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Preparative Isolation of six Anti-Tumour Biflavonoids from *Selaginella Doederleinii* Hieron by High-Speed Counter-Current Chromatography

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ABSTRACT:

Introduction – Biflavonoids are the primary constituents of *Selaginella doederleinii* Hieron, to which different bioactivities have been attributed, including anti-cancer, anti-inflammatory, anti-oxidant, anti-fungal and anti-virus activity. However, effective methods for separation of these compounds are not currently available.

Objective – To develop a high performance and bioassay-guided method for preparative isolation of biflavonoids from *S. doederleini* via high-speed counter-current chromatography (HSCCC).

Methods – The anti-proliferation effects of four fractions (70% ethanol, petroleum ether, dichloromethane and acetic ether extracts) of *S. doederleinii* on five human cancer cells were monitored. The dichloromethane and acetic ether extracts showed good cytotoxicities to the studied cancer cell lines, guiding the subsequent separation. Two solvent systems composed of *n*-hexane:ethyl acetate:methanol:water (1:2:1.5:1.5, v/v) and *n*-hexane:ethyl acetate:methanol:water (3:2:3:2, v/v) were developed for separation of the active fractions, respectively. Identification of the biflavonoids was performed by EI-MS^n, ^{1}H- and ^{13}C-NMR.'

Results – Under the optimised conditions, 12.6 mg amentoflavone (91.4%), 6.6 mg robustaflavone (90.4%), 7.5 mg 2″, 3″-dihydro-3′, 3‴-biapigenin (98.2%) and 7.3 mg 3′, 3‴-binaringenin (90.3%) from acetic ether extract (500 mg) and 6.3 mg heveaflavone (93.5%) and 5.3 mg 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone (94.5%) from dichloromethane extract (200 mg) were obtained, respectively. The anti-proliferation effects of the six biflavonoids on the five human cancer cells were further verified.

Conclusion – The study provides methodological references for simultaneously preparative isolation of several bioactive biflavones from the herbal family of *Selaginella*. It is the first report discovering 2″, 3″-dihydro-3′, 3‴-biapigenin and 3′, 3‴-binaringenin from this herb and describing their cytotoxicities to human cancer cell lines. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: Activity-guided preparative isolation; HSCCC; biflavonoids; cytotoxicity; *Selaginella doederleinii* Hieron

Introduction

Selaginella doederleinii Hieron, also called Shishangbai in Chinese and belonging to the familiy *Selaginellaceae*, has been traditionally used as a folk medicine in China for the treatment of different tumours, especially for nasopharyngeal carcinoma, lung cancer and trophoblastic tumour (Editorial Committee of Chinese Materia Medica, 1999; Zhou *et al*., 2006, 2008). Pharmacological studies disclosed that the ethanol extract of *S. doederleinii* can trigger the mitochondrial/caspase apoptotic pathway in tumour cells *in vitro* (Liu *et al*., 2011a, 2011b). *In vivo*, the extracts inhibited tumour growth in mice (Liu *et al*., 2011a, 2011b). Therefore, it is expected and also interesting to obtain natural bioactive ingredients with the potential of preventing cancers from this plant resource. Furthermore, to perform the various *in vitro* and *in vivo* studies about their effects and metabolism and to evaluate the quality of various *S. doederleinii* with a reference standard, large quantities of purified bioactive ingredients from this herbal medicine are required.

However, so far there has been little study on the active components from this herbal medicine. Cao *et al*. (2010) reported the purification of four flavonids and nine biflavonids from *S. doederleinii* by traditional phytochemical tools. The conventional separation and purification methods required a multi-step protocol including solvent extractions, repeated thin-layer chromatography (TLC) and silica-gel column chromatography (SGC), polyamide column chromatography (PAC) and Sephadex LH20 gel chromatography (LH20 GC), etc., which were tedious, time-consuming and cost-expensive, and also resulted in a relatively lower preparative capacity due to irreversible adsorptions of compounds onto the stationary phase materials during separation (Ito, 1981; Wang *et al*., 2011, 2013; Yoon *et al*., 2011). In addition, the ingredients obtained from the herbal medicine studied are numerous, possibly

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causing aimlessness and time-consuming screening process for evaluation of their bioactivity. Hence, simple and convenient methods have gained growing importance in the activity-guided preparation separation for not only *S. doederleinii*, but also the other herbal medicines (Frighetto *et al*., 2005; Wang *et al*., 2012; Xian *et al*., 2012).

