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Identification of phenolic antioxidants from Sword Brake fern (*Pteris ensiformis* Burm.)

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Abstract

Sword Brake fern (*Pteris ensiformis* Burm.) is one of the most common ingredients of traditional herbal drinks in Taiwan. In an effort to identify antioxidants from the aqueous extract of Sword Brake fern (SBF), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity-guided isolation was employed. Three new compounds, kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (**3**) and hispidin 4-*O*-β-D-glucopyranoside (**4**), together with five known compounds, kaempferol 3-*O*-α-L-rhamnopyranosid-7-*O*-β-D-glucopyranoside (**2**), caffeic acid (**5**), 5-*O*-caffeoylquinic acid (**6**), 3,5-di-*O*-caffeoylquinic acid (**7**) and 4,5-di-*O*-caffeoylquinic acid (**8**) were isolated and determined on the basis of spectroscopic analyses. HPLC with UV detector was further employed to analyze the content of each compound in SBF based on the retention time by comparison with isolated pure compounds. It was found that the most abundant phenolic compound was compound **3**, followed by compounds **7** and **4**. The di-*O*-caffeoylquinic acids (**7** and **8**) have the strongest DPPH scavenging potential with IC_{50} around 10 μM and the highest Trolox equivalent antioxidant capacity (TEAC) about 2 mM. This data indicates that SBF is rich in phenolic antioxidants.
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Keywords: *Pteris ensiformis*; DPPH; TEAC; Caffeic acid; Hispidin

1. Introduction

Botanicals have been used for treatment or prevention of various human diseases throughout history. Many indigenous herbal plants of regional interest have been used popularly as folk medicines in Taiwan or other Asian countries; however, their active phytochemicals or biological effects remained to be elucidated. Sword Brake fern (*Pteris ensiformis* Burm.) is one of the most popular herbs used in beverages in Taiwan. It has been previously demonstrated that the aqueous extract of Sword Brake fern (SBF) exerts immunomodulatory effect by inhibiting the release of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nitric oxide (NO), and prostaglandin E2 (PGE_2) in lipopolysaccharide (LPS)-activated RAW264.7 cells (Wu, Wang, Weng, & Lian, 2005). In view that a variety of anti-inflammatory natural components also possess antioxidant activity (Chamundeeswari, Vasantha, Gopalakrishnan, & Sukumar, 2003; Lo, Liang, Lin-Shiau, Ho, & Lin, 2002; Wu, Yen, Wang, & Weng, 2004; Wu, Huang, Lian, Kou, & Wang, 2005) the possibility that SBF has a free radical scavenging activity arose.

Phytochemical investigations on the *Pteris* genus have yielded various phenolic compounds (Imperato, 1994; Lu,

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Hu, Zhang, & Tan, 1999) and diterpenes (Deng & Liang, 2005; Woerdenbag, Lutke, Bos, & Stevens, 1996). Phenolic compounds are potent antioxidants that play an important role in human nutrition as preventive agents against several diseases and protecting the body tissues from oxidative stress. Epidemiological evidences indicate an inverse relationship between the intake of polyphenol-rich foods and the risk of coronary heart disease as well as some types of cancer (Scalbert, Johnson, & Saltmarsh, 2005). The diterpene isolated from *Pteris* has been shown to be a potent antitumor agent by inducing apoptosis (Chen et al., 2004; Liu, Chen, et al., 2005; Liu, Ng, et al., 2005).

The aim of this study is to search for the antioxidant principals using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity as an index. Our result demonstrated that the aqueous extract of Sword Brake fern (SBF) had strong antioxidant activity. Eight phenolic compounds were identified (**1**–**8**) (Fig. 1) from the *n*-butanol layer and kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (**3**), and hispidin 4-*O*-β-D-glucopyranoside (**4**) are novel compounds. The Trolox equivalent antioxidant capacity (TEAC) of each compound was further analyzed using the (ABTS/H_2O_2/HRP) methods.

2. Materials and methods

2.1. General procedures

Optical rotations were measured on a JASCO DIP-370 digital polarimeter. UV spectra were obtained on a Hitachi 220-20 spectrophotometer. IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Inova 500, Varian Unity Plus 400 MHz, or Varian Gemini 200 MHz spectrometer using TMS as an internal standard. Chemical shifts were reported in parts per million (δ) and coupling constants (J) were expressed in Hertz. LR-EI-MS were collected on a Bruker APEX II mass or a Quattro GCMS spectrometer having a direct inlet system. LR-ESI-MS and HR-ESI-MS were measured on a Bruker APEX II mass spectrometer. Purospner STAR RP-18e (Merck KGaA, Darmstadt, Germany), Silica gel 60 (230–400 mesh, Merck), Sephadex LH-20 (GE Healthcare UK Ltd., Buckinghamshire, England), and Diaion HP20SS (Mitsubishi Chemical Co., Japan) were used for column chromatography. Spots were detected by spraying TLC with 50% H_2SO_4 followed by heating on a hot plate.

