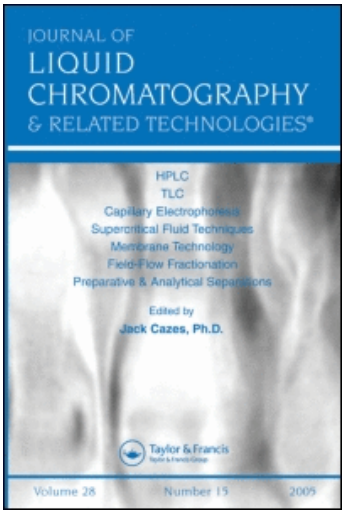

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Single-Step Preparative Isolation and Separation of Three Flavonones from *Sophora flavescens* using High-Speed Countercurrent Chromatography with Stepwise Increase in the Mobile Phase Flow Rate

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Single-Step Preparative Isolation and Separation of Three Flavonones from *Sophora flavescens* using High-Speed Countercurrent Chromatography with Stepwise Increase in the Mobile Phase Flow Rate

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Abstract: Three flavonoids, including sophoraflavanone G (SFG), kushenol I, and kurarinone were isolated and separated by high-speed countercurrent chromatography (HSCCC) from *Sophora flavescens*, a traditional Chinese medicinal herb. Preparative HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratio of 1:1:1:1 (v/v/v/v) was successfully performed by increasing the flow rate of the mobile phase from 1.0 mL/min to 2.0 mL/min after 120 min. The total isolation produced 22.5 mg SFG, 39.3 mg kushenol I and 83.5 mg kurarinone from 350 mg crude extract (containing SFG 6.7%, kushenol I 11.9%, kurarinone 25.7%) in one-step isolation with purities of 95.6%, 97.3%, and 99.4%, respectively, as determined by high performance liquid chromatography (HPLC), and the recoveries of the targets were 91.7%, 91.8%, and 92.3%, respectively. The chemical structural identification was performed by UV, IR, MS, ^{1}H NMR, and ^{13}C NMR.

Keywords: Preparative chromatography, Countercurrent chromatography, Flavonoid, *Sophora flavescens*, Plant material

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INTRODUCTION

Sophora flavescens (*Kushen* in Chinese), a famous traditional Chinese medicine herb, is cold by nature with bitter taste, entering three channels of heart, spleen and kidney. It has been used as a diuretic and for the treatment of acute dysenteric gastrointestinal hemorrhage and eczema[1–3] for thousands of years from *Sheng Nong's Herbal Classic*, a famous ancient medical literature, and now listed in the Chinese Pharmacopoeia. The major active ingredients are considered to be a number of quinolizidine alkaloids and a series of flavonoids.[1,2] Now, pharmacological tests have revealed the flavonoids of *S. flavescens* showed some beneficial actions.[2]

Sophoraflavanone G (SFG), kushenol I, and kurarinone are three prenylated flavonoids (shown in Fig. 1). Now, pharmacological tests revealed that these prenylated flavonoids showed strong antimicrobial activities against *Streptococcus mutans*, *Pythium vanterpooli*, *P. graminicola*,[4,5] and a strong inhibition effect to Tyrosinase;[6–8] they also showed anticancer actions against human myeloid leukemia HL-60 cells and human hepatocarcinoma HepG2 cells.[9] Furthermore, SFG exhibited strong inhibition activities to A549, HeLa, K562, and L1210 cancer cell cultures[10] and strong anti-inflammatory activity.[11,12] In view of these beneficial effects, an efficient method for the preparative separation and purification of SFG, kushenol I and kurarinone from natural sources is warranted.

