Journal of Food Quality

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Research Article

Journal of Food Quality Evaluation of Effect of Extraction Solvent on Selected Properties of Olive Leaf Extract

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The quest for natural preservatives and functional foods with health benefits has seen an increasing demand for natural products having therapeutic value. Herein, we investigated the influence of ethanol, methanol, acetone (50%, 70%, and 90% v/v), and distilled water on selected properties of olive leaf extract and determined the yield, total phenolic content (TPC), antioxidant activity, and antimicrobial activity. Extracts were analyzed for their oleuropein, hydroxytyrosol, and tyrosol contents by high-performance liquid chromatography (HPLC). The highest extraction yield of 20.41% was obtained when using 90 vol% methanol, while the highest total polyphenol contents of 232 and 231 $mg_{gallic\text{-}acid\text{-}equivalent}/100$ g were obtained for 90 vol% methanol and 90 vol% ethanol, respectively. Antioxidant activity was determined using the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging assay, by determining the ferric reducing antioxidant power (FRAP), and using the Fe^{2+}-chelating activity assay, which provided the highest values when 90 vol% methanol was used (33.84%, 0.75, and 12.91%, respectively). HPLC analysis showed that the highest oleuropein contents corresponded to the extracts obtained using 90 and 70 vol% methanol (26.10 ± 0.20 and 24.92 ± 1.22 g/L, respectively), and the highest antimicrobial activity was observed for 90 vol% methanol and distilled water. Olive leaf extracts using 90 vol% methanol had high levels of polyphenols and were highly antioxidant and antimicrobial. The results of this study facilitate the commercial applications of natural extracts with antioxidant and antibacterial activities and are expected to establish a foundation for further optimization studies.

1. Introduction

The increasing demand for natural preservatives and new functional foods with health benefits has inspired numerous studies on biologically active compounds found in plant extracts and the by-products of plant processing [1–3]. Among these compounds, phenol derivatives (phenolics) exhibit a wide range of physiological effects, including antiallergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, anticancer, cardioprotective, and vasodilatory activities [4]. Since phenolics are typically extracted from natural matrices or food industry by-products that are usually discarded or used for animal feed production [5], the influence of solvent on the extraction of phenolics from vegetable substrates has been extensively researched [6]. For example, solvent polarity is known to strongly affect extraction efficiency and other parameters [7–9]. The differences in the structures of phenolic compounds determine the solubilizing abilities of solvents whose polarities are different. Therefore, the type of extraction solvent and separation procedure can have an important influence on the amounts of polyphenols extracted from plant substances. Although the phenolic contents of food have been widely investigated and extraction conditions optimized for antioxidant activity, some studies have shown that the optimum separation procedure normally depends on the characteristics of the plant [10, 11].

Olive *(Olea europaea)* fruit, oil, and leaves have a long-standing history of medicinal and nutritional use [12]. Olive leaves are a by-product of olive processing, accounting for up to 10% of the total olive weight, and are considered to be an inexpensive raw material source of antioxidant compounds [13]. Olive leaves have traditionally been used in

animal feed, but because they contain high-value compounds with antioxidant and antibacterial properties, they have recently been used as food additives, in functional foods and in pharmaceuticals. [14, 15]. Olive leaves, in particular, exhibit antioxidant, antihypertensive, and anti-inflammatory activities and are effective against hypoglycemia and hypocholesterolemia [16, 17]. The antioxidant activities of olive leaf extracts have been ascribed to the presence of phenolics such as oleuropein, luteolin, and hydroxytyrosol [18]. For example, oleuropein, the main component of olive leaf extract, exhibits antihypotensive, anti-inflammatory, and strong antioxidant activities [19–21]. Consequently, there is a growing interest in recovering phenolic compounds from olive leaves [22]. However, the conditions currently used for the extraction of biologically active compounds need to be improved in order to increase extraction efficiency, decrease extraction costs, and preserve functional activity in a better way [23]. In view of the above, in this study, we investigated the effect of the solvent (water, aqueous methanol, aqueous ethanol, and aqueous acetone) used to extract olive leaves on yield, as well as the antioxidant and antimicrobial activities of the extract.

2. Materials and Methods

Olive leaves were imported from Spain (Teetraum, Wollenhaupt Co., Ltd., Germany) and purchased through CJ mall in Korea. Oleuropein, hydroxytyrosol, and tyrosol standards, and α,α-diphenyl-β-picrylhydrazyl (DPPH) were obtained from the Sigma-Aldrich Chemical Co., Korea.

2.1. Preparing the Olive Leaf Extract. Distilled water (DW), aqueous ethanol (50, 70, and 90 vol%), aqueous methanol (50, 70, and 90 vol%), and aqueous acetone (50, 70, and 90 vol%) were used as extraction solvents. Typically, a mixture of dried olive leaf powder (5.0 g) and the solvent of choice (100 mL) were agitated in a shaking incubator at room temperature (25°C) and 250 rpm for 1 h and then centrifuged at 10000 rpm for 10 min. The supernatant was concentrated in vacuo at 50°C using a rotary evaporator, and the residue was freeze-dried.

2.2. Determining Extraction Yield. Extraction yield (%) was calculated as follows: $100\% \times m_{\text{Extract}}/m_{\text{Powder}}$, where m_{Extract} and m_{Powder} are the masses of the extract and olive leaf powder (g), respectively.

2.3. Determining Total Polyphenol Content (TPC). TPC was determined using a slight modification of the method reported by Wei et al. [24]. In brief, the test solution (100 μL) was treated with the Folin–Ciocalteu reagent (100 μL, 1 N) and incubated at room temperature for 3 min. The mixture was then treated with aqueous Na_2CO_3 (300 μL, 1 N), incubated at room temperature for 90 min, and diluted with DW (1 mL). The absorbance of the resulting solution was measured at 725 nm using an OPTIZEN 2120 UV spectrophotometer (Mecasys Co., Ltd., Korea). A standard curve was prepared using 100, 250, 500, and 1000 ppm gallic acid, the results of which were used to calculate the TPC, which is expressed as milligrams of gallic acid equivalents (GAEs) per 100 g ($mg_{GAE}/100$ g) of the sample. Gallic acid was used as the standard in these experiments. The linear equation for the gallic acid calibration curve can be written as follows: $y = 0.0057x - 0.2484$ ($R^2 = 0.991$), where y is the TPC and x is the absorbance value.

2.4. α,α-Diphenyl-β-Picrylhydrazyl Radical Scavenging Activity (DPPH), Ferric Reducing Antioxidant Power (FRAP), and Fe^{2+}-Chelating Activity. DPPH radical scavenging activity was determined using the method of Blois [25]. In brief, a solution of DPPH in MeOH (1 mL, 1.5×10^{-4} M) was added to the test solution (4 mL) with stirring, and the resulting mixture was incubated at room temperature for 30 min after which absorbance was measured at 517 nm using the abovementioned spectrophotometer.

FRAP was determined using a slightly modified method reported by Oyaizu [26]. In brief, samples were mixed with sodium phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and $K_3Fe(CN)_6$ (2.5 mL, 1% (w/v)). The obtained mixture was incubated at 50°C for 20 min and treated with trichloroacetic acid (2.5 mL, 10% (w/v)). The upper layer of the mixture (2.5 mL) was diluted with DW (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% (w/v)), after which absorbance was measured at 700 nm using the abovementioned spectrophotometer.

Fe^{2+}-chelating activity was determined using the method of Dinis et al. [27]. Briefly, 0.5 mL of the sample was mixed with a solution of $FeCl_2$ (2 mL, 1 mM) in 95 vol% ethanol. The reaction was initiated by the addition of aqueous ferrozine (2.5 mL, 2 mM), and the mixture was vortexed for 10 min, filtered through a nylon syringe filter (0.45 μm), after which absorbance was measured at 562 nm using the abovementioned spectrophotometer. Fe^{2+}-chelating activity (%) was calculated as follows:

$$100\% \times (\text{absorbance control} - \text{absorbance sample})/(\text{absorbance control}). \quad (1)$$

2.5. Quantitating Oleuropein by High-Performance Liquid Chromatography (HPLC). Oleuropein, hydroxytyrosol, and tyrosol were quantified by HPLC (Agilent 1100 series, USA) after sample filtration through a 0.45 μm PVDF membrane filter (Pall Life Science). The mobile phase contained 5% formic acid (A) and methanol (B), and the following gradient was used: 5% B, then 15% B after 3 min, 25% B after 13 min, 35% B after 25 min, 45% B after 35 min, 50% B after 40 min, 100% B after 45 min, 5% B after 46 min, and re-equilibrate to the initial composition for 4 min. The flow rate was 0.9 mL/min, and elution was performed at room temperature. The injection volume was 10 μL. A Supelcosil LC-ABZ column (250 mm × 4.6 mm, 5 μm) was used, and the absorbance detector was operated at 280 nm.

2.6. Determining Antimicrobial Activity. Total viable counts (TVC) were determined on 3M™ Petrifilm™ aerobic count plates (3M, Seoul, Korea) incubated at 35°C for 24 h.

Coliforms and *Escherichia coli* (*E. coli*) were determined on 3M™ Petrifilm™ *E. coli*/coliform count plates (3M, Seoul, Korea) after incubation for 24 h at 35°C. Colonies were identified and counted as per manufacturer's instructions.

2.7. Statistical Analysis. All experiments were performed in triplicate. Experimental data were analyzed by one-way analysis of variance using SPSS/PC Statistics 23.0 software (SPSS Inc., Chicago, IL, USA). The obtained results are presented as means with corresponding standard deviations. Tukey's multiple range tests were used to determine significant differences between mean values, and $P < 0.05$ was taken as an indicator of statistical significance.

3. Results and Discussion

3.1. Extraction Yields. Table 1 lists extraction yields and selected properties of the extracts obtained using each solvent. The highest extraction yields of 20.41 and 18.88% were observed for 90 vol% methanol and DW, respectively, while the lowest yield of 10.83% was observed for 90 vol% acetone, which is similar to the trend reported by Butsat and Siriamornpun; when olive leaves were extracted for 6 h using 80% methanol, 80% ethanol, 80% acetone, and DW, the highest extraction yield was observed for 80% methanol and the lowest for 80% acetone [28]. These yields were ascribed to the effect of solvent polarity on the solubilities of the extract components, i.e., proteins and carbohydrates are more soluble in water and methanol than in ethanol or acetone [29].

3.2. Total Polyphenol Content (TPC). Figure 1 shows the effect of solvent on TPC and reveals that the highest values of 231.98 and 230.61 $mg_{gae}/100\,g$ were obtained for 90 vol% methanol and 90 vol% ethanol, respectively. The lowest TPC of 192.03 $mg_{gae}/100\,g$ was observed for DW and was significantly different to the values obtained using the other solvents ($p < 0.05$). TPC was observed to decrease with increasing water content for each solvent, in agreement with previously reported results. Thus, when compared to the other extraction solvents, water results in a higher non-phenolic compound content (e.g., carbohydrates and terpenes) because some phenolic compounds soluble in methanol, ethanol, and acetone can be extracted through complex formation. Hence, compounds that contain more phenol groups or have higher molecular weights than simple phenols are found in the water extract [30]. Moreover, compounds extracted with methanol have been reported to exhibit higher antioxidant activities and phenolic contents than those prepared using other solvents [29, 31], which is consistent with the results of this study. Some researchers have revealed that methanol is typically preferred for the effective extraction of phenolic compounds from plants [32, 33] and that methanol decreases the degeneration of phenols in plant extracts by controlling polyphenol oxidative enzyme activity [34]. Moreover, moudache et al. showed that the TPC content of an olive leaf extract increases with increasing organic content in the extraction solvent [15].

3.3. Antioxidant Activity. The effects of the various solvents on the DPPH radical scavenging activity, FRAP, and Fe^{2+}-chelating activity of the extracts are summarized in Table 1. The first of these parameters is primarily used to quantify the FRAP of natural antioxidants. The original violet color of the DPPH radically changes to yellow when reduced to the corresponding stable diamagnetic molecule. Consequently, determining DPPH radical scavenging activity by observing this color change allows one to characterize numerous samples within a short period, and the method is sensitive enough to detect active ingredients at low concentrations [35]. The highest DPPH radical scavenging activity of 33.84% was observed for 90 vol% methanol; however, the values of 32.97 and 33.27% obtained for 50 and 70 vol% methanol, respectively, were not significantly different ($P > 0.05$). For aqueous ethanol and aqueous acetone, statistically similar ($P > 0.05$) values of ~31% were observed, irrespective of water content. The lowest DPPH scavenging activity of 26.75% ($P < 0.05$) was observed for DW.

FRAP is a parameter that quantifies antioxidant activity related to the electron-donating capability of a molecule. The Fe^{3+} in $K_3Fe(CN)_6$ is reduced to Fe^{2+} in the presence of an antioxidant, which results in the initial yellow test solution turning green or blue [36]. High FRAP values were obtained for all solvents in this study, with the exception of DW, and decreased in the order: aqueous methanol > aqueous acetone > aqueous ethanol >> pure water. The olive leaf methanol extract was determined have strong antioxidant properties; hence, the compounds in this methanol extract are outstanding electron donors capable of terminating oxidation chain reactions by reducing oxidized intermediates to stable forms [37].

Determining Fe^{2+}-chelating activity relies on the ability of the extract to complex Fe^{2+} ions, thereby inhibiting the formation of the Fe^{2+}-ferrozine complex. The highest Fe^{2+}-chelating activity was obtained for 90 vol% methanol, while the lowest value was obtained for DW ($P < 0.05$). These findings show that the radical scavenging activity of the olive leaf extract depends on the polarity of the solvent used, in agreement with previous results [38]. Sepúlveda-Jimenez et al. demonstrated that extracts of the same plant origin obtained using methanol exhibited higher antioxidant activities than those extracted with water [39]. Franco et al. showed that the polarity of the extraction solvent strongly influences the extraction efficiency and the antioxidant activities of *Rosa rubiginosa* and *Gevuina avellana* extracts [40]. Fractions with different antioxidant activities could be separated on the basis of the polarity of the extracting solvent, with oxygenated compounds selectively extracted in accordance with their chemical structures, polarities, and solubilities [41].

3.4. Analyzing Olive Leaf Extract by HPLC. The oleuropein, hydroxytyrosol, and tyrosol contents of the olive leaf extracts were quantified by HPLC (Table 2, Figure 2), which revealed that 90 vol% methanol was best able to extract these phenolic compounds. Oleuropein has previously been identified as an important component of olive leaf extract [42, 43]. The

Table 1: Extraction yields and antioxidant activities of olive leaf extracts obtained using various solvents.

Solvent	Extraction yield (%)	DPPH radical scavenging activity (%)	FRAP	Fe^{2+}-chelating activity
Ethanol				
50%	17.55 ± 0.88^{bc}	31.17 ± 0.10^{b}	0.71 ± 0.01^{bc}	9.78 ± 0.12^{bc}
70%	17.08 ± 0.77^{bc}	31.68 ± 0.40^{b}	0.69 ± 0.01^{c}	10.93 ± 0.24^{b}
90%	17.44 ± 1.13^{bc}	31.56 ± 0.19^{b}	0.59 ± 0.02^{d}	10.06 ± 0.07^{bc}
Methanol				
50%	17.17 ± 0.25^{bc}	32.97 ± 0.23^{a}	0.75 ± 0.01^{a}	8.81 ± 0.67^{cd}
70%	16.45 ± 0.64^{bc}	33.27 ± 0.41^{a}	0.76 ± 0.00^{a}	9.68 ± 0.81^{bcd}
90%	20.41 ± 0.63^{a}	33.84 ± 0.47^{a}	0.75 ± 0.01^{a}	12.91 ± 0.37^{a}
Acetone				
50%	16.47 ± 0.84^{bc}	31.83 ± 0.13^{b}	0.72 ± 0.00^{b}	9.12 ± 0.30^{cd}
70%	16.03 ± 1.29^{c}	31.67 ± 0.35^{b}	0.70 ± 0.00^{bc}	8.38 ± 0.77^{de}
90%	10.83 ± 0.13^{d}	31.90 ± 0.22^{b}	0.72 ± 0.01^{b}	9.19 ± 0.37^{cd}
100% DW	18.88 ± 1.83^{ab}	26.75 ± 0.46^{c}	0.64 ± 0.00^{e}	7.22 ± 0.17^{e}

Means with different superscripts ($^{a\text{-}e}$in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations from three replicates.

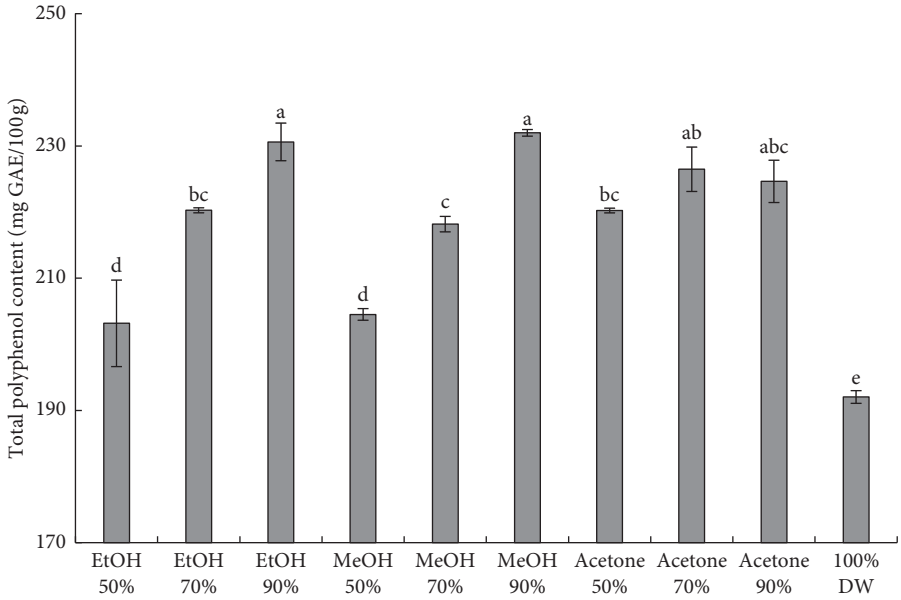

Figure 1: Effect of solvent on the TPC of olive leaf extract. Means with different superscripts ($^{a-e}$in the same column) differ significantly ($P < 0.05$). All values represent means ± standard deviations for three replicates.

highest oleuropein content of 26.10 g/L was obtained when extracted with 90 vol% methanol ($P < 0.05$), while the lowest content of 5.36 g/L was observed for DW, which is the same as the TPC trend. Hydroxytyrosol and tyrosol were detected in considerably smaller amounts, which is in agreement with previous results [44]. Thus, among the tested solvents, methanol was found to be most favorable for extracting oleuropein from olive leaves, which is in agreement with the findings of Bouaziz and Sayadi [18].

3.5. Antimicrobial Activity. Table 3 shows the antimicrobial activities of fractions extracted with various solvents, which reveals that antimicrobial activity decreases in the order: DW > 90 vol% methanol >70 vol% methanol >90 vol% ethanol >90 vol% acetone. The above extracts were examined for their effectiveness against experimental microorganisms. All extract did not detect in coliform count plate and *E. coli* count plate (data not shown). These findings are in agreement with the previously reported abilities of olive leaf extract to inhibit the growth of certain pathogenic bacteria [45, 46]. The observed antimicrobial activities are attributable to the phenolic contents of the extracts [47, 48]; the high contents of oleuropein and other phenolic compounds identified in the extracts contribute to the observed antibacterial properties. In this study, the total viable counts of the 90 vol% methanol, ethanol, and acetone extracts are low because of the high phenol contents of these extracts. Oleuropein has been reported to improve the production of nitric oxide in a dose-dependent manner (it is known to be cytotoxic to various pathogenic bacteria) in endotoxin-challenged mouse macrophages [49]. The effects of oleuropein and its derivatives contribute to the in vivo defense system against bacterial infection. The total phenolic content and the amount of oleuropein determined by HPLC in the DW extract were the lowest; however, this extract exhibited the highest antimicrobial

Table 2: Major phenolic compounds in olive leaf extracts obtained using various solvents.

Solvent	Compound (g/L)		
	Hydroxytyrosol	Tyrosol	Oleuropein
Ethanol			
50%	0.61 ± 0.09^{abc}	0.10 ± 0.00^{ab}	19.72 ± 1.27^{bc}
70%	0.63 ± 0.12^{abc}	0.11 ± 0.02^{a}	21.96 ± 1.96^{b}
90%	0.62 ± 0.09^{abc}	0.07 ± 0.01^{c}	21.89 ± 1.13^{b}
Methanol			
50%	0.72 ± 0.05^{abc}	0.07 ± 0.00^{c}	19.47 ± 0.85^{bc}
70%	0.77 ± 0.03^{a}	0.08 ± 0.01^{bc}	24.92 ± 1.22^{a}
90%	0.74 ± 0.02^{ab}	0.07 ± 0.00^{c}	26.10 ± 0.20^{a}
Acetone			
50%	0.54 ± 0.07^{bc}	0.08 ± 0.00^{c}	17.70 ± 0.47^{c}
70%	0.52 ± 0.06^{c}	0.03 ± 0.01^{d}	17.66 ± 0.64^{c}
90%	0.61 ± 0.10^{abc}	0.02 ± 0.00^{d}	17.79 ± 0.48^{c}
100% DW	0.25 ± 0.02^{d}	0.03 ± 0.00^{d}	5.36 ± 0.78^{d}

Means with different superscripts ($^{a\text{-}d}$in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations for three replicates.