2.2. Plant

Sword Brake fern (*Pteris ensiformis* Burm.) was obtained from the Taitung District Agricultural Research and Extension Station, Taitung, Taiwan in Dec. 2004. A voucher specimen (PE001) was deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

2.3. Extraction and isolation

Fresh whole plants (8.1 kg wet weight) of Sword Brake fern were cut into small pieces and extracted with boiling water (3 × 20 l). A small portion of the combined aqueous extract was lyophilized to yield a dark-brown powder, which was redissolved in deionized water prior to use. It was denoted as the crude aqueous extract, SBF.

The rest of the aqueous extract was concentrated to a small volume and partitioned with chloroform to yield the chloroform and aqueous layers. The resulting aqueous layer was further partitioned with *n*-butanol to give *n*-butanol and aqueous layers. The *n*-butanol layer (30.0 g) was then passed through Diaion HP20SS chromatography and was eluted with water and methanol (1:0–0:1) to give five fractions (A1–A5).

Fr. A2 (7.6 g) was passed through silica gel column chromatography and eluted with chloroform–methanol (4:1) followed by preparative reverse-phase HPLC (Purospner, 20 ×250 mm, methanol: water = 1:3, flow rate 3 ml/min) to yield compounds **1** (37.5 mg) and **2** (30.0 mg).

Compound **3** (ca. 100.0 mg) was obtained by re-crystallization of the marc of the Fr. A3 (3.6 g) with methanol and water (1:1). The rest of Fr. A3 was then passed through preparative reverse-phase HPLC (Purospner, 20 × 250 mm, methanol: water = 1:1, flow rate 3 ml/min) to yield compound **4** (ca. 100.0 mg).

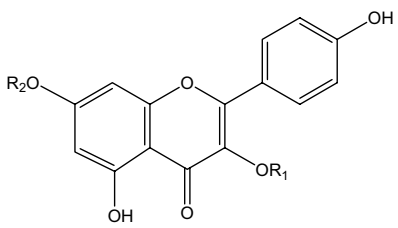
Fr. A4 (6.2 g) was chromatographed on Sephadex LH-20 (eluted with 100% methanol) followed by preparative reverse-phase HPLC (Purospner, 20 × 250 mm, methanol: water = 1:1, flow rate 3 ml/min) to yield compounds **5** (30.1 mg), **6** (20.2 mg), **7** (19.8 mg), and **8** (18.0 mg).

2.4. Acid hydrolysis of compounds *1* and *2*

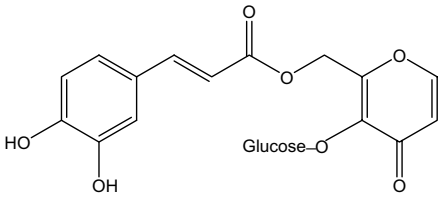
A solution of each compound (3.0 mg) in 6% aqueous HCl (3.5 ml) was refluxed for 2 h. The reaction mixture was diluted with water and then extracted with ethyl acetate. The resulting aglycones were identified by their ^{1}H NMR spectra.

2.5. Acetylation of compounds *1* and *2*

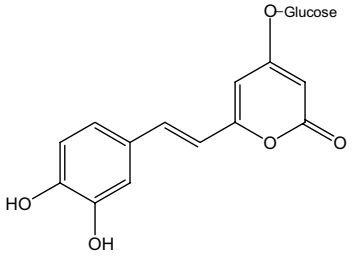
Compounds **1** or **2** (2.0 mg of each) was heated in a sealed vial for 24 h at 80 °C in 2% methanol–HCl (2 ml). The mixture was extracted with ethyl acetate. The aqueous hydrolysate was neutralized with Na_2CO_3 and filtered. L-Rhamnose, D-glucose, and D-apiose in the filtrate were acetylated with pyridine/Ac_2O. The acetylated sugar residues were compared with the acetylated references, L-rhamnose, D-glucose, and D-apiose with GC–MS. The results showed that peracetylrhamnose, peracetylglucose, and peracetylapiose were derived from **1** and **2**.



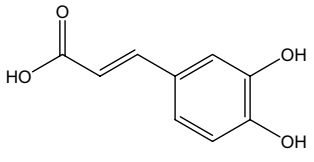
1 R_1 = rha R_2 = glc (2-1) api
2 R_1 = rha R_2 = glc



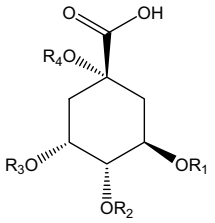
3 7-*O*-Caffeoylhydroxymaltol *O*-*β*-D-glucopyranoside



4 Hispidin 4-*O*-*β*-D-glucopyranoside



5 Caffeic acid



6 R_1 = Caffeoyl R_2 = H R_3 = H R_4 = H
7 R_1 = Caffeoyl R_2 = H R_3 = Caffeoyl R_4 = H
8 R_1 = Caffeoyl R_2 = Caffeoyl R_3 = H R_4 = H

Fig. 1. Structures of phenolic compounds **1**–**8** isolated from the aqueous extract of Sword Brake fern (*Pteris ensiformis* Burm.).

