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Journal of Chromatography A, 1097 (2005) 33–39
JOURNAL OF CHROMATOGRAPHY A
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Preparative isolation and purification of five compounds from the Chinese medicinal herb *Polygonum cuspidatum* Sieb. et Zucc by high-speed counter-current chromatography

Xin Chu, Ailing Sun, Renmin Liu *

Department of Chemistry, College of Chemistry and Chemical Engineering, Liaocheng University, No. 34 Wenhua Road, Liaocheng, Shandong 252059, China

Received 14 July 2005; received in revised form 1 August 2005; accepted 3 August 2005
Available online 19 August 2005

Abstract

High-speed counter-current chromatography (HSCCC) was applied to the separation and purification of five compounds from the Chinese medicinal herb *Polygonum cuspidatum* Sieb. et Zucc. The crude extracts from *P. cuspidatum* Sieb. et Zucc were treated with light petroleum–ethyl acetate–methanol–water (2:5:4:6, v/v). Sample 1 was obtained from the lower phase and sample 2 from the upper phase. The sample 1 was separated with light petroleum–ethyl acetate–water (1:5:5, v/v) and yielded 19.3 mg of piceid, 17.6 mg of anthraglycoside B from 200 mg of sample 1. The sample 2 was separated with light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) and light petroleum–ethyl acetate–methanol–water (3:5:7:3, v/v) in a gradient elution and yielded 18.5 mg of resveratrol, 35.3 mg of emodin and 8.2 mg of physcion from 220 mg of sample 2. The purity of each compound is over 95% as determined by HPLC. The chemical structures of these components were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: *Polygonum cuspidatum* Sieb. et Zucc; HSCCC; Piceid; Anthraglycoside B; Resveratrol; Emodin; Physcion

1. Introduction

Polygonum cuspidatum Sieb. et Zucc, a well-known traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia [1], has been traditionally used for treatment of various inflammatory diseases, hepatitis, tumors, and diarrhea in East Asian countries such as China, Korea, Taiwan, and Japan [2]. Extracts of *P. cuspidatum* has also been mentioned to inhibit several kinds of virus [3]. Moreover, it possessed the antiviral activity against HBV [4]. The major components of *P. cuspidatum*, including piceid, resveratrol, anthraglycoside B, emodin and physcion, each have specific pharmaceutical activity. Resveratrol and piceid have effects of inhibiting the copper-catalyzed oxidation of low-density lipoprotein [5], inhibiting platelet clotting and arachidonate metabolism, reducing liver injury from peroxidized oil [6], and having cancer-chemopreventive activities [7]. Anthraglycoside B has been used for the treatment of acute hepatitis and symptoms of the reduction of leucocytes [8]. Some findings indicated that emodin is phytoestrogen with an affinity to human estrogen receptor [9]. Piceid, resveratrol and anthroglycosides B with high purity are needed for the quality control of products from *P. cuspidatum* or other related products. So it is important to develop the method for isolation and purification of all these compounds. The chemical structures of them are shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a kind of support-free all-liquid partition chromatography which was first invented by Ito [10]. Successful application of HSCCC for the separation and purification of resveratrol, piceid and anthraglycoside B from the Chinese herb *P. cuspidatum* has been reported previously [11,12]. The sepa-

* Corresponding author. Tel.: +86 6358230600.
E-mail address: renminliu@lctu.edu.cn (R. Liu).

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doi:10.1016/j.chroma.2005.08.008

