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Isolation and purification of coumarin compounds from the root of *Peucedanum decursivum* (Miq.) *Maxim* by high-speed counter-current chromatography

Renmin Liu [a,b], Qinghua Sun [b], Yunrong Shi [a], Lingyi Kong [a,*]

[a] *Department of Natural Medicinal Chemistry, China Pharmaceutical University, Nanjing 210009, China*
[b] *College of Chemistry and Chemical Engineering, Liaocheng University, Liaocheng 252059, China*

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of coumarin compounds from the Chinese medicinal plant *Peucedanum decursivum* (Miq.) *Maxim* (Zihuaqianhu in Chinese) was successfully established by using light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v) as the two-phase solvent system. The upper phase of light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v) was used as the stationary phase of HSCCC. Nodakenetin (2.8 mg), 6.1 mg of Pd-C-IV, 7.3 mg of Pd-D-V, 4.7 mg of ostruthin, 7.8 mg of decursidin and 11.2 mg of decursitin C with the purity of 88.3%, 98.0%, 94.2%, 97.1%, 97.8% and 98.4%, respectively, were separated successfully in one-step separation from 150 mg of crude sample from *P. decursivum* (Miq.) *Maxim*. After purified by HSCCC again with light petroleum–ethyl acetate–methanol–water (5:5:4:5, v/v) as the two-phase solvent system, the purity of (I) can reach 99.4%. The structures of all the compounds were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; Preparative chromatography; *Peucedanum decursivum* (Miq.) *Maxim*; Nodakenetin; Pd-C-IV; Pd-D-V; Ostruthin; Decursidin; Decursitin C

1. Introduction

Radix Peucedani, known as Qianhu in Chinese, has been used as a traditional Chinese medicine for centuries and is officially listed in the Chinese Pharmacopoeia [1]. The herb is frequently used to dispel wind and remove heat, relieve cough and resolve phlegm [2]. *Peucedanum decursivum* (Miq.) *Maxim*, known as Zihuaqianhu in Chinese, is an important species of *Peucedani*, and many coumarin constituents extracted from it have been reported to carry strong biological activity [3]. Further studies to better understand the biochemical properties of other related coumarins need the development of efficient, preparative-scale separation and purification methods for these compounds.

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of sample components that is often the case with solid supports [4]. So, this technique is gaining increasing interest recently.

The present paper developed a HSCCC method for the separation and purification of six coumarin compounds including nodakenetin, Pd-C-IV, Pd-D-V, ostruthin, decursidin and decursitin C from the light petroleum extract of *P. decursivum* (Miq.) *Maxim*. Ostruthin was separated from *P. decursivum* (Miq.) *Maxim* for the first time. Pd-D-V, named by us, was isolated in the form of pure compound for the first time. The chemical structures of the compounds are given in Fig. 1.

* Corresponding author. Tel.: +86 25 85391289.
E-mail address: lykong@jlonline.com (L. Kong).

