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Preparative isolation and purification of coumarins from *Peucedanum praeruptorum* Dunn by high-speed counter-current chromatography

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of coumarins from *Peucedanum praeruptorum* Dunn (Baihuaqianhu in Chinese) was successfully established by using light petroleum–ethyl acetate–methanol–water as the two-phase solvent system in gradient elution mode. The upper phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the stationary phase of HSCCC. The mobile phase used in HSCCC was the lower phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) and light petroleum–ethyl acetate–methanol–water (5:5:6.5:3.5, v/v) that was changed in gradient. Four kinds of coumarins and another unknown compound were obtained and yielded 5.3 mg of qianhucoumarin D, 7.7 mg of Pd–Ib, 35.8 mg of (+)-praeruptorin A, 31.9 mg of (+)-praeruptorin B and 6.4 mg of unknown compound with the purity of 98.6%, 92.8%, 99.5%, 99.4% and 99.8% in one-step separation, respectively. The structures of the coumarins were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; Preparative chromatography; *Peucedanum praeruptorum* Dunn; (+)-Praeruptorin A; (+)-Praeruptorin B; Qianhucoumarin D

1. Introduction

Baihuaqianhu, the dried roots of *Peucedanum praeruptorum* Dunn, is one of the most popular traditional medicinal herb which has been widely used in traditional Chinese medicine for over 1500 years [1] and is officially listed in the Chinese Pharmacopoeia [2]. Coumarins are major bioactive components in *P. praeruptorum* Dunn including (+)-praeruptorin A, (+)-praeruptorin B, Pd–Ib, qianhucoumarin D etc. Their chemical structures are given in Fig. 1. Original studies indicated that these compounds have prominent effect on digestive system, respiratory system and hemal system [3]. Recently, human and animal studies demonstrated that they also have the effect of anticancer [4,5], antileukemia [6], vasodilatation, protection of cardiac muscles from anoxia, reduced platelet aggregation and thrombus formation [7–13]. The conventional separation method of coumarins in *P. praeruptorum* Dunn is silica gel column chromatography, which is time consuming and has the peril of loss of compounds due to the violent adsorptive effect of the solid matrix.

High-speed counter-current chromatography (HSCCC), first invented by Y. Ito [14], a liquid–liquid partition chromatographic technique that uses no support matrix and the liquid stationary phase is immobilized by centrifugal force, eliminates irreversible adsorption of sample onto the solid support used in the conventional chromatographic column. What's more, it has a large of sample injection, multiform relative pure substances can obtain at one time in large amount. So, it is especially suitable for separation and purification of active components from natural products [15–22].

The present paper describes the separation and purification of coumarins from light petroleum extract of

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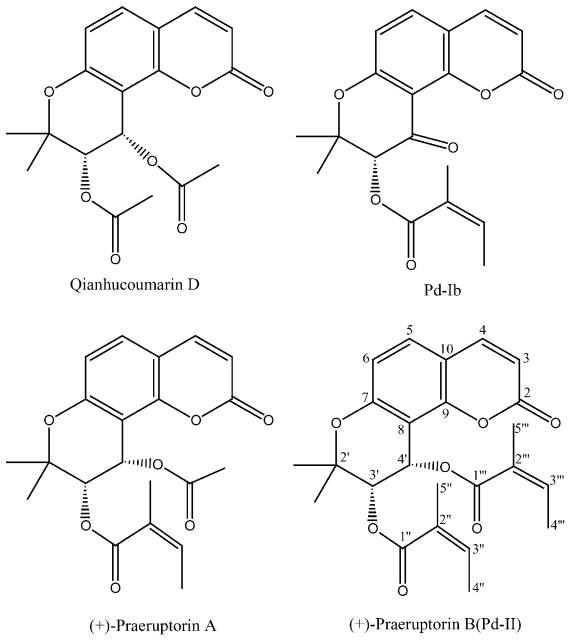

Fig. 1. The chemical structures of coumarins in *Peucedanum praeruptorum* Dunn.

P. praeruptorum Dunn by HSCCC. In the process of separation, two-phase solvent system composed of light petroleum–ethyl acetate–methanol–water at volume ratios of 5:5:5:5, 5:5:6.5:3.5 were used in gradient elution. Four kinds coumarins including qianhucoumarin D (I), Pd–Ib (II), (+)-praeruptorin A (III), (+)-praeruptorin B (IV) and another unknown compound were obtained with the purity of 98.6%, 92.8%, 99.5%, 99.4% and 99.8% in one-step separation, respectively.