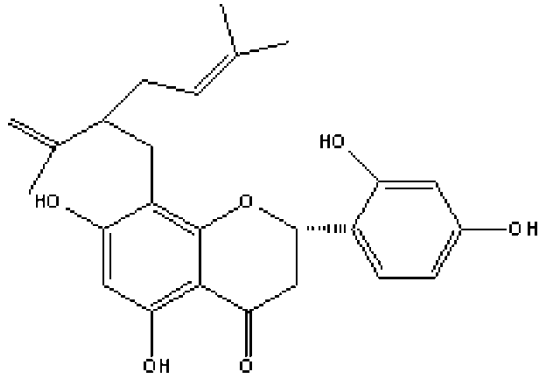
The preparative separation of SFG, kushenol I and kurarinone from medicinal plants by some classical methods are tedious, time consuming, and require multiple chromatographic steps. High-speed countercurrent chromatography (HSCCC), a support free liquid-liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto a solid support and exhibits excellent sample recovery. The method permits direct introduction of crude samples into the column without prior preparation, so it has been successfully applied to isolate and purify a number of natural products.[13–16] However, there have been no reports of using HSCCC to isolate and separate SFG, kushenol I and kurarinone from *S. flavescens*.

The aim of the present paper, therefore, was to develop an efficient method for the isolation and purification of SFG, kushenol I, and kurarinone from *S. flavescens* by high-speed countercurrent chromatography.

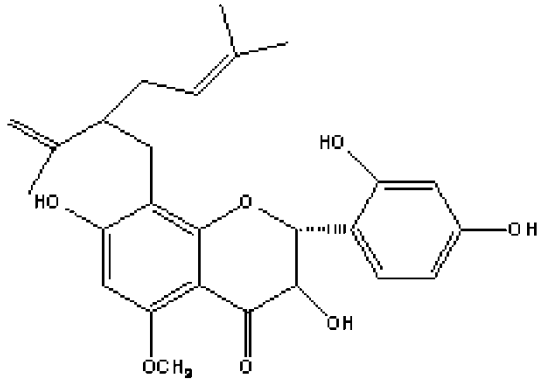
EXPERIMENTAL

Chemicals

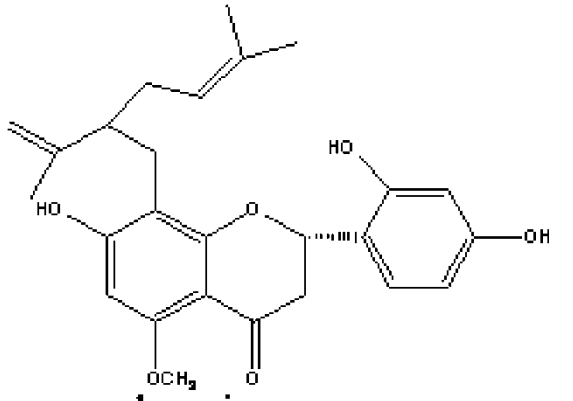
Ethyl acetate, chloroform, *n*-hexane, methanol, ethanol, and acetic acid were analytical grade and were purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was HPLC grade (Merck, Germany).



sophoraflavanone G (SFG)



kushenol I



kurarinone

Figure 1. The chemical structures of sophoraflavanone G (SFG), kushenol I, and kurarinone.

Reverse osmosis Milli-Q water, 18 MΩ, (Millipore, USA) was used for all solutions and dilutions.

The *S. flavescens* was purchased from a local drug store (Shanghai, China) and identified by Doctor Luping Qin, Department of Pharmacognosy, College of Pharmacy, the Second Military Medical University, Shanghai, P. R. China.

Preparation of the Crude Extract

The *S. flavescens* was ground to a powder. A 4,000 mL volume of 80% aqueous ethanol was added into a bottle (5,000 mL) which contained 500 g of the powder for extraction. The extraction was reflux, two times, and 1.5 h for each. The filtrates were combined and evaporated to remove ethanol under reduced pressure at 60°C and a residue was obtained; then, the residue was re-dissolved in water, which was added into a glass column (6.0 cm × 80.0 cm) which contained 300 g polyamide. Water was first used to elute the polyamide until the elution was nearly colorless, then, 95% ethanol (4,000 mL) was used to elute the target compounds, and 10 elution fractions (400 mL each) were collected. Four elution fractions (4 to 7) were combined, based on HPLC analysis, and evaporated to dryness. This was used for further HSCCC isolation and separation.