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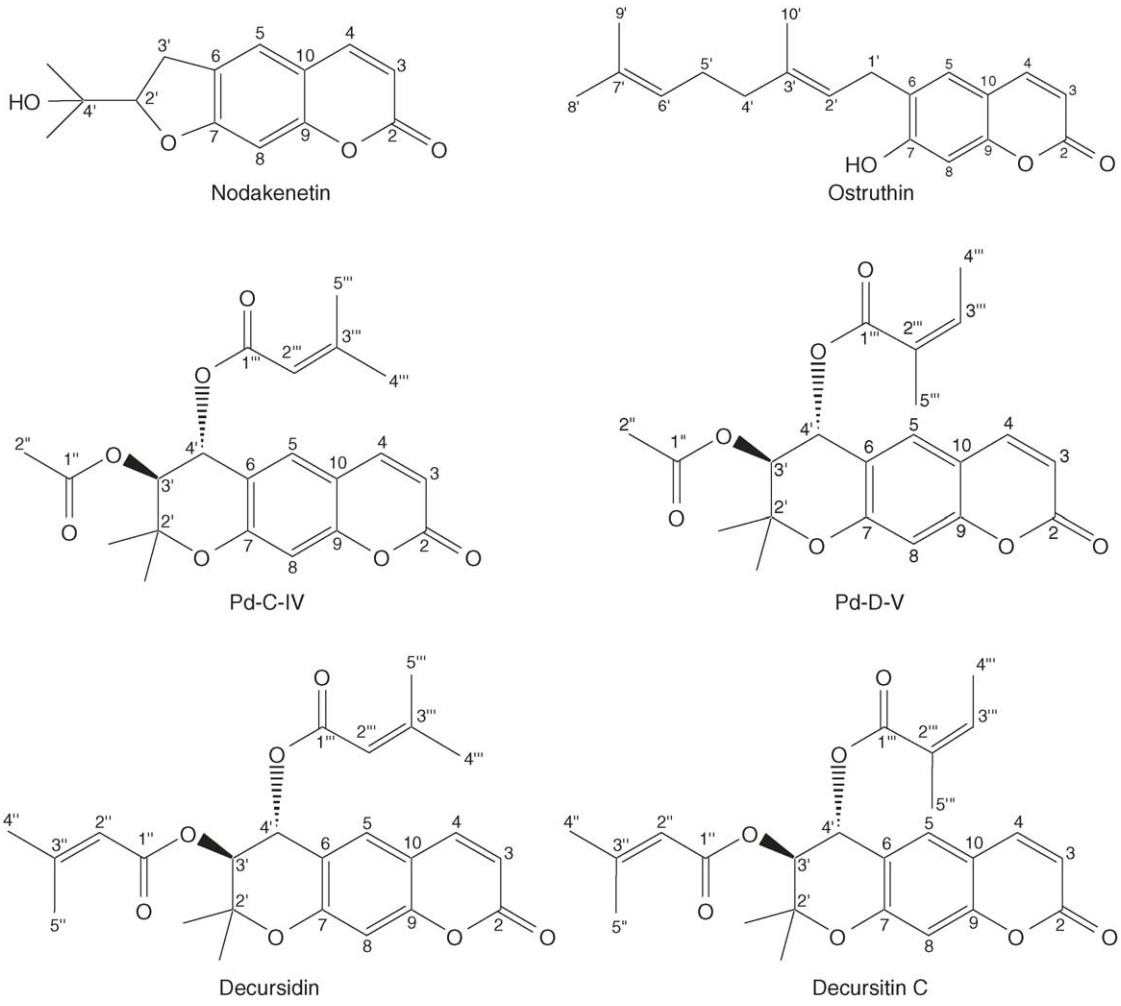

Fig. 1. Chemical structures of some coumarin compounds of *Peucedanum decursivum* (Miq.) *Maxim*.

2. Experimental

2.1. Reagents and materials

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Acetonitrile used for HPLC was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water.

The roots of *P. decursivum* (Miq.) *Maxim*, herborized from Anhui, was provided by professor Shoujin Liu (Anhui University of Traditional Chinese Medicine, Hefei, China).

2.2. 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 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. A 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 were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd., Hangzhou, China).

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

The HPLC equipment used was Agilent 1100 HPLC system including a G1311A Quat pump, a G1315B DAD, a Rheodyne 7725i injection valve with a 20 μl loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., USA). EIMS spectra were measured with a HP5989A instrument.

2.3. Preparation of crude sample

The roots of *P. decursivum* (Miq.) *Maxim* were dried constant at 60 °C and then pulverized to about 30-mesh with a disintegrator. The powder (100 g) was extracted with 500 ml of boiling light petroleum (boiling range, 60–90 °C) under reflux for three times. The extraction time was 2, 2 and 1 h, respectively. The extracts were combined together. After filtrated with ceramic filter, the filtrate was evaporated by rotary vaporization at 40 °C under reduced pressure. About 9.0 g of yellow oil syrup was obtained. It was stored in a refrigerator (−4 °C) for the subsequent HSCCC separation.

