Journal of the Science of Food and Agriculture

J Sci Food Agric **85**:615–621 (2005)
DOI: 10.1002/jsfa.2017

The phenolic constituents and free radical scavenging activities of *Gynura formosana* Kiamnra

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Abstract: *Gynura formosana* Kiamnra (Compositae) is a herbal folk medicine that is a popular vegetable in Taiwan. The free-radical scavenging activities of a 70% aqueous acetone extract from the herb *G formosana* were evaluated. Bioassay-guided fractionation, column separation on Diaion, Toyopearl HW 40(C), Sephadex LH-20 and MCI CHP20P, and high-performance liquid chromatography (HPLC) were used to isolate for the first time in *G formosana* four potent phenolics [caffeic acid (I), quercetin 3-*O*-rutinoside (II), kaempferol 3-*O*-rutinoside (III) and kaempferol 3-*O*-robinobioside (IV)]. The IC_{50} values of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity for compounds I–IV were 6.7, 7.7, 300.3 and 286.7 μM, respectively, and, for superoxide radical scavenging activity, they were 187.3, 25.8, 55.3 and 87.4 μM, respectively. Using a spin trapping electron spin resonance (ESR) method, caffeic acid (I) and quercetin 3-*O*-rutinoside (II) exhibited good hydroxyl radical activity. The free radical scavenging activity of *G formosana* phenolics may improve the economic value of this herb and assist in its development as a health food.

Keywords: free-radical scavenging; *Gynura formosana*; caffeic acid; quercetin 3-*O*-rutinoside; kaempferol 3-*O*-rutinoside; kaempferol 3-*O*-robinobioside

INTRODUCTION

Reactive oxygen species (ROS) are highly reactive molecules; they include the hydroxyl radical (·OH), the superoxide anion radical ($O_2^{\cdot -}$), and hydrogen peroxide (H_2O_2). ROS generate metabolic products that may attack lipids in cell membranes or DNA. Free radical chain reaction processes are associated with several types of biological damage, including DNA damage, carcinogenesis and cellular degeneration related to aging. An imbalance between ROS-generating and -scavenging systems can damage cells. Thus, ROS play an important role in the pathogenesis of human diseases including cancers, arteriosclerosis and cardiovascular diseases.[1] Most ROS are scavenged by endogenous defense systems such as catalase, superoxide dismutase (SOD), and the peroxidase/glutathione system, but these systems are not completely efficient, making it desirable to isolate exogenous antioxidants from natural sources. Various plant sources have been evaluated for antioxidant activity; the ability of components of these plants to remove oxidative stresses is of interest in the development of health foods, nutritional supplements and herbal medicines. Recent studies have also indicated that many plant products, including flavonoids, anthraquinones,[2] tannins, proanthocyanidins and other phenolics, as well as substances derived from fruit, vegetables, and various plant or herbal extracts, have radical-scavenging activity.[3,4] Therefore, these substances could be proposed as health-beneficial products.[5–8]

Gynura formosana Kiamnra (Compositae) is a herbal folk medicine that grows at lower altitudes on the north, south and east coasts of Taiwan. The plant is popular as a vegetable and as a treatment for such diseases as hypertension, diabetes mellitus and cerebral infarction. The literature has reported the presence of several interesting components in plants of the *Gynura* genus, such as pyrrolizidine alkaloids, a terpene coumarin, and several new spirostene derivatives.[9–11] It has also been shown that an ethyl acetate extract of *G formosana* contains an optically active chromanone.[12] However, there are few reported phytochemical examinations of this species. In this study, we isolated the phenolic constituents of *G formosana* and assessed their 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and hydroxyl radical scavenging activities.

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(*Received 14 November 2003; revised version received 29 March 2004; accepted 24 August 2004*)
Published online 26 November 2004

EXPERIMENTAL

Plant material

The herbs of *G formosana* were obtained from a Taipei market in September 2001 and were identified by Mr Mu-Cun Gao, Department of Botany, National Taiwan University. Voucher specimens have been deposited at the Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan.

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), dihydronicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were purchased from Sigma Chemical Co (St Louis, MO, USA). Hydrogen peroxide (H_2O_2) was obtained from Wako (Osaka, Japan). The other chemicals and reagents used in the study were high-grade commercial products.

