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Comprehensive separation and identification of chemical constituents from *Apocynum venetum* leaves by high-performance counter-current chromatography and high performance liquid chromatography coupled with mass spectrometry

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A B S T R A C T

High-performance counter-current chromatography (HPCCC) and high performance liquid chromatography coupled with mass spectrometry (HPLC–MS) was efficiently utilized for the separation and identification of the chemical components with a wide range of polarity from the mixed extract of Chinese medicinal herb *Apocynum venetum*. For HPCCC separation, four sets of solvent systems, *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:4.5, v:v:v:v), ethyl acetate–methanol–water (5:2:5, v:v:v) and *n*-butanol–methanol–water (5:1:5, v:v:v) were used for the one-step separation by four stages. The HPCCC separation was initiated by filling the column with the lower phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) as a stationary phase followed by elution with the upper phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) to separate the hydrophobic compounds (tail to head). Then the mobile phase was switched to the upper phase of ethyl acetate–acetonitrile–water (5:3:7, v:v:v) to eluted the moderate hydrophobic compounds, then the mobile phase was switched to the upper phase of ethyl acetate–methanol–water (5:2:5, v:v:v) to eluted the moderate hydrophilic compounds, and finally the hydrophilic compounds still retained in the column was eluted by the upper phase of *n*-butanol–methanol–water (5:1:5, v:v:v). A total of 16 named compounds including adhyperforin, hyperforin, amentoflavone, biapigenin, quercetin, avicularin, acetylated isoquercetin, acetylated hyperoside, astragalin, trifolin, isoquercetin, hyperoside, querciturone, rutin, chlorogenic acid and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside were successfully separated *via* the four sets of solvent systems in one step operation for 130 min. The compounds separated by HPCCC were identified by comparing with mixed standards data of HPLC–MS as well as NMR data.
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1. Introduction

Counter-current chromatography (CCC) is essentially a form of liquid–liquid partition chromatography, CCC applications were mostly performed using immiscible organic–aqueous two-phase solvent systems [1–4]. Due to the CCC separation principle of the compounds distribution between two phase [5], the polarity range of the compounds separated by conventional CCC technique are very short, and hence most reports have focused on several main compounds in a certain fraction [6–11]. In practice, three-phase solvent systems of HSCCC (high speed counter-current chromatography) have been applied successfully to separation plant metabolite [12,13]. More recently, we have demonstrated a simpler and faster novel four stage gradient elution mode for the systematic separation of chemical compounds in a broad range of polarity (Fig. 1) from the mixed extracts of *A. venetum via* HPCCC (high performance counter-current chromatography), This being far simpler than any other preparative HPLC isolation approaches. In order to rapidly identify and characterize a large number of constituents and commonly existing isomers in plants, liquid chromatography coupled with mass spectrometry (HPLC–MS) has become one of the most powerful techniques. Consequently investigating the fragmentation pathways of constituents to obtain important structural information is important in this research field [14–16].

The leaves of *A. venetum* are highly valued ingredient in Chinese and Japanese traditional medicine and have been used in many herbal preparations either singly or in combination with other plants. Pharmacological research indicated that *A. venetum* has widely useful activities including lower blood pressure [17], antidepressant [18] antinephritis [19] and antineurasthenia [20]. Chemical research indicated that the major constituents are complex mixtures of phenolic acids [21] and flavonoids [22].

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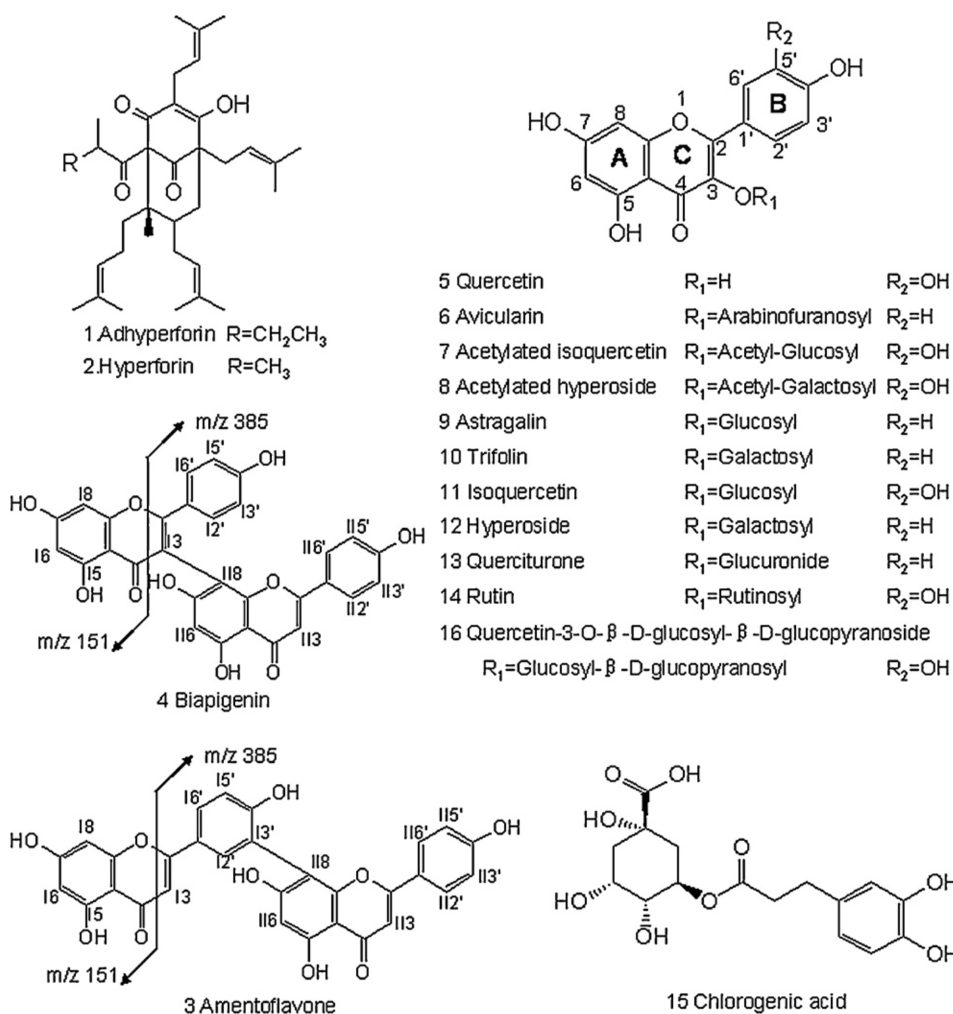