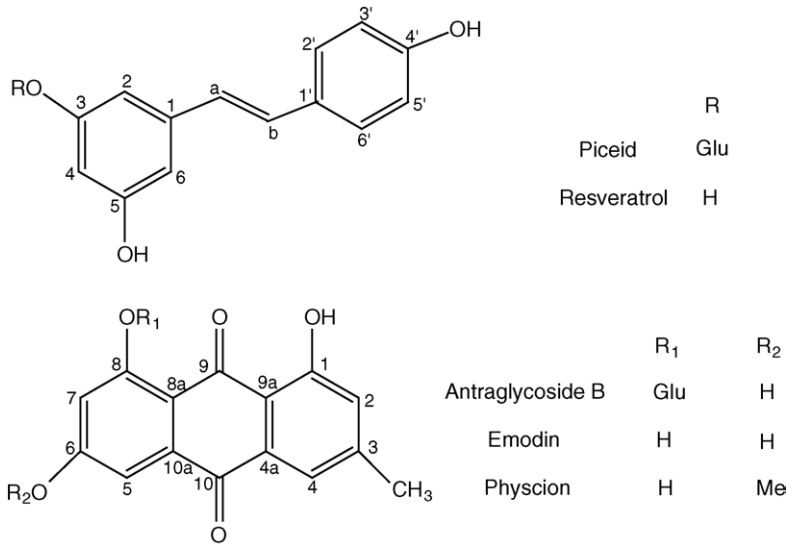

Fig. 1. Chemical structures of target compounds from *P. cuspidatum*.

ration was used with a two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v) and piceid was separated by a two-step HSCCC separation. Chloroform is a kind of deleterious organic solvent, which polluted the environment seriously and is not suitable for the separation of the Chinese traditional medicine in industry. In this paper, the two-phase solvent system composed of light petroleum–ethyl acetate–water (1:5:5, v/v) was applied to the separation and purification of piceid (I) and anthraglycoside B (II); a gradient elution with a pair of two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 3:5:4:6 and 3:5:7:3 was used for the separation and purification of resveratrol (III), emodin (IV) and physcion (V). The solvents used in this experiment were propitious to the protection of the environment. The purity of piceid, anthraglycoside B, resveratrol, emodin and physcion was 97.5%, 97.1%, 98.7%, 99.3% and 98.5%, respectively, as determined by HPLC.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Shanghai Tauto Biotech Co. Ltd., Shanghai, China) with three multilayer coil separation column connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at 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. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. A ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including G1311A QuatPump, G1315B UV–vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μl loop, G1332 degasser and Agilent HPLC workstation (Agilent Technologies, Germany).

The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian Inc., USA).

A FZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for disintegration of the sample.

2.2. Reagents and materials

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). The boiling point range of the light petroleum used for all experiments was 60–90 °C). Methanol used for HPLC was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water.

P. cuspidatum was purchased from a local drug store and was identified as the dried root of *P. cuspidatum* Sieb. et Zucc by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of crude sample

The dried roots of *P. cuspidatum* Sieb. et Zucc were ground to powder (about 40 mesh) by using the FZ102 plant disintegrator. One hundred grams of the powder was extracted with 500 ml of 95% methanol for 24 h at room temperature. The extraction procedure was repeated three times. The extracts were combined and evaporated to dryness under reduced pressure, which yielded 11.3 g of crude extracts. The crude extracts were dissolved in the mixture of 500 ml of the lower phase and 500 ml of the upper phase of light petroleum–ethyl acetate–methanol–water (2:5:4:6, v/v). The two phases were separated after the phase separation reached. The remained lower phase solution was then extracted with 500 ml of the upper phase for twice. 4.6 g extracts (sample 1) was obtained after evaporated the lower phase extracted solution to dryness and 6.7 g extracts (sample 2) was obtained after combined and evaporated the upper phase extracted solutions to dryness.

Sample 1 was used for HSCCC separation of piceid, anthraglycoside B and sample 2 for resveratrol, emodin and physcion.

2.4. Selection of the two-phase solvent systems

The composition of the two-phase solvent system was selected according to the partition coefficient (K) of target compounds of crude example. The partition coefficients were determined by HPLC as follows: suitable amount of crude example was dissolved in 2 ml of aqueous phase of the pre-equilibrated two-phase solvent system. The solution was determined by HPLC and the peak area was recorded as A_1. Then equal volume of the organic phase was added to the solution and mixed thoroughly. After the equilibration was established, the aqueous phase was determined by HPLC again and the peak area was recorded as A_2. The partition coefficient (K) was obtained by the following equation: $K = (A_1 - A_2)/A_2$.

