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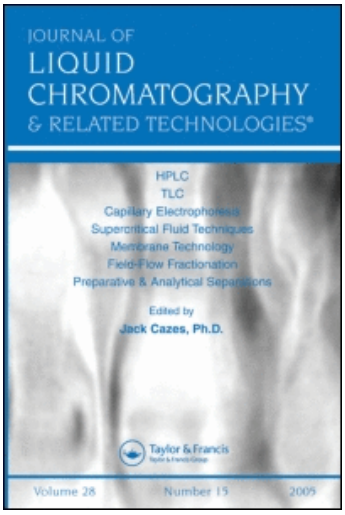

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Isolation and Purification of Three Flavonoids from the Hawthorn Leaves by High Speed Countercurrent Chromatography, Combined with Isocratic Preparative Reversed-Phase High Performance Liquid Chromatography

Honglei Xu*[a]; Tingting Zhou*[a]; Jun Wen[a]; Guorong Fan[a]; Yutian Wu[a]
[a] Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, Shanghai, P.R. China

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Isolation and Purification of Three Flavonoids from the Hawthorn Leaves by High Speed Countercurrent Chromatography, Combined with Isocratic Preparative Reversed-Phase High Performance Liquid Chromatography

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Shanghai Key Laboratory for Pharmaceutical Metabolite Research, School of Pharmacy, Second Military Medical University, Shanghai, P.R. China

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Abstract: Three flavonoids including 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin, and 4″-O-glucosylvitexin were isolated and purified from the hawthorn leaves by high speed countercurrent chromatography (HSCCC), combined together with isocratic preparative reversed-phase high performance liquid chromatography. Analytical HSCCC was used for the preliminary selection of a suitable solvent system composed of *n*-butanol-water (1:1, v/v). After two useful fractions were obtained by HSCCC using the optimal solvent system, one of them, fraction II, was further purified by reversed-phase semipreparative high performance liquid chromatography. From 500 mg partially purified extract of the hawthorn leaves, 188.4 mg 2″-O-rhamnosylvitexin, 28.5 mg 4‴-O-rhamnosylrutin, and 55.3 mg 4″-O-glucosylvitexin with purities of over 97%, respectively, were obtained. The structure identification of all pure fractions was carried out by UV, MS, ^{1}H NMR and ^{13}C NMR.

Keywords: 2″-O-rhamnosylvitexin, 4″-O-glucosylvitexin, 4‴-O-rhamnosylrutin, Countercurrent chromatography, Hawthorn leaves, Preparative chromatography

*Authors contributed equally to this work.

Correspondence: Dr. Guorong Fan, Second Military Medical University, School of Pharmacy, 325 Guohe Road, Shanghai 200433, P.R. China. E-mail: Guorfan@yahoo.com.cn

INTRODUCTION

Hawthorn (*Crataegus*) is widely used as a medicinal plant for a long time both in folk and official medicine and plays an important role in the prevention and treatment of cardiovascular diseases such as hypertension, hyperlipidemia, and congestive heart failure.[1–3] These beneficial effects may result from the presence of hawthorn leaves flavonoids (HLF). 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin, and 4″-O-glucosylvitexin, the main three flavonoids of HLF, possess anti-ischemia/reperfusion injury, anti-arrhythmic, hypolipidemic, and hypotensive effects, hypolipidemic and antihypertensive activities.[4–9] Further studies on the evaluation of the clinical efficacy of 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin, and 4″-O-glucosylvitexin necessitate the development of an efficient method for the preparative separation and purification these compounds. Such a method will also facilitate quality control and improvement of quality of existing hawthorn products.

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However, the preparative separation and purification of 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin, and 4″-O-glucosylvitexin from hawthorn leaves by conventional methods, such as crystallization and column chromatography, is tedious and usually requires multiple chromatography steps.[10,11] Existing high performance liquid chromatography (HPLC) methods are not suitable for large scale isolation of those three flavonoids.

High speed countercurrent chromatography (HSCCC), a support free liquid liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto the solid support,[12] and has been widely used in the preparative separation of natural products.[13–16] However, the complexity of the crude extract and similar polarity of compounds always compromise the resolution of HSCCC. It is not always successful to achieve very high purities of all target compounds by HSCCC and further purification of target compounds by prep HPLC is needed.[17]

In this paper, we developed a HSCCC combined with prep HPLC method to isolate and purify 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin, and 4″-O-glucosylvitexin from hawthorn. The chemical structures of the three compounds are shown in Figure 1.

EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study is a Model TBE-30A analytical high speed countercurrent chromatograph (Tauto Biotechnique Company, Shanghai, China), with three multilayer

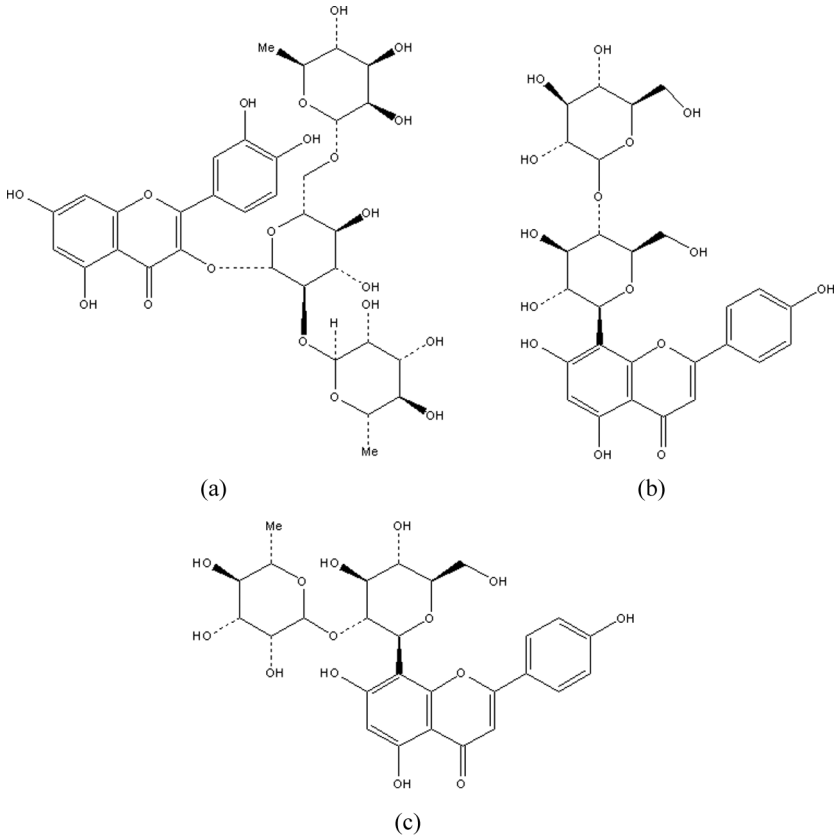

Figure 1. The chemical structures of (a) 4‴-O-rhamnosylrutin, (b) 4″-O-glucosylvitexin, and (c) 2″-O-rhamnosylvitexin.

coil separation columns connected in series (i.d. of the tubing = 1.6 mm, total volume = 18 mL) and a 100 μL sample loop.

Preparative HSCCC was carried out with a model TBE–300A high speed countercurrent chromatograph (Tauto Biotechnological Company, Shanghai, China). The apparatus was equipped with three preparative polytetrafluoroethylene coils (diameter of tube, 2.6 mm, total volume, 300 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 value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a model S constant flow pump (Beijing Boyikang Lab Implement, Beijing, China), with a flow rate in the range between 0.01 and 9.99 mL/min, a model UV-Vis

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detector (Shanghai Institute of Biochemistry, Shanghai, China), and a model N2010 workstation (Zhejiang University, Hangzhou, China).

A Varian ProStar HPLC instrument (Varian Corporation, USA), equipped with a ProStar 210 solvent delivery module and a Prostar 330 photodiode array detector (DAD) was used. The analysis and preparative was accomplished with the coupled YMC C_{18} column at room temperature; the analytical YMC C_{18} column (4.6 mm × 250 mm, 5 μm, Japan) and the preparative YMC C_{18} column (10 mm × 250 mm, 5 μm, Japan).

Materials and Reagents

n-Butanol, ethanol, and acetic acid for HSCCC were of analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was of HPLC grade (Merck, Darmstadt, Germany).

D101 macroporous resin (Tianjin Agricultural Chemical Co. Ltd., Tianjin, China), a kind of milk white spherical granule, shows stronger intension, larger absorption capability, and more easy activation than some other sorbents.

The hawthorn leaves was purchased from a local drug store and identified by Professor Luping Qin (Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, China).

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Preparation of Partially Purified Sample and Sample Solution

Dried hawthorn (400 g) was extracted two times by reflux with 3200 mL of 60% ethanol in a haven for 2 h. Extracts were filtered and concentrated to dryness at reduced pressure, using a rotary evaporator at 60°C. A total of 600 mL residue was obtained and redissolved in water, which was then chromatographed on a glass column (4.0 cm × 60 cm, which contained 350 g D101 macroporous resin). Thousand milliliters of water and 1500 mL of 10% ethanol were first used to elute the resin until the elution was nearly colorless. Then 2000 mL 20% ethanol was then used to yield target compounds, and 95% ethanol was used to activate the resin for another use.

Selection of HPLC Conditions

When the partially purified extract was analyzed by HPLC, an excellent separation was achieved by the following separation conditions: the mobile phase composed of aceonitrile-water-acetic acid (15:85:0.5,

v/v/v) was isocratically eluted at a flow rate of 0.6 mL min^{-1}, and UV detection was set at 254 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the partially purified extract was shown in Figure 2a.