Fig. 1. Structures of compounds separated and identified from leaves of *Apocynum venetum* L.

The aim of the present paper, therefore, was to develop an efficient comprehensive method for the separation of the compounds with high purities using HPCCC and identified by HPLC–MS, ^{1}H NMR (proton nuclear magnetic resonance spectrometry) as well as UV (ultra-violet) spectral. This paper describes the successful comprehensive separation and identification of 16 compounds (Fig. 1) from *A. venetum*.

2. Experimental

2.1. Apparatus

High-performance counter-current chromatograph was performed on DE Spectrum HPCCC (Dynamic Extractions, Slough, UK). The multilayer coil separation column was prepared by winding a 28 m × 2.6 mm I.D. PTFE tube directly onto one of the holders forming multiple coiled layers to give a total capacity of 146 ml. The β-value varied from 0.33 at the internal terminal to 0.58 at the external terminal ($R = 8$ cm, $\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The rotation speed was adjustable in a range from 0 to 1600 rpm and 800 rpm was used in the present study. Electrospray ionization mass spectrometry (ESI-MS) used was a Finnigan LCQ ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). High-performance liquid chromatography (HPLC) used was a Waters 2695 coupled with a Waters 2998 Diode array detector (DAD) (Milford, MA, USA). Nuclear magnetic resonance spectrometer spectra used was a Bruker AV 500 (Bruker BioSpin, Rheinstetten, Germany)

2.2. Reagents and materials

Ethyl acetate, acetonitrile, *n*-hexane, *n*-butanol, methanol and ethanol were of analytical grade (Beijing Chemicals, Beijing, China). Water was purified on a Milli-Q water purification system (Millipore, Boston, USA). Acetonitrile, acetic acid and deuterated methanol were of HPLC grade (Fisher Scientific, Pittsburg, PA, USA). Standard compounds, quercetin, astragalin, hyperoside, rutin and chlorogenic acid were purchased from the Chinese Authenticating Institute of Material Medical and Biological Products (Beijing, China); acetylated hyperoside, adhyperforin, hyperforin and amentoflavone were from Sigma–Aldrich Shanghai Co. (Shanghai, China); quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside, biapigenin, trifolin and isoquercetin were isolated by the central laboratory of Changchun normal university and identified by NMR, ESI-MS, UV and HPLC (Changchun, China). *A. venetum* leaf was harvest from Lop Nor of Xinjiang province (Lop Nor, China) and identified by Yuchi Zhang (Changchun Normal University, Changchun, China). D-101 macroporous resin (Chemical Plant of Nankai University, Tianjin, China).

2.3. Preparation of sample and standard solutions

About 100 g of dried *A. venetum* powder was extracted (refluxed) with 1000 ml of a 50% ethanol solution. After filtration, the

extracted materials were again extracted with 500 ml of ethyl acetate *via* sonication for 40 min. After filtration, the two extractions were combined and concentrated to dryness under reduced pressure (60 °C). The combined extract was loaded on D-101 macroporous resin column (35 cm × 3.4 cm, the volume of column was 1000 ml) and eluted with 5000 ml water and 5000 ml 75% ethanol, respectively. The 75% ethanol effluent was collected and evaporated to dryness. The residue was stored in a refrigerator (5 °C) for HPCCC separation and HPLC–DAD–MS analysis. The mixed standards solution was prepared by dissolving adhyperforin (2.10 mg), hyperforin (2.08 mg), amentoflavone (2.03 mg), biapigenin (1.98 mg), quercetin (2.18 mg), acetylated hyperoside (2.16 mg), astragalin (2.01 mg), trifolin (1.43 mg), isoquercetin (2.24 mg), hyperoside (2.29 mg), rutin (2.35 mg), chlorogenic acid (2.09 mg) and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside (2.17 mg) in methanol and made the total volume of 50 ml. The mixed standards solution was stored in a refrigerator (5 °C) for HPLC–DAD–MS analysis.

