Evidence-Based Complementary and Alternative Medicine

The Health Effects of Dietary Supplements

Lead Guest Editor: Alessandra Durazzo
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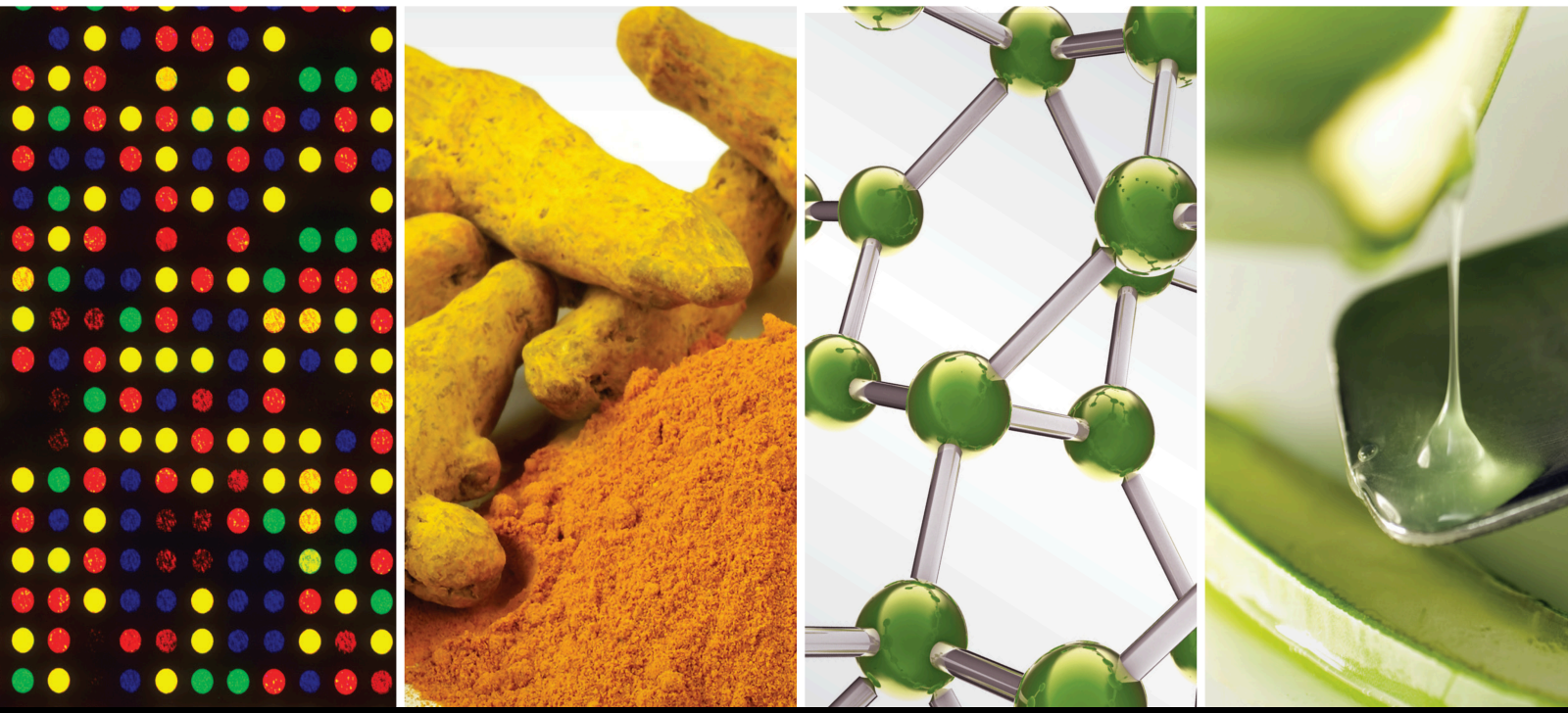

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Editorial

The Health Effects of Dietary Supplements

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Traditional medicinal plants and herbs are commonly used for health purposes and have been extensively studied [1–4]. Nowadays, dietary supplements have received particular interest worldwide as they are valuable health tools in disease management [5–8]. They are relatively easy to use. Moreover, they are cost-effective when compared with chemical entities obtained from synthesis. Botanicals, one of the most emerging classes of dietary supplements, are made of herbs. They are also created by mixing different herbs from raw materials, from whole plants, or from certain parts. This includes flowering herbs, leaves, leaf exudates, fruits, berries, roots, and rhizomes.

Current methodologies enable us to isolate, standardize, and characterize fractions of medicinal plants with specific bioactivities. However, there is a need to investigate further methodologies. Nowadays, research focuses on new formulations and the health properties of dietary supplements. Moreover, dietary supplement research is progressively integrating multidisciplinary research approaches. Current and novel research includes emerging technologies such as nuclear magnetic resonance (NMR) spectroscopy, isotopic ratio mass spectrometry, multielemental analysis, fluorescence, near-infrared (NIR) spectroscopy, mid-infrared (MIR) spectroscopy, and mass spectrometry combined with chemometrics [9–12].

This Special Issue aimed at bringing together original research and review articles discussing our current knowledge of the health effects of dietary supplements. Multidisciplinary approaches, with particular focus on the investigation of the quality assessment and control of dietary supplements, have been explored into following topics: (i) isolation and quantification of natural products used for dietary supplements (e.g., standardized fractions and emerging technologies involving chemometrics); (ii) *in vitro* and *in vivo* research investigating the potential health properties of natural products used in dietary supplements; (iii) classification and categorization of dietary supplements; (iv) quality assessment and control of dietary supplements, with particular focus on the metrological approach.

In this context, Wu et al. [13] elucidated the role of cinnamic acid in amelioration of nonalcoholic fatty liver disease by suppressing hepatic lipogenesis and promoting fatty acid oxidation. A systematic review and meta-analysis of 10 randomized controlled trials on the improving effect and safety of probiotic supplements on patients with osteoporosis and osteopenia was carried out by Zeng et al. [14]. Yarizadeh et al. [15] studied the effects of omega-3 supplementation on resting metabolic rate in a systematic review and meta-analysis of clinical trials. On the other hand, edible *Cyanobacterium Arthrospira platensis* collected from the south Atlantic coast of Morocco was studied and proposed as a promised source of dietary supplements [16].

New functionalities of foods are also considered. For instance, the work of Salamatullah et al. [17] explored the

bioactive properties of coffee beans, with particular regards to the effect of roasting. In another work, Salamatullah et al. [18] studied the effects of different solvents extractions on total polyphenol content, HPLC analysis, antioxidant capacity, and antimicrobial properties of peppers (red, yellow, and green *Capsicum annum* L.). Another example is given by Elhadef et al. [19] who studied pistachio hull extract as a practical strategy to extend the shelf life of raw minced beef: compared to synthetic antioxidants, the pistachio hull extract could be a clean-label alternative that can protect and enhance the quality of meat products. Song et al. [20] studied the biological functions of diallyl disulfide, a garlic-derived natural organic sulfur compound.

de Morais Lima et al. [21] studied the effects of "Bacuri" seed butter (*Platonia insignis* Mart.) on metabolic parameters in hamsters with diet-induced hypercholesterolemia: Bacuri seed butter at doses of 25 and 50 mg/kg/day has positive repercussions on the lipid profile, more precisely on plasma HDL-c and LDL-c, and additionally promotes reduction in the risk of atherosclerosis in hamsters.

All articles, part of this Special Issue, reflect new trends and promote new ideas for future collaborative network and infrastructure in perspective of update, and standardize the study approach and quality control, adding new information and sharing data.

We hope that the readers will find this Special Issue interesting and inspiring.

Conflicts of Interest

The editors declare no conflicts of interest.

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Alessandra Durazzo
Daniel Dias Rufino Arcanjo
Massimo Lucarini

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Research Article

Effects of Different Solvents Extractions on Total Polyphenol Content, HPLC Analysis, Antioxidant Capacity, and Antimicrobial Properties of Peppers (Red, Yellow, and Green (*Capsicum annum* L.))

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Plants possessing various bioactive compounds and antioxidant components have gained enormous attention because of their efficacy in enhancing human health and nutrition. Peppers (*Capsicum annuum* L.), because of their color, flavor, and nutritional value, are considered as one of the most popular vegetables around the world. In the present investigation, the effect of different solvents extractions (methanol, ethanol, and water) and oven drying on the antioxidant and antimicrobial properties was studied of red, yellow, and green peppers. The green pepper water extract showed the highest total polyphenol content (30.15 mg GAE/g DW) followed by red pepper water extract (28.73 mg GAE/g DW) and yellow pepper water extract (27.68 mg GAE/g DW), respectively. The methanol extracts of all the pepper samples showed higher TPC as compared to the ethanol extract. A similar trend was observed with the total flavonoid content (TFC). The antioxidant assays (DPPH scavenging and reducing power) echoed the findings of TPC and TFC. In both antioxidant assays, the highest antioxidant activity was shown by the water extract of green pepper, which was followed by the water extract of red pepper and yellow pepper. Furthermore, all extracts were assessed for their potential antimicrobial activity against bacterial and fungal pathogens. Aqueous extracts of all three pepper samples exhibited slightly higher inhibition zones as compared to their corresponding ethanolic and methanolic extract. Minimum inhibitory concentration (MIC) values ranged from 0.5 to 8.0 mg/ml. The lowest MIC values ranging from 0.5 to 2.0 mg/ml concentration were recorded for aqueous extracts of green pepper. High-performance liquid chromatography (HPLC) analysis revealed tannic acid as the major phenolic compound in all three pepper samples. Thus, it is envisaged that the microwave drying/heating technique can improve the antioxidant and antimicrobial activity of the pepper.

1. Introduction

Antioxidants protect biological processes by delaying, controlling, or inhibiting the oxidative stress caused by free radicals [1]. Free radicals' accumulation in the human body could disturb the normal functions of cells and organs that successively result in the onset of noncommunicable diseases (NCDs) [2]. Plants with a variety of bioactive

compounds and antioxidant components are gaining popularity as a result of their efficacy in enhancing human health and nutrition [3, 4]. They have been linked to lower cancer and heart disease incidence and in turn the mortality rates [5, 6].

Peppers (*Capsicum annuum* L.) are a member of the Solanaceae family known by various other names too, such as bell pepper, chili, and capsicum. Because of their color, flavor, and nutritional value, peppers are considered one of the most popular vegetables around the world. The plant, which is native to North and South America, thrives in hot, dry climates and is used in Africa and other parts of the world for both medicinal and culinary purposes [7]. They are thick-walled bell-shaped vegetables, comprising three or four lobes, and are found in different sizes and colors depending on the genotype or seasonal period of breeding [8]. The chlorophyll and carotenoids give peppers their green color [9, 10], and carotene, zeaxanthin, lutein, and cryptoxanthin are liable for giving the yellow-orange hue of pepper [10]. Capsanthin, capsorubin, and capsanthin 5,6-epoxides are carotenoid pigments that give peppers their red color [11]. The difference in levels of these compounds, changes during ripening, the genotype, and the seasonal period of breeding are the various factors responsible for the differences in the colors of peppers. The taste and flavor of each pepper can be influenced by the color of the fruit. Red, yellow, and orange peppers, for example, are sweeter than green peppers as a result of higher glucose content during the ripening period [12]. Bell peppers are good sources of vitamins, such as vitamins C and E, provitamin A, and carotenoids [13–15]. They were also found to be a good source of phenolic or flavonoids, such as quercetin, luteolin, and capsaicinoids [16]. Types and quantities of bioactive compounds differ among different colored peppers.

Studies have shown the efficacy of the antioxidative components of several pepper species [17, 18]. They are effective in reducing the risk of various degenerative, mutagenic, and chronic diseases [19–21]. It has also been used for alleviating toothaches and in the management of the respiratory disease [22]. Loizzo et al. 2008 reported the inhibitory effect of *C. annuum* var. Acuminatum on the enzyme acetylcholinesterase, which is a therapeutic method for the symptomatic management of Alzheimer's disease [23]. In animal assays, peppers have shown hypocholesterolemic properties [24, 25]. Capsaicin, the main representative of the pungent components, is a lipophilic alkaloid and because of its analgesic and anti-inflammatory activity has been used in clinical practice. An analysis on rats' revealed peppers antioxidant capacity, which has defensive effects on the brain cells [26]. It is critical to study the phytochemicals found in common vegetables and fruits in order to learn more about their possible health benefits. The extraction solvents used may have an impact on the precision with which bioactive compound concentrations are measured [27, 28]. In natural foods, the concentration and activity of bioactive compounds can be directly related to solvent properties such as lipophilic and hydrophilic solvents and their respective polarity [14, 29]. A study on the efficiency of different extraction solvents (hexane, ethyl acetate, acetone, methanol, and methanol-water mixture) using high-performance liquid chromatography (HPLC) on the bioactivity of nonpungent peppers demonstrated that solvent chemical properties such as polarity can differentially influence the efficacy of recovering bioactive compounds from foods, and this might eventually result in differences in estimated biological activity, such as antioxidant capacity [30]. In another study, in comparison with green and yellow sweet peppers, the orange and red sweet peppers extracted with hexane showed the highest TPCs and antioxidant activities, likely caused by carotenoids as the compounds were mainly extracted by nonpolar solvents [31]. Perishable products face losses due to enzymatic and microbial degradation which are active at suitable temperature and storage problems. Drying comprises concurrent transient heat, mass, and momentum transport, and it is one of the most widely used methods for the preservation of food [32]. Dried food products can be stored at ambient temperatures for longer periods due to their low moisture content, which reduces the microbiological activity and allows the availability of the product even in off-season. Studies have indicated that as substitutes to the conventional drying procedures (sun drying), the use of microwaves drying/heating techniques can improve the antioxidant activity of the plant materials by reducing the thermal damages of antioxidant components [33, 34].

Exploring antimicrobial properties along with the antioxidant activity of the plant are an important aspect as there is a growing need to replace existing synthetic food additives with those of natural origin. Various studies have demonstrated the antibacterial potential of different species of *Capsicum* spp. Methanolic extracts of *C. annuum* and *C. frutescens* were found effective against food-borne pathogens *Staphylococcus aureus*, *Vibrio cholerae*, and Salmonella Typhimurium. Recently, an aqueous extract of yellow-colored *C. annuum* was found to demonstrate the highest antimicrobial activity against pathogen *P. aeruginosa*. In another study, phenolic compounds capsaicin, dihydrocapsaicin, and chrysoeriol isolated from the hexane and acetonitrile extracts of fruit, peel, and seed of *C. frutescens* demonstrated promising antimicrobial activity against three Gram-negative bacteria (*E. coli*, *P. aeruginosa,* and *K. pneumoniae*), three Gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis,* and *S. aureus*), and yeast (*C. albicans*). Flavanoid chrysoeriol was found to possess potent antimicrobial potential as compared to the other two isolated compounds.

This study was undertaken to investigate the effect of different solvents extractions (methanol, ethanol, and water) on the antioxidant and antimicrobial properties of oven-dried red, yellow, and green peppers.

2. Materials and Methods

2.1. Plant Materials and Reagents. Fresh red, yellow, and green peppers were procured from the local market in Riyadh, Saudi Arabia, in January 2021. The experiments were performed immediately after procurement. Gallic acid, Folin–Ciocalteu reagent, and 2,2-diphenyl-1-picrylhydrazyl

(DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample Preparation. The peppers were rinsed in water and dried on paper towels. The stem and seeds were removed, and edible parts were collected. These portions were cut in almost equally shaped small pieces (2 × 2 cm) and a lot was dried through a hot air oven. All experiments were performed in triplicate, each using 200 g of pepper.

2.3. Drying. Two hundred grams of sliced peppers were placed in a hot air oven and dried at 60°C for 4 consecutive days. The dried peppers were allowed to cool down at room temperature for 15 min, and then slices were ground using an electronic blender to obtain peppers powder. Finally, the powder sample was packed in low-density polyethylene (LDPE) bags.

2.4. Extraction. Two grams of dried pepper samples were extracted individually with 100 mL of ethanol, methanol, and water solvents. The contents were sonicated at room temperature for 30 min in an ultrasonic bath (frequency, 40 Hz; power, 300 W; SD-350H; Seong Dong, Seoul, Korea) and then filtered using Whatman No. 4 filter paper.

2.5. Total Polyphenol Content (TPC). In this study, TPC was detected by Folin–Ciocalteu (FC) method as described earlier [35]. Firstly, 25 μL of the extract was added to 125 μL of undiluted FC reagent, and then 1500 μL nanopure water was added to the mixture. The mixture was allowed to shake for 1 min at room temperature and then 20% sodium carbonate (375 μL) and 475 μL of water were added, and the final volume of the mixture was made to 2500 μL. Finally, the prepared mixture was incubated at room temperature for 30 min. Phenol's detection was accomplished spectroscopically at 760 nm (Jasco, V-630 spectrophotometer, USA). The TPC was expressed as gallic acid equivalent per Gram dry weight of the sample (mg GAE/g DW).

2.6. Total Flavonoid Content (TFC). The TFC was determined according to the precisely described method used by [35]. Thousand μL of water was added to 250 μL of extract. After that, 75 μL of each 5% (w/v) sodium nitrite and 10% (w/v) aluminum chloride was added to the mixture and incubated for 5 min at room temperature. Then, the mixture was vortexed after adding 500 μL of 1 M sodium hydroxide and 600 μL of water. The blank was prepared following the same procedure without extract. The absorbance was measured spectroscopically at 510 nm (Jasco, V-630 spectrophotometer, USA). TFC was expressed as mg catechin equivalent per Gram dry weight of the sample (mg CE/g DW).

2.7. DPPH Scavenging. The free radical scavenging capacity of the extract was determined using DPPH according to the standard method with some modifications [36]. Firstly, 0.1 mM DPPH solution was prepared and then 130 μL of the extract was mixed with 2000 μL of DPPH solution. The mixture was allowed to rest in the dark for 30 min and then absorbance was measured at 510 nm (Jasco, V-630 spectrophotometer, USA). Control was prepared in the same manner, but ethanol was used instead of extract. Methanol was used as a blank. The scavenging percentage was calculated as

$$\text{DPPH scavenging \%} = \frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}} \times 100. \quad (1)$$

2.7.1. Reducing Power. The ferric reducing power of the sample was estimated according to the method of Hayat et al. [33]. Half (0.5) mL extract was mixed with 1.25 mL of potassium ferricyanide, and 1.25 mL buffer (0.2 M, pH 6.6). The mixture was then incubated for 20 min at 50°C. After the incubation of 20 minutes, trichloroacetic acid (1.25 mL) was added and the mixture was centrifuged at 3000 ×g for 10 min at room temperature. Finally, an aliquot (1.25 mL) was taken from the supernatant, to which 1.25 mL water and 0.25 mL of ferric chloride were added. Blank was also prepared following the same protocol but without extract. The absorbance was recorded at 700 nm (Jasco, V-630 spectrophotometer, USA).

2.8. Determination of Phenolic Compounds. In the present study, we utilize HPLC with the method described previously [37]. Phenolic compounds (tannic acid, resorcinol, 1,2-dihydroxybenzene, chlorogenic acid, caffeic acid, vanillin, acetylsalicylic acid, 3,5-dinitro salicylic acid, salicylic acid, and quercetin) quantification in three pepper (green, yellow, and red) samples was carried out using HPLC analysis, as described earlier with some modification [37]. The HPLC (Prominence) system Shimadzu (Kyoto, Japan) was equipped with an LC-20AB binary pump and variable Shimadzu SPD-10A UV detector. The column used was Zorbax SB-C18 (250 × 4.6 mm, 5 μm; Agilent, Santa Clara, CA, USA) and the mobile-phase was Milli Q water (1% acetic acid, A) and methanol (B). The binary gradient program used was 0–10 min, 15–30% B; 10–20 min, 30–40% B; 20–30 min, 40–50% B; 30–41 min, 50–60% B; and 41–45 min, 15% B. The flow rate was 1.0 mL/min. The injection volume was 10 μL, and the detector was set at 280 nm. Compounds in pepper samples were identified by comparing their peak retention time with those of standards. All samples were analyzed in duplicate and arithmetical mean ± standard error was reported.

2.9. Antimicrobial Activity of Pepper Extracts. Antimicrobial activity of red, green, and yellow pepper extracts was assessed against *Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans* using agar well diffusion assay [38]. Briefly, 0.1 ml of overnight grown cultures was spread onto Mueller Hinton Agar (MHA) plates, agar wells were punched, and 6 mg/ml concentration of the prepared

extracts was loaded in each well. Solvent (5% DMSO) and Mueller Hinton Broth (MHB) were used as negative controls and antibiotics were used as a positive control. Plates were incubated for 18–24 h at 37°C and observed for halo zones of inhibition around the well. All the samples were analyzed in triplicates.

2.10. Assessment of Minimum Inhibitory Concentration (MIC). MIC of the pepper extracts was determined using the microbroth dilution method described previously [39].

2.11. Statistical Analysis. Statistical analysis was performed using SAS (Version 9.2, 2000–2008; SAS Institute Inc., Cary, NC, USA) for data analysis. All the analyses were carried out in triplicate. The results were expressed as mean ± standard deviation (SD). The differences among the treatment groups were analyzed using one-way analysis of variance (ANOVA) at a significance level of $p \leq 0.05$, and a post hoc analysis using Duncan's multiple range tests was performed if differences were found significant between the groups.

3. Results and Discussion

3.1. Total Polyphenol Content (TPC). The effect of different extraction (ethanol, methanol, and water) solvents on the total polyphenol content of green, yellow, and red peppers are shown in Figure 1. The green pepper water extract showed the highest total polyphenol content (30.15 mg GAE/g DW) followed by red pepper water extract (28.73 mg GAE/g DW) and yellow pepper water extract (27.68 mg GAE/g DW), respectively. The methanol extracts of all the pepper samples showed higher TPC as compared to the ethanol extract. For example, the TPC of methanol extract of green, yellow, and red pepper was 22.69, 24.33, and 22.76, while that of the ethanol extract was 19.63, 15.55, and 17.1 mg GAE/g DW. Our results are contrary to the findings of Sun et al. [14] who reported a higher TPC of red peppers than the green peppers. The TPC of the methanolic extract of green, yellow, orange, and red peppers was documented as 2.4, 3.3, 3.4, and 4.2 micromol catechin equivalent/g fresh weight, respectively. Another study also reported that the methanolic extract of red sweet pepper cultivar/rootstock (Fascinato/Robusto) had a higher concentration of total phenols of 111.26 mg/100 g of dry weight as compared to the green pepper (Sweet/Robusto) which showed the lowest content, averaging 70.39 mg/100 g of dry weight. Moreover, the total phenol content depended on the variety as well as the color of the bell peppers and the highest content was recorded in colored peppers than in the green, values being highest in red, followed by yellow, and then by orange peppers [18]. But our results are in line with the findings of Blanco-Ríos et al. [40], who found that the variety Orion (green) had the highest concentration of phenolic compounds, while no differences were detected between the varieties Mazurca (red), Simpaty (orange), and Taranto (yellow). Ahmad et al. [41] reported that the solvents (acetone, ethanol, and water) established a significant role in the extraction of phenolic compounds from 27 samples of pepper from different origins. Kumar et al. [42] reported that the fresh green bell peppers showed a TPC of 64.58 mg GAE/g. The extraction for the TPC measurement in this study was performed by homogenizing the fresh bell peppers with water.

3.2. Total Flavonoid Content (TFC). Figure 2 shows the total flavonoid content of pepper samples extracted with three different solvents. TFC showed almost a similar trend as that of the TPC. The water extract of green pepper exhibited the highest (13.04 mg CE/g DW), while the ethanol extract of red pepper showed the lowest (5.11 mg CE/g DW) total flavonoid content among all the samples. Statistically, the total flavonoid contents of the methanol extract of green pepper (5.74 mg CE/g DW) and ethanol extracts of green (5.72 mg CE/g DW) and yellow pepper (5.82 mg CE/g DW) were not significantly different from each other ($p > 0.05$), while the TPC of the water extracts of yellow and red peppers were also statistically similar to each other. Kumar et al. [41] reported the TFC of water extract of green bell-pepper as 11.95 mg quercetin equivalent (QE)/g sample. In an earlier study, the TFC of the water, methanol, and ethanol extract of the pepper (*C. annum* L.) was determined as 78.2, 67.2, and 82.3 mg QE/100 g DW, respectively, and the values were not significantly different ($p > 0.05$) from each other [43]. Previous study reported that the TFC values of extract from *Capsicum annuum* L. averaged from 121 to 130 mg QE/100 DW [44]. It is well known that water is more polar than ethanol and methanol. Some of the plant bioactive compounds, like O-methylated are less polar than the non-methylated flavonoids [45]. Based on the different TFC valued obtained by solvent used, the results of our study might be explained that the peppers have different group of flavonoids soluble in different polarities. Moreover, the different values of bioactive compounds of pepper reported in the literature might be due to the varietal, agronomical, environmental, and analytical factor. Hallman and Rembialkowska [46] reported that the phenolic content was influenced by the crop, as the organic system gave higher values than did the conventional one.

3.3. Antioxidant Activity. The antioxidant potential of different extracts of green, yellow, and red peppers are as assessed by 2,2-diphenyl-1-picrylhydrazyl scavenging and ferric reducing power is shown in Figure 3, respectively. The antioxidant activity potential of the extracts echoed the aforementioned results of TPC and TFC. The significantly highest DPPH scavenging was shown by the water extract (0.02 g/mL) of green pepper (72.76%) ($p < 0.05$), which was followed by the water extract of red pepper (70.26%) and yellow pepper, respectively. But statistically, there was no difference ($p > 0.05$) between the DPPH scavenging of the water extract of the red and yellow pepper. The ethanol extract of red pepper showed the lowest DPPH scavenging (18.31%) among all the samples. Figure 4 depicts the reducing power of the pepper extracts. As can be seen, the highest reducing power was exhibited by the water extract of green pepper (2.305) followed by the water extract of yellow

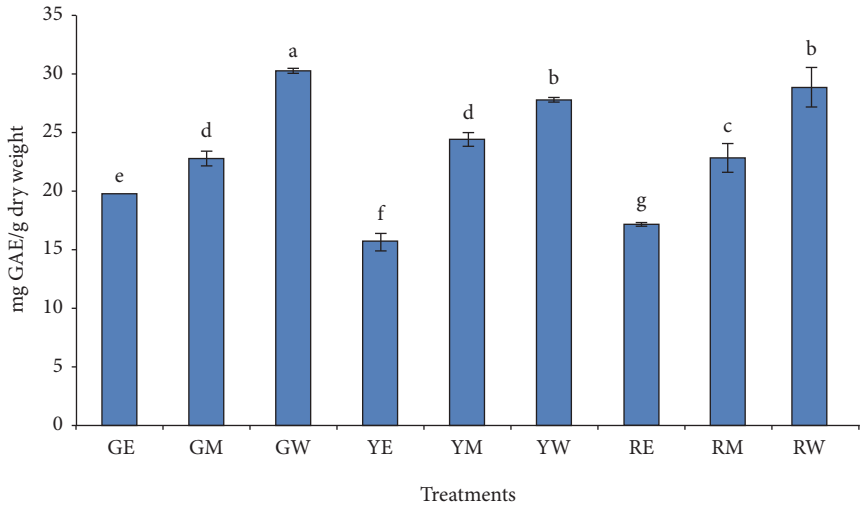

Figure 1: Effect of different solvent extractions on the total polyphenol content of peppers. The treatment codes denoted by two letters represent the green (G), yellow (Y), and red pepper (R), extracted with ethanol (E), methanol (M), and water (W).

pepper (1.905) and red pepper (1.857), respectively. The lowest reducing power was shown by the ethanol extract of yellow pepper (0.696) among all the samples. The higher antioxidant activity of the water extract could be due to the leaching of hydrophilic phenolic compounds in the extract [47, 48].

A recent study reported the DPPH scavenging of 88.35% for the water extract (0.25 g/mL) of green bell pepper [42]. In another study, the methanolic extract (0.04 g/mL) of red bell pepper dried at 50°C and 70°C exhibited the DPPH scavenging of 67.02% and 73.25%, respectively [49]. The free radical scavenging ability of peppers determined by the DPPH method was the lowest for the green pepper but not significantly different from the other 3 peppers (yellow, orange, and red) (Sun et al.) [14]. In another study, the TPC, TFC, and DPPH scavenging of red and green sweet bell peppers processed at various temperatures were evaluated. The methanolic extract of red peppers showed higher DPPH scavenging under all the processing conditions as compared to the green peppers (Yazdizadeh Shotorbani et al.) [50]. Chávez-Mendoza et al. [18] evaluated the antioxidant activity by DPPH of the 80% ethanolic extract of different cultivar/rootstock combinations of bell peppers and found that Fascinato/Robusto red colored had the highest antioxidant activity with an average of 79.65%, while yellow colored Jeanette/Terrano presented the lowest activity of 64.90%. The average antioxidant activity of the cultivar/rootstock combinations is diminished as follows: (red) Fascinato/Robusto > (red)Fascinato/Terrano > (green) Sweet/Robusto > (orange)Orangela/Terrano > (yellow)Jeanette/Terrano.

3.4. Reducing Power

3.4.1. Antimicrobial Studies. Solvent extracts of red, yellow, and green pepper were examined for their potential antimicrobial activity against bacterial and fungal pathogens. Aqueous extracts of all the three pepper samples exhibited slightly higher inhibition zones as compared to their corresponding ethanolic and methanolic extract (Figure 5). Aqueous extract of green pepper extract demonstrated the highest inhibition zone of 15, 13, 15, 14, and 12 mm against *S. aureus, L. monocytogenes*, *E. coli, P. aeruginosa,* and *C. albicans*, respectively. Similarly, the zone of inhibition for green pepper (ethanol extract) was recorded as 13, 12, 15, 15, and 13 mm against *S. aureus, L. monocytogenes, E. coli, P. aeruginosa,* and *C. albicans*, respectively, while methanolic extract of green pepper demonstrated inhibition zone ranging from 10 to 13 mm against the test pathogens. Red pepper (alcoholic extracts) showed inhibition zones ranging from 10 to 14 mm, while the aqueous extract of the red pepper demonstrated inhibition zones of 12–15 mm against the test pathogens. In the case of yellow pepper, extract from methanolic samples showed the highest zone of 11 mm against *E. coli*, *L. monocytogenes*, and *P. aeruginosa,* and the lowest zone of 8 mm was recorded against *C. albicans*. Almost similar results were observed with the ethanolic extract of yellow pepper. Slightly higher inhibition zones ranging from 10 to 12 mm were recorded with the aqueous extract of yellow pepper samples. Antibiotics chloramphenicol and fluconazole (antifungal) were used as positive controls. Our findings are in sync with those reported with methanolic extracts of *C. annuum* and *C. frutescens*. Both extracts were found effective against food-borne pathogens *Staphylococcus aureus, Vibrio cholerae,* and *Salmonella typhimurium* [51]. Recently, an aqueous extract of yellow-colored *C. annuum* was found to demonstrate the highest antimicrobial activity against pathogen *P. aeruginosa* [52]. In another study, phenolic compounds capsaicin, dihydrocapsaicin, and chrysoeriol isolated from the hexane and acetonitrile extracts of fruit, peel, and seed of *C. frutescens* demonstrated promising antimicrobial activity against three Gram-negative bacteria (*E. coli*, *P. aeruginosa,* and *K. pneumoniae*), three Gram-positive bacteria (*Enterococcus faecalis*, *Bacillus subtilis,* and *S. aureus*), and yeast (*C. albicans*) [53].

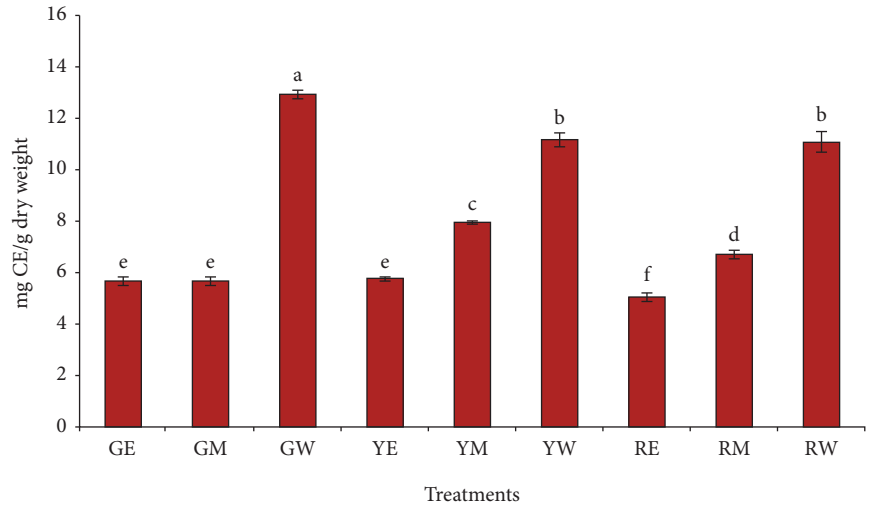

Figure 2: Effect of different solvent extractions on the total flavonoid content of peppers. The treatment codes denoted by two letters represent the green (G), yellow (Y), and red pepper (R), extracted with ethanol (E), methanol (M), and water (W).

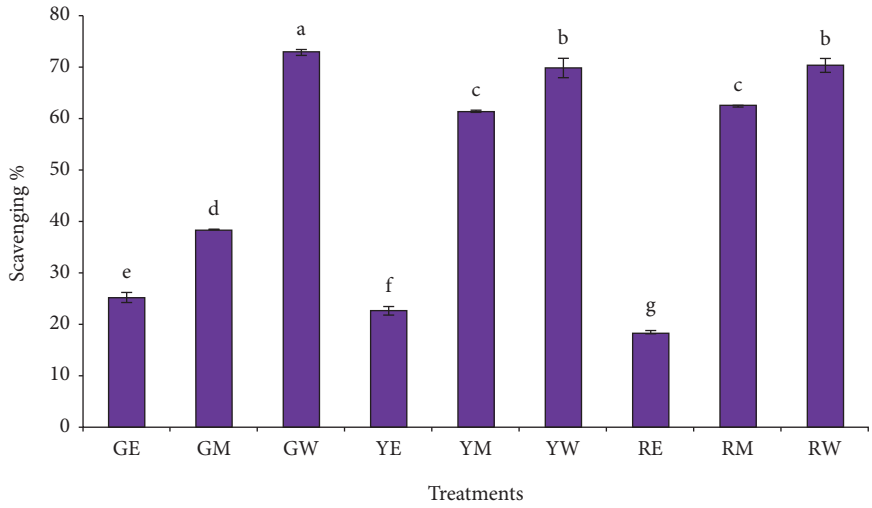

Figure 3: Effect of different solvent extractions on the DPPH scavenging activity of peppers. The treatment codes denoted by two letters represent the green (G), yellow (Y), and red pepper (R), extracted with ethanol (E), methanol (M), and water (W).

MIC and MBC values of all the prepared extracts were determined against all test pathogens. Extracts of water demonstrated lower MIC and MBC values as compared to the alcoholic extracts (Table 1). The lowest MIC values ranging from 0.5 to 2.0 mg/ml concentration were recorded for aqueous extracts of green pepper, while the highest MICs (4–8 mg/ml) and MBCs (8–16 mg/ml) were observed with the alcoholic extracts of yellow pepper. The antimicrobial action of the pepper extracts can be attributed to the presence of polyphenols and capsaicinoids as demonstrated previously by Mokhtar et al. [54]. Our results demonstrate slightly higher MIC values against Gram-positive bacteria as compared to Gram-negative bacteria. This finding is on the expected lines as the structure and composition of the cell wall of Gram-positive bacteria differs from Gram-negative bacteria. The cell wall of the Gram-positive bacteria comprises a thick layer of peptidoglycan with covalently bound teichuronic and teichoic acid making them less susceptible to the action of plant extracts.

3.5. HPLC Analysis of Phenolic Compounds. The effect of different extracting solvents on the phenolic compounds of three (green, yellow, and red) pepper (*Capsicum annuum* L.) samples that were analyzed by high-performance liquid chromatography (HPLC) representative overlay chromatograms is shown in Figure 6 and the average values are reported in Table 2. Phenolic substances' type and concentration are responsible for biological activities. The analysis and characterization of phenolic compounds with modern techniques potentially open the door for the discovery of biologically active compounds. The factors which affect the phenolic compounds are the production system, climate conditions, fruits, cultivars' maturation state, and

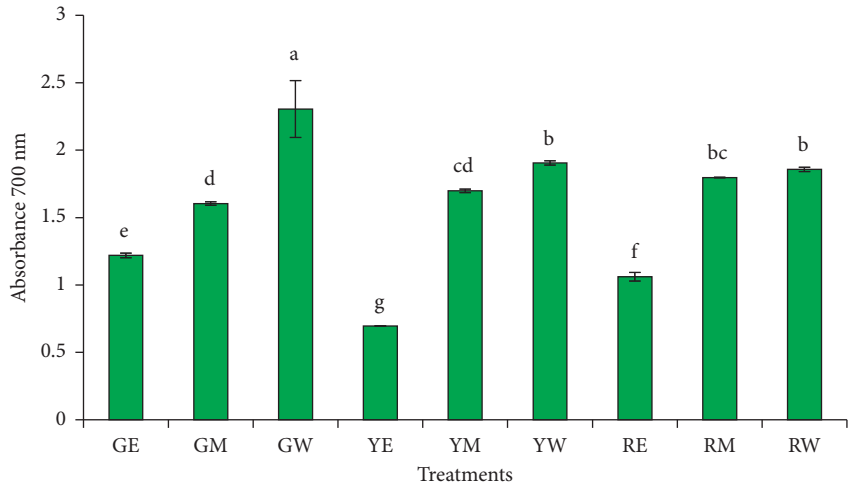

Figure 4: Effect of different solvent extractions on the reducing power of peppers. The treatment codes denoted by two letters represent the green (G), yellow (Y), and red pepper (R), extracted with ethanol (E), methanol (M), and water (W).

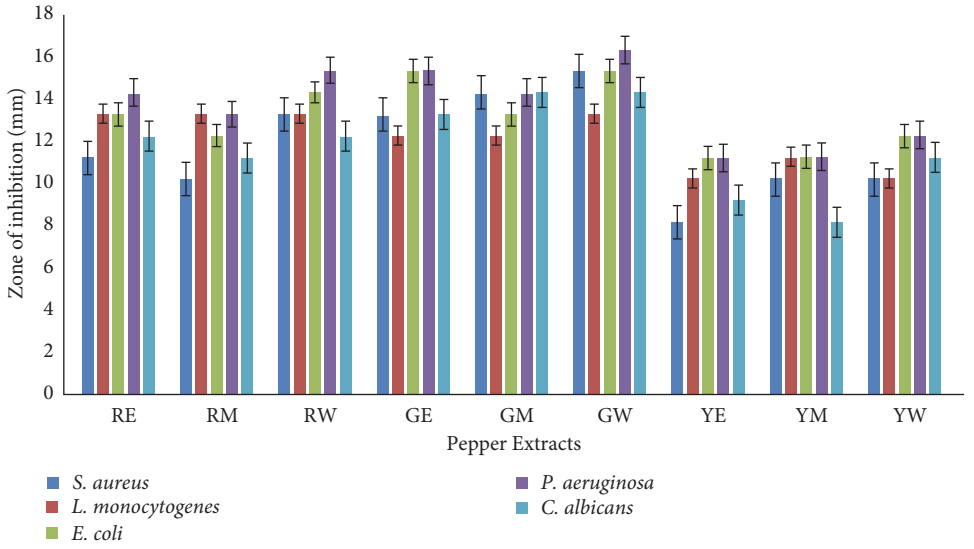

Figure 5: Antimicrobial activity of pepper extracts. The treatment codes denoted by two letters represent the green (G), yellow (Y), and red pepper (R), extracted with ethanol (E), methanol (M), and water (W).

Table 1: MIC and MBC values of different pepper extracts against test pathogens.

Sample	Pathogens									
	S. aureus		*L. monocytogenes*		*E. coli*		*P. aeruginosa*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
RE	4	8	4	8	1	2	2	4	8	16
RM	4	8	4	8	1	2	2	4	8	16
RW	2	8	2	4	1	2	1	2	2	4
GE	2	4	2	4	1	2	2	4	4	8
GM	2	4	2	4	1	2	2	4	4	4
GW	1	2	1	1	0.5	1	0.5	1	2	2
YE	8	16	8	16	4	8	4	4	8	16
YM	8	8	8	16	4	8	4	4	8	16
YW	4	8	4	8	2	4	2	4	4	8

MIC and MBC values are given in mg/ml.

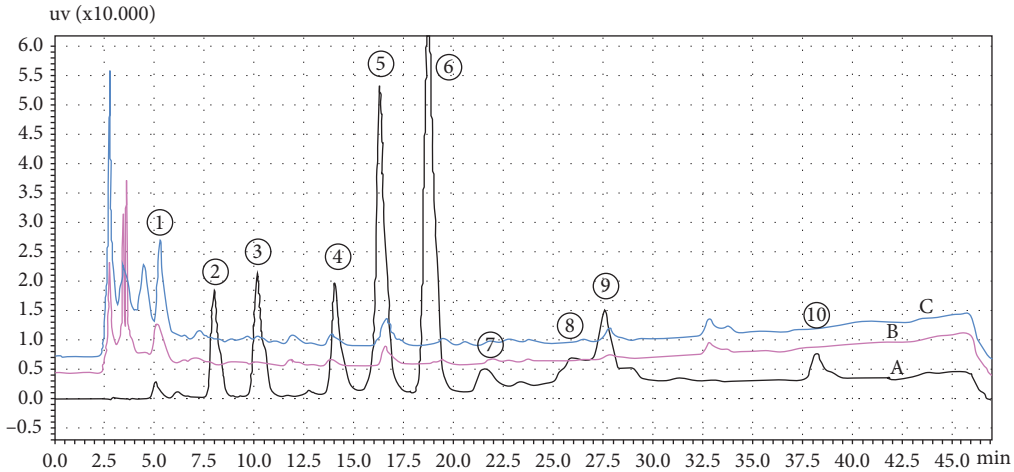

Figure 6: Representative HPLC chromatogram of phenolic compound analysis. 1 = tannic acid, 2 = resorcinol, 3 = 1,2-DHB, 4 = chlorogenic acid, 5 = caffeic acid, 6 = vanillin, 7 = acetyl salicylic acid, 8 = 3,5-DNSA, 9 = salicylic acid, 10 = quercetin. A = phenolic compound standards (50 μg/ml), B = green pepper ethanol extraction, and C = green pepper methanol extraction.

Table 2: Phenolic compounds of pepper (green, yellow, and red) HPLC (mg/100 g) dry weight (dw).

Sample	Tannic acid	Resorcinol	1,2-DHB	Chlorogenic acid	Caffeic acid	Vanillin	Acetyl salicylic acid	3,5-DNSA	Salicylic acid
GE	1028.67 ± 1.38	ND	10.57 ± 0.10	19.03 ± 0.28	16.45 ± 0.19	1.43 ± 0.02	34.94 ± 0.76	5.72 ± 0.08	13.53 ± 0.15
GM	1689.40 ± 1.37	ND	14.34 ± 0.11	25.42 ± 0.51	24.20 ± 0.32	2.44 ± 0.03	22.77 ± 0.25	9.15 ± 0.14	27.83 ± 0.38
GW	2284.25 ± 1.84	ND	21.47 ± 0.09	28.42 ± 0.13	23.05 ± 0.10	2.42 ± 0.00	28.07 ± 0.03	9.92 ± 0.07	17.83 ± 0.22
YE	2577.62 ± 1.57	ND	13.88 ± 0.01	10.44 ± 0.01	18.89 ± 0.04	0.70 ± 0.04	11.88 ± 0.37	4.10 ± 0.03	2.78 ± 0.02
YM	3501.16 ± 1.23	ND	13.31 ± 0.00	13.81 ± 0.10	28.79 ± 0.10	2.01 ± 0.08	ND	17.06 ± 0.01	5.07 ± 0.02
YW	2618.90 ± 3.54	14.45 ± 0.18	18.40 ± 0.04	15.03 ± 0.02	26.70 ± 0.03	1.64 ± 0.01	ND	18.31 ± 0.11	2.96 ± 0.01
RE	2559.68 ± 1.19	ND	12.67 ± 0.00	7.99 ± 0.08	21.36 ± 0.06	0.68 ± 0.02	9.94 ± 0.04	6.10 ± 0.02	1.06 ± 0.00
RM	2940.58 ± 1.05	ND	17.92 ± 0.07	13.14 ± 0.00	30.23 ± 0.03	0.55 ± 0.01	ND	18.51 ± 0.09	0.85 ± 0.00
RW	1933.00 ± 3.57	10.42 ± 0.16	21.15 ± 0.26	16.15 ± 0.09	27.47 ± 0.07	1.61 ± 0.09	ND	15.01 ± 0.02	0.50 ± 0.00

DHB = dihydroxy benzene; DNSA = dinitro salicylic acid; G = green pepper; Y = yellow pepper; R = red pepper; E = ethanol; M = methanol; W = water; ND = not detected.

postharvest treatment [17]. Tannic acid is the major phenolic compound in all three pepper samples ranging within 1028.67–3501.16 mg/100 g dw. Chlorogenic acid, 19.03–28.42 mg/100 g dw, is high in green pepper as compared to yellow and red pepper samples. Other individual phenolic compound ranges are resorcinol 10.42–14.45, 1,2-DHB 10.57–21.47, caffeic acid 16.45–30.23, acetylsalicylic acid 9.94–34.94, 3, 5 DNSA 4.10–18.51, and salicylic acid 0.5–27.83 mg/100 g dw. In general, yellow pepper samples show higher phenolic compounds as compared to red and green peppers. Green pepper phenolic compounds are higher in water extraction than methanol and ethanol extraction. Guilherme et al. [17] reported the higher content of chlorogenic acid in green pepper. Hallmann and Rembialkowska [46] reported chlorogenic acid 877.0 mg/kg dw in organic and 749.0 mg/kg in conventional grown bell pepper. Lee et al. [55] reported that the fresh pepper contains the total soluble phenolic compound from 178 to 384.9 mg chlorogenic acid equivalent per 100 gram off fresh weight. Caffeic acid in four cultivars of red sweet pepper are a little higher to this study, in the range of 38–63 μg/kg [56]. Different values in the literature may be due to different cultivars, different extraction methods, and the ways to express the results [57].