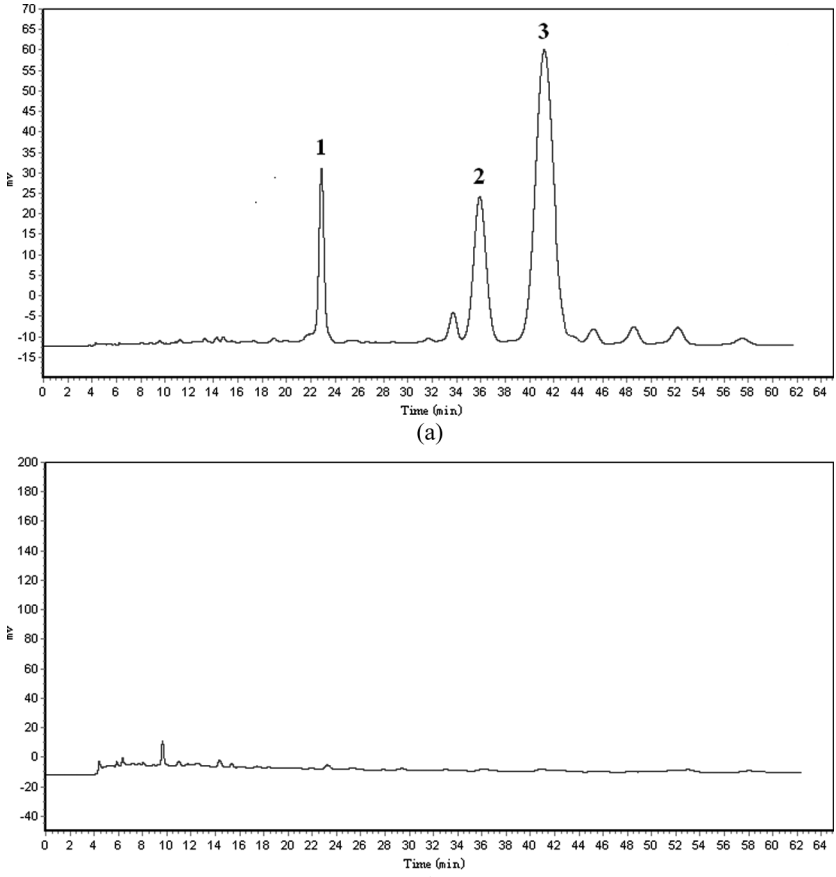

Figure 2. Chromatograms of partially purified extract and HSCCC fractions from hawthorn leaves by HPLC analysis. Conditions: column: reversed-phase YMC C_{18} column (4.6 mm × 250 mm i.d. 5 μm); mobile phase: acetonitrile-water-acetic acid (15:85:0.5, v/v/v); flow rate: 0.6 mL min^{-1}; detection at 254 nm; injection solvent: mobile phase; inject volume: 20 μL. (a) the partially purified extract; (b) fraction I of HSCCC; (c) fraction II of HSCCC; (d) fraction III of HSCCC; (e) fraction IV of HSCCC; (f) the stationary phase remained in the coil. Peak 1:4‴-O-rhamnosylrutin; Peak 2:4″-O-glucosylvitexin; Peak 3: 2″-O-rhamnosylvitexin.

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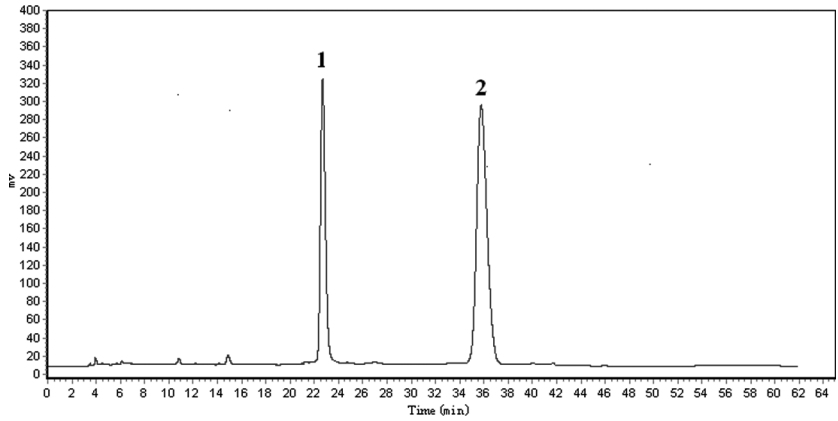
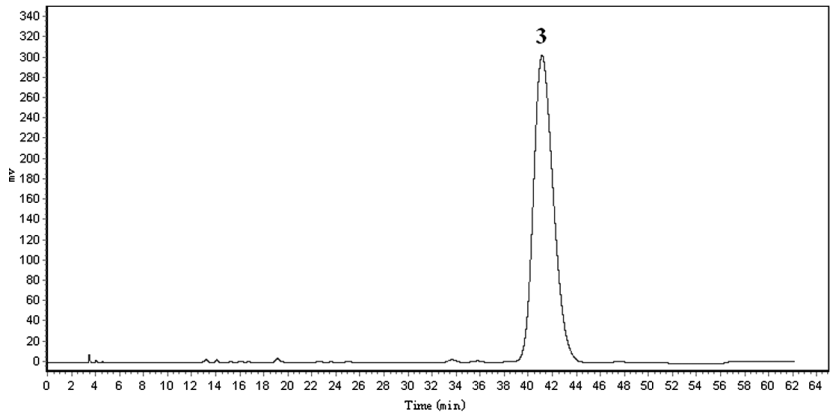