2.4. Measurement of partition coefficient

Among the 16 sets of solvent systems described in the following text, *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:1.5:3.5, 1.5:3.5:2:4.5, 1.5:3.5:2.5:4.5, 1.5:3.5:2:5 and 1.5:3.5:2.5:5.5, v:v:v:v), ethyl acetate–acetonitrile–water (5:2:4, 5:2:5 and 5:3:7, v:v:v), ethyl acetate–methanol–water (5:1:5, 5:2:5, 5:2.5:5 and 5:3:5, v:v:v) and *n*-butanol–methanol–water (5:0:5, 5:1:5, 5:1.5:5 and 5:2:5, v:v:v) were selected for performing partition coefficient evaluation. The partition coefficient values of compounds with a wide range of polarity in *A. venetum* were measured either between upper phase and lower phase of *n*-hexane–ethyl acetate–acetonitrile–water for hydrophobic compounds, between upper phase of ethyl acetate–methanol–water and lower phase of *n*-hexane–ethyl acetate–acetonitrile–water we choose for moderate hydrophobic, between upper phase of ethyl acetate–acetonitrile–water and lower phase of *n*-hexane–ethyl acetate–acetonitrile–water we choose for moderate hydrophilic compounds, between upper phase of *n*-butanol–methanol–water and lower phase of *n*-hexane–ethyl acetate–acetonitrile–water for hydrophilic compounds. About 2 ml of each phase was delivered into a test tube to which about 1.5 mg of the *A. venetum* mixed extract was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers were formed, 0.2 ml of each phase was diluted with 2 ml methanol to measure the HPLC peak area at 254 nm using a Waters 2695 HPLC coupled with a Waters 2998 DAD.

2.5. Separation by high-speed counter-current chromatography

The separations of the constituents in *A. venetum* were performed *via* four stage gradient elution mode as follows. After filling the column with the lower phase (LP) of *n*-hexane–ethyl acetate–acetonitrile–water as a stationary phase, the coiled column was rotated at 800 rpm, and then the upper phase (UP) of *n*-hexane–ethyl acetate–acetonitrile–water was pumped into the column in the tail to head direction at a flow-rate of 2.0 ml/min. After the hydrodynamic equilibrium between the two phases was established in the rotating column, 6 ml of a sample solution (a mixture of 3 ml of each phase containing 40 mg of extract) was injected into the column. After the hydrophobic compounds were eluted, the mobile phase was then switched to the upper phase of ethyl acetate–methanol–water and ethyl acetate–acetonitrile–water respectively to elute the moderately hydrophobic compounds and hydrophilic compounds from the column, and finally the hydrophilic compounds still remained in the column were eluted out with upper phase of *n*-butanol–methanol–water.

2.6. HPLC analysis and identification of HPCCC peak fractions

For the *K* evaluation and purity examination, the crude extract of *A. venetum* and HPCCC peak fractions were each analyzed by HPLC–DAD. The analyses were performed on an Agilent C_{18} column (250 mm × 4.6 mm I.D., 5 μm) with a DAD system (Waters model 2998). Column temperature: 25 °C. Mobile phases: (A) acetonitrile and (B) 0.5% acetic acid in water. Linear gradients: 0–10 min, 10% A; 20–80 min, 10–100% A; 80–90 min, 100% A, flow rate: 1.2 ml/min, detection wavelength: 254 nm.

2.7. LC–ESI-MS and MS/MS analysis

A Finnigan LCQ ion-trap mass spectrometer with an electrospray ion source was coupled to the HPLC system described in Section 2.6. The mass spectrometer conditions were optimized for rutin and hyperoside detection prior to sample analyses, which was performed in order to achieve the maximum sensitivity. As a result, the following mass spectrometer conditions were chosen: sheath gas flow rate: 60 bar; auxiliary gas flow rate: 10 bar; electrospray voltage of the ion source: 5 kV; capillary voltage: 10 V; capillary temperature: 260 °C. The full scan of ions ranging from 100 to 2000 molecular weight units in the negative ion mode was carried out. MS/MS experiments were performed to obtain detailed structural information of the constituents.

2.8. NMR experiments

NMR spectra were recorded at 25 °C on a Bruker AV 500 operating at a ^{1}H frequency of 500.13 MHz. Chemical shifts (δ) were expressed in ppm and coupling constants (J) were reported in Hz. Samples were dissolved in deuterated methanol (CD_3OD).

3. Results and discussion

3.1. Selection of solvent systems

Successful separation by HPCCC depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (K) for the targeted compounds. The partition coefficients of the compounds by four stages from the mixed extract of *A. venetum* were measured. The K values of hydrophobic compounds including adhyperforin, hyperforin, amentoflavone, biapigenin and quercetin between the upper and lower phase of *n*-hexane–ethyl acetate–acetonitrile–water were obtained, the data are shown in Table 1, indicating that the K values of hydrophobic compounds were from 0.02 to 4.72. The K value data indicated that when *n*-hexane–ethyl acetate–acetonitrile–water by volume ratio of 1.5:3.5:1.5:3.5 or 1.5:3.5:2:4.5 was used as the two-phase solvent system, the five compounds could not be separated. When *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2.5:5.5, v:v:v:v) was used, the five compounds can be well separated, but the separation time was long. When *n*-hexane–ethyl acetate–acetonitrile–water by volume ratio of 1.5:3.5:2.5:4.5 or 1.5:3.5:2:5 was used, five compounds were well separated and the separation time was also acceptable. So *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) was used as the initial two-phase solvent system. K values of moderate hydrophobic compounds (avicularin, acetylated isoquercetin, acetylated hyperoside and astragalin) and K for moderate hydrophilic compounds (trifolin, isoquercetin, hyperoside and querciturone) between the upper phase of ethyl acetate–acetonitrile–water or ethyl acetate–methanol–water and lower phase of *n*-hexane–ethyl

Table 1
Partition coefficient values ($K_{UP/LP}$) of the major compounds from *Apocynum venetum* leaves.

