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Target-guided isolation and purification of antioxidants from *Selaginella sinensis* by offline coupling of DPPH-HPLC and HSCCC experiments

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ABSTRACT

Selaginella sinensis (Selaginellaceae) is used extensively in traditional Chinese medicine (TCM) for the treatment of many kinds of chronic diseases. In this study, fractionation of the methanol extract of *S. sinensis* by different polarity solvents indicated the ethyl acetate fraction exhibited an potent 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging activity with the IC_{50} value of 44.9 μM. In order to evaluate the scientific basis, antioxidant peaks were firstly screened using DPPH spiking test through high performance liquid chromatography (DPPH-HPLC). Under the target-guidance of DPPH-HPLC experiment, two flavonoids and six biflavonoids, quercetin (**1**), apigenin (**2**), amentoflavone (**3**), robustaflavone (**4**), 2,3-dihydroamentaflavone (**5**), hinokiflavone (**6**), 4′-*O*-methyl-robustaflavone (**7**) and ginkgetin (**8**) were separated by high-speed counter-current chromatography (HSCCC) method using *n*-hexane–ethyl acetate–methanol–water (8:8:9:7) as the solvent system with purities 98.2%, 97.6%, 99.4%, 92.3%, 98.5%, 98.9% and 99.6%, respectively. The structures were identified by electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR) analysis. Antioxidant activity of eight isolated compounds was assessed by the radical scavenging effect on DPPH radical, compound **1** showed strongest antioxidant activities with IC_{50} values of 3.2 μM, while compounds **2**–**8** showed weak antioxidant activities. This is the first report on simultaneous separation of eight antioxidant compounds from *S. sinensis* by HSCCC, moreover, apigenin and 4′-*O*-methyl-robustaflavone were first identified from this plant. Results of the present study indicated that the combinative method using DPPH-HPLC and HSCCC could be widely applied for rapid screening and isolating of antioxidants from complex TCM extract.

1. Introduction

Selaginella sinensis (Selaginellaceae) is mainly distributed in north and northeast China [1], which has been used widespread in traditional Chinese folk medicine for the treatment of many kinds of diseases, especially for chronic tracheitis [2]. Thus, chemical and pharmacological studies of this species are valuable. Previous phytochemical investigations have indicated that seven biflavonoids (e.g. amentoflavone, 7″-*O*-methylamentoflavone, 4′,7″-di-*O*-methylamentoflavone, 2,3-dihydroamentoflavone, robustaflavone, ginkgetin, hinokiflavone) [2–6], two flavonoids (e.g. genistin and quercetin) [2,5], lignans [7,8] and phenols [9] exist in *S. sinensis*. However, no detailed bioactivity for *S. sinensis* crude extract has been reported to date. Because antioxidants have received a great amount of attention as being primary preventive ingredients against various diseases [10], different solvent fractionations of *S. sinensis* were actively screened based on the DPPH radical scavenging activity, and its ethyl acetate extract exhibited considerable antioxidant effect. Therefore, the ethyl acetate fraction might be a good candidate for further development as antioxidant remedies, which prompted us to perform a detailed target-guided chemical investigation on ethyl acetate extract of *S. sinensis*.

The conventional activity-guided fractionation of complex plant extracts is a time-consuming, labor intensive and expensive process, and often leads to loss of activity during the isolation and purification procedures due to dilution effects or decomposition especially for antioxidants [11]. In order to avoid the above-mentioned problems, simple, rapid and effective methods to screen and purify potential antioxidants from complex plant extract are essential. As for screening method, recent development HPLC-based on-line post-column free radical assays have been successfully utilized to fast screen antioxidants from complex extracts [12–15]. However, this method could not be adopted widely because it needs special equipment and technical skill for

