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Separation and purification of echinacoside from *Penstemon barbatus* (Can.) Roth by recycling high-speed counter-current chromatography

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ABSTRACT

Echinacoside is an important bioactive compound extracted from *Cistanche tubulosa* which was endangered by overexploitation. It is imperative to find an alternative source. Echinacoside was isolated from *Penstemon barbatus* (Can.) Roth for the first time. The peak contents of echinacoside are 9.09 ± 0.32 mg/g and 7.25 ± 0.36 mg/g respectively in the leaves and roots annually. The methanolic extracts from 20 g of dried powder of the roots of *P. barbatus* were pre-purified by AB-8 resin and the fraction containing echinacoside was further purified by conventional high-speed counter-current chromatography (HSCCC) and recycling HSCCC with the solvent system *n*-butanol–water (1:1, v/v). Totally 42.0 mg echinacoside with a purity of 96.3% was recovered. The recovery rate of echinacoside by recycling HSCCC reached 91.0%. The structure of our echinacoside confirmed by IR, ^{1}H NMR and ^{13}C NMR is identical to the standard sample. This indicates that *P. barbatus* might be ideal source for preparation of large scale of echinacoside.

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1. Introduction

Echinacoside (Fig. 1.), a caffeic acid derivative, can protect collagen from free radical-induced degradation. It possesses a spectrum of beneficial activities, such as neuroprotective, hepatoprotective, anti-inflammatory and antineoplastic. Moreover, this compound can boost the immunoprotective efficacy and is more potent than other caffeic acid derivatives, such as caftaric acid, chlorogenic acid, caffeic acid, cynarin, cichoric acid, and so on, to scavenge DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl) radicals [1].

Echinacoside was firstly reported to be extracted from *Echinacea angustifolia*, Compositae, [2] with a content around 0.2% [3] and subsequently prepared from the aerial part of *Penstemon crandallii* A. Nels, Scrophulariaceae, with a content of 0.09% [4]. Currently, echinacoside is largely extracted from *Cistanche tubulosa*, Orobanchaceae, with the content of no less than 1.0% [5], a perennial parasitical plant on the roots of *Haloxylox ammodendron* and *H. persicum*, Chenopodiaceae. The content of echinacoside is the only quality standard for the herbal medicine of *C. tubulosa* in Pharmacopoeia of the People's Republic of China. *C. tubulosa* is a slow-growing plant endemic to arid lands and deserts and recalcitrant to domestication. Increasing demands for the soaring medication value of echinacoside further endangered *C. tubulosa*. New alternative source other than *C. tubulosa* for echinacoside is urgent both for commercial demands and environmental protection. *Penstemon barbatus* (Can.) Roth, Scrophulariaceae, an American and Mexican aborigine, abounds in echinacoside. *P. barbatus* can tolerate low temperature, drought [6], and saline alkali and is a widely grown landscape plant across China. The annual content dynamics of echinacoside in the leaves and the roots of *P. barbatus* were determined by high performance liquid chromatography (HPLC) to justify its in-depth exploitation potential. HSCCC was employed to purify echinacoside. The structure of echinacoside was confirmed by IR, ^{1}H NMR and ^{13}C NMR.

2. Experimental

2.1. Chemicals and reagents

All solvents used for preparation of enriched extract and for HSCCC separation were of analytical grade (Kelong Chemical Reagent Factory, Chengdu, China). Methanol and acetonitrile used for HPLC were of chromatographic grade (Honeywell Burdick & Jackson, USA). Reference standard of echinacoside was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The purity of the authentic sample determined by HPLC is 99.1%.

P. barbatus has been grown in the Botanical Garden of School of Life Science, Southwest University, China, and was authenticated

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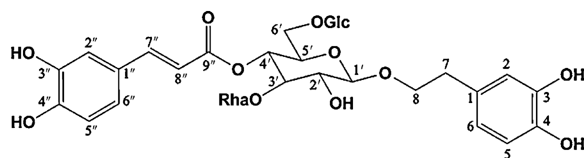

Fig. 1. Chemical structure of echinacosiden.

by Yucheng Liu, a professor in the same school, by morphological analysis. The aerial parts and the roots of the plant were collected from the garden and the voucher specimens have been deposited in the Herbarium, Department of Botany, School of Life Science, Southwest University, Chongqing, PR China.

