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Article

Separation of Four Phenylpropanoid Glycosides from a Chinese Herb by HSCCC

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Four phenylpropanoid glycosides (PPGs), such as echinacoside, wiedemannioside C, forsythoside B and verbascoside, were isolated and purified from the Tibetan medicinal herb *Pedicularis longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong by high-speed counter-current chromatography (HSCCC) combined with macroporous resin (MR) column separation for the first time. In the present study, the two-phase solvent system composed of ethyl acetate–*n*-butanol–water (10 : 6:15, v/v/v) was used for HSCCC separation. A total of 3.5 mg of echinacoside, 12.6 mg of wiedemannioside C, 22.7 mg of forsythoside B and 48.7 mg of verbascoside with the purity of 93.6, 97.9, 97.8 and 98.1%, respectively, were obtained from 120 mg of crude sample. The HSCCC fractions were analyzed by HPLC, and the chemical structures were identified by ^{1}H NMR and ^{13}C NMR. The results demonstrate that MR coupled with HSCCC is a powerful technique for separation of PPGs from natural products.

Introduction

Pedicularis longiflora Rudolph. var. *tubiformis* (Klotz) Tsoong is a traditional Tibetan medicine plant, distributed mainly in the high altitude area of Tibet, Qinghai, Sichuan and Gansu province of China (1). It has been proved to be effective in the treatment of hepatitis, cholecystitis, edema, spermatorrhea and tinnitus (2). The major active constituents of this herb are phenylpropanoid glycosides (PPGs), including echinacoside (I), wiedemannioside C (II), forsythoside B (III) and verbascoside (IV) (Figure 1). Echinacoside and related PPGs are a group of water-soluble natural products widely distributed in the plant kingdom (3–7), and have been reported to have various biological activities, including antihepatotoxic (8), anti-inflammatory (9), antibacterial (10), antinociceptive (11), antioxidant and antigenotoxic (12) activities.

In view of their wide pharmacological activities, large quantities of pure compounds are urgently needed as chemical reference standards and for further pharmacological studies. With regard to the methods available for the separation of organic compounds, silica and gel chromatography are two classical methods due to their different separation principles. However, these separation methods are tedious, require large amounts of organic solvents and usually require numerous steps resulted in lower recovery and higher cost. Especially, the four compounds with similar structure and polarity present a number of practical difficulties for the separation by silica and gel chromatography. Therefore, effective methods for the isolation and purification of the above compounds become necessary.

High-speed counter-current chromatography (HSCCC), invented by Ito and Conway (13), a support-free liquid–liquid partition chromatographic technique based on partitioning of compounds between two immiscible liquid phases without support matrix, eliminates irreversible adsorption in the solid support. A number of compounds with high purity could be obtained in a one-step separation. Furthermore, HSCCC could easily realize the separation of compounds with similar polarity and structures (14, 15). Compared with the scale-up of conventional column chromatography, the separation process is simple, the solvent needed is 75% less and the purity of the target compounds is higher in the separated fractions (16). Macroporous resin (MR), as the adsorption material for column chromatography, has been widely applied in combination with HSCCC for preseparation due to its low cost, high efficiency, easy recycling and simple scaling-up performance.

To date, few HSCCC separations of PPGs have been successfully implemented (17–22), and no report has been published on the use of HSCCC for the separation and purification of the four PPGs from *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong. The two-phase solvent system composed of ethyl acetate–*n*-butanol–water (10 : 6: 15, v/v/v). Here, a convenient and efficient HSCCC method combined with MR for the separation and purification of echinacoside, wiedemannioside C, forsythoside B and verbascoside from the partially purified extract of traditional Tibetan medicinal herb *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong has been successfully established.

Experimental

Reagents and materials

All organic solvents used for sample preparation and HSCCC were of analytical grade and purchased from the Tianjin Chemical Factory (Tianjin, China). Methanol used for HPLC analysis was of chromatographic grade, and was purchased from Yuwang Chemical Ltd (Shandong, China). The water used in this experiment was prepared by a Milli-Qplus purification system (Millipore Corporation, Billerica, MA, USA).

