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Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography

Jinyong Peng, Guorong Fan *, Zhanying Hong, Yifeng Chai, Yutian Wu

Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai 200433, China

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Abstract

High-speed counter-current chromatography (HSCCC) with a solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) was used to isolate and separate two C-glycosylflavones from *Patrinia villosa* Juss, a traditional Chinese medicine. The separation produced 42.9 mg isovitexin and 20.1 mg isoorientin with purities of 99.3% and 98.5%, respectively as determined by high-performance liquid chromatography (HPLC) in one step elution from 250 mg crude extract, and identification was performed by MS, ^{1}H NMR and ^{13}C NMR. It is the first report of discovering isovitexin and isoorientin from the plant of *Patrinia* genus.

Keywords: Preparative chromatography; Counter-current chromatography; Isovitexin; Isoorientin; *Patrinia villosa* Juss

1. Introduction

Patrinia, a genus of about 20 species, is mainly distributed in central to east of Asia and northeast of North America, 10 of which growing in China. *Patrinia* species have been used as medicinal plants for more than 2000 years from Shen-NongBenCaoJing, a famous ancient Chinese medicinal literary, and some of them still used in folk medicine as anti-virus and anti-bacteria [1,2], especially two species, *Patrinia scabiosaefolia* Fisch and *Patrinia villosa* Juss (BaiJiangCao in Chinese).

With regard to the chemical constituents of this genus, we have found more research about *P. scabiosaefolia* Fisch [3], *Patrinia scabra* [4] and *Patrinia gibbosa* [5] than *P. villosa* Juss. A literature search did not yield any reference to earily report on the chemical study from *P. villosa* Juss except for some iridoids [6,7].

Isovitexin and isoorientin (structures shown in Fig. 1), two C-glycosylflavones (6-C-glucosyl luteolin and 6-C-glucosyl apigenin), have been separated from *Drosophyllum lusitanicum* [8], *Cucumis sativus* [9], *Gnidia involucrata* [10] and so on. Previous study indicated that isoorientin exhibited significant hepatoprotective effect [11], and caused concentration dependent inhibition of the amplitude and the frequency of the phasic contractions of rats and guinea-pig uterus [12], and isovitexin had antioxidant properties [13]. In view of these beneficial effects, an efficient method for the preparative separation and purification of isovitexin and isoorientin from natural sources is warranted.

The preparative separation of isovitexin and isoorientin from medicinal plants by classical methods are tedious, time consuming, requiring multiple chromatographic steps on silica gel, polyamide column, etc. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support [14], has an excellent sample recovery. The method permits directly introduction of crude samples into the column without more preparation, so it has been successfully applied to isolate and purify a number of natural products [15–19].

The aim of the present paper, therefore, was to develop an efficient method for the isolation and purification isovitexin

* Corresponding author. Tel.: +86 21 2507 0388; fax: +86 21 2507 0388.
E-mail address: guorfan@yahoo.com.cn (G. Fan).

