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Full Length Research Paper

Whole-plant profiling of total phenolic and flavonoid contents, antioxidant capacity and nitric oxide scavenging capacity of *Turnera subulata*

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Turnera subulata **is a medicinal plant used as an herbal remedy for coughs and bronchitis. Unlike two other members of the genus,** ***Turnera diffusa*** **and** ***Turnera ulmifolia*****, which dominate the ethnopharmacological and pharmacological literature, very little is known about the bioactive properties of** ***T. subulata*****. In this study, whole-plant profiling of the total phenolic and flavonoid contents as well as antioxidant capacity of** ***T. subulata*** **was accomplished by analysing the leaf, stem, root, fruit and flower extracts. Analyses of total phenolic and flavonoid contents, free radical scavenging activity and ferric reducing ability revealed that the leaf extract had the highest antioxidant potential. Comparison of total phenolic contents and antioxidant parameters between** ***T. subulata*** **leaf extract and the extracts of other medicinal plants also indicated** ***T. subulata*** **to be a rich source of antioxidant activities. Strong, positive correlations were found between total phenolic and flavonoid contents and three antioxidant parameters, namely Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and nitric oxide scavenging activity. Our results indicate that the total pool of phenolic compounds, including flavonoids, in the** ***T. subulata*** **extracts were capable of both scavenging free radicals and reducing oxidants.**

Key words: Ferric reducing antioxidant power, flavonoids, free radical scavenging activities, nitric oxide, phenolics, trolox equivalent antioxidant capacity, *Turnera subulata.*

INTRODUCTION

Turnera subulata Sm. is native to Tropical America, but has been widely naturalised outside its native range, including South-east Asia (Short, 2011). While there are about 120 species in the genus *Turnera* (Short, 2011), the ethnopharmacological and pharmacological literature on the genus is dominated by *Turnera diffusa* Willd. ex Schult (synonym *Turnera aphrodisiaca* Ward), followed by *Turnera ulmifolia* L (Kumar et al., 2005). The medicinal potential of other *Turnera* species is largely under-explored and much work is needed to investigate their therapeutic potential (Kumar et al., 2005). *T. diffusa* is reputed for its use in traditional medicine as aphrodisiac. It is also prescribed as nerve tonic and used as herbal remedies for disorders of the digestive, respiratory and reproductive systems. Likewise, *T. ulmifolia* has been used as remedies for a broad range of ailments in traditional medicines (Kumar et al., 2005). By contrast, very little is known about the medicinal potential of *T. subulata*, although in Brazil, *T. subulata* is used as a medicinal herb. The entire plant is used to prepare syrup, which works as expectorant to treat bronchitis and coughs. Alternatively, its roots are used to prepare decoction used to treat amenorrhoea and dysmenorrhoea (Agra et al., 2007).

Free radical scavenging capacities and total phenolic contents of the leaf extracts of *T. ulmifolia* (Nascimento et al., 2006) and leaf, stem and root extracts of *T. diffusa* (Salazar et al., 2008) have been reported. Potent antioxidant capacity detected in the two species was positively correlated with their total phenolic contents

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Abbreviations: DPPH, 1,1-Diphenyl-2-picrylhydrazyl; **ABTS,** 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); **FRAP,** ferric reducing antioxidant power; **TEAC,** trolox equivalent antioxidant capacity.

(Nascimento et al., 2006; Salazar et al., 2008). These findings prompted us to hypothesise that *T. subulata* may also possess high phenolic contents and potent free radical scavenging activities.

In this study, whole-plant profiling of total phenolic and flavonoid contents as well as antioxidant capacity of *T. subulata* was accomplished by analysing the extracts prepared from five different plant organs (leaf, stem, root, fruit and flower). We have determined the nitric oxide scavenging activity of the extracts, which has not been reported in any *Turnera* species. Evaluation of the nitric oxide scavenging activity of the extracts is pertinent in view of the traditional use of the plant as remedy for coughs and bronchitis (Agra et al., 2007). These two conditions are associated with airway inflammation (Kinsey, 2010), which is associated with increased nitric oxide production (van der Vliet et al., 1999). To our knowledge, this paper represents the first report on the antioxidant capacity of *T. subulata* extracts.

