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Separation of patuletin-3-O-glucoside, astragalin, quercetin, kaempferol and isorhamnetin from *Flaveria bidentis* (L.) Kuntze by elution-pump-out high-performance counter-current chromatography

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ABSTRACT

Flaveria bidentis (L.) Kuntze is an annual alien weed of Flaveria Juss. (Asteraceae) in China. Bioactive compounds, mainly flavonol glycosides and flavones from *F. bidentis* (L.) Kuntze, have been studied in order to utilize this invasive weed, Analytical high-performance counter-current chromatography (HPCCC) was successfully used to separate patuletin-3-O-glucoside, a mixture of hyperoside (quercetin-3-O-galactoside) and 6-methoxykaempferol-3-O-galactoside, astragalin, quercetin, kaempferol and isorhamnetin using two runs with different solvent system. Ethyl acetate–methanol–water (10:1:10, v/v) was selected by analytical HPCCC as the optimum phase system for the separation of patuletin-3-O-glucoside, a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside, and astragalin. A Dichloromethane–methanol–water (5:3:2, v/v) was used for the separation of quercetin, kaempferol and isorhamnetin. The separation was then scaled up: the crude extract (ca 1.5 g) was separated by preparative HPCCC, yielding 12 mg of patuletin-3-O-glucoside at a purity of 98.3%, yielding 9 mg of a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside constituting over 98% of the fraction, and 16 mg of astragalin (kaempferol-3-O-glucoside) at a purity of over 99%. The pump-out peaks are isorhanetin (98% purity), kaemferol (93% purity) and quercitin (99% purity). The chemical structure of patuletin-3-O-glucoside and astragalin were confirmed by MS and ^{1}H, ^{13}C NMR.

1. Introduction

Flaveria bidentis (L.) Kuntze, is an annual alien weed of Flaveria Juss (Asteraceae), recently found in China. It is native to South America, mainly Argentina, including *F. bidentis* var. angustifolia Kuntze as its synonym [1,2]. It may be a potentially invasive plant because it has very strong reproductive and survival abilities. *F. bidentis* is now widely distributed in Hebei and Shandong province in China. This exotic weed has the ability to survive under different environmental conditions [3]. *F. bidentis* is a highly dangerous exotic weed damaging native ecosystems and causing great economic loss [4]. In addition to controlling this plant, developing commercial uses for it might be a way to provide economic benefit. However, little attention has been paid to the separation and identification of its chemical components in China. Isolating and characterizing bioactive compounds is one approach.

Flavonol glycosides are the major bioactive components in *F. bidentis* [5,6]. To evaluate the biological effects of these bioactive compounds, particularly by in vivo trials, it is necessary to obtain large quantities of highly purified compounds. Isolation and purification of bioactive compounds from *F. bidentis* using column chromatography have been reported [2,5], but the method was time-consuming and required multiple steps. Additionally, this method often generated large amount of organic solvent waste and had the problem of irreversible adsorption of some components.

High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition method, eliminates irreversible adsorption of some elements of the sample onto the solid support [7], and has been widely used in the preparative separations of natural products [8–10]. High performance counter-current chromatography (HPCCC) is a recent development in counter-current chromatography in which separations are provided in minutes rather than the hours generally experienced with HSCCC. The equipment is more robust than previous HSCCC instruments, and the scale-up to pilot level has been shown to be quick and easy

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Table 1
Partition coefficient (*K*) values of the flavonol glycosides in different solvent systems.

Ethyl acetate–methanol–water ratio (v/v)[a]	Partition coefficient (*K*)			
	Patuletin-3-O-glucoside	Hyperoside	6-Methoxykaempferol-3-O-galactoside	Astragalin
5:2:5	0.9407	1.2893	1.1983	1.9149
5:1:5	0.8736	2.1487	2.0590	2.8767
10:1:10	1.0279	1.6156	1.6850	3.5167
25:1:25	0.7227	1.1401	1.1598	2.5004

[a] Solvent ratios were by volume (35 °C).

