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Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa*

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Abstract

Bidens pilosa has been used as a folk medicine in various medications and as a popular ingredient in herb teas. Chemopreventive activities of crude and fractionated plant extracts of *Bidens pilosa* were evaluated in this study. Ethyl acetate and butanolic fractions, partitioned from the total crude extract of *Bidens pilosa*, exhibited significant scavenging free radical activity (IC_{50} values ≅ 14–17 μg/mL) comparable to that of α-tocopherol. Strong effects on the inhibition of LPS-mediated nitric oxide production in RAW 264.7 cells were also observed for the EA and BuOH fractions. Detectable cytotoxicity on RAW 264.7 cells, however, was observed for the EA fraction at a dose >100 μg/ml. The metabolite profile and major constituents of the BuOH fraction were studied and characterized using various spectroscopic analyses. A new compound, heptanyl 2-*O*-β-xylofuranosyl-(1→6)-β-glucopyranoside (**1**), and eight phenolic compounds, namely quercetin 3-*O*-rabinobioside (**2**), quercetin 3-*O*-rutinoside (**3**), chlorogenic acid (**4**), 3,4-di-*O*-caffeoylquinic acid (**5**), 3,5-di-*O*-caffeoylquinic acid (**6**), 4,5-di-*O*-caffeoylquinic acid (**7**), jacein (**8**), centaurein (**9**) were for the first time isolated from *Bidens pilosa*. Compounds **2–7** are the major antioxidative constituents in the *Bidens pilosa* extract.

Keywords: *Bidens pilosa*; Chemopreventive bioactivities; Metabolite profile; Phenolic compounds

1. Introduction

Reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl, nitric oxide and peroxynitrite radicals, play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system; however, oxidative stress is generated when equilibrium favors free radical generation as a result of a depletion of antioxidant levels. Oxidative damage, caused by the action of free radicals, may initiate and promote the progression of a number of chronic diseases, such as cancer, cardiovascular diseases, neurodegenerative disorders, and ageing (Finkel and Holbrook, 2000; Esposito et al., 2002). Nitric oxide (NO), a diatomic free radical, is synthesized in biological systems by constitutive and inducible nitric oxide synthase (cNOS and iNOS) (Vanvaskas and Schmidt, 1997). Excess generation of NO by iNOS has been found to contribute to many diseases, such as carcinogenesis, septic shock, cerebral injury, atherosclerosis, rheumatoid arthritis, cell apoptosis and necrosis (Sheu et al., 2001). Moreover, under pathological conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously to form a peroxynitrite anion ($ONOO^-$), which is more toxic than O_2^- or NO to biological systems, by causing modification of proteins (Van der Vliet et al., 1995) or nucleic acid

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(Yermilov et al., 1995). Thus, reducing the NO generation in excess amounts is now accepted widely as an important goal for the chemoprevention of various diseases, e.g., cancer.

Search for natural antioxidants, especially from plant sources, as a potential preventive intervention for free-radical mediated diseases has already turned into an attractive research field and a very important social issue for improvement of the quality of human life. Polyphenols, including phenolic acids, flavonoids, tannins, lignans and others, are widespread, in plant foods and in different medicinal plants. Plant phenolics may function as potent free radical scavengers, reducing agents, quenchers of ROS, and protect against lipid peroxidation (Montoro et al., 2001; Osawa, 1994). Currently, the antioxidative properties of plant phenolics have been suggested to play an important role in the maintenance of human health and prevention of several diseases. The application of antioxidative activities in reducing oxidative stress in vivo have promoted the investigation of potent antioxidant constituents from plant sources (Montoro et al., 2001; Morand et al., 1998).

Bidens pilosa Linn. var. *radiata* (Compositae) is a plant widely found in tropical and sub-tropical regions of the world and is used traditionally as an ingredient in anti-inflammatory, diuretic, anti-rheumatic, antibiotic, or anti-diabetes folk medicines (Brandão et al., 1998). Previous studies on the genus *Bidens* reported the isolation of chalcones (Redl et al., 1993), phenylpropanoid glucosides (Sashida et al., 1991), polyacetylenes (Brandão et al., 1997; Chang et al., 2000; Redl et al., 1994; Ubillas et al., 2000), a diterpene (Zulueta et al., 1995), flavonoids (Wang et al., 1997a, 1997b; Sarker et al., 2000), and flavone glycosides (Brandão et al., 1998). Bioactivities of *Bidens pilosa* plant extracts, including anti-hyperglycemic (Ubillas et al., 2000), anti-hypertensive (Dimo et al., 2001; Dimo et al., 2002), anti-ulcerogenic (Tan et al., 2000), hepatoprotective (Chih et al., 1996), immunosuppressive and anti-inflammatory (Jager et al., 1996; Pereira et al., 1999), anti-leukemic (Chang et al., 2001a), anti-malarial (Brandão et al., 1997), anti-bacterial (Rabe and vanStaden, 1997), and anti-microbial (Khan et al., 2001) effects have been reported. However, the antioxidative benefits of *Bidens pilosa* have not been studied to date.

In this study, we investigated the scavenging activities for free radicals and superoxide anions, inhibition of NO production from LPS-stimulated macrophage RAW 264.7 cells, and phytochemical characteristics of *Bidens pilosa* extracts. Purification of a BuOH fraction, by column chromatography, resulted in the isolation of one new compound, heptanyl 2-*O*-β-xylofuranosyl-(1→6)-β-glucopyranoside (**1**), together with eight known phenolic compounds (**2–9**) that have been isolated for the first time from *Bidens pilosa* (Fig. 1). The chemical profile of the BuOH fraction, which exhibited significant antioxidative activities, was also determined using the analyses of HPLC and LC/MS.

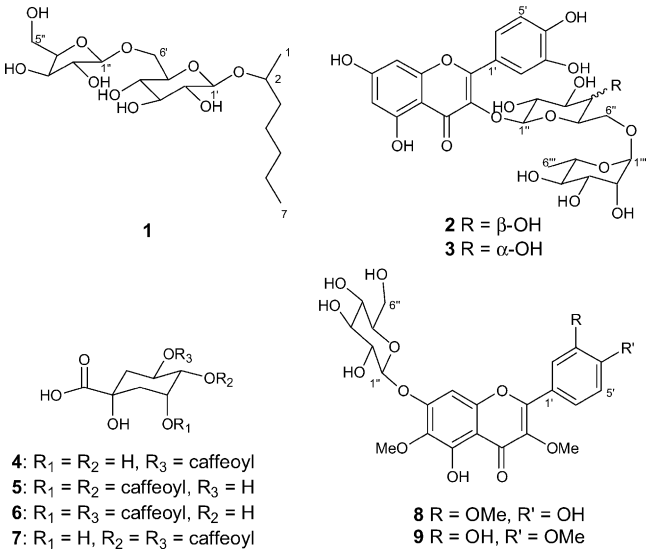

Fig. 1. The structures of compounds isolated from the BuOH fraction of *Bidens pilosa*.

