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Bioactive Food Components and their Chronic Diseases Prevention Effects

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Review Article

Extraction of Flavonoids from Corn Silk and Biological Activities *In Vitro*

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When harvesting corn, corn silk was discarded as waste, including the compounds isolated and identified from corn silk such as flavonoids, sterols, alkaloids, polysaccharides, organic acids, volatile oils, trace elements, and multivitamins. It not only pollutes the environment but also wastes resources. In this paper, extraction methods commonly used for extracting flavonoids from corn silk were reviewed, such as reagent method, enzymatic method, microwave, supercritical CO_2 extraction, ultrasonic, and microwave-assisted ultrasonic. Flavonoids are natural antioxidants and have application value in scavenging free radicals, inhibiting bacteria, and regulating blood lipids. The *in vitro* biological activities of flavonoids from corn silk extracted by different extraction methods were also compared.

1. Introduction

During the metabolism of the human body, substances such as reactive oxygen species and free radicals with strong oxidizing properties will be produced [1, 2]. If these substances are not removed in time, they would cause damage to biological membranes and cell functions and then cause aging, cardiovascular disease, and cancer [3]. Antioxidants could scavenge free radicals, thereby reducing their harm to the human body [4]. However, due to the potential safety hazards of synthetic antioxidants, the development of low-toxic, safe, and efficient natural antioxidants has become a research hotspot [5]. In recent years, many researchers have found that flavonoids are important secondary metabolites that were widely present in plant tissues [6–8]. Biological activities in vitro aroused great interest of many researchers. At present, the main problems on the antioxidant activity of flavonoids are the low purity of the extracts and the low content of potent flavonoids [9–11].

Flavonoids commonly found in plants are an indispensable part of the human diet, especially for compounds found in corn silk, which have antioxidant properties and potential health benefits, and have attracted extensive attention [12]. Corn silk, also named as Maydis stigma (*Zea mays* hairs), which refers to the stigmas of the maize female flowers, is frequently used in traditional Chinese herbal medicines [13]. Corn silk is made from stigmas, the yellowish thread-like strands from the female flower of corn with various therapeutic values and no documented toxicity [14]. Corn silk contains abundant flavonoids. The total amount of flavonoids varies greatly with the variety, and the content is from less than 0.1% to 3% [15, 16]. Flavonoids show a variety of biological activities, such as antioxidant, antibacterial, antidiabetic, and antifatigue, and also have some clinical applications [16]. Microwave, ultrasonic, supercritical fluid extraction, and multiple extraction technologies were used to extract flavonoids effectively [16, 17]. As shown in Table 1, to date, several flavonoids such as maysin, apigmaysin, 3-methoxymaysine, ax-4-OHmaysin, and isoorientin-2″-O-a-L-rhamnoside have been isolated and identified from corn silk [12, 17, 18]. In order to clarify the extraction method and chemical composition of flavonoids in corn silk, this paper systematically studied the chemical components of corn silk and its biological activities *in vitro*.

2. Extraction Methods of Flavonoids from Corn Silk

About 29 flavonoids have been isolated from corn silk. Most of them are C-glycoside compounds and have the same

Table 1: Structures and molecular weight of flavonoids or flavone glycosides from corn silk.

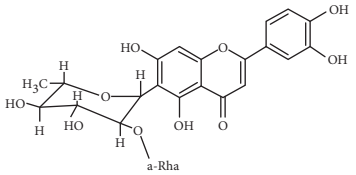
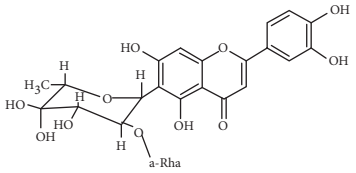
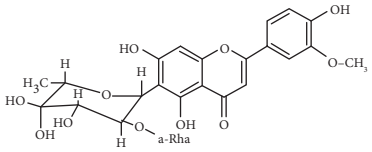
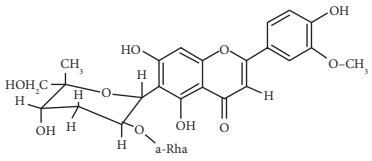
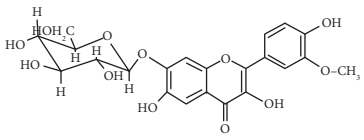
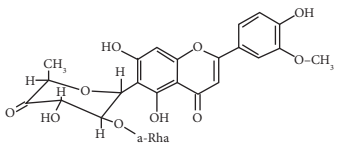
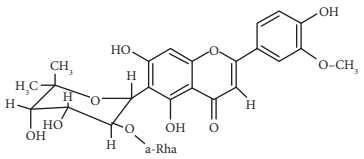
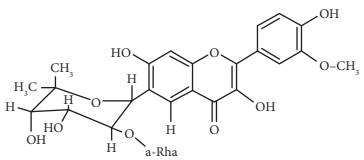
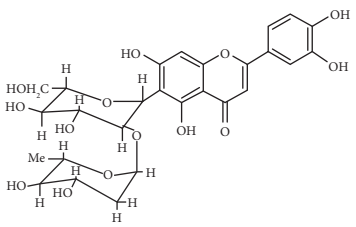
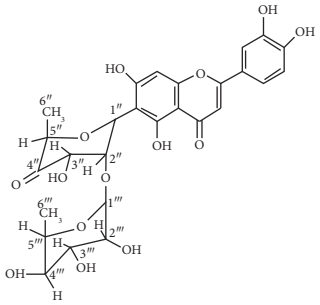
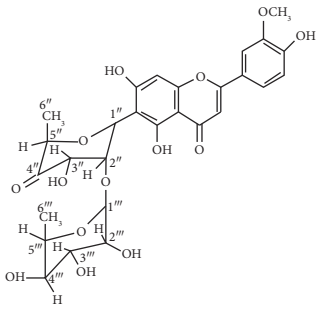
Structures of flavonoids or flavone glycosides	Chemical ingredients	Molecular weight
	2″-O-α-L-Rhamnosyl-6-C-quinovosylluteolin	570
	2″-O-α-L-Rhamnosyl-6-C-fucosylluteolin	586
	2″-O-α-L-Rhamnosyl-6-C-fucosyl-3′-methoxyluteolin	600
	2″-O-α-L-Rhamnosyl-6-C-3″-deoxyglucosyl-3′-methoxyluteolin	608
	6,4′-Dihydroxy-3′-methoxyflavone-7-O-glucosides	451
	ax-5″-Methane-3′-methoxymaysin	584
	ax-4″–OH–3′-Methoxymaysin	586
	7,4′-Dihydroxy-3′-methoxyflavone-2″-O-α-L-rhamnosyl-6-C-fucoside	602
	Isoorientin-2-2″-O-α-L-rhamnoside	632

Table 1: Continued.

Structures of flavonoids or flavone glycosides	Chemical ingredients	Molecular weight
	Maysin	576
	Maysin-3′-methyl ether	590

parent nucleus of luteolin. The flavonoids of corn silk show strong biological activities [19]. They are usually combined with sugars in corn silk to form C-glycosides, and a small part exists in a free form [20]. Flavonoids are easily soluble in organic solvents such as ethanol, methanol, ethyl acetate, and ether. Therefore, the extraction of flavonoids usually uses the ethanol reflux extraction method [21]. As shown in Figure 1, different steps may be involved in the preparation of flavonoids of corn silk. In recent years, with the rapid development of microwave, ultrasonic, supercritical fluid extraction, and multiple extraction technologies, many researchers have used them as an auxiliary technology for flavonoid extraction in order to save time and maximize the flavonoid extraction rate [22, 23]. The advantages and disadvantages of various extraction methods are compared in Table 2.

2.1. Ethanol Reflux Extraction. Ethanol reflux extraction is a traditional method for flavonoid extraction, which is widely used in the extraction of flavonoids. Flavonoid aglycones are generally insoluble or insoluble in water and easily soluble in organic solvents such as methanol, ethanol, ethyl acetate, and ether. Therefore, taking advantage of the low price and nontoxicity of ethanol, the method of refluxing ethanol is used to remove flavonoids from corn silk [24]. The factors affecting the extraction of corn flavonoids mainly include extraction time, extraction temperature, extraction times, material-liquid ratio, and ethanol concentration. The optimized extraction process is relatively simple, and the extraction rate of corn flavonoids is relatively stable. Although the ethanol reflux extraction method has the advantages of simple operation and better extraction effect, it is associated with a large amount of extraction liquid and a long extraction time. It is not conducive to saving energy and cost and hence not suitable for large-scale production. High extraction yield (5.32 mg·g^{-1}) of total flavonoids was obtained under the optimal extraction conditions, which are 80°C for 3 h with corn silk over 60% ethanol ratio of 1 : 30 [25].

2.2. Aqueous Extraction. Aqueous extracts of corn silk showed a broad spectrum of antibacterial activity on the tested microorganisms. These derivatives could be potential alternatives to the traditional control of bacteria and fungi during food storage [26].

For aqueous extraction, 50 g of air-dried powder was placed in distilled water and boiled for 6 h. At intervals of 3 h, the solvent was removed in vacuum. The supernatant was collected. After 6 h, the supernatant was concentrated to make the final volume to 100 ml. Finally, 50 g of material was extracted in 100 ml of distilled water giving a concentration of 500 mg/ml [27].

2.3. Microwave-Assisted Extraction. Microwaves are electromagnetic waves with a frequency between 300 MHz and 300 GHz and a wavelength between 1 mm and 1 m. During microwave extraction, polar substances in plant cells absorb microwave energy to generate a large amount of heat, which causes the intracellular temperature to rise rapidly and generate pressure [28]. The pressure breaks the cell membrane and cell wall, and the intracellular material is dissolved and released into the extract. Microwave-assisted extraction methods are either better or comparable with conventional solvent extraction methods [29, 30].

The process of microwave-assisted extraction (MAE) of total flavonoids from corn silk and the hypolipidemia in animal models were studied. The influence of solvent

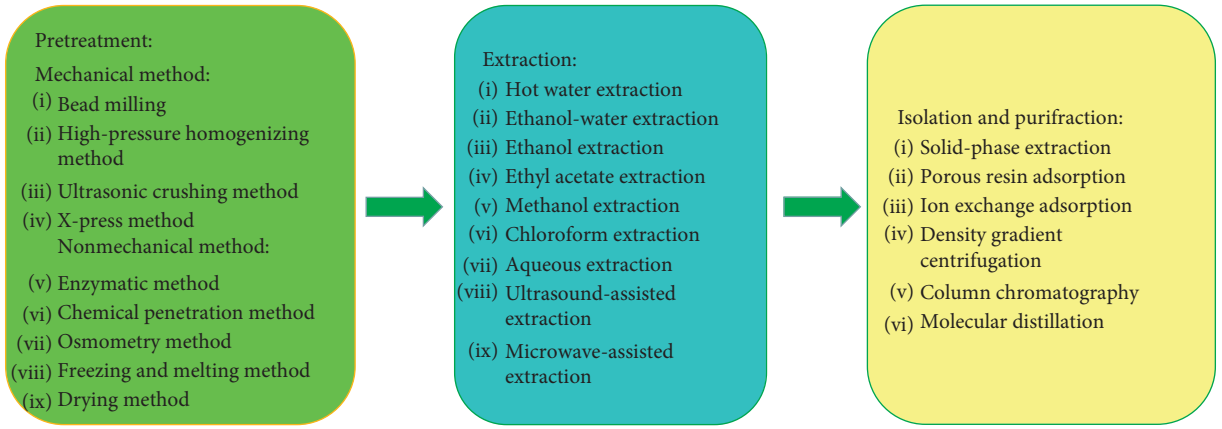


Figure 1: Different steps that may be involved in the preparation of flavonoids of corn silk.

Table 2: The advantages and disadvantages of various extraction methods.

Extraction methods	Advantages	Disadvantages
Ethanol reflux extraction	Less dosage, short extraction time, less impurities, complete extraction	Volatile and toxic
Aqueous extraction	Simple operation, low cost, suitable for large-scale production	More impurities
Microwave-assisted extraction	High selectivity, fast and efficient, stable quality	The extraction time is too long and the temperature is too high, but the extraction rate decreases
Ultrasonic-assisted extraction	Short extraction time and high yield without heating	Small extraction scale, not suitable for large-scale production
Supercritical fluid extraction	Low extraction temperature, high extraction rate, low solvent consumption, no pollution	The separation selectivity is reduced, it is not easy to obtain a pure substance, the investment is large, and the operating component is high

concentration, microwave power, extraction time, and dose of solvent was investigated, and then, the orthogonal experiments were performed. Animal models of hyperlipidemia induced by a high-fat diet were established. The serum levels were, respectively, measured, including total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). The optimum extraction parameters were determined as follows: ethanol concentration (60%), power (600 W), extraction time (16 min), ratio of plant material to solvent (M/S) (1 : 20), and yield of extract (4.55%). Corn silk total flavonoids (CSTFs) significantly lowered the serum TC, TG, and LDL-C levels. Furthermore, the serum lipid level was decreased by CSTF in hyperlipidemic animal models in a dose-dependent manner [31].

2.4. Ultrasonic-Assisted Extraction. Ultrasonic wave is a kind of sound wave with a frequency higher than 20000 Hz. Ultrasonic waves form mechanical vibrations of medium particles in the material medium, which can cause the interaction of the medium [32]. Cavitation and thermal effects are the main effects of ultrasonic waves in the liquid. This is beneficial to increase the dissolution of flavonoids. The ultrasonic-assisted extraction method does not require any additional chemical reagents and does not destroy the flavonoid structure, so it is considered a good auxiliary extraction method [33]. Through a three-level, three-variable Box–Behnken design of response surface methodology (RSM) adopting yield as response, the optimal conditions were determined as follows: ultrasonic power of 500 W, extraction time of 20 min, material solvent ratio of 1 : 20, and ethanol concentration of 30%. Under the optimum conditions, the extraction yield of total flavonoids was 1.13% [34].

2.5. Supercritical Fluid Extraction. Supercritical fluid carbon dioxide (SF-CO_2) extraction (SFE) of flavonoids from corn silk and its nitrite scavenging ability were investigated. The effects of extraction time, particle size, and cosolvent composition in terms of water content in ethanol were first optimized. Then, a Box–Behnken design combined with response surface methodology (RSM) was employed to study the effects of three independent variables (temperature, pressure, and cosolvent amount) on the extraction yield of flavonoids. A maximal extraction yield of flavonoids of approximately 4.24 mg/g of corn silk by SFE was obtained under optimal conditions (a temperature of 50.88°C, a pressure of 41.80 MPa, a cosolvent amount of 2.488 mL/g, and an extraction time of 120 min with 0.4 mm particle sizes and 20% aqueous ethanol as the cosolvent). Furthermore, the nitrite scavenging ability of the flavonoid-enriched SFE extracts was assessed using the Griess reagent. The flavonoid-enriched SFE extracts exhibited the highest scavenging

ability on nitrite (88.1 ± 3.04%) at the concentration of 500 g/ml and at pH 3.0. The nitrite scavenging ability of the extracts appeared to be concentration-dependent but negatively correlated with the pH [35].

3. Biological Activities of Corn Silk

As shown in Table 3, corn silk shows a variety of biological activities, such as antioxidant, antibacterial, antidiabetic, and antifatigue, and also has some clinical applications [36, 37].

3.1. Antioxidant Activity of Corn Silk. Radicals are molecules or fragments of molecules that possess an unpaired electron in their outer orbital. As a result of this molecular instability, radicals are highly reactive and can promote damaging oxidation reactions with cellular proteins, lipids, or DNA, leading to oxidative stress and impaired cellular function [38].

The flavonoids in corn silk have good antioxidant effects, which are mainly manifested in reducing free radical generation and scavenging free radicals [39]. Antioxidant activity of corn silk 70% acetone-water extract was determined by ferric reducing antioxidant power (FRAP) test. The results showed that the antioxidant activity of corn silk extract had a good correlation with total polyphenol content ($R^2 = 0.9306$) [40]. The relationships between phytochemicals and antioxidant activity in corn silk were evaluated and it was found that antioxidant activity was correlated with total phenolics and total flavonoids [41]. The influence of variety and harvest maturity on phytochemical content and antioxidant activity in corn silk was studied. The results showed that different parts of corn silk also differ in antioxidant activity, and high pigmentation generally occurred at the early maturity phase, and it was related directly to high antioxidant activity [42]. The nutritional compositions and antioxidative capacity from the silk obtained by immature corn and mature corn were studied [16]. The result showed that both ethanol extracts of immature and mature silks possessed strong free radical scavenging capacity compared to the water and ethyl acetate extract. It was reported that the antioxidant and free radical scavenging activities of extract and fractions from corn silk were evaluated by using *in vitro* antioxidant models. The results showed that *N*-butanol fraction, which demonstrated the highest total phenolic and flavonoid contents, exhibited the highest antioxidant and free radical scavenging activities rather than petroleum ether fraction, ethyl acetate fraction, and water fraction [4]. Antioxidant activity of the flavonoid-rich extract from corn silk was studied *in vitro* and *in vivo*. The results showed that the IC50 values of the extract for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity were 50.73 and 0.23 mg/mL, respectively. Furthermore, the extract could significantly enhance total superoxide dismutase (T-SOD) activity, reduce malondialdehyde (MDA) content in the serum, increase glutathione content, and decrease protein carbonyl content in the liver tissue [43]. Antioxidant activity of crude flavonoid extract using STZ-induced diabetic mice was studied. The results showed that the MDA values were significantly reduced [15].

3.2. Antibacterial Activity of Corn Silk. Neucere reported that water extract from corn silk could inhibit the growth of *Aspergillus*. The active ingredients were showed to contain chitosan and dextran according to the analysis of electrophoresis, both of which had antibacterial activity [44]. The antibacterial activity of corn silk extracts against seven kinds of common pathogens and food spoilage bacteria was investigated. Various extracts were studied on the antibacterial properties, and the results showed that 90% ethanol extract had the best antibacterial effect. In addition, acidic and neutral environment could enhance the antibacterial effects of the extracts. The antibacterial activity of ethanol extracts from corn silk was also studied [45]. Two flavonoid glycosides (compounds I and II) were isolated from *n*-butanol fraction of methanol extract of corn silk. The chemical structures of the compounds were elucidated as maysin (I) and maysin-3′-methyl ether (II) by means of different analytical methods such as UV, IR, NMR, and MS (ESI) analyses and by comparison with those reported in the literature for the compounds. The antimicrobial activities of maysin (I) and maysin-3′-methyl ether (II) were studied against twelve bacteria and one yeast. The sensitivity of the compounds (2.0 mg/mL) towards bacteria was compared with that of standard gentamycin (50 μg/mL). Flavonoid glycosides showed a wider range of activity towards Gram-positive and Gram-negative bacteria. Comparatively, compound I exerted the highest antibacterial activity towards Gram-positive bacteria than compound II. In comparison with gentamycin, compound I showed significantly ($p < 0.05$) higher activity against the tested bacteria except *Enterobacter aerogenes*, *Salmonella paratyphi*, and *Proteus mirabilis* where it exerted statistically ($p < 0.05$) similar activity with gentamycin. But it showed lower activity towards *Pseudomonas aeruginosa* than gentamycin. Maysin-3′-methyl ether (II) showed comparatively lower activity than I; it seems that the presence of methoxyl substitution on the C-3′ position slightly decreases the sensitivity towards bacteria [46].

3.3. Antidiabetic Activity of Corn Silk. Antidiabetic activity of corn silk flavonoids (CSFs) was studied on STZ-induced diabetic mice. The results revealed that treatment with 300 mg/kg or 500 mg/kg of CSFs significantly increased the body weight, water consumption, and especially the blood glucose concentration of diabetic mice, which indicated their potential antidiabetic activities. Antidiabetic activity of corn silk crude polysaccharides on streptozotocin-induced hyperglycemic rats was studied, and its effects on hyperlipidemia, nerve excitability, and fatigue resistance were investigated. The results showed that the polysaccharides had a certain hypoglycemic effect on diabetic rats, and it also improved the symptoms of emaciation and polydipsia in diabetic rats, but the hypoglycemic effect was not as obvious as that of positive control metformin [47]. Antidiabetic activity of corn silk methanolic extract on glucose uptake by

Table 3: Bioactivity and extraction method of flavonoids or flavone glycosides from corn silk.

Bioactivity	Extraction method	Results	Producing area	References
Antioxidant activity	Ethanol-water extraction	The percentage of DPPH radical scavenged by CS extract was 92.6 at a concentration of 1.6 mg/ml^{-1}	Iran	[54]
	Three separate solvents (ethyl acetate, ethanol, and water)	The ABTS inhibition of immature and mature silks was ranged from 13.4% to 35% and 15.56% to 44.36%, respectively	Malaysia	[16]
	Ultrasound-assisted extraction	FRAP value of total flavonoids extracted from corn silk was 467.59 μmol/L	China	[34]
Antibacterial activity	The petroleum ether (PECS), chloroform (CECS), and methanol (MECS) extractions	PECS, MECS, and flavonoids were active against eleven bacteria out of twelve bacteria. CECS was active only against five bacteria	Malaysia	[46]
	Ethanol, chloroform, and methanol extractions	Ethanol extract showed significant antimicrobial activity against both Gram-positive bacteria (13.17 to 9.45 mg/ml) and Gram-negative bacteria (12.36 to 8.15 mg/ml) bacteria	Bangladesh	[55]
	Aqueous extraction	The antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* is 500 mg/ml and 62.5 mg/ml. The minimum inhibitory concentration to *Candida albicans* is 125 mg/ml	China	[27]
Antidiabetic activity	Ethanol extraction	Treatment with 300 mg/kg or 500 mg/kg of corn silk flavonoids significantly reduced the body weight loss, water consumption, and especially the blood glucose (BG) concentration of diabetic mice	China	[15]
Antifatigue activity	Hot ethanol continuous extraction	Swimming exercise results indicated that corn silk flavonoids had antifatigue activity of mice by inhibiting the production of blood lactic acid	China	[17]
	Hot ethanol continuous extraction	Corn silk flavonoids could elevate the exercise tolerance of mice and provide protection against oxidative stress induced by exhaustive exercise in mice	China	[14]

isolated rat hemidiaphragm was studied. The results showed that the treatment of corn silk increased the uptake of glucose by isolated rat hemidiaphragm significantly and was found to be more effective than insulin [48]. Corn silk aqueous extract was also shown to exhibit potent and moderate inhibitory potential against α-amylase and α-glucosidase *in vitro*, respectively [49]. Corn silk aqueous extract treatment markedly reduced hyperglycemia in alloxan-induced diabetic mice. According to the study, the mechanism of the hypoglycemic effect was through increasing insulin level and repairing the injured β-cells instead of increasing glycogen and inhibiting gluconeogenesis [50]. The hypoglycemic effect of corn silk flavonoids was studied on alloxan-induced diabetic mice, and the results showed that corn silk flavonoids could significantly reduce the level of blood glucose of diabetic mice and significantly slowed down the weight loss of diabetic mice. In addition, corn silk flavonoids had an obvious improving effect on the activity of superoxide dismutases in the diabetic mice and had a certain inhibitory effect on the production of lipid peroxide (MDA), which indicated that the hypoglycemic effect might be associated with antioxidant activity by improving the function of islet cells, especially β-cells. It was found that the combination of corn silk and binahong leaves could be more effective in repairing renal damage caused by oxidative stress [51].

3.4. Antifatigue Activity of Corn Silk. In order to clarify the mechanism of antifatigue activity of corn silk flavonoids, blood biochemical parameters were measured in the swimming-treated mice. The swimming exercise was known to induce blood biochemical changes [52]. Blood biochemical parameters are shown in Table 3. It was found that the blood lactate and blood urea nitrogen concentrations of treatment groups were significantly lower than those of the control group ($p < 0.05$). The results suggest that corn silk flavonoids can inhibit the production of blood lactic acid during exercise and retard the formation of blood urea nitrogen (BUN) after exercise. It was known that the endurance capacity of the body markedly decreased if energy was exhausted. As glycogen was the important resource of energy during exercise, the increase of glycogen stored in the liver is advantageous to enhance the endurance of the exercise [53]. It was found that the hepatic glycogen concentration of treatment groups was higher than that of the control group ($p < 0.05$), and the increasing rates were 260.71 and 281.25%, respectively. The results suggested that corn silk flavonoids could elevate hepatic glycogen concentration during exercise. However, the detailed mechanism was not clear. The possible reason is that corn silk flavonoids may increase the content of postexercise hepatic glycogen of mice by improving glycogen reserve, or by reducing the consumption of glycogen during exercise, or both. It still needs further study [17].

4. Conclusions

The biological activity process involves many physiological challenges, which are significantly affected by biologically active substances. In this paper, ethanol, microwave, ultrasonic, supercritical fluid extraction, and multiple extraction technologies were reviewed as impactful technologies for flavonoid extraction of corn silk. Lots of studies have shown the potential benefits of ingested flavonoids against some major selected risk factors. The antioxidant, antibacterial, antidiabetic, and antifatigue activities are also described, while *in vitro* and *in vivo* studies are mainly related to the possible mechanisms of action of flavonoids, which represent a closer approach for the substantiation of the effects of these molecules in humans and allow health benefits to be claimed for foods containing them. Corn silk shows a variety of biological activities, such as antioxidant, antibacterial, antidiabetic, and antifatigue and has some clinical applications. In the future, the structure-activity relationship of corn flavonoids should be explored in depth, and the advantages of multiple sciences should be used to provide a strong scientific basis for the further development of natural and effective new drugs, as well as a basis for the full development and utilization of corn flavonoid resources.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Research Article

Preliminary Study on Ultrasonic Ageing Zhenjiang Vinegar Mechanism Based on Maillard Simulation System

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In this study, ultrasonic technology was used to treat Zhenjiang vinegar, and the effects on the physicochemical characteristics of Zhenjiang vinegar were investigated. The influences of ultrasound time and power on the number of induced hydroxyl radicals and superoxide radicals were also investigated. Besides, the novel simulation system of the Maillard reaction was built to research the effects of different ultrasonic times and power treatment on Zhenjiang vinegar. The results show that, under the conditions of ultrasonic treatment, the changes of Zhenjiang vinegar physiochemical index, such as color, reducing sugar, and amino acid, are consistent with those of natural ageing. In addition, ultrasound can produce a cavitation effect and cracking water molecules to produce hydroxyl radicals and superoxide radicals, so as to achieve the ageing effect of vinegar.

1. Introduction

Fermented vinegar has widely been used in China and other Asian countries as a food condiment for hundreds of years [1, 2]. Zhenjiang Vinegar, which is one of the most famous traditional fermented vinegar types in China, is world-famous for its special elegant and complex aroma [3]. It is widely accepted like wine because of the harsh taste, pungent smell, and some possible harmful side-effects; fresh vinegar needs a specific process known as ageing in which a specific time period was used to produce high-quality vinegar [4]. Traditionally, ageing vinegar in barrel ageing systems is a common practice used for improving vinegar quality and stability. However, considering the disadvantages of natural ageing such as high production cost and long time period, it is critical and essential to study innovative ageing technology.

Recently, scientists have put great attempts into promoting brand new ageing techniques and enhancing present techniques. Present studies showed that there are diverse physical methods displaying considerable potential for accelerating the ageing process, including ultrasonic wave, electric fields, ultra-high pressure, and gamma irradiation [5–10]. It was found that the ultrasound (16–60 kHz) was adept to expedite oxidation, condensation, and polymerization of ethanol, aldehydes, esters, and olefins in wines [11]. Zheng et al. [10] found an optimum treatment for speeding up fresh red wines ageing, which enhanced wines with excellent taste and flavor. Reference [12] found that the high-pressure intervention in the scope from 80 MPa to 120 MPa not beyond 2 hours was shown to considerably improve the wine taste. Better apprehension of each divisor that impacts the quality of vinegar ageing process combined the economic and operational reasons; ultrasonic is a potential physical method to accelerate the vinegar ageing process [13].

Ultrasound, especially with high power and low frequency, has the advantage of pollution-free and being speedy. Over the last decades, abundant works have

concentrated on the utilization of ultrasound for food processing, like extraction, freezing, oxidization, sterilisation, and desiccation [14, 15]. Evidence from different research [16–18] showed that the influence of ultrasound on vinegar ageing processing was possibly associated with the acoustic cavitation [19, 20], which includes the generation, expansion, and breakdown of microbubbles. The violent breakdown of bubbles can generate exceedingly steep heat and pressure. Many scientists have studied that during ageing processing, the vinegar undergoes many complicated changes, including Maillard reaction, oxidation reaction, and esterification reaction. Pripis-Nicolau et al. [21] revealed that, under the appropriate wine conditions, the production of odoriferous compositions or strong-smelling by-products would enhance Strecker and Maillard reactions. Casale et al. Reference [22] found that the reaction of oxidization occurs and oxygen boosts a series of chemical and enzymatic reactions that change the vinegar; thus they found an affinity between spectral variables and store time-changes in vinegar. Maillard reaction is defined as the interaction of carbonyl compounds (e.g., reducing sugars) with free amino groups (usually amino acid) leading to flavor compounds and melanoidins [23]. Maillard reaction is of great importance in food quality, especially in heat-process foods, affecting not only the flavor but also the color and nutritional values. Previous work [9] in our laboratory showed that vinegar treated with ultrasound was concluded to be tantamount to 2-3 years aged traditionally Zhenjiang vinegar under the optimum experimental conditions (ultrasonic power 50 W/100 mL, time 75 min, and ethanol addition 0.75% (V/V)). Nevertheless, little work has been done with Zhenjiang vinegar, especially the mechanism of the chemical reactions during the ageing process.

In view of the wide variety of substance in vinegar, Maillard reactions develop complex intermediates and final reaction products, in order to clarify each substance in the ultrasound induced by the specific changes and the mechanisms is very difficult or even almost impossible. Therefore, the complex vinegar system was replaced for the simulation system [21, 24, 25], to study the mechanism of ultrasound to accelerate the ageing process. Thus, the aim of this study was to investigate the relevant physicochemical indicators and the ageing mechanism based on the Zhenjiang vinegar simulation system. This study is of practicable interest since it provides beneficial information for vinegar industry in order to optimize the process of vinegar.

2. Materials and Methods

2.1. Chemicals. Chemicals used in this study were furfural, n-Octane, fructose, glycine, methylene blue, vanillin, anhydrous ethanol, and aniline which were purchased from Sinopharm Group Chemical Co., Ltd. In this study, all chemicals and solvents used were of analytical grade.

2.2. Samples. The simulated Maillard reaction system was constructed to be 100 mL of 0.1 mol/L fructose and glycine aqueous solution. All the vinegar samples of different storing times used in this study were collected from Jiangsu Hengshun Vinegar Co., Ltd. Samples of vinegar were divided into 2 kinds of different treatments. One is the naturally aged vinegar samples, which were aged traditionally for different periods such as 12, 18, 32, and 44 months and kept in glasses. The samples of fresh and aged vinegar were stored at 4°C in a refrigerator. Samples with ultrasonic were treated according to the method of Wang et al. [9].

2.3. Color Evaluation. Color is one significant sensory characteristic of vinegar, which affects the consumers' overall acceptableness. The change of color can well reflect the process of Maillard reaction. Color measurement is measured by Hunterlab Spectrophotometer. The L^*, a^*, and b^* values (CIELAB parameters) were monitored by using the software CromaLab [26], which better test the color of wines and permit better differentiation [27–30]. In this study, the CIE Lab system was used to define the color of vinegar. Record the color difference value of the vinegar L^*, a^*, and b^*, measured three times, and take the average. Value of L^* indicates the brightness, the value of 0–100; the greater the value the greater the brightness; a^* represents the values of red/green, positive partial red, and negative partial green; b^* represents the yellow/blue value, positive yellow, and negative blue. The value ΔE^* is measured as the Euclidean distance between two points in the three-dimensional space defined by L^*, a^*, and b^* and tells from color differences:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}. \quad (1)$$

2.4. Determination of Reducing Sugar. The content of reducing sugar in the samples was analyzed as stated in the GB/T 5009.7–2016 method including different ages of traditional aged vinegar and optimum ultrasonic experimental conditions treated vinegar (ultrasonic power 50 W/100 mL, time 75 min, and ethanol addition 0.75% (V/V)). All vinegar samples were determined in triplicate.

2.5. Determination of Free Amino Acid. Automatic amino acid analyzer was exploited to measure the content of free amino acids in the vinegar samples. The vinegar sample to be tested was diluted 20 times with 1% sulfosalicylic acid solution, centrifuged at 10000 rpm for 15 min, and the supernatant was clarified through 0.22 μmmicroporous membrane filter for the determination of the use of the machine.

Amino acid analysis chromatographic conditions are fluorescence detector, 4.0 × 125 mm C18 column; column temperature, 40°C; flow rate, 1.0 mL/min; and detection wavelength, Ex 440 nm, Em 570 nm.

2.6. Determination of Hydroxyl Radicals. The hydroxyl radical evoked by the cavitation of ultrasound was assessed indirectly by using methylene blue as the radical scavenger and ultraviolet-visible spectrophotometer as the testing method. [31, 32] The methylene blue was handled with

dissimilar ultrasonic treatments and the maximum absorption wavelength was measured by UV-visible spectrophotometer full-wavelength scanning. The hydroxyl radical produced by ultrasound was also measured by the decrease of absorbance at the maximum absorption wavelength.

2.7. Determination of Superoxide Radicals. Vanillin aniline fluorescent reagent synthesis method: 1.5 g vanillin was dissolved in 20 mL of anhydrous ethanol; 0.9 g of aniline was added dropwise, refluxed at 80°C, for 5 h; most of the solvent was distilled off; and a pale yellow solid was obtained after cooling. Recrystallize from anhydrous ethanol and vacuum dry. 2×10^{-4} mol/L vanillin aniline ethanol solution was configured.

In a 10 mL colorimetric tube, add 2 mL of vanillin aniline ethanol solution, 2 mL of sonicated aqueous solution, and 1.5 mL of Tris-HCL buffer solution and bring the volume to the mark. The fluorescence intensity was measured under the excitation wavelength of 282 nm, and the emission wavelength range was 250–400 nm. Excitation and emission slit widths were measured at 5 nm and 10 nm, respectively.

2.8. Determination of Maillard Reaction Process. In view of a large variety of components in vinegar, it is difficult or even impossible to find out the specific changes and mechanisms of each substance under ultrasonic induction; therefore, the content of vinegar should be higher when studying the ultrasonic accelerated vinegar ageing mechanism. The representative substances that have an important influence on the quality of vinegar were studied as objects. The results of this study are also easy to compare with the relatively clear mechanism of change in the natural maturation process of these substances. The Maillard reaction is one of the most important reactions in the vinegar ageing process. Therefore, it is necessary to construct the Maillard simulation system to study the mechanism of ultrasound on its action.

Maillard reaction model system is the 100 mL of 0.1 mol/L fructose and glycine solution. The degree of browning and the amount of intermediate product of the Maillard reaction were measured according to the way of Gu et al. [33]. According to the literature, absorbance at 294 nm was served to examine the formation of the intermediate compounds of the Maillard reaction and the size of the UV-visible absorption at 420 nm in the Maillard reaction system is related to the final stages of the reactions [34, 35]. The browning indices were evaluated by spectrophotometry, recording the absorbance at 420 nm and 294 nm ($A_{420\ \mathrm{nm}}$ and $A_{294\ \mathrm{nm}}$) of each model system against distilled water. The results were recorded on a UV software (Beijing Beifen-Ruili) spectrophotometer using a 1 cm path length quartz cell. All samples were done three times in parallel.

2.9. Data Analysis. All model systems were prepared in duplicate and the analysis was performed in triplicate. Excel 2010, Origin 8.0, and SPSS Statistics 17.0 were applied to complete the data analysis. The significant differences between the different samples were obtained through a one-way ANOVA analysis with a level of $P < 0.05$.

3. Results and Discussion

3.1. Color Evaluation. As Table 1 shows, the color of the vinegar was significantly affected by the ageing time and different storage times have a significant effect on vinegar L^*, a^*, b^*. For the traditionally natural aged vinegar samples with the extension of storage time, the values showed a downward trend. Lightness is the attribute of a visual sensation according to which a given visual stimulus appears to be more or less light, ranging from "light" to "dark" Gómez-m Guez [26]. Table 1 shows that all the L^* values of vinegar samples were investigated varying from 2.07 to 0.49 units; a^* values were between 5.54 and 1.12 units. Besides, b^* values were between 4.21 and 0.95. According to Oszmianski [36, 37], these minute dissimilarities between the vinegar samples were by reason to the oxidation instead of the enzymatic treatment. However, the previous literature [38, 39] reported that the ultrasound can accelerate oxidation, esterification, and other reactions. The values of the ultrasonic treated vinegar ($L^* = 2.41$, $a^* = 6.37$, $b^* = 5.25$) were higher as compared to nonultrasonic treated vinegar. The value of ΔE^* was found between the ultrasonic treated vinegar and fresh vinegar ($\Delta E^* = 0.94$). These colorimetric data present that the ultrasonic treated vinegar samples exhibited mainly red and yellow tint, which is also a consumer favor. The result shows that a tester can distinguish the color of these vinegar samples. In addition, the reducing sugar in vinegar declining with the extension of ageing time, combined with the later experimental verification Maillard reaction products, increased after ultrasonic treatment, which is consistent with the results of the color change of vinegar treatment.

3.2. Effect of Ultrasound on Hydroxyl Radicals. Figures 1 and 2 show the methylene blue solution UV-visible absorption curve along with the change of ultrasonic time and power. Besides, these results showed that methylene blue solution has the maximum absorption peak at 664 nm and gradually decreases with the increase of ultrasonic time and power. These results show that the concentration of methylene blue solution gradually decreased to prove that the ultrasonic treatment of hydroxyl radicals generated and hydroxyl radical concentration gradually increased over ultrasonic time and power.

As shown in Figure 3, it is not hard to see that in the initial period of time, the production of hydroxyl radical increased rapidly. As the time extended to 75 min later, the rate of increase slowed down. The reason here might be that the increased time for ultrasonic treatment increases the vinegar temperature too high. The vapor pressure in bubbles increases and consequently bubbles closed to enhance the buffer effect and the cavitation role becomes weakened. Therefore, in this study, the ultrasonic time is 75 min.

As shown in Figure 4, with the increase of ultrasonic power, the intensity of the ultrasonic wave generated in unit

Table 1: Effect of color change and reducing sugar in the natural ageing process of vinegar and ultrasound treated vinegar.

Age (month)	CIE lab color change				Reducing sugar (g/100 mL)
	L^*	a^*	b^*	ΔE^*	
0	2.07 ± 0.38^{c}	5.54 ± 0.17^{d}	4.21 ± 0.23^{dc}	0.00	4.06 ± 0.15^{d}
12	1.24 ± 0.24^{b}	5.31 ± 0.20^{d}	3.23 ± 0.10^{dc}	0.85	3.39 ± 0.14^{c}
18	0.89 ± 0.02^{b}	4.56 ± 0.13^{c}	2.05 ± 0.11^{b}	1.03	2.51 ± 0.16^{ab}
32	0.64 ± 0.04^{a}	2.22 ± 0.11^{b}	1.83 ± 0.09^{b}	2.79	2.92 ± 0.18^{b}
44	0.49 ± 0.02^{d}	1.12 ± 0.02^{a}	0.95 ± 0.02^{a}	1.00	2.24 ± 0.14^{a}
Ultrasonic treated	2.41 ± 0.01^{d}	6.37 ± 0.02^{d}	5.25 ± 0.13^{d}	0.94	3.03 ± 0.45^{c}

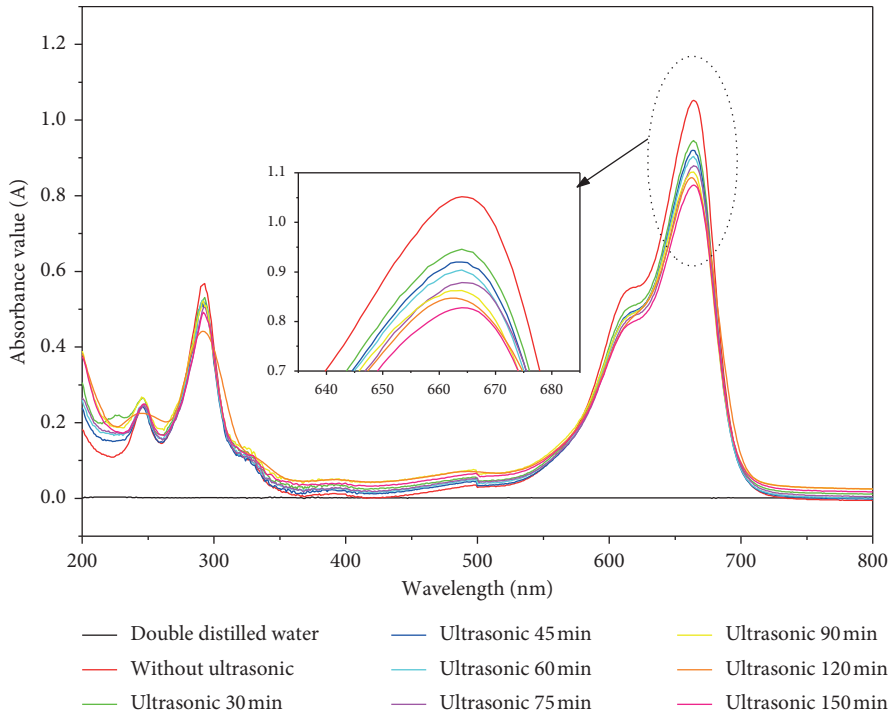

Figure 1: UV-visible spectra of methylene blue solution at different ultrasonic times.

time increases, and the cavitation effect produced is stronger [40]. Therefore, when the cavitation bubbles generated by the aqueous solution are crushed, high temperature and high-pressure environment occurs; the number of free radicals generated will be increased sufficiently.

Hydroxyl radical is the most active oxygen free radical [41]. Ultrasound-induced hydroxyl radicals can cause oxidation of sugars, amino acids, proteins, and esters in vinegar [42]. The production of free radicals can speed up the breakage and formation of various substances in vinegar, accelerate the ripening of vinegar, and shorten the ageing time [43].

3.3. Effect of Ultrasound on Superoxide Radica. From Figures 5 and 6, it can be seen that superoxide radicals in the solution that has not been sonicated are not detected; however, a large number of free radicals are generated in the aqueous solution after sonication, which proves that the ultrasonic treatment produces superoxide radicals in the solution and has a significant effect. According to Figure 5, the number of superoxide radicals generated increases with the increase of the ultrasound time and reaches the maximum level at 40 min after sonication. Figure 6 shows that the number of superoxide radicals produced by ultrasound gradually decreases with the increase of ultrasonic power of 10 W/100 mL. The generation of superoxide radicals is due to the action of ultrasound on the oxygen molecules in the aqueous solution. The reason for the above research results is that, on the one hand, it is finally converted into hydroxyl radicals through a series of reactions which is consistent with the results of the previous studies on hydroxyl radicals and, on the other hand, superoxide anions can dismutate to produce hydrogen peroxide and oxygen [44].

Superoxide radicals in water can be considered as a base, which can accept H^+ from a protonated superoxide radical HOO•. In one way, the mechanism is largely to convert it into other more active oxygen ions. The base achieves the promotion of chemical reaction.

3.4. Effect of Ultrasound on Browning Degree of Maillard Reaction. Table 1 shows that after the ultrasonic treatment, the color of the vinegar becomes lighter. The content of

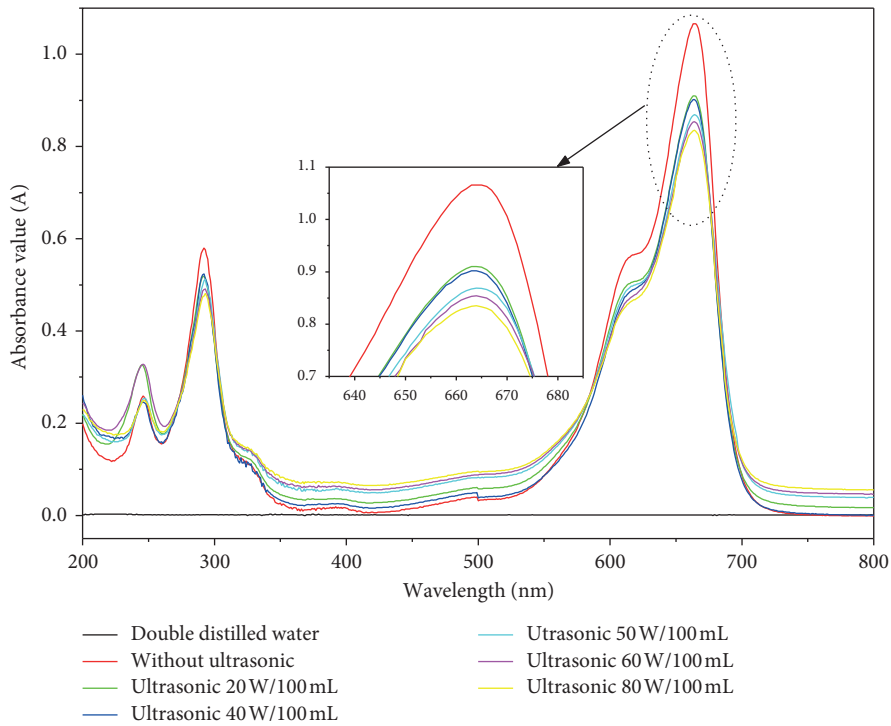

Figure 2: UV-visible spectra of methylene blue solution at different ultrasonic powers.

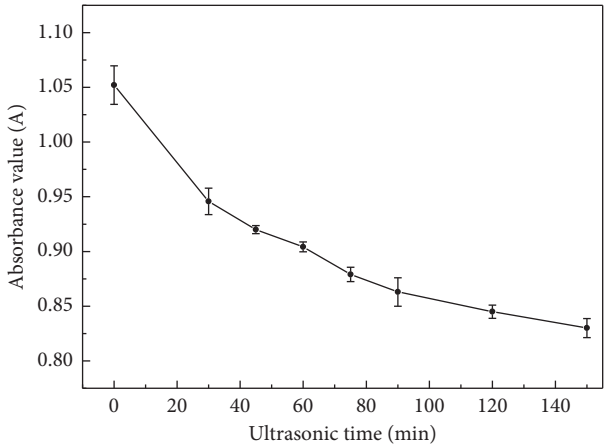

Figure 3: The maximum absorption intensity (664 nm) of the methylene blue solution in the UV-visible light varies with the ultrasonic time.

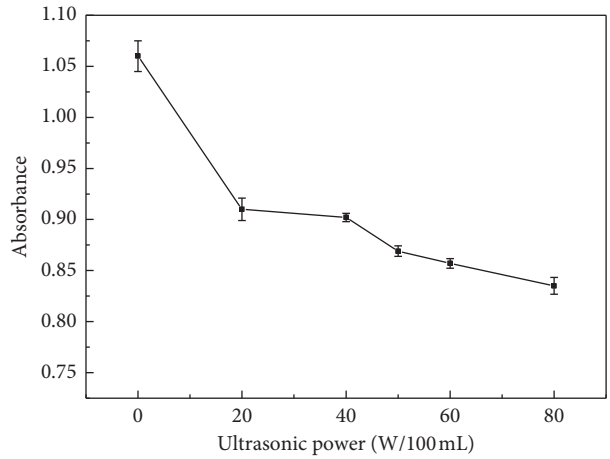

Figure 4: The maximum absorption intensity (664 nm) of the methylene blue solution in the UV-visible light varies with the ultrasonic power.

reducing sugar decreased by nearly 50% from 4.06 g/100 mL to 2.24 g/100 mL. Besides, during the traditionally natural ageing process, the changes of vinegar free amino acids are shown in Table 2. It is revealed that as the ageing time becomes longer, the content of free amino acids decreased by 1041.35 mg/100 mL down to 757.87 mg/100 mL. Moreover, after ultrasonic treatment, the content of amino acids in vinegar decreased, consistent with the trend of natural ageing. And ultrasound can produce lots of hydroxyl radicals. Combined with the above experimental results analysis, we speculate that the changes are related to the progress of the Maillard reaction. The monosaccharides in Zhenjiang vinegar is mainly glucose and fructose. And glycine in the vinegar free amino acid mass fraction is relatively high. In particular, fructose-glycine can form a kind of Amadori compounds [45, 46]. Therefore, in this experiment, the fructose-glycine simulation system was used to verify that ultrasound may speed up the Maillard reaction. As far as we know, the melanoidin produced during the Maillard reaction can make the color of the vinegar darker, so that the degree of browning can be directed to the extent of the Maillard reaction.

As can be seen from Figure 7, the degree of browning of the fructose-glycine simulation system gradually increased with the ultrasonic time rise. And the results showed that

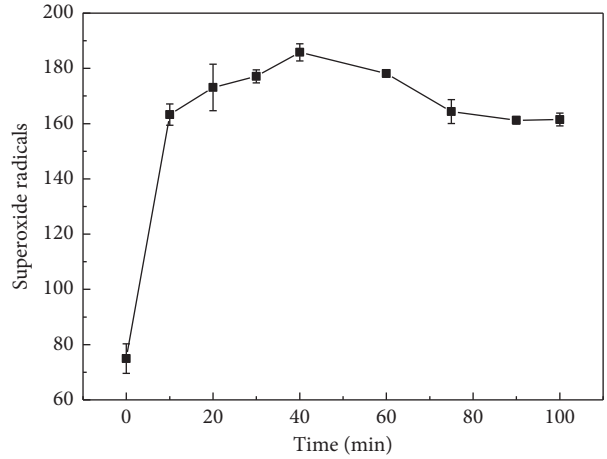

Figure 5: The effect of ultrasonic time on superoxide radicals.

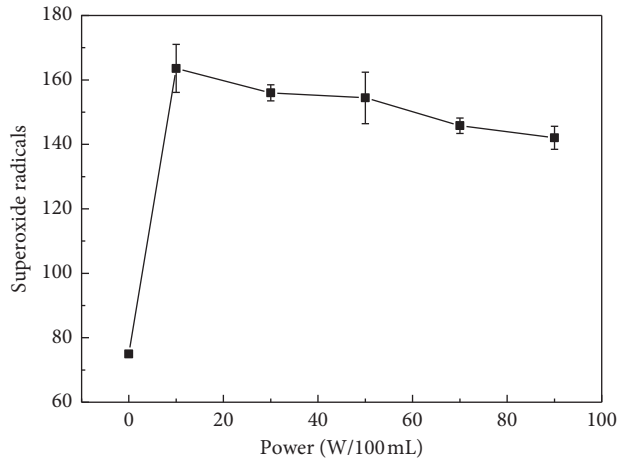

Figure 6: The effect of ultrasonic power on superoxide radicals.

Table 2: Changes in the free amino acids in the vinegar during the ageing process.

Amino acids	Amino acid content (mg/100 mL)						Taste
	Fresh	12 months	18 months	32 months	44 months	Ultrasonic treated	
Asp	82.43	67.24	51.90	48.03	46.76	52.22	Sour
Glu	115.64	114.61	120.12	127.85	134.72	128.96	Fresh
Val^{E}	77.15	66.66	63.48	58.54	48.86	65.65	Astringent
Leu^{E}	107.87	92.73	85.18	82.42	78.73	89.50	Astringent
Gly	91.65	70.70	66.49	63.85	61.84	64.73	Strong sweet
Ala	118.64	103.56	94.81	90.06	83.65	94.46	Strong sweet
Thr^{E}	42.13	41.30	38.39	35.48	32.73	38.57	Sweet
Ser	52.49	45.15	44.46	41.94	40.89	42.27	Sweet
Met^{E}	13.36	14.39	13.65	12.57	9.29	12.06	Sweet
Phe^{E}	34.18	31.83	45.29	39.79	45.45	39.56	Sweet
Pro	61.46	38.97	43.16	32.81	35.68	36.89	Sweet
Lys^{E}	45.65	36.55	37.30	27.95	21.19	27.21	Slight sweet
Ile^{E}	47.59	51.91	45.73	40.61	37.45	46.39	Bitter
Arg	62.87	50.75	32.20	34.40	29.21	33.86	Bitter
Tyr	49.81	35.48	30.72	31.44	27.78	31.29	Slight bitter
His	27.77	15.00	16.18	11.57	10.12	16.44	Slight bitter
Trp^{E}	10.48	14.32	17.63	17.54	13.52	14.88	Slight bitter
Total	1041.35	891.18	836.69	796.85	757.87	843.94	

E represents the essential amino acids, *T* represents the total amino acids, and N represents the nonessential amino acids.

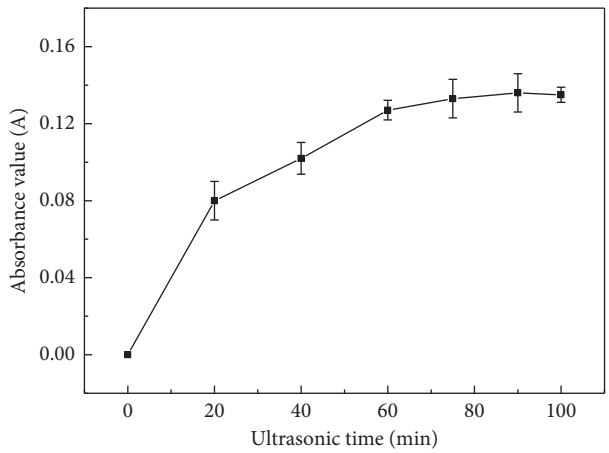

Figure 7: The effect of ultrasonic time on browning intensity.

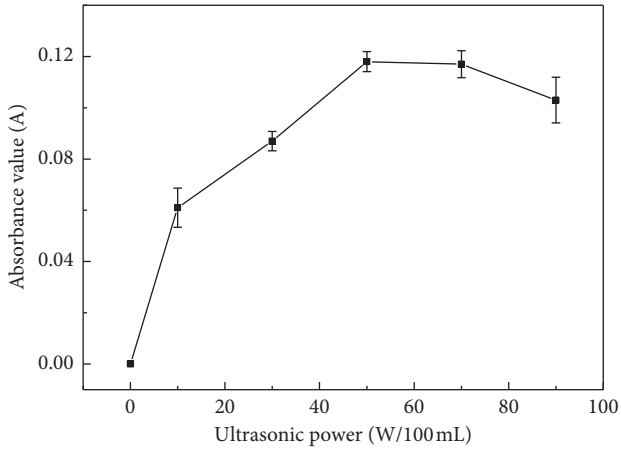

Figure 8: The effect of ultrasonic power on browning intensity.

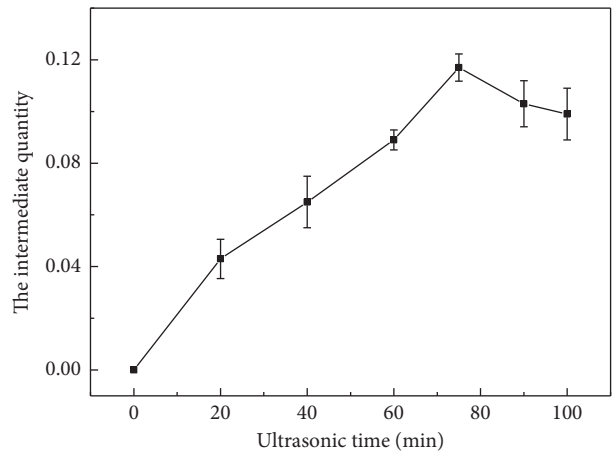

Figure 9: The effect of ultrasonic time on intermediate quantity.

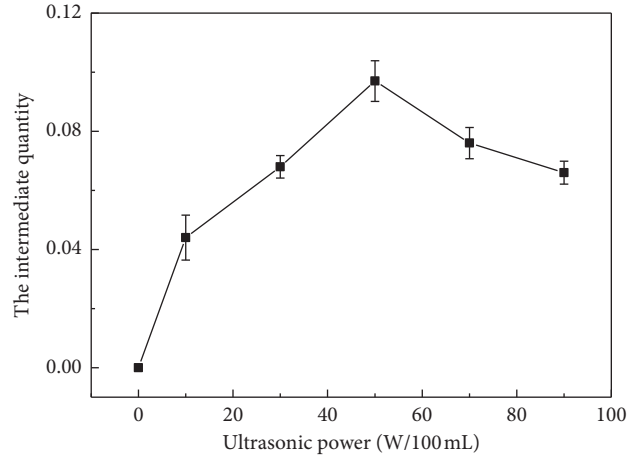

Figure 10: The effect of ultrasonic power on intermediate quantity.

the degree of browning increased slowly after 60 min of sonication. In Figure 8, with the ultrasonic power rise, the degree of browning of the simulated system showed a tendency to increase first and then decrease and reached the maximum when the ultrasonic power density was 50 W/100 mL. This result is consistent with our previous experimental conclusions [9]. The apparent increase in the absorbance indicates that the midterm product of the Maillard reaction is accumulating. This difference could be related to the acoustic cavitation. Since this cavitation activity can be viewed as a dramatic concentration of acoustic energy resulting in localized high stresses, temperatures, and/or fluid velocities, its biological consequences should be understood by those who are trying to either optimize or minimize its effects [47]. Additionally, the violent collapse of bubbles can generate exceedingly steep heat and pressure, which can produce free radicals and so forth [48], and then small molecules can rearrange [4]. The results show that the ultrasonic wave can strengthen the fructose-glycine Maillard reaction, promote the system browning, and induce the middle stage and the formation of advanced stage products.

3.5. Effect of Ultrasound on the Amount of the Intermediate Products in Maillard Reaction. In the Maillard reaction process, there are some noncolor intermediates, such as small molecules of ketones and aldehydes, which are also important indicators of the Maillard reaction [34]. As can be seen from Figures 9 and 10, without ultrasonic processing, the intermediate product of the Maillard reaction has not been detected in the simulation system solution. Applying the ultrasonic vinegar sample, however, the intermediate product of the reaction significantly altered. Results (Figures 9 and 10) showed that accompanied with the ultrasonic time and ultrasonic power rise, the median product of the Maillard reaction increased gradually, reaching the maximum at 75 min and 50 W/100 mL, respectively. The change of Maillard intermediates may largely be due to the fact that ultrasound can induce some oxygen ions and hydroxyl radicals in the aqueous solution [49], and the production and rearrangement of these oxygen ions and hydroxyl radicals can promote the Maillard reaction of the fructose-glycine simulation system, leading to the production of intermediates. The degree of browning and the number of intermediate products in the ultrasonic treatment

simulation system can be used to prove the ultrasonic catalytic Maillard reaction.

4. Conclusion

In this study, under the conditions of ultrasonic processing, the changes of Zhenjiang vinegar physiochemical index, such as color, reducing sugar, and amino acid, are consistent with those of natural ageing. In addition, the sonicated vinegar produces hydroxyl radicals, which generally appear to increase with increasing ultrasound time and ultrasound power. The present study reveals that the mechanism of ultrasonic ageing lies in the cavitation of ultrasound to induce the generation of radicals, such as hydroxyl radicals and superoxide radicals, catalyze the oxidation reaction in vinegar, and verify it by the fructose-glycine Maillard simulation system. The results showed that ultrasonic treatment had positive impacts on the vinegar ageing process. For the future, much research is needed to determine the exact role of ultrasound in vinegar ageing.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

All authors declare no conflicts of interest.

Authors' Contributions

Xiaoke Ma and Tingting Li contributed equally to this study.

Acknowledgments

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Research Article

The Effects of Carbendazim on Acute Toxicity, Development, and Reproduction in *Caenorhabditis elegans*

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Carbendazim, as a fungicide, was commonly used to control fungal diseases in agriculture, forestry, and veterinary medicines. In this study, the acute and reproductive toxicity of carbendazim was assessed using *Caenorhabditis elegans* (*C. elegans*) as a model in order to preliminarily evaluate the potential risks of this fungicide in agricultural production and application. The results showed that the growth of *C. elegans* was inhibited by 0.01 μg/L carbendazim. The treatment of 0.1 μg/L carbendazim caused a significant decrease in locomotion behavior and significant damage to the reproductive and antioxidant system, causing the lifespan of nematodes to be drastically shortened. These results provide a better understanding of the environmental risk of carbendazim and raise new concerns about safety.

1. Introduction

Pesticides, a kind of chemical or biological reagents, are widely used in agriculture to regulate plant growth and control diseases and insect pests, which can promote crop growth and improve crop yield [1]. However, the widespread use of pesticides will lead to different degrees of residues in crops or food and thus affect human health [2]. The problems of pesticide residues have not only attracted great attention of consumers but also become one of the key factors affecting food safety [3].

Carbendazim, as a broad-spectrum fungicide, has been used to control fungal diseases in agriculture, forestry, and veterinary medicines [4]. However, carbendazim is categorized in the hazardous category of chemicals by the World Health Organization [5] and has been classified in the priority list of endocrine-disrupting chemicals by the European Commission [6]. In recent years, it is obvious that the widespread use of carbendazim with over-range and overdose and the fact that carbendazim is hard to be degraded both lead to the problem of carbendazim residues in agriculture [7]. Although the toxic effect of carbendazim has been reported since the 1980s, the toxicity of carbendazim becomes a hot topic because of the increasing concern about environmental endocrine disruptors [4]. Carbendazim has been banned in several countries because of its negative impacts on the environment and health such as development and reproductive disturbances, toxicity, and mutagenicity [8]. The adverse effects of carbendazim on the biochemical, histopathological, and hematological parameters in the liver, kidney, and endocrine glands and their hormonal levels have been illustrated in rats [4]. Additionally, it needs further study on low concentration due to the residues of carbendazim.

Caenorhabditis elegans (*C. elegans*), as an important research model, is widely used to do some assessment. According to Amrit et al. [9], *C. elegans* has many advantages, such as small size, rapid generation time, easy of culturing on laboratory, and short adult lifespan. *C. elegans* was chosen in this study as the model organism to evaluate the toxicity of low concentration of carbendazim, which may be considered as a reference value for the application of carbendazim in agriculture.

2. Materials and Methods

2.1. Chemicals and Strains. Carbendazim (purity ≥ 99%; Aladdin® Biochemical Technology Co., LTD, Shanghai, China) was dissolved in N, N-dimethylformamide (DMF; Sinopharm Chemical Reagent Co., LTD, Shanghai, China) to produce 1 g/L carbendazim original solution. The concentrations of DMF were 0.1% in final exposure solutions (0.01, 0.1, 1, 10, 100 μg/L). 0.1% DMF without carbendazim was the control group. *C. elegans* (wild-type N2) were originally obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, MN, USA). The nematodes were cultivated on nematode growth medium (NGM) plates that were seeded with *Escherichia coli* OP50 at 20°C as described [10]. L1-larval *C. elegans* were collected by washing the gravid nematodes with a bleaching mixture (1 M NaOH, 10% NaHOCl).

2.2. Lethality. Carbendazim original solutions (1 g/L) were diluted with S liquid medium (1.12 g K_2HPO_4, 5.92 g KH_2PO_4, and 5.85 g NaCl were diluted with 1 L water) to get the final carbendazim concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mg/L, which contained 0.1% DMF. S liquid medium with 0.1% DMF was the control group. 30 nematodes (L4) were tested in 96-well plates for each concentration. The survival of nematodes was counted under a microscope after culturing for 24 hours in the incubator. The process of the test is based on the method of Xiang et al. [11]. Three parallel experiments were needed.

2.3. Locomotion Behavior. At least 10 *C. elegans* (L4) were picked randomly from each concentration to determine the locomotive behavior, which was recorded by both the head-thrash frequency and the body bend times [12]. The number of head-thrash frequency was counted by changes in the direction of bending at the midbody of *C. elegans* in 1 min. Measurement of body bend was defined as the times of the direction changes of the part of the nematodes cultured in NGM without *E. coli* OP50 in the 30 s.

2.4. Growth and Development Assays. *C. elegans* exposed to carbendazim for 24 h was analyzed. The body length of nematodes exposed to carbendazim was assessed by the Image J software. The offspring of each *C. elegans* from L4 larvae to day 1 was recorded at the L3 stage after individually transferring to a new plate every day until reproduction ceased [13]. At least three parallel tests were carried out.

2.5. Lifespan Analysis. All of *C. elegans* tested for lifespan were cultured in the same condition at 20°C. The synchronized *C. elegans* were cultivated in NGM plates with different concentrations of carbendazim until day 4. The tested nematodes then would be transferred into new NGM plates every 2 days. Surviving and dead *C. elegans* were recorded daily (beginning on the first day of adulthood) until all nematodes for each concentration had died [11]. At least three parallel tests were carried out.

2.6. Determination of Oxidative Damage. Intracellular ROS was measured with 2′,7′-dichlorodihydrofluoroscein diacetate (H_2DCFH-DA), which is the most common and sensitive reactive oxygen detection probe by far. The wild-type N2 *C. elegans* were washed in M9 buffer and then ultrasonically disrupted. The supernatant was analyzed the ROS level following the instruction of the ROS kit. The final working concentration of H_2DCHE-DA was 10 μM [11]. The excitation and emission absorbance wavelengths were 485 nm and 535 nm, respectively. At least three parallel tests were carried out.

Intracellular total superoxide dismutase (T-SOD) was determined according to the instruction of the T-SOD kit purchased from the Nanjing Jiancheng Bioengineering Institute. After washed with M9 three times, the examined nematodes were ultrasonically disrupted and reacted with a T-SOD kit. The absorbance wavelength was 550 nm. In addition, the supernatant was used to detect the level of protein for each concentration, in which absorbance wavelength was 595 nm. At least three parallel tests were carried out.

2.7. Data Analysis. All data were given as mean ± standard error of the mean (SEM) by using one-way ANOVA. Graphs were presented using Origin 8.5 and GraphPad Primer 7, and statistical analysis was performed using the SPSS 19.0 software. The statistical significance level was conducted using $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Determination of the Locomotion Behavior of C. elegans after Carbendazim Acute Exposures. LC_{50} *C. elegans* were exposed to carbendazim for 24 hours to assess its acute toxic effects. Data are represented as shown in Table 1, and the obtained linear fitting equation was $y = 2.180x - 0.223$ through data analysis. The obtained LC_{50} is 0.867 mg/L.

