Journal of Liquid Chromatography & Related Technologies®, 29: 1619–1632, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600678357

Preparative Separation and Isolation of Three Flavonoids and Three Phloroglucinol Derivatives from *Hypericum japonicum* Thumb. using High-Speed Countercurrent Chromatography by Stepwise Increasing the Flow Rate of the Mobile Phase

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Abstract: A preparative high-speed counter-current chromatography (HSCCC) was used to isolate and separate chemical compounds from the medicinal plant *H. japonicum*. First, the ethanol extract of *H. japonicum* was directly isolated by HSCCC without any preparation and three flavonoid glycosides including isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside were successfully purified using a two-phase solvent system composed of ethyl acetate–ethanol–water at the volume ratio of 5:1:5 (v/v) by increasing the flow rate of the mobile phase from 1.0 mL/min to 2.0 mL/min after 120 min. After 400 min, the remaining compounds, mainly containing three phloroglucinol derivatives including sarothialen A, sarothralen B, and sarothalin G in the HSCCC column were forced out by pressurized nitrogen gas, which were further successfully separated by HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the volume ratio of 1:1.2:1.2:1 (v/v) by increasing the flow rate of the mobile phase from 1.2 mL/min to 2.2 mL/min after 120 min. The fractions obtained from HSCCC were analyzed by high performance liquid chromatography, and the separation produced a total of 124 mg isoquercitrin, 85 mg quercitrin, 68 mg quercetin-7-O-rhamnoside, 24 mg sarothialen A, 18 mg sarothralen B, and 58 mg sarothalin G from 750 mg ethanol extract, with the purities

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of 98.6%, 95.8%, 95.6%, 96.5%, 95.4%, and 98.6%, respectively. The chemical structural identification was carried out by MS, ^{1}H NMR, and ^{13}C NMR.

Keywords: Preparative chromatography, Countercurrent chromatography, *Hypericum japonicum* Thumb., Plant material, Flavonoid, Phloroglucinol derivatives

INTRODUCTION

H. japonicum. (*Tianjihuang* in Chinese), a herblet and stem slender, small and yellow, bitter and acrid in taste, and mild in nature, is a famous traditional Chinese medicinal herb used for the treatment of bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal hemorrhage, and tumors.[1–3] The herb grows widely in the warm and humid southern China (Jiangxi, Fujian, Guangdong, Guangxi, Sichuan, and Guizhou provinces). It is a profile producer of secondary metabolites, and the major chemical constituents are considered to be flavonoids mainly including isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside (Figure 1), and phloroglucinol derivatives mainly including sarothialen A, sarothralen B, and sarothalin G (Figure 1). These pure products from medicinal plants have been widely isolated and separated by some conventional techniques including silica gel, polyamide, macroporous resin, preparative liquid chromatography, and thin layer chromatography. However, these classical methods have some shortcomings, such as tedious, time consuming, needing multiple steps, and causing samples loss. So, an efficient method for the preparative purification and isolation natural materials from raw resources is warranted. High-speed countercurrent chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support, and has an excellent sample recovery. The method permits direct introduction of crude samples into the column without more preparation, so it has been successfully applied to isolate and purify a number of natural products.[4–8] However, there have been no reports of using HSCCC to isolate and separate isoquercitrin, quercitrin, quercetin-7-O-rhamnoside, sarothialen A, sarothralen B, and sarothalin G from *Tianjihuang* through a literature search.

The aim of the present paper, therefore, was to develop an efficient method to isolate and purify isoquercitrin, quercitrin, quercetin-7-O-rhamnoside, sarothialen A, sarothralen B, and sarothalin G from the medicinal plant *H. japonicum* by HSCCC.

