Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: Rosidah, Mun Yam, Amirin Sadikun & Mohd. Asmawi (2008) Antioxidant Potential of *Gynura procumbens*, Pharmaceutical Biology, 46:9, 616-625, DOI: 10.1080/13880200802179642

To link to this article: https://doi.org/10.1080/13880200802179642

Published online: 20 Oct 2008.

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Pharmaceutical Biology
2008, Vol. 46, No. 9, pp. 616–625

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Antioxidant Potential of *Gynura procumbens*

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Abstract

The leaves of *Gynura procumbens* (Merr.) Compositae, commonly called "sambung nyawa" in Malaysia, are often eaten raw with rice. The methanol extract was prepared from the dried leaves using a Soxhlet apparatus. The methanol extract was then fractionated into chloroform, ethyl acetate, *n*-butanol, and aqueous fractions using a separating funnel. In the current study, the antioxidant potency of *G. procumbens* extract and fractions were investigated, employing various established *in vitro* systems, such as trolox equivalent antioxidant capacity, β-carotene–linoleic acid model system, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, reducing power, and xanthine oxidase inhibitory activity. Based on the results obtained, the extract and fractions showed different antioxidant potential. Among the fractions, the ethyl acetate fraction displayed higher antioxidant properties. The contents of the potential antioxidant component of the extract and fractions were also determined using HPTLC densitometric and spectrophotometric (using Folin-Ciocalteu reagent) methods. HPTLC study revealed that the methanol extract and the ethyl acetate and *n*-butanol fractions contain 0.74% and 2.9%, 7.76% and 12.75%, and 4.52% and 0.33% of kaempferol-3-*O*-rutinoside and astragalin, respectively. The total phenolic content of the extract and fractions varied from 4.37% to 23.43% of dry weight, expressed as gallic acid equivalents (GAE). With further data analysis, it was found there was a significant correlation ($p < 0.05$) between the total phenolic content of the sample and its DPPH scavenging activity and reducing power with correlation coefficients (r) of 0.891 and 0.926, respectively. These results suggest that phenolics in these plants provide substantial antioxidant activity.

Keywords: Antioxidant, astragalin, free radical, *Gynura procumbens,* HPTLC, kaempferol-3-*O*-rutinoside, reducing power.

Introduction

Oxidation is an important process for living organisms. The uncontrolled production of oxygen free radicals and the unbalanced mechanism of antioxidant protection result in the onset of many diseases, namely cancer, diabetes, Alzheimer's disease, coronary heart diseases, and aging (Hertog et al., 1995; Keli et al., 1996; Deng et al., 1998; Geleijnse et al., 1999, 2000; Erlund et al., 2001; Yang et al., 2004). Antioxidants are regarded as possible protection agents reducing oxidative damage in the human body. Therefore, there is a growing interest in the substances exhibiting antioxidant properties that are available to human and animal organisms as food components or as specific pharmaceutics. There are two basic categories of antioxidants, namely, synthetic and natural ones. Restriction on the use of synthetic antioxidants is being imposed because of their carcinogenicity (Grice, 1986, 1988). Recently, natural antioxidants have become a major area of scientific research (Demo et al., 1998; Sanchez-Moreno et al., 1999). Owing to safety concerns of synthetic antioxidants and effectiveness of natural antioxidants, the public prefers to take natural antioxidant sources from edible materials such as fruits, spices, herbs, and vegetables. Therefore, the development and use of more effective antioxidants of natural origin is desired.

Gynura procumbens (Merr.) (Compositae) is an annual evergreen shrub with a fleshy stem and purple tint. In Southeast Asia, especially Indonesia, Malaysia, and Thailand, the plant has been traditionally used for treatment of eruptive fevers, rash, kidney disease, migraines, constipation, hypertension, diabetes mellitus, and cancer (Perry, 1980). Recently, pharmacologic studies reported that *G. procumbens* has anti–Herpes simplex virus, anti–hyperglycemic, anti–hyperlipidemic, anti–inflammatory, analgesic, and reduced blood hypertension properties (Lam et al., 1998; Nawawi et al., 1999; Akowuah et al., 2001, 2002; Iskander et al., 2002). However, by reviewing the current literature, we know of no previous research on the

Accepted: January 31, 2008

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DOI: 10.1080/13880200802179642 © 2008 Informa UK Ltd.

antioxidant study of this plant. Therefore, experiments were carried out to test the antioxidant potential of *G. procumbens.* In the experiment, antioxidant properties of *G. procumbens* were assayed in terms of inhibitory ability on xanthine oxidase and β-carotene, scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′azino-bis(3-ethybenzthiazoline-6-sulfonic acid) (ABTS), and reducing power. The contents of the potential antioxidant components (kaempferol-3-*O*-rutinoside and astragalin) of the extract and fractions were also determined using HPTLC densitometric and spectrophotometric methods.