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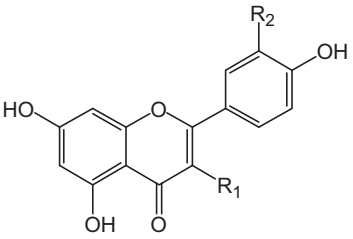
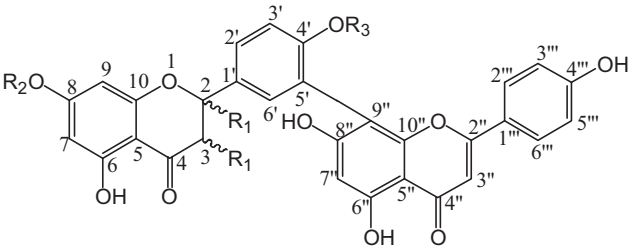
analysis. Then, lately a simple and useful approach by spiking complex extract with DPPH test has been successfully developed [16,17]. As for preparative method, high-speed counter-current chromatography (HSCCC) is an optimal choice, which is a continuous liquid–liquid partition chromatography based on partitioning of compounds between two immiscible liquid phases without support matrix, no irreversible adsorption, low risk of sample denaturation, total sample recovery, large load capacity, and low cost [18], and has been successfully applied to isolate and purify many bioactive compounds from natural products [19–22]. In general, the split screening method and preparative means were disadvantageous to the fast isolating active compounds from TCM.

As part of our continuous efforts on rapid screening and isolating antioxidants from TCM [13,23], the present paper describes an efficient method by offline coupling of DPPH-HPLC and HSCCC experiments to screen and purify antioxidant from *S. sinensis* ethyl acetate extract. Two flavonoids and six biflavonoids, quercetin (**1**), apigenin (**2**), amentoflavone (**3**), robustaflavone (**4**), 2,3-dihydroamentaflavone (**5**), hinokiflavone (**6**), 4′-*O*-methyl-robustaflavone (**7**) and ginkgetin (**8**) were target-guided purified (Fig. 1), and their antioxidant activities were estimated. This is the first report on screening and simultaneous separation of eight antioxidant compounds from *S. sinensis* by DPPH-HPLC and HSCCC, moreover, apigenin and 4′-*O*-methyl-robustaflavone were first reported from this plant.



1 $R_1 = R_2 = OH$ quercetin

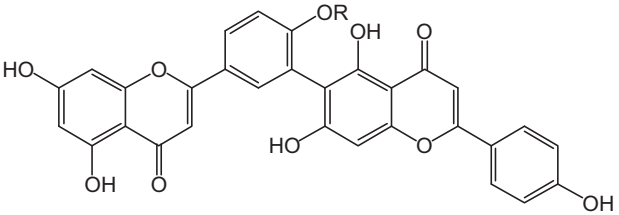
2 $R_1 = R_2 = H$ apigenin



3 $R_1 = \Delta$ $R_2 = R_3 = H$ amentoflavone

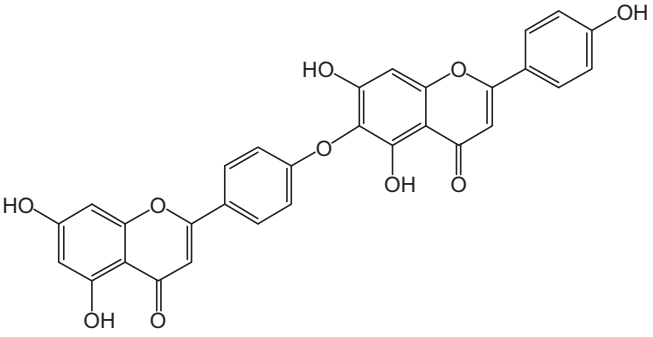
5 $R_1 = H$ $R_2 = R_3 = H$ 2,3-dihydroamentoflavone

8 $R_1 = \Delta$ $R_2 = R_3 = CH_3$ ginkgetin



4 R = H robustaflavone

7 $R = CH_3$ 4'-*O*-methyl-robustaflavone



6 hinokiflavone

Fig. 1. Structures of the identified compounds from *S. sinensis* by HSCCC.

2. Experimental

2.1. Chemicals and reagents

Petroleum ether (60–90 °C), ethyl acetate, *n*-hexane, *n*-butanol and methanol for preparation of active fractions and HSCCC separation were analytical grade and purchased from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Methanol and acetic acid used for analytical HPLC was of chromatographic grade (Merk, Darmatadt, Germany). All aqueous solutions were prepared with pure water produced by Milli-Q water (18.2 MΩ) system (Millipore, Bedford, MA, USA). DPPH (95%) was bought from Sigma–Aldrich (Shanghai Division), and DPPH radical solutions were freshly prepared in methanol every day and kept protected from light. Multi-well plates (Greiner) and multi-well plates readers (Bio-Tek Instruments, USA) were used in the antioxidant activity experiments. Sephadex LH-20 gel was purchased from Pharmacia Fine Chemicals.

