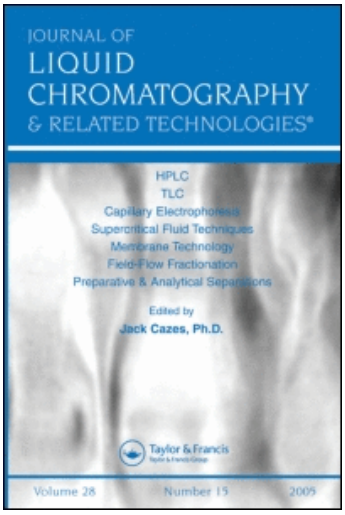

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Abstract: Stepwise flow rate preparative high-speed countercurrent chromatography (HSCCC) was successfully used for isolation and purification of imperatorin, oxypeucedanin, and isoimperatorin from a crude root extract of A*ngelica dahurica* (Fisch. ex Hoffm) Benth. et Hook. The separation was performed with a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water at volume ratio of 5:5:4:6, v/v/v/v, which had been selected by analytical HSCCC. About a 600 mg amount of the crude extract was separated in a one step operation, yielding 35.6 mg of imperatorin, 16.4 mg of oxypeucedanin, and 22.7 mg of isoimperatorin, all at a high purity of over 98%.

Keywords: Imperatorin, Oxypeucedanin, Isoimperatorin, *Angelica dahurica*, High-speed countercurrent chromatography, Stepwise flow

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INTRODUCTION

Angelica dahurica (Fisch. ex Hoffm) Benth. et Hook is a useful traditional Chinese herb. All members of this genus contain furocoumarins, which increase skin sensitivity to sunlight often causing dermatitis.

Coumarins and structurally related compounds have been recently shown to inhibit human immunodeficiency virus, type 1 (HIV-1) activity. Among them, the imperatorin strongly suppresses cyclin D1 expression and arrests the cells at the G1 phase of the cell cycle. These results highlight the potential of Sp1 transcription factor as a target for natural anti-HIV-1 compounds, such as furanocoumarins, that might have a potential therapeutic role in the management of AIDS.[1]

Five furanocoumarins, including byakangelicin, phellopterin, imperatorin, isoimperatorin, and oxypeucedanin, from the roots of *Angelica dahurica* (Umbelliferae) have been isolated, and the effects of these compounds on lipopolysaccharide (LPS) induced prostaglandin E2 (PGE2) production in rat peritoneal macrophages were examined.[2] Among these compounds, imperatorin showed the most potent inhibitory activity on the LPS induced PGE2 production.

Imperatorin was found to induce apoptosis in human promyelocytic leukaemia, HL-60 cells. DNA fragmentation assay, morphology based evaluation, and flow cytometric analysis demonstrated that imperatorin at micromolar concentrations was able to trigger apoptosis of HL-60 cells. Neither necrosis nor differentiation was observed at cytotoxic micromolar concentrations of imperatorin.[3]

The separation of these active compounds from natural sources, however, may encounter various problems. High-speed countercurrent chromatography (HSCCC), being a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support,[4] and has been widely used in preparative separation of natural products.[5,6] The present paper describes the successful preparative separation and purification of imperatorin, oxypeucedanin, and isoimperatorin from the crude extract of *Angelica dahurica* by stepwise flow rate high-speed countercurrent chromatography.

EXPERIMENTAL

Apparatus

The present study employed two different types of the multilayer coil planet centrifuge for performing high-speed countercurrent chromatography (HSCCC). The analytical HSCCC instrument (a Model GS 20, Beijing Institute of New Technology Application, Beijing, China) holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm from the

central axis of the centrifuge. The separation column consisted of 50 m long and, 0.85 mm I.D. PTFE (polytetrafluoroethylene) tubing, which was directly wound onto the holder hub forming multiple coiled layers with a total capacity of 40 mL. The β values varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R, the distance between the holder axis and central axis of the centrifuge). Although, the revolution speed of the apparatus is regulated with a speed controller up to 2,000 rpm, an optimum speed of 1,800 rpm was used in the present studies. A manual sample injection valve with a 1.0 mL loop was used for sample loading.

The preparative separation was performed using an HSCCC multilayer coil planet centrifuge (a Model S10, Beijing Institute of New Technology Application, Beijing, China) equipped with a multilayer coil of 110 m × 1.6 mm I.D PTFE tubing with a total capacity of 250 mL. The β values of this preparative column range from 0.5 at the internal terminal to 0.8 at the external terminal. A Model NS-1007 constant flow pump was used to elute the mobile phase, while a Model 8823A-UV Monitor was used for continuous monitoring of the effluent at 254 nm. A manual six port valve with a 20 mL loop was used for sample injection and a portable recorder for drawing the chromatogram.

