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Research Article

Flavonoids in Different Parts of *Lysimachia clethroides* Duby Extracted by Ionic Liquid: Analysis by HPLC and Antioxidant Activity Assay

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To establish methods for simultaneous determination of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers of *Lysimachia clethroides* Duby, respectively, the methods were ultrasound-assisted extraction combined with RP-HPLC, and ionic liquid was used as the extraction solvent. Meanwhile, the antioxidant activity of the different extracts of *L. clethroides* was evaluated. Purospher STAR RP-C_{18} column (4.6 mm × 250 mm, 5 μm) was used for analysis. The flow rate was 0.6 mL·min^{-1}, and the column temperature was 25°C. The detection wavelength was 360 nm. The mobile phases a and b consisted of acetonitrile-0.4% phosphoric acid (18 : 82, v/v), methanol (A), and 0.4% phosphoric acid (B), respectively. Linear ranges were 0.068~1.64, 0.060~1.44, 0.0080~0.19, and 0.0077~0.18 μg for isoquercitrin, astragalin, quercetin, and kaempferol, respectively. The average recoveries of the four constituents were 99.17%, 98.39%, 100.68%, and 98.81%, respectively. The antioxidant activity of the extracts was detected by DPPH, ABTS, and FRAP. Under the optimized conditions, all the test solutions showed a certain antioxidant activity and the ionic liquid extracts were better than that of extract of methanol. Ionic liquid used as the extraction solvent had the potential to extract active ingredients efficiently from *L. clethroides*, and this method improved the antioxidant activity with accurate and reliable results.

1. Introduction

Lysimachia clethroides Duby belongs to the Primulaceae family and is abundant and widely distributed in China. The root or the whole plant of *L. clethroides* was used as traditional Chinese herbal medicine with many health benefits in the treatment of edema, jaundice, dysentery, rheumatic fever, amenorrhea, bruises, fractures, traumatic bleeding, mastitis, boils, and snakebite [1]. Furthermore, its tender stems and leaves serve as popular vegetable in some areas [2]. Pharmacological research showed that *L. clethroides* had many pharmacological activities such as antitumor [3, 4], antioxidant [5], hypoglycemic [6], and hepatoprotective effect [7]. The main chemical components of *L. clethroides* were flavonoids, triterpenes, and organic acids [8–11]. The flavonoids are responsible for the bioactivity of *L. clethroides* [12, 13].

Ionic liquids (ILs) are solely composed of cations and anions as liquid near room temperature [14]. ILs own a large amount of excellent properties, including negligible vapour pressure, good thermal stability, wide liquid range, good dissolving, and high electric conductivity [15]. ILs are expected to be greener alternatives to traditional volatile organic solvents with the characteristic of being environment-friendly. ILs are promising solvents for extracting the active ingredients from traditional Chinese medicine, which are not only environment-friendly but also highly efficient [16, 17]. However, it unavoidably involved some organic solvents during the extraction. ILs in traditional Chinese medicine extraction are applied in the initial stage; it is necessary to conduct in-depth research.

Our group had reported the content of four flavonoids in *L. clethroides* [18], and on the basis of other studies [19–21],

the contents of isoquercitrin, astragalin, quercetin, and kaempferol in different parts of *L. clethroides* using high-performance liquid chromatography (HPLC) were compared and a rapid, effective, and environment-friendly ionic liquid-based ultrasonic-assisted approach was established to improve the extraction yield. In addition, the antioxidant activities of the different extracts of *L. clethroides* were measured in order to prove the efficient advantage of ionic liquid in vitro.

2. Materials and Methods

2.1. Reagents and Materials. Isoquercitrin, astragalin, quercetin, and kaempferol of purity greater than 98% were purchased from Chengdu Pufei De Biotech Co., Ltd. Acetonitrile used for HPLC was purchased from Avantor Performance Materials, Inc. (USA). Methanol used for HPLC was purchased from Tianjin Shield Fine Chemicals Co., Ltd. The water was Wahaha pure water.

1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF_4), 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6), 1-hexyl-3-methylimidazolium hexafluorophosphate ([HMIM]PF_6), and 1-butyl-3-methylimidazolium bromide ([BMIM]Br) were obtained from Merck KGaA.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Tokyo Chemical Industry Co., Ltd. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Fluka. 2,4,6-Tri(2-pyridinyl)-1,3,5-triazine (TPTZ), butylated hydroxytoluene (BHT), butyl-p-hydroxyanisole (BHA), and gallic acid propyl (PG) were obtained from Sigma. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical Co., Ltd.

Air-dried plant was collected from Huaxi District, Guizhou Province of China, in June 2014. The plant was identified as *L. clethroides* of Primulaceae family by Professor Changqin Li (Henan University, Kaifeng, China). The voucher specimens were deposited in the Institute of Natural Products, Henan University (number 20140612).

2.2. Preparation of Standard Solution. The four standard solutions of isoquercitrin, astragalin, quercetin, and kaempferol were prepared in methanol at a concentration of 1365, 1200, 160, and 154 $\mu g \cdot mL^{-1}$ and stored at 4°C, respectively. The standard working solutions were prepared by appropriate dilution of the four stock standard solutions with methanol to the required concentrations. Standard working solutions (a and b) were prepared by diluting isoquercitrin and astragalin and quercetin and kaempferol stock solutions at proper concentrations, respectively.

