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Preparative Separation of Methylswertianin, Swerchirin and Decussatin from the Tibetan Medicinal Plant *Swertia Mussotii* Using High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – Xanthones, the primary constituents of *Swertia mussotii*, are known to possess a variety of biological activities, including anti-depressant, anti-leukaemic, anti-tumour, anti-tubercular, choleretic, diuretic, anti-microbial, anti-fungal, anti-inflammatory, anti-viral, cardiotonic and hypoglycemic properties. However, high performance, environmentally friendly methods for isolating and purifying xanthones from *S. mussotii* are not currently available.

Objective – To develop a high performance and environmentally friendly method for the preparative separation of xanthones methylswertianin, swerchirin and decussatin from *S. mussotii* using high-speed counter-current chromatography (HSCCC).

Methodology – A solvent system composed of *n*-hexane:ethyl acatate:methanol:water (5:5:10:4, v/v/v/v) was developed for the separation method. The upper phase was used as the stationary phase, and the lower phase was used as the mobile phase at a flow rate of 1.5 mL/min, a rotation speed of 800 rpm and a temperature of 25 °C.

Results – Using the described method, 8 mg of methylswertianin, 21 mg of swerchirin and 11 mg of decussatin with purities of over 98% could be isolated from a 150 mg crude sample. They were identified by ^{1}H-NMR and ^{13}C-NMR analysis.

Conclusion – Three xanthones in *Swertia mussotii* could be systematically isolated and purified using HSCCC. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: high-speed counter-current chromatography; xanthone; methylswertianin; swerchirin; decussatin; *Swertia mussotii*

Introduction

Swertia mussotii, in the family Gentianaceae, is a traditional Tibetan folk medicine called 'Zang yin chen' used to treat diseases of the liver and gall. There are about 170 species in the genus *Swertia*, with approximately 79 species present in China (Yang, 1991). Chemical and pharmacological studies of *S. mussotii* demonstrated that the major bioactive constituents are xanthones and iridoids (Brahmachari *et al*., 2004; Shang *et al*., 2008; Zhang *et al*., 2009). Xanthones (primarily 1, 3, 5, 8- and 1, 3, 7, 8-tetraoxygenate forms) exhibit various biological activities, including anti-depressant, anti-leukaemic, anti-tumour, anti-tubercular, choleretic, diuretic, anti-microbial, anti-fungal, anti-inflammatory, anti-viral, cardiotonic, and hypoglycaemic properties (Pant *et al*., 2000).

Methylswertianin, swerchirin and decussatin (Fig. 1), the major bioactive constituents in *S. mussotii*, have been isolated and purified using classic methods such as silica gel, which requires large amounts of organic, environmentally hazardous solvents. In addition, conventional separation methods require multiple steps and are therefore time consuming and unsuitable for large-scale isolation. Thus, it is necessary to develop a more efficient method to separate and purify the material in bulk for quality control, pharmacological research, and new product development based on the plant *S. mussotii*.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography technique that requires no solid support matrix (Ito and Conway, 1984). The major advantage of HSCCC is that it overcomes the irreversible adsorptive loss of samples onto the solid support matrix often employed in conventional methods. It also has the unique features of high recovery, high efficiency and ease of scale-up (Liu *et al*., 2008). This method has been applied successfully to the analysis and separation of several natural products (Tong *et al*., 2006; Liu *et al*., 2008; Urbain *et al*., 2008; Destandau *et al*., 2009; Kim and Ha, 2009). There has been a report describing xanthones separation using HSCCC (Zhang and Shan, 2010).

To the best of our knowledge, the use of HSCCC for the isolation and purification of methylswertianin, swerchirin and decussatin from a herbal medicinal plant has not been reported.

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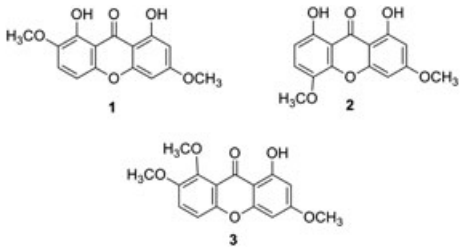

Figure 1. Structures of (1) methylswertianin, (2) swerchirin and (3) decussatin.

We report an optimised, general procedure for the isolation and purification of these xanthones by HSCCC capable of producing pure compounds that can be used as 'reference compounds' for quality control in *S. mussotii* medicinal products.

