

Light intensity effects on production and antioxidant activity of flavonoids and phenolic compounds in leaves, stems and roots of three varieties of *Labisia pumila* Benth

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Abstract

This research was performed to evaluate the effect of light intensity (310 and 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$) on production and antioxidant activity of flavonoids and phenolic compounds from the leaves, stems and roots in three varieties of *Labisia pumila* Benth. The experiment was carried out base on randomized complete block design with a factorial arrangement. The results revealed that total phenolic and flavonoid content as well as antioxidant activity in all three varieties had consistently higher values when exposed to high irradiance (70% IR). The highest amount of these components was accumulated in the leaves followed by the roots and stems in all three varieties. The results showed that total flavonoid accumulation was highest in the leaves of *L. pumila* var *pumila* (2.94±0.11 mg rutin equivalent /g DW) under 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity and total phenolics was highest in *L. pumila* var *alata* (3.92±0.06 mg Galic acid equivalent (GAE)/g DW) under the same light intensity. HPLC analyses of phenolics and flavonoids in all three varieties revealed the presence of gallic acid, caffeic acid, kaempferol, naringin and myricetin in the leaves of all three varieties of *L. pumila* Benth and these compounds increased under 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity. Antioxidant activities determined by 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and ferric reduction antioxidant power (FRAP) assays in all varieties. The results showed significantly ($p \leq 0.05$) higher activity with increasing total phenolics and flavonoids in all plant organs under 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity compared to 310 $\mu\text{mol m}^{-2}\text{s}^{-1}$. This study indicates the ability of different light intensities to enhance the secondary metabolites such as flavonoid and phenolic compounds as well as their antioxidant activities in all three varieties of *L. pumila* Benth.

Keywords: light intensity, *Labisia pumila* Benth, Antioxidant Activity, Total phenolics and flavonoids.

Abbreviations: IR_Irradiance; GAE_galic acid equivalents; DW_dry weight; HPLC_High performance liquid chromatography; DPPH_1,1-diphenyl-2-picryl hydrazyl; FRAP_Ferric Reducing Antioxidant Power.

Introduction

Labisia pumila, locally known as Kacip Fatimah, is a forest-floor herbal plant that has tremendous potential in the herbal industry (Ibrahim et al., 2012). It is one of the five herbal plants identified by the government as one of the national key economic areas to be developed for commercial purposes. In Malaysia, it is an important and popular medicinal plant that has long been recognized and much demanded for its value as female tonics and health products (Ibrahim et al., 2010). With the recent discovery of estrogenic activity the demand for *L. pumila* is expected to soar (Jaafar et al, 2008). As little has been done to domesticate *L. pumila*, the current high rate of demand and methods of harvesting, particularly from the wild, has made research on domestication, propagation and cultivation of robust high quality plants urgent to ensure prolonged richness of the tropical biodiversity and to avoid extinction of natural forest populations from over harvesting (Jaafar et al, 2008). Natural phytochemicals obtained from plants have been reported to have a wide range of biological activities, including antioxidant, anti-inflammatory and antimicrobial actions (Ao et al., 2008; Brunet et al., 2009). Among them, phenolic acids and flavonoids are important secondary metabolites. A great number of studies have been

directed towards biological activities of traditional medicinal plants. The antioxidant activity may be due to these compounds, which act as free radical scavengers and metal chelators (Ao et al., 2008; Brunet et al., 2009). Positive correlations have been established between high phenolic content and strong antioxidant activity (Barros et al., 2007; Karimi et al., 2011a). Natural antioxidants neutralize the free radicals harmful effect in the human body and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (Oskoueian et al., 2011).

The world is rich with natural and unique medicinal plants which have been developed and considered for a large range of purposes, including medicine, nutrition, flavourings, beverages, dyeing, repellents, fragrances, cosmetics and industrial uses (Safaei-ghomi et al., 2010). Currently, medicinal plant extracts are very attractive not only in modern phytotherapy but also in the food industry (Brunet et al., 2009). Raising *L. pumila* under greenhouses, where micro-climate could be manipulated, appears to be a promising alternative for controlling levels of phytochemicals and producing targeted quality raw materials in a sustainable way (Jaafar et al., 2008). However, the existence of inter-

Table 1. Accumulation of total phenolics in leaf, stem and root of three varieties of *Labisia pumila* Benth. under different light intensities.

Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Total phenolic (mg gallic acid/g dry weight)			
	Plant Part	<i>Alata</i>	<i>Pumila</i>	<i>Lanceolata</i>
310	Leaf	3.56±0.03 ^b	3.46±0.3 ^{bc}	3.27±0.007 ^{bc}
	Root	1.56±0.06 ^d	1.18±0.2 ^e	1.15±0.11 ^e
	Stem	1.22±0.02 ^e	1.14±0.03 ^e	1.10±0.047 ^e
630	Leaf	3.92±0.06 ^a	3.59±0.04 ^b	3.31±0.04 ^c
	Root	1.58±0.26 ^d	1.21±0.01 ^e	1.21±0.07 ^e
	Stem	1.23±0.01 ^e	1.18±0.02 ^e	1.17±0.08 ^e

All analyses are the mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $P \leq 0.05$.

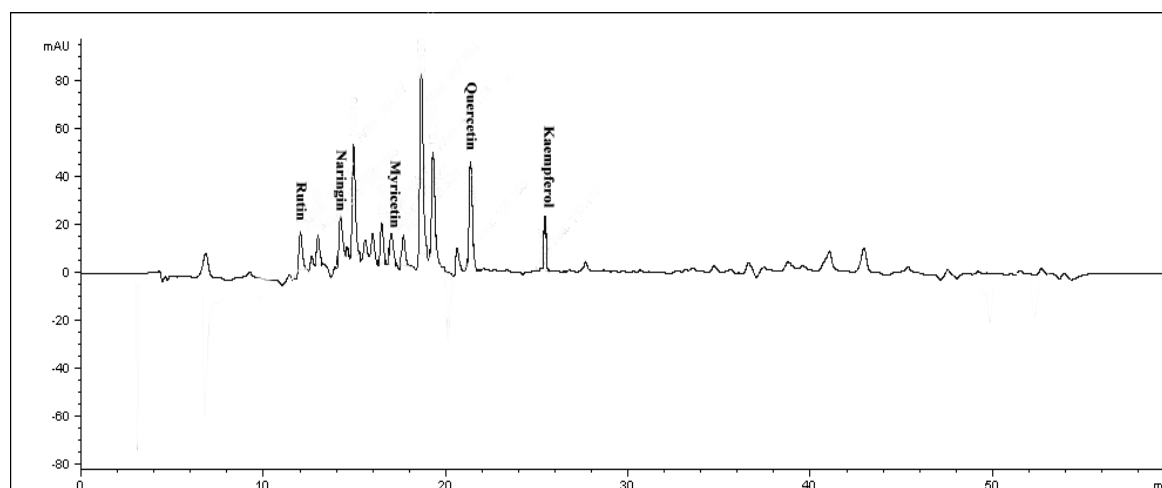


Fig 1. The RP-HPLC chromatogram of flavonoid compounds in the leaves of *Labisia pumila* var. *pumila*. under $310 \mu\text{mol m}^{-2}\text{s}^{-1}$. Identification of compounds: rutin, naringin, myricetin, quercetin and kaempferol.

specific differences to micro-environment may influence plant accumulation and distribution of total phenolics and flavonoid compounds (Jaafar et al., 2008). Previous studies by Karimi et al. (2011b) have demonstrated and documented the phytochemical properties (flavonoids, phenolics, antioxidant activity) of *L. pumila*. Since microenvironment of the greenhouse may influence the phytochemical content in plants, it would be interesting to examine the effect of different greenhouse irradiance levels on the accumulation and distribution of the metabolites. The aim of the present work was to determine the effect of two levels of greenhouse irradiance (310 and $630 \mu\text{mol m}^{-2}\text{s}^{-1}$) on the accumulation and concentration of phenolics and flavonoid compounds as well as their antioxidant activity in the leaf, stem and root of three varieties of *L. pumila* Benth. The findings would provide useful information for future studies on production of higher quality *L. pumila* with improved greenhouse irradiance.