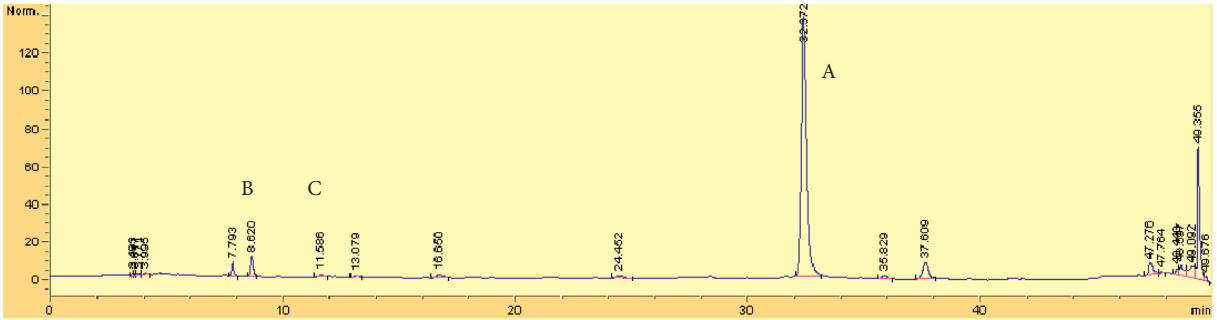

Figure 2: HPLC trace of phenolic compounds extracted from olive leaves with 90 vol% methanol: (A) hydroxytyrosol, (B) tyrosol, and (C) oleuropein.

Table 3: Total viable counts (TVCs) in olive leaf extracts obtained using various solvents.

Solvent	Antimicrobial (CFU/mL)
Ethanol	
50%	771.67 ± 34.03^{b}
70%	756.67 ± 91.70^{b}
90%	145.00 ± 56.35^{c}
Methanol	
50%	598.33 ± 117.30^{b}
70%	116.67 ± 2.89^{c}
90%	36.67 ± 18.93^{c}
Acetone	
50%	1070.00 ± 105.00^{a}
70%	780.00 ± 173.86^{b}
90%	165.00 ± 17.32^{c}
100% DW	33.33 ± 16.07^{c}

Means with different superscripts ($^{a\text{-}c}$in the same column) differ significantly ($P < 0.05$). All values are means ± standard deviations for three replicates.

activity of 33.33 CFU/mL. According to previous reports, antimicrobial activity is not only related to the total phenol content, but also to the types and relative distributions of the phenolic components, which are important for biological activity. Bacterial resistance is also related to the structure of the polyphenol. Therefore, the compounds in the DW extract need to be identified through further studies.

4. Conclusions

We determined optimal conditions for olive leaf extraction by examining the effect of extraction solvent on selected extract properties. The highest extraction efficiency of 20.41% was obtained using 90 vol% methanol, while the lowest value of 18.88% was obtained using DW, and the highest total polyphenol contents were obtained with 90 vol% methanol and 90 vol% ethanol, while the lowest was obtained using DW ($P < 0.05$). The highest antioxidant activity was observed for the extract obtained using 90 vol% methanol, and the oleuropein content was highest when 90 and 70 vol% methanol were used as the extraction solvents. Finally, extracts with the highest antimicrobial activities were obtained using 90 vol% methanol and DW. Thus, we conclude that 90 vol% methanol is the optimal extraction solvent, affording extracts with high antioxidant and antibacterial activities in high yields. The results of this study are expected to be of importance for the development of a wide range of products based on olive leaf extract.

Data Availability

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

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Polyphenol-Rich Extracts of Traditional Culinary Spices and Herbs and Their Antibacterial Activity in Minced Beef

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This study was conducted to elucidate minced beef stabilization properties of hydroalcoholic extracts of commonly used culinary spices from Pakistan against meat oxidative stress and microbial spoilage. Hydroalcoholic extracts of six selected spices, namely, onion, ginger, turmeric, coriander, fennel, and mint, were evaluated to inhibit microbial growth in minced beef under refrigerated storage (4°C) of nine days. Maximum phenolic concentration, i.e., 70.8 mg GAE/100 g, and free radical scavenging activity (75.9%) were anticipated by hydromethanolic extracts of ginger. The results propose that the addition of hydroalcoholic extracts of ginger and coriander @ 6.0% anticipate significantly ($p < 0.05$) higher inhibitory effects against *Staphylococcus aureus* and *Escherichia coli*. The results of this research conclude that the utilization of hydroalcoholic extracts may serve as a promising approach to preserve microbiological as well as the oxidative quality of minced beef and products of meat origin.

1. Introduction

Spices have a long history for culinary application as seasoning ingredients in various cultures, e.g., garlic, onion, cinnamon, anise, clove, and red pepper are preferred seasoning agents of Chinese culture while coriander and black pepper are likely consumed in the East Indian region [1]. Spice extracts and essential oils have been extensively explored for shelf stability of raw and processed chicken [2, 3], shallow and deep-fried meat [4], fermented meat [5], meat sausages [6], and dried cured meat [7].

Microbiological food safety in the meat distribution system can be achieved to a greater extent with natural ingredients of plants and animal origins like organic acids, plant extracts, and essential oils [8]. Antimicrobial features of spices are predominantly associated with phytochemicals like flavonoids, flavones, isoflavones, and anthocyanins that anticipate significant free radicals and free metal ion binding properties in food systems [9, 10]. Active ingredients of spices have also been attributed to bringing about changes in cellular membrane permeability leading to intracellular matrix leakage and cell lysis [11]. Typical characteristics of spices defining their role as potential antimicrobial agents have been embedded in rendering bacteria to poorly synthesize microbial nucleotides, i.e., DNA and RNA, which could further halt microbial growth and proliferation [12].

Spices primarily provide a convenient and reasonable choice towards minimizing household and industrial use of synthetic additives and to add value to the consumer good. Spices could further substantially contribute to reducing the adverse effects of synthetic additives on product quality and consumer's health. The objectives of the present study entail

investigation into assessing the antioxidant potential of turmeric, onion, ginger, fennel, coriander, and mint extracts and their role as antimicrobials in inhibiting microbial growth in the minced beef model under refrigerated storage.

2. Material and Methods

2.1. Procurement of Raw Materials and Chemicals. Fresh ginger (*Zingiber officinale*) rhizomes, onion (*Allium cepa*) bulbs, turmeric (*Curcuma longa*) stems, coriander (*Coriandrum sativum*) seeds, fennel (*Foeniculum vulgare*) seeds, and peppermint (*Mentha piperita*) leaves were purchased from the local market of Multan, Pakistan. Samples were maintained at −18°C until drying. All reagents unless specified including solvents, sodium acetate buffer (pH = 3.6), DPPH (2, 2-diphenyl-1-picryl-hydroxyl) reagent, Folin–Ciocalteu phenol reagent (FCR), gallic acid, sodium carbonate, TPTZ [2, 4, 6-tri (2-pyridyl)-s-triazine], and ascorbic acid were analytical grade and purchased from Sigma-Aldrich Inc., USA.

2.2. Development of Spice Powders. Green spices were procured from the local vegetable market, washed with potable water, sorted, graded, and dehydrated in a cabinet dryer at 70°C to 15–17% moisture contents. Dehydrated spices were ground to 70 mm mesh size, sieved, and stored in airtight plastic containers at 25°C for further applications [13].

2.3. Physicochemical Analysis of Spice Powders. Fine powders of spices were analyzed for moisture, fat, ash, and protein contents in accordance with the procedure laid down in AOAC [14, 15]. Carbohydrate contents were estimated as nitrogen-free extract (NFE) using formula, i.e., NFE% = 100 − (moisture + crude protein + total ash + crude fat + -crude fiber). Mineral contents (Na, Ca, and K) of spice powders were analyzed using a flame photometer in accordance with the method laid down by AOAC [14].

2.4. Total Phenolic Contents. Spice powders were soaked in hydromethanolic and ethanolic solvents (70 : 30) (solvent: distilled water) for 8 hours. Orbital shaking was performed at 40°C for 3 hours. The supernatant was filtered via filter paper no. 41 followed by rotary evaporation at 40°C. Extracts' concentrates were freeze-dried and stored at −18°C for further assay. Total phenolic contents in spice extracts were determined by the method adopted by Manzoor et al. [16]. Plant extracts (i.e., 100 ppm) were prepared with solvents, i.e., methanol and ethanol. An aliquot of 0.5 mL was transferred to the test tubes followed by the addition of 10-fold diluted FCR. 2 mL of sodium carbonate (7.5%) was added, and samples were subjected to react for 30 min at 25°C. Absorbance was measured spectrophotometrically (UV-Vis 3000, ORI, Germany) at 760 nm using gallic acid standard (10–100 ppm), and results were expressed as mg GAE/100 g.

2.5. 2, 2-Diphenyl-1-Picryl-Hydrazyl (DPPH) Assay. Free radical scavenging activity of hydroalcoholic extracts was determined using DPPH assay [17]. Different concentrations of spice extracts ranging from 50–100 ppm were prepared. Aliquots (50–100 μL) were pipetted in labeled test tubes, and the final volume was adjusted to 100 μL with methanol. 5 mL of DPPH reagent (0.1 mM) was added to each test tube. The contents of test tubes were vortexed and incubated for 20 min at 27°C. Absorbance was measured spectrophotometrically at 517 nm. Free radical scavenging activity was calculated using the following formula:

$$\text{radical scavenging activity (\%)} = \frac{\text{control Abs.} - \text{sample Abs.}}{\text{control Abs.}} \times 100. \quad (1)$$

2.6. Microbiological Analysis

2.6.1. Bacterial Cultures and Inocula Preparation. Bacterial isolates from minced beef were spread onto specific microbial culture media including mannitol salt agar (*Staphylococcus aureus*), MacConkey agar (*Escherichia coli*), and SS agar (*Salmonella* spp.). Confirmed colonies of each test microorganisms were shifted to phosphate buffer saline and incubated at 37°C for 3–6 hours to achieve 0.5 McFarland turbidity standard.

2.6.2. Antimicrobial Assay (Disc Diffusion Method). Antimicrobial screening of spice extracts was performed in accordance with the method developed by Adetunde et al. [18]. Microbial cultures vis. *S. aureus*, *E. coli*, and *Salmonella* spp. were evenly spread on Muller Hinton Agar (MHA) plates. Sterilized discs were aseptically placed over the inoculated MHA media plates. Spice extracts (50 μL) of 150 ppm strength were loaded onto the discs. Solvent and standard drugs, i.e., gentamycin and penicillium (20–30 μg), were taken as negative and positive controls, respectively. MHA plates were subjected to incubation at 37°C for 24 hours, and zones of inhibitions (mm) were computed.

2.6.3. Microbiological Inhibition Properties of Spice Extracts in Minced Beef. Freshly purchased minced beef was decontaminated using sodium hypochlorite (20 ppm). Hundred-gram minced beef sample with no decontamination treatment was designated as a negative control. Twenty-five grams of minced beef was marinated with hydromethanolic extracts of spices including onion, turmeric, ginger, coriander, fennel, and mint at the rate of 1.5%, 3%, and 6%. Marinated samples were stomached for 2 min (Stomacher® 400 Circulator). Stomached samples were further inoculated with 100 μL (1.5×10^8 CUF/mL~0.5 McFarland turbidity standard) cultures of *S. aureus* and *E. coli*. Microbial spiked minced beef samples were stored at 4 ± 2°C, and total counts of *S. aureus* and *E. coli* of minced beef samples were enumerated on 0, 3rd, 6th, and 9th day of storage. Results were expressed as $\log_{10}$ CFU/g [19, 20].

2.7. Statistical Analysis. All experiments were performed twice, and the results were expressed as mean ± SD. Data

were statistically analyzed with Statistics 8.1 software using a two-way analysis of variance (ANOVA) technique at $p < 0.05$. Means were compared using the least significant difference (LSD) test.

3. Results and Discussion

3.1. Physicochemical Properties of Spices and Their Extracts. Data on the nutritional composition of spices powder are presented in Tables 1 and 2. A significant difference in ash contents was detected in turmeric (6.5%) and mint powder (1.9%). Maximum fat contents were recorded in fennel while coriander depicted the highest concentration of protein. A significantly higher amount of carbohydrates was recorded in onion powder.

The appreciable concentration of sodium, calcium, and potassium was observed in coriander, fennel, and ginger powder, respectively (Table 2). Average spice consumption from various modes in the Indian subcontinent has been reported around 10 g that can anticipate ~1.2–8% of daily energy requirements [21]. In addition to create appeal and anticipate functional properties, compositional analysis of spices thus suggests their supplementary role in improving the nutritional value of the finished goods.

3.2. Total Phenolic Contents and Antioxidant Activity. The extracts' yield, total phenolic contents, and antioxidant potential of spices are presented in Table 3. Significant ($p < 0.05$) effect of solvent and type of spices was revealed on phenolic recovery. The highest total phenolic contents with a mean value of 70.8 mg GAE/100 g were recovered from ginger followed by turmeric extracts, i.e., 70 mg GAE/100 g, while onion and fennel hydroethanolic extracts were bearing lower phenolics recovery rate, i.e., 36 mg GAE/100 g and 35 mg GAE/100 g, respectively. Relatively lower total phenolic contents were reported in spices by Kumari and Gupta [22] wherein the phenolic recovery rate was in a range between 20–78 mg GAE/100 g. Hydroalcoholic extraction of plant phenolics has variable recovery rates that depend on the type of solvent, combinations of solvents like water : alcohol ratio (70 : 30) and solvent/solid ratio, part of the plant, i.e., leaves, roots, seeds, fruit, flower, and bark, particle size or the surface area of the plant matter, and extraction conditions like pressure (30–250 bar), extraction time (3-4 hours), and extraction temperature, i.e., ~25°C [23].

Significantly higher antioxidant properties were observed in the ginger extract in comparison with extracts of other spices under investigation (Table 3). Hydroalcoholic extracts yielded higher free radical scavenging properties with ginger followed by turmeric, i.e., 75.9%, while hydroethanolic extracts presented higher DPPH radical scavenging activities for ginger (66.3%) and coriander (51.7%). Higher DPPH free radical scavenging properties of ginger and turmeric correlates with their higher phenolic contents as compared to the onion extract. DPPH free radical scavenging property of ginger extracts has been previously cited between 67–78% [22]. The considerably higher concentration of hydroxyl rich total phenolics and synergistic role of spices extracts could be achieved by their application as additives in meat and meat-based products.

3.3. Antimicrobial Screening of Spice and Herb Extracts. The antimicrobial activity of spice extracts against various pathogenic microbes at 150 ppm concentration is presented in Table 4. Inhibition zones of various extracts against Gram-negative and Gram-positive bacteria including *E. coli, Salmonella* spp., and *S. aureus* were determined for methanolic and ethanolic extracts of ginger, turmeric, onion, coriander, fennel, and mint extracts at 150 ppm concentration (30–40 μg extracts disc). In comparison with gentamycin and penicillin discs, both methanolic and ethanolic extracts of onion generated larger zones of inhibition against the tested pathogens. Onion extracts generated wider inhibition zones, i.e., 17.1 mm, 16.5 mm, and 15.5 mm, for *E. coli*, *S. aureus*, and *Salmonella* spp., respectively. Comparatively lower antimicrobial activities against tested pathogens were reflected by hydroethanolic extracts of mint, fennel, and coriander extracts.

3.4. Effect of Extracts' Supplementation on E. coli Counts in Minced Beef. *E. coli* counts under refrigeration of minced beef treated with hydromethanolic extracts of spices were estimated in $\log_{10}$ CFU/g during 0–9 days of storage. Interpretation of the data presented in Figure 1 suggests significant ($p < 0.05$) reductions in *E. coli* counts of minced beef on treatment with varying levels of spice extracts at different storage intervals. In comparison to the negative control where *E. coli* counts were found to increase from 5.72 $\log_{10}$ CFU/g to 6.29 $\log_{10}$ CFU/g, coriander extracts' supplementation in *E. coli* inoculated minced beef presented peak inhibitory properties, i.e., from 4.8 $\log_{10}$ CFU/g to 5 $\log_{10}$ CFU/g, during 9 d refrigerated storage. Around 0.23 $\log_{10}$ CFU/g increase in *E. coli* counts was observed in minced beef supplemented with 6% ginger extracts as compared to 0.56 $\log_{10}$ CFU/g and 0.60 $\log_{10}$ CFU/g for negative and positive control under similar study conditions. Fennel and mint extracts were also found equally efficacious in inhibiting the pathogenic load of *E. coli*. Furthermore, methanolic extracts of fennel, mint, and coriander increased the lag period in relation to the normal control. Comparable role of turmeric and onion extracts were noticed against *E. coli* inhibition. Pearson correlation (r=0.96) shows that the extracts' amount and storage duration suggested higher *E. coli* inhibitory properties of spice extracts at an extended amount of supplementation.

Antimicrobial activities of spices have been attributed to flavonoids, saponins, glucosinolates, thiosulfinates, and saponins [24]. Ginger bioactive compounds that exhibit antimicrobial activity include ar-curcumin, caryophyllene, β-sesquiphellandrene, α-farnesene, and zingiberene [25]. Coriander methanolic extracts have been already reported effective against human pathogens including *E. coli* and *Salmonella typhi* [26]. An earlier study carried out by Bali et al. [27] endorsed coriander application at the rate of 2–5% in beef sausages to attribute improved meat quality parameters under refrigerated storage for a period of 14

Table 1: Proximate composition of spice powders on dry weight basis (g/100 g).

Spices	Moisture	Ash	Fat	Protein	Fiber	Carbohydrates†
Onion	9.5 ± 1.3^{b}	3.4 ± 0.5^{c}	1.5 ± 0.7^{c}	2.5 ± 0.6^{c}	2.2 ± 1.59^{e}	80.9 ± 3.3^{a}
Ginger	10.0 ± 0.3^{b}	5.4 ± 0.4^{b}	1.9 ± 0.2^{c}	6.2 ± 0.6^{b}	6.0 ± 2.7^{cd}	70.5 ± 2.7^{b}
Turmeric	8.8 ± 0.7^{b}	6.5 ± 0.6^{a}	3.8 ± 0.2^{b}	4.0 ± 0.7^{c}	4.8 ± 1.0^{de}	72.2 ± 4.0^{b}
Coriander	9.2 ± 0.6^{b}	2.5 ± 0.1^{cd}	6.8 ± 0.3^{a}	13.2 ± 0.3^{a}	14.3 ± 0.9^{b}	63.1 ± 2.0^{c}
Fennel	9.4 ± 0.8^{b}	5.9 ± 0.4^{b}	7.1 ± 0.6^{a}	3.9 ± 0.2^{c}	20.3 ± 0.9^{a}	53.4 ± 0.9^{c}
Mint	12.3 ± 1.0^{a}	1.9 ± 0.9^{d}	1.3 ± 0.2^{c}	2.2 ± 0.2^{c}	8.4 ± 1.6^{bc}	74.0 ± 3.3^{b}

Mean ± SD; means bearing same letters in a column are statistically nonsignificant at $p < 0.05$. †Calculations on dry weight basis as 100−(Ash + Protein + Fiber + Fat).

Table 2: Mineral composition of spice powders on dry weight basis (mg/kg^{-1}).

Spices	Calcium	Potassium	Sodium
Onion	5.0 ± 0.0^{d}	135.0 ± 0.0^{c}	14.3 ± 0.6^{c}
Ginger	6.0 ± 0.0^{d}	229.7 ± 0.6^{a}	16.0 ± 0.0^{c}
Turmeric	6.3 ± 0.6^{d}	169.0 ± 0.0^{b}	16.3 ± 0.6^{c}
Coriander	18.3 ± 2.5^{c}	94.7 ± 4.9^{e}	95.3 ± 5.5^{a}
Fennel	32.0 ± 2.6^{a}	116.3 ± 5.0^{d}	42.7 ± 2.5^{b}
Mint	24.7 ± 1.5^{b}	89.7 ± 0.6^{e}	18.3 ± 0.6^{c}

Mean ± SD; means bearing same letters in a column are statistically nonsignificant at $p < 0.05$.

Table 3: Extracts' yield, total phenolic contents, and antioxidant potential of spices.

