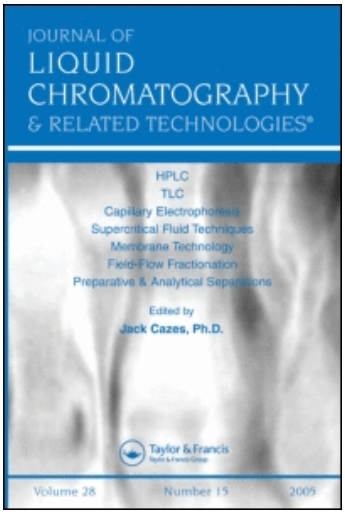

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SEPARATION AND PURIFICATION OF FLAVONOIDS FROM BLACK CURRANT LEAVES BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY AND PREPARATIVE HPLC

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SEPARATION AND PURIFICATION OF FLAVONOIDS FROM BLACK CURRANT LEAVES BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY AND PREPARATIVE HPLC

Dajun He,[1,2,3] Yun Huang,[1,3] Amatjan Ayupbek,[1,3] Dongyu Gu,[1,3] Yi Yang,[1,3] Haji Akber Aisa,[1] and Yoichiro Ito[4]

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□ *High-speed countercurrent chromatography (HSCCC) has been successfully used for the preparative isolation of flavonoids from the ethyl acetate extracts of black currant leaves. The HSCCC separation was performed with a two-phase solvent system composed of n-hexane/EtOAc/MeOH/H_2O (1:10:1:10, v/v) at a flow rate of 1.5 mL/min. When the flow rate was increased from 1.0 to 3.0 mL/min, the retention of stationary phase decreased from 60.3% to 39.7% resulting in a loss of peak resolution, while the stationary phase retention is stable with an increase in sample size from 25 to 200 mg. From 100 mg of the crude sample, HSCCC separation yielded 4.0 mg of kaempferol 3-O-galactoside, 6.0 mg of kaempferol 3-O-glucoside, and 9.0 mg of fraction I containing a mixture of hyperoside and isoquercitrin. Then, from 18 mg of fraction I, 3.0 mg of hyperoside and 11.0 mg isoquercitrin were separated by preparative HPLC by successive sample injection at every 100 min interval. Chemical structures of all these compounds were confirmed by MS and NMR.*

Keywords black currant, high-speed countercurrent chromatography (HSCCC), hyperoside, isoquercitrin, kaempferol 3-O-galactoside, kaempferol 3-O-glucoside

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INTRODUCTION

The leaves of black currant (*Ribes nigrum* L.) have received considerable attention for its putative human health benefits. Most studies have been focused on the condensed fractions rich in tannins such as prodelphinidins and proanthocyanidins due to their anti-inflammatory activity.[1–3] Jessica Tabart demonstrated that black currant leaves (in June) had a higher content in phenolics and antioxidants than fully ripened berries where the total phenolic level was correlated with antioxidant activity.[4] These phenolic compounds, such as flavonoids, have long been recognized to possess antiallergenic, anti-inflammatory, antiviral, and antiproliferative activities,[5,6] and antioxidative[7,8] and anticarcinogenic effects,[9] and they can reduce the risk of coronary heart disease,[10,11] and stroke.[12] In order to provide a better understanding of these pharmacological functions and to further exploit the important plant's resource, isolation and structure elucidation of the lead structures are essential.

High-speed countercurrent chromatography (HSCCC) is a support free liquid-liquid partition chromatographic technique, and eliminates irreversible adsorption of sample onto the solid support. Since the 1980s, the method has been widely applied for the separation and purification of various natural compounds.[13–16] In the present study, kaempferol 3-O-galactoside, kaempferol 3-O-glucoside, and fraction I containing hyperoside and isoquercitrin, were successfully separated by HSCCC, and hyperoside and isoquercitrin were further separated by prep HPLC.

EXPERIMENTAL

Apparatus

The preparative HSCCC instrument employed in this study was a model TBE-300A high speed countercurrent chromatograph (Tauto Biotech, Shanghai, China) with three polytetrafluoroethylene (PTFE) preparative coils (tubing ID: 2.6 mm; total volume: 290 mL). The revolution radius or the distance between the holder axis and the 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). An optimum speed of 800 rpm was used throughout in the present study. The solvent was pumped into the column with a model TBP-50A constant-flow pump (Tauto Biotech, Shanghai, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor at 254 nm, and a manual sample injection valve with a 20 mL loop for the preparative HSCCC was used to introduce the sample into the column.

Model N2000 workstation (Zhejiang University, Hangzhou, China) was used to draw the chromatogram.

The analytical high performance liquid chromatography (DIONEX, USA) equipment used was a DIONEX Ultimate 3000 system. The analysis was carried out with an inertsil ODS-SP column (5 μm, 4.6 × 250 mm, GL Sciences Inc, Japan). Evaluation and quantification were made on a Chromeleon WorkStation.

