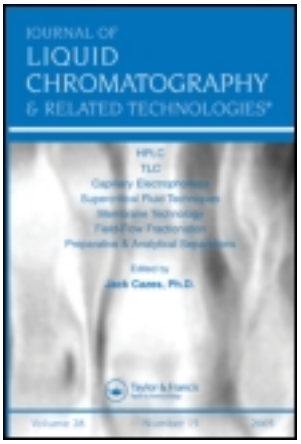

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SEMI-PREPARATIVE SEPARATION AND PURIFICATION OF THREE FLAVONOIDS FROM *PEDICULARIS LONGIFLORA* VAR. *TUBIFORMIS* (KLOTZSCH) P. C. TSOONG BY HSCCC

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□ *An effective high–speed counter–current chromatography (HSCCC) method was established for semi-preparative isolation and purification of bioactive chemical constituents from the Tibetan medicinal plant* Pedicularis longiflora *var.* tubiformis *(Klotzsch) P. C. Tsoong. With a two-phase solvent system composed of chloroform–methanol–water (8:4:5, v/v), 40 mg of extract was separated to yield luteolin (12.5 mg), apigenin (9.6 mg), and chrysoeriol (4.8 mg), with purities of 99.3, 98.2, and 98.6%, respectively, as determined by high-performance liquid chromatography (HPLC). The chemical structures of these three components were identified by* ^{1}H *NMR and* ^{13}C *NMR.*

Keywords apigenin, chrysoeriol, flavonoid, HSCCC, luteolin, *pedicularis longiflora* var. *tubiformis* (Klotzsch) P. C. Tsoong

INTRODUCTION

Pedicularis longiflora var. *tubiformis* (Klotzsch) P. C. Tsoong (Chinese name, Banchunmaxianhao, BCM) is a traditional Tibetan medicine plant, distributed mainly in Sichuan, Yunnan, Qinghai and Xizang in China.[1] It has been proven to be effective in the treatment of hepatitis, cholecystitis, edema, spermatorrhea, and tinnitus.[2] Luteolin, apigenin, and chrysoeriol are the major bioactive constituents of BCM.

Luteolin, apigenin, and chrysoeriol, which belong to the flavonoid family, were found to possess excellent antitumor, antioxidant, anti-inflammatory, and many other pharmacological activities.[3–10] For example, apigenin was recently reported to exhibit strong cytotoxic activity in

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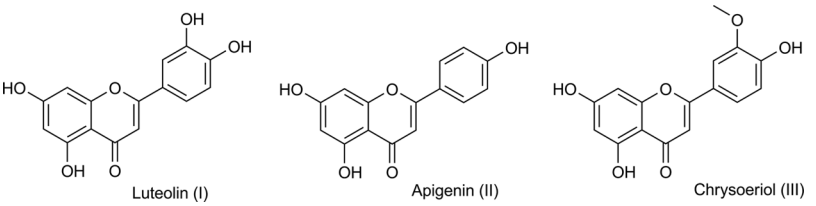

FIGURE 1 The chemical structures of luteolin (I), apigenin (II), and chrysoeriol (III).

numerous types of cancer cells including hepatocarcinogenesis,[11] neuroblastoma,[12] breast cancer,[13] esophageal squamous cell carcinoma,[14] colon cancer,[15] lung cancer,[16] and prostate cancer cells through the impairment of cell mitosis or promotion of cell apoptosis.[17] In view of these beneficial effects, an efficient method for semi-preparative separation and purification of luteolin, apigenin, and chrysoeriol from BCM is necessary.

High-speed counter-current chromatography (HSCCC), invented by Ito and Conway,[18] is a support-free liquid–liquid partition chromatographic technique, and eliminates irreversible adsorption or chemical reaction that occur in solid support used in conventional column chromatography and high-performance liquid chromatography (HPLC). In addition, this technique permits excellent sample recovery and can be employed for semi-preparative-scale separation in a completely straightforward manner. Therefore, it has been widely used for the separation and purification of active components with similar chemical structures of natural products, especially for flavonoids.[19,20] The present paper describes a successful semi-preparative separation and purification of luteolin, apigenin, and chrysoeriol from the partially purified extract of traditional Tibetan medicinal herb BCM by HSCCC. Above all, no report has been published on the isolation and purification of chrysoeriol by HSCCC. The chemical structures of these three flavonoids are shown in Figure 1.