Spices	Solvent	Extracts yield (%)	TPC (mg GAE/100 g)	DPPH (%)
Onion	MeOH	9.5 ± 1.5^{a}	55.9 ± 3.2^{bc}	54.4 ± 3.8^{e}
	EtOH	5.9 ± 0.5^{bc}	36.0 ± 4.6^{hi}	43.1 ± 4.4^{g}
Ginger	MeOH	5.5 ± 1.6^{bc}	70.8 ± 3.3^{a}	75.9 ± 3.9^{a}
	EtOH	3.2 ± 0.9^{ef}	54.1 ± 3.4^{bcd}	66.3 ± 5.0^{bc}
Turmeric	MeOH	4.8 ± 0.1^{cd}	51.6 ± 1.6^{cde}	61.4 ± 1.9^{cd}
	EtOH	6.2 ± 1.2^{b}	42.7 ± 6.0^{fgh}	46.1 ± 4.0^{fg}
Coriander	MeOH	2.4 ± 0.3^{f}	$69.8 \pm 1.^{6a}$	70.5 ± 2.5^{ab}
	EtOH	3.1 ± 0.0^{ef}	49.0 ± 0.5^{def}	51.7 ± 1.6^{ef}
Fennel	MeOH	5.4 ± 0.1^{bc}	46.3 ± 5.2^{efg}	56.2 ± 5.2^{de}
	EtOH	4.0 ± 0.0^{de}	34.8 ± 4.7^{i}	41.2 ± 1.2^{g}
Mint	MeOH	3.9 ± 0.1^{de}	59.1 ± 5.7^{b}	62.1 ± 3.2^{c}
	EtOH	3.0 ± 0.0^{ef}	40.6 ± 2.9^{ghi}	42.6 ± 1.2^{g}

Mean ± SD; means bearing same letters in a column are statistically nonsignificant at $p < 0.05$. MeOH = hydromethanolic extracts; EtOH = hydroethanolic extracts; TPC = total phenolic content; DPPH = diphenyl picrylhydrazyl.

days. The study further suggested coriander application to anticipate ~1.18 log inhibition of total bacterial count in comparison with normal control during extended refrigerated storage of 21 days. Relatively lesser antibacterial activity of ginger, turmeric, and onion extracts was observed in ground beef that may be associated with poor distribution of spice extracts in a beef matrix. A study conducted by Gupta and Ravishankar [28] revealed that antimicrobial activity of pure pastes of ginger, garlic, and turmeric against *E. coli* O157:H7 was found higher than that observed in beef, thus suggesting a partial reduction in bactericidal properties of extracts in food system. Ginger extracts have been also reported as proteolytic because they enhance the antimicrobial characteristics against Gram-negative and positive pathogens including *E. coli* and *L. monocytogenes* [29].

3.5. Effect of Extracts' Supplementation on S. aureus Counts in Minced Beef. In comparison with both the positive and negative controls, methanolic extracts of tested spices significantly ($p < 0.05$) inhibited *S. aureus* growth in minced beef during 0–9 days of the study period. Data presented in Figure 2 showed that minced beef marinated with spice extracts at the rate of 1.5–6.0% offered better shelf stability and reduced pathogen growth. Least *S. aureus* count increment, i.e., 0.24 $\log_{10}$ CFU/g, was observed on 9th day of storage in minced beef marinated with 6% ginger extracts, whereas positive control inoculated with *S. aureus* at same inoculation levels as of treatment groups was observed with 0.77 $\log_{10}$ CFU/g increase in pathogen counts at the end of the study. Methanolic extracts of coriander and turmeric also delivered pronounced inhibition in *S. aureus* proliferation during 9 d storage with

Table 4: Antimicrobial activity of spice extracts against various pathogenic microbes at 150 ppm concentration.

Spices	Extracts	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella* spp.
Gentamycin	—	21.5 ± 0.7^{a}	22.0 ± 0.7^{a}	24.0 ± 0.0^{a}
Penicillin	—	19.5 ± 0.7^{b}	20.5 ± 1.4^{a}	23.5 ± 0.7^{a}
Onion	MeOH	17.1 ± 0.6^{c}	16.3 ± 1.1^{bc}	15.5 ± 3.5^{b}
	EtOH	14.5 ± 0.7^{def}	16.5 ± 0.7^{bc}	15.3 ± 0.4^{b}
Ginger	MeOH	11.5 ± 0.7^{hi}	14.5 ± 0.7^{bcde}	9.8 ± 0.4^{f}
	EtOH	13.5 ± 2.1^{efg}	16.0 ± 1.4^{bc}	11.0 ± 1.4^{ef}
Turmeric	MeOH	14.8 ± 0.4^{def}	15.5 ± 2.1^{bc}	14.0 ± 1.4^{bcd}
	EtOH	16.0 ± 1.4^{cd}	15.0 ± 1.4^{bcd}	15.5 ± 0.7^{b}
Coriander	MeOH	15.2 ± 0.2^{de}	15.5 ± 0.7^{bc}	14.3 ± 0.4^{bcd}
	EtOH	13.7 ± 0.2^{efg}	14.0 ± 0.0^{cde}	12.0 ± 0.0^{def}
Fennel	MeOH	13.2 ± 0.3^{fgh}	15.0 ± 0.0^{bcd}	14.8 ± 0.4^{bc}
	EtOH	12.3 ± 0.4^{ghi}	12.0 ± 1.4^{e}	13.1 ± 0.2^{bcde}
Mint	MeOH	16.0 ± 0.7^{cd}	16.6 ± 1.3^{b}	14.1 ± 0.4^{bcd}
	EtOH	11.0 ± 0.7^{i}	12.7 ± 1.4^{de}	12.7 ± 0.2^{cde}

Mean ± SD; means bearing same letters in a column are statistically nonsignificant at $p < 0.05$. MeOH = hydromethanolic extracts; EtOH = hydroethanolic extracts.

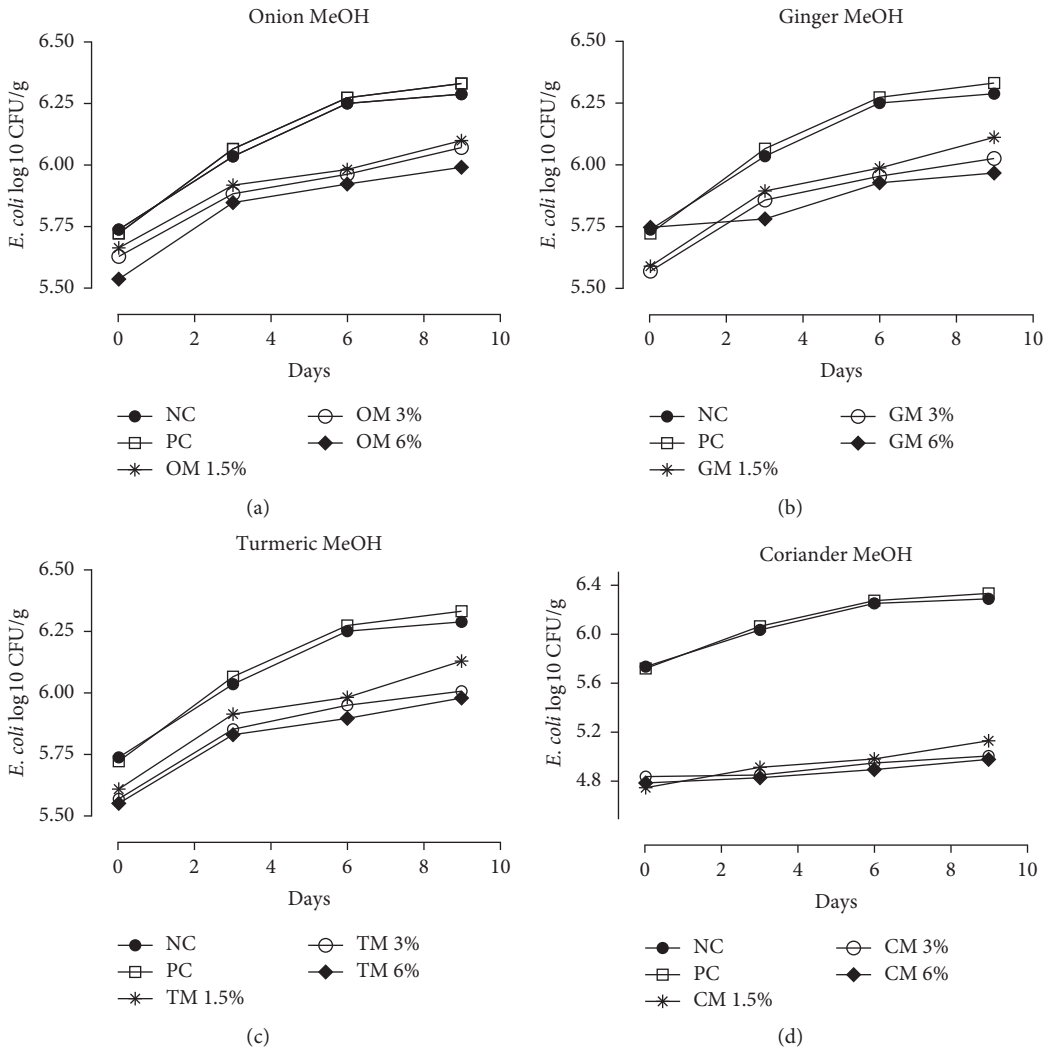

Figure 1: Continued.

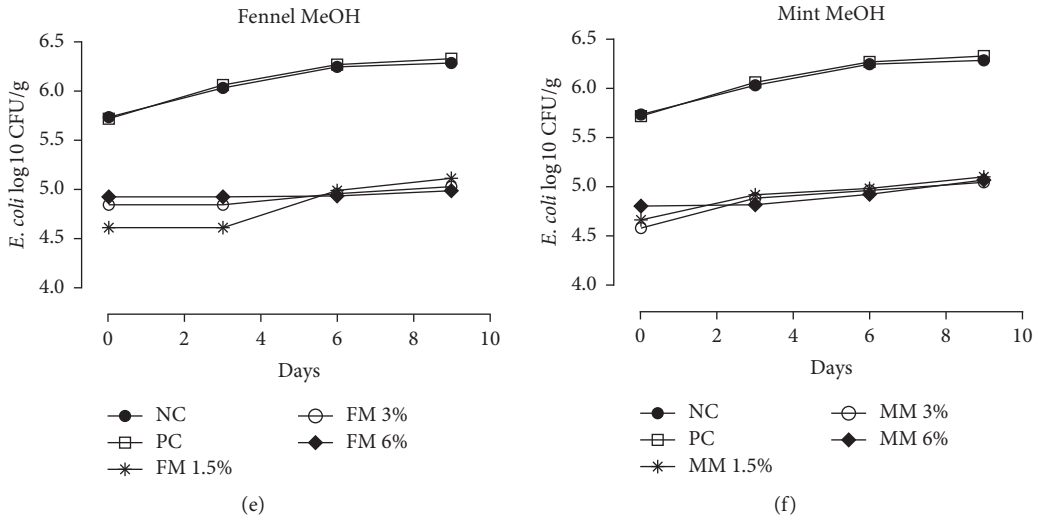

Figure 1: *E. coli* inhibitory activity of spice extracts in marinated minced beef under refrigerated (4°C) 9 d storage. NC: negative control, PC: positive control, OM: onion methanolic extracts, GM: ginger methanolic extracts, TM: turmeric methanolic extracts, CM: coriander methanolic extracts, FM: fennel methanolic extracts, and MM: mint methanolic extracts.

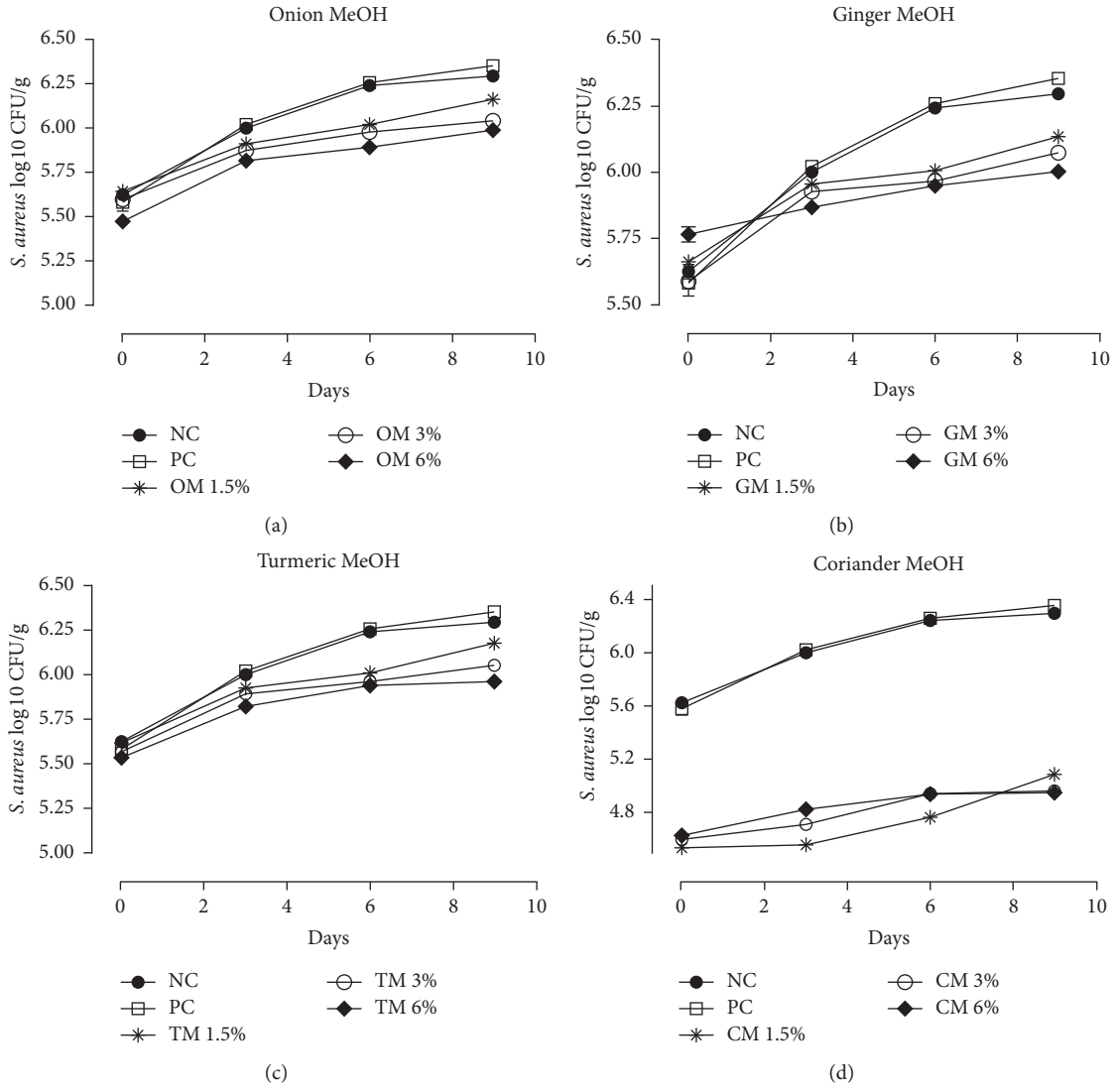

Figure 2: Continued.

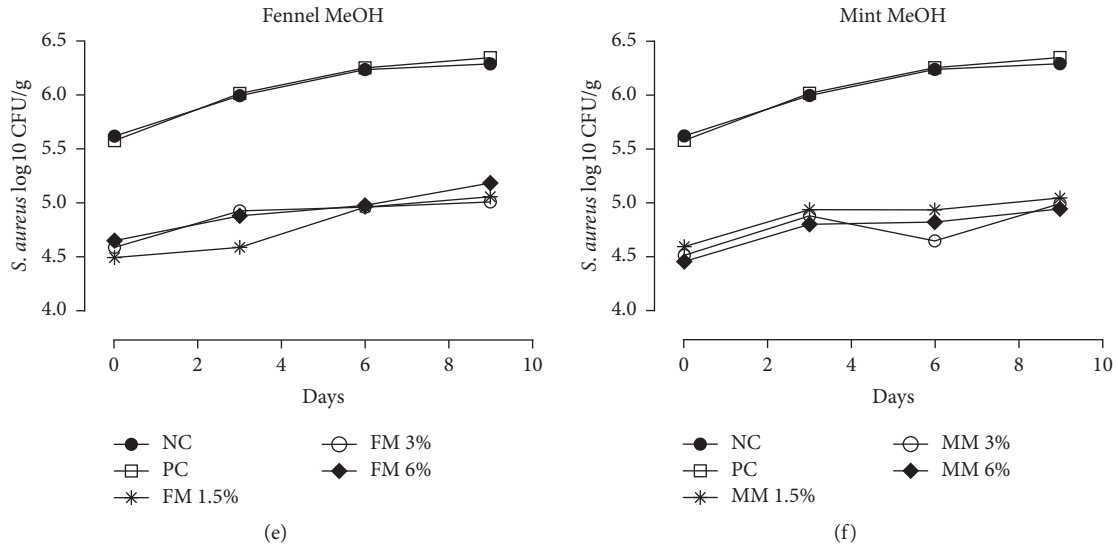

Figure 2: *S. aureus* inhibitory activity of spice extracts in marinated minced beef under refrigerated (4°C) 9 d storage. NC: negative control, PC: positive control, OM: onion methanolic extracts, GM: ginger methanolic extracts, TM: turmeric methanolic extracts, CM: coriander methanolic extracts, FM: fennel methanolic extracts, and MM: mint methanolic extracts.

up to 0.32 and 0.43 $\log_{10}$ CFU/g upsurge for coriander and turmeric extracts, respectively. Fennel, mint, and onion extracts also exhibited significant ($p < 0.05$) *S. aureus* inhibition in comparison with the normal and positive control (Figure 2).

Ginger extracts have been reported efficacious against both the Gram-positive and Gram-negative bacteria including *S. aureus* and *E. coli* [30]. In an earlier study wherein ginger extracts were applied as natural preservatives in frozen beef sausages, the extracts' application at the rate of 1.0% was found to increase product shelf stability by significantly inhibiting microbial growth and lipid oxidation [31]. The study in question declares the application of a relatively higher amount of ginger extracts, i.e., 6.0%, to deliver strong microbial inhibitory properties under refrigerated storage. Ginger extracts have also been reported to disrupt and extensively break muscle fibers [32]. This feature enables spice extracts to deliver higher microbicidal activities alongside meat tenderizing properties.

The complex composition of minced beef, i.e., carrying a higher amount of lipids, protein, water, and salts, makes it more resistant towards both the synthetic and natural antimicrobials. Hence, higher concentration and amount of spice extracts are desired to offer microbiological inhibitory properties in food in comparison with the microbial growth medium [10]. A study on 43 different spices used in meat-based cuisines of 36 countries concludes that the spices application as food cleanser and their utilization increases in cookeries [33]. Earlier work on spice application in meat-based products suggests palatability of cooked recipes at relatively higher doses of spices than recommended in recent study. Supplementing 10% extracts of myrtle, rosemary, lemon balm, nettle leaves, green tea, and ginger in beef patties, cooked pork meat, and stewed pork had been suggested as organoleptically acceptable [34, 35]. Variability of different spice extracts in presenting microbicidal properties against both *E. coli* and *S. aureus* as observed in this study is associated with their varied antimicrobial property-bearing phytochemical profile and growth conditions. Findings from this recent study also suggest relatively higher amount of extract (i.e., 6.0%) supplementation as marinades in meat-based system to offer antimicrobial and other quality enhancement and preservative properties.

4. Conclusions

Development of natural ingredient-based blends as preservatives for the meat industry serves emerging challenges including microbiological and oxidative spoilage, pathogenicity, and risks of synthetic additive-associated toxicity. This research demonstrates culinary spices and herbs commonly used in South East Asia and Central Asia as a potential source of antibacterial compounds that might anticipate a broad range of functional and biological properties in meat and meat-based edible goods. This study defines the correlation among phenolic contents and antioxidant activity of spice extracts. However, unique antimicrobial response identified for spices bearing comparatively lower phenolic pool and antioxidant activity is expected to be an outcome of their particular chemical composition with strong bactericidal properties. The findings of this research work are suggestive of spice and herb extracts' application in beef as culinary agent up to a level of 6% to contribute as a viable strategy in preventing pathogen growth and proliferation under refrigerated storage.

Data Availability

The dataset supporting the conclusions of this article is included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Optimization of Phenolic Compound Extraction from Chinese *Moringa oleifera* Leaves and Antioxidant Activities

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Rich in phenolic compounds, *Moringa oleifera* leaf extract (ME) exhibits significant antioxidant activity both in vitro and in vivo. ME has already been widely used in fields of medicine, functional food, and cosmetics. Ultrasonic extraction (UE) method has been improved to be one of the most effective ways to extract phenols from *M. oleifera* leaves. The purpose of this study was to optimize ultrasonic extraction of phenols by response surface methodology (RSM). Four parameters were discussed, such as ethanol concentration, solvent-sample ratio, extraction temperature, and extraction time. Also, purification methods of the crude ME by organic solvent extraction and column chromatography were examined. Antioxidant activities of ME and each fraction were evaluated by DPPH, ABTS, and hydroxy radical-scavenging activities and reducing power. The phenol content of the purified ME reached up to 962.6 mg RE/g, extremely higher than the crude extract 107.22 ± 1.93 mg RE/g. The antioxidant activity of the purified ME was also significantly improved. Furthermore, phenols were identified by using the HPLC-MS method, and the results showed that there were 6 phenolic acids and derivatives and 7 flavonoids in ME. Quercetin-3-O-β-D-glucoside isolated from ME showed excellent DPPH and ABTS radical-scavenging abilities, which were comparable to V_C.