A Shimadzu LC-20A system for HPLC analysis includes two LC-20A solvent delivery units, an SPD-M20A UV-VIS photodiode array detector, a Model 7725 injection valve with a 20 μL loop, an SCL-20A system controller, and a Class-VP-LC workstation (Shimadzu Corporation, Kyoto, Japan).

Reagents

Organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China). Methanol used for HPLC analysis was of chromatographic grade and purchased from Tianjin Huaxi Special Reagent Factory (Tianjin, China).

Roots of *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook were purchased from a local store (Tong Ren Tang Shop, Beijing, China).

Preparation of Sample

About 500 g of dried *Angelica dahurica* was ground, and a 100 g amount of this dried powder was extracted by refluxing for 4 hours with 500 mL of 95% ethanol solution, and concentrated to dryness under vacuum. The dried material was again extracted with 200 mL of ethyl acetate with sonication for 20 min, and dried under vacuum, yielding 4.5 g of a crude sample, which contained imperatorin, oxypeucedanin, and isoimperatorin as target compounds. The purity of each compound was determined by HPLC (Figure 1).

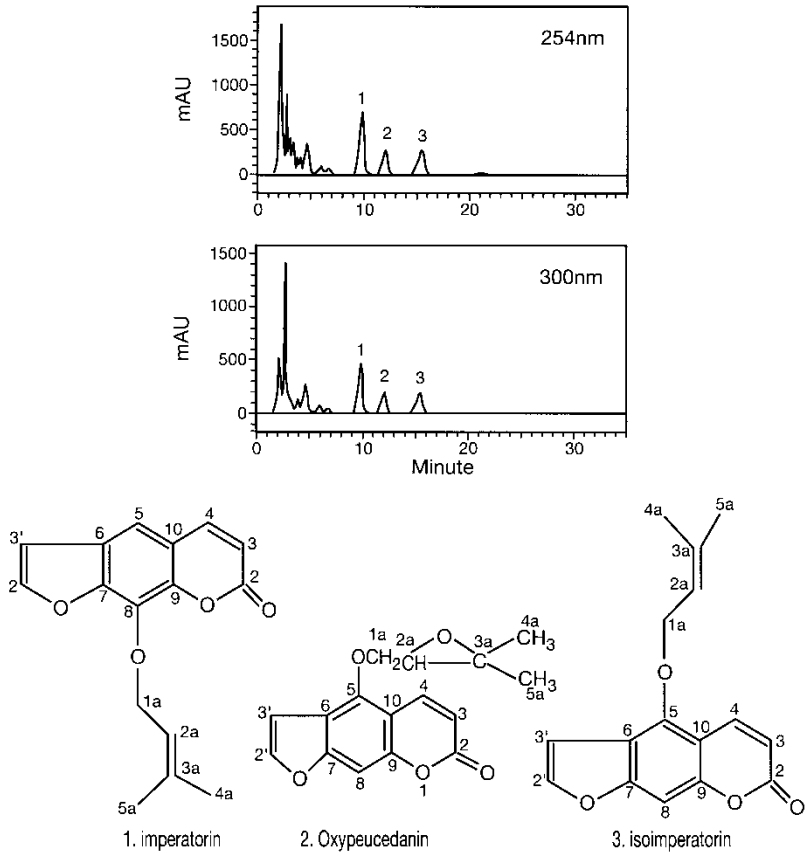

Figure 1. HPLC analyses of the crude extract from *Angelica dahurica* with the chemical structures of imperatorin, oxypeucedanin, and isoimperatorin. HPLC conditions: Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 35°C. The mobile phase composed of methanol: water (60:40, v/v) was isocratically eluted at a flow rate of 1.0 mL/min, and the effluent monitored at 254 nm and 300 nm by a PAD detector.

Preparation of Two-Phase Solvent System and Sample Solutions

Two-phase solvent systems for HSCCC were prepared by mixing n-hexane-ethyl acetate-methanol-water at various volume ratios of 1:1:1:1, 5:5:4.8:5.2, 5:5:4.5:5.5, and 5:5:4:6, v/v/v and thoroughly equilibrating the mixture in a separatory funnel at room temperature, two phases being separated shortly before use.

The sample solutions were prepared by dissolving the crude extract in the lower phase of the above solvent system used for the separation, each at a suitable concentration according to the analytical or preparative purpose.

