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Short communication

Application of preparative high-speed counter-current chromatography for separation and purification of arctiin from Fructus Arctii

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Abstract

Following an initial clean-up step on the AB-8 resin (polystyrene resin, 0.3–1.25 mm: NanKai Chemical Factory, Tianjin, China), high-speed counter-current chromatography (HSCCC) was used to purify an arctiin from an extract of the fruits of the *Arctium lappa* L. Arctiin is a major lignan compound in the traditional Chinese medicinal herb *A. lappa* L. The two-phase solvent system used was composed of ethyl acetate–*n*-butanol–ethanol–water at an optimized volume ratio of 5:0.5:1:5 (v/v/v/v). The upper phase was used as the mobile phase in the head to tail elution mode. A total amount of 159 mg of arctiin at 98% purity was obtained from 350 mg of the crude extract (containing 49% arctiin) with 91% recovery. The preparative isolation and purification of arctiin by HSCCC was completed in 5 h in a separation. Identification of the target compound was performed by LC–electrospray ionization MS and ^{13}C-NMR. The structure of the product was further confirmed by comparison with authentic sample (National Institute of the Control of Pharmaceutical and Biological Products, Beijing, China).
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Keywords: *Arctium lappa*; Counter-current chromatography; Arctiin; Preparative chromatography

1. Introduction

Fructus Arctii (Niubangzi in Chinese), the dried fruits of *Arctium lappa* L. (Compositae), is one of the most popular traditional Chinese medicines and is officially listed in the Chinese Pharmacopoeia [1,2]. It has been widely used for dispelling pathogenic wind-heat, promoting eruption, relieving sore throat, removing toxic substances and subduing swelling [1,2]. More interestingly, arctiin (Fig. 1), a major lignan compound in Fructus Arctii, has been found to has protective effects on 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced carcinogenesis particularly in the mammary gland in the promotion period by testing on Sprague–Dawley rats [3] and a remarkable anti-tumor-promoting effect on carcinogenesis test of mouse skin tumors induced by 7,12-dimethylbenzanthracene [4]. Recently, Huang et al. reported that arctiin could significantly induce cell detachment and decreases the cell numbers via the up-regulation of MUC-1 mRNA and protein in PC-3 cells [5]. Thus, it is important to develop an efficient method to separate and purify arctiin.

2. Experimental

2.1. Reagents and materials

All organic solvents used for high-speed counter-current chromatography (HSCCC) were of analytical grade and purchased from Tianjin Guangcheng Chemical Factory, Tianjin, China. Acetonitrile used for HPLC analysis was of chromatographic grade and purchased from Tianjin Siyou Special Reagent Factory, Tianjin, China. The standard arctiin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The arctiin stock solution was 1.00 mg/ml, which

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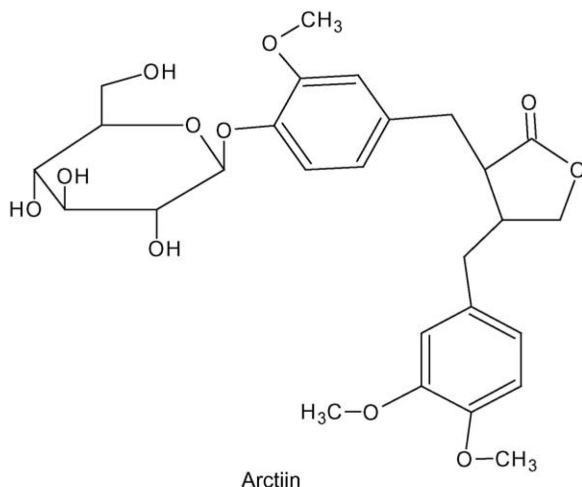

Fig. 1. Chemical structure of arctiin.

was prepared by dissolving 10.0 mg of arctiin in 10.00 ml methanol and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with methanol. All other solutions were prepared by dissolving appropriate amounts of commercially available chemicals in water.

The dried seeds of *A. lappa* L. were purchased from a local drug store.