4. Conclusions

The current study investigated the effect of different solvents extractions (methanol, ethanol, and water) and oven drying on the antioxidant and antimicrobial properties of red, yellow, and green peppers. All solvent extracts impacted the biological properties of the pepper samples. Among all the samples tested, an aqueous extract of green pepper was found to possess the highest TPC, TFC, antioxidant, and antimicrobial activity. HPLC analysis revealed tannic acid as the major phenolic compound in all three pepper samples, while chlorogenic acid was found to be in higher amounts in green pepper samples as compared to red and yellow pepper. It is postulated that envisaged that the microwave drying/heating technique can improve the antioxidant and antimicrobial activity of the pepper, but the exact mechanism needs to be unearthed in future studies. Further, the findings of this study could be exploited in the processing, storage, and consumption of pepper.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Ahmad Mohammad Salamatullah, Khizar Hayat, and Fohad Mabood Husain were responsible for writing of the original draft and supervision. Mohammed Asif Ahmed, Shaista Arzoo, and Nawal Albader carried out formal analysis and data curation. Mohammed Musaad Althbiti, Abdulhakeem Alzahrani, Hiba-Allah Nafidi, and Mohammed Bourhia were responsible for writing, reviewing, and editing. Bandar Ali M Al-Zaied and Heba Kahlil Alyahya performed data validation.

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Review Article

The Effects of Omega-3 Supplementation on Resting Metabolic Rate: A Systematic Review and Meta-Analysis of Clinical Trials

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Background. It is uncertain if omega-3 polyunsaturated fatty acids are associated with increase in resting metabolic rate (RMR) in adults. *Objective*. The aim of the present study was to evaluate the overall effects of omega-3 on RMR. *Methods*. Both PubMed and Scopus libraries were searched up to April 2021. Study quality was assessed using the Jadad scale. Random- and fixed-effects models were utilized in order to obtain pooled estimates of omega-3 supplementation impacts on RMR, using weight mean difference (WMD). *Results*. Seven studies including a total of 245 participants were included. There was significantly higher FFM-adjusted RMR in the intervention group than the control group (WMD: 26.666 kcal/kg/day, 95% CI: 9.010 to 44.322, $p = 0.003$). Study quality showed that four of seven included studies were of high quality. However, there was no significant difference in results in the subgroup analysis according to the quality of studies. Subgroup analyses revealed significant changes for sex (for women: WMD = 151.793 kcal/day, 95% CI = 62.249 to 241.337, $p = 0.001$) and BMI (for BMI > 25: WMD = 82.208 kcal/day, 95% CI = 0.937 to 163.480, $p = 0.047$). Influence analysis indicated no outlier among inclusions. *Conclusion*. The current study depicted that omega-3 polyunsaturated acids can significantly increase RMR in adults. However, further assessments of omega-3 supplementation therapy are critical to monitor its long-term outcomes and potential clinical application.

1. Introduction

The global proportion of the aging population is increasing and predicted to reach more than 22% by 2050. Critical changes that appear during aging are increase in fat mass and the reduction of either fat free mass (FFM) or resting metabolic rate [1]. These changes may increase susceptibility to different diseases, particularly diabetes and cardiovascular diseases, which can affect quality of life [2, 3]. As a solution, several studies have suggested increasing the intake of

omega-3 polyunsaturated fatty acids (n-3 PUFAs) that exerts beneficial effects by reducing body weight and fat mass through stimulating energy expenditure [4], which may ultimately help elevating the resting metabolic rate (RMR) of individuals.

n-3 PUFAs are natural antioxidants and cofactors for mitochondrial enzymes [5]. Existing evidence has considered n-3 PUFAs as a therapeutic component that influences the metabolic processes of some tissues. For example, it is believed that a higher intake of omega-3 increases the whole-body energy expenditure in the skeletal muscle fibers by changing the activity of membrane-bounded proteins [6–8]. In addition, omega-3 is involved in fat metabolism by changing the expression of proteins such as fatty acid translocase [9]. Considering these properties, n-3 PUFAs may increase the whole-body RMR and promote a shift towards fatty acid oxidation.

However, data provided by human studies have been conflicting. Some studies have indicated that n-3 PUFAs have positive effects on RMR [9, 10]. A study by Christopher et al. indicated that, in a group of healthy young men, supplementation of omega-3s for 12 weeks increased RMR [10]. In contrast, another study revealed that fish oil supplementation did not alter RMR [11]. Therefore, we conducted this systematic review and meta-analysis of the available clinical trials to assess the efficacy of n-3 PUFAs supplementation on RMR in adults.

2. Methods

This study was carried out based on the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement [12].

2.1. Search Strategy. We performed a literature search of the online bibliographic databases (PubMed and Scopus) for relevant publications up to April 2021. In order to find relevant publication, we used the combination of following medical subject headings (MeSH) and non-MeSH keywords: ("Fatty Acids, Unsaturated" OR "Fatty Acids, Omega-3" OR "Fish Oils" OR "Eicosapentaenoic Acid" OR "n-3 Polyunsaturated Fatty Acid "OR "n-3 PUFA ("AND ("Energy Metabolism" OR "Basal Metabolism") AND ("Clinical Trials as Topic" OR "Clinical Trial" OR "randomized"). Databases were searched by two independent investigators (HY and SA). We also searched for systematic reviews from the abovementioned databases and hand-searched reference lists to identify studies that might have been missed.

2.2. Selection of Studies. After removal of duplications, the search results were evaluated by one investigator (SA). Selected studies based on review of the title or abstract were retrieved and reviewed by two investigators. The arguing studies were passed to the third researcher (DJ) for a definite decision of rejection.

2.3. Inclusion and Exclusion Criteria for Studies. Eligible publications were included based on the following criteria: (1) investigating population was adults (over 18 years of age); (2) all studies assessed the effects of omega-3 supplementation on RMR or resting energy expenditure (REE); (3) the control group received non-n-3 PUFA (such as olive oil and oleic acid); (4) studies with a design of randomized, controlled clinical trial; (5) human studies; and (6) manuscripts published in English language.

Studies that met the following criteria were excluded: (1) participants younger than 18 years of age; (2) non-RCT designs (observational studies, crossover design studies, letters, review articles, and meta-analysis); (3) studies that did not provide enough data; and (4) studies on specific diseases (such as spinal cord injury (SCI) and acquired immunodeficiency syndrome (AIDS)).

2.4. Data Extraction. The study selection and data extraction from each eligible study were conducted independently by two reviewers (HY and SA), and any disagreements were discussed. Data of interest from each individual study were extracted as follows: participant characteristics (first author, year of publication, study population, sample size, age, sex, weight, and BMI) and supplement and placebo details (presence of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), dose, and intervention duration) (Table 1). For three studies which graphically presented their data, the mean and standard deviation were extracted using the GetData Graph Digitizer 2.24 software.

2.5. Assessment of Study Quality. Study quality was assessed by a modified Jadad scale [13], in which the total score ranges from 0 to 5 values based on the following criteria: (1) randomization, (2) method of randomization, (3) double blinding, (4) method of double blinding, and (5) report of dropouts and withdrawals. Any discrepancies were resolved by discussion. We defined high-quality publications as those with a Jadad score of 3 or more (Table 1).

2.6. Data Synthesis and Statistical Analysis. Data were analyzed using Stata software version 14 (Stata Corp Lp, College Station, TX, USA). Random- and fixed-effects models were utilized in order to obtain pooled estimates of omega-3 supplementation impacts on RMR, using weight mean difference (WMD). Studies that reported two or more interventions of different omega-3 dosages were entered as separate studies. We performed three analyses to compare the effect of omega-3 on (1) RMR; (2) RMR adjusted for body weight; and (3) RMR adjusted for FFM. Within-group mean change was calculated using the difference between baseline and final time point values of RMR (with 95% confidence interval) for either intervention or control groups. Some studies provided a standard error of mean which was used to compute standard deviation according to the formula SD = SEM × square root of N. Then, we calculated SD of the mean difference as follows: SD change = square root $[(\text{SDbaseline}^2 + \text{SDfinal}^2) - (2 \times R \times$

Table 1: Demographic characteristics of the included studies.

Study first author (year)	Study population	Gender	Mean age I and P (years)	Mean weight I and P (kg)	Mean BMI I and P	Sample size I and P	Duration (week)	Placebo	Assessment methods of FFM and RMR	Omega-3 daily dose (g)	Jaded score
Moses. AWG (2004)	Cachectic patients with advanced pancreatic cancer	Male and female	68	NR	21–20	7–12	8	n-9 fatty acid (oleic)	RMR: Schofield equations FFM: BIA	2.2 g (EPA)	5
Gerling. C.J (2014)	Healthy active male	Male	22.7–20	82.1–79.0	24	21–9	12	Olive oil	RMR: Péronnet and Massicotte equation FFM: NR	3 g (EPA: 2 and DHA: 1)	2
Lalia. A.Z (2014)	Insulin-resistant humans	Male and female	35.3–32.6	105.3–99.6	35.5–35.2	14–11	26	Softgels oil (oleic)	RMR: NR FFM: DXA	3.9 g (EPA: 2.7 and DHA: 1.2)	4
Noreen (2010)	Healthy adults	Male and female	33–35	71.3–71.1	NR	22–22	6	Safflower oil	RMR: NR FFM: Bod Pod	4 g (EPA: 2.7 and DHA: 1.3)	3
Huerta. A.E (2015)	Overweight and obese women during weight loss	Female	38–39	88.4–84.6	Between 27.5 and 40	18–22	10	Sunflower oil	RMR: Weir equation FFM: DXA	1.3 g (EPA)	5
Huerta. A.E (2015)	Overweight and obese women during weight loss	Female	39–38	84.9–83.5	Between 27.5 and 40	17–20	10	α-Lipoic acid	RMR: Weir equation FFM: DXA	1.3 g (EPA: 1.3 and α-lipoic acid:0.3)	5
Logan. S.L (2015)	Healthy older women	Female	66	72.9–69.1	28–26	12–12	6	Olive oil	RMR: Harris and Benedict equations FFM: BIA	3 (EPA: 2 and DHA:1)	2
Logan. S.L (2015)	Healthy older women	Female	66	72.9–69.1	28–26	12–12	12	Olive oil	RMR: Harris and Benedict equations FFM: BIA	3 (EPA: 2 and DHA: 1)	2
Jannas-Vela. S (2017)	Healthy young man	Male	23–22	77.5–77.8	24	13–13	6	Olive oil	RMR: Péronnet and Massicotte equation FFM: NR	3 (EPA: 2 and DHA: 1)	2
Jannas-Vela. S (2017)	Healthy young man	Male	23–22	77.5–77.8	24	13–13	12	Olive oil	RMR: Péronnet and Massicotte equation FFM: NR	3 (EPA: 2 and DHA: 1)	2

I: intervention; P: placebo; RMR: resting metabolic rate; FFM: fat-free mass; BMI: body mass index; NR: not reported: DXA: dual X-ray absorptiometry; BIA: bioelectrical impedance analyzer.

SD baseline × SD final)]. Moreover, we determined a correlation coefficient of 0.9 as R-value that ranges between 0 and 1. Between-study heterogeneity was examined using the I-square (I^2) test. To assess the influence of each study on the overall mean difference, we used a sensitivity analysis by the one-study removal approach. Publication bias was assessed by visual evaluation of the funnel plot and Egger's test. p values <0.05 were considered significant.

3. Results

3.1. Study Selection. According to the selected search terms, a total of 1512 articles were identified from electronic databases, of which 65 papers were potentially eligible for inclusion after reading the titles and abstracts. Subsequently, 7 studies were found eligible and, therefore, included in the meta-analysis. The remaining articles were excluded due to inaccessibility of the data, additional interventions performed on the participants, missing the control group among others. A flow diagram of the literature search procedure is shown in Figure 1.

3.2. Study Characteristics. A total of 245 individuals (with a mean age range of 20 to 68 years) enrolled in the trials, which included 70 men and 131 women; however, gender was not reported for 44 subjects. Of the seven studies in the meta-analysis, two studies were exclusively conducted on women, two exclusively included men, and three studies recruited both sexes. The mean BMI of participants ranged between 20 and 40 kg/m^2. Most of the participants were healthy adults. The study population comprised of normal weight, overweight, obese, and also, insulin resistance persons. However, a couple of cachectic patients with advanced pancreatic cancer were included. The dose of intervention ranged from 2.2 to 4 grams. The study duration was between 6 and 26 weeks. Additionally, some studies used EPA alone and most of the studies prescribed combination of EPA and DHA (with a ratio of 2 : 1, respectively) (Table 1).

3.3. Quality Assessment and Risk of Bias. The quality score of included studies ranged from 2 to 5. Three trials were categorized as low-quality publications (Jadad score <3) and four trials were classified as high quality (Jadad score ≥3). All studies were randomized trials, but four studies did not explain the randomization procedure. Among the seven included studies, three studies were single blind (no study was double blind). All studies reported details concerning with the number of participants that dropped out.

Visual assessment of the funnel plot denoted no publication bias for RMR, RMR adjusted for body mass, and RMR adjusted for FFM (Figure 2). Accordingly, Egger's test also did not provide evidence of publication bias for RMR ($p = 0.085$), RMR adjusted for body mass (Egger's test $p = 0.084$), and RMR adjusted for FFM ($p = 0.080$).

3.4. Outcomes. The pooled effect size of 7 studies demonstrated a significant increase of RMR adjusted for FFM (WMD: 26.666 kcal/kg/day, 95% CI: 9.010 to 44.322, $p = 0.003$) following the intervention (Figure 3). In contrast, all changes in RMR (WMD: 47.225 kcal/day, 95% CI: −2.437 to 96.887, $p = 0.062$) and RMR adjusted for body mass (WMD: 0.237 kcal/day, 95% CI: −0.268 to 0.741, $p = 0.358$) were not statistically significant (Figure 4). The results of the influence analysis did not change the significance level of our findings after the removal of each trial. Furthermore, elimination of a study carried out by Moses et al. in pancreatic cancer patients did not change the statistical outcomes of the study (Figure 5). Finally, the between-study heterogeneity was significant for RMR (I^2: 54.3%, $p = 0.032$).

3.5. Subgroup Analysis. To identify the potential sources of heterogeneity, subgroup analysis was conducted according to sex, age, BMI, quality of studies, and dosage of supplement, as well as intervention duration. Significant sources were explored in our meta-analysis including sex (for women: WMD = 151.793 kcal/day, 95% CI = 62.249 to 241.337, $p = 0.001$) and BMI (for BMI > 25: WMD = 82.208 kcal/day, 95% CI = 0.937 to 163.480, $p = 0.047$).

4. Discussion

This is the first systematic review and meta-analysis, to the best of our knowledge, which investigated the effects of omega-3 supplementation on resting metabolic rate in adults. Our results illustrated that the intervention did not significantly change RMR in the study population. Since there was heterogeneity among studies, the subgroup analysis was applied to eliminate heterogeneity. Improvements in subgroup analysis were observed in females and those with a BMI of over 25 kg/m^2 (overweight and obese individuals). Additionally, significant outcomes were not observed when RMR was adjusted for body mass. Interestingly, omega-3 supplementation led to significantly increased RMR when adjusted for FFM compared to the control group.

Body weight consists of two main parameters: fat-free mass and fat mass. Conflicting studies point to the key role of one of these two parameters as the main determinant of RMR [14]. In contrast, numerous studies have demonstrated that total body weight directly affects RMR [15]. Our data found a significant p value for the independency of increased RMR from FFM following omega-3 supplementation. The elevated RMR was significant when we separately analyzed the FFM-adjusted RMR data. Additionally, we found that increase in RMR was no longer statistically significant when RMR was adjusted for body weight after omega-3 intervention. However, studies conducted by Gerling et al. showed that increase in RMR was not affected by body weight [9]. Indeed, RMR changes caused by omega-3 consumption maybe influenced by fat mass, but there was

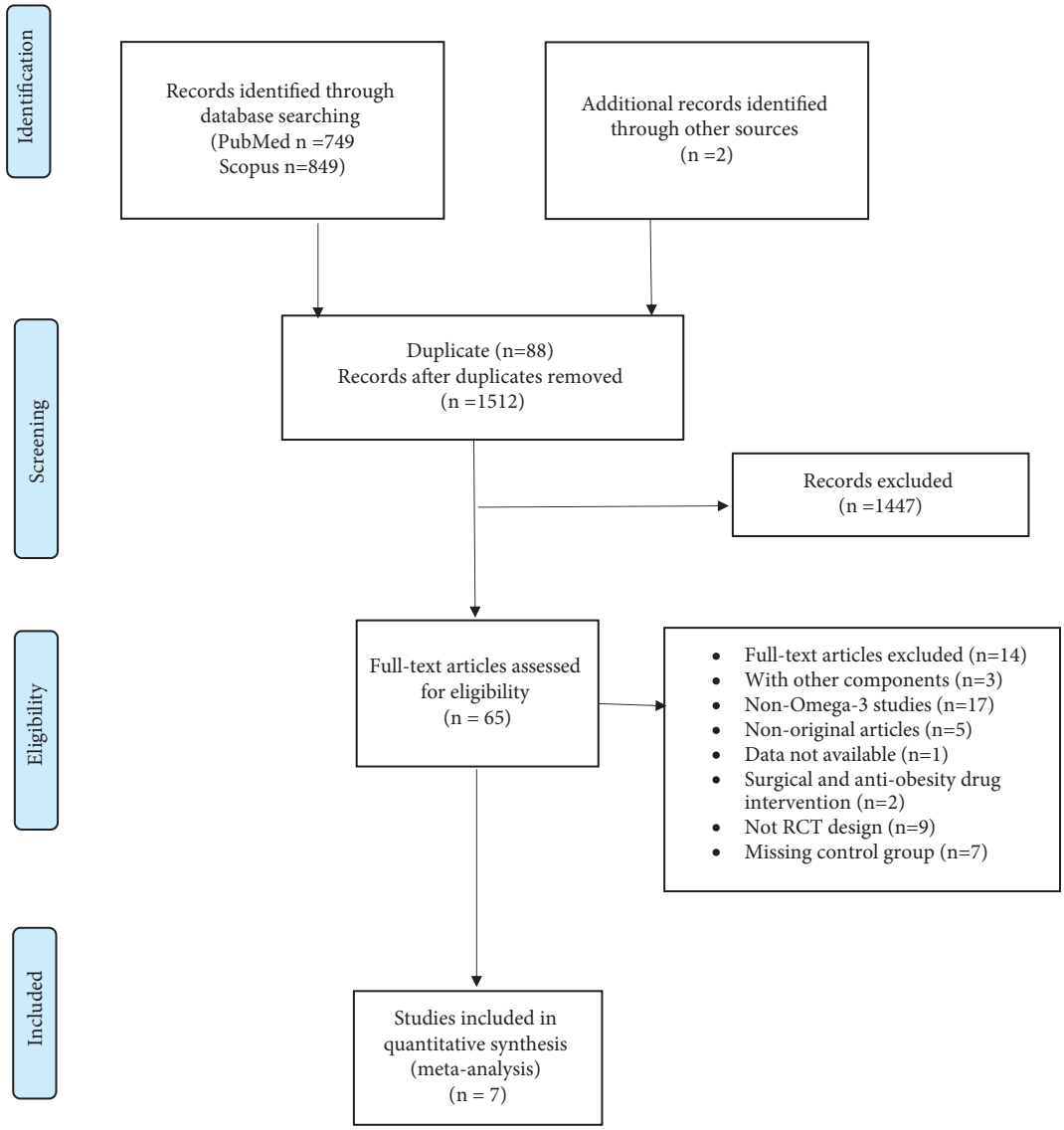

Figure 1: Flow diagram of studies' screening and selection in literature search.

insufficient evidence of fat mass-adjusted RMR data to confirm this hypothesis.

We noticed that omega-3 affects females and people with BMI > 25 more efficiently than males and normal-weight individuals. The molecular mechanism behind the positive effects of omega-3 supplementation on RMR could possibly underlay on the fact that omega-3 increases insulin sensitivity of the tissues without influencing the body weight [4]. In consensus with this explanation, insulin resistance was already reported in omega-3-deficient rats [16] and also in obese individuals who had lower concentration of omega-3 [17]. Furthermore, omega-3 is believed to activate the peroxisome proliferator-activated receptor (PPAR) family [18], and then, the whole complex upregulates the following genes which contribute, particularly, in the metabolism of fatty acids: (1) intra- and extracellular fatty acid transporters (fatty acid-binding protein [19] and fatty acid translocase [20]); (2) ion symporters (such as mitochondrial uncoupling protein 3 which protects the mitochondria from oxidative stress by increasing the proton gradient of the intermembrane space [21, 22]); (3) fatty acid oxidative enzymes [23]; and eventually, (4) a transcriptional coactivator named peroxisome proliferator-activated receptor gamma coactivator 1-alpha as the master regulator of energy metabolism in the mitochondria [24, 25]. Improved glucose tolerance concomitant with higher energy expenditure of the cells generally leads to higher oxygen consumption and metabolic rate.

In line with our findings, previous investigations have also demonstrated that women and overweight people have lower insulin sensitivity [26]. A previous study in healthy females ($n = 257$) has frequently monitored the amount of homeostasis model assessment for insulin resistance (HOMA-IR) in order to explain insulin resistance. They found HOMA-IR in positive correlation with estradiol and progesterone produced in menstrual cycle. However, there were concerns how accurately HOMA-IR altered the insulin resistance in females [27]. HOMA, together with fat mass

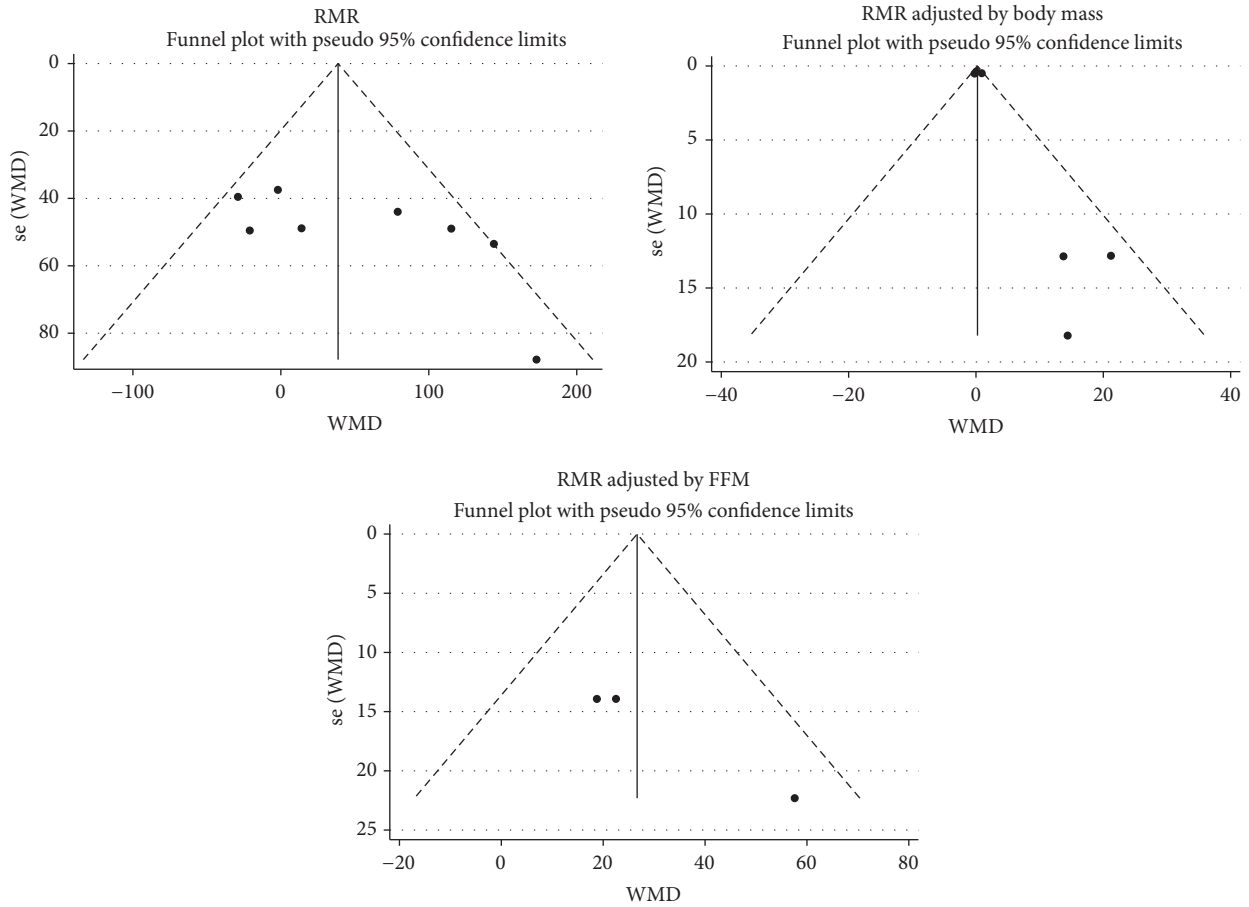

Figure 2: Funnel plot for evaluating publication bias for RMR, RMR adjusted for body mass, and RMR adjusted for FFM.

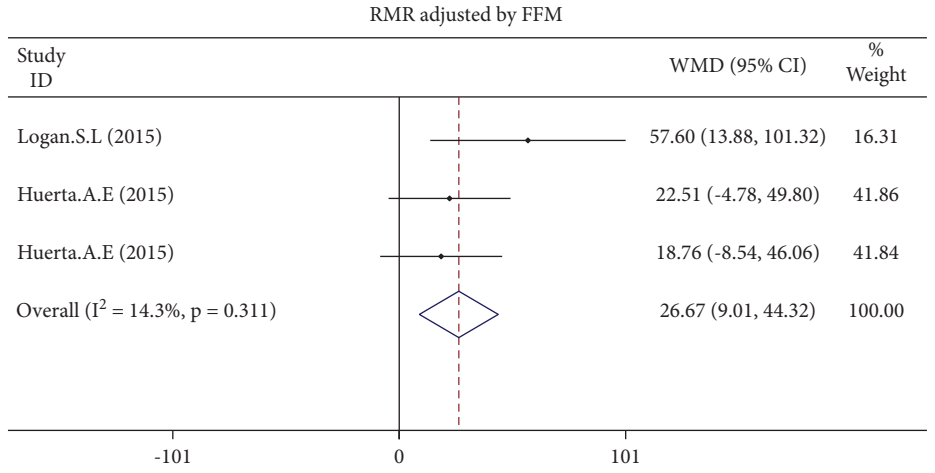

Figure 3: Forest plot presenting weighted mean difference (WMD) and 95% confidence intervals for RMR adjusted for FFM.

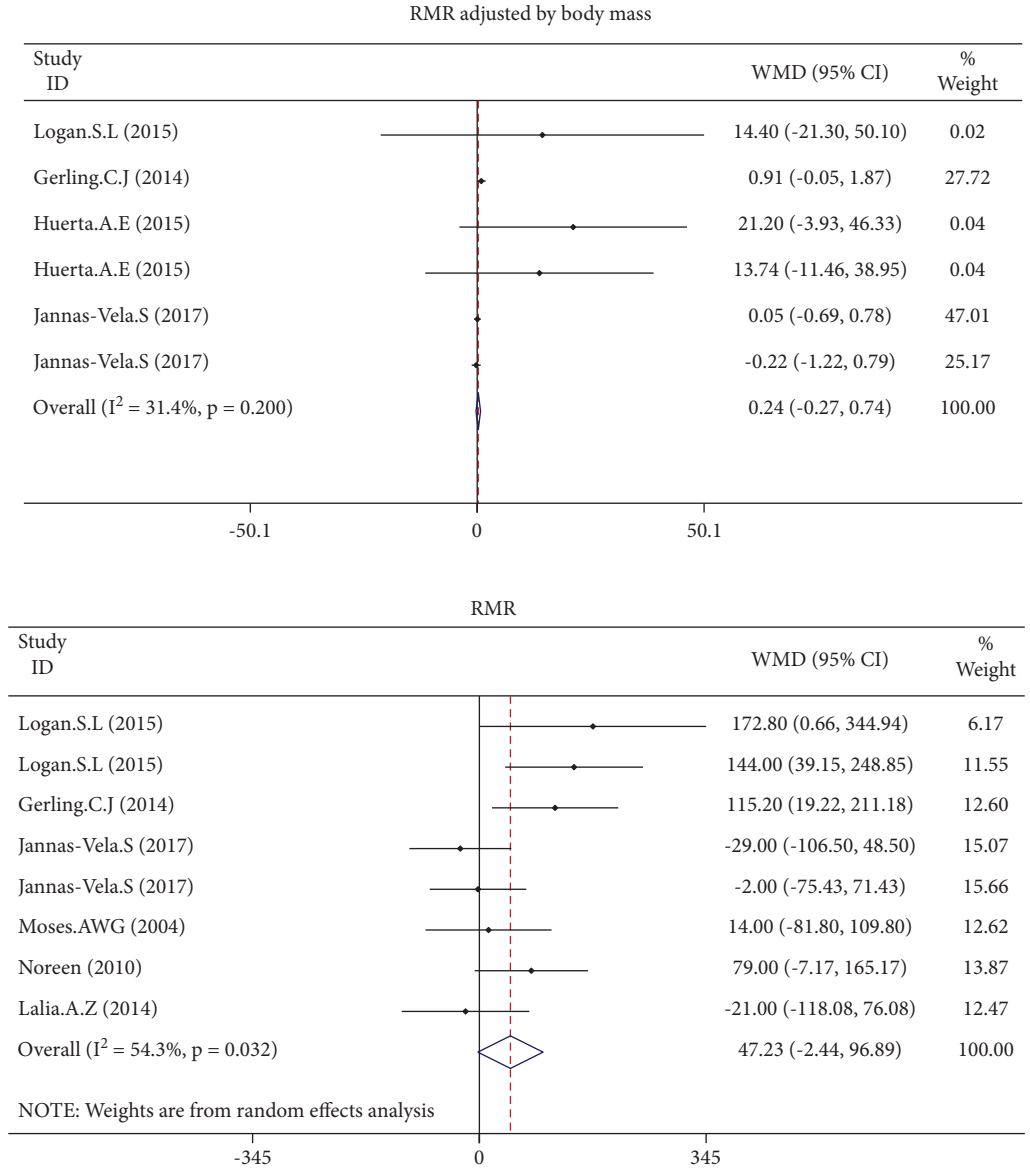

Figure 4: Forest plot presenting weighted mean difference (WMD) and 95% confidence intervals for RMR adjusted for body mass.

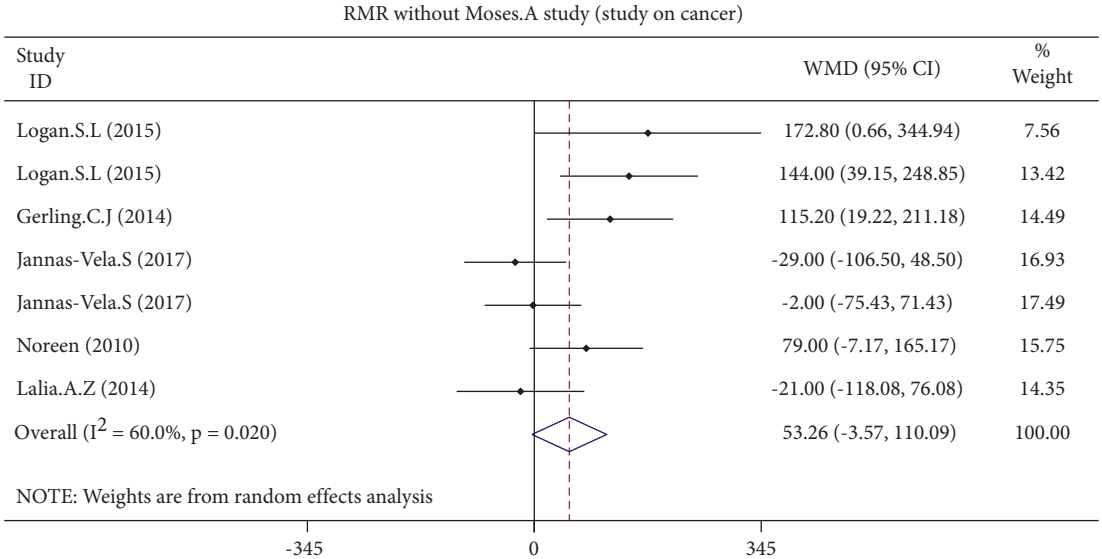

Figure 5: Forest plot presenting weighted mean difference (WMD) and 95% confidence intervals for RMR and intentional elimination of a study carried out by Moses et al. with a population of pancreatic cancer patients.

and their association with estradiol level [28], was shown to be negatively affected by omega-3 in children [29] as well as adults [30]. According to previous findings, our data suggest that n-3 PUFAs increase RMR level, perhaps through change in HOMA-IR and balanced sensitivity of insulin. However, this needs further evaluation of RMR of n-3 PUFA-consuming subjects with reference to the HOMA, insulin, and glucose level.

The findings should be considered with a few limitations in mind. Firstly, all studies controlled the dietary regiment of participants for three months except for one study. However, attendants in different studies did not use the same dietary intake. Secondly, studies did not use the same equation to calculate the RMR. Additionally, some of the studies had prescribed different amounts of the EPA and DHA. The main strength of this study is that it is, to our knowledge, the first systematic review and meta-analysis which investigated the effects of omega-3 supplementation on RMR.

5. Conclusions

Present meta-analysis demonstrated that omega-3 polyunsaturated fatty acids increased the RMR in adult participants, especially in females and those with a BMI of over 25 kg/m^2 (overweight and obese individuals). Additionally, RMR was shown to be body mass dependent. In contrast, omega-3 supplementation significantly increased RMR when adjusted for FFM compared to the control group. Overall, these data suggest that omega-3 supplementation maybe a healthy approach to increase RMR, consequently preventing from chronic metabolic diseases. However, further long-term studies are needed to evaluate RMR in response to omega-3 with reference to insulin level changes and also metabolism controlling gene expression.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

HY, KJ, SAB, and KM designed the research; HY, SA, and SHS conducted the research; HY analyzed data; HB and LS wrote the paper; KJ and KhM had primary responsibility for the final content; and SI, a native English speaker, improved the grammar and readability. All authors read and approved the final manuscript.

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Research Article

Effects of "Bacuri" Seed Butter (*Platonia insignis* Mart.) on Metabolic Parameters in Hamsters with Diet-Induced Hypercholesterolemia

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This study aimed to evaluate the effects of the treatment with bacuri seed butter (BB) on body weight, growth, body mass index, lipid profile, atherosclerotic indices, and liver function in dyslipidemic hamsters. Freshly weaned, male hamsters were divided into four groups: (1) normal group (NG)—maintained with standard chow (AIN-93G); (2) dyslipidemia group (DG)—maintained with hyperlipidemic chow (AIN-93G modified) throughout the follow-up period; (3) bacuri seed butter 25 mg/kg/day (BB-25); and (4) bacuri seed butter 50 mg/kg/day (BB-50). BB groups (25 and 50 mg/kg/day) were also maintained with hyperlipidemic chow throughout the follow-up period, and the treatment started after 21 days receiving a hyperlipidemic diet to induce hypercholesterolemia and maintained for 28 days. No significant differences in triglycerides and total cholesterol were observed for BB-25 and BB-50 groups when compared with NG and DG groups. On the contrary, BB-25 and BB-50 induced both increase of HDL-c (51.40 ± 1.69 and 51.00 ± 2.34, respectively) and decrease of LDL-c (103.80 ± 6.87 and 100.50 ± 3.95, respectively) when compared with DG (41.00 ± 2.94 and 132.70 ± 9.41, respectively). In addition, BB promoted a reduction in the risk of atherosclerotic disease by decreasing ($p < 0.05$) the atherogenic index, coronary artery risk index, and LDL/CT ratio ($p < 0.05$) and increasing HDL/CT ratio. On the contrary, no changes were observed in total cholesterol and triglyceride levels or in body weight, growth, body mass index, or liver function parameters. Thus, bacuri seed butter at doses of 25 and 50 mg/kg/day has positive repercussions on the lipid profile, more precisely on plasma HDL-c and LDL-c, and additionally promotes reduction in the risk of atherosclerosis in hamsters.

1. Introduction

Cardiovascular diseases (CVDs) continue to be a major cause of disability and mortality in developed and developing countries. About 45% of all deaths from chronic noncommunicable diseases (NCDs) in the world are caused by cardiovascular diseases [1–3], and in low- and middle-income countries, they account for 88% of premature deaths [4]. Atherosclerotic disease is characterized by lipid accumulation and formation of atheromatous plaques within the endothelium, with consequent impairment of the elastic capacity of the smooth muscle tissue. In this context, dyslipidemia, as a result of hypertriglyceridemia and hypercholesterolemia [5] with elevated levels of low-density lipoprotein cholesterol (LDL-c) and reduced levels of high-density lipoprotein cholesterol (HDL-c), represents a key factor for its development [6].

Pharmacological therapy for the treatment of dyslipidemia is based on the use of statins, resins, and ezetimibe, among others, which help to regulate serum cholesterol levels, reducing the synthesis of cholesterol in the liver and its intestinal absorption [6, 7]. On the contrary, the use of these drugs is limited because of their adverse effects, among them, myalgia, increased hepatic transaminases, and changes in intestinal motility, such as constipation or diarrhea [8, 9]. It is worth mentioning the systematic review for the 2020 US Department of Veterans Affairs and US Department of Defense Guidelines for the management of dyslipidemia published in *Annals of Internal Medicine* by Reston et al. [10]: even if the strength of evidence for most interventions was low or very low, intensified patient care and rechallenging with the same or a different statin (or a lower dose) appear to represent favorable options for improving statin adherence.

In this context, the use of medicinal plants and their products in the treatment of dyslipidemia has been increasing because natural products present a lower cost when compared to synthetic, as well as their obtention is suitable [11]. Generally, medicinal plants and herbs are widely being used as sources of nutraceutical active compounds for the management of several types of diseases [12, 13]. The nutraceutical approach to dyslipidemia has been described in different papers as a possible alternative to the conventional drug-based therapy and/or adjuvant therapy using promising natural agents [13–15].

In addition, it is noteworthy that dietary interventions, especially those that provide a large intake of foods with functional properties, can delay or reduce the risk of the development and progression of chronic diseases by modulation of body physiological functions [16]. Studies have shown that plant foods and their derived extracts, for example, can act on a variety of intermediate markers of cardiometabolic risk, including blood pressure, glucose-insulin homeostasis, blood lipids and lipoproteins, endothelial function, inflammation, and oxidative stress. These products have gained increasing notoriety in the last decade due to emerging evidence of their role in important pathways, modulating responses capable of promoting cardiovascular health benefits [3, 17].

"Bacurizeiro" (*Platonia insignis* Mart.) is a plant typical of Cerrado, belonging to the family Clusiaceae and to the genus *Platonia* [18, 19], and has been used in folk medicine in the treatment of diarrhea [20], wounds, and other skin conditions [21]. Some studies have investigated different biological activities of bacuri, and they are identified as antioxidant [22, 23], anti-inflammatory [24], wound healing [25, 26], anticonvulsant [27, 28], antileishmanial [18, 29, 30], and immunomodulatory [31], among other effects. Moreover, formulation of bacuri-based functional products is being investigated [24, 26, 32].

There have been no studies to date on the effects of bacuri seed butter (*Platonia insignis* Mart.) on experimental dyslipidemia, and then considering its wide pharmacological and nutraceutical potentials, the present work aimed to investigate its effects in hamsters with diet-induced hypercholesterolemia.

2. Materials and Methods

2.1. Materials. Bacuri seed butter (*Platonia insignis* Mart.) was supplied by Amazon Oil Indústria e Comércio Ltda (Ananindeua, PA, Brazil). This butter is cold extracted from wild species that grow naturally in the Amazon rainforest, sustainably extracted without using pesticides and fertilizers. No preservatives, additives, or any other chemical substances are added (see https://www.amazonoil.com.br/pt/perfil/).

2.2. Animals and Diets. Freshly weaned, male hamsters (*Mesocricetus auratus*) were obtained from AniLab Laboratory Animals Ltd. (Paulínia, SP, Brazil). Hamsters were kept in individual cages under controlled conditions: temperature $24 \pm 2^{\circ}$C; 12 h light/dark cycle; humidity (55%); water and chow *ad libitum*. After twenty days of adaptation, the animals were divided into four groups: (1) normal group (NG; $n = 8$)—maintained with standard chow (AIN-93G); (2) dyslipidemia group (DG; $n = 8$)—maintained with hyperlipidemic chow (AIN-93G modified) throughout the follow-up period; (3) bacuri seed butter 25 mg/kg/day (BB-25; $n = 10$); and (4) bacuri seed butter 50 mg/kg/day (BB-50; $n = 10$). BB groups (25 and 50 mg/kg/day) were also maintained with hyperlipidemic chow throughout the follow-up period, and the treatment started after 21 days receiving a hyperlipidemic diet to induce hypercholesterolemia. The animals were kept with standard or experimental (modified) chows produced according to the American Institute of Nutrition (AIN) 93G [33]. The composition of experimental diets is listed in Table 1.

Bacuri butter (25 or 50 mg/kg/day) was administered orally once daily, dissolved in 0.1% Tween 80 in distilled water (5 ml/kg volume). Normal and dyslipidemia groups received daily vehicle volume (0.1% Tween 80 in distilled water). After 28 days of treatment, the animals were euthanized by an overdose of sodium thiopental (100 mg/kg) mixed with lidocaine (10 mg/mL) i.p., and blood samples were collected.

Table 1: Composition of experimental diets (g/kg).

Ingredients	AIN-93G	AIN-93G modified
Casein	200.0	221.0
L-cystine	3.0	3.0
Corn starch	397.5	427.5
Dextrinized corn starch	132.0	—
Sucrose	100.0	50.0
Fiber	50.0	100.0
Soybean oil	70.0	20.0
Coconut oil	—	130.0
Cholesterol	—	1.0
tert-Butylhydroquinone	0.014	0.024
Mineral mix (S10022G)	35.0	35.0
Vitamin mix (V10037)	10.0	10.0
Choline bitartrate	2.5	2.5

Food intake was monitored every two days and body weight every three days. The nasoanal length was determined on the first day of induction of hypercholesterolemia, on the first day of treatment, and on the day of euthanasia. From these data, the Lee index was calculated by the following formula: Lee = $(\sqrt[3]{\text{weight}} \div \text{NL}) \times 10,000$, where "NL" stands for nasoanal length, while the body mass index (BMI) was obtained dividing weight by the square of nasoanal length.

All procedures performed were approved by the Ethics Committee on the Use of Animals of the Federal University of Piauí (CEUA/UFPI) (authorization no. 197/16).

For analyses of the centesimal composition of the standard and hypercholesterolemic diets (Table 2), the moisture, ash, lipid, and protein contents were determined according to the method described by the Association of Official Analytical Chemists (AOAC) [34]. The total carbohydrate content of the samples was estimated by difference: [100 − (moisture + ash + protein + lipids)]. Data were expressed in g/100 g of dry matter (energy conversion factors: protein 17 kJ/g; fat 37 kJ/g; total carbohydrates 17 kJ).

2.3. Lipid Profile and Liver Function. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALK) levels were analyzed using diagnostic kits, purchased from Labtest (São Paulo, BR), according to the manufacturer's specifications and Labmax Plenno Automated Chemistry Analyzer. Low-density lipoprotein cholesterol (LDL-c) values were obtained from the Friedewald formula: LDL − c = total cholesterol − (HDL cholesterol + triglyceride÷5) [35].

2.4. Atherosclerosis Indices. The coronary artery risk index (CRI) was calculated by dividing plasma levels of LDL-c by the HDL-c levels, according to Draper et al. [36]. Atherogenic index (AI) was calculated according to Roth et al. [1] by dividing the triglyceride levels by the HDL-c levels.

The HDL/TC ratio was calculated by the following formula: HDL − c/TC ratio = HDL − c÷TC, while the LDL-c/TC ratio was calculated by LDL − c/TC ratio = LDL −c÷TC, according to Lee et al. [37].

Table 2: Centesimal composition of rations (g/100 g dry matter).

Components	Standard	Hypercholesterolemic
Moisture (%)	10.20 ± 0.10	$6.85 \pm 0.20^{*}$
Ash (%)	7.80 ± 0.10	$4.80 \pm 0.20^{*}$
Lipids (%)	3.30 ± 0.10	$14.61 \pm 0.10^{*}$
Protein (%)	20.90 ± 0.40	19.06 ± 1.00
Total carbohydrate (%)	$57.80 \pm 0.30^{\#}$	$54.68 \pm 0.80^{\#}$
TEV ($kJ \cdot g^{-1}$)	14.42	17.85

Mean ± standard deviation. TEV: total energy value. #Carbohydrate calculated by difference, including fibers. $^{*}p < 0.05$ when compared with normolipidemic feed, Student's *t*-test.

2.5. Statistical Analyses. The values were represented as mean ± standard deviation of the mean. Statistical analysis was performed by one-way ANOVA followed by Tukey's posttest for multiple comparisons. The level of significance was set at $p < 0.05$.