The prep HPLC system equipment used was a DIONEX system including a P680 pump, an ASI-100 Automated sample injector, a TCC-100 thermostatted column compartment, and a UVD170U detector. The separations were carried out on a Prep C_{18} column (5 μm, 10 × 250 mm, X-Bridge^{TM}, Waters, USA). Evaluation and quantification were made on a Chromeleon WorkStation.

Reagents

All organic solutions used for HSCCC were of analytical grade and purchased from Tianjin Chemical Factory (Tianjin, China). Methanol and acetonitrile used for HPLC were of HPLC grade and purchased from Fisher Scientific Company (Fair Lawn, NJ, USA).

The black currant leaves were collected from Emin County, Xinjiang, China in August, 2008.

Preparation of Crude Sample

Dry black currant leaves (1 kg) were extracted with 5 L of methanol for 24 h. The extraction procedure was repeated three times. After concentration under reduced pressure, the extract was diluted with water to a total volume of 1 L and partitioned with petroleum ether (3 × 1 L) first, and then the aqueous phase was extracted with ethyl acetate (3 × 1 L) to give ethyl acetate fraction. This fraction was concentrated by a rotary evaporator at 40°C, yielding 5.8 g of a crude sample, which was subjected to HSCCC separation and purification.

Measurement of Partition Coefficient (K)

The two-phase solvent system was selected according to the partition coefficient (K) of the target components. Different ratios of n-hexane-ethyl acetate-methanol-water (HEMW) were prepared and equilibrated in a separation funnel at room temperature. The K values were determined by HPLC analysis as follows: A suitable amount of samples (1 mg) was added to 4.0 mL of the mixture containing equal volume of each phase

of the two-phase solvent system in a test tube, and the contents were mixed thoroughly. After the equilibration was completed, the upper phase and the lower phase were each separately analyzed by HPLC. The peak area of the upper phase was recorded as A_U and that of the lower phase, as A_L. The K value was calculated according to the equation, $K = A_U/A_L$.

HSCCC Separation

The preparative HSCCC was performed with a model TBE-300A HSCCC instrument as follows: The multilayer coil separation column was first entirely filled with the upper phase as the stationary phase. The lower phase was then pumped into the head end of the column at a given flow rate, while the apparatus was run at a revolution speed of 800 rpm. After hydrodynamic equilibrium had been reached, the sample solution (100 mg of the crude sample in 4 mL of a mixture of upper and lower phases) was injected through the sample port. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions, I, II, and III, were manually collected according to the chromatogram. When the separation was completed, revolution was stopped and the column contents were collected into a graduated cylinder by connecting the column inlet to a nitrogen cylinder. The retention of stationary phase was computed by dividing the volume of the recovered stationary phase with the total volume collected.

Preparative HPLC

The prep HPLC separation was performed with a Prep C_{18} column (5 μm, 10 × 250 mm, X-Bridge™, Waters, USA) at column temperature of 35°C. The mobile phase solvents consisted of isocratic methanol and 0.2% formic acid (20:80, v/v). The flow rate was 0.8 mL/min and 50 μL of sample solution (70 mg/μL of fraction I from HSCCC separation in methanol) was injected through the sample injector. The effluent from the outlet of the column was monitored at 254 nm. The peak fractions were collected according to the chromatogram.

HPLC Analysis and Identification of Crude Sample and the Peak Fraction from HSCCC and Prep-HPLC

The crude sample and the peak fractions from HSCCC and prep HPLC were analyzed by HPLC. The analyses were performed with an inertsil ODS-SP column (4.6 × 250 mm, 5 μm) at column temperature of 35°C. The mobile phase was eluted with a linear gradient of acetonitrile (A),

methanol (B), and 0.2% formic acid (C) as follows: A-B-C (10:10:80, v/v) to A-B-C (15:15:70, v/v) in 15 min, A-B-C (0:55:45, v/v) in 35 min, then to A-B-C (0:80:20, v/v) in 6 min, and finally to A-B-C (0:80:20, v/v) in 4 min. The flow rate was 1.0 mL/min and the effluent was monitored at 254 nm by a UV detector.

Identification of the HSCCC peak fractions was carried out by MS, ^{1}H-, and ^{13}C-NMR.

RESULTS AND DISCUSSION

The crude sample of black currant was analyzed by HPLC first. The result indicated that it contained several flavonoids, including hyperoside (retention time: 15.7 min), isoquercitrin (retention time: 16.1 min), kaempferol 3-O-galactoside (retention time: 18.8 min), kaempferol 3-O-glucoside (retention time: 20.0 min), and some other unknown compounds as shown in Figure 1a.

