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Extraction and preparative purification of tanshinones from *Salvia miltiorrhiza Bunge* by high-speed counter-current chromatography

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ABSTRACT

A method for extraction and preparative separation of tanshinones from *Salvia miltiorrhiza Bunge* was successfully established in this paper. Tanshinones from *Salvia miltiorrhiza Bunge* were extracted using ethyl acetate as the extractant under reflux. The extracts were then purified by high speed counter-current chromatography (HSCCC) with light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) as the two phase solvent system. The upper phase was used as the stationary phase and the lower phase as the mobile phase. 8.2 mg of dihydrotanshinone I, 5.8 mg of 1,2,15,16-tetrahydrotanshiquinone, 26.3 mg of cryptotanshinone, 16.2 mg of tanshinone I, 25.6 mg of neo-przewaquinone A, 68.8 mg of tanshinone IIA and 9.3 mg of miltirone were obtained from 400 mg of extracts from *Salvia miltiorrhiza Bunge* in one-step HSCCC separation, with the purity of 97. 6%, 95.1%, 99.0%, 99.1%, 93.2%, 99.3% and 98.7%, respectively, as determined by HPLC area normalization method. Their chemical structures were identified by ^{1}H NMR.

1. Introduction

Salvia miltiorrhiza Bunge has been widely used as a traditional Chinese medicine and officially listed in the Chinese Pharmacopoeia [1]. An earlier study indicated that it has many effects such as promoting blood circulation, relieving vexation, nourishing blood, tranquilizing the mind and relieving carbuncle [2]. Recent studies also demonstrated that it also has an anticancer effect [3], prevention of angina pectoris and myocardial infarction [4], antileukaemia [5], vasodilatation, protection of cardiac muscles from anoxia, reduced platelet aggregation and thrombus formation [6]. The major active constituents of this herb are tanshinones. Many tanshinone compounds have been isolated from *Salvia miltiorrhiza Bunge* by conventional methods and many of them were shown to have biological activities including antitumor and antimicrobial activities [7]. Some of the tanshinone compounds are used as reference standards in the quality control of *Salvia miltiorrhiza Bunge* and its products. Therefore, high purity preparation of them is of great interest for pharmacological study and good manufacturing practice.

High-speed counter-current chromatography (HSCCC), a liquid–liquid partition chromatographic technique that uses no support matrix and the stationary phase is immobilized by centrifugal force, eliminates irreversible adsorption of the sample onto the solid support used in a conventional chromatographic column [8]. Moreover, it has a large scale of injection. Multiform relatively pure substances can be obtained at one time in large amounts. So it is suitable for separation and purification of active components from natural products. Many successful applications have been reported on the purification of different kinds of active compounds from natural products, such as alkaloids [9–12], flavonoids [13–17], coumarins [18–21], anthraquinones [22–24], polyphenols [25], diterpenoids [26–29], saponins [30–32], and so on. Separation and purification of tanshinones from *Salvia miltiorrhiza Bunge* by HSCCC has been reported previously [27–29]. Tian and co-workers reported a HSCCC method using stepwise elution and three compounds including Tanshinone IIA, tanshinone I and cryptotanshinone were obtained in a single run [27]. Tian and co-workers also reported a multidimensional HSCCC method and four major components including tanshinone IIA, tanshinone I, dihydrotanshinone I and cryptotanshinone were isolated [28]. Li and Chen reported another HSCCC method with the two-phase solvent systems A composed of *n*-hexane–ethanol–water (10:5.5:4.5, v/v) and B composed of *n*-hexane–ethanol–water (10:7:3, v/v) in a stepwise elution mode and six relatively pure compounds including dihydrotanshinone I, cryptotanshinone, methylenetanshiquinone, tanshinone I, tanshinone IIA and danshenxinkun B