2.6. Quantification of phenolic compounds with HPLC

The contents of phenolic compounds in the aqueous extract of Sword Brake fern (SBF) were analyzed by HPLC. Dried whole plants of SBF (1 g) were dispersed and ground in boiling water (10 ml × 3). A total of 30 ml filtrate was transferred into a volumetric flask and then passed through a C18-E SPE cartridge (500 mg/3 ml) (Merck). The cartridge was eluted successively with 3 ml of 10% methanol/water (v/v), and 3 ml of 60% methanol/water (v/v).The 60% methanol fraction was collected and subjected to HPLC analysis. HPLC analysis was performed using Shimadzu LC-10AT pumps, SPD-10A UV–Vis detector, and TSK-GEL ODS-80TM (250 × 4.6 mm i.d.). The wavelength of the UV detector was set to be 297 nm and the flow rate was 1 ml/min. The stepwise HPLC conditions with methanol/water as mobile phase were as follows: initial methanol composition, 20%, increased to 40% in 20 min, and increased to 60% in 5 min.

2.7. DPPH scavenging capacities

The crude aqueous extract of Sword Brake fern (SBF), different layers and isolated compounds were evaluated for activities to scavenge the stable DPPH radical (0.1 mM, Sigma Chemical, St. Louis, MO, USA) according to the method (Dinis, Maderia, & Almeida, 1994). The affinity of the test material to quench the DPPH free radical was evaluated according to the equation: scavenging % = $(A_c - A_s)/A_c \times 100\%$. A_s and A_c are absorbance at 517 nm of the reaction mixture with sample and control, respectively. The IC_{50} values were obtained through extrapolation from linear regression analysis and denoted the

concentration of sample required to scavenge 50% of DPPH radicals. All experiments were repeated at least three times.

2.8. Assay of Trolox equivalent antioxidant capacity (TEAC)

The antioxidant activity of each compound (**1**–**8**) was further measured using the TEAC assay as described by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993) with minor modification. The TEAC value is based on the ability of samples to scavenge the blue-green $ABTS^{\cdot+}$ radical cation relative to the ability of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). $ABTS^{\cdot+}$ is generated by the interaction of ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, 100 μM), H_2O_2 (50 μM), and horse radish peroxidase (Sigma, 4.4 U/mL). After 1 ml of $ABTS^{\cdot+}$ was added to samples or Trolox, the absorbance at 734 nm was recorded after 10 min of incubation. TEAC is defined as the concentration (mM) of Trolox having the antioxidant equivalent to a 1.0 mM of the compound under investigation. To calculate the TEAC, the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot for the antioxidant in question is divided by the gradient of the plot for Trolox (Re et al., 1999).

3. Results and discussion

3.1. Identification of compounds 1–8

The aqueous extract of Sword Brake fern (*Pteris ensiformis* Burm.) was partitioned successively with chloroform and *n*-butanol. The IC_{50} values for DPPH scavenging of crude extract (SBF), chloroform, *n*-butanol, and aqueous layers were 50.3, >200, 15.7, and 61.5 μg/ml, respectively. This result indicates that SBF is a strong antioxidant and most of the active components may exist in the *n*-butanol layer.

Further isolation of compounds from the *n*-butanol layer was carried out with Diaion HP20SS, Sephadex LH-20, silica gel column chromatography, and preparative reverse-phase HPLC. This led to the isolation of eight phenolic compounds, whose structures were determined to be kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**), kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (**2**), 7-*O*-caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (**3**), hispidin 4-*O*-β-D-glucopyranoside (**4**), caffeic acid (**5**), 5-caffeoylquinic acid (**6**), 3,5-di-caffeoylquinic acid (**7**), and 4,5-di-caffeoylquinic acid (**8**), respectively, by detailed spectroscopic analysis and comparing with literature data. Many phenolic compounds have been isolated from *Pteris* genus (Imperato, 1994; Lu et al., 1999); however, compounds **1**–**8** were identified from the genus *Pteris* for the first time possibly due to the high biodiversity of *Pteris* genus in terms of phenolic composition. Compounds **2**, **3**, and **4** are new compounds. Their structures are shown in Fig. 1.

Kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**): $C_{32}H_{38}O_{19}$, yellow amorphous solid, LR-ESI-MS *m/z*: 749.0 [100%, $M+Na]^+$; HRESIMS *m/z*: 749.1906 (calcd. for $C_{32}H_{38}O_{19}Na^+$, 749.1899), $[\alpha]_D^{25}$: −60° (*c* 0.35, MeOH), UV λ_{max}^{MeOH} ($\log\varepsilon$): 337 (3.8), 227 (3.9) nm, IR ν_{max} (neat): 3410, 1650 cm^{-1}, ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) are given in Table 1.

Kaempferol 3-*O*-α-L-rhamnopyranosid-7-*O*-β-D-glucopyranoside (**2**): $C_{27}H_{30}O_{15}$, yellow amorphous solid, LR-ESI-MS *m/z*: 617.0 [100%, $M+Na]^+$, HR-ESI-MS *m/z*: 617.1486 (calcd for $C_{27}H_{30}O_{15}Na^+$, 617.1482), $[\alpha]_D^{25}$: −72° (*c* 0.42, MeOH), UV λ_{max}^{MeOH} ($\log\varepsilon$): 349 (3.9), 263 (4.1) nm, IR ν_{max} (Neat): 3400, 1654 cm^{-1}, ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz) are given in Table 1, NMR data are consistent with (El-Sayeda, Awaadb, Hifnawyb, & Mabryc, 1999).

