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Isolation, identification and antioxidative capacity of water-soluble phenylpropanoid compounds from *Rhodiola crenulata*

Danjun Chen, Junting Fan, Peng Wang, Lanying Zhu, Yang Jin, Yan Peng, Shuhu Du *

School of Pharmacy, Nanjing Medical University, Nanjing 210029, China

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ABSTRACT

Six water-soluble phenylpropanoid compounds obtained from *Rhodiola crenulata* (*R. crenulata*) were fractionated by high-speed counter-current chromatography (HSCCC), and purified by semi-preparative high-performance liquid chromatography (Semi-prep HPLC). The purities of the six compounds were all above 98.0% and their structures were identified by spectroscopic methods. Among them, a new compound, 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**), together with two known phenylpropanoids, *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**) and picein (**4**) were isolated from *R. crenulata* for the first time. Meanwhile, the contents of six isolated ingredients from the crude extract of *R. crenulata* had been simultaneously detected, with satisfactory results. Furthermore, the antioxidant activities of the six compounds were accessed by measuring the radical scavenging activity against 2,2-diphenyl-1-picrylhydrazy (DPPH), and four compounds exhibited potent antioxidative activity.

1. Introduction

Rhodiola crenulata (Hook. f. et Thoms) H. Ohba, as an important member of the Crassulaceae family, is mainly distributed in the high cold region of Yunnan and Sichuan province as well as the Tibetan Autonomous Region. Its roots have been usually used as a health food, antidepressant and antifatigue for a long time. Modern pharmacological studies have demonstrated that the genus *Rhodiola* possessed the functions of reinforcing immunity (Mishra, Padwad, Jain, Karan, Ganju, & Sawhney, 2006), improving memory (Fan, Tezuka, Namba, & Kadota, 1999; Tezuka, Fan, Kasimu, & Kadota, 1999), scavenging active-oxygen species (Furmanowa, Skopińska-Różewska, Rogala, & Hartwich, 1998; Ohsugi et al., 1999), lowering blood glucose (Kwon, Jang, & Shetty, 2006; Yang, Liu, Feng, Jiang, & Zhang, 2012), resisting tumour (Dement'eva & Iaremenko, 1987; Udintsev & Schakhov, 1991), and so on. Especially, *R. crenulata* has the bi-directional function of accommodating central nervous system and endocritic system, and keeps the body in excellent balance situation (You et al., 2003). Additionally, extracts of *R. crenulata* are usually made into pharmaceutical preparations or functional foods used by astronauts, pilots, divers and sports who work under special circumstances (Shen, Jiang, Gu, Qiu, Shen, & Yang, 2010; Zhao et al., 1998). Owing to the important virtues of *R. crenulata*, its study is a subject of great interest. Previous phytochemical studies have reported the isolation of many compounds from this plant, including phenols, phenylpropanoids, phenyethanoids, flavonoids, monoterpenoids, cyanogens and their corresponding glycosides (Du & Xie, 1994; Nakamura, Li, Matsuda, & Yoshikawa, 2008; Peng, Ma, & Ge, 1995; Wu, Guo, Guo, Li, Wang, & Ma, 2008; Yang et al., 2012). Among the isolated compounds, tyrosol, gallic acid and crenulatin had been proved to be good radical scavengers, based on the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazy (DPPH) free radical (Domínguez, Torres, & Núñez, 2001; Lee et al., 2000). The above investigation suggests that it would also be a very interesting and plentiful source of antioxidants. Therefore, further chemical research from this herb is warranted for new pharmaceutical preparations or functional products and DPPH radical scavenging capacity assay.

The classical methods of preparative separation and purification of compounds from complex plant extracts usually require multiple chromatographic steps on silica gel, polyamide, Sephadex LH-20 column, and so on. These methods often lead to the loss of active components (due to the dilution effects and/or decomposition of the components, especially antioxidants during the isolation and purification processes) (Hostettmann, Wolfender, & Terreaux, 2001). In order to avoid the problems mentioned above, it is necessary to develop more efficient methods for separating the bioactive compounds from natural products. High-speed counter-current chromatography (HSCCC), being a continuous liquid–liquid partition chromatographic method, eliminates complications such as irreversible adsorption on the solid support, denaturation of

* Corresponding author. Tel./fax: +86 25 86862762.
E-mail address: shuhudu@njmu.edu.cn (S. Du).

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sample, tailing of the solute peaks, etc. (Ito, 2005). Based on the advantages of the HSCCC system, it has been successfully applied to the separation of active constituents of various herbs (Du, Li, & Ito, 2011; Han, Zhang, Wei, Cao, & Ito, 2002).

The purpose of this work was to set up a very convenient and efficient method for the rapid separation of water-soluble phenylpropanoid compounds from *R. crenulata* and for screening their antioxidative activities. Firstly, water-soluble phenylpropanoid compounds from *R. crenulata* were isolated by offline coupling of HSCCC and semi-preparative high-performance liquid chromatography (Semi-prep HPLC). Secondly, the chemical structures of the isolated compounds were elucidated by ultraviolet spectra (UV), infrared spectrum (IR), high resolution mass spectrum (HRMS) and nuclear magnetic resonance spectrum (NMR). Moreover, the method of simultaneous determination of isolated compounds from the crude extract of *R. crenulata* was developed. Finally, the antioxidant activities of the pure compounds were determined by measuring the radical scavenging activity against DPPH, which may give support for the traditional use of the herb.