Separation Procedure

Analytical HSCCC separation was initiated by filling the column entirely with the upper phase. The lower phase was then pumped into the head end of the column at a flow rate of 1.0 mL/min, while the apparatus was run at a revolution speed of 1,800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (5 mg in 1 mL of lower phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm and each peak fraction was collected according to the chromatogram. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column after the separation was completed.

Preparative HSCCC was performed with a Model GS 10 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column inlet at a flow rate of 2.0 mL/min, while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (600 mg in 10 mL of lower phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254 nm. Each peak fraction was collected according to the chromatogram. After target fractions 1 and 2 were eluted, the flow rate was increased to 5.0 mL/min to facilitate elution of fraction 3.

HPLC Analyses and Identification of HSCCC Peak Fractions

The crude root extract of *Angelica dahurica* and HSCCC peak fractions were each analyzed by HPLC using a Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 35°C. The mobile phase composed of methanol: water (60:40, v/v) was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 254 nm and 300 nm by a PAD detector.

Identification of the target compounds (imperatorin, oxypeucedanin, and isoimperatorin) was based on MS, ^{1}H-NMR, and ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

Figure 1 shows HPLC analysis of the crude root extract of *Angelica dahurica* in which the purity of imperatorin, oxypeucedanin, and isoimperatorin is estimated at 30.8%, 15.1%, and 16.9%, respectively, based on the percentage of their peak area.

In order to achieve an efficient resolution of target compounds, a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water

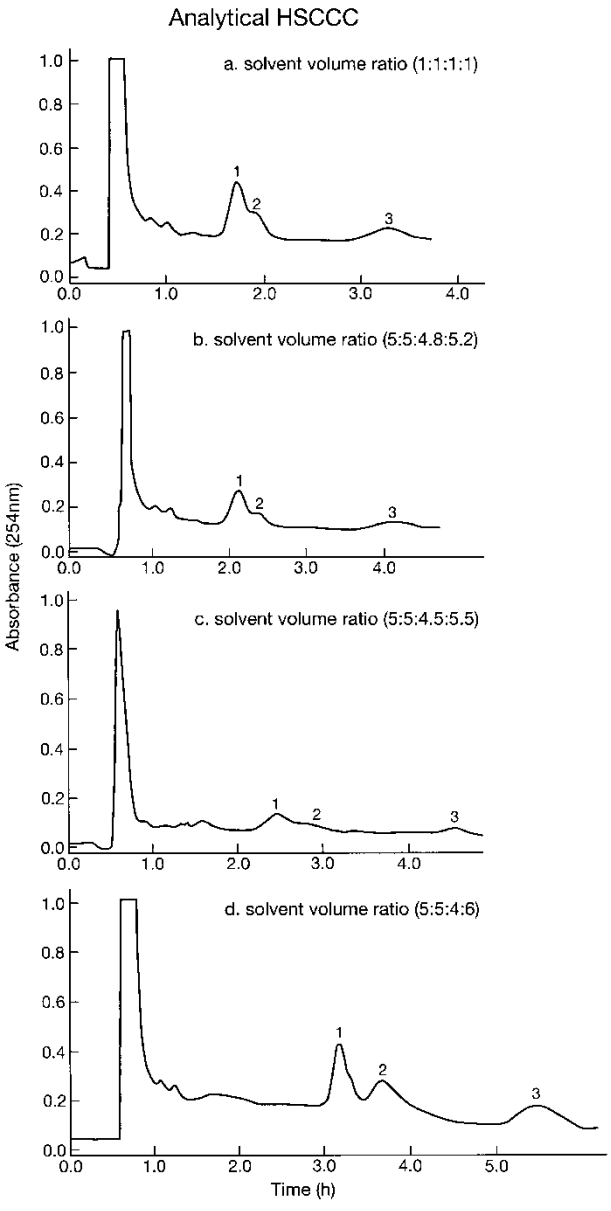

Figure 2. Chromatogram of the crude extract from *Angelica dahurica* by analytical HSCCC. Solvent system: A. n-hexane-ethyl acetate-methanol-water (1:1:1:1, v/v/v/v); B. n-hexane-ethyl acetate-methanol-water (5:5:4.8:5.2, v/v/v/v); C. n-hexane-ethyl acetate-methanol-water (5:5:4.5:5.5, v/v/v/v); D. n-hexane-ethyl acetate-methanol-water (5:5:4:6, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1.0 mL/min; revolution speed: 1800 rpm; sample: 5 mg dissolved in 1.0 mL of lower phase.

was examined using analytical HSCCC by varying the mutual volume ratio, since this solvent system has been successfully applied to various samples with a moderate degree of polarity. The results are illustrated in Figure 2a–d.

