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Research Article

Extraction, Enrichment, and Quantification of Main Antioxidant Aglycones of Flavonoids and Tannins from *Melastoma Dodecandrum* Lour.: Guided by UPLC-ESI-MS/MS

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The objective of this work was to determine the main antioxidant aglycones of flavonoids and tannins in antioxidant extracts from *Melastoma dodecandrum* Lour. (MD), based on its extraction and purification process optimization and component identification by UPLC-ESI-MS/MS. Firstly, the ultrasound-assisted extraction (UAE) process for antioxidants was established by using single factor tests and response surface optimization. Evaluating by DPPH radical scavenging assay, the antioxidant capacity of MD could reach 2742.27 ± 93.86 (μmol Trolox/g) under the optimized conditions including methanol concentration (61%, v/v), extraction time (45 min), liquid-solid ratio (0.46 ml/mg), and extraction temperature (25°C). Then, the antioxidants in the solution were enriched by using macroporous resins. Because of the highest adsorption capacity and desorption ratio, HPD 500 was selected out of 14 macroporous resins for further study based on the static adsorption and desorption tests. The adsorption mechanism of the HPD 500 resin presented that pseudo-first-order kinetics model and Freundlich isotherm model could adequately explain the adsorption process. After the antioxidants were dynamically saturated by HPD 500, the column was eluted with different concentrations of ethanol (0, 25, 50, 75, and 100%, v/v). Among the above five ethanol fractions, 50% ethanol fraction showed the strongest antioxidant activity and more than 95% antioxidants adsorbed in HPD500 could be eluted by 75% ethanol. Guided by analysis results of UPLC-ESI-MS/MS, main antioxidant aglycones of flavonoids and tannins in each ethanol elution fraction were quantitatively detected. Finally, according to the correlation analysis between the antioxidant capacity of each ethanol elution fraction and its content of 10 main flavonoids and phenolic acids, the antioxidant activities of MD could mainly contribute to tannins containing phenolic units such as ellagic acid and/or gallic acid. The results of this work would provide useful information for the production of antioxidants from MD. Thus, the analysis methods could also be a quality control tool for material or products related with MD.

1. Introduction

Melastoma dodecandrum Lour. (MD), an important source material for food and pharmacological industrial in southern China, exhibits a variety of biological activities, such as anti-inflammatory [1], hypoglycemic [2], and hypolipidemic effects [3]. It can also be used to prevent and treat gastrointestinal diseases [4]. MD is rich in flavonoids and tannins with high-antioxidant activity. In our previous study, an ellagitannin named casuarinin was isolated from MD and was found to have significant anti-inflammatory activity [5]. Under physiological conditions in vivo, ellagitannins can be hydrolyzed to ellagic acid by intestinal microbes and then further be metabolized into urolithin A [6],

a metabolite that can significantly enhance gut barrier function through the Nrf2 pathway according to a research in 2019 [7]. This innovative research has attracted great attention [8–10]. In addition, many reports have revealed the antioxidant [11], anti-inflammatory [12], and hypoglycemic activities [13] of flavonoids which are also found to be abundant in MD. Thus, it can be seen the biological activities of MD may be related with its antioxidants including flavonoids and tannins.

However, there is little research on extraction and purification process of antioxidants from MD, which is not conducive to its development and utilization. Furthermore, the reports on detail chemical composition of antioxidants from MD are also very scare. With the aim to solve above problem, the influence of methanol concentration, extraction time, liquid-solid ratio, and extraction temperature on extraction yield of antioxidants from MD was firstly investigated, and an extraction process of antioxidants from MD was established. In addition, following the enrichment of antioxidants from MD by macroporous resins and guided by the analysis result of UPLC-ESI-MS/MS, main aglycones of major flavonoids and tannins from MD were quantified by HPLC-UV, and their contributions to antioxidant capacity of MD were calculated.

2. Materials and Methods

2.1. Materials. The fresh aerial parts of *Melastoma dodecandrum* Lour. were obtained from Lishui city, Zhejiang province of China, in September 2017 and identified by Professor Ping Wang (cxb20170906033). The materials were dried in an air-circulating oven at 40°C and then ground to sift through a 50-mesh screen stencil, and about 2 kg of powder were collected and kept in a 4°C refrigerator for the following experiments.

2.2. Chemicals. The reference substances including *p*-coumaric acid, gallic acid, ellagic acid, apigenin, naringenin, luteolin, kaempferol, quercetin, vitexin, and isovitexin were obtained from Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). Their purity was all greater than 98% (w/w). Trolox and 1,1-diphenyl-2-picrylhydrazyl radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), 2.2′-azino-bis(3-ethylbenzenothiazoline6-sulfonic acid) (ABST), and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Yuanye Biological Technology Co., Ltd. (Shanghai, China). Acetonitrile and methanol (HPLC grade) were obtained from Tedia Company (USA). Deionized water was prepared by a Milli-Q plus purification system (Millipore, Billerica, MA, USA). All the other chemicals were reagent grade.

AB-8, ADS-17, D101, HPD100, HPD500, HPD600, HPD950, LSA-10, LSA-21, LX-11, LX-8, NKA-2, X-5, and XDA-8 macroporous resins were supplied by Shaanxi Lebo Biochemical Technology Co., Ltd. (Shaanxi, China), and the detail physical characteristics of these fourteen resins are shown in Table 1. Their moisture contents in Table 1 were determined by drying at 105°C until constant weight. Before using, they were firstly shaken for 24 hours by soaking in 95% ethanol and then washed with deionized water thoroughly.

2.3. Tests for Antioxidant Activity of MD. The antioxidant activity of MD extracts was evaluated by ferric-reducing antioxidant power (FRAP) assay, ABTS$\bullet^+$, and DPPH radical scavenging assay. By using Trolox (TE) as a reference standard, the antioxidant activity of MD was calculated and represented as μmol Trolox equivalents for dry sample (g).

2.3.1. FRAP Assay. It was carried out by Santosh with minor modifications [14]. Firstly, 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in HCL (40 mM), and $FeCl_3$,$6H_2O$ (20 mM) with the ratio of 10 : 1 : 1 were mixed to form FRAP reagent. Then, 0.4 mL of MD extracts were reacted with 3.5 mL of the FRAP reagent for 15 min under room temperature. The absorbance was recorded at 595 nm using a UV1800 spectrophotometer (Shimadzu, Kyoto, Japan).

2.3.2. DPPH Radical Scavenging Assay. The antioxidant capacities of MD extracts were evaluated by DPPH radical scavenging assay described by Maira with some modifications [15]. Firstly, 3.5 mL of 0.07 mM DPPH solution was mixed with 0.4 mL of extracts and then incubated in the dark at room temperature for 30 min. The absorbance at 517 nm was recorded by a UV1800 spectrophotometer (Shimadzu, Kyoto, Japan).

