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Isolation and purification of Pseudostellarin B (cyclic peptide) from *Pseudostellaria heterophylla* (Miq.) Pax by high-speed counter-current chromatography

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Abstract

Pseudostellarin B (cyclic peptide) was isolated and purified from the herbs of *Pseudostellaria heterophylla* (Miq.) Pax for the first time by high-speed counter-current chromatography (HSCCC) using a two-phase solvent system consisting of *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v). The technique can isolate mg levels of the target compound per run with purity better than 96%. The chemical structure of the compound has been positively confirmed by electrospray ionization time of flight (TOF) MS, ^{1}H–NMR, ^{13}C–NMR and ^{1}H–^{13}C–COSY analyses.

Keywords: *Pseudostellaria heterophylla* (Miq.) Pax; HSCCC; Pseudostellarin B; Preparative chromatography

1. Introduction

Pseudostellaria heterophylla (Miq.) Pax, or Taizishen has been used as a traditional Chinese medicine for over 1000 years. The herb is frequently used to treat diseases such as lung and spleen tonic [1]. Cyclic peptides extracted from *P. heterophylla* (Miq.) Pax have been a subject of considerable interest because many individual species in this compound class exhibited potent inhibitory activities towards tyrosinase and melanin production [2–4]. The preparative separation and purification of cyclic peptides from many plants by conventional methods are tedious and usually require repeated chromatographic steps on a silica gel column [2–4]. The overall yield of this method is usually poor because the target species tend to strongly adsorbed onto the solid support during separation.

High-speed counter-current chromatography (HSCCC) is a support-free all liquid chromatographic technique that has been successfully applied to the separation and isolation of many natural products [5–11]. It relies on the use of centrifugal force for the retention of the liquid stationary phase, which is continuously eluted by the counter-flowing mobile phase driven by external pumping [12]. Compared to other liquid–liquid techniques, HSCCC is advantageous because of its shorter separation time, wider range of selection of solvent systems and quantitative material recovery.

In this paper, the development of HSCCC method for the separation and purification of Pseudostellarin B (a cyclic peptide) from the crude extract of *P. heterophylla* (Miq.) Pax will be described. The chemical structure of the compound was elucidated by electrospray ionization time of flight (TOF) MS, ^{1}H–NMR, ^{13}C–NMR and ^{1}H–^{13}C–COSY analyses.

2. Experimental

2.1. Materials

Dried *P. heterophylla* (Miq.) Pax roots, whose species was identified by Mr. Wenshen Wang (B&C Technology Inc., Xiamen, China) according to morphological characteristics.

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Acetonitrile is of chromatographically grade and purchased from Sigma. The water used in mobile phase mixtures was treated with a Milli-Q water purification system (Millipore, Molsheim, France). All organic solvents used for HSCCC were of analytical grade and purchased from Shanghai chemical reagent corporation, Shanghai, China.

2.2. Preparation of the crude extract of P. heterophylla (Miq.) Pax

Roots of died *P. heterophylla* (Miq.) Pax (400 g), were milled to powder (ca. 60 mesh) by a disintegrator made in Hangzhou Chunjiang Pharmacy Machine Co. It was extracted three times (3× 45 min), each with 2000 ml of methanol by sonication using a SK3200LH ultrasonic cleaning instrument (Shanghai Kudos Ultrasonic Instrument Co., Shanghai, China). The combined methanolic extract was evaporated to dryness under vacuum with a Model SENCO R-201 rotary evaporator (Shanghai Shensheng Biotech Co., Shanghai, China). The dried residue obtained was then dissolved in 400 ml of water. The aqueous solution was placed in a funnel, and was extracted three times each with 300 ml of anhydrous ethyl ether, and then the aqueous solution was extracted three times each with 300 ml of water-saturated butanol. The combined butanolic extract was evaporated to dryness under vacuum at 50 °C to obtain the crude extract of *P. heterophylla* (Miq.) Pax. The crude extract was further purified by LSA-20 macroporous resin (Xi'an Lanshen Technology Corporation, Xi'an China). The fraction obtained by ethanol–water (3:2, v/v) elution was concentrated under reduced pressure and freeze-dried with a LABCONCO freeze dry system (USA) to yielded 1835 mg of the crude extract. The crude extract was stored at −4 °C before HSCCC separation.

