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Profiling and Characterization Antioxidant Activities in *Anoectochilus formosanus* Hayata

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Phytochemical characteristics and antioxidant activities of the crude and fractionated plant extracts of *Anoectochilus formosanus* were evaluated using five different assay systems. An acid-treatment (2 N HCl in 95% ethanol) was employed to treat a butanol fraction (BuOH), creating an acid-hydrolyzed BuOH fraction. The IC_{50} values for DPPH radicals in the BuOH and acid-hydrolyzed BuOH fractions were 0.521 and 0.021 mg/mL, respectively. The acid-hydrolyzed BuOH exhibited approximately 5-fold higher activity in scavenging superoxide anion than catechin. The acid-hydrolyzed BuOH fraction also effectively protected ϕ x174 supercoiled DNA against strand cleavage induced by H_2O_2 and reduced oxidative stress in HL-60 cells. Metabolite profiling showed that the aglycones of flavonoid glycosides in BuOH were produced after acid hydrolytic treatment, and this resulted in a significant increase in antioxidant activities of acid-hydrolyzed BuOH. One new diarylpentanoid, kinsenone, and three known flavonoid glycosides and their derivatives were identified for the first time from *A. formosanus*, with strong antioxidant properties.

KEYWORDS: ***Anoectochilus formosanus*; antioxidant activity; acid treatment; flavonoid glycosides**

INTRODUCTION

Reactive oxygen species (ROS) encompass a spectrum of diverse chemical species, including superoxide anions, hydrogen peroxide, hydroxyl radicals, nitric oxide, peroxynitrite, and others, which can play a variety of important physiological roles in both animal and plant systems. For instance, some of the ROS have been shown to be involved in mechanisms of cell-to-cell signaling, cell growth regulation, specific cellular physiology, and energy production (*1*). However, the oxidation of lipid, DNA, protein, carbohydrate, and other biological molecules by toxic ROS may cause DNA mutation or/and serve to damage target cells or tissues, and this often results in cell senescence and death. In human systems, such oxidative stress or damages are considered to be strongly associated with the aging process and certain degenerative diseases including various cancers, cognitive dysfunctions, and coronary heart disease (*2*). Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health and is now considered by many physicians, clinicians, and researchers as a key strategy for inhibiting, delaying, or even reversing the process of carcinogenesis (*3*). Moreover, knowledge and application of such potential antioxidant activities in reducing oxidative stresses in vivo have prompted many investigators to search for potent and cost-effective antioxidants from various plant sources (*4*−*8*). These research activities have contributed to new or renewed public interests worldwide in herbal medicines, health foods, and nutritional supplements. Unfortunately, many important and popular indigenous herbal plants of some regional interest have not been systematically studied. Therefore, local or regional putative medicinal plants that potentially contain high-level antioxidant metabolites or phytochemicals need to be scientifically studied.

Anoectochilus formosanus Hayata (Orchidaceae) is an indigenous and valuable Taiwanese medicinal plant and has been used popularly as a nutraceutical herbal tea in Taiwan and other Asian countries. This herbal plant is also called "King Medicine" because of its diverse pharmacological effects such as liver protection, cancer prevention, and diabetes and for treatment of cardiovascular diseases (*9*). However, limited scientifically proven information is available on the bioactivity, physiological function, and specific clinical efficacy of this herbal orchid plant. Sporadic studies on the potential reduction of acetaminophen-induced liver oxidative damage and increase of hepatoprotective

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activity on carbon tetrachloride damaged liver in test rats (*10*, *11*) and on the arachidonate metabolism (*12*) have been reported. Two phytochemical studies have been reported for *A. formosanus*. A butanoic acid glucoside (3-*O*-β-D-glucopyranosyl-(3*R*)-4-dihydroxybutanoic acid) (*13*), kinsenoside (2-β-D-glucopyranosyloxybutanolide), and megastigma glycosides (*14*) have been isolated from the plant. In the current study, in vitro antioxidant activities and the phytochemistry of crude plant extract and bio-organically fractionated metabolites of *A. formosanus* were evaluated. Results obtained from metabolite profiling analysis and antioxidant activity assays suggest that an acid pretreatment procedure employed on the plant extract can significantly increase the antioxidant activity of the test extracts. A new compound, kinsenone, and a number of known flavonoid glycosides were identified for the first time from *A. formosanus* plants. These phytochemicals and their acidic hydrolysis derivatives were evaluated for specific antioxidant activities.

MATERIALS AND METHODS

Plant Materials and Chemicals. In the flowering stage of fresh *A. formosanus*, plants (September 1999) were purchased from the reputable Anoectochilus Cultural Station in Nan-tou County of Taiwan, as officially recommended by Dr. Hsin-Sheng Tsay of the Taiwan Agricultural Research Institute, via the National Science and Technology Program in Agriculture Biotechnology, National Science Council, Taiwan. Authenticity of the plant species of *A. formosanus* was validated by the specific morphological and anatomical features of the flowers as reported by Li et al. (*15*). The leaves of *Ginkgo biloba*, used as a reference plant, were collected from the Experimental Forest of National Taiwan University, Taiwan, in 1999. Chemicals and reagents including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), trichloroacetic acid (TCA), quercetin, ascorbic acid, and ϕ x174 RF1 DNA were purchased from Sigma Chemical Co. (MO). Agarose was obtained from Bio-Rad (CA) and RPMI 1640 from Gibco BRL. All other chemicals and solvents used in this study were of reagent grade or HPLC grade.