2.4. Selection of the two-phase solvent system

Light petroleum–ethyl acetate–methanol–water was used as the two-phase solvent system of HSCCC. The composition of the two-phase solvent system was selected according to the partition coefficient (*K*) of the target compounds of crude sample extracted from *P. decursivum* (Miq.) *Maxim*. The *K*-values were determined by HPLC as follows: suitable amount of crude sample was dissolved in 5 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. Preparation of two-phase solvent system and sample solution

Light petroleum–ethyl acetate–methanol–water two-phase solvent systems with the volume ratios of 5:5:7:4 and 5:5:4:5 were prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking repeatedly. Then the upper phase and the lower phase were separated and degassed by sonication for 30 min prior to use.

Crude extract sample (150 mg) was dissolved in 5 ml of the lower phase of light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v).

2.6. HSCCC separation procedure

The upper phase (stationary phase) and the lower phase (mobile phase) of the two-phase solvent system, light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v), were pumped into the multilayer-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 2.0 ml min^{-1}, at the same time, the HSCCC apparatus was run at a revolution speed of 900 rpm. After hydrodynamic equilibrium was reached (about half an hour), the sample solution (150 mg of crude sample dissolved in 5 ml of the lower phase) 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 30 min after the sample injected. 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.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude light petroleum extract of *P. decursivum* (Miq.) *Maxim* and each of the peak fractions collected from HSCCC separation were analyzed by HPLC. The HPLC analyses were performed with a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. The mobile phase was acetonitrile and water in gradient mode as follows: 0–24 min, 50% acetonitrile; 24–40 min, 65% acetonitrile. The flow-rate was 1.5 ml min^{-1} and the effluent was continuously monitored at 254 nm.

Identification of each HSCCC peak fractions was performed by ^{1}H NMR and ^{13}C NMR. 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 HPLC conditions

Different kinds of solvent system were used as the mobile phase and different elution modes were employed to analyze the crude extract from *P. decursivum* (Miq.) *Maxim* by HPLC. The results indicated that when acetonitrile and water were used as the mobile phase in gradient mode (acetonitrile: 0–24 min, 50%; 24–40 min, 65%), six major peaks can be obtained and each peak got baseline separation. The peak purification of HPLC was analyzed with Agilent 1100 workstation and the results were satisfactory. The HPLC chromatogram of the crude extract from *P. decursivum* (Miq.) *Maxim* was given in Fig. 2A.

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

In order to determine the optimal two-phase solvent system for the HSCCC separation, a series of experiments were performed in the present study. Ethyl acetate–water, ethyl acetate–methanol–water, light petroleum–ethyl

