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Full Length Research Paper

Evaluation of Antioxidative Properties in Leaf Extracts of *Labisia pumila*, a Traditional Malaysian Herb

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A study was undertaken to examine the presence of antioxidative activities of two varieties of *Labisia pumila*; *L. pumila* var. Alata and *L. pumila* var. *Pumila* using DPPH, FRAP and -carotene bleaching methods. In addition, ascorbic acid, - carotene, anthocyanin, total flavonoid and total phenolic content were also analyzed. In eight methods studied, six of them showed high activities of antioxidant in *L. pumila* var. Alata compared to that of *L. pumila* var. *Pumila*. The results obtained showed that *L. pumila* var. *Alata* contained higher antioxidative activities in all three methods applied compared to var. Pumila. In DPPH, FRAP and -carotene bleaching methods, *L. pumila* var. Alata had high antioxidant activities with 299.84 M trolox/g db, 164.16 M trolox/g db and 89.22%, respectively. The same pattern of antioxidant activities also can be observed in ascorbic acid, -carotene and anthocyanin in *L. pumila* var. Alata compared to var. Pumila with 0.022, 3.175 and 0.328 mg/g FW, respectively. *L. pumila* var. Pumila had higher total flavonoid content than *L. pumila* var. Alata with 1.281 mg/g FW. For total phenolic content, no significant different was observed because the amount of total phenolic content ranging from 2.53 to 2.55 mg/g FW. There is a positive correlation between antioxidant capacities and individual antioxidative compounds in the following order -carotene>flavonoid>vitamin C>total anthocyanins >phenolics.

Keywords: *Labisia pumila,* antioxidants, -carotene, flavonoid, vitamin C, anthocyanin, phenolics.

INTRODUCTION

Labisia pumila is a popular herb from the family Myrsinaceae locally known as Kacip Fatimah used to induce and facilitate childbirth as well as a post partum medication to help contract the birth channel, to tone the abdominal muscles and to regain body strength (Wan Ezumi et al., 2007). Other traditional uses include treatment for dysentery, rheumatism and gonorrhea. *L. pumila* products are widely available commercially as health supplements. In spite of the wide usage of this plant as a traditional herb no information on its chemical constituents has been documented. However, it was reported that plants from the same genus (Myrsinaceae) are being used to treat respiratory tract infections and menstrual disorders (Huang et al., 2000). Many herbal plants are known to contain high bioactive compounds such as saponins, ste-roids and flavonoids. Results of previous studies (Huang et al., 2003; Jansakul et al., 1987; De Tommasi et al., 1993) have shown that steroid and triterpenoid saponins have been isolated from *Ardisia* plants (Myrsinaceae). Several studies on the antioxidative activities properties from herbal plants have been reported (Djeridane et al., 2006; Chanwitheesuk et al., 2005; Saha et al., 2004; Velioglu et al., 1998). Plant antioxidants are believed to play a role in protection against a variety of diseases and delaying ageing processes. The health promoting effect of antioxidants from plants could be due to their protect-tive effects by counter acting reactive oxygen species (ROS) (Wong et al., 2006). There are several compounds which contribute to the antioxidative properties, these include polyphenols (Marinova et al., 2005), vitamin C (Chanwitheesuk et al., 2005), - carotene (Lisiewska et al., 2006), anthocyanins (Longo and Vasapollo, 2006) and flavonoids (Harborne and Williams, 2000). There are three varieties of *L. pumila* namely, *L. pumila* var. *Pumila*, *L. pumila* var. *Alata* and *L. pumila* var. *Lanceolata*. How-ever, only two varieties were available, *L. pumila* var. *Pu-*

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mila and *L. pumila* var. *Alata*. The antioxidative capacities of *L. pumila* plants have not been reported. A study was, therefore, undertaken to examine the presence of antioxidative activities of *L. pumila.* The information will be useful in preparing the herbal formulations for health supplements.