[11,12]. HPCCC is an advanced technique that is useful for scale-up separation with minimum sample pre-treatment and cleanup procedures.

The present paper describes a method using HPCCC for purifying flavonol glycosides and flavone from *F. bidentis*. Analytical HPCCC was used to select a two-phase solvent system of ethyl acetate–methanol–water (10:1:10, v/v) for the separation and purification of patuletin-3-O-glucoside, a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside, and astragalin. A dichloromethane–methanol–water (5:3:2, v/v) phase system was required for the separation of quercetin, kaempferol and isorhamnetin. The crude extract was separated by preparative HPCCC, yielding 12 mg of patuletin-3-O-glucoside at a purity of 98.3%, yield 9 mg of a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside constituting over 98% of the fraction, and 16 mg of astragalin (kaempferol-3-O-glucoside) at a purity of over 99%. The pump-out peaks were isorhanetin, kaemferol and quercitin with high purity.

2. Experimental

2.1. Apparatus

The Analytical high-performance counter-current instrument used was a DE-Mini multilayer coil planet centrifuge (Dynamic Extractions Ltd., Plymouth Road, Slough, UK) equipped with a 0.8 mm I.D. PTFE (polytetrafluoroethylene) multilayer coil of 17.1 ml total capacity.

The preparative high-performance counter-current instrument was a DE-Midi multilayer coil planet centrifuge equipped with a 110 m × 4 mm I.D. PTFE (polytetrafluoroethylene) multilayer coil with a total capacity of 912.5 ml also supplied by Dynamic Extractions.

HPLC was performed on a Waters Alliance 2695 separations module (Empower software) connected to a Waters 2996 photodiode array (DAD) detector (210–400 nm) using a Sunfire C18 column (150mm × 4.6 mm I.D., 5 μm) (Waters, Milford, MA, USA).

Identification of preparative HPCCC peak fractions was carried out by MS (Finnigan MAT711), ^{1}H NMR and ^{13}C NMR spectra (av 600).

2.2. Reagents

F. bidentis plant was supplied by the Chinese Academy of Agricultural Sciences and identified by Dr. Guoliang Zhang. Reference standards Kaempferol and isorhamnetin were purchased from Fluka. Quercetin, patuletin-3-O-glucoside and astragalin were purchased from Sigma–Aldrich (Gillingham, UK). All organic solvents used for sample preparation and HPCCC were of analytical grade and purchased from Fisher Chemicals (Loughborough, UK). Methanol used for HPLC analysis was of chromatography grade and purchased from Fisher Chemicals (Loughborough, UK).

2.3. Preparation of crude extract from F. bidentis

The aerial part of *F. bidentis* was dried under sunlight, and ground in a crusher. 100 g *F. bidentis* powder was extracted (refluxed) with 80% ethanol (3 l) for 75 min, filtered and re-extracted the same way. The various extracts were combined and concentrated to dryness under reduced pressure in a rotary evaporator, yielding 16.9 g crude sample. The concentration of flavonol glycosides and flavone was determined by HPLC based on external standard curves.

2.4. Preparation of two-phase solvent and sample solution for HPCCC

Selection of the two-phase solvent systems was based on determining partition coefficient (*K*) of the target components of flavonol glycosides and flavones in a range of solvent systems: ethyl acetate–methanol–water (5:2:5, 5:1:5, 10:1:10 and 25:1:25, v/v) and dichloromethane–methanol–water (4:3:2, 5:3:2, v/v). The procedure was this: approximately 20 mg of crude extract was weighed in a 10 ml test tube into which 3 ml of each phase of the pre-equilibrated two-phase solvent system was added. The test tube was capped and shaken vigorously for 1 min, and allowed to stand until it separated completely. A 1 ml of each layer was taken out and evaporated separately to dryness under vacuum. The residue was dissolved in 1 ml methanol and analyzed by HPLC for determining the partition coefficient (*K*) value of target. The *K* value was expressed as the peak area of target compound in the upper phase divided by that in the lower phase. The results were shown in Tables 1 and 2. Phases were equilibrated in a separating funnel at room temperature and then thoroughly mixed. Phases were separated shortly before use. The sample solution for HPCCC was prepared by dissolving the crude extract in equal volumes of lower phase and upper phase at suitable concentrations for the analytical or preparative HPCCC. Sample solutions for HPLC analysis were prepared by dissolving the dried HPCCC peak fractions in the mobile phase of HPLC at suitable concentration followed by filtration (0.45 μm filter membrane).

