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Preparative isolation and purification of chemical constituents from the root of *Polygonum multiflorum* by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography methods, combined with solvent partition, were applied to the systematic separation and purification of chemical components from Chinese medicinal herb *Polygonum multiflorum* extract. The aim of this paper is summing up the rules of solvent system selection for diverse fractions of herbal extract, and establishing the systematic pattern to screen the bioactive constituents rapidly. Nine compounds including emodin, chrysophanol, rhein, 6-OH-emodin**,** emodin-8-β-D-glucoside, polygonimitin B, 2,3,5,4′-tetrahydroxystilbene-2-β-D-glucoside, gallic acid and an unkown glycoside, which differed in quantity and polarity remarkably, were obtained. The purities of them were all above 97% as determined by high-performance liquid chromatography (HPLC), and their structures were identified by ^{1}H NMR and electrospray ionization mass spectrometry (ESI–MS). The results demonstrated that HSCCC is a speedy and efficient technique for systematic isolation of bioactive components from traditional medicinal herbs.

Keywords: High-speed counter-current chromatography; *Polygonum multiflorum*; Systematic isolation and purification

1. Introduction

Dried root of *Polygonum multiflorum* Thunb. (Polygonaceae), well known as Heshouwu, is one of the most popular traditional medicinal herb in China, and is officially listed in the Chinese Pharmacopoeia [1]. It is frequently used as a tonic and purgative in China and Japan. The main active constituents of the herb have been reported to be hydroxyanthraquinones, stilbenes, other phenolic compounds and their glycosides [2].

High-speed counter-current chromatography (HSCCC), which was first invented by Ito [3], is a form of liquid-liquid partition chromatography. It is considered as a suitable alternative for separation of phenolic compounds such as hydroxyanthraquinones and stilbenes [4–6]. Only a few CCC studies [7] have targeted a range of compounds in a single procedure, and most reports focus on several main compounds in certain fraction. In the present paper, diverse methods of HSCCC were established for systematic separation of different fractions of *Polygonum multiflorum* Thunb. Nine compounds including emodin, chrysophanol, rhein, 6-OH-emodin**,** emodin-8-β-D-glucoside, polygonimitin B, 2,3,5,4′-tetrahydroxystilbene-2-β-D-glucoside, gallic acid and an unkown glycoside, were obtained as shown in Fig. 1.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study was TBE-300 high-speed counter-current chromatography (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation column connected in series (I.D. of the tubing = 1.5 mm, total volume = 300 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the β values (β = r/R, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. The system was

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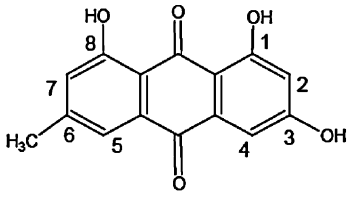

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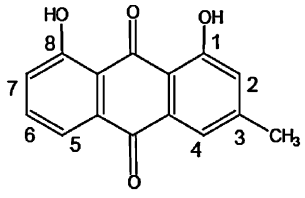

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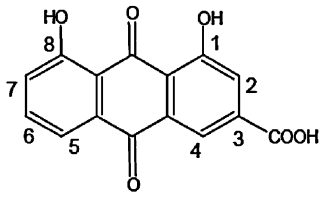

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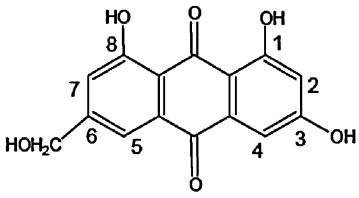
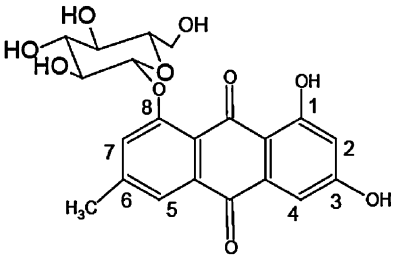
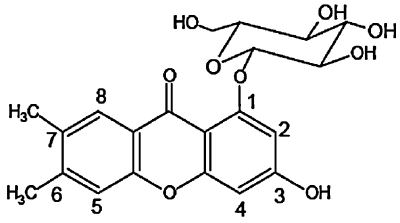


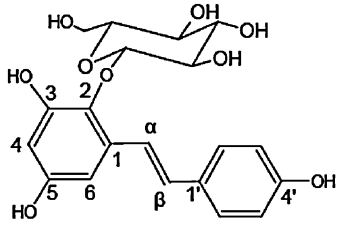
6-OH-emodin



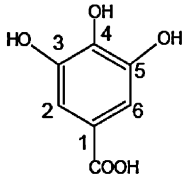
emodin-8-β-D-glucoside



polygonimitin B



2, 3, 5, 4'-tetrahydroxystilbene-2-β-D-glucoside



gallic acid

Fig. 1. Chemical structures of compounds from *Polygonum multiflorum* Thunb.

also equipped with a S-1007 constant flow pump (Shenyitong Tech & Exploitation, Beijing, China), a Model 8823B-UV monitor (Bingdayingchuang Sci & Tech, Beijing, China). The data were collected with model N2000 chromatography workstation (Zhejiang University, Hangzhou, China). The HPLC equipment used was Shimadzu LC-2010C HT system and Shimadzu HPLC workstation (Shimadzu, Japan). Nuclear magnetic resonance (NMR) spectrometer used here was Bruker AM-500 MHz (Bruker, Switzerland). Electrospray ionization mass spectrometry (ESI–MS) used was Agilent 1100 Series LC–MS Trap SL (Agilent, USA).