2. Materials and methods

2.1. Apparatus

The HSCCC apparatus employed in the current study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation columns connected in series (i.d. of the tubing is 1.6 mm, total volume is 260 ml) and a 20 ml manual sample loop. The revolution speed of the instrument could be regulated with a speed controller in the range from 0 to 1000 rpm. An HX 1050 constant temperature circulator (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. An ÄKTA prime system (GE Healthcare, Uppsala, Sweden) was used to pump the two-phase solvent system and performed the UV absorbance measurement. The detection wavelength was 280 nm. An automatic fraction collector (GE Healthcare, Uppsala, Sweden) was used to collect the fractions. The data was collected with N2000 chromatography workstation (Hangzhou Zhida Science Apparatus Company, Hangzhou, China).

Analytical high-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-10A system equipped with a UV detector (monitoring at 275 nm) and Shimadzu LC-20A system equipped with SPD-M20A detector. The semi-preparative high-performance liquid chromatography (Semi-prep HPLC) equipment used consisted of two LC-6A pumps with a UV–visible detector (monitoring at 275 nm) and a Phenomenex C_{18} column (250 × 10.0 mm, 8 μm).

The IR spectrum was obtained on a Tensor 27 FT-IR spectrophotometer with KBr pellet (Bruker, Ettlingen, Germany). The UV spectrum was obtained on a UV-2501 spectrometer (Shimadzu, Toyko, Janpan). HRMS was recorded on a 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Santa Clara, USA). The NMR spectrometer used was AV-300 MHZ NMR (Bruker, Fällanden, Switzerland). The reference compound tetramethylsilane (TMS) was used as an internal standard for the determination of chemical shifts. Gas chromatograph (GC) analysis was performed on a HP5890 gas chromatograph (Agilent Technologies, Massy, France) equipped with a 30QC2/AC-5 quartz capillary column (30 × 0.32 mm, 0.25 μm) and a flame ionisation detection (FID). The optical rotation was determined on a p-1020 polarimeter (Jasco, Tokyo, Japan). 96 Well microtiter plates (Costar, New York, USA) and a Spectra Max M2 microplate reader (Molecular Devices, Sunnyvale, USA) were used in the radical scavenging activity experiments.

2.2. Regents and materials

All solvents used for HSCCC were of analytical grade (Merck, Darmstadt, Germany). Methanol used for analytical HPLC was of chromatographic grade (Merck, Darmstadt, Germany). Deionized and purified water was prepared using a water system (Millipore, Bedford, USA). D-101 Macroporous resin was purchased from the chemical plant of Nankai University (Tianjin, China). 2,2-Diphenyl-1-picrylhydrazy (DPPH), D-glucose and L-glucose were purchased from Sigma–Aldrich (Steinheim, Germany). DPPH radical solution was freshly prepared in methanol every day and kept protected from light. Rutin standard was supplied by National Institute of the Control of Pharmaceutical and Biological Products (Beijing, China).

The dried roots of *R. crenulata* (Hook. f. et Thoms) H. Ohba were collected from the Tibetan Autonomous Region of China in August, 2009. The botanical origin of the material was identified and the voucher specimens were deposited at the School of Pharmacy, Nanjing Medical University (Nanjing, China).

2.3. Preparation of crude extract

The dried roots of *R. crenulata* (2.0 kg) were pulverised and extracted three times (1 h for each time), each time with 6000 ml of 80% ethanol. The extract solutions were combined and concentrated by rotary evaporator at 65 °C to form a brownish syrup (about 400 g). This was dissolved in hot water by sonication, statically cooled down and filtered. The filtrate was partitioned three times with equal volume of ethyl acetate. The ethyl acetate fractions (upper layer) were removed. Subsequently, a suitable volume of the lower layer was added into a glass column of macroporous resin (1.8 × 70 cm, contained 80 g of D-101 macroporous resin). The resin was washed by water until the effluent almost colorless, and sequentially eluted with 30% ethanol. The elution fractions were collected and evaporated to dryness under reduced pressure. Finally, 15 g of crude extract was obtained, which was stored in the refrigerator for the further HSCCC isolation.

2.4. Procedure of HSCCC pre-separation

The two-phase solvent system composed of ethyl acetate–*n*-butanol–water (0.5:4.5:5, v/v/v) was used for HSCCC separation. Each component of the solvent system was added to a separatory funnel and thoroughly equilibrated by shaking at room temperature. The upper phase and lower phase were separated and degassed by sonication for 30 min shortly before use.

The sample solution for the HSCCC pre-separation was prepared by dissolving 150 mg of the dried powder of crude extract in 20 ml of the lower phase of two-phase solvent system. The HSCCC pre-separation was performed as follows: the coiled column was first pumped with the upper phase of the solvent system and then the lower phase was pumped into the column at a flow rate of 2.0 ml/min, meanwhile the apparatus was rotated at 850 rpm. After a hydrodynamic equilibrium was reached, 20 ml of the sample solution were injected into the separation column. The effluent was monitored with UV detector at 280 nm and the peak fractions were automatically collected according to the chromatographic profile.