MATERIALS AND METHODS

Plant materials

The *T. subulata* plant samples were collected from the campus ground of Universiti Tunku Abdul Rahman in September 2011. The plant was authenticated by Professor Dr Ong Hean Chooi at the Institute of Biological Sciences, University of Malaya, Malaysia and Dr Richard Chung of Forest Research Institute Malaysia. Voucher specimens were deposited at Department of Chemical Science, Universiti Tunku Abdul Rahman.

Preparation of aqueous extracts

Whole plants were separated into leaves, stems, roots, flowers and young fruits immediately after they were collected and processed separately. Plant samples were oven-dried for 48 h at 45°C before they were pulverized in a Waring blender. They were then extracted with autoclaved deionized water at a 1:10 (dry weight: volume) ratio at 90°C for 1 h (Kumaran and Joel karunakaran, 2006). The heat-incubated homogenates were then vacuum-filtered and the filtrate was clarified by centrifugation at 9000 rpm and 4°C for 10 min. The supernatant obtained, taken as 100 mg/ml in concentration, was immediately aliquoted (500 µl each) and stored at -20°C until used.

Determination of total phenolic contents

Total phenolic contents of the extracts were evaluated using a Folin-Ciocalteu colorimetric assay (Waterhouse, 2001). A mixture containing 0.2 ml of extract, 0.8 ml of deionised water and 0.1 ml of Folin-Ciocalteu reagent was first incubated at room temperature for 3 min. After adding in 0.3 ml of Na_2CO_3 (20% w/v), the mixture was further incubated at room temperature for 120 min. Absorbance of the mixture was read at 765 nm. Total phenolic contents were expressed in mg gallic acid equivalents (GAE)/g dry matter, calculated from a standard curve prepared with 0 to 100 mg/l gallic acid.

Determination of total flavonoid contents

Total flavonoid contents of the extracts were determined using a colorimetric assay described by Zou et al. (2004). A mixture of 0.2 ml of extract and 0.15 ml of $NaNO_2$ (5% w/v) was first incubated at room temperature for 6 min. Next, 0.15 ml of $AlCl_3.6H_2O$ (10% w/v) was added to the mixture, which was then kept at room temperature for 6 min. Then, 0.8 ml of NaOH (10% w/v) was added and the absorbance of the mixture was read at 510 nm after standing at room temperature for 15 min. For the blank, the extracts were replaced with water. To correct for background absorbance, each sample measurement was accompanied with a simultaneous reaction in which $AlCl_3.6H_2O$ was replaced with water. A standard curve was prepared with 0 to 500 µg/ml quercetin (in 80% ethanol). Total flavonoid content was expressed in mg quercetin equivalents (QE)/g dry matter.

Determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of the extracts was assessed using an assay modified from Lim and Quah (2007). A mixture of 0.5 ml of DPPH (0.10 mM in methanol) and 0.5 ml of extract was kept in the dark for 30 min. Its absorbance was then read at 517 nm. A blank was prepared for each sample in which the DPPH solution was replaced with methanol. DPPH radical scavenging activity (%) was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = (1- [A_{sample} / A_{control}]) \times 100$$

where $A_{control}$ is the absorbance of control reaction (without plant extract) and A_{sample} is the absorbance in the presence of a plant extract.