In HSCCC, successful separation depends upon the selection of a suitable two-phase solvent system, which requires the following considerations: retention of the stationary phase should be satisfactory, which is indicated by the short settling time of the solvent system in a test tube (<25 sec), and the partition coefficient of the target compound is between 0.4–2.5.[17,18] A series of experiments were performed to optimize the two-phase solvent system for HSCCC separation using several different volume ratios of n-hexane-ethyl acetate-methanol-water. The K values of four target compounds in these solvent systems are shown in Table 1. The two-phase solvent system of n-hexane-ethyl acetate-methanol-water (1:10:1:10, v/v) was found to be suitable for the separation of compounds **3** and **4**. But since the K values of compounds **1** and **2** were very similar, it was impossible to separate them in any of these two-phase solvent systems. Unfortunately, hyperoside and isoquercitrin often coexist in the natural resource. In the past study, several polyphenols were separated from ethyl acetate extract of apple pomace by HSCCC. However, hyperoside and isoquercitrin were not separated using the two-phase solvent system composed of n-hexane-ethyl acetate–1% acetic acid (1:9:10, v/v) at a flow rate of 2 mL/min.[19] In the past, quercitrin, quercetin, hyperoside, and isoquercitrin were separated from *Hypericum perforatum* L. by HSCCC. It also failed to purify the last two compounds, hyperoside and isoquercitrin.[20] These studies indicate that hyperoside and isoquercitrin are very difficult to be separated by HSCCC. So we decided to separate the compound 3, 4, and a mixture of compounds 1 and 2 (fraction I) first by HSCCC, and then the fraction I is further separated by prep HPLC.

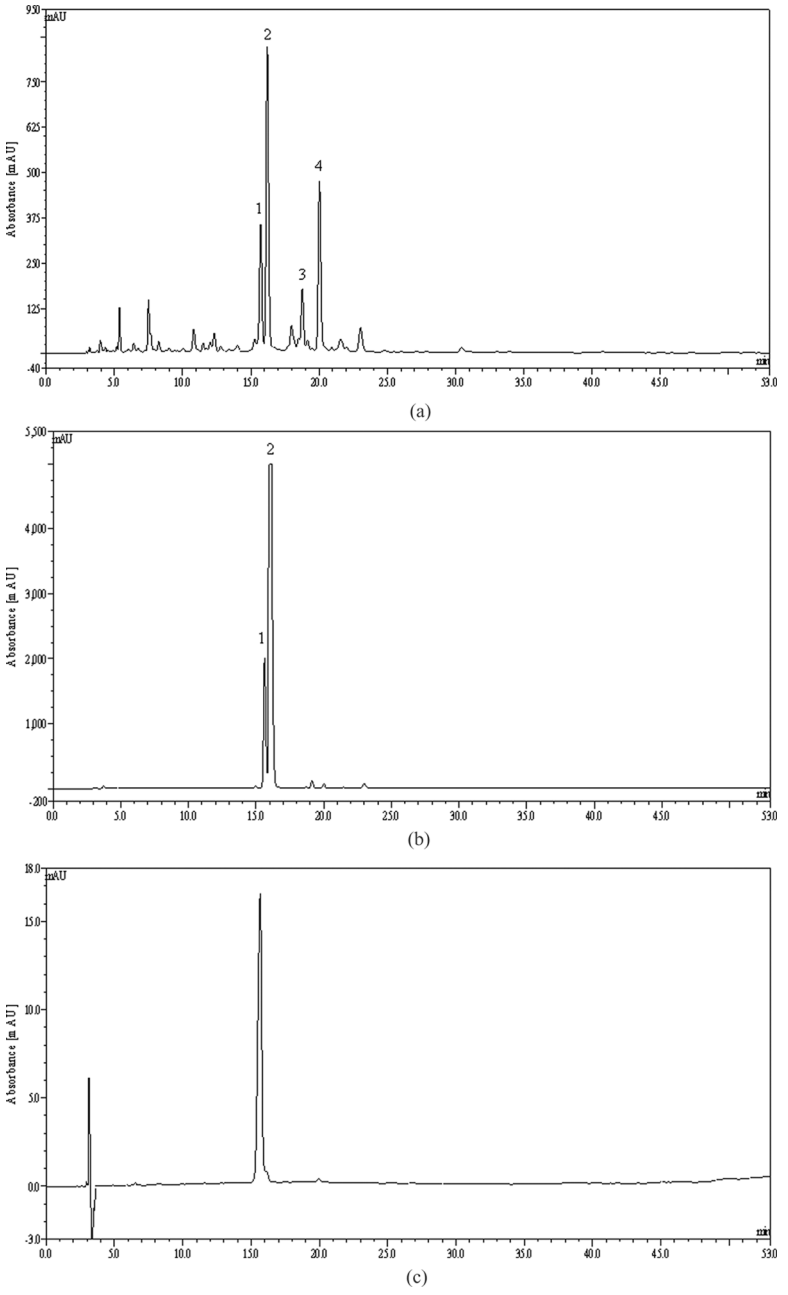
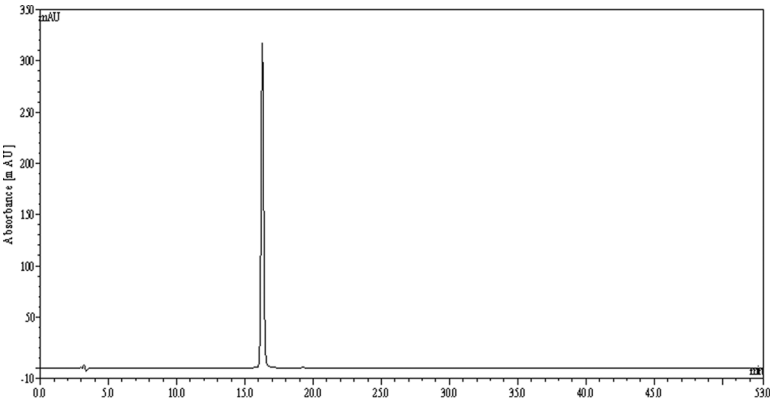
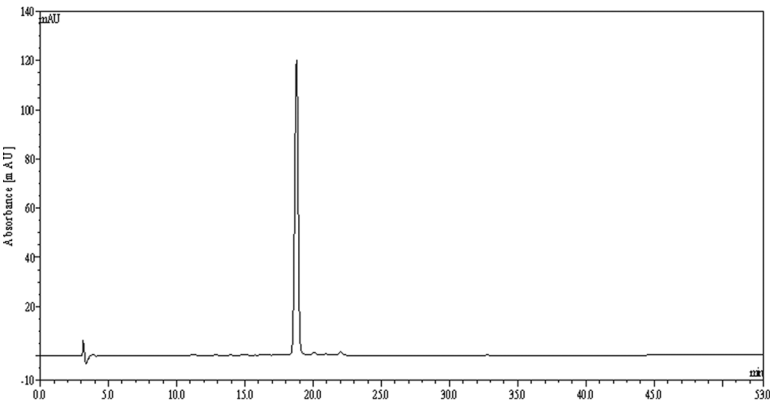