Materials and Methods

Chemicals and reagents

Polyoxyethylene sorbitan monopalmitate, β-carotene, xanthine oxidase, xanthine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), allopurinol, sodium phosphate (dibasic), sodium phosphate (monobasic), potassium persulfate, linoleic acid, 2,2′azino-*bis*(3-ethybenzthiazoline-6-sulfonic acid), 1,1-diphenyl-2-picrylhydrazyl, Folin-Ciocalteu reagent, phosphate-buffered saline (PBS) (pH 7.4), quercetin, gallic acid, and sodium carbonate were purchased from Sigma (St. Louis, MO, USA). Trolox was purchased from Calbiochem (Darmstadt, Germany). Chloroform, petroleum ether (60–80°C), methanol, ethyl acetate, and *n*-butanol were purchased from Merck (Darmstadt, Germany). Potassium ferricyanide was purchased from R&M Marketing (Essex, UK). Kaempferol-3-*O*-rutinoside and astragalin (kaempferol-3-*O*-glucoside) were purchased from ChromaDex Inc. (California, USA).

Extraction and fraction procedure

The *Gynura procumbens* was obtained from Penang Island, Malaysia in (December 2004). A voucher specimen (no. 10833) was deposited at the herbarium of the School of Biological Sciences, Universiti Sains Malaysia, by a botanist. The leaves of *G. procumbens* were dried in an oven at 45°C and ground into powder. The dried powdered leaves (500 g) were first defatted with 5 L petroleum ether (60–80°C) and then further extracted with methanol in a Soxhlet apparatus. The methanol extract (ME) was concentrated by a rotary evaporator (Büchi-RE121, Flawil, Switzerland) under vacuum (Büchi-B169), and the concentrated extract was dried in a freeze-dryer (HETO-Hetovac VR-1, Birkerød, Denmark) (yield 12.2% of dried leaf). The ME was then resuspended in water and fractionated to chloroform fraction (CF), ethyl acetate fraction (EF), *n*-butanol fraction (BF), and aqueous fraction (AF) using a separating funnel. The yields of CF, EF, BF, and AF were 20.4%, 6.7%, 11.8%, and 60.8% of ME, respectively.

HPTLC procedure

Chromatography was performed on a preactivated (100°C) silica gel $60F_{254}$ TLC plate (20 × 10 cm; 0.25 mm layer thickness; Merck). The Camag densitometry (Camag Model-3 TLC scanner equipped with Camag CATS 4 software; Mutten, Switzerland) and a reflectance spectrometer (190–700 nm) were employed for the analysis. The slit was set to 8 × 0.4 mm, and data acquisition and processing were performed using winCATS software. Four-microliter samples were applied to the layer at 8-mm-width bands, positioned 10 mm from the the bottom of the plate, using a Camag Linomat IV automated TLC applicator with nitrogen flow providing delivery from the string at a speed of 10 μL/s that of was maintained for all analyses. The HPTLC plates were developed in a Camag twintrough glass tank presaturated with the mobile phase [ethyl acetate:methanol:water (100:13.5:10)] for 2 h at room temperature (22–24°C). Solvent was allowed to run the plate to a height of 8 cm. After development, the TLC plate was dried, and the components were visualized by UV light at 365 and 254 nm for kaempferol-3-*O*-rutinoside and astragalin, respectively.

The quantitative determination was performed by winCATS software program using the external calibration method. The calibration curve was prepared with kaempferol-3-*O*-rutinoside and astragalin in the range from 1000 to 15.63 μg/mL in methanol. The *G. procumbens* extract and fractions (10 mg/mL) in methanol were subjected to HPTLC analysis.

Assessment of total antioxidant activity

The total antioxidant activity (TAA) value was estimated by the trolox equivalent antioxidant capacity (TEAC) test (Re et al., 1999; Yam et al., 2007). 2,2′Azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) was dissolved in deionized water to a 7 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature (22–24°C) for 12–16 h before use. The concentrated $ABTS^{+}$ solution was diluted with PBS pH 7.4 to a final absorbance of 0.70 ± 0.02 at 734 nm at 30°C. Stock solution of trolox (0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mM) and *G. procumbens* extract and fractions were prepared in methanol. The spectrophotometer (Hitachi U-2000, Tokyo, Japan) was first blanked with PBS. Ten microliters of antioxidant-containing solution was added to 2 mL $ABTS^{\cdot+}$ solution. The decrease of absorbance was measured at 734 nm 6 min after addition of the trolox and *G. procumbens* extract and fractions. All determinations were carried out in triplicate. The TEAC of the *G. procumbens* extract and fractions was calculated by relating this decrease in absorbance to that of a trolox solution on a molar basis.