2.3. Preparation of Test Sample Solution. Leaves (0.50 g) and flowers (0.25 g) of *L. clethroides* were mixed with 5 mL of [BMIM]BF_4 methanol solution (0.5 $mol \cdot L^{-1}$) in glass vessel, respectively. The samples of leaves and flowers were extracted by ultrasonic-assisted extraction (20 and 45 min, 500 W) and then centrifuged (4000 and 5000 $r \cdot min^{-1}$, resp.). Then, the extract was filtrated through a 0.22 μm microporous membrane. The subsequent filtrate was the test sample solutions a and b (the optimum extraction solvent, concentration of selected extraction solvent, mesh, solid-liquid ratio, ultrasonic time and power, and centrifugation speed were systematically investigated in the study).

2.4. HPLC Analysis. LC-20AT high-performance liquid chromatography (Shimadzu) was equipped with a degasser, a quaternary gradient low pressure pump, the CTO-20A column oven, a SPD-M20A UV-detector, and a SIL-20A autosampler. The data were acquired and processed using LC-Solution chromatography data processing system.

The two chromatographic separations were all performed on a Purospher STAR RP-C18 column (4.6 mm × 250 mm, 5 μm) at a column temperature of 25°C. The flow rate of mobile phase was 0.6 $mL \cdot min^{-1}$. The UV detection wavelength was set at 360 nm. All injection volumes were 10 μL. Mobile phase a consists of acetonitrile-0.4% phosphoric acid (18 : 82, v/v). Mobile phase b consists of methanol (A) and 0.4% phosphoric acid (B), and the gradient program was as follows: 0~25 min, 52% A; 25~30 min, 52%~65% A; 30~46 min, 65% A. The HPLC chromatograms of the standard solution and the extract of sample were shown in Figure 1.

2.5. Experimental Procedure. The schematic plot of the experimental procedure was shown in Figure 2.

2.6. Antioxidant Activity In Vitro

2.6.1. Preparation of the Sample Solution. According to the optimized conditions, methanol and ionic liquid sample solutions were prepared and concentrated to 1 mL, respectively.

2.6.2. DPPH Assay. The sample was diluted into a range of concentrations with methanol. 10 μL of sample solution (or methanol) and 175 μL of DPPH methanol solution (200 μM) were added to 96-well microplate and mixed. The solution was measured at 515 nm using a Multiskan GO microplate reader after 20 min in the dark place, with PG, BHA, and BHT as positive controls. All the experiments were performed in triplicate. The percentage of radical scavenging rate was calculated using formula (1). IC_{50} value was calculated from the concentration-effect linear regression curve [22].

$$\text{DPPH radical scavenging rate (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100\%. \quad (1)$$

Note. A_0 was the absorbance of the DPPH itself; A_1 was the absorbance of sample and the positive control. IC_{50} represents the concentration of sample in $\mu g \cdot mL^{-1}$ which inhibits DPPH radical by 50%.

2.6.3. ABTS Assay. According to literature [23], the ABTS radical working solution was prepared. Then, the sample was diluted into a range of concentrations with methanol. 10 μL of sample solution (or methanol) and 200 μL of ABTS solution

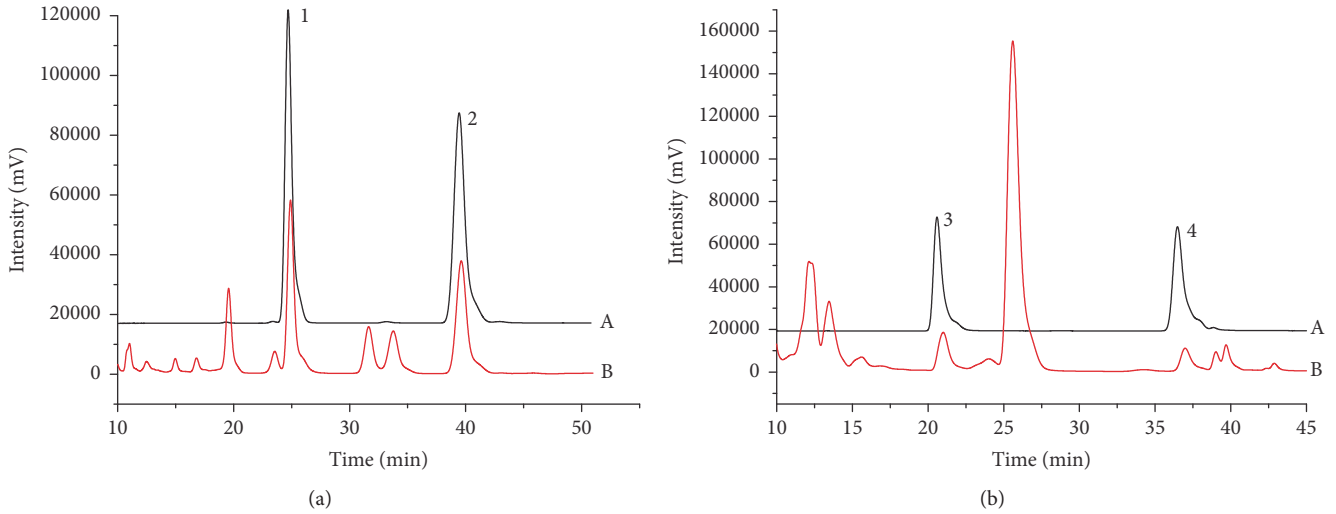

Figure 1: HPLC chromatograms of the standard solution (A) and the sample solution (B): (1) isoquercitrin, (2) astragalin, (3) quercetin, and (4) kaempferol.

