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Activities of antioxidants, α-Glucosidase inhibitors and aldose reductase inhibitors of the aqueous extracts of four *Flemingia* species in Taiwan

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ABSTRACT. The aim of this study was to examine the possible antioxidant and antidiabetic effects of the aqueous extracts of four *Flemingia* species in Taiwan. A number of methods were employed for this investigation, including ABTS radical monocation scavenging, FRAP (ferric reducing antioxidant power) method, DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging, total polyphenol content, total flavonoid content, total flavonol content, and inhibition of α-glucosidase and aldose reductase methods. The results showed that the aqueous extract of *Flemingia macrophylla* (WFM) had the strongest antioxidant activity in comparison with the other extracts. We also found that WFM had higher contents of polyphenol compounds, flavonoids and flavonols than the other extracts. The correlation coefficient (R^2) values of TEAC (trolox equivalent antioxidant capacity) and FRAP showed high correlations (R^2=0.83). The R^2 values of TEAC and total polyphenol content showed a higher correlation (R^2=0.66). The R^2 values of TEAC and total flavonoid content for the aqueous extracts was 0.94. The antidiabetic activities of the four *Flemingia* species were studied *in vitro* using α-glucosidase and aldose reductase (AR) inhibitory methods. WFM had the highest inhibitory activities on α-glucosidase and aldose reductase, with IC_{50} (concentration with 50% inhibition) of 153.92 ± 0.20 μg/mL and 79.36 ± 3.20 μg/mL respectively. The positive control (genistein) had higher inhibitory activities on α-glucosidase and aldose reductase (IC_{50} 16.65 ± 0.92 μg/mL and 45.62 ± 2.16 μg/mL) respectively. In *LC-MS-MS* analyses, the chromatograms of WFM with the highest antioxidant and antidiabetic activity were established. Genistein might be an important bioactive compound in WFM extract. This experiment suggests that WFM might serve as a good resource for future development of antioxidant and antidiabetic drugs.

Keywords: Aldose reductase; Antioxidant; α-glucosidase; *Flemingia*; Free radical; Polyphenol.

INTRODUCTION

Diabetes mellitus is a common disease with many complications such as atherosclerosis, cardiac dysfunction, retinopathy, neuropathy, and nephropathy (Sowers et al., 2001). α-glucosidase (EC 3.2.1.20) catalyzes the final step in the digestive process of carbohydrates. Its inhibitors can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia and could be useful for treating diabetic and/or obese patients (Toeller, 1994). α-Glucosidase inhibitors such as acarbose, miglitol, and voglibose are known to reduce postprandial hyperglycemia primarily by interfering with the carbohydrate digestive enzymes and by delaying glucose absorption. Aldose reductase (E.C.1.1.1.21, AR) is the first enzyme in the polyol pathway; it catalyzes the reduction of D-glucose from the aldehyde form into D-sorbitol with concomitant conversion of NADPH to $NADP^+$ (Kador et al., 1985a, b). It is generally accepted that this polyol pathway plays an important role in the development of some degenerative complications of diabetes. The elevated blood glucose level, characteristic of diabetes mellitus, causes significant fluxes of glucose through the polyol pathway in tissues such as nerves, retina, lens, and kidneys, where glucose uptake is independent of insulin (Chihiro, 1998). Thus, AR inhibitors have attracted attentions in therapeutic researches of diabetic complications.

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The inhibitory effects of plant phytochemicals, including polyphenols, against carbohydrate hydrolyzing enzymes contribute to the lowering of postprandial hyperglycemia in diabetic management as observed *in vivo* (Griffiths and Moseley, 1980). Further evidence that polyphenolic compounds is linked to prevention of diabetic complications stems from *in vivo* studies with diabetic rats; polyphenolic compounds in plant materials are capable of reducing oxidative stress by scavenging reactive oxygen species and preventing cell damage (Fukuda et al., 2004). The polyphenolic compounds in edible plants are currently regarded as natural antioxidants, and their antioxidant activities are important for human health (Sabu et al., 2002).

Plants constitute a rich source of bioactive chemicals (Kador et al., 1985a, b; Williamson et al., 1992). Since many plants are largely free from adverse effects and have excellent pharmacological actions, they could possibly lead to the development of new classes of safer antidiabetic agents or diabetic complication resolving agents. In addition, some flavonoids and polyphenols as well as sugar derivatives are found to be effective in inhibiting α-glucosidase and aldose reductase (Haraguchi et al., 1996; Lee and Kim, 2001). Therefore, much effort has been focused on plants to produce potentially useful products such as commercial α-glucosidase inhibitors and aldose reductase inhibitors or lead compounds.

The *Flemingia* genus, known as 'I-Tiao-Gung' in Chinese, is distributed in tropical areas. The traditional usages of the roots of *Flemingia* species have been for the treatment of rheumatism, arthropathy, leucorrhea, menalgia, menopausal syndrome, chronic nephritis, and improvement of bone mineral density (Li et al., 2008). Only few studies have confirmed the pharmacological activity of members in the *Flemingia* genus. For example, it was reported that the extract of the root of *F. philippinensis* (*F. prostrata* (FP)) exhibited anti-oxidative, anti-inflammatory, estrogenic, and anti-estrogenic activities (Li et al., 2008). The stems of *F. macrophylla* have been used in traditional medicine as an antirheumatic and anti-inflammatory agent and for improving blood circulation. Furthermore its flavonoids have inhibitory effects on A β-induced neurotoxicity (Shiao et al., 2005).