EXPERIMENTAL

Reagents and Materials

BCM was collected from Gangcha, QingHai, China, in August 2010 and identified by Prof. Guichen Chen (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). The specimen (BCM-G, 2 kg) was stored in the Laboratory sample cabinet.

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatography grade

and purchased from Yuwang Chemical Ltd. (Shandong, China), and water used was purified using a PAT-125 (Chengdu, China) laboratory ultra-pure water system (0.4-μm filter) prior to use.

Apparatus

The HSCCC instrument used for the present study is the TBE–300A high–speed counter–current chromatography (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three PTFE (polytetrafluoroethylene) semi-preparative coils (diameter of tube, 1.6 mm, total volume, 280 mL) and a 20-mL sample loop. The revolution 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 ranged from 0.5 at 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 revolution speed of the apparatus could be adjusted with a speed controller in a range between 0 and 1000 rpm. The separation temperature was controlled by an HX–1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China). An ÄKTA prime system (GE Co. Ltd., USA) 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 was collected and analyzed with an N2000 workstation (Zhejiang University Star Information Technology Co., Ltd., Hangzhou, Zhejiang, China). The HPLC equipment used was an Agilent 1200 system, equipped with 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). The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury-400BB NMR (Varian Co. Ltd., USA) with tetramethylsilane (TMS) as internal standard.

Preparation of the Crude Sample

The dried BCM (500 g) was powdered and extracted three times with 70% EtOH under reflux (each time for 2 hr). After concentration under vacuum, the residues were suspended in distilled water and extracted with light petroleum (b.p. 60–90°C, 2.5 L), EtOAc (5 L), and *n*-butanol (5 L), respectively. The EtOAc solutions were evaporated to dryness under vacuum at 45°C to generate 20 g EtOAc extract. In order to enrich the target components, the extract of EtOAc (20 g) was then loaded on silica gel column (60 cm × 3.5 cm, contained 200 g silica gel) eluted with chloroform–methanol (5:1, v/v) to give 28 portions and 1.2 g crude sample was obtained from No 9-20 portions for HSCCC sample.

Measurement of Partition Coefficient

The composition of the two-phase solvent system was selected according to *K* value of each target component in the crude sample. The partition coefficient is the ratio of solute distributed between the mutually equilibrated two solvent phases. The partition coefficients were determined by HPLC as follows: in brief, a suitable amount of crude sample was added into a series of pre-equilibrated two-phase-solvent systems, followed by shaking the solution fully. Then, the same volume of upper and the lower phases were separately evaporated to dryness. The residues were diluted into 2 mL methanol and analyzed by HPLC. 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.

Preparation of the Two Phase Solvent System and Sample Solution

The two-phase solvent system composed of chloroform–methanol–water (8:4:5, *v*/*v*) was used for the HSCCC separation. The solvent mixture was thoroughly equilibrated in a separation funnel at room temperature and the two phases were separated immediately before use. The upper phase was used as the stationary phase and the lower phase was used as the mobile phase. The sample solution for HSCCC separation was prepared by dissolving 40 mg of dried crude powder in 10 mL of the upper phase.

HSCCC Separation Procedure

The multilayer coil column was first entirely filled with the upper phase (stationary phase). Then the apparatus was rotated at 800 rpm, while the lower phase (mobile phase) was pumped into the column at a flow rate of 1.8 mL/min. After reaching hydrodynamic equilibrium, as indicated by the emergence of the mobile phase front, 10 ml sample solution containing 40 mg of crude powder was injected into the column through the injection valve by an ÄKTA prime system. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and the chromatogram was recorded. The temperature of the apparatus was set at 25°C. The peak fractions were collected manually according to the elution profile and evaporated under reduced pressure. Then, the residues were dried and the yields were measured. In addition, the residues were dissolved in methanol for subsequent purity analysis by HPLC.

HPLC Analysis and Identification of HSCCC Peak Fractions

The crude sample purified by silica-gel column chromatography and each peak fraction obtained by HSCCC were analyzed by HPLC with DAD detection. The HPLC analysis was accomplished with a reversed-phase Agilent Eclipse XDB–C_{18} (5 μm, 4.6 × 150 mm) analytical column at a column temperature of 25°C. Methanol–water (60:40, *v*/*v*) was used as the mobile phase. The flow-rate and detection wavelength were set at 1.0 mL/min and 254 nm, respectively. Identification of the HSCCC peak fractions was performed by ^{1}H NMR and ^{13}C NMR.