1. Introduction

Moringa oleifera Lam. has been widely used as a nutritional supplement to reduce malnutrition and some ailments [1]. *M. oleifera* was authorized as a new food resource by Ministry of Health of China in 2012 [2]. Rich in phenolic acids and flavonoids, *M. oleifera* extract exhibits significant antioxidant activity both in vitro and in vivo [3, 4]. Especially *M. oleifera* leaves have highest phenols and highest antioxidant activity, compared with roots, barks, flowers, and seed [5]. At present, the *M. oleifera* extract has been widely used in fields of medicine, functional food, and cosmetics [6–8].

Many extraction methods have been studied for phenolic compounds extraction from *M. oleifera*, such as ultrasonic extraction (UE), subcritical water/ethanol extraction, and microwave-assisted extraction [9–11]. These are several heat-sensitive hydroxyl-type substituents existing in ME, such as kaempferol diglycoside and its acetyl derivatives [12, 13]. In the subcritical water/ethanol extraction method, these bioactive compounds may be destroyed by high temperature in subcritical conditions [14]. In addition, microwave-assisted extraction always employs a temperature higher than 150°C. The UE method has been proved to be the most effective way to extract phenolic compounds from *M. oleifera* [3]. It has been reported that there were significant differences in the phenolic profile, nutritional value, and antioxidant activity of *M. oleifera* from many different cultivars [15, 16].

In this study, extraction conditions of phenolic compounds from Chinese *M. oleifera* leaves were optimized by using response surface methodology (RSM). Afterwards, the crude ME was further purified by organic solvent extraction and column chromatography. Antioxidant activity and total phenol content of ME and each fraction were evaluated. The antioxidant activity was evaluated in vitro by DPPH, ABTS,

and hydroxyl radical-scavenging activity and reducing power. In addition, phenolic compounds were identified by using the HPLC-MS method.

2. Materials and Methods

2.1. Raw Materials and Chemicals. Samples of *M. oleifera* leaves were collected from the Moringa farm (100 km southeast of Dehong) (June 2016) in Yunnan Province (Southwest of China). *M. oleifera* leaves were dried in the open air and then ground to fine powder with a grinder.

All chemical reagents were of analytical grade. Ethanol, gallic acid, rutin, petroleum ether, ethyl acetate, *n*-butanol, Folin–Ciocalteu reagent, vitamin C (V_C), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, trichloroacetic acid, potassium ferricyanide, salicylic acid, ferrous sulfate, and hydrogen peroxide were obtained from Sigma-Aldrich (Shanghai, China). HPLC-grade methanol was from Merck (Germany).

2.2. Extraction Method Optimization

2.2.1. Extraction Method. *M. oleifera* leaves powder (50 g) was extracted with 70% aqueous ethanol (1.5 L) UE at 50°C for 42 min, using an ultrasonic circulating extraction equipment (KQ-5200B, Gongyi Yuhua Co., Ltd.) at 300W. And then, the mixture was centrifuged at 3500 r/min for 15 min, concentrated by rotary evaporation at 50°C, and further dried by the vacuum freeze-drying method. The resulting extract was stored at −20°C to avoid degradation until use.

2.2.2. Experiment Design of Response Surface Methodology. The effect of extraction conditions on phenol yield was studied and optimized by RSM. A flour-factor three-level Box–Behnken design (BBD) was employed to discuss four independent variables: extraction time (X_1), extraction temperature (X_2), solvent/solid ratio (X_3), and ethanol concentration (X_4). Each factor was fixed at 3 levels (−1, 0, and 1), with X_1 (30, 45, and 60 min), X_2 (40, 50, and 60°C), X_3 (20 : 1, 30 : 1, and 40 : 1 mL/g), and X_4 (60, 70, and 80%). All experiments were conducted in triplicate, and the mean values were fitted to a second-order polynomial model equation as follows:

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

where Y is the response (phenols content); X_i and X_j are independent variables; and β_0, β_i, β_{ii}, and β_{ij} are intercept, linear, quadratic, and cross-product terms regression coefficients, respectively.

Analysis of variance (ANOVA) with 95% confidence interval was used for the analysis of the model and the optimization of extraction conditions of phenols. The regression coefficient (R^2) was used to test the adequacy of the model. Finally, experimental results and predicted values were compared to estimate the validity of the model.

2.3. Isolation Method. 10 g of the crude ME was dissolved into 100 mL water. The obtained solution was fractionated with petroleum ether, ethyl acetate, and *n*-butanol, respectively, so that 4 different fractions were obtained as listed in Table 1. The *n*-butanol fraction was further purified by polyamide column (100–200 mesh) chromatography and eluted with a gradient of ethanol-water (0%, 30%, 50%, and 70% ethanol) to produce 4 fractions (BA, BB, BC, and BD) monitored by TLC. Fraction BB was separated over a Sephadex LH-20 column (MeOH/H_2O) to produce 2 major fractions (BB1 and BB2). Fraction BB2 was purified over semipreparative HPLC (MeOH/H_2O) to afford compound H. Each fraction was collected and measured by phenol content and antioxidant activity.

2.4. Phenol Content Determination. The total flavonoid (TF) content was measured by a minor modification of the aluminum chloride colorimetric method [17]. In brief, ME solution (500 μL and 1000 μg/mL) was mixed with aluminum chloride solution (500 μL, 2%, w/v). After incubating at 30°C for 30 min, the absorbance was measured at 410 nm. Rutin was used as reference, and TF was expressed as mg of rutin equivalent gram of sample (mg RE/g).

The total phenol (TP) content was determined by using the Folin–Ciocalteu method [18]. In brief, ME solution (200 μL, 0.1 mg/mL in ethanol) was mixed with the Folin–Ciocalteu reagent (500 μL) and diluted 10 times. The mixture was left for 5 min at room temperature before being mixed with Na_2CO_3 solution (800 μL, 60 mg/mL). After placing at room temperature and darkness for 2 h, the absorbance of the mixture was measured at 725 nm. Using gallic acid as reference, the concentration was recorded as mg of gallic acid equivalents gram of sample (mg GAE/g).

2.5. HPLC-MS Analysis. HPLC analysis was carried out using an Agilent 1200 Series HPLC system (Agilent, USA) equipped with a diode array detector (DAD) and Zorbax Eclipse Plus C18 column (150 mm × 4.6 mm, 1.8 μm) (Agilent, USA). The column was controlled at 25°C ± 0.6°C, and the detection was performed at 330 nm. Separation was achieved using a gradient of acidified water (1%, v/v) (solvent A) and methanol (solvent B) at 1.0 mL/min. The linear gradient was as follows: 0–10 min, 5%–25% B; 10–20 min, 25%–40% B; 20–30 min, 40%–50% B; and 30–45 min, 50%–100% B [3].

HPLC-MS analysis was performed on the HPLC system coupled to a G6310 mass spectrometer (Bruker Daltonik GmbH, Germany) equipped with electrospray ionization (ESI) ion source. The mass spectrometer was operated in the negative ion mode with a capillary voltage of 2.5 kV and a mass range of 100–1000 m/z. Nitrogen was used as the nebulizer and drying gas. The pressure of the nebulizer gas was 30.0 psi. The drying gas flow rate was 600.0 L/h, and the drying gas temperature was 350°C.

Table 1: Total quality, TF purity, TF quality, and TF yield of isolated fractions.

Item	Total quality (mg)	TF content (mg RE/g)	TF quality (mg)	TF yield (%)
Petroleum ether fraction	303.52 ± 35.84	107.22 ± 1.93	32.54	1.84
Ethyl acetate fraction	648.21 ± 22.57	375.89 ± 5.24	243.66	13.77
n-Butanol fraction	3067.25 ± 207.17	466.01 ± 12.92	1429.34	80.75
Water fraction	5981.16 ± 209.16	5.53 ± 1.38	32.60	1.84
BA	1118.41 ± 89.34	7.64 ± 0.81	8.50	0.61
BB	784.25 ± 21.44	765.76 ± 6.47	600.58	42.96
BC	457.54 ± 38.13	713.94 ± 3.79	326.64	23.36
BD	208.82 ± 24.20	594.20 ± 7.25	124.08	8.88
BB1	324.76 ± 27.85	642.92 ± 8.40	208.79	10.70
BB2	246.95 ± 21.39	962.6 ± 3.92	237.81	13.44

2.6. Antioxidant Activity Analysis

2.6.1. DPPH Radical-Scavenging Activity. The DPPH radical-scavenging activity was measured as described in [19]. In brief, DPPH solution (3.0 mL, 20 μM, ethanol as solvent) was mixed with the aqueous ME sample (1.0 mL). The mixture was kept in darkness for 30 min, and then the absorbance was measured at 517 nm. Vitamin C solution was prepared and used as an equivalent calibration standard. The radical-scavenging activity of each solution was calculated as the inhibition percentage with the following formula:

$$\text{DPPH-scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100, \quad (2)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.6.2. ABTS Radical-Scavenging Activity. The ABTS radical-scavenging activity was determined according to [20]. The ME sample (0.4 mL) was mixed with ABTS solution (1.5 mL, 7 mM) and potassium persulfate solution (1.5 mL, 2.45 mM). The mixture was kept in darkness for 2 h, and then the absorbance was measured at 732 nm. The radical-scavenging activity of each solution was calculated as the inhibition percentage with the following formula:

$$\text{ABTS-scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100, \quad (3)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.6.3. Hydroxyl Radical-Scavenging Activity. The hydroxyl radical-scavenging activity was determined according to [21]. The ME sample (1 mL) was mixed with $FeSO_4$ solution (1 mL, 9 mM), salicylic acid ethanol (70%) solution (1 mL, 9 mM), and H_2O_2 solution (1 mL, 8.8 mM). The mixture was kept in darkness for 0.5 h, and then the absorbance was measured at 510 nm. The radical-scavenging activity of each solution was calculated as the inhibition percentage with the following formula:

$$\text{hydroxyl-scavenging activity (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100, \quad (4)$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

2.6.4. Reducing Power. The reducing power was carried out as described in [22]. Firstly, 1 mL ME solution was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was kept at 50°C for 20 min in water bath, after which trichloroacetic acid (2.5 mL, 10%) was added. And then, the mixture was centrifuged at 3000 r/min for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). The absorbance of the mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing powder.

2.7. Statistical Analysis. All experiments were repeated for triplication, and the results were expressed as mean-± standard deviation. ANOVA procedure and Duncan's multiple range method were used to evaluate the significant differences between treatments ($p < 0.05$).

3. Results and Discussion

3.1. Fitting the Model. The experimental design and results of RSM are listed in Table 2. Based on the ANOVA results of total flavonoid (TF) content and total phenol (TP) content (Tables 3 and 4), two models were both remarkably significant ($p < 0.0001$) for TF and TP. The lack of fit of each model was not significant (0.8488 for TF and 0.2844 for TP), R^2 was close to 1 (0.9910 for TF, 0.9113 for TP), and adjusted R^2 was close to 1 (0.9821 for TF and 0.8226 for TP), indicating that both two models had good linear fitting.

3.2. Optimization of Extraction Conditions

3.2.1. TF. Table 3 shows that the linear effect of ethanol concentration (X_1) and quadratic (X_1^2, X_2^2, X_3^2, and X_4^2) had remarkably significant negative influence on TF ($p < 0.001$). Solvent-to-sample ratio (X_2) had significant positive influence on TF ($p < 0.05$), while extraction temperature (X_3) had significant negative influence ($p < 0.05$). According to the regression coefficient values (β), X_1^2 had a major influence, followed by X_2^2, X_3^2, X_1X_2, X_1, X_4^2, X_1X_3, X_1X_4, X_2, and X_3.

The second-order polynomial equation of TF yield was expressed as follows:

Table 2: Response surface experimental design and results.

No.	Ethanol concentration (%) X_1	Solid-liquid ratio (g/mL) X_2	Extraction temperature (°C) X_3	Extraction time (min) X_4	TF (%)	TP (%)
1	70	1 : 30	60	60	4.63	2.33
2	70	1 : 30	50	45	4.88	2.49
3	60	1 : 30	40	45	4.38	2.45
4	80	1 : 30	50	60	4.37	2.23
5	60	1 : 30	50	60	4.48	2.38
6	70	1 : 20	50	30	4.59	2.25
7	70	1 : 20	40	45	4.53	2.42
8	70	1 : 30	50	45	4.79	2.47
9	60	1 : 30	50	30	4.42	2.35
10	70	1 : 30	40	30	4.71	2.27
11	70	1 : 30	50	45	4.82	2.45
12	70	1 : 30	40	60	4.67	2.40
13	70	1 : 30	50	45	4.83	2.41
14	70	1 : 20	60	45	4.50	2.26
15	60	1 : 20	50	45	4.36	2.31
16	70	1 : 20	50	60	4.55	2.32
17	70	1 : 40	60	45	4.52	2.42
18	70	1 : 40	40	45	4.57	2.37
19	60	1 : 30	60	45	4.41	2.25
20	60	1 : 40	50	45	4.27	2.28
21	70	1 : 30	50	45	4.85	2.47
22	70	1 : 30	60	30	4.68	2.29
23	70	1 : 40	50	60	4.61	2.36
24	70	1 : 40	50	30	4.66	2.44
25	80	1 : 30	50	30	4.43	2.25
26	80	1 : 30	40	45	4.32	2.21
27	80	1 : 30	60	45	4.22	2.34
28	80	1 : 20	50	45	4.15	2.12
29	80	1 : 40	50	45	4.31	2.38

Table 3: ANOVA of the predicted regression model for TF.

Source	Sum of squares	df	Mean square	F value	p value	Significance
Model	1.09	14	0.078	110.70	<0.0001	***
X_1	0.023	1	0.023	32.12	<0.0001	***
X_2	$5.633E-003$	1	$5.633E-003$	8.03	0.0133	*
X_3	$4.033E-003$	1	$4.033E-003$	5.75	0.0310	*
X_4	$2.700E-003$	1	$2.700E-003$	3.85	0.0700	
X_1X_2	0.016	1	0.016	22.28	0.0003	**
X_1X_3	$4.225E-003$	1	$4.225E-003$	6.02	0.0278	*
X_1X_4	$3.600E-003$	1	$3.600E-003$	5.13	0.0399	*
X_2X_3	$1.000E-004$	1	$1.000E-004$	0.14	0.7114	
X_2X_4	$2.500E-005$	1	$2.500E-005$	0.036	0.8530	
X_3X_4	$2.500E-005$	1	$2.500E-005$	0.036	0.8530	
X_1^2	0.91	1	0.91	1296.97	<0.0001	***
X_2^2	0.23	1	0.23	323.38	<0.0001	***
X_3^2	0.097	1	0.097	137.64	<0.0001	***
X_4^2	0.010	1	0.010	14.43	0.0020	***
Residual	$9.820E-003$	14	$7.014E-004$			
Lack of fit	$5.300E-003$	10	$5.300E-004$	0.47	0.8488	
Pure effort	$4.520E-003$	4	$1.130E-003$			
Cor. total	1.10	28				
$R^2 = 0.9910$, adj. $R^2 = 0.9821$, adeq. precision = 36.173, and CV% = 0.58						

No significant difference ($p > 0.05$). *Significantly different ($p < 0.05$). **Highly significantly different ($p < 0.01$). ***Remarkably significantly different ($p < 0.001$).

Table 4: ANOVA of the predicted regression model for TP.

Source	Sum of squares	d*f*	Mean square	*F* value	*p* value	Significance
Model	0.22	14	0.015	10.27	<0.0001	***
X_1	0.020	1	0.020	13.35	0.0026	**
X_2	0.027	1	0.027	18.07	0.0008	***
X_3	$4.408E-003$	1	$4.408E-003$	2.94	0.1084	
X_4	$2.408E-003$	1	$2.408E-003$	1.61	0.2256	
X_1X_2	0.021	1	0.021	14.03	0.0022	**
X_1X_3	0.027	1	0.027	18.17	0.0008	***
X_1X_4	$6.250E-004$	1	$6.250E-004$	0.42	0.5288	
X_2X_3	0.011	1	0.011	7.36	0.0168	*
X_2X_4	$5.625E-003$	1	$5.625E-003$	3.75	0.0731	
X_3X_4	$2.205E-003$	1	$2.205E-003$	1.35	0.2645	
X_1^2	0.072	1	0.072	47.95	<0.0001	***
X_2^2	0.022	1	0.022	14.44	0.0020	**
X_3^2	0.015	1	0.015	9.87	0.0072	**
X_4^2	0.028	1	0.028	18.43	0.0007	***
Residual	0.021	14	$1.499E-003$			
Lack of fit	0.017	10	$1.730E-003$	1.88	0.2844	
Pure effort	$3.680E-003$	4	$9.200E-004$			
Cor. total	0.24	28				
$R^2 = 0.9113$, adj. $R^2 = 0.8226$, adeq. precision = 11.632, and CV% = 1.65						

No significant different ($p > 0.05$). *Significantly different ($p < 0.05$). **Highly significantly different ($p < 0.01$). ***Remarkably significantly different ($p < 0.001$).

$$Y = 4.83 - 0.043X_1 + 0.022X_2 - 0.018X_3 - 0.015X_4 - 0.063X_1X_2 - 0.033X_1X_3 - 0.030X_1X_4 - 0.005X_2X_3 - 0.0025X_2X_4 - 0.0025X_3X_4 - 0.37\,X_1^2 - 0.19\,X_2^2 - 0.12\,X_3^2 - 0.04X_4^2. \quad (5)$$

The interaction of ethanol concentration and solvent to sample ratio (X_1X_2) had highly significant positive influence on TF ($p < 0.01$). Figure 1(a) shows the effect of ethanol concentration, solvent-to-sample ratio, and their interaction on TF yield at 50°C and 45 min. The shape of contour plots was elliptical, which indicated that the interaction was significant [23]. The maximum TF yield was achieved at a solvent-to-sample ratio of 30 : 1–35 : 1 and ethanol concentration of 65–70%. The TF yield gradually increased with the increase in solvent-to-sample ratio from 20 : 1 to 30 : 1. In some extent, increase in the solvent-to-sample ratio could enhance the TF yield. A relatively higher concentration gradient of solute between the inside and outside of the cell could help the solute to dissolve into the solvent. Appropriate ethanol concentration was also important for phenol extraction. Most phenols in ME are of medium and high polarity. Too high concentration will lead to decrease in the dissolution of phenols because of the dissolution of lipid soluble substances. Also, too low concentration will increase the dissolution of water-soluble impurities such as sugars and proteins, reducing the extraction rate of phenols. Therefore, 70% ethanol was more suitable for phenol extraction, compared with other solutions. This result was in accordance with previous studies [11].

The interaction of ethanol concentration and extraction temperature (X_1X_3) had a significant negative effect on TF yield ($p < 0.05$). As it could be seen in Figure 1(b), the ethanol concentration had more important influence on TF than extraction temperature. The maximum TF yield was achieved at an ethanol concentration of 70% and extraction temperature of 50°C.

The interaction of ethanol concentration and extraction time (X_1X_4) revealed a significant negative effect on TF ($p < 0.05$). As shown in Figure 1(c), the ethanol concentration had more important influence on TF than extraction time. Also, phenols almost kept constant with the increasing extraction time, which indicated that extraction time was an insignificant variable in RSM optimization of phenol extraction ($p > 0.05$).