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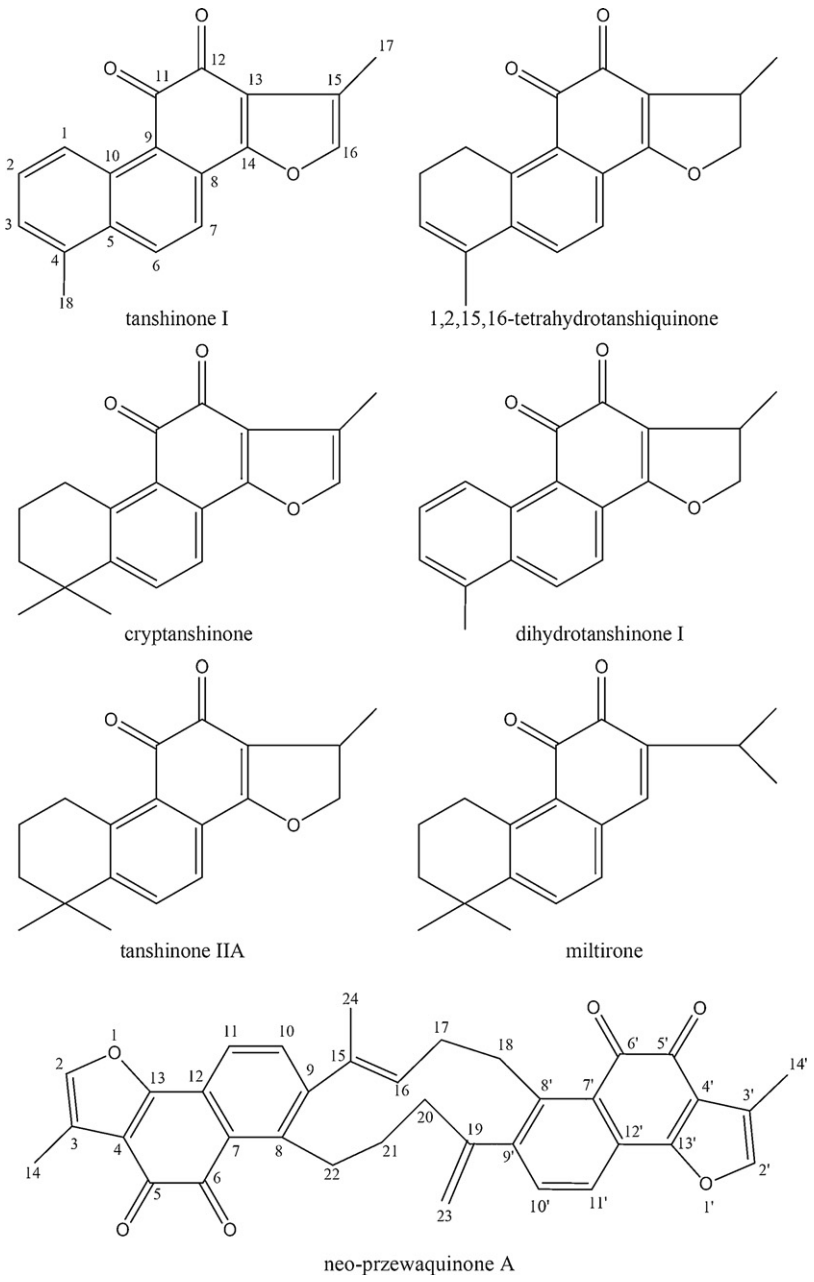

Fig. 1. Chemical structures of the separated target compounds from *Salvia miltiorrhiza Bunge*.

were obtained [29]. Gu and co-workers developed fingerprinting of *Salvia miltiorrhiza Bunge* by HSCCC [33–36]. They also separated and identified crypototanshinone, tanshinone I and tanshinone II A from *Salvia miltiorrhiza Bunge*.

In the present paper, a new HSCCC method for separation of tanshinones from *Salvia miltiorrhiza Bunge* was established. Seven kinds of tanshinone compounds including dihydrotanshinone I, 1,2,15,16-tetrahydrotanshiquinone, cryptotanshinone, tanshinone I, neo-przewaquinone A, tanshinone IIA and miltirone were obtained with light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) as the two phase solvent system in a one-step separation. 1,2,15,16-Tetrahydrotanshiquinone, neo-przewaquinone A and miltirone were first separated by HSCCC. The purities of the separated compounds were all over 95% as determined by HPLC area normalization method. The chemical structures of the separated compounds shown in Fig. 1 were identified by ^{1}H NMR.

2. Experimental

2.1. Apparatus

The HSCCC instrument used was a TBE-300A high-speed counter-current chromatography (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The total dead volume (volume between the injection valve and the inlet of the separation column and the volume between the outlet of the separation column and the detector) is 12 ml. The rotational 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 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 rotational speed of the apparatus can be regulated with a speed controller

in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulator (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature using water as the circulating media. A ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

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

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

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

2.2. Reagents

All solvents used for the preparation of crude extracts and HSCCC separations were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical Plant, Yucheng, China), and water used was distilled water.