Next, we assayed the determination of the locomotive behavior of *C. elegans* after carbendazim acute exposures by analyzing the data about head-thrash frequency and body-bending times of nematodes (Figures 1(a) and 1(b)). Both of them showed significant decreases at the carbendazim concentrations ranging from 0.01 μg/L to 100 μg/L ($p < 0.01$). Additionally, the head thrashes of nematodes exposed to 100 μg/L decreased to 68.27%. For the body bends test, when the carbendazim concentrations were 10 μg/L and 100 μg/L, it had a significant inhibitory effect on the body bends of *C. elegans* by 36.77% and 35.48% compared with the control one, respectively.

3.2. Determination of the Growth and Development of C. elegans after Carbendazim Acute Exposures. Compared with the control group (Figures 1(c) and 1(d)), body length and body surface area were significantly ($p < 0.01$) reduced in the exposure groups from 0.01 μg/L to 100 μg/L. Both of them were decreased by 19.16% and 22.15% at the treatment of 0.01 μg/L compared with the control group, respectively. The concentration of 10 μg/L presented the most negative

Table 1: Effects of carbendazim on LC_{50} *C. elegans* by 24-h acute exposures.

Concentration (mg/L)	Total	Survival
0	30	28
0.2	30	25
0.4	30	21
0.6	30	18
0.8	30	16
1.0	30	15

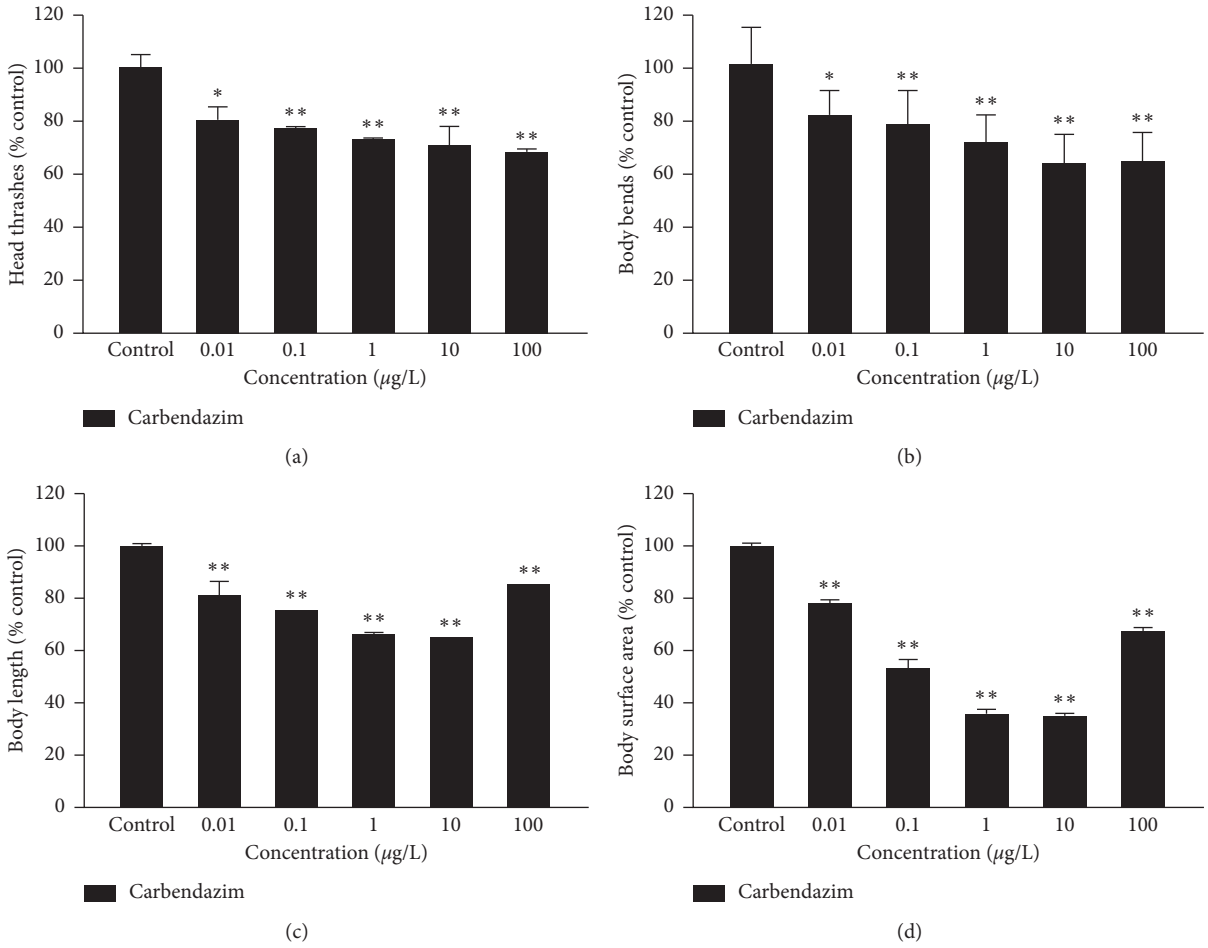

Figure 1: Effects of *C. elegans* on physiological traits exposed to carbendazim. (a) The head thrashes of *C. elegans* after carbendazim exposure; (b) the body bends of *C. elegans* after carbendazim exposure; (c) the body length of *C. elegans* after carbendazim exposure; and (d) the body surface area of *C. elegans* after carbendazim exposure. Data (mean ± SEM) are shown in figures as the percentage values compared with the control group. The asterisks present the significances between the each exposure group and control group ($^{*}p < 0.05$ and $^{**}p < 0.01$).

impacts, and body length and body surface area of *C. elegans* were decreased by 35.21% and 65.22% compared with the control group, respectively.

3.3. Determination of the Brood Sizes of C. elegans after Carbendazim Acute Exposures. According to Figure 2, brood sizes of nematodes had a significant decrease ($p < 0.01$) in the treatment groups from 0.1 μg/L to 100 μg/L. The brood sizes of *C. elegans* decreased most significantly, which decreased to 43.71% with the treatment of 10 μg/L compared with the control group.

3.4. Determination of the Lifespan of C. elegans after Carbendazim Acute Exposures. C. elegans lifespan was significantly inhibited by 0.01 μg/L to 100 μg/L carbendazim according to the lifespan curve shown in Figure 3. The results presented that *C. elegans* lifespan was decreased from 24 to 20 days with the treatment of 0.01 μg/L carbendazim. The

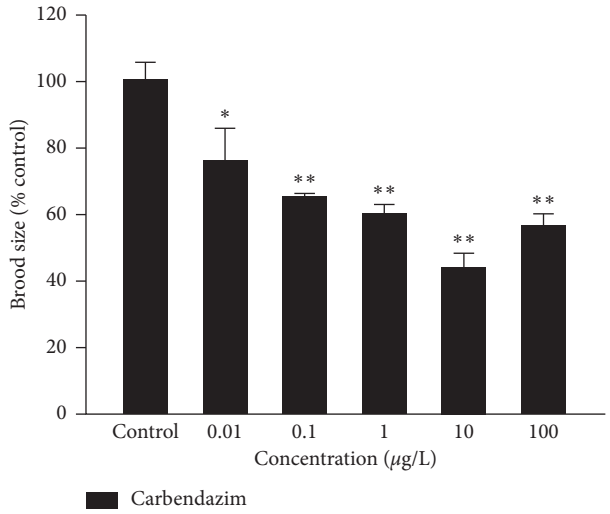

Figure 2: Effects of *C. elegans* on brood size exposed to carbendazim. Data (mean ± SEM) are shown as the percentage value compared with the control group. The asterisks present the significance between the each exposure group and control group ($^{*}p < 0.05$ and $^{**}p < 0.01$).

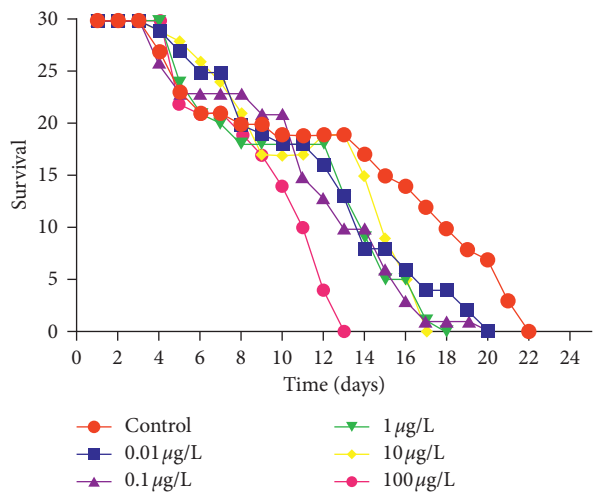

Figure 3: Effects of *C. elegans* on survival exposed to carbendazim. Data (mean ± SEM) are shown as the percentage value compared with the control group. The asterisks present the significance between the each exposure group and control group ($^{*}p < 0.05$ and $^{**}p < 0.01$).

lifespan of nematodes treated with 0.01 μg/L carbendazim was reduced by 20.00%. When the carbendazim exposure concentration was 100 μg/L, the lifespan of *C. elegans* was reduced the most by 45.83%.

3.5. Effects of Acute Exposure to Carbendazim on the Antioxidant System of C. elegans. The ROS levels of control and treated *C. elegans* at carbendazim exposure of different concentrations are shown in Figure 4(a). It was indicated that the level of intracellular ROS was significantly increased ($p < 0.01$) at ranges from 0.01 μg/L to 100 μg/L carbendazim. Compared with the control one, the ROS level was increased at most by 70.60% with the treatment of 10 μg/L. According to the results of intracellular SOD levels, it had an increase at the treatment from 0.01 μg/L to 100 μg/L carbendazim (Figure 4(b)). The SOD level was increased by 10.70% at 0.1 μg/L carbendazim compared with the control group.

4. Discussion

Carbendazim, as the fungicides, is widely used in agriculture to inhibit the growth of fungus. Carbendazim has been prohibited to use in Australia, most of the European Union, and the USA because of its severe toxicology and persistent nature [14]. For this study, it was the first time to use *C. elegans* as the model organisms to evaluate the effects of carbendazim on locomotive behavior, growth and development, reproduction, lifespan, and antioxidant systems. Moreover, results showed that it had a negative influence on *C. elegans*.

According to the 24 h-LC_{50}, the acute toxicity concentration of *C. elegans* exposed to different concentrations of carbendazim is 0.867 mg/L. The 96-h LC_{50} of carbendazim in response to zebrafish has been illustrated as 1.75 mg/L [15]. The eggs of Prussian carp *Carassius gibelio* has shown the toxicity effects at the concentration of 0.036 mg/L [16, 17]. Studies presented that the growth and development of *Navicula* sp. is inhibited by carbendazim with a 24 h-EC_{50} value of 2.18 mg/L. Though the rate of algal growth is recovered after 72-h exposure, the chlorophyll-a content remains significantly decreased when the treatment of carbendazim was beyond 0.5 mg/L [18]. In this present, *C. elegans* exposed to a low concentration of carbendazim were selected to evaluate its effects depending on the actual concentration of human daily exposure. Low concentrations of carbendazim do not mean it is safe. Carbendazim shows negative biological impacts at much lower doses in some studies.

Locomotive behavior was evaluated to assess the neurotoxicity of *C. elegans* (L4 larva) after 24-h exposure to carbendazim. The results showed that carbendazim could have negative effects on locomotive behavior through the detection of head thrashing and body bending of *C. elegans*, which both were more sensitive at the higher exposure group. The locomotive behavior of zebrafish embryos exposed to carbendazim is sensitive [15]. Previous studies have shown that fish have an abnormal behavior when sublethal concentrations of carbendazim are 0.22–0.43 mg/L [19].

Developmental malformations could also be one reason for abnormal locomotion [20]. The growth and development of *C. elegans* were assayed in our study. Results showed that the body length and body area of *C. elegans* were significantly narrowed at the treatment exceeding 0.01 μg/L carbendazim. The normal growth of vertebrates is related to the metabolic thyroid hormone homeostasis [21, 22]. Williams et al. [23] have indicated that carbendazim could cause sperm loss after implantation, fetal malformation, and slow growth and development.

The reproductive toxicity of carbendazim has been demonstrated that carbendazim could inhibit the microtubule polymerization of fungal and mammalian cells, causing disruption of microtubule assembly by acting with

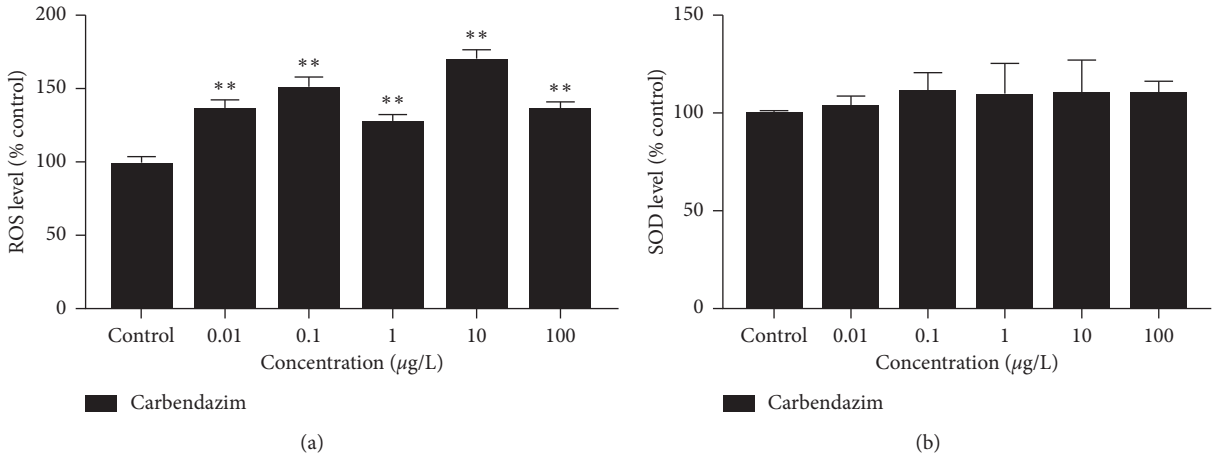

Figure 4: Effects of *C. elegans* on antioxidative system exposed to carbendazim. (a) The ROS level of *C. elegans* after carbendazim exposure; (b) the SOD level of *C. elegans* after carbendazim exposure. Data (mean ± SEM) are shown as the percentage value compared with the control group. The asterisks present significance between the each exposure group and control group ($^{*}p < 0.05$ and $^{**}p < 0.01$).

β-tubulin, which results in impairing the segregation of chromosomes in the process of cell division [24]. The formation of microtubules by noncovalent bindings of α- and β-tubulin is responsible for chromosome segregation in the process of mitosis and meiosis [24]. The brood size of *C. elegans* significantly decreases at 0.1 μg/L carbendazim concentration. Carbendazim has been found to affect the reproduction systems in Japanese quails [25] and hamsters [23]. It was concluded that *C. elegans* lifespan was significantly decreased with carbendazim concentration of $\geq$0.01 μg/L based on our study. Studies have shown that carbendazim has led to infertility and developmental toxicity and manifests embryo toxicity, germ cell apoptosis, and teratogenesis in different mammalian species [17, 18, 24].

Apoptosis is a complex programmed cell death, which is a highly regulated phenomenon characterized by a series of cellular processes [26, 27]. Many studies have presented that the production of ROS induced by oxidative stress is related to apoptotic cell death [28]. Our study found that carbendazim could induce a significant increase in the level of ROS values and a little increase in the level of SOD values. Oxidative stress caused by environmental pollution induces the increased expression of ROS and subsequently damages the antioxidant defense system [29]. SOD is responsible for the detoxification of toxic free radicals and their activities, which is used to evaluate the oxidative stress level and cellular antioxidant status [17, 24]. Metalloenzyme SOD accelerates the transformation of endogenous cytotoxic superoxide radicals to H_2O_2, and the increase of SOD expression levels may contribute to improving the enzyme activities in order to eliminate the superoxide radicals induced by carbendazim and to prevent the occurrence of cellular dysfunction during exposure of carbendazim [24, 29]. Higher exposure concentrations of carbendazim could cause severe oxidative stress, which subsequently destroys the balance of cell homeostasis and promote apoptosis [30]. However, carbendazim in low concentrations could still significantly damage the reproductive system according to our results.

5. Conclusion

As far as we know, the present study evaluated the safety of carbendazim exposed to *C. elegans* for the first time. It demonstrated that carbendazim could have a harmful effect on the locomotive behavior, development and growth, reproduction, lifespan, and antioxidant system of *C. elegans*. Hope that it needs to pay more attention to the application of carbendazim based on the results. In addition, the safety of carbendazim for use needs to evaluate further, especially the bioaccumulation toxicity and potential genotoxic effects.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Influence of Heat Treatments on the Content of Bioactive Substances and Antioxidant Properties of Sweet Potato (*Ipomoea batatas* L.) Tubers

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Heat treatment can affect the bioactive compounds in sweet potato (SP). In this work, we monitored the influence of heat treatment (boiling, steaming, microwaving, and baking) on the total polyphenols content (TPC), total antioxidant capacity (TAC), total anthocyanins content (TANTC), and phenolics acids (chlorogenic (CGA), neochlorogenic (neo-CGA), and *trans*-ferulic (tFA)) in two SP varieties grown in Slovakia and Croatia. TPC, TAC, and TANT were determined spectrophotometrically and phenolic acids by HPLC. TPC ranged from 576 (Beauregard, Croatia; Be/HR) to 3828 (414-purple, Slovakia; Pu/SK) mg/kg DW in the raw SP tubers. After heat treatment, TPC increased, most in steamed SP (8438 mg GAE/kg DW; Pu/SK), while only in boiled SP (Be/HR), TPC decreased (353 mg GAE/kg DW). TAC varied from 0.848 (Be/HR) to 8.67 (Pu/HR) (μmol TE/g DW) in raw SP. The TAC increased by heat treatment (max. 14.2 μmol TE/g DW; cooking Be/SK), except for Pu/HR. The TANT ranged from 151 (raw Pu/SK) to 1276 (microwaved Pu/SK) mg CyE/kg FW. Heat treatment had a negative effect on phenolic acid content; the largest reduction was after boiling: CGA by 29% (Pu/SK), neo-CGA by 69% (Pu/HR), and tFA by 29% (Be/HR). The influence of heat treatment on the monitored quantities is not definite.

1. Introduction

Sweet potatoes belong to the most economically important crops in the world with corn, wheat, rice, and potatoes. In 2019, their world production amounted to almost 92 million tons. The largest producer was Asia (75%), while the production of China was 53 mil. tons, which represented 57.6% of world production. In Europe, 93.4 thousand tons of SP were grown [1]. Sweet potatoes are tropical plants and do not tolerate frost. At temperatures below 1°C, their vegetative growth and productivity decline [2]. However, they quickly adapt to high temperatures and drought, making them an attractive crop, especially in countries with limited agricultural resources [3]. In developing countries, they are the fifth most important food crop [4]. For human consumption, sweet potato tubers are preferably prepared in various ways: cooked, baked, fried, and dried in the form of sticks or slices. They are also used as a raw material in the production of pasta, in alcoholic beverages, as a source of natural pigments, or in the production of paper, cosmetics, and adhesives tissues [5, 6]. Sweet potatoes are a crop with high nutritional value; they contain large amounts of carbohydrates, fiber, vitamins, and minerals [7]. Carbohydrates are the predominant part of sweet potato tubers. The sweet taste

of sweet potatoes is caused by the presence of glucose (2.7–4.7 mg/g DW), fructose (1.4–4.0 mg/g DW), sucrose (56.9–60.0 mg/g DW), and nondetectable maltose [8]. The major polysaccharide of SP is starch making up 80% of the dry matter [9]. Sweet potatoes are also a cheap and rich source of fiber. Its content depends on the variety; the amount of crude fiber is in the range of 0.6 to 1.5% [10]. The total dietary fiber content of sweet potatoes is enough to affect the glycemic index elicited by these roots, and we cannot rule out a bioactive effect of the protein components. It may prove beneficial for diabetic patients who consume sweet potatoes [11]. In general, sweet potato roots contain 1.73%–9.14% (dry basis) proteins. The main protein in sweet potato is sporamin, which can be divided into two subgroups: sporamin A (contains 219 amino acid residues) and sporamin B (contains 216 amino acid residues). The sweet potato protein contains 18 different amino acids, about 40.7% of the sweet potatoes protein consists of the essential amino acids Ile, Leu, Met, Phe, Thr, Val, Lys, and Trp. The last two of them are the primary limiting amino acids [12]. Sweet potato tubers contain a small amount of lipids (from 0.72% to 1.44%), while the crude fat content is significantly affected by a variety [13]. The primary fatty acids are palmitic acid (C16 : 0), linoleic acid (C18 : 2), and linolenic acid (C18 : 3), while a smaller proportion is stearic acid (C18 : 0), oleic acid (C18 : 1), and arachidic acid (C20 : 0) [14]. Sweet potatoes are an excellent source of minerals. The most represented are Ca, K, and P and from trace elements Cu, Fe, Zn, Mn, Na, and Mg. In comparison with the other vegetables, sweet potatoes have more Mg, Na, P, and Fe than carrots or cabbage [5, 15]. According to [16], SP are also crucial in terms of the content of vitamins and other bioactive substances (polyphenolic compounds, phenolic acids, and anthocyanins and beta-carotene), which have antioxidant effects [4, 17, 18]. Sweet potato roots comprise hydrophilic vitamins (B1, B2, B6, niacin, pantothenic acid, biotin, and vitamin C) and lipophilic vitamins E and A [8, 15]. The precursor of vitamin A is beta-carotene with two beta-ionone rings, which also possesses antioxidant activity [19]. Beta-carotene is mainly present in orange varieties of SP. In 2016, because of the high content of vitamin A, the orange SP were recognized as a food able to improve nutrition in many households in sub-Saharan Africa [19–21]. Another antioxidant vitamin, ascorbic acid, also occurs in high amounts in the roots. The high content of vitamin B6 (pyridoxine) in the roots helps in reducing the blood levels of homocysteine, which is associated with the increased risk of cardiovascular diseases [19]. The characteristic color of purple SP is caused by the presence of anthocyanins. Together with phenols, they represent the main bioactive substances in purple SP. Anthocyanins create a group of water-soluble flavonoids; in sweet potato, they occur as mono- or diacylated forms of cyanidin and peonidin [22]. It is supposed that anthocyanins—natural pigment—can have many positive effects on human health, such as antioxidant, anti-inflammatory, anticarcinogenic, chemoprotective, and antihyperglycemic effects, and they can help avoid oxidation of LDL-cholesterol. They are present mainly in purple varieties of sweet potato—in peel and also in the flesh [23, 24]. Phenolic compounds are antioxidant molecules with at least one aromatic ring and one or more hydroxyl groups, including their functional derivatives. These slightly various substances are essential for the growth and reproduction of plants, and they act as antipathogenic agents [18]. The main components of the sweet potato phenolic compounds are chlorogenic acids, which belong to the group of ester compounds. They are formed by the condensation of quinic acid and *trans*-cinnamic acids, which include coffee acid, *p*-coumaric acid, and ferulic acid [25]. 3-O-Caffeoylquinic acid is the most common of chlorogenic acids [17, 26, 27]. For example, in sweet potato leaves, twenty CQA phenolic acids, such as 3-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, and tri-CQA, were identified by LC-MS2 analysis [25]. The phenolic hydroxyl of chlorogenic acids easily reacts with free radicals, making the free radicals lose activity. Chlorogenic acids in plants have high antioxidant activities, such as reducing power, metal chelating, and lipid peroxidation-inhibiting activity [27]. Thus, in addition to antimutagenic and anticarcinogenic effects *in vitro* and *in vivo*, chlorogenic acid can prevent the formation of hydroxyl radicals, remove free radicals, and eliminate oxidative activity [28]. However, there are only a few studies about the differences in antioxidant activities between different chlorogenic acids, which does not provide sufficient theoretical support for the application of antioxidant activities [27]. Sweet potatoes are commonly prepared before consumption in different ways: baking, boiling, microwaving, steaming, or frying. Heat treatment improves their digestibility [29], induces significant changes in the chemical composition, and thus influences the concentration and bioavailability of compounds [18, 30]. Generally, the total phenolic content of sweet potatoes increases; on the other hand, some phenolic derivatives such as caffeic acids are decreased by heat treatment. Concerning the variation of these constituents, the antioxidant activity of sweet potatoes also shows similar variation during thermal processes [30]. The aim of our study was to research changes in the content of bioactive substances and their antioxidant activity due to heat treatments which are commonly used in the preparation of sweet potato tubers.

2. Materials and Methods

2.1. Plant Material. Two varieties of sweet potatoes with different flesh colors were used for the analyses—Beauregard (orange) and 414-purple (purple). Both cultivars were grown in the cadastral area of Šoporňa in the Slovak Republic (Be/SK, Pu/SK) and the east part in Croatia (cadastral area of Vukovar) (Be/HR, Pu/HR). After plowing using the adjusted plow machine, sweet potatoes from Croatia were sorted and cured at 25°C for 4 to 5 days. After, they were stored at a temperature of 13–16°C and a maximum humidity of 70% (max. 10 days). After transport to Slovakia, they were immediately delivered to our workplace and used for sample preparation. Sweet potatoes from Slovakia were taken directly in the field and then brought to the workplace, where we cleaned them. The next day, they were used for sample preparation. About 3 kg of plant material was taken from the

given sampling sites for each cultivar. Raw and heat-treated sweet potatoes were used for analyses.

2.2. Chemicals. MetOH (80%), MetOH (99.8%), Folin–Ciocalteu reagents, DPPH (2,2′-diphenyl-1-picrylhydrazyl), Trolox (2,5,7,8-tetramethylchroman-2-carboxylic acid), HCl (36%), buffer pH 3.5 (Na_2HPO_4, $c = 0.2$ mol/L; citric acid, $c = 0.1$ mol/L), EtOH (80%), authentic standards of chlorogenic acid (purity ≥ 95.0%), neochlorogenic acid (purity ≥ 95.0%), *trans*-ferulic acid (purity ≥ 95.0%), acetonitrile (gradient HPLC grade), and phosphoric acid (ACS grade) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); gallic acid (p.a.) and Na_2CO_3 were purchased from Merck (Germany); gallic acid (p.a.) was provided by Merck (Germany); double deionized water (ddH_2O) was treated (0.054 μS/cm) in a Simplicity 185 purification system (Millipore, UK).

2.3. Preparation of Samples. All tubers were washed (first with drinking water and then distilled water) and peeled. The peeled tubers were rewashed (with distilled water) and cut into slices of the thickness of 3 mm. The slices from each tuber were divided into five approximately equal parts. One part of the tuber (crude) was mixed (Grindomix GM2000 Retsch, 2000 rpm, 30 sec) and then the sample was homogenized. Other sections were variously heat-treated: cooked in water (10 min), steamed (15 min, 97 ± 2°C), and microwaved (5 min, 800 W) as well as baked (15 min, 200°C). After cooling, slices of sweet potatoes were mixed (Grindomix GM2000 Retsch, 2000 rpm, 15 sec) and then homogenized.

2.4. Preparation of Extracts. 25 g of homogenized sample (raw flesh and boiled, steamed, baked, and microwaved flesh, respectively) was taken and poured into 50 mL of 80% MetOH at laboratory temperature and extracted by horizontal shaker (Unimax 2010; Heidolph Instrument GmbH, Germany) for 12 hours. Sample was filtered through Munktell No. 390 paper (Munktell & Filtrac, Germany) and stored in closed 66 mL vial tubes. Determination of TPC, TAC, TANTC, and phenolics acids was performed eight times.

2.5. Determination of Total Polyphenols Content. The total polyphenols content (TPC) was determined using the colorimetric Folin–Ciocalteu method [31] by spectrophotometric analysis (spectrophotometer Shimadzu UV-1800). The aliquot portion of extract (0.1 mL) was pipetted into a 50 mL flask. Then 2.5 mL of Folin–Ciocalteu reagent was added; after 3 minutes, 5 mL of 20% sodium carbonate aqueous was added; and distilled water was added to mark. Also, blank with distilled water was prepared by the same procedure and the calibration curve was prepared with standard solutions of gallic acid. After mixing and leaving for 2 hours in the laboratory, the complex forming was ended and the absorbance of blue solutions was measured in cuvettes of 1 cm width at a wavelength of 765 nm. The content of total polyphenols in the sample was expressed as the content of gallic acid in mg/kg of fresh matter and calculated to dry weight (mg GAE/kg DW).

2.6. Determination of Total Antioxidant Capacity. For the determination of total antioxidant capacity (TAC), the method based on the radical reaction of 2,2′-diphenyl-1-picrylhydrazyl (DPPH) according to [32] was used. To obtain a stock solution, 0.025 g of DPPH was diluted to 100 mL with MetOH (99.8%) and kept in a cold and dark place. Immediately before the analysis, a 1 : 10 dilution of the stock was made with methanol. For the analysis, 3.9 mL of the DPPH working solution was added to a cuvette and the absorbance at a wavelength of 515.6 nm was measured (A_0) by UV-VIS 1800 spectrophotometer (Shimadzu, Japan). Subsequently, 0.1 mL of the extract was added to the cuvette with DPPH, and the absorbance was measured after 10 min (A_{10}). An increasing amount of antioxidants present in the methanol extract of the sample reduced DPPH and faded the color of the solution in a correlation proportional to the antioxidant concentration. The percentage of DPPH inhibition was calculated according to the following equation:

$$\%\ \text{in } h.\ \text{DPPH} = \frac{(A_0 - A_{10})}{A_{10}} \times 100, \quad (1)$$

where A_0 is absorbance at time $t = 0$ min (solution of DPPH) and A_{10} is absorbance at time $t = 10$ min.

The antioxidant activity was calculated using a standard curve with known concentrations of Trolox and expressed in terms of μmols of Trolox equivalents per Gram dry weight.

2.7. Determination of Total Anthocyanins Content. Total anthocyanin content (TANTC) was determined according to the pH differential spectroscopic method. Aliquot portions of extract (0.1 mL) were pipetted into two test tubes and 1 mL of 0.01% HCl in 80% EtOH was added to each tube. Subsequently, 10 mL of HCl (2%) was added to the first tube and 1 mL pH 3.5 buffer (Na_2HPO_4, $c = 0.2$ mol/L and citric acid, $c = 0.1$ mol/L) was added to the second tube. After 30 minutes of staining the solutions at room temperature, absorption was measured at 520 nm (Shimadzu UV-VIS 1800, Japan). Total anthocyanins content was calculated and expressed in mg CyE/kg (cyanidin eq/kg) FW.

2.8. Determination of Phenolic Acids Content (Chlorogenic, Neochlorogenic, and trans-Ferulic Acid). Aliquot volumes of supernatants from sweet potato extracts were filtered through a membrane filter of 0.22 μm. Separation of chlorogenic and neochlorogenic acids was carried out by reversed-phase HPLC with a column CORTECS C18 (150 × 4.6 mm; 2.7 μm). The column oven temperature was set at 30°C. A sample volume of 8 μL was injected onto the column using a Waters 717 autosampler connected to a Waters pump (Waters Corp., Milford, MA). Phenolic acids were eluted using a mobile phase A 0.1% (v/v) phosphoric acid, B acetonitrile, and C methanol. Detected peaks were

identified and quantified by comparing to retention times and areas of peaks of known standards. The gradient program was set, as shown in Table 1. The results were quantified by using a Waters 2965 UV detector at wavelength 325 nm.

Peaks were identified and quantified by comparison of retention times and areas of peaks of known standards. Results were quantified by using Waters 2965 UV detector at wavelength 326 nm.

Table 1: Gradient profile.

Minutes	*A* (%)	*B* (%)	*C* (%)
0	90	0	10
3	80	10	10
5	40	25	5
8	50	30	20
10	10	80	10
12	0	90	10

2.9. Statistical Analysis. All measurements were done in quadruplicate and presented as mean ± SD ($n = 4$). Results were statistically evaluated by one-way analysis of variance (ANOVA—Multiple Range Tests, method: 95.0 percent LSD) using statistical software STATGRAPHICS (Centurion XVI.I, USA) and a regression and correlation analysis (Microsoft Excel) was used. Differences at $p < 0.05$ were considered to be significant.

3. Results and Discussion

3.1. Total Polyphenols Content (TPC). Phenolic compounds are the primary antioxidants found in fruits, vegetables, and grains [28]. Sweet potatoes, which are also a source of many other bioactive substances, are also characterized by a high content of polyphenolic compounds [33]. Raw and heat-treated sweet potatoes were analyzed for the total polyphenols content and other bioactive compounds. TPC ranged from 576 mg/kg DW (169 mg/kg FW, resp.) in variety Be/HR to 3828 mg/kg DW in variety Pu/SK (750 mg/kg FW Be/HR, resp.) (Table 2) in the raw sweet potato tubers. The wide range of TPC values in raw flesh of SP can be attributed to several factors. Many authors [17, 34–36] mention the variety as the most significant factor. In particular, purple sweet potatoes are rich in phenolic substances. White, yellow, and orange varieties have a lower content of this group of compounds compared to purple varieties [7].

Comparable polyphenol contents were determined in [30] (0.93–1.05 mg/g DW), [4] (28.4 mg/100 g FW), and [18] (1300–1930 mg/kg DW). Higher TPC were determined in [37] in 15 varieties from Tenerife Island and in 15 varieties of SP from La Palma Island (90–166, resp., 78.8–161 mg phenolic compounds/100 g FW), [17] in orange SP (0.130–0.472 mg/g FW) and purple SP (0.477–0.949 mg/g FW); [26] in orange SP (284.1 mg/100 g DW) and purple SP (757.4 mg/100 g DW). As in our case, the authors report lower TPC in orange sweet potatoes than in purple sweet potatoes. The variation can be attributable to phenolics extraction methods, sweet potato genotypes, and growing conditions [38]. The content of polyphenols in the flesh was significantly affected by the method of treatment of sweet potatoes. There are statistically significant differences between the TPC of raw and heat-treated SPs in all varieties. However, it can not be clearly stated which heat treatment had the greatest effect on the change of TPC in sweet potatoes. In all heat-treated SP except for Be/HR (boiled), TPC was higher than in raw flesh. Only in SP Be/HR cooked in water was TPC 39% lower compared to raw SP. In this variety, the effect of heat treatment was the least (maximum 24% in baked SP). In the Pu/SK variety, the TPC in microwaved sweet potatoes was up to 5.5 times higher than in raw flesh. The slight increase of phenolic content in cooked samples can be attributed to the release of bound phenolics and inactivation of polyphenol oxidase affected by heat treatment. Besides, some phenolics can be degraded by polyphenol oxidase during slicing of raw sweet potatoes samples [38]. C. Dincer et al. [30] report the highest TPC in the boiled samples for all cultivars (up to 1.98 mg GAE/g DW). The authors explained this definite increase of TPC as the release of phenolics by hydrolysis of glycoside bonds during treatment and the induction of TPC oxidation in fresh samples through the catalytic activity of the enzyme polyphenol oxidase. C. M. Donado-Pestana et al. [18] determined the content of total phenolic compounds in boiled SP from 1.33 to 2.05 mg/g DW and in steamed SP from 1.05 to 1.56 mg/g DW; while boiling increased TPC in 2 varieties and decreased TPC in 2 varieties, steaming decreased TPC in all varieties. Their results suggest that the methods of heat treatment sometimes led to a significant loss of the total phenolic content in sweet potato tubers. Y. Tang et al. [7] reported a decrease of TPC in orange SP of 60.6% (boiled SP) and 15.9% (steamed SP), resp., and in purple SP of 31.3% (boiled SP) and 6.0% (steamed SP). The study in [39] determined in raw pith 2.1 mg TPC/g DW and in microwaved (boiled, baked) potato root tissues 1.8 (1.6, 2.1) mg TPC/g DW. The study in [40] studied 12 different ways of preparation (freezing, cooking, baking, etc.) of 15 types of food. Most of the studied processes produced a wide range of retentional factors (ratio of concentration of polyphenol in processed food to centration of polyphenol in raw food). Home cooking methods of common plant foods caused significant losses of polyphenols (median RF = 0.45–0.70).

3.2. Total Antioxidant Capacity (TAC). Variety and locality are essential factors influencing an antioxidant activity. The purple SP showed higher TAC, whereas TAC in Pu/HR was 2.7 times higher than in Pu/SK. On the other hand, TAC was 2.2 times higher in the Beauregard variety grown in Slovakia than in Be/HR. There are statistically significant differences between TAC values (Table 3).

However, the impact of the technological treatment method on TAC can not be clearly stated. Based on the DPPH test, the raw SP flesh Be/HR showed the lowest antioxidant activity (0.848 μmol TE/g DW, 212 μg TE/g DW) and the highest TAC had boiled SP Be/SK (14.2 μmol TE/g

Table 2: The total polyphenols content (mg GAE/kg DW).

	Raw	Boiled	Steamed	Microwaved	Baked
Be/SK	$1102 \pm 21.2^{a,B}$	$1762 \pm 1762^{c,B}$	$1770 \pm 34.4^{c,B}$	$1542 \pm 22.0^{b,B}$	$2065 \pm 38.2^{d,B}$
Pu/SK	$2056 \pm 114^{a,C}$	$3667 \pm 26.0^{b,C}$	$8438 \pm 839^{d,D}$	$11338 \pm 371^{e,D}$	$7212 \pm 256^{c,D}$
Be/HR	$576 \pm 53.6^{b,A}$	$353 \pm 67.0^{a,A}$	$692 \pm 81.4^{c,A}$	$710 \pm 63.6^{c,A}$	$714 \pm 51.2^{c,A}$
Pu/HR	$2444 \pm 72.0^{a,D}$	$4748 \pm 40.5^{c,D}$	$5461 \pm 185^{e,C}$	$5070 \pm 109^{d,C}$	$3692 \pm 93.8^{b,C}$

GAE: gallic acid equivalent; Be/SK: cv. Beauregard from Slovakia; Be/HR: cv. Beauregard from Croatia; Pu/SK: cv. 414-purple from Slovakia; Be/SK: cv. 414-purple from Croatia.

Table 3: Total antioxidant capacity (μmol TE/g DW).

	Raw	Boiled	Steamed	Microwaved	Baked
Be/SK	$1.81 \pm 0.239^{a,B}$	$14.2 \pm 0.178^{e,D}$	$10.9 \pm 0.195^{d,C}$	$5.50 \pm 0.073^{b,B}$	$5.80 \pm 0.062^{c,D}$
Pu/SK	$3.23 \pm 0.433^{a,C}$	$6.20 \pm 0.041^{c,B}$	$10.9 \pm 0.293^{e,C}$	$8.63 \pm 0.104^{d,C}$	$5.35 \pm 0.076^{b,C}$
Be/HR	$0.848 \pm 0.225^{a,A}$	$1.85 \pm 0.249^{b,A}$	$4.60 \pm 0.430^{c,A}$	$5.25 \pm 0.225^{d,A}$	$5.17 \pm 0.186^{d,B}$
Pu/HR	$8.67 \pm 0.097^{d,D}$	$11.3 \pm 0.120^{e,C}$	$7.14 \pm 0.735^{c,B}$	$5.19 \pm 0.039^{b,A}$	$4.74 \pm 0.051^{a,A}$

The values in the row marked with different lowercase letters are significantly different ($p < 0.05$); the values in the column marked with different uppercase letters are significantly different ($p < 0.05$). TE: Trolox equivalent.

DW, 3546 μg TE/g DW). Except for Pu/HR (steamed, microwaved, and baked), the highest TAC was in all heat-treated SP. The study in [38] determined TAC in raw flesh of orange SP in the range from 1.1 to 2.0 μmol TE/g FW and in the boiled SP from 1.7 to 2.7 μmol TE/g FW, which is an increase of 1.28–1.55 times of TAC after heat treatment. According to [41], there was no significant difference between the DPPH radical scavenging capacity of steamed and boiled sweet potato and untreated sweet potato. L. E. Steed and V.-D. Truong [22] determined the highest antioxidant activity (87.4 μmol TE/g FW) in raw peels. There was no significant difference between DPPH values in raw (75.5 μmol TE/g FW) and steamed SP (77.1 μmol TE/g FW). In comparison with our result, [7] determined higher values of TAC in sweet potatoes (orange—raw: 25.07, purple—raw: 27.79 μmol TE/g FW). The heat treatment resulted in a reduction of TAC (orange—boiled: 23.62, steamed: 15.22; purple—boiled: 22.11, steamed: 26.04 μmol TE/g FW). Individual heat treatments have different effects on the chemical composition. That is, after boiling and microwaving, 63.82% and 32.25% decreases of antioxidant activity were observed, while the antioxidant activity increased by 81.40%, 30.09%, respectively, and by 85.82% (frying). An increase of 9.44% in total polyphenol content was observed after steaming, while decreases of 30.51%, 25.70%, and 15.73% were observed after boiling, microwaving, and frying [25]. Heat treatment of SP possibly leads to a reduction of bioactive compounds content and, consequently, to a decrease of antioxidant capacity determined by the DPPH and ABTS tests. The antioxidant capacity can also be associated with the presence of phenolic acids in sweet potato roots. The processing of SP tubers (peeling, boiling, frying, and steaming) can also reduce the antioxidant capacity by the enzyme polyphenol oxidase, which catalyses the oxidative polymerization of phenolic acids [18]. Compared to raw SP (92.0−—132.3), heat-treated SP showed a decrease of antioxidant capacity (DPPH, IC50) to 74.1–88.2 in boiled and to 62.2–86.3 mg/mL in baked SP [30]. The authors report that the differences between TAC values in different varieties treated by the same method may be associated with various polyphenolic compounds that might have different sensitivity to heat treatment.

3.3. Total Anthocyanins Content (TANTC). The content of anthocyanins was determined only in purple varieties 414-purple grown in Slovakia and Croatia. The content of anthocyanins in PU/HR was almost 1.6 times higher than in Pu/SK, which indicates a significant effect of locality—the differences in TANTC in raw SP are statistically significant (Table 4). C. C. Teow et al. [17] determined anthocyanins in purple varieties SP from 0.246 to 0.531 mg/g FW, while the study in [42] reported the average content of anthocyanins in purple SP from 110 mg to 210 mg/100 g; the study in [26] reported 57.9 mg/100 g DW; and the study in [8] reported 32 to 1390 mg/100 g DW.

Anthocyanins are highly reactive molecules sensitive to degradation reactions. Their stability is affected by the structure and concentration of anthocyanins, the presence of enzymes, oxygen, pH, or temperature [43, 44]. Most authors report the reduction of anthocyanin content due to various heat treatments [7, 45]. Y. Tang et al. [7] determined a 34% lower content of anthocyanins in steamed SP (10.35 mg CyE/g) and a 41% lower content of anthocyanins in boiled SP (9.24 mg CyE/g) compared to raw deep purple SP. On the contrary, the results of our analyses show an increase in the content of anthocyanins after heat treatment of SP. The average TANT in raw SP Pu/SK (Pu/HR) increased 3.7 (2.4) times by boiling in water, 4.3 (4.0) times by steaming, 6.6 (3.7) times by baking, and 8.5 (4.8) times by microwaving. Cooking in the microwave oven had the most significant effect. The content of anthocyanins in the microwaved SP was 1276 mg/kg FW in Pu/SK and 1194 mg/kg FW in Pu/HR. Paper [23] reported a significant increase in the total anthocyanin content of boiled colored potatoes compared to fresh uncooked tubers of five studied cultivars. The highest increases were observed in cultivars with low TAC (11.1 times and 10.6 times), while in the cultivars with high TAC,

Table 4: Total anthocyanins content (mg CyE/kg FW).

	Raw	Boiled	Steamed	Microwaved	Baked
Pu/SK	$151 \pm 4.55^{a,A}$	$552 \pm 30.7^{b,A}$	$690 \pm 51.4^{c,A}$	$1276 \pm 9.16^{e,B}$	$1004 \pm 10.4^{d,B}$
Pu/HR	$238 \pm 28.4^{a,B}$	$575 \pm 13.7^{b,A}$	$959 \pm 14.4^{d,B}$	$1147 \pm 10.1^{e,A}$	$874 \pm 16.0^{c,A}$

The values in the row marked with different lowercase letters are significantly different ($p < 0.05$); the values in the column marked with different uppercase letters are significantly different ($p < 0.05$). CyE: cyanidin equivalent.

an increase was relatively low (3.44 times and 3.20 times). Paradoxically, most anthocyanins have greater stability at higher temperatures used in the processing of fruit and vegetables. This phenomenon is explained by the protective effect of the various system components and the condensation of monomers. In these reactions, more stable oligomeric pigments are formed, the amount of which increases with temperature [46]. The thermal stability of anthocyanins is affected by other factors; one of the main factors in the processing of fruit and vegetables is pH value. Anthocyanins of purple SP achieved the highest stability at pH value from 3 to 4 [8]. The red cultivar of sweet potato grown in the Andean region has been reported to have higher antioxidant activity and phenolic content than blueberry cultivar, the fruit with a high level of antioxidants [47]. The antioxidant capacity of sweet potato roots can vary widely depending on the flesh color, which can differ among sweet potato cultivars from white and yellow to orange and even purple [17].

On the contrary, there are significantly fewer studies where the authors describe the preservation or increase of their content due to cooking, baking, steaming, or microwave heating. The stability of anthocyanins in berries is lower, such as the stability of anthocyanins in radish, red potatoes, red cabbage, and purple SP [48–50] because, in PSP, anthocyanins occur mainly in acylated forms [51]. Acylation with various phenolic (cinnamic, *p*-coumaric, caffeic, and ferulic) and aliphatic (acetic, malonic, and oxalic, as well as succinic) acids makes these anthocyanins more resistant to pH, sensitivity to light and heat [52, 53]. The study in [51] determined the stability of twelve individual anthocyanins in raw P40 and cooked P40 via various cooking conditions. Although some heat treatments caused the degradation of total anthocyanins, the content of individual anthocyanins increased several times, for example, by microwave cooking the content of cyanidin 3-*p*-hydroxybenzoyl sophoroside-5-glucoside 3.81-fold and peonidin 3-*p*-hydroxybenzoyl sophoroside-5-glucoside 4.57-fold, or by baking the content of cyanidin 3-(6′′-feruloyl sophoroside)-5-glucoside 2.75-fold. Monoacylated anthocyanins showed higher heat resistance than di- and nonacylated ones. Similarly, [54] report an almost 50% reduction in total PSP Shinzani anthocyanins after steaming, but only slightly after baking, and a tendency to increase some acylated anthocyanins (cyanidin 3-(6′-feruloyl sophoroside)-5-glucoside: 1.23-fold). The study in [55] investigated the effects of several representative home cooking methods on the content and composition of anthocyanins. Air-fried, fried, and stir-fried cooking resulted in a reduction in anthocyanin content and the effect of steaming and microwaving on anthocyanin content was inconclusive, but cooking increased anthocyanin content by 6.55%. The application of different cooking methods caused changes in the content of individual anthocyanins. For example, the content of cyanidin 3-dicaffeoyl sophoroside-5-glucoside (the main component of the analyzed SP GZ9) increased by 8.83% after cooking. Interestingly, nearly all monoacylated anthocyanins increased significantly after boiling and microwaving. On the other hand, total diacylated anthocyanins decreased significantly after frying, air-frying, and stir-frying. The effect of SP (steamed and kneaded sweet potato flour) treatment was manifested by a five-to-six-time increase in the content of anthocyanins in red peeled (RP) and white peeled (WP) SP, a 1.3-fold increase in the content of flavonoids in RP, and a 2–13-fold increase in the total phenols contents of all genotypes [35]. Effect of three cooking methods on the content of anthocyanins in potato tubers with red- and purple-fleshed has been studied [56]. Cooking treatments resulted in a significant increase in total anthocyanin content in all cultivars in comparison with raw tubers. In the average of all cultivars, the greatest TAC increase was found as a result of the boiling of tubers (3.79 times against the value of raw tubers), followed by the microwave treatment (3.06 times), and the lowest increase in baked tubers (2.94 times) has been observed. While the most significant impact on TAC increase by boiling was observed in all cultivars except for Violette, higher TAC increase by microwaving was found only in three of the five cultivars. As the authors further state, there appears to be a consensus on the loss of anthocyanins upon exposure to heat (there appears to be a consensus on the loss of anthocyanins when exposed to heating). Therefore, it is necessary to continue doing the experiments, while the results will be the subject of our further research.

3.4. Phenolic Acids: Chlorogenic (CGA), Neochlorogenic (Neo-CGA), and trans-Ferulic (tFA) Acid. Phenolic acids show varied biological activity in the human body; among others, they take an active part in the removal of free radicals and metal ion chelation as well as affecting enzyme activity and protein availability [57]. Chlorogenic acid and its derivatives are esters of caffeic acid. These acids are the main component of sweet potato phenolic compounds. Chlorogenic acid and isochlorogenic acid are the primary acids [17, 26, 28]. Precision or repeatability of retention times and peak areas from five standards was calculated using percent relative standard deviation (% RSD) and is presented in Table 5.

The proportion of phenolic acids in the raw SP was different. The CGA content was the highest in the variety of Pu/HR (468 mg/kg) and decreased in order Be/HR > Be/SK > Pu/SK (147 mg/kg). The content of neo-CGA and *trans*-ferulic acid decreased in reverse order as follows: Pu/SK (7.26 and 10.4 mg/kg) > Be/SK > Be/HR > Pu/HR (0.768

Table 5: Validation parameters HPLC-DAD, analytical characteristics, and method validation data for HPLC using a Cortex 2.7 μm, 4.6 × 150 mm column.

Compound	Accuracy (% recovery)	Precision (% RSD)	Rt (min)	LOD (μg/mL)	LOQ (μg/mL)
tFA	98	2.897	5.2	0.0251	0.0753
CGA	115	5.564	2.3	0.0149	0.0447
Neo-CGA	113	4.326	2.8	0.0072	0.0216

RSD: relative standard deviation; RT: retention time; LOD: limit of detection; LOQ: limit of quantification.

Table 6: Content of phenolics acid (mg/kg FW).

	Raw	Boiled	Steamed	Microwaved	Baked
Chlorogenic acid (CGA)					
Be/SK	$336 \pm 21.9^{b,B}$	$234 \pm 28.7^{a,B}$	$347 \pm 75.5^{b,B}$	$212 \pm 13.1^{a,B}$	$238 \pm 33.0^{a,B}$
Pu/SK	$147 \pm 16.2^{c,A}$	$104 \pm 5.26^{a,A}$	$114 \pm 5.89^{ab,A}$	$109 \pm 6.15^{ab,A}$	$118 \pm 10.8^{b,A}$
Be/HR	$362 \pm 22.4^{c,C}$	$281 \pm 52.7^{a,C}$	$344 \pm 7.55^{bc,B}$	$302 \pm 5.97^{a,C}$	$330 \pm 4.93^{b,C}$
Pu/HR	$468 \pm 26.1^{c,D}$	$441 \pm 17.0^{b,D}$	$414 \pm 8.55^{a,C}$	$414 \pm 6.26^{a,D}$	$470 \pm 11.1^{c,D}$
Neochlorogenic acid (neo-CGA)					
Be/SK	$6.23 \pm 0.268^{d,C}$	$3.29 \pm 0.107^{a,D}$	$5.38 \pm 0.143^{c,C}$	$4.51 \pm 0.152^{b,C}$	$3.29 \pm 0.098^{a,C}$
Pu/SK	$7.26 \pm 0.164^{d,D}$	$2.60 \pm 0.326^{a,C}$	$5.35 \pm 0.099^{c,C}$	$6.31 \pm 0.150^{d,D}$	$4.31 \pm 0.076^{b,D}$
Be/HR	$3.30 \pm 0.103^{d,B}$	$1.25 \pm 0.069^{a,B}$	$2.13 \pm 0.060^{b,B}$	$2.27 \pm 0.065^{c,B}$	$2.17 \pm 0.092^{b,B}$
Pu/HR	$0.768 \pm 0.055^{e,A}$	$0.235 \pm 0.033^{a,A}$	$0.710 \pm 0.050^{d,A}$	$0.488 \pm 0.048^{c,A}$	$0.413 \pm 0.015^{b,A}$
trans-Ferulic acid (tFA)					
Be/SK	$8.58 \pm 0.120^{d,C}$	$7.28 \pm 0.021^{a,C}$	$8.33 \pm 0.022^{c,C}$	$8.18 \pm 0.066^{b,C}$	$7.31 \pm 0.061^{a,C}$
Pu/SK	$10.4 \pm 0.155^{d,D}$	$7.86 \pm 0.111^{a,D}$	$9.68 \pm 0.129^{c,D}$	$9.55 \pm 0.221^{c,D}$	$8.13 \pm 0.087^{b,D}$
Be/HR	$5.85 \pm 0.078^{e,B}$	$4.14 \pm 0.066^{a,B}$	$4.91 \pm 0.026^{c,B}$	$5.63 \pm 0.083^{d,B}$	$4.64 \pm 0.144^{b,B}$
Pu/HR	$3.30 \pm 0.044^{e,A}$	$4.14 \pm 0.066^{a,B}$	$3.24 \pm 0.023^{d,A}$	$3.17 \pm 0.025^{c,A}$	$2.96 \pm 0.040^{b,A}$

The values in the row marked with different lowercase letters are significantly different ($p < 0.05$); the values in the column marked with different uppercase letters are significantly different ($p < 0.05$).

and 3.30 mg/kg). These differences confirm not only the influence of variety but also the influence of growing conditions (Table 6).

The heat treatment of SP had a negative effect on the content of phenolic acids. The most considerable decrease of CGA content was recorded in the variety of Pu/SK (boiled SP: −29%). In the variety Pu/HR, the CGA content in the microwaved SP was comparable to raw SP and in the variety Be/SK (steamed SP), the CGA content was 3% higher than in raw SP. However, these differences are not statistically significant and it can not be clearly stated which method of heat treatment has the least/most significant effect on the CGA content. In the case of the other two phenolic acids, cooking in the water had the greatest effect on their content. The neo-CGA content in boiled SP decreased by 47% (64, 62, and 69) in the variety Be/SK (Pu/SK, Be/HR, and Pu/HR, respectively). The largest reduction of tFA acid content was in the Be/HR variety (−29%). In general, its content in SP decreased in the order: raw > steamed > microwaved > baked > boiled. A similar effect of heat treatment is reported by [57]. Home cooking methods of SP caused a significant loss of CGA and ferulic acid. The greatest decrease of CGA content compared to raw SP (463.7 mg/100 g DW) was observed after baking (38.52%), followed by boiling and cooking in the microwave oven (21.04% and 20.01%, resp.) while cooking in steam caused only a slight increase ($p < 0.05$). Compared to ferulic acid content determined in raw SP (24.36 mg/100 g DW), steaming and microwaving caused only a slight change in its content, while other cooking methods induced a significant decrease ($p < 0.05$). M. S. Padda [39] reported a significant decrease in CGA content after processed sweet potato pith tissue. Its content (mg/g DW) decreased as a result of heat treatment in the order: raw (0.46) > microwaved (0.34) > baked (0.26) > boiled (0.20). The authors of [28] evaluated the effect of heat treatment with distilled water and methanol A on several phenolic acids, e.g., vanillic acid, protocatechuic acid, gallic acid, chlorogenic acid, and caffeic acid. After heat treatment with distilled water, as protocatechuic acid and chlorogenic acid content decreased, on the contrary, the content of minor phenolic acids of SP—vanillic acid, gallic acid, and caffeic acid—increased. Paper [58] reported the most significant reduction of CGA content in boiled SP and the smallest in microwaved SP (raw 45.7, microwaved 33.9, boiled 19.7, and baked 26.5 mg/100 g DW). In contrast, [41] reported a significant increase in CGA content in SP after steaming (7.99 ± 0.45 mg/g DW) and boiling (5.16 ± 0.18 mg/g DW) compared to untreated SP (2.34 ± 0.09 mg/g DW). F. Rautenbach et al. [59] determined 28.7 and 47.1% higher CGA content in boiled SP of variety Resisto and W-119 compared to raw SP (69.6 ± 4.8, 89.3 ± 5.4 μg/g FW). Increased contents of CGA and neo-CGA after different cooking methods were determined in three potato cultivars (Bintje, Piccolo, and Purple Majesty) by [60]. The higher CGA content was in baked potatoes and decreased in the order steamed > boiled > microwaved > raw. The neo-CGA content decreased in the order boiled > steamed > baked > microwaved > raw. L. E. Steed and V.-D. Truong [22]

determined 5.1–9.3 (raw flesh) and 10.6–16.2 (cooked flesh) mg CGA/100 g in three SP varieties. They also report higher contents of other phenolic acids (CQA, 4,5-di-CQA, 3,5-di-CQA, and 3,4-di-CQA) due to cooking. They hypothesize that these changes in the content of individual phenolic acids in SP affect the antioxidant properties, including ABTS and DPPH radical scavenging activities. It can be assumed that these changes in the content of individual phenolic acids in sweet potatoes according to cultivar and heat treatment conditions affect the antioxidant properties, including DPPH radical scavenging activities [28].

4. Conclusions

Based on obtained results, it can be stated that specific methods of SP treatment differently affected the content of bioactive compounds such as polyphenols, phenolic acids, and anthocyanins and their antioxidant effects. Due to boiling, steaming, microwaving, and baking, the content of polyphenolic and anthocyanins and also antioxidant activity increased almost in all samples compared to raw sweet potatoes. In contrast, the content of CGA, neo-CGA, and tFA was lower in heat-treated than in raw sweet potatoes. In addition to degradation, their losses are caused by transport from tissue to the surrounding water and also by the type of phenolic acid. It is not possible to determine which method of heat treatment is the gentlest for the preservation of bioactive substances in sweet potatoes.

Data Availability

All basic data supporting the results of this study are available from the corresponding author.

Conflicts of Interest

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

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Research Article

Antioxidant Activity of *Lactobacillus plantarum* DY-1 Fermented Wheat Germ Extract and Its Influence on Lipid Oxidation and Texture Properties of Emulsified Sausages

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The nutrient compositions and in vitro antioxidant activities of water-soluble extract from *Lactobacillus plantarum* DY-1 fermented wheat germ and its effect on the lipid oxidation and texture properties of emulsified sausages were investigated. The optimal hydroxyl radical scavenging capacity of 72.8 ± 2.9% was demonstrated for fermented wheat germ extract (FWGE) by terms of the fermentation conditions as follows: fermentation time of 26 h, fermentation temperature of 35°C, initial pH of 3.0, solid to liquid ratio of 1/10, and inoculum amount of 0.48 g. The enhancement in FWGE content could improve the oxidation stability of emulsified sausages by retarding the formation of thiobarbituric acid-reactive substances (TBARSs) during 7 days of storage at 4°C. However, a higher FWGE content (2.14%) resulted in 78% of increase in cooking loss ($p < 0.05$) and 41.4% of decrease in hardness ($p < 0.05$) of emulsified sausages. It was suggested that the biotransformation of wheat germ with lactic acid bacteria could improve its nutritional quality and functional properties.

1. Introduction

Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butylhydroquinone (TBHQ) have widely been used in retarding lipid oxidation, their safety has been questioned due to toxicity and possible carcinogenicity [1]. Thus, development of natural antioxidants has been of interest. Peptides, flavonoids, and other phenolic compounds from natural sources have been carried out to be potential natural antioxidants in food industry [2–6]. As cereals are good source of protein and phenolic compounds, utilizing the ingredients in cereals as the natural antioxidants was a concern for many researchers [7–9]. However, the compounds with potential antioxidant capacities in cereals are usually bounded into the matrix of cereals [10]. This hinders their antioxidant capacities. To improve the antioxidant properties of cereals, microbial bioprocessing was used to release antioxidant peptides and phenolic compounds from cereals [11–13].

Wheat germ, which is the by-product of flour milling industry, contains a lot of components with a high nutritional value. After extracting the wheat germ oil, the defatted wheat germ still contains components including protein and phenolic compounds. Both peptides released from wheat germ protein by commercial enzyme [14] and phenolic compounds [15] extracted from wheat germ have showed antioxidant activities. However, these studies concerned on only peptides or phenolic compounds. Little has been done to take use of both protein and phenolic compounds. And combining the antioxidant peptides and phenolic compounds has shown to improve the oxidation stability of food emulsions [16].

Microbial biotransformation using *Bacillus subtilis* or *Aspergillus oryzae* has been turn out to be able to convert the protein to peptides [17] and release phenolic compounds [18] from wheat germ. The antioxidant activities of wheat

germ fermentation were facilitated by fermentation. And the fermented wheat germ was able to improve the quality of bread [19]. However, few studies have been done on using lactic acid bacteria fermentation to improve the antioxidant activities of wheat germ. Also, using the fermented products as a functional food ingredient to retard lipid oxidation of food is rare.

The objective of present study was to optimize the antioxidant properties of water-soluble extract from fermented defatted wheat germ prepared with the lactic acid bacteria of *Lactobacillus plantarum* DY-1 and assess its role on lipid oxidation of emulsified sausages.

2. Materials and Methods

2.1. Materials. *Lactobacillus plantarum* DY-1 direct-vat-starter (DVS) with a cell density of 10^{10} cfu/g was screened and prepared by our group [20]. Defatted wheat germ power was purchased from Mantianxue Co. (Henan, China). Ferrozine was purchased from Sigma Chemical Co. (St. Louis, USA). Fresh pork and pork fat were purchased from local supermarket. All other reagents were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2. Experiment Design and Fermentation. Defatted wheat germs were dispersed into 100 mL of citrate buffer in 250 mL conical flasks, followed by mixing with *Lactobacillus plantarum* DY-1 DVS. After sealing with gauze, the defatted wheat germs were fermented in the shaker at the speed of 120 r/min. Uniform experimental design [21] with five factors and 12 of runs was used to analyze the fermentation parameters by terms of chemical compositions and antioxidant activities, as shown in Table 1. The fermentation parameters including fermentation time (X_1), temperature (X_2), pH (X_3), ratio of solid to liquid (X_4), and inoculation amount (X_5) were evaluated. The experiments were carried out in random order. And the response functions including soluble protein content (Y_1), total phenolic content (Y_2), total sugar content (Y_3), reducing sugar content (Y_4), Fe^{2+} chelating activity (Y_5), hydroxyl free radical scavenging capacity (Y_6), and superoxide anion radical scavenging capacity (Y_7) of fermented wheat germ extract (FWGE) were assessed.

The relationship between independent variables and responses was expressed with second-order polynomial regression model as follows:

$$Y = \beta_0 = \sum_{i-1}^{m} \beta_i X_i + \sum_{i-1}^{m} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j + e, \quad (1)$$

where Y is the response variable; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients; and X_i and X_j are the independent variables.

2.3. Preparation of Fermented Wheat Germ Extract (FWGE). Fermented wheat germs were centrifuged at 8000 g for 15 min by Avanti J-26S (Beckman Coulter, Inc., CA, USA). The supernatants were collected and freeze-dried for 48 h. The lyophilized water-soluble fermented wheat germ extracts were stored at −20°C before further analysis.

2.4. Radical Scavenging Activities of FWGE

2.4.1. Superoxide Anion Radical Scavenging Activity. The superoxide anion scavenging activity of FWGE solutions was determined by the method of Tang et al. [22] and the methods description partly reproduces their wording. The mixture of 1 mL FWGE (2 mg/mL) and 1.8 mL of Tris-HCl buffer (50 mM, pH 8.2) was incubated at 25°C for 10 min, followed by adding 0.1 mL of pyrogallol solution (10 mM). The absorbance of solution was recorded every 15 s in 3 min at the wavelength of 320 nm. The slope of the linear regression equation according to the absorbance and time was used to indicate the oxidation rate of pyrogallol (V_s). Deionized water was used as the control to determine the autoxidation rate of pyrogallol (V_0). The superoxide anion radical scavenging activity was expressed as $[(V_0 - V_s)/V_0] \times 100\%$.

2.4.2. Hydroxyl Radical Scavenging Activity. Hydroxyl radical scavenging activity of FWGE solutions was measured according to the method of Li et al. [23] and the methods description partly reproduces their wording. One milliliter of 200 mM phosphate buffer (pH 7.4) was mixed with 0.5 mL of 1,10-phenanthroline (0.75 mM) and 0.5 mL of $FeSO_4$ (0.75 mM), followed by adding 0.5 mL of H_2O_2 (0.01%) and 0.5 mL of FWGE (20 mg/mL). After holding at 37°C for 60 min, the absorbance of the mixture was recorded at 536 nm. The hydroxyl radical scavenging activity was expressed as $[(A_s - A_1)/(A_0 - A_1)] \times 100\%$, where As is the absorbance value of the samples; A_1 is the absorbance value of control solution containing 1,10-phenanthroline, $FeSO_4$, and H_2O_2; and A_0 is the absorbance value of blank solution containing 1,10-phenanthroline and $FeSO_4$.

2.5. Fe2+ Chelating Activity. Ferrous metal ion chelating activity of FWGE solutions was determined according to the method of Cheng et al. [24] and the methods description partly reproduces their wording. Two milliliters of ferrozine (500 μM) was mixed with 1 mL of $FeCl_2$ (20 μM), followed by adding 0.5 mL of FWGE solutions (8 mg/mL). The absorbance of the mixture was recorded at 562 nm (A_s) after 10 min incubation. Deionized water was used as the control to determine the absorbance of the mixture (A_0). The Fe^{2+} chelating activity was calculated as $[(A_0 - A_s)/A_0] \times 100\%$.

2.6. Chemical Analysis of FWGE. The content of total phenols was evaluated using in FWGE Folin–Ciocalteu method, as described by Zhang et al. [20]. The content of soluble protein, total sugar, and reducing sugar was determined by the biuret method [25], anthrone-sulfuric acid colorimetric method, and dinitrosalicylic acid method [26], respectively.

Table 1: Uniform design for lactic acid bacteria fermentation of wheat germ.