EXPERIMENTAL

Apparatus

Preparative HSCCC was carried out with a model TBE–300A high-speed countercurrent chromatography (Shenzhen, Tauto Biotech, China). The

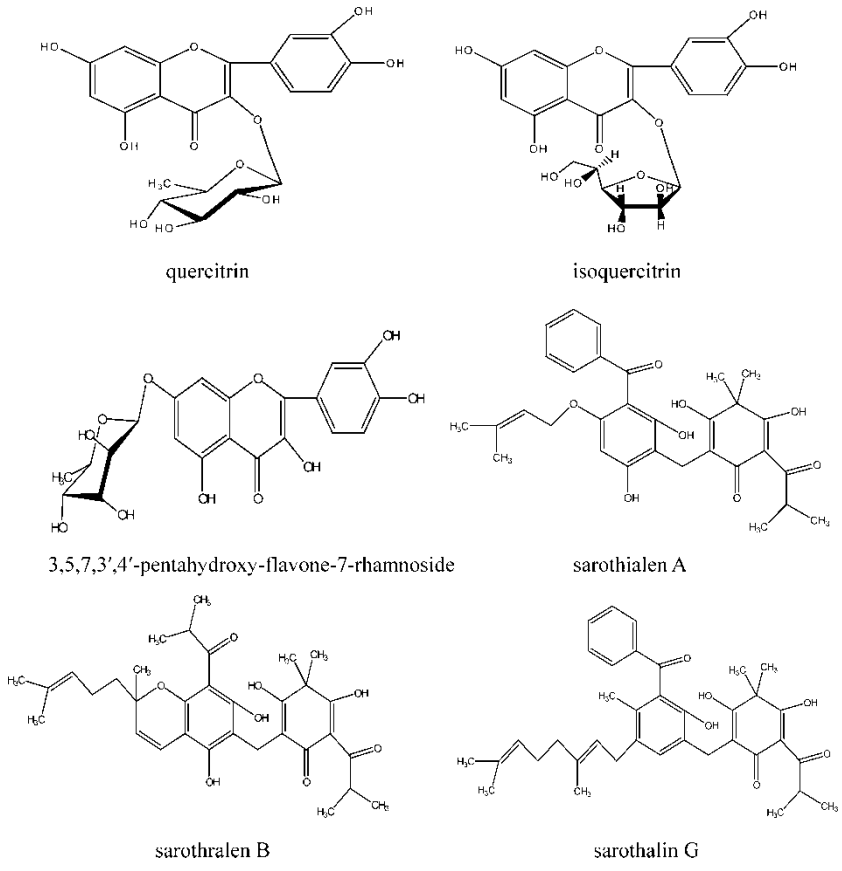

Figure 1. Chemical structures of isoquercitrin, quercitrin, quercetin-7-O-rhamnoside, sarothialen A, sarothralen B, and sarothalin G.

apparatus was equipped with three polytetra-fluoroethylene preparative 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, a model UV-II detector operating at 280 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by an HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). The analytical HPLC system used throughout this study consisted of LC–10AT pump and a SPD–10A UV-Vis detector (Shimadzu, Japan), and a model N2000 workstation (Zhejiang University, Hangzhou, China).

Reagents

Ethyl acetate, *n*-hexane, methanol, ethanol, acetic acid, were analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The *H. japonicum* was purchased from a local drug store (Shanghai, China).

Preparation of the Crude Extract

The *H. japonicum* was ground into powder, 500 g of the powder was first extracted by reflux with 40% aqueous ethanol and filtered. Then the residue was re-extracted with 95% aqueous ethanol. The extraction solution was filtered and combined, and then evaporated to dryness by rotary vaporization at 60°C under reduced pressure. The light yellow powder was obtained, which was used for HSCCC isolation and purification.

Preparation of Two-Phase Solvent System and Sample Solution

Two kinds of solvent systems were selected and used in the present paper. One was composed of ethyl acetate–ethanol–water (5:1:5, v/v/v) and used to isolate the flavonoids, and the other was composed of *n*-hexane–ethyl acetate–ethanol–water (1:1.2:1.2:1, v/v) used for phloroglucinol derivatives separation. The solvent mixtures were thoroughly equilibrated in a separated funnel at room temperature, and the two phases were separated shortly before use. The sample solution was prepared by dissolving the crude sample in the solvent mixture of lower phase and upper phase (1:1, v/v) of the solvent system used for isolation, because the sample was not easy dissolved in either phase.