Experimental

Reagents and materials

All organic solvents used for HSCCC separation were of analytical grade and purchased from the Jinan Reagent Factory (Jinan, China). The chromatographic grade methanol used for HPLC analysis was purchased from Yuwang Chemical Ltd (Shandong, China). Deionised water was used throughout the experiment. Column chromatography silica gel was purchased from the Qingdao Ocean Chemical Factory (Shandong, China). Dimethyl sulphoxide (DMSO-d_6) was used as the solvent for NMR determination. *Swertia mussotii* was collected from Qinghai Province and positively identified by Professor Guichen Chen (Northwest Institute of Plateau Biology, Chinese Academy of Sciences).

Apparatus

High-speed counter-current chromatography. HSCCC was performed using a TBE-300A high-speed counter-current chromatography unit (Tauto Biotechnique Company, Shanghai, China) with a series of three multilayer coil separation columns (i.d. of the tubing = 1.6 mm, total volume = 280 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β-values of the preparative column ranged from 0.5 (at the internal terminal) to 0.8 (at the external terminal), where $\beta = r/R$, with r the distance from the coil to the holder shaft and R the revolution radius or the distance between the holder axis and central axis of the centrifuge. The HSCCC instrument was assembled by ÄKTAprime (GE Co. Ltd, USA) with a UV absorbance detector to monitor the effluent, an N2000 chromatography workstation (Zheda Information Project Company, Hang Zhou, China) to collect the data, and a HX 1050 constant-temperature module (Beijing Boyikang Laboratory Instrument Company, Beijing, China) for temperature control. The revolution speed of the apparatus can be adjusted from 0 to 1000 rpm.

High-performance liquid chromatography. The HPLC system was an Agilent 1200 system, consisting of a G1354A solvent delivery unit, a G1315B UV–vis photodiode array detector, a G1316A column thermostat, a G1313A autosampler, an Eclipse XDB-C_{18} 5 μm, 4.6 × 150 mm analytical column, and an Agilent HPLC workstation (Agilent Technologies Co. Ltd., USA). Nuclear magnetic resonance spectra were obtained using an AM400 system (Bruck Co. Ltd) at the State Key Laboratory of Applied Organic Chemistry, Lanzhou University.

Preparation of the crude sample

Whole *S. mussotii* plants (0.5 kg) were ground into a powder and extracted three times with 75% ethanol. The extraction times were 2 h, 2 h and 1 h, respectively. The extracts were combined and evaporated to dryness by rotary evaporation at 60 °C under vacuum, which yielded 0.11 kg of dry powder. The residues were then suspended in distilled water (3 L) and extracted with light petroleum ether (b.p. 60–90 °C, 6 L), chloroform (6 L) and *n*-butanol (6 L). After the chloroform layer was concentrated to dryness, 26 g of extract was obtained. The extract of chloroform (26 g) was subjected to silica gel (1000 g) CC eluted with petroleum ether and increasing proportions of ethyl acetate to give five fractions, and 6.4 g of crude sample was obtained from fraction 4 for HSCCC separation.

Preparation of the two-phase solvent system and sample solution

The two-phase solvent system was prepared by mixing *n*-hexane:ethyl acatate:methanol:water (5:5:10:4, v/v/v/v) thoroughly equilibrated in a separation funnel at room temperature. Two phases were separated shortly before use and degassed by sonication for 20 min. The sample for HSCCC separation was prepared by dissolving 150 mg of the dried crude powder sample in 20 mL of the upper phase.

HSCCC separation procedure

The HSCCC was performed as follows: the multilayer column was first completely filled with the upper (stationary) phase using the ÄKTAprime plus system. Then the apparatus was rotated at 800 rpm (forward direction), and the lower phase was pumped into the column at a flow rate of 1.5 mL/min when the revolution velocity was smooth. After hydrodynamic equilibrium was reached, indicated by the emergence of the mobile phase front, 20 mL of the sample solution was injected into the column through the injection valve. The effluent from the tail end of the column was continuously monitored by a UV detector at 254 nm and the chromatogram was recorded. Peak fractions were manually collected according to the chromatogram data, and each fraction was evaporated to dryness under reduced pressure and dissolved in methanol for subsequent purity analysis by HPLC.