Table 2
Partition coefficient (*K*) values of flavones in solvent system.

Dichloromethane–methanol–water ratio (v/v)[a]	Partition coefficient (*K*)		
	Quercetin	Kaempferol	Isorhamnetin
4:3:2	1.23	0.57	0.30
5:3:2	3.38	1.00	0.61

[a] Solvent ratios were by volume (35 °C).

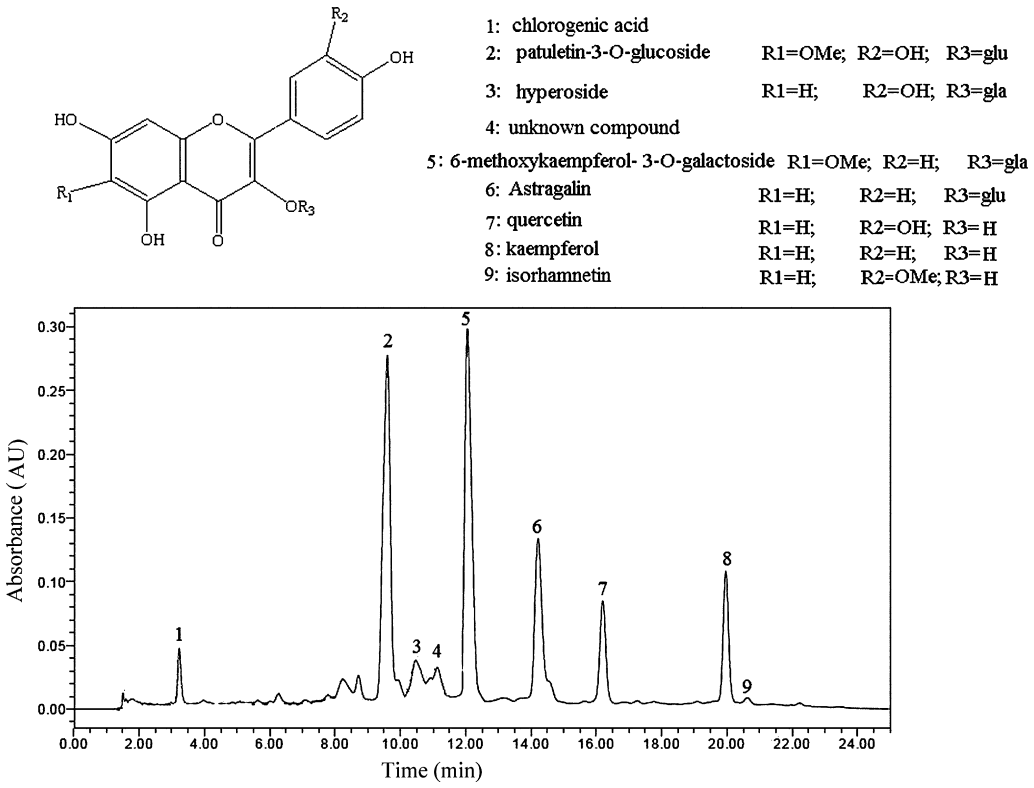

Fig. 1. HPLC chromatogram of the crude extract from *F. bidentis* (L.) Kuntze with the chemical structure of the flavonol glycosides and flavones. HPLC conditions: a reversed phase C18 column (4.6 mm × 150 mm, 5 μm, Sunfire) was used. Gradient elution was performed using an A eluent MeOH and a B eluent with 0.05% (v/v) TFA in water in the following linear gradient combinations: at time 0 min, 70% B; at time 15 min, 50% B; at time 20 min, 40% B; at time 25 min, 70% B. UV wavelength is 360 nm, temperature: 40 °C. Flow rate was 1 ml/min, and 40 μl was injected into the column. Peak 1: chlorogenic acid, peak 2: patuletin-3-O-glucoside, peak 3: hyperoside (quercetin-3-O-galactoside), peak 4: unknown compound, peak 5: 6-methoxykaempferol-3-O-galactoside, peak 6: kaempferol-3-O-glucoside (astragalin), peak 7: quercetin, peak 8: kaempferol, peak 9: isorhamnetin.

2.5. Analytical HPCCC separation procedure

Analytical HPCCC separation was performed 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 column at a flow rate of 0.5 ml/min, while the apparatus was run at a rotational speed of 2100 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 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.

2.6. Flow optimization on the preparative scale HPCCC

