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Research Article

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

Beibei Zhao,[1] **Jiawen Deng**,[1] **Hua Li**,[1] **Yaqiang He**,[1] **Tao Lan**,[2] **Di Wu**,[1] **Haodi Gong**,[1] **Yan Zhang**,[1] **and Zhicheng Chen**[1]

[1]*College of Food Science and Engineering, Henan University of Technology, Zhengzhou, Henan 450002, China*
[2]*China National Institute of Standardization, Beijing, China*

Correspondence should be addressed to Zhicheng Chen; chen_1958@163.com

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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

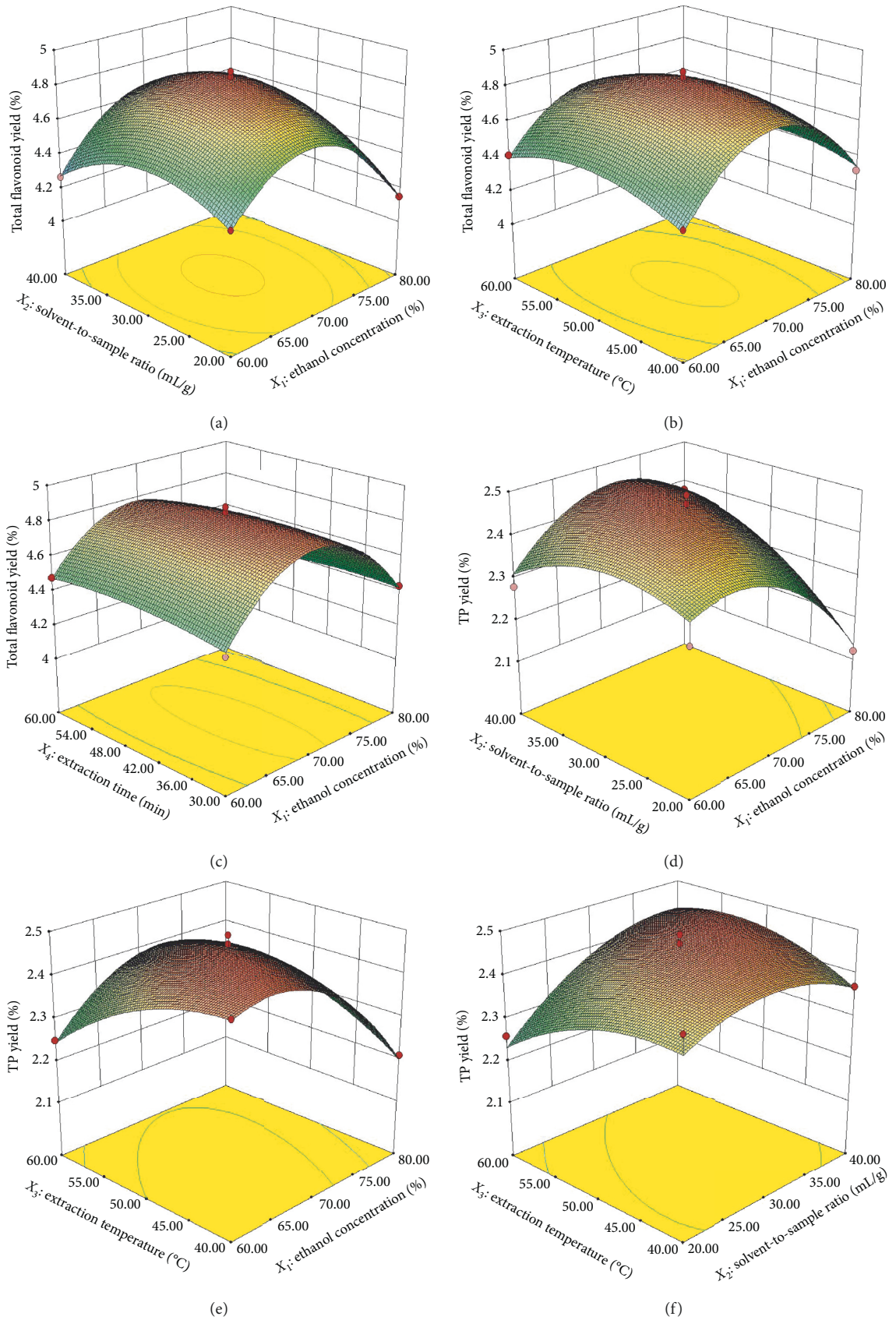

Figure 1: Interactive effect of extraction variables on (a–c) TF and (d–f) TP.