2.3.3. ABTS$\bullet^+$ Radical Scavenging Assay. The ABTS assay was performed following Jorge [16]. 7 mM ABTS solution was mixed with 150 mM potassium persulfate and kept in the dark at room temperature for 12–16 to get ABTS radical cation (ABTS$\bullet^+$). Then, the mixture was diluted with ethanol to obtain an absorbance of about 0.70 at 734 nm. After that, 400 μL of MD extracts was reacted with 4 mL of ABTS$\bullet^+$ solution and incubated at 30°C for 30 min. The absorbance at 734 nm was recorded by a spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Ultrasound-Assisted Extraction of Antioxidants in MD

2.4.1. Extraction Process. An ultrasonic extractor (DL-720J, Shanghai Zhixin Instrument Co., Ltd.) was used to extract antioxidants from MD. Firstly, about 0.1 g MD powder was weighted accurately and mixed with various volumes (10, 20, 30, 40, and 50 mL) of different concentrations of methanol aqueous solution (0, 25, 50, 75, and 100%, $V_{\text{methanol}}/V_{\text{water}}$), and then the mixtures were treated with different times (15, 30, 45, 60, and 75 min) at certain temperatures (25, 35, 45, and 55°C). After extraction, the samples were cooled to room temperature and centrifuged at 3500 rpm for 15 min. The suspensions were collected to evaluate their antioxidant capacities by DPPH radical scavenging assay (μmol TE/g).

Table 1: Physicochemical properties and the static adsorption and desorption parameters of antioxidants on fourteen resins.

Resins	Polarity	Structure	Particle size (mm)	Specific surface area (m^2/g)	Pore diameter (nm)	Moisture contents (%)	Adsorption capacity (μmol TE/g)	Desorption ratio (%)
AB-8	Weak-polar	Polystyrene	0.3–1.25	480–520	12–16	41.27	1552.41 ± 89.5	89.55 ± 4.82
ADS-17	Middle-polar	Acrylic acid	0.3–1.25	90–125	25–30	46.55	1989.47 ± 117.46	77.12 ± 4.59
D101	Nonpolar	Polystyrene	0.3–1.2	650–700	8.5–9	41.86	1718.29 ± 139.84	82.72 ± 4.02
HPD100	Nonpolar	Polystyrene	0.3–1.25	500–550	10–11	46.95	1938.47 ± 138.49	89.56 ± 0.69
HPD500	Strong-polar	Polystyrene	0.3–1.2	500–550	10–11	55.54	2132.45 ± 51.53	89.74 ± 2.49
HPD600	Strong-polar	Polystyrene	0.3–1.2	500–600	8	45.37	2063.16 ± 84.36	82.39 ± 5.92
HPD950	Nonpolar	Polystyrene	0.3–1.2	493	8	41.4	1843.88 ± 26.47	66.61 ± 3.98
LSA-10	Middle-polar	Methacrylic	0.3–1.2	500–540	8.4–9.4	49.02	1980.7 ± 82.9	84.05 ± 1.62
LSA-21	Nonpolar	Polystyrene	0.3–1.2	650–700	8.5–9	44.44	1388.96 ± 125.8	87.94 ± 4.35
LX-11	Nonpolar	Polystyrene	0.3–1.2	550	30.1	71.62	1699.18 ± 128.67	82.91 ± 4.35
LX-8	Nonpolar	Polystyrene	0.3–1.2	650–700	8.5–9	45.38	1837.91 ± 80.1	91.71 ± 2.15
NKA-2	Polar	Polystyrene	0.3–1.25	160–200	14–16	40.86	1864.74 ± 54.07	86.04 ± 4.21
X-5	Nonpolar	Polystyrene	0.3–1.25	500–600	29–30	26.14	1657.58 ± 34.01	84.42 ± 6.9
XDA-8	Middle-polar	Polystyrene	0.37–1.25	1000–1100	—	36.88	1601.24 ± 77.28	78.74 ± 4.51

Note. All the analytical results are expressed as mean ± SD ($n = 3$).

2.4.2. Box–Behnken Design (BBD) Optimization. Based on the single-factor experiments, response surface methodology (RSM) was further applied to optimize the ultrasound-assisted extraction conditions in this work by using Design-expert 11 software. Factors of methanol concentration (X_1), extraction time (X_2), and liquid-solid ratio (X_3) were carried out at three levels to check their influence on the antioxidant capacities of MD extraction solutions. A full quadratic equation formula was utilized to analyze the data from BBD as follows:

$$Y = \alpha_0 + \sum_{i=1}^{3} \alpha_i X_1 + \sum_{i=1}^{3} \alpha_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \alpha_{ij}^2 X_i X_j. \quad (1)$$

where Y and α_0 represent the response function and regression coefficients and α_i, α_{ii}, and α_{ij} are the linearity, square, and intercept and interaction terms, respectively.

2.5. Antioxidants Enrichment by Using Macroporous Resins

2.5.1. Preparation of Sample Solutions. Based on the optimized extraction conditions by BBD, the extracted solution of MD powder was dried in vacuum at 40°C by a rotary evaporator (Heidolph Instruments GmbH & Co. KG, Germany), then the dried extracts of MD were dissolved by distilled water, and the ratio of distilled water and MD powder was about 100 mL/g.

2.5.2. Screening of Macroporous Resins by Static Adsorption and Desorption Experiments. The fourteen fresh resins (equal to 0.2 g dry resin) were weighted and mixed with 30 mL of sample solutions, and then the flasks were shaken at 160 rpm at 25°C for 180 min in a thermostatic oscillator. After full adsorption, the antioxidant capacities of the filtrate solutions were determined by DPPH radical scavenging assay and represented as μmol Trolox equivalents for 1 mL of sample solution. In desorption experiments, the above fourteen fresh resins were washed with distilled water in triplicate and 10 mL of ethanol was added for desorption at 160 rpm at 25°C for 180 min. Next, the antioxidant capacities of ethanol solutions were analyzed. The adsorption capacity, desorption capacity, and desorption ratio were calculated using the following formulas:

$$Q_e = \frac{(C_0 - C_e) \times V_i}{W},$$
$$Q_d = \frac{C_d \times V_d}{W}, \quad (2)$$
$$D = \frac{Q_d}{Q_e} \times 100\%,$$

where Q_e is the adsorption capacity at equilibrium (μmol TE/g dry resin), Q_d is the desorption capacity (μmol TE/g dry resin), D is the desorption ratio (%), C_0 and C_e are the initial and absorption equilibrium concentrations of antioxidant capacities of sample solutions (μmol TE/mL), V_i and V_d are the volume of the initial sample and desorption solution (mL), and W is the dry resin weight (g).