A flow optimization study was performed as described by Ignatova and Sutherland [13]. Using the Midi HPCCC centrifuge, the coil was initially filled with the upper phase (the intended stationary phase). The centrifuge was then rotated at 1200 rpm and the mobile lower phase set at the lowest flow rate of 10 ml min^{-1}. The eluent was collected in a measuring cylinder and the volumes of upper and lower phase displaced were measured. Once equilibrium was established (i.e., when no more stationary phase was eluted) the flow was increased by a further 10 ml min^{-1}. A new equilibrium was established and the additional stationary phase displaced was measured. This procedure was repeated in increments of 10 ml min^{-1} until the flow reached 50 ml min^{-1}. The volume of stationary phase eluted is the sum of the extra coil volume (i.e., the volume of the

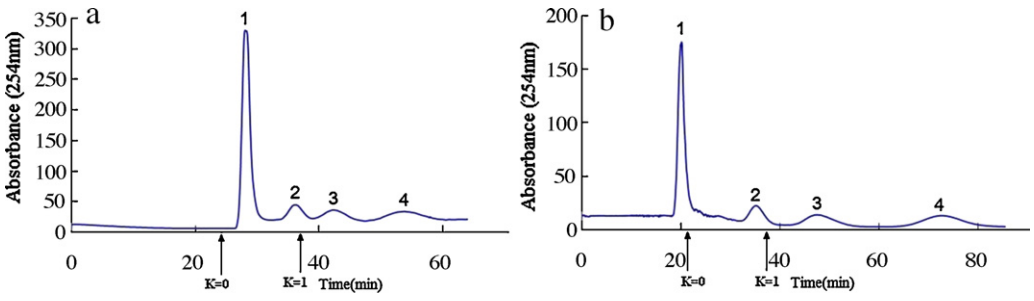

Fig. 2. Chromatogram of the crude extract from *F. bidentis* (L.) Kuntze by analytical HPCCC. Solvent system: ethyl acetate–methanol–water (a) 5:1:5 (v/v), and (b) 10:1:10 (v/v), stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 0.5 ml/min; revolution speed: 2100 rpm; sample: 40 mg dissolved in 0.5 ml upper phase and 0.5 ml lower phase. Peak 2 = patuletin-3-O-glucoside, peak 3 = a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside, peak 4 = astragalin.

inlet and outlet leads) and the volume of mobile phase in the coil (which has displaced the stationary phase which was initially in the coil).

2.7. Preparative HPCCC separation procedure

The preparative HPCCC separation procedure was the same as the analytical one except the flow rate was 50 ml/min and rotational speed was 1200 rpm. All the preparative fractions were collected every minute (50 ml per tube). After one hour elution, the retained sample was pumped out with stationary phase at a flow rate of 100 ml/min, auto collecting 35 tubes at 30 ml/tube. All the fractions were analyzed by HPLC.