Plant Extracts Preparation. The air-dried and powdered whole plant materials of *A. formosanus* (300 g) were extracted twice by boiling for 2 h with three volumes of distilled water. The total crude plant extract was collected by centrifugation at 24000*g* for 20 min at 4 °C and further extracted stepwise with 50%, 75%, and 87.5% (v/v) ethanol. The ethanol-insoluble fractions were collected by centrifugation at 24000*g* for 20 min at 4 °C. The final 87.5% ethanol-soluble fraction, designated as S fraction, was then evaporated to dryness by rotavapor (ca. 6.8 g solute), resuspended in 100 mL of H_2O, and followed by a successive extraction with ethyl acetate (EtOAc) and *n*-butanol (BuOH), yielding three subfractions denoted as the EtOAc (ca. 0.4 g), BuOH (ca. 1.2 g), and water (ca. 4.9 g) fractions.

Acid Hydrolysis of BuOH Fraction. The acid hydrolysis procedures are based on the Harborne method (*16*) with some modifications. A 500 mg sample of the BuOH fraction was dissolved in 50 mL of 95% ethanol/2 N HCl (1:1, v/v) solution in a securely sealed reaction bottle and then heated to 60 °C for 60 min. After repeated cycles of liquid−liquid partition with water/EtOAc (1:1), the acid-hydrolyzed BuOH fraction (acid-hydrolyzed BuOH) was then obtained from the pool of EtOAc extracts.

Free Radical Scavenging Activity. Scavenging activity on diphenyl-2-picrylhydrazyl (DPPH) radicals of *A. formosanus* extracts or the derived compounds was measured according to the method reported by Chang et al. (*17*) with minor modifications. Assays were performed in 3 mL reaction mixtures containing 2.0 mL of 0.1 mM DPPH/ethanol solution, 0.9 mL of 50 mM Tris-HCl buffer (pH 7.4), and 0.1 mL of deionized H_2O (as control) or test plant extracts. After 30 min of incubation at room temperature, absorbances of the reaction mixtures at 517 nm were taken. The inhibitory effect of DPPH was calculated according to the following formula:

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

IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

Superoxide Scavenging Activity. The superoxide anion was generated by xanthine/xanthine oxidase and detected by the nitroblue tetrazolium (NBT) reduction method (*18*, *19*). Reagents in this study were prepared in 50 mM KH_2PO_4/KOH buffer, pH 7.4. The reaction mixture contained 20 μL of 15 mM Na_2EDTA (pH 7.4), 50 μL of 0.6 mM NBT, 30 μL of 3 mM hypoxanthine, 50 μL of xanthine oxidase solution (1 unit in 10 mL of buffer), and 150 μL of various concentrations of *A. formosanus* plant extracts or 150 μL of KH_2PO_4/KOH buffer (as a control). The reaction was initiated by the addition of xanthine oxidase at 25 °C, and the absorbance at 405 nm was recorded every 20 s for 5 min using an ELISA reader (Labsystems Multiskan MS). Results were expressed as a percentage of inhibition relative to the control, given by

$$\left[\frac{(\text{rate of control}) - (\text{rate of sample reaction})}{\text{rate of control}}\right] \times 100$$

Lipid Peroxidation Assay. The thiobarbituric acid (TBA) test was employed for measuring the activity for peroxidation of lipid (*7*, *17*). The brain tissues (1 g) obtained from 8-week-old Balb/c mice were dissected, minced, and homogenized with 9 mL of ice-cold 20 mM Tris-HCl buffer (pH 7.4) to prepare a 1:10-fold diluted homogenate. The brain homogenate was centrifuged at 12000*g* at 4 °C for 15 min, and the supernatant was used for the in vitro lipid peroxidation assays. The reaction mixture (1.5 mL) contained 1 mL of the supernatant, 10 μM $FeSO_4$, 0.1 mM ascorbic acid, and 0.1 mL of various concentrations of *A. formosanus* extracts or deionized water (as control). The reaction was performed at 37 °C for 1 h and then stopped by addition of 1.0 mL of 28% (w/v) TCA and 1.5 mL of 1% (w/v) TBA and followed by heating at 100 °C for 15 min. The precipitated debris was removed by centrifugation, and the malondialdehyde (MDA)−TBA complex with a pink color in the supernatant was detected by its absorbance at OD 532 nm, using a Beckman DU 640 spectrophotometer. The inhibition ratio (%) was calculated using the following formula,

$$\text{inhibition ratio (\%)} = \left(\frac{A - A_1}{A}\right) \times 100$$

where A was the absorbance of the control and A_1 was the absorbance of test samples.

Analysis of Hydroxyl-Radical-Induced DNA Strand Scission. This assay was done according to the method of Keum et al. (*20*). The reaction mixture (30 μL) contained 0.3 μg of ϕ x174 RF1 DNA, 30 mM H_2O_2, and 10 mM Tris-EDTA buffer (pH 8.0) in the presence or absence of plant extracts. Various amounts of *A. formosanus* extracts dissolved in 10 μL of ethanol were added to the reaction mixture prior to the addition of H_2O_2. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 30 cm with a 12 W UV lamp (Spectroline, Spectronics Co.) at room temperature for 10 min. The reaction was terminated by addition of a loading buffer (0.02% bromophenol blue tracking dye and 40% sucrose), and the reaction mixtures were then analyzed by 0.8% submarine agarose gel electrophoresis. The gels were stained with ethidium bromide, destained with water, and photographed on a transilluminator.