2.2. Apparatus

Preparative HSCCC was carried out using a Model GS10A-2 multilayer coil of 1.6 mm i.d. and 110 m in length with a total capacity of 230 ml. The β values of this preparative column range from 0.5 at internal to 0.8 at the external ($\beta = r/R$, where r is the rotation radius or the distance from the coil to the holder shaft, and R ($R = 8$ cm) is the revolution radius or the distances between the holder axis and central axis of the centrifuge) (Beijing Institute of New Technology Application, Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823 A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 254 nm. A manual sample injection valve with a 15-ml loop (for the preparative HSCCC) (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Waters Millennium^{32} system including a Waters 996 Photodiode Array Detection (DAD) system, a Waters 600 Multisolvent Delivery System, a Waters 600 System controller, a Waters 600 pump, and a Millennium^{32} work-station (Waters, Milford, USA).

2.3. Extraction of crude arctiin

The dried fruits of *A. lappa* L. (4 kg) were extracted three times with 20 l of 70% ethanol. Then, the extract was combined and evaporated to dryness under reduced pressure, which yield 500 g of dry powder. About 300 g of the residue obtained from the combined extract was dissolved with 2 l of water. After filtration, the aqueous solution was extracted three times with 5 l each of water-saturated light petroleum (bp 60–90 °C), ethyl acetate and *n*-butanol successively which yielded 30 g of light petroleum extract, 40 g of ethyl acetate extract and 100 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. Then, the *n*-butanol extract was chromatographed on AB-8 resin (polystyrene resin, 0.3–1.25 mm: NanKai Chemical Factory, Tianjin, China) with 30% ethanol. Portions of the above 30% ethanol extract were subjected to HSCCC.

2.4. Selection of two-phase solvent system

A number of two-phase solvent systems were tested by changing the volume ratio of the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values. The partition coefficient values were determined according to the literature [6,7]. In brief, 2 ml of each phase of the equilibrated two-phase solvent system was added to approximately 1 mg of crude arctiin placed in a 10-ml test tube. The test tube was caped, and was shaken vigorously for 1 min to equilibrate the sample thoroughly. An equal volume of each phase was then analyzed by HPLC to obtain the partition coefficients. The partition coefficient value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

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

The selected solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly and degassed by sonication prior to use. The upper phase was used as the stationary phase, while the lower phase was used as the mobile phase. The sample solution was prepared by dissolving the crude arctiin in the mixture solution of lower phase and upper phase (1:1, v/v) of the solvent system.

2.6. HSCCC separation

The multilayer-coiled column was first entirely filled with the upper phase. The lower aqueous phase was then pumped into the head end of the column at a suitable flow rate of 1 ml/min for Model GS10A-2 while the apparatus was rotated at an optimum speed of 800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (350 mg of the crude arctiin in 15 ml of both phases)

Table 1
The K (partition coefficient) values of arctiin in different solvent systems

Solvent system	K value[a]
n-Hexane–ethyl acetate–methanol–water (1:4:2:5, v/v/v/v)	0.10
n-Hexane–ethyl acetate–methanol–water (1:4:2:4, v/v/v/v)	0.11
Ethyl acetate–ethanol–water (5:1:5, v/v/v)	0.54
Ethyl acetate–ethanol–water (5:0.5:5, v/v/v)	0.77
Ethyl acetate–*n*-butanol–ethanol–water (5:0.5:1:5, v/v/v/v)	1.50
Ethyl acetate–*n*-butanol–ethanol–water (5:0.2:1:5, v/v/v/v)	1.10
Ethyl acetate–*n*-butanol–ethanol–water (5:1:1:5, v/v/v/v)	2.99
Ethyl acetate–*n*-butanol–water (5:1.5:6, v/v/v)	5.20

[a] K value was expressed as the peak area of the compound in the upper phase divided by the peak area of the compound in the lower phase.

was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV–vis detector at 254 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and determined by HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas.

2.7. HPLC analysis and identification of arctiin

The crude extract, arctiin (standard) and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a Shim-pack VP-ODS column (250 mm × 4.6 mm i.d.) column at a column temperature of 25 °C. The mobile phase composed of acetonitrile–water (30:70, v/v) was isocratically eluted at a flow rate of 1.0 ml/min. The effluent was monitored at 280 nm to facilitate the simultaneous detection of arctiin and the other compounds. Chromatographic peaks were identified by comparing the retention time and the UV–vis spectra from 210 to 400 nm by DAD against the standard. Routine sample calculations were made by comparison of the peak area with that of the standard.

