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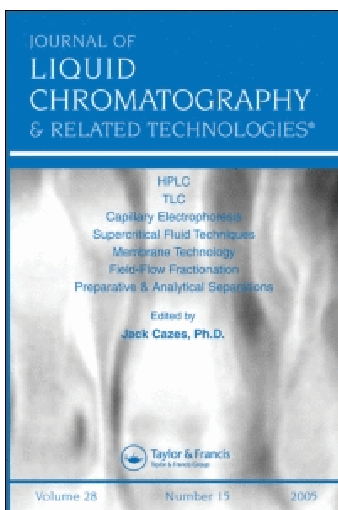

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Preparative Separation of Axifolin-3-Glucoside, Hyperoside and Amygdalin from Plant Extracts by High Speed Countercurrent Chromatography

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Abstract: High speed countercurrent chromatography (HSCCC) was successfully used to isolate three bioactive compounds, i.e., amygdalin from bitter almond and taxifolin-3-glucoside and quercetin-3-galactoside (hyperoside) from water extract of *A. pilosa* Ledeb, respectively. From 1 g of the crude extract 65 mg of amygdalin was isolated at 97% purity using a two phase solvent system composed of ethyl acetate-n-butanol-water (5:2:5, v/v) by preparative HSCCC. From a 400 mg amount of crude extract of *A. pilosa* Ledeb, 11 mg of taxifolin-3-glucoside and 8 mg of hyperoside were isolated at 96% purity, using a two phase solvent system composed of ethyl acetate-methanol-water (25:1:25, v/v) similarly by preparative HSCCC. The final structural identification was performed by MS, ^{1}H-NMR, and ^{13}C-NMR spectra.

Keywords: *Agrimonia pilosa* Ledeb, Amygdalin, High speed countercurrent chromatography (HSCCC), Hyperoside, Taxifolin-3-glucoside

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INTRODUCTION

Bitter almond is a traditional Chinese medicine, containing about 48% fat, 30% protein, 6% carbohydrate, 3% amygdalin, and other nutritions.[1] Amygdalin is a commercially available natural cyanogenic glycoside, which can dispel the phlegm, relieve the cough, is an anticancer drug, can improve immunity, reduce the blood sugar, prevent chronic gastritis and withering gastritis.[2,3] The molecular formula of amygdalin is $C_{20}H_{27}NO_{11}$ and the chemical structure is shown in Figure 1. The separation of amygdalin has been performed by recrystallization and macroporous resin.[4,5] However, the processes need several steps and are time consuming.

Agrimonia pilosa Ledeb is a native plant that grows in many parts of China. It contains flavonoids and phenolic compounds. A pharmaceutical compound of *A. pilosa* Ledeb for treating or preventing hepatitis

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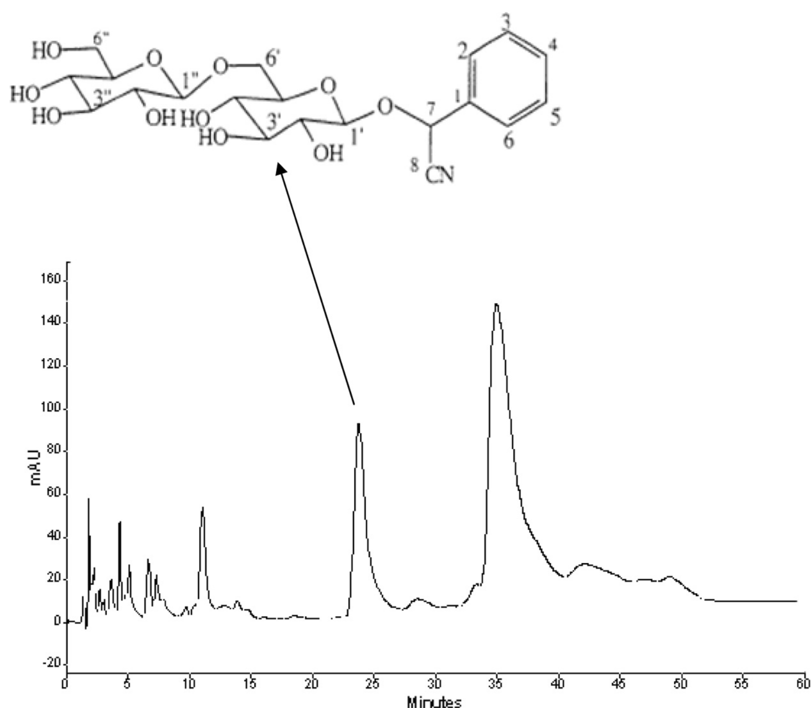

