fluorescent images of nematodes captured by ImageXpress Micro System with 20% transmitted light and FITC filter cubes, respectively. The nematodes were pretreated with *A. sinensis* peptides (AsiPeps) for 24 h prior to exposure to 2 mM paraquat. Scale bars: 200 μm. (b) Effect of *A. sinensis* peptides (AsiPeps) on ROS level in nematodes exposed to 2 mM paraquat. (c) Effect of *A. sinensis* peptides (AsiPeps) on ROS level in nematodes undergoing senescence (Day 10). The DCF fluorescence intensity in (b) and (c) was detected by a microplate reader at 485 nm excitation and 520 nm emission, and the data are shown as mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

extend lifespan and decrease intestinal autofluorescence in *C. elegans* [40].

As lifespan is regarded as an unequivocal anti-aging index [22], we further investigated the effect of AsiPeps on the lifespan of *C. elegans* and showed that the lifespan of the nematodes was indeed extended after treatment with 2.0 and 4.0 mg/mL of the peptides (Figure 5(b)). Since the nematodes may cut down their consumption of food if a test sample is distasteful, dietary restriction, which is well known to retard development and delay aging in *C. elegans* [41], may contribute to the lifespan-extending effect of AsiPeps. To investigate this probability, extinction, which represents the optical density and internal structure, and TOF, which indicates the axial length of the nematodes, are measured to estimate nematode size and development [23, 42]. The pharyngeal pumping rate, which can be used to directly reflect food intake, was also determined. As shown in Figure 5(c), after treatment with a series of concentrations of AsiPeps (0.25–6.0 mg/mL), no obvious changes of extinction, TOF, and pharyngeal pumping rate were observed. Taken

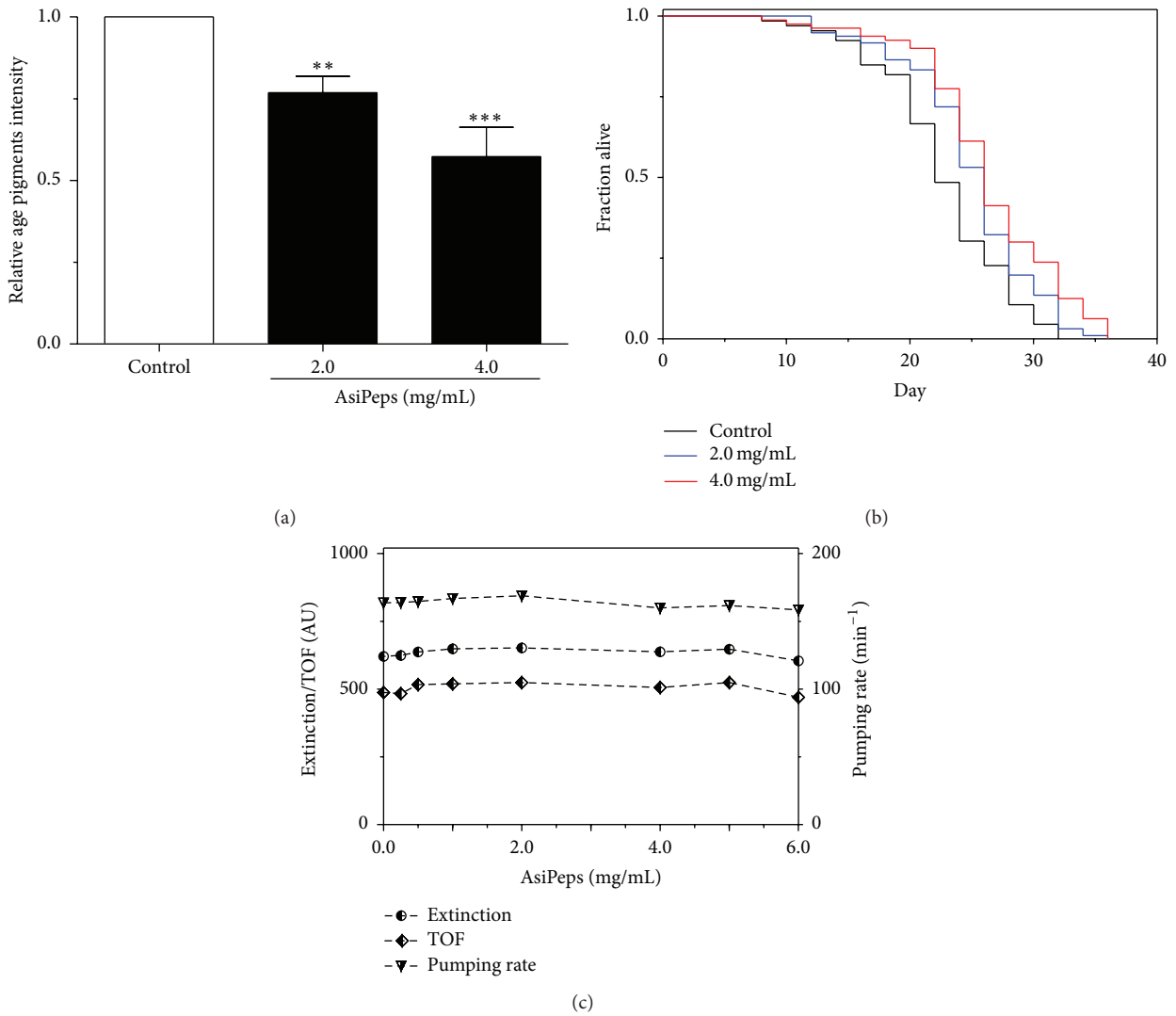

FIGURE 5: Effect of *A. sinensis* peptides on age pigments content, lifespan, and food-intake behavior of *C. elegans*. (a) Relative level of intestinal age pigments in senescent nematodes treated with *A. sinensis* peptides (AsiPeps). The fluorescence intensity of age pigments was detected at Day 10 by a microplate reader at 355 nm excitation and 460 nm emission. Data are shown as mean ± SD of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$. (b) Lifespan of nematodes with or without AsiPeps treatment. Representative Kaplan-Meier survival curves are shown from three independent experiments (>100 animals in each group). (c) Extinction, TOF, and pharyngeal pumping rate of the nematodes with or without AsiPeps treatment. AU: arbitrary units.

together, our data demonstrate that the *A. sinensis* peptides (AsiPeps) were able to extend the lifespan of *C. elegans* under physiological conditions, independent of dietary restriction.

4. Conclusion

In this paper, we isolated the peptides (AsiPeps) from *A. sinensis* protein hydrolyzate and demonstrate that AsiPeps were capable of not only improving oxidative survival of *C. elegans* models exposed to 70 mM paraquat but also decreasing endogenous ROS level, increasing antioxidant enzyme activities, and reducing lipid peroxidation product content of nematodes treated with 2 mM paraquat or undergoing senescence. AsiPeps were also shown to reduce age pigments content and extend lifespan of *C. elegans* but did not affect the food-intake behavior of the nematodes. Together, our data demonstrate that the *A. sinensis* peptides (AsiPeps) were able to delay aging in *C. elegans* through antioxidant activities but independent of dietary restriction.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Qiangqiang Wang and Yunxuan Huang contributed equally to this work.

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Research Article

Bioactive Flavonoids, Antioxidant Behaviour, and Cytoprotective Effects of Dried Grapefruit Peels (*Citrus paradisi* Macf.)

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Grapefruit (*Citrus paradisi* Macf.) is an important cultivar of the *Citrus* genus which contains a number of nutrients beneficial to human health. The objective of the present study was to evaluate changes in bioactive flavonoids, antioxidant behaviour, and *in vitro* cytoprotective effect of processed white and pink peels after oven-drying (45°C–60°C) and freeze-drying treatments. Comparison with fresh grapefruit peels was also assessed. Significant increases in DPPH, FRAPS, and ABTS values were observed in dried grapefruit peel samples in comparison with fresh peels, indicating the suitability of the treatments for use as tools to greatly enhance the antioxidant potential of these natural byproducts. A total of thirteen flavonoids were quantified in grapefruit peel extracts by HPLC-MS/MS. It was found that naringin, followed by isonaringin, was the main flavonoid occurring in fresh, oven-dried, and freeze-dried grapefruit peels. *In vivo* assay revealed that fresh and oven-dried grapefruit peel extracts (45°C) exerted a strong cytoprotective effect on SH-SY5Y neuroblastoma cell lines at concentrations ranging within 0.1–0.25 mg/mL. Our data suggest that grapefruit (*Citrus paradisi* Macf.) peel has considerable potential as a source of natural bioactive flavonoids with outstanding antioxidant activity which can be used as agents in several therapeutic strategies.

1. Introduction

Today, there is increasing demand for natural bioactive compounds as people express more concern about their health, especially in connection with health-giving diets. Epidemiological studies suggest that high dietary intake of phytochemicals, in particular of polyphenols, is associated with a reduced risk of a multitude of chronic diseases.

In this connection, fruits of the *Citrus* genus are recognized as being a healthful source of bioactive compounds such as vitamins, carotenoids, fibre, and phenolic compounds [1–3]. Worldwide agricultural citrus production, including oranges, mandarins, lemons, bergamots, limes, pummelos, and grapefruits, has been increasing strongly in the last decades, reaching over 100 million metric tons per year [4]. About a third of citrus fruits go to produce fresh juice or citrus-based drinks. The juice yield of citrus fruits accounts for half of the fruit weight, and hence a very large amount of pulp and peel waste is produced worldwide every year [5].

It has been found that peels are the main sources of polyphenols in citrus fruits [6]. Peel residues from sweet and bitter oranges, lemons, and mandarins have proved to

be an important source of phenolic acids and flavonoids, chiefly polymethoxyflavones (PMFs), flavanones, and glycosylated flavanones [7–10]. These bioactive compounds are strongly associated with therapeutic properties including antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, anticarcinogenic, antithrombotic, cardioprotective, and vasodilatory effects [11–18]. Many of these pharmacological activities of citrus polyphenols are a consequence of their ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) [19].

Since oxidative stress is involved in all the abovementioned pathological conditions, the outstanding antioxidant role of natural polyphenols has received much attention from many researchers. In this regard, *Citrus* flavonoids have recently attracted considerable interest as potential therapeutic agents in numerous *in vitro* and *in vivo* studies. Naringin, high levels of which occur in several varieties of citrus fruits and citrus byproducts, has demonstrated anti-inflammatory, anticarcinogenic, lipid-lowering, and antioxidant activities [20–23]. Hesperidin, one of the main flavanone glycosides, which occurs in oranges, has been shown to exert a wide range of therapeutic effects such as antioxidant, anti-inflammatory, and anticarcinogenic properties [20]. Moreover, it has been found to significantly reduce ROS generation in cells [23, 24] and to restore mitochondrial enzyme activity [25].

Citrus flavonoids may also exert neuroprotective effects since they are involved in the modulation of neuronal activities and mental health including brain plasticity, behaviour, mood, depression, and cognition [20, 22]. In this regard, it has been demonstrated that hesperidin can protect neurons against various types of insults associated with many neurodegenerative diseases [26]. Also, naringin has proven to exert neuroprotective effects through anti-inflammatory activity on the survival of dopaminergic neurons and on the integrity of the nigrostriatal pathway in animal models of Parkinson's disease [27–29]. Natural flavonoids would therefore seem to have important potential as medicaments in the field of mental health, although their use in clinical practice is still a long way off [30].

The peel from *Citrus* fruits is also a source of Polymethoxylated Flavones (PMFs), flavonoids substituted by methoxy groups, which rarely occur in other plants [31]. PMFs are more physiologically active than their methylated derivatives. For instance, research data have demonstrated that nobiletin possesses a wide range of therapeutic applications including antioxidant, antitumor properties, in both *in vitro* and *in vivo* models [32–36]. Moreover, it has recently been reported that a novel citrus tangeretin derivative, 5-acetyl-6,7,8,4′-tetramethylnortangeretin, can inhibit MCF-7 breast cancer cell proliferation [37]. These data provide new insights into the role that citrus polyphenols can play in the prevention of diseases.

In recent years, white and pink grapefruits (*Citrus paradisi* Macf.) have attracted much attention because of their nutritional and antioxidant properties [38]. High levels of bioactive flavanones glycosides, namely, naringin and narirutin, have been reported in seed and peel residues released after grapefruit juice extraction [38, 39], although further research is required to explore the composition of this fruit variety and its byproducts in more detail.

Several treatments, including far-infrared radiation, ultrasound-assisted alkaline hydrolysis, enzyme treatment, and heat treatment, have been proposed to release more bioactive glycosylated flavonoids and low molecular weight phenolic compounds from several species of citrus genus [1, 4, 40]. In this connection it has been found that dried orange, mandarin, and lemon peel extracts contained much higher concentrations of phenolic compounds than fresh ones and hence exhibited greater antioxidant activity [14, 41, 42].

However, little is known about the bioactive flavonoids in treated grapefruit (*Citrus paradisi* Macf.) peel as a source of health-promoting phytochemicals. To our knowledge, only Xu et al. 2007 [42] have discussed the extractable phenolic fraction of grapefruit (*Citrus paradisi Changshanhuyou*), in a cultivar located in southern China, showing increases in the individual phenolic compounds and enhancements of antioxidant capacity after heat treatment.

Based on these results, then, it is easy to understand the interest of new comprehensive studies to determine the potential of treated grapefruit (*Citrus paradisi* Macf.) peel as a natural product that can serve as an outstanding low-cost antioxidant source. Treated grapefruit peel residues could play an important role in the development of nutraceutical products or as therapeutic agents for use in various pharmacological *in vitro* or *in vitro* approaches.

For all these reasons, the objectives of this research were as follows: (i) to describe and quantify the flavonoid profiles and antioxidant activities of processed white and pink grapefruit peels (*Citrus paradise* Macf.) after oven-drying and freeze-drying; (ii) to study the *in vitro* cytoprotective effectiveness of grapefruit peel extracts on SH-SY5Y neuroblastoma cell lines.

2. Material and Methods

2.1. Fruit Peel Materials. White and pink grapefruits (*Citrus paradise*) were grown in several Valencia areas (Spain) corresponding with the cropping areas. They were purchased at Corte Ingles supermarket and possess certified geographical origin.

2.2. Oven-Dried and Freeze-Dried Grapefruit Peels Treatment. Grapefruits were cleaned with distilled water in the laboratory and they were immediately peeled. White and pink grapefruits peels were cut into pieces (sized from approximately 0.5 × 0.5 cm thick). Sliced peel was divided into four portions: (i) one fresh peel portion to be directly analyzed; (ii) two fractions which were dried in an oven at 45°C and 60°C, respectively, until their water content was within 9–12%; (iii) one grapefruit peel fraction that was freeze-dried.

2.3. Extraction of Phenolic Compounds. Extraction of grapefruits peels was carried out by means of an accelerated solvent extractor ASE 200 (Dionex Corp, Sunnyvale, CA, USA). Extractions were performed using 5 g of grapefruits peel which was placed into inox extraction cells of 22 mL. Every

cell was filled with methanol and raised to 60°C. Then, two static extraction phases lasting for 10 min were carried out under 1500 psi. Between extractions, a rinse of the complete system was performed to avoid any carry-over.

Extracts were evaporated using a rotavapor with a vacuum controller (Heidolph, Schwabach, Germany) at 40°C. Samples were redissolved with 5 mL of methanol and they were filtered through a Whatman Number 1 filter paper. Samples were kept at −20°C prior to being used to determine antioxidant activity and phenolic compounds.

2.4. HPLC-DAD-ESI-MS Analysis. HPLC separation and identification and quantification of phenolic compounds were performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany), equipped with a DAD photodiode detector (G1315B) and a LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI/MSn) system, both coupled to an Agilent Chem Station (version B.01.03) for data processing.

The samples, after filtration (0.20 μm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany), were injected in duplicate on a reversed-phase narrow-bore column Zorbax Eclipse XDB-C18 (2.1 × 150 mm; 3.5 μm particle; Agilent) protected by a guard column Zorbax Eclipse XDB-C8 (2.1 × 12.5 mm; 5 μm particle; Agilent), both thermostated at 40°C.

The solvents were as follows: solvent A (acetonitrile/water/formic acid, 3 : 88.5 : 8.5, v/v/v), solvent B (acetonitrile/water/formic acid, 50 : 41.5 : 8.5, v/v/v), and solvent C (methanol/water/formic acid, 90 : 1.5 : 8.5, v/v/v). The flow rate was 0.190 mL/min. The linear solvents gradient was as follows: 0 min, 99% A and 1% B; 8 min, 97% A and 3% B; 37 min, 70% A, 17% B, and 13% C; 40 min, 50% A, 30% B, and 20% C; 51 min, 10% A, 40% B, and 50% C; 56 min, 50% B and 50% C; 59 min, 50% B and 50% C; and 65 min, 99% A and 1% B.

For identification, ESI-MSn was used in both positive and negative modes, setting the following parameters: dry gas, N2, 11 mL/min; drying temperature, 350°C; nebulizer, 65 psi; capillary, −2500 V (positive ionization mode) up to 42 minutes and +2500 V (negative ionization mode) until the end of the chromatogram; target mass, 600 m/z; compound stability, 40% (negative ionization mode) and 100% (positive ionization mode); trap drive level, 100%; and scan range, 50–1200 m/z.

The identification of flavonoid compounds was carried out by comparing their retention times and mass spectra provided with those of authentic standard (from Sigma St. Louis, MO) when available and spilling the samples with standard solutions. This was the case of hesperidin, neohesperidin, naringin, naringenin, nobiletin, and tangeretin.

The identification of compounds where the standards were not available was performed by comparing the UV spectra and the $[M + H]^+$, $[M - H]^-$ m/z with those reported in the literature. Quantification was made by means of external standard calibration lines and was expressed as milligrams of compounds per gram of dry weight (DW). Quantitative results for compounds without chemical standard were expressed in mg·g^{-1} naringin equivalents.

2.5. DPPH Radical Scavenging Assay. The DPPH assay was carried out according to the method proposed by [43] where 1,1-diphenyl-2-picrylhydrazyl radical was used as a stable radical. One hundred microliters of different dilutions of extracts was added to 2.9 mL of a 0.06 mM methanol DPPH radical solution. Methanol was used to adjust the zero and the decrease in absorbance was measured at 515 nm every minute for 25 min in a UV-vis spectrophotometer (Helios, Thermo Spectronic, Cambridge, UK). Only values between 20% and 80% of the initial absorbance of the radical DPPH were taken into consideration. Concentrations were calculated from a calibration curve in the range between 0.1 and 0.8 mM trolox. Results were expressed in milligrams of trolox per gram of dry weight.

2.6. $ABTS^{\bullet+}$ Radical Scavenging Assay. The method used was the $ABTS^{\bullet+}$ (radical cation azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) decolourisation assay according to [44]. The assay is based on the ability of an antioxidant compound to quench the $ABTS^{\bullet+}$ relative to that of a reference antioxidant such as trolox. A stock solution of $ABTS^{\bullet+}$ radical cation was prepared by mixing ABTS solution and potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 12–16 h before use. The working $ABTS^{\bullet+}$ solution was produced by dilution in ethanol (1 : 90 v/v) of the stock solution to achieve an absorbance value of 0.7 (±0.02) at 734 nm. An aliquot of 20 μL of diluted extract was added to $ABTS^{\bullet+}$ working solution (3 mL). For the blank and standard curve, 20 μL of ethanol or trolox solution was used, respectively. Absorbance was measured by means of a UV-vis spectrophotometer at 734 nm immediately after addition and rapid mixing ($At = 0$) and then every minute for 5 min. Readings at $t = 0$ min ($At = 0$) and $t = 5$ min ($At = 5$) of reaction were used to calculate the percentage inhibition value for each extract.

A standard reference curve was constructed by plotting % inhibition value against trolox concentration (0.1–0.8 mM). The radical scavenging capacity of extracts was quantified as milligrams of trolox per gram of dry weight.

2.7. FRAP Assay. The FRAP assay (Ferric Reducing Ability of Plasma) was performed as previously described by Alañón et al. (2011a) and Benzie and Strain, 1999 [43, 45], with some modifications. This spectrophotometric assay measures the ferric reducing ability of antioxidants. The experiment was conducted at 37°C and pH 3.6. In the FRAP assay, antioxidants present in the extract reduce Fe (III)-tripyridyltriazine complex to the blue ferrous form, with an absorption maximum at 593 nm. The assay was performed by means of an automated microplate reader (Tecan GENios Pro (Tecan Ltd., Dorset, UK)) with 96-well plates. Reagents included 300 mM acetate buffer pH 3.6, 40 mM hydrochloric acid, 10 mM TPTZ solution, and 20 mM ferric chloride solution. The working FRAP reagent was prepared fresh on the day of analysis by mixing acetate buffer, TPTZ solution, and ferric chloride solutions in the ratio 10 : 1 : 1 and the mixture was incubated at 37°C. Diluted extract (30 μL) and prewarmed FRAP reagent (225 μL) were put into each well. The absorbance at time

Table 1: Total Polyphenol Index (TPI), DPPH, FRAP, and ABTS assays mean values and standard deviation (SD) of untreated, oven-dried, and freeze-dried grapefruit peel extracts.

	TPI	DPPH	FRAP	ABTS
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Fresh white grapefruit	$49.14^{a} \pm 7.91$	$32.46^{a} \pm 0.80$	$60.30^{a} \pm 3.25$	$122.34^{a} \pm 6.22$
Fresh pink grapefruit	$27.95^{b} \pm 0.83$	$25.18^{a} \pm 8.52$	$44.82^{b} \pm 5.35$	$99.46^{a} \pm 12.09$
White grapefruit dried at 45°C	$52.95^{a} \pm 4.83$	$48.05^{b} \pm 3.75$	$71.57^{a} \pm 0.60$	$194.81^{b} \pm 3.80$
Pink grapefruit dried at 45°C	$42.29^{a} \pm 3.30$	$35.26^{a} \pm 3.62$	$65.86^{a} \pm 5.28$	$175.87^{b} \pm 5.64$
White grapefruit dried at 60°C	$63.35^{c} \pm 0.84$	$86.76^{c} \pm 8.40$	$105.86^{c} \pm 22.39$	$339.66^{c} \pm 33.61$
Pink grapefruit dried at 60°C	$49.36^{ac} \pm 3.57$	$50.07^{b} \pm 2.26$	$79.43^{ac} \pm 5.16$	$210.78^{b} \pm 2.19$
Freeze-dried white grapefruit	$84.60^{d} \pm 10.80$	$122.83^{d} \pm 15.95$	$181.80^{d} \pm 25.97$	$537.48^{d} \pm 36.10$
Freeze-dried pink grapefruit	$66.70^{e} \pm 1.54$	$110.98^{d} \pm 13.76$	$207.74^{d} \pm 14.65$	$455.38^{e} \pm 1.95$

TPI are expressed as mg gallic acid equivalents per gram of dry weight.
DPPH, FRAP, and ABTS assays are expressed as mg trolox per gram of dry weight.
[a,b,c,d,e]Different letters in the same column denote a significant difference according to the Student-Newman-Keuls test, at $p < 0.05$.

zero and after 4 min was recorded at 593 nm. The calculated difference in absorbance is proportional to the ferric reducing/antioxidant power of the extract. For quantification, a calibration curve of trolox was prepared with dilutions within 0–1.5 mM. The final results were expressed as milligrams of trolox per gram of dried grapefruit peel.

2.8. Total Phenol Index (TPI). The total phenol content of extracts was determined according to the Folin-Ciocalteu procedure described by Singleton and Rossi [46]. Deionized water (1.8 mL) was added to 0.2 mL of each extract. Folin-Ciocalteu reagent (0.2 mL) was then added and tubes were shaken vigorously. After 3 min, 0.4 mL sodium carbonate solution (35% w/v) was added, along with 1.4 mL of deionized water. Samples were well mixed and left in the dark for 1 h. The absorbance was measured at 725 nm using a UV-vis spectrophotometer (Lambda 5, Perkin-Elmer, Seer Green, UK) and the results were expressed in gallic acid equivalents, GAE, using a gallic acid standard curve (0–0.2 mg mL^{-1}). Extracts were further diluted if the absorbance value measured was above the linear range of the standard curve.

2.9. Cell Culture and Drug Treatment Procedures. SH-SY5Y cultures were grown as described previously by Jordán et al., 2004 [47], in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 20 units·mL^{-1} penicillin, 5 mg·mL^{-1} streptomycin, and 15% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The SH-SY5Y cells (1×10^{6}/mL) were seeded 24 h before the experiments in a 96-well plate and they were grown in a humidified cell incubator at 37°C under a 5% CO_2 atmosphere. For treatments, extracts from white and pink grapefruit peels (fresh, dried, and freeze-dried) were directly added to the culture medium at different concentrations (0.1, 0.25, 0.50, 0.75, and 1 mg/mL) for 24 h. The corresponding controls were treated with the same concentration of ethanol, which was always below 0.1% (final concentration).

2.10. Viability Assay: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Cell viability was measured by the ability to reduce 3-(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the blue formazan product. The culture medium was removed after 24 h of treatment. 150 μL of MTT (1 mg·mL^{-1} in normal culture medium) was added to the plates, and the cells (control and treated) were incubated for 2 h at 37°C. The medium was then replaced with DMSO, and MTT absorption was measured in a VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were expressed as the percentage of MTT reduction, assuming that the absorbance of the control SH-SY5Y cells was 100%.

2.11. Statistical Analysis. Analysis of variance and multivariate analysis were performed using *SPSS* 15.0 for Windows statistical package. Differences among means were determined for significance at $p \leq 0.05$ using the Student-Newman-Keuls test. Principal Component Analysis was performed to classify the samples into groups according to phenolic composition and antioxidant activity.

3. Results and Discussion

3.1. Effect of Processing on the Antioxidant Activities and Total Polyphenol Index of Grapefruit Peel Extracts. The effects of oven-drying and freeze-drying treatments on the antioxidant activity of grapefruit peels extracts were determined by DPPH, $ABTS^{\bullet+}$, and FRAPS tests (see Table 1). It is interesting to note that freeze-drying enhanced antioxidant activity in all cases.

The DPPH assay showed significantly higher levels of antioxidant capacity ($p < 0.05$) in freeze-dried than in fresh grapefruit peels. Freeze-dried white and pink grapefruit peels registered 122.83 and 110.98 mg trolox/g DW, while extracts from fresh peels registered values of 32.46 and 25.17 mg trolox/g DW, respectively. This effect is probably a consequence of the freeze-drying process. This process

Table 2: Spectral data of flavonoids in grapefruit peel extracts.

Tentative identification	RT (min)	UVmax λ (nm)	MS $[M - H]^-$ (m/z)	MS $[M + H]^+$ (m/z)	Products ions (m/z)
Isonaringin [32, 41]	31.43	217, 284, 331	579		271, 151
Naringin [32, 41]	33.58	224, 283, 331	579		459, 271
Hesperidin [32, 41]	38.02	225, 284, 328	609		301
Neohesperidin [41]	40.19	228, 283, 331	609		301, 489
Unknown-1	44.65	283, 328		617	465, 303
Unknown-2	46.57	249, 257, 324		595	449, 287
Naringenin [42]	48.04	226, 284, 325		273	153
Hesperetin [40]	51.98	225, 285, 329		303	285
Isosinensetin [40]	53.46	249, 270, 342		373	357, 343, 327
Sinensetin [32, 40, 43]	54.54	243, 264, 333		373	358, 343, 312
Unknown-3	56.22	250, 272, 335		403	388, 373
Nobiletin [40, 43]	57.60	248, 268, 334		403	388, 373
Tangeretin [32, 40]	59.42	271, 322		373	358, 343, 325, 297

References given in brackets are taken from papers with matching spectral data:
Angeloni et al. 2012 [32]; Rivas et al. 2008 [40]; Jeong et al. 2004 [41]; Xu et al. 2007 [42]; Alañón et al. 2011 [43].

has been associated with high production of redox-active metabolites which play an important role in adsorbing and neutralizing free radicals or decomposing peroxides, as previously reported by other researchers [48].

After oven-drying at 45°C and 60°C, DPPH values of both white and pink grapefruit peels were significantly greater than those of fresh peel extracts (Table 1). These increases denote increased antioxidant activity, particularly in the case of white grapefruit peel heated at 60°C. They are presumably a consequence of the relationship between the generation of breakdown antioxidant products and the increasing temperatures to which the grapefruit peels were subjected, and they are consistent with data reported for other citrus varieties subjected to comparable heat treatments [41, 42, 49]. The $ABTS^{\bullet+}$ assay showed the same tendency, revealing a significant increase of free radical scavenging activity in white and pink freeze-dried grapefruit peel extracts ($p < 0.05$), which reached 537.45 and 455.38 mg trolox/g DW, versus 122.34 and 99.46 mg trolox/g DW in the case of fresh samples.

$ABTS^{\bullet+}$ activity was also greater in grapefruit peel dried at 45°C and 60°C than in fresh extracts, although the rise was less pronounced than in the case of freeze-dried peel. For instance, scavenging ability was significantly ($p < 0.05$) greater in white grapefruit peel extracts than in fresh extracts (from 122.34 to 194.81 and 339.66 mg trolox/g DW) as a result of treatment at 45°C and 60°C, respectively. ABTS also increased in the case of pink grapefruit peel but did not differ significantly between 45°C and 60°C. This behaviour is consistent with reports for extracts of dried citrus peel of other varieties [42, 49].

FRAP chelating values for white and pink grapefruit peels oven-dried at 60°C reached 105.86 and 79.43 mg trolox/g DW, respectively, that is, 1.7 times the values for fresh peels extracts (Table 1). In the case of freeze-dried samples, results varied from 60.30 and 44.82 for white and pink fresh grapefruit peel extracts to 181.80 and 207.74 mg trolox/g DW for freeze-dried grapefruit peels, respectively.

In general, the scale of the antioxidant activity observed in freeze-dried white and pink grapefruit peels suggested that this treatment might produce not only dissociation or liberation of some phenolic compounds from biological structures but also chemical changes enabling the conversion of insoluble phenols into more soluble and free forms, as indicated by the data from other freeze-dried vegetable extracts [50, 51].

The results of the Folin-Ciocalteu Total Phenol Index (TPI), a preliminary screening factor to establish the antioxidant capacities of treated grapefruit peels, are shown in Table 1. Total phenols in white and pink grapefruit peels dried at 45°C, and particularly at 60°C (63.35 and 49.36 mg GAE/g dry weight, resp.), were significantly higher than in fresh samples (49.14 and 27.95 mg GAE/g dry weight), most probably due to the cleaving of glycosylated bonds in various phenolic compounds.

Note also that TPI values from white and pink freeze-dried grapefruit peel extracts were 58% and 42% higher, respectively, than in fresh samples. This trend is consistent with reports in previous works carried out on lyophilized fruits, tubers, vegetables, and fungi [50, 52, 53].

The same trend was observed in the behaviour of TPI, ABTS, FRAP, and DPPH values, which were significantly enhanced after oven-drying (45°C, 60°C) and especially freeze-drying treatments. This suggests that both processes could be successfully used to enhance antioxidant activity in grapefruits peels for use as natural sources of antioxidants, with major attendant environmental and economic benefits. As natural products, with outstanding antioxidant power, processed grapefruit look very promising for use in the development of new therapeutic strategies.

3.2. Effect of Oven-Drying and Freeze-Drying on Flavanone and Polymethoxylated Flavones of Grapefruit Peels. A total of thirteen compounds were quantified, based on their UV-data spectra and $[M + H]^+$, $[M - H]^-$ m/z (Table 2), and were

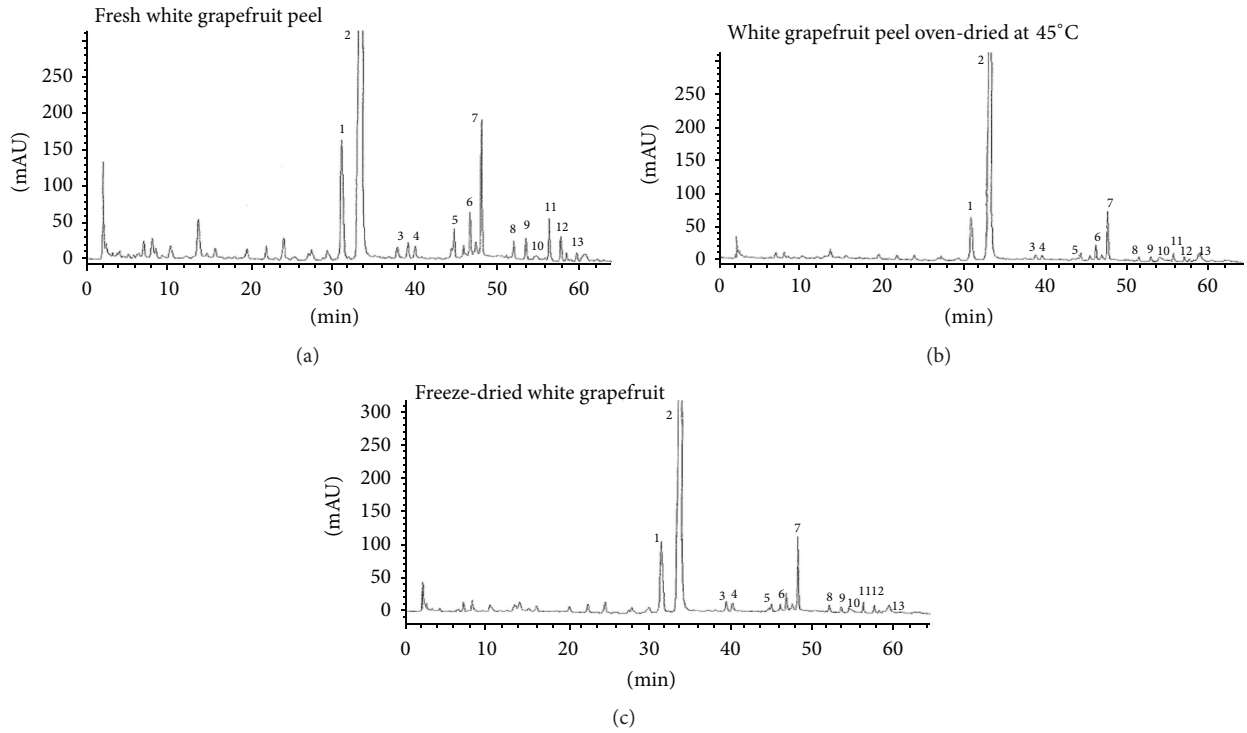

Figure 1: HPLC chromatogram of white grapefruit peels. Peak assignments: (1) isonaringin; (2) naringin; (3) hesperidin; (4) neohesperidin; (5) unknown-1; (6) unknown-2; (7) naringenin; (8) hesperetin; (9) isosinensetin; (10) sinensetin; (11) unknown-3; (12) nobiletin; (13) tangeretin.

quantified by HPLC-MS. These were as follows: four glycosylated flavanones (FGs), namely, isonaringin, naringin, hesperidin, and neohesperidin; four polymethoxylated flavones (PMFs), namely, isosinensetin, sinensetin, nobiletin, and tangeretin; two flavanone aglycones: hesperetin and naringenin; and three unknown compounds. Typical MS total ion current chromatograms with numbered peaks are shown in Figure 1.

Naringin and to a much lesser extent isonaringin were the main FGs in all grapefruit peels extracts (Table 3). Naringin levels in fresh pink and white grapefruits varied within 142–160 mg/g DW, respectively, while isonaringin ranged between 11.85 and 13.42 mg/g DW. Moreover, naringenin was the most abundant flavanone aglycone in fresh white and pink grapefruit peels (Table 3).

Levels of polymethoxylated flavones, sinensetin, nobiletin, and tangeretin ranged from 1.03 to 3.45 mg/g DW in fresh grapefruit peels. Results were very similar to reports in the literature for peels of mandarin and thirteen citrus species [53–56]. However, one of the most outstanding findings in this study was the flavonoid losses in processed grapefruit peels. Oven-dried and freeze-dried grapefruit peels had similar flavonoid profiles, although concentrations were lower than in fresh samples. After oven-drying of grapefruit peels at 45°C and 60°C, the concentrations of most FGs, PMFs, and flavanone aglycones declined sharply, 3–6-fold with respect to fresh grapefruit peel extracts (Table 3). The same behaviour has been reported in dried citrus peels from other varieties [42, 49]. However, it is important to note that naringin concentrations in processed grapefruit peels were between two and four times higher than reported in treated byproducts from other citrus species [42, 57, 58].

The same downward trend was observed when comparing flavonoid contents in freeze-dried grapefruit peels and fresh grapefruit peel extracts (Table 2). For instance, isonaringin decreased from 13.42 mg/g DW in fresh white grapefruit peels to 7.17 mg/g DW in freeze-dried white grapefruit peels. The decreases were most pronounced (from 13.42 to 4.57 and 4.02) after oven-drying at 45°C and 60°C, respectively.

It is interesting to note that the effects of oven-drying and freeze-drying on FG and PMF levels were opposite to their effects on TPI values and free radical scavenging activities. This may be because the TPI assay evaluates the totality of phenols, that is, all flavonoids and nonflavonoid phenolic compounds, which suggests that some phenolic compounds other than flavones and flavanones are involved in the antioxidant activities of grapefruit peel extracts. This tends to confirm some recent studies which described progressive increases of cinnamics and benzoics acids levels in orange peels dried from 60°C to 120°C in comparison with untreated samples due to the cleaving of esterified bond and glycosylated bond [42, 49]. By the other hand, our results are in a good agreement with decreases of naringin, hesperidin, and neohesperidin reported in dried orange peels in comparison with untreated samples [42]. Heat treatment of grapefruit peels is thus closely associated with releases of bound phenolic acids, including hydroxybenzoic and hydroxycinnamic acids, producing higher levels of free

Table 3: Flavanones glycosides, flavanones, and polymethoxylated flavones content (milligrams per gram of dry weight) and relative standard deviations (RSD) both for white and pink grapefruit peel extracts.

	Fresh white grapefruit		White grapefruit oven-dried at 45°C		White grapefruit oven-dried at 60°C		Freeze-dried white grapefruit		Fresh pink grapefruit		Pink grapefruit oven-dried at 45°C		Pink grapefruit oven-dried at 60°C		Freeze-dried pink grapefruit	
Isonaringin*	13.42^{a}	(13.92)	4.57^{b}	(1.71)	4.02^{b}	(0.22)	7.17^{c}	(2.06)	11.85^{a}	(10.73)	3.72^{b}	(5.65)	3.55^{b}	(0.32)	5.22^{b}	(4.74)
Naringin	160.25^{a}	(18.24)	59.41^{b}	(1.96)	51.16^{b}	(0.25)	95.08^{c}	(5.39)	142.39^{a}	(16.81)	45.92^{b}	(6.39)	43.50^{b}	(0.34)	50.40^{b}	(3.12)
Hesperidin	3.23^{a}	(15.81)	0.51^{cd}	(2.20)	0.37^{cd}	(5.82)	0.65^{cd}	(1.90)	2.68^{b}	(6.31)	0.21^{d}	(6.29)	0.13^{d}	(0.26)	0.95^{c}	(2.77)
Neohesperidin	3.30^{a}	(9.93)	0.79^{c}	(2.34)	0.67^{cd}	(0.48)	1.39^{e}	(5.45)	2.93^{b}	(7.40)	0.30^{d}	(6.41)	0.27^{d}	(0.39)	0.79^{c}	(2.09)
Unknown-1*	4.39^{a}	(5.26)	0.76^{c}	(2.17)	0.76^{c}	(0.08)	0.74^{c}	(1.26)	3.88^{b}	(4.83)	0.93^{c}	(10.65)	0.84^{c}	(0.12)	0.61^{c}	(5.59)
Unknown-2*	5.36^{a}	(6.71)	1.67^{bc}	(2.28)	1.46^{b}	(1.80)	2.20^{c}	(7.01)	5.05^{a}	(8.82)	1.60^{bc}	(4.70)	1.29^{b}	(0.08)	1.09^{b}	(1.40)
Naringenin	8.49^{a}	(9.95)	5.05^{b}	(2.16)	4.54^{b}	(1.10)	7.83^{a}	(4.78)	8.14^{a}	(15.83)	2.87^{c}	(5.29)	2.49^{c}	(0.29)	2.35^{c}	(0.35)
Hesperetin*	2.93^{a}	(2.24)	0.54^{c}	(1.94)	0.43^{cd}	(1.17)	0.87^{e}	(1.25)	2.55^{b}	(3.03)	0.42^{cd}	(5.57)	0.39^{d}	(1.40)	0.47^{cd}	(6.99)
Isosinensetin*	3.02^{a}	(3.71)	0.56^{b}	(3.44)	0.45^{b}	(0.59)	0.60^{a}	(4.96)	3.12^{a}	(10.67)	0.41^{b}	(1.34)	0.43^{b}	(2.07)	0.24^{b}	(6.16)
Sinensetin*	2.10^{a}	(14.48)	1.82^{a}	(0.13)	1.78^{a}	(0.68)	1.74^{a}	(2.68)	2.07^{a}	(5.30)	0.63^{b}	(0.77)	0.63^{b}	(0.69)	0.79^{b}	(7.69)
Unknown-3*	4.07^{a}	(9.42)	1.14^{b}	(4.56)	0.99^{b}	(0.87)	1.46^{b}	(3.69)	3.86^{a}	(9.83)	0.32^{c}	(5.50)	0.32^{c}	(3.68)	0.41^{c}	(8.13)
Nobiletin	2.36^{a}	(14.26)	1.22^{c}	(1.29)	1.35^{c}	(13.02)	2.09^{a}	(12.63)	3.45^{b}	(11.67)	0.63^{d}	(2.74)	0.58^{d}	(2.61)	1.24^{c}	(10.19)
Tangeretin	2.45^{a}	(10.48)	2.05^{c}	(1.11)	1.98^{c}	(5.29)	1.96^{c}	(2.51)	1.03^{b}	(3.34)	0.97^{b}	(0.38)	0.97^{b}	(0.46)	1.06^{b}	(8.32)

*Compounds tentatively identified. Data are expressed as naringin equivalents (mg/g).

[a,b,c,d,e]Different letters in the same column denote a significant difference according to the Student-Newman-Keuls test, at $p < 0.05$.

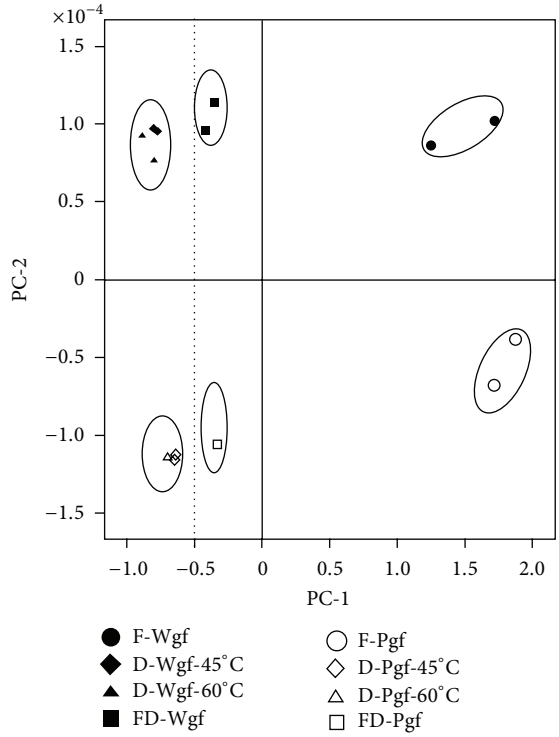

Figure 2: Principal Component Analysis performed considering duplicates of the flavanones glycosides, flavones, and polymethoxylated flavones contents on fresh, oven-dried and freeze-dried, and white and pink grapefruit peel extracts. F-Wgp: fresh white grapefruit; F-Pgf: fresh pink grapefruit; D-Wgf-45°C: dried white grapefruit at 45°C; D-Pgf-45°C: dried pink grapefruit at 45°C; D-Wgf-60°C: dried white grapefruit at 60°C; D-Pgf-60°C: dried white grapefruit at 60°C; FD-Wgf: freeze-dried white grapefruit; FD-Pgf: freeze-dried pink grapefruit.

phenolic acids. Therefore, the increase in the total phenol index and DDP, FRAP, and ABTS values would appear to be caused by the free fraction of phenolic acids.

To obtain more detailed information on the individual flavanone glycosides, flavanones, and polymethoxylated flavones that occur in processed white and pink grapefruit peels, the data matrix was processed by Principal Component Analysis (PCA). The two-dimensional projection of variables is presented in Figure 2.

The first principal component axis explains 83.47% of the total variation and clearly isolated fresh grapefruit peel extracts which were grouped on the right side of the plot, correlating closely with higher levels of hesperidin, neohesperidin, isonaringin, naringin, nobiletin, and unknowns-1-2.

PC-1 also exhibited correlation with freeze-dried samples plotted on the negative area of PC-1 (Figure 2). The fact that amounts of isosinensetin, hesperetin, hesperidin, isonaringin, neohesperidin, unknown-3, naringin, and nobiletin in freeze-dried grapefruit peels were higher than in extracts from oven-dried peels indicates a good degree of discrimination and also suggests that freeze-drying is more effective in preserving bioactive compounds than oven-drying. However, extracts from grapefruit peels dried at 45°C and 60°C were located too close together on the x-axis for discrimination.

PC-2 explains 10.04% of the total variation and is particularly important in terms of differentiating grapefruit varieties. PC-2 showed positive loadings for tangeretin, sinensetin, and naringenin, grouping white grapefruit peel extracts at the top of the axis and pink grapefruit peel extracts at the bottom.

3.3. Viability. SH-SY5Y cell viability results were influenced by two parameters; firstly the treatment of grapefruit peels (fresh, oven-drying, or freeze-drying) and secondly the concentration of bioactive compounds. Our results showed that the potential cell protection and/or cell cytotoxicity of grapefruit peel extracts was determined by these two factors together.

In general, cell viability decreased with increasing concentrations of grapefruit peel extracts, whether they were fresh/processed or white/pink. On the other hand, in the case of SH-SY5Y cells viability percentages were higher in fresh than in treated grapefruit peel extracts.

It is important to stress that fresh white grapefruit peel extracts at concentrations between 0.1 and 0.25 mg/mL clearly exerted a protective effect on the SH-SY5Y cell line, reaching viabilities of 100% (Figure 3). Similar effects were reported by Chen et al. 2012 [59] on Hep G2 cells after contact with fresh *Citrus sinensis* peel extracts at concentrations ranging within 0.01–0.5 mg/mL, which significantly protected against tertiary butyl hydroperoxides t-BHP.

The cytoprotective effect observed in the current is most probably attributable to the levels of bioactive flavonoids (FGs and PMFs), mainly naringin, isonaringin, and naringenin, which largely occur in fresh white grapefruit peel extracts (Table 2). Similar finding have also been reported, revealing a relationship between naringin and naringenin and neuroprotection and oxidative stress delay [60]. In the present case, fresh white grapefruit peel extracts, which registered the highest flavonoid contents, also scored best for viability. On the other hand, freeze-dried peel extracts, containing less flavonoids (Table 2), registered the lowest cell viability ratios.

Also, SH-SY5Y cell viability decreased to 75% after the following: (i) 24-hour incubation with fresh grapefruit peel extracts at 0.75 mg/mL; (ii) incubation with oven-dried grapefruit peels extract (45°C and 60°C) at 0.25 mg/mL; and (iii) contact with freeze-dried grapefruit peel extract at concentrations ranging within 0.1–0.25 mg/mL (Figure 3).

Finally, it was found that oven-dried grapefruit peels (45°C and 60°C), at concentrations ranging from 0.75 mg/mL to 1 mg/mL, induced cell death by 75–95% in both white and pink grapefruits. This effect was especially pronounced after cell contact with freeze-drying extracts (0.75 mg/mL and 1 mg/mL) which triggered 90% and 96%, respectively, of apoptosis in SH-SY5Y-cells. This suggests that cell cytoprotection and/or apoptosis, expressed as cell viability, can be influenced in a dose-dependent way by flavonoids.