2. Materials and methods

2.1. Plant materials and chemicals

Bidens pilosa Linn. var. *radiata* (Compositae), as authenticated by Dr. Sheng-Yang Wang, Institute of BioAgricultural Sciences, Academia Sinica, was collected on the campus of Academia Sinica, Taiwan, in 2001, and plant specimens were deposited as BP001 in Institute of BioAgricultural Sciences, Academia Sinica. All plant materials chemicals and reagents, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), caffeic acid, chlorogenic acid, quercetin, α-tocopherol, Vitamin C, and lipopolysaccharide (LPS) were purchased from Sigma (St. Louis, MO, USA). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech. All other chemicals and solvents used in this study were of reagent or HPLC grade.

2.2. Plant extracts preparation and analysis

The fresh whole plant of *Bidens pilosa* was crushed to give 2.5 kg of raw material, which was extracted with 25 L of 70% ethanol at room temperature. The total crude extract was evaporated in vacuo to yield a residue (130 g) that was then suspended in water (1 L) and successively partitioned with ethyl acetate (1 L × 3 times) and *n*-butanol (1 L × 3 times), yielding EA, BuOH, and water fractions. Each fraction was evaporated on a rotary evaporator, under reduced pressure, to remove organic solvent and then lyophilized until dry and weighed to determine the yields. The yields of the EA, BuOH and water fractions were 14.1, 15.2, and 69.0%, respectively, of the total crude extract in dry weight. The BuOH fraction was chromatographed over a RP-18 silica gel col-

umn (Cosmosil 75C_{18}-OPN) and TLC analysis of plant extracts was performed on RP-18 F_{254S} TLC aluminium sheets (Merck), with compounds visualized by spraying with 10% (v/v) H_2SO_4.

2.3. Free radical scavenging activity

The scavenging activity for DPPH radicals by plant extracts and phytocompounds from *Bidens pilosa* was measured according to the method reported by Chang et al. (2001b) with minor modifications. Compounds, namely ascorbic acid, α-tocopherol, and quercetin, were used as positive controls in this study. Assays were performed in 300 μL reaction mixtures, containing 200 μL of 0.1 mM DPPH–ethanol solution, 90 μL of 50 mM Tris–HCl buffer (pH 7.4), and 10 μL of deionized H_2O (as control) or test plant extracts. After 30 min of incubation at room temperature, absorbance (540 nm) of the reaction mixtures was taken by a plate reader (Labsystems Multiskan MS). The inhibitory effect of DPPH was calculated according to the following formula:

$$\text{Inhibition (\%)} = \frac{\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{control}}} \times 100$$

IC_{50} represents the levels at which 50% of the radicals were scavenged by test samples.

2.4. Superoxide scavenging activity

Superoxide anion was generated by xanthine–xanthine oxidase and detected by using the nitroblue tetrazolium (NBT) reduction method (Halliwell, 1985; Kirby and Schmidt, 1997). Twenty microliters of 15 mM Na_2EDTA in buffer (50 mM KH_2PO_4/KOH, pH 7.4), 50 μL of 0.6 mM NBT in buffer, 30 μL of 3 mM hypoxanthine in 50 mM KOH, 5 μL of test samples in ethanol (final concentrations were 1, 2, 5, 10, 25, 50, and 100 μg/mL, respectively), and 145 μL of buffer were mixed in 96-well microplates (Falcon). The reaction was started by adding 50 μL of xanthine oxidase solution in buffer (1 unit in 10 mL of buffer) to the mixture. The reaction mixture was incubated at 25 °C, and the absorbance at 570 nm was determined every 20 s up to 20 min using a plate reader (Labsystems Multiskan MS). The control was 5 μL of ethanol instead of the sample solution. Quercetin was used as a positive control. Results were expressed as a percentage of inhibition relative to the control, given by:

$$\frac{\text{rate of control} - \text{rate of sample reaction}}{\text{rate of control}} \times 100\%$$

2.5. Cell line and cell culture

RAW 264.7 cells, a murine macrophage cell line, were obtained from ATCC and cultured at 37 °C in Dulbecco's modified essential medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator, as recommended by ATCC.

2.6. Nitric oxide (NO) inhibition and cell viability assays

RAW 264.7 cells grown in T150 culture flasks were harvested and seeded in 96-well plates at a density of 2×10^5 cells/well. Adhered cells were incubated for 24 h with (positive control) or without (blank) 1 μg/mL LPS, in the absence or presence of test extracts. Nitrite concentration, as a parameter of NO synthesis, in the culture supernatant of RAW 264.7 cells was measured by the Griess reaction (Schmidt and Kelm, 1996). Briefly, 100 μL cell culture supernatants were reacted with 100 μL of Griess reagent [1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and absorbance was recorded using an ELISA reader (Labsystems Multiskan MS) at 540 nm. Results were expressed as a percentage of inhibition relative to the control (cell treated with LPS alone). In parallel to the Griess assays, RAW 264.7 cells treated with or without the extracts were tested for cell viability using the MTT based colorimetric assay as described elsewhere (Scudiero et al., 1988).

2.7. Compound isolation

The BuOH fraction (15 g) of *Bidens pilosa* was chromatographed over an open column packed with RP-18 silica gel, eluting with a MeOH/H_2O gradient solvent system, to give a total of 13 subfractions (Ba–Bm). A RP-18 HPLC column [Phenomenex Luna 5μ C18 (2), 250 mm × 10 mm] coupled with various solvent systems was employed for further isolation of single compounds. Compound **4** was isolated from the Bb fraction (eluant of 10% MeOH/H_2O from the open column) using 7% acetonitrile (MeCN) in H_2O as the solvent system; compounds **2**, **3**, **5**, and **6** were isolated from the Bd fraction (eluant of 30% MeOH/H_2O from the open column) using 15% MeCN in H_2O as solvent; and compounds **8** and **9** were isolated from the Bf fraction (eluant of 50% MeOH/H_2O from the open column) using 25% MeCN in H_2O as solvent. Compounds **1** and **7** were isolated from the Be fraction (eluant of 40% MeOH/H_2O from the open column) by a Sephadex LH-20 column chromatography using MeOH as a solvent system.