Determination of antioxidant assay using the β-carotene–linoleic acid method

The procedure described by Sun and Ho (2005) was used for evaluating the antioxidant activity of the *G. procumbens* leaf extract and fractions. One milliliter of β-carotene (1 mg in 1 mL chloroform) was added to a conical flask with 20 mg linoleic acid and 200 mg polyoxyethylene sorbitan monopalmitate (Tween-40). Chloroform was removed under vacuum at 40°C (using a rotary evaporator), and the resultant mixture was diluted with 10 mL of water, mixed well, and followed by addition of oxygenated water (40 mL) to form a solution. The aliquots (4 mL) were pipetted into different test tubes containing 0.2 mL of extracts, quercetin, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and quercetin (0.2 mg/mL, in ethanol), respectively, and the absorbance was measured with a spectrometer (PerkinElmer Lambda 45, Massachusetts, USA) at 470 nm immediately and to 120 min (15-min intervals), against a blank solution without the β-carotene. All determinations were carried out in triplicate. The antioxidant activity (AA) was evaluated following the formula

$$\mathrm{AA} = 100\left[1 - (\mathrm{A}_0 - \mathrm{A}_\mathrm{t})/\left(\mathrm{A}_0^0 - \mathrm{A}_\mathrm{t}^0\right)\right]$$

where A_0 and A_0^0 are the absorbance value measured at zero time of the incubation for test sample and control, respectively, and A_t and A_t^0 are the absorbance value measured after incubation for test sample and control, respectively. The results were expressed in percentage.

Determination of xanthine oxidase inhibition

The xanthine oxidase activities with xanthine as the substrate were measured spectrophotometrically, based on the procedure reported by Noro et al. (1983) and Sweeney et al. (2001), with minimum modification. The extract, fractions (500 μL of 0.1 mg/mL), and allopurinol (in methanol) were mixed with 1.3 mL phosphate buffer (pH 7.5) and 0.2 mL 0.2 unit/mL xanthine oxidase solution. After 10 min of incubation at room temperature (22–24°C), the mixture was added with 1.5 mL 0.15 M xanthine substrate solution. The mixture was incubated for 30 min at room temperature (22–24°C), and then the absorbance was measured at 293 nm using a spectrometer (PerkinElmer Lambda 45) against a blank (0.5 mL methanol, 1.3 mL phosphate buffer, 0.2 mL xanthine oxidase). The solution of 0.5 mL methanol, 1.3 mL phosphate buffer, 0.2 mL xanthine oxidase, and 1.5 mL xanthine substrate solution was used as a control. Each sample was measured in triplicate and was averaged. Percentage of inhibition was calculated using the formula

$$\text{Percentage of inhibition} = [1 - (A_\mathrm{s}/A_\mathrm{c})] \times 100$$

Where A_s and A_c are the absorbance value of test sample and control, respectively.

Reducing capacity

Reducing capacity was determined through a method used by Oyaizu (1986). One milliliter of different concentration of *G. procumbens* extract and fractions (0.25, 0.125, and 0.0625 mg/mL) (in methanol) was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm (Eppendorf 5403, Engelsdorf, Germany) for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction indicated increased reducing power.

Determination of DPPH scavenging activity

The free radical scavenging activity of *Gynura procumbens* extract, fractions, and BHT were measured in terms of hydrogen donating or radical scavenging ability using the stable DPPH (Kumaran & Karunakaran, 2006). One milliliter each of 0.025, 0.05, 0.1, 0.2, and 0.4 mg/mL *Gynura procumbens* extract, fractions, and BHT (in methanol) were placed in different tubes. To this mixture, 2 mL 0.1 mM DPPH was added. After 60 min of incubation at room temperature (22–24°C), absorbance was measured at 517 nm by using a spectrometer (PerkinElmer Lambda 45) against methanol as the blank. A control contained 1 mL methanol and 2 mL 0.1 mM DPPH methanol. Free radical scavenging activity of the extract and fractions was determined according to the following formula:

$$\text{Free radical scavenging activity (\%)} = (A_\mathrm{c} - A_\mathrm{s})/A_\mathrm{c} \times 100$$

Where A_s is the absorbance of DPPH and sample, and A_c is the absorbance of control.

Determination of total phenolic content

The total phenolic content of the methanol extract and its fractions was determined by the Folin-Ciocalteu method with some modification (Slinkard & Singleton, 1977).

Table 1. Percentage of kaempferol-3-*O*-rutinoside and astragalin in *G. procumbens* extract and fractions.