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

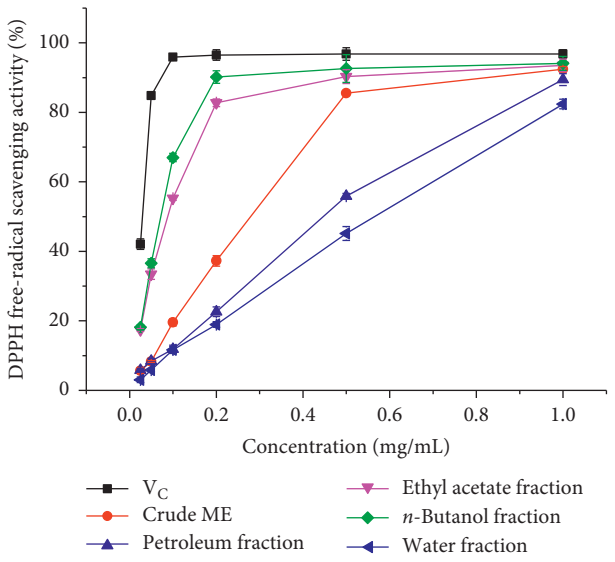

Figure 2: DPPH-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

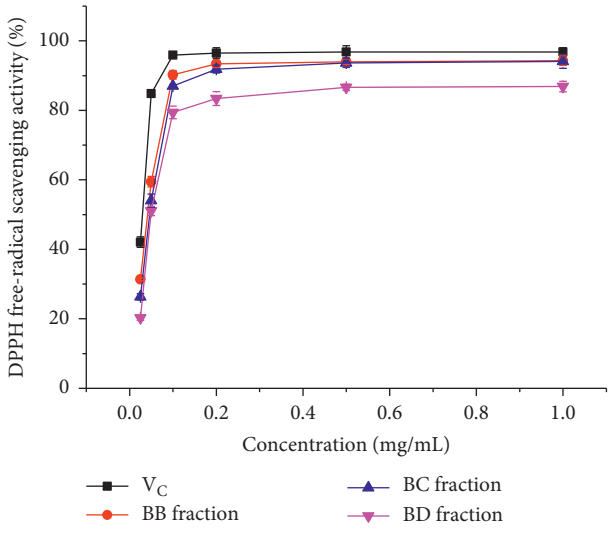

Figure 3: DPPH-scavenging activity of V_C, BB, BC, and BD fractions.

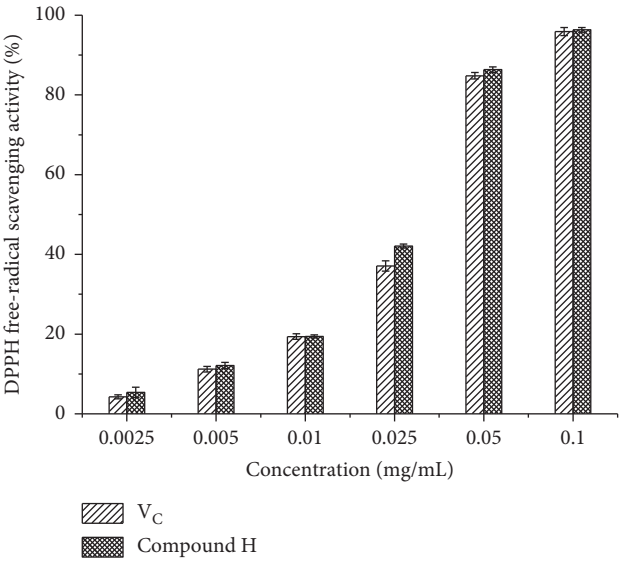

Figure 4: DPPH-scavenging activity of compound H and V_C.