MATERIALS AND METHODS

Chemicals and reagents

Gallic acid, Folin-Ciocalteau phenol reagent, 2, 2-diphenyl-1-picryl-hydrazyl radical (DPPH), 2, 4, 6 - tri (2-pyridyl) 2, s-trizine (TPTZ) and kaemferol were purchased from Sigma Chemical Co. Ltd (USA). The 2, 6-dichlorophenolindophenol disodium salt was obtained from Sigma (USA). All other chemicals and solvents used in this study were of analytical grades.

Plant materials

Two varieties of *L. pumila,* var. *Pumila* and var. *Alata* were used. The first fully expanded leaves from one-year old plants, grown in the greenhouse, were sampled. Leaf samples were dried in a convection oven at 45°C for 72 h or until there is no change in weight. The samples were placed in air tight plastic bags and kept in the refrigerator (-20 °C) until ready to be analyzed.

Extraction method for antioxidant activity

For DPPH and FRAP assay, 0.2 g of fresh weight were extracted in 10 ml of double distilled water. The mixture was allowed to stand at room temperature for 1 h in the dark with occasional agitation. The aqueous extract was obtained by filtering the mixture through Whatman No. 1 filter paper and used for analysis. Ground sample (1 g) was extracted for -carotene bleaching assay, 25 ml of 80 % methanol on orbital shaker for 120 mins at room temperature. The mixture was filtered using Whatman No. 1 filter paper and the filtrate was assayed for antioxidant activity.

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

The DPPH free radical scavenging activity of each sample was determined according to the method described by Wong et al. (2006). A solution of 0.1 mM DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm. An aliquot (40 µl) of an extract was added to 3 ml of methanolic DPPH solution. The change in absorbance at 515 nm was mea-sured after 30 mins. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmole Trolox equivalent per gram of dried plant material.

Ferric reducing antioxidant potential (FRAP) assay

The ability to reduce ferric ions was measured using modifying methods of Wong et al. (2006), Benzie and Strain (1996). An aliquot (200 l) of the extract with appropriate dilution was added to 3 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM $FeCl_3 .6H_2O$ solution) and the reaction mixture was incubated in a water bath at 37°C. The increase in absorbance at 593 nm was measured after 30 mins. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as µmole Trolox equivalents per gram of plant material based on dry weight.

ß-Carotene bleaching assay

The - carotene bleaching method (Velioglu et al., 1998) was carried out to measure the antioxidant activity. - carotene (0.2 mg/ ml) dissolved in chloroform, was added to round bottom flasks (50 ml) containing 20 µl of linoleic acid and 200 µl of Tween 20. A volume of 200 µL of 80% MeOH (as control) or corresponding plant extract or BHT (as standard) was added to the mixture. The concentrations of the BHT and plant extract are same (40 mg/ml). After evaporation to dryness under vacuum at room temperature, oxygenated distilled water (50 ml) was added and the mixture was shaken to form a liposome solution. The samples were then sub-jected to thermal autoxidation at 50°C for 2 h. The absorbance of the solution was monitored at 470 nm by taking measurements at 10 mins intervals for 120 min and the rate of bleaching of ß-caro-tene was recorded.

Ascorbic acid content

The ascorbic acid content was measured using a modified method of Davis and Masten (1991) . The leaf samples were extracted in 1% of phosphate-citrate buffer, pH 3.5 using chilled pestle and mortar. The homogenate was filtered. The filtrate was added to the 1 ml of 1.7 mM 2, 6-dichloroindophenol (2, 6-DCPIP) in 3 ml cuvette. The absorbance at 520 nm was read within 10 mins of mixing the reagents. The extraction buffer was used as a blank.

β-Carotene content

β-carotene content was determined using the method described by (Harborne, 1973). Fresh leaves were ground in the presence of cold acetone and light petroleum. The extract was applied on to Thin Layer Chromatography (TLC) plate silica gel 60 F_{254} plate (20x20 cm). The plate was developed with hexane: acetone (1:1). The spot was scraped and diluted back to the extraction solvent. The absorbance was read at 451 nm. The amount of β -carotene was determined as mg of β-carotene equivalent by using an equa-tion obtained from the standard curve of β-carotene (Merck, Darm-stadt, Germany).