Extraction, isolation and identification

Fresh herbs (5.0 kg) were extracted with 70% aqueous acetone and the extracts were concentrated under reduced pressure. The 70% aqueous acetone extract (300 g) was chromatographed over Diaion HP-20 gel, eluting with water containing increasing concentrations of methanol and finally with a mixture of acetone–water (7:3) to obtain five fractions (Fr A–Fr E). The DPPH free radical scavenging activity was evaluated to identify the active fractions. Repeated chromatography of the fractions with monitoring of bioactivity and HPLC analysis led to the isolation of active constituents. Fractions B and C were applied to Toyopearl HW-40 (C) columns, and elution was with a gradient solvent system of methanol in H_2O ($H_2O \rightarrow$ 20% MeOH $\rightarrow$ 40% MeOH $\rightarrow$ 60% MeOH $\rightarrow$ 100% MeOH).

Five fractions were obtained from each of the two columns (Fr B-1–Fr B-5 and Fr C-1–Fr C-5, respectively). Fraction B-1 was then applied to a column packed with Sephadex LH-20 (100 μm, Pharmacia Fine Chemicals Co Ltd, Germany) and subjected to semi-preparative HPLC to yield compound **I**. Fraction C-1 was rechromatographed on a MCI CHP 20P column and eluted with a gradient solvent system of methanol in H_2O to give six fractions (Fr C-2-1–Fr C-2-6). Fraction C-2-1 and Fr C-2-6 were purified by semi-preparative HPLC to yield compounds **II, III** and **IV**.

*Caffeic acid (**I**)*
FABMS *m/z* 181 $[M+H]^+$; ^{1}H-NMR (methanol-d_4, 500 MHz), δ 7.46 (*d*, J = 15.8 Hz, H-β), 7.01 (*d*, J = 1.9 Hz, H-2), 6.91 (*dd*, J = 1.9, 8.2 Hz, H-6), 6.76 (*d*, J = 8.15 Hz, H-5), 6.22 (*d*, J = 15.8 Hz, H-α); ^{13}C-NMR (methanol-d_4, 125 MHz), δ 115.0 (C-5), 115.7 (C-2), 116.9 (C-α), 122.6 (C-6), 128.1 (C-1), 146.0 (C-3), 149.2 (C-4), 146.8 (C-β), 171.9 (COOH).[13]

Quercetin 3-O-*rutinoside (rutin) (**II**)*
FABMS *m/z* 611 $[M+H]^+$; ^{1}H-NMR (methanol-d_4, 500MHz), δ 7.66 (1H, *d*, J = 2.1 Hz, H-2′), 7.62 (1H, *dd*, J = 2.1, 8.5 Hz, H-6′), 6.87 (1H, *d*, J = 8.5 Hz, H-5′), 6.40 (1H, *d*, J = 2.1 Hz, H-8), 6.21 (1H, *d*, J = 2.1 Hz, H-6), 5.10 (1H, *d*, J = 7.7 Hz, glc H-1), 4.51 (1H, *d*, J = 1.6 Hz, rha H-1), 3.79 (1H, *dd*, J = 3.7, 10.8 Hz, glc H-6), 3.40 (1H, *dd*, J = 3.0, 10.5 Hz, glc H-6), 1.16 (3H, *d*, J = 6.0 Hz, rha H-6), 3.27–3.80 (sugar proton); ^{13}C-NMR (methanol- d_4, 125MHz), δ 179.4 (C-4), 166.1 (C-7), 163.0 (C-5), 159.4 (C-9), 158.5 (C-2), 150.0 (C-4′), 145.8 (C-3′), 135.6 (C-3), 123.6 (C-1′), 123.1 (C-6′), 117.7 (C-5′), 116.1 (C-2′), 105.6 (C-10), 104.7 (glc C-1), 102.9 (rha C-1), 100.0 (C-6), 94.9 (C-8), 78.2 (glc C-3), 77.3 (glc C-5), 75.7 (glc C-2), 73.9 (glc C-4), 72.3 (rha C-3), 72.1 (rha C-2), 71.4 (rha C-4), 69.7 (rha C-5), 68.6 (glc C-6), 17.9 (rha C-6).[14]