It should be noted that the results for SH-SY5Y cell viability after contact with grapefruit peel extracts did not really match expectations in the light of the polyphenol index (TPI) and DPPH, FRAP, and ABTS here reported. There seemed to be an inverse correlation between antioxidant

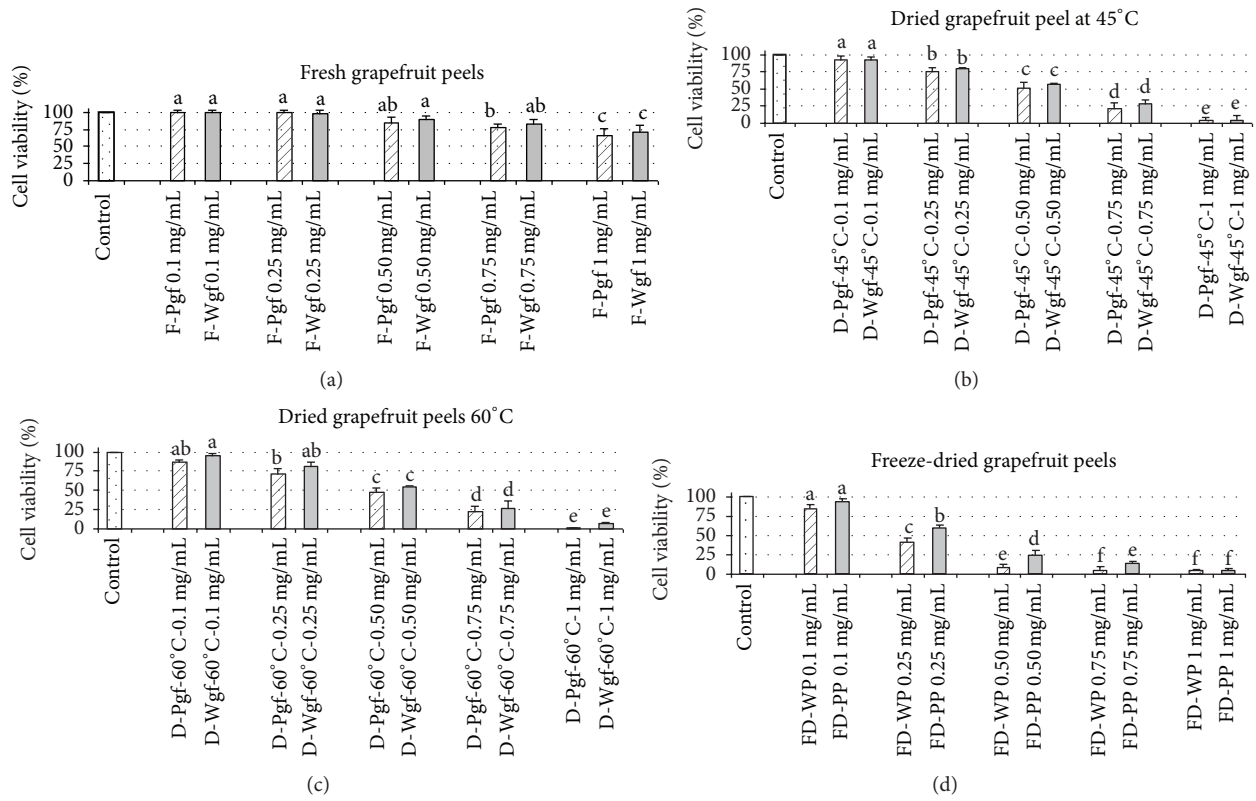

Figure 3: Effects of different concentrations of red and white grapefruit extracts in cell viability of SH-SY5Y cell cultures for 24 h. Data were expressed as the percentage of live cells relative to total cells. Data are presented by means ± SD ($n = 3$). F-Wgp: fresh white grapefruit; F-Pgf: fresh pink grapefruit; D-Wgf-45°C: dried white grapefruit at 45°C; D-Pgf-45°C: dried pink grapefruit at 45°C; D-Wgf-60°C: dried white grapefruit at 60°C; D-Pgf-60°C: dried white grapefruit at 60°C; FD-Wgf: freeze-dried white grapefruit; FD-Pgf: freeze-dried pink grapefruit. a, b, c, d, e, f: different letters in the same column denote a significant difference according to the Student-Newman-Keuls test, at $p < 0.05$.

activity and cell viability since the extracts with the highest antioxidant capacities were the most closely associated with cell cytotoxicity. The explanation of this singular behaviour probably lies in the amounts of phenolic acids, which would surely increase after drying at 45°C and 60°C and more so after freeze-drying, producing considerable increases in the overall antioxidant activity and TPI index. However, it has also been reported that phenolic acids, due to their chemical structure and depending on certain conditions, are involved in prooxidant reactions associated with damage to molecules such as DNA [61, 62]. These last findings would seem to indicate that phenolic acid levels are strongly associated with cell cytotoxicity and apoptosis, which supports the findings in the present work.

4. Conclusions

Our results indicate that oven-drying (45°C, 60°C) and especially freeze-drying can be used to significantly enhance the antioxidant power of grapefruit peels, thus realizing their outstanding potential for biomedical use.

Fresh and processed grapefruit peel wastes are a natural source of valuable bioactive flavonoids, mostly naringin, that could be incorporated as food ingredients or as therapeutic agents as part of pharmacological strategies.

Finally, the *in vitro* cytoprotection demonstrated by fresh and oven-dried (45°C) grapefruit peels opens up new possibilities for these natural extracts; however, further research into action mechanisms, animal models, clinical trials, and dose-effect will be needed.

Conflict of Interests

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

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Research Article

Antioxidant and Antifatigue Properties of the Aqueous Extract of *Moringa oleifera* in Rats Subjected to Forced Swimming Endurance Test

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The effects of the aqueous extract of *Moringa oleifera* on swimming performance and related biochemical parameters were investigated in male Wistar rats (130–132 g). Four groups of rats (16 per group) were fed a standard laboratory diet and given distilled water, 100, 200, or 400 mg/kg of extract, respectively, for 28 days. On day 28, 8 rats from each group were subjected to the forced swimming test with tail load (10% of body weight). The remaining 8 rats per group were subjected to the 90-minute free swim. Maximum swimming time, glycemia, lactamia, uremia, triglyceridemia, hepatic and muscle glycogen, hematological parameters, and oxidative stress parameters (superoxide dismutase, catalase, reduced glutathione, and malondialdehyde) were measured. *Results*. *M. oleifera* extract increased maximum swimming time, blood hemoglobin, blood glucose, and hepatic and muscle glycogen reserves. The extract also increased the activity of antioxidant enzymes and decreased the blood concentrations of malondialdehyde. Furthermore, it decreased blood concentrations of lactate, triglycerides, and urea. In conclusion, the antifatigue properties of *M. oleifera* extract are demonstrated by its ability to improve body energy stores and tissue antioxidant capacity and to reduce the tissue build-up of lactic acid.

1. Introduction

Fatigue is best defined as difficulty in initiating or sustaining voluntary activities [1]. Fatigue is accompanied by a feeling of extreme physical or mental tiredness, resulting from severe stress and hard physical or mental work [2]. Physical fatigue is thought to be accompanied by deterioration in performance [1, 3]. There are several theories about the mechanisms of physical fatigue. These include the exhaustion theory, the clogging theory, the radical theory, and the hemoglobin theory. The exhaustion theory suggests that during exercise, many energy sources, such as glucose and liver glycogen, will be exhausted, thus leading to physical fatigue [4]. Prolonged, continuous utilization of carbohydrates depletes hepatic and muscle glycogen reserves (at 60 to 80% of maximal oxygen consumption (VO_2max)) and considerably diminishes blood glucose concentration to subphysiological concentrations, resulting in fatigue [5, 6]. The radical theory suggests that intense exercise can produce an imbalance between the body's oxidation system and its antioxidation system. The accumulation of reactive-free radicals will put the body in a state of oxidative stress and bring injury to the body by attacking large molecules and cell organs. The mechanisms and cellular systems responsible for oxidative stress include mitochondria, leucocytes, and ischemia-reperfusion, and recovery from exercise-induced fatigue requires damage repair and elimination of the accumulated metabolic products [1, 4, 7–10]. The clogging theory suggests that exercise can cause the production and accumulation of metabolism-related substances such as lactic acid and urea in the body, which affect cellular homeostasis. The resulting acute or chronic acidosis triggers muscle cramps, muscular pain, acute

respiratory distress, inhibition of enzymatic systems, and eventually fatigue [11–13]. The hemoglobin theory on its part suggests that myoglobin and an energy metabolic system coenzyme leak out into the blood from cells and tissues damaged by exercise, and destruction of red blood cells occurs [14].

Moringa oleifera Lam. is a plant of Indian origin which is now widespread in Asia and Africa. It belongs to the family Moringaceae with about 13 species [15]. It is commonly referred to as "tree of life," "miracle tree," or "divine plant" due to its numerous nutritive, medicinal, and industrial potentials [16, 17]. The leaves are widely consumed as a legume and used in traditional medicine in Africa in general and in Cameroon in particular. The leaves are an excellent source of protein (19–35% dry matter) [18–26] and are rich in metabolisable energy (2273–2978 kcal/kg DM) [18, 25], vitamins (A, B, C, and E), minerals (0.6–11.2% DM), for example, iron, calcium, zinc, selenium, and β-carotene [19, 27], and fats (2.3–10% DM) and contain the 10 amino acids essential to man [19, 20, 24, 25]. Crude protein levels of 30.3% and 19 amino acids have been reported in the South African ecotype of the plant [28]. *M. oleifera* leaves are used in traditional medicine to treat malnutrition, fever, headaches, nerve pain, and diabetes [24, 29]. The leaves are used in Benin as food supplements for HIV patients [30] and as antipyretic and antibiotic [31]. In part one of a comprehensive review of the medical evidence for its nutritional, therapeutic, and prophylactic properties, *M. oleifera* has been cited for its numerous applications in disease treatment and prevention. These include antibiotic, antitrypanosomal, hypotensive, antispasmodic, antiulcer, anti-inflammatory, hypocholesterolemic, and hypoglycemic activities, as well as having considerable efficacy in water purification by flocculation, sedimentation, antibiosis, and even reduction of schistosoma cercariae titer. The plant family is rich in compounds containing the simple sugar, rhamnose, and also rich in a fairly unique group of compounds called glucosinolates and isothiocyanates, some with anticancer activity [32, 33]. Torres-Castillo et al. [34] have provided an overview of the histological organization and the composition of some biochemical components (e.g., enzymatic inhibitors, phytochemicals, enzymatic profiles, and antifungal potential) of different plant tissues of *M. oleifera*, associating these components with the physiology and defense mechanisms of the plant.

Nutrient supplementation to improve exercise performance has involved the use of high fat diets, carbohydrate supplements, and various dietary supplements or "tonics" of plant origin to enhance exercise capacity. In the fight against fatigue, more and more sports professionals and athletes are turning to plant extracts as sources of energy in replacement of banned doping substances. Studies have revealed the widespread use of plant extracts in many African countries for performance enhancement [35]. It is therefore important to develop efficient and safe plant-based antifatigue products that can enhance exercise performance without deleterious effects on the health of the users. It has been widely claimed that "ounce-for-ounce, Moringa leaves contain more Vitamin A than carrots, more calcium than milk, more iron than spinach, more Vitamin C than oranges, and more potassium than bananas" and that the protein quality of Moringa leaves rivals that of milk and eggs. Given the rich nutrient, phytochemical, and organoleptic potential of *M. oleifera*, we designed the present experiment to study the antifatigue potency of the aqueous extract in rats subjected to the forced swimming test.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material and Preparation of Aqueous Extract of Leaves from M. oleifera. The fresh leaves of *M. oleifera* were harvested from the North Region of Cameroon in December 2014 and identified in the National Herbarium (Yaoundé) where a voucher specimen No. 49178/HNC exists. The leaves were cleaned immediately after harvest, cut into small pieces, and dried in the shade for about 2 weeks. The dried material was ground into a powder using an electrical homogenizer (Zaiba®). The aqueous extract was prepared as described by Thilza et al. [36]. 100 g of ground plant material was macerated in 1.5 L of boiled distilled water for one hour. The mixture was filtered through Whatman filter paper No. 3 and filtrate obtained was evaporated to dryness using a rotator evaporator at 45°C. The extract obtained (22, 9% yield) was stored at 4°C. Extract solution was prepared in distilled water each time prior to experimentation.

2.1.2. Animals and Grouping

(1) Animals. Male albino rats of Wistar strain weighing 130–132 g were obtained from the Animal House of the National Institute of Youth and Sports in Yaoundé. They were placed in plastic cages in a room under standard laboratory conditions (temperature 20 to 30°C, relative air humidity 45 to 55%, and 12/12 h light/dark cycle). The rats were fed with a basal diet and water *ad libitum*. The feed was a standard rat chow (National Veterinary Laboratory (LANAVET), Cameroon) composed of carbohydrates (52%), protein (22%), fat (6.5%), water (12%), ash (6%), and fiber (4.5%). The authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethics Committee (Reg. No. FWA-IRBoooo1954). The use, handling, and care of animals were done in adherence to the European Convention (Strasbourg, 18.III.1986) for the protection of vertebrate animals used for experimental and other purposes (ETS-123), with particular attention to Part III, articles 7, 8, and 9. The animals were transferred to the laboratory at least 1 hour before the start of the experiment. The experiments were performed during the day (11:00–17:00 hr).

(2) Animal Grouping. After two weeks of acclimatization, sixty-four rats were divided randomly into four groups of sixteen rats each: group 1: a control group which received the vehicle (distilled water) only, and three treatment groups (groups 2, 3, and 4) which received 100, 200, and 400 mg/kg, respectively, of *M. oleifera* extract orally once a day for 28 days. The quantity of food and water consumed by each group of rats, as well as body weights were measured every two

days during 28 days, between 11:00 AM and 12:00 AM before extract administration.

2.2. Methods

2.2.1. Weight Loaded Force Swimming Test. The weight loaded force swimming test was performed as described previously [37–41] but with some modifications. Briefly, 30 minutes after the last dose of extract on day 28 of treatment, eight rats taken from each group were subjected to the force swimming exercise. Each animal was supplied with a constant load (corresponding to 10% of the body weight) tagged to the tail and placed individually in a swimming pool (90 cm × 45 cm × 45 cm), filled with water to a depth of 35 cm [42, 43] and maintained at 25 ± 1°C [41]. Exhaustion was determined by observing loss of coordinated movements and failure to return to the surface within 10 sec [41, 44] and swimming time was recorded immediately. The rats were then removed from the pool, dried with a paper towel, and returned to their original cages. The pool water was replaced after each session.

2.2.2. The 90-Minute Free Swimming Test. Thirty minutes after the final extract treatment, the remaining eight rats from each group were subjected to the 90-minute free swimming experience without a weight load. At the end of the swim, the rats were rested for an hour and then sacrificed (under ether anesthesia) by cutting through the jugular vein. Blood samples were taken into sterile tubes and serum was prepared (centrifuging at 3000 rpm for 10 min) for the analysis of blood glucose (Glu), triglycerides concentration (TG), blood lactic acid (BLA), and blood urea nitrogen (BUN). Another part of blood collected into bottles containing EDTA as anticoagulant was used to determine blood cell count. Vital body organs (spleen, fatty mass, heart, lungs, kidneys, and testicles) were cleaned using 0.9% saline and then weighed using a sensitive electronic balance. Tissue samples of liver and gastrocnemius muscle were taken and stored frozen at −20°C awaiting determination of glycogen and antioxidant status parameters.

2.2.3. Measurement of Serum Biochemical Parameters and Blood Cell Count. Blood cell count in all rat groups was measured with the help of blood analyzer (Hospitex Diagnostic Hema Screen 18). The serum levels of glucose were estimated using a glucometer (Reader Accu-CHEK® Active). Blood lactic acid concentrations and levels of BUN were measured using an L-lactate assay kit (Abcam 65331 L-Lactate assay kit) and a colorimetric and enzymatic method (Bioassay System, CA Kit), respectively. Triglyceride concentrations were measured using a commercial kit for measurement of triglycerides in serum or plasma (Enzymatic Trinder Method).

2.2.4. Measurement of Tissue Glycogen. Liver and muscle glycogen contents were measured calorimetrically using anthrone reagent [45]. Briefly, after hydrolysis of the liver and gastrocnemius muscle samples in 30% KOH at 100°C for 30 min, 1.5 mL of anhydrous ethanol was added to the vials. After centrifugation at 4000 ×g for 15 min, the supernatants were discarded. 0.5 mL of distilled water and 1 mL of 0.2% anthrone were added, and the vials were placed in a boiling water bath for 20 min. The absorbance of the solution in vials was determined at 620 nm using a spectrophotometer (V-530, Jasco Co., Japan).

2.2.5. Measurement of Antioxidant Status in Liver and Gastrocnemius Muscle. The liver and gastrocnemius muscle tissue (1 g of each) was homogenized in 4 mL of Tris/HCl. These tissue homogenates were centrifuged at 4000 g for 15 min at 4°C and the supernatants were assessed for the antioxidant status. Lipid peroxidation (the level of thiobarbituric acid reactive substances in terms of malondialdehyde) was measured as described previously [46], and total glutathione (GSH) content was measured according to the method of Ellman [47]. Catalase activity was measured according to the method of Sinha [48]. Superoxide dismutase (SOD) activity was measured using the pyrogallol autoxidation method [49].

2.2.6. Statistical Analysis. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons and P values less than 0.05 were considered significant. The results are expressed as mean ± standard error of mean (SEM).

3. Results and Discussion

The forced swimming test represents a valid animal model for screening antifatigue potency of various bioactive compounds [3, 50–52]. Administration of *M. oleifera* extract did not bring about significant differences in food and water intake (Table 1). In addition, we did not observe significant differences in final body weights (207–213 g) (Figure 1) and organ weights (Table 2) following 28 days of extract administration. Body weight gain ranged between 77.0 and 80.8 g for the four treatment groups. This result was in contrast to the results obtained by Osman et al. [53] who reported up to 14% changes in body weight of rats given *M. oleifera* extract for 21 days, attributing these changes to the rich nutrient quality of the extract.

In this study, the forced swimming capacity test in mice was employed to evaluate the effect of leaf aqueous extract from *M. oleifera* on exercise durability of rats with 10% tail load. The forced swimming capacities are shown in Figure 2. The results showed that the swimming time to exhaustion of each extract-treated group was significantly longer ($P < 0.05$) than that recorded for the control group. The maximum forced swimming times were 135.12 ± 35.62, 140.5 ± 32.17, and 131.25 ± 38.64 seconds, respectively, for the 100, 200, and 400 mg/kg extract-treated groups compared with 89.75 ± 17.19 seconds for the control group. The 200 mg/kg dose was most effective. The shortness of the length of the exhaustive swimming time indicates the degree of fatigue [54]. The results therefore indicated that extract of *M. oleifera* enhanced the swimming capacity by delaying the onset of physical fatigue in rats. Similar results have been obtained by other

Table 1: Effect of *M. oleifera* extract on food (g/group/week) and water (mL/group/week) intake in rats.

	Control	*M. oleifera* (100 mg/kg)	*M. oleifera* (200 mg/kg)	*M. oleifera* (400 mg/kg)
Food intake				
Week 1	327.67 ± 40.81	252.00 ± 37.40	269.00 ± 32.08	319.33 ± 19.03
Week 2	285.67 ± 14.64	231.33 ± 4.93	257.67 ± 17.62	304.00 ± 42.33
Week 3	299.67 ± 16.86	243.67 ± 33.86	232.33 ± 35.53	290.00 ± 28.58
Week 4	335.00 ± 22.01	249.25 ± 29.65	286.25 ± 29.77	321.75 ± 37.85
Water intake				
Week 1	341.33 ± 33.50	319.00 ± 14.8	341.00 ± 30.51	322.67 ± 24.42
Week 2	396.67 ± 16.77	343.00 ± 34.65	381.33 ± 14.74	390.00 ± 38.43
Week 3	332.33 ± 46.01	285.33 ± 19.55	337.67 ± 26.63	308.67 ± 12.22
Week 4	346.25 ± 26.36	322.01 ± 31.76	353.02 ± 16.58	323.75 ± 33.63

Each value represents the mean ± SEM, $n = 8$.

Table 2: Effect of *M. oleifera* extract on relative organ weights of the rats.

Organ weight (% body weight)	Control	*M. oleifera* (100 mg/kg)	*M. oleifera* (200 mg/kg)	*M. oleifera* (400 mg/kg)
Heart	0.28 ± 0.04	0.30 ± 0.04	0.29 ± 0.05	0.34 ± 0.04*
Liver	3.74 ± 0.27	3.24 ± 0.69*	3.38 ± 0.24	3.53 ± 0.17
Lungs	0.73 ± 0.12	0.93 ± 0.23	0.89 ± 0.41	0.85 ± 0.07
Spleen	0.24 ± 0.06	0.32 ± 0.08	0.28 ± 0.17	0.34 ± 0.09
Left kidney	0.28 ± 0.03	0.27 ± 0.05	0.28 ± 0.03	0.31 ± 0.04
Right kidney	0.30 ± 0.02	0.26 ± 0.04	0.26 ± 0.05	0.32 ± 0.06
Left testis	0.50 ± 0.14	0.47 ± 0.08	0.61 ± 0.14	0.59 ± 0.08
Right testis	0.55 ± 0.14	0.44 ± 0.10	0.51 ± 0.07	0.59 ± 0.07
Fatty mass	1.65 ± 0.32	1.43 ± 0.55	2.27 ± 1.07	1.67 ± 0.37

Each value represents the mean ± SEM, $n = 8$. *$P < 0.05$ compared with control group.

workers who tested the antifatigue potential of various plant extracts [3, 50–52].

Serum biochemical parameters are shown in Table 3. Results show that after the swimming test, blood glucose levels were significantly and dose-dependently higher ($P < 0.05$–$P < 0.01$) in the extract-treated groups compared with the controls. On the contrary, blood lactate levels were significantly ($P < 0.05$–$P < 0.01$) and dose-dependently reduced (20.9–36.7%) by extract treatment compared with the controls. Blood lactate is the glycolysis product of carbohydrates under anaerobic conditions and glycolysis is the main energy source for intense exercise over a short time. Therefore, lactate concentrations serve as indicators for judging the intensity of the exercise or the degree of fatigue. With the accumulation of blood lactate, blood and muscle tissue pH reduces, a condition which is harmful to some vital organs and which also causes fatigue [55–57]. Blood lactate levels are therefore representative of the degree of postexercise fatigue and the condition of recovery [44, 58]. Antifatigue agents have been shown to effectively work by delaying lactate accumulation either by reducing the glycolytic process or by increasing the rate of removal of blood lactate [56, 59]. The leaf extract of *M. oleifera* could effectively delay the onset of fatigue through one or both of these mechanisms.

The importance of muscle glycogen levels in endurance exercise has been demonstrated and it is suggested that depletion of muscle glycogen is an important factor in fatigue and exhaustion [60]. However, there is evidence that energy provision for intense prolonged aerobic muscular work relies mainly on fat utilization [61]. In this study, plasma TG levels were significantly lowered ($P < 0.05$) in all extract-treated groups compared with the controls, while extract treatment raised blood glucose levels (Table 3). In addition, following the swim test, extract-treated rats at all dose levels maintained significantly higher concentrations of muscle and hepatic glycogen compared with the controls (Table 4). Hepatic and muscle glycogen reserves constitute reliable determinants of fatigue on which endurance capacity relies [62], and the prolonged exercise-induced hypoglycemia can be harmful to nervous function [63, 64]. The concomitant significant drop in serum triglyceride concentrations and elevated blood glucose levels after endurance exercise suggest that *M. oleifera* extract preferentially promoted the utilization of fat during prolonged exercise, a glycogen-sparing mechanism that delays the onset of fatigue [65, 66]. There is experimental evidence that endurance can be improved by increasing the availability of fatty acids and that this effect is mediated by a slowing of glycogen depletion [67].

Blood urea nitrogen (BUN) levels are shown in Table 3. BUN concentrations of extract-treated rats (26.4–28.2 mg/dL) were significantly lower ($P < 0.01$) following the experimental swimming exercise compared with controls (45.0 mg/dL). The positive correlation between

Table 3: Effect of *M. oleifera* extract on rat serum biochemical parameters.

	Control	*M. oleifera* (100 mg/kg)	*M. oleifera* (200 mg/kg)	*M. oleifera* (400 mg/kg)
Glycemia (mg/dL)	119.9 ± 24.87	145.01 ± 16.25*	147.4 ± 21.35*	163.9 ± 16.10***
Lactamia (mmol/L)	31.27 ± 3.21	24.14 ± 2.41*	19.37 ± 4.53***	18.87 ± 6.77***
Urea (mg/dL)	45.03 ± 13.26	26.78 ± 8.77**	26.41 ± 8.19**	28.21 ± 9.37**
Triglycerides (mg/dL)	176.77 ± 26.61	75.05 ± 7.63***	125.28 ± 12.40***	58.42 ± 7.22***

Each value represents the mean ± SEM, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control group.

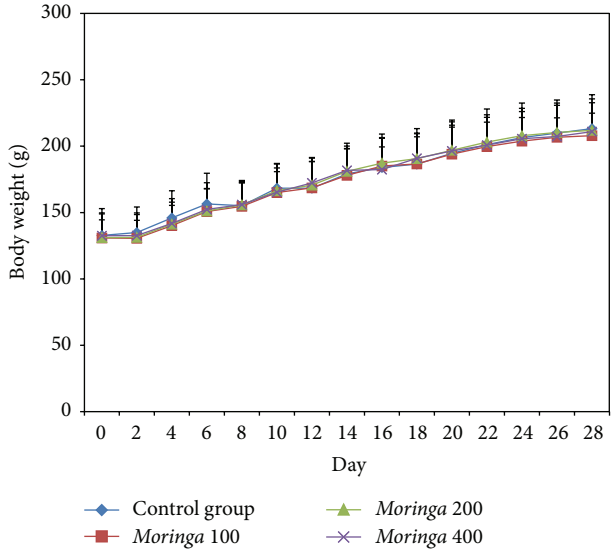

Figure 1: Body weight change of the rats during the experimentation. The values are expressed as mean ± SEM, $n = 16$. Control group = rats treated with distilled water; *Moringa* 100, *Moringa* 200, and *Moringa* 400 group = rats treated with 100, 200, and 400 mg/kg, respectively, of *Moringa oleifera* extract.

Table 4: Effect of *M. oleifera* extract on rat hepatic and muscle glycogen.

Groups	Glycogen (mg/g)	
	Liver	Muscle
Control	17.68 ± 1.74	1.19 ± 0.46
M. oleifera (100 mg/kg)	21.57 ± 2.45*	2.06 ± 0.77*
M. oleifera (200 mg/kg)	22.69 ± 3.44**	2.76 ± 0.78***
M. oleifera (400 mg/kg)	22.64 ± 3.69**	2.68 ± 0.68***

Each value represents the mean ± SEM, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control group.

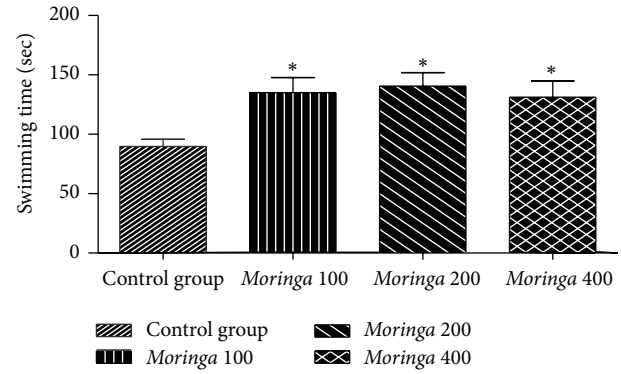

Figure 2: Effect of the *M. oleifera* aqueous extract on swimming time in rats. Data are presented as mean ± SEM, $n = 8$. Control group = rats treated with distilled water; *Moringa* 100, *Moringa* 200, and *Moringa* 400 group = rats treated with 100, 200 and 400 mg/kg of *M. oleifera* extract, respectively. $^{*}P < 0.05$ compared with control group.

BUN levels and the degree of exercise tolerance is well known [60, 68, 69]. The less adapted or tolerant the body is to prolonged exercise, the more significant the rise in BUN levels following protracted exercise [70, 71]. The results therefore suggest that treatment with *M. oleifera* extract for 28 days can contribute to fatigue retardation by reducing hepatic amino acid and protein catabolism during exercise.

Intense physical exercise also causes oxidative stress in the body due to excessive generation of oxygen-derived free radicals. During exercise, a large amount of oxygen is consumed and 4-5% of the total oxygen consumed during respiration is incompletely reduced to water and therefore results in the acceleration of free radical generation. These radicals, in turn, oxidatively degrade biomolecules such as lipids, proteins, and nucleic acids and therefore affect the homeostatic environment of cells. A vast amount of evidence indicates that reactive oxygen species (ROS) are responsible for exercise-induced protein oxidation and contribute strongly to muscle fatigue [72]. As shown in Table 5, MDA concentrations in liver and gastrocnemius muscle of rats treated with *Moringa* extract were significantly lower ($P < 0.05$) compared with the controls, while the activities of SOD, GPx, and CAT in liver and gastrocnemius muscle of control rats were significantly lower ($P < 0.05$) compared with the extract-treated groups. MDA is one of the degradation products in the lipid peroxidation process [71]. Earlier studies have shown that lipid peroxidation in liver and muscle tissues increases during intense physical exercise [44]. Peroxidation is an important indicator of oxidative stress that results from degradation of cell membrane by free radicals. The results of the present study indicated the antiperoxidation capacity of *M. oleifera* extract. Enzymatic antioxidant systems, such as GPx, SOD, and CAT, are important in scavenging free radicals and their metabolites [73]. SOD protects cells by catalyzing the conversion of superoxide radicals to O_2 and H_2O_2. This toxic H_2O_2 is further decomposed into O_2 and H_2O by catalase. GPx catalyzes the reduction of hydroperoxides by glutathione. These antioxidant defense mechanisms become weaker during chronic fatigue and other disease

Table 5: Effect of *M. oleifera* extract on rat hepatic and muscle antioxidant parameters.

	Control	*M. oleifera* (100 mg/kg)	*M. oleifera* (200 mg/kg)	*M. oleifera* (400 mg/kg)
Hepatic parameters				
SOD (U/mg protein)	31.09 ± 3.15	35.35 ± 2.61*	38.42 ± 3.22***	39.11 ± 3.48***
MDA (μmol/g)	52.00 ± 5.66	42.13 ± 3.64***	41.63 ± 4.91***	42.32 ± 4.43***
CAT (U/mg protein)	25.13 ± 2.17	27.52 ± 1.64	29.33 ± 1.92**	28.34 ± 2.67*
GPx (μmol/mg protein)	3.62 ± 0.71	5.63 ± 1.13**	6.22 ± 1.12***	6.95 ± 1.74***
Muscle parameters				
SOD (U/mg protein)	6.55 ± 0.98	8.95 ± 1.06***	9.23 ± 1.54***	9.12 ± 0.87***
MDA (μmol/g)	33.24 ± 5.24	24.13 ± 4.57**	22.28 ± 4.63***	22.14 ± 4.54***
CAT (U/mg protein)	0.27 ± 0.05	0.32 ± 0.04	0.38 ± 0.03***	0.39 ± 0.05***
GLU (μmol/mg de protein)	0.88 ± 0.37	2.98 ± 0.26**	4.09 ± 1.97***	3.75 ± 1.04***

Each value represents the mean ± SEM, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control group.

Table 6: Effect of *M. oleifera* aqueous extract on hematological parameters of rats.

	Control	*M. oleifera* (100 mg/kg)	*M. oleifera* (200 mg/kg)	*M. oleifera* (400 mg/kg)
WBC $\times 10^3/mm^3$	10.70 ± 4.45	9.08 ± 1.67	9.60 ± 2.47	11.32 ± 4.31
RBC $\times 10^6/mm^3$	6.52 ± 0.25	6.57 ± 0.70	6.13 ± 0.16	6.3575 ± 0.2
Hb (g/dL)	12.30 ± 1.01	13.00 ± 0.22*	12.38 ± 0.262	12.225 ± 0.29
HCT (%)	35.12 ± 0.22	33.82 ± 0.85	32.55 ± 1.35**	34.2 ± 1.09
PLA $\times 10^3/mm^3$	559.25 ± 40.79	543.75 ± 189.50	649.25 ± 113.84	497.75 ± 58.26
%LYM (%)	54.82 ± 23.35	70.87 ± 6.42*	63.7 ± 7.44	62.17 ± 3.55
%MON (%)	10.13 ± 5.05	13.72 ± 4.19	13.15 ± 2.92	14.02 ± 1.27
%GRA (%)	24.88 ± 6.70	15.4 ± 3.75*	23.15 ± 7.75	23.8 ± 4.75
LYM $\times 10^3/mm^3$	8.10 ± 2.20	6.30 ± 0.70*	6.05 ± 1.61*	5.43 ± 1.03
MON $\times 10^3/mm^3$	1.20 ± 0.89	1.22 ± 0.58	1.17 ± 0.22	1.17 ± 0.27
GRA $\times 10^3/mm^3$	3.00 ± 1.73	1.55 ± 0.50*	2.37 ± 1.26	2.22 ± 0.61

Each value represents the mean ± SEM, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.0.1$ compared with control group.

conditions [1, 72]. Thus, the improvement in the activities of these defense mechanisms can help to fight against fatigue. Our results indicated that the antifatigue effect of *M. oleifera* extract probably occurs through protection of corpuscular membranes by preventing lipid oxidation via modifying activities of several enzymes. These results are in accordance with the findings by Wang and Yan [74], which demonstrated similar effects of ginseng polysaccharides on MDA and GPx levels. Extract effect was maximal at doses 100, 200, and 400 mg/kg, respectively, for blood hemoglobin (Table 6), hepatic/muscle glycogen (Table 4), and blood glucose concentrations (Table 3). However, the dose-dependent increase in blood glucose levels was not translated into a corresponding dose-dependent increase in maximum swimming time which peaked at dose 200 mg/kg. This limited response may be explained by the effect of extract on hepatic and muscle antioxidant parameters which peaked at dose 200 mg/kg (Table 5). These results respond to the notion that antioxidants can paradoxically become prooxidant when administered at excessive doses. It is for this reason that food-derived antioxidants are preferably taken in the form of a composite mixture of many antioxidants with complimentary activity rather than a massive supply of a single antioxidant [75]. The results obtained here may therefore reflect an excessively massive import of a high concentration of one or more potent antioxidant components of the extract at the dose of 400 mg/kg.

Hematological parameters of the rats measured after exercise are presented in Table 6. There were significant increases ($P < 0.05$) in hemoglobin (Hb) and percentage lymphocytes in rats given 100 mg/kg of extract compared with the controls. Hemoglobin is the main component of erythrocytes whose main function is to serve as the carrier for oxygen and carbon dioxide. Hb also plays a role in the maintenance of the body fluid's acid/alkali balance [76]. Therefore, it can directly affect energy metabolism, body function, and exercise ability, the loading capacity of the exercise and consequently fatigue [77]. Hb normally is one of the indicators that reflect the degree of recovery from fatigue after exercise, and higher levels of Hb can improve exercise ability [44]. Our results are in accordance with the findings by Okwari et al. [78], who demonstrated similar effects of the leaf aqueous extract of *M. oleifera* on hemoglobin levels in rats subjected to thermooxidized palm oil diet-induced toxicity. Table 6 also shows that extract treatment also decreased hematocrit, percentage of granulocyte, and lymphocytes at 100 and 200 mg/kg doses ($P < 0.05$). Many studies describe changes induced by physical exercise on subtypes of blood mononuclear cells (neutrophiles, lymphocytes, and monocytes) [79, 80]. In general, during and immediately

after intense exercise, total circulating numbers of leucocytes (polynuclear and mononuclear) increase in proportion to the intensity and the duration of the exercise [81] but disappear 24 hours after the exercise.

In conclusion, the leaf aqueous extract of *M. oleifera* possesses antifatigue properties. It improved the swimming ability of rats by delaying the accumulation of blood lactate and blood urea nitrogen, by increasing the mobilization and use of body fats, and by slowing the depletion of glycogen stores. The antifatigue potential may be expressed through mechanisms that involve the antioxidant activity of the extract. Further studies are needed to determine the effect of the extract on chronic physical activity.

Conflict of Interests

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

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Research Article

Phenolic Rich Extract from *Clinacanthus nutans* Attenuates Hyperlipidemia-Associated Oxidative Stress in Rats

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Clinacanthus nutans is used as traditional medicine in Asia but there are limited scientific studies to support its use. In this study, the stem and leaf of *C. nutans* were extracted using solvents of differing polarities, and their antioxidant capacities were determined using multiple antioxidant assays. The water and aqueous methanolic leaf extracts were further fractionated and their antioxidant capacities and phenolic compositions were tested. Furthermore, the efficacies of the water and aqueous methanolic leaf extracts were tested against hyperlipidemia-induced oxidative stress in rats. Serum and hepatic antioxidant and oxidative stress markers were tested after feeding the rats with high fat diet together with the extracts or simvastatin for 7 weeks. The results indicated that both leaf extracts attenuated oxidative stress through increasing serum antioxidant enzymes activity and upregulating the expression of hepatic antioxidant genes. Multiple phenolic compounds were detected in the extracts and fractions of *C. nutans*, although protocatechuic acid was one of the most abundant and may have contributed significantly towards the bioactivities of the extracts. However, synergistic effects of different phenolics may have contributed to the overall bioactivities. *C. nutans* can be a good source of functional ingredients for the management of oxidative stress-related diseases.

1. Introduction

Hypercholesterolemia is a lipoprotein metabolic disorder characterized by altered metabolism of cholesterol, which promotes the production of reactive oxygen species (ROS) through modulation of the activities of enzymes like NADPH oxidase and xanthine oxidase. The altered activities of these enzymes have been demonstrated to disturb the endothelial superoxide anion production resulting in c-Jun-N-terminal kinase-mediated inactivation of endothelium-derived nitric oxide and subsequent increases in oxygen radical production and inflammation [1–3]. Conversely, oxidative stress due to an imbalance between the generation of ROS and the endogenous antioxidant systems has been reported to lead to increased lipid peroxidation, which in turn is involved in the etiology of several chronic diseases such as cardiovascular diseases (CVD), diabetes, obesity, and cancer [4].

Antioxidants attenuate or inhibit the oxidation of lipids or other biomolecules and thus prevent or repair the damage to body cells due to oxidation by free radical species [5]. Free radical species can be neutralized through dismutation or reduction by endogenous antioxidants like superoxide dismutase (SOD) and catalase, respectively, and through direct scavenging or electron transfer by exogenous antioxidants like vitamins C and E, respectively [6]. Additionally, plant bioactive compounds including polyphenols and flavonoids have been demonstrated to attenuate oxidative stress through multiple mechanisms including the regulation of free radical-induced oxidation of biomolecules [7]. Moreover, these plants have received heightened attention due to their perceived

cost-effectiveness and lesser side effects compared to synthetic pharmaceutical agents [8]. Additionally, the presence of phenolic compounds correlates with the antioxidant activities of plants and thus there have been suggestions that antioxidant tests can be used as the major determinants for evaluating the antioxidant potentials of herbs [9]. Phenolic compounds are also known for their wide range of physiological properties including cardioprotection, anticancer, and neuroprotection [7].

Clinacanthus nutans belongs to the family of Acanthaceae and is widely used in Thailand and Indonesia as traditional medicine. In fact, it is categorized as an essential medicinal plant for primary health care of the Thai Ministry of Public Health [10]. This plant has been traditionally used in Asia to treat oxidative stress-related diseases such as CVD, diabetes, and various kinds of cancers. It is not surprising, therefore, that in recent years its extracts have been demonstrated to have blood glucose-lowering [11], antioxidant [12, 13], antiproliferative [13], and anti-inflammatory effects [14]. Numerous bioactives have been demonstrated in *C. nutans*, including phenolics, flavonoids, β-sitosterol, stigmasterol, and chlorophyll derivatives [15]. However, an extensive investigation of its phenolic compounds and their correlation with the antioxidative effects of this plant has not been demonstrated. This is important since the plant has potent antioxidative potentials likely mediated by its phenolics [12, 13]. Furthermore, the preparation of bioactive-rich fractions, having a lead compound and lesser amounts of other bioactive compounds, has conveniently gained momentum due to possible synergistic actions of different bioactives compared with the effects of individual bioactive compounds [16].

Thus, we prepared protocatechuic acid- (PCA-) rich fractions from *C. nutans*, followed by the determinations of their antioxidant activities and compositional analyses. We also evaluated their effects against oxidative stress in hypercholesterolemic rats. The findings could pave the way for development of functional ingredients from *C. nutans* for management of oxidative stress associated with hypercholesterolemia.

2. Materials and Methods

2.1. Reagents and Chemicals. Chemicals used for extraction including hexane, methanol, and ethyl acetate were of analytical grade and purchased from Thermo Fisher Scientific (Massachusetts, USA). The compounds, 2,2-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu's phenol reagent, potassium ferricyanide ($K_3[Fe(CN)_6]$), sodium dodecyl sulphate (SDS), ferric chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), 2,2′-azinothiazoline-6-sulphonic acid (ABTS), and potassium persulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenolic acid standards (Vanillic, protocatechuic acid, Cinnamic acid, Chlorogenic, Gallic, Caffeic, and *p*-Coumaric), hydrochloric acid (37%) (HCl), and trolox were obtained from Sigma-Aldrich (Hamburg, Germany), while methanol, acetic acid, acetonitrile, and phosphoric acid used in the HPLC analyses were of HPLC grade and purchased from Thermo Fisher Scientific, (Loughborough, and Leicestershire, UK).

2.2. Collection of Plant Materials and Sample Preparation. Twenty kg of *C. nutans* was collected randomly from three different parts of the *C. nutans* garden at the YPL Herbal Farm, Jelebu, Seremban, Negeri Sembilan, Malaysia. Authentication was made by Dr. Shamsul Khamis at the Biodiversity unit of the Institute of Bioscience, Universiti Putra Malaysia, where the voucher specimen SK 2002/12 was deposited. The collected plant was washed thoroughly with running tap water and repeatedly with deionized water. The leaves and stem were individually freeze-dried (VirTis benchtop K, Bieleveld, Germany) and stored at −80°C prior to further analyses.

2.3. Solvent Extraction and Fractionation of Crude Extracts. The leaf and stem of *C. nutans* were, respectively, pulverised into fine powder using a stainless steel blender (Waring Commercial, Torrington, CT, USA) and passed through a mesh opening of 35 mm sieve. The leaf and stem were separately mixed with solvents of differing polarities: hexane, ethyl acetate, 80% methanol, water, and hot water (70°C) at the ratio of 1 : 10 (w/v). Then, these mixtures were sonicated for 60 min at 25°C in an ultrasonicator water bath (Power sonic 505, Hwa Shin Technology Co., Seoul, Korea). The mixtures were then individually filtered through Whatman filter paper No. 1 and the entire extraction process was repeated twice on the residue obtained from the previous filtration process. Subsequently, solvents were removed under reduced pressure (Rotavapor R210, Buchi, Flawil, Switzerland) followed by lyophilization (Virtis Benchtop K Freeze Dryer, SP Industries, Warminster, PA, USA). Following antioxidant activity tests (as detailed below), the hot water (AL) and 80% methanolic (AML) leaf extracts of *C. nutans* showed the best activities, and thus they were further fractionated as depicted in Figure 1. Briefly, the crude extracts (10 g of AML and AL each) were dispersed separately into 100 mL of double distilled water, and the solutions were partitioned with 100 mL of n-hexane by stirring for 60 min at room temperature to remove any residual lipids. Subsequently, the mixtures were left at room temperature until both solvent layers were well-separated from each other. After separation from the n-hexane layer, the aqueous layer was homogenized with 100 mL of ethyl acetate by magnetic stirrer for 60 min at room temperature. Consequently, the mixtures were left at room temperature until both solvent layers were well-separated from each other. Then, the aqueous layer after partitioning with n-hexane and ethyl acetate was homogenized with 100 mL of n-butanol. The partitioning procedure was repeated twice at each step and the solvents were pooled and concentrated to dryness under reduced pressure (Rotavapor R210, Buchi, Flawil, Switzerland). The remaining aqueous fraction (Aq) was subjected to lyophilization (Virtis Benchtop K Freeze Dryer, SP Industries, Warminster, PA, USA). The dried crude extracts and the fractions were stored at −80°C until further analysis.

2.4. Determination of Total Phenolic Contents (TPC) of Crude Extracts. The TPC were determined using Folin-Ciocalteu reagent as described by Ainsworth and Gillespie [17], and

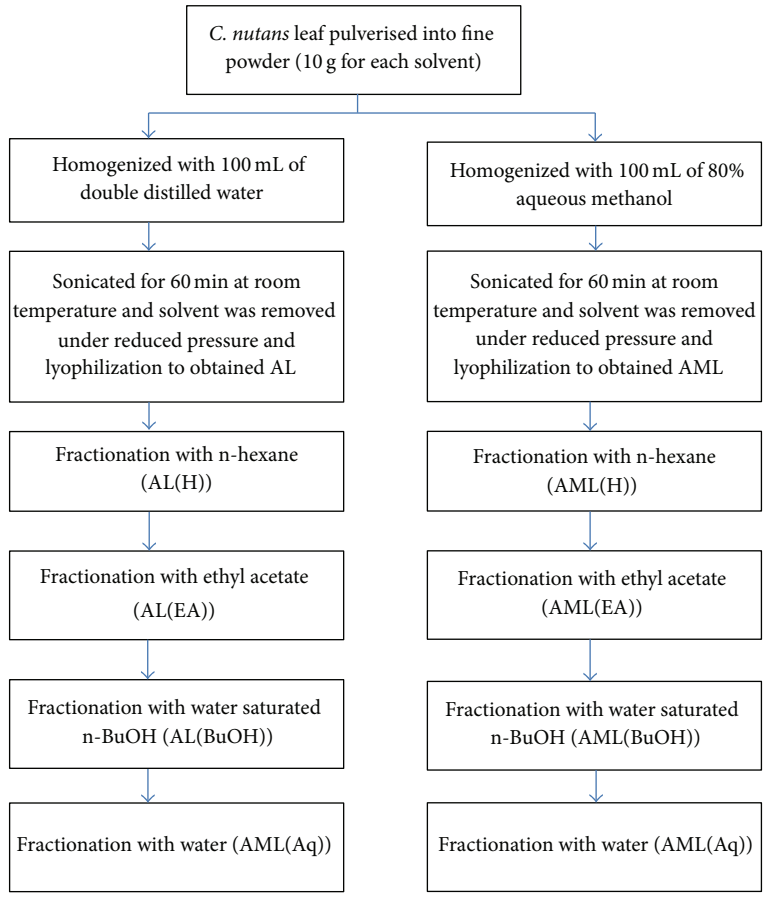

Figure 1: Flow chart showing the preparation of crude leaf extracts of *Clinacanthus nutans* (AL and AML), which were defatted using hexane (AL[H], AML[H]), ethyl acetate (AL[EA] and AML[EA]), and n-butanol (AL[BuOH] and AML[BuOH]), to obtain the final aqueous fractions (AL[Aq] and AML[Aq]).

the results ($n = 3$) were expressed as milligram Gallic acid equivalent per gram extract (mg GAE/g extract).

2.5. Antioxidant Activity Assays of Crude Extracts and Fractions of the AL and AML Extracts

2.5.1. DPPH Free Radical Scavenging Activity. The antioxidant activities of the extracts and fractions were determined using the DPPH radical assay according to the method described by Chan et al. [18], with Trolox as the standard. The radical scavenging activity of each extract was expressed as percentage of activity over the blank ($n = 3$).

2.5.2. ABTS Radical Cation-Scavenging Activity. The ABTS free radical scavenging activity of each sample was determined as previously described by Iqbal et al. [19], and the results ($n = 3$) were expressed as milligram Trolox equivalents per gram extract (mg TE/g extract).

2.5.3. Ferric Reducing Antioxidant Power (FRAP). Total reducing capacity was determined by using ferricyanide method as described by Berker et al. [20], and the results ($n = 3$) were expressed as milligram Gallic acid equivalents in 1 gram extract (mg GAE/g extract).

2.6. Analysis of Selected Phenolic Compounds in Crude Extracts and Fractions of the AL and AML Extracts by HPLC-DAD. HPLC analysis was performed using Agilent G1310A pump linked with diode array detector (Agilent, Stevens Creek Blvd Santa Clara, USA). Chromatographic separations were performed on a LUNA C-18 column (5 mm, 250 × 4.6 mm) (Phenomenex, Torrance, CA, USA). The solvent composition and gradient elution conditions were described previously by Mariod et al. [21] with some modifications. The mobile phase was composed of solvent (A) water-acetic acid (94 : 6, v/v, pH 2.27) and solvent (B) acetonitrile. The solvent gradient was as follows: 0–15% B in 40 min, 15–45% B in 40 min, and 45–100% B in 10 min. A flow rate of 0.5 mL/min was used and 20 μL of sample was injected. Samples and mobile phases were filtered through a 0.22 μm Millipore filter, type GV (Millipore, Bedford, MA) prior to HPLC injection. Each extract and fraction were analyzed in three replicates. The standards used were protocatechuic acid, Ferulic acid, Gallic acid, *p*-Coumaric, Chlorogenic acid, Vanillic acid, and Caffeic acid and were measured at 320 nm. Phenolic compounds were identified and quantified by comparing their retention times with authenticated standards.

2.7. Animal Handling and Feeding. The high fat and high cholesterol (HFHC) diet was formulated according to Imam

et al. [22], with minor modifications. Every kg of HFHC formulation contained 500 g ground standard rat chow, 25 g of cholesterol, 200 mL palm oil (instead of corn oil), 60 g fine sugar, 200 g Nespray full cream milk, and 50 g of starch (to cement the pellet together). The HFHC diet was dried in an oven at 60°C for 24 hours, cut into small equal-sized pieces, and fed to the rats to induce hypercholesterolemia.

Furthermore, healthy male Sprague-Dawley rats weighing about 200 g–250 g were housed in large stainless steel spacious cages, with free access to food and water. The animal house was ventilated with a 12-hour light/dark cycle at the ambient temperature of 25–30°C, throughout the experimental period. Rats were allowed to adapt to their environment for at least 10 days before the initiation of experiment. All experiments and protocols described in the study were approved by the Animal Ethics Committee (Project approval number: UPM/FPSK/PADS/BR-UUH/00484) of the Faculty of Medicine and Health Science, Universiti Putra Malaysia, Malaysia. The rats were randomly divided into nine groups of seven rats each; the normal control (NC) received normal pellet, while the control group received HFHC and the STATIN groups received HFHC + oral gavage of 10 mg/kg/day simvastatin. The aqueous leaf extract (AL) and aqueous methanolic leaf extract (AML) groups were given HFHC + oral gavage of 500, 250, or 125 mg/kg/day/rat of the respective extracts. At the end of the experimental period (49 days), the animals were fasted overnight and sacrificed by dissection method. The liver was excised immediately and washed with ice-cold saline prior to storage in RCL2 Solution (ALPHELYS, France) at −80°C. Blood was collected by cardiac puncture after an overnight fast and centrifuged at 3000 rpm for 10 min at 4°C to separate the serum.

2.8. Antioxidant Markers

2.8.1. Serum Antioxidant Markers. Serum total antioxidant status (TAS), glutathione peroxidase (GPx), and SOD were analyzed using Randox analytical kits according to the manufacturer's instructions using Selectra XL instrument (Vita Scientiĕc, Dieren, Netherlands).