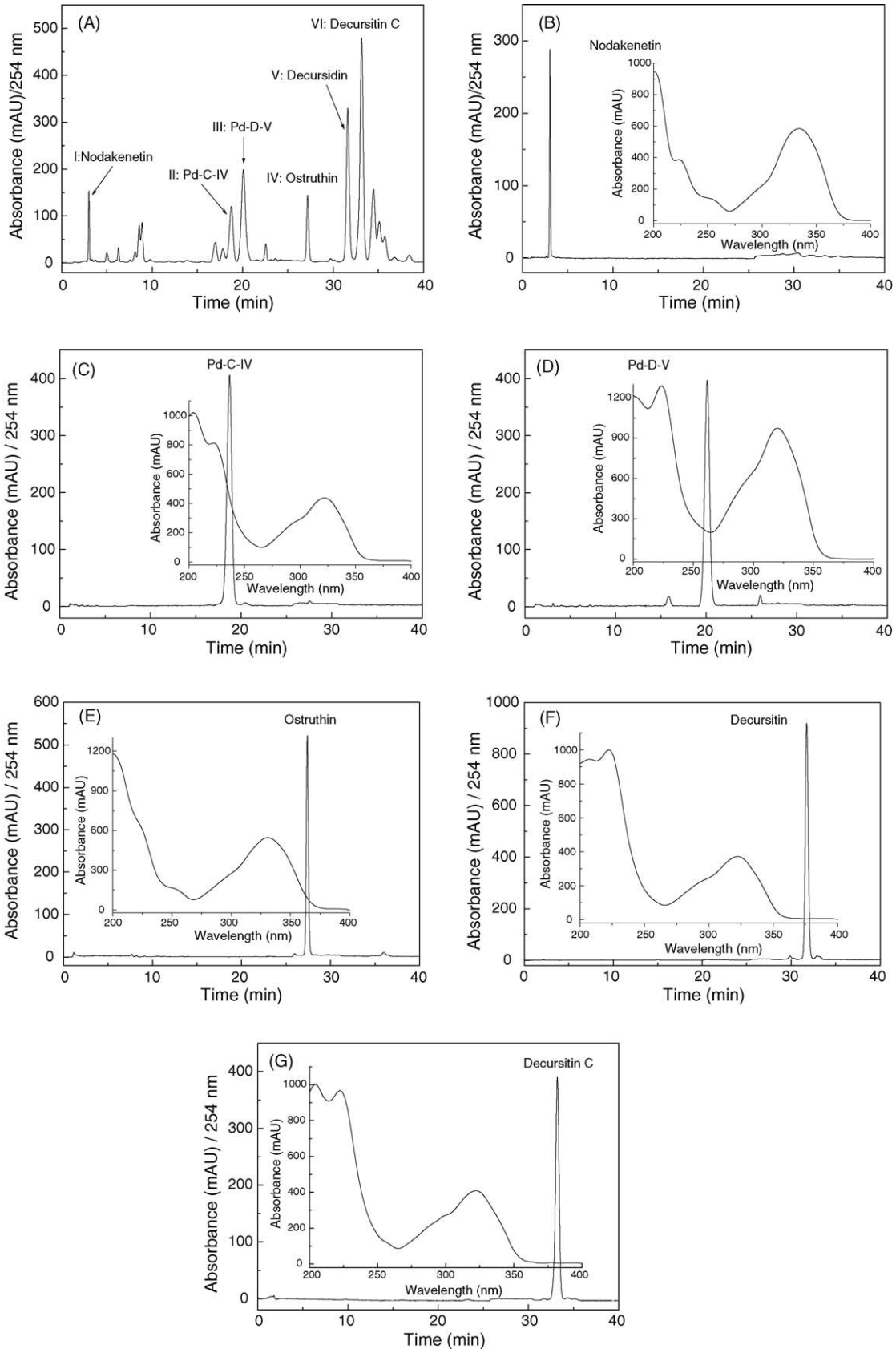

Fig. 2. HPLC chromatogram of crude extract from *Peucedanum decursivum* (Miq.) *Maxim* and HSCCC peak fractions. Conditions: column: reversed phase SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: acetonitrile and water in gradient mode as follows: 0–24 min, 50% acetonitrile; 24–40 min, 65% acetonitrile; flow-rate: 1.5 ml min^{-1}; detection wavelength: 254 nm. (A) Crude extract from *Peucedanum decursivum* (Miq.) *Maxim*; (B)–(G) HSCCC peak fractions I–VI in Fig. 3.

Table 1
The *K*-values of the compounds in different two-phase solvent systems

Solvent system	*K*-value					
	I	II	III	IV	V	VI
Ethyl acetate–water (5:5, v/v)	11.6	–	–	–	–	–
Ethyl acetate–methanol–water (5:1:5, v/v)	5.72	–	–	–	–	–
Ethyl acetate–methanol–water (5:2:5, v/v)	3.03	–	–	–	–	–
Ethyl acetate–methanol–water (5:3:5, v/v)	2.72	–	–	–	–	–
Light petroleum–ethyl acetate–methanol–water (5:5:4:5, v/v)	0.42	–	–	–	–	–
Light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v)	0.25	7.52	12.5	–	–	–
Light petroleum–ethyl acetate–methanol–water (5:5:6:5, v/v)	0.17	4.68	6.12	11.3	–	–
Light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v)	0.07	1.01	1.41	2.18	3.19	4.24
Light petroleum–ethyl acetate–methanol–water (5:5:7:3, v/v)	0.02	0.83	0.93	1.34	1.87	2.15

The symbol '–' means the partition coefficient is too large that cannot be evaluated.

acetate–methanol–water were used as the two-phase solvent system to optimize the HSCCC separation condition. The partition coefficients of the compounds of crude sample in these two-phase solvent systems were given in Table 1. It can be seen from Table 1 that light petroleum–ethyl acetate–methanol–water with the volume ratio of 5:5:7:4 may be suitable for HSCCC separation. So it was used as the two-phase solvent system in HSCCC separation.