Kaempferol 3-O-*rutinoside (nicotiflorin) (**III**)*
FABMS *m/z* 595 $[M+H]^+$; ^{1}H-NMR (methanol-d_4, 500 MHz), δ 8.06 (2H, *d*, J = 8.9 Hz, H-3′, H-5′), 6.90 (2H, *d*, J = 8.9 Hz, H-2′, 6′), 6.40 (1H, *d*, J = 2.2 Hz, H-8), 6.21 (1H, *d*, J = 2.0 Hz, H-6), 5.12 (1H, *d*, J = 7.4 Hz, glc H-1), 4.53 (1H, *d*, J = 1.6 Hz, rha H-1), 3.80 (1H, *dd*, J = 1.0, 10.4 Hz, glc H-6), 3.38 (1H, *dd*, J = 4.7, 10.8 Hz, glc H-6), 1.11 (3H, *d*, J = 6.2 Hz, rha H-6); 3.25–3.62 (sugar proton); ^{13}C-NMR (methanol- d_4, 125 MHz), δ 179.4 (C-4), 166.0 (C-7), 163.0 (C-5), 161.5 (C-4′), 159.4 (C-9), 158.6 (C-2), 135.5 (C-3), 132.4 (C-2′, C-6′), 122.8 (C-1′), 116.2 (C-3′, C-5′), 105.7 (C-10), 104.6 (glc C-1), 102.6 (rha C-1), 100.0 (C-6), 94.9 (C-8), 78.2 (glc C-3), 77.2 (glc C-5), 75.8 (glc C-2), 73.9 (rha C-4), 72.3 (rha C-3), 72.0 (rha C-2), 71.5 (glc C-4), 69.7 (rha C-5), 68.6 (glc C-6), 17.9 (rha C-6).[15]

Kaempferol 3-O-*robinobioside (**IV**)*
FABMS *m/z* 595 $[M+H]^+$; ^{1}H-NMR (methanol-d_4, 500 MHz), δ 8.09 (2H, *d*, J = 9.0 Hz, H-2′, H-6′), 6.88 (2H, *d*, J = 9.0 Hz, H-3′, 5′), 6.41 (1H, *d*, J = 2.0 Hz, H-8), 6.21 (1H, *d*, J = 2.0 Hz, H-6), 5.03 (1H, *d*, J = 7.8 Hz, gal H-1), 5.33 (1H, *d*, J = 1.8 Hz, rha H-1), 3.71 (1H, *dd*, J = 5.6, 10.2 Hz, gal H-6), 3.38 (1H, *dd*, J = 6.7, 10.2 Hz, gal H-6), 1.17 (3H, *d*, J = 6.2 Hz, rha H-6), 3.49–3.79 (sugar proton); ^{13}C-NMR (methanol- d_4, 125MHz), δ 179.6 (C-4), 166.1 (C-7), 163.0 (C-5), 161.6(C-4′), 159.4 (C-9), 158.6 (C-2), 135.7 (C-3), 132.5 (C-2′, 6′), 122.7 (C-1′), 116.1 (C-3′, 5′), 105.6 (C-10), 105.5 (gal C-1), 101.9 (rha C-1), 100.0 (C-6), 94.9 (C-8), 75.4 (gal C-5), 75.1 (gal C-3), 73.9 (rha C-4), 73.0 (rha C-2), 72.3 (rha C-3), 72.1 (gal C-2), 70.2 (gal C-4), 69.7 (rha C-5), 67.4 (gal C-6), 18.0 (rha C-6).[16]

HPLC analysis

An LC-10A pump (Shimadzu Corporation Chromatographic Instruments Division, Kyoto, Japan) was connected to an SPD-6A ultraviolet spectrophotometric detector set at UV 280 nm. The column

consisted of a Licrospher RP-18e, 10.0 × 250 mm (Merck, Darmstadt, Germany). The solvent systems were 0.05 M H_3PO_4–0.05 M KH_2PO_4–acetonitrile (40:40:20 or 45:45:10). The flow rate was 3.0 ml min^{-1}. The solvent was degassed prior to use.

