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Full Length Research Paper

Antioxidation and cytotoxic activities of selected medicinal herbs used in Malaysia

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Free radicals, with their single electrons and reactive nature, could easily interact with different cellular compartments and cause oxidative damage. Prolonged oxidative stress could lead to DNA mutations, neurodegenerative diseases and heart disorders. These disorders could deteriorate our overall well-being and prove to be fatal. Medicinal herbs, rich in their secondary metabolites, could provide the antioxidants and scavengers needed to eliminate the free radicals. In this study, we tested six medicinal herbs used in Malaysia for their scavenging activities, in three different radical scavenging assays. We also tested their total phenolic and flavonoid levels. Among the herbs tested, *Scutellaria barbata, Prunella vulgaris* and *Lophatherum gracile* consistently demonstrated the highest activities in all three radical scavenging assays and trolox equivalent antioxidant capacity. Higher levels of total phenolic and flavonoid contents were also detected in these three herbs. Additionally, brine shrimp lethality assays also indicated low cytotoxicity in these herbs. In short, our results provided evidence for the safe use of these herbs as treatment for ailment in which free radical damage is implicated.

Key words: Cytotoxicity, flavonoid, free radical scavenging activities, medicinal herbs, phenolic, trolox equivalent antioxidant capacity.

INTRODUCTION

Free radicals are compounds with unpaired electrons, and they could be generated during the normal body metabolism or from the ingestion of foreign chemicals and pollutants. Because of their reactive nature, free radicals could interact with and cause oxidative damage on different cellular compartments, including cell membranes and DNA. Prolonged oxidative stress will induce permanent damage on vital body organs, which could eventually lead to arterial disorders, heart and neurodegenerative diseases (Uttara et al., 2009; Sugamura and Keaney, 2011). Accumulated mutations, as a result of oxidative damage by free radicals, have been linked to the formation of cancers too (Chahar et al., 2011). If excess free radicals in our bodies could be eliminated, it could prove very beneficial to our overall well-being. Plants, rich in their phytochemical compounds, are good sources of antioxidants and radical scavengers (Gescher et al., 1998). Medicinal plants are especially rich in various secondary metabolites, including but not limited to polyphenols and flavonoids, which are capable of eliminating free radicals (Ren et al., 2003). Both *in vitro* and *in vivo* experiments have shown the ability of these plants-derived compounds in neutralizing free radicals (Etsuo, 2010; Park et al., 2010).

Medicinal herbs have long been utilized by different ethnic groups all over the world for thousand of years. The herbs are ingested, in a variety of forms, for therapeutic or health-promoting purposes. Many of these medicinal herbs may exert their beneficial properties, partly by eliminating excess free radicals like reactive oxygen and nitrogen species from our bodies. Modern science has offered more concrete evidence to support this claim (Hou, 2003; Lipinski, 2011). Some of these medicinal herbs with paramount antioxidant and radical scavenging activities have further been developed into commercial anti-tumor drugs. For instance, Vinblastine and Vincristine, alkaloid drugs widely used in

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **NO,** nitric oxide; **TEAC,** trolox equivalent antioxidant capacity.

chemotherapy, are derived originally from *Catharanthus roseus* (Madagascar periwinkle), a plant endemic to Madagascar in Africa (Facchini and De Luca, 2008). Moreover, camptothecin analogs such as Irinotecan and Topotecan, drugs used for treatment of cancers, were originally derived from *Camptotheca acuminata* (Asian happy tree) (Lorence and Nessler, 2004; Sriram et al., 2005).

Although many medicinal herbs have long been utilized by the local community as health promoting tonics, the health benefits of many herbs have not been scientifically studied. Frequently, their cytotoxicity or lethality is not well established. In this paper, we aimed to investigate the antioxidation and radical scavenging activities of selected medicinal herbs used in Malaysia. We focused on six species of medicinal herbs originated from four plant families, namely *Houttuynia cordata*, *Imperata cylindrica*, *Lophatherum gracile*, *Polygonatum odoratum*, *Prunella vulgaris* and *Scutellaria barbata*. These herbs are frequently used as health-promoting tonics and supplements, as well as treatments for body detoxification and cleansing (Yang, 2007). In addition, *L. gracile* is used for postnatal care (Zakaria and Mohd, 2010). We tested these six herbs for their radical scavenging activities using *in vitro* assays. Their corresponding levels of total phenolic and total flavonoid were also quantified and compared. Additionally, we also assessed the medicinal herbs' cytotoxicity in a brine shrimp assay. With this work, we aimed to contribute to the understanding and discovery of powerful antioxidants, with low cytotoxicity and safe for large dosage consumption.