Although the revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 1000 rpm, the speed of 900 rpm was used invariably in the present study. The influence of the flow-rate of mobile phase was also investigated. The results indicated that reducing flow-rate could improve the reservation of the stationary phase in some degree, but the chromatogram peaks were extended at the same time. At last, a flow-rate of 2.0 ml min^{-1} was employed in the experiment.

The crude samples from *P. decursivum* (Miq.) *Maxim* were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram was shown in Fig. 3. Nodakenetin (2.8 mg), 6.1 mg of Pd-C-IV, 7.3 mg of Pd-D-V, 4.7 mg of ostruthin, 7.8 mg of decursidin and 11.2 mg of decursitin C were obtained from 150 mg of the crude sample by one-step HSCCC separation. The purity of them was 88.3%, 98.0%, 94.2%, 97.1%, 97.8% and 98.4%, respectively, as determined by HPLC. Peak I was purified by HSCCC again with light petroleum–ethyl acetate–methanol–water (5:5:4:5, v/v) as the two-phase solvent system. The purity of it can reach 99.4% after the second separation step. The chromatograms of HPLC and UV spectra of all these compounds were shown in Fig. 2.

3.3. Structural identification

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

Peak I: ^{1}H NMR (400 MHz, $CDCl_3$): 6.18 (1H, d, J = 9.2 Hz, H-3), 7.58 (1H, d, J = 9.2 Hz, H-4), 7.21 (1H, s, H-5), 6.74 (1H, s, H-8), 4.74 (1H, t, J = 8.8 Hz, H-2′), 3.24 (2H, m, H-3′), 1.38 (3H, s, C-4′-CH_3), 1.24 (3H, s, C-4′-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): 161.4 (C-2), 112.2 (C-3), 143.7 (C-4), 123.4 (C-5), 125.1 (C-6), 163.1 (C-7), 97.9 (C-8), 155.6 (C-9), 112.7 (C-10), 91.1 (C-2′), 29.4 (C-3′), 71.6 (C-4′), 26.1 (C-4′-CH_3), 24.3 (C-4′-CH_3). Compared with the data given in [5], peak I was identified as nodakenetin.

Peak II: ^{1}H NMR (400 MHz, $CDCl_3$): 6.25 (1H, d, J = 9.6 Hz, H-3), 7.60 (1H, d, J = 9.6 Hz, H-4), 7.37 (1H, s, H-5), 6.81 (1H, s, H-8), 5.25 (1H, d, J = 6.0 Hz, H-3′), 6.04 (1H, d, J = 6.0 Hz, H-4′), 1.45 (3H, s, C-2′-CH_3), 1.39 (3H, s, C-2′-CH_3), 2.10 (3H, s, H-2″), 5.70 (1H, br s, H-2‴), 1.94 (3H, s, H-4‴), 2.24 (3H, s, H-5‴); ^{13}C NMR (100 MHz, $CDCl_3$): 160.8 (C-2), 113.8 (C-3), 143.1 (C-4), 129.0 (C-5), 117.0 (C-6), 156.0 (C-7), 104.8 (C-8), 155.3 (C-9), 113.3 (C-10), 77.7 (C-2′), 72.7 (C-3′), 66.1 (C-4′), 25.0 (C-2′-CH_3), 22.4 (C-2′-CH_3), 170.0 (C-1″), 20.8 (C-2″), 166.0 (C-1‴), 114.9 (C-2‴), 159.9 (C-3‴), 20.5 (C-4‴), 27.6 (C-5‴). Compared with the data given in [6,7], peak II was identified as Pd-C-IV.