Gas chromatographic analysis

GC/MS analysis was carried out using a HP-5890/5989B system equipped with a HP-1 capillary column (15 m × 0.25 mm × 0.25 μm). Injector and detector temperatures were 250 and 300 °C, respectively. The temperature program was 60–140 °C (25 °C min^{-1}) and then to 250 °C (10 °C min^{-1}). Helium was used as carrier gas (0.7 bar, 1 ml min^{-1}). The MS was taken at 70 eV. Scanning speed was 0.84 scans s^{-1} and the scanning period was from 40 to 550 s.[17] Sample volume was 3 μl. Split: 1:40.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Free radical scavenging activity

The DPPH radical scavenging activity was evaluated as reported previously.[18] Briefly, each test was conducted by placing a sample and 100 μM DPPH radical in Tris–HCl buffer solution (pH 7.4). After a 20 min incubation period at room temperature in the dark, absorbance was read at 517 nm.

Measurement of superoxide radical scavenging activity

Superoxide radical-scavenging activity was determined by the PMS–NADH superoxide generating system.[19] The tested samples were added to a solution containing 80 μM PMS, 200 μM NBT and 624 μM NADH in phosphate buffer (0.1 M, pH 7.4). After incubating for 2 min at room temperature, absorbance was measured at 560 nm. The superoxide radical scavenging activity was calculated using the following equation:

$$\text{Scavenging activity (\%)} = [1 - (\text{absorbance of sample at 560 nm})/(\text{absorbance of control at 560 nm})] \times 100$$

Hydroxyl radical scavenging activity determined by electron paramagnetic resonance (EPR) spectrometry

Hydroxyl radicals were generated by the Fenton reaction according to the method of Kohno *et al*.[20] In brief, the solution consisted of 0.1 mM H_2O_2, 0.1 mM $FeSO_4$, 5 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and the sample. The spectrum of the DMPO-OH spin adduct was measured after the addition of $FeSO_4$.[21] Deionized water was used instead of the sample solution for control experiments. All EPR spectra were recorded at ambient temperature (298 K) on a Bruker EMX-6/1 EPR spectrometer equipped with WIN-EPR SimFonia software (version 1.2). The EPR instrument settings were as follows: center field, 348.2 ± 5.0 mT; microwave power, 2 mW (2.354 GHz); modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 0.6 s; conversion time, 83 ms.

Data analysis

The data are presented as the mean ± standard deviation (SD) of each triplet test.

RESULTS AND DISCUSSION

The herbs of *G formosana* were homogenized in 70% acetone. After the evaporation of acetone, the 70% acetone extract was then fractionated with a Diaion HP-20 column. The active fractions were evaluated for DPPH radical-scavenging activity. DPPH is a stable radical that has often been used to evaluate the antioxidant activity of plant extracts.[22] When the concentration of the extracts was 25 μg ml^{-1}, the scavenging effects were 18.8, 39.3, 73.9, 34.2 and 10.6% for Fr A–E, respectively (Fig 1).

Both activity- and HPLC-directed purification of Fr B and Fr C by column and semi-preparative HPLC methods led to isolation of the principal active constituents. The chemical structures were identified by spectral data [FAB-MS, ^{1}H- and ^{13}C-NMR, heteronuclear multiple-bond correlation (HMBC), and heteronuclear multiple-quantum correlation (HMQC)] and the sugar portions were analyzed by GC-MS spectroscopy. The isolated flavonoid glycosides were methanolysed after methylation and subjected to GC-MS analyses. After comparison with the standards and reports in the literature, compounds **I**–**IV** were characterized as caffeic acid (**I**),[13] quercetin 3-*O*-rutinoside (rutin) (**II**),[14] kaempferol 3-*O*-rutinoside (nicotiflorin) (**III**),[15] and kaempferol 3-*O*-robinobioside (**IV**),[16] respectively. The structures of these compounds are shown in Fig 2; this is the first

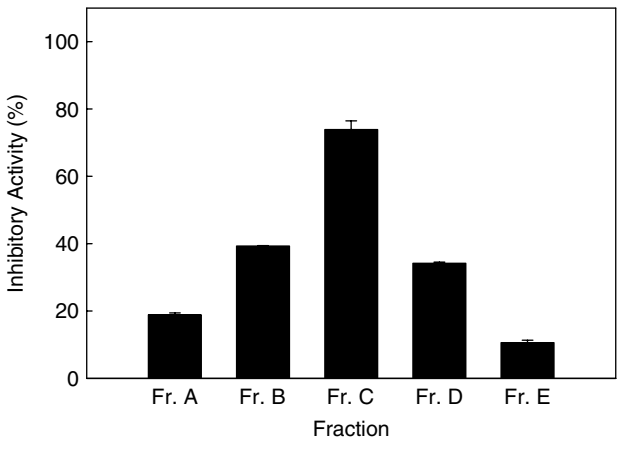

Figure 1. DPPH inhibitory activity of each fraction (25 μg ml^{-1}) of *G formosana*.