FIGURE 1 HPLC chromatogram of the crude sample and HSCCC and prep HPLC fractions from black currant leaves. Column: inertsil ODS-SP column (4.6 mm × 250 mm, 5 μm); the mobile phase A: acetonitrile, B: methanol, C: 0.2% formic acid, the gradient was as follows: A-B-C (10:10:80, v/v) to A-B-C (15:15:70, v/v) in 15 min, then to A-B-C (0:55:45, v/v) in 35 min, then to A-B-C (0:80:20, v/v) in 6 min, and finally to A-B-C (0:80:20, v/v) in 4 min, detection: 254 nm; flow rate: 1.0 mL/min. (a) ethyl acetate fraction from black currant, (b) mixture of hyperoside (**1**) and isoquercitrin (**2**), (c) hyperoside (**1**), (d) isoquercitrin (**2**), (e) kaempferol 3-O-galactoside (**3**), (f) kaempferol 3-O-glucoside (**4**).





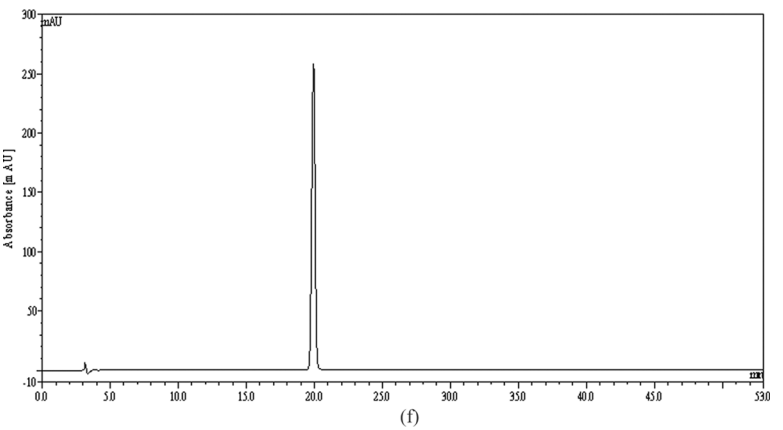


FIGURE 1 Continued.

TABLE 1 K Values of Compounds 1, 2, 3, and 4 in the HEMW Solvent System

	K			
Ratio (v/v)	1	2	3	4
3:5:3:5	0.05	0.03	0.04	0.06
2:5:2:5	0.11	0.08	0.11	0.15
1:5:1:5	0.30	0.28	0.38	0.54
1:10:1:10	0.66	0.63	0.88	1.23
0:5:0:5	1.18	1.18	1.62	2.22

In order to obtain good peak resolution within acceptable separation time, the flow rate and sample size were evaluated. The flow rate was tested from 1 mL/min to 3 mL/min with the 50 mg sample size. The results showed that the high flow rate decreased resolution (Rs) between fraction II and III (Fig. 2), apparently due to lower retention of stationary phase (Fig. 3), though the retention time was reduced. With an increased flow rate from 1.0 to 3.0 mL/min, the stationary phase retention decreased from 60.3% to 39.7%. At the flow rate of 1.0 mL/min, peaks I – III were eluted within 390 minutes with the retention of stationary phase at 60.3% as shown in Figure 2e. When the flow rate was increased to 3.0 mL/min, the whole retention time was shortened to 120 minutes (Figure 2a) while the retention of stationary phase was reduced to 39.7%, resulting in loss of peak resolution between peaks II and III containing compounds 3 and 4, respectively. These results indicate that retention of the stationary phase is sharply decreased by increased flow rate in this HSCCC separation. Therefore, the flow rate of 1.5 mL/min was used for the separation because of the acceptable resolution of target fractions (Fig. 2d).