HSCCC Separation and Isolation

Preparative HSCCC was carried out with a model TBE–300A high-speed countercurrent chromatograph (Shenzhen, Tauto Biotech, China). The apparatus was equipped with three polytetrafluoroethylene 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-Vis 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).

In the HSCCC experiment, the coil column was first entirely filled with the upper phase of the solvent system, composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratio of 1:1:1:1 (v/v/v/v). 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. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution (350 mg crude extract dissolved in 15 mL lower-phase) was injected through the injection valve. After 120 min, the flow-rate of the

mobile-phase was increased to 2.0 mL/min. The effluent of the column was continuously monitored at 280 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 30°C.

HPLC Analysis

The analytical HPLC system used throughout this study consisted of a Model 515 pump (Waters, USA), Model 2487 detector (Waters, USA), a Model N2000 workstation (Zhejiang University, Hangzhou, China), and a 20 μL sample loop. The crude sample and each peak fraction obtained by HSCCC were analyzed by high performance liquid chromatography. The column used was a reversed-phase Lichrospher C_{18} (6.0 mm × 150 mm i.d., 5 μm) (Hanbang Science, Jiang-Su Province, China) with a pre-column filled with the same stationary phase. In the present study, some organic-aqueous based mobile phases were tested on a reversed-phase C_{18} column, including methanol-water and acetonitrile-water in combination with acetic acid, phosphate buffer, or phosphoric acid. The flow rate of the mobile phase and the temperature of the column, which might affect the separation, were also tested.

Identification of the Obtained Fractions from HSCCC

The nuclear magnetic resonance (NMR) spectrometer used for this work was a Varian Unity Inova−500 NMR system with TMS as internal standard and the mass spectrometer (MS) was a quadrupole-time of flight (Q-TOF) tandem MS system equipped with a turbo-ion spay source (APIQ-STAR Pulsar *i*, Applied Biosystems, Concord, Ont., Canada). Negative ion modes of ESI were used for structural analyses using the following conditions: a syringe pump at a flow rate of 200 μL/min; drying gas was nitrogen at 7.0 L/min; and nebulizer pressure was set at 5 psi. ESI-MS (negative mode) parameters: capillary: +4500 V, and plate +4000 V, cap exit −90 V, cap exit offset −60 V, skim1 −30 V, skim 2 −10 V. UV (Cary-50) and IR (Hitachi 275-50) were also used.

RESULTS AND DISCUSSION

HPLC Analytical Conditions

The crude extract was first analyzed by HPLC. As a result, an excellent separation was achieved by the following separation conditions: the mobile phase composed of acetonitrile−water−acetic acid (50:50:2, v/v/v) was isocratically eluted at a flow rate of 1.0 mL/min and 30°C, and UV detection was set at 280 nm. Complex gradient of mobile phase and buffer were unnecessary. The HPLC chromatogram of the crude extract is shown in Fig. 2, in which

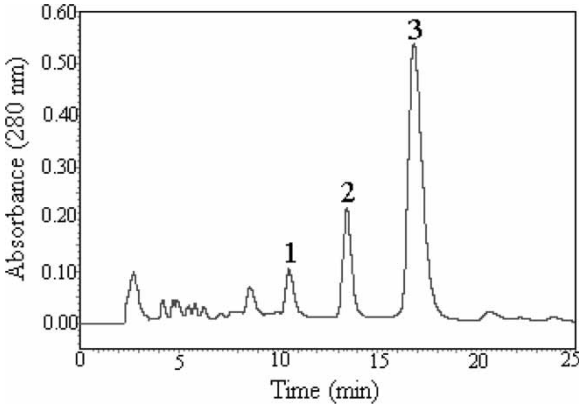


Figure 2. HPLC of the crude extract from *S. flavescens* after polyamide column chromatography. Peak 1: SFG; peak 2: kushenol I; peak 3: kurarinone.

SFG (peak 1), kushenol I (peak 2), and kurarinone (peak 3) presented at contents of 6.7%, 11.9%, and 25.7%, respectively.