2.2. Reagents

All solvents used for HSCCC were of analytical grade (Hanbon Sci & Tech, Jiangsu, China). Methanol and acetonitrile used for HPLC were of chromatographic grade (Hanbon Sci & Tech). D-101 macroporous resin (Chemical Plant of Nankai University,

Tianjin, China) was used for sample preparation and water used was distilled water.

The dried roots of *Polygonum multiflorum* were purchased from a local drug store and identified by Professor Mingjian Qing, Department of Medicinal Plant, China Pharmaceutical University.

2.3. Preparation of crude extract

Five hundred grams of dry slice (thickness: 1.0 cm, breadth: 0.8 cm) of *Polygonum multiflorum* Thunb. was extracted with 5 L 95% ethanol and then evaporated to form a syrup. The syrup was dissolved in 300 ml of water by sonication and partitioned with ether and *n*-butanol of equal volume three times successively. Both ether and *n*-butanol solution was vacuum evaporated at 65 °C. About 8.0 g residue of ether and 7.5 g residue of *n*-butanol were obtained, respectively. In order to enrich the target components, the residue of *n*-butanol was then loaded on D-101 macroporous resin column (35 cm × 3.4 cm, the volume of the column was 170 ml) and eluted with 1700 ml of distilled water and 3400 ml of 30% ethanol, respectively. The effluent of 30% ethanol was collected and evaporated at 65 °C under vacuum and about 3.5 g residue was obtained. Finally the fraction of water was evaporated at 90 °C under vacuum and about 10.0 g residue was obtained. All the residues were stored in a refrigerator (5 °C) for further use.

2.4. Preparation of two-phase solvent system and sample solution

In the present study, the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v), *n*-hexane–ethyl acetate–methanol–water (9:1:5:5, v/v/v/v), ethyl acetate–methanol–water (50:1:50, v/v/v) and ethyl acetate–*n*-buthanol–water (20:1:20, v/v/v) were used for HSCCC separation. Each component of the solvent system was added to a separatory funnel and thoroughly equilibrated at room temperature. The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

For HSCCC separation of ether fraction, the sample solution was prepared by dissolving 200 mg of ether extract in 5 ml of the lower phase of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v). The sample solution for HSCCC separation of *n*-butanol fraction was prepared by dissolving 130 mg of refined *n*-buthanol extract in 5 ml of the lower phase of ethyl acetate–methanol–water (50:1:50, v/v/v), and the sample solution of water fraction was prepared with 100 mg extract in 5 ml of the lower phase of ethyl acetate–methanol–water (20:1:20, v/v/v).

2.5. HSCCC separation

In each separation process, the separation column of HSCCC was first entirely filled with the upper organic phase (stationary phase). Then the apparatus was rotated at 800 rpm, while the lower aqueous phase (mobile phase) was pumped into the column at the flow rate of 2.0 ml/min. After the mobile phase front emerged (about half an hour later) and the system established hydrodynamic equilibrium, the sample solution was injected into the separation column through the injection valve. The effluent from the outlet of the column was monitored with the UV detector at 254 nm. The whole process of separation was under room temperature (25.0–27.5 °C). Each peak fraction was manually collected according to the chromatogram and evaporated under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

2.6. HPLC analysis and identification of HSCCC peak fractions

The HPLC analysis of every fraction of *Polygonum multiflorum* Thunb. and HSCCC peak fraction were performed with a Shimadzu VP-ODS column (150 mm × 4.6 mm I.D., 5 μm) at room temperature. For analysis of the fraction of ether, the mobile phase was methanol and water in gradient mode as follows: 5:95 to 100:0 in 60 min, The effluent was monitored at 254 nm and the flow rate was set at 1.2 ml/min constantly. When the fraction of *n*-buthanol purified by macroporous resin column was analyzed, the mobile phase was acetonitrile and water in gradient mode as follows: 1:99 to 100:0 in 45 min, The effluent was monitored at 254 nm and the flow rate was kept at 0.8 ml/min constantly.

3. Results and discussion

In the present study, D-101 macroporous resin was used to purify crude extract of *n*-butanol fraction. Water was used to remove the hydrosoluble chemicals, such as pigments and saccharides which had no or little retention on D-101 macroporous resin, at first. Then 30% ethanol was used to elute the target compounds, which was prepared for further HSCCC isolation and purification. At last, 95% ethanol was used to activate the macroporous resin. Consequently, 3.5 g of refined *n*-butanol fraction was obtained from 500 g *Polygonum multiflorum*. A roadmap of the whole work is given in Fig. 2 and the HSCCC chromatograms are shown in Fig. 3.