2. Experimental

2.1. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, 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 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. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. An ÄKTA prime (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 can be used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd., Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including G1311A solvent delivery unit, G1315B UV–vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μl loop, G1332A degasser and Agilient HPLC workstation.

The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian Inc., America). Optical rotations were recorded with a PE-241 MC polarimeter at the sodium D-line and electron impact ionization (EI) MS spectra were measured with a HP5989 A instrument.

2.2. Reagents

All organic solvent used for preparation of crude sample and HSCCC separation was of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was Chromatographic grade (Yucheng Chemical Plant, Yucheng, China), and water was distilled water.

The dried roots of *P. praeruptorum* Dunn were purchased from a local drug store and identified by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.3. Preparation of sample

The dried roots of *P. praeruptorum* Dunn were shattered to powder (about 30 mesh) and extracted with 1500 mL of boiling light petroleum (boiling range 60–90 °C) four times. The extraction time was 2 h, 2 h, 1 h and 1 h, respectively. Then the light petroleum solutions were combined and evaporated to about 100 mL by rotary vaporization at 40 °C under reduced pressure. The enriched liquid was frozen under −4 °C for 24 h. The deposit was separated and dried, 5.27 g of crude sample were obtained. It was stored in a refrigerator for subsequent HSCCC separation. The sample solutions were prepared by dissolving the crude extracts in the stationary phase of HSCCC.

2.4. Selection of two-phase solvent system

Light petroleum–ethyl acetate–methanol–water was used as the two-phase solvent system. The composition of the two-phase was selected according to the partition coefficient (K). The K-values were determined by HPLC as follows: about 0.1 mg of sample was added to a test tube, to which 2 mL of each phase of the two-phase solvent system was added. The test tube was shaken violently for several minutes, then the upper and lower phase were analyzed by HPLC. The partition coefficients of all components in sample were obtained according to the peak areas.

2.5. Separation procedure

The upper phase (stationary phase) and the lower phase (mobile phase) of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) were pumped into the multilayer-coiled column simultaneously by using an Äkta prime system, according to the volume ratio of 55:45. When the column was totally filled with the two phases, only the lower phase was pumped at a flow rate of 2.0 mL min^{-1}, and at the same time, the HSCCC apparatus was run at a revolution speed of 900 rmp. After hydrodynamic equilibrium was reached, 5 mL of crude sample solution (22 mg mL^{-1}) was injected into the separation tube through the sample port, and at the same time the gradient elution started. The volume ratio of the lower phase of 5:5:5:5 system and 5:5:6.5:3.5 system changed as follows: 0–150 min, 100:0; 150–300 min, 100:0 to 0:100; after 300 min, 0:100. The effluent from the tail end of the column was continuously detected with the Äkta prime system at 254 nm. The chromatogram was recorded 20 min after sample injection. Each peak fraction was collected according to the chromatogram.

2.6. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each peak fraction of HSCCC were analyzed by HPLC with a Spherigel ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm) at room temperature. Methanol and water were used as the mobile phase in gradient mode. The volume ratio of methanol/water was changed from 75:25 to 80:20 in 30 min. The flow-rate of the mobile phase was 0.5 mL min^{-1}, and the effluents were monitored at 254 nm by a photodiode array detector.

Identification of HSCCC peak fraction was performed by optical rotation data, ^{1}H NMR, ^{13}C NMR and EI-MS spectra. The UV spectra were taken from the HPLC three-dimensional spectrum of absorbance versus time and wavelength.

3. Results and discussion

3.1. Optimization of conditions of HPLC

A series of elution systems were tested in separation of the coumarins in crude extract sample of *P. praeruptorum* Dunn. Methanol–water and methanol–acetonitrile–water at various volume ratio and different gradient mode were tested. The results indicated that when methanol–water system was used as the mobile phase in gradient mode (75:25 to 80:20 in 30 min) with the flow rate of 0.5 mL min^{-1}, satisfying separation results could be obtained. The crude sample and the peak fractions separated by HSCCC were analyzed by HPLC under the optimum conditions. The chromatograms are shown in Fig. 2.