2.8.2. Liver Electron Spin Resonance (ESR) Spectroscopy. The hepatic antioxidant capacities of rats against hydroxyl radical was measured using ESR spectrometer (Jeil FA100; Tokyo, Japan) as described by Imam et al. [22], and DMSO was used as standard.

2.9. Oxidative Stress Markers

2.9.1. Thiobarbituric Acid Reactive Substances (TBARS). TBARS was determined using the method described by Chan et al. [23], and MDA was used as the standard ($y = 0.1982x - 0.1898$, $R^2 = 0.9947$).

2.9.2. F_2-Isoprostane. Serum from blood collected in plain tubes was used for measurements of Serum F_2-isoprostane using the respective ELISA kits according to the manufacturers' instructions. Absorbances were read on BioTeK Synergy H1 Hybrid Reader (BioTek Instruments Inc., Winooski, VT, USA) at the appropriate wavelengths (450 nm). The results were analyzed on http://www.myassays.com/ using four-parametric test curve; F_2-isoprostane ($R^2 = 1$).

2.10. Hepatic mRNA Expression Level. Hepatic RNA was isolated using the Total RNA Isolation Kit (Vivantis, Malaysia) according to the kit protocol. Primers were designed on the GenomeLab eXpress Profiler software using *Rattus norvegicus* sequences adopted from the National Center for Biotechnology Information GenBank Database (http://www.ncbi.nlm.nih.gov/nucleotide/). The genes of interest, housekeeping genes, and an internal control are shown in Table 1. The forward and reverse primers had universal sequences (tags) in addition to nucleotides that were complementary to the target genes. Primers were supplied by First Base Ltd. (Selangor, Malaysia) and diluted in 1x Tris-EDTA buffer. Reverse transcription and multiplex PCR of RNA samples (50 ng each) were done in an XP Thermal Cycler (BIOER Technology, Hangzhou, China) according to the kit protocol, while PCR products (1 μL each) were mixed with 38.5 μL of sample loading solution and 0.5 μL of DNA size standard 400 (Beckman Coulter, Inc., Miami, FL, USA) in a 96-well sample loading plate and analyzed in the GeXP machine (Beckman Coulter, Inc., Miami, FL, USA). The results were analyzed using the Fragment Analysis module of the GeXP system software and then imported into the analysis module of eXpress Profiler software. Normalization was done with GAPDH.

2.11. Statistical Analyses. Data were reported as mean ± standard deviation ($n = 3$ for antioxidant assays and 7 for animal study). Difference between each group was assessed by ANOVA accompanied by Duncan's multiple range test (SPSS for windows, version 17), and $p < 0.05$ was regarded as significant.

3. Results

3.1. Extraction Yield and TPC of Crude Extracts. The leaf and stem of *C. nutans* were extracted using solvents with differing polarities including hexane, ethyl acetate, 100% methanol, 80% methanol, hot water, and ambient water. The extraction yield increased with increasing solvent polarity, and the highest extraction yields were from the hot water leaf and stem extracts, while the lowest were the hexane leaf extract and ethyl acetate stem extract (Table 2). Generally, the leaf extracts showed higher TPC in comparison with the stem extracts (Table 2). The AML extract showed the highest TPC but was not significantly different from those of the water leaf extracts (HAL and AL), which were comparatively high ($p > 0.05$). A different trend was observed for the stem extracts, in which the EAS showed the highest TPC followed by the hot water extract (HAS), aqueous methanol extract (AMS), water extract (AS), 100% methanolic extract (MS), and hexane extract (HS).

3.2. Antioxidant Capacities and Phenolic Compositions of the Crude Leaf and Stem Extracts of C. nutans. As can be recalled, antioxidant activities were determined by DPPH, ABTS, and

Table 1: Gene name, accession number, and sequences of primers used in multiplex panel analysis.

Gene name		Primer sequence (with universal tag)	
		Forward primer	Reverse primer
CAT	NM_012520	AGGTGACACTATAGAATACATTCTATACGAAGGTGTTG	GTACGACTCACTATAGGGAGGTGTGAATTGCATTCTTAG
SOD1	NM_017050	AGGTGACACTATAGAATATCAATATGGGGACAATACAC	GTACGACTCACTATAGGGATACTTTCTTCATTTCCACCTT
SOD2	NM_017051	AGGTGACACTATAGAATAACTTTGGGTCTTTTGAGAA	GTACGACTCACTATAGGGATTCACTTCTTGCAAACTATG
GPx1	NM_030826	AGGTGACACTATAGAATAGGCAAGAATGAAGAGATTC	GTACGACTCACTATAGGGACTACCAGGAACTTCTCAAAG
GSR	NM_053906	AGGTGACACTATAGAATAAGCCTGGGGATAACCAGTGA	GTACGACTCACTATAGGGAAATGTAACCGGCACCCACAA
PPIA[a]	NM_017101	AGGTGACACTATAGAATATTCTGTAGCTCAGGAGAGCA	GTACGACTCACTATAGGGATTGAAGGGGAATGAGGAAAA
GAPDH[a,*]	NM_017008	AGGTGACACTATAGAATAATGACTCTACCCACGGCAAG	GTACGACTCACTATAGGGAAGCATCACCCCATTTGATGT
KanR[b]			

[a]Housekeeping gene. [b]Internal control. [*]Normalization gene. Reverse transcription (RT) and PCR were done according to manufacturer's instructions; RT reaction was at 48°C for 1 min; 37°C for 5 min; 42°C for 60 min; 95°C for 5 min and then held at 4°C, while PCR was as follows: initial denaturation at 95°C for 10 min, followed by two-step cycles of 94°C for 30 sec and 55°C for 30 sec, ending in a single-extension cycle of 68°C for 1 min. CAT: catalase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GPx: glutathione peroxidase; GSR: glutathione reductase; KanR: kanamycin resistant; PPIA: cyclophilin A; SOD: superoxide dismutase.

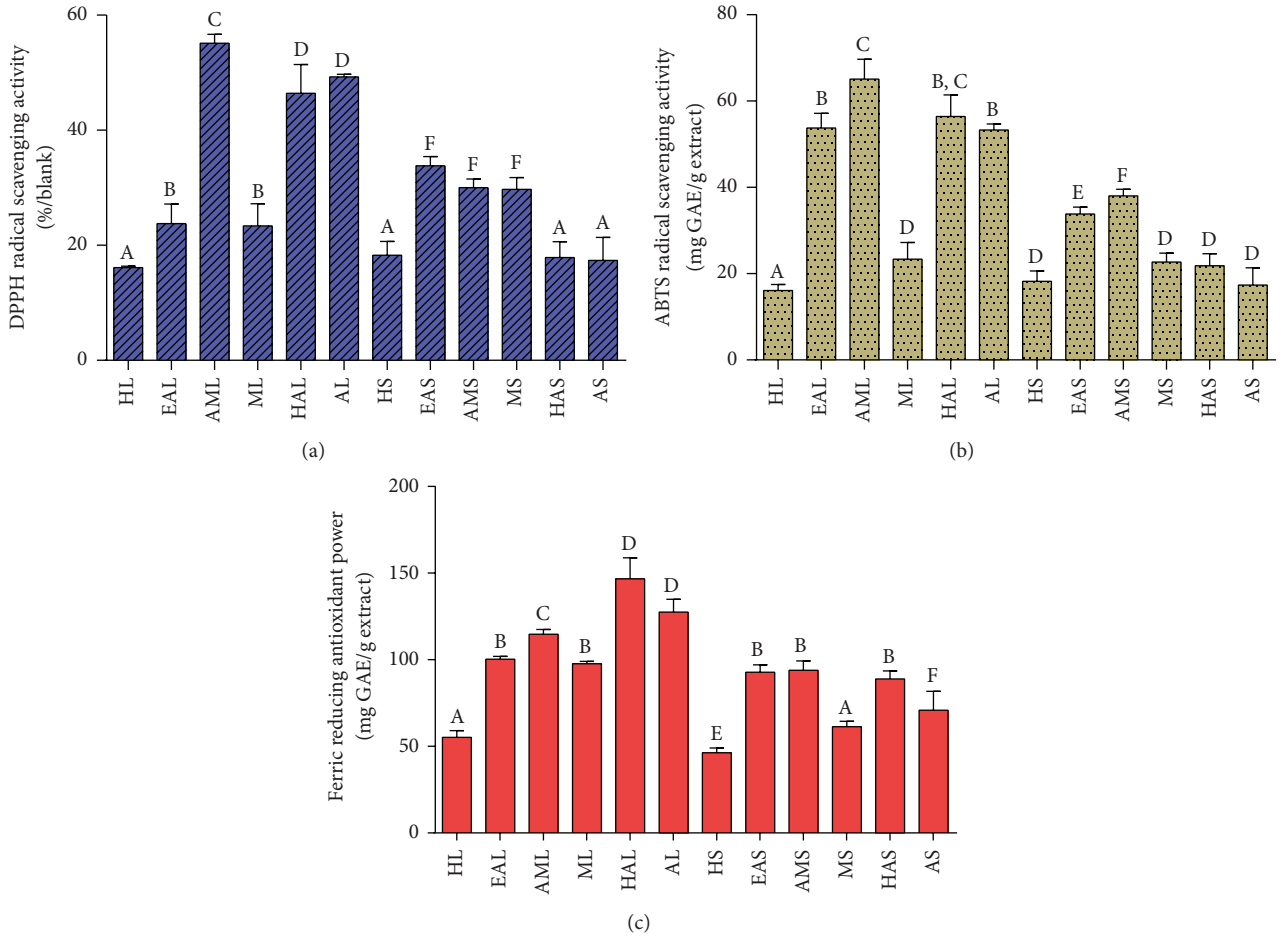

Figure 2: Antioxidant capacities of different solvent extracts of the leaf and stem of *Clinacanthus nutans* determined by (a) DPPH, (b) ABTS, and (c) FRAP Assays. Determinations for DPPH were expressed in percentage of radical scavenging activity over blank (%/blank), and those of ABTS assay were expressed as equivalent of Trolox, while, for FRAP assay, Gallic acid was used as calibration standard. Data are means of three replicates and are reported as mean ± standard deviation. Bars with different letters in each panel differ significantly ($p < 0.05$) according to Duncan's multiple range test. Groups are the same as Table 2.

FRAP assays and expressed as mg GAE or TE/g extract. The estimated values of antioxidant activities by DPPH radical varied from 16.12 to 55.12% (Figure 2(a)). The highest DPPH radical scavenging activity was exhibited by the AML extract followed by the AL, HAL, EAS, AMS, MS, EAL, ML, HS, HL, HAS, and HS extracts. A similar trend was observed for the ABTS radical scavenging activities of the leaf and stem extracts. However, the EAL extract showed good ABTS radical scavenging activity almost comparable to those of the HAL and AL extracts, in contrast to the results of the DPPH radical scavenging activity (Figure 2(b)). A different trend was observed for FRAP, whereby the HAL extract showed the highest activity followed by those of the AL, AML, EAL, ML, and other stem extracts (Figure 2(c)).

The three most potent extracts (in terms of antioxidant capacities), which were the HAL, AL, and AML extracts, were subjected to phenolic acid composition analyses by HPLC-DAD (Figure 3, Table 3). Eight phenolic acids were tested, including Cinnamic acid, PCA, Vanillic acid, Gallic acid, Caffeic acid, Ferulic acid, Chlorogenic acid, and *p*-Coumaric acid. In all the three extracts, PCA was detected to be the major phenolic acid, followed by Chlorogenic acid and trace amounts of Ferulic acid and Caffeic acid. However, *p*-Coumaric acid, Vanillic acid, and Gallic acid were not detected in all the 3 tested extracts. Cinnamic acid was detected in trace amounts in the AML and HAL extracts, but not detected in the AL extract. Furthermore, the TPC detected by HLPC-DAD were 432.50, 409.54, and 398.54 mg/g extract for AML, HAL, and AL, respectively.

3.3. Antioxidant Capacities and Phenolic Compositions of the AML and AL Fractions. For practical reasons, the two extracts (AML and AL) with the highest TPC, PCA, and antioxidant capacities were chosen for fractionation. The yield and TPC contents of the AL and AML fractions are presented in Table 4. The TPC was found to be high in all the tested fractions, with the AL(EA) fraction being the highest followed by the AML(EA), AL(BuOH), AML, AML(BuOH), AL, and aqueous fractions (AL(Aq) and AML(Aq)), while the lowest were the AL(H) and AML(H) fractions, respectively ($p < 0.05$).

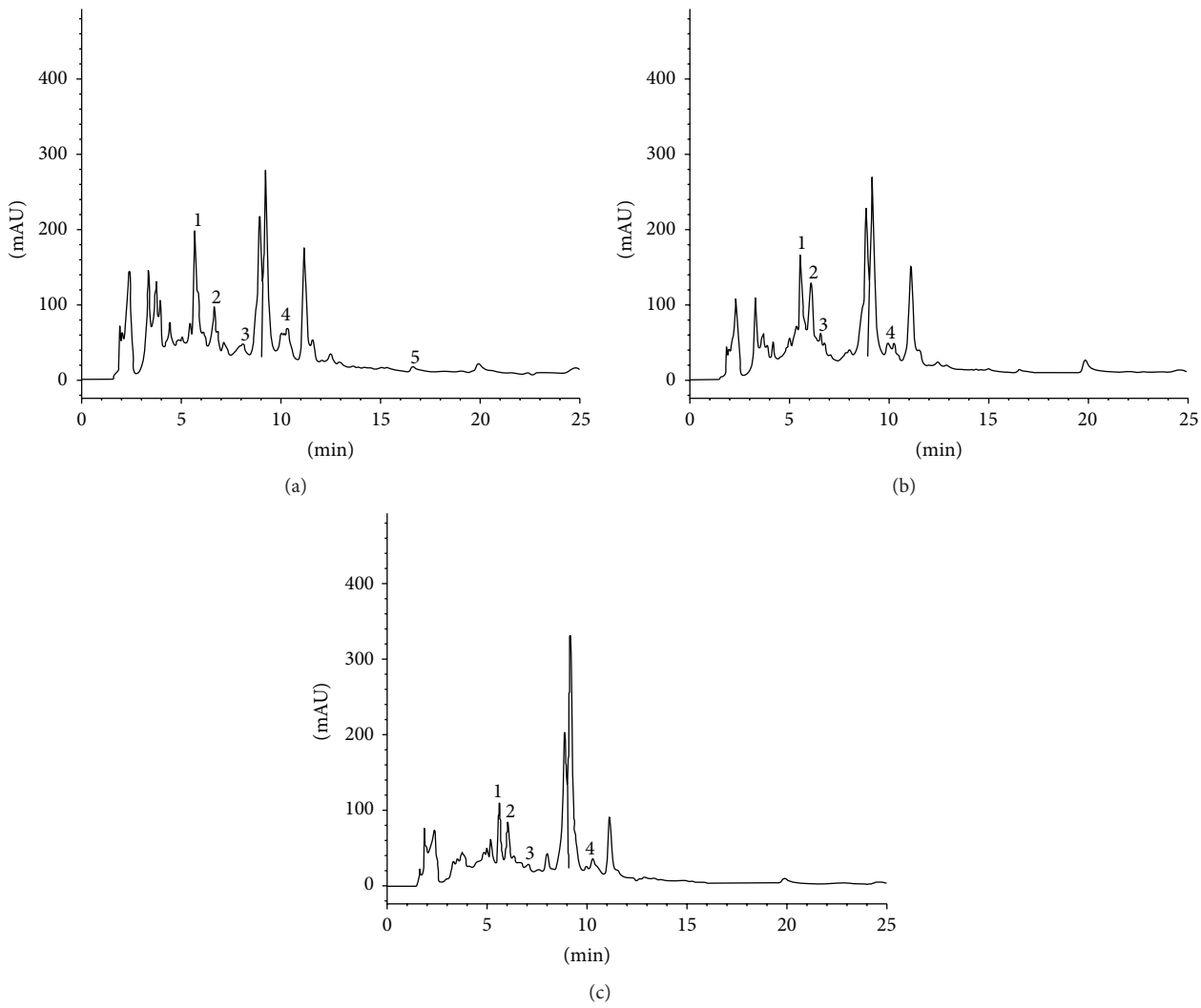

Figure 3: HPLC-DAD chromatogram of aqueous methanol leaf (a), aqueous leaf (b), and hot aqueous leaf (c) extract from *Clinacanthus nutans*. Peaks labelled with 1: protocatechuic acid, 2: Chlorogenic acid, 3: Caffeic acid, 4: Ferulic acid, and 5: Cinnamic acid.

Furthermore, the antioxidant capacities of the AL and AML fractions of *C. nutans* were determined using multiple assays based on different mechanistic principles (Figure 4). Specifically, the DPPH radical scavenging activities of *C. nutans* extracts (Figure 4(a)) were in the order of EA > BuOH > Aq > crude (AL and AML) > hexane ($p < 0.05$). The EA fractions of both AL (90.13 ± 3.13%) and AML (78.12 ± 2.35%) showed significantly higher DPPH radical scavenging activities than other fractions ($p < 0.05$). Interestingly, both EA-AL and EA-AML showed 2-fold higher DPPH radical scavenging activities compared with the crude extracts (AL and AML), respectively ($p < 0.05$). Similar patterns were observed for the ABTS radical scavenging activities (Figure 4(b)), whereby the EA fractions showed the most radical scavenging capacity compared with the other fractions ($p < 0.05$). In this case, the ABTS radical scavenging activity of the AL-EA fraction was 7-fold greater than that of the AL extract, whereas that of the AML-EA fraction was 3-fold greater compared to that of the AML extract ($p < 0.01$). No significant differences were observed between the EA fractions of the AL and AML extracts ($p > 0.05$). Different trends were observed for the FRAP of the *C. nutans* extracts (Figure 4(c)), in which the AL-BuOH fraction showed the highest activity followed by the AL-EA, AML-EA, AML-BuOH, AL, AML, AL-H, AL-Aq, AML-Aq, and AML(H) fractions ($p < 0.05$).

Similarly, the phenolic composition of the AML and AL fractions of the AL and AML were analysed on HPLC (Table 5). HPLC-DAD detected PCA, Cinnamic acid, Gallic acid, Caffeic acid, Ferulic acid, and *p*-Coumaric acid in the AL fractions, in contrast with the AL crude extract which had PCA, Chlorogenic acid, Caffeic acid, and Ferulic acid. Also, the AML fractions had Vanillic acid, Gallic acid, and *p*-Coumaric acid, which were not in the AML crude extract. Interestingly, both crude extracts of *C. nutans* showed high amounts of PCA (33.28 ± 0.01 mg/g extract), while the PCA concentrations in both EA fractions were significantly

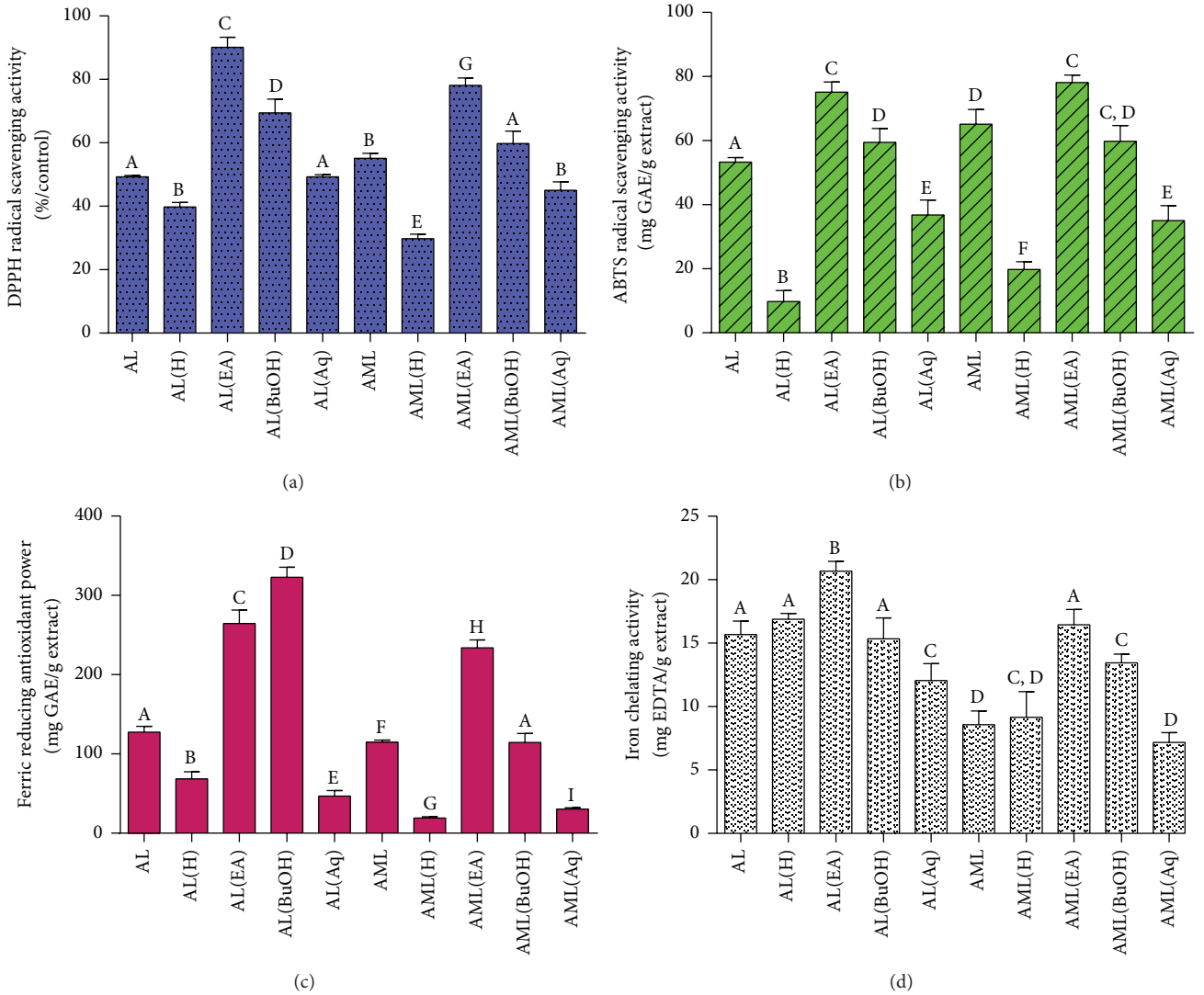

Figure 4: Antioxidant activities of crude leaf extracts and fractions of *C. nutans* determined by DPPH (a), ABTS (b), FRAP (c), and Iron Chelating (d). Determination for DPPH was expressed in percentage of radical scavenging activity over control (%/control), while, in ABTS and FRAP, Gallic acid and Trolox were used as calibration standards, respectively. Data are means of three replicates and data is reported as mean ± standard deviation. Bars with different letters (A–G) in each panel differ significantly ($p < 0.05$) according to Duncan's multiple range test. Groups are the same as Figure 1.

concentrated at around 6-fold higher for AL-EA and 3.6-fold higher for AML-EA in comparison with the respective AL and AML crude extracts ($p < 0.01$).

Results from various antioxidant assays and phenolic composition showed that the most active fractions of the AL and AML crude extracts were the EA fractions, which had PCA contents concentrated up to 6-fold in comparison with crude extracts, thus the choice of the name PCA-rich fraction.

3.4. Animal Study

3.4.1. C. nutans Extracts Slowed the Rate of Weight Gain Induced by HFHC Diet. Figure 5 shows the caloric intakes of the different animal groups throughout the experimental period. Food consumption (g/100 g/day) was significantly lower ($p < 0.05$) in the groups that received HFHC compared with the NC group, although the total calories consumed were the same for all groups. Weight changes are depicted in Figure 6, which showed progressive weight gain in all groups with time. In particular, the HFHC group showed significant increase in body weight gain throughout the intervention period, in comparison with the AL- and AML-treated experimental rats, which had dose-dependent reductions in weight gain ($p < 0.05$). Interestingly, at the end of the intervention period, the STATIN, AL(H), and AML(H) groups had the least weight gains ($p < 0.05$).

3.4.2. Lipid Profile Analysis. The lipid profiles obtained at the end of the intervention are shown in Table 6. From the results, it was observed that the HFHC caused significant elevation of the TC level by 202% compared with the NC group. On the other hand, there was significant reduction of TC by 37%,

Table 2: Extraction yield and total phenolic contents (TPC) of the crude leaf and stem extracts of *Clinacanthus nutans*.

Sample extracts	Extraction yield (g/100 g sample)	TPC (mg GAE/g extract)
HL	3.07 ± 0.14^{a}	27.60 ± 3.88^{a}
EAL	3.96 ± 0.97^{a}	50.14 ± 1.69^{b}
AML	19.6 ± 0.77^{b}	73.33 ± 12.18^{c}
ML	12.24 ± 0.74^{c}	48.84 ± 1.33^{b}
HAL	22.50 ± 1.85^{b}	69.73 ± 3.69^{c}
AL	20.51 ± 0.14^{b}	63.77 ± 7.31^{c}
HS	2.04 ± 0.16^{d}	23.15 ± 2.78^{a}
EAS	1.00 ± 0.07^{e}	52.91 ± 0.27^{b}
AMS	12.06 ± 0.97^{c}	40.91 ± 0.17^{c}
MS	11.45 ± 0.74^{c}	30.67 ± 3.07^{d}
HAS	22.30 ± 1.85^{b}	44.48 ± 4.64^{e}
AS	10.67 ± 0.14^{c}	$35.45 \pm 10.93^{a,c,d,e}$

Data for extraction yield and TPC are means of three replicates and data are reported as mean ± standard deviation ($n = 3$). Means within each column with different letters differ significantly ($p < 0.05$) according to Duncan's multiple range tests.

HL: hexane leaf extract; EAL: ethyl acetate leaf extract; AML: aqueous methanol leaf extract; ML: 100% methanol extract; HAL: hot aqueous leaf extract; AL: aqueous leaf extract; HS: hexane stem extract; EAS: ethyl acetate stem extract; AMS: aqueous methanol stem extract; MS: 100% methanol stem extract; HAS: hot aqueous stem extract; AS: aqueous stem extract.

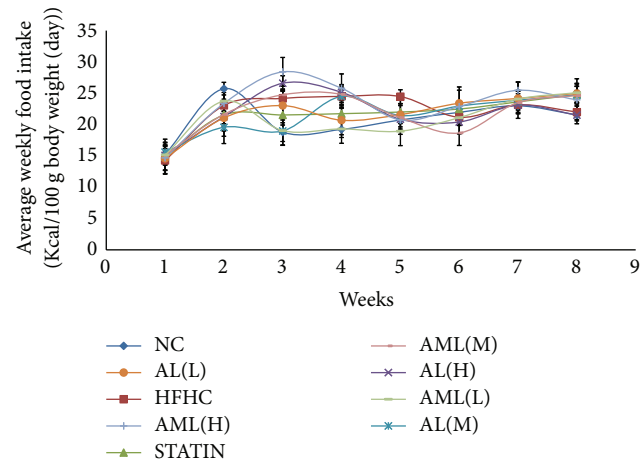

Figure 5: Average weekly food intake in high fat and high cholesterol fed rats after 7 weeks of intervention. Values are means ± SD ($n = 7$). NC: normal control group; HFHC: high fat and high cholesterol group; STATIN: HFHC + simvastatin (10 mg/kg/day/rat); AL(H): HFHC + high dose aqueous leaf extract (500 mg/kg/day/rat); AL(M): HFHC + medium dose aqueous leaf extract (250 mg/kg/day/rat); AL(L): HFHC + low dose aqueous leaf extract (125 mg/kg/day/rat); AML(H): HFHC + high dose aqueous methanol leaf extract (500 mg/kg/day/rat); AML(M): HFHC + medium dose aqueous methanol leaf extract (250 mg/kg/day/rat); AML(L): HFHC + low dose aqueous methanol leaf extract (125 mg/kg/day/rat).

28%, and 18% in the STATIN, AML(H), and AL(H) groups, respectively, compared with the HFHC group. Furthermore, there was a 3-fold decrease in HDL of the HFHC group compared with the NC group. On the other hand, the treated groups showed significantly higher HDL levels compared with the HFHC group except the AL(M) and AL(L). The LDL levels were similarly elevated in the HFHC group by 184% compared with the NC group, and there were dose-dependent decreases in the AL groups, while the AML groups did not show such differences. The serum VLDL of the HFHC group was elevated by 92% compared with the NC group. The treated groups showed significant reductions in VLDL levels compared with the HFHC group, except for the AL(L) group. TG levels in the HFHC group were slightly higher than in the treated groups, although only the AML(H) had significantly lower level ($p < 0.05$), which was comparable to that of the NC group.

3.4.3. Serum and Hepatic Markers of Antioxidant Status. The consumption of the HFHC diet significantly reduced antioxidant capacity based on the serum TAS, SOD and GPx, and hepatic OH radical scavenging activity in comparison with the control group ($p < 0.05$) (Figure 7). The serum total antioxidant level was significantly elevated for the all the treated groups. Interestingly, antioxidant capacities for the high dose groups of the AL and AML extracts were as high as that of the NC group. There was a significant inhibition of the serum antioxidant status in the HFHC group, as shown by the reduced SOD activity, which was significantly enhanced in groups treated with *C. nutans* ($p < 0.05$) and higher than that of the simvastatin-treated group ($p < 0.05$). Similarly, the serum GPx activity was elevated in the *C. nutans*-treated groups compared with the HFHC group (except the AML(L) group). Additionally, the hepatic hydroxyl radical scavenging activity was evaluated, which indicated that long term HFHC diet reduced the scavenging activity by 2-fold in comparison with the NC group. *C. nutans*-treated groups showed significantly elevated hepatic hydroxyl radical scavenging activities especially in the AL(H) group.

3.4.4. Serum and Hepatic Markers of Oxidative Stress. The extent of lipid peroxidation in the liver was determined using the MDA content. This method was also used to evaluate the redox equilibrium in the rats fed with the HFHC diets. Hepatic MDA content in the HFHC group was elevated by 3.5-fold in comparison with the NC group (Figure 8(a)). All the treated groups showed significantly lower levels of hepatic MDA, dose-dependently, compared with the HFHC group ($p < 0.05$). Serum lipid peroxidation was determined by F_2-isoprostanes (Figure 8(b)). Figure 8(b) shows that the serum F_2-isoprostanes levels were increased approximately 4-fold in the HFHC group, while the treated groups showed significantly lower levels of F_2-isoprostanes except the AL(L) and AML(L) groups. The AL(H) group had the lowest level of F_2-isoprostanes.

3.4.5. Effects of C. nutans Crude Extracts on mRNA Levels of Antioxidant Genes. The mRNA levels of hepatic antioxidant genes (SOD 1, SOD 2, CAT, G-Px, and GSR) were studied using Multiplex GeXP genetic analysis system, with KanR

Table 3: Phenolic composition of the extracts from the leaf of *Clinacanthus nutans*.

Phenolic compound	Individual phenolic content in *C. nutans* extracts (mg/g extract)		
	Aqueous methanol leaf (AML)	Hot aqueous leaf (HAL)	Aqueous leaf (AL)
Cinnamic acid	0.64 ± 0.01^{a}	1.00 ± 0.02^{a}	ND
Protocatechuic acid	33.28 ± 0.01^{b}	33.28 ± 0.12^{b}	33.28 ± 0.12^{a}
Caffeic acid	3.62 ± 0.04^{c}	5.32 ± 0.09^{c}	5.11 ± 0.04^{b}
Ferulic acid	1.33 ± 0.02^{d}	10.39 ± 0.58^{d}	1.49 ± 0.06^{c}
Chlorogenic acid	21.38 ± 0.61^{e}	25.24 ± 5.14^{b}	22.84 ± 9.14^{d}
Total phenolic	60.25 ± 0.69	75.23 ± 0.58	62.72 ± 9.26

Data of phenolic compositions are means of three replicates and the data is reported as mean ($n = 3$) ± standard deviation. Means within each column labelled with different letters are significantly different ($p < 0.05$) according to Duncan's multiple range test. ND: non detected. Groups are the same as Table 2.

Table 4: Extraction yield and total phenolic contents (TPC) of the AL and AML fractions.

Sample fractions	Extraction yield (g/100 g sample)	Total phenolic content (TPC) (mg GAE/g extract)
AL	20.51 ± 0.14^{a}	63.77 ± 7.31^{a}
AL(H)	2.04 ± 0.16^{b}	13.15 ± 2.78^{b}
AL(EA)	1.00 ± 0.37^{c}	128.83 ± 0.58^{c}
AL(BuOH)	6.06 ± 0.97^{d}	80.91 ± 0.49^{d}
AL(Aq)	10.45 ± 0.74^{e}	38.67 ± 4.07^{e}
AML	19.60 ± 0.77^{a}	73.33 ± 12.18^{f}
AML(H)	2.07 ± 0.14^{b}	27.60 ± 3.88^{g}
AML(EA)	1.36 ± 0.97^{c}	120.14 ± 1.69^{h}
AML(BuOH)	3.06 ± 0.80^{e}	66.34 ± 3.69^{i}
AML(Aq)	12.24 ± 0.74^{f}	48.84 ± 1.33^{j}

Data of extraction yield and TPC is means of three replicates and data is reported as mean ± standard deviation. Means within each column with different letters differ significantly ($p < 0.05$) according to Duncan's multiple range tests. Groups are the same as Figure 1.

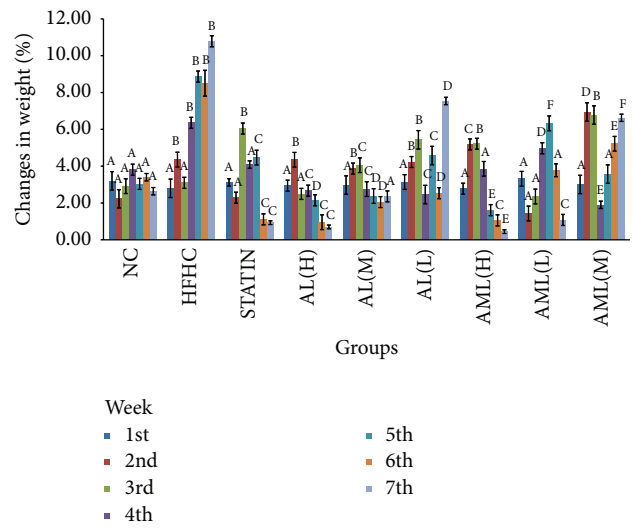

Figure 6: Weekly body weight changes in high fat and high cholesterol fed rats after 7 weeks of intervention. Values are means ± SD ($n = 7$). Bars representing weekly mean weights labelled with different letters (A–F) are significantly different between the groups ($p < 0.05$) according to Duncan's multiple range test. Groups are the same as Figure 5.

as the internal control. As shown in Figure 9, the AL and AML groups upregulated the expression of the antioxidant genes in comparison to the HFHC and NC groups. There was significant upregulation of the SOD 1 gene in the treated rats compared to the HFHC group ($p < 0.05$). Similarly, SOD 2 was upregulated in the treated groups although not different from the HFHC group except for the statin group ($p > 0.05$). The mRNA levels of CAT in the AML and AL groups were found to be higher than in the HFHC group, in a dose-dependent manner. Interestingly, the statin group showed a significantly higher level compared to the other groups. The expression levels of GPx in the AL-treated groups were significantly upregulated up to the level of the NC group. However, for the AML-treated group, even though the expression levels showed increases in a dose-dependent manner, no significant differences were observed among the different dose-treated groups ($p > 0.05$). A different trend was observed in the expression levels of GSR, whereby the AML groups showed upregulation in a dose-dependent manner, but only the AL(H) group was significantly different compared to the HFHC group.

4. Discussion

As can be recalled, based on the TPC results and those of other antioxidant assays (DPPH, ABTS, and FRAP), the AML, AL, and HAL extracts had the most antioxidant potentials, hence their detailed compositional analyses and subsequent use for the animal study. Moreover, numerous findings have shown that high phenolic content correlates with good antioxidant capacity and better attenuation of oxidative stress-related chronic diseases [5, 24]. Based on the strong antioxidant potentials, we hypothesized that the extracts would have potent antioxidative effects. Moreover, the phenolic compounds detected in the three most active extracts (HAL, AL, and AML), PCA, Chlorogenic acid, Cinnamic acid, Ferulic acid, Vanillic acid, Gallic acid, and Caffeic acid have all been reported to possess potent antioxidant properties. Accordingly, PCA has been demonstrated to prevent oxidative stress-related diseases such as CVD and

Table 5: Phenolic composition of the crude leaf extracts and fractions of *Clinacanthus nutans*.

Extract	Individual Phenolic Content in *C. nutans* extracts (mg/g extract)								
	Cinnamic acid	Protocatechuic	Vanillic acid	Gallic acid	Caffeic acid	Ferulic acid	Chlorogenic acid	*p*-Coumaric acid	Total phenolic content
AL	ND	33.28 ± 0.32^{a}	ND	ND	5.11 ± 0.04^{a}	1.49 ± 0.06^{a}	22.84 ± 9.14^{a}	ND	$\mathbf{62.68 \pm 9.26}^{a}$
AL(EA)	2.12 ± 0.03^{a}	198.06 ± 0.62^{b}	ND	4.50 ± 0.05^{a}	ND	33.09 ± 0.26^{b}	ND	39.80 ± 0.21^{a}	$\mathbf{278.00 \pm 1.18}^{b}$
AL(BuOH)	2.64 ± 0.02^{a}	66.17 ± 1.23^{c}	ND	11.20 ± 0.01^{b}	9.72 ± 0.51^{e}	22.94 ± 0.81^{c}	ND	43.23 ± 0.59^{d}	$\mathbf{155.90 \pm 4.35}^{c}$
AL(Aq)	ND	13.70 ± 1.56^{d}	ND	2.49 ± 0.02^{c}	ND	0.44 ± 0.03^{d}	ND	0.96 ± 0.04^{b}	$\mathbf{17.59 \pm 1.65}^{d}$
AML	0.64 ± 0.01^{c}	33.28 ± 0.01^{a}	ND	ND	3.62 ± 0.04^{c}	1.33 ± 0.02^{e}	21.38 ± 0.61^{b}	ND	$\mathbf{60.25 \pm 0.69}^{e}$
AML(EA)	6.12 ± 0.03^{d}	122.23 ± 1.24^{e}	42.90 ± 0.17	16.49 ± 0.28^{d}	6.53 ± 0.04^{d}	25.16 ± 0.09^{f}	15.44 ± 0.12^{d}	12.72 ± 0.24^{c}	$\mathbf{247.59 \pm 2.21}^{f}$
AML(BuOH)	3.74 ± 0.26^{e}	46.83 ± 1.10^{f}	ND	12.03 ± 0.11^{e}	ND	35.16 ± 0.07^{g}	31.00 ± 0.81^{e}	32.93 ± 0.07^{d}	$\mathbf{161.69 \pm 2.42}^{g}$
AML(Aq)	ND	17.77 ± 10.02^{g}	ND	ND	4.28 ± 0.04^{e}	0.62 ± 0.02^{h}	40.28 ± 0.03^{f}	ND	$\mathbf{62.95 \pm 10.11}^{e}$

Data of phenolic compositions are means of three replicates and data is reported as mean ($n = 3$) ± standard deviation ($n = 3$). Means within each column labelled with different letters are significantly ($p < 0.05$) different according to Duncan's multiple range test. ND: nondetected. Groups are the same as Figure 1.

Table 6: Effect of *Clinacanthus nutans* on lipid profile in high fat and high cholesterol fed rats after 7 weeks of intervention.

Group	Lipid profile (mmol/L)				
	TC	HDL	LDL	VLDL	TG
NC	1.06 ± 0.14^{a}	0.33 ± 0.02^{a}	1.15 ± 0.31^{a}	0.14 ± 0.01^{a}	0.30 ± 0.03^{a}
HFHC	3.21 ± 0.2^{b}	0.14 ± 0.01^{b}	3.27 ± 0.08^{b}	0.27 ± 0.04^{b}	0.49 ± 0.07^{b}
STATIN	2.01 ± 0.10^{c}	0.29 ± 0.05^{a}	1.91 ± 0.18^{c}	0.17 ± 0.05^{a}	$0.37 \pm 0.10^{a,b}$
AL(H)	2.62 ± 0.34^{d}	0.20 ± 0.03^{c}	2.37 ± 0.22^{d}	0.18 ± 0.04^{a}	$0.39 \pm 0.10^{a,b}$
AL(M)	2.84 ± 0.09^{d}	$0.20 \pm 0.07^{b,c}$	2.83 ± 0.19^{e}	0.19 ± 0.01^{c}	0.42 ± 0.03^{b}
AL(L)	$3.04 \pm 0.27^{b,d}$	$0.16 \pm 0.06^{b,c}$	$3.18 \pm 0.57^{b,e}$	$0.19 \pm 0.05^{a,b,c}$	$0.43 \pm 0.10^{a,b}$
AML(H)	$2.31 \pm 0.24^{c,d}$	$0.27 \pm 0.09^{a,c}$	$2.44 \pm 0.31^{d,e}$	$0.12 \pm 0.03^{a,c}$	0.33 ± 0.02^{a}
AML(M)	2.62 ± 0.06^{d}	0.22 ± 0.03^{c}	$2.63 \pm 0.41^{d,e}$	0.19 ± 0.03^{c}	0.43 ± 0.07^{b}
AML(L)	2.77 ± 0.17^{d}	0.21 ± 0.01^{c}	$2.56 \pm 0.32^{d,e}$	0.21 ± 0.01^{c}	0.46 ± 0.03^{b}

Values were means ± SD ($n = 7$). Means within each column labelled with different letters (a–c) are significantly ($p < 0.05$) different according to Duncan's multiple range test. Groups are the same as Figure 5.

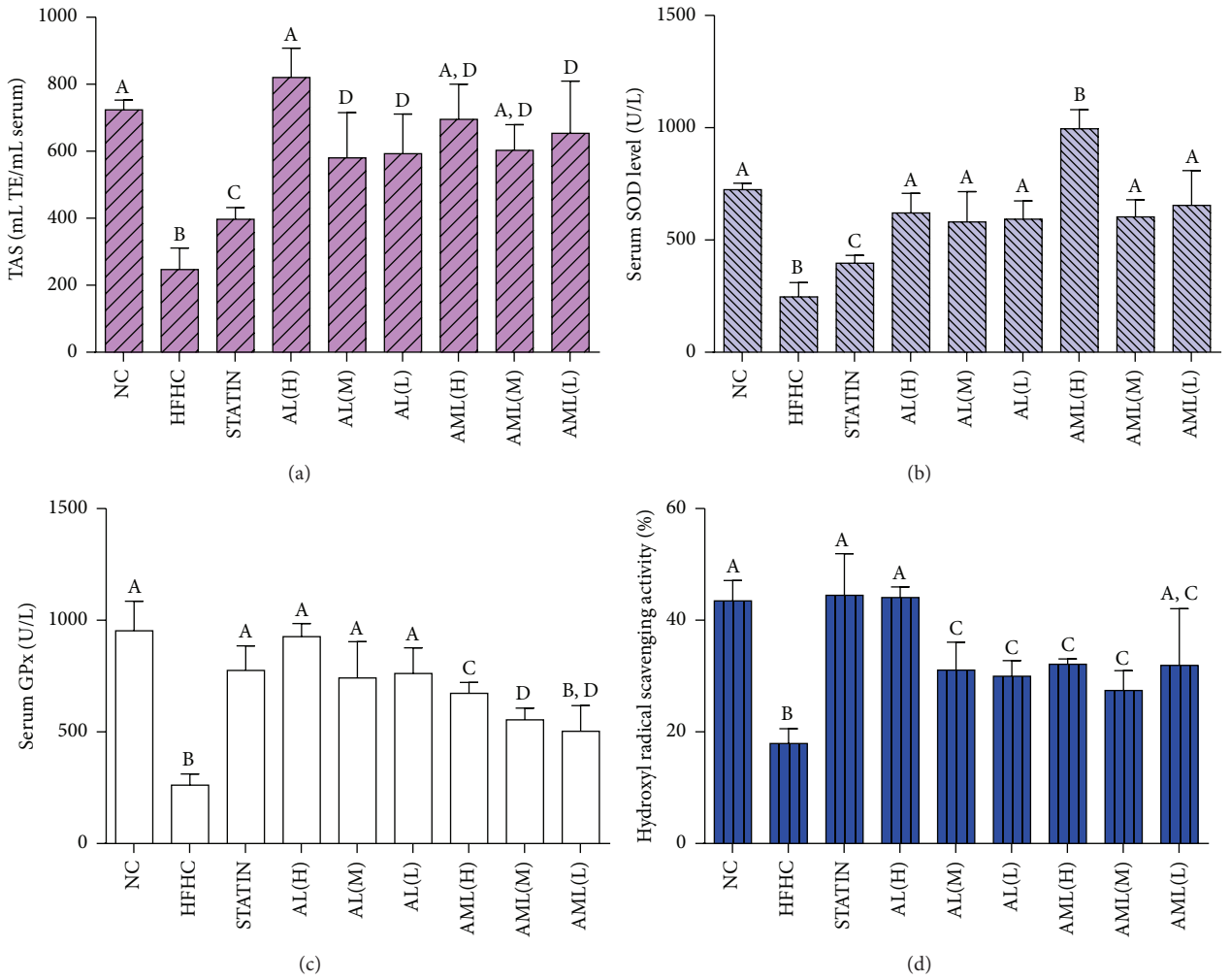

Figure 7: Effects of *Clinacanthus nutans* extracts on (a) serum total antioxidant status (TAS), (b) serum superoxide dismutase (SOD), (c) serum glutathione peroxidase (GPx), and (d) liver hydroxyl radical scavenging activity, in high fat and high cholesterol fed rats after 7 weeks of intervention. Bars and error bars represent mean ± standard deviation ($n = 7$). Bars representing different groups in each panel with different letters are significantly different ($p < 0.05$). Groups are the same as Figure 5.

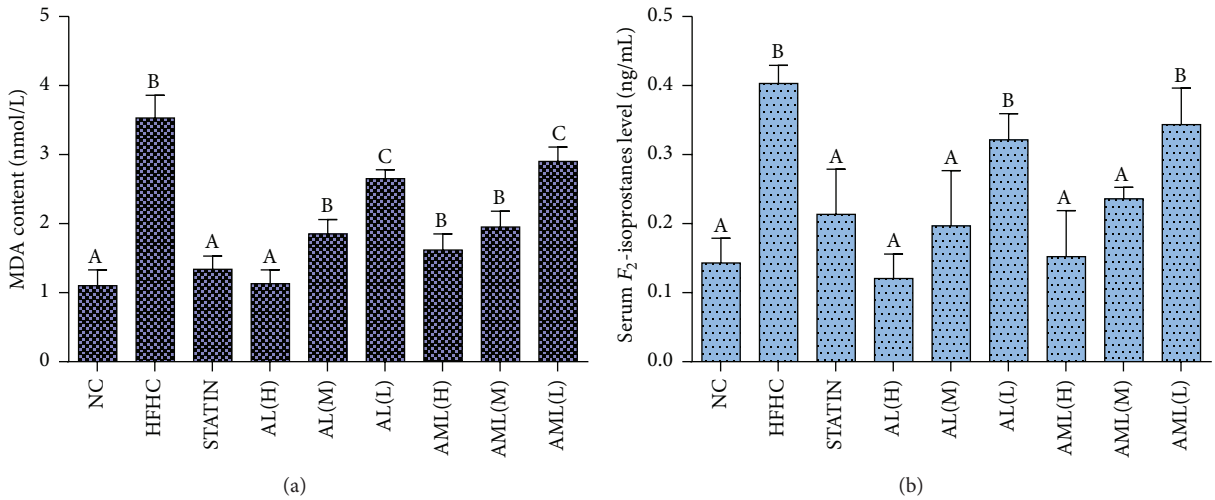

Figure 8: Effects of *Clinacanthus nutans* extracts on (a) hepatic MDA content, (b) serum F_2-isoprostanes level in high fat and high cholesterol fed rats after 7 weeks of intervention. Bars and error bars represent mean ± standard deviation ($n = 7$). Bars representing different groups in each panel with different letters are significantly different ($p < 0.05$). Groups are the same as Figure 5.

cancer [25, 26]. Chlorogenic acid is also known to have potent antioxidant effects, which may explain the superior DPPH and ABTS radical scavenging of the AML extract compared with the HAL and AL extracts [27]. Additionally, Cinnamic acid may have contributed to the antioxidant properties of the HAL and AML extracts [28], while Vanillic acid, which was only detected in the AL extract, may have only contributed minimally to the antioxidant activity of the extract. Moreover, Prince et al. [29] had demonstrated that Vanillic acid is a potent antioxidant capable of protecting against lipid peroxidation. In general, however, based on the presence of multiple phenolics in the extracts of *C. nutans*, it is likely that synergism played a role in their overall bioactivities. The presence of PCA as the major phenolic in the EA fractions suggested that it may have contributed significantly to the bioactivity of the *C. nutans* extracts since the A fractions also showed potent antioxidant effects. Increased release of bound phenolics during partitioning may have also contributed to the high amounts of phenolics detected in the fractions.