2.2. Apparatus

The HSCCC instrument was TBE-300A high-speed counter-current chromatography (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation column connected in series (i.d. 1.5 mm, total capacity 260 ml), equipped with a 20 ml sample loop, a S-1007 pump (Beijing Shenyitong Technology Co. Ltd.), a 8823B UV detector (Beijing Bindayingchuang Technology Co. Ltd.), and a model N2010 workstation (Zhejiang University, China). HPLC was carried out on a Shimadzu LC-20A system and a Shimadzu SPD-20A UV detector. Rotational vacuum concentrator RVC 2-33 was used for concentration (Martin Christ, Germany). The IR experiment was performed on Perkin-Elmer Spectrum One NTS (PerkinElmer, USA) FT-IR Spectrometer. The ^{1}H NMR and ^{13}C NMR experiment was performed on a VARIAN INOVA-600 (Varian Corporation, USA) NMR spectrometer.

2.3. The determination of the content of echinacoside

2.3.1. HPLC condition

The analyses were carried out on a Phenomenex ODS column (150 mm × 4.6 mm, 5 μm) with a C_{18} guard column using the isocratic elution of (A) methanol and acetonitrile (3:2, v/v) and (B) aqueous phosphoric acid solution (0.1%) (22:78, v/v) as mobile phase. The flow rate was 1.0 ml/min. The eluent was monitored at 330 nm. The column temperature was set at 30 °C. 20 μl of each sample was injected in HPLC instrument in triplicate.

2.3.2. Preparation of standard solution and sample solution

Preparation of the stock standard solution of echinacoside: ca. 50.0 mg of echinacoside was accurately weighed, placed into a 50 ml glass volumetric flask, and dissolved with diluent of 50% aqueous methanol. Finally, it was added to the constant volume and then, it was diluted into five standard concentrations, 25, 50, 100, 250, and 500 μg/ml, which were prepared for the construction of the calibration curve.

Preparation of sample solution: the leaves and the roots of *P. barbatus* were harvested every two months from March, 2009 to January, 2010, dried at 40 °C in a forced-air oven, ground, and then sifted through a 0.45 mm sieve. 1 g of material was accurately weighed, then extracted with 18 ml of diluent [5] at room temperature under sonication for three times with the interval of 10 min. The supernatant solution was filtered under vacuum and transferred to a 25 ml volumetric flask, and the final volume was set at 25 ml and then determined by HPLC.

2.3.3. Precision and accuracy

The precision of the method was defined by repeatability and intermediate precision. Repeatability was examined by six evaluations of the same concentration sample, on the same day, under the same experimental conditions. The intermediate precision was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy of the method was evaluated by recovery test. 0.6, 1.0, 1.4 times of the amount of the reference standard echinacoside was added to the sample of known concentration of echinacoside.

2.3.4. Limits of detection (LOD) and quantitation (LOQ)

The LOD was calculated with standard solution on the basis of a signal-to-noise ratio (S/N) of 3:1. The LOQ was defined as the analyte mass resulting in a S/N of 10:1.

2.4. Isolation and purification of echinacoside

2.4.1. Preparation of sample for HSCCC

20 g of powder of the roots of *P. barbatus* was extracted with 200 ml of 50% aqueous methanol under sonication (40 kHz, 200 W) three times consecutively. The filtrate was combined and evaporated under vacuum to form syrup. Then, the syrup was dissolved with some water, subjected to AB-8 resin (polystyrene resin, 0.3–1.25 mm: NanKai Chemical Factory, Tianjin, China) and eluted with distilled water, 10%, 30% and 50% aqueous methanol in turn. Eluent of 50% aqueous methanol was concentrated to dryness in the rotational vacuum concentrator and the residue (S1) was stored in a refrigerator (4 °C) for further isolation by HSCCC.

2.4.2. HSCCC separation procedure

The conventional HSCCC: the apparatus was switched on until the entire coiled column has been impregnated with the stationary phase at a flow rate of 9 ml/min. The mobile phase was pumped into the head-end of the column at a flow rate of 2 ml/min when the rotate speed reached 800 rpm with the forward mode. The temperature was set at 25 °C. After the mobile phase was eluted from the tail outlet and the hydrodynamic equilibrium of the two phases had been established, samples dissolved in 20 ml mobile phase was injected. The effluent from the outlet of the column was continuously monitored at 280 nm and collected into test tubes with a fraction collector set at 2 min for each tube. The purity of echinacoside within each concentrated fraction was determined by HPLC.

The difference between the conventional HSCCC and the recycling HSCCC is that the latter iteratively injects the effluent by channeling the outlet of the detector to the inlet of the pump.