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

The crude sample purified by silica-gel column chromatography was first analyzed by HPLC, so selecting an appropriate condition for HPLC was required. In the present study, different mobile phases, flow rates, column temperatures, and detection wavelengths were all tested. The result indicated that the satisfactory separation could be obtained when methanol–water (60:40, *v*/*v*) was used as the mobile phase, and the flow rate, column temperature, and detection wavelength were set at 1 mL/min, 25°C and 254 nm, respectively. The crude sample and peak fractions purified by HSCCC were analyzed by HPLC under the optimized conditions and their chromatograms are shown in Figure 2.

Selection of Two-Phase-solvent System and Other Conditions of HSCCC

Successful separation by HSCCC depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient ($0.2 < K < 2$) for the target compounds.[21] Large *K* values tend to produce excessive band broadening, while small *k* values usually result in a poor peak resolution.[22] In the present experiment, several two-phase solvent systems including *n*-hexane–ethyl acetate–methanol–water, ethyl acetate–*n*-butanol–water, and chloroform–methanol–water were tested according to the procedure described in the Measurement of Partition Coefficient section.[23–26] The *K*-values of the target compounds in different solvent systems were determined by HPLC and the results were shown in Table 1. When two-phase solvent systems comprised of *n*-hexane–ethyl acetate–methanol–water (5:5:5:5, *v*/*v*) and ethyl acetate–*n*-butanol–water (4:2:5, *v*/*v*; 2:3:5, *v*/*v*; and 5:3:4, *v*/*v*) were employed, the *K* values were too large and would result in broad peaks and long separation time.

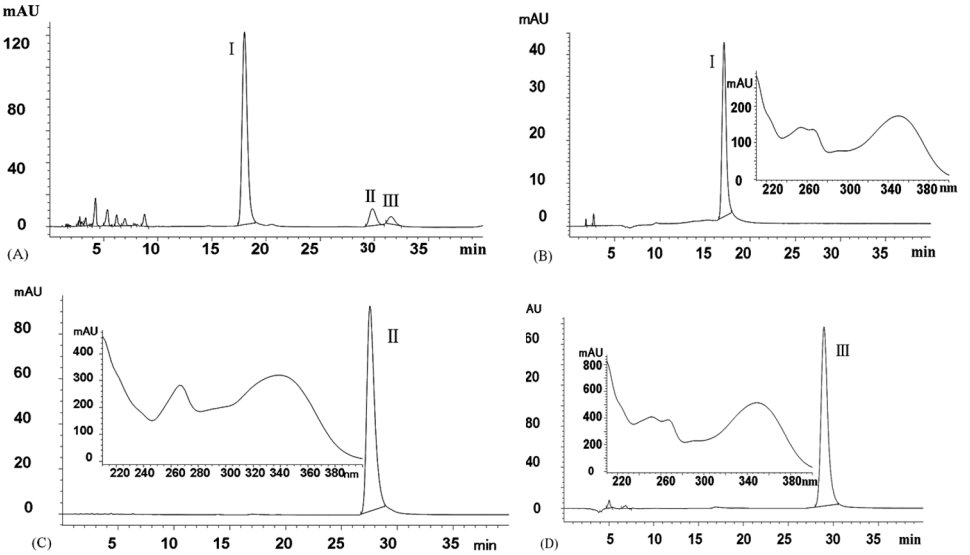

FIGURE 2 HPLC chromatograms of the crude extract from BCM (A) after cleaning up by silica-gel column chromatography and the three targeted compounds (peak fractions I, II, and III, B–D) purified by HSCCC. Conditions: column: Eclipse XDB–C_{18} column (5 μm, 4.6 × 150 mm); mobile phase: methanol–water (60:40, *v*/*v*); flow-rate: 1.0 mL/min; column temperature: 25°C; detection wavelength: 254 nm.

When chloroform–methanol–water (6:4:5, *v*/*v* and 7:5:5, *v*/*v*) were used as two-phase solvent system, the *K* values were small and might lead to poor retention of target compounds in the upper phase. Further optimization suggested that an appropriate *K* value with good resolution could be obtained by using two-phase solvent system of chloroform–methanol–water at a ratio of 8:4:5 (*v*/*v*).