2.5.3. Adsorption Kinetics on Selected Resin. The fresh selected resin (equivalent to 2.0 g dry resin) mixed with 300 mL of sample solution was added to a flask, shaking on a thermostatic oscillator (160 rpm) at 25°C for 160 min. At each time points of 0, 2, 4, 6, 8, 12, 16, 20, 25, 30, 40, 50, 60, 70, 80, 100, 120, 140, and 160 min, 1 mL of solution was

obtained to determine its antioxidant capacity (μmol TE/mL) and the kinetic adsorption curve was plotted.

2.5.4. Adsorption Thermodynamics on Selected Resin. Thirty milliliters of sample solutions with various contents of antioxidants (11.62, 14.52, 17.42, 20.33, and 23.23 μmol TE/mL, respectively) was poured into the flasks which were loaded with hydrated resins (equal to 0.20 g dry resins). Then, the flasks were shaken at 160 rpm at 25, 30, and 35°C for 160 min. Subsequently, the antioxidant capacities in the filtrate solutions were determined.

2.5.5. Enrichment of Antioxidants. The selected resin was packed into a column with a bed volume (BV) of 3 mL, and then 70-fold BV of sample solution was loaded at a flow rate of 0.5 mL/min. Subsequently, the column was eluted with 5-fold BV of distilled water, 25% (v/v), 50% (v/v), 75% (v/v), and 100% (v/v) ethanol sequentially at 0.5 mL/min. After evaluating the antioxidant contents in each fraction, they were, respectively, concentrated in vacuum at 40°C and stored at −20°C with the names of MD-0%, MD-25%, MD-50%, MD-75%, and MD-100%.

2.6. UPLC-ESI-MS/MS Analysis. UPLC-ESI-MS/MS analysis was carried out on an ultra-high-performance liquid chromatography (Waters ACQUITY I Class) coupled with the Waters Xevo G2-XS-Q-TOF mass spectrometer. The phytochemicals were separated by Waters BEH C_{18} (2.1 mm $*$ 50 mm, 1.7 μm) at a flow rate of 0.43 mL/min at 40°C. The mobile phase was made up by acetonitrile (A) and 0.1% formic acid aqueous solution (B), using a linear gradient program: 0–4 min, from 2 to 5% (A); 4 to 12 min, 5 to 11% (A); 12 to 18 min, 11 to 17% (A); 18 to 28 min, 17 to 98% (A) and then followed by 98% (A) for 7 min. The ESI source was operated in the positive and negative modes with full scan mode from 50 to 1500 m/z, and the conditions were as follows: ion source temperature, 120°C; cone gas flow, 50 L/h; desolvation gas flow, 595 L/h; and capillary voltage, 2 kV.

2.7. Simultaneous Quantification of Main Flavonoids and Phenolic Acids in MD by HPLC-DAD. The extracts named MD-0%, MD-25%, MD-50%, MD-75%, and MD-100% were hydrolyzed with various volumes of 10% hydrochloric acid (HCl) aqueous solution at 80°C for 3 h, and DMSO solutions were added if the extracts could not be dissolved by 10% HCl. After the solutions were cooled to room temperature, they were filtered through 0.25 μm membrane filters and directly injected into an Agilent 1260 HPLC equipped with a diode array detector (DAD) (Agilent Technologies, USA). The column used in this work was an Agilent Zorbax Extend C_{18} reserved phase column (4 μm, 150 mm × 4.6 mm), the sample injection volume was 5 μl, the flow rate was 1.0 mL/min, the column temperature was 30°C, and the detection wavelength was set from 200–400 nm. The mobile phase was mixed with solvent A (methanol), solvent B (acetonitrile), and solvent D (0.1% formic acid aqueous solution). The gradient program is shown in Table 2.

Table 2: The gradient program for simultaneous quantification of main flavonoids and phenolic acids in MD.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent D (%)
0	5	2.5	92.5
6	18	2.5	79.5
12	21	2.5	76.5
12.5	23	2.5	74.5
18	23	2.5	74.5
19	39	2.5	58.5
23	39	2.5	58.5
24	47	2.5	50.5
30	41	2.5	56.5
31	50	2.5	47.5
40	50	2.5	47.5
45	97.5	2.5	0

3. Results and Discussion

3.1. Effect of the Extraction Conditions on the Yields of Antioxidants from MD. Experiments were carried out to investigate the influence of methanol concentration on extraction yield. As shown in Figure 1(a), the yield of antioxidants gradually increased from 1997.68 ± 8.97 to 2492.23 ± 122.67 μmol TE/g as the methanol concentration was changed from 0 to 75% (v/v), while the other extraction parameters were set as follows: extraction temperature 25°C, extraction time 30 min, and liquid-solid ratio 0.3 mL/mg; but the yield of antioxidants significantly decreased to 1209.91 ± 77.45 μmol TE/g when the methanol concentration was 100%. This might be due to the low solubility of antioxidants including flavonoids and tannins in high-concentration methanol. In addition, high-concentration of methanol might also cause protein denaturation and further prevent the dissolution of antioxidants [17].

The effect of extraction temperature variations (25, 35, 45, and 55°C) on the yields was inspected and is shown in Figure 1(b) as the other extraction conditions were as follows: methanol concentration 75%, extraction time 30 min, and liquid-solid ratio 0.3 mL/mg; as the temperature increased, the extraction yields gradually decreased and the maximal yield was 2688.55 ± 95.71 μmol TE/g at 25°C, which was close to room temperature. The result demonstrated that polyphenols such as tannins were not stable under high-temperature conditions, which was consistent with Makkar's report [18].

The influence of extraction time (15, 30, 45, 60, and 75 min) on the yields of antioxidants is displayed in Figure 1(c). These experiments were performed at 25°C with a 0.3 mL/mg liquid-solid ratio by using 75% aqueous methanol solution as extraction solvent. As seen from Figure 1(c), the extraction yield reached a maximal value of 2471.85 ± 74.75 μmol TE/g at 30 min.

By selecting 75% aqueous methanol solution as extraction solvent, experiments were conducted to determine the optimal liquid-solid ratio at 25°C for 30 min. As shown in Figure 1(d), the extraction yields gradually increased from 0.1 to 0.4 mL/mg and reached a maximal value of 2515.34 ± 86.2 μmol TE/g at 0.4 mL/mg; then, the yields were nearly constant from 0.4 to 0.5 mL/mg.