2.8. General methods for compound structure elucidation

The structures of compounds **1–9** were elucidated using spectroscopic analysis. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 983G spectrophotometer. ^1H and ^{13}C NMR spectra were run on a Varian Unity Plus 400 spectrometer. EIMS were obtained on a JEOL JMS-HX 300 mass spectrometer. FABMS were obtained on a JEOL JMS-H110 mass spectrometer. Melting points were determined with a

Yanagimoto micromelting point apparatus and are uncorrected.

*2.8.1. Heptanyl 2-O-β-xylofuranosyl-(1→6)-β-glucopyranoside (**1**)*

Colorless oil; IR ν_{max}: 3380, 1072, 1010 cm^{-1}; $[\alpha]_D^{35}$ −22.2° ($CHCl_3$, *c* 0.5); ^1H- and ^{13}C-NMR data shown in Table 1; FABMS *m/z* (rel intensity): 433 $[M + Na]^+$ (100); HRFABMS *m/z*: 433.2041 (calcd for $C_{18}H_{34}O_{10}Na$, 433.2050).

*2.8.2. Quercetin 3-O-rabinobioside (**2**)*

Pale yellow solid, mp 193–194 °C; IR ν_{max}: 3399, 1659, 1607, 1204, 1060, 813 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 633 $[M + Na]^+$ (46), 611 $[M + H]^+$ (100), 465(36), 303(62); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Rastrelli et al., 1995).

*2.8.3. Quercetin 3-O-rutinoside (**3**)*

Pale yellow solid, mp 192–194 °C; IR ν_{max}: 3378, 1657, 1608, 1203, 1065, 809 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 633 $[M + Na]^+$ (27), 611 $[M + H]^+$ (100), 465(31), 303(41); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Rastrelli et al., 1995; Price et al., 1998).

*2.8.4. Chlorogenic acid (**4**)*

Amorphous yellow solid; IR ν_{max}: 3369, 1725, 1692, 1605, 1280, 1182, 978, 814 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 355 $[M + H]^+$ (100), 163(28), 149(41); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Lin et al., 2000).

*2.8.5. 3,4-Di-O-caffeoylquinic acid (**5**)*

Amorphous yellow solid; IR ν_{max}: 3336, 1701, 1607, 1283, 1120, 981, 814 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 517 $[M + H]^+$ (39), 499 $[M + H - H_2O]^+$ (100); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Tatefuji et al., 1996).

*2.8.6. 3,5-Di-O-caffeoylquinic acid (**6**)*

Amorphous yellow solid; ESI-MS *m/z* (rel intensity, positive ion mode): 517 $[M + H]^+$ (25), 499 $[M + H - H_2O]^+$ (100); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Tatefuji et al., 1996).

*2.8.7. 4,5-Di-O-caffeoylquinic acid (**7**)*

Amorphous yellow solid; IR ν_{max}: 3394, 1696, 1603, 1265, 1163, 980, 815 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 517 $[M + H]^+$ (37), 499 $[M + H - H_2O]^+$ (100); ^1H and ^{13}C NMR data (CD_3OD), in good agreement with the published data (Tatefuji et al., 1996).

*2.8.8. Jacein (**8**)*

Yellow solid, mp 203–205 °C; IR ν_{max}: 3369, 1653, 1602, 1074, 807 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 523 $[M + H]^+$ (100), 361 (25); ^1H NMR (CD_3OD, 400 MHz): aglycon signals, δ 3.81, 3.88, 3.93 (each 3 H, s, 3-OMe, 6-OMe, 3′-OMe, respectively), 6.93 (1H, d, *J* = 8.4 Hz, H-5′), 6.93 (1H, s, H-8), 7.66 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 7.72 (1H, d, *J* = 2.0 Hz, H-2′); sugar signals, δ 3.39 (1H, dd, *J* = 9.6, 8.8 Hz, H-4″), 3.50 (1H, t, *J* = 8.8 Hz, H-3″), 3.56 (1H, m, H-5″), 3.56 (1H, dd, *J* = 8.8, 8.0 Hz, H-2″), 3.69 (1H, dd, *J* = 12.0, 6.0 Hz, H_a-6″), 3.93 (1H, dd, *J* = 12.0, 2.0 Hz, H_b-6″), 5.10 (1H, d, *J* = 8.0 Hz, H-1″). ^{13}C NMR (CD_3OD, 100 MHz): aglycon signals, δ 56.6 (3′-OMe), 60.6 (3-OMe), 61.5 (6-OMe), 95.8 (C-8), 108.2 (C-10), 113.1 (C-2′), 116.5 (C-5′), 122.7 (C-1′), 124.0 (C-6′), 133.9 (C-6), 139.6 (C-3), 149.0 (C-3′), 151.4 (C-4′), 153.4 (C-5), 153.8 (C-9), 157.9 (C-7), 158.6 (C-2); sugar signals, δ 62.6 (C-6″), 71.4 (C-4″), 74.8 (C-2″), 78.0 (C-3″), 78.6 (C-5″), 102.1 (C-1″).

*2.8.9. Centaurein (**9**)*

Yellow solid, mp 208-209 °C; IR ν_{max}: 3396, 1655, 1605, 1271, 1074, 809 cm^{-1}; ESI-MS *m/z* (rel intensity, positive ion mode): 523 $[M + H]^+$ (100), 361 (26); ^1H NMR (CD_3OD, 400 MHz): aglycon signals, δ 3.80, 3.88, 3.93 (each 3 H, s, 3-OMe, 6-OMe, 4′-OMe, respectively), 6.89 (1H, s, H-8), 7.05 (1H, d, *J* = 8.4 Hz, H-5′), 7.61 (1H, d, *J* = 2.4 Hz, H-2′), 7.65 (1H, dd, *J* = 8.4, 2.4 Hz, H-6′); sugar signals, δ 3.41 (1H, dd, *J* = 9.6, 8.8 Hz, H-4″), 3.52 (1H, t, *J* = 8.8 Hz, H-3″), 3.56 (1H, m, H-5″), 3.56 (1H, dd, *J* = 8.8, 7.2 Hz, H-2″), 3.71 (1H, dd, *J* = 12.0, 6.0 Hz, H_a-6″), 3.94 (1H, dd, *J* = 12.0, 2.0 Hz, H_b-6″), 5.11 (1H, d, *J* = 7.2 Hz, H-1″). ^{13}C NMR (CD_3OD, 100 MHz): aglycon signals, δ 56.4 (3′-OMe), 60.6 (3-OMe), 61.5 (6-OMe), 95.6 (C-8), 108.2 (C-10), 112.4 (C-2′), 116.3 (C-5′), 122.4 (C-6′), 124.1 (C-1′), 133.9 (C-6), 139.9 (C-3), 147.7 (C-3′), 151.9 (C-4′), 153.4 (C-5), 153.8 (C-9), 157.9 (C-7), 158.3 (C-2); sugar signals, δ 62.6 (C-6″), 71.3 (C-4″), 74.8 (C-2″), 78.0 (C-3″), 78.5 (C-5″), 102.0 (C-1″).