MATERIALS AND METHODS

Preparation of medicinal herb extracts

Medicinal herbs (*H. cordata*, *I. cylindrica*, *L. gracile*, *P. odoratum*, *P. vulgaris* and *S. barbata*) were purchased from local food market and herb store in May and June of 2011. The medicinal herbs were identified morphologically by referring to the literature (Duke et al., 2002; Yang, 2007; Zakaria and Mohd, 2010). Either the aerial parts (*S. barbata, P. vulgaris, L. gracile and H. cordata*) or the rhizomes (*I. cylindrica and P. odoratum*) were used in this study, to reflect their actual usage by the local people. The herbs were incubated in an oven at 40°C for 48 h or until constant weight was observed. Each dried herb was then macerated for 24 h in 90% ethanol [1:10 (w/v)] at room temperature. After removing the ethanol extract, maceration was repeated with fresh 90% ethanol. Ethanol extracts were combined and filtered. Filtered extract was then concentrated and dried under reduced pressure. Crude extract was stored in -20°C until testing.

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

DPPH radical scavenging activity was assessed as described previously with modifications (Thaipong et al., 2006; Lim and Quah, 2007). DPPH stock solution was prepared by dissolving 24 mg of DPPH into 100 ml ethanol and stored at -20°C until needed. DPPH working solution was prepared by mixing 10 ml of stock solution with 45 ml ethanol. To 1 ml of DPPH working solution, 50 µl of extract was added. The mixture was left in the dark for 30 min before its absorbance was read at 517 nm. A blank was prepared for each sample in which the DPPH solution was replaced with ethanol. DPPH radical scavenging activity was calculated using the following formula:

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

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. Trolox was used as reference. Results are also presented as EC_{50} values, which represent concentrations of extracts required to scavenge 50% of the DPPH radicals.

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

ABTS radical cation ($ABTS^+$) scavenging activity of the extracts was determined as described in Re et al. (1999) with modifications. To prepare the $ABTS^+$ stock solution, an equal volume of ABTS solution (8 mg/ml) was first mixed with potassium persulfate (1.32 mg/ml). The mixture was kept in the dark for 12 h at room temperature. Then, an $ABTS^+$ working solution was prepared by diluting the $ABTS^+$ stock solution with potassium phosphate buffer (100 mM, pH 7.4) to obtain an absorbance of 0.700 ± 0.005 at 734 nm. For measurements, 0.1 ml of extract was added to 1 ml of $ABTS^+$ working solution. The mixture was kept in the dark for 10 min before its absorbance was read at 734 nm. $ABTS^+$ radical scavenging ability (%) was calculated as shown below:

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

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. Relative antioxidant capacities of the extracts are also presented as Trolox Equivalent Antioxidant Capacity (TEAC) values (mM Trolox equivalents/100 g dry matter), calculated from a standard curve prepared with 0 to 0.25 mM Trolox.

Determination of nitric oxide (NO) scavenging activity

NO scavenging activity of the extracts was determined as described in 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 bulb).

Then, 0.6 ml of the mixture was transferred to a new tube containing 0.6 ml of freshly prepared Griess Reagent (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. NO radical scavenging activity (%) was calculated as follows:

$$\text{NO scavenging activity (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

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 total phenolic content

The concentrations of total phenolic in the extracts were determined using a Folin-Ciocalteu colorimetric assay, as described previously with modifications (Ainsworth and Gillespie, 2007). A mixture of extract (0.1 ml) and 10% (v/v) Folin-Ciocalteu reagent (0.2 ml) was first incubated at room temperature for 3 min. Next, 0.8 ml of 700 mM Na_2CO_3 was added and the mixture was incubated at room temperature for 2 h. Absorbance of the mixture was read at 765 nm. A standard curve was prepared from 0 to 42 mg/L gallic acid. Total phenolic content was expressed in mg gallic acid equivalents/g dry matter.