Selaginella sinensis was purchased from Guilin, Guangxi province, China, in April 2008, and identified by Prof. Guangzhao Li, Guangxi Institute of Botany, Chinese Academy of Sciences. A voucher specimen (SS0804) is deposited at the school of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan, China.

2.2. Preparation and fractionation of crude extracts

The pulverized material of *S. sinensis* (2.0 kg) was extracted with methanol under reflux for 3 h and concentrated under reduced pressure to give a brown syrup (136 g). A portion of this syrup (120 g) was then dissolved in water (500 mL) and submitted to liquid–liquid fractioning using solvents with increasing polarities. This procedure produced petroleum ether (1.9 g), ethyl acetate (12.6 g) and *n*-BuOH (20.7 g) fractions.

2.3. Evaluation of antioxidant activity

The DPPH radical assay was performed as described [24]. The free radical scavenging efficiency of the fractions and isolated compounds was determined by decoloration of the DPPH radical. In brief, 25 μl of diluted sample (4 mg/ml dissolved in methanol) mixed with 40 μl DPPH methanol solution (0.4 mg/ml) and made up with methanol to a final volume of 250 μl. The methanol solution of DPPH was served as a control. The absorbance was measured at 517 nm after the mixture was incubated at 37 °C for 30 min. The antioxidant activity is expressed as percentage of DPPH radical elimination calculated according to the following formula: $[(A_{blank} - A_{sample})/A_{blank}] \times 100\%$, where A_{blank} is the absorbance of the DPPH radical solution and A_{sample} is the absorbance of the DPPH radical solution after the addition of the sample. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage. All tests were run in triplicate, and the average value was calculated.

2.4. DPPH-HPLC experiment

Ethyl acetate fraction of *S. sinensis* was reacted with DPPH in methanol by the same procedure of colorimetric analysis as described in Section 2.3, and then passed through 0.45 μm filter and subjected to a HPLC analysis. A blank of ethyl acetate fraction with methanol was used as a control. The peaks with antioxidant activities will be reduced or disappeared after reaction. Analytical HPLC was consisted of two LC-8A pumps, a Prominence SPD-M20A diode array detector performing the wavelength scanning from 190 to 950 nm, a manual injection valve with a 20 μl loop and an LC Solution workstation (Shimadzu, Japan). The samples were separated and analyzed by a reversed phase Symmetry® C_{18} (150 mm × 3.9 mm i.d., 5 μm, Milford, MA, USA) column and a security guard C_{18} ODS (4.0 mm × 3.0 mm i.d.) from Phenomenex (Torrance, CA, USA). The mobile phase was consisted of A (0.05% acetic acid in water) and B (0.05% acetic acid in methanol), which was programmed as follows: 0–8 min, 50–70% B; 8–25 min, 70–75% B; 25–28 min, 75–85% B; 28–35 min, 85% B. The flow rate was 1.0 ml/min while the ambient temperature was controlled at 20 °C by air conditioner. Spectra were recorded from 200 to 400 nm (peak width 0.2 min and data rate 1.25 s^{-1}) while the chromatogram was acquired at 335 nm.

2.5. HSCCC separation

The preparative HSCCC was performed on a seal-free high-speed counter-current chromatography by Prof. Qizhen Du (Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China). The apparatus was equipped with one polytetrafluoroethylene (PTFE) layer coil column with an average of I.D.2.6 mm and a total volume of 420 ml. The column revolves around its own axis at the angular velocity in the same direction. The revolution speed of the apparatus could be regulated between 0 and 1000 rpm. The revolution radius or the distance between the holder axis and central axis of the centrifuge was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79. $\beta = r/R$, where r is the distant from the coil to the holder shaft and R is the revolution radius or the distant between the holder axis and central axis of the centrifuge. The solvent was pumped into the tubing with a FMI pump (Zhejiang Instrument Factory, Hangzhou, China). The effluent was continuously monitored with a variable wavelength PC300 detector at 335 nm and the chromatogram with a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China). A manual sample injection valve with a 20 ml loop was adjusted to the system. The suitable solvent systems were evaluated by HPLC according to the partition coefficients (K). The solvent system composed of *n*-hexane–ethyl acetate–methanol–water (8:8:9:7) was used for the separation of ethyl acetate fraction of *S. sinensis* with the upper phase as the stationary phase. The sample solution was prepared by dissolving 400 mg of the active fraction in 20 ml of the lower phase of the solvent system for isolation and purification. HSCCC was performed as follows: the multiplayer coiled column was first entirely filled with the upper phase; the lower mobile phase was then pumped into the inlet of the column at the flow rate of 1.2 ml/min, while the apparatus was run at 1000 rpm; after a clear mobile phase eluted at the tail outlet and the hydrodynamic equilibrium was reached, sample was then injected into the injection valve; the effluent from the outlet of the column was continuously monitored with a UV detector at 335 nm and the peak fractions were collected manually according to the chromatographic profile; after target compounds were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase; the effluent was collected for purity analysis.