2.9. LC/MS analysis

LC/MS was employed to analyze the metabolite profiling of the BuOH fraction of *Bidens pilosa*. LC/MS was carried out using a ThermoFinnigan LCQ Advantage ion trap mass spectrometer (California, USA) equipped with an Agilent 1100 series liquid chromatograph at the LC/MS core facility of the Institute of BioAgricultural Sciences, Academia Sinica, Taiwan. The BuOH fraction of *Bidens pilosa* was directly analyzed by the LC/MS instrument with a 5 μL sample injection (1 mg/mL) after a solid phase extraction (SPE) with a C18-E cartridge (Phenomenex). The HPLC profile of the BuOH fraction was performed using a RP-18 column [Phenomenex Luna 3μ C18 (2), 150 mm × 2.0 mm] at a flow rate of 0.2 mL/min and measured by a UV detector at 330 nm. The solvent gradient for HPLC was 0.05% TFA/acetonitrile (solvent B) in 0.05% TFA/H_2O (solvent A): 10–11% from 0 to 10 min, 11–19% from 10 to 15 min,

Table 1
NMR data for compound **1** (100 and 400 MHz in $CDCl_3$, *J* in Hz)

Position	δ_C, mult	δ_H	HMBC	COSY	NOESY
1	22.1 q	1.21 d (6.0)		H-2	H-2, H_a-3, H-1′
2	77.8 d	3.80[a]	H_3-1, H_2-3, H-1′	H_3-1, H_2-3	H_3-1, H_2-3, H-1′
3	37.7 t	1.42 m (H_a)	H_3-1	H-2, H_2-4	H_3-1, H-2, H_b-3, H_2-4, H-1′, H-2, H_a-3, H_2-4, H-1′
		1.59 m (H_b)			
4	26.1 t	1.32[a]	H_b-3	H_2-3	H_2-3
5	33.2 t	1.32[a]	H_2-4, H_2-6, H_3-7		
6	23.7 t	1.32[a]	H_3-7	H_3-7	H_3-7
7	14.4 q	0.90 t (6.8)	H_2-6	H_2-6	H_2-6
Glu 1′	104.1 d	4.31 d (8.0)	H-2, H-2′	H-2′	H_3-1, H-2, H_2-3, H-2′, H-3′, H-5′
2′	75.3 d	3.15 dd (9.6, 8.0)	H-1′	H-1′, H-3′	H-1′, H-4′
3′	78.0 d	3.34[a]	H-1′	H-2′	H-1′
4′	71.7 d	3.34[a]	H-2′, H-5′, H_b-6′	H-5′	H-2′, H-5′, H_2-6′
5′	76.7 d	3.40[a]	H_a-6′	H-4′, H_2-6′	H-1′, H-4′, H_2-6′
6′	69.5 t	3.72 dd (11.2, 5.6, H_a)	H-1″	H-5′, H_b-6′	H-4′, H-5′, H_b-6′, H-1″
		4.06 dd (11.2, 2.4, H_b)		H-5′, H_a-6′	H-4′, H-5′, H_a-6′
Xyl 1″	105.0 d	4.32 d (6.4)	H_2-6′, H-2″, H-3″	H-2″	H_a-6′, H-2″, H-3″, H-4″
2″	72.3 d	3.59 dd (8.8, 6.4)	H-3″	H-1″	H-1″
3″	74.1 d	3.52[a]	H-2″, H-4″	H-4″	H-1″
4″	69.4 d	3.80[a]	H_2-5″	H-3″	H-1″, H_2-5″
5″	66.5 t	3.52[a] (H_a)		H-4″, H_b-5″	H-4″, H_b-5″
		3.85 dd (12.0, 3.6, H_b)		H-4″, H_a-5″	H-4″, H_a-5″

[a] Data obtained from HMQC spectrum.

19–21% from 15 to 35 min, 21–28% from 35 to 47 min, 28–100% from 47 to 55 min, maintained at 100% from 55 to 57 min, 100–10% from 57 to 60 min, and re-equilibration with 10% from 60 to 62 min. Positive ion electrospray ionization (ESI) was used for the detection of the analytes without solvent splitting. Conditions for MS analysis of each HPLC peak included a capillary voltage 4.5 kV, a sheath gas flow rate at 40 arbitrary units, an auxiliary gas flow rate at 20 arbitrary units, and the ion transfer capillary temperature at 300 °C.

3. Results

3.1. Radical scavenging activities of Bidens pilosa extracts

Radical scavenging activities of the total crude extract and its derived fractions of *Bidens pilosa* were assessed by DPPH and NBT/hypoxanthine superoxide assays. The magnitude of the scavenging activities for DPPH radicals (IC_{50}, μg/mL) from the total crude extract and sub-fractions were in the following order: quercetin (1.98 μg/mL) > ascorbic acid (6.34 μg/mL) > α-tocopherol (8.97 μg/mL) > EA fraction (13.83 μg/mL) > BuOH fraction (16.69 μg/mL) > total crude extract (80.93 μg/mL) > water fraction (>100 μg/mL) (Fig. 2). Superoxide scavenging activities (IC_{50}, μg/mL) were in the following order: quercetin (1.49 μg/mL) > BuOH fraction (11.43 μg/mL) > EA fraction (59.73 μg/mL) > total crude extract (98.25 μg/mL) and water fraction (>100 μg/mL) (Fig. 3). These results demonstrated that the EA and BuOH fractions exhibit significant radical scavenging activities.