HPLC analysis and identification of target compounds

The crude extract powder and each fraction corresponding to portions of the major peaks identified by HSCCC were analysed by HPLC. The HPLC analysis was performed on a reversed-phase Agilent Eclipse XDB C_{18}-column (150 mm × 4.6 mm i.d., 5 μm) with gradient elution at a column temperature of 25 °C. The gradient elution began with 80% methanol and linearly increased to 85% methanol in 2.5 min and to 95% methanol within the following 6.5 min at a constant flow rate of 1.0 mL/min, with the detector measuring at 254 nm. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalised to the sum of all observed peaks.

Target compounds were identified using ^{1}H-NMR and ^{13}C-NMR spectra. ^{1}H-NMR and ^{13}C-NMR spectra were measured in DMSO-d_6 on a Mercury-400BB NMR spectrometer with tetramethylsilane (TMS) as the internal standard. All were run at room temperature.

Results and Discussion

Selection of solvent system and other HSCCC conditions

A suitable solvent system is the key factor for successful HSCCC separation. In this study, a series of experiments were performed to optimise the two-phase solvent system for HSCCC separation. The K values of the three target compounds in the crude sample are shown in Table 1. Two-phase solvent systems with chloroform:water (5:5, v/v) and chloroform:methanol:water (4:7:5, v/v/v and 6:9:7, v/v/v) were tested first, resulting in small K values and poor retention of target compounds in the upper phase. Then, two-phase solvent systems comprised of ethyl acetate:water (5:5, v/v) and ethyl acetate:methanol:water (8:1:4,

Table 1. The *K* values of the target compounds in different solvent systems

	Solvent system	Volume ratio	Methylswertianin	Swerchirin	Decussatin
1	Chloroform:water	5:5	∞	<0.01	0.03
2	Chloroform:methanol:water	4:7:5	0.01	<0.01	<0.01
3	Chloroform:methanol:water	6:9:7	0.017	0.014	0.014
4	Ethylacetate:water	5:5	>100	∞	∞
5	Ethyl acetate:methanol:water	8:1:4	69.9	>1001	>100
6	Ethyl acetate:methanol:water	7:2:3	14.67	18.67	14.08
7	*n*-hexane:ethyl acetate:methanol:water	5:5:5:5	15.50	32.10	4.41
8	*n*-hexane:ethyl acetate:methanol:water	5:5:7:5	9.44	6.44	1.53
9	*n*-hexane:ethyl acetate:methanol:water	5:5:6:4	5.32	7.68	1.68
10	*n*-hexane:ethyl acetate:methanol:water	5:5:9:4	2.07	2.78	1.01
11	*n*-hexane:ethyl acetate:methanol:water	5:5:10:4	1.32	1.95	0.50

Table 2. Comparison of separation time, stationary phase retention and purities of the three xanthones studied under different flow rates

Flow rate (mL/min)	Separation time (min)	Retention (%)	Purity (%)		
			Methylswertianin	Swerchirin	Decussatin
1.5	360	68.7	99.1	98.9	99.0
2.0	280	65.5	98.9	92.5	97.3
2.5	220	61.7	98.9	91.1	97.2

v/v/v and 7:2:3, v/v/v) were tested. However, the resulting *K* values were too high (larger than 10), and the long time that the target compounds required for elution resulted in poor resolution.

The addition of *n*-hexane to the ethyl acetate:methanol:water system could improve the phase separation. This solvent system has been successfully applied to many samples with moderate polarity (Liu *et al*., 2004; Wang *et al*., 2004, 2005; Wei and Ito, 2006). Five *n*-hexane:ethyl acetate:methanol:water solvent solutions with the following volume ratios were tested in HSCCC separation: 5:5:5:5, 5:5:7:5, 5:5:6:4, 5:5:9:4 and 5:5:10:4. The results indicated that a ratio of 5:5:10:4 (v/v/v/v) was suitable for the separation of the three target compounds from the crude sample, and appropriate resolution and retention of the stationary phase (about 69%) was obtained.