Table 1
Antioxidant activities of fractionations of different polarities and isolated compounds in DPPH assay.

Samples	DPPH (IC_{50}, μM)[a]
Petroleum ether fraction	>75.0
Ethyl acetate fraction	44.9 ± 0.4
n-BuOH fraction	72.3 ± 0.6
Quercetin (**1**)	3.2 ± 0.02
Apigenin (**2**)	>75.0
Amentoflavone (**3**)	>75.0
robUstaflavone (**4**)	>75.0
2,3-Dihydroamentaflavone (**5**)	>75.0
Hinokiflavone (**6**)	>75.0
4′-*O*-Methyl-robustaflavone (**7**)	>75.0
Ginkgetin (**8**)	>75.0
Rutin[b]	3.8 ± 0.03

[a] Each value is mean ± SD (n = 3).
[b] Used as control.

2.6. Analysis and identification of target compounds

The target compounds obtained by HSCCC were analyzed by HPLC by the same conditions as described in Section 2.4, and identified by their mass data and NMR spectra. ESI-MS data were acquired in the negative ion mode from Bruker Esquire 3000+ ESI ion trap instrument (Faellanden, Switzerland). NMR experiments were performed on a Varian INOVA-400 (Varian Corporation, USA) NMR spectrometer. The reference compound TMS was used as internal standard for the determination of chemical shifts.

3. Results and discussion

3.1. Antioxidant activity of different fractions of S. sinensis

Solvent extraction is usually used for isolating antioxidants from TCM, and antioxidant activity of extracts is strongly dependent on the solvent [25]. Solvents with different polarities were used to fraction crude methanol extract of *S. sinensis*. The petroleum ether, ethyl acetate and *n*-BuOH fractions were then evaluated by DPPH radical scavenging activity. The ethyl acetate fraction showed potent capacity to scavenge DPPH radical compared with the IC_{50} value of 44.9 μM (Table 1). The result implies that there have antioxidants present in the ethyl acetate fraction, then successive DPPH-HPLC and HSCCC experiments was applied to screen and isolate them.

3.2. Screening antioxidants by DPPH-HPLC analysis

The DPPH-HPLC method could be used for a rapid screening of radical scavenging in complex mixtures, particularly plant extract with a minimum of sample preparation. The peak areas of compounds with potential antioxidant activities will be reduced or disappeared in the HPLC chromatogram after their reaction with DPPH, and for those without antioxidant activities, the peak areas was almost no change.

The separation of all compounds in complex extract is the first and challenging tasks in analytical HPLC. To our best knowledge, the *Selaginella* genus is rich in biflavonoids with similar structure skeleton. Since acid is known to achieve better separation for flavonoid derivatives by reducing the tailing of the peaks, acid must be added into the mobile phase. In the course of optimizing the conditions of separation, the system conditions including the mobile phase (methanol–acetic acid, acetonitrile–water and different concentrations of acetic acid in water and organic phase were compared to get the most suitable mobile phase), gradient program (gradient time, gradient shape and initial composition of the mobile phase), column temperature and detection wavelength (relatively higher