3.1. HPLC analysis and identification of HSCCC peak fractions

Each peak fraction of HSCCC was analyzed by HPLC. According to the adopted methods the purity of eight compounds were all above 97%. The HPLC chromatograms were shown in Fig. 4. Identification of the HSCCC peak fractions was based on retention time together with ^{1}HNMR and ESI–MS. The latter is a technology of soft ionization used to give the exact mass/charge (*m*/*z*) of quasi-molecular ion and valuable fragment peaks of losing residual group of glucose.

3.2. Selection of two-phase solvent system and other conditions of HSCCC

Successful separation by HSCCC largely depends upon the selection of suitable two-phase solvent system. In this experi-

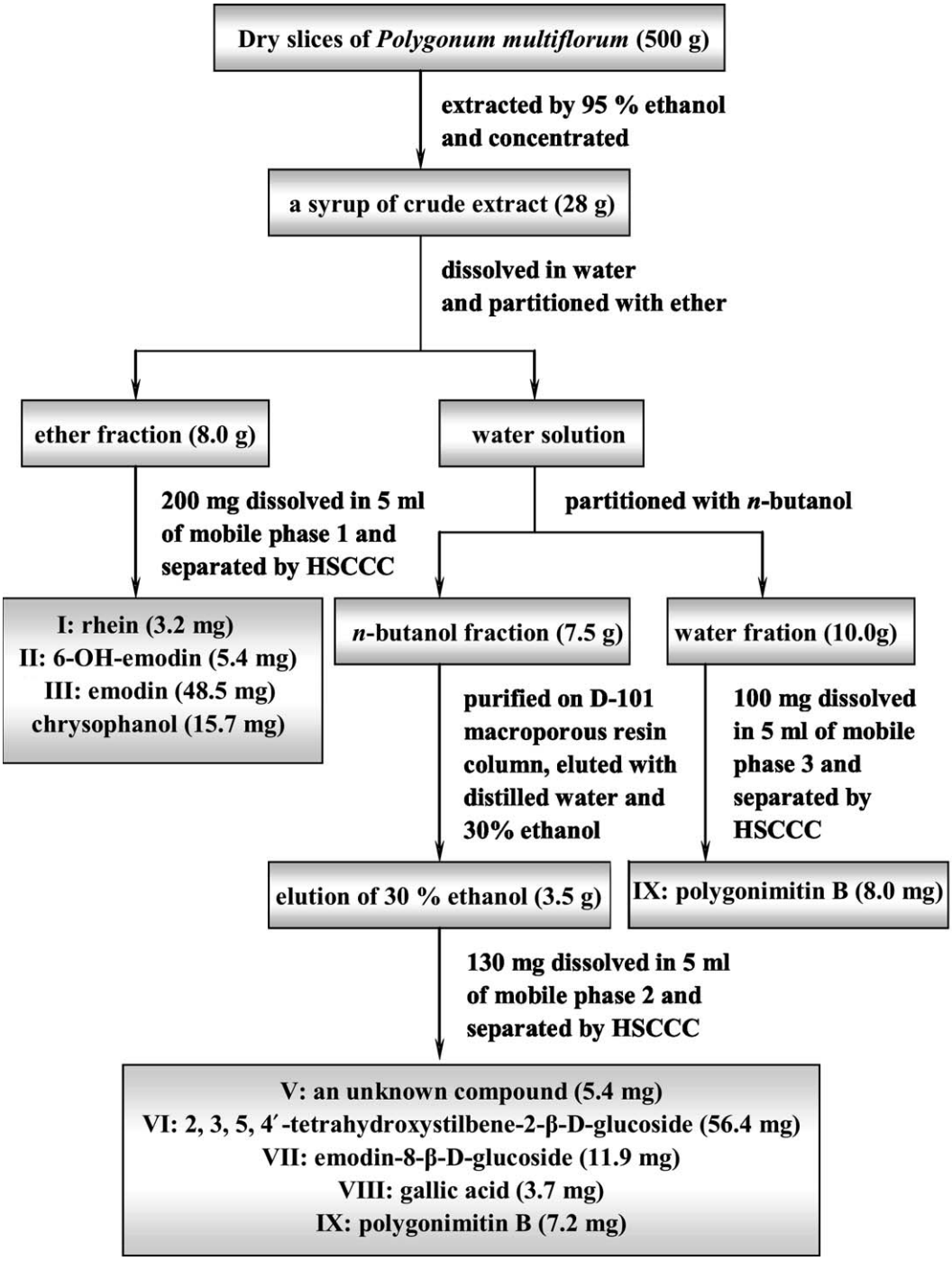


Fig. 2. Roadmap of extraction and separation. Mobile phase 1: the lower phase of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v); mobile phase 2: the lower phase of ethyl acetate–methanol–water (50:1:50, v/v/v); mobile phase 3: the lower phase of ethyl acetate–*n*-buthanol–water (20:1:20, v/v/v).