HSCCC Separation Procedure

In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 1.0 mL/min in the first step for three flavonoids isolation and 1.2 mL/min in the second step for three phloroglucinol derivatives separation. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 16 mL sample solutions containing 750 mg of the ethanol extract for the first isolation and 280 mg of the remaining compounds after the first HSCCC purification, were injected through the injection value. After 120 min, the flow rate was increased to 2.0 mL/min in the first step and 2.2 mL/min in the second step. The effluent of the column was continuously

monitored with a UV-Vis detector at 280 nm. Peak fractions were collected according to the elution profile.

HPLC Analysis and Identification of CCC Peak Fractions

The crude samples and the peak fractions obtained by HSCCC were analyzed by high performance liquid chromatography. The column used was a Lichrospher C_{18} (4.6 mm × 250 mm i.d., 5 μm) (Hanbang Science, Jiang-Su province, China) with a pre-column equipped with the same stationary phase, the mobile phases used in the present paper were CH_3CN-H_2O-HAC (20:80:1, v/v/v) for flavonoids analysis and CH_3CN-H_2O-HAC (55:45:1, v/v/v) for phloroglucinol derivatives analysis. The flow rate was set at 1.0 mL/min, and the effluent was monitored at 280 nm.

Identification of the CCC peak fractions was carried out by MS (Varian–1200L), ^{1}H NMR, and ^{13}C NMR spectra (Varian Unity Inova–500).

RESULTS AND DISCUSSION

Optimization of HPLC Method

Isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside are three glycosides and could be dissolved in some polar solutions. So, low concentration of methanol or acetonitrile was used to separate the targets on a reversed-phase HPLC column. However, sarothialen A, sarothralen B, and sarothalin G are three non-polar compounds, and high concentration of methanol or acetonitrile was required in HPLC analysis. In the present paper, different mobile phases (methanol–water, acetonitrile–water) with different concentrations of acetic acid, different flow rates, and column temperatures were all tested. The results indicated that the mobile phase was acetonitrile–water–acetic acid at a volume ratio of 20:80:1 (v/v/v), and the flow rate, column temperature, and detection wavelength were set at 1.0 mL/min, 30°C and 280 nm, which were most suitable for flavonoids analysis. While the volume ratio of acetonitrile–water–acetic acid was changed into 55:45:1 (v/v/v), and the flow rate, column temperature, and detection wavelength were used the same as above depiction, which were most suitable for phloroglucinol derivatives analysis. The ethanol extract from *H. japonicum* was analyzed by the two kinds of analytical conditions to detect the flavonoids and the phloroglucinol derivatives, and the HPLC chromatograms are shown in Figure 2A and B. It is obvious that the six compounds all obtained baseline separation, and the separation times were less than 25 min for flavonoids and 35 min for phloroglucinol derivatives. Peaks 1, 2, 3, 4, 5, and 6 correspond to isoquercitrin, quercitrin, quercetin-7-O-rhamnoside, sarothialen A, sarothralen B, and sarothalin G, respectively.

