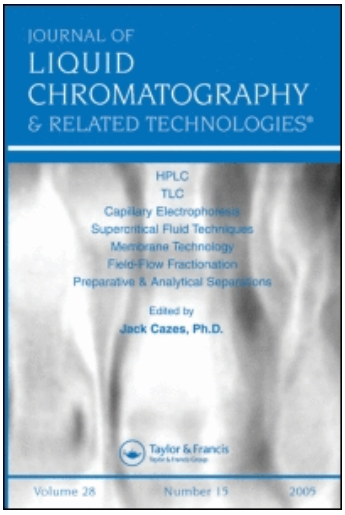

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Preparative Isolation and Purification of Two Coumarins from *Edgeworthia chrysantha* Lindl by High Speed Countercurrent Chromatography

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Abstract: Two bioactive coumarins including 7-hydroxycoumarin (umbelliferone) and 7-hydroxyl-6-methoxy-3, 7′-dicoumarylether (daphnoretin) were separated from the ethyl acetate extract of the stems of *Edgeworthia chrysantha* Lindl (*E. papyrifera*) by high speed countercurrent chromatography (HSCCC). The two-phase solvent system used was composed of *n*-hexane-ethyl acetate–methanol–water at an optimized volume ratio of 4 : 6 : 4 : 6 (v/v/v/v). Preparative HSCCC yielded, from 317 mg of the crude extract, 21 mg umbelliferone and 41 mg of daphnoretin each, at over 95% purity by high performance liquid chromatography (HPLC) analysis. Their structures were identified by EI-MS and ^{1}H NMR.

Keywords: Countercurrent chromatography, Preparative chromatography, *Edgeworthia chrysantha* Lindl, Plant materials, Pharmaceutical analysis, Umbelliferone, Daphnoretin

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INTRODUCTION

Edgeworthia chrysantha Lindl *(E. papyrifera S. et Z., Thymelaeaceae)* is a traditional Chinese medicine used for treating ocular malfunction in humans. In our past studies,[1] the bioactive compound syringin and the coumarin glycoside edgeworoside C were successfully separated from the partially purified fraction of *Edgeworthia chrysantha* Lindl by HSCCC with high purity. The present paper reports our following studies on the one step separation of the two bioactive coumarins (see Fig. 1.), namely umbelliferone and daphnoretin, from the acetate ethyl extract of the stems of *Edgeworthia chrysantha* Lindl by preparative HSCCC. Though umbelliferone had been reported to be isolated by analytical HSCCC with the solvent system chloroform-methanol-water,[2] chloroform is a very toxic solvent and it is harmful to our environment. The friendly solvent system *n*-hexane-ethyl acetate–methanol–water was employed during our separation of the two coumarins.

Biological effects of coumarins observed include antibacterial, antithrombotic and vasodilatory, antimutagenic, lipoxygenase and cycloxygenase inhibition, scavenging of reactive oxygen species, and antitumourigenic effects, including malignant melanoma.[3–5] A recent study has shown that umbelliferone inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. This knowledge may lead to its use in cancer therapy.[6]Daphnoretin has showed strong suppressive effects on the expression of the hepatitis B surface antigen in human hepatoma Hep3B cells,[7] antineoplastic against Ehrlich ascitic carcinoma,[8,9] antitumor activity,[10] and inhibition of oncogene product enzyme activity, as an approach to cancer chemoprevention.[11]

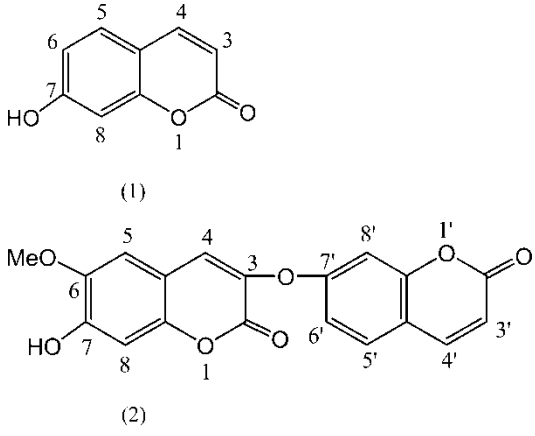

Figure 1. Chemical structures of umbelliferone (1) and daphnoretin (2).