Acid hydrolysis of compounds **1** and **2** released kaempferol, identified by ^1H and ^{13}C NMR spectroscopy. The gas chromatographic analysis of the acetylation products of both compounds shows the presence of glucose, rhamnose, and apiose in the ratio 1:1:1. The molecular formula of **1** and **2**, $C_{32}H_{38}O_{19}$ and $C_{27}H_{30}O_{15}$, respectively, were established by HR-ESI-MS (**1**: $[M+Na]^+$ *m/z* 749.1906, calcd. 749.1899, **2**: $[M+Na]^+$ *m/z* 617.1486, calcd. 617.1477). The complete structures of **1** and **2** were elucidated by 1D and 2D NMR experiments. The ^1H NMR spectrum of compound **1** showed the presence of two *meta*-coupled aromatic protons at δ 6.47 and 6.72 (both d, $J = 2$ Hz), which were assigned to the protons at H-6 and H-8 respectively; as well as *ortho*-coupled aromatic protons at δ 8.12 (d, $J = 8.0$ Hz, H-2′ and H-6′) and 6.90 (d, $J = 8.0$ Hz, H-3′ and H-5′). The ^{13}C NMR shifts of the aglycone moiety of **1** corresponded to the shifts for kaempferol with significant differences on C-3 and C-7. These shifts were analogous to those reported for the glycosylation of hydroxy group in a flavonol glycoside (Agrawal, 1989). Three anomeric protons were easily identified in the spectra of **1** at δ_H 5.12 (d, $J = 7.5$ Hz), 5.39 (d, $J = 1.5$ Hz), 5.46 (d, $J = 1.5$ Hz) and correlated with carbons at δ_C 100.1, 103.5, and 110.8, respectively. From the assigned aglycone and sugar values, it was apparent that one saccharide and one disaccharide unit were attached to C-3 and C-7 of the aglycone. The structure of the sugar chain was assigned by a combination of COSY, HMQC, and HMBC experiments. Starting from the anomeric protons of each sugar unit, all of the hydrogens could be identified using a combination of COSY experiments (Table 1). The assignments of all proton resonances for the sugar moieties allowed us to assign the resonances of the linked carbon atoms by HMQC experiment (Table 1). Information about the sequence of the oligosaccharide chain was deduced from HMBC experiments. Key correlation peaks were observed between the anomeric proton of the rhamnose (δ 5.39) and the C-3 of the kaempferol (δ 136.4), between the anomeric proton signal of the glucose (δ 5.12) and C-7 of the kaempferol (δ 164.4) as well as between the anomeric proton of the apiose (δ 5.46) and

Table 1
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compound **1** and **2** in CD_3OD (δ in ppm, J in Hz)

Position	^{1}H NMR		^{13}C NMR[a]		HMBC
	1	**2**	**1**	**2**	**1**
2			159.8	159.7	H-2′, H-6′
3			136.4	136.4	H-1″
4			179.7	179.7	
5			162.8	162.7	H-6
6	6.47 (1H, d, 2)	6.47 (1H, d, 2)	100.6	100.8	H-8
7			164.4	164.6	H-6, H-8, H-1‴
8	6.72 (1H, d, 2)	6.72 (1H, d, 2)	95.7	95.7	H-6
9		158.0	158.0	H-8	
10		107.6	107.6	H-6, H-8	
1′		122.3	122.3	H-3′, H-5′	
2′	7.79 (1H, d, 8.0)	7.78 (1H, d, 8.0)	132.1	132.0	H-6′
3′	6.93 (1H, d, 8.0)	6.94 (1H, d, 8.0)	116.5	116.5	H-5′
4′		161.7	161.7	H-2′, H-6′	
5′	6.93 (1H, d, 8.0)	6.94 (1H, d, 8.0)	116.5	116.5	H-3′
6′	7.79 (1H, d, 8.0)	7.78 (1H, d, 8.0)	132.1	132.0	H-2′
Rhamnoside					
1″	5.39 (1H, s)	5.39 (1H, s)	103.5	103.4	
2″	4.23 (1H, br s)	4.23 (1H, br s)	71.2	71.2	H-1″
3″	3.94 (1H, m)	3.94 (1H, m)	72.1	72.1	H-4″, H-5″
4″	3.43 (1H, m)	3.45 (1H, m)	73.1	73.1	H-5″, H-6″
5″	3.64 (1H, m)	3.50 (1H, m)	71.8	71.8	H-4″, H-6″
6″	0.92 (3H, d, 6.2)	0.92 (3H, d, 6.2)	17.6	17.6	
Glucoside					
1‴	5.12 (1H, d, 7.2)	5.06 (1H, d, 7.2)	100.1	101.5	H-2‴, H-3‴
2‴	3.66 (1H, m)	3.42 (1H, m)	78.2	75.1	H-3‴, 1⁗
3‴	3.43 (1H, m)	3.40 (1H, m)	78.6	78.0	H-2‴
4‴	3.31 (1H, m)	3.31 (1H, m)	72.1	72.1	H-5‴, H-6‴
5‴	3.19 (1H, m)	3.19 (1H, m)	78.4	78.3	H-3‴, H-6‴
6‴	3.51 (2H, m)	3.51 (2H, m)	62.4	62.4	
Apioyl					
1⁗	5.46 (1H, s)		110.8		H-4a⁗
2⁗	3.96 (1H, s)		78.1		H-1⁗, H-4a⁗
3⁗			80.7		H-1⁗, H-2⁗, H-4a⁗
4a⁗	3.82 (1H, d, 10.0)		75.4		H-1⁗, H-2⁗
4b⁗	4.04 (1H, d, 10.0)				
5⁗	3.55 (2H, br s)		65.4		H-2⁗, H-4b⁗