TABLE 1 *K* Values of the Target Compounds in Different Solvent Systems

Solvent systems	Ratio (*v*/*v*)	*K* values		
		I	II	III
n-hexane–ethyl acetate–methanol–water	5:5:5:5	35.84	41.49	3.99
ethyl acetate–*n*-butanol–water	4:2:5	109.89	∞	∞
ethyl acetate–*n*-butanol–water	2:3:5	>1000	65.36	∞
ethyl acetate–*n*-butanol–water	5:3:4	95.24	109.89	113.64
chloroform–methanol–water	7:5:5	1.35	0.32	0.09
chloroform–methanol–water	6:4:5	1.42	0.32	0.11
chloroform–methanol–water	7:4:5	1.66	0.35	0.19
chloroform–methanol–water	8:4:5	1.66	0.55	0.20
chloroform–methanol–water	5:5:5	1.98	0.85	0.56

The symbol "∞" means the partition coefficient is too large to be evaluated.

In an addition to a suitable two-phase solvent system, other factors such as the flow rate of mobile phase and the revolution speed were also investigated. In the present study, different flow rates (1.5, 1.8, and 2.2 mL/min) of the mobile phase were first tested for their effect on separation time, retention of the station phase, and the purity of targeted compounds (Table 2). The results indicated that the low flow-rate of the mobile phase, which could improve the peak resolution and be beneficial to increase the retention of stationary phase, led to a long separation time; a higher mobile phase flow-rate could speed up the elution of the peaks, resulting in the decline of the peak resolution and the reduction of the retention of the stationary phase. The purity of compounds II and III was only 91.1% and 96.2%, respectively, under the highest flow rate (2.2 mL/min). Considering the separation time and the purity of the target compounds, a flow rate of 1.8 mL/min was used in subsequent HSCCC separation procedures. In addition, the revolution speed could also impact the retention of the stationary phase, and the 800 r/min speed in our isolation procedure was mainly considered. The high revolution speed is prone to produce emulsification.[27–31]

Under the optimized conditions, three fractions (I, II, and III) were obtained in one step separation within 8 hr (HSCCC chromatogram is shown in Figure 3), which is luteolin (I, 12.5 mg), apigenin (II, 9.6 mg), and chrysoeriol (III, 4.8 mg). As shown in Figure 2, the HPLC-DAD analysis of each HSCCC fractions revealed that the purities of these three compounds were 99.3, 98.2, and 98.6%, respectively. In addition, each HSCCC fraction was further analyzed by HPLC with ELSD (drift tube temperature 50°C; nebulizing gas flow-rate 2.6 L/min), and the purities of these three compounds were 98.7, 99.1, and 98.2%, respectively.

Structure Identification

The chemical structures of the peak fractions separated by HSCCC were determined by ^{1}H NMR and ^{13}C NMR. The NMR data of luteolin (I), apigenin (II), and chrysoeriol (III) were in agreement with published data.

TABLE 2 Comparison of Separation Time, Stationary Phase Retention, and Purities of Three Compounds Under Different Flow Rate

			Yield (mg/40 mg crude sample)/Purity (%)		
Flow-rate (mL/min)	Separation-time(min)	Retention(%)	I	II	III
1.5	420	69.1	12.1/99.4	9.0/98.7	4.6/98.9
1.8	360	67.9	12.5/99.3	9.6/98.2	4.8/98.6
2.2	300	61.8	13.0/98.7	10.7/91.1	5.2/96.2