	Percentage (%)	
	Kaempferol-3-*O*-rutinoside	Astragalin
Methanol extract	0.74 ± 0.03	2.92 + 0.05
Ethyl acetate fraction	7076 ± 0.2	12.75 ± 2.04
Butanol fraction	4.52 ± 0.13	0.33 ± 0.03

Each value represents a mean of % ± SD (n = 3)

Five hundred microlitres of 1 mg/mL extract and fractions (in methanol) was added to 500 μL 2 N Folin-Ciocalteu reagent. After 4 min of incubation at room temperature (22–24°C), 1 mL 20% (w/v) sodium carbonate was added followed by 6 mL distilled water. After 2 h of incubation at room temperature (22–24°C), absorbance of the mixture was measured at 760 nm using a spectrometer (PerkinElmer Lambda 45). A calibration curve, using gallic acid in a concentration range of 0.001–0.1 mg/mL, was prepared. The total phenolic content of the samples was expressed as gallic acid equivalents (GAE), which reflected the phenolic content as amount of gallic acid in sample. Experiments were performed in triplicate.

Statistical analysis

The experimental data were expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by least significant difference (LSD) multiple range test was carried out to determined significant differences ($p < 0.05$), and correlation coefficients (r) were quantified by SPSS (version 10.01).

Results and Discussion

HPTLC analysis

The quantitative analysis of the *G. procumbens* extract and fractions, made using high performance thinlayer liquid chromatography coupled with Camag densitometry, is presented in Table 1, and representative chromatograms are presented in Figure 1. Phenolic compounds were identified and quantified at 365 and 254 nm as kaempferol-3-*O*-rutinoside and astragalin, respectively. The results revealed that ME, EF, and BF contain 0.74%, and 2.9%, 7.76%, and 12.75%, and 4.52%, and 0.33% kaempferol-3-*O*-rutinoside

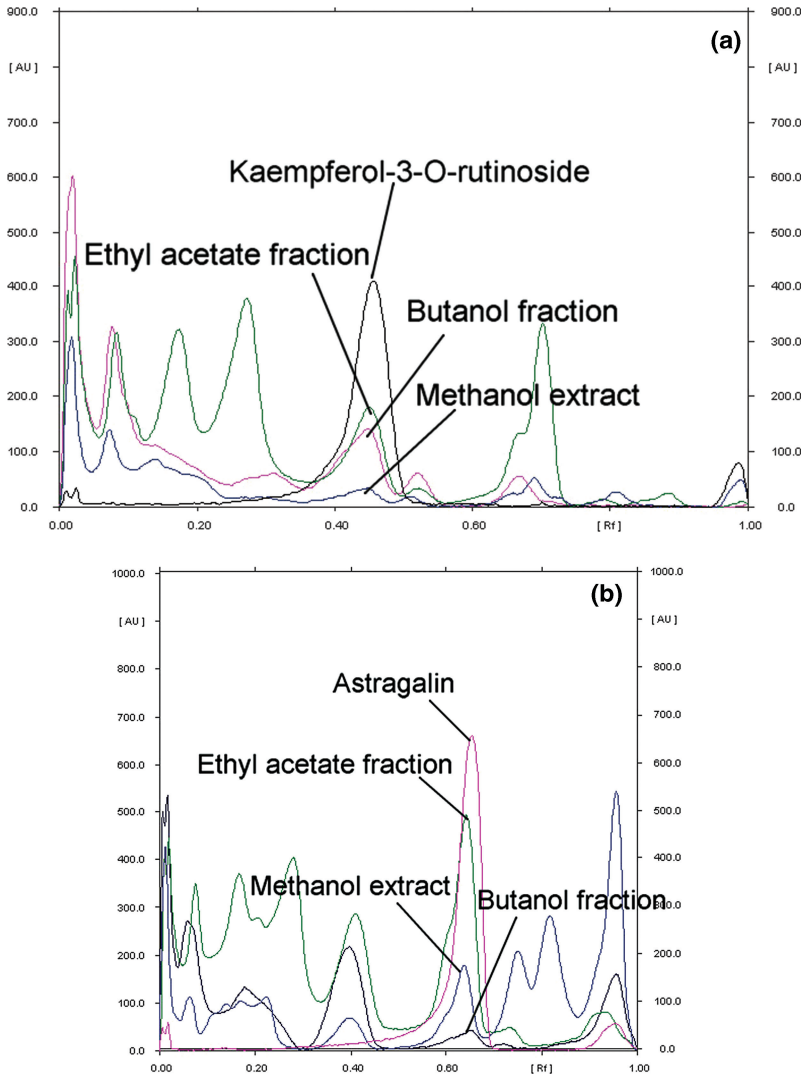

Figure 1. HPTLC profiles (overly chromatograms) of methanol extract, ethyl acetate fraction, and butanol fraction. Eluent: ethyl acetate:methanol:water (100:13.5:10) (v/v). (a) Detection, 365 nm; (b) detection, 254 nm.