2.5. Characterization of echinacoside

The structure of echinacoside obtained by the recycling HSCCC was characterized by analysis of the spectral data of FT-IR and NMR and comparing the data with those reported in literature. The FT-IR spectrum was detected with KBr tablet and NMR spectra were determined with MeOD as solvent.

3. Results and discussion

3.1. Linearity, precision, accuracy, LOQ and LOD

The calibration curves for echinacoside were linear in the range of 25–500 μg/ml. The linear equation was $y = 1206661x + 23141$ ($r^2 = 0.9995$), where, x is concentration and y is the peak area. The RSD value of repeatability was 0.33% ($n = 6$). The RSD value of inter-day precision was 0.51%. The RSD between-analysts precision was 0.67%. In accuracy tests, the recovery rates of echinacoside were 99.1%, 99.5%, and 101.4% in three groups of fortified samples to which echinacoside was added to the sample at ca. 0.6, 1.0, and 1.4 times of the estimated mass of echinacoside contained in the sample, respectively, in triplicate. The results demonstrated that the

Table 1
The annual content dynamics of echinacoside in the roots and leaves of *P. barbatus*.

	Jan.	Mar.	May	Jul.	Sep.	Nov.
Leaves	3.30 ± 0.19	7.39 ± 0.26	9.09 ± 0.32	4.89 ± 0.21	3.33 ± 0.27	2.61 ± 0.16
Roots	3.78 ± 0.22	3.05 ± 0.24	2.45 ± 0.21	2.83 ± 0.21	3.09 ± 0.28	7.25 ± 0.36

Data are expressed as mean ± S.D. For each sample n = 3.
Data are expressed as mg/g.

method was reliable within the expected range. The values calculated for LOD and LOQ were 2.4 μg/ml and 7.8 μg/ml, respectively.

3.2. The content of echinacoside in P. barbatus

Table 1 shows the dynamic variation of the content of echinacoside in the roots and leaves within one year. In November, echinacoside reached 7.25 ± 0.36 mg/g in the roots and in May, it even reached its highest level of as much as 9.09 ± 0.32 mg/g in the leaves, which indicates that the content of echinacoside in *P. barbatus* is almost the same as reported earlier in *C. tubulosa.*

3.3. Sample preparation

The crude extract pre-purified by AB-8 was analyzed by HPLC (shown in Fig. 2). The result indicated that most of the impurities were excluded and the recovery of echinacoside pre-purified by AB-8 was 82.9%.

3.4. Selection of the solvent system of HSCCC

A successful HSCCC separation depends largely on a suitable solvent system. An ideal partition coefficient (*K*) in the range of 0.6–1.5 can both separate more efficiently and shorten the running time. In our experiment, the *K* values of a serial of solvent systems [7–9] were tested according to the method described in previous article [10] and the results were summarized in Table 2. Solvent system N1 to N4 were not suitable for isolation and purification of echinacoside, for their *K* values were smaller than 0.6. Despite that the *K* value of N5 was ideal, the retention of the stationary phase of this solvent system was less than 10%. Although the *K* value of N7 was suitable, it was not the ideal solvent system since when the collected eluent was concentrated at 40 °C, the addition of modifier, acetic acid, was concentrated meanwhile. Consequently, echinacoside was hydrolyzed and completely degraded. Finally, N6 was chosen as solvent system for isolation and purification of echinacoside from *P. barbatus* in our experiment.

Table 2
The partition coefficient (*K*) values of echinacoside in different solvent systems.

Number	Solvent system	Ratio	*K*
N1	Chloroform–methanol–water	4:3:2	0.08
N2	Ethyl acetate–acetonitrile–water	2:3:5	0.04
N3	Ethyl acetate–*n*-butanol–water	4:1:5	0.10
N4	Ethyl acetate–*n*-butanol–ethanol–water	5:0.5:1:5	0.50
N5	Ethyl acetate–*n*-butanol–ethanol–water	5: 5:1:10	0.84
N6	*n*-Butanol–water	1:1	0.78
N7	*n*-Butanol–acetic acid–water	4:1:5	1.11

