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Research Article

Separation and purification of five phenylpropanoid glycosides from *Lamiophlomis rotata* (Benth.) Kudo by a macroporous resin column combined with high-speed counter-current chromatography

Five phenylethanoid glycosides (PhGs), forsythoside B, verbascoside, alyssonoside, isoverbascoside, and leucosceptoside B, were isolated and purified from *Lamiophlomis rotata* (Benth.) Kudo by high-speed counter-current chromatography (HSCCC) combined with macroporous resin (MR) column separation. In the present study, the two-phase solvent system composed of ethyl acetate/*n*-butanol/water (13:3:10, v/v/v) was used for HSCCC separation. A total of 27 mg of forsythoside B, 41 mg of verbascoside, 29 mg of alyssonoside, 23 mg of isoverbascoside, and 13 mg of leucosceptoside B with purities of 97.7, 99.2, 99.5, 99.3, and 97.3%, respectively, were obtained in a one-step separation within 4 h from 150 mg of crude extract. The recoveries of the five PhGs after MR-HSCCC separation were 74.5, 76.5, 72.5, 76.4, and 77.0%, respectively. The chemical structures of all five compounds were identified by ^{1}H and ^{13}C NMR spectroscopy.

Keywords: HSCCC / *Lamiophlomis rotata* (Benth.) Kudo / Macroporous resins / Phenylpropanoid glycosides / Traditional Chinese medicines
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1 Introduction

The genus *Lamiophlomis* (family Lamiacea), distributed mainly in the Qinghai-Tibet Plateau in northwest of China, comprises only one species, *L. rotata* (Benth.) Kudo. *L. rotata* (Benth.) Kudo, as a well-known traditional Chinese medicine, has been widely used to relieve pain and tumescence, promote blood circulation, subdue swelling, and remove blood stasis [1]. Previous phytochemical and pharmacological studies have demonstrated that iridoid glucosides, phenylethanoid glycosides (PhGs), and flavonoids are the main components of the plant [2–7]. Among these constituents, five PhGs, forsythoside B, verbascoside, alyssonoside, isoverbascoside, and leucosceptoside B, have recently attracted much attention due to their pharmacological properties; these properties include anti-inflammatory [8], antioxidant [9,10], antifungal [11], antibacterial [12], antiviral [13], antihyperalgesic [14], antinociceptive [15], and neuroprotective activities [16].

In view of their wide pharmacological activities, large quantities of pure compounds are urgently needed as chemical reference standards and for further pharmacological studies and clinical applications. Therefore, effective methods for the isolation and purification of PhGs from natural sources are necessary. The preparative separation and purification of PhGs by traditional methods including preparative HPLC [17, 18], silica-gel column chromatography [19, 20], and gel filtration chromatography [21] are time consuming, require high amounts of organic solvents, and typically require numerous chromatographic steps resulting lower recovery and higher cost.

High-speed counter-current chromatography (HSCCC), a support-free liquid–liquid partition chromatographic technique based on the partitioning of compounds between two immiscible liquid phases without a support matrix, eliminates irreversible adsorption in the solid support and has been widely used in the preparative separation of natural products. Macroporous resin (MR), as the adsorption material for column chromatography, has been widely applied in combination with HSCCC for preseparation due to its low cost, high efficiency, easy recycling, and simple scaling-up performance.

To date, a small number of studies about the separation of phenylpropanoid glycosides by HSCCC have been published [22–29], and no report has been published on the use of HSCCC for the separations of PhGs from *L. rotata* (Benth.) Kudo. The present paper describes the successful

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Abbreviations: **HSCCC**, high-speed counter-current chromatography; **MR**, macroporous resin; **PhGs**, phenylethanoid glycosides