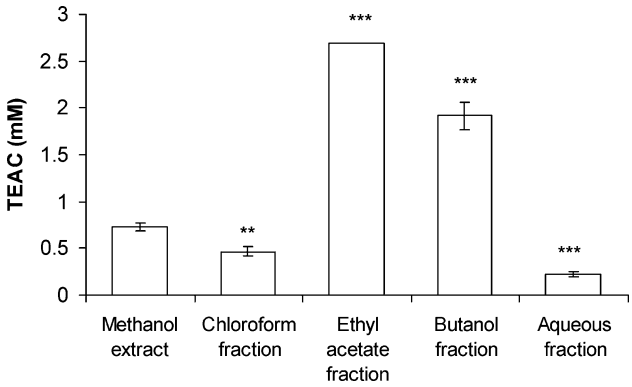

Figure 2. Trolox equivatent antioxidant capcity (TEAC) of *Gynura procumbens* extract and fraction. Measurments were carried out in triple. Each value a mean ± S D (n = 3).** and *** represent indicate significant difference compared with methanol extract at p < 0.001, respectively.

and astragalin respectively. The chromatogram showed the kaempferol-3-*O*-rutinoside and astragalin with symmetrical peak, at R_f= 0.43 and R_f = 0.72, respectively.

Total antioxidant capacity

Reaction of the *G. procumbens* extract and fractions with ABTS radicals was examined. The TEAC assay is applied to assess the total amount of ABTS radical that can be scavenged by *G. procumbens* extract and fractions. The total antioxidant activity of *G. procumbens* extract and fractions was expressed as mM trolox equivalents. As shown in Figure 2, EF and BF were 2.69 ± 0.004 and 1.92 ± 0.15 mM of trolox equivalent, respectively, resulting in significantly higher ABTS radical scavenging than that of methanol extract ($p < 0.001$). The antioxidant activity of putative antioxidants have been attributed to various mechanisms, namely, prevention of chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, prevention of hydrogen abstraction, and radical scavenging (Diplock, 1997; Yilidirim et al., 2001).

β-Carotene–linoleic acid antioxidant capacity

The antioxidant activity of *G. procumbens* extract and fractions, BHA, BHT, and quercetin, as measured by the bleaching of β-carotene, is presented in Figure 3. It can be seen that *G. procumbens* extract and fractions exhibited varying degrees of antioxidant activity. The mechanism of bleaching of β-carotene is a free radical–mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. β-Carotene, in this model system, undergoes rapid discoloration in the absence of antioxidant. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecule. As β-carotene molecules lose their double bonds by oxidation, the compound loses it's chromophore (orange color). The antioxidant of the extract and fractions of *G. procumbens* and standard increased in the order BHA > BHT > quercetin > EF > CF > ME > BF > AF.

Xanthine oxidase inhibition

Xanthine oxidase is a highly versatile enzyme and widely distributed within the various tissues of mammals. It is an important source of oxygen free radicals. Xanthine oxidase

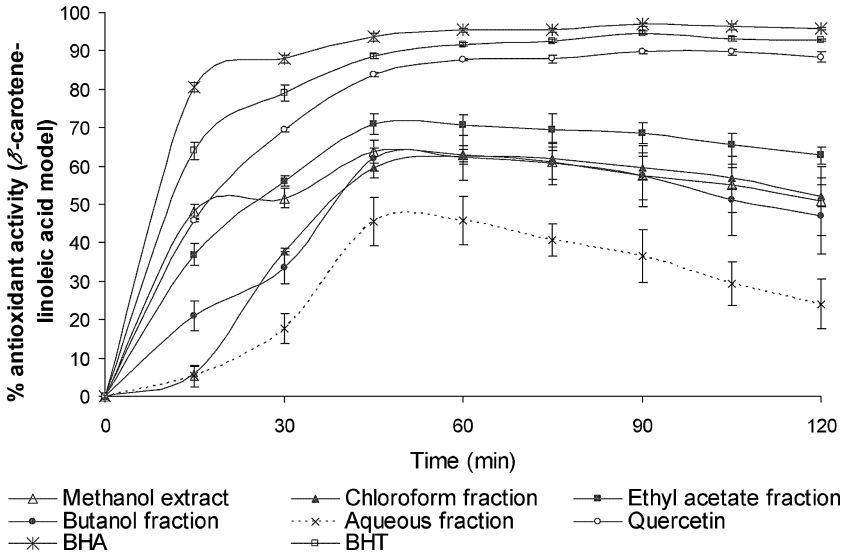

Figure 3. β-Carotene inhibition activity of the *Gynura procumbens* extract and fraction at different concentrations. Each value represents a mean ± SD (n = 3).