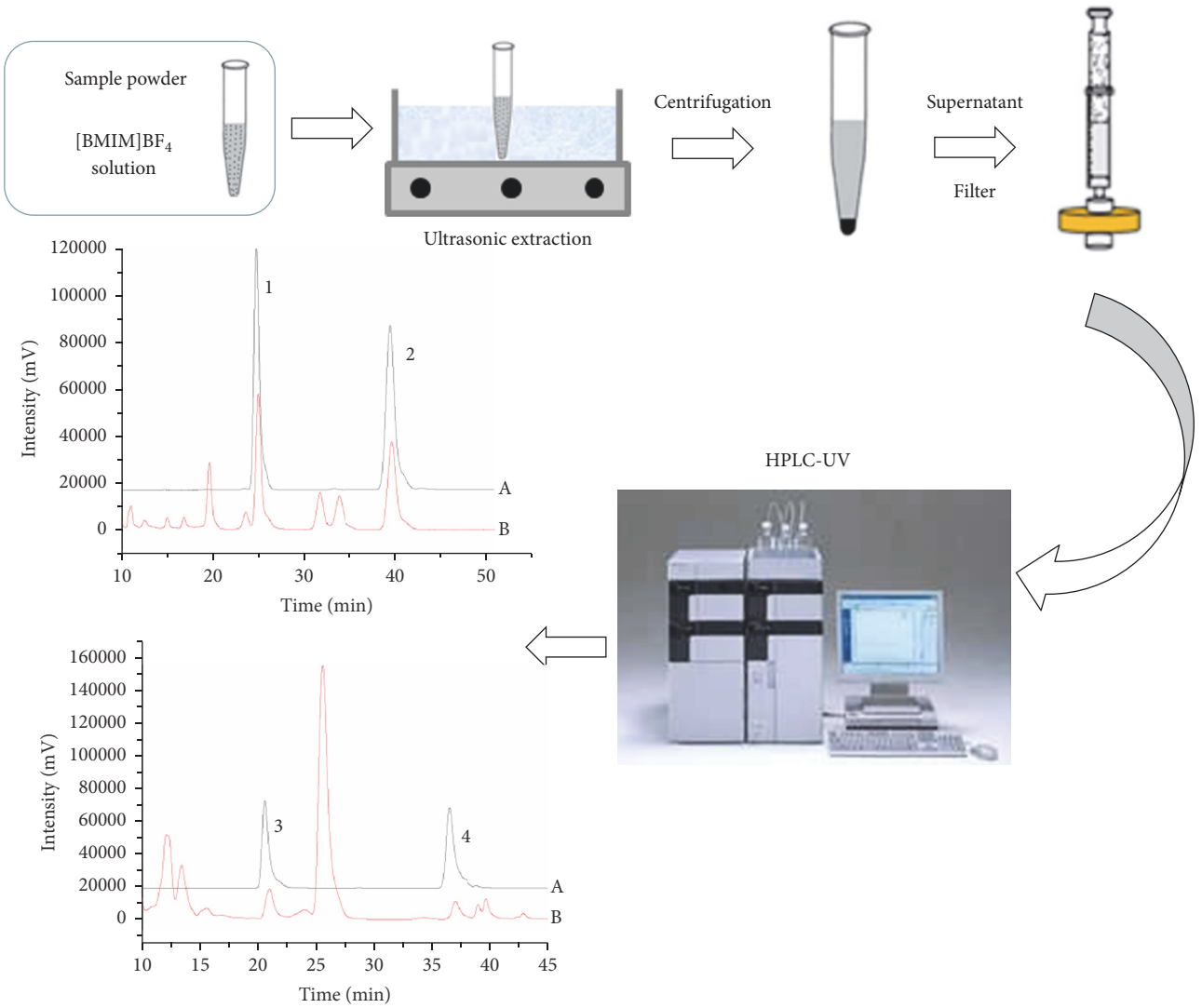

Figure 2: Schematic plot of the experimental procedure.

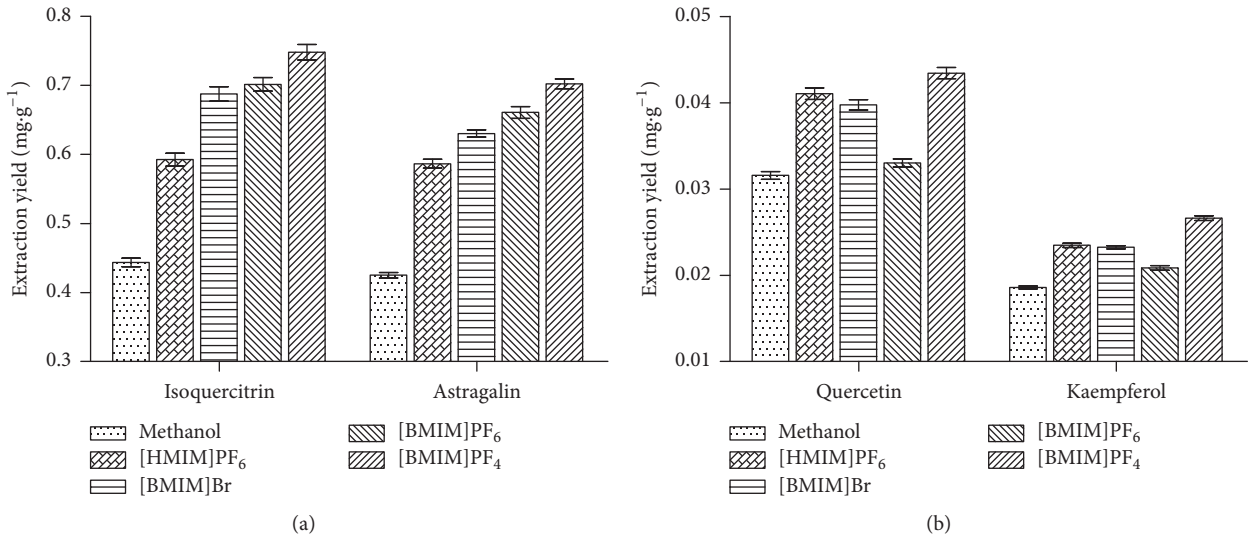

Figure 3: Effect of type of ILs on extraction yields. Concentration of the four ILs in methanol solution: 0.5 mol·L^{-1}; the particle size: 50 mesh; solid-liquid ratio: 1 : 10 g·mL^{-1}; ultrasound time: 30 min; ultrasound power: 500 W; centrifugation speed: 5000 r·min^{-1}.

were added to 96-well microplate and mixed. The solution was measured at 405 nm after 20 min in the dark place. All the experiments were performed in triplicate. Calculate the percentage of radical scavenging rate and IC_{50} value.