Anthocyanin content

Anthocyanin content was determined according to Bharti and Khurana (2003). Fresh leaves were added in 10 ml acidic methanol (1% v/v HCl) and incubate overnight. Anthocyanin was partitioned from chlorophyll with 10 ml chloroform, followed by adding 9 ml of double deionised water. The test tubes containing the samples were shaken gently and allowed the mixture to settle down. The absorbance was read at 505 nm. Petunidin was used as a stan-dard.

Total flavonoid content

The total flavonoid content was determined using the aluminium chloride assay (Zhishen et al., 1999). The fresh samples were added in 2 ml HCl in test tube and reflux it at 100°C for 30- 40 min. The hydrophilic extract was top up to 5 ml with distilled water. The mixture was added with 5% w/v $NaNO_2$ and 10 % $AlCl_3$ was added. 1 M NaOH was added a min later. Absorption reading was read at 510 nm. Kaemferol was used as the standard.

Total phenolic content

The total phenolic content of leaves was determined by using the Folin-Ciocalteau method (Singleton and Rossi, 1965). The dried

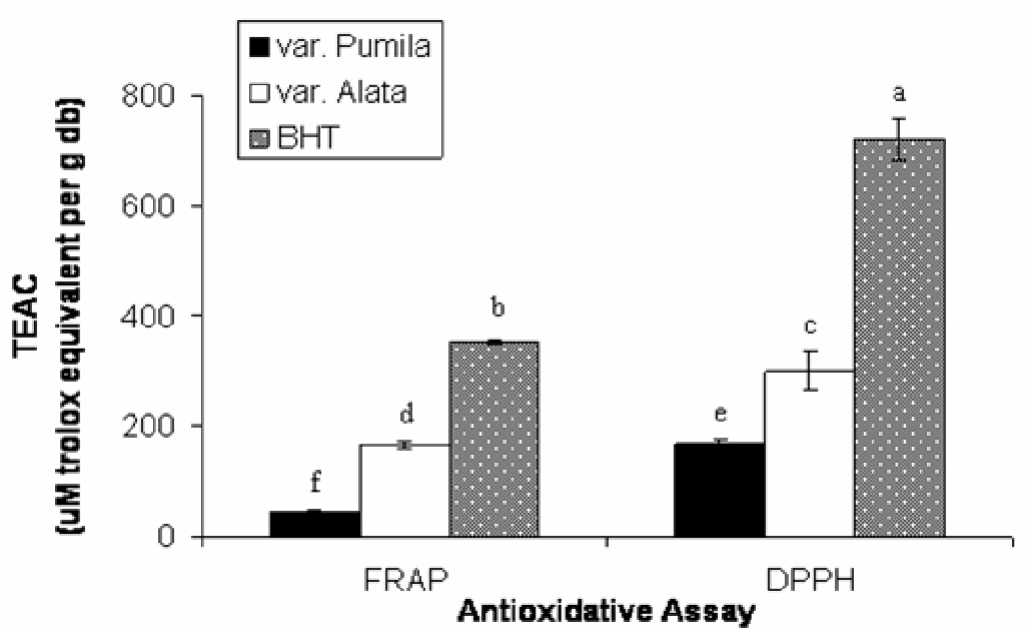

Figure 1. Antioxidant activities of *L. pumila* leaf extracts based on their abilities to reduce the ferric ion-TPTZ complex (FRAP) and scavenge DPPH free radicals. Data were analyzed using One-Way ANOVA and the different contrasted using Tukey's multiple range tests. Different letter(s) indicate the values are significantly different ($p < 0.05$).

sample was extracted with the 80 % methanol that contains 1 % HCl at room temperature on an orbital shaker set at 200 rpm. The mixture was centrifuged at 1000 g for 15 min. The extract was added to the 0.2 ml Follin- Ciocalteau reagent and mixed tho-roughly. After 4 mins, 15 % Na_2CO_3 was added. After 2 h, the absorbance was read at 760 nm with spectro uv-vis auto, LaboMed. Inc. The amount of the total phenolic content was determined as mg of gallic acid equivalent using the standard curve.

Statistical analysis

Data were analyzed using One–Way ANOVA and the different contrasted Tukey's multiple range tests. Different letter(s) indicate the values are significantly different ($p < 0.05$).

