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PREPARATIVE SEPARATION AND PURIFICATION OF SESQUITERPENOIDS FROM *Tussilago farfara* L. BY HIGH-SPEED COUNTER-CURRENT CHROMATOGRAPHY

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Preparative high-speed counter-current chromatography (HSCCC) was successfully applied for separation and purification of sesquiterpenoids from an extract of *Tussilago farfara* L. with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.5:1.1:0.3, v/v/v/v). The separation produced a total of 32 mg of tussilagone, 18 mg of 14-acetoxy-7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'-methyl butyryloxy)-notonipetranone and 21 mg of 7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'- methyl butyryloxy)-3,14-dehydro-Z-notonipetranone from 500 mg of the crude extract in one step separation with the purity of 99.5, 99.4 and 99.1%, respectively, as determined by HPLC. The structures of these compounds were identified by ESI-MS, ^{1}H-NMR and ^{13}C-NMR.

Keywords: high-speed counter-current chromatography; sesquiterpenoids; *Tussilago farfara* L.

INTRODUCTION

Tussilago farfara L., one of the most popular Chinese traditional medicines, widely spread in China, North Africa, Siberia, and Europe. The flower buds of *Tussilago farfara* L. were used in the treatment of cough, bronchitis, and asthmatic disorders.[1,2] Sesquiterpenoids in the flower buds of *Tussilago farfara* L. have been reported to have antimicrobial activity,[3] inhibitory activity on platelet activating factor,[4] stimulative activity on cardiovascular and respiratory systems.[5] The preparative separation and purification of those compounds from the flower buds of *Tussilago farfara* L. by traditional column methods are tedious and usually require multiple chromatographic steps.[6] Therefore, it is important to develop an efficient method to separate and purify them. High-speed counter-current chromatography (HSCCC) is a support free liquid-liquid partition chromatographic technique and eliminates irreversible adsorption of the sample onto the solid support. With a large volume of sample injection, multiform relative pure compounds can be obtained at one step in large amount. It is especially suitable for separation and purification of active components from natural products.[7] To the best of our knowledge, there has been no report of using HSCCC to isolate chemical compounds from *Tussilago farfara* L. The aim of the present paper was to develop an efficient method for the preparative separation and purification of sesquiterpenoids with high purity from *Tussilago farfara* L. by HSCCC. Three compounds were successfully separated and their chemical structures were shown in Figure 1.

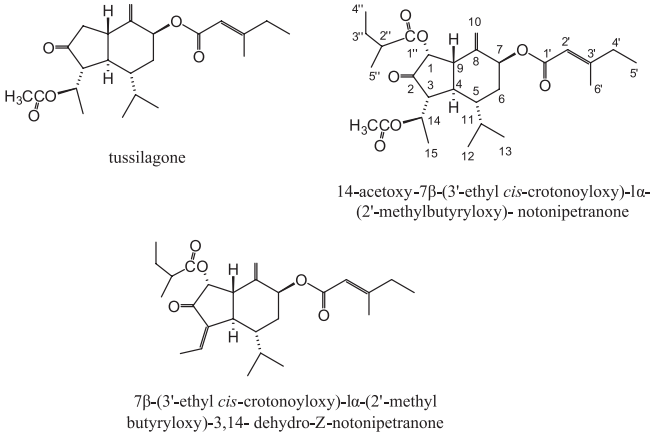

Figure 1. *Chemical structures of three compounds isolated from Tussilago farfara* L.

EXPERIMENTAL

Apparatus

HSCCC was carried out using a Model GS10A-2 instrument (Beijing Emilion Science & Technology Co., Beijing, China), with a multilayer PTFE coil of 1.6 mm i.d. and 110 m in length with a total capacity of 230 mL. The β values of the column range from 0.5 at internal to 0.8 at the external (β = *r*/*R*, where *r* is the rotation radius or the distance from the coil to the holder shaft, and *R* (*R* = 8 cm) is the revolution radius or the distances between the holder axis and central axis of the centrifuge). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Emilion Science & Technology Co., Beijing, China). Continuous monitoring of the effluent was carried out with a Model 8823A-UV detector (Beijing Emilion Science & Technology Co., Beijing, China). A manual sample injection valve with 20 mL loop (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column. A Model 3057 portable recorder (Yokogawa, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

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The high-performance liquid chromatography (HPLC) used throughout this study consisted of a Waters 996 photodiode array detection (DAD), a Waters 600 Multisolvent Delivery, a Waters 600 system controller, a Waters 600 pump, and a Millennium32 workstation (Waters, Milford, USA).