3. Results and Discussion

Here, main findings are highlighted and contextualized as follows: (i) food consumption, body weight, growth, and body mass index; (ii) lipid profile of hamsters with diet-induced hypercholesterolemia; (iii) cardiovascular risk indexes of hamsters with diet-induced hypercholesterolemia; (iv) liver function of hamsters with diet-induced hypercholesterolemia.

3.1. Food Consumption, Body Weight, Growth, and Body Mass Index. Hyperlipidemic diet has caused a significant reduction ($p < 0.05$) in food intake in dyslipidemic animals when compared to normal animals receiving standard chow (Table 3). Despite this, hypercholesterolemic hamsters did not differ ($p > 0.05$) in relation to body weight, length, and body mass indices (BMI and Lee) at the end of the experimental period. According to Kretschmer et al. [38], animals fed a diet high in fat/carbohydrates and were able to detect the energy content of the food and compensate for this with a lower intake, which may explain the lower consumption observed in groups BB and DG. Although there is a discrepancy between the results reported in the literature [39–41], there is a consensus that this difference in consumption may be strongly related to the duration of the animals' maintenance on that diet and the time of initiation of treatment [38, 41].

These results can be attributed to the duration of the experimental period since the total time of the hyperlipidemic diet was only 7 weeks and other studies used protocols of longer duration to induce obesity in hamsters, with 12 to 16 weeks, and despite this, there were no differences in relation to weight gain among groups, probably due to the higher amount of kcal/g in hyperlipidemic diet [42–44].

3.2. Lipid Profile of Hamsters with Diet-Induced Hypercholesterolemia. Hyperlipidemia induced by high-fat diet in rodents is a widely used model for the evaluation of

Table 3: Food intake, body weight, and nasoanal length indices in hamsters (*Mesocricetus auratus*) after 28 days of treatment with bacuri seed butter (25 or 50 mg/kg/day).

Parameters	Groups (mean ± SEM)			
	NG	DG	BB-25	BB-50
Daily food intake (g)	9.42 ± 0.21	7.49 ± 0.18a	7.47 ± 0.38a	7.44 ± 0.26[a]
Initial body weight (g)	114.37 ± 4.08	113.25 ± 6.43	112.44 ± 5.24	112.87 ± 4.14
Final body weight (g)	132.12 ± 4.52	134.87 ± 4.41	132 ± 5.36	129.25 ± 5.60
Weight gain (g)	83.57 ± 4.67	74.29 ± 3.83	73.71 ± 2.61	79.11 ± 4.46
Initial nasoanal length (cm)	15.24 ± 0.26	15.00 ± 0.35	14.66 ± 0.88	15.70 ± 0.25
Final nasoanal length (cm)	16.75 ± 0.43	17.12 ± 0.41	17.2 ± 0.33	17.35 ± 0.14
Lee index	3,073.00 ± 33.33	3,020.00 ± 102.70	3,016.00 ± 32.15	2,970.00 ± 37.87
BMI	0.48 ± 0.01	0.46 ± 0.02	0.46 ± 0.01	0.43 ± 0.01

NG: normal group; DG: dyslipidemia group; BB-25: bacuri seed butter 25 mg/kg/day; BB-50: bacuri seed butter 50 mg/kg/day; [a] $p < 0.05$ in relation to NG according to Tukey's *post hoc* test.

compounds with alleged hypolipidemic effect [45]. In this context, coconut oil and cholesterol were used as sources of lipids for induction of hypercholesterolemia in this study. Coconut oil is rich in saturated fatty acids and produces elevation of triglyceride, total cholesterol, and LDL cholesterol levels [46, 47]. Similarly, cholesterol intake promotes elevation of total cholesterol (TC) levels, contributing for induction of dyslipidemia [48, 49].

The lipid metabolism of *Mesocricetus auratus* makes this species one of the best models for the study of dyslipidemia due to the similarity to that of humans, in which the transport of cholesterol in blood occurs mainly in the form of LDL cholesterol; and the elevation of dietary lipid intake is followed by an increase in triglyceride levels, unlike other rodents [50–53].

Thus, the effects of bacuri butter on the lipid profile of hypercholesterolemic hamsters were evaluated (Figure 1). It was observed that hyperlipidemic diet promoted a significant increase ($p < 0.05$) in the levels of triglycerides, total cholesterol, HDL-c, and LDL-c. In addition, BB was shown to have an atheroprotective effect by increasing ($p < 0.05$) HDL-c levels and reducing ($p < 0.05$) LDL-c levels in BB-treated animals when compared to the dyslipidemic group.

HDL cholesterol is initially synthesized in the liver in the form of apolipoprotein A1 and transferred to the bloodstream where it binds to phospholipids and cholesterol, as well as promotes efflux of cholesterol stored in cells and subsequently carries cholesterol to the liver to be excreted in the feces [54]. The reduction of LDL cholesterol is associated with the reduction of cardiovascular risk due to its ability to cross the vascular endothelium and accumulate, undergoing oxidation and initiating the formation of atherosclerotic lesions [55]. In this sense, the risk of atherogenicity was assessed using cardiovascular risk indices of animals treated with bacuri seed butter.

The oil extracted from the bacuri seed predominantly contains saturated fatty acids, such as palmitic acid, and monounsaturated acids, such as oleic and palmitoleic acids [56]. Saturated fatty acids' intake leads to an increase in total cholesterol and LDL cholesterol levels by increasing the synthesis of hepatic cholesterol and by reducing the activity of LDL receptors, while unsaturated (poly- or monounsaturated) acids promote the increase in the activity and in the amount of LDL receptors, as well as in its mRNA, thus increasing its turnover [57]. In this sense, it was suggested that the effects of BB on the lipid profile were at least in part due to its content of unsaturated fatty acids.

Bacuri is rich in secondary metabolites, especially xanthones and chemical precursors thereof, such as polyisoprenylated benzophenones [22, 29]. Miura et al. [58] investigated the effects of mangiferin, a xanthone, on mice with hypercholesterolemia induced by high cholesterol intake and observed a reduction in total cholesterol levels in treated animals. In another study, Bao et al. [59] demonstrated that dimethoxyxanthone and trimethoxyxanthone improved lipid metabolism in obese rats induced by a high-fructose diet by reducing total cholesterol, triglyceride, and LDL cholesterol levels and raising HDL cholesterol levels. In this context, xanthones may have contributed to BB effects on the lipid profile of hypercholesterolemic hamsters.

3.3. Cardiovascular Risk Indexes of Hamsters with Diet-Induced Hypercholesterolemia. Additionally, BB was found to reduce the risk of cardiovascular disease by promoting reduction ($p < 0.05$) in atherogenic and coronary artery risks (Table 4). These results are worth noting since high AI values correlate with elevated blood pressure and metabolic dysfunctions and diseases, such as hyperinsulinemia [60].

Furthermore, untreated hypercholesterolemic hamsters were found to have lower HDL/TC and higher LDL/TC ratios, which indicate a higher risk of atherosclerosis severity in the dyslipidemia group when compared to the groups treated with BB. Similarly, but using different animal species, Basu et al. [61] observed that *Hippophae rhamnoides* seed oil, popularly known as common sea buckthorn, promoted an increase in the HDL/TC ratio and reduced the risk of atherosclerosis in hypercholesterolemic rabbits after 30 days of supplementation with 1% cholesterol.

3.4. Liver Function of Hamsters with Diet-Induced Hypercholesterolemia. Afterwards, it was verified that the ingestion of hyperlipidemic diet did not cause change of the levels of AST, ALT, and ALK at the end of the experimental period (Table 5). Likewise, BB did not produce changes in liver function, which indicates that its administration does

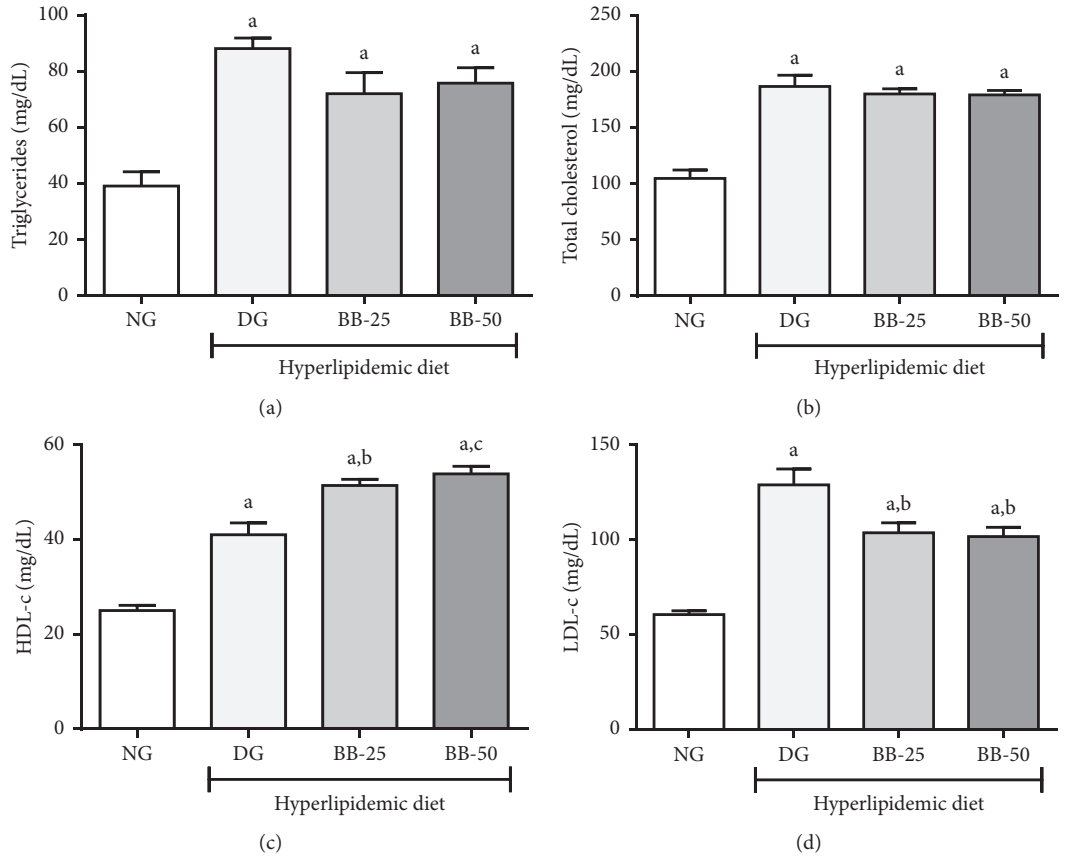

Figure 1: Triglycerides, total cholesterol, HDL-c, and LDL-c serum levels in hamsters (*Mesocricetus auratus*) after 28 days of treatment with bacuri seed butter (25 or 50 mg/kg/day). NG: normal group; DG: dyslipidemia group; BB-25: bacuri seed butter 25 mg/kg/day; BB-50: bacuri seed butter 50 mg/kg/day. [a] $p < 0.01$ in relation to NG; [b] $p < 0.05$ in relation to DG; [c] $p < 0.01$ in relation to DG according to Tukey's *post hoc* test.

Table 4: Effects of bacuri seed butter (25 or 50 mg/kg/day) on the atherogenic index (AI), coronary artery risk index (CRI), HDL/TC ratio, and LDL/TC ratio in hamsters (*Mesocricetus auratus*) with diet-induced hypercholesterolemia.

Groups	Parameters (mean ± SEM)			
	AI	CRI	HDL/TC ratio	LDL/TC ratio
NG	1.47 ± 0.16	2.42 ± 0.19	0.238 ± 0.01	0.578 ± 0.04
DG	2.14 ± 0.16[a]	3.14 ± 0.19[a]	0.219 ± 0.02	0.691 ± 0.07[a]
BB-25	1.4 ± 0.16[b]	2.01 ± 0.11[b]	0.285 ± 0.01[a.b]	0.576 ± 0.03[b]
BB-50	1.4 ± 0.15[b]	1.88 ± 0.09[a.b]	0.300 ± 0.01[a.b]	0.568 ± 0.01[b]

NG: normal group; DG: dyslipidemia group; BB-25: bacuri seed butter 25 mg/kg/day; BB-50: bacuri seed butter 50 mg/kg/day; AI: atherogenic index; CRI: coronary artery risk index. [a] $p < 0.05$ in relation to NG. [b] $p < 0.05$ in relation to DG according to Tukey's *post hoc* test.

Table 5: Serum levels of AST, ALT, and ALP (U/L) in hamsters (*Mesocricetus auratus*) after 28 days of treatment with bacuri seed butter (25 or 50 mg/kg/day).

Groups	Parameters (mean ± SEM)		
	AST (U/L)	ALT (U/L)	ALP (U/L)
NG	61.42 ± 5.69	73.66 ± 6.95	267.66 ± 41.97
DG	69.85 ± 14.36	65.50 ± 7.93	363.6 ± 78.83
BB-25	71.87 ± 5.11	68.16 ± 4.94	353.66 ± 53.53
BB-50	80.28 ± 6.28	83.28 ± 4.8	320.62 ± 13.25

NG: normal group; DG: dyslipidemia group; BB-25: bacuri seed butter 25 mg/kg/day; BB-50: bacuri seed butter 50 mg/kg/day; AST: aspartate transaminase; ALT: alanine aminotransferase; ALP: alkaline phosphatase. No significant differences were observed according to Tukey's *post hoc* test.

not produce systemic toxicity. Similarly, however, using a hypercholesterolemic diet composed of a standard diet plus 1% cholesterol, Martinello et al. [62] observed that high-fat intake, for 10 weeks, did not promote an increase on levels of AST and ALT in hamsters fed by a high-fat diet. On the contrary, Lai et al. [63], using a hypercholesterolemic diet composed of standard diet supplemented with 11.5% coconut oil, 11.5% corn oil, and 1% cholesterol, observed that hypercholesterolemic hamsters with dyslipidemia induced for 12 weeks presented increased levels of AST and ALT compared to the normal group. Also, in a different way, Yang et al. [64] found that hamsters fed by a high-fat diet of 94.9% of standard feed, 5% of Ching-Shan oil, and 0.1% of cholesterol showed a significant increase in serum levels of AST and ALT.

This study has a pioneering character in relation to the use of bacuri butter in dyslipidemia models, and although bacuri is a monotype fruit of Amazonian origin, the

Clusiaceae family encompasses approximately 1000 species belonging to 47 genera [65, 66], which leads to the possibility of further research being conducted with different species distributed in the most diverse parts of the world.

Bacuri butter is industrially obtained through cold pressing of the fruit seeds, and it is worth noting that, although pre- and postharvest factors can influence the composition of the fruits, the compositional analyses available in the literature show that no important nutritional variations occur as a result of these factors, although the physical-chemical characteristics may be influenced by the industrial treatment received during the process of obtaining the final product, which may originate virgin or clarified butter [67–69].

Furthermore, future research using longer clinical trial protocols will be needed for a better understanding of outcomes related to cardiovascular health in order to develop safe guidelines for an effective indication as a preventive or auxiliary agent in the treatment of dyslipidemia in humans.

4. Conclusion

Bacuri seed butter, at the doses and schedule used in this study, has positive repercussions on the lipid profile, more precisely on the fractions of HDL-c and LDL-c, and additionally promotes reduction in the risk of atherosclerosis in hamsters. Furthermore, BB did not produce deleterious effects on liver enzyme activity, weight gain, growth, body mass indices, or food intake in hamsters fed by a high-fat diet. Future directions could be the application of nanotechnologies to an improvement of functional properties of the bacuri component in the perspective of nanonutraceutical science [70].

Abbreviations

AI: Atherogenic index
AIN: American Institute of Nutrition
ALP: Alkaline phosphatase
ALT: Alanine transaminase
AOAC: Association of Official Analytical Chemists
AST: Aspartate aminotransferase
BB: Bacuri seed butter
BMI: Body mass index
CNCDs: Chronic noncommunicable diseases
CRI: Coronary artery risk index
CVD: Cardiovascular disease
DG: Dyslipidemia group
HDL-c: High-density lipoprotein cholesterol
LDL-c: Low-density lipoprotein cholesterol
NG: Normal group
TC: Total cholesterol
TEV: Total energy value
TG: Triglycerides.

Data Availability

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

Ethical Approval

This study was approved by the Ethics Committee on the Use of Animals of the Federal University of Piauí (CEUA/UFPI) (authorization no. 197/16).

Disclosure

Daniel Dias Rufino Arcanjo and Maria do Carmo de Carvalho e Martins share senior authorship.

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

Biological Functions of Diallyl Disulfide, a Garlic-Derived Natural Organic Sulfur Compound

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Garlic is widely accepted as a functional food and an excellent source of pharmacologically active ingredients. Diallyl disulfide (DADS), a major bioactive component of garlic, has several beneficial biological functions, including anti-inflammatory, antioxidant, antimicrobial, cardiovascular protective, neuroprotective, and anticancer activities. This review systematically evaluated the biological functions of DADS and discussed the underlying molecular mechanisms of these functions. We hope that this review provides guidance and insight into the current literature and enables future research and the development of DADS for intervention and treatment of multiple diseases.

1. Introduction

Plants are excellent sources of pharmacologically active ingredients. Garlic has been commonly accepted as a functional food and traditional herb for the prevention and treatment of several diseases, especially cancer and infectious diseases [1–4]. It is believed that organic sulfur compounds are responsible for most of the biological activities of garlic [5]. Diallyl disulfide (DADS; structure: two sulfur atoms with two allyl groups; see Figure 1) is a major organosulfur compound of garlic [6, 7]. Studies have shown that DADS has many biological functions, including anti-inflammatory, antioxidant, anticancer, and detoxifying effects, which may be determined by its chemical structure [4, 7–9]. Previous reviews have discussed the promising value of DADS in the prevention and treatment of a wide range of diseases [6]. In this work, we performed a systematic review of the biological functions of DADS based on the cellular and molecular mechanisms, hoping to provide an updated scientific basis and insight for future experiments.

2. Methodologies

We made a search in PubMed, Web of Science, and GeenMedical up to June 2021 for the existing literature on DADS. We also searched the International Clinical Trials Registry Platform and ClinicalTrials.gov for potentially relevant clinical trials. References of included papers and reviews were manually searched to make a supplement.

3. Biological Functions of DADS

3.1. Anti-Inflammatory Activity. Inflammation is an adaptive response of the host to adverse stimuli such as trauma, toxicity, and microbial infection. A proper inflammatory response can eliminate harmful stimuli and promote tissue healing [10]. However, uncontrolled inflammation leads to sustained damage of the tissues and organs often resulting in pathological changes to these systems [11]. Researchers have reported that DADS can inhibit inflammation in several diseases, such as enteritis, arthritis, and pancreatitis [12–14]. Fasolino et al. [15] demonstrated that edema of the mucosa

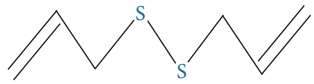

Figure 1: Chemical structure of DADS.

and submucosa was significantly reduced in the colon of rats treated with DADS. Furthermore, a low dose of DADS (between 0.3 and 10 mg/kg) was observed to suppress increases in the colon weight/colon length ratios that represent dinitrobenzene sulfonate-induced intestinal inflammation/damage. In a recent animal study, the anti-inflammatory and antioxidant effects of DADS were further confirmed using a carrageenan injection-induced acute inflammatory response mouse paw model [9].

DADS plays an essential role in inflammatory response by modulating immune cells. Hashizume et al. [16] reported that DADS could modulate the circulating number of total lymphocytes, leukocytes, and monocytes in both dose- and time-dependent manners. Immune cells usually activate intracellular signaling pathways to respond appropriately to adverse stimuli. One of the most important pathways is the nuclear factor kappa B (NF-κB) signaling pathway. A study showed that DADS attenuated the development of cerulein-induced pancreatitis and its associated lung injury in mice by suppressing the transcriptional activity of NF-κB p65 and the degradation of IκB [14], which were consistent with other study findings [17, 18]. Further research revealed that DADS inhibited glycogen synthase kinase (GSK)-3β, which suppressed the NF-κB pathway and further prevented prolonged inflammation, cellular transformation, and tissue damage [19]. DADS affected the expression of signal transducer and activator of transcription 1 (STAT 1), which could inhibit the enhancement of NF-κB signaling by binding to the target of tumor necrosis factor (TNF)-α [20]. In addition, DADS was shown to suppress the receptor activator of NF-κB ligand-induced inflammatory osteolysis by inhibiting STAT3 and NF-κB signaling both *in vitro* and *in vivo* [21].

One of the most prominent features of the inflammatory response is the release of inflammatory mediators. One study provided strong evidence that DADS could inhibit the lipopolysaccharide (LPS)-induced production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Cox-2) in RAW 264.7 cells [22], which thus led to the reduction in NO and prostaglandin E2 (PGE2) in activated cells [23–25]. Another study on LPS-stimulated neurogenic innate immune cells, BV2 microglia, also found that treatment with DADS significantly inhibited several proinflammatory cytokines and chemokines, including interleukin (IL)-1β, IL-6, TNF-α, and monocyte chemoattractant protein-1 [26]. Recent studies have further confirmed this physiological effect in animal models of neuroinflammation [27].

3.2. Antioxidant Activity. Antioxidants are substances that prevent, reduce, or repair tissue damage caused by reactive oxygen species (ROS). Over the past two decades, several studies have shown that DADS has a range of antioxidant properties [9, 28–30]. This includes a direct effect on ROS production, which was identified in an *in vitro* study demonstrating that DADS reduced deoxycholic acid-induced ROS levels in Barrett's epithelial cells when introduced within an effective concentration range [31]. Another study showed that treatment with DADS significantly reduced ROS levels in IL-1β-treated bone marrow mesenchymal stem cells [17]. However, Filomeni et al. [32] found that DADS induced oxidative stress in neuroblastoma SH-SY5Y cells, which was consistent with the findings of a study on human lung carcinoma cells [33]. This finding aligned with that of other sulfur-containing compounds from garlic, such as diallyl trisulfide (DATS), which induced the apoptosis of human breast cancer cells through ROS accumulation and inhibited it in high glucose-induced cardiomyocytes by reducing ROS production [34, 35]. The discrepancies in these studies may be attributed to the specificity of the tumor cells and differences in the therapeutic dosage of DADS employed in each study. A study on PC12 neuronal cells found that treatment with 20 μM DADS did not exert any evident effect on cell activity. However, the levels of free radicals and membrane lipid peroxidation increased significantly when these cells were treated with concentrations above 50 μM. In addition, there was an increased risk for cytotoxicity when 100 μM of DADS was administered to these cells [36]. The results of this study were similar to those observed in the neuron cell line, N18D3 [37].

Treatment with DADS can activate antioxidant enzymes, such as glutathione S-transferase (GST), catalase, heme oxygenase-1 (HO-1), and superoxide dismutase, which can convert peroxides into less toxic or harmless substances via oxidation reduction, thereby protecting a wide range of cells and tissues from ROS [30, 38, 39]. Treatment with DADS significantly increased nuclear factor-erythroid-2-related factor 2 (Nrf2) and HO-1 levels in acute ethanol-intoxicated mice, ethanol-induced human normal liver cells [40], and LPS-stimulated RAW264.7 cells [41]. Lee et al. [18] demonstrated that DADS promoted the transcription of antioxidant enzymes by dose-dependently enhancing the stability and nuclear translocation of Nrf2 in the cytoplasm. The effects of DADS on Nrf2 also exerted anti-inflammatory effects by deactivating the redox-sensitive proinflammatory NF-κB pathway [42]. In addition, DADS could restore the reduced catalase activity associated with hydrogen peroxide treatment in intestinal porcine epithelial cells [43].

3.3. Antimicrobial Activity

3.3.1. Antibacterial Activity. In recent years, antibiotic resistance has become a major health problem. Garlic is believed to be an alternative or complementary medicine for antibiotics owing to its extensive antibacterial properties [44, 45]. Studies have demonstrated that garlic extracts could weaken the formation of *Pseudomonas aeruginosa* biofilms and sensitize them to tobramycin and phagocytosis by polymorphonuclear leukocytes [46]. According to further research, DADS reduced the production of virulence factors, such as elastase, pyocyanin, and swarming motility, in *Pseudomonas aeruginosa* by blocking the inactivation of

quorum-sensing (QS) genes [47–49]. The anti-QS effect of DADS was also found to inhibit *Hafnia alvei* H4 by downregulating the expression of luxI and luxR genes [50]. However, the mechanisms by which QS regulates these functions remain unclear. DADS was reported to prevent methicillin-resistant *Staphylococcus aureus* infection in diabetic mice [51], inhibit the growth of *Escherichia coli* as an adjunct of gentamicin [52, 53], suppress the activity of *Helicobacter pylori* both *in vitro* and *in vivo* [54, 55], and reduce the pathogenicity of common microorganisms isolated from ear infections [56]. In addition, several recent studies have demonstrated that DADS could modulate the gut microbiota. H_2S gas released after treatment with DADS prevented or reversed the naproxen-induced changes in the composition of the intestinal microbiota [57]. According to an *in vivo* study, when a low dose of DADS was added to the normal diet of mice, the bacterial level of *Bacteroides* in their intestinal tract decreased, while that of *Firmicutes* increased [58].

3.3.2. Antifungal Activity. To evaluate the antifungal effect of DADS, Alam et al. [59] administered DADS to mice infected with *Candida albicans*. They found that the niosomal formulation of DADS markedly decreased the secretion of protease and phospholipase from *Candida albicans* and increased the survival of the infected animals. DADS was also reported to inhibit the growth of *Aspergillus versicolor* and its toxic metabolites [60].

3.3.3. Antiviral Activity. The antiviral effect of DADS was first reported in 1993. In this research, DADS inhibited the proliferation of HIV-1-infected cells [61]. In addition, DADS exerted anti-inflammatory and antioxidant effects in a dengue virus study, reducing the symptoms and severity of the disease [62]. In recent reports, garlic has been recommended as a potential medicine for COVID-19 based on the findings of several preclinical and clinical studies [63, 64]. In the molecular docking test, garlic essential oil also showed a good inhibitory effect on SARS-CoV-2 [65, 66]. However, it is still unclear whether DADS plays a role in the anti-SARS-CoV-2 effect of garlic, and further studies are needed.

3.4. Detoxification. Numerous studies have shown that DADS can protect organs from the harmful effects of several chemical compounds [4]. For instance, DADS can reduce the hearing loss caused by aminoglycoside drugs [67], attenuate the side effects of gentamicin and cisplatin [67–69], positively affect carbon-tetrachloride-induced hepatic damage [18, 70], relieve haemorrhagic cystitis induced by cyclophosphamide in rats [71, 72], decrease cyclophosphamide-induced developmental toxicity [73], and greatly alleviate the methotrexate-induced decline in kidney function and subsequent kidney damage [74].

DADS can promote detoxification of the body, which is believed to be related to the activation of antioxidant enzymes and phase II enzymes via the Nrf2/ARE pathway. DADS was found to significantly boost the activities of phase II enzymes, including GST, quinone reductase, microsomal epoxide hydrolase, and UDP-glucuronosyltransferase in the liver, intestine, kidney, and lungs [75, 76]. DADS also upregulated the expression of the pi class of GST through JNK/AP-1 and ERK/AP-1 signaling pathways. GST is known to combine with electrophilic compounds in cells to cause detoxification [77].

It has been reported that DADS primarily suppresses the carcinogenic effects of chemical compounds via two mechanisms: the modulation of cytochrome P450 (CYP)-dependent monooxygenase to inhibit carcinogen activation and the induction of phase II enzymes to accelerate carcinogen degradation. DADS exerted its anticarcinogenic effect by inhibiting CYP2E1 levels in humans, and CYP2A3 and CYP2A3 levels in rats induced by methyl-n-pentylnitrosamine [78]. Based on animal studies, the administration of DADS to rats through gastric intubation reduced the amount of liver CYP2E1 protein by 25% [79]. Furthermore, treatment with DADS was found to induce the activation of phase II enzymes by protecting Nrf2 from proteasomal degradation of Keap1 and promoting Nrf2 nuclear accumulation, thereby inhibiting the occurrence of chemical-induced papilloma in mice [80]. A study showed that DADS inhibited cell proliferation, G2/M arrest, H_2O_2 formation, and DNA damage induced by ben-zo[a]pyrene, thereby inhibiting the occurrence of breast cancer [81]. DADS also inhibited the expression of serotonin N-acetyltransferase genes and proteins, leading to the reduction of N-acetyl-2-aminofluorene-DNA adducts, which could reduce the risk of cancer associated with exposure to environmental carcinogens [82, 83].

3.5. Cardiovascular Protection. The intake of garlic can effectively reduce the risk factors associated with cardiovascular diseases [3, 84, 85]. Based on existing research, DADS plays a critical role in the cardiovascular protective effect exhibited by garlic, by acting as an angiogenesis inhibitor. Exposure to DADS significantly inhibited the angiogenic differentiation of endothelial cells by reducing the activation of matrix metalloproteinases (MMPs) and the secretion of tissue inhibitor of metalloproteinase-1 in endothelial morphogenesis [86, 87]. DADS was also found to effectively downregulate both the transcription and expression of vascular endothelial growth factors in HL-60 cells in time- and dose-dependent manners [88, 89]. Increasing evidence suggests that both connexins and gap junctions are involved in cardiovascular diseases [90]. DADS was observed to improve rat liver epithelial cell gap-junctional intercellular communication, regulate vascular smooth muscle cell proliferation, and significantly increase connexin 43 expression, which is very important for maintaining normal vascular function [91, 92]. Furthermore, DADS is an effective agent against atherosclerosis as it can protect low-density lipoprotein (LDL) from oxidation and glycation [93, 94]. DADS also protected endothelial cells from oxidized LDL (ox-LDL) damage by reversing the inactivation of endothelial NOS (eNOS) by ox-LDL [95].

DADS induces vasodilation by activating perivascular sensory nerve endings [96]. A recent study found that DADS strongly inhibited angiotensin-converting enzyme, upregulated the expression of prostacyclin and Cox-2 in SVEC4-10 cells, and reduced the level of ROS, thereby playing a role in vasodilation [22]. According to reports, DADS could downregulate intercellular adhesion molecule-1 and MMP-9 and block the inactivation of eNOS [95, 97], which has been demonstrated to relieve pulmonary hypertension [98]. Of note, impaired endogenous H_2S production may be one of the mechanisms underlying hypertension. As DADS is an H_2S-releasing agent, it could be considered a promising drug for the treatment of cardiovascular disease [99].

Various studies have suggested that DADS protects the heart, and treatment with DADS was found to improve cardiac dysfunction by inhibiting death receptor-dependent and mitochondrial-dependent apoptotic pathways and enhancing the PI3K/Akt pathway in diabetic rats [100]. Furthermore, DADS ameliorated myocardial hypertrophy by enhancing the biogenesis and biological function of mitochondria in the rat heart [101]. The mitochondrial lipid peroxidation product, tans-crotonaldehyde, is known to cause myocardial ischemia by damaging mitochondrial genes [102]. However, DADS eliminated the toxic effect of trans-crotonaldehyde by interaction with its –C=C–C– and –CH=O groups [103].

3.6. Neuroprotection. Garlic and garlic extracts are believed to provide therapeutic benefits in neurological disorders owing to their antioxidant, anti-inflammatory, and neuroprotective effects [3, 104]. A recent study found that DADS (40 or 80 mg/kg) effectively improved LPS-induced depression-like behaviours in mice, with treatment effects comparable to those of imipramine (10 mg/kg), a clinical antidepressant [105]. However, in young mice, especially during the neural growth stages, high doses of DADS may adversely affect hippocampal neurogenesis, the proliferation of neural progenitor cells, and neurocognitive functions by regulating ERK and brain-derived neurotrophic factor (BDNF)/cAMP response element-binding protein (CREB) signaling, resulting in significant memory deficits [106]. Besides, another recent animal study has shown that DADS played a role in the inhibition of neuropathic pain via the H_2S/BDNF/Nrf2 pathway [107].

Several previous studies have suggested that DADS may be an effective drug for the treatment of neurodegenerative disorders, such as Alzheimer's disease (AD). Animal studies have shown that DADS could ameliorate the learning and memory of AD mouse models by increasing the number of hippocampal dendritic spines and synapses [108]. DADS derivatives, 7k and 7l, inhibited Aβ-induced neuronal cell death and reverse scopolamine-induced cognitive impairment in rats via their antioxidative and metal-chelating effects [109, 110]. Moreover, DADS exerted anti-amyloidogenic and anti-inflammatory effects and inhibited conformational alteration in tau protein induced by phosphorylation via the GSK-3β pathway [111]. A clinical trial found that the severity of some neurodegenerative diseases, such as AD, was associated with H_2S levels. Therefore, as DADS is an H_2S donor; it may play a role in the treatment of AD [112].

3.7. Anticancer Activity

3.7.1. Inhibition of Invasion and Migration. The inhibitory effect of DADS on cancer cell movement and invasiveness is identified to be linked to the enhancement of tight junctions and the decrease in MMPs activity [113, 114]. Increases in transepithelial electrical resistance confirmed that DADS enhances the tight junctions of human prostate cancer cells [113]. DADS was found to block the migration and invasion of human colon cancer 205 cells by inhibiting the expression of MMP-9, MMP-2, and MMP-7 [115]. Additional evaluations revealed that the effect of DADS on MMPs was regulated through the NF-κB and PI3K/Akt pathways [116]. Previous studies had shown that DADS could reduce TNF-α-induced C–C motif chemokine ligand 2 release, thereby blocking monocyte recruitment and inhibiting malignant tumor invasion [117, 118].

The prevention of epithelial-mesenchymal transition (EMT) is a new hotspot in tumor metastasis research. Inhibiting Ras-related C3 botulinum toxin substrate (Rac)-1 and β-catenin expression can inhibit EMT in tumor cells [119]. According to studies by Su et al. [120], DADS suppressed the activities of Rac1, β-catenin, p21 activated kinase-1, and Rho kinase-1, leading to the inhibition of gastric tumor cell growth, invasion, and metastasis. Furthermore, DADS regulated MMP-9 expression and reversed EMT by inhibiting the β-catenin pathway to reduce breast cancer cell metastasis [121]. Inhibition of the LIMK1-cofilin1 pathway by DADS also inhibited EMT, migration, and invasion of gastric cancer cells, which are closely associated with the formation of invasive pseudopods [122]. Notably, these findings were also confirmed using colon cancer cells [123, 124]. Fibronectin, an extracellular matrix component, also causes EMT in tumors. However, treatment with DADS has been reported to reverse the EMT induced by fibronectin in tumors [125]. The deglycase-1 (DJ-1) protein is another promising target for cancer therapy owing to its roles in invasion, migration, and chemoresistance, and several reports have suggested that inhibition of Src phosphorylation by DADS could downregulate DJ-1 expression, thereby inhibiting leukemic cell migration and invasion [126].

3.7.2. Regulation of Cell-Cycle Arrest. DADS was found to inhibit the proliferation of tumor cells partly because of its ability to reduce the cell ratio in the G1 phase and increase the cell ratio in the G2/M phase [127]. During treatment with DADS, the proportion of G2/M cells increased with increasing concentration and exposure time. Further molecular analysis indicated that the reduced level of cyclin B1, cell division cycle (cdc) 25C, cdc2, and phosphorylated-cdc2 proteins may have contributed to the blockage of the G2/M phase in DADS-treated esophageal squamous cell carcinoma cells [128]. Studies have shown that DADS increased the mRNA and protein levels of p21 and p53 in carcinoma cells

and activated the p53/p21 signaling pathway, thereby inducing cell-cycle arrest and cell apoptosis [128, 129]. A previous study indicated that the ability of DADS to block the cell cycle was also associated with histone acetylation [130]. Further research demonstrated that DADS induced an increase in histone H3 and H4 acetylation in the CDKN1A promoter, ultimately leading to an increase in CDKN1A gene expression and $p21^{WAF1}$ protein levels [131]. Moreover, DADS resisted the activation of the G2/M gene damage checkpoints by relying on Mec1 (ATR) and Tel1 (ATM) to inhibit DNA repair, which could improve the efficacy of DNA damage-based cancer therapies [132].

Some garlic extracts, including DADS, exert antimitotic effects by impairing microtubules and hindering the assembly of mitotic spindles. Aquilano et al. [133] reported the obvious loss of the microtubule network, with an irregular accumulation of soluble β-tubulin and reduction of the cytoskeletal counterpart in neuroblastoma SH-SY5Y treated with DADS. In addition, DADS-derived superoxide was observed to actively participate in the oxidation of actin and tubulin, which eventually led to the breaking of the microfilaments and microtubules.

3.7.3. Induction of Apoptosis and Autophagy. Inducing apoptosis in cancer cells is the main anticancer mechanism employed by most chemotherapeutic drugs [134]. DADS-induced apoptosis was observed to be accompanied by an increase in Ca^{2+} levels and a decrease in mitochondrial membrane potential. Increased Ca^{2+} led to the activation of caspase-3 and the release of cytochrome C from the mitochondria, resulting in proteolysis and apoptosis [135]. In addition to caspase-3, caspase-9 and caspase-10 were also activated by DADS [136]. Exposure to DADS increased the expression of p53, p38, and p21; decreased the level of antiapoptotic protein, Bcl-2; and upregulated the levels of the proapoptotic proteins, Bax and Bad [127, 137–139]. Moreover, inhibition of histone deacetylation and the ERK pathway and activation of the SAPK/JNK pathway were also found to influence the proapoptotic effect of DADS in human breast cancer [140]. Several studies have also revealed that treatment with DADS could lead to an increase in ROS levels, resulting in the apoptosis of human leukemia HL-60 cells [141, 142]. However, cells with an ROS buffer system, such as adenocarcinoma gastric cells (rich in glutathione peroxidase) or copper-overexpressing neuroblastoma cells, were shown to be resistant to DADS treatment [143, 144]. One animal experiment showed that pretreatment with 10 μM DADS resulted in an increase in the radiation sensitivity of HeLa cells and significantly promoted radiation-induced apoptosis. Such findings indicated that DADS is a potential radiosensitive agent for human cervical cancer [145].

In addition to inducing apoptosis, some chemotherapeutic drugs induce autophagy, which is another cell death pathway. Studies have reported that exposure to DADS significantly increased the autophagic flux of RAW264.7 cells, and the effects of DADS on autophagy were likely the result of inhibition of the phosphorylation of mTOR and P70S6k/S6K1 [146]. DADS-induced autophagy increases the death of tumor cells, including leukemia and osteosarcoma cells, by inhibiting the PI3K/Akt/mTOR signaling [147–149]. It has been suggested that histone deacetylase (HDAC) inhibitors also play an antitumor role through autophagy [150]; thus, the inhibitory effect of DADS on HDAC activity may partially induce autophagy. However, there is a paucity of research in the field of DADS-induced autophagy, and more investigations are warranted.

3.7.4. Induction of Cell Differentiation. DADS-induced differentiation of human leukemia HL-60 cells was found to be related to the decrease in DJ-1 and calreticulin (CRT) contents [151]. DJ-1 has been reported to play a role in cell differentiation by acting as a cofactor-binding protein or transcription factor [152]. DADS significantly decreased the expression of cluster of differentiation 33 (CD33) and increased the expression of CD11b by downregulating CRT, ultimately inducing the differentiation of human leukemia HL-60 cells [153]. Furthermore, the DADS-induced reduction of CRT could upregulate the mRNA expression of CCAAT enhancer-binding protein-α, thereby affecting cell differentiation [154]. Moreover, treatment with DADS was found to increase the acetylation level of core nucleosome histones (H3 and H4) and accelerate the differentiation of human leukemia cells [155, 156] and liver cancer cells [157].

3.7.5. Effect on Epigenetics. The blocking of normal histone acetylation or abnormal histone acetylation is believed to be the root cause of several cancers. DADS was found to enhance the acetylation of histones H3 and H4 in normal colon cells both *in vitro* and *in vivo* [158]. Druesne et al. [159] reported that treatment with DADS alone increased the transient acetylation of histone H3K14 in human colon tumor cells. However, unlike in normal colon cells, DADS had no effect on histone H4 acetylation in colon tumor cells, regardless of the cell culture conditions. Furthermore, DADS induced an increase in histone acetylation of the CDKN1A promoter, which in turn led to an increased level of the $p21^{WAF1}$ protein; this process is known to inhibit tumor proliferation and induce G2/M phase arrest and apoptosis [160]. Notably, these effects of DADS were only observed at high concentrations. Further research is thus needed to confirm whether the HDAC inhibitory effect of DADS can result in primary anticancer effects when normal human diet doses are administered.

3.8. Regulation of Metabolism

3.8.1. Regulation of Glycose Metabolism. Several *in vivo* studies have shown a dose-dependent increase in blood glucose concentration and free fatty acid levels in rats treated with DADS. Such findings suggest that DADS affects glucose metabolism [161]. However, another study reported that garlic oil, rather than DADS, had beneficial effects on glycemic control in streptozotocin-induced diabetic rats [162]. As a result, the specific effects of DADS on glucose metabolism, under both healthy and diabetic conditions, need to

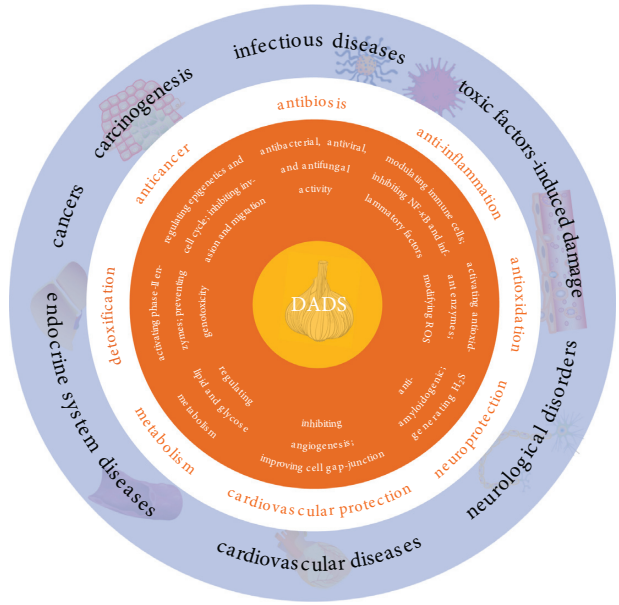

Figure 2: Overview of the biological functions of DADS.

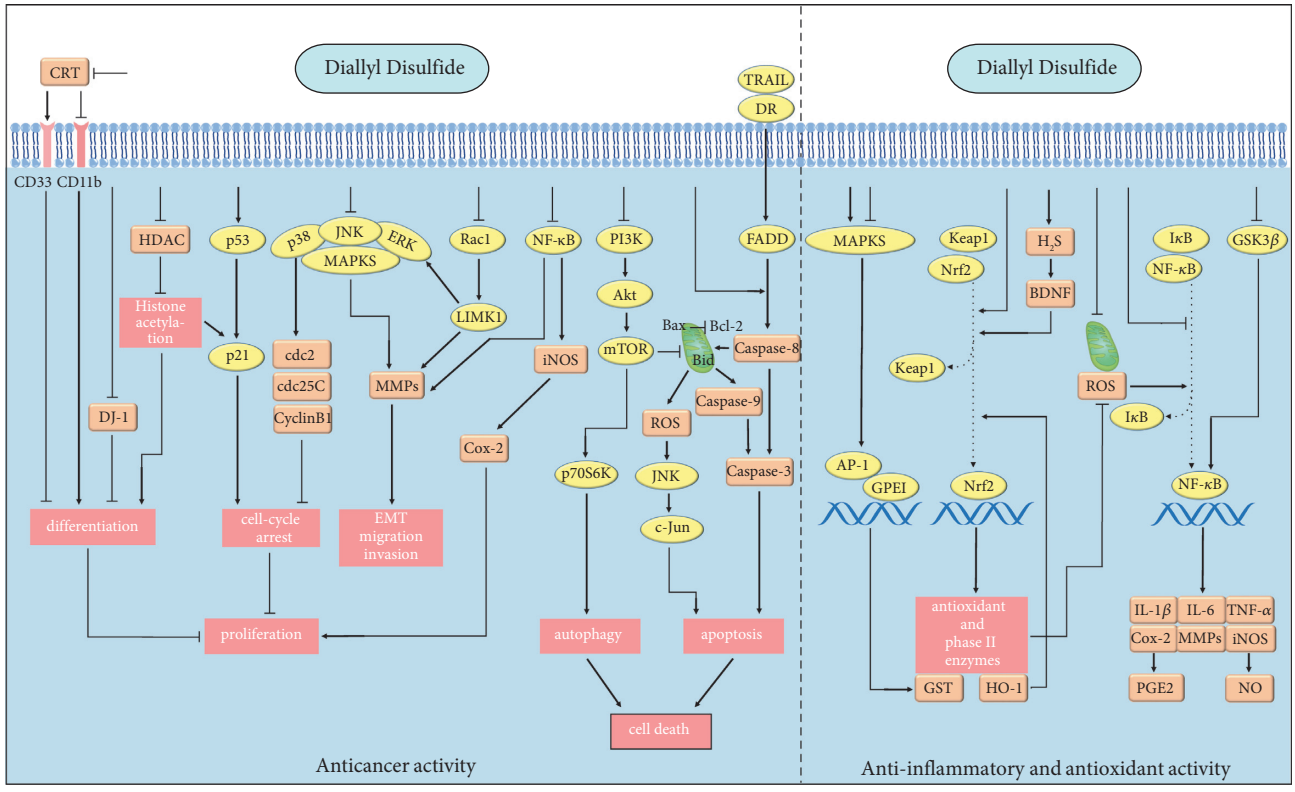

Figure 3: The signaling pathways affected by DADS.

be further elucidated. In addition, DADS was observed to suppress glucose metabolism of breast cancer stem cells by inhibiting the CD44/pyruvate kinase M2/AMPK pathway [163].