Determination of total flavonoid content

The concentrations of total flavonoid in the extracts were determined using an assay modified from Zou et al. (2004). Plant extract (0.2 ml) was added to 0.15 ml of $NaNO_2$ (5% w/v) and the mixture was 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 left at room temperature for 6 min. Next, 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, a blank was prepared for each sample in which the $AlCl_3.6H_2O$ was replaced with water. A standard curve was prepared from 0 to 500 μg/ml quercetin dissolved in 80% ethanol. Total flavonoid content was expressed in mg quercetin equivalents/g dry matter.

Brine shrimp cytotoxicity assay

To assess the toxicity of each extract, brine shrimp cytotoxicity assays were performed as previously described with modifications (McLaughlin and Rogers, 1998; Ayuko et al., 2009). Eggs of brine shrimp (*Artemia franciscana*) originated from Great Salt Lake (USA) were purchased from Sera and mixed with artificial seawater (prepared by dissolving 38 g sea salt per liter of water). The eggs were allowed to hatch at room temperature for 48 to 72 h. Larvae (nauplii) were attracted to one side of the vessel with a light source and collected with plastic pipette. To determine if the herbal extracts were cytotoxic, different concentrations of each extract (4000, 2000, 1000, 500, 200, 100 and 50 μg/ml) were tested, in triplicates. Ten brine shrimp larvae were then added to each vial containing the aforementioned extracts. After incubating for 24 h at room temperature, the numbers of dead and surviving brine shrimps were counted. LC_{50} (concentration required to kill 50% of the brine shrimps) was determined for each herbal extract. Potassium dichromate was used as positive control.

RESULTS AND DISCUSSION

In this paper, a total of six medicinal herbs used in Malaysia were selected for study. The six herbs represent members of four plant families, namely *Lamiaceae* (*S. barbata, P. vulgaris*), *Poaceae* (*L. gracile, I. cylindrica*), *Saururaceae* (*H. cordata*) and *Asparagaceae* (*P. odoratum*). Either the aerial parts (*S. barbata, P. vulgaris, L. gracile and H. cordata*) or the rhizomes (*I. cylindrica and P. odoratum*) were used in this study, to reflect their actual usage by the local people. These six medicinal herbs are frequently used as health-promoting tonics and incorporated into the local diets in a variety of forms. We reported here their radical scavenging activities using three different types of assays, their total phenolic and flavonoid contents, and corresponding cytotoxic activities.

DPPH radical scavenging activity and EC_{50} values

To evaluate their radical scavenging potential, the extracts of these medicinal herbs were first tested in a DPPH assay. All herbal extracts exhibited DPPH radical scavenging activity in a concentration-dependent manner, in the range from 1 to 10 mg/ml. *S. barbata* was found to have the highest DPPH scavenging activity (99%), followed by *P. vulgaris* (91%) and *L. gracile* (66%), while *I. cylindrica*, *H. cordata* and *P. odoratum* possessed 10% or lower scavenging activity, compared at the concentration of 10 mg/ml (Figure 1). The corresponding EC_{50} values, which represent the concentration of extracts required to scavenge 50% of the DPPH radicals, were also determined (Table 1). The high antioxidation potential of *S. barbata*, *P. vulgaris* and *L. gracile* was reflected by their low EC_{50} values (<8 mg dry matter/ml). While *I. cylindrica* and *H. cordata* had EC_{50} values which were 23 and 179 folds higher, respectively, compared to that of *S. barbata*.

The lowest DPPH radical scavenging activity was observed in *P. odoratum*, as shown by its large EC_{50} value, which were 1541 folds higher than that of *S. barbata*.