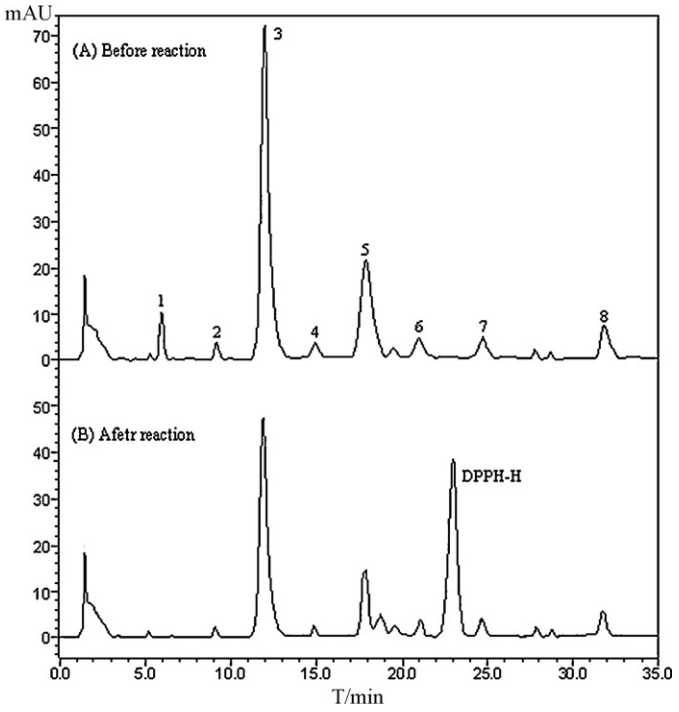


Fig. 2. HPLC-UV and DPPH-HPLC-UV of *S. sinensis* ethyl acetate fraction. HPLC conditions: column, reversed-phase Symmetry® C_{18} (150 mm × 3.9 mm i.d., 5 μm); mobile phase, consisted of A (0.05% acetic acid in water) and B (0.05% acetic acid in methanol), which was programmed as follows: 0–8 min, 50–70% B; 8–25 min, 70–75% B; 25–28 min, 75–85% B; 28–35 min, 85% B; flow rate, 1.0 ml/min; UV wavelength, 335 nm; column temperature, 20 °C. Peaks 1–8, see Fig. 1.

absorption) were investigated. The final results showed that best resolution and shortest analysis time were achieved when a gradient elution mode composed of solvent A 0.05% acetic acid in water and solvent B 0.05% acetic acid in methanol was programmed as follows: 0–8 min, 50–70% B; 8–25 min, 70–75% B; 25–28 min, 75–85% B; 28–35 min, 85% B. The flow rate was 1.0 ml/min, the column temperature was set at 20 °C by air conditioner, and the 335 nm was selected as the detection wavelength. Under the optimum gradient elution, the compounds of the *S. sinensis* ethyl acetate fraction reached base-line separation (Fig. 2(A)).

The chromatogram of *S. sinensis* ethyl acetate fraction spiking with DPPH at 335 nm is shown in Fig. 2(B), which presented that peak area of compound **1** disappeared after spiking with the DPPH solution, while peak areas of eight compounds (**2**–**8**) a little reduced. Therefore, compound **1** possesses strong antioxidant activity, and compounds **2**–**8** possess weak antioxidant activities. By analysis of the UV spectra, compounds **1**–**8** had two maximum absorption bands at 250–270 nm and 340–370 nm, which were the typical spectra of flavonoid derivatives. Flavonoid derivatives are known to exist widespread in TCM and to possess a variety of biological activities, particular to their antioxidant activity. In order to obtain these active compounds, HSCCC was used to isolate and purify them under the guidance with the DPPH-HPLC analysis.

3.3. Preparative HSCCC experiments

HSCCC targeted on isolation of eight potent antioxidants in DPPH-HPLC experiments. More than 60% of free flavonoid derivatives were obtained by HSCCC using *n*-hexane–ethyl acetate–methanol–water as the solvent system [26]. To our best knowledge, attempts to separate flavonoid derivatives from *Selaginella* genus have been reported by three reports. Yuan et al. [27,28] obtained amentaflavone, robustaflavone, bilobetin, hinokiflavone, isocryptomerin and apigenin-diglucoside from *S. tamariscina* by HSCCC using the solvent system *n*-heptane–ethyl acetate–methanol–water (2:3:2:3). A solvent system composed of *n*-hexane–ethyl acetate–methanol–water (8:8:9:7) was used to separate amentoflavone, hinokiflavone, podocarpusflavone A and ginkgetin from *S. moellendorffii* in our previous report [23]. However, there was no report about the comprehensive separation and purification of active compounds from *S. sinensis*, and most of the published studies were focused on the purification of less than eight (bi)flavonoids derivatives by HSCCC from TCM [27–29]. This is an attempt to purify eight potent antioxidants from *S. sinensis* by HSCCC for the first time.