Determination of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation scavenging activity

$ABTS^+$ radical scavenging activity of the extracts was determined as described by Re et al. (1999) with modifications. The $ABTS^+$ radical stock solution was prepared by mixing an equal volume of ABTS solution (8 mg/ml) with potassium persulfate (1.32 mg/ml). The mixture was kept in the dark for 12 h at room temperature. An $ABTS^+$ radical working solution was prepared by diluting the $ABTS^+$ radical stock solution with potassium phosphate buffer (100 mM, pH 7.4) to obtain an absorbance of 0.700 $\pm$ 0.005 at 734 nm. For measurements, 0.1 ml of extract was added to 1 ml of $ABTS^+$ radical working solution.

The mixture was kept in the dark for 10 min before its absorbance was read at 734 nm. $ABTS^+$ radical scavenging activity (%) was calculated as follow:

$$ABTS^+ \text{ radical scavenging activity (\%)} = (1- [A_{sample} / A_{control}]) \times 100$$

where $A_{control}$ is the absorbance of control reaction (without plant extract) and A_{sample} is the absorbance in the presence of a plant extract. Antioxidant capacities of the 1 mg/ml extracts are also presented as TEAC values (mmole Trolox equivalents (TE)/100 g dry matter), calculated from a standard curve prepared with 0 to 0.25 mM Trolox.

Determination of nitric oxide scavenging activity

Nitric oxide scavenging activity of the extracts was determined as described by Sreejayan and Rao (1997) with modifications. Briefly, a mixture of 0.8 ml of extract and 0.2 ml of freshly prepared sodium nitroprusside (5 mM, in phosphate buffered saline, pH 7.4) was kept at room temperature for 150 min under light source (24 W compact fluorescent light bulbs). Then, 0.6 ml of the mixture was transferred to a new tube containing 0.6 ml of freshly prepared Griess Reagent

Table 1. Total phenolic and flavonoid contents of the extracts of *T. subulata.*

Extract	Total phenolic content (mg GAE / g dry matter)	Total flavonoid content (mg QE / g dry matter)
Leaf	23.43 ± 0.56^{a}	53.11 ± 1.82^{a}
Stem	18.08 ± 0.11^{b}	37.59 ± 0.81^{b}
Root	8.07 ± 0.15^{c}	11.19 ± 0.08^{c}
Flower	11.06 ± 0.24^{d}	6.81 ± 0.18^{d}
Fruit	6.95 ± 0.14^{e}	$8.50 \pm 0.24^{c, d}$

Data are expressed as mean ± SE values (n=3). Different super indexes indicate significant differences within a column when compared by the Fisher's least significant difference (LSD) test ($P < 0.05$).

(1% sulphanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 5% phosphoric acid). This mixture was then allowed to stand at room temperature in darkness for 10 min. Its absorbance was then read at 546 nm. To correct for background absorbance, each sample measurement was accompanied with a simultaneous reaction in which sodium nitroprusside solution and Griess Reagent were replaced with water. Nitric oxide radical scavenging activity (%) was calculated as follows:

$$\text{Nitric oxide scavenging activity (\%)} = (1- [A_{sample} / A_{control}]) \times 100$$

where $A_{control}$ is the absorbance of control reaction (without plant extract) and A_{sample} is the absorbance in the presence of a plant extract.

Determination of FRAP

Ferric reducing capacities of the extracts were assessed with the FRAP assay (Benzie and Strain, 1996). FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-tripyridyl-s-triazine (10 mM), and $FeCl_3.6H_2O$ (20 mM) in a 10:1:1 (v:v:v) ratio and pre-warmed to 37°C before use. A reaction mixture containing 0.2 ml of 1 mg/ml extract and 1.2 ml of FRAP reagent was incubated at 37°C for 5 min. Absorbance of the mixture was then read at 593 nm. Reducing power is presented in mmole Fe^{2+} equivalents, calculated from a standard curve prepared with 0 to 0.40 mM $FeSO_4.7H_2O$.

Data analysis

All experiments were carried out in triplicates and data reported are mean ± standard errors. Statistical analysis was performed using SAS (Version 9.2), except correlation analyses, which were carried out using Microsoft Office Excel 2003. Data were analyzed by the ANOVA test and means of significant differences were separated using Fisher's least significant difference test at the 0.05 level of probability.