2.8. HPLC analysis

HPLC conditions were as follows: a reversed phase C18 column (4.6 mm × 150 mm, 5 μm, Sunfire) was used. Gradient elution was performed using an A eluent MeOH and a B eluent with 0.05% (v/v) TFA in water in the following linear gradient combinations: at time 0 min, 70% B; at time 15 min, 50% B; at time 20 min, 40% B; at time 25 min, 70% B. UV wavelength was 360 nm, temperature: 40 °C. Flow rate was 1 ml/min, and 40 μl was injected into the column.

2.9. MS and NMR

Identification of HPCCC peak fractions was carried out by ESI-MS and ^{1}H, ^{13}C NMR spectra. ESI mass spectra (MS) and nuclear magnetic resonance (NMR) spectra were obtained by analysts at the Center of Analysis, Beijing University of Chemical Technology. A Waters Micromass Quattro Premier MS was used with an ESI source. NMR spectra were performed in DMSO-d_6, $CD_3COCD_3 + D_2O$ and DMSO-$d_6 + D_2O$ respectively using a Bruker high-resolution AV600NMR spectrometer at 600 MHz (Bruker Biospin Corporation, USA).

3. Results and discussion

As shown in Fig. 1, the HPLC analysis of the crude extract of *F. bidentis* indicated that it contained several compounds. Based on external standard curves, patuletin-3-O-glucoside (2), hyperoside (quercetin-3-O-galactoside) (3), 6-methoxykaempferol-3-O-galactoside (5), astragalin (6), quercetin (7), kaempferol (8), and isorhamnetin (9) were present at 0.94%, 0.45%, 0.26%, 1.17%, 0.28%, 0.52% and 0.07% in the crude extract, respectively.

Successful separation by HPCCC depends upon the selection of suitable two-phase solvent systems in which the target compounds must be stable and soluble, and the two phases settle quickly. Furthermore, the partition coefficient (K) of the target compounds should be in the range 0.2–5.0 [14]. Analytical HPCCC was used to optimize the resolution of the target flavonol glycosides by varying the volume of methanol as shown in Fig. 2. Only one peak was eluted at a volume ratio of 5:2:5 (v/v) because only 6.4% of the stationary phase was retained, that is, everything has been eluted at the $K = 1$ point as there was virtually no retention of stationary phase. At the volume ratio of 5:1:5, peaks 2 and 3 (Fig. 2a) could not be baseline resolved with a retention value of stationary phase of 41%. Baseline resolution between peaks 2 and 3 was obtained at a volume ratio of 10:1:10 (Fig. 2b) with 50.3% retention of stationary phase. Although the retention of stationary phase was initially 59.1% for the volume ratio of 25:1:25, there was loss of stationary phase following sample injection. Therefore the ethyl acetate–methanol–water system (10:1:10, v/v) was selected for the HPCCC purification of the flavonol glycosides.

In order to elute isorhanetin, kaemferol and quercitin with mobile phase and isolate them from the extract, a dichloromethane–methanol–water (5:3:2, v/v) phase system was required. Fig. 3 shows the analytical HPCCC separation obtained from 40 mg of the crude extract of *F. bidentis*. Peak 9 yielded trace amount of isorhamnetin at a purity of 98% based on the HPLC analysis. Peak 8 yielded 0.2 mg kaempferol at over 93% purity, and peak 7 gave 0.1 mg quercetin at a purity of over 99%. All these compounds were identified by standard addition HPLC analysis method. Although the CCC preparation of these three compounds could have been scaled up using this solvent system we chose to isolate them by preparative HPCCC by pump-out after flavonol glycosides were eluted using with the ethyl acetate–methanol–water system (10:1:10, v/v), as described below, rather than using an additional CCC process with the dichloromethane–methanol–water phase system. Isolation of these flavones is less important than the flavonol glycosides, which are much more important compounds than flavones because they are rich in natural products resulting in many majority medicinal properties and biological effects. In addition they have been obtained from other plants using HSCCC [15], As a first step to developing a preparative CCC process, the dependence of the retention of the stationary phase on flow rate was examined to determine how high a flow rate could be used in preparative HPCCC. Fig. 4 shows the variation of the volume of stationary phase retained in the coil as a function of the mobile phase flow rate. It was noted that significant retention of stationary phase could be obtained up to a flow rate of 50 ml min^{-1}.

