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Preparative isolation and purification of bergapten and imperatorin from the medicinal plant *Cnidium monnieri* using high-speed counter-current chromatography by stepwise increasing the flow-rate of the mobile phase

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Abstract

A high-speed counter-current chromatography (HSCCC) method was developed for the preparative separation and purification of bergapten and imperatorin from the Chinese medicinal plant *Cnidium monnieri* (L.) Cusson. The crude extract was obtained by extraction with ethanol from the dried fruits of *Cnidium monnieri* (L.) Cusson under sonication. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (5:5:5:5, v/v/v/v) was successfully performed by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml min^{-1} after 180 min. The components purified and collected were analyzed by high-performance liquid chromatography. The method yielded 45.8 mg of bergapten at 96.5% purity and 118.3 mg of imperatorin at 98.2% purity from 500 mg of the crude extract in a single run. The recoveries of bergapten and imperatorin were 92.1 and 93.7%, respectively.

Keywords: *Cnidium monnieri*; Counter-current chromatography; Preparative chromatography; Pharmaceutical analysis; Plant materials; Bergapten; Imperatorin

1. Introduction

Cnidium monnieri (L.) Cusson (Shechaungzi in Chinese) is one of the most popular traditional Chinese medicinal herbs. Its fruits have been used for the treatment of impotence, frigidity and skin-related diseases [1,2], and have exhibited strong antiallergic, antipruritic, antidermatophytic, antibacterial, antifungal and antiosteoporotic activities [3–8]. The major bioactive components of the fruits of *Cnidium monnieri* (L.) Cusson are coumarins that mainly include bergapten, imperatorin, osthol and xanthotoxol. Bergapten possesses anti-inflammatory and analgesic activities [9], while imperatorin exhibits strong cytotoxic activity on human leukemia, chemopreventive effects on hepatitis and skin tumor, and anti-inflammatory activity [10–13]. The chemical structures of bergapten and imperatorin are shown in Fig. 1A.

Although osthol and xanthotoxol have been purified from *Cnidium monnieri* (L.) Cusson by HSCCC at high purity [14], preparative separation and purification of bergapten and imperatorin from *Cnidium monnieri* (L.) Cusson, however, has not been explored. The present paper describes successful preparative separation and purification of bergapten and imperatorin from the crude alcohol extract of *Cnidium monnieri* (L.) Cusson by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (inner diameter of tube, 2.6 mm; total volume, 325 ml). The rev-

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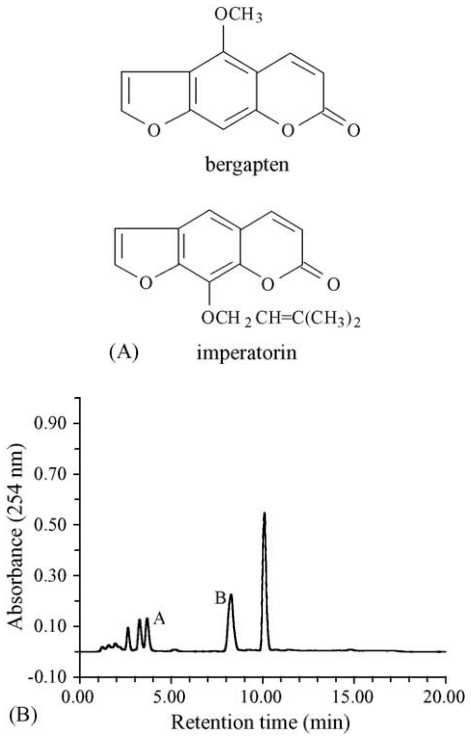

Fig. 1. (A) Chemical structure of bergapten and imperatorin. (B) Chromatogram of crude extract from *Cnidium monnieri* (L.) Cusson by HPLC analysis. Peaks: (A) bergapten, (B) imperatorin. *Conditions*: column, reversed-phase symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase, methanol (solvent A)–water (solvent B) in the gradient mode as follows: 0–5 min, 60% A; 5–14 min, 60–80% A; 14–15 min, 80–60% A; flow-rate, 1.0 ml min^{-1}; detection at 254 nm.

olution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β-value varied from 0.47 at the internal terminal to 0.73 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 Series II HPLC pump (Pharma-Tech Research), a Model SPD-10Avp UV–vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10-ml sample loop.

2.2. Reagents

All solutions were prepared with analytical grade chemicals. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethyl acetate, *n*-hexane, ethanol and methanol were obtained from BDH (Poole, UK). The standard coumarins were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. The coumarin stock solution was at 1.00 mg ml^{-1} which was prepared by dissolving 10.0 mg of the coumarin in 10.00 ml methanol, and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solution with methanol.

The dried fruits of *Cnidium monnieri* (L.) Cusson were obtained from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.3. Preparation of crude extract from Cnidium monnieri (L.) Cusson

Preparation of crude extracts was carried out according to the literature [15]. In brief, the dried fruits of *Cnidium monnieri* (L.) Cusson were ground to powder. The powder (350 g) was extracted with 1000 ml ethanol under sonication for 1 h. The mixture was filtered with 0.22 μm filter membrane of Type GV (Millipore, USA), and the residue was then extracted twice (1000 ml each time). The filtrate was combined, and the extract was evaporated to dryness by rotary vaporization at 45 °C. The dried crude extract (5.648 g) was stored in a refrigerator for subsequent HSCCC separation.