(c)



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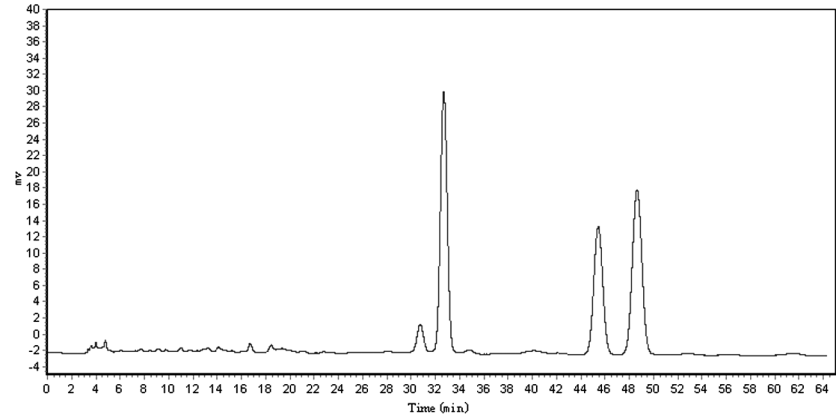

(e)

Figure 2. Continued.

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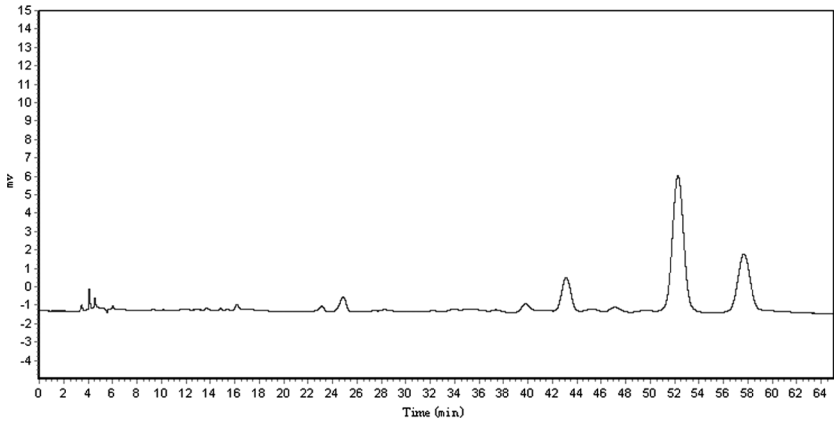

(f)

Figure 2. Continued.

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Selection of Two Phase System

The selection of a suitable solvent system is the first and most important step in performing preparative HSCCC. In the present studies, analytical HSCCC was used for selecting a suitable solvent system for the separation of the target compound. In each analytical separation, the coiled column was first entirely filled with the organic stationary phase, and then the apparatus was rotated at 1600 rpm, while the aqueous mobile phase was pumped into the column at a flow rate of 0.5 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, 100 μL of the sample solution containing 1 mg of the partially purified extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. The solvent systems including *n*-butanol-water (1:1, 1:1.5, 1.5:1, 2:1, v/v), chloroform-methanol-water (4:3:2, v/v), and *n*-butanol-acetic acid-water (2:1:1, v/v) were tested. The solvent system *n*-butanol-water (1:1, v/v) was found to be satisfactory (Figure 3).

HSCCC Separation Procedure

For the present study, a two-phase solvent system composed of n-butanol-water (1:1, v/v) was prepared. The coil column was first entirely filled with the upper phase. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow

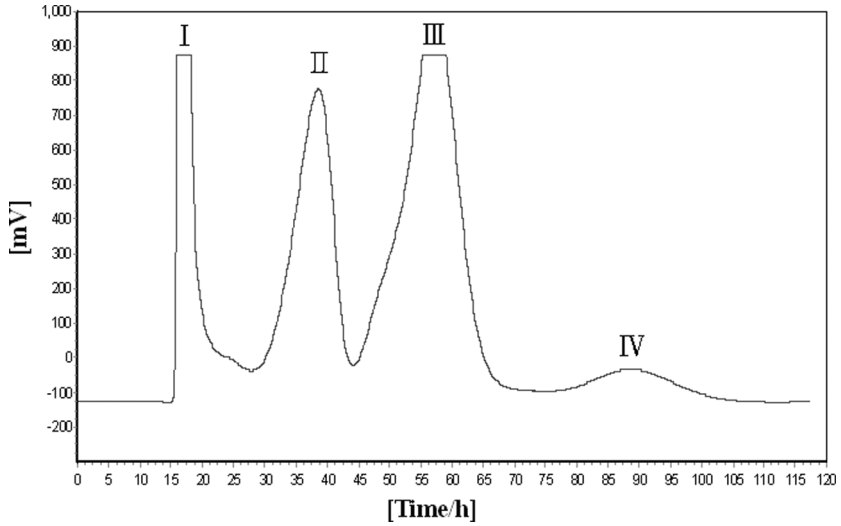

Figure 3. Chromatogram of partially purified extract from hawthorn leaves by analytical HSCCC separation, β = 2″-*O*-rhamnosidevitexin. Conditions: column: multilayer coil of 1.6 mm I.D. PTFE tube with a total capacity of 18 mL; rotary speed: 1600 rpm; solvent system: n-butanol-water (1:1, v/v); mobile phase: lower phase; flow rate: 0.5 mL·mL^{-1}; detection at 254 nm; sample size: 1 mg; injection solvent: mobile phase; inject volume: 100 μL; retention of the stationary phase: 40%.

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rate of 2.0 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution (200 mg of the partially purified extracts dissolved in the 15 mL lower phase of solvent system) was injected through the injection value. After 120 min, the flow rate of the mobile phase was decreased to 1.8 mL/min. The effluent of the column was continuously monitored with a UV-Vis detector at 254 nm. Four fractions were obtained in one step HSCCC (Figure 4) and according to the elution profile, each peak fraction together with the stationary phase remaining in the coil were manually collected, and analyzed by HPLC.

Preparative HPLC Purification Procedure

The fraction II containing 4‴-O-rhamnosylrutin and 4″-O-glucosylvitexin was evaporated to dryness by rotary vaporization and purified by pre-HPLC. In our pre-HPLC procedure, the fraction α was prepared using acetonitrile-water-acetic acid (18:85:0.5, v/v/v) as the

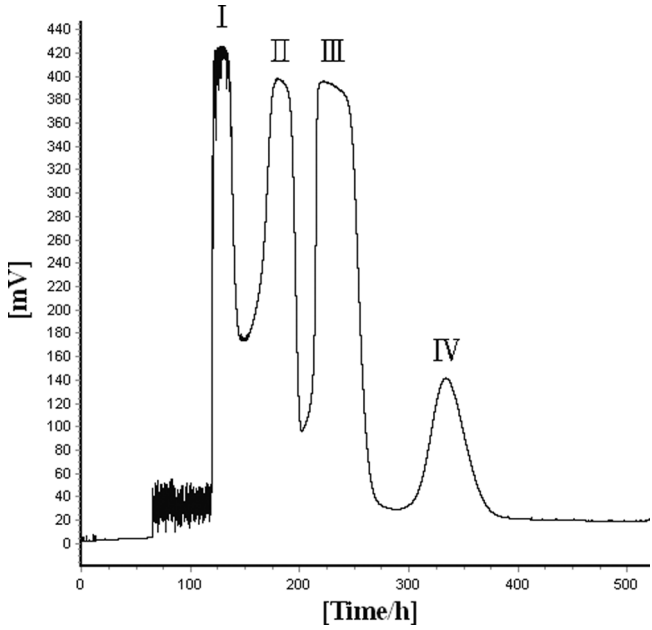

Figure 4. Chromatogram of partially purified extract from hawthorn leaves by preparative HSCCC separation, $\beta = 2''$-*O*-Rhamnosidevitexin. Conditions: column: multilayer coil of 1.6 mm I.D. PTFE tube with a total capacity of 300 mL; solvent system:n-butanol-water (1:1, v/v); mobile phase: lower phase; elution mode: 0–120 min, flow rate 2 mL/min, rotation speed 800 rpm; 120–500 min, flow rate 1.8 mL/min, rotation speed 860 rpm; detection at 254 nm; sample size: 200 mg; injection solvent: mobile phase; inject volume: 15 mL; retention of the stationary phase: 40%.

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solvent at a flow of 3 mL/min, and the effluent was monitored at 254 nm (Figure 5).

HPLC-DAD Analysis and Identification of HSCCC Peak Fractions and Preparative HPLC Fraction

The four fractions of HSCCC and the stationary phase which remained in the coil were analyzed by the HPLC using acetonitrile and water in certain volume ratios (Figures 2b–f). The five fractions were analyzed by analytic HPLC using acetonitrile–water–acetic acid (15:85:0.5, v/v/v) as the solvent at a flow of 0.6 mL/min. 4‴-O-rhamnosylrutin and 4″-O-glucosylvitexin were found in fraction α, which would be further purified by prep HPLC, while fraction β contained 2″-O-rhamnosylvitexin.