2.6.4. FRAP Assay. According to literature [24], the TPTZ working solution was prepared. Then, the sample was diluted into a range of concentrations with methanol. 10 μL of sample solution (or methanol) and 200 μL of TPTZ solution were added to 96-well microplate and mixed. The solution was measured at 593 nm after 20 min at 37°C. All reactions were carried out with three replications. Calculate the reducing ability of Fe^{3+} of the sample. The results were expressed in μmol Trolox equivalents (TE)/g sample. The standard curve was linear when the concentration of Trolox was between 50 and 1600 μM. Further dilution of the sample was needed if the FRAP value measured exceeded the linear range of the standard curve.

2.7. Statistical Analysis. All the grouped data were statistically evaluated with SPSS 19.0 software. All results are expressed as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Optimization of Extraction Conditions. The contents of isoquercitrin, astragalin, quercetin, and kaempferol in the roots, stems, leaves, and flowers of *L. clethroides* were determined according to the method of literature [18]. The results showed that the contents of the four flavonoids were different, and the contents of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were the highest, respectively. Therefore, the extraction conditions of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers of *L. clethroides* were chosen to optimize the conditions of extraction.

3.1.1. Type of ILs. The structure of IL has a significant effect on its physicochemical properties including solubility in water, the viscosity, and extraction capacity, which might have great impact on the extraction efficiency of target compounds [25]. In our study, four kinds of ILs were used as the extraction solvents, including water-soluble and water-insoluble types. In addition, methanol was selected as extraction solvent for evaluating the extraction yield compared with the developed method. In Figure 3, the addition of ILs to methanol obviously improved the extraction efficiency of the four flavonoid compounds compared with methanol as solvent. These might be due to the stronger multi-interactions between the analytes and ILs including hydrogen bonding and van der Waals interaction energy [26]. Among the four types of ILs, the extraction efficiency of [BMIM]BF_4 was the highest, but others did not show a certain regularity. The reason for the above phenomenon may be the fact that other ingredients in leaves and flowers had an influence on the extraction of target analytes. Therefore, [BMIM]BF_4 was chosen as the component of extraction solvent in the following studies.

3.1.2. [BMIM]BF_4 Concentration. The concentration effect of [BMIM]BF_4 on the extraction was shown in Figure 4. The extraction yields continued to rise when the concentration increased from 0.1 to 0.5 mol·L^{-1} and then different degrees of decrease appeared with the further increase of the concentration. This was because the solubility of the ionic liquid solution increased with the increasing concentration, but the diffusion and transfer capability of extraction solvent decreased, which led to declining of the solvent's penetration power into the interior of sample matrix [27, 28].

3.1.3. Particle Size. The effect of the particle sizes of sample on extraction efficiency was investigated. In Figure 5, the highest extraction yields of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were achieved at 24 and

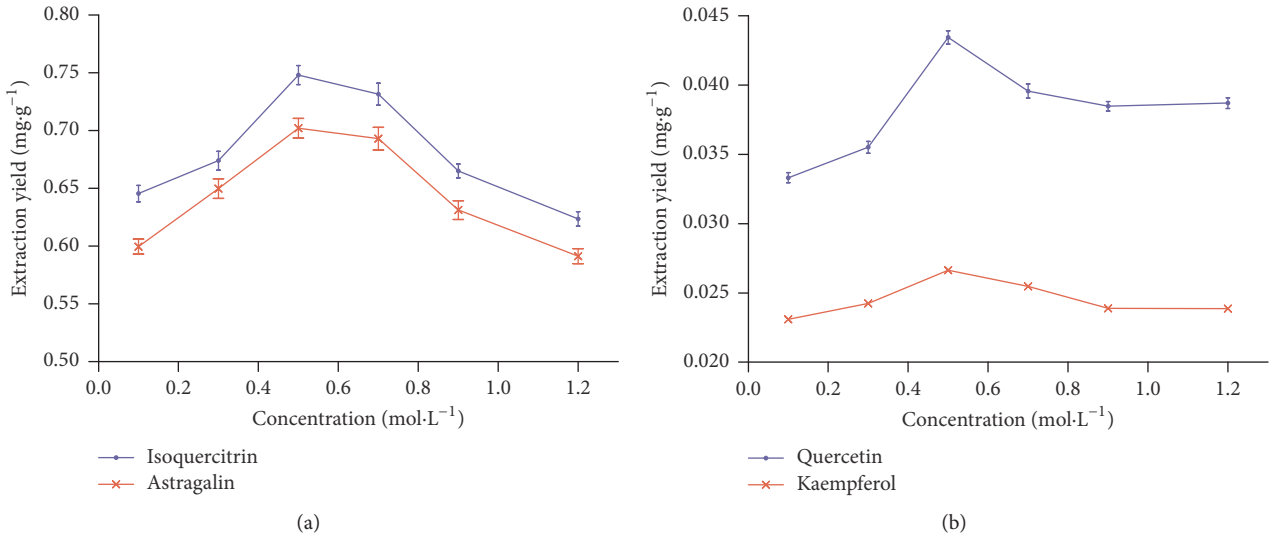

Figure 4: Effect of the concentration of IL on extraction yields. Type of IL: [BMIM]BF_4; the particle size: 50 mesh; solid-liquid ratio: 1 : 10 g·mL^{-1}; ultrasound time: 30 min; ultrasound power: 500 W; centrifugation speed: 5000 r·min^{-1}.