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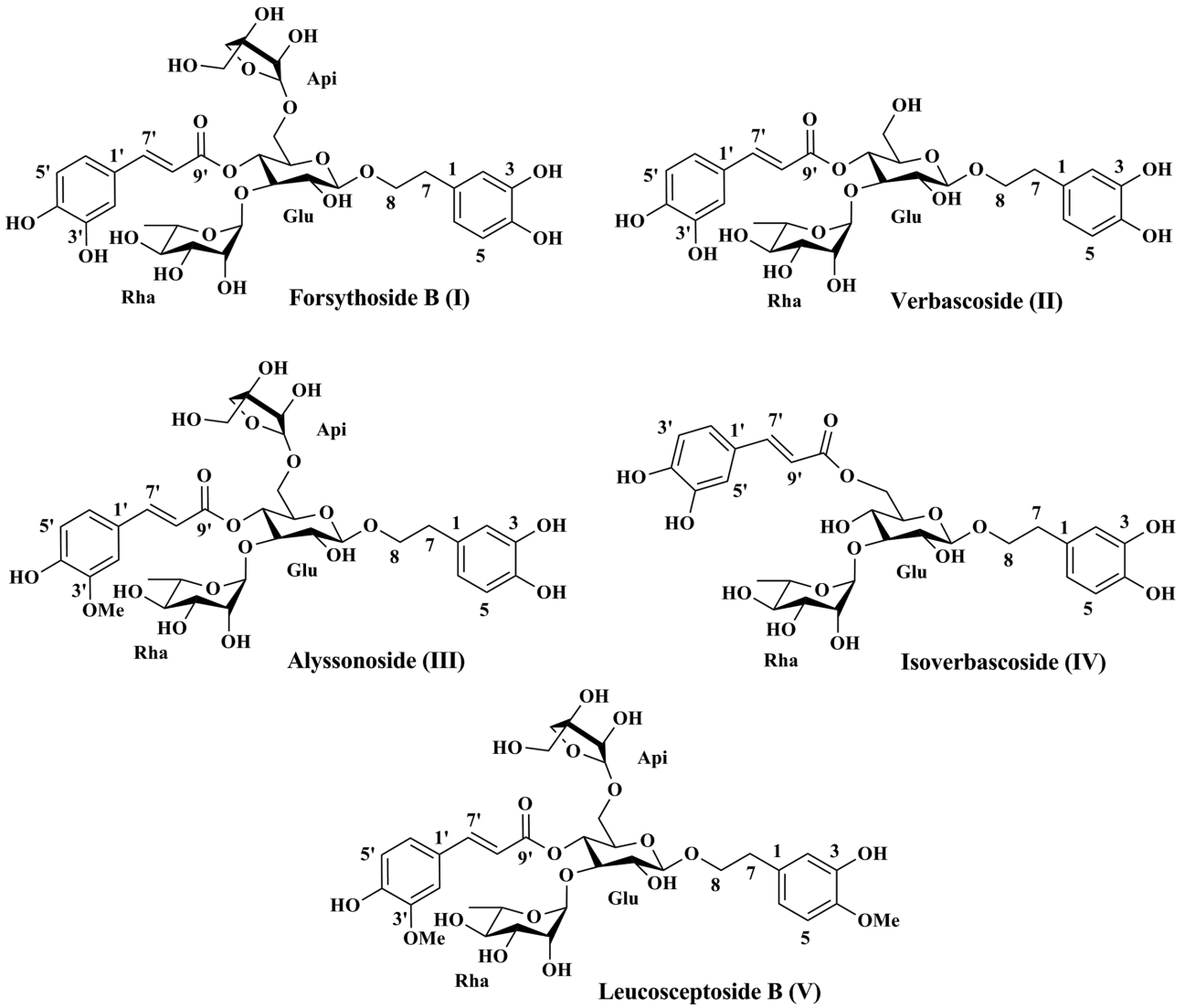

Figure 1. The chemical structures of forsythoside B (I), verbascoside (II), alyssonoside (III), isoverbascoside (IV), and leucosceptoside B (V).

preparative separation and purification of five PhGs, forsythoside B, verbascoside, alyssonoside, isoverbascoside, and leucosceptoside B, from *L. rotata* (Benth.) Kudo by HSCCC. To the best of our knowledge, this is the first report of the isolation of forsythoside B, alyssonoside, and leucosceptoside B from a herbal medicinal plant by HSCCC. The chemical structures of the five PhGs are shown in Fig. 1.

2 Materials and methods

2.1 Reagents and materials

L. rotata (Benth.) Kudo was collected from Yushu, Qinghai, China in September 2011, and identified by Professor Li-Juan Mei of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences. The voucher specimen (Mei 201109) was deposited in the herbarium of the Northwest Institute of Plateau Biology, Xining, Qinghai province.

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from the Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatographic grade and was purchased from Yuwang Chemical (Shandong, China). The water used was purified using a PAT-125 (Chengdu ultra Technology, Chengdu, China) laboratory ultrapure water system with a 0.4 μm filter.