Reduction of Oxidative Stress in HL-60 Cells. In this study the effect of acid-hydrolyzed BuOH from *A. formosanus* on reduction of oxidative stress in human promyelocytic leukemia HL-60 cells was evaluated on the basis of the method reported by Simizu et al. (*21*). HL-60 cells (1×10^6) were obtained from American Type Culture Collection (ATCC) and cultured at 37 °C in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS) and 100 units/mL penicillin and streptomycin in a 5% CO_2 incubator as recommended by ATCC. A total of 1.5 mL of the cell suspensions, at a concentration of 3×10^5 cells/well, was seeded in 6-well plates (Falcon) and incubated with varying dosages of acid-hydrolyzed BuOH at 37 °C for 40 min. Cells were then co-incubated at 37 °C with 25 μM 2′,7′-dichloro-

fluorescein diacetate (DCFH-DA) in the absence or presence of 25 μM H_2O_2 in darkness for 20 min. After incubation, cells were collected and washed once with ice-cold phosphate-buffered saline (PBS) buffer, were resuspended in 1 mL of fresh PBS buffer on ice in the dark, and were then ready for flow cytometry analysis (Coulter EPICS XL flow cytometer; Beckman/Coulter). The percent reduction on the H_2O_2-induced DCFH-DA fluorescence was calculated using the following formula,

$$\text{reduction (\%)} = 100 - \left(100 \times \frac{I_{\text{extract}+H_2O_2} - I_{\text{control}}}{I_{H_2O_2} - I_{\text{control}}}\right)$$

where $I_{\text{extract}+H_2O_2}$, $I_{H_2O_2}$, and I_{control} are the mean of DCF fluorescence values in cells treated with extract + H_2O_2, in cells treated with H_2O_2 alone, and in untreated cells, respectively.

Compound Identification. The BuOH extract was further fractionated with a Sephadex LH-20 column (80 cm × 2 cm) by elution sequentially with H_2O, 50% ethanol (EtOH), and 100% EtOH, resulting in the collection of another 12 subfractions (B1−B12). Compounds **1** (15 mg), **2** (5 mg), **3** (12 mg), and **4** (10 mg) were isolated and purified from the B9−B11 subfractions, using a semipreparative HPLC on a Si-60 column (250 mm × 10 mm, Hibar Lichrosorb, Merck): mobil phase, chloroform/MeOH/H_2O = 10/5/1; flow rate, 1.5 mL/min at retention times of 13.2, 25.3, 31.0, and 34.8 min, respectively. The yields of compounds **1**, **2**, **3** and **4** were approximately of 0.05%, 0.016%, 0.04%, and 0.033% (w/w), respectively, in whole plant tissues of *A. formosanus*. The structure of compounds **1**−**4** were then elucidated using spectroscopic analysis. UV spectra of test compounds were recorded with a Jasco V-550 spectrometer, and IR spectra were obtained from a Bio-Rad FTS-40 spectrophotometer. Electron-impact mass spectrometry (EIMS) and high-resolution electron-impact mass spectrometry (HREIMS) data were collected with a Finnigan MAT-958 mass spectrometer, and NMR spectra were recorded with Bruker Avance 500 and 300 MHz FT-NMR spectrometers, at 500 MHz (^{1}H) and 75 MHz (^{13}C).

The constituents of acid-hydrolyzed BuOH were analyzed by using semipreparative HPLC on a C-18 column (250 mm × 10 mm, Vydac): mobile phase, solvent A = 20% MeOH, solvent B = 100% MeOH. Elution conditions were the following: 0−20 min of 10−50% A to B (linear gradient); 20−40 min of 50−80% A to B (linear gradient); 40−50 min of 80−100% A to B (linear gradient); flow rate, 1.5 mL/min. The major compounds in acid-hydrolyzed BuOH were obtained at retention times of 38 min (**5**), 41 min (**6**), and 46 min (**7**). The compound structures shown in **Figure 1** were characterized by using mass spectrometric and NMR analysis.

Kinsenone (1). Light-yellow amorphous solid. UV (MeOH), λ_{max}, nm (log ϵ): 311 (3.10), 280 (3.05), 237 (3.06). IR (KBr), ν_{max}, cm^{-1}: 3434, 3050, 3010, 1662, 1600, 1502, 1439, 1379. HREIMS, *m*/*z*: 266.2936, [M]$^+$ calcd 266.2993 for $C_{17}H_{14}O_3$. ^{1}H NMR (CD_3OD, 500 MHz): δ 7.50 (2H, d, J = 8.8 Hz, H-2″ and H-6″), 7.49 (1H, d, J = 15.9 Hz, H-1), 7.35 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.70 (1H, d, J = 12.8 Hz, H-5), 6.69 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 6.64 (2H, d, J = 8.8 Hz, H-3″ and H-5″), 6.18 (1H, J = 15.9 Hz, H-2), 5.67 (1H, d, J = 12.8 Hz, H-4). ^{13}C NMR (CD_3OD, 100 MHz): δ 171.1 (C-3), 161.0 (C-4′), 159.9 (C-4), 146.2 (C-1), 143.5 (C-5), 133.3 (C-2″ and C-6), 130.8 (C-2′ and C-6′), 127.8 (C-1′ and C-1″), 116.6 (C-4), 116.1 (C-3′ and C-5′), 115.9 (C-2), 115.0 (C-3″ and C-5″).