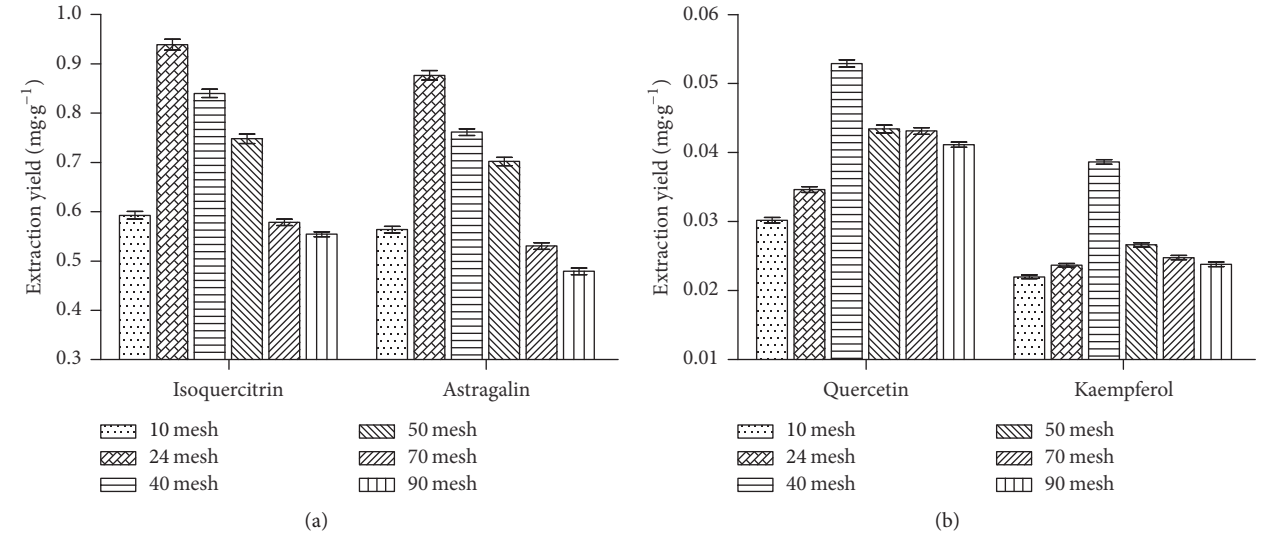

Figure 5: Effect of the particle size on extraction yields. Type of IL: [BMIM]BF_4; concentration: 0.5 mol·L^{-1}; solid-liquid ratio: 1 : 10 g·mL^{-1}; ultrasound time: 30 min; ultrasound power: 500 W; centrifugation speed: 5000 r·min^{-1}.

40 mesh, respectively. It might be the smaller the particle size, the easier the extraction, but the samples of leaves and flowers can easily be conglomerated by ionic liquid when the particle size was too small [29]. In addition, the different optimum particle sizes of leaves and flowers might be caused by the difference of organizational structures.

3.1.4. Solid-Liquid Ratio. Six different ratios of solid-liquid (1 : 5, 1 : 10, 1 : 20, 1 : 30, 1 : 50, and 1 : 80 g·mL^{-1}) were chosen to evaluate the effect of solid-liquid ratio on the extraction yield. The results in Figure 6 indicated that the maximum extraction rates of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were obtained at 1 : 10 and 1 : 20 g·mL^{-1}, respectively. Results showed that the target analytes in the sample had basically been extracted. When the proportion of extraction agent was increased, the extraction efficiencies were constant or decreased slightly; this finding might be related to the viscosity of ionic liquid.

3.1.5. Ultrasound Time. To investigate the influence of ultrasound time on the extraction efficiency, six time points ranging from 10 to 60 min were chosen. In Figure 7, the maximum extraction efficiencies of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were obtained at 20 and 45 min, respectively. Nevertheless, the ultrasound time was further extended; the extraction yields of target

