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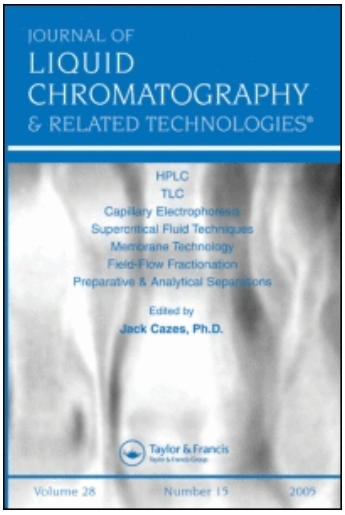

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Purification of Eremophilane-Type Sesquiterpenes from *Ligularia atroviolacea* by High Speed Countercurrent Chromatography

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Abstract: Following an initial cleanup step on the C_{18} open column chromatography, a preparative high speed countercurrent chromatography (HSCCC) method for isolation and purification of 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide, and furanoeremophil-3-en-15,6α-olide from the Chinese medicinal plant *Ligularia atroviolacea* was successfully established by a one-step separation, using *n*-hexane-ethyl acetate-ethanol-water (4:1:4:1, v/v/v/v) as the two phase solvent system. The upper phase was used as the mobile phase in the head to tail elution mode. HPLC analysis of the fractions collected on the preparative HSCCC of 600 mg of crude extracts showed that the purity of 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide (54.7 mg) was 98.1% and that of furanoeremophil-3-en-15,6α-olide (41.8 mg) was 98.4%. The chemical structures of the two eremophilane-type sesquiterpenes were identified by ESI-MS, ^{1}H-NMR, and ^{13}C-NMR analysis. To the best of our knowledge, 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide was obtained from *L. atroviolacea* for the first time, while furanoeremophil-3-en-15,6α-olide was first isolated as a natural product.

Keywords: Preparation, High speed countercurrent chromatography, *Ligularia atroviolacea*, Eremophilane, Sesquiterpene

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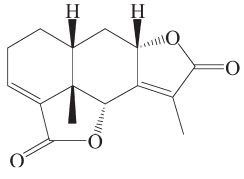
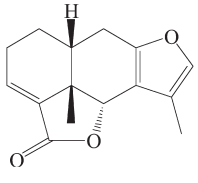
INTRODUCTION

A recent review shows that *ca* 111 of the 130 species of *Ligularia* occur in China.[1] Most of *Ligularia* plants have been used as folk remedies for the treatment of influenza, coughs, ulcers, and tuberculosis since ancient times,[2] and many bioactive eremophilane type sesquiterpenes have been found.[3–6]

Ligularia atroviolacea (Franch.) Hand.-Mazz. (Asteraceae) is a plant indigenous to Yunnan Province of China.[7] We have isolated and separated four new eremophilenolides from it.[8] A deep literature search yielded only one reference to an early report on the study of chemicals from the medicinal herb *L. atroviolacea*,[9] and our pharmacological research indicated that eremophilenolides from *L. atroviolacea* have anti-tumor activity.[10–12] So, further chemical research and discovery from *L. atroviolacea* is warranted for exploitation of new products and pharmacological tests. 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide (Figure 1), two major eremophilane type sesquiterpenes in *L. atroviolacea*, could be isolated by some conventional methods including silica gel, sephadex, and preparative high performance liquid chromatography, which is pretty easy to enlarge the scale of separation. However, these methods are tedious, time consuming, needing multiple chromatographic steps, and requiring large amounts of solvent and resin. Furthermore, these methods would cause adsorbing effects on a stationary phase and artifact formation. Then, setting up an easy system capable of separating the bioactive compounds access to pure and well characterized reference compounds is necessary. High speed countercurrent chromatography (HSCCC), first invented by Ito,[13] is a support free liquid-liquid partition chromatographic technique with no solid support matrix, which could eliminate irreversible



8β-H-eremophil-3,7(11)-dien-12,8α;15,6α-diolide



furanoeremophil-3-en-15,6α-olide

Figure 1. The chemical structures of 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide.

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adsorption of samples onto solid support in conventional column chromatography.[14] This method has been successfully applied to separation and isolation of many natural products.[15–20] However, no report has been published on the use of HSCCC for the isolation and purification of bioactive components from *L. atroviolacea*.

In the present study, an efficient method was developed for the isolation and purification of 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide with high purities from *L. atroviolacea* by HSCCC. The chemical structures of the two target compounds were elucidated by ESI-MS, ^{1}H-NMR, and ^{13}C-NMR. As far as we know, 8β-H-eremophil-3,7(11)-dien-12,8α;15,6α-diolide was obtained from *L. atroviolacea* for the first time, and furanoeremophil-3-en-15,6α-olide was first isolated as a natural product.