The plants of *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong were collected from Datong county, Qinghai Province, China and identified by Prof. Li-Juan Mei (Northwest Institute of Plateau Biology, Chinese Academy of Sciences). The specimen (BCM-G, 2 kg) was stored in the laboratory sample cabinet.

Apparatus

A TBE-300A high-speed counter-current chromatography (HSCCC) instrument (Shanghai Tauto Biotech Co., Ltd, Shanghai, China) with

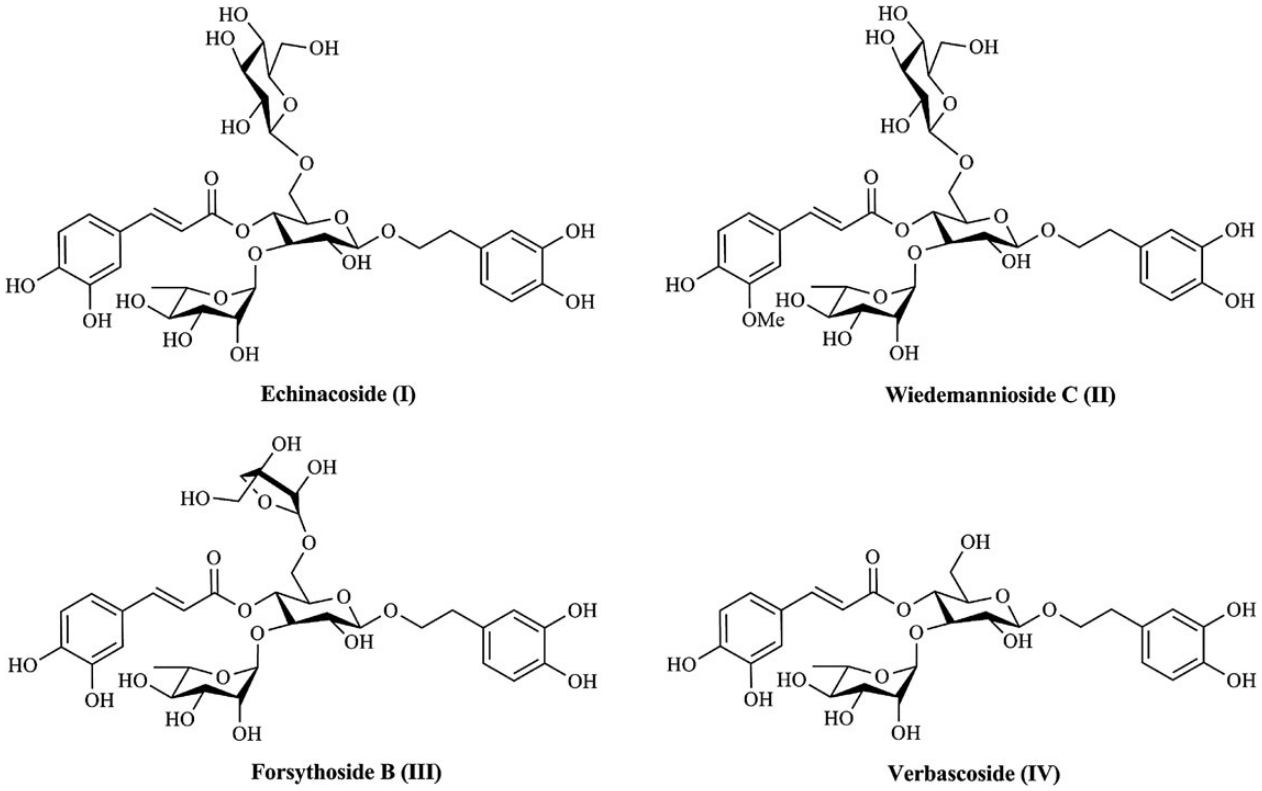

Figure 1. The chemical structures of echinacoside (I), wiedemannioside C (II), forsythoside B (III) and verbascoside (IV).