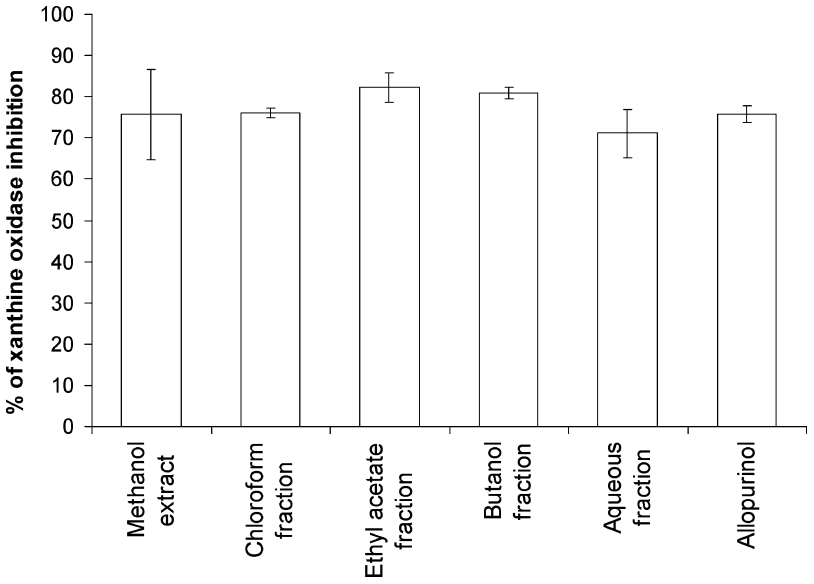

Figure 4. Xanthine oxidase in inhibition activity of the *Gynura procumbens* extract and fraction at different concentrations. Each value represents a mean ± SD (n = 3).

catalyzes the reduction of O_2, leading to the formation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), and it has been proposed as a central mechanism of oxidative injury (Massey et al., 1969; McCord, 1985; Zweier et al., 1988). The xanthine oxidase inhibitory ability of *G. procumbens* extract, fractions, and known standard compound, viz., allopurinol, is presented in Figure 4. The extract and fractions of *G. procumbens* exhibited xanthine oxidase inhibitory activity. This activity increased up to 82.2%, 80.9%, and 76.0% after the crude extract was fractionated with ethyl acetate, *n*-butanol, and chloroform, respectively. There were no significant differences found between xanthine oxidase inhibition ability of *G. procumbens* extract or fractions and allopurinol. Xanthine oxidase inhibitory activity of the samples followed the order EF > BF > CF > allopurinol > ME > AF.

Reducing power

Reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron (Dorman et al., 2003). The reducing capacity of a plant extract or compound may

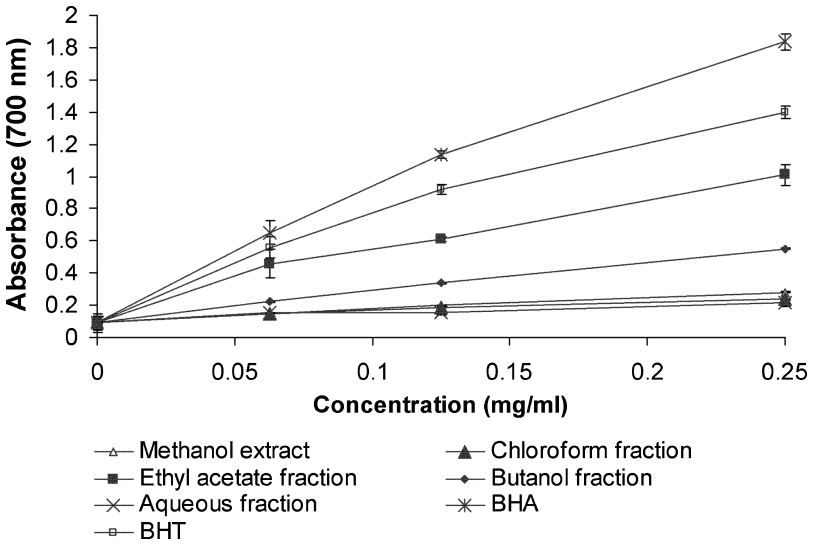

Figure 5. Reducing powers of the *Gynura procumbens* extract and fraction at different concentrations. Each value represents a mean ± SD (n = 3).