Then, the effect of the sample size was further investigated at the flow rate of 1.5 mL/min. With increased sample size from 25 to 200 mg, the stationary phase retention was very stable and always maintained at about 60%. However, when the amount of sample exceeded 100 mg, the resolution of peak II and III was decreased (Fig. 4).

The HSCCC separation was performed with the sample size of 100 mg at the flow rate of 1.5 mL/min. The HSCCC run yielded fractions of three separated peaks, which were each combined to obtain fraction I (135–150 min), fraction II (172–188 min), and fraction III (206–228 min), respectively, as shown in Fig. 2d. The fractions II and III corresponded to compound **3** and **4**, and their purities were 97.1 and 98.5%, respectively, as shown in Figs. 1e and f. The fraction I was a mixture of compounds **1** and **2**, their purities being 16.6% and 80.0%, respectively, as shown in Fig. 1b. From 100 mg of the crude sample, 9.0 mg of a mixture of

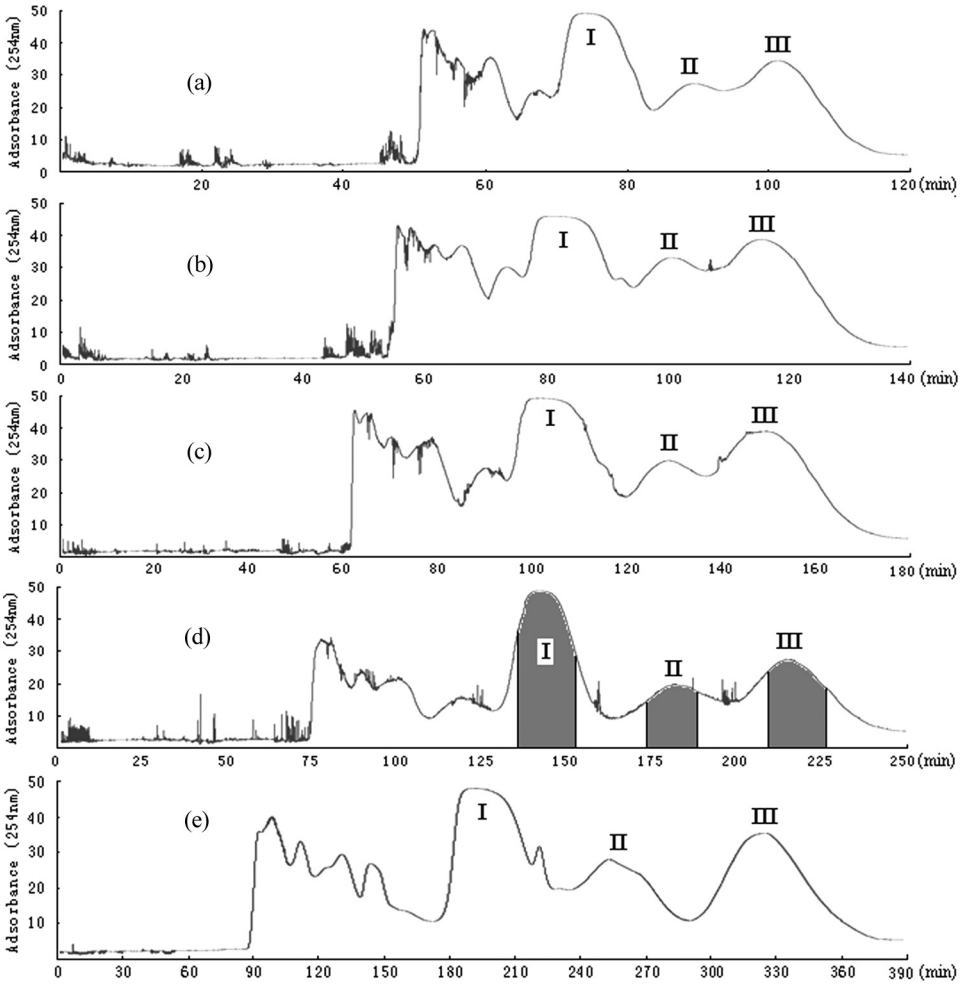


FIGURE 2 Comparison of performance of HSCCC chromatograms from black currant leaves at the different given flow rates. Flow rates (mL/min): (a) 3.0, (b) 2.5, (c) 2.0, (d) 1.5, (e) 1.0. Solvent system: HEMW (1:10:1:10 v/v); stationary phase: upper organic phase; rotational speed: 800 rpm; detection wavelength: 254 nm; sample size: 50 mg.