RESULTS

Free Radical Scavenging Activity in Plant Extracts of *A. formosanus*. The free radical scavenging activity of total crude extract and its derived fractions of *A. formosanus* was quantitatively determined using a DPPH assay. The IC_{50} value for scavenging DPPH radicals of the total crude extract of *A. formosanus* was 0.994 mg/mL, which is very similar to that of *Ginkgo biloba* leaf extract (0.932 mg/mL), as was determined in parallel in this study. The dosage of extract (or compound) is expressed in milligrams of dry weight of the extract (compound) per milliliter of the assay mixture. The IC_{50} value represents the concentration of test extract or compound where the inhibition of test activity reached 50%. *G. biloba* extract is known to exhibit a high level of antioxidant activity (*22*), and this in turn has been speculated to contribute to the clinical efficacy of this popular medicinal herb. A 2-fold decrease in the capability of inhibiting free radical activity was detected in the 87.5% ethanol-soluble fraction (S fraction) of the crude extract, whereas the EtOAc and BuOH fractions exhibited a 2- to 3-fold decrease in the IC_{50} value. The IC_{50} for EtOAc, BuOH, and S fractions were 0.311, 0.521, and 0.992 mg/mL, respectively. These results indicate that the free radical scavenging activity of *A. formosanus* extract can be effectively enriched in the BuOH and EtOAc fractions. The BuOH fraction had 3-fold more mass than the EtOAc fraction and was further investigated for its phytochemical characteristics and in vitro and cellular biological antioxidant activities.

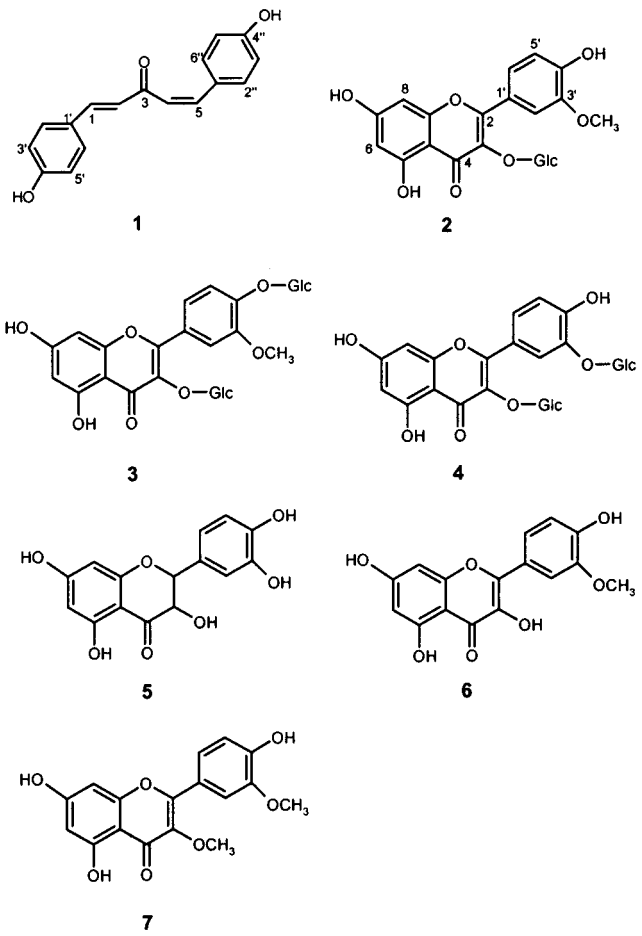

Figure 1. Structures of compounds from *A. formosanus*: kinsenone (**1**); isorhamnetin 3-*O*-β-D-glucopyranoside (**2**); isorhamnetin 3,4′-*O*-β-D-diglucopyranoside (**3**); quercetin 3,3′-*O*-β-D-diglucopyranoside (**4**); dihydroquercetin (**5**); isorhamnetin (**6**); quercetin 2,4′-dimethyl ether (**7**).

Comparison of the Free Radical and Superoxide Anion Scavenging Activities of BuOH and Acid-Hydrolyzed BuOH Fractions. In this study, a defined acid treatment procedure for the BuOH fraction was performed to generate a hydrolyzed product fraction, designated as acid-hydrolyzed BuOH. The scavenging DPPH radical activity of acid-hydrolyzed BuOH fraction was characterized and compared with those of the total crude extract and BuOH fraction. The IC_{50} value for DPPH in the acid-hydrolyzed BuOH fraction was 0.021 mg/mL, which is approximately a 50- and 26-fold decrease in comparison with that of the crude extract and BuOH fraction, respectively (**Table 1**).