High-speed counter-current chromatography (HSCCC) is a liquid–liquid extraction technique based on hydrodynamic equilibration of the two-phase solvent system in the separation column (a centrifuged coil of tubing). Due to the absence of any solid stationary phase, adsorption losses are minimised compared to that caused by conventional column chromatography, such as SGC, PAC and LH20 GC. Meanwhile, the sample loading capacity can reach gram level in one run of HSCCC, which allows preparative-scale separation of natural active components from herbal medicines. In addition, the two-phase solvent system for HSCCC requires less organic solvent than that for the convenient column chromatography mentioned above, which makes the cost relatively less for one run of HSCCC. Therefore, HSCCC is an ideal tool of preparative-scale separation for various natural or synthetic products, such as essential oils (Chen *et al*., 2011), flavonoids (Liu *et al*., 2011a, 2011b; Zhang *et al*., 2011, 2012; Dai *et al*., 2013), anthraquinones (Tong and Yan, 2007) and phenolic compounds (Regalado *et al*., 2011; Shi *et al*., 2012).

In this study, a bioassay-guided HSCCC method was developed for preparative isolation of the anti-tumour active biflavonoids from *S. doederleinii*. The anti-proliferation effects of four extraction fractions (70% ethanol, petroleum ether, dichloromethane and acetic ether extracts) of *S. doederleinii* on five human cancer cells, including K562, A549, CNE-2, PC-9 and HL60, were at first monitored. The results guided the subsequent preparative isolation of six active biflavonoids (Fig. 1) using HSCCC with optimised conditions. The anti-proliferation effects of the obtained biflavonoids on human cancer cells were then verified. It is the first report discovering 2″, 3″-dihydro-3′, 3‴-biapigenin and 3′, 3‴-binaringenin from this herbal medicine and describing their cytotoxicities to human cancer cell lines. The present study provides methodological references for simultaneously preparative purification of several biflavonoids from the herbal family of *Selaginella* for the sake of further studies *in vitro* or *in vivo*.

Experimental

Reagents and materials

All solvents used for preparation of crude samples and HSCCC separation were of analytical grade and obtained from Sinopharm Chemical Reagents (Shanghai, China). Acetonitrile used for HPLC analysis was of chromatographic grade (Merck, Darmstadt, Germany). All aqueous solutions were prepared with double distilled water.

Selaginella doederleinii Hieron was purchased from a local drug store in Fuzhou (China) and the voucher specimens, identified by Professor Yonghong Zhang, were deposited in the Photochemistry Laboratory, Fujian Medical University (Fuzhou, China).

Sample preparation

The dry aerial whole plants were cut into small pieces, ground to powder and passed through a 20-mesh sieve. A 5.0 kg sample powder was first extracted with an eightfold volume of 70% ethanol (40000 mL) under reflux twice (2 h per time). The extracts were merged and concentrated by rotary evaporation at 55°C under reduced pressure and about 800 g ethanol extract was obtained. Of which, 100 g of extract was then suspended with 1000 mL water, followed by extraction with twofold volume of petroleum ether (b.p., 60–90°C), dichloromethane and acetic