The influence of flow rate on the mobile phase and revolution speed were also investigated. The flow rate of the mobile phase determines the separation time, i.e., the retention of the stationary phase in the column and peak resolution (Ito, 2005). Under the same conditions of revolution speed, temperature and sample loading volume, different flow rates (1.5, 2.0 and 2.5 mL/min) were tested for their effect on separation time, retention of the solid phase and the sample purity (Table 2). At a flow rate of 1.5 mL/min the target compounds required 360 min to elute, and the retention of the stationary phase was 68.7%. An increased flow rate of 2.0 mL/min resulted in a shorter separation time (280 min) and lower retention of the stationary phase (about 65.5%). At a flow rate of 2.5 mL/min, the elution required only 220 min, but the retention of the stationary phase decreased to 61.7%. While the elution time decreased with an increased flow rate, the retention of the solid phase and the resolution of target compounds decreased, particularly for compounds two and three. The purity of compounds two and three were only 91.1% and 97.2%, respectively, under the highest flow rate (2.5 mL/min). We used a flow rate of 1.5 mL/min in subsequent HSCCC separation procedures despite the longer separation time and mobile phase. In addition, the revolution speed can also have an impact on the retention of the stationary phase, and the 800 rpm speed in our isolation procedure was mainly considered emulsification. High rotary velocity can damage the separation pipelines.

HSCCC separation

The crude sample (150 mg) was dissolved in 20 mL of the mobile phase. The sample solution was separated and purified by HSCCC according to the procedure described above. The upper phase was used as the stationary phase while the lower phase was used as the mobile phase in the head to tail elution mode. The retention of the stationary phase was 69%, and the total separation time was about 360 min. The HSCCC fractions were analysed by HPLC, and their absorbance was measured at 254 nm to construct the elution curve (Fig. 2). Based on HPLC

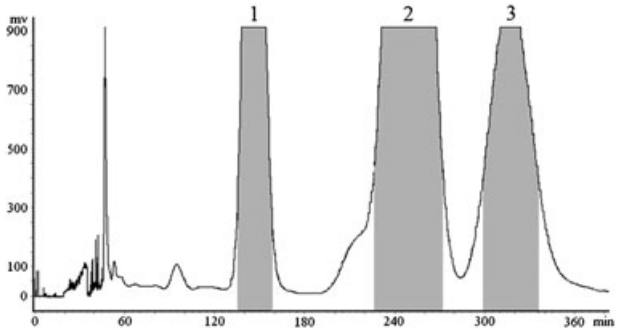

Figure 2. Chromatogram of the crude sample from *Swertia mussotii* by preparative HSCCC. Two-phase solvent system: *n*-hexane:ethyl acatate: ethanol:water (5:5:10:4, v/v/v); stationary phase, upper phase; mobile phase, lower phase; revolution speed, 800 rpm; sample size, 150 mg; detection wavelength, 254 nm;flow rate, 1.5 mL/min.

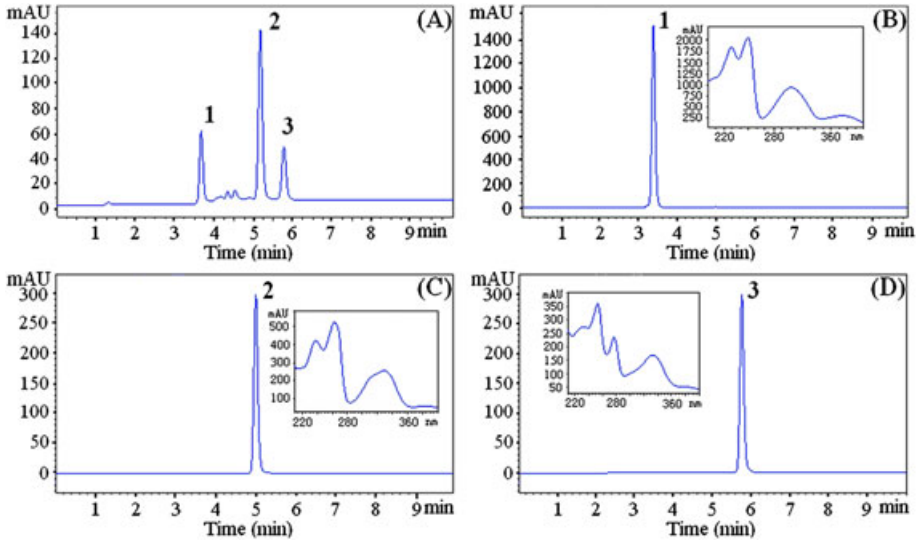

Figure 3. (A) HPLC chromatogram of the crude sample from *Swertia mussotii* and (B–D) HPLC chromatograms and UV spectra of compounds **1–3**. Column: Eclipse XDB C_{18}-column (150 mm × 4.6 mm i.d., 5um); mobile phase: methanol and water in gradient mode (methanol concentration raised from 80% to 85% in the beginning 2.5 min and increased to 95% within the following 6.5 min); flow rate: 1.0 mL/min; column temperature: 25 °C; UV wavelength: 254 nm.