The objectives of this work were to investigate the antioxidant and antidiabetic properties of the aqueous extracts of *Flemingia macrophylla* (Willd.) Kuntze *ex* Prain (FM), *Flemingia prostrata* Roxb (FP), *Flemingia lineata* (L.) Roxb. (FL), and *Flemingia strobilifera* (L.) R. Br. Ex Ait. (FS) by comparing them with chemical compounds such as glutathione (GSH) or genistein, and to find out the levels of their inhibitory activities on α-glucosidase and aldose reductase through a series of *in vitro* tests.

MATERIALS AND METHODS

Materials

GSH, potassium peroxodisulfate ($K_2S_2O_8$), DPPH, Tris (hydroxylmethyl) aminomethane, potassium ferricyanide ($K_3Fe(CN)_6$), TCA, ferric chloride ($FeCl_3$), aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), sodium bicarbonate ($NaHCO_3$), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), genistein standard and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu solution and 95% ethanol were purchased from Merck Co. (Santa Ana, CA, USA). Plant materials were collected from Taichung, Nantou, and Hsinchu counties in Taiwan. They were identified and authenticated by Dr. Chao-Lin Kuo, Associate professor and Chairman, Department of Chinese Medicine Recourses, China Medical University, Taichung, Taiwan.

Preparing aqueous extracts of plant materials

Dried herb roots (100 g for each species) were boiled with 1 L deioned water for 1 hour. Filtration and collection of the extracts were done three times. The resulting decoction (about 1 L) was evaporated to 10 mL and dried in vacuum at 50°C. The dried extract was weighted and dissolved in distilled water (stock 4 mg/mL) and stored in -20°C for the usage in later steps. For each sample, the yield was calculated in percentage by dividing the quantity of dry mass obtained after extraction by the dry weight of the herb used (100 g).

Determining antioxidant activities by $ABTS^{\cdot+}$ scavenging ability

The $ABTS^{\cdot+}$ scavenging ability was determined according to the method of Chang et al. (2007a, b). Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 h in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). For each sample, an aliquot (20 μL) of sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution, and then the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard. A standard curve was constructed for Trolox at 0, 15.625, 31.25, 62.5, 125, 250, 500 μM concentrations. TEAC was expressed in millimolar concentration of trolox solution with the antioxidant equivalent to a 1000 ppm solution of the sample under investigation.

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay of the crude extracts was carried out according to the method of Huang et al. (2008). This assay measured the change in absorbance at 593 nm due to the action of electron donating antioxidants, changing the colorless oxidized Fe^{3+} into blue colored Fe^{2+}-tripyridyltriazine compound. To prepare the FRAP reagent, a mixture of 0.1 M acetate buffer (pH 3.6), 10 mM 2, 4, 6-tris(2-pyridyl)-*s*-triazine (TPTZ), and 20 mM ferric chloride (10:1:1, v/v/v) was

made. For each sample, an aliquot (10 μL) of sample solution (125 μg/mL concentration) was mixed with 300 μL FRAP reagent, and the absorbance was read at 593 nm after 15 min. A standard curve was constructed for $FeSO_4 \cdot 7H_2O$ at 0, 31.25, 62.5, 125, 250, 500, 1000 μg/mL concentrations. In the FRAP assay, the antioxidant efficiencies of the samples were calculated according to the reaction signal given by an Fe^{2+} solution of known concentration, which represented an one-electron exchange reaction. The results were corrected for dilution and expressed in μmol Fe^{2+}/mg.

Determining antioxidant activity by DPPH radical scavenging ability

The effects of crude extracts and positive controls (GSH and genistein) on DPPH radicals were estimated according to the method of Huang et al. (2007). Aliquots of crude extracts (20 μL) at various concentrations were each mixed with 100 mM Tris-HCl buffer (80 μL, pH 7.4) and then with 100 μL of DPPH in ethanol to a final concentration of 250 μM. All the mixtures were shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solutions were measured spectrophotometrically at 517 nm. The DPPH decolorizations of the samples were calculated in percentage according to the equation: % decolorization = $[1- (ABS_{sample} / ABS_{control})] \times 100$. IC_{50} value was the effective concentration in which DPPH 50% of radicals were scavenged and was obtained by interpolation with linear regression analysis. A lower IC_{50} value indicated a greater antioxidant activity.

