Separation and Purification of Rutin and Acaciin from the Chinese Medicinal Herb *Herba Cirsii* by Combination of Macroporous Absorption Resin and High-Speed Counter-Current Chromatography

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of rutin and acaciin from the Chinese medicinal herb *Herba Cirsii* was successfully established. The crude extracts obtained from *Herba Cirsii* by water under reflux were subjected to a macroporous resin column and eluted with 10% and 60% ethanol, respectively. The fraction of 60% ethanol was used as the sample for HSCCC separation of rutin and acaciin. The two-phase solvent system used for the separation was ethyl acetate–*n*-butanol–water (5:1.5:5, v/v) and the upper phase was used as the stationary phase. Rutin (25.2 mg) and acaciin (21.8 mg) with a purity of 99.2% and 99.6%, respectively, were purified successfully from 60 mg of sample. The chemical structures of rutin and acaciin were identified by ^{1}H-NMR and ^{13}C-NMR.

Introduction

Herba Cirsii, the dry aerial parts of *Cirsium setosum* (Willd.) MB., which is a kind of plant in the family Compositate, is one of the most popular traditional medicinal herbs in China and is officially listed in the Chinese Pharmacopoeia (1). It is used to stanch blood, for detumescence, and to treat acute and chronic inflammation. The water extraction of *Herba Cirsii* can cure infectivity icterus and chronic hepatitis. The flower of *Cirsium setosum* (Willd.) MB. has a good effect on dormancy and is a kind of hypnogenetic Chinese herb with curative effects. Pharmacological tests revealed that extraction of *Herba Cirsii* has the functions of accelerating blood solidification, strengthening heart constriction, restrain tubercle bacillus, and reducing the cholesterol level (2). Rutin and acaciin are the main active ingredients in *Herba Cirsii*. Therefore, the separation and purification of rutin and acaciin from *Herba Cirsii* is of great interest. The chemical structures of rutin and acaciin are shown in Figure 1.

High-speed counter-current chromatography (HSCCC), invented by Y. Ito (3), is a kind of support-free all-liquid partition chromatography. It eliminates irreversible absorptive loss of samples onto the solid support matrix used in conventional chromatography. The technique allows complete recovery of the sample. Thus, it is suitable for separation and purification of active compounds from traditional Chinese medicinal herbs and other natural products (4–11). Analytical separation of rutin and other compounds from extracts of commercial tea leaves by HSCCC has been published previously by A. Yanagida et al. (12). But up to now, there has been no report about the preparative separation and purification of rutin and acaciin from *Herba Cirsii* by HSCCC.

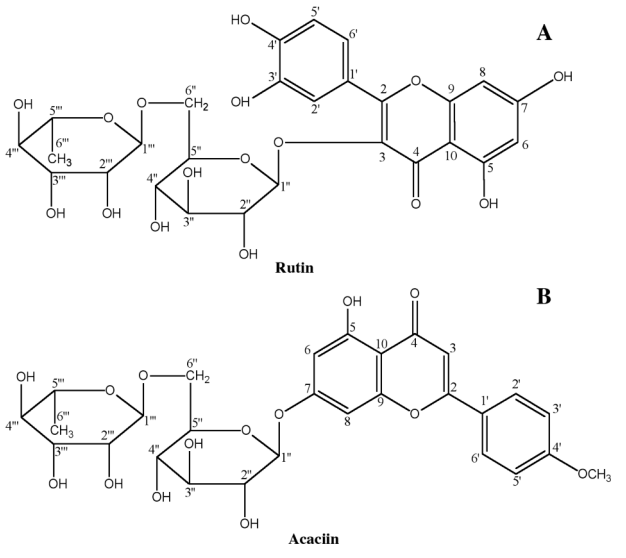

Figure 1. Chemical structures of rutin (A) and acaciin (B).

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In the present study, an HSCCC method for preparative purification of rutin and acaciin from *Herba Cirsii* was established by using ethyl acetate–*n*-butanol–water (5:1.5:5, v/v) as the two-phase solvent system. The crude extracts obtained from *Herba Cirsii* by water under reflux were subjected to a macroporous resin column and eluted with 10% and 60% ethanol, respectively. The fraction of 60% ethanol was used as the sample for HSCCC separation of rutin and acaciin. The present work establishes the first successful HSCCC method for the separation and purification of rutin and acaciin from *Herba Cirsii*.