Table 1. Free Radical (DPPH) and Superoxide Anion Scavenging Activities of Total Crude Extract and Derived Fractions of *A. formosanus*[a]

extract	DPPH radical, IC_{50} (mg/mL)	superoxide anion, IC_{50} (mg/mL)
total extract	0.994 ± 0.0007	1.452 ± 0.001
BuOH fraction	0.521 ± 0.0004	0.663 ± 0.0005
acid-hydrolyzed BuOH fraction	0.021 ± 0.0000	0.115 ± 0.0001
EtOAc fraction	0.311 ± 0.0002	ND
S fraction	0.992 ± 0.0007	>1.60
quercetin[b]	2.3 ± 0.02[b]	3.4 ± 0.03[b]

[a] Each assay was performed in triplicate. ND: not determined. [b] Quercetin (μg/mL) was used as a reference compound for this experiment.

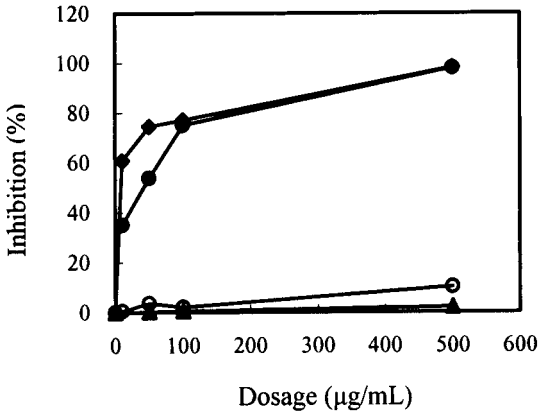

Figure 2. Effects of *A. formosanus* extracts on lipid peroxidation using an assay with mouse brain tissue homogenates: ◆, acid-hydrolyzed BuOH fraction; ▲, BuOH fraction; ○, hot water extracts of *A. formosanus*; ●, catechin. Results are mean ± SD ($n = 3$).

The superoxide anion scavenging activities in total crude extract, BuOH, and acid-hydrolyzed BuOH fractions were also compared by using an NBT reduction method. The concentration for inhibiting 50% superoxide activity (IC_{50}) was determined as 0.115, 0.663, and 1.452 mg/mL for the acid-hydrolyzed BuOH, BuOH, and total extract of *A. formosanus*, respectively (**Table 1**). Furthermore, in the presence of 500 μg/mL of crude plant extract, BuOH, or acid-hydrolyzed BuOH fractions in the reaction mixture, the inhibition for superoxide radicals were 10%, 22%, and 76%, respectively, relative to the control without plant extracts. The IC_{50} values of quercetin, used as a reference, for DPPH radical and superoxide anion scavenging activities were 2 and 3 μg/mL, respectively.

Effect on Lipid Peroxidation. Figure 1 shows that the acid-hydrolyzed BuOH extract of *A. formosanus* can dramatically inhibit the peroxidation of mouse brain lipids. The IC_{50} value was determined to be 8.2 μg/mL. Approximately 60% of lipid peroxidation was inhibited by the extract at 10 μg/mL. At a dosage of 0.5 mg/mL, the crude extract and the BuOH fraction exhibited only <10% inhibition of lipid peroxidation (**Figure 2**). In comparison, we show that catechin, a well-known and highly effective antioxidant isolated from green tea (*23*), was less effective ($IC_{50} = 40$ μg/mL) than the acid-hydrolyzed BuOH extract ($IC_{50} = 8.2$ μg/mL), as determined in parallel within the same experiment (**Figure 2**).

Inhibition of Hydroxyl-Radical-Induced DNA Strand Scission. The protective effect of acid-hydrolyzed BuOH extract from *A. formosanus* on φ x174 RF1 DNA strand scission caused by UV photolysis of H_2O_2 was investigated. As shown in **Figure 3**, there was no DNA cleavage when observed with UV irradiation alone (negative control, lane 2) or incubated with plant extract (acid-hydrolyzed BuOH) alone (lanes 3 and 4). Treatment of the supercoiled form φ x174 RF1 DNA with both H_2O_2 and UV resulted in >90% conversion of the supercoiled DNA form into its open circular form (lane 5). The acid-hydrolyzed BuOH extract of *A. formosanus*, however, was able to greatly reduce the DNA strand scissions in a dosage-dependent manner (lanes 6−11). The DNA cleavage was virtually protected in total in the presence of 5−10 mg/mL of acid-hydrolyzed BuOH extract (lanes 6 and 7). A significant protection of DNA cleavage was even detected at 10 μg/mL (lane 11), compared to the result of the φ x174 RF1 DNA sample that was treated with both H_2O_2 and UV without co-incubation with the extract (lane 5). In comparison, BuOH extract at 5 mg/mL (lane 12) exhibited much less effect on DNA scission protection when compared to the same dose of acid-hydrolyzed BuOH extract (lane 6).

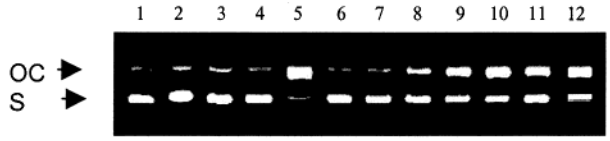

Figure 3. Protection effect of acid-hydrolyzed BuOH fraction of *A. formosanus* on φ x174 RF1 DNA strand scission induced by UV photolysis of H_2O_2. Results were analyzed by 0.8% agarose gel electrophoresis. Lane 1: native supercoiled DNA sample without any treatment. Lanes 2–4: DNA samples treated with UV irradiation alone, 10 mg/mL, and 1 mg/mL of plant extract alone, respectively. Lane 5: DNA treated with H_2O_2 and UV irradiation. Lanes 6–11: DNA samples treated with H_2O_2 + UV irradiation in the presence of 10, 5, 1, 0.5, 0.1, and 0.01 mg/mL acid-hydrolyzed BuOH, respectively. Lane 12: native DNA treated with 5 mg/mL of BuOH and H_2O_2 + UV irradiation. Arrows indicate distinct forms of the DNA: OC (open circular) and S (supercoiled).

