

Antioxidant activities and polyphenol contents of six folk medicinal ferns used as “Gusuibu”

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ABSTRACT. In the traditional Chinese system of medicine, the folk remedy “Gusuibu,” renowned for its therapeutic effects on bone is sourced from six different ferns. However, no scientific investigation has been carried out so far, to evaluate the comparative values of these sources. In the present report, ethanol and aqueous extracts of these six sources were characterized for their antioxidant, scavenging activities, reducing power, total polyphenols, flavonols, flavonoids, condensed tannins, and proanthocyanidin contents. Results showed wide variation among the six sources. Most samples in aqueous extracts had higher antioxidant potencies and polyphenol contents than the ethanol extracts, indicating that the aqueous preparation of “Gusuibu” is more potent than the ethanol one. EC₅₀ values of reducing capacities, and scavenging activities against DPPH radicals showed significant variation among the six sources, within ethanol or aqueous extracts and between the two solvents. The maximum (1.27) Trolox Equivalent Antioxidant Capacity (TEAC) was recorded in aqueous extract of fern *Davallia mariesii*. The correlation coefficient (R²) values of TEAC and total polyphenol contents showed a higher correlation (aqueous extract, R² = 0.971; ethanol extract, R² = 0.981).

Keywords: Antioxidant activity; Gusuibu; Medicinal fern; Polyphenols contents; Radical scavenging activity; Reducing capacity.

Abbreviations: RSC, radical scavenging capacity; DPPH, 1,1-diphenylpicrylhydrazyl free radical; TEAC, Trolox equivalent antioxidant capacity.

INTRODUCTION

In the Chinese traditional medicine system, “Gusuibu” has long been used in the treatment of bone injuries. It has been proved very effective for the treatment of inflammation, hyperlipemia, and arteriosclerosis (Editorial Board of Zhong Hua Ben Cao [China Herbal], State Administration of Traditional Chinese Medicine, 1999). Also, several recent studies have claimed the medicine has therapeutic effects on bone healing (Ma et al., 1996; Sun et al., 2004; Jeong et al., 2005). However, in the Chinese Material Medica, different herbs from different areas with similar common names have been mentioned for treatment of the same disease. This is evident from field records, visits to local traditional doctors, review of specimens in herbaria, and the available literature. Rhizomes of *Drynaria fortunei* (Kze.) J. Sm., *Pseudodrynaria coronans*

(Wall. ex Mett.) Ching (both from Polypodiaceae), *Davallia divaricata* Bl., *Davallia mariesii* Moore ex Bak, *Davallia solida* (Forst.) Sw., and *Humata griffithiana* (Hk.) C. Chr. (from Davalliaceae) are used as or called “Gusuibu” or “Shibu” in Taiwan. These have been claimed to cure physique ache, inflammation, cancer, ageing, blood stasis, and bone injuries. In a publication by the Pharmacopoeia Commission of the People’s Republic of China, only *Drynaria fortunei* has been reported to be a source of “Gusuibu” (ChPC, 2005). However, no systematic investigation has been carried out so far to evaluate the comparative values of these different sources.

Over the past few years, investigations for phenolic compounds in medicinal herbs have gained importance due to their high antioxidative activity (Zhu et al., 2004). A large number of reports have demonstrated that these compounds are of great value in preventing the onset and/or progression of many human diseases (Parshad et al., 1998; Lee et al., 2000). Polyphenols have many favorable effects on human health, like inhibiting the oxidation of low-density proteins (Frankel et al., 1993), thereby decreasing the risk of heart disease (Williams and Elliot,

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1997). These compounds have anti-inflammatory and anti-carcinogenic properties (Carrol et al., 1999; Maeda-Yamamoto et al., 1999). Also, flavonoids and many other phenolic compounds of plant origin have been reported as scavenger reactive oxygen species (ROS), and are viewed as promising therapeutic drugs for free radical pathologies (Parshad et al., 1998; Lee et al., 2000). Thus, measurement of the polyphenols and antioxidant activity in herbs have become important tools to understand the relative values of plant species, especially from a health point of view.

The main objective of the present study was to characterize the antioxidative potencies, scavenging activities against DPPH radical, reducing power, and estimation of polyphenol contents in six sources of "Gusuibu."

MATERIALS AND METHODS

Plant materials

Plant materials of six sources of "Gusuibu" were collected from the counties of Hsinchu, Taichung, Nantou, and Taitung in Taiwan. These were identified and authenticated by Professor Chung-Chuan Chen of the Institute of Chinese Pharmaceutical Science, China Medical University, Taichung, Taiwan. Particulars of these sources including species, families, and their uses have been listed in Table 1.

Extractions

Ethanol extracts: Dried rhizome (100 g each) was macerated with 1,000 ml ethanol for 24 h at room temperature. Filtration and collection of the extract was

done thrice. Then, the ethanol with crude extract (3,000 ml) was evaporated to 10 ml and dried *in vacuo* at 40°C. The dry extract was weighted and dissolved in ethanol (stock 5mg/ml) and stored in -20°C until further use.