2.2 Apparatus

The HSCCC instrument used in the present study was a TBE-300B HSCCC instrument (Shanghai Tauto Biotech, Shanghai, China) with three preparative polytetrafluoroethylene coils (tube diameter: 2.6 mm, total volume: 300 mL), and

a 20 mL sample loop. The revolution radius or the distance between the holder axis and the central axis of the centrifuge (R) was 5 cm. The β values of the multilayer coil ranged from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The HSCCC system was equipped with a model TBP-5002 constant-flow pump (Shanghai Tauto Biotech), a model UV500 detector (Shanghai Tauto Biotech) operating at 330 nm, and a model of N2010 workstation (Zhejiang University Star Information Technology, Hangzhou, China). The separation temperature was controlled by a DC-0506 constant-temperature circulating instrument (Shanghai Shunyuhengping Science Instruments, Shanghai, China).

The HPLC equipment was an Agilent 1200 system equipped with a G1354A solvent delivery unit, a G1315B UV/vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an Eclipse XDB–C_{18} 4 μm, 4.6 × 250 mm analytical column, and an Agilent HPLC workstation (Agilent Technologies, USA). The NMR spectrometer was a Mercury-600BB NMR (Varian, Palo Alto, CA, USA) with tetramethylsilane as the internal standard.

2.3 Extraction of the medicinal plant

The dried whole plant of *L. rotata* (Benth.) Kudo (4 kg) was extracted three times using 65% ethanol under reflux in a small extracting tank, each time for 2 h. The extracts were concentrated at 50°C under reduced pressure in a rotary evaporator. The concentrated extracts were not fully dried and suspended in distilled water. Then, the suspension was extracted with light petroleum (boiling point: 60–90°C, 9 L), ethyl acetate (9 L), and *n*-butanol (10 L), respectively [17]. The *n*-butanol solutions were evaporated to dryness under vacuum at 65°C to generate 400 g of *n*-butanol extract.

2.4 MR column chromatography

D101 MR is widely used in the separation of phenylpropanoid glycosides [29, 30], and thus in this study it was selected for the preseparation in order to enrich the targeted components. The extract of *n*-butanol (400 g) was dissolved in deionized water, loaded into a MR column (140 × 12 cm, containing 10 kg D101 MR), and eluted with various proportions of a mixture of water/ethanol (100:0, 95:5, 90:10, 70:30, 60:40, 40:60, and 20:80 v/v; about 15 L for each gradient). The water/ethanol (60:40) fraction was concentrated to produce 10 g of crude sample for subsequent HSCCC isolation and purification.

2.5 Measurement of partition coefficient

The partition coefficients were determined by HPLC as follows. About 3 mg of crude sample was added into a series of pre-equilibrated two-phase solvent systems (100 mL), and the solution was then fully shaken to reach the partition equilibrium. Subsequently, the same volumes of upper and lower phase were each evaporated to dryness. The residues were diluted into 2 mL methanol and then analyzed by HPLC. The K value was defined as the peak area of the component in the upper phase divided by the peak area of the component in the lower phase.

2.6 Preparation of the two-phase solvent system and sample solution

In the present study, a two-phase solvent system composed of ethyl acetate/*n*-butanol/water (13:3:10, v/v/v) was used for HSCCC separation. Each solvent was added to a separatory funnel and thoroughly equilibrated at room temperature. The upper phase and the lower phase were separated shortly before use. The sample solution for HSCCC separation was prepared by dissolving 150 mg of the dried powder of the crude extract after cleaning up by D101 MR in 10 mL of the lower phase of the two-phase solvent system.

2.7 HSCCC separation procedure

First, the multilayer coil column was entirely filled with the upper phase (stationary phase). Then the apparatus was rotated at 950 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 3.0 mL/min. After hydrodynamic equilibrium was reached, as indicated by the emergence of the mobile phase, 10 mL of sample solution containing 150 mg of the crude extract was injected into the column through the injection valve. The eluant from the tail end of the column was continuously monitored with a UV detector at 330 nm. The peak fractions were collected manually according to the elution profile. Evaporation under reduced pressure was then carried out, and the residues were dissolved in methanol for subsequent purity analysis by HPLC. The purity was obtained by HPLC peak area calculation.