RESULTS

Total antioxidant activities

DPPH, FRAP and -carotene bleaching methods were carried out to determine the total antioxidative potential of two varieties of *L. pumila* leaves. The results obtained showed that *L. pumila* var. Alata contained higher anti-oxidative activities for all the methods employed.

DPPH and FRAP assays

DPPH is a very stable free radical and it is widely used to evaluate antioxidant activities in a relatively short time (Mokbel and Hashinaga, 2006). The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance to drop at 515 nm. In this study, the anti-xidant activity was expressed as Trolox equivalents per gram of plant material on a dry basis. Wong et al. (2006) reported that this method is a more meaningful and descriptive expression than the assay that used percent of the antioxidant to reduce ferric ions of the extract though both methods are expressed as mol Trolox equivalents per gram of plant material on dry weight basis. The control used was butylated hydroxytoluene (BHT). The DPPH free radical scavenging activity (Figure 1) showed that *L. pumila* var. *Alata* had higher sca-venging activity (299.84±34.05 M trolox equivalent per g db) as compared to *L. pumila* var. *Pumila* (167.6 ± 68.44 M trolox equivalent per g db). This accounts to an increase of scavenging power by almost two-fold in *L. pumila* var. *Alata.* The FRAP values were also expressed as Trolox equivalent capacity (TEAC) of the two varieties as shown in Figure 1. A similar trend was obtained whereby the *L. pumila* var. *Alata* showed higher $TEAC_{FRAP}$ values (164.16 ± 6.89 M trolox equivalent per g db) followed by var. *Pumila* (45.47±3.02 uM trolox equivalent per g db). This further indicates that *L. pumila* var. *Alata* had the higher ability to reduce ferric ions. The procedure of FRAP assay is relatively simple and easy to be standardized. But, this assay has been reported not to react fast enough with some antioxidants, such as gluta-thione (Guo et al., 2003). Although the trend for both DPPH and FRAP free radical scavenging activity appear-ed the same the absolute values obtained were higher in DPPH assay. The $TEAC_{DPPH}$ values were consistently higher than those obtained for $TEAC_{FRAP}$. Gil et al. (2002) reported that the FRAP values were higher than the

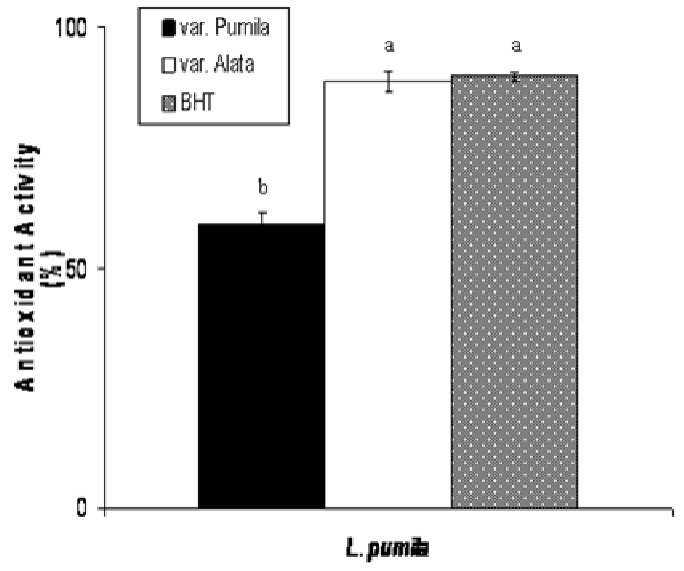

Figure 2. Percent antioxidant activities (%) of *L. pumila* leaf extract using -carotene bleaching method. Data were analyzed using One-Way ANOVA and the different contrasted using Tukey's multiple range tests. Different letter(s) indicate the values are significantly different ($p < 0.05$).