Experimental

Reagents and materials

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water.

Herba Cirsii was purchased from a local drugstore (Limin-drugstore) and identified by professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

Apparatus

The HSCCC instrument employed in the present study is a TBE-300A high-speed counter-current chromatograph (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 mL) and a 20-mL sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (*R*) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal (β = *r*/*R*, where *r* is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. An ÄKTA prime system (GE Healthcare Bio-Sciences) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

The HPLC equipment used was Agilent 1100 HPLC system including G1311A QuatPump, G1315B UV-vis photodiode array detector, Rheodyne 7725i injection valve with a 20 µL loop, G1332 degasser, and Agilent HPLC workstation (Agilent Technologies, Waldbronn, Germany).

The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian, Palo Alto, CA).

Preparation of crude sample

Herba Cirsii herbs (300 g) were dried constant at 50°C, cut into small pieces, and extracted with 2500 mL of water for 30 min under reflux. The extraction procedure was repeated, and the extracts were combined together. After filtration with a ceramic filter, the filtrate was subjected to a glass column (5 × 80 cm) packed with macroporous resin (D-101, 400 g) and washed with 5000 mL of 10% ethanol and 5000 mL of 60% methanol, respectively. The eluent of 60% ethanol was concentrated in vacuum using a rotary evaporator to give dried powder. The dried powder was stored in a refrigerator (–4°C) for the subsequent HSCCC separation.

Preparation of two-phase solvent system and sample solution

Ethyl acetate–*n*-butanol–water (5:1.5:5, v/v) was used as the two-phase solvent system for the purification of rutin and acaciin. It was prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrating by shaking repeatedly. Then the upper phase and the lower phase were separated and degassed by sonication for 30 min prior to use.

The sample solution for HSCCC separation was prepared by dissolving 60 mg of crude sample in 5 mL of the upper phase of ethyl acetate–*n*-butanol–water (5:1.5:5, v/v).

HSCCC separation

The HSCCC separation procedure was carried out as follows: the upper phase (stationary phase) and the lower phase (mobile phase) of ethyl acetate–*n*-butanol–water (5:1.5:5, v/v) were pumped into the separation column simultaneously using an ÄKTA prime system, according to the volume ratio of 50:50. After the column was entirely filled with the two phase solvent, only the lower phase was pumped into the column at the flow rate of 2.0 mL/min, and at the same time, the HSCCC apparatus was run at the revolution speed of 800 rmp. The separation temperature was controlled at 20°C. After hydrodynamic equilibrium was reached (~ 30 min), the sample solution was injected into the separation column. The effluent from the outlet of the column was continuously monitored at 254 nm. The chromatogram was recorded 50 min after sample injection. Different fractions were collected according to the obtained chromatogram and were evaporated to dryness under reduced pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

HPLC analysis and identification of HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. Analysis was accomplished with a YWG C18 column (200 × 4.6 mm i.d., 10 µm) at room temperature. Methanol–water was used as the mobile phase in gradient elution mode as follows: 0–10 min, 10% methanol to 40% methanol; 10–20 min, 40% methanol to 60% methanol; after 20 min, 60% methanol. The flow-rate of the mobile phase was 1.0 mL/min. The effluents were monitored at 254 nm by photodiode array detector.

Identification of HSCCC peak fractions was performed by ^{1}H-NMR and ^{13}C-NMR, and were recorded on a Mercury Plus 400 NMR.

Results and Discussions

Optimization of HPLC method

In the present work, all samples were analyzed by HPLC. The purities of the target compounds separated by HSCCC were also determined by HPLC. Thus, an HPLC method for analysis of crude sample was established at first. In order to select an appropriate elution system for the HPLC separation of the crude sample, different kinds of solvents were employed. When methanol–water was used as the mobile phase in gradient mode as follows: 0–10 min, 10% methanol to 40% methanol; 10–20 min, 40% methanol to 60% methanol; after 20 min, 60% methanol; major peaks could be obtained, and rutin and acaciin could be separated well with other components. The chromatograms for analysis of the sample are shown in Figure 2.