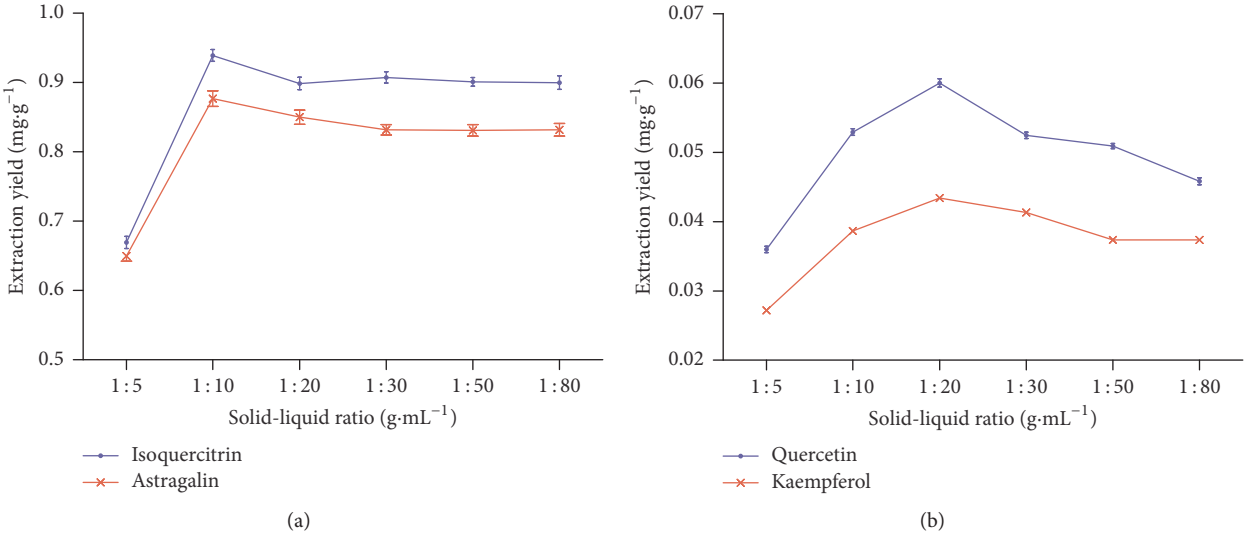

Figure 6: Effect of solid-liquid ratio on extraction yields. Type of IL: [BMIM]BF_4; concentration: 0.5 mol·L^{-1}; the particle size: 24 mesh (a) and 40 mesh (b); ultrasound time: 30 min; ultrasound power: 500 W; centrifugation speed: 5000 r·min^{-1}.

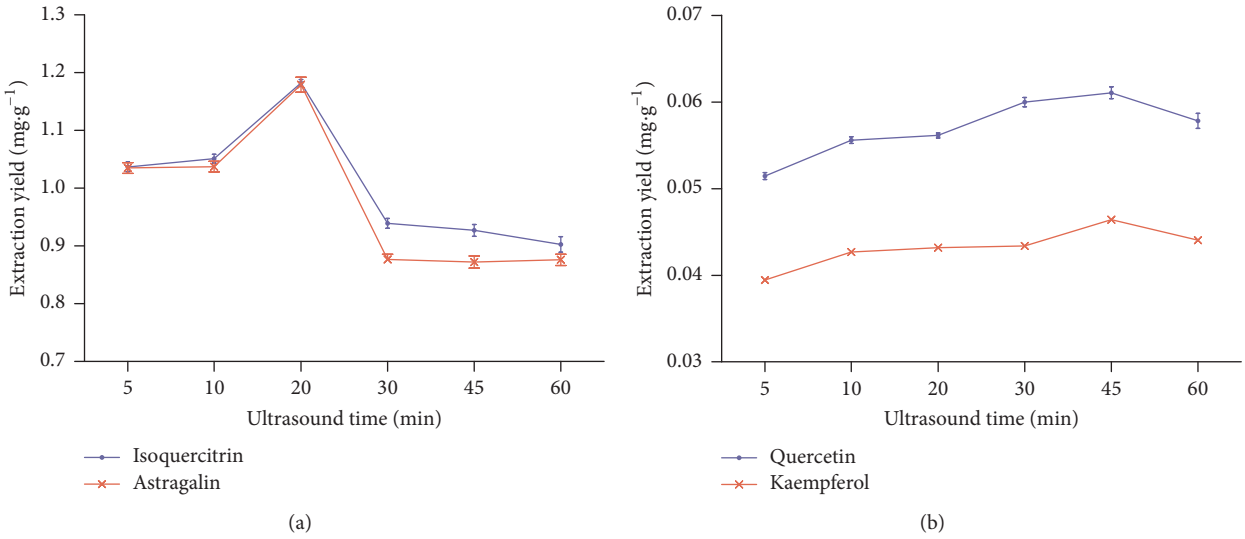

Figure 7: Effect of ultrasound time on extraction yields. Type of IL: [BMIM]BF_4; concentration: 0.5 mol·L^{-1}; the particle size: 24 mesh (a) and 40 mesh (b); solid-liquid ratio: 1 : 10 g·mL^{-1} (a) and 1 : 20 g·mL^{-1} (b); ultrasound power: 500 W; centrifugation speed: 5000 r·min^{-1}.

analytes had a certain decrease; the reason for the above phenomenon may be the fact that abundant sonication heat destroys the structure of the analytes [30].

3.1.6. Ultrasonic Power. The ultrasonic power had a significant effect on the extraction of the analytes. In our study, the effect of different ultrasonic powers on extraction efficiency was investigated from 200 to 500 W (in Figure 8). Results showed that the extraction efficiencies were increased with the increase of ultrasonic power. Therefore, 500 W was chosen as ultrasonic power in this study.

3.1.7. Centrifugation Speed. The influence of centrifugation speed on the extraction yield of the target analyte was investigated. The centrifugation time was set at 5 min, which was a reasonable time for productivity of the method [31]. In Figure 9, results showed that the maximum extraction yields of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were obtained at 4000 and 5000 r·min^{-1}, respectively. The reason might be that the higher centrifugal speed affects the solubility of the analytes, so that the solubility of the analytes is reduced.

3.2. Method Validation

3.2.1. System Suitability Test (SST). System suitability test (SST) of HPLC includes the theoretical plate number (n), the resolution (Rs), the tailing factor (T), and the repeatability.