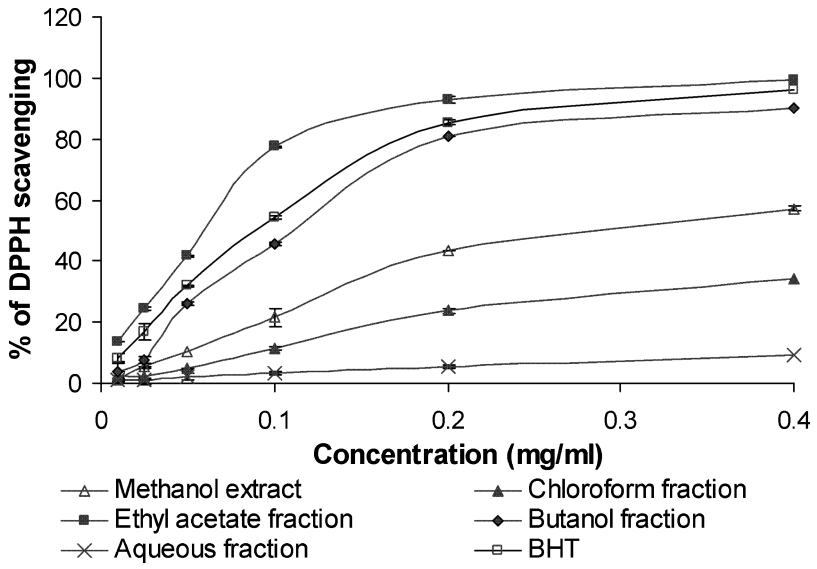

Figure 6. DPPH scavenging activity of the *Gynura procumbens* extract and fraction at different concentrations. Each value represents a mean ± SD (n = 3).

serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). Previous studies also reported that there is a direct correlation between the antioxidant effect and reducing capacity of some plant extracts (Yilidirim et al., 2001).

Figure 5 shows the reductive capabilities of *G. procumbens* extract and fractions compared with BHA and BHT. Reducing power of *G. procumbens* extract and fractions and standard compounds followed the order BHA > BHT > EF > BF > CF > ME > AF. According to the results in the current study, it is suggested that EF is a good electron donor and has a potency to convert the free radicals to nonreactive form and terminate the free radical reaction.

Free radical scavenging activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction in DPPH radical was determined by the decrease of its absorbance at 517 nm by antioxidants. All the extract and fractions of *G. procumbens* showed DPPH scavenging effect at all amounts (Fig. 6). The DPPH radical scavenging effects of the extract and fractions of*G. procumbens* and standard compounds increased in the order EF > BHT > BF > ME > CF > AF. From Figure 6, we observe that a dose-response relationship is found in DPPH radical scavenging activity; the activity increased as the concentration increased for each extract and fraction of *G. procumbens*. These results were consistent with total antioxidant and reducing power.

Total phenolic compounds

The phenolic compounds are rich in hydroxyl groups and are believed to have the ability of free radical scavenging and antioxidation. Polyphenols are multifunctional and can act as reducing agents, as hydrogen atom–donating antioxidants, chelating metal ions and as singlet oxygen quenchers. The results for phenolic content in the studied *G. procumbens* extract and fractions are presented in Figure 7. The data clearly outlines the phenolics sources: EF [23.4 ± 0.26% GAE (w/w)], ME [9.4 ± 0.08% GAE (w/w)], BF [8.3 ± 0.87% GAE (w/w)], CF [6.25 ± 0.5% GAE (w/w)], and AF [4.4 ± 0.79% GAE (w/w)]. However, in this study, the fractionation product may be fractionated to different amounts of total phenolic content. Our results demonstrated a significantly higher ($p < 0.001$) amount of phenolics when the ethyl acetate fraction was used compared with crude extract. The ability of phenolic compounds of *G. procumbens*

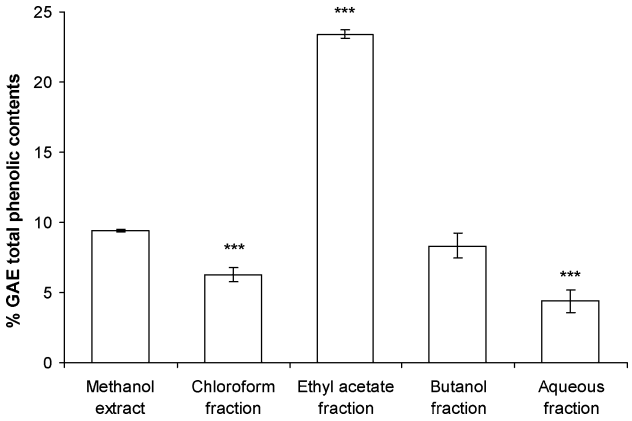

Figure 7. Gallic acid equivant total phenolic contents of the *Gynura procumbens* extract and fraction. Each value represents a mean ± SD (n = 3).*** indiactes significat differences comared with the methanol extract at $p < 0.05$.