Materials

Petroleum ether (60-90°C), *n*-hexane, methanol, ethanol and ethyl acetate were of analytical grade (Juye Chemical Factory, Jinan, China). Methanol used for HPLC analysis was of chromatographic grade (Siyou Special Reagent Factory, Tianjin, China). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions.

The flower buds of *Tussilago farfara* L. was obtained from a local drug store and identified by Dr Li jia (College of Pharmacy, Shandong University of Traditional Chinese Medicine, Shandong, China).

Sample preparation

The dried flower buds of *Tussilago farfara* L. were ground to powder. The powder (500 g) was extracted three times with petroleum ether (60-90°C), and the combined extracts were concentrated under reduced pressure which yielded 21.8 g of crude extract for the further separation and purification.

Selection of two-phase solvent system

The composition of the two-phase solvent system was selected according to the partition coefficient (*K*) of the target compounds of the samples. The partition coefficient was determined by HPLC as follows: 10 mg of the crude extract 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. Equal volumes of each phase were then analyzed by HPLC to obtain the partition coefficients (*K*). The *K*-value was defined as the peak area of compound in the upper phase divided by the peak area of compound in the lower phase (Figure 2).[8]

Preparation of the two-phase solvent system and sample solution

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane-ethyl acetate-methanol-water (1:0.5:1.1:0.3, v/v/v/v). After thoroughly equilibrating the mixtures in a separation funnel at room temperature, two phases were separated shortly before use. The upper organic was used as stationary phase, and the lower phase as mobile phase. The sample solution was prepared by dissolving the crude sample in the mixture solution of organic phase and aqueous phase (1:1, v/v) of the solvent system used for HSCCC separation.

HSCCC separation

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase, and then the lower phase was pumped into the head end of the column inlet at a flow rate of 1.0 mL/min, while the column was rotated at 800 rpm. After hydrodynamic equilibrium was reached as indicated by a clear mobile phase eluting from the tail outlet, the sample solution (500 mg dissolved in 10 mL mixture consisting of equal volumes of each phase of the solvent system) was injected through the sample port. The effluent from the outlet of the column was continuously monitored by UV detector at 254 nm,

