ANTIOXIDANT AND ANTITYROSINASE PROPERTIES OF MALAYSIAN BAMBOO LEAF EXTRACTS

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MOHD ILHAM, A., VIMALA, S., ABDULL RASHIH, A., ROHANA, S., JAMALUDDIN, M. & JULIZA, M. 2008. Antioxidant and antityrosinase properties of Malaysian bamboo leaf extracts. The aqueous and ethanolic extracts of Malaysian bamboo species, namely, *Gigantochloa scortechinii, G. levis, G. ligulata* and *Schizotachyum zollingeri* were tested for their antioxidant properties using three bioassay systems: (1) ferric thiocyanate (FTC) method, (2) xanthine/xanthine oxidase superoxide scavenging activity (X/XOD) method and (3) free radical scavenging activity (2,2-diphenyl-2-picrylhydrazyl, DPPH) method. Tyrosinase inhibitory assay was used to evaluate the skin whitening properties of the four bamboo species studied. The aqueous of all species studied showed very high (> 90%) superoxide scavenging activity and the activity increased with an increase in the total phenolic content (TPC). Ethanolic extracts of *G. ligulata* and *S. zollingeri* at the concentration of 1.0 mg/ml each demonstrated very high tyrosinase inhibitory activities, 98.9 and 100% respectively. This indicated that these strong active extracts have the ability to inhibit the enzymatic pathway of melanin pigment biosynthesis. The high performance liquid chromatography (HPLC) fingerprint developed for the four bamboo species revealed the presence of ρ-coumaric acid as major phenolic compound in the extracts.

Keywords: Superoxide and free radical scavenging, skin whitening, phenolic compounds, ρ-coumaric acid

MOHD ILHAM, A., VIMALA, S., ABDULL RASHIH, A., ROHANA, S., JAMALUDDIN, M. & JULIZA, M. 2008. Sifat antioksida dan antitirosinase ekstrak daun buluh Malaysia. Ekstrak akues dan ekstrak etanol spesies buluh Malaysia iaitu *Gigantochloa scortechinii, G. levis, G. ligulata* and *Schizotachyum zollingeri* diuji sifat antioksida masing-masing menggunakan tiga jenis sistem biocerakin iaitu (1) kaedah ferik tiosianat (FTC), (2) kaedah aktiviti pembilasan superoksida xanthina/xanthina oksidase (X/XOD) dan (3) kaedah aktiviti pembilasan radikal bebas (2,2-difenil-2-pikrilhidrazil, DPPH). Cerakinan rencatan tirosinase digunakan untuk menilai sifat pemutih kulit keempat-empat ekstrak buluh tersebut. Ekstrak akues semua spesies buluh yang dikaji menunjukkan aktiviti pembilasan superoksida yang tinggi (> 90%) dan peningkatan aktiviti ini adalah selari dengan peningkatan jumlah sebatian fenol (TPC) dalam ekstrak tersebut. Ekstrak etanol *G. ligulata* dan *S. zollingeri* masing-masing telah menunjukkan aktiviti rencatan tirosinase yang tinggi iaitu 98.9% and 100% pada kepekatan 1.0 mg/ml. Ini menunjukkan bahawa ekstrak aktif ini mempunyai keupayaan untuk merencat laluan biosintesis pigmen melanin. Cap jari kromatografi turus cecair prestasi tinggi (HPLC) yang diperoleh untuk keempat-empat ekstrak spesies buluh ini menunjukkan kehadiran asid ρ-kumarik yang bertindak sebagai sebatian fenol utama.

INTRODUCTION

Bamboo leaves have been reported to have anti-free radical activity and are comparable with the leaf of *Ginkgo biloba*, which is one of the resources of natural antioxidant and free radical scavenger (Hibatallah *et al.* 1999, Hu *et al.* 2000). The antioxidant bamboo leaves extract was reported to have the ability to block chain reaction of lipid auto-oxidation, chelate metal ions of transient state, scavenge nitrite compounds and to block the synthetic reaction of nitrosamine (Zhang *et al.* 2007b). Based on these characteristics, antioxidant bamboo leaves (AOB) have recently been listed by the Ministry of Health, People Republic of China as a kind of food antioxidant and are permitted to be used in food, dairy and meat products as well as oils (Zhang *et al.* 2007a).