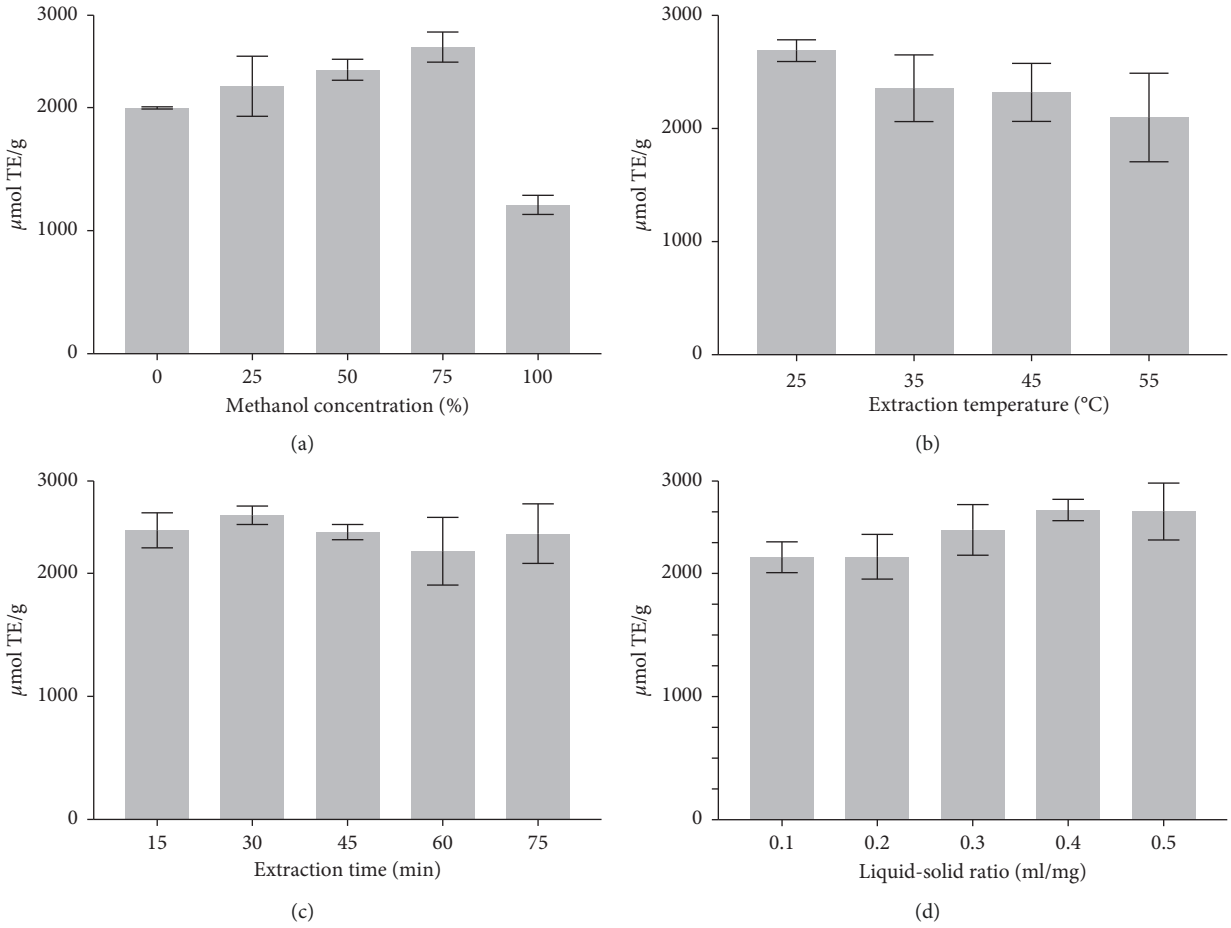


Figure 1: Influence of the methanol concentration (a), extraction temperature (b), extraction time (c), and liquid-solid ratio (d) on the extraction yield of antioxidant contents.

3.2. Optimization of the Extraction Parameters by Response Surface Methodology (RSM). Three individual factors, including extraction time, methanol concentration, and liquid-solid ratio, were further optimized by the response surface methodology by using Design-expert 11 software. 17 experiments were carried out by Box–Behnken design, and the results are listed in Table 3 [19, 20]. As displayed in Table 3, the extraction yields ranged from 1214.48 ± 211.17 to 2779.49 ± 291.99 μmol TE/g. Moreover, the analysis of variance (ANOVA) of the response surface model is displayed in Table 4. The P value was used to test the significance of a coefficient. Basically, a P value, smaller than 0.05, indicated that the model terms were significant. As shown in Table 4, the individual factor (X_1) and interaction coefficient (X_2X_3) could significantly influence the extraction yield because the P values were smaller than 0.05. The P value of the regression model was <0.0001, suggesting that the model was significant. The "Lack of fit P value" of $0.9453 > 0.05$ indicated that the model, which was chosen and shown as follows, was adequately accurate in predicting the relevant response:

$$\begin{aligned} Y = {} & 2570.47 - 560.49X1 + 65.52X2 - 4.68X3 \\ & - 120.35X1X2 - 30.65X1X3 + 154.50X2X3 \\ & - 607.92X12 - 86.23X22 - 141.46X32. \end{aligned} \quad (3)$$

In addition, the adjusted coefficient of determination ($R_{adj}^2 = 0.9441$) indicated that the model could explain 94.41% of the response value changes. This affirmed that the predicted model was highly significant and could sufficiently match the observed values.

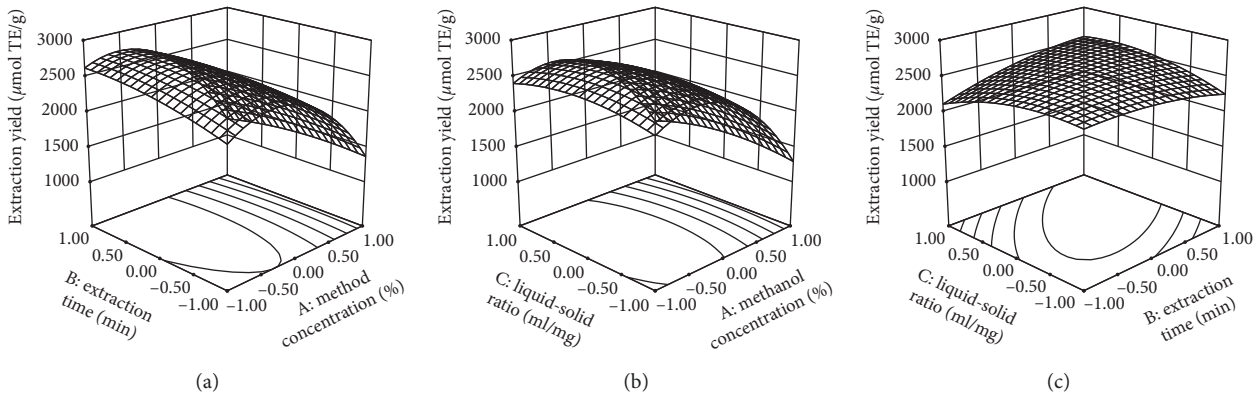
Three-dimensional response surface plots are displayed in Figure 2. From Figures 2(a) and 2(b), we could clearly observe that the extraction time and liquid-solid ratio had a significantly weaker effect on extraction yield than methanol concentration. However, higher yields could be gained by increasing the extraction time at high liquid-solid ratios (Figure 2(c)). The optimal conditions for antioxidants predicted by the response surface software were as follows: 60.75% methanol concentration, 45 min, 0.459 ml/mg, and 25°C. The final conditions were slightly modified: 61% methanol concentration, 45 min, 0.46 ml/mg, and 25°C. Under these conditions, the extraction yield of antioxidants was 2742.27 ± 93.86 μmol TE/g, which was not significantly different from the predicted value of 2789.81 μmol TE/g. The antioxidant capacities of sample solutions extracted under the optimized condition were further evaluated by ABTS$^{\bullet+}$ radical scavenging assay and FRAP assay. The values for these two assay methods were 2689.33 ± 75.46 and 1892.29 ± 100.14 μmol TE/g, respectively.