ment, several kinds of solvent systems including *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, 9:1:5:5, 1:1:1:1, 1:9:5:5, 7:3:5:5, v/v/v/v), ethyl acetate–methanol–water (50:1:50, 10:1:10, 1:0:1, v/v/v), and ethyl acetate–*n*-buthanol–water (20:1:20, 4:1:5, v/v/v) were tested. The results indicated that the target compounds have appropriate *K*-values (0.5–2) basically in four systems, which were listed in Table 1. In general, small *K*-values usually result in a poor peak resolution, while large *K*-values tend to produce excessive sample band broadening. For the components of large *K*-values, other modes of elution, such as dual mode, reversed mode elution or expelling solution from column could be employed to shorten separation time [8].

For separation of the fraction of ether, *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v) was used as the two-phase solvent system of HSCCC. The anterior peaks were separated well, while the retention time of the remainder was so long that the stepwise elution mode had to be employed. The lower phase of *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, v/v/v/v) and (9:1:5:5, v/v/v/v) were used as the mobile

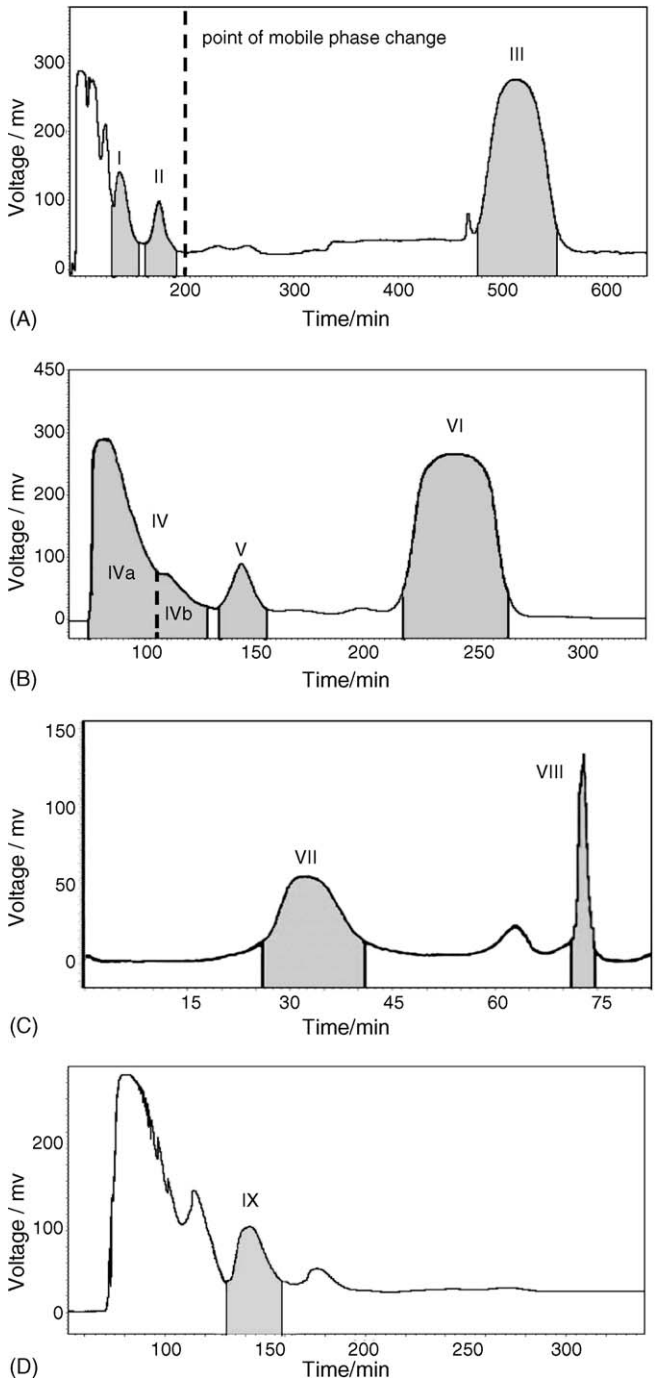


Fig. 3. HSCCC chromatograms of two fractions of ether (A), *n*-butanol (B, C) and its second separation (D) from *Polygonum multiflorum* Thunb. Conditions of A: Two-phase solvent system: *n*-hexane–ethyl acetate–methanol–water (3:7:5:5, 9:1:5:5, v/v/v/v) in stepwise elution; Stationary phase: upper organic phase of 3:7:5:5; mobile phase: lower phase of 3:7:5:5 and 9:1:5:5 used in stepwise elution mode as follows: 0–200 min, the lower phase of 3:7:5:5 system; after 200 min, the lower phase of 9:1:5:5 system; flow rate: 2.0 ml/min; revolution speed: 800 rpm; detection wavelength: 254 nm; sample size: 200 mg of ether extracts dissolved in 5 ml of the lower phase of 3:7:5:5 system; retention of the stationary phase: 56%; Peak I: rhein; Peak II: 6-OH-emodin; Peak III: emodin. Conditions of B & C: Two-phase solvent system: ethyl acetate–methanol–water 50:1:50 (v/v/v), after 450 min the direction of revolution was reversed, mobile phase: lower aqueous phase (0–450 min, B) and upper organic phase (after 450 min, C); flow rate: 2.0 ml/min (0–450 min, B) and 1.5 ml/min (after 450 min, C); revolution speed: 800 rpm; detection wavelength: 254 nm; sample size: 130 mg of refined *n*-buthanol extract dissolved in 5 ml of the lower phase, retention of the stationary phase: 46%; Conditions of D: Two-phase solvent system: ethyl acetate–*n*-buthanol–water 20:1:20 (v/v/v); mobile phase: the lower phase; flow rate: 2.0 ml/min; revolution speed: 800 rpm; detection wavelength: 254 nm; the sample was from the eluent of peak IV of B; retention of the stationary phase: 44%; Peak V: an unknown glucoside; Peak VI: 3,5,4′-tetrahydroxystilbene-2-β-D-glucoside; Peak VII: emodin-8-β-D-glucoside; Peak VIII: gallic acid; Peak IX: polygonimitin B. Separation temperature: room temperature (25.0–27.5 °C).