[a] Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC.

the C-2 of the glucose (δ 78.2). From these considerations, the structure of kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (Fig. 1) was assigned to **1**. The ^{1}H NMR spectrum of **2** also showed a kaempferol derivative. The signals in the ^{1}H, ^{13}C, and 2D NMR spectra were superimposable to those of **1**, as a result, the structure of **2** was kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (Fig. 1), which was reported previously (El-Sayeda et al., 1999; Sharaf, El-Ansari, & Saleh, 1997) (Table 2).

7-*O*-Caffeoylhydroxymaltol 3-*O*-β-D-glucopyranoside (**3**): $C_{21}H_{22}O_{12}$, colorless amorphous solid, LR-ESI-MS m/z: 489.0 $[M+Na]^+$; HR-ESI-MS m/z: 489.1006 (calcd. for $C_{21}H_{22}O_{12}Na^+$, 489.1003, $[\alpha]_D^{25}$: −20.5° (c 0.62, MeOH), UV λ_{max}^{MeOH} ($\log\varepsilon$): 324 (3.57), 270 (3.71) nm, IR ν_{max} (Neat): 3370, 1710, 1650 cm^{-1}, ^{1}H NMR (C_5D_5N, 400 MHz), aglycone signals: δ 6.48 (1H, d, $J = 5.0$, H-5), 7.94 (1H, d $J = 5.0$, H-6), 5.56 (1H, d, $J = 14.0$, H-7a), 5.62 (1H, d, $J = 14.0$, H-7b), 7.46 (1H, s, H-2′), 7.10 (2H, s, H-5′, H-6′), 6.49 (1H, d, $J = 15.6$, H-7′) 7.86 (1H, d, $J = 15.6$, H-8′), sugar signals: δ 5.52 (1H, d, $J = 7.6$, H-1″), 3.39 (1H, m, H-2″), 4.15 (1H, m, H-3″), 4.06 (1H, m, H-4″), 4.02 (1H, m, H-5″), 4.32 (1H, m, H-6″), and ^{13}C NMR (C_5D_5N, 100 MHz): ppm 157.6 (C-2), 143.2 (C-3), 175.4 (C-4), 117.2 (C-5), 155.7 (C-6) 58.1 (C-7), 126.4 (C-1′) 115.3 (C-2′), 147.8 (C-3′) 146.9 (C-4′), 122.2 (C-5′), 116.3 (C-6′), 113.3 (C-7′), 166.6 (C-8′), 104.4 (C-1″), 75.0 (C-2″), 77.6 (C-3″), 70.6 (C-4″), 78.6 (C-6″), 62.0 (C-6″), key HMBC correlations: H-1″/C-3, H-7/C-9′.

Hispidin 4-*O*-β-D-glucopyranoside (**4**): $C_{19}H_{20}O_{10}$, yellow powder, LR-ESI-MS m/z: 431.1 $[M+Na]^+$; HR-ESI-MS m/z: 431.0952 (calcd for $C_{19}H_{20}O_{10}Na^+$, 431.0949), $[\alpha]_D^{25}$:-65.1° (c 0.52, MeOH), UV λ_{max}^{MeOH} ($\log\varepsilon$): 263 (4.53), 339 (3.90) nm, IR ν_{max} (Neat): 3400, 2942, 1702 cm^{-1}, ^{1}H NMR (CD_3OD, 400 MHz), aglycone signals: δ 5.73 (1H,

Table 2
^{13}C NMR (100 MHz) spectral data of compound **5**, **6**, **7**, and **8** in CD_3OD (δ in ppm)

Position	^{13}C NMR[a]					
	5	**6**	**7**		**8**	
1		78.0	74.7		76.3	
2		38.9	36.1		38.4	
3		72.6	72.5		69.5	
4		75.0	69.9		75.8	
5		73.1	72.8		69.0	
6		40.6	37.9		39.5	
7		180.9	177.0		177.2	
Coffeoyl						
1′	127.7	127.8	127.7	127.8	127.7	127.6
2′	115.1	115.5	114.7	114.7	114.7	114.7
3′	146.8	146.8	146.7	146.7	146.7	146.7
4′	149.4	149.5	149.6	149.6	149.6	149.6
5′	116.5	116.5	116.5	116.5	116.5	116.5
6′	122.8	122.9	123.2	123.1	123.2	123.1
7′	147.1	147.0	147.7	147.5	147.7	147.5
8′	115.5	115.1	114.6	114.6	114.6	114.6
9′	171.1	169.1	168.5	168.2	168.5	168.2

[a] Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, NOESY, HMQC and HMBC.