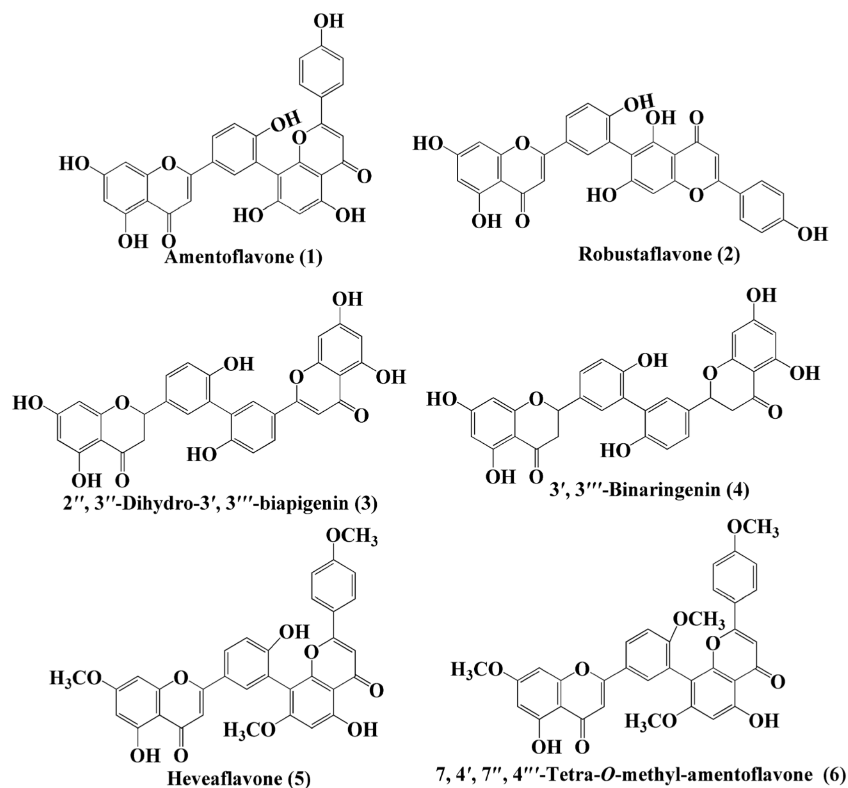

Figure 1. Chemical structures of the six biflavonoids obtained from *S. doederleinii*.

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ether thrice, respectively in turn. The extracts were concentrated with rotary evaporation at 55°C under reduced pressure and about 80 g petroleum ether extract, 40 g dichloromethane extract and 8.5 g acetic ether extract were obtained.

Cell lines and culture

Human lung-cancer cell lines (A549 and PC-9), human acute promyelocytic leukaemia cell line (HL60), human erythroleukemia cell line (K562) and human nasopharyngeal carcinoma line (CNE2) were obtained from the Department of Pharmacology of Fujian Medical University. All cell lines were grown and maintained in a humidified incubator at 37°C and in 5% CO_2 atmosphere. Roswell Park Memorial Institute (RPMI-1640) culture medium supplemented with 5–10% foetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin was used as the culture medium for A549, PC-9, CNE2, HL-60 and K562. The extracts of petroleum ether, dichloromethane and ethyl acetate, amentoflavone, robustaflavone 2″, 3″-dihydro-3′, 3‴-biapigenin, 3′, 3‴-binaringenin, heveaflavone and 7, 4′, 7″, 4″-tetra-*O*-methyl-amentoflavone were pre-solubilised in dimethyl sulphoxide (DMSO), respectively, and the final concentration of DMSO in culture medium did not exceed 0.1%.

3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay

Cytotoxicity was assessed with the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay method (Yao *et al*., 2011). Briefly, after being harvested from culture flasks, the cells were counted using a haemocytometer and the cell viability was determined by trypan blue exclusion. For all the cell lines, 2×10^3 cells were seeded in 96-well plates containing 100 μL of the growth medium per well. The cells were permitted to incubate for 24 h, and then treated with various concentrations of compounds for 48 h, with 20 μL of 5 mg/mL MTT in PBS being added to each well, and the plate was then incubated at 37°C for 4 h. The medium was removed and 100 μL of DMSO was added to each well. After incubation at 37°C for 15 min, the absorbance at 550 nm of the dissolved solutions was detected by a microplate reader (Bio-Tek ELX800, Bio Tek Instruments, Inc., Lanzhou, China). The absorbance of control cells (treated with 0.1% DMSO) was considered as 100%. A chemotherapeutic anti-cancer drug, adriamycin hydrochloride (purity ≥ 98%, Sigma-Aldrich Co., St. Louis, MO, USA) was used as the positive control.

High-speed counter-current chromatography

The HSCCC was performed with a TBE-300A(Shanghai Tauto Biotechnique, Shanghai, China) equipped with preparative column, consisting of 1.8 mm i.d. polytetrafluoroethylene (PTFE) tubing with a total capacity of 280 mL and a 20 mL sample loop. The β value of the preparative columns ranged from 0.5 to 0.8 ($\beta = r/R$, with $R = 5$ cm for preparative columns, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 to 1000 rpm. The system was equipped with a UV detector at 254 nm (Model TBD-23, Shanghai Tauto Biotech), and a N2000 chromatography workstation (Zhejiang University, Hangzhou, China) was employed to record the chromatogram. A HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature.

The two-phase solvent systems of *n*-hexane:ethyl acetate:methanol:water (1:2:1.5:1.5, v/v) and *n*-hexane:ethyl acetate:methanol:water (3:2:3:2, v/v) were used for HSCCC analysis of ethyl acetate and dichloromethane extracts, respectively. The systems were prepared by adding the solvents to a separation funnel according to the volume ratios and fully equilibrated. Then, the upper phase and the lower phase of each system were separated and degassed for 30 min prior to use. Five hundred milligrams of ethyl acetate extract and 200 mg of dichloromethane extract were dissolved in 20 mL of each two-phase solvent system, respectively.