Table 3: Box–Behnken design and result of response surface method analysis.

Run	Methanol concentration (X_1) (%)	Extraction time (X_2) (min)	Liquid-solid ratio (X_3) (ml/mg)	Extraction yield (μmol TE/g)
1	75	30	0.4	2346.21 ± 225.54
2	100	30	0.3	1323.4 ± 181.8
3	50	30	0.5	2380.09 ± 28.83
4	75	30	0.4	2509.85 ± 72.34
5	75	45	0.3	2277.51 ± 155.14
6	100	45	0.4	1214.48 ± 211.17
7	100	30	0.5	1254.07 ± 96.16
8	75	30	0.4	2575.83 ± 113.58
9	75	45	0.5	2575.85 ± 110.16
10	75	15	0.3	2418.72 ± 15.54
11	75	30	0.4	2640.97 ± 429.23
12	50	45	0.4	2632.44 ± 128.83
13	100	15	0.4	1360.9 ± 144.1
14	50	30	0.3	2326.82 ± 55.19
15	75	30	0.4	2779.49 ± 291.99
16	50	15	0.4	2297.45 ± 225.81
17	75	15	0.5	2099.04 ± 89.07

Table 4: Variance analysis of regression equations.

Source	Sum of squares	df	Mean square	F value	P value	
Model	$4.46E+06$	9	$4.95E+05$	31.04	<0.0001	Significant
X_1	$2.51E+06$	1	$2.51E+06$	157.51	<0.0001	
X_2	34344.27	1	34344.27	2.15	0.1858	
X_3	174.84	1	174.84	0.011	0.9196	
X_1X_2	57938.9	1	57938.9	3.63	0.0984	
X_1X_3	3757.69	1	3757.69	0.24	0.6423	
X_2X_3	95487.18	1	95487.18	5.98	0.0443	
X_1^2	$1.56E+06$	1	$1.56E+06$	97.52	<0.0001	
X_2^2	31310.57	1	31310.57	1.96	0.204	
X_3^2	84252.09	1	84252.09	5.28	0.0552	
Residual	$1.12E+05$	7	15956.21			
Lack of fit	9037.78	3	3012.59	0.12	0.9453	Not significant
Pure error	$1.03E+05$	4	25663.92			
Cor total	$4.57E+06$	16				



(a) (b) (c)

Figure 2: Response surface (3D) showing the influence of different extraction parameters on extraction yield.

3.3. Static Adsorption and Desorption Parameters of Antioxidants from MD on Fourteen Resins. Static adsorption and desorption tests were carried out to screen the optimum resin. The antioxidant constituents in MD including flavonoids and tannins process abundant hydrogen groups, exhibiting higher polarity. As shown in Table 1, because of their similar polarity with the target compounds, most of resins with strong-polarity or middle-polarity, including

HPD500, HPD600, ADS-17, and LSA-10, had high adsorption capacities while nonpolar resins such as LSA-21 which displayed low adsorption capacities [21]. Considering adsorption and desorption abilities, HPD500 was selected for separation of antioxidants in MD.

3.4. Static Adsorption Kinetics of the Antioxidants from MD on HPD-500 Resin. The curves of the adsorption kinetics for HPD500 are displayed in Figure 3. As we see, the adsorption of antioxidants on HPD500 increased rapidly for first 30 minutes, especially for first 16 minutes. Then, the adsorption elevated slowly in the next 50 minutes and reached equilibrium.

To explain the adsorption process of antioxidants on HPD500, the pseudo-first-order (equation (4)), pseudo-second-order (equation (5)), and particle diffusion kinetics models (equation (6)) were adopted.

According to the correlation coefficients, both pseudo-first-order ($R^2 = 0.992$) and pseudo-second-order kinetics models ($R^2 = 0.9804$) could describe the adsorption antioxidants on HPD500:

$$\ln\left(Q_e - Q_t\right) = -k_1 \times t + \ln\left(Q_e\right), \tag{4}$$

$$Q_t = Q_e \times \left(1 - e^{-k_2 \times t}\right), \tag{5}$$

$$Q_t = k_t \times t^{0.5} + C. \tag{6}$$

where Q_t is the adsorption capacity (μmol TE/g) at time t, k_1, k_2, and k_t are the rate constants of pseudo-first-order, pseudo-second-order, and particle diffusion kinetics models in adsorption process, respectively, and C refers to the constant in the particle diffusion kinetics model.

3.5. Static Thermodynamics of Antioxidants on HPD-500 Resin. Langmuir and Freundlich models, two most common theoretical models, were used to fit equilibrated relationship between the concentrations of antioxidants in the fluid phase and HPD500 resin at a given temperature. These two equations are as follows:

$$Q_e = \frac{Q_0 K_L C_e}{1 + K_L C_e}, \tag{7}$$

$$Q_e = K_F C_e^{1/n}, \tag{8}$$

where Q_0 is the maximum adsorption capacity of HPD500 (μmol TE/g), K_L reflects the adsorption energy (L/μmol), K_F is the parameter related to the adsorption capacity of HPD500 ((μmol TE/g) (L/μmol TE)$^{1/n}$), and $1/n$ represents the adsorption intensity of HPD500.

As shown in Table 5, the correlation coefficients in Langmuir and Freundlich models were both higher than 0.9, indicating that both two models could characterize the isothermal adsorption processes of antioxidants on HPD500 resin and the adsorption processes may belong to monolayer coverage adsorption.