2.3. High-speed counter-current chromatography (HSCCC)

2.3.1. Instrumentation

HSCCC was performed with a Model TBE-300A HSCCC system manufactured by Tauto Biotech Co. Ltd., Shanghai, China. The multi-layer coil planet centrifuge was prepared by winding 1.8 mm i.d. PTFE tubing coaxially onto the column holder with a total capacity of 350 ml. The β-value varied from 0.42 at the internal terminal to 0.63 at the external terminal. $\beta = r/R$, where r is the distant from the coil to the holder shaft and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The rotation speed is adjustable from 500 to 1000 rpm; and 800 rpm was used in the present study. The constant temperature control in this system was performed by a constant temperature circulating implement with the aim to eliminate the harmful effect of temperature variation on separation efficiency.

The solvent was pumped into the column with a Model S1007 constant-flow pump (Beijing Shengyitong Technology Development, Beijing, China). The effluent was detected on-line at 213 nm with a Model 8823A UV detector (Beijing Institute of New Technology Application). A manual sample injection valve with a 20 ml loop was used to introduce the sample into the column. A Model 3057 portable recorder (Sichuan Instrument Factory, Chongqing, China) was used to record the chromatogram. Moreover, the data was also displayed and analysed simultaneously on a Model Sepu 3000 chromatographic data station provided by Hangzhou Puhui Scientific Technology.

2.3.2. HSCCC separation procedure

A mixture of *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v) was shaken vigorously in a separatory funnel and let stand at room temperature until there were two clearly separated phases. The two-phases were then used in the HSCCC after they reached equilibrium. The multi-layer coiled column was first filled completely with the upper layer solvent which serves as the stationary phase, then the lower layer (mobile phase) was pumped into head end of the column at a flow-rate of 1.6 ml/min while the column was rotating at 800 rpm. A sample (60 mg) dissolved in 8 ml of the upper phase was loaded into the injection valve after the system reached hydrodynamic equilibrium. The column effluent was monitored with a UV detector at 213 nm as stated earlier, and each peak fraction was collected manually according to the chromatographic profile displayed on the recorder.

2.4. High-performance liquid chromatography conditions

An Agilent Technology 1100 Series HPLC system equipped with a quaternary pump, a degasser, a thermostatic auto-sampler and a photodiode array detector (DAD), was used for the analysis of Pseudostellarin B in the crude extract and fractions collected from the HSCCC separation. The analysis was carried out with a SinoChrom ODS-BP C_{18} column (4.6 mm × 200 mm, 5 μm, Dalian Elite Analytical Instruments). The binary mobile phase consisted of acetonitrile and pure water. All solvents were filtered through a 0.45 μm filter prior to use. The flow-rate was kept constant at 1.0 ml/min for a total run time of 40 min. The percentage of acetonitrile in the mobile phase was programmed as follows: 2% (0 min)–10% (10 min)–45% (30 min)–55% (40 min). Elution was monitored at 213 nm by a DAD detector. The purity of the isolated constituent was determined based on the peak area normalized to all observed HPLC peak area.

2.5. ESI-TOF-MS for identification

The MS instrument used to perform the studies was an electrospray ionization time of flight mass spectrometer Agilent MSD TOF (Agilent Technologies), using the operational parameters included in Table 1. The data recorded was processed with the Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application specific additions from Agilent MSD TOF software. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: 121.0509 and 922.0098 *m/z*. Spectra were acquired over the *m/z* 100–2000 range at a scan rate of 1 s per spectrum.

2.6. NMR for identification

The ^{1}H–NMR, ^{13}C–NMR, and ^{1}H–^{13}C–COSY spectra were recorded on a Bruker 500 MHz nuclear magnetic resonance

Table 1
TOF-MS operational parameters in positive ESI ion mode

Parameter	Value
Capillary voltage	3500 V
Nebulizer pressure	45 psig
Drying gas	12 l/min
Gas temperature	350 °C
Fragmentor voltage	200 V
Skimmer voltage	60 V
Mass range (*m/z*)	100–2000
Resolution	9500 ± 500 (922.0098)
Reference masses	121.0509, 922.0098

spectrometer (Bruker BioSpin Ltd., Canada). TMS was an internal standard. Approximately 8 mg of the purified compound was dissolved in 500 μl of pyridine-d_5 in NMR measurement.

3. Results and discussion

3.1. HSCCC separation of Pseudostellarin B from the crude extract

In a HSCCC experiment, selection of the two-phase solvent system is the first and critical step; a good solvent system can provide an ideal partition coefficient (*K*) for the target compounds. The key of solvent optimization is first to find a solvent combination in which the samples is freely soluble, then to adjust this solvent combination to ensure that the *K* value of the target compounds is close to 1 [13,14]. The *K* value of a two-phase solvent system is critical for efficient separation. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution; if the *K* value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time [5].