phase in stepwise elution. The lower phase of 3:7:5:5 system was used as the mobile phase during 0–200 min. After 200 min, the mobile phase was changed to the lower phase of 9:1:5:5 system, which was saturated with the upper phase of 3:7:5:5 system before use. Three pure peaks, marked as peak I, peak II and peak III in Fig. 3A, could be obtained. They were identified as rhein (3.2 mg), 6-OH-emodin (5.4 mg) and emodin (48.5 mg) in turn. Finally the residual solution in the separation column was blown out and another compound, which could not be shown in Fig. 3A, was acquired and identified as chrysophanol (15.7 mg).

Ethyl acetate–methanol–water (50:1:50, v/v/v) was used as the two-phase solvent system for separation of the refined *n*-butanol fraction. The upper organic phase was used as the stationary phase and the lower aqueous phase as the mobile phase. When the refined *n*-butanol fraction was analyzed by TLC (developer: ethyl acetate-acetic acid (9:1)), a big spot with distinct green fluorescence (254 nm) and small Rf value was found and naturally it was regarded as one of the target compounds. During this HSCCC separation process, peak IVa and IVb in Fig. 3B were collected and analyzed by TLC, respectively. The results indicated that both IVa and IVb all contained the spot with green fluorescence and other impurities. So peak IVa and IVb were combined together as peak IV and further separated with another solvent system, ethyl acetate-*n*-buthanol–water 20:1:20 (v/v/v) for better resolution and higher recovery. Then peak IX in Fig. 3D corresponding to the compound with green fluorescence was obtained and identified as polygonimitin B (7.2 mg). After peak VI in Fig. 3B was eluted out, the rotation direction of the HSCCC apparatus was changed to reversed rotation and mobile phase to the upper phase. Meanwhile the flow rate was reduced from 2.0 to 1.5 ml/min. Then another two peaks were obtained, which were marked as peak VII and peak VIII in Fig. 3C. Peak V, VI, VII and VIII were identified as an unknown glucoside (5.4 mg), 2,3,5,4′-tetrahydroxystilbene-2-β-D-glucoside (56.4 mg), emodin-8-β-D-glucoside (11.9 mg) and gallic acid (3.7 mg) in turn.

The fraction of water, which contained polygonimitin B mainly and other water-soluble impurity like pigments and saccharides, could be separated by the same solvent system of ethyl acetate–*n*-buthanol–water (20:1:20, v/v/v) and relevant conditions by HSCCC to obtain polygonimitin B (about 8 mg) too. The chromatogram was very similar to Fig. 3D. So it is not given in this paper.

3.3. The structural identification

The structural identification of peak fractions was performed with ESI–MS (negative ion mode) and ^{1}H NMR with TMS

Table 1
The *K*-values of target components measured in different solvent systems

Solvent system	*K*-value			
	n-Hexane–ethyl acetate–methanol –water (3:7:5:5, v/v/v/v)	*n*-Hexane–ethyl acetate–methanol – water (9:1:5:5, v/v/v/v)	Ethyl acetate–methanol –water (50:1:50, v/v/v)	Ethyl acetate–*n*-buthanol –water (20:1:20, v/v/v)
Rhein	0.83	0.46	–	–
6-OH-Emodin	1.12	0.68	–	–
Emodin	7.34	4.55	–	–
Chrysophanol	14.87	11.30	–	–
Polygonimitin B	–	–	0.74	0.95
The unknown glucoside	–	–	0.92	1.16
2,3,5,4'-Tetrahydroxystilbene-2-β-D-glucoside	–	–	2.26	3.15
Emodin-8-β-D-glucoside	–	–	4.74	6.97
Gallic acid	–	–	7.46	10.91