compounds **1** and **2** (fraction I), 4.0 mg of compound **3** (fraction II), 6.0 mg of compound **4** (fraction III) were separated by one step HSCCC separation. The HSCCC separation was repeated twice in order to prepare enough amount of fraction I for prep HPLC separation and the eluate was detected at 254 nm. After successively loading the sample five times each at every 100 min interval, prep HPLC separation yielded 3.0 mg of compound **1** and 11.0 mg of compound **2** from 18.0 mg of fraction I in 785 min, as shown in Fig. 5. The purity of compounds **1** and **2** was 98.1% and 99.4%,

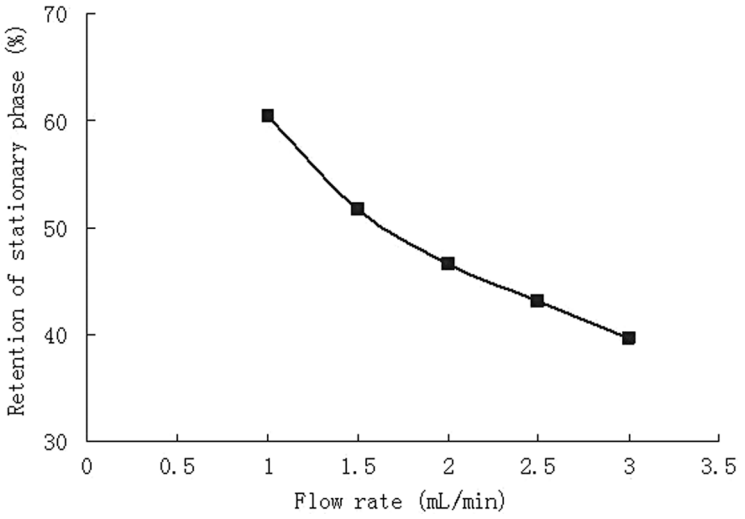


FIGURE 3 Comparison of retention of stationary phase at the different given flow rates by HSCCC. Solvent system: HEMW (1:10:1:10 v/v); stationary phase: upper organic phase; rotational speed: 800 rpm; detection wavelength: 254 nm; sample size: 50 mg.

respectively, as shown in Figs. 1c and d. Compounds **1**, **2**, **3**, and **4** were confirmed to be hyperoside, isoquercitrin, kaempferol 3-O-galactoside, and kaempferol 3-O-glucoside, respectively, by ESI–MS and NMR analysis, and their structures are shown in Fig. 6.

The identification of compounds **1**, **2**, **3**, and **4** was carried out by ESI-MS, ^{1}H-, and ^{13}C-NMR as follows:

Quercetin 3-O-β-D-galactopyranoside (hyperoside) (**1**): ESI-MS, m/z: 463 $[M-H]^-$. ^{1}H NMR (400 MHz, DMSO) δ: 7.63 (1H, dd, $J=8.4$ and 2.4 Hz, H-6′), 7.55 (1H, d, $J=1.6$ Hz, H-2′), 6.80 (1H, d, $J=8.4$ Hz, H-5′), 6.35 (1H, s, H-8), 6.13 (1H, s, H-6), 5.36 (1H, d, $J=7.2$ Hz, H-1″), 3.09∼3.60 (6H, m, H-2″∼6″). ^{13}C NMR (100 MHz, DMSO) δ:156.2 (C-2), 133.5 (C-3), 177.5 (C-4), 161.2 (C-5), 98.7 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 121.1 (C-1′), 115.9 (C-2′), 144.8 (C-3′), 148.5 (C-4′), 115.2 (C-5′), 122.0 (C-6′), 103.9 (C1″), 71.2 (C-2″), 73.2 (C-3″), 67.9 (C-4″), 75.9 (C-5″), 60.1 (C-6″). The ^{1}H NMR and ^{13}C NMR spectral data were in agreement with literature.[21]

Quercetin 3-O-β-D-glucopyranoside (isoquercitrin) (2): ESI-MS, m/z: 463 $[M-H]^-$. ^{1}H NMR (400 MHz, DMSO) δ: 12.63 (1H, s, H-5(OH)), 7.58 (2H, d, $J=6.4$ Hz, H-2′, 6′), 6.84 (2H, d, $J=8.8$ Hz, H-5′), 6.39 (1H, s, H-8), 6.18 (1H, s, H-6), 5.46 (1H, d, $J=7.2$ Hz, H-1″), 3.09∼3.60 (6H, m, H-2″6″); ^{13}C NMR (100 MHz, DMSO) δ:155.86 (C-2), 133.21 (C-3), 176.21 (C-4), 161.18 (C-5), 99.63 (C-6), 164.75 (C-7), 93.63 (C-8), 156.35