In HSCCC procedure, the coil column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the inlet column at a flow rate of 1 mL/min, while the apparatus was rotated at 850 rpm. After reaching hydrodynamic equilibrium, as indicated by a clear mobile phase eluting at the tail outlet, the prepared sample solution was injected into the column through the injection valve. The effluent was monitored with a UV–Vis detector at 254 nm and the fractions were collected manually according to the chromatographic peak profiles displayed on the recorder. After running, the solvents in the column were pushed out and the retention of the stationary phase was measured.

High-performance liquid chromatography

The extracts and each purified fraction from the preparative HSCCC separation were analysed using a Shimadzu 20A HPLC system including a LC-20A pump, a SPD-20A UV detector, at 203 nm and 254 nm with a column temperature of 30°C and a flow rate of 1 mL/min. Analysis was carried out on an a Ultimate™ XB-C_{18} column (Welch Materials Inc.; 4.6 mm × 250 mm, 5.0 μm) with a gradient elution programme using a mixture of water and acetonitrile as the mobile phase within 75 min. The elution programme was optimised and conducted as follows: 10–42% in 0–30 min; 42–60% in 30–60 min; 100% in 60–70 min. The re-equilibrium took 10 min, giving a total run time of 80 min. The injection volume was 10 μL.

Structure identification

Electrospray ionisation mass spectrometry (ESI/MS) and nuclear magnetic resonance (NMR) were used for identification of the isolated compounds. The six compounds were dissolved in ethanol to produce 100 μg/mL solutions for ESI/MS experiments, and were dissolved in DMSO-*d6* to form 10 mg/mL solutions for NMR experiments.

All ESI/MS experiments were performed on a Finnigan LCQ Deca XP MAX (Thermo Finnigan, San Jose, CA, USA). NMR spectra were recorded on a Bruker Avance 400 NMR instrument (Karlsruhe, Germany) with 100 MHz for ^{13}C-NMR measurements in DMSO-*d6*, respectively.

Results and discussion

Anti-proliferation effects of extracts

The anti-proliferation effects of 70% ethanol, petroleum ether, dichloromethane and ethyl acetate extracts were evaluated on A549, PC-9, k562, HL60 and CNE2 in order to search for the bioactive fractionations. As shown in Table 1, all four extracts showed cytotoxicity to some of the five cell lines. Especially, the ethyl acetate extract possessed strong cytotoxicity to all the five cell lines. In addition, dichloromethane extract also possessed cytotoxicity to all the five cell lines. The results identified that the extracts of dichloromethane and ethyl acetate contained anti-proliferative ingredients to all the five cancer cell lines.

Activity-guided preparation isolation with HSCCC

As a result of the anti-proliferation investigation, the extracts of dichloromethane and ethyl acetate were submitted to further preparative isolation for obtaining bioactive ingredients. Before HSCCC analysis, the two-phase solvent system must be investigated by HPLC for estimation of partition coefficients of compound peaks in HSCCC. A number of solvent systems were tested, including *n*-hexane–water, *n*-heptane–water, ethyl acetate–water, *n*-hexane–ethyl acetate–methanol–water and

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Table 1. The inhibition effects of the extracts on human cancer cell lines (mean ± SD, $n = 3$)

Cell lines	IC_{50} values of extracts (μg/mL)			
	70% ethanol	Petroleum ether	Dichloromethane	Ethyl acetate
A549	196.8 ± 3.0	> 100	87.5 ± 1.9	15.4 ± 0.6
PC-9	81.1 ± 9.3	25.8 ± 0.5	29.01 ± 4.5	38.9 ± 6.3
K562	8.63 ± 0.87	32.27 ± 4.3	50.36 ± 2.2	18.43 ± 4.8
HL60	226.9 ± 4.0	> 100	22.3 ± 0.7	43.9 ± 4.2
CNE2	63.15 ± 6.8	41.34 ± 2.3	72.12 ± 3.3	36.1 ± 4.5

n-heptan–-ethyl acetate–methanol–water. Briefly, 5 mg of each extract was dissolved in 5 mL of the two-phase systems mentioned above, respectively, by shaking vigorously for 5 min to equilibrate the components thoroughly between the two phases. The two-phase solvents were separated and an equal volume of each was analysed by HPLC (Fig. 2). The partition coefficients($K1$ to $K6$)of peaks **1–6** were calculated by the ratios of each peak area in the upper phase against that in the lower phase, respectively (Table 2).