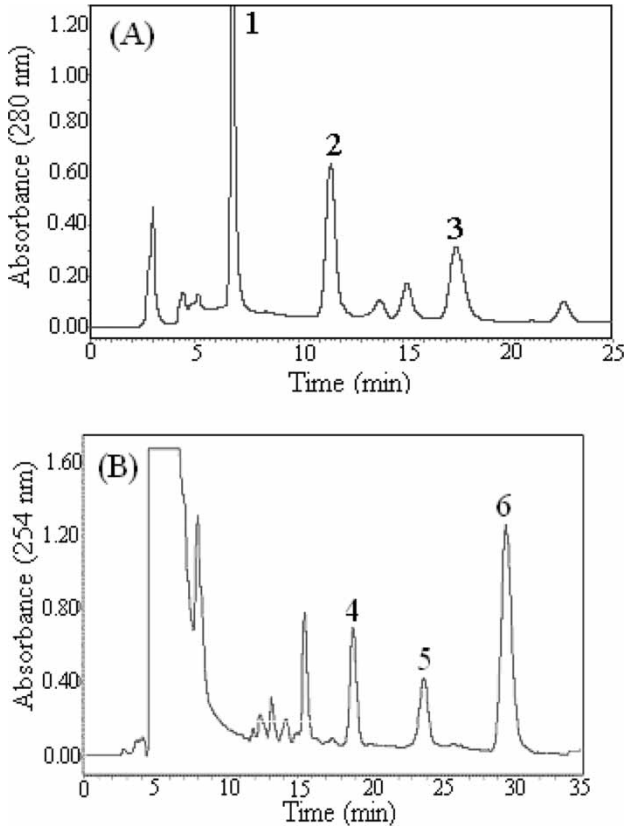

Figure 2. HPLC chromatograms of the ethanol extract from *H. japonicum* under different analytical conditions. (A) Column: reversed-phase Lichrospher C_{18} (4.6 mm × 250 mm i.d., 5 μm); mobile phase: $CH_3CN–H_2O–HAC$ (20:80:1, v/v/v); flow rate: 1.0 mL/min; UV wavelength: 280 nm; column temperature: 30°C; 1: isoquercitrin; 2: quercitrin; 3: quercetin-7-O-rhamnoside. (B) Column: reversed-phase Lichrospher C_{18} (4.6 mm × 250 mm i.d., 5 μm); mobile phase: $CH_3CN–H_2O–HAC$ (55:45:1, v/v/v); flow rate: 1.0 mL/min; UV wavelength: 280 nm; column temperature: 30°C; 4: sarothialen A; 5: sarothralen B; 6: sarothalin G.

Selection of Two-Phase Solvent Systems for HSCCC Isolation

In HSCCC, the selection of the two-phase solvent system is the most important for successful separation, and is also the most difficult step; it is estimated that about 90% of the entire work in HSCCC is spent on this. If only one compound needs to be separated from the others, the standard HSCCC method, which uses a constant flow rate of the mobile phase, could be used. In order to isolate more different compounds, stepwise elution or stepwise increasing the flow rate of the mobile phase might be adopted.[9,10]

First, we intended to simultaneously isolate and separate the six target compounds from the ethanol extract of the medicinal plant, and a lot of tests have been carried out including selection of suitable solvent systems, suitable flow rate of the mobile phase, column temperature, and revolution speed of the apparatus, and even the modes of stepwise elution and stepwise increasing the flow rate of the mobile phase were all examined. Especially, the solvent system composed of *n*-hexane–ethyl acetate–methanol–water, which could be used to isolate and separate a widely range of compounds by modifying the four kinds of solvents, was carefully examined. However, the results were not satisfactory. So, simultaneous isolation and separation of isoquercitrin, quercitrin, quercetin-7-O-rhamnoside, sarothialen A, sarothralen B, and sarothalin G from the ethanol extract could not be achieved by HSCCC in our research. Finally, we planed to use a two-step HSCCC separation mode to isolate the target compounds. A suitable solvent system was first selected to separate isoquercitrin, quercitrin and quercetin-7-O-rhamnoside from the ethanol extract, then the non-polar compounds, including sarothialen A, sarothralen B and sarothalin G retained in the HSCCC column was further separated by another suitable solvent system.

In the first step, the HSCCC experiment was carried out with the two-phase solvent system composed of *n*-butanol–water (1:1, v/v) to isolate the flavonoids. Although isoquercitrin could be separated from the crude sample, it was difficult to purify the other two compounds. Then, chloroform–methanol–water (4:3:2, v/v/v) was used. It was, however, not practical to purify the flavonoids from the crude extract, because the time they were retained in the column was too long. Subsequently, a two phase solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) was tested. Although the peak resolution was improved, and isoquercitrin could be separated from others, it was difficult to purify quercitrin and quercetin-7-O-rhamnoside, which were eluted out together in one fraction. With the solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v/v), the peak resolution was improved at 1.0 mL/min of the flow rate of the mobile phase, but quercetin-7-O-rhamnoside was retained in the column for a long period of time (>10 h). Finally, the method with stepwise increasing the flow rate of the mobile phase was developed with this kind of solvent system. That is, the flow rate of the mobile phase was kept at 1.0 mL/min before 120 min, and subsequently, increased to 2.0 mL/min after 2 h. The isolation of the target compounds was achieved with good peak resolution, and the retention of the stationary phase was 42%. After isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside were eluted, the remaining compounds in the HSCCC column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase. The selected solution was evaporated to dryness by rotary vaporization at 60°C under reduced pressure. A black solid (280 mg) was obtained and subjected to further HSCCC isolation. Figure 3 shows the preparative HSCCC