RESULTS

Total phenolic and flavonoid contents

Total phenolic contents of the five extracts ranged from 6.95 to 23.43 mg GAE/g dry matter. In descending order, relative abundance of total phenolics in the extracts was as follows: leaf > stem > flower > root > fruit (Table 1). The total phenolic contents of leaf and stem extracts were 3.4-fold and 2.6-fold higher than that of fruit extract, respectively. Total flavonoid contents varied between 6.81 and 53.11 mg QE/g dry matter and were in the descending order of leaf > stem > root > fruit, flower (Table 1). Total flavonoid contents of the fruit extract was 1.25-fold higher compared with flower extract, but the difference was not statistically different. The average of total flavonoid contents of flower and fruit extracts was 7.66 mg QE/g dry matter. Leaf and stem flavonoid contents were 7-fold and 5-fold higher than this average value, respectively.

DPPH, $ABTS^+$ and nitric oxide scavenging activity

Free radical scavenging capacities of the extracts were assessed using the DPPH radical, $ABTS^+$ radical and Nitric acid scavenging assays. At extract concentration 5 mg/ml, all extracts exhibited at least 90% DPPH radical scavenging activities (Figure 1). At extract concentration 1 mg/ml, DPPH radical scavenging activities varied from 54 to 93%. DPPH radical scavenging activities of the 1 mg/ml extracts in descending order was as follows: stem, leaf > root > flower, fruit. DPPH radical scavenging activities were not statistically different between leaf and stem extracts and between flower and fruit extracts. There was a 1.7-fold difference between the average scavenging activities of leaf and stem extracts and the average scavenging activities of flower and fruit extracts. All extracts exhibited at least 87% $ABTS^+$ radical scavenging activities at extract concentration 5 mg/ml (Figure 2). At extract concentration 1 mg/ml, $ABTS^+$ radical scavenging activity varied from 24 to 71% and ranked in the descending order of leaf > stem > flower > root > fruit. The $ABTS^+$ scavenging activities of 1 mg/ml leaf and stem extracts were about 3-fold and 2.3-fold higher than that of the fruit extract, respectively.

Nitric oxide scavenging activities ranging from 30 to 64% and from 52 to 70% were detectable in the 1 and 5 mg/ml extracts, respectively (Figure 3). Based on both extract concentrations, the efficacy of the extracts in nitric oxide scavenging in descending order was leaf > stem > root, flower, fruit. Nitric oxide scavenging activities of root, flower and fruit extracts were not statistically different. At 1 mg/ml, average nitric oxide scavenging activities of root,

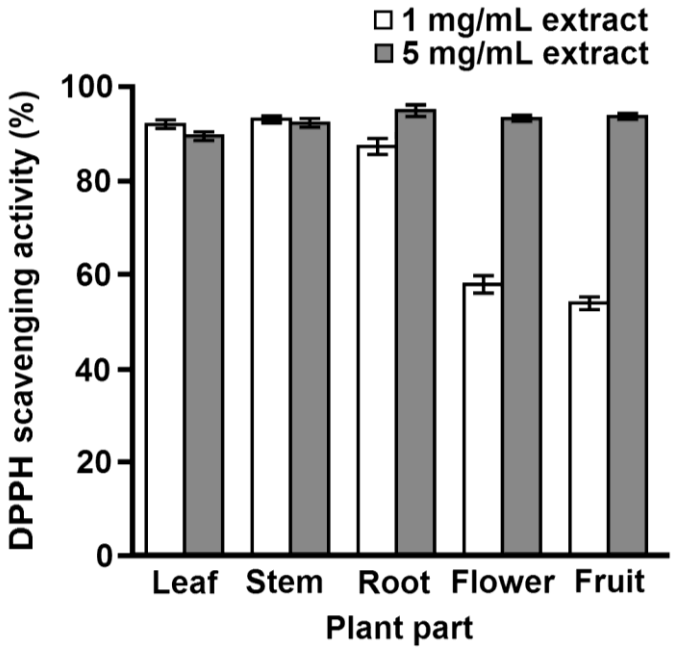

Figure 1. DPPH radical scavenging activities of aqueous extracts of different parts of the *T. subulata* plant. Data are expressed as mean ± SE values (n=3).