a set of three preparative PTFE (polytetrafluoroethylene) coils (tube diameter: 1.6 mm and total volume: 305 mL) and a 20-mL sample loop were used for HSCCC. The revolution radius, or the distance between the holder axis and the central axis of the centrifuge (R), was 5 cm. The β-values of the multilayer coil ranged 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 revolution speed of the apparatus could be adjusted between 0 and 1,000 rpm using a speed controller. The separation temperature was controlled by an HX-1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China). An ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Uppsala, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. The system contained a switch valve and a mixer, which were used for gradient formation. The data were collected and analyzed with an N2000 workstation (Zhejiang University Star Information Technology Co., Ltd, Hangzhou, Zhejiang, China). An Agilent 1200 HPLC equipped with a Gemini C_{18} column (5 μm, 4.6 × 250 mm, Phenomenex Corp. Ltd, USA) was used for HPLC analysis. The nuclear magnetic resonance (NMR) spectrometer was a Mercury-400BB NMR (Varian Co., Ltd, Palo Alto, CA, USA) with tetramethylsilane as the internal standard.

Preparation of crude sample

The plants (500 g) were powdered and extracted three times using 70% ethanol under reflux, each time for 2 h. Then, the extracts were concentrated under vacuum. The residues (about 64 g) were then suspended in deionized water (500 mL) and extracted five times with ethyl acetate (2.5 L). The aqueous fraction was loaded into a MR column (90 cm × 4.5 cm, containing 400 g D101 MR), and eluted with various proportions of a mixture of water–ethanol (100 : 0, 80 : 20, 40 : 60 and 10 : 90 v/v; about 3,000 mL for each gradient). The water–ethanol (80 : 20) fraction was concentrated to produce 8.0 g of crude sample for subsequent HSCCC isolation and purification.

Measurement of partition coefficient

The partition coefficient was the ratio of the solute distributed between the mutually equilibrated two solvent phases. The partition coefficients were determined by HPLC as follows: a suitable amount of crude sample was added into a series of pre-equilibrated two-phase solvent systems, and the solution was then shaken thoroughly. Subsequently, the same volumes of upper and lower phases were each evaporated to dryness. The residues were diluted into 2 mL of methanol and then analyzed by HPLC. The *K*-value was measured at room temperature and was defined as the peak area of the component in the upper phase divided by the peak area of the component in the lower phase.

Preparation of two-phase solvent system and sample solution

The two-phase solvent system composed of ethyl acetate–*n*-butanol–water (10 : 6 : 15, v/v/v) was finally selected for

HSCCC separation. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated immediately before use. The sample solution for HSCCC separation was prepared by dissolving 120 mg of the crude extract in 5 mL of the lower phase.

Procedure of HSCCC separation

First, the multilayer coil column was entirely filled with the upper phase (stationary phase). Then, the apparatus was rotated at 950 rpm, while the lower phase (mobile phase) was pumped into the head of the column at a flow rate of 2.5 mL/min. After hydrodynamic equilibrium was reached, as indicated by the emergence of the mobile phase front, 5 mL of sample solution containing 120 mg of the crude extract 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 330 nm, and the chromatogram was recorded. The temperature of the apparatus was set to 40°C. The peak fractions were collected manually according to the elution profile. Evaporation under reduced pressure was then allowed, and the residues were dissolved in methanol for subsequent purity analysis by HPLC. The purity was obtained by HPLC peak area calculation.

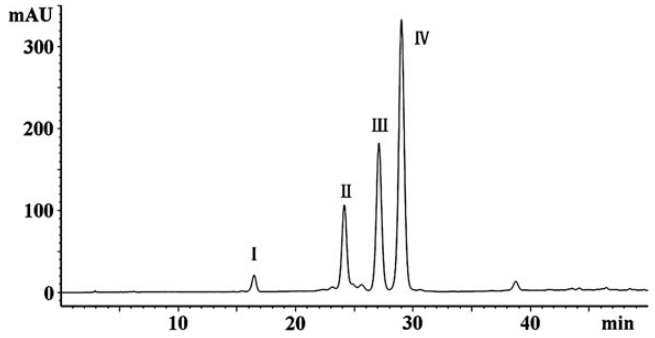

Figure 2. The HPLC chromatogram of the crude sample. Conditions: Gemini C_{18} column (5 μm, 4.6 × 250 mm, Phenomenex Corp. Ltd); mobile phase: methanol–0.01% phosphoric acid in water (methanol: 0–30 min, 28–36%; and 30–50 min, 36–50%); flow rate: 1 mL/min; column temperature: 25°C; detection wavelength: 330 nm.

