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Preparative isolation of imperatorin, oxypeucedanin and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook using multidimensional high-speed counter-current chromatography

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Abstract

Preparative high-speed counter-current chromatography (CCC) was successfully used for isolation and purification of imperatorin, oxypeucedanin and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook using multidimensional counter-current chromatography with a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water at volume ratios of 1:1:1:1 (v/v) and 5:5:4.5:5.5 (v/v), which had been selected by analytical high-speed counter-current chromatography (HSCCC). Using two preparative units of the CCC centrifuge, which are connected by a column switching valve, about a 300 mg amount of the crude extract was separated, yielding 19.9 mg of imperatorin, 8.6 mg of oxypeucedanin and 10.4 mg of isoimperatorin all at a high purity of over 98%.
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Keywords: Multidimensional counter-current chromatography; Isolation and purification; Imperatorin; Oxypeucedanin and isoimperatorin; *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook; bai zhi

1. Introduction

Angelica dahurica (Fisch. ex Hoffm) Benth. et Hook is a useful traditional Chinese herb, its usefulness is not connected to dermatitis: all members of this genus contain furocoumarins, which increase skin sensitivity to sunlight.

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 Acquired Immunodeficiency Syndrome (AIDS) [1].

Five furanocoumarins, byakangelicin, phellopterin, imperatorin, isoimperatorin, and oxypeucedanin, from the roots of *A. 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.

The separation of these active compounds from natural sources, however, may encounter various problems. Counter-current chromatography (CCC), being a support-free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support [3], and has been widely used in preparative separation of natural products [4,5]. Multidimensional counter-current chromatog-

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raphy (MDCCC) has been reported on separation of flavone aglycones by Yang et al. [6]. The present paper describes the successful preparative separation and purification of imperatorin, oxypeucedanin and isoimperatorin from the crude extract of *A. dahurica* by multidimensional counter-current chromatography.

2. Experimental

2.1. Apparatus

The analytical CCC instrument employed in the present study is a Model GS 20 analytical counter-current chromatograph. The apparatus 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 multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm I.D. Polytetrafluoroethylene (PTFE) tube directly onto the holder hub forming multiple coiled layers with a total capacity of 40 ml. The β-value 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 revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 2000 rpm, an optimum speed of 1800 rpm was used in the present studies. A manual sample injection valve with a 1.0 ml loop was used.

An MDCCC system (Fig. 1) was used with two Model GS10A2 multilayer coil planet centrifuges, each equipped with a PTFE multilayer coil of 110 m × 1.6 mm I.D. with a total capacity of 250 ml. The β-value of the preparative column ranges from 0.5 to 0.8. Two model NS-1007 constant-flow pumps were used to elute the mobile phase while continuous monitoring of the effluent was achieved with two model 8823A-UV Monitors at 254 nm. Two manual six-port valves, one with a 20 ml loop used as the injection valve and the other without loop used as the switching valve were used to introduce the sample into the column. Two portable recorders were used to draw the chromatogram.

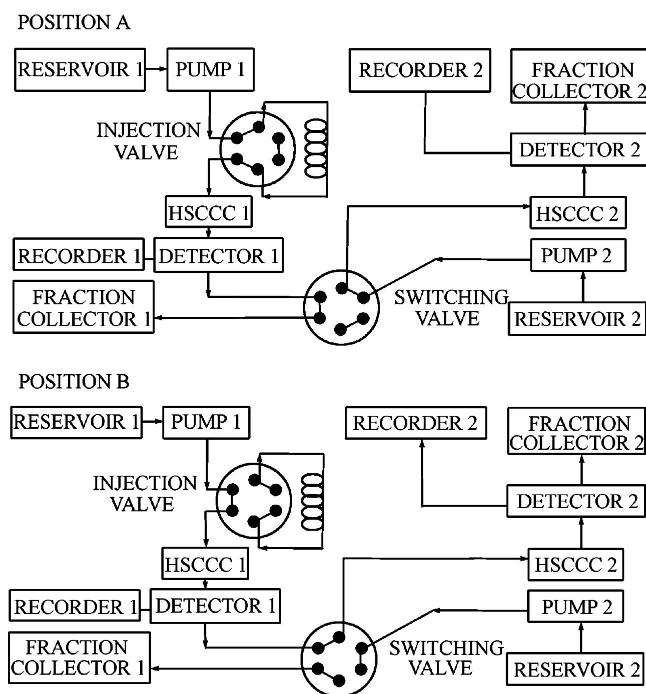

Fig. 1. Schematic diagram of the multidimensional counter-current chromatography (MDCCC) system with two sets of counter-current chromatography (CCC) system, a six port injection valve and a six port switching valve.