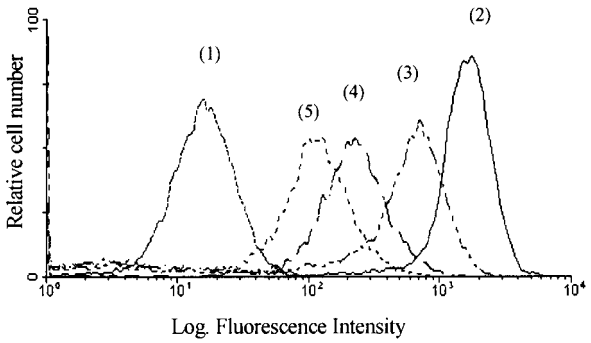

Figure 4. Cellular biological antioxidant activity of the acid-hydrolyzed BuOH fraction of *A. formosanus*: (1) control, mean = 16; (2) cells treated with H_2O_2 alone, mean = 1560; (3–5) cells treated with 0.1, 0.25, 0.5 mg/mL of the acid-hydrolyzed BuOH fraction before H_2O_2 was added. The mean values for spectra 3, 4, 5 were 672, 169, and 94, respectively.

Effective Reduction of Oxidative Stress in HL-60 Cells. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), a free-radical-sensitive indicator, was used for evaluating the effectiveness of *A. formosanus* plant extracts on the oxidative stress in mammalian cells based on the method reported by Simizu et al. (*21*). Upon oxidation of the nonfluorescent DCFH-DA molecule by reactive oxygen species, e.g., H_2O_2, the 2′,7′-dichlorofluorescein (DCF) molecules are generated as products that emit green fluorescence. The fluorescence intensity relevant to the H_2O_2 concentration can thus be measured quantitatively by using flow cytometric analysis. **Figure 4** shows that an 83-fold increase in fluorescence intensity in human promyelocytic leukemia HL-60 cells was detected when the cells were treated with 25 μM H_2O_2 (spectrum 2) relative to the untreated cells

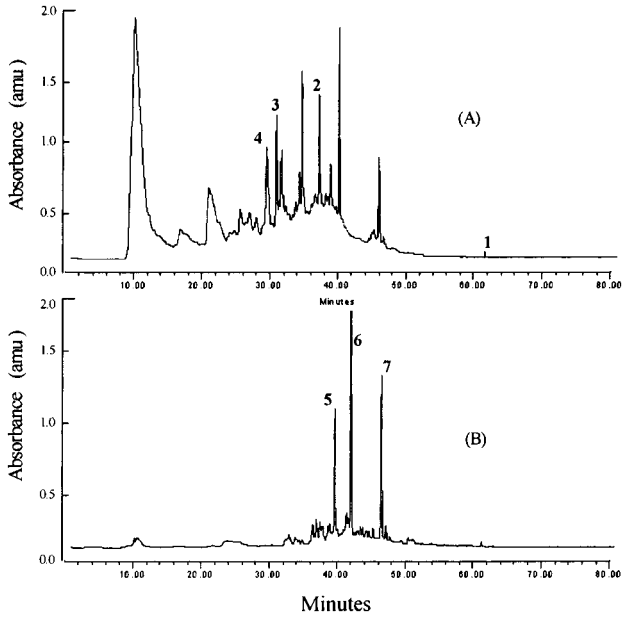

Figure 5. HPLC chromatograms of BuOH fraction (A) and its acid-treated product (acid-hydrolyzed BuOH) (B) from *A. formosanus*: **1**, kinsenone; **2**, isorhamnetin 3-*O*-β-D-glucopyranoside; **3**, isorhamnetin 3,4′-*O*-β-D-diglucopyranoside; **4**, quercetin 3,3′-*O*-β-D-diglucopyranoside; **5**, dihydroquercetin; **6**, isorhamnetin; **7**, quercetin 2,4′-dimethyl ether.

(spectrum 1). Levels of 69% (spectrum 3), 88% (spectrum 4), and 94% (spectrum 5) reduction in the total increased DCF fluorescence intensity in 25 μM H_2O_2 treated HL-60 cells were observed when co-incubated with 0.1, 0.25, and 0.5 mg/mL of the acid-hydrolyzed BuOH fraction, respectively. These results demonstrate that the levels of intracellular reactive oxygen species in HL-60 cells were effectively reduced when the cells were treated with the acid-hydrolyzed BuOH fraction, suggesting that the damage from oxidative stress in test cells could be greatly alleviated by acid-hydrolyzed BuOH extract.