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

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude extract in the solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system used for separation because the sample was not easily dissolved in either phase alone.

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) was pumped into the column at a flow-rate of 1.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, approximately 10 ml of the sample solution, containing 500 mg of the crude extract was injected through the injection valve. After 180 min, the flow-rate of the mobile phase was increased to 2.0 ml min^{-1}. 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.

2.6. HPLC analysis

The crude extract and each peak fraction obtained by HSCCC were analyzed by HPLC according to the literature [16,17]. The HPLC system used throughout this study consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millennium chromatogra-

phy data system (Waters). The column used was a reversed-phase symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm, Waters). The mobile phase was methanol (solvent A)–water (solvent B) in the gradient mode as follows: 0–5 min, 60% A; 5–14 min, 60–80% A; 14–15 min, 80–60% A. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 254 nm. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

The crude extract obtained from *Cnidium monnieri* (L.) Cusson was analyzed by HPLC, and the chromatogram is shown in Fig. 1B. The contents of bergapten and imperatorin were 9.6 and 24.8%, respectively.

HSCCC is a very effective tool for the preparative separation and purification of natural products. The extracts from plant materials usually contain a high number of different compounds with a broad range of hydrophobicity. Most often, only one component needs to be separated from the others, and the standard HSCCC method, which uses a constant flow-rate of the mobile phase, is used. In order to separate more different compounds, stepwise elution or stepwise increasing the flow-rate of the mobile phase might be adopted [18,19]. Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of *n*-hexane–ethanol–water at a volume ratio of 10:5:5 or 10:6:4. Although imperatorin could be separated from other compounds, it was difficult to separate bergapten. In the subsequent studies, another two-phase solvent system was tested.

Performance of the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water was evaluated in terms of peak resolution. With the two-phase solvent system at volume ratios of 5:5:6:4 and 5:5:7:3, bergapten still was not well separated, although the separation of imperatorin could be achieved. At a ratio of 4:6:5:5 with the same two-phase solvent system, imperatorin was retained in the column for a long period of time (10 h) and the separation of bergapten was still poor. Finally, the two-phase solvent system at a ratio of 5:5:5:5 was attempted at a flow-rate of 1.0 ml min^{-1}, which was subsequently increased to 2.0 ml min^{-1} after 180 min, the separation of the both compounds was achieved with satisfactory peak resolution, and the retention of the stationary phase was good (about 58%). After imperatorin was eluted out, in order to save solvents and time, the remaining compounds in the column were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was not to be reused. Fig. 2 shows the preparative HSCCC separation of 500 mg of crude extract using the solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a ratio of 5:5:5:5 by increasing the flow-rate of the mobile phase stepwise from 1.0 to 2.0 ml min^{-1} after 180 min. This separation yielded 45.8 mg of bergapten at 96.5% purity and 118.3 mg of imperatorin at 98.2% purity according to HPLC analysis. The recoveries of bergapten and imperatorin were 92.1 and 93.7%, respectively.

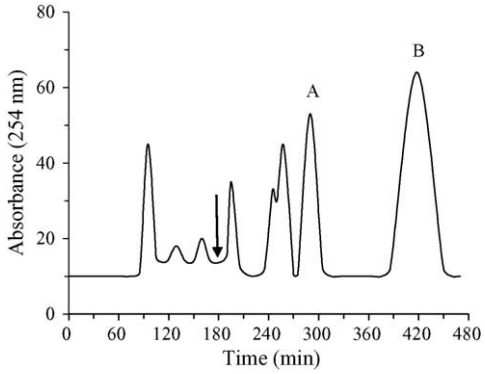

Fig. 2. Chromatogram of crude extract from *Cnidium monnieri* (L.) Cusson by HSCCC separation. Peaks: (A) bergapten, (B) imperatorin. *Conditions*: column, multilayer coil of 2.6 mm i.d. PTFE tube with a total capacity of 325 ml; rotary speed, 1000 rpm; solvent system, *n*-hexane–ethyl acetate–ethanol–water (5:5:5:5, v/v/v/v); mobile phase, lower phase (water phase); flow-rate, 0–180 min, 1 ml min^{-1}, and 180–480 min, 2 ml min^{-1}; detection at 254 nm; sample size, 500 mg; retention of the stationary phase, 58%. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 to 2.0 ml min^{-1} after 180 min.

In conclusion, an HSCCC method for the preparative separation and purification of bergapten and imperatorin from the medicinal plant *Cnidium monnieri* (L.) Cusson was developed by stepwise increasing the flow-rate of the mobile phase. The 45.8 mg of bergapten at 96.5% purity and 118.3 mg of imperatorin at 98.2% purity could be obtained from 500 mg of the crude extract in a one-step separation, and their recoveries as high as 92.1 and 93.7% were achieved, respectively. The present study indicates that HSCCC is a very powerful technique for the preparative separation and purification of bioactive components from plant materials.

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