Figure 1. HPLC analyses of the crude extract from bitter almond with the chemical structure of amygdalin. HPLC conditions: reversed phase C_{18} column (250 × 4.6 mm, 5 μm, SUPELCOSIL™), column temperature: 30°C. Mobile phase: MeOH (eluent A)-water (eluent B) (5:95, v/v). Flow rate: 1.0 mL/min, monitored at 254 nm.

is reported. Bioassays showed that flavonoid effectively controls fungal pathogens in vitro, including *Alternaria alternate* and *Fusarium avenaceum*, although the antifungal activity of this compound in the plant is limited.[6] Hyperoside (quercetin-3-galactoside) also decreases blood pressure transiently.[7] However, the separation of these active compounds from natural sources has always been difficult to achieve. The ethanol extract of *A. pilosa* Ledeb has been studied in our lab,[8] and the water extract is further studied in this paper.

High Speed countercurrent chromatography (HSCCC), being a support free liquid–liquid partition method, eliminates irreversible adsorption of sample onto the solid support, and has been widely used in preparative separations of natural products.[9,10] The aim of this study was to develop a method for purifying natural amygdalin and bioactive constituents from the water extract of *A. pilosa* Ledeb. The present paper describes the successful preparative separation and purification of amygdalin from the crude extract of bitter almond and taxifolin-3-glucoside and hyperoside from the water extract of *A. pilosa* Ledeb by HSCCC.

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EXPERIMENTAL

Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high speed countercurrent chromatograph designed and constructed in Beijing Institute of New Technology Application (Beijing, China). 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. PTFE (polytetrafluoroethylene) tube directly onto the holder hub, forming multiple coiled layers with a total capacity of 30 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 to 2000 rpm, a speed of 1600 rpm was used in the present studies. A manual sample injection valve with a 1.0 mL loop was used.

The preparative high speed countercurrent instrument was used with a Model GS10 multilayer coil planet centrifuge equipped with a PTFE (polytetrafluoroethlene) multilayer coil of 110 m $\times$ 1.6 mm I.D. with a

total capacity of 230 mL, which is also designed and constructed in Beijing Institute of New Technology Application (Beijing, China).

The high performance liquid chromatography (HPLC) equipment used was a Shimadzu LC-20AVP system with two LC-20AT solvent delivery units, an SPD-M20AVP UV–VIS photodiode array detector (DAD), a Model 7725 injection valve with a 20μL loop and auto sampler, an SCL-20AVP system controller, and a Class-VP-LC work station (Shimadzu, Tokyo, Japan).

The ^{1}H-NMR and ^{13}C-NMR Spectra were recorded on a "amms 600" Varian Inova 600 (600 MHz) spectrometer.

Reagents

All organic solvents used for sample preparation and HSCCC were of analytical grade while methanol used for HPLC analysis was of chromatography grade. These solvents were all purchased from Beijing Chemical Factory (Beijing, China). Bitter almond was purchased from a company in ZuoQuan, Shanxi Province, China. *A. pilosa* Ledeb was purchased from a local store (Tong Ren Tang Shop, Beijing, China). Hyperoside standards were purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China).

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Preparation of Sample

Bitter almond dross was obtained through the process of enzyme destruction, crushing, and defatting. The extraction condition of amygdalin from bitter almond dross was optimized by orthogonal design. About 30 g of bitter almond dross was extracted (refluxed) with 180 mL 80% ethanol for 45 min 3 times. The obtained liquid was combined and concentrated to dryness under reduced pressure, yielding 7.4 g of crude sample in which amygdalin purity was determined by HPLC based on the external standard curve (Figure 1).

About 200 g of dried *A. pilosa* Ledeb was extracted (refluxed) for 3 h, three times, with 500 mL of petroleum ether (b.p.: 60–90°C), and then filtered by six layer pledget. The residues were extracted with 500 mL of 95% ethanol solution, and then filtered by six layer pledget. The residues were again extracted with 500 mL of water, and concentrated to dryness under reduced pressure, yielding 12 g of a crude sample, which contained taxifolin-3-glucoside and hyperoside at purity of 3.1% and 3.3%, respectively, as determined by HPLC based on the external standard curve.