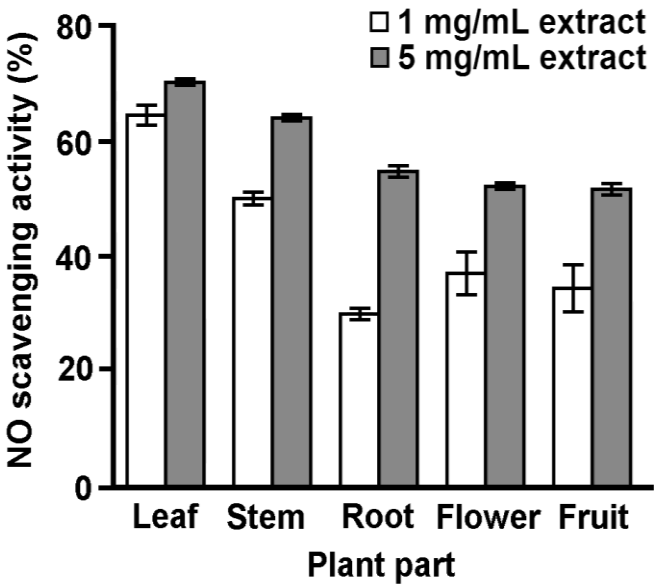

Figure 3. Nitric oxide scavenging activities of aqueous extracts of different parts of the *T. subulata* plant. Data are expressed as mean ± SE values (n=3).

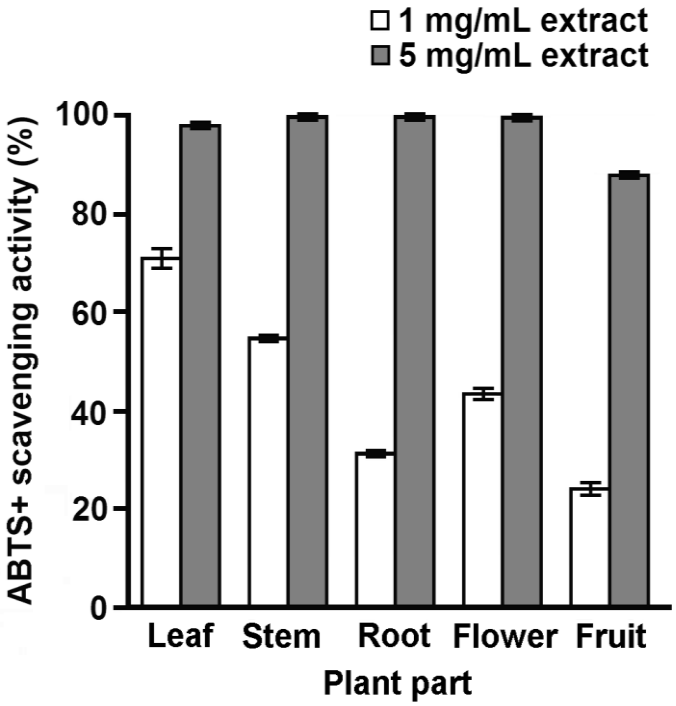

Figure 2. $ABTS^{+}$ radical scavenging activities of aqueous extracts of different parts of the *T. subulata* plant. Data are expressed as mean ± SE values (n=3).

flower and fruit extracts was 34%. The nitric oxide scavenging activities of leaf and stem extracts were 1.9-fold and 1.5-fold higher than this average value, respectively. At 5 mg/ml, average nitric oxide scavenging activities of root, flower and fruit extracts was 53%. The nitric oxide scavenging activities of leaf and stem extracts were 1.3-fold and 1.2-fold higher than this average value, respectively.