HSCCC Isolation Conditions

In HSCCC, the selection of a two-phase solvent system is 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 solvent system selection. 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 a greater number of different compounds, step-wise elution or stepwise increase of the flow-rate of the mobile phase might be adopted.[17,18] Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of chloroform–methanol–water (4:3:2, v/v/v). Although kurarinone could be separated from the other compounds, it was difficult to purify kushenol I and SFG.

In the following work, a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water, which could be used to isolate and separate a broad range of hydrophobicity by modifying the volume ratio of the four solvents at the volume ratio of 1:2:2:1 (v/v/v/v) was first used. It was, however, very difficult to isolate and separate the three target compounds from the crude extract, because the time during which they were retained in the column was short. Subsequently, the two-phase solvent system at a volume ratio of 1:0.7:0.7:1 (v/v/v/v) was also tested. Although the peak resolution was improved and SFG could be separated, it was difficult to separate the other two compounds. When the volume ratio was converted to 1:1:1:1 (v/v/v/v), the peak resolution was improved, but

kurarinone was retained in the column for a long time (10 h). Finally, the method with stepwise increase in the flow rate of the mobile phase was attempted with this two-phase solvent system. That is, the flow-rate of the mobile phase was kept at 1.0 mL/min for the first 120 min, and subsequently increased to 2.0 mL/min. The isolation of the target compounds was achieved with good peak resolution. After SFG, kushenol I, and kurarinone were eluted, in order to conserve solvent and time, the remaining compounds in the column were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase, because the stationary phase was not going to be reused.

The influence of the separation temperature and the revolution speed were also investigated. The temperature has a significant effect on K values, the retention of stationary phase, and the mutual solvency of the two-phase system. After being tested at 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C, it can be seen that good results can be obtained when the separation temperature was controlled at 30°C. The revolution speed has a great influence on the retention of stationary phase; a high rotary speed can increase the retention of the stationary phase. In our experiments, the revolution speed was set at 800 rpm.

Under the optimized conditions, three fractions (I, II, and III) were obtained in one-step elution in less than 6 hours (HSCCC chromatogram shown in Fig. 3), and the retention of the stationary phase was 65%.

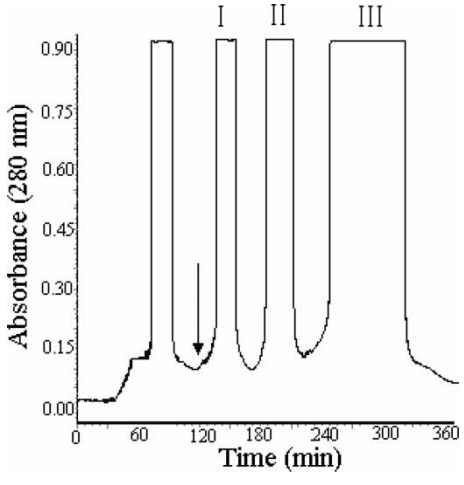


Figure 3. Chromatogram of the crude extract by preparative HSCCC. solvent system: *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/v/v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 0–2 h, 1.0 mL/min and 2–6 h, 2.0 mL/min; revolution speed: 800 rpm; separation temperature: 30°C; sample size: 350 mg; sample loop: 20 mL; detection wavelength: 280 nm; retention of the stationary phase: 65%. The arrow indicates the flow-rate of the mobile phase was increased stepwise from 1.0 mL/min to 2.0 mL/min after 2 h.