Preparation of Two Phase Solvent System and Sample Solution

For the separation of amygdalin the solvent system utilized was prepared by mixing ethyl acetate-n-butanol-water (5:2:5, 5:1:5, 10:1:10, or 50:1:50, v/v), or n-butanol-water (1:1, v/v). For the separation of water extract of *A. pilosa* Ledeb the solvent system was prepared by mixing ethyl acetate-methanol-water (25:1:25, 10:1:10 or 5:1:5, v/v), and thoroughly equilibrating the mixture in a separatory funnel at room temperature, two phase 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.

Separation Procedure

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Analytical HSCCC was performed with a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower phase was then 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 1600 rpm. After hydrodynamic equilibrium was established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (12 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 detector at 280 nm. Each peak fraction was collected according to the chromatogram.

Preparative HSCCC separation was performed as follows: the multiplayer coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the column 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 established, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 254/280 nm. Each peak fraction was collected according to the chromatogram. The retention of the stationary phase was computed from the volume of the stationary phase collected from the column after the separation was completed.

HPLC Analyses and Identification of HSCCC Peak Fractions

HPLC conditions of the crude extract of bitter almond with the chemical structure of amygdalin are as follows: the solvent system consisted of

water as mobile phase A and methanol as mobile phase B (95:5, v/v). Flow rate was 1.0 mL/min, and 10 μL portions were injected into the column. Amygdalin was detected by absorbance at 254 nm.

The water extract of *A. pilosa* Ledeb and HSCCC peak fractions were each analyzed by HPLC. The analyses were performed with a YMC ODS column (150 × 4.6 mm I.D.). The mobile phase composed of methanol-water (50:50, v/v) was isocratically eluted at a flow rate of 1.0 mL/min and the effluent monitored at 280 nm by a DAD detector. Identification of the target compounds was based on MS, ^{1}H-NMR; and ^{13}C-NMR spectra.

RESULTS AND DISCUSSION

The HPLC analyses of the crude extract of bitter almond indicated that it contains several compounds as shown in Figure 1. An amount of amygdalin in the crude extract is 10.9% based on the external standard curve as determined by HPLC. Although amygdalin is a water soluble compound, it is more soluble in n-butanol than in water according to the HPLC analysis. In order to achieve an efficient resolution of target compound, the two phase solvent system of n-butanol-water (1:1, v/v) was first used (Figure 2a). However, none of the two peaks was found to be amygdalin, which was still retained in the column. Then, two phase solvent systems composed of ethyl acetate-water were investigated. A certain volume of n-butanol was gradually added to make ethyl acetate-n-butanol-water at volume ratios of 50:1:50, 10:1:10, 5:1:5, 5:2:5, v/v. (shown in Figure 2b, c, d, e). As n-butanol concentration was increased, the partition coefficient of the solute in the upper phase to lower phase increased and the volume ratio of 5:2:5 was considered to be most suitable for the HSCCC run for purification of amygdalin, which yields the baseline separation (Figure 2b). Then, the volume ratio of 5:3:5 system was examined in the test tube, but it showed a long settling time, indicating that it would produce a low level of the stationary phase retention in the column. In addition, the volume of the upper phase is 1.5 times that of the lower phase, which is a waste of solvent. Therefore, the volume ratio of 5:2:5 was chosen as the separation. Figure 3 shows the result obtained from 1 g of the crude extract of bitter almond by preparative HSCCC. The second peak was cut and concentrated, yielding 65 mg of amygdalin at 97% purity based on the HPLC analysis (Figure 3).

As shown in Figure 4, the HPLC analysis of the crude extract of *A. pilosa* Ledeb shows several compounds where the purity of taxifolin-3-glucoside and hyperoside in the crude extract is 3.1% and 3.3%, respectively, based on the HPLC external standard