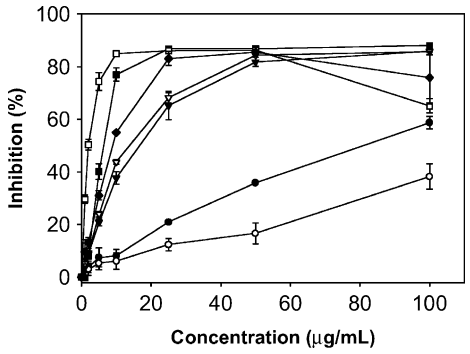

Fig. 2. Free-radical scavenging activities of total crude extract and derived subfractions of *Bidens pilosa* measured using the DPPH assay. (●) total crude extract; (▽) EA fraction; (▼) BuOH fraction; (◯) water fraction; (■) ascorbic acid; (□) quercetin; (♦) α-tocopherol. Results are mean ± S.D. (*n* = 3).

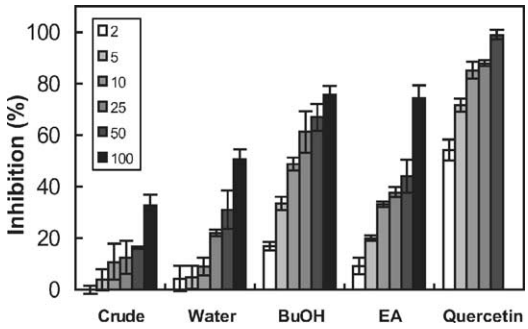

Fig. 3. Superoxide scavenging activities of total crude extract, EA, BuOH and water fractions of *Bidens pilosa* measured using the NBT assay at various concentrations (μg/mL) as indicated. Quercetin was used as the positive control in this experiment. Results are mean ± S.D. (*n* = 3).

3.2. Inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells

The effects of total crude extract and derived fractions from *Bidens pilosa* on NO synthesis in RAW 264.7 macrophages were now investigated. As shown in Fig. 4A, EA and BuOH fractions exhibited significant dose-dependent inhibition of nitrite production. Amongst the test extracts, the EA fraction exhibited the most significant inhibitory activity (IC_{50} = 36.2 μg/mL) with the BuOH fraction exhibiting comparatively moderate activity (IC_{50} = 250.8 μg/mL). However, a high concentration of the EA fraction had a strong cytotoxic effect on RAW 264.7 cells (Fig. 4B). Less than 30% of tested cells were detected as viable after treatment with 200 μg/mL EA fraction for 24 h. In contrast, no or little cytotoxic effects were observed from the treatment of total crude extract, BuOH, or water fraction to RAW 264.7 cells at the same dosage (Fig. 4B). In fact, more than 90% of LPS-stimulated cells were viable when treated with the BuOH fraction at concentrations up to 500 μg/mL. These results indicate that the discrepancy for anti-RAW 264.7 macrophages proliferation by the EA and BuOH fractions may be due to the difference in compound constituents in these two fractions. The polyacetylene compounds may contribute to the cytotoxic effect of EA fraction (data not shown, Brandão et al., 1997; Chang et al., 2000; Redl et al., 1994; Ubillas et al., 2000). The BuOH fraction of *Bidens pilosa* with little concerns regarding cytotoxicity at cellular level was further systematically investigated for its phytochemical characteristics.

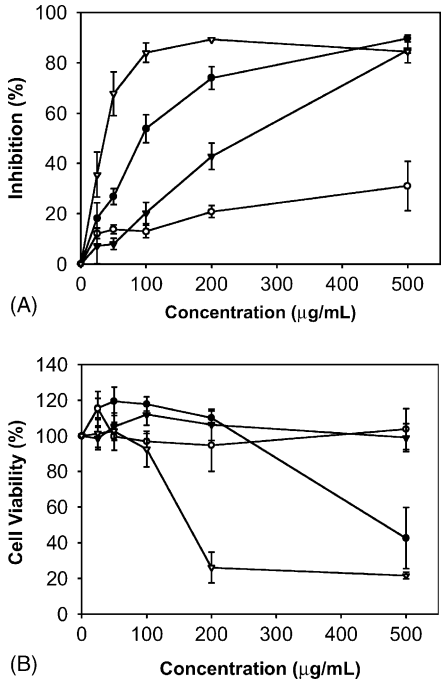

Fig. 4. Dose-response curves for inhibition of nitrite production (A) and cell viability (B) by total crude extract and derived subfractions of *Bidens pilosa* in LPS-activated RAW 264.7 macrophages. (●) Total crude extract; (▽) EA fraction; (▼) BuOH fraction; (○) water fraction. Results are mean ± S.D. (n = 3).

3.3. Compound characteristics in the BuOH fraction of Bidens pilosa

The major constituents in the BuOH fraction were isolated using several repeated procedures of RP-18 silica gel high performance liquid column chromatography. The structure of one new compound, heptanyl 2-*O*-β-xylofuranosyl-(1→6)-β-glucopyranoside (**1**), along with eight known compounds, quercetin 3-*O*-rabinobioside (**2**), quercetin 3-*O*-rutinoside (**3**), chlorogenic acid (**4**), 3,4-di-*O*-caffeoylquinic acid (**5**), 3,5-di-*O*-caffeoylquinic acid (**6**), 4,5-di-*O*-caffeoylquinic acid (**7**), jacein (**8**), and centaurein (**9**) (Fig. 1) were determined mainly using IR, MS, and NMR analyses. None of these compounds have been previously reported from *Bidens pilosa*. In summary, the structures of compounds **2–8** were elucidated from their ^{1}H and ^{13}C NMR spectra with the aid of HMQC, HMBC, COSY, and NOESY experiments in this study and, in parallel, by comparison with existing data from previous reports (Flamini et al., 2001; Lin et al., 2000; Price et al., 1998; Rastrelli et al., 1995; Tatefuji et al., 1996). The complete assignments of compounds **2**, **3**, and **8** were performed because the ^{13}C NMR data of compounds **2** and **3** were slightly different here from that published previously (Rastrelli et al., 1995; Price et al., 1998); full assignments of NMR data in CD_3OD regarding compound **8** was not available previously (Flamini et al., 2001). In addition, due to lack of reported NMR spectral data for compound **9** and we observed in our preliminary studies that the chemical structure of compound **9** was very similar to that of jacein (**8**), therefore, we compared the ^{1}H and ^{13}C NMR data of **9** with those known for jacein (**8**). Our results suggest that **9** possesses the same skeletal structure as that of **8** with a minor difference on the C ring. NOESY correlations between δ_H 3.93 (4′-OMe) and 7.05 (H-5′) demonstrated that the methoxy group on the C ring of compound **9** is located at C-4′, whereas the methoxy group on the C ring of compound **8** is located at C-3′.