Peak III: ^{1}H NMR (400 MHz, $CDCl_3$): 6.26 (1H, d, J = 9.2 Hz, H-3), 7.60 (1H, d, J = 9.2 Hz, H-4), 7.40 (1H, s,

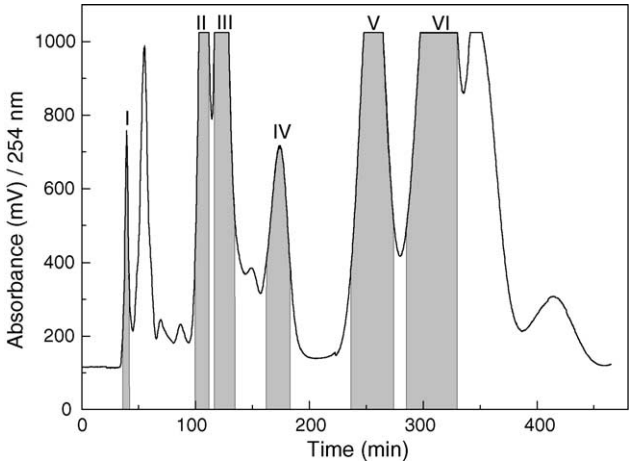

Fig. 3. HSCCC chromatogram of crude extract from *Peucedanum decursivum* (Miq.) *Maxim*. Two-phase solvent system: light petroleum–ethyl acetate–methanol–water (5:5:7:4, v/v); mobile phase: the lower phase; flow-rate: 2.0 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; sample size: 150 mg of crude sample dissolved in 5 ml of the lower phase; separation temperature: 25 °C. The shading indicates what was collected.

H-5), 6.82 (1H, s, H-8), 5.28 (1H, d, J = 6.0 Hz, H-3′), 6.10 (1H, d, J = 6.0 Hz, H-4′), 1.47 (3H, s, C-2′-CH_3), 1.40 (3H, s, C-2′-CH_3), 2.10 (3H, s, H-2″), 6.21 (1H, br q, J = 7.2 Hz, H-3‴), 2.06 (3H, br d, J = 7.2 Hz, H-4‴), 1.88 (3H, br s, H-5‴); ^{13}C NMR (100 MHz, $CDCl_3$): 160.8 (C-2), 113.9 (C-3), 143.1 (C-4), 129.2 (C-5), 116.8 (C-6), 156.1 (C-7), 105.0 (C-8), 155.4 (C-9), 113.4 (C-10), 77.6 (C-2′), 72.4 (C-3′), 66.5 (C-4′), 24.9 (C-2′-CH_3), 22.4 (C-2′-CH_3), 169.7 (C-1″), 20.8 (C-2″), 169.7 (C-1‴), 126.7 (C-2‴), 140.9 (C-3‴), 16.0 (C-4‴), 20.5 (C-5‴). EIMS m/z: 386 (M^+), 327, 311, 287, 245, 229, 228, 213, 83 (100), 55. The compound was isolated in the form of pure compound for the first time, named Pd-D-V by us. In the literature [6], Pd-C-V was a mixture which composed of two compounds including Pd-D-V and another compound.

Peak IV: ^{1}H NMR (400 MHz, $CDCl_3$): 6.24 (1H, d, J = 9.6 Hz, H-3), 7.64 (1H, d, J = 9.6 Hz, H-4), 7.20 (1H, s, H-5), 6.97 (1H, s, H-8), 3.40 (2H, d, J = 7.2 Hz, H-1′), 5.30 (1H, m, H-2′), 2.08–2.14 (4H, m, H-4′, H-5′), 5.10 (1H, m, H-6′), 1.69 (3H, s, H-8′), 1.61 (3H, s, H-9′), 1.75 (3H, s, H-10′); ^{13}C NMR (100 MHz, $CDCl_3$): 162.2 (C-2), 112.4 (C-3), 144.1 (C-4), 128.3 (C-5), 125.5 (C-6), 158.5 (C-7), 103.3 (C-8), 154.2 (C-9), 112.3 (C-10), 28.5 (C-1′), 120.8 (C-2′), 138.9 (C-3′), 39.7 (C-4′), 26.4 (C-5′), 123.8 (C-6′), 131.9 (C-7′), 25.8 (C-8′), 17.7 (C-9′), 16.2 (C-10′). Compared with the data given in [8–10], peak IV was identified as ostruthin. This compound was separated from *P. decursivum* (Miq.) *Maxim* for the first time.