In our experiment, the crude Pseudostellarin B extract was used as the testing material and five series of solvent systems according to the solubility of the target compound was selected. LC was used to measure the sample concentration in each phase, from which the *K* values of the Pseudostellarin B were calculated. The measured *K* values for Pseudostellarin B in these different solvent systems are summarized in Table 2.

Under the conditions of 800 rpm revolution speed, 1.6 ml/min flow-rate, and 25 °C coil planet centrifuge temperature, the following these five solvent systems were evaluated: (1) *n*-butanol–ethyl acetate–acetic acid–water (0.5:4.5:0.5:6, v/v); (2) *n*-butanol–ethyl acetate–methanol–water (0.5:4.5:1:4, v/v); (3) *n*-butanol–ethyl acetate–ethanol–water (0.5:4.5:0.2:4, v/v); (4) *n*-butanol–ethyl acetate–ethyl ether–water (0.5:4.5:0.5:6, v/v); (5) *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v). The upper phases were used as the stationary phase while the lower phases were used as the mobile phase in all tests. 59.6%, 57.3%, 15.8%, 77.3% and 45.2% of stationary phase retentions were obtained for solvent systems (1), (2), (3), (4) and (5), respectively.

Based on the results of *K* values and stationary phase retentions values, two systems selected for further evaluation were *n*-butanol–ethyl acetate–acetic acid–water (0.5:4.5:0.5:6, v/v) and *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v).

Table 2
The *K* values (partition coefficient) of Pseudostellarin B in different two-phase solvent systems used in HSCCC[a]

Solvent system	*K* value
n-Butanol–ethyl acetate–acetic acid–water (0.5:4.5:0.5:6, v/v)	0.86
n-Butanol–ethyl acetate–methanol–water (0.5:4.5:1:4, v/v)	0.72
n-Butanol–ethyl acetate–ethanol–water (0.5:4.5:0.2:4, v/v)	0.79
n-Butanol–ethyl acetate–ethyl ether–water (0.5:4.5:0.5:6, v/v)	0.64
n-Butanol–ethyl acetate–water (0.6:4.4:5, v/v)	1.12

[a] Experimental procedure: approximately 1 mg of the sample was weighed in a 10 ml test tube into which 2 ml of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. An aliquot of 200 μl of each layer was taken out and evaporated separately to dryness invacuo at <40 °C. The residue was dissolved in 200 μl methanol and analysed by LC for determining the partition coefficient (*K*) of compound Pseudostellarin B. The *K* value was expressed as the peak area of target compound in the upper phase vs. in the lower phase.

Table 3 lists the distributions of the 10 peaks (Fig. 2(A)) in the two solvent systems of: (1) *n*-butanol–ethyl acetate–acetic acid–water (0.5:4.5:0.5:6, v/v) and (2) *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v). In system (1), the *K* values of peak 6 compared with peaks 4 and 7 are so similar, and may result in poor separation. In system (2), the value of peak 6 is significantly different from the other peaks. The *K* values of the peaks 3, 7, 8 and 10 were too greater than 1, they were retained in the CCC column and may lead to extended elution time [5]. The results thus suggest that solvent system (2) is suitable for the separation of Pseudostellarin B, as shown in Fig. 1. This solvent system was therefore used for all later HSCCC runs. The HPLC chromatogram of a crude extract of *P. heterophylla* (Miq.) Pax is given in Fig. 2(A). It can be seen that the crude extract gives many peaks among which Pseudostellarin B is the major peak. The peaks I–IV in Fig. 1 were individually collected and analysed by HPLC. Peak I consists of two peaks (peaks 1 and 2) in the HPLC chromatogram (Fig. 2(A)); Peak II consists of two peaks (peaks 4 and 5) in the HPLC chromatogram (Fig. 2(A)); Peak III in the HPLC chromatogram is peak 6 (Fig. 2(B)), in which a clean peak of the target species is observed; Peak IV in