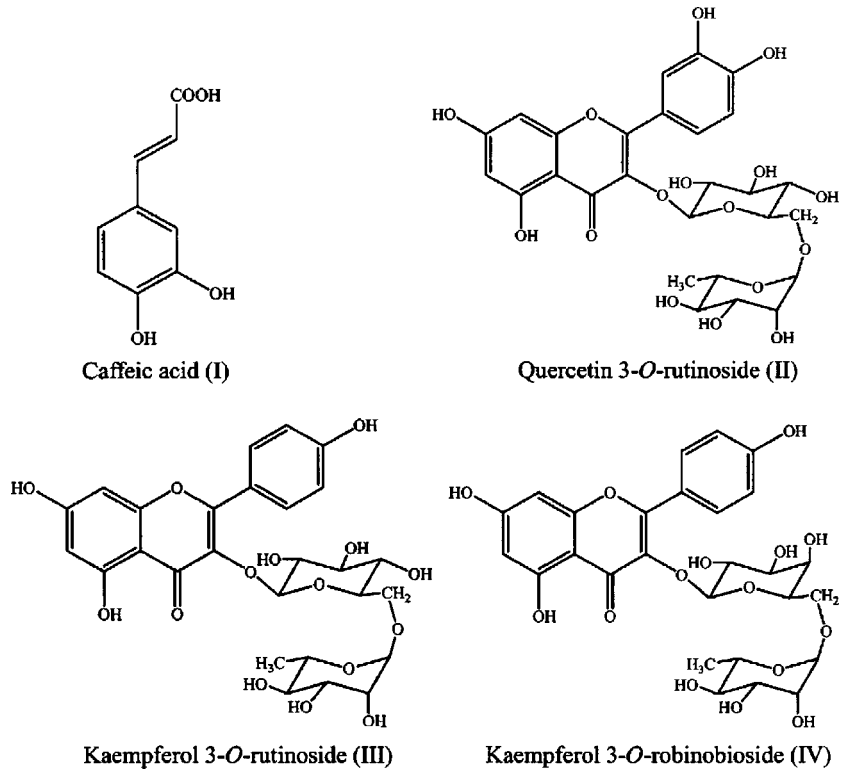

Figure 2. The structures of the isolated phenolic constituents.

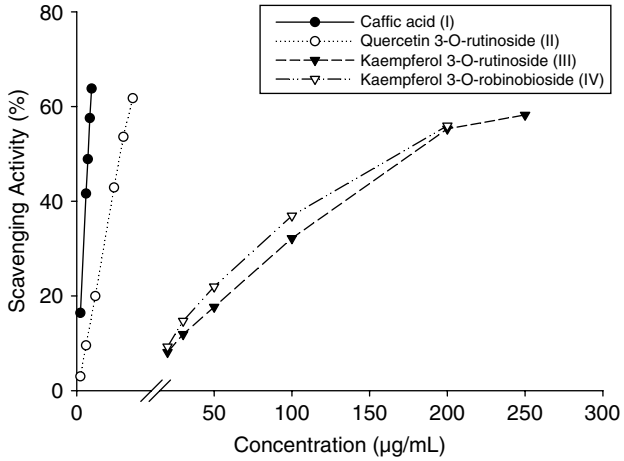

Figure 3. The scavenging activity of the four constituents against the DPPH radical.