Table 1
The *K*-values of target components in light petroleum–ethyl acetate–methanol–water

Solvent system (v/v)	*K* (I)	*K* (II)	*K* (III)	*K* (IV)
5:5:5:5	1.10	1.74	4.48	10.32
5:5:5.5:4.5	0.83	1.27	2.81	7.65
5:5:6:4	0.43	0.64	2.18	4.99
5:5:6.5:3.5	0.24	0.31	1.28	2.33

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

Lots of experiments were performed to optimize the two-phase solvent system for the HSCCC separation. The partition coefficients of the target compounds in different solvent systems are shown in Table 1. According to the *K* shown in Table 1, some solvent systems were tested. The results indicated that when light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the solvent system, very long time was needed for the separation and the shape of the posterior peaks were very bad. When light petroleum–ethyl acetate–methanol–water (5:5:6.5:3.5, v/v) was used, the shape of the posterior peaks improved very much, but the anterior peaks could not be separated. So gradient elution was used in the HSCCC separation. When the upper phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the stationary phase, and the lower phases of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) and light petroleum–ethyl acetate–methanol–water (5:5:6.5:3.5, v/v) were used as the mobile phase in gradient elution mode (0–150 min, only the lower phase of 5:5:5:5; 150–300 min, the lower phase of 5:5:6.5:3.5 changed from 0% to 100%), good separation results could be obtained and the separation time was acceptable.

The influence of flow rate of mobile phase, the separation temperature and revolution speed were also investigated. The results indicated that reducing flow speed can improve separation impact in some degree, but at the same time the chromatogram peaks were extended. Considering the two aspects, the flow rate of the mobile phase was set at 2 mL min^{-1} in this experiment. The temperature has significant effect on *K* (partition coefficient), the retention of percentage of stationary phase and the mutual solvency of the two-phase. After tested at 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, it can be seen that good results can be obtained when the separation temperature was controlled at 35 °C. The rotary speed of the separation coil tube has great influence on the retention percentage of the stationary phase. Expediting the rotary can increase the retention of the stationary phase. In this experiment, all separations were performed at 900 rpm.

The crude samples from *P. praeruptorum* Dunn were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram is shown in Fig. 3. Four kinds of coumarins and another unknown compound were obtained and yielded 5.3 mg of qianhucoumarin D (I), 7.7 mg of Pd–Ib