High fat diet feeding can cause disorders like hyperlipidemia, insulin resistance, and CVD, which resemble the human metabolic syndrome closely [30–33]. The present study demonstrated that rats fed a HFHC diet showed approximately 200%, 79%, and 95% higher concentrations of serum TC, LDL, and TG, respectively, and 36% lower HDL compared with rats fed a normal rat chow diet ($p < 0.05$), confirming hyperlipidemic condition (Table 6). Additionally, *C. nutans* supplementation in the rats showed evidence of attenuating hyperlipidemia-associated oxidative stress [34]. Moreover, there were improvements in antioxidant enzymes activities in serum (Figure 7) with underlying transcriptional changes in the antioxidant genes (Figure 9), which may have been the basis for the improved antioxidant capacities of the serum and liver of the rats. Furthermore, we observed significant increases in oxidative stress markers (MDA and F_2 isoprostane) in the HFHC group (Figure 8), in keeping with the reduced antioxidant status due to hyperlipidemia-induced oxidative stress [5, 34]. Conversely, administration of the phenolic-rich extracts from *C. nutans* significantly decreased the hyperlipidemia-induced oxidative stress markers in rats. The attenuation of lipid peroxidation in the *C. nutans*-treated animals, especially at the higher dose, suggested that the rats had reduced risk of cardiometabolic diseases, since lipid peroxidation is known to promote the diseases [35]. Moreover, F_2-isoprostanes which are clinically relevant and potent indicators of oxidative stress [36] were attenuated by *C. nutans* supplementation. The ability of the *C. nutans* extracts to attenuate hyperlipidemia-induced oxidative stress is indicative of their rich phenolic contents. Moreover, phenolics such as PCA, Chlorogenic acid, and Caffeic acid have been shown to reduce biomarkers of oxidative stress like MDA and F_2-isoprostanes and increase the activities of antioxidant enzymes like CAT, SOD, GPx, or Gsr [7, 9].

The use of single, mostly synthetic, antioxidant compounds has not proved effective in reducing overall chronic disease burden and mortality likely due to the limited effects on metabolic processes or the metabolic compensation from other systems in the presence of the single antioxidants [37]. This has generated interests in bioactive-rich fractions, which have been demonstrated to be more effective than single compounds, and can potentially have effects across different metabolic pathways, thus reducing the chances of any metabolic compensatory process canceling out the effects of the antioxidants in the rich-fraction [16]. In this study, the different phenolic compounds in the extracts may have acted through different mechanisms including the scavenging of free radicals, electron transfer, and neutralization of the free radicals to attenuate the hyperlipidemia-induced oxidative stress. These may have prevented the oxidation of biomolecules, as suggested by the reduced oxidative stress products in the *C. nutans*-treated groups in this study,

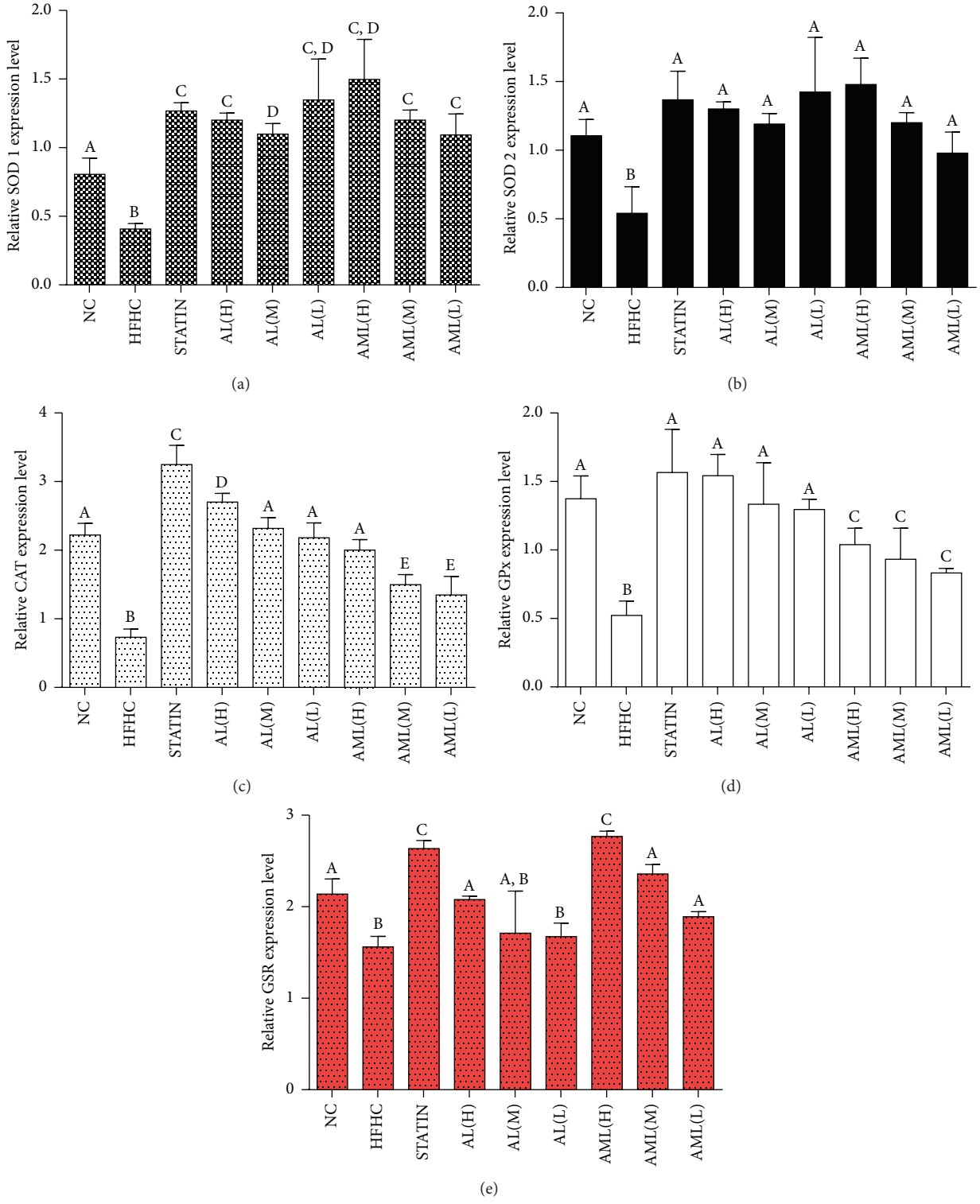

FIGURE 9: Effects of *Clinacanthus nutans* extracts on the mRNA levels of hepatic antioxidant genes in high fat and high cholesterol fed rats after 7 weeks of intervention. Bars and error bars represent mean ± standard deviation ($n = 7$). Bars representing different groups in each panel with different letters are significantly different ($p < 0.05$). CAT: catalase; GPx: glutathione peroxidase; GSR: glutathione peroxidase; SOD: superoxide dismutase. Groups are the same as Figure 5.

in addition to the regulation of cholesterol levels possibly through modulating its metabolism. Similarly, the phenolic compounds in the extracts may have regulated the c-Jun-N-terminal kinase, thereby modulating oxygen radical production and inflammation [3, 6].

5. Conclusions

The present study demonstrated the antioxidant capacities of *C. nutans* extracts and their efficacies against hypercholesterolemia-induced oxidative stress in rats. The results indicated that *C. nutans* is rich in multiple natural antioxidants, and hence its effects may be contributed by multiple bioactive compounds. Thus, *C. nutans* may be a good source of functional ingredients that can be used for managing oxidative stress-related diseases. However, future clinical studies including detailed toxicity analyses are needed to determine the usefulness of this plant in managing such diseases.

Abbreviations

AL: Aqueous leaf
AML: Aqueous methanol leaf
HFHC: High fat and high cholesterol
CVD: Cardiovascular disease
PCA: Protocatechuic acid
FRAP: Ferric reducing antioxidant power
TPC: Total phenolic content
HPLC: High performance liquid chromatography.

Conflict of Interests

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

Authors' Contribution

Mustapha Umar Imam, Der-Jiun Ooi, Kim Wei Chan, Norhaizan Md Esa, Norhasnida Zawawi, and Maznah Ismail contributed equally to this work.

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Review Article

Sulforaphane and Other Nutrigenomic Nrf2 Activators: Can the Clinician's Expectation Be Matched by the Reality?

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The recognition that food-derived nonnutrient molecules can modulate gene expression to influence intracellular molecular mechanisms has seen the emergence of the fields of nutrigenomics and nutrigenetics. The aim of this review is to describe the properties of nutrigenomic activators of transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2), comparing the potential for sulforaphane and other phytochemicals to demonstrate clinical efficacy as complementary medicines. Broccoli-derived sulforaphane emerges as a phytochemical with this capability, with oral doses capable of favourably modifying genes associated with chemoprevention. Compared with widely used phytochemical-based supplements like curcumin, silymarin, and resveratrol, sulforaphane more potently activates Nrf2 to induce the expression of a battery of cytoprotective genes. By virtue of its lipophilic nature and low molecular weight, sulforaphane displays significantly higher bioavailability than the polyphenol-based dietary supplements that also activate Nrf2. Nrf2 activation induces cytoprotective genes such as those playing key roles in cellular defense mechanisms including redox status and detoxification. Both its high bioavailability and significant Nrf2 inducer capacity contribute to the therapeutic potential of sulforaphane-yielding supplements.

1. Introduction

Whilst early 20th century nutrition science resolved issues related to micronutrient deficiency states and the latter part focused more on macronutrient excesses [1], the first decade of the 21st century has already seen old paradigms challenged and new theories proposed. The recognition that food-derived nonnutrient molecules can modulate intracellular molecular mechanisms has seen the emergence of the fields of nutrigenomics and nutrigenetics, disciplines derived from the interweaving of the sciences of nutrition, biochemistry, molecular biology, and genomics. It has been estimated that there are more than 5000 different phytochemicals present in food [2] and our current knowledge is limited to a reasonable understanding of the function of just a few.

Against this background sits the quest to identify biomolecules with significant nutrigenomic potential. A growing body of research highlights one such biomolecule, sulforaphane, an isothiocyanate (ITC) derived from the cruciferous vegetable family and in particular from *Brassica oleracea* [3]. Although the plant kingdom is the source of thousands of phytochemicals, little is known about the way in which food-derived phytochemicals support the maintenance of human health and especially those associated with cellular defense mechanisms. As the science of nutrigenomics evolves and our understanding of the many interactions between phytochemicals and endogenous cytoprotective mechanisms grows, the significance of plant foods in human health becomes clearer.

A critical review of the formulations of some available supplements reveals numerous flaws, shedding doubt on their potential efficacy [4]. There are few published clinical trials using phytochemicals as the intervention material and only a small number of these withstand scientific scrutiny. However, even when benefit for a compound has been demonstrated, it is common for a commercial product to include the ingredient at a dose manyfold lower than that shown to be efficacious in either clinical trials or as it was traditionally employed by cultures of the past. As a further trap for the unwary consumer or uninformed clinician, supporting

commentary may include citations for in vitro and animal studies, giving the reader a false impression of the product's likely efficacy as a supplement for humans.

Because it appears that many consumers have accepted a role for complementary medicines in their personal health management, it is important to review the evidence on whether plant-derived supplements can assist in modifying various biochemical and physiological risk factors for disease. The aim of this review is to describe the properties of nutrigenomic activators of Nrf2, focusing on the potential for sulforaphane and other activators of gene expression to demonstrate clinical efficacy as complementary medicines.

2. Beyond Nutritional Deficiencies and Excesses

2.1. Nutrigenetics and Nutrigenomics. The interlinked sciences of nutrigenetics and nutrigenomics provide the clinician with a more targeted opportunity to personalise a patient's treatment programme [5], revealing those genetic polymorphisms which may compromise individual biochemical function. Even without access to sophisticated genome profiling, a clinician's knowledge that potent food-derived biomolecules can interact with intracellular signaling pathways provides another dimension to clinical management and disease prevention processes.

The realization that food-derived molecules are in constant conversation with complex intracellular control systems via signaling pathways has unveiled the role of food as so much more than a source of micro- and macronutrients [6]. What becomes immediately apparent in this model is that no multinutrient supplement can substitute for the enormous diversity in phytochemicals present in a balanced human diet. Also evident is that the health benefits of the popular polyphenolic phytochemicals such as those found in green tea, grape seed, red wine, curcumin, pomegranate, and olives are unlikely to be due to direct-acting antioxidant effects demonstrated by these molecules in numerous in vitro studies [7, 8]. Polyphenols are typically large bulky molecules which are poorly absorbed and poorly bioavailable [9], so that it is unlikely that the intracellular micromolar concentrations necessary to scavenge free radicals can be achieved. Polyphenols can also behave as either antioxidants or prooxidants depending on the experimental conditions [10]. In addition, newer evidence suggests polyphenols and other phytochemicals may function hormetically, whereby dose response is characterised by low dose stimulatory response and high dose inhibition [11].

In a bioactive-specific approach, a recent comprehensive review of phytochemicals indicated for cardiovascular disease focused on both preclinical and clinical beneficial effects of four commonly supplemented compounds [12]. The review concluded that there are few definitive trials in this area and in some studies the exact dose used is not clear. However, the authors confirm the findings of others in that the use of a very high dose is associated with the most protective effects for a few phytochemicals, whereas the lowest dose turns out to be the most effective for other compounds.

As with vitamin "antioxidants," the notion that ingested polyphenol supplements act as "antioxidants" in human cells is called into question [7]. Emerging evidence suggests that polyphenols or their metabolites exert their systemic intracellular effects not as direct "antioxidants" per se but as modulators of signaling pathways.

2.2. Cruciferous Vegetables Harbor Nutrigenomic Potential. The classification, cruciferous vegetables (crucifers), includes species predominantly from Brassicaceae family and the more common members are cultivars not only of *Brassica oleracea* genus including broccoli, cabbage, cauliflower, Brussels' sprout, and kale but also of *Raphanus* genus which includes various types of radish. Although these vegetables are good sources of micronutrients, their value to human health would seem to be at least partly due to the nature of the phytochemicals they contain and in particular the glucosinolates [13], the enzymatic hydrolysis products of which are capable of modifying gene expression [14]. Although vegetables such as broccoli are not popular dietary choices [15], the unique health-promoting value of crucifers continues to be reaffirmed [16]. A recent review [17] investigating the effect of crucifers on total and cardiovascular mortality found that several prospective studies showed no association for total vegetable consumption but did show a significant inverse association for cruciferous vegetable consumption. The potential benefits of green leafy vegetables in general and cruciferous vegetables in particular are not limited to their effects in cancer and cardiovascular disease. In a 27-year prospective cohort study on cognitive decline in ageing women (n = 15,080), those in the highest quintile of cruciferous vegetable intake declined more slowly than those in the lowest quintile, with an evident linear dose response [18]. Those in the highest quintile of green leafy vegetable intake also experienced slower cognitive decline. The association did not change when data for participants with cardiovascular disease and diabetes were excluded.

Most research on crucifers has focused on broccoli, *Brassica oleracea* (both vegetable and sprouts), as a source of bioactive compounds with nutrigenomic potential. The last two decades have seen accelerating interest in the role of broccoli in human health following evidence that induction of detoxification enzymes might be responsible for the majority of the observed health benefits of vegetables [19, 20]. After isolating broccoli-derived sulforaphane, Zhang's group showed that sulforaphane was a major and very potent Phase II enzyme inducer. The group of induced enzymes includes NAD(P)H:NQO1 (quinone reductase) and the family of glutathione-S-transferases (GSTs), both of which are required for the detoxification of steroids and the ubiquitous environmental toxin, benzo(a)pyrene [21–23]. Zhang et al. concluded that the induction of detoxification enzymes by sulforaphane may significantly contribute to the anticarcinogenic action of broccoli. The way that sulforaphane demonstrably increased target enzymes is indicative of a nutrigenomic effect, even though the precise mechanism to explain such gene expression was not known at the time. It would be another two years before the mechanism to explain the effect of sulforaphane would be elucidated [24].

3. Influencing Signaling Pathways

3.1. Nrf2 as "Master Regulator" of Cell Defense. Although sulforaphane interacts in a number of mammalian biochemical pathways, its effect on the redox-sensitive transcription factor, Nrf2 (nuclear factor erythroid 2-related factor 2), appears to be responsible for its greatest clinical potential when administered at practical oral doses [25]. Reference to Nrf2 first appeared in the scientific literature in 1994 and has subsequently been the subject of over 5,500 MEDLINE published papers [24]. In the ensuing two decades, Nrf2 has emerged as a key modulator of the cell's primary defense mechanism, countering many harmful environmental toxicants and carcinogens [26]. Considerable research has focused on Nrf2's role in preventing the activation of carcinogens to toxic metabolites, especially by induction of the Phase II detoxification enzyme, NAD(P)H:quinone reductase (NQO1) [27].

The elucidation of the mechanism by which Nrf2 acts as a cytoplasmic "switch" to activate a battery of cytoprotective genes arguably heralds a new paradigm in nutrition science. Identification of Nrf2 gave the first real clue that bioactive diet-derived compounds like sulforaphane had the potential to coordinately influence large banks of function-specific genes [28].

Nrf2 has been variously described as an activator of cellular defense mechanisms [29], the master redox switch [30] and a guardian of health span and gatekeeper of species longevity [31]. As a mediator for amplification of the mammalian defense system against various stressors, Nrf2 sits at the interface between our prior understanding of oxidative stress and the endogenous mechanisms cells use to deal with it. What has become clear is that although attempts to counter oxidative stress by "antioxidant" vitamin supplementation have been disappointing [32], many phytochemicals have the capacity to activate Nrf2 and thereby induce genes [33] which collectively regulate much of the cell's endogenous defense system, enhancing its survival [34]. This finding may be clinically significant in that diseases known to be underpinned by oxidative stress may prove to be more responsive to such amplification of cellular defenses via Nrf2 activation compared to by the administration of direct-acting antioxidant supplements [35].

3.2. Sulforaphane, an Inducer of Nrf2 Target Genes. Notably and perhaps surprisingly, given its significant cytoprotective potential, sulforaphane does not exhibit a direct antioxidant effect; instead it is weakly prooxidant [36]. As further evidence to support the critical role of redox signaling in cellular defense mechanisms, the ability of sulforaphane to induce NQO1 and cell cycle arrest in prostate cancer cell lines was shown to have been completely abrogated by pretreatment with the glutathione (GSH) precursor, N-acetylcysteine [37, 38]. This finding has implications for the regular ingestion of readily available supplements of N-acetylcysteine.

Sulforaphane [1-isothiocyanato-(4R)-(methylsulfinyl) butane: $CH_3S(O)(CH_2)_4$-N=C=S] is a small (MW = 177.29) aliphatic lipophilic organosulfur molecule which is not present in cruciferous or other plants (Supplementary Data, Figure 1, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/7857186). Instead, plants of *Brassica* genus contain a biologically inactive precursor compound, glucoraphanin (GRN), which is contained within a plant cell vacuole together with an enzyme, myrosinase (MYR), which is separately compartmentalized [39]. It is when the plant cell ruptures and the GRN and MYR come into contact that sulforaphane is enzymatically produced [40] (Supplementary Data, Figure 2). Compared with its stable GRN precursor, the resulting sulforaphane aglycone is relatively unstable [41]; this has implications for culinary applications of broccoli and other cruciferous vegetables. Broccoli is not the only crucifer which yields sulforaphane but it yields the highest amounts, with its GRN content around 75% [42] of total glucosinolates. Notably, glucosinolate-containing plants contain variable quantities of both precursor and enzyme [43]. As a result, the yield of sulforaphane and other isothiocyanates can vary widely.

Cutting, chewing, or otherwise disrupting the broccoli plant cell structure initiates the synthesis of sulforaphane which, compared to its stable GRN precursor, begins degrading soon after synthesis [44]. For consumers to take advantage of the cytoprotective benefits of broccoli and other crucifers, steps must be taken to conserve the integrity of the sulforaphane released.

Sulforaphane belongs to one of nine identified classes of chemical Nrf2 activator [45]. Structurally varied, the only property shared by all inducers is their ability to react with sulfhydryl (-SH) groups. Nrf2 therefore is intimately tied to sulfur chemistry and provided dietary protein is adequate, a balanced diet should furnish sufficient sulfur. However, there are concerns that sulfur intake in many may be marginal [46], with some researchers suggesting that deficiency of sulfur amino acids can compromise GSH synthesis to a greater extent than for protein synthesis in both the presence and absence of inflammatory stimuli [47]. Whilst vegan diets may provide significant levels of phytochemicals [48], there may be a need for vigilance regarding sulfur adequacy, given that the sulfur-containing amino acids are least abundant in plant proteins and that vegans typically consume about half of the sulfur consumed by those consuming a mixed balanced diet [46].

3.3. Broccoli Sprout versus Broccoli Vegetable. Much of the clinically relevant *Brassica* research relates to broccoli sprouts [49] rather than to the mature vegetable, with most of the early work in this field done by a group at the Johns Hopkins University beginning in the early 1990s. The group found that 3-day-old sprouts of cultivars of certain crucifers contained 10–100 times higher concentration of GRN than the corresponding mature plants [49]. With a focus on identifying plants with cancer chemopreventive properties, they found that the sprouts were highly effective in reducing the incidence, multiplicity, and rate of development of mammary tumors in dimethylbenz(a)anthracene-treated rats. Broccoli sprouts also had the added advantage of containing mostly the methylsulfinylalkyl glucosinolate (75% of the total) and very little of the indole glucosinolate found in the mature plant, which is a potential tumor promoter [50]. Their

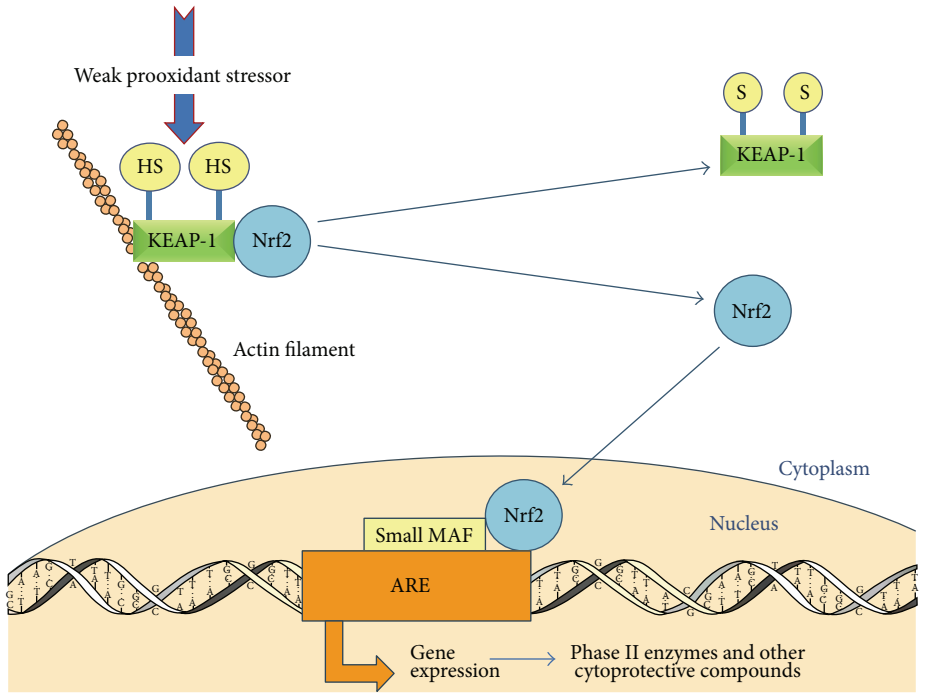

Figure 1: The mechanism by which Nrf2 activation increases the expression of genes with an Antioxidant Response Element (ARE) in their promoter regions. Human Keap-1 contains 27 cysteine residues providing sulfhydryl groups (-SH) which act as sensors of ARE inducers including oxidative stress [69]. Small Maf protein is essential for Nrf2 function [170]. Figure adapted from Kensler et al., 2003, with permission [51].

realization was that small quantities of broccoli sprouts may protect against cancer as effectively as much larger quantities of the vegetable stimulated subsequent research.

3.4. How Nrf2 Activators Influence Gene Expression. Although the complexity of Nrf2 pathways has not yet been fully elucidated, the principal elements are depicted in Figure 1 [51]. Essentially, Nrf2 is sequestered in the cytoplasm by the actin-bound cytosolic repressor Keap-1 (Kelch-like ECH-associated protein 1), a cysteine-rich protein which also acts as a sensor of variations in cytoplasmic redox status. When the appropriate signal is detected by cysteine thiols within Keap-1, its ability to bind and retain the transcription factor Nrf2 in the cytoplasm is lost. Keap-1 typically responds to an electrophilic or oxidative stress signal [51].

Thus released, Nrf2 translocates to the nucleus where it aligns with a short nucleotide base sequence in the promoter region of its target genes; this sequence is commonly known as the Antioxidant Response Element (ARE) or the Electrophilic Response Element (EpRE), with the latter being considered a more correct descriptor, although the terms are used interchangeably [52]. To bind, Nrf2 dimerizes with other basic leucine zipper (bZIP) proteins such as small Maf proteins (Maf G) to form a transactivation complex that binds to AREs [53].

When an electrophilic or oxidative stressor challenges the cell, Keap-1 senses the disturbance to its cytoplasmic redox equilibrium. After release from Keap-1, Nrf2 levels rapidly rise in the nucleus, upregulating a battery of cytoprotective genes, each containing at least one ARE. Of significance is the effect of Nrf2 on induction of the rate-limiting enzyme for (GSH) synthesis, γ-glutamyl-cysteine synthetase, thereby elevating tissue GSH levels [54].

For the Nrf2-Keap-1 pathway to such a play a key role in cytoprotection, its activity must be capable of being regulated in tandem with the ever-changing cellular environment. Under basal nonstressed conditions, Nrf2 is continuously degraded via the ubiquitin-proteasome pathway [55]. With a half-life of around 20 minutes [56], Nrf2 is maintained at a low cellular level [57]. Exposure to stressors inactivates Keap-1 by direct modification of cysteine thiol residues, thereafter releasing Nrf2 in a derepression-type stress response [58].

The clinical significance of this mechanism is apparent when considering the hepatotoxic effects of acetaminophen, a drug responsible for considerable drug-induced liver injury [59]. Excessive doses of this common analgesic/antipyretic drug rapidly deplete intracellular GSH reserves. However, the cell activates an adaptive response whereby Keap-1 senses the acetaminophen metabolite, N-acetyl-p-benzoquinone imine (NAPQI), subsequently activating Nrf2 [60]. GSH is synthesised rapidly along with a battery of other Nrf2 target genes. This mechanism may not be adequate to increase GSH levels in an acute care setting, given that translation times for protein synthesis of various Nrf2 target genes can take hours. A study investigating the effect on gene expression of cytoprotective heme oxygenase-1 (HO-1) in neurons after subarachnoid haemorrhage showed that Nrf2 levels increased ~4-fold at 12 hours, peaking at >4.5-fold at 24 hours, with

HO-1 levels increased to >3-fold at 12 hours and peaking at >4.5-fold at 24 hours [61].

3.5. Phase II Enzymes and the Detoxification Mechanisms. The mechanisms that cells use to detoxify potentially harmful compounds, often carcinogens [62, 63], can involve a Phase I component associated with monoamine oxidases of the Cytochrome P450 family and a Phase II component where the intermediate compound produced by Phase I is metabolised in a way that permits ready excretion. A compound which activates Phase I and Phase II enzymes is known as a bifunctional inducer; however, if it activates only Phase II enzymes, it is a monofunctional inducer [64]. Phase II enzymes are induced by Nrf2 and as such are integral to this discussion. For safe and efficient detoxification, a toxin will ideally undergo a relatively slow Phase I reaction followed by a more rapid Phase II; this tends to prevent accumulation of the Phase I metabolite which can be more toxic than its precursor [65].

Therefore, for an optimal cellular detoxification environment, Phase II reactions should be at a rate which prevents intermediate products of Phase I from accumulating. Aliphatic sulforaphane acts as a monofunctional inducer, whereas the indole ITCs from mature broccoli are bifunctional inducers derived from the glucosinolate, glucobrassicin [49]. Of clinical significance is the finding that Phase II enzymes have a relatively long half-life, so that upregulated expression of these proteins can remain for several days. In a study using human adult retinal pigment epithelial cells (ARPE-19), NAD(P)H:quinone reductase remained active for more than 5 days [66].

4. Inducers of Nrf2 Target Genes

Nrf2 can be activated by a variety of inducers, not all of which are obtained orally. For example, the prooxidant signals generated by the reactive oxygen species released during exercise [67] or from inhaled environmental chemicals [68] are capable of upregulating the cellular endogenous defenses, provided exposure is sufficiently modest that it does not overwhelm the cell's defenses.

4.1. Diet-Derived Nrf2 Inducers. Although a number of phytochemicals have been investigated in relation to their Nrf2 inducer ability, the mechanistic studies to explain the nature of the induction are limited. A review paper focused on molecular mechanisms of phytochemicals in chemoprevention suggested that only three plant-derived molecules, sulforaphane, carnosol, and quercetin, have been mechanistically investigated in this regard and only sulforaphane has been studied for its roles in multiple mechanisms [69]. Given the more extensive literature on sulforaphane, we hereafter consider its potential as a supplement of clinical significance and where the data exist, comparing its potential with that of popular and widely available phytochemical supplements.

4.2. Sulforaphane: In Vitro Effects. Sulforaphane is a potent Nrf2 inducer with consequent induction of cellular defenses [70]. The effect is rapid in cell culture with activation by sulforaphane occurring within 30 minutes in human bronchial epithelial BEAS-2B cells [71]. Using microarray analysis to investigate the effect of sulforaphane in the wild-type murine liver, Hu et al. showed that expression levels of 1725 genes were increased after 3-hour exposure and 3396 genes were changed after 12 hours [33]. Comparing expression patterns at different time points, they also showed that maximal change occurred 12 hours after a single administration of sulforaphane, based on fold changes greater than 2-fold. The identified Nrf2 target genes can be classified broadly as those coding for a range of cytoprotective proteins, including antioxidants (enzyme and nonenzyme), drug-metabolising enzymes, drug-efflux pumps, heat shock proteins, NADPH regenerative enzymes, growth factors and growth factor receptors, heavy metal binding proteins, and various nuclear receptors including PPAR-γ, as well as for Nrf2 itself [33].

Vitamin D's protective effects on human cells are well recognized [72]; it may be nutritionally significant that the vitamin D receptor (VDR) is Nrf2 target gene inducible by sulforaphane [73]; in turn, Vitamin D can increase Nrf2 expression [74]. To further illustrate this diversity, Nrf2 target genes include those coding for β-defensin-2 (HBD-2), an antimicrobial peptide associated with innate immunity, protecting the intestinal mucosa against bacterial invasion. HBD-2 can be induced by sulforaphane [73] and was shown in a cell culture study using human Caco-2 cells to be significantly induced 1.6-fold at 24 hours and 2-fold at 48 hours by sulforaphane concentrations of >5 μM. These results may have relevance in disorders of the intestinal epithelium but systemically an intracellular concentration of 5 μM is probably higher than what can be readily achieved by diet or even via practical doses of available oral sulforaphane-yielding supplements.

The downstream enzyme products of Nrf2 target genes are efficient and versatile. They include those which constitute the glutathione and thioredoxin systems, the major cellular reducing systems in the body [75]. Several reasons explain their efficiency and versatility [76]: (1) they are not consumed stoichiometrically, as are direct-acting antioxidants such as ascorbate and tocopherols; (2) their duration of action is long with half-lives measured in days, so their induction need not be continuous; (3) they restore the endogenously produced direct-acting antioxidants like coenzyme Q10 and the tocopherols by returning them to the reduced state (in particular via NQO1 because both coenzyme Q10 and tocopherols are quinones). Major products of Nrf2 target genes and their roles in cytoprotection are listed in Supplementary Data, Table 1.

5. Quinone Reductase (NQO1), a Tool to Evaluate Inducer Capacity

Initially considered as an Nrf2-activated Phase II enzyme associated with detoxification pathways, the function of NQO1 is now considered to be much broader [77]. NQO1 has been described as a "quintessential cytoprotective enzyme" and is coded by what is considered "one of the most consistently and robustly inducible genes within its class"

[77]. Furthermore, its activity declines with age whilst upregulation of its activity by Nrf2 induction is described as an avenue for maintaining cellular defenses with advancing age [31]. Furthermore, animal studies show significant decline in Nrf2 activity between youth and old age [78–80]. Humans genetically deficient in NQO1 are more susceptible to the carcinogenicity of benzene exposure [81]. NQO1 is highly active in pulmonary tissues [82] as well as in epithelial and endothelial cells in general [25], suggesting that it could act defensively against compounds absorbed via the airways, gut, and bloodstream. NQO1 activity is used as a rapid screening procedure and a biomarker of the anticarcinogenic activity of phytochemicals [45, 83]. The assay [20] uses cells defective in Phase I function to provide the means for selectively distinguishing monofunctional inducers that elevate Phase II enzymes [84].

5.1. CD Values as a Comparative Marker. The term "CD value" describes the concentration required to double NQO1 activity in murine hepatoma cells [85]. A CD value is also useful for comparing the potential in vivo nutrigenomic effects of an ingestible bioactive compound. The CD value has also been used [19, 83] to classify *Brassica* spp. according to their relative "anticancer potential." When several crucifers were compared for their Nrf2 inducer effect [86], ITCs of cabbage, kale, and turnips exhibited less NQO1 inducer capacity than broccoli-derived sulforaphane. Sulforaphane returned ~33,000 units NQO1 inducer activity/g fresh weight for broccoli, cabbage returned ~11,000 units, and kale returned ~10,000 units with turnip returning ~2,000 units. This property may partly explain why broccoli is researched more extensively than other *Brassica* spp.

5.2. Clinical Significance of CD Value. In data from studies comparing CD values of well-known phytochemicals, sulforaphane showed the highest potency, with a concentration as low as 0.2 μM required to double the activity of NQO1 [19, 85]. The comparative CD values of other phytochemicals have been documented by others [87–90], with lower micromolar concentrations representing those with the higher inducer activity (Figure 2).

CD values are available for phytochemicals used in common oral supplements [83, 87–89, 91]: sulforaphane (0.2 μM), andrographolides (1.43), quercetin (2.50), β-carotene (7.2 μM), lutein (μM), resveratrol (21 μM), indole-3-carbinol from mature broccoli vegetable (50 μM), chlorophyll (250 μM), α-cryptoxanthin (1.8 mM), and zeaxanthin (2.2 mM). An earlier study conducted in a different laboratory [91] had shown curcumin (2.7 μM), silymarin (3.6 μM), tamoxifen (5.9 μM), genistein (16.2 μM), epigallocatechin-3-gallate (EGCG) (>50 μM), and ascorbic acid (>50 μM). The comparative NQO1 inducer activity of these phytochemicals is sulforaphane > andrographolides > quercetin > curcumin > silymarin > tamoxifen > beta-carotene > genistein > lutein > resveratrol > I-3-C > chlorophyll > α-cryptoxanthin > zeaxanthin.

Notably, the CD value of sulforaphane is 13.5-fold greater than that of curcumin, 18-fold greater than silymarin, and 105-fold greater than resveratrol, all phytochemicals which

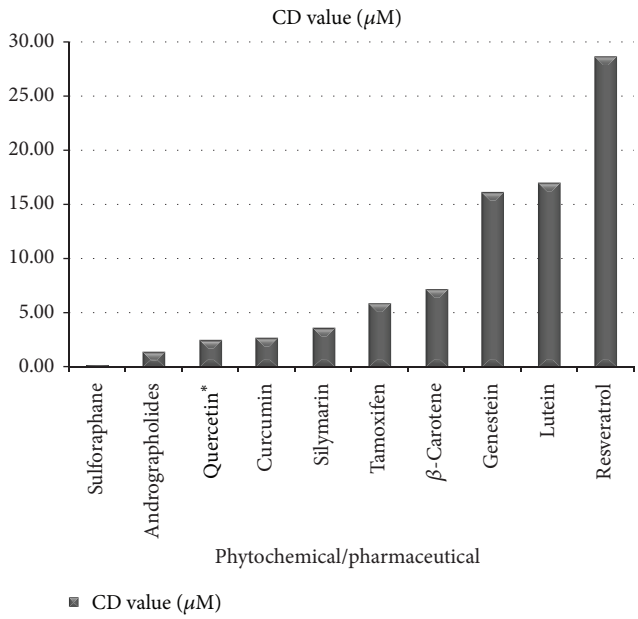

Figure 2: CD values of popular phytochemicals used as supplements and a commonly prescribed pharmaceutical. CD values refer to the concentration of a compound required to double the activity of the Phase II detoxification enzyme, quinone reductase [83, 87–89, 91].

are extensively promoted for their claimed health-promoting properties. It may be useful for relevant oral supplements to be evaluated in relation to the CD value of their primary ingredient(s), given that an Internet search will readily reveal many self-select and clinician-recommended supplements claiming to "enhance detoxification" and "promote longevity," even though supporting evidence is not apparent. Many such supplements claiming to target "detox" are based on ingredients such as chlorophyll and vitamin C, both of which have comparatively low NQO1 inducer capacity.

5.3. Comparing Effects of Indole Glucosinolates. Indole-3-carbinol (I-3-C), the ITC found in mature broccoli vegetable (but not significantly in the sprout), required >50 μM to double NQO1 activity [83]. In vivo, I-3-C must be dimerized in the acidic environment of the stomach to 3,3′-diindolymethane (DIM) to be active [92]. This has certain clinical implications as synthetic molecules of both I-3-C and DIM are available as supplements. With significantly lower inducer capacity than sulforaphane [91], it bears mention that DIM is also a bifunctional inducer of the detoxification pathway, thus limiting its cytoprotective potential. Early research on broccoli sprouts suggested potential limitations to the use of indole glucosinolates such as I-3-C as chemoprotectors in humans [49]. Not only are they weak inducers of Phase II enzymes but also, as bifunctional inducers, they simultaneously activate Phase I enzymes. They may also have estrogen receptor binding activity, adding to their potential as tumor promoters [49].

Interestingly, DIM is sometimes recommended clinically for patients with compromised estrogen metabolism, the theory being that DIM inhibits CYP1B. Inhibition of CYP1B1

shifts estrogen metabolism towards 4-hydroxyestrone, a metabolite which can contribute to carcinogenesis [93]. Not all data agree; a 2007 cell culture study analyzed gene expression using microarray profiling and quantitative real time-polymerase chain reaction in MCF7 breast cancer cells treated simultaneously with estradiol and DIM [94]. CYP1B1 was upregulated with a fold change of 3.93 ± 0.25. Such findings would tend to suggest that DIM may not protect against the metabolism of estrogen to the 4-hydroxy metabolites. Such conflicting data indicate that clinical trials are required to establish the in vivo effects of such an intervention when using a clinically relevant dose of a readily available supplement.

To illustrate the differences in potency between sulforaphane and I-3-C in a study using a prostate cell line, it was found that both compounds inhibited the proliferation of the prostate cancer cells in a dose-dependent manner but the inhibitory concentration of sulforaphane required was just 10% that of I-3-C [95]. There may also be safety issues which require caution in the recommendation of I-3-C supplements, available at many times the quantity of I-3-C achievable from broccoli vegetable consumption. Although I-3-C administered one week after the last dose of the carcinogen has been shown in rats to result in a latency delay of mammary tumor formation, it did not alter tumor incidence or multiplicity among survivors [96]. Any research showing a preventive benefit of this compound must be considered against the risk that it may promote liver and colon cancer [96].

5.4. Other Modes of Activating Nrf2. Although our focus is to compare the inducer capacity of phytochemicals, Nrf2 in human cells is activated by a range of stressors, not all of which are chemical in nature. The diverse nature of Nrf2 activators is highlighted in the three examples which follow. We use several examples of pharmaceuticals with pleiotropic Nrf2-inducing effects. Furthermore, we illustrate that when pharmaceutical Nrf2 activation occurs at supraphysiological levels, the outcome may be unexpected, indicating that the significantly lower inducer capacity of diet-derived Nrf2 activators may represent a hormetic effect [97].

5.4.1. Mechanical Effects. The mechanical effects of blood flow in regions where arteries are exposed to high shear stress are protected from inflammation and atherosclerosis. By contrast, low-shear regions are susceptible and this effect has been shown to be due to the effect of Nrf2 in reducing activation of the endothelium at atherosusceptible sites [98].

5.4.2. Pharmaceutical Drugs. The pharmaceutical tamoxifen, commonly prescribed to women following treatment for breast cancer, is an NQO1 inducer but its CD value is 30-fold lower than that for sulforaphane [99]. Nrf2 inducer activity may play some role in this drug's therapeutic profile in addition to its primary role as a selective estrogen receptor modulator (SERM) [99]. These comparative data may be clinically significant when considering the potential value of a drug or supplement with cytoprotective potential. A number of other pharmaceuticals pleiotropically activate Nrf2. The redox-modulating activity of the frequently prescribed statins and ACE inhibitors has been attributed to their Nrf2 inducer ability [100]. Similarly, gold salts, once the mainstay of treatment for rheumatoid arthritis, are Nrf2 inducers [101]. Indomethacin, now seldom used in reducing the symptoms of inflammatory joint diseases, has Nrf2-inducing properties, illustrating that nonsteroidal anti-inflammatory drugs (NSAIDs) exhibit properties other than their anti-inflammatory effects [102].

A relatively new pharmaceutical, Bardoxolone Methyl (BARD), was shown to enhance estimated glomerular filtration rate (eGFR) in patients with chronic kidney disease, a disease characterised by significant oxidative stress [103–105]. BARD is a synthetic analogue of oleanolic acid, a triterpenoid found extensively in edible plants [106], with broader cytoprotective properties attributed to Nrf2 induction [107]. The Phase 3 BEACON Trial [108] was halted in October 2012 following adverse events including 57 deaths out of 2185 participants in the BARD arm [105]. In comparing the inducer activity of BARD with that of SFN, a 2005 study comparing a range of triterpenoids showed that BARD was 230-fold more potent than SFN as a NQO1 inducer [109]. The adverse effects demonstrated by the synthetic triterpenoid analogue in the BEACON trial may be representative of a hormetic response at the upper end of a bifunctional dose response. By contrast, phytochemicals at the doses provided by foods are typically nontoxic [97].

5.4.3. Exercise. Exercise is associated with an increased flux of glucose and oxygen through the mitochondria, a process which increases levels of ROS such as superoxide. An essential role for exercise-induced ROS formation in activating transcription factors and coactivators has been proposed [110]. Ristow et al. demonstrated that typical exercise-related changes in gene expression were almost completely abrogated by daily ingestion of supplements of vitamins C and E at doses of 1000 mg and 400 IU, respectively.

A review highlighted 23 studies showing that antioxidant supplementation interferes with exercise training-induced adaptations [111]. An emerging theme [112] supports the view that because Nrf2 is activated by a mild prooxidant signal, high doses of antioxidant supplements may blunt signals required to activate endogenous defenses [113, 114]. Ristow's assertion that antioxidant supplementation blocks many of the beneficial effects of exercise is supported by such evidence.

5.5. Other Actions of NQO1 Which Can Be Influenced by Sulforaphane. NQO1 exhibits broad substrate specificity extending well outside its better known role as a Phase II inducer; its other roles as described in the following section may contribute to its cytoprotective capacity. Its actions include the following: (1) it can protect against benzene-derived quinones such as benzo(a)pyrene, a carcinogen found commonly in petrochemical exhaust gases and in barbecued meats [115]; (2) NQO1 can reduce catechol estrogen quinones to catechol estrogens, a process associated with lowering breast cancer risk due to elevated estrogen metabolites [116]; (3) NQO1 can scavenge superoxide, albeit at a lower order of

magnitude than does superoxide dismutase (SOD) [117], (4) NQO1 stabilises p53, the tumor suppressor gene [77]; and (5) NQO1 restores oxidized coenzyme Q10 (ubiquinone) and the tocopherols to their reduced forms [77].

Several NQO1 polymorphisms exist and these have been associated with risk of carcinogenesis. The C609T gene variant is one of very few common single nucleotide polymorphisms known to almost completely eliminate enzymatic activity; consequently, NQO1 is attracting considerable research attention given its multiple effects in cellular defenses [118].

5.6. Other Mechanisms: Animal Studies. Although a large volume of the published sulforaphane research is associated with its Nrf2 inducer potential, some studies point to other mechanisms. A recent study used broccoli sprout juice as the intervention material in stroke-prone spontaneously hypertensive rats to investigate possible effects on renal damage [119]. After 4 weeks, the animals were shown to have been largely protected against renal damage. Mechanistically, the effect was shown to be independent of systemic blood pressure but to parallel stimulation of the AMPK/SIRT1/PGC1a/PPARa/UCP2 axis. Whether this can be replicated in humans at practical doses has not yet been investigated.

6. The Issue of Bioavailability

6.1. Comparative Effects of Popular Phytochemical Supplements. Aside from wide variation in Nrf2 inducer capacity, a second barrier to clinical efficacy is bioavailability. When bioavailability is low, cell culture studies may significantly overestimate the intracellular concentration that ingestion of such a compound can achieve, being unlikely to demonstrate the expected clinical benefit indicated by the in vitro work [120, 121]. In considering the potential clinical efficacy of a phytochemical, the active compound and/or any of its active metabolites must reach the cells of the target organ(s) in appropriate concentration. Oral bioavailability of polyphenols is typically <10%, ranging between 2 and 20% [122], with many closer to 1%; cooking and processing significantly reduce polyphenol content [123]. By comparison, a pharmacokinetic animal study showed that sulforaphane was rapidly absorbed with its absolute bioavailability 82% [124].

Many phytochemical-containing supplements contain polyphenolic molecules such as curcumin (turmeric), catechins (green tea), resveratrol (grapes), ellagic acid (berries and pomegranate), and hydroxytyrosol and oleuropein (olives). Much of the evidence used to promote these supplements is from either in vitro or animal studies, with limited clinical evidence to support the assertions. Supplements of these phytochemicals frequently bear an "antioxidant" claim, even though the amount of polyphenol reaching the circulation or target cells is seldom adequate to alter redox status [7, 125]; gene expression studies have helped in quantifying likely systemic responses. Preclinical cell culture or animal studies may involve very high doses of an isolated polyphenol. Such doses are seldom clinically practical, considering average dietary intake of mixed polyphenols in food is approximately 1 gram per day of poorly bioavailable compounds [126].

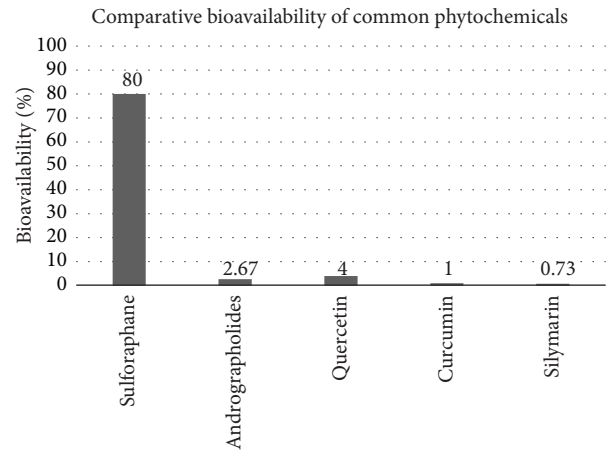

Figure 3: Comparative bioavailability of phytochemicals commonly used in supplements [90, 124, 127–129, 153].

Curcumin, resveratrol, and silybin are examples of popular polyphenol supplements for which preclinical findings cannot be readily extrapolated to the clinical environment. Figure 3 compares the bioavailability of several polyphenols with that of sulforaphane (native curcumin at ~1% [127], resveratrol <1% [128], and silybin ~0.73% [129]). In each case, the systemic bioavailability compares the plasma concentration of an oral dose to an intravenous dose and is expressed as a percentage, where F is bioavailability [90]:

$$F^{\text{oral}} = \frac{\left(\text{AUC}^{\text{oral}}/\text{Dose}^{\text{oral}}\right)}{\left(\text{AUC}^{\text{i.v.}}/\text{Dose}^{\text{i.v.}}\right)} \times 100\%. \quad (1)$$

The high intracellular concentrations of polyphenols required to replicate in vitro findings are difficult to achieve in humans with practical oral doses.

There is some evidence to suggest that the activity of some polyphenols may instead reside in their metabolites [130], so that small quantities absorbed intracellularly act as signaling molecules and may act synergistically with other biomolecules [131]. It is likely that any direct antioxidant effects occur only within the lumen of the gut [10, 132] and not systemically.

Quercetin naturally found in onions, watercress, tea, and other plants is a popular oral supplement typically promoted as an "antioxidant" or "anti-inflammatory" agent. Some studies suggest that quercetin may have anticancer potential [133] but other studies describe potential for risk [134], given that quercetin may exhibit prooxidant effects, especially in a GSH-depleted cellular environment [135].

Specifically, quercetin can exert an inhibitory effect on the metabolism of catechols via the catechol-O-methyltransferase enzyme (COMT) [136]. This may have implications in estrogen-related disorders where inappropriately metabolised estrogens can form DNA adducts [93]. Whether oral doses of quercetin have these effects in humans is not known but the issue has been flagged as "concerning"

since readily available quercetin supplements represent up to 100 times the quantity typically available in a Western diet [137].

6.2. Curcumin. Curcumin is regarded as having in vitro anti-inflammatory activity by virtue of its ability to inhibit the transcription factor, NF-κB [138]. In a study investigating inflammation in human tenocytes, high concentrations of 5–20 μM were required to inhibit IL-1β-induced inflammation [139]. However, very high oral doses in humans (up to 8 g) yielded curcumin peak intracellular levels of only 0.5–2.0 μM, clearly not attaining a concentration of the same order; commonly recommended supplemental doses of up to 180 mg were undetectable in plasma [140, 141]. Laboratory findings demonstrating an impressive and diverse array of cytoprotective effects for curcumin may not generally apply to practical oral doses in humans [142].

By contrast, there is evidence for an effect in gastrointestinal tissue, where transport occurs across a single enterocyte membrane [143, 144]. Patients with colorectal cancer were administered doses up to 3.6 g curcumin daily [145]. M_1G, a marker of DNA damage, decreased 38% in the colorectal tissue, showing that a dose of 3.6 grams daily achieves pharmacologically efficacious levels in colonocytes but with negligible distribution outside the gut, confirming its poor systemic bioavailability.