Table 1. DPPH, superoxide, and hydroxyl radical-scavenging activities of isolated phenolic constituents of *Gynura formosana*

	IC_{50} μM[a]		
Compound	DPPH	Superoxide	Hydroxyl
Caffeic acid (**I**)	6.6	187.3	4.4
Quercetin 3-*O*-rutinoside (**II**)	7.7	25.8	7.5
Kaempferol 3-*O*-rutinoside (**III**)	300.3	55.3	—
Kaempferol 3-*O*-robinobioside (**IV**)	286.7	87.4	—

[a] The molecular weights of caffeic acid, quercetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside, and kaempferol 3-*O*-robinobioside are 181 $(M+H)^+$, 611 $(M-H)^+$, 595 $(M+H)^+$ and 595 $(M+H)^+$, respectively.

time that these substances have been identified from this plant.

The four compounds **I**–**IV** were evaluated for their free radical scavenging activities. The four compounds showed dose-dependent curves when evaluated using the DPPH method (Fig 3). The IC_{50} values of the DPPH radical were 6.7, 7.7, 300.3, and 286.7 μM for compounds **I**–**IV**, respectively (Table 1). The IC_{50}s of caffeic acid (**I**) and quercetin 3-*O*-rutinoside (**II**) were significantly lower than that of Trolox (IC_{50}, 100.8 μM), which was used as a positive control. The DPPH is a stable free radical; the ability to scavenge the DPPH radical is related to proton-donating ability and is one of several methods of antioxidation. The results imply that the antioxidative activity of caffeic acid (**I**) and quercetin 3-*O*-rutinoside (**II**) may be attributed to their proton-donating ability.

The superoxide radical scavenging activities of the four compounds were determined by the PMS–NADH generating system. Commercial recombinant human superoxide dismutase was used as a positive control. ΔA560 nm min^{-1} was negatively correlated ($r^2 = 0.998$) with superoxide dismutase added (20, 40 and 60 units, respectively, for 19.8, 43.0 and 61.3% scavenging activities). The IC_{50} values were 187.3, 25.8, 55.3, and 87.4 μM for compounds **I**–**IV**, respectively (Table 1). The IC_{50} values of compounds **I**–**IV** were equivalent to 1.4, 3.1, 1.5 and 0.9 unit μg^{-1} recombinant human superoxide dismutase, respectively. Superoxide ($\cdot O_2^-$) is the one-electron reduced form of molecular oxygen, which is a precursor to

active free radicals that have the potential of reacting with biological cells and inducing tissue damage. The results reveal that the four isolated compounds are scavengers of superoxide radicals and have SOD-like ability.

The scavenging ability of the four compounds against the hydroxyl radical was investigated by EPR spectrometry. Hydroxyl radicals were generated by the Fenton reaction[23] and trapped by DMPO to form DMPO-OH adducts. The intensities of the DMPO-OH spin signal in EPR spectrometry were used to evaluate the scavenging activity of the isolated compounds. When the concentration was 5 μM, the four isolated compounds exhibited hydroxyl radical-scavenging activity as shown in Fig 4. Kaempferol 3-*O*-rutinoside (**III**) and kaempferol 3-*O*-robinobioside (**IV**) did not have significant inhibitory activities against the hydroxyl radical. Figure 5 shows the scavenging activity against the hydroxyl radical with different concentrations of caffeic acid (**I**) and quercetin 3-*O*-rutinoside (**II**). On the basis of DMPO-OH signal intensities, when the concentrations were 1, 2, 5 and 7 μM, caffeic acid produced 31, 49, 72 and 78% decreases in hydroxyl radical levels (**I**). When the concentrations were 5, 7, 10 and 15 μM, quercetin 3-*O*-rutinoside (**II**) caused decreases in hydroxyl radicals of 37, 48, 60 and 87%, respectively. The IC_{50} values of caffeic acid (**I**) and quercetin 3-*O*-rutinoside (**II**) were 4.4 and 7.5 μM, respectively (Table 1).