2.8 HPLC analysis and identification of HSCCC peak fractions

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC. HPLC analysis was performed on a RP Agilent Eclipse XDB-C18 (4 μm, 4.6 × 250 mm) analytical column with gradient elution, at a column temperature of 30°C. Methanol/water in the following percentage and corresponding time was used as the mobile phase: methanol: 0–30 min, 36–45%. The flow rate and detection wavelength were set at 1.0 mL/min and 330 nm, respectively. The crude sample and peak fractions separated by HSCCC were analyzed by HPLC under the optimum analytical conditions, and the chromatograms are presented in Fig. 2.

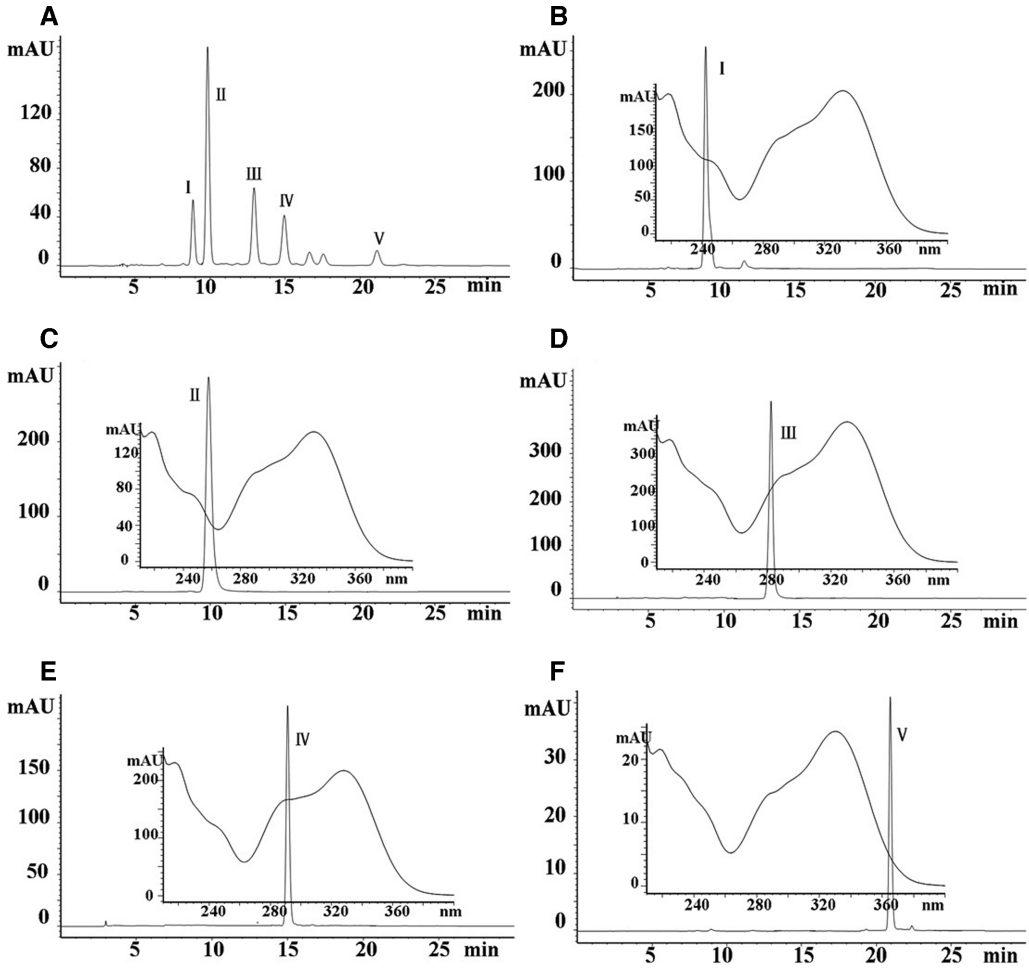

Figure 2. HPLC chromatograms of the crude extract from *L. rotata* (Benth.) Kudo (A) after cleanup by MR column chromatography and the five targeted compounds (peak fractions I, II, III, IV and V, B–F) purified by HSCCC. Conditions: column: Eclipse XDB-C_{18} column (250 × 4.6 mm id, 4 μm); mobile phase: methanol/water (methanol: 0–30 min, 36–45%); flow rate: 1.0 mL/min; column temperature: 30°C; and detection wavelength: 330 nm.