Salvia miltiorrhiza Bunge was purchased from a local drug store and was identified as the roots of *Salvia miltiorrhiza* by professor Yongqing Zhang, according to its plant morphology, plant classification and root trait identification. The voucher specimen of the plant material was stored in Shandong University of Traditional Chinese Medicine, Jinan, China.

2.3. Extraction of tanshinones from Salvia miltiorrhiza Bunge

The dried *Salvia miltiorrhiza Bunge* samples were shattered to powder by a plant disintegrator, and 500 g of the powder was dunked into 5000 ml of ethyl acetate at room temperature for 1 h and then extracted under reflux for 30 min. The extraction procedure was then repeated twice again. The extracts were combined together and evaporated to dryness by rotary vaporization at 45 °C under reduced pressure, yielding 11.80 g of crude sample that was stored in a refrigerator (−4 °C) for subsequent HSCCC separation.

2.4. Selection of two-phase solvent systems

The two-phase solvent system was selected according to the partition coefficient (K) of each target component. The K value was determined by HPLC analysis. Suitable amount of crude extract was dissolved in the lower phase of two-phase solvent system. The solution was analysed by HPLC. The peak area was recorded as A_1. Then equal volume of the upper phase was added to the solution and the two phases were mixed thoroughly. After partition equilibration, the lower phase solution was determined by HPLC again, and the peak area was recorded as A_2. The K value was calculated according to the following equation: $K = (A_1 - A_2)/A_2$.

2.5. Preparation of two-phase solvent system and sample solutions

Light petroleum–ethyl acetate–methanol–water solvent system (6:4:6.5:3.5, v/v) was used as the two-phase solvent system for HSCCC separation. It was prepared by adding the solvents to a separation funnel according to the volume ratios and mixed thoroughly. After the equilibration was established, the upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before using.

The sample solution for HSCCC separation was prepared by dissolving 400 mg of the crude extracts in 5 ml of the upper phase of the two-phase solvent system.

2.6. HSCCC separation procedure

The multilayer coiled column was first entirely filled with the upper phase and the lower phase of light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) system in the proportion of 50:50. Then the lower phase was pumped into the head end of the inlet column at a flow-rate of 2.0 ml/min, while the apparatus was run at 900 rpm. After hydrodynamic equilibrium was reached, 5 ml of the sample solution were injected into the separation column. The separation temperature was controlled at 25 °C. The effluent from the tail end of the column was continuously detected with a UV monitor at 254 nm. The chromatogram was recorded 50 min after the sample injected. Each peak fraction was manually collected according to the chromatogram and evaporated to dryness under reduced pressure. After HSCCC separation finished, the HSCCC instrument was turned off and the two phase solvent system blown out. The volume of the upper phase and lower phase was measured and the retention of stationary phase was calculated. The upper phase was separated and evaporated to dryness under reduced pressure. All the residuals of each peak fraction and upper stationary phase were dissolved in methanol for subsequent HPLC analysis.

2.7. HPLC analysis and identification

The crude extracts and each peak fraction from HSCCC were analysed by HPLC. The HPLC analysis was performed with a SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm) at room temperature. Methanol and water with the ratio of 75:25 was used as the mobile phase. The flow-rate was 1.0 ml/min, and the effluent was monitored at 254 nm by a photodiode array detector.

Identification of HSCCC peak fraction was performed by ^{1}H 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

Selection of two-phase solvent system and the determination of the purities of the target compounds separated by HSCCC were performed by HPLC. So an HPLC method for analysis of *Salvia miltiorrhiza Bunge* was established at first. In order to select an appropriate elution system, methanol–water was tested for HPLC analysis. When methanol–water with the volume ratio of 75:25 was used as the mobile phase, seven major peaks were obtained, and each peak got good separation. Fig. 2(A) shows the HPLC chromatogram of crude extracts from *Salvia miltiorrhiza Bunge*.