Determination of total polyphenol content

The total polyphenol content of the crude extracts were determined according to the method of Huang et al. (2005). For each sample, 20 μL of the extract (125 μg/mL) was added to 200 μL distilled water and 40 μL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min, and then 40 μL of 20% sodium carbonate was added to the mixture. The resulting blue complex was measured at 680 nm. Catechin was used as a standard for the calibration curve. The polyphenol content was calibrated using the calibration curve based linear equation. The total polyphenol content was expressed as mg catechin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

Determination of total flavonoid content

The total flavonoid contents of the crude extracts were determined according to the method of Huang et al. (2004a, b). For each sample, an aliquot of 1.5 mL extract was added to an equal volume of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 mL methanol) solution. The mixtures were vigorously shaken, and the absorbances at 430 nm were read after 10 min of incubation. Rutin was used as the standard for the calibration curve. The total flavonoid content was calibrated using the linear equation based on the calibration curve. The total flavonoid content was expressed as mg rutin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

Determination of total flavonol content

The total flavonol content of the crude extracts was determined according to the method of Chang et al. (2007a, b). For each extract, an aliquot of 200 μL was added to 1 mL of 0.1% *p*-dimethylaminocinnamaldehyde (DMACA) in methanol/HCl (3:1, v/v). All the mixtures were vigorously shaken, and the absorbances were read after 10 min of incubation at 640 nm. Catechin was used as a standard for the calibration curve. The total flavonol content was calibrated using the linear equation based on the calibration curve. The total flavonol content was expressed as mg catechin equivalent/g dry weight. The dry weight indicated was the sample dry weight.

Inhibition assay for alpha-glucosidase activity

The alpha-glucosidase inhibitory effects of the aqueous extracts of the four *Flemingia* species were assayed according to the procedure described previously by Matsui et al. (2001) with minor modifications. Briefly, the enzyme reaction was performed using *p*-Nitrophenyl-alpha-D-glucopyranoside (PNP-glycoside) as a substrate in 0.1 M piperazine- N, N′-bis (2-ethanesulfonic acid) (PIPES) buffer, pH 6.8. The PNP-glycoside (2.0 mM) was premixed with samples at various concentrations. Each mixture was added to an enzyme solution (0.01 unit) to make 0.5 ml of final volume. The reaction was terminated by adding 1 ml of 0.64% *N*-(1-naphthyl) ethylenediamine solution (pH 10.7). Enzymatic activity was quantified by measuring the *p*-nitrophenol released from PNP-glycoside at 405 nm wave length. All reactions were carried out at 37°C for 30 min with three replications. Acarbose was used as a positive control. One set of mixtures prepared with an equivalent volume of PIPES buffer instead of tested samples was used as control. The concentration of the extracts required to inhibit 50% of α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

Measuring aldose reductase activity *in vitro*

Crude AR was prepared as in the following steps: lenses were removed from Sprague-Dawley (SD) rats weighing 250-280 g, and were kept frozen until use. A homogenate of rat lens was prepared in accordance with the method described by Hayman and Kinoshita (1965). A partially purified enzyme, with a specific activity of 6.5 U/mg, was routinely used in the evaluations of enzyme inhibition. The partially purified material was separated into 1.0 mL aliquots, and stored at -40°C. The AR activity was spectrophotometrically assayed by measuring the decrease in NADPH absorption at 340 nm over a 4 min period, using DL-glyceraldehyde as a substrate. Each 1.0 mL cuvette containing equal units of enzyme, 0.1M sodium phosphate buffer (pH 6.2) and 0.3 mM NADPH either with

or without 10 mM substrate and inhibitor was prepared (Lim et al., 2006). One set of mixtures prepared with an equivalent volume of sodium phosphate buffer instead of tested samples was used as control. The concentration of the extracts required to inhibit 50% of AP activity under the assay conditions was defined as the IC_{50} value.

Analyses of genistein and WFM by *LC-MS-MS*

Moderate amounts of WFM were weighed and dissolved in water. At first, the solutions were filtered through 0.45 μm PVDF filters. The *LC-MS-MS* (Waters 2695 separations module; detector: Waters 996 photodiode array detector; with a ES-D609 mass spectrometer) analysis was carried out under the following conditions: the Waters Cosmosil 5C18-AR-II column (5 μm, 4.6 × 150 mm) was used with 0.25% methanol for mobile phase A, acetonitrile was used as mobile phase B, and water was used as mobile phase C. The ratio of A:B:C was 20:20:60, and the gradient elution was ran at a flow rate of 0.5 mL/min. The injection volume was 10 μL, and a wavelength of 254 nm was used for detection. Pure genistein, was also analyzed using *LC-MS-MS* under the same conditions, and the retention time was used to identify the genistein in the samples.

Statistical analyses

Experimental results were presented as the mean ± standard deviation (SD) of three parallel measurements. The statistical analyses were performed by one-way ANOVA, followed by Dunnett's *t* test. The difference was considered to be statistically significant when the p value was less than 0.05.

RESULTS AND DISCUSSION

Extraction yields

The yields in the aqueous extracts of the four *Flemingia* species were given in Table 1. The yield percentages of aqueous extracts (code as W) in decreasing order were as follows: WFL (12.57%) > WFS (11.67%) > WFM (10.65%) > WFP (9.88%).

Table 1. Extraction yields from the aqueous extracts of four *Flemingia* species.