Aqueous extracts: Dried rhizome (100 g each) was used for decoction with 1,000 ml distilled water for 1 h. Filtration and collection of extract was done thrice. The resulting decoction (about 1,000 ml) was evaporated to 10 ml and dried *in vacuo* at 50°C. Each dry extract was weighted, dissolved in distilled water (stock 5 mg/ml) and stored in -20°C until further use.

For each sample, yields were calculated in percentages on the basis of dry weight of rhizome used (100 g) and quantity of dry mass obtained after extraction.

Antioxidant activity by ABTS assay

The assay was carried out as described earlier (Re et al., 1999). An aqueous solution of 2, 2-Azinobis [3-ethylbenzothiazoline-6-sulfonate] (ABTS) (7 mM) was oxidized using potassium peroxodisulfate (2.45 mM) for 16 h in dark. The ABTS⁺ solution was diluted with ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). A standard calibration curve was constructed for Trolox at 0, 0.1, 0.2, 0.5, 1.0, 2.0 mM concentration. An aliquot (10 µl) of each sample (100 µg ml⁻¹ concentration) was mixed with 1.0 ml of ABTS⁺ radical cation solution in cuvette and absorbance was read at 734 nm after 1 min. Antioxidant properties of "Gusuibu" extracts were expressed as Trolox Equivalent Antioxidant Capacity (TEAC), calculated from at least three different concentrations of extract tested in the assay giving a linear response.

Table 1. Particulars of six sources of medicinal fern used as "Gusuibu" and their comparative yields in ethanol and aqueous extracts.

Species	Common name	Ethanol extract		Aqueous extract		Medical use/ disease treated
		Code	Yield (%) ^a	Code	Yield (%)	
<i>Drynaria fortunei</i> (Polypodiaceae)	Gusuibu	DFE	11.2	DFW	12.8	Inflammation, hyperlipemia and arteriosclerosis (ChPC, 2005); Cancer (Cai et al., 2004).
<i>Pseudodrynaria coronans</i> (Polypodiaceae)	Gusuibu	PCE	15.0	PCW	18.6	Bone injuries, tinnitus and lumbago (Editorial Board of China Herbal, State Administration of Traditional Chinese Medicine, 1999).
<i>Davallia divaricata</i> (Davalliaceae)	Dayegusuibu	DDE	14.6	DDW	20.4	Bone injuries, tinnitus and lumbago (Editorial Board of China Herbal, State Administration of Traditional Chinese Medicine, 1999); Joint pain.(Hwang et al., 1989)
<i>Davallia mariesii</i> (Davalliaceae)	Haizhougusuibu	DME	8.5	DMW	29.0	Common cold, neuralgia, stomach cancer, lumbago, rheumatism, odontalgia and tinnitus (Cui et al., 1990)
<i>Davallia solida</i> (Davalliaceae)	Koyegusuibu	DSE	12.7	DSW	14.5	Physique ache, inflammation, cancer, and bone injuries.
<i>Humata griffithiana</i> (Davalliaceae)	Begaigusuibu	HGE	6.7	HGW	16.1	Physique ache, inflammation, cancer, and bone injuries.

^aDry weight basis.

Antioxidant activity by Dot-Blot and DPPH staining

An aliquot (3 μ l) of each sample was carefully loaded on a 20 cm \times 20 cm TLC layer (silica gel 60 F₂₅₄; Merck) and allowed to dry (3 min). Drops of each sample were loaded in order of decreasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rivas et al. (2000). The sheet bearing the dry spots was placed upside down for 10 s in a 0.4 mM DPPH solution. The excess solution was then removed with a tissue paper, and the layer was dried with a hair-dryer blowing cold air. A stained silica layer revealed a purple background with white spots at the location where radical scavenger capacity was present. The intensity of the white color depends upon the amount and nature of radical scavenger present in the sample. The GSH was used for the positive control in the aqueous extract, and the BHT was used for the positive control in ethanolic extracts.

Determination of “Reducing power”

The reducing power of the extracted samples (dissolved in ethanol or distilled water), glutathione (GSH, dissolved in distilled water) or butylated hydroxytoluene (BHT, dissolved in ethanol) were determined according to the method of Jayaprakasha et al. (2002). Different concentrations (12.5, 25, 50, 100, 200 and 400 μ g ml⁻¹) of each extract, GSH or BHT were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, to reduce ferricyanide into ferrocyanide. Thereafter, an equal volume of 1% trichloroacetic acid was added to the mixture and centrifuged at 6,000 rpm for 10 min. The upper layer of the solution was collected and mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:0.2. Absorbance was measured to determine the amount of ferric ferrocyanide (Prussian Blue) formed at 700 nm against a blank in a DU 640® UV/Vis spectrophotometer (Beckmann). The GSH was used for the positive control in the aqueous extract, and the BHT was used for the positive control in ethanolic extracts. The reducing power tests were run in triplicate. Increase in absorbance of the reaction indicated the reducing power of the samples. EC₅₀ value (μ g extract ml⁻¹) was the effective concentration at which the absorbance was 0.5 for reducing capacity and was obtained by interpolation from linear regression analysis.