Identification of the HSCCC peak fractions was performed by ^{1}H and ^{13}C NMR spectroscopy.

3 Results and discussion

3.1 Selection of solvent system and other HSCCC conditions

Successful HSCCC separation depends upon the selection of a suitable two-phase solvent system, which requires the following considerations [31, 32]: (i) the settling time of the solvent system should be short (<30 s); (ii) the partition coefficient (K) of the target compound should fall within a suitable range (i.e. usually between 0.2 and 5); and (3) the separation factor ($\alpha = K_1/K_2$, $K_1 > K_2$) between any two compounds should be greater than 1.5.

In this experiment, different two-phase solvent systems, such as ethyl acetate/*n*-butanol/water and *n*-hexane/ethyl acetate/methanol/water, were tested according to the polarity of the target compounds. The K values of the five target compounds corresponding to peak fractions I, II, III, IV, and V in different solvent systems were determined by HPLC and the results are shown in Table 1. The two-phase solvent systems with *n*-hexane/ethyl acetate/methanol/water (10:12:9:12 and 9:14:9:10, v/v/v/v) were tested first; small K values and poor retention of target compounds in the upper phase resulted. Then, two-phase solvent systems comprising ethyl acetate/*n*-butanol/water (8:8:10, 10:6:15, 8:4:15, 9:7:13, 9:7:12, 10:6:12, 12:4:10, and 13:3:10, v/v/v) were further investigated. When a ratio of 8:8:10, 10:6:15, 8:4:15, or 9:7:13 v/v/v was used for the separation, the resulting K values were too large and would result in longer separation time and broader peaks [33]. When a ratio of 9:7:12, 10:6:12, or 12:4:10 v/v/v was tested, the separation factors between compounds I, II, III, IV, and V were too small and not suitable for the separation of the five target compounds from the crude sample. Fortunately, when a ratio of 13:3:10 v/v/v was tested, the K values of the

Table 1. The *K* values of the target compounds in different solvent systems

Solvent systems	Ratio (v/v)	Settling time (s)	*K* values				
			I	II	III	IV	V
n-Hexane/ethyl acetate/methanol/water	10:12:9:12	13	0.02	0.02	0.02	0.03	0.02
n-Hexane/ethyl acetate/methanol/water	9:14:9:10	17	$-\infty$	$-\infty$	$-\infty$	$-\infty$	$-\infty$
Ethyl acetate/*n*-butanol/water	8:8:10	25	7.62	22.52	∞	5.79	3.31
Ethyl acetate/*n*-butanol/water	10:6:15	19	3.50	16.47	8.53	7.35	2.16
Ethyl acetate/*n*-butanol/water	8:4:15	14	1.37	5.90	2.07	8.76	3.19
Ethyl acetate/*n*-butanol/water	9:7:13	13	1.26	9.14	4.54	5.46	2.67
Ethyl acetate/*n*-butanol/water	9:7:12	13	0.99	2.54	2.75	1.74	1.38
Ethyl acetate/*n*-butanol/water	10:6:12	12	1.65	5.98	4.23	3.67	2.44
Ethyl acetate/*n*-butanol/water	12:4:10	13	0.74	3.58	1.11	5.78	1.82
Ethyl acetate/*n*-butanol/water	**13:3:10**	**22**	**0.33**	**1.96**	**0.61**	**3.16**	**1.19**

five target compounds were between 0.2 and 5, and the separation factors were large enough for the five phenylpropanoid glycosides to exhibit large resolutions relative to one another.

In addition to screening a suitable two-phase solvent system, other factors such as separation temperature and flow rate of the mobile phase were also investigated. Firstly, the effects of separation temperature on the retention of the stationary phase were tested (Fig. 3). The results demonstrated that the retention of the stationary phase was improved with an increase in separation temperature, but high temperature would often trap a lot of air in the apparatus and decrease the separating efficiency of the apparatus to some degree. Therefore, the separation temperature was set at 35°C in this experiment. Then, different flow rates of the mobile phase (2.5, 3.0, and 3.5 mL/min) were tested in order to determine their effect on separation time, stationary phase retention, and targeted compound purity (Table 2). The results indicated that a low flow rate of the mobile phase could improve the peak resolution and increase stationary phase retention, but more time and more mobile phase would be required, and the chromatogram peak was extended. Considering the elucidated separation time and the purity of the targeted compounds, a flow rate of 3.0 mL/min was used.