Run	Independent variables (coded and uncoded)					Responses						
	X_1	X_2	X_3	X_4	X_5	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7
1	(1) 6	(3) 25	(4) 3.5	(9) 1/8	(12) 0.6	364.7	5.8	403.5	162. 7	18.9	16.4	19.1
2	(2) 6	(6) 30	(8) 4.5	(5) 1/6	(11) 0.6	275.0	6.4	583.7	115.5	7.5	53.4	10.4
3	(3) 12	(9) 40	(12) 5.5	(1) 1/2	(10) 0.5	304.9	6.5	404.3	190.0	12.1	33.5	9.0
4	(4) 12	(12) 45	(3) 3.5	(10) 1/8	(9) 0.5	265.6	9.0	604.6	126.5	2.3	27.8	2.0
5	(5) 18	(2) 20	(7) 4.5	(6) 1/6	(8) 0.4	273.3	7.4	587.7	137.5	14.7	24.9	6.1
6	(6) 18	(5) 30	(11) 5.5	(2) 1/2	(7) 0.4	333.5	6.9	480.7	158.9	5.5	32.1	5.2
7	(7) 24	(8) 35	(2) 3.0	(11) 1/10	(6) 0.3	345.9	9.7	364.9	157.3	1.4	68.9	28.6
8	(8) 24	(11) 45	(6) 4.0	(7) 1/7	(5) 0.3	375.1	8.0	331.1	152.7	25.9	26.8	13.2
9	(9) 30	(1) 20	(10) 5.0	(3) 1/4	(4) 0.2	244.1	8.9	526.6	151.8	23.9	65.0	10.7
10	(10) 30	(4) 25	(1) 3.0	(12) 1/10	(3) 0.2	347.9	10.5	615.1	55.7	0.2	58.7	17.6
11	(11) 36	(7) 35	(5) 4.0	(8) 1/7	(2) 0.1	224.9	8.1	611.9	123.9	7.0	12.3	1.6
12	(12) 36	(10) 40	(9) 5.0	(4) 1/4	(1) 0.1	291.1	9.0	582.1	54.0	2.2	17.6	6.0
Control	Unfermented wheat germ					154.6	3.4	845.0	36.0	64.4	39.5	−29.6

$X1$: fermentation time (h); X_2: fermentation temperature (°C); X_3: initial pH; X_4: solid/liquid ratio (g/mL); X_5: inoculum amount (g); Y_1: soluble protein content (mg/g); Y_2: total phenolic content (mg/g); Y_3: total sugar content (mg/g); Y_4: reducing sugar content (mg/g); Y_5: Fe^{2+} chelating activity (%); Y_6: hydroxyl radical scavenging capacity (%); Y_7: superoxide anion scavenging capacity (%).

2.7. Preparation of Emulsified Sausages. The emulsified sausages were prepared as described by Nieto et al. [27] and the methods description partly reproduced their wording. Lean and pork fat were minced through 5 mm and 3 mm plate, respectively, using a meat grinder. The grounded lean and fat were mixed with crushed ice, FWGE, chilled water (2°C), and salt according to the formula in Table 2. The fat in emulsified sausages was replaced with 0, 2%, 5%, 7%, and 10% of FWGE. After being chopped for 5 min to form meat emulsions, the mixture was stuffed into manufactured collagen casings with the diameter of 29 mm by a stuffer (MGJ-090, Demas Network Technology Co., Ltd, Foshan, China). The samples were then tied and cut into sausages with the length of 10 cm by hand. The sausages were heated in a steamer until their central temperature was kept at 80 ± 2°C for 20 min. The central temperature of sausages was measured with thermocouple thermometer. The cooked sausages were cooled with cold water, sealed in polyethylene bags using vacuum packing, and maintained at 4°C for 7 days to estimate the oxidation stability.

2.8. Cooking Loss. The emulsified sausages were weighed before the heating process and after the cooling process. The cooking loss was expressed as the weight loss between initial (W_0) and final (W_f) weight of the sausages and calculated as $[(W_0 - W_f)/W_0] \times 100\%$.

2.9. Thiobarbituric Acid-Reactive Substances (TBARSs). The method of Nieto et al. [27] was used to assess the lipid oxidation of sausages and the methods description partly reproduced their wording. TBARS value was determined by the following equation: TBARS (mg/kg) = $(A_{532}/W_s) \times 9.48$, where A_{532} was the absorbance of the red TBA reaction product at 532 nm, W_s was the sausage sample weight (g), and 9.48 was a constant derived from the dilution factor and the molar extinction coefficient ($1.52 \times 10^3\ M^{-1} \cdot cm^{-1}$) of the red TBA reaction product.

Table 2: Formulation of emulsified sausages.

Formula	Treatment (fat replacement ratio with FWGE)				
	0%	2%	5%	7%	10%
Fat (g)	107	104.86	101.65	99.51	96.3
Lean (g)	249.5	249.5	249.5	249.5	249.5
FWGE (g)	0	2.14	5.35	7.49	10.7
Water (g)	86	86	86	86	86
Ice (g)	50	50	50	50	50
NaCl (g)	7.5	7.5	7.5	7.5	7.5
Total (g)	500	500	500	500	500

2.10. Texture Profile Analysis (TPA). TPA was performed at room temperature using a texture analyzer (TA-XT Plus, Stable Micro Systems Ltd., Surrey, UK) with the compression distance of 10.0 mm using P50 probe. The pretest speed, test speed, and posttest speed were 2.0, 1.0, and 1.0 mm/s, respectively, as described by Cheng et al. [28]. All sausage samples were stripped of the casings and cut into cylinders with the length of 20 mm before analysis.

2.11. Statistical Analyses. All assays were done in triplicate. Two different batches of raw materials were used to prepare emulsified sausages at different days. The data were analyzed using DPS statistical software (V9.5, Hangzhou, China) [29] in one-way analysis of variance (ANOVA). The differences between means were compared with the LSD test, and significant difference was considered when $p < 0.05$. Uniform design and its regression model assays were also carried out with DPS statistical software.

3. Results and Discussion

3.1. Composition Analysis of FWGE. The chemical compositions of FWGE at different fermentation conditions by terms of uniform design are listed in Table 1. Fermentation with *Lactobacillus plantarum* DY-1 increased the content of soluble protein, total phenolic, and reducing sugar while

resulted in decrease in the content of total sugar. These results can be explained by the fact that levels of bioactive compounds can be modified by the biotransformation activity of microbes. Also, fermentation might result in breakdown of cell walls in wheat germ cells and release their endogenous enzymes. Enzymes such as amylases, xylanases, and proteases derived from the microbes and cereals might contribute to the conversion of compositions in cell of cereals [30]. The metabolism of *Lactobacillus plantarum* DY-1 could lead to the liberation and/or synthesis of various bioactive compounds. It was demonstrated that fermentation has a positive influence on total phenolic content and antioxidant activity of cereals [12, 14]. And the degree of influence depended on microorganism species [31]. Our result was consisted with previous research of Zhang et al. [20] that total phenolic content was enhanced by fermentation with *Lactobacillus plantarum* DY-1.

3.2. Antioxidant Activities of FWGE. The results of antioxidant activities (Table 1) showed that fermentation improved the superoxide anion radical scavenging activity of wheat germ water extracts while weakened their Fe^{2+} chelating activity. And the hydroxyl radical scavenging activity could be enhanced at the selected fermentation conditions.

The Pearson correlation was used to display the relation between chemical compositions and antioxidant activities of FWGE. As shown in Table 3, soluble protein content of FWGE was in significant positive correlation with its superoxide anion radical scavenging activity. Endogenous proteases of wheat germ and proteases from DY-1 could hydrolyze the wheat germ protein into protein hydrolysate. This would result in improvement of the solubility of wheat germ protein. It has been showed by many research studies that protein hydrolysates exhibit higher antioxidant capacities than their original proteins. However, it was strange that the total phenolic content of FWGE was not significantly correlative with their antioxidant capacities. It suggested that the wheat germ protein hydrolysate might contribute more to their antioxidant activities. It was interesting that the soluble protein content of FWGE showed significant negative correlation with its total sugar content. It seemed that wheat germ protein was bounded with polysaccharides in wheat germ. Fermentation released the wheat germ protein bounded from the matrix of polysaccharides.

3.3. Optimizing Fermentation Conditions. To optimize the fermentation condition for better antioxidant activities, quadratic polynomial regression equation was used to express the relationship between fermentation conditions and antioxidant activities of FWGE. The details of regression equations are shown in Table 4. Since only the model for regression equation of hydroxyl radical scavenging activity was significant, it was used for further analysis. The Durbin–Watson value of 1.96 for that model was close to 2. And the R^2 of the model was 0.9997. All the results indicated that regression equation of hydroxyl radical scavenging activity was able to well predict the change in hydroxyl radical scavenging activity by terms of fermentation parameters. The predicted model could be described as follows:

$$Y_1 = -1628.8 + 3324.6X_3 - 71455.4X_4 + +3.0X_1 * X_4 - 5.4X_1 * X_5 - 1.34X_2 * X_3 + 4.6X_2 * X_5 + 17215.0X_3 * X_4 + 0.058X_2 * X_2 - 656.8X_3 * X_3 - 32971.6X_4 * X_4. \quad (2)$$

Based on the regression model, an optimum condition of fermentation was demonstrated as follows: fermentation time of 26 h, fermentation temperature of 35°C, initial pH of 3.0, solid to liquid ratio of 1/10, and inoculum amount of 0.48 g. The verification experiment at the above conditions

Table 3: Pearson correlation of chemical compositions and antioxidant capacities of FWGE.

	$y1$	$y2$	$y3$	$y4$	$y5$	$y6$	$y7$
$y1$	1	−0.044	−0.693*	0.042	0.099	0.088	0.669*
$y2$		1	0.277	−0.536	−0.414	0.441	0.213
$y3$			1	−0.662*	−0.465	−0.081	−0.582*
$y4$				1	0.495	0.075	0.055
$y5$					1	−0.118	0.031
$y6$						1	0.585*
$y7$							1

*Significant at $p < 0.05$. Y_1: soluble protein content (mg/g); Y_2: total phenolic content (mg/g); Y_3: total sugar content (mg/g); Y_4: reducing sugar content (mg/g); Y_5: Fe^{2+} chelating activity (%); Y_6: ·OH scavenging capacity (%); Y_7: $^{\cdot}O_2^-$ scavenging capacity (%).

Table 4: Significance of regression coefficients and regression equations for antioxidant activities of lactic acid bacteria fermented wheat germ extract.

Coefficients	Y_5	Y_6	Y_7
β_0	−1893.5	−1628.8	73.5
β_2	22.4*		
β_3		3324.6*	−76.4*
β_4		−71455.4*	
β_5	−66.1		171.4*
β_{12}			0.112*
β_{14}	654.5*	3.0	
β_{15}		−5.39*	
β_{23}		−1.34*	−0.138
β_{24}	26.4*		−9.78*
β_{25}		4.64*	
β_{34}	−4257.8*	17215.0*	346.6*
β_{35}	378.8*		−28.8*
β_{45}	37154.6*		1389.7*
β_2^2	−0.43*	0.058*	
β_3^2		−656.8*	
β_4^2	−3782.8*	−32971.6*	−3083.8*
β_5^2	−1585.6*		−34.5
R^2 of model	0.998	0.9997	0.997
Adjusted R^2	0.976	0.997	0.973
Significant of model	0.114	0.040	0.121
Durbin–Watson	2.11	1.96	2.32

*Significant at $p < 0.05$. Y_5: Fe^{2+} chelating activity (%); Y_6: ·OH scavenging capacity (%); Y_7: $^{\cdot}O_2^-$ scavenging capacity (%).

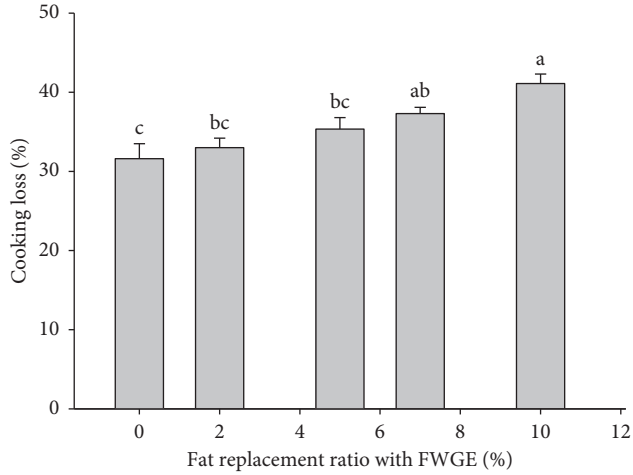

Figure 1: Cooking loss of emulsified sausages with different ratios of fat replacement using FWGE.

Table 5: Texture properties analysis of emulsified sausages with different concentrations of FWGE.

FWGE (%)	Hardness (g)	Resilience (%)	Springiness (%)	Cohesiveness	Chewiness
0	2098.1 ± 161.0^{a}	19.9 ± 2.7^{a}	73.4 ± 2.3^{a}	0.47 ± 0.03^{a}	706.0 ± 62.4^{a}
0.43	2514.5 ± 341.3^{a}	17.7 ± 5.6^{a}	74.2 ± 2.2^{a}	0.44 ± 0.08^{ab}	828.0 ± 237.8^{a}
1.07	2049.0 ± 259.5^{a}	14.1 ± 2.1^{a}	70.4 ± 0.9^{a}	0.38 ± 0.03^{ab}	550.9 ± 113.1^{a}
1.50	1606.8 ± 788.2^{ab}	12.4 ± 4.6^{a}	68.5 ± 1.5^{a}	0.34 ± 0.08^{ab}	358.5 ± 114.2^{a}
2.14	1478.7 ± 203.7^{b}	11.4 ± 1.4^{a}	55.4 ± 16.1^{a}	0.33 ± 0.04^{b}	300.5 ± 111.7^{a}

All values are presented as mean ± standard deviation of three replicates ($n = 2$). [a,b]Means within a column with different superscript letters are significantly different ($p < 0.05$).

was carried out in two replicates. And the hydroxyl radical scavenging capacity and Fe^{2+} chelating capacity of FWGE were $72.8 \pm 2.9\%$ and $20.5 \pm 3.6\%$, respectively. The optimized hydroxyl radical scavenging capacity of FWGE was 1.84 times higher than the wheat germ water-soluble extract.

3.4. Effect of FWGE Content on Cooking Loss. The effect of the replacement of pork fat with FWGE on the cooking loss of the emulsified sausages is shown in Figure 1. The replacement of pork fat at the ratio lower than 5% did not change the cooking loss of the sausages ($p > 0.05$). However, increasing the replacement ratio of 7–10% would lead to enhancement in the cooking loss of sausages ($p < 0.05$). The cooking loss of the sausages with 10% of fat replacement was increased by 1.30 times.

3.5. Effect of FWGE Content on Texture of Emulsified Sausages. As shown in Table 5, addition of FWGE had little effect on the textural properties of emulsified sausages including hardness, resilience, springiness, cohesiveness, and chewiness. Increasing the content of FWGE seemed to reduce the hardness of emulsified sausages. When ten percent of fat was replaced with FWGE, the hardness and cohesiveness of the emulsified sausages were reduced by 41.2 and 42.4% ($p < 0.05$), respectively. The reason was that FWGE might hinder the formation of protein gel network of emulsified sausages and weakened the gel properties of them. Emulsified sausage is a model of emulsion filled myofibrillar protein gel. The hardness of emulsified sausage is usually influenced by the filler of oil droplet and the matrix of protein gel network. As FWGE was not able to form the viscoelastic particles, the replacement of oil with FWGE might reduce the viscoelastic property of the filler. This might result in the weak hardness of the sausages. The phenols in the FWGE might combine with myofibrillar protein and prevent its aggregation. This might lead to destruction in the formation of myofibrillar protein network. Also, the free amino acid released might have negative effect on the texture of protein gel [32, 33]. Thus, the hardness and cohesiveness of the emulsified sausages might decrease.

3.6. Effect of FWGE Content on Lipid Oxidation of Emulsified Sausages. FWGE was able to retard lipid oxidation of emulsified due to its antioxidant activities. The emulsified sausages with FWGE demonstrated a higher oxidation stability than that without FWGE. The effect of the FWGE on retarding lipid oxidation of emulsified sausages during 7 days of storage at 4°C is shown in Figure 2. At 0 days of storage, the formation of TBARS was inhibited by 36.4 and 78.3% at the FWGE content of 1.07 and 2.14%, respectively ($p < 0.05$). At 7^{th} days of storage, the formation of TBARS in the emulsified sausages with 1.07 and 2.14% of FWGE decreased by 27.6 and 48.6% ($p < 0.05$).

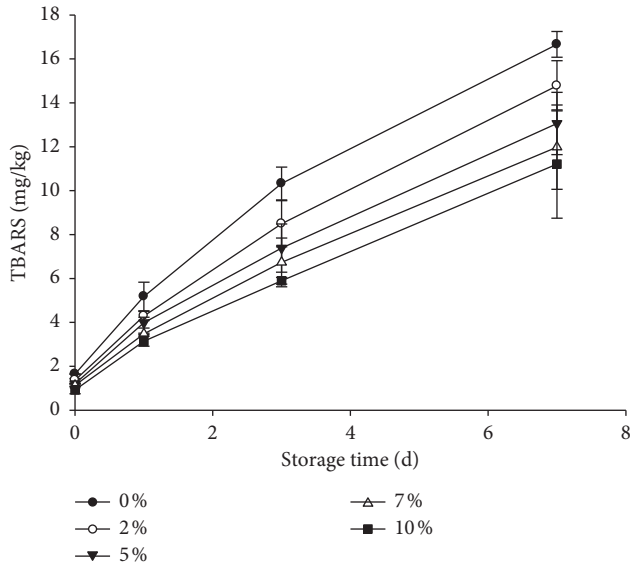

Figure 2: Oxidation stability of emulsified sausages with different ratios of fat replacement using FWGE during 7 days of storage at 4°C.

Although increasing the FWGE content was able to retard the lipid oxidation in emulsified sausages, it leads to increase in cooking loss and hardness of emulsified sausages. When compared with the TBARS formed at 0 days of storage, it increased by 9.1, 9.8, 9.8, 9.6, and 11.1 times at 7 days of storage under the FWGE content of 0.43, 1.07, 1.50, and 2.14%, respectively ($p < 0.05$). It suggested that FWGE was prone to hinder the lipid oxidation at the early stage due to their Fe^{2+} chelating capacity and free radical scavenging capacity. Increasing the content of FWGE, the antioxidant properties of FWGE would enhance due to a higher content of antioxidant wheat germ protein hydrolysate and free polyphenols. They might show a higher Fe^{2+} chelating capacity and free radical scavenging capacity. This might result in diminishing the oxidation factors in the sausages. Our results suggested that coexistence of wheat germ protein hydrolysate and free polyphenols in FWGE might lead to synergic effect on antioxidant activities.

4. Conclusions

The antioxidant properties of defatted wheat germ were improved by fermentation with lactic acid bacteria (*Lactobacillus plantarum* DY-1). The enhancement in the antioxidant properties of fermented wheat germ extract (FWGE) might be due to the increase in their soluble protein content. The optimized fermentation conditions by terms of hydroxyl radical scavenging capacity were as follows: fermentation time of 26 h, fermentation temperature of 35°C, initial pH of 3.0, solid to liquid ratio of 1/10, and inoculum amount of 0.48 g. The optimal hydroxyl radical scavenging capacity of FWGE was 72.8 ± 2.9%. FWGE was able to retard lipid oxidation of emulsified sausage. Replacing pork fat with 5% FWGE resulted in better properties of emulsified sausage. The results suggested that biotransformation with lactic acid bacteria could be a promising way to enhance the bioactive properties of wheat germ. FWGE might have potential application in food industry. Further studies on isolation and purification of efficient antioxidants from FWGEs and the in vivo antioxidant activity tests are needed. And the enzymes involved in the fermentation of wheat germ on accumulation of the bioactive ingredients could be concerned.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Antihyperglycemic, Vasodilator, and Diuretic Activities of Microencapsulated Bioactive Product from *Moringa stenopetala* Leaves Extract

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Moringa stenopetala has nutritional and medicinal values, which is widely used by the local communities. The study aimed to evaluate the antihyperglycemic, vasodilator, and diuretic activities of the microencapsulated bioactive product from *M. stenopetala* leaves extract. Microencapsulation of the extract was done by spray drying technique using maltodextrin and pectin as coating materials with the core: coating ratio of 1 : 6. Then, the antihyperglycemic, diuretic, and vasodilator activities were evaluated after the product was administered to experimental animals at different doses and compared with the control groups. There were no observed physical, behavioral, and physiological changes on the mice during the acute toxicity test. The results also indicated no toxicity signs and death occurrence in the experimental animals up to 5000 mg/kg administered dose. Therefore, microencapsulated *M. stenopetala* leaves extract does not produce adverse effects in experimental mice. The study also showed that the microencapsulated bioactive product exhibited significant antihyperglycemic, vasodilator, and diuretic activities as the doses increase. Therefore, the study showed that microencapsulated bioactive product has significant medicinal values. Further detailed studies are recommended on chronic toxicity tests and to understand the possible mechanism of actions on the antihyperglycemic, vasodilator, and diuretic activities of the microencapsulated product.

1. Introduction

Moringa stenopetala plant has biologically active compounds that have beneficial medicinal attributes in addition to food sources and feed. Traditional treatment using different plant extracts is undertaken for many diseases and health disorders, in particular, in the developing countries [1]. This is due to the presence of various pharmacological active compounds in the plants. About thirty percent of the marketed drugs contain compounds derived from the plants [2]. Although there are many modern antihypertensive medicines, many peoples prefer to use traditional medicines [3]. *M. stenopetala* is a plant that has medicinal and nutritional importance for the community and reported to have antihypertensive effects [4] and antidiabetic activity [5,6]. Similarly, moringa extract also showed hepato- and kidney protective effects due to its protective action against lipid peroxidation and reactive oxygen species [7]. Furthermore, the toxicity and the medicinal values of the *M. stenopetala* leaves extract have been evaluated using experiments in animal models. The toxicity test of the extract was declared as it does not show toxic signs [2,8].

Phenolic and flavonoid compounds are the major constituents reported from aqueous and alcoholic extracts of

M. stenopetala leaves [9,10]. These constituents exhibit higher antioxidant activity and are claimed to be responsible for a number of biological activities [11–13]. However, these bioactive constituents are significantly reduced during plant material processing [14,15] and storage [16]. Encapsulation of medicinal plant extracts can be considered as an option to improve the stability of bioactive compounds during processing and storage. Microencapsulation is a technology for packaging of extracts and thereof active ingredients in microparticle matrices [17]. The wall material forms a physical barrier between the active compounds and the external environment and controls the release of the active compounds [18]. Furthermore, it masks the bitter taste and odor of active extracts or compounds [17]. Considering the medicinal importance of *M. stenopetala,* the leaves extract was formulated into microencapsulate using spray drying technology. This study, therefore, aimed to evaluate the antihyperglycemic, diuretic, and vasodilator activities of the microencapsulated product of the extract of *M. stenopetala* leaves.

2. Materials and Methods

2.1. Chemicals and Reagents. All chemicals and reagents were of analytical grades. Maltodextrin (Eisse Food Co. Ltd., Korea), high methoxyl pectin (RS-400, Danisco Inc., USA), glacial acetic acid (Labort Fine Chemicals, India), glibenclamide (Remedica, Cyprus), ethanol (WINLAB, UK), D-glucose anhydrous and sodium hydrogen carbonate (Eurostar Scientific Ltd., Liverpool, UK), streptozotocin and potassium phosphate monobasic (Sigma Aldrich, St. Louis, USA), potassium chloride (Park Scientific Ltd., Northampton, UK), calcium chloride (Allied Chemical, New Jersey, USA), magnesium chloride (lot no. 86F-3524, Sigma Chemical Company, St. Louis, USA), magnesium sulfate (BDH Lab Chem Division, UK), sodium chloride (Riedel-de Haen, Germany), and furosemide (ERIS Pharma, Australia) were used in the study.

2.2. Microencapsulated Product Development. The coating material solutions were prepared according to the method described by Dadi et al. [19]. Spray-dried microencapsulated product of *M. stenopetala* leaves extract was developed using maltodextrin (9%) and pectin (1%) as a composite coating material with a core: coating ratio of 1 : 6 and an inlet air temperature of the spray dryer at 140°C. The product was then packed in the aluminum package to protect it from environmental contacts and stored in the refrigerator at 4°C until it is used for the experiment.

2.3. Experimental Animal Preparation. The experimental animals were bred at the Ethiopian Public Health Institute. All animals were used under controlled laboratory conditions of temperature (22 ± 2°C), humidity, and 12 h day and 12 h night and had free access to a standard pellet diet and water *ad libitum*. The institutional ethical review board ethically approved the studies. The animals were treated throughout the study period according to the guideline for the use and care of animals [20].

2.4. Acute Toxicity Test. The experiment was performed on adult, healthy female Swiss albino mice with a weight range of 25–30 g. The acute toxicity of microencapsulated product from *M. stenopetala* leaves extract was evaluated as follows: the mice were divided into seven groups and five mice were allocated in each group per cage. The animals had fastened for 4–5 h before taking the initial weight measurement. The administration was done orally with single doses of the microencapsulated product which was dissolved in distilled water at different doses (500, 1000, 1500, 2000, and 5000 mg/kg) for five treatment groups and two control groups (coating material for the first group and distilled water for the second group).

2.5. In Vivo Antihyperglycemic Test. Diabetic induction of the mice was done according to the method described by Furman [21]. Briefly, before inducing the streptozotocin (STZ), the animals fasted for 4 h for which only water was provided as normal. Then, a single-dose STZ solution was freshly prepared in citrate buffer (pH = 4.5) at a concentration of 200 mg/kg of the body weight of the mice. The STZ solution was induced using intraperitoneal (IP) injection. Five days after STZ injection, the animals were fastened for 6 h and the blood was taken from the tail vein to measure the glucose level using glucometer (Prodigy, OK Biotech, Hsinchu, Taiwan). Then, the mice were considered as diabetic when their blood glucose level was read as > 150 mg/dl. The antihyperglycemic mice were grouped into six groups, and five mice were put in each cage. The microencapsulated product and the standard drug (glibenclamide) were administered orally using gavage. Groups 1, 2, and 3 were considered as treatment groups which were administered the microencapsulates at the dose of 500, 750, and 1000 mg/kg, respectively. Group 4 was administered the standard drug, glibenclamide, at the dose of 5 mg/kg which was considered as a positive control group. Group 5 was considered as a negative control group in which diabetic mice stayed without treatment. Group 6 was administered distilled water (0.5 mL/100 g) which was considered as a normal control group. The administration was done for 21 days. The body weight and the fastened blood glucose level were measured on the 1st, 7th, 14th, and 21st days.

2.6. In Vitro Vasodilator Activity Test. The *in vitro* vasodilatory activity of the microencapsulated product of *M. stenopetala* leaves extract was done using isolated guinea pig thoracic aorta according to the methods described by Vogel [22]. The guinea pig was sacrificed by gentle cervical dislocation and the thoracic cavity was opened to identify the aorta. The descending thoracic aorta was then taken and placed in Krebs physiological solution maintained at 37°C. Excess adherent fat and connective tissues were trimmed off and cleaned. The aorta was cut spirally using a heparinized capillary tube with plastic sealing and a strip of about 2 mm

wide and 4 cm long using a heparinized capillary tube with plastic sealing. The strip was immediately mounted in an organ bath containing 30 mL Krebs-Henseleit physiological solution. The strip was attached to isometric transducers connected to a polygraph and a resting tension of 1 g was applied to strip. The aorta ring was mounted under this resting tension onto two L shaped stainless steel wire hooks gently inserted into the lumen to avoid damage to the endothelium in Krebs-Henseleit solution and allowed to stabilize for about an hour before commencing an experiment. The physiological solution was allowed to pass through a warm water jacket to maintain its temperature and continuous aeration with carbogen (95% O_2 + 5% CO_2 gas mixture) at a pH of 7.4. The pH of the buffer was checked every 60 minutes of aeration with carbogen.

After the aortic strip equilibration period of 1 h under a resting tension of 1 g, tissue viability was confirmed by adding 80 mM KCl. After equilibration and tissue viability was checked, contraction of the thoracic aorta strip was induced by the addition of 80 mM KCl into the organ bath during which period it was washed by overflowing every 15 min. Once a contraction plateau was achieved, microencapsulated product was administered cumulatively every 15 min to assess their capacity of reducing thoracic aorta strip contraction induced, and tension responses of the tissue were recorded isometrically with Grass transducers and displayed on a Grass model 7 polygraph. At the end of the experiment, the percent relaxation was determined before and after extract administration. Relaxation, a measure of inhibition of contraction in aorta ring precontracted with a contracting agent, was measured in percentage and calculated using

$$\%\ \text{relaxation}\ (R_{\max}) = \frac{T_c - T_t}{T_c} * 100, \quad (1)$$

where T_c stands for tension in Newton due to contracting agents, while T_t stands for change in tension in Newton after adding encapsulates at different dose level.

2.7. In Vivo Diuretic Activity Test. The diuretic activity of the microencapsulated product was evaluated according to the method described by Vogel [22]. The Wisteria male rats with the body weight in the range of 204–254 g were taken and acclimatized for 7 days. They were kept in the cage with free access to water and standard feed in pellet form. The rats were grouped into five groups; within each cage, six rats were put. Group 1 is a positive control group which was provided furosemide standard drug, group two is negative control group which was provided a saline solution, and the rest three groups were treatment groups in which different doses (500, 750, and 1000 mg/kg) of the microencapsulated products of *M. stenopetala* leaves extract were provided. The rats were fastened overnight prior to administration. The sample and the standard drug were dissolved in 0.9% saline solution in the form of 5 mL/kg of the rat's weight. Then, a normal saline solution (50 mL/kg of the rat's weight) was provided for all rats and kept in the metabolic cage for 8 h. All the administration was done orally using gavage. Then, the urine volume was measured every 2 h. Finally, the electrolyte concentration in the urine was measured and analyzed.

The urine output, urinary excretion, diuretic index, diuretic activity, natriuretic activity, saluretic activity, carbonic anhydrase inhibition, Na^+, K^+, and Cl^- excretion were the parameters determined in order to compare the effects of the test doses of the extract with furosemide on diuresis. The percent of the urinary excretion was calculated as the total urinary output divided by total liquid administered (equation (2)). The ratio of urinary excretion in the test group to urinary excretion in the control group was used as a measure of the diuretic action of the diuretics (equation (3)). The diuretic activity was calculated as the ratio of the diuretic action of the test substances to that of the standard drug (equation (4)):

$$\text{urinary excretion}\,(\%) = \frac{\text{total urine output}\,(\text{ml})}{\text{total liquid administered}\,(\text{ml})} * 100, \quad (2)$$

$$\text{diuretic index} = \frac{\text{urinary excretion of treated group}\,(\text{ml})}{\text{urinary excretion of control group}\,(\text{ml})}, \quad (3)$$

$$\text{diuretic activity} = \frac{\text{diuretic excretion test drug}\,(\text{ml})}{\text{diuretic action of standard drug}\,(\text{ml})}. \quad (4)$$

2.8. Determination of Ion Concentrations in the Excreted Urine. Urinary Na^+, K^+, and Cl^- ions concentrations of the animals (experimental, positive control, and normal control groups) were measured using ion-selective electrodes (ISE) analysis (AVL 9180 Electrolyte Analyzer, Roche, Germany). Dilutions of the urine samples were made as required to bring electrolyte content in the range that can be determined by the electrolyte analyzer.

2.9. Statistical Data Analysis. All the experimental values were expressed as mean ± standard deviation. The results were analyzed using analysis of variance (ANOVA) by JMP software (Version 13.0, 2016, SAS Institute Inc., Cary, NC, USA). Tukey's HSD test was used and the difference was considered at a significance level of $P < 0.05$.

3. Results and Discussion

3.1. Acute Toxicity. The result of the acute toxicity of microencapsulated product from *M. stenopetala* leaves extract indicated that there was no death within 24 hours up to maximum doses (5000 mg/kg body weight). In addition, there was no sign of behavioral and physical changes and also body weight reduction within 14 days. Besides, there were no gross pathological changes in color and size. These overall parameters show that the microencapsulated bioactive product of *M. stenopetala* leaves did not show any visible sign of toxicity. Similar results were found for

Table 1: Effects of the microencapsulated bioactive product of *M. stenopetala* on the body weight of the mice.

Treatment groups	Fastened body weight of the mice (g)			
	1st day	7th day	14th day	21st day
Dose 500 mg/kg	30.03 ± 4.16^{aA}	31.12 ± 4.37^{aAB}	32.84 ± 4.90^{aAB}	33.56 ± 4.92^{aAB}
Dose 750 mg/kg	32.92 ± 3.02^{aA}	32.40 ± 3.40^{aAB}	33.01 ± 3.43^{aAB}	33.12 ± 3.77^{aB}
Dose 1000 mg/kg	33.84 ± 3.07^{aA}	33.56 ± 3.75^{aAB}	32.48 ± 2.76^{aAB}	33.08 ± 2.96^{aB}
Positive control	30.57 ± 2.65^{aA}	31.04 ± 1.89^{aAB}	31.66 ± 2.36^{aAB}	32.06 ± 2.03^{aBC}
Negative control	29.88 ± 1.71^{aA}	28.49 ± 1.09^{abB}	27.34 ± 1.03^{bcB}	26.58 ± 1.72^{cC}
Normal control	34.08 ± 3.99^{aA}	36.10 ± 4.10^{aA}	37.74 ± 3.53^{aA}	39.76 ± 3.46^{aA}

Superscript letters with small letters indicate the presence of significant differences ($P < 0.05$) in a raw (in different days), whereas the capital letters indicate the presence of significant differences in a column (different doses of the microencapsulates and control groups).

Table 2: Antihyperglycemic effects of microencapsulated bioactive product of *M. stenopetala* leaves extract.

Treatment groups	Fastened blood glucose level (mg/dl)			
	1st day	7th day	14th day	21st day
Dose 500 mg/kg	337.60 ± 63.93^{aA}	329.90 ± 65.33^{aB}	319.90 ± 68.30^{aB}	281.80 ± 67.05^{aB}
Dose 750 mg/kg	271.10 ± 35.86^{aA}	257.40 ± 37.60^{aAB}	241.60 ± 37.32^{aAB}	228.20 ± 33.80^{aAB}
Dose 1000 mg/kg	300.20 ± 47.94^{aA}	263.60 ± 29.50^{aAB}	258.90 ± 48.17^{aA}	242.00 ± 47.41^{aAB}
Positive control	291.90 ± 51.95^{aA}	265.70 ± 51.07^{aA}	248.00 ± 49.03^{aA}	221.70 ± 48.08^{aA}
Negative control	223.90 ± 37.59^{b}	316.20 ± 31.68^{a}	330.70 ± 24.11^{a}	347.10 ± 36.06^{a}
Normal control	99.00 ± 5.70^{a}	99.20 ± 6.72^{aC}	98.20 ± 5.54^{aC}	98.20 ± 5.81^{a}

Superscript letters with small letters indicate the presence of significant differences ($P < 0.05$) in a raw (in different days), whereas the capital letters indicate the presence of significant differences in a column (different doses of the microencapsulates and control groups).

unencapsulated aqueous and ethanol extracts of *M. stenopetala* leaves [2,5,6]. Therefore, the result indicates that the processing conditions and coating materials employed (maltodextrin and pectin) are safe.

3.2. Body Weight. The body weight of the mice had shown significant differences depending on the treatment type. The change in body weight was higher in the normal control group, in which the hyperglycemic mice followed were treated by the glibenclamide and microencapsulated products of low and middle dose levels (Table 1). The change in body weight of microencapsulated product might be attributed to the presence of the antioxidant activity, which may scavenge the free radicals produced by STZ. King [23] stated that STZ is a source of free radicals, which damage the DNA and cause cell destruction. Moreover, the microencapsulate of *M. stenopetala* leaves extract also has nutritional components due to the presence of macro- and micronutrients, which might be used as food supplements. On the contrary, the body weight of the untreated hyperglycemic mice (negative control group) was lost significantly ($P < 0.05$), which could be attributable to the physiological disorder in the diabetic mice as a result of the loss of amino acids and inaccessibility of carbohydrates as an energy source.

3.3. Blood Glucose Level. As shown in Table 2, the blood glucose level of the fastened mice indicates that the dose of the microencapsulated product had considerable effects on blood glucose level reduction. The negative control group, which were STZ-induced untreated mice, attended as diabetic mice whose blood glucose level was kept rising as compared to the normal control group. In addition, two of them were dead (the first one on the 12th and second on the 16th day). There was a significant reduction of the blood glucose level after the diabetic mice were treated by the microencapsulated product and standard drug, although the blood glucose level was not returned back to normal level in 21 days. Thereafter, on the 21st day, the blood glucose level reduction was not different significantly at different doses. The same trends were shown for ethanol extract of *M. stenopetala* leaves in alloxan-induced diabetic mice [6] and STZ-induced diabetic rat [5].

Daily oral administration of microencapsulated product from *M. stenopetala* leaves extract had shown a significant reduction of the blood glucose level. Moreover, a significant prevention action on the blood glucose level increment was also shown when it was compared to the untreated diabetic mice. The standard drug (glibenclamide, 5 mg/kg/day) reduced the blood glucose level significantly ($P < 0.05$) when it was compared to the microencapsulated product. Glibenclamide is an antihyperglycemic drug, which results in the increment of intracellular calcium in the β-cell, which leads to stimulating for the release of insulin [24]. The result of microencapsulated product is in agreement with the report of Toma et al. [5]. On the contrary, Nardos et al. [6] found out that the extract reduced the blood glucose level higher than the standard drug.

The effectiveness in the reduction of the blood glucose level in the mice was a dose-dependent manner. Lower blood glucose levels were observed in the middle and higher dose level as indicated in Table 2. Furthermore, the daily oral administration of the highest dose of the microencapsulate (1000 mg/kg) reduced the blood glucose level comparable to that of glibenclamide on the 14th day of treatment (Table 2).

Table 3: Vasodilation effect of the microencapsulated bioactive product from *M. stenopetala* leaves extract on the guinea pig thoracic aorta.

Concentration (mg/mL)	Relaxation of the precontracted aorta (%)	
	Extract	Encapsulated product
2.5	10.00 ± 1.08^{e}	4.50 ± 0.77^{g}
5	26.27 ± 1.95^{d}	8.00 ± 0.55^{f}
10	53.03 ± 2.45^{c}	14.33 ± 1.03^{e}
20	66.53 ± 1.73^{b}	24.67 ± 0.82^{d}
40	99.13 ± 2.02^{a}	38.50 ± 1.97^{c}
80	–	64.83 ± 1.83^{b}
160	–	74.17 ± 2.04^{a}

Superscripted letters in the column indicate the presence of the significant differences ($P < 0.05$) among the values which showed different concentrations of the samples.

This is due to the increase of the active compounds at a higher dose, which may in turn elevate the antioxidant activity of the microencapsulates. Kim [25] described that high methoxyl pectin contributes to decreasing the rate of glucose absorption due to its viscous nature. The increment of the concentration of the coating material also improves the lowering of the blood glucose level. The same trend was also reported for *M. stenopetala* leaves extract [5,6] and *Sasa borealis* extract [26]. The ethanolic extract of *Zygophyllum album* at high dose also resulted in a significant reduction of the blood glucose level in STZ-induced diabetic mice [27]. This probable action of the indicated activity might be attributed to the activation of protein kinase (AMPK) and enhancement of insulin signaling by the active compounds [26]. In addition, the decrease in the blood glucose level in animals might be related to the inhibition of glycogenolysis as proposed by increased liver glycogen [27].

3.4. Vasodilator Activity. The cardiovascular disorder is among the major causes of morbidity and mortality that contributes to a strong public burden on treatment and therapeutic management [28]. Vasodilation is the relaxation of the smooth muscle of blood vessels, which results in normal blood pressure. Nitric oxide is one of the most important vasodilators or endothelium-derived relaxing factors [29]. Ferreira et al. [30] found that the relaxation of the contracted aortic strip was due to the release of nitric oxide when it was treated by *Hancornia speciosa* extract. Similar result was also reported for *Viscum album* extract [31]. This shows that phytochemical constituents of the plant extracts may increase the release of nitric oxide; consequently, the vasodilation properties of nitric oxide are improved.

On the other hand, when the smooth muscle is being contracted, it results in increased blood pressure [29]. The results are shown in Table 3; the unencapsulated extract had significantly ($P < 0.05$) higher relaxation against KCl-induced contraction of the guinea pig thoracic aorta compared to the microencapsulated product. The high relaxation (99.13%) was found from the unencapsulated extract at the concentration of 40 mg/mL. Microencapsulated product gave the maximum relaxation of 74.17% at the concentration of 160 mg/mL (Table 3). This is probably due to the variation in the concentration of the active compounds and effects of processing temperature and coating materials. Even if the pure extract had a more relaxing effect, the microencapsulated product has also shown its vasodilation effect in a dose-dependent manner.

3.5. Diuretic Activity. Diuretics also produce antihypertensive effect and have clinical application [32]. Diuretics increase the urinary sodium excretion, thereby reducing the plasma volume, which in turn lower the blood pressure, extracellular fluid volume, and cardiac output [33], though the mechanism of action of the diuretics was not known exactly.

3.5.1. Urine Output. The effect of microencapsulated product of *M. stenopetala* leaves extract on the urine output is shown in Table 4. Accordingly, the urine output of the rats treated with the microencapsulated product was considerably higher when compared to the normal control group (the group treated with normal saline solution). This showed that the microencapsulated product of *M. stenopetala* leaves extract could increase urine excretion.

The urine output was increased with increase in time until 8 h. The urine output of rats treated with the standard drug (furosemide) was significantly higher than the microencapsulated product at 2 h and 4 h. Thereafter, the urine output of the microencapsulated product was comparably increased at 8 h with furosemide. This indicates that the diuresis action of the furosemide drug occurred earlier than the microencapsulated product. Roush and Sica [32] described that the duration of action of the furosemide drug is 4-5 h. The urine output was higher when the dose of the microencapsulated product was increased. The same trend was observed as reported earlier for the 70% ethanol extract of *M. stenopetala* leaves [34] and aqueous extract of *T. schimperi* leaves [3].

3.5.2. Urinary Excretion. The percentage of urinary excretion from the rats treated with different doses of microencapsulated bioactive product was the highest (54 and 56%) at the dose of 750 and 1000 mg/kg at 8 h, respectively, whereas the lowest was found in the untreated rats along the time intervals (Table 5). On the other hand, the percentage of urinary extraction was increased with the increment of the dose of the microencapsulated product as the time is getting longer to 8 h (Table 5). The result was in agreement with the reports of Geleta et al. [34] and Fekadu et al. [35] for *M. stenopetala* leaves extract.

When the standard drug (furosemide) was considered, the high urinary excretion (23%) was found in the first 2 h. Thereafter, it was continued with the same trend as the microencapsulated treated rats for the rest time intervals due to the aforementioned reason (Table 5). The microencapsulate took a longer time, probably due to the time required for the release of active compounds from the coating

Table 4: Urine output from the rats treated with microencapsulated product of *M. stenopetala* leaves extract.

Treatment	Urine output (mL)			
	2 h	4 h	6 h	8 h
Dose 500 mg/kg	1.77 ± 0.84^{cBC}	3.62 ± 1.00^{bAB}	5.30 ± 1.30^{abAB}	6.28 ± 1.23^{aAB}
Dose 750 mg/kg	1.72 ± 0.55^{cBC}	4.70 ± 0.82^{bA}	5.88 ± 0.86^{abA}	6.98 ± 0.80^{aA}
Dose 1000 mg/kg	2.07 ± 0.48^{cAB}	4.80 ± 0.60^{bA}	6.57 ± 0.93^{aA}	7.45 ± 0.90^{aA}
Positive control	3.06 ± 1.03^{cA}	4.63 ± 1.28^{bcA}	5.75 ± 0.76^{abAB}	6.48 ± 0.78^{aAB}
Normal control	0.70 ± 0.62^{bC}	2.67 ± 1.65^{abB}	3.90 ± 1.70^{aB}	4.88 ± 1.58^{aB}

All values with different superscripted small letters in each row indicate the presence of the significant differences ($P < 0.05$) in different hours, whereas the superscripted capital letters in each column indicate the presence of significant difference at different doses and control groups.

Table 5: Diuretic activity of the rat treated by the microencapsulated bioactive product.

Treatment	Urinary excretion (%)				Diuretic index				Diuretic activity			
	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h	2 h	4 h	6 h	8 h
Dose 500 mg/kg	13	26	38	45	2.34	1.25	1.23	1.14	0.56	0.76	0.88	0.92
Dose 750 mg/kg	14	37	46	54	2.43	1.73	1.46	1.36	0.58	1.05	1.05	1.09
Dose 1000 mg/kg	15	35	48	56	2.74	1.63	1.55	1.42	0.66	0.99	1.11	1.14
Positive control	23	35	44	49	4.17	1.65	1.40	1.24	1	1	1	1
Normal control	6	21	32	39	1	1	1	1	–	–	–	–

Table 6: Electrolyte extraction in the treated rats using the microencapsulated bioactive product.

Treatment	Excreted electrolytes (mmol/L)			Natriuretic activity	Saluretic activity	Carbonic anhydrase inhibition	Saluretic index		
	Na^+	K^+	Cl^-	Na^+/K^+	$Na^+ + Cl^-$	$Cl^-/(Na^+ + K^+)$	Na^+	K^+	Cl^-
Dose 500 mg/kg	137.83 ± 14.82^{bc}	79.73 ± 3.36^{bc}	166.67 ± 14.35^{ab}	1.73 ± 0.38^{a}	304.50 ± 29.13^{ab}	0.76 ± 0.04^{a}	1.52	1.20	1.34
Dose 750 mg/kg	180.50 ± 9.40^{ab}	104.15 ± 8.75^{ab}	245.35 ± 21.89^{a}	1.76 ± 0.20^{a}	425.85 ± 30.76^{a}	0.86 ± 0.08^{a}	1.99	1.57	1.97
Dose 1000 mg/kg	187.33 ± 15.33^{ab}	103.32 ± 8.32^{ab}	243.00 ± 31.92^{a}	1.82 ± 0.19^{a}	430.33 ± 46.76^{a}	0.82 ± 0.12^{a}	2.06	1.56	1.96
Positive control	191.00 ± 13.81^{a}	113.62 ± 9.19^{a}	253.37 ± 27.23^{a}	1.73 ± 0.38^{a}	444.37 ± 46.76^{a}	0.82 ± 0.13^{a}	2.10	1.72	2.04
Normal control	90.83 ± 5.07^{c}	66.25 ± 4.97^{c}	124.28 ± 7.16^{b}	1.42 ± 0.40^{a}	215.12 ± 11.80^{b}	0.79 ± 0.06^{a}	1	1	1

All values indicated in different superscript letters in each column indicate the presence of significant differences ($P < 0.05$) in the dose of the microencapsulated product and control groups.

material. The values of the diuretic index and diuretic activity showed the same trends with the urinary excretion (Table 5). The diuretic activity depended on the dose of the microencapsulated bioactive product. As the dose was increased, the diuretic activity was higher. The results showed that microencapsulated product of *M. stenopetala* leaves extract has a significant diuretic activity. Consequently, this microencapsulated product may be used to minimize the abnormal accumulation of fluid. The diuretics are the most commonly prescribed agents for the management of hypertension [32].

3.5.3. Electrolyte Concentration. The microencapsulated product of *M. stenopetala* leaves extract had a significant ($P < 0.05$) effect on the excretion of Na^+, K^+, and Cl^- ion (Table 6). A high electrolyte excretion was observed in furosemide-provided rats followed by the microencapsulate-treated rats at the doses of 1000 and 750 mg/kg, respectively. The lowest electrolyte excretion was observed for the control group (untreated rats). The highest dose of the microencapsulated product had shown significant electrolyte excretion. The *T. schimperi* extract also showed that the electrolyte extraction increased as the dose was increased [3]. This experimental data showed that the microencapsulated product has a role in antihypertensive activity. This effect may have advantages in the case of salt-sensitive hypertension due to the excretion of sodium. The removal of sodium may lead to a drop in systolic blood pressure of 10 mm Hg or more [32].

Moreover, the natriuretic, saluretic, and carbon anhydrase inhibition activities were not significantly ($P < 0.05$) different at different doses of the microencapsulated product with the exception of the saluretic activity at which a significant difference was found at lower doses (500 mg/kg) of the microencapsulated product. The removal of these

electrolytes in the body may lead to the minimization of the cardiac work by reducing the amount of plasma, thereby leading to the return of the venous to the heart [34].

The common feature of diuretics is their natriuretic action, which leads to a decrease in total body sodium [36]. The observation was made in this study that the maximum value of natriuretic activity was 1.82, which was found from the administration of a dose of 1000 mg/kg microencapsulated product. This indicated a favorable natriuretic effect. The natriuretic activity was considered to be favorable when the value is greater than or equal to two [36]. When the value for natriuretic activity is more than ten, it is determined to have potassium sparing effect [22]. Therefore, natriuretic activity value of the treatment group indicates favorable potassium sparing although there is a tendency for excretion of potassium.

The carbonic anhydrase inhibition values of the microencapsulated bioactive product are in the range of 0.76 to 0.86 (Table 6). Carbonic anhydrase inhibitor is used to increase the urinary excretion of the bicarbonate [36]. Carbonic anhydrase inhibition can be excluded if the ratio is between 0.8 and 1 [22]. All the values in the current study were in this range except the microencapsulate at dose of 500 mg/kg. Thus, the diuretic activity of the microencapsulated product did not possess carbonic anhydrase inhibition action. The diuretic activity is claimed to be effective as the carbonic anhydrase inhibition ratio is going to be decreased [22,36].

4. Conclusions

The microencapsulated product from *M. stenopetala* leaves extract is shown to be safe as determined in the acute toxicity study. The microencapsulated bioactive product possessed significant vasodilator (up to 74.17% of relaxation) and diuretic (up to 56% of urinary excretion) activities. The blood glucose levels of the STZ-induced diabetic mice were significantly reduced when the microencapsulated product of *M. stenopetala* leaves extract was administered at the doses of 500, 750, and 1000 mg/kg. The vasodilator, increased urinary excretion, and saluretic effects are indicative of the antihypertensive effect of the microencapsulated bioactive product of *M. stenopetala* leaves extract. Therefore, the microencapsulated product may be considered as a potential nutraceutical for prediabetic and prehypertensive treatments. Further study, however, needs to be undertaken to elucidate the possible mechanism of actions on the antihyperglycemic, vasodilator, and diuretic activities of the microencapsulated product.

Data Availability

All the data generated and analyzed during this study are included in this paper.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Digestive Enzyme Inhibition of Different Phenolic Fractions and Main Phenolic Compounds of Ultra-High-Pressure-Treated Palm Fruits: Interaction and Molecular Docking Analyses

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The purpose of the present work was to evaluate the inhibitory effects of different phenolic extracts from non- and ultra-high-pressure- (UHP-) treated palm fruits and their main phenolic compounds against pancreatic lipase and α-glucosidase and to further analyze the interaction and inhibitory mechanisms of two main phenolics (caffeic acid and catechin). Results showed that the free, esterified, and insoluble-bound phenolic fractions from the non- and UHP-treated fruits demonstrated good inhibitory effects towards two enzymes. The insoluble-bound phenolic fraction, regardless of UHP treatment, presented the strongest inhibitory capacities, and UHP treatment significantly upgraded the inhibitory effects of these phenolic fractions (lipase IC_{50}: 78.01 vs. 72.50 μg/mL; α-glucosidase IC_{50}: 76.42 vs. 64.51 μg/mL). Catechin and caffeic acid, main phenolic compounds detected in all phenolic fractions of the fruits, showed similar efficiencies on inhibiting the two enzymes, which were consistent with the findings observed by molecular docking analysis. Moreover, these two phenolic compounds exhibited a synergy effect on inhibiting pancreatic lipase and α-glucosidase at a relatively high combination concentration with the ratio of 1 : 1. Therefore, the present work may be helpful for further application of palm fruits as food supplements or nutraceuticals to control energy intake to improving some chronic metabolic diseases.

1. Introduction

With the continuous improvement of people's material life, long-term consumption high-glucose and high-fat diet results in glucose and lipid metabolic disorders, causing severe problems to human health [1]. Glucose and lipid metabolic disorders, a cluster of metabolic syndrome, are closely related with many chronic disease occurrences, such as obesity and diabetes [2]. An epidemiological survey suggested that more than three million people bear the obesity torture in the USA, and about one billion people either being overweight or obesity worldwide [3]. Diabetes (the fifth health killer) threatens the human health by damaging the blood vessels, nerves, and organs [4]. The most common drug to control or improve obesity and diabetes available on the market are orlistat and acarbose. Orlistat can reduce the lipid absorption by inhibiting the lipase activity [5], and acarbose controls postprandial blood glucose by reducing the glucose absorption with inhibiting the α-glucosidase activity [6]. Nevertheless, some unpleasant effect may take place, such as abdominal discomfort and flatulence [5, 6]. Therefore, excavating natural inhibitors of pancreatic lipase and α-glucosidase to control obesity and diabetes is the research hotspots. Many studies have proved that phenolic compounds possess inhibitory effect on digestive enzymes, such as pancreatic lipase and α-glucosidase [7–9]. Meanwhile, phenolic compounds could also be absorbed into human body to directly exert their health benefits [10].

Oil palm (*Elaeis guineensis* Jacq.) fruits, a common economic oil crop for pressing oil [11], are not only abundant with oil but also rich in phenolic compounds [12]. However, to the best of our knowledge, few studies have been performed to evaluate the inhibitory effects of the free, esterified and insoluble-bound phenolic fractions (F, *E*, and IB) from oil palm fruits against the pancreatic lipase and α-glucosidase. Meanwhile, ultra-high-pressure (UHP) treatment, one of the newest food processing methods, has been widely used in food processing industry. UHP greatly improved bioaccessibility and bioactivity of the bioactive compounds in food materials [13]. In our previous study, we found that the phenolic and flavonoid contents of the F, *E*, and IB phenolic fractions of oil palm fruits were significantly upgraded after being treated by UHP [12]. However, the influence of UHP treatment on the pancreatic lipase and α-glucosidase inhibitory activities of the different phenolic fractions from oil palm fruits is still unclear.

Therefore, the pancreatic lipase and α-glucosidase inhibitory activities of the different phenolic fractions (F, *E*, and IB) from nontreated and UHP-treated oil palm fruits were comparatively evaluated, and then the interactions between the two main phenolic compounds (detected in all phenolic fractions) on inhibiting lipase and α-glucosidase activities were further investigated. Finally, molecular docking analyses were used to delineate the underlying inhibition mechanisms of those two predominant phenolic compounds.

2. Materials and Methods

2.1. Chemicals and Reagents. Pancreatic lipase (from porcine pancreas, 163 U/mg, EC: 3.1.1.3), α-glucosidase (from *Saccharomyces cerevisiae*, Type I, ≥10 U/mg protein), *p*-nitrophenyl laurate (PNP), *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG), acarbose, and orlistat were purchased from Sigma (Sigma-Aldrich, Shanghai, China). The purity of authentic standards of phenolic compounds (catechin and caffeic acid) were ≥98% and obtained from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Other reagents used were of analytical grade.

2.2. Preparation and UHPLC-MS/MS Analysis of Samples. Oil palm fruits were obtained in 2017, from a local market (Haikou City, Hainan Province, P.R. China) and kept at low temperature (4°C–6°C) throughout transportation and stored at −20°C after being received. The methods used for UHP pretreatment of oil palm fruits and the extraction of F, *E*, and IB phenolic fractions were performed in the same way as in our previous report [12]. Mesocarps were vacuum-packed before UHP treatment and then processed with HHP-600 (Baotou Kefa High Pressure Technology Limited Company, Baotou, China) for 10 min at 500 Mpa. Then, all samples were lyophilized and smashed into powder with a high-speed grinder (Lingdan LD-T300, Shanghai, China) for extraction. The phytochemical compositions of *F*, *E*,, and IB phenolic fractions were analyzed by UHPLC-MS/MS, which were similar with the results reported in our previous work [12]; catechin and caffeic acid were the main phenolic compounds detected in all phenolic fractions of non- and UHP-treated fruits.

2.3. Inhibition of Pancreatic Lipase Activity. The assay of pancreatic lipase inhibition was performed according to the method reported earlier [14]. Briefly, pancreatic lipase was dissolved into 150 mg/mL with distilled water, and the supernatant was obtained by centrifuged with 10,000 g for 5 minutes at 4°C. The 1% PNP and 1% Triton X-100 were added in sodium acetate solution (5 mM pH 5.0) to prepare the substrate solution. The absorbance value of each sample was obtained at 400 nm by a SpectraMax M5 microplate reader (Molecular device, USA). All the experiments were conducted three times.

2.4. Inhibition of α-Glucosidase Activity. The inhibition of α-glucosidase activity was conducted as per a previously described method [15]. In this assay, *p*-NPG served as substrate, which was dissolved into 2.5 mM with PBS (PH = 6.8). α-Glucosidase solution also prepared with PBS, by dissolving 1 mg α-glucosidase with 0.5 mL PBS and then diluting ten times when needed. The absorbance value of each reaction mixture was measured at 405 nm by a SpectraMax M5 microplate reader. All the experiments were conducted three times.

2.5. Interaction of Catechin and Caffeic Acid on Inhibition of Pancreatic Lipase and α-Glucosidase. According to the phytochemical analysis [12], catechin and caffeic acid were the predominant phenolic compounds detected in all samples. Therefore, the interaction of catechin and caffeic acid on inhibition of pancreatic lipase and α-glucosidase was determined by CalcuSyn software (Biosoft, Ferguson, MO, USA) as in the previous report [16]. For facilitating calculation and analysis of phenolic interaction types, the inhibitory effects of combined catechin and caffeic acid on pancreatic lipase and α-glucosidase were evaluated by three different ratios, 2 : 1, 1 : 2, and 1 : 1, respectively. The interaction types were evaluated based on CI (combination index; $CI \leq 0.90$: synergistic effect; $0.90 < CI < 1.10$: additive effect; and $CI \geq 1.10$: antagonistic effect).

2.6. Molecular Docking. Molecular docking of pancreatic lipase and α-glucosidase with phenolic standards (catechin and caffeic acid) was conducted by SYBYL-X 2.1.1 (Tripos, Inc., St. Louis, MO, USA) according to the previous methods [14, 15]. The 3D configurations of the catechin and caffeic acid compounds were obtained from PubChem (http://www.ncbi.nlm.nih.gov/pccompound), and the 3D structure of lipase was downloaded from RCSB PDB (http://www.rcsb.org/pdb/home.do). Since the 3D structure of α-glucosidase of *S. cerevisiae* is unavailable, isomaltase (PDB code: 3A4A) from the same organism, which shares high similarity with α-glucosidase, were used for homology modeling in the molecular docking analysis in this study.

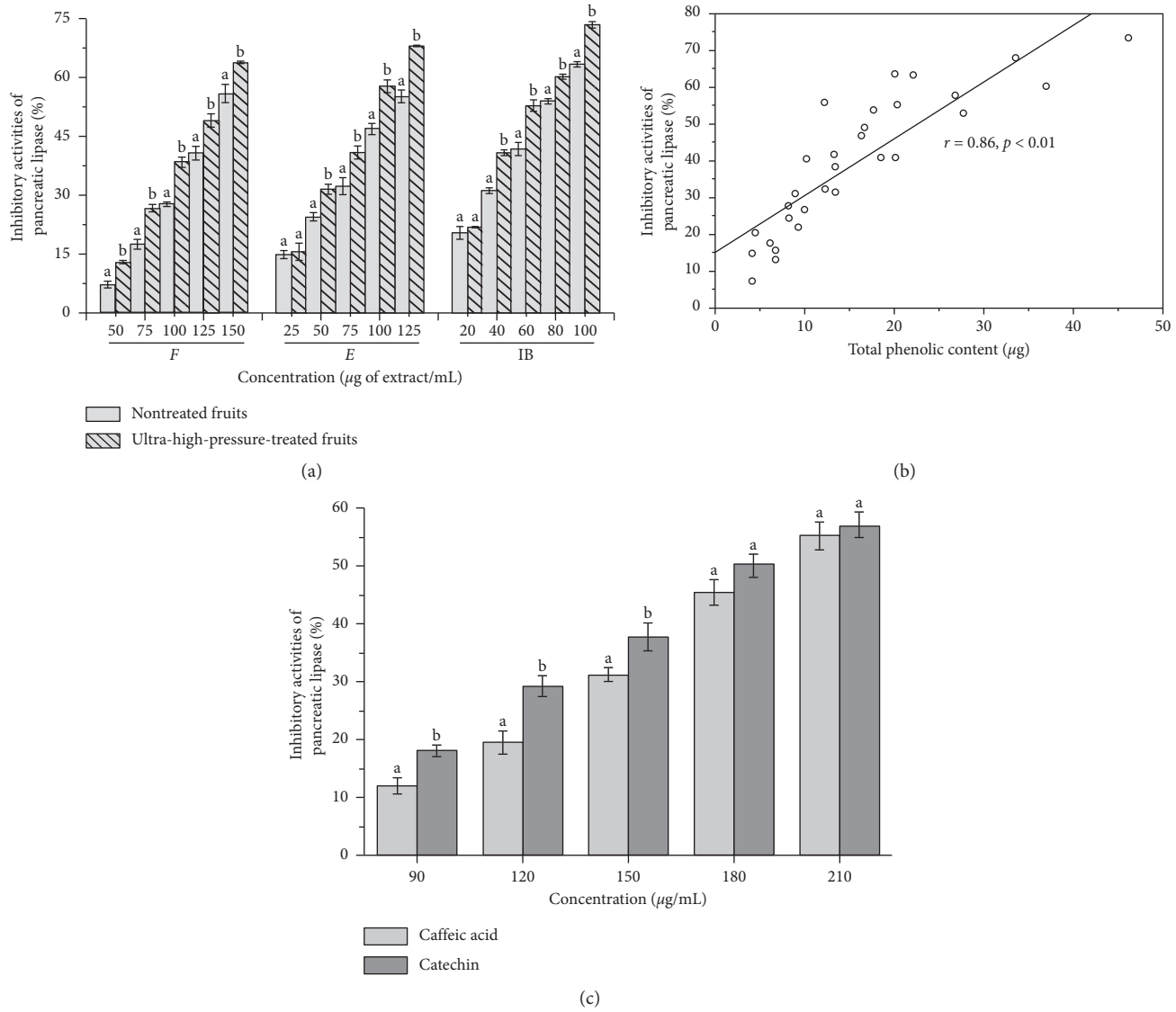


Figure 1: (a) The lipase inhibitory effects of free, esterified and insoluble-bound phenolic fractions from nontreated and ultra-high-pressure-treated oil palm fruits. (b) The correlation analysis of TPC and pancreatic lipase inhibitory effects. (c) Two main phenolic compound standards (catechin and caffeic acid). "*F*": free phenolic fraction; "*E*": esterified phenolic fraction; "IB": insoluble-bound phenolic fraction.