3.8.2. Regulation of Lipid Metabolism. According to reports, DADS may regulate lipid metabolism by: (a) regulating sterol regulatory element-binding protein-1c, apolipoprotein A1, CREB-H, and fibroblast growth factor 21; (b) preventing lipotoxicity by increasing peroxisome proliferator-activated receptor-α and inhibiting stearyl coenzyme A desaturase enzyme-1; and (c) significantly inhibiting lipid peroxidation by regulating malondialdehyde and superoxide dismutase [164–166]. Additional studies have reported that the lipid metabolism-regulating activity of DADS may have significant hepatoprotective effects [3]. Additionally, DADS could inhibit

the accumulation or activation of mesenteric adipose tissue macrophages and the release of monocyte chemoattractant protein-1, suppressing the inflammatory response induced by obesity [167].

3.9. Other Effects. Oral administration of DADS increased the activity of the natural antibody in broiler serum [168]. Moreover, DADS induced chromosome aberration and sister chromatid exchange in the Chinese hamster ovary [169]. DADS could also change iron homeostasis by regulating the expression of ferritin and transferrin receptor genes in hepatocytes *in vitro* and *in vivo* [170] (see Figure 2).

4. Conclusions and Prospects

DADS, a natural organic sulfur compound, is commonly used as a food additive. Current research suggests that DADS is a promising drug agent for the prevention and treatment of several diseases. This review sought to systematically identify the biological functions of DADS and summarize the underlying molecular mechanisms employed by this compound. The biological functions of DADS can be divided into two categories: the protective effects on normal tissues and the inhibitory effect on disease status. The anti-inflammatory and antioxidant effects of DADS are the basis for maintaining tissue homeostasis (such as neurovascular protection and metabolic regulation) and fighting infections (antibiosis). There are interlinks between the anti-inflammatory and antioxidant effects, with NF-κB and ROS signaling playing key roles. DADS alters the biological properties of cancer cells via specific intracellular and intercellular mechanisms. As a result, DADS exerts significant anticancer effects, such as inducing apoptosis, autophagy, and differentiation. In addition, DADS can also improve efficacy and reduce the negative effects of chemotherapy drugs.

Anti-inflammatory and antioxidant signaling mediators, such as NF-κB, TNF-α, ROS, Nrf2, AP-1, JNK, and STAT, play important roles in the biological functions of DADS. Apoptosis and autophagy-associated pathways, such as PI3K, Akt, mTOR, MAPKs, Bcl-2, and Bax, also contribute to the anticancer action of DADS. Notably, the signaling pathways affected by DADS are similar between normal tissue cells and cancer cells. However, different dosages and methods of administration may produce different effects, which requires more experiments to fully verify (see Figure 3).

There are some clinical trials focusing on garlic and its biological effects, including anticancer, anti-inflammatory, antioxidant, and antiviral activities [64, 171–173]. These clinical studies have shown that garlic can be used as an adjunct in the management of several diseases, but with limited effects. Further clinical trials on solitary compounds are necessary to identify the specific active ingredients and thus enhance their medicinal value. Although animal and *in vitro* experiments have shown that DADS has comparable biological activity with garlic, clinical trials of DADS have not yet been conducted. Therefore, it is still unclear whether DADS is an active ingredient in the use of garlic in humans and how it exerts its effect. It should be noted that there are some non-negligible issues that need to be solved before conducting clinical trials of DADS. The first is that DADS is rapidly metabolized after being taken into the body and has low bioavailability [6]. The second is the technical difficulties of processing DADS, such as characterization, optimization, and the production of suitable delivery systems [174, 175]. The most important issue is the pharmacokinetic studies of DADS, and its metabolites should be refined. Currently, countries are probing different strategies to prevent and treat COVID-19, which has had a negative impact on global public health and economies. Readily available natural plant products could be a promising starting point for the discovery of new therapeutic drugs. Research on the biological function of DADS may bring us new hope.

Conflicts of Interest

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

Authors' Contributions

Xiuxiu Song and Ziqi Yue contributed equally to this work.

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Research Article

Effect of Microwave Roasting and Extraction Solvents on the Bioactive Properties of Coffee Beans

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Coffee is an intricate mixture of thousands of chemical compounds that are accountable for its flavor and aroma. Roasting is a key step in the processing of coffee beans. This study assessed the effect of microwave roasting (MW) and extraction solvents (ES) on the total polyphenol content, total flavonoid content, and antioxidant activity of coffee beans. The untreated and microwave-roasted (MR) coffee beans showed a total polyphenol content of 40.40 and 35.15 mg GAE/gm DW, respectively, when methanol was used as the solvent for extraction. Similarly, for the untreated coffee beans, the methanol extracted coffee had a significantly ($p < 0.05$) higher total flavonoid content (39.34 mg CE/g DW) as compared to ethanol (34.82 mg CE/g DW). The obtained IC_{50} for the untreated and microwave-roasted samples as extracted by methanol were 4.13 and 5.68 mg/mL, respectively, while the IC_{50} values of untreated and microwave-roasted samples extracted by ethanol were 4.59 and 6.24 mg/mL, respectively. Untreated coffee beans exhibited a higher reducing power (1.237) than that of the microwave-roasted ones (0.839) when extracted with methanol. Chlorogenic acid was the major (2.31–2.68%) phenolic compound found in all the coffee samples whether it was untreated or microwave-roasted. Vanillin demonstrated the lowest (0.118–0.166%) phenolic compound found in the coffee bean samples. These results might be helpful for obtaining the maximum health benefits from coffee.

1. Introduction

Plant-derived foods have been used since the dawn of humankind for promoting health and preventing diseases. People from different cultures worldwide have revered coffee not only for its aromatic compounds but also because of its stimulating and health-promoting properties [1]. In addition to being the world's most highly consumed beverage, coffee has high nutritional value [2]. Many countries grow coffee as a primary crop as well as a valuable commodity [3–5]. Even though studies have reported inconsistent results in connection with coffee consumption, the general consensus is that regular, moderate coffee consumption by healthy individuals is either benign or slightly beneficial [6–10]. Coffee health benefits include reduction in the risk of metabolic syndrome, and protection against noncommunicable diseases such as liver disease, diabetes, cancer, and Parkinson's disease [11–13]. Polyphenols are abundant micronutrients in our diets, and evidence for their role in the prevention of noncommunicable diseases is growing. The bioavailability of polyphenols in coffee differs from one type to another [14]. High amounts of antioxidants and bioactive compounds are found in coffee, and it is the major source of chlorogenic acid [15]. Intake of chlorogenic acid varies widely but may be very

high, up to 800 mg/d among coffee drinkers [16]. A single cup of Arabica coffee contains around 70 to 200 mg of chlorogenic acid [17]. Coffee contains a large number of bioactive compounds which are presented in Table 1 [18–20].

Raw coffee beans undergo a chemical transformation during roasting and various factors can influence the biochemical composition of the end product. These factors include but are not limited to the type of beans, method of preparation and degree of roasting [21,22]. The coffee roasting process creates a special aroma and taste [23]. In addition, aroma and flavor are affected by temperature and time and the roasting parameters of coffee [18]. Longer roasting of coffee influences the level of bioactive compounds [24]. In a study by Alkaltham, the total phenol content of green coffee beans was reduced by 13.59% and 16.66% on microwave and oven roasting respectively [25]. There is an inverse relationship between the degree of roasting and antioxidant content [26]. Thus, the coffee roasting process is a key factor for maintaining nutritional value [18]. The phytochemicals in the food matrix have the property of being soluble in specific solvents, although, there is no universally accepted procedure available for measuring the antioxidant and phenolic contents of food. This in turn increases the need for careful selection of the extracting solvent [27,28]. Coffee extraction is a process, which states to dissolving the soluble components of coffee beans powder in a liquid solvent. In a study on comparing the results from water and methanolic extracts of coffee from different countries, the highest amount of phenolics, caffeine, reducing power, ability to chelate Fe2+, inhibition of linoleic acid peroxidation, and inhibition of lipoxygenase was determined for a methanolic extract of coffee [29]. An efficient method of preserving the bioactive compounds is needed [30]. Solid-liquid extraction with different solvents or solid-phase extraction (SPE) followed by high-performance liquid chromatography (HPLC) for the determination of phenolic compounds is reported [31]. HPLC is commonly used for the qualitative and quantitative determination of phenolic compounds in coffee beans and HPLC based methods use mainly C18 with 5 μm particle size packing materials as the stationary phase [32]. A lot of studies have been conducted on coffee; thus, data on the effects of microwave roasting with different extraction solvents on the bioactivity of coffee grown in Saudi Arabia is hardly available. As a result, this study focused on analyzing the effect of microwave roasting (MW) and extracting solvents (ES) on the bioactivity of coffee beans. Therefore, the total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant activity (AA) in terms of DPPH (2, 2-diphenyl-1-picryl-hydrazyl), reducing power, and identification of phenolic compounds will be detected.

Table 1: Composition of bioactive compounds of roasted coffee bean [18–20].

Bioactive compound	Traditional roasted
Caffeine	526 ± 1.97
Gallic	117 ± 0.57
Chlorogenic	600 ± 1.83
Quercetin	7 ± 0.03
Kaempferol	6 ± 0.04
Caffeic	5 ± 0.07
Salicylic	12.1 ± 0.16
Epigallocatechin gallate	28 ± 0.19
Quercetin-3-O-glucoside	2.8 ± 0.1
Kaempferol-3-O-glucoside	35 ± 0.15
Caffeoylquinic acid	3530 ± 0.02
Caffeine	2840 ± 0.00
Melanoidins	2380 ± 0.77
Trigonelline	1000 ± 0.00
Redutores	100 ± 0.00
Cafestol	742 ± 41
Nicotinic acid	19 ± 3
Kahweol	465 ± 18

*Results are expressed in means ± standard deviation and given in mg/100 g of sample.

2. Materials and Methods

2.1. Materials. *Coffea arabica* was obtained from the Jazan region located in the Kingdom of Saudi Arabia. The coffee beans were sun-dried until their moisture level reached 11.2% of the dry weight. The samples were then ground and passed through a 60-mesh (250 μm) sieve. Coffee bean powder (4 g) was heated using a microwave oven at 720 W for 6 min. An unheated sample was used as a control.

2.2. Extraction of the Samples. Two grams of coffee bean powder was extracted with 20 mL of either 50% methanol or 50% ethanol using an ultrasonic bath at (20°C) for 45 min. Afterward, the sample mixture was centrifuged for 10 minutes at 3000 rpm and the temperature of the machine was set at 20°C. Finally, the obtained supernatant was filtered through filter paper (Whatman filter paper No. 2). The coffee bean extract (CBE) was maintained at 4°C and used for antioxidant assays. Steps followed in preparation of coffee bean extract are presented in Figure 1.

2.3. Total Polyphenol Content. The method suggested by Hayat [33] was used to analyze TPC. In brief, 25 μL of CBE was mixed with 1500 μL of water. After this, 125 μL undiluted Folin-Ciocalteu reagent was added to the mixture. After 1 min, 375 μL of 20% sodium carbonate and 475 μL of water were added and the mixture was incubated for 30 min at 23°C. Finally, the absorbance of the mixtures was read at 760 nm and the result was represented as mg gallic acid equivalent per gram dry matter (mg GAE/g DW).

2.4. Total Flavonoid Content. The procedure used by Hayat [33] was followed for analyzing TPC in CBE. Two hundred and fifty μL of CBE was mixed with 1000 μL of water, and then 75 μL of both NaNO2 and AlCl3 was added. The mixture was held for 5 min at 23°C, and then 500 μL of 1 M NaOH and 600 μL of water were added. The absorbance of the solution was recorded at 510 nm. The result was represented as mg catechin equivalents per gram dried extract (mg CE/g DW).

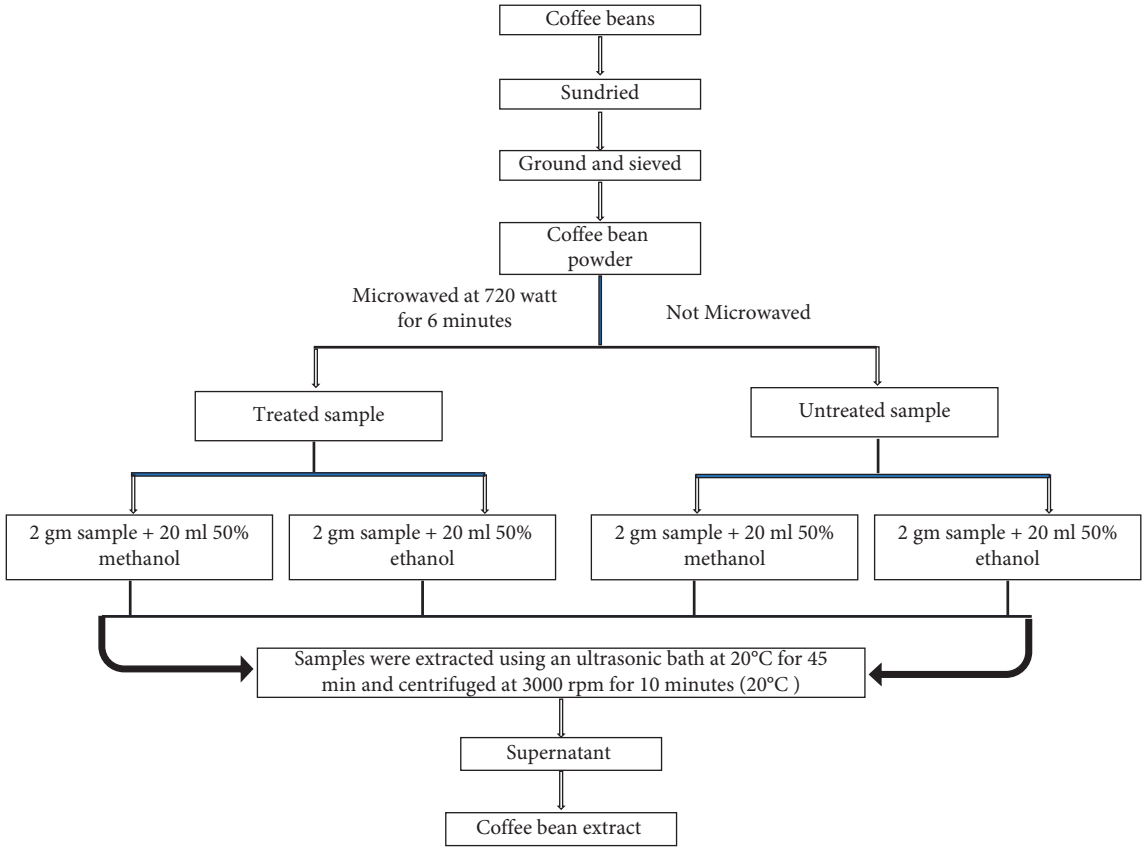

Figure 1: Schematic representation of preparation of coffee bean extract.

2.5. DPPH Scavenging. The free radical scavenging capacity of the CBE was determined with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution as described by Noreen et al.[34] with slight modification. Briefly, an aliquot of extract (130 μL) and 0.1 mM DPPH solution was mixed thoroughly and allowed to stand in a dark place for 30 min. Afterward, the absorbance of the sample and control was recorded at 510 nm. The control was prepared in the same manner except that methanol was used instead of the coffee extract. Methanol was used as a blank. The DPPH scavenging percentage was measured as follows:

$$\text{DPPH scavenging \%} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100. \quad (1)$$

The outcome was illustrated as 50% inhibitory concentration (IC_{50}) of the CBE.

2.6. Reducing Power. The ferric reducing power of CBE was estimated according to the method of Hayat et al. [35]. Coffee bean extract (0.5 mL) was mixed thoroughly with 1.25 mL buffer (0.2 M, pH 6.6) and 1.25 mL of potassium ferricyanide and incubated for 20 min at 50°C. Then, 1.25 mL of trichloroacetic acid (TCA) was added. Afterward, the CBE mixture was centrifuged for 10 min (at room temperature) at 3000 ×g. An aliquot (1.25 mL) was taken from the supernatant, to which 1.25 mL water and 0.25 mL of ferric chloride were added, respectively. In the end, the absorbance of the sample was measured at 700 nm.

2.7. HPLC Analysis of Phenolic Compounds. In the current study, utilizing HPLC with the method described previously [36], the phenolic (chlorogenic acid, gallic acid, vanillin, salicylic acid, and caffeic acid) compounds in CBE were quantified. In HPLC system Shimadzu, prominence (Kyoto, Japan) equipped with an LC-20AB binary pump, variable Shimadzu SPD-10A UV-Vis detector was used. The column used was Zorbax SB-C18 (250 × 4.6 mm, 5 μm) (Agilent, Santa Clara, CA, USA) and the mobile was (0.1% formic acid, A) and MeOH (0.1% formic acid, B). The gradient program was the following: 0 min 5% B; 4 min 5%B; 20 min 73% B; 50 min 95% B; 57 min 1% B; 58 min 1% B; 60 min 5% B; at a low rate of 0.7 mL/min. The injection volume was 10 μL, and the detector was set at 280 nm. Compounds were identified by comparing their retention time with those of the standard (Figure 2). All samples were analyzed in duplicate.

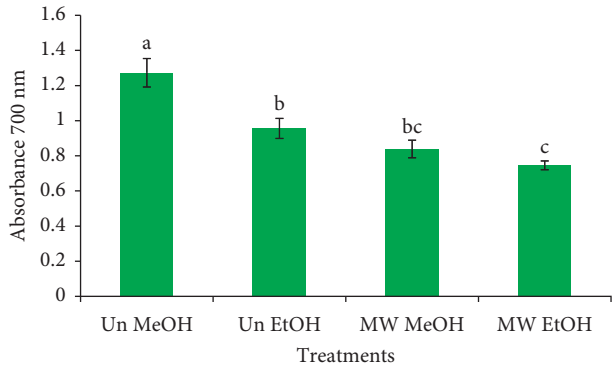

Figure 2: Impact of microwave roasting and extraction solvents on the reducing power of coffee beans. Bars with different small letters are significantly different from one another ($p < 0.05$). Un MeOH: untreated sample extracted with 50% (v/v) methanol, Un EtOH: untreated sample extracted with 50% (v/v) ethanol, MW MeOH: microwave-roasted sample extracted with 50% (v/v) methanol, and MW EtOH: microwave-roasted sample extracted with 50% (v/v) ethanol.

2.8. Statistical Analysis. Each test was performed in triplicate. The results are illustrated as the mean ± SD (standard deviation). Using SAS statistical software (version 9.2, 2000–2008; SAS Institute Inc., Cary, NC, USA), one-way analysis of variance (ANOVA) was applied to groups, and Duncan's multiple range tests were used to calculate significant differences among the parameters.

3. Results and Discussion

3.1. Effect of Microwave Roasting (MW) and Extracting Solvents (ES) on the Total Polyphenols of Coffee Bean Extracts. Figure 3 depicts the effect of MW and ES (ethanol and methanol) on the TPC of untreated (raw) and microwave-roasted coffee beans. In the current study, it was found that the untreated CBE exhibited higher TPC as compared with that of microwave-roasted coffee beans as extracted with both methanol and ethanol. It was found that in the coffee beans extracted with methanol, the TPC of the untreated sample was 40.40 mg GAE/g DW, which was higher than the TPC found in the MW sample (35.15 mg GAE/g DW). Previous studies on the impact of roasting on TPC and antioxidant activity have reported inconsistent findings. In accordance with the current report, another study demonstrated that green coffees (Arabica and Robusta) had higher TPC and possessed enhanced antioxidant activity compounds in comparison to roasted coffees [37]. These results are also in accordance with the findings of Nebesny and Budryn who found that green coffee exhibited a higher antioxidant activity as compared to the conventional and microwave-roasted samples [38]. The degradation, polymerization, and auto-oxidation of the phenolic compounds during roasting process might be the cause of a decrease in their content [39]. In another study, in contradiction of the above mentioned studies, statistically insignificant differences were reported between the TPC of raw and roasted coffee beans [40]. However, during the process of roasting, Divišet al.[41] reported an upsurge in the TPC of green coffee beans, and Król et al.[19] reported that the highest TPC was determined in coffees roasted under light and medium roasting conditions.

When the extracting solvent was taken into account, it was found that compared to ethanol, the other solvent (methanol) extracted a significantly higher content ($p < 0.05$) of polyphenols from both the untreated and MW coffee beans. The TPC of untreated coffee beans extracted by methanol and ethanol was measured as 40.40 and 36.92 mg GAE/g DW, respectively. The outcomes of this study are in accordance with the findings of Jaiswal et al.[42] who reported the highest TPC in methanolic extract compared to others, that is, water, acetone, and ethanol extracts.

3.2. Effect of Microwave Roasting (MW) and Extraction Solvents (ES) on the Total Flavonoid Content of Coffee Beans. The TFC of CBE is shown in Figure 4. The MW, as well as ES, showed a similar trend for the TFC of coffee beans as was that for the TPC. Untreated coffee beans and methanol as an ES showed a higher TFC as compared to MW coffee beans and ethanol, respectively. The TFCs of untreated and microwave-roasted coffee beans extracted with ethanol were found as 34.82 and 25.59 mg CE/g DW, respectively. When comparing the results of this study to the findings of Ghafoor et al.[43], it was found that roasting has similar effects on other plant materials as he reported a decrease in the TFC of poppy seeds and oil upon roasting in the microwave oven at 720 W for 5 min. In contrast, Al-Juhaimi et al. [44] indicated that the TFC of apricot kernels was increased by roasting them at 320 W, 540 W, but decreased when the microwave power was increased to 720 W. Microwave heating breaks open the cell walls of the plant materials, allowing phytochemicals to be released more easily, and increasing the availability of bioactive materials [45].

Methanol extracted resulted in a significantly ($p < 0.05$) higher total flavonoid content when compared to ethanol. For instance, the TFC of untreated coffee beans as extracted by methanol and ethanol was 39.34 and 34.82 mg CE/g DW, respectively. In an earlier study, extraction was done with acetone, water, ethanol, and methanol and highest level of TFC was obtained using methanol as an ES, which corroborates well with the findings of the present study [42]. Polarity is responsible for the extraction of phenolic compounds from solvents. Alkalthamet al. reported higher TPC and TFC in coffee beans and pulp samples extracted with methanol as compared to ethyl acetate [46]. Similarly, in another study conducted on green coffee beans extracted in ethanol and ethyl acetate, the ethyl acetate extract exhibited a lower TPC [47].

3.3. Effect of Microwave Roasting (MW) and Extraction Solvents (ES) on the DPPH Scavenging of Coffee Beans. The antioxidant activity of the untreated and MW coffee beans was measured using the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging method and represented as 50% inhibitory concentration (IC_{50}) of the coffee extract. The results expressed in Figure 5 showed that MW and the ES

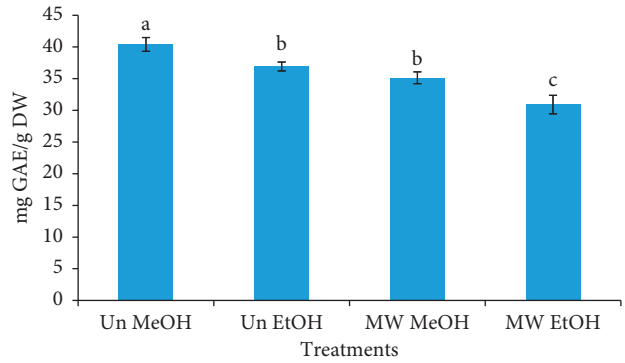

Figure 3: Impact of microwave roasting and extraction solvents on the total polyphenol content of coffee beans. Bars with different small letters are significantly different from one another ($p < 0.05$). Un MeOH: untreated sample extracted with 50% (v/v) methanol, Un EtOH: untreated sample extracted with 50% (v/v) ethanol, MW MeOH: microwave-roasted sample extracted with 50% (v/v) methanol, and MW EtOH: microwave-roasted sample extracted with 50% (v/v) ethanol.

had a significant ($p < 0.05$) effect on the DPPH scavenging of the coffee beans. In comparison to the MW coffee beans, the untreated samples of CBE exhibited a significantly higher antioxidant activity with lower IC_{50} values. For example, the obtained IC_{50} values for untreated and MW samples as extracted by methanol were 4.13 and 5.68 mg/mL, respectively. Similarly, Doğan et al.[48] found that the roasting of coffee exerted a negative effect on its antioxidant potential. In another study, green (raw) coffee also resulted in a higher antioxidant activity when compared to the conventional and microwave-roasted samples [38]. In contrast, Ludwig et al., [49] stated an upsurge in the DPPH scavenging of the roasted coffee. The DPPH scavenging of some other plant materials like citrus peels and pomace, fennel seeds [45], and apricot kernels [44] was increased by microwave roasting. High-temperature processing is thought to result in an increase of TPC and TFC in the plant materials, which leads to their increased antioxidant activity [50].

In addition, the methanol extracted coffee bean samples had significantly higher antioxidant activity in comparison with their ethanol extracted counterparts. The IC_{50} values for MR samples extracted using methanol and ethanol were 5.68 mg/mL and 6.24 mg/ml, respectively. The methanol extraction of coffee silver skin yielded a higher DPPH quenching ability than that extracted with other solvents [46].

3.4. Effect of Microwave Roasting (MW) and Extraction Solvents (ES) on the Reducing Power of Coffee Beans. The outcome of the effect of MW and ES on the reducing power of coffee beans is described in Figure 2. Untreated coffee beans exhibited higher reducing power (1.237) than that of the microwave-roasted ones (0.839) when extracted with methanol. Extraction solvents also showed a significantly different ($p < 0.05$) effect on the reducing power of coffee beans. It was noted that the reducing power of the untreated coffee beans sample extracted with ethanol (0.956) was less than those extracted with methanol (1.237). The results of antioxidant assays (DPPH scavenging, reducing power) echoed the results of TPC and TFC showing that the antioxidant potential of coffee beans was at least in part due to the polyphenol and flavonoid contents.

A previous study [51] reported a negative trend of the antioxidant capacity of *Coffea arabica* with increasing roasting degrees, which is in accordance with the results of the present study. Likewise, our findings find support from the observations made with green coffee beans decline that demonstrated a decline in the antioxidant capacity during the roasting process [52]. However, in contrast to our results, when compared to green coffee, Liang et al.[53] reported an increase in the antioxidant capacity of roasted beans. Such inconsistencies could be accredited to the intricacy of the chemical reactions during the roasting process. The plausible reason could be that during the roasting process of coffee beans, some of the bioactive compounds like chlorogenic acids are degraded which in turn could reduce the antioxidant activity of the coffee beans [52]. In addition, such degradations may also result in the release of other bioactive compounds such as hydroxycinnamates and quinic acid, which contribute to increased antioxidant potential [54]. Moreover, the Maillard reaction may also take place due to high temperature during the roasting process, generating a number of compounds, which can contribute to the elevated antioxidant potential of the product [55]. Consequently, all of the substances appearing during roasting process can either compensate for the loss of some compounds or even contribute towards the enhancement in the antioxidant potential [49].

3.5. HPLC Analysis of Phenolic Compounds. The average quantitative data reported in this study (Table 2) show that chlorogenic acid was the major (2.31–2.68%) phenolic compound found in all the coffee samples (untreated and microwave-roasted) followed by caffeic acid (0.997–1.18%). Vanillin demonstrated the lowest (0.118–0.187%) phenolic compound found in the coffee bean samples. Typical chromatogram of HPLC for standards and the sample is shown in Figure 6. An insignificant influence of MW and ES on the phenolic compounds of the coffee beans was observed. There is no

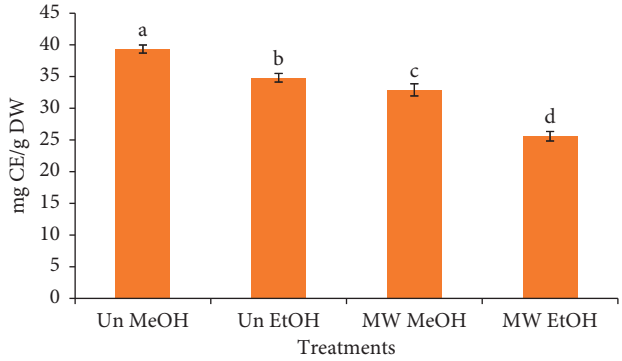

Figure 4: Impact of microwave roasting and extraction solvents on the total flavonoid content of coffee beans. Bars with different small letters are significantly different from one another ($p < 0.05$). Un MeOH: untreated sample extracted with 50% (v/v) methanol, Un EtOH: untreated sample extracted with 50% (v/v) ethanol, MW MeOH: microwave-roasted sample extracted with 50% (v/v) methanol, and MW EtOH: microwave-roasted sample extracted with 50% (v/v) ethanol.

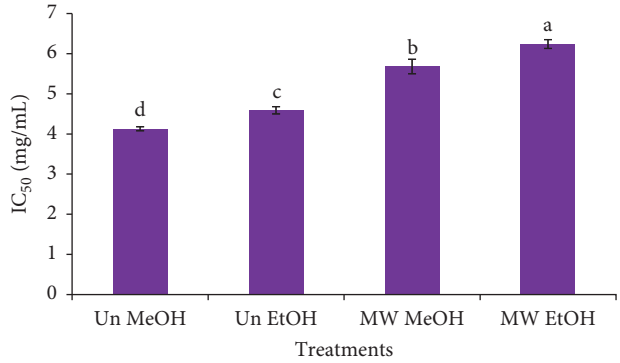

Figure 5: Impact of microwave roasting and extraction solvents on the DPPH scavenging of coffee beans. Bars with different small letters are significantly different from one another ($p < 0.05$). Un MeOH: untreated sample extracted with 50% (v/v) methanol, Un EtOH: untreated sample extracted with 50% (v/v) ethanol, MW MeOH: microwave-roasted sample extracted with 50% (v/v) methanol, and MW EtOH: microwave-roasted sample extracted with 50% (v/v) ethanol.

Table 2: Concentration of phenolics (gm/100 gm) dry weight basis.

Sample	Chlorogenic acid	Caffeic acid	Vanillin
Untreated MeOH	2.31 ± 0.37	0.997 ± 0.33	0.118 ± 0.65
Untreated EtOH	2.31 ± 0.58	0.997 ± 0.67	0.166 ± 0.27
MW MeOH	2.68 ± 0.63	1.18 ± 0.91	0.187 ± 0.63
MW EtOH	2.55 ± 0.87	1.09 ± 0.65	0.166 ± 0.87

Results are expressed as mean ± SD.

significant difference between the untreated methanolic (0.997–2.31 g/100 g) or ethanolic (0.997–2.31 g/100 g) extraction and microwave methanolic (0.187–2.68 g/100 g) or microwave ethanolic (0.166–2.55 g/100 g) treatments on the phenolic compounds. Similarly, the individual phenolic compounds, chlorogenic acid, caffeic acid, and vanillin, have close similarity in concentrations of all extraction and treatment methods (Table 2). Traditional heating at a high temperature of 120°C even for a short time results in the loss of 15–36% of the bioactive compounds [56]. Similar results have been reported in Ethiopian and Ugandan with roasted coffee where chlorogenic acid was present in the highest concentrations [44]. In another study, chlorogenic acid was reported as the major phenolic acid in spent coffee grounds extract as confirmed by HPLC [45]. Higher chlorogenic acid levels were extracted by multistep whole coffee fruit extracts than by the single-step extract [46].

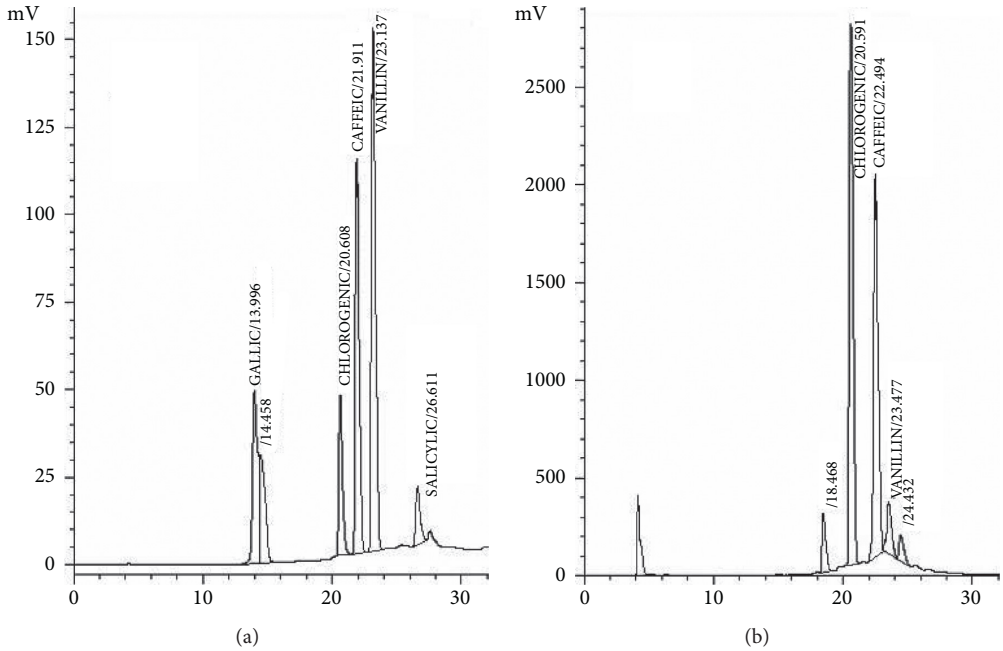

Figure 6: Typical HPLC chromatogram of standards (a) and coffee sample (b).

4. Conclusions

Roasting is an important step in the processing of coffee before it is consumed as a beverage. Therefore, the effects of MW and ES on the TPC, TFC, and antioxidant activity of coffee beans were evaluated. Compared to the untreated coffee beans, the MR coffee beans showed significantly lower TPC, TFC, and antioxidant activity. Similarly, the methanol showed better results compared to ethanol for both untreated and microwave-roasted samples. Irrespective of the ES and MW treatment, all major and important phenolic compounds were present with no significant difference. Chlorogenic acid is the key phenolic acid in untreated, microwave, methanol, and ethanolic extracts and can reap health benefits. This information might be helpful for the processing of coffee beans and can be exploited by the beverage industry. There are certain inconsistencies in the findings as compared to previous studies, but these can be attributed to factors such as method of extraction, microwave treatment, and climatic conditions. Moreover, future studies need to focus on the underlying mechanism of action of roasting on the studied parameters.

Data Availability

Data used to support the findings are included within the text.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Cinnamic Acid Ameliorates Nonalcoholic Fatty Liver Disease by Suppressing Hepatic Lipogenesis and Promoting Fatty Acid Oxidation

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Background. Cinnamic acid (CA) has been shown to have many beneficial effects including regulating lipid metabolism and reducing obesity. However, its effect on nonalcoholic fatty liver disease (NAFDL) has not been investigated in detail. Thus, we performed this study in order to explore CA's effect on hepatic lipid metabolism and the underlying mechanisms. *Method*. Oleic acid (OA) was used to induce lipid accumulation in HepG2 cells. After coincubation with CA, the cells were stained with oil red O and the triglyceride (TG) content was assessed. Key genes in lipogenesis and fatty acid oxidation pathways were tested. Additionally, db/db and wt/wt mice were divided into three groups, with the wt/wt mice representing the normal group and the db/db mice being divided into the NAFLD and CA groups. After 4 weeks of oral treatment, all mice were sacrificed and the blood lipid profile and liver tissues were assessed. *Results*. CA treatment reduced the lipid accumulation in HepG2 cells and in db/db mouse livers. ACLY, ACC, FAS, SCD1, PPARγ, and CD36 were significantly downregulated, while CPT1A, PGC1α, and PPARα were significantly upregulated. *Conclusion*. CA's therapeutic effect on NAFLD may be attributed to its ability to lower hepatic lipid accumulation, which is mediated by suppression of hepatic lipogenesis and fatty acid intake, as well as increased fatty acid oxidation.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), the incidence of which often parallels the trends in obesity, type II diabetes, dyslipidemia, and other metabolic diseases, has become a major health problem worldwide and the most common chronic liver disease in recent years [1]. The global prevalence of NAFLD is approximately 25.24% [2] and the rate continues to increase [3]. NAFLD begins with simple hepatic steatosis and can develop into nonalcoholic steatohepatitis, potentially leading to hepatic fibrosis and cirrhosis and causing severe complications such as hepatocellular carcinoma [1]. It reflects disrupted body energy homeostasis, which is the common pathological change in metabolic diseases.

Intrahepatic triglyceride (IHTG) accumulation indicates imbalanced hepatic energy metabolism and it can be regarded as a biomarker of NAFLD [4, 5]. Abnormally high IHTG levels in individuals with NAFLD may be attributed to increased *de novo* lipogenesis (DNL) and decreased fatty acid oxidation. Fatty acids are synthesized in the liver through DNL and are esterified by glycerol-3-phosphate to

produce triglyceride (TG). DNL is mediated by several lipogenic enzymes, the transcription of which is governed by transcription factors such as carbohydrate-responsive element-binding protein (ChREBP), sterol regulatory element-binding protein 1c (SREBP1c), and liver X receptors (LXRs) [6]. Fatty acid catabolism in the liver mainly takes place within mitochondria, generating ATP and ketone bodies. Long-chain fatty acid- (LCFA-) CoA translocates into mitochondria, which is mediated by carnitine palmitoyltransferase-1A (CPT1A), the rate-limiting enzyme for fatty acid β-oxidation [7]. Altered activity of the above pathways in individuals who consume excess calories results in NAFLD and other metabolic diseases [8]. Thus, regulating energy metabolism, especially the fatty acid synthesis and oxidation pathways in hepatocytes, plays an important role in controlling excess IHTG levels and, eventually, improves the condition of NAFLD.

Cinnamic acid (CA) is a natural polyphenol that comprises nine carbon atoms (C6-C3) (Figure 1(a)). It occurs in many fruits such as citrus fruits and grape and vegetables such as spinach and celery, and CA and its many derivatives are permitted to be used as flavor compounds in numerous regions by authorities [9, 10]. However, it is mostly enriched in *Cinnamomum cassia* (Chinese cinnamon) [11, 12], which has been used as a traditional herb as well as a flavoring material in many countries for thousands of years [13]. It has been demonstrated that CA possesses multiple therapeutic effects, including antimicrobial [14], anticancer [15, 16], anti-inflammatory [17], antioxidant [18, 19], and antidiabetic [10] effects. Moreover, recent studies have highlighted CA's effects on lipid metabolism and obesity. It was reported that CA lowered the serum lipid levels in streptozotocin- (STZ-) induced diabetic rats [20] and in animal models of high fat diet-induced obesity [21, 22]. *In vitro* studies showed that CA stimulated white fat browning in 3T3-L1 adipocytes and activated HIB1B brown adipocytes [23]. It also lowered the TG levels in 3T3-L1 cells [24] and lipid accumulation in oleic acid- (OA-) treated HepG2 hepatocytes [22]. Moreover, in a previous study on a palmitic acid-induced triglyceride accumulation cell model, CA treatment elevated CPT1 protein expression, suggesting CA may accelerate lipid oxidation *in vitro* [25].

Although CA has exhibited hypolipidemic effects in both *in vivo* and *in vitro* experiments, the underlying mechanisms of CA's effect on NAFLD are still poorly understood. Thus, we investigated the effect of CA on OA-stimulated HepG2 cells and db/db mice, a commonly used genetic model for NAFLD [26, 27], and we explored the alterations in the expression of transcription factors and key enzymes in the lipogenesis and fatty acid oxidation pathways in cells and mice after CA treatment.

2. Materials and Methods

2.1. Chemicals and Reagents. Cinnamic acid (≥99.5% purity) was purchased from Shanghai Yuanye (Shanghai, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Carlsbad, CA, USA). OA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) and 0.5% carboxymethyl cellulose (CMC) sodium salt solution were purchased from Solarbio Science & Technology (Beijing, China) and Coolaber Science & Technology (Beijing, China), respectively. CA was dissolved in 0.5% CMC buffer solution before being orally administered to the mice.

2.2. Cell Culture. HepG2 cells were purchased from KeyGEN Biotech (Nanjing, China). The cells were cultured in DMEM with 10% FBS and maintained in a humidified, 37°C, 5% CO_2 environment, with the media changed every 24 hours. Based on previous studies, 0.5 mM OA was used to establish a high-fat model *in vitro* [28, 29]. After reaching 80% confluence, the cells were placed in 6-well plates and stimulated with 0.5 mM OA media with or without CA of different concentrations. Cells cultured without OA were used as normal controls. The OA/BSA complex used for cell stimulation was prepared as follows: OA was dissolved in 0.1 M NaOH and heated at 70°C for 30 min to form a 100 mM OA solution. This was further mixed with 10% BSA in phosphate-buffered saline to acquire a 10 mM OA/BSA complex. This complex was diluted in DMEM with 10% FBS to a concentration of 0.5 mM for final use. All cell experiments were performed in at least three replicate wells.

2.3. Cell Viability Assay. The effect of CA on the viability of HepG2 cells was assessed by Cell Counting Kit- (CCK-) 8 assays. CCK-8 assays were conducted as previously described [22, 30] with slight modification. Briefly, 200 μl HepG2 cells were seeded at a density of 1×10^4/well in a 96-well plate and cultured for 12 h. Thereafter, the cells were treated with CA (diluted to 12.5, 25, 50, 100, or 200 μM) for 24 h, followed by changing the media to 200 μL DMEM containing 10 μL CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) and incubating for 1 h in 37°C. Absorbance at 450 nm was measured using a microplate reader (Promega BioSystems Sunnyvale, Sunnyvale, CA, USA). Cells cultured in DMEM with 10% FBS (without CA) were used as normal controls.

2.4. Oil Red O Staining and TG Assessment. After treatment for 24 h, HepG2 cells were stained with Oil red O to determine the intracellular lipid accumulation. The staining was performed using a commercial kit (Solarbio Science & Technology) according to the manufacturer's instructions. TG content of HepG2 cells was determined using a TG measuring kit (Nanjing Jiancheng Bioengineering, Nanjing, China) following the manufacturer's instructions. Cells cultured in DMEM with 10% FBS were used as normal controls.

2.5. Animal Experiments. The animal experiments conducted in this study were approved by the Animal Care and Ethics Committee of Beijing University of Traditional Chinese Medicine (approval no. BUCM-4-20190931002-1088). A total number of 14 5-week-old male C57BL/KsJ-db/db mice and 7

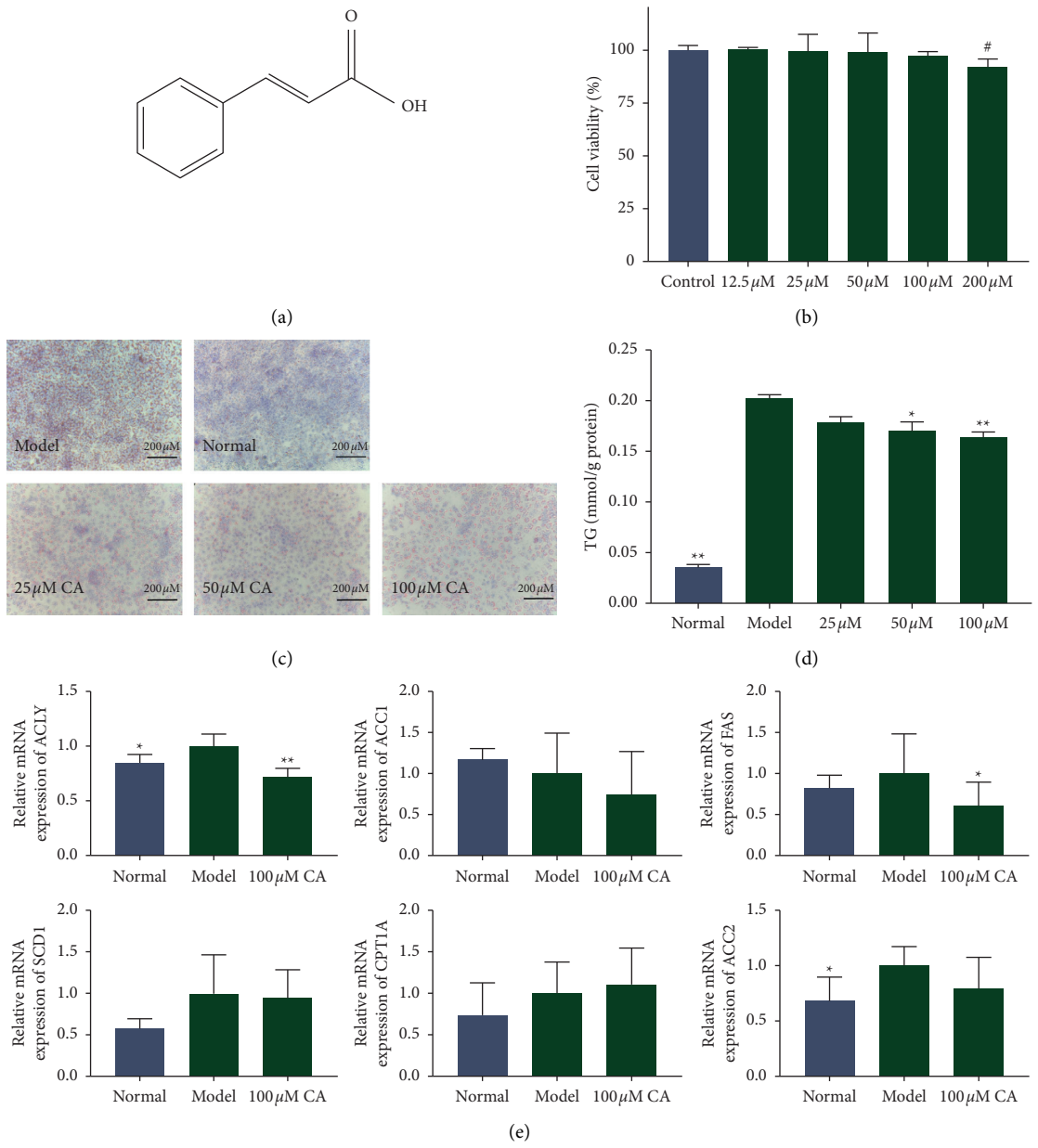

Figure 1: (a) Chemical structure of cinnamic acid (CA). (b) CCK-8 assay results. Control group: HepG2 cells cultured with 10% FBS in DMEM. 25 μM group: cells cultured with 25 μM CA, 10% FBS in DMEM. 50 μM group: cells cultured with 50 μM CA, 10% FBS in DMEM. 100 μM group: cells cultured with 100 μM CA, 10% FBS in DMEM. 200 μM group: cells cultured with 200 μM CA, 10% FBS in DMEM. (c) Images of oil red O staining of HepG2 cells (200X magnification). (d) Triglyceride (TG) content of HepG2 cells after treatment for 24 h. (e) mRNA expression of ATP-citrate lyase (ACLY), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1), carnitine palmitoyltransferase-1A (CPT1A), and ACC2 in HepG2 cells. Normal group: cells cultured with 10% FBS in DMEM. Model group: cells cultured with 0.5 mM oleic acid (OA). 25 μM group: cells cultured with 0.5 mM OA and 25 μM CA. 50 μM group: cells cultured with 0.5 mM OA and 50 μM CA. 100 μM group: cells cultured with 0.5 mM OA and 100 μM CA. Data are presented as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the model group; $^{\#}p < 0.05$, compared to control group.

age-matched male C57 BL/KsJ-wt/wt mice were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China) and housed in specific-pathogen-free conditions under a 12/12 h light/dark cycle, with free access to water and food. The bodyweight of mice was measured every week. After 1 week of acclimatization, all mice were fasted overnight and blood glucose was measured by a portable glucometer (Glucocard 01-mini; Arkray, Kyoto, Japan) using blood collected from the tail vein.