When considering both CD value and bioavailability, native curcumin with bioavailability of ~1% would appear to be less clinically relevant than sulforaphane which shows both high inducer activity and high bioavailability. Even enhanced forms of curcumin with ~7-fold higher bioavailability still exhibit comparatively low bioavailability [146]. Investigating physiologically achievable doses of curcumin, Lao et al. administered from 500 to 12,000 mg of a curcumin powder; no curcumin was detected in any of 74 participants taking up to 8,000 mg; low serum levels in the ng/mL range were detected only for doses >8,000 mg, with doses below 4,000 mg barely detected [147]. Similarly, curcumin was not detected in normal liver or colorectal liver metastases in patients receiving 3.6 g/d for 1 week [145]. Howells et al. conclude that in vitro studies with curcumin in the high 10 μmol/L range or below might have human physiological relevance but that its role as a chemopreventive agent may lie primarily within the gastrointestinal tract [141].

6.3. Resveratrol. Resveratrol achieved international acclaim after studies in mice and lower organisms indicated that it was responsible for a longevity effect [148]. Only mice injected with resveratrol from birth lived longer; those that started at middle age had no longevity benefit [149]. The benefit appeared due to enhanced expression of survival genes, a number of which are also expressed during caloric restriction [150].

The longevity effect has never been tested in humans, so an appropriate dose is not known nor even if a longevity benefit is likely [151, 152]. Although well absorbed, resveratrol displays low bioavailability; at least 70% of an oral 25 mg dose in human subjects was shown to appear as resveratrol metabolites in plasma, with most of the oral dose subsequently recovered in the urine [128]. Like curcumin, resveratrol is readily absorbed by enterocytes/colonocytes [153], showing potential benefit to intestinal tissues. A daily resveratrol dose of 3000 mg administered to overweight or obese men with nonalcoholic fatty liver disease (NAFLD) over 8 weeks did not significantly improve any of the features of NAFLD over placebo [154].

A review of 3650 publications on resveratrol concluded that the evidence is not sufficiently strong to justify a recommendation for resveratrol to humans beyond the dose which can be obtained from dietary sources, which is estimated to be ~4 mg daily for adults [155].

6.4. Silymarin. Silymarin, the major flavonoid complex in *Silybum marianum*, has a long history of traditional use in liver disorders [156]. Silymarin supplements claiming to target human liver detoxification mechanisms are readily available. Silibinin, the most bioactive of the complex, is insoluble in water and not lipophilic with low bioavailability of 0.73% in rats [129]. Its CD value ranks next below curcumin and third after sulforaphane. Where optimising Phase II detoxification is the desired outcome, there may be value in considering both CD values and bioavailability. Such evidence sheds considerable doubt on the likely efficacy of many such phytochemicals at doses typically found in commercially available supplements. Nevertheless, published trials show that silymarin exhibits hepatoprotective properties in humans, indicating that other mechanisms may be responsible [156, 157].

6.5. Sulforaphane. Sulforaphane's lipophilic nature and low molecular weight readily enable passive diffusion into cells [124]. It is rapidly absorbed, peaking in plasma as early as 1 hour after ingestion [158]. Predictably, dose-dependent pharmacokinetics in rats reveals that bioavailability decreases with increasing dose [124]. The doses corresponded to ~0.5 mg, 1.0 mg, and 5.0 mg/kg of pure sulforaphane which is relatively high for humans who typically consume *Brassica* vegetable and not pure sulforaphane. It is unlikely that humans through diet would ingest such high quantities of SFN. By calculation, MYR-active whole broccoli sprout supplement yielding 1% SFN could deliver 10 mg SFN per gram of powder, corresponding to ~12 grams of fresh broccoli sprouts (dried powder retains ~8% moisture). Administering 5.0 mg/kg of sulforaphane to a 70 kg human at the upper end of the animal dose range represents an intake of 350 mg or 35-fold the quantity that a human might reasonably ingest dietary fresh sprouts. Clearly, these quantities are not a practical means of providing a broccoli sprout supplement for human use.

6.6. Dose Considerations in Humans. An indication of what might be practically achievable with supplementation is illustrated by several human studies. Ye et al. showed that after a single 200 μmol oral dose of sulforaphane both sulforaphane and its metabolites were detected in plasma and erythrocytes in just 15 minutes, peaking in all four subjects at ~2.00 μM after 1 hour and declining with first-order kinetics, with a mean half-life of 1.77 ± 0.13 hours [159]. To investigate effects

in systemic tissue, Cornblatt et al. showed that, one hour after a single 200 μmol oral dose of sulforaphane administered to 8 women, metabolites were detected in resected left and right breast tissue at concentrations of 1.45 ± 1.12 and 2.00 ± 1.95 pmol/mg tissue, respectively. This proof-of-principle study observed a significant induction of NQO1 enzymatic activity in the same tissue [158]. In another example, a dose escalation placebo-controlled study investigated Phase II gene expression in human airways mucosa, showing that a 200-gram broccoli sprout homogenate delivering 102 μmol of sulforaphane increased NQO1 mRNA expression by almost 200% [160].

Given that oral doses appear to be capable of increasing NQO1, we consider whether it may be possible that a sulforaphane-yielding broccoli sprout powder might deliver a plasma concentration of ~2.00 μM. By calculation, a 1% powder yields 56.4 μmol sulforaphane per gram. Ye et al. showed that a single 200 μmol dose resulted in a peak plasma concentration of ~2.0 μM after 1 hour. As Ye et al. [159] had shown that a 200 μmol oral dose had resulted in a plasma concentration of ~2.0 μM and Riedl et al. [160] had shown that 102 μmol had increased NQO1 mRNA expression by ~200%; these orders of magnitude could be achievable with a sulforaphane-yielding broccoli sprout powder. Theoretically and by calculation, an individual could consume around 2 grams of a 1% sulforaphane-yielding broccoli sprout powder to achieve what Riedl et al. achieved with 200-gram broccoli homogenate and 4 grams to achieve what Ye et al. achieved with a single 200 μmol dose.

7. Factors Governing Sulforaphane Yield

7.1. The Role of Myrosinase. Glucosinolates as *Brassica*-derived precursor compounds are converted to their bioactive forms only under the action of MYR because GRN has no inherent bioactivity. Investigating the metabolic fate of ingested broccoli phytochemicals, Shapiro et al. showed that MYR-inactivated broccoli resulted in 10–20% lower conversion to ITCs. When the colonic microfloras were reduced, recovery of ITCs in a MYR-free environment was negligible. It may be inferred that MYR is essential for sulforaphane synthesis and that the colonic microflora may exhibit MYR-like activity.

The colonic microfloras appear to be capable of limited MYR activity, with conversion to the bioactive ITC varying from 1% to 40% of the dose [161]. Several genera of human microflora such as *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* have been reported to possess MYR-like activity [162] but with wide variability in their population; the ability to hydrolyse glucosinolates cannot be reliably estimated. So unpredictable is this factor that a large clinical trial using MYR-inactive broccoli sprout extract could not achieve statistical significance [163]. Many available broccoli sprout supplements are MYR-inactive extracts which claim their clinical benefit is due to the alleged conversion to sulforaphane by the colonic microflora. Neither consumers nor clinicians have any way of knowing if an individual harbors MYR-active microflora.

7.2. The Nitrile Factor. Among crucifers, broccoli contains significant amounts of epithiospecifier protein (ESP), a non-catalytic inhibitor of MYR activity [164]. ESP produces inactive sulforaphane nitrile. Under certain conditions, the nitrile pathway is favoured, with the hydrolysis product constituting as much as 75% nitrile. The colonic microfloras also support nitrile formation, thereby further limiting the potential of MYR-inactive supplement [165]. ESP deactivation can significantly enhance sulforaphane yield, illustrating that broccoli and broccoli sprout products cannot be meaningfully evaluated on the basis of their GRN content alone. It is likely that clinical trials using either fresh or powdered broccoli sprouts may give conflicting results when the presence or absence of nitrile has not been considered. The presence of ESP means that assayed measurement of the sulforaphane yield is critical in order to estimate the real efficacy of a broccoli sprout powder intended for a supplement; measurement only of GRN and MYR does not allow for the effect of ESP on enzyme activity.

8. Clinical Implications

8.1. Cruciferous Vegetable Consumption. The presence of unquantified amounts of ESP in raw broccoli has clinical implications; as a salad vegetable, raw broccoli may not be an efficient means of obtaining the benefits conferred by sulforaphane. Similarly, cooking has been shown to destroy the enzyme in as little as 3 minutes of steaming [166]. Five minutes of microwave cooking resulted in 74% loss of glucosinolates from broccoli florets with high-pressure cooking and boiling leading, respectively, to 33% and 55% losses [83]. Even consumers and clinicians conscious of the importance of cruciferous vegetables in the diet may be unaware that open-air storage of broccoli as occurs during transport and in retail environments may lose 55% of its glucosinolates after 3 days and storage in plastic bags at 22°C may result in similar losses over 7 days [83].

Also of significance is the fact that broccoli cultivars for vegetable production are not selected on the basis of their sulforaphane yield. It is possible that the cultivars available to consumers are not good sources of cruciferous bioactives. Until Food Law allows appropriate health claims to be associated with cruciferous vegetables, there is no incentive for growers to select higher yielding cultivars. In short, neither a clinician nor a consumer has the information needed to make an appropriate choice.

8.2. Supplements Derived from Cruciferous Vegetables. Similarly, it is not generally known if a producer of broccoli sprout powder as a supplement ingredient has deactivated the ESP. If two supplements contain high levels of GRN but one has had the ESP deactivated, the comparative sulforaphane yield from these broccoli sprout powders may be markedly different. Ideally, a sulforaphane-yielding supplement would be characterised on the basis of the various determining factors: the presence of quantifiable GRN and active MYR together with the inhibitory ESP.

These concerns are reflected in a recent study which compared a commercially available supplement labelled as

containing 30 mg "sulforaphane glucosinolate" per dose with a quantity of fresh sprouts containing the same amount of GRN [167]. The study showed that consumption of MYR-devoid broccoli supplement when compared with broccoli sprouts produced 7-fold lower plasma concentrations of the bioactive ITC metabolites in the subjects. Clarke et al. concluded that these findings have implications for people who consume the recommended dose of such MYR-inactive broccoli supplement believing they are obtaining equivalent doses of ITCs. This is significant in that the available broccoli sprout supplements are dominated by the MYR-inactive "extracts," even though MYR-active whole broccoli sprout supplements do exist.

There is a further strong case for a whole food broccoli sprout supplement on the grounds that although GRN is the primary glucosinolate found in broccoli and broccoli sprouts, it is not the only one; erucin and iberin comprise most of the remaining 25% of the glucosinolate content of broccoli. Recently, it was found that erucin and sulforaphane are interconvertible, so that the clinical effects are likely to be due to the combined effects of all the glucosinolate hydrolysis products [167].

9. Standardisation

To compound the difficulties associated with determining the clinical potential of a sulforaphane-yielding supplement, variations in nomenclature add to the problem. The term "*sulforaphane glucosinolate*" which has recently appeared in the scientific literature is now associated with and specified for commercially available MYR-inactive extracts derived from broccoli seed or sprout extracts [167, 168]. Since "sulforaphane glucosinolate" describes only the quantity of "glucoraphanin," this nomenclature could erroneously lead both clinicians and consumers to believe that the material will deliver sulforaphane when consumed.

9.1. Commercial Assay Protocols. Various methods to describe a sulforaphane supplement are commonly used in industry. To evaluate and compare different broccoli sprout powders intended as supplements or for use in clinical trials, assay methodologies must be standardised. There are several common practices for reporting the sulforaphane derived from a broccoli sprout sample but because the assay protocol is almost never specified for a commercial product there is no way to reliably compare these values from one product to another.

9.2. Sulforaphane Potential. Sulforaphane potential is a calculated value by measuring GRN and then assuming 100% conversion to sulforaphane, whether or not MYR has been retained after processing. Based on relative molecular weights, the measured amount of GRN is multiplied by 0.406 to arrive at a sulforaphane potential. No provision is made for the presence or absence of either MYR or ESP. Where ESP has not been fully deactivated, calculating sulforaphane potential will overestimate the amount of sulforaphane that could be produced on ingestion. Broccoli sprout powdered ingredients or supplements which claim sulforaphane potential and for which the ESP has not been deactivated may yield limited sulforaphane.

9.3. Sulforaphane Yield with Addition of Exogenous MYR. By adding enough exogenous MYR to ensure full conversion of GRN to sulforaphane, this method overcomes the possibility that the starting material may contain only GRN and may be completely or partially MYR-inactive. The assay results may not specify that exogenous MYR was added, so that the reader may incorrectly conclude that the material will yield sulforaphane on ingestion.

9.4. Sulforaphane Yield due to Endogenous MYR. This method more closely resembles the in vivo situation after ingestion of the supplement, in that conversion to sulforaphane is entirely dependent on the quantities of ESP and MYR retained after processing. It may provide a lower sulforaphane value when compared with the other methods, even though it may be the method which most reliably approximates sulforaphane's metabolic fate in human physiology.

The same supplement assayed by each of these procedures is likely to produce quite different results and, more importantly, only supplements which have retained MYR activity are likely to demonstrate in vivo effects. Methods which assess sulforaphane's inducer capacity in cell culture may more reliably evaluate the clinical potential of a supplement or enable comparison of supplements. PCR array and pathway analysis studies provide gene expression data which is another closer step to establishing the clinical effects of a supplement [169].

10. Conclusion

The evolving science of nutrigenomics is in many ways legitimizing the important role of plant foods in human health, not just as sources of nutrients but as a huge library of phytochemicals capable of interacting with intracellular biomolecules to influence gene expression. Of the many thousands of phytochemicals in the food supply, sulforaphane exhibits properties which may make it an ideal cytoprotective biomolecule, deliverable in practical doses as a whole food supplement. Commercial attempts to produce sulforaphane-releasing supplements have resulted mostly in forms with little or no bioactivity, typically seed or sprout extracts. The ideal sulforaphane-releasing supplement retains both its glucoraphanin precursor and its myrosinase enzyme in the form of a whole broccoli sprout ingredient with nothing but water removed. When compared with other phytochemicals widely used in dietary supplements, sulforaphane is significantly more bioavailable than polyphenols such as curcumin, resveratrol, and silymarin. It is also significantly more able to induce NQO1, a Phase II enzyme essential in the metabolism of a number of exogenous toxins, oxidized nutrients, and endogenous metabolites. Such comparative findings call into question the clinical efficacy of many of the supplements popular among consumers. Alleged benefits of such supplements appear to require much higher intracellular concentrations than can be achieved with reasonable oral intake.

Initial attempts to produce high-potency pharmaceutical Nrf2 inducers have so far been unsuccessful. Given the prevalence of diet-related disease and the evidence that many consumers have accepted a role for complementary medicines in their personal health management, appropriately validated sulforaphane-releasing supplements may provide another avenue for supporting human health. Such supplements will need to demonstrate sufficient nutrigenomic potential that they can modify key biochemical and physiological risk factors for disease.

Conflict of Interests

Christine A. Houghton is the managing director of Cell-Logic Pty Ltd., a company that manufactures a broccoli sprout ingredient. Robert G. Fassett and Jeff S. Coombes declare that they have no conflict of interests regarding the publication of this paper.

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Research Article

Polydatin Attenuates H_2O_2-Induced Oxidative Stress via PKC Pathway

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Oxidative stress plays an important role in the pathogenesis of endothelial dysfunction, which is found to precede the development of diverse cardiovascular diseases (CVDs). The aim of this study was to observe the protective effects of PD against H_2O_2-induced oxidative stress injury (OSI) in human umbilical vein endothelial cells (HUVECs) and the possible mechanism of PD in OSI treatment. HUVECs were subjected to H_2O_2 in the absence or presence of PD. It turned out that PD improved cell viability and adhesive and migratory abilities, inhibited the release of lactate dehydrogenase (LDH) and reactive oxygen species (ROS), and elevated the content of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). TUNEL, fluorometric assays, and Western blotting showed that OSI upregulated the apoptosis ratio, the activity of caspase-3 and the level of proapoptotic protein Bax and decreased the level of antiapoptotic protein Bcl-2. However, PD treatment partially reversed these damage effects and Protein Kinase C (PKC) activation by thymeleatoxin (THX) in turn eliminated the antiapoptotic effect of PD. Furthermore, PD attenuated the H_2O_2-induced phosphorylation of PKCs α and δ and increased the phosphorylation of PKC ε. Our results indicated that PD might exert protective effects against OSI through various interactions with PKC pathway.

1. Introduction

Endothelial cells maintain vascular homeostasis through inhibiting platelet aggregation, preventing adhesion of leukocytes, and limiting proliferation of vascular smooth muscle and through balancing vasodilators and vasoconstrictors to manipulate vascular tone. Endothelial dysfunction is reported to be the initial step of many cardiovascular pathophysiological progresses, such as hypertension [1], coronary artery disease [2], or heart failure [3]. Meanwhile, clinical intervention to reserve or augment the endothelium function is recommended to be an appropriated method to CVD treatments. The latest studies show that oxidative stress plays a main character in the pathogenesis of endothelial dysfunction [4], and oxidative stress induces apoptosis [5].

Polydatin, 3,4′,5-trihydroxystilbene-3-β-mono-d-glucoside, is extracted from the root stem of a traditional Chinese herbal medicine named *Polygonum cuspidatum* Sieb. A large number of evidences show that PD is involved in a wide range of biological functions, such as antiplatelet aggregation [6] and antiatherosclerosis [7]. It has been demonstrated that PD acts as antioxidant agents to prevent severe diseases. Particularly the PD induced antioxidative activity is reported to reverse renal injury by attenuating oxidative stress-related inflammatory responses in fructose-induced urate nephropathic [8]. Besides, PD could eliminate ischemia reperfusion (I/R) damage toward brain [9] and lung [10], in which the antioxidative stress ability was thought to be one of the key regulators. PD is proved to exert inhibitory effects on I/R-induced apoptosis in myocardium [11]. Resveratrol (RSV), the deglycosylation form of (PD), could mitigate oxidative stress-induced apoptosis in C2C12 cells and primary neonatal rat cardiomyocytes [12] and increase viability of dopaminergic neurons against neurotoxicity [13]. PKCs are involved in the effects of PD on cardiac ischemia [14] and hypoxic pulmonary hypertension [15]. However, whether PKC cellular circuit is

involved in the prevention by PD from H_2O_2-induced OSI has not been elucidated to date.

The Protein Kinase C (PKC) is a group of serine/threonine kinases and important intracellular signal transduction molecules. It possesses multiple functions in regulating cellular differentiation [16], proliferation [17], and apoptosis [18]. PKCs are particularly important in redox reaction because their regions are susceptible to redox modifications, since variations of redox state have consequences on their activity [19]. According to the N-terminal regulatory domains, PKCs are classified into three subfamilies: conventional (α, βI, βII, and γ), novel (δ, ε, η, θ, and μ), and atypical (ζ and λ). The expression of PKCs in cardiac tissue differs with species and cell type, but most adult mammals express PKC α, β, δ, and ε [20]. PKC α, the first found conventional PKC, is expressed at a high level in cardiovascular system and is activated in cardiomyocytes subjected to oxidative stress [21]. PKC δ is the representative of novel PKC and is widely involved in oxidative stress events [22]. Although PKCs δ and ε are highly homologous, PKC ε is known for its antioxidative ability [23] and cardioprotection in the I/R model [24] which differs from the function of PKC δ.

In this study, we investigated the role of PD in H_2O_2-induced OSI of HUVECs. Then we explored whether PD protected HUVECs against OSI injury via regulation of PKC signaling pathway. Furthermore the possible involvement of PKCs α, δ, and ε, the representative and unique isoforms, was demonstrated in PD treatment of the oxidative stress injured HUVECs.

2. Materials and Methods

2.1. Chemicals and Reagents. The PD was purchased from WeiJia Technology Company (Xi'an, China), and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], PKC pathway activator thymeleatoxin (THX), and 2′,7′-dichlorofluorescein diacetate (2′,7′-DCFH-DA) were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kits were purchased from Roche Company (Mannheim, Germany). The kits for the detection of LDH, GSH-Px, and SOD concentrations and caspase-3 activity were purchased from the Institute of Jiancheng Bioengineering (Nanjing, Jiangsu, China). The PKC α, PKC δ, PKC ε, Bcl-2, Bax, and GAPDH antibodies were purchased from Bioss (Beijing, China). The p-PKC α/β (Thr638/641) and p-PKC δ (Tyr311) antibodies were purchased from Proteintech Group (Chicago, USA) and the p-PKC ε (Ser729) antibody was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). The secondary antibodies (goat anti-rabbit and anti-mouse) were purchased from Zhongshan Company (Beijing, China).

2.2. Cell Culture and Treatments. HUVECs (ATCC CRL-1730; Shanghai Tiancheng Technology Company) were cultured in RPMI 1640 medium (Hyclone, Utah, USA) containing heat-inactivated 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL (100 U/mL) streptomycin at 37°C in 5% CO_2. The PD stock solution was prepared in 1% dimethylsulfoxide (DMSO) and diluted with culture medium immediately prior to the experiment. The cells were treated with H_2O_2 (400 μM) [25] alone or in combination with PD for 4 h. After the treatments, the cells were prepared for further studies.

2.3. Analysis of Cell Viability and Morphology. The viability of HUVECs was assessed using the MTT assay referring to a previous study [25]. Briefly, cells were seeded in 96-well plates. After being treated with H_2O_2 and PD of different concentrations for 4 h, the cells were washed for 3 times with PBS, then 100 μL of MTT solution (0.5 mg/mL) was added directly to the culture medium, and the cells were incubated for 4 h at 37°C. After MTT removal, 100 μL of N,N-dimethylformamide was added to each well to dissolve the formazan crystals, and the plates were shaken for 15 min at 37°C. Absorbance of samples was measured at 490 nm using a Microplate Reader (SpectraMax 190, Molecular Device, USA), and the results were expressed as % cell viability by the analysis of an OD value.

2.4. Cell Adhesion Assay. Cell adhesion test was assessed as previously described [26]. After different treatments, cells (1×10^4 cell per well) were resuspended in culture medium, placed on fibronectin-coated 6-well plates, and incubated for 30 min at 37°C. Then the cells were gently washed with PBS for 3 times. The adherent cells were counted by independent and blinded investigators. The results were expressed as the percentage of control.

2.5. Wound Healing Assay. As previously described [27], HUVECs were seeded in 6-well plates and cultured for 24 h. Subsequently, we scratched confluent cell monolayers with a P200 pipette tip to make three parallel "wounds" in each well and washed the cells with serum-free medium. After the cells were incubated with different treatments for 8 h, the migrated cells were photographed using an inverted/phase-contrast microscopy (Olympus BX61, Japan). The mean distance between the two ends of the scratch was quantified by manual measurements.

2.6. Measurement of the Content of ROS. Measurement of intracellular ROS was based on ROS-mediated conversion of nonfluorescent 2′,7′-DCFH-DA into fluorescent DCFH, as previously described [25]. After being seeded and incubated with different treatments for 4 h in black 96-well plates, the cells were washed with PBS and subsequently incubated with DCFH-DA (20 microM) in PBS at 37°C for 2 h. At the end of incubation, the fluorescence of the cells was measured using an FLX 800 microplate fluorescence reader (the emission wavelength, 530 nm; the excitation wavelength, 485 nm) (Biotech Instruments Inc., USA). The background was from cell-free conditions. The results were expressed as the fold of the fluorescence intensity compared to the control group.

2.7. Measurement of LDH Release. LDH release, which could reflect the cell membrane integrity, was detected with an assay kit (A020-1, Jiancheng, Nanjing, China) according to

the manufacturer's instructions. Briefly, after incubation with different treatments for 4 h, the cells were centrifuged at 300 ×g for 10 min. Then 60 μL supernatant was added with 30 μL LDH substrate solution for incubation for 30 min at 37°C. The activity of enzyme was expressed as units per liter, and the absorbance was read at 440 nm.

2.8. Measurement of the Intracellular Contents of GSH-Px and SOD. Commercially available kits for the measurement of SOD (A001-3, Jiancheng, Nanjing) and GSH-Px (A061-2, Jiancheng, Nanjing, China) were used according to the manufacturer's instructions. After being lysed by the compound lysis buffer and centrifuged at 12000 r/mim for 5 min, the cell supernatant was collected for detecting the contents of GSH and SOD that were expressed as units/mg protein. One unit of SOD was equal to the amount that reduced the absorbance by a half at 550 nm. The GSH-Px activity assay was performed by quantifying the oxidation rate of reduced GSH to oxidized form by H_2O_2 and catalyzed by GSH-Px. One unit of GSH-Px was equal to the amount that reduced the level of GSH at 412 nm by 1 mM in one min per mg protein.

2.9. Cellular Apoptosis Assay. The apoptosis of HUVECs was analyzed by performing a TUNEL assay using a commercial kit (11684817910, Roche Company, Germany) according to the manufacturer's instructions. After being fixed in paraformaldehyde (4%) for 24 h, the nuclei of the apoptotic cells were stained green, and all of the nuclei were stained blue with 4′,6-diamino-2-phenylindole (DAPI). The TUNEL positive ratio was expressed as the ratio of positively stained cells to the total number of HUVECs.

2.10. Measurement of Caspase-3 Activity. Apoptotic cell death was determined by caspase-3 activation via a fluorometric kit (G007, Jiancheng, Nanjing, China). Briefly, after being seeded and treated in 96-well plates, endothelial cells were harvested using caspase lysis buffers (50 mM HEPES, pH 7.4, 0.1% Chaps, 5 mM dithiothreitol, 0.1 mM EDTA, and 0.1% Triton X-100) for 5 min on ice and centrifuged at 12000 rpm for 10 min at 4°C. Then the supernatant (50 μg) was saved and incubated with 10 μL caspase-3 substrate (Ac-DEVDpNA) for 1 h at 37°C. The fluorescence emission was measured using a Biotech microplate fluorescence reader at an excitation wavelength of 400 nm (emission wavelength, 505 nm). The fluorescence intensity was set as 100% in the control group.

2.11. Western Blot Assay. HUVECs were washed with PBS and lysed with 100 μL RIPA Lysis buffer (Beyotime, Shanghai, China, P0013C) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% SDS for 30 min on ice. Then cells were scraped and centrifuged for 10 min at 4°C and the concentration was qualified with a BCA protein Assay Kit (Lot#PI208677, Thermo, Rochford, USA). Proteins of a total content of 50 μg were separated on SDS-polyacrylamide gel electrophoresis gels (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and then incubated with primary antibodies (PKC δ and PKC ε with a dilution of 1 : 2000, PKC α, p-PKC ε, Bcl-2, Bax and GAPDH with a dilution of 1 : 1000, p-PKC α/β with a dilution of 1 : 300, and p-PKC δ with a dilution of 1 : 100) and horseradish peroxidase-conjugated secondary antibody (Zhongshan Jinqiao, Beijing). The blot was developed with a Supersignal Chemiluminescence detection kit (Solarbio, Beijing) and observed with ChemiDoc XRS+ Molecular Imager (BIO-RAD).

2.12. Statistical Analysis. All values in the text and figures are presented as mean ± SEM of at least three independent experiments. Analysis of variance of parametric difference among experimental data was performed by one-way ANOVA and Dunnett's test was then performed for multiple comparisons [16]. Western blot densities were analyzed with Kruskal-Wallis ANOVA test [28]. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of PD on the Cell Viability, Adhesive Ability, and Migratory Ability of OS-Injured HUVECs. To investigate whether PD could prevent endothelium from OSI, HUVECs were subjected to H_2O_2 (400 μM) in the absence or presence of PD of various concentrations (0.1–10 μg/mL) for 4 hours. The cell viability decreased to 48% after H_2O_2 treatment. Although the difference between H_2O_2 group and the low dose group (0.1 μg/mL) was not manifest, the cell viability elevated to 72% when the concentration of PD was 3 μg/mL or higher (Figure 1(a)). Based on these results, treatment with 400 μM H_2O_2 and 3 μg/mL PD for 4 hours was selected for the further experiments. As shown in Figure 1(b), H_2O_2 treatment resulted in the change of cell morphology, such as remarkable cell shrinkage and reduced refraction. Meanwhile, the cellular adhesion rate in the H_2O_2 group was reduced by more than 50% compared to the control group. The PD treatment significantly improved the cellular adhesion rate but the cell shape did not evidently recover compared to the H_2O_2 group. As demonstrated in Figure 1(c), the distance between the scratches increased significantly after treatment with H_2O_2 compared to the control group but curtailed almost 40% after PD treatment compared to the H_2O_2 group, indicating that PD enhanced the migratory ability of HUVECs injured by H_2O_2.

3.2. Effects of PD on LDH, ROS, GSH-Px, and SOD Measured in OS-Injured HUVECs. To explore the effects of PD on the oxidative stress of H_2O_2-injured HUVECs, we next measured the levels of intracellular LDH, ROS, GSH-Px, and SOD. H_2O_2 treatment elevated the level of LDH to 5 times (Figure 2(a)), doubled ROS release (Figure 2(b)), and reduced the content of GSH-Px and SOD by a half (Figures 2(c) and 2(d)) compared to the control group. However, PD replenishment induced a remarkable decrease in the level of LDH by more than 50% (Figure 2(a)) and ROS by 18% and significantly attenuated the changes in the content of GSH-Px by 33% (Figure 2(c)) and SOD by 60% (Figure 2(d)) compared to the H_2O_2 group, indicating the protective activity of PD.

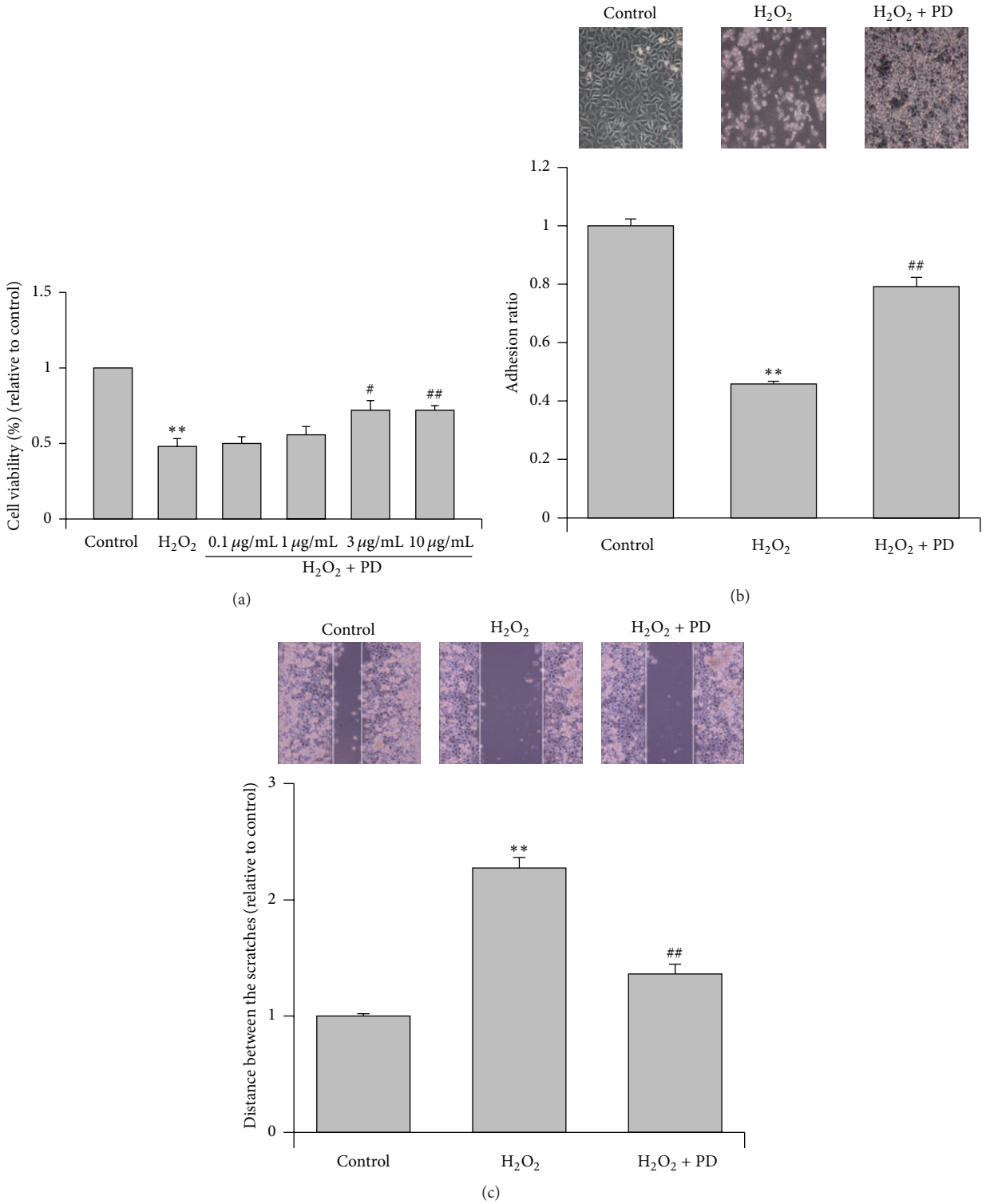

Figure 1: Effects of PD on the viability, adhesive ability, and migratory ability of OS-injured HUVECs. (a) HUVECs were subjected to H_2O_2 (400 μM) in the absence or presence of PD of different concentrations for 4 hours. The cell viability of HUVECs was measured by the MTT assay. (b) The adhesive ability of the HUVECs was assessed by performing an adhesion assay, and cell adhesion was expressed as an adhesion ratio. The number of adherent cells in the control group was set at 100%. The cell morphology was observed using inverted/phase-contrast microscopy, and images were obtained. (c) The migratory ability of the HUVECs was assessed by performing a wound healing assay, and the migratory ability was expressed as the mean distance between the two ends of the scratch. The mean distance in the control group was set at 100%. The results are expressed as mean ± SEM, $n = 3$ ($^{**}p < 0.01$, compared to the control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to the H_2O_2 group). Significant differences between groups were analyzed with one-way ANOVA.

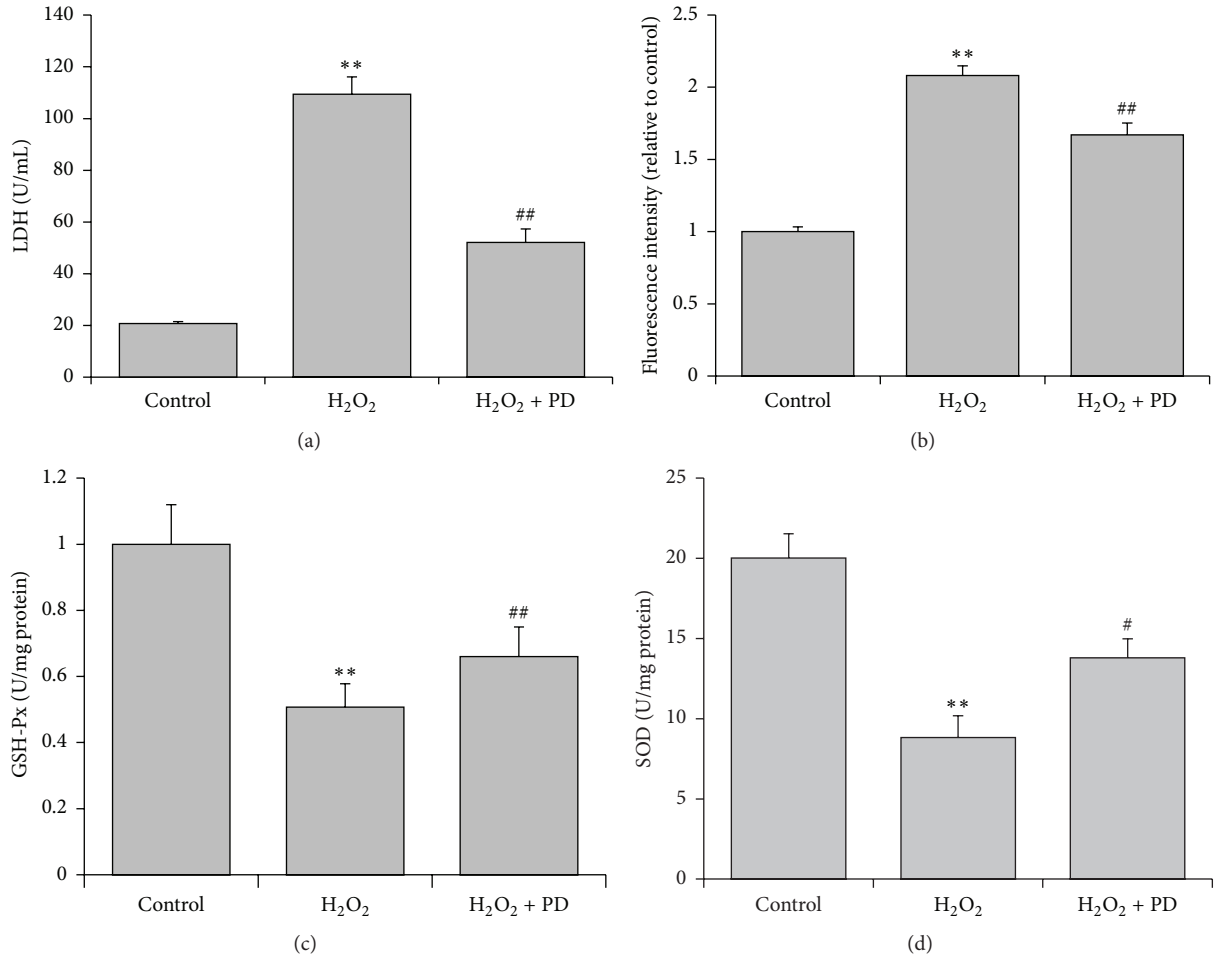

Figure 2: Effects of PD on LDH, ROS, GSH-Px, and SOD in OS-injured HUVECs. HUVECs were treated with H_2O_2 (400 μM) alone or with PD (3 μg/mL). (a) The release of LDH from HUVECs was detected using an assay kit. (b) The intensity of DCFH fluorescence was measured to evaluate the intracellular ROS level. (c) The intracellular GSH-Px level of HUVECs. (d) The intracellular SOD level of HUVECs. The results are expressed as mean ± SEM, $n = 3$ ($^{**}p < 0.01$, compared to control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to H_2O_2 group). Significant differences between groups were analyzed with one-way ANOVA.

3.3. Effects of PD and THX on the Apoptosis of OS-Injured HUVECs. The antiapoptotic effect of PD has been demonstrated in previous studies [11, 12]. We therefore explored whether PD could attenuate the apoptosis of H_2O_2-injured HUVECs and the possible involvement of PKC signaling. As shown in Figure 3(a), the TUNEL positive ratio increased from less than 1% to 50% after H_2O_2 treatment and decreased to 25% when PD was replenished. Furthermore, the activity of caspase-3 increased by 200% after H_2O_2 treatment compared to the control group and decreased by almost 30% when PD was added (Figure 3(b)). Nonetheless, after the forced activation of PKC by THX (100 nM) [29], the apoptosis ratio and caspase-3 activity increased to a degree even higher than those of the H_2O_2-treated group (Figures 3(a) and 3(b)). In line with this observation, the attenuated expression of proapoptotic molecule Bax and the corresponding enhanced expression of antiapoptotic signal Bcl-2 were noticeable when the cells were treated with H_2O_2 and PD compared to the H_2O_2 group, whereas THX treatment suppressed the effects of PD on Bax and Bcl-2 expression (Figure 3(c)).

3.4. Effects of PD on Phosphorylation Level of PKC in OS-Injured HUVECs. Our above data collectively raise the possibility that PD may exert its antiapoptotic effect on endothelial cells through PKC dependent pathway. Since PKC α and PKC δ have been reported to be activated by oxidative stress [30] and PKC ε is an important molecule in the protection of OSI [24], we determined whether PD attenuates OSI in HUVECs through regulation of these three PKCs. As shown in the left panel of Figure 4, HUVECs expressed all the three isoforms of PKC. No obvious differences in the total amount of PKCs α, δ, and ε were observed among the three groups as indicated in the left panel. It is worth noting that the level of proteolytic fragment of PKC δ (41 kDa) significantly increased along with H_2O_2 stimulation (<0.01) but abated remarkably after PD incubation (<0.05). A ratio of phospho-PKC to the total PKC was calculated to evaluate the activation of PKC upon stimulation to oxidative stress (the right panel of Figure 4). An increased phosphorylation level of both PKC α (by 65%) and PKC δ (by 100%) was observed after H_2O_2 treatment, while PD replenishment was able to decrease the

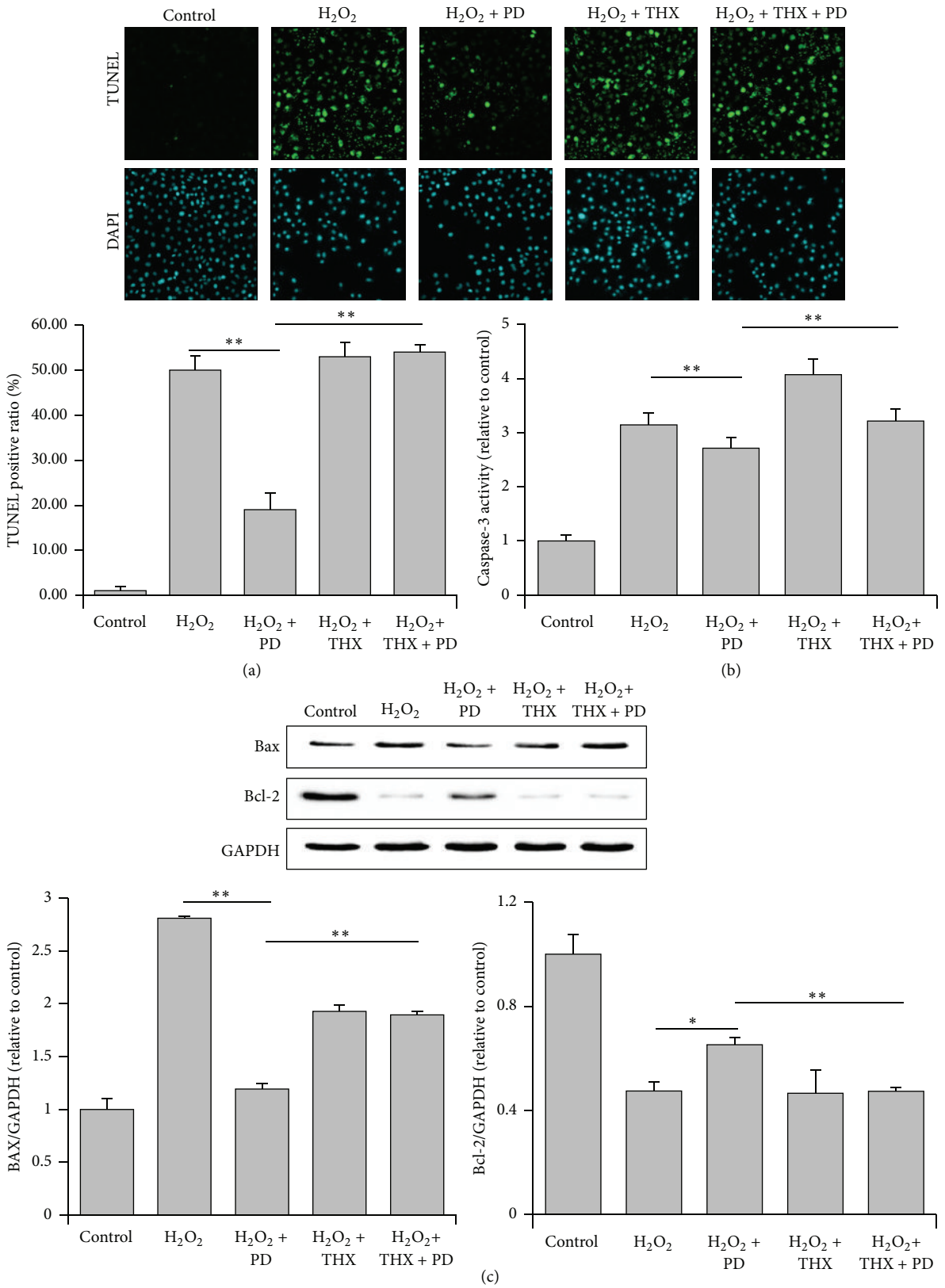

Figure 3: Effects of PD and THX on the apoptosis of OS-injured HUVECs. (a) The apoptosis of the HUVECs was assessed by the TUNEL assay. TUNEL staining was performed to stain the nuclei of the apoptotic cells (green), and DAPI was used to stain all of the nuclei (blue). The apoptotic index was expressed as the percentage of positively stained apoptotic cells out of the total number of cells counted. (b) The caspase-3 activity was measured via a fluorometric kit. (c) The Western blots images of Bcl-2 and Bax are, respectively, shown in the upper panel. The lower panels represent the densitometric analysis of the data. GAPDH was used as a loading control. The results are expressed as mean ± SEM, $n = 3$ ($^{*}p < 0.05$, $^{**}p < 0.01$). Significant differences between groups were analyzed with one-way ANOVA.

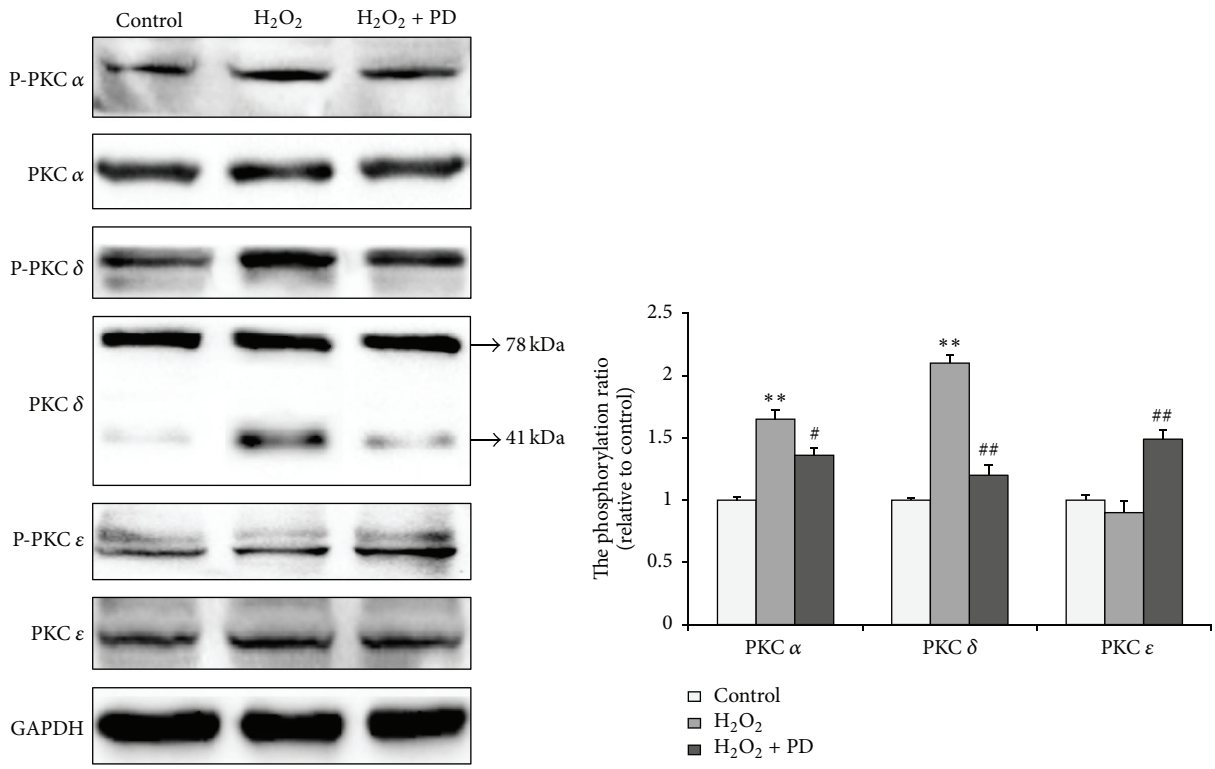

Figure 4: Effects of PD on the levels of p-PKC δ of OS-injured HUVECs. The Western blots images of p-PKC α, p-PKC δ, p-PKC ε, and total PKCs α, δ, and ε are shown in the left panel. The right panel represents the densitometric analysis of the data. Expression of each p-PKC was normalized to total PKC content, and GAPDH was used as a loading control. The result are expressed as mean ± SEM, $n = 3$ ($^{**}p < 0.01$, compared to the control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to the H_2O_2 group). Significant differences between groups were analyzed with one-way ANOVA.

phosphorylation level of PKC α (by 17.6%) and PKC δ (by 42.8%) compared to the H_2O_2 group. As to the content of p-PKC ε, little difference was found between the control group and H_2O_2 single treated group (>0.05). But compared with H_2O_2 group, the level of p-PKC ε was increased by 50.5% after being treated in combination with PD compared to H_2O_2 single treated group.

4. Discussion

It is well documented that endothelial dysfunction is a crucial event in the development of CVDs including atherosclerosis, myocardial ischemia reperfusion, and hypertension. The endothelial cells are very sensitive to OSI, which is involved in endothelial dysfunction [31]. In the present study, we established H_2O_2-induced OSI model to study the protective effect of PD against endothelial damage. We confirmed that PD conferred protection to HUVECs against H_2O_2 by improving cell viability and adhesive and migratory abilities. These findings concur with previous studies where resveratrol, the deglycosylation form of PD, significantly attenuates the decrease of cell viability in OS injured HUVECs [32] and the methamphetamine-induced neurotoxicity in mouse mesencephalic neurons [13] and evidently enhance activities of proliferation, adhesion, and migration of endothelial progenitor cells [33]. Thus, our results suggest that supplementation with PD could ameliorate endothelial dysfunction induced by OSI.