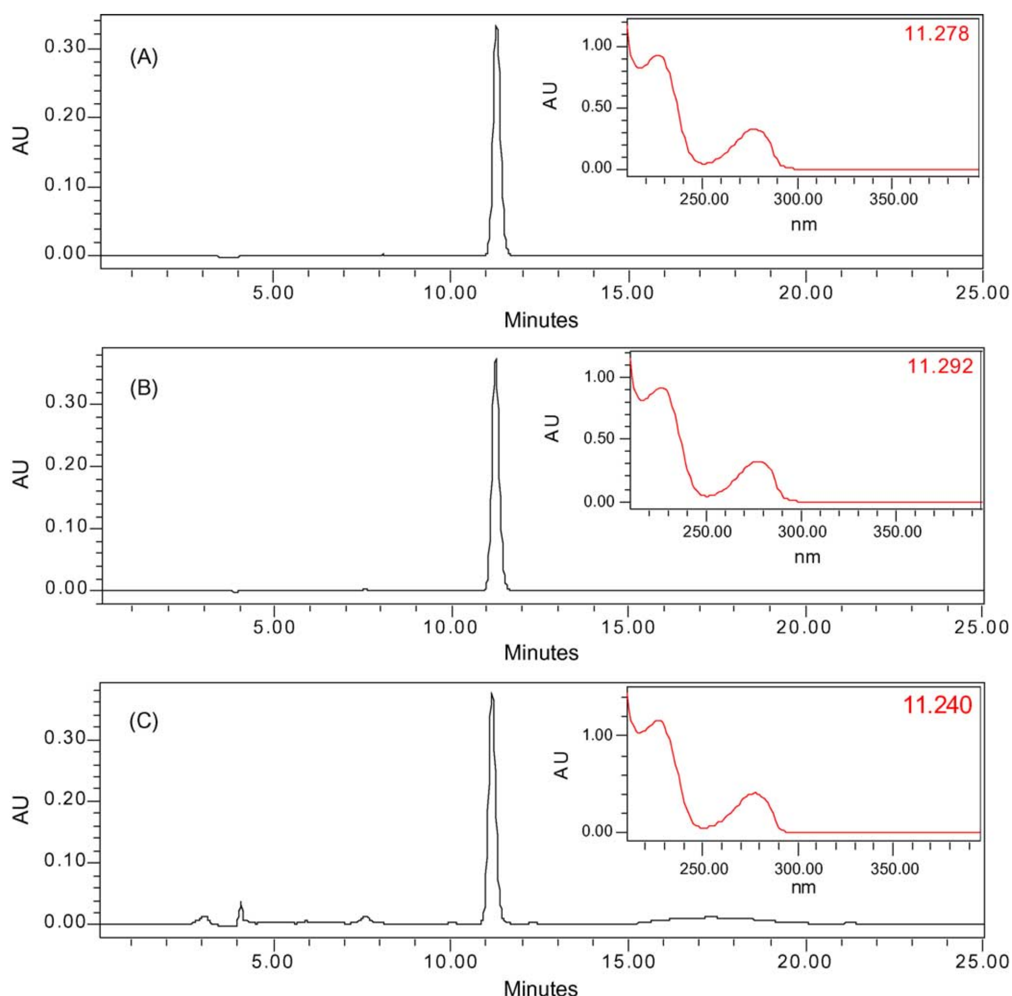

Fig. 2. (A) HPLC analyses and UV spectrum of authentic arctiin; (B) HPLC analyses and UV spectrum of the purified arctiin by HSCCC; (C) HPLC chromatogram of the crude arctiin extracted from *A. lappa* L. after cleaning up by AB-8 resin. Experimental conditions: a Shim-pack VP-ODS column (250 mm × 4.6 mm i.d.); column temperature, 25 °C; mobile phase, acetonitrile–water (30:70, v/v); flow rate, 1.0 ml/min; detection, 280 nm; injection volume, 10 μl.

The identification of HSCCC peak fractions was further confirmed, respectively, by LC–electrospray ionization (ESI)-MS on an Agilent 1100/MSD and by ^{13}C-NMR spectra on an INOVA 600 MHz NMR spectrometer.