3.2.2. TP. As shown in Table 4, the linear effect of the solvent to sample ratio (X_2) exhibited remarkably a significant positive effect ($p < 0.001$). Ethanol concentration (X_1) and the quadratic (X_1^2, X_2^2, X_3^2, X_4^2) exhibited a highly significant ($p < 0.01$) negative effect. TP depended mostly on X_1X_3, followed by X_1X_2, X_4^2, X_2^2, X_2X_3, X_3^2, X_2, X_1, and X_1^2. The fitted second-order polynomial equation of TP yield was as follows:

$$Y = 2.46 - 0.041X_1 + 0.047X_2 - 0.019X_3 + 0.014X_4 + 0.073X_1X_2 + 0.083X_1X_3 - 0.012X_1X_4 + 0.052X_2X_3 - 0.0037X_2X_4 - 0.022X_3X_4 - 0.11\,X_1^2 - 0.058\,X_2^2 - 0.048\,X_3^2 - 0.065X_4^2. \quad (6)$$

The interactive effects of ethanol concentration and solvent-to-sample ratio (X_1X_2) had highly significant positive effect ($p < 0.01$). As shown in Figure 1(d), the maximum

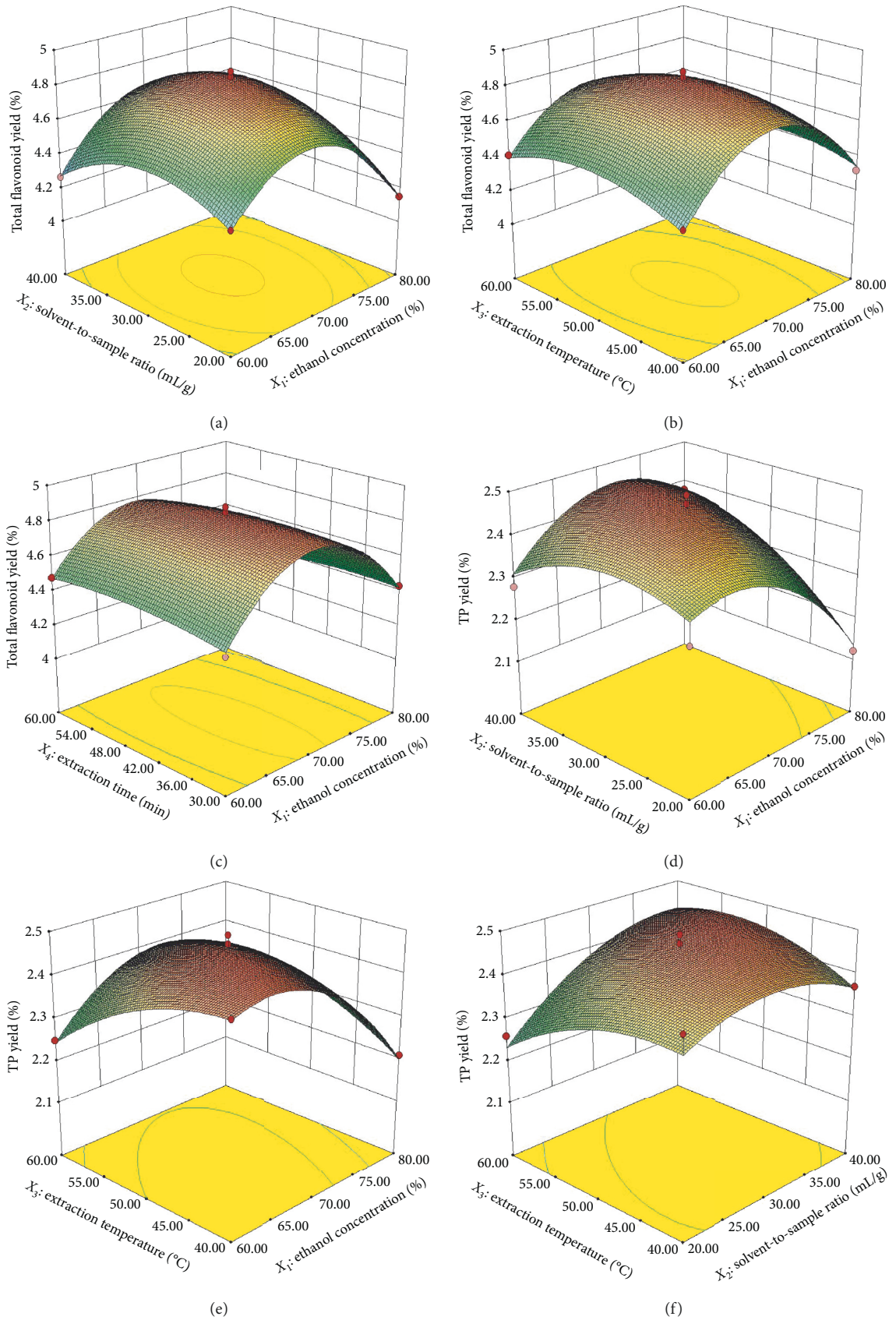

Figure 1: Interactive effect of extraction variables on (a–c) TF and (d–f) TP.

TP yield was achieved at an ethanol concentration of 70% and solvent-to-sample ratio of 30 : 1.

The interactive effects of ethanol concentration and extraction temperature (X_1X_3) had remarkably significant positive influence ($p < 0.001$). As shown in Figure 1(e), at a lower level of ethanol concentration, TP yield gradually decreased with increasing extraction temperature. Also, at a lower level of extraction temperature, TP yield decreased as the ethanol concentration raised.

Table 3 shows that interactive effects (X_2X_3) had a significant positive influence ($p < 0.05$). Figure 1(f) shows that TP yield at low level of extraction temperature and high level of solvent-to-sample ratio was higher than that at a low level of solvent-to-sample ratio and a high level of extraction temperature. These indicated that solvent-to-sample ratio had more significant influence than extraction temperature.

3.3. Model Validation. The optimum conditions for phenol extraction were predicted using Design Expert 10.0.4 : 70% of ethanol concentration, 30 : 1 of solvent-sample ratio, 50°C of extraction temperature, and 42 min of extraction time. Afterwards, the model validation was evaluated. TF and TP yields were approximated at 4.83 ± 0.07% and 2.44 ± 0.10%, respectively, which did not show significant differences ($p > 0.05$) with the experimental value of TF yield (4.83%) and TP yield (2.44%).

M. oleifera leaves from Africa had a TP content of 4.7 g/100 g [3]. TF and TP contents of *M. oleifera* leaves from Nicaragua reached up to 11.04–12.33 g/100 g and 10.14–14.07 g/100 g, respectively, while those from sub-Saharan Africa were only 0.11–1.26 g/100 g and 0.05–0.67 g/100 g, respectively [11, 24]. Differences in TF and TP contents depended on cultivar, growing environment, sample treatment method, leaf maturity, and so on [25].

3.4. Isolation of M. oleifera Leaf Extract. The TF value of ME extracted by ethanol : water (70 : 30) was measured as 107.22 ± 1.93 mg RE/g. So total flavonoid quality of the crude ME (10 g) was calculated as 1.77 g. ME (10 g) was dissolved in water and then fractionated with petroleum ether, ethyl acetate, and *n*-butanol, respectively. TF of the obtained petroleum ether, ethyl acetate, *n*-butanol, and water fractions was measured, and total flavonoid quality and yield were also calculated, as shown in Table 1. Total flavonoid quality of *n*-butanol fraction was 1429.34 mg, much higher than that of other fractions. Most of the phenols in ME (80.75%) were enriched in *n*-butanol fraction, and TF of *n*-butanol fraction was 2.63 times of ME. It was indicated that *n*-butanol had a good enrichment effect on flavonoids. *n*-Butanol fraction was then subjected to a polyamide column and eluted with a gradient of ethanol-water (water and 30%, 50%, and 70% ethanol). TF of BB (30% ethanol fraction) and BC (50% ethanol fraction) fractions were 765.76 ± 6.47 and 713.94 ± 3.79 mg RE/g, respectively. The total flavonoid quality of BB fraction (600.58 mg) was also higher than that of BC fraction (326.64 mg). BB fraction was then separated over a Sephadex LH-20 column so that BB1 and BB2 fractions were obtained. TF of BB2 fraction reached up to 962.6 ± 3.92 mg RE/g. BB2 fractions (237.81 mg) were purified over semipreparative HPLC ($MeOH/H_2O$), and then compound H (158 mg) was obtained.

3.5. Antioxidant Activity of M. oleifera Leaf Extract and Each Fraction

3.5.1. DPPH-Scavenging Activity. DPPH and ABTS are two kinds of traditional free radical commonly used to evaluate the free radical-scavenging activity. As shown in Figure 2, the DPPH-scavenging activity of crude ME and each fraction increased with the increasing concentration. The scavenging activity of *n*-butanol and ethyl acetate fractions at 0.5 mg/mL were 92.62% and 90.27%, respectively, higher than crude ME (85.51%) and comparable to V_C (96.82%). But the scavenging activity of petroleum ether and water fractions at 0.5 mg/mL was only 55.89% and 45.13%, respectively, rather lower than crude ME (85.51%). The scavenging activity of petroleum ether and water fractions at 1.0 mg/mL reached up to 89.43% and 82.40%, respectively. The results indicated that the DPPH-scavenging activity sequence was V_C > *n*-butanol fraction > ethyl acetate fraction > petroleum ether fraction > water fraction ($p < 0.05$), with EC_{50} of 0.020, 0.067, 0.082, 0.353, and 0.439 mg/mL, respectively. Therefore, both *n*-butanol and ethyl acetate fractions showed excellent scavenging activity that may be due to its enrichment of active components.

As shown in Figure 3, the DPPH-scavenging activity of BB, BC, and BD fractions increased rapidly with increasing concentration. The scavenging activity of BB, BC, and BD fractions at 0.025 mg/mL were all less than 50%, while that of BB, BC, and BD fractions at 0.2 mg/mL reached 93.37%, 91.87%, and 83.41%, respectively. There was no significant difference on the DPPH-scavenging activity between BB and BC fractions at 0.5–1.0 mg/mL ($p > 0.05$), which was a little lower than that of V_C ($p < 0.05$). The scavenging activity decreased as $V_C > BB > BC > BD$, with an EC_{50} value of 0.023, 0.036, 0.042, and 0.053 mg/mL, respectively.

DPPH-scavenging activity of compound H at 0.1 mg/mL reached 96.33%. Compound H showed excellent scavenging ability, with an EC_{50} value of 0.022 mg/mL, comparable to V_C (0.023 mg/mL). As shown in Figure 4, there was no significant difference of the DPPH scavenging ability between compound H and V_C ($p > 0.05$).

3.5.2. ABTS-Scavenging Activity. As shown in Figure 5, the ABTS radical-scavenging activity of each fraction increased with the increase in concentration. The scavenging activity of *n*-butanol and ethyl acetate fractions at 0.2 mg/mL reached 99.46% and 97.49%, respectively, higher than crude ME (77.82%) and comparable to V_C (99.62%). With the same concentration, the scavenging activity of petroleum ether and water fractions was 42.22% and 33.50%, respectively. There was no significant difference of ABTS-scavenging activity between *n*-butanol fraction and V_C at 0.2–1.0 mg/mL ($p > 0.05$), as well as between V_C, BB, and BC fractions at 0.5–1.0 mg/mL ($p > 0.05$). The results indicated that the ABTS-scavenging activity sequence was V_C > *n*-butanol

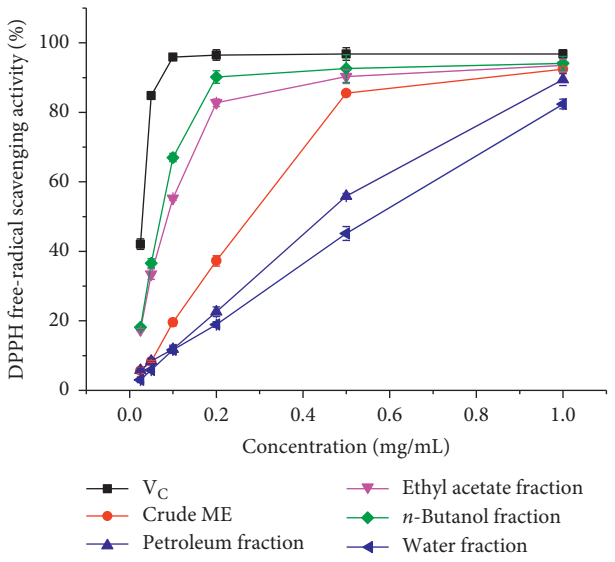

Figure 2: DPPH-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

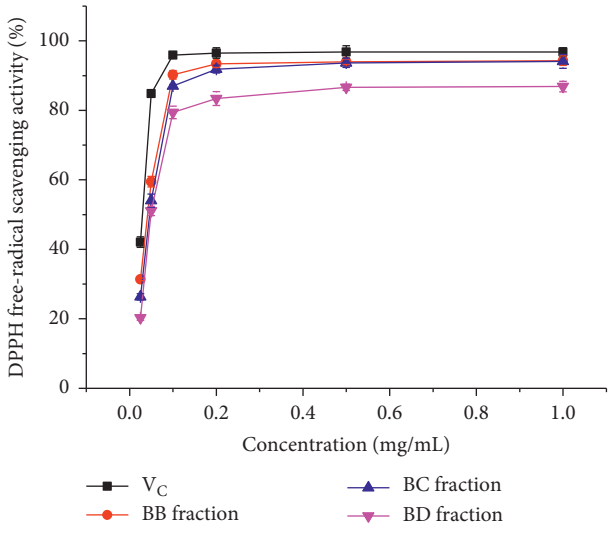

Figure 3: DPPH-scavenging activity of V_C, BB, BC, and BD fractions.

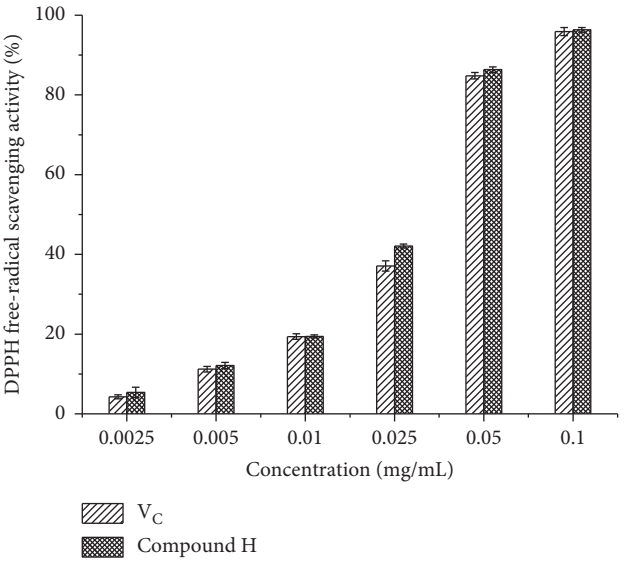

Figure 4: DPPH-scavenging activity of compound H and V_C.

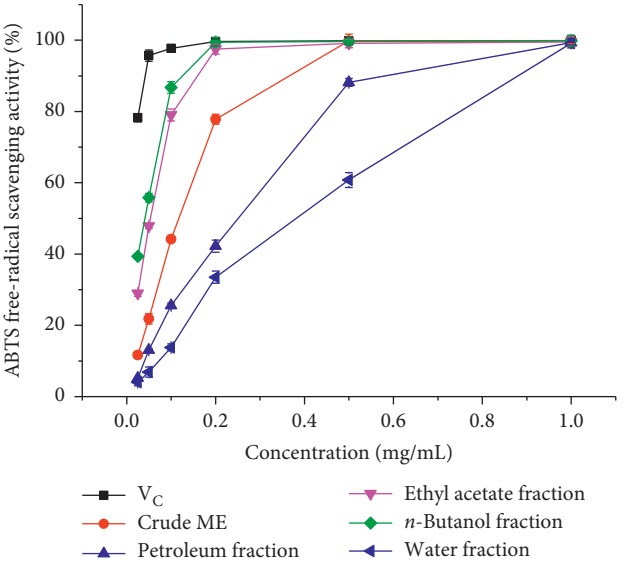

Figure 5: ABTS-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

fraction > ethyl acetate fraction > petroleum ether fraction > water fraction, with an EC_{50} value of 0.013, 0.036, 0.046, 0.181, and 0.285 mg/mL, respectively.

The ABTS-scavenging activity of BB, BC, and BD fractions all increased with the increasing sample concentration significantly ($p > 0.05$) (Figure 6). ABTS-scavenging activity of BB, BC, and BD fractions at 0.1 mg/mL reached 96.84%, 92.95%, and 90.19%, respectively. There was no significant difference between BB and BC fractions and V_C at 0.2–1.0 mg/mL ($p > 0.05$). The scavenging activity decreased as V_C > BB > BC > BD, with an EC_{50} value of 0.009, 0.022, 0.026, and 0.030 mg/mL, respectively.

As shown in Figure 7, the radical-scavenging activity of compound H was slightly higher than V_C, with an EC_{50} value of 0.007 and 0.009 mg/mL, respectively.

3.5.3. Hydroxyl Radical-Scavenging Activity. Hydroxyl radical ($OH^{\cdot}$) is a kind of reactive oxygen-free radical with strong oxidability, which can react with lipids, amino acids, sugars, and other substances. It is toxic to biological cells, DNA, and other macromolecules, thus causing pathological changes of the body.

As shown in Figure 8, the hydroxyl radical-scavenging activity of *n*-butanol and ethyl acetate fractions were remarkably higher than petroleum ether and water fractions ($p < 0.05$). The hydroxyl radical-scavenging activity of *n*-butanol and ethyl acetate fractions at 1.5 mg/mL reached up to 94.46% and 80.68%, respectively, while that of petroleum ether and water fractions were both less than 20%. The hydroxyl radical-scavenging activity of *n*-butanol and ethyl

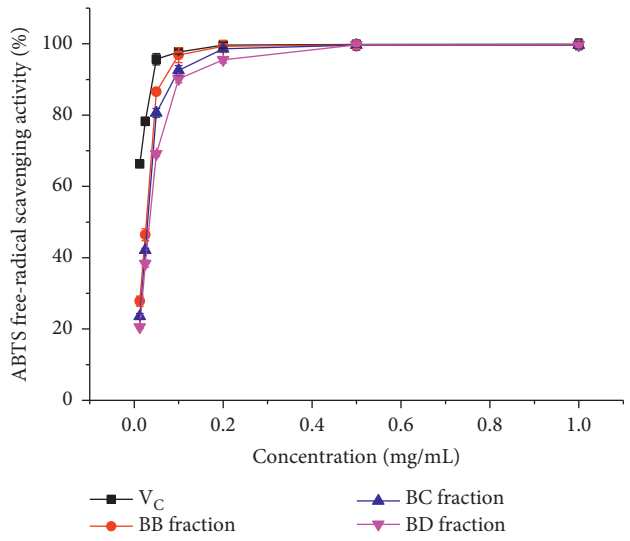

Figure 6: ABTS-scavenging activity of V_C, BB, BC, and BD fractions.

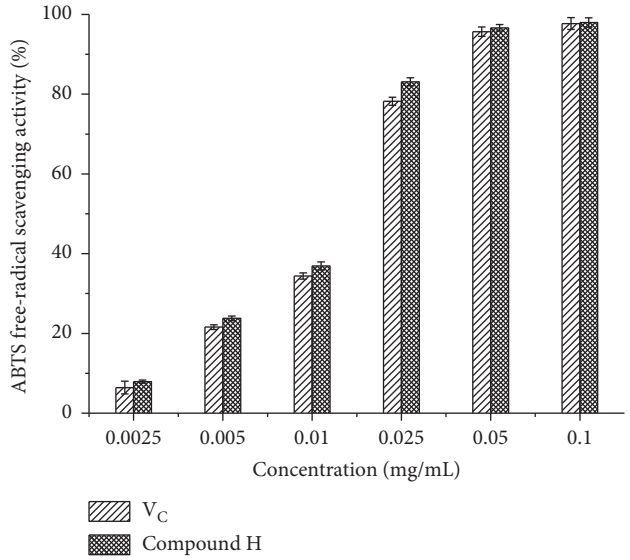

Figure 7: ABTS-scavenging activity of compound H and V_C.

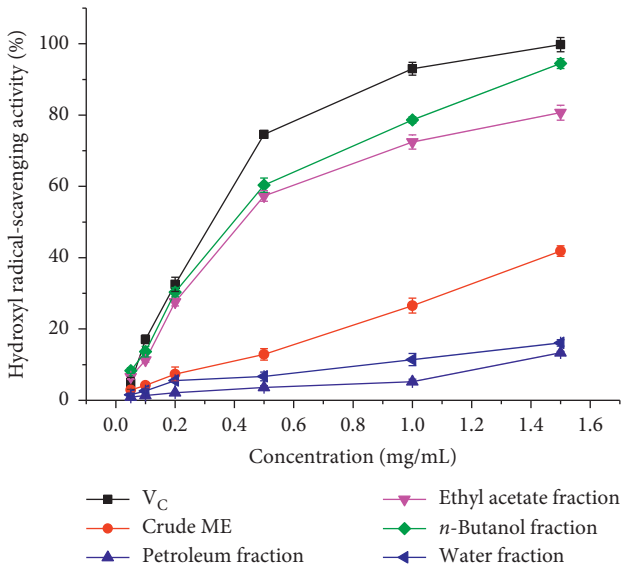

Figure 8: Hydroxyl radical-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

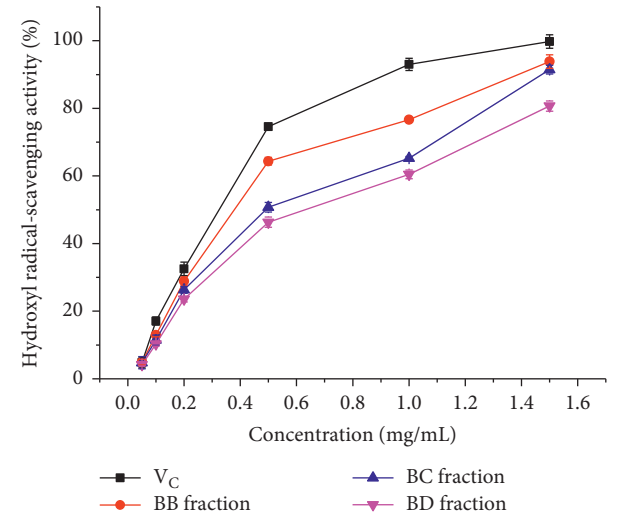

Figure 9: Hydroxyl radical-scavenging activity of V_C, BB, BC and BD fractions.

acetate fractions increased steadily with the increase in concentration, but that of petroleum ether and water fractions both increased slightly.

Figure 9 shows that BB, BC, and BD fractions all exhibited good hydroxyl radical-scavenging activity, and that of the three fractions all increased with the sample concentration gradually. The hydroxyl radical-scavenging activity sequence was $V_C > BB > BC > BD$ ($p < 0.05$), with an EC_{50} value of 0.262, 0.358, 0.462, and 0.573 mg/mL, respectively.

As shown in Figure 10, the hydroxyl radical-scavenging activity of V_C was slightly higher than compound H, with an EC_{50} value of 0.262 and 0.349 mg/mL, respectively.