$ABTS^+$ and NO radical scavenging activity

Radical scavenging activities of the 6 herbal extracts were also tested in two additional assays, namely $ABTS^+$ and NO scavenging assays. All extracts showed $ABTS^+$ and NO radical scavenging activities in a concentration-dependent manner, in the range from 1 to 10 mg/ml (Figures 2 and 3). Both $ABTS^+$ and NO scavenging results demonstrated a trend similar to that observed in DPPH scavenging assay. At extract concentration 10 mg/ml, *S. barbata* showed the highest $ABTS^+$ and NO scavenging activities (99.6% and 78.8% respectively), followed by *P. vulgaris* (43.7 and 47.2%, respectively) and *L. gracile* (55.7% and 55.6%, respectively). Lower than 20% activities were observed in *I. cylindrica* and *H. cordata*, in both $ABTS^+$ and NO radical scavenging assays. The lowest activity was observed with *P. odoratum*, with activities which were 23 folds ($ABTS^+$ scavenging assay) and 25 folds (NO scavenging assay) lower, compared to that of *S. barbata*. TEAC values for these medicinal herbs in descending order were *S. barbata* > *L. gracile* > *P. vulgaris* > *H. cordata* > *P. odoratum* > *I. cylindrica* (Table 1). The TEAC value of *S. barbata* was about 11 folds higher than that of *I. cylindrica*.

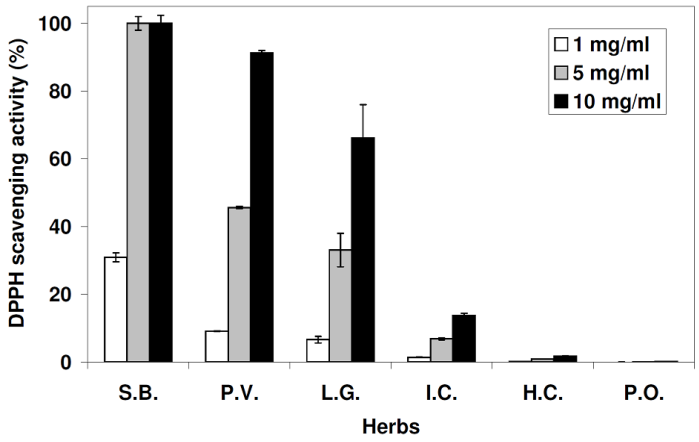

Figure 1. DPPH radical scavenging activities of herb extracts at different concentrations. Data are reported as mean ± SE values (n=3). S.B. (*S. barbata*), P.V. (*P. vulgaris*), L.G.(*L. gracile*), I.C. (*I. cylindrica*), H.C.(*H. cordata*), P.O.(*P. odoratum*).

Table 1. Antioxidant capacity of the medicinal herbs based on DPPH and $ABTS^+$ radical scavenging assays.

Herbs	DPPH EC_{50} [1] (mg dry matter/ml)	TEAC [2] (mmole TE/ 100 g dry matter) [3]
Scutellaria barbata	1.62 ± 0.07 [4]	11.76 ± 0.35
Prunella vulgaris	5.48 ± 0.04	2.88 ± 0.53
Lophatherum gracile	7.88 ± 1.04	4.57 ± 0.42
Imperata cylindrica	36.63 ± 1.87	1.09 ± 0.44
Houttuynia cordata	290.05 ± 19.65	1.85 ± 0.57
Polygonatum odoratum	2496.44 ± 27.52	1.21 ± 0.31

[1]DPPH EC_{50}, concentration of extract required to scavenge 50% of DPPH radicals. [2]TEAC, Trolox equivalent antioxidant capacity, determined by means of the $ABTS^+$ radical scavenging assay using Trolox as standard, [3]TE, Trolox equivalents, [4]Values reported as mean ± SE values (n=3).

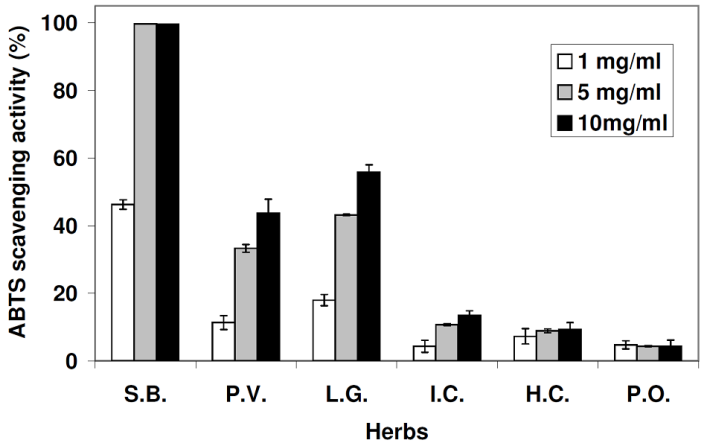

Figure 2. $ABTS^+$ radical scavenging activities of herb extracts at different concentrations. Data are reported as mean ± SE values (n=3). S.B. (*S. barbata*), P.V. (*P. vulgaris*), L.G.(*L. gracile*), I.C. (*I. cylindrica*), H.C.(*H. cordata*), P.O.(*P. odoratum*).