There are about 70 known bamboo species in Malaysia of which 50% are in Peninsular Malaysia, 30% in Sabah and 20% in Sarawak. However, only

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12 species are being utilized commercially (FAO 1997). These species grow wild in the forests and are also cultivated by villagers in rural areas. Four species, namely, *Gigantochloa scortechinii*, *G. levis*, *G. ligulata* and *Schizotachyum zollingeri* are the most commonly exploited for commercial purposes (furniture and handicraft). However, the industrial and medicinal uses of the leaves of these four species are still not fully exploited in this country. In addition, no report has been published yet for antioxidant and skin whitening properties of the leaf extracts of these Malaysian bamboo species.

The aim of this study was to evaluate antioxidant and skin whitening properties of aqueous and ethanolic leaf extracts of *G. scortechinii*, *G. levis*, *G. ligulata* and *S. zollingeri* and to evaluate their various mechanistic pathways as well as to quantify the ρ-coumaric acid as a major phenolic compound in the extracts.

MATERIALS AND METHODS

Reagents and standard

Methanol, ethanol and acetonitrile of high performance liquid chromatography (HPLC) quality were purchased from MERCK (New Jersey, USA). High-purity water was obtained by a Millipore system. Sample and solvents were filtered using 0.45 µm membrane filters (Millipore, Massachusetts, USA). All solutions were degassed prior to use. Chemicals, reagents of analytical grade, standards and enzymes were obtained from SIGMA (St. Louis, USA). Xanthine oxidase (XOD) was supplied by Roche (New Jersey, USA).

Plant material

Leaves of *G. scortechinii*, *G. levis*, *G. ligulata* and *S. zollingeri* were collected from the Bamboo Arboretum at the Forest Research Institute Malaysia (FRIM), Kepong. Herbarium specimen of each species was deposited in the herbarium at FRIM. The leaves were left to dry at room temperature. After drying all samples had water contents below 10%. They were ground to fine powder in a mechanical blender and kept at room temperature prior to extraction.

Bamboo leaf powder was refluxed with 1 l water for three hours at 100 ˚C or soaked for 48 hours in 600 ml ethanol at room temperature. The extracts were filtered through Whatman No. 42 filter paper. The ethanol extract was evaporated at low temperature under reduced pressure in a rotavapor. The water liquid extract was concentrated to one-third of the initial volume before freeze-drying.

Analyses

Analyses were carried out in a liquid chromatograph equipped with two pumps (Waters Delta 600), an automated gradient controller (model 600), an injector (Rheodyne) with a 20 µl loop and a photodiode array detector (Waters model 996) operated at λ = 280 nm and a Millennium 32 software. The analytical column, Phenomenex-Luna 5uC18 100A (250.0 × 4.6 mm, i.d. 5.0 mm), was kept at room temperature. Samples were quantified by using peak heights.

The mobile phase used for the separation was water (solvent A) containing 0.1% H_3PO_4 and acetonitrile (solvent B). Samples were eluted according to the following gradient: the gradient was started with 15% of B in order to reach 25% B at 10 min, and 15% B at 11 and 35 min. The flow-rate was 1.0 ml/min. The injection volume was 20 µl.

Six commercially available phenolic compounds, namely, 3,4-dihydroxybenzoic acid (protocatechuic acid), ρ-coumaric acid (CA), ferulic acid, vanillic acid, *trans*-cinnamic acid and ellagic acid were used as standards. To prepare the standard solutions, stock solutions (1 mg/ml) were made in acetonitrile and stored in darkness at 4 °C. Dilutions of different concentrations ranging from 5–100 µg/ml were made from these solutions using acetonitrile as solvent. Calibration graphs were obtained using the standards at different concentrations.

Bioassay systems

Ferric thiocyanate (FTC) method

The autoxidation assay was performed based on the method of Osawa and Namiki (1981). A sample solution containing 4.0 mg plant extract in 4.0 ml of 99% ethanol, 4.1 ml of 5.0% linoleic acid in 99.5% ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of distilled water was placed in a columnar vial (diameter

40 mm, height 75 mm) with a screw cap and incubated in the dark at 40 °C for one week. To 0.1 ml of this sample solution were added 9.7 ml of 75% ethanol and 0.1 ml of 30% of ammonium thiocyanate. Ferrous chloride (0.1 ml of 2×10^{-2} M) in 3.5% hydrochloric acid was then added to the reaction mixture. Exactly 3 min later the absorbance of the red colour developed was measured at 500 nm. Antioxidant activity was judged from the decrease in the absorbance value relative to the negative control which was incubated without plant sample. Butylated hydroxyl toluene, BHT (4 mg) was used as a positive control.