s, H-3), 6.25 (1H, s, H-5), 6.61 (1H, d, $J = 15.6$, H-7) 7.31 (1H, d, $J = 15.6$, H-8), 7.03 (1H, d, $J = 1.2$, H-2), 6.95 (1H, dd, $J = 8.0$, 1.2, H-5), 6.77 (1H, d, $J = 8.0$, H-6), sugar signals: δ 4.66 d (1H, d, $J = 7.6$, H-1″), 3.38 (1H, m, H-2″), 3.40 (1H, m, H-3″) 3.31 (1H, m, H-4″), 3.22 (1H, m, H-5″), 3.75 (1H, m, H-6″), and ^{13}C NMR (C_5D_5N, 100 MHz): ppm 166.6 (C-2), 92.3 (C-3), 171.6 (C-4), 101.1 (C-5), 161.7 (C-6), 116.7 (C-7), 137.5 (C-8), 128.7 (C-9), 114.8(C-10), 148.7 (C-11), 146.8 (C-12),116.5 (C-13), 122.0 (C-14), 100.8 (C-1′), 74.4 (C-2′), 77.6 (C-3′), 70.9 (C-4′), 78.5 (C-5′), 62.0 (C-6′), key HMBC correlation: H-1′/C-4.

The HMBC spectrum of compound **3** showed the correlations of H-5/C-4, H-5/C-3, H-6/C-4, H-6/C-2, H-7/C-2, and H-7/C-3, which confirmed the presence of the hydroxymaltol moiety. The HMBC correlation of H-7ab/C-9′ was observed corresponding to the linkage between the hydroxymaltol and the caffeoyl moiety (Guo, Koike, Li, Guo, & Nikaido, 2004). Furthermore, the β-glucopyranose group was identified by direct comparison with spectroscopic data (Guo et al., 2004). An obvious correlation between δ_H 5.52 (anomeric proton) and δ_C 143.2 (C-3) has been observed in the HMBC experiment. This evidence confirmed that the glucose was linked at C-3 of hyroxymaltol. Thus, the proposed structure **3** was determined as hydroxymaltol 7-*O*-caffeoyl-3-*O*-β-D-glucopyranoside. The aglycone part of **4** was a known compound, hispidin, isolated from *Inonotus hispidus*. The structure was identified by the NMR data, which were reported in the literature (Ali, Jansen, Horst, Liberra, & Lindequist, 1996). The HMBC spectrum correlations of δ_H 5.52 (anomeric proton) and δ_C 171.6 (C-4) has been observed. This evidence confirmed that the glucose was linked at C-4 of hispidin. Thus, the proposed structure of hispidin 4-*O*-β-D-glucopyranoside (**4**) was determined unambiguously.

Caffeic acid (**5**): $C_9H_8O_4$, yellow powder, EI-MS: *m*/*z* 181.1 $[M+H]^+$, ^{1}H NMR (400 MHz, CD_3OD): δ 6.31 (1H, d, $J = 16.0$ Hz, H-8), 6.75 (1H, d, $J = 8.0$ Hz, H-5), 6.93 (1H, dd, $J = 2.0$, 8.0 Hz, H-6), 7.04 (1H, d, $J = 2.0$ Hz, H-2), 7.57 (1H, d, $J = 16.0$ Hz, H-7); ^{13}C NMR (CD_3OD, 100 MHz) were given in Table 1, identical to data in the literature (Kumaran & Karunakaran, 2007).

5-*O*-Caffeoylquinic acid (**6**): $C_{16}H_{18}O_8$, yellow powder, ESI-MS: *m*/*z* 355.5 $[M+H]^+$.^{1}H NMR (400 MHz, CD_3OD): δ 2.00 (2H, m, H-2, H-6), 2.13 (2H, m, H-2, H-6), 3.63 (1H, dd, $J = 3.0$, 9.0 Hz, H-4), 4.14 (1H, s, H-3), 5.34 (1H, m, H-5), 6.30 (1H, d, $J = 16.0$ Hz, H-8′), 6.76 (1H, d, $J = 8$ Hz, H-5′), 6.93 (1H, dd, $J = 2.0$, 8.0 Hz, H-6′), 7.04 (1H, d, $J = 2.0$ Hz, H-2′), 7.58 (1H, d, $J = 16$ Hz, H-7′); ^{13}C NMR (CD_3OD, 100 MHz) were given in Table 1, identical to NMR data in the literature (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004).

3,5-Di-*O*-caffeoylquinic acid (**7**): $C_{25}H_{24}O_{12}$, yellow power, ESI-MS: *m*/*z* 539.0 $[M+Na]^+$, ^{1}H NMR (CD_3OD, 400 MHz): δ 2.13–2.34 (4H, m, H-2, -6), 4.16 (1H, m, H-4), 5.12 (1H, m, H-5), 5.63 m (1H, m, H-3), 6.26 and 6.31 (1H each, d, $J = 16.0$ Hz, H-8′,-8″), 6.71 and 6.75 (1H each, d, $J = 8.0$ Hz, H-5′, -5″), 6.96 and 6.97 (1H each, dd, $J = 2.0$, 8.2 Hz, H-6′, -6″), 7.06 and 7.07 (1H each, d, $J = 2.0$ Hz, H-2′, -2″), 7.57 and 7.61 (1H each, d, $J = 16.0$ Hz, H-7′, -7″); ^{13}C NMR (CD_3OD, 100 MHz) were given in Table 1. The NMR data were consistent with the literature (Iwai et al., 2004; Zhu, Zhang, & Lo, 2004).