Solvent system (volume ratio)	*K* value/ Compounds [a]															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
n-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:1.5:3.5, v:v:v:v)	4.72	3.29	2.81	2.68	1.19	0.22	0.01	—[b]	—	—	—	—	—	—	—	—
n-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:4.5, v:v:v:v)	3.70	2.51	1.72	1.62	0.92	0.08	—	—	—	—	—	—	—	—	—	—
n-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2.5:4.5, v:v:v:v)	3.52	2.28	1.49	1.36	0.79	0.03	—	—	—	—	—	—	—	—	—	—
***n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v)[d]**	**3.50**	**2.13**	**1.36**	**0.97**	**0.49**	**0.01**	—	—	—	—	—	—	—	—	—	—
n-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2.5:5.5, v:v:v:v)	2.84	1.15	0.84	0.12	0.02	—	—	—	—	—	—	—	—	—	—	—
ethyl acetate–acetonitrile–water (5:2:4, v:v:v)[c]	—	—	23.65	14.68	8.16	3.84	3.05.	2.21	1.34	1.05	0.72	0.43	0.21	0.04	—	—
ethyl acetate–acetonitrile–water (5:2:5, v:v:v)	—	—	19.26	12.67	6.39	3.34	2.87	2.06	1.12	0.70	0.53	0.29	0.14	0.01	—	—
ethyl acetate–acetonitrile–water (5:3:7, v:v:v)	—	**24.12**	**14.35**	**10.39**	**5.94**	**2.84**	**2.04**	**1.42**	**0.93**	**0.52**	**0.45**	**0.12**	**0.08**	**0.01**	—	—
ethyl acetate–methanol–water (5:1:5, v:v:v)[c]	—	—	—	—	23.14	14.98	9.78	4.36	2.19	1.80	1.32	0.86	0.48	0.20	0.16	0.06
ethyl acetate–methanol–water (5:2:5, v:v:v)	—	—	—	—	**22.07**	**13.68**	**8.74**	**3.62**	**1.79**	**1.04**	**0.86**	**0.56**	**0.42**	**0.19**	**0.10**	**0.04**
ethyl acetate–methanol–water (5:2.5:5, v:v:v)	—	—	—	24.12	20.84	12.47	7.59	3.24	1.58	0.87	0.48	0.29	0.13	0.10	0.06	0.03
ethyl acetate–methanol–water (5:3.5:5, v:v:v)	—	—	—	22.96	18.71	11.97	7.47	2.83	1.37	0.60	0.23	0.09	0.02	0.08	0.04	0.01
n-butanol–methanol–water (5:0:5, v:v:v)[c]	—	—	—	—	—	—	—	—	—	—	22.87	14.67	8.27	4.57	3.10	2.04
***n*-butanol–methanol–water (5:1:5, v:v:v)**	—	—	—	—	—	—	—	—	—	—	**20.89**	**13.45**	**7.58**	**2.24**	**1.62**	**0.81**
n-butanol–methanol–water (5:1.5:5, v:v:v)	—	—	—	—	—	—	—	—	—	—	19.64	13.07	7.45	2.01	1.22	0.39

[a] 1, adhyperforin; 2, hyperforin; 3, amentoflavone; 4, biapigenin; 5, quercetin; 6, avicularin; 7, acetylated isoquercetin; 8, acetylated hyperoside; 9, astragalin; 10, trifolin; 11, isoquercetin; 12, hyperoside; 13, querciturone; 14, rutin; 15, chlorogenic acid; 16, quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside. The first Gitternetzlinien represent the *K* evaluation of compounds 1–5 (hydrophobic compound) using solvent system of *n*-hexane–ethyl acetate–acetonitrile–water, and so on.

[b] Compounds dissolve in the upper or lower phase totally and dissolve in the other phase scarcely.

[c] Partition coefficient values between the upper phase of ethyl acetate–acetonitrile–water or ethyl acetate–methanol–water or *n*-butanol–methanol–water and the lower phase of *n*-hexane–ethyl acetate–methanol–water (1.5:3.5:2:5, v:v:v:v).