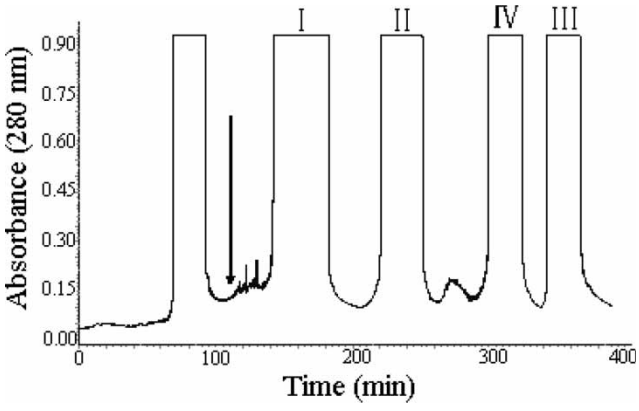

Figure 3. HSCCC chromatogram of the ethanol extract from *H. japonicum*. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate, 0–120 min, 1.0 mL/min and 120–400 min, 2.0 mL/min; detection wavelength: 280 nm; sample size: 750 mg; retention of stationary phase: 42%; separation temperature: 30°C; revolution speed: 800 rpm; sample loop: 20 mL. The arrow indicates the flow rate of the mobile phase was increased stepwise from 1.0 to 2.0 mL/min after 2 h.

isolation of 750 mg of ethanol extract, using the solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v/v), by increasing the flow rate of the mobile phase from 1.0 mL/min to 2.0 mL/min after 120 min. Four fractions (I, II, III, IV) were obtained according to the elution profile. Figure 4 shows the HPLC chromatogram of the remaining compounds

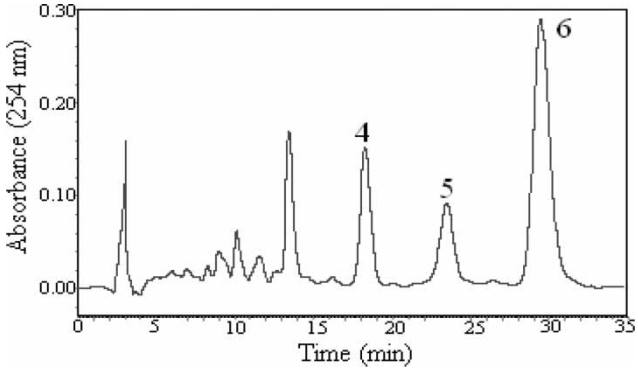

Figure 4. HPLC chromatogram of the remaining compounds in the HSCCC column after the first step isolation. HPLC conditions and the peaks were the same as shown in Fig. 2(B) .

retained in the HSCCC column after the first-step separation, which mainly contained sarothialen A, sarothralen B, and sarothalin G.

In the second step, a solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the volume ratio of 1:0.4:0.5:1 (v/v) was first examined, but no pure compound could be obtained in 10 h because of their long retention time in the column. The volume ratio of the solvent system was changed to 1:2:2:1 (v/v), however, it was very difficult to purify the phloroglucinol derivatives, because of their short retention time in the column. Subsequently, a solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the volume ratio of 1:1.2:1.2:1 (v/v) was tested at 1.2 mL/min of the flow rate of the mobile phase. The peak resolution was improved and sarothialen A was obtained, but sarothalin G was retained in the column for a long time (>10 h). Finally, the method with stepwise increasing the flow rate of the mobile phase was developed with this kind of solvent system. That is, the flow rate of the mobile phase was kept at 1.2 mL/min before 120 min, and subsequently increased to 2.2 mL/min after 2 h. The isolation of the target compounds was achieved with good peak resolution, and the retention of the stationary phase was 64%. After sarothialen A, sarothralen B, and sarothalin G were eluted out, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized

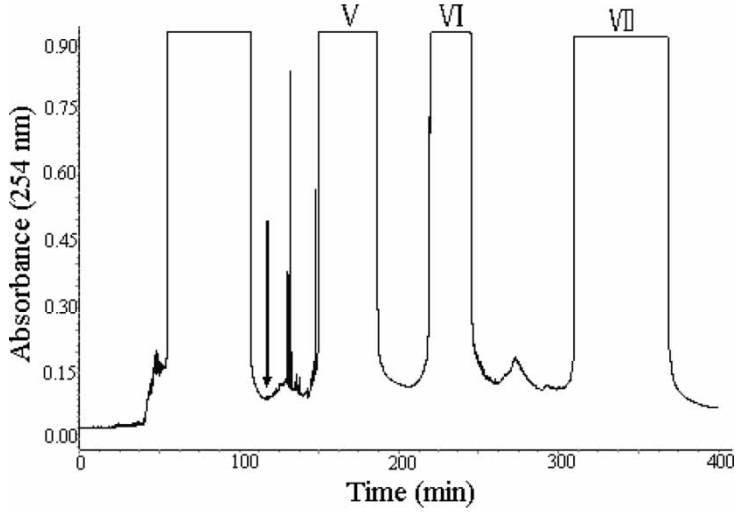

Figure 5. HSCCC chromatogram of the remaining compounds after the first step HSCCC separation. Solvent system: *n*-hexane–ethyl acetate–ethanol–water (1:1.2:1.2:1, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate, 0–120 min, 1.2 mL/min and 120–400 min, 2.2 mL/min; detection wavelength: 280 nm; sample size: 280 mg; retention of stationary phase: 64%; separation temperature: 30°C; revolution speed: 800 rpm; sample loop: 20 mL. The arrow indicates the flow rate of the mobile phase was increased stepwise from 1.2 to 2.2 mL/min after 2 h.

nitrogen gas instead of eluting them with the mobile phase, because the mobile phase was not to be reused. Figure 5 shows the preparative HSCCC isolation of 280 mg of crude extract after the first HSCCC isolation, using the solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the volume ratio of 1:1.2:1.2:1 (v/v) by increasing the flow rate of the mobile phase from 1.2 mL/min to 2.2 mL/min after 120 min. Three fractions (V, VI, VII) were obtained according to the elution profile.