3. Results and discussion

3.1. Selection of suitable two-phase solvent system

Successful separation by HSCCC largely depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K) for the targeted sample [8,9]. Small K values usually result in a poor peak resolution, while large K values tend to produce excessive sample band broadening [10–12]. Several two-phase solvent systems were tested and their K values were measured and summarized in Table 1. When *n*-hexane–ethyl acetate–methanol–water was used as the two-phase solvent system, their K values were small. Arctiin and some other compounds were eluted together closely near the solvent front and resulted in a poor resolution. When ethyl acetate–ethanol–water at the ratios of 5:1:5 and 5:0.5:5 (v/v/v) was used as the two-phase solvent system, the K values were suitable and arctiin could be well separated from the other compounds. However, such solvent systems were not appropriate for separating a large amount of sample due to the poor solubility of arctiin at high concentrations. When ethyl acetate–*n*-butanol–ethanol–water was used as the two-phase solvent system as shown in Table 1, all K values were suitable and arctiin could be well separated from the other compounds. Furthermore, arctiin is freely soluble in these solvent systems. From the above, the two-phase solvent system composed of ethyl acetate–*n*-butanol–ethanol–water at a ratio of 5:0.5:1:5 (v/v/v/v) was found to be the best.

3.2. Separation of arctiin by HSCCC

The crude sample (350 mg) after cleaning up by AB-8 resin was dissolved in 15 ml of both phases. The sample solution was separated and purified by HSCCC according to the procedure described earlier. The lower phase was used as the stationary phase while the upper phase was used as the mobile phase in the head to tail elution mode. The retention of the stationary phase was 30%, and the total separation time was about 5 h. The HSCCC fractions were analyzed by HPLC, and their absorbance was measured at 254 nm to draw the elution curve (Fig. 3). Based on the HPLC analysis and the elution curve of the preparative HSCCC, all collected fractions were combined into different pooled fractions. Fig. 2(C) shows the HPLC analysis of the combined fractions. Peak d (Fig. 2(B) and Fig. 3) was identified as arctiin by congruence its retention time and UV spectra with that of authentic arctiin (Fig. 2(A)).

The crude extract from *A. lappa* L. was analyzed by HPLC (Fig. 2(C)). The result indicated that the crude sample contained several compounds among which arctiin represented 49% of the total. After only one step of operation by HSCCC, a total amount of 159 mg of arctiin (98% purity) was yielded with 91% recovery. These results demonstrate the high resolving power of HSCCC.

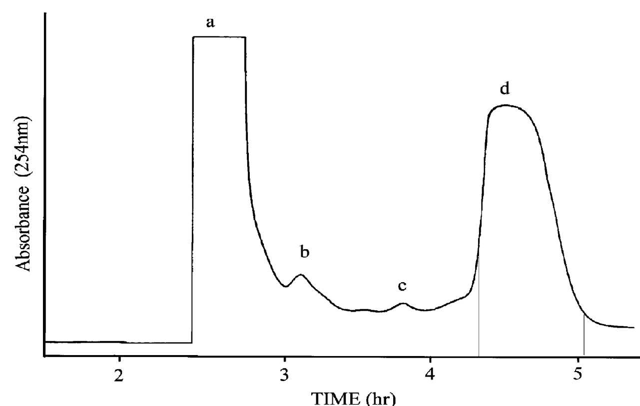

Fig. 3. Chromatogram of the crude extract by preparative HSCCC. Experimental conditions: column, multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 230 ml; revolution speed, 800 rpm; solvent system, ethyl acetate–*n*-butanol–ethanol–water (5:0.5:1:5, v/v/v/v); stationary phase, upper organic phase; mobile phase, lower aqueous phase; flow rate, 1.0 ml/min; detection, 254 nm; sample size, 350 mg; injection volume, 15 ml; retention of the stationary phase, 30%; d, arctiin.

3.3. Identification of the components of arctiin

The structures of the product (peaks d in Fig. 3) were further confirmed by LC–ESI-MS and ^{13}C-NMR spectra. The LC–ESI-MS of product d (peaks d in Fig. 3) in the negative mode gave m/z 553 as the deprotonated molecular ion $[M-\mathrm{H}]^-$, which confirmed the molecular mass as 554, the same as that for arctiin. The ^{13}C-NMR data of product d matched with the reported NMR data for arctiin [13].

4. Conclusion

Using HSCCC, we were able to purify arctiin efficiently and quickly. From a 350 mg mixture, we obtained 159 mg of arctiin with a purity of 98% in 5 h. The results of the present study clearly demonstrated that HSCCC is very useful in the preparative separation of arctiin from the crude extract of *A. lappa* L.

Acknowledgements

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