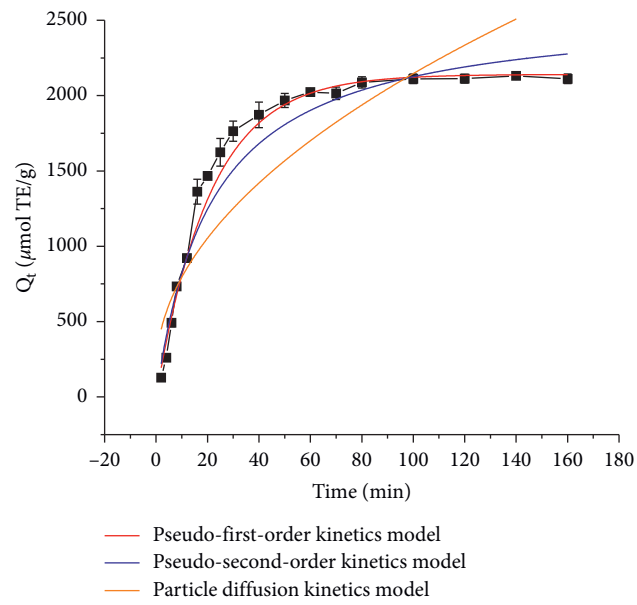


Figure 3: Adsorption kinetics of antioxidants on HPD500.

Thermodynamic parameters for the adsorption of antioxidants on HPD500 resin were further estimated to account for the adsorption process. The thermodynamic parameters, including enthalpy (ΔH), entropy (ΔS), and free energy (ΔG), were calculated according to the following equation:

$$\ln\left(K_L\right) = -\frac{\Delta G}{RT} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}, \tag{9}$$

where R is the gas constant (J/mol K) and T is the absolute temperature (°K).

As displayed in Table 5, the values of the free energy (ΔG) of adsorption were −3.461, −3.639, and −2.452 kJ/mol at 25, 30, and 35°C, correspondingly implying that the adsorption process was spontaneous and feasible. The enthalpy changes (ΔH) were also negative, suggesting that the adsorption process was exothermic, and low temperature was favorable to the adsorption process. Moreover, the absolute value of ΔH was less than 43 kJ/mol, showing that the adsorption of antioxidants in MD on HPD500 was controlled by physical mechanisms rather than chemical mechanisms.

3.6. Enrichment of Antioxidants from MD by HPD-500 Resin Column Chromatography. The antioxidant adsorbed on the resin was eluted with different concentrations of ethanol solution. As shown in Table 6, the contents of antioxidants in five fractions were in the order of MD-50% (1406.85 ± 45.37 μmol TE) > MD-25% (570.87 ± 23.94 μmol TE) > MD-75% (540.18 ± 17.66 μmol TE) > MD-100% (82.42 ± 4.09 μmol TE) > MD-0% (68.09 ± 2.74 μmol TE). About 52.72% antioxidants were eluted in the 50% ethanol fraction while only 3.09 and 2.55% antioxidants were in 100% and 0% ethanol fraction, respectively. The contents of antioxidants in the MD-25% and 75% fraction were similar, 21.39% and 20.24%, respectively.

Table 5: Langmuir model, Freundlich model, and thermodynamic parameters of the antioxidants in MD on HPD500 resin.

Temperature (°C/K)	Langmuir model			Freundlich model			Thermodynamic parameters		
	K_L	Q_m	R^2	K_F	n	R^2	ΔH	ΔS	ΔG
25/298.15	4.040	2137.717	0.940	1708.098	9.864	0.994			−3.461
30/303.15	4.235	2075.722	0.905	1651.770	9.613	0.996	−33.302	−0.099	−3.639
35/308.15	2.604	2037.162	0.9889	1580.325	9.993	0.953			−2.452

Table 6: The contents of antioxidants and ten main flavonoids and phenolic acids in five fractions from MD.

	Calibration curves	Test range (μg)	R^2	MD-0%	MD-25%	MD-50%	MD-75%	MD-100%
Gallic acid**	$y = 1603.6x + 0.4019$	0.0115–0.1842	0.9999	81.91 ± 2.46	120.69 ± 3.11	377.73 ± 5.49	126.55 ± 6.3	15.28 ± 1.06
Coumaric acid	$y = 781.24x + 1.7639$	0.0447–0.3579	0.9999	—	—	—	—	—
Vitexin	$y = 1632.6x + 6.0558$	0.0222–0.3553	0.9998	2.68 ± 0.38	18.92 ± 4.99	173.84 ± 8.16	174.79 ± 8.01	183.87 ± 0.72
Isovitexin	$y = 1468.4x + 1.3678$	0.0125–0.2000	0.9999	4.38 ± 1.59	9.79 ± 3.31	128.48 ± 9.6	227.49 ± 10.98	447.33 ± 4.22
Ellagic acid*	$y = 9669.8x + 26.979$	0.0392–0.6275	0.9999	192.92 ± 3.72	248.73 ± 4.87	1303.79 ± 9.42	300.53 ± 12.3	199.55 ± 3.73
Quercetin	$y = 3370.3x + 2.1296$	0.0107–0.1711	0.9999	—	—	16.67 ± 2.88	21.99 ± 2.25	55.64 ± 0.9
Naringenin	$y = 471.59x + 2.1251$	0.0429–0.6868	0.9998	—	—	—	—	—
Luteolin	$y = 4226.9x + 3.4346$	0.0103–0.1645	0.9999	—	—	—	—	5.27 ± 0.84
Kaempferol	$y = 2558.7x + 2.9206$	0.0184–0.2947	0.9999	—	—	—	22.93 ± 3.23	133.66 ± 1.08
Apigenin	$y = 2492.2x + 2.0216$	0.0087–0.1395	0.9999	—	—	—	—	80.31 ± 1.43
Trolox equivalent (μmol)				68.09 ± 2.74	570.87 ± 23.94	1406.85 ± 45.37	540.18 ± 17.66	82.42 ± 4.09

**Correlation coefficient of gallic acid vs antioxidants was 0.968 ($P = 0.007 < 0.01$); *Correlation coefficient of ellagic acid vs antioxidants was 0.929 ($P = 0.023 < 0.05$); —not detected.