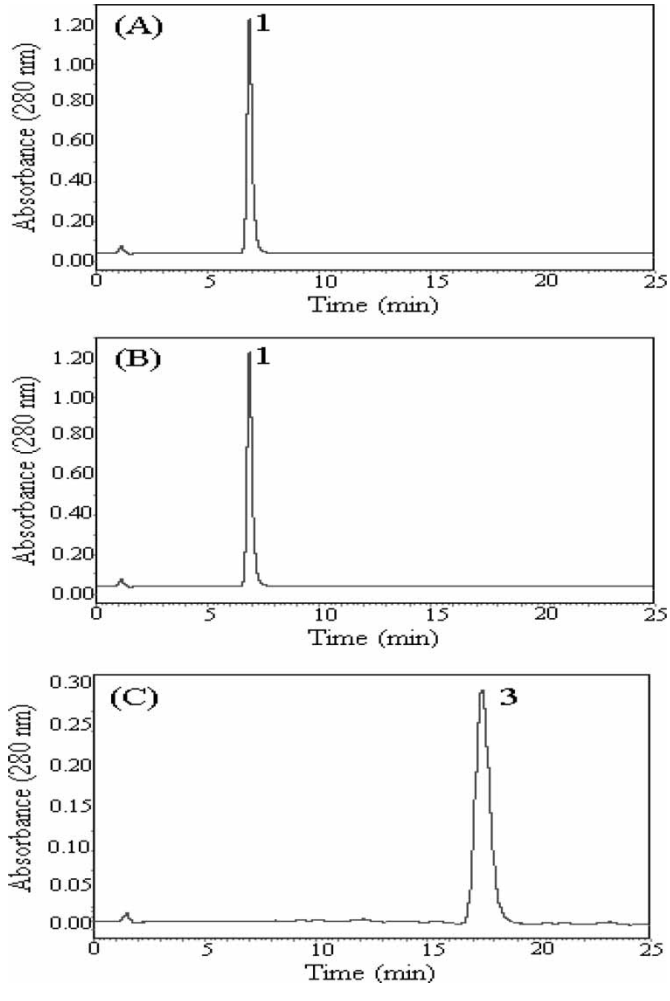

Figure 6. HPLC chromatograms of isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside purified from the ethanol extract by HSCCC. HPLC conditions and the peaks were the same as shown in Fig. 2 (A). A: fraction I purified by HSCCC; B: fraction II purified by HSCCC; C: fraction III purified by HSCCC.

HPLC Analysis and Chemical Structure Identification

The fractions obtained from HSCCC were all analyzed by HPLC. In the first step isolation, three fractions (I, II, III) contained only one peak with the purities of 98.6%, 95.8%, and 95.6%, respectively (Figure 6), and one (IV) was not a pure fraction and contained several peaks (chromatogram not shown). In the second step separation, the obtained three fractions (V, VI, VII) contained only one peak with the purities of 96.5%, 95.4%, and 98.6%, respectively (Figure 7).

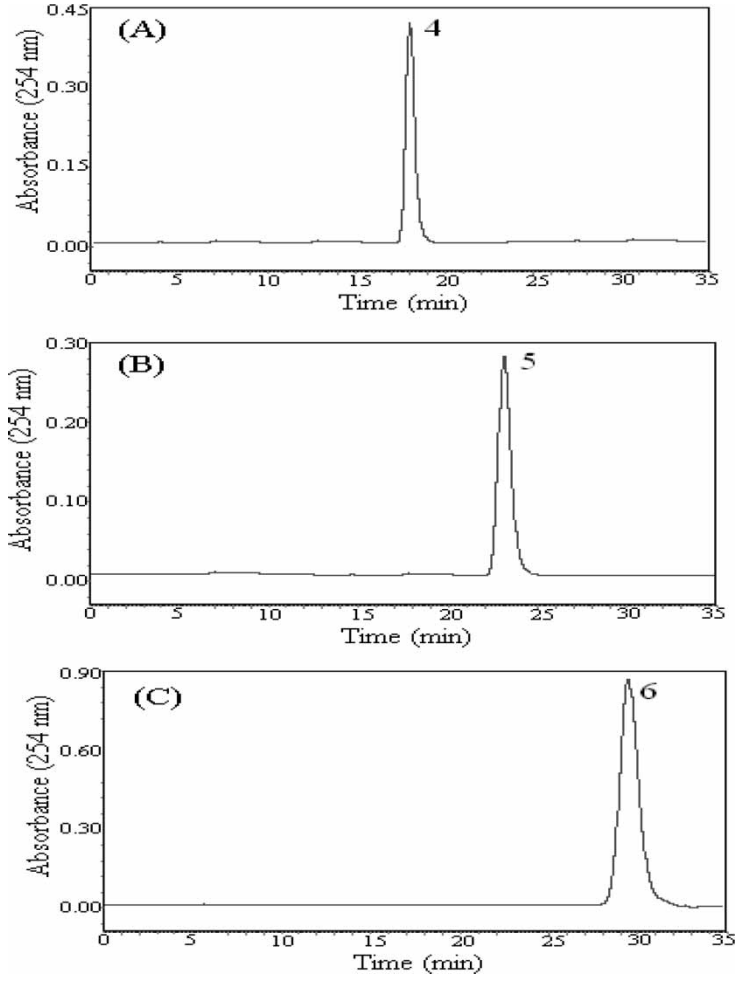

Figure 7. HPLC chromatograms of sarothialen A, sarothralen B, and sarothalin G purified from the remaining compounds after the first step HSCCC purification. HPLC conditions and the peaks were the same as shown in Fig. 2 (B). A: fraction V purified by HSCCC; B: fraction VI purified by HSCCC; C: fraction VII purified by HSCCC.