Xanthine /xanthine oxidase superoxide scavenging activity (X/XOD) method

The method of Chang *et al.* (1996) was used with slight modification. Nitro blue tetrazolium (NBT) solution (100 ml of 4.1 mM) was prepared by adding 3.15 g Tris HCl , 0.1 g $MgCl_2$, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4-nitro blue tetrazolium chloride to 100 ml of distilled water. The reaction mixture (100 ml) was prepared by dissolving 0.53 g Na_2CO_3 (pH 10.2), 4.0 mg ethylene diamine-tetraacetic acid (EDTA) and 2.0 mg xanthine in 0.025 mM NBT solution. The mixture was kept refrigerated at 4 °C. The reaction mixture (999 µl) was transferred into a microcuvette and placed in a 25 °C cell holder of a spectrometer. Generation of superoxide was initiated by adding 0.1 µl of XOD (1×10^{-3} U/ml). The optical density (OD) measurement was taken at 560 nm for 120 s using a Lamda 2S spectrophotometer. The reaction mixture (979 µl) was transferred into a microcuvette and placed in the 25 °C cell holder of a spectrometer. A total of 0.1 µl superoxide dismutase (SOD) (1.16 U/m) was added into the reaction mixture and thoroughly mixed. The 0.1 µl of XOD (1×10^{-3} U ml) was added to start the generation of oxyradicals. OD was taken at 560 nm for 120 s at intervals of 10 s. Plant extracts were dissolved in the reaction mixture to a concentration of 250 µg/ml. The stock solution (5 µl) was added to 995 µl of the reaction mixture and placed in the cell holder to autozero. XOD (1×10^{-3} U/ml) was then added to the stock solution and thoroughly mixed before being measured for XOD and SOD curves.

Free radical scavenging activity (2,2-diphenyl-2-picrylhydrazyl, DPPH) method

The effect of plant extracts on DPPH radical was estimated according to the method of Blois (1958). Extracts (4.0 ml of 0.5 mg/ml) were added to 1.0 ml of DPPH (1.0 mM in methanolic solution) in a 5.0 ml bottle with screw cap. The mixture was shaken and left to stand at room temperature for 10.0 min. Similarly, the absorbance of the resulting solution was measured at 520 nm.

Tyrosinase inhibitory assay

The method of Tomita *et al.* (1990) was slightly modified. A pre-incubation mixture consisting of 1.8 ml of 0.1 M phosphate buffer (pH 6.5), 0.6 ml of H_2O_2, 0.1 ml of the sample solution (1 mg/ml in ethanol) and 0.1 ml of the aqueous solution of mushroom tyrosinase (9600 U/ml) was pre-incubated at 25 °C for 5 min. Then 0.4 ml of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 100 s. L-cystine (8 mg/ml) was used as the positive control. The negative control has ethanol instead of the sample.

Total phenolic content

The amount of total phenolic content in extracts was determined according to the Follin-Ciocalteu procedure used by Kahkonen *et al.* (1999). Samples (300 µl in triplicates) were dispensed into test tubes followed by 1.5 ml Follin-Ciocalteu's reagent (10× dilution) and 1.2 ml sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorption was measured at 765 nm. Total phenolic content was expressed as gallic acid equivalent (GAE) in mg/100 g material.

RESULTS AND DISCUSSION

Table 1 shows the percentage of yields of ethanol and aqueous extracts and antioxidant activities of the four bamboo species in this study. With the exception of *S. zollingeri* and *G. ligulata* the extracts showed high antioxidant activities, i.e. > 70% inhibition in all three pathways (lipid peroxidation inhibitory, superoxide scavenging and DPPH scavenging activities).