Oxidative stress results from an imbalance between oxidants and antioxidants within cells [4]; namely, the enhanced ROS overwhelms the antioxidative capacity and subsequently leads to endothelial dysfunction [34]. GSH-Px, SOD, and other enzymatic and nonenzymatic antioxidants play pivotal roles in preventing the cellular damage caused by ROS. In the present study, an obvious elevation of LDH and ROS production along with significant decrease in GSH-Px as well as SOD was observed in HUVECs after subjected to H_2O_2, indicating the shift of the balance between oxidative systems and antioxidant defenses. Nonetheless, when HUVECs were supplied with PD, the changes of these indexes induced by H_2O_2 were partially reversed. This is in agreement with previous reports confirming that administration of PD significantly limits LDH release from the I/R infracted myocardial tissue [14] and elevates the activity of SOD in I/R injured lung [10]. Similarly, PD has also been reported to exert antioxidative activity in kidney [8] and brain [9] as indicated before. These results clearly suggest that PD is a potent antioxidant against OSI related diseases.

Given that PD has antiapoptotic abilities [35, 36], we confirmed these findings of the change of several apoptosis related indexes including TUNEL staining positive ratio, caspase-3 activity, and Bax and Bcl-2 levels. Here, we found that the treatment with PD could significantly restrain H_2O_2-induced apoptosis, as verified by the reduction

of TUNEL positive ratio, the caspase-3 activity, and Bax expression as well as by the upregulation of Bcl-2 expression. There is abundant evidence to support that PKC signaling plays a crucial role in OSI events. For instance, PKC is activated in redox stress in cardiac muscle cells [37] and endothelial cells [38]. As expected, the aforementioned antiapoptotic effect of PD vanished after PKC consistent activation by THX. Collectively, these results indicated that forced activation of PKC signaling could abolish the antiapoptotic effects of PD under oxidative stress condition. Our study demonstrated for the first time that PD may suppress apoptosis in H_2O_2-injured HUVECs through inhibiting PKC signaling pathway. These findings are in line with other studies, in which PD exerts protective effects against myocardial I/R and pulmonary hypertension through interaction with PKC signaling [14, 15].

Among PKC isoforms, PKCs α, β, δ, and ε are mainly involved in the pathogenesis of CVDs [20]. In the present study, conventional isoform α, novel isoforms δ and ε, and their phosphorylation were examined in HUVECs. PKC α has been validated to enhance superoxide production via NADPH oxidase [39] and to be activated by reactive oxygen species production in endothelial cells, which in turn activated the apoptosis pathway [40]. Upon activation, PKC δ mediates oxidative stress and cell death in a dietary model of nonalcoholic steatohepatitis [22] and in endothelia cells of lungs [41]. It is of special interest to investigate whether PD attenuates OSI in HUVECs through inhibition of PKCs α and δ cellular circuit. Our observation of significant phosphorylation of PKCs α and δ in HUVECs after exposure to H_2O_2 is consistent with the previous results obtained in COS-7 cells [30].

Meanwhile, as our results showed, the phosphorylation of PKCs α and δ decreased with PD replenishment. Interestingly, an enhanced 41 kDa proteolytic fragment below the total PKC δ protein band appeared when the cells were subjected to H_2O_2. This proteolytic fragment is probably caused by the elevated caspase-3 activity during H_2O_2 stimulation, which is consistent with other articles showing that caspase-3 cleaves the native PKC δ into a 41 kDa catalytically active fragment and a 38 kDa regulatory fragment to keep activating the kinase [42, 43]. The cleavage fragment has high catalytic activity and plays a key role in promoting apoptotic cell death. Thus, attenuation of PKC δ proteolytic activation may be also involved in the antiapoptotic effect of PD.

PKC ε, another member of novel PKC subfamily, is highly homologous to PKC δ. However, PKC ε and PKC δ sometimes have opposite effects. For example, PKC ε activation involved in isoflurane pretreatment could activate downstream signaling pathways and exert cardioprotection, which is directly opposed to that of PKC δ [24]. As shown in our results, there was little difference in PKC ε phosphorylation between the control group and H_2O_2-treated group, but the p-PKC ε significantly increased after being treated with PD. Similarly, previous research has shown that decrease in the apoptosis of cardiocytes damaged by H_2O_2 is partially attributed to the increased p-PKC ε [44]. Altogether, these studies reveal that PD protects H_2O_2-induced OSI through activation of PKC ε.

5. Conclusions

In summary, the present study demonstrates that PD could prevent HUVECs from H_2O_2-induced OSI, as evidenced by improvements of cell viability, adhesive and migratory ability, the rebalance between intracellular oxidants and antioxidants, and the inhibition of apoptosis. Nevertheless, the protective effects of PD are totally reversed when PKC signaling is compulsively activated by THX. Moreover, the phosphorylation of PKCs α and δ significantly decreases while the phosphorylation of PKC ε conspicuously increases after PD replenishment. Hence, PD may exert its antioxidative stress effects on H_2O_2-injured HUVECs through PKC dependent pathway.

Conflict of Interests

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

Authors' Contribution

Huilian Qiao and Hao Chen contributed equally to this work.

Acknowledgments

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Research Article

Effects of *Bauhinia forficata* Tea on Oxidative Stress and Liver Damage in Diabetic Mice

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This study was designed to evaluate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (BF) tea on oxidative stress and liver damage in streptozotocin (STZ)-induced diabetic mice. Diabetic male mice have remained 30 days without any treatment. BF treatment started on day 31 and continued for 21 days as a drinking-water substitute. We evaluated (1) BF chemical composition; (2) glucose levels; (3) liver/body weight ratio and liver transaminases; (4) reactive oxygen species (ROS), lipid peroxidation, and protein carbonylation in liver; (5) superoxide dismutase (SOD) and catalase (CAT) activities in liver; (6) δ-aminolevulinate dehydratase (δ-ALA-D) and nonprotein thiols (NPSH) in liver; (7) Nrf2, NQO-1, and HSP70 levels in liver and pancreas. Phytochemical analyses identified four phenols compounds. Diabetic mice present high levels of NQO-1 in pancreas, increased levels of ROS and lipid peroxidation in liver, and decrease in CAT activity. BF treatment normalized all these parameters. BF did not normalize hyperglycemia, liver/body weight ratio, aspartate aminotransferase, protein carbonyl, NPSH levels, and δ-ALA-D activity. The raised oxidative stress seems to be a potential mechanism involved in liver damage in hyperglycemic conditions. Our results indicated that BF protective effect could be attributed to its antioxidant capacity, more than a hypoglycemic potential.

1. Introduction

Historically, basic therapy for treating several diseases includes the use of medicinal plants. Vegetable species with medicinal power have considered complex mixtures of biologically active products, and usually many of them are responsible for their biological properties [1]. Therefore, many plants considered medicinal have been used in folk medicine to treat *diabetes mellitus* (DM) [2]. Among these is *Bauhinia forficata* (BF) (Leguminosae, Fabaceae), popularly known as "paw of cow" [3].

In Brazil, the tea (infusion) of BF leaves is an important alternative treatment for people with DM [2]. The BF genus comprises about 300 species found especially in the tropical regions of the planet [3]. Besides their possible hypoglycemic potential, considerations about the antioxidant and hepatoprotective activities of some *Bauhinia* species have been postulated. For example, extracts of *Bauhinia forficata* Link and *Bauhinia cheilandra* showed antidiabetic activity in STZ and alloxan-induced diabetic rats [4–6]. Already, the antioxidant and hepatoprotective activity was previously demonstrated for *Bauhinia forficata* Link, *Bauhinia racemosa*

Lam, and *Bauhinia variegata* [7–9]. However, we did not find in scientific literature studies with mice or rats that investigate the same *Bauhinia* species that we use here (*Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin).

Biological properties of *Bauhinia* species have been attributed to its phenolic compounds. In this context, *Bauhinia forficata* Link subsp. *pruinosa* are able to scavenge reactive oxygen species (ROS) because it contains flavonoids among its constituents (especially derivatives of quercetin and kaempferol) [10, 11]. These characteristics can be extremely important in diseases where there is an increase in oxidative stress, as in DM and its complications.

Indeed, chronic hyperglycemia in DM has related to a bigger ROS production and severe oxidative damage in different tissues, including the liver (for a review see [12]). Increased ROS has been known to induce changes in expression and activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), as well as thiol oxidation and lipid peroxidation [12]. Furthermore, previous reports showed that, in experimental models of DM, the sulfhydryl-containing enzyme δ-aminolevulinate dehydratase (δ-ALA-D) was inhibited [13–15].

Moreover, increases in ROS production, both in liver and in pancreas, stimulate expression of factors related to cellular antioxidant response, such as NF-E2-related nuclear factor erythroid-2 (Nrf2), NADPH quinone oxidoreductase 1 (NQO-1), and heat shock protein 70 (HSP70) [16, 17]. According to Yeo et al. [18], antioxidant and chemical stress, including chemical DM induction in mice, increases NQO-1 expression.

Therefore, the aim of this study was to evaluate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin (infusion) (BF tea) treatment on oxidative stress and liver damage in diabetic mice. Among the parameters evaluated were the glycaemia, ROS production, lipid peroxidation, protein carbonylation, and nonprotein thiols levels in liver, as well as the activities of enzymes δ-ALA-D, SOD, and CAT in liver. Moreover, we evaluate the Nrf2, NQO-1, and HSP70 expression in liver and, additionally, in pancreas.

2. Materials and Methods

2.1. Chemicals. Sigma-Aldrich Chemical Co. (St. Louis, MO) supplied Ellman's reagent (DTNB) and streptozotocin. Labtest (Minas Gerais, Brazil) supplied commercial kits. Other reagents were obtained from local suppliers.

2.2. Bauhinia forficata (BF) Preparation. BF leaves were collected in September (spring) of 2014 in southern Brazil ($29^\circ 44' 58.8'' S$ $57^\circ 05' 01.7'' W$). Botanical identification of BF leaves samples was confirmed and a voucher specimen (number ICN 167491; *B. forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin) was deposited at ICN Herbarium of Federal University of Rio Grande do Sul (Brazil).

BF tea was prepared with naturally dried leaves in a 1 mg/mL proportion (weight of dried leaves/volume of water), described by Salgueiro et al. [11].

2.3. Chromatographic Analyses. Chromatographic analyses by HPLC were conducted, described in [11, 19], using a Prominence Liquid Chromatograph (Shimadzu, Kyoto, Japan). This apparatus is equipped with an SLC-10A controller, LC-20AD pump, SIL-10AF autosampler, and SPD-M10A PDA detector. An ODS-Hypersil Thermo Scientific C18 column (250 × 4.6 mm i.d., 5 μm particle size) (Bellefonte, United States) was used. Mobile phase consisted of water containing 0.05% phosphoric acid (A) and acetonitrile (B) at a flow rate of 0.8 mL min^{-1} using the following gradients: 0.1–23 min, 10–40% of solvent B in A and 23.01–40 min, 10% solvent B, and 90% solvent A. Detection was done on a diode array detector (DAD) set at 340 nm and the injection volume was 20 μL. The HPLC system was operated at $25 \pm 1^\circ C$. Runs were made in triplicate. The reference standard chemical composition for BF tea was established previously by our group, identifying the compounds quercetin-3-O-(2-rhamnosyl) rutinoside, kaempferol-3-O-(2-rhamnosyl) rutinoside, quercetin-3-O-rutinoside, and kaempferol-3-O-rutinoside [11, 19].

2.4. Diabetes Mellitus (DM) Induction and BF Treatment. Committee on the Ethics of Animal Experiments approved this study (permit number 001/2012). All experiments were conducted with the minimum number of animals and in obedience to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology. Animals were maintained in an enriched environment with a room-controlled temperature, 12 h light-dark cycle, and food and water available *ad libitum*.

Three-month male Swiss albino mice (30–35 grams) were divided into four different groups with six animals for each group:

(1) Control received only citrate buffer intraperitoneally (*i.p.*) and drank water throughout the period.

(2) DM received a single STZ dose (150 mg/kg) *i.p.* and drank water throughout the period.

(3) BF received only citrate buffer *i.p.*, drank water during 30 days, and afterwards drank BF tea (1 mg/mL) during 21 days.

(4) DM + BF received a single STZ dose (150 mg/kg) *i.p.*, drank water during 30 days, and afterwards drank BF tea (1 mg/mL) during 21 days.

STZ was freshly prepared in citrate buffer (0.05 M, pH 4.5), and before STZ administration the animals were fasted for a period of four hours. STZ dose was established, proposed by Animal Models of Diabetic Complications Consortium [20], in order to induce a severe hyperglycemia in mice. Five days after STZ injection the hyperglycemia was confirmed by collecting a tail drop of blood and using ACCU-Check Active (Roche Diagnostics) glucometer.

The BF concentration (approximately 313 mg/kg of body weight) was established after evaluation of liquid intake of diabetic mice in metabolic cage (9.4 ± 2.24 mL of tea per day) and body weight mean (0.030 kg). This dose is in accordance with previous studies that investigated the hypoglycemic and hepatoprotective activity of other *Bauhinia* species [4–6, 8, 9].

BF treatment started on day 31 and continued for 21 days in drinking water.

2.5. Tissue Preparation for Biochemical Analyses. After the period of treatment, animals were killed by cardiac puncture. This procedure was performed under enough ether anesthesia to ameliorate mice suffering. Mice livers were removed and carefully washed, and part of them were weighted and homogenized in 1 : 10 ratio of tissue to cold NaCl (0.9%). The homogenates were centrifuged at 4000 g for 10 min at 4°C and the supernatants (S1) collected for biochemical analyses. All the biochemical analyses were performed in the same day of euthanasia. The liver and body weight were used to evaluate the liver weight/body weight ratio.

2.6. Analysis of Glucose Levels and Liver Transaminases. Blood was collected in heparinized tubes by cardiac puncture after fasting for 6 hours. After centrifugation, levels of glucose and liver transaminases were determined in plasma using a commercial kit (Labtest, Minas Gerais/Brazil).

2.7. Assessment of 2,7-Dichlorofluorescein (DCFH) Oxidation. Indirect quantification of reactive oxygen species (ROS) production was determined in S1 samples by evaluation of dichlorofluorescein reactive species (DCF-RS) levels, proposed by Myhre et al. [21]. Briefly, an aliquot of S1 (100 μL) were added to a medium containing Tris-HCl buffer (0.01 mM, pH 7.4) and DCFH-DA (7 μM). This medium was incubated in the dark for 1 h until fluorimetric analysis (Ex: 488 nm; Em: 520 nm). The results were stated as DCF fluorescence intensity, corrected by protein content, and expressed as percentage of control.

2.8. Thiobarbituric Acid Reactive Species (TBA-RS) Levels. Lipid peroxidation was assayed by adding S1 samples (100 μL) to a medium containing 8.1% sodium dodecyl sulfate, acetic acid buffer (pH 3.5), and 0.8% aqueous solution of thiobarbituric acid, proposed by Ohkawa et al. [22]. After heating at 95°C for 60 min, the red pigment produced was measured spectrophotometrically at 532 nm. The results were calculated using a standard curve constructed with malondialdehyde (MDA) at known concentrations and corrected by protein content. The results were expressed as nanomoles of MDA per milligram of protein.

2.9. Protein Carbonyl Levels. Protein carbonyl was measured in S1 samples, proposed by Levine et al. [23]. Briefly, an aliquot of S1 (200 μL) were derivatized using 2,4-dinitrophenylhydrazine (DNPH). DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with an ethanol/ethyl acetate mixture (1 : 1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution. Protein carbonyl levels were determined spectrophotometrically at 370 nm, against blanks. The results were calculated using the molar extinction coefficient of DNPH, corrected by protein content, and expressed as nanomoles of carbonyl per milligram of protein.

2.10. Superoxide Dismutase (SOD) Enzyme Activity. SOD enzyme activity was determined in S1 samples, proposed by Kostyuk and Potapovich [24]. This method is based on the capacity of SOD in inhibiting quercetin autooxidation. Briefly, S1 aliquots (25 μL) were added to a medium containing 0.016 M phosphate buffer, 0.8 mM N,N,N',N'-Tetramethylethylenediamine, and 0.08 mM EDTA (final pH of the medium was 10). The kinetic analysis of SOD was measured spectrophotometrically at 406 nm after quercetin addition (1.5 mg of quercetin in 10 mL of N,N-Dimethylformamide). The results were corrected by protein content and expressed as unit per milligram of protein. One unit of SOD activity is defined as the amount of enzyme that inhibited the quercetin oxidation reaction by 50% of maximal inhibition. Fifty percent inhibition was produced by approximately 1.5 ng/mL of pure enzyme [17].

2.11. Catalase (CAT) Enzyme Activity. CAT enzyme activity was determined in S1 samples, proposed by Aebi [25]. This method is based on the rate of hydrogen peroxide (H_2O_2) degradation by the action of CAT. Briefly, S1 aliquot (20 μL) was added to a medium containing potassium phosphate buffer (50 mM, pH 7.4) and H_2O_2 (1 mM). The kinetic analysis of CAT was measured spectrophotometrically at 240 nm after H_2O_2 addition. The results were calculated using the molar extinction coefficient of H_2O_2, corrected by protein content, and expressed as nmol H_2O_2/mg protein/min.

2.12. Delta-Aminolevulinic Acid Dehydratase (δ-ALA-D) Enzyme Activity. δ-ALA-D enzyme activity was determined in S1 samples, according to Sassa [26]. This method is based in analysis of porphobilinogen (PBG) formation after δ-aminolevulinic acid addition. Briefly, S1 samples (100 μL) were mixed with δ-aminolevulinic acid (12 mM initial concentration). The tubes were incubated for 60 min at 37°C. The reaction was stopped by adding 10% trichloroacetic acid with 10 mM mercuric chloride. After centrifugation, an equal volume of Ehrlich reagent was added to the supernatant, and absorbance at 555 nm was recorded. The results were corrected by protein content and expressed as nanomoles of PBG per milligram of protein per hour of incubation.

2.13. Nonprotein Thiols (NPSH) Levels. NPSH levels were determined according to Ellman [27]. Briefly, the S1 samples were precipitated with 10% trichloroacetic acid (1 : 1) and centrifuged at 4000 g for 10 min at 4°C to obtain supernatants (S2). S2 (100 μL) samples were added to a medium containing phosphate buffer (TFK 0.25 mM, pH 7.4), and Ellman reagent (DTNB 1 mM). The yellow pigment produced was measured spectrophotometrically at 420 nm. The results were calculated in relation to a standard curve constructed with glutathione (GSH) at known concentrations and corrected by protein content. The results were expressed as nanomoles of SH per milligram of protein.

2.14. Protein Content Determination. Protein content was determined in S1 samples, proposed by Bradford [28], and measured spectrophotometrically at 595 nm. Bovine serum

albumin at known concentrations was used to construct a standard curve.

2.15. Western Blot Analysis. Western blotting was performed according to Posser et al. [29] with minor modifications. Part of the liver and pancreas were homogenized at 4°C in a medium containing 50 mM Tris, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride, and protease inhibitor cocktail (Sigma, MO), pH 7.0. The homogenates were centrifuged at 1000 g for 10 min at 4°C and the supernatants (S1) collected. After protein determination (following Bradford [28]) using bovine serum albumin as standard, β-mercaptoethanol and glycerol were added to samples to a final concentration of 8 and 25%, respectively, and the samples were frozen in −80°C until further analysis. Proteins (2 mg/mL) were separated using SDS-PAGE with 10% gels and then electrotransferred to nitrocellulose membranes as previously described [29]. Membranes were washed in Tris-buffered saline with Tween (TBST; 100 mM Tris-HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5) and incubated overnight (4°C) with different primary antibodies (Santa Cruz Biotechnology, TX), all produced in rabbit (anti-Nrf2, anti-NQO-1, and anti-HSP70 anti-β-actin; 1 : 1000 dilution in TBST). Following incubation, membranes were washed in TBST and incubated for 1 h at 25°C with HRP-linked anti-rabbit-IgG secondary specific antibodies (Sigma, MO). The immunoblots were visualized in the Image Station 4000MM PRO using ECL reagent (Promega, WI). Immunoreactive bands were quantified using the Scion Image software and expressed as percentage of untreated controls.

2.16. Statistical Analysis. Data were expressed as mean ± SEM of the number of animals used in each experiment. Statistical analysis was performed using two-way ANOVA and Tukey post hoc test. Values of $p < 0.05$ were considered statistically significant. GraphPad prism 6 software was used for statistical analysis and for plotting graphs.

3. Results

3.1. Chromatographic Profile. HPLC analysis of BF tea revealed the following main compounds kaempferol-3-O-(2-rhamnosyl) rutinoside (2) > quercetin-3-O-(2-rhamnosyl) rutinoside (1) > quercetin-3-O-rutinoside (3) > kaempferol-3-O-rutinoside (4) (Figure 1).

3.2. Glucose Levels. Diabetic mice had a significant increase in the serum glucose levels, which were not reduced by BF (Figure 2).

3.3. Liver Toxicity Evaluation. The liver/body weight ratio was increased in diabetic mice when compared to control group. These changes were not modified by BF treatment. BF *per se* did not affect this parameter (Figure 3). Diabetic mice had a significant increase in aspartate aminotransferase (AST) level (Figure 4(a)) when compared to the control group. This change was not modified by BF treatment.

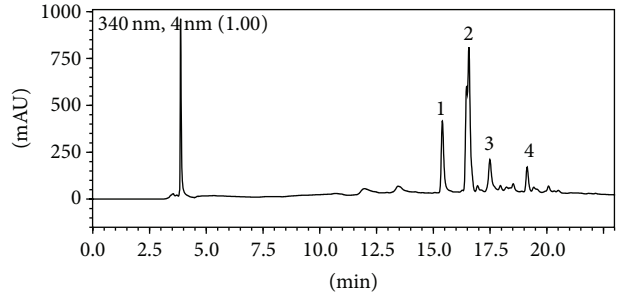

Figure 1: Chromatographic profile of *B. forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin tea. Chemical compounds identified Peak 1: quercetin-3-O-(2-rhamnosyl) rutinoside (retention time: 15.60 min); Peak 2: kaempferol-3-O-(2-rhamnosyl) rutinoside (retention time: 16.70 min); Peak 3: quercetin-3-O-rutinoside (retention time: 17.40 min); Peak 4: kaempferol-3-O-rutinoside (retention time: 19.10 min).

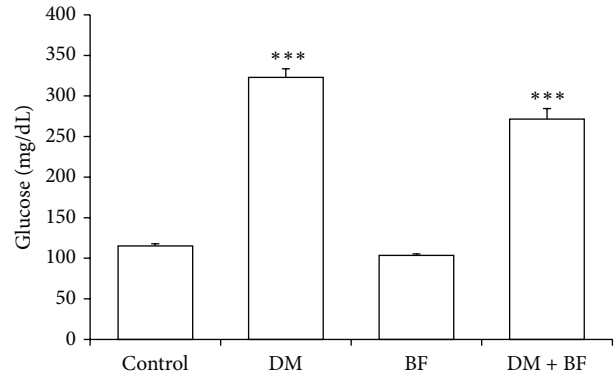

Figure 2: Glucose levels (mg/dL) at the end of treatment. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

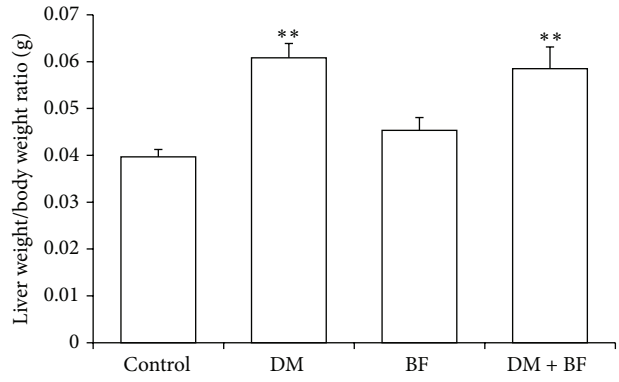

Figure 3: Liver/body weight ratio (g) of diabetic mice treated with BF. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

Alanine aminotransferase (ALT) level (Figure 4(b)) was not changed by any treatment.

3.4. Liver Oxidative Stress Evaluation. BF treatment was effective in normalizing the increases in ROS (DCF-RS) and lipid

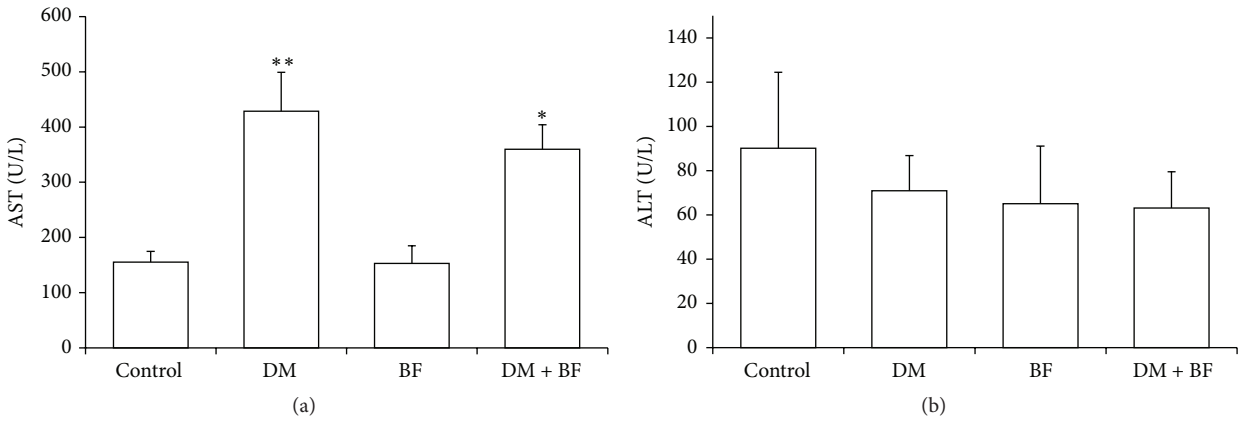

Figure 4: Alanine aminotransferase (a) and aspartate aminotransferase (b) levels (U/L) of diabetic mice treated with BF. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

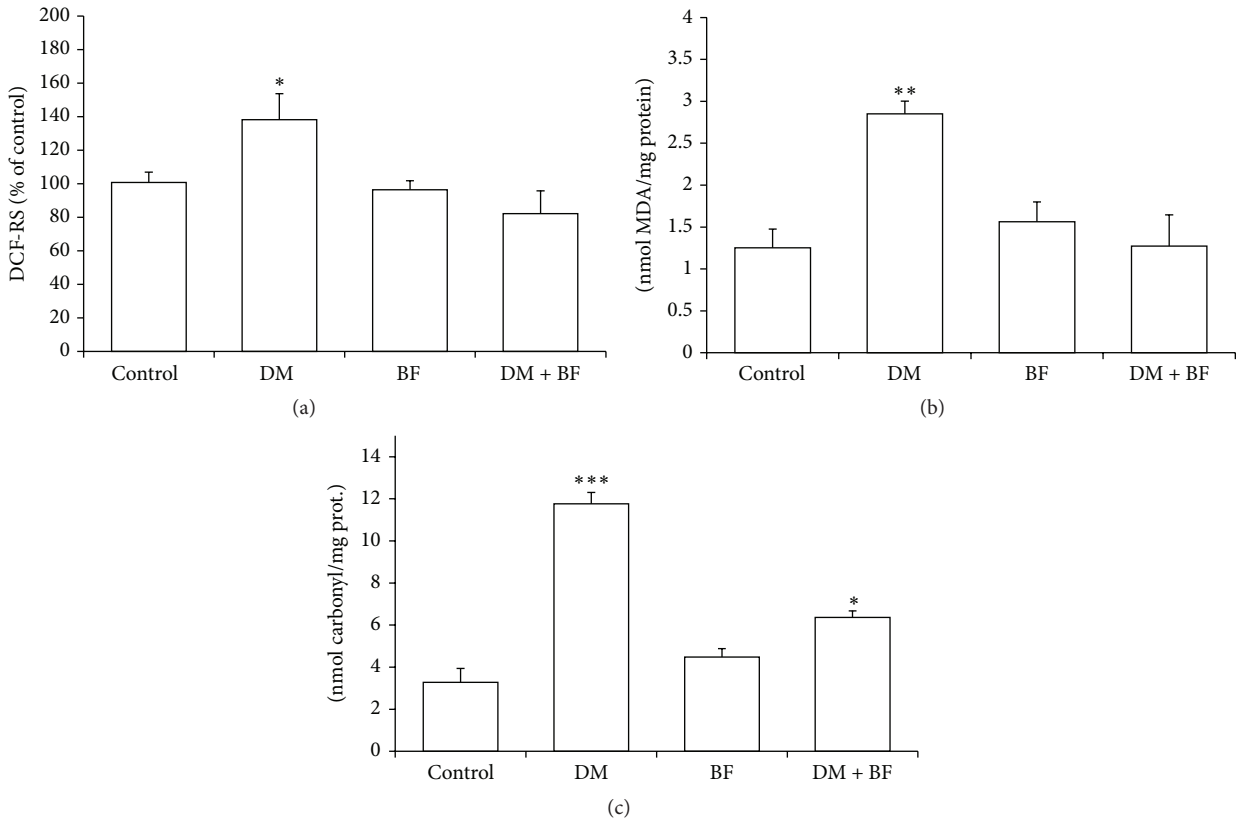

Figure 5: Liver dichlorofluorescein reactive species (DCF-RS) (a); thiobarbituric acid reactive species (TBA-RS) (b); and protein carbonyl levels (c) of diabetic mice treated with BF. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

peroxidation (TBA-RS) levels observed in diabetic mice, to the control levels (Figures 5(a) and 5(b), resp.). Furthermore, diabetic mice had an increase in the carbonylated protein levels (Figure 5(c)) that were only partially reduced by BF treatment.

No difference in SOD activity was observed among the groups (Figure 6(a)). However, the diabetic mice had a significant decrease in CAT activity when compared to the control group. This decrease was attenuated by BF treatment (Figure 6(b)).

The activity of liver δ-ALA-D was inhibited in diabetic mice. The inhibition of δ-ALA-D enzyme activity was not modified by BF treatment (Figure 7(a)). Addition of a thiol donor dithiothreitol (DTT) partially reactivated δ-ALA-D, however, without restoring the basal activity of δ-ALA-D (data not shown). The levels of nonprotein thiol groups

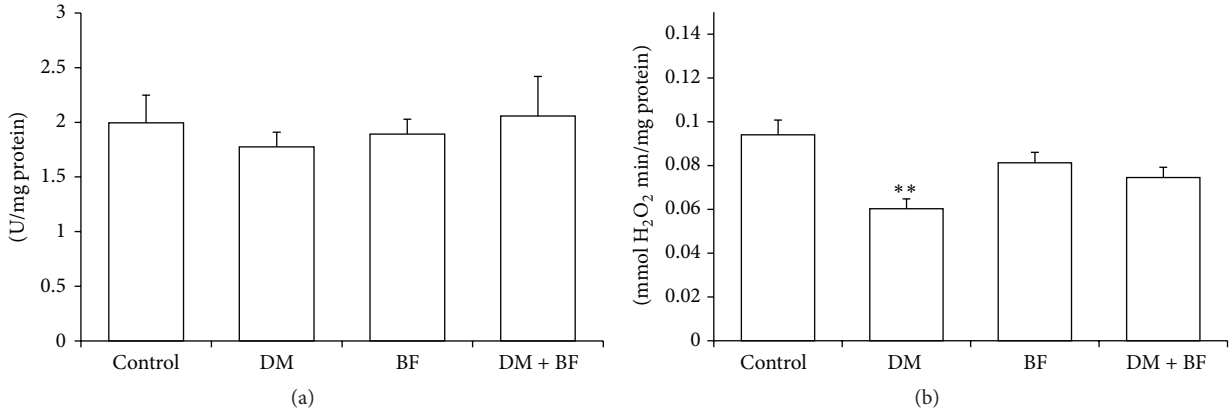

Figure 6: Liver superoxide dismutase (SOD) (a) and catalase (CAT) (b) activities of diabetic mice treated with BF. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

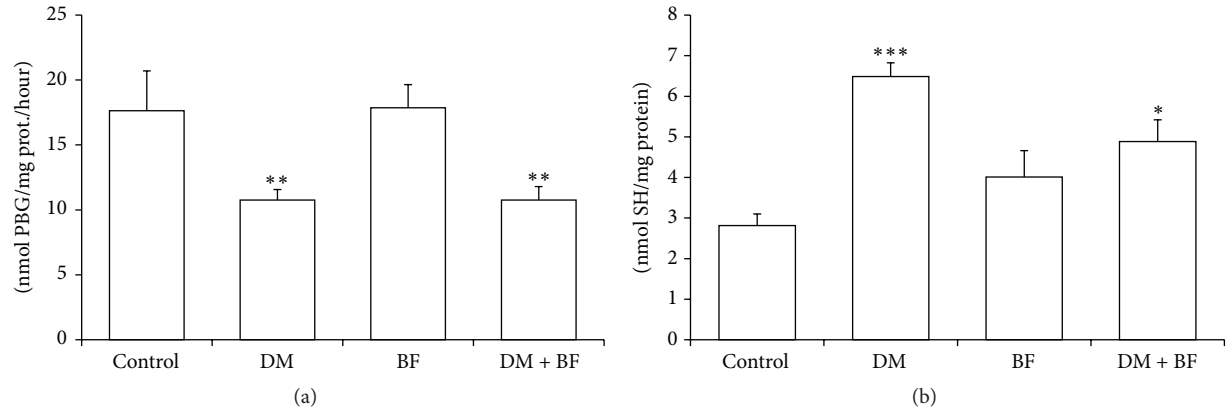

Figure 7: Liver delta-aminolevulinic acid dehydratase (δ-ALA-D) activity (a) and nonprotein SH (NPSH) levels (b) in diabetic mice treated with BF. The ∗ indicates significant difference in comparison to control group ($p < 0.05$).

(NPSH) were increased in diabetic mice and BF treatment restores only partially this increase (Figure 7(b)).

3.5. Western Blot Analysis. Liver western blot showed that Nrf2, NQO-1, or HSP70 protein levels were not altered in diabetic mice or BF treatment when compared to the control (Figure 8). In pancreas, an increase in NQO-1 levels was observed, and BF treatment reduce these at levels lower than the control group (Figure 9). No differences in the levels of Nrf2 and HSP70 were observed among the groups in pancreas.

4. Discussion

The present study was designed to investigate the effects of *Bauhinia forficata* Link subsp. *pruinosa* (Vogel) Fortunato & Wunderlin (BF) tea against oxidative stress and liver damage in diabetic mice. In folk medicine, various species of BF have been used to treat *diabetes mellitus* (DM) [2], especially due to their possible hypoglycemic effect. Our results show BF tea reduced liver oxidative stress in diabetic mice, although it did not change the glycaemia.

In this context, the absence of hypoglycemic action of BF tea may be due to the nonextraction of some compounds in the aqueous fraction (infusion) or due to absence of kaempferitrin compound (kaempferol-3,7-O-(r)-dirhamnoside), pointed out as responsible for hypoglycemic action in other *Bauhinia* species [10].

Our results show BF tea *per se* does not determine abnormal hepatic growth or transaminases changes, indicating possible absence of toxicity (Figures 3 and 4(a), 4(b)). On the other hand, we had an increase in AST levels and in liver/body weight ratio in diabetic mice. The increase in liver/body weight ratio may be due to the reduction of body weight (data not shown), common in untreated diabetes [30]. Regarding transaminases, both AST and ALT are highly concentrated in the liver. However, ALT is localized only in the cellular cytoplasm, whereas AST is cytosolic in a minor portion and mitochondrial in a major portion. Furthermore, AST is highly concentrated in zone 3 of the hepatic acinus, and damage to this zone may indicate ischemic or toxic events, resulting in greater AST levels [31]. In case of diabetes, hepatic toxic events may occur in response to an excess in free fatty acids [32] results of insulin impairment. Known mechanisms

for hepatic toxics events that increase transaminases levels in diabetic state include cell membrane disruption, mitochondrial dysfunction, toxin formation, oxidative stress, and recruited inflammatory cells [32].

We observe an increase in reactive oxygen species (ROS) and lipid peroxidation levels (Figures 5(a) and 5(b), resp.), indicating oxidative damage in liver. The assessment of DCF-RS is well accepted to determine ROS levels, as well as reactive nitrogen species able to oxidize the DCFH, a general index of oxidative stress. Similarly, TBA-RS assay is a known biomarker used to estimate lipid damage from cells and tissues, and its increased levels are an indirect evidence of high ROS production. Although BF tea treatment did not modify the changes in liver/body weight ratio and AST levels in diabetic mice, the plant was effective in reducing DCF-RS and TBA-RS levels. These findings reinforce our previously reported antioxidant activity of BF tea even at low concentrations [11]. The antioxidant activity of BF extracts has been attributed to high levels of polyphenols and flavonoids present in its composition [11, 33]. Here, we identify four major compounds (Figure 1) that were previously reported using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) [11, 19]. Among the chemical constituents identified in our extract, the quercetin and kaempferol derivate have been extensively studied to have antioxidant properties, such as reduction of TBA-RS levels and control of antioxidant response [10, 11].

Our results also showed that there is an increase in liver carbonylated protein levels (Figure 5(c)). This increase is reduced only partially by BF treatment, and it is not related with ROS levels that were controlled by BF treatment (Figure 5(a)). Probably, a longer BF treatment might reduce the protein carbonyl levels to control levels. This is possible whereas carbonylated proteins have a long half-life and take longer to suffer degradation when compared to normal proteins.

Concerning liver antioxidant enzymes, we observed a significant decrease in CAT activity in diabetic mice, which was reverted by BF tea treatment (Figure 6(b)). No changes were observed in liver SOD activity in diabetic mice. For instance, changes in antioxidant enzymes activities or its return to normal values following a previous decrease may occur as a compensatory mechanism in response to a constant exposure to increased oxidative stress, such as those determined by prolonged hyperglycemia. This could explain the decreases in SOD activity observed by some researchers and the normal SOD activity observed by other investigators (for a review see [12]).

We also observed a decrease in δ-ALA-D enzyme activity (Figure 7(a)), not related to a decrease in NPSH levels (Figure 7(b)), in diabetic mice. Several studies report that the δ-ALA-D enzyme activity is reduced in hyperglycemic conditions [13, 14, 34]. This occurs due to presence of thiol groups in its structure, which are sensitive to oxidation. This characteristic explains its use as a good oxidative stress biomarker [13, 14]. In diabetic mice, we observed an increase in thiol levels, probably due to a physiological compensatory effect. In this context, the NPSH levels, glutathione (GSH) as a major compound, increase to counteract the high ROS production [11]. GSH is a ubiquitous cellular three-peptide antioxidant that acts as an intracellular buffer being responsible for the maintenance of the thiol redox balance [35]. In this line, mainly three functional changes may lead to a δ-ALA-D enzyme activity reduction, namely, removal of divalent zinc from its catalytic site (1); oxidation of its critical thiol groups (2); or protein oxidation (3) [13, 14, 34]. Taking into account that there were no thiol levels compromising and that the SH donor dithiothreitol (DTT) only partially reactivated δ-ALA-D (data not shown) we believe that the mechanism of inhibition was linked to protein oxidation. In fact, reducing sugars can interact with critical lysine residues of δ-ALA-D catalytic site, oxidizing the lysine residues to disulfides and inactivating the enzyme [34]. In this context, inhibition of δ-ALA-D in diabetes may be related to hyperglycemia [13, 14].

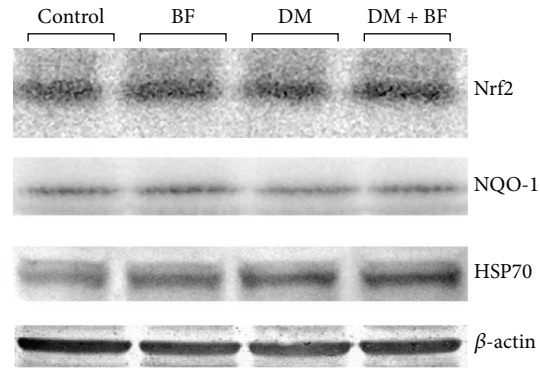

Figure 8: Liver Nrf2, NQO-1, and HSP70 protein levels in diabetic mice treated with BF. The data were normalized with β-actin expression and expressed as % of control.

Here, the oxidative damage in liver seems to occur without changes in Nrf2, HSP70, or NQO-1 protein levels (Figure 8). Different in pancreas (target organ of STZ), we observed changes in NQO-1 expression (Figure 9) that were minimized by BF treatment. The enzyme NQO-1 is generally considered as a detoxification enzyme and has been known to protect β-cells against stressors, including the diabetogenic agent STZ [18, 36]. There is evidence that NQO-1 knockout mice present increased pancreatic β-cell death induced by STZ [18]. Furthermore, both STZ and hyperglycemia have been known to increase ROS production [12], and NQO-1 enzyme plays an important role as a superoxide scavenger that may provide an additional level of protection against ROS toxicity [36]. The increase observed in pancreas NQO-1 could be associated with a possible response against the xenobiotic injury determined by STZ. However, more studies are necessary to highlight the reasons for increased expression of NQO-1 in pancreas but not in liver.

Although we did not observe changes in Nrf2 protein levels, the NQO-1 upregulation in pancreas and the elevated levels of GSH in liver suggest an early activation of Nrf2-antioxidant response element (ARE) pathway, probably in response to increase in ROS levels. In fact, under stressing condition, the transcription factor Nrf2 interacts with ARE and upregulates antioxidative genes including NQO-1, antioxidant enzymes, and GSH levels, which are very important components of the cellular antioxidant defense

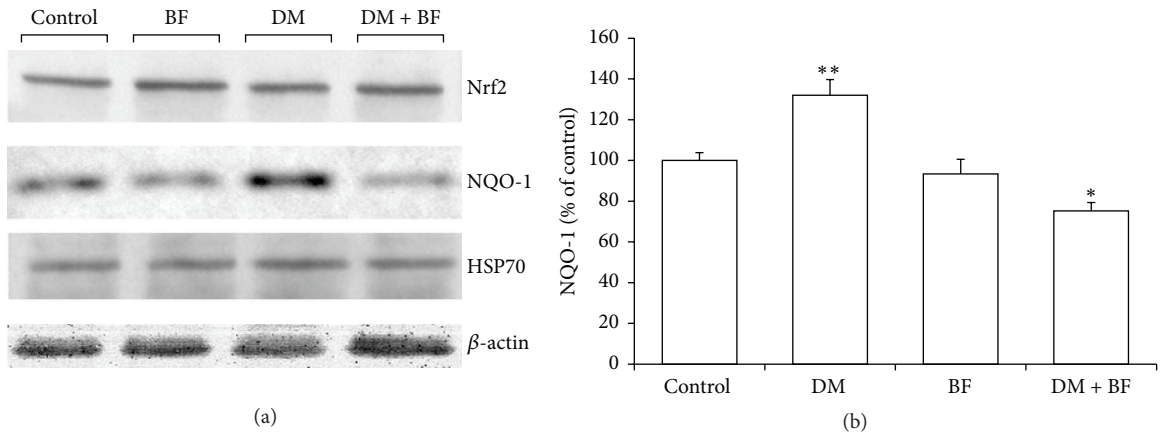

Figure 9: Pancreas Nrf2, NQO-1, and HSP70 protein levels in diabetic mice treated with BF (a). Graphical representation of NQO-1 pancreas protein levels (b). The data were normalized with β-actin expression and expressed as % of control. The $*$ indicates significant difference in comparison to the control group ($p < 0.05$).

[37]. However, while NQO-1 is a stable protein (half-life greater than 18 hours in wild type cells) [38], Nrf2 is a highly unstable protein and its half-life is about 15 min under nonstress condition [39] to 100 min under stress condition [40]. In fact, according to Nguyen et al. [39], even in stress condition, Nrf2 has a short life and is still subject to a high rate of degradation. The same has been observed with the HSP70, which has a half-life of approximately 2 hours [41]. This rapid degradation rate occurs, presumably, to prevent its accumulation in an uncontrolled manner [39] and may be the reason why we cannot observe differences in the levels of this protein in our study.

We highlight that although our objective was to investigate effects of BF tea (crude aqueous extract) on liver damage in diabetic mice, some points are extremely relevant and deserve further attention in future investigations, in particular, deeper analysis of the pancreas, serum insulin concentration, analysis of BF compounds concentration in the plasma, and the role/effect of their isolated bioactive components.

5. Conclusion

Taken together, our observations suggested that diabetic mice present an increase in liver oxidative damage and in pancreas NQO-1 expression, which were modulated by BF treatment. Since BF tea decreased liver oxidative injury but does not change glycaemia, we believe that BF protective effect may be attributed especially to its antioxidant capacity.

Conflict of Interests

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

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Research Article

Chemical Characterization, Free Radical Scavenging, and Cellular Antioxidant and Anti-Inflammatory Properties of a Stilbenoid-Rich Root Extract of *Vitis vinifera*

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Dietary stilbenoids are receiving increasing attention due to their potential health benefits. However, most studies concerning the bioactivity of stilbenoids were conducted with pure compounds, for example, resveratrol. The aim of this study was to characterize a complex root extract of *Vitis vinifera* in terms of its free radical scavenging and cellular antioxidant and anti-inflammatory properties. HPLC-ESI-MS/MS analyses of the root extract of *Vitis vinifera* identified seven stilbenoids including two monomeric (resveratrol and piceatannol), two dimeric (trans-ε-viniferin and ampelopsin A), one trimeric (miyabenol C), and two tetrameric (r-2-viniferin = vitisin A and r-viniferin = vitisin B) compounds which may mediate its biological activity. Electron spin resonance and spin trapping experiments indicate that the root extract scavenged 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, galvinoxyl, and superoxide free radicals. On a cellular level it was observed that the root extract of *Vitis vinifera* protects against hydrogen peroxide-induced DNA damage and induces Nrf2 and its target genes heme oxygenase-1 and γ-glutamylcysteine synthetase. Furthermore, the root extract could induce the antiatherogenic hepatic enzyme paraoxonase 1 and downregulate proinflammatory gene expression (interleukin 1β, inducible nitric oxide synthase) in macrophages. Collectively our data suggest that the root extract of *Vitis vinifera* exhibits free radical scavenging as well as cellular antioxidant and anti-inflammatory properties.

1. Introduction

Stilbenoids are secondary plant metabolites which are mainly present in *Vitis vinifera* L. species, the latter belonging to the plant family Vitaceae [1]. *Vitis vinifera* derived stilbenoids exist as monomers, that is, *trans*-resveratrol or piceatannol, and oligomers [1] and mostly occur in the plant kingdom as *trans*-isomers (*E*) [1]. The stilbene aglycone consists of two aromatic rings linked by an ethylene bridge [1]. Beside *Vitis vinifera*, dietary sources of resveratrol (*trans*-3,5,4′-trihydroxystilbene) and its oligomers are tea, peanuts, and pistachios [1–4]. Table 1 summarizes the occurrence of different stilbenoids in cell suspension culture, berries, stems, leaves, roots, and wine of *Vitis vinifera* according to Pawlus et al. [5]. Stilbenoids exhibit antimicrobial properties and as phytoalexins they play an important role in plants defending pathogens [5].

Up to now, the majority of studies concerning stilbenoids were conducted with resveratrol as a purified standard compound. However, studies in which a complex stilbene extract of *Vitis vinifera* was applied are scarce. The use of an extract may lead to synergistic effects of the various stilbenoids as far as their bioactivity is concerned. Stilbenoids are known to exhibit potential health benefits, that are, antioxidant [3, 6], anti-inflammatory [7, 8], anticancerogenic [9], antiatherogenic [10], antiviral [11], and neuroprotective properties [12].

Table 1: Stilbenoids in cell suspension culture, berries, stems, leaves, roots, and wine of *Vitis vinifera* according to Pawlus et al. [5].

Plant part	Stilbenoids
Cell suspension culture	*Monomer*: *E*-/*Z*-Astringin, E-/*Z*-Piceid, E-/*Z*-Resveratrol, *E*-/*Z*-Resveratrol-3,4′-*O*-*β*-diglucoside, *E*-/*Z*-Resveratrol-3,5-*O*-*β*-diglucoside, *Z*-Resveratrol-3,5,4′-*O*-*β*-triglucoside, *E*-/*Z*-Resveratroloside *Dimer*: Pallidol, *E*-*δ*-Viniferin, *E*-*δ*-Viniferin-11-*O*-*β*-D-glucopyranoside, *E*-*δ*-Viniferin-11′-*O*-*β*-D-glucopyranoside
Berries	*Monomer*: *E*-Piceatannol, E-/*Z*-Piceid, *E*-Pterostilbene, *E*-Resveratrol
Stems	*Monomer*: *E*-Piceatannol, E-/*Z*-Piceid, E-/*Z*-Resveratrol, *E*-Resveratrol-2-*C*-glucoside *Dimer*: (+)-Ampelopsin A and F, (−)-Malibatol A, Pallidol, Scirpusin A, Viniferifuran, (+)-*E*-*ε*-Viniferin, *E*-*ε*-Viniferin *Trimer*: *E*-*trans*-Miyabenol C, (+)-Viniferol D *Tetramer*: Hopeaphenol, Isohopeaphenol, (+)-Viniferol A, B and C, (+)-Vitisifuran A and B, Vitisin A, *E*-Vitisin B and C
Leaves	*Monomer*: *E*-Piceid, *E*-Pterostilbene, E-/*Z*-Resveratrol *Dimer*: Ampelopsin D, Pallidol, Quadrangularin A, *E*-*δ*-Viniferin, *Z*-*ε*-Viniferin, (+)-*E*-*ε*-Viniferin, *E*-*ε*-Viniferin, *E*-/*Z*-*ω*-Viniferin *Trimer*: *E*-/*Z*-*trans*-Miyabenol C, *E*-*cis*-Miyabenol C, *α*-Viniferin *Tetramer*: Ampelopsin H, Hopeaphenol, Isohopeaphenol, Vaticanol C isomer
Roots	*Dimer*: (+)-Viniferether A and B, *E*-*ε*-Viniferin *Trimer*: Gnetin H *Tetramer*: Hopeaphenol, (+)-Viniferol E, *E*-Vitisin B
Wine[a]	*Monomer*: *E*-/*Z*-Astringin, *E*-Piceatannol, E-/*Z*-Piceid, E-/*Z*-Resveratrol, *E*-Resveratrol-2-*C*-glucoside, 2,4,6-Trihydroxyphenanthrene-2-*O*-glucoside *Dimer*: Pallidol, Pallidol-3,3″-diglucoside, Pallidol-3-*O*-glucoside, Parthenocissin A, *E*-*δ*-Viniferin, *Z*-*ε*-Viniferin, *E*-*ε*-Viniferin, *E*-/*Z*-*ε*-Viniferin-diglucoside *Tetramer*: Hopeaphenol

[a]Not distinguished between red wine and white wine.