Sample	Aqueous extract yield (% w/w)[a]
WFL	12.57
WFM	10.65
WFP	9.88
WFS	11.67

[a]Dried weight basis.

Antioxidant activity estimated by ABTS and FRAP assay

ABTS assay is often used in evaluating the total antioxidant power of single compound and complex mixtures of various plants (Huang et al., 2004a, b; Huang et al., 2006). In this assay, ABTS radical monocations were generated directly from the stable form of potassium peroxodisulfates. Radicals were generated before the antioxidants were added to prevent interference of compounds which could have affected radical formation. This modification made the assay less susceptible to artifacts and prevented overestimation of antioxidant power (Sanchez-Moreno, 2002). Antioxidant samples were added to the reaction medium when the absorbance became stable, and then the antioxidant activity was measured in terms of decolorization.

Results of the ABTS assay were expressed in TEAC value. A higher TEAC value meant that the sample had a stronger antioxidant activity. TEAC values of the four *Flemingia* species were determined from the calibration curve, as shown in Table 2. Antioxidant activities of the aqueous extracts of the four *Flemingia* species were in the following decreasing order: WFM (1.13 ± 0.05 mM/mg extract) > aqueous extract of *Flemingia prostrata* (WFP) (0.48 ± 0.02 mM/mg extract) > aqueous extract of *Flamingia lineate* (WFL) (0.36 ± 0.01 mM/mg extract) > aqueous extract of *Flamingia strobilifera* (WFS) (0.32 ± 0.01 mM /mg extract). The antioxidant potency of genistein (positive control) was 0.31± 0.01 mM/mg extract.

The FRAP values of the aqueous extracts of the four *Flemingia* species were in the following order: WFM (1.53 ± 0.02 μmol Fe^{2+}/mg extract) > WFL (0.77 ± 0.03 μmol Fe^{2+}/mg extract) > WFS (0.42 ± 0.01 μmol Fe^{2+}/mg extract) > WFP (0.40 ± 0. 02 μmol Fe^{2+}/mg extract). The antioxidant potency of genistein was 0.23 ± 0.02 μmol Fe^{2+}/mg extracts (Table 2). Thus, it showed that WFM had the highest activity.

Both FRAP and TEAC assays were used to estimate the total antioxidant power because they were quick and simple to perform, and the reactions were reproducible and linearly related to the molar concentration of the antioxidants (Benzie et al., 1999). FRAP assay was initially developed to assay the plasma antioxidant capacity; however it can also be used to measure the antioxidant capacity of a wide range of biological samples, pure compounds, fruits, wines, and animal tissues (Katalinic et al., 2006).

Table 2. Total antioxidant activity assessed by TEAC and FRAP.

Species and positive control	TEAC (mM/mg extract)*	FRAP (μmol Fe^{2+}/mg extract)*
WFL	0.36 ± 0.01	0.77 ± 0.03
WFM	1.13 ± 0.05	1.53 ± 0.02
WFP	0.48 ± 0.02	0.40 ± 0.02
WFS	0.32 ± 0.01	0.42 ± 0.01
Genistein	0.31 ± 0.01	0.23 ± 0.02

*All values represent means ± S.D. of triplicate tests. (n = 3).

The correlation coefficients (R^2) of the FRAP and TEAC assays conducted on the aqueous extracts of the four *Flemingia* species were shown in Figure 1. The R^2 values of FRAP and TEAC assay showed high correlation (R^2=0.83).

Scavenging activity against 1, 1-diphenyl-2-picrylhydrazyl radicals

The relatively stable organic DPPH radicals are widely used in model systems to investigate the scavenging activities of several natural compounds, such as phenolics and anthocyanins, or crude mixtures. A DPPH radical is scavenged by antioxidants through the donation of a proton to form the reduced DPPH. The color changes from purple to yellow after reduction, which could be quantified by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increases when the percentage of free radical inhibition increases. Table 3 shows the IC_{50} values for the radical-scavenging activities of the four *Flemingia* species, GSH, and genistein using the DPPH colorimetric method. It was found that WFM had the lowest IC_{50} value (36.34 ± 1.22 μg/mL), followed by WFL (187.34 ± 1.28), WFS (197.97 ± 1.41 μg/mL), WFP (204.76 ± 0.57 μg/mL). The four extracts showed significant differences (p<0.05) in radical-scavenging activity. As demonstrated by the above results, the most active sample was WFM, however, its antioxidant capacity was still stronger than GSH and genistein positive controls (71.77 ± 1.39 μg/mL and 368 ± 5.35 μg/mL) in DPPH assay.

Total polyphenol, flavonoid, and flavonol content

The total polyphenol, flavonoid, and flavonol contents of the four *Flemingia* species are shown in Table 4. The total polyphenol content was expressed as μg of catechin equivalent per milligram of dry weight. The total polyphenol contents of the extracts of the four *Flemingia* species ranged from 45.46 to 197.73 μg CE/mg, and decreased in the following order: WFM > WFL > WFP > WFS. WFM had the highest polyphenolic content.