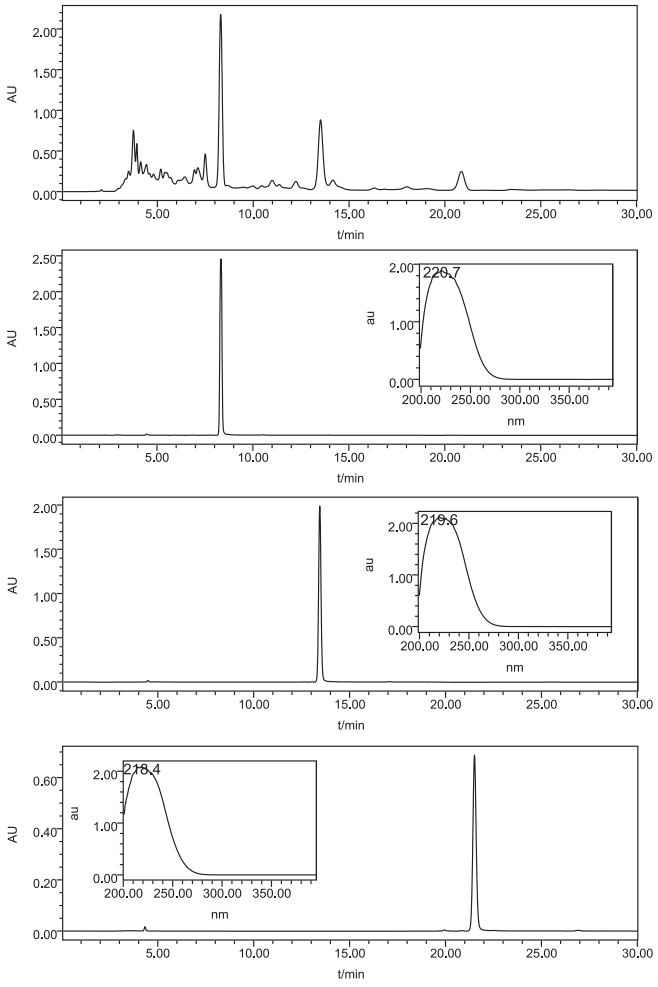

Figure 2. *(a) HPLC chromatograms of the crude extract from Tussilago farfara L.; (b) HPLC analysis and UV spectrum of the tussilagone (peak I in Figure 3) purified with HSCCC; (c) HPLC analysis and UV spectrum of the 14-acetoxy-7β-(3'-ethyl cis- crotonoyloxy)-lα-(2'-methyl butyryloxy)-notonipetranone (peak II in Figure 3) purified with HSCCC; (d) HPLC analysis and UV spectrum of the 7β-(3'-ethyl cis- crotonoyloxy)-lα-(2'-methyl butyryloxy)-3,14-dehydro-Z-notonipetranone (peak III in Figure 3) purified with HSCCC. Conditions: a Shim-pack VP-ODS column (250×4.6 mm, i.d., 5 μ); column temperature: 25 °C; Mobile phase: methanol-water (75:25, v/v); flow rate: 1.0 mL/min; detection: 220 nm; injection volume: 10 μL*

and the peak fractions were collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

Analysis and identification of HSCCC peak fractions

The crude sample and each purified fraction from the HSCCC separation were analyzed by HPLC with a Shim-pack VP-ODS column (250 x 4.6 mm, i.d., 5 μ) and column temperature of 25 °C. The mobile phase, a solution of methanol and water (75:25, v/v), was set at a flow-rate of 1.0 mL/min. The effluent was monitored by DAD at 220 nm.

The identification of HSCCC peak fractions were performed by mass spectrometry (ESI-MS) with an Agilent 1100/MSD (California, USA), and NMR spectra with a Varian-600 spectrometer (Varian, Palo Alto, CA, USA) with $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard.

RESULTS AND DISCUSSION

Optimization of HSCCC conditions

The selection of the two-phase solvent system is the most important, and is also the most difficult step. It is estimated that about 90% of the entire work in HSCCC is invested in solvent system selection.[9,10] Partition coefficient (*K*) is the most important parameter in solvent system selection. Successful separation by HSCCC needs a suitable *K*-value. Large *K*-value usually tends to produce excessive sample band broadening, while small *K*-value results in a poor peak resolution.[11]

In this work, selection of a suitable solvent system was carried out according to the polarity of the target compound.[12] If the sample is a petroleum ether extract (relatively hydrophobic solvent), the solvent system may first select *n*-hexane–ethyl acetate–methanol–water. Whereas if the sample is a methanol extract (polar solvent), the solvent system may first select butanol–water. Since the flower buds of *Tussilago farfara* L. were extracted with petroleum ether, the selection of solvent system was started with the two-phase solvent system consisted of *n*-hexane–ethyl acetate–methanol–water, and the volume ratios in Table 1 were tested. As shown in Table 1, it was difficult to separate compound 1 and 2 for the close *K*-value in the solvent system of *n*-hexane-ethyl acetate-methanol-water (1:0.2:1:0.2, v/v/v/v). The *K*-value of *n*-hexane-ethyl acetate-methanol-water (1:0.4:1:0.4, v/v/v/v) and (1:0.5:1:0.5, v/v/v/v) was too big and would result in a long separation time. Among these solvents, the ratio of *n*-hexane-ethyl acetate-methanol-water (1:0.5:1.1:0.3, v/v) was the best to separation. Figure 3 shows the separation of HSCCC using this solvent system.