HPLC analysis and identification of HSCCC peak fractions

The crude extract and each HSCCC peak fraction were analyzed by HPLC. HPLC analysis was performed on a reversed-phase Agilent Gemini C_{18} (5 μm, 4.6 × 250 mm) analytical column with gradient elution, at a column temperature of 25°C. Methanol–0.1% phosphoric acid in water in the following percentages and corresponding times was used as the mobile phase: (methanol: 0–30 min, 28–36%; and 30–50 min, 36–50%). The flow rate and detection wavelength were set at 1.0 mL/min and 330 nm, respectively. The crude sample and peak fractions separated by HSCCC were analyzed by HPLC under the optimum analytical conditions, and the chromatograms are presented in Figures 2 and 3. Identification of HSCCC peak fraction was performed by ^{1}H and ^{13}C NMR.

Results

With a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (10 : 6 : 15, v/v/v), a total of 3.5 mg of echinacoside, 12.6 mg of wiedemannioside C, 22.7 mg of forsythoside B and 48.7 mg of verbascoside were isolated and purified from 120 mg of crude sample. According to ^{1}H NMR and ^{13}C NMR data, the chemical structures of the peak fractions separated by HSCCC were identified. Through comparison with reference data, peaks I, II, III and IV were effectively identified as echinacoside, wiedemannioside C, forsythoside B and verbascoside, respectively.

Discussion

Selection of two-phase solvent system and other conditions of HSCCC procedure

Successful separation by HSCCC depends on the selection of a suitable two-phase solvent system, which requires the following

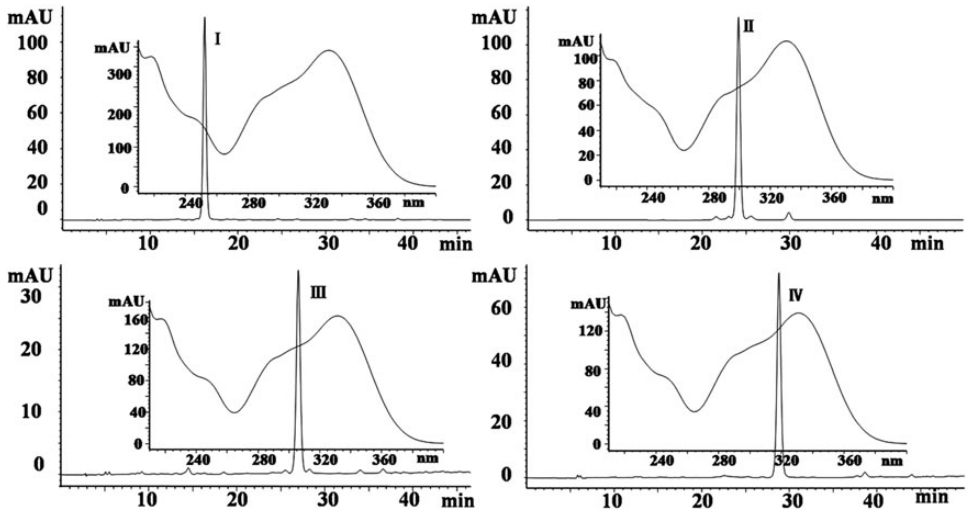

Figure 3. The HPLC chromatograms of the four targeted compounds (peak fractions I, II, III and IV) purified by HSCCC. Conditions: Gemini C_{18} column (5 μm, 4.6 × 250 mm, Phenomenex Corp. Ltd); mobile phase: methanol–0.01% phosphoric acid in water (methanol: 0–30 min, 28–36%; and 30–50 min, 36–50%); flow rate: 1 mL/min; column temperature: 25°C; detection wavelength: 330 nm.