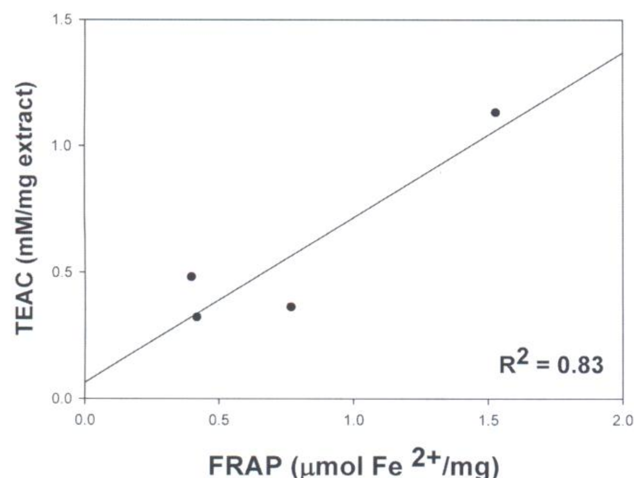

Figure 1. Correlation coefficients (R^2) of TEAC and FRAP in the aqueous extracts of the four *Flemingia* species.

Table 3. IC_{50} values of the aqueous extracts of four *Flemingia* species in DPPH radical scavenging activity.

Species and positive controls	IC_{50} (μg/mL)[a]
WFL	187.34 ± 1.28
WFM	36.34 ± 1.22
WFP	204.76 ± 0.57
WFS	197.97 ± 1.41
GSH	71.77 ± 1.39
Genistein	368 ± 5.35

[a]Values represent means ± S.D. of three parallel measurements.

Table 4. Contents of phytochemicals extracted from the aqueous extracts of four *Flemingia* species.

Species	Total phenols[a] (μg CE/mg)	Total flavonoids[b] (μg RE/mg)	Total flavonols[a] (μg CE/mg)
WFL	139.42 ± 1.59	0.53 ± 0.02	2.11 ± 0.10
WFM	197.73 ± 1.05	0.75 ± 0.04	2.76 ± 0.64
WFP	125.00 ± 0.58	0.58 ± 0.01	2.05 ± 0.04
WFS	45.46 ± 0.18	0.74 ± 0.04	2.08 ± 0.04

*All data are expressed as means ± S.D. of triplicate tests. (n = 3) (p < 0.05).

[a]Data expressed in μg catechin equivalent/mg dry weight (μg CE/mg).

[b]Data expressed in μg rutin equivalent/mg dry weight (μg rutin/mg).

The total flavonoid contents were expressed as μg of rutin equivalent per milligram of dry weight. The total flavonoid contents of the extracts of the four *Flemingia* species ranged from 0.53 to 0.75 μg RE/mg, and decreased in the following order: WFM > WFS > WFP > WFL. WFM had the highest flavonoid content.

The total flavonol contents were expressed as μg of catechin equivalent per milligram of dry weight. The total flavonol contents of the extracts of the four *Flemingia* species ranged from 2.05 to 2.76 μg CE/mg, and decreased in the following order: WFM > WFL > WFS > WFP. WFM had the highest flavonols.

Both flavonoid and flavonol are polyphenolic compounds. Polyphenolic compounds have important roles in stabilizing lipid oxidation and are associated with antioxidant activities (Yen et al., 1993). The phenolic compounds may contribute directly to antioxidative actions (Duh et al., 1999). It is suggested that 1.0 g of polyphenolic compounds from a daily diet rich in fruits and vegetables has inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al., 1998). The antioxidative activities observed could be ascribed both to the different mechanisms exerted by various phenolic compounds and to the synergistic effects of different compounds. The antioxidant assay used in

this study measured the oxidative products at the early and final stages of oxidation. The antioxidants had different functional properties, such as reactive oxygen species scavenging, e.g. quercetin, rutin, and catechin (Liu et al., 2008); free radical generation inhibitions and chain-breaking activity, for example *p*-coumaric acids (Laranjinha et al., 1995) and metal chelation (Van-Acker et al., 1998). These antioxidative compounds are usually phenolic compounds that are effective in donating protons, such as tocopherols, flavonoids, and other organic acids. However, the active contents responsible for the antioxidative activities of the four *Flemingia* species are still unclear. Therefore, further work must be performed to isolate and identify these components.

Relationship between the total antioxidant power and the total polyphenol, flavonoid, and flavonol content

The correlation coefficients (R^2) of the total antioxidant power (TEAC) and the total polyphenol, TEAC and the total flavonoid, and TEAC and the total flavonol of the aqueous extracts were shown in Figure 2. The R^2 value of TEAC and the total polyphenol content of the water (Figure 2A) extracts was 0.66. Similarly, R^2 value of TEAC and the total flavonoid content of the aqueous (Figure 2B) extracts was 0.26. R^2 value of TEAC and total flavonol content of the aqueous (Figure 2C) extracts was 0.94. Among the above 3 statistics, we could see that there were high correlations between TEAC and the total polyphenol and also TEAC and the total flavonol.