Under the optimized conditions, five fractions (I, II, III, IV, and V) were obtained in a one-step separation within 4 h, which were forsythoside B (I, 27 mg), verbascoside (II, 41 mg), alyssonoside (III, 29 mg), isoverbascoside (IV, 23 mg), and leucosceptoside B (V, 13 mg). HPLC analysis of each HSCCC fraction revealed that the purities of these five compounds were 97.7, 99.2, 99.5, 99.3, and 97.3%, respectively. The HSCCC chromatogram is shown in Fig. 4.

3.2 The structure identification of the HSCCC peak fractions

According to ^{1}H and ^{13}C NMR spectra, the chemical structures of the peak fractions separated by HSCCC were identified. In comparison with reference data, peaks I–V were effectively identified as forsythoside B, verbascoside, alyssonoside, isoverbascoside, and leucosceptoside B, respectively [34–37].

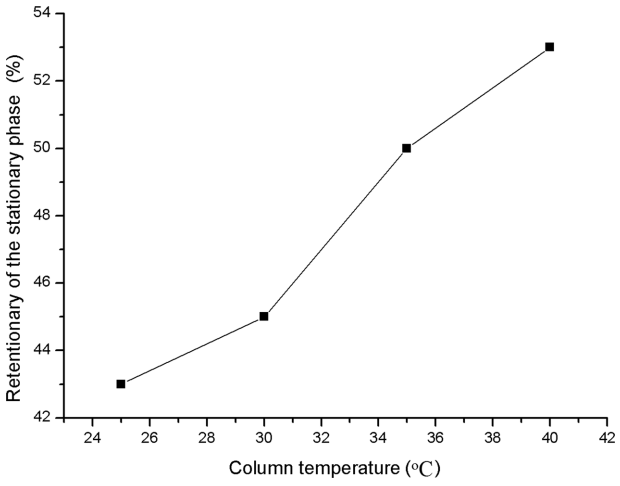

Figure 3. Comparison of stationary phase retention under different seperation temperature.

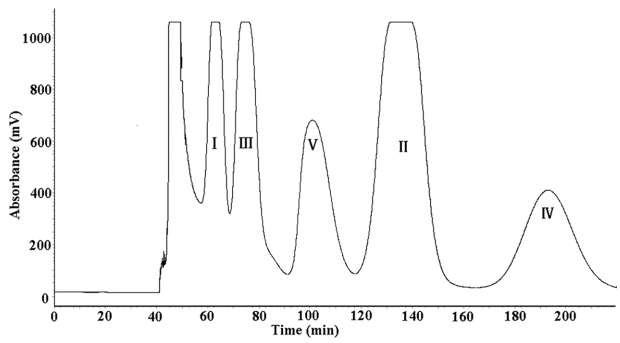

Figure 4. HSCCC chromatogram of the crude extract from *L. rotata* (Benth.) Kudo after cleanup by MR column chromatography. Two-phase solvent system: ethyl acetate/*n*-butanol/water 13:3:10 v/v/v; stationary phase: upper phase; mobile phase: lower phase; flow rate: 3.0 mL/min; revolution speed: 950 rpm; detection wavelength: 330 nm; sample size: 150 mg of crude sample dissolved in 10 mL of the lower phase; and separation temperature: 35°C.

Table 2. Comparison of separation time, stationary phase retention, and purities of five compounds under different flow rates

Flow rate (mL/min)	Separation time (min)	Retention (%)	Purity (%)				
			I	II	III	IV	V
2.5	280	54.9	98.1	99.6	99.7	99.5	97.9
3.0	**220**	**50.0**	**97.7**	**99.2**	**99.5**	**99.3**	**97.3**
3.5	190	48.3	93.0	95.0	95.5	97.3	92.4

4 Concluding remarks

A convenient and efficient method for the preparative separation and purification of forsythoside B, verbascoside, alyssonoside, isoverbascoside, and leucosceptoside B from the partially purified extract of *L. rotata* (Benth.) Kudo has been successfully developed by HSCCC in a one-step separation within 4 h. The present study indicates that HSCCC is a very powerful technique for the preparative separation and purification of bioactive components from plant materials.

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The authors have declared no conflict of interest.

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