2.5. HPLC analysis of HSCCC peak fractions

Each fraction isolated from HSCCC was analysed by analytical HPLC, which was performed with Phenomenex C_{18} column (250 × 4.6 mm, 5 μm). The mobile phase was composed of methanol–water (12:88, v/v) and the flow rate was 1.0 ml/min. All sample solutions were filtered through a 0.45 μm microporous

membrane before injection. The injection volume was 20 μl and the detection was carried out at 275 nm.

2.6. HPLC purification of HSCCC peak fractions

Each fraction isolated from HSCCC was purified by Semi-prep HPLC, which was performed with a Phenomenex C_{18} column (250 × 10.0 mm, 8 μm). The mobile phase (ratio of methanol to water) was determined based on the analytical HPLC results and the flow rate was 4.0 ml/min, the column temperature was at 25 °C. All sample solutions were filtered through 0.45 μm filter before delivering into the system. The injection volume was 200 μl and the chromatograms recorded at 275 nm.

2.7. Identification of the purified compounds

A Tensor 27 FT-IR spectrometer with a resolution of 2 cm^{-1} and a spectral range of 4000–500 cm^{-1} was employed to examine infrared spectra of samples by a pressed tablet (sample:KBr = 1:14 in mass). UV spectrum was recorded on a UV 2501 spectrometer. HRMS data was measured on a 6520 Accurate-Mass Q-TOF LC/MS with an ESI source, a negative ion mode was employed. The ^1H NMR, ^{13}C NMR and 2D NMR experiments were performed on a AV-300MHZ NMR spectrometer using DMSO-d_6 as solvent.

2.8. Acid hydrolysis of compound *1*

The isolated compound **1** (4.0 mg) was hydrolysed with 2 M HCl in 1,4-dioxane (1:1, 4 ml) under reflux at 80 °C for 6 h. Each reaction mixture was extracted with $CHCl_3$ three times (2 ml × 3). The aqueous layer was neutralised with 2 M NaOH and then dried to give one or more monosaccharides. The dried powders were dissolved in pyridine (2 ml), L-cysteine methyl ester hydrochloride (1.5 mg) was added, and the mixture was heated at 60 °C for 1 h. Subsequently, trimethylsilylimidazole (1.5 ml) was added to the above mixture in ice-cold water and kept at 60 °C for another 30 min. An aliquot of the supernatant (4 μl) was directly subjected to GC analysis under the following conditions: column temperature, 180–280 °C; programmed increase, 3 °C/min; carrier gas, N_2 (1.0 ml/min); injector and detector temperature, 250 °C; split ratio, 1:50. The configuration of D-glucose for compound **1** was determined by comparing the retention times with the derivatives of references (D-glucose and L-glucose).

2.9. Validation study of analytical HPLC

2.9.1. Calibration curves

Stock solution containing six reference compounds (compounds **1**–**6**) was prepared and diluted to appropriate concentrations for construction of calibration curves. Each concentration of the mixed standard solution was injected in duplicate, and then the calibration curves were constructed by plotting the peak area versus the concentration of each analyte.

2.9.2. LOD and LOQ

The stock solution containing six reference compounds (compounds **1**–**6**) was diluted to a series of appropriate concentrations, and an aliquot of the diluted solution was injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (*S*/*N*) of about 3 and 10, respectively.

2.9.3. Precision, accuracy and repeatability

Intra- and inter-day variations were selected to determine the precision of the method. For intra-day variability test, the mixed standard solutions were analysed for six replicates within one day, while for the inter-day variability test, the solutions were detected in duplicates for consecutive three days. Variations were expressed in terms of relative standard deviation (RSD).

The recovery was used to evaluate the accuracy of the method. A known amount of six reference compounds (compounds **1**–**6**) were added into a certain amount of crude extract solution. Three replicates were performed in the test. The precision was expressed in terms of RSD.

To confirm the repeatability, six replicates of the same sample (crude extract) were analysed as mentioned above. The RSD value was calculated as a measurement of the method repeatability.

2.10. Analysis of crude extract

The crude extract (0.10 g) was transferred into 50 ml volumetric flask which was made up to its volume with 30% methanol and filtered through a 0.45 μm nylon membrane filter prior to injection into the HPLC system. Then the contents of the isolated compounds (compounds **1**–**6**) from the crude extract were determined by HPLC, which was performed with Phenomenex C_{18} column (250 × 4.6 mm, 5 μm). The mobile phase was composed of methanol–water (15:85, v/v) and the flow rate was 1.0 ml/min. The injection volume was 20 μl and the detection was carried out at 275 nm.