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EXPERIMENTAL

Apparatus

A seal free HSCCC instrument was built in the Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China. The apparatus was equipped with a polytetrafluoroethylene preparative layer coil with a 2.6 mm average I.D. and a 20 mL sample loop (total volume, 420 mL). The column revolves around its own axis at the angular velocity in the same direction. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79. $\beta = r/R$, where r is the distance from the coil to the holder shaft. The rotation speed is adjustable from 0 to 1000 rpm. The HSCCC system was equipped with a constant flow pump (Zhejiang Instrument Factory, Hangzhou, China) and a variable wavelength PC300 detector operating at 240 nm and a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China).

The analytical HPLC equipment used throughout this study was a Waters Alliance 2695 separations module equipped with a quaternary pump, a column temperature control module, an automatic sampler, a 2699 photodiode array detector, and *Empower pro* data handling system (Waters Corporation, Milford, MA01757, USA).

Chemicals and Reagents

All organic solvents used for preparation of crude extracts and HSCCC separation were of analytical grade (The Second Institute of Oceanography, Zhejiang, China). Methanol used for HPLC was of chromatographic grade (Merk, Darmstadt, Germany). All aqueous solutions were prepared with

pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Bedford, MA, USA). The reversed phase C_{18} resin was purchased from Merck (Darmatadt, Germany).

The roots of *L. atroviolacea* were collected from Lijiang, Yunnan province in August, 2001, and were identified by Prof. Hua Peng (Kuming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (LSP200108-04) was deposited in the Department of Traditional Chinese Medicine and Natural Drug Research, College of Pharmaceutical Sciences, Zhejiang University.

Preparation of the Crude Extract

The air dried roots of *L. atroviolacea* (3.0 Kg) were powdered and extracted three times with 95% EtOH under reflux for 3 h and concentrated under reduced pressure to give a brown residue (328 g). A portion of this residue (300 g) was suspended in water and then partitioned with light petroleum (60–90ºC), ethyl acetate, and *n*-BuOH, successively, which afford 59 g of ethyl acetate extract after being combined and evaporated to dryness under reduced pressure. Then the ethyl acetate extract was subjected to a reversed phase C_{18} open column chromatograph (50 cm × 4 cm, contained 300 mL C_{18} resin) and eluted with methanol-water step gradients. Methanol (40%) was utilized to elute the column until the elution was nearly colorless; then 60% methanol aqueous solution was subsequently used as the eluent. Portions of the above 60% methanol extract were subjected to HSCCC.

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Selection of the Two Phase Solvent Systems

The solvent system for HSCCC separation was selected according to the partition coefficients (K) of the target components of the crude sample extracted from *L. atroviolacea* in various solvent systems. The K-values were determined by HPLC as follows: a suitable amount of crude sample was added to the mixture of equal volume of the upper phase and the lower phase of the two phase solvent system. The solution was then mixed thoroughly. After the equilibration was established, the upper phase and lower phase were separated and analyzed by HPLC, respectively. The K-values of the target component in the sample were calculated as the peak area of the solute in the upper phase divided by that in the lower phase.

Preparation of Two Phase Solvent System and Sample Elution

In the present study, the two phase solvent system was composed of *n*-hexane-ethyl acetate-ethanol-water at volume ratio of 4:1:4:1 (v/v/v/v). The solvent mixture was thoroughly equilibrated in a separation funnel at room temperature and the two phases were separated shortly before use.

The upper phase and the lower phase were separated and degassed by sonication for 30 min shortly before use.

The sample solution for HSCCC separation was prepared by dissolving 600 mg of the crude extract in 20 mL in the solvent mixture of the lower phase and upper phase (1:1, v/v) of the solvent system for isolation and purification, because the sample was not easily dissolved in either phase alone.

HSCCC Separation Procedure

In the crude sample isolation and separation, the multiplayer coil column was first entirely filled with the upper phase (stationary phase) of the solvent system. Then the lower phase (mobile phase) was pumped into the inlet of the column at the flow rate of 1.5 mL/min, while the apparatus was rotated at 800 rpm. After the mobile phase was eluted from the tail outlet and the two phases had established the hydrodynamic equilibrium throughout the column, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 240 nm and the peak fractions were collected according to the chromatogram. After two peaks were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase.

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HPLC Analysis and Identification of HSCCC Peak Fractions

Samples were conducted on a Symmetry® C_{18} column (150 mm × 3.9 mm i.d., 5 μm) column using a isocratic elution of 0.1% acetic acid and methanol (30:70, v/v) as mobile phase. The analysis was carried out using a flow rate 0.8 mL/min at 30°C. Chromatograms were recorded at 240 nm.

The pooled fraction was concentrated by a rotary evaporator and each fraction was analyzed by analytical HPLC to check the purity prior to characterization. The purities of the collected fractions were determined by HPLC based on the peak area of the target species normalized to the sum of all observed peaks.