The db/db mice were then randomly divided into two groups with fasting blood glucose and body weight of which at the same baseline: the CA and NAFLD groups. The CA mice were orally treated with 20 mg/kg body weight CA every day. The dosage was chosen based on previous studies. The NAFLD model mice were treated with the same volume

of the vehicle (0.5% CMC buffer). The wt/wt mice were used as the normal control group and treated the same as NAFLD mice. After 4 weeks of treatment, the mice were sacrificed. Blood samples were collected and centrifuged to acquire serum. Additionally, liver and epididymal adipose tissues were removed and weighed. A small slice of each tissue was fixed in 4% paraformaldehyde solution (Solarbio Science & Technology) and the remaining tissues were immediately frozen in liquid nitrogen.

2.6. Assessment of Serum Lipid Profile and IHTG. The serum was stored at −20°C before the total cholesterol (TC), TG, high-density lipoprotein (HDL), low-density lipoprotein (LDL), free fatty acid (FFA), and glucose levels were tested using an automated chemistry analyzer (AU480; Beckman Coulter, Brea, CA, USA). IHTG levels (mmol/g protein) of the mice were measured using a commercial kit (Nanjing Jiancheng Bioengineering, Nanjing, China).

2.7. Histological Analysis of Liver and Adipose Tissues. Liver tissues and epididymal adipose tissues fixed in 4% paraformaldehyde were embedded in paraffin, cut into 4 μm thick slices using a microtome, and then stained with hematoxylin and eosin (H&E). Immunohistochemical (IHC) staining of liver tissues was used to examine protein expression. Primary antibodies against SREBP1 (ab191857; Abcam, Cambridge, UK) and CPT1A (ab234111; Abcam) were used, followed by incubation with horseradish peroxidase- (HRP-) labeled anti-rabbit IgG secondary antibody for 20 min. The tissues were then stained with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. The IHC staining images were analyzed based on the mean optical density using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.8. Quantitative Real-Time PCR (RT-qPCR). Total RNA was extracted from HepG2 cells and the liver tissues using a commercial kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The RNA was then homogenized by RNase-free water to the same concentration of 0.5 ng/tube and cDNA was synthesized using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan). Thereafter, the RT-qPCR analyses were conducted on Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using a GoTaq®qPCR Master Mix Kit (Promega). The expression levels of target genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the results were calculated using the relative quantitative ($2^{-\Delta\Delta CT}$) method. The primers used are listed in Table 1.

2.9. Western Blot Analysis. Total protein was extracted from the liver tissues as follows. The tissues were homogenized and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor, phosphatase inhibitor, and phenylmethanesulfonyl fluoride (KeyGEN Biotech). The concentration of each extracted protein solution was then measured using a Bradford protein quantitative assay with Coomassie brilliant blue G-250 (Solarbio Science & Technology). Protein samples were prepared at a concentration of 1 μg/μl and denatured at 100°C for 5 min in loading buffer (Solarbio Science & Technology). The proteins were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and separated according to their molecular weights. They were then transferred onto methanol-soaked 0.45 μm polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). These membranes were then blocked using Blocking-One solution (Nacalai Tesque, Kyoto, Japan) for 1 h and then incubated with primary antibodies against the following proteins: ATP-citrate lyase (ACLY; #13390; Cell Signaling Technology, USA), acetyl-CoA carboxylase (ACC; #3676; Cell Signaling Technology), fatty acid synthase (FAS; #3180; Cell Signaling Technology), stearoyl-CoA desaturase 1 (SCD1; #2794; Cell Signaling Technology), CPT1A (ab234111; Abcam), and β-actin (#4970; Cell Signaling Technology) at 4°C overnight. After washing with Tris-buffered saline with Tween 20 (TBST; Beijing Applygen Technologies, Beijing, China), the membranes were incubated with HRP-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (Proteintech Group, Rosemont, IL, USA) for 1 h and washed with TBST again. Enhanced chemiluminescence (ECL) reagent (Solarbio Science & Technology) was reacted with the HRP to generate fluorescence. The gray values of the protein bands were calculated by normalization to the endogenous control protein β-actin.

2.10. Statistical Analysis. All data are presented as mean ± standard deviation (SD). SPSS 23.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Normally distributed data were compared between two groups using Student's t-test. Normally distributed data were compared between three or more groups using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for groups with equal variances or Dunnett's test for groups with unequal variances. Nonnormally distributed data were compared between two groups using the Mann-Whitney U test. Nonnormally distributed data were compared between three or more groups using the nonparametric Kruskal–Wallis test. A statistically significant difference was defined as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Effect of CA on HepG2 Cell Viability. CA treatment at doses of 12.5, 25, 50, or 100 μM for 24 h had no significant impact on the viability of HepG2 cells (Figure 1(b)). The viability of the cells in the 200 μM CA group was lower than that of the control group ($p < 0.05$). Therefore, 25, 50, and 100 μM CA were chosen for further investigations.

3.2. Effect of CA on Lipid Accumulation in HepG2 Cells. Oil red O staining showed that OA dramatically increased the lipid accumulation in HepG2 cells and CA treatment ameliorated this increase. The lipid droplets were remarkably reduced in the CA group compared to the NAFLD

Table 1: Sequences of primers used in RT-qPCR.

Gene	Forward primer	Reverse primer
ACC1	GATGAACCATCTCCGTTGGC	GACCCAATTATGAATCGGGAGTG
ACC2	CGCTCACCAACAGTAAGGTGG	GCTTGGCAGGGAGTTCCTC
ACLY	ACCCTTTCACTGGGGATCACA	GACAGGGATCAGGATTTCCTTG
BDK	ACATCAGCCACCGATACACAC	GAGGCGAACTGAGGGCTTC
CD36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
ChREBP	GAACCGCCTCTTCTGCT	CAACTCCATACAACCCTCG
CPT1A	AGATCAATCGGACCCTAGACAC	CAGCGAGTAGCGCATAGTCA
FAS	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
GAPDH	AAGATGGTGAAGGTCGGTGT	GCTTCCCATTCTCAGCCTTG
LXRα	CTCAATGCCTGATGTTTCTCCT	TCCAACCCTATCCCTAAAGCAA
PGC1α	TTCAAGATCCTGTTACTACT	ACCTTGAACGTGATCTCACA
PPARα	ACGCGAGTTCCTTAAGAACCTG	GTGTCATCTGGATGGTTGCTCT
PPARγ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
PPM1K	TTATCAGCGGCCTTCATTACTTT	GGATGGAGCTTAACAACACTCTC
SCD1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
SREBP1c	CAAGAAGCGGATGTAGTCG	GAGCCGTGGTGAGAAGC
GAPDH (human)	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
ACC1 (human)	ATGTCTGGCTTGCACCTAGTA	CCCCAAAGCGAGTAACAAATTCT
ACC2 (human)	CAAGCCGATCACCAAGAGTAAA	CCCTGAGTTATCAGAGGCTGG
ACLY (human)	TCGGCCAAGGCAATTTCAGAG	CGAGCATACTTGAACCGATTCT
CPT1A (human)	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
FAS (human)	AAGGACCTGTCTAGGTTTGATGC	TGGCTTCATAGGTGACTTCCA
SCD1 (human)	TCTAGCTCCTATACCACCACCA	TCGTCTCCAACTTATCTCCTCC

group (Figure 1(c)). This was further confirmed by the results of the TG content examination. The TG content was significantly increased by OA, and CA reduced the TG content in a dose-dependent manner (Figure 1(d)). qPCR results indicated that CA treatment significantly suppressed the expression of ACLY and FAS. It also showed the tendency to decrease the expression of ACC1, ACC2, and SCD1 and increase the expression of CPT1A (statistically insignificant, Figure 1(e)).

3.3. Effect of CA on Body Weight, Blood Glucose, and Liver and Fat Mass of db/db Mice. Mice in the CA group were lighter after 4 weeks than those in the NAFLD group (Figures 2(a) and 2(b)). Oral administration of CA significantly reduced body weight gain of db/db mice from the third week. Notably, CA decreased the liver mass and liver index (liver mass/body weight) in the obese db/db mice ($p < 0.05$ and $p < 0.01$, respectively) (Figures 2(c) and 2(d)). CA also exhibited a hypoglycemic effect in the db/db mice, as at the end of the treatment, the glucose levels in CA group were significantly lower than those in the NAFLD group despite the same baseline (Figure 2(e)), confirming its previously reported antidiabetic effect [31, 32]. Furthermore, CA reduced the mean weight of epididymal fat, though the data was not significant according to one-way ANOVA (Figure 2(f)).

3.4. Effect of CA on Serum Lipid Profile and IHTG Levels in db/db Mice. The serum TG, IHTG, and FFA levels in db/db mice were significantly lowered and the HDL level was raised by CA treatment (Figures 3(b), 3(c), 3(e), and 3(f)), suggesting that CA improved lipid metabolism and may have a beneficial effect on NAFLD. However, CA had no noticeable effect on serum TC or LDL levels (Figures 3(a) and 3(d)).

3.5. Histological Observation Results. The liver tissues and epididymal adipose tissues were fixed in paraffin and stained with H&E for observation of histological changes. Compared to normal wt/wt mice, db/db mice in the NAFLD group had severe hepatic steatosis and disruption of hepatocyte structure (Figure 4(a)). These changes were ameliorated by CA treatment. Adipocytes in the NAFLD group were dramatically larger than those in the normal control group, consistent with the abovementioned increased epididymal fat mass. H&E staining showed that 4 weeks of CA treatment reduced the size of the adipocytes (Figure 4(b)). The IHC analysis showed less SREBP1c-positive staining in the CA group than the NAFLD group (Figure 4(c)). In contrast, the CPT1A-positive area was larger in the CA group than the NAFLD group (Figure 4(d)). The IHC results were further quantified and verified based on mean optical density using ImageJ software (Figure 4(e)).

3.6. CA Downregulated Lipogenesis Transcription Factors and Lipogenic Enzymes in db/db Mice. The relative mRNA expression of transcription factors that regulate genes that encode lipogenic enzymes was downregulated and, consistently, the mRNA and protein expression of lipogenic enzymes including ACLY, ACC, FAS, and SCD1 were decreased (Figure 5). The mRNA expression of factors related to the expression and regulation of transcription factors, such as branched-chain ketoacid dehydrogenase kinase (BDK) and branched-chain ketoacid dehydrogenase phosphatase (PPM1K), was also investigated by qPCR.

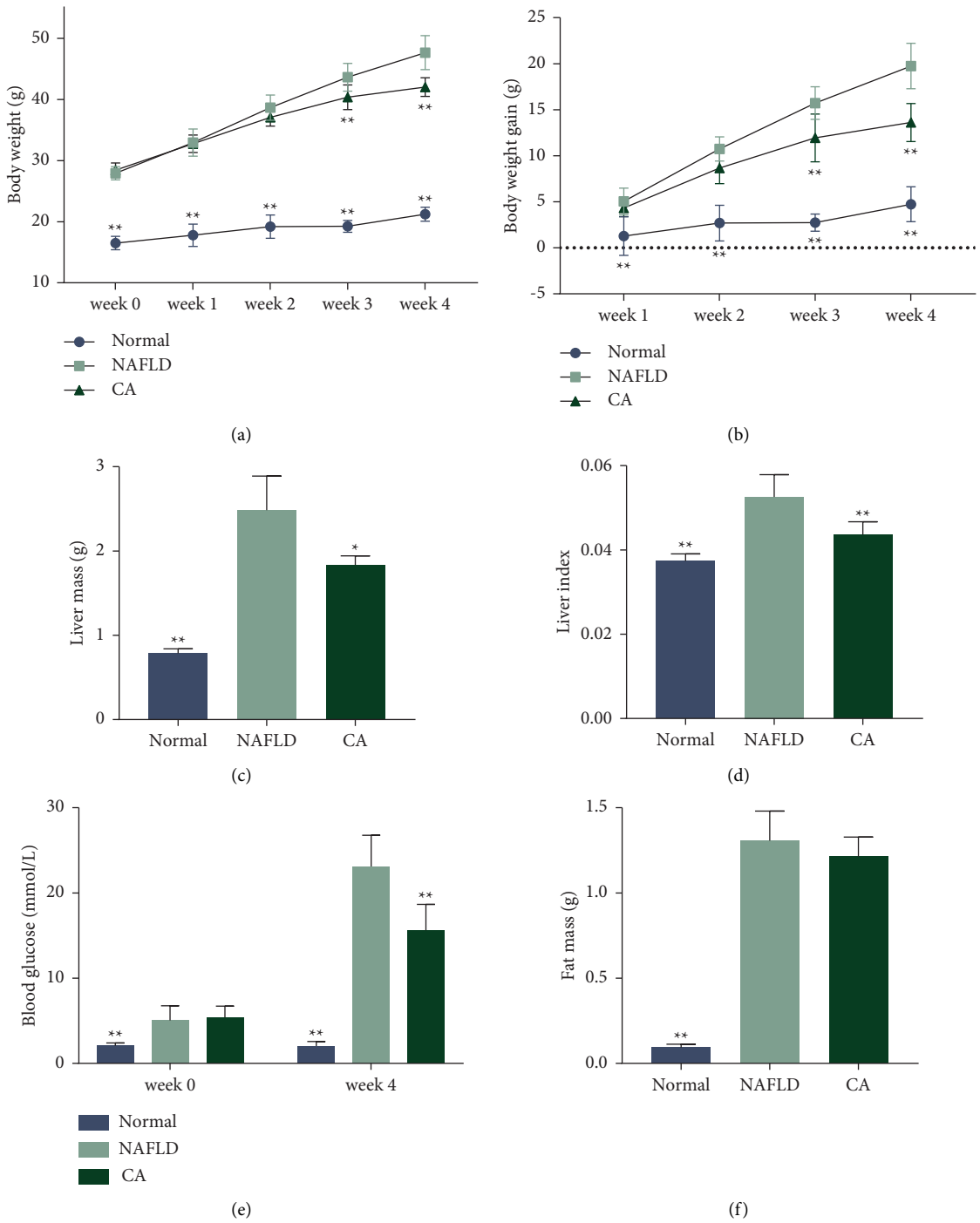

Figure 2: Cinnamic acid (CA) reduced body weight gain of db/db mice, liver mass, liver index, and blood glucose levels. (a) Body weight of mice compared among groups of mice. (b) Body weight gain of mice compared to initial body weight at week 0. (c) Liver mass of mice. (d) Liver index (liver mass/body weight) of mice. (e) Blood glucose levels from week 0 (tested by portable glucose monitor using blood from tail vein) and week 4 (tested by automated chemistry analyzer using blood collected from abdominal aorta). (f) Epididymal fat (from the left side of mice) mass. Normal group: wt/wt mice treated with vehicle. NAFLD group: db/db mice treated with vehicle. CA group: db/db mice treated with CA 20 mg/kg/day. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the model group.

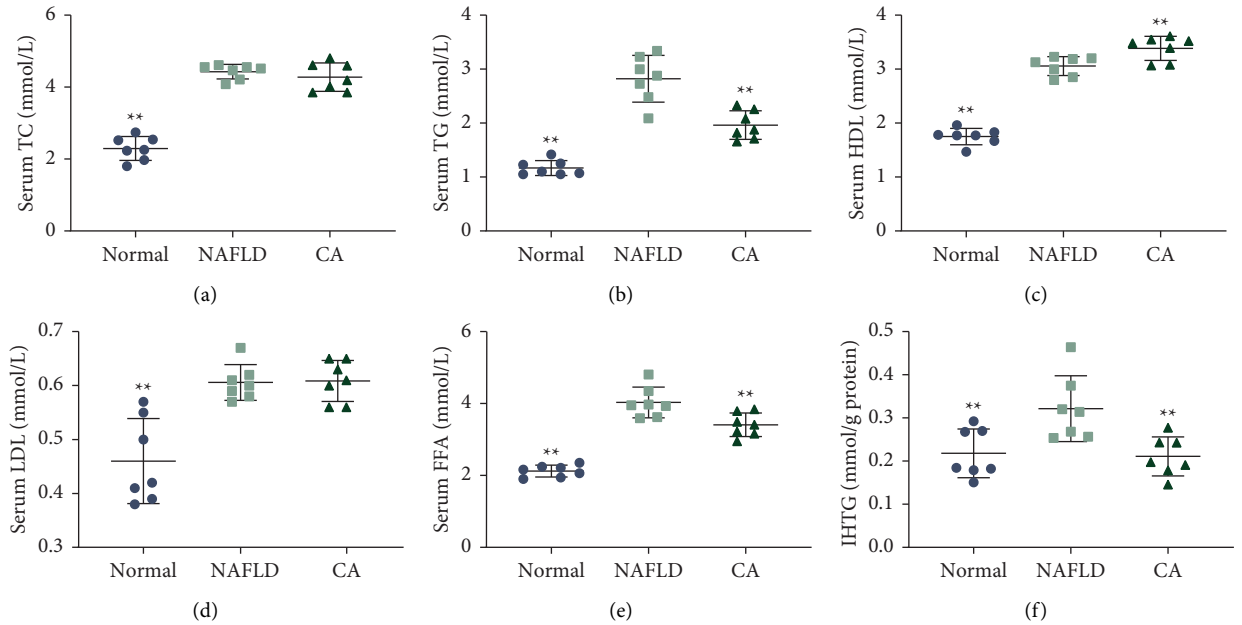

Figure 3: Serum lipid profile and intrahepatic triglyceride (IHTG) levels in mice. Serum total cholesterol (TC) levels (a), serum triglyceride (TG) levels (b), serum high-density lipoprotein (HDL) levels (c), serum low-density lipoprotein (LDL) levels (d), serum-free fatty acid (FFA) levels (e), and IHTG levels (f) in mice. Normal group: wt/wt mice treated with vehicle. NAFLD group: db/db mice treated with vehicle. CA group: db/db mice treated with CA 20 mg/kg/day. Data are presented as mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the NAFLD group.

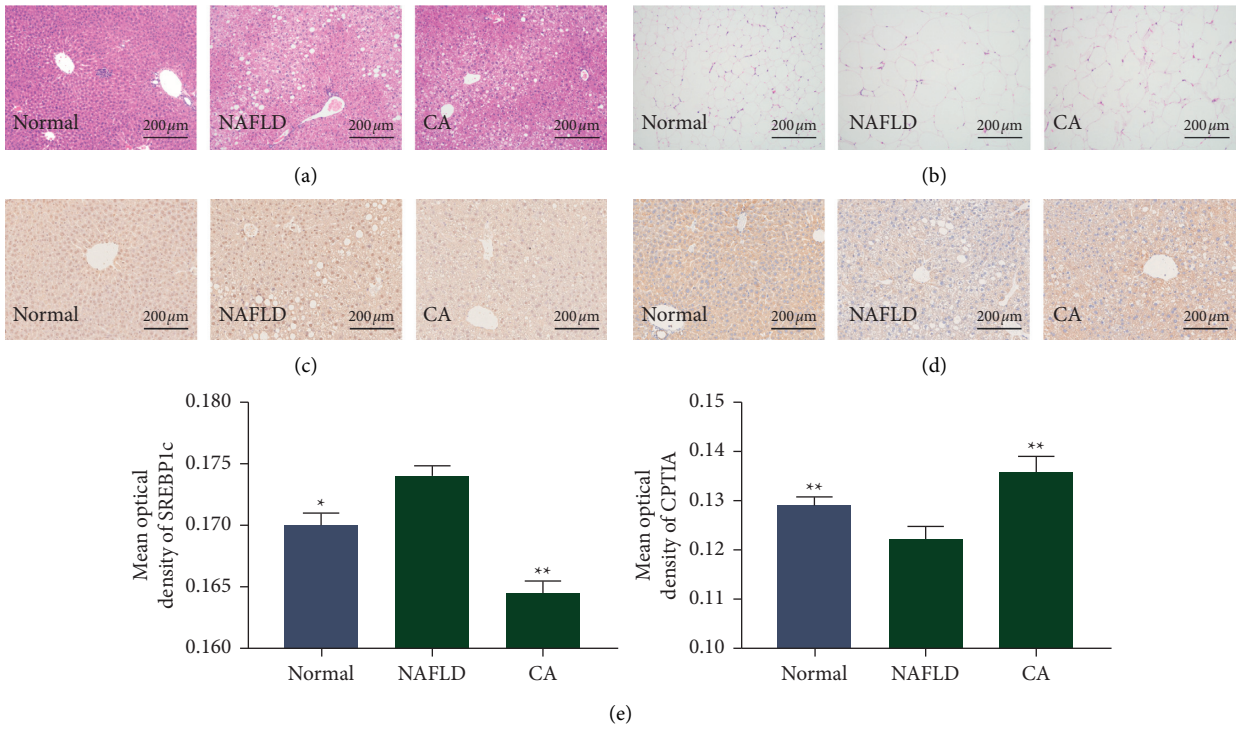

Figure 4: Histological observation results (200× magnification). (a) Images of H&E staining of liver tissues. (b) Images of H&E staining of epididymal fat tissues. (c) Immunohistochemical (IHC) staining of hepatic sterol regulatory element-binding protein 1c (SREPB1c) in mice. (d) IHC staining of hepatic carnitine palmitoyltransferase-1A (CPT1A) in mice. (e) Mean optical density of IHC staining images. Normal group: wt/wt mice treated with vehicle. NAFLD group: db/db mice treated with vehicle. CA group: db/db mice treated with CA 20 mg/kg/day. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the NAFLD group.

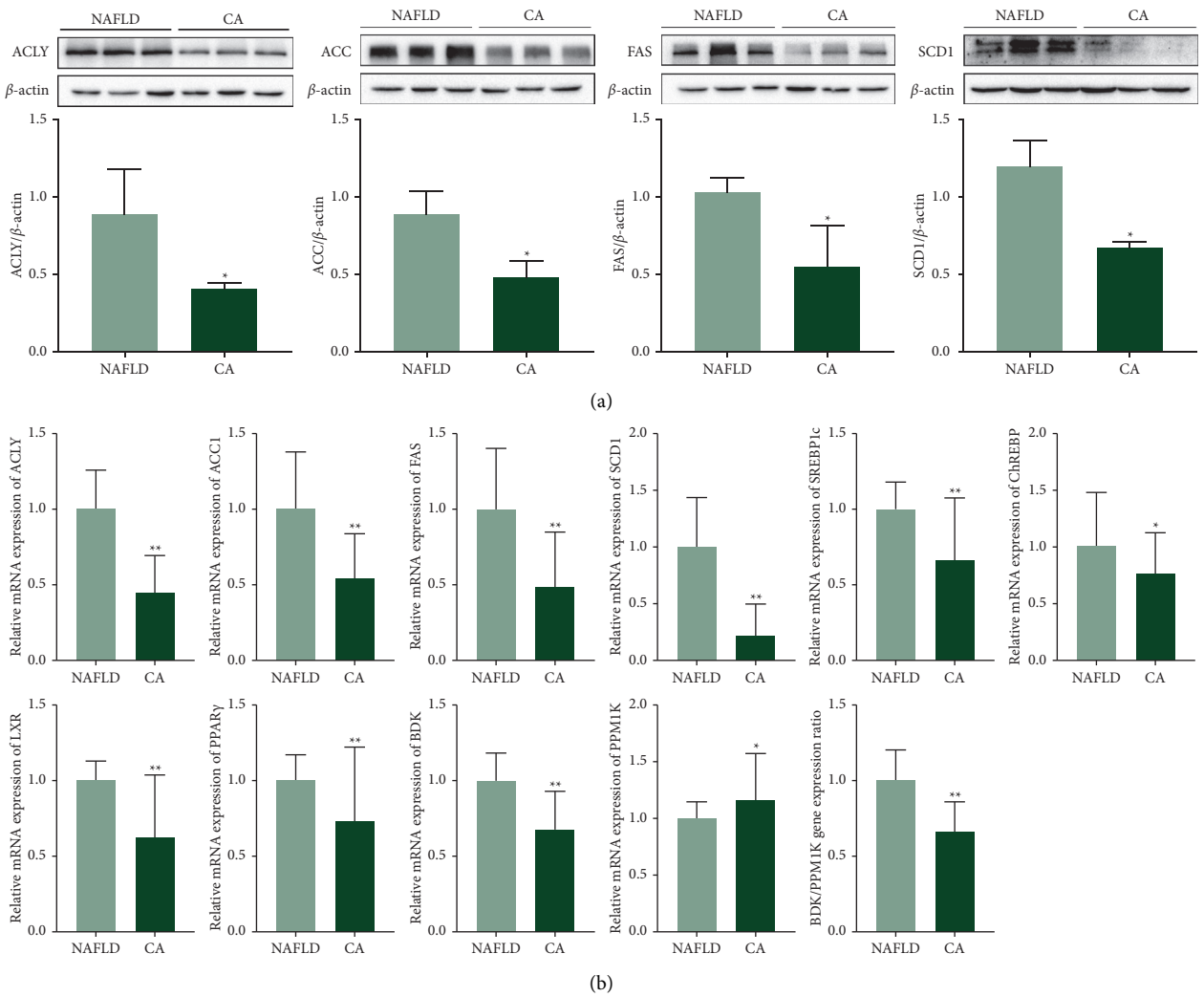

Figure 5: Cinnamic acid (CA) downregulated lipogenic transcription factors and other lipogenic genes in db/db mice. (a) Protein expression of ATP-citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD1) in db/db mice assessed by western blotting. (b) mRNA expression of ACLY, ACC1, FAS, SCD1, sterol regulatory element-binding protein 1c (SREBP1c), carbohydrate-responsive element-binding protein (ChREBP), liver X receptor α (LXRα), peroxisome proliferator-activated receptor γ (PPARγ), branched-chain ketoacid dehydrogenase kinase (BDK), and branched-chain ketoacid dehydrogenase phosphatase (PPM1K) in db/db mice assessed by qPCR. NAFLD group: db/db mice treated with vehicle. CA group: db/db mice treated with CA 20 mg/kg/day. Data are presented as mean ± SD, $n = 3$ for western blotting analyses, $n = 5$ for PCR analyses. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the NAFLD group.

Moreover, BDK and PPM1K mRNA expression was decreased and increased by CA, respectively. This led to a significantly decreased BDK : PPM1K in the CA group.

3.7. CA Upregulated Fatty Acid Oxidation Pathway in db/db Mice. Compared to the NAFLD group, mice in the CA group had higher CPT1A protein expression, which indicates that fatty acid oxidation was upregulated in the CA group. Additional factors from fatty acid metabolism pathways were also examined by qPCR. The results showed that CA increased the mRNA expression of peroxisome proliferator-activated receptor α (PPARα) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) and decreased the mRNA expression of ACC2 and CD36 (Figure 6).

4. Discussion

As the most common chronic liver disease, NAFLD affects nearly one-third of the adult population and 10% of children in developed countries, and the prevalence continues to increase in concert with the increasing number of obese individuals [33, 34]. In Asian countries, the current prevalence of NAFLD is around 25%, which is also increasing [35]. Unhealthy lifestyles, especially overconsumption of high-calorie foods and sedentary lifestyles and lack of exercise, lead to disorders of energy metabolism, causing

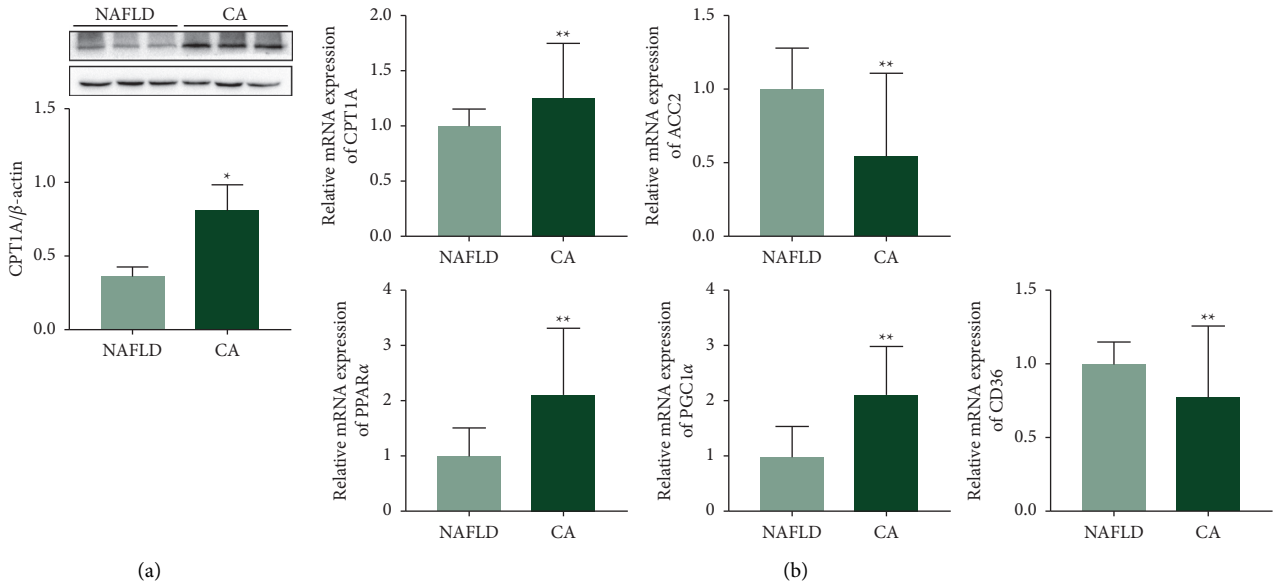

Figure 6: CA upregulated the fatty acid oxidation pathway and reduced CD36 mRNA expression in db/db mice. (a) Protein expression of carnitine palmitoyltransferase-1A (CPT1A) in db/db mice assessed by western blotting. (b) mRNA expression of CPT1A, acetyl-CoA carboxylase 2 (ACC2), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), and CD36 in db/db mice assessed by qPCR. NAFLD group: db/db mice treated with vehicle. CA group: db/db mice treated with CA 20 mg/kg/day. Data are presented as mean ± SD, $n = 3$, for western blotting analyses, $n = 5$ for PCR analyses. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the NAFLD group.

dyslipidemia, obesity, metabolic syndrome, and type 2 diabetes [36].

CA, a chemical almost ubiquitous in plants and particularly enriched in cinnamon, has been reported to improve lipid metabolism and ameliorate diabetes and obesity [20, 21, 23, 31, 32, 37–39]. Despite recent research progress, CA's effects on NAFLD and the possible mechanisms remain to be elucidated. The development of NAFLD is linked to the abnormal accumulation of hepatic lipids, manifested as an excessive level of IHTG [33]. In our study, CA treatment significantly decreased OA-induced lipid accumulation in HepG2 cells and significantly reduced the TG content in a dose-dependent manner. In db/db mice, 4 weeks of CA treatment at 20 mg/kg/day significantly decreased body weight gain and the blood glucose level of db/db mice. CA treatment also improved the serum lipid profile of db/db mice, as indicated by lower FFA and TG levels compared to those in the NAFLD group. Most importantly, the liver weight and liver index, as well as the IHTG levels, were significantly decreased, showing that CA treatment exerted effects against NAFLD *in vivo*.

As the increased IHTG is caused by increased DNL and fatty acid uptake rates (TG synthesis) and a lower fatty acid oxidation rate (TG break down), we investigated the effect of CA on the lipogenesis and fatty acid oxidation pathways. Aiming to explore the underlying mechanisms of the effects of CA on NAFLD model animals, only the NAFLD group and CA group were included in our PCR and western blotting analyses, as comparing the differently expressed factors within these two groups would be sufficient for the demonstration.

Abundant carbohydrates are converted into fatty acids and then esterified to form TGs in the liver and adipose tissues via DNL [40]. DNL in the liver is mediated by a series of coordinated lipogenic enzymes [41]. Briefly, citrate, which is generated from glucose via glycolysis and the tricarboxylic acid cycle, is transformed by ACLY into acetyl-CoA. Acetyl-CoA from the first step of DNL is carboxylated by ACC to form malonyl-CoA. Mammals have two types of ACC proteins, ACC1 in the cytoplasm and ACC2 on the mitochondrial outer membrane [42]. Malonyl-CoA is believed to inhibit CPT1A, and suppression of ACC1 and ACC2 decreases the hepatic malonyl-CoA levels, increases fatty acid oxidation, and improves NAFLD in diet-induced rat models [43, 44]. Malonyl-CoA is further converted into palmitic acid by FAS. Palmitic acid is elongated on the endoplasmic reticulum to form LCFAs, which are subsequently desaturated by SCD1 to generate unsaturated fatty acids. Palmitic acid, LCFAs, and unsaturated fatty acids are all fatty acid products of DNL, and they can be esterified to generate IHTG [40, 44, 45]. In this study, CA downregulated the mRNA expression of the lipogenesis genes ACLY, ACC, FAS, and SCD1, suggesting that CA suppresses fatty acid synthesis *in vivo*. The western blotting results showed that CA also significantly decreased the related protein expression. As an *in vitro* model of human liver steatosis, OA-induced lipid accumulation was suppressed by CA in HepG2 cells. The results showed that 100 μM of CA significantly downregulated several key genes' expression levels in lipogenesis.

The genes encoding lipogenesis proteins are primarily governed by transcription factors [6]. SREBP1c binds to sterol regulatory elements, ChREBP binds to carbohydrate

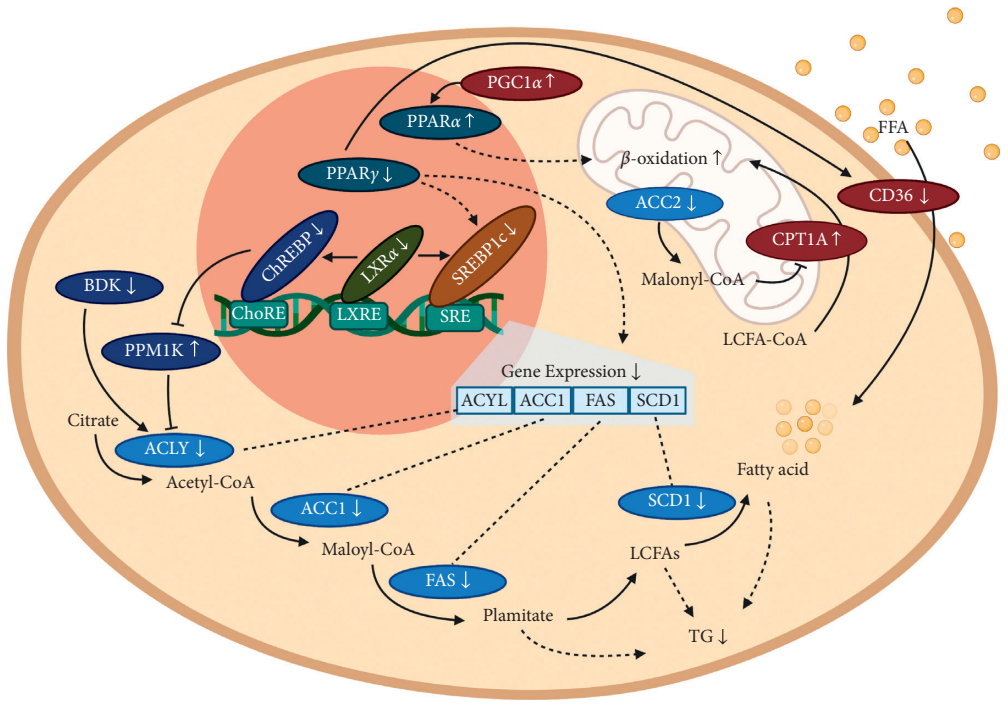

Figure 7: Effect of cinnamic acid (CA) on hepatic lipid metabolism pathways investigated in this study. According to this study, in hepatocytes, CA downregulates the following factors: ATP-citrate lyase (ACLY), acetyl-CoA carboxylase 1 (ACC1), ACC2, fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), sterol regulatory element-binding protein 1c (SREBP1c), carbohydrate-responsive element-binding protein (ChREBP), liver X receptor α (LXRα), peroxisome proliferator-activated receptor γ (PPARγ), branched-chain ketoacid dehydrogenase kinase (BDK), and CD36. CA upregulates the following factors: branched-chain ketoacid dehydrogenase phosphatase (PPM1K), carnitine palmitoyltransferase-1A (CPT1A), peroxisome proliferator-activated receptor α (PPARα), and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α). The suppressed lipogenesis decreased fatty acid intake and boosted fatty acid oxidation contribute to the decreased triglyceride (TG) level in the liver. LCFA: long-chain fatty acid; FFA: free fatty acid; ↑: upregulated; ↓: downregulated.

response elements, and LXRs bind to LXR response elements, all located in the promoter region of lipogenesis genes, and thereby upregulate their target genes [6, 46, 47]. LXRα is mainly expressed in the liver and other lipogenic tissues [48] and may also activate SREBP1c and ChREBP [49, 50]. Furthermore, PPARγ is also considered to be an agonist of SREBP1c, as hepatic deletion of PPARγ downregulated SREBP1c, SCD1, and ACC [51]. Our experiments revealed that CA decreased the expression of transcription factors of lipogenesis genes, consistent with the downregulation of their target genes. In addition, BDK and PPM1K are two kinases that coregulate ACLY activity, and hepatic BDK overexpression may lead to excessive DNL. The BDK : PPM1K ratio is believed to be a bioindicator of DNL and metabolic disorder phenotype, and the ratio can be increased by ChREBP [52]. CA downregulated BDK and upregulated PPM1K, which led to a decreased BDK : PPM1K ratio. This may also indicate its inhibitory effect on the transcription factor ChREBP, as well as reflecting CA's therapeutic effects against obesity.

TG accumulation may also be attributed to an increased exocellular fatty acid intake rate. Fatty acids are taken up by hepatocytes through several membrane proteins such as CD36, fatty acid translocase, and fatty acid-binding proteins [53]. In obese rats, CD36 mRNA expression is positively related to the IHTG level and liver steatosis severity [54]. As PPARγ promotes CD36 transcription in the liver [55, 56] and PPARγ was downregulated in the CA group, we investigated the expression of its transcriptional target, CD36. The results showed that CD36 mRNA was significantly downregulated as predicted, indicating that CA may also inhibit the fatty acid intake of hepatocytes.

Fatty acids are principally consumed in the liver, via fatty acid β-oxidation, which mainly takes place in the mitochondria [8]. LCFAs are activated by converting them to LCFA-CoA by long-chain acyl-CoA synthetases [57] and they are then transferred to mitochondria by CPT1A [58]. PGC1α is a coactivator of PPARα, which plays an important role in fatty acid oxidation [59]. Deletion of PPARα decreased hepatic fatty acid β-oxidation and aggravated hepatic steatosis in mice [60]. In our db/db mice, CA treatment was shown to remarkably increase the CPT1A mRNA and protein expression, as well as increasing the mRNA expression of PGC1α and PPARα. Additionally, CA treatment downregulated ACC2 mRNA expression, which, as discussed above, generates malonyl-CoA and inhibits the activity of CPT1A (Figure 7).

In addition, CA is believed to be of relatively low toxicity [13, 15, 22], and novel formulations are being developed to boost its bioavailability [11]. Thus, CA may be an ideal alternative treatment for NAFLD. However, further experiments, especially comprehensive investigations, such as

omics investigations, are still required to explore the mechanisms of the beneficial effects in more detail. It is also necessary to determine the toxic and effective dosages of CA prior to its clinical use.

5. Conclusion

In conclusion, this study examined CA's effect on lipid metabolism in OA-treated HepG2 cells and mice with NAFLD and investigated the underlying mechanisms. CA showed a therapeutic effect against hyperlipidemia both *in vitro* and *in vivo*. In terms of the possible mechanisms, CA downregulated the transcription factors PPARγ, SREBP, LXRα, and ChREBP, and their target genes ACLY, ACC, FAS, and SCD1. CA also upregulated PGC1α, PPARα, and CPT1A and downregulated CD36. Taking all the evidence together, it is presumed that CA suppresses IHTG accumulation and ameliorates NAFLD by inhibiting lipogenesis and fatty acid intake, as well as promoting hepatic fatty acid oxidation.

Abbreviations

ACC: Acetyl-CoA carboxylase
ACLY: ATP-citrate lyase
BDK: Branched-chain ketoacid dehydrogenase kinase
BSA: Bovine serum albumin
CA: Cinnamic acid
ChREBP: Carbohydrate-responsive element-binding protein
CMC: Carboxymethyl cellulose
CPT1A: Carnitine palmitoyltransferase-1A
DAB: 3,3′-Diaminobenzidine
DNL: *De novo* lipogenesis
EDTA: Ethylenediaminetetraacetic acid
FAS: Fatty acid synthase
FFA: Free fatty acid
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HDL: High-density lipoprotein
IHTG: Intrahepatic triglyceride
LCFA: Long-chain fatty acid
LDL: Low-density lipoprotein
LXRs: Liver X receptors
NAFLD: Nonalcoholic fatty liver disease
PGC1α: Peroxisome proliferator-activated receptor-γ coactivator 1α
PPARα: Peroxisome proliferator-activated receptor α
PPARγ: Peroxisome proliferator-activated receptor γ
PPM1K: Branched-chain ketoacid dehydrogenase phosphatase
RIPA: Radioimmunoprecipitation assay
SCD1: Stearoyl-CoA desaturase 1
SREBP1c: Sterol regulatory element-binding protein 1c
TC: Total cholesterol
TG: Triglyceride.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. A preprint of the manuscript has previously been published [61].

Ethical Approval

All animal experiments were approved by the Animal Care and Ethics Committee of Beijing University of Traditional Chinese Medicine (approval code no. BUCM-4-20190931002-1088).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Y.W. and M.W. conceptualized the study. Y.W., M.W., and T.Y. developed the methodology. L.Q. performed validation. Y.W., M.W., T.Y., Y.H., and D.Z. performed investigation. Y.W. wrote the original draft. T.Y. and L.Q. reviewed and edited the article. Y.W. and T.Y. performed visualization. L.W. and T.L. supervised the study. L.W. and T.L. were responsible for funding acquisition. All the authors have read and agreed to the published version of the manuscript. You Wu, Minghui Wang, and Tao Yang contributed equally and share the first authorship.

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Research Article

Pistachio Hull Extract as a Practical Strategy to Extend the Shelf Life of Raw Minced Beef: Chemometrics in Quality Evaluation

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The agricultural processing industry produces a notable quantity of by-products rich in bioactive compounds, which can be exploited for agri-food applications. From pistachio industrial processing, pistachio's hull is one of the major by-products. This work aimed to evaluate the potential of pistachio hull, as a potential source of natural antioxidant, to preserve the meat quality. Here, we investigated the impact of aqueous pistachio hull extract (PHE) at 0.156% (PHE1), 0.312% (PHE2), and 0.625% (PHE3) on the quality of raw minced beef meat stored for 14 days at 4°C. At the end of storage, mesophilic total viable plate, psychotropic and Enterobacteriaceae counts, showed significantly lower ($P < 0.05$) microbial count in PHE samples. PHE3 revealed a powerful inhibitory effect on lipid/protein oxidation, and sensory characteristics were positively ($P < 0.05$) affected. Principal component analysis and heat map indicated complex and close synchronized relations among lipid/protein oxidation processes, microbial loads, and sensory attributes. Obtained results using univariate and multivariate statistical analysis underlined the importance of using different mathematical approaches, which are complementary to each other and could provide considerable information about the minced beef meat treated by PHE. Therefore, compared to synthetic antioxidants, PHE could be a clean-label alternative that can protect and enhance the quality of meat products.

1. Introduction

Extending the shelf life of meat and meat products through the control of chemical processes and microbial contamination, both within and upon product surfaces, is important to guarantee that the safety, quality, and nutritional status of products are preserved throughout the distribution chain for as long as possible, effectively attaining consumers for consumption [1–3]. Nowadays, the use of antioxidants from plant matrices and their by-products emphasizes the necessity of antioxidant solutions for the meat industry since consumers perceive them as safe and are Generally Recognized as Safe (GRAS) [4, 5]. This strategy is becoming an attractive strategy in order to enhance quality- and health-related characteristics of meat products. Some plants such as *Hibiscus sabdariffa* L. [6], *Punica granatum* [7], *Ephedra alata* [8], *Vachellia nilotica* [9], and *Ilex meserveae* [10] extracts are incorporated in different beef meat products as "natural antioxidants." These active compounds can maintain characteristics of beef meat by retarding chemical oxidation and minimizing microbial spoilage caused by aerobic microorganisms and therefore prolonging the shelf life.