2.5. HSCCC separation

In each separation process, the upper phase (stationary phase) and the lower phase (mobile phase) were pumped into the multiplayer-coiled column simultaneously by using ÄKTA prime system, according to the volume ratio of 50:50. When the column was totally filled with the two phases, only the lower phase was pumped at a flow rate of 1.5 ml min^{-1}, and at the same time, the HSCCC apparatus was run at a revolution speed of 700 rpm. After hydrodynamic equilibrium was reached (about half an hour later), the sample solution was injected into the separation column. The separation temperature was controlled at 20 °C. The effluent from the outlet of the column was continuously monitored at 254 nm. The chromatogram was recorded 80 min after sample injection. 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.

2.5.1. Separation of piceid and anthraglycoside B by HSCCC

A solvent system consisting of light petroleum–ethyl acetate–water (1:5:5, v/v) was used for piceid (I) and anthraglycoside B (II) HSCCC separation. 19.3 mg of piceid and 17.6 mg of anthraglycoside B were obtained from 200 mg of the sample 1 with the purity of 97.5% and 97.1%, respectively, as determined by HPLC.

2.5.2. Separation of resveratrol, emodin and physcion by HSCCC

The gradient elution with a pair of two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 3:5:4:6 and 3:5:7:3 was used for resveratrol (III), emodin (IV) and physcion (V) HSCCC separation. The gradient elution was as follows: in the first 120 min, only the lower phase of light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) was pumped. Then the volume ratio of the lower phase of light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) and (3:5:7:3, v/v) was continuously changed from 100:0 to 0:100 in 100 min.

2.6. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. The analysis was accomplished with a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. Methanol–water system was used as the mobile phase in gradient mode as follows: methanol: 0–15 min, 30–50%; 15–35 min, 50–90%; 35–45 min, 95%. The flow-rate of the mobile phase was 1.0 ml min^{-1}. The effluents were monitored at 254 nm by a photodiode array detector.

Identification of the HSCCC peak fractions was performed by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Mercury Plus 400 NMR.

3. Results and discussion

3.1. Optimization of HPLC conditions

Several elution systems were tested in HPLC separation of crude sample, such as gradient elution of methanol–water, acetonitrile–water, methanol–acetonitrile–water, etc. When methanol–water was used as the mobile phase in gradient mode (methanol: 0–15 min, 30–50%; 15–35 min, 50–90%; 35–45 min, 95%), good results could be obtained. The crude samples and peak fractions separated by HSCCC were analyzed by HPLC under the optimum analytical conditions. The chromatograms were shown in Fig. 2.