The isolation and purification produced a total of 124 mg isoquercitrin, 85 mg quercitrin, 68 mg quercetin-7-O-rhamnoside, 24 mg sarothialen A, 18 mg sarothralen B, and 58 mg sarothalin G from 750 mg ethanol extract of *H. japonicum* using this two-step preparation procedure.

The chemical structure identification was carried out by MS, ^{1}H NMR, and ^{13}C NMR as follows. Fraction I: light yellow powder, ESI-MS: 472.1 $[M + Na]^{+}$. Fraction II: light yellow powder. ESI-MS: 487.1 $[M + Na]^{+}$. Fraction III: yellow powder, ESI-MS: 448.0 $[M]^{+}$. Fraction V: light yellow plate, ESI-MS: 533.4 $[M - H]^{-}$. Fraction VI: yellow plate, ESI-MS: 565.3 $[M - H]^{-}$. Fraction VII: light yellow semi-solid, ESI-MS: 583.4 $[M\text{-}H]^{-}$. All ESI-MS spectra of the six fractions obtained from HSCCC are shown in Figure 8. Their ^{1}H NMR and ^{13}C NMR data are in agreement with those in the literatures.[2,11–16]

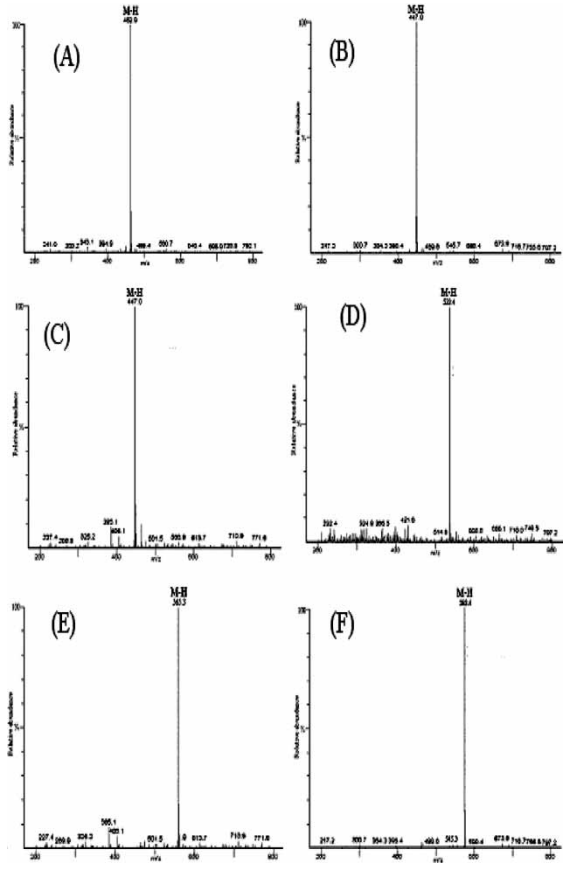

Figure 8. The ESI-MS spectra of the obtained compounds from *H. japonicum* by HSCCC.

CONCLUSION

The outcome indicated that an efficient method has been developed by us to isolate three flavonoids and three phloroglucinol derivatives from the medicinal plant *H. japonicum* using HSCCC by stepwise increasing the flow rate of the mobile phase. Three flavonoids, including isoquercitrin, quercitrin, and quercetin-7-O-rhamnoside, were successfully purified using HSCCC with a two-phase solvent system composed of ethyl acetate–ethanol–water at the volume ratio of 5:1:5 (v/v) by increasing the flow rate of the mobile phase from 1.0 mL/min to 2.0 mL/min after 120 min. Three phloroglucinol derivatives, including sarothialen A, sarothralen B, and sarothalin G, were successfully separated using HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at the volume ratio of 1:1.2:1.2:1 (v/v) by increasing the flow rate of the mobile phase from 1.2 mL/min to 2.2 mL/min after 120 min. Our results also indicated that HSCCC is a powerful tool to isolate and separate natural products from medicinal plants.

ACKNOWLEDGMENT

Financial support from Ministry of Science and Technology of China (863 project) is gratefully acknowledged.

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Received January 6, 2006
Accepted January 31, 2006
Manuscript 6809