The structure identification of HSCCC peak fractions was carried out by ESI-MS, ^{1}H-NMR, and ^{13}C-NMR. ESI-MS data were measured on an Apex III instrument (Bruker Daltonics Corporation, USA). The ^{1}H-NMR and ^{13}C-NMR experiments were performed on a VARIAN INOVA-400 (Varian Corporation, USA) and CD_3OD and $CDCl_3$ was used as solvent for the NMR spectrometer.

RESULTS AND DISCUSSION

HPLC Analysis of the Crude Sample

The enriched extracts obtained from *L. atroviolacea* were analyzed by HPLC and the chromatogram is shown in Figure 2. Two major peaks were separated and detected.

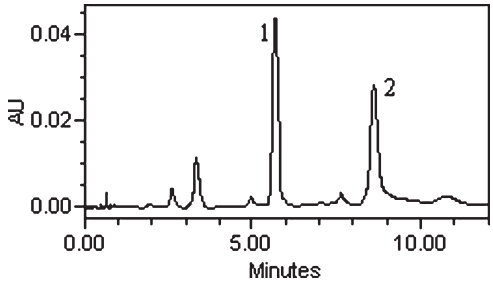

Figure 2. HPLC chromatogram of the enriched extracts from *L. atroviolacea*. HPLC conditions: reversed-phase Symmetry® C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase, 0.1% acetic acid-methanol (30:70, v/v); flow rate, 0.8 mL/min; UV wavelength, 240 nm; column temperature, 30°C. Peaks 1 and 2 correspond to 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide, respectively.

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Selection of the Two Phase Solvent System

The appropriate solvent system plays an important role in separation by HSCCC. Partition coefficient (K) is the most important parameter in solvent system selection, which should be close to 1 to get an efficient separation and a suitable run time. If it is much smaller than 1, the solutes will be eluted close to each other near the solvent front, which may result in loss of peak resolution, however, if the K value is much greater than 1, the solutes will be eluted in excessively broad peaks, and may lead to extended elution time.[21]

In the experiment, the two phase solvent systems composed of *n*-hexane-ethyl acetate-ethanol-water at different volume ratios was evaluated according to K-values and peak resolution. The measured K-values for furanoeremophil-3-en-15,6α-olide and 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide are shown in Table 1. It can be observed, that the K-values of the target compounds increased along with the increasing of the ratio of ethyl acetate.

Table 1. The partition coefficients (K) of the target components in different ratio of volume in *n*-hexane-ethyl acetate-ethanol-water solvent system (component **1**, 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide; component **2**, furanoeremophil-3-en-15,6α-olide)

n-Hexane-ethyl acetate-ethanol-water	Component **1**	Component **2**
7:3:7:3	2.12	4.25
7:3:8:2	1.18	2.67
4:1:4:1	0.61	1.59
9:1:8:2	0.23	0.74

Among the solvent systems, *n*-hexane-ethyl acetate-ethanol-water (4:1:4:1, v/v/v/v) gave suitable partition coefficients for 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide (0.61) and furanoeremophil-3-en-15,6α-olide (1.59). Finally, *n*-hexane-ethyl acetate-ethanol-water (4:1:4:1, v/v/v/v) was used for the HSCCC of the crude extracts from *L. atroviolacea*; good separation results could be obtained and the separation time was acceptable.

Apart from a suitable two phase solvent system, the influence of revolution speed and flow rate of the mobile phase was also investigated. The result showed that when the flow rate was 1.5 mL/min, resolution speed was 800 rpm, retention percentage of the stationary phase could reach 54.2%, and good separation results could be obtained.

Under the optimum conditions, two fractions (I, II) were obtained in one step elution and in less then 6 h (HSCCC chromatogram is shown in Figure 3), which is 54.7 mg of fraction I (collected during 189–220 min) and 41.8 mg of fraction II (collected during 252–280 min).

As shown in Figure 4, the HPLC analysis of each HSCCC fraction revealed that two pure eremophilane type sesquiterpenes could be obtained from the crude extracts in one step. The purities of these two compounds were 98.1% and 98.4%, respectively.

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Identification of the Separated Peaks

The structural identification of peak fractions were all performed with ESI-MS, ^{1}H NMR, and ^{13}C NMR spectra as follows:

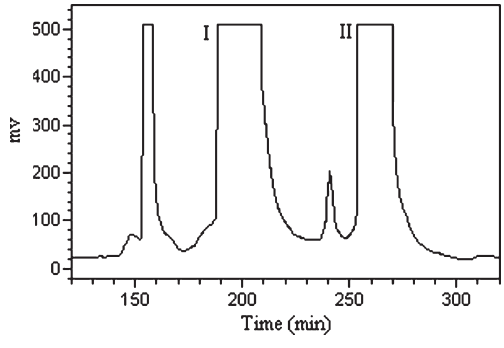

Figure 3. Preparative HSCCC separation of the crude extracts from *L. atroviolacea* after cleaning up by C_{18} resin. Experimental conditions: revolution speed, 800 rpm; solvent system, *n*-hexane-ethyl acetate-ethanol-water (4:1:4:1, v/v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow rate, 1.5 mL/min; retention of the stationary phase, 54.2%; detection wavelength, 240 nm. Fractions I and II correspond to 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide, respectively.