Scavenging activity against DPPH radical

The effect of each extract sample on the DPPH radical was estimated according to the method reported by Blois (1958) with minor modification. Stock solution (500.0 mg ml⁻¹) of each sample was diluted to the concentrations of 400, 200, 100, 50, 25 and 12.5 mg ml⁻¹, in ethanol or distilled water. An aliquot of each sample (20 μ l) was mixed with 100 mM Tris-HCl buffer (80 μ l, pH 7.4), and then 100 μ l of the DPPH in ethanol with a final concentration of 200 μ M was added. The mixture was

shaken vigorously and left to stand at room temperature for 20 min in dark. The absorbance was measured at 517 nm against a blank in a DU 640® UV/Vis spectrophotometer (Beckmann). The percentage of DPPH discoloration of the sample was calculated according to the equation: % discoloration = (1 - Abs sample / Abs control) \times 100. EC₅₀ value (μ g extract/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. The GSH was used for the positive control in the aqueous extract, and the BHT was used for the positive control in ethanolic extracts.

Total polyphenols content (TPC)

Total phenolic compounds were estimated using the Folin-Ciocalteu method (Ragazzi and Veronese, 1973). Twenty μ l of each extract (100 μ g ml⁻¹) was added to 200 μ l distilled water and 40 μ l of Folin-Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrunn, Germany). The mixture was allowed to stand at room temperature for 5 min, and then 40 μ l of 20% sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. The TPC was expressed as μ g catechin equivalent/mg dry weight by reference to the (+)-catechin standard calibration curve.

Total flavonoid content

The AlCl₃ method (Lamaison and Carnet, 1990) was used for estimation of the total flavonoids content of the extracted samples. An aliquot of 100 μ l of each extract (100 μ g ml⁻¹) was added individually to equal volumes of solution of 2% AlCl₃·6H₂O (2 g in 100 ml methanol). The mixture was vigorously shaken, and after 10 min of incubation, absorbance was taken at 430 nm. Flavonoids contents were calculated from the calibration curve of rutin standard solutions, and expressed as μ g rutin equivalent/mg dry weight.

Total flavanol content

The total flavanol content was estimated using the p-di methylaminocinnamaldehyde (DMACA) method (Arnous et al., 2001). This method has a great advantage over the widely used vanillin method since there is no interference by anthocyanins. Furthermore, it provides higher sensitivity and specificity (Li et al., 1996). Forty μ l of each extract (100 μ g/ml) was added to 200 μ l of DMACA solution [0.1% in methanol/HCl (3:1, v/v)]. The mixture was vortexed and allowed to react at room temperature for 10 min. The absorbance at 640 nm was then read against a blank prepared without DMACA. The concentration of total flavanols was estimated from a calibration curve. Results are expressed as μ g catechin equivalent/mg dry weight.

Condensed tannin content

Condensed tannin content was estimated using the vanillin assay method (Julkunen-Titto, 1985). Twenty

five μl of extract ($100 \mu\text{g ml}^{-1}$) was added to 750 μl of vanillin/methanol solution (4%, w/v) and vortexed. Then, concentrated HCl (375 μl) was added and allowed to react at room temperature for 20 min. The absorbance at 550 nm was then read against a blank. Concentration of tannins was calculated as μg catechin equivalent/mg dry weight from a calibration curve.

Proanthocyanidin content

The proanthocyanidin content was estimated using HCl/butanol assay method (Porter et al., 1986). An aliquot (0.2 ml) of each extract (stock $100 \mu\text{g/ml}$) was added to 0.2 ml solution of 0.04 M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in 2 M HCl) in a 1.5 ml centrifuge tube, followed by 0.8 ml of butanol. The tube was incubated for 30 min at 95°C . The absorbance of the red colouration was read at 550 nm. Results were expressed in mg cyanidin chloride g^{-1} fresh weight.

Statistical analysis

All analyses were carried out in triplicate. Data are expressed as mean \pm standard deviation (SD). Differences were estimated by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test. Probability values of less than 0.05 were considered statistically significant. All statistical analyses were performed using SAS statistical software package (SAS[®] Inc., 2001).

RESULTS

Extraction yields

Ethanol extract yields of six sources of “Gusuibu” are given in Table 1. Species-wise percentages of ethanol extract yields in decreasing order were as follows: *Pseudodrynaria coronans* (PCE) (15.0), *Davallia divaricata* (DDE) (14.6), *Davallia solida* (DSE) (12.7), *Drynaria fortunei* (DFE) (11.2), *Davallia mariesii* (DME) (8.5), and *Humata griffithiana* (HGE) (6.7). The aqueous extract yields in decreasing order were as follows: *Davallia mariesii* (DMW) (29.0), *Davallia divaricata* (DDW) (20.4), *Pseudodrynaria coronans* (PCW) (18.6), *Humata griffithiana* (HGW) (16.1), *Davallia solida* (DSW) (14.5), *Drynaria fortunei* (DFW) (12.8).