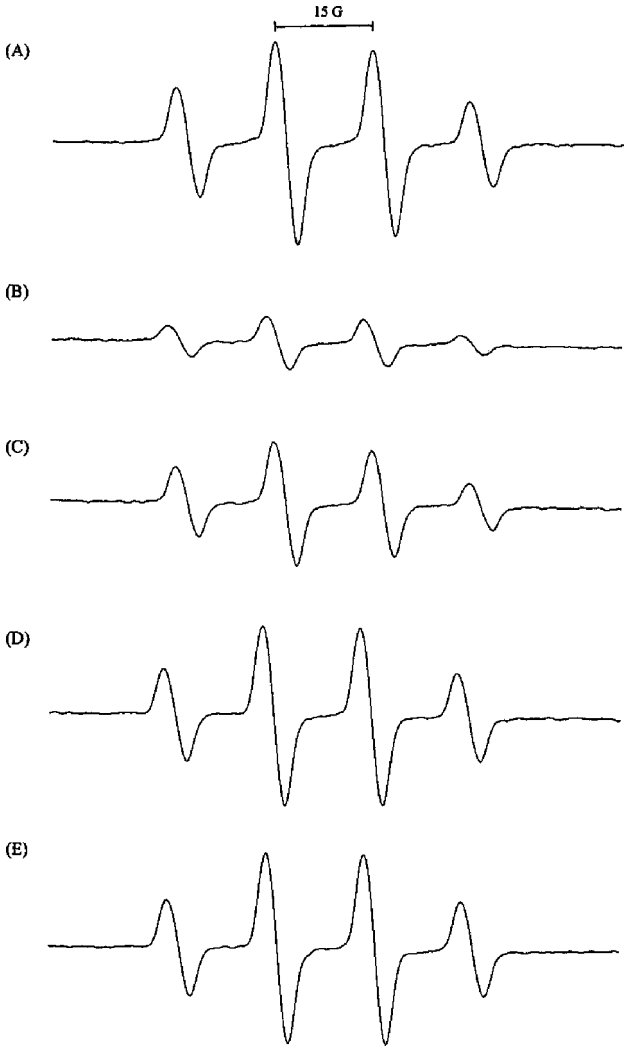

Figure 4. ESR spectra of the scavenging activity of the isolated constituents at 5 μM against hydroxyl radicals. (A) Control, (B) caffeic acid (**I**), (C) quercetin 3-*O*-rutinoside (**II**), (D) kaempferol 3-*O*-rutinoside (**III**), and (E) kaempferol 3-*O*-robinobioside (**IV**).

Hydroxyl radicals are among the strongest free radicals; they have damaging effects on living cells. They produce other kinds of cell-damaging free radicals and oxidizing agents,[24] which can attack DNA to cause strand scission. In biochemical systems, superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions such as iron or copper, especially iron,[25] or by UV photolysis.[26] In this experiment, caffeic acid (**I**) showed the most active effect; the EPR signal was significantly decreased in comparison to the control. Fig 5 provides the first piece of evidence that the four isolated compounds exhibited scavenging activity against the hydroxyl radical as shown by EPR spectrometry.

The four isolated compounds are phenolics. Compounds **II**, **III** and **IV** are related to 3-substituted flavone glycosides. There are two sugars in the structures of these three flavonoids. The sugar constituents of compounds **II** and **III** are rhamnose and glucose; compound **IV** includes rhamnose and galactose. The aglycon portion of compound **II** is quercetin, and that of compounds **III** and **IV** is kaempferol. A comparative analysis of the antioxidant activity of flavonoid glycosides and their flavonoid aglycones has demonstrated that the aglycone form can confer a higher antioxidant activity. Caffeic acid (**I**) and quercetin 3-*O*-rutinoside (**II**) showed good activity against DPPH and hydroxyl radicals. This may be attributed to the structure of catechol; the phenolic portions of the structures may be important determinants of antioxidant activity.

CONCLUSION

In Taiwan, *G formosana* is a cultured and popular vegetable; it is also used as a folk medicine. This study demonstrated that a 70% aqueous acetone extract of *G formosana* has free-radical scavenging activity. It is well known that free radicals contribute to the causation of several diseases, such as coronary heart disease, atherosclerosis and various cancers. Phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects, including antioxidant activity.[27–29] In the current study, we examined the phenolic content of *G formosana*. Four phenolic constituents from *G formosana* were identified. None of these phenolic compounds had been previously reported in *G formosana*. It would be interesting and worthwhile to further investigate the potential effectiveness or usage