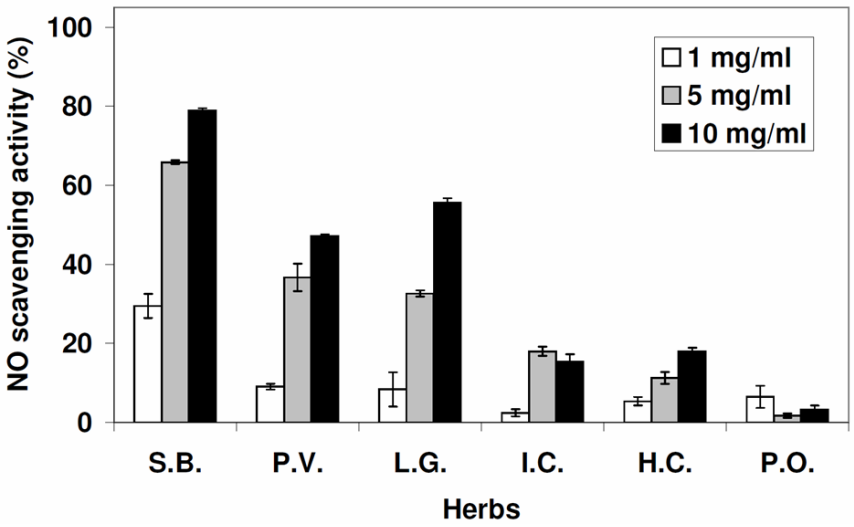

Figure 3. NO radical scavenging activities of herb extracts at different concentrations. Data are reported as mean ± SE values (n=3). S.B. (*S. barbata*), P.V. (*P. vulgaris*), L.G.(*L. gracile*), I.C. (*I. cylindrica*), H.C.(*H. cordata*), P.O.(*P. odoratum*).

Table 2. Total phenolic and total flavonoid contents of the six medicinal herbs.

Herbs	Total phenolics (mg GAE/g dry matter)[(1)]	Total flavonoids (mg QE/g dry matter)[(2)]
Scutellaria barbata	18.22 ± 0.15[(3)]	3.34 ± 0.24
Prunella vulgaris	15.13 ± 0.06	2.33 ± 0.05
Lophatherum gracile	12.06 ± 0.09	1.56 ± 0.12
Imperata cylindrica	3.05 ± 0.02	1.02 ± 0.13
Houttuynia cordata	2.79 ± 0.04	0.82 ± 0.14
Polygonatum odoratum	0.61 ± 0.03	0.13 ± 0.03

[(1)]GAE, gallic acid equivalents, [(2)]QE, quercetin equivalents, [(3)]Values reported as mean ± SE values (n=3).

Total phenolic and total flavonoid contents

As the antioxidation capacities of medicinal herbs were often linked to their levels of phenolic and flavonoid contents, we tested these herbal extracts for their total phenolic and flavonoid levels. Consistently, extracts with high contents of total phenolic were also found to possess high levels of total flavonoid. The highest levels of total phenolic and total flavonoid were detected in *S. barbata*, followed by *P. vulgaris* and *L. gracile* (Table 2). Significantly lower levels of phenolic and flavonoid were detected in *I. cylindrica* and *H. cordata*. Their phenolic and flavonoid contents are 5-6 folds and 3-4 folds lower, respectively, when compared to the levels observed in *S. barbata*. We also noticed that herbs (*S. barbata, P. vulgaris, L. gracile*) with the highest levels of total phenolic and flavonoid contents were found to demonstrate the highest radical scavenging activities. This direct relationship was observed in all DPPH, NO and $ABTS^+$ radical scavenging assays. Only trace amounts of phenolic (30 folds lower) and flavonoid (26 folds lower) were detected in *P. odoratum*, compared to *S. barbata*. Consistently, *P odoratum* also demonstrated the lowest radical scavenging activity.