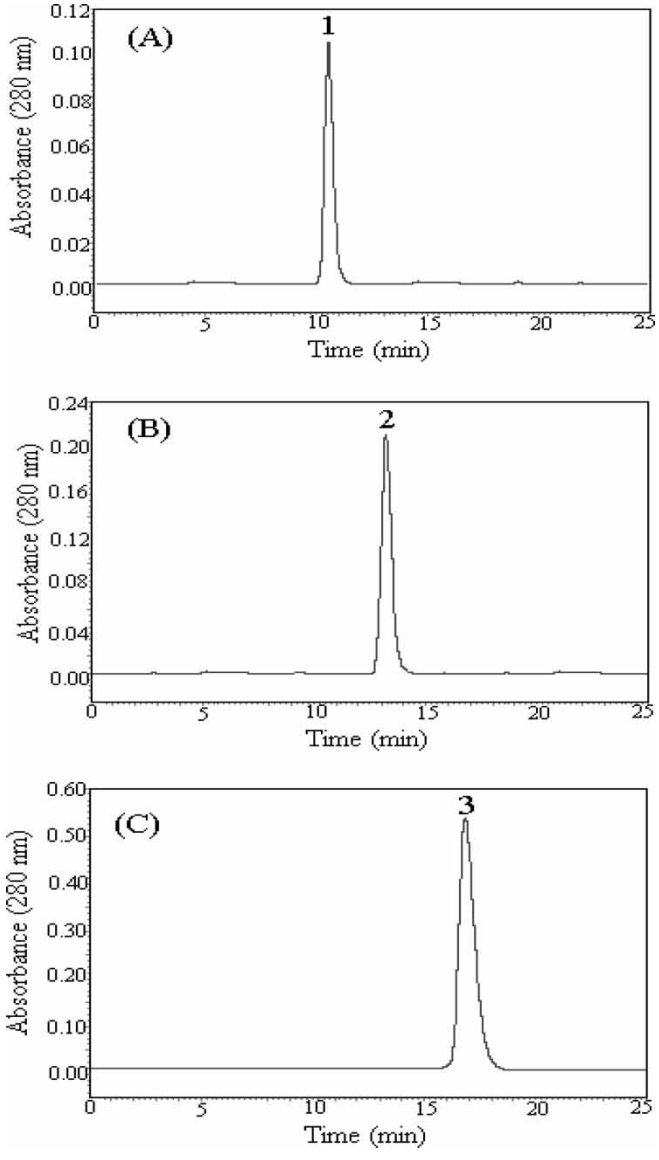


Figure 4. The HPLC analysis of the fractions obtained by HSCCC. A: fraction I; B: fraction II; C: fraction III; peak 1: SFG; peak 2: kushenol I; peak 3: kurarinone.

The isolation procedure yielded 22.5 mg SFG, 39.3 mg kushenol I, and 83.5 mg kurarinone from 350 mg crude extract, in a one-step isolation, with the purities of 95.6%, 97.3%, and 99.4%, respectively, as determined by high performance liquid chromatography (HPLC chromatogram is shown in Fig. 4). As expected, the HPLC analysis of each fraction revealed that the