Inhibitiory assay for alpha-glucosidase activity

The α-glucosidase inhibitory activity of the aqueous extracts of the four *Flemingia* species are shown in Table 5. The effectiveness of enzymatic inhibition of the aqueous extracts of the four *Flemingia* species were determined by calculating IC_{50}. The lower the value, the higher the quality of enzymatic inhibition. The IC_{50} of the four *Flemingia* species in inhibiting α-glucosidase ranged from 153.92 to 1468.60 μg/mL, and its effectiveness was ranged as in the following increasing order:: WFM > WFL > WFP > WFS. WFM had the highest α-glucosidase inhibitory activity (IC_{50} = 153.92 ± 0.20 μg/mL). The positive controls against α-glucosidase inhibitory activity were genistein (IC_{50} = 16.65 ± 0.92 μg/mL) and acarbose (IC_{50} = 2596.04 ± 0.56 μg/mL).

The IC_{50} of positive control for alpha-glucosidase inhibitor (acarbose) is found much higher in the present assay which is similar to many previous literatures. Little articles had discussed it in detail (Youn et al., 2004; Shinde et al., 2008). When compared to acarbose as the control, only mammalian enzyme was inhibited. This was expected since acarbose has been shown to be a potent inhibitor of

Table 5. IC_{50} values of the aqueous extracts of four *Flemingia* species in α-glucosidase inhibition.

Species and positive controls	IC_{50} (μg/mL)[a]
WFL	269.66 ± 0.40
WFM	153.92 ± 0.20
WFP	1091.91 ± 1.63
WFS	1468.60 ± 2.10
Acarbose	2596.04 ± 0.56
Genistein	16.65 ± 0.92

[a]Values represent means ± S.D. of three parallel measurements.

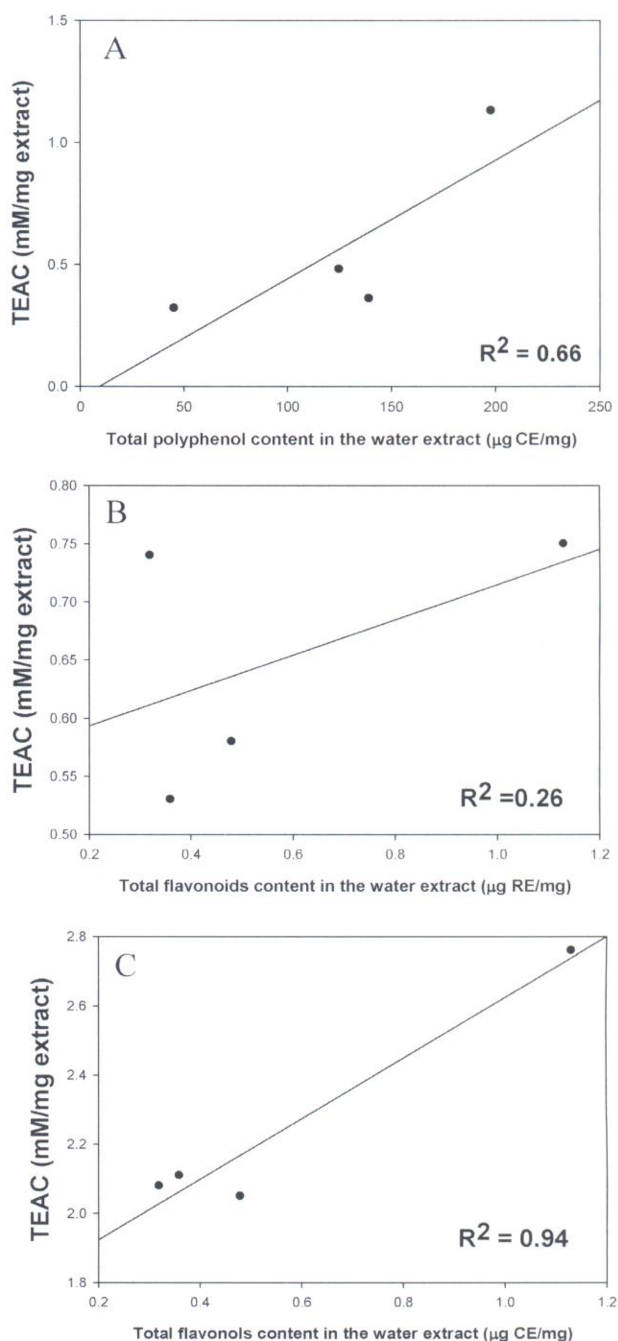

Figure 2. Correlation coefficients (R^2) of TEAC and total polyphenol (A), flavonoid (B) and flavonol (C) contents in the aqueous extracts of the four *Flemingia* species.

mammalian sucrase and maltase and inactive against yeast and bacterial forms (Kim et al., 2004).

Polyphenolic compounds in plants have long been recognized to inhibit the activities of digestive enzymes because of their ability to bind with proteins (Griffiths and Moseley, 1980). Various *in vitro* assays have shown that many plant polyphenols possess carbohydrate hydrolyzing enzyme inhibitory activities. These compounds include green tea polyphenols which inhibit the activities of α-glucosidase and sucrase (Hara and Honda, 1992), sweet potato polyphenols which inhibit the activities of α-glucosidase (Matsui et al., 2001), and berry polyphenols which inhibit the activities of α-glucosidase and α-amylase (McDougall and Stewart, 2005).