3.7. Identification of Chemical Constituents of Antioxidants from MD. MD solutions used in UPLC-ESI-MS/MS experiments were prepared under the optimized conditions as shown in Section 3.2. According to a recent paper about the chemical characterization of MD using UPLC-ESI-Q-Exactive Focus-MS/MS [22], various chemical classes including tannins, flavonoids, fatty acids, triterpenoids, sterols, and others were reported. Many researches had presented that polyphenols including tannins and flavonoids had higher antioxidant activities by comparing with fatty acids, triterpenoids, and sterols [23–26], so this work mainly focused on tannins and flavonoids from MD. In general, 39 tannins and 16 flavonoids were tentatively identified and shown in Table S1 including their retention times (R_t), molecular formulas, detected accurate mass in negative mode, error in ppm, and the MS/MS fragment ions. The codes of above 54 compounds are marked in Figure 4, the base peak chromatogram in negative ionisation modes. The compounds were identified by comparing the observed MS data and MS/MS spectra in this work with those found in the literatures, especially the constituents from MD and its same botanical family. In addition, the following public databases were also reviewed: ChemSpider (https://www.chemspider.com), Spectral Database for Organic Compounds SDBS (http://sdbs.db.aist.go.jp), and *m/z* cloud (https://www.mzcloud.org).

3.7.1. Tannins. Tannins are the most abundant compounds in MD, and they could be eluted in shorter time under reversed-phase chromatographic conditions because they exhibited very strong polarity with many hydroxyl groups. In this work, 39 tannins had been identified from MD. In MS^2 spectra of tannins, ions at *m/z* 169.0135 and/or *m/z* 301.0004 could be commonly detected, implying the presence of galloyl and/or hexahydroxydiphenolic acid (HHDP) in tannins. This result was further confirmed by the mass loss of 302 or 152 Da in MS^2 spectra, due to the loss of the HHDP and/or galloyl units [27–30]. Figure 5 shows the characteristic fragmentation pathway of a tannins (A23 in Figure 4.), an isomer mono-O-ellagic-tri-O-galloyl glucoside.

3.7.2. Flavonoids. Another kind of main antioxidant compounds from MD were flavonoids, and their characterization of fragmentation pathways have been revealed and recognized as the typical retro-Diels–Alder fissions [31]. In this work, 15 flavonoid O-glycosides were determined from MD and their aglycons were considered as apigenin, naringenin, luteolin, kaempferol, or quercetin, due to the observation of ions at *m/z* 151 and their $[M\text{-}H]^-$ ions [32, 33]. According to our previous research [5], flavonoid C-glycosides, such as vitexin and isovitexin, were the most abundant flavonoids in MD, and their structures were identified based on comparison of MS^2 spectra obtained in this work and fragmentation pattern characteristic reported in the literatures [22, 34–37].

3.8. Simultaneous Determination of Major Flavonoids and Phenolic Acids in Antioxidants in MD. Based on the result of UPLC-ESI-MS/MS, ten phenolic units including *p*-coumaric acid, gallic acid, ellagic acid, apigenin, naringenin, luteolin,

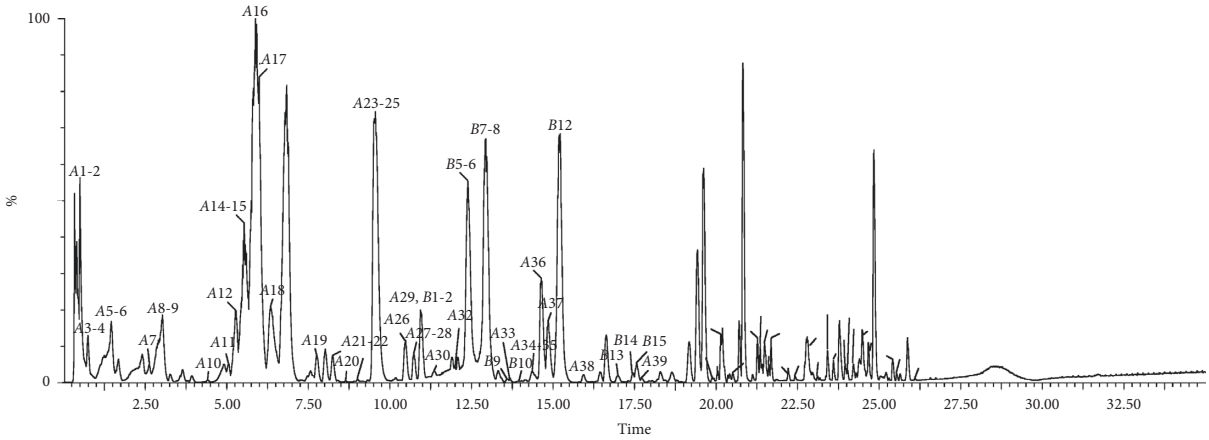


Figure 4: LC-MS chromatograms of extracts from MD in negative-ion mode.

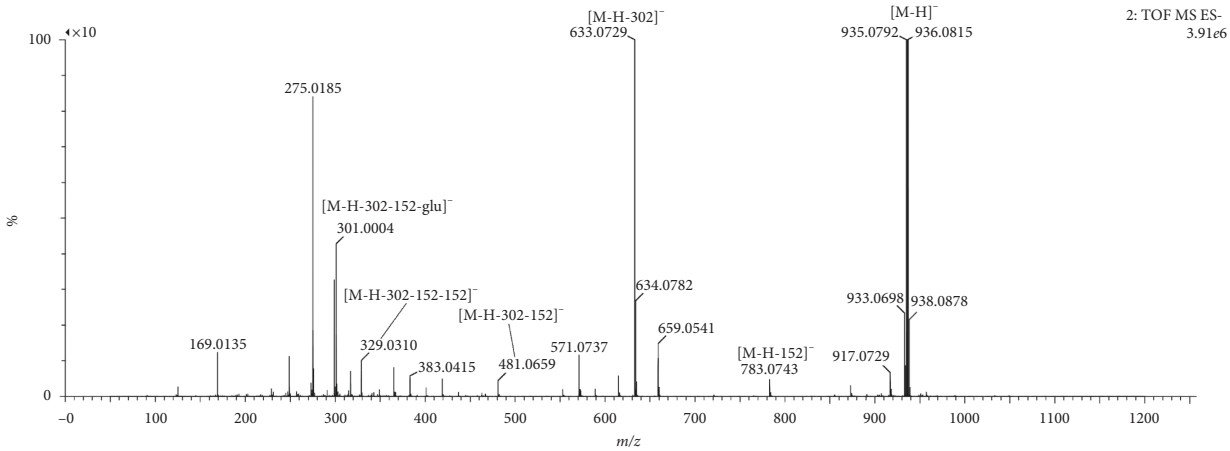


Figure 5: The MS^2 spectra and fragmentation pathway of mono-O-ellagic-tri-O-galloyl glucoside.

kaempferol, quercetin, vitexin, and isovitexin were identified. To further confirm the relationship between these 10 phenolic units and antioxidant capacity of MD, the contents of above 10 phenolic units were quantified. In addition to ellagic acid which was dissolved in DMSO, all standards were dissolved in methanol and kept as stock solutions. The above ten stock solutions were mixed in proportion and then diluted to five appropriate concentrations for the construction of calibration curves. Each concentration of solution was injected in triplicate. As demonstrated in Table 6, all calibration curves between the peak areas and the concentration of each standard showed good linear regression ($R^2 > 0.999$) within test ranges. The contents of these 10 compounds in five fractions named MD-0%, MD-25%, MD-50%, MD-75%, and MD-100% were calculated based on the calibration curves.