In this experiment, various solvent systems based on *n*-hexane–ethyl acetate–methanol–water were conducted partition coefficient tests. After comparison of different ratios, *n*-hexane–ethyl acetate–methanol–water (8:8:9:7) reported in our previous manuscript [19] gave suitable partition coefficients for eight objective antioxidants, quercetin (0.16), apigenin (0.23), amentoflavone (0.29), robustaflavone (0.34), 2,3-dihydroamentaflavone (0.45), hinokiflavone (0.57), 4′-*O*-methyl-robustaflavone (0.76) and ginkgetin (1.08). Eventually, *n*-hexane–ethyl acetate–methanol–water (8:8:9:7) was used for the HSCCC of the ethyl acetate fraction of *S. sinensis*.

Other factors such as the resolution speed of the separation column and the flow rate of the mobile phase were also investigated. Different flow rate (1.0, 1.2, 1.5, 2.0 and 2.5 ml/min) of the mobile phase and different revolution speed (500, 600, 700 and 800, 1000 rpm) of the selected system were examined in the present paper, which can vary the retention of the stationary phase. The retention of the stationary phase is one of the most important parameters in HSCCC. Successful separation in HSCCC largely depends on the amount of stationary phase retained in the column. In general, the higher the retention of the stationary phase, the better the peak resolution. It was clear that high flow rate was unfavorable to the retention of the stationary phase, and the slow flow speed can produce a good separation, but more time and more mobile phase will be needed, and the chromatogram peak was extended. High rotary speed can increase the retention of the stationary phase. The result showed that when the flow rate was 1.2 ml/min, resolution speed was 1000 rpm, retention percentage of the stationary phase could reach 63% and good separation results could be obtained.

Under the optimum conditions, eight fractions (I–VIII) were obtained in one-step elution less than 6.5 h as shown in Fig. 3(A), which is 9.7 mg of fraction I (collected during 149–166 min), 3.2 mg of fraction II (collected during 171–188 min), 96.3 mg of fraction III (collected during 190–212 min), 4.9 mg of fraction IV (collected during 218–228 min), 24.4 mg of fraction V (collected during 228–250 min), 6.2 mg of fraction VI (collected during 261–274 min), 7.9 mg of fraction VII (collected during 300–315 min) and 11.2 mg of fraction VIII (collected during 350–375 min).

3.4. Analysis and structural identification of HSCCC fractions

The purities of fractions I–VIII corresponded to peaks 1–8 in Fig. 1 were 98.2%, 97.6%, 99.4%, 92.3%, 98.5%, 98.9% and 99.6%, respectively, determined by HPLC peak area percentage (Fig. 3(B)). Then, compounds **1**–**8** were further purified by Sephadex LH-20 column chromatography using methanol as mobile phase before structural identification and antioxidant activities assay.

The ESI-MS of compounds **1**–**8** yielded ions with *m*/*z* 301 $[M-H]^-$, 269 $[M-H]^-$, 537 $[M-H]^-$, 537 $[M-H]^-$, 539 $[M-H]^-$, 537 $[M-H]^-$, 551 $[M-H]^-$ and 565 $[M-H]^-$, respectively. Comparing the NMR data (Tables 2 and 3) with the reported data in literature [13,23,27,30,31], compounds **1**–**8** were identified as quercetin, api-

Table 2
^{1}H NMR spectral data of compounds **1**–**8**[a] (TMS, δ ppm, 400 MHz).