2.7. Statistical Analysis. Each experiment was conducted three times, and results were expressed as mean ($n = 3$) ± standard deviation (SD). The significance analysis ($p < 0.05$) of all data was conducted by one-way ANOVA and Tukey's procedure by using Origin 8.5 software (OriginLab, Northampton, MA, USA). Pearson's correlation coefficient was performed by SPSS 20.0 (SPSS, Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Lipase Inhibition of Extracts from Nontreated and UHP-Treated Oil Palm Fruits. The results of lipase inhibitory effects of the extracts from nontreated and UHP-treated oil palm fruits are presented in Figure 1(a). All of phenolic fractions, regardless of UHP treatment, showed good inhibitory activities towards the pancreatic lipase and presented in a dose-dependent manner at the tested concentrations. Among the three fractions of nontreated oil palm fruits, the insoluble-bound phenolic fraction showed the strongest inhibition, with the IC50 value of 78.01 ± 1.04 μg/mL. The following is the esterified phenolic fraction, whose IC50 value was 112.24 ± 1.63 μg/mL, and the free phenolic fraction possessed the weakest inhibition, i.e., IC50 = 141.01 ± 2.43 μg/mL. As for UHP-treated oil palm fruits, the inhibitory effects coincided with the order shown in nontreated oil palm fruits, insoluble-bound phenolic fraction > esterified phenolic fraction > free phenolic fraction, and their IC50 value was 72.50 ± 1.36 μg/mL, 95.68 ± 2.37 μg/mL, and 127.57 ± 2.17 μg/mL, respectively. The insoluble-bound phenolic fraction showed the highest inhibitory rate, regardless of UHP pretreatment, which may

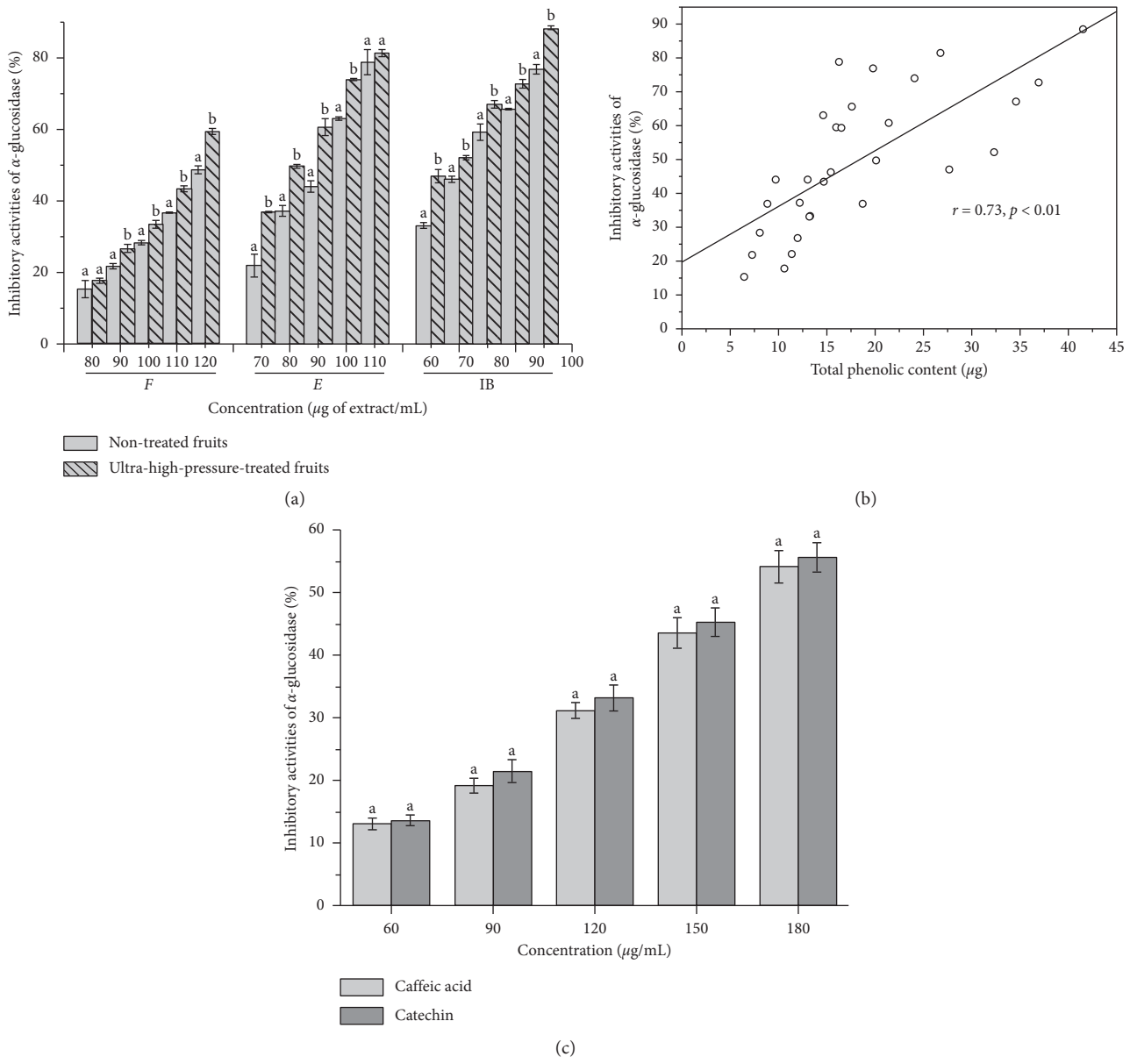


Figure 2: (a) The α-glucosidase inhibitory effects of free, esterified, and insoluble-bound phenolic fractions from nontreated and ultra-high-pressure-treated oil palm fruits. (b) The correlation analysis of TPC and α-glucosidase inhibitory effects. (c) Two main phenolic compound standards (catechin and caffeic acid). "*F*": free phenolic fraction; "*E*": esterified phenolic fraction; "IB": insoluble-bound phenolic fraction.

be because the composition of this phenolic fraction was abundant and the content was comparatively higher than other two parts according to our previous report [12]. A previous study also reported that the insoluble-bound phenolic extract of Sophia seeds had the best inhibitory effect against pancreatic lipase [17]. However, some other studies found that the free phenolic fraction of chia meal or Chinese sumac fruits had the strongest inhibition towards lipase [14, 18]. Those results indicated that the inhibitory effect of lipase may be profoundly affected by the differences of phenolic composition or raw material. Compared with nontreated oil palm fruits, the UHP pretreatment significantly enhanced the inhibitory effect ($p < 0.05$). After UHP treatment, the IC_{50} values of free, esterified, and insoluble-bound phenolic fractions were declined by about 7.06%, 14.75%, and 9.53%, respectively. Cai et al. [19] reported fermentation could also be used as an efficient method to enhance the inhibition of oat towards the pancreatic lipase.

Many previous studies have indicated that there was a close correlation between enzyme inhibition and total phenolic content (TPC) [19,20]. In this work, a positive relationship between lipase inhibitory rates and TPC was also observed ($r = 0.86, p < 0.01$, Figure 1(b)), which suggested phenolic compounds may also respond to the lipase inhibition of the oil palm fruits. According to our previous work, catechin and caffeic acid were the main phenolics presented in all six phenolic fractions of nontreated and UHP-treated oil palm fruits [12]. As seen in Figure 1(c),

these two phenolic standards also exhibited good inhibitory effects against lipase in a dose-dependent manner. The IC_{50} values of catechin and caffeic acid were $189.02 \pm 5.56\,\mu g/mL$ and $197.26 \pm 4.40\,\mu g/mL$ respectively, both of which were significant higher than that of orlistat (positive control, $IC_{50} = 46.22 \pm 0.84\,\mu g/mL$) ($p < 0.05$) and had no significant difference with each other ($p > 0.05$). While, in Tan et al. [21] report, the IC_{50} values of caffeic and catechin acid were much higher than our values obtained in this study, with the IC_{50} values of 0.94 ± 0.09 mg/mL and 0.53 ± 0.05 mg/mL, respectively. In another report, the IC_{50} value of caffeic acid was $32.60\,\mu g/mL$ [5]. These discrepancies may be caused by the differences of assay methods and enzyme batch used.

3.2. α-Glucosidase Inhibition of Extracts from Nontreated and UHP-Treated Oil Palm Fruits. The α-glucosidase inhibitory results of different phenolic fraction from nontreated and UHP-treated oil palm fruits are presented in Figure 2(a). It is apparent that all the phenolic fractions displayed inhibition to α-glucosidase in a dose-dependent manner. In terms of nontreated oil palm fruits, the inhibitory tendency of the free, esterified, and insoluble-bound phenolic fraction towards α-glucosidase was similar to that towards pancreatic lipase; the insoluble-bound phenolic fraction possessed the strongest inhibition, followed by esterified fraction, while the free fraction had the weakest inhibitory effect ($p < 0.05$). The IC_{50} values of those three fractions were $76.42 \pm 0.94\,\mu g/mL$, $93.42 \pm 1.53\,\mu g/mL$, and $122.82 \pm 2.01\,\mu g/mL$, respectively. The UHP pretreatment significantly enhanced the α-glucosidase inhibitory effects of the three different phenolic fractions and the insoluble-bound phenolic fraction also showed the strongest inhibition ($p < 0.05$). The IC50 values of insoluble-bound, esterified, and free phenolic fractions were $64.51 \pm 2.41\,\mu g/mL$, $82.43 \pm 0.65\,\mu g/mL$, and $114.10 \pm 0.96\,\mu g/mL$, respectively, which were enhanced by about 15.58%, 11.76%, and 7.10%, respectively, by comparison with the corresponding fraction without UHP. A previous study showed that esterified phenolic of pomegranate seeds possessed the strongest α-glucosidase inhibitory effect [22]. Another study reported that the inhibitory effect of chia meal-free phenolic against α-glucosidase was the strongest [18]. Therefore, the inhibitory effects of α-glucosidase largely depend on phenolic composition and raw materials. In this work, UHP pretreatment significantly enhanced the α-glucosidase inhibitory capacity. Feng et al. [23] found that solid-state fermentation also greatly increased the α-glucosidase inhibition of tartary buckwheat.

There is also a clear correlation between α-glucosidase inhibitory rates and TPC in the present work ($r = 0.73$, $p < 0.01$), suggesting phenolic compounds may take a responsibility to the α-glucosidase inhibition of the oil palm fruits (Figure 2(b)). Catechin and caffeic acid, as the primary phenolics in all sample, showed good α-glucosidase inhibitory activities with IC_{50} values of $163.07 \pm 3.93\,\mu g/mL$ and $165.29 \pm 5.47\,\mu g/mL$, respectively (Figure 2(c)). According to the IC_{50} values of these two phenolics, they showed a similar efficiency on inhibiting the α-glucosidase and also had no significant difference with each other ($p > 0.05$). However, their inhibitory effects were dramatically weaker than that of acarbose ($IC_{50} = 5.83 \pm 0.65\,\mu g/mL$) ($p < 0.05$).

3.3. Interactions between Catechin and Caffeic Acid on Inhibition of Pancreatic Lipase and α-Glucosidase. As shown in the above results of pancreatic lipase or α-glucosidase inhibition, the inhibitory effect of single phenolic was significantly weaker than that of any of the phenolic fractions. According to our previous work, several phenolics have been detected in oil palm fruits, and each phenolic fraction included more than one compound [12]. So, we speculated that the interaction between different phenolics may be taken when evaluating the enzyme inhibitory effects of different phenolic fractions. Since catechin and caffeic acid were two main phenolics detected in all phenolic fractions of nontreated and UHP-treated oil palm fruits [12], the interaction between these two phenolic compounds was evaluated by CalcuSyn software (Biosoft, Ferguson, MO, USA). Three different combination ratios, 1 : 2, 2 : 1, and 1 : 1, were used to explore the interaction types, and the best combination ratio was found. The interacted inhibitory effects of catechin and caffeic acid on pancreatic lipase are presented in Table 1 and on α-glucosidase are summarized in Table 2. According to the results of pancreatic lipase inhibition (Table 1), when catechin and caffeic acid are combined at a ratio of 1 : 2, the CI value gradually decreases with the increasing concentration, and interaction type between catechin and caffeic acid varies from antagonistic (low concentration) to additive effect (high concentration). When the combination ratio of catechin and caffeic acid was 2 : 1, they showed the same trend as observed at the ratio of 1 : 2. When catechin and caffeic acid was combined at a ratio of 1 : 1, the synergistic inhibitory effect against pancreatic lipase was observed at relative high concentrations (185 and $210\,\mu g/mL$).

Results of interactions between catechin and caffeic acid on inhibiting αα-glucosidase at different combination ratios, which are presented in Table 2, exhibit similar phenomena in the findings observed in lipase inhibition assay. As shown in 2, when catechin and caffeic acid were combined at the ratio of 1 : 2, the CI value decreased with the increasing concentration. At the highest concentration, the interaction type between those two phenolic compounds transformed from antagonistic effect into addictive effect. Similar behaviors found at ratio of 1 : 2 was also observed when the combination ratio between catechin and caffeic acid was set at 2 : 1. When catechin and caffeic acid were combined at a ratio of 1 : 1, the interaction type varied from additive effect to synergistic effect on inhibiting α-glucosidase with increasing concentration. Many previous literatures have reported that the combined samples work better than each sample working alone [24, 25]. Zhang et al. [16] reported that combining myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside dramatically increased the inhibitory effect to lipase. The present work also suggested that interaction between different phenolic compounds on inhibiting pancreatic lipase and α-glucosidase may happen, and the

Table 1: The interactions between catechin and caffeic acid on inhibition of pancreatic lipase.

Combination doses	Catechin	Caffeic acid	Inhibition ratio	CI[a]	Fa[b]	CI of Fa	Catechin	Caffeic acid
	Combination test (μg/mL)		(%)		(%)		Calculation at corresponding Fa (μg/mL)	
	45.0	90.0	13.70 ± 0.23	1.27	25.0	1.16	62.88	125.76
	60.0	120.0	21.00 ± 1.63	1.22	50.0	1.01	97.54	195.09
1 : 2	75.0	135.0	32.30 ± 1.22	1.14	75.0	0.93	151.31	302.63
	90.0	185.0	46.17 ± 1.21	1.08	90.0	0.87	234.72	469.45
	105.0	210.0	55.76 ± 2.14	0.99				
	90.0	45.0	15.14 ± 1.36	1.24	25.0	1.17	121.38	60.69
	120.0	60.0	22.45 ± 1.99	1.19	50.0	0.98	190.72	95.36
2 : 1	135.0	75.0	33.74 ± 2.10	1.10	75.0	0.87	299.68	149.84
	185.0	90.0	47.61 ± 1.82	1.05	90.0	0.83	470.89	235.44
	210.0	105.0	57.21 ± 2.53	0.92				
	90.0	90.0	41.64 ± 2.31	1.06	25.0	1.15	45.11	45.11
	120.0	120.0	54.10 ± 1.44	0.97	50.0	0.99	113.64	113.64
1 : 1	135.0	135.0	57.40 ± 1.92	0.95	75.0	0.84	286.28	286.28
	185.0	185.0	64.28 ± 2.13	0.90	90.0	0.78	521.24	521.24
	210.0	210.0	66.43 ± 2.37	0.88				

[a]CI, combination index. CI ≤ 0.90, 0.90 < CI < 1.10, or CI ≥ 1.10 represent synergistic, additive, and antagonistic effects, respectively. [b]Fa, fraction affected means the dose of combination of catechin and caffeic acid effect on inhibition rate. CI25, CI50, CI75, and CI90 represent the corresponding CI value when Fa is 25%, 50%, 75%, and 90%.

Table 2: The interactions between catechin and caffeic acid on inhibition of α-glucosidase

Combination doses	Catechin	Caffeic acid	Inhibition ratio	CI[a]	Fa[b]	CI of Fa	Catechin	Caffeic acid
	Combination test (μg/mL)		(%)		(%)		Calculation at corresponding Fa (μg/mL)	
	30.0	60.0	12.42 ± 0.39	1.30	25.0	1.21	48.47	96.95
	45.0	90.0	20.41 ± 1.03	1.24	50.0	1.09	84.48	168.96
	60.0	120.0	32.24 ± 1.76	1.16	75.0	0.95	147.24	294.48
1 : 2	75.0	135.0	44.50 ± 1.43	1.12	90.0	0.89	256.62	513.23
	90.0	180.0	54.91 ± 1.82	1.07				
	60.0	30.0	15.05 ± 0.62	1.28	25.0	1.20	89.12	44.56
	90.0	45.0	23.04 ± 0.88	1.22	50.0	1.02	160.43	80.22
	120.0	60.0	34.87 ± 1.71	1.13	75.0	0.94	288.81	144.41
	150.0	75.0	47.13 ± 1.46	1.09	90.0	0.87	519.92	259.96
2 : 1	180.0	90.0	57.54 ± 2.03	1.04				
	60.0	60.0	34.68 ± 1.08	1.16	25.0	1.23	45.02	45.02
	90.0	90.0	43.83 ± 1.22	1.10	50.0	1.02	98.72	98.72
	120.0	120.0	56.73 ± 2.36	0.99	75.0	0.85	215.59	215.59
1 : 1	150.0	150.0	65.51 ± 2.14	0.93	90.0	0.76	470.83	470.83
	180.0	180.0	69.98 ± 1.75	0.90				

[a]CI, combination index. CI ≤ 0.90, 0.90 < CI < 1.10, or CI ≥ 1.10 represent synergistic, additive, and antagonistic effects, respectively. [b]Fa, fraction affected, means the dose of combination of catechin and caffeic acid effect on inhibition rate. CI_{25}, CI_{50}, CI_{75}, and CI_{90} represent the corresponding CI value when Fa is 25%, 50%, 75%, and 90%.

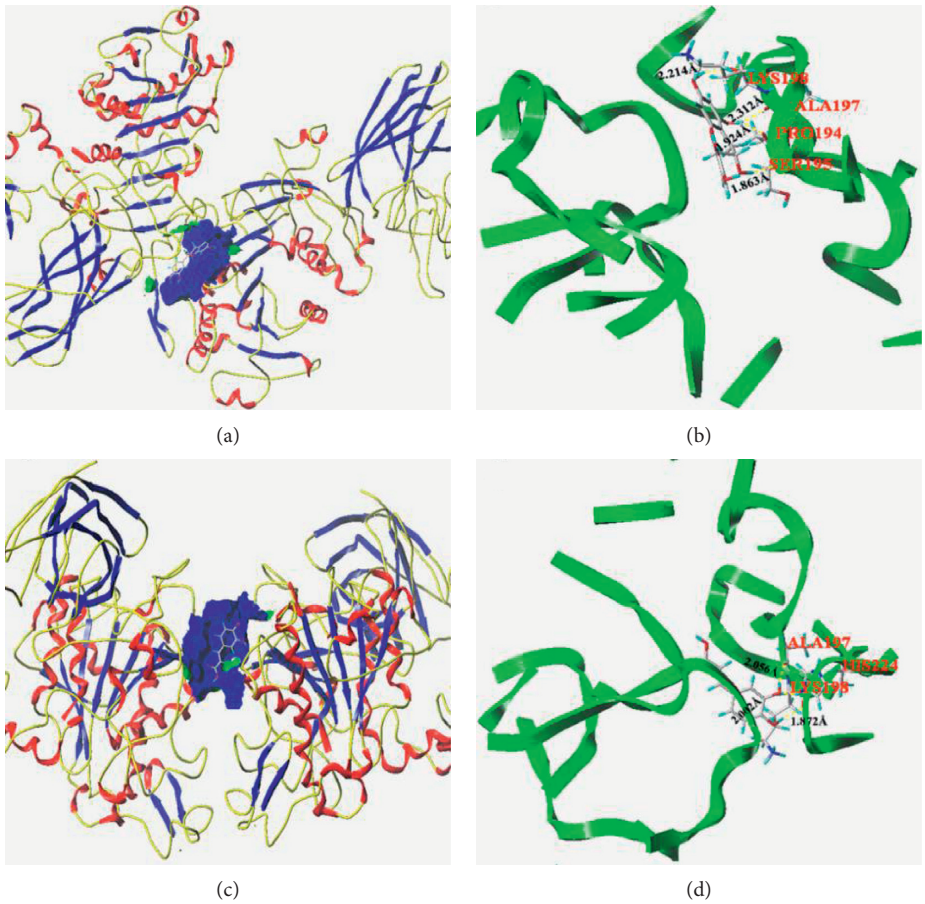
interaction type varies with the variation of the combination concentrations and ratios.

3.4. Molecular Docking Results of Pancreatic Lipase and α-Glucosidase. Computational modeling soft SYBYL-X 2.1.1 (Tripos, Inc., St. Louis, MO, USA) was used to analyze the enzyme inhibitory mechanism of catechin and caffeic acid. The interaction was evaluated by some relevant parameters, for example, C-Score and T-Score. The value of C-Score is used to evaluate if the docking result is credible, which should be no less than four, and the T-Score reveals the ability of binding affinity, which is an important index to value the docking results [14].

The docking results of pancreatic lipase are summarized in Table 3 and Figure 3(a). The C-Scores of two main phenolics (catechin and caffeic acid) were four, suggesting the docking results were effective and credible (Table 3). The T-Scores in Table 3 indicated that catechin may have a better binding affinity to lipase than caffeic acid. As shown in the Figures 3(a) and 3(b), catechin bound with four amino acid residues (Ala197, Pro194, Ser195, and Lys198) in the pancreatic lipase active site. Ala197, Pro194, and Ser195 served as an H-bond receptor, the hydrogen bond distances of

Table 3: The score results of two phenolic compounds docking with pancreatic lipase and α-glucosidase, respectively.

	C-Score	T-Score	PMF-Score	CHEM-Score	G-Score	D-Score
Pancreatic lipase						
Catechin	4	4.4351	27.2574	−15.5057	−131.1809	−85.8563
Caffeic acid	4	3.4707	22.5169	−17.6857	−113.1308	−67.2611
α-Glucosidase						
Catechin	5	4.8871	19.9048	−14.4315	−87.8383	−106.1603
Caffeic acid	4	4.0400	−13.7135	−13.0411	−51.2488	−74.2849



(a) (b) (c) (d)

Figure 3: The molecule docking results of catechin and caffeic acid with pancreatic lipase. (a) Catechin binds with the active site of pancreatic lipase. (b) Catechin interacts with amino acid residues. (c) Caffeic acid binds with the active site of pancreatic lipase. (d) Caffeic acid interacts with amino acid residues. Two phenolic standards appeared in ball and stick models, amino acid residues are represented by green capped stick models, and short dotted yellow line stands for hydrogen bonds.

which were 2.312 Å, 1.924 Å, and 1.863 Å, respectively. Lys198 was an H-bond donor, and the hydrogen bond length was 2.124 Å. The average hydrogen bond distance was 2.056 Å. In Figures 3(c) and 3(d), caffeic acid bound with the pancreatic lipase active site by interacting with three amino acid residues, namely, Ala197, His224, and Lys198. Ala197 and His224 served as an H-bond receptor, and Lys198 acted as an H-bond donor. The hydrogen bond distance between caffeic acid and these amino acid residues was 2.065 Å, 1.872 Å, and 2.022 Å, respectively. The average hydrogen bond distance was 1.986 Å. According to those results, both catechin and caffeic acid interacted with amino acid residues Lys198 and Ala197, suggesting these two amino acids may play a key role in the pancreatic lipase activity. Zhang et al. [15] also reported that cyandin-3-O-glucoside may exert its lipase inhibition through binding with Lys198 and Ala197. Another study reported that myricitrin and quercitrin interacted with Asp 80 and Gly 77 to inhibit the activity of pancreatic lipase [14]. According to those previous results and the current findings, they clearly indicated that different phenolic compounds may interact with different amino acids to exert their lipase inhibitory effects. Although T-scores indicated that catechin may have a better binding affinity to lipase than caffeic acid, the number and distance of hydrogen bonds are also very important for phenolics inhibiting the lipase activity. The more and/or the shorter

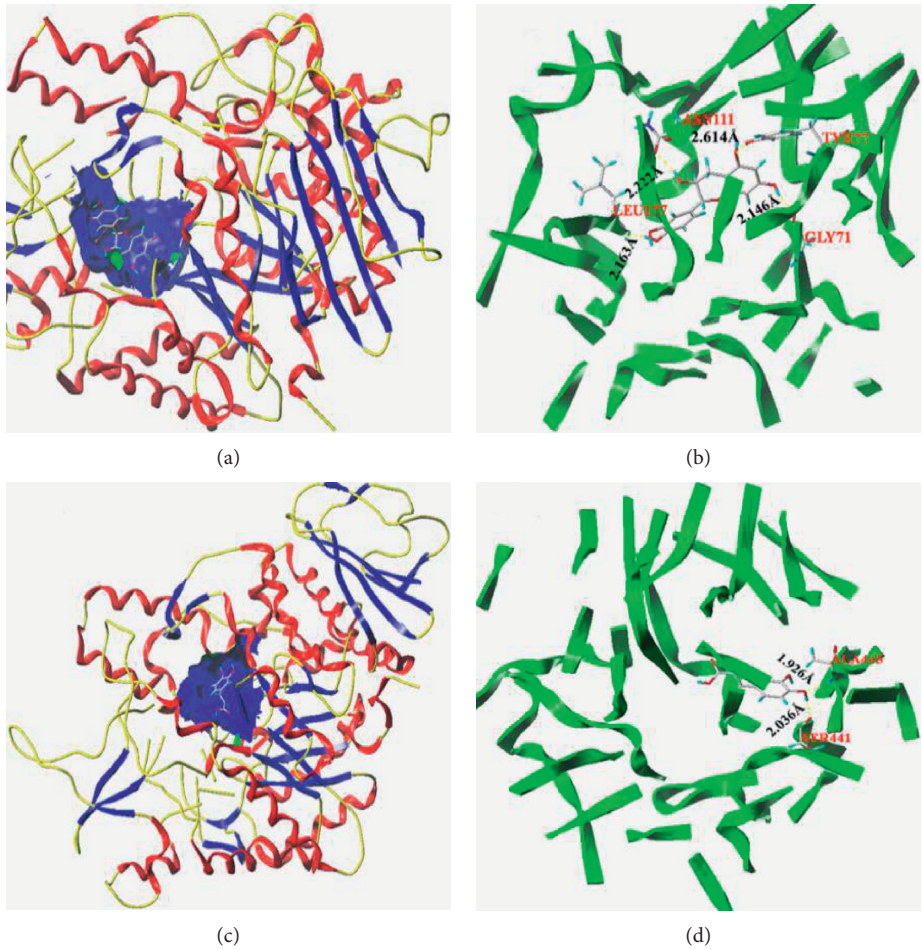


Figure 4: The molecule docking results of catechin and caffeic acid with α-glycosidase. (a) Catechin binds with the active site of α-glucosidase. (b) Catechin interacts with amino acid residues. (c) Caffeic acid binds with the active site of α-glucosidase. (d) Caffeic acid interacts with amino acid residues. Two phenolic standards appeared in ball and stick models, amino acid residues are represented by green capped stick models, and short dotted yellow line stands for hydrogen bonds.

hydrogen bonds represent a tighter binding and thereby a stronger inhibition [26]. In the present work, the average hydrogen bond distance of catechin was longer than that of caffeic acid, while the hydrogen bond number of catechin was one more than that of caffeic acid. The one more hydrogen bond of catechin may counteract the disadvantage of slight longer hydrogen bond distance of catechin on inhibiting pancreatic lipase, which could explain why catechin and caffeic acid had a similar efficiency on inhibiting lipase.

For molecular docking of α-glucosidase, the C-Score results of two phenolics were also no less than four (Table 3). The catechin also had a higher T-score than caffeic acid, indicating that catechin may also have a better binding affinity to α-glucosidase than caffeic acid (Table 3). In Figures 4(a) and 4(b), four hydrogen bonds were formed between catechin and four amino acids (Leu177, Asn111, Tyr77, and Gly71) in the α-glucosidase active pocket. Among the three H-bond receptors (Leu177, Asn111, and Gly71), the Asn111 possessed the longest hydrogen bond with a distance of 2. 222 Å, and the hydrogen bond distances of Leu177 and Gly71 were 2.163 Å and 2.146 Å, respectively. The Tyr77 acted as an H-bond donor, and the hydrogen bond distance was 2.614 Å. The average distance of these four hydrogen bonds was 2.286 Å. Two amino acid residues were observed between caffeic acid and α-glucosidase active pocket at Figures 4(c) and 4(d), namely, Ala445 and Ser441. Ala445, as an H-bond donor, generated an H-bond with caffeic acid with a distance of 1.926 Å, while Ser441 served as an H-bond receptor and the hydrogen bond length was 2.036 Å. The average hydrogen bond distance was 1.981 Å. The average hydrogen bond distance of caffeic acid was much shorter than that of catechin, while the hydrogen bond number of catechin was two more than that of caffeic acid. As mentioned above in the docking analysis of pancreatic lipase, the disadvantage of longer hydrogen bond of catechin on inhibiting α-glucosidase may be remedied by more hydrogen bond number when compared with that of caffeic acid. Therefore, catechin and caffeic acid had a similar efficiency on inhibiting α-glucosidase. A previous study found that both rutin and isorhamnetin-3-*O*-rutinoside formed H-bonds with the amino acid residues Ile 440, Arg 213, and Arg 315 of α-glucosidase [15]. Another study found that the binding sites of apigenin were Ser311 and Gly309

[26]. Gallocatechin gallate was reported to form hydrogen bonds with Arg 315 and Phe 303 to effectively inhibit the catalytic activity of α-glucosidase [27]. Those different results also obviously suggested that the structures of phenolic compounds may profoundly affect their binding sites on α-glucosidase.

4. Conclusions

The results of the present study showed that the free, esterified, and insoluble-bound phenolic fractions from nontreated and UHP-treated oil palm fruits demonstrated good inhibitory effects towards pancreatic lipase and α-glucosidase. Moreover, the inhibitory effects of these three different phenolic fractions on lipase and α-glucosidase were significantly upgraded after being pretreated with UHP ($p < 0.05$). The insoluble-bound phenolic fraction, regardless of UHP treatment, presented the strongest enzyme inhibitory capacities, both in lipase and α-glucosidase. Catechin and caffeic acid, as the main phenolic compounds detected in all samples, also showed a dose-dependent inhibition toward the two enzymes and exhibited a synergistic effect on inhibiting pancreatic lipase and α-glucosidase at a high combination concentration with the ratio of 1 : 1. Moreover, catechin and caffeic acid showed similar efficiencies on inhibiting the two digestive enzymes, which were consistent with the findings observed by molecular docking analysis. This study suggested that different phenolic extracts of oil palm fruits, especially the UHP-treated fruits, could be potentially developed as nutraceuticals or functional foods for controlling or alleviating diseases related to over intake of glucose and/or lipid, such as diabetes, hyperlipidemia, cardiovascular diseases, and obesity.

Data Availability

All data included in this study are available upon request by contacting the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Qingfeng Zhou and Jiexin Zhou contributed equally to this work.

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Research Article

Different Phenolic Extracts of Oil Palm Fruits and Caffeic Acid Prevent Palmitic Acid-Induced Lipotoxicity in HepG2 Cells via Improving Mitochondrial Function

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Oil palm (*Elaeis guineensis* Jacq.) fruits are rich in antioxidative phenolic compounds, which exert various health improving effects. This study aimed to comparatively study the protective effects of different phenolic fractions from the fruits treated with or without ultrahigh pressure (UHP) and their most abundant phenolic substance, caffeic acid, against the palmitic acid-induced lipotoxicity in HepG2 cells and to clarify the potential mechanisms. Results showed that the TG accumulation, intracellular reactive oxygen species content, cell apoptosis, and mitochondrial membrane potential depolarization were relieved by the administration of those phenolic fractions and caffeic acid. The protection of insoluble-bound (IB) phenolic fraction was the best, and UHP treatment significantly enhanced its protective effect on lipotoxicity. The lipotoxicity preventive effect may be achieved by alleviating intracellular oxidative stress, downregulating the expression of P-P38, COX-2, and iNOS to relieve inflammation, and downregulating Bax and cytochrome C and upregulating Bcl-2 to suppress the mitochondrial-mediated apoptosis. Therefore, the oil palm fruits phenolic fractions, especially the IB phenolic fraction, might be utilized as dietary antioxidants to relieve the adverse effects of a high-fat diet on the body, and UHP treatment is a potential method to increase this bioactivity.

1. Introduction

High-fat diet consumption is very common in modern society, which could easily lead to high level-free fatty acid (FFA) in the body circulation system and excessive fat deposition in organs [1]. Excessive intake of FFA accompanied by large amounts of fat (mainly triglyceride, TG) accumulated in cells of different organs can lead to chronic injury and dysfunction of those organs, which is defined as lipotoxicity [2, 3]. The lipotoxicity has been proved to be closely related to many metabolic dysfunction diseases, such as hyperlipidemia, fatty liver, and diabetes [4, 5]. For instance, when hepatocytes ingest a large amount of FFA, on the one hand, the FFA is stored as triglycerides in hepatocytes, resulting in hepatic steatosis [6]; on the other hand, the FFA is oxidized, leading to many reactive oxygen species (ROS) production and thereby damaging hepatocytes; and these will lead to the occurrence of fatty liver or/and steatohepatitis [6, 7]. Some natural compounds in dietary plants with a good free radical scavenging activity can prevent oxidative stress-induced liver injury [8].

Among the FFAs, palmitic acid (PA) is abundant in the human body and widely distributed in daily diet. Based on these, PA has been used to induce lipotoxicity in the in vitro studies, especially in cells model, such as hepatocytes, islet β-cells, and endothelial cells [9]. Inflammation response, endoplasmic reticulum stress, mitochondrial damage, and cell apoptosis were proved to be involved in PA induced lipotoxicity [2, 5, 9, 10]. During the excessive PA intake, massive reactive oxygen species (ROS) were generated along with the lipid peroxidation, which probably stimulates inflammation response and cell apoptosis, and eventually leads

to physiological dysfunction [7, 11]. Previous researches reported that ROS could cause an inflammatory response by the activation of the P38 mitogen activated protein kinases (MAPK) pathway and regulate the secretion of inflammatory cytokines, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [12, 13]. Besides, under the stimulation of ROS and inflammation, cell apoptosis could be activated, and the mitochondrial pathway was involved in the possible mechanism [11, 14]. In the mitochondrial pathway, the B cell lymphoma-2 (Bcl-2) family could interact with mitochondria members to disrupt mitochondrial membrane potential (MMP) and mitochondrial permeability. Bax, a proapoptosis of the Bcl-2 family, could be activated by inflammation and then bind with the mitochondrial membrane to open the permeability transition channels. Meanwhile, the antiapoptosis Bcl-2 could be suppressed [15, 16]. In a case like this, cell apoptosis was activated and markers of apoptosis were released, like cytochrome C, a protein that has been widely accepted as the marker of apoptosis [17].

Since the negative effect of high FFAs consumption on health has been widely proved, and no effective clinical drug is available, it is necessary to explore bioactive compounds from food materials to decrease the damage of FFAs. Among the numerous natural compounds, antioxidative phenolic compounds showed effectively protective effects against lipotoxicity induced by FFAs, such as PA [18]. In our previous research, we reported that oil palm fruit (OPF) was rich in phenolic compounds, especially caffeic acid, and exhibited good antioxidant activity in vitro; moreover, we found that ultrahigh pressure (UHP) treatment increased the phenolic compounds bioaccessibility and the antioxidant of OPF [19]. Therefore, we hypothesized that OPF and its main phenolic compound, caffeic acid, might effectively prevent FFA-induced lipotoxicity and OPF treated by UHP may have a better effect, which has not been investigated. In the present study, the free (F), esterified (E) and insoluble-bound (IB) phenolic fractions were extracted from UHP-treated or nontreated OPF. Their protective effects and the caffeic acid on PA-induced lipotoxicity would be comparatively investigated by the analyses of triglyceride (TG) accumulation, intracellular ROS scavenging ability, cell apoptosis, and MMP. Moreover, the expression levels of several key markers in important signaling pathways of inflammation and apoptosis were also testified by western blot to illuminate the potential mechanism. The results of this study might provide scientific information for the devolvement of OPF product as a functional food to prevent or alleviate FFA-induced lipotoxicity.

2. Materials and Methods

2.1. Materials and Chemical Reagent. The OPF was obtained from a local market in Haikou city (Hainan province, China). The human hepatocellular carcinoma cell line (HepG2) was obtained from Kunming cell bank (Chinese Academy of Sciences, China). Fetal bovine serum (FBS), penicillin, streptomycin, and Dulbecco's modified Eagle's medium (DMEM) were supplied by Gibco (Grand Island, NY). Palmitic acid, methylthizol-2-yl-2,5- diphenyl tetrazolium bromide (MTT), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (Shanghai, China). Bovine serum albumin (BSA) was purchased from BioDee Biotech Co., Ltd. (Beijing, China). Annexin V-FITC/PI apoptosis kit was obtained from Beijing 4A Biotech Co., Ltd. (Beijing, China). Cell lysis buffer, TG assay kit, BCA protein assay kit, and mitochondrial membrane potential assay kit (JC-1) were purchased from Shanghai Beyotime Co., Ltd. (Shanghai, China). Caffeic acid standards were supplied by Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). All antibodies were procured from Abcam (Cambridge, UK), and other reagents used were of analytical grade.

2.2. Preparation and Analysis of Different Phenolic Fractions from OPF. The UHP pretreatment of OPF and preparation of different phenolic fractions (F, E, and IB) were conducted as a previous report [19]. Briefly, chopped mesocarps were vacuum-sealed and then pretreated by a UHP equipment (HHP-600, Baotou Kefa High Pressure Technology Limited Company, Baotou, China) with 500 MPa pressure for 10 min. Subsequently, the F, E, and IB phenolic fractions were extracted from OPF with or without a UHP pretreatment, respectively. The phenolic compositions of different fractions were analyzed by UHPLC-ESI-HRMS/MS in negative mode. As we reported previously, a total of 13 phenolic compounds were detected, including caffeic acid, catechin, gallic acid, protocatechuic acid and kaempferol, and caffeic acid was the predominant phenolic compound in all fractions [19].

2.3. PA/BSA Complex Solution Preparation. The PA/BSA complex solution was prepared according to the previous method with some modifications [20]. Briefly, PA was dissolved with 0.1 M NaOH into 100 mM in a shaking water bath at 70°C for 30 mins. Then, 50 μL PA solution was mixed with 950 μL BSA (10%) and incubated in a water bath at 70°C for six hours. Finally, the PA/BSA complex solution was sterile filtered with 0.22 μm membrane filter and stored at −20°C as stock. DMEM was used to dilute the stock into appropriate concentration for the experiment, and the same concentration of BSA/NaOH complex was used as a vehicle in each assay.

2.4. Determination of PA and Sample Cytotoxicity. The HepG2 cell was cultured in DMEM supplemented with 10% FBS and 5% penicillin-streptomycin at an atmosphere of 5% CO_2 at 37°C. The culture medium was changed every two days. Cells grown up to 90% confluence were used for subsequent experiments. MTT method was conducted as our previous report to evaluate the cytotoxicity of PA and different phenolic fractions on HepG2 [19]. In brief, after being seeded in a plate (1×10^5/mL) with a 24-hour incubation, cells were stimulated with PA/BSA complex solution at the concentrations of 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM or different concentrations of the sample ranging

from 40.0 μg/mL to 200.0 μg/mL. For another 16-hour (PA) or 24-hour (sample) incubation, the treatment medium was removed, and cells were washed twice with PBS solution. Then, 0.5 mg/mL MTT solution was added, and then cells were incubated for another four hours. Thereafter, MTT solution was discarded and 150 μL of DMSO was added into each well to completely solubilize the formazan. The absorbance of each well was recorded by SpectraMax M5 microplate reader (Molecular Device, USA) at 570 nm. The cells treated with DMEM were used as the corresponding control group. The control group was considered as 100% viability. Each group was set with six repeating wells, and each experiment was conducted three times.

2.5. Measurement of Intracellular TG Level in PA-Induced HepG2 Cells. The intracellular TG level in PA-induced HepG2 cells was determined according to a published method with some modifications [9]. Briefly, HepG2 cells were seeded in a six-well plate at a density of 1×10^5/mL with 2 mL per well. After incubation for 24 hours, 200 μL of F, E, IB phenolic fractions or caffeic acid dissolved in DMEM with 120.0 μg/mL were separately added into the corresponding well for another 24-hour incubation, while cells in Control (CK), BSA, and PA groups were still cultured with DMEM. Thereafter, HepG2 cells in sample and PA groups were exposed to 0.3 mM of PA/BSA complex solution for 16 hours, and cells in BSA and CK groups were incubated with the corresponding concentration of BSA/NaOH complex solution and DMEM, respectively. Then, HepG2 cells were washed twice with PBS, lysed with lysis buffer (200 μL per well), homogenized with ultrasonic cell crusher (Scientz-II D, Ningbo Scientz Biotech Co., Ltd. Ningbo, China) and centrifuged at 4°C with 6000 g for five minutes. The supernatant was collected for determining protein and TG contents with BCA protein assay kit and TG assay kit, respectively, according to the manufacturer's instructions. Each group was set with three repeating wells, and each experiment was conducted three times.

2.6. Inhibition of Intracellular ROS. HepG2 cells were cultured and treated as mentioned in Section 2.5. After incubation, cells in all groups were washed with PBS for three times, and then harvested and labeled with DCFH-DA. Thereafter, FBS-free medium was used to wash the uncombined dyes and resuspend the cells. The fluorescence was detected with a flow cytometer (Guava easy Cyte 6-2L, Millipore, Billerica, USA) as the previous report [19]. The relative amount of intracellular ROS in each group was calculated by taking the CK group as 100% for reference.

2.7. Analysis of Apoptosis. HepG2 cells were treated as mentioned in Section 2.5. After 16-hour incubation in PA/BSA complex solution, the apoptosis of cells was analyzed with Annexin V-FITC/PI apoptosis kit [19]. Briefly, harvested cells were washed with PBS, and then 1.0 mg/mL Annexin V-FITC and 20.0 μg/mL PI were successively applied to stain the cells. Thereafter, the apoptosis rate was detected by a Guava easy Cyte 6-2L flow cytometer as the previous report.

2.8. Analysis of Cellular MMP. HepG2 cells were treated as mentioned in Section 2.5. After 16-hour incubation in PA/BSA complex solution, the MMP of HepG2 cells was analyzed by a mitochondrial membrane potential assay kit (JC-1) based on the instructions of the manufacturer. After harvest, cells were labeled with 1 mL JC-1 (1X) and incubated at 37°C. About 30 mins later, JC-1 (1X) was removed, and 1 mL ice-cold JC-1 buffer (1X) was added to wash the cells twice. Then cells were resuspended with 1 mL FBS-free medium and determined with a Guava easy Cyte 6-2L flow cytometer within 30 mins. The mitochondrial membrane depolarization level was reflected by the ratio of red fluorescence intensity to green fluorescence intensity.

2.9. Western Blot Analysis. The cell treatments were as the same as mentioned in Section 2.5. The target proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis, electrotransferred to the nitrocellulose membrane (Gelman Laboratory, Ann Arbor, USA), and blocked with 5% BSA for 1 h. Then, the membrane was incubated with the corresponding primary antibody overnight at 4°C. After being washed three times with TBS-Tween 20 (0.1%, v/v), the membrane was incubated with secondary antibody for another 1 h at room temperature and washed with TBS-Tween 20 again. Thereafter, the image of each protein was captured by a VILBER Fusion FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France) with enhanced chemiluminescent detection reagent (Millipore, Billerica, MA, USA).

2.10. Statistical Analysis. All experiments were conducted at least three times. Data were presented as mean ± standard deviation (SD) and analyzed with Origin 8.5 software (OriginLab, Northampton, MA, USA). The significant differences between groups were analyzed with One-Way ANOVA followed by Tukey's test ($p < 0.05$).

3. Results and Discussion

3.1. Phenolic Fractions and Caffeic Acid Attenuated the PA-Induced TG Accumulation. In the present work, the appropriate concentration of PA used for inducing lipotoxicity in HepG2 cells was investigated by MTT assay. As shown in Figure 1(a), the cell viability decreased gradually with the increase of PA concentration. In order to successfully establish the lipotoxicity cell model for subsequent research, the cell viability needs to be maintained at a certain extent. In this work, the cell viability was about 70% and 60% at 0.3 mM and 0.4 mM of PA, respectively, which indicated that cells suffered a moderate lipotoxicity. Therefore, both 0.3 mM and 0.4 mM of PA can be used to establish the lipotoxicity cell model, and in this work, 0.3 mM of PA was applied. Moreover, the MTT assay suggested that all samples showed no toxicity to HepG2 cells at the concentrations up

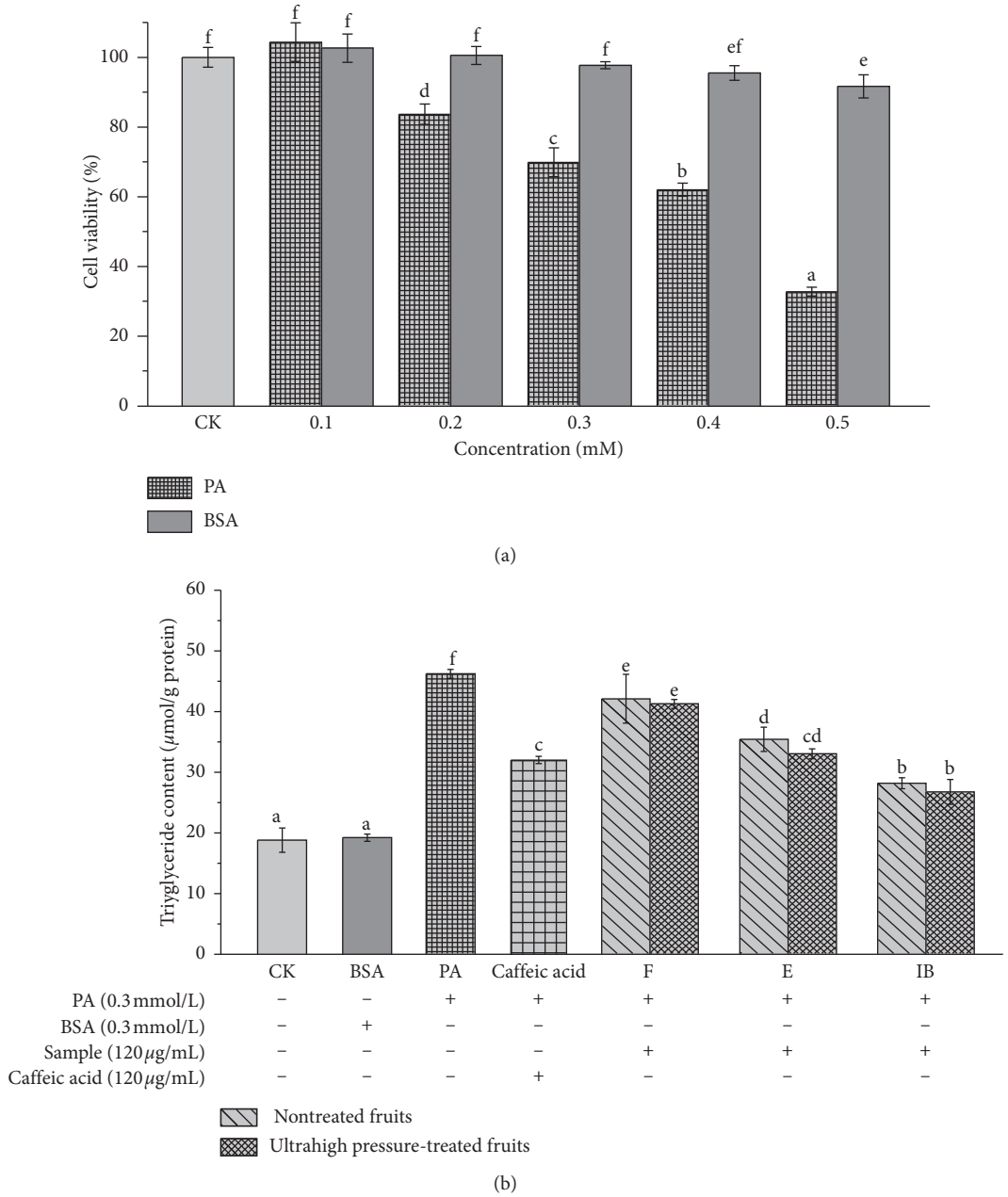


Figure 1: (a) The MMT result of PA, (b) the effects of phenolic fractions from oil palm fruits with or without ultrahigh pressure treatment and caffeic acid on PA-induced TG accumulation. Data are expressed as mean ± SD ($n = 3$). Mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA: PA/BSA complex solution; "F" free phenolic fraction; "E" esterified phenolic fraction; "IB" insoluble-bound phenolic fraction.

to 120 μg/mL, which was consistent with our previous report [19].

Long term and excessive fat intake are easy to result in lipotoxicity to cause fat deposition and eventually lead to obesity, fatty liver disease, or other chronic diseases [21]. Thus, the effects of different phenolic fractions from OPF on TG deposition in HepG2 cells stimulated by PA were presented in Figure 1(b). The TG content of control group and BSA group was similar ($p > 0.05$), with values of 18.82 ± 2.00 μmoL/g of protein and 19.32 ± 0.61 μmoL/g of protein, respectively, while the TG level in PA group was about 46.18 ± 2.05 μmoL/g of protein, which was approximately two times more than that in the CK group or BSA group ($p < 0.05$) and indicated significant fat deposition in hepatic cells. When treated with different phenolic fractions of OPF, all groups showed significantly lower TG levels than that of the PA group ($p < 0.05$), indicating significant effects on inhibition of TG deposition. Regardless of UHP treatment, the IB fraction exhibited the best inhibition effect among the three phenolic fractions on TG accumulation. With the administration of IB fraction, the intracellular TG level was 28.16 ± 0.90 μmoL/g of protein in nontreated fruit group,

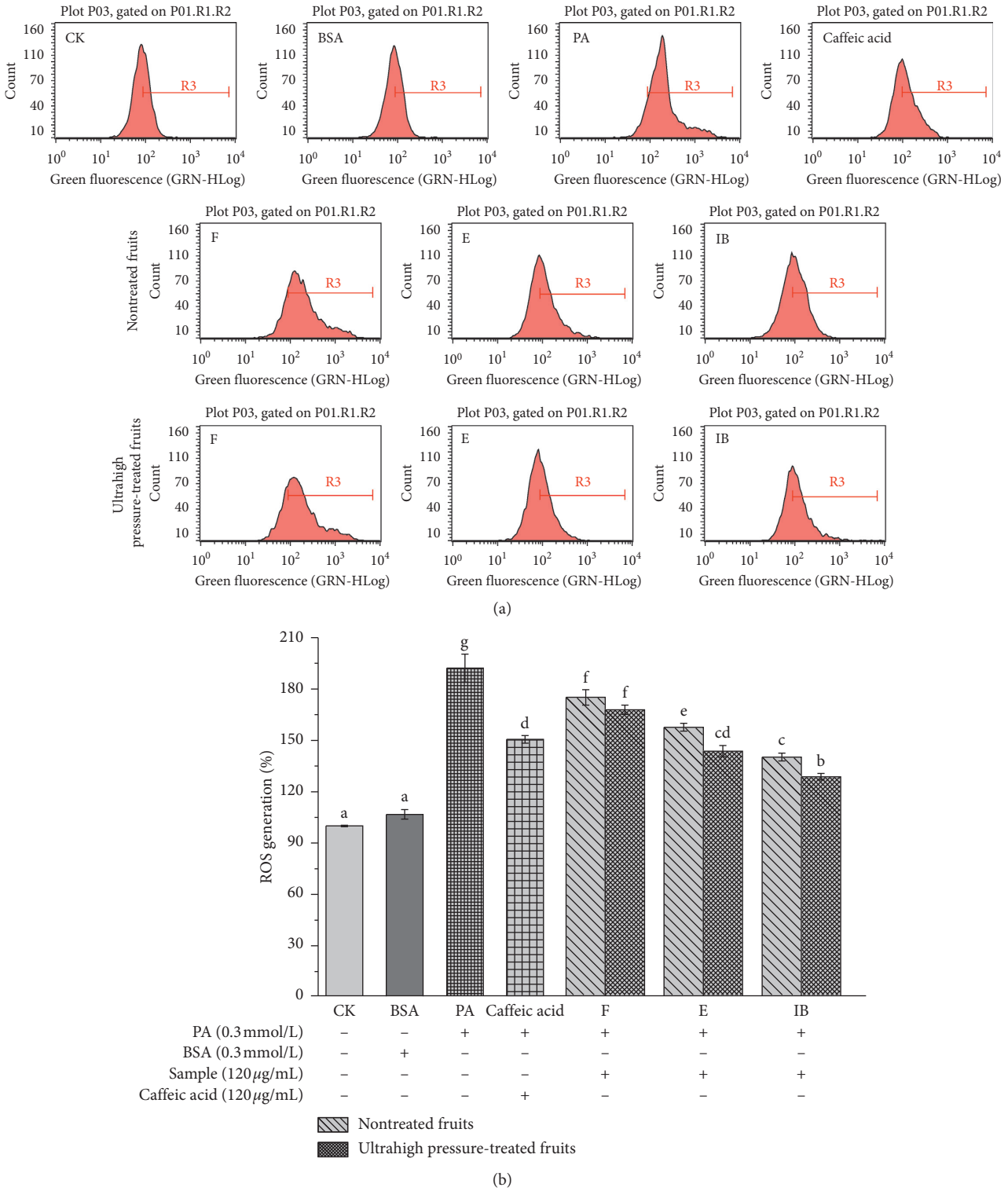


Figure 2: The inhibitory effects of phenolic fractions from oil palm fruits with or without ultrahigh pressure treatment and caffeic acid on PA-induced ROS generation: flow cytometry analysis (a) and the relative amount of ROS of different groups (b). Data are expressed as mean ± SD ($n = 3$). In Figure 2(b), mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA: PA/BSA complex solution; "F" free phenolic fraction; "E" esterified phenolic fraction; "IB" insoluble-bound phenolic fraction.

and 26.17 ± 2.05 μmoL/g of protein in UHP-treated fruit group, both of which were about 40% lower than that of the PA group ($p < 0.05$). However, UHP treatment did not significantly enhance the inhibitory effects of those three phenolic fractions on TG deposition in PA-induced HepG2 cells ($p > 0.05$).

Caffeic acid was the most abundant phenolic compound in both of UHP-treated and nontreated OPF [19]. Therefore, the inhibitory effect of caffeic acid on TG deposition was also clarified. In contrast with the PA group, the caffeic acid administration also significantly lowered TG content by approximately 30.45 % ($p < 0.05$). This result indicated that caffeic acid can effectively attenuate hepatic lipid accumulation, which was in accordance with the previous research: it was proved that the TG clearance was over 50% in oil acid induced HepG2 cells with the treatment of 40 μg/mL of caffeic acid [22]. Moreover, a previous study found that caffeic acid moiety in chlorogenic acid and its analogues were supposed to play a key role in the lipid-lowering activities of those compounds [23]. Therefore, the suppression effects of OPF phenolic fractions on hepatic fat accumulation probably attributed to caffeic acid and/or compounds with a similar structure.

3.2. Phenolic Fractions and Caffeic Acid Reduced PA-Induced ROS Generation. ROS was a cluster of high energy charged free radicals that are generally supposed to be responsible for many biological dysfunctions, such as inflammation, cells apoptosis, and MMP disruption [24, 25]. A previous study has verified that PA administration would lead to oxidative stress by producing lipid peroxides, accompanied by an increase of ROS level [26]. As presented in Figure 2(a), it was obvious that the fluorescence intensity of the PA group moved toward the right by comparison with that of the control group and BSA group (Figure 2(a)). And the ROS level in the PA group was 191.53 %, which was almost two times over that of the CK group or BSA group ($p < 0.05$, Figure 2(b)), indicating serious oxidative stress under PA induction. When compared with the PA treatment, the fluorescence peak of each OPF phenolic fraction treatment gradually moved toward the left (Figure 2(a)), and the ROS generation of each treatment was correspondingly decreased ($p < 0.05$, Figure 2(b)). Specifically, the ROS clearance effects of F, E, and IB fractions in nontreated fruits group were gradually increased, which reduced the ROS content to 174.14%, 156.65%, and 139.25% respectively. The ROS inhibition trend of three phenolic fractions in UHP-treated fruit was similar to that in nontreated fruit. The scavenging effect of F fraction was weaker than the other two fractions, decreased the ROS amount to 166.86%, and has no significant difference with the corresponding part in nontreated fruits ($p > 0.05$). The E and IB fractions declined the ROS amount to 142.72% and 127.79%, respectively, both of which were significantly lower than the counterpart in nontreated fruits ($p < 0.05$). Generally, the UHP treatment significantly enhanced the ROS clearance capacity of OPF in PA-induced HepG2 cells, which was consistent with the previous result observed in H_2O_2-induced HepG2 cells [19], but no matter UHP treated or not, the ROS production of IB fraction was the lowest and was followed by the E fraction.

As one of the major phenolic compounds in OPF, caffeic acid administration significantly attenuated the PA-induced ROS generation by 21.8% ($p < 0.05$, Figure 2(b)), which was markedly better than that of the F fraction treatment, but had significantly lesser effect than that of the IB fraction treatment ($p < 0.05$). Besides caffeic acid, some other phenolics in the IB fraction may also contribute to its ROS inhibitory capacity. For example, catechin and protocatechuic acid were also the primary phenolic compounds detected in an insoluble-bound fraction [19]. Wong et al. [27] reported that 0.3 mM catechin reduced the amount of ROS by 38 % in PA-induced astrocytes. In another study, 10 μM catechin can decrease 54% ROS generation in PA-induced HepG2 cells [28]. Moreover, it also found that protocatechuic acid could significantly attenuate ROS generation by activating endogenous antioxidant enzymes [29].

3.3. Phenolic Fractions and Caffeic Acid Prevented PA-Induced Cell Apoptosis. The excessive ROS not only leads to oxidative damage but also impairs cells and thereby induces cell apoptosis, which partly accounts for the autoimmune disorders and cancers [30–32]. As OPF phenolic fractions exhibited significant ROS scavenge ability, the inhibition effects of them on PA-induced apoptosis were further evaluated by flow cytometry assay. As shown in Figure 3(a), the apoptotic cells appear in two parts of each panel of Figure 3(a): the cells in late apoptosis are presented in the upper right gate, and the early apoptosis cells are showed in the lower right gate [33]. Based on the results in Figure 3(a), the corresponding cell apoptotic proportion was calculated in Figure 3(b). As shown in Figure 3(b), the cell apoptotic rate of the PA group was about 43.06%, which was significantly higher than that of the CK group or the BSA group ($p < 0.05$). The amounts of normal cells in the CK group was 92.42% and the BSA group was 88.69%; the slight decrease in the survival rate of the BSA group may be due to the introduction of NaOH with the BSA/NaOH complex solution. With the treatment of the OPF phenolic fractions, the PA-induced cell apoptosis was attenuated. Regardless of UHP treatment, the IB fraction exhibited the best protective effect against the PA-induced cell apoptosis, followed by the E fraction ($p < 0.05$), while the F fraction from OPF showed the weakest protective activity ($p < 0.05$). When OPF was pretreated with UHP, the IB fraction exerted significantly better protection against cell apoptosis than the counterpart of OPF without UHP treatment ($p < 0.05$). However, UHP treatment did not significantly enhance the protective activities of the F and E fractions from OPF. These results indicated that the inhibitory effect of IB fraction on cell apoptosis was upgraded after UHP treatment, which is probably due to its higher phenolic content [19] and the better antioxidant ability (Figure 2).

The result of caffeic acid inhibiting the PA-induced cell apoptosis was also determined (Figure 3). In contrast with PA treatment, caffeic acid significantly inhibited cell

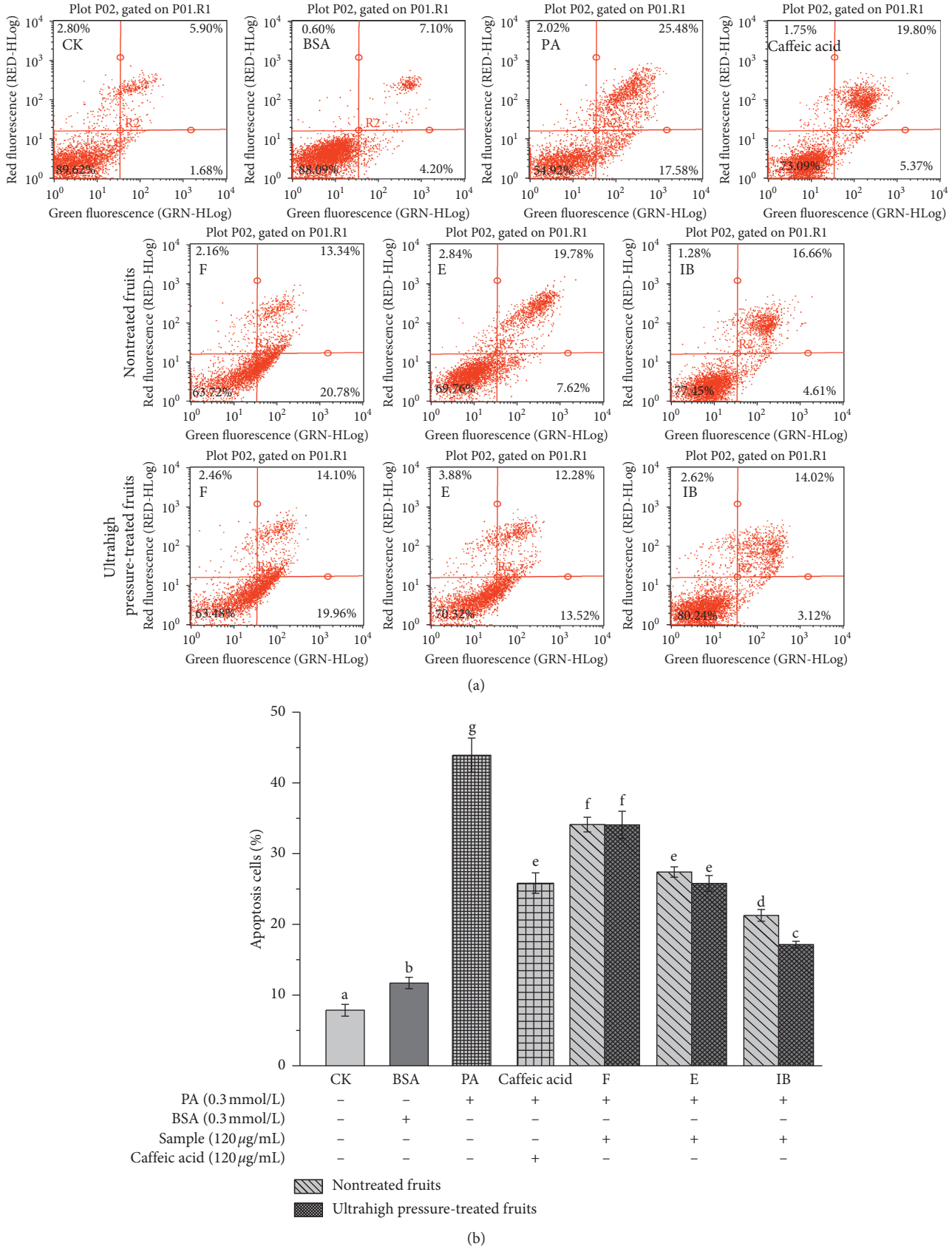


Figure 3: The inhibitory effects of phenolic fractions from oil palm fruits with or without ultrahigh pressure treatment and caffeic acid on PA-induced cell apoptosis: (a) flow cytometry analysis, (b) the relative apoptosis rate of different groups. Data are expressed as mean ± SD ($n = 3$). In Figure 3(b), mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA: PA/BSA complex solution; "F" free phenolic fraction; "E" esterified phenolic fraction; "IB" insoluble-bound phenolic fraction.

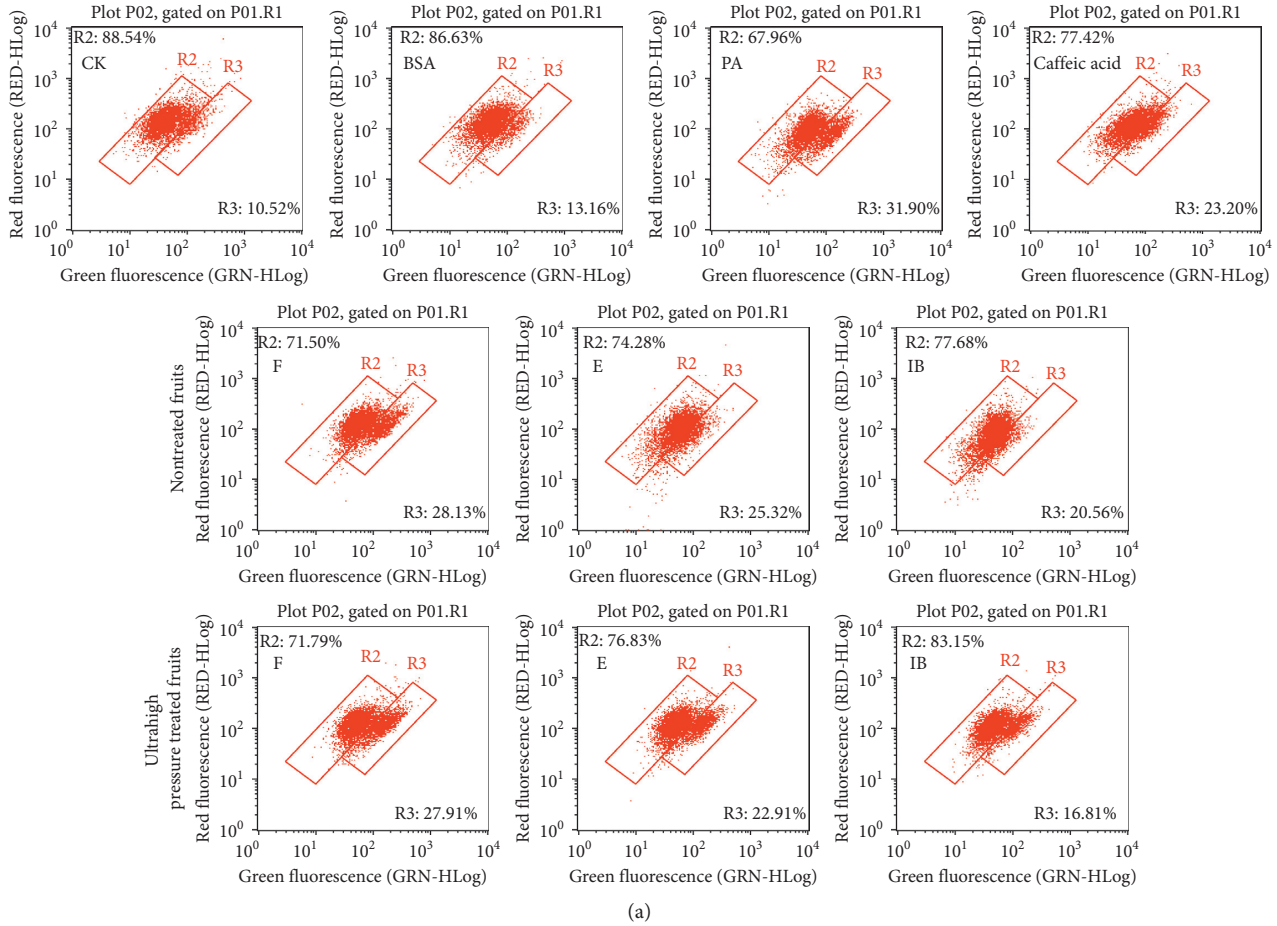
apoptosis by 41.55% ($p < 0.05$), indicating caffeic acid might be the main functional compound in OPF to inhibit PA-induced cell apoptosis. Besides, a previous study reported that kaempferol, also detected in IB fraction, could increase the cell viability from 68% to 98% in the PA-induced pancreatic β-cell [10]. Moreover, it is worth noting that the protective effect of the IB fraction, regardless of UHP treatment, was significantly better than that of caffeic acid. Therefore, those results indicated that other phenolic compounds in OPF may also contribute to the prevention against PA-induced cell apoptosis, and those phenolic compounds may interact with each other to exert a better protective activity.

3.4. Phenolic Fractions and Caffeic Acid Ameliorated PA-Induced MMP Depolarization. The mitochondrion is a crucial organelle, that not only undertakes the energy regulation but also controls the balance of ROS generation [9]. Under the condition of lipotoxicity, the massive ROS could rapidly diffuse to the mitochondrion, lead to MMP depolarization, and thereby mediate cell apoptosis [29, 34]. Therefore, bioactive compounds with ROS scavenge ability may inhibit cell apoptosis through mitochondria protection [35]. In this study, the MMP of each group was monitored by a flow cytometer with the probe of JC-1. JC-1 could penetrate into mitochondria and show different fluorescence to reveal the MMP. Briefly, the red fluorescence was triggered when the MMP was high, while the green fluorescence was produced when the MMP decreased [36]. Based on these, the mitochondrial membrane depolarization level could be calculated as the ratio of the red fluorescence intensity to the green fluorescence intensity.