Ethanolic and aqueous extracts of *S. zollingeri* showed very high level of inhibitory activity (> 90%) towards lipid peroxidation. Interestingly, the aqueous extract of all four bamboo species studied showed very high (> 90%) superoxide scavenging activity. The total phenolic contents for ethanolic and aqueous bamboo extracts are shown in Figure 1. All aqueous bamboo extracts except *G. ligulata* showed higher total phenolic content compared with their respective ethanolic extracts. It is noteworthy to mention that higher scavenging activities observed in aqueous extract than that of ethanolic extract corresponded to the high amounts of TPC in aqueous extract. The superoxide anion ($O_2^{\cdot -}$) scavenging properties of *G. scortechinii*, *G. levis*, *G. ligulata* and *S. zollingeri* aqueous extracts may be attributed to both neutralization of $O_2^{\cdot -}$ radicals via hydrogen donation and inhibition of xanthine oxidase by various phenolics present in the extracts. Polyphenols such as catechin, epicatechin, epigallocatechin, catechin gallate, epicatechin gallate and epigallocatechin gallate have been reported to be widely distributed in plants and algae and these phenolic antioxidants have been found to act as free radical scavengers (Sanchez-Moreno *et al.* 1999, Yoshie *et al.* 2002). Recently, Kurosumi *et al.* (2007) developed a

Table 1 Antioxidant and tyrosinase inhibitory activities of four species of Malaysian bamboo

Species	Extract	Extraction yield (%)	Lipid peroxidation inhibitory activity (%)	Superoxide scavenging activity (%)	DPPH scavenging activity (%)	Tyrosinase inhibition activity (%)
Gigantochloa scortechinii	Ethanolic	4.5	75.6 ± 0.4	76.6 ± 2.15	75.1 ± 2.4	25.3 ± 1.5
	Aqueous	8.5	74.8 ± 1.75	93.2 ± 2.5	75.4 ± 4.75	24.1 ± 2.1
G. levis	Ethanolic	3.5	76.0 ± 1.35	76.8 ± 2.25	86.4 ± 1.05	24.1 ± 0.3
	Aqueous	6.0	76.5 ± 3.5	99.0 ± 1.05	91.1 ± 0.1	24.1 ± 2.1
G. ligulata	Ethanolic	4.4	73.2 ± 0.65	53.5 ± 0.0	87.4 ± 0.1	98.8 ± 1.2
	Aqueous	8.3	77.3 ± 0.2	98.9 ± 1.15	83.4 ± 1.25	33.7 ± 0.4
Schizotachyum zollingeri	Ethanolic	5.5	96.0 ± 0.2	57.1 ± 1.0	52.2 ± 0.5	100.0 ± 0.0
	Aqueous	6.0	98.5 ± 0.2	94.0 ± 1.25	71.9 ± 0.15	29.7 ± 2.95

Values are means of triplicates per treatment (± SE). Final concentrations of samples used were 0.25 µg/ml , 250 µg/ml, 400 µg/ml and 0.17 mg/ml for lipid peroxidation, superoxide scavenging, DPPH scavenging and tyrosinase inhibition test respectively. Positive control used for each test showed 100% inhibition.

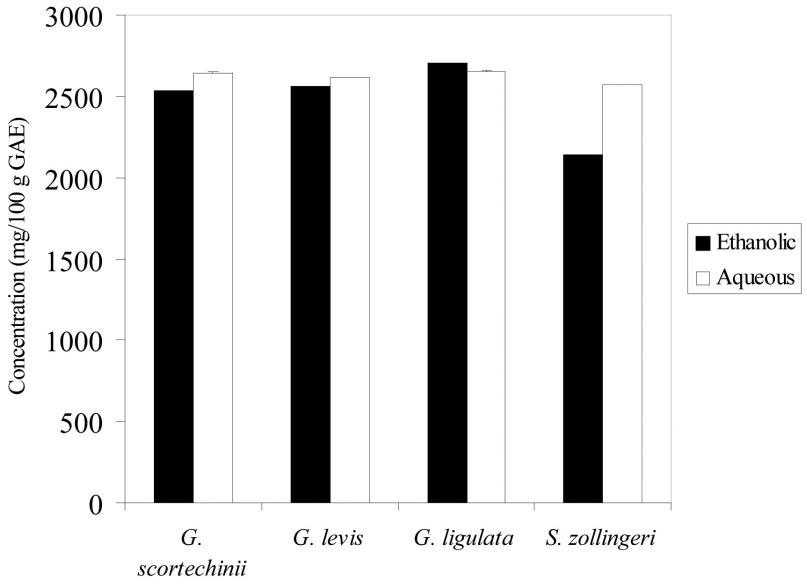

Figure 1 Total phenolic content of bamboo leaf extracts. Results are expressed as gallic acid equivalents (GAE) in mg/100 g material (n = 3).