3.5.4. Total Reducing Power. The total reducing power was measured by the reduction of the Fe^{3+}/ferriccyanide complex, which was reduced to its ferrous form by gaining an electron from antioxidants.

As shown in Figure 11, *n*-butanol and ethyl acetate fractions had better reducing power than petroleum ether and water fractions. Reducing powder of crude ME and each fraction decreased in order of $V_C > n$-butanol fraction > ethyl acetate fraction > crude ME > petroleum ether fraction > water fraction. As shown in Figure 12, reducing power of V_C, BB, BC, and BD fractions decreased in the same order with the hydroxyl radical-scavenging activity. As shown in Figure 13, the reducing power of V_C was slightly better than that of compound H ($p < 0.05$).

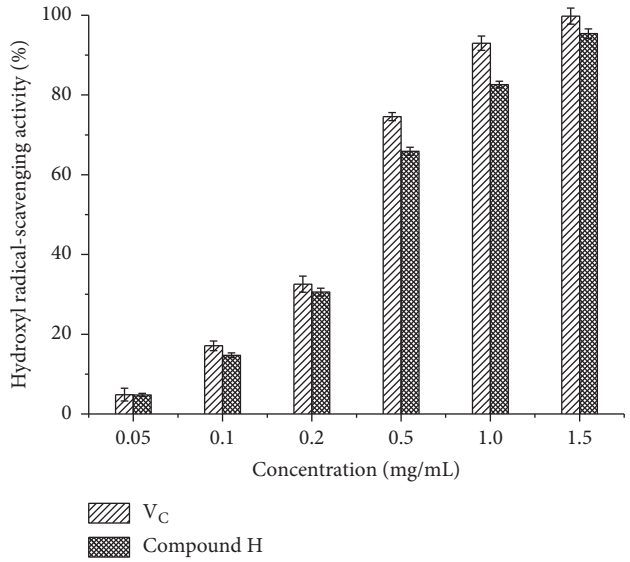

Figure 10: Hydroxyl radical-scavenging activity of compound H and V_C.

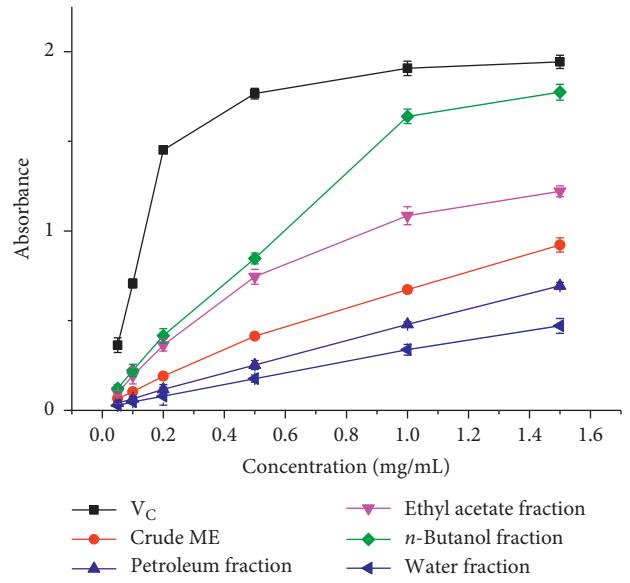

Figure 11: Reducing power of crude ME, V_C, and organic extraction fractions extracted from ME.

3.6. Characterization of the M. oleifera Leaf Extract. HPLC-DAD results of ME and isolated fractions are shown in Figures 14 and 15, respectively. A total of 14 representative peaks were obtained in ME. The HPLC-ESI-MS results of the compounds are summarized in Table 5. However, peak 1 was still unknown, needing further research. Peaks 2–7 were tentatively identified as phenolic acids, such as 3-caffeoylquinic acid, 4-caffeoylquinic acid, coumaroylquinic acid isomers, and caffeoylquinic acid isomer. The most abundant compound within phenolic acids was 3-caffeoylquinic acid (peak 2), representing 46.93% of the total phenols. Previous studies showed that 3-caffeoylquinic acid was the most abundant compound in ME of seven different cultivars [16]. The second most abundant phenolic acid was

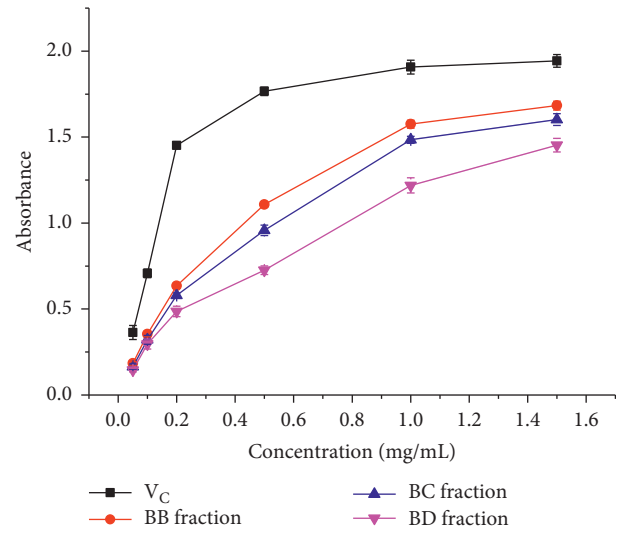

Figure 12: Reducing power of V_C, BB, BC, and BD fractions.

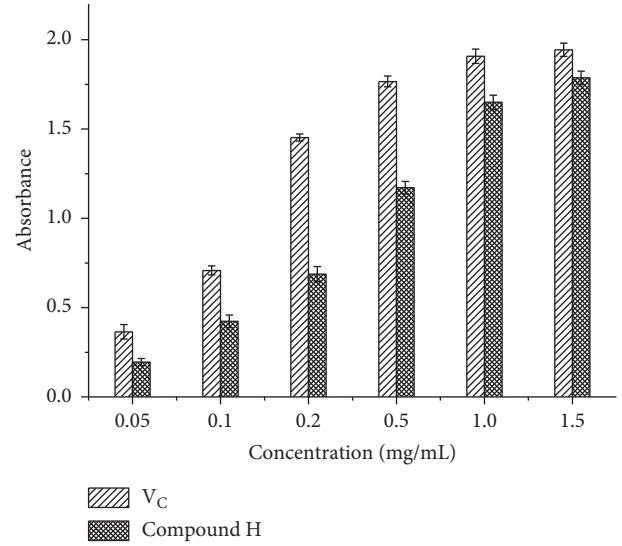

Figure 13: Reducing power of compound H and V_C.

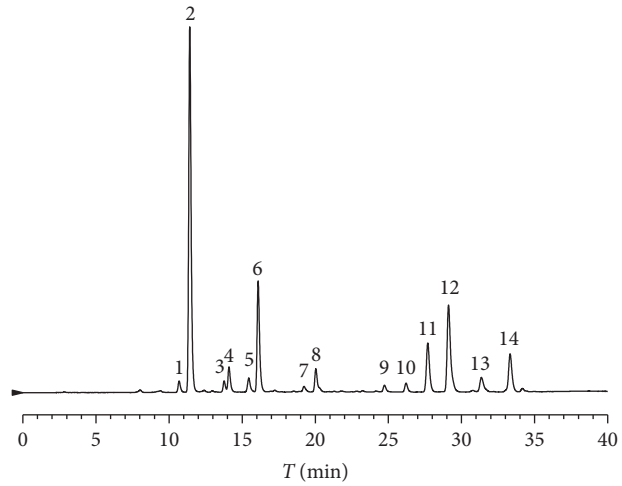

Figure 14: HPLC chromatogram profile of ME extracted by using the UE method.

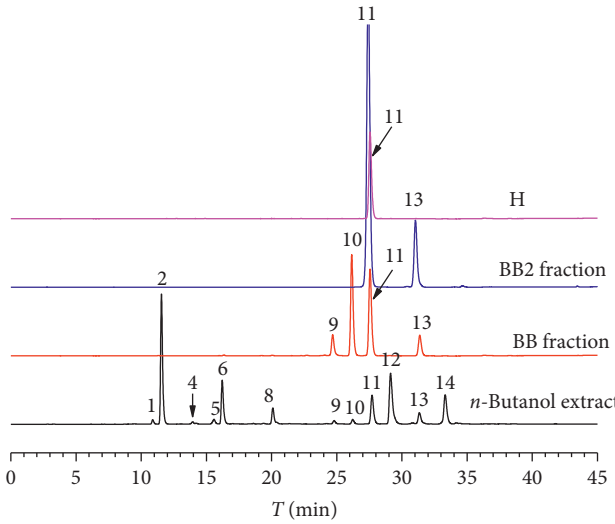

Figure 15: HPLC chromatogram profile of isolated fractions.

Table 5: HPLC-ESI-MS data of the compounds identified in ME.

Peak	RT (min)	$[M-H]^-$ (m/z)	Fragment (m/z)	Compound	Molecular formula	Content (%)
1	10.7	611.9	369.9 258.7 290.7	Unknown	Unknown	1.92
2	11.4	353.0	190.7 134.7	3-Caffeoylquinic acid	$C_{16}H_{18}O_9$	46.93
3	13.7	336.9	162.7 176.7 118.7	Coumaroylquinic acid isomer 1	$C_{16}H_{18}O_8$	1.53
4	14.1	337.0	162.9 118.7 190.7	Coumaroylquinic acid isomer 2	$C_{16}H_{18}O_8$	3.83
5	15.4	352.8	190.7	Caffeoylquinic acid isomer 1	$C_{16}H_{18}O_9$	1.94
6	16.1	352.8	172.7 134.7	4-Caffeoylquinic acid	$C_{16}H_{18}O_9$	14.37
7	19.2	336.8	190.7 172.7	Coumaroylquinic acid isomer 3	$C_{16}H_{18}O_8$	0.77
8	20.0/20.1	593.0	473.0 352.9	6,8-Di-*c*-glucosyl apigenin	$C_{27}H_{30}O_{15}$	3.83
9	24.7	430.9	310.7 340.8 282.7	Apigenin glucoside isomer 1	$C_{21}H_{20}O_{10}$	0.76
10	26.2	430.8	310.8 340.8 412.8	Apigenin glucoside isomer 2	$C_{21}H_{20}O_{10}$	0.96
11	27.7	462.9	300.9	Quercetin-3-O-β-D-glucoside	$C_{21}H_{20}O_{12}$	5.75
12	29.1	505.1	300.9 462.9	Quercetin-acetyl-glycoside	$C_{23}H_{22}O_{13}$	10.54
13	31.3	446.9	284.9	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	2.11
14	33.2	489.0	284.9	Kaempferol-acetyl-glycoside	$C_{23}H_{22}O_{12}$	4.79

4-caffeoylquinic acid (peak 6), with a content of 14.37%. Coumaroylquinic acid isomers (peaks 3 and 4) represented 5.36% of the total phenols. However, the phenolic compounds of ME in our experiment did not include feruloylquinic acid, which was detected in most kinds of *M. oleifera* leaves [3, 16].

Peaks 8–14 were tentatively identified as flavonoids, such as 6,8-Di-C-glucosylapigenin, apigenin glucoside isomers, quercetin-3-O-β-D-glucoside, quercetin-acetyl-glycoside isomer, kaempferol-3-O-glucoside, and kaempferol-acetyl-glycoside isomer. Quercetin-acetyl-glycoside (peak 12) appeared as the highest concentration, constituting 10.54% of the total phenols. Quercetin-3-O-β-D-glucoside (peak 11) and kaempferol-acetyl-glycoside (peak 14) represented an amount of 5.75 and 4.79%, respectively. Other existing flavonoids were 6,8-Di-C-glucosylapigenin (peak 8) (3.83%), apigenin glucoside isomers (peaks 9 and 10) (1.72%), and kaempferol-3-O-glucoside (peak 13) (2.11%). Most common and existing in abundance flavonoids in ME were kaempferol-O-glycosides, quercetin-O-glycosides, and apigenin-C-glycosides [12]. There were significant differences in flavonoid composition between *M. oleifera* leaves collected from different varieties. The profile of flavonoids in *M. oleifera* leaves may be dependent on the cultivar, growing environment, sample treatment method, and leaf maturity [16, 25].

Isolated fractions of crude ME were also analyzed by HPLC-ESI-MS. As shown in Figure 15, there were 4 flavonoids in BB fraction, such as apigenin glucoside isomers, quercetin-3-O-β-D-glucoside, and kaempferol-3-O-glucoside. BB2 fraction contained 2 flavonoids, such as quercetin-3-O-β-D-glucoside and kaempferol-3-O-glucoside. The compound H was identified as quercetin-3-O-β-D-glucoside.

4. Conclusion

In the present study, the optimization of UE was established for improving the phenolic compounds from *M. oleifera* leaves. RSM was successfully applied to optimize the extraction process. The optimum extraction condition was as follows: ethanol concentration of 70%, solvent-to-sample ratio of 30 : 1, extraction temperature of 50°C, and extraction time of 40 min, with a TF yield of 4.83% and TP yield of

2.44%. The crude ME obtained at the optimized conditions was then further isolated by organic solvent extraction and column chromatography. HPLC-DAD-MS results showed that there were 6 phenolic acids and derivatives and 7 flavonoids in ME. Antioxidant property results showed that the scavenging activity sequence was *n*-butanol fraction > ethyl acetate fraction > ME > petroleum ether fraction > water fraction. Quercetin-3-O-β-D-glucoside isolated from ME showed excellent DPPH and ABTS radical-scavenging abilities, which were comparable to V_C.

Data Availability

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

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

Phenolic Analysis for Classification of Mulberry (*Morus* spp.) Leaves according to Cultivar and Leaf Age

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Phenolic compounds in mulberry leaves harvested from three cultivars (Buriram 60, BR 60; Sakonnakhon, SK; and Khunphai, KH) at different leaf ages (tips, young, and old leaves) were identified and quantified using HPLC-DAD and HPLC-ESI/MS. A total of 13 phenolic compounds, which were mainly as caffeoylquinic acids and flavonol glycosides, were detectable. Predominant phenolic compounds were 5-*O*-caffeoylquinic acid (3.5–13.1 mg/g dry weight), 4-*O*-caffeoylquinic acid (1.3–2.4 mg/g dry weight), and quercetin-3-*O*-rutinoside (1.0–4.4 mg/g dry weight). Qualitative and quantitative differences in phenolic compounds in mulberry leaves were investigated among cultivars and leaf ages. Principal component analysis and hierarchical cluster analysis were used for classification of the mulberry leaves. Based on the similarity of phenolic compounds, mulberry leaves were clustered into three groups: (1) tips of leaves from all cultivars; (2) young and old leaves of mulberry cv. BR 60; (3) young and old leaves of mulberry cv. SK and KH. Therefore, according to phenolic compounds in mulberry leaves, tips of leaves from all cultivars should be intended for production of functional healthy foods.

1. Introduction

Mulberry is a fast-growing plant belonging to genus *Morus* of family Moraceae. Mulberry has been widely cultivated in Asian countries such as China, Japan, Korea, and Thailand in order to utilize its leaves as food for silkworms in sericulture. The leaves are also used as supplements for feeding livestock to improve milk yield and quality [1]. Mulberry leaves have been traditionally applied as folk medicine to treat fever, protect the liver, strengthen the joints, facilitate the discharge of urine, and lower blood pressure. Moreover, the leaves have been processed as human food such as mulberry leaf tea and seasoning power. Several research studies have revealed pharmacological activities of mulberry leaves including hypoglycemic effect [2] and anti-inflammatory [3] and antihypertensive properties [4] which might be due to their bioactive compounds. Significant amounts of phenolic compounds, 1-deoxynojirimycin (DNJ) and melatonin [5–7], were quantified in mulberry leaves. Therefore, mulberry leaves could be a potent functional ingredient for production of healthy foods. To our knowledge, there is limited information of mulberry leaf selection for their utilization. Selection of mulberry leaves is an important approach that could affect the quality of mulberry leaf-based products. Most of previous studies focused on only the effect of cultivars on phenolic compounds in mulberry leaves. However, there has been reported that melatonin content in mulberry leaves were influenced by the combination effects of cultivars and leaf ages [7]. Moreover, both cultivars and leaf ages were also affected on phenolics in spinach and berries [8, 9]. Considering these two factors, this study aims to investigate phenolic profiles and contents of mulberry leaves harvested from three commercial cultivars in Thailand including Buriram 60 (BR 60), cv. Sakonnakhon (SK), and cv. Khunphai (KH) at different three stages of leaf ages (tips, young, and old leaves) using HPLC-DAD and HPLC-ESI/MS. Furthermore, chemometric

analysis (principle component analysis (PCA) and hierarchical cluster analysis (HCA)) was also used for classification of mulberry leaves. The information obtained from this study could benefit both growers and food processers who would like to use the mulberry leaves as an alternative healthy ingredient for development of functional foods and supplements.

2. Materials and Methods

2.1. Plant Materials. Mulberry leaves from three commercial Thai cultivars (BR 60, SK, and KH) were manually harvested at Queen Sirikit Sericulture Center, Saraburi, Thailand (latitude 14°41′18.6″N × longitude 100°53′39.4″E) in March 2012. For each cultivar, three stages of leaf ages (tips, young, and old leaves) were collected. The tips of leaves were selected from the leaves at positions 1 to 3 from the top of each branch; young leaves were from positions 4 to 6; and old leaves were from positions 7 to 10. The averages of horizontal and vertical lengths of leaves were 8.4 ± 1.6 and 11.5 ± 2.5 cm for tips, 12.2 ± 2.0 and 16.1 ± 2.8 cm for young leaves, and 15.1 ± 2.4 and 18.8 ± 3.0 cm for old leaves. For all cultivars, a thousand leaves of each leaf age were collected. The sampling was carried out in duplicate for each sample. For sample preparation, the leaves were washed, cut into small pieces, immediately frozen in liquid nitrogen, and freeze-dried (Model: Gamma 2–16 LSC, Christ, Germany; freezing condition: temperature −50°C, pressure 0.1 mbar). The dried sample was ground, sieved with a 40 mesh sieve, and stored at −20°C.

2.2. Chemicals. The chemicals, 5-caffeoylquinic acid (chlorogenic acid), 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), quercetin-3-*O*-rutinoside (rutin), and kaempferol-3-*O*-rutinoside, were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). Analytical ethanol was purchased from Labscan (Bangkok, Thailand). HPLC grade acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). The water used in all experiments was purified using a Milli-Q system from Millipore Corp. (Bedford, MA, USA).

2.3. Extraction. The extraction method was modified from Pothinuch et al. [10]. Freeze-dried powder of sample (1 g) was extracted twice with 80% ethanol (25 mL) using ultrasonication for 30 min while cooled with ice. After centrifugation at 10,000 g at 4°C for 30 min, the supernatant was filtered and evaporated under vacuum. The aqueous extract was stored at −20°C until use. Each sample was extracted in triplicate.

2.4. Identification and Quantification of Phenolic Compounds. Phenolic compounds were identified and quantified using HPLC-DAD and HPLC-ESI/MS systems, following the methods of Pothinuch et al. [10]. The HPLC-PDA system consisted of a reverse phase 5 μm Symmetry® (4.6 × 250 mm) column (Waters, Milford, MA, USA) and an HPLC (Waters, Milford, MA, USA) equipped with a Waters 2707 autosampler, a Waters 600 pump, and a Waters 2998 PDA detector. The mobile phase used was acetonitrile (A) and 1% formic acid in water (B). The solvent gradient was performed as follows: 0–5 min with 5–10% A; 5–10 min with 10–15% A; 10–30 min with 15–25% A; 30–40 min with 25–50% A; 40–45 min with 50–5% A; and 45–50 min with 5% A. The flow rate was 1.0 mL/min, and injection volume of extract was 20 μL. The detection wavelength of the PDA detector was set in range of 200–500 nm. Mass spectral (MS) analysis was carried out with HPLC-ESI/MS under API-ES positive and negative modes. The ionization condition was set at 350°C and 3,000 V for capillary temperature and voltage, respectively. The nebulizer pressure was 60 psig, and flow rate of nitrogen gas was 13 L/min. The full scan mass was range from *m/z* 50 to *m/z* 1000. Identification of phenolic compounds was based on retention times, UV spectra, and MS information. Quantification of phenolic compounds was performed using reference standards of 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-rutinoside. The contents were expressed as mg/g DW and determined in triplicate.

2.5. Statistical Analysis. Data were expressed as mean ± SEM. Statistical analysis was performed by using a two-way analysis of variance (ANOVA) followed by a Tukey simultaneous test. Significant difference was statistically considered at $p < 0.05$. PCA and HCA were performed to standardize data in order to classify different mulberry leaf samples. PCA was used to observe interrelationships of sample and analyzed parameters. HCA was applied to cluster different samples by considering hierarchical associations using Euclidean distance and Ward's method as dissimilarity measure and agglomeration method, respectively.