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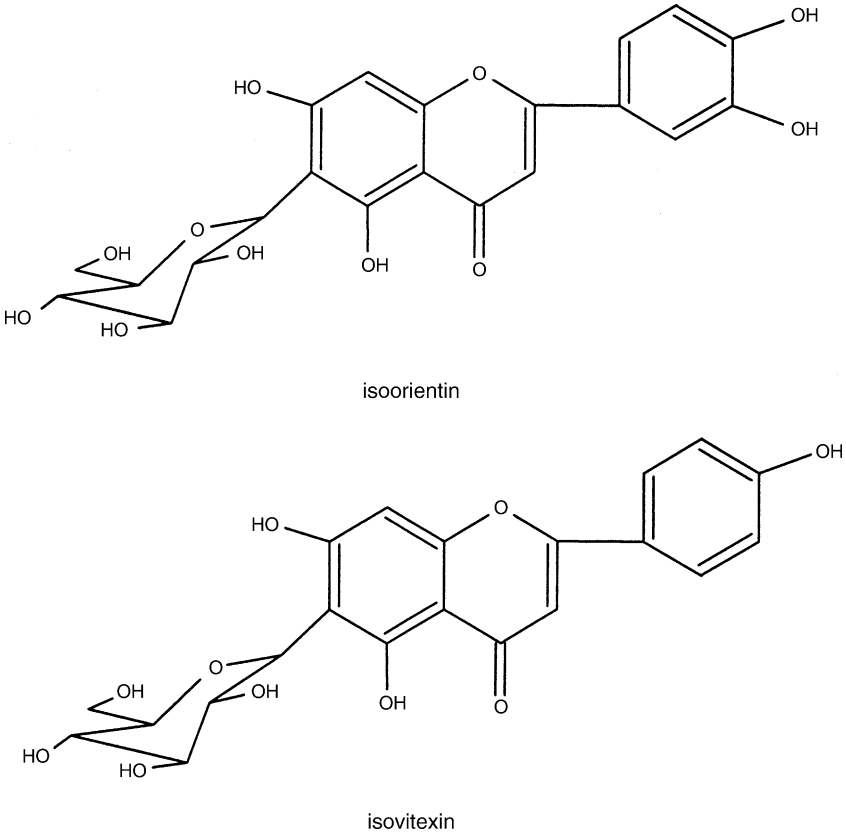

Fig. 1. Chemical structures of isovitexin and isoorientin.

and isoorientin with high purities from *P. villosa* Juss by high-speed counter-current chromatography. As far as we know, this is the first report of discovering isovitexin and isoorientin from the plant of *Patrinia* genus.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a model TBE-300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 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 value varied 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 HSCCC system was equipped with a model S constant-flow pump, a model UV-II detector operating at 254 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China).

The analytical high-performance liquid chromatography (HPLC) system used throughout this study consisted of LC-10AT pump (Shimadzu, Japan), a SPD-10A UV–vis detector (Shimadzu, Japan), and a model N2000 workstation (Zhejiang University, Hangzhou, China).

The nuclear magnetic resonance (NMR) spectrometer used here was a Varian Unity Inova-500 NMR system with TMS as internal standard and mass spectrometer (MS) was a quadrupole-time-of-flight (Q-TOF) tandem MS system equipped with a turbo-ion spay source (APIQ-STAR Pulsar *i*, Applied Biosystems, Concord, Ont., Canada). Positive and negative ion modes of ESI were used for structural analyses.

The 1300 macroporous resin (Shanghai Institute of Pharmaceutical Industry, Shanghai, China), a kind of milk-white spherical granule, cinnamene and hydrophobic sorbent, shows stronger intension, larger adsorption capability and more easy activation than some other sorbents.

2.2. Reagents

Ethyl acetate, chloroform, potassium dihydrogen phosphate, sodium hydroxide, aluminium trichloride, acetone, ethanol, *n*-butanol, phosphoric acid, acetic acid were analytical grade and purchased from WuLian Chemical Factory, Shanghai, China. Aceonitrile and methanol was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions.

The *P. villosa* Juss was purchased from a local drug store and identified by Doctor Luping Qin (Department of

Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

2.3. Preparation of the crude extract

The *P. villosa* Juss was ground into powder, 300 g of the powder was added to a bottle and extracted by reflux with 1500 ml volume of 60% aqueous ethanol in a haven for 2 h. The mixture was filtered, and the filtrate was collected. The extract was then concentrated to dryness by rotary vaporization at 60 °C under reduced pressure and redissolved in water. The water soluble was then chromatographed on 1300 macroporous resin to obtain the crude extract for HSCCC isolation. Water and different concentration of aqueous ethanol were tested to a gradient elution and each elution was analyzed by TLC, which performed with chloroform–acetone–methanol–acetic acid (2:1:2:0.05, v/v) on polyamide plates (WuLian Chemical Factory, Shanghai, China) in saturated chambers. The detection was achieved by spray 1% aluminium trichloride ethanol solution.