2.11. Evaluation of antioxidative activity

The antioxidative activities of compounds **1**–**6** were assessed on the basis of the scavenging activity of the stable DPPH free radical (Brand-Williams, Cuvelier, & Berset, 1995; Shi, Zhao, Zhou, Zhang, Jiang, & Huang, 2008). Typically, isolated compounds and reference compound were dissolved in methanol to form various concentrations of test samples, respectively. 100 μl of test samples mixed with 100 μl DPPH methanol solution (100 μg/ml) in 96 well microtiter plates, respectively. Meanwhile, the methanol solution of DPPH was served as a control, which was prepared freshly every day. The absorbance was measured at 517 nm after the mixture was kept in the dark for 30 min at room temperature. The antioxidant activity was expressed as radical scavenging percentage (RSP) calculated according to the following equation:

$$\mathrm{RSP} = \frac{A_1 - A_2}{A_1} \times 100\%$$

where A_1 is the absorbance of the DPPH solution and A_2 is the absorbance of the DPPH solution after the addition of samples. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage. All tests were operated in triplicate.

3. Results and discussion

3.1. Optimisation of suitable HSCCC solvent system

The optimum of the separation conditions for HSCCC is related to various parameters including a two-phase solvent system, revolution speed, flow rate, column temperature, etc. In order to select the optimum two-phase solvent system, a series of tests were performed in this study. According to the rules in selecting the optimal conditions introduced by Ito (Ito, 2005), the solvent system of ethyl acetate–*n*-butanol–water would be suitable for water-soluble compounds (i.e., salidroside, tyrosol, picein, etc.). Also, several types of the solvent systems composed of ethyl acetate–*n*-butanol–water at different volume ratios were chosen and assessed for *K*-values. The *K*-values of compounds **1**–**6** for the solvent system under the volume ratios of 3:2:5, 4:1:5, 1:4:5 and 0.5:4.5:5 were summarised in Table 1, respectively. It could be seen that

Table 1
The *K* values of the target components in ethyl acetate–*n*-butanol–water.

Solvent system (v/v/v)	*K*					
	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6**
3:2:5	0.19	0.39	0.33	0.33	0.73	0.68
4:1:5	0.15	0.37	0.30	0.32	0.72	0.66
1:4:5	0.26	0.63	0.48	0.51	0.84	0.81
0.5:4.5:5	0.29	0.68	0.49	0.52	0.85	0.83

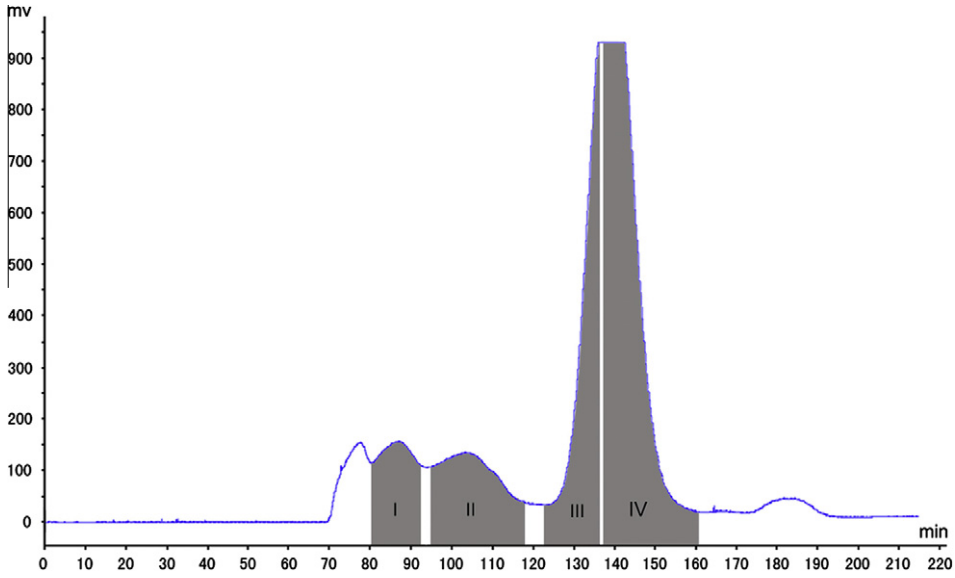

Fig. 1. HSCCC chromatogram of the crude extract from *R. crenulata*. Experimental conditions: two-phase solvent system, ethyl acetate–*n*-butanol–water (0.5:4.5:5, v/v/v); stationary phase, upper phase; mobile phase, lower phase; elution mode, head to tail; flow rate, 2.0 ml/min; revolution speed, 850 rpm; separation temperature, 30 °C; detection wavelength, 280 nm; sample loading, 150 mg/20 ml; retention of stationary phase: 68.9%.