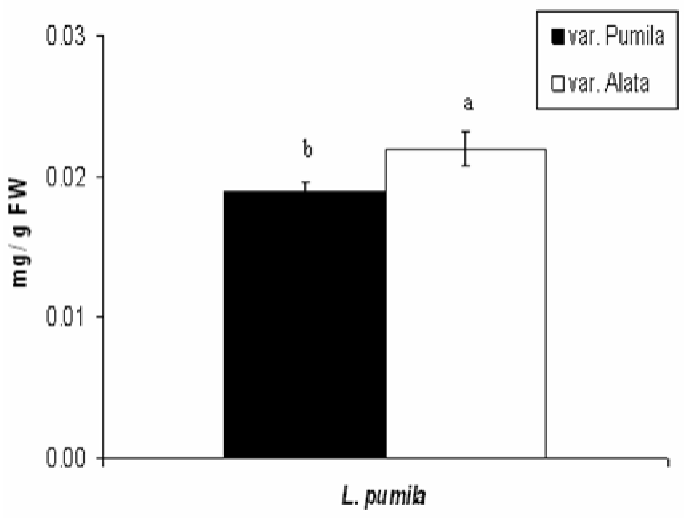

Figure 3. Vitamin C content (mg/ g FW) of *L. pumila* leaf extracts. Data were analyzed using One-Way ANOVA and the different contrasted using Tukey's multiple range tests. Different letter(s) indicate the values are significantly different ($p < 0.05$).

DPPH free radical scavenging activities of stone fruits. Similarly, Wong et al. (2006) reported that the ferric ions reducing activities of 25 tropical plants, expressed as Trolox equivalent antioxidant capacity were higher to that of DPPH free radical scavenging activities. In this study the reason is probably due to the difference in com-pounds that are reactive towards the two different me-thods. Wong et al. (2006) suggested that there are some compounds such as polyphenols which may be more efficient as reducing agents for ferric ions but some may not scavenge DPPH free radicals due to steric differences. In both cases, however, the antioxidative ability of the control BHT was significantly higher than in the *L. pumila* leaf extracts.

-Carotene bleaching assay

Besides the two methods described above - carotene bleaching method expressed as percent (%) of total antioxidant activity (Figure 2) was used in determining antioxidant activity. Again the values indicated that *L. pumila* var. Alata has higher percentage of antioxidative activity (89.72 ± 0.95%) than *L. pumila* var. *Pumila* (59.09 ± 2.24%). *L. pumila* var. *Alata* had exceptionally high antioxidant level.

Ascorbic acid content

Ascorbic acid also known as vitamin C is one of the most abundant antioxidants in plant where the role of ascor-bate is to protect plants against oxidative stress (Smirnoff, 2000). It is a powerful water soluble antioxidant and its established role is to prevent scurvy (Padayatty et al., 2003). The result in Figure 3 further showed that there is a slight different in vitamin C content and *L. pumila* var*. Alata* appeared to have a higher vitamin C content (0.022 ± 0.001 mg/g FW) when compared with var. *Pumila* (0.019 ± 0.001 mg/g FW).

-Carotene content

Carotenoids are also classified among the basic constituents of the antioxidative effect (Duthie et al., 2003; Kidmose et al., 2001). Figure 4 shows the -carotene content of *L. pumila* var. *Alata* and *L. pumila* var. *Pumila.* The highest content of -carotene is in *Labisia pumila* var. *Alata* followed by var. *Pumila,* 3.175 ± 0.12 mg/g FW and 2.34 ± 0.16 mg/g FW, respectively. The result indicated that - carotene content was higher in var. *Alata*. The red colored leaves of var*. Alata* may contribute to the high - carotene content as opposed to *var. Pumila* where the leaves appear greener. This finding is in parallel with the report by Gil et al. (2002) which reports that total carotenoids is higher in yellow-colored nectarines compared to that of white-flesh. -carotene is a precursor of vitamin A, which is important in human vision and also to prevent certain types of cancer (Craft et al., 1993; Colditz, 1987).