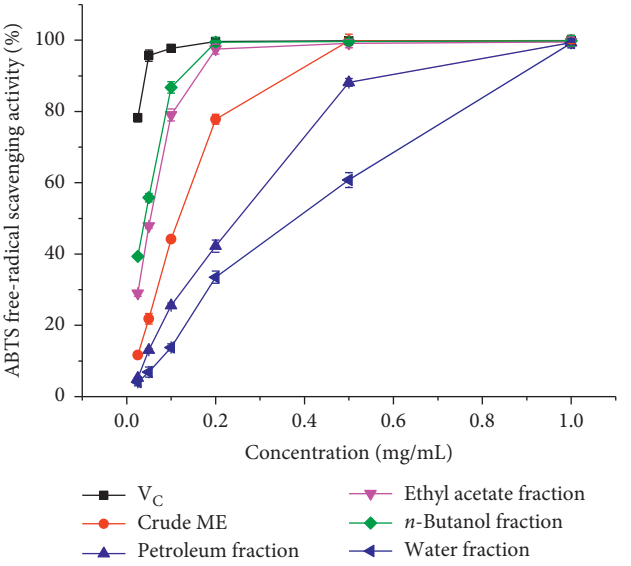

Figure 5: ABTS-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

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

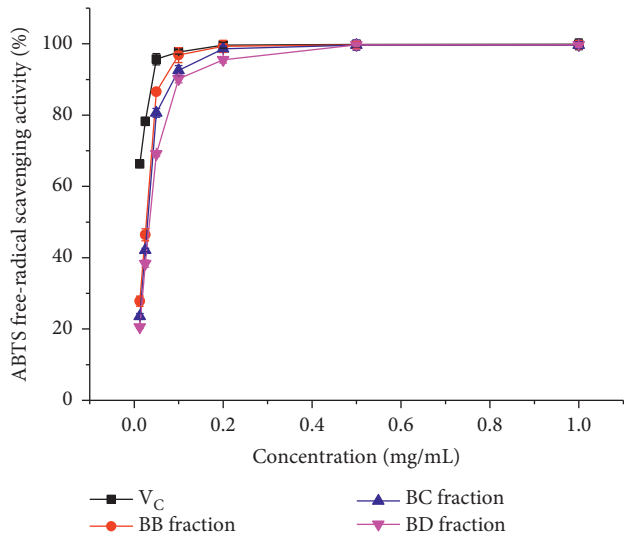

Figure 6: ABTS-scavenging activity of V_C, BB, BC, and BD fractions.

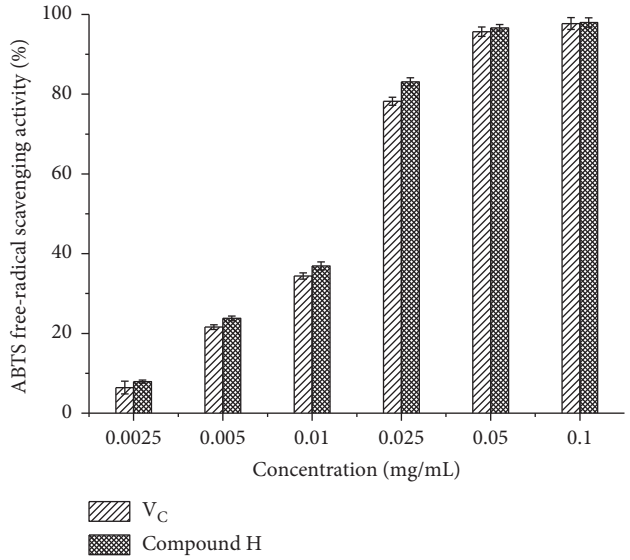

Figure 7: ABTS-scavenging activity of compound H and V_C.

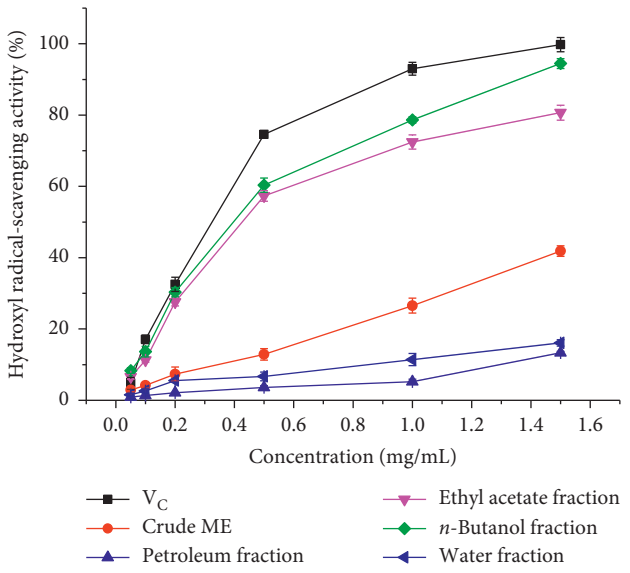

Figure 8: Hydroxyl radical-scavenging activity of crude ME, V_C, and organic extraction fractions extracted from ME.