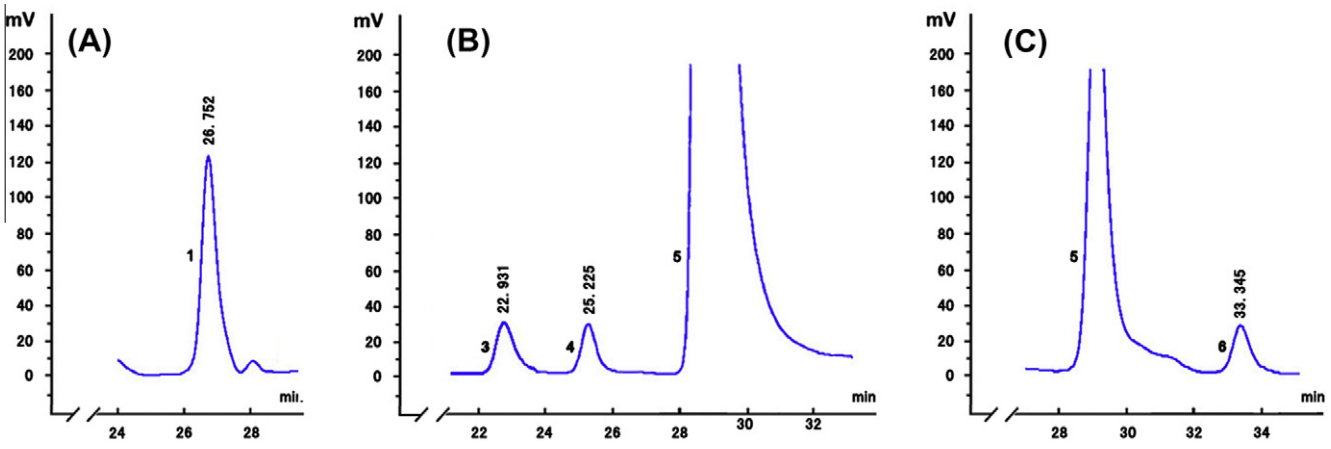

Fig. 2. Semi-prep HPLC chromatogram of compound **1** (A), compound **3**, compound **4** and compound **5** (B), and compound **6** (C). Experimental conditions: column, Phenomenex C_{18} column (250 × 10.0 mm, 8 μm); mobile phase, methanol–water (12:88, v/v) (A), methanol–water (11:89, v/v) (B) and methanol–water (10:90, v/v) (C), respectively; flow rate, 4.0 ml/min; detection wavelength, 275 nm; column temperature, 25 °C.

3:2:5, 4:1:5 had minor *K*-value and when performed on HSCCC, the peak was overlapped. The other 1:4:5 and 0.5:4.5:5 had appropriate *K*-values. Further HSCCC separation was performed and the results showed that the solvent system composed of ethyl acetate–*n*-butanol–water (0.5:4.5:5, v/v/v) could achieve a better retention of stationary phase (60.9%) for preparative HSCCC, and the four fractions (1–4) could be separated along with acceptable separation time. So, it was selected as the solvent system of HSCCC in the following studies.

In the 0.5:4.5:5 solvent system, several kinds of flow rates (1.0, 1.5, 2.0 and 2.5 ml/min) were tested. The results showed that high flow rate (2.5 ml/min) was not good for the retention of the stationary phase and reducing the flow rate (1.0 and 1.5 ml/min) could improve the retention of the stationary phase to some degree, but the separation time would be extended. Thus, the flow rate was set at 2.0 ml/min in the experiment.

In general, the revolution speed of the apparatus also has an influence on the retention percentage of the stationary phase and

Table 2
NMR data (300 MHz, DMSO-d_6) and HMBC correlations of compound **1**.

Position	δ_C	Mult.	δ_H (J in Hz)	HMBC[a]
1	127.6	C		
2, 6	129.6	CH	6.98, d (8.4)	3, 4, 5, 7
3, 5	115.2, 115.3[b]	CH	6.59, d (8.4)	1, 4
4	156.7	C		
7	34.8	CH_2	2.70, t (7.5)	1, 2, 6, 8
8	69.9	CH_2	3.86, dd (7.5, 17.1) 3.58, overlap	1′, 1
1′	102.7	CH	4.16, d (7.8)	8
2′	73.3	CH	2.95, overlap	1′, 3′
3′	76.7	CH	3.14, m	2′, 4′
4'	70.0	CH	3.05, overlap	3′
5′	75.7	CH	3.30, m	
6′	68.4	CH_2	3.97, brd (10.5) 3.58, overlap	1″
1″	103.3	CH	4.25, d (7.8)	6′
2″	73.5	CH	2.95, overlap	1″, 3″
3″	76.6	CH	3.10, overlap	2″, 4″, 5″
4″	70.1	CH	3.10, overlap	5″
5″	76.8	CH	3.05, overlap	3″
6″	61.0	CH_2	3.66, brd (11.1) 3.43, m	4″

[a] HMBC correlations are from proton (s) stated to the indicated carbon.
[b] The signals of C-3 and C-5 could be changed.

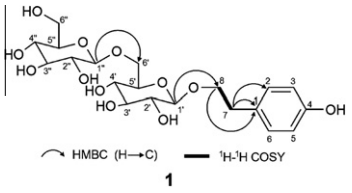

Fig. 3. Selected ^{1}H–^{1}H COSY and HMBC corrections of compound **1**.