Experimental protocol: 4 ml of each phase of the equilibrated two-phase solvent system was added to approximately 8 mg of crude sample placed in a 10 ml test tube. The test tube was caped and shaken vigorously for 2 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficient (*K*). The partition coefficient (*K*) value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

as internal standard. Data of each compound were given as follows:

Peak I in Fig. 3A: ESI–MS (*m/z*): 283 ($[M-H]^-$). ^1H NMR (500 MHz, CD_3OD): 8.07 (1H, s, H-4), 7.81 (1H, d, $J=7.5$ Hz, H-7), 7.76 (1H, s, H-2), 7.71 (1H, m, H-6), 7.38 (1H, d, $J=8.5$ Hz, H-5). The results were similar to those in ref. [9], and peak I corresponded to rhein.

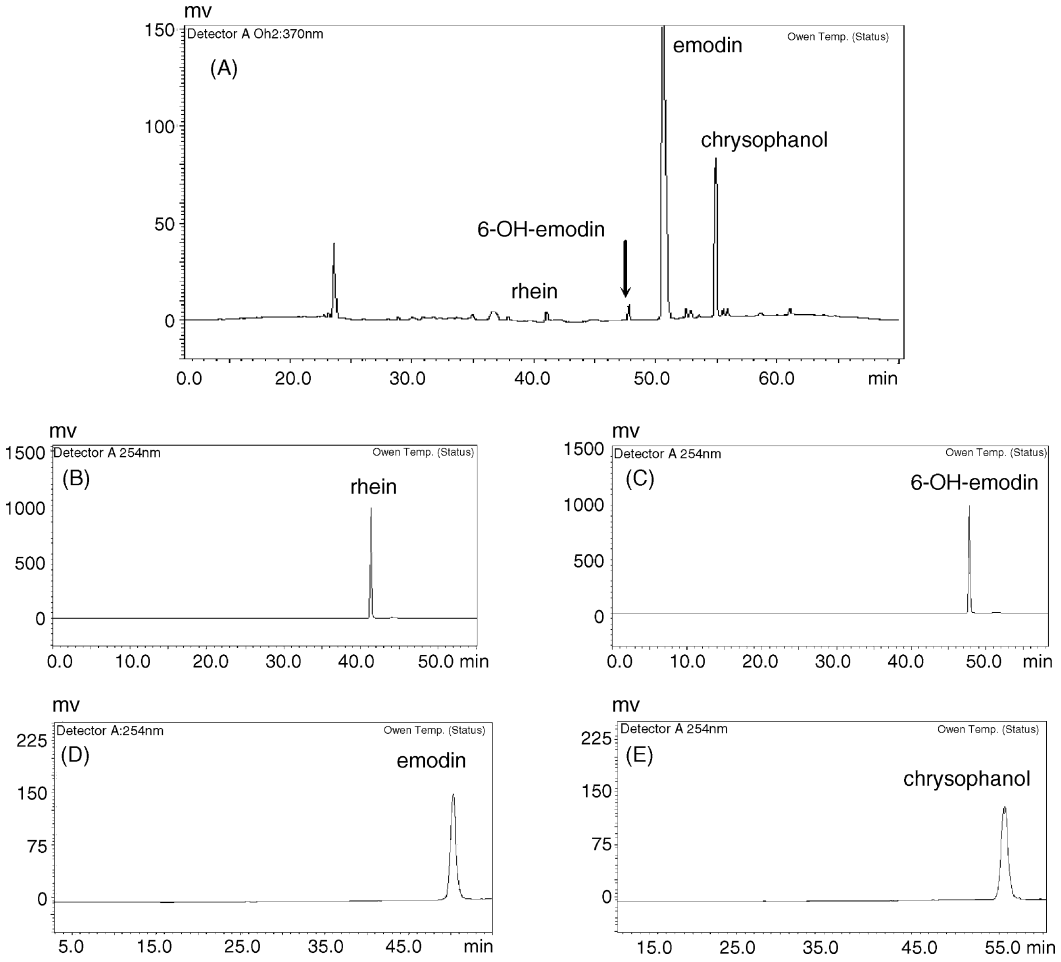


Fig. 4. HPLC chromatograms of two fractions of ether (A) and *n*-butanol (F) from *Polygonum multiflorum* Thunb. and HSCCC peak fractions of them (B–E, G–H) Column: Shimadzu VP-ODS (150 mm × 4.6 mm I.D., 5 μm); mobile phase (A–E): methanol–water (methanol: 5% to 100% in 60 min); flow rate: 1.2 ml/min; detection wavelength: 254 nm; mobile phase (F–H): acetonitrile–water (acetonitrile: 1% to 100% in 45 min); flow rate: 0.8 ml/min; detection wavelength: 254 nm.