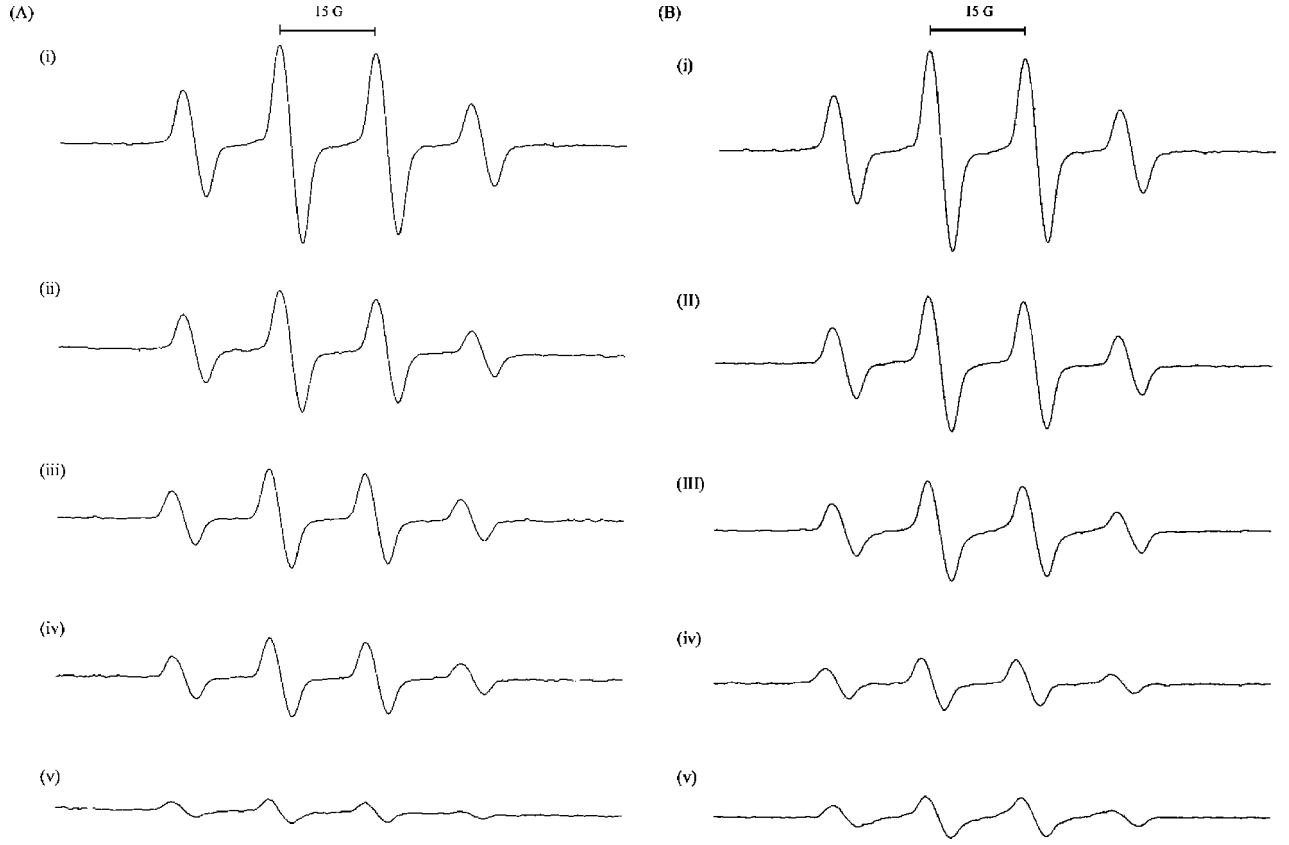

Figure 5. ESR spectra of the scavenging activity of (A) caffeic acid (**I**) and (B) quercetin 3-*O*-rutinoside (**II**) against hydroxyl radicals. (A) (i) control, (ii) 1 μM, (iii) 2 μM, (iv) 5 μM and (v) 7 μM; (B) (i) control, (ii) 5 μM, (iii) 7 μM, (iv) 10 μM and (v) 15 μM.

of *G formosana* in preventing diseases caused by the overproduction of radicals; this plant may prove to be a useful source of dietary antioxidants in the future.

ACKNOWLEDGEMENTS

We thank Mu-Cun Gao, Department of Botany, National Taiwan University, for plant identification. This study was supported by a grant from the Taipei Medical University (grant no. TMU91-Y05-A126). The authors also express thanks for financial support for this work from the Juridical Person of Yen's Foundation, Taiwan.

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