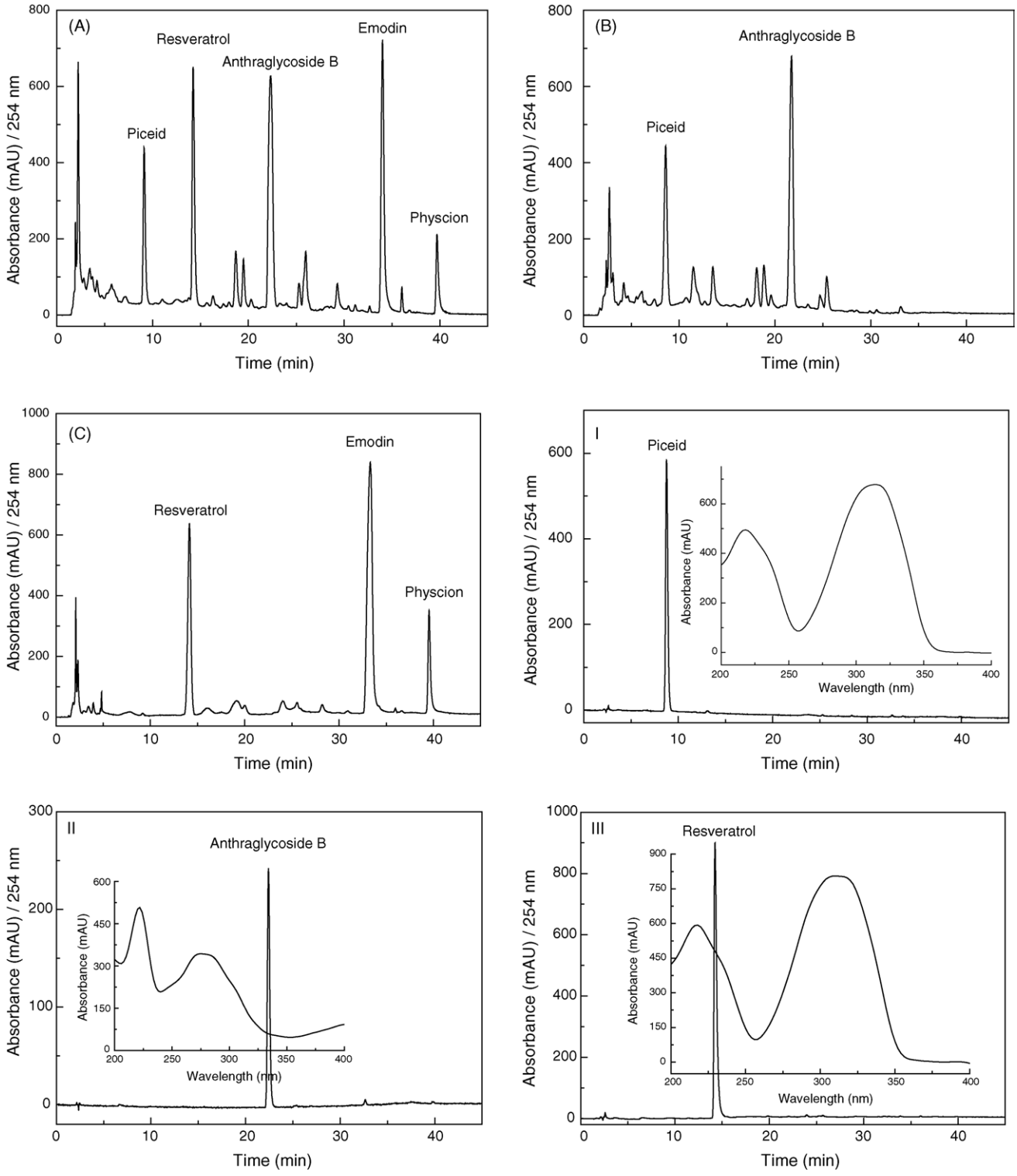

Fig. 2. HPLC chromatograms of crude extract (A), sample 1 (B), sample 2 (C) from *P. cuspidatum*, and HSCCC peak fractions (I–V), Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 10 μm); mobile phase: methanol–water (methanol: 0–15 min, 30–50%; 15–35 min, 50–90%; 35–45 min, 95%); flow rate: 1.0 ml min^{-1}; detection wavelength: 254 nm.