The high-performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-20A system including two LC-20A solvent delivery units, an SPD-M20A UV–vis photodiode array detection (DAD), a Model 7725 injection valve with a 20 μl loop, an SCL-20A system controller, and a Class-VP-LC workstation (Shimadzu, Kyoto, Japan). A rotary evaporator was also used.

2.2. Reagents

All organic solvents used for CCC 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).

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

2.3. Preparation of sample

About 500 g of dried *A. dahurica* was ground, and a 100 g amount of this dried powder was extracted (refluxed) 4 h with 500 ml of 95% ethanol solution, and concentrated to dryness under reduced pressure. The dried material was again extracted with 200 ml of ethyl acetate by sonication for 20 min, and concentrated to dryness under reduced pressure yielding 4.5 g of a crude sample, which contained imperatorin, oxypeucedanin and isoimperatorin. The purity of each compound was determined by HPLC (Fig. 2).

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

The solvent system utilized in the present study was prepared by mixing *n*-hexane–ethyl acetate–methanol–water (1:1:1:1 or 5:5:4.8:5.2 or 5:5:4.5:5.5, 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 at suitable concentrations according to the analytical or preparative purpose.

2.5. Separation procedure

Analytical CCC was performed with a Model GS 20 CCC instruments as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then

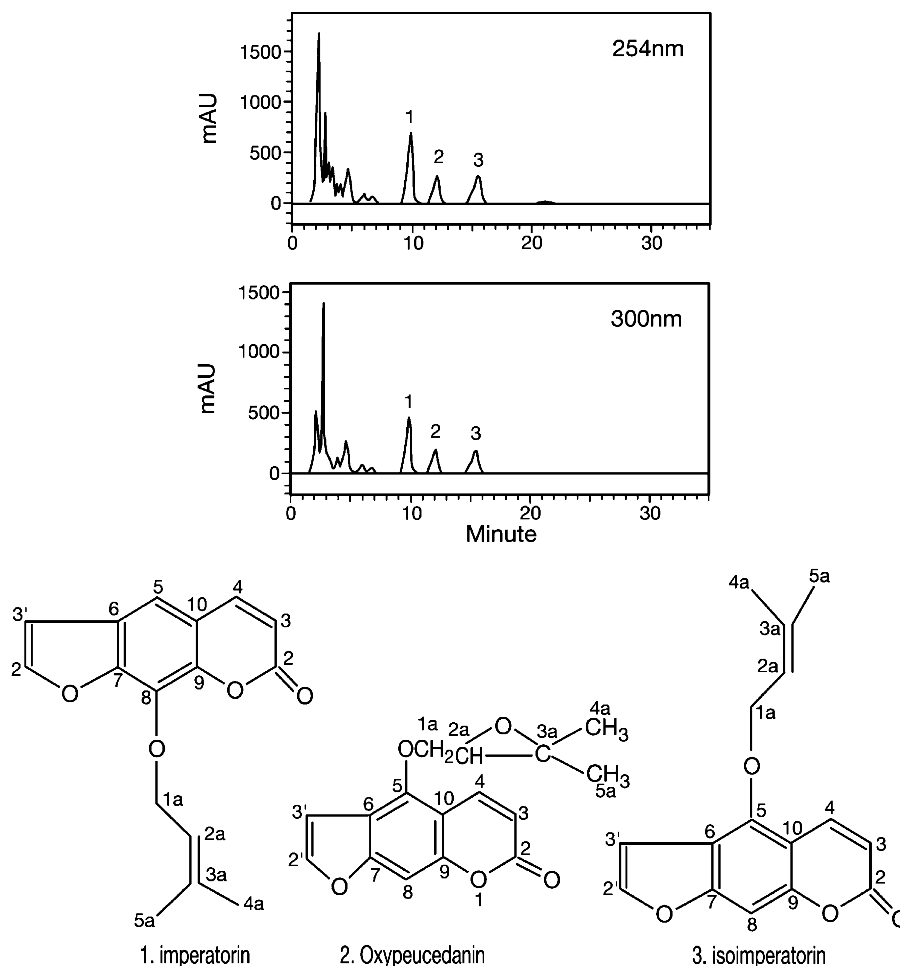

Fig. 2. HPLC analyses of the crude extract from *Angelica dahurica* with the chemical structure of imperatorin, oxypeucedanin and isoimperatorin. HPLC conditions: Polaris ODS column (250 mm × 4.6 mm I.D.) at column temperature of 35 °C. The mobile phase composed of methanol: water (60:40, v/v) was isocratically eluted at a flow-rate of 1.0 ml/min and the effluent monitored at 254 and 300 nm by a DAD detection.