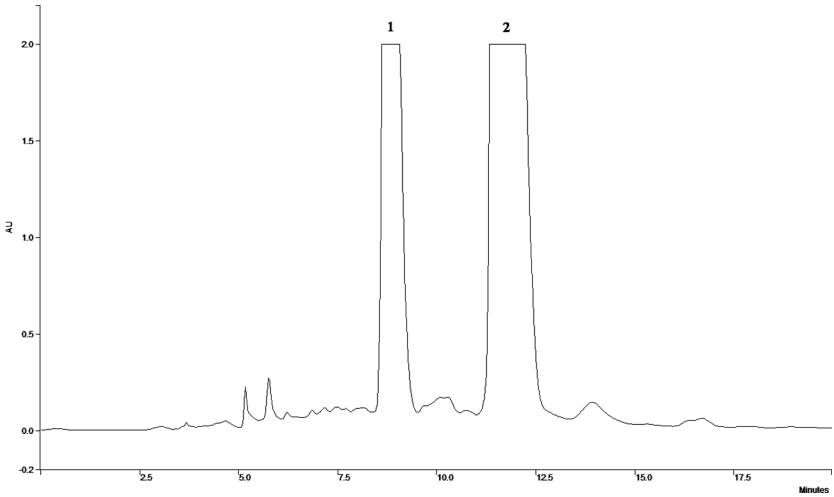

Figure 5. Semi-preparative chromatogram of 4″-O-Rhamnosylvitexin and 4‴-O-Rhamnosylrutin after separating by HSCCC. 1 = 4″-O-Rhamnosylvitexin; 2 = 4‴-O-Rhamnosylrutin. Column: reversed-phase YWC C_{18} (10.0 mm × 250 mm i.d., 5 μm); mobile phase: acetonitrile-water-acetic acid (18:85:0.5, v/v/v); injection volume: 500 μL; flow rate: 3.0 mL min^{-1}; UV wavelength: 254 nm.

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In addition, the peak fractions obtained by pre-HPLC were also analyzed by HPLC under the same chromatographic conditions. Identification of the HSCCC pure fraction β and preparative HPLC factions were carried out by UV, MS, ^{1}H NMR, and ^{13}C NMR.

RESULTS AND DISCUSSION

Optimization of HSCCC Conditions

Since little information was available in the literature on the use of HSCCC to separate hawthorn leaves, we conducted an extensive, systematic study of the generic, biphasic solvent systems that could be used.

Analytical HSCCC with its speedy separation and minimum solvent consumption offers a very promising way to carry out rapid solvent system selection and method development for preparative HSCCC separations.[18]

In our research, the two-phase solvent system composed of n-butanol-water at different volume ratios (1:1, 1:1.5, 1.5:1, 2:1, v/v), chloroform-methanol-water (4:3:2, v/v/v) and n-butanol-acetic acid-water(2:1:1, v/v/v) were tested. Among them, when chloroform-methanol-water (4:3:2, v/v/v) was used as the two-phase solvent system,

it was difficult to separate 2″-O-Rhamnosidevitexin from other compounds. When *n*-butanol-water (2:1, 1.5:1, v/v) were used, the retention of the stationary phase was poor (<20%), so it was not suitable for the separation. When *n*-butanol-water (1:1.5, v/v) was tested, 2″-O-rhamnosidevitexin could be well separated from the other compounds using these solvent systems. However, the separation time was long. At last, the two solvent systems n-butanol-water (1:1, v/v) and n-butanol-acetic acid-water (2:1:1, v/v/v) were found to be suitable for the separation of 2″-O-Rhamnosidevitexin from the partially purified extract. After analytical HSCCC optimization, n-butanol-water (1:1, v/v) was selected for our experiment.

Although the selection of the two-phase system is critical, the flow rate of the mobile phase may also affect the separation, and stepwise elution or stepwise increasing or decreasing the flow rate of the mobile phase might be applied to get better resolution. In our study, the preparative HSCCC experiment was carried out at a constant flow rate of 2 mL/min and then the apparatus was rotated at 800 rpm. As a result, the stationary phase missing was serious, which would affect the reservation of the target compounds. Therefore, at last, the improved separation conditions were as follows: 0–120 min, 2 mL/min, rotation speed 800 rpm; 120–500 min, 1.8 mL/min, rotation speed 860 rpm.

Under the optimum HSCCC conditions, the partially purified extracts were separated and purified. The typical HSCCC chromatogram was shown in Figure 4. Of 2″-O-rhamnosylvitexin (β), 188.4 mg were obtained from 500 mg of the partially purified extract in a one step separation with good resolution, and the retention of stationary phase was 40%. The purity of 2″-O-rhamnosylvitexin was 97.2%.

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The Excellent 100% Recovery of HSCCC

The most prominent advantage of HSCCC is no irreversible adsorption of the sample onto the solid support, which contributes to the 100% recovery of sample. In our paper, all of the four fractions obtained in HSCCC, in addition to the stationary phase, remained in the coil of the HSCCC and were in good agreement with the compounds in the partially purified extraction from hawthorn leaves. The above results had been shown in Figure 2.