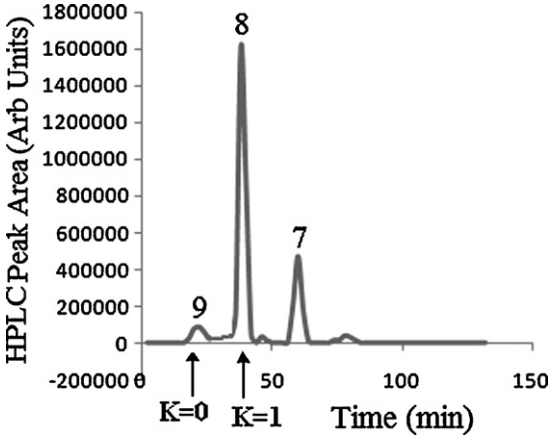

Fig. 3. Chromatogram of the crude extract from *F. bidentis* (L.) Kuntze by analytical HPCCC. Solvent system: dichloromethane–methanol–water (5:3:2, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 0.5 ml/min; revolution speed: 2100 rpm; sample: 40 mg dissolved in 0.5 ml upper phase and 0.5 ml lower phase. HPLC conditions are the same as Fig. 1. Peak 7 = quercetin, peak 8 = kaempferol, Peak 9 = isorhamnetin.

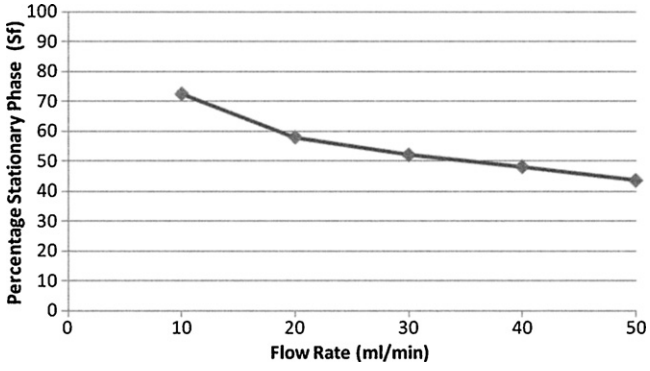

Fig. 4. Variation of the volume of stationary phase retained in the coil as a function of the mobile phase flow rate for the preparative HPCCC centrifuge running at 1200 rpm using an ethyl acetate–methanol–water (10:1:10, v/v) phase system in reverse phase mode with lower (aqueous) phase as the mobile phase.