In the current study, we investigated potential free radical scavenging and cellular antioxidant and anti-inflammatory activities of the root of *Vitis vinifera*, which may be highly enriched with various stilbenoids. A standardized ethanol extract of the root of *Vitis vinifera* purified with ethyl acetate/*n*-hexane was applied for all studies. The qualitative and quantitative stilbenoid composition was analyzed by HPLC-ESI-MS/MS and HPLC-PDA.

Plant bioactives may prevent the oxidation of lipids, proteins, and DNA either directly by free radical scavenging or indirectly by induction of endogenous antioxidant defense mechanisms. Free radical scavenging activity was monitored by ESR spectroscopy and as spin trapping and the prevention of DNA damage was determined by the Comet assay.

The redox sensitive transcription factor nuclear factor erythroid 2-related factor-2 (Nrf2) partly regulates the expression of genes encoding antioxidant enzymes. Nrf2 is bound in the cytoplasm to its inhibitor Keap1 (Kelch-like ECH-associated protein 1). When Nrf2 is activated by electrophiles, it is released from its cytosolic protein Keap1 and binds to the antioxidant response element of the DNA in the nucleus thereby regulating the transcription of target genes including *γ*-glutamylcysteine synthetase (*γ*GCS) and heme oxygenase-1 (HO-1) [13]. Nrf2 transactivation and its target genes HO-1 and *γ*GCS were determined by a reporter gene assay, real-time PCR, and Western blotting, respectively. Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL) associated enzyme which is primarily synthesized in the liver [14]. PON1 prevents low-density lipoproteins (LDL) from oxidation and thereby mediats antiatherogenic effects [14]. PON1 transactivation was measured by a reporter gene assay. Biomarkers of inflammation including interleukin 1*β* (IL-1*β*) and inducible nitric oxide synthase (iNOS) were determined in cultured cells by real-time PCR.

2. Materials and Methods

2.1. Chemicals. Methanol, HPLC grade was purchased from VWR (Leuven, Belgium) and methanol, LC-MS grade was purchased from Fisher Chemical (Loughborough, UK). Acetic acid, HPLC grade was obtained from AppliChem (Darmstadt, Germany). Doubly deionized water using a Nanopure resin (Nanopure, Barnstead) was used for high-performance liquid chromatography (HPLC) analyses.

Sodium dihydrogen phosphate dihydrate and galvinoxyl radical were purchased from Wako Chemicals (Osaka, Japan). Disodium hydrogen phosphate, hypoxanthine, xanthine oxidase, and ethanol were obtained from Nacalai Tesque (Kyoto, Japan). Hydrogen peroxide (35%) was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Sigma-Aldrich (St. Louis, MO, USA (Tokyo branch)). Ultrapure water was prepared by PWE-500 (Advantec, Tokyo, Japan) for electron spin resonance spectroscopy (ESR).

Dulbecco's modified Eagle's medium high glucose (4.5 g/L) (with sodium pyruvate and L-glutamine), Dulbecco's modified Eagle's medium high glucose (4.5 g/L) (with sodium pyruvate), Dulbecco's modified Eagle's medium high glucose (4.5 g/L), fetal bovine serum, L-glutamine 200 mM (100x), penicillin/streptomycin (100x; 10,000 U/mL

penicillin; 10 mg/mL streptomycin), G-418 sulfate (50 mg/mL), fetal bovine serum, and Dulbecco's phosphate-buffered saline (DPBS) without Ca and Mg were obtained from PAA (Cölbe, Germany). Neutral red and glacial acetic acid (99–100%) were purchased from Carl Roth (Karlsruhe, Germany) and ethanol (absolute) was purchased from Merck (Darmstadt, Germany). Resveratrol and dimethyl sulfoxide were obtained from Sigma (Steinheim, Germany). Lipopolysaccharide (LPS) from *Salmonella enterica* serotype Enteritidis was obtained from Sigma. Dual-luciferase reporter assay system, phRL-TK, and passive lysis buffer were purchased from Promega (Mannheim, Germany). JetPEI and peqGOLD TriFast were obtained from Peqlab Biotechnologie GmbH (Erlangen, Germany). Primers for heme oxygenase-1 (HO-1), γ-glutamylcysteine synthetase (γGCS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were ordered from Eurofins Genomics (Ebersberg, Germany). SensiMix one-step kit was obtained from Bioline (Luckenwalde, Germany). Heme oxygenase-1 antibody was purchased from Stressgen (Michigan, USA), immun-star goat anti-rabbit (GAR)-HRP conjugate secondary antibody was purchased from Bio-Rad (Munich, Germany), and Pierce ECL Western blotting substrate, spectra multicolor broad range protein ladder, and Pierce BCA protein assay kit were purchased from Thermo Scientific (Rockford, USA). GAPDH antibody and donkey anti-goat IgG HRP secondary antibody were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

Vitisin grapevine root extract (*Vitis vinifera* cultivated from vines of the area Bordeaux) was kindly provided by Wolfgang Loersch (Breko, Bremen, Germany).

2.2. HPLC-Photodiode Array Detector (HPLC-PDA). HPLC analysis was done according to Macke [15]. HPLC system from Jasco (Groß-Umstadt, Germany) was used which consisted of a pump (PU-2080 Plus, Intelligent HPLC Pump), degasser (DG-2080-53, 3-Line Degasser), ternary gradient unit (LG-2080-02), autosampler (Intelligent Sampler AS-2057 Plus), and PDA (MD-2010 Plus). The separation was done on a Kromasil 100-5 C18 5 μm (250 mm × 4.6 mm i.d.) column (Eka Chemicals AB, Bohus, Sweden) protected with a guard column of the same material (4 mm × 4 mm). The mobile phase consisted of 1% aqueous acetic acid (A) and methanol (B). The following gradient was used for separation: 0 min 20% B, 5 min 30% B, 15 min 30% B, 18 min 37% B, 29 min 37% B, 35 min 50% B, 57 min 50% B, 58 min 100% B, 71 min 100% B, 72 min 20% B, and 75 min 20% B. The flow rate was set at 0.8 mL/min and the injection volume was 20 μL. HPLC chromatograms were recorded at $\lambda = 280$ nm. The root extract of *Vitis vinifera* was dissolved in methanol/water (80/20, v/v). Monomeric stilbenoids in the root extract of *Vitis vinifera* were quantified as *trans*-resveratrol equivalents and oligostilbenoids as *trans*-ε-viniferin equivalents. The measurements were repeated five times.

2.3. HPLC-Electrospray Ionization-Tandem Mass Spectrometry (HPLC-ESI-MS/MS). A HPLC analysis was performed according to Macke [15]. HPLC system from Agilent Technologies (Waldbronn, Germany) composed of a pump (1100 Series, BinPump G1312A), autosampler (1200 Series), PDA (1100 Series, DAD G1315B), and mass spectrometer (HCT Ultra; Bruker Daltonics, Bremen, Germany) was used. The ESI conditions were as follows: ion polarity: negative, scan range: 100–3000 *m/z*, dry gas temperature: 330°C, dry gas flow: 10 L/min, nebulizer pressure: 50 psi, capillary voltage: 3500 V, capillary exit: −3500 V, and end plate: −500 V. HPLC separation was achieved on a Luna 3u C18 100 A 3 μm (150 mm × 2.0 mm i.d.) column protected with a guard column of the same material (4 mm × 4 mm) (Phenomenex, Aschaffenburg, Germany). Mobile phases and gradient elution were as described above. The flow rate was set at 0.2 mL/min and the injection volume at 5 μL. HPLC chromatograms were recorded at $\lambda = 280$ nm.

2.4. Free Radical Scavenging Activity Measured by Electron Spin Resonance Spectroscopy (ESR). ESR and spin trapping measurements were conducted according to Esatbeyoglu et al. [16] using a JEOL JES-FR30EX free radical monitor (JEOL Ltd., Akishima, Japan). The amplitude was set at 200 for DPPH radicals, 250 for galvinoxyl, 400 for hydroxyl radicals, and 500 for superoxide radicals.

2.4.1. DPPH and Galvinoxyl Radical Scavenging Experiments. To a reaction mixture containing 360 μL distilled water, 500 μL ethanol, 100 μL 1.875 mM DPPH (in ethanol), or 100 μL 375 μM galvinoxyl radical (in ethanol), 40 μL of the root extract of *Vitis vinifera* (1.3, 10, 13, 40, 100, and 130 mg/mL; in case of galvinoxyl radical, 1, 1.33, 2, 4, 10, and 1000 mg/mL) was added and stirred for a few seconds. After incubating the solution for 10 min (3 h for galvinoxyl), ESR spectra were recorded.

2.4.2. Hydroxyl Radical Scavenging Experiments. To the reaction mixture of 25 μL 200 mM DMPO, 20 μL 50 mM hydrogen peroxide, 35 μL distilled water, and 10 μL 0.5 mM ferric chloride, 10 μL of the root extract of *Vitis vinifera* (1, 1.25, 1.67, 2.5, 5, and 10 mg/mL) was added. The reaction mixture was stirred, vortexed, and put on ice for 10 sec.

2.4.3. Superoxide Radical Scavenging Experiments. To the reaction mixture of 30 μL 2 mM hypoxanthine, 30 μL 4 M DMPO, 26 μL 200 mM DPBS buffer solution (pH 7.4), and 4 μL 1 U/mL xanthine oxidase, 10 μL of the root extract of *Vitis vinifera* (2, 10, 12.5, 25, 50, and 1000 mg/mL) was added. The combined reaction mixture was incubated at 30°C for 1 min.

2.5. Cell Lines. The detailed cell culture conditions regarding the human liver hepatoma cell line Huh7, stably transfected PON1-Huh7 cells, and human colonic adenocarcinoma cell line HT-29 are described by Esatbeyoglu et al. [16].

Murine RAW264.7 macrophages (obtained from the Institute of Applied Cell Culture, Munich, Germany) were cultured in Dulbecco's modified Eagle's medium high glucose (4.5 g/L) containing sodium pyruvate and L-glutamine supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin and grown in a humidified atmosphere of 5% CO_2 at 37°C.

For all cell culture studies, 100 mg/mL stock solutions of the root extract of *Vitis vinifera* in ethanol and resveratrol in DMSO were prepared and stored at −80°C until further use. LPS from *Salmonella enterica* serotype Enteritidis (Sigma) was dissolved in DPBS to a stock solution of 1 mg/mL and stored at −20°C until further use.

2.6. Cytotoxicity (Neutral Red Assay). Cell viability was determined using the colorimetric neutral red assay [17]. PON1-Huh7, Huh7, HT-29, and RAW264.7 cells (0.15×10^6 cells/well, 0.15×10^6 cells/well, 0.4×10^6 cells/well, and 0.08×10^6 cells/well) were seeded in a 24-well plate for 24 h. The cells were treated with the root extract of *Vitis vinifera* at various concentrations (PON1-Huh7 and Huh7 1–100 μg/mL; RAW264.7 1–50 μg/mL) for 24 h (PON1-Huh7 for 48 h).

2.7. Oxidative DNA Damage (Comet Assay). HT-29 cells were treated with 50 μg/mL root extract of *Vitis vinifera* and 50 μM resveratrol as positive control for 14 h at 37°C. Subsequently, cells were treated with 25 μM H_2O_2 in DPBS for 15 min to induce DNA damage. Oxidative DNA damage in HT-29 cells was measured using the Comet assay as described earlier [16].

2.8. Nrf2 Transactivation (Dual-Luciferase Reporter Gene Assay). Transient transfection and luciferase reporter gene assay for measuring Nrf2 transactivation were conducted as described elsewhere [16].

Transiently transfected Huh7 cells were incubated with the root extract of *Vitis vinifera* (1, 5, 10, 25, and 50 μg/mL) and 25 μM resveratrol was used as a positive control.

2.9. Determination of Nrf2 Target Genes Heme Oxygenase-1 (HO-1) and γ-Glutamylcysteine Synthetase (γGCS) (RNA Isolation and Real-Time PCR). Human Huh7 liver cells were seeded in a 6-well plate at a density of 0.9×10^6 cells/well for 24 h. Subsequently, cells were treated with 1, 10, 25, and 50 μg/mL root extract of *Vitis vinifera* for 6 h. Cells were washed with DPBS and RNA was isolated using peqGOLD TriFast via phenol-chloroform extraction according to manufacturer's description.

Primers for genes of human origin were designed by Primer3 software: HO-1, F: 5′-CCAGGCAGAGAATGCTGAGT-3′, R: 5′-GTAGACAGGGGCGAAGACTG-3′; γGCS, F: 5′-TTTGGTCAGGGAGTTTCCAG-3′, R: 5′-TGAACAGGCCATGTCAACTG-3′; GAPDH, F: 5′-CAATGACCCCTTCATTGACC-3′, R: 5′-GATCTCGCTCCTGGAAGATG-3′. All primers were ordered from Eurofins Genomics (Ebersberg, Germany).

SensiMix one-step kit (Quantace, Berlin, Germany) was used for real-time PCR. Human GAPDH was used as housekeeping gene.

2.10. Inhibition of LPS-Mediated Interleukin-1β (IL-1β) and Inducible Nitric Oxide Synthase (iNOS) (RNA Isolation and Real-Time PCR). RAW264.7 macrophages were seeded in 12-well plates at a density of 0.2×10^6 cells/well for 24 h. Afterwards, cells were treated with 20 μg/mL of the root extract of *Vitis vinifera* for 24 h. LPS (10 ng/mL) was added to the cells for 4 h. RNA was isolated by peqGOLD TriFast according to manufacturer's protocol. Remaining DNA was lysed using DNAse according to manufacturer's instructions (New England Biolabs, Ipswich, USA).

Primers for murine genes were designed by Primer3 software and ordered from Eurofins Genomics (Ebersberg, Germany): Interleukin-1β (IL-1β), F: 5′-CAGCTATGGCAACTGTTCCT-3′, R: 5′-CTGGATGCTCTCATCAGGAC-3′; inducible nitric oxide synthase (iNOS), F: 5′-GGCAGCCTGTGAGACCTTTG-3′, R: 5′-GCATTGGAAGTGAAGCGTTTC-3′; GAPDH, F: 5′-CCGCATCTTCTTGTGCAGT-3′, R: 5′-GGCAACAATCTCCACTTTGC-3′.

SensiMix one-step kit (Quantace, Berlin, Germany) was used for real-time PCR. Gene expression was normalized to the housekeeping gene GAPDH.

2.11. HO-1 Protein Levels (Western Blot Analysis). Whole cell extracts, total protein determination, and Western blot analysis were performed according to Wagner et al. [18] and Esatbeyoglu et al. [16].

2.12. PON1 Transactivation (Luciferase Reporter Gene Assay). Luciferase reporter gene assay for measuring PON1 transactivation was described in Schrader et al. [19]. PON1-Huh7 cells were seeded at a density of 0.15×10^6 cells/well in 24-well plates and incubated with 1, 2.5, 5, 15, and 25 μg/mL of the root extract of *Vitis vinifera* and 25 μmol/L resveratrol.

2.13. Statistical Analyses. Data obtained from cell culture experiments were expressed as means + standard error of the mean (SEM) or standard deviation (SD) of three independent experiments and compared to untreated cells (control) or LPS-stimulated control cells. HPLC analyses of stilbenes were expressed as means + standard deviation of five injections. Statistical analysis was performed by PASW Statistics Software Version 18 (IBM, Chicago, Illinois, USA). Data were tested for normality of distribution (Shapiro-Wilk test). Significant differences between groups were analyzed by Student's t-test. In case of not normally distributed data the non-parametric Mann-Whitney U test was applied. Significance was accepted at $p < 0.05$.

3. Results

3.1. Characterization and Quantification of the Vitis vinifera Root Extract. The grapevine root extract derived from *Vitis vinifera* was analyzed by HPLC-ESI-MS/MS and quantified by HPLC-PDA. HPLC-ESI-MS/MS analyses were performed using electrospray ionization operated in negative ion mode. Seven stilbenoids including two monomeric (resveratrol and piceatannol), two dimeric (*trans*-ε-viniferin and ampelopsin A), one trimeric (miyabenol C), and two tetrameric (r-2-viniferin = vitisin A and r-viniferin = vitisin B) were detected in the root extract of *Vitis vinifera*. Chemical structures of all detected compounds are given in Figure 1. A representative HPLC chromatogram at $\lambda = 280$ nm is shown in Figure 2. The main compounds in the root extract of *Vitis vinifera* were the dimer *trans*-ε-viniferin (125.1 g/kg), a dehydrodimer of resveratrol, and the tetramer

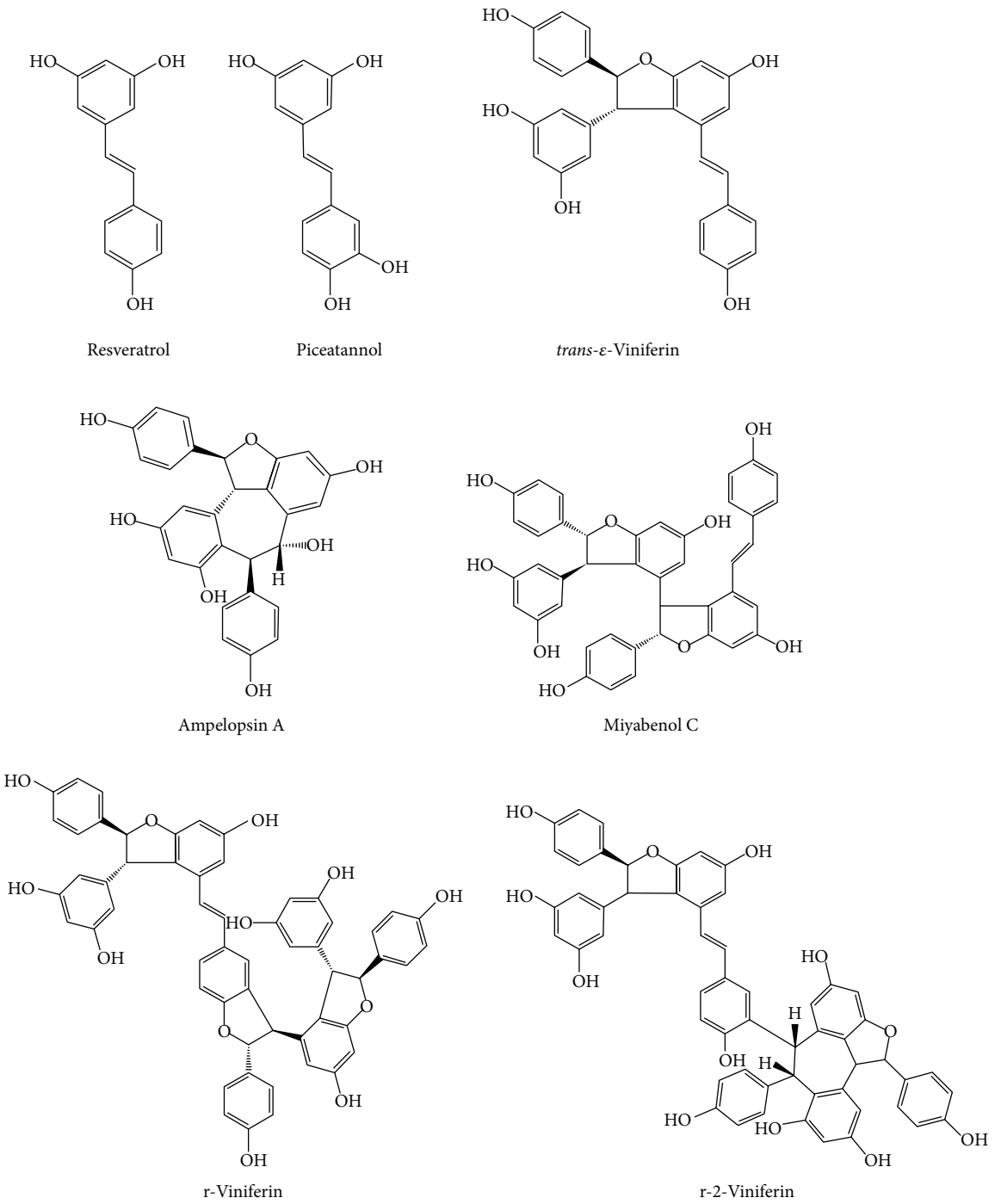

Figure 1: Chemical structures of monomeric and oligomeric stilbenoids of the root extract of *Vitis vinifera*.

r-2-viniferin (87.1 g/kg), composed of two resveratrol dimers (+)-ε-viniferin and ampelopsin B, followed by the monomer resveratrol (46.3 g/kg) (Table 2). The monomer piceatannol, the dimer ampelopsin A, the trimer miyabenol C, and the tetramer r-viniferin were present in considerably lower amounts (~4–16 g/kg) (Table 2).

3.2. Radical Scavenging Activity of the Root Extract of Vitis vinifera. The free radical scavenging activity of the root extract of *Vitis vinifera* was determined by ESR and spin trapping analysis. The root extract of *Vitis vinifera* exhibited relatively potent free radical scavenging activity in terms of DPPH, hydroxyl, and galvinoxyl radicals and scavenged these free radicals in a dose-dependent manner (Figures 3(a)–3(c)). Superoxide radicals were scavenged at higher concentrations of the root extract (Figure 3(d)).

3.3. Cytotoxic Effects of the Vitis vinifera Root Extract in Huh7, PON1-Huh7, HT-29, and RAW264.7 Cells. Cytotoxicity measurements of the root extract of *Vitis vinifera* at different

Table 2: Quantification of monomeric stilbenoids in the root extract of *Vitis vinifera* as *trans*-resveratrol equivalents and oligostilbenoids as *trans*-ε-viniferin equivalents by HPLC-PDA at $\lambda = 280$ nm ($n = 5$).

Peak	Compound	Retention time (t_R) [min]	Molecular ion $[M-H]^-$ m/z	Fragment ions m/z	Content [g/kg]	SD [g/kg]
1	Ampelopsin A	16.2	469	451, 363	15.6	0.41
2	Piceatannol	22.3	243	225, 201, 181, 175, 159	4.20	0.34
3	r-Viniferin (Vitisin B)	25.5	905	887, 799, 705, 675, 545, 451, 359	11.1	0.35
4	Resveratrol	36.2	227	212, 185, 159, 141, 107	46.3	0.85
5	r-2-Viniferin (Vitisin A)	37.3	905	887, 811, 705, 675, 545, 451, 359	87.1	1.31
6	Miyabenol C	40.4	679	661, 637, 585, 479, 451, 345	12.7	1.30
7	*trans*-ε-Viniferin	42.8	453	435, 411, 359, 347	125.1	1.23

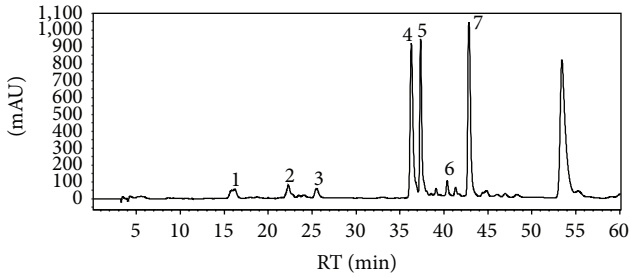

Figure 2: HPLC chromatogram of the root extract of *Vitis vinifera* at $\lambda = 280$ nm. For peak numbers, compare Table 2.

concentrations were carried out by the neutral red assay. Huh7, PON1-Huh7, HT-29, and RAW264.7 cells were treated with increasing concentrations of the root extract of *Vitis vinifera* for 24 h (PON1-Huh7 for 48 h). The root extract of *Vitis vinifera* was not cytotoxic up to a concentration of 50 μg/mL in Huh7, PON1-Huh7, and HT-29 cells and up to 20 μg/mL in RAW264.7 cells (data not shown). The root extract of *Vitis vinifera* was used in noncytotoxic concentrations in the subsequent cell culture experiments.

3.4. Prevention of Oxidative DNA Damage Induced by Hydrogen Peroxide. Comet assay was used to determine the effects of the root extract of *Vitis vinifera* counteracting H_2O_2-induced DNA damage in HT-29 cells. Treatment of HT-29 cells with 50 μg/mL of root extract of *Vitis vinifera* for 14 h resulted in a moderate but significant protection of H_2O_2-induced DNA damage as shown in Figure 4.

3.5. Induction of Antioxidant Defense Mechanisms through Nrf2 Transactivation. Treatment of Huh7 cells with the root extract of *Vitis vinifera* at concentrations of 25 μg/mL and 50 μg/mL significantly ($p < 0.001$) upregulated Nrf2 transactivation. This effect was comparable with the Nrf2 inducing activity of 25 μM resveratrol (Figure 5). Moreover, mRNA and protein levels of the Nrf2 target genes HO-1 and γGCS were analyzed by real-time PCR and Western blotting in human liver Huh7 cells. The root extract of *Vitis vinifera* (50 μg/mL) significantly induced HO-1 both on the mRNA ($p < 0.001$) (Figure 6(a)) and protein levels (Figure 6(b)). Accordingly, a significant induction of γGCS was observed at 50 μg/mL root extract of *Vitis vinifera* ($p < 0.05$; Figure 7).

3.6. PON1 Transactivation. Under the conditions investigated, luciferase reporter gene activity of stably transfected PON1-Huh7 cells was significantly ($p < 0.001$) induced by the root extract of *Vitis vinifera* in a dose-dependent manner (Figure 8).

3.7. Inhibition of Proinflammatory Biomarkers like IL-1β and iNOS due to Vitis vinifera Root Extract. Furthermore, the root extract of *Vitis vinifera* (20 μg/mL) significantly decreased the NF-κB target genes IL-1β (Figure 9(a)) and iNOS (Figure 9(b)) on the mRNA level in LPS-stimulated murine RAW264.7 macrophages.

4. Discussion

Stilbenoids are currently receiving increasing attention due to their potential health benefits [1, 6, 7, 10–12, 20]. In this study, we combined ESR and spin trapping measurements with cellular assays in order to determine the free radical scavenging and antioxidant and anti-inflammatory properties of a root extract of *Vitis vinifera*.

Our analyses indicate that the root extract of *Vitis vinifera* contained substantial amounts of dimeric and oligomeric stilbenoids including the dimer *trans*-ε-viniferin and the tetramer r-2-viniferin which may have contributed to its free radical scavenging properties. The free radical scavenging activity of stilbenoids seems to be partly related to proton abstraction as previously reported [21, 22].

Since the root extract contained a portfolio of various stilbenoids, these compounds may interact synergistically thereby exhibiting free radical scavenging and antioxidant activity [23].

On a cellular level, free radicals are inactivated by endogenous antioxidant and stress response mechanisms. We found that root extract of *Vitis vinifera* exhibited HO-1 and γGCS inducing activity which is most likely due to Nrf2 activation. Oxidized LDL plays a central role in atherogenesis [24]. Stilbenoids, such as resveratrol, have been shown to prevent

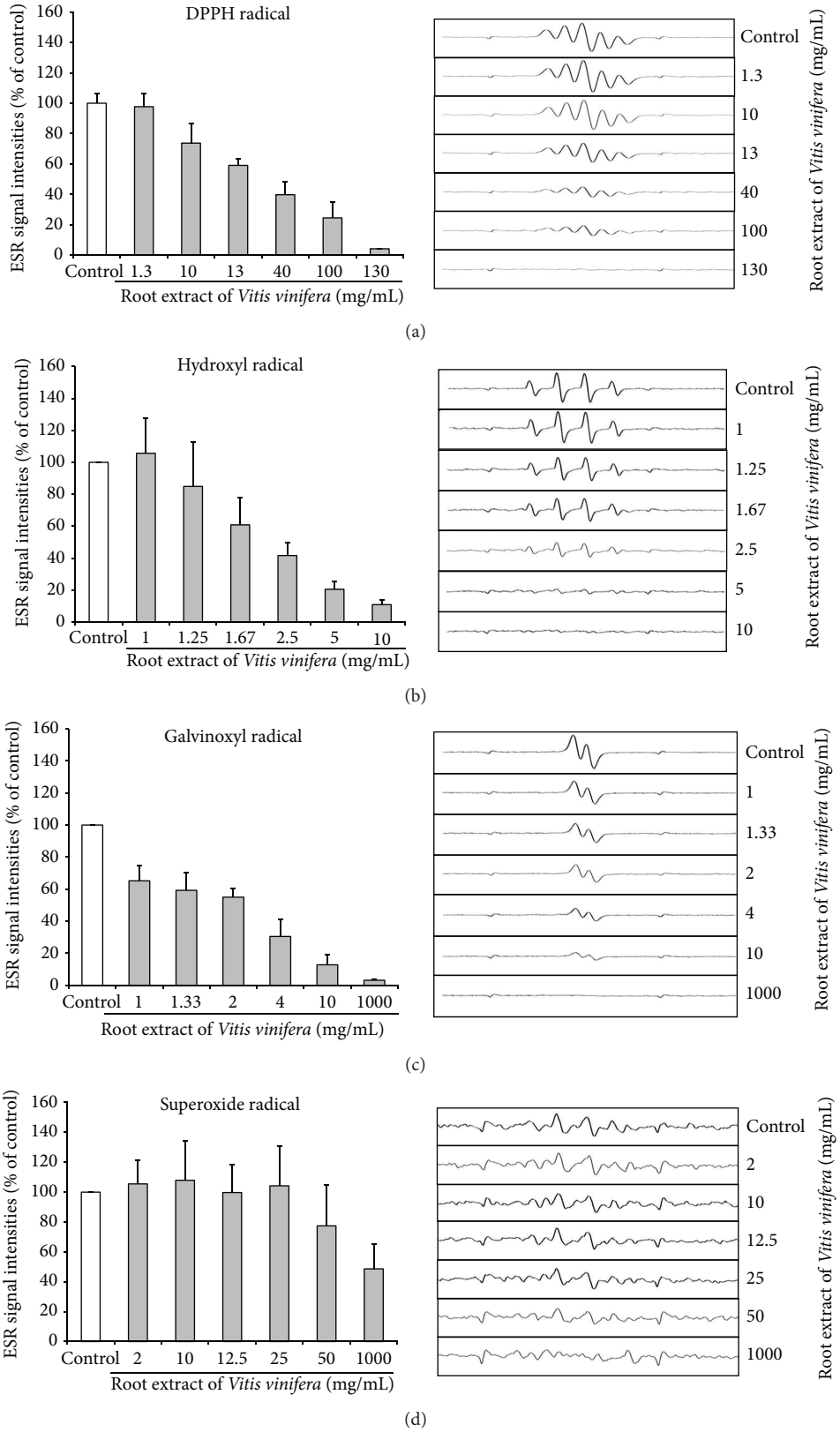

Figure 3: The scavenging effect of the root extract of *Vitis vinifera* on DPPH (a), hydroxyl (b), galvinoxyl (c), and superoxide free radical (d) measured by electron spin resonance spectroscopy (ESR). ESR spectra were recorded three times. Data are means + SD.

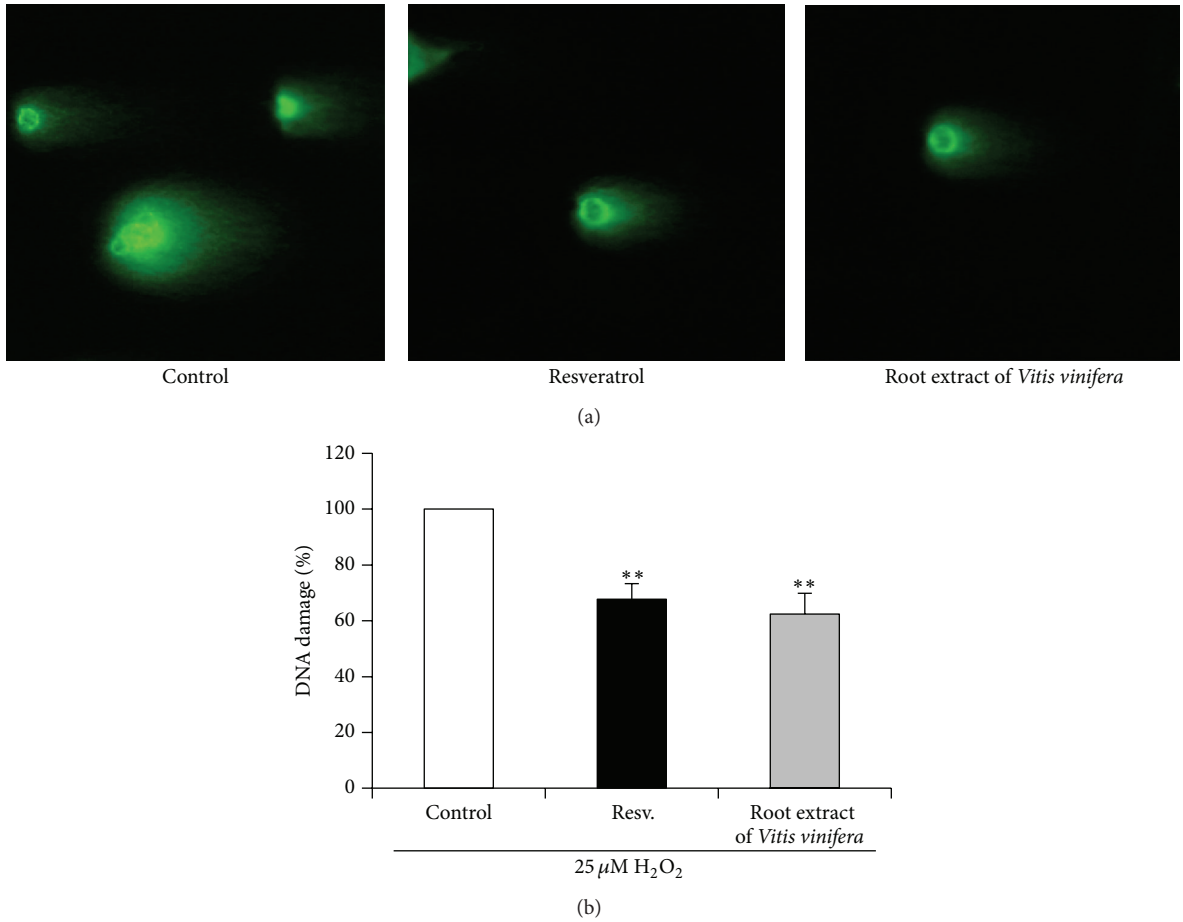

Figure 4: Effect of 50 μg/mL root extract of *Vitis vinifera* on H_2O_2-induced DNA damage in HT-29 cells after 14 h of incubation. Following treatment cells were stressed with 25 μM H_2O_2 for 15 min. Resveratrol (Resv., 50 μM) was used as a positive control. DNA damage was measured by the Comet assay. The photographs represent the comet tails (a) and the inhibition of DNA damage is shown as percentage of control damage (damage of control = 100%; (b)). Each bar represents the mean of three independent experiments + SD. ** indicates significant differences compared to untreated control cells; $p < 0.01$, Student's t-test.

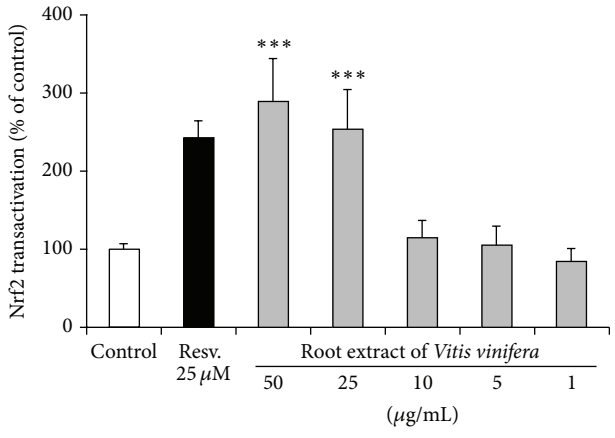

Figure 5: Effect of the root extract of *Vitis vinifera* on Nrf2 transactivation in transiently transfected Huh7 liver cells. Resveratrol (Resv., 25 μM) was used as positive control. Data are mean + SEM of at least three experiments performed in triplicate. * * * indicates significant differences compared to control; $p < 0.001$, Mann-Whitney U test.

copper mediated LDL oxidation *in vitro* through free radical scavenging activity [10]. Alternatively, our data indicate that a stilbenoid-rich extract may prevent LDL oxidation via cell signaling due to PON1 induction. Thus, stilbenoids may exhibit antiatherogenic properties due to both free radical scavenging and induction of antioxidant defense mechanisms. Interestingly, HO-1, γGCS, and PON1 decrease with age [25]. Thus, it is tempting to speculate that our root extract may counteract an aging phenotype which warrants further investigations in appropriate *in vivo* models such as laboratory rodents. Furthermore, other age-related molecular targets including sirtuins [25, 26] and FOXO [26] as well as autophagy related pathways [27] should be taken into account since they have been reported to be modulated by resveratrol in cultured cells and various model organisms.

Recent studies suggest cross talk between Nrf2 and proinflammatory gene expression. Nrf2 counteracts inflammatory processes by downregulating NF-κB [28, 29]. In the present study, the root extract of *Vitis vinifera* significantly decreased the expression of the NF-κB target genes IL-1β and iNOS in

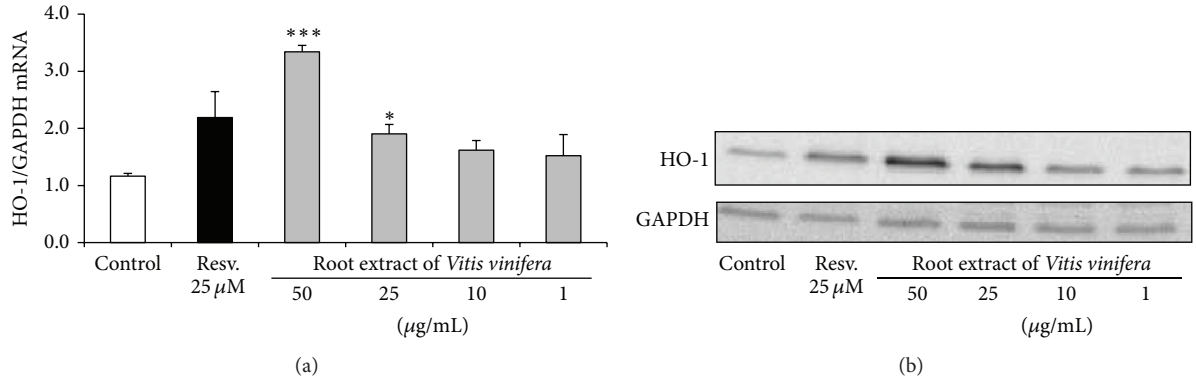

Figure 6: (a) HO-1 mRNA levels in Huh7 liver cells following 6 h of incubation with the root extract of *Vitis vinifera* compared to untreated control cells. Resveratrol (Resv., 25 μM) was used as positive control. Data are means + SEM of at least three experiments. $*$ indicates significant differences compared to untreated control; $p < 0.05$, Student's t-test, and $***$ indicates significant differences compared to untreated control cells; $p < 0.001$. (b) Western blotting of HO-1 in Huh7 whole cell extracts following 24 h of incubation with the root extract of *Vitis vinifera*. Resveratrol (Resv., 25 μM) was used as positive control and GAPDH was used as loading control. One representative Western blot out of three is shown.

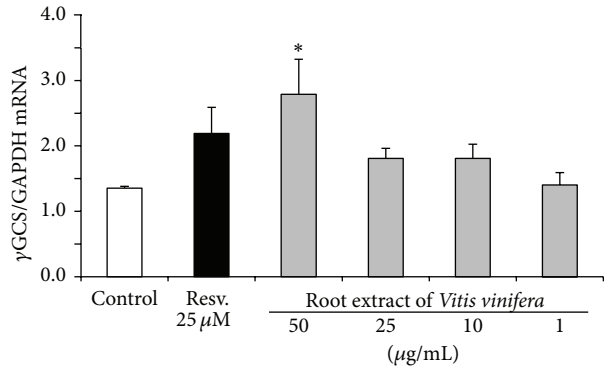

Figure 7: γGCS mRNA levels in Huh7 liver cells following 6 h of incubation with the root extract of *Vitis vinifera* compared to untreated control cells. Resveratrol (Resv., 25 μM) was used as positive control. Data are means + SEM of at least three experiments. $*$ indicates significant differences compared to untreated control cells; $p < 0.05$, Student's t-test.

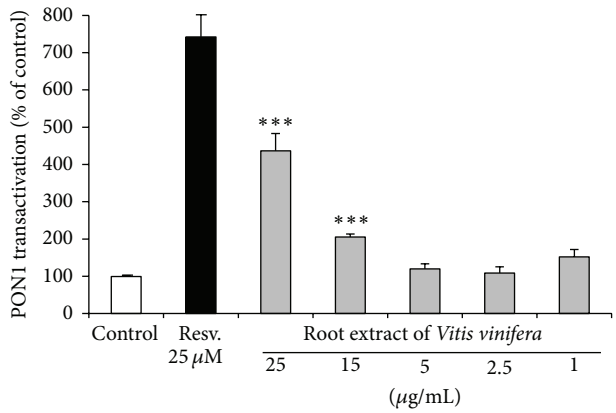

Figure 8: Effects of the root extract of *Vitis vinifera* on PON1 transactivation in stably transfected PON1-Huh7 cells. Resveratrol (Resv., 25 μM) was used as positive control. Data are means + SEM of at least three independent experiments performed in triplicate. $***$ indicates significant differences compared to control; $p < 0.001$, Mann-Whitney U test.

murine macrophages suggesting anti-inflammatory activity. These anti-inflammatory properties of the *Vitis vinifera* root extract and other related plant extracts may be beneficial in pathologies characterized by an overproduction of nitric oxide and inflammatory cytokines [30]. Additionally, other biological properties of the root extract including its effect on platelet aggregation [31], smooth muscle cell proliferation [32], cellular adhesion [33], and vasodilation [34] should be taken into consideration.

5. Summary and Conclusion

In this study, seven stilbenoids including resveratrol, piceatannol, *trans*-ε-viniferin, ampelopsin A, miyabenol C, r-2-viniferin = vitisin A, and r-viniferin = vitisin B were identified in the root extract of *Vitis vinifera* by HPLC-PDA. The root extract of *Vitis vinifera* scavenged DPPH, hydroxyl, galvinoxyl, and superoxide free radicals. Accordingly, a protection against hydrogen peroxide-induced DNA damage was observed in cultured cells. Furthermore, Nrf2 and its target genes HO-1 and γ-GCS as well as PON1 were induced by *Vitis vinifera* root extract. Moreover, the root extract downregulated proinflammatory gene expression including IL-1β and iNOS in cultured macrophages. To sum up, our results suggest free radical scavenging and cellular antioxidant and anti-inflammatory activities of the *Vitis vinifera* root extract *in vitro*. However, little is known about the bioavailability, metabolism, and bioactivity of root-derived stilbenoids *in vivo*. Therefore, future studies should address the question to which extent stilbenoids from the roots of *Vitis vinifera* are bioavailable and may exhibit potential health benefits in humans.

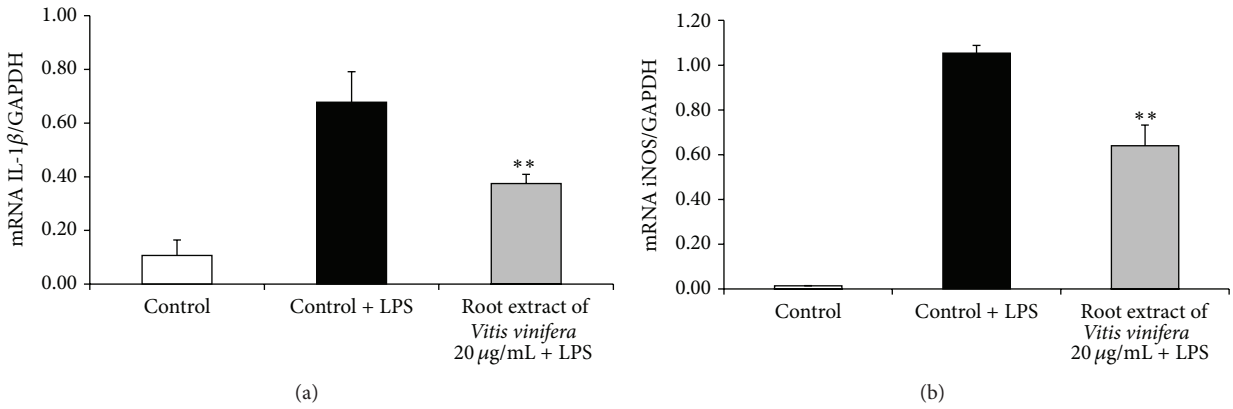

Figure 9: Effect of the root extract of *Vitis vinifera* on the inflammatory biomarkers IL-1β (a) and iNOS (b) in murine macrophages. RAW264.7 murine macrophages were incubated with the root extract of *Vitis vinifera* (20 μM) for 24 h and stimulated with 10 ng/mL lipopolysaccharide (LPS) for 4 h. mRNA levels of IL-1β and iNOS were examined with real-time PCR. Each bar represents the mean (SEM) of at least three independent experiments measured in duplicate. $**$ indicates significant differences compared to stimulated control; $p < 0.01$, Mann-Whitney U test (a) and Student's t-test (b).

In addition, both the food industry and the consumer exhibit an increasing demand for natural antioxidants [35]. Thus, further studies are needed to elucidate to which extent the stilbenoid-rich *Vitis vinifera* root extracts could prevent oxidation processes, such as lipid peroxidation, in the food matrix. Additionally, it needs to be established whether the present *Vitis vinifera* root extract may be used as a nutraceutical in functional foods.

Conflict of Interests

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

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Research Article

Modulation of Tamoxifen Cytotoxicity by Caffeic Acid Phenethyl Ester in MCF-7 Breast Cancer Cells

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Although Tamoxifen (TAM) is one of the most widely used drugs in managing breast cancer, many women still relapse after long-term therapy. Caffeic acid phenethyl ester (CAPE) is a polyphenolic compound present in many medicinal plants and in propolis. The present study examined the effect of CAPE on TAM cytotoxicity in MCF-7 cells. MCF-7 cells were treated with different concentrations of TAM and/or CAPE for 48 h. This novel combination exerted synergistic cytotoxic effects against MCF-7 cells via induction of apoptotic machinery with activation of caspases and DNA fragmentation, along with downregulation of Bcl-2 and Beclin 1 expression levels. However, the mammalian microtubule-associated protein light chain LC 3-II level was unchanged. Vascular endothelial growth factor level was also decreased, whereas levels of glutathione and nitric oxide were increased. In conclusion, CAPE augmented TAM cytotoxicity via multiple mechanisms, providing a novel therapeutic approach for breast cancer treatment that can overcome resistance and lower toxicity. This effect provides a rationale for further investigation of this combination.

1. Introduction

Breast cancer is the most prevalent cancer among the female population [1]. Despite the evolution in treating breast cancer, it is still the principal cause of cancer death in females [1, 2].

Tamoxifen (TAM) is a widely used antiestrogenic drug for treating breast cancer patients [3]. Although there are many satisfying outcomes from the endocrine therapy with TAM, not all treated patients get the hopeful result. High concentration of TAM showed several hormonal dependent and independent effects. TAM can induce apoptosis of cancer cells via the involvement of a mitochondria-dependent pathway, the amendment of signaling proteins such as protein kinase C, and/or the upregulation of p53 [4]. Hence, a combination of lower concentrations of TAM with other drugs of synergistic antitumor effect might be of priority in the therapy of breast carcinomas.

Caffeic acid phenethyl ester (CAPE) is a polyphenolic compound existing in numerous medicinal plants and in propolis [5]. It is subjected to the action of blood esterase due to its aryl ester structure. The pharmacokinetic profiles of CAPE in rat tissues showed high values of volume of distribution and short elimination half-life after its systemic administration [6].

CAPE has a diversity of important biological activities including antibacterial, antiviral, and anticancer ones [7]. Moreover, at low doses, CAPE inhibits lipid peroxidation [8] and shows antioxidant activities [9].

Several reports have shed light on the impact of CAPE on cell cycle progression, cell proliferation, induction of cell cycle arrest, and apoptosis [10]. The *in vitro* and *in vivo* inhibitory effects of CAPE were predictable in plenty of cancer models, such as colon [11], lung cancers [12], and pancreatic carcinoma [13]. A remarkable finding is the ability of CAPE to exhibit differential toxicity against tumor cells without affecting normal cells. In this context, CAPE has no cytotoxic effect on normal nonmalignant cells as MCF-10A mammary cells [5, 14].