From Table 2, the solvent systems *n*-hexane:water (1:1), methyl *tert*-butyl ether:water (1:1) and ethyl acetate:water (1:1) were eliminated out based on the K values. As the ratio of the solvent system *n*-hexane:ethyl acetate:methanol:water was 1:2:1.5:1.5, the K values were suitable for isolation of peaks **1** to **4** from ethyl acetate extract, and as the ratio of the solvent system *n*-hexane:ethyl acetate:methanol:water was 3:2:3:2, the K values were suitable for isolation of peaks **5** and **6** from dichloromethane extracts. Thus, the ratios of *n*-hexane:ethyl acetate:methanol:water were selected at 1:2:1.5:1.5 and 3:2:3:2 for HSCCC analysis of the ethyl acetate and dichloromethane extracts, respectively.

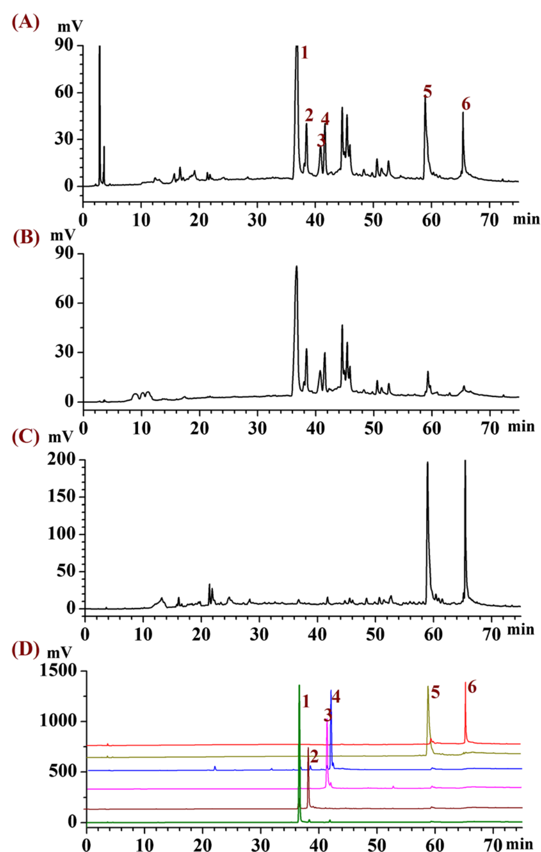

Figure 2. HPLC–UV chromatograms of (A) 70% ethanol extract, (B)ethyl acetate, (C) dichloromethane and (D) the six purified biflavonoids. **1**, amentoflavone; **2**, robustaflavone; **3**, 2″, 3″-dihydro-3′, 3‴-biapigenin; **4**, 3′, 3‴-binaringenin; **5**, heveaflavone; **6**, 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone.

In addition, using the selected solvent systems, the flow rates (0.6, 0.8, 1 and 1.2 mL/min) and the rotation rates of the column (700, 750, 800 and 850 rpm) were also examined. Finally, as the flow rate of 1 mL/min and the rotation rate of 850 rpm were used, good separation was obtained and the chromatogram is shown in Fig. 3.

Preparative separation of the six peaks (Fig. 2) by HSCCC was performed according to the above analysis. The sample amount of the injection for separation was 20 mL of the two-phase mixture solvent containing 500 mg of ethyl acetate extract and 200 mg of dichloromethane extract. The two lower phases of *n*-hexane:ethyl acetate:methanol:water (1:2:1.5:1.5 and 3:2:3:2) as mobile phases, respectively. The upper of each solvent system was used as the stationary phase, respectively. Every fraction was automatically collected every 3 min for 300 min. The retention rates of the stationary phase for the two solvent systems (1:2:1.5:1.5 and 3:2:3:2) were 57.4% and 52.7%, respectively. Fractions 1–4 were collected from fractions 20–26, 33–43, 50–60 and 81–97 by one HSCCC run, and evaporated under reduced pressure, followed by lyophilisation yielding 12.6 mg of compound **1** (91.4%), 6.6 mg of compound **2** (90.4%), 7.5 mg of compound **3** (98.2%) and 7.3 mg of compound **4** (90.3%). Fractions 5 and 6 were collected from fractions 56–63 and 75–81 yielding 6.3 mg of compound **5** (93.5%), 5.3 mg of compound **6** (94.5%) by one HSCCC run.