Antioxidant activities estimated by ABTS assay method

TEAC values determined from the calibration curve for six sources of “Gusuibu” are shown in Figure 1. Antioxidant activities of both aqueous and ethanol extracts of the six sources were in the following decreasing order: DMW (1.27 mM) > DDW (0.96 mM) > DSW (0.91 mM) > PCW (0.77 mM) > DSE (0.32 mM) > DFW (0.26 mM) > HGE (0.18 mM) > DME and DDE (0.17 mM) > PCE (0.11 mM) > DFE (0.07 mM). Thus, it was observed that most samples in aqueous extracts had higher antioxidant potencies than ethanol extracts.

Antioxidant activities estimated by “dot-blot” method

In order to visualize semi-quantitatively and for rapid screening, each diluted sample was applied as a dot on a TLC plate which was then stained with DPPH solution (Figure 2). White spots of strong intensity appeared quickly up to the dilution of $25 \mu\text{g ml}^{-1}$ for DSE (the final amount in the spot: $0.075 \mu\text{g}$ dry matter), 50-100 $\mu\text{g/ml}$ for PCE, PCW, DDE, DDW, DSW and HGE (final amount: 0.15-0.3 μg dry matter); and the lowest intensities of DFE, DFW and HGW. For antioxidant analysis, fast-reacting and strong intensity white spots appeared up to dilutions of 50-100 $\mu\text{g ml}^{-1}$ for BHT and GSH (final amount: 0.15-0.3 μg dry matter). Appropriate dilutions react positively with DPPH, depending upon their free radical scavenging capacity (RSC) and nature (Chang et al., 2002) and result in dots of a certain diameter and color intensity, which indicate radical scavenging capacity. Darker dots indicate higher RSC values. According to the color intensities,

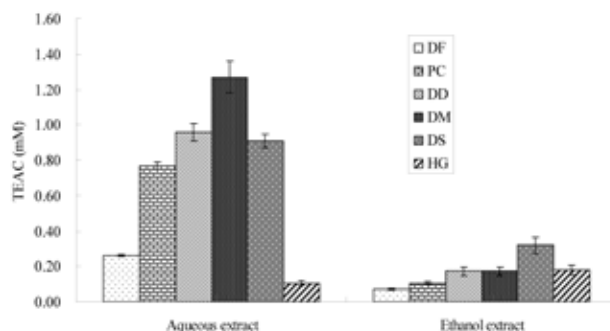


Figure 1. Trolox equivalent antioxidant capacity (TEAC) values of aqueous and ethanol extracts of six sources of Gusuibu. DF: *D. fortunei*; PC: *P. coronans*; DD: *D. divaricata*; DM: *D. mariesii*; DS: *D. solida*; HG: *H. griffithiana*. In text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code.

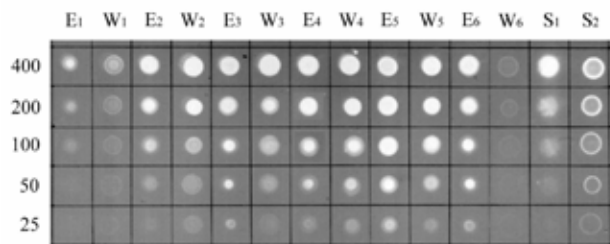


Figure 2. Dot blot assay of free radical scavenging capacity (RSC) on a silica sheet stained with a DPPH solution in methanol. Each 3 μl of plant extract [400, 200, 100, 50, and 25 $\mu\text{g ml}^{-1}$] was applied from top to down. Dots from left to right are: *D. fortunei* (E₁, W₁), *P. coronans* (E₂, W₂), *D. divaricata* (E₃, W₃), *D. mariesii* (E₄, W₄), *D. solida* (E₅, W₅) and *H. griffithiana* (E₆, W₆). Antioxidants; butylated hydroxytoluene (BHT) (S₁), glutathione (GSH) (S₂) were used as positive controls. (E₁-E₆: Ethanol extracts; W₁-W₆: Aqueous extracts).

RSC values of ethanol extracts in decreasing order were as follows: DSE (E_5) > DDE (E_3) > HGE (E_6) > DME (E_4) > PCE (E_2) > DFE (E_1) while RSC values of aqueous extracts in the decreasing order were as follows: DMW (W_4) > DSW (W_5) > DDW (W_3) > PCW (W_2) > DFW (W_1) > HGW (W_6). Between the two standards, BHT (S_1) had higher RSC compared to glutathione (S_2) (Figure 2).