3.2. Selection of extraction method for extraction of tanshinones

In order to select a suitable method for extraction of tanshiones from *Salvia miltiorrhiza Bunge*, ultrasonic extraction and reflux extraction were tested with different extractants. The ratio of solid to solvent solution was kept at 1:10. *Salvia miltiorrhiza Bunge* samples were dunked in the extractant for 1 h and then extracted for 30 min for all tests. The extraction procedure was repeated twice and the solutions were combined together and evaporated to dryness. The amounts of extracts, the contents of total tanshinones of the extracts and the contents of the representative compounds

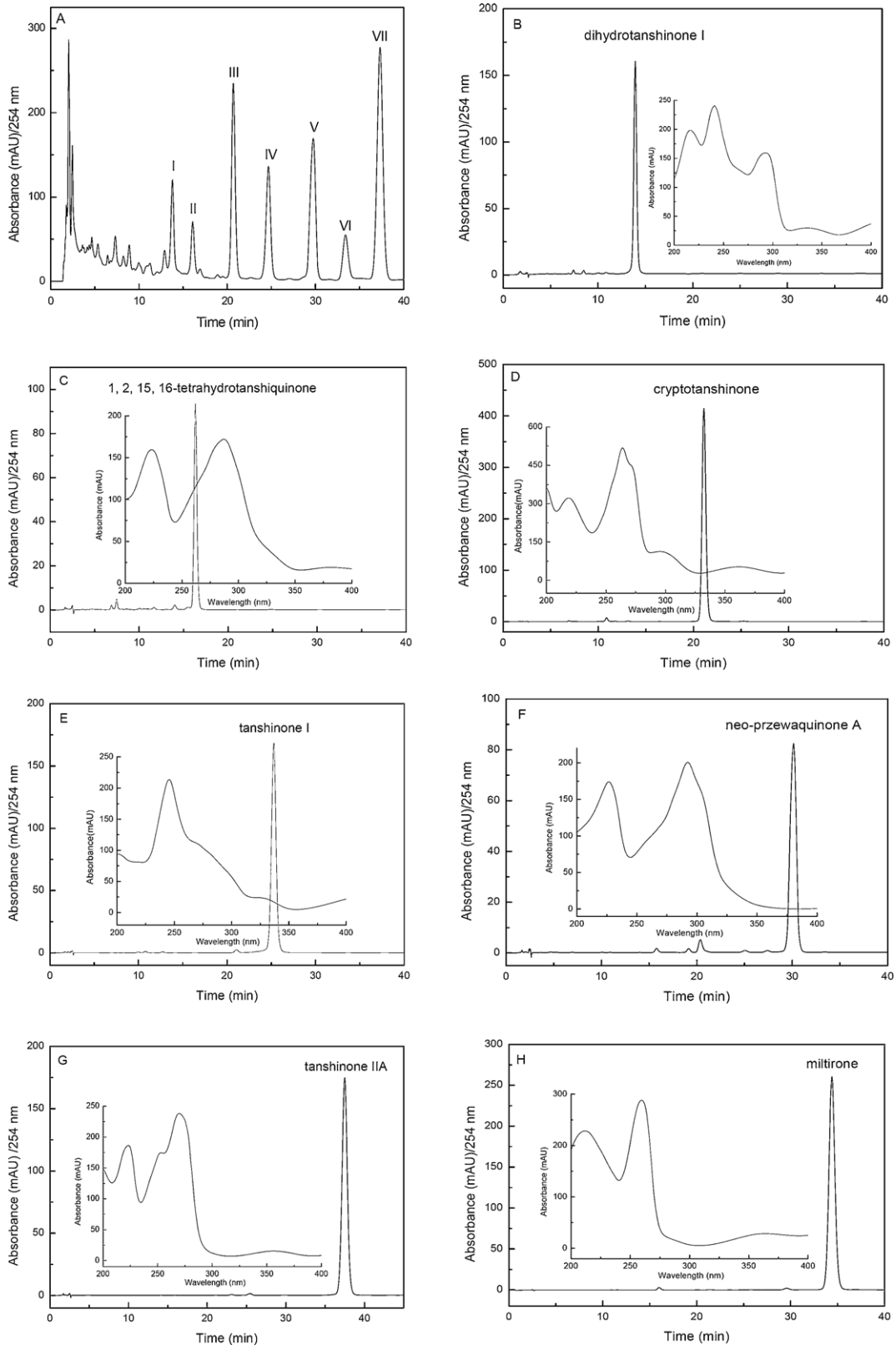

Fig. 2. HPLC chromatogram of the crude extract and HSCCC peak fractions. Conditions: column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D., 5 μm); mobile phase: methanol and water with volume ratio of 75:25; flow rate: 1.0 ml/min; detection wavelength: 254 nm. (A) Crude extracts; (B)–(G) HSCCC peak fractions I–V, VII in Fig. 3; (H) stationary phase fraction.