Compound identification

Compound **1** was obtained as a pale yellow amorphous powder with the following properties: (−)ESI/MS m/z 537.2 $[M-H]^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 8.01 (1H, dd, $J = 8.4$, 1.6 Hz, 6‴-H), 8.0 (1H, d, $J = 1.6$, 2‴-H), 7.57 (2H, d, $J = 8.8$ Hz, 2′-H, 6′-H), 7.15 (1H, d, $J = 8.4$ Hz, 5‴-H), 6.84 (1H, s, 3-H), 6.80 (1H, s, 3″-H), 6.72 (2H, d, $J = 8.8$ Hz, 3′-H, 5′-H), 6.47 (1H, d, $J = 1.6$ Hz, 8″-H), 6.41 (1H, s, 5-H), 6.19 (1H, d, $J = 1.6$ Hz, 6″-H). The ^{1}H-NMR profiles of compound **1** matched the reported NMR data for amentoflavone (Markham *et al.*, 1987, 1990).

Compound **2** was obtained as a pale yellow amorphous powder with following properties: (−)ESI/MS m/z 537.4 $[M-H]^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 8.25 (1H, $J = 1.6$ Hz, 2‴-H), 7.89 (2H, d, $J = 8.8$ Hz, 2′-H, 6′-H), 7.71 (1H, dd, $J = 8.8$, 1.6 Hz, 6‴-H), 7.28 (1H,d, $J = 8.8$ Hz, 5‴-H), 6.92 (2H, d, $J = 8.8$ Hz, 3′-H, 5′-H), 6.79 (2H, s, 3-H, 3″-H), 6.56(1H, s, 8″-H), 5.79(1H, s, 6″-H). The ^{1}H-NMR profiles of compound **2** matched with the reported NMR data for robustaflavone (Silva *et al.*, 1995; He *et al.*, 1996).

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Table 2. Partition coefficients *(K)* of six peaks in different solvent systems

Solvent system (v/v)	*K*1	*K*2	*K*3	*K*4	*K*5	*K*6
n-Hexane:water (1:1)	ND	ND	ND	ND	ND	ND
Methyl *tert*-butyl ether:water (1:1)	ND	ND	ND	ND	ND	ND
Ethyl acetate:water (1:1)	ND	ND	ND	ND	ND	ND
n-Hexane:ethyl acetate:methanol:water (1:1:1:1)	0.19	0.25	0.69	1.22	6.54	10.2
n-Hexane:ethyl acetate:methanol:water (1:2:1:1)	1.75	1.42	1.84	2.85	17.7	25.3
n-Hexane:ethyl acetate:methanol:water (1:1.2:1.2:1)	0.16	1.53	0.34	0.08	4.84	19.0
n-Hexane:ethyl acetate-methanol-water (1:2:1.5:1.5)	1.01	1.17	1.62	2.73	22.6	31.7
n-Hexane-ethyl acetate:methanol-water (3:2:3:2)	ND	ND	ND	0.02	0.38	0.21
n-Hexane:ethyl acetate:methanol:water (4:4:4.5:3.5)	0.02	0.05	0.08	0.19	1.87	2.53
n-Hexane:ethyl acetate:methanol:water (3:5:3:5)	5.16	7.22	19.4	18.7	16.6	15.5
n-Hexane:ethyl acetate:methanol:water (3.5:4:4.5:4.5)	0.03	0.04	0.09	0.25	3.26	9.89
n-Hexane:ethyl acetate:methanol:water (4:4:4.5:3.5)	0.05	0.08	0.19	0.25	1.51	1.19

ND, peaks **1–6** were not observed in the upper phase or in the lower phase.