On the basis of data obtained from high-resolution fast atom bombardment mass spectrometry (HRFABMS) and ^{13}C NMR, the molecular formula of compound **1** is $C_{18}H_{34}O_{10}$, indicating two indices of hydrogen deficiency (IHD). The IR spectrum indicated the presence of hydroxyl groups (3300 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) exhibited signals for a doublet methyl group [δ_H 1.21 (d, J = 6.0 Hz, H_3-1)], a triplet methyl group [δ_H 0.90 (t, J = 6.8 Hz, H_3-7)], a multiplet signal for the H_2-3 at δ_H 1.59 and 1.42, and complex absorption for the H_2-4, H_2-5, and H_2-6 centered at δ_H 1.32 forming a broad singlet. The down field region showed eight oxymethine protons [δ_H 3.1–3.8 (H-2, H-2′, H-3′, H-4′, H-5′, H-2″, H-3″, and H-4″)], four oxymethylene protons [δ_H 3.52 and 3.85 (H_2-5″); δ_H 3.72 and 4.06 (H_2-6′)], and two anomeric protons [δ_H 4.31 (d, J = 8.0 Hz, H-1′) and 4.32 (d, J = 6.4 Hz, H-1″)]. The ^{13}C NMR spectrum (Table 1) exhibited 18 signals (2 CH_3, 6 CH_2, and 10 CH)

including 10 oxygenated carbons and 2 anomeric carbons (δ_C 104.1 and 105.0). Heptanyl 2-*O*-β-D-glucopyranoside isolated from *Lessingia glandulifera* (Jolad et al., 1988) has similar ^{1}H NMR data to that of **1** on the aglycone moiety. From the molecular formula and above evidence, compound **1** was considered to consist of a heptanyl, a glucosyl, and a pentose unit. The arrangement of the oligosaccharide moiety was determined using a combination of long-range ^{13}C–^{1}H correlations (HMBC), two-dimensional ^{1}H correlations (COSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY). The COSY experiments allowed the sequential assignment (Table 1) of most resonance's for each sugar ring from anomeric proton signals. The long-range ^{13}C–^{1}H correlations, observed as C-2/H-1′, C-1′/H-2, C-6′/H-1″ and C-1″/H$_2$-6′, established the sequential arrangement of the disaccharide moiety (Fig. 5A). NOESY correlations (Fig. 5B) for H-1′/H$_3$-1, H-2, H$_2$-3, H-3′, H-5′ and H-1″/H$_2$-6′, H-3″, H-4″ established the β-xylofuranosyl and β-glucopyranosyl moieties. Thus, the structure of **1** was heptanyl 2-*O*-β-xylofuranosyl-(1→6)-β-glucopyranoside. However, the absolute configuration of the aglycone center was not determined.

3.4. Free radical scavenging activity of the secondary metabolites isolated from Bidens pilosa

To evaluate the contribution of the identified compounds in antioxidative activity of BuOH fraction, free radical scavenging activity of the individual phyto-compounds were characterized. The results in Fig. 6 show that quercetin 3-*O*-rabinobioside (**2**), quercetin 3-*O*-rutinoside (**3**), chlorogenic acid (**4**), 4,5-di-*O*-caffeoylquinic acid (**5**), and 3,4-di-*O*-caffeoylquinic acid (**7**) possess significant DPPH-radical scavenging activities. The IC$_{50}$ values of these compounds are in the range of 3–7 μM, whereas heptanyl 2-*O*-β-xylofuranosyl-(1→6)-β-glucopyranoside (**1**), jacein (**8**) and centaurin (**9**) have little or no effect in scavenging DPPH radicals even at concentrations of 30 μM (Table 2). The IC$_{50}$ for quercetin and caffeic acid, as reference controls in this experiment, were determined as 2.56 and 8.9 μM, respectively.

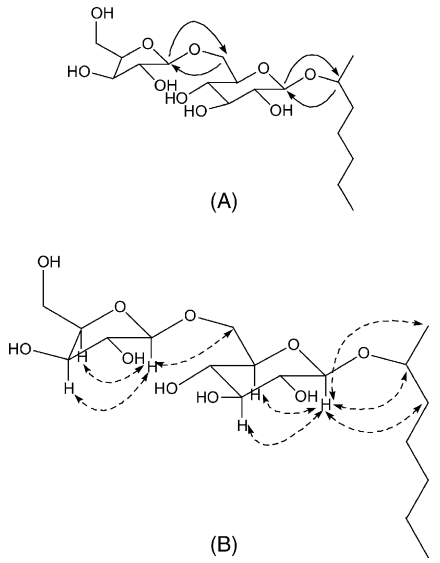

Fig. 5. Selected HMBC (A) and NOESY (B) correlations of **1**.

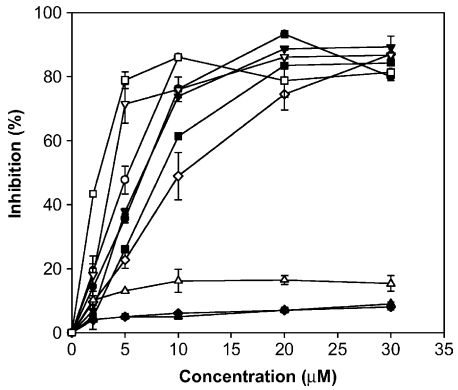

Fig. 6. Comparison of antioxidant activities of phytocompounds isolated from *Bidens pilosa*, as measured by the DPPH radical scavenging assay. Heptanyl 2-*O*-β- xylofuranosyl-(1→6)-β-glucopyranoside, **1** (♦); quercetin 3-*O*-rabinobioside, **2** (○); quercetin 3-*O*-rutinoside, **3** (●); chlorogenic acid, **4** (◊); 3,4-di-*O*-caffeoylquinic acid, **5** (▼); 4,5-di-*O*-caffeoylquinic acid, **7** (▽); jacein, **8** (▲); centaurin, **9** (△); caffeic acid (■); and quercetin (□).