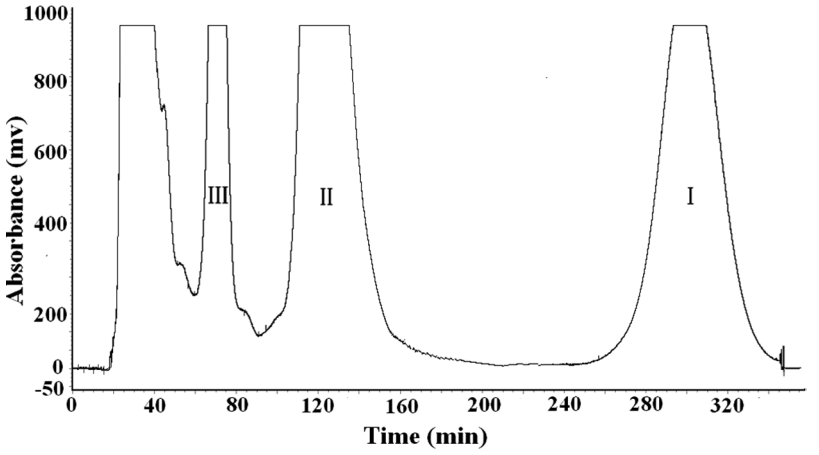

FIGURE 3 HSCCC chromatogram of the crude extract from BCM after cleaning up by silica-gel column chromatography. Two-phase-solvent system: chloroform–methanol–water at a ratio of 8:4:5 (*v*/*v*/*v*); stationary phase: upper phase; mobile phase: the lower phase; flow-rate: 1.8 mL/min; revolution speed: 800 rpm; detection wavelength: 254 nm; sample size: 40 mg of crude sample dissolved in 4 mL of the upper phase; separation temperature: 25°C.

Peak fraction I: yellow needles, ^{1}H NMR (DMSO-d_6, 400 MHz): $\delta = 12.99$ (1H, s, 5-OH), 7.41 (1H, d, $J = 8.3$ Hz, H-6′), 7.40 (1H, s, H-2′), 6.88 (1H, d, $J = 8.3$ Hz, H-5′), 6.68 (1H, s, H-8), 6.44 (1H, s, H-3), 6.19 (1H, s, H-6). ^{13}C NMR (DMSO-d_6, 400 MHz) $\delta = 165.0$ (C-2), 103.5 (C-3), 182.3 (C-4), 162.1 (C-5), 99.5 (C-6), 164.6 (C-7), 94.5 (C-8), 158.0 (C-9), 104.3 (C-10), 122.1 (C-1′), 114.0 (C-2′), 150.5 (C-3′), 146.4 (C-4′), 116.7 (C-5′), 118.6 (C-6′). In comparson with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with that of luteolin in the literature.[32,33]

Peak fraction II: yellow needles, ^{1}H NMR (DMSO-d_6, 400 MHz): $\delta = 12.98$ (1H, s, 5-OH), 10.61 (1H, s, 7-OH), 7.93 (2H, d, $J = 8.4$ Hz, H-2′, 6’), 6.94 (2H, d, $J = 8.8$ Hz, H-3′, 5’), 6.79 (1H, s, H-3), 6.49 (1H, d, $J = 2.1$ Hz, H-8), 6.20 (1H, d, $J = 2.1$ Hz, H-6). ^{13}C NMR (DMSO-d_6, 400 MHz) $\delta = 164.1$ (C-2), 102.8 (C-3), 181.7 (C-4), 161.4 (C-5), 98.8 (C-6), 163.7 (C-7), 93.9 (C-8), 157.3 (C-9), 103.7 (C-10), 121.2 (C-1′), 128.4 (C-2′), 115.9 (C-3′), 161.1 (C-4′), 115.9 (C-5′), 128.4 (C-6′). In comparison with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with that of apigenin in the literature.[34]

Peak fraction III: yellow needles, ^{1}H NMR (DMSO-d_6, 400 MHz): $\delta = 7.55$ (1H, d, $J = 8.4$ Hz, H-6′), 7.54 (1H, s, H-2′), 6.93 (1H, d, $J = 8.4$ Hz, H-5′), 6.87 (1H, s, H-3), 6.48 (1H, s, H-8), 6.17 (1H, s, H-6), 3.81 (3H, s, H-OCH_3). ^{13}C NMR (DMSO-d_6, 400 MHz) $\delta = 166.2$ (C-2), 103.3 (C-3), 181.5 (C-4), 161.4 (C-5), 99.1 (C-6), 163.4 (C-7), 94.1 (C-8), 157.4 (C-9), 103.0 (C-10), 121.3 (C-1′), 110.1 (C-2′), 150.9 (C-3′), 148.0 (C-4′), 115.8

(C-5′), 120.3 (C-6′). Im comparison with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with that of chrysoeriol in the literature.[35]

CONCLUSION

Three main bioactive flavonoids including luteolin, apigenin, and chrysoeriol from the traditional Tibetan medicine *Pedicularis longiflora* var. *tubiformis* (Klotzsch) P. C. Tsoong were successfully separated and purified with chloroform–methanol–water (8:4:5, *v*/*v*) as the two-phase-solvent system of HSCCC in one-step separation within 8 hr. The compounds obtained may be used as reference substances for chromatographic purposes without additional clean-up.

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