The petroleum ether (60-90°C) extract (500 mg) from the flower buds of *Tussilago farfara* L. was isolated and purified under the optimum HSCCC conditions. The retention of the stationary phase was 62%, and the total separation time was about 7 h. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 220 nm to draw the elution curve (Figure 3). Based on the HPLC analysis, three compounds were obtained in one step separation and yielded 32 mg of tussilagone (peak I in Figure 3), 18 mg of 14-acetoxy-7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'-methyl butyryloxy)- notonipetranone (peak II in Figure 3) and 21 mg of 7β-(3'-ethyl *cis*- crotonoyloxy)-lα-(2'-methyl butyryloxy)-3,14-dehydro-Z-notonipetranone (peak III in Figure 3). The purity was 99.5, 99.4 and 99.1%, respectively (Figure 2).

Table 1. Partition coefficients (K) of three compounds

Solvent system (n-hexane-ethyl acetate-methanol-water)	*K*		
	Compound 1	Compound 2	Compound 3
1:0.2:1:0.2	0.79	0.85	5.23
1:0.4:1:0.4	2.46	9.01	13.76
10.5:1:0.5	5.48	14.92	31.91
1:0.5:1.1:0.3	1.57	2.60	6.96

The structural identification

The structural identification of the three compounds was performed with ESI-MS, ^{1}H and ^{13}C-NMR spectra.

Compound corresponding peak I: ESI-MS (positive mode), *m/z* 413.2 $[M+Na]^+$. ^{1}H and ^{13}C-NMR Data are given in Table 2. Comparing the data with references, the obtained compound was identified as a sesquiterpenoid, i.e. tussilagone (14-acetoxy-7β-(3′-ethyl crotonoyloxy)- notonipetranone).[12,13]

Compound corresponding peak II: ESI-MS (positive mode), *m/z* 513.2 $[M+Na]^+$. ^{1}H and ^{13}C-NMR Data are given in Table 2. Comparing the data with references, the obtained compound was identified as a sesquiterpenoid, i.e. 14-acetoxy-7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'-methyl butyryloxy)-notonipetranone.[6,13]

Compound corresponding peak III: ESI-MS (positive mode), *m/z* 883.3 $[2M+Na]^+$. ^{1}H and ^{13}C-NMR Data are given in Table 2. Comparing the data with references, the obtained compound was identified as a sesquiterpenoid, i.e. 7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'-methyl butyryloxy)-3,14-dehydro-Z-notonipetranone.[6,13]

The total above results showed HSCCC is an excellent method to separate sesquiterpenoids from the extract of flower buds of *Tussilago farfara* L.

CONCLUSION

An HSCCC technique has been developed and successfully applied to the separation and purification of the flower buds of *Tussilago farfara* L. Three compounds including tussilagone, 14-acetoxy-7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'- methyl butyryloxy)-notonipetranone and 7β-(3'-ethyl *cis*-crotonoyloxy)-lα-(2'-methyl butyryloxy)-3,14-dehydro-Z-notonipetranone were separated and purified in one step separation. The present study demonstrates that HSCCC are very useful techniques for the isolation and purification of sesquiterpenoids from natural products.