2.4. Measurement of partition coefficient and separation factor

Approximately 2 mg of the crude extract was weighted in a 10 ml test tube to which 4.0 ml of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then the two-phase was separated and evaporated to dryness under reduced pressure. The residue was diluted with 20% CH_3CN and analyzed by HPLC. The partition coefficient (K) value was expressed as the peak area of target components in the upper phase divided by that in the lower phase. The separation factor (α) = K_2/K_1, $K_2 > K_1$.

2.5. Preparation of two-phase solvent system and sample solution

Two-phase solvent systems were used in the present study, ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the sample in the 10 ml lower phase of solvent system for isolation and purification.

2.6. HSCCC separation procedure

In the crude sample isolation and separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 2.4 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml sample solution containing 250 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV–vis detector at 254 nm. Peak fractions were collected according to the elution profile.

2.7. HPLC analysis and identification of CCC peak fractions

The crude sample and each peak fraction obtained by HSCCC were analyzed by high-performance liquid chromatography. The HPLC system consisted of LC-10AT pump (Shimadzu, Japan), a SPD-10A UV–vis detector (Shimadzu, Japan), a model N2000 recorder for evaluation and quantification and a 20 μl sample loop. The column used was a reversed-phase LiChrospher C_{18} (6.0 mm × 150 mm i.d., 5 μm) (Hanbang Science, Jiang-Su Province, China) with a pre-column equipped with the same stationary phase. In the present study, some organic aqueous-based mobile phases were tested on a reversed-phase C_{18} column, including methanol–water and acetonitrile–water in combination with acetic acid, phosphate buffer, phosphoric acid. The flow rate of the mobile phase and the column temperature, which might affect the separation, were also tested.

Identification of the CCC peak fractions was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra.

3. Results and discussion

In 1300 macroporous resin column chromatography, 10% ethanol was first used to remove some un-target chemicals, which have no or little retention on 1300 macroporous resin, 40% ethanol was then used to yield target sample, and 95% ethanol was used to activate the resin for another use. 2.5 g crude extract was obtained using this extraction protocol from 300 g medicinal plant.

Subsequently, the crude extract was analyzed by HPLC. It was found that an excellent separation was achieved by the following separation conditions: the mobile phase composed of acetonitrile–water–acetic acid (20:80:1, v/v/v) was isocratically eluted at a flow rate of 1.0 ml/min and 30 °C, and UV detection was set at 254 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the crude extract is shown in Fig. 2, it contained several compounds including isoorientin and isovitexin (peak 1 and peak 2).

In HSCCC separation, a suitable two-phase solvent system is critical, which requires the following considerations [20–22]: (1) retention of the stationary phase should be satisfactory; (2) the settling time of the solvent system should be short (i.e. <30 s); and (3) the partition coefficient (K) of the target compound should be close to 1, and the separation factor (α) between two components should be greater

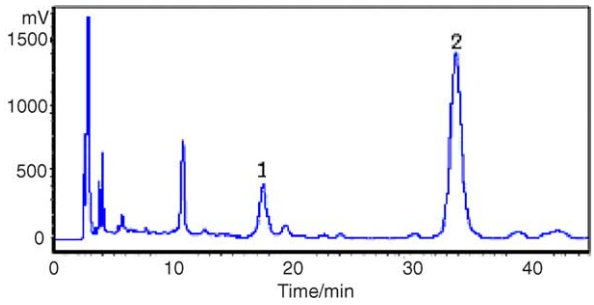

Fig. 2. HPLC chromatogram of crude extract from *Patrinia villosa* Juss after cleaning-up by 1300 macroporous resin. Column: reversed-phase Lichrospher C_{18} (6.0 mm × 150 mm i.d. 5 μm); mobile phase: CH_3CN:HAC:H_2O = 20:1:80; flow rate: 1.0 ml/min; UV wavelength: 254 nm; 1: isoorientin; 2: isovitexin.