of *Salvia miltiorrhiza Bunge*, tanshinone IIA, were determined and used for evaluation of the extraction method. All the results are given in Table 1. It can be seen that the highest total recoveries and contents of total tanshinones and tanshinone IIA could be obtained when ethyl acetate was used as the extractant by reflux extraction. So ethyl acetate was chosen as the extractant and reflux extraction method was employed for extraction of tanshinones from *Salvia miltiorrhiza Bunge* in this paper.

Table 1
Extraction results of tanshinones from *Salvia miltiorrhiza Bunge* by different extraction methods ($n = 3$).

Extractant	Amount of extracts from 100 g of sample (g)		Content of total tanshinones of extracts (%)		Content of tanshinone IIA of extracts (%)	
	Ultrasonic	Reflux	Ultrasonic	Reflux	Ultrasonic	Reflux
95% Ethanol	2.52	2.77	33.58	32.32	14.86	13.82
Ethyl acetate	2.18	2.36	38.76	39.96	18.05	18.71
Dichloromethane	1.90	2.03	37.80	38.50	17.40	18.61

3.3. Optimization of two-phase solvent system and other conditions of HSCCC

The selection of the two-phase solvent system is the most important step in performing HSCCC. Light petroleum–ethyl acetate–methanol–water system was widely used as the two-phase solvent system in HSCCC separation due to its wide polarity range through changing the proportion of the four solvents. Considering the properties of the target compounds of tanshinones, light petroleum–ethyl acetate–methanol–water with different volume ratios were tested for HSCCC separation in this paper. In order to choose the optimum two-phase solvent system for HSCCC separation, the partition coefficients (*K*-values) of the target compounds in different solvent systems were determined and the *K*-values are shown in Table 2. When light petroleum–ethyl acetate–methanol–water (5:5:5:5, v/v) was used as the two phase solvent system, the *K*-values of compounds III–VII were very high. So the proportion of methanol and light petroleum were adjusted to reduce the *K*-values of the target compounds. When light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) was used as the two phase solvent system, only the *K*-value of compound VI was a little high. The *K*-values of other compounds were suitable for separation. If the proportion of methanol was increased further, the *K*-values of compound VI could be reduced, but compound V and VII could not be separated well. So light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) was chosen as the two phase solvent system. In this manner, compound VI kept in stationary phase while other compounds could be separated well.

The influences of the rotational speed, the separation temperature and the flow rate of the mobile phase on HSCCC separation were also investigated. The rotational speed of the separation coil tube has a great influence on separation. Increasing the rotational speed can increase the retention of the stationary phase and improve the separation efficiency. In this experiment, all separations were performed at 900 rpm. The temperature has significant effect on *K*-values, the retention percentage of stationary phase and the mutual solvency of the two-phase. When the separation temperature was controlled at 25 °C, good results could be obtained. Lower flow speed can improve separation to 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. When the flow rate was 2.0 ml/min, the rotational speed was 900 rpm, and separation temperature was 25 °C, good separation results were obtained. Under these conditions, the retention percentage of the stationary phase is 50%.

The crude samples from *Salvia miltiorrhiza Bunge* were separated under the optimum HSCCC conditions. The typical HSCCC chromatogram is shown in Fig. 3. The *K* values of the compounds I, II, III, IV, V and VII were measured from the chromatogram. They were 0.71, 0.96, 1.56, 2.04, 3.26, 5.11, respectively. They were in accordance with the test tube *K* values given in Table 1. Finally, seven compounds including 8.2 mg of dihydrotanshinone I, 5.8 mg of 1,2,15,16-tetrahydrotanshiquinone, 26.3 mg of cryptotanshinone, 16.2 mg of tanshinone I, 25.6 mg of neo-przewaquinone A, 68.8 mg of tanshinone IIA and 9.3 mg of miltirone were obtained from 400 mg of the crude sample by one-step HSCCC separation. The purity of them was 97.6%, 95.1%, 99.0%, 99.1%, 93.2%, 99.3% and 98.7%, respectively, as determined by HPLC area normalization method. The chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 2(B–H).