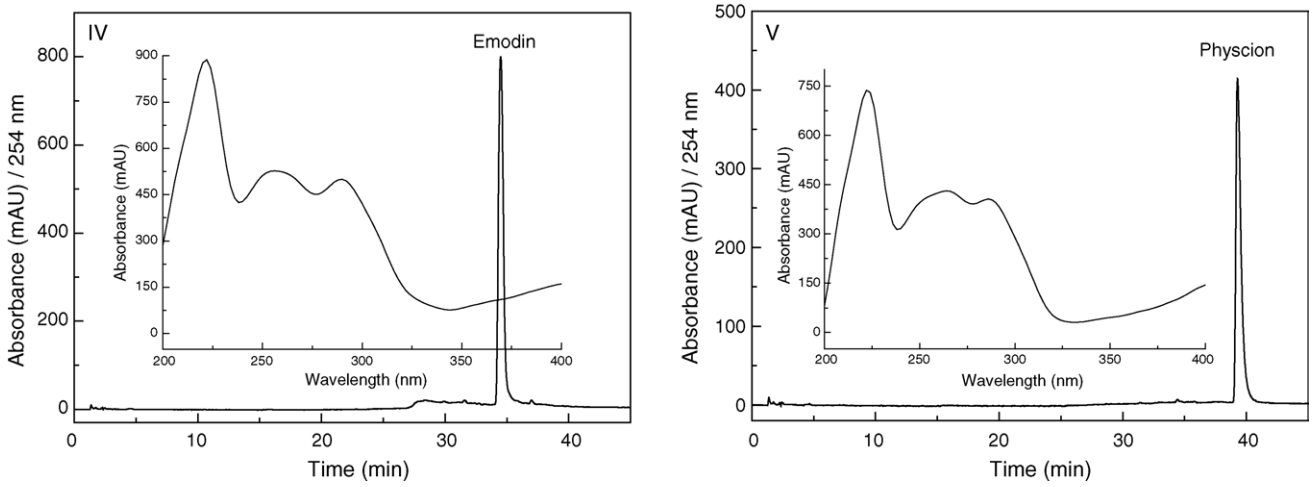

Fig. 2. (*Continued*).

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

Successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (*K*) for the targeted compounds. Several two-phase solvent systems were tested and the *K*-values were measured and summarized in Table 1.

It can be seen from Table 1 that the *K*-values of all the target compounds were too big when ethyl acetate–water and ethyl acetate–methanol–water were used as the two-phase solvent system. The *K*-values could be reduced to some degree by increase the volume ratio of methanol. But the phase separation became unsatisfactory when the volume ratio of methanol was high. So ethyl acetate–methanol–water system was unsuitable for separation of target compounds from *P. cuspidatum*.

The addition of light petroleum to ethyl acetate–methanol–water system could improve the phase separation. When light petroleum-ethyl acetate–methanol–water (1:5:1:5, v/v) was used for HSCCC separation, the *K*-values of piceid and anthraglycoside B were suitable for HSCCC separation, but the *K*-values of resveratrol, emodin and physcion were too big. When light petroleum–ethyl acetate–methanol–water (1:5:4:5, v/v) was used for HSCCC separation, the *K*-values of piceid and anthraglycoside B were very small while the *K*-values of emodin and physcion were very big. When light petroleum–ethyl acetate–methanol–water (1:5:7:3, v/v) was used as the two-phase solvent system, the *K*-values of emodin and physcion were suitable, but that of piceid, resveratrol and anthraglycoside B were very small. So HSCCC separation could not be performed with the single two-phase solvent system mentioned above. The gradient elution mode was tested for the HSCCC separation with light petroleum–ethyl acetate–methanol–water (1:5:1:5, v/v) and light petroleum–ethyl acetate–methanol–water (1:5:7:3, v/v). The stationary phase of HSCCC lost seriously in gradient elution mode. A pair of two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 2:5:1:9 and 2:5:7:3 was also tested for HSCCC separation in gradient elution. The stationary phase of HSCCC still lost

Table 1
K-values of the target compounds in several two-phase solvent systems

Solvent system (v/v)	*K*				
	Piceid	Resveratrol	Anthraglycoside B	Emodin	Physcion
E–W (5:5)	2.43	24.52	6.81	150.3	–
E–M–W (5:1:5)	2.25	21.34	5.22	138.5	–
E–M–W (5:2:5)	2.12	17.88	4.35	121.9	–
L–E–W (1:5:5)	0.41	41.2	1.55	–	–
L–E–M–W (1:5:1:5)	0.36	31.4	1.46	–	–
L–E–M–W (1:5:4:5)	0.06	1.83	0.42	21.8	30.3
L–E–M–W (1:5:7:3)	0.01	0.05	0.01	1.20	4.08
L–E–M–W (2:5:1:9)	0.09	38.3	0.62	–	–
L–E–M–W (2:5:4:6)	0.01	1.73	0.09	42.8	53.9
L–E–M–W (2:5:7:3)	0.01	0.05	0.01	1.08	3.83
L–E–M–W (3:5:4:6)	0.03	1.28	0.04	34.6	40.9
L–E–M–W (3:5:7:3)	0.01	0.04	0.01	0.99	1.56

"–" means the *K*-value is very big. E: ethyl acetate; W: water; L: light petroleum; M: methanol.

seriously. Light petroleum–ethyl acetate–methanol–water with the volume ratio of 3:5:4:6 and 3:5:7:3 were also used as the two-phase solvent system. But the *K*-values of piceid and anthraglycoside B were very small in these systems. So the separation of the five target compounds by gradient elution in a one-step separation cannot be achieved.