analysis and the elution curve of the preparative HSCCC, collected fractions were combined into different pooled fractions. After only one round of HSCCC, 8 mg methylswertianin, 21 mg swerchirin and 11 mg decussatin were obtained from the 150 mg crude extract and their purities were 99.1%, 98.9% and 99.0%, respectively. Figure 3 shows the HPLC analysis of the combined fractions and the crude sample from *S. mussotii*.

Identification of the separation peaks

The chemical structures of the peak fractions separated by HSCCC were identified according to their ^{1}H-NMR and ^{13}C-NMR data. By comparison with reference data, peak 1, peak 2, and peak 3 were identified as methylswertianin, swerchirin and decussatin, respectively. The results for each peak fraction were as follows.

Peak 1 (Fig. 2): yellow needles. ^{1}H-NMR (DMSO-d_6,400 MHz) δ: 11.82 (1H, s,1-OH), 11.80 (1H, s,8-OH),7.51 (1H,d, J = 9.2 Hz, H-6), 6.98 (1H, d, J = 9.2 Hz, H-5), 6.60 (1H, d, J = 2.0 Hz, H-4), 6.39 (1H, d, J = 2.0 Hz, H-2), 3.89 (3H, s, OCH_3), 3.84 (3H, s, OCH_3). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 184.3 (C = O), 167.2 (C-3), 161.9 (C-1), 157.6 (C-4a), 149.1 (C-4b), 148.8 (C-8), 142.6 (C-7), 121.3 (C-6), 107.4 (C-8a), 105.7 (C-5), 101.7 (C-8b), 97.3 (C-2), 92.9 (C-4), 56.6 (OCH_3), 56.3 (OCH_3). By comparison of this profile with reference data (Pan *et al*., 2002), peak 1 was identified as methylswertianin (1,8-dihydroxy-3,7-dimethoxyxanthone).

Peak 2 (Fig. 2): yellow needles. ^{1}H-NMR (DMSO-d_6,400 MHz) δ: 11.85 (1H, s,1-OH), 11.20 (1H, s,8-OH), 7.48 (1H,d, J = 8.8 Hz, H-6), 6.75 (1H, d, J = 8.8 Hz, H-7), 6.60 (1H, s), 6.35 (1H, s), 3.90 (3H, s, OCH_3), 3.89 (3H, s, OCH_3).^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 183.8 (C = O), 167.2 (C-3), 162.0 (C-1), 157.4 (C-4a), 152.7 (C-5), 144.6 (C-4b), 139.6 (C-8), 120.7 (C-6), 109.2 (C-7), 107.6 (C-8a), 102.1 (C-8b), 97.8 (C-2), 93.1 (C-4),56.6 (OCH_3), 56.3 (OCH_3). By comparison of this profile with reference data (Shi *et al*., 2004), peak 2 was identified as swerchirin (1,8-dihydroxy-3,5-dimethoxyxanthone).

Peak 3 (Fig. 2): yellow needles. ^{1}H-NMR (DMSO-d_6,400 MHz) δ: 13.21 (1H, s,1-OH),7.61 (1H,d, J = 9.6 Hz, H-6), 7.30 (1H, d, J = 9.2 Hz, H-5), 6.50 (1H, s, H-4), 6.32 (1H, s, H-2), 3.92 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 3.82 (3H, s, OCH_3). ^{13}C-NMR (DMSO-d_6, 100 MHz) δ: 180.5 (C = O), 166.1 (C-3), 162.8 (C-1), 156.6 (C-4a), 149.9 (C-4b), 149.1 (C-7), 147.8 (C-8), 120.9 (C-6), 114.8 (C-8a), 112.8 (C-5), 103.1 (C-8b), 96.7 (C-2), 91.9 (C-4), 61.0 (OCH_3), 56.6 (OCH_3), 56.0 (OCH_3). By comparison of this profile with reference data (Chakravarty *et al*., 1994), peak 3 was identified as decussatin (1-hydroxy-3,7,8-trimethoxyxanthone).

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