3.4. Structural identification

The chemical structures of the separated compounds were identified according to ^{1}H NMR spectra.

Compound I (Peak I in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 9.29 (1H, d, J = 9.2 Hz, H-1), 7.59 (1H, q, J = 9.2 Hz, 6.8 Hz, H-2), 7.42 (1H, d, J = 6.8 Hz, H-3), 8.34 (1H, d, J = 8.4 Hz, H-6), 7.79 (1H, d, J = 8.8 Hz, H-7), 3.60–3.80 (1H, m, H-15), 4.98 (1H, t, H-16), 4.45 (1H, q, H-16), 1.42 (3H, d, J = 6.8 Hz, H-17), 2.72 (3H, s, H-18). Comparing the above

Table 2
The *K* values of target compounds in several solvent systems.

Solvent system	*K*						
	I	II	III	IV	V	VI	VII
L^1–E^2–M^3–W^4 (5:5:5:5)	3.01	3.80	6.42	11.14	11.73	19.20	17.55
L–E–M–W (5:5:6:4)	1.05	1.44	2.46	4.20	4.60	9.57	7.32
L–E–M–W (6:4:6:4)	1.01	1.43	2.46	3.73	6.72	11.02	8.96
L–E–M–W (6:4:6.5:3.5)	0.58	0.76	1.45	2.58	3.47	5.62	4.71
L–E–M–W (6:4:7:3)	0.34	0.57	0.87	1.46	2.21	3.96	2.52

1. L: light petroleum; 2. E: ethyl acetate; 3. M: methanol; 4. W: water.

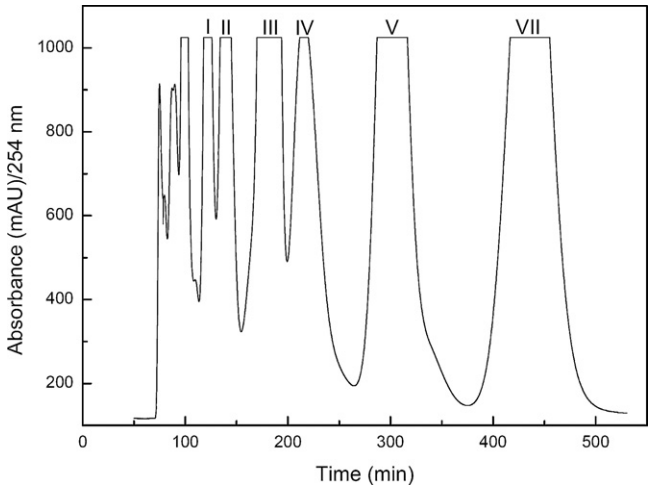

Fig. 3. HSCCC chromatogram of crude extracts from *Salvia miltiorrhiza Bunge*. Conditions: column: multilayer coil of 1.6 mm I.D. PTFE tube with a total capacity of 260 ml; solvent system: light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v); stationary phase: the upper phase; mobile phase: the lower aqueous; flow-rate: 2.0 ml/min; rotational speed: 900 rpm; detection wavelength: 254 nm; separation temperature: 25 °C; sample size: 400 mg dissolved in 5 ml of upper phase; retention of the stationary phase: 50%. I: dihydrotanshinone I (collected during 70–80 min); II: 1,2,15,16-tetrahydrotanshiquinone (collected during 82–100 min); III: cryptotanshinone (collected during 116–148 min); IV tanshinone I (collected during 155–182 min); V: neo-przewaquinone A (collected during 225–275 min); VII: tanshinone IIA (collected during 350–420 min).

data with Refs. [37,38], the obtained compound was identified as dihydrotanshinone I.

Compound II (Peak II in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 3.35–3.40 (2H, m, H-1), 2.25–2.28 (2H, m, H-2), 6.11 (1H, t, H-3), 7.53 (1H, d, J = 8.0 Hz, H-6), 7.43 (1H, q, J = 8.0 Hz, H-7), 3.60–3.66 (1H, m, H-15), 4.89 (1H, t, H-16), 4.40 (1H, m, H-16), 1.38 (3H, d, J = 6.8 Hz, H-17), 2.08 (3H, s, H-18). Comparing the above data with Ref. [39], the obtained compound was identified as 1,2,15,16-tetrahydrotanshiquinone.