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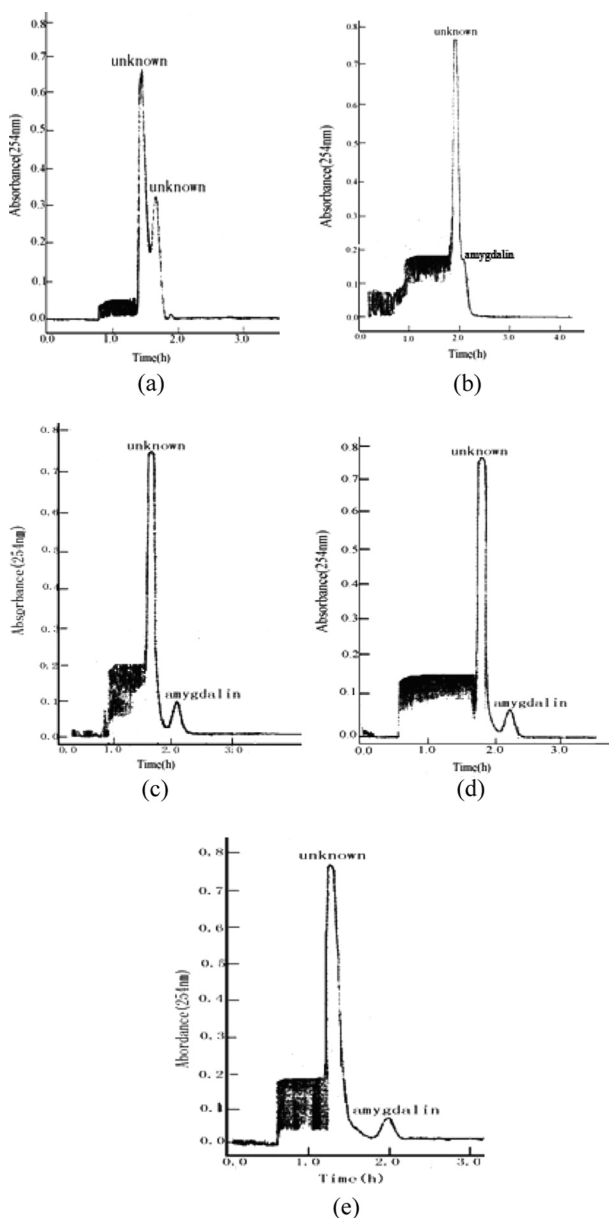

Figure 2. Chromatogram of the crude extract from bitter almond by HSCCC. Solvent system: n-butanol-water (1:1, v/v) (a); ethyl acetate-1-butanol-water (50:1:50, v/v) (b), (10:1:10, v/v) (c), (5:1:5, v/v) (d), (5:2:5, v/v) (e); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm. Sample: 300 mg dissolved in 10 mL of lower phase.

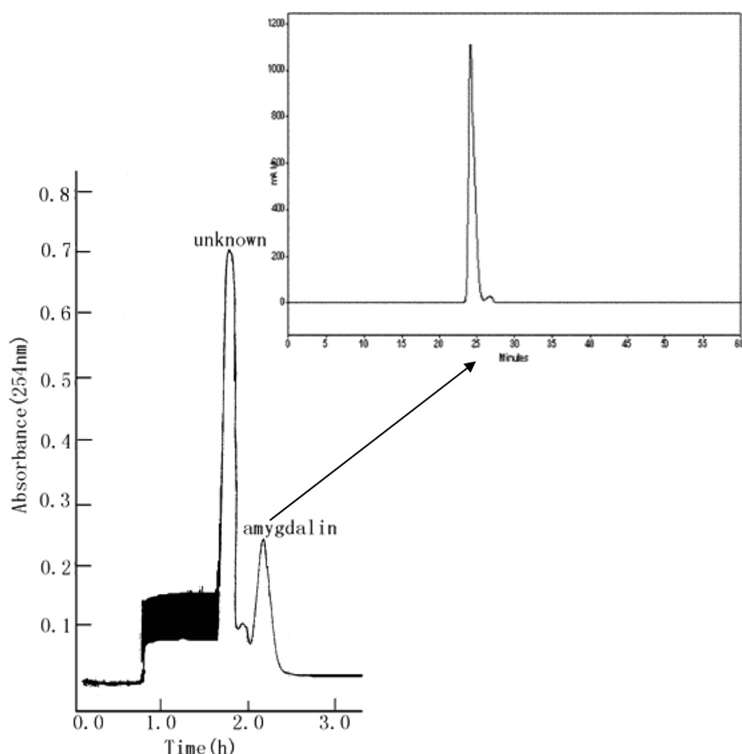

Figure 3. Chromatogram of the crude extract from bitter almond by HSCCC. Solvent system: ethyl acetate-n-butanol-water (5:2:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm. Sample: 1 g dissolved in 10 mL of lower phase. Retention of the stationary phase is 50.8%.