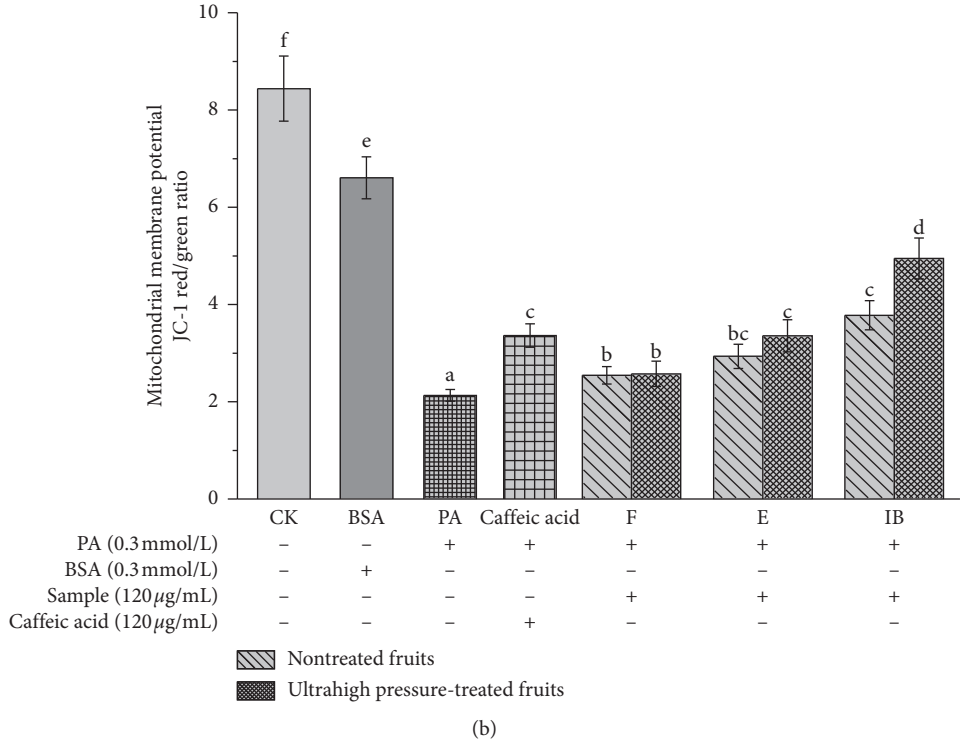
As shown in Figure 4, the CK group has strong red fluorescence intensity and a stable MMP with a red-to-green ratio of about 8.5, and this ratio in the BSA group was about 6.8. The minor decline of the ratio in the BSA group may be due to the effect of NaOH in BSA/NaOH complex solution. When treated with PA, the red fluorescence intensity was obviously declined (Figure 4(a)), and the red-to-green ratio of the PA group was significantly decreased in comparison with that of the CK group or the BSA group ($p < 0.05$, Figure 4(b)), indicating that PA treatment decreased MMP and thereby caused a serious mitochondrial membrane depolarization. Pretreatment of OPF phenolic fractions showed a protective effect on PA-induced mitochondria damage. As demonstrated in Figure 4, the red-to-green ratios of three phenolic fractions in nontreated fruits were about 2.54, 2.93, and 3.79, respectively. As for UHP-treated fruits, the red-to-green values of the corresponding phenolic fractions were 2.57, 3.35, and 4.95. It was obvious that, no matter UHP treatment or not, all the three phenolic fractions of OPF can effectively alleviate the depolarized mitochondrial membrane potential caused by PA, and the effect of the IB fraction was the best ($p < 0.05$). Meanwhile, the results clearly showed that UHP treatment significantly enhanced the protective effect of the IB fraction against PA-induced the depolarization of MMP ($p < 0.05$). Therefore, the lipotoxicity preventive effect of OPF phenolic fractions may be partly achieved by protecting mitochondrial function. The ameliorative effect of phenolic compounds on mitochondrial membrane depolarization has been previously reported. Jin et al. [6] manifested that Chinese propolis treatment can rescue the MMP decrease induced by PA in HepG2 cells, and composition analysis showed that Chinese propolis contained many phenolic compounds, including ferulic acid, caffeic acid, and kaempferol, and these phenolics has also been detected in the different phenolic fractions from OPF [19]. Moreover, it is found that catechin pretreatment could almost protect the MMP of HepG2 cells from the influence of PA treatment [28]. In our previous study, caffeic acid was found to be the most abundant phenolic compound in all the OPF phenolic fractions, while the information about its effect on MMP maintenance of the lipotoxicity condition has not been investigated. Result in the current study showed that the red fluorescence intensity of caffeic acid treatment was significantly higher than that of the PA group with the mean value of the red-to-green ratio rising from 3.10 to 3.58, indicating that the mitochondrial membrane depolarization was significantly improved by caffeic acid treatment ($p < 0.05$, Figure 4). This result may provide evidence that caffeic acid was one of the bioactive compounds contributing to the MMP protection of the different phenolic fractions from OPF.

3.5. Preventive Mechanisms of the IB Fractions and Caffeic Acid in PA-Induced Lipotoxicity. According to the above-mentioned results, regardless of UHP treatment, IB phenolic fraction showed the best effects on TG clearance, ROS reduction, inhibition of cells apoptosis, and MMP maintenance among the three OPF phenolic fractions. Therefore, the underlying mechanisms of IB phenolic fractions from OPF with or without UHP treatment and caffeic acid on alleviating PA-induced lipotoxicity in HepG2 cells were comparatively investigated by Western blot analysis.

Massive ROS would be generated with PA induction, which may lead to an inflammatory response and finally result in cell apoptosis [11]. COX-2 and iNOS are two characteristic inflammation cytokines that expression levels sensitively increase with excessive ROS production [37]. Under the stimulation of inflammation cytokines, the P38 protein would be phosphorylated, which may activate the P38-MAPK pathway to regulate physiological and pathological processes, such as inflammation, cell growth, and apoptosis [12, 13]. As shown in Figure 5, in contrast with the CK group, the expressions of P-P38, COX-2, and iNOS proteins in the PA group increased significantly ($p < 0.05$). And the expression levels of those proteins in the PA group were about four times, seven times, and three times of those in the CK group, respectively. However, the administration of IB phenolic fractions, regardless of UHP treatment, significantly inhibited the expressions of those proteins in comparison with the counterpart of the PA group ($p < 0.05$). Besides, the IB fraction from the UHP-treated OPF exhibited better effect in inhibiting COX-2 expression than that of the IB fraction from the nontreated OPF (Figure 5, $p < 0.05$). The antioxidant ability of the IB fraction increased



(a)



(b)

Figure 4: The inhibitory effects of phenolic fractions from oil palm fruits with or without ultrahigh pressure treatment and caffeic acid on PA-induced MMP depolarization: (a) flow cytometry analysis, (b) the ratio of red fluorescence to green fluorescence of different groups. Data are expressed as mean ± SD ($n = 3$). In Figure 4(b), mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA: PA/BSA complex solution; MMP: mitochondrial membrane potential; "F" free phenolic fraction; "E" esterified phenolic fraction; "IB" insoluble-bound phenolic fraction.

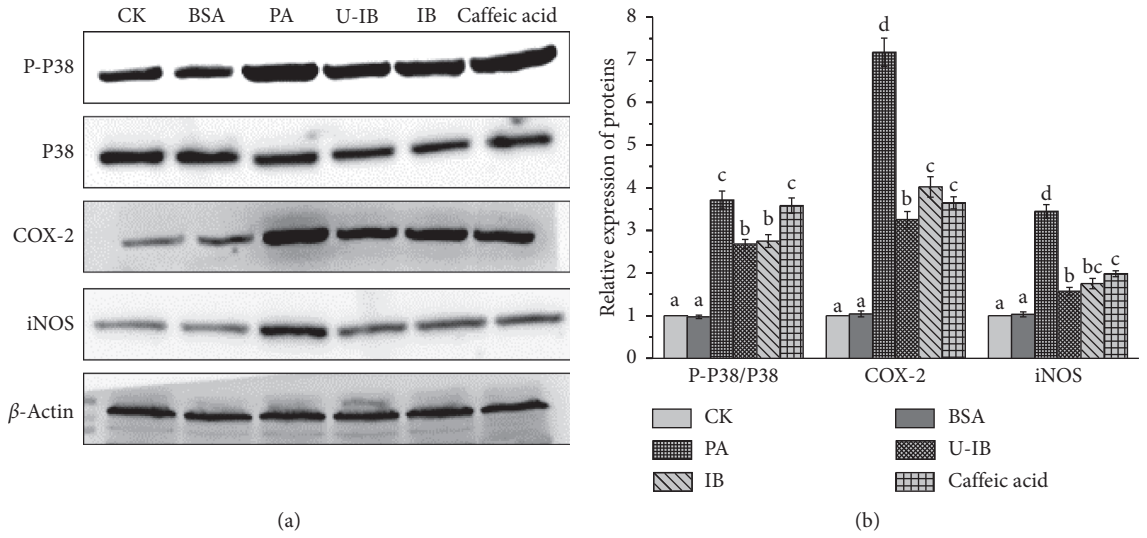


Figure 5: Effects of insoluble-bound fraction and caffeic acid on the protein expression levels of P38, COX-2, and iNOS (a) and the relative protein expression levels (b). Protein expression levels were normalized with β-actin, according to grayscale. Data are expressed as mean ± SD ($n = 3$). In Figure 5(b), mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA, PA/BSA complex solution; P38, P38 mitogen activated protein kinases; COX-2: cyclooxygenase-2; iNOS: inducible nitric oxide synthase; IB: insoluble-bound phenolic fraction; U-IB: UHP-treated insoluble-bound phenolic fraction.

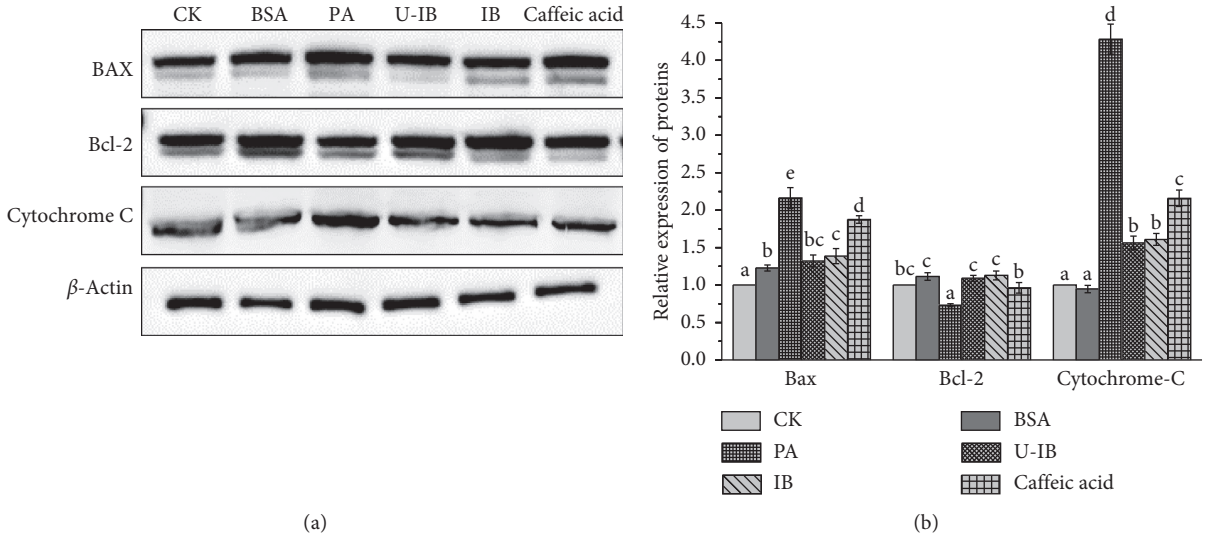


Figure 6: (a) Effects of insoluble-bound fraction and caffeic acid on the protein expression levels of Bax, Bcl-2, and cytochrome C, (b) the relative protein expression levels. Protein expression levels were normalized with β-actin according to grayscale. Data are expressed as mean ± SD ($n = 3$). In Figure 6(b), mean values with different letters are significantly different ($p < 0.05$). CK: control group; BSA: BSA/NaOH complex solution; PA: PA/BSA complex solution; Bax: Bcl-2-associated X protein; Bcl-2: B cell lymphoma-2; IB: insoluble-bound phenolic fraction; U-IB: UHP-treated insoluble-bound phenolic fraction.

significantly [19], and the generation of ROS was remarkably suppressed (Figure 2(b)) after the UHP treatment, which may further lower the oxidative stress of cells to cause lesser expression of inflammatory cytokines and thereby exhibit better cell protection. Caffeic acid administration significantly inhibited the expressions of COX-2 and iNOS proteins when compared with that of the corresponding protein in the PA group ($p < 0.05$). However, caffeic acid did not remarkably reduce the expression ratio of P-P38/P38 ($p > 0.05$).

In addition to inflammatory pathways, the mitochondrial pathway is also mediated by ROS and may lead to cell apoptosis [11, 38]. Bax and Bcl-2 were apoptotic related proteins of the Bcl-2 family, which played an important role in the mitochondrial pathway. A phenolic compound like naringin was proved to inhibit cell apoptosis by regulating the Bax/Bcl-2 pathway [39]. In the PA treatment group of the present study, the expression level of Bax was increased significantly when compared with that in the CK group or BSA group (Figure 6), which indicated the proapoptosis protein was activated and

would interact with mitochondrial membrane to decline MMP. Meanwhile, the Bcl-2 expression was declined remarkably by the PA treatment (Figure 6), which indicated that the bioactivity of this antiapoptosis protein may reduce. With the effects of the PA treatment on Bax and Bcl-2, the MMP would be disrupted and the mitochondrial permeability might be damaged, and thereby finally stimulate cell apoptosis, which could be proved as the increased expression level of cytochrome C in this study. When cells were incubated with IB fraction, regardless of UHP treatment, the expression of Bax was significantly inhibited while the expression of Bcl-2 was markedly promoted in contrast with the PA group (Figure 6, $p < 0.05$), which provide possible pathways for IB fraction administration to achieve a higher MMP, decrease mitochondrial membrane depolarization level, and prevent the PA induced cell apoptosis. However, UHP treatment did not significantly enhance the effects of the IB phenolic fraction on the expressions of the Bax, Bcl-2, and cytochrome C proteins ($p > 0.05$). Caffeic acid also significantly inhibited the expressions of the Bax and cytochrome C proteins and upgraded the Bcl-2 expression when compared with that of the counterpart in the PA group ($p < 0.05$). However, the effect of caffeic acid was weaker than those of the IB phenolic fractions with or without UHP treatment, indicating some other phenolic compounds in the IB phenolic fraction may also contribute to the antiapoptosis, which needs to be proved by further experiments.

4. Conclusions

The results of this study revealed that the OPF phenolic fractions could prevent the PA induced lipotoxicity. Among the three phenolic fractions, the IB phenolic fraction showed the best effect to inhibit TG accumulation, reduce intracellular ROS, decrease the apoptotic rate, and prevent MMP depolarization. After the UHP treatment, the protective effect of the IB phenolic fraction was increased. Inhibition of inflammation by downregulating the expression of P-P38, COX-2, and iNOS, and suppression of mitochondrial mediated apoptosis by downregulating Bax and cytochrome C and upregulating Bcl-2 might be the potential mechanism pathway for the IB phenolic fraction to achieve the lipotoxicity preventive effect. Besides, as the most abundant phenolic compound in OPF phenolic fractions, caffeic acid exhibited similar effects as the IB fraction, and probably achieved such effect through the same pathway. Therefore, the OPF phenolic fractions, especially the IB phenolic fraction, might be utilized as natural materials for the development of antilipotoxicity functional food. The UHP treatment might be the potential method to increase this bioactivity of OPF.

Data Availability

All data included in this study are available upon request by contacting the corresponding author.

Conflicts of Interest

There are no conflicts of interest regarding the publication of this article.

Authors' Contributions

Xiaojing Liu and Ou Wang contributed equally to this work.

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Review Article

Chemical Constituents and Health Benefits of Four Chinese Plum Species

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Prunus is a large genus in the Rosaceae family of flowering plants, comprising over 340 species inhabiting variable landscapes in the world. Over 500 listed phytochemicals have been isolated from this single genus so far. The present study focused four Chinese *Prunus* species, viz., *Prunus cerasifera*, *Prunus domestica*, *Prunus salicina*, and *Prunus spinosa*, due to their uses, demand, nutritional value, medicinal importance, and diverse biological potential. The current review article highlights the details about the active phytochemicals and various pharmacological activities already reported. Almost 212 compounds, the majority of which are flavonoids, phenolic acids, anthocyanins, and their derivatives, which have been isolated from these four *Prunus* species fall in different categories and are helpful to evade chronic oxidative stress-mediated diseases. A huge variation exists in the total phytochemicals composition in different *Prunus* species, making these species to have different biological activities in multiple disease conditions, and even the same variety growing under different edaphic conditions may have different antioxidant capacities. It is suggested to perform extensive and indepth studies to find new phytochemicals from these four Chinese *Prunus* species which could boost the local industry to fulfill the increasing demands.

1. Introduction

Prunus is a large genus (340 species) comprising variable habit of plants belonging to family Rosaceae of flowering plants. The major representatives of the genus *Prunus* include plums, cherries, peaches, apricots, and almonds [1]. Over 500 listed phytochemicals have been isolated from this single genus so far from this genus. In the past few decades, plums have been explained as health-promoting foods due to their anti-inflammatory actions, improving neurological disorders and strong antioxidant nature mainly because of phenolic compounds and anthocyanins [2].

Prunus species are rich in biologically active ingredients, e.g., apigenin bears strong anticancer properties as a study by Jiang and colleagues showed that it inhibits hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF) expression in human ovarian cancer cells [3]. Moreover, Apigenin inhibited tumorigenesis as examined by Matrigel plug assay and chorioallantoic membrane assay (CAM assay) [3]. Similarly, another active ingredient luteolin in *Prunus* species, which is also reported to show

strong anticancer activity by inhibiting the growth and invasion of prostate cancer (PC3) cells, is used to check lung metastasis in an *in vivo* study, and hence, it is highlighted that luteolin targets E-cadherin and may be a useful way to cure invasive prostrate cancer [4]. Chrysin shows anticancer effects by potentially inhibiting HIF-1α [5], while kaempferol, quercetin, and multiforin extracted from *Prunus* species showed strong antioxidant potential in the DPPH radical scavenging assay [6–8]. Traditionally, plums are dried and processed for a prolonged shelf life of plums. Considering the dried fruits in terms of the phytochemical constituents, the presence of antioxidants has been reported to be the maximum compared with other dried fruits or vegetables [9].

Reactive oxygen/nitrogen species (ROS/RNS) are important physiological molecules involved, among others, in cell signaling and host defense [10]. However, an uncontrolled and excessive ROS/RNS production may overwhelm the antioxidant capacity of the cells and lead to deleterious oxidative stress, which is implicated in the pathogenesis of numerous chronic diseases [11]. Various polyphenolic compounds or plant extracts, as natural antioxidants, are regarded as a potential treatment option in prophylaxis and adjunctive therapy of CVD, and their mechanisms of action, including possible interactions with ROS/RNS, are closely looked into the restriction of onset of oxidative stress-mediated disease [12].

1.1. Aims and Objectives. As the genus *Prunus* is one of the largest ones, there is no authentic and detailed study describing the biological activities of its constituents in four Chinese *Prunus* species, viz., *P. cerasifera*, *P. domestica*, *P. salicina*, and *P. spinosa*. Hence, the current review article will highlight the details about the active phytochemicals and various pharmacological activities already reported.

2. Chemical Constituents of Four Plum Species

Almost 212 compounds, the majority of which were flavonoids and phenolic acids, isolated from different *Prunus* species have been classified under different categories and summarized in Tables 1–3.

2.1. Flavonoids. Approximately 113 flavonoids compounds (Table 1, Figure 1) including 9 anthocyanins (**1**–**9**), 33 proanthocyanins (**10**–**42**), 55 flavone and flavonols (**43**–**92, 105**–**106, 110**–**111, 113**), 13 dihydroflavonols (**93**–**104**), 3 isoflavonoids (**107**–**109**), and 1 dihydrochalcone (**112**) were identified from *P. cerasifera*, *P. domestica*, *P. salicina*, and *P. spinosa*.

2.2. Phenolic Acids. The analysis of phenolic acids (Table 2, Figure 2) indicated that chlorogenic acid was the predominant compound. Seven benzoic acid derivatives (**114**–**120, 140**, **141**), 7 cinnamic acid derivatives (**121**–**127, 148**, **149**), 6 caffeoylquinic acid derivatives (**128**–**133**), 2 feruloylquinic acid derivatives (**134**, **135**), 3 comaroylquinic acid derivatives (**136**–**139**), 2 shikimic acid (**142**, **143**), ellagic acid (**145**), 2 propionic acid (**146**, **147**), 2 abscisic acid (**152**, **153**), and 3 abscisic acid derivatives (**154**–**158**) were identified from *P. cerasifera*, *P. domestica*, *P. salicina*, and *P. spinosa*.

2.3. Others. A total of 16 individual phenolic compounds have been identified from *P. domestica*. The major components identified and quantitated by HPLC-ESI-MS comprise (**163**, **166**, **167**, **180**), along with a neolignane (**171**), two guajacyl-glycerin-coniferyl aldehyde isomers (**172**, **173**) and dehydro-diconiferyl aldehyde (**174**). Three cyanogenic glycosids (**167**-**169**) were identified from *P. spinosa*. Four organic acids (**175**-**178**) and vitamin C (**179**) contents of three plum species (*P. cerasifera*, *P. domestica*, and *P. spinosa*.) were determined by the HPLC method. Two carbaldehyde derivatives (**180**, **181**), 3 steroids (**185**–**187**), 4 terpenoids (**188**-**191, 204, 205**), 8coumarin derivatives (**193**-**201**), 3 cinnamic acid derivatives (**202**, **203**, **209**), and 2 glucosyl terpenates (**205**, **208**) were identified from different *Prunus* species (Table 3, Figure 3).

3. Biological Activities of Four Plum Species

Various compounds have been isolated from different *Prunus* species and classified under different categories, viz., polyphenols, flavonoids, anthocyanins, alkaloids, and terpenes. The pharmacological properties of all four Chinese plum species have been discussed in the next sections.

3.1. Prunus cerasifera. Cherry plum (*P. cerasifera* Ehrh. and Rosaceae) or "Myrobalan plum" is a well-known medicinal plant and is a rich source of polyphenolics, anthocyanins, carotenoids, flavonoids, various organic acids, aromatic compounds, tannins, minerals, vitamins, and antioxidant compounds [13, 71]. The Myrobalan plum fruits are rich in health-promoting phytochemicals that help prevent the onset of different diseases. The *P. cerasifera* fruit has strong antibacterial and antifungal potential of pathogenic class for medical sciences and agricultural product-related pathogens [72]. The purple Myrobalan plum fruit peels have high amounts of anthocyanins and phenolic compounds due to which it showed high antioxidant activity [24]. Considerable differences have also been reported for *P. cerasifera* accessions with regard to total phenolics and antioxidant activity [73]. Another report highlighted that tannins found in *P. cerasifera* have high antioxidant properties and showed potential inhibition of tyrosinase activity and, thus, could be used as a strong inhibitor for the onset of melanogenesis [29]. Gunduza and Saracoglu studied various fruit characteristics (total phenolics and antioxidant activity) in different accessions of *P. cerasifera* from Turkey and reported considerable variation for these characters among the accessions, but the phenolic contents are comparable to many other plum species [73]. The antioxidant capability of plums mainly depends on the level of ripening, and this trend is entirely opposite to that of the phenolic contents [74]. The leaves of *P. cerasifera* are enriched with biologically active ingredients, notably tannins, flavonoids, and phenolic acids,

Table 1: Flavonoids compounds isolated and identified from different *Prunus*.

No.	Compounds	Sources	Ref.
1	Cyanidin-3-galactoside	*P. cerasifera Ehrh.*, *P. salicina Lindl.*	[13–17]
2	Cyanidin-3-glucoside	*P. cerasifera Ehrh.*, *P. salicina Lindl.*, *P. domestica L.*, *P. spinosa L.*	[13–15, 18–27]
3	Cyanidin-3-rutinoside	*P. cerasifera Ehrh.*, *P. salicina Lindl.*, *P. domestica L.*, *P. spinosa L.*	[13–27]
4	Cyanidin-3-acetylglucoside	*P. cerasifera Ehrh.*	[13]
5	Cyanidin-3-xyloside	*P. cerasifera Ehrh.*, *P. domestica L.*	[13, 23, 27]
6	Cyanidin-3-pentoside	*P. spinosa L.*	[21, 28]
7	Peonidin-3-rutinoside	*P. spinosa L.*, *P. domestica L.*	[18, 21–25, 27]
8	Peonidin-3-glucoside	*P. spinosa L.*, *P. domestica L.*	[19–25, 27]
9	Peonidin-3-acetylglucoside	*P. spinosa L.*	[21]
10	Afzelechin	*P. cerasifera Ehrh.*, *P. spinosa L.*	[29, 30]
11	Epiafzelechin	*P. cerasifera Ehrh.*, *P. spinosa L.*	[29–31]
12	(+)-Catechin	*P. salicina Lindl.*, *P. domestica L.*, *P. cerasifera Ehrh.*, *P. spinosa L.*	[8, 15, 16, 24, 29–39]
13	(−)-Epicatechin	*P. salicina Lindl.*, *P. domestica L.*, *P. spinosa L.*	[15, 16, 24, 29–32, 35, 36, 38–42]
14	Epigallocatechin	*P. spinosa L.*	[30]
15	Gallocatechin	*P. spinosa L.*	[30]
16	Procyanidin B1	*P. salicina Lindl.*, *P. domestica L.*	[15, 16, 35, 36, 39]
17	Procyanidin B2	*P. salicina Lindl.*, *P. domestica L.*	[15, 16, 28, 35, 36, 39]
18	Epicatechin 3-*O*-gallate	*P. domestica L.*	[39]
19	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-catechin	*P. spinosa L.*	[30, 31, 43]
20	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-catechin 5,7,11,12,5′11′,12′-heptamethyl ether-3,3′-diacetate	*P. spinosa L.*	[31, 43]
21	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-catechin-nona-acetate	*P. spinosa L.*	[43]
22	*Ent*-epigallocatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin	*P. spinosa L.*	[30]
23	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epiafzelechin	*P. spinosa L.*	[30, 43, 44]
24	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epiafzelechin 7,12,5′,12′-tetramethyl ether	*P. spinosa L.*	[44]
25	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin	*P. spinosa L.*, *P. spinosa L.*	[32, 36, 43]
26	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin 5,7,11,12,5′11′,12′-heptamethyl ether-3,3′-diacetate	*P. spinosa L.*	[43]
27	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin 7,11,12,5′11′,12′-hexamethyl ether-3,5,3′-triacetate	*P. spinosa L.*	[43]
28	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin-nona-acetate	*P. spinosa L.*	[43]
29	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin	*P. spinosa L.*	[30, 32, 43–45]
30	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epiafzelechin 7,12,5′,12′-tetramethyl ether-3,5,3′-triacetate	*P. spinosa L.*	[44]
31	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epiafzelechin-hepta-acetate	*P. spinosa L.*	[43, 44]
32	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin 7,12,5′,11′,12′-pentamethyl ether	*P. spinosa L.*	[44]
33	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin-octa-acetate	*P. spinosa L.*	[44]
34	*Ent*-epiafzelechin -(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin 5,7,12,5′11′,12′-hexamethyl ether-3,3′-diacetate	*P. spinosa L.*	[43]
35	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin 7,12,5′11′,12′-pentamethyl ether-3,5,3′-triacetate	*P. spinosa L.*	[43]
36	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin-3′-*O*-gallate	*P. spinosa L.*	[30]
37	*Ent*-epicatechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-epicatechin-3′-*O*-gallate	*P. spinosa L.*	[30]
38	*Ent*-epiafzelechin-(2$\alpha\longrightarrow O\longrightarrow$7; 4$\alpha\longrightarrow$8)-catechin	*P. spinosa L.*	[30, 45]

Table 1: Continued.

No.	Compounds	Sources	Ref.
39	*Ent*-epiafzelechin-(2α⟶O⟶7; 4α⟶8)-catechin-octa-acetate	*P. spinosa L.*	[43]
40	Proanthocyanidin oligomer	*P. domestica L.*	[46]
41	Epicatechin-4,8′-epicatechin-4′,8″-epicatechin	*P. domestica L.*	[36]
42	Epicatechin-4,8′-epicatechin-(2′α⟶O⟶7″; 4′α⟶8″)-epicatechin	*P. domestica L.*	[36]
43	Quercetin	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[28, 30–32, 36, 38, 47–50]
44	Quercetin-3-O-α-L-arabinofuranoside	*P. spinosa L.*, *P. spinosa L.*	[32, 33, 47]
45	Quercetin-3-rutinoside	*P. salicina Lindl.*, *P. domestica L.*, *P. cerasifera Ehrh.*, *P. spinosa L.*	[8, 15, 16, 21, 22, 24, 26, 28, 30, 35–39, 42, 49–55]
46	Quercetin-3-O-glucoside	*P. salicina Lindl.*, *P. domestica L.*, *P. spinosa L.*	[15–17, 19, 21, 22, 24, 26, 28, 35, 36]
47	Quercetin-3-O-xyloside	*P. salicina Lindl.*, *P. cerasifera Ehrh.*	[15–17, 56]
48	Quercetin-3-O-arabinoside	*P. salicina Lindl.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[15, 16, 24, 56]
49	Quercetin-3-O-rhamnoside	*P. salicina Lindl.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[15, 16, 28, 32, 35, 36, 56]
50	Quercetinl-7-O-α-L-rhamnopyransoide	*P. domestica L.*	[32]
51	Quercetin-3-O-β-D-galactoside	*P. salicina Lindl.*, *P. domestica L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[17, 19, 24, 26, 28, 32, 35, 36, 56]
52	Quercetin-3-O-α-D-xylopyranoside	*P. domestica L.*	[32]
53	Quercetin-3-O-α-D-glucopyranoside	*P. domestica L.*	[32]
54	Quercetin-pentoside	*P. domestica L.*	[28, 36, 39]
55	Quercetin-pentoside-rhamnoside	*P. domestica L.*	[36]
56	Quercetin pentosyl-hexoside	*P. salicina Lindl.*, *P. domestica L.*	[15, 16, 39]
57	Quercetin pentosyl-pentoside	*P. salicina Lindl.*	[15, 16, 39]
58	Quercetin-acetylhexoside	*P. salicina Lindl.*, *P. domestica L.*	[15, 16, 39]
59	Quercetin-deoxyhexose	*P. domestica L.*	[39]
60	Quercetin-3-O-(4″-O-β-D-glucopyranosyl)-α-L-rhamnopyransoide	*P. domestica L.*	[32]
61	Quercetin-3-O-(6″-O-α-L-rhamnopyranosyl)-β-D-glucopyranoside(rutin)	*P. spinosa L.*	[32]
62	Quercetin-3-O-(2″-O-β-D-glucopyranosyl)-α-L-arabinofuranoside	*P. spinosa L.*	[32, 33, 47]
63	Hyperin	*P. cerasifera Ehrh.*	[50]
64	Kaempferol	*P. spinosa L.*, *P. domestica L.*, *P. spinosa L.*, *P. cerasifera Ehrh.*	[30–33, 47–50, 57, 58]
65	Apigenin	*P. cerasifera Ehrh.*	[50]
66	Kaempferol-3-O-α-D-glucoside	*P. cerasifera Ehrh.*	[50, 56]
67	Kaempferol-3,7-di-O-α-L-rhamnopyranoside	*P. spinosa L.*	[30–32, 47]
68	Kaempferol-7-O-α-L-rhamnopyransoide	*P. spinosa L.*	[32, 33, 47]
69	Kaempferol-3-O-α-L-arabinofuranoside-7-O-α-L-rhamnopyransoide	*P. spinosa L.*	[32, 33, 47]
70	Kaempferol-3-O-α-L-arabinofuranoside	*P. spinosa L.*, *P. cerasifera Ehrh.*	[30, 32, 33, 47, 56]
71	Kaempferol-3-O-arabinoside-7-O-rhamnoside	*P. spinosa L.*	[30, 32]
72	Kaempferol-3-O-α-D-xylopyranoside-7-O-α-L-rhamnopyransoide(lepidoside)	*P. spinosa L.*	[32, 33]
73	Kaempferol-3-O-α-D-xylopyranoside	*P. spinosa L.*, *P. cerasifera Ehrh.*	[32, 56]
74	Kaempferol-3-O-α-L-rhamnopyransoide	*P. spinosa L.*, *P. cerasifera Ehrh.*	[32, 33, 56]
75	Kaempferol-3-O-(2″-E-p-conmaroyl)-α-L-arabinofuranoside	*P. spinosa L.*	[32]
76	Kaempferol-3-O-rutinoside	*P. spinosa L.*	[21, 28]
77	Kaempferol-3-O-α-L-arabinopyranoside-7-O-α-L-rhamnopyransoide	*P. spinosa L.*	[33]
78	5,4′-Dihydroxyflavone-7-O-α-D-glucoside	*P. domestica L.*	[59]
79	Kaempferol-hexoside	*P. domestica L.*	[28, 39]
80	Kaempferol-pentoside-rhamnoside	*P. domestica L.*	[28]

Table 1: Continued.

No.	Compounds	Sources	Ref.
81	Kaempferol-pentoside	*P. domestica L.*	[28]
82	Kaempferol-3-*O*-(4″-*O*-*β*-D-glucopyranosyl)-*α*-L-rhamnopyransoide	*P. spinosa L.*	[32]
83	Kaempferol-3-*O*-(2″-E-*p*-conmaroyl)-*α*-L-arabinofuranoside-7-*O*-*α*-L-rhamnopyransoide	*P. spinosa L.*	[32, 33, 47]
84	Myricetin	*P. salicina Lindl.*, *P. domestica L.*	[20, 35, 40, 48, 49]
85	Luteolin	*P. spinosa L.*, *P. cerasifera Ehrh.*	[41, 50]
86	Luteolin-4′-*O*-*α*-D-glucoside	*P. cerasifera Ehrh.*	[50]
87	Prudomestin	*P. domestica L.*	[60]
88	3,5,7-Trihydroxy-4′-methoxyflavanone	*P. domestica L.*	[57]
89	Isorhamnetin-3-*O*-rutinoside	*P. domestica L.*	[24, 28, 35]
90	Isorhamnetin-3-*O*-glucoside	*P. domestica L.*	[24, 26]
91	Isorhamnetin-3-*O*-galactoside	*P. domestica L.*	[26]
92	Quercetin-4′-*O*-*α*-D-glucoside	*P. cerasifera Ehrh.*	[50]
93	Hesperetin	*P. salicina Lindl.*	[40]
94	Isosakuranetin	*P. domestica L.*	[60]
95	Dihydrokaempferide	*P. domestica L.*	[60]
96	Naringenin	*P. domestica L.*	[60]
97	3,5,7-Trihydroxy-8,4′-dimethoxyflavanone	*P. domestica L.*	[58, 60]
98	3,5,7-Trihydroxy-6,4′-dimethoxyflavanone	*P. domestica L.*	[59, 60]
99	5,7,4′-Trihydroxy-3-methoxyflavanone.	*P. domestica L.*	[60]
100	7,4′-Dimethylaromadendrin	*P. domestica L.*	[57]
101	5,7-Dihydroxy-4′-methoxy-dihydroflavonol	*P. domestica L.*	[57]
102	5,7-Dihydroxy-8,4′-dimeth-oxyllavonol	*P. domestica L.*	[57]
103	Dihydrokaempferol-3-*O*-*α*-L-rhamnoside	*P. cerasifera Ehrh.*	[56]
104	Dihydrokaempferol-3-*O*-*α*-D-glucoside	*P. cerasifera Ehrh.*	[56]
105	Prudomestiside A	*P. domestica L.*	[59]
106	Prudomestiside B	*P. domestica L.*	[59]
107	Purunuside A	*P. domestica L.*	[61]
108	Purunuside B	*P. domestica L.*	[61]
109	Purunuside C	*P. domestica L.*	[61]
110	Prunusins A	*P. domestica L.*	[58]
111	Prunusins B	*P. domestica L.*	[58]
112	Phloridzin	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
113	5,2′-Dihydroxy-7,5′-dimethoxyflavanone	*P. domestica L.*	[59]

Table 2: Phenolic acids compounds isolated and identified from different *Prunus* species.

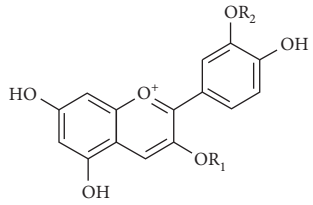
No.	Compounds	Sources	Ref.
114	Vanillic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37, 41, 49, 52, 53, 62, 63]
115	Protocatechuic acid	*P. spinosa L.*, *P. domestica L.*, *P. spinosa L.*	[6, 30, 39, 41, 42, 48, 52–54, 62]
116	*p*-Hydroxybenzoic acid	*P. spinosa L.*, *P. domestica L.*	[41, 49, 53]
117	Vanillic acid-*β*-glucoside	*P. domestica L.*	[6, 36]
118	Vanillic acid-*α*-D-glucopyranoside	*P. domestica L.*	[52]
119	Gallic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*, *P. spinosa L.*	[19, 30, 37, 49, 50, 53, 54, 62]
120	Syringic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[34, 37, 38, 41, 49, 53]
121	Caffeic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*, *P. spinosa L.*	[6, 8, 19, 20, 30, 32, 34, 37, 42, 48, 49, 51, 52, 54]
122	Caffeic acid methyl ester	*P. domestica L.*	[52, 63]

Table 2: Continued.

No.	Compounds	Sources	Ref.
123	Ferulic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37, 48, 49, 62]
124	*p*-Coumaric acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[6, 8, 24, 32, 34, 37, 39, 41, 42, 49, 51, 52]
125	Cinnamic acid	*P. domestica L.*	[49]
126	Ferulic acid-β-D-glucopyranoside	*P. domestica L.*	[52, 63]
127	Coniferine	*P. domestica L.*	[52, 63]
128	3-*O*-Caffeoylquinic acid (neochlorogenic acid)	*P. salicina Lindl.*, *P. domestica L.*, *P. spinosa L.*	[8, 15–17, 21, 22, 24–26, 28, 32, 33, 35, 36, 42, 48, 51, 53, 54]
129	4-*O*-Caffeoylquinic acid	*P. spinosa L.*, *P. domestica L.*	[21, 22, 24, 28, 32, 33, 35, 36, 42, 51–53]
130	5-*O*-Caffeoylquinic acid	*P. salicina Lindl.*, *P. domestica L.*, *P. cerasifera Ehrh.*, *P. spinosa L.*	[15, 16, 22, 25, 26, 28, 32, 34–38, 42, 48, 49, 51–54, 62, 64]
131	3-*O*-Caffeoylquinic acid methyl ester	*P. domestica L.*	[36, 52, 63]
132	4-*O*-Caffeoylquinic acid methyl ester	*P. domestica L.*	[36, 52, 63]
133	5-*O*-Caffeoylquinic acid methyl ester	*P. domestica L.*	[36]
134	3-*O*-Feruloylquinic acid	*P. spinosa L.*, *P. domestica L.*	[21, 32, 33, 35, 36]
135	4-*O*-Feruloylquinic acid	*P. spinosa L.*	[32]
136	3-*O*-Coumaroylquinic acid	*P. spinosa L.*, *P. domestica L.*	[8, 15, 16, 21, 25, 28, 32, 33, 36, 53]
137	4-*O*-Comaroylquinic acid	*P. spinosa L.*	[32]
138	5-*O*-Comaroylquinic acid	*P. spinosa L.*.	[32]
139	3-Coumaroylquinic acid methyl ester	*P. domestica L.*	[36]
140	Salicylic acid	*P. domestica L.*	[49]
141	2,3-Dimethylbenzoic acid	*P. domestica L.*	[38]
142	Shikimic acid	*P. domestica L.*	[39]
143	3-Caffeoylshikimic acid	*P. domestica L.*	[35, 36]
144	2-(3′,4′-Dihydroxyphenyl)acetic acid	*P. spinosa L.*	[65]
145	Ellagic acid	*P. domestica L.*	[49]
146	3-(4′-Hydroxyphenyl)propionic acid	*P. spinosa L.*	[65]
147	3-(3′,4′-Dihydroxyphenyl)propionic acid	*P. spinosa L.*	[65]
148	Caffeoyl hexoside	*P. domestica L.*	[39]
149	*p*-Coumaroyl-hexoside	*P. domestica L.*	[39]
150	3,4-Dihydroxybenzoyl-glucoses	*P. domestica L.*	[36]
151	Rosmarinic acid	*P. domestica L.*	[49]
152	Abscisic acid	*P. domestica L.*	[52, 66]
153	β-D-glusosylabscisate	*P. domestica L.*	[52, 66]
154	Rel-5-(1*R*,5*S*-dimethyl-3*R*,4*R*,8*S*-trihydroxy-7-oxabicyclo (3,2,1)-oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid	*P. domestica L.*	[52, 66]
155	Rel-5-(1*R*,5*S*-dimethyl-3*R*,4*R*,8*S*-trihydroxy-7-oxa-6-oxobicyclo (3,2,1) oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid	*P. domestica L.*	[52, 66]
156	Rel-5-(3*S*,8*S*-dihydroxy-1*R*,5*S*-dimethyl-7-oxa-6-oxobicyclo (3,2,1) oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid	*P. domestica L.*	[52, 66]
157	Rel-5-(3*S*,8*S*-dihydroxy-1R,5*S*-dimethyl-7-oxa-6-oxobicyclo s oct-8-yl)-3-methyl-2*Z*,4*E*-pentadienoic acid-3′-*O*-α-D-glucopyranoside	*P. domestica L.*	[52, 66]
158	(6*S*,9*R*)-roseoside	*P. domestica L.*	[52, 66]

Table 3: Others compounds isolated and identified from different *Prunus*.

No.	Compounds	Sources	Ref.
159	*p*-Hydroxybenzaldehyde	*P. domestica L.*	[53]
160	Benzoic acid	*P. spinosa L.*, *P. domestica L.*	[36, 38, 49, 53, 62]
161	Syringaldehyde	*P. domestica L.*	[53]
162	Hydroxy-4-methoxybenzaldehyde	*P. domestica L.*	[38]
163	Vanillin	*P. spinosa L.*, *P. domestica L.*	[49, 53, 62]
164	Protocatechuic aldehyde	*P. spinosa L.*	[62]
165	Coniferyl aldehyde	*P. domestica L.*	[53]
166	Dimethoxycinnamaldehyde	*P. domestica L.*	[53]
167	Amygdalin	*P. cerasifera Ehrh. P. domestica L.*	[53, 67]
168	Prunasin	*P. cerasifera Ehrh.*	[67]
169	Sambunigrin	*P. cerasifera Ehrh.*	[67]
170	Coniferyl aldehyde	*P. domestica L.*	[52]
171	Pinoresinol-*O*-*α*-D-glucopyranoside	*P. domestica L.*	[52]
172	Guajacyl-glycerin-coniferyl aldehyde-1	*P. domestica L.*	[53]
173	Guajacyl-glycerin-coniferyl aldehyde-2	*P. domestica L.*	[53]
174	Dehydro-diconiferyl aldehyde	*P. domestica L.*	[53]
175	Fumaric acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
176	Malic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
177	Succinic acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
178	Citric acid	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
179	Vitamin C	*P. spinosa L.*, *P. domestica L.*, *P. cerasifera Ehrh.*	[37]
180	2-(5-Hydroxymethyl-2′,5′-dioxo-2′,3′,4′,5′-tetrahydro-1′H-1,3′-bipyrrole)-carbaldehyde	*P. domestica L.*	[52]
181	Hydroxymethylfurfural	*P. domestica L.*	[52]
182	Benzyl-*β*-primeveroside	*P. domestica L.*	[52, 63]
183	Vanillin diglucoside	*P. domestica L.*	[53]
184	4-Amino-4-carboxychroman-2-one	*P. domestica L.*	[6]
185	*β*-Sitosterol	*P. domestica L.*, *P. cerasifera Ehrh.*	[50, 61]
186	Daucosterol	*P. cerasifera Ehrh.*	[50]
187	Stigmasterol	*P. cerasifera Ehrh.*	[50]
188	Ursolic acid	*P. cerasifera Ehrh.*	[50]
189	Arjunolic acid	*P. cerasifera Ehrh.*	[50]
190	Niga-ichigoside F1	*P. cerasifera Ehrh.*	[50]
191	Lupeol	*P. cerasifera Ehrh.*	[50]
192	3-(*α*-D-glucopyranosyloxymethyl)-2-(4-hydroxy-3-methoxyphenyl)-5-(3-hydroxypropyl)-7-methoxy-(2*R*,3*S*)-dihydrobenzofuran	*P. domestica L.*	[52, 66]
193	5-Hydroxy-6-methoxy-7-*O*-*β*-D-glucosyl coumarin	*P. spinosa L.*	[30, 31]
194	5-Hydroxy-6-methoxy-7-*O*-*α*-D-rhamnosyl coumarin	*P. spinosa L.*	[30]
195	5,7-Dimethoxy-6-hydroxy-coumarin	*P. domestica L.*	[57, 68]
196	7-Methocycoumarin	*P. domestica L.*	[42]
197	Esculin	*P. domestica L.*	[36]
198	Scopolin	*P. domestica L.*	[69]
199	Scopoletin	*P. domestica L.*	[52, 63, 70]
200	Magnolioside	*P. domestica L.*	[52, 63]
201	6,7-Methylenedioxy-8-methoxycoumarin	*P. domestica L.*	[61]
202	(3-*O*-*cis*-p-Coumaroyl-*α*-D-fructofuranosyl)-(2⟶1)-*α*-D-glucopyranoside	*P. domestica L.*	[69]
203	(3-*O*-*trans*-p-Coumaroyl-*α*-D-fructofuranosyl)-(2⟶1)-*α*-D-glucopyranoside	*P. domestica L.*	[69]
204	2,7-Dimethyl-2*E*,4*E*-octadienedioic acid	*P. domestica L.*	[69]
205	*α*-D-Glucopyranosyl 7-carboxy2-methyl-2*E*,4*E*-octadienate	*P. domestica L.*	[69]
206	1*S*-(4-*α*-D-glucopyranosyl-3-methoxyphenyl)-2*R*-[4-(3-hydroxypropyl)-2-methoxyphenoxy]-1,3-propanediol	*P. domestica L.*	[69]
207	*α*-D-glucopyranosyl 9-carboxy-8-hydroxy-2,7-dimethyl-2*E*,4*E*-nonadienate	*P. domestica L.*	[69]
208	8-Hydroxy-2,7-dimethyl-2*E*,4*E*-decadienedioic acid 1-*α*-D-glucopyranyl ester 10-Methyl ester	*P. domestica L.*	[69]
209	*α*-D-glucopyranosyl cinnamate	*P. domestica L.*	[69]
210	(-)-Dihydrodehydrodiconiferyl alcohol	*P. domestica L.*	[70]
211	(-)-Ficusal	*P. domestica L.*	[70]
212	(*E*)-3, 3′-dimethoxy -4,4′-dihydroxystilbene	*P. domestica L.*	[70]



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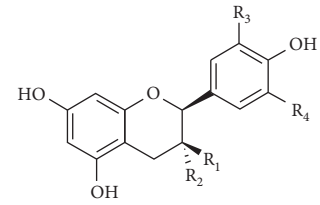
2 R_1 = glucosyl; R_2 = H
3 R_1 = α-L-rhamnosyl-β-D-glucose; R_2 = H
4 R_1 = acetylglucosyl; R_2 = H
5 R_1 = Xyl; R_2 = H
6 R_1 = pentosyl; R_2 = H
7 R_1 = α-L-rhamnosyl-β-D-glucose; R_2 = CH_3
8 R_1 = glucosyl; R_2 = CH_3
9 R_1 = acetylglucosyl; R_2 = CH_3



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12 R_1 = H; R_2 = OH; R_3 = OH; R_4 = H
13 R_1 = OH; R_2 = H; R_3 = OH; R_4 = H
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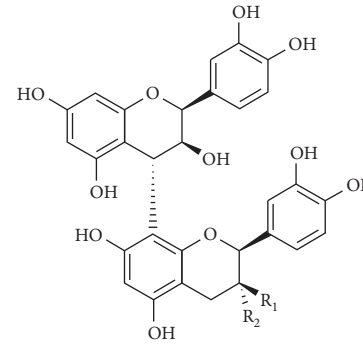

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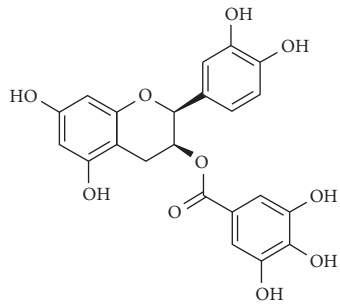

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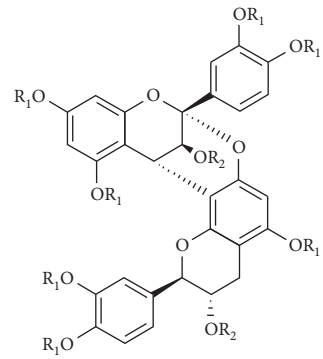
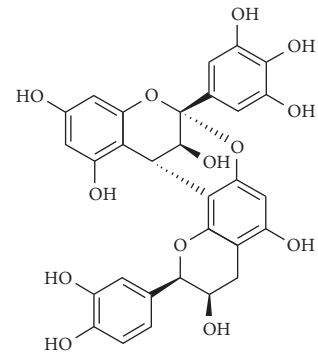


19 R_1 = H; R_2 = H
20 R_1 = CH_3; R_2 = Ac
21 R_1 = Ac; R_2 = Ac



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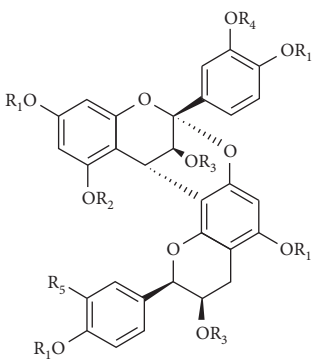
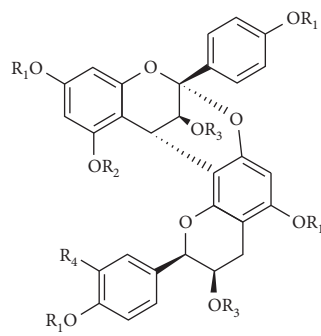
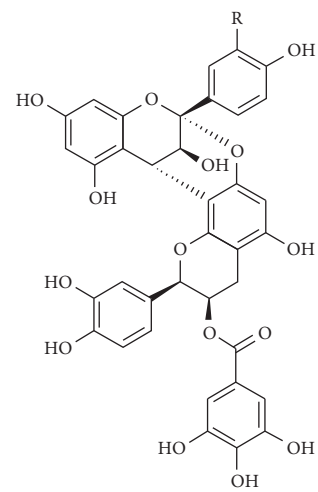


23 R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = H
24 R_1 = CH_3; R_2 = H; R_3 = H; R_4 = H; R_5 = H
25 R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = OH
26 R_1 = CH_3; R_2 = CH_3; R_3 = Ac; R_4 = CH_3; R_5 = OCH_3
27 R_1 = CH_3; R_2 = Ac; R_3 = Ac; R_4 = CH_3; R_5 = OCH_3
28 R_1 = Ac; R_2 = Ac; R_3 = Ac; R_4 = Ac; R_5 = OAc



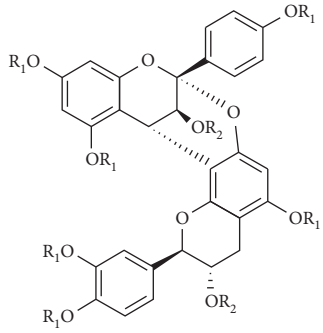
29 R_1 = H; R_2 = H; R_3 = H; R_4 = OH
30 R_1 = CH_3; R_2 = Ac; R_3 = Ac; R_4 = H
31 R_1 = Ac; R_2 = Ac; R_3 = Ac; R_4 = H
32 R_1 = CH_3; R_2 = Ac; R_3 = Ac; R_4 = OCH_3
33 R_1 = Ac; R_2 = Ac; R_3 = Ac; R_4 = OAc
34 R_1 = CH_3; R_2 = CH_3; R_3 = Ac; R_4 = OCH_3
35 R_1 = CH_3; R_2 = Ac; R_3 = Ac; R_4 = OCH_3



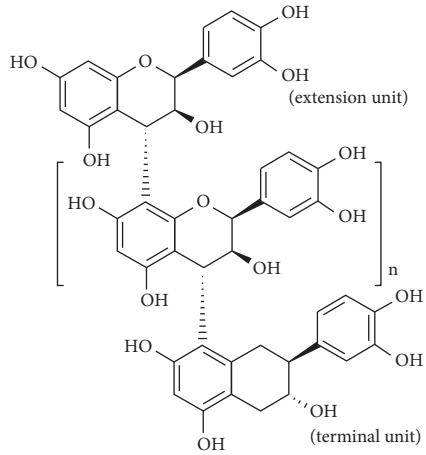
36 R = H
37 R = OH

(a)

Figure 1: Continued.



38 R_1 = H; R_2 = H
39 R_1 = Ac; R_2 = Ac



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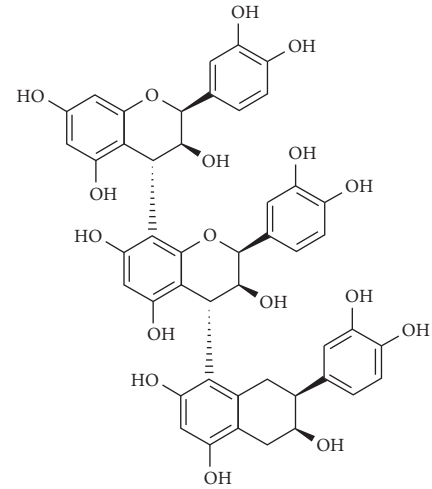

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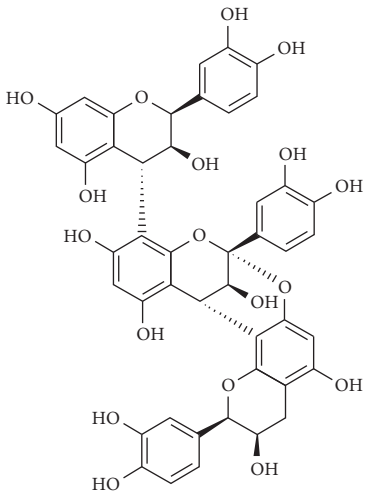

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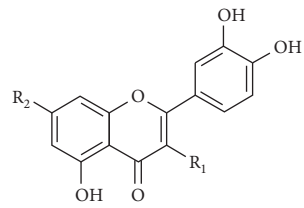
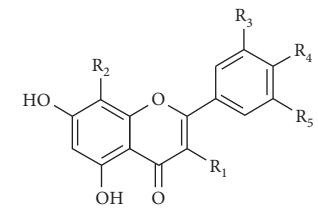
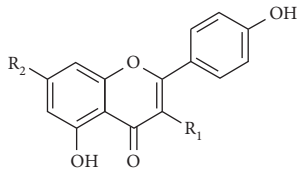


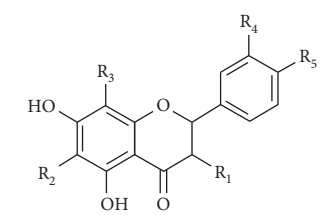
43 R_1 = OH; R_2 = OH
44 R_1 = *O*-L-arabinofuranose; R_2 = OH
45 R_1 = α-L-rhamnosyl-β-D-glucose; R_2 = OH
46 R_1 = α-D-Glu; R_2 = OH
47 R_1 = Xyl; R_2 = OH
48 R_1 = arabinoside; R_2 = OH
49 R_1 = α-L-rhamnopyranosyl; R_2 = OH
50 R_1 = OH; R_2 = α-L-rhamnopyranosyl
51 R_1 = *O*-β-D-Gal; R_2 = OH
52 R_1 = *O*-α-D-Xyl; R_2 = OH
53 R_1 = β-D-Glu; R_2 = OH
54 R_1 = pentosyl; R_2 = OH
55 R_1 = pentosyl-α-L-rhamnopyranosyl; R_2 = OH
56 R_1 = pentosylhexoside; R_2 = OH
57 R_1 = pentosyl-pentosyl; R_2 = OH
58 R_1 = acetylhexoside; R_2 = OH
59 R_1 = deoxylhexoside; R_2 = OH
60 R_1 = 4″-β-D-glucopyrnosyl-α-L-rhamnopyrnosyl; R_2 = OH
61 R_1 = 6″-α-L-rhamnopyrnosyl-β-D-glucopyrnosyl; R_2 = OH
62 R_1 = 2″-β-D-glucopyrnosyl-α-L-arabinofuranose; R_2 = OH
63 R_1 = β-D-galactopyranosyloxy; R_2 = OH



84 R_1 = OH; R_2 = H; R_3 = OH; R_4 = OH; R_5 = OH
85 R_1 = H; R_2 = H; R_3 = OH; R_4 = OH; R_5 = H
86 R_1 = H; R_2 = H; R_3 = OH; R_4 = b-D-Glu; R_5 = H
87 R_1 = OH; R_2 = OCH_3; R_3 = H; R_4 = OCH_3; R_5 = H
88 R_1 = OH; R_2 = H; R_3 = H; R_4 = OCH_3; R_5 = H
89 R_1 = *O*-α-L-Rha-β-D-Glu; R_2 = H; R_3 = OCH_3; R_4 = OH; R_5 = H
90 R_1 = *O*-Glu; R_2 = H; R_3 = OCH_3; R_4 = OH; R_5 = H
91 R_1 = *O*-Gal; R_2 = H; R_3 = OCH_3; R_4 = OH; R_5 = H
92 R_1 = OH; R_2 = H; R_3 = OH; R_4 = β-D-Glu; R_5 = H



64 R_1 = OH; R_2 = OH
65 R_1 = H; R_2 = OH
66 R_1 = β-D-Glu; R_2 = OH
67 R_1 = α-L-rhamnopyranosyl; R_2 = rhamnopyranosyl
68 R_1 = OH; R_2 = α-L-rhamnopyranosyl
69 R_1 = α-L-arabinofuranose; R_2 = α-L-rhamnopyranosyl
70 R_1 = α-L-arabinofuranose; R_2 = OH
71 R_1 = arabinofuranose; R_2 = rhamnopyranosyl
72 R_1 = O-β-D-xylopyranose; R_2 = α-L-rhamnopyranosyl
73 R_1 = O-β-D-xylopyranose; R_2 = OH
74 R_1 = α-L-rhamnopyranosyl; R_2 = OH
75 R_1 = 2″-E-p-conmaroyl-α-L-arabinofuranose; R_2 = OH
76 R_1 = α-L-rhamnosyl-β-D-glucose; R_2 = OH
77 R_1 = α-L-arabinofuranose; R_2 = α-L-rhamnopyranosyl
78 R_1 = H; R_2 = β-D-Glu
79 R_1 = hexosyl; R_2 = OH
80 R_1 = pentosyl-rhamnopyranosyl; R_2 = OH
81 R_1 = pentosyl; R_2 = OH
82 R_1 = 4″-β-D-glucopyrnosyl-α-L-rhamnopyrnosyl; R_2 = OH
83 R_1 = 2″-E-p-conmaroyl-α-L-arabinofuranose; R_2 = α-L-rhamnopyrnosyl



93 R_1 = H; R_2 = H; R_3 = H; R_4 = OH; R_5 = OCH_3
94 R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = OCH_3
95 R_1 = OH; R_2 = H; R_3 = H; R_4 = H; R_5 = OCH_3
96 R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = OH
97 R_1 = OH; R_2 = H; R_3 = OCH_3; R_4 = H; R_5 = OCH_3
98 R_1 = OH; R_2 = OCH_3; R_3 = H; R_4 = H; R_5 = OCH_3
99 R_1 = OCH_3; R_2 = H; R_3 = H; R_4 = H; R_5 = OH
100 R_1 = OCH_3; R_2 = H; R_3 = H; R_4 = H; R_5 = OCH_3
101 R_1 = H; R_2 = H; R_3 = H; R_4 = H; R_5 = OCH_3
102 R_1 = H; R_2 = H; R_3 = OCH_3; R_4 = H; R_5 = OCH_3
103 R_1 = O-a-L-Rha; R_2 = H; R_3 = H; R_4 = H; R_5 = OH
104 R_1 = O-β-D-Glu; R_2 = H; R_3 = H; R_4 = H; R_5 = OH

(b)

Figure 1: Continued.

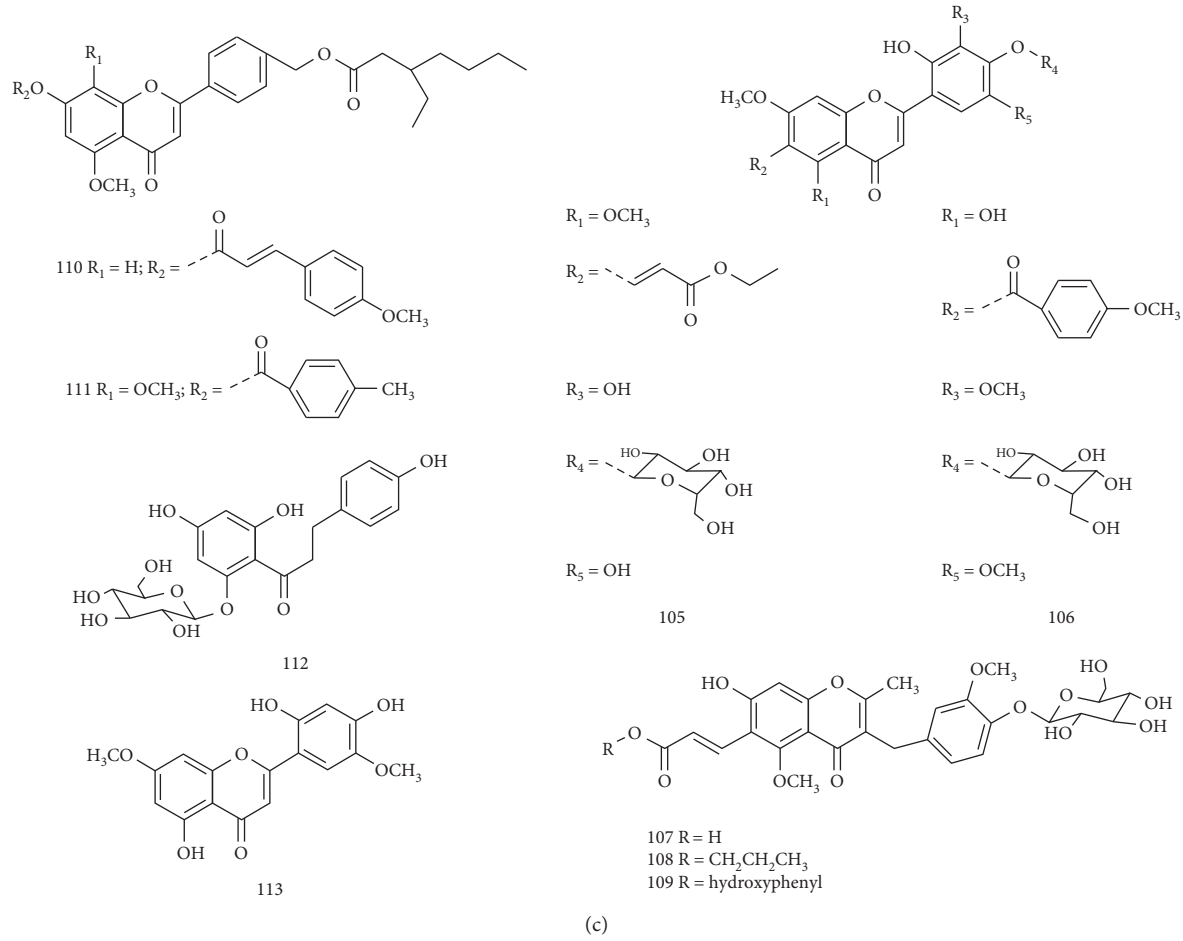


Figure 1: Chemical structures of flavonoids from *Prunus* species.

all of which show high antioxidant activity [50, 75]. Excessive presence of various natural and edible pigments in leaves and branches of *P. cerasifera* make it more demanding in the world market, especially the anthocyanins rich in red edible pigments for beverage industry [29, 76–78].

3.2. Prunus domestica. The Cherry plum (*Prunus domestica* L., Rosaceae) is famous as "Mirabelle plum" or "Myrobalan," and its fruits display a huge variation in size, shape, taste, and appearance [24]. Fresh fruits of *P. domestica* are processed into dried functional food so that they keep its potential health effects shown by the presence of plenty of phenolics and antioxidants [54]. *Prunus* kernels which actually are the dried form of the fruits are achieved at 85–90°C for 18 hours [79] and have been utilized in medicine for centuries [63]. In different parts of the world, especially in Southeast Asia, prunes are being administered alone or in combination with other medicines to cure menstrual disorders, leucorrhoea, and debility after the miscarriage [80]. Moreover, plums have a laxative effect due to high fibre contents and higher phenolic contents [81, 82]. Certain studies reported that high phenolics may pose positive health effects on the development and strengthening of bones and memory-related issues, reduce inflammations, release constipation, and scavenge ROS [83–87]. The higher antioxidant activity of extracts of *P. domestica* is mainly because of the presence of phenolic compounds, especially the isomers of caffeoylquinic acid [6, 63].