Total anthocyanin content

Anthocyanins are the naturally occurring phenolic compounds responsible for the color of many flowers, fruits, and berries (Cooper-Driver, 2001). It is the most impor-

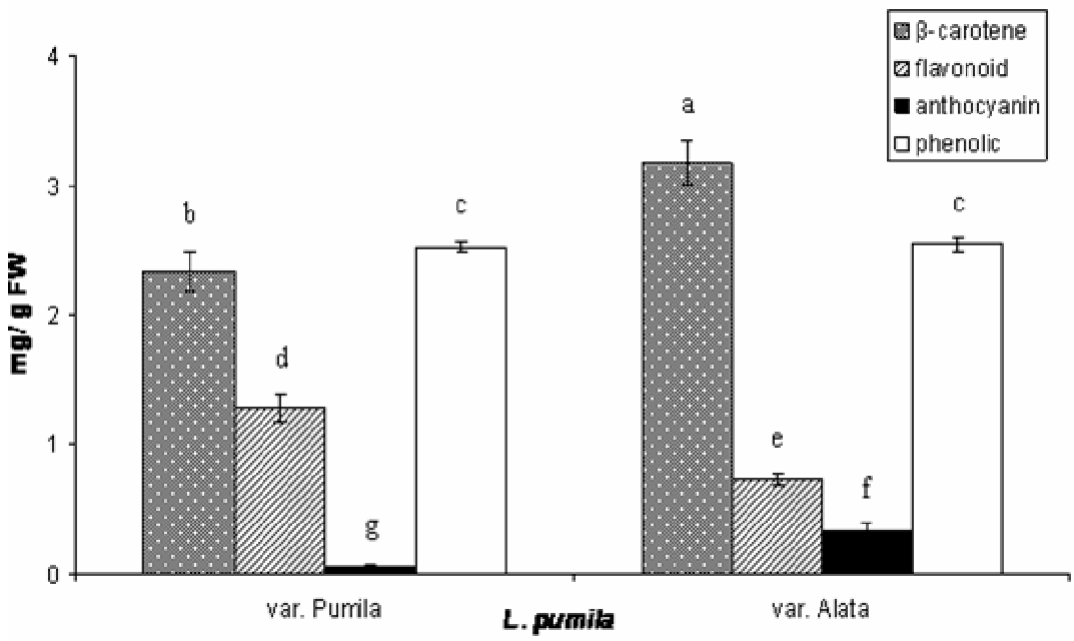

Figure 4. -Carotene, flavonoid, anthocyanin, phenolic content (mg/g FW) of *L. pumila* leaf extracts. Data were analyzed using One-Way ANOVA and the different contrasted using Tukey's multiple range tests. The result are the mean ± SE. (n = 5). Different letter(s) indicate the values are significantly different ($p < 0.05$).

tant group of water soluble-pigments in plants and had the beneficial health effects as antioxidant and anti-inflammatory agents (Wang and Jiao, 2000; Wang et al., 1999; Tamura and Yamagami, 1994). The total anthocyanin content (Figure 4) was higher in *L. pumila* var. *Alata* (0.328 ± 0.07 mg/g FW) compared to that of *L. pumila* var. *Pumila* (0.058 ± 0.01 mg/g FW). From this result, it shows that anthocyanin- rich samples generally showed very strong activities (var. *Alata*) and confirm that anthocyanins possess strong antioxidant activities (Wang et al., 1997; Tsuda et al., 1994). Anthocyanins are pro-bably the largest group of phenolics compounds in the human diet, and their strong antioxidant activities suggest their importance in maintaining health. Anthocyanin is also important as antioxidant, which have roles in pro-moting good health and reducing the risk of chronic disease and also as anti-inflammatory agents. It was re-ported by (Wang and Jiao 2000; Wang et al., 1999; Tamura and Yamagami, 1994) that anthocyanins pos-sess some positive therapeutic effects, mainly associated with their antioxidant activities.