Trolox equivalent antioxidant capacity (TEAC)

TEAC values of the 1 mg/ml extracts were calculated from $ABTS^{+}$ radical scavenging assay calibrated using Trolox, a water-soluble analogue of tocopherol. TEAC is a measurement of effective antioxidant activity of the extracts analysed. Higher TEAC values signify higher antioxidant activity. TEAC values of the extracts varied between 6.06 and 17.99 mmole TE/100 g dry matter and were in the descending order of leaf > stem > flower > root > fruit (Table 2). TEAC values of leaf and stem extracts were 3-fold and 2.3-fold higher than that of fruit extract, respectively.

Ferric reducing antioxidant power (FRAP)

FRAP values of the 1 mg/ml extracts ranged between 5.98 and 22.50 mmole Fe^{2+} equivalents/100 g dry matter and decreased in following order: leaf > stem > root > flower, fruit (Table 2). FRAP values of the flower extract was 10% higher compared with fruit extract, although the

Table 2. TEAC and FRAP of the extracts of *T. subulata.*

Extract	TEAC (mmole TE / 100 g dry matter)	FRAP (mmole Fe^{2+} equivalents / 100 g dry matter)
Leaf	17.99 ± 0.49^{a}	22.50 ± 0.47^{a}
Stem	13.87 ± 0.07^{b}	17.25 ± 0.78^{b}
Root	7.92 ± 0.12^{c}	9.81 ± 0.32^{c}
Flower	10.99 ± 0.29^{d}	6.58 ± 0.07^{d}
Fruit	6.06 ± 0.42^{e}	5.98 ± 0.02^{d}

TEAC and FRAP values were both computed from measurements made on the 1 mg/ml extracts. Data are expressed as mean ± SE values (n=3). Different super indexes indicate significant differences within a column when compared by the Fisher's least significant difference (LSD) test ($p < 0.05$).

Table 3. Correlation between total phenolic and flavonoid contents and three bioactive parameters.

Parameter	Correlation of determination (R^2)		
	TEAC values	FRAP values	NO scavenging activity
Total phenolics	0.99	0.98	0.90
Total flavonoids	0.87	0.95	0.73

R^2 values presented below are all statistically significant ($p < 0.05$).

difference was not statistically significant. The average FRAP value for the flower and fruit extracts was 6.28 mmole Fe^{2+} equivalents/100 g dry matter. FRAP values of leaf and stem extracts were 3.6- and 2.7-fold higher than this average value, respectively.

Correlation between total phenolic and flavonoid contents with other bioactive parameters

Total phenolic contents were strongly and positively correlated with three bioactive parameters analysed, namely TEAC value, FRAP value and nitric oxide scavenging activity (Table 3). Coefficient of determination (R^2) values for the correlation between total phenolic contents and the three bioactive parameters were between 0.90 and 0.99 ($p < 0.05$). R^2 values for the correlations between total flavonoid contents and the three bioactive parameters were similarly significant, ranging between 0.73 and 0.95 ($p < 0.05$).

DISCUSSION

Our analyses of total phenolic and flavonoid contents, free radical (DPPH, $ABTS^{+}$ and nitric oxide) scavenging activity and ferric reducing ability showed that the leaf extract of *T. subulata* had the highest antioxidant capacities compared with extracts of other plant parts. This thus suggests that when the entire plant is used in traditional medicine (Agra et al., 2007), the antioxidant activities of the preparation are mainly contributed by the leaf extract.