In the world, *Pistacia vera* L. is the most industrialized variety; nevertheless, it produces important quantities of by-products, which are considered as waste and can lead to

environmental problems [11]. Conversely, these by-products, especially the hull, have diverse valuable phytochemical groups. Based on the chemical composition, many studies are conducted on pistachio hull extracts displaying that it contains different types of antioxidants, including anthocyanins, flavan-3-ols, proanthocyanidins, flavonols, isoflavones, flavanones, stilbenes, and phenolic acids [11, 12]. In addition, some functional properties of pistachio hull extracts have been previously studied in terms of antioxidant [13–15] and antimicrobial activity [16]. Pistachio hull extracts could be considered as a suitable additive in food industries due to the presence of valuable compounds that established multiple functional effects. Recently, Abolhasani et al. [14] and Fattahifar et al. [17] revealed that pistachio hull can be useful in prolonging browning reactions in some foodstuffs due to its the antityrosinase activity. Furthermore, the antioxidant and antimicrobial properties of pistachio hull extracts have been studied in some food formulations such as chicken burger [18]. It was also reported that it could delay soybean oil fat oxidation [15, 19] and rheological and sensory properties of marmalade [20].

Actually, continuous research studies are in progress to elaborate efficient and healthy natural substrates for application in meat preservation and in light of the multiple issues that pistachio hull extract (PHE) could resolve it. The present study investigated the benefit that PHE might provide in terms of the chemical, microbiological, and sensory attributes of minced beef meat. The study aimed to understand the links between quality parameters and analyses by PCA and heat map in order to provide more information about sample distribution at different storage time periods.

2. Materials and Methods

2.1. Plant Material and Extraction. Pistachio (*Pistacia vera* L) hulls were harvested and sampled in August 2020 from farms located in Sfax (N: 34.4426°, E: 10.4537°). The hulls were ground to a fine powder using an electric grinder (Moulinex, France). Then, the powder was extracted by mixing with distilled water (ratio of 1 : 8) and stirring overnight at room temperature using a magnetic stirrer. Later, all the samples were centrifuged (Sorvall Biofuge Stratos, ThermoScientific, Hanau, Germany) at 12,000x for 20 min. The aqueous supernatant was freeze-dried (Martin Christ, Alpha 1–2 LD plus Germany) and stored at −20°C for more application and analysis.

PHE was used as biopreservative in minced beef for its richness in phenolics.

Antioxidant and antimicrobial properties of PHE were previously demonstrated by Elhadef et al. [13]. Taking into account its originality, total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and total anthocyanin content (TAC) are 182.11 mg GAE/g, 15.54 mg QE/g, 68.24 mg CE/100 g, and 40.98 μg cyanidin-3-O- glucoside/g, respectively. Regarding antioxidant activity, evaluated by ABTS and DPPH scavenging assays, concentrations providing 50% of radical scavenging activity (EC50) of PHE are 0.19 and 0.09 mg/mL. For antibacterial activity, assessed by the agar diffusion method and evaluated by measuring the diameters of circular inhibition zones around the wells, PHE displayed the diameters of inhibition zones 14.5, 17.25, 16.25, 14.25, 14.25, and 15.75 mm, respectively, against *S. aureus*, *L. monocytogenes*, *B. cereus*, *S. enterica*, *E. coli,* and *P. aeruginosa*, respectively [13].

2.2. Preparation of Minced Beef Meat Samples. We bought fresh beef meat from a regional slaughterhouse located in Sfax. The beef meat was grounded using a sterile meat grinder. Then, we divided the raw minced beef meat in five lots: lot 1 and lot 2 were used as controls (lot 2 was supplemented with BHT at 0.01%), PHE was added to the minced beef at three concentrations equivalent to MIC (0.156% (*v*/*w*) (PHE1)), 2 × MIC (0.312% (*w*/*v*) (PHE2)), and 4 × MIC (0.625% (*w*/*v*) (PHE3)) against *L. monocytogenes* ATCC19117 [13]. We followed the same protocol described by Elhadef et al. [8] to make a homogeneous mixture of each treatment and then we kept them under vacuum using plastic bags to contribute three replicates. Finally, all aliquots were saved for 14 days at 4°C ± 1°C, and quality characteristics were analyzed in days 0, 3, 7, 10, and 14.

The total number of analyzed samples was 225 (75 × 3). For microbiological physicochemical and sensory tests, 75 trials (5 × 3 × 5) were used, obtained as follows: five treatments (C, BHT, PHE1, PHE2, and PHE3) for three subsamples and for each ageing period (five storage periods: 0, 3, 7, 10, and 14 days).

2.3. Analysis of Meat Samples

2.3.1. Microbiological Analysis. 25 g samples were mixed in 225 mL of sterile 0.85% NaCl solution for 10 min. The aerobic plate count (APC) was enumerated on plate count agar (PCA, Oxoid, UK) incubated at 30°C for 48 h [21, 22]. The aerobic psychrotrophic count (PTC) was determined on plate count agar (PCA) incubated at 7°C for 10 days [23]. Enterobacteriaceae count was enumerated on violet red bile glucose medium (VRBG, Oxoid, UK), incubated at 37°C for 24 h [24]. Results were calculated and expressed as $\log_{10}$ CFU (colony forming units)/g of meat.

2.3.2. Physicochemical Analysis

(1) Protein Oxidation. Protein oxidation was estimated in terms of formation rate of metmyoglobin (MetMb %) and carbonyl groups:

(1) *Metmyoglobin (MetMb %).* MetMb was evaluated following the procedure described by Krzywicki [25]. 5 g of each aliquot was mixed with 25 mL of 0.04 M K_3PO_4 buffer (pH 6.8). Homogenates were held in an ice bath for 1 h to process complete extraction and centrifuged at 4500 ×g for 30 min. Finally, the absorbance was calculated at 525 (A525), 572 (A572), and 700 (A700) nm. The MetMb% was quantified using the following equation:

$$\text{MetMb\%} = \left[-2.51\left(\frac{A_{572}}{A_{525}}\right) + 0.777\left(\frac{A_{565}}{A_{525}}\right) + 0.8\left(\frac{A_{545}}{A_{525}}\right) + 1.098\right] \times 100. \quad (1)$$

(2) *Determination of Carbonyls*. Carbonyl groups were detected by their reactivity with 2,4-dinitrophenylhydrazine (DNPH) to form protein hydrazones following the method of Ariga [26]. 1 g was suspended in phosphate buffer (20 mM, pH 6.0) to 5 mg/mL. Two samples (400 μL/each) were collected from each aliquot solution: one sample was combined with 800 μL of 2 M HCl including DNPH at 0.2% (w/v); the other sample was combined with 800 μL of 2 M HCl (blank sample). After that, aliquot was precipitated with 400 μL of trichloroacetic acid (10%, w/v) and centrifuged at 5000 × g for 5 min. The pellet was homogenized with 1 mL of ethanol-ethyl acetate solution (1 : 1, v/v) and centrifuged under the same condition. This process was repeated twice. Then, 1.5 mL of 20 mM NaH_2PO_4 (pH 6.5) including 6 M guanidine hydrochloride was added. The absorbance was calculated at 370 nm. The protein carbonyl content was calculated in accordance with a molar extinction coefficient (22000 M^{-1} cm^{-1}) and expressed in nmol carbonyl mg/protein.

(2) Lipid Oxidation. Lipid oxidation was estimated based on the primary lipid oxidation compound (peroxide value (PV) and conjugated dienes (CD)) and secondary lipid oxidation products (malondialdehyde):

(1) *Peroxide Value (PV)*. PV was assessed according to the ISO 960:2 (2007) [27]. Fatty fraction was extracted with chloroform, and later, oxidation of potassium iodate to iodine form was done by active oxygen in the presence of acetic acid. The amount of iodine generated was then determined by volumetric titration with sodium thiosulphate, and values were expressed in meq of peroxide/kg of meat.

(2) *Conjugated Dienes Hydroperoxides (CDs)*. 0.5 g of each sample of beef meat was suspended in 5 mL of distilled water and mixed. A 0.5 mL sample of this suspension was mixed with 5 mL of extracting solution, hexane: isopropanol, at 3 : 1 (v/v) for 1 min and centrifuged at 2000 × g for 5 min. Absorbance of the supernatant was measured at 233 nm. CD was calculated using the molar extinction coefficient of 25200 M^{-1} cm^{-1}, and the results were expressed as μmol/mg [28].

(3) *Thiobarbituric Acid Reactive Substances (TBARS) Value*. Two grams of the sample, combined with 100 μL of butylated hydroxyl toluene in ethanol (1 g/L) and 16 mL of TCA at 50 g/L, was mixed for 10 min and filtered. 2 mL of the filtrate (or 2 mL of TCA for blank) was added to 2 mL of thiobarbituric acid solution (20 mol/L). Absorbance was measured against the blank at 508, 532, and 600 nm. The absorbance was corrected for the baseline drift as follows:

$$A_{532\,\text{nm corrected}} = A_{532\,\text{nm}} - \left[\left(A_{508\,\text{nm}} - A_{600\,\text{nm}}\right) \times \frac{(600-532)}{(600/508)}\right] - A_{600\,\text{nm}}. \quad (2)$$

TBARS values were expressed as mg of malonaldehyde equivalent per kg of sample (mg/kg) with the molar extinction coefficient of the MDA-TBA adducts at 532 nm (1.56 × 10^5 M^{-1} cm^{-1}) [29]. MDA was determined using the following equation:

$$\text{MDA}\left(\frac{\text{mg}}{\text{kg of meat}}\right) = \left[\frac{A_{\text{corrected}} \times \text{VTCA} \times 2 \times \text{MMDA} \times 0.01}{1.56 \times \text{m}}\right]. \quad (3)$$

2.3.3. Sensory Evaluation. Twenty trained members of the panel conducted sensory evaluation. Sensory attributes including color, appearance, odor, and overall acceptability (OA) were assessed on days 0, 3, 7, 10, and 14 of storage at 4°C by using a 9-point hedonic scale. Attribute scales varied from 1 to 9 with 9 being very good, 5 being the limit of acceptability, and 1 being very bad. A score below 5 indicated the sample to be unacceptable [30].

2.4. Statistical Analysis. Measurements were done 0, 3, 7, 10, and 14 days, and experiments with five treatments were used in a randomized complete block design. All analytical determinations were performed in triplicate. A two-way analysis of variance (ANOVA) with two factors (treatments and storage time) was carried out using SPSS 19 statistical package (SPSS Ltd., Woking, UK). Means and standard deviations were calculated and a probability level of $P < 0.05$ was used in testing the statistical significance of all experimental data. Tukey's post hoc test was used to determine significance of mean values for multiple comparison at $P < 0.05$.

To group samples based on chemical oxidation microbial counts and sensory traits during storage, all variables were autoscaled prior to chemometrics application. By using XLSTAT software for Windows (v.2014.1.08, Addinsoft, New York, USA), principal component analysis (PCA) and heat maps were performed to distinguish between samples at 0, 3, 7, 10, and 14 days. For all samples, dendrograms were established to obtain a two-dimensional projection of the dissimilarity or similarity of the entire sample set.

3. Results and Discussion

3.1. Microbial Analysis. During storage time, we observed a significant growth of all microbial counts ($P < 0.05$), mainly in control and BHT samples (Table 1). Also, a significant decrease in APC growth rate ($P < 0.05$) was induced by PHE

Table 1: Effect of PHE on the microbial load of aerobic plate count (APC), psychrotrophic count (PTC), and Enterobacteriaceae count of raw minced meat beef stored at 4°C.

	Day	Control	BHT	PHE1	PHE2	PHE3
APC	0	2.22 ± 0.10^{aA}	2.21 ± 0.09^{aA}	2.23 ± 0.07^{aA}	2.22 ± 0.10^{aA}	2.22 ± 0.09^{aA}
	3	5.34 ± 0.25^{dB}	4.95 ± 0.23^{bB}	5.17 ± 0.18^{cB}	4.9 ± 0.22^{bB}	4.07 ± 0.2^{aB}
	7	7.65 ± 0.37^{dC}	6.27 ± 0.31^{bC}	6.86 ± 0.33^{cC}	5.69 ± 0.27^{aC}	5.5 ± 0.19^{aC}
	10	8.24 ± 0.41^{eD}	6.65 ± 0.30^{bC}	7.07 ± 0.32^{dCD}	6.47 ± 0.31^{cD}	6.12 ± 0.27^{aD}
	14	11.95 ± 0.59^{dE}	7.19 ± 0.29^{bcD}	7.27 ± 0.26^{cD}	7.09 ± 0.34^{bE}	6.68 ± 0.32^{aE}
PTC	0	2.08 ± 0.08^{aA}	2.05 ± 0.09^{aA}	2.05 ± 0.07^{aA}	2.02 ± 0.03^{aA}	2.02 ± 0.04^{aA}
	3	4.32 ± 0.17^{dB}	3.72 ± 0.17^{cB}	3.47 ± 0.17^{bB}	3.39 ± 0.16^{abB}	3.25 ± 0.10^{aB}
	7	6.32 ± 0.31^{eC}	5.54 ± 0.26^{cC}	5.89 ± 0.22^{dC}	5.03 ± 0.24^{bC}	4.36 ± 0.16^{aC}
	10	7.36 ± 0.29^{dD}	5.78 ± 0.27^{Bc}	6.1 ± 0.29^{cC}	5.93 ± 0.28^{bcD}	5.2 ± 0.22^{aD}
	14	9.25 ± 0.42^{dE}	6.18 ± 0.30^{bD}	6.51 ± 0.31^{cD}	6.26 ± 0.26^{bE}	6.04 ± 0.25^{aE}
Enterobacteriaceae counts	0	<1	<1	<1	<1	<1
	3	2.22 ± 0.1^{dA}	1.49 ± 0.07^{cA}	1.22 ± 0.06^{bA}	1.15 ± 0.05^{abA}	1.09 ± 0.04^{aA}
	7	2.89 ± 0.14^{cB}	1.92 ± 0.09^{bB}	1.89 ± 0.09^{bB}	1.33 ± 0.06^{aA}	1.29 ± 0.06^{aB}
	10	3.21 ± 0.16^{dC}	2.29 ± 0.1^{cC}	2.11 ± 0.09^{bcB}	1.89 ± 0.1^{bB}	1.51 ± 0.08^{aC}
	14	3.54 ± 0.17^{bD}	2.57 ± 0.12^{bD}	2.41 ± 0.11^{bC}	1.91 ± 0.1^{aB}	1.88 ± 0.09^{aD}

Values with a different letter (a–c) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–D) of the same concentration are significantly different.

addition. The microbial spoilage of meat occurs when APC and PTC reach 6.7-log CFU/g [31]. Control samples touched limits of shelf life on the 7th day; however, PHE3 reached on day 14. PTC registered for PHEs was noted to lower the detection limits until the 14th day. On the other hand, PHE reduced successfully the Enterobacteriaceae counts in meat. After 14 days, PHE1, PHE2, and PHE3 delayed Enterobacteriaceae counts to 1.13, 1.63, and 1.66 log CFU/g, therefore extending the shelf life until 14 days. In the same way, Elhadef et al. [13] mentioned that aqueous pistachio hull extracts contained phenolic compounds that have an inhibitory effect on various food-borne pathogens. Furthermore, TPC, TFC, and TAC have been exceedingly associated with antibacterial activity. These authors, also, demonstrated that *Ephedra alata* aqueous extract, used at 0.156, 0.312, and 0.624%, has an antimicrobial potential on minced beef meat during its refrigeration and storage.

3.2. Oxidative Stability Evolution

3.2.1. Protein Oxidation. Color is the most important factor in meat products influencing the consumer purchase decision and affecting the perception of freshness. Purchasing intent of fresh meat by consumers is based largely on Mb content of muscles and it is often implicated in its color stability. In fact, higher Mb concentrations lead to rapid oxidation and a decrease in color stability in beef muscles. The consumer rejection occurred at 40% MetMb in meat products [2]. As shown in Figure 1, antioxidants (BHT and PHE) are efficient ($P < 0.05$) in avoiding MetMb oxidation and maintaining the red color of beef meat until the 14th day of storage. Direct oxidation of the side chains from Lys, Thr, Arg, and Pro can module carbonyls (ketones and aldehydes) in proteins [32]. Control and BHT samples presented significantly ($P < 0.05$) higher amounts of protein carbonyls as compared to the treated ones during sampling days (Figure 2). Similarly, the decline in carbonyl groups was previously disclosed using beef patties [33], beef meat balls [34], and minced beef meat [7, 8, 13]. Thus, protein degradation, denaturation, and loss of functionality are due to the formation of protein carbonyls from amino acid side chains caused by the impairment of myofibrillar protein conformation [35].

3.2.2. Lipid Oxidation. In order to evaluate the PHE impact on lipid oxidation, primary (PV and CD) and secondary (TBARS) product concentrations were measured. During storage, in all treatment, PV increased significantly ($P < 0.05$). Except day 0, meat samples incorporated with 0.156, 0.325, and 0.625 mg/g showed significantly lower PV than control samples (Figure 3). On the other hand, we noticed that PV did not exceed the detection limit (25 meq O_2 kg/lipid), which was reported by Sallam et al. [36].

Regarding CD, formed by polyunsaturated acid oxidation, we distinguished a continuous decrease in their formation in meat treated with PHE at 0.325 and 0.625 mg/g (Figure 4). These results were in accordance with studies done by Elhadef et al. [8].

TBARS values resulted from their reaction with free amino acids, proteins, and peptides that are present in the minced meat, to form Schiff's bases, apart from the breakdown of the malonaldehyde due to tertiary degradation [37]. The increase of TBARS depended on the time of storage (Figure 5). Throughout the whole period of storage, TBARS value of control samples was greater than that of PHE groups. Remarkably, after 14 days, TBARS value of treated samples (BHT and PHEs) seemed to be significantly lower ($P < 0.05$) than in control and they were lower than 2 mg/kg (the acceptable sensory threshold limit) [38]. PV, CD, and TBARS were significantly ($P < 0.05$) lower in meat treated with PHE compared to control and BHT samples. The observed differences could be explicated by the presence of antioxidants in the PHE, which delay the lipid oxidation processes [11].

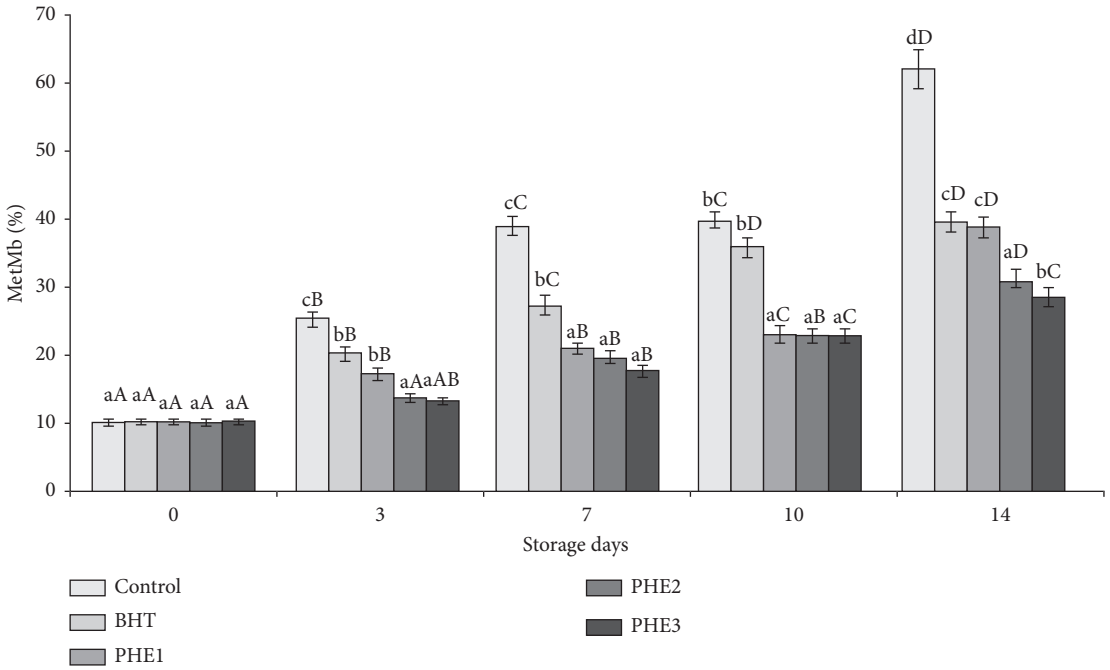

Figure 1: Effect of PHE on MetMb of raw minced meat beef stored at 4°C. Values with a different letter (a-b) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–D) of the same concentration are significantly different.

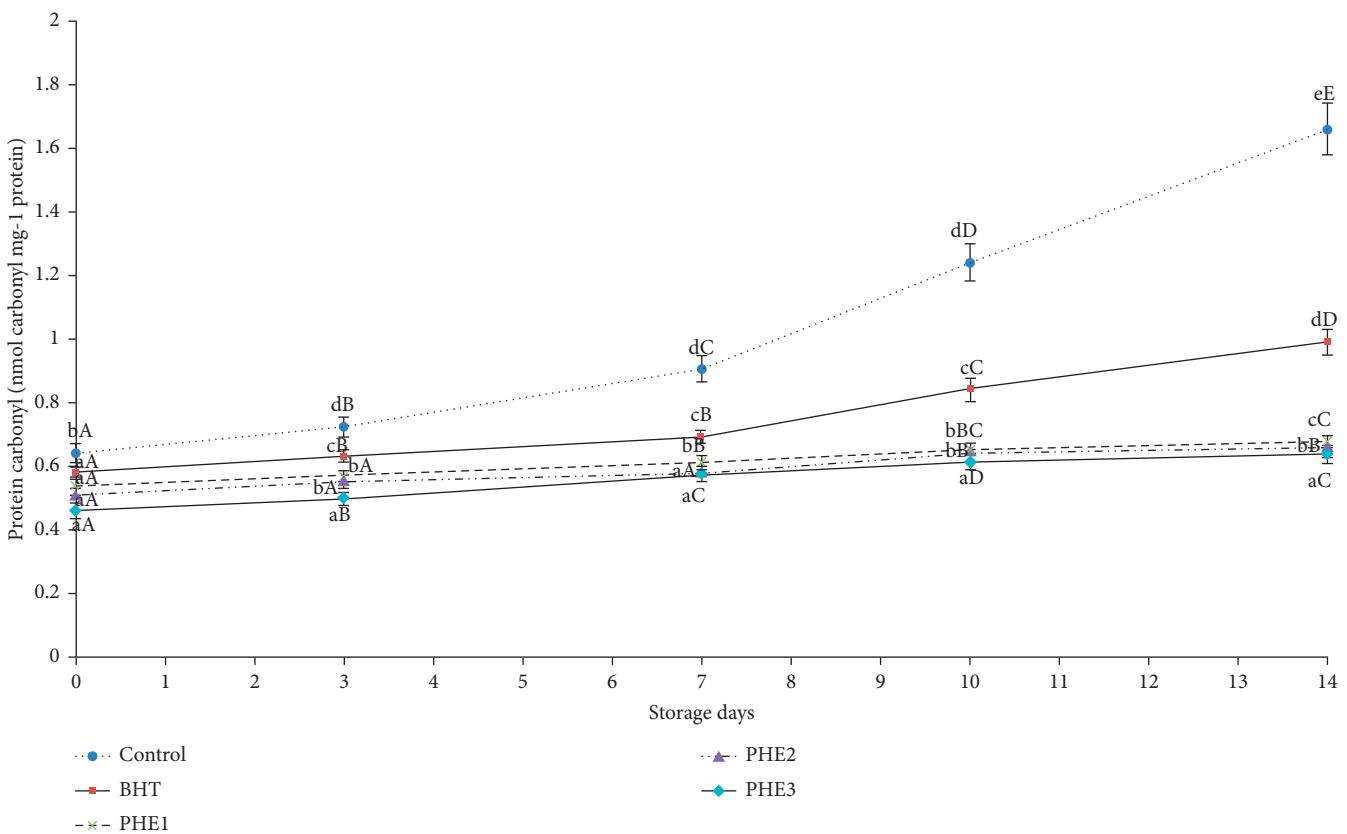

Figure 2: Effect of PHE on protein carbonyl (nmol carbonyl/mg of protein) of raw minced meat beef stored at 4°C. Values with a different letter (a–d) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–D) of the same concentration are significantly different.

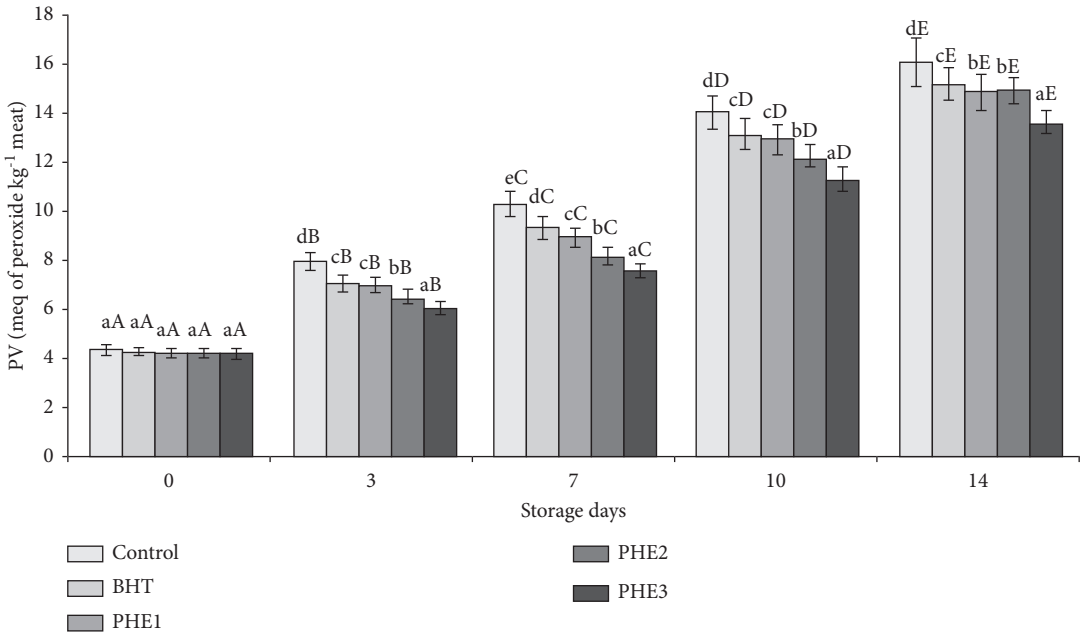

Figure 3: Effect of PHE on peroxide values (meq of peroxide/kg of meat) of raw minced meat beef stored at 4°C. Values with a different letter (a–d) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–E) of the same concentration are significantly different.

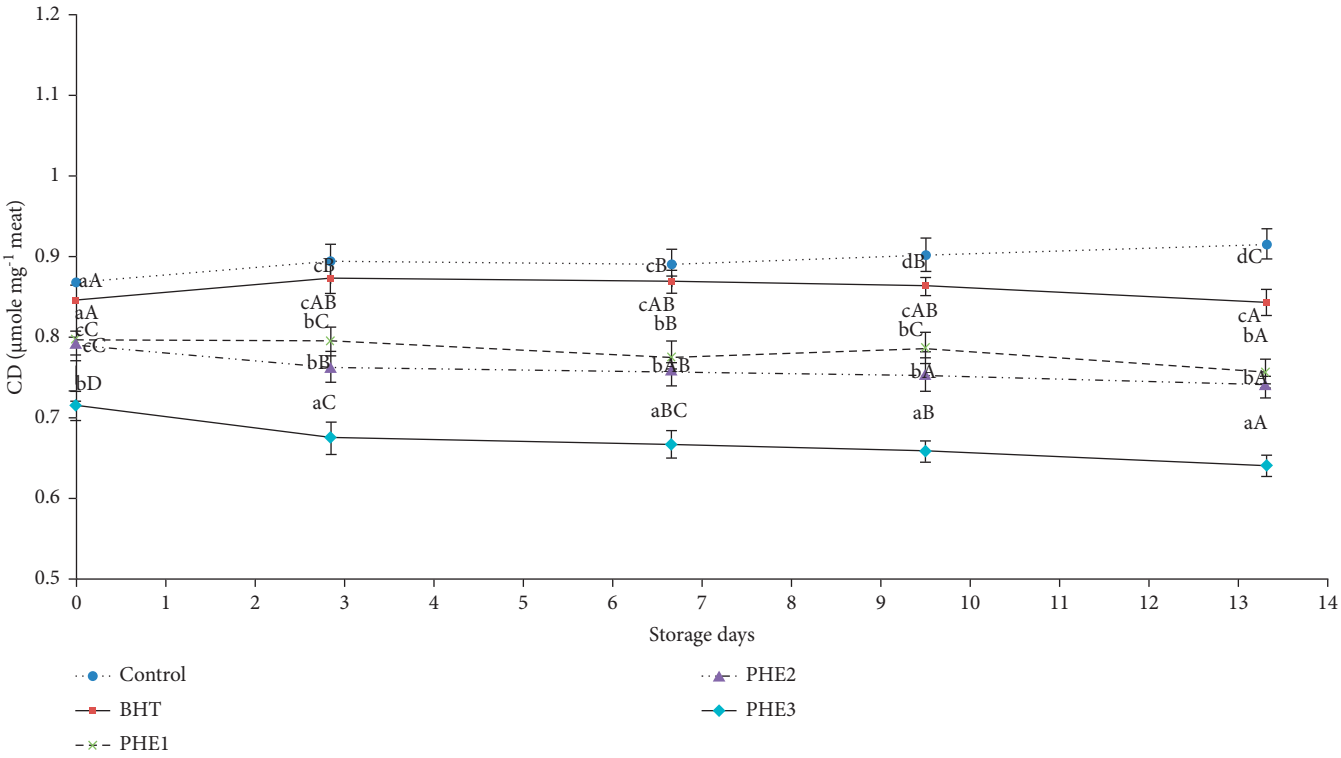

Figure 4: Effect of PHE on conjugated diene hydroperoxides (μmole/mg meat) of raw minced meat beef stored at 4°C. Values with a different letter (a–d) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–D) of the same concentration are significantly different.

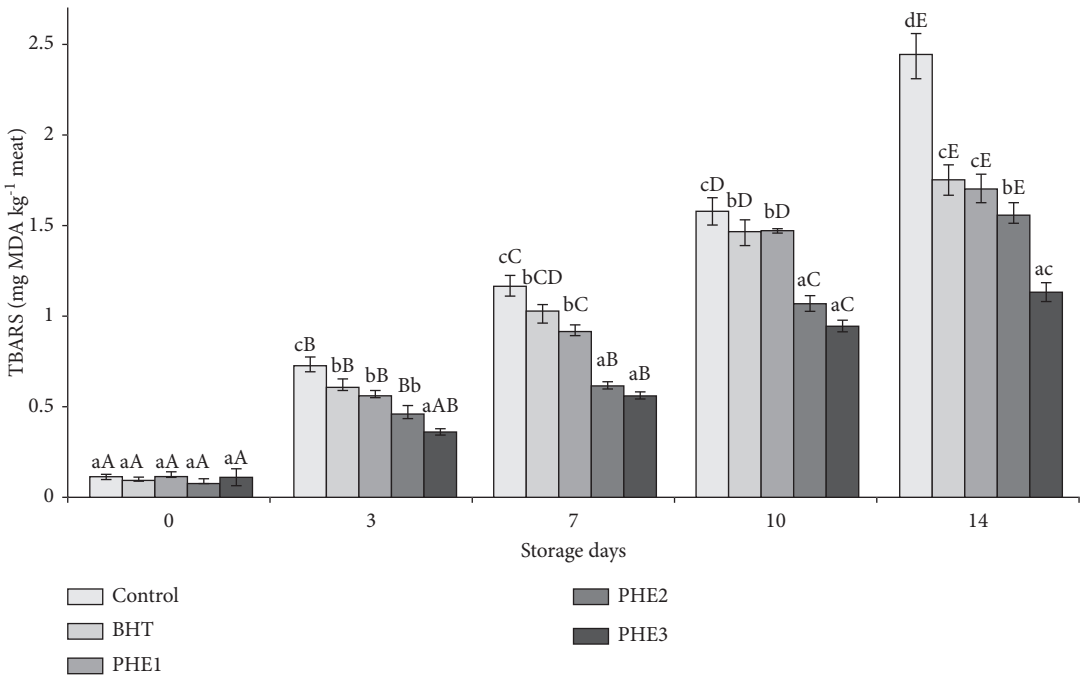

Figure 5: Effect of PHE on TBARS (mg/kg meat) of raw minced meat beef stored at 4°C. Values with a different letter (a–d) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–E) of the same concentration are significantly different.

Table 2: Effect of PHE on appearance, color, odor, and overall acceptability of raw minced meat beef stored at 4°C.

	Day	Control	BHT	PHE1	PHE2	PHE3
Appearance	0	6.63 ± 0.32^{aE}	6.6 ± 0.31^{aD}	6.61 ± 0.3^{aC}	7.05 ± 0.31^{Be}	7.05 ± 0.3^{bD}
	3	6.2 ± 0.30^{aD}	6.28 ± 0.29^{aC}	6.5 ± 0.26^{bC}	6.75 ± 0.27^{cD}	6.9 ± 0.31^{dD}
	7	5 ± 0.24^{aC}	5.7 ± 0.24^{bB}	6.1 ± 0.28^{cB}	6.15 ± 0.24^{cC}	6.21 ± 0.22^{cC}
	10	4.2 ± 0.21^{aB}	5.2 ± 0.22^{bA}	5.26 ± 0.19^{bA}	5.6 ± 0.26^{cB}	5.69 ± 0.2^{cB}
	14	3.1 ± 0.15^{aA}	5.13 ± 0.14^{bA}	5.19 ± 0.12^{bA}	5.2 ± 0.21^{bA}	5.26 ± 0.18^{bA}
Color	0	6.55 ± 0.29^{aE}	6.49 ± 0.23^{aE}	6.6 ± 0.27^{aD}	6.72 ± 0.29^{aD}	6.78 ± 0.3^{aC}
	3	6.1 ± 0.27^{aD}	6.09 ± 0.22^{aD}	6.25 ± 0.28^{bC}	6.33 ± 0.24^{bC}	6.54 ± 0.29^{cC}
	7	5.75 ± 0.27^{aC}	5.75 ± 0.19^{aC}	6 ± 0.27^{bB}	6.2 ± 0.22^{cC}	6.5 ± 0.29^{dC}
	10	4.8 ± 0.21^{aB}	5.25 ± 0.22^{bB}	5.25 ± 0.23^{bA}	5.4 ± 0.22^{cB}	5.8 ± 0.26^{dB}
	14	3.2 ± 0.14^{aA}	4.5 ± 0.22^{bA}	5.15 ± 0.23^{cA}	5.16 ± 0.19^{cA}	5.5 ± 0.19^{dA}
Odor	0	6.2 ± 0.21^{aE}	6.19 ± 0.21^{aC}	6.42 ± 0.29^{bE}	6.75 ± 0.3^{cE}	6.91 ± 0.31^{dE}
	3	5.3 ± 0.2^{aD}	5.9 ± 0.26^{bC}	$6.1 \pm 0.27b^{cD}$	6.27 ± 0.28^{cD}	6.3 ± 0.28^{cD}
	7	5 ± 0.11^{aC}	5.45 ± 0.21^{bB}	5.75 ± 0.25^{cC}	5.87 ± 0.24^{cC}	6.1 ± 0.27^{dC}
	10	4.15 ± 0.18^{aB}	5.1 ± 0.22^{bA}	5.4 ± 0.22^{cB}	5.4 ± 0.2^{cB}	5.6 ± 0.25 dB
	14	3.5 ± 0.15^{aA}	5 ± 0.21^{bA}	5 ± 0.22^{bA}	5.16 ± 0.23^{bcA}	5.33 ± 0.27^{cA}
Overall acceptability	0	6.52 ± 0.29^{aD}	6.52 ± 0.29^{aD}	6.57 ± 0.22^{aD}	6.6 ± 0.29^{aD}	6.61 ± 0.28^{aD}
	3	5 ± 0.2^{aC}	5.95 ± 0.27^{bcC}	5.8 ± 0.26^{bC}	6.1 ± 0.27^{cC}	6.18 ± 0.27^{cC}
	7	4.3 ± 0.19^{aBC}	5.63 ± 0.25^{bB}	5.58 ± 0.24^{bB}	5.75 ± 0.25^{bB}	5.81 ± 0.26^{bB}
	10	4 ± 0.18^{Ab}	5.15 ± 0.1^{bA}	5.25 ± 0.11^{bcA}	5.33 ± 0.23^{cA}	5.52 ± 0.21^{dA}
	14	3.3 ± 0.11^{Aa}	5 ± 0.11^{bA}	5.14 ± 0.14^{bcA}	5.25 ± 0.24^{cA}	5.52 ± 0.22^{dA}

Values with a different letter (a–c) of the same storage day are significantly different ($P < 0.05$); values with a different letter (A–D) of the same concentration are significantly different.

3.3. Sensory Analysis. Sensory results of minced beef meat were assessed during all the storage periods (Table 2). If the sensory score >5, the meat samples are considered suitable for human consumption [39]. Appearance, color, odor, and overall acceptability were given unacceptable scores by the 7th day for control group, 10th day for BHT group, and up to 14th day for PHE groups. Oxidative changes, related to protein and lipid oxidation, and microbial growth influence the sensory quality, which can be enhanced by PHE addition. Earlier researchers have reported a similar trend of quality change [18, 33].

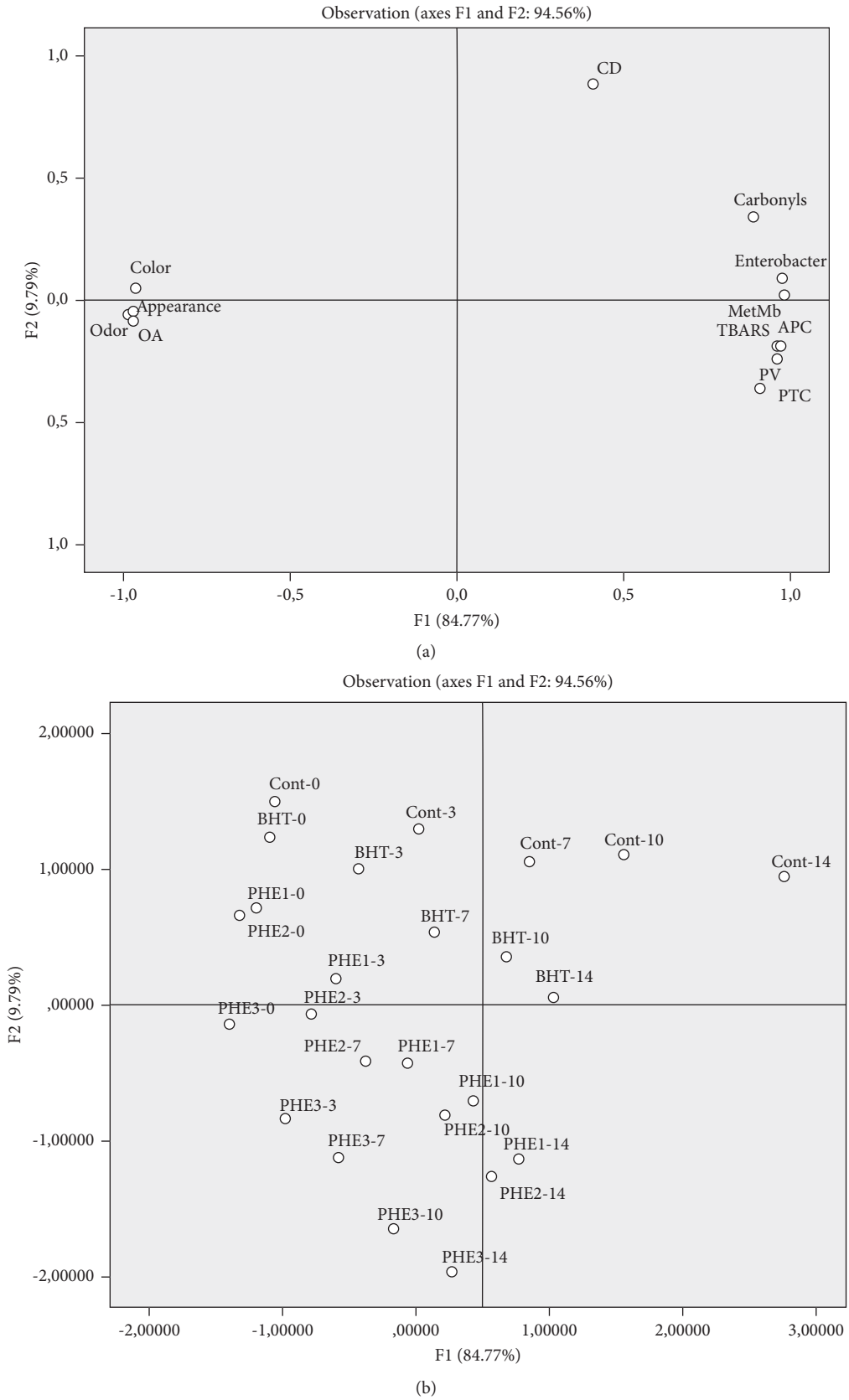

Figure 6: Principal component analysis (PCA) plots of physicochemical parameters, microbial loads, and sensory characteristics of different treated and untreated samples at each storage time: (a) variable-loading plot of PCA; (b) observation score plot of PCA.

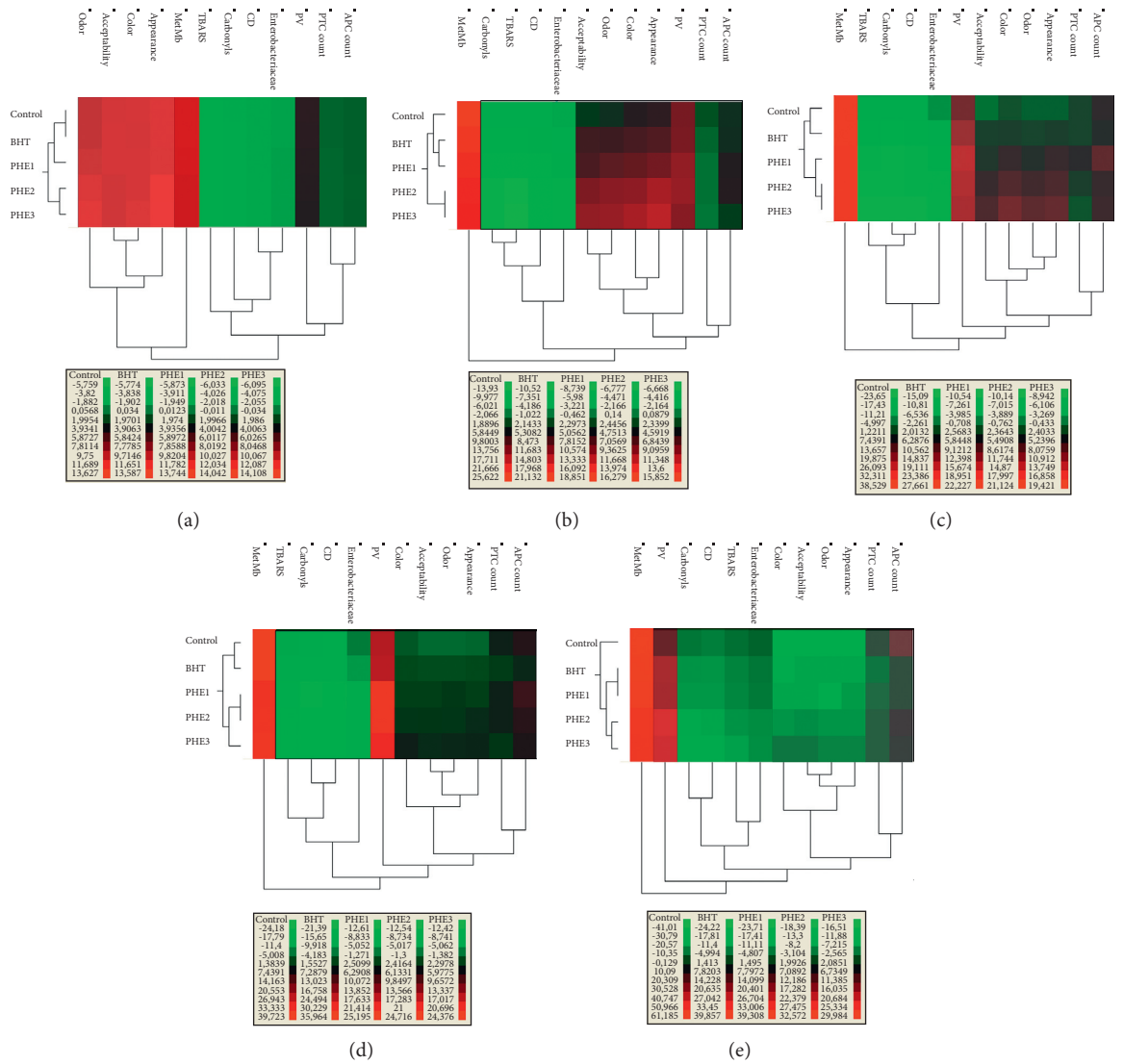

FIGURE 7: Agglomerative hierarchical cluster analysis (HCA) and heat map of physicochemical parameters, microbial loads, and sensory characteristics of different treated and untreated samples at each storage time periods: (a) 0 days, (b) 3 days, (c) 7 days, (d) 10 days, and (e) 14 days.