4,5-Di-*O*-caffeoylquinic acid (**8**): $C_{25}H_{24}O_{12}$, yellow powder, ESI-MS: *m*/*z* 539.1 $[M+Na]^+$. ^{1}H NMR (CD_3OD, 400 MHz): δ 1.94–2.34 (4H, m, H-2, -6), 4.36 (1H, s, H-3), 5.11 (1H, m, H-4), 5.55 (1H, m, H-5), 6.19 and 6.29 (1H each, d, $J = 16.0$ Hz, H-8′, -8″), 6.74 and 6.76 (1H each, d, $J = 8.0$ Hz, H-5′, -5″), 6.90 and 6.92 (1H each, dd, $J = 2.0$, 8.0 Hz, H-6′, -6″), 7.01 and 7.03 (1H each, d, $J = 2.0$ Hz, H-2′, -2″), 7.52 and 7.60 (1H each, d, $J = 16.0$ Hz, H-7′, -7″); ^{13}C NMR (CD_3OD, 100 MHz) were given in Table 1. The NMR data were consistent with the literature (Iwai et al., 2004; Zhu et al., 2004).

3.2. The contents of compounds *1–8*

The contents of phenolic compounds **1**–**8** in the aqueous extract of Sword Brake fern (SBF) was analyzed by HPLC as described in Section 2. A representative HPLC chromatogram is shown in Fig. 2. The purified compounds **1**–**8** were used in parallel to obtain the retention time for quantification analysis. Table 3 shows the contents of compounds **1**–**8** in SBF. It was found that caffeic acid (**5**) and its derivatives (**3**, **7**, and **8**) were the major class of phenolic acids in SBF, with levels of 2.64 ± 0.23, 9.99 ± 0.17, 4.47 ± 0.15 and 3.68 ± 0.22 mg/g dry weight, respectively.

Hispidin 4-O-β-D-glucopyranoside (**4**) is another important composition with content of 4.11 ± 0.08 mg/g dry weight. Kaempferol glycosides (**1** and **2**) account for the

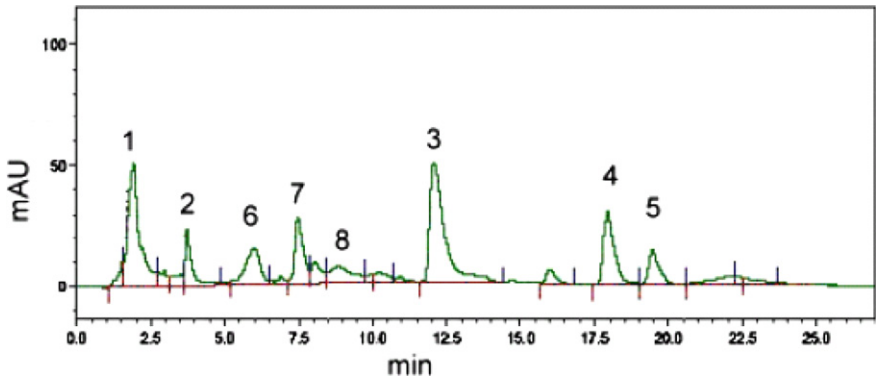


Fig. 2. A representative HPLC chromatograms of phenolic compounds **1**–**8** from the aqueous extract of Sword Brake fern (*Pteris ensiformis* Burm.).

Table 3
Contents of the isolated phenolic compounds in Sword Brake fern

Samples	Amounts (mg/g dry weight)[a]
Kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**)	2.41 ± 0.04
Kaempferol 3-*O*-α-L-rhamnopyranosid-7-*O*-β-D-glucopyranoside (**2**)	2.62 ± 0.02
7-*O*-caffeoylhydroxymaltol 3-O-β-D-glucopyranoside (**3**)	9.99 ± 0.17
Hispidin 4-*O*-β-D-glucopyranoside (**4**)	4.11 ± 0.08
Caffeic acid (**5**)	2.64 ± 0.23
5-*O*-Caffeoylquinic acid (**6**)	0.86 ± 0.09
3,5-Di-*O*-caffeoylquinic acid (**7**)	4.47 ± 0.15
4,5-Di-*O*-caffeoylquinic acid (**8**)	3.68 ± 0.22

[a] Values were determined from integration of HPLC signals and response factors calculated from isolated pure compounds. Values represent means ± SEM ($n = 3$).

major flavonols in SBF with 2.41 ± 0.04 and 2.62 ± 0.02 mg/g dry weight, respectively.