No.	1[b]	2[b]	3[b]	4[b]	5[c]	6[b]	7[b]	8[b]
2	–		–	–	5.42 (dd, 2.6, 13.0)	–	–	–
3	–	6.73 (s)	6.83 s	6.84 s	3.20 (dd, 13.0, 16.0) 2.75 (dd, 5.6, 16.0)	6.85 s	6.89 s	6.96 s
6	6.41 (d, 2.0)	6.19 (d, 2.0)	6.19 s	6.19 (d, 2.0)	5.91 (d, 2.0)	6.19 (d, 2.0)	6.20 (d, 2.0)	6.34 s
8	6.20 (d, 2.0)	6.48 (d, 2.0)	6.46 s	6.48 (d, 2.0)	5.87 (d, 2.0)	6.49 (d, 2.0)	6.51 (d, 2.0)	6.75 s
2′	7.68 (d, 2.0)	7.92 (d, 8.5)	8.02 (d, 2.0)	7.81 (d, 2.0)	7.44 (d, 2.0)	8.02 (d, 8.5)	7.84 (d, 2.0)	8.09 s
3′	–	6.93 (d, 8.5)	–	–	–	7.03 (d, 8.5)		
5′	6.89 (d, 8.4)	6.93 (d, 8.5)	7.13 (d, 8.5)	7.03 (d, 8.5)	7.06 (d, 8.5)	7.03 (d, 8.5)	7.25 (d, 8.5)	7.35 (d, 9.0)
6′	7.54 (dd, 2.0, 8.4)	7.92 (d, 8.5)	8.00 (dd, 2.0, 8.5)	7.92 (dd, 2.0, 8.5)	7.46 (dd, 2.0, 8.5)	8.02 (d, 8.5)	7.92(dd, 8.5, 2.0)	8.19 (d, 9.0)
3″	–		6.78 s	6.80 s	6.58 s	6.85 s	6.84 s	6.78 s
6″	–		6.38 s	–	6.35 s	–		6.41 s
8″	–		–	6.63 s	–	6.72 s	6.65 s	–
2‴	–		7.58 (d, 8.5)	7.97 (d, 8.0)	7.57 (d, 8.0)	7.97 (d, 8.0)	7.97 (d, 8.5)	7.50 (d, 8.5)
3‴	–		6.71 (d, 8.5)	6.95 (d, 8.0)	6.79 (d, 8.0)	6.94 (d, 8.0)	6.95 (d, 8.5)	6.73 (d, 8.5)
5‴	–		6.71 (d, 8.5)	6.95 (d, 8.0)	6.79 (d, 8.0)	6.94 (d, 8.0)	6.95 (d, 8.5)	6.73 (d, 8.5)
6‴	–		7.58 (d, 8.5)	7.97 (d, 8.0)	7.57 (d, 8.0)	7.97 (d, 8.0)	7.97 (d, 8.5)	7.50 (d, 8.5)
–OCH_3	–		–	–	–	–	3.81 s	3.81 s
–OCH_3	–		–	–	–	–		3.79 s

[a] Chemical shifts in ppm units.
[b] Measured in DMSO-d_6.
[c] Measured in CD_3OD.

genin, amentoflavone, robustaflavone, 2,3-dihydroamentaflavone, hinokiflavone, 4′-*O*-methyl-robustaflavone and ginkgetin, respectively. Among them, apigenin and 4′-*O*-methyl-robustaflavone were first isolated from *S. sinensis*.

3.5. Antioxidant activities of target-isolated compounds

The antioxidant activities of target-isolated compounds from *S. sinensis* ethyl acetate fraction were measured spectrophotometrically by DPPH radical scavenging activity assay in comparison with rutin as standard antioxidant. As shown in Table 1, compound **1** exhibited effective antioxidant activity against DPPH with IC_{50} value of 3.2 μM, which were stronger than the positive control, rutin with IC_{50} value of 3.8 μM, and compounds **2**–**8** exhibited a DPPH scavenging activities up to 75 μM, which were in accordance with the DPPH-HPLC experiment. Previously, some biflavonoids including amentoflavone (**3**), robustaflavone (**4**), hinokiflavone (**6**) and ginkgetin (**8**) have

Table 3
^{13}C NMR spectral data of compounds **1**–**8**[a] (TMS, δ ppm, 100 MHz).