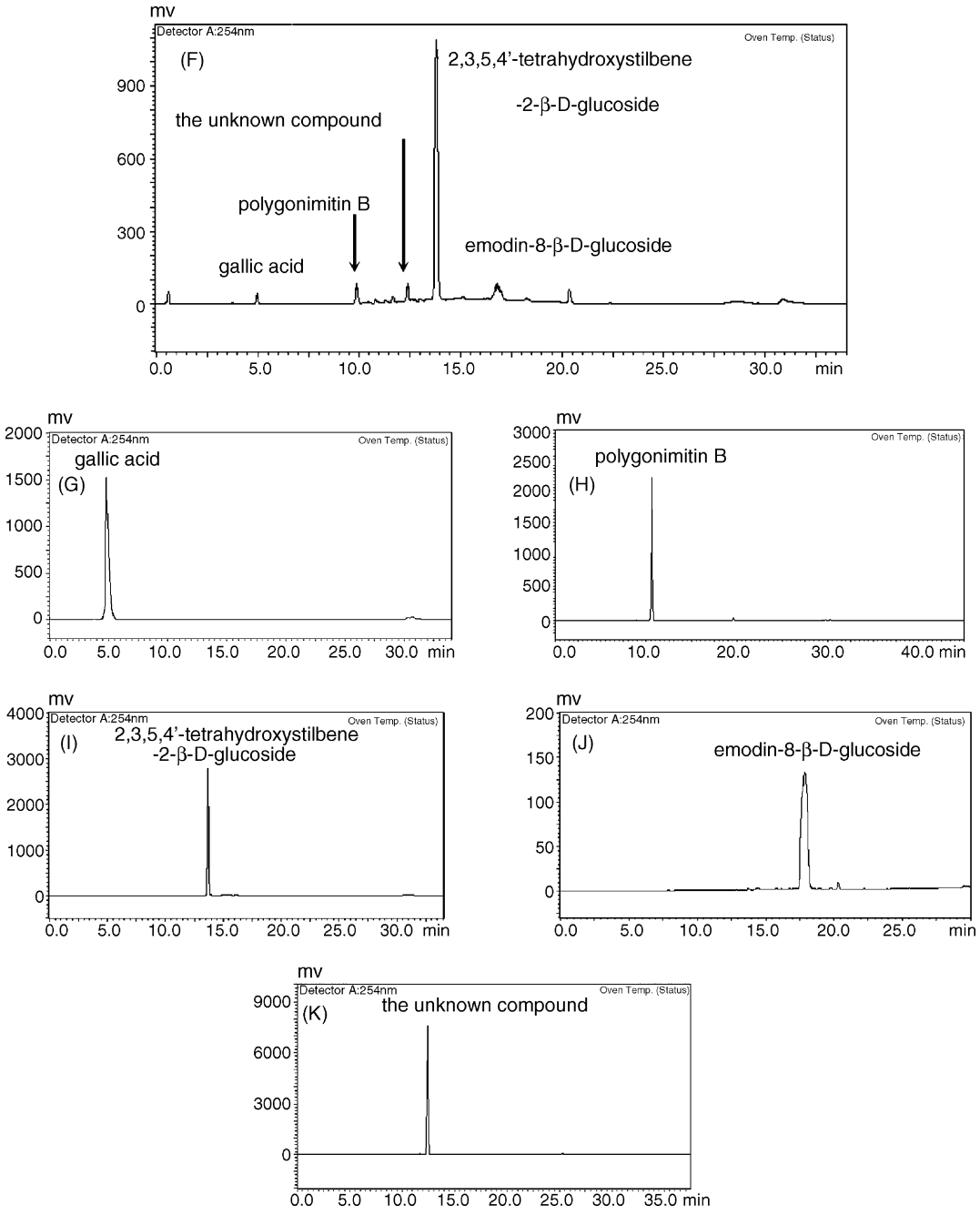


Fig. 4. (*Continued*).

Peak II in Fig. 3A: ESI–MS (*m/z*): 285 ($[M-H]^-$). ^{1}H NMR (500 MHz, CD_3OD): 7.67 (1H, d, $J=2.2$ Hz, H-5), 7.21 (1H, d, $J=2.2$ Hz, H-7), 7.01 (1H, $J=3.1$ Hz, H-4), 6.60 (1H, d, $J=3.1$ Hz, H-2), 4.62 (2H, s, 6-CH_2OH). The results were similar to those in ref. [10], and peak II corresponded to 6-OH-emodin.

Peak III in Fig. 3A: ESI–MS (*m/z*): 269 ($[M-H]^-$). ^{1}H NMR (500 MHz, CD_3OD): 7.64 (1H, s, H-5), 7.30 (1H, d, $J=2.4$ Hz, H-4), 7.09 (1H, s, H-7), 6.70 (1H, d, $J=2.4$ Hz, H-2), 2.45 (3H, s, 6-CH_3). The results were similar to those in ref. [11], and peak III corresponded to emodin.