Peak V: ^{1}H NMR (400 MHz, $CDCl_3$): 6.25 (1H, d, J = 9.2 Hz, H-3), 7.60 (1H, d, J = 9.2 Hz, H-4), 7.39 (1H, s, H-5), 6.81 (1H, s, H-8), 5.27 (1H, d, J = 5.6 Hz, H-3′), 6.05 (1H, d, J = 5.6 Hz, H-4′), 1.46 (3H, s, C-2′-CH_3), 1.39 (3H, s, C-2′-CH_3), 5.68 (1H, br s, H-2″), 1.90 (3H, s, H-4″), 2.16 (3H, s, H-5″), 5.70 (1H, br s, H-2‴), 2.24 (3H, s, H-4‴), 1.93 (3H, s, H-5‴); ^{13}C NMR (100 MHz, $CDCl_3$): 160.9 (C-2), 113.6 (C-3), 143.2 (C-4), 129.3 (C-5), 117.0 (C-6), 156.2 (C-7), 104.8 (C-8), 155.3 (C-9), 113.2 (C-10), 77.9 (C-2′), 71.1 (C-3′), 66.1 (C-4′), 24.9 (C-2′-CH_3), 22.5 (C-2′-CH_3), 164.9 (C-1″), 115.0 (C-2″), 159.2 (C-3″), 20.4 (C-4″), 27.6 (C-5″), 165.9 (C-1‴), 115.1 (C-2‴), 159.4 (C-3‴), 20.5 (C-4‴), 27.6 (C-5‴). Compared with the data given in [5], peak V was identified as decursidin.

Peak VI: ^{1}H NMR (400 MHz, $CDCl_3$): 6.26 (1H, d, J = 9.2 Hz, H-3), 7.61 (1H, d, J = 9.2 Hz, H-4), 7.42 (1H, s, H-5), 6.82 (1H, s, H-8), 5.30 (1H, d, J = 5.6 Hz, H-3′), 6.11 (1H, d, J = 5.6 Hz, H-4′), 1.47 (3H, s, C-2′-CH_3), 1.41 (3H, s, C-2′-CH_3), 5.69 (1H, br s, H-2″), 1.92 (3H, s, H-4″), 2.16 (3H, s, H-5″), 6.18 (1H, br q, J = 7.2 Hz, H-3‴), 2.04 (3H, br d, J = 7.2 Hz, H-4‴), 1.86 (3H, br s, H-5‴); ^{13}C NMR (100 MHz, $CDCl_3$): 160.9 (C-2), 113.7 (C-3), 143.2 (C-4), 129.3 (C-5), 117.0 (C-6), 156.2 (C-7), 104.9 (C-8), 155.3 (C-9), 113.3 (C-10), 77.8 (C-2′), 71.1 (C-3′), 66.6 (C-4′), 24.9 (C-2′-CH_3), 22.6 (C-2′-CH_3), 164.9 (C-1″), 114.9 (C-2″), 159.5 (C-3″), 20.4 (C-4″), 27.5 (C-5″), 167.2 (C-1‴), 126.9 (C-2‴), 140.5 (C-3‴), 15.9 (C-4‴), 20.5 (C-5‴). Compared with the data given in [5], peak VI was identified as decursitin C.

4. Conclusion

HSCCC has been successfully applied to the separation and purification of six coumarin compounds from the light petroleum extract of the roots of *P. decursivum* (Miq.) *Maxim*. Among them, Pd-D-V was isolated in the form of pure compound for the first time and ostruthin was isolated from *P. decursivum* for the first time. The results illustrated that HSCCC as a preparative separation technique was an effective method for the isolation and purification of bioactive components from *P. decursivum* (Miq.) *Maxim*. HSCCC thus provides an attractive method for the preparative-scale separation and purification of bioactive components in herbal extracts.

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