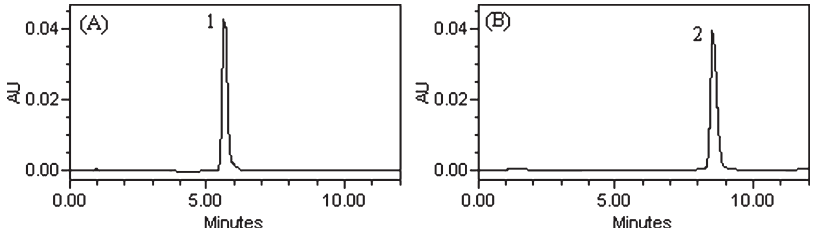

Figure 4. HPLC chromatogram of the fractions obtained by HSCCC. HPLC conditions: reversed-phase Symmetry® C_{18} column (150 mm × 3.9 mm i.d., 5 μm); mobile phase, 0.1% acetic acid-methanol (30:70, v/v); flow rate, 0.8 mL/min; UV wavelength, 240 nm; column temperature, 30°C. (A) fraction "I" obtained by HSCCC; (B) Peaks fraction "II" obtained by HSCCC; peak 1: 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide; peak 2: furanoeremophil-3-en-15,6α-olide, respectively.

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Peak I: ESI-MS m/z: 261 $[M + H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.06 (1H, m, H-9a),1.41 (3H, s, H-14), 1.72 (1H, m, H-1a), 1.98 (3H, d, $J = 2.0$ Hz, H-13), 2.03 (1H, m, H-1b), 2.12 (1H, m, H-10β), 2.16 (1H, m, H-2a), 2.21 (1H, m, H-9b), 2.37 (1H, m, H-2b), 4.70 (1H, dd, $J = 9.2$, 2.8 Hz, H-8β), 5.14 (1H, brs, H-6β), 6.84 (1H, t, $J = 3.2$ Hz, H-3). ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 9.4 (q, C-13), 21.7 (t, C-1), 21.9 (t, C-2), 26.9 (q, C-14), 32.9 (t, C-9), 33.5 (d, C-10), 43.9 (s, C-5), 81.7 (d, C-8), 77.3 (d, C-6), 125.2 (s, C-11), 129.5 (s, C-4), 136.9 (d, C-3), 155.9 (s, C-7), 168.3 (s, C-15), 173.4 (s, C-12). Comparing the data with the literature,[22,23] peak I was identified as 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide.

Peak II: ESI-MS m/z: 261 $[M + H]^+$. ^{1}H-NMR (CD_3OD, 400 MHz): δ 1.45 (3H, s, H-14), 1.79 (1H, m, H-1a), 2.06 (3H, d, $J = 1.2$ Hz, H-13), 2.21 (1H, m, H-1b), 2.34 (1H, m, H-9a), 2.42 (1H, m, H-10β), 2.47 (2H, m, H-2), 2.56 (1H, m, H-9b), 5.37 (1H, br s, H-6β), 6.84 (1H, t, $J = 3.6$ Hz, H-3), 7.15 (1H, s, H-12). ^{13}C-NMR (CD_3OD, 100 MHz): δ 8.1 (q, C-13), 22.6 (t, C-1), 23.3 (t, C-2), 24.4 (t, C-9), 27.6 (q, C-14), 37.2 (d, C-10), 41.6 (s, C-5), 83.4 (d, C-6), 117.1 (s, C-11), 120.9 (s, C-7), 132.8 (s, C-4), 137.8 (d, C-3), 139.8 (d, C-12), 153.6 (s, C-8), 172.1 (s, C-15). Peak II was identified as furanoeremophil-3-en-15,6α-olide according to the literature.[24]

CONCLUSIONS

In combination with a suitable extraction and cleanup procedure prior to HSCCC separation, two eremophilane type sesquiterpenes including 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide and furanoeremophil-3-en-15,6α-olide from *L. atroviolacea* are obtained on a preparative scale. The results obtained in the present study clearly demonstrate that HSCCC is a powerful technique for the isolation of pure compounds

from natural plants. As we know, 8β-H-eremophil-3,7(11)-dien-12,8α; 15,6α-diolide was obtained from *L. atroviolacea* for the first time, while furanoeremophil-3-en-15,6α-olide was first isolated as a natural product.

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