3.3. DPPH radical scavenging assay and TEAC analysis

DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay and Trolox equivalent antioxidant capacity (TEAC) are two widely used methods to evaluate antioxidant capacity in a short time (Blois, 1958; Re et al., 1999). Table 4 shows the DPPH scavenging activities and TEAC values of compounds **1**–**8** isolated from SBF. All of these compounds exhibited considerable DPPH and $ABTS^{\cdot+}$ radical cation scavenging activities. Among them, caffeic acid (**5**) and its derivatives (**3**, **6**, **7**, and **8**) as well as hispidin glucoside (**4**) displayed stronger or equal DPPH scavenging activities as compared with the common antioxidant supplement, α-tocopherol. This result indicates that the structure prerequisite to reinforce DPPH scavenging is catechol moiety. In addition to OH moieties in the structural arrangements, the resonance of electrons between rings may also be important for their DPPH scavenging activities.

Among the isolated compounds, di-*O*-caffeoylquinic acids (**7**, **8**) were both the strongest DPPH and $ABTS^{\cdot+}$ radical cation scavengers with IC_{50} and TEAC value about 10 μM and 2 mM, respectively. It is worthy of noting that the antioxidant activity increased with the number of caffeoyl moeity in the molecule and this agreed with a previous report (Iwai et al., 2004).

Phenolic acids, known as a kind of multipurpose bioactive agent, frequently occur in herbal plants (He, 2000). Besides the antibacterial and antifungal activity, phenolic acids were considered in recent years as potentially protective compounds against cancer and heart disease, in part because of their antioxidant properties (Morton, Caccetta, Puddey, & Croft, 2000). Caffeic acid (3,4-dihydroxycinnamic acid) is among the major hydroxycinnamic acids present in nature and is a potent antioxidant (Gulcin, 2006). In this research, it was found that caffeic acid derivatives (compounds **3**, **5**, **6**, **7** and **8**) were the major antioxidant phenolics in SBF.

Hispidin inhibits protein kinase C (PKC) β-isoform (IC_{50} = 2 μM) and is preferentially cytotoxic to cancer cells (Gonindard et al., 1997). The derivatives and analogs

Table 4
The antioxidant activities of phenolic compounds isolated from *P. ensiformis*

Samples	IC_{50} (μM)[a]	TEAC (mM)[b]
Kaempferol 3-*O*-α-L-rhamnopyranoside-7-*O*-[α-D-apiofuranosyl-(1-2)-β-D-glucopyranoside] (**1**)	104.27 ± 2.36	0.58 ± 0.01
Kaempferol 3-*O*-α-L-rhamnopyranosid-7-*O*-β-D-glucopyranoside (**2**)	128.78 ± 2.52	0.52 ± 0.02
7-*O*-caffeoylhydroxymaltol 3-O-β-D-glucopyranoside (**3**)	21.06 ± 0.81	0.89 ± 0.02
Hispidin 4-*O*-β-D-glucopyranoside (**4**)	28.21 ± 1.51	0.85 ± 0.04
Caffeic acid (**5**)	17.48 ± 0.33	0.92 ± 0.04
5-*O*-Caffeoylquinic acid (**6**)	21.63 ± 1.37	1.27 ± 0.03
3,5-Di-*O*-caffeoylquinic acid (**7**)	10.71± 1.13	1.99 ± 0.02
4,5-Di-*O*-caffeoylquinic acid (**8**)	10.30 ± 0.72	2.19 ± 0.03
Kaempferol (reference)	25.70 ± 1.02	1.19 ± 0.02
Hispidin (reference)	29.97 ± 2.84	0.91 ± 0.02
α-Tocopherol (reference)	28.08 ± 2.69	
Trolox (reference)		1.00 ± 0.01

[a] IC_{50}: concentration (in μM) necessary for reduction 50% DPPH radical. Values represent means ± SEM ($n = 3$).

[b] TEAC: concentration (in mM) of Trolox having the antioxidant equivalent to 1.0 mM of the tested compound. Values represent means ± SEM ($n = 3$).

of hispidin have also been shown to be potent free radical scavengers (Lee, Seok, Kim, & Yun, 2006; Lee & Yun, 2006). In this research, we identified a new compound, hispidin 4-*O*-β-D-glucopyranoside (**4**), exhibited compatible DPPH scavenging activity and TEAC value as compared with its aglycone, hispidin.

It has been suggested that flavonols with a free 3-OH group, are the strongest antioxidants among flavonoids (Burda & Oleszek, 2001; Hollman & Katan, 1997). The isolated flavonol glycosides (**1**–**2**) in SBF displayed much weaker DPPH radical scavenging activity ($IC_{50} > \approx 100$ μM) and TEAC values (≈0.5 mM) than the aglycone, kaempferol ($IC_{50} = 25.70 \pm 1.02$ μM; TEAC ≈ 1 mM). Current result supported previous notion that *O*-glycosylation had a tremendous negative effect on free radical scavenging of kaempferol (Cardoso, Silva, Castro-Gamboa, & Bolzani, 2005).

Previously, it has been demonstrated that SBF inhibited inflammatory mediator production in LPS-activated RAW264.7 cells. From current research, it suggests that the health promotion effect of SBF may be the result of combinatory activities, including antioxidant and antiinflammatory. This result supports the hypothesis that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their health benefits (Liu, 2003). In conclusion, we demonstrated for the first time that SBF exhibited strong antioxidant activity, attributed to the phenolic compounds, especially derivatives of caffeic acid, hispidin and kaempferol.

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