As seen in Figure 2a, the separation of imperatorin (peak 1) and oxypeucedanin (peak 2) were only partially resolved at the volume ratio (1:1:1:1), while isoimperatorin (peak 3) was completely separated and eluted in $3\frac{1}{2}$ hr. On the other hand, the solvent ratio (5:5:4:6) improved the peak resolution between imperatorin and oxypeucedanin while isoimperatorin (peak 3) was completely separated and eluted in $5\frac{1}{2}$h, as shown in Figure 2d. Based on the results of the above analytical HSCCC experiment, 600 mg of the crude root extract of *Angelica dahurica* was separated by preparative HSCCC using the above solvent system at a volume ratio of (5:5:4:6) in Figure 3, where elution of the last peak (peak 3) required for 13 h.

In order to improve the separation time, after the first two target peaks were eluted the flow rate was increased stepwise at 5 mL/min to elute the last peak (peak 3). This strategy shortened the total elution time from 13 h to less than 10 h as shown in Figure 4, which indicated that a two-phase solvent system composed of n-hexane-ethyl acetate-methanol-water (5:5:4:6, v/v/v), combined with the stepwise flow rate elution, enables efficient preparative purification of three target compounds, imperatorin, oxypeucedanin and isoimperatorin, from a crude root extract of *Angelica dahurica* by one step elution within a reasonable elution time.

The structural identification of imperatorin, oxypeucedanin, and isoimperatorin was carried out by MS, ^{1}H-NMR and ^{13}C-NMR Spectra as follows: the EI-MS: m/z 270, 202, 174, 145, 118, 89, 69, 53, 41. It showed

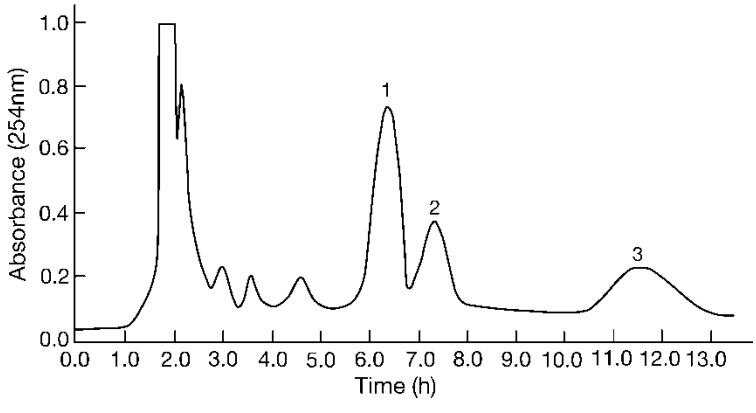

Figure 3. Chromatogram of the crude extract from *Angelica dahurica* by preparative high-speed countercurrent chromatography. Solvent system: n-hexane-ethyl acetate-methanol-water (5:5:4:6, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; sample: 600 mg dissolved in 10 mL of lower phase.