Table I
The *K*-Values of the Target Compounds in Different Solvent Systems

Solvent systems	Ratio (v/v)	Settling time (s)	*K*-values			
			I	II	III	IV
Chloroform–*n*-butanol–methanol–water	1 : 1 : 1 :1	28	11.26	7.04	5.66	2.12
Chloroform–*n*-butanol–methanol–water	12 : 9 : 12 : 12	24	13.76	8.50	7.41	2.96
Chloroform–*n*-butanol–methanol–water	12 : 9 : 12 : 18	18	19.64	13.05	12.77	4.21
Chloroform–*n*-butanol–methanol–water	12 : 9 : 12 : 15	21	15.87	10.78	9.14	3.17
Chloroform–*n*-butanol–methanol–water	12 : 9 : 11 : 10	28	8.54	5.40	2.25	1.19
Chloroform–*n*-butanol–methanol–water	12 : 8 : 12 : 10	28	8.97	6.41	6.00	2.81
Chloroform–*n*-butanol–methanol–water	15 : 9 : 15 : 12	26	5.47	4.21	3.81	1.72
Chloroform–*n*-butanol–methanol–water	8 : 10 : 12 : 10	29	2.78	1.84	1.86	1.12
Ethyl acetate–*n*-butanol–water	12 : 6 : 18	17	0.17	0.26	0.75	2.81
Ethyl acetate–*n*-butanol–water	10 : 6 : 18	17	0.27	0.43	1.37	6.23
Ethyl acetate–*n*-butanol–water	10 : 6 : 17	17	0.25	0.43	1.36	6.17
Ethyl acetate–*n*-butanol–water	10 : 6 : 16	18	0.25	0.41	1.24	4.31
Ethyl acetate–*n*-butanol–water	**10 : 6 : 15**	18	**0.22**	**0.39**	**1.16**	**4.11**

This solvent system was selected as the suitable two-phase solvent system (highlighted in bold).

considerations (23, 24): (i) the setting time of the solvent system should be short (i.e., <30 s); (ii) the partition coefficient (*K*) of target compound should fall within a suitable range (i.e., usually between 0.2 and 5) and (iii) the separation factors ($\alpha = K1/K2$, $K1 > K2$) between any two compounds all should be greater than 1.5.

In this experiment, different two-phase solvent systems, such as ethyl acetate–*n*-butanol–water and chloroform–*n*-butanol–methanol–water, were tested according to the polarity of the target compounds. The *K*-values of the target compounds in different solvent systems were determined by HPLC and the results are given in Table I. The two-phase solvent systems with chloroform–*n*-butanol–methanol–water (1 : 1 : 1 : 1, 12 : 9 : 12 : 12, 12 : 9: 12 : 18, 12 : 9 : 12 : 15, 12 : 9 : 11 : 10 and 12 : 8 : 12 : 10, v/v/v/v) were tested first; the *K*-values were too large, which lead to a long separation time and broad peaks. When a ratio of chloroform–*n*-butanol–methanol–water (8 : 10 : 12 : 10, v/v/v/v) was tested, the separation factors between compounds I, II, III and IV were too small and not suitable for the separation of the four target compounds from the crude sample. Then, two-phase solvent systems comprised of ethyl acetate–*n*-butanol–water (12 : 6 : 18, 10 : 6 : 18, 10 : 6 : 17, 10 : 6 : 16, 10 : 6 : 15, v/v/v) were further investigated. When a ratio of (12 : 6 : 18, 10 : 6 : 18, or 10 : 6 : 17, v/v/v) was tested, the separation factors between these four compounds were large enough. Nevertheless, the *K*-value of compound I or IV was not suitable. Fortunately, when a ratio of (10 : 6 : 15, v/v/v) was tested, the *K*-values of the four target compounds were between 0.2 and 5, and the separation factors were large enough for the four PPGs to exhibit large resolutions relative to one another.