[d] Bold values represents the solvent systems were choose finally for four stage elution.

acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) were obtained, the data indicating that the *K* values of moderate hydrophobic compounds were from 0.93 to 3.84 by using of upper phase of ethyl acetate–acetonitrile–water as mobile phase, and they were eluted easily, but the *K* values of moderate hydrophilic compounds were from 0.08 to 1.05, so they eluted hardly, at the different ratios of ethyl acetate–acetonitrile–water (5:2:4, 5:2:5 and 5:3:7, v:v:v), the series of solvent system of ethyl acetate–acetonitrile–water (5:3:7, v:v:v) are the most advantageous: the compounds separated well and the separation time was less than 30 min. So we chose the upper phase of ethyl acetate–acetonitrile–water (5:3:7, v:v:v) as the following mobile phase for elute the moderate hydrophobic compounds. Because the *K* values of moderate hydrophilic compounds were too low, we switch mobile phase to the upper phase of ethyl acetate–methanol–water, The *K* values of trifolin, isoquercetin, hyperoside and querciturone between the upper phase of ethyl acetate–methanol–water (5:0:5, 5:1:5 5:1.5:5 and 5:2:5, v:v:v) and the lower phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) were obtained. The data indicate that ethyl acetate–methanol–water (5:2:5, v:v:v) are most suitable solvent system for elute the moderate hydrophilic compounds due to the *K* values from 0.42 to 1.04. For comprehensive separation, solvent system of *n*-butanol–methanol–water was used for separation of hydrophilic compounds (rutin, chlorogenic acid and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside). The *K* value data indicate that when the upper phase of *n*-butanol–methanol–water (5:0:5, v:v:v) was used as the mobile phase, the compounds could be dissolved in the upper phase easily but scarcely in the lower phase. When *n*-butanol–methanol–water (5:2:5, v:v:v) was used, the solvent system can not be distributed into two phases, and system of *n*-butanol–methanol–water with a volume ratio of 5:1.5:5 can be distributed into the two phases but

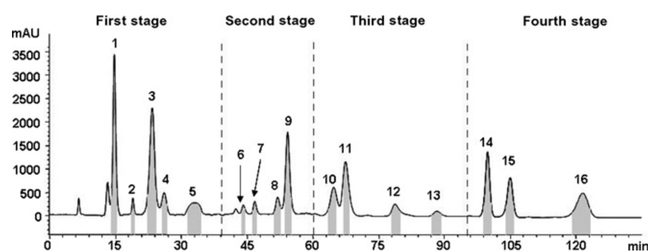

Fig. 2. HPCCC chromatogram of the mixed extract of *A. venetum* at 254 nm. 1, adhyperforin; 2, hyperforin; 3, amentoflavone; 4, biapigenin; 5, quercetin; 6, avicularin; 7, acetylated isoquercetin; 8, acetylated hyperoside; 9, astragalin; 10, trifolin; 11, isoquercetin; 12, hyperoside; 13, querciturone; 14, rutin; 15, chlorogenic acid; 16, quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside.

the distribution time was too long and the two-phase system was unstable (easy to convert to single layer). So we chose the upper phase of *n*-butanol–methanol–water (5:1:5) as the mobile phase to elute the hydrophilic compounds. The *K* values were from 0.81 to 2.24 indicated that the hydrophilic compounds can elute easily and separated well. The experiment also indicated that the hierarchical time of the upper phase of *n*-butanol–methanol–water (5:1:5) with lower phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:4.5, v:v:v:v) was short (15 s, 25 °C), and the system was very stable. So *n*-butanol–methanol–water (5:1:5, v:v:v) was chosen for the last stage of gradient elution of HPCCC (Table 1).

3.2. HPCCC separation of major compounds with wide range of hydrophobicities

The HPCCC procedure was modified as follows: the column was first filled with a lower phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) followed by elution with the upper phase in a tail to head direction at a flow rate of 2.0 ml/min while the column was rotating at 800 rpm. After the hydrodynamic equilibrium of the two-phase system was reached in the rotating column, 6 ml of sample solution composed of 3 ml of every phase containing 10.31 mg of extract was injected into the column *via* a sample injector. Fig. 2 shows a comprehensive separation of the constituents having a broad range of polarity detected by the diode array detector. The upper phase of *n*-hexane–ethyl acetate–acetonitrile–water (1.5:3.5:2:5, v:v:v:v) as a starting mobile phase was eluted for 40 min, five hydrophobic *n*-hexane soluble compounds including adhyperforin, hyperforin, amentoflavone, biapigenin and quercetin were eluted. For the purpose of the complete elution, the mobile phase was switched to the upper phase of ethyl acetate–acetonitrile–water (5:3:7, v:v:v) eluted for 20 min and upper phase of ethyl acetate–methanol–water (5:2:5, v:v:v) eluted for 35 min respectively, four moderate hydrophobic compounds including avicularin, acetylated isoquercetin, acetylated hyperoside, astragalin and four moderate hydrophilic compounds including trifolin, isoquercetin, hyperoside, querciturone were eluted. Then the mobile phase was switched to the upper phase of *n*-butanol–methanol–water (5:1:5, v:v:v). Three hydrophilic compounds including rutin, chlorogenic acid and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside were eluted continuously. With the help of the gradient elution mode of HPCCC, the 13 compounds were well separated within 130 min.