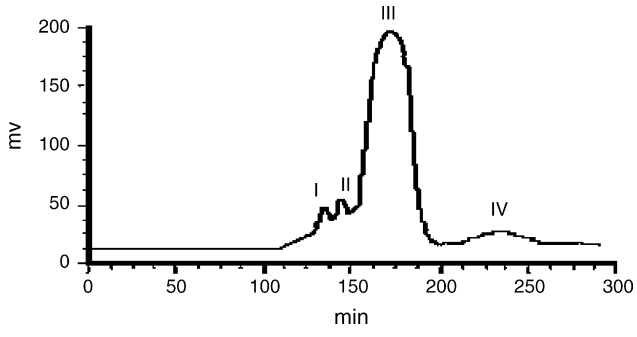

Fig. 1. HSCCC chromatogram of the crude extract of *P. heterophylla* (Miq.) Pax; solvent system: *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v); stationary phase: upper phase; mobile: lower phase; flow-rate: 1.6 ml/min; revolution speed: 800 rpm; temperature: 25 °C; sample size: 60 mg extract dissolved in 8 ml upper phase of solvent system; detection at 213 nm. (The fraction III were collected from 160 to 175 min and purified to over 96% estimated by HPLC analyses).

Table 3
Partition coefficient of 10 compounds in two solvent systems

	Peaks									
	1	2	3	4	5	6	7	8	9	10
Solvent systems 1 (1)	0.61	0.67	3.16	1.14	0.86	1.18	1.37	4.68	2.07	3.92
Solvent systems 1 (2)	0.68	0.71	4.65	0.76	0.80	1.12	3.96	5.16	2.36	4.32

Note: solvent systems: (1) *n*-butanol–ethyl acetate–acetic acid–water (0.5:4.5:0.5:6, v/v) and (2) *n*-butanol–ethyl acetate–water (0.6:4.4:5, v/v).

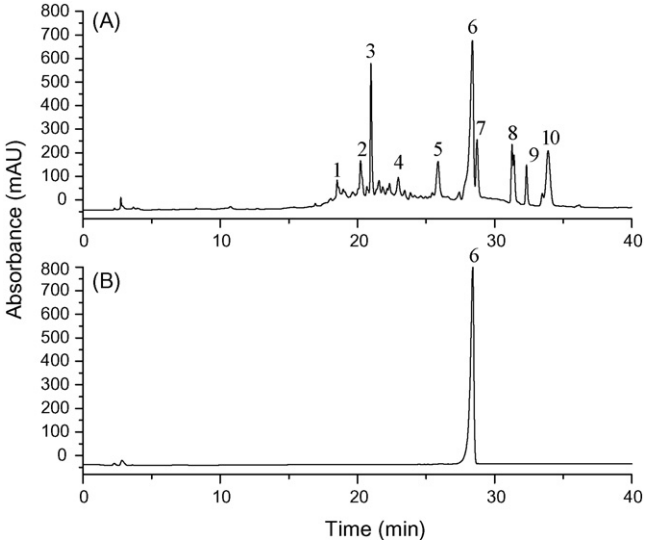

Fig. 2. HPLC chromatograms of the crude sample of (A) *P. heterophylla* (Miq.) Pax and (B) the purified Peak III (Pseudostellarin B); column: SinoChrom ODS-BP C_{18} (4.6 mm × 200 mm, 5 μm, Dalian Elite Analytical Instruments) at room temperature; elution: acetonitrile and pure water, the percentage of acetonitrile in the mobile phase was programmed as follows: 2% (0 min)–10% (10 min)–45% (30 min)–55% (40 min); flow-rate: 1.0 ml/min; detection at 213 nm. (Peak I in the HPLC chromatogram is peak 1 and peak 2; Peak II in the HPLC chromatogram is peaks 4 and 5; Peak III in the HPLC chromatogram is peak 6; Peak IV in the HPLC chromatogram is peak 9).

the HPLC chromatogram (Fig. 2(A)) is peak 9. Since Peaks I, II and IV were no major peak, and the overall contents of them were very low, no further study was therefore carried out. The yield of Pseudostellarin B amount 3 mg (5.0%, w/w) from 60 mg

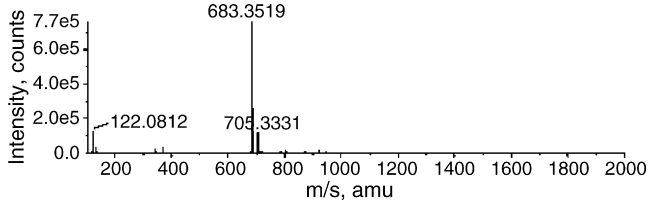

Fig. 3. TOF-MS spectrum of Pseudostellarin B $[M+H]^+$.