According to the *K*-values of the target compounds in light petroleum–ethyl acetate–methanol–water (2:5:4:6, v/v), piceid and anthraglycoside B mainly distributed in the lower phase, while resveratrol, emodin and physcion mainly in the upper phase. So the crude extracts were treated with light petroleum–ethyl acetate–methanol–water (2:5:4:6, v/v) system first and divided into two parts: sample 1 and sample 2. Sample 1 was used for separation of piceid and anthraglycoside B, and sample 2 for resveratrol, emodin and physcion.

Many two-phase solvent systems could be used for the separation of piceid and anthraglycoside B from sample 1, according to the *K*-values given in Table 1. When light petroleum–ethyl acetate–methanol–water (1:5:1:5, v/v) was used as the two-phase solvent system, the purity of piceid was lower than 90%. When light petroleum–ethyl acetate–water (1:5:5, v/v) was used as the two-phase solvent system, good separation results could be obtained. So light petroleum–ethyl acetate–water (1:5:5, v/v) was chosen for separation and purification of piceid and anthraglycoside B from sample 1.

The *K*-value of resveratrol, emodin and physcion shown in light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) was 1.28, 34.6 and 40.9, respectively, and 0.04, 0.99 and 1.56 in light petroleum–ethyl acetate –methanol–water (3:5:7:3, v/v). There is no possibility for separation and purification of these compounds by using a single two-phase solvent system. So the gradient elution mode was tested. The upper phase of light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) was used as the stationary phase. When the mobile phase was the lower phase of light petroleum–ethyl acetate–methanol–water (3:5:4:6, v/v) and the lower phase of light petroleum–ethyl acetate–methanol–water (3:5:7:3, v/v) in gradient elution mode (0–100 min, only the lower phase of 3:5:4:6 system; 100–200 min, the volume ratio of 3:5:7:3 system was continuously changed from 0% to 100%), good separation results could be obtained.

Other conditions such as the flow rate of the mobile phase, the revolution speed of the separation column were also investigated. When the flow rate of 2.0 ml min^{-1}, revolution speed of 900 rpm were employed in HSCCC separation, the stationary phase lost seriously. Reducing the flow rate of the mobile phase and the revolution speed of the separation column could improve the maintenance of the stationary phase. At last, a flow rate of 1.5 ml min^{-1}, revolution speed of 700 rpm was employed in HSCCC separation.

Samples 1 and 2 were separated and purified under the optimum HSCCC conditions. The typical HSCCC chromatograms were shown in Fig. 3. 19.3 mg of piceid (I), 17.6 mg of anthraglycoside B (II) were obtained from 200 mg of sample 1 in a one-step separation. 18.5 mg of resveratrol (III), 35.3 mg of emodin (IV) and 8.2 mg of physcion (V) were obtained from 220 mg of sample 2. The purity of piceid, anthraglycoside B, resveratrol, emodin and physcion was 97.5%, 97.1%, 98.7%, 99.3% and 98.5%, respectively, as determined by HPLC. The chromatograms of HPLC and UV spectra of these compounds were shown in Fig. 2.