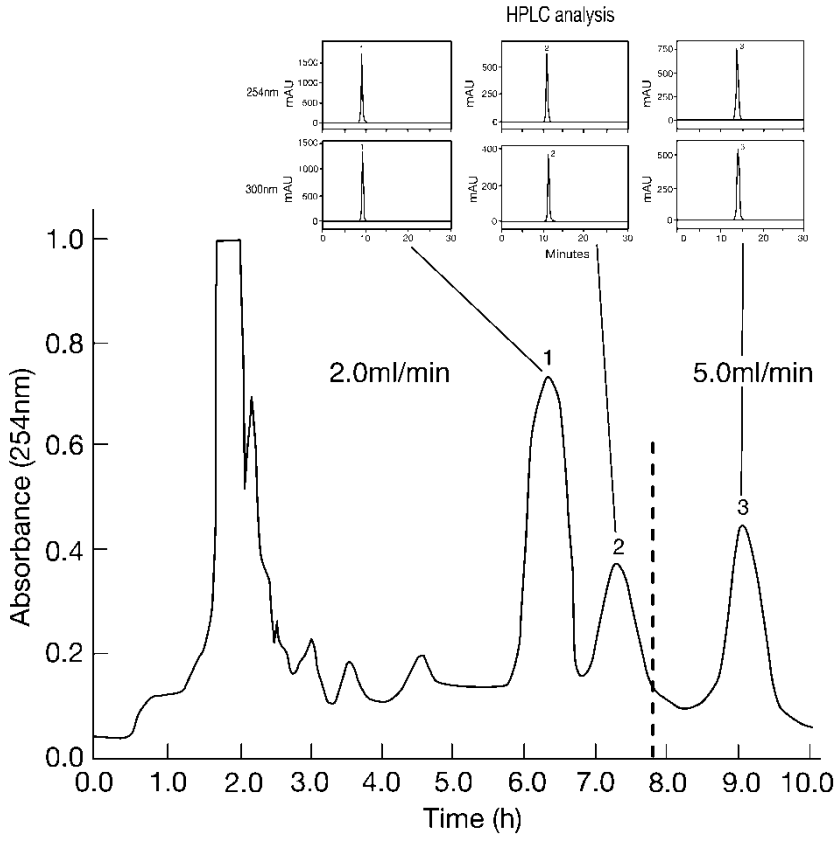

Figure 4. Chromatogram of the crude root extract from *Angelica dahurica* by stepwise flow rate preparative high-speed countercurrent chromatography. Solvent system: n-hexane-ethyl acetate-methanol-water (5:5:4:6, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate changed from 2.0 mL/min to 5.0 mL/min after peak 2 eluted; revolution speed: 800 rpm; sample: 600 mg dissolved in 10 mL of lower phase. HPLC conditions: Polaris ODS column (250 × 4.6 mm I.D.) at column temperature of 35°C. The mobile phase composed of methanol:water (60:40, v/v) was isocratically eluted at a flow rate of 1.0 mL/min, and the effluent monitored at 254 nm and 300 nm by a PAD detector.

the molecular ion at m/z 270, which is in agreement with the molecular formula $C_{16}H_{14}O_4$ of imperatorin.[7] The EI-MS: m/z 286, 215, 202, 173, 157, 145, 129, 101, 89. It showed the molecular ion at m/z 286, which is in agreement with the molecular formula $C_{16}H_{14}O_5$ of oxypeucedanin.[8] And the EI-MS: m/z 270, 202, 174, 69. It showed the molecular ion at m/z 270, which is in agreement with the molecular formula $C_{16}H_{14}O_4$ of isoimperatorin.[9] Imperatorin: ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 6.290 (3C-H), 7.731 (4C-H), 7.293 (5C-H), 7.670 (2′C-H), 6.782 (3′C-H), 5.561

(–CH═), 1.793 (CH_3-H), 4.981 (OCH_2-H). Imperatorin: ^{13}C-NMR (500 MHz, $CDCl_3$) δ ppm: 159.825 (2-C), 114.203 (3-C), 145.345 (4-C), 114.127 (5-C), 125.723 (6-C), 147.832 (7-C), 130.560 (8-C), 143.209 (9-C), 116.385 (10-C), 146.433 (2′-C), 107.093 (3′-C), 69.375 (1a-C), 119.696 (2a-C), 139.119 (3a-C), 17.849 (4a-C), 25.493 (5a-C). The results were similar to those in reference.[10,11]

Oxypeucedanin: ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 6.243 (3C-H), 8.153 (4C-H), 7.111 (8C-H), 7.601 (2′C-H), 6.897 (3′C-H), 3.912 (–CH═), 1.312 (CH_3-H), 4.568 (CH_2-H). Oxypeucedanin: ^{13}C-NMR (500 MHz, $CDCl_3$) δ ppm: 161.033 (2-C), 113.141 (3-C), 139.012 (4-C), 148.691 (5-C), 114.419 (6-C), 158.238 (7-C), 94.833 (8-C), 152.711 (9–C), 107.478 (10-C), 145.766 (2′-C), 104.782 (3′-C), 71.766 (1a-C), 73.744 (2a-C), 74.763 (3a-C), 26.653 (4a-C), 25.321 (5a-C). The results were similar to those in reference.[11]

Isoimperatorin: ^{1}H-NMR (500 MHz, DMSO) δ ppm: 6.405 (3C-H), 8.085 (4C-H), 7.072 (8C-H), 7.077 (2′C-H), 6.425 (3′C-H), 5.532 (–CH═), 1.762 (CH_3-H), 4.926 (OCH_2-H). Isoimperatorin: ^{13}C-NMR (500 MHz, DMSO) δ ppm: 161.189 (2-C), 112.433 (3-C), 139.496 (4-C), 148.986 (5-C), 114.811 (6-C), 158.113 (7-C), 95.012 (8-C), 152.667 (9–C), 107.413 (10-C), 144.910 (2′-C), 105.087 (3′-C), 69.711 (1a-C), 119.181 (2a-C), 139.733 (3a-C), 18.233 (4a-C), 25.778 (5a-C). The results were similar to those in reference.[9,11–13]

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