3.3. Identification of the components from A. venetum by LC–MS and NMR

The HPLC peak fractions (peaks 1–3, 5, 8, 9, 12, 14 and 15 for *A. venetum* sample were identified by compared with those of available reference compounds analyzed under the identical LC–MS conditions in order to compare the retention time and mass spectra, as well as the UV spectral (Fig. 3). For compounds without available reference, ^{1}H NMR experiments appear to be a powerful tool for their characterization. Tables 2 and 3 present the LC–MS and NMR data obtained for all the peaks, including the retention time, UV λ_{max}, MS/MS as well as NMR data. The experiment reveals that the HPLC–MS peaks 1 and 2 belong to phloroglucinols, peaks 3 and 4 belong to biflavone, peak 5 belongs to flavone, peaks 6–14 and 16 belongs to the kind of flavone-O-glycosides and peaks 15 belong to organic acid. Peaks 1 and 2 were identified to be same kind of phloroglucinols by their LC retention time (82.45, 78.08 min), UV λ_{max} at 204 and 276 as well as MS/MS data, the ion $[M-H]^-$ of peak 1 is at *m*/*z* 549 and the ions of its MS/MS were at *m*/*z* 480, 411, 397, the $[M-H]^-$ of peak 2 is at *m*/*z* 535 and its MS/MS were at *m*/*z* 466, 397, 383, which shows losses of 69, 138 and 152 Da, so the cleavage pathway above mentioned were corresponding to $[M-C_5H_9-H]^-$, $[M-H-C_5H_9-C_5H_9]^-$ and $[M-H-C_5H_9-C_6H_{11}]^-$ respectively, and the above mentioned MS data agree with published reports [23–26], we identified peaks 1 and 2 were adhyperforin and hyperforin. Both peaks 3 and 4 show molecular ion $[M-H]^-$ at *m*/*z* 537, and their $-MS^2[M-H]^-$ gave ions at *m*/*z* 443, 417, 385 ($[M-C_7H_4O_4-H]^-$) and 151 ($[M-C_{23}H_{14}O_6-H]^-$) (Fig. 1), they both show UV λ_{max} at 268 and 333 nm (Table 2), we identified the two compounds to be the kind of biflavone, by comparing the LC retention time and their cleavage pathway with those of the authentic standard, we determined that peaks 3 and 4 were assigned to amentoflavone (I3′, II8-biapigenin) and biapigenin (I3, II8-biapigenin) respectively, the cleavage pathway of ions at *m*/*z* 386 and 152 being consistent with the literature [27,28]. Peaks 7 and 8 both show a molecular ion $[M-H]^-$ at *m*/*z* 505, and the MS^2 of their $[M-H]^-$ both show ion at *m*/*z* 463 and *m*/*z* 300. The ^{1}H NMR data of the portion under peak 7 indicate that H-2′, H-6′ and H-5′ on ring B [ABX system signals at δ 7.64 (1H, *dd*, *J* = 8.47, 1.81 Hz, H-6′), 7.51 (1H, *d*, *J* = 1.89 Hz, H-2′), 6.81(1H, *J* = 8.47 Hz, H-5′)], corresponding to a quercetin aglycon δ1.73(*s*, 3H) was confirm to be methyl proton of acetyl group, so peaks 7 and 8 were identified as acetylated isoquercitrin and acetylated hyperoside respectively. Peaks 9 and 10 were identified as kaempferol-3-*O*-glucoside (astragalin) and kaempferol-3-*O*-galactoside (trifolin) by a neutral loss of 162 Da

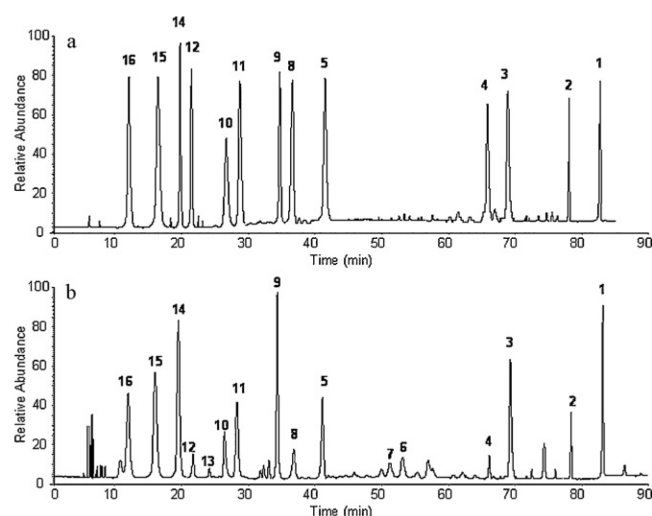

Fig. 3. Total ion chromatogram of HPLC-MS of authentic standards mixture (a), *A. venetum* leaf extract (b). The numbers of HPLC peaks were consistent with Fig. 2. Column: Agilent C_{18} (250 mm × 4.6 mm I.D., 5 μm) with a DAD system (Waters model 2998), Column temperature: 25 °C. Mobile phase: (A) acetonitrile; (B) 0.5% acetic acid in water. The linear gradient was: 0–10 min, 10% A; 20–80 min, 10–100% A; 80–90 min, 100% A, flow rate of 1.2 ml/min. Detection wavelength: 254 nm.

Table 2
UV and ^{1}H NMR data of the compounds separated by HPCCC.