Compound III (Peak III in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 3.22 (2H, t, H-1), 1.64–1.67 (2H, m, H-2), 1.76–1.82 (2H, m, H-3), 7.64 (1H, d, J = 8.4 Hz, H-6), 7.50 (1H, d, J = 8.4 Hz, H-7), 3.57–3.63 (1H, m, H-15), 4.89 (1H, t, H-16), 4.37 (1H, q, H-16), 1.36 (3H, d, J = 6.8 Hz, H-17), 1.31 (6H, s, H-18, H-19). Comparing the above data with Ref. [38], the obtained compound was identified as cryptotanshinone.

Compound IV (Peak IV in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 9.25 (1H, d, J = 8.8 Hz, H-1), 7.56 (1H, q, J = 8.8 Hz, 6.8 Hz, H-2), 7.36 (1H, d, J = 6.8 Hz, H-3), 8.31 (1H, d, J = 8.8 Hz, H-6), 7.82 (1H, d, J = 8.8 Hz, H-7), 7.31 (1H, s, H-16), 3.20 (3H, s, H-17), 2.70 (3H, s, H-18). Comparing the above data with Refs. [37,38], the obtained compound was identified as tanshinone I.

Compound V (Peak V in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 7.23 (1H, d, J = 1.2 Hz, H-2), 7.26 (1H, d, J = 1.2 Hz, H-2′), 7.43 (1H, d, J = 8.0 Hz, H-10), 7.54 (1H, d, J = 8.0 Hz, H-10′), 7.58 (1H, d, J = 8.0 Hz, H-11), 7.89 (1H, d, J = 8.0 Hz, H-11′), 6.05 (1H, m, H-16), 2.28 (2H, m, H-17), 3.34 (2H, t, H-18), 2.51 (2H, t, H-20), 1.88 (2H, m, H-21), 3.29 (2H, t, H-22), 5.51 (1H, s, H-23α), 5.08 (1H, s, H-23β), 2.27 (6H, s, H-14, H-14′), 2.07 (3H, d, J = 1.6 Hz, H-24). Comparing the above data with Ref. [40], the obtained compound was identified as neo-przewaquinone A.

Compound VI (preserving in stationary phase): ^{1}H NMR (400 MHz,$CDCl_3$): 3.15–3.19 (2H, m, H-1), 1.78–1.80 (2H, m, H-2), 1.63–1.66 (2H, m, H-3), 7.60 (1H, d, J = 8.0 Hz, H-6), 7.11 (1H, d, J = 8.0 Hz, H-7), 7.08 (1H, s, H-14), 3.02 (1H, m, H-15), 1.16 (6H, d, J = 6.8 Hz, H-16, H-17), 1.30 (6H, s, H-18, H-19). Comparing the above data with Ref. [41], the obtained compound was identified as miltirone.

Compound VII (Peak VII in Fig. 3): ^{1}H NMR (400 MHz,$CDCl_3$): 3.18 (2H, t, H-1), 1.64–1.67 (2H, m, H-2), 1.78–1.80 (2H, m, H-3), 7.64 (1H, d, J = 8.4 Hz, H-6), 7.55 (1H, d, J = 8.0 Hz, H-7), 7.29 (1H, s, H-16), 2.26 (3H, d, H-17), 1.31 (6H, s, H-18, H-19). Comparing the above data with Refs. [37,38], the obtained compound was identified as tanshinone IIA.

4. Concluding remarks

In conclusion, HSCCC was successfully used for the isolation and purification of tanshinones from *Salvia miltiorrhiza Bunge* using light petroleum–ethyl acetate–methanol–water (6:4:6.5:3.5, v/v) as the two phase solvent system, which yielded 8.2 mg of dihydrotanshinone I, 5.8 mg of 1,2,15,16-tetrahydrotanshiquinone, 26.3 mg of cryptotanshinone, 16.2 mg of tanshinone I, 25.6 mg of neo-przewaquinone A, 68.8 mg of tanshinone IIA and 9.3 mg of miltirone were obtained from 400 mg of extracts from *Salvia miltiorrhiza Bunge* in one-step HSCCC separation. The present paper indicated that HSCCC is of great use for samples with complex composition.

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