Optimization of macroporous absorption resin column separation

The crude sample extracted from *Herba Cirsii* contained too many impurities. This made the HSCCC separation of rutin and acaciin impossible. Therefore, macroporous absorption resin was employed for pretreatment of the crude sample. After filtration with the ceramic filter, the water extraction was subjected to a D-101 macroporous resin column and washed with a different concentration of ethanol. When the sample was first eluted with 10% ethanol first and then with 60% methanol, most impurities present in the crude sample could be removed and rutin and acaciin were mainly present in the eluent of 60% ethanol. The HPLC chromatogram of the eluent of 60% ethanol is shown in Figure 2B. Thus, the sample obtained from the eluent of 60% ethanol was used for subsequent HSCCC separation.

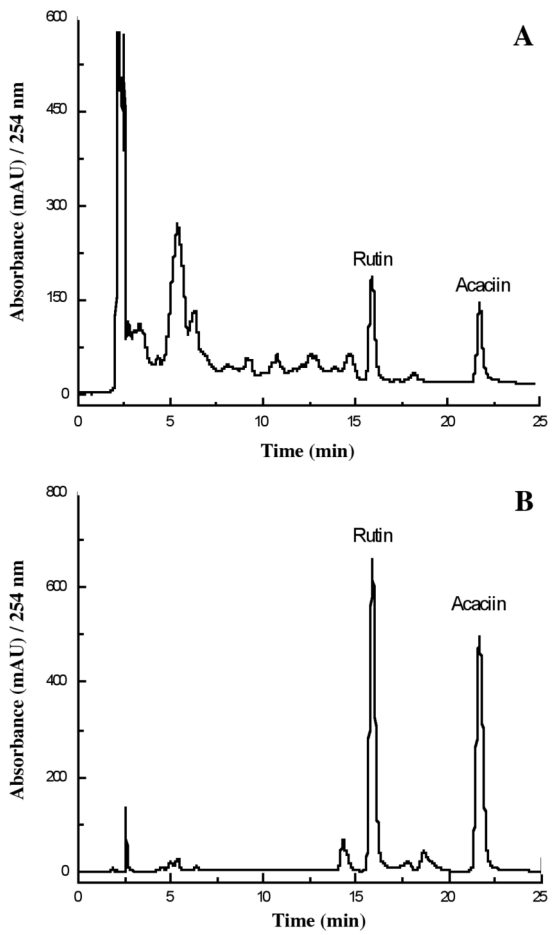

Figure 2. HPLC chromatograms of sample from *Herba Cirsii*. Column: YWG C18 column (200 mm × 4.6 mm i.d., 10 µm); mobile phase: methanol–water in gradient as follows: 0–20 min, methanol–water 10:90–55:45; flow rate: 1.0 mL/min; detection wavelength: 254 nm. Crude sample (A); crude sample separated by macroporous absorption resin (B).

Optimization of two-phase solvent system of HSCCC

Several two-phase solvent systems such as ethyl acetate–water, ethyl acetate–methanol–water, *n*-butanol–water, *n*-butanol–acetic acid water, and ethyl acetate–*n*-butanol–water were tested for the separation. When ethyl acetate–water and ethyl acetate–methanol–water were used as the two-phase solvent system, rutin and acaciin were mainly present in the aqueous phase and they could not be separated. When a more polar solvent system, such as *n*-butanol–acetic acid–water and *n*-butanol–water, was used as the two-phase solvent system, rutin and acaciin were mainly present in the organic phase, and the separations were not as agreeable. Thus, the solvent system of ethyl acetate–*n*-butanol–water was tested, and good separation results were obtained when ethyl acetate–*n*-butanol–water (5:1.5:5, v/v) was employed as the two-phase solvent system of HSCCC separation.

The crude extracts obtained from *Herba Cirsii* by water under reflux were subjected to a macroporous resin column and eluted with 10% and 60% ethanol, respectively. The sample obtained from the fraction of 60% ethanol was then separated and purified by HSCCC under the optimized HSCCC conditions. The HSCCC chromatogram is shown in Figure 3. Rutin (25.2 mg) and acaciin (21.8 mg) at purities of 99.2% and 99.6%, respectively, were determined by HPLC and were obtained from 60 mg of sample.