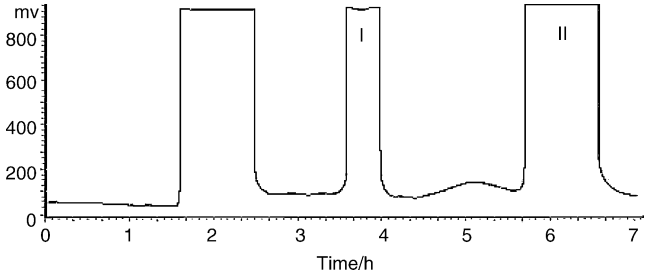

Fig. 3. HSCCC chromatogram of the crude extract from *Patrinia villosa* Juss after cleaning-up by 1300 macroporous resin. Solvent system: ethyl acetate–*n*-butanol–water (2:1:3, v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.4 ml/min; revolution speed: 800 rpm; sample size: 250 mg; retention of stationary phase: 49%; sample loop: 20 ml; detection wavelength: 254 nm.

than 1.5. Large *K* values tend to produce excessive sample band broadening, while small *K* values usually result in a poor peak resolution. Isovitexin and isoorientin are not soluble in non-polar solvent, but have some solubility in ethyl acetate, *n*-butanol, methanol and ethanol. According these properties of isovitexin and isoorientin, some two-phase solvent systems were tested, their *K* and *α* values are listed in Table 1. Among them, the two-phase solvent system, including ethyl acetate– *n*-butanol–water (1:4:5, 2:3:5, 3:2:5, v/v/v), chloroform–methanol–water (4:3:2, 8:10:5, v/v/v) and *n*-butanol–water (1:1, v/v) had large *K* values. When they were used for HSCCC separation, excessive isovitexin and isoorientin band broadening would be produced. Thus, these solvent systems were not suitable for the isolation isovitexin and isoorientin from the crude extract. The other two solvent system ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) and *n*-butanol–ethanol–water (4:1:4, v/v/v) had suitable *K* and *α* values. However, when *n*-butanol–ethanol–water (4:1:4, v/v/v) was used, the setting time of the solvent system was long (>30 s), so it was not suitable for the separation isovitexin and isoorientin from the crude extract. At last, the two-phase solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) was found to be satisfactory for the separation isovitexin and isoorientin from the crude extract. Fig. 3 shows the separation of HSCCC using the solvent system. As a result, two fractions ("I" and "II") were obtained from 250 mg crude extract in less than 7 h and the retention of stationary phase was 49%.

Analytical HPLC was used to determine the purities of the two fractions obtained by HSCCC (shown in Fig. 4). The result indicated that the fraction "I" only contained one peak (t_R = 17.5 min) with purity of 98.5% and "II" contained one peak (t_R = 33.8 min), too, with purity of 99.3%. As expected, the HPLC analysis of each fraction revealed that the components eluted in the order of peaks 1 (isoorientin) and 2 (isovitexin).

Identification of the pure products obtained by HSCCC was performed by MS, ^{1}H NMR and ^{13}C NMR analysis as follows.

Isoorientin (fraction "I"): yellow powder, UVλ_{max}^{MeOH}: 348, 270, 255. TOF-MS: 447 $[M-H]^-$, 895 $[2M-H]^-$, 471 $[M+Na]^+$, 919 $[2M+Na]^+$, 449 $[M+H]^+$, 487 $[M+K]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 13.55 (1H, brs, 5-OH), 7.44 (1H, dd, J = 2.5 Hz, 9.0 Hz, 6′-H), 7.38 (1H, d, J = 2.5 Hz, 2′-H), 6.90 (1H, d, J = 9.0 Hz, 5′-H), 6.64 (1H, S, 3-H), 4.58 (1H, d, J = 10.0 Hz, 1″-H). ^{13}C NMR (500 MHz, DMSO-d_6) δ: 163.44 (C-2), 102.38 (C-3), 181.45 (C-4), 160.59 (C-5), 108.88 (C-6), 163.44 (C-7), 93.73 (C-8), 156.27 (C-9), 102.79 (C-10), 121.56 (C-1′), 112.92 (C-2′), 145.95 (C-3′), 150.44 (C-4′), 116.00 (C-5′), 118.82 (C-6′), 73.18 (C-1″), 70.50 (C-2″), 78.95 (C-3″), 70.19 (C-4″), 81.35 (C-5″), 61.34 (C-6″).