Preparation of 4‴-O-Rhamnosylrutin and 4″-O-Glucosylvitexin by HPLC

In order to obtain 4‴-O-rhamnosylrutin and 4″-O-glucosylvitexin with high purity, prep-HPLC was used to further purify fraction α using

acetonitrile-water-acetic acid (18:85:0.5, v/v/v) as the solvent at a flow of 3 mL/min. The peak fractions α were evaporated to dryness and 62.6 mg of refined sample was obtained. The effluent of target compounds were collected and analyzed by HPLC-DAD. The results indicated that 4‴-O-rhamnosylrutin and 4″-O-glucosylvitexin with purity of over 98% could be obtained successfully by preparative HPLC separation under the above optimized conditions, yielding 28.5 mg of 4‴-O-rhamnosylrutin and 55.3 mg of 4″-O-glucosylvitexin. The purities of 4‴-O-rhamnosylrutin and 4″-O-glucosylvitexin were 98.1% and 99.5%, respectively, and the chromatograms were shown in Figure 6.

Structural Identification

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Identification of the pure products was performed by MS, ^{1}H NMR, and ^{13}C NMR analysis as follows:

2″-O-rhamnosylvitexin: $UV^{\lambda_{max}^{MeOH}}$ nm: 340, 271. ESI-MS: 579 $[M+H]^+$, 417, 399 (M-glu). ^{13}C NMR (500 MHz, CD3OD) δ: 166.05 (C-2), 103.42 (C-3), 183.76 (C-4), 162.51 (C-5), 99.79 (C-6), 164.01 (C-7), 105.43 (C-8), 157.54 (C-9), 105.85 (C-10), 123.38 (C-1′), 129.88 (C-2′), 116.76 (C-3′), 162.49 (C-4′), 116.89 (C-5′), 129.94 (C-6′), 73.52 (C-1″), 77.99 (C-2″), 81.47 (C-3″), 72.12 (C-4′), 82.67 (C-5″), 62.66 (C-6″), 102.28 (C-1‴), 72.41 (C-2″), 71.81 (C-3‴), 73.25 (C-4‴), 69.82 (C-5‴), 17.93 (C-6‴).

4‴-O-rhamnosylrutin: $UV^{\lambda_{max}^{MeOH}}$: 349, 256. ESI-MS: 757 $[M+H]^+$, 779 $[M+Na]^+$. ^{13}C NMR (500 MHz, CD3OD) δ: 158.3 (C-2), 134.2 (C-3), 179.1 (C-4), 162.8 (C-5), 99.7 (C-6), 165.6 (C-7), 94.8 (C-8), 158.8 (C-9), 105.7 (C-10), 123.4 (C-1′), 117.3 (C-2′), 146.1 (C-3′), 149.2 (C-4′), 117.2 (C-5′), 123.4 (C-6′), 102.5 (C-1″), 73.9 (C-2″), 78.8 (C-3″), 71.6 (C-4″), 77.2 (C-5″), 68.3 (C-6″), 102.1 (C-1‴), 72.3 (C-2‴) 72.1 (C-3‴), 80.2 (C-4‴), 69.7 (C-5‴), 17.7 (C-6⁗), 100.4 (C-1⁗), 71.8 (C-1⁗), 72.3 (C-1⁗), 73.8 (C-1⁗), 69.8 (C-1⁗), 17.4 (C-1⁗).

4″-O-glucosylvitexin: $UV^{\lambda_{max}^{MeOH}}$: 338, 266. ESI-MS: 595 $[M+H]^+$, 617 $[M+Na]^+$. ^{13}C NMR (500 MHz, DMSO-d6) δ: 163.8 (C-2), 105.2 (C-3), 182.2 (C-4), 156.1 (C-5), 98.1 (C-6), 159.8 (C-7), 103.6 (C-8), 162.4 (C-9), 103.8 (C-10), 121.7 (C-1′), 128.6 (C-2′), 115.8 (C-3′), 160.7 (C-4′), 116.0 (C-5′), 128.8 (C-6′), 81.9 (C-1″), 70.1 (C-2″), 71.6 (C-3″), 80.9 (C-4″), 78.5 (C-5″), 61.4 (C-6″), 102.5 (C-1‴), 74.3 (C-2‴), 76.1 (C-3‴), 69.4 (C-4‴), 76.5 (C-5‴), 60.4 (C-6‴).