Data of the compound blown out from the column during the separation of ether fraction: ESI–MS (*m/z*): 255 ($[M-H]^-$); ^{1}H NMR (500 MHz, CD_3OD): 7.87 (1H, d, $J=7.2$ Hz, H-5), 7.69 (1H, m, H-6), 7.64 (1H, s, H-4), 7.32 (1H, d, $J=8.4$ Hz, H-7), 7.13 (1H, s, H-2), 2.50 (3H, s, 3-CH_3). The results were similar to those in ref. [9], it corresponded to chrysophanol.

Peak V in Fig. 3B: ESI–MS fragment peak (*m/z*: 325) showed the losing of glucose from quasi-molecular ion (*m/z*: 487), and corresponding ^{1}H NMR signals of glucose in the region of 3.30–4.50 ppm were found. Peak V was determined as a glucoside temporarily and further structural identification will be done in the further research work.

Peak VI in Fig. 3B: ESI–MS (*m/z*): 405 ($[M-H]^-$), 243 ($[M-H-162]^-$). ^{1}H NMR (500 MHz, CD_3OD): 7.76 (1H, d, $J=16.0$ Hz, H-α), 7.44 (2H, d, $J=8.2$ Hz, H-3′, 5′), 6.92 (1H,

d, $J = 16.0$ Hz, H-β), 6.80 (2H, d, $J = 8.2$ Hz, H-2′, 6′), 6.62 (1H, $J = 3.1$ Hz, H-6), 6.24 (1H, $J = 3.1$ Hz, H-4), 4.50 (1H, d, $J = 7.0$ Hz, H-$1_{2\text{-O-Glc}}$), 3.40–3.80 (6H, m, protons of glucose). The results were similar to those in ref. [11], and peak VI corresponded to 2,3,5,4′-tetrahydroxystilbene-2-β-D-glucoside.

Peak VII in Fig. 3C: ESI–MS (*m*/*z*): 431 ($[M-H]^-$), 269 ($[M-H-162]^-$). ^{1}H NMR (500 MHz, CD_3OD): 7.45 (1H, br, s, H-4), 7.28 (1H, d, $J = 2.1$ Hz, H-5), 7.14 (1H, br, s, H-2), 6.99 (1H, d, $J = 2.1$ Hz, H-7), 5.04 (1H, d, $J = 6.2$ Hz, H-$1_{8\text{-O-Glc}}$), 3.30–3.70 (6H, m, protons of glucose), 2.40 (3H, s, 6-CH_3). Compared with the data given in ref. [11], and peak VII corresponded to emodin-8-β-D-glucoside.

Peak VIII in Fig. 3C: ESI–MS (*m*/*z*): 169 ($[M-H]^-$). Data of ^{1}H NMR (500 MHz, CD_3OD) matched with the reported the data given in ref. [12], and peak VIII corresponded to gallic acid.

Peak IX in Fig. 3D: ESI–MS (*m*/*z*): 417 ($[M-H]^-$), 255 ($[M-H-162]^-$). ^{1}H NMR (500 MHz, CD_3OD): 7.26 (1H, s, H-8), 6.78 (1H, s, H-4), 6.74 (1H, d, $J = 2.2$ Hz, H-2), 6.17 (1H, s, H-5), 4.54 (1H, $J = 7.2$ Hz, H-$1_{1\text{-O-Glc}}$), 3.44 ∼ 3.90 (6H, m, protons of glucose), 2.73 (3H, s, 7-CH_3), 2.53 (3H, s, 6-CH_3). The results were similar to those in ref. [13], and peak IX corresponded to polygonimitin B.

4. Conclusions

Some of the chemical constituents of *Polygonum multiflorum*, one of the most important Chinese medicinal herbs, were isolated and purified systematically by HSCCC. The results of the research provided a successful pattern for isolation of chemical compounds from low polarity such as aglycones to high polarity such as glycosides by HSCCC. Because there are too many constituents present in natural herbs, and their polarity differed very much, it is comparatively difficult to isolate all the constituents of crude extract with only one solvent system. So it is necessary to obtain a few main fractions from low polarity to high polarity by using different solvent extraction. For low polarity fraction, such as aglycones, *n*-hexane could be used as the major solvent of organic phase, and *n*-hexane-methanol (ethanol)-water or *n*-hexane-ethyl acetate-methanol (ethanol)-water could be used as the two-phase solvent system. For moderately polar fraction, just like a part of glycosides with single sugar, ethyl acetate could be used as the major solvent of organic phase, and ethyl acetate-methanol (ethanol)-water or ethyl acetate-*n*-buthanol-water could be used as the two-phase solvent system. If the polarity of fraction was higher, just like general glycosides with polysaccharide, *n*-buthanol could be used as the major solvent of organic phase, and the volume ratio of every solvent should be adjusted properly according to the actual separation demand to exert advantages of HSCCC.

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