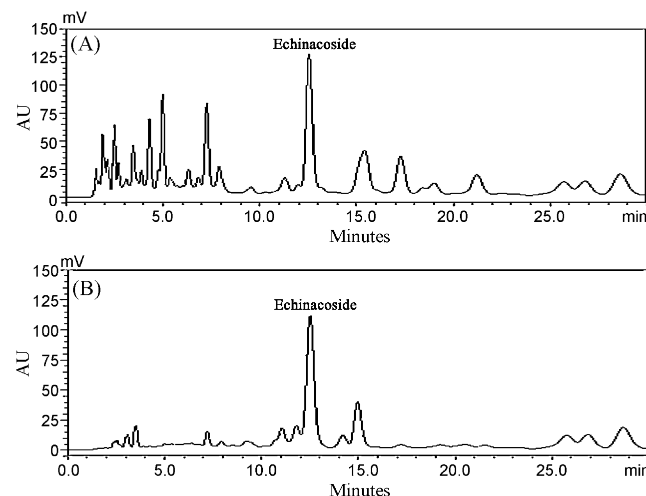

Fig. 2. HPLC chromatograms of methanolic extract of *P. barbatus* (A) and the sample pre-purified by AB-8 resin (B). Column, Phenomenex ODS (150 mm × 4.6 mm 5 μm); mobile phase, (A) methanol and acetonitrile (3:2, v/v) and (B) aqueous phosphoric acid solution (0.1%) (22:78, v/v); flow rate, 1.0 ml/min; detection wavelength, 330 nm; column temperature, 30 °C.

3.5. HSCCC separation and HPLC analysis

S1 was separated by the conventional HSCCC. (Shown in Fig. 3A.) Peak1 and peak 2 was not baseline separated. Therefore, peak 1 and 2 were collected, concentrated and reinjected. Compared with the

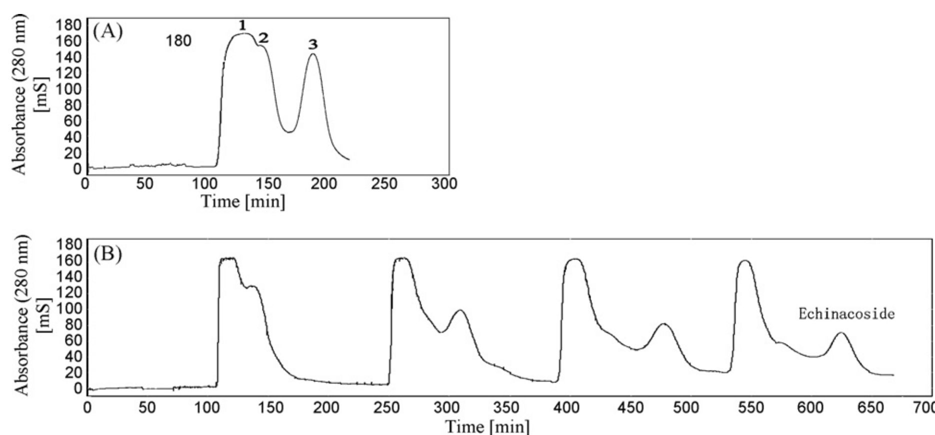

Fig. 3. The conventional HSCCC (A) and the recycling HSCCC chromatograms (B). Coil column, 260 ml; rotation speed, 800 rpm; flow rate, 2 ml/min; sample injection volume, 20 ml; detection wavelength, 280 nm; solvent system, *n*-butanol–water (1:1, v/v).

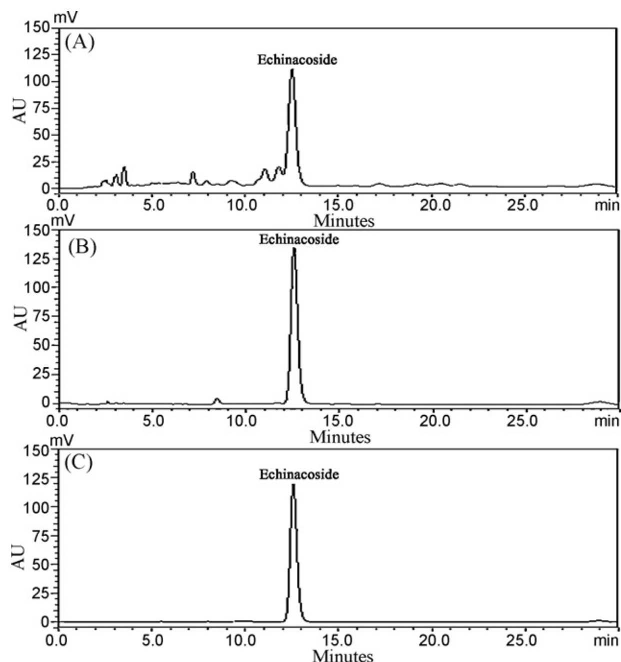

Fig. 4. HPLC chromatograms of the sample after the conventional HSCCC (A), the finally purified echinacoside after the 4th cycle of recycling HSCCC (B), and the authentic sample of echinacoside (C). Column, Phenomenex ODS (150 mm × 4.6 mm 5 μm); mobile phase, (A) methanol and acetonitrile (3:2, v/v) and (B) aqueous phosphoric acid solution (0.1%) (22:78, v/v); flow rate, 1.0 ml/min; detection wavelength, 330 nm; column temperature, 30 °C.