Results and Discussion

Total phenolics (TP) content

The results of total phenolic content are shown in Table 1. The results indicate that TP accumulation and partitioning in the plant was considerably affected by different light intensities. The different light intensities had a significant ($p \leq 0.05$) effect on total phenolic content (Table 1). The results showed that in all three varieties of *L. pumila*, the leaves had a higher total phenolic content (3.92 ± 0.06 mg gallic acid equivalent (GAE)/g DW in *L. pumila* var. *alata*, 3.59 ± 0.04 mg gallic acid equivalent (GAE)/g DW in *L. pumila* var.

pumila and 3.31 ± 0.04 mg gallic acid equivalent (GAE)/g DW in *L. pumila* var. *lanceolata*) under a light intensity of $630 \mu\text{mol m}^{-2}\text{s}^{-1}$ compared to $310 \mu\text{mol m}^{-2}\text{s}^{-1}$. The highest amount of TP after three months planting was observed in leaves and followed by roots and stems. The light treatment imposed significant effects ($p \leq 0.05$) on TP accumulation and partitioning in all three varieties of *L. pumila* Benth. (Table 1). Similar enhancement in TP with increased light intensity was reported in two varieties of *Zingiber officinale* (Halia Bentong and Halia Bara) (Ghasemzade et al., 2010). It was also reported that the leaves had higher TP content compared to the rhizomes and stems. Kumari et al. (2009) reported that Phenylalanine ammonia-lyase (PAL) is an important enzyme in the biosynthesis of phenolic acids, and that the activity of this enzyme increased while this enzyme was induced by high light intensity. Therefore, increasing production of phenolic acids could be related to increasing PAL enzyme activity.

Total flavonoids (TF) content

The results on the TF content of *L. pumila* indicated that TF was considerably affected by the different light intensities (Table 2). Differing light intensity had a significant ($p \leq 0.05$) effect on TF content similar to TP content. The results showed that the leaves in all three varieties had a higher content of TF with 2.73 ± 0.14 mg rutin equivalent /g DW in *L. pumila* var. *alata*, 2.94 ± 0.11 mg rutin equivalent /g DW in *L. pumila* var. *pumila* and 2.54 ± 0.11 mg rutin equivalent /g DW in *L. pumila* var. *lanceolata* compared to roots and stems under $630 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light intensity. Between varieties, *L. pumila* var. *pumila* had the highest content of

Table 2. Accumulation of total Flavonoids in leaf, stem and root of three varieties of *Labisia pumila* Benth. under different light intensities.

Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Plant Part	Total flavonoid (mg rutin/g dry weight)		
		<i>Alata</i>	<i>Pumila</i>	<i>Lanceolata</i>
310	Leaf	2.51±0.11 ^{cd}	2.84±0.11 ^a	2.43±0.13 ^d
	Root	0.79±0.05 ^{fg}	0.72±0.04 ^{fgh}	0.66±0.08 ^{gh}
	Stem	0.67±0.05 ^{gh}	0.69±0.01 ^{fgh}	0.65±0.03 ^{gh}
630	Leaf	2.73±0.14 ^b	2.94±0.11 ^a	2.54±0.11 ^c
	Root	0.79±0.07 ^f	0.74±0.04 ^{fgh}	0.68±0.01 ^h
	Stem	0.68±0.01 ^{fgh}	0.70±0.01 ^e	0.65±0.01 ^{gh}

All analyses are the mean of triplicate measurements ± standard deviation. Means not sharing a common letter were significantly different at $P \leq 0.05$.