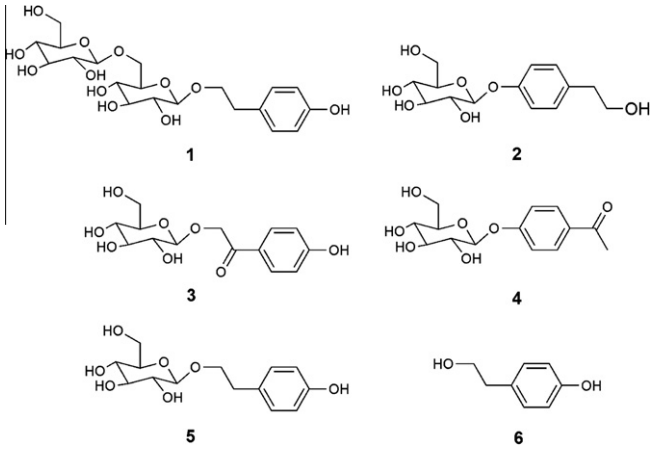

Fig. 4. The chemical structures of six water-soluble phenylpropanoid compounds. 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**), icariside D_2 (**2**), *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**), picein (**4**), salidroside (**5**), tyrosol (**6**).

expediting the rotary can increase the retention of the stationary phase. Although the revolution speed of the apparatus could be performed with a speed controller in a range from 0 to 1000 rpm, complete separation was invariably achieved at 850 rpm in this test. Meanwhile, the influence of column temperature was investigated. After tested at 20, 25, 30 and 35 °C, it could be seen that good results would be obtained when the column temperature was controlled at 30 °C.

Finally, when the solvent system of ethyl acetate–*n*-butanol–water at a volume ratio of 0.5:4.5:5, flow rates of 2.0 ml/min, revolution speed of 850 rpm and column temperature of 30 °C were used, respectively, four fractions (1–4) were obtained by one-step elution and the whole separation time was less than 3 h (see Fig. 1). As shown in Fig. 1, the HPLC analysis of HSCCC fraction "1" (peak I, collected during 80.5–92.5 min) revealed that it contained compound **1** and a minor unknown compound. Fraction "2" (peak II, collected during 95–118 min) contained pure compound **2** (26.5 mg), the purity of which was 98.3%. Fraction "3" (peak III, collected during 122.5–137 min) included compounds **3**, **4** and **5**. Fraction "4" (peak IV, collected during 138–160.5 min) consisted of compounds **5** and **6**.

3.2. HPLC purification of HSCCC peak fractions

In order to obtain pure compounds, the samples of fraction "1", fraction "3" and fraction "4" were subjected to Semi-prep HPLC to be purified further (Fig. 2). The conditions of Semi-prep HPLC experiments were directly transferred from the analytical HPLC results. The effluent of each target compound was collected and analysed by analysis HPLC-DAD. The results indicated that the purities of compound **1** (20.9 mg), **3** (11.1 mg), **4** (10.2 mg), **5** (120.2 mg) and **6** (16.2 mg) were 99.6%, 98.2%, 98.7%, 99.1% and 98.9%, respectively.

3.3. Structure elucidation of the purified compounds

Compound **1** was obtained as a white powder with a negative specific rotation ($[\alpha]_D^{25} - 34.4°$). Its molecular formula was established as $C_{20}H_{30}O_{12}$ on the basis of negative-mode HRMS (m/z 461.1669 $[M-H]^-$, calcd 461.1664), indicating 6 degrees of unsaturation. Its UV spectrum showed the existence of a phenyl group based on the absorptions at 228, 278 nm. The IR absorption bands indicated the presence of hydroxyl (3385 cm^{-1}) and aromatic (1614, 1516 cm^{-1}) groups. In the ^{1}H NMR spectrum (Table 2), two aromatic proton signals resonated at [δ_H 6.98 (2H, d, J = 8.4 Hz, H-2, 6), δ_H 6.59 (2H, d, J = 8.4 Hz, H-3, 5)] indicating a *para*-substituted benzene ring. The ^{13}C NMR and DEPT spectrum of **1** displayed 20 carbon signals corresponding to six aromatic carbons, two methylene carbons, and twelve carbons arising from two glucopyranosyl units. The ^{1}H NMR and ^{13}C NMR showed spectral features similar to those of 3′-*O*-*β*-D-glucopyranosylsalidroside (Machida, Ohkawa, Ohsawa, & Kikuchi, 2009). In the heteronuclear multiple quantum correlation (HMBC) spectrum, the methylene signal at δ_H 2.70 (H-7) was attached to C-1, as deduced from HMBC correlations of H-7 with C-1, C-2, and C-6. Then, the ^{1}H–^{1}H COSY correlations of H-8 (δ_H 3.58, 3.86) with H-7, as well as the HMBC correlation of H-8 with C-1, suggested the linkage of C-8 and C-7. As for the glucopyranosyl moieties of **1**, HMBC correlations of the anomeric proton (δ_H 4.16, d, J = 7.8 Hz, H-1′) (*β*-form) with C-8, and of H-8 with C-1′ indicated the glucose unit was attached to C-8. Moreover, the H-1″ signal at δ_H 4.25 (d, J = 7.8 Hz) (*β*-form) showed a HMBC correlation with C-6′ (δ_C 68.4), which implied a (1 → 6) linkage of the two glucose units (Fig. 3). Furthermore, based on the molecular mass or fragment mass of **1** together with the apparent downfield shift of C-4 (δ_C 156.7), a hydroxy group was assigned to be located at C-4. Acid hydrolysis of **1** yielded tyrosol (**6**) and D-glucose. The absolute configuration of glucose was determined by GC analysis of its corresponding trimethylsilylated L-cysteine adduct. Therefore, from these structural elements we conclude that compound **1** is a phenylethanoid disaccharide and defined as 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside.