Brine shrimp cytotoxic activity

The herbal extracts were tested in brine shrimp assay to determine their cytotoxic activities. The results were reported as LC_{50} values, the extract concentrations required to kill 50% of a group of brine shrimps. Among the herbs tested, *H. cordata* was found to show the highest cytotoxic activity, with a LC_{50} of 1442.4 µg/ml. The cytotoxic activities for the medicinal herbs in descending order were *H. cordata* > *S. barbata* > *L.*

Table 3. Brine shrimp cytotoxicity assay.

Herbs	LC_{50}[(1)] (µg/ml)
Scutellaria barbata	1913.9 ± 104.7[(2)]
Prunella vulgaris	3223.9 ± 95.0
Lophatherum gracile	2348.1 ± 223.0
Imperata cylindrica	5490.2 ± 556.8
Houttuynia cordata	1442.4 ± 60.5
Polygonatum odoratum	4162.9 ± 281.7
Potassium dichromate	47.7 ± 4.8

[(1)]LC_{50}, concentration required to kill 50% of a group of shrimps, [(2)]Values reported as mean ± SE values (n=3).

gracile > *P. vulgaris* > *P. odoratum* > *I. cylindrica* (Table 3). *S. barbata* (LC_{50}: 1913.9 µg/ml) and *P. vulgaris* (LC_{50}: 3223.9 µg/ml), both from the *Lamiaceae* family, have cytotoxic activities which are 40.1 and 67.6 folds lower, respectively, compared to potassium dichromate (LC_{50}: 47.7 µg/ml).

Medicinal herbs used in the study

Among the medicinal herbs tested, *S. barbata* and *P. vulgaris* were consistently found to have the highest radical scavenging activities, and the highest levels of total phenolic and flavonoid contents. Coincidentally, both *S. barbata* and *P. vulgaris* are from the *Lamiaceae* family, which comprises many aromatic medicinal herbs, including basil, lemon balm, oregano, rosemary and others (Matkowski et al., 2008). Many members of *Lamiaceae* have previously been reported as rich in phenolic acids, with caffeic and chlorogenic acids as the main constituents (Zgorka and Glowniak, 2001; Wink, 2003). However, it remains to be determined whether the two aforementioned phenolic compounds are actually responsible for the radical scavenging activities observed with *S. barbata* and *P. vulgaris*.

Scavenging activities were also detected in *L. gracile*, a member of *Poaceae* family. The aerial part of *L. gracile* is used by the locals for relieving heat irritation and promoting urination (Zhang et al., 2009). Previously, *Phleum pratense* (Sarker et al., 2005), *Sasa borealis* (Park et al., 2007) and *Cymbopogon jawarancusa* (Dar et al., 2011), all from the *Poaceae* family, have been reported to possess radical scavenging activities. For *S. borealis* and *P. pratense*, the detections of antioxidant flavone glycosides (Park et al., 2007) and caffeic acid (Sarker et al., 2005) have been reported. Additionally, detailed constituent analysis of *L. gracile* has resulted in the isolation of triterpenes and other chemical compounds (Zhang et al., 2009), although it remains to be determined their functional roles in the scavenging activity demonstrated by *L. gracile*. Among the six herbs tested in this study, *P. odoratum* possessed the lowest phenolic and flavonoid contents, as well as demonstrating the lowest radical scavenging activities. During the extraction process, the rhizome of *P. odoratum* was used, to reflect its actual usage by the local people, as a health promoting tonic. It remains unclear whether the choice of which part of the herb to use may influence the levels of phenolic and flavonoid detected, though it is possible that the choice of extraction method may play a critical role too. As recently reported, the reflux extraction method yielded the highest yield, followed by the ultrasonication method, in the isolation of isoflavones from *P. odoratum* (Wang et al., 2011).

Conclusions

Among the six medicinal herbs analyzed in this study, *S. barbata* and *P. vulgaris* consistently demonstrated the highest activities, in all three scavenging assays tested. The highest levels of total phenolic and flavonoid contents were also detected in *S. barbata* and *P. vulgaris*. In conclusion, *S. barbata* and *P. vulgaris*, and possibly other members of the *Lamiaceae* family, possess potent antioxidation capacities. Considering the herbs' low cytotoxicity, further work in this direction could potentially lead to the discovery of powerful antioxidants that are safe for large dosage consumption.

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