In addition to developing a suitable two-phase solvent system, other factors were also investigated: separation temperature, flow rate of the mobile phase and revolution speed of the apparatus. First, effects of separation temperature on the retention of the stationary phase were investigated (Figure 4). The retention values of the stationary phase at different separation temperatures were 42.3 ± 0.41, 46.2 ± 0.38, 50.2 ± 0.29 and 53.4 ± 0.39, and the RSD values were 0.97 0.82, 0.58 and 0.73%, respectively. The results demonstrated that the retention of the stationary phase improved with an increase in separation temperature. The high temperature is mostly like to lead to a short service life of the apparatus. Therefore, we set the separation temperature to 40°C in

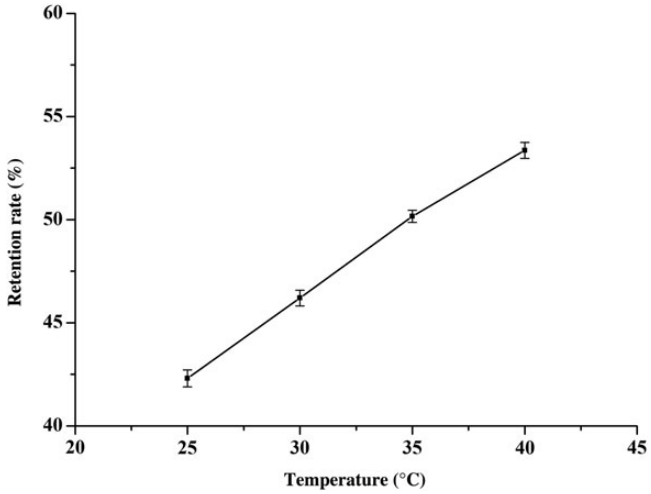

Figure 4. Comparison of stationary phase retention under different separation temperatures.

this experiment. Then, different flow rates of the mobile phase (2.0, 2.5 and 3.0 mL/min) were tested in order to determine their effect on separation time, stationary phase retention and targeted compound purity (Table II). Results indicated that the low flow rate of the mobile phase led to a lengthy separation time; a low flow rate of the mobile phase could improve the peak resolution and increase stationary phase retention. The increase of the flow rate in the mobile phase resulted in peak resolution decline and a reduction in stationary phase retention; the increase of the flow rate in the mobile phase could speed up the elution of peaks. Under the highest flow rate (3.0 mL/min), the purity of compounds I and II was only 89.5 and 94.5%, respectively. Considering the elucidated separation time and the purity of the targeted compounds, a flow rate of 2.5 mL/min was used in subsequent HSCCC separation procedures. Additionally, the revolution speed could also impact stationary phase retention, and the high revolution speed is likely to cause emulsification. Thus, we mainly considered the 950 r/min speed in our isolation procedure.

Under the optimized conditions, four fractions (I, II, III and IV) were obtained in one-step separation within 6 h (HSCCC chromatogram is shown in Figure 5), including echinacoside (peak

I, collected during 70–76 min, 3.5 mg), wiedemannioside C (peak II, collected during 80–92 min, 12.6 mg), forsythoside B (peak III, collected during 121–149 min, 22.7 mg) and verbascoside (peak IV, collected during 309–405 min, 48.7 mg). As shown in Figure 3, the HPLC analysis of each HSCCC fraction revealed that the purities of these four compounds were 93.6, 97.9, 97.8 and 98.1%, respectively. To the best of our knowledge, this is the first report of separation and purification of echinacoside, wiedemannioside C, forsythoside B and verbascoside from *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong by HSCCC combined with MR column separation.

Table II
Comparison of Separation Time, Stationary Phase Retention and Purities of Target Compounds Under Different Flow Rates

Flow rate (mL/min)	Separation time (min)	Retention (%)	Purity (%)			
			I	II	III	IV
2	520	57	97.2	98.4	97.8	98.7
2.5	400	53	93.6	97.9	97.8	98.1
3	320	47	89.5	94.5	97.4	96.4

Conclusion

In this study, four similar structure and polarity PPGs including echinacoside, wiedemannioside C, forsythoside B and verbascoside from the traditional Tibetan medicinal herb *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong were successfully separated and purified with ethyl acetate–*n*-butanol–water (10 : 6 : 15, v/v/v) as the two-phase solvent system of HSCCC in one-step separation within 6 h. With the exception of echinacoside, the compounds such as obtained may be used as reference substances for chromatographic purposes without additional clean-up. The present study indicates that HSCCC is a very powerful