Compounds **2**–**6** (Fig. 4) were identified by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and specific rotation measurements (figures of ^{1}H NMR of compounds **2**–**6** in supplementary information). Their spectral and

Table 3
Linear regression data, limit of detection (LOD), limit of quantification (LOQ) of the isolated compounds.

Compounds	Linearity range (μg/ml)	Linear regression equation	Correlation coefficient (r^2)	LOD (μg/ml)	LOQ (μg/ml)
1	1.50–31.12	$y = 24.452x + 1.302$	0.9997	0.04	0.25
2	2.00–56.03	$y = 49.790x + 2.265$	0.9998	0.05	0.30
3	0.50–16.81	$y = 34.785x - 0.043$	0.9997	0.01	0.10
4	0.50–12.56	$y = 30.623x - 0.051$	0.9996	0.01	0.08
5	12.00–180.21	$y = 81.012x + 3.923$	0.9999	0.10	0.80
6	1.50–36.76	$y = 48.603x + 1.589$	0.9998	0.05	0.28

Table 4
Recoveries for the assay of six isolated compounds from crude extract.

Analytes	Originals (μg)	Spiked (μg)	Found[a] (μg)	Recovery[b] (%)	RSD (%)
		60.1	134.0	98.2	2.2
Compound **1**	75.0	75.1	149.9	99.7	2.0
		90.1	163.8	98.6	1.9
		118.6	255.5	98.8	2.3
Compound **2**	138.3	148.0	286.8	100.3	1.8
		173.4	311.6	99.9	2.0
		33.1	72.1	99.7	2.7
Compound **3**	39.1	42.1	80.6	98.6	2.9
		50.8	89.6	99.4	2.4
		25.4	54.6	99.6	3.0
Compound **4**	29.3	30.2	59.4	99.7	2.5
		38.1	67.1	99.2	2.4
		376.4	841.9	99.7	1.9
Compound **5**	466.7	470.0	937.2	100.1	1.5
		564.3	1027.6	99.4	1.1
		80.2	180.2	101.6	2.1
Compound **6**	98.7	100.1	198.1	99.3	2.4
		121.4	218.5	98.7	2.7

[a] The data was average of three determinations.
[b] Recovery (%) = (amount found − original amount)/amount spiked × 100%.

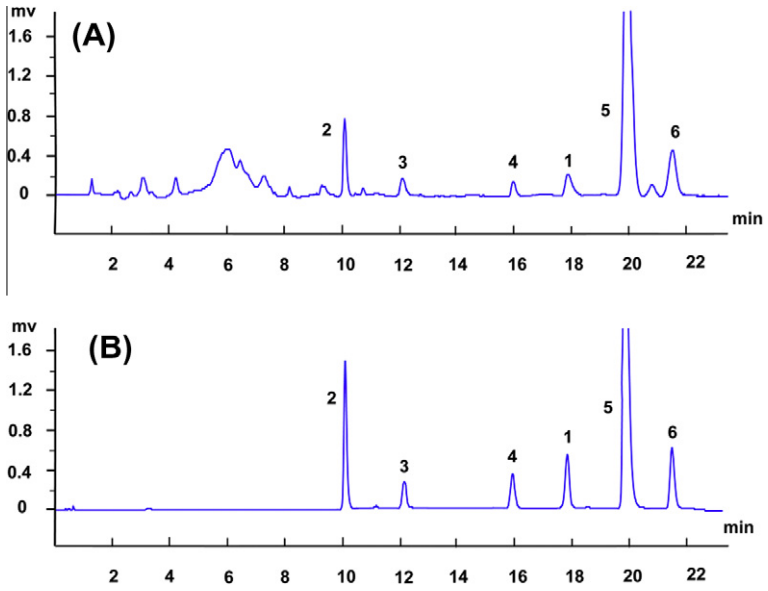

Fig. 5. Typical HPLC chromatograms of crude extract of *R. crenulata* (A) and mixed reference compounds (compounds **1**–**6**) (B). 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**), icariside D_2 (**2**), *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**), picein (**4**), salidroside (**5**), tyrosol (**6**). Experimental conditions: column, Phenomenex C_{18} column (250 × 4.6 mm, 5 μm); mobile phase, methanol–water (15:85, v/v); flow rate, 1.0 ml/min; detection wavelength, 275 nm; column temperature, 25 °C.

physical data are in agreement with previous literature data: icariside D_2 (Peng, Luo, Lu, Chen, Xie, Chen, 2009), *p*-hydroxyphenacyl-*β*-D-glucopyranoside (Peng et al., 2009), picein (Peng et al., 2009), salidroside (Lalonde, Wong, & Tsai, 1976), tyrosol (Lalonde et al., 1976), respectively.

3.4. Validation of method

The linearities, regressions and linear ranges of six target analytes were performed using the developed HPLC method (Table 3). The correlation coefficient values ($r^2 > 0.9996$) indicated

Table 5
The contents of isolated compounds **1**–**6** from crude extract of *R. crenulata*.