curve. Two phase solvent systems composed of ethyl acetate-water were then investigated. Methanol was gradually added to make various volume ratios of ethyl acetate-methanol-water at 25:1:25, 10:1:10, and 5:1:5 as shown in Figure 5a, b, c. It was found that the volume ratio of 25:1:25 was most suitable for the analytical HSCCC run for purification of taxifolin-3-glucoside and quercetin-3-galactoside, which gave base line separation (Figure 5a). Figure 6 shows the result obtained from 400 mg of the extract of Agrimonia pilosa Ledeb by preparative HSCCC. Peak 1 and peak 2 were cut and concentrated yielding 11 mg of taxifolin-3-glucoside and 8 mg of quercetin-3-galactoside each at 96% purity based on the HPLC analysis.

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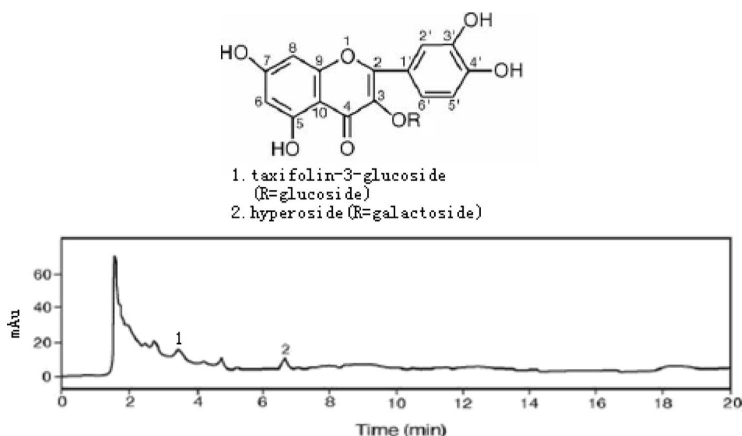

Figure 4. HPLC analyses of the crude extract from *Agrimonia pilosa* Ledeb with the chemical structure of taxifolin-3-glucoside and quercetin-3-galactoside. HPLC conditions: reversed phase C_{18} column (150 × 4.6 mm, 5 μm, YMC), column temperature: 30°C. Mobile phase: MeOH (eluent A)-water (eluent B) (50:50, v/v). Flow rate: 1.0 mL/min, monitored at 280 nm.

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The structural identification of amygdalin was carried out by MS, ^{1}H-NMR and ^{13}C-NMR spectra as follows: EI-MS (pos. m/z): 458 $[M+H]^+$, 325 $[\text{diglucoside}]^+$, 117[benzene and –CN]. The results were similar to those in reference.[11]

Amygdalin: ^{1}H-NMR (600 MHz, CD_3COCD_3) δ: 7.614 (2H, m) and 7.491 (3H,m)—ArH; 5.945 (1H, s, CHCN); 2.815–4.497(12H, sugar-H), 4.497(1H, d, H-1″), 4.432 (1H, d, H-1′), 4.276 (1H, dd, Hz, H-5″), 4.195 (1H, dd, Hz, H-5′), 2.815 (1H, dd, H-6′), 3.711 (3H, m), 3.347(4H,m). ^{13}C-NMR (600 MHz CD_3COCD_3): δ61.59 (C-6″), 69.13 (C-6′), 69.28 (C-7), 70.10 (C-4′), 70.49 (C-4″), 73.67 (C-2′),74.05 (C-2″), 76.40 (C-3′, C-5′), 76.73 (C-3″, C-5″), 102.51 (C-1′), 103.67 (C-1″), 119.37 (C-8), 128.16 (C-2,C-6), 129.98 (C-3, C-5), 130.93 (C-4), 133.61 (C-1). The results were similar to those in reference,[12] and the compound was identified as amygdalin.