3.5. Application of LC/MS in analysis of metabolite profiling of plant extracts

In this study, LC/MS was employed to analyze the metabolite profile and major constituents of the BuOH fraction of *Bidens pilosa*. The overall metabolite profile of the BuOH fraction, as determined using reverse phase HPLC, is shown in Fig. 7A. Trifluoroacetic acid (TFA) was added to the mobile phase of the HPLC analysis to suppress potential on-column ionic dissociation of pytocompounds of *Bidens pilosa*, as most of the compounds were determined to contain phenolic hydroxyl groups, which are in acidic forms in solution. The acidity of the mobile phase suppressed negative ion formation and facilitated the formation of protonated molecules;

Table 2
IC$_{50}$ values for DPPH radicals of compounds isolated from *Bidens pilosa*[a]

Compound	DPPH radical IC$_{50}$ (μM)
Heptanyl 2-*O*-*β*-xylofuranosyl- (1→6)-*β*-glucopyranoside (**1**)	ND
Quercetin 3-*O*-rabinobioside (**2**)	5.29
Quercetin 3-*O*-rutinoside (**3**)	6.77
Chlorogenic acid (**4**)	10.45
3,4-Di-*O*-caffeoylquinic acid (**5**)	3.29
4,5-Di-*O*-caffeoylquinic acid (**7**)	3.79
Jacein (**8**)	ND
Centaurin (**9**)	ND
Caffeic acid[b]	8.90
Quercetin[b]	2.56

ND: not determined.
[a] Each assay was performed in triplicate.
[b] Quercetin and caffeic acid were used as reference compound in this experiment.

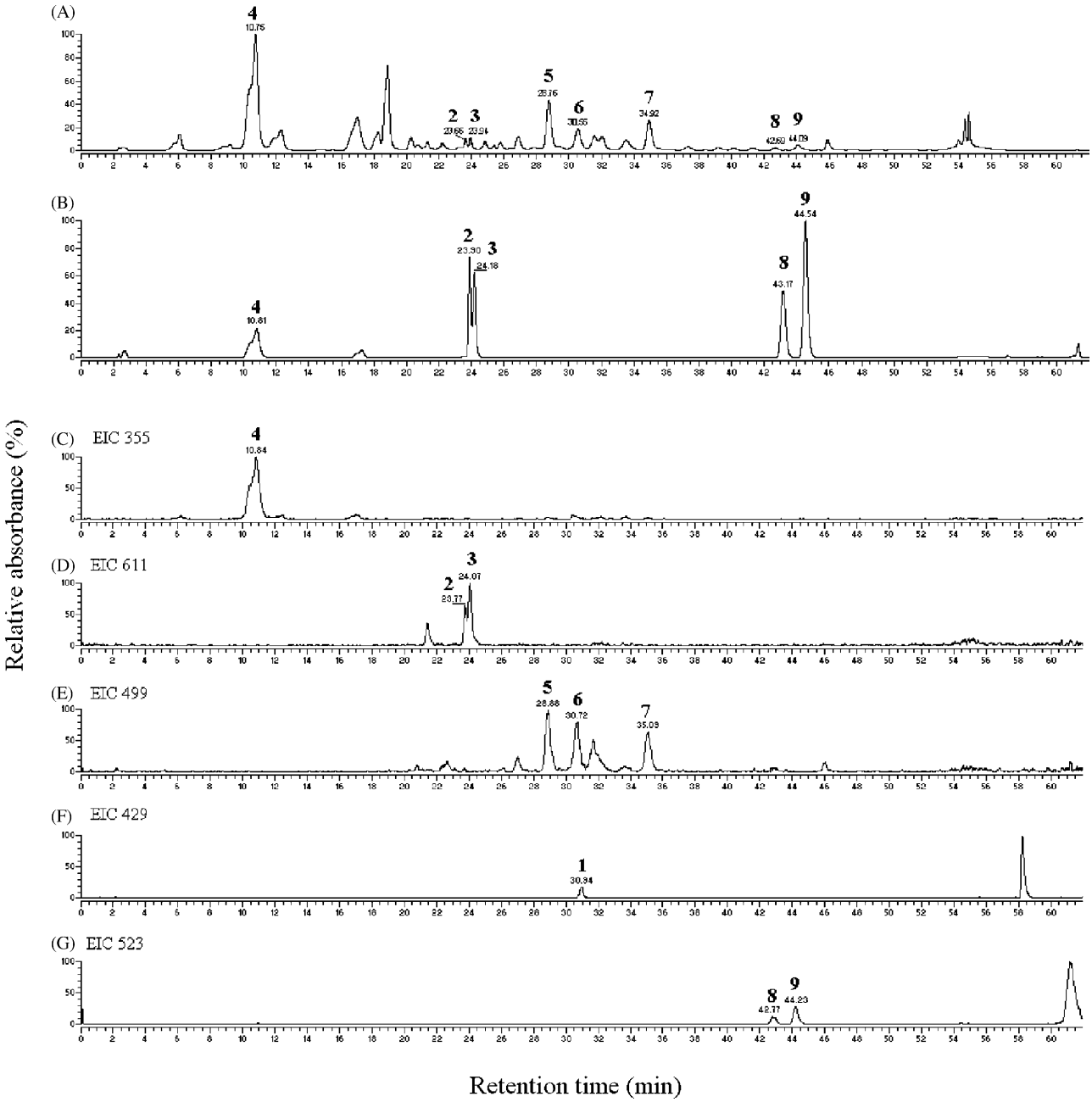

Fig. 7. HPLC chromatograms: (A) Metabolite profile of the BuOH fraction from *Bidens pilosa* at 330 nm; (B) Profile of a mixture of compounds **1**, **2**, **3**, **4**, **8**, and **9** at 330 nm. LC/MS profile of specific phytocompounds isolated from the BuOH fraction responded at *m/z* 355 (C), *m/z* 611 (D), *m/z* 499 (E), *m/z* 429 (F), and *m/z* 523 (G) in a LCQ Advantage ion trap mass spectrometer.