Genistein belongs to the isoflavonoid family. Most previous studies have focused on the pharmacological activities of genistein as a tyrosine kinase inhibitor, and its chemoprotectant activities against cancers and cardiovascular disease. Recently, Dong-Sun et al. also have reported that genistein could be a potent α-glucosidase inhibitor (Lee and Lee, 2001).

Measurement of aldose reductase activity *in vitro*

AR, the principal enzyme of the polyol pathway, has been shown to play an important role in the complications associated with diabetes. The AR inhibitory activity of aqueous extracts of the four *Flemingia* species are shown in Table 6. The IC_{50} of the extracts of the four *Flemingia* species AR inhibitory activities ranged from 79.36 μg/mL to 172.41 μg/mL, and increased as in the following order: WFM > WFS > WFL> WFP. WFM had the highest AR inhibitory activity (IC_{50} = 79.36 ± 3.20 μg/mL). The positive control in the AR inhibitory activity assay was genistein (IC_{50} = 45.62 ± 2.16 μg/mL).

Many natural compounds have been tested for AR inhibitory activities. Medicinal plants are particularly likely to be non-toxic and may be useful for the prevention and treatment of diabetes-related complications (Preet et al., 2006). In addition to its antioxidant properties, genistein has an inhibitory effect on the formation of advanced glycation end products (Jang et al., 2006). Further evidence that genistein can inhibit diabetic related

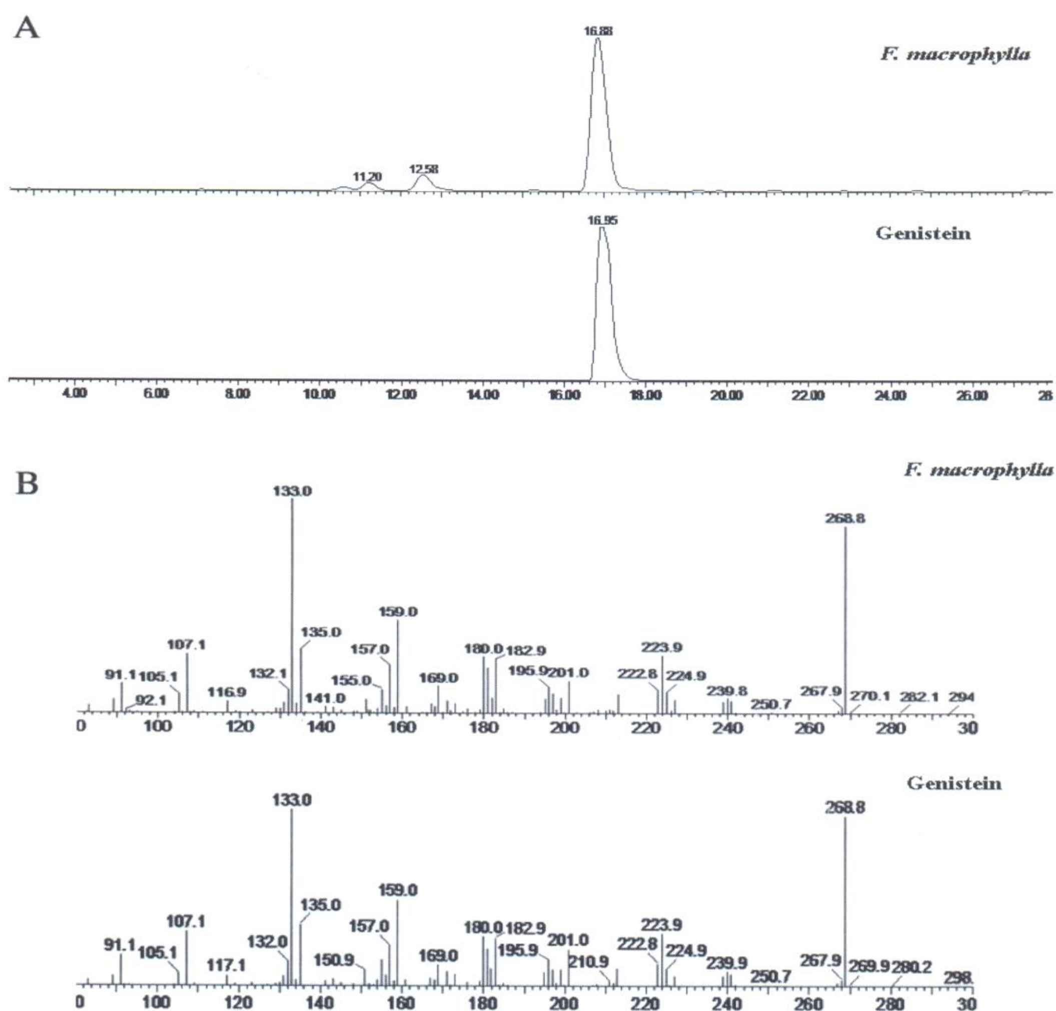

Figure 3. (A) High performance liquid chromatogtraphic profile of genistein in WFM and genistein standard; (B) Daughter ion spectrum of major peaks in WFM (Rt = 16.88) and genistein standard (Rt = 16.95).

problems stems from studies with type 2 diabetic animals; genistein has been shown to decrease blood glucose and glycated hemoglobin levels ($HbA1_{IC}$) and increase the glucagon/insulin ratio (Lee, 2006).