Batch no.[a]	Contents (mg/g) (RSD%, $n = 3$)					
	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**	Compound **6**
1	1.46 (2.2)	2.69 (2.1)	0.76 (2.6)	0.57 (2.9)	9.08 (1.6)	1.92 (1.3)
2	1.51 (1.9)	2.78 (1.1)	0.82 (2.0)	0.64 (2.5)	9.12 (1.3)	1.85 (1.8)
3	1.39 (2.8)	2.89 (1.6)	0.79 (2.1)	0.61 (2.8)	9.57 (1.1)	1.78 (2.4)

[a] Three batches of crude extract of *R. crenulata*, which was collected from the same region (Tibetan, China).

Table 6
Antioxidant activity of isolated compounds **1**–**6** by DPPH assay radical scavenging assay.

Compounds	IC_{50} (μM)
Crude extract	7.49 ± 0.52[a]
2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**)	12.13 ± 0.54
Icariside D_2 (**2**)	>40
p-hydroxyphenacyl-*β*-D-glucopyranoside (**3**)	13.45 ± 0.79
Picein (**4**)	>40
Salidroside (**5**)	10.97 ± 0.89
Tyrosol (**6**)	8.10 ± 0.98
Rutin[b]	4.12 ± 0.54

Each value is the mean of three independent experiments ± standard deviation (SD).
[a] The unit of IC_{50} of crude extract is μg/ml.
[b] Used as positive control.

good correlations between the tested compound concentrations and their peak areas within the test ranges. The LOD and LOQ were less than 0.10 and 0.80 μg/ml, and the overall intra- and inter-day variations (RSD) of the six analytes were less than 1.6% and 2.9%, respectively. The recoveries were between 98.2% and 101.6% (Table 4), and the repeatabilities were presented as RSD ($n = 6$) were between 1.2% and 3.0%. These results showed that the developed method had good accuracy and repeatability.

3.5. Analysis of the crude extract

The developed method was applied to simultaneous analysis of compounds **1**–**6** from the crude extract of *R. crenulata*. The typical HPLC chromatogram of crude extract was shown in Fig. 5, and the data was listed in Table 5. Among these investigated compounds, the content of compound **5** (salidroside) in the three batches of crude extract was the highest and the content of compound **4** (picein) in the three batches of crude extract was the lowest. The results indicated that the proposed HPLC method could be used to monitor the quality of the crude extract of *R. crenulata*.

3.6. Radical scavenging activity of isolated compounds

The effect of antioxidants on DPPH radical scavenging is generally based on their hydrogen-donating ability. The antioxidant activities of the target isolated compounds and crude extract from *R. crenulata* were determined spectrophotometrically by the DPPH radical scavenging activity assay and expressed in terms of the antioxidant concentration (μM) required to reduce the initial DPPH concentration. Rutin (Zhang, Shi, Wang, & Huang, 2011) was used as reference antioxidant. As shown in Table 6, the crude extract (IC_{50} 7.49 μg/ml) showed potent antioxidative activities in comparison with the reference antioxidant rutin (IC_{50} 4.12 μM, which is equal to 2.52 μg/ml). Also, the antioxidant activity of the crude extract was higher than that of the six isolated compounds. One main reason maybe is a synergistic effect of the phenolics. The other reason is likely that the most active component is not one of the isolated compounds, such as organic acids and flavonoids (Lee et al., 2000; Ohsugi et al., 1999), which have been reported to have better antioxidant activity. Among the six isolated compounds, compound **6** (IC_{50} 8.10 μM) showed the highest antioxidative activity. Compound **5** (IC_{50} 10.97 μM), **1** (IC_{50} 12.13 μM) and **3** (IC_{50} 13.45 μM) showed moderate antioxidative activity. These compounds might be contributed to the strong antioxidative activity of *R. crenulata*, which can be explained by the presence of hydroxybenzene.

4. Conclusion

We have demonstrated that HSCCC combined with Semi-prep HPLC is an efficient and superior separation strategy for water-soluble phenylpropanoids over other chromatographic methods for complementary action between the two methods. In this work, initial preparation by HSCCC enriched some minor water-soluble phenylpropanoids from *R. crenulata*, followed by further purification using high resolution Semi-prep HPLC. Six high purity phenylpropanoid compounds, namely 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**), icariside D_2 (**2**), *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**), picein (**4**), salidroside (**5**) and tyrosol (**6**) were obtained from the crude extract of *R. crenulata*. 2-(4-Hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**) is a new compound, and *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**) and picein (**4**) are isolated from the plant of *R. crenulata* for the first time. Meanwhile, a simple and rapid HPLC method for the simultaneous determination of the six phenylpropanoid ingredients from the crude extract of *R. crenulata* has also been developed for the first time. Furthermore, the antioxidant capacities of the six phenylpropanoid compounds were evaluated by using an established biochemical assay *in vitro*, the DPPH free radicals scavenging assay. It was found that 2-(4-hydroxyphenyl)-ethyl-*O*-*β*-D-glucopyranosyl-6-*O*-*β*-D-glucopyranoside (**1**), *p*-hydroxyphenacyl-*β*-D-glucopyranoside (**3**), salidroside (**5**) and tyrosol (**6**) possess significant inhibitory effects on the DPPH free radicals. Hence, these results suggested that *R. crenulata* could be a promising source of natural antioxidants.

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