3.4. Chemometric Analysis

3.4.1. PCA. In order to classify the studied samples according to the traits described above, PCA was used to confirm the cluster analysis results (Figure 6). Thus, we performed a PCA as a means to reduce the multidimensional structure of the data and to provide a two-dimensional map explaining the observed variance. The PCA accounted for 94.56% of the variance of the original data (Dim 1 : 84.77%, Dim 2 : 9.79%) (Figure 6(a)). A high correlation was observed between protein oxidation (carbonyls and MetMb), lipid oxidation (TBARS and PV), and microbial load (PTC, APC, and Enterobacteriaceae counts) which support the suggested interaction between lipid/protein oxidation and microbial growth. In addition, the increase of the storage time led to the disposition of the samples towards the right side of the PCA, which were designated by a high concentration of primary and secondary lipid and protein oxidation products and high microbial load (Figure 6(b)). In this regard, a recent research paper indicates that protein/lipid oxidation and microbial growth occur simultaneously [8]. Protein oxidation generates protein aggregates through the formation of disulfide bonds, which can delay with muscle proteolysis. This latter phenomenon induces the formation of small molecular components, principally composed by polypeptides, peptides, free amino acids, and amines and further enzymatic and chemical reactions leading to the release of nonprotein nitrogen compounds [40]. Furthermore, these authors demonstrated that aldehyde moieties from lipid oxidation products such as malondialdehyde can covalently bind to amino acid residues, resulting in indirect protein oxidation. On the other hand, the release of free fatty acids from meat lipids is facilitated by the synergistic action of endogenous enzymes

and bacterial lipolytic enzymes [41]. With a shorter storage time (0–3 days), a significant and positive correlation was detected between control, BHT, PHE1, and PHE2 samples, and color. Meanwhile, remarkably, high scores of appearance, odor, and overall acceptability were closer with PHE3 at any storage time (0, 3, 7, 10, and 14 days) (Figure 6(b)). Interestingly, the use of PHE3 in minced beef meat prevents lipid/protein oxidation and allows a larger extent of proteolysis, leading to maintaining oxidation products and sensory attributes till the end of refrigerated storage time.

3.4.2. Heat Map. To summarize quantitative data of the samples regarding the lipid/protein oxidation, microbial growth, and sensory parameters at each storage time, we used the heat map represented in Figure 7. In this regard, the high number of extra correlations is corroborated by the heat map depiction of the correlation analysis. Each parameter was associated with a color: from green for low concentrations to red for high concentrations. At day 0, the present study indicated that MetMb % was the main contributor to the sensory attributes, which can also be influenced by the variation of lipid oxidation, carbonyl contents, and microbial growth. According to the color scale, at day 0, Figure 7(a) expresses four different clusters with a high similarity between control and BHT samples; moreover, PHE1, PHE2, and PHE3 presented dissimilarity in their composition. At days 3 and 7, dendrograms indicated the presence of four clusters: clusters I (Control), II (BHT), III (PHE1), and IV (PHE2 and PHE3). In these sampling days, it is clear that the nodes accumulation of CD, TBARS, and carbonyl contents was influenced by the growth of Enterobacteriaceae count (Figures 7(b) and 7(c)). In addition, dependency relation (PV-sensory attributes) was shown at day 3; however, at day 7, the relation was more significant between ((APC and PTC)-sensory attributes). At the end of storage, four groups were discriminated: clusters I (Control), II (BHT-PHE1), III (PHE2), and IV (PHE3). It should be noted that sensory traits were controlled directly by (APC and PTC), which also can indirectly be influenced by CD and carbonyl contents (Figure 7(e)). In this vein, Elhadef et al. [8], Fourati et al. [7], Nishad et al. [42], and Bouaziz et al. [43] studied the applicability of chemometrics for quality control and authentication of several types of meat and derived products (minced beef and turkey meat) incorporated by various plant (pomegranate peel, *Ephedra alata,* nutmeg, and citrus peel and date palm seeds) extracts.

4. Conclusion

The results of our study revealed that the addition of PHE can decrease lipid and protein oxidation and it can also increase microbiological stability and enhance sensory traits of raw minced beef meat stored at 4°C. During the storage time, a multitude of interactions among compounds derived from lipid/protein oxidation and microbial change contributed, therefore, to the intensification of sensory attributes. The results enabled discrimination of the meat samples, showing a great impact of the extract at three concentrations of PHE on the quality of meat samples. By the end of storage, factors including PV, APC, and PTC play a key role in modulating the sensory profile of the final product. Thus, industrial wastes like pistachio hull could be effectively used to extend the shelf life of refrigerated meat and derived products.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

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Review Article

The Improving Effect and Safety of Probiotic Supplements on Patients with Osteoporosis and Osteopenia: A Systematic Review and Meta-Analysis of 10 Randomized Controlled Trials

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Aim. Probiotics are considered to be bone metabolism regulators, and their efficacy as an adjuvant treatment option for osteoporosis is still controversial. The purpose of this study is to compare the available data from randomized controlled trials (RCT) of probiotics in the treatment of osteoporosis and osteopenia. *Methods*. As of June 2021, databases such as Medline, Embase, Web of Science, and Central Cochrane Library have been used for English-language literature searches and CNKI and China Biomedical Database have been used for Chinese-language literature searches. RevMan 5.3 was used for bias risk assessment, heterogeneity detection, and meta-analysis. This research has been registered in PROSPERO (CRD42020085934). *Results*. This systematic review and meta-analysis included 10 RCTs involving 1156. Compared with the placebo, the absolute value of lumbar spine's BMD was not statistically significant (WMD 0.04 (−0.00, 0.09), $P = 0.07$, random effect model), while the percentage of lumbar spine's BMD was higher (SMD 1.16 (0.21, 2.12), $P = 0.02$, random effect model). Compared with the control group, the percentage of total hip's BMD was not statistically significant (SMD 0.52 (−0.69, 1.73), $P = 0.40$, random effect model). The safety analysis showed that, compared with control group, the adverse events in the experimental group were not statistically significant (RR 1.02 (0.92, 1.12), $P = 0.70$, fixed effect model). *Conclusion*. Probiotics may be safety supplements to improve the lumbar spine's BMD of patients with osteoporosis and osteopenia. More large-sample, random-controlled, high-quality RCTs are needed to further verify the effectiveness and safety of probiotics in intervening osteoporosis or osteopenia.

1. Introduction

Osteoporosis is one of the diseases most closely related to the aging of the social population. It is a common bone disease characterized by bone loss and bone tissue structural degradation [1]. Osteoporotic bone loss usually has no obvious clinical manifestations in the early stage of the disease. However, as the disease progresses and bone mass is continuously lost, the bone microstructure will become more severely destroyed, and patients will have a series of clinical manifestations. For example, patients with osteoporosis can have bone pain, which can occur in the bones of the whole body, or only low back pain; when osteoporosis develops to a serious degree, hunchback and compression fractures can occur. The most serious complication of osteoporosis is osteoporotic fractures, and if such fractures have occurred, the risk of refractures increases significantly [2, 3]. The main cause of osteoporosis is that bone resorption dominated by osteoclasts is greater than bone formation dominated by osteoblasts; that is, bone remodeling has a negative balance [4, 5]. Osteoporosis is currently mainly divided into secondary osteoporosis and primary osteoporosis [6, 7]. The

etiology of secondary osteoporosis is relatively clear, mainly endocrine factors, nutritional factors, disuse factors, genetic factors, immune factors, drug factors, etc. [6]. The onset of primary osteoporosis is related to heredity, aging, hormone levels, immunity, environmental factors, and nutritional status [7, 8]. According to the pathogenesis of osteoporosis, the current treatment needs to be combined with lifestyle adjustment, bone health supplement addition, drug intervention, and rehabilitation [9–11].

Recent studies have found that the intestinal flora is related to the loss of bone mass and the incidence of osteoporosis in the human body. These microorganisms may change the relative activity of osteoclasts and osteoblasts through their own metabolites, affect host metabolism and immune system, and thus affect bone metabolism. Probiotics are currently proven to have an effect on bone metabolism [12–14]. Many studies have also shown that probiotics have health-promoting effects in preventing and curing diseases. For example, probiotics can prevent or treat acute, antibiotic-related and *Clostridium-difficile*-related diarrhea [15, 16], improve inflammatory bowel disease and irritable bowel syndrome (IBS) [17, 18], reduce the risk of late-onset sepsis and necrotizing enterocolitis in newborns [19, 20], and have neuroprotective effects on neurodegenerative diseases (such as Parkinson's) [21, 22]. The same research on the treatment of osteoporosis with probiotics shows that supplementing with probiotics can prevent osteoporosis and bone loss [23, 24]. A number of clinical studies have shown that probiotics can improve the bone condition of patients with osteopenia. However, there is no systematic evaluation and summary of these clinical trials, which makes the evidence scattered and inconsistent, unable to provide new evidence for the clinic and provide new reference value for the next clinical trial design [25–35]. Therefore, this study would conduct a systematic review and meta-analysis to assess the effectiveness and safety of probiotics on postmenopausal women with osteoporosis or osteopenia for the first time, in order to provide new clinical reference information in the future.

2. Materials and Methods

2.1. Protocol. The systematic review and meta-analysis were conducted strictly in accordance with the protocol registered in PROSPERO (CRD42020085934) and PRISMA-guidelines (Supplementary Materials).

2.2. Selection Criteria. (1) Participants are patients who have osteoporosis or osteopenia or may suffer from osteopenia. (2) Intervention: the intervention of the experimental group is probiotics with various preparations and dosages. The intervention of the control group is a placebo or other nonprobiotic intervention methods. (3) Outcomes: primary outcomes are bone mineral density (BMD), adverse events; secondary outcomes are I collagen carboxy terminal peptide (CTX), osteoprotegerin (OPG), Receptor Activator of Nuclear Factor-κ B Ligand (RANKL), and Osteocalcin (OC). (4) Study design is RCTs. (5) Exclusion criteria are Non-RCT.

2.3. Literature Search Strategy. Web of Science, MEDLINE Complete, VIP Database for Chinese Technical Periodicals, Wanfang Database on Academic Institutions in China, PubMed, China Biology Medicine (CBM), and China National Knowledge Infrastructure (CNKI) were utilized for literature search with the retrieval time up to June 2021. The search strategy of PubMed and Embase is shown in Table S1 as an example.

2.4. Literature Screening. The two reviewers read independently, preliminary screening based on the title and abstract of the article, and read the full text if it is an RCT. The RCTs that meet the standards will be classified and evaluated and cross-checked by the two reviewers. When opinions differ, they will be discussed with all reviewers to decide whether to include the article.

2.5. Data Extraction and Risk of Bias Assessment. According to the selection criteria, data are extracted from RCTs' countries, sample size, intervention measures, baseline data, and research duration. Two evaluators independently perform data extraction, entry, and cross-check after completion to ensure data accuracy. The risk of bias of RCTs was assessed according to Cochrane Handbook for Systematic Reviews of Interventions [36]. The content of the evaluation includes random sequence generation, allocation concealments, blinding, incomplete outcomes, selective reporting, and other biases.

2.6. Statistical Analysis. Data analysis was performed using RevMan 5.3 statistical software provided by the Cochrane Collaboration. The measurement data use mean difference (MD) as the effect size, and the effect size is expressed in a 95% confidence interval (CI). The enumeration data are expressed by Risk Ratio (RR) and 95% CI. The $\chi 2$ test is used to evaluate the heterogeneity of the RCTs. When $P \geq 0.05$ or I $2 \leq 50\%$, the fixed effects model is used for analysis; otherwise, the source of heterogeneity is analyzed first, and the random effects model is used when the source of heterogeneity cannot be eliminated.

3. Results

3.1. Results of the Search. The total records identified through database searching and other sources were 439. According to the search strategy, a total of 13 articles were obtained through preliminary search. By eliminating duplicate documents, carefully reading the title and abstract, a total of 426 articles were excluded. After carefully reading the full text and comparing the selection criteria, 11 records (10 RCTs) were screened out and finally included [25–35] (Figure 1). Among the excluded research, the study by Zhang et al. did not use randomization [37].

3.2. Description of Included Trials. The 10 RCTs are all from different countries, and the research scale is about 40–100 participants. The intervention measures of the 10 RCTs are

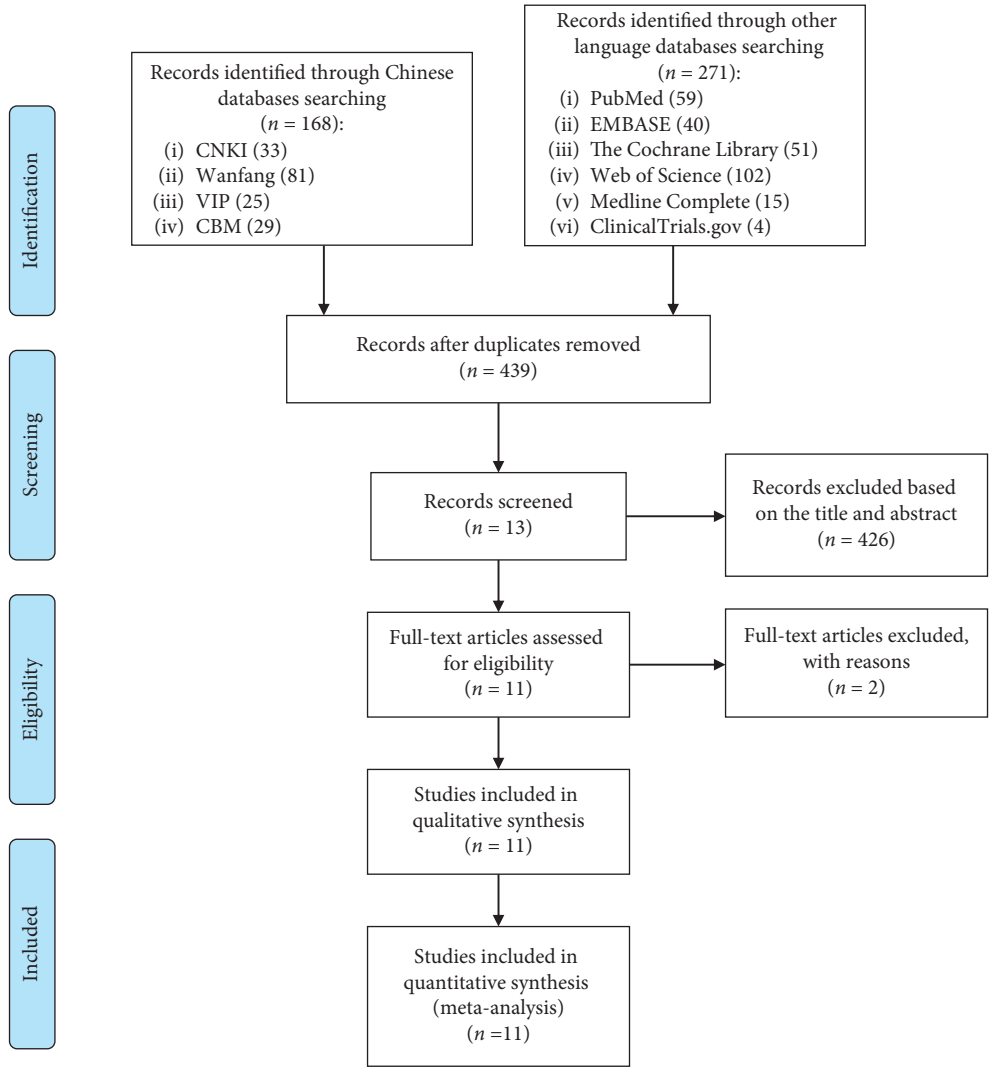

Figure 1: Flow diagram.

all probiotics, but the sources of probiotics are different. The details of study characteristics are presented in Table 1.

3.3. Risk of Bias of Included Studies. The summary and graph of risk of bias are shown in Figure 2.

3.3.1. Sequence Generation and Allocation Concealment. Three RCTs describe their random sequence generation method: Lambert et al. and Jafarnejad et al. [26] and Jansson et al. [30] used computer-generated random numbers; Derwa et al. [18, 19] used a website (http://www.randomization.com) to generate random sequences. Li et al. [31], Wang et al. [32], and Guo et al. [33] used the random number table method to generate random numbers. Therefore, these RCTs were assessed as low risk of bias. Takimoto et al. [27], Liu [34], and Song et al. [35] did not describe the method of random sequence generation, so its risk of bias was assessed as unclear.

Lambert et al. [25], Jansson et al. [30], and Guo et al. [33] used tablets with the same taste and appearance and packaged them in identical, sealed, white cardboard boxes. The random sequence of Jafarnejad et al. [26] was generated by computer, and the researchers who recruited the subjects could not predict the distribution. The researchers and patients of Takimoto et al. [27] were not aware of the distribution during the study period. The experimental group and control group of Nilsson et al. [28, 29] used the same outer packaging. Therefore, those RCTs were considered to have implemented allocation concealment and were assessed as low risk of bias. Li et al. [31], Wang et al. [32], Liu [34], and Song et al. [35] did not mention allocation concealment, and the risk of bias was not clear.

3.3.2. Blinding, Incomplete Outcome Data, and Selective Reporting. Five RCTs [25–30] describe the process of blind implementation to patients and researchers and are therefore considered to be a low risk of bias. Five RCTs [31–35] did not mention whether to use blinding, but their outcomes are objective indicators and would not be affected by not using blinding, so they are assessed as low-risk bias.

Table 1: The characteristics of the included studies.

Study	Country	Participant	Sample size		Intervention		Relevant outcomes	Mean age (years)		BMI		Duration
			Trial group	Control group	Trial group	Control group		Trial group	Control group	Trial group	Control group	
Lambert et al. [25]	Denmark	Postmenopausal osteopenic women	38	40	Red clover extract (RCE) (rich in isoflavone aglycones and probiotics)	Placebo [made by 90 L of water mixed with 250 g brown food coloring (ammoniated caramel) (Kavli)]	BMD, CTX, OPG, RANKL, OC, adverse events	60.84 ± 1.07	62.85 ± 0.99	24.84 ± 0.62	26.65 ± 0.81	12 months
Jafarnejad et al. [26]	Iran	Postmenopausal osteopenic women	20	21	Multispecies probiotic supplement (GeriLact capsule)	Placebo	BMD, CTX, RANKL, OPG, OC	58.85 ± 0.68	57.29 ± 0.72	24.86 ± 0.41	23.82 ± 0.38	6 months
Takimoto et al. [27]	Japan	Healthy postmenopausal women	31	30	Probiotic *Bacillus subtilis* C-3102 (C-3102)	Placebo	BMD, adverse events	57.5 ± 4.3	57.8 ± 5.4	22.2 ± 3.3	22.1 ± 2.7	6 months
Nilsson et al. [28, 29]	Sweden	Postmenopausal women with low bone mineral density	45	45	Freeze-dried *L. reuteri* 6475 (BioGaia AB, Stockholm, Sweden)	Placebo (maltodextrin powder)	BMD, adverse events	76.4 ± 1.0	76.3 ± 1.1	25.5 ± 3.5	25.3 ± 3.3	12 months
Jansson et al. [30]	Sweden	Healthy postmenopausal women	126	123	Three lactobacillus strains (*L. paracasei* 8700 : 2 (DSM 13434), *L. plantarum* heal 9 (DSM 15312), and *L. plantarum* heal 19 (DSM 15313))	Placebo	BMD, adverse events	59.1 ± 3.8	58.1 ± 4.3	24.2 ± 2.7	23.9 ± 2.6	12 months
Li et al. [31]	China	Postmenopausal osteopenic women	73	73	Bifidobacterium quadruple viable bacteria tablets 0.5 g Tid + oral alendronate sodium 10 mg Qd + subcutaneous or intramuscular injection of salmon calcitonin 50 IU Qd.	Oral alendronate sodium 10 mg Qd + subcutaneous or intramuscular injection of salmon calcitonin 50 IU Qd.	BMD, CTX, OC, adverse events	68.15 ± 22.36	69.82 ± 21.47	26.31 ± 8.36	24.85 ± 7.40	6 months
Wang et al. [32]	China	Senile osteoporosis	75	75	Bifidobacterium triple live bacteria capsules 840 mg Bid + conventional therapy	Conventional therapy	BMD	71.52 ± 5.46	71.68 ± 5.41	—	—	2 months

Table 1: Continued.

Study	Country	Participant	Sample size		Intervention		Relevant outcomes	Mean age (years)		BMI		Duration
			Trial group	Control group	Trial group	Control group		Trial group	Control group	Trial group	Control group	
Guo [33]	China	Postmenopausal osteopenic women	30	24	Dry Probio-M8 lactic acid bacteria	Placebo	BMD, CTX, OC	61.91 ± 6.37	6.34 ± 5.71	23.59 ± 3.43	23.86 ± 3.19	6 months
Liu [34]	China	Diabetic osteoporosis	42	45	Bifidobacterium triple viable enteric-coated capsules + conventional therapy	Conventional therapy	BMD, OC	70.5 ± 6.8	69.8 ± 6.4	—	—	6 months
Song et al. [35]	China	Diabetic osteoporosis	100	100	Quadruple bifidobacterium live bacteria tablets + conventional therapy	Conventional therapy	BMD, CTX, OC	68.20 ± 12.78	69.76 ± 12.09	25.21 ± 2.55	26.90 ± 2.39	12 months

Figure 2: Risk of bias assessment. (a) Risk of bias graph; (b) risk of bias summary.

Although the 10 RCTs [25–35] have missing data, the reasons for the missing and the number are balanced or utilized intention-to-treat analysis, so they are considered low risk of bias in "incomplete outcomes." All RCTs do not have selective reporting and are therefore considered to be a low risk of bias.

3.3.3. Other Potential Bias. Other sources of bias were not observed in 10 RCTs; therefore, the risks of other bias of the RCTs were low.

3.4. Primary Outcomes

3.4.1. BMD. Seven RCTs reported the absolute value of BMD, and 3 RCTs reported the percentage of BMD improvement:

(1) The absolute value of lumbar spine's BMD: the heterogeneity test results showed I2 = 53% and P = 0.10 (in postmenopausal woman), suggesting that the heterogeneity is medium, and the random effects model is used. In postmenopausal woman subgroup, the improvement of BMD in the experimental group was not statistically significant compared with the control group (WMD 0.01 (−0.03, 0.06), P = 0.48, random effect model). In senile osteoporosis, the improvement of BMD in the experimental group was higher (WMD 0.13 (0.06, 0.20), P = 0.0003, random effect model). In diabetic osteoporosis, the improvement of BMD in the experimental group was not statistically significant compared with the control group (WMD 0.06 (0.00, 0.11), P = 0.05, random effect model) (Figure 3). The summary results also showed that the improvement of BMD in the experimental group was not statistically significant compared with the control group (WMD 0.04 (−0.00, 0.09), P = 0.07, random effect model).

(2) The percentage of lumbar spine's BMD improvement: since the data unit of this indicator is not uniform, standardized MD (SMD) is used for

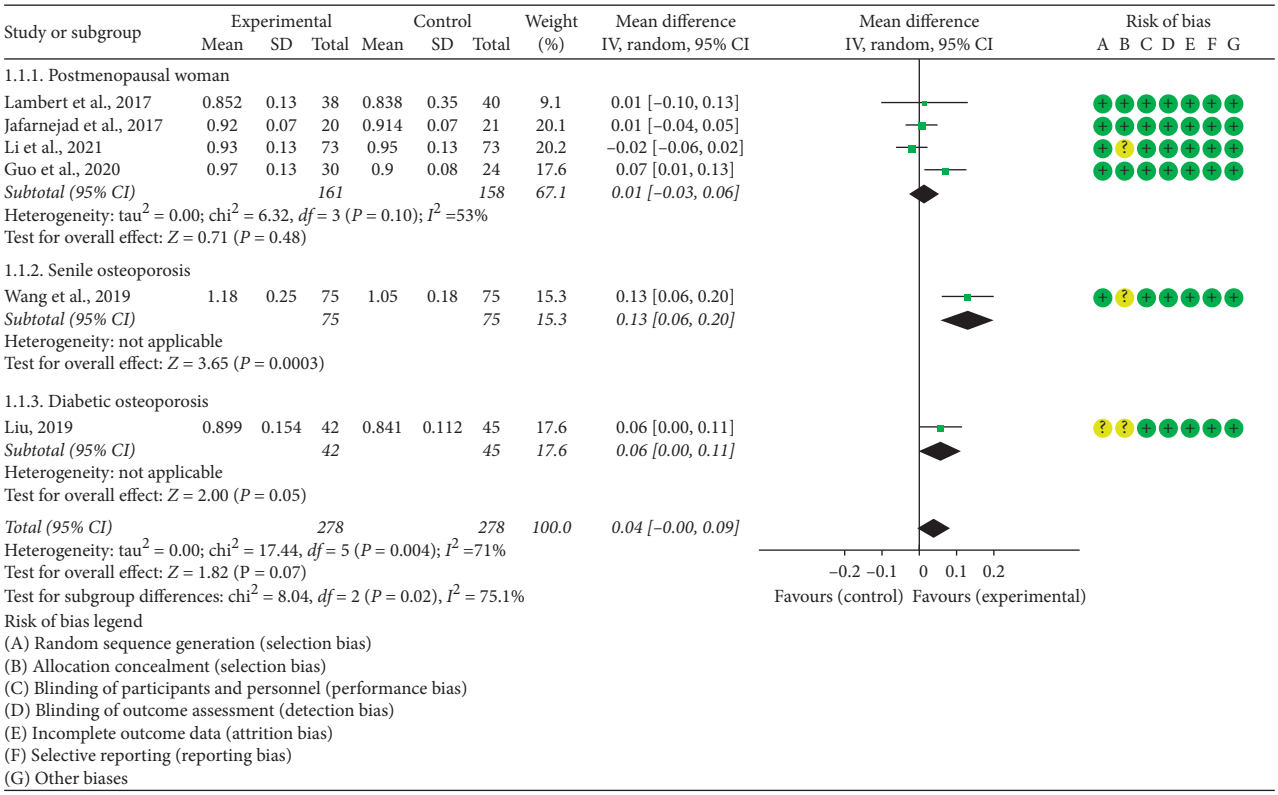

Figure 3: The absolute value of lumbar spine's BMD.

analysis. The heterogeneity test results showed I2 = 94% and $P < 0.00001$, suggesting that the heterogeneity is high, and the random effects model was used. The summary results showed that, compared with the control group, the improvement of BMD in the experimental group was higher (SMD 1.16 (0.21, 2.12), $P = 0.02$, random effect model) (Figure 4).

(3) The percentage of total hip's BMD improvement: since the data unit of this indicator is not uniform, standardized MD (SMD) is used for analysis. The heterogeneity test results showed I2 = 96% and $P < 0.00001$, suggesting that the heterogeneity is high, and the random effects model is used. The summary results showed that, compared with the control group, the improvement of BMD in the experimental group was of no statistical significance (SMD 0.52 (−0.69, 1.73), $P = 0.40$, random effect model) (Figure 5).

(4) Jafarnejad et al. [26] reported the absolute value of forearm BMD; they found that the improvement of total hip's BMD in the experimental group was not statistically significant compared with the control group ($P = 0.725$). Lambert et al. [25] reported the absolute value of femoral neck and trochanter's BMD; they found that compared with control group, the improvement of BMD in the experimental group was higher (femoral neck: $P = 0.0059$; trochanter: $P = 0.03$). Song et al. [35] reported the absolute value of total hip's BMD. It showed that, compared with the control group, the BMD of both forearms improved significantly ($P < 0.05$).

3.5. Secondary Outcomes

3.5.1. CTX. Five RCTs reported CTX. The heterogeneity test results showed I2 = 92% and $P < 0.00001$, suggesting that the heterogeneity is high, and the random effects model is used. The summary results showed that, compared with the control group, the CTX in the experimental group was lower (SMD −0.83 (−1.50, −0.16), $P = 0.02$, random effect model) (Figure 6).

3.5.2. OPG and RANKL. Two RCTs reported OPG and RANKL. (1) OPG: the heterogeneity test results showed I2 = 82% and $P = 0.02$, suggesting that the heterogeneity is high, and the random effects model is used. The summary results showed that the improvement of OPG in the experimental group was not statistically significant compared with the control group (WMD −0.10 (−1.00, 0.79), $P = 0.82$, random effect model) (Figure 7). (2) RANKL: the heterogeneity test results showed I2 = 86% and $P = 0.007$, suggesting that the heterogeneity is high, and the random effects model is used. The summary results showed that the improvement of RANKL in the experimental group was not statistically significant compared with the control group (SMD −0.25 (−0.72, 0.22), $P = 0.29$, random effect model) (Figure 8).

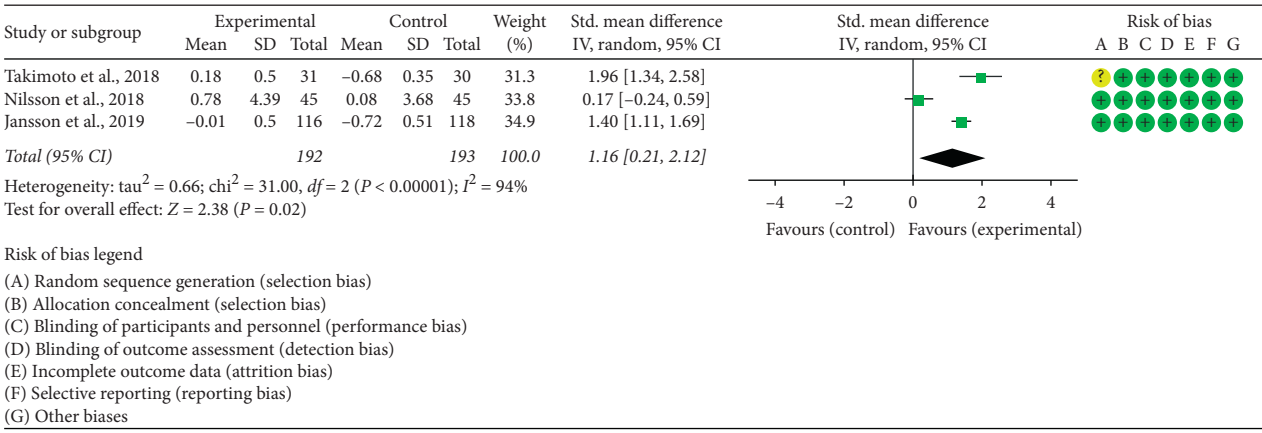

Figure 4: The percentage of lumbar spine's BMD improvement.

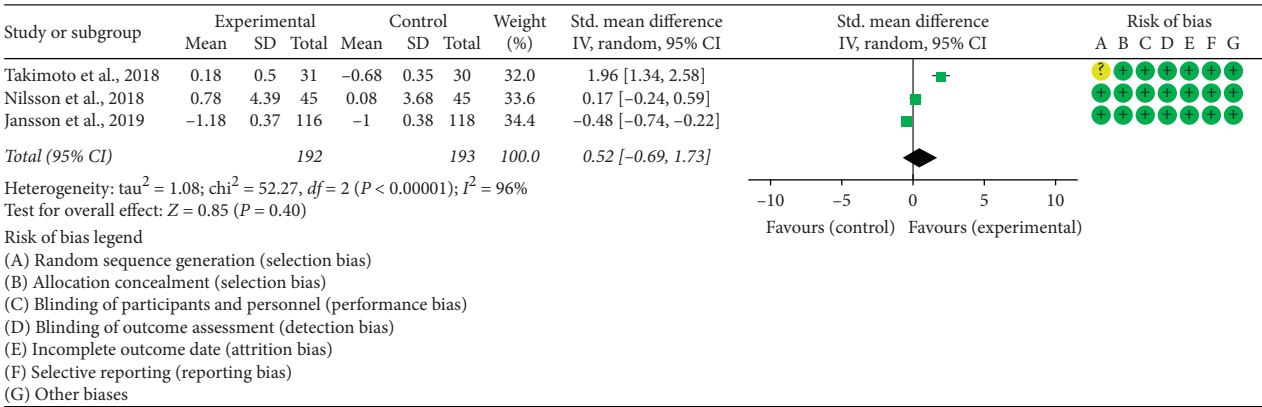

Figure 5: The percentage of total hip's BMD improvement.

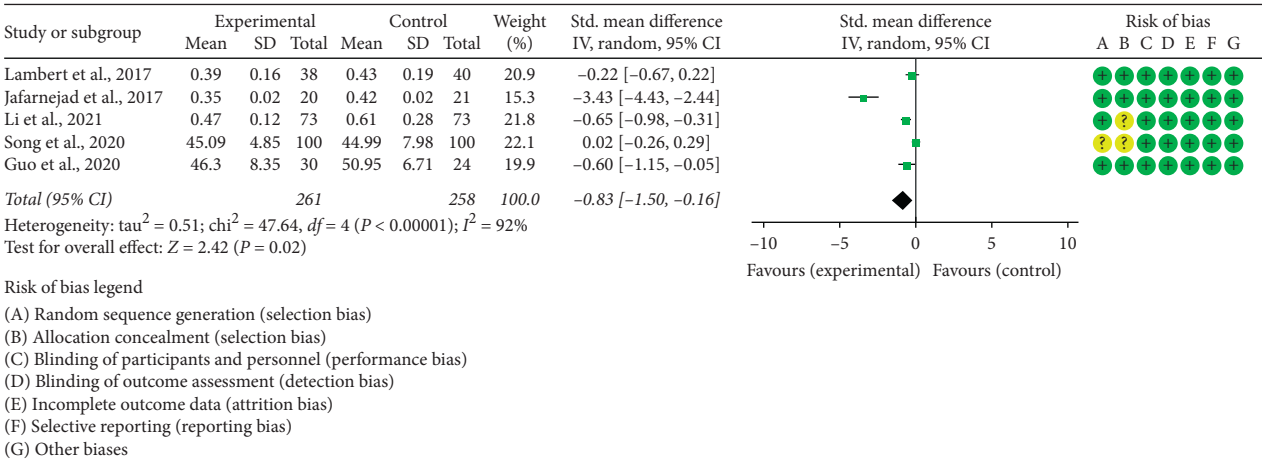

Figure 6: The results of CTX.

3.5.3. OC. Six RCTs reported OC. The heterogeneity test results showed that (1) in postmenopausal women subgroup: I2 = 79% and $P = 0.003$; (2) in diabetic osteoporosis subgroup, I2 = 91% and $P = 0.0008$, suggesting that the heterogeneity is high, and the random effects model is used. In postmenopausal women subgroup, the improvement of OC in the experimental group was not statistically significant compared with the control group (SMD 0.33 (−0.18, 0.85), $P = 0.21$, random effect model). In diabetic osteoporosis subgroup, the OC in the experimental group was lower (SMD −1.06 (−1.96, −0.17), $P = 0.02$, random effect model). The summary results showed that the improvement of OC in

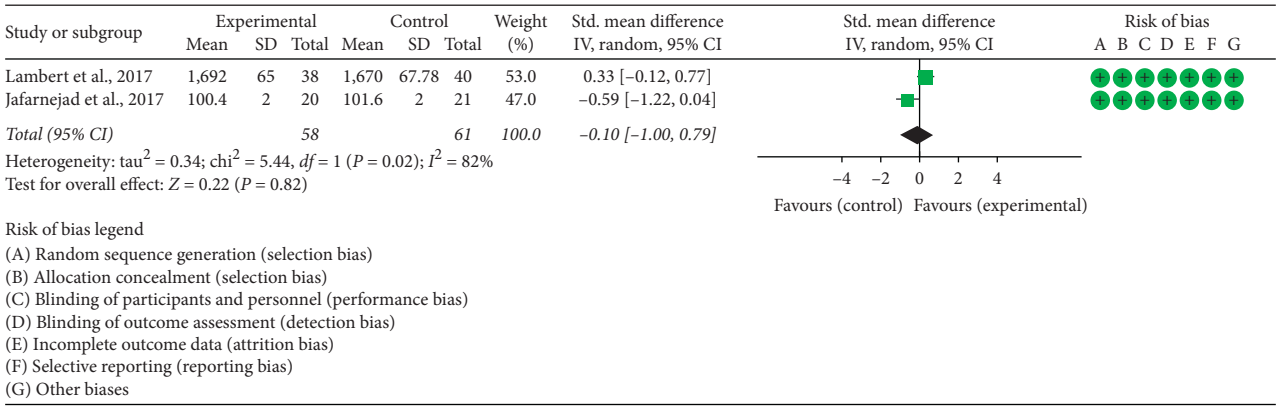

Figure 7: The results of OPG.

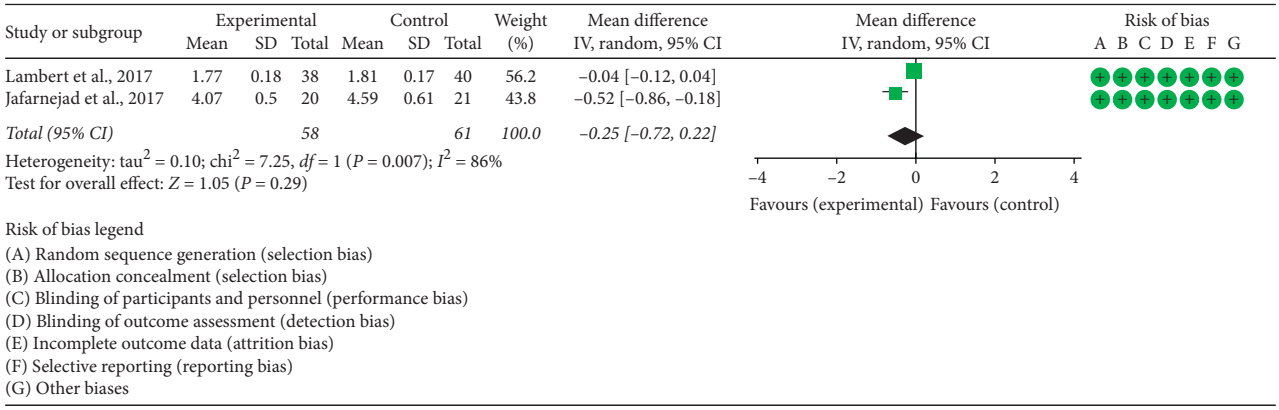

Figure 8: The results of RANKL.

the experimental group was not statistically significant compared with the control group (SMD −0.12 (−0.85, 0.61), $P = 0.75$, random effect model) (Figure 9).

3.6. Adverse Events. Five RCTs reported the adverse events. The heterogeneity test results showed I2 = 0% and $P = 0.44$, suggesting that the heterogeneity is low, and the fix effects model is used. The summary results showed that the adverse events in the experimental group were not statistically significant compared with the control group (RR 1.02 (0.92, 1.12), $P = 0.70$, fixed effect model) (Figure 10).

4. Discussion

In this paper, the clinical studies of probiotics intervention for osteopenia mostly use radiographical indicators (BMD) and biochemical indicators (CTX, OPG, RANKL, OC), which are more objective. Therefore, the efficacy criteria can be considered reliable. The 10 studies included in this study are all RCTs of different preparations of probiotics that interfere with osteoporosis or osteopenia. The results of the meta-analysis showed that, compared with the control group, the difference in some of the primary outcomes was statistically significant, suggesting that probiotics have a certain effect on antiosteoporosis. The specific results are (1) in postmenopausal woman, compared with control group; the improvement of absolute value of lumbar spine's BMD was of no statistical significance, but that of the percentage of lumbar spine's BMD was higher. In senile osteoporosis, the improvement of absolute value of lumbar spine's BMD in experimental group was higher, while in diabetic osteoporosis, that was of no statistical significance. (2) Compared with control group, the improvement of absolute value and percentage of total hip's BMD was of no significance in postmenopausal woman. (3) The CTX level in the experimental group was lower. In postmenopausal women subgroup, the improvement of OC in the experimental group was not statistically significant; however, in diabetic osteoporosis subgroup, the OC in the experimental group was lower. This suggest that probiotics have bone protection. (4) Compared with control group, there was no statistical difference in the changes of OPG, RANKL in the experimental group. (5) The incidence of adverse events in the experimental group was not statistically different from that in the control group, suggesting that the use of probiotics would not increase the incidence of adverse events.

In terms of improving lumbar spine BMD in postmenopausal woman, the improvement of its absolute value is different from that of its percentage, and the heterogeneity between RCTs is medium. Since those RCTs are from different countries, we suspect that the main reason for this

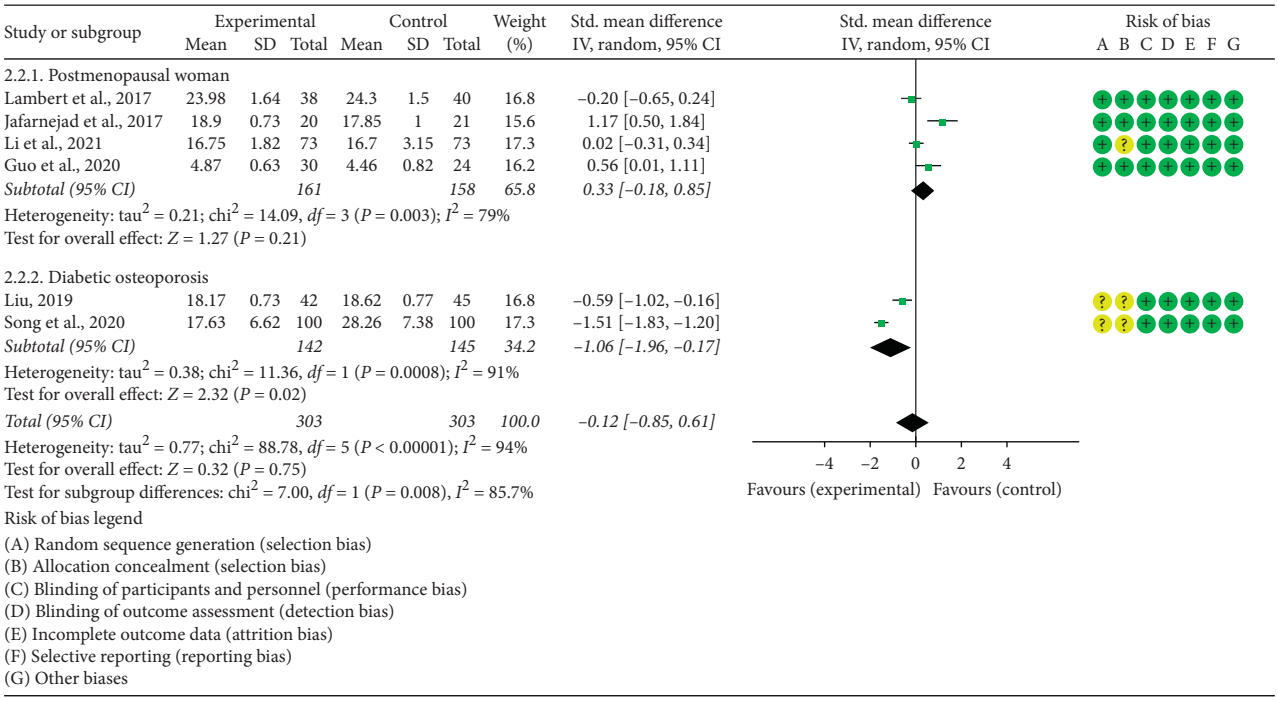

Figure 9: The results of OC.

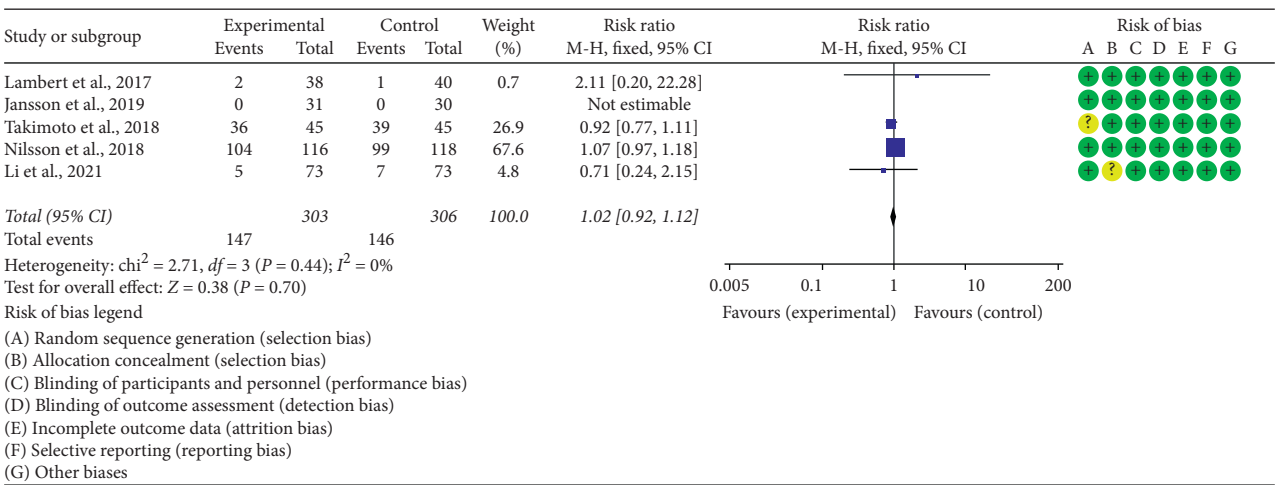

Figure 10: Adverse events.

result may be related to the different reactions of different nationalities to probiotics. This study also showed that, in postmenopausal woman, probiotics have an improvement effect on lumbar spine BMD, but the improvement effect on total hip BMD is not obvious. This suggests that probiotics have different effects on the bones of different parts of postmenopausal women. However, further long-term studies are needed to explore the obvious bone site-specific effects of probiotic treatment on postmenopausal women. The RCT on diabetic osteoporosis also showed that probiotics can improve the BMD of the forearm, but the improvement of the lumbar spine BMD is not obvious. However, because diabetic osteoporosis involves only 1–2 RCTs, more relevant RCTs are needed to further verify or modify the conclusion. In addition, although these RCTs utilized probiotics, the bacterial species used in each study are different, so this difference may also be related to the different bacterial species. Among the biochemical indicators OPG, RANKL, and OC, the heterogeneity between RCTs is relatively large, which may be related to individual differences. However, due to the fact that there are fewer RCTs including probiotics to interfere with osteoporosis and osteopenia and the control group is placebo, more RCTs with large samples, uniform probiotic preparation, and different control drugs are needed to verify the improvement effect of probiotics on osteoporosis or bone loss. In the incidence of adverse events, there was no statistical difference between the probiotic group and the control group.