therefore, positive ion mode was used for all mass spectral analyses. A mixture of the isolated compounds **1**, **2**, **3**, **4**, **8**, and **9**, was further subjected to the HPLC procedure as used previously. Fig. 7B shows the specific corresponding peaks and retention times of these compounds to those in the overall metabolite profile (Fig. 7A). The profiles of the mass spectra respective to the specific compounds in the BuOH fraction were further characterized using their pseudo molecular weights after mass spectrometer analyses electrospray ionization at *m/z* 355 ($[M + H]^+$; T_R = 10.8 min, compound **4**, Fig. 7C), *m/z* 611 ($[M + H]^+$; T_R = 23.8 and 24.1 min for compounds **2** and **3**, respectively, Fig. 7D), *m/z* 499 ($[M + H - H_2O]^+$; T_R = 28.9, 30.7, and 35.1 min for compounds **5**, **6**, and **7**, respectively, Fig. 7E), *m/z* 429 ($[M + H + H_2O]^+$; T_R = 30.9 min for compound **1**, Fig. 7F), and *m/z* 523 ($[M + H]^+$; T_R = 42.8 and 44.2 min for compounds **8** and **9**, respectively, Fig. 7G). Due to structural instability, upon storage at room temperature, the corresponding peaks of compounds **5**, **6**, and **7** in the HPLC profile were identified by tracing their mass spectrometric responses at *m/z* 499 and comparing these with the HPLC profiles of the Be and Bf subfractions (data not shown) derived from the total BuOH fraction, as

described in Section 2. These results demonstrated that the major compounds isolated in this study could then be relatively easily identified from the plant extracts of *Bidens pilosa* using LC/MS data.

The content of chlorogenic acid (**4**) was quantatively determined in this study for use as a candidate index compound of the specific BuOH fraction from *Bidens pilosa*, which exhibits strong antioxidative activities. A standard calibration curve (peak area versus concentrations) was prepared for chlorogenic acid ranging from 20 to 100 μg/mL. The compound absorbance at λ = 330 nm increased linearly against the tested concentrations. The amount of chlorogenic acid (**4**) in the BuOH fraction was hence determined as 2.99% (w/w) the dry weight of the fraction.

4. Discussion and conclusions

Phenolic compounds are one of the most diverse groups of secondary metabolites in edible plants. Epidemiological studies have indicated that naturally occurring phenolic compounds may play an important role in the maintenance of human health and prevention of several diseases (Rose and Kasum, 2002). This is also believed to be correlating to the anti-oxidative properties of phenolics. In particular, flavonoids, a group of phenolic compounds with diverse chemical structure and characteristics, have a broad spectrum of antioxidant properties. It has been previously reported that the presence of hydroxyl substituents on a flavonoid nucleus enhances the inhibition on lipid peroxidation, whereas substitution by methoxy groups or glycogens diminishes the antioxidant activity (Arora et al., 1998). Based on structure and activity relationship analysis, we observed that the substitution of the C3 hydroxyl group with glycosides, for instance in the cases of quercetin 3-*O*-rabinobioside (**2**) and quercetin 3-*O*-rutinoside (**3**), resulted in approximately a 2-fold increase in IC_{50} values for DPPH radicals relative to that of quercetin, containing only free form of hydroxyl groups in the C3 position. Jacein (**8**) and centaurein (**9**) possessing substituted groups, either glycoside or methoxy, at their C3, C6, C7, C3′ and/or C4′ positions, exhibit very low free radical scavenging activities in comparison to compounds **2**, **3** or quercetin (Table 2).

The antioxidative activity of polyphenols is generally ascribed to their hydroxyl groups. The presence of two hydroxyl groups in the *ortho* and *para* positions of phenolic compounds is known to increase anti-oxidative activity due to additional resonance stabilization and *ortho*- or *para*-quinone formation (Cuvelier et al., 1992; Graf, 1992). For instance, caffeic acid contains *para*- and *ortho*-hydroxyl groups exhibiting potent antioxidant activity (IC_{50} for DPPH = 8.9 μM) as characterized in this study (Table 2) and elsewhere (Chen and Ho, 1997). Our results show that compounds **5** and **7**, containing two caffeoyl moieties, exhibit stronger (2.3- to 3.2-fold decrease in IC_{50} values) antioxidative activity than caffeic acid, indicating the direct contribution of the free hydroxyl groups in these compounds. Chlorogenic acid, an ester of caffeic acid with quinic acid, exhibits significant, however, a less activity for scavenging DPPH radicals (IC_{50} = 10.45 μM) relative to that of caffeic acid (Table 2). Although slight difference in scavenging free radical activities were observed for compounds of caffeoyl derivatives (**4**, **5**, and **7**) and flavonoid glycosides (**2** and **3**), these compounds can be considered as potent antioxidants, as the IC_{50} values for these compounds were all detected as <10 μM, and play important roles in the observed antioxidant activities of *Bidens pilosa* extract.

LC/MS experimental system was employed in this study to aid quality control measures for the lot-to-lot preparation of plant extract(s), before or in parallel with bioactivity assays. In addition, the HPLC chromatogram and the mass profile of specific chemical constituents in the plant extract (shown in Fig. 7) provide means for characterization of compound identity in a mixture of plant compounds. None of the phenolic compounds identified here have been previously reported from *Bidens pilosa* and they can serve as candidate indexing compounds in the active BuOH fraction of the plant.

Bioactivities of caffeoylquinic acid derivatives have been previously reported, for instance, they enhance the spread and mobility of murine macrophages (Tatefuji et al., 1996), exhibit hepatoprotective activity in CCl_4-induced liver cell injury in cultured rat hepatocytes (Basnet et al., 1996), and potently and selectively inhibit human immunodeficiency virus type 1 (HIV-1) integrase (King et al., 1999). Several medicinal properties of flavonoids, for instance, antioxidant activity, anti-inflammatory activity and the ability to lower the risk of coronary heart disease, have been reported (Simonetti et al., 2001). In this study we observed that plant extracts of *Bidens pilosa* could serve as a good source of caffeoylquinic acid derivatives and flavonoid glycosides. We have also demonstrated that the plant extracts of *Bidens pilosa* exhibited significant antioxidant activity and an inhibitory effect on NO production (an inflammatory mediator) in macrophages. The metabolism and bioavailability of these phenolic compounds in vivo are suggested to be correlating well to their observed antioxidant properties in vitro (Morand et al., 1998; Rose and Kasum, 2002; Simonetti et al., 2001). Therefore, we propose here the potential dietary value and benefits of *Bidens pilosa* extract on the basis of the phytochemical characteristics and the observed bioactive properties. Naturally occurring phytocompounds possessing antioxidative and anti-inflammatory properties appear to contribute to their chemopreventive or chemoprotective activity. Further studies of the plant extracts and/or the identified compounds from *Bidens pilosa* on the pharmacokinetics or mode of action on mechanisms of chemo-preventive properties are warranted.

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