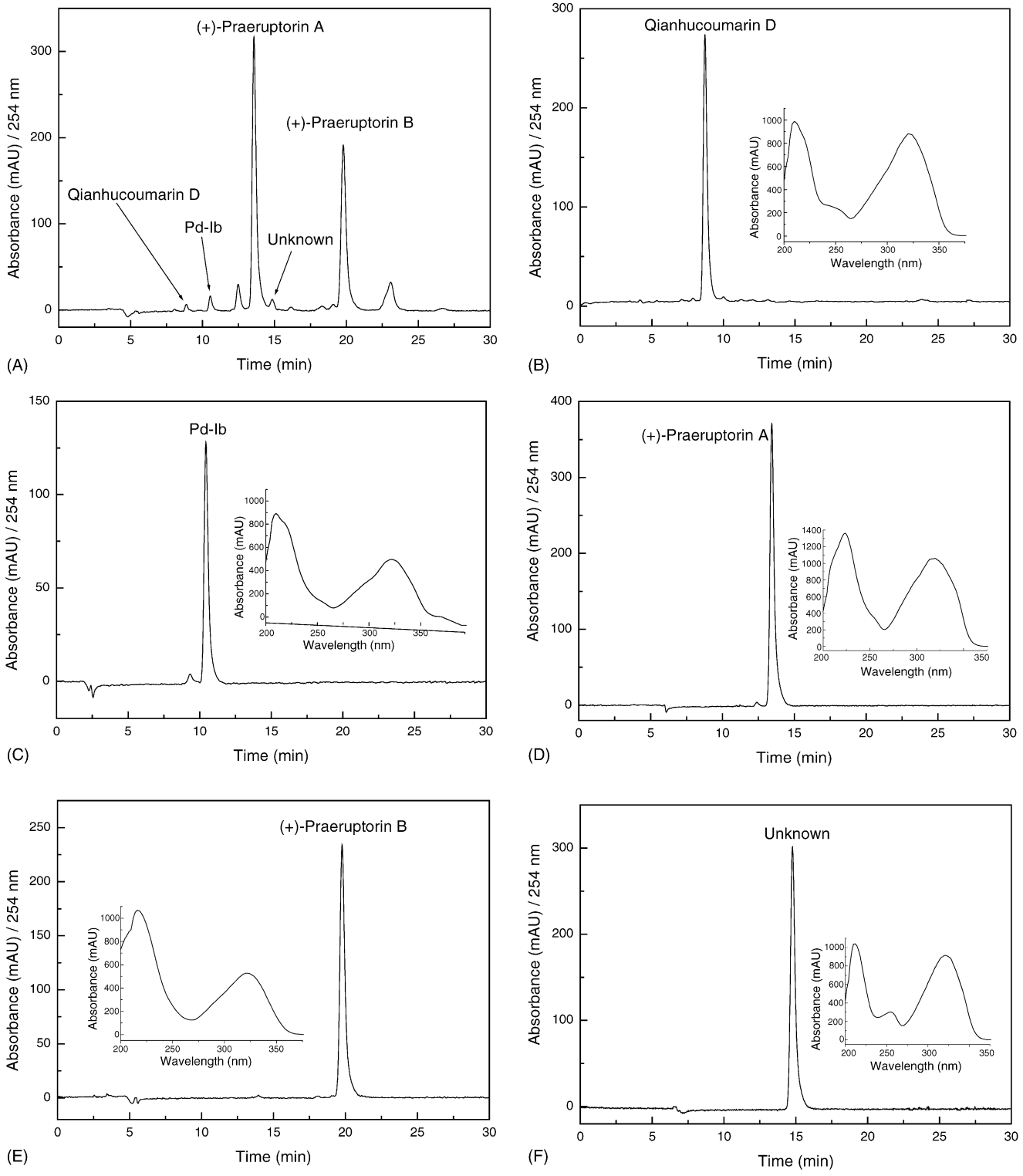

Fig. 2. The HPLC chromatograms of crude extract and HSCCC peak fractions of crude extract from *Peucedanum praeruptorum* Dunn; (B)–(E), (F) HSCCC peak fractions I–IV and *X* in Fig. 3., column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm i.d., 5 μm); column temperature: 25 °C; mobile phase: methanol–water in gradient mode as follows: 75:25 to 80:20 in 30 min, flow rate: 0.5 mL min^{-1}, detection wavelength: 254 nm.

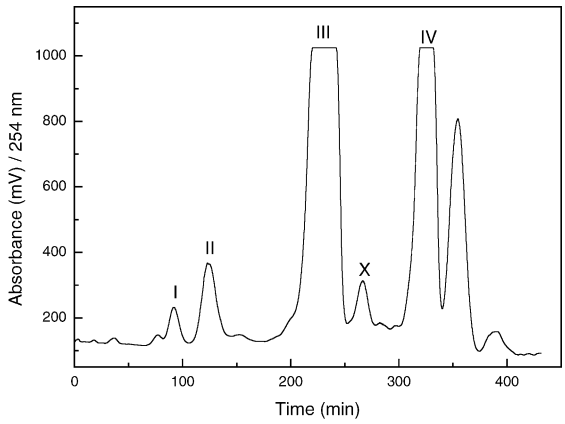

Fig. 3. Chromatogram of crude extract from *Peucedanum praeruptorum* Dunn by HSCCC. Stationary phase: upper phase of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v); mobile phase: lower phases of light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) and (5:5:6.5:3.5, v/v) in gradient elution (0–150 min, only the lower phase of 5:5:5:5; 150–300 min, the proportion of 5:5:5:5 changed from 100 to 0); flow-rate: 2.0 mL min^{-1}; revolution speed: 900 rpm; sample size: 110 mg dissolved in 5 mL of stationary phase; retention percentage of the stationary phase: 55%.

(II), 35.8 mg of (+)-praeruptorin A (III), 31.9 mg of (+)-praeruptorin B (IV) and 6.4 mg of unknown compound (*X*) with the purity of 98.6%, 92.8%, 99.5%, 99.4% and 99.8% in one-step separation, respectively. The chromatograms of HPLC and UV spectra of these compounds were shown in Fig. 2.

3.3. The structural identification

The structural identification of peak fractions of HSCCC was performed according to optical rotation data, ^{1}H NMR data, ^{13}C NMR data and EIMS spectra.

Peak I: $[\alpha]_D^{25}$ +9.5° (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): 6.25 (1H, d, *J* = 9.4 Hz, H-3), 7.60 (1H, d, *J* = 9.4 Hz, H-4), 7.36 (1H, d, *J* = 8.6 Hz, H-5), 6.80 (1H, d, *J* = 8.6 Hz, H-6), 5.31 (1H, d, *J* = 5.0 Hz, H-3′), 6.54 (1H, d, *J* = 5.0 Hz, H-4′), 1.42 (3H, s, C-2′–CH_3), 1.45 (3H, s, C-2′–CH_3), 2.14 (3H, s, H-2″), 2.11 (3H, s, H-2‴). EI-MS *m*/*z*: 346 (M^+), 286, 245, 244, 230, 229 (100), 213, 204, 191, 162, 134, 72 and 43. Comparing the above data with Ref. [23], the obtained product was identified as qianhucoumarin D.