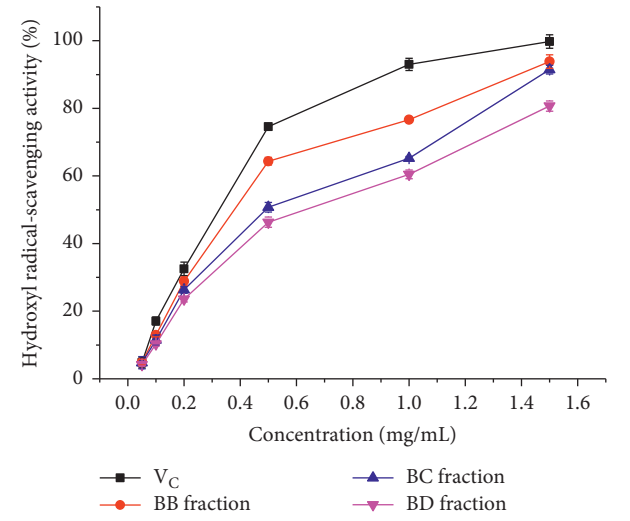

Figure 9: Hydroxyl radical-scavenging activity of V_C, BB, BC and BD fractions.

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 ($p < 0.05$).

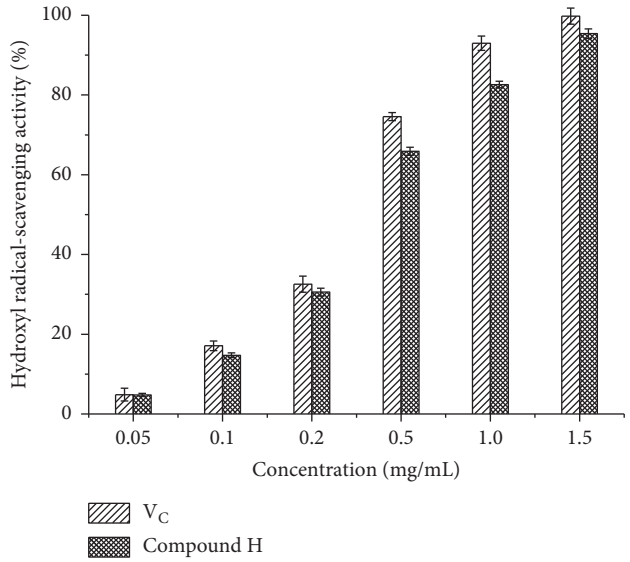

Figure 10: Hydroxyl radical-scavenging activity of compound H and V_C.

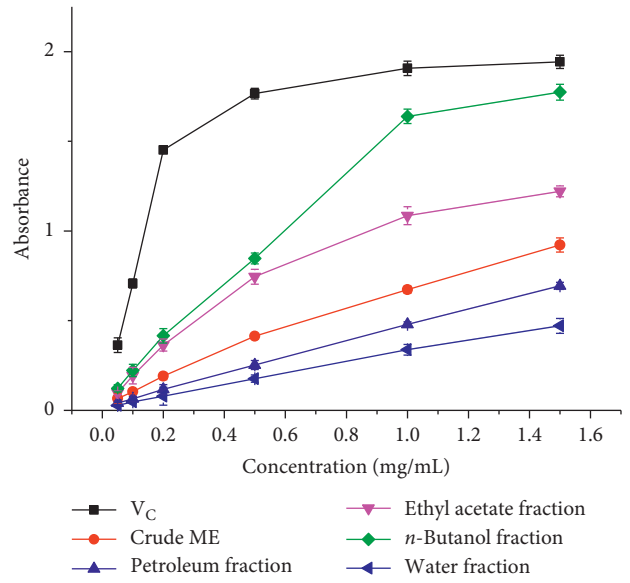

Figure 11: Reducing power of crude ME, V_C, and organic extraction fractions extracted from ME.