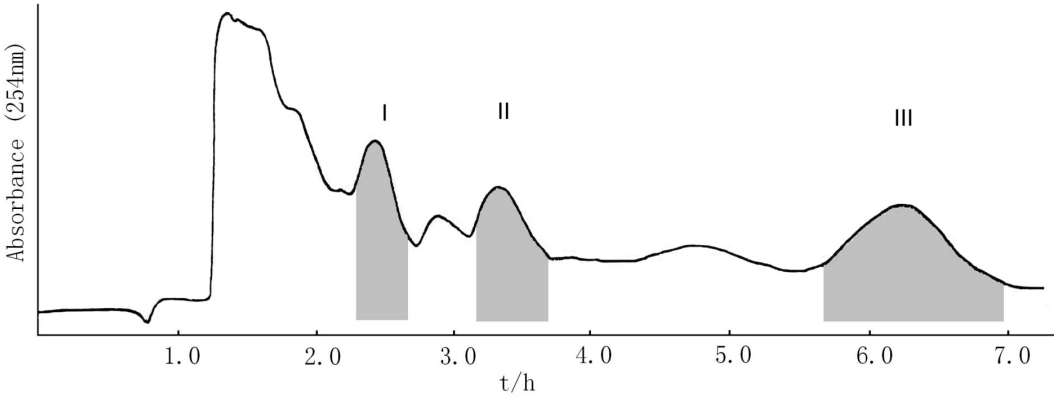

Figure 3. *Chromatogram of the crude extract by HSCCC. Two-phase solvent system: n-hexane-ethyl acetate-methanol-water (1:0.5:1.1:0.3, v/v/v/v); Mobile phase: the lower phase; flow rate: 1.0 mL/min; revolution speed: 800 rpm; detection wave length: 254 nm; sample size: 500 mg; injection volume: 10 mL; retention of stationary phase: 62%; I: tussilagone; II: 14-acetoxy-7β-(3'-ethyl cis-crotonoyloxy) -lα-(2'-methyl butyryloxy)-notonipetranone; III: 7β-(3'-ethyl cis-crotonoyloxy)-lα- (2'-methyl butyryloxy)-3,14-dehydro-Z-notonipetranone*

Table 2. ^{1}H and ^{13}C-NMR Data of compounds **1**-**3** (600 and 150 MHz in $CDCl_3$)

No.	**1**		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1α	2.17 m	42.6		72.5		72.4
1β	2.38 dd (16.8, 6.0)		5.45 d (3.6)		5.55 d (4.0)	
2		214.9		208.1		200.2
3β	2.49 dd (10.8, 2.4)	57.2	2.60 m	56.5		139.1
4α	1.47 m	49.1	2.02 m	41.2	2.79 m	44.7
5β	1.97 m	43.9	1.91 m	44.0	2.10 m	40.9
6α	1.45 m	31.2	1.50 m	30.3	1.55 m	29.7
6β	2.06 m		2.00 m		1.95 m	
7α	5.58 t (2.7)	72.9	5.54 br s	73.4	5.52 d (3.3)	73.3
8		146.1		140.6		140.9
9β	2.59 m	42.3	2.58 m	46.7	2.70 m	45.8
10	5.15 s	110.1	4.78 s	113.5	5.17 s	112.6
10′	4.79 s		5.18 s		4.81 s	
11	2.30 m	27.6	2.37 m	27.6	2.02 m	27.5
12	0.98 d (7.2)	21.4	1.00 d (6.6)	21.3	0.98 d (6.2)	26.8
13	0.78 d (7.2)	15.4	0.82 d (6.6)	15.4	0.88 d (7.2)	15.5
14	5.10 m	69.6	5.16 m	69.6	6.39 q (7.2)	136.8
15	1.22 d (6.6)	15.2	1.23 d (6.6)	15.8	2.18 d (7.2)	15.1
1′		166.0		165.8		165.9
2′	5.63 s	114.6	5.61 s	114.5	5.63 s	114.5
3′		162.0		162.2		162.1
4′	2.18 m	33.8	2.16 m	33.8	2.18 m	33.8
5′	1.07 t (7.8)	11.9	1.07 t (7.8)	11.6	1.07 t (7.2)	11.9
6′	2.15 d (1.2)	18.9	2.10 br s	18.9	2.15 s	19.0
1″				175.0		175.5
2″			2.37 m	44.1	2.40 m	40.4
3″			1.60 m	26.7	1.65 m	11.5
4″			0.89 t (7.2)	11.9	0.90 t (7.2)	16.5
5″			1.15 t (6.6)	16.8	1.13 d (7.2)	21.3
$CO\underline{C}H_3$	2.11 s	21.6	2.14 s	21.4		
$\underline{C}OCH_3$		171.0		170.9		

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