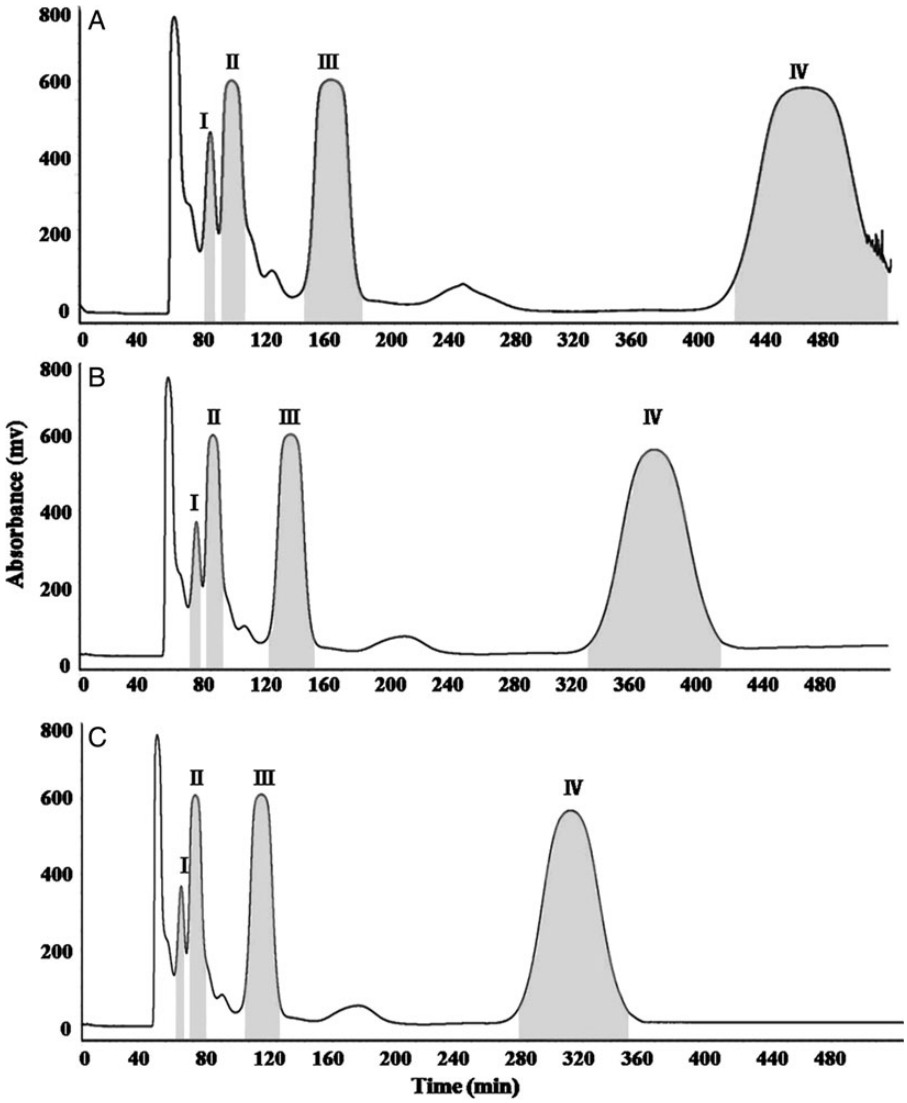

Figure 5. HSCCC chromatogram of the crude sample from *P. longiflora* Rudolph. var. *tubiformis* (Klotz) Tsoong. Two-phase solvent system: ethyl acetate–*n*-butanol–water (10 : 6 : 15, v/v/v); stationary phase: upper phase; mobile phase: the lower phase; flow rate: 2 mL/min (A), 2.5 mL/min (B) and 3 mL/min (C); revolution speed: 950 rpm; detection wavelength: 330 nm; sample size: 120 mg of crude sample dissolved in 5 mL of the lower phase; and separation temperature: 40°C.

technique for the preparative separation and purification of bioactive components from plant materials.

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