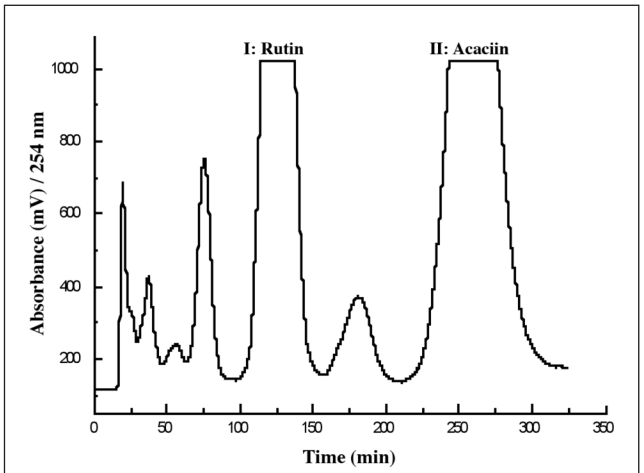

Figure 3. HSCCC chromatogram of sample from *Herba Cirsii*. Two-phase solvent system: ethyl acetate–*n*-butanol–water (5:1.5:5, v/v); mobile phase: the upper phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm; detection wavelength: 254 nm; sample: obtained from the fraction of 60% ethanol of macroporous resin separation; sample size: 60 mg of sample dissolved in 5 mL of the upper phase; separation temperature: 20°C; retention percentage of the stationary phase: 46%.

The structural identification

The chemical structures of each peak fraction of HSCCC were identified according to 1H-NMR and 13C-NMR spectra.

Peak I: ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.55 (^{1}H, d, H-6'), 7.54 (^{1}H, dd, H-2'), 6.82 (1H, d, H-5'), 6.36 (1H, d, H-8), 6.17 (^{1}H, d, H-6), 5.32 (^{1}H, d, H-1''), 5.02 (^{1}H, d, H-1'''), 1.01 (^{3}H, d, H-6'''). ^{13}C-NMR (400 MHz, DMSO-d_6): δ 156.4 (C-2), 133.3 (C-3), 177.4 (C-4), 161.2 (C-5), 98.7 (C-6), 164.1 (C-7), 93.6 (C-8), 156.6 (C-9), 104.0 (C-10), 121.2 (C-1'), 115.2 (C-2'), 144.8 (C-3'), 148.4 (C-4'), 116.3 (C-5'), 121.6 (C-6'), 101.2 (C-1''), 74.1 (C-2''), 75.9 (C-3''), 70.1 (C-4''), 76.5 (C-5''), 67.0 (C-6''), 100.7 (C-1'''), 70.4 (C-2'''), 70.5 (C-3'''), 71.9 (C-4'''), 68.2 (C-5'''), 17.7 (C-6'''). Comparing the data with the literature (13), peak I was identified as rutin.

Peak II : ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.92 (^{1}H, s, 5-OH), 8.04 (^{2}H, d, H-2', 6'), 7.13 (^{2}H, d, H-3', 5'), 6.94 (^{1}H, s, H-3), 6.78 (^{1}H, d, H-8), 6.43 (^{1}H, d, H-6), 5.47 (^{1}H, s, H-1''), 5.07 (^{1}H, d, H-1'''), 3.84 (^{3}H, s, –OCH_3), 1.03 (^{3}H, d, H-6'''). ^{13}C-NMR (400 MHz, DMSO-d_6): δ 164.4 (C-2), 104.3 (C-3), 182.5 (C-4), 161.6 (C-5), 100.4 (C-6), 161.4 (C-7), 95.3 (C-8), 158.4 (C-9), 105.9 (C-10), 123.2 (C-1'), 128.9 (C-2', 6'), 115.2 (C-3', 5'), 162.9 (C-4'), 101.0 (C-1''), 73.5 (C-2''), 76.7 (C-3''), 71.2 (C-4''), 76.1 (C-5''), 66.6 (C-6''), 101.1 (C-1'''), 70.1 (C-2'''), 70.8 (C-3'''), 72.5 (C-4'''), 68. 8 (C-5'''), 18.2 (C-6'''), 56.1 (–OCH_3). Comparing the data with the literature (14), peak II was identified as acaciin.

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Manuscript received October 24, 2007;
revisioin received February 20, 2008.