No.	1[b]	2[b]	3[b]	4[b]	5[c]	6[b]	7[b]	8[b]
2	147.0	164.1	163.8	164.5	80.8	163.3	163.3	163.9
3	136.0	102.8	102.8	103.3	44.1	103.7	103.4	104.1
4	176.1	181.7	181.6	182.2	197.9	181.9	181.6	182.2
5	160.9	157.3	161.3	161.9	165.5	161.4	161.3	161.4
6	98.4	98.8	98.7	99.3	97.2	99.0	98.8	98.4
7	164.1	163.7	164.0	164.4	168.7	164.3	164.1	163.8
8	93.6	93.9	93.9	94.5	96.4	94.1	94.0	93.0
9	156.3	161.4	157.3	157.8	157.3	157.6	157.3	157.6
10	103.2	103.7	103.6	104.1	103.4	104.2	103.5	103.9
1′	122.2	121.2	120.6	121.5	131.1	124.2	122.3	121.5
2′	115.8	128.4	131.3	131.3	128.7	128.4	130.2	131.2
3′	145.3	115.9	120.2	121.1	121.1	115.4	121.1	122.7
4′	147.9	161.1	159.9	159.5	157.4	160.6	160.6	160.9
5′	116.4	115.9	116.3	116.7	117.2	115.4	111.6	112.0
6′	120.2	128.4	127.6	127.9	132.6	128.4	127.8	128.6

No.	3[b]	4[b]	5[c]	6[b]	7[b]	8[b]
2″	163.5	164.5	166.0	164.1	163.6	165.5
3″	102.5	103.2	103.3	102.7	102.8	102.8
4″	181.9	182.3	184.3	182.1	181.9	182.3
5″	160.4	161.6	162.4	153.0	158.8	161.4
6″	98.7	109.5	100.5	124.6	108.5	98.9
7″	161.3	164.0	164.9	153.9	161.8	162.0
8″	104.1	94.0	106.8	94.6	93.4	105.0
9″	154.4	156.8	156.5	157.0	156.3	154.6
10″	103.4	103.8	105.2	103.9	103.7	103.9
1‴	121.3	121.7	123.4	121.2	122.5	122.0
2‴, 6‴	128.1	128.9	129.5	128.7	128.4	128.3
3‴, 5‴	115.6	116.5	116.9	116.0	115.9	116.1
4‴	160.9	160.4	162.7	161.3	161.1	160.9
–OCH_3	–	–	–	–	55.7	56.3
–OCH_3	–	–	–	–	–	56.2

[a] Chemical shifts in ppm units.
[b] Measured in DMSO-d_6.
[c] Measured in CD_3OD.

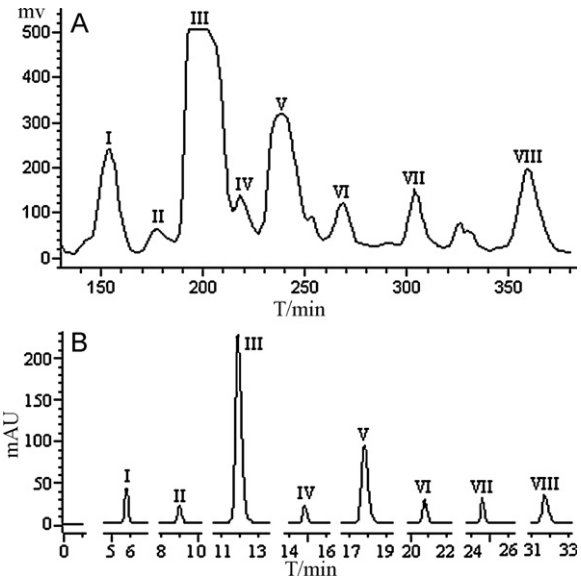


Fig. 3. (A) Preparative HSCCC separation of *S. sinensis* ethyl acetate fraction. Experimental conditions: revolution speed: 1000 rpm; solvent system: *n*-hexane–ethyl acetate–methanol–water (8:8:9:7); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.2 ml/min; retention of the stationary phase: 63%; detection wavelength: 335 nm. Fractions I–VIII corresponded to compounds **1**–**8** in Fig. 1; (B) Part of the HPLC chromatograms of eight target-isolated compounds (there have no other components found in any of these chromatograms). The conditions were the same with that in Fig. 2.

been reported for their DPPH inhibitory activities [23,32], but to our best knowledge, there has been no prior report for other biflavonoids.

4. Conclusions

DPPH-HPLC method followed by HSCCC experiments was successively developed for the fast screening and purification of potent radical scavengers from *S. sinensis* ethyl acetate fraction. The best advantage of this method is that the active compounds can be screened from a chromatographic separation, and then target-guided purifications can be operated by HSCCC. The described method has a broad applicability and is rapid, robust and suitable for fast screening and preparing radical scavengers from crude plant extracts.

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