EXPERIMENTAL

Apparatus

A Model TBE-300A high speed countercurrent chromatography (Shanghai Tauto Biotechnique, Shanghai, China) equipped with three preparative multilayer coils (270 mL, wound with 1.6 mm I.D. PTFE tubing) was used. The β values of this column range from 0.46 to 0.73 ($\beta = r/R$, $R = 6.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1000 rpm. The columns of the HSCCC were installed in a vessel that was maintained at 25°C by a Model HX-1050 constant temperature controller (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant flow pump (Beijing Shengyitong Technique Co. Ltd., Beijing, China). Continuous monitoring of the effluent was achieved with a Model UV-II Detector Monitor (Shanghai Institute of Biochemistry of Academy of Science, Shanghai, China) at 254 nm. A manual sample injection valve with a 20 mL loop (Shanghai Tauto Biotechnique, Shanghai, China) was used to introduce the sample into the column. Sepu3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-100 fraction collector (Shanghai Huxi Tech, Shanghai, China), 6 mL for each fraction.

The high performance liquid chromatograph (HPLC) used was a CLASS-VP Ver. 6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver. 6.1 workstation.

Reagents and Materials

All organic solvents used for HSCCC were of analytical grade and were purchased from Hangzhou HuiPu Chemical Factory, Hangzhou, China. Acetonitrile used for HPLC analysis was of chromatographic grade. Raw stems of *Edgeworthia chrysantha* Lindl were cultivated and collected from the botanical garden of the university.

Extraction of Crude Samples

Air dried stems and barks of *Edgeworthia chrysantha* Lindl (0.5 kg) were chopped into small pieces and extracted with ethyl acetate (1 l × 3) under

reflux. The combined ethyl acetate extracts were concentrated under vacuum. The residue (5.6 g) obtained from the combined extract was directly subjected to HSCCC.

Preparation of Two-phase Solvent System and Sample Solutions

For the present study, we selected a two-phase solvent system composed of *n*-hexane-ethyl acetate–methanol–water (4 : 6 : 4 : 6, v/v/v/v), which was selected by a partition experiment of the crude extract in a series of solvent systems composed of *n*-hexane-ethyl acetate–methanol–water at different volume ratios (see Table 1). The experiment of selecting the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values of the target compounds was performed as in the literature.[12] The solvent mixture was thoroughly equilibrated in a separation funnel at the same temperature as in the vessel of HSCCC, and the two phases separated shortly before use.

The sample solution was prepared by dissolving the acetate ethyl extract in the mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system used for HSCCC separation.

Separation Procedure

HSCCC was performed as follows. The multilayer coiled column was first entirely filled with the upper phase as a stationary phase. The lower aqueous mobile 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 reached, as indicated

Table 1. The K (partition coefficient) values of umbelliferone (K_1) and daphnoretin (K_2) in different solvent systems

Solvent system	K_1	K_2
Ethyl acetate-water (1 : 1)	—	—
Ethyl acetate-ethanol-water (50 : 1 : 50)	—	—
Ethyl acetate-ethanol-water (10 : 1 : 10)	792.69	—
n-Hexane-ethyl acetate–water (1 : 5 : 6)	—	—
n-Hexane-ethyl acetate–water (5 : 5 : 10)	95.61	153.25
n-Hexane-ethyl acetate–methanol–water (5 : 5 : 5 : 5)	0.23	0.27
n-Hexane-ethyl acetate–methanol–water (4 : 5 : 4 : 5)	0.32	0.41
n-Hexane-ethyl acetate–methanol–water (2 : 5 : 2 : 5)	0.98	1.31
n-Hexane-ethyl acetate–methanol–water (4 : 6 : 4 : 6)	0.64	0.90

Note: "—" shows that the K value is very large.

by a clear mobile phase eluting at the tail outlet, the sample solution [317 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system] 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 automatically 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 which were forced out of the column with pressurized air.

HPLC Analysis and Identification of HSCCC Peak Fractions

The ethyl acetate extract from the stems of *Edgeworthia chrysantha* Lindl, and each peak fraction from HSCCC, were analyzed by HPLC. The analyses were performed with a Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm I.D.). The mobile phase composed of acetonitrile–water was eluted with linear gradient elution (acetonitrile: 0–10 min, 35%; 10–30 min, 35–65%, 30–60 min, 65%). The flow rate was 0.6 mL/min and the effluent was monitored by a Shimadzu SPD10Avp UV detector at 254 nm.

Identification of HSCCC peak fractions was carried out by EI-MS (electron impact mass spectrum) and ^{1}H NMR spectra. NMR spectra were recorded on a Bruker Advance 400 MHz spectrometer with TMS (tetramethylsilane) as internal standard. EI-MS was obtained on a HP5989B mass spectrometer.