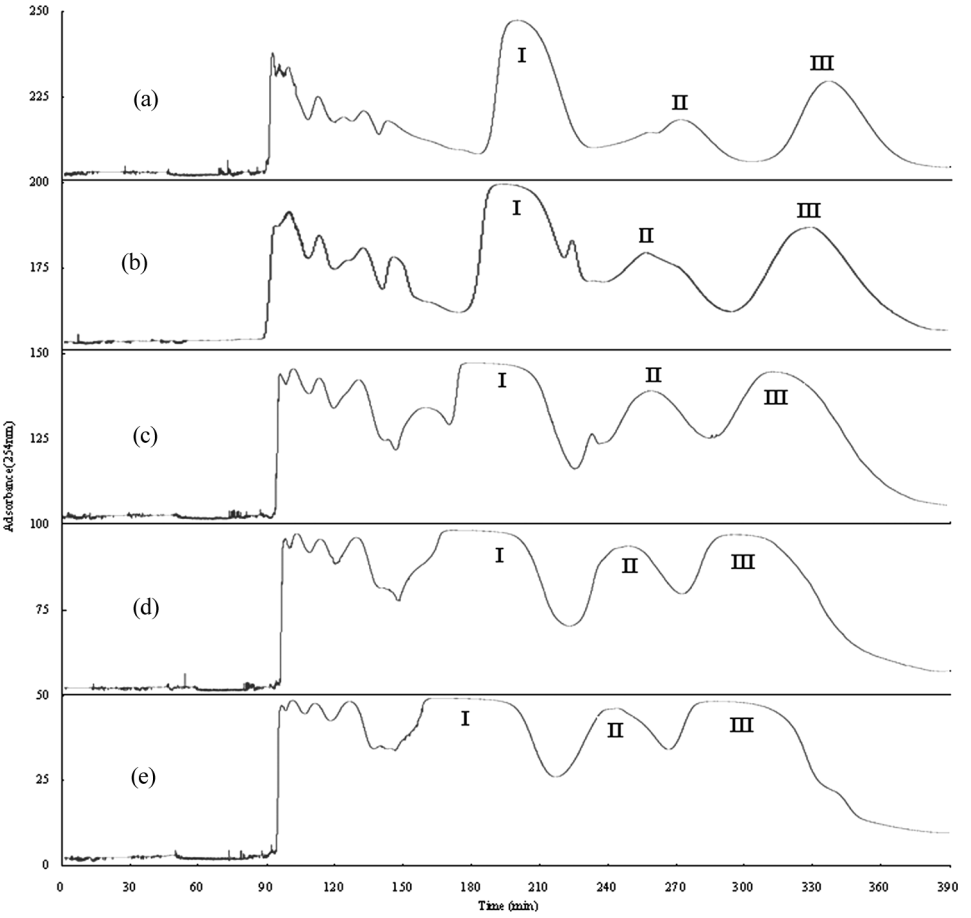


FIGURE 4 Comparison of performance of HSCCC with different sample sizes from black currant leaves. Sample size: (a) 200 mg, (b) 150 mg, (c) 100 mg, (d) 50 mg, (e) 25 mg. Solvent system: HEMW (1:10:1:10 v/v); stationary phase: upper organic phase; rotational speed: 800 rpm; flow rates: 1.0 mL/min; detection wavelength: 254 nm.

(C-9), 103.65 (C-10), 121.52 (C-1′), 115.23 (C-2′), 144.90 (C-3′), 148.63 (C-4′), 116.14 (C-5′), 121.05 (C-6′), 100.89 (C-1″), 74.08 (C-2″), 77.55 (C-3″), 69.91 (C-4″), 76.50 (C-5″), 60.94 (C-6″). The ^{1}H NMR and ^{13}C NMR spectral data were in agreement with the literature.[22,23]

Kaempferol 3-O-β-D-galactopyranoside (3): ESI-MS, m/z: 447 $[M-H]^{-}$. ^{1}H NMR (400 MHz, DMSO) δ: 12.62 (1H, s, H-5(OH)), 8.07 (2H, d, $J=8$ Hz, H-2′, 6′), 6.86 (2H, d, $J=8$ Hz, H-3′, 5′), 6.43 (1H, s, H-8), 6.20 (1H, s, H-6), 5.39 (1H, d, $J=8$ Hz, H-1″), 3.17∼5.19 (6H, m, H-2″6″); ^{13}C NMR (100 MHz, DMSO) δ: 156.28 (C-2), 133.20 (C-3), 177.48 (C-4), 161.18 (C-5), 98.73 (C-6), 164.65(C-7), 93.58(C-8), 156.34 (C-9), 103.83