As shown in Table 6 and Figure 6, gallic acid, vitexin, isovitexin, and ellagic acid could be detected in each fraction, while coumaric acid and naringenin could not be determined due to the low detection limit of HPLC-UV. Flavonoids were mainly concentrated in MD-75% and MD-100%, while the contents of gallic acid and ellagic acid in MD-50% could reach 377.73 ± 5.49 and $1303.79 \pm 9.42\,\mu g$, respectively, which were much higher than other fractions.

Correlation analysis of antioxidant activity versus the contents of ten phenolic molecules illustrated that antioxidant activities of MD fractions had a significant correlation with gallic acid ($r = 0.968$, $P = 0.007 < 0.01$) and ellagic acid ($r = 0.929$, $P = 0.023 < 0.05$). The following two factors may be relevant with the above result: (1) The contents of gallic acid and ellagic acid in the antioxidants absorbed onto HPD500 resin were significantly higher than other flavonoids, which could totally reach $722.16\,\mu g$ and $2245.22\,\mu g$, respectively. (2) More importantly, there was ortho-hydroxyl group in gallic acid and ellagic acid which were considered to be the most important structural feature for anti-DPPH free radicals [38, 39]. That is why the contents of vitexin and isovitexin were similar to gallic acid, but the contributions to antioxidant activities of MD fractions were less. Although flavonoids such as luteolin and quercetin also had an ortho-dihydroxy group, their activities markedly reduced due to glycosylation as displayed in Table S1 [40–42]. Based on the above results, the method of correlation analysis was a useful tool to discover

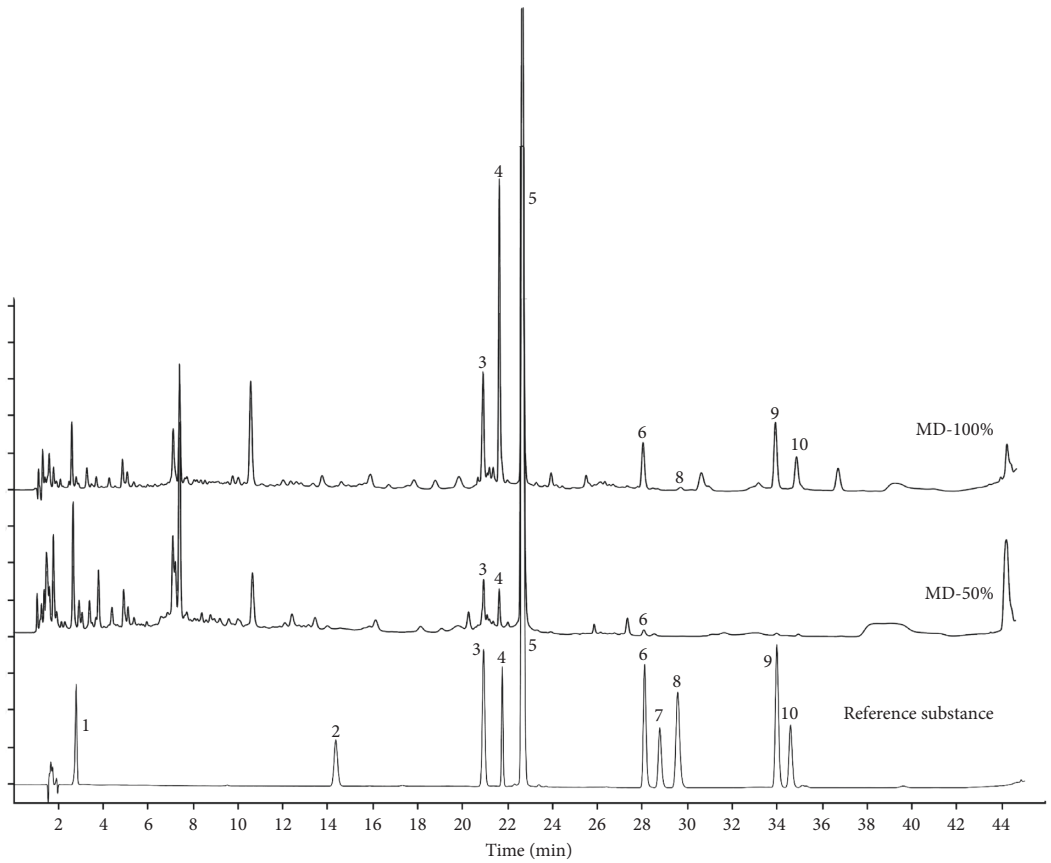


Figure 6: Representative HPLC chromatograms of standard solution and eluted fractions (1, gallic acid; 2, *P*-coumaric acid; 3, vitexin; 4, isovitexin; 5, ellagic acid; 6, quercetin; 7, naringin; 8, luteolin; 9, kaempferol; 10, apigenin).

the key antioxidant units in complex mixtures without isolation.

4. Conclusions

In summary, an efficient extraction and purification method was established to enrich antioxidants from MD by combining ultrasonic microwave-assisted extraction and macroporous resin chromatography. The optimal extraction conditions for microwave-assisted extraction were as follows: methanol concentration 61%, extraction time 45 min, liquid-solid ratio 0.46 ml/mg, and extraction temperature 25°C. Under these conditions, the extraction yield of antioxidants could reach 2742.27 ± 93.86 (DPPH assay), 2689.33 ± 75.46 (ABST assay), and $1892.29 \pm 100.14\,\mu$mol TE/g (FRAP assay), respectively. HPD 500 macroporous resin was adopted to purify the antioxidants in MD solution due to its effective adsorption and desorption. More than 95% antioxidants adsorbed in HPD500 could be eluted by 75% ethanol, and the 50% ethanol fraction showed the strongest antioxidant activity. Guided by UPLC-ESI-MS/MS, the contents of ten aglycones of flavonoids and tannins were detected by HPLC-DAD and the key antioxidant phenolic units in MD were found in gallic acid and ellagic acid by correlation analysis. The study provides helpful information for antioxidants production from MD and reveals a new way for the quality control of material or products related with MD. In addition, further research can be carried out to develop functional food or cosmetics using the antioxidants from MD.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Table S1: the tannins and flavonoids of *Melastoma dodecandrum* Lour. identified by UPLC-ESI-MS/MS in negative modes. (*Supplementary Materials*)

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