It has been well-acknowledged that plant-derived extracts and phytochemicals are potential alternatives to synthetic inhibitors against AR and α-glucosidase (Lee, 2006). Currently, AR inhibitor and α-glucosidase inhibitor compounds isolated from plants are classified as diterpene-, triterpene-, and flavonoid-related compounds. In this study, the active component isolated from WFM against aldose reductase and α-glucosidase was identified as genistein, even though the inhibitory responses varied with concentrations.

Compositional analyses of genistein and WFM by *LC-MS-MS*

To verify whether genistein was present in WFM or not, both WFM and genistein standard were separated through HPLC column separately under the same conditions. A prominent peak appeared in WFM (Rt = 16.88) which was equivalent to the genistein peak with Rt = 16.95 (Figure 3A). In order to confirm the identity of genistein in WFM, the two peaks were then subject to ESI/MS/MS analyses. The daughter ion spectrum of the major peak in WFM (Rt = 16.88) was found to be identical to that of the genistein standard. (Figure 3B). *LC-MS-MS* analyses confirmed the existence of genistein in WFM.

In conclusion, the results from *in vitro* experiments, including ABTS radical monocation scavenging, FRAP method (Table 2), DPPH radical scavenging (Table 3), total polyphenol content, total flavonoid content and total flavonol content (Table 4), α-glucosidase inhibition (Table 5), AR inhibition (Table 6) and *LC-MS-MS* assay (Figure 3) demonstrated that the phytochemicals in the aqueous extracts of the four *Flemingia* species might have significant antioxidant and anti-diabetic activities, directly related to the total amount of polyphenols and flavonols. Hence, the four *Flemingia* species could be used as easy accessible sources of natural antioxidants in pharmaceutical and medical industries. For this reason, further work should be performed to isolate and identify the antioxidative or anti-diabetic components of the FM.

Table 6. IC_{50} values of the aqueous extracts of four *Flemingia* species in aldose reductase inhibition.

Species and positive controls	IC_{50} (μg/mL)[a]
WFL	108.69 ± 1.40
WFM	79.36 ± 3.20
WFP	172.41 ± 3.13
WFS	112.12 ± 2.32
Genistein	45.62 ± 2.16

[a] Values represent means ± S.D. of three parallel measurements.

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臺灣產四種佛來明屬植物水萃取物之抗氧化，α-葡萄糖苷酶抑制劑和醛糖還原酶抑制劑活性

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本實驗針對臺灣產四種佛來明屬植物之水萃取物進行抗氧化及抗糖尿病活性研究，分析方法包括ABTS自由基的清除、FRAP方法、DPPH自由基的清除、總多酚類含量、總類黃酮類含量、總黃酮醇類含量以及抑制 *α*- 葡萄糖苷酶和醛糖還原酶活性。結果顯示佛來明屬植物之水萃取物中以大葉千斤拔具有最強之抗氧化活性。我們也發現大葉千斤拔的水萃取物之多酚類、類黃酮和黃酮醇含量最高。在TEAC與FRAP之相關係數 (R^2) 顯示具有高的相關性 (R^2 值爲 0.83)，而TEAC與多酚類含量之相關係數 (R^2) 也顯示具有較高的相關性 (R^2=0.66)，又TEAC與水萃取物中類黃酮類的 R^2 值也顯示具有高的相關性 R^2=0.94。在四種佛來明屬植物之水萃取物抗糖尿病體外活性試驗，分析方法爲抑制 *α*- 葡萄糖苷酶和醛糖還原酶活性。結果顯示水萃取物中以大葉千斤拔具有最強之抑制 *α*- 葡萄糖苷酶和醛糖還原酶活性，抑制酶活性50%之濃度爲 153.92 ± 0.20 μg/mL 和 79.36 ± 3.20 μg/mL 正對照組金雀異黃酮 genistein 也具有相當強的抑制 *α*- 葡萄糖苷酶和醛糖還原酶活性 (IC_{50} =16.65 ± 0.92 μg/mL 和 45.62 ± 2.16 μg/mL)。由於大葉千斤拔其水萃取物具有最高的抗氧化及抗糖尿病活性，利用LC-MS-MS的分析建立指紋圖譜，發現金雀異黃酮 genistein 可能是大葉千斤拔水萃取物中的一個重要活性成分。因此，本實驗結果建議大葉千斤拔未來可以做爲一個天然抗氧化劑和抗糖尿病的藥物來源。

關鍵詞：抗氧化；醛糖還原酶；α- 葡萄糖苷酶；佛來明屬；自由基；多酚類。