EC₅₀ of scavenging activity against DPPH radical

Each sample extract exhibited the scavenging activity and was found to be dose-dependent (data not shown). The EC₅₀ values calculated at 20 min incubation are given in Table 2. It was found that ethanol extract of fern species *Davallia solida* (DSE) had the highest radical-scavenging activity (expressed as $\mu\text{g extract/ml}$) (26.89), followed by DDW (52.81), DDE (87.95), HGE (93.50), PCW (98.23), DSW (166.72), DMW (173.03) and PCE (190.81). Lower radical-scavenging activities (>400) were observed in case of DFE, DFW and HGW. In this assay condition the scavenging activity of GSH (89.64) against DPPH radicals is not as effective as that of BHT (42.28).

EC₅₀ of reducing power

Extracts of all six sources of “Gusuibu” exhibited the reducing powers and were concentration dependent (data not shown). The reducing powers (expressed as $\mu\text{g extract/ml}$) of the samples studied have been given in Table 2. Lower EC₅₀ values means higher reducing capacity. It was found that the aqueous extract of fern species *Davallia mariesii* (DMW) had a higher degree of reducing power (26.58), followed by DSW (32.81), PCW (38.96), DDW (43.36), DSE (185.10), DFW (193.55), HGE (317.8), DDE

(349.46) and DME (364.55). DFE, PCE and HGW showed lower reducing power (>400).

Total polyphenol content

The total polyphenols, flavonoids, flavonols, condensed tannin, and proanthocyanidin in ethanol and aqueous extracts of six sources of “Gusuibu” are given in Table 3. Total polyphenol contents (expressed as $\mu\text{g catechin equivalent/mg dry weight}$) varied significantly among the ethanol or aqueous extracts of six sources of “Gusuibu.” The quantities of total polyphenols ranged from a minimum 53.44 μg in ethanol extract of *Drynaria fortunei* (DFE) to the maximum 1635.16 μg in aqueous extract of *Davallia mariesii* (DMW) (Table 3). Depending upon species (source of Gusuibu), the quantities of total polyphenols in aqueous extracts were 5-12 times higher compared to ethanol extracts. The only exception was fern species *Humata griffithiana* (HG), where the margin of difference in total polyphenols in both the solvents was narrow.

Total flavonoid contents (expressed as $\mu\text{g rutin equivalent/mg dry weight}$) ranged from 0.0 to 122.44 μg in ethanol extracts and from 3.91 μg to 123.98 μg in the aqueous extracts of the six sources. The ethanol extracts of fern species *Pseudodrynaria coronans* (PC), *Davallia mariesii* (DM) and *Davallia solida* (DS) had no flavonoid content (Table 3).

Quantities of total flavonols in ethanol and aqueous extracts of six sources of “Gusuibu” varied significantly (Table 3). With the exception of fern species *Humata griffithiana* (HG), aqueous extracts had 3 to 22 times higher total flavonol contents compared to ethanol extracts. However, the ethanol extract of *Humata griffithiana*

Table 2. EC₅₀ values of ethanol and aqueous extracts in antioxidant properties of six sources of Gusuibu.

Sources of Gusuibu ^b	EC ₅₀ value ($\mu\text{g extract ml}^{-1}$) ^a			
	Reducing capacity		Scavenging ability on DPPH radicals	
	Ethanol extract	Aqueous extract	Ethanol extract	Aqueous extract
<i>Drynaria fortunei</i> (DF)	>400	193.55 ± 1.31 A	>400	>400
<i>Pseudodrynaria coronans</i> (PC)	>400	38.96 ± 0.14 CD	190.81 ± 4.15 A	98.23 ± 14.36 B
<i>Davallia divaricata</i> (DD)	349.46 ± 2.53 A ^c	43.36 ± 1.53 C	87.95 ± 0.88 B	52.81 ± 1.01 C
<i>Davallia mariesii</i> (DM)	364.55 ± 15.83 A	26.58 ± 0.26 E	95.87 ± 8.42 B	173.03 ± 10.68 A
<i>Davallia solida</i> (DS)	185.10 ± 15.25 D	32.16 ± 0.04 DE	26.89 ± 0.53 D	166.72 ± 7.24 A
<i>Humata griffithiana</i> (HG)	317.80 ± 19.90 B	>400	93.50 ± 8.60 B	>400
Glutathione (GSH)	N.D ^d	135.11 ± 12.25 B	N.D	89.64 ± 2.88 B
2, 6-Di-tert-butyl-4-methylphenol (BHT)	229.01 ± 0.60 C	N.D	42.28 ± 0.87 C	N.D

^a EC₅₀ value: the effective concentration at which the absorbance was 0.5 for reducing capacity; 1, 1'-diphenyl 2-picrylhydrazyl (DPPH) radicals were scavenged by 50%. EC₅₀ value was obtained by interpolation from linear regression analysis.

^b In text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code.

^c Each value is expressed as mean ± standard deviation (n=3). Means with different letters within a column are significantly different (p<0.05).

^d N.D: Not detected.