The competency to increase response and reduce chemoresistance of cancer therapeutics via the use of the

combination therapy might thus be a significant advantage for cancer patients. Combination therapies promoting the effectiveness of TAM have been previously investigated in several studies, using compounds as vitamin E [15] and green tea [16].

Accordingly, this study examined the efficacy of TAM and CAPE with regard to multiple targets as apoptosis, autophagy, angiogenesis, and oxidative stress in breast cancer cells.

2. Materials and Methods

2.1. Drugs. TAM was a kind gift from El Amirya Pharmaceuticals Company (Alexandria, Egypt). It was dissolved in dimethyl sulfoxide (DMSO) at concentration 1 : 1 and stored at −20°C.

CAPE was purchased from Sigma-Aldrich Chemical Co. (ST. Louis, MO, USA). The compound was dissolved in DMSO at 100 mM concentration and stored at −20°C. Serial dilutions of both drugs were made in cell culture medium just prior to use, so that the final concentration of DMSO was about 0.1% (v/v).

2.2. Chemicals. Fetal bovine serum (FBS, F6178), L-glutamine, penicillin/streptomycin antibiotic, RPMI-1640 medium (R8758), and trypsin-EDTA were purchased from Biowest, France. Agarose and DMSO were purchased from Sigma-Aldrich Chemical Co., USA. Antibodies used for the detection of caspase-9 (primary mouse anti-human caspase-9 monoclonal antibody), microtubule-associated protein light chain 3- (LC3-) II (primary rabbit LC3-II oligoclonal antibody), and β-actin (primary rabbit anti-human β-actin monoclonal antibody) were obtained from eBioscience (Austria), Invitrogen (USA), and Sigma-Aldrich (USA), respectively. The primer sequences for Bcl-2, Beclin 1, vascular endothelial growth factor (VEGF), and GADPH were supplied by R&D systems (Minneapolis, MN, USA). Thermo Scientific Gene JET RNA Purification Kit (UK), Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (UK), and Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix kit (UK) were used in quantitative real time PCR analysis. Caspase-3 activity was measured using the colorimetric assay kit (R&D systems, USA). DNA fragmentation was performed by QIAamp DNA Mini Kit (QIAGEN, USA) using a suitable DNA marker (Gibco, BRL, Life technologies, USA). Other reagents were of analytical grade or the highest quality available.

2.3. Human Cancer Cell Line and Cell Culture. Human breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were cultured in RPMI-1640 medium supplemented with heat-inactivated 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified 5% CO_2 incubator.

2.4. Cytotoxicity Assay. Cytotoxicity was determined using SRB method as described by Skehan et al. [17]. In brief, cells were seeded at a density of 3×10^3 cells/well in 96-well microtiter plates. They were left to attach for 24 h before incubation with drugs. The old medium was discarded and was replaced with a fresh one containing drugs added alone or simultaneously with the specified concentrations.

In brief, the cells were treated with different concentrations of TAM (10, 20, 30, 40, and 50 μM), CAPE (0.1, 1, 10, 100, and 200 μM), or their combination. Two different regimens have been designed to approach the most effective concentrations:

(i) Cross matching combination regimen, TAM (10, 20, 30, 40, and 50 μM) with matched reversed doses of CAPE (0.1, 1, 10, 100, and 200 μM).

(ii) Fixed dose combination regimen, 10 μM of TAM with different concentrations (0.1–200 μM) of CAPE.

For each sample, three wells were used for every concentration and incubation was continued for 48 h. The same volume (200 μL/well) of DMSO (1% v/v) was used as the vehicle control. At the end of incubation, cells were fixed with 20% trichloroacetic acid (TCA), stained with 0.4% sulforhodamine-B (SRB), and rinsed with 1% acetic acid. The bound protein stain was solubilized with Tris base (10 mM, pH 10.5) and the optical density (OD) of each well was measured spectrophotometrically at 570 nm using ELISA microplate reader (TECAN sunrise, Germany). The experiment was repeated 3 times and the mean values were estimated as fraction of cell survival as follows: OD (treated cells)/OD (control cells).

The IC_{50} value (the required concentration to produce 50% inhibition of cell growth) of each drug was calculated using sigmoidal dose response curve-fitting models (GraphPad Prism software, version 5).

2.5. Evaluation of Drugs Interaction. The interaction between CAPE and TAM was evaluated by the isobologram equation: the combination index (CI) = $d1/D1 + d2/D2$ [18].

$d1$ and $d2$ signify the respective concentrations of TAM and CAPE used in combination to produce a fixed level of inhibition, while $D1$ and $D2$ represent their concentrations that are alone able to produce the same magnitude of effect. If "CI" is less than 1, the effect of combination is synergistic, whereas if CI = 1 or >1, the effect is additive or antagonistic, respectively.

2.6. Determination of TAM Uptake by MCF7 Cells. MCF7 cells 10×10^3/well were seeded in RPMI-1640 medium and left for 24 h. The plate was divided into 2 groups as follows:

(a) Group I was treated with 10 μM TAM.

(b) Group II was treated with 10 μM TAM and 4 μM CAPE.

The medium was then aspirated after 0, 2, 4, and 24 h intervals and centrifuged and the supernatant was stored at −20°C till HPLC assay.

2.7. Sample Extraction and Preparation for Liquid Chromatography-Tandem Mass Spectrometry. 200 μL of the medium was mixed thoroughly with 200 μL acetonitrile (Alliance Bio, USA) and centrifuged at 1400 rpm for 15 min at 4°C. 10 μL

of the resultant clear supernatant was then injected into AB SCIEX LC/MS/MS system (AB SCIEX 3200 Q TRAP, Germany) equipped with electrospray ionization (ESI) source and an Agilent 1260 affinity HPLC system, consisting of a vacuum degasser, a binary pump, and an autosampler to determine the concentration of TAM. Analyst 1.5.2 software was used for data acquisition and processing. The analytical column used was Agilent Poroshell 120-C18 (50 mm × 3 mm × 2.7 μm, Agilent, Germany) at 25°C. The mobile phase consists of 0.1% formic acid/water (solvent A) and 0.1% formic acid/acetonitrile (solvent B), delivered at a flow rate of 0.5 mL/min. Mass spectrometric analysis was performed in the positive ion mode.

2.8. Western Blotting Analysis. Cells were seeded, cultured, and treated with TAM and CAPE and their combination for 24 h and 48 h. At time of harvest, control and treated cells were collected and lysed in the lysis buffer (150 mM NaCl, 10 mM Tris, 0.2% Triton X-100, 0.3% NP-40 (nonyl phenoxypolyethoxyl ethanol), 0.2% Na_3VO_4, and protease inhibitor cocktail, pH 7.4) (Bio Basic Inc., Canada). After centrifugation at 14,000 rpm for 15 min (at 4°C), the protein concentration in the supernatant was measured by Bradford method using Coomassie Protein Assay Kit (Pierce, USA) [19]. Proteins were then separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked with 5% w/v nonfat dry milk and incubated overnight with the specific primary antibodies {mouse anti-human for caspase-9 monoclonal antibody (1 : 1000), rabbit LC_3-II oligoclonal antibody (1 : 500), and rabbit anti-human β-actin monoclonal antibody (1 : 1000)}. This step was followed by incubation with the appropriate diluted secondary antibody (1 : 2000 in PBS-T) for 1 h at room temperature. The blots were developed with Amersham ECL western blotting detection reagents and analysis system (GE Healthcare, UK) according to the manufacturer's protocol. Protein loading was corrected for β-actin and quantitation of band intensity was performed using Image J Software.

2.9. Determination of the Enzymatic Activity of Caspase-3 in Cell Lysate. Caspase-3 activity was measured colorimetrically using Caspase-3 Colorimetric Assay kit (R&D, USA, Catalog number BF3100) according to the manufacturer's instructions based on the method of Fernandes-Alnemri et al. [20]. The cleavage of the peptide by the caspase released the chromophore pNA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. Caspase-3 activity was expressed as optical density.

2.10. Flow Cytometric Analysis of FITC Annexin V Staining. FITC Annexin V Apoptosis Detection kit (BD Bioscience Pharmingen, San Jose, CA) was used as per manufacturer's recommendation. Approximately 5×10^5 cells were plated in T-75 flasks on Day −1 and left to adhere. On Day 0 the medium was replaced with fresh standard medium, and 10 μM TAM, 4 μM CAPE, or both of them were added. Untreated control received ethanol solvent (0.1%). Cells were harvested after 48 h (Day 2). Cells were trypsinized and then washed in PBS and centrifuged at 1200 rpm for 5 min. The pellets were resuspended in 100 μL of staining solution (containing annexin V-fluorescein and propidium iodide in buffer) and were mixed gently and incubated for 15 min at room temperature (15–25°C) in the dark. Finally, 400 μL of binding buffer was added. FACScan analysis was performed using a Becton Dickinson FAC Scan analyzer (Becton Dickinson, Heidelberg, Germany).

Table 1: Primer sequences for quantitative real time PCR.

Gene	Sequences
Bcl-2	Forward 5′-TCT GAC GGC AAC TTC AAC TG-3′
	Reverse 5′-TGG GTG TCC CAA AGT AGG AG-3′
Beclin 1	Forward 5′-ATC CTG GAC CGT GTC ACC ATC CAG G-3′
	Reverse 5′-GTT GAG CTG AGT GTC CAG CTG G-3′
VEGF	Forward 5′-TCC TCA CAC CAT TGA AAC CA-3′
	Reverse 5′-GAT CCT GCC CTG TCT CTC TG-3′
GAPDH	Forward 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′
	Reverse 5′-GTC CAC CAC CCT GTT GCT GTA G-3′

2.11. DNA Fragmentation Analysis. DNA was extracted using QIAamp DNA Mini Kit according to the manufacturer's instructions. DNA was electrophoresed and visualized under ultraviolet light using 0.8% agarose gel stained with ethidium bromide.

2.12. Quantitative Real Time PCR Analysis. Total RNA was extracted from cell culture by utilizing Thermo Scientific Gene JET RNA Purification Kit, following the manufacturer's protocol. cDNA was generated with M-MuLV reverse transcriptase using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. For real time PCR quantification, Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix kit was used. Briefly, in a 25 μL reaction volume, 12.5 μL of master mix, 2.5 μL of primer assay, and 10 μL of template cDNA (100 ng) were added to each well. Sequences of primers were described in Table 1. The PCR plate was subjected to 40 cycles of the following conditions: PCR activation at 95°C for 5 min, denaturation at 95°C for 5 sec, and annealing/extension at 60°C for 10 sec.

The values of RT-PCR products were normalized with respect to GAPDH and then compared to controls. The relative expression was calculated from the $2^{-\Delta\Delta CT}$ formula [21].

2.13. Determination of Glutathione (GSH). Reduced glutathione was determined as described by Ellman [22]. In brief, cells were collected by trypsinization after treatment with TAM, CAPE, and their combination, as well as the control. Samples were then centrifuged at 1200 rpm for 5 min and the resultant cell pellet was suspended in 1 mL saline.

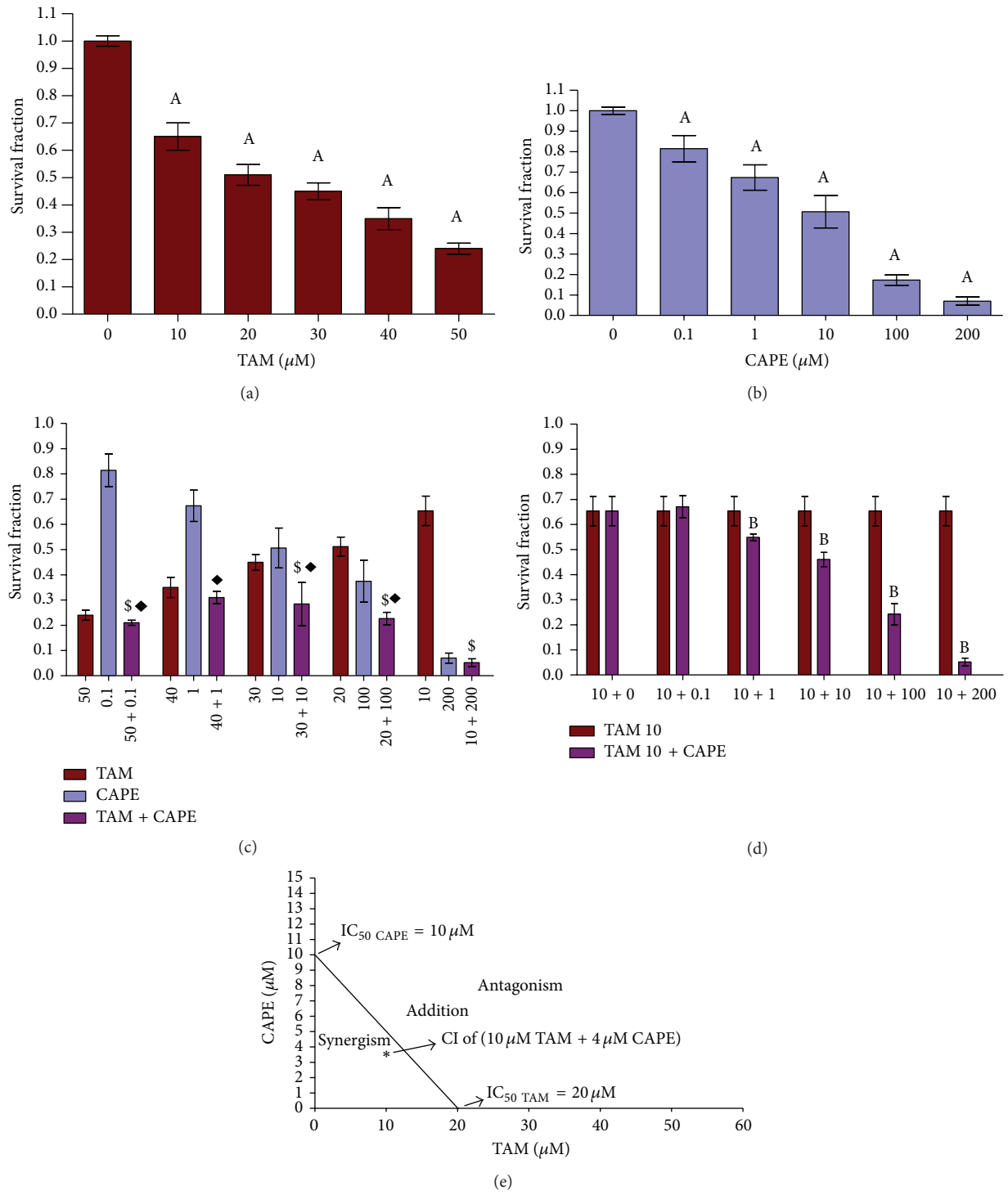

Figure 1: Survival fraction in MCF-7 cells after 48 h treatment with TAM, CAPE, and their combination. Cells were treated with various concentrations of (a) TAM, (b) CAPE, (c) TAM + CAPE, and (d) 10 μM TAM + CAPE for 48 h. (e) Isobologram analysis of MCF-7 cell growth inhibition by TAM and CAPE after 48 h of treatment. The IC_{50} value of each drug after 48 h is plotted on the axes; the solid line represents the additive effect, while the asterisk (∗) located below the connecting line points to the synergistic effect of the concentrations of TAM and CAPE used in the combination. Results were expressed as means ± SD of 3 independent experiments performed in triplets. P value < 0.05 is considered significant. A: significantly different from the respective concentration of control. B or \$: significantly different from the respective concentration of TAM. ◆: significantly different from the respective concentration of CAPE.

Next, 500 μL of cell suspension was mixed well with 25 μL trichloroacetic acid (TCA) and the tubes were centrifuged at 3000 rpm for 10 min at 4°C. 100 μL of the resultant supernatant was mixed thoroughly with 850 μL of phosphate buffer followed by addition of 50 μL Ellman's reagent. After 5 min, the absorbance was measured spectrophotometrically at 405 nm against a blank. Glutathione content was expressed as nmoles of GSH/mg protein.

2.14. Determination of Nitric Oxide (NO). Nitric oxide produced in cell culture media was estimated spectrophotometrically [23]. Briefly, cells were collected by trypsinization after treatment with TAM, CAPE, and their combination as well as the control. Next, 50 μL of zinc sulfate solution was added to 250 μL media and centrifuged at 17000 rpm. The resultant supernatant was treated with vanadium chloride (0.8% in 1 M HCl) and Griess reagent (prepared by mixing equal volumes of N-1-(naphthyl)ethylenediamine {0.1% in bidistilled water} and sulfanilamide {2% in 5% HCl}). Total NO content was expressed as μg/mL.

2.15. Statistical Analysis. Data were expressed as means ± SD. Differences among groups were tested using one-way analysis of variance (ANOVA) followed by a Tukey post hoc correction for multiple comparisons using SPSS (version 17.0). Significant differences were considered at P value < 0.05. All figures were established using GraphPad Prism, version 5.

2.16. Ethical Aspects. There is no need for ethical approval or informed consent since all experiments were carried out using MCF-7 breast cancer cell line.

3. Results

3.1. Growth Inhibition of TAM, CAPE, and Their Combination against MCF-7 Cell Line. Figure 1(a) shows the effect of different concentrations of TAM on the survival fraction of MCF-7 cells after 48 h exposure. As evident, there was a significant dose dependent decrease in the survival fraction compared to each respective control value. At 50 μM, maximum cytotoxicity (76%) of TAM against MCF-7 was reached, whereas the IC_{50} value was obtained at 20 μM.

Figure 1(b) depicts the effect of treating MCF-7 cells with different concentrations of CAPE. After 48 h, the number of surviving cells was significantly decreased in a dose dependent manner compared to the respective control values. The effect of CAPE on cell survival reached its maximum (93%) at 100 μM, with IC_{50} value of 10 μM.

When examining the cytotoxic effect of different combinations of TAM and CAPE (Figure 1(c)), we observed some cytotoxic actions upon decreasing the concentration of TAM and increasing that of CAPE. When combining the smallest dose of TAM (10 μM) with different concentrations of CAPE, the IC_{50} of CAPE decreased to a value ranging between 1 μM and 10 μM (Figure 1(d)). An isobologram analysis illustrated the synergistic effect of 10 μM TAM and 4 μM CAPE (CI = 0.9).

3.2. Effect of CAPE on the Cellular Uptake of TAM. Figure 2 showed that majority of the TAM was taken by the cells after

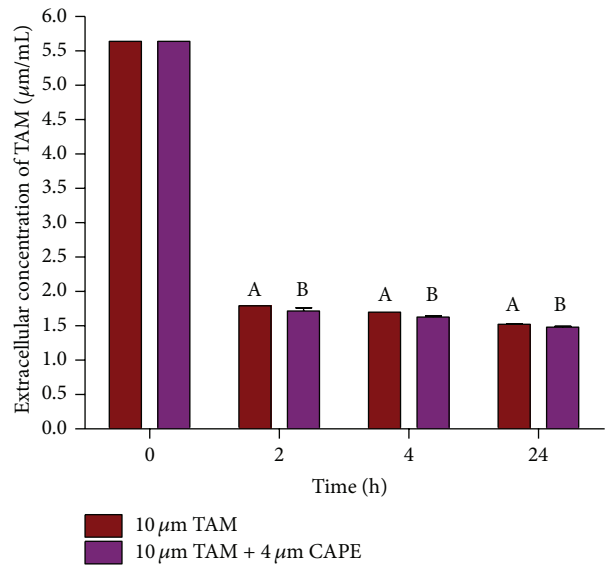

Figure 2: Effect of CAPE on cellular uptake of TAM from the culture medium after different time intervals. Results were expressed as means ± SD of 2 independent experiments. P value < 0.05 is considered significant. A: significantly different from TAM alone at $P < 0.05$. B: significantly different from TAM + CAPE at $P < 0.05$.

2 h treatment. Meanwhile, no significant change in cellular uptake of TAM was achieved upon cotreatment with CAPE, as compared with the respective TAM treated group in all the studied time intervals.

3.3. Effect of TAM, CAPE, and Their Combination on Caspase-9 and LC3-II Protein Levels in MCF-7 Cell Line. As illustrated in Figures 3(a) and 3(b), incubation of MCF-7 cells with 10 μM TAM, 4 μM CAPE, and their combination for different time intervals (24 h and 48 h) caused activation and subsequent cleavage of caspase-9. On the other hand, no change in the protein level of LC3-II was detected.

3.4. Effect of TAM, CAPE, and Their Combination on Caspase-3 Activity in MCF-7 Cell Line. Treatment of MCF-7 cells with 10 μM TAM produced 1.5- and 2.5-fold increases in caspase-3 enzymatic activity after 24 h and 48 h, respectively, as compared to the control values. Furthermore, 4 μM CAPE exhibited approximately 2.5-fold increases at both treatment periods compared to the control groups. Upon treating the cells with both TAM and CAPE, the activity of caspase-3 was considerably enhanced almost 3- and 4-fold, respectively, as compared to the control values (Figures 4(a) and 4(b)).

3.5. Effect of TAM, CAPE, and Their Combination on Apoptosis as Detected by Annexin Binding Assay. As shown in Figure 5, TAM, CAPE, and their combination enhanced apoptosis of MCF7 cancer cells by 59.54%, 45%, and 61%. It is worthy noting that the combination regimen exhibited the pronounced effect in this regard.

3.6. Effect of TAM, CAPE, and Their Combination on DNA Fragmentation in MCF-7 Cell Line. Gel electrophoresis

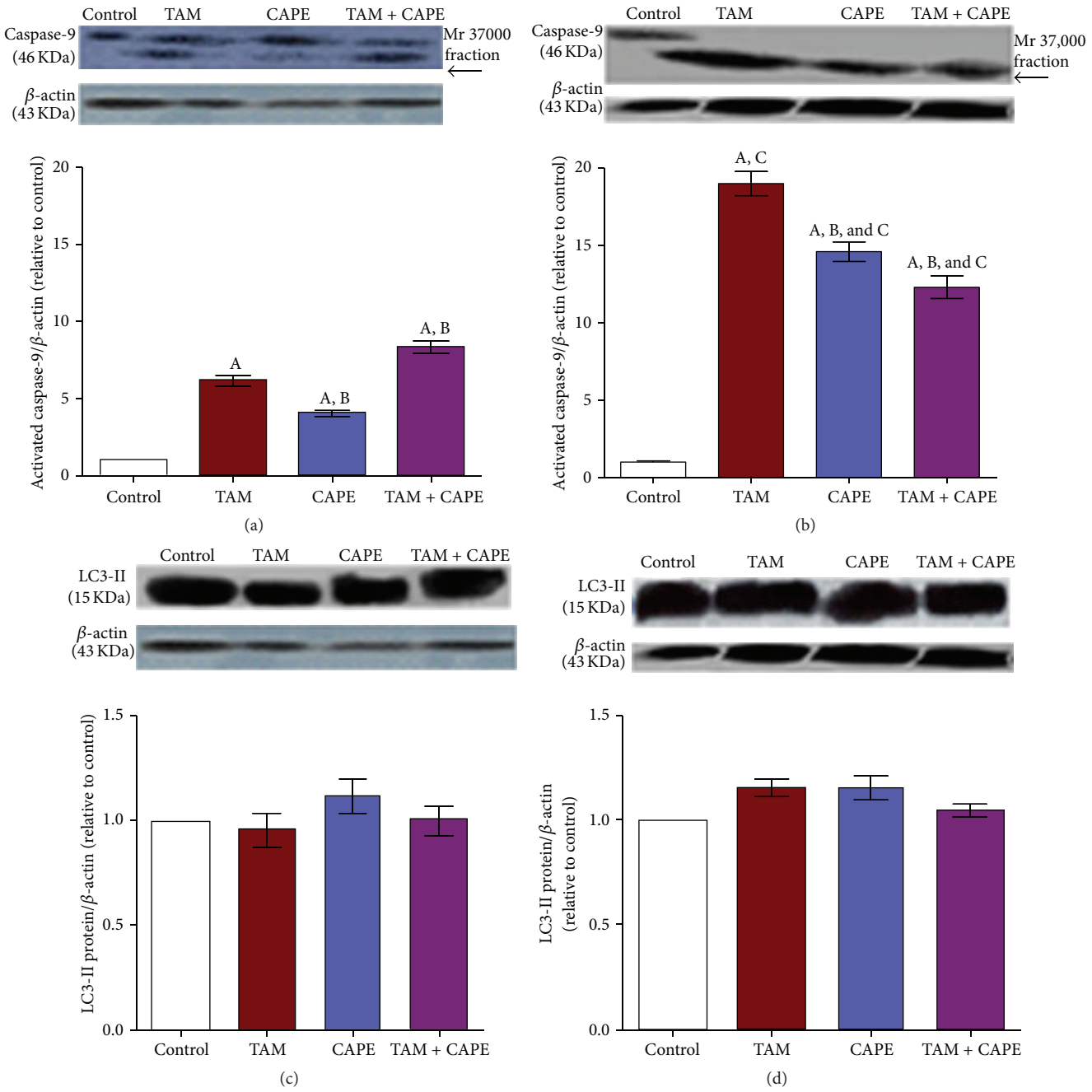

Figure 3: Western blot of caspase-9 and LC3-II in MCF-7 cells. Western blot analysis of caspase-9 and LC3-II after 24 h (a and c) and 48 h (b and d) treatments with TAM (10 μM), CAPE (4 μM), and their combination. Results were expressed as means ± SD of 3 independent experiments performed in triplets. P value < 0.05 is considered significant. A: significantly different from the respective concentration of control at $P < 0.05$. B: significantly different from the respective concentration of TAM at $P < 0.05$. C: significant difference of 48 h treatment from the respective concentration at 24 h at $P < 0.05$.

revealed that treatment of MCF-7 cells with 10 μM TAM, 4 μM CAPE, and their combination for 48 h triggered degradation of DNA into oligonucleosomal fragments as detected by DNA laddering (Figure 6).

3.7. Gene Expression Profile. All treatments displayed a trend of downregulation in both Bcl-2 and Beclin 1 expression levels. As shown in Figure 7(a), 24 h treatment with either TAM or CAPE reduced Bcl-2 level almost 7- and 4-fold, respectively. Meanwhile, the combination afforded 8-fold decrease relative to the corresponding control values. On the other side, the 48 h (Figure 7(b)) treatment reduced its level 4-, 6-, and 15-fold for TAM, CAPE, and TAM + CAPE, respectively. Regarding Beclin 1 expression, treatment with TAM decreased its expression almost 2-fold after both periods. Meanwhile, the decrease was amounted to 1-fold in case

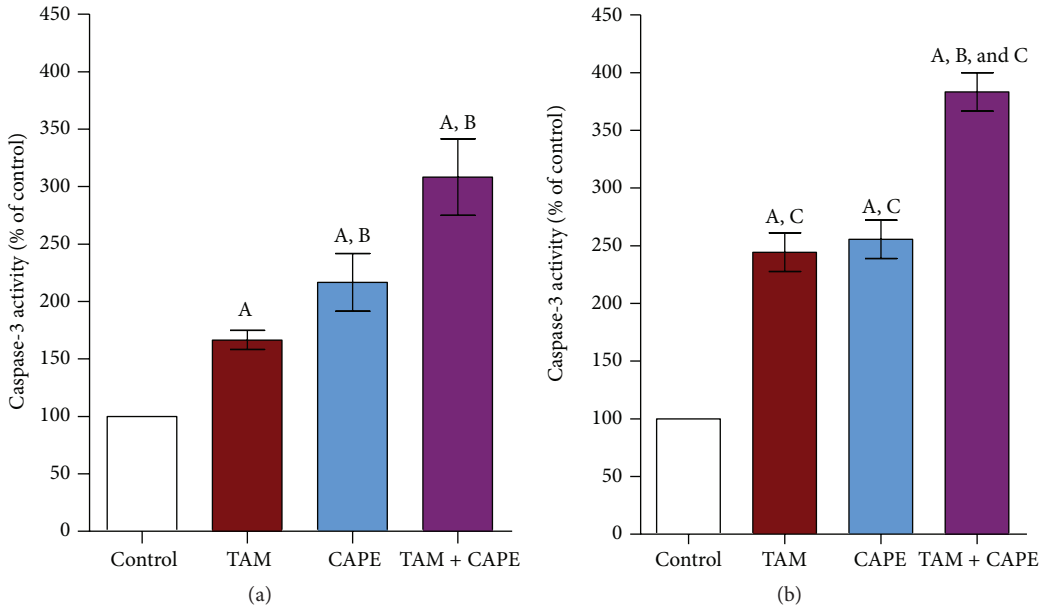

Figure 4: Caspase-3 activity in MCF-7 cells. Caspase-3 activity after 24 h (a) and 48 h (b) treatments with (10 μM), CAPE (4 μM), and their combination. Results were expressed as means ± SD of 3 independent experiments performed in triplets. P value < 0.05 is considered significant. A: significantly different from the respective concentration of control at $P < 0.05$. B: significantly different from the respective concentration of TAM at $P < 0.05$. C: significant difference of 48 h treatment from the respective concentration at 24 h at $P < 0.05$.

of CAPE. Nevertheless, the combination regimen reduced its level 1-fold after 24 h and 4-fold after 48 h (Figures 7(c) and 7(d)). VEGF gene expression was also downregulated time dependently in all treated groups with the combination regimen showing the most potent effect (Figures 7(e) and 7(f)).

3.8. Effect of TAM, CAPE, and Their Combination on GSH Content in MCF-7 Cells. As shown in Figure 8(a), 10 μM TAM when used alone resulted in significant increase in cellular GSH content to approximately 2-fold as compared to the control. On the other hand, treatment with 4 μM CAPE resulted in insignificant increase in GSH content relative to the control values. However, the combination of TAM and CAPE significantly increased GSH level almost 3-fold when compared to the control cells.

3.9. Effect of TAM, CAPE, and Their Combination on NO Production in MCF7 Cells. TAM and CAPE significantly increased NO content as compared to vehicle-treated cells (Figure 8(b)). However, the combination significantly increased the NO values compared to either single treatment group.

4. Discussion

Since cancer is still a major threat to health worldwide, there is global demand for more affordable and effective therapeutic alternatives. Perhaps, combining anticancer drugs with natural nutrients will be promising in the therapy of cancer patients.

According to this background, we aimed to explore the effect of TAM and CAPE, as a novel combination regimen in treating human breast cancer, and also to determine their supposed mechanisms of action.

The data of the present study revealed a concentration-dependent cytotoxic effect of TAM on MCF-7. This result supports the previously reported data elucidating the effectiveness and usefulness of TAM as a chemotherapeutic agent in human MCF-7 cells [24]. Similarly, the cytotoxic effect in MCF-7 treated with CAPE was evidenced herein. This finding concurs with the report of Wu et al. [14] demonstrating a diversity of oncolytic effects of CAPE in preclinical models of human breast cancer. Based on that, it is plausible that the combination of TAM and CAPE produced a synergistic cytotoxic effect in MCF-7 cells as indicated by CI.

Regarding the cellular uptake of TAM, the present data showed that the majority of the TAM was taken up by the cells after 2 h treatment and that no significant change in its uptake was achieved upon cotreatment with CAPE as compared with TAM treated group in all the studied time intervals. This can be explained on the basis of the fact that the lipophilicity of TAM is the controlling factor responsible for its cellular uptake [25]. TAM was taken up by the cells through simple diffusion until establishing equilibrium after 2 h, as suggested by the present data. De Santana et al. have previously reported that TAM citrate reached maximum cellular uptake after 4 h when tested in dogs [26]. This finding somewhat concurs with the current one.

Our data also revealed that the incubation of MCF-7 cells with TAM, CAPE, and their combination provoked the activation of caspase-9 and increased significantly caspase-3 activity. These results pointed to the contribution of the intrinsic pathway of apoptosis where cytosolic cytochrome c binds with procaspase-9 and Apaf-1, forming the apoptosome that triggers the cleavage of procaspase-9. Active caspase-9

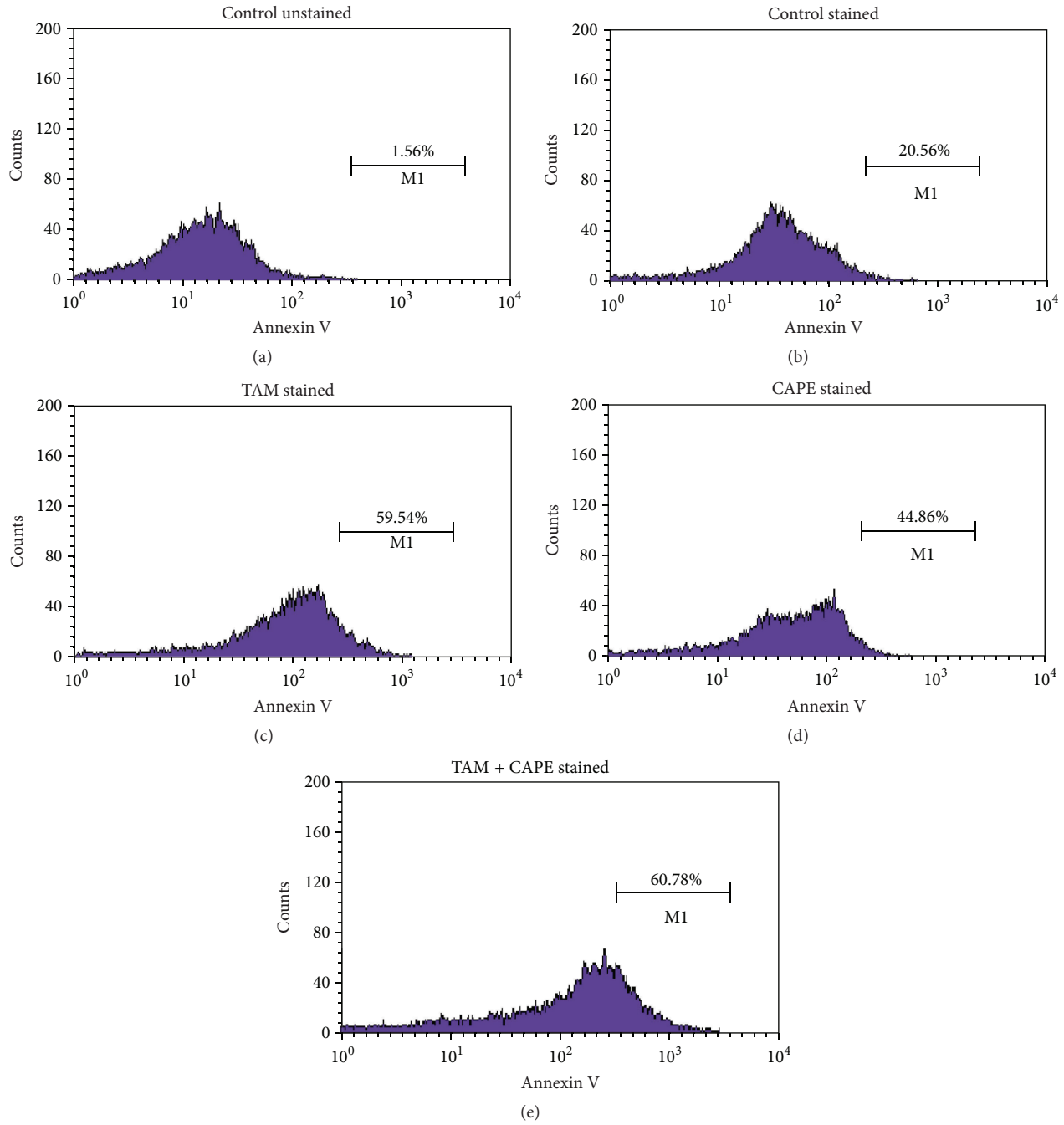

Figure 5: Flow cytometric analysis of TAM, CAPE, and their combination in MCF-7 after 48 h treatment. (a) Untreated unstained MCF-7 cells. (b) Untreated stained MCF-7 cells. (c) MCF-7 cells treated with 10 μM TAM for 48 h. (d) MCF-7 cells treated with 4 μM CAPE for 48 h. (e) MCF7 cells treated with both drugs for 48 h.

then switches on downstream executioner caspases, including caspase-3 [27].

The roles of mitogen-activated protein kinases (MAPK), mitochondrial permeability transition, and ceramide generation have been formerly implicated in TAM-induced apoptosis [28]. Likewise, activation of Fas- and Bax-mediated apoptosis and associated DNA fragmentation was detected in MCF-7 human breast cancer cells following treatment with CAPE [29]. Thus, it is obvious that CAPE could potentiate both the antiproliferative and apoptotic effects of TAM.

It is worthy noting that despite the significant increase in caspase-9 activity, seen in the combination regimen after 24 h, there was a decrease in its activity after 48 h when compared to either TAM or CAPE alone. This result might be attributed to the increased GSH level observed in this group. Actually, increased GSH level decreased the availability of free radicals, leading to attenuation of mitochondrial-dependent apoptosis.

TAM- and CAPE-triggered apoptosis was also supported by DNA fragmentation in MCF-7 cells following treatments.

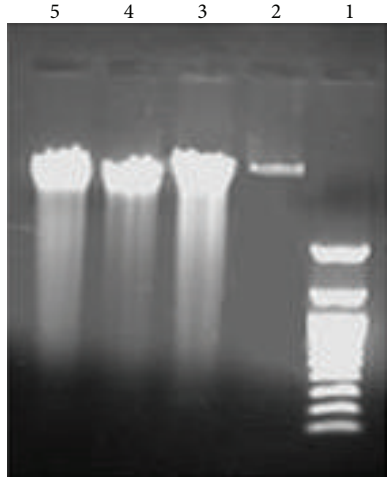

Figure 6: DNA gel electrophoresis in MCF-7 cells. DNA gel electrophoresis was performed after 48 h treatment with TAM (10 μM), CAPE (4 μM), and their combination. Lane (1): DNA ladder; lane (2): the control group; lane (3): TAM; lane (4): CAPE; lane (5): the combination therapy.

This effect might be attributed to the caspases activation mentioned before. Virtually, DNA fragmentation is a consequence of the activation of a specific DNase (CAD, caspase activated DNase), found complexed with ICAD (inhibitor of CAD) in proliferating cells. When cells undergo apoptosis, caspases, in particular caspase-3, dissociate the CAD-ICAD complex, freeing CAD that cleaves chromosomal DNA [30].

Beclin 1 level plays a crucial role in controlling autophagy [31]. Another important factor involved in this process is the antiapoptotic Bcl-2, which is highly expressed in 40–80% of breast cancer patients [32]. Bcl-2 suppresses autophagy by directly targeting Beclin 1 [33].

In the present investigation, both Bcl-2 and Beclin 1 expression levels were downregulated in all treated groups. This finding pointed to their interaction. Interestingly, Beclin 1 was found to contain a well-recognized α-helical BH3 domain that allows it to dock into the hydrophobic groove of Bcl-2 [34]. Furthermore, decreased Beclin 1 expression level might be related to the plausible role of caspases in inactivating Beclin 1-induced autophagy. According to Wirawan et al. [34], caspases mediate cleavage of Beclin 1, abrogate its autophagic function, and generate a Beclin 1-C fragment that can enhance apoptosis by promoting the release of proapoptotic factors from the mitochondria. By this way, Beclin 1 switches from a proautophagic to a proapoptotic protein. In this context, Qadir et al. [35] have demonstrated that when autophagy is compromised in TAM treated MCF-7 cells, activation of the mitochondrial apoptotic pathway takes place and increased apoptosis is achieved, at least in part, through caspase-9.

Herein, no change in the protein level of LC3-II was detected in all treated groups. These results were in contrast with those of Hwang et al. [36] who found increased levels of LC3-II in MCF-7 cells exposed to TAM. Similarly, CAPE triggered activation of the autophagic response in C6 glioma cells by inducing an increase in LC3 [37].

The discrepancy between our result and other researchers may be due to time of detection of autophagosomes and interplay between autophagy and other players as oxidative stress, metabolites, and apoptosis.

The major problem was to quantify precisely autophagy levels in cancer cells. Indeed, LC3-II levels have been described as an accurate marker of the number of autophagosomes in cells. However, an accumulation of these intracellular vesicles can be also linked to an increase of autophagy induction or an inhibition of autophagosome degradation by the lysosomes. To our knowledge, no autophagy marker is currently available in order to discriminate between an increase or a decrease of overall autophagy flux *in vivo* [38].

The present study also wanted to clarify whether, or not, the tumor inhibitory effect of TAM + CAPE is mediated via angiogenesis inhibition in breast cancer. Previously, low serum VEGF levels were reported in breast carcinoma patients after TAM therapy [39]. However, the marked decrease in VEGF levels when combined with CAPE could be related to the additional angiostatic activity exerted by CAPE. According to El-Refaei and El-Naa [40], CAPE controls tumor growth by elevating the angiostatic factors and inhibiting the angiogenic ones. Moreover, CAPE was able to inhibit NF-κB in human breast cancer MCF-7 cells [29]. Blockade of NF-κB signaling has been suggested to inhibit angiogenesis and tumorigenicity in different types of cancer cells by suppressing the expression of VEGF [41].

Unexpectedly, the current study demonstrated that both TAM and CAPE alone increased GSH content, whereas their combination enhanced this effect compared to either treatment alone. These data suggested that increased cellular GSH content might have a role in TAM and CAPE-mediated effect. In line, Loo et al. [42] and Moreira et al. [43] have demonstrated that TAM exhibited an antioxidant effect and was able to induce the NO synthesis *in vitro*. Likewise, Singhal et al. [44] have revealed that low concentrations of polyphenolics can enhance the activity of γ-glutamyl cysteinyl synthetase and other GSH-linked detoxifying enzymes, preserving thus GSH level.

With respect to the effect of TAM and CAPE on NO production, we found an increase in NO level. Previously, TAM treatment has prevented the transformation of C3H10T1/2 murine fibroblast cell line and has augmented NO production via the induction of inducible nitric oxide synthase (iNOS) at concentrations blocking the cell transformation [42]. NO was found to exert a cytotoxic effect on tumor cells via inhibition of cellular proliferation [45]. Moreover, Ignarro et al. [46] have confirmed the inhibitory effect of arginine-NO pathway in vascular smooth muscle proliferation and have attributed their finding to the capacity of NO to inhibit ornithine decarboxylase. Furthermore, it has been reported that NO possesses a cytotoxic effect caused by its interaction with the Fe-containing rate-limiting enzyme of DNA synthesis, ribonucleotide reductase [47], and [Fe-S] cluster enzymes, as mitochondrial aconitase [48, 49]. Moreover, Watts et al. [50] have reported that the cytotoxic and the antiproliferative effects of NO were attributed to its ability to induce iron and GSH efflux from tumor cells via the GSH transporter multidrug resistance-associated protein 1 (MRP1).

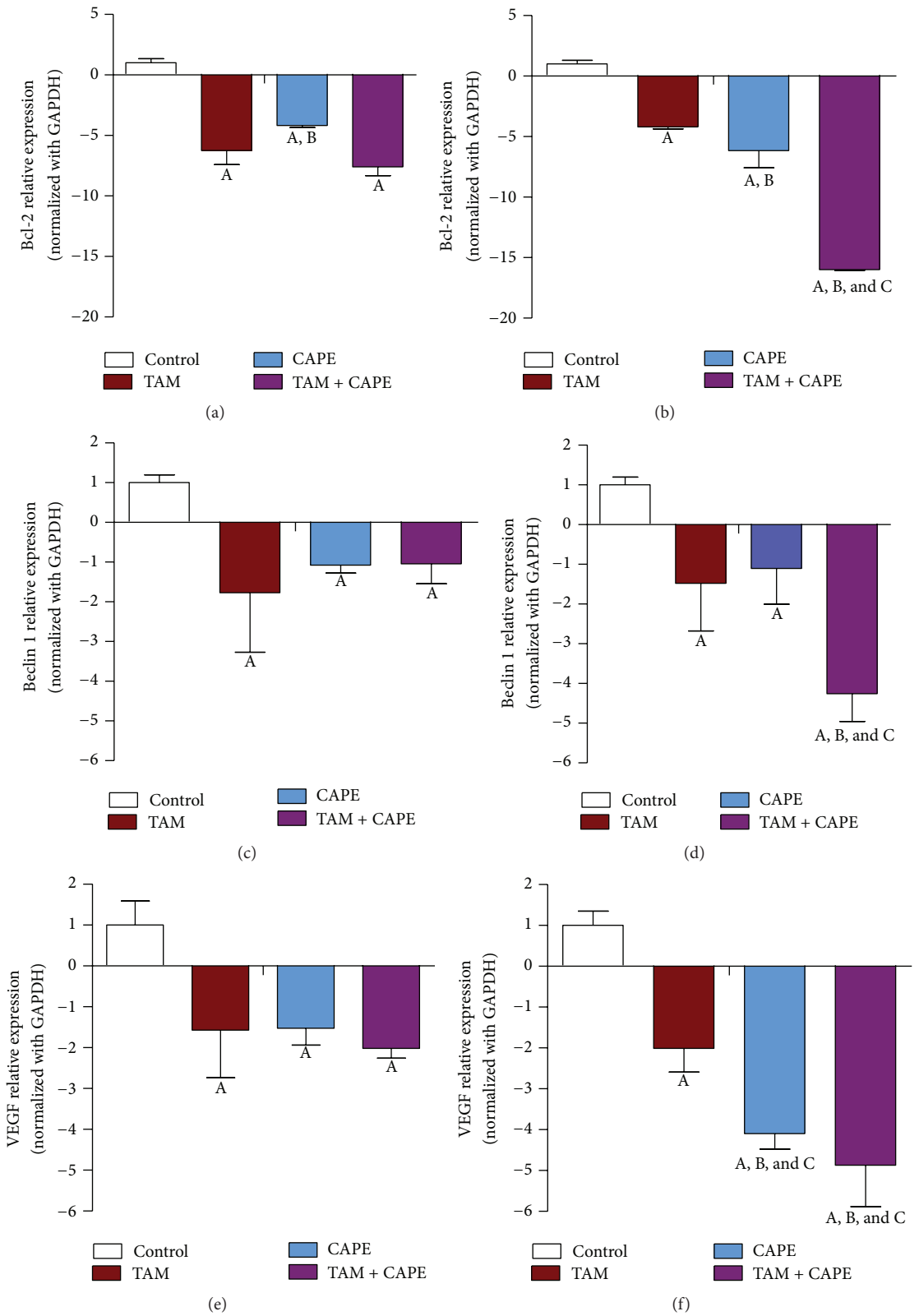

FIGURE 7: Expression levels of Bcl-2, Beclin 1, and VEGF in MCF-7 cells. Expression levels of Bcl-2, Beclin 1, and VEGF after 24 h (a, c, and e) and 48 h (b, d, and f) treatments with TAM (10 μM), CAPE (4 μM), and their combination. Results were expressed as relative expression of 3 independent experiments performed in triplets. Values of each bar are means ± SD. GAPDH was used as an internal control for calculating mRNA relative expression. A: significantly different from the respective concentration of control at $P < 0.05$. B: significantly different from the respective concentration of TAM at $P < 0.05$. C: significant difference of 48 h treatment from the respective concentration at 24 h at $P < 0.05$.

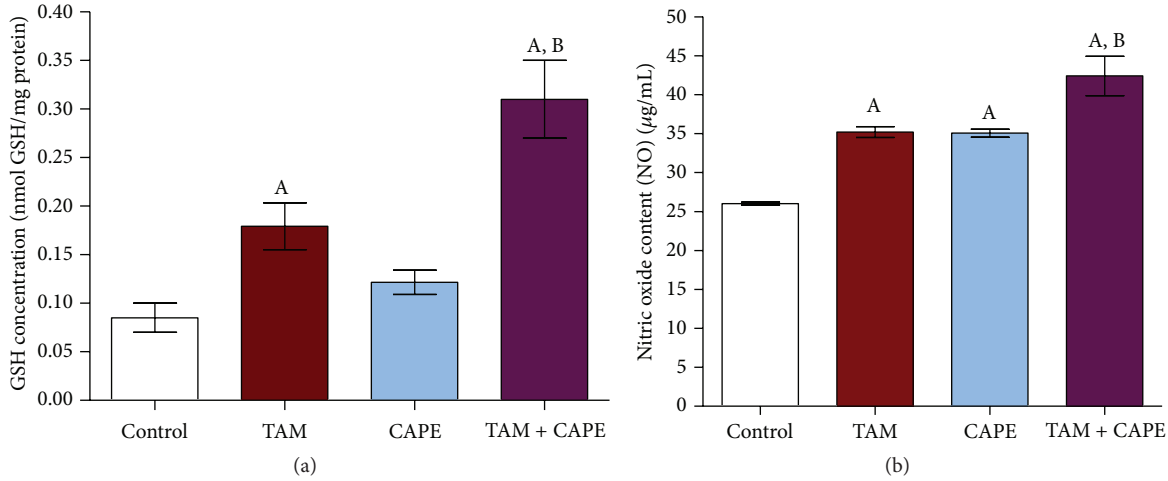

Figure 8: Glutathione (GSH) and nitric oxide (NO) levels in MCF7 cells following 48 h treatment. (a) Effect of treatment with 10 μM TAM, 4 μM CAPE, and their combination on glutathione (GSH) level in MCF-7 cells. The columns represent nmol GSH/mg protein. (b) Effect of treatment with 10 μM TAM, 4 μM CAPE, and their combination on nitric oxide (NO) level in MCF-7 cells. The columns represent μg/mL nitrate. The values were represented as means ± SD of three separate experiments. A: significantly different from the control group at $P < 0.05$. B: significantly different from TAM group at $P < 0.05$.

In conclusion, this study demonstrates that CAPE enhanced TAM cytotoxicity via multitarget approach, including weakening of autophagy, strengthening of both apoptotic and angiostatic potentials, and finally augmentation of both cellular GSH and NO levels. In addition, the ability of CAPE to lower the effective dose of TAM provides a rationale for further experimental and clinical investigations of this combination.

Conflict of Interests

There is no conflict of interests.

Acknowledgments

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