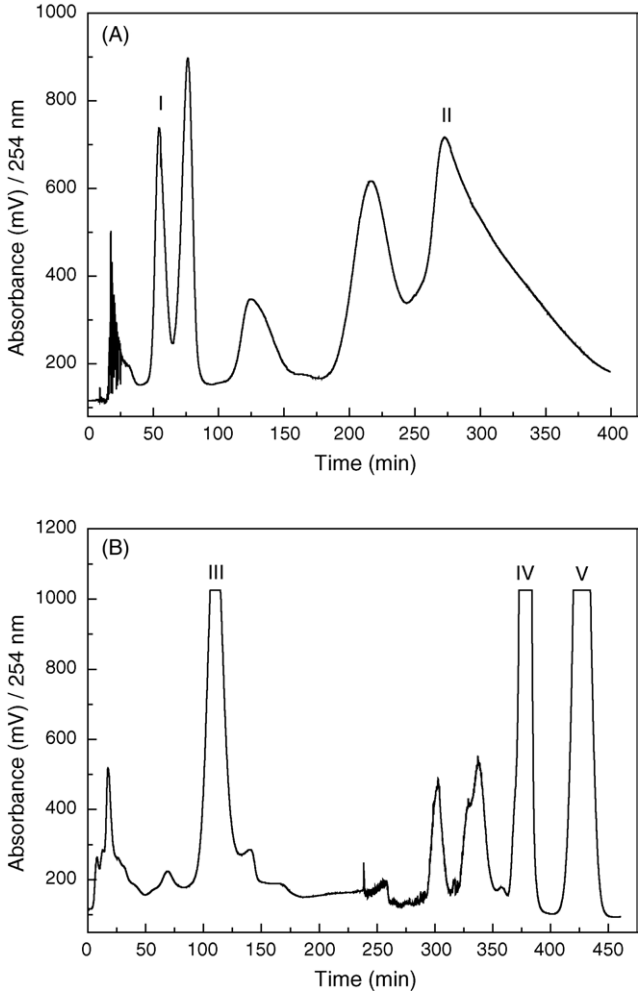

Fig. 3. HSCCC chromatograms of sample 1 (A) and sample 2 (B) from *P. cuspidatum*. Conditions of A: Two-phase solvent system: light petroleum–ethyl acetate–water (1:5:5, v/v); mobile phase: the lower phase; flow rate: 1.5 ml min^{-1}; revolution speed: 700 rpm; detection wavelength: 254 nm; sample size: 200 mg of the sample 1 dissolved in 2 ml of the upper phase and 2 ml of the lower phase; separation temperature: 20 °C. Conditions of B: Two-phase solvent system: light petroleum–ethyl acetate–methanol–water (3:5:4:6, 3:5:7:3, v/v) in gradient elution; stationary phase: upper organic phase of 3:5:4:6; mobile phase: lower aqueous phase of 3:5:4:6 in 0–100 min, lower aqueous phase from 3:5:4:6 to 3:5:7:3 in 100–200 min; flow rate: 1.5 ml min^{-1}; revolution speed: 700 rpm; detection wavelength: 254 nm; sample size: 220 mg of sample 2 dissolved in 2 ml of the upper phase and 2 ml of the lower phase; separation temperature: 20 °C.

3.3. The structural identification

The chemical structure of each peak fraction of HSCCC was identified according to its ^{1}H NMR and ^{13}C NMR data.

Peak I: ^{1}H NMR (400 MHz, DMSO) δ: 9.46 (2H, s, –OH), 7.38 (2H, d, H-2′, 6′), 7.02 (1H, d, J = 16.4 Hz, H-b), 6.84 (1H, d, J = 16.4 Hz, H-a), 6.74 (1H, d, H-3′, 5′), 6.70 (1H, s, H-2), 6.54 (1H, s, H-6), 6.31 (1H, s, H-4), 4.62–5.27 (sugar –OH), 3.16–3.70 (sugar C–H). ^{13}C NMR (100 MHz, DMSO) δ: 139.4 (C-1), 107.2 (C-2), 158.9 (C-3), 104.8 (C-4), 158.3 (C-5), 109.3 (C-6), 125.2 (C-a), 128.0 (C-b), 128.6 (C-1′), 127.9 (C-2′), 115.5 (C-3′), 157.3 (C-4′), 115.5 (C-5′), 127.9 (C-6′), sugar C1-6: 102.8, 73.3, 77.1, 69.8, 76.7, 60.8. Compared with the data given in references [7,8], peak I corresponded to piceid.