3. Results and Discussion

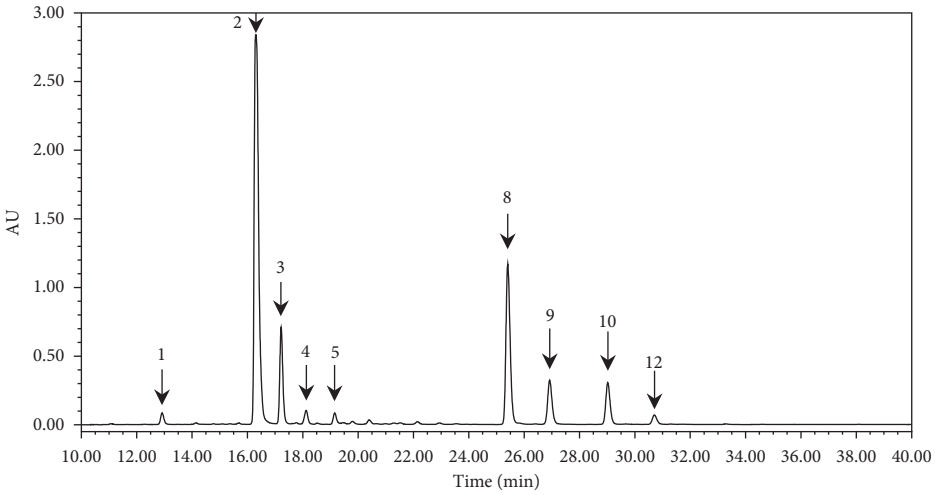
Mulberry cv. BR 60, cv. SK, and cv. KH are commercially promoted by The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives, Thailand. BR 60 is a hybrid cultivar between mulberry cv. Liu Jio 44 (male) and cv. Noi (native Thai cultivar, female), while SK is a hybrid cultivar between mulberry cv. Luin Jio No. 40 (male) and cv. KH (native Thai cultivar, female). KH is a native Thai cultivar. The leaf production of mulberry cv. BR 60, cv. SK, and cv. KH were 6.9, 4.0, and 3.2 kg/km^2/year, respectively. Mulberry cv. BR 60 can grow only in the area that has sufficient amount of water, while mulberry cv. SK and cv. KH can be cultivated in any area in Thailand. All cultivars also have highly resistant against leaf mosaic and root rot diseases. In this study, HPLC profile and contents of phenolic compounds were investigated in order to characterize mulberry leaves according to cultivars and leaf ages.

3.1. Identification of Phenolic Compounds in Mulberry Leaves. Phenolic compounds in mulberry leaves were identified using HPLC-DAD and HPLC-ESI/MS. HPLC chromatograms of mulberry leaves from three cultivars at

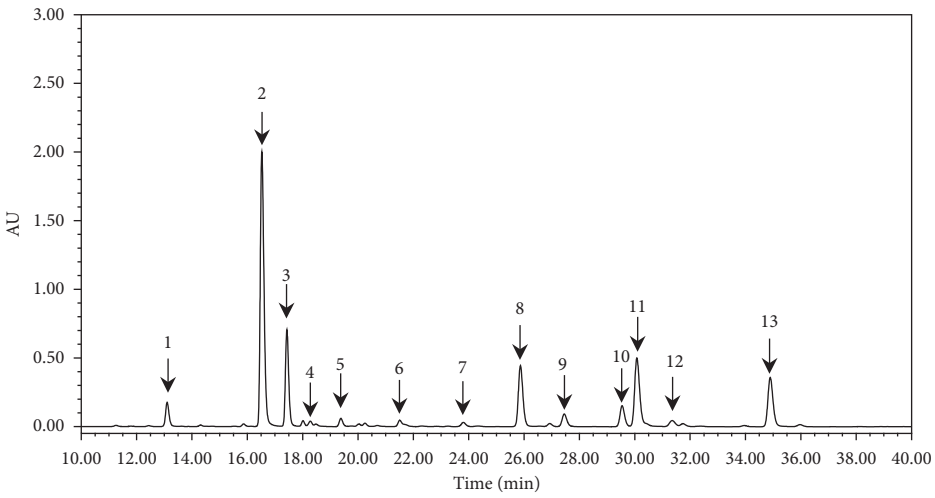
different three stages of leaf ages are illustrated in Figures 1(a)–1(i). A total of 13 peaks were detected at a wavelength of 350 nm in young and old leaves of mulberry cv. BR 60, whereas other mulberry leaf samples showed only 9 peaks in their chromatograms. The combination of distinct UV spectra and MS characteristics in both positive and negative ionization modes was used to identify individual phenolic compounds in mulberry leaves. External reference standards including 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-rutinoside were used for spiking and confirmation of the authenticity of peaks 2, 3, 8, and 10, respectively. For other peaks, comparison of λ_{max} and MS data with data obtained from previous studies was used for identification. The results showed that two groups of phenolic compounds were identified in the mulberry leaves: (1) caffeoylquinic acids and (2) flavonol glycosides. Peak identification of individual compounds is shown in Table 1 and mentioned below:

(1) *Caffeoylquinic Acids*. Peaks 1, 2, 3, and 5 were identified as caffeoylquinic acid isomers. These peaks were detected in the first 20 min of running time and had similar UV spectra with λ_{max} at 237–241 and 317–325 nm. Observation of the base ions at *m/z* 355 in the positive mode ($[M+H]^+$) and 353 in the negative mode ($[M-H]^-$) in the four peaks indicates that their molecular weight equals 354, corresponding to the molecular weight of monocaffeoylquinic acid. Peaks 2 and 3 were identified as 5-*O*-caffeoylquinic acid (chlorogenic acid) and 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), respectively, by comparing retention time of reference standards and confirming spiking of the reference standard. To differentiate individual isomers of caffeoylquinic acid, MS data were used by considering patterns of negative fragment ions identified by LC-MS^4, as explained in the previous study [11]. Peak 1 was likely to be 3-caffeoylquinic acid (neochlorogenic acid) due to the existence of the fragment ion at *m/z* 179, and this peak was first eluted from the C-18 column, similarly to the results of Nakatani et al. [12] who used a similar system of reverse phase HPLC. Peak 5 was also assigned as an isomer of caffeoylquinic acid. This peak produced same fragment ions as found in peak 2. These two peaks might be different in three-dimensional orientation of their atoms (*cis*- and *trans*-forms), which could not be differentiated based on our analysis. According to the results reported by Xie et al. [13], peak 5 might be assigned as the *cis*-form of 5-*O*-caffeoylquinic acid since *cis*-form of 5-*O*-caffeoylquinic acid retained longer than its *trans*-form. The *cis*-5-*O*-caffeoylquinic acid has not been reported in mulberry leaves, but this compound was found in mulberry fruits [14]. Naturally, the *trans*-form of 5-*O*-caffeoylquinic acid was predominantly produced in plant tissue over its *cis*-form; however, under UV light exposure, the *trans*-form could be converted to the *cis*-form as observed in tobacco leaves [15, 16].

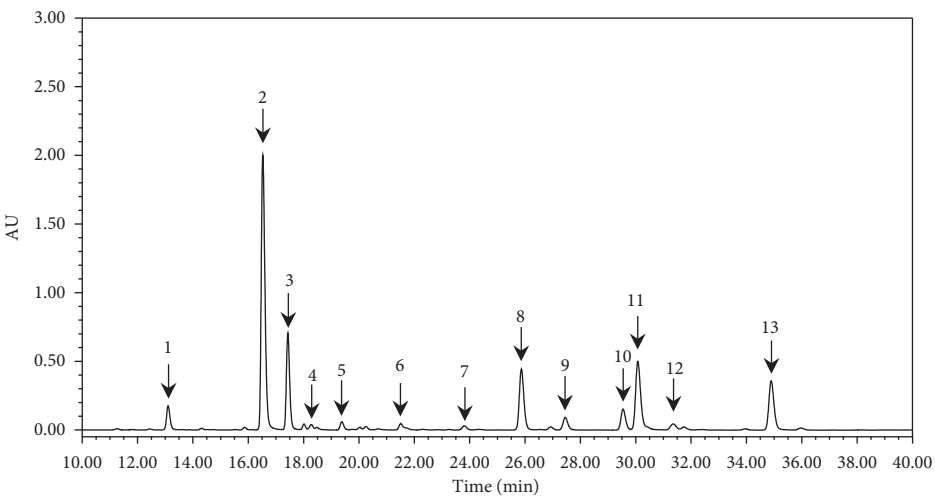
(2) *Flavonol Glycosides*. Peaks 4 and 6–13 were identified as glycosides of flavonols (quercetin and kaempferol). These peaks were eluted after 20 min of running time, except for peak 4. Peaks 6, 8, 9, and 11 were considered to be quercetin derivatives since these peaks had similar UV spectra with λ_{max} at 256 and 353-354 and also produced a positive ion at *m/z* 303, suggesting the existence of quercetin residue. Peak 8 was identified as quercetin-3-*O*-rutinoside by spiking and comparing with the reference standard. Also, production of $[M+H]^+$ at *m/z* 611 and $[M-H]^-$ at *m/z* 609 in peak 8 indicated its molecular weight of 610 which matched quercetin-3-*O*-rutinoside. Peak 6 generated similar positive fragment ions as detected in peak 8. Moreover, peak 6 possibly produced the fragment ion at *m/z* 611 by cleavage rhamnose (molecular mass 146 amu) from $[M+H]^+$. Thus, peak 6 might consist of quercetin-rutinoside and rhamnosyl residues. Regarding MS data and its possible molecular weight of 756, peak 6 might match quercetin-3-(rhamnosyl-glucoside)-7-*O*-rhamnoside or quercetin-3-*O*-(2-rhamnosyl) rutinoside [17], which have never reported in mulberry leaves. Peak 9 produced $[M+H]^+$ and $[M-H]^-$ at *m/z* 465 and 463, representing a molecular weight equal to 464, respectively. The fragment ion at *m/z* 303 was also found in this peak, which possibly was generated by loss of hexoside residue (a molecular mass of 162 amu) from $[M+H]^+$. Therefore, this peak was supposed to contain quercetin and hexosyl group in its structure, which could be either quercetin-glucoside or quercetin-galactoside. However, only quercetin-3-*O*-glucoside has been reported in mulberry leaves [18–20]. Peak 11 was tentatively identified as quercetin-(malonyl)hexoside by comparing with the results of Ruiz et al. [21]. This peak generated $[M+H]^+$ and $[M-H]^-$ at *m/z* 551 and 549, respectively, indicating its molecular weight of 550. Its molecular weight might belong to quercetin-(malonyl)glucoside or quercetin-(malonyl)galactoside. However, only quercetin-(malonyl)glucoside was determined in mulberry leaves [5, 19, 20, 22]. Peaks 4, 7, 10, 12 and 13 were identified as kaempferol derivatives because these peaks had similar UV spectra with λ_{max} at 265 and 345–347 nm and also produced the fragment ion at *m/z* 287 (kaempferol residue). Peak 10 was identified as kaempferol-3-*O*-rutinoside by comparing with the reference standard and confirming by spiking with its reference standard. Based on MS data, peak 7 was likely to be derivative of kaempferol-rutinoside because this peak produced same fragment ions as presented in peak 10. Also, the fragment ion at *m/z* 611 was produced by loss of rhamnose (molecular mass 146 amu). Thus, peak 7 possibly consisted of kaempferol-rutinoside and rhamnosyl residues [18]. The fragment ion at *m/z* 287 was also found in peak 12, which possibly was generated by loss of the hexosyl group



(a)

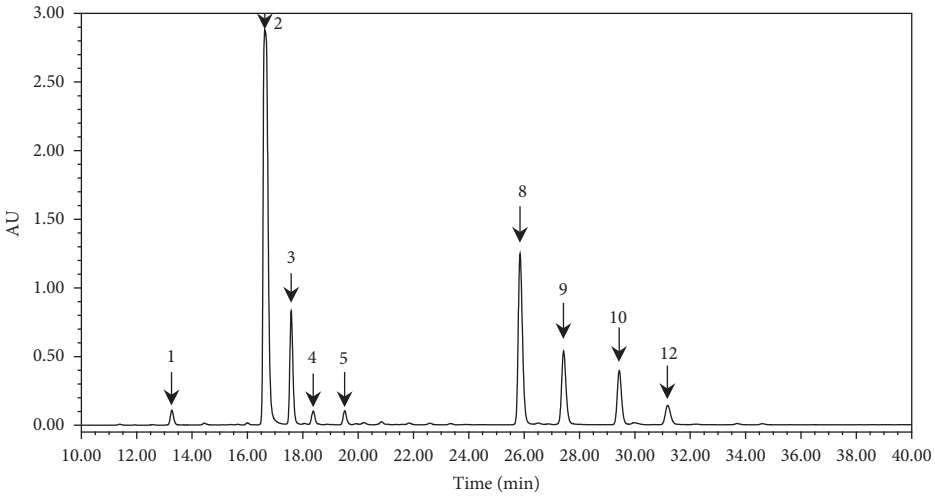


(b)



(c)

Figure 1: Continued.



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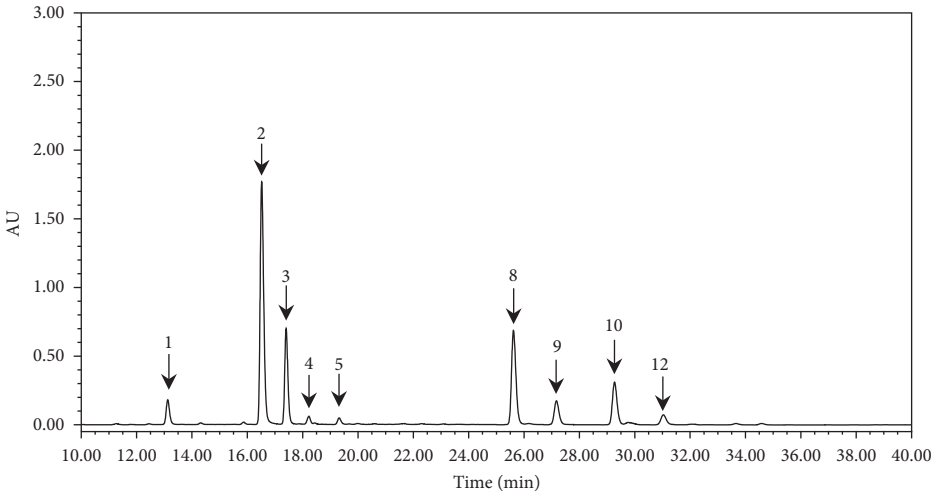

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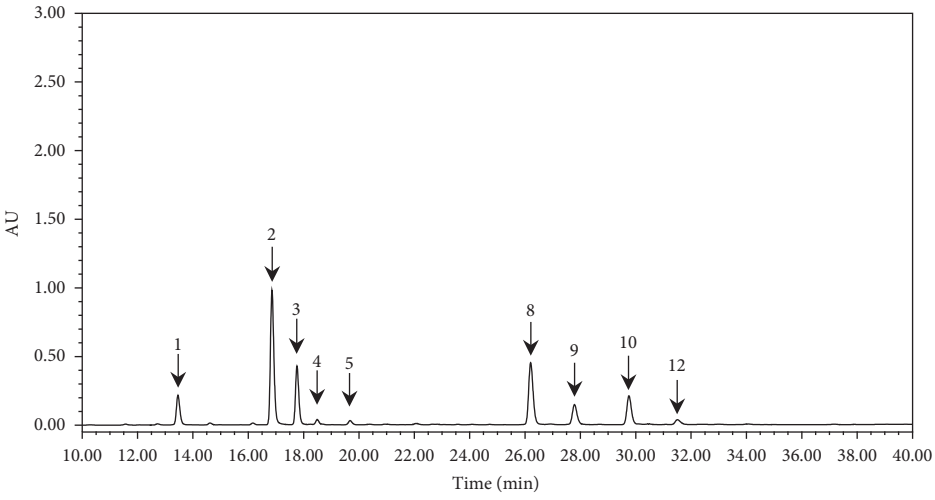

(f)

Figure 1: Continued.

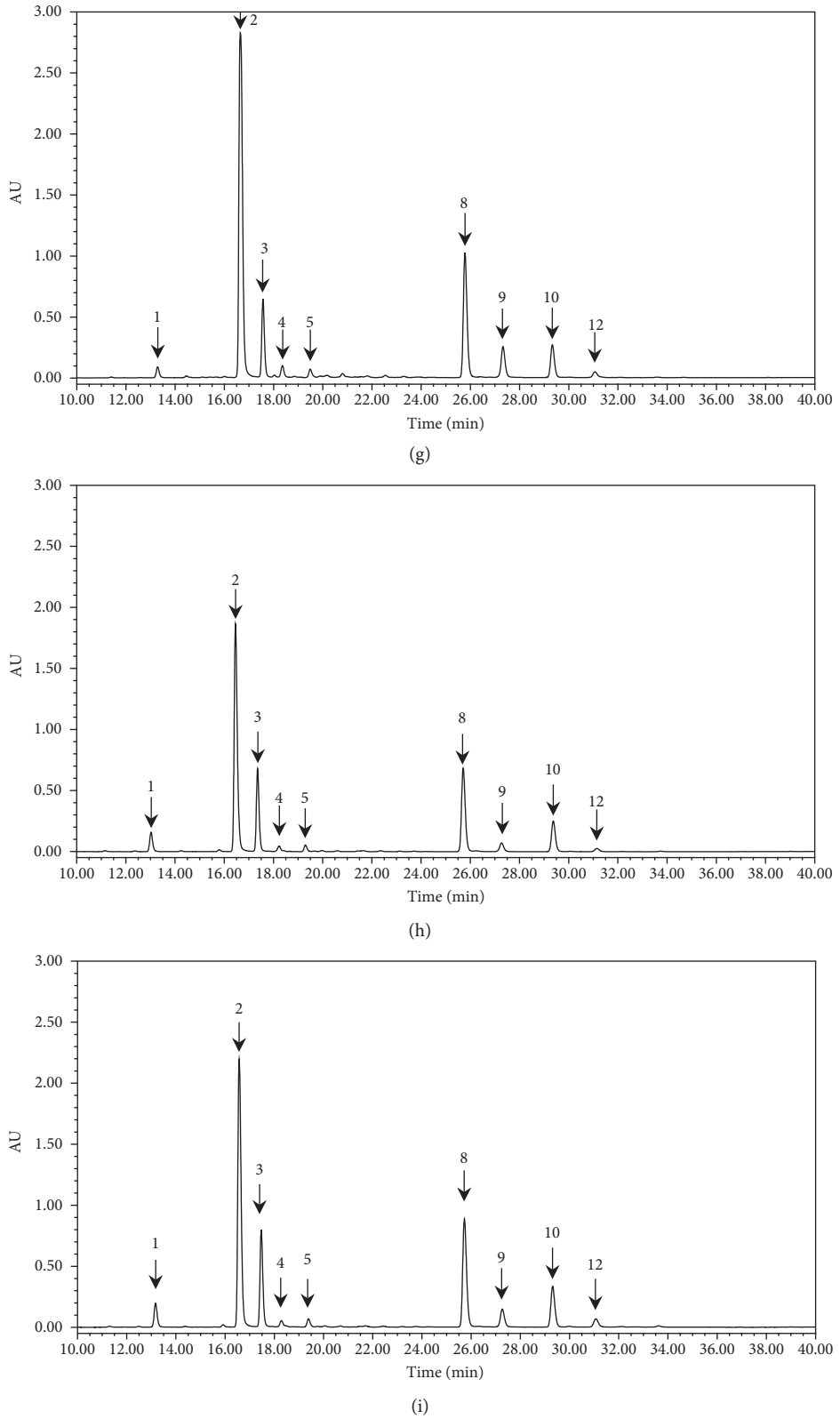

Figure 1: Typical HPLC chromatograms of phenolic compounds of different mulberry leaves: tip of leaves cv. Buriram 60 (a), young leaves cv. Buriram 60 (b), old leaves cv. Buriram 60 (c), tip of leaves cv. Sakonnakhon (d), young leaves cv. Sakonnakhon (e), old leaves cv. Sakonnakhon (f), tip of leaves cv. Khunphai (g), young leaves cv. Khunphai (h), and old leaves cv. Khunphai (i), detected at 350 nm and zoom-in spectrum range of 10–40 min. The peak numbers are corresponding to UV spectrum and MS data in Table 1.

Table 1: Identification of phenolic compounds of mulberry leaves and the data taken from HPLC-DAD and HPLC-MS.

Peak	RT (min)	λ_{max} (nm)	Mass spectra				Tentative compound*
			Positive ion (m/z)		Negative ion (m/z)		
			$[M+H]^+$	Fragment ions	$[M-H]^-$	Fragment ions	
Caffeoylquinic acid isomers							
1	13.1	239, 325	355	163, 135	353	191, 179	Caffeoylquinic acid isomer I[2]
2	16.5	241, 325	355	163, 135	353	191	5-*O*-Caffeoylquinic acid[1]
3	17.4	240, 325	355	163, 135	353	191, 179, 173	4-*O*-Caffeoylquinic acid[1]
5	19.5	237, 317	355	163, 135	353	191	Caffeoylquinic acid isomer II[2]
Flavonol derivatives							
4	18.2	265, 347	757	611, 287, 449	755	609	Kaempferol-hexoside derivative[2]
6	21.4	256, 354	757	303, 611, 627, 465	755	625	Quercetin-rutinoside derivative[2]
7	23.7	265, 354	741	595, 287, 449	739	—	Kaempferol-rutinoside derivative[2]
8	25.6	256, 354	611	465, 303	609	—	Quercetin-3-*O*-rutinoside[1]
9	27.1	256, 353	465	303	463	—	Quercetin-hexoside[2]
10	29.1	265, 347	595	449, 287	593	—	Kaempferol-3-*O*-rutinoside[1]
11	29.6	256, 354	551	303	549	505	Quercetin-(malonyl)-hexoside[2]
12	31.0	265, 347	449	287	447	—	Kaempferol-hexoside[2]
13	34.3	265, 347	535	287	533	489	Kaempferol-(malonyl)-hexoside[2]

*Identification of phenolic compound. [1]According to UV spectrum and MS data in combination with retention time of the reference standard. [2]According to UV spectral and MS data comparison with the data obtained from previous studies.