Substance	Peak	UV λ_{max}	Purity (%)	^{1}H NMR data (CD_3OD) Chemical shift (ppm) multiplicity (coupling constant *J* (Hz))
Adhyperforin	1	204, 276	94.4	n.d[a]
Hyperforin	2	204, 276	98.2	n.d.
Amentoflavone	3	268, 333	99.4	n.d.
Biapigenin	4	268, 333	98.7	13.09 (1H, s, OH-5(I)), 12.82 (1H, s, OH-5(II)), 7.60 (2H, d, *J* = 9.0 Hz, H-20(I), H-60(I)), 7.41 (2H, d, *J* = 9.0 Hz, H-20(II), H-60(II)), 6.85 (2H, d, J = 9.0 Hz, H-30(I),H-50(I)), 6.72 (2H, d, *J* = 9.0 Hz, H-30(II),H-50(II)), 6.60 (1H, s, H-3(II)), 6.58 (1H, d, *J* = 2.2 Hz, H-8(I)), 6.35 (1H, d, *J* = 2.2 Hz, H-6(I)), 6.30 (1H, s, H-6(II))
Quercetin	5	254, 368	99.9	n.d.
Avicularin	6	228, 254, 360	97.3	6.21 (*d*, 2.0, H-6), 6.40 (*d*, 2.0, H-8), 7.54 (*d*, 2.1, H-2′), 6.92 (*d*, 8.3, H-5′), 7.50 (*dd*, 2.1, 8.3, H-6′), 5.48 (*d*, 1.0, H-1′′), 4.35 (*dd*, 1.0, 3.0, H-2′′), 3.93 (*dd*, 3.0, 5.0, H-3′′), 3.89 (*m*, H-4′′) 3.52 (*dd*, 4.5, 11.5, H-5′′a), 3.48 (*dd*, 3.6, 11.5, H-5′′b)
Acetylated isoquercetin	7	230, 254, 355	98.4	12.60, 10.85, 9.70 and 9.18 (4H, *s*, phenolic hydroxyl protons), 7.54 (1H, *dd*, *J* = 8.71, 1.93 Hz, H-6′), 7.52(1H, *d*, *J* = 1.89 Hz, H-2′), 6.82(1H, *d*, *J* = 8.66 Hz, H-5′), 6.40(1H, *d*, *J* = 2.00 Hz, H-8), 6.20(1H, *d*, *J* = 2.00 Hz, H-6), 5.40–3.10 (6H, *m*, sugar protons), 5.35 (1H, *d*, *J* = 7.20 Hz, terminal group proton of sugar), 1.73 (3H, *s*, methyl proton)
Acetylated hyperoside	8	230, 254, 355	93.3	n.d.
Astragalin	9	235, 265, 350	92.3	n.d.
Trifolin	10	235, 265, 350	97.9	6.10 (1H, *d*, H-6),6.44 (1H, *d*, H-8), 6.86 (2H, *d*, H-3′,5′), 8.07(2H, *d*, H-2′, 6′), 12.62 (5-OH), 10.88 (*s*, 7-OH), 10.17 (*s*, 4′-OH) 5.40 (1H, *d*, H-1′′)
Isoquercetin	11	228, 254, 346	98.4	12.26 (1H, *s*, OH-5'), 8.00 (1H, *d*, *J* = 1.7 Hz, H-2′), 7.56 (1H, dd, $J_{6',5'}$ = 8.4 Hz, $J_{6',2'}$ = 1.7 Hz, H-6′), 6.96 (1H, *d*, *J* = 8.4 Hz, H-5′), 6.51 (1H, *s*, H-8), 6.30 (1H, *s*, H-6) 5.03 (1H, *d*, *J* = 7.9′Hz, H-1′′)
Hyperoside	12	228, 254, 356	97.1	n.d.
Querciturone	13	237, 270, 355	98.8	6.42 (*d*, 2.0, H-6), 6.23 (*d*, 2.0, H-8), 7.72 (*d*, 1.9, H-2′′), 6.87 (*d*, 8.4, H-5′′), 7.60 (*dd*, 1.9, 8.4, H-6′), 5.38 (*d*, 7.4, H-1′′), 3.56 (*m*, H-2′′), 3.50 (*m*, H-3′′), 3.61 (*m*, H-4′′) 3.78 (*m*, H-5′′a)
Rutin	14	228, 265, 360	97.8	n.d.
Chlorogenic acid	15		98.8	n.d.
Quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside	16	225, 257, 355	98.9	12.68 (5-OH), 10.84 (7-OH), 9.19 (3′-OH), 9.70 (4′-OH), 6.19 (1H, *d*, *J* = 1.5 Hz, H-6), 6.40 (1H, *d*, *J* = 1.5 Hz, H-8), 7.55 (1H, *s*, H-2′),6.87 (1H, *d*, *J* = 8.5 Hz, H-5′), 7.60 (1H, *d*, *J* = 8.5 Hz, H-6′), 5.48 (1H, *d*,*J* = 7.0 Hz, H-1′′), 4.60 (1H, *d*, *J* = 7.5 Hz,H-1′′′)

[a] Identified by comparison with the authentic standard HPLC–MS and UV data.

which indicated that lose of one hexose molecule, and both precursor ions scanned were at *m*/*z* 284 according to their MS/MS data. Discrimination between astragalin and trifolin can be based on the differences of the glycoside protons chemical shifts, especially that one of the H-1′′ proton (δ 5.40 for terminal proton of galactoside signal and δ 5.46 for terminal proton of glucoside signal respectively). Peaks 14 and 16 had a molecular ion [M−H]$^-$ at *m*/*z* 625 and 609 respectively, and they (−MS^2 [M−H]$^-$) both gave a single ion at *m*/*z* 301 (Table 3), indicating that it is a quercetin derivative with two sugar units, therefore these two compounds were identified as rutin and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside respectively [29,30]. Peaks 5 and 15 were identified as quercetin and chlorogenic acid [31,32]. Peaks 11 and 12 were identified as isoquercetin and hyperoside respectively. Discrimination between two compounds can be based on the differences of the glycoside protons chemical shifts of ^{1}H NMR, especially that one of the H-1′′

Table 3
HPLC–MS data of major constituents identified in the extract of *A. venetum*.