pumped into the head end of the inlet column at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1800 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 *detection* at 254 nm. 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.

MDCCC separation was performed as follows: The switching valve shown in Fig. 1 is initially set in position A, and CCC systems 1 and 2 are simultaneously filled with the upper stationary phase using pumps 1 and 2, respectively (Fig. 1). Both apparatuses are rotated at 800 rpm, while the lower phase is eluted through CCC systems 1 and 2 using their respective pumps at a flow-rate of 2.0 ml/min. After hydrodynamic equilibrium is reached in each column, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (300 mg in 10 ml of lower phase) is injected into CCC 1 through the injection valve while pump 2 is stopped. The effluent from the outlet of CCC 1 was continuously monitored with UV detection 1 at 254 nm, and collected according to the chromatogram. When the target peak appeared, the effluent from CCC 1 was cut and introduced into the CCC 2 column by turning the switching valve to position B. After the target peak was completely introduced from CCC 1 to CCC 2 columns, the switching valve was returned to position A, while restarting pump 2 to resume the elution of the target peak with detection 2 and recorder 2.

2.6. *HPLC analyses and identification of CCC peak fractions*

The crude extract of *A. dahurica* and CCC peak fractions were each analyzed by HPLC. The analyses were performed with a Polaris ODS column (250 mm × 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 and 300 nm by a DAD detection.

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

3. Results and discussion

As shown in Fig. 2, the HPLC analysis of the crude extract of radix Angelicae Dahuricae shows several compounds where the purity of imperatorin, oxypeucedanin and isoimperatorin in crude extract is 30.8, 15.1, and 16.9%, respectively, based on HPLC peak area percentage.

In order to achieve an efficient resolution of target compounds, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water was examined using analytical CCC by varying the mutual volume ratio, since this solvent system has been successfully applied to various samples with a moderate degree of polarity. Although partition coefficient measurement is the important first step for separation by CCC, it may be substituted by analytical CCC, if the additional apparatus is available. While the latter may require a much longer time for analysis, it gives not only partition coefficient of each solute, but also indicates other important parameters such as the retention of the stationary phase and peak resolution, which may vary according to the physical properties of the solvent system. The results are illustrated in Fig. 3a–c.

As seen in Fig. 3a, 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 three and half hours. On the other hand, the solvent ratio (5:5:4.5:5.5) improved the peak resolution between imperatorin and oxypeucedanin as shown in Fig. 3c. As suggested by these results of analytical CCC experiment, when 300 mg of the crude extract of *A. dahurica* was separated by preparative CCC using the above solvent system at a volume ratio of (1:1:1:1), peaks 2 and 3 were only partially resolved as shown in Fig. 4.

These results suggest that the combined use of these two solvent systems would provide satisfactory purification of these three compounds by the multidimensional chromatographic method. This strategy was successfully demonstrated in Figs. 4 and 5 where the crude extract was eluted with the solvent system at a volume ratio of 1:1:1:1 using preparative CCC. After three and half hours when peak 1 (shaded) started to elute, the effluent from CCC 1 was cut and introduced into the CCC 2 column by turning the switching valve to position B (see Fig. 1). After peak 1 was completely introduced from CCC 1 into CCC 2 column (about 30 min), the switching valve was returned to position A and the elution of the cut peak 1 was resumed by pump 2 with the second solvent system at a volume ratio of 5:5:4.5:5.5, while the rest of the components (peaks 2 and 3) still remaining in the CCC 1 column was continuously eluted with the solvent system at a volume ratio of 1:1:1:1 using pump 1.