The oral administration of the *Prunus* fruit extracts (75, 100, 150 mg/kg) to male mice gave much higher learning and memory enhancement [88]. The chlorogenic acid isolated from *P. domestica* reduces the anxiolytic-like effect *in vitro* which is linked with anxiety behaviour and provides protection to granulocytes by avoiding ROS efflux [64]. The improvement of bone structure and its biomechanical properties is linked with the usage of higher doses of plums that downregulates the expression of TNF-α in lymphocytes [89], as well as retention of bone calcium ions [86].

The fruit flesh extract of *P. domestica* inhibited the cell proliferation of breast cancer cells (MCF-7, MDA-MB-453, and MCF-10A cell lines), as well as reduces the toxicity levels in the normal cells [84]. Furthermore, prunes are also found to reduce various other cancers such as colon cancer by inducing apoptosis without any harm to the normal neighbouring cells [90–92]. For human liver cancer cells

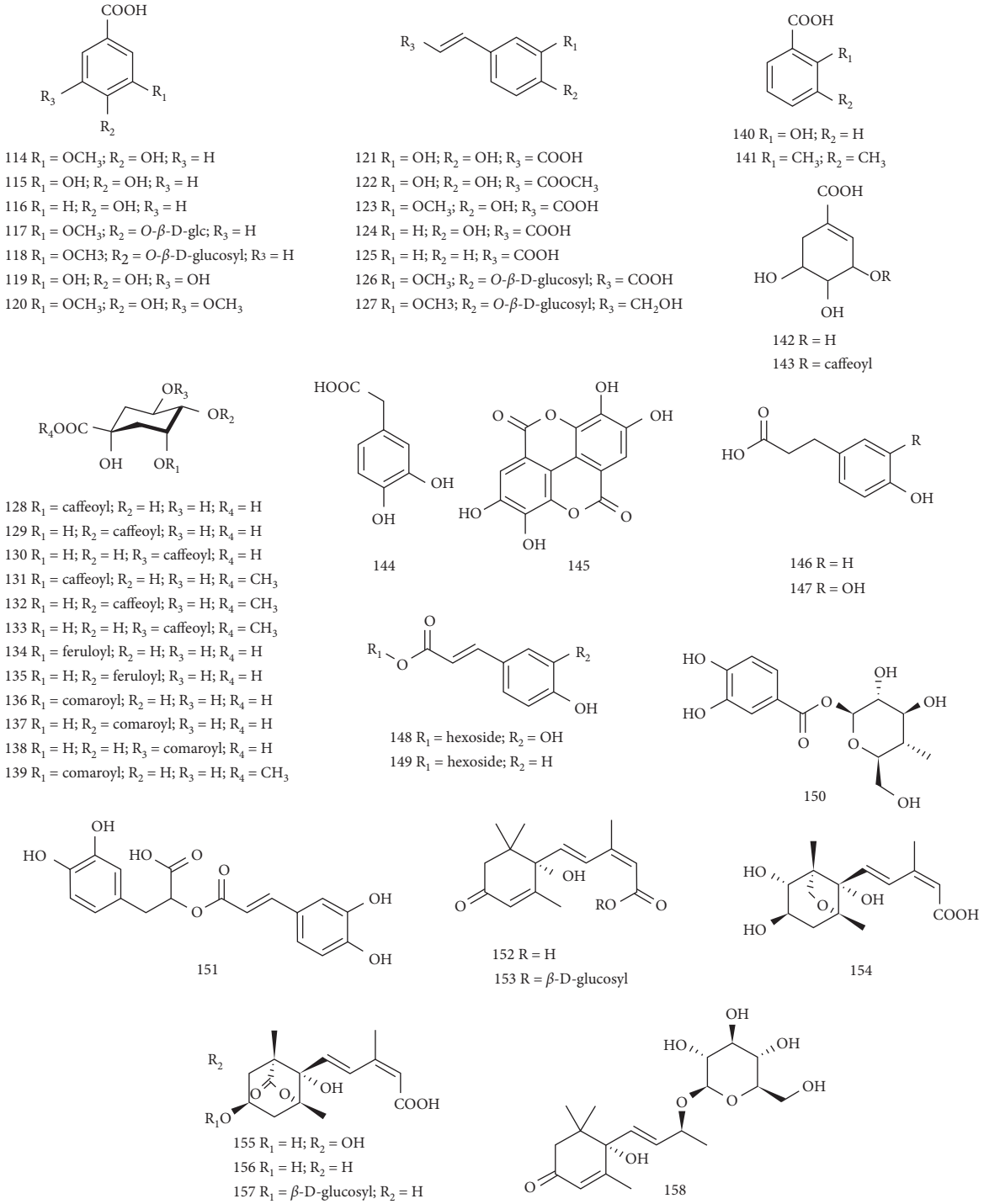
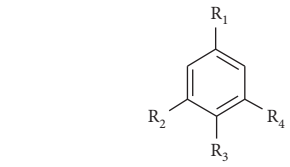


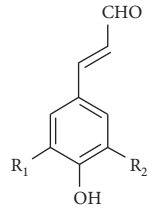
Figure 2: Chemical structures of phenolic acids from *Prunus* species.

(HepG2), prunes aid in atntiproliferation activity [90]. Moreover, the polyphenolics in prunes help in reduction in inflammatory markers such as Cyclooxygenase 2 (COX-2) [92]. The fruit extract of dried plums have been reported to be rich in polyphenolic compounds and showed a huge reduction in the inflammatory markers such as nitric oxide and melondialdehyde production in a dose-dependent manner [93]. On the other hand, another class of phyto-compounds reported in *P. domestica* is polysaccharides, which reduces and completely inhibited gastric lesions in a rat model [94].

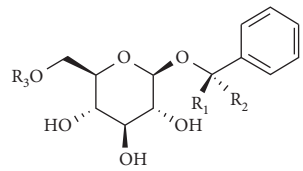
The fruit extracts of *P. domestica* showed positive results against peptic ulcer in Wistar albino rats, and it was said that due to the presence of higher amounts of polyphenolics in the fruit juices of plumes, it showed strong antioxidant potential which reduces the oxidative stress and engages various acids to neutralize the corrosive effects of various acids and, hence, appeared as antiulcerogenic [95]. Phenolic



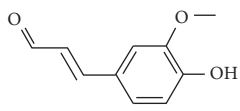
159 R_1 = CHO; R_2 = H; R_3 = OH; R_4 = H
160 R_1 = COOH; R_2 = H; R_3 = H; R_4 = H
161 R_1 = CHO; R_2 = OCH_3; R_3 = OH; R_4 = OCH_3
162 R_1 = OCH_3; R_2 = OH; R_3 = CHO; R_4 = H
163 R_1 = CHO; R_2 = OCH_3; R_3 = OH; R_4 = H
164 R_1 = CHO; R_2 = OH; R_3 = OH; R_4 = H



165 R_1 = H; R_2 = OCH_3
166 R_1 = OCH_3; R_2 = OCH_3



167 R_1 = CN; R_2 = H; R_3 = β-D-glucosyl
168 R_1 = CN; R_2 = H; R_3 = H
169 R_1 = H; R_2 = CN; R_3 = H



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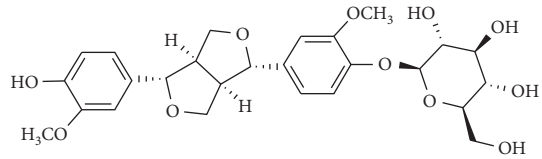

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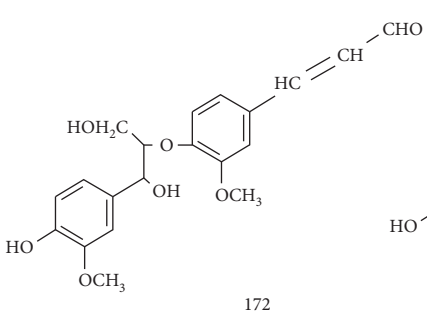

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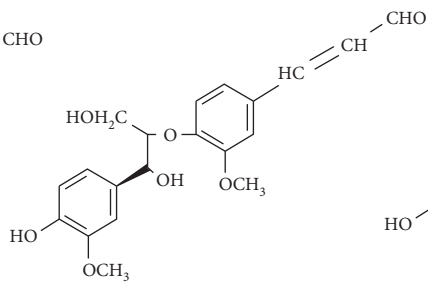

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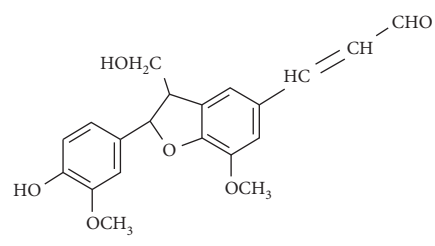

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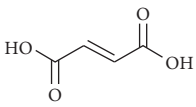

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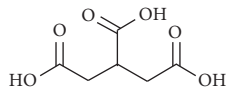

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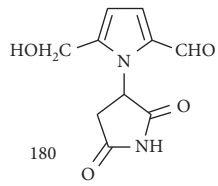

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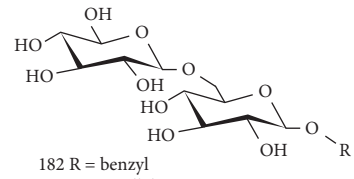
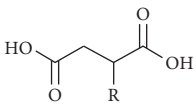
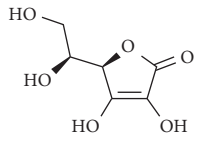


182 R = benzyl
183 R = vanillyl



176 R = OH
177 R = H



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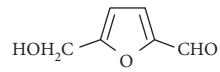

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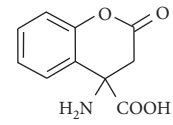

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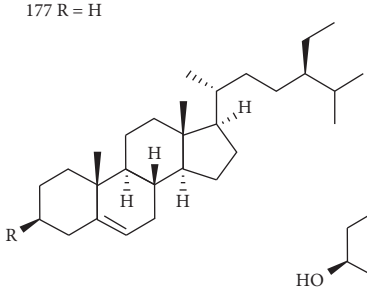
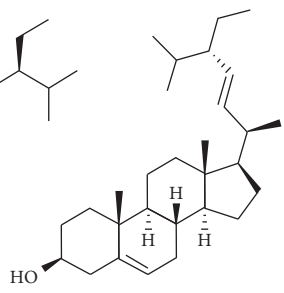


185 R = OH
186 R = β-D-glucosyl



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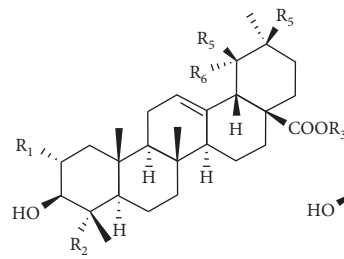
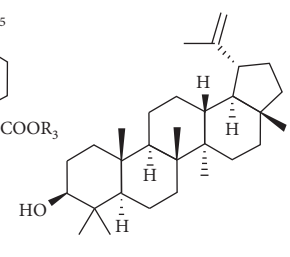


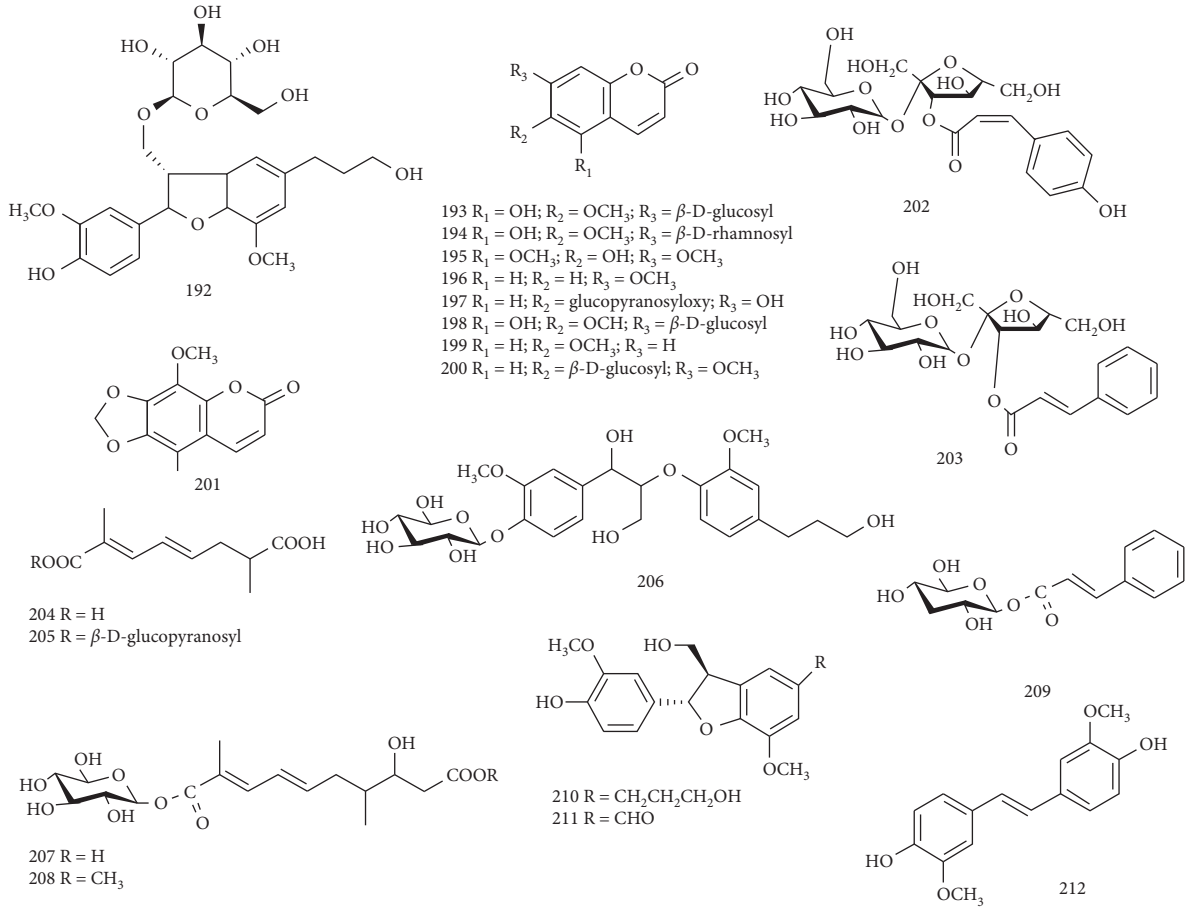


191

188 R_1 = H; R_2 = CH_3; R_3 = H; R_4 = H; R_5 = CH_3; R_6 = H
189 R_1 = OH; R_2 = CH_2OH; R_3 = H; R_4 = CH_3; R_5 = H; R_6 = H
190 R_1 = OH; R_2 = CH_2OH; R_3 = β-D-Glu; R_4 = H; R_5 = CH_3; R_6 = OH

(a)

Figure 3: Continued.



(b)

Figure 3: Chemical structures of other compounds from *Prunus* species.

compounds reported in *P. domestica* minimized the H_2O_2-induced oxidative stress through reducing the intracellular ROS accumulation in granulocytes [96]. Prunes are stated as highly useful to protect against cardiovascular disorders to regulate or modulate blood pressure, prevent atherosclerosis, and boost high-density lipoproteins (HDL) [97]. *P. domestica* fruit juice (juice concentrate + prune puree + water + 7% fructose) has laxative effects on bowel functions in individuals suffering gastrointestinal issues and increases flatulence [98]. In another study, *P. domestica* fruit extracts were studied for their antiallergic responses, and it was reported that it reduces the type-I allergic symptoms in mice by adjusting type-1 helper T-cell/type-2 helper T-cell balance and suppression of mast cell degranulation [99].

3.3. Prunus salicina Lindl. The oriental plum (*Prunus salicina* Lindley, *Rosaceae*), locally called as Chinese plum, is reported to be a rich source of various pigments such as anthocyanins and polyphenolic compounds. Foods enriched with polyphenolic compounds using oriental plum can improve the symptoms of neurodegenerative diseases by reducing the brain cholesterol levels and upregulation of neurodegenerative-related proteins [100]. The crude extracts of the peels and flesh of *P. salicina* fruit prepared in acetone have strong anticancer properties as reported for inducing apoptosis in MDA-MB-231 cells [53, 101]. Moreover, *P. salicina* fruits are rich in phenolic antioxidants (82%), of which a nitrogenous compound amygdalin is in higher quantity, which is banned by the FDA as a cancer chemotherapeutic agent [53].

The *P. salicina* fruits extracts were prepared at various levels of fruit maturity, i.e., immature, partial-mature, and fully mature fruits, and these extracts showed strong anticancer potential against various cancer cell lines, viz. HepG2, Kato11, Hela, U937 lukaemia cells, MCF-7 cells, and MDA-MB-231 human breast cancer cell lines [101]. The mechanism for this anticancer activity was due to the cytotoxic effects of the *P. salicina* fruit extracts containing polyphenolics that activate apoptotic pathway leading to the programmed cell death [101]. In another study, *P. salicina* fruit juices showed antiadipogenic effects and reduce inflammations, blood glucose levels, triglycerides, and high-density lipoproteins (HDL) cholesterol levels in obese rats [102]. The

fruit extracts of *P. salicina* are rich bioactive compounds when mixed with the food supplements which help reduce mite allergic responses [99].

3.4. Prunus spinosa L. Blackthorn or sloe (*Prunus spinosa* L., Rosaceae) is widely cultivated throughout the world [103]. *P. spinosa* is resistant to cold, drought, and calcareous soils and represents one of the ancestors of *P. domestica* [104]. Like all other Chinese prunes, *P. spinosa* also bears strong biological constituents. Active components of the plant are believed to be polyphenols, including flavonoids and A-type proanthocyanidins, anthocyanins, coumarins, and phenolic acids, forming unique and diversified profiles in particular organs, among which the flowers are the least characterized [30, 33, 43, 47, 105–108]. The unique composition of phytochemicals in the *P. spinosa* plant may correspond to the distinctive activity profile reported by traditional Chinese medication systems. Other than traditional Chinese medicine, huge literature reports ethnomedicinal and ethnopharmacological uses of *P. spinosa*, which showed its potential benefits to cure various diseases [1, 32]. *P. spinosa* fruits and its juice could be considered as a valuable source of antioxidant compounds for nutritional supplementation, as well as of herbal medicine [18]. *P. spinosa* L. flowers are a traditional herbal medicine recommended for the adjunctive treatment of oxidative stress-related diseases [65].

4. Conclusions and Further Prospective

The major phytochemicals have been isolated from four Chinese plum species including polyphenols, flavonoids, and anthocyanins. There occur huge variations in the total phytochemicals contents in different species, which make these different species to have different biological activities in multiple disease conditions, and even the same variety growing under different edaphic conditions may have different antioxidant capacities. Moreover, 212 known compounds have been reported to be present in these four Chinese plum species, which are helpful to evade chronic oxidative stress-mediated diseases. Moreover, it is suggested to perform some extensive and in-depth studies to find new phytochemicals from these four Chinese plum species which could boost the local industry to fulfill the increasing demands.

Data Availability

No data were used to support this study.

Conflicts of Interest

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

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Research Article

Effects of Bitter Melon Saponin on the Glucose and Lipid Metabolism in HepG2 Cell and *C. elegans*

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This study tried to explore how saponins from bitter melon (BMS) affect the glucose and lipid metabolism in palmitic acid-treated HepG2 cell and glucose-treated *Caenorhabditis elegans* (*C. elegans*). Results showed that BMS could effectively accelerate glucose consumption and elevate the levels of glycogen and ATP in palmitic acid-treated HepG2 cell, while significantly decreasing the triglyceride (TG) content. qRT-PCR data indicated that BMS might promote fatty acid β-oxidation by AMPK-ACC2-CPT1 pathway and glucose uptake by upregulating GLUT4 expression. In the model of glucose-treated *C. elegans*, we observed that BMS obviously inhibited fat accumulation, along with no toxicity towards some physical activities. The potential mechanism of BMS in the metabolism involved the suppression of synthesis of polyunsaturated fatty acids and enhancement of fatty acid β-oxidation. Taken together, BMS exhibited ability of regulating energy metabolism in HepG2 cell line and *C. elegans*.

1. Introduction

In the last few decades, obesity and its related metabolic disorders have emerged as public health issues worldwide [1]. Obesity is developed with the evidence of body weight gain, insulin resistance, hyperlipaemia, and so on, increasing the risk of II type diabetes, nonalcoholic fatty liver disease, and some other metabolic syndromes [2]. Nowadays, much attention has been paid to some natural nutrients to early intervene the obesity. Bitter melon (*Momordica charantia* L.) is commonly used as a medicinal and edible plant for disease prevention, particularly obesity and diabetes [3]. Saponin is one of the main active components in bitter melon, whose structure consists of triterpenes and steroids [4]. Large amounts of mechanism studies have shown that the saponins of bitter gourd have physiological activities such as lowering blood glucose and improving insulin resistance, which are recognized as insulin-like [5, 6]. What is more, the fat-lowering effect of BMS is another bioactivity, which interacts with the glucose metabolism to improve the insulin resistance. *In vitro* study suggested bitter melon triterpenoid reduced preadipocyte viability and lipid accumulation by downregulating PPARγ in 3T3-L1 cells [7]; *in vivo* study reported that saponin extract from bitter melon significantly suppressed body weight gain and visceral fat accumulation in the PPARα- and PPARγ-mediated pathways in obese mice [8].

Caenorhabditis elegans (*C. elegans,* also referred to as 'nematode' hereinafter), is a whole-system organism for the evaluation and mechanism study of toxicity or bioactivity. Recently, *C. elegans* has become an excellent model for studying the mechanism of lipid metabolism due to its high homology to mammals and rich genes-deficient mutants [9]. Lipid metabolism includes fatty acid synthesis, oxidation, unsaturation, and elongation, which are deeply evolutionarily conserved in *C. elegans* to the mammals. Additionally, in the obese, liver is one of the important insulin-sensitive tissues and HepG2 cell line is commonly applied to study the lipid metabolism in hepatic function by stimulation with fatty acids.

Therefore, this study aimed to figure out the impacts of BMS on ameliorating insulin sensitivity and fat deposition in hepatic cell. Based on this, we further confirm the fat-lowering effects of BMS in *C. elegans* and preliminarily

reveal the potential mechanism in the models we used in this current study.

2. Materials and Methods

2.1. Materials and Chemicals. Fresh bitter melons were collected from Lvjian Agricultural Station (Yangzhong City, China) and authenticated by Jiangsu Academy of Agricultural Science. HepG2 cell was purchased from Shanghai institute of biochemistry and cell biology. *Escherichia coli OP50* and N2 strain of *C. elegans* was obtained from *Caenorhabditis* Genetics Center, University of Minnesota, USA. Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO Company, USA. Metformin and palmitic acid were bought from Sigma, USA. MTT assay, Oil Red O staining assay, glucose and glycogen contents determination, ATP and TG contents determination, and protein assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. TaKaRa MiniBEST Universal RNA extraction kit, PrimeScript™ RT Master Mix kit, and SYBR Premix Ex Taq™ kit were purchased from Takara Bio Company, Japan. All primers were purchased from Sangon Biotech Company, China. All of the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., China and were of either analytical or chromatographic grade.

2.2. BMS Preparation. Unripe bitter melons were washed thoroughly in water and the seeds were removed. Then, the pulp was thinly sliced, freeze-dried, and milled (diameter < 100 μm). Bitter melon powder was added with 75% ethanol for reflux extraction twice at 80°C, and the filtrate was collected. Then, n-butanol saturated with water was applied to extract from the filtrate for 3 times until the concentrated n-butanol phase was brown and sticky. Methanol and acetone were added to produce precipitation, which then was frozen as the BMS.

2.3. HepG2 Cell Culture and HepG2-IR Cell Model. The cells were cultured in monolayers up to 80% confluence in DMEM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin at 37°C in a humidified incubator supplied with 5% CO_2. Cells in the logarithmic growth phase were used for all the studies described below.

For model of insulin resistance (IR), the supernatant of HepG2 cells inoculated in 96 plates was discarded and replaced with serum-free DMEM medium for 12 hours to keep fasting. Then, the supernatant was discarded, washed twice with PBS, and added with serum-free culture medium containing palmitic acid (the final concentration was 0.25 mM) for 12 hours to induce HepG2-IR cell model.

2.4. Cell Viability and Glucose Consumption Determination. Based on the establishment of HepG2-IR cell model, BMS at different concentrations of 50, 100, 250, 500, 750, 1000, and 2000 μg/L were incubated together with palmitic acid after 12 h fasting. The cell viability was detected by MTT method [10].

For glucose consumption determination, groups were divided into blank group (without cell), normal group, IR model group, and IR model groups with BMS or 2 mM metformin. After 12 h treatment together with palmitic acid, the glucose content determination kit and MTT assay kit were used to measure the glucose consumption, calculated by the following formula:

$$\text{relative glucose consumption}\ (\%) = \frac{(\text{glucose contents of blank group} - \text{IR model groups})/\text{cell viability of IR model groups}}{(\text{glucose contents of blank group} - \text{normal group})/\text{cell viability of normal group}} \times 100\%. \quad (1)$$

2.5. Glycogen, TG, and ATP Contents Determination for Cells. For glycogen, TG, and ATP contents determination, groups were divided into normal group, IR model group, IR model groups with BMS or 2 mM metformin. After 12 h treatment together with palmitic acid, the kits for glycogen, TG, ATP, and protein contents determination were applied. Formulas were as the following:

$$\text{relative glycogen content}\,(\%) = \frac{(\text{glycogen content of IR model groups})/\text{protein content of IR model groups}}{(\text{glycogen content of normal group})/\text{protein content of normal group}} \times 100\%, \quad (2)$$

$$\text{relative TG content}\,(\%) = \frac{(\text{TG content of IR model groups})/\text{protein content of IR model groups}}{(\text{TG content of normal group})/\text{protein content of normal group}} \times 100\%, \quad (3)$$

$$\text{relative ATP content}\,(\%) = \frac{(\text{ATP content of IR model groups})/\text{protein content of IR model groups}}{(\text{ATP content of normal group})/\text{protein content of normal group}} \times 100\%. \quad (4)$$

2.6. C. elegans Culture. M9 buffer, S-complete, and nematode growth medium (NGM) used for *C. elegans* culture were prepared as previously described [11]. Glucose plates were prepared by adding glucose (1M, sterile filtered) into the autoclaved NGM after cooling. Worms were synchronized according to standard protocols [12]. For maintenance, worms were grown on NGM plates and *E. coli OP50* was freshly provided as the food source. For assays, *C. elegans* were treated by ddH_2O or BMS for 48 h from L1 to L4 in the NGM with or without glucose (1 mM final concentration). Groups were divided to normal, model, and model with BMS 100 and 250 μg/L.

2.7. Oil Red O Staining for C. elegans. Synchronized L1 worms were treated with BMS (100 or 250 μg/mL) for 48 h to late L4 stage followed by Oil Red O staining. Oil Red O staining (~1000 worms per each group) was conducted by washing animals with 1x phosphate-buffered saline (PBS). Worms were washed with 1x PBS three times, fixed with 4% formaldehyde for 15 min and dehydrated with 60% isopropanol for 15 min. Oil Red O solution (0.5 g/100 mL in isopropanol) was diluted in double distilled water to 60% working solution and filtered. Fixed worms were incubated in the working solution overnight at room temperature. Dye was removed after allowing worms to settle, and 200 μL of 1x PBS 0.01% Triton X-100 was added. 20–30 Oil Red O stained worms were randomly selected for imaging with a Leica microscope outfitted with DIC optics (Leica, Wetzlar, Germany) under identical settings and exposure times.

2.8. TG Assay for C. elegans. Synchronized L1 worms were treated with BMS (100 or 250 μg/mL) for 48 h to late L4 stage followed by TG measurement. *C. elegans* (~2000 worms per each group) were broken by an ultrasonic cell disrupter and centrifuged to obtain the supernatant. The supernatant was reacted with the kit and the absorbance was measured at a wavelength of 510 nm. The TG and the protein assays were conducted according to the manufacturer's instructions. TG content was normalized with protein concentration determined by Bradford assay.

2.9. Locomotive Activity, Brood Size, and Lifespan Assay. For head thrashing, worms were washed with M9 buffer and dropped on another NGM plate. The head thrashing frequency (~30 worms in each group) was observed under a stereo microscope as a change in the direction of bending at the midbody and the number of times was recorded in one minute. The experiment was repeated at least 3 times.

For body bend assay, the examined nematodes (~30 worms in each group) were picked onto another NGM plate and scored for the times of bodies bends in an interval of 20 sec. A body bend was counted as a change in the direction of the part of nematodes corresponding to the posterior bulb of the pharynx along the y-axis, assuming that nematode was moving along the x-axis. The experiment was repeated at least 3 times.

For brood size, 1 random worm in each group was transferred to a new NGM plate with OP 50, and each group has three duplicates. Worms were transferred to fresh NGM plates during the reproduction period, and the eggs left were allowed to hatch and grow to L4 stage before counting the number of progeny of each worm.

For lifespan, about 100 worms in each group were transferred every day to the corresponding fresh NGM plates from L4 stage until all died. The number of surviving worms were counted and the actual number in the statistical results varies slightly due to the loss of dead or censored animals. Indicators of death included lack of movement, the stress movement of the parasite, and a lack of pharynx contraction after one or two attempts at gentle touching.

2.10. Quantitative Real-Time PCR (qRT-PCR) for Cells and C. elegans. RNA of HepG2 cell and *C. elegans* samples were extracted by the TaKaRa MiniBEST Universal RNA Extraction Kit. Prime Script™ RT Master Mix Kit was applied to synthesis cDNA according to the manufacturer's protocol. cDNA was amplified and quantified in a CFX96-PCR detection system using the SYBR Premix Ex Taq II Kit. Primer sequences are listed in Table 1. Data were normalized to GAPDH gene in cells and β-actin gene in *C. elegans*, analyzed using the $\triangle\triangle$Ct method [13].

2.11. Statistical Analysis. Data are presented as means ± *S*E and analyzed with the Statistical Analysis System (SAS Institute, NC, USA). Statistical analysis was performed using one-way analysis of variance, followed by Tukey's multiple range test to compare between groups. The significance of differences was defined at the $p < 0.05$ level.

3. Results and Discussion

3.1. Influences of BMS on the Cell Viability and Glucose Consumption. Figure 1(a) showed the effect of BMS at different concentrations on the survival rate of HepG2 cells for 12 h. There were no significant effects of BMS concentration from 50 μg/mL to 250 μg/mL on cell viability ($p > 0.05$), while the concentrations of BMS at 500, 750, 1000, and 2000 μg/mL were obvious ($p < 0.05$), indicating that BMS concentration greater than 500 μg/mL had a significant inhibitory effect on the cell growth. Therefore, the concentration range of BMSE was determined to be between 50 and 250 μg/mL.

The effects of BMS at different concentrations on glucose consumption in HepG2 cells were shown in Figure 1(b). With the palmitic acid induction, the glucose consumption of cells in the model group was significantly lower than that in the normal group ($p < 0.05$), indicating that the HepG2-IR cell model was successfully established. Similar to the effect of Met, the BMS concentration at 100 and 250 μg/mL, not 50 μg/mL, obviously enhanced the glucose consumption of HepG2 insulin resistant cells ($p < 0.05$), which was largely due to the stimulation of GLUT4 transferring to the membrane [14].

Table 1: Primer sequences for cells and *C. elegans*.

Models	Genes	Forward sequences (5′-3′)	Reverse sequences (5′-3′)
HepG2 cells	AMPK	ACAGGCATATGGTGGTCCATAGAGA	TTGGGTGAGCCACAACTTGTTC
	ACC2	GCAAGAACGTGTGGGGTTACT	TCGTAGTGGGCTTGCTGAAAA
	CPT1	GGTGAACAGCAACTATTATGTC	ATCCTCTGGAACTGCATC
	GLUT4	GCTACCTCTACATCATCCATCTC	ACCCGGCTACAAAGACC
	PGC-1α	GGGGCTCTCCAGAACATCAT	TCAAGGGGTCTACATGGCAA
	GAPDH	GGGGCTCTCCAGAACATCAT	TCAAGGGGTCTACATGGCAA
C. elegans	Fat-5	CGGCCGCCCTCTTCCGTTAC	TGGCTGCCATCCGACCCAGT
	Fat-6	TCAACAGCGCTGCTCACTAT	TTCGACTGGGGTAATTGAGG
	Fat-7	CAACAGCGCTGCTCACTATT	CACCAACGGCTACAACTGTG
	Nhr-49	AGGCTCGTGTCAATCAAGAGATGTG	ATGCCGATGCTCCAGAATCACTTC
	β-Actin	GCCGGAGACGACGCTCCACGCG	GCCTCGTCTCCGACGTACGAGTC

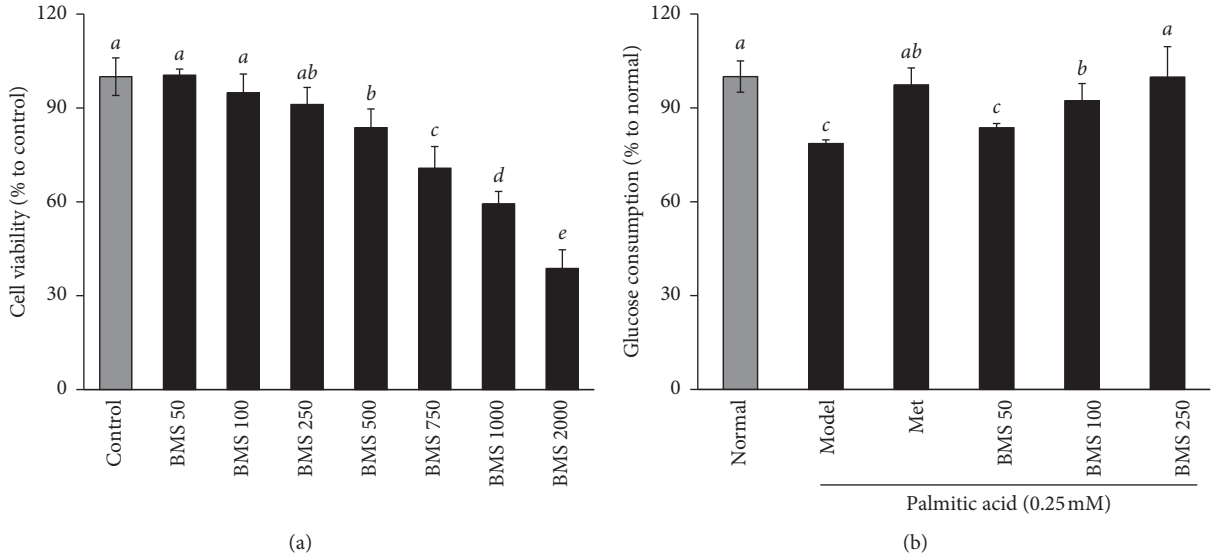

Figure 1: Effects of different concentrations of BMS on the survival rate (a) and glucose consumption (b) of HepG2 cells. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. Different superscripts were considered significantly different. Met: metformin; BMS: bitter melon saponins.

3.2. BMS Altered the Levels of Glycogen, TG, and ATP in Insulin-Resistance HepG2 Cell. The liver is the main part of the body that regulates blood glucose. As shown in Figure 2(a), the glycogen content in model group was significantly decreased compared to the normal group ($p < 0.05$), suggesting that the glucose metabolism, especially the gluconeogenesis, was disordered after palmitic acid induction. However, BMS at 100 and 250 μg/mL remarkably normalized the glycogen level of HepG2-IR cells compared with model group, as well as the Met ($p < 0.05$). Consistent with the results from Min et al. found that triterpenoids isolated from bitter melon could inhibit gluconeogenesis in both L6 myotubes and 3T3-L1 adipocytes to decrease the glucose level [15].

Long-term treatment with palmitic acid in HepG2 cells accelerated the fat accumulation, characterized by the increase of TG level, as shown in Figure 2(b). Supplementation with BMS at 100 and 250 μg/mL concentrations effectively decreased the TG content compared to the model group, suggesting BMS could inhibit the fat deposition of HepG2-IR cells ($p < 0.05$). In 3T3-L1 cell line, it was reported that bitter melon triterpenoid extract could reduce preadipocyte viability and lipid accumulation [7].

ATP level reflects the status of energy metabolism. Palmitic acid can lead to the uncoupling of mitochondrial respiratory chain of cells and reduce the ATP level, causing the idling state of oxidative phosphorylation [16]. Compared with the normal group, the ATP content in the model group was strikingly declined ($p < 0.05$). Only BMS at 250 μg/mL exhibited improved ATP level, even not the Met, which illustrated that BMS not only recovered the levels of glycogen and TG, but also improved the disorder of energy metabolism. Metformin failed to increase ATP content, probably due to its abilities to inhibit mitochondrial respiratory chains, therefore reducing ATP production and declining the ratio of ATP/AMP, which in turn activates AMPK [17].

3.3. Effects of BMS on the Expressions of Glucose and Lipid Metabolism Related Genes in HepG2-IR Cells. To discover the potential mechanism of BMS ameliorating the indexes of metabolism, we applied qRT-PCR to check the expressions of related genes. From Figure 3(a), for genes related fatty

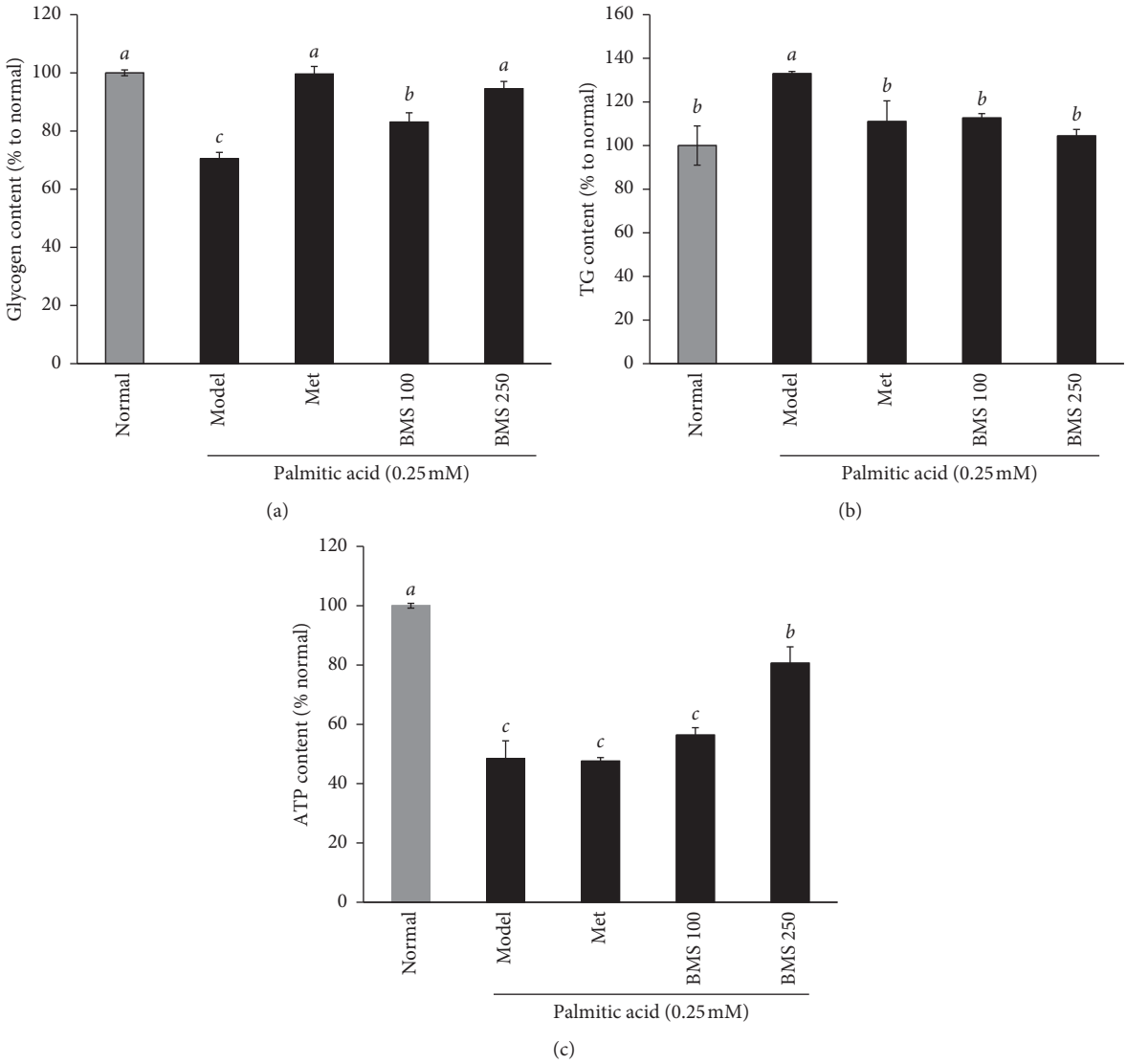

Figure 2: Effect of BMS on the levels of glycogen (a), TG (b), and ATP (c) of HepG2-IR cells. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. Different superscripts were considered significantly different. HepG2-IR cells: HepG2 insulin resistance cells; TG: triglyceride; Met: metformin; BMS: bitter melon saponins.

acid β-oxidation, BMS at 250 μg/mL concentration largely stimulated the mRNA expression of AMPK and CPT1, while decreasing the ACC2 mRNA level ($p < 0.05$), which enhanced fatty acid β-oxidation [18]. For genes related glucose intake, BMS could augment the expressions of PGC-1α and GLUT4, similar to the effect of Met. Increased GLUT4 translocation from the cytoplasm to the membrane promotes glucose uptake [19]. In line with Han's study, triterpenoids in bitter melon improved glucose homeostasis by upregulating GLUT4 in streptozotocin-induced diabetic mice [20]. Based on these results, we speculated the potential pathways of BMS involving in the metabolism included the fatty acid β-oxidation and glucose intake (Figure 3(b)).

3.4. BMS Altered the Fat Accumulation of Glucose-Treated C. elegans. *C. elegans* has been widely used as an in vivo model for exploring the genetic regulation of fat storage, due to the fact that many aspects of fat synthesis and breakdown pathways characterized in humans are conserved in this easy-handling organism [21]. The intestine in *C. elegans* consists of 20 cells and is responsible for food digestion and nutrient absorption as well as the synthesis and storage of fat [22]. As BMS exhibited strong effects on the lipid metabolism in *vitro*, we further assessed the impact of BMS on the overall fat and lipid metabolism in *C. elegans* by Oil Red O staining and TG assay to confirm it. Figure 4 indicated that 1 mM glucose could dramatically stimulate the fat accumulation in *C. elegans* compared to the normal group, while BMS at both 100 and 250 μg/mL concentrations obviously decreased the overall fat in the body of *C. elegans* ($p < 0.05$). Likewise, a striking reduction in TG level was observed in BMS groups than the model group, which further confirmed lipid-lowering efficacy of BMS both in *vitro* and in *vivo*.

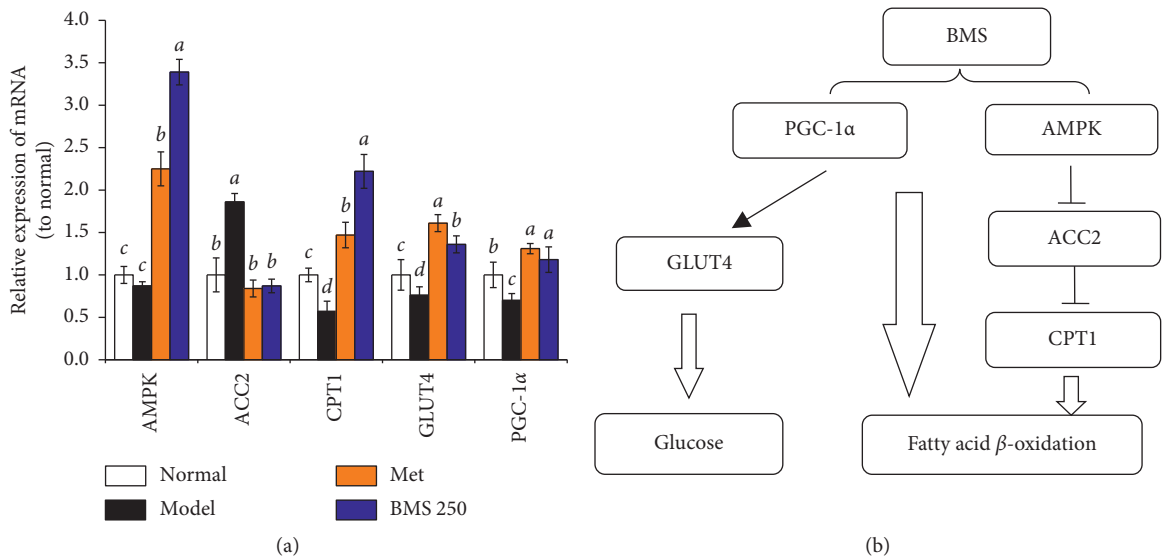

Figure 3: Effect of BMS on fatty acid β-oxidation and glucose intake. (a) mRNA expressions of AMPK, ACC2, CPT1, GLUT4, and PGC-1α of HepG2-IR cells; (b) potential pathways of BMS involved in the glucose and lipid metabolism. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. Different superscripts were considered significantly different. Met: metformin; BMS: bitter melon saponins.

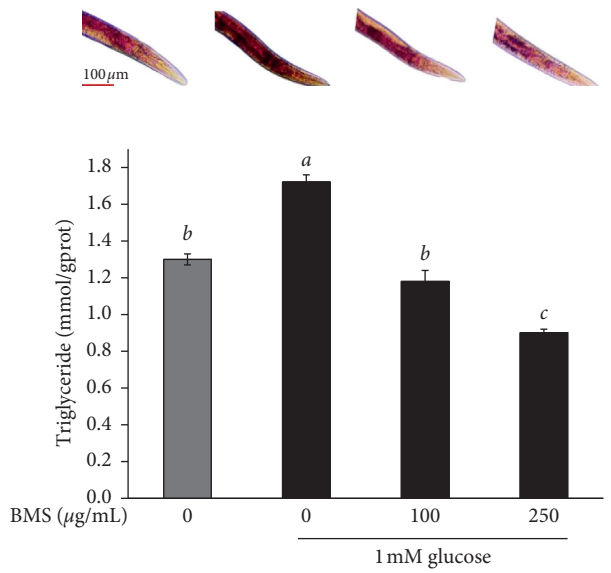

Figure 4: Effect of BMS on lipid accumulation (overall fat and TG content) in glucose-treated *C. elegans*. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. Different superscripts were considered significantly different. BMS: bitter melon saponins.

3.5. Influences of BMS on the Locomotive Activity, Progeny Production, and Longevity in C. elegans. Genetic and environmental factors, including food, have been shown to affect the physical activities of *C. elegans* and could also play a large role in fat accumulation [21]. Thus, we needed to figure out whether BMS affected the basic growth and development of *C. elegans*. As shown in Figures 5(a) and 5(b), we found that BMS had no effect on the body bends of *C. elegans,* but it improved the frequency of head thrashes in glucose-treated worms, which inferred that BMS might slightly enhance the energy expenditure to lower the overall fat. Longevity of *C. elegans* is closely related with many factors, including environmental, dietary, and genetic factors [23, 24]. High concentration of glucose is shown to exhibit a negative impact on the lifespan of nematodes [25]. Regarding the progeny production and longevity, data suggested that BMS showed no influence on the brood size, while extending the median lifespan of glucose-treated worms, which indicated that both 100 and 250 μg/mL BMS exhibited no toxicity to the growth and development of *C. elegans*.

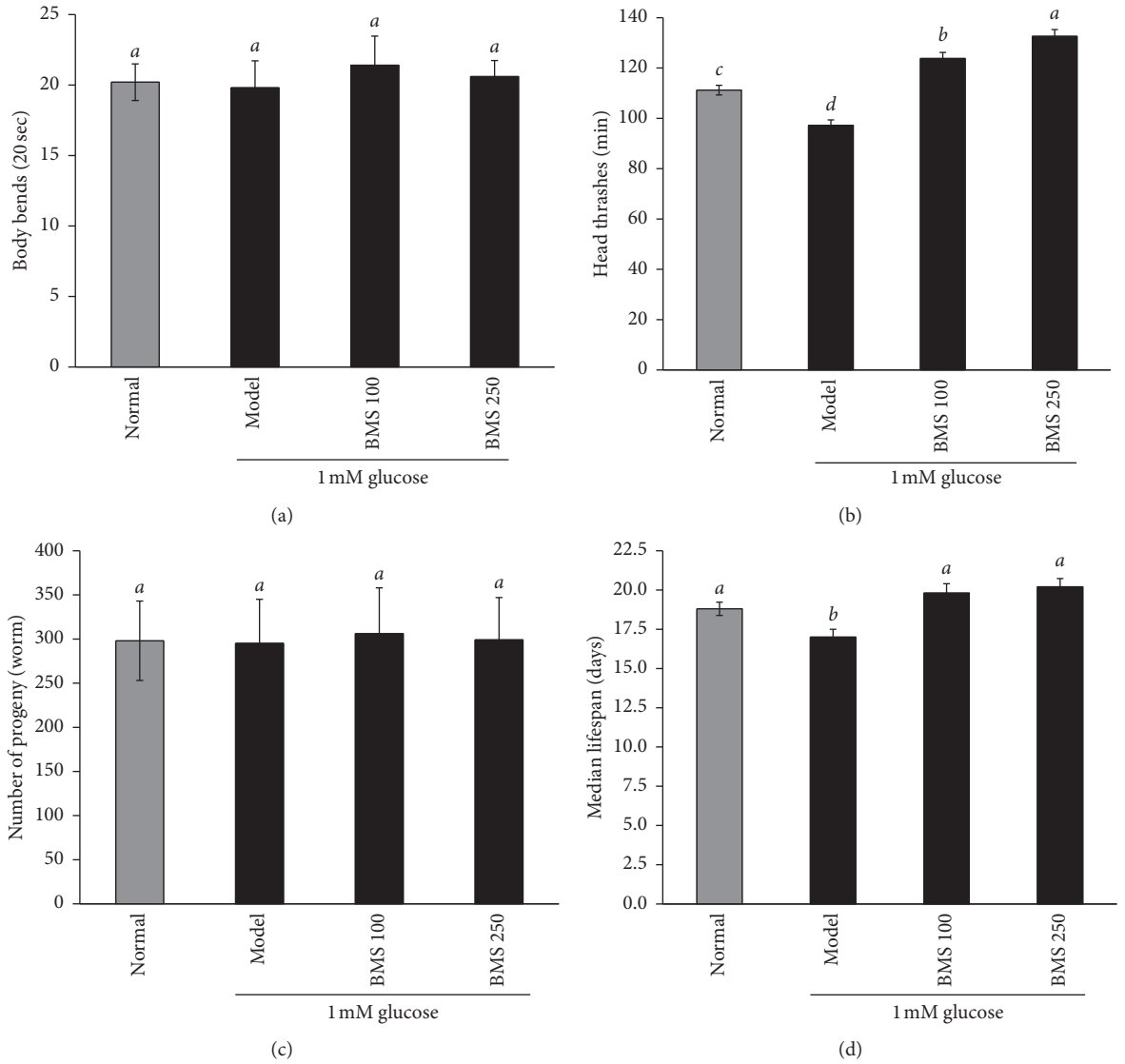

Figure 5: Effects of BMS on the locomotive activity, brood size, and lifespan of glucose-treated C *elegans*. (a) Body bends, (b) head thrashes, (c) progeny production, (d) median lifespan. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. Different superscripts were considered significantly different. BMS: bitter melon saponins.

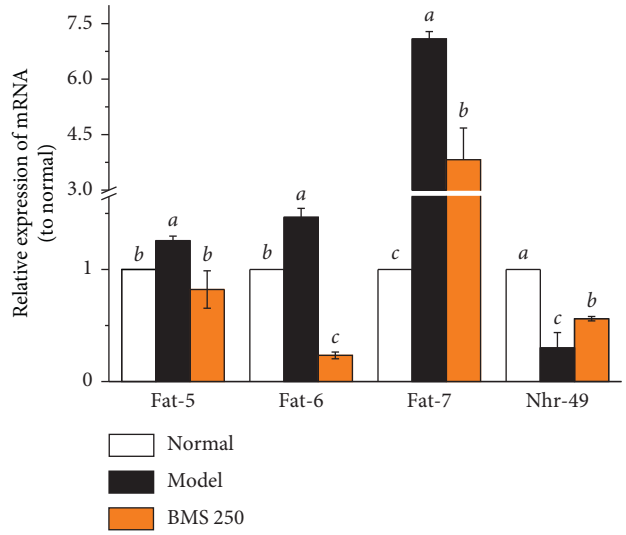

Figure 6: Effect of BMS on expressions of lipid metabolism related genes in *C. elegans*. Values are expressed as means ± SE. Statistical analysis was performed using ANOVA. The means with different superscript were considered significantly different. BMS: bitter melon saponins.

3.6. BMS Regulated the Expressions of Genes Involved in Lipid Metabolism in C. elegans. To discover the potential molecular pathways involved in the effect of BMS on fat reduction, we determined some important genes related lipid metabolism by qRT-PCR. In our current study, we found that BMS treatment profoundly decreased the mRNA expressions of desaturase genes, namely, fat-1, fat-5, and fat-7 (Figure 6), which are known as involved in the de novo synthesis of polyunsaturated fatty acids (PUFAs) [26]. Additionally, we tested a key gene expression involved fatty acid β-oxidation, the nuclear hormone receptor nhr-49 [27]. We found that 1 mM glucose impaired the expression of nhr-49 compared to that in normal group, while BMS significantly upregulated nhr-49 gene expression, indicating that BMS might act on nhr-49 to regulate catabolism of fatty acids by mediating the β-oxidation pathway, thereby reducing fat deposition in *C. elegans*. This was in accordance with the effects of BMS on enhancing β-oxidation in HepG2 cells.

4. Conclusions

In conclusion, BMS exerted beneficial effects on maintaining energy homeostasis, evidenced by enhancing glucose uptake and consumption in HepG2-IR model through upregulating GLUT4 expression and inhibiting fat accumulation in both HepG2 cell and *C. elegans* via promoting fatty acid β-oxidation. Prospectively, saponins from bitter melon might be a potential prebiotic to manage clinical patients with obesity or obesity-related metabolic syndrome.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

The Impact of Polyphenol on General Nutrient Metabolism in the Monogastric Gastrointestinal Tract

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Polyphenols are bioactive compounds occurring in plant foods, which are considered significant owing to their contribution to human health and the prevention of chronic diseases. Phenolic compounds mainly depend on plant food structure and the interaction with other food constituents, mostly proteins, lipids, and carbohydrates. The interaction with the food matrices can obstruct or enhance nutrient accessibility and availability and even impair others. Food digestion is a complex process where ingested foods are converted to nutrients via mechanical and enzymatic alterations. The absorption of nutrients predominantly occurs in the small and large intestine, respectively. The metabolised product, however, is the main bioactive component due to their ability to enter the systemic circulation and reach the targeted organs. There is limited knowledge on the cellular uptake, phenolic metabolite, and polyphenolic effect in the gastrointestinal ecosystem. Therefore, improved understanding of the biological properties and stages of dietary phenols is essential for the effective utilization of their therapeutic potentials. This review will explore, summarise, and collate current information on how polyphenols influence nutrient metabolism, bioavailability, and the biotransformation stages.

1. Introduction

Digestion is an intricate process in which foods ingested are further converted into nutrients through enzymatic and mechanical adjustments, whereby nutrients are further absorbed into the bloodstreams. Naturally, food fragmentation occurs in the mouth and the stomach, whiles nutrients and water absorption, as well as enzymatic digestion predominantly, occur in the large and small intestines [1]. Food composition and antioxidant activity are of great importance in our daily dietary ways owing to their contribution to disease prevention, radical-scavenging [2], and anti-inflammatory activities [3]. Epidemiological research shows that consumption of foods high in bioactive components and antioxidant activities, including phytochemicals, vitamins, and mainly phenolic compounds, such as anthocyanin and carotenoids, has positive merit on human health and could reduce the chances of diseases such as cancer, Alzheimer's, stroke, heart disease, diabetes, and age-related functional decades [4]. Therefore, determination of the phenolic metabolome in the human will not only aid reveal the availability of specific phenolic molecules in the human but also helps to be aware of the relationship between food eaten today and the chances of obtaining certain chronic diseases in the near future [5]. Chronic diseases, including diabetes, cancer, and cardiovascular diseases, are related to the inadequate consumption of plant food. Polyphenols are derived from plants, which include fruits and vegetables, legumes, and plant-based products such as coffee, chocolate, tea, and other beverages. Therefore, it can be tagged as a major constituent of daily human food. On average, the daily consumption of polyphenol in our diet is 1 g [6, 7]. Growing evidence and research on polyphenols prove that plant foods play a higher role in the protection of human health and also

influence several metabolic activities as well as decrease the risk of cardiovascular diseases, among others [7, 8]. Is concluded through several animal studies (in vivo) and in vitro research that polyphenol exhibits oxidative, neuroprotective and chemopreventive activities and anti-inflammatory characteristics, leading to plant food's health protection properties. Hence, it remains vital and necessary to utilize the health merits of these plant foods. In support of this, epidemiological education, the World Health Organization (WHO), and several health organizations encourage the intake of more fruits, vegetables, fibre, and leguminous plants to aid reducing disease risks [9–11]. To further understand the effect of polyphenol on nutrient metabolism and degradation, in vitro digestion models have extensively been used. Still, the greater part of the works has excluded the colonic reactions such as fermentation and others [12, 13]. This review explores, summarises, and collates the current information on how bioactive compounds (polyphenols) aids in nutrient metabolism as well as its bioavailability.

2. Food Composition

2.1. Nutrients and Bioactive Compounds. Apart from the primary known essential nutrients required to ensure that the human body has all it takes for good health, i.e., for the effective functioning of the central nervous and immune system, as well as disease prevention, [14]. There are other natural and synthetic compounds present in our daily diets that as well interfere with food digestion, enzyme activities, and the absorption of nutrients. They are termed antinutrients and include the following: protease inhibitors, amylase inhibitors, and lipase inhibitors. [15]. Bioactive compounds are molecules that present therapeutic potential with an influence on oxidative stress, metabolic disorders, energy intake, and reducing proinflammatory state [4]. Recent studies acknowledge the need for nonnutrients (bioactive), which are supposed to be of health benefits. Bioactive compounds are regarded to be different from nutrients because it is not essential for life compared with macro- or micronutrients but have an effect on the body as a whole or specific cells and tissues. It is believed that such compounds from both animal and plant sources have a positive impact on human health. These compounds include non-pro-vitamin A carotenoids, polyphenols, peptides, phytosterols, and fatty acids. Bioactive compounds act as antioxidants and further provide the defence that prevents disease spread and enhance body repairs [15–20].

2.2. Phenolic Profiles. Phenols can as well be called phenolics, which are natural compounds occurring in plant foods such as cereals, coffee, fruits, vegetables, and wine. Chemically, polyphenols are class of aromatic compounds characterised by hydroxyl groups attached to an aromatic hydrocarbon group. Thus, polyphenol is a term explaining natural products having not less than double phenyl rings bearing single or additional hydroxyl substituents, while phenols are used in expressing a phenyl ring bearing single or extra hydroxyl substituents (i.e., phenol is in a monomeric form while polyphenols are in the polymeric form). Based on their substituting groups and the number of phenolic rings, polyphenols are categorised into nonflavonoids, phenolic acids, and flavonoids. Generally, phenols have double or more hydroxyl groups, which are the biologically active substance that occurs in food plants mostly consumed by a considerable number of people [21–24]. Polyphenols constitute most subclasses of the active biological phytochemicals, including flavonoids (e.g., isoflavones and anthocyanins), nonflavonoids (e.g., tannins, stilbenes, and lignans) and phenolic acids (e.g., hydroxybenzoic acids and hydroxycinnamic acids) [25]. Flavonoids are the most studied phenolic groups, with over 9000 identified different structures in nature. It forms a major heterogeneous subgroup with a variety of compounds bearing similar diphenyl propane skeleton (C6-C3-C6). Sequentially, it is further subclassified due to their structural variation, including proanthocyanins, dihydroflavonols, flavonols, flavan-3-ols or flavanols, anthocyanidins, isoflavones, flavanones, and flavones [26–28] Table 1 presents the classes of polyphenols and chemical structures as well as their respective known food sources and compounds [25, 29–32].

2.3. Bioavailability and Bioaccessibility of Bioactive Compounds (Polyphenol) within the GI Tract (Gastrointestinal Tract). Bioactive compounds with diverse chemical structures affect bioavailability and biological properties, while the antinutritional factor can as well decrease or inhibit digestion enzymes [33]. Therefore, to get a detailed understanding of absorption and metabolism of phenols within the GI tract, it is better to consider the chemical structure of a phenol, since different chemical structures affect their redox potential. Comparably, polyphenols with double close hydroxyl groups are better and have free radical scavenging ability to those with a single hydroxyl group per [34]. Bioactive compounds can alter metabolic processes and give out positive functions such as inhibition of receptor activities, induction of enzymes, antioxidant effect, and inhibition of gene expression [35]. The changes in bacterial microbiota in the gastrointestinal tract are associated with metabolic disorders including, diabetes, obesity, or nonalcoholic fatty liver disease [36]. The in vitro test is repeatedly used for the determination of almost all biological activities, using polyphenol in their natural states, i.e., as it is in food without any breakdown. However, polyphenols are extensively metabolised both in tissues and by the colonic microflora [6]. Bioavailability of polyphenols within the gastrointestinal tract depends on the phenolic secondary microbial metabolites acting within the colons. Therefore, it is vital to explore the metabolites as well as biological properties and activities. A current finding reveals that there was a good correlation between in vivo and in vitro bioavailability of the same compound under the concentration-time curve. Therefore, cocultures can be used to copycat absorption in an in vivo test [13]. The bioaccessibility of a polyphenol is solely dependent on the bioavailability of the food matrix after successful absorption into the bloodstream [37]. For that reason, bioavailability can be defined as the proportion of bioactive compounds that are successfully absorbed into the bloodstream

Table 1: Detailed classification of natural polyphenols.

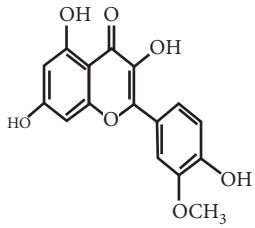
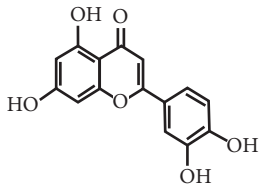
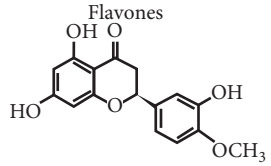
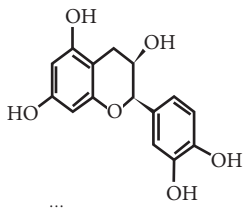
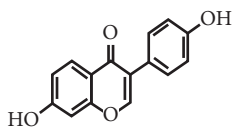
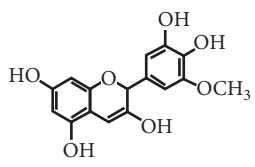
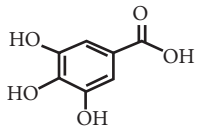
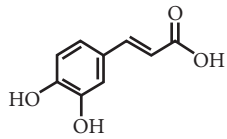
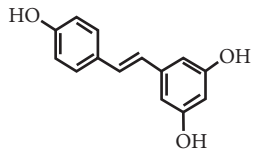
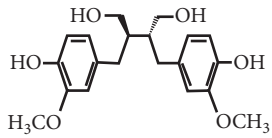
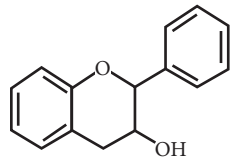
Classification of polyphenols	Subclasses and structure	Food sources	Compounds
Flavonoids	 Flavonols	Black tea, onion, wine, walnuts, apple, shallots, green tea, blueberries, almonds, oranges, chocolate, spinach	Flavonols, kaempferol, quercetin, isorhamnetin, myricetin
	 Flavones	Grain, vegetable, oils, celery, onion, pepper, garlic, parsley, herbs, citrus, thyme	Apigenin, luteolin, wogonin, tangeretin, baicalein, nobiletin
	 Flavanones	Tomatoes, citrus fruits, grapefruit Black tea, green tea, red wine, blueberries, berries, nuts, apple with peel, almonds, dark chocolate, cereals	Naringenin, hesperetin, hesperidin, neohesperidin, naringin, rutinosides, narirutin
	 Flavan-3-ols		Gallocatechin, catechin, epicatechin, epigallocatechi, epiafzelechin, ogallate
	 Isoflavones	Leguminous plants, e.g., soybeans, and soy products, e.g., tofu	Naringenin, genistein, hesperetin, naringin, daidzein, neohesperidin, rutinosides, glycitein, narirutin, hesperidin
	 Anthocyanins	Black soybean, onion, cabbage, currants, beans, potatoes, grapes red wine, berries, pomegranate, blue corn, cherries, oranges, rice	Pelargonidi, cyanidin, pelargonidi, delphinidin, malvidin, peonidin, petunidin

Table 1: Continued.