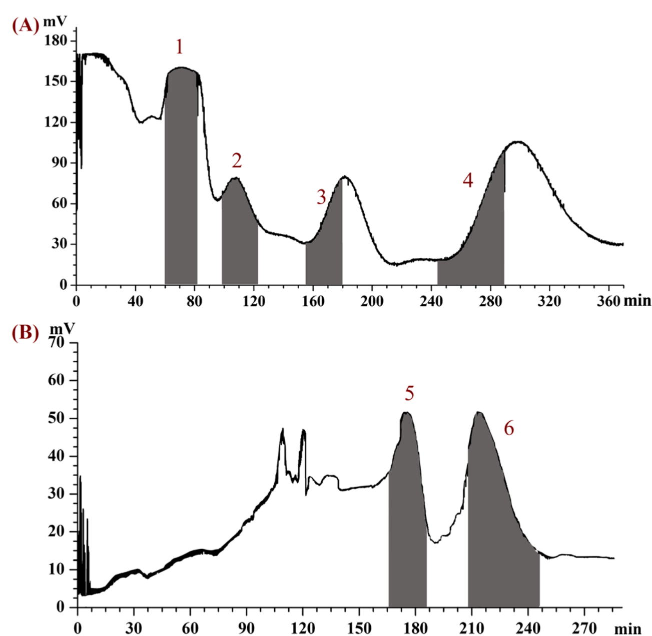

Figure 3. The HSCCC chromatograms of (A) ethyl acetate and (B) dichloromethane extracts. Ingredients in fractionations: **1**, amentoflavone; **2**, robustaflavone; **3**, 2″, 3″-dihydro-3′, 3‴-biapigenin; **4**, 3′, 3‴-binaringenin; **5**, heveaflavone; **6**, 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone.

Compound **3** was obtained as a pale yellow amorphous powder with the following properties: (−)ESI/MS *m/z* 539.1 [M − H]$^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 7.86 (1H, d, *J* = 1.6 Hz, 2′-H), 7.68 (1H, dd, *J* = 8.0, 1.6 Hz, 6′-H), 7.48 (1H, d, *J* = 1.6 Hz, 2‴-H), 7.21 (1H,dd, *J* = 8.0, 1.6 Hz, 6‴-H), 6.67 (1H, d, *J* = 8.0 Hz, 5′-H), 6.63 (1H, s, 3-H), 6.57 (1H, d, *J* = 8.0 Hz, 5‴-H), 6.45 (1H, d, *J* = 1.6 Hz, 8-H), 6.11(1H, d, *J* = 1.6 Hz, 6-H), 5.87 (1H, d, *J* = 1.6 Hz, 8″-H), 5.81 (1H, d, *J* = 1.6 Hz, 6″-H), 5.50 (1H, dd, *J* = 2.8, 2.8 Hz, 2″-H), 2.75 (1H, dd, *J* = 16.4, 2.8 Hz, 3″-H). The ^{1}H-NMR profiles of compound **3** matched with the reported NMR data for 2″, 3″-dihydro-3′, 3‴-biapigenin (Seeger *et al*., 1993), which is the first time it has been discovered in *S. doederleinii*.

Compound **4** was obtained as a pale yellow amorphous powder with following properties: (−)ESI/MS *m/z* 541.2 [M − H]$^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 7.30 (2H, d, *J* = 1.6 Hz, 2′, 2‴-H), 7.27 (2H, dd, *J* = 8.0, 1.6 Hz, 6′, 6‴-H), 6.85(2H, d, *J* = 8.0 Hz, 5′, 5‴-H), 5.89 (2H, dd, *J* = 8.0, 1.6 Hz, 8, 8″-H), 5.87 (2H, dd, *J* = 8.0, 1.6 Hz, 6, 6″-H), 5.50 (2H, dd, *J* = 2.4, 2.4 Hz, 2-H, 2″-H), 2.75 (2H, dd, *J* = 16.8, 2.4 Hz, 3″-H). The ^{1}H-NMR profiles of compound **4** matched with the reported NMR data for 3′, 3‴-binaringenin (Silva *et al*., 1995), which is the first time it has been discovered in *S. doederleinii*.

Compound **5** was obtained as a pale yellow amorphous powder with the following properties: (−)ESI/MS *m/z* 579.2 [M − H]$^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 8.05 (1H, dd, *J* = 8.4, 1.6 Hz, 6‴-H), 8.02 (1H, d, *J* = 1.6, 2‴-H), 7.67 (2H, d, *J* = 8.8 Hz, 2′-H, 6′-H), 7.17 (1H, d, *J* = 8.4 Hz, 5‴-H), 6.94 (2H, 3, 3″-H), 6.91 (2H, d, *J* = 8.8 Hz, 3′, 5′-H), 6.76 (1H, d, *J* = 1.6 Hz, 8″-H), 6.68 (1H, s, 5-H), 6.36 (1H, d, *J* = 1.6 Hz, 6″-H). The ^{1}H-NMR profiles of compound **5** matched with the reported NMR data for heveaflavone (Cheng *et al*., 2008).