The structural identification of taxifolin-3-glucoside and quercetin-3-galactoside was carried out by MS, ^{1}H-NMR, and ^{13}C-NMR Spectra as follows: EI-MS: m/z 464(M^+), 302 (100), 274, 217, 153, 137; it showed the molecular ion at m/z 464, which is in agreement with the molecular formula $C_{21}H_{20}O_{12}$ of quercetin-3-galactoside.[13] EI-MS: m/z 466(M^+), 304, 276, 137, which is in agreement with the molecular formula $C_{21}H_{22}O_{12}$ of taxifolin-3-glucoside.[14]

Quercetin-3-galactoside: ^{1}H-NMR (500 MHz, DMSO) δppm: 5.351 (1H, H-100), 6.191 (1H, H–6), 6.402 (1H, H-8), 6.811 (1H, H-50), 7.533

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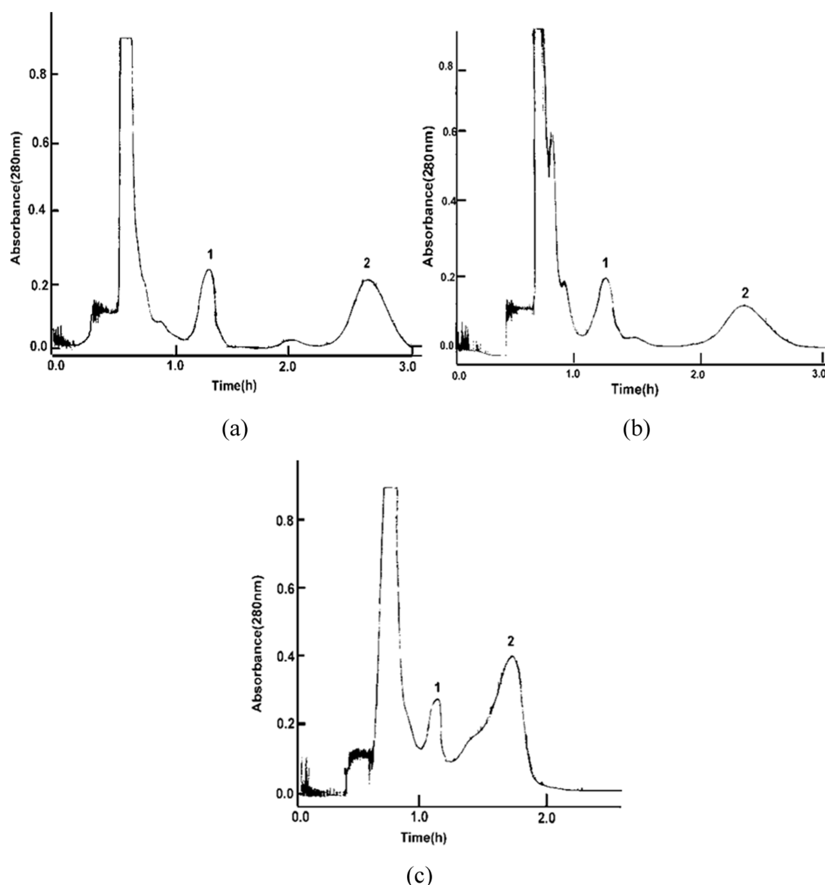

Figure 5. Chromatogram of the water extract of *Agrimonia pilosa* Ledeb by analytical HSCCC. Solvent system: ethyl acetate-methanol-water (25:1:25, v/v) (a), (10:1:10, v/v) (b), (5:1:5, v/v) (c); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 1.0 mL/min; revolution speed: 1600 rpm. Sample: 30 mg dissolved in 5 mL of lower phase.

(1H, H-20), 7.632 (1H, H-60). 13C-NMR (500 MHz, DMSO) δppm: 156.305(C-2), 133.502(C-3), 177.411(C-4), 161.210(C-5), 98.702(C-6), 164.102(C-7), 93.604(C-8), 156.302(C-9), 104.104(C-10), 121.101(C-10), 115.212(C-20), 144.810(C-30), 148.403(C-40), 116.003(C-50), 121.903 (C-60), 101.905(C-10), 71.306(C-20), 73.311(C-30), 68.012(C-40), 75.906(C-50), 60.211(C-60).[15,16]

Taxifolin-3-glucoside: ^{1}H-NMR (500 MHz, DMSO) δppm: 8.59 (1H, HO-5), 6.61-6.49 (3H, Ar-H), 5.73 (2H, H-8 and H-6), 5.43 (1H, H-2), 4.98 (1H, H-3), 4.7 (1H, H-1″), 4-3 (5H, H of glucoside).[14]