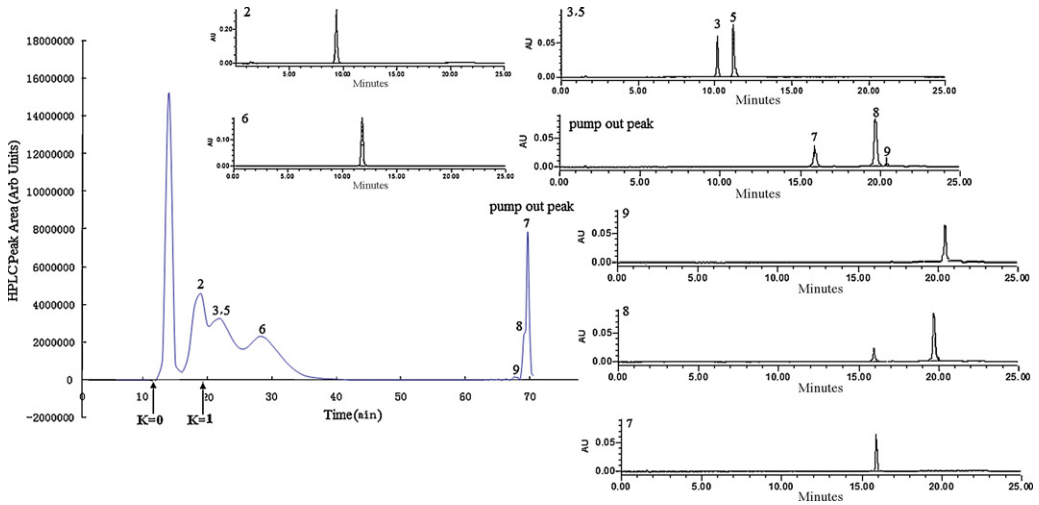

Fig. 5. Chromatogram of the crude extract from *F. bidentis* (L.) Kuntze by Preparative HPCCC. Solvent system: ethyl acetate–methanol–water (10:1:10, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow rate: 50 ml/min; revolution speed: 1200 rpm; Retention of the stationary phase is 41.9%; sample: 1.5 g dissolved in 10 ml of lower phase. HPLC conditions are the same as Fig. 1. Peak 2 = patuletin-3-O-glucoside, peaks 3 and 5 = a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside, peak 6 = astragalin, peak 7 = quercetin, peak 8 = kaempferol, peak 9 = isorhamnetin.

The separation was scaled up loading 1.5 g of crude extract onto the preparative scale HPCCC (Fig. 5). Peak 2 yielded 12 mg of patuletin-3-O-glucoside at a purity of 98.3% based on HPLC analysis. Peak 3, 5 yield 9 mg of a mixture of hyperoside (quercetin-3-O-galactoside) and 6-methoxykaempferol-3-O-galactoside constituting over 98% of the fraction. Peak 6 yielded 16 mg of astragalin at a high purity of over 99%. These purities are similar to those reported for separations by HSCCC [16], However, these latter purifications were obtained at lower loads (400 mg compared with 1500 mg in the present study) and at considerably longer run time (6 h compared with the 1.5 h reported here); throughput of 67 mg/h or 2 mg/min for HSCCC compared with that of 1 g/h or 16.7 mg/min obtained in this study for HPCCC.

The HPLC analysis of the pump out showed peaks of isorhanetin, kaemferol and quercitin eluting in that order, yielding 0.9 mg, 6.7 mg and 3.6 mg, respectively.

The structural identification of the patuletin-3-O-glucoside and astragalin was confirmed by ESI-MS, ^{1}H NMR, ^{13}C NMR analysis.

Patuletin-3-O-glucoside: ESI (Electro Spray Ionization)-MS: *m/z* 492.9 $[M-H]^-$, *m/z* 331.1 ($[M-H]^- - 162$, loss of glucose) and *m/z* 316.1 ($[M-H]^- - 162 - 16$, loss of $-CH_3$). ^{1}H NMR (600 MHz, DMSO-d_6 + D_2O): δ ppm 7.590 (dd, 1H, J = 1.8, 9.0 Hz, H-6′), 7.576 (d, 1H, J = 1.8 Hz, H-2′), 6.855 (d, 1H, J = 9.0 Hz, H-5′), 6.523 (s, 1H, H-8), 3.755 (s, 3H, 6-OMe), 5.486 (d, 1H, J = 7.2 Hz, H-1″), 3.090–3.595 (m, 5H, H-2″-6″ of glucoside). ^{13}C NMR (125 MHz, DMSO-d_6): 178.156 (C-4), 157.797 (C-9), 156.776 (C-2), 152.875 (C-5), 151.974 (C-7), 148.919 (C-4′), 145.272(C-3′), 133.467 (C-3), 131.686 (C-6), 122.069 (C-1′), 121.698 (C-6′), 116.674 (C-2′), 115.667 (C-5′), 104.845 (C-10), 101.320 (C-1″), 94.212 (C-8), 93.611 (C-8), 78.025 (C-5″), 76.975 (C-3″), 74.562 (C-2″), 70.425 (C-4″), 61.464 (C-6″), 60.451 (6-OMe). The peaks assigned in ^{1}H NMR and ^{13}C NMR corresponded to the reference [17,18], and the glucoside NMR data was matched with that in Ref. [19].

Mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside: ESI-MS: *m/z* 463.0, 476.9 $[M-H]^-$, MS^2 yielded ions at *m/z* 301.1 and 315.0, respectively ($[M-H]^- - 162$, loss of galactose), and the 315.0 ion further produced *m/z* 299.1 ($[M-H]^- - 162 - 15$, loss of $-CH_3$). ^{1}H NMR (600 MHz, CD_3COCD_3 + D_2O): δ ppm 8.12 (d, 2H), 7.91 (d, 1H), 7.57 (dd, 1H), 6.96 (d, 2H), 6.95 (d, 1H), 6.61 (s, 1H), 6.52 (d, 1H), 6.27 (d, 1H), 5.28 (d, 1H), 5.27 (d, 1H), 3.86 (s, 3H), 3.09–3.71 (m, H of galactoside).

Astragalin: ESI-MS: *m/z* 446.8 $[M-H]^-$, *m/z* 331.1 ($[M-H]^- - 162$, loss of glucose). ^{1}H NMR (600 MHz, DMSO-d_6 + D_2O): δ ppm 8.161 (d, 2H, J = 9.0 Hz, H-2′, 6′), 6.847 (d, 2H, J = 9.0 Hz, H-3′, 5′), 6.551 (d, 1H, J = 2.4 Hz, H-8), 6.308 (d, 1H, J = 2.4 Hz, H-6), 5.293 (d, 1H, J = 7.8 Hz, H-1″), 3.279–3.677 (m, 5H, H-2″-6″ of glucoside). ^{13}C NMR (125 MHz, DMSO-d_6): 177.940 (C-4), 164.590 (C-7), 161.691 (C-5), 160.415 (C-4′), 156.845 (C-9), 156.724 (C-2), 133.668 (C-3), 131.339 (C-2′, 6′), 121.367 (C-1′), 115.567 (C-3′, 5′), 104.480 (C-10), 101.346 (C-1″), 99.147 (C-6), 94.110 (C-8), 77.958 (C-5″), 76.898 (C-3″), 74.685 (C-2″), 70.372 (C-4″), 61.314 (C-6″). The peaks assigned in ^{1}H NMR and ^{13}C NMR corresponded to those Refs. [10,19,20].

4. Conclusions

In the present study, HPCCC was successfully used for the separation of flavonol glycosides and flavones from *F. bidentis*. Analytical HPCCC was used to select a two-phase solvent system of ethyl acetate–methanol–water (10:1:10, v/v) for the separation and purification of patuletin-3-O-glucoside, a mixture of hyperoside and 6-methoxykaempferol-3-O-galactoside, and astragalin. A dichloromethane–methanol–water (5:3:2, v/v) phase system was required for the elution of quercetin, kaempferol and isorhamnetin. The separation was scaled up using preparative scale HPCCC. From a load of 1.5 g of extract 12 mg of patuletin-3-O-glucoside (purity 98.3%), 9 mg of a mixture of hyperoside (quercetin-3-O-galactoside) and 6-methoxykaempferol-3-O-galactoside constituting over 98% of the fraction, and 16 mg of astragalin (kaempferol-3-O-glucoside)(purity 99%) were obtained. The pump-out peaks were isorhanetin, kaemferol and quercitin with high purity and eluting in that order. The results of this study demonstrate that HPCCC is a very efficient method for the rapid preparative separation of flavonol glycosides and flavones from *F. bidentis* giving an improvement of over 10-fold in crude material processed (mg/min) compared with HSCCC.

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