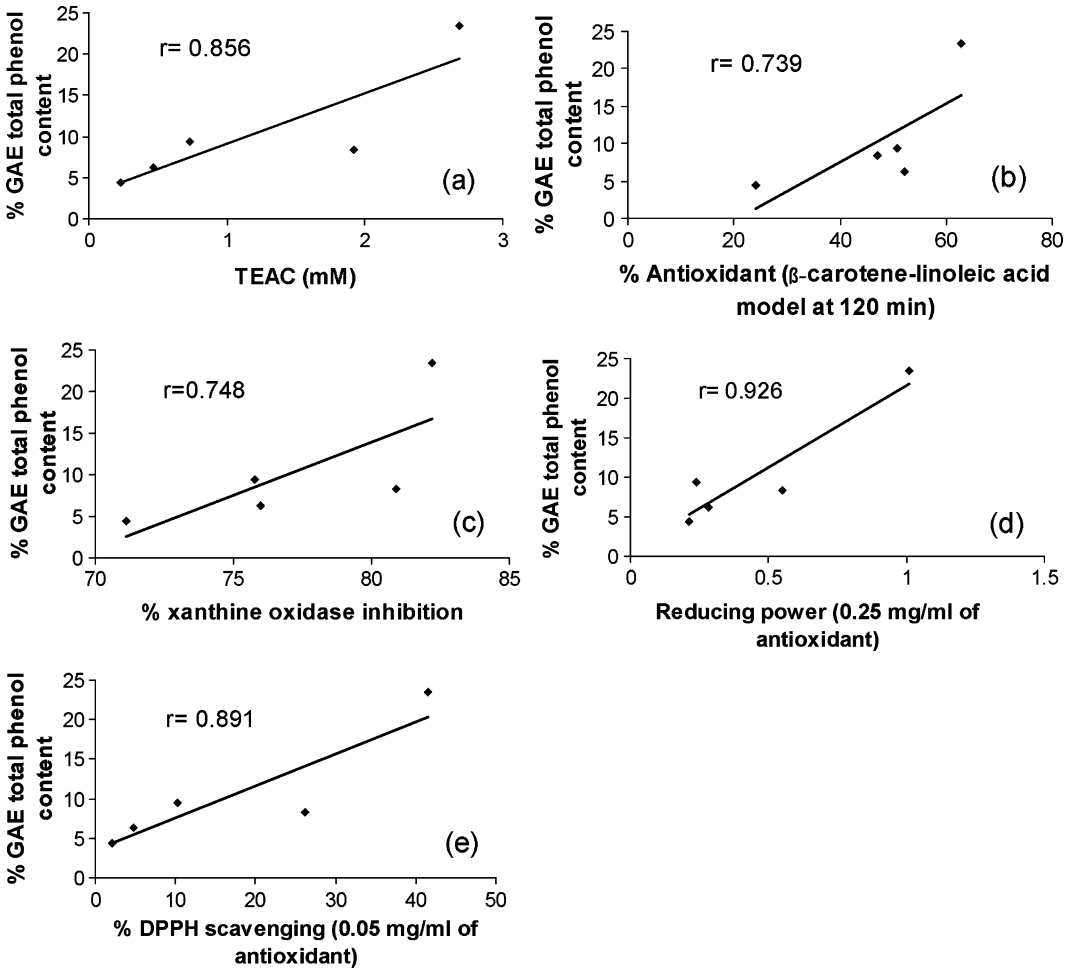

Figure 8. Linear correlation between the total phenolic contents and (a) trolox equivant antioxidant captacity (TEAC), (b) β-carotene–linoleic acid antioxidant model, (c) xanthine oxidase inhibition, (d) reducuing power, and (e) DPPH seaveing activites of *G. procumbents* extract and fractions.

extract and fractions to scavenge free radicals and the reducing power were in direct agreement with earlier reported literature (Behera et al., 2006). Correlation coefficients (r) of total phenolic content and reducing power and scavenging ability of DPPH were 0.926 ($p < 0.05$) and 0.891 ($p < 0.05$), respectively (Fig. 8).

Overall, EF and BF were better in reducing power, total antioxidant capacity, xanthine oxidase inhibition, and DPPH scavenging activities, and higher in total phenol content. Kaempferol-3-*O*-rutinoside and astragalin are the most representative phenolic compounds in EF and BF (Table 1); 58.4% and 87.6% of total phenolic compound in EF and BF, respectively. It seems that contents of total phenol, especially kaempferol-3-*O*-rutinoside and astragalin were highly associated with antioxidant properties (Chiang et al., 1994; Kim et al., 1999; Plumb et al., 1999; Han et al., 2004).

Conclusions

In conclusion, all the extract and fractions of *G. procumbens* in this research exhibited different extents of antioxidant activity. The results of the current study showed that the ethyl acetate fractions, which contain the highest amount of phenolic compounds, exhibited the greatest antioxidant activity. The high scavenging property of *G. procumbens* may be due to hydroxyl groups existing in the chemical structure of the phenolic compound that can provide the necessary component as a radical scavenger and antioxidant. Research is still going on in our laboratory to isolate and verify the mechanism of antioxidative effect of kaempferol-3-*O*-rutinoside, astragalin and other phenolic compounds from the ethyl acetate fraction of *G. procumbens* methanol extract.

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