Table 3. Phenolic contents in ethanol and aqueous extracts of six sources of Gusuibu.

Sources of Gusuibu ^d	Total polyphenols ^a	Total flavonoids ^b	Total flavonols ^a	Condensed tannins ^a	Proanthocyanidins ^c
Ethanol extract					
<i>Drynaria fortunei</i> (DF)	53.44 ± 2.78	2.36 ± 1.54	0.75 ± 0.00	3.17 ± 5.63	89.17 ± 0.00
<i>Pseudodrynaria coronans</i> (PC)	108.18 ± 3.21	0	12.91 ± 0.21	32.73 ± 11.26	103.62 ± 1.28
<i>Davallia divaricata</i> (DD)	134.15 ± 12.13	0	49.39 ± 2.24	108.76 ± 14.07	128.63 ± 1.11
<i>Davallia mariesii</i> (DM)	140.64 ± 8.50	0	30.41 ± 0.86	53.85 ± 11.26	128.63 ± 1.11
<i>Davallia solida</i> (DS)	291.86 ± 7.00	122.44 ± 1.78	66.14 ± 2.61	260.82 ± 22.52	180.88 ± 2.12
<i>Humata griffithiana</i> (HG)	166.62 ± 5.79	11.64 ± 0.00	31.03 ± 0.86	79.19 ± 11.26	168.09 ± 3.85
Aqueous extract					
<i>Drynaria fortunei</i> (DF)	326.18 ± 12.13	16.28 ± 0.00	16.51 ± 0.56	108.76 ± 5.63	132.15 ± 3.39
<i>Pseudodrynaria coronans</i> (PC)	1120.29 ± 20.51	41.01 ± 1.54	145.31 ± 2.61	636.73 ± 14.07	341.13 ± 2.56
<i>Davallia divaricata</i> (DD)	1203.78 ± 12.55	3.91 ± 1.54	263.81 ± 4.85	1029.54 ± 30.97	258.13 ± 3.85
<i>Davallia mariesii</i> (DM)	1635.16 ± 48.39	3.91 ± 0.00	295.70 ± 3.33	1422.35 ± 14.07	467.85 ± 2.56
<i>Davallia solida</i> (DS)	1400.45 ± 34.87	123.98 ± 5.42	246.93 ± 5.02	1164.70 ± 25.34	429.32 ± 3.85
<i>Humata griffithiana</i> (HG)	183.32 ± 49.88	14.22 ± 1.78	12.041 ± 1.07	32.73 ± 14.07	106.21 ± 1.28

^aExpressed as µg catechin equivalent/mg dry weight.

^bExpressed as µg rutin equivalent/mg dry weight.

^cExpressed as µg cyanidin chloride equivalent/mg dry weight.

^dIn text, all ethanol extracts have been designated as E and aqueous extracts as W after the species code.

showed a 2.5-times higher quantity than aqueous extract. The minimum quantity of total flavonols (0.75 µg) was found in *Drynaria fortunei* (DFE), and the maximum (295.70 µg) was in *Davallia mariesii* (DMW) (Table 3).

In the case of condensed tannins and proanthocyanidin, trends more or less similar to total flavonols were observed among the six sources. Aqueous extracts had several times higher values compared to ethanol extracts with the exception of *Humata griffithiana* (Table 3).

Relationship between antioxidant activity and total polyphenols, and total flavonoid contents

Correlation coefficients (R^2) of antioxidant capacity (TEAC) and total polyphenols, and TEAC and total flavonoids of both aqueous and ethanol extracts are shown in Figures 3 and 4. R^2 values for TEAC and total polyphenol contents for aqueous (Figure 3A) and ethanol (Figure 3B) extracts were 0.971 and 0.981, respectively. Similarly, R^2 values for TEAC and total flavonoid contents