Here it may be worthwhile to discuss the advantage of multidimensional CCC applied in the present study. In CCC separation when two peaks are overlap, it is a common practice that

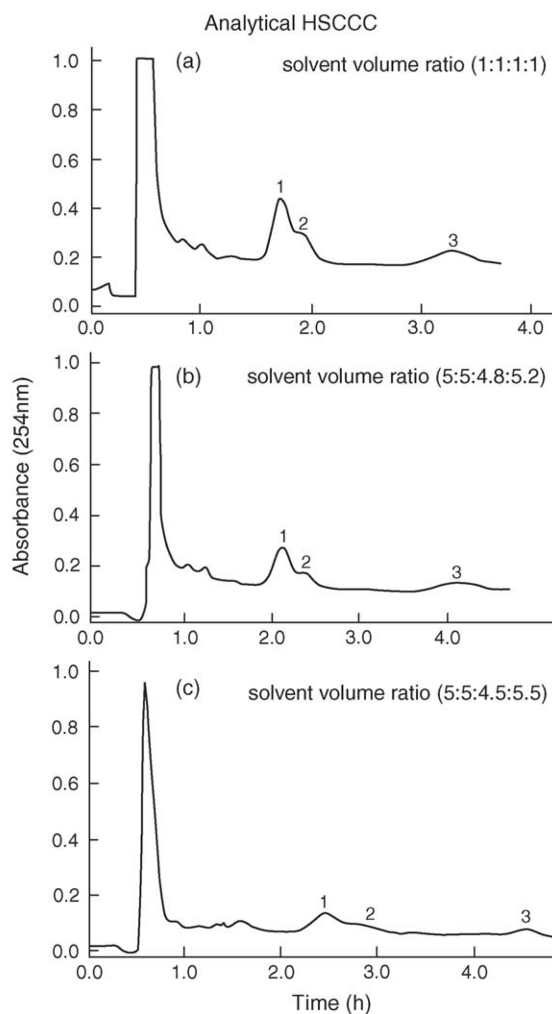

Fig. 3. Chromatogram of the crude extract from *Angelica dahurica* by analytical *CCC*. Solvent system: (a) *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v); (b) *n*-hexane–ethyl acetate–methanol–water (5:5:4.8:5.2, v/v); (c) *n*-hexane–ethyl acetate–methanol–water (5:5:4.5:5.5, v/v); stationary 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 lower phase.

each peak fraction is pooled, dried and rechromatographed with the same or a slightly modified solvent system to improve the yield of pure fraction. This is possible because the yield of a target compound in CCC depends on the amount of the impurities in the fraction, i.e., the smaller the amount of impurity, the higher the yield of target compound. Of course, purification of the partially resolved two compounds in this method each requires another individual run and separation time. The multidimensional CCC described above improves both yield and separation time by introducing the first peak in the second column to separate it in tandem. The cut and introduced portion of the peak into the second column will yield improved peak resolution at 2½ times of the original separation without extra separation time. If the yield of the second peak to be improved, one can cut the first peak deeper into the second peak, the loss of the second peak being recovered in the second column after the first peak is eluted.