novel extraction method of antioxidant phenolic compounds from *Sasa palmata* (Bean) Nakai, a bamboo plant whose leaves are commonly used to wrap foodstuffs in Japan. This proves the food preservative properties of bamboo leaves.

Radical scavenging activities of ethanolic and aqueous extracts of the four selected Malaysian bamboo were determined from the reduction in the optical absorbance at 520 nm due to the scavenging of stable DPPH radical. All extracts except for ethanolic extract of *S. zollingeri* showed moderate to high DPPH scavenging activities (Table 1). Except for *G. levis*, it was observed that the aqueous extracts showed higher DPPH scavenging activities than the ethanolic extracts and the activities were increased with an increased content of TPC in the aqueous extract. Several flavonoids and polyphenols have been isolated from plant extracts with potent DPPH scavenging activities such as piceatannol, *trans*-resveratrol, apigenin and scirpusin A (Lee *et al.* 1998).

Ethanolic extracts of *G. ligulata* and *S. zollingeri* demonstrated very high tyrosinase inhibitory activities (98.9 and 100% respectively) at the concentration of 1.0 mg/ml (Table 1). These strong active extracts have the ability to inhibit the catalytic pathway of melanin pigment biosynthesis and thus we feel could be applied as skin whitening materials for cosmetics or as browning-prevention agents for food. Ethanol has also been shown to be an effective solvent for extracting tyrosinase-inhibiting materials from plant species (Soo *et al.* 2003, Masuda *et al.* 2007). Chinese traditional medicines with tyrosinase inhibitory activity have been reported to be used as cosmetics for beauty treatment (Miao *et al.* 1997). Determining the active constituents present in the leaf extract would be interesting and deserves further study.

For comparison purposes, attempt was made to evaluate the antioxidant potential and antityrosinase property of six commercially available phenolic compounds, namely, 3,4-dihydroxybenzoic acid (protocatechuic acid), ρ-coumaric acid (CA), ferulic acid, vanillic acid, *trans*-cinnamic acid and ellagic acid (Table 2). It was observed that 3,4-dihydroxybenzoic acid and ellagic acid exerted high DPPH and superoxide scavenging activities. Both phenolics also showed very high tyrosinase inhibitory suggesting that they have the ability to inhibit the catalytic pathway of melanin pigment biosynthesis. 3,4-dihydroxybenzoic acid and ellagic acid are natural phenolic compounds found in many edible and medicinal plants which have been reported to scavenge the reactive oxygen species associated with the initiation of lipid peroxidation (Meyer *et al.* 1998, Nunez-Selles 2005). In another study, ellagic acid exhibited skin whitening property on guinea pig, suppressing melanogenesis by reacting with activated melanocytes without injuring the cells (Shimogaki *et al.* 2000). In the current study, it was observed that CA showed very low antioxidant activity toward DPPH radical and showed no superoxide anion scavenging activity (Table 2).

An HPLC fingerprint of the four bamboo species studied was developed. A wavelength of 330 nm was used for detection. Results indicated that the chromatographic patterns of aqueous and ethanolic extracts of the four species were generally consistent although the absorption intensities of some peaks were different (Figure 2). Peak 1 at 18.07 min (average) was the highest peak in the chromatograms representing the major compound of chemical components in the aqueous extracts of the bamboo species. The ethanolic extracts of *G. scortechinii* and *S. zollingeri*

Table 2 Antioxidant potential and whitening properties of six commercially available phenolic compounds