Substance	Peak	t_R (min)	MW	Substance MS data		Authentic standard MS data	
				MS [M−H]$^-$	MS/MS[a]	MS [M−H]$^-$	MS/MS[a]
Adhyperforin	1	82.45	550	549	480, 411, 397	549	480, 411, 397
Hyperforin	2	78.08	536	535	466, 397, 383	535	466, 397, 383
Amentoflavone	3	69.01	538	537	443, 385, 417, 151	537	443, 385, 417, 151
Biapigenin	4	67.87	538	537	443, 385, 417, 151	537	443, 385, 417, 151
Quercetin	5	41.84	302	301	271, 255, 151	301	271, 255, 179, 151
Avicularin	6	53.74	434	433	301, 271, 179, 151	–	–
Acetylated isoquercetin	7	51.24	506	505	463, 300, 301, 255	–	–
Acetylated hyperoside	8	36.75	506	505	463, 301, 255	505	463, 301, 255, 179
Astragalin	9	34.14	448	447	284	447	284
Trifolin	10	26.45	448	447	284	447	284
Isoquercetin	11	28.24	464	463	301	463	300, 301, 271, 179
Hyperoside	12	21.81	464	463	301, 271, 255	463	301, 271, 255, 179
Querciturone	13	24.03	478	477	301	–	–
Rutin	14	19.28	610	609	463, 301	609	463, 301
Chlorogenic acid	15	17.94	354	353	191, 179	353	191, 179
Quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside	16	11.87	626	625	463, 301	–	–

[a] Listed by order of abundance.

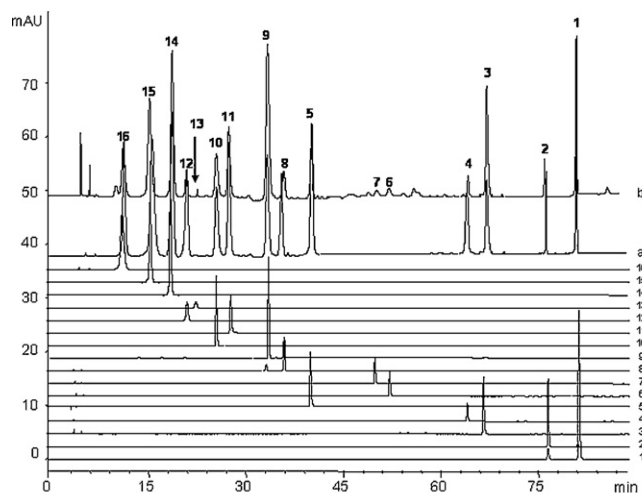

Fig. 4. HPLC chromatogram of *A. venetum* leaf extract and HPCCC peak fractions. The numbers of HPLC peaks were consistent with Fig. 2(a) HPLC chromatogram of mixed standards. (b) HPLC chromatogram of crude extract.

proton of 5.10 (1H, *d*, *J* = 7.3 Hz) for isoquercitrin [33]. Peak 6 was identified as avicularin [34]. Peak 13 was identified as querciturone [35,36]. Data were shown in Table 2.

3.4. Purity analyses by HPLC

Each peak fraction of the extract of *A. venetum* by HPCCC was analyzed by HPLC–DAD. According to the adopted methods the purities of 16 compounds were all above 92.0% (purities of peaks 2–5, 7, 11, 13, 15 and 16 were above 98.0%, purities of peaks 1, 6, 8–10, 12 and 14 were from 92.3% to 98.0%). The HPLC chromatograms of each fraction are shown in Fig. 4. Identification of the HPCCC peak fractions was based on UV spectral, HPLC retention time as well as HPLC–MS data. Peak fractions of 4, 6, 7, 10, 11, 13 and 16 were also identified with the help of ^{1}H NMR experiment.

4. Conclusion

The results of the present studies indicate that the novel HPCCC technique using gradient elution of the four sets of solvent systems is very useful for the separation of multiple compounds with a broad range of polarity. Ten compounds which separated by HPCCC were identified by HPLC–MS with authentic standards and five compounds were identified by ^{1}H NMR. A total of 16 compounds including adhyperforin, hyperforin, amentoflavone, biapigenin, quercetin, avicularin, acetylated isoquercetin, acetylated hyperoside, astragalin, trifolin, isoquercetin, hyperoside, querciturone, rutin, chlorogenic acid and quercetin-3-*O*-β-D-glucosyl-β-D-glucopyranoside with the purities of 94.4%, 98.1, 99.41%, 98.7, 99.9, 97.3, 98.4, 93.3, 92.3, 97.9%, 98.4%, 97.1% 98.82% and 98.9% respectively in *A. venetum* were comprehensively separated in one step operation in 130 min. If we were to separate the above sixteen compounds by conventional HPCCC mode, it would be necessary to use a system of two or three different solvents and multi-stage separation, which is tedious and time consuming.

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