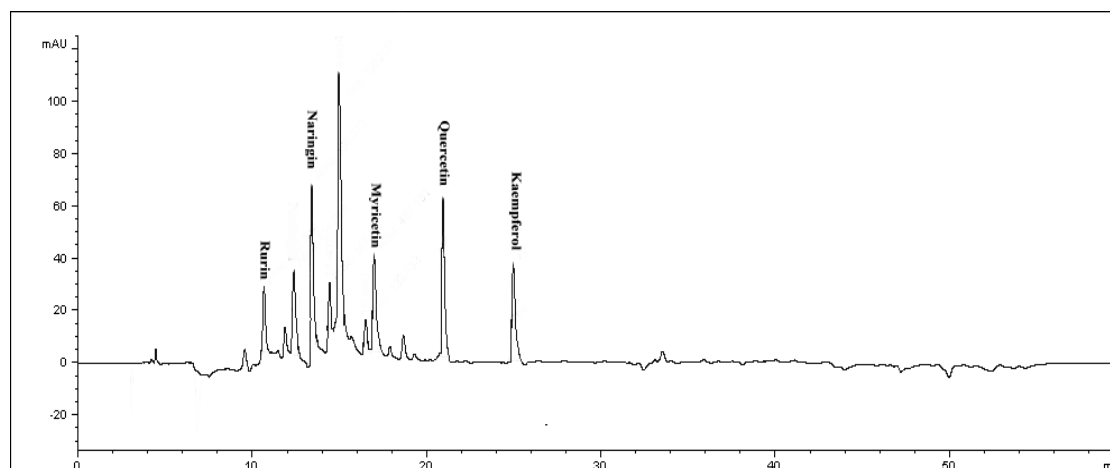


Fig 2. The RP-HPLC chromatogram of flavonoid compounds in the leaves of *Labisia pumila* var. *pumila*. under $630 \mu\text{mol m}^{-2}\text{s}^{-1}$. Identification of compounds: rutin, naringin, myricetin, quercetin and kaempferol.

flavonoids under the two different light intensities compared to the other two varieties. Chen et al. (2002) indicated that vegetable leaves that are exposed to shade had much greater concentrations of flavones and flavonols. Bergquist's et al. (2007) had also demonstrated that the flavonoids concentration and composition in baby spinach under shade netting was more effective and acceptable. Increasing light intensity increases primary photosynthate, which leads to an increase in phenolic concentration in the plant (Warren et al., 2003). Flavonoids are the most readily-produced phenolics in the epidermal cells of plants exposed to high light intensity. They are antioxidants, and their production is considered as a response toward protecting the plant against oxidative damage. Studies have shown an increase in flavonoid content of various plant species grown under high light conditions compared to those in the shade.

In hemlock, the concentration of various phenolics has been found to be lower in plants grown under shade than those found in full sun (Zobel and Clarke, 1999). This suggests that different plant species have different levels of sensitivity to light intensities. Flavonoid compounds in fruits and vegetables have been believed to function as therapeutic agents with beneficial health effects, such as protection against certain cancers, cardiovascular diseases and aging (Ross and Kasum, 2002).

However, flavonoids and other plant metabolites are not evenly distributed throughout the plant tissues. Their concentration and distribution in plants are not only a function of genetics, but are also found to be influenced by various environmental factors such as light, humidity and soil fertility (Mannfried, 1993). Effects of these factors on the concentration of plant metabolites are very important and

need to be considered in assessing and evaluating propagation of medicinal plant materials. Light is the most imperative factor among all the ecological factors. Flavonoid and phenolic biosynthesis require light or are enhanced by light. Flavonoid formation is absolutely light-dependent, and its biosynthetic rate is related to light intensity and density (Ghasemzadeh and Ghasemzadeh, 2011).

Profiling of phenolic and flavonoid compounds by HPLC

Various types of phenolic and flavonoid components were identified based on the standards using high performance liquid chromatography (HPLC) (Tables 3 and 4). The analysis for phenolics and flavonoids was performed on the leaves of all three varieties of *L. pumila* Benth due to the higher total phenolic and flavonoid contents in the leaf tissues as compared to the roots and stems. It is apparent that phenolic and flavonoid accumulation and partitioning in all three varieties of *L. pumila* Benth was considerably affected by the different light intensities. The methanolic extracts of the leaves of all varieties exhibited variable patterns of flavonoid and phenolic compounds under different light intensities.

Kaempferol, naringin and myricetin were the main flavonoid compounds available in all three varieties. Meanwhile, gallic acid and caffeic acid were present as the major phenolic acid compounds in all extracts. Among all varieties, *L. pumila* var. *alata* showed a greater variation in phenolic compounds compared to *L. pumila* var *pumila* and *lanceolata*, but *L. pumila* var *pumila* exhibited high concentrations of the flavonoid compounds compared to *L.*

Table 3. Concentration of different phenolic compounds in the leaves of three varieties of *Labisia pumila* Benth. under different light intensities.

Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Phenolic contents ($\mu\text{g/g}$ dry sample)			
	Variety	Gallic acid	Pyrogallol	Caffeic acid
310	<i>Alata</i>	512.5 \pm 0.19 ^b	845.5 \pm 0.21 ^b	52.5 \pm 0.1 ^e
	<i>Pumila</i>	259.2 \pm 0.16 ^f	ND	42.8 \pm 0.06 ^f
	<i>Lanceolat</i>	385.8 \pm 0.12 ^d	ND	98.1 \pm 0.13 ^b
630	<i>Alata</i>	577.3 \pm 0.22 ^a	912.3 \pm 0.36 ^a	85.3 \pm 0.05 ^c
	<i>Pumila</i>	266.5 \pm 0.11 ^e	ND	61.7 \pm 0.02 ^d
	<i>Lanceolat</i>	412.6 \pm 0.15 ^c	ND	135.2 \pm 0.18 ^a

ND: not detected. All analyses were mean of triplicate measurements \pm standard deviation. Means not sharing a common letter were significantly different at $P \leq 0.05$.

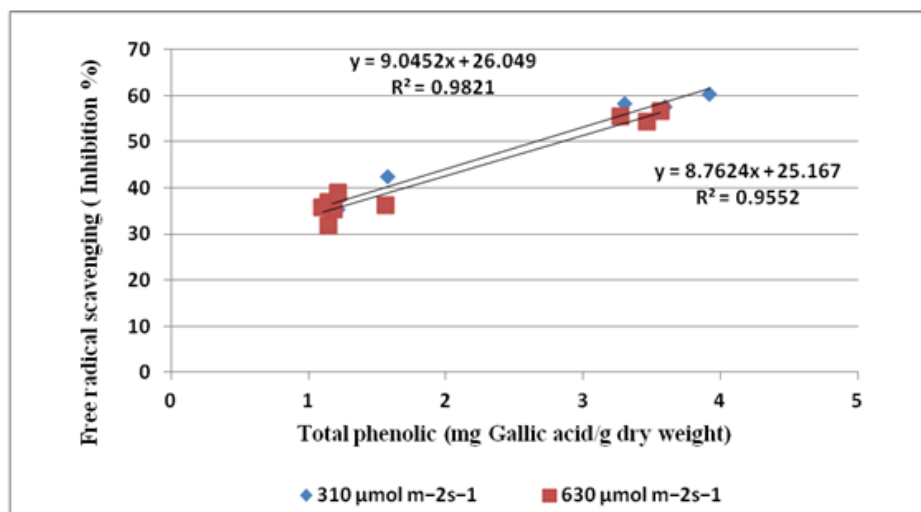


Fig 3. Relationship between total phenolic and total antioxidant activities of *Labisia pumila* under different light intensities (310 and 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$).

pumila var *alata* and *lanceolata*. The HPLC chromatograms from the leaf extracts show some of the flavonoids compounds found in *L. pumila* var *pumila* under different levels of greenhouse irradiance (310 and 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$) (Figs 1 and 2). Increasing the light intensity from 310 to 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ resulted in an enhancement in phenolics such as gallic acid and caffeic acid as well as flavonoid compounds such as quercetin, rutin, myricetin, kaempferol and naringin in the leaves of all three varieties of *L. pumila* Benth. On the other hand pyrogallol was only observed in *Labisia pumila* var. *alata* (845.5, 912.3 $\mu\text{g/g}$ dry sample) and rutin was only recorded in *Labisia pumila* var. *pumila* (38.4, 56.1 $\mu\text{g/g}$ dry sample) under 310 and 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$, respectively.

These results are in agreement with Ghasemzade and Ghasemzade (2011) who reported the accumulation of phenolic acids like gallic acid with high light intensities. It was also indicated that myricetin was absolutely light dependent and its biosynthetic rate was related to light intensity. However, it was also shown that increasing the light intensity reduced some flavonoids compounds such as quercetin, apigenin and luteolin in leaves of two varieties of ginger (*Halia Bentong* and *Halia Bara*) (Ghasemzade and Ghasemzade, 2011). Several previous studies have also shown that varying light intensities resulted in changes in plant morphological and