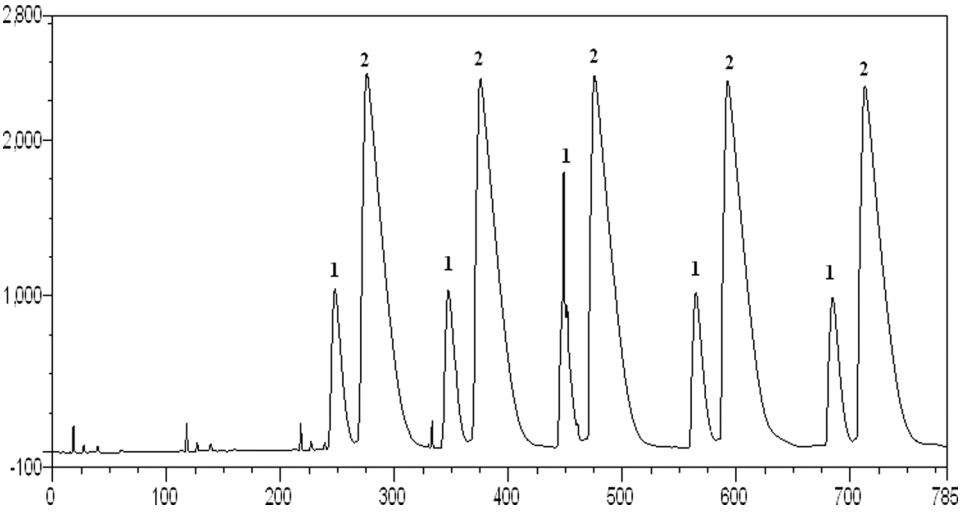
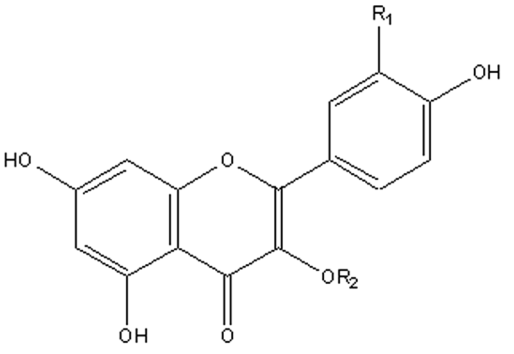


FIGURE 5 Prep-HPLC chromatogram of fraction I (hyperoside and isoquercitrin) separation. Column: Prep C_{18} column (5 μm, 10 mm × 250 mm, X-Bridge™, Waters, USA); Column temperature: 35°C; Mobile phase: MeOH–0.2% formic acid (20:80, v/v), flow rate: 0.8 mL/min; detection: 254 nm; sample size of each time: 50 μl; interval time of sample loading: 100 min. the number of sample loading: 5 times.

(C-10), 120.86 (C-1′), 130.97 (C-2′, 6′), 159.94 (C-4′), 115.05 (C-3′, 5′), 101.66 (C-1″), 71.19 (C-2″), 73.08 (C-3″), 67.86 (C-4″), 75.77 (C-5″), 60.17 (C-6″). The ^{1}H NMR and ^{13}C NMR spectral data were in agreement with the literature.[24]



1 R1=H, R2=Glu; Kaempferol 3-O-glucopyranoside (Astragalin)
2 R1=H, R2=Gal; Kaempferol 3-O-galactopyranoside
3 R1=OH, R2=Glu; Quercetin 3-O-glucopyranoside (Isoquercitrin)
4 R1=OH, R2=Gal; Quercetin 3-O-galactopyranoside (Hyperoside)

FIGURE 6 The chemical structures of compounds 1–4 from black currant leaves isolated by HSCCC and prep-HPLC.

Kaempferol 3-O-β-D-glucopyranoside (astragalin) (**4**): ESI-MS, m/z: 447 $[M-H]^-$. ^{1}H NMR (400 MHz, DMSO) δ: 12.61 (1H, s, H-5(OH)), 8.04 (2H, d, J= 8.8 Hz, H-2′, 6′), 6.89 (2H, d, J= 8.8 Hz, H-3′, 6′), 6.43 (1H, s, H-8), 6.20 (1H, s, H-6), 5.47 (1H, d, J= 7.6 Hz, H-1″), 3.09∼3.58 (6H, m, H-2″6′); ^{13}C NMR (100 MHz, DMSO) δ: 156.18 (C-2), 133.15 (C-3), 177.42 (C-4), 161.14 (C-5), 98.74 (C-6), 164.32 (C-7), 93.38 (C-8), 156.33 (C-9), 103.89 (C-10), 120.89 (C-1′), 130.88 (C-2′, 6′), 159.95 (C-4′), 115.10 (C-3′, 5′), 100.86 (C-1″), 74.20 (C-2″), 77.49 (C-3″), 69.88 (C-4″), 76.41 (C-5″), 60.82 (C-6″). The ^{1}H NMR and ^{13}C NMR spectral data were in agreement with literature.[22–24]

CONCLUSIONS

High-speed countercurrent chromatography (HSCCC) combined with prep HPLC has successfully been applied for separation of hyperoside, isoquercitrin, kaempferol 3-O-galactoside, and kaempferol 3-O-glucoside. In the HSCCC separation, the effects of flow rate and sample size on the stationary phase retention and peak resolution were studied. The results indicated that sample size has little effect on stationary phase retention, while the flow rate is the major factor to determine the stationary phase retention and the peak resolution. A lower flow rate provides a higher retention level of the stationary phase, hence improving the peak resolution, although it requires a longer separation time. Overall results of our studies demonstrate that the combined use of HSCCC and prep HPLC is an excellent method for purification of natural products.

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