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

Various kinds of elution systems with different volume ratios were tried in HPLC separation of the crude extract, including isocratic elution of methanol-water (30 : 70, 40 : 60, 50 : 50, 60 : 40, 80 : 20, v/v), acetonitrile-water (15 : 85, 20 : 80, 50 : 50, v/v), methanol-acetonitrile-water (5 : 20 : 75, v/v), methanol-acetonitrile-0.025 mol/L phosphoric acid (30 : 20 : 48, v/v), and gradient elution of the above elution systems. Finally, when acetonitrile-water was used as the mobile phase in gradient mode (acetonitrile: 0–10 min, 35%; 10–30 min, 35–65%, 30–60 min, 65%), a good separation could be achieved within 30 min.

The ethyl acetate extract *Edgeworthia chrysantha* Lindl and peak fractions obtained from HSCCC were analyzed by HPLC under the optimum analytical conditions (see Fig. 3A.). The result indicates that the chemical constituents of the crude extract is very complex, in which umbelliferone represented 6.89% of the total and daphnoretin about 18.87% of the total.

Selection of the HSCCC Solvent Systems

Successful separation in using HSCCC depends upon the selection of a suitable two-phase solvent system in which the partition coefficient (K) values of the target compound should fall within a suitable range (i.e., usually between 0.5 and 1).[12] A series of experiments were performed to determine the optimal solvent two-phase system for the HSCCC separation. The following systems at different volume ratios were tested and the K values of the two target compounds are listed in Table 1: ethyl acetate-water (1 : 1); ethyl acetate–ethanol–water (50 : 1 : 50) and (10 : 1 : 10); *n*-hexane-ethyl acetate–water (1 : 5 : 6) and (5 : 5 : 10); *n*-hexane-ethyl acetate–methanol–water (5 : 5 : 5 : 5), (4 : 5 : 4 : 5), (2 : 5 : 2 : 5) and (4 : 6 : 4 : 6). The K values of the three solvent systems including ethyl acetate-water, ethyl acetate–ethanol–water, and *n*-hexane-ethyl acetate–water are too large, and HSCCC is unsuitable for separating the target compounds. When the methanol was added to the solvent systems, *n*-hexane-ethyl acetate–water, the K values of both compounds decreased sharply when the volume ratio was (5 : 5 : 5 : 5). So, it is feasible to obtain the satisfying K values if volume ratio for methanol was modulated to an appropriate range. Finally, the solvent system *n*-hexane-ethyl acetate–methanol–water (4 : 6 : 4 : 6, v/v/v/v) gave the best K values of both compounds, which resulted in good separation of the target compounds by HSCCC.

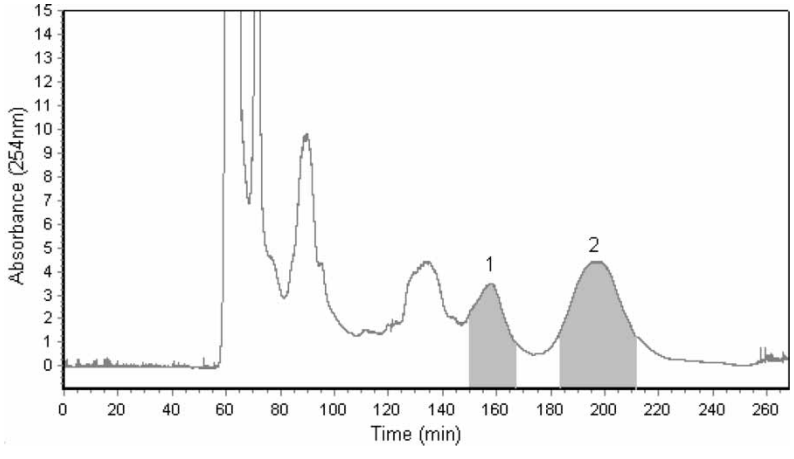

Figure 2. Chromatogram of the crude extract from the stems of *Edgeworthia chrysantha* Lindl by preparative HSCCC. Peak 1: umbelliferone; Peak 2: daphnoretin. Solvent system: *n*-hexane-ethyl acetate–methanol–water (4 : 6 : 4 : 6, v/v/v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; sample: 317 mg dissolved in 10 mL mixture solution of lower phase and upper phase (1 : 1, v/v) of the solvent system; retention of the stationary phase: 46.3%.