Total flavonoid content

Flavonoids are naturally occurring substances in plants that are thought to have positive effects on human health (Montoro et al., 2005). The most important function of flavonoids is the antioxidants properties. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals (Bravo, 1998). The flavonoid content was higher in *L. pumila* var. *Pumila* (1.281 ± 0.11mg/g FW) compared to that of *L. pumila* var. *Alata* (0.732 ± 0.05 mg/g FW) (Figure 4). There are different classes of flavonoids and the protective activity may differ with each of the compounds present. It was reported that quercetin, kaemferol, catechin and taxifolin were shown to suppress the cytotoxicity of O_2 and H_2O_2 on Chinese hamster V79 cells in a protective manner (Nakayama et al., 1993). Flavonoid distribution in plants depends on the several factors including variation according to plant phyla/order/family and population variations within species (Harborne, 1986). The distribution pattern de- pends on the degree of accessibility to light and previous illumination because formation of the higher oxidized flavornoids is accelerated by light (Smith et al., 2000). Lee et al. (2005) reported that immature fruit generally con-tained lower levels of lutein and xeaxanthin than mature, color fruit, but the differences were not always statistically significant. The antioxidant property of flavonoids was the first mechanism of the action studied, particularly with regard to their protective effects against cardiovascular diseases. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing mole-cules, including singlet oxygen and various free radicals (Bravo, 1998) that are probably involved in several dis-eases. Harborne and Williams (2000) suggested that additional benefit of flavonoids is their ability to stabilize membranes by decreasing membrane fluidity.

Total phenolic content

Phenolics are class of low molecular weight secondary

Table 1. Correlation (R^2) between antioxidant activities ($TEAC_{DPPH}$ and $TEAC_{FRAP}$) and -carotene, flavonoid, vitamin C, anthocyanin and phenolic content (R^2) in *L. pumila* var. *Alata*

	DPPH (R^2)	FRAP (R^2)
Beta-carotene	0.99	0.96
Flavonoid	0.97	0.95
Vitamin C	0.92	0.94
Anthocyanin	0.87	0.86
Phenolic	0.81	0.88

metabolite that found in most land plants (Akowuah et al., 2004). Many phenolic compounds have been reported to possess potent antioxidant activity and have anti-cancer or anti-carcinogenic, anti -bacterial, anti-viral or anti-inflammatory activities to a greater or lesser extent (Chung et al., 1998; Cassidy et al., 2000; Gao et al., 2000; Tapiero et al., 2002). The phenolic content of *L. pumila* was shown in Figure 4. There was no significant different of total phenolic content in *L. pumila*. The con-tent of phenolics is more or less the same among the two varieties which ranged from 2.53 ± 0.04 mg/g FW to 2.55 ± 0.06 mg/g FW (Figure 4).

DISCUSSION

The antioxidant properties of *L. pumila* have not been reported previously. Its use as a traditional herb for women appears to have some basis in relation to the total antioxidant content and in relation to report that an *Alectoria crispa* plants which comes from the family as *L. pumila* have utero wall-contracting saponins (Jansakul et al., 1987). Further studies on the specific components such as the saponins, enzymatic antioxidants will be carried out. *L. pumila* var. *Alata* had higher total antioxidant activity compared to that of var. *Pumila* in all the three different methods which is FRAP, DPPH and - carotene bleaching assays. There is a positive correlation between total antioxidant activities and the individual antioxidant compounds with a range correlation coefficient of R^2 = 0.81 to 0.99 (Table 1). The highest correlation based on DPPH/FRAP assays is with -carotene (0.99/0.96) followed by flavonoids (0.97/0.95), vitamin C, (0.92/0.94) anthocyanins (0.87/0.86) and total phenolics (0.81/0.88). It is therefore, appears that the antioxidative activities are primarily due to -carotene and flavonoids. Such correlations have been observed in other studies. Chanwitheesuk et al. (2005) reported that the contents of vitamins C and E, total carotenes, total xanthophylls, tannins and total phenolics of some plants are correlated with antioxi-dant activities. Such correlations have been widely repor-ted. Studies on the antioxidant activities of Algerian medi-cinal plants linear correlation of Trolox equivalent antioxi-dant capacity (TEAC) with respect to the total and flavor-noid content (Djeridane et al., 2006) where there is a positive correlation between the total phenolic content of a given sample and its antioxidant activity. However the value is R^2= 0.7931. There was a strong correlation (0.93-0.96) between total phenolics and antioxidant acti-vity in stone fruits nectarines, peaches and plums but low correlation with vitamins C and carotenoids (Gil et al., 2002). A similar correlation was obtained by Wong et al. (2006) . Further studies to identify the different flavonoids will be identified as each flavonoid exhibits different scavenging capacities (Cai et al., 2006).

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