Table 1
The partition coefficient and separation factor of isovitexin and isoorientin in different solvent systems

Solvent system	Isoorientin (K_1)	Separation factors (α)	Isovitexin (K_2)
Ethyl acetate–*n*-butanol–water (1:4:5)	4.41	2.11	9.32
Ethyl acetate–*n*-butanol–water (2:3:5)	3.94	1.96	7.72
Ethyl acetate–*n*-butanol–water (3:2:5)	2.71	3.21	8.70
Ethyl acetate –*n*-butanol–water (2:1:3)	0.38	2.84	1.08
Chloroform–methanol–water[a] (4:3:2)	9.51	4.42	2.15
Chloroform–methanol–water[a] (8:10:5)	7.86	4.20	1.87
n-Butanol–water (1:1)	0.02	4.50	0.09
n-Butanol–ethanol–water (4:1:4)	0.41	2.68	1.10

[a] The separation factor (α) = K_1/K_2.

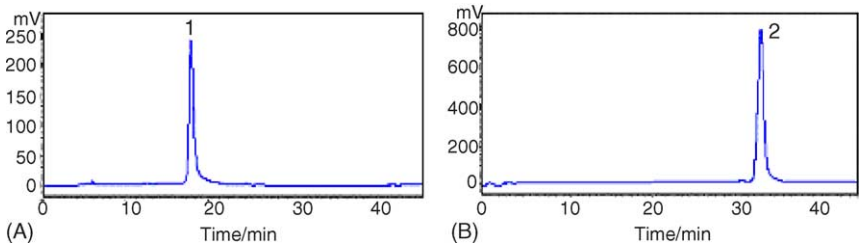

Fig. 4. HPLC chromatogram of HSCCC fractions. Column: reversed-phase Lichrospher C_{18} (6.0 mm × 150 mm i.d. 5 μm); mobile phase: CH_3CN:HAC:H_2O = 20:1:80; flow rate: 1.0 ml/min; UV wavelength: 254 nm; A: fraction "I"; B: fraction "II"; 1: isoorientin; 2: isovitexin.

Isovitexin (fraction "II"): yellow powder, UVλ_{max}^{MeOH}: 334, 270, 302. TOF-MS: 455 $[M+Na]^+$, 433 $[M+H]^+$, 471 $[M+K]^+$, 887 $[2M+Na]^+$, 903 $[2M+K]^+$. ^{1}H NMR (500 MHz, DMSO-d_6) δ: 13.46 (1H, brs, 5-OH), 7.85 (2H, d, J = 8.5 Hz, 3′, 5′-H), 6.94 (2H, d, J = 8.4 Hz, 2′, 6′-H), 6.71 (1H, s, 3-H), 6.42 (1H, s, 8-H), 4.56 (1H, d, J = 9.8 Hz, 1″-H). ^{13}CNMR (500 MHz, DMSO-d_6) δ: 163.32 (C-2), 102.60 (C-3), 181.73 (C-4), 160.64 (C-5), 108.95 (C-6), 163.32 (C-7), 93.79 (C-8), 156.31 (C-9), 102.95 (C-10), 121.00 (C-1′), 128.35 (C-2′, 6′), 116.01 (C-3′, 5′), 161.32 (C-4′), 73.13 (C-1″), 70.52 (C-2″), 78.93 (C-3″), 70.20 (C-4″), 81.38 (C-5″), 61.37 (C-6″).

Comparing with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with those of isovitexin and isoorientin in literatures [23–25].

4. Conclusion

Our study demonstrates that HSCCC is a powerful method in separating, isolating and purifying bioactive components from natural sources. Using HSCCC two C-glycosylflavones including isovitexin and isoorientin are separated from *P. villosa* Juss with a two-phase solvent system comprising of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v). 42.9 mg isovitexin and 20.1 mg isoorientin could be obtained from 250 mg crude extract in one-step elution in less than 7 h. The method is simple, fast and without complex solvent system or gradient elution. It is the first time of discovering isovitexin and isoorientin from the plant of *Patrinia genus*.

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