Compound **6** was obtained as a pale yellow amorphous powder with following properties: (−)ESI/MS *m/z* 593.4 [M − H]$^-$. ^{1}H-NMR (DMSO-*d6*, TMS) δ ppm: 8.23 (1H, dd, *J* = 8.4, 1.6 Hz, 6‴-H), 8.10 (1H, d, *J* = 1.6, 2‴-H), 7.61 (2H, d, *J* = 8.8 Hz, 2′, 6′-H), 7.38 (1H, d, *J* = 8.4 Hz, 5‴-H), 7.06 (1H, s, 3-H), 6.95 (1H, s, 3″-H), 6.93 (2H, d, *J* = 8.8 Hz, 3′, 5′-H), 6.78 (1H, d, *J* = 1.6 Hz, 8″-H), 6.70 (1H, s, 5-H), 6.37 (1H, d, *J* = 1.6 Hz, 6″-H), 3.84(3H, s, 4‴-OMe), 3.83 (3H, s, 7-OMe), 3.80 (3H, s, 4′-OMe), 3.76 (3H, s, 7″-OMe). The ^{1}H-NMR profile of compound 6 matched with the reported NMR data for 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone (Markham *et al*., 1987; Yang *et al*., 2011).

Anti-proliferation effects of the purified bioflavonoids

Cytotoxicities of the six compounds obtained for five human cancer cell lines after 48 h exposure at dosages from 3.125 to 50 μg/mL are shown in Table 3. Doxorubicin was used as a positive control at dosages from 0.313 to 5 μg/mL. From Table 2, amentoflavone has obvious cytotoxicities for the five human cancer cell lines, which suggests that amentoflavone was one of the main anti-tumour active ingredients in *S. doederleinii*; 2″, 3″-dihydro-3′, 3‴-biapigenin, heveaflavone and 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone showed strong cytotoxicities to human lung cancer cell, suggesting that they are the main responding components for the therapeutic effect of *S. doederleinii* on lung cancer. Meanwhile, heveaflavone (IC_{50} = 15.8 ± 2.9 μg/mL) could be partly responsible for the therapeutic

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Table 3. The inhibition effects of the six compounds on human cancer cell lines (mean ± SD, $n = 3$)

Cell lines	IC_{50} values of compounds (μg/mL)					
	1	**2**	**3**	**4**	**5**	**6**
A549	36.3 ± 5.3	> 50	42.0 ± 1.2	19.3 ± 2.3	> 50	> 50
PC-9	6.41 ± 1.9	37.4 ± 10.0	8.12 ± 1.0	49.7 ± 0.37	6.74 ± 2.1	9.46 ± 2.8
K562	5.25 ± 0.87	> 50	39.29 ± 2.2	> 50	> 50	> 50
HL60	46.3 ± 4.3	48.0 ± 5.6	> 50	> 50	46.0 ± 4.6	49.2 ± 1.9
CNE2	17.3 ± 1.7	42.8 ± 0.2	> 50	> 50	15.8 ± 2.9	> 50

1, amentoflavone; **2**, robustaflavone; **3**, 2″, 3″-dihydro-3′, 3‴-biapigenin; **4**, 3′, 3‴-binaringenin; **5**, heveaflavone; **6**, 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone.

effect of the medical plant on nasopharyngeal carcinoma. In addition, robustaflavone also showed cytotoxicities (median inhibition concentration (IC_{50}) < 50 μg/mL) against human cancer cell lines, PC-9, HL60 and CNE2. Thus, further studies *in vitro* and *in vivo* should be carried out to disclose the molecular and biological mechanism of this anti-tumour effect. also In addition, the six compounds should be considered as the markers of quality control for *S. doederleinii*.

Summary

In this study, a bioassay-guided HSCCC method was used for preparative isolation of the anti-tumour active biflavonoids from *S. doederleinii*. As a result, milligram levels of six anti-tumour active biflavonoids, amentoflavone, robustaflavone, 2″, 3″-dihydro-3′, 3‴-biapigenin, 3′, 3‴-binaringenin, heveaflavone and 7, 4′, 7″, 4‴-tetra-*O*-methyl-amentoflavone with high purities (> 90%) were obtained by HSCCC in 300 min. This is the first report of 2″, 3″-dihydro-3′, 3‴-biapigenin and 3′, 3‴-binaringenin from this herb and of their cytotoxicities to human cancer cell lines. The present study also provides methodological references for simultaneous preparative isolation of multiple bioactive biflavonoids from the herbal family of *Selaginella* for further studies *in vitro* and *in vivo* of the bioactive ingredients.

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