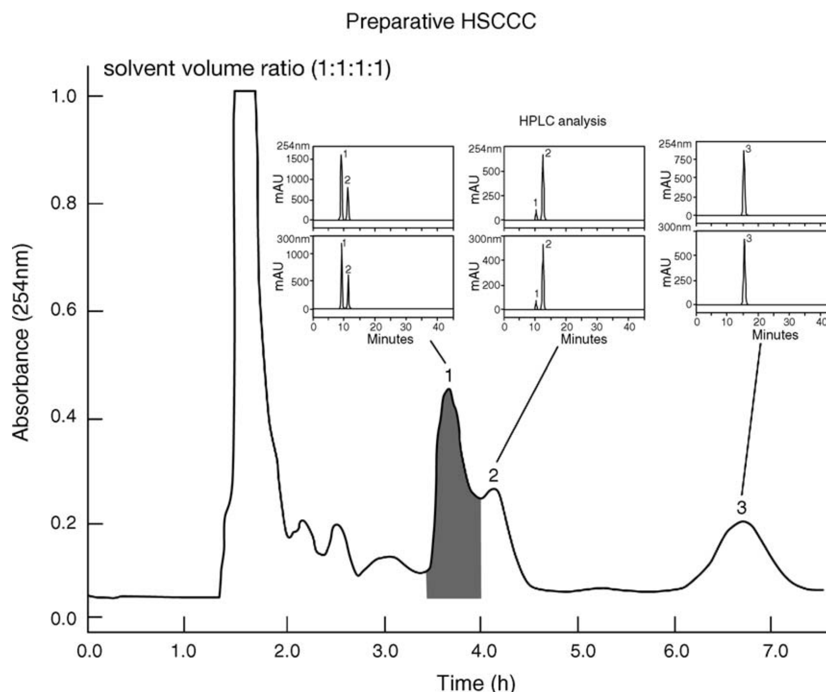

Fig. 4. Chromatogram of the crude extract from *Angelica dahurica* by preparative counter-current chromatography. Solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 300 mg dissolved in 10 ml of lower phase. HPLC conditions: Polaris ODS column (250 mm × 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 and 300 nm by a DAD detection.

Fig. 5A shows the chromatogram obtained from CCC 1 with recorder 1 yielding 8.6 mg of oxypeucedanin and 10.4 mg of isoimperatorin. The chromatogram in Fig. 5 B was obtained by the cut fraction of CCC 1 (the shaded part of the peak in Fig. 4) introduced into and eluted from the CCC 2 column. This separation yielded 19.9 mg of imperatorin at over 98% purity based on HPLC analysis. In CCC-2, the Peak 2 was eluted after 9 h, it appeared as a small peak at nine and quarter hours which is not shown in the figure. The results illustrated in Fig. 5 indicated that a pair of two-phase solvent systems composed of *n*-hexane–ethyl acetate–methanol–water with the volume ratios of 1:1:1:1 and 5:5:4.5:5.5 is suitable for the MDCCC method for purification of imperatorin, oxypeucedanin and isoimperatorin from *A. dahurica*.

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 and 41. It showed 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 and 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 and 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].

The results of our studies demonstrated that multidimensional counter-current chromatography is a useful method for

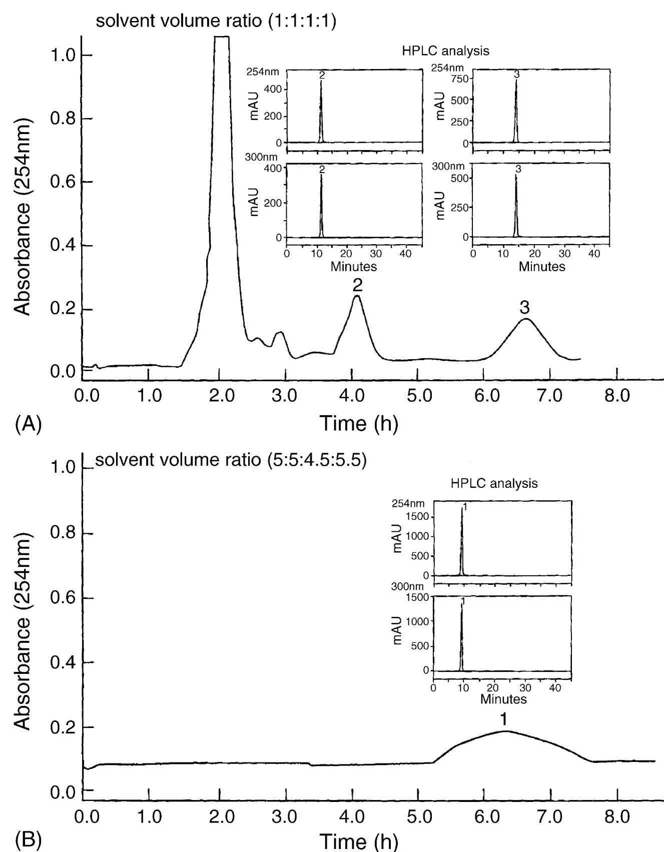

Fig. 5. Chromatogram of the crude extract from *Angelica dahurica* by multidimensional counter-current chromatography (MDCCC). Solvent system: *n*-hexane (1:1:1:1, and 5:5:4.5:5.5 v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 300 mg dissolved in 10 ml of lower phase. (A) Chromatogram obtained by CCC 1 with peak 1 cut out (peak 2 and peak 3 of A). (B) Chromatogram of the cut fraction (the shaded part of the peak in Fig. 4) obtained by CCC 2. HPLC conditions: Polaris ODS column (250 mm × 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 and 300 nm by a DAD detection.

the preparative separation of imperatorin, oxypeucedanin and isoimperatorin from *A. dahurica* (Fisch. ex Hoffm) Benth. et Hook.

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