Identification of the three pure products was performed by UV, MS, ^{1}H NMR, and ^{13}C NMR analysis. Comparing with the reported data, the UV, MS, ^{1}H NMR, and ^{13}C NMR data agree with those of 4‴-O-rhamnosylrutin, 2″-O-rhamnosylvitexin and 4″-O-glucosylvitexin.[19–21]

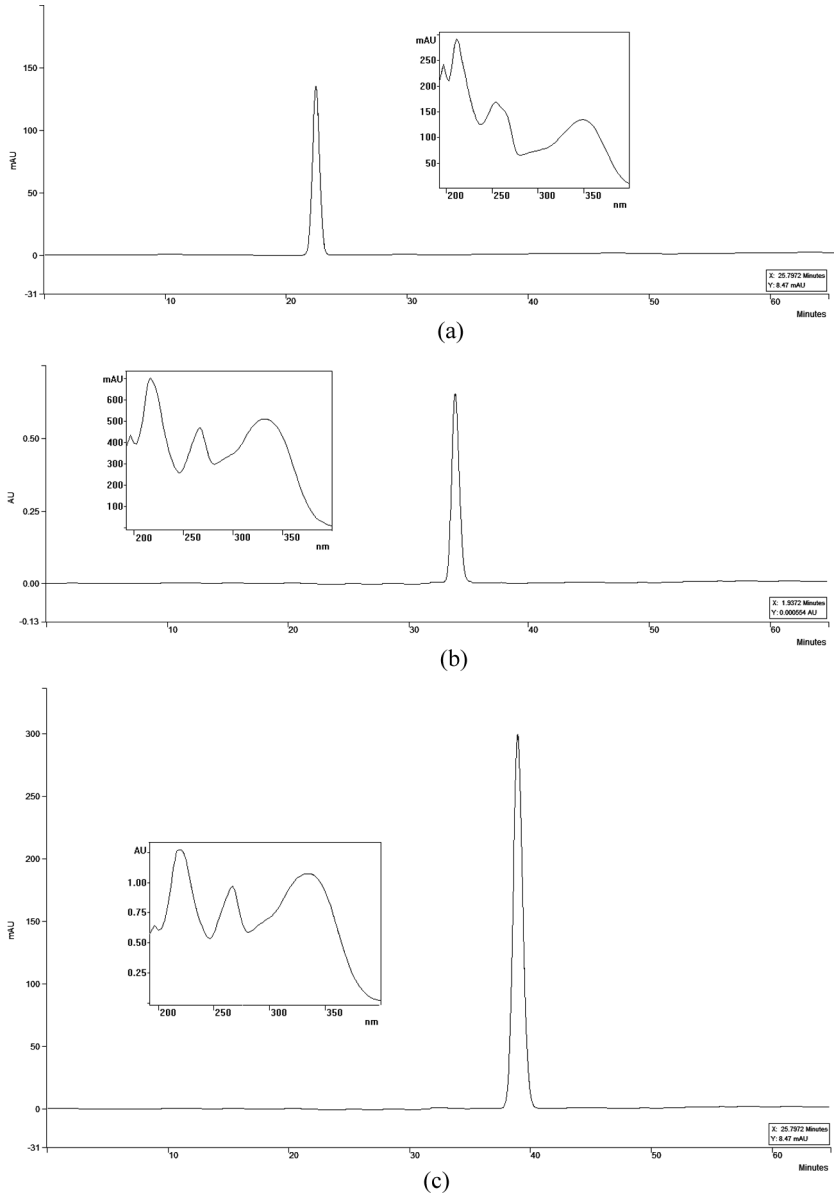

Figure 6. HPLC chromatogram and UV spectrum of the three flavonoids purified from hawthorn leaves. Conditions: column: YMC ODS (4.6 mm×250 mm, 5 μm); mobile phase: acetonitrile-water-acetic acid (15:85:0.5, v/v/v); flow rate: 0.6 mL min^{-1}; detection at 254 nm. (a) 4″-O-rhamnosylvitexin; (b) 4‴-O-rhamnosylrutin; (c) 2″-*O*-rhamnosidevitexin.

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Significant Advantages of HSCCC Combined with Prep-HPLC Over Single Prep-HPLC in Separation Efficiency

Compared with HSCCC, prep-HPLC was far more expensive and tedious with low sample loadability and recoveries. To minimize the flushing time that the system would require to remove strongly retained species, resolutions of components with similar relative migration rates would be poor in the mode of isocratic preparative HPLC separations. Therefore, HSCCC was the first choice. After extensive attempts to separate the two compounds in the fraction II by a second run of HSCCC failed, we adopted a HSCCC combined with the prep-HPLC approach. As were shown in the Figures 2c and 2d, peak 1 and peak 2 in fraction II had been extracted from the crude sample, and peak 3 in fraction III was also obtained as a pure compound by one step of HSCCC, making the further purification by prep-HPLC far more convenient. Therefore, the sample loadability and cycle time of prep-HPLC after HSCCC had been significantly improved compared with that of prep-HPLC without HSCCC.

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CONCLUSIONS

The results of our studies clearly demonstrated the potential of novel HSCCC combined with pre-HPLC for the isolation of the three components at high purity from hawthorn leaves. Four fractions in narrow polarity from the partially purified extracts were obtained in one step HSCCC separation. Subsequently, the fraction α was further purified by pre-HPLC. It is envisioned that this approach could be adopted as a new technical platform for a wide range of different HSCCC-pre-HPLC purification systems to isolate chemical constituents from TCM in general.

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