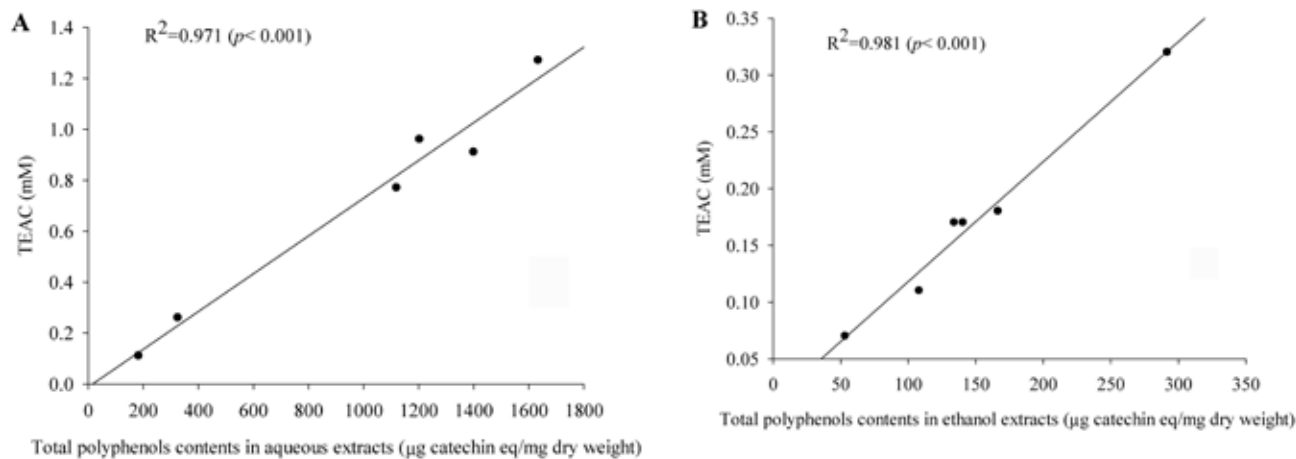


Figure 3. Correlation coefficients (R^2) of TEAC and total polyphenol contents in aqueous (A) and ethanol (B) extracts.

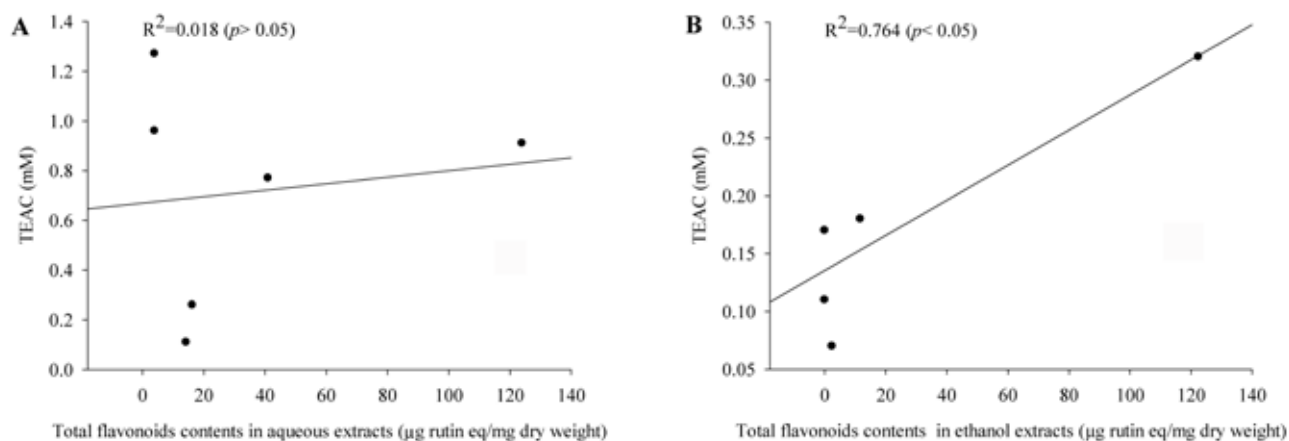


Figure 4. Correlation coefficients (R^2) of TEAC and total flavonoid contents in aqueous (A) and ethanol (B) extracts.

for aqueous (Figure 4A) and ethanol (Figure 4B) extracts were 0.018 and 0.764, respectively.

DISCUSSION

Antioxidants have been defined as substances that, when present at low concentrations compared with oxidizable compounds (e.g. DNA, protein, lipid, or carbohydrate), delay or prevent oxidative damage due to the presence of reactive oxygen species (ROS). These ROS undergo redox reactions with phenolics, resulting in inhibition of antioxidant activity in a concentration dependant manner (Halliwell and Gutteridge, 1990). Thus, measurement of total polyphenols and its constituents along with antioxidant activity has increasingly been used in plant samples and has become an important tool for investigation. The TEAC assay is one of the most frequently used analytical strategies for antioxidant activity. The assay shows a good correlation with the other methods as well, such as the 2, 2'-diphenyl-1-picrylhydrazyl assay (DPPH), the total radical-trapping antioxidant parameter assay (TRAP), the photochemiluminescence assay (PCL), and the ferric reducing ability of plasma assay (FRAP). In the present study, it was observed that the greatest antioxidant activities (measured as TEAC values) more or less had direct correlation with quantities of total polyphenols both in aqueous as well as ethanol extracts. Similar findings have been reported earlier (Zhu et al., 2004) suggesting a causative relationship between total polyphenols content and antioxidant activity and indicating that phenolic compounds present in the extracts of six sources of "Gusuibu" may be responsible for these antioxidant properties.