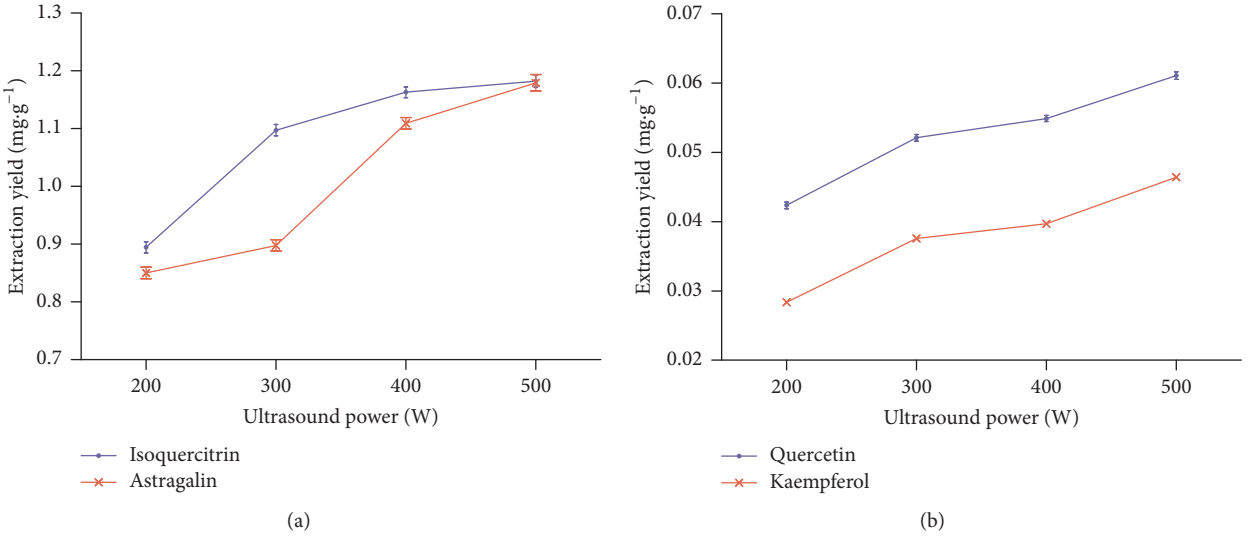

FIGURE 8: Effect of ultrasound power on extraction yields. Type of IL: [BMIM]BF_4; concentration: 0.5 mol·L^{-1}; the particle size: 24 mesh (a) and 40 mesh (b); solid-liquid ratio: 1 : 10 g·mL^{-1} (a) and 1 : 20 g·mL^{-1} (b); ultrasound time: 20 min (a) and 45 min (b); centrifugation speed: 5000 r·min^{-1}.

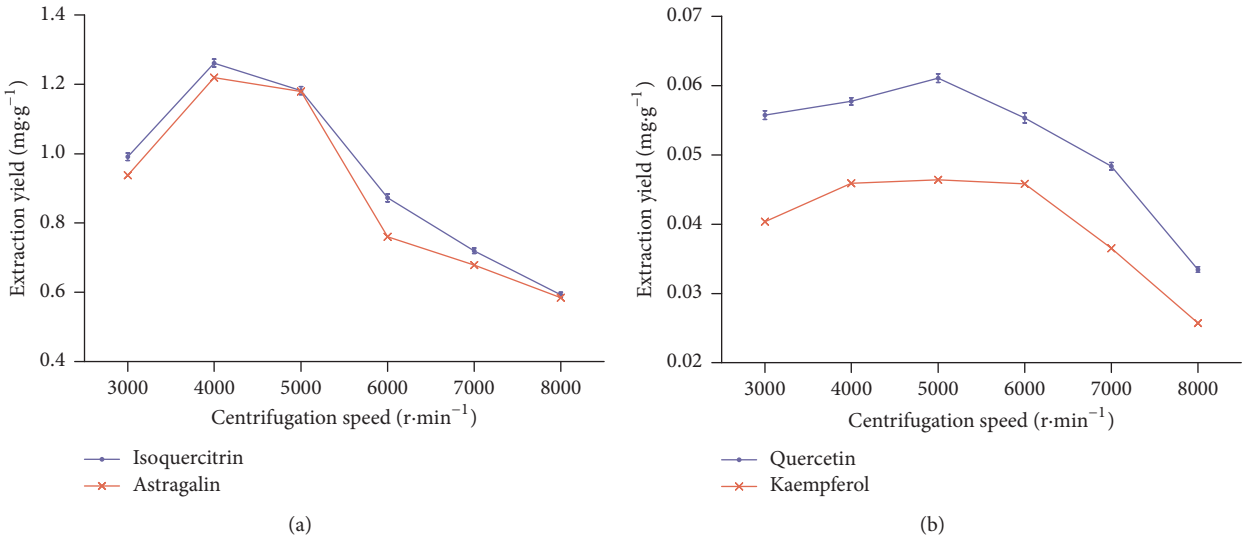

FIGURE 9: Effect of centrifugation speed on extraction yields. Type of IL: [BMIM]BF_4; concentration: 0.5 mol·L^{-1}; the particle size: 24 mesh (a) and 40 mesh (b); solid-liquid ratio: 1 : 10 g·mL^{-1} (a) and 1 : 20 g·mL^{-1} (b); ultrasound time: 20 min (a) and 45 min (b); ultrasound power: 500 W.

The repeatability was represented by the relative standard deviation of the peak area (RSD%). The results of SST were shown in Table 1, which shows that the chromatographic systems had good suitability.

3.2.2. Linearity. The stock standard solutions a and b were accurately injected 0.5, 1, 2, 4, 6, 8, 10, and 12 μL to chromatographic instrument for the construction of calibration curves, respectively. The calibration curves were constructed by plotting the peak areas versus the injection volume of each compound. The results were demonstrated in Table 2. r values were in the range from 0.9996 to 1.0000, which indicated that the methods had good linearity.