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

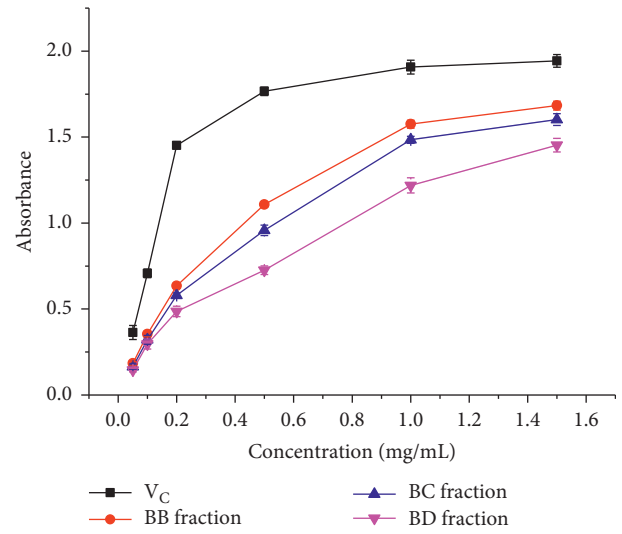

Figure 12: Reducing power of V_C, BB, BC, and BD fractions.

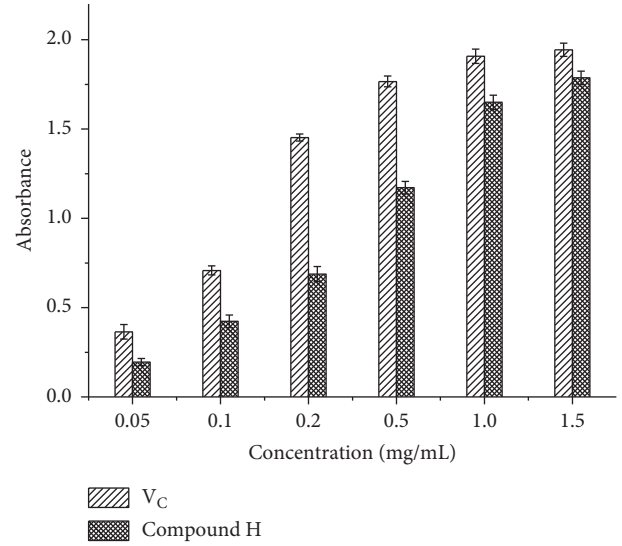

Figure 13: Reducing power of compound H and V_C.

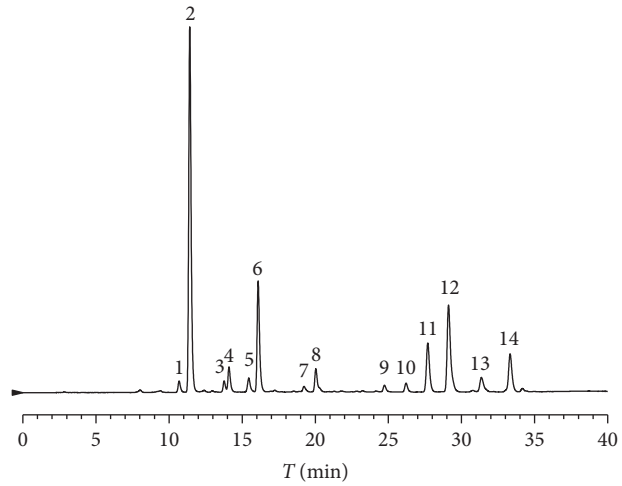

Figure 14: HPLC chromatogram profile of ME extracted by using the UE method.

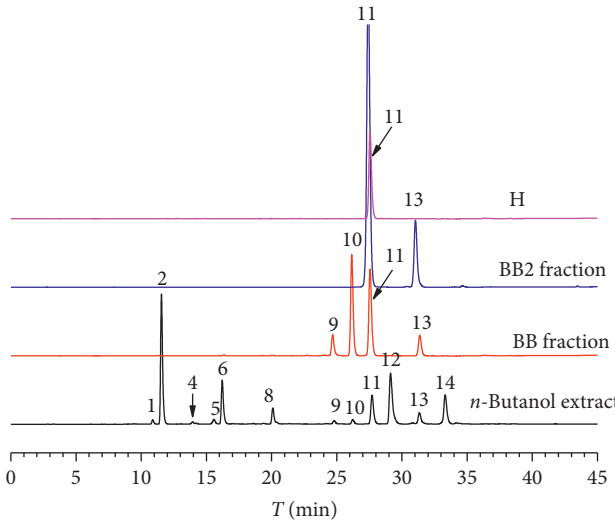

Figure 15: HPLC chromatogram profile of isolated fractions.

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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