Several methods have been used to measure free radical scavenging capacities (RSC), regardless of the individual compounds which contribute to the total capacity of a plant product in scavenging free radicals. Most methods are based on the inhibition of the accumulation of oxidized products, since the generation of free radical species

is inhibited by the addition of antioxidants resulting in reduction of the end point by scavenging free radicals. The reliable method to determine RSC involves the measurement of the disappearance of free radicals, such as the 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic) acid radical ($\text{ABTS}^{\cdot+}$), the 2, 2-diphenyl-1-picrylhydrazyl radical ($\text{DPPH}^{\cdot+}$), or other colored radicals, with a spectrophotometer (Miller and Rice-Evans, 1997). The DPPH radical has been widely used as a model system to investigate the scavenging activities of several natural compounds including phenolic compounds, flavonoids, or crude mixtures such as ethanol or water extracts of plants. DPPH radical is scavenged by antioxidants through the donation of a hydrogen atom, forming the reduced DPPH-H. The color changes from purple to yellow after reduction, and this can be quantified by its decrease in absorbance at wavelength 517 nm. In the present study, all the samples irrespective of solvent showed scavenging activity in a concentration dependent manner. Stained silica layer revealed a purple background with white spots at the location of drops, which showed radical scavenging capacity. The intensity of the white color depended upon the amount and nature of radical scavenger present in the samples. Thus, TLC screening and DPPH staining methods demonstrated that ethanol and aqueous extracts had significant variations in free radical scavenging capacities.

Most samples in water extract were found to have higher antioxidant potencies than ethanol extracts. This observation is in conformity with an earlier report, which observed that the antioxidant activities of more-polar solvent extracts (BuOH and water extracts) exceeded those of non-polar solvent extracts (hexane and EtOAc extracts). The much higher antioxidant activity of the water extracts of "Gusuibu" preparations indicates that the herb may be most effective when taken with water. In contrast to this report, alcoholic preparations of the herbal drug *Uncaria tomentosa* showed higher antioxidant activity than water extract (Pietta et al., 1998). Thus it is essential to carry out a study on different solvents.

In the present study, all the extracts of six sources exhibited the reducing power in a concentration dependent manner. The reducing properties are generally associated with the presence of reductones (Duh, 1998). It has been reported that the antioxidant action of reductone was based on the breaking of the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. It was reported earlier that the antioxidative properties of extracts from *Garcinia subelliptica* and *G. arcinia mangostana* were due to presence of various xanthenes with phenolic functional groups (Minami et al., 1996; Mahabusarakam et al., 2000). These compounds act similarly to reductones by donating the electrons and reacting with free radicals to convert them to a more stable product, and by terminating the free radical chain reaction. Results in the present study demonstrate that aqueous extracts exert a reducing activity 2-14 times higher than the ethanol extracts, indicating that some enzymatic protein molecules may be involved in the ferricyanide reduction. In fact, several enzymes such as the cytochrome c reductase (Rafferty and Malech, 1996) or the lactate dehydrogenase may catalyze the ferricyanide reduction.

It has been reported that the antioxidant activity of many compounds of botanical origin is proportional to the phenolic content (Rice-Evans et al., 1997), suggesting a causative relationship between total phenolic content and antioxidant activity (Veglioglu et al., 1998). Cai et al. (2004) showed a linear correlation between antioxidant activity and total phenolic content (R^2 values > 0.95) in the 112 traditional Chinese medicinal plants associated with anticancer properties. In the present study, higher correlation coefficients (R^2) values for TEAC and total polyphenol contents for aqueous or ethanol extracts were observed compared to R^2 values for total flavonoid contents. The results suggest that the total polyphenol compounds in extracts of six folk medicinal ferns used as "Gusuibu" contributed significantly to the antioxidant capacities.

CONCLUSION

The present investigations provide useful information on antioxidant properties and polyphenolic contents of six sources of "Gusuibu." Antioxidant activities of both ethanol and aqueous extracts among the six sources varied significantly and had a strong correlation with total polyphenol contents. The methods employed in the present study are easily used and provide reproducible results. Much higher antioxidant activities of the aqueous extracts have given evident assumption that the water preparation of "Gusuibu" is more potent than the ethanol one from a medical point of view.

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骨碎補類藥材之六種民俗藥用蕨類植物的 抗氧化活性及其多酚類組成

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在傳統中草藥系統中，共有六種藥用蕨類植物被當做骨碎補類藥材，主要用於治療“骨”相關的疾病，但卻尚未有相關的科學研究進行綜合性的評估。本研究，分析這六種植物之乙醇及水萃取物的總抗氧化能力、清除 DPPH 自由基能力、還原力、總多酚類、總黃酮類、總黃酮醇類、總縮合單寧及原花青素類成分，結果顯示這六種來源植物間有極大的差異。大部分的水萃取物比乙醇萃取物具有較高的抗氧化潛力及較多的多酚類含量。比較六種來源植物之乙醇萃取物或水萃取物、兩者之間的還原力及清除 DPPH 自由基能力，在 EC₅₀ 的數據上均顯示具有顯著的差異性。海州骨碎補的水萃取物具有最高的總抗氧化活性 (1.27 mM TEAC)。總抗氧化活性與總多酚類成分間具有極高的相關係數（水粗萃物 R²=0.971 及乙醇粗萃物 R²=0.981）。

關鍵詞：抗氧化活性；骨碎補；藥用蕨類；多酚類組成；清除自由基活性；還原力。