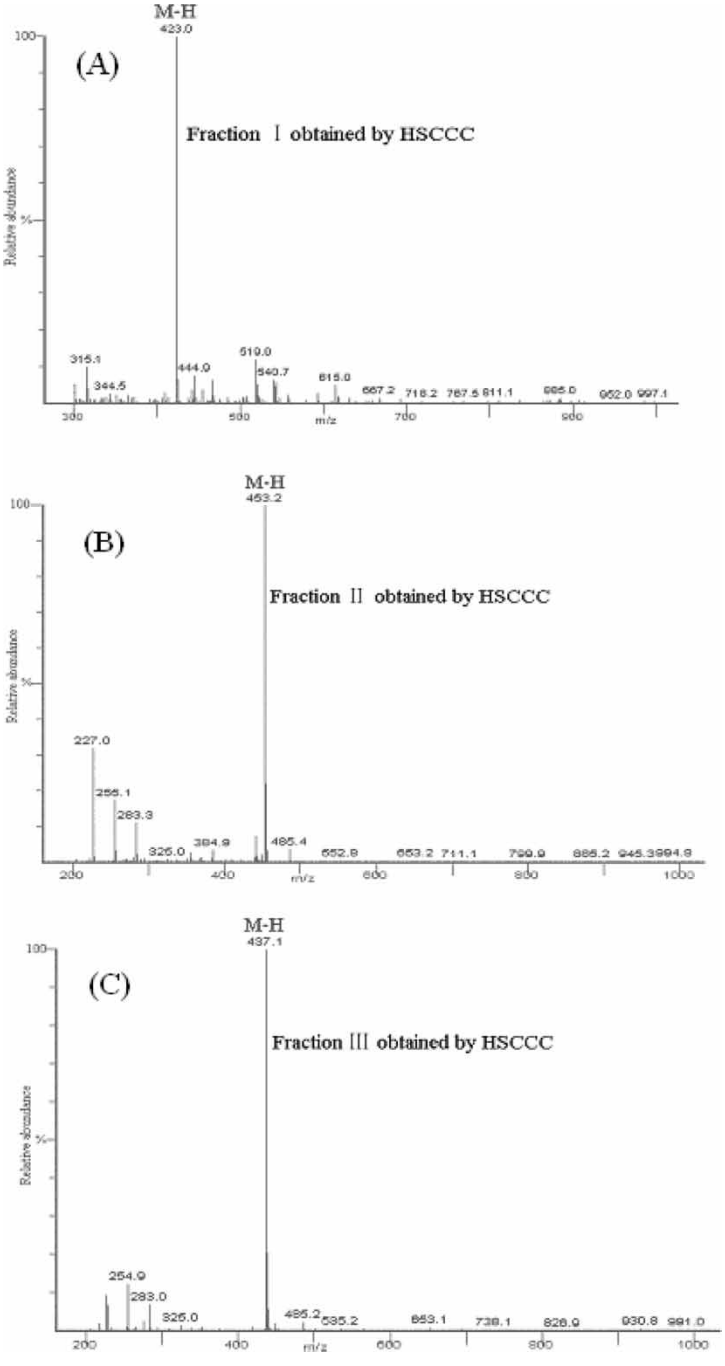


Figure 5. The ESI-MS spectra of the obtained fractions from HSCCC. A: fraction I; B: fraction II; C: fraction III.

components eluted in the order of peaks 1 (SFG), 2 (kushenol I), and 3 (kurarinone).

Chemical Structural Identification

The structural identification of the isolated components was performed by MS, ^{1}H NMR, ^{13}C NMR, UV, and IR as follows. Fraction "I" (SFG): a white amorphous powder; mp 128 $\sim$ 130°C; UV λ_{max}^{MeOH} (logε): 217.5 (4.64), 251.2 (4.20), 276.5 (3.53). IRυ_{max} (KBr) cm^{-1}: 3490 (OH), 1593 (aromatic); ESI-MS: 423 [M-H]^{M} (Fig. 5). Fraction "II" (kushenol I): light yellow powder, mp 115 $\sim$ 116°C; UV λ_{max}^{MeOH} (logε): 218.0 (4.62), 254.2 (4.32), 274.5 (3.57). IRυ_{max} (KBr) cm^{-1}: 3470 (OH), 1610 (aromatic); ESI-MS: 453 [M-H]$^{-}$ (Fig. 5). Fraction "III"(kurarinone): a white amorphous powder; mp 86 $\sim$ 88°C; UV λ_{max}^{MeOH} (logε): 219.0 (4.61), 251.5 (4.06), 273.0 (3.59); IRυ_{max} (KBr) cm^{-1}: 3360 (OH), 1615, 1595 (aromatic); ESI-MS: 437 [M-H]$^{-}$ (Fig. 5). Compared with the reported data, the MS, ^{1}H NMR, and ^{13}C NMR data are in agreement with those of SFG, kushenol I, and kurarinone in the literature.[19–23]

CONCLUSION

In the present paper, three prenylated flavonoids, including SFG, kushenol I, and kurarinone are separated from the Chinese medicinal plant *S. flavescens* by HSCCC with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:1:1:1, v/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. 22.5 mg SFG, 39.3 mg kushenol I and 83.5 mg kurarinone with high purities from 350 mg crude extract were obtained in a single isolation step. Our study demonstrates that HSCCC is a powerful method for separating and isolating bioactive components from natural sources.

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