3.2.3. Precision. The stock standard solutions a and b were accurately injected 10 μL to chromatographic instrument, respectively. And they were analyzed in six replicates within one day for determining the precision of the developed assay. The RSDs of peak areas for isoquercitrin, astragalin, quercetin, and kaempferol were 0.46%, 0.83%, 0.23%, and 0.24%, respectively. The results indicated that the methods were precise for quantitative analysis of isoquercitrin, astragalin, quercetin, and kaempferol.

3.2.4. Stability. The sample solutions were prepared under the optimum extraction conditions and placed at room temperature and then were injected 10 μL to chromatographic

Table 1: System suitability test (SST) of HPLC.

Compound	Theoretical plate number	Resolution	Tailing factor	Repeatability
Isoquercitrin	8392	1.52	1.31	0.87
Astragalin	8834	10.95	1.18	0.64
Quercetin	7071	5.50	1.25	1.13
Kaempferol	13956	8.54	1.35	1.02

Table 2: Regression equations and linear ranges for four compounds.

Compound	Regressive equation	r	Linear range/μg
Isoquercitrin	$Y = 3.19 \times 10^6 X - 2.92 \times 10^4$	0.9996	0.068~1.64
Astragalin	$Y = 3.00 \times 10^6 X - 2.32 \times 10^4$	0.9997	0.060~1.44
Quercetin	$Y = 7.42 \times 10^6 X - 9.58 \times 10^3$	1.0000	0.0080~0.19
Kaempferol	$Y = 8.90 \times 10^6 X - 1.49 \times 10^4$	1.0000	0.0077~0.18

Y: peak area; X: injection amount (μg).

Table 3: Antioxidant activity of the different extracts of *L. clethroides*.

Sample	DPPH		ABTS		FRAP
	Radical scavenging rate/%	IC_{50}/μg·mL^{-1}	Radical scavenging rate/%	IC_{50}/μg·mL^{-1}	TEAC/μmol·g^{-1}
Leaf's methanol extract	93.91	1403 ± 80	97.01	144 ± 5	38.58 ± 0.72
Leaf's ionic liquid extract	88.48	638 ± 60	93.23	50 ± 2	105.79 ± 7.02
Flower's methanol extract	93.92	1004 ± 12	98.04	151 ± 1	50.69 ± 1.10
Flower's ionic liquid extract	89.77	460 ± 36	96.36	51 ± 2	105.83 ± 1.25
[BMIM]BF_4	−4.43	NT	0.03	NT	NT
BHA	76.00	15 ± 1.46	99.94	2.06 ± 0.08	5919.11 ± 14.10
BHT	73.06	12 ± 0.48	99.42	6.56 ± 0.10	589.20 ± 59.20
PG	95.61	4.01 ± 0.08	95.80	0.69 ± 0.05	9441.33 ± 69.27

Note. NT means not available because of low activity; BHA, BHT, and PG are positive controls.

instrument at 0, 3, 6, 9, 12, and 24 h, respectively. The RSDs of peak areas for isoquercitrin, astragalin, quercetin, and kaempferol were 0.67%, 0.83%, 1.65%, and 1.74%. The results indicated that the sample solution was basically stable at room temperature within 24 h.

3.2.5. Repeatability. Six test sample solutions a and b were processed under the optimum extraction conditions and then injected 10 μL to chromatographic instrument for analysis. The RSDs of peak areas for the four compounds were 2.03%, 1.84%, 1.15%, and 1.40%, indicating that the analytical methods had an acceptable level of repeatability.

3.2.6. Recovery. Nine batches of leaves and flowers samples of *L. clethroides* were prepared and divided into three groups, respectively. Then the four standard substances at three different amounts were added to the leaves and flowers samples. The spiked samples were prepared according to the optimum extraction conditions. All the calculated recovery values of the analytes ranged from 98.39 to 100.68% and the RSDs were less than 2.20%. The results demonstrated that the methods were reasonable and feasible.

3.3. Antioxidant Activity of the Different Extracts of L. clethroides In Vitro. The antioxidant activities of methanol and ionic liquid sample solutions were evaluated by the DPPH, ABTS, and FRAP assay comprehensively, and the results were shown in Table 3. Results showed that the ionic liquid extract had stronger antioxidant activity than that of the methanol extract, and the ionic liquid itself had no antioxidant activity. Results indicated that the ionic liquid used as extraction solvent had more advantages for extracting ingredients.

3.4. Sample Analysis. Ionic liquid was successfully applied for the determination of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers of *L. clethroides* by the optimized conditions, respectively. The mass fractions of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers were 1.262, 1.219, 0.061, and 0.046 mg·g^{-1} ($n = 3$), respectively.

4. Conclusion

The method ionic liquid-based ultrasonic-assisted extraction had been developed for the extraction of isoquercitrin, astragalin in leaves, quercetin, and kaempferol in flowers of *L. clethroides*. Compared with the conventional extraction with methanol, the proposed approaches provided higher extraction efficiencies. Results demonstrated that the methods

were simple, rapid, green, and effective. ILs can be recycled by some methods, such as vacuum distillation, membrane filtration, salting out, and liquid-liquid extraction [32]. Considering the unique properties of ILs, the methods have a great promising prospect in sample preparation of Chinese herbal medicine.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Wen-yi Kang and Jin-feng Wei conceived the research idea. Zhi-juan Zhang and Li-li Cui conducted the experiments, collected the plant specimens, analyzed and interpreted the data, and prepared the first draft. Wen-yi Kang, Zhi-juan Zhang, and Jin-feng Wei critically read and revised the paper. All the authors read and approved the paper before its final submission.

Acknowledgments

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