(molecular mass 162 amu) from its $[M+H]^+$. It indicated that peak 12 was supposed to contain the hexosyl group, which might be kaempferol-glucoside or kaempferol-galactoside. Only kaempferol-3-*O*-glucoside has been identified in mulberry leaves [23], whereas there have been no previous studies reporting kaempferol-galactoside in mulberry leaves. Peak 4 produced similar fragment ions as found in peak 12. It is possible that peak 4 might be the kaempferol-hexoside derivative. Moreover, fragment ion at m/z 611 was also found in this peak, which is possibly generated by cleavage of rhamnose (molecular mass 146 amu) from $[M+H]^+$. The results suggested that peak 4 was possibly comprised of kaempferol-hexoside and rhamnosyl residue. Comparing with previous results reported by El-Desoky et al. [24] and Stobiecki et al. [25], this peak might be kaempferol-3-*O*-glucosyl-(1 ⟶ 2)-rhamnoside-7-O-glucoside or kaempferol-3-*O*-glucosyl-glucoside-7-*O*-rutinoside. Based on survey of literatures, there was no study that identified this compound in mulberry leaves. Peak 13 generated $[M+H]^+$ at m/z 535 and $[M-H]^-$ at m/z 533, indicating molecular weight equal to 534. The fragment ion at m/z 489 was also yielded in the negative mode. When compared with the results from a previous study reported by Llorach et al. [26], this peak might be kaempferol-(malonyl)hexoside. However, only kaempferol-(malonyl)glucoside was found in mulberry leaves [20]. Thus, peak 10 was tentatively identified as kaempferol-(malonyl)glucoside.

3.2. Quantification of Phenolic Compounds in Mulberry Leaves. Quantitative analysis of phenolic compounds in mulberry leaves is shown in Table 2. The 5-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, and kaempferol-3-*O*-rutinoside were quantified using external reference standards. Caffeoylquinic acid isomers I and II were quantified as chlorogenic acid equivalents. Quercetin derivatives were quantified as quercetin-3-*O*-rutinoside equivalents, whereas kaempferol derivatives were reported as kaempferol-3-*O*-rutinoside equivalents. The results showed that total caffeoylquinic acid and total flavonol derivative contents of mulberry leaves ranged from 5.7 to 16.3 and 2.9 to 9.1 mg/g DW, respectively (Table 2). Among all samples, tip of leaves from mulberry cv. SK had the highest content of total caffeoylquinic acids and total flavonol glycosides. For young leaves, total caffeoylquinic acids and total flavonol derivatives were higher in mulberry leaves cv. BR 60 than other cultivars. No significant difference of total caffeoylquinic acids was found in old leaves; however, total flavonol glycosides of old leaves were significantly higher in mulberry leaves cv. BR 60 when compared to cv. SK and KH. Total caffeoylquinic acids and total flavonol derivatives decreased as mulberry leaves aged. The results indicated that phenolic compounds in mulberry leaves were distinctly influenced by cultivars and leaf ages, which is in agreement with the results determined in pear leaves [27]. Variation of phenolic compounds in different cultivars could be related to genetic diversity which might be involved in synthesis and metabolism of phenolic compounds [28]. Concerning the effect of leaf ages, decreasing phenolic contents as leaves age might lessen the activity of phenylalanine ammonia-lyase, a key enzyme in

Table 2: Quantification of phenolic compounds detected in mulberry leaves from 3 commercial Thai cultivars at different leaf ages.

Parameter	Buriram 60			Sakonnakhon			Khunphai		
	Tips	Young leaves	Old leaves	Tips	Young leaves	Old leaves	Tips	Young leaves	Old leaves
Caffeoylquinic acid isomer I[1]	$0.32 \pm 0.01^{B,c}$	$0.60 \pm 0.01^{NS,a}$	$0.55 \pm 0.01^{B,b}$	$0.39 \pm 0.01^{A,b}$	$0.68 \pm 0.02^{NS,a}$	$0.68 \pm 0.02^{A,a}$	$0.33 \pm 0.01^{B,b}$	$0.64 \pm 0.06^{NS,a}$	$0.73 \pm 0.01^{A,a}$
5-*O*-Caffeoylquinic acid	$11.03 \pm 0.53^{B,a}$	$6.76 \pm 0.14^{A,b}$	$3.88 \pm 0.12^{NS,c}$	$13.12 \pm 0.24^{A,a}$	$5.04 \pm 0.80^{B,b}$	$3.50 \pm 0.36^{NS,c}$	$10.72 \pm 0.13^{B,a}$	$5.73 \pm 0.85^{B,b}$	$5.03 \pm 0.91^{NS,b}$
4-*O*-Caffeoylquinic acid	$1.91 \pm 0.11^{B,a}$	$2.13 \pm 0.05^{NS,a}$	$1.26 \pm 0.03^{B,b}$	$2.45 \pm 0.05^{A,a}$	$1.80 \pm 0.25^{NS,b}$	$1.38 \pm 0.11^{B,c}$	$1.84 \pm 0.01^{B,ns}$	$1.99 \pm 0.09^{NS,ns}$	$1.88 \pm 0.17^{A,ns}$
Caffeoylquinic acid isomer II[1]	$0.23 \pm 0.02^{B,a}$	$0.18 \pm 0.00^{A,b}$	$0.10 \pm 0.00^{NS,c}$	$0.33 \pm 0.02^{A,a}$	$0.13 \pm 0.02^{B,b}$	$0.10 \pm 0.01^{NS,b}$	$0.23 \pm 0.02^{B,a}$	$0.15 \pm 0.01^{B,b}$	$0.14 \pm 0.02^{NS,b}$
Total caffeoylquinic acids	$13.49 \pm 0.66^{B,a}$	$9.68 \pm 0.20^{A,b}$	$5.79 \pm 0.17^{NS,c}$	$16.29 \pm 0.16^{A,a}$	$7.59 \pm 0.45^{B,b}$	$5.65 \pm 0.47^{NS,c}$	$13.13 \pm 0.08^{B,a}$	$8.51 \pm 0.69^{A,b}$	$7.78 \pm 0.97^{NS,b}$
Kaempferol-hexoside derivative[3]	$0.30 \pm 0.02^{A,a}$	$0.09 \pm 0.01^{B,b}$	$0.07 \pm 0.00^{C,b}$	$0.32 \pm 0.00^{A,a}$	0.13 ± 0.01	0.11 ± 0.01	0.29 ± 0.00	0.16 ± 0.00	0.01 ± 0.00
Quercetin-rutinoside derivative[2]	ND	0.14 ± 0.00^{ns}	0.15 ± 0.02^{ns}	ND	ND	ND	ND	ND	ND
Kaempferol-rutinoside derivative[3]	ND	0.10 ± 0.00^{b}	0.13 ± 0.00^{a}	ND	ND	ND	ND	ND	ND
Quercetin-3-*O*-rutinoside	$3.65 \pm 0.26^{B,a}$	$1.55 \pm 0.05^{B,b}$	$1.02 \pm 0.04^{B,b}$	$4.39 \pm 0.05^{A,a}$	$1.97 \pm 0.18^{B,b}$	$1.56 \pm 0.12^{B,c}$	$3.45 \pm 0.02^{B,a}$	$2.42 \pm 0.20^{A,b}$	$2.22 \pm 0.29^{A,b}$
Quercetin-hexoside[2]	$1.00 \pm 0.08^{B,a}$	$0.28 \pm 0.02^{B,b}$	$0.05 \pm 0.00^{B,c}$	$2.03 \pm 0.05^{A,a}$	$0.46 \pm 0.05^{A,b}$	$0.38 \pm 0.05^{A,b}$	$0.94 \pm 0.03^{B,a}$	$0.36 \pm 0.0^{5B,b}$	$0.36 \pm 0.05^{A,b}$
Kaempferol-3-*O*-rutinoside	$1.11 \pm 0.07^{B,a}$	$0.61 \pm 0.02^{B,b}$	$0.41 \pm 0.01^{C,c}$	$1.59 \pm 0.01^{A,a}$	$1.07 \pm 0.09^{A,b}$	$0.84 \pm 0.07^{B,c}$	$1.05 \pm 0.00^{B,ns}$	$1.14 \pm 0.08^{A,ns}$	$1.11 \pm 0.09^{A,ns}$
Quercetin-(malonyl)-hexoside[2]	ND	2.00 ± 0.08^{a}	1.46 ± 0.06^{b}	ND	ND	ND	ND	ND	ND
Kaempferol-hexoside[3]	$0.25 \pm 0.02^{B,a}$	$0.22 \pm 0.01^{NS,a}$	$0.14 \pm 0.01^{NS,b}$	$0.73 \pm 0.02^{A,a}$	$0.17 \pm 0.01^{NS,b}$	$0.14 \pm 0.00^{NS,b}$	$0.23 \pm 0.01^{B,ns}$	$0.20 \pm 0.04^{NS,ns}$	$0.19 \pm 0.04^{NS,ns}$
Kaempferol-(malonyl)-hexoside[3]	ND	1.67 ± 0.20^{a}	1.31 ± 0.17^{b}	ND	ND	ND	ND	ND	ND
Total flavonols	$6.31 \pm 0.42^{B,a}$	$6.66 \pm 0.22^{A,a}$	$4.74 \pm 0.17^{A,b}$	$9.06 \pm 0.14^{A,a}$	$3.67 \pm 0.28^{B,b}$	$2.92 \pm 0.22^{B,b}$	$5.67 \pm 0.05^{B,a}$	$4.12 \pm 0.33^{B,b}$	$3.88 \pm 0.47^{B,b}$

Values are expressed as mean ± SEM. Different capital letters indicate significant differences between cultivars within the same leaf age at $p < 0.05$. Different small letters indicate significant differences between leaf ages within the same cultivar at $p < 0.05$. ND: none detectable; NS: no significant difference among cultivars at $p < 0.05$; ns: no significant difference among leaf age at $p < 0.05$. [1]Content expressed as mg chlorogenic acid equivalent/g dry weight. [2]Content expressed as mg quercetin-3-rutinoside equivalent/g dry weight. [3]Content expressed as mg kaempferol-3-rutinoside equivalent/g dry weight.

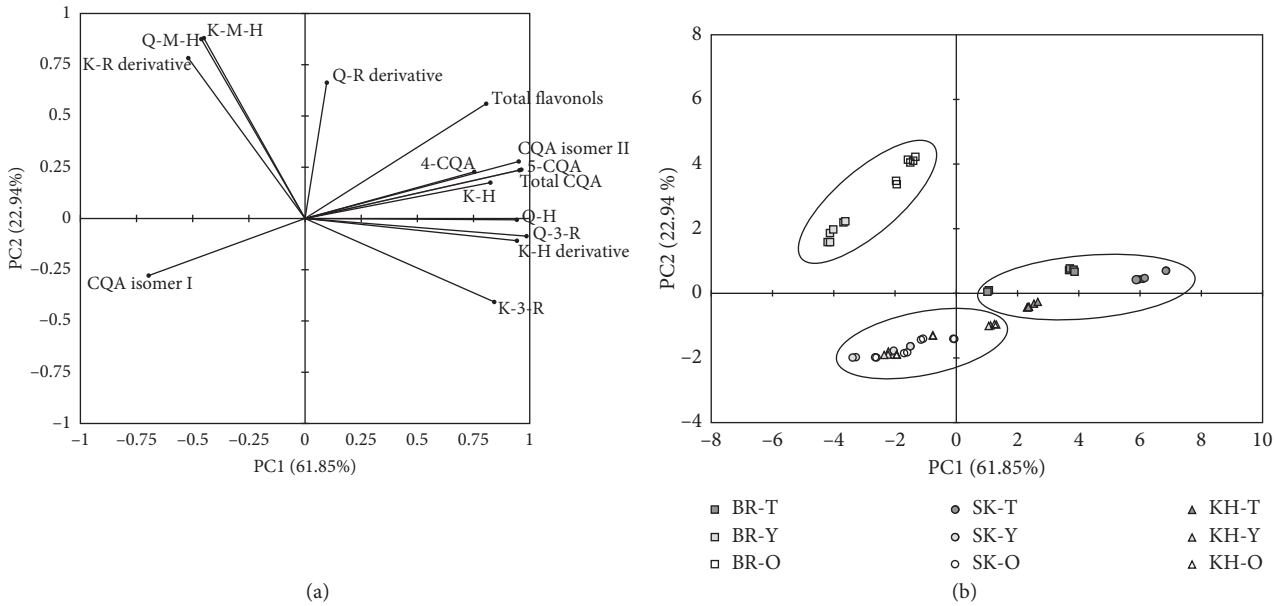

Figure 2: Principal component analysis based on phenolic compounds of different 9 samples of mulberry leaves: (a) scattering plot of loadings on principle components PC 1 and PC 2; (b) sample map of scores on PC1 and PC 2 as function of cultivars and leaf age. 4-CQA: 4-*O*-caffeoylquinic acid; 5-CQA: 5-*O*-caffeoylquinic acid; CQA isomers I, II; caffeoylquinic acid isomers I, II; total CQA: total caffeoylquinic acids; K-H: kaempferol-hexoside; Q-H: quercetin-hexoside; Q-3-R: quercetin-3-*O*-rutinoside; K-3-R: kaempferol-3-*O*-rutinoside; K-M-H: kaempferol-(malonyl)hexoside; Q-M-H: quercetin-(malonyl)hexoside; K-R: kaempferol-rutinoside; Q-R: quercetin-rutinoside. Cultivar's codes are BR 60: Buriram 60, SK: Sakonnakhon, and KH: Khunphai. Leaf age's codes are T: tips, Y: young leaves, and O: old leaves.

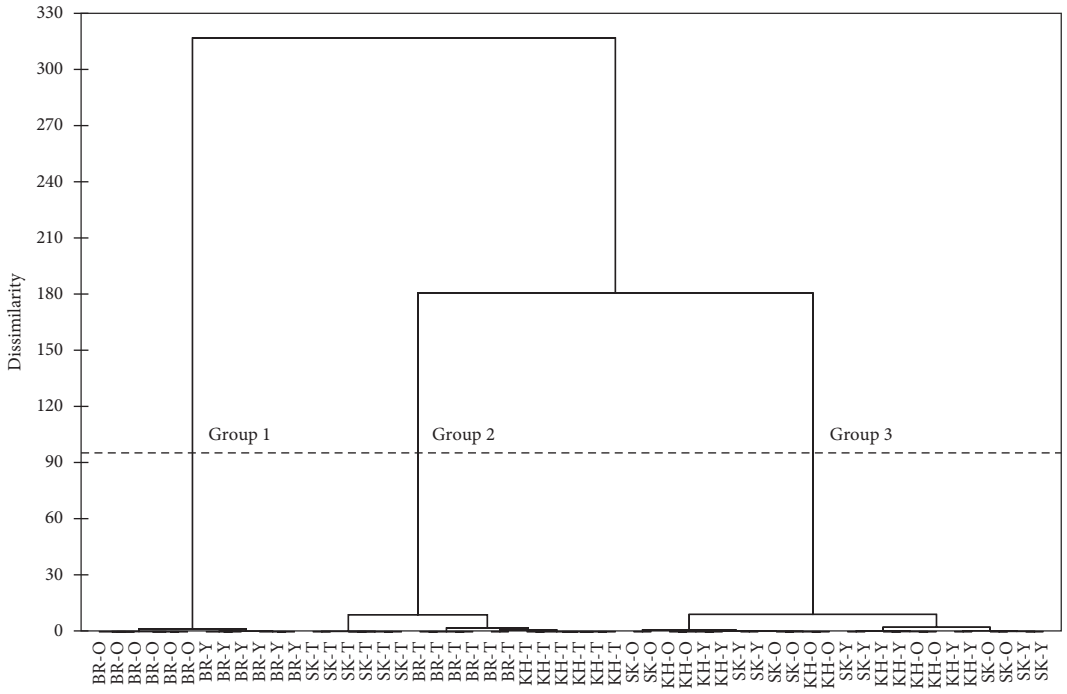

Figure 3: Dendrogram of hierarchical cluster analysis among different samples of mulberry leaves from three cultivars at different mulberry leaves. Cultivar's codes are BR 60: Buriram 60, SK: Sakonnakhon, and KH: Khunphai. Leaf age's codes are T: tips, Y: young leaves, and O: old leaves.

phenolic synthesis and metabolites, as reported in apple leaves [29]. The result also showed that 5-*O*-caffeoylquinic acid is the most abundant phenolic compound of mulberry leaves, contributing up to 57%. Similarly, this compound was predominantly found in the leaves of mulberry cultivated in Korea [18], Spain [19], and Tunisia [20]. The mulberry leaves in this study contained greater 5-*O*-caffeoylquinic acid content than that contained in the Korean and

Tunisian mulberry leaves but lower than that of the Spanish mulberry leaves. Quercetin-3-*O*-rutinoside and 4-*O*-caffeoylquinic acid were also major phenolic compounds, which contributed up to 19.2% and 16.1%, respectively. Quercetin-(malonyl)hexoside (12.2–13.8%) and kaempferol-(malonyl)hexoside (10.2–12.4%) were contributed only in young and old leaves of mulberry cv. BR 60. Quercetin 3-(malonyl)glucoside was reported as a major phenolic compound in Japanese and Korean mulberry reported by Katsube et al. [5] and Lee and Choi [18], respectively. Furthermore, quercetin-rutinoside derivative and kaempferol-rutinoside derivative were observed only in young and old leaves of mulberry cv. BR 60 with very low amount (<1%). Considering individual phenolic compounds, their contents significantly decreased when the leaves aged, except for caffeoylquinic acid isomer I (3-*O*-caffeoylquinic acid).

3.3. Classification of Mulberry Leaves. PCA was applied to the data set of 9 different samples of mulberry leaves harvested from three cultivars (BR 60, SK, and KH) at different 3 stages of leaf ages (tips, young, and old leaves) in order to achieve understanding of characteristics of these leaf samples. The results showed that the first two principal components (PCs) explained 84.79% of the total variation of data set. PC1 explained 61.34% of total variance and had high contribution of 5-*O*-caffeoylquinic acid, quercetin-3-*O*-rutinoside, caffeoylquinic acid isomer II, and total caffeoylquinic acids (Figure 2(a)). The nine samples of mulberry leaves from different cultivars and leaf ages were clearly divided into three groups (Figure 2(b)). The first group had very high positive scores for PC1, which consisted of tips of leaves from all cultivars. This group can be best described by high content of phenolic compounds, especially 5-*O*-caffeoylquinic acid (>10 mg/g DW) and quercetin-3-*O*-rutinoside (>3.4 mg/g DW). The second group had negative scores for PC1 and positive scores for PC2 including young and old leaves of mulberry cv. BR 60. This group was best characterized by the presence of quercetin-(malonyl)hexoside and kaempferol-(malonyl) hexoside. The third group contained young and old leaves of mulberry cv. SK and KH, which had negative scores for PC1 and PC2. This group corresponds to low phenolic contents. HCA also applied for grouping the mulberry leaves based on the similarities of phenolic compounds. Three groups of mulberry leaves were clustered by HCA (Figure 3). It showed clearly that the results from HCA were very similar to the results from PCA. Summarizing the PCA and HCA, these chemometric analyses revealed that the predominant phenolic compounds, 5-*O*-caffeoylquinic acid and quercetin-3-*O*-rutinoside, can be used to classify tips of leaves from young and old leaves. However, the difference of tips of leaves among cultivars could not be classified. Besides, the results from this study also suggested that mulberry leaf samples in the same group might be used interchangeably because the leaves provided similar phenolic compounds. Previous study reported that mulberry leaves were clustered by genetic characters such clones and species [19, 30]. This study exhibited that cultivars and leaf ages were firstly considered for classification of mulberry leaves which is the new evidence exhibiting the important of the two factors in mulberry leaf utilization.

4. Conclusions

Caffeoylquinic acids and flavonol glycosides were main phenolic compounds in mulberry leaves. The predominant compounds were 5-*O*-caffeoylquinic acid and quercetin-3-*O*-rutinoside. Cultivars and leaf ages significantly influenced phenolic compounds in mulberry leaves; therefore, the two factors should be used as criteria for selection of mulberry leaves.

According to the similarity of phenolic compounds, three groups of mulberry leaves were classified using PCA and HCA. Tips of mulberry leaves from all cultivars should be intended for production of functional healthy because of their higher phenolic contents. Therefore, cultivars and leaf ages can be used as the quality index for selection of mulberry leaves in their utilization.

Data Availability

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

Conflicts of Interest

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

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