Identification of Secondary Metabolite Compounds in the BuOH and Acid-Hydrolyzed BuOH Fractions of *A. formosanus*. It has been reported that certain flavonoid glycosides may be less effective as antioxidants than their aglycone forms under in vitro assay conditions (*24*). The secondary metabolite profiles of the BuOH and acid-hydrolyzed BuOH fractions were characterized using HPLC and 1D and 2D NMR spectrometry. Parts A and B of **Figure 5** show the HPLC chromatograms of BuOH and acid-hydrolyzed BuOH fractions, respectively. Four major phenolic compounds (**1**−**4**) were isolated and characterized from the BuOH fraction. Compound **1** was obtained as a light-yellow amorphous solid material with a molecular formula of $C_{17}H_{14}O_3$ based on high-resolution electron-impact mass (HREIMS) analysis. The IR spectrum of **1** showed absorptions at 3434 cm^{-1} (hydroxyl), 1662 cm^{-1} (conjugated ketone), and 1600 and 1505 cm^{-1} (aromatic ring). The ^{1}H NMR signals (in CD_3OD) of **1** showed an (*E*)-coumaroyl moiety (δ 7.49 and 6.18, each 1H, d, J = 15.9 Hz; δ 7.35 and 6.69, each 2H, d, J = 8.8 Hz) and a (*Z*)-coumaroyl moiety (δ 6.70 and 5.67, each 1H, d, J = 12.8 Hz; δ 7.50 and 6.64, each 2H, d, J = 8.6 Hz). The ^{13}C NMR spectrum of **1** showed a carbonyl at δ 171.1, the high field of the carbonyl signal indicating cross-conjugation with two double bonds. Heteronuclear multiple-bond correlation (HMBC) analysis showed that four olefinic proton signals (δ 6.18, 7.46, 6.69, and 7.50) were correlated to the carbonyl at δ 171.1 (C-3), indicating that the (*E*)-coumaroyl and (*Z*)-coumaroyl moieties are colocated on the same carbonyl group. The ^{13}C NMR data were assigned by HMQC and HMBC as follows: δ 143.5 (C-5), 146.2 (C-1), 159.9 (C-4), 161.0 (C-4′), 130.8 (C-2′ and C-6′), 133.3 (C-2″ and C-6″), 127.8 (C-1′ and C-1″), 146.2 (C-1′), 116.6 (C-4), 116.1 (C-3′ and C-5′), 116.1 (C-4′), and 115.0 (C-3″ and C-5″). The structure of **1** was also further confirmed by the nuclear Overhauser effect spectrometry (NOESY) and HMBC correlations. Compound **1** was assigned the structure (1*E*,4*Z*)-1,5-bis(4-hydroxyphenyl)-1,4-pentadien-3-one, and we refer to it hereafter as kinsenone. Kinsenone is a new compound and is reported here as a new natural product.

According to the negative ion MS and NMR analyses, compounds **2**−**4** in the BuOH fraction were identified as isorhamnetin 3-*O*-β-D-glucopyranoside (*25*, *26*), isorhamnetin 3,4′-*O*-β-D-diglucopyranoside (*25*, *27*), and quercetin 3,3′-*O*-β-D-diglucopyranoside (*28*), respectively. The main constituents of the acid-hydrolyzed BuOH fraction were determined to be dihydroquercetin (**5**) (*29*), isorhamnetin (**6**) (*30*), and quercetin 2,4′-dimethyl ether (**7**) (*31*). These results demonstrate that the 2 N HCl treatment on the BuOH fraction caused the hydrolysis of glycosidic linkages in compounds **2**−**4**, resulting in the formation of the respective aglycone forms of the compounds.

DPPH Radical Inhibition by Phenolic Metabolites. The scavenging activities for DPPH radicals (%) of quercetin 3,3′-*O*-β-D-diglucopyranoside (**4**), kinsenone (**1**), isorhamnetin 3-*O*-β-D-glucopyranoside (**2**), and isorhamnetin 3,4′-*O*-β-D-diglucopyranoside (**3**), at a concentration of 30 μg/mL, were 37%, 20%, 8.3%, and 6.5%, respectively. Quercetin, used as a control reference, was found to have approximately 80% of the DPPH radical scavenging activity at the same dosage.

DISCUSSION

Four major phenolic compounds in the BuOH fraction from *A. formosanus*, including one new diarylpentanoid and three known flavonoid glucosides, were identified in this study. None of these phenolic compounds have been previously reported from the *Anoectochilus* genus. A comparative analysis of antioxidant activity on flavonoid glycosides and their flavonoid aglycones has demonstrated that the aglycone form can confer a higher antioxidant activity than their glycoside-conjugated counterparts. At a dose of 30 μg/mL, 2-fold higher DPPH-scavenging activity for quercetin compared with that of quercetin 3,3′-*O*-β-D-diglucopyranoside (**4**) was observed. We further showed that the glucose-conjugated forms of isorhamnetin, i.e., compounds **2** and **3**, exhibited a 4.5- to 6-fold lower antioxidant activity than quercetin glycoside (**4**). Therefore, quercetin has a 10- to 12-fold higher activity than isorhamnetin 3-*O*-β-D-glucopyranoside (**2**) or isorhamnetin 3,4′-*O*-β-D-diglucopyranoside (**3**). These results show that the glucose moiety of the flavonoid glycosides can apparently contribute to the decrease of free-radical scavenging activity of the compounds and, in combination with the methylation of hydroxyl group at the C-3 position, it can result in a much reduced effect on antioxidant activity. Cinnamic acid derivatives, such as caffeic acid, *p*-coumaric acid, and ferulic acid, have been demonstrated to exhibit significant antioxidant activities (IC_{50} = 0.5−10 μg/mL). The new diarylpentonoid, kinsenone (**1**) isolated from *A. formosanus*, contains two coumaroyl groups (*E* and *Z* forms) but was not found to have a comparable radical-scavenging activity relative to that of quercetin or *p*-coumaric acid. DPPH radicals were only inhibited by 20% using 30 μg/mL of kinsenone.