Peak II: ^{1}H NMR (400 MHz, DMSO) δ: 13.23 (1H, s, –OH), 7.43 (1H, s, H-4), 7.23 (1H, s, H-5), 7.13 (1H, s, H-2), 6.94 (1H, s, H-7), 4.61–5.09 (sugar –OH), 3.21–3.71 (sugar C–H), 2.35 (3H, s, –CH_3). ^{13}C NMR (100 MHz, DMSO) δ: 164.2 (C-1), 119.6 (C-2), 148.9 (C-3), 124.2 (C-4), 109.3 (C-5), 161.2 (C-6), 109.3 (C-7), 161.7 (C-8), 186.2 (C-9), 182.3 (C-10), 132.2 (C-4a), 113.2 (C-8a), 114.4 (C-9a), 136.5 (C-10a), 21.4 (CH_3), sugar C1-6: 101.0, 73.3, 76.3, 69.4, 77.3, 60.6. Compared with the data given in reference [13], peak II corresponded to anthraglycoside B.

Peak III: ^{1}H NMR (400 MHz, DMSO) δ: 9.52 (1H, s, –OH), 9.17 (2H, s, –OH), 7.39 (1H, d, H-2′, 6′), 6.90 (1H, d, J = 16.4 Hz, H-b), 6.78 (1H, d, J = 16.4 Hz, H-a), 6.73 (2H, d, H-3′, 5′), 6.37 (2H, d, H-2, 6), 6.11 (1H, s, H-4). ^{13}C NMR (100 MHz, DMSO) δ: 139.2 (C-1), 104.3 (C-2), 158.5 (C-3), 101.7 (C-4), 158.5 (C-5), 104.26 (C-6), 125.6 (C-a), 127.8 (C-b), 128.0 (C-1′), 127.8 (C-2′), 115.5 (C-3′), 157.2 (C-4′), 139.2 (C-5′), 104.3 (C-6′). Compared with the data given in references [7,8], peak III corresponded to resveratrol.

Peak IV (emodin): ^{1}H NMR (400 MHz, DMSO) δ: 11.96, 12.04 (1H, each, s, OH), 7.42 (1H, s, H-4), 7.11 (1H, s, H-5), 7.07 (1H, s, H-2), 6.56 (1H, s, H-7), 2.38 (3H, s, CH_3). ^{13}C NMR (100 MHz, DMSO) δ: 164.4 (C-1), 120.4 (C-2), 148.2 (C-3), 124.0 (C-4), 108.8 (C-5), 161.4 (C-6), 107.9 (C-7), 165.6 (C-8), 189.6 (C-9), 181.2 (C-10), 132.7 (C-4a), 108.7 (C-8a), 113.3 (C-9a), 135.0 (C-10a), 21.5 (–CH_3). Compared with the data given in reference [14], peak IV corresponded to emodin.

Peak V: ^{1}H NMR (400 MHz, DMSO + $CDCl_3$) δ: 12.19, 12.06 (1H each, s, OH), 7.64 (1H, s, H-4), 7.32 (1H, s, H-5), 7.09 (1H, s, H-2), 6.74 (1H, s, H-7), 3.92 (3H, s, OCH_3), 2.42 (3H, s, CH_3). ^{13}C NMR (100 MHz, DMSO + $CDCl_3$) δ: 164.7 (C-1), 120.7 (C-2), 148.0 (C-3), 124.0 (C-4), 107.8 (C-5), 161.9 (C-6), 106.1 (C-7), 166.1 (C-8), 190.2 (C-9), 181.6 (C-10), 132.7 (C-4a), 110.2 (C-8a), 113.1 (C-9a), 134.7 (C-10a), 55.7 (OCH_3), 21.7 (–CH_3). Compared with the data given in references [14,15], peak V corresponded to physcion.

Acknowledgements

Jichun Cui was greatly acknowledged for his help in structure identification. We also thank all of our colleagues for their excellent assistance.

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