Separation of Umbelliferone and Daphnoretin by HSCCC

A 317 mg quantity of the ethyl acetate extract was separated by HSCCC. The retention of the stationary phase was 46.3%, and the separation time was 220 min for a separation run. Figure 2 shows the result obtained from 317 mg of the acetate ethyl extract of *Edgeworthia chrysantha* Lindl by preparative HSCCC. After this separation, the fractions containing umbelliferone and daphnoretin were collected, respectively. The analysis of these fractions indicated that the Peak 1 fraction contained umbelliferone, which weighed 21 mg, at over 95% purity, and the Peak 2 fraction

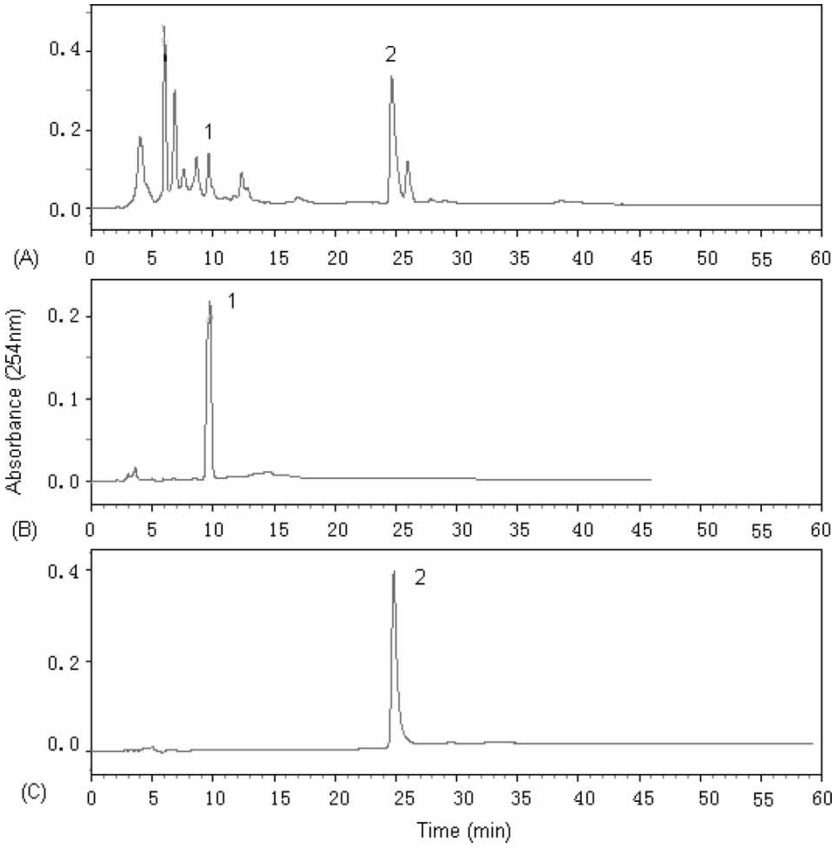

Figure 3. Results of HPLC analyses of the crude extract from the stems of *Edgeworthia chrysantha* lindl and its HSCCC fractions. Column: Shim-Pack CLC-ODS C_{18} column (250 mm × 6 mm I.D.); mobile phase: acetonitrile–water in linear gradient elution (acetonitrile: 0–10 min, 35%; 10–30 min, 35–65%, 30–60 min, 65%); flow rate: 0.6 mL/min; UV detector: 254 nm (A) The original sample; (B) HSCCC fraction from Peak 1 (Fig. 2); (C) HSCCC fraction from Peak 2 (Fig. 2).

contained daphnoretin, which weighed 41 mg, at over 98% purity, as determined by HPLC (Fig. 3B and C).

Structural Identification

The structural identification of the two components was carried out by EI-MS and ^{1}H NMR spectra.

Peak 1: EI-MS m/z: 162, 84, 66, 46. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 7.93 (1H, d, $J = 9.44$ Hz, H-4), 6.20 (1H, d, $J = 9.44$ Hz, H-3), 7.52 (1H, d, $J = 8.52$ Hz, H-5), 6.78 (1H, dd, $J = 8.44$, 2.30 Hz, H-6), 6.71 (1H, d, $J = 2.30$ Hz, H-8), 10.56 (1H, s, OH-7). After comparing the data with spectral information from literature,[13] the first component was confirmed as umbelliferone.

Peak 2: EI-MS m/z: 352, 179, 89, 63. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.05 (1H, d, $J = 9.56$ Hz, H-4′), 6.38 (1H, d, $J = 9.48$ Hz, H-3′), 7.88 (1H, s, H-4), 7.71 (1H, d, $J = 8.60$ Hz, H-5′), 7.12 (1H, dd, $J = 8.58$, 2.35 Hz, H-6′), 7.22 (1H, s, H-5), 7.19 (1H, d, $J = 2.16$ Hz, H-8′), 6.87 (1H, s, H-8), 10.31 (1H, s, OH-7), 3.82 (3H, s, OMe-6). Compared with the reported data, the spectra data of the second component was in agreement with those of daphnoretin.[14]

The result of our studies described above clearly demonstrated that HSCCC is very successful in the preparative separation of umbelliferone and daphnoretin from ethyl acetate extract of *Edgeworthia chrysantha* Lindl.

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