Classification of polyphenols	Subclasses and structure	Food sources	Compounds
Phenolic acids	 Hydroxybenzoic acids	Green and black tea, raspberries, strawberries, pomegranate, mango, blackberries, persimmon, grapes, wine, mangoes, nuts	Gallic acid, vanillic acids, protocatechuic acid
	 Hydroxycinnamic acids	Fruits (e.g., grapes, cherries, blueberries, apple, and kiwis), coffee, cereals, vegetables, red wine	Curcumin, p-coumaric acid, caffeic acid, ferulic acid, sinaptic acid
Nonflavonoids	 Stilbenes	Red wine, grape fruits	Resveratrol
	 Lignans	Cereals, flaxseed, linseed, algae, pumpkin, potato, leguminous crops, cereals, vegetables, fruits	Syringaresinol, ecoisolariciresinol, Secoisolariciresinol, matairesinol, medioresinol, sesamin, pinoresinol, lariciresinol
	 Tannin	Leguminous plant, grape	Gallotannins (gallic acid), Ellagitannins (ellagic acid, punicalin, and punicalagin)

for metabolic utilization [38]. However, bioaccessibility can as well be defined as the fraction of bioactive compounds released from the food matrix after digestion and becoming available for intestinal absorption [39]. In this case, the antioxidants will only be readily available for absorption after successful digestion. Before absorption, the majority of dietary polyphenols are metabolised by the colonic microbiota, and a small proportion will be directly absorbed in the upper gastrointestinal tract [40]. Hence, the microbial metabolism is a prerequisite for absorption. Again, gut bacteria can modulate polyphenols via several mechanical means including demethylation, decarboxylation, hydrolysis, reduction, and ring-cleavage as well as modulate the biological activities of a compound. Dietary polyphenols systematically depend on the synergistic action that polyphenols may exert after entering circulation, endogenous factors, and other dietary constituents [41, 42]. Several factors interfere with the absorption and release of polyphenols, and the difficulty in nutrient absorption within the intestine is due to the changes in the interaction of other compounds, chemical nutrients from the food, suppressors in the food composition, and overall metabolism. Several studies aimed at defining the bioavailability after digestion, since the effectiveness of all polyphenols or antioxidants in vivo depends on the concentration of the food matrix as well as both bioaccessibility and bioavailability [43–45]. Figure 1 presents a summary of the bioavailability and bioaccessibility determination methods [46].

3. Role of Polyphenol in the Gastrointestinal Tract

The gastrointestinal tract (GIT) is the host for over one thousand different microbial species, which include both

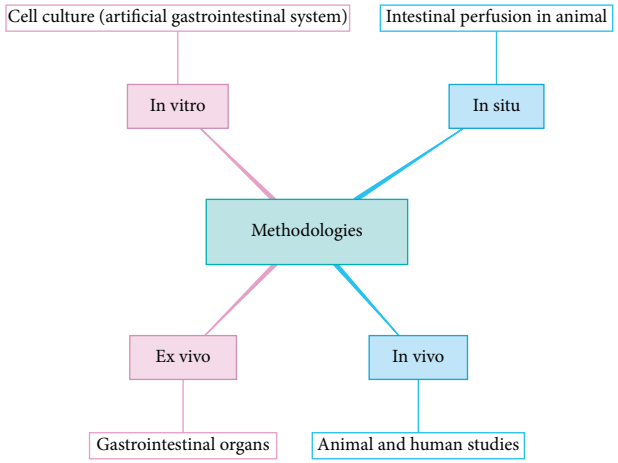


Figure 1: Bioavailability and bioaccessibility determination methods of bioactive compounds.

biotic and symbiotic bacterial species. At present, it is estimated that 500–1000 species hinder the GIT from attaining the highest concentration in the colon. Yet, out of these bacterial species (e.g., *Eubacterium*, *actinobacteria*, *Escherichia coli*, *Proteobacteria*, *Lactobacillus*, *Bifidobacterium*, *Bacteroidetes*, *Bacteroides*, *and Firmicutes*), few are known to aid in the phenolic metabolic activities [47, 48]. Though the modulation and formation of bioactive polyphenol-derived metabolites, and the colonic microbiota might both merit the host cells through nutrient processing, boosting of the immune system, and gastrointestinal protection; the mechanism is yet to be defined [49, 50]. The natural process of digestion begins in the mouth by chewing and emitting of bioactive compounds from food substance. For successful bioaccessibility of nutrients during gastrointestinal digestion, the hydrolytic enzymes, gastric acids, the physicochemical properties of the secretion act on the food bolus, resulting in the stabilisation of the phenolic components as well as facilitating the release of compounds from the food matrix [51, 52]. The human body recognises the absorbed phenolic components as a xenobiotic substance, and their absorption is quite low compared with macro- and micronutrients and thereby has to metabolise it to increase their bioavailability rate. Its metabolism starts from the upper intestinal epithelia and proceeds to the lower intestine, the liver, and then to the peripheral tissues, which include adipose tissue and the kidneys. To facilitate the excretion of urine, the hepatic enzymes convert the molecules by increasing or reducing hydroxyl groups (first phase) and inflecting them to other molecules (second phase), thereby maximising their water solubility. Cytochrome P450 monooxygenases are one of the key enzyme systems that aid in metabolising xenobiotic substances [53, 54]. The human gastrointestinal system begins digestion with the simulation of gastric acids (pepsin-HCL), followed by the breakdown of food macromolecules within the small intestine with the aid of bile and pancreatic juice and finally with dialysis [55]. Figure 2 presents the main mechanisms within the human digestive system [46].

3.1. Biotransformation Process of Polyphenol within the Mouth, Stomach, Small Intestine, and Colon. There is limited knowledge concerning the cellular uptake of phenolic metabolites in the gastrointestinal tract due to the complex processes in their absorption [56]. The route of phenolic absorption can either be via the stomach and small intestine or possibly absorbed by the colon after chemical modification by the colonic microbiota. During the transformation process, dietary polyphenols under conjugation and catabolic reactions change and get absorbed into the bloodstream and thereafter get excreted either in the urine or via the bile. The substrate or unabsorbed is passed out into faeces [57]. Recent research shows that out of 100% total polyphenolic intake, only 5–10% will be absorbed within the small intestine (i.e., monomeric, dimeric structure, and aglycones can be absorbed in the small intestine) and the 90–95% will be in the large intestine lumen together with other conjugates excreted by the bile. They are then exposed to the intestinal enzymes and gut microbiota. Therefore, the colonic microbiota is in charge of further breaking down of the polyphenolic structures into smaller molecules to aid easy absorption [58, 59]. The incorporation of polyphenol is variable within the digestive tract. Phenolic concentration in the food matrix does not affect the bioavailability rate but relatively depends on the functional group present and the chemical structure of the dietary polyphenols. Besides, they may be other biologically active molecules present in the body, which can as well influence the absorption and metabolic rate as well as its rapid elimination to reach the blood circulation and the targeted organs [60–62]. Naturally, polyphenols in the human body are in glycoside, ester, and polymeric forms that need to be hydrolysed to facilitate rapid absorption by either gut microbiota or intestinal enzymes. With the aid of β-glucosidase during the oral cavity salivation process, hydrolysation is likely to begin in the mouth though not thoroughly researched. Once absorbed into the small intestine, phenolic compounds with less complex structures undergoes extensive phase I biotransformation (oxidation, reduction, or hydrolysis) and converts into water-soluble metabolite in the enterocytes before reaching the liver. The complex phenolic compounds not absorbed in the small intestine reach the colon. Once in the large intestine, glycosides are hydrolysed by the gut microbiota resulting in the formation of aglycones through opening the heterocycle. This catabolic action decreases the complex structure of the phenolic hydroxyl group into low-molecular-weight phenolic metabolites that can be absorbed. When absorbed, the molecules reach the liver via the hepatocytes, where they are again subjected to phase II biotransformation process (conjugation) which includes methylation, glucuronidation, and sulfation or sulfate derivatives. The conjugation stage enhances easy absorption (hydrophobicity) of the molecules and aids in rapid elimination. The metabolites then enter the systemic circulation where they are distributed to the targeted organs or eliminated in the urine [58, 63]. Figure 3 shows the metabolic route for dietary polyphenol in the human gastrointestinal tract.

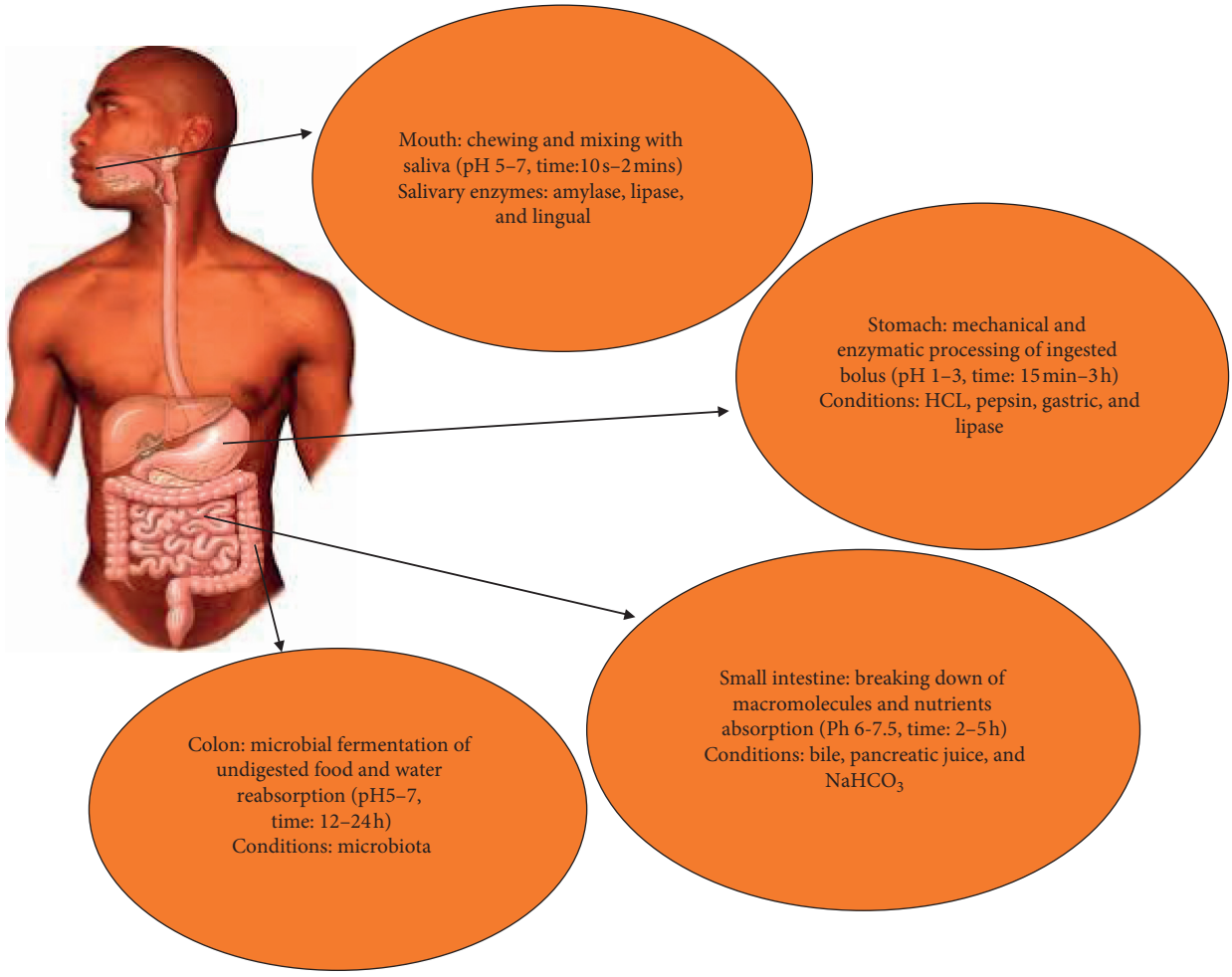


Figure 2: The main mechanisms of the human digestive system.

3.2. Influence of Polyphenol on Gut Microbiota. Recent studies show the connection between polyphenol and the intestinal microbiota and how parallel rich phenolic-foods can influence the activity and composition of the microbiota. Polyphenol intake improves the health effects of the gastrointestinal microbiota by activating the intestinal immune function, SCFA (short-chain fatty acids) excretion, and other physiological processes [32]. Though the chemistry and biotransformation process of polyphenols in the digestive tract is well defined [64], little is known of the effect polyphenol possesses on the gut's ecosystem. Recent advances confirm that the rate of bacterial growth in the guts ecosystem is influenced by the dose and structure of the polyphenol in the diet as well as the bacteria's species or strain [65]. The larger composition of bacteria is known to produce high resistance to polyphenolic influence than fewer bacteria composition due to the differences in cell structures [66]. However, it is impossible to predict the required dosage that will yield a beneficial or consequential effect in vivo [57]. The antibacterial effect is influenced by several factors, including cell wall damaging [67], permeability of cell wall [68], or production of H_2O_2 [69]. The microbiota colonizing the gastrointestinal tract of human is mainly in charge of modulating the immune system [70] and also contributes to the production of several vitamins including vitamins K and B. It can as well contribute to a negative health effect. Atypical microflora action can influence the growth of neoplasm in the gut due to mutagen and carcinogen production (*Bacteroides*, *E. coli*, *and faecalis*) [71]. In Frejnagel and Juskiewicz's study [72] on the effect of green tea, blue-berried honeysuckle and chokeberry's phenolic extract on rat caecal fermentation process were assessed. The outcome of the studies indicates that the addition of the three-extract resulted in an increment in weight of the gut wall as compared with the control animals. However, only rat-fed diet supplemented with green tea extract polyphenol yielded a significant ($P < 0.05$) increment in gut-weight content. Also, the experiment showed a decrease in pH and concentration of ammonia in rat fed with honeysuckle-supplemented polyphenolic extracts. But Leverat et al. [73] used 1% Quebracho tannins to supplement the diet of a rat, and it recorded an increase in the rat's digester pH. Two-way variance analysis confirmed there is a dose-dependent decrement in comparison with the control animals. On the contrary, honeysuckle extract in rat diets elevated the enzyme activity of the caeca while chokeberry extract in rat diets recorded a dose-dependent reduction in all enzyme-tested activities. It was only green tea extract in

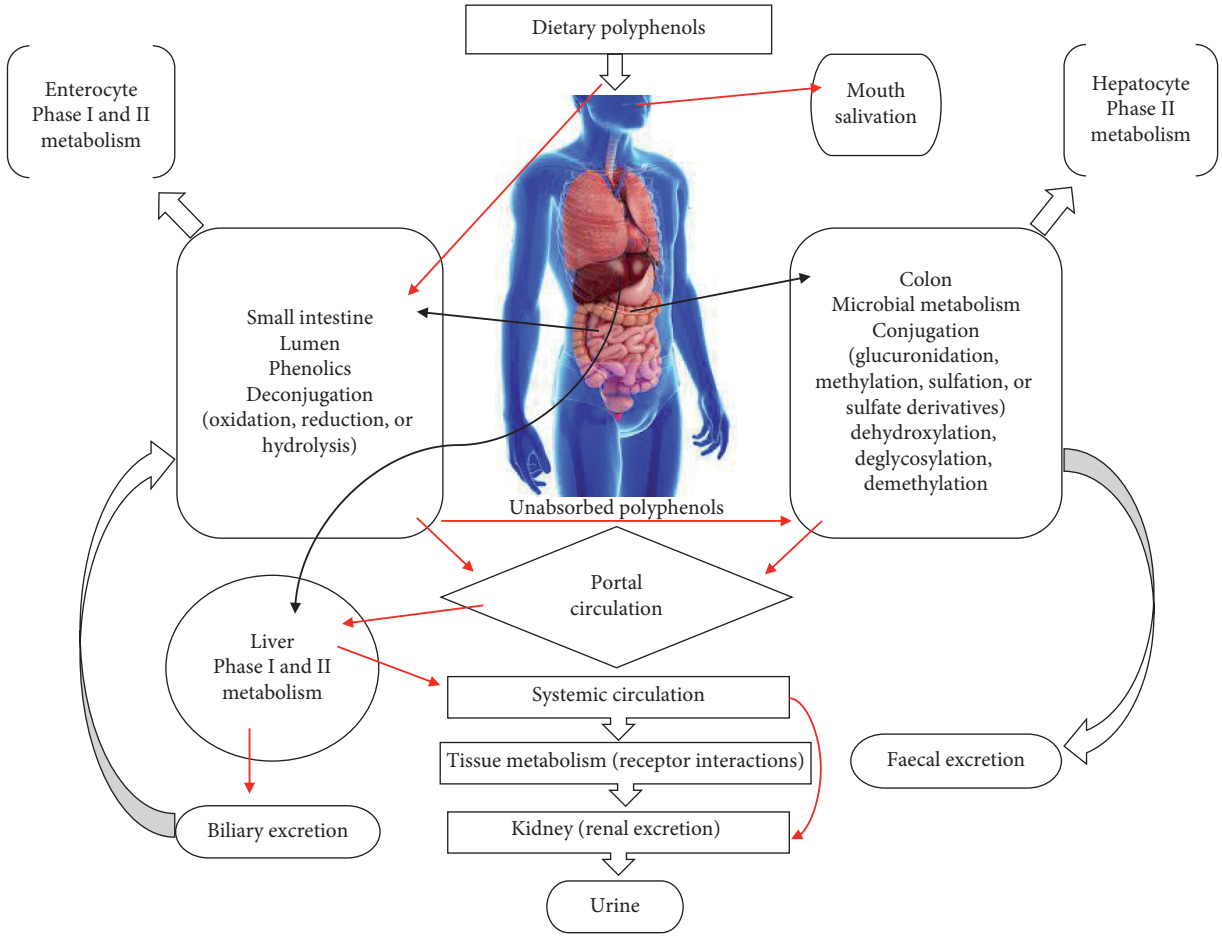


Figure 3: Metabolic route for polyphenol in the human gastrointestinal tract.

rat diets that resulted in a significant ($P < 0.05$) inhibition of enzymatic activity. The results indicated that supplementation of diets with phenolic extracts could physically and biochemically affect caecal parameters in rats [72]. Also, research by Smith et al. on bacterial mechanisms in overcoming the inhibitory effect of dietary tannins reveals that rats fed with tannin-rich diet had a significant increase in *Bacteroides* groups with a decrease clostridium leptum cluster [74]. The research by Tzonuis et al. [75], on flavonol monometer-induced changes to the human faecal microflora, was also reviewed. The study shows that (+) catechin significantly inhibits the growth rate of *Clostridium histolyticum* and improves the growth rate of *E. coli* and members of the *Clostridium coccoides-Eubacterium rectale* group, while the growth rate of *Lactobacillus* spp. and *Bifidobacterium* remains relatively unaltered. Varricchio et al. [76] also analysed the influence of polyphenols from olive mill wastewater on the intestine, alveolar macrophages, and blood leukocytes of pigs. They found an increase in the number of lamina propria and intraepithelial leukocytes present in the treated animal's gastrointestinal tracts in contrast with the control group. Immunopositive cells were found mainly in the infiltrate leukocytes in the intraepithelial leukocytes from the gastrointestinal tracts of the controls, with zero amount of immunopositive cells present in the treated animal. The study carried out by Puupponen-Pimia et al. [77], on the antimicrobial effect of phenolic compounds from berries, reveals that lactic acid bacteria were more resistant to polyphenol compounds except for myricetin, which inhibited the growth rate of all lactic acid bacteria with no effect on salmonella. The extract exhibited a growth-inhibiting effect on gram-negative bacteria. The results indicated that the synergistic action of polyphenols is the cause of the inhibition. This proves that gut microbiota plays a significant role in both nutrient and polyphenols metabolism and can as well influence their interactions.

3.3. Influence of Polyphenol on Nutrient Metabolism. Polyphenols are biologically active compounds that solely depend on plant foods structure and interactions with other food constituents, mainly the macronutrients (proteins, lipids, and carbohydrates). This interaction with food matrices can affect or enhance phenolic accessibility and availability and even impair others [78]. A substantial amount of dietary proteins, carbohydrates, and all fats are absorbed before reaching the large intestine. Few human types of research have explored the influence of polyphenols on bacterial metabolism of macronutrients as they are highly absorbed in the early stage of the gut, and modification

observed seems to reflect on the balance between these digestible macronutrients [79].

3.3.1. Influence on Protein. The influence of phenolic compounds on the activities of protease and protein substrate accessibility determines the digestibility of protein. These are due to the binding of phenolic compounds onto the endogenous protein, which includes the gastric and intestinal mucus, digestive enzymes, and protein saliva. which are the main factors influencing protein digestibility and metabolism. It can as well hinder both the catalytic site and the substrate-binding site, which will end up reducing proteolytic activities. Phenolic compounds can influence protein hydrolysis via the interaction with various protein substrates and protease. The binding activity of phenolic compounds is linked with the binding affinity to enzymes since enzymes are as well protein. Therefore, their molecular size, structure, and composition of an amino acid are all determined by the enzymatic affinity to polyphenols. Generally, the bulkiness and structure of a phenol mainly influence the structure of the protein by either stabilising or loosening the structure, which, in a way, affects enzyme activity [80]. The protein type also affects microbiota composition, which, on the other hand, influences phenolic activities [81]. Data from human and animal studies on phenolic and microbiota influence on dietary protein were thoroughly reviewed. A result of studying the effect of dietary protein source on rat metabolic effect has been evaluated [81]. The effect of 20% protein from casein, soy, and fish was evaluated in rat diets for 16 weeks. The authors reported a difference in the caecal short-chain fatty acids (SCFA); that is, the fish protein diet recorded an increase in the total SCFA and lactic acid, which was twice that of soy and casein protein diets. The concentration of butyrate was also greater in animals fed with soy diet in contrast with the other two groups. Caecal indole concentration was twice higher with the soy and fish diets as compared with casein diets. Ammonia was higher in the fish diet compared with other diets. Phenol and H_2S were as well higher with fish and soy diets against the casein diets. The result from in vivo studies with rats revealed that the digestion of whey proteins could be obstructed by the reactions with some phenolic compounds, mainly chlorogenic acids [82]. The authors focused on determining whether chlorogenic acid can influence the nutritional quality of β-lactoglobulin and protein digestibility. The results showed a decrease in nitrogen digestibility on higher derivatization with chlorogenic acid, in contrast with zero or lower derivatization at all. It was concluded that good nutritional quality of whey proteins could only be threatened at high-level derivatization with chlorogenic acids. In addition to this, rat fed with soy protein derivatives displayed an increase in urinary and faecal nitrogen and true nitrogen digestibility, and the total utilization of protein was affected. The protein digestibility-corrected amino acid score (PDCAAS) for tryptophan, lysine, and sulfur-containing amino acids were all decreased [83]. Therefore, the reduction in protein digestibility as an impact of covalent interaction or binding with polyphenol plays a role in the decreasing of polyphenol released from food matrices during digestion, thereby reducing bioavailability. Also, the binding to protein phenolic compounds is protected from adverse stomach condition [80].

3.3.2. Influence on Carbohydrates. Carbohydrate is made up of amylose, which is a linear α-1,4-linked glucose polymer, and a highly branched amylopectin consisting of a linear α-1,4-linked glucose chain with α-1,6-linked branch chains. Pancreatic and salivary α-amylases catalyse the endo-hydrolysis of α-1,4-glucosidic linkages releasing mainly maltose, maltotriose, and related α-1,6-oligomers. In addition, the main enzymes responsible for the breakdown of carbohydrates into glucose are α-amylase and α-glucosidase [8]. The metabolism of carbohydrates begins in the oral cavity and continues in the pancreas and the small intestine. Any disturbance in metabolism can yield health concerns, including diabetes, obesity, and even dental caries [78]. The majority of carbohydrate ingested is entirely digested in the small intestine; a small proportion escapes and reaches the colon. They are known as resistant starch (RS), it is subclassified into four forms of starch, i.e., chemically modified starch, physically inaccessible starch, retrograded starch, and certain granular starch that are resistant to enzyme digestion. The RS not only does inhibit metabolism but can as well provide the colonic microbiota with the largest single-source dietary-derived energy [79]. Besides drugs, polyphenols can as well aid in the inhibition of carbohydrate's metabolic enzyme activity. The structure of polyphenol can be disadvantageous to enzymatic actions, especially via the hydroxylation process. That is, during the carbon atom hydroxylation process, at some point in rings, it can inhibit enzymatic activities and the glycosylation of the hydroxyl group can as well increase enzyme activities [78]. There have been several in vivo and in vitro studies on the impact of polyphenol on carbohydrates metabolism as well as glucose homeostasis. A study indicated that phenolic extract from chokeberry could serve as an effective α-glucosidase inhibitor, which will subsequently decrease the blood glucose level and provide protection against diabetes. Therefore, continuous consumption of chokeberry could aid in the reduction of blood glucose in mild hypercholesterolemia patients and as well decrease the levels of glycated haemoglobin of noninsulin-dependent diabetes patients [84, 85]. Hence, phenolic compounds could act as a potential antidiabetic agent. A series of animal experiments have shown inhibition of polyphenols to α-amylase and α-glucosidase activities. The inhibitory phenols include catechins, isoflavones, anthocyanins, flavonols, flavanones, flavones, ellagitannins, and proanthocyanins [8]. The study carried by Adisakwattana et al. [86] analysed α-amylase, α-glucosidase, and protein glycation inhibitory activities of edible plants. It was observed that hypoglycaemic effects of tannins extracted from grape seed could originate not only from the inhibition of digestive enzymes but can as well be obtained from the inhibition of the glycation process. Again, a human study involving the consumption of coffee with a high carbohydrate or high-fat meal and nondairy creamier was observed

to impede the absorption of gut microbiota catabolites [87, 88]. A phenolic (resveratrol) rat experiment has been suggested to increase glucose uptake through the enhancement of estrogenic receptor-α, which in turn promotes Glut 4 expression via the AKT pathway and phosphatidyl inositol-3 kinase (PI3K) [89]. Finally, Yamashita's [90] study on the comparison between low- and high-degree of polymerization procyanidin and of antihyperglycemic activities was also reviewed. And, it was observed that procyanidins with a high-degree polymerisation have a strong influence on α-glucosidase inhibition as compared with less polymerised ones. Therefore, an antihyperglycemic impact on procyanidins with a small degree of polymerization could be attributed to the stimulation of glucose uptake by glucose transporter in the skeletal muscle. Though phenolic compounds can inhibit the performance of carbohydrate digestive enzymes (α-glucosidase and α-amylase), yielding to a delay in digestion, it can as well modulate glucose uptake after consumption of carbohydrate-rich meals.

3.3.3. Influence on Lipids. Compared with other macronutrients like protein-polyphenol interactions, lipids interactions with polyphenol are not well known and have less attention, with the exception of plant-derived oil, i.e., mainly olive oil, which has recently gained attention owing to its best source of oleic fatty acids as well as total monounsaturated fatty acids (MUFA) [78]. Polyphenol is a known antioxidant soluble to water that moves through the portal veins and lipids as well have less influence on the absorption of hydrophilic molecules (polyphenols). Other authors suggested that bioavailability and absorption of anthocyanins from the gastrointestinal might be hindered by the presence of lipids, sugar, and carbohydrates in the whole blueberry extract [91]. Mullen et al.'s [92] study on the metabolism of strawberries with and without cream in humans was reviewed. The study confirmed a delay in digestion of anthocyanins from strawberries when they were consumed with cream, but its bioavailability was not altered. The study conducted on the effect of curcumin on adipogenesis in 3T3-L1 adipocytes and angiogenesis and obesity in C57/BL mice was reviewed. It was observed via the activation of AMPK (adenosine monophosphate-activated protein kinase) that curcumin impeded the synthesis of glycerol lipids by hindering the activity of (GPAT-1) glycerol-3-phosphate acyl transferase-1, which in turn esterifies fatty acids into glycerol to form triglycerides for storage [93]. Peng et al. as well affirmed in recent research that vitexin, i.e., a flavonoid glycoside (an apigenin-8-C-glucoside), activated AMPKα, which is a key enzyme in controlling and reducing fat accumulation [94–96]. Other animal and cell culture studies revealed that the addition of a phenolic compound (resveratrol) to rat isolated hepatocytes turns to hinder the synthesis of triglycerides and fatty acid, i.e., through the lowering effect of lipids. Again, it lowers adipogenesis in human isolated adipocytes and as well encourages lipolytic activity in adipocytes via the induction of cAMP [97]. Finally, a study that evaluated the effect of polyphenol-rich diet on the composition postprandial lipoprotein in individuals at high cardiometabolic risk was reviewed, and the study revealed a decreased level of cholesterol and triglycerides. The authors concluded that polyphenol-rich diet reduces the postprandial lipid content of very-low-density lipoproteins (VLDL) and as well modifies the composition of low-density lipoproteins (LDL) particles- which in turn became richer in triglycerides and of which high-density lipoproteins (HDL) became triglyceride poor [98]. Therefore, polyphenolic interaction in the cell membranes can cause changes to the lipid profile.

4. Conclusions

Polyphenols are biologically active compounds that are well-thought-out as one of the essential antinutritional factors. The reviewed animal, human, and cell culture studies present the influence of the interactions between polyphenols and nutrients in the monogastric gastrointestinal tract as well as their consequence after metabolism. The interactions can reflect on the metabolism and absorption of interacting compounds, which depends on the food matrices and its biotransformation by the component of the gastrointestinal microbiota. Though the metabolism of polyphenol is considered to aid in the protection of other food compounds and maintain the digestive health via the stimulation and modulation of microbial balance, it can as well impact the organoleptic properties of the food matrices either positively or negatively depending on the phenolic dosage. Again, the complexity of phenolic biotransformation and the association between food matrices and the intestinal microbiome is incompletely understood. Therefore, future researches are essential to investigate the safety required phenolic dosage and the catabolic reactions of polyphenols and conduct a well-designed and controlled human trial, as well as assess the microbial activities at all stages of biotransformation of polyphenol compounds and the impact on human health in order to understand completely.

Data Availability

No data were used to support this study, and all references were duly acknowledged.

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

Bioavailability and Bioactivity of Alkylresorcinols from Different Cereal Products

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Cereal products are the most important dietary source for energy intake and several bioactive compounds with high concentrations in the bran and the germ. Different cereal products provide a rich source of bioactive phytochemicals, namely, phenolic acids, carotenoids, tocopherols, alkylresorcinols, benzoxazines, phytosterols, and lignans. The bioactive substance alkylresorcinols (ARs) present in the whole cereal can inhibit enzyme activity, prevent bacterial or fungal infection, reduce cholesterol absorption, prevent cancer, and resist oxidation. In this paper, we discussed the biological activity of ARs in whole cereal products. Understanding the effects of processing on cereal phytochemicals will help us to develop improved processes for processing cereal foods with higher retention rates of bioactive compounds.

1. Introduction

Polyphenolic compounds from cereal products are an indispensable part of human diet, especially from whole wheat flour, whole rye flour, and whole triticale flour, which have antioxidant properties and potential health benefits, and have attracted extensive attention [1]. Alkylresorcinols (ARs) only exist in wheat and rye bran and are related to preventing and reducing the risks of related chronic diseases such as diabetes, cardiovascular diseases, obesity, and cancer [2–4]. According to a case-control study report, only whole cereal intake dominated by rye consumption may be very important for the prevention of type 2 diabetes, which indicates that ARs have certain effects on the prevention of diabetes [5, 6]. In addition, ARs can also inhibit digestive enzyme activity, reduce oxidative stress, reduce cholesterol absorption, prevent bacterial or fungal infection, reduce cholesterol absorption, and regulate triglyceride metabolism [7–9]. As a phenolic lipid, ARs contain amphiphilic 1, 3-dihydroxybenzene derivatives (Figure 1) with an odd alkyl chain at the 5-th position of the benzene ring, where the length of the saturated alkyl tail varies between 15 and 27 carbons. As shown in Table 1, the homologues are C17 : 0, C19 : 0, C21 : 0, C23 : 0, and C25 : 0 [11, 12]. Therefore, C21 : C19 can be used to determine whether wheat is a whole wheat or a whole wheat product.

ARs in wheat bran samples were extracted with four different solvents, and ARs in bread were extracted with hot 1-propanol and quantified by gas chromatography-mass spectrometry (GC/MS) [13]. It was found that ARs can be 3, 5-dihydroxybenzoic acid (DHBA) and 3-(3, 5-dihydroxyphenyl)-1- C ionic acid (Population and Health Action Plan) in the hepatic metabolism after being absorbed by the human body. Several studies have reported a positive correlation between whole cereal intake and the presence of ARs in the plasma or its urine metabolites [14–22]. However, the physical and chemical properties of whole cereal foods are also closely related to the ARs content in cereals.

2. The Effect of ARs Content on Whole Cereal Products

The content of ARs in different cereal products is also different. According to the research on different varieties of Italian hard and soft wheat products, the ARs content of whole wheat bread made of hard and soft red wheat is significantly higher than that of other kinds of wheat [13]. The processing of whole wheat flour has a great influence on

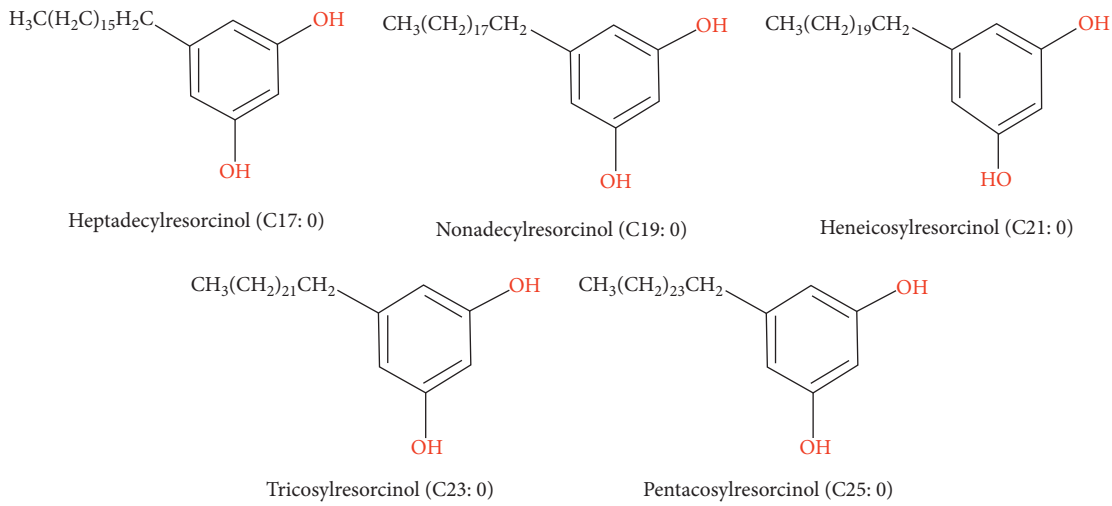

Figure 1: Alkylresorcinol found in wheat, rye, and *Triticum aestivum* [10].

Table 1: Structure and molecular weight of alkylresorcinol found in wheat, rye, and triticale.

Alkylresorcinol	Abbreviation	R	Molecular weight (g/mole)
5-n-Heptadecylresorcinol	(C17 : 0)	$C_{17}H_{35}$	348
5-n-Nonadecylresorcinol	(C19 : 0)	$C_{19}H_{39}$	376
5-n-Heneicosylresorcinol	(C21 : 0)	$C_{21}H_{43}$	404
5-n-Tricosylresorcinol	(C23 : 0)	$C_{23}H_{47}$	432
5-n-Pentacosylresorcinol	(C25 : 0)	$C_{25}H_{51}$	460

ARs. The results showed that the ARs level was significantly reduced when preparing whole wheat bread and rye bread [23]. It is speculated that the decrease of the ARs content may be due to the combined effect of fermentation and baking. The results showed that the baking temperature of bread was 218°C, when the intermediate compound starts sublimation at 196°C–250°C. However, the whole cereal products prepared after whole cereal fermentation showed that the ARs concentration increased slightly, indicating that ARs did not degrade during baking and showed expected stability during cereal preparation, which indicated that fermentation had a significant impact on ARs in whole cereal products [24]. Compared with whole wheat products, crisp bread has the highest ARs content in whole wheat or rye products, and its range is similar to that of whole wheat and rye products [25]. Therefore, the ARs content in bread products can be used to distinguish comprehensive and rye products.

It was found that the combined effect of fermentation and germination on rye can effectively enhance the contents of folic acid, total phenol, free phenolic acid, and ARs in whole wheat products, with synergistic effect. Therefore, the nutritional value of whole wheat products can be further enhanced through specific processing. The high content of ARs (0.5–1%) was added to the dough to improve the fermentation performance and increase the volume of rye bread [26]. The result showed that adding ARs could reduce bread quality and bread volume by 26–39% because rye bran contains about 4.3% salt, and it affects the baking of the whole wheat rye bread. Although the ARs content of bread baked with black wheat bran was ten times lower, the size and porosity of the bread were not affected. These results showed that compared with the study, the content of natural ARs in bread was too low, which had no practical influence on the quality of bread [27]. Bread is baked with wheat flour and 20% or 30% fine black bran (not extracted and extracted). The moisture content in the baking test (Table 2) is the optimum amount of each flour mixture.

3. Bioactivity of ARs in Different Cereal Products

ARs have a variety of physiological and biological activities, such as bactericidal, antioxidant, affecting membrane phospholipid activity and enzyme activity, and inhibiting tumors. The role of ARs in the body is closely related to its absorption and metabolism. The small intestine can partially absorb or metabolize ARs. The concentration of ARs in the plasma was 1.47–124 μg/L. The biological activity of ARs is related to its alkyl chain length, mainly because the longer the alkyl chain, the better the fat solubility.

3.1. Antibacterial Activity of ARs in Different Cereal Products. ARs with high concentration have been proved to have antibacterial and antifungal activities and the ability to kill mollusks. This is mainly because alkylresorcinol inhibits phenol oxidase in mollusks. ARs had a strong and persistent effect on Gram-positive bacteria, but they have little effect on Gram-negative bacteria [28]. C15 : 0 has an inhibitory effect on the bread mold *Aspergillum* parasitic bacteria in the

Table 2: Water amount added to different flour mixtures.

Flour mixture	Water amount (mL)
Wheat +	
30% nonextracted rye bran	141
30% finely milled nonextracted rye bran	143
30% extracted rye bran	142
30% finely milled extracted rye bran	144
Wheat +	
20% nonextracted rye bran	138
20% finely milled nonextracted rye bran	141
20% extracted rye bran	138
20% finely milled extracted rye bran	141

United States and *Penicillium chrysogenum*, but only at high concentration (10 mg/ml agar medium) [29]. Carol has an obvious effect on malaria parasites. The side chain was found to be important for the antiallergic effect [30]. Antibacterial activity of ARs in more detail can be seen in [28]. In addition, ARs also play an important role in protecting wheat seedlings from fungal infection [29].

3.2. Antioxidant Properties of ARs in Different Cereal Products. The free radical mechanism is considered to be one of the mechanisms of oxidative aging. Some in vitro experiments have shown that 5-alkylresorcinol has antioxidant activity, which is mainly due to the two indirect positions of hydroxyl groups on the benzene ring which can scavenge free radicals and generate protons. When studying the oxidation resistance of 5-alkylresorcinol on biofilms, 5-alkylresorcinol exhibits strong oxidation resistance, and the alkyl chain may play an important role in this process. 5-Alkylresorcinol exhibits strong oxidation resistance on membranes, mainly because it can be inserted into membranes and bind to adjacent phospholipids in the form of hydrogen bonds [31], thereby interfering with the structure of these membranes, affecting its performance, and resisting oxidation. To some extent, this theory can be used to explain the relationship between antioxidant performance and the alkyl chain length of 5-alkylresorcinol. The longer the alkyl chain is, the stronger the antioxidant activity is.

Most of the cereal foods that people need in daily life need to undergo production and processing. Processing affects the flavor and nutritional value of cereal products. This process may affect the active substance content and bioavailability in whole grain foods [32]. Research by Ross et al. [33] showed that ARs can be extracted from baked bread. Ross et al. [14] studied the combined effects of fermentation and germination on rye and found that the combination of these two processing methods can very effectively enhance the content of folic acid, total phenol, free phenolic acids, and ARs in whole wheat products. Therefore, the nutritional value of whole wheat products can be further enhanced through specific processing. Ross et al. [34] measured the ARs content of yeast, dough, and bread at different stages of wheat and rye whole flour through research, and the research showed that the ARs content decreased significantly during fermentation and baking. At the same time, Weipert and EIBaya reported that when flour is made into bread, the amount of ARs decreases significantly [35, 36]. Ross et al. [37] found that natural whole wheat increased its ARs content to a certain extent during fermentation. Winata, and Lorenz [38] compared alkylresorcinol, fatty acids, and antioxidant activities of wheat processed by traditional and modern plants and found that whole grains showed higher ARs content and resistance to the corresponding crushed products oxidative activity, a reduction in total ARs content was observed after pulverization, and the lowest amount was detected in the semolina-like portion. Yu et al. [39] reported the effects of different peeling levels on wheat flour ARs. Studies have shown that peeling treatment has a significant effect on nutrients in wheat nutrition powder, and the ARs content gradually decreases with increasing peeling time.

3.3. Anticancer Properties of ARs in Different Cereal Products. Wheat bran plays an important role in preventing the occurrence and development of colon cancer. There is evidence that eating wheat bran can reduce the risk of colon cancer. Most of the animal and human tests have shown the relationship between wheat bran and reduction in the risk of colon cancer [10, 40, 41]. The main component of wheat bran oil, 5-alkylresorcinol, has a strong inhibitory effect on the proliferation of colon cancer cells [42], but the relevant mechanism has not been fully elucidated.

ARs can reduce the induction of some indirectly induced substances. Compared with anthocyanin, ARs can effectively inhibit the induction rate and frequency of inducers in the lymphocyte culture. The induction effect of ARs on reducing four standard inducers was found through the Ames test, and it was found that its effect is very significant [43]. In the sister chromosome hybridization test, ARs can significantly reduce the frequency of chromosome exchange. Further research shows that ARs can accelerate the death rate of damaged cells with genotoxicity and inhibit the formation of cancer cells. Later, the anticancer effect of ARs is attributed to the ability to increase apoptosis of cells damaged by genetic toxins [44]. Chain length seems to be very important for the cytotoxicity of cancer cells in vitro95 and inhibition of the formation of cancer, while shorter chain lengths (C15 : 0 and C17 : 0) may not be the most effective [45], further confirming that oxidative damaged DNA has strong induction and carcinogenic effects. Studies have shown that ARs inhibit oxidative damage caused by hydrogen peroxide to colon cancer cells. By adding 5-alkylphloroglucinol directly related to colon cancer, reproductive toxicity of cancer cell excreta is also reduced. It can be proved that 5-alkylisophenol is a natural DNA polymer, which can be used as an inhibitor.

3.4. Inhibition of ARs on Enzymes in Different Cereal Products. Sileshi et al. [46] proposed that ARs and related compounds may have inhibitory effects on certain metabolic enzymes. C15 : 0 can inhibit the key enzyme GPDH of triacylglycerol synthesis, and the inhibitory effect is related to the alkyl chain length [47]. The metabolized alkylresorcinol can inhibit the action of phosphorylase and activate the synthesis

of glycogen [48]. For example, 5-pentadecylresorcinol can inhibit glycerol 3-phosphate glycerol dehydrogenase activity. In a study, mice were fed with high concentrations of purified rye ARs (0.1%~0.4%). It was found that the level of VE in the liver was increased and hepatic cholesterol was reduced by 47% relative to the control group when the mice were fed with 0.4% of ARs [34]. The reduction in liver cholesterol is 47% [34]. The decrease of liver cholesterol may be due to the fact that ARs can interfere with lipid absorption or liver sterol synthesis, but its mechanism of action requires further research to clarify.

ARs are natural alpha-glycosidase inhibitors in whole grain foods. Wheat bran ARs can reduce fasting blood glucose in obese mice, induced by high-fat and high-sugar diets, increase glucose tolerance and insulin sensitivity in mice, increase fecal cholesterol excretion in mice, and reduce cholesterol concentrations in the blood [47].

Maria et al. [49] studied the effect of phenolic lipids extracted from whole wheat on the activity of acetylcholinesterase. The experimental results show that phenolic lipids can inhibit the activity of acetylcholinesterase, and the effect of phenolic lipids and the structural characteristics of its hydrophilic part and the alkyl chain length is related.

4. Conclusion

The combined effect of fermentation and germination on rye can effectively enhance the contents of folic acid, total phenol, free phenolic acid, and ARs in whole wheat products, with synergistic effect. The nutritional value of whole wheat products can be further enhanced through specific processing. High content of ARs (0.5–1%) was added to the dough to improve the fermentation performance and increase the volume of rye bread. ARs have a variety of physiological and biological activities, such as bactericidal, antioxidant, affecting membrane phospholipids' activity and enzyme activity, and inhibiting tumors. The role of ARs in the body is closely related to its absorption and metabolism. The small intestine can partially absorb or metabolize ARs. There is also a certain concentration of ARs in the plasma. The biological activity of ARs is related to its alkyl chain length, mainly because the longer the alkyl chain, the better the fat solubility.

Conflicts of Interest

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

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Research Article

Optimization of Enzymatic Conditions of Sturgeon Muscles and Their Anti-Inflammatory Potential

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The objective of this study was to investigate the effects of different enzymolysis conditions on the NO inhibition rate and DH (degree of hydrolysis) of sturgeon hydrolysates (SH) prepared by Alcalase. The NO inhibition rate of 60.23% was attained under the optimum enzymolysis conditions as follows: pH 9.0, enzymolysis time of 4.92 h, enzymolysis temperature of 55°C, solid/liquid ratio of 1 : 20, and enzyme additive amount of 7674.22 U/g protein, which was well matched with the predicted value 61.44% of the Box–Behnken design model. After the ultrafiltration of SH, SH-3 (SH < 3 kDa) could significantly decrease the levels of NO and proinflammatory cytokine level IL-6. Also, we found that the obtained SH-3 contained good properties of emulsification and possessed good WHC and OHC. SH-3 demonstrated appreciable antioxidant potential on DPPH and ABTS radical scavenging activities. These results suggested that SH-3 derived from sturgeon muscles could potentially be used as a promising ingredient against inflammatory and oxidative stress-associated diseases.

1. Introduction

Fish and fishery products provide not only essential micronutrients, but also high-value protein. However, approximately 50% of protein-rich fish byproducts are used as fishmeal and animal feed or discarded, causing a lot of waste. Enzymatic hydrolysis is the most efficient method for recovering value-added proteins from fish byproducts and keeping the value of nutrition. According to previous studies, fish protein-derived hydrolysates possess different bioactivities, such as antioxidative, antitumor, anti-inflammatory, neuroprotective, and antidiabetic effects [1–3].

Inflammation is a defense mechanism triggered by pathogen invasion or tissue damage caused by biological, physical, or chemical damage [4, 5]. The activation of macrophages is an important part of initiating defensive responses, and the release by macrophages of inflammatory mediators such as nitric oxide (NO) and proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) can enhance the defense ability [6, 7]. NO is an important signaling molecule in neurotransmission, vasodilation, and host immune defense [8]. However, overproduction of NO and inflammatory cytokines is related to various diseases, such as atherosclerosis, rheumatoid arthritis, and endotoxin-induced organ injury [9, 10]. Some investigators found that the anti-inflammatory effects were mainly related to the antioxidant activities [11, 12]. Besides, plenty of research studies have reported that anti-inflammatory hydrolysates can be obtained from fish protein hydrolysates [13, 14]. For example, salmon byproduct protein hydrolysates and fish scale collagen peptides possess anti-inflammatory activity [14, 15].

In China, sturgeon caviars are expensive and in short supply; sturgeon muscles are found in large quantities and most of them are discarded as waste. Sturgeon muscles have been used in traditional Chinese medicine to help people

maintain overall wellness for thousands of years. However, in recent years, sturgeon muscles have received limited attention as a potential resource of bioactive hydrolysates. Thus, exploring the anti-inflammatory activity of sturgeon muscles hydrolysates is significant to expand the application in food industries.

The objectives of this study were to optimize the enzymatic hydrolysis conditions of sturgeon muscles and investigate its anti-inflammatory and anti-oxidant effects to verify the possibility for application of functional food materials.

2. Materials and Methods

2.1. Materials. Alcalase were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) were obtained from Aladdin Industrial Corporation (Ontario, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Biological Industries (Kibbutz Beit Haemek, Israel). 3-(4,5-Dimethylthizaol-2-yl)-2,5-diphenylterazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin, streptomycin, lipopolysaccharide (LPS), and Griess Reagent were from Beyotime Biotechnology (Shanghai, China). Interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Fcmacs (Nanjing, China). All other chemicals were of reagent grade.

2.2. Single-Factor Experiments. In order to obtain the optimal enzymatic hydrolysis conditions of sturgeon muscles by using Alcalase, the effect of pH, time, temperature, solid/liquid ratio, and enzyme additive amount was tested by single-factor experiment. The chosen pH is 7.5, 8.0, 8.5, 9.0, and 9.5. The chosen hydrolysis times are 2, 3, 4, 5, and 6 h. The chosen reaction temperatures are 45, 50, 55, 60°C, and 65°C. The chosen solid/liquid ratios are 1 : 25, 1 : 20, 1 : 15, 1 : 10, and 1 : 5. The selected enzyme additive amounts are 1000, 2000, 4000, 6000, 8000, and 10,000 U/g protein. SH was prepared according to previous reports with slight modification [16, 17]. Sturgeon muscles were initially homogenized with distilled water. The suspension was added with Alcalase and the pH was adjusted to hydrolyze. Afterwards, the reaction was terminated by heating the mixture in a water bath at 95°C for 20 min. The supernatant was collected and freeze-dried for further analysis.

2.3. Response Surface Method. According to the single-factor experimental results, the range of level values of each factor was determined. The response surface method with three-level and four test variables (pH (X_1), time (X_2), temperature (X_3), and enzyme additive amount (X_4)) was employed in optimization procedure. The NO inhibition rate was chosen as the response variable of the experiments. The range of independent variables and levels are shown in Table 1. A second-order polynomial model [18] was used to describe the optimal hydrolysis conditions of sturgeon muscles.

Table 1: Independent variables and levels used in response surface design.

Independent variables	Units	Levels		
		−1	0	1
pH (X_1)		8.5	9.0	9.5
Time (X_2)	h	4	5	6
Temperature (X_3)	°C	45	50	55
Enzyme additive amount (X_4)	U/g	6000	8000	10000

Design Expert (8.0.6) software was used for the design and statistical analysis.

2.4. Ultrafiltration. SH under the optimal enzymatic conditions was filtered through ultrafiltration membranes with a molecular weight cut-off (MWCO) of 10 and 3 kDa (Millipore Co., USA). All fractions including SH-1 (>10 kDa), SH-2 (3–10 kDa), and SH-3 (<3 kDa) were freeze-dried for further experiment.

2.5. Determination of the Degree of Hydrolysis. The DH was estimated by the o-phthaldialdehyde (OPA) method [19].

2.5.1. Cell Culture. The murine macrophage RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were grown in a humidified incubator with 5% CO_2 at 37°C.

2.5.2. Cell Viability. RAW264.7 cells were seeded into a 96-well plate overnight and pretreated with different concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 mg/mL) of SH for 24 h. MTT solution was added to each well and incubated for another 4 h at 37°C. Then, the supernatant was discarded, 150 μL DMSO was added to each well, and the absorbance was detected at 570 nm using a microplate reader (Tecan Infinite PRO TWIN 200, Switzerland). Viability was expressed as a percentage of the control.

2.6. Determination of Nitric Oxide (NO) and IL-6. RAW264.7 cells were cultured in 24-well plates overnight and pretreated with SH for 12 h. Then, cells were stimulated with LPS (2 μg/mL) for 24 h. The nitrite concentration was measured with Griess reagent. Inflammatory cytokine IL-6 was assessed by ELISA kit following the manufacturer's instructions.

2.7. Functional Properties of SH

2.7.1. Emulsifying Properties. Emulsifying activity index (EAI) and the emulsion stability index (ESI) were measured according to previous reports with slight modifications [20]. 30 mL of 1% SH solution with different pH (2, 4, 6, 8, and 10) was mixed with 10 mL soybean oil and homogenized at 15,000 rpm for a minute. 50 μL of the emulsion was pipetted from the bottom of the container at 0 and 10 min after homogenization and diluted with 5 mL of 0.1% sodium

dodecyl sulfate solution. The absorbance of the solution was measured at 500 nm using a spectrophotometer. EAI and ESI were calculated as follows:

$$\mathrm{EAI}\left(\mathrm{m}^2/\mathrm{g}\right) = \frac{2 \times 2.303 \times \mathrm{dil} \times A}{(C \times \Phi \times 10000)}, \quad (1)$$

$$\mathrm{ESI}\,(\mathrm{min}) = \frac{(A_0 \times 10)}{\Delta A}, \quad (2)$$

where A is absorption at 500 nm; dil is dilution factor (100); C is protein concentration in aqueous phase (g/mL); Φ is oil volume fraction (0.25); and $\Delta A = A_0 - A_{10}$.

2.7.2. Foaming Capacity and Foam Stability. Foaming capacity (FC) and foam stability (FS) were determined according to the previous methods with some modifications [21]. 0.4 g of the sample was placed in 40 mL distilled water and stirred at room temperature for 30 min. The foam was prepared using a homogenizer at 15,000 rpm for 2 min. The foam volume was noted immediately after 2 min. FS was calculated by measuring the fall of the foam volume after every 1 min. FC and FS were calculated as follows:

$$\text{Foaming capacity} = \left[\frac{V_2 - V_1}{V_1}\right] \times 100, \quad (3)$$

where V_1 is volume before whipping and V_2 is volume after whipping.

$$\text{Foam stability} = \left[\frac{\text{Foam volume after time}}{\text{initial foam volume}}\right] \times 100. \quad (4)$$

2.7.3. Water Holding Capacity. WHC was estimated by the centrifuge method according to the previous study with some modifications [22]. 0.5 g of SH was dissolved in 10 mL of distilled water and vortexed for 30 s. The solution was allowed to stand for 6 h at room temperature and then centrifuged at 5,000 g for 30 min. The supernatant was then filtered and the volume collected was noted. The difference between the initial volume of distilled water added to the sample and the volume of the supernatant was measured, and the results were calculated as milliliters of water absorbed per gram of SH.

2.7.4. Oil Holding Capacity. OHC was determined by the centrifuge method according to the reported method with slight modifications [22]. 0.5 g of SH was added to 10 mL of soybean oil and vortexed for 30 s. The oil dispersion was then centrifuged at 2,800 g for 30 min. The volume of oil separated from the hydrolysate was measured, and OHC was calculated as the grams of oil absorbed per gram of SH.

2.8. Determination of Antioxidant Activity

2.8.1. DPPH Radical Scavenging Activity. DPPH free radical-scavenging activity of SH was measured according to the method described by previous studies with some modifications [23, 24]. 2 mL of each sample with different concentrations was added to 2 mL of 0.1 mM DPPH in 99.5% ethanol and incubated for 30 min at 37°C in dark conditions; the absorbance was determined at 517 nm with spectrophotometer. In addition, a control sample containing DPPH solution without sample was prepared. In blank sample, DPPH solution was substituted with ethanol. The DPPH radical scavenging activity was evaluated by the inhibition percentage of DPPH radical as in the following equation:

$$\text{DPPH radical scavenging activity}\,(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%, \quad (5)$$

where A_1 was the absorbance of the sample; A_0 was the absorbance of the control group; and A_2 was blank absorbance.

2.8.2. ABTS Radical Scavenging Activity. Radical scavenging ability was measured as described previously with slight modifications [25]. The ABTS radical cation was generated by mixing 7.4 mM ABTS with 2.45 mM $K_2S_2O_8$ and exposing the resultant mixture to the dark at room temperature for at least 12 h. The ABTS radical solution was diluted in phosphate buffered saline (PBS) (pH 7.0) to an absorbance of 0.70 ± 0.02 at 734 nm. 1 mL of diluted ABTS free radical solution was mixed with 1 mL of the sample at different concentrations. After 10 minutes, the absorbance was measured at 734 nm against the corresponding blank. The ABTS scavenging activity was calculated as in the following equation:

$$\text{ABTS scavenging activity}\,(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%, \quad (6)$$

where A_{control} was the absorbance without sample and A_{sample} was the absorbance with sample.

2.9. Statistical Analysis. Data are expressed as means ± standard deviation of more than two replicates. The statistical difference among groups was evaluated by one-way ANOVA (SPSS for win 8.0, SPSS Inc., Chicago, IL). The results were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. Cell Viability. In order to evaluate cytotoxic effects of SH on RAW264.7 cells, MTT assay was performed. Figure 1(a) showed that SH did not have cytotoxic effects up to 2.0 mg/mL. Therefore, the concentration of 0.5 mg/mL of SH was selected for further study.

3.2. The Analysis of Single-Factor Experiments

3.2.1. The Effect of pH on DH and NO Inhibition Rate. As shown in Figure 2(a), the effect of enzymolysis pH on DH and NO inhibition rate of SH was investigated. The pH was

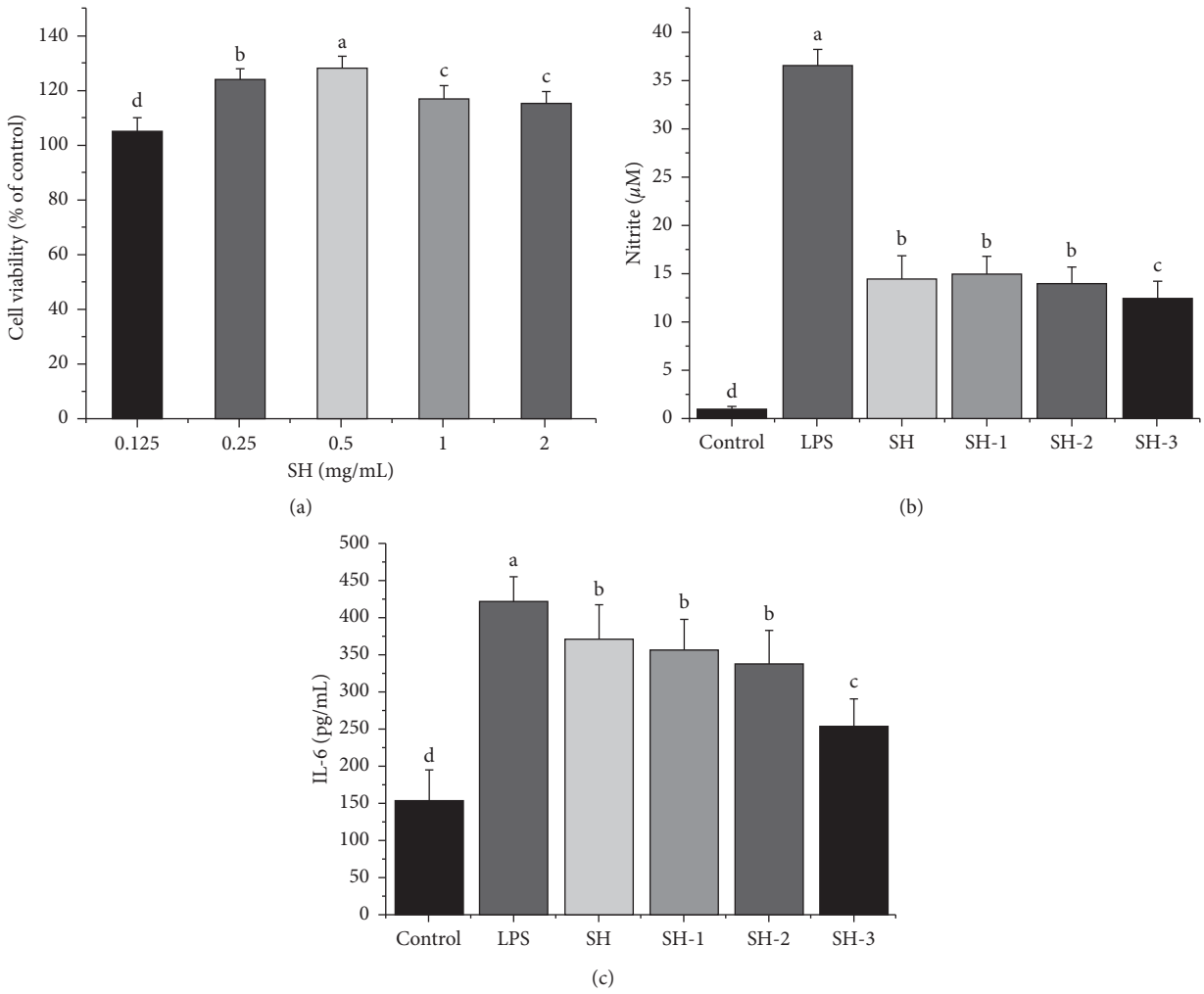


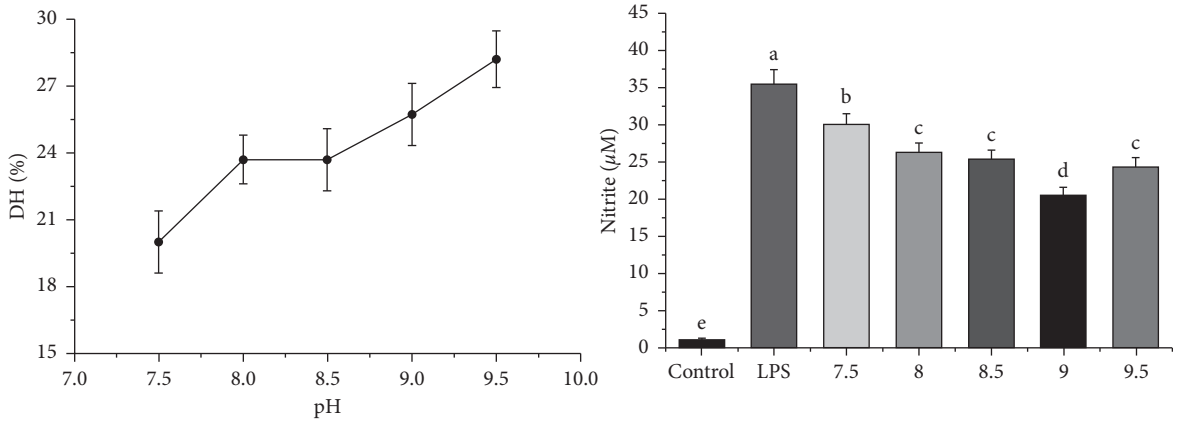
Figure 1: SH supplementation inhibited LPS-stimulated inflammation in macrophages: cell viability (a), nitrite level (b), and IL-6 level (c) in RAW 264.7 cells. Data are expressed as means ± SEM. Values without a common letter are significantly different at $P < 0.05$.

changed from 7.5 to 9.5, while other variables were set as follows: enzymolysis temperature 50°C, enzymolysis time 6 h, solid/liquid ratio 1 : 25, and enzyme additive amounts 2000 U/g protein. The DH was increased from 20.0% to 28.2% as the pH increased from 7.5 to 9.5. However, the NO inhibition rate of SH increased with pH until it was up to 9.0 and then began to decrease. The maximum NO inhibition rate was 42.36% at the pH of 9.0. Such a decline in the NO inhibition rate might be due to the change of spatial structure of the enzyme or the interference of the ionic properties of the substrate, which could reduce the ability of the substrate to bind the enzyme, resulting in a decrease or even loss of enzyme activity [20].

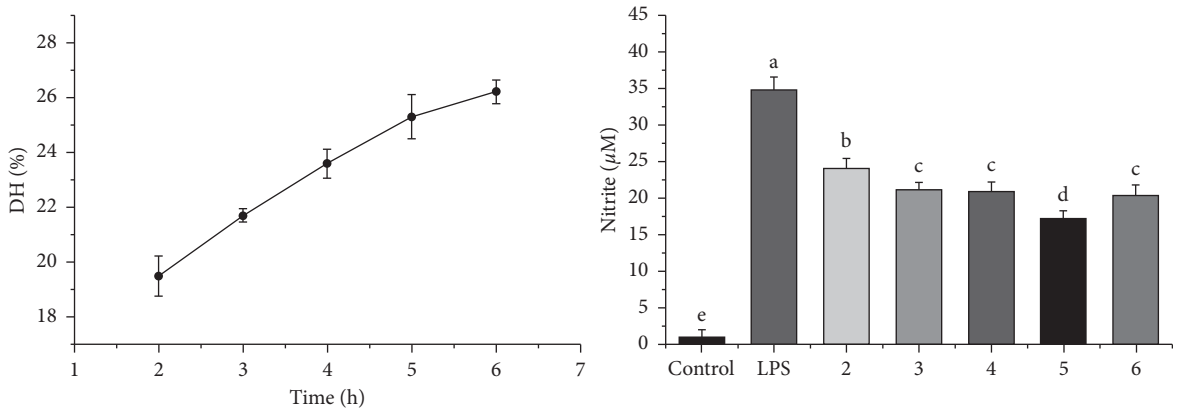
3.2.2. The Effect of Enzymolysis Time on DH and NO Inhibition Rate. Figure 2(b) showed the effects of different enzymolysis times on the DH and NO inhibition rate of SH when the pH was 9.0 and other three factors remained unchanged. The NO inhibition rate increased when the enzymolysis time varied from 2 h to 5 h peaked at 50.66% and then decreased. While DH kept increasing with the increase of time. This might be attributed to the fact that longer enzymolysis time could lead to excessive hydrolysis of SH.

3.2.3. The Effect of Enzymolysis Temperature on DH and NO Inhibition Rate. The reaction temperature of SH was set with the range of 45 to 65°C. Enzymolysis time was 5 h while the other three conditions were unchanged. As shown in Figure 2(c), the NO inhibition rate of SH peaked when the temperature was 50°C. When the temperature exceeded 50°C, the rate decreased. This phenomenon could be attributed to the possibility that the protease might be denatured and inactivated under high temperature conditions, the stability of the enzyme will decline, and its role of cutting the peptide chain will be lost [26].