Phenolic compound	DPPH radical scavenging (%)		Superoxide scavenging (%)		Tyrosinase inhibitory activity (%)	
Trans-cinnamic acid	2.3 ± 0.71	L	29.1 ± 3.50	L	> 100 ± 0.00	H
3,4–Dihydroxybenzoic acid	99.1 ± 0.07	H	92.1 ± 1.40	H	92.5 ± 1.35	H
ρ-Coumaric acid	11.9 ± 0.91	L	0.0 ± 0.00	NIL	76.4 ± 0.75	H
Ferulic acid	99.3 ± 0.11	H	42.4 ± 1.10	L	65.6 ± 1.15	M
Vanillic acid	31.1 ± 0.96	L	14.7 ± 1.65	L	97.9 ± 0.01	H
Ellagic acid	> 100 ± 0.00	H	93.7 ± 0.20	H	94.7 ± 0.90	H

H – high; M – medium; L – low. Values are means of triplicates per treatment (± SE). Final concentrations of samples used were 400 µg/ml, 250 µg/ml and 0.17 mg/ml for DPPH scavenging, superoxide scavenging and tyrosinase inhibition test respectively.

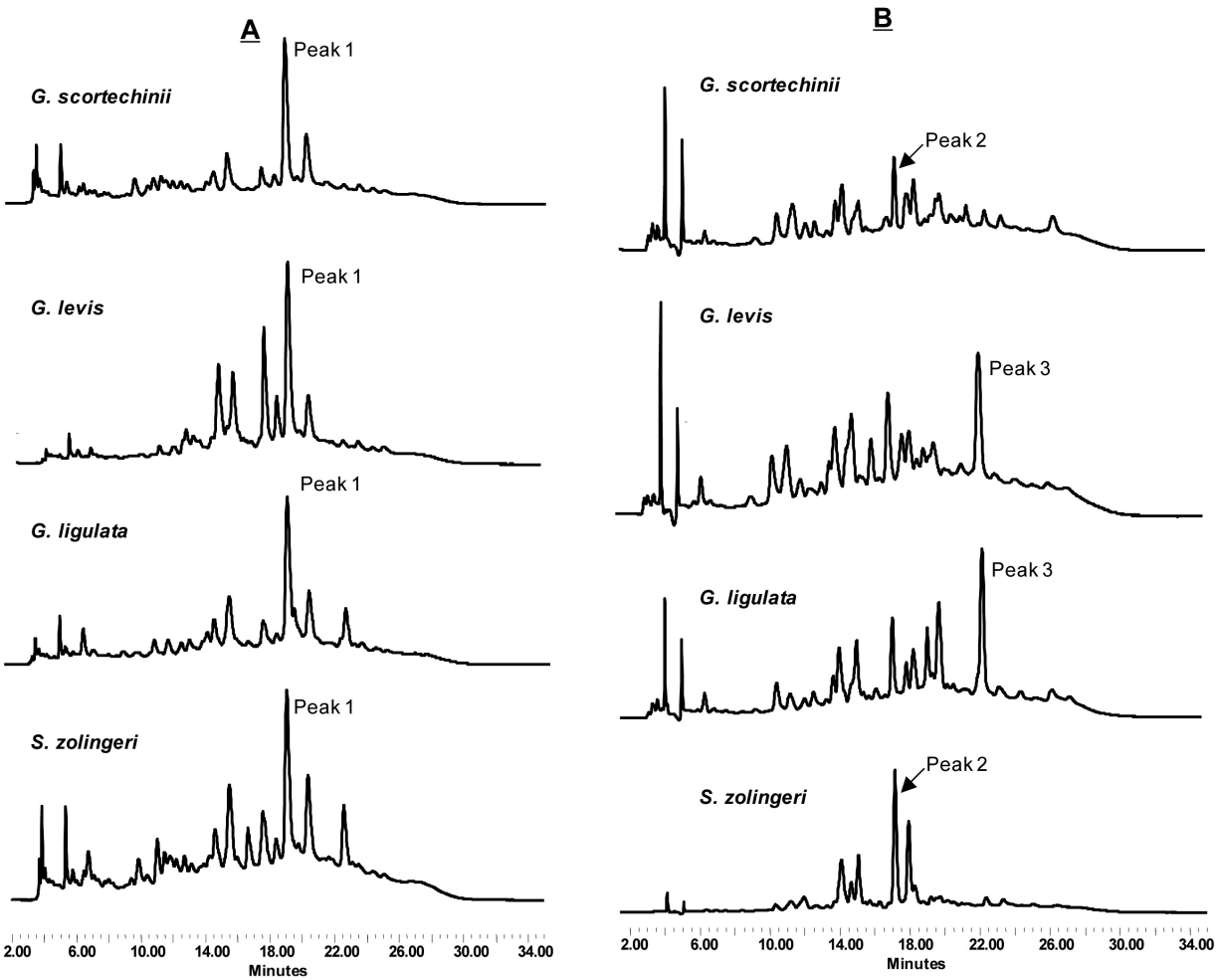

Figure 2 HPLC chromatogram of aqueous (A) and ethanolic (B) extracts of *G. scortechinii, G. levis, G. ligulata* and *S. zollingeri*. Peaks I, 2 and 3 are highest peaks in the chromatograms representing major compounds of chemical components in the extracts.

exhibited main chemical component at peak 2 (average retention time = 16.44 min), whereas *G. levis* and *G. ligulata* showed similar main chemical compound at peak 3 (average retention time = 21.81 min).