conventional HSCCC, the advantage of recycling HSCCC is dramatically improved resolution. This methodology requires prolonged preparation, but the solvent consumption and the volume separated remain the same. This is particularly suitable for separation of analogues [11]. After four cycles in the recycling HSCCC (Fig. 3B), echinacoside was basically isolated from the impurity. The eluent corresponding to peak 2 was collected and a total amount of 42.0 mg of echinacoside was obtained with a purity of 96.3% (shown in Fig. 4). The recovery of echinacoside in the separation by recycling HSCCC was 91.0%. Further, it was subjected to structural identification.

3.6. Identification of echinacoside obtained by the recycling HSCCC

The spectral data of the product collected after the recycling HSCCC are listed as follows. IR ν_{max} cm^{-1} (KBr): 3422 (OH), 2927 (C—H), 1698 (conj. ester), 1630 (CH=CH), 1604, 1525 (aromatic ring). ^{1}H-NMR (MeOD, 600 MHz) δ: 7.59 (1H, d, J = 19.1 Hz, H-7″), 7.05 (1H, d, J = 2.1 Hz, H-2″), 6.94 (1H, dd, J = 8.3,2.1 Hz, H-6″), 6.77 (1H, d, J = 8.3 Hz, H-5″), 6.70 (1H, d, J = 2.1 Hz, H-2), 6.69 (1H, d, J = 8.5 Hz, H-5), 6.58 (1H, dd, J = 8.5,2.1 Hz, H-6), 6.27 (1H, d, J = 19.1 Hz, H-8″), 5.17(1H, d, J = 1.2 Hz, Rha-H-1), 5.00(1H, t, J = 9.5 Hz, H-4′), 4.38(1H, d, J = 9.5 Hz, H-1′), 4.29 (1H, d, J = 9.3 Hz, Glc-H-1), 3.91 (1H, m, Hb-8), 3.79(1H, dd, J = 10.5,3.6 Hz, H-3′), 3.56 (1H, m, Ha-8), 2.79 (2H, t, J = 5.7 Hz, H-7), 1.07 (1H, d, J = 7.4 Hz, Rha-H-6). ^{13}C-NMR: δ (MeOD): 168.5 (C-9″), 149.9 (C-3″), 148.3 (C-7″), 146.9 (C-4″), 146.1 (C-4), 144.7 (C-3), 131.5 (C-1), 127.5 (C-1″), 123.3 (C-6″), 121.3 (C-6), 117.2 (C-5), 116.5 (C-2), 116.4 (C-5″), 115.3 (C-8″), 114.7 (C-2″), 104.7 (Glc-C-1), 104.2 (C-1′), 103.1 (Rha-C-1), 81.7 (C-3′), 78.0 (Glc-C-3), 77.9 (Glc-C-5), 76.2 (C-2′), 74.8 (C-5′, Glc-C-2), 73.8 (Rha-C-4), 72.4 (C-8, Rha-C-2), 72.1 (Rha-C-3), 71.5 (Glc-C-4), 70.6 (C-4′, Rha-C-5), 69.4 (C-6′), 62.7 (Glc-C-6), 36.6 (C-7), 18.5 (Rha-C-6). The spectral data above matched the data of echinacoside reported in the literature [12]. Therefore, the compound prepared by us was identified as echinacoside.

4. Conclusion

42.0 mg of echinacoside with a purity of 96.3% was obtained from *P. barbatus* by recycling HSCCC. The structure of the obtained echinacoside was confirmed by IR, ^{1}H NMR and ^{13}C NMR. The content of it varied within one year and it was found to be as much as 9.09 ± 0.32 mg/g in the leaves and 7.25 ± 0.36 mg/g in the roots which indicates that the content of echinacoside in *P. barbatus* is almost the same as that in *C. tubulosa.* Potentially, it might be a good candidate of substitute for *C. tubulosa* to yield echinacoside.

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