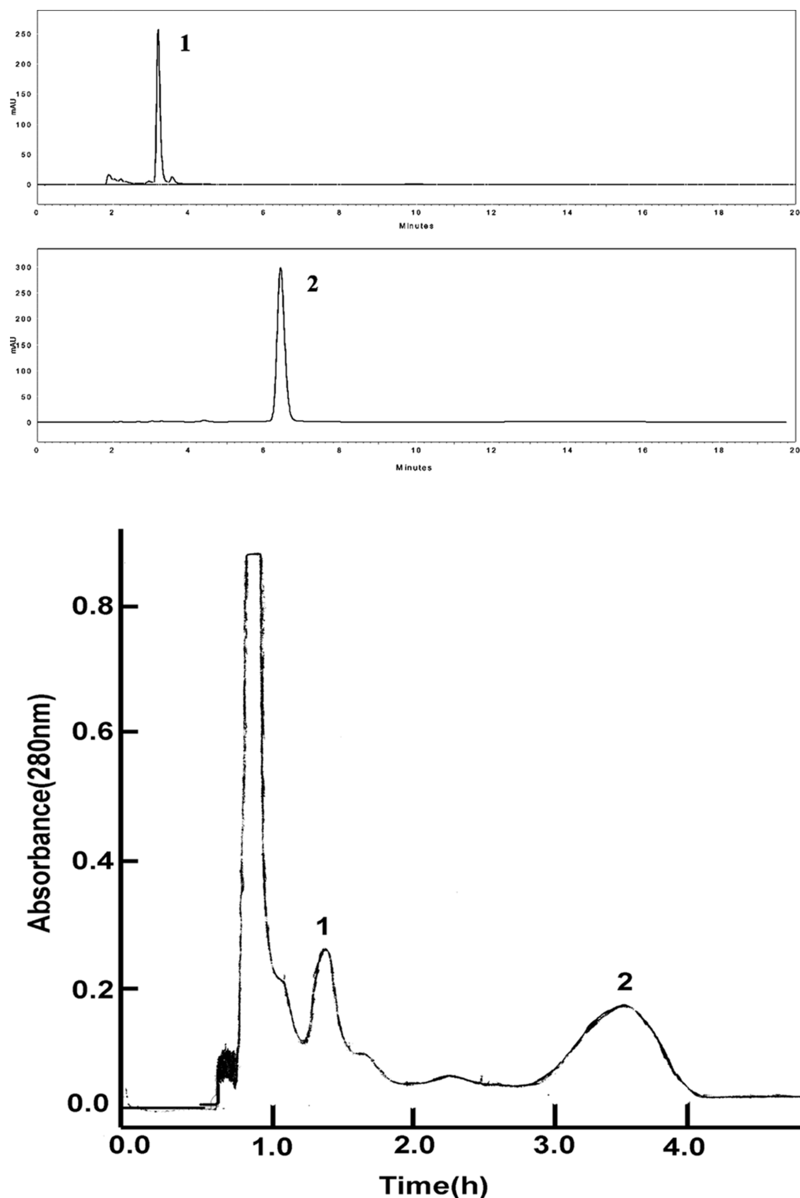

Figure 6. Chromatogram of taxifolin-3-glucoside and quercetin-3-galactoside from the crude extract of *Agrimonia pilosa* Ledeb by preparative HSCCC. Solvent system: ethyl acetate-methanol-water (25:1:25, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 2.0 mL/min; revolution speed: 800 rpm. Sample: 400 mg dissolved in 10 mL of lower phase. Retention of the stationary phase is 50.4%.

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CONCLUSION

In the present study, about a 1 g amount of the crude extract of amygdalin was isolated to yield 65 mg of amygdalin at 97% purity using a two phase solvent system composed of ethyl acetate- n-butanol-water (5:2:5, v/v) by preparative HSCCC. About a 400 mg amount of the crude extract of *A. pilosa* Ledeb was isolated to yield 11 mg of taxifolin-3-glucoside and 8 mg of hyperoside, each at 96% purity, using a two phase solvent system composed of ethyl acetate-methanol-water (25:1:25, v/v) by preparative HSCCC. Several kinds of two phase solvent systems, different volume ratios of ethyl acetate-n-butanol-water or ethyl acetate-methanol-water, were tested in the solvent selection process by analytical HSCCC. The result of this study demonstrates that HSCCC is very useful for the preparative separation of amygdalin, taxifolin-3-glucoside, and quercetin-3-galactoside from crude plant extracts.

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