We also carried out HPLC analyses of six commercially available phenolic compounds and determined their maximum absorption wavelengths (chromatogram not shown). Further HPLC analyses of peak 1 in the aqueous extracts of the four bamboo species revealed that they have maximum absorption wavelength similar to that of CA (λ_{max}= 226.9 nm and 309.6.nm) (Figure 3). The retention time and spectra of peak 1 were also consistent with those of CA. The work to identify chemical component of peak 2 and 3 is ongoing.

CA was found to be higher in the aqueous extract compared with the ethanol extract (Figure 4). Aqueous extract of *G. levis* had the highest concentration of CA. CA can be widely found in fruits, such as apples and pears, and in vegetables and plant products, such as beans, potatoes, tomatoes and tea. It is an intermediate product of the phenylpropanoid pathway in plants (Bremner *et al.* 2000). However, compared with findings by other workers, e.g. Castelluccio *et al.* (1996), this study showed low antioxidant activities of CA (Table 2). The exact reason for the low antioxidant activities is not known. However, the *trans-cis* isomerism of CA used in the study may have contributed to these lower values. Other workers have reported that CA exhibited antioxidant properties and was able to provide antioxidant protection to low-density lipoprotein (LDL) *in vitro* as a result of its chain-breaking activity (Loake *et al.* 1992, Castelluccio *et al.* 1996, Laranjinha *et al.* 1996). Antioxidant properties of