Peak II: $[\alpha]_D^{25}$ +41.6° (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, C^2HCl_3): 6.33 (1H, d, *J* = 9.6 Hz, H-3), 7.61 (1H, d, *J* = 9.6 Hz, H-4), 7.55 (1H, d, *J* = 8.6 Hz, H-5), 6.88 (1H, d, *J* = 8.6 Hz, H-6), 5.67 (1H, d, *J* = 5.0 Hz, H-3′), 1.43 (3H, s, C-2′–CH_3), 1.60 (3H, s, C-2′–CH_3), 6.21 (1H, br q, *J* = 7.2 Hz, H-3″), 2.05 (3H, br d, *J* = 7.2 Hz, H-4″), 1.98 (3H, br s, H-5″). EI-MS *m*/*z*: 342 (M^+), 242, 189, 188, 160, 83 (100), 55 and 39. Comparing the above data with Ref. [24], the obtained product was identified as Pd–Ib.

Peak III: $[\alpha]_D^{25}$ +48.2° (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): 6.24 (1H, d, *J* = 9.6 Hz, H-3), 7.61 (1H, d, *J* = 9.6 Hz, H-4), 7.36 (1H, d, *J* = 8.6 Hz, H-5), 6.81 (1H, d, *J* = 8.6 Hz, H-6), 5.40 (1H, d, *J* = 5.0 Hz, H-3′), 6.55 (1H, d, *J* = 5.0 Hz, H-4′), 1.44 (3H, s, C-2′–CH_3), 1.48 (3H, s, C-2′–CH_3), 6.14 (1H, br q, *J* = 7.2 Hz, H-3″), 1.96 (3H, br d, *J* = 7.2 Hz, H-4″), 1.87 (3H, br s, H-5″), 2.11 (3H, s, H-2‴). ^{13}C NMR (100 MHz, $CDCl_3$): 159.99 (C-2), 113.10 (C-3), 143.34 (C-4), 129.14 (C-5), 114.34 (C-6), 156.68 (C-7), 106.96 (C-8), 153.91 (C-9), 112.48 (C-10), 77.66 (C-2′), 69.70 (C-3′), 60.95 (C-4′), 22.89 (C-2′–CH_3), 24.86 (C-2′–CH_3), 166.42 (C-1″), 126.85 (C-2″), 139.88 (C-3″), 15.75 (C-4″), 20.47 (C-5″), 169.89 (C-1‴), 20.63 (C-2‴). EI-MS *m*/*z*: 386 (M^+), 286, 245, 244, 229, 191, 190, 83 (100), 55 and 43. Comparing the above data with Refs. [25,26], the obtained product was identified as (+)-praeruptorin A.

Peak IV: $[\alpha]_D^{25}$ +14.5° (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): 6.21 (1H, d, *J* = 9.6 Hz, H-3), 7.58 (1H, d, *J* = 9.6 Hz, H-4), 7.35 (1H, d, *J* = 8.6 Hz, H-5), 6.80 (1H, d, *J* = 8.6 Hz, H-6), 5.44 (1H, d, *J* = 4.8 Hz, H-3′), 6.69 (1H, d, *J* = 4.8 Hz, H-4′), 1.45 (3H, s, C-2′–CH_3), 1.48 (3H, s, C-2′–CH_3), 6.11 (1H, br q, *J* = 7.2 Hz, H-3″), 6.02 (1H, br q, *J* = 6.8 Hz, H-3‴), 1.98 (3H, d, *J* = 7.2 Hz, H-4″), 1.95 (3H, d, *J* = 6.8 Hz, H-4‴), 1.84 (3H, br s, H-5″), 1.82 (3H, br s, H-5‴). ^{13}C NMR (100 MHz, $CDCl_3$): 159.75 (C-2), 113.23 (C-3), 143.15 (C-4), 129.14 (C-5), 114.32 (C-6), 156.65 (C-7), 107.49 (C-8), 154.3 (C-9), 112.42 (C-10), 77.41 (C-2′), 70.09 (C-3′), 60.95 (C-4′), 22.47 (C-2′–CH_3), 25.34 (C-2′–CH_3), 166.45 (C-1″), 127.29 (C-2″), 139.88 (C-3″), 15.75 (C-4″), 20.39 (C-5″), 166.22 (C-1‴), 127.00 (C-2‴), 138.45 (C-3‴), 15.56 (C-4‴), 20.31 (C-5‴). EI-MS *m*/*z*: 426 (M^+), 326, 245, 229, 191, 83 (100), 55 and 43. Comparing the above data with Ref. [25], the obtained product was identified as (+)-praeruptorin B.

The results of our study clearly demonstrate that HSCCC with gradient elution can provides highly efficient preparative separation of coumarins from crude extract of *P. praeruptorum* Dunn.

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