In this study, the plant material was extracted with hot water to reflect the use of water in the preparation of *T. subulata* in traditional medicine. Although total phenolic contents and some other antioxidant parameters have been reported for *T. ulmifolia* (Nascimento et al., 2006) and *T. diffusa* (Salazar et al., 2008), the samples analysed were hydro-ethanolic and methanolic extracts, respectively. Different extraction approaches used between the aforementioned studies (Nascimento et al., 2006; Salazar et al., 2008) and ours preclude meaningful, quantitative comparison between studies. Nonetheless, based on total phenolic contents, TEAC values and FRAP values, *T. subulata* leaf extracts appeared to be a potent source of antioxidant activities when compared with other medicinal plants. The total phenolic content of the leaf extract was higher compared with the aqueous extracts of 21 vegetables (Wong et al., 2006) and 84 anticancer medicinal plants (Cai et al., 2004). TEAC value of the leaf extract, on the other hand, was greater compared with aqueous extracts of 112 anticancer medicinal plants (Cai et al., 2004). Comparison of FRAP values with other studies also found *T. subulata* leaf extract to have greater ferric reducing ability than the aqueous extracts of 27 other medicinal plants (C.C. Wong et al., 2006).

Strong, positive correlations between TEAC and FRAP values with total phenolic compounds in the *T. subulata* extracts accords with studies carried out on numerous other medicinal plants (Cai et al., 2004; Wong et al., 2006; Guo et al., 2008; Lizcano et al., 2010). Our results imply that 99% of free radical scavenging activity (based on TEAC values) and 98% of ferric reducing activity (based on FRAP values) can be attributed to the contribution of phenolic compounds. Total flavonoid contents also correlated with these bioactive parameters significantly,

but to a lower extent. Such differences in the strength of correlations between total phenolic and flavonoid contents with antioxidant parameters have been previously reported (Lizcano et al., 2010). Taken together, the outcome of correlation analyses implies that high contents of total phenolics and flavonoids are important determinants of antioxidant activity in the *T. subulata* extracts. The total pool of phenolic compounds in the extracts was apparently capable of both scavenging free radicals and reducing oxidants (Fe^{3+} ions). However, certain non-phenolic and non-flavonoid compounds in the extracts probably concurrently contribute to the overall antioxidant potential of the extracts.

Correlation analysis revealed that 90 and 73% of Nitric oxide scavenging activities of the extracts may be accounted for by total phenolic and flavonoid contents, respectively. The ability of phenolic and flavonoid compounds to directly scavenge reactive nitrogen species, including nitric oxide has been discussed in several reviews (Nijveldt et al., 2001; Dai and Mumper, 2010; Procházková et al., 2011).

Free radical, including nitric oxide scavenging activity of the *T. subulata* extracts that we have demonstrated in this study may at least partly explain the beneficial effects of the plant when used to treat coughs and bronchitis (Agra et al., 2007). Bronchitis is an inflammation of the bronchial tubes, a condition associated with irritation of the airways and persistent coughs (Kinsey, 2010). Excessive nitric oxide production during inflammation can lead to respiratory tract injury through interaction between nitric oxide and reactive oxygen species to form more toxic reactive nitrogen species (van der Vliet et al., 1999). Future studies are required to confirm the relationship between the therapeutic properties of *T. subulata* and its antioxidant capacity *in vivo*.

Conclusions

Analyses of total phenolic and flavonoid contents, free radical scavenging activity and ferric reducing ability showed that the aqueous extracts of *T. subulata* leaf was the greatest source of antioxidant potential compared with extracts of other plant organs. Notably, based on total phenolic contents, TEAC and FRAP values, *T. subulata* leaf extracts also appeared to be a more potent source of antioxidant activities compared with many other medicinal plants. Our study suggests that *T. subulata* leaf extract is potentially a rich source of phenolic compounds with antioxidant activity. Further investigation on the phytochemical constituents of the leaf extract of *T. subulata* as well as isolation and purification of antioxidant compounds is thus warranted. On the other hand, the relationship between the therapeutic properties of the plant and its antioxidant capacity *in vivo* merits further investigation.

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