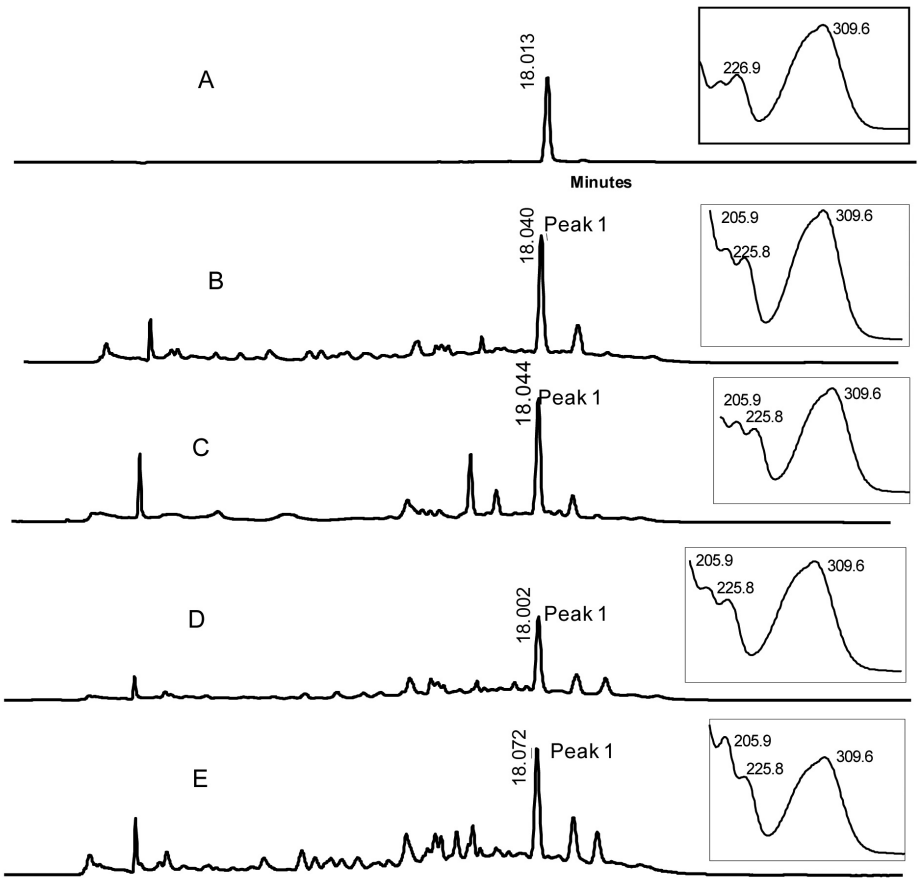

Figure 3 Typical chromatogram and UV spectra of (A) ρ-coumaric acid and peak 1 in the bamboo leaf aqueous extracts of (B) *G. scortechinii*; (C) *G. levis*; (D) *G. ligulata* and (E) *S. zollingeri*

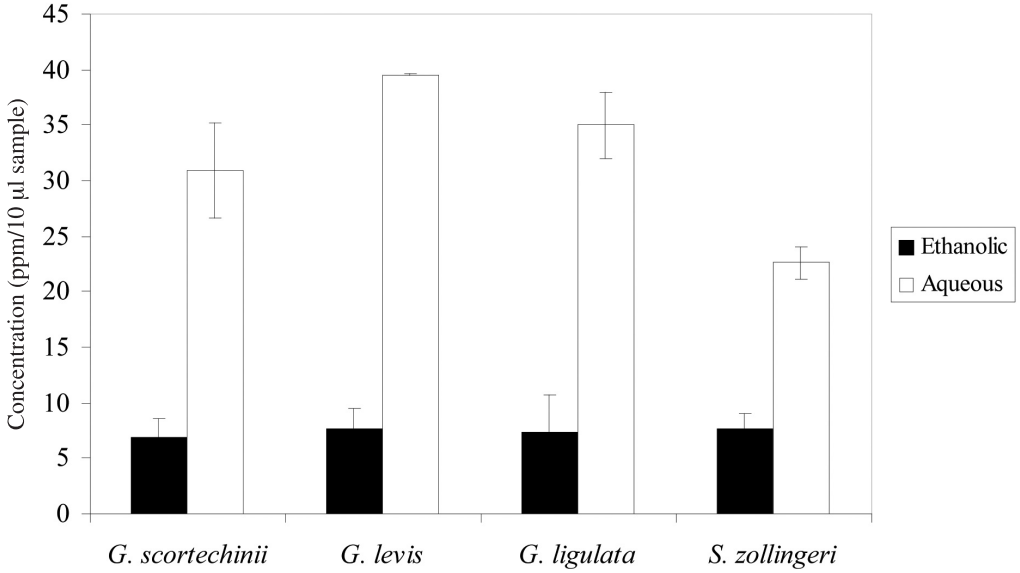

Figure 4 ρ-Coumaric acid (CA) content in bamboo leaf extracts (n = 3). Bars indicate SD.

CA may involve the direct scavenging of reactive oxygen species, which consist of free radicals such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl (HO.) radicals and non-free radical species such as H_2O_2 and singled oxygen. They are different forms of activated oxygen that can react with most biological molecules including proteins, lipids, lipoproteins and DNA (Halliwell & Gutteridge 1999, Lun-Yi *et al.* 2000). The antioxidant activity of CA and the ability to scavenge free radicals have been reported recently for bamboo leaf extract (Zhang *et al.* 2007a). Bamboo extracts with antioxidant activity may act as electron donors and can react with free radicals to convert them into more stable products and terminate radical chain reaction (Mu *et al.* 2004).

Results from this study suggest that CA is not the only active radical scavenging compound in leaves of *G. scortechinii*, *G. levis*, *G. ligulata* and *S. zollingeri* that possesses beneficial antioxidant properties against free radicals as well as LDL oxidation. The activities could be due to the presence of other compounds or CA reacts synergistically with these compounds to exert the antioxidant activities. The presence of CA as a major compound detected by HPLC in the bamboo leaf aqueous extracts can be used as a chemical or biological marker in the preparation of standardized natural antioxidant and skin whitening extracts.

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