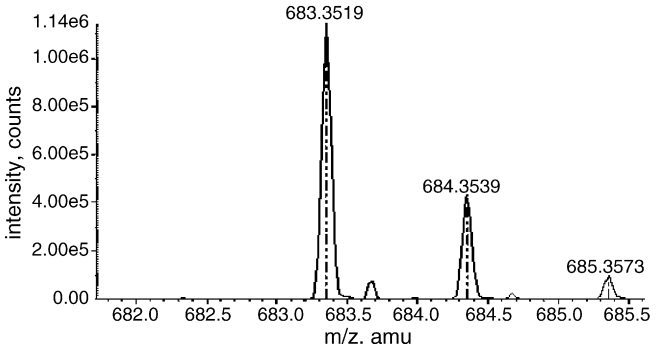

Fig. 4. Pseudostellarin B $[M+H]^+$ isotopics compared with calculated isotopics.

of the crude extract sample and the purity of Pseudostellarin B (Fig. 2(B)) estimated by HPLC analyses is 96.2%.

3.2. Identification by TOF-MS and NMR experiments

The cyclic peptide Pseudostellarin B discussed in this paper has been previously identified in literature report [3]. To confirm the identity of the HSCCC peak as described above, the isolated material were collected and measured by ESI-TOF-MS and ^{1}H–NMR, ^{13}C–NMR and ^{1}H–^{13}C–COSY. The ESI-TOF-MS mass spectrum of peak 6 in the positive mode gave *m/z*

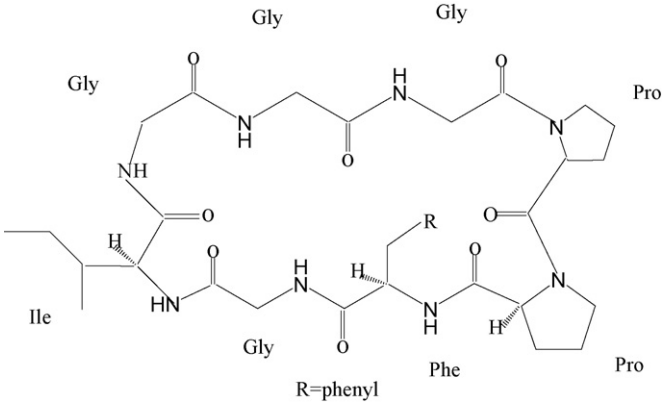

Fig. 5. The structure of Pseudostellarin B.

Table 4
Exact mass measurement and elemental composition of Pseudostellarin B

Compound	Formula	Selected ion	Experimental *m/z*	Calculated *m/z*	DBE	Error	
						mda	ppm
Pseudostellarin B	$C_{33}H_{46}N_8O_8$	$[M+H]^+$	683.3519	683.3518	15	0.1	−0.3131

683.3519 as the protonated molecular ion $[M+H]^+$ (Fig. 3) and the *m/z* 705.3331 peak was sodium adduct molecular ion $[M+Na]^+$, showed a molecular formula $C_{33}H_{46}N_8O_8$, indicating 15 degrees of unsaturation, the same as that for Pseudostellarin B [3]. Fig. 4 shows that theoretical isotopics of the compound can perfectly match it of actual compound and the mass error under 1 ppm (Table 4). The ^{1}H–NMR, ^{13}C–NMR and ^{1}H–^{13}C–COSY results agree completely with the literature [3]. Based on the combined results of TOF-MS and NMR, peak 6 is positively identified as Pseudostellarin B with its structure given in Fig. 5.

4. Conclusion

HSCCC technique has been developed and successfully applied to the separation and purification of Pseudostellarin B in crude extract of *P. heterophylla* (Miq.) Pax. Combined ESI-TOF-MS and NMR analyses were employed to positively identify the isolated target species, which is a known bioactive species in *P. heterophylla* (Miq.) Pax. HSCCC thus provides an attractive alternative to HPLC for the semi-preparative scale separation and purification of bioactive components in herbal extracts. In previous studies in our laboratory, HSCCC has been successfully developed for the preparative separation of bioactive flavonoids inflacoumarin A and licochalcone A from the crude extract of *Glycyrrhiza inflata* Bat and salvianolic acids from *Salvia miltiorrhiza* Bunge. The current study further illustrates the effectiveness of HSCCC as a semi-preparative separation technique for the isolation and purification of bioactive components from herbal extracts.

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