We have hypothesized that the plant extracts containing mainly the flavonoid glycoside forms of the compound may

not be able to exhibit an effective antioxidant activity before a metabolic conversion of these molecules into their aglycone counterparts. Harborne (*16*) has demonstrated that the aglycone moieties of flavonoid glycosides can be liberated by acid hydrolysis. In this study we therefore employed an acid-hydrolysis treatment on the BuOH fraction and then subjected the product fraction (acid-hydrolyzed BuOH) to assays for various antioxidant activities. We have observed that both DPPH-scavenging and superoxide-anion-scavenging activities of the BuOH fraction were greatly enhanced after it was acid-hydrolyzed (**Table 1**). The scavenging DPPH radical activity in the acid-hydrolyzed BuOH fraction was 26-fold and 50-fold higher than that obtained from the BuOH fraction and the total crude extract, respectively. Results obtained from comparative analysis of metabolite profiles of the BuOH and acid-hydrolyzed BuOH fractions show that the aglycone moieties of flavonoid glycosides are released after an acid treatment (**Figure 5**). These non-glucose-conjugated forms of flavonoids were not detected in the BuOH fraction. We suggest that this prehydrolysis procedure may therefore serve as a very useful, routine procedure step for evaluating antioxidant activity in various plant extracts or other natural products.

It is known that oxidation of polyunsaturated fatty acids in biological membranes often leads to the formation and propagation of lipid radicals, uptake of oxygen, rearrangement of the double bonds in unsaturated lipids, and even destruction of membrane lipids. Many of these biochemical activities can lead to the production of breakdown products that are highly toxic to most mammalian cell types (*7*). Effects on the inhibition of lipid peroxidation by the BuOH and acid-hydrolyzed BuOH fractions of *A. formosanus* were also investigated in this study using mouse brain homogenates as a lipid source. A drastic increase in the capability of inhibiting lipid peroxidation was detected in the acid-hydrolyzed BuOH fraction compared to the BuOH fraction. Significantly, the combination of phytochemicals existing mainly as flavonoids in the acid-hydrolyzed BuOH fraction ($IC_{50} = 8.2$ μg/mL) can confer a potency higher than that of a well-known antioxidant, catechin ($IC_{50} = 40$ μg/mL). These results suggest that the hydrolyzed BuOH fraction of *A. formosanus* can effectively inhibit the activity of both free radicals and superoxide anions radicals and can also confer very efficient inhibition against lipid peroxidation.

In biochemical systems, the superoxide radical can be converted into hydrogen peroxide by the action of superoxide dismutase and the H_2O_2 can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions (e.g., iron and copper) or by UV photolysis (*1*). Hydroxyl radicals can attack DNA molecules to cause strand scission. Incubation of ϕ x174 RF1 DNA with both H_2O_2 and UV can result in a conversion from most of the supercoiled DNA (>90%) to its open circular form (**Figure 3**). We show that the acid-hydrolyzed BuOH fraction, at a dose of 5 mg/mL, can dramatically inhibit the DNA strand cleavage under the same UV/H_2O_2 treatment. Keum et al. (*20*) have demonstrated that a methanolic extract of heat-processed ginseng can completely protect the DNA strand scission at a dosage of 30 mg/mL. In this study, a 6-fold lower dosage requirement was observed for the acid-hydrolyzed BuOH fraction of *A. formosanus* than for the methanol-extracted heat-processed ginseng extract (*20*). These results demonstrate that the acid-hydrolyzed BuOH fraction of *A. formosanus* is a very effective protector for double-strand DNA molecules against oxidative damage. Furthermore, we have demonstrated that the acid-hydrolyzed BuOH fraction can also significantly reduce the oxidative stress resulting from reactive oxygen species (ROS) in human promyelocytic leukemia HL-60 cells (**Figure 4**). Approximately 94% of H_2O_2-derived free radicals in test cells were reduced by treatment with 0.5 mg/mL of acid-hydrolyzed BuOH.

Experimental results obtained in this study effectively support our conclusion that a strong and broad spectrum of antioxidant activities, mainly contributed by phytochemicals existing as specific phenolic compounds, are present in *A. formosanus* extracts. This study has provided a simple, rapid acid hydrolysis protocol, using a 2 N HCl/alcohol solution, for profiling and evaluation of potential antioxidation activity for medicinal herbs and nutraceuticals. On the basis of the studies using in vitro biochemical and cell and molecular biological assays, we have defined the specific bioactivities in the plant extracts of *A. formosanus* against various antioxidative stresses. We suggest that this herbal plant can serve as a good candidate as food supplement for human health care. For future studies, it will be desirable to employ experimental conditions that can more specially reflect the various gastric/intestinal microenvironments, which are pertinent to the uptake of nutrient and/or plant secondary metabolites in human systems.

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