Limited to the number of included RCTs, there is no enough evidence to verify the incidence of adverse events in treatment of probiotics and whether probiotics combined with antiosteoporosis drugs can reduce the incidence of adverse events. The occurrence of adverse events is often related to drugs and treatment methods. Because most patients with osteoporosis and osteopenia are elderly patients, they often have other medical conditions. Therefore, while applying probiotics to treat osteoporosis, in addition to choosing the right treatment, it is also necessary to take preventive measures against possible adverse events.

Osteoporosis is an epidemic metabolic bone disease characterized by bone loss and structural destruction [38], which easily leads to fractures and disability. It is affected by a variety of genetic factors and environmental factors, such as genetics, diet, hygiene, and the use of antibiotics. With the aging of the population becoming more and more serious, there are more than 200 million people in the world suffering from osteoporosis, and its incidence has jumped to the sixth place among common and frequently-occurring diseases, becoming a global public health problem [39]. Among the population with osteoporosis, postmenopausal women are the majority. The lack of estrogen in postmenopausal women increases their risk of osteoporosis. Postmenopausal osteoporosis not only has a high incidence, but also has serious complications. It has always been the focus of prevention and treatment [40].

Current studies have found that the intestinal flora is related to the loss of bone mass and the incidence of osteoporosis in the human body. These microorganisms may change the relative activity of osteoclasts and osteoblasts through their own metabolites, affecting host metabolism and immune system, thereby affecting bone metabolism [41]. Used in an appropriate amount, probiotics have been shown to change and synthesize the metabolites of the intestinal flora [41] and regulate the immune response in the host [42, 43]. Importantly, probiotics can enhance the epithelial barrier function. These effects explain the beneficial effects of probiotics [44, 45]. Among them, *Lactobacillus acidophilus* is a kind of *Lactobacillus*. After fermentation in the intestine, it can produce lactic acid, butyric acid, and acetic acid, which can improve the utilization of calcium, phosphorus, and iron and promote the absorption of iron and vitamin D [46]. Another widely studied probiotic is *Lactobacillus rhamnosus* (LGG), which also belongs to the genus Lactobacillus, a third-generation probiotic. Current research reports show that *Lactobacillus rhamnosus* can prevent bone loss induced by ovariectomy, reduce intestinal permeability, and improve intestinal and systemic inflammation [47].

In addition, the intestinal flora is considered a virtual "endocrine organ" because it affects host hormone levels. And because some bacteria can produce and secrete hormones, including serotonin and dopamine, and sex hormones, they may regulate bone remodeling by affecting hormone levels [48]. In particular, for the treatment of postmenopausal osteoporosis, intestinal flora, especially intestinal probiotics, has been proven to be a potential therapeutic strategy [49]. Among them, *Lactobacillus rhamnosus* can stimulate bone formation by increasing estrogen [50]. The study found that prebiotics can increase the number of probiotics such as lactobacilli and butyric acid bacteria to promote the secretion of more short-chain fatty acids, thereby reducing the intestinal PH value and increasing the solubility of calcium in the intestinal lumen, thus increasing the bone mineral content and bone mineral density of young people [51]. Prebiotics are indigestible and fermentable food ingredients that not only promote the growth of intestinal probiotics, but also promote the production of probiotic metabolites [52], and a variety of substrates that can be metabolized by bacteria besides sugars [53]. Many studies have proven that prebiotics enhance human calcium absorption [54].

Compared with previous systematic review and meta-analysis [55], this study is the newest systematic review and meta-analysis strictly based on the PRISMA guidelines with preregistered plans. This study also covers a wider population (postmenopausal women, senile osteoporosis, and diabetic osteoporosis) than previous reviews. Meanwhile, this study included 7 RCTs about postmenopausal women involving 719 participants; the applicable population of the conclusion has also been extended to the East Asian population. This study also found that in the same population, probiotics can improve the BMD of different body parts differently. For different groups of people, the improvement of BMD of the same body part by probiotics is also different. These may inspire future research. The advantage of this study is that this is the first systematic review and meta-analysis of RCTs in which probiotics interfere with osteoporosis or osteopenia. The disadvantage is that the results may be affected due to the lack of included RCTs: (1) because different studies use different probiotics as clinical interventions, they may be interest related, and this inconsistency may certainly affect the strength of the argument. (2) Although RCTs come from different countries, they are all single-center, small-sample clinical trials.

5. Conclusion

Probiotics may improve BMD and reduce CTX and OC, but there is no difference in improving OPG and RANKL. This may be due to the small number of included RCTs and the influence of many factors, and further research is needed. The incidence of adverse events in the probiotic group is comparable to that in the control group and can be considered a safe intervention. In future research, we should pay attention to the standardization of clinical research evidence-based medicine methodology and optimize clinical treatment plans. In the future, more large-sample, random-controlled, high-quality RCTs are needed to further verify the effectiveness and safety of probiotics in intervening osteoporosis or osteopenia.

Data Availability

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

Disclosure

Ganpeng Yu and Liuting Zeng should be considered the co-first authors.

Conflicts of Interest

The authors have no conflicts of interest.

Supplementary Materials

PRISMA 2009 checklist: checklist; Table S1: search Strategies for PubMed and Embase. (*Supplementary Materials*)

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Research Article

Physicochemical Evaluation of Edible Cyanobacterium *Arthrospira platensis* Collected from the South Atlantic Coast of Morocco: A Promising Source of Dietary Supplements

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The cyanobacterium *Arthrospira platensis (A. platensis)*—a genus of nonheterocystous filamentous cyanobacteria—is used in industrial applications and as a food supply. The current research work aims to study the physicochemical characteristics of *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune (Foum El Oued lagoon). The contents of proteins, carbohydrates, vitamins, lipids, minerals, heavy metals, energy value, humidity, ash, pigments, and tannins in *A. platensis* were investigated using protocols as described in the earlier literature. The values of protein, carbohydrate, and lipid contents in *A. platensis* were 58.9 ± 0.07, 14.67, and 45.54% respectively. The values of vitamins B_2 and B_3 dosed in *A. platensis* were 1.31 ± 0.19 and 30.8 ± 0.001 mg/kg, respectively. The values of heavy metals including lead and chromium were 70 ± 4.5 and 5 ± 0.5 PPB (parts-per-billion), respectively; however, no trace concerning cadmium was detected. The values of energy value, humidity, and ash content were 346.48 ± 0.21, 11.6 ± 0.17%, and 9.1 ± 0.21% kcal/100 g, respectively. The results of pigment content showed the presence of chlorophyll b, chlorophyll a, and carotenoids of 37.506 ± 3.38, 26.066 ± 3.08, and 9.52 ± 0.22 mg/g, respectively. The results obtained revealed that *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune was found to be very rich in proteins, carbohydrates, vitamins, minerals, ash, and pigments and lower in heavy metals and saturated fats when compared with species investigated in the literature. Thus, *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune fulfills the requirements for being used as dietary supplements.

1. Introduction

Microalgae comprising large photosynthetic plants whose vegetative system is called "thallus." They have variable shapes and dimensions. Some of them are microscopic, and others are macroscopic, but they share structural and genetic similarities [1]. Overall, microalgae are subdivided into several classes including 30,000 to 40,000 species. Microalgae

present a large morphological and physiological diversity, which helps them create an aerobic atmosphere necessary for the development of life [2]. The majority of microalgae species are micromicroalgae, which account for more than ten million [3]. Microalgae are mainly aquatic living in fresh or marine waters, and some of them on the high mountains [4]. Microalgae are recognized for their ability to withstand high temperatures in the waters of thermal springs [4].

Nowadays, microalgae seem to be one of the best solutions for producing high-quality food supplements [5]. Microalgae were the first photosynthetic living things that have appeared on the Earth about 3 to 4 billion years ago through cyanobacteria. According to their color pigments, microalgae are usually classified into green, brown, and red. As a result, these microalgae are divided into four classes: green microalgae (chlorophytes), blue microalgae (cyanobacteria), red microalgae (rhodophytes), and brown microalgae (chromophytes) (see [2] and [6]).

In recent times, the use of photosynthetic microorganisms has progressively increased. They have been used in different fields, including food dyes, cosmetics, dietetics, and biotechnology [7]. Cyanobacteria are prokaryotes that accomplish oxygenic photosynthesis and form a wide taxonomic group within eubacteria. Cyanobacteria are morphologically subdivided into unicellular or filamentous organisms. Functionally, these microorganisms can be classified into N2-fixing and non-N2-fixing. [8]. *Arthrospira* is a genus belonging to nonheterocystous filamentous cyanobacteria that live in an alkaline environment [9]. Even though these microorganisms constitute a special taxonomic unit, many *Arthrospira* species were classified in the genus *Spirulina*, and some of them are still being named under this name [10]. Anyway, the current taxonomy asserts that the name "Spirulina" used to indicate strains used as food supplements is unsuitable and there is accordance that *Arthrospira* is a distinct genus including more than 30 different species [11].

Arthrospira species are rich in nutrients like essential fatty acids, minerals, vitamins, and pigments [12]. Thus, they have largely been used as food supplements, feedstock in both agriculture and aquaculture [13]. It has become an interesting source of organic material, beta-carotene, and natural food dyes [14]. In addition to their important nutritional value, *Arthrospira* species have also the requirement for being introduced to serve heath by exhibiting interesting pharmacological activities like anti-inflammatory, antioxidant, and immunomodulating ([15] and [16]). *Arthrospira platensis* (*A. platensis*) is recommended for being applied in environmental sectors for wastewater treatment (metals, nitrogen, phosphorus) [16].

Arthrospira species have ecologically valuable criteria such as alkali and salt tolerance. This organism can grow where several species cannot even under high salt concentrations of 1.5-fold higher than seawater as reported in earlier works [17]. These photosynthetic organisms are often live in lakes with high pH and carbonate levels [18].

It was reported that the Moroccan *A. platensis* has been used in the Mediterranean diet for many decades. In this sense, *A. platensis* requires processing into an acceptable product before it can be used. However, the physicochemical composition of species indigenous to the Moroccan Atlantic coast at Laayoune (Foum El Oued lagoon) has not yet been investigated. It is thus fitting that the present research work aimed to achieve this goal by studying the physicochemical criteria of *A. platensis* collected from this local cultivar.

2. Material and Methods

2.1. Organism. *Arthrospira platensis* was obtained from the culture collection at the Moroccan Foundation for Advanced Science, Innovation and Research, which was originally isolated from the Atlantic coast at Laayoune (Foum El Oued lagoon)—south of Morocco (Figure 1) (027° 06′ 00.0″ N, 013° 25′ 00.0″ W) before being cultured at the Faculty of Sciences Ain Chock, University Hassan II of Casablanca, Morocco. Briefly, the cells obtained were cultured in Zarrouk's medium (Zarrouk, 1966) at 31 ± 1°C, pH = 9, irradiated with 40 mol m^{-2} s^{-1} of cool-white fluorescent light (12-h:12-h light:dark cycle) and aerated with ambient air (360 ppmv CO_2). Samples in the exponential growth phase were used to perform the analysis (Figure 2).

2.2. Physicochemical Characteristics of the Study Area (Foum El Oued Lagoon)

2.2.1. Temperature. The temperature of the collection area fluctuated between 16.1°C and 17.2°C at time sampling. Lagoon water was generally warmer than that of the ocean. Similarly, the seasonal variation was pronounced, with warmer water in September (21.5–24.4°C) than in February (16.3–19.5°C) [20].

2.2.2. Salinity. Salinity showed an increasing gradient from downstream to upstream. Salinity gradually increased inside the lagoon with values close to those of the ocean (34–35 PSU). Salinity was higher in September than in February as reported in earlier works [20].

2.2.3. Dissolved Oxygen. Dissolved oxygen concentrations were variable according to stations (6.9–8.5 mg l^{-1}). A strong concentration gradient was noted from downstream to upstream in the lagoon [20].

2.2.4. Nitrates. The lagoon was found to be generally richer in nitrates in February than in September. The concentration of nitrates decreased from downstream to upstream in the lagoon with values ranging from 80 μg l^{-1} (H4) to 9.9 μg l^{-1} [20].

2.2.5. Phosphates. The spatial distribution of phosphates showed two trends depending on the tide and the season. This distribution was more homogeneous across the lagoon with 97 μg l^{-1}, and the water was generally richer in phosphates in February than in September as reported elsewhere [20].

2.3. Protein Content Determination

2.3.1. Quantitative Determination. A zero-point seventy-five gram of *A. platensis* dried sample was introduced into a

Figure 1: Area of collection (Laayoune-Foum El Oued lagoon-Morocco) [19].

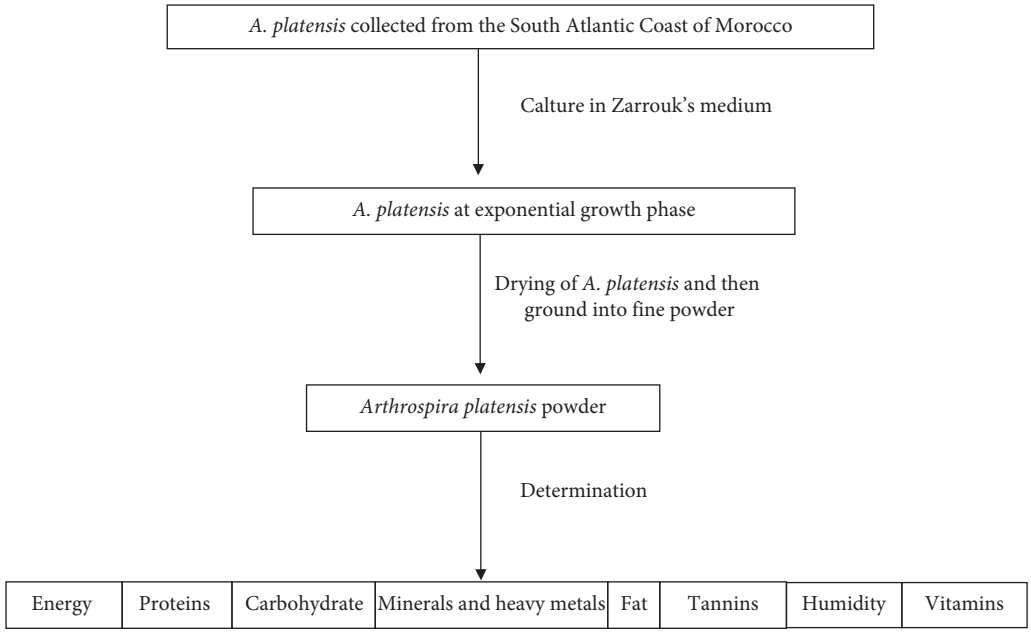

Figure 2: Scheme of the study design.

flask containing 7.5 g of catalyst (100 g of potassium sulfate K_2SO_4, 10 g of copper sulfate $CuSO_4.5H_2O$) and 15 mL of sulfuric acid H_2SO_4 (0.1 N). The assay was carried out in duplicate. The mixture was subjected to mineralization using a mineralization ramp apparatus (Büchi) for 4 hours until reaching a maximum production of ammonium sulfate $(NH_4)_2SO_4$. After cooling, the volume of the mineralized sample was mixed with 50 mL of distilled water. Afterward, 85% NaOH solution (65 mL) was added to the mineralized sample before being distilled, and then the solution was trapped in boric acid (H_3BO_3, 4%). Next, the ammoniacal distillate was titrated with sulfuric acid (0.1 N) to perform analysis [21].

The total protein content was calculated using the following formula:

$$\%\ \text{of the protein content} = \frac{(VV \times 0.0014 \times F)}{PE} \times 100. \quad (1)$$

Here, *F*: conversion factor (6.25), VV: volume of the sulfuric acid solution, and PE: test portion.

2.3.2. Qualitative Determination of Amino Acids. The determination of the amino acid composition (valine, glutamate, arginine, threonine, methionine, and phenylalanine) of *A. platensis* was carried out using high-performance liquid chromatography (HPLC). Briefly, 5 g of *A. platensis* were dried in an oven set at 40°C for 24 h before being added to 40 mL of sulfuric acid H_2SO_4 (2N). After maceration for 3 hours, the extract obtained was filtered before being analyzed using HPLC (Agilent 1100) (mobile phase: 43% KH_2PO_4 and 57% methanol; precolumn: o-phthaldialdehyde (OPA); column: C18; volume injection: 20 μL; UV detection at a wavelength of 333 nm; flow rate: 0.8 mL/min) [22].

The concentration of amino acids was calculated using the following formula:

$$[\mathrm{XE}] = \frac{([\mathrm{St}] \times \mathrm{AE})}{\mathrm{AS} \times \mathrm{FD}}. \quad (2)$$

Here, AE: area of the sample, AS: area of the standards, FD: dilution factor, [St]: concentration of standards, and [XE]: concentration of the sample in ppm.

2.4. Determination of Carbohydrate Content

2.4.1. Quantitative Determination of Carbohydrate Content. Total carbohydrate content contained in the test portion of *A. platensis* was calculated using the formula:

Carbohydrate content = 100 − (humidity + mineral matter + fat + proteins) [21].

2.4.2. Qualitative Determination of Carbohydrate Content. Five grams of *A. platensis* sample was extracted with 50 mL of demineralized water for 2 hours. Afterward, the carbohydrate concentration of the filtrate obtained was dosed using HPLC (column: silica grafted with NH_2: 25*4, 6*5 cm*mm*μm; mobile phase: acetonitrile/water (80/20, respectively); flow rate: 1 mL/min) [21].

2.5. Fat Determination

2.5.1. Quantitative Determination. Five grams of *A. platensis* sample was mixed with 31.5 mL of hydrochloric acid supplemented with 125 mL of distilled water for 2 hours (12 N). After filtration, the residues obtained were placed in an oven set at 105°C overnight. Next, the residues obtained were extracted again with 300 mL petroleum ether using a Soxhlet for 4 hours [21].

After removing the solvent under reduced pressure, the measures were performed using the following formula:

$$\text{Fat content (\%)} = \frac{T2 - T1}{\mathrm{PE}} \times 100. \quad (3)$$

Here, *T1*: the weight of empty flasks, *T*2: the weight of flasks containing fat, and PE: test portion.

2.5.2. Analysis of Fatty Acid Composition. The fatty substance was esterified with methanol. Next, the fatty acid methyl esters were separated through a polar column using gas chromatography (GC) (Annex 5). Briefly, 2 mL of isooctane and 0.1 mL of methyl KOH (2N) were added to 0.5 g of the extracted fat to prepare methyl esters. Afterward, the mixture was stirred for one minute before adding 2 mL of NaCl (40 grams/100 mL). Next, one gram of sodium bisulfate was added to the recovered supernatant before proceeding with the gravimetric analysis [21].

2.6. Determination of Minerals and Heavy Metals

2.6.1. Dosage of Cu, Fe, Mn, Ca, Mg, K, Na, Pb, Cr, Cd. Concentrations of calcium (Ca) and magnesium (Mg) were determined by adding 2 mL of lanthanum chloride La_2O_3 (50g/L) to the mother solution. Potassium (K) determination was conducted by adding 2 mL of cesium chloride. Next, the mineral content was measured using atomic absorption spectrophotometry with flame (Varian SpectrAA 220FS Spectrometer FLAME AA with Varian SIPS-10 Sample Introduction Pump System with Varian SPS-5 Sample Preparation System) [21].

2.6.2. Dosage of P. One milliliter of mother solution was mixed with 10 mL of the monovanado-molybdic reagent. The analysis was conducted using a UV spectrophotometer at 430 nm [23]. The mineral concentration was calculated according to the following formula:

$$\text{Percentage of minerals} = (L - B) \times 10^{-3} \times \frac{\mathrm{VR}}{1000} \times \frac{100}{\mathrm{PE}} \times \mathrm{FD}. \quad (4)$$

Here, *L*: reading, *B*: blank, VR: recovery volume, PE: test portion, and FD: dilution factor (g).

2.7. Determination of Vitamins. Vitamins B_2 (riboflavin) and B3 (nicotinamide) contained in *A. platensis* were determined by using HPLC (NM 08.1.264 (2009). Briefly, 2 g of *A. platensis* sample was added to 40 mL of sulfuric acid (0.1 mol/L) before being stirred for 15 min. Afterward, the mixture was completed with sulfuric acid to reach 100 mL as a final volume. The concentration of vitamins was measured with HPLC (mobile phase: mixture of 970 mL of n-octane sulfonic acid 7 mmol/L (pH = 3), 30 mL of acetonitrile; stationary phase: Hypersil HyPURITY C18 5 μm 250*4.6 mm; injection volume: 50 μL; flow rate: 1 mL/min; fluorimetric detection: 375nm–525 nm; UV detection: 261 nm; gradient in min: 0-9-9.1-22-22.1 and 35).

The concentration of vitamins B_2 and B_3 was calculated using the following formula:

$$\text{Percentage of vitamins} = \frac{\mathrm{AEch}}{\mathrm{ASt} \times \mathrm{FD} \times \mathrm{CSt}}. \quad (5)$$

Here, AEch: peak area of the sample, ASt: peak area of the standard solution, FD: dilution factor = (final volume/test

sample), and CSt: concentration of vitamin standard solution (0.2 for B2 and 5 for B3) [21].

2.8. Energy Value Determination. The evaluation of *A. platensis* energy value was based on the calorific value of different components (proteins, lipids, and carbohydrates) [24].

The energy value was calculated according to the following formula:

$$\text{Energy Value}\left(\frac{K\text{cal}}{100\,g}\right) = (4\,\text{Carbohydrates} + 9\,\text{Fat} + 4\,\text{Proteins}) \times 100. \quad (6)$$

2.9. Humidity Determination. Two grams of *A. platensis* were placed in previously weighed glass capsules before being introduced into an oven set at 105°C for 4 h. Next, the humidity percentage was calculated according to the following formula:

$$\text{Humidity percentage} = \frac{(T2 - T3)}{(T2 - T1)} \times 100. \quad (7)$$

Here, $T1$: the weight of the empty capsule, $T2$: the weight of capsule containing the fresh sample, and $T3$: the weight of capsule containing the dry sample [25].

2.10. Ash Content Determination. Three grams of *A. platensis* sample was introduced into a capsule before being placed in a muffle oven set at 550°C for six hours [26]. After cooling for fifteen minutes, the remaining mineral matter was weighed. The ash content was calculated according to the following formula:

$$\text{Ash content}\,(\%) = \frac{(T3 - T1)}{\text{PE}} \times 100. \quad (8)$$

Here, $T1$: the weight of empty capsule, $T2$: the weight of capsule containing the fresh sample, $T3$: the weight of capsule containing the dry sample, and PE: test sample = $T2 - T1$.

2.11. Determination of Pigments. The content determination of carotenoids, chlorophyll a, and chlorophyll b was done according to the earlier reported data [27]. Briefly, 0.5 g of *A. platensis* sample was extracted with 10 mL of acetone under ultrasound (130 W, 20 KHz) for 15 min. After filtration, the mixture was centrifuged at 3000 rpm for 10 min. The pigment content (carotenoids, chlorophyll a, and chlorophyll b) was determined according to the following formula:

$$\begin{aligned} \text{Chl}\,a &= 13.36 \times A664 - 5.19, \\ \text{Chl}\,b &= 27.43 \times \text{A}648 - 8.12, \\ \text{content of carotenoids} &= \frac{(1000 \times A470 - 1.63 \times \text{chl}\,a - 104.96 \times \text{chl}b)}{\text{Chl}\,a\text{: Chlorophyll}\,a, \text{Chl}\,b\text{: Chlorophyll}\,b}. \end{aligned} \quad (9)$$

2.12. Determination of Tannins. The content of tannins was determined according to the previously reported method [28].

2.13. Statistical Analysis. Data were expressed as means of triplicate assays ± SD (standard deviation). The significant differences were investigated using the t-test. The Mann–Whitney test was used as a post doc test to perform the comparison. Statistically, a significant difference was considered at $P < 0.05$.

3. Results and Discussion

3.1. Protein Content Determination. The value of protein content in *A. platensis* was 58.9 ± 0.07%. Besides, this species was quantitatively rich in essential amino acids as reported in our findings (Table 1). The characterization of *A. platensis* total amino acid content with high-performance liquid chromatography showed the presence of threonine and phenylalanine as major constituents of essential amino acids. Methionine was the most sulfur amino acid detected in the studied species. *A. platensis* also found to be rich in non-essential amino acids like glutamic acid (Table 1).

In the current research work, we investigated *A. platensis* as one of the most recommended foods to prevent diseases. The findings of chemical composition showed that *A. platensis* collected from the South Atlantic Coast of Morocco has a significant amount of proteins (58.9 ± 0.07%). This species contains protein twice as much as soybeans and thrice as much as meat [29, 30]. A promising rate of essential amino acids was found in the investigated species including glutamic and threonine acids (5042.29 ± 374.19 mg/kg and 131.39 ± 1.96 mg/kg, respectively), which quantitatively

Table 1: Amino acids content in *A. platensis*.

Amino acid	Content in mg/kg
Methionine	43.71 ± 0.68
Threonine	131.39 ± 1.96
Phenylalanine	44.97 ± 2.64
Valine	63.05 ± 4.73
Arginine	34.82 ± 1.15
Glutamic acid	5042.29 ± 374.19

exceeded those reported in species from different cultivars [30]. Our results showed that the methionine content in local *A. platensis* was 43.71 ± 0.68 mg/kg. Therefore, we can confirm that *A. platensis* collected from the Moroccan Atlantic coast at Laayoune belongs to the rare cyanobacteria that contain a high amount of methionine. These results agree with those reported elsewhere since it was stated that *Arthrospira* constitutes a promising source of protein not only because of its high rate in the dry biomass but also due to the composition of amino acids and high digestibility. It is worth reporting that *Arthrospira* biomass possesses all 8 exogenic and 12 endogenic amino acids [31].

3.2. Determination of Carbohydrate Content. High-performance liquid chromatography analysis showed that the value of carbohydrate content in dry *A. platensis* was 14.67 ± 0.001%. The characterization of *A. platensis* total carbohydrate content showed the presence of a significant amount of glucose (3.17 ± 1.01 g/L), unlike fructose, sucrose, and maltose, which were detected in very little quantity.

The present results showed that *A. platensis* has a low content of carbohydrates (14.67 ± 0.001%) with a very little amount of simple carbohydrates (glucose and fructose). These findings were in agreement with those reported in earlier works [32]. *Arthrospira* was described by the presence of two specific polysaccharides including sodium spirulan and calcium spirulan, which are involved in antiviral, anticoagulant, and immunostimulatory activities of *A. platensis* as reported elsewhere [31, 33].

The total lipid content of *A. platensis* was determined at 5.8 ± 0.21% using gravimetric analysis. The characterization of *A. platensis* total lipid content revealed the presence of monounsaturated and saturated fatty acids with 53 ± 0.003% and 45.54 ± 0.15%, respectively. The total lipid content of the presently studied species was majorly constituted of palmitoleic acid (45.52 ± 0.01%), palmitic acid (37.06 ± 0.502%), and oleic acid (7 ± 0.003%) (Table 2).

3.3. Fat Determination. Regarding the total lipid content in the local *A. platensis,* the results obtained showed that this species was majorly constituted of saturated and unsaturated fatty acids with a total percentage of 5.8 ± 0.25%. *A. platensis* possessed both monounsaturated and saturated fatty acids with values of 53 ± 0.003% and 45.54 ± 0.15% respectively. Our findings are in accordance with those reported in earlier works, which showed that lipid in *Arthrospira* varies from 1.5 to 12% of dry mass [34].

Table 2: Total lipid content in *A. platensis*.

Compound name	Percentage (%)
Lauric acid	0.86 ± 0.01
Myristic acid	1.90 ± 0.41
Palmitic acid	37.06 ± 0.502
Palmitoleic acid	46.52 ± 0.15
Margaric acid (17:0)	4.6 ± 0.03
Margaric acid (17:1)	0.25 ± 0.001
Stearic acid	0.9 ± 0.01
Pentadecylic acid	0.23 ± 0.0005
Vaccenic acid	7 ± 0.003
Linoleic acid	1.20 ± 0.001
γ-Linolenic acid	0.26 ± 0.001
Arachidic acid	0.05 ± 0.01
Gondoic acid	0.23 ± 0.0001

3.4. Determination of Minerals and Heavy Metals. The mineral composition analysis with flame atomic absorption spectroscopy revealed that *A. platensis* collected from the South Atlantic Coast of Morocco have important mineral elements (Table 3) and very few or no heavy metals (Table 4). The values of lead and cadmium content in *A. platensis* were lower than the largest tolerated values in the food according to the World Health Organization (WHO). Statically, there was a significant difference between the content of heavy metals detected in *A. platensis* and WHO threshold values ($P < 0.05$). Moreover, *A. platensis* was free of cadmium as shown in Table 4.

Arthrospira largely meets the nutritional requirements for the body since it is rich in essential mineral elements. The present findings showed that this species has important mineral elements (Ca, Mg, K, Na, P) (Table 4). Therefore, we can confirm that these results were in accordance with those reported in earlier works [29]. Our species was screened for potential heavy metals whose results showed very few or no heavy metals (Fe, Zn, Mn, Cu) (Table 5). According to the results obtained in the present study and those previously reported on the phytochemical screening of *A. platensis*, we can confirm that this species is rich in minerals, and therefore, *A. platensis* can be considered a good choice for nutritional supplement product since the minerals discussed in this study play an important role in the function of the body. More specifically, potassium (K) helps prevent hypertension and improve bone health, whereas phosphorus (P) is required for skeletal mineralization [35]. Furthermore, magnesium (Mg) is a cofactor for a variety of metabolic activities and is essential for bone mineralization and muscle relaxation [36], and iron (Fe) prevents anemia by generating hemoglobin and myoglobin. It is also involved in the production of enzymes and other iron-containing enzymes [37].

Lead and chromium were found to be present in the local species with values of 70 ± 4.5 PPB and 5 ± 0.5 PPB, respectively; however, no trace was detected for cadmium. These values are lower than those of the World Health Organization threshold (mercury, 5 μg/kg/week; lead, 25 μg/kg/week; cadmium 7 μg/kg/week).

3.5. Determination of Vitamins. The results obtained showed that *A. platensis* possesses vitamins B_2 and B_3 with values of 1.31 ± 0.19 and 30.8 ± 0.001 mg/kg, respectively, according to

Table 3: Mineral elements contained in *S. platensis*.

Minerals	Wavelength	Content in mg/kg
Cu	324.8	6.95 ± 0.07
Fe	248.3	836.7 ± 131.8
Mn	279.5	47.28 ± 1.17
Zn	213.9	89.7 ± 54.7
Ca	423	1579.22 ± 68.7
Mg	202	2729.71 ± 46.25
K	767.2	16954.85 ± 305.8
Na	589.6	
P	430	16954.85 ± 29.2

Table 4: Heavy metals detected in *A. platensis*.

Heavy metals	Content in PPB	Max. Level in PPB (WHO)
Lead	70 ± 4.5	658
Cadmium	0	472
Chromium	5 ± 0.5	30

Table 5: Vitamins detected in *A. platensis*.

Sample	Percentage (%)
Standard Vit B_3	100.000
Standard Vit B_2	53.687
Sample Vit B_2	18.0055
Sample Vit B_3	55.836

the high-performance liquid chromatography analysis (Table 5).

Since it contains significant levels of fat-soluble vitamins (vitamins A, D, E, and K) and water-soluble vitamins (vitamin B_2: 1.31 ± 0.19 mg/kg; vitamin B_3: 30.8 ± 0.001 mg/kg), *A. platensis* could cover the requirements of vitamins, which the body is unable to synthesize. Thus, these results were in agreement with those reported in earlier literature [38].

3.6. Energy Value Determination. The findings of energetic values assessed in the current research work showed that *A. platensis* from the local cultivar possesses a high energetic value (346.48 ± 0.21 kcal/100 g). The remarkable value of total lipid content (5.8 ± 0.25%) found in A. *platensis* could be responsible for its high energetic value (348.6 ± 0.21 kcal/100 g). In this sense, arachidonic as derived acid from palmitic acid plays a key role in the synthesis of prostaglandins and leukotrienes [39]. Moreover, the findings of chemical analysis showed that the studied species was found to be higher in polyunsaturated fatty acids including omega-3 and 6 that are involved in the prevention of cholesterol accumulation in the body [38, 39].

3.7. Moisture Determination. The findings obtained in the current research showed that the value of moisture content determined in the studied organism was 11.6% ± 0.17. Moisture content is defined as a quantity of water that exists in the biomass. Moisture plays a key role in food storage due to its either direct or indirect effect on microorganism development. In the present work, the moisture content defined in *A. platensis* was 11.6% ± 0.17. Therefore, these results were partially in agreement with those stated in earlier works, which showed that the moisture content in *A.* platensis was 12.5% corresponding to 56% relative humidity [40].

3.8. Ash Content Determination. The results reported in the present study showed that the ash content determined in *A. platensis* was estimated at 9.1% ± 0.21. The ash is a measure of mineral content in biomass. In food, ash content is an important part of food quality analysis. Herein, *A. platensis* was also investigated in terms of ash content. As reported in the current research, the ash content was estimated at 9.1% ± 0.21. This finding was supported by the earlier found data, which reported that *Arthrospira* grown in Zarrouk's medium acquired the highest percentage of ash [41].

3.9. Determination of Pigments. The analysis of pigment content in *A. platensis* showed the presence of chlorophyll *a*, chlorophyll B, and carotenoid with values of 26.066 ± 3.08 mg/g, 37.506 ± 3.38 mg/g, and 9.52 ± 0.22 mg/g, respectively. Regarding the pigment production, the analyzed sample evidenced the presence of chlorophyll b, chlorophyll a, and carotenoids with values 37.506 ± 3.38 mg/g, 26.066 ± 3.08 mg/g, and 9.52 ± 0.22 mg/g, respectively. Thus, these findings were in accordance with those reported in earlier works, which showed that the values of chlorophyll and carotenoid content in the genus *Arthrospira* were 26 mg/g and 3 mg/g DM, respectively. *Arthrospira* cells possessing carotenoids in different forms including α-carotene, β-carotene, cryptoxanthin, zeaxanthin, xanthophylls, echinenone, and lutein as reported elsewhere [32]. Therefore, we can confirm that this species can be a promising source of pigments like chlorophylls, carotenoids, and phycocyanins as reported in the earlier literature [31].

3.10. Determination of Tannins. Qualitative analysis of *A. platensis* extracts revealed a low tannin content. Gallic tannins were also present in little amount. Tannins are belonging to the secondary metabolites synthesized by plants and microorganisms to accomplish ecological functions. Our results showed that our organism has no important amount of tannins. Hence, these results were in contrast with the previously reported literature, which revealed the presence of promising tannin content in the genus *Arthrospira* [6].

3.11. Comparison of A. platensis Indigenous to the Moroccan Atlantic Coast at Laayoune with the Same Species from Different Collection Areas in Terms of Physicochemical Characteristics. Species of *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune possess unique features in terms of physicochemical contents when compared with the same species collected from different collection areas as reported in earlier works [25]. The studied species in

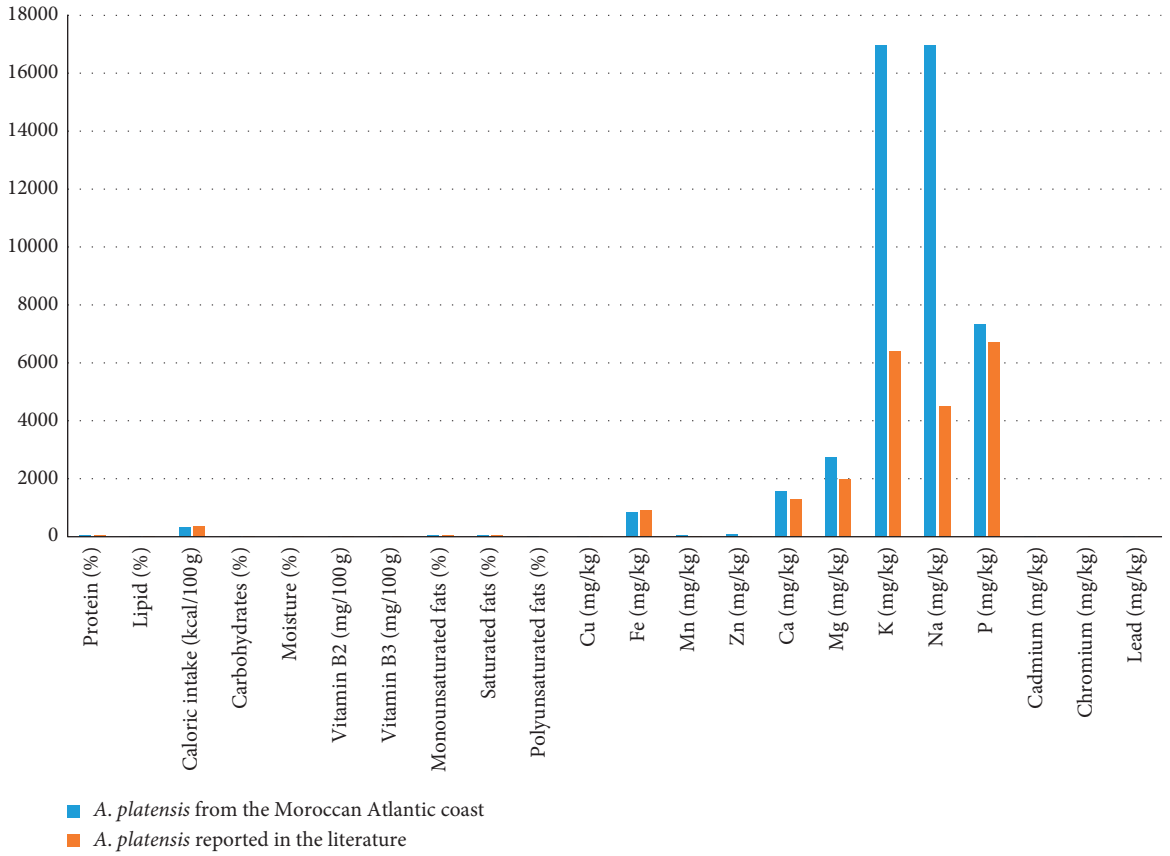

Figure 3: Physicochemical characteristics of *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune in comparison with those of the same species reported in the literature.

the present work was found to be higher in the following parameters: proteins, carbohydrates, monounsaturated fats, moisture, vitamin B_2, vitamin B_3, ash, Mn, Zn, Ca, Mg, K, Na, and P when compared with the same species indigenous to other areas. Our species was also found to be lower in heavy metals (lead, chromium, and cadmium) and saturated fats as nonrequired parameters in foods (Figure 3; Table 6).

Malnutrition is a public health problem throughout the world over the past decades. Several people worldwide have suffered malnutrition and food-related chronic diseases. In Africa, more than 30% of the deaths of less than five-year-old children result directly or indirectly from malnutrition, which is coupled with deficiencies in vitamins and minerals. It is thus fitting that people across the world have looked for natural products to improve health or to remedy deficiencies. Around fifty microalgae are currently consumed worldwide. The most common in the trade are sea lettuce, dulse, sea beans, nori, wakame as well as spirulina, and chlorella. [2]. The consumption of spirulina as a portion of food could back to many years ago. The nutritional value of spirulina can be due to its chemical composition, which is constituted of fibers, minerals, and proteins in large part, not that only but also the presence of secondary metabolites (vitamins, tannins), which are known to possess antioxidant and antibacterial effects. The chemical composition of spirulina exhibits other important benefits such as cosmetic, pharmacological, and therapeutic values [6].

Our results are in accordance with those reported by Jourdan, who showed that *A. platensis* possessing about 50 to 70% protein, 15 to 25% carbohydrates, and 11% for lipids, vitamins, minerals, as well as chlorophyll [44]. In this sense, it was reported that *A. platensis* is a potential source of several water-soluble vitamins (B2, B3, B5, and B9), which act as coenzymes for mitochondrial enzymes and play important roles in cell metabolism and energy production according to prior research [45].

A. platensis possesses an interesting protein family that is recognized by its activities such as antioxidant, anticoagulant, antihypertensive, immunomodulatory, and antimicrobial [29]. Species of *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune have unique features in terms of physicochemical contents when compared with the same species investigated elsewhere. The obtained results showed that our studied species were higher in proteins, polyunsaturated and monounsaturated fats, minerals, vitamins (B_2, B_3), ash, and pigment contents when compared with species studied by Bensehaila, (2015). Moreover, our species was lower in heavy metals and saturated fats when compared with those studied by Bensehaila, (2015), and Falquet, (2012). Therefore, we could confirm that *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune is a promising source of food supplement due to their high values concerning proteins, unsaturated fats, carbohydrates, minerals, vitamins, and pigments and their low values

Table 6: Comparison of *A. platensis* indigenous to the Moroccan Atlantic coast with the same species from different collection areas in terms of physicochemical characteristics.

Parameter	*A. platensis* indigenous to the Moroccan Atlantic coast	*A. platensis* reported in the literature	Publications used
Protein (%)	58.9 ± 0.07^{a}	52.86 ± 2.92^{a}	[25]
Lipid (%)	5.8 ± 0.25^{a}	7.28 ± 0.021^{a}	[25]
Calorie intake (kcal/100 g)	346.48^{a}	369.28^{a}	[25]
Carbohydrates (%)	14.67 ± 0.001^{a}	13.6^{a}	[25]
Moisture (%)	$11.6\% \pm 0.17^{a}$	5.42 ± 0.031^{b}	[25]
Vitamin B_2 (mg/100 g)	1.31 ± 0.19^{a}	0.009^{b}	[42]
Vitamin B_3 (mg/100 g)	30.8 ± 0.001^{a}	0.053^{b}	[42]
Monounsaturated fats (%)	53 ± 0.003^{a}	40.1^{b}	[42]
Saturated fats (%)	45.54 ± 0.15^{a}	55.72^{b}	[43]
Polyunsaturated fats (%)	1.46 ± 0.01	—	[42]
Cu (mg/kg)	6.95 ± 0.07^{a}	8^{a}	[42]
Fe (mg/kg)	836.7 ± 131.8^{a}	900^{a}	[42]
Mn (mg/kg)	47.28 ± 1.17^{a}	25^{b}	[42]
Zn (mg/kg)	89.7 ± 54.7^{a}	21^{b}	[42]
Ca (mg/kg)	1579.22 ± 68.7^{a}	1300^{b}	[42]
Mg (mg/kg)	2729.71 ± 46.25^{a}	2000^{b}	[42]
K (mg/kg)	16954.85 ± 305.8^{a}	6400^{b}	[42]
Na (mg/kg)	16954.85 ± 29.2^{a}	4500^{b}	[42]
P (mg/kg)	7335.35 ± 123.6^{a}	6700^{b}	[42]
Cadmium (mg/kg)	0.005 ± 0.0005^{a}	14.2^{b}	[42]
Chromium (mg/kg)	0	≤1	[42]
Lead (mg/kg)	0.07 ± 0.0045^{a}	5 ± 00.5^{b}	[42]

The results were given as average ± standard deviation. The reported values with the same letter in the same line did not differ significantly at $P < 0.05$.

regarding heavy metals and saturated fats. *A. platensis* is reported to be of high nutritional value and pharmacological and biological potentials so that they can be used for medicinal purposes for humans or animals [46]. In addition to their nutritional and pharmacological properties, *A. platensis* has a biofuel production potential, and therefore, it can significantly contribute to economic performance [47].

A. platensis cannot be free of toxins. In this sense, the rate of trace elements (B, Ba, Li, Ni, Sr, V) alongside toxic metals (Al, Cd, Pb) was detected in *A. platensis* samples. The highest element concentration was detected in the powder format, except for Li. When element levels in *A. platensis* exceed the tolerable weekly intake (TWI), the consumer would place his health at risk. The consumption of spirulina contributes largely to the Al intake by a value higher than TWI determined at 1 mg/kg bw/w (body weight/week), followed by Cd exceeding its TWI set at 2.5 μg/kg bw/w was reported in previous works [48]. Pb intake with a value higher than the TWI level can be associated with nephrotoxicity and cardiovascular effects. However, this literature suggests that spirulina consumption does not place the consumer at risk as far as exposure to toxic metals (Al, Cd, Pb) is regarded as a concern. However, the presence of trace elements and toxic metals in spirulina destined for food purposes should be monitored to ensure its quality and safety. In contrast, lead and chromium were found in our species with values of 70 ± 4.5 PPB and 5 ± 0.5 PPB, respectively; however, no trace was detected for cadmium. These values are lower than those of the World Health Organization threshold (mercury, 5 μg/kg/week; lead, 25 μg/kg/week; cadmium 7 μg/kg/week). Therefore, *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune (Foum El Oued lagoon) can be considered safe for being ingested.

4. Conclusion

The present research work aims to assess the nutritional value of *A. platensis* collected from the Moroccan Atlantic coast at Laayoune. The obtained results showed that *A. platensis* indigenous to the Moroccan Atlantic coast at Laayoune was found to be very rich in proteins, carbohydrates, vitamins, minerals, ash, and pigments and lower in heavy metals and saturated fats when compared with species investigated in the literature. Therefore, we could confirm that *A. platensis* indigenous of the Moroccan Atlantic coast at Laayoune can be a very promising source of dietary supplements. Overall, *A. platensis* should be optimized further, and processing strategies based on the optimization approaches can be developed.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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