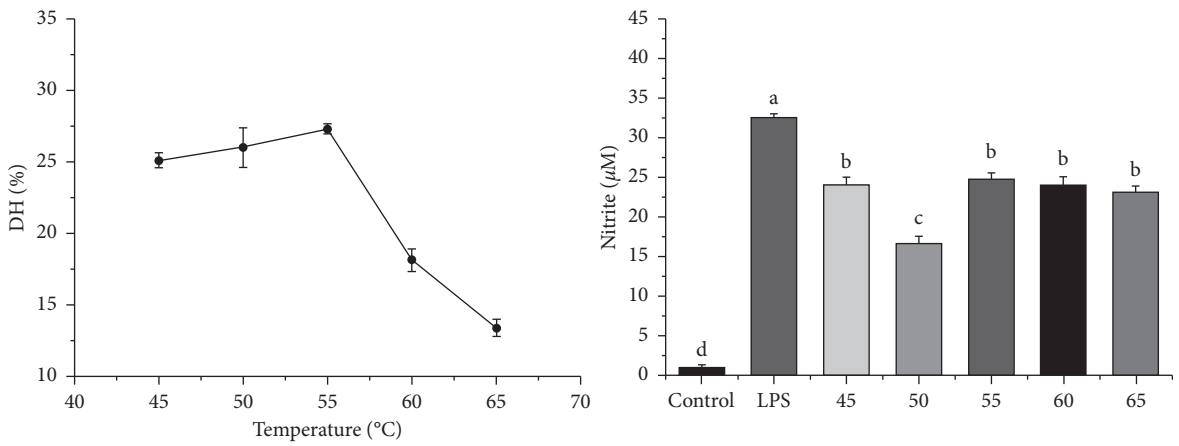
3.2.4. Effect of Solid/Liquid Ratio on DH and NO Inhibition Rate. The solid/liquid ratio of SH was assessed within the range of 1 : 5 to 1 : 25. Enzymolysis temperature was 50°C while the other three conditions were unchanged. The results



(a)



(b)



(c)

Figure 2: Continued.

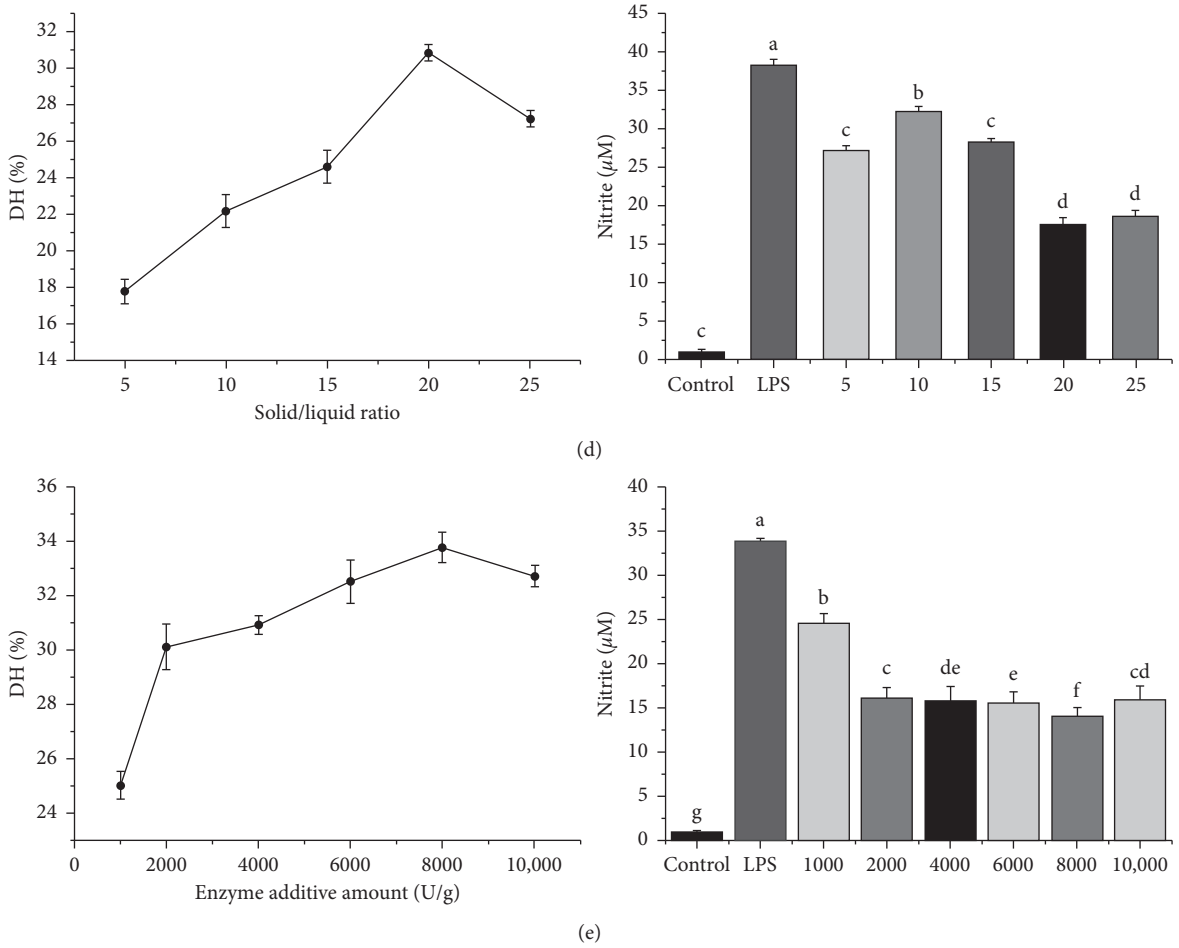


Figure 2: Effects of enzymolysis conditions on the DH and NO inhibition rate of SH: (a) pH; (b) enzymolysis time; (c) enzymolysis temperature; (d) solid/liquid ratio; and (e) enzyme additive amount. Data are expressed as means ± SEM. Values without a common letter are significantly different at $P < 0.05$.

were displayed in Figure 2(d). When the S/L ratio is 1 : 20, the NO inhibition rate is relative higher, which was 53.94% and the DH reached the peak. Considering the subsequent mass production, 1 : 20 of the S/L ratio was selected for further optimization.

3.2.5. Effect of Enzyme Additive Amounts on DH and NO Inhibition Rate. The effect of enzyme additive amounts on DH and NO inhibition rate was shown in Figure 2(e). It was set at 1000, 2000, 4000, 6000, 8000, and 10,000 U/g protein, respectively. Solid/liquid ratio was 1 : 20 and the other three conditions were unchanged. When the enzyme additive amount was 8000 U/g protein, NO inhibition rate and DH reached 58.73% and 33.76%, respectively. However, there was a decrease when the enzyme additive amount continued to rise. This finding is probably attributed to the increase of enzyme molecular collision and aggregation, leading to the enzyme autolysis, which is not conducive to the enzymatic reaction. This result indicated that the enzyme additive amount of 8000 U/g protein was sufficient to obtain a high NO inhibition rate.

3.3. Response Surface Analysis. The response surface analysis of the experimental data was shown in Tables 2 and 3. The response variable and the test variables were related by the following a second-order regression equation:

$$\begin{aligned}\text{NO inhibition rate} = {} & 58.53 - 2.39X_1 + 1.20X_2 + 2.46X_3 \\ & - 0.59X_4 + 3.30X_1X_2 + 2.98X_1X_3 \\ & + 2.42X_1X_4 - 1.33X_2X_3 + 2.18X_2X_4 \\ & - 0.14X_3X_4 - 4.05{X_1}^2 - 3.24{X_2}^2 + 0.39X_3^2 \\ & - 2.82{X_4}^2,\end{aligned} \tag{7}$$

where Y is the NO inhibition rate of SH, X_1 is pH, X_2 is enzymolysis time, X_3 is enzymolysis temperature, and X_4 is enzyme additive amount. As presented in Table 3, a high F-value (10.31) and a low p-value (<0.0001) indicated that the regression model was significant. In addition, the value of the determination coefficient ($R^2 = 0.9116$), the adjusted coefficient of determination ($R^2_{\text{adj}} = 0.8332 > 0.8$), and the coefficient of variation (C.V. = 3.35%) indicated a high

Table 2: Box–Behnken experimental design with independent variables.

Run	Coded variable levels				NO inhibition rate (%)
	X_1	X_2	X_3	X_4	
1	−1	−1	0	0	56.51
2	1	−1	0	0	45.24
3	−1	1	0	0	52.24
4	1	1	0	0	54.17
5	0	0	−1	−1	54.37
6	0	0	1	−1	60.24
7	0	0	−1	1	53.84
8	0	0	1	1	59.17
9	−1	0	0	−1	57.57
10	1	0	0	−1	49.51
11	−1	0	0	1	49.04
12	1	0	0	1	50.64
13	0	−1	−1	0	49.04
14	0	1	−1	0	56.51
15	0	−1	1	0	57.57
16	0	1	1	0	59.7
17	−1	0	−1	0	58.64
18	1	0	−1	0	46.2
19	−1	0	1	0	55.94
20	1	0	1	0	55.44
21	0	−1	0	−1	53.31
22	0	1	0	−1	49.04
23	0	−1	0	1	49.91
24	0	1	0	1	54.37
25	0	0	0	0	58.64
26	0	0	0	0	59.7
27	0	0	0	0	57.44
28	0	0	0	0	59.44
29	0	0	0	0	57.44

degree of correlation between the NO inhibition rate and the four variables. Furthermore, the p-value of the lack of fit was 0.1113 ($p > 0.05$), which indicated that the factors other than these four factors had less interference with the test results and the differences were not significant, suggesting good reliability of the regression model. Moreover, the linear coefficients and quadratic term coefficients (X_1, X_2, and X_3) (X_1^2, X_2^2, and X_4^2) (X_1X_2, X_1X_3, X_1X_4, and X_2X_4) were significant ($p < 0.05$).

Response surfaces were plotted using Design-Expert to study the effects of variables and their interactions on NO inhibition rate [27]. The 3D response surface plots and 2D contour plots were shown in Figure 3. It provided a method to visualize the relationship between the responses and experimental levels of each variable and the type of interactions between the two test variables. The shapes of the contour plots indicate the significance of the interactions between two tested variables, where a circular contour plot indicates no significant interaction, and an elliptical or saddle contour plot suggests a significant interaction. As shown in Figure 3, the interactions of the variables (pH and enzymolysis time, pH and enzymolysis temperature, pH and enzyme additive amount, and enzymolysis time and enzyme additive amount) were significant ($p < 0.05$).

According to the regression model, the optimal enzymolysis conditions were as follows: pH = 9.0, enzymolysis time = 4.92 h, enzymolysis temperature = 55°C, and enzyme additive amount = 7674.22 U/g protein. Under these conditions, the theoretical NO inhibition rate of SH was 61.44%. In order to confirm the validity of the regression model, an additional experiment was conducted under the optimal enzymolysis conditions. The NO inhibition rate of SH was 60.23%, almost approaching the predicted value 61.44%, which meant that the regression model was valid.

Table 3: ANOVA for the response surface quadratic model of NO inhibition rate.

Source	SS	Df	MS	F value	p-value	
Model	480.18	14	34.3	10.31	<0.0001	Significant
X_1	68.83	1	68.83	20.7	0.0005*	
X_2	17.4	1	17.4	5.23	0.0382*	
X_3	72.32	1	72.32	21.75	0.0004*	
X_4	4.17	1	4.17	1.25	0.2819	
X_1X_2	43.56	1	43.56	13.1	0.0028*	
X_1X_3	35.64	1	35.64	10.72	0.0055*	
X_1X_4	23.33	1	23.33	7.02	0.0191*	
X_2X_3	7.13	1	7.13	2.14	0.1652	
X_2X_4	19.05	1	19.05	5.73	0.0312*	
X_3X_4	0.073	1	0.073	0.022	0.8844	
X_1^2	106.34	1	106.34	31.98	<0.0001*	
X_2^2	68.1	1	68.1	20.48	0.0005*	
X_3^2	0.99	1	0.99	0.3	0.5935	
X_4^2	51.41	1	51.41	15.46	0.0015*	
Residual	46.55	14	3.33			
Lack of fit	41.97	10	4.2	3.66	0.1113	Not significant
Pure error	4.59	4	1.15			
Total variation	526.73	28				
R^2	0.9116					
Adj R^2	0.8332					
C.V. (%)	3.35					

3.4. LPS-Stimulated Inflammation Was Inhibited by SH in Macrophages. In RAW264.7 cells, LPS-stimulation increased the secretion of NO and IL-6 (Figures 1(b) and 1(c)). SH dramatically inhibited the secretion of NO and IL-6, with SH-3 (SH < 3 kDa) showing the best inhibitory effect by 66.24% and 39.54%, respectively. This result indicated that SH could inhibit the inflammatory response in macrophage cells, and thus SH-3 was used for further investigation.

3.5. Functional Properties

3.5.1. Emulsifying Properties. SH is a surface-active material that contains both hydrophilic and hydrophobic groups which could promote the formation of an oil-in-water emulsion. Protein ability can be determined through the formation and stabilization of the emulsion [21, 28]. As shown in Figures 4(a) and 4(b), the EAI and ESI of SH-3 were affected by pH, where the EAI and ESI were the worst near the isoelectric point (pH = 4) and better under acidic and alkaline conditions that deviated from the isoelectric point. Several studies have shown that a range of pH at 6–10

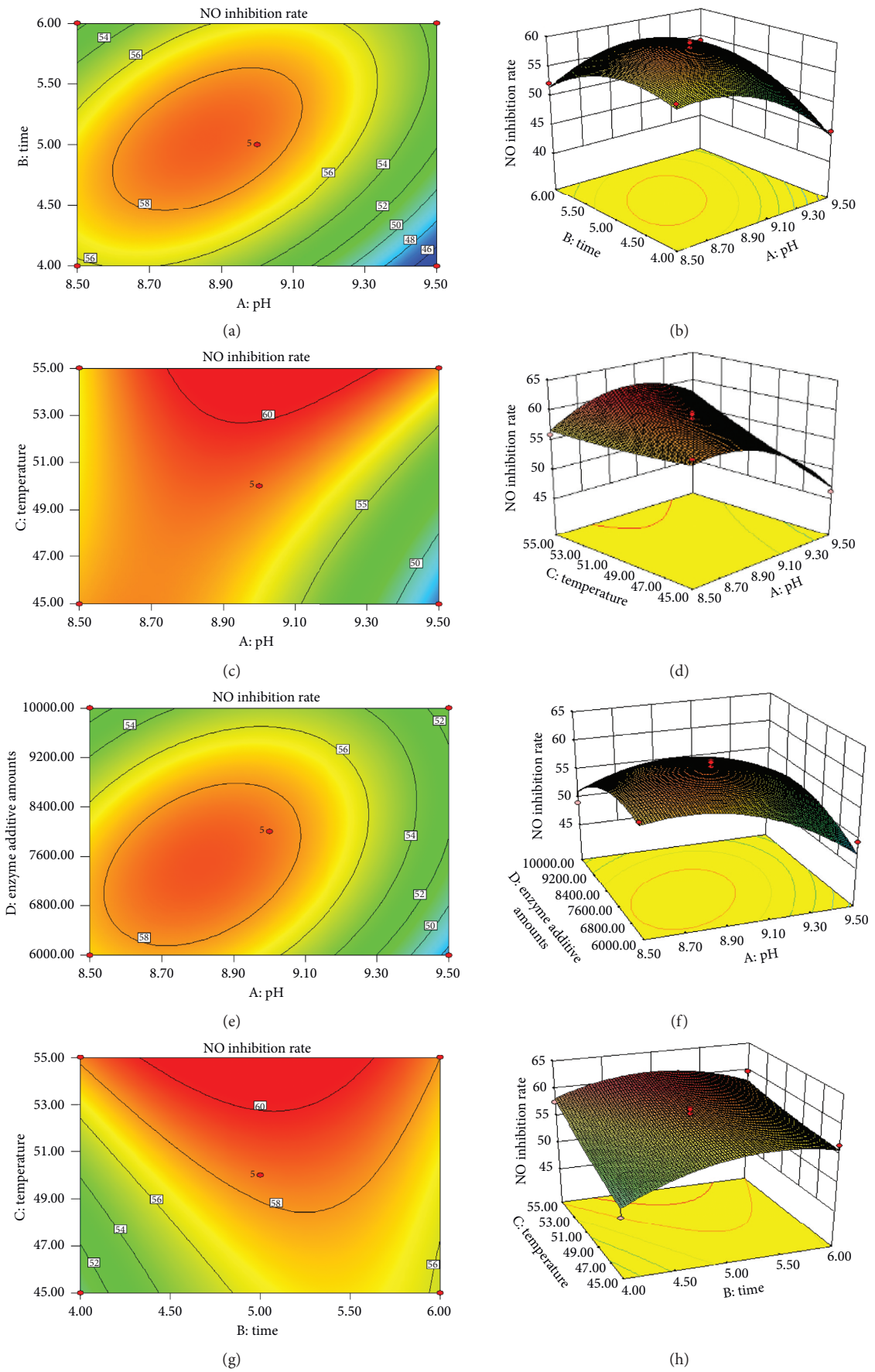


Figure 3: Continued.

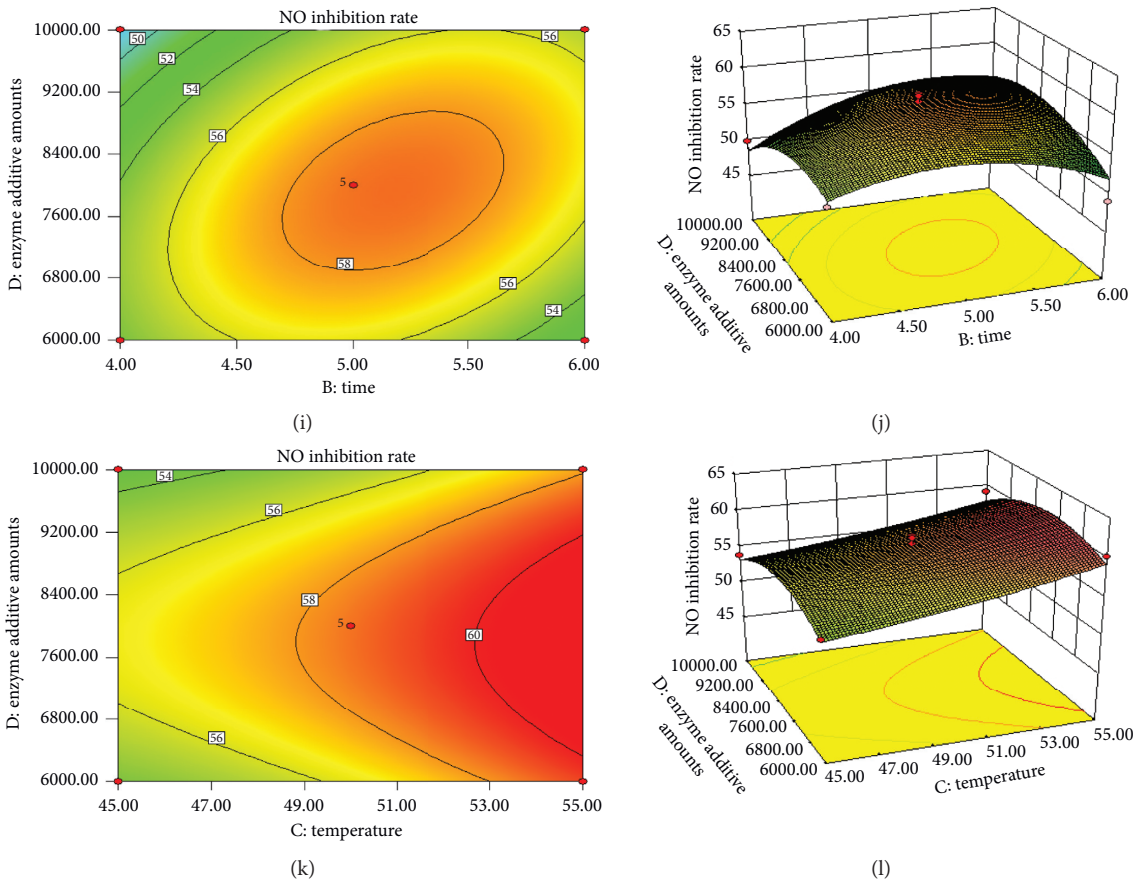


Figure 3: Response surface (3D) and contour plots (2D) showing interaction effect of (a) pH, (b) enzymolysis time, (c) enzymolysis temperature, and (d) enzyme additive amount on NO inhibition rate.

produced the highest EAI, with the lowest EAI at pH 4 [29, 30]. According to Taheri et al. [29], polypeptides unfold at highly alkaline pH due to their negative charges. Repulsion caused by this change allows a better orientation at the interface. Therefore, this condition makes the exposure of hydrophilic and hydrophobic peptide residues more effective and promotes significant interactions at the oil–water interface.

3.5.2. Foaming Capacity and Foam Stability. Foaming capacity is one of the important functional properties of proteins and peptides. The surface tension at the air–water interface is lower by the proteins or peptides which leads to the formation of a stable foam [31]. Generally, foam formation follows three major steps, transportation, penetration, and restructuring of the molecules at the air–water interface [32]. FC and FS of SH-3 are shown in Table 4 and Figure 4(c), respectively. FC of SH-3 was 73.75 ± 2.58%. Regarding FS, it was 17.50% after 1 min and 0.63% after 4 min at pH 6, and the foam faded afterwards. Low FS possibly arises from the formation of free amino acids during hydrolysis [33]. Nalinanon et al. [34] reported that low-molecular-weight (~1 kDa) peptides were unable to maintain a well-ordered, interface orientation of the molecule. Besides, water has fluidity under gravity; gas diffusion between foams will cause gas imbalance, which is the main factor of foam instability [35].

3.5.3. Water and Oil Holding Capacity. The solubility of protein affects both WHC and OHC because high solubility means smaller molecular size, which influences the absorption of water and oil [28]. As shown in Table 4, the values of WHC and OHC in SH-3 were 1.90 ± 0.12 and 2.35 ± 0.05 g/g, respectively.

3.5.4. Antioxidant Activity. Oxidative stress is an imbalance between reactive oxygen species (ROS) generation and scavenging by antioxidative agents [36, 37]. In addition to natural antioxidants (carotenoids, phenolic compounds, vitamin C, vitamin E, and so on), the hydrolysates and peptides have also been recognized for their antioxidative activity [38, 39]. As free radical compounds, DPPH and ABTS are extensively employed to estimate the antioxidant capacity of various samples [40]. As shown in Table 5, the IC_{50} values of DPPH and ABTS of SH were 1.215 ± 0.081 mg/mL and 1.563 ± 0.080 mg/mL. After ultrafiltration of SH, DPPH radical scavenging activity and ABTS radical scavenging activity increased with the decrease of molecular weight, indicating that low molecular weight possessed higher antioxidant activity, which were in agreement with

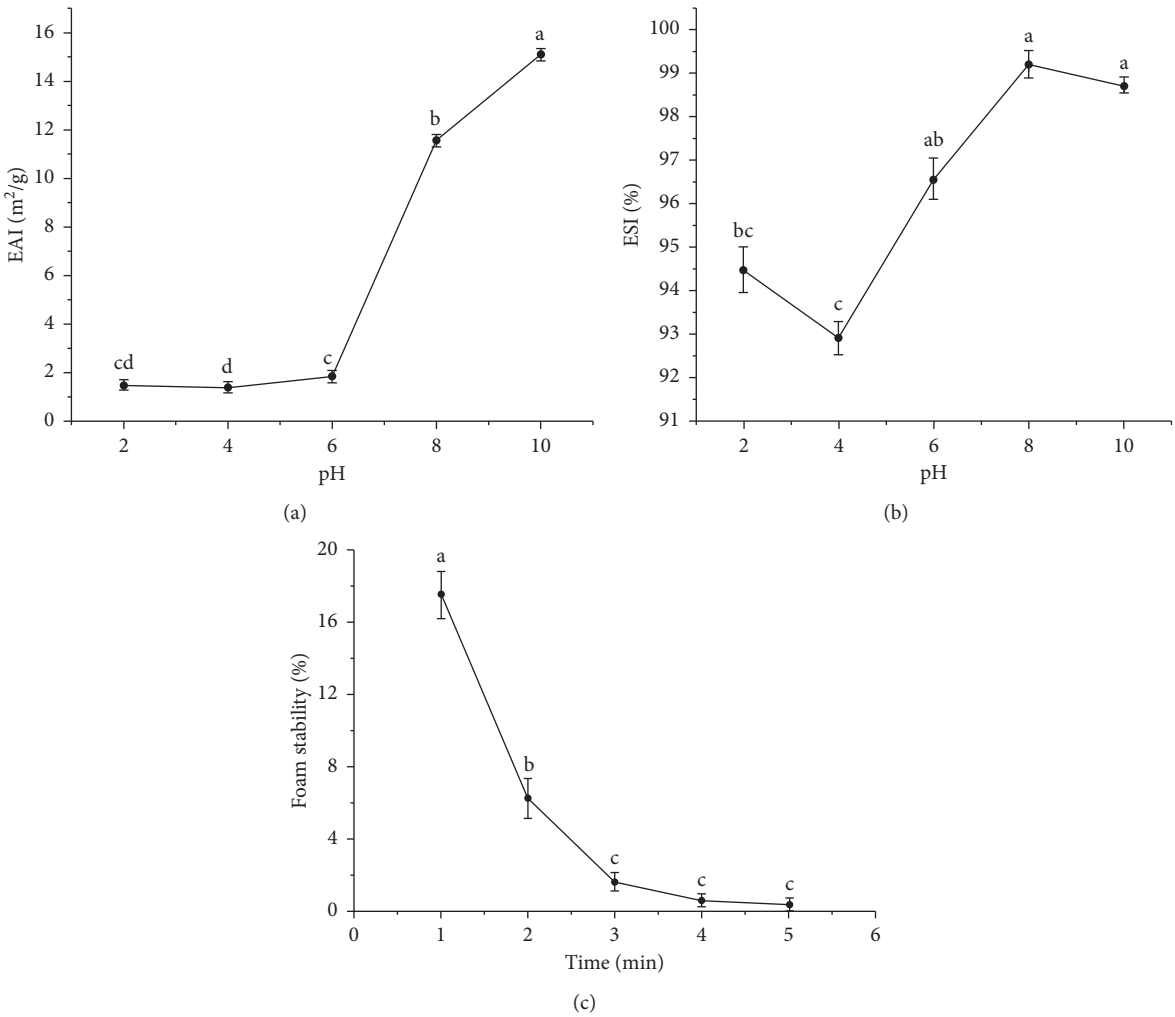


Figure 4: Functional properties of SH-3 at different pH: (a) emulsion activity index; (b) emulsifying stability index; and (c) foam stability at different time. Data are expressed as means ± SEM. Values without a common letter are significantly different at $P < 0.05$.

Table 4: Functional properties of SH-3 obtained under optimal enzymolysis conditions.

Functional property	Capacity
WHC (g water/g protein)	1.90 ± 0.12
OHC (g oil/g protein)	2.35 ± 0.05
Foam capacity (%)	73.75 ± 2.58

Table 5: IC_{50} value of SH and its fractions by ultrafiltration on DPPH radical scavenging activity and ABTS radical scavenging activity.

Fraction	IC_{50} DPPH radical scavenging activity (mg/mL)	IC_{50} ABTS radical scavenging activity (mg/mL)
SH	1.215 ± 0.081^{b}	1.563 ± 0.080^{B}
>10 kDa	1.968 ± 0.095^{a}	2.215 ± 0.109^{A}
3–10 kDa	1.264 ± 0.105^{b}	1.603 ± 0.063^{B}
<3 kDa	1.043 ± 0.090^{c}	1.396 ± 0.074^{C}
GSH	0.007 ± 0.001^{d}	0.009 ± 0.002^{D}

GSH was used as positive control. Data are expressed as means ± SEM. Values without a common letter are significantly different at $P < 0.05$.

the published reports on antioxidant peptides [41]. However, GSH showed higher antioxidative ability compared to SH.

4. Conclusion

In this study, underutilized sturgeon muscles were successfully transformed to bioactive hydrolysates by enzymatic hydrolysis. Based on the single-factor experiments, RSM was used to optimize the experimental variables. The optimum enzymolysis conditions were as follows: pH = 9.0, enzymolysis time = 4.92 h, enzymolysis temperature = 55°C, solid/liquid ratio = 1 : 20, and enzyme additive amount = 7674.22 U/g protein. Under these conditions, the NO inhibition rate of SH was 60.23%. After ultrafiltration of SH, SH-3 (SH < 3 kDa) showed the best inhibitory effect of NO and proinflammatory cytokine IL-6 by 66.24% and 39.54%, respectively. In addition, we found that SH-3 had good properties of emulsification and possessed good WHC and OHC. SH-3 was also found to exhibit stronger antioxidant capacity in DPPH and ABTS radical scavenging activity. These results suggest that SH-3 might be the potential safer alternative ingredient possessed of anti-inflammatory and antioxidative activities. However, the structure characterization and anti-inflammatory mechanism need to be further investigated.

Data Availability

All data generated or used during the study appear in the submitted article.

Conflicts of Interest

There are no conflicts of interest associated with the authors of this manuscript.

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Research Article

Anti-Inflammatory Effects of Essential Oils of *Amomum aromaticum* Fruits in Lipopolysaccharide-Stimulated RAW264.7 Cells

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Inflammation is a vital physiologic response of cellular injury, infection, or autoimmune activation. Overproduction of proinflammatory mediators may result in the chronic inflammation that leads to many diseases such as rheumatoid arthritis, asthma, multiple sclerosis, and atherosclerosis. In this study, we assessed for the first time the anti-inflammatory effects of the essential oils of *Amomum aromaticum* fruits (AAE) in RAW264.7 murine macrophage model. As a result, AAE potently inhibited the production of nitric oxide in LPS-induced RAW264.7 cells with the IC_{50} value of $0.45 \pm 0.11\,\mu g/ml$. AAE also dose-dependently reduced the expression of two proinflammatory proteins iNOS and COX-2 in the stimulated cells. Phytochemical analysis revealed that major compositions of the volatile oils including 1,8 cineole (48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), and α-terpineol (2.28%) may contribute greatly to the inhibition effects due to their anti-inflammatory properties. The results suggest for the potential uses of AAE in chronic inflammation prevention.

1. Introduction

Chronic inflammation is an undesirable phenomenon of a prolonged inflammatory response. Overproduction of proinflammatory mediators such as cytokines, interleukins, nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX-2) may result in various diseases such as rheumatoid arthritis, asthma, multiple sclerosis, and atherosclerosis [1]. Therefore, control of proinflammatory responses is a wise strategy to prevent the development of inflammatory diseases. Since the ancient time, food was determined as an important source for prevention of diseases. There has been accumulation of evidence that increases consumption of certain foods might lower the risk of cardiovascular disease, cancer, and inflammation [2, 3].

Amomum aromaticum Roxb. is a species of the Zingiberaceae family, which is a common spice and food flavoring agent in Vietnam and other Asian countries. The fruits of this plant have been used in traditional medicine for the treatment of cough, abdominal pain, vomiting, diarrhea, and malaria. The oils of seeds have been used in India for benefiting the digestive system, applied to the eyelids to eliminate the inflammation [4, 5]. To date, there has been only few studies about of the phytochemicals as well as the biological activities of this plant. Recently, *A. aromaticum* essential oils are shown as promising antileishmanial agent in a screening program of 37 plants of Vietnam flora [6]. The methanolic extract of *A. aromaticum* exhibited significant antimicrobial activity against *Enterococcus faecalis*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Proteus mirabilis*,

and *Pseudomonas aeruginosa* with the MIC values ranging from 3.41 to 9.63 mg/ml [7].

In this study, we investigated the phytochemical contents of essential oils of the *A. aromaticum* fruits and its anti-inflammatory properties including NO production inhibition assay and inhibitory effects on the expression of two key enzymes of inflammation process: inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW264.7 cells stimulated with LPS.

2. Materials and Methods

2.1. Plant Materials and Essential Oil Preparation. The fruits *A. aromaticum* were freshly collected in Ha Giang province in November 2019. The samples were taxonomically identified by Dr. Nguyen the Cuong, Institute of Ecology and Biological Resources (VAST), and voucher specimens were deposited in the Institute of Marine Biochemistry. The samples (500 g) were hydrodistilled in a Clevenger-type apparatus for 4 h, after which the essential oils were separated and dried with anhydrous Na_2SO_4. The obtained oils (AAE) were stored at −5°C until used.

2.2. GC/MS Analysis of Essential Oils. GC/MS analysis was performed using an Agilent GC7890A apparatus coupled to a mass selective detector (Agilent 5976C). A HP-5MS fused silica capillary column (60 $m \times 0.25$ mm id. $\times$ 0.25 μm film thickness) was used. Helium was the carrier gas with a flow rate of 1.0 ml/min. The inlet temperature was 240°C, and the oven temperature program was as follows: 60°C to 220°C at 4 °C/min and then at 20°C/min to 240°C. The split injection mode was 1 : 142, the detector temperature was 240°C, and the injection volume was 0.1 μl. The MS interface temperature was 240°C, MS mode, E.I. detector voltage 1300 V, and mass range 40–400 Da at 1.0 scan/s. Identification of components was achieved based on their retention indices and by comparison of their mass spectral fragmentation patterns with those stored on the MS library (NIST08, Wiley09). Component relative contents were calculated based on total ion current without standardization. Data processing was MassFinder4.0.

2.3. Cell Culture. Murine macrophage RAW264.7 cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and penicillin (100 units/ml)-streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA). Cultures were maintained in a CO_2 incubator-humidified atmosphere 5% CO_2 at 37°C.

2.4. Assay for Inhibition of NO Production. The effects of samples on the NO production in LPS-stimulated RAW264.7 macrophage cells were examined as described previously [8]. The cells were seeded in 96-well plate at 2×10^5 cells/well and incubated for 18 h. The plates were pretreated with AAE (from 0.1 μg/ml to 100 μg/ml) for 30 min and then incubated for another 24 h with or without 1 μg/ml LPS (*Escherichia coli* 0111: B4; Sigma Aldrich, USA). 100 μl of the culture supernatant was transferred to other 96-well plates, and 100 μl of Griess reagent was added. The absorbance of the reaction solution was read at 570 nm with a XMark microplate reader (BioRad, USA). The remaining cell solutions in cultured 96-well plate were used to evaluate cell viability by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [9]. Cardamonin, a known NO production inhibitor, was used as a positive control [10].

2.5. Western Blot Analysis. The RAW264.7 cells were harvested and lysed in a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% NP-40, 5 mM sodium orthovanadate, and protease inhibitors cocktail (BD Biosciences)) and then centrifuged for 10 min at 4°C and 15,000 rpm. An equal amount of protein was separated onto SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to a PVDF membrane (Millipore, Germany). The membranes were blocked in 5% nonfat skim milk for 1 h at room temperature, probed with the appropriate primary antibodies, washed, and then incubated with the corresponding secondary antibodies. α-Tubulin was used as the loading control. The signal was developed using the ECL (enhanced chemiluminescence) system (GE Healthcare, UK) and detected in a gel imaging system Azure c300 (Azure Biosciences, UK). The captured images were analyzed and quantified using ImageJ *v*. 1.53a (NIH, Maryland, USA).

2.6. Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Statistical significance was assessed by the two-tailed unpaired Student's t test, and P values less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Phytochemicals of AAE Analyzed by GC/MS. The essential oils of *A. aromaticum* fruits (AAE) obtained by hydrodistillation yields 1.49% based on a calculation with the dry weight of fruits. A total of 25 compounds were identified by using GC/MS data in combination with the MS library analyses (Table 1). The major chemical group of AAE is monoterpene with more than 81% of the total contents. Of which, 1,8-cineole (or eucalyptol, 48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), α-terpineol (2.28%), and β-pinene (2.18%) are among the most abundance monoterpenes of the fruit oils. Four aliphatic aldehydes was found including n-octanal, 2-octenal, (*E,E*)-decenal, and (*E,E*)-dodecanal which comprise about 8.26% of the AAE content. Meanwhile, only one sesquiterpene *E*-nerolidol (1.69%) was found. Our results were in good agreement with the previous report which shows monoterpenes as the major contents together with the presence of aliphatic aldehyde and sesquiterpene groups [6]. The fruit essential oils contained 55.2% of 1,8-cineole which was slightly higher than that of our findings. The distribution of relative quantities of major

Table 1: Chemical compositions of essential oils of *A. aromaticum* fruits.

No.	Compounds	RI	Relative percentage (%)
1	α-Thujene	930	0.16
2	α-Pinene	939	2.43
3	Sabinene	978	0.54
4	β-Pinene	984	2.18
5	Myrcene	991	0.46
6	*n*-Octanal	1003	0.47
7	α-Phellandrene	1010	1.43
8	*O*-Cymene	1029	0.51
9	Limonene	1034	2.9
10	1,8-Cineole	1038	48.22
11	(*E*)-β-cymene	1048	0.83
12	2-Octenal	1058	0.95
13	γ-Terpinene	1063	0.3
14	Linalool	1101	0.37
15	Isoneral	1166	0.19
16	δ-Terpineol	1174	0.21
17	Isogeranial	1184	0.3
18	Terpinen-4-ol	1185	0.92
19	α-Terpineol	1198	2.28
20	Neral	1246	6.72
21	Geraniol	1256	1.33
22	(*E,E*)-Decenal	1264	4.9
23	Geranial	1275	9.24
24	(*E,E*)-Dodecanal	1470	1.94
25	*E*-Nerolidol	1570	1.69
	Total identified (%)		**91.47**
	Yield[a] (%)		**1.49**

[a]Yield calculated based on the fresh materials; RI: retention index.

monoterpenes in both studies was found to be similar. The difference of quantities of individual compounds in both samples may be due to the variation of the origin of samples, seasons of collection, or the environmental factors. The presence of high content of 1,8-cineole was not only found in *A. aromaticum* but also in some other *Amomum* species including *A. tsao-ko* (23.87%–45.24%) [11–13] and *A. subulatum* Roxb (20%–89%) [14–16] depending on parts of the plant used for analysis.

3.2. AAE Reduced the NO Production in LPS-Induced RAW264.7 Cells by Inhibiting the Expressions of iNOS and COX-2. NO is an important signaling molecule in various physiological and pathophysiological responses [17]. Searching for inhibitors of NO production in LPS-stimulated macrophages has been a worldwide effort for the development of anti-inflammatory agents. The in vitro anti-inflammatory activity of AAE was investigated by determining its NO production inhibitory effect in LPS-stimulated RAW264.7 cells. The primary screening results showed that AAE inhibited potently the NO production (about 100%) in the stimulated cells at concentration of 100 μg/ml. We further evaluated the potency of the inhibitory activity of AAE by determining its IC_{50} value. As the results, the IC_{50} value of AAE was determined as 0.45 ± 0.11 μg/ml which was slightly higher than the positive control, cardamonin (0.59 ± 0.18 μg/ml). Treatment of AAE at the screening concentration after 24 h had no impact on the cell viability (data not shown). Next, we investigated the effects of AAE on the two key enzymes of inflammation process: iNOS (inducible nitric oxide synthase), mainly responsible for the production of NO and COX-2 (cyclooxygenase-2), in charge of production inflammatory mediators such as PGE2 (prostaglandin E2). The western blot analysis revealed that AAE dose-dependently inhibited the expression of both enzymes. Remarkably, at a concentration of 0.3 μg/ml, the inhibitory effects of AAE against iNOS and COX-2 expressions were still observed significantly (Figure 1(b)). To our knowledge, this is the first report of this potent anti-inflammatory activity of the *A. aromaticum* essential oils.

The phytochemicals are considered as the major contributors to the biological activity of a plant samples. In our study, we found that the fruit essential oils showed remarkable anti-inflammatory effects. The major composition of AAE, as indicated, is 1,8-cineole which comprises about 48% of the total oil content. Interestingly, 1,8-cineole was demonstrated as a very promising anti-inflammatory agent. Molecular mechanism studies indicated that 1,8-cineole effectively reduced the expression of proinflammatory cytokines such as TNF-IL-1β and IL-6 with the IC_{50} values ranging from 0.2 to 7.0 μM. It was found to be a potent inhibitor of NF-κB activation [18]. 1,8-cineole also displayed its anti-inflammatory properties in various animal models. This compound was advanced to clinical trials for bronchial asthma. When administered as an adjunct therapy with prednisolone, 1,8-cineole showed a significant improvement in respiratory volume and quality of asthma. The effect was still maintained when the dosage of prednisolone was decreased by 36% [19]. Other major compositions of AAE such as α-pinene [20], α-terpineol [21], geraniol [22], neral, and geranial [23] also exhibited their effects of anti-inflammation. It is demonstrated that the chief monoterpenes of AAE seem to greatly contribute to the anti-inflammatory activity of the fruit oils.

The anti-inflammatory properties of essential oils of some other *Amomum* species were reported. The fruit extract of *A. tsao-ko* displayed potent anti-inflammatory effects in RAW264.7 cells stimulated with LPS [24]. Further studies showed that the effects were achieved because this extract induced the expression of heme oxygenase-1 which consequently increased the Nrf-2 activation. The similar effects were also obtained from different extracts and isolated compounds from *A. tsao-ko* [25–27]. Agnihotri et al. investigated the topical anti-inflammatory effect of the fruit essential oils of *A. subulatum*. The results showed that the volatile oils exhibited moderate activities compared with standard drug, diclofenac [14]. The extracts of *A. compactum*, *A. xanthoides*, and *A. vilosum* also demonstrated their anti-inflammatory activities in vitro and in vivo [28–30]. Interestingly, there have been very few studies on the *Amomum* essential oils with anti-inflammation. In our study, we have reported that AAE is a promising anti-inflammatory agent by potently inhibiting the production of nitric oxide, the expressions of iNOS and COX-2 in LPS-induced RAW264.7 murine macrophages. Notably, *A. aromaticum* has been traditionally used as a common spice suggesting its safety effects in therapeutic use.

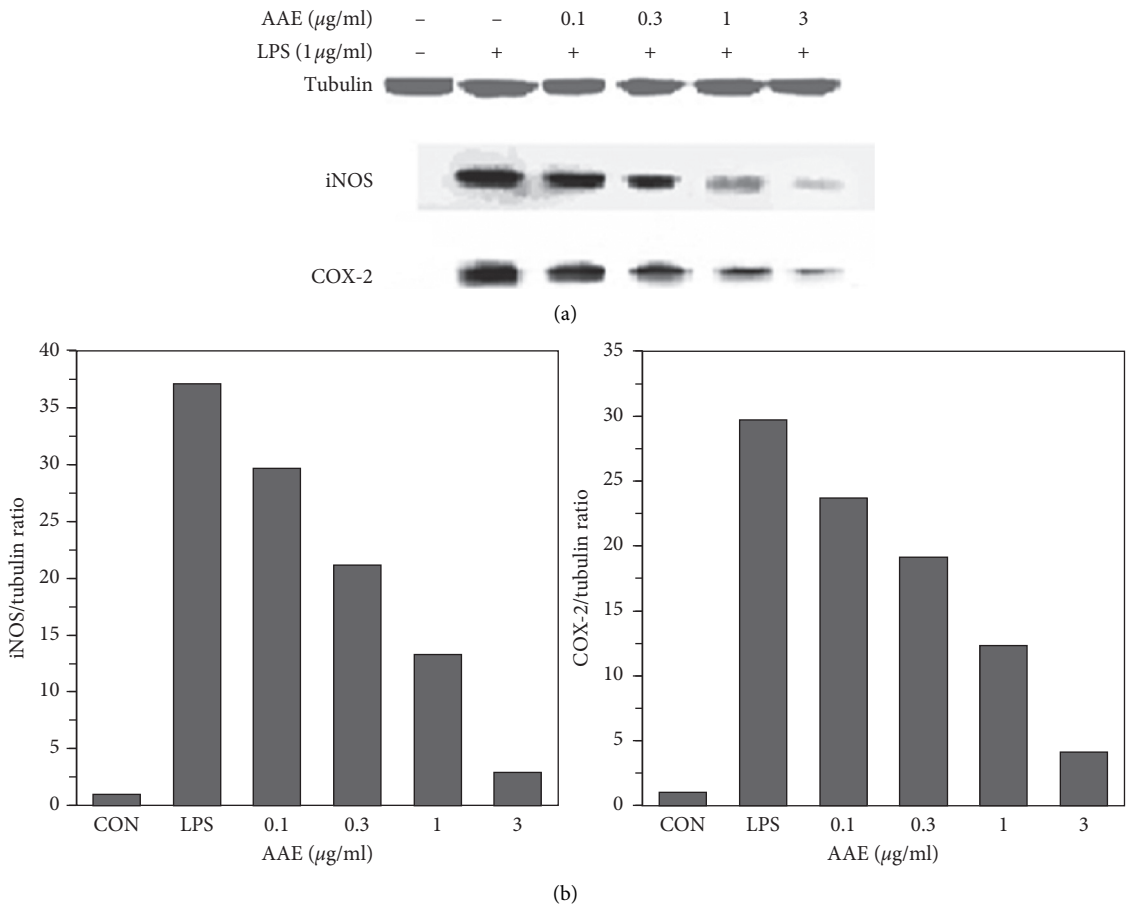

Figure 1: Inhibitory effect of AAE on LPS-induced iNOS and COX-2 expression in RAW264.7 murine macrophages. (a) Western blot analysis of iNOS and COX-2 expression in LPS-induced RAW264.7 cells after 24 h of treatment with AAE in different concentrations; (b) quantitation was analyzed by ImageJ 1.53a (NIH, USA). The ratio of the relative intensity of iNOS or COX-2 to tubulin is expressed; CON : control, LPS : lipopolysaccharide.

4. Conclusions

For the first time, the anti-inflammatory properties of the fruit essential oils of *Amomum aromaticum* Roxb. were investigated. The volatile oils displayed potent inhibitory effects against the production of nitric oxide; the expression of two proinflammatory enzymes iNOS and COX-2 in RAW264.7 macrophages was stimulated with LPS. Phytochemical investigation revealed that the essential oils contain various anti-inflammatory compositions including 1,8 cineole (48.22%), geranial (9.24%), neral (6.72%), α-pinene (2.43%), and α-terpineol (2.28%). These findings suggest that essential oils of *A. aromaticum* fruits can be an alternative natural source for prevention of chronic inflammation. Further studies are necessary for evaluation of anti-inflammation mechanisms of action and in vivo assessments of the very promising essential oils.

Data Availability

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

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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Research Article

Chemical Composition of *Pistacia lentiscus* Seeds' Oil from Moroccan High Atlas Mountain

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Pistacia lentiscus is an aromatic member of the Anacardiaceae family, endemic to the Mediterranean regions, which produces red and black oleaginous seeds in maturity. Our study focuses on the characterization of *Pistacia lentiscus* seed oils from three localities of High Atlas Mountain in the Azilal region of Morocco. Our results showed a very appreciable oil yield reaching more than 21% which clearly differs between the three sites studied (21.33 ± 0.17% in Boizoghrane, 15.22 ± 0.10% in Tighanimine, and 7.67 ± 0.29% in Tawjanizm locality); the analysis of the total fatty acids composition revealed a predominance of the unsaturated fatty acids represented essentially by the oleic and linoleic fatty acids, and also the triglyceride composition was dominated mainly by POO + SOL, POL + SLL + PoOP, and OOO. This potential of the chemical composition offers the possibility of producing nutraceutical oil, which represents a source of income capable of insisting vulnerable local populations to protect this species from deforestation, thus maintaining biodiversity, and to reduce by this interest the migratory flow from marginal areas.

1. Introduction

Pistacia lentiscus, known as Mastic tree, is named in Morocco as "Drou" or "Tidit." This plant is a membership of the Anacardiaceae family. It is a three-meter-high branched shrub with a resin odor [1]. Its leaves are paripennial and persistent and are found in lowland, low, and medium mountains in Morocco, under semiarid, humid, subhumid, and perhumid bioclimates [2]. Moreover, lentisk is known for its medicinal properties since antiquity. In fact, it used and practiced in traditional medicines for treatment of ulcers, eczema, hypertension, sore throat, cough, and kidney stones [3]. Mastic seed oil is often used as an application remedy to treat burns or back pain [4].

Benhammou et al. [5] reported that this oil has good nutritive quality because of its content in unsaturated fatty acids (70%) and saturated fatty acids (26%). The triglyceride (TAG) composition of lentisk oil showed that the majority of TAGs of this oil are of mono- and polyunsaturated forms, and the main constituents are SOL + POO (27.58 ± 2.36%) followed by SLL + POL (21.50 ± 2.06%) [6]. According to Arab et al. [7], the yield of phenolic compounds obtained from *Pistacia lentiscus* fruit is 61.34% (vegetable powder), whereas the concentration of the phenolic fruit extract, expressed as gallic acid, is 31.81 mg/ml.

The study of acute toxicity realized by Boukeloua et al. [8] showed a low toxicity of *Pistacia lentiscus* fixed oil. The high values of oral and intraperitoneal lethal doses of both *P. lentiscus* fixed oil administered in mice, respectively, (LD50 = 37 ml/kg body wt., p. o and LD50 = 2.52 ml/kg body wt., i. p.) show their low acute toxicity. The subchronic toxicity test conducted in rabbits at different doses (0.5, 1, and 2 mL kg−1) for 28 days via oral route also did not result in any signs of toxicity. The biochemical results indicate that this oil maintains the rates of aspartate amino transferase (AST) and alanine amino transferase (ALT) in the physiological norms [9].

However, until now, no studies have been made on the composition of the lentisk seeds, especially the chemical composition of the lipid profile, in the Azilal region, having a particular forest on the High Atlas Mountains with a favorable microclimate for several plants with high added values and also with a vulnerable population of which this plant can constitute a source of income in a spirit of protection of the biodiversity, and this region is characterized by a semiarid climate in the North and subhumid climate in the South. Therefore, the main objective of the presented work is to highlight the chemical composition of Moroccan lentisk seeds under the influence of pedoclimatic conditions of the study area.

2. Materials and Methods

2.1. Plant Material. *Pistacia lentiscus* seeds were harvested at full maturity (identical red color of seeds) from natural populations in three localities: Tawjanizm (TA), Boizoghrane (BO), and Tighanimine (TI) of the Azilal region (Morocco) (Table 1), during December 2015. Identification of the species was confirmed in regional herbarium MARK faculty of Sciences Semlalia in Marrakech (Morocco). A voucher specimen (MARK 10938) was deposited at the herbarium of this faculty. The seeds are taken manually from small and large plants of different heights (top and bottom) on the two sides of mountains and then mixed from each sampling site. Then, the seeds were sorted and thoroughly cleaned of all impurities, and after drying at 40°C, the seeds were ground to obtain a powder.

2.2. Extraction and Oil Content. The extraction of the oil was carried out for 6 hours with hexane in a Soxhlet extraction system, and at the end of the extraction, the solvent is evaporated in a rotary evaporator under vacuum, with slight heating (+30°C). The residual traces of hexane were removed by bubbling the extracted oil with nitrogen. The extracted oils are stored under nitrogen in the refrigerator until analysis.

2.3. Fatty Acid Composition. The total fatty acid composition of the lentisk oils was determined according to the AFNOR, T60-233, and T60-234 method. The methyl esters were then analyzed by gas chromatography (GC) using a Varian CP 3380 chromatograph with a flame ionization detector equipped with a capillary column packed with a stationary phase: CPWAX 52 CB (length: 25 m, inner diameter: 0.25 mm, and outer diameter: 0.39 mm). The temperature of the oven is 180°C, the temperature of the injector is 200°C, and the temperature of the detector is 210°C. The carrier gas is nitrogen.

2.4. Analysis of Triglycerides by HPLC. The method of triglyceride determination was according to the official method of the Commission of the European Union (1991). The analysis of triglycerides was carried out in a Jasco PU-2080 LC as well as an intelligent HPLC pump equipped with a Jasco CO-2065 in addition to the furnace column and a Jasco RI-930 refractive index detector equipped with Jasco AS-2055 autosampler. The column used was an omnisphere 5 μm C18, length 250 mm, and 4.6 mm ID. The conditions of the analysis were 50 : 50 v/v acetone/acetonitrile solvent, 1.2 mL/min flow rate, and 40°C oven temperature.

2.5. Statistical Analysis. All experiments were conducted in triplicate with SPSS Inc. software (version 13.0). One-way analysis of variance (ANOVA) was used to determine significant differences among means, with the significance level taken at $a = 0.05$. Tukey's HSD test was used to perform multiple comparisons among means.

A principal component analysis was studied using factor analysis of XLSTAT statistical software version 2011. The relationships between harvest sites and the parameters studied were also evaluated by Pearson's product moment correlation at $P \leq 0.05$.

3. Results and Discussion

3.1. Oil Content. The seeds show a good oil yield, and the average values obtained from studied localities are shown in Figure 1. The highest oil content corresponded to Boizoghrane seed at $21.33 \pm 0.17\%$. This value is almost consistent with that found by Boukeloua et al.'s study [8] of lentisk seeds from west of Skikda (Algeria) ($20.25\% \pm 0.10$), followed by Tighanimine at $15.22 \pm 0.10\%$. This yield is higher than that reported by Charef et al. [10] (11.72%) for red mastic fruit of lentisk collected from a forest located 70 km from the Algerian capital, and the lowest value was obtained for Tawjanizm seed oil at $7.67 \pm 0.29\%$, which is close to the value obtained for *P. lentiscus* harvested in France by Ferlay (9.8%).

The oilseeds of the lentisk from Boizoghrane and Tighanimine can be classified as oil-rich such as sunflower oil, peanut oil, palm oil, and soybean; therefore, these seeds can be used as a source of vegetable oil. On the contrary, the lentisk seeds taken from Tawjanizm, in which the oil content does not exceed 12%, are classified as seeds moderately poor in fat.

From these results, it can be said that the oil yield of lentisk seeds is influenced by the sampling zone, and the large difference in the oil yield between the three localities can be explained by the maturity of the lentisk seeds (in fact, the amount of oil increases during the seed maturation phase) as reported by Charef et al. [10] (11.70% for red fruits and 32.8% for black fruits) and also can be explained by the different bioclimatic conditions close to each site [11].

3.2. Fatty Acid Composition. The fatty acid composition of *Pistacia lentiscus* seeds is shown in Table 2. The lentisk oils of the three localities had high amounts of monounsaturated fatty acids (MUFA) with values between 52.43% and 53.67% of total fatty acids (TFA). It was followed by polyunsaturated fatty acids (PUFA) between 22.27% and 25.32% and then saturated fatty acids (SFA) representing 22.25 to 24.07% of TFA. The major FA was oleic acid (C18 : 1) with the highest

Table 1: Geographical parameters of the three localities of lentisk seeds.

Localities	Latitude	Length	Level of the sea (m)	Annual rain (mm)	Minimum average temperatures (°C)	Maximum average temperatures (°C)
TA	31°54′58′74″N	6°35′12′77″O	1422.86			
BO	31°54′44′47″N	6°35′40′08″O	1410.44	260.3	4 to 10°C	20 to 40°C
TI	31°54′8′33″N	6°35′47′78″O	1416.06			

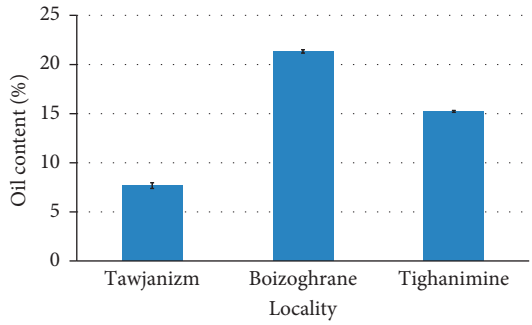

Figure 1: Oil content of lentisk seeds from the three localities.

amount of Boizoghrane seed oil (53.23%), followed by Tawjanizm at 52.50%. The lowest value corresponded to Tighanimine seed oil at 51.56%. These values were similar to those reported by Dhifi et al. [6] and Charef et al. [10]. The oleic FA is reputed for its role in the preservation of cardiovascular diseases and its nutritional value [12]. Furthermore, linoleic acid (C18 : 2), an essential FA, accounted for 20.95% to 23.77% of whole FA; it has favorable nutritional implications and beneficial physiological effects in the prevention of coronary heart disease and cancer [13], and it provides lipids necessary for cell membrane repair and cellular respiration [14]. For palmitic acid (C16 : 0), the highest value was detected in Boizoghrane seed oil at 23.01% followed by Tawjanizm oil (22.55%) and 20.51% for Tighanimine oil. The content of oleic acid, linoleic acid, and palmitic acid from three localities studied is consistent with the results obtained by Dhifi et al. [6].

The values obtained in unsaturated fatty acids (UFA) for the three localities are higher than those obtained by Dhifi et al. [6] and lower than those reported by Charef et al. [10]. This is explained by the absence of linolenic acid in the oils studied by Charef et al. [10]. The percentage of saturated fatty acids (SFA) is higher than that reported by Charef [10] and lower than those obtained by Dhifi et al. [6]. The low saturated/unsaturated FA ratio (0.35) reveals a high content in UFA which may give it nutritiona and, dietetic virtues and curative properties. The FA composition of *Pistacia lentiscus*seeds' oil is similar to that of *Pistacia vera* [15] and *Pistacia atlantica* [16]. The existence of omega 6 and 9 in this oil makes it as an alternative source of these essential FA. Furthermore, the profile of FA confirms the similarity between *Pistacia lentiscus* oils and other edible vegetable oils such as rapeseed, olive, sunflower, and cotton. The fatty acid profile of three oil samples is in accordance with the standards of the Codex Alimentarius for rapeseed oil concerning specially stearic acid, oleic acid, linoleic acid, and linolenic acid, and it also corresponds to that of olive oil in terms of the composition of palmitoleic acid (C16 : 1) and stearic acid (C18 : 0). On the contrary, the composition of the lentisk oils studied exceeds that of olive oil containing palmitic acid, linoleic acid, and linolenic acid. Thus, higher values of palmitic and palmitoleic acid compared with rapeseed oil are recorded. These findings are in agreement with the results reported by Dhifi et al. [6], but the percentage values were different. This inconsistency could be attributed to different causes, such as genotype and growing conditions.

3.3. Analysis of Triglycerides. The triglyceride composition of seeds' oil in the three localities revealed the existence of 16 triglycerides and had high amounts of POO + SOL, OOO, and POL + SLL + PoOP. The triglyceride (TAG) composition of lentisk showed that the majority of the TAGs is in mono- and polyunsaturated forms (Table 3). Considering the fatty acid composition, the main constituents were stearoyloleyl-linoleylglycerol (SOL) and palmitoyl-dioleylglycerol (POO) for 21.24 to 24.71% of the total TAGs. Stearoyl-dilinoleoylglycerol (SLL) and palmitoyl-oleyllinoleol glycerine (POL) accounted for 16.37 to 16.47% of the total TAGs, while trioleylglycerol (OOO) and dioleyl-linoleylglicerin (OOL) were significantly represented with quantities, respectively, of 15.28 to 16.02%, and 11.57 to 14.44%. These results are in agreement with those reported in the literature for a study on grape seed oils [6].

It should be noted that our oils are rich in triglycerides formed by the combination of oleic, linoleic, and palmitic fatty acids, which confirmed the composition results of fatty acids.

3.4. Statistical Analysis. The Pearson correlation coefficients between the oil content (TH) and fatty acids values (C16 : 1, C17 : 1, C18 : 0, C18 : 1, and C18 : 2) and triglycerides (OLL, SOS, SOO, OOO, POP, POL, POL + SLL + POOP, and OOL + PLnP + PoOP) were calculated. Then, the analysis was performed to determine the strength of the relationship between the three sampling localities and the analytical parameters.

The correlation circle (Figure 2) represents the evolution and the relationship between the variables studied. Three distinguished groups of components were found. The first group corresponded to the correlation between oil content (TH), cis-10-heptadecanoic acid (C17 : 1), and oleyl-dilinoleoyl-glycerol (OLL). The second group showed a strong correlation between the two harvest sites Boizoghrane (BO) and Tawjanizm (TA) in terms of the composition in triglyceride (SOO). The third group consisted of triglycerides palmitoyl-dilinoleoyl-glycerol (PLL) and triglyceride (SOS)

Table 2: *Pistacia lentiscus* seed oil fatty acids composition (%).

	TA	BO	TI	Dhifi et *al.*, 2013 (Tunisia)	Charef et *al.*, 2011 (Algeria)		Olive oilCOI, 2013	Rapeseed oil CODEX STAN 210-1999
					Pistacia.L (black seeds)	Pistacia.L (red seeds)		
C16 : 0	22.55 ± 0.02	23.01 ± 0.04	20.51 ± 0.11	23.52 ± 3.01	19.5	16.3	7.50–20.00	1.5–6.0
C16 : 1	0.38 ± 0.16	0.44 ± 0.13	0.53 ± 0.23	1.19 ± 0.12	2.1	1.0	0.30–3.50	ND-3.0
C18 : 0	0.98 ± 0.20	1.05 ± 0.10	1.3 ± 0.05	1.41 ± 0.02	1.7	0.7	0.50–5.00	0.5–3.1
C18 : 1	52.5 ± 0.35	53.23 ± 0.53	51.56 ± 0.21	51.06 ± 4.37	55.3	53.5	55.0–83.00	8.0–60.0
C18 : 2	22.14 ± 0.44	20.95 ± 0.45	23.77 ± 0.38	20.71 ± 2.25	21.4	28.5	3.50–21.00	11.0–23.0
C18 : 3	1.45 ± 0.06	1.31 ± 0.02	1.54 ± 0.01	0.47 ± 0.10	—	—	<1.00	5.0–13.0
MUFA	52.88 ± 0.06	53.67 ± 0.02	52.43 ± 0.12	52.4 ± 7.18	57.4	54.5		
PUFA	23.59 ± 0.51	22.27 ± 0.47	25.32 ± 0.38	21.18 ± 2.23	21.4	28.5		
SFA	23.53 ± 0.65	24.07 ± 0.56	22.25 ± 0.48	26.42	21.2	17.0		
MUFA/ PUFA	2.24 ± 0.05	2.41 ± 0.05	2.07 ± 0.04	2.47	2.68	1.91		
SFA/UFA	0.31 ± 0.01	0.32 ± 0.01	0.29 ± 0.01	0.35	0.27	0.2		

MUFA monounsaturated fatty acids, MUFA polyunsaturated fatty acids, SFA saturated fatty acids, and UFA unsaturated fatty acids.

Table 3: Triglyceride composition of the seed oil of *Pistacia lentiscus* by HPLC (%).

	TA	BO	TI	Dhifi et al. (2013)
LLL	2.64	2.26	3.40	1.32 ± 0.28
PLLn	0.79	0.71	0.83	
OLL	0.50	6.21	8.77	5.67 ± 1.62
PLL	7.28	6.94	7.27	7.97 ± 1.86
POLn	0.29	0.28	0.23	
OOL + PLnP + PoOO	11.57	11.57	14.44	9.83 ± 2.03
POL + SLL + PoOP	16.37	16.39	16.47	21.50 ± 2.06
PPL	3.96	3.96	3.55	5.58 ± 1.12
OOO	15.74	16.02	15.28	12.04 ± 1.43
POO + SOL	24.14	24.71	21.24	27.58 ± 2.36
POP	7.91	8.31	6.42	8.51 ± 1.09
GaOO	0.40	0.30	0.03	
SOO	1.39	1.36	1.05	
POS	0.94	0.97	0.99	
AOO	0.05	0.03	0.03	
SOS	0.04	0.00	0.02	

LLL: trilinoleoyl-glycerol, OLLn: oleyl-linoleoyllinolenoylglycerol, OLL: oleyl-dilinoleoyl-glycerol, PLL: palmitoyl-dilinoleoyl-glycerol, OOL: dioleyllinoleoylglcerol, SLL: stearoyl-dilinoleoylglycerol, POL: palmitoyl-oleyl linoleoylglycerol, PPL: dipalmitoyllinoleoylglycerol, OOO: trioleylglycerol, SOL: stearoyloleyl-linoleoylglycerol, P OO: palmitoyl-dioleylglycerol, and PPO: dipalmitoyl-oleylglycerol.

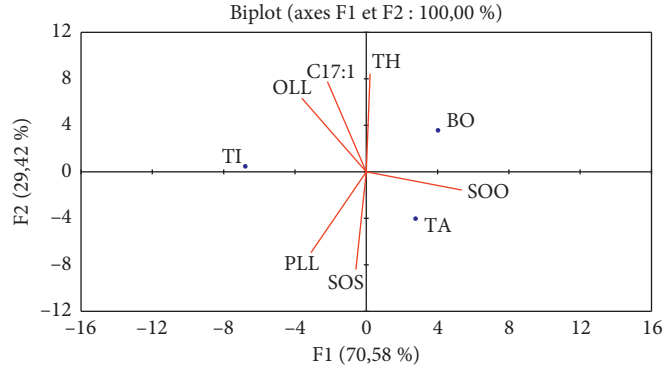

Figure 2: Correlation circle of variables according to PCA of the seed oil of *Pistacia lentiscus* and according to oil content fatty acid and triglyceride composition.

that were highly correlated. The dispersion of the variables in this way showed a significant variability of the parameters studied under the effect of the sampling site.

The positive correlation is highly significant between the oil content and the C17 : 1 fatty acid, thus between the oil content and the OLL triglyceride on one side. On the contrary, the negative correlation is highly significant between the oil content and triglycerides (AOO, SOS and PLL). The strong correlation ($r > 0.7$) between cis-10-heptadecanoic acid and oleyl-dilinoleoyl-glycerol and their positive relationship with oil content and the negative relationship with triglycerides (AOO, SOS, and PLL) showed the importance of these five parameters in the characterization of three oils studied.

The correlation circle and the biplot showed that the oils from three sampling sites analyzed were characterized by a very interesting composition diversity defined by three types (oil content, fatty acid composition, and triglyceride composition) of the lentisk oils and discriminated by the oil content that is the variable.

4. Conclusion

On the basis of our study, it may be concluded that the results of this work showed that the seeds of lentisk can be used as an edible oil source. This oil had an interesting oil yield and presents a high content in the unsaturated fatty acids. It is rich in C18 : 1 whose dietary virtues are well established and also rich in C18 : 2 which is an essential FA with beneficial physiological effects. Those compounds may be used to study the potential use of oils from these seeds in pharmaceuticals, foodstuffs, and cosmetic industry. In a further work, we will try to complete this research by valorizing unsaponifiable matters in the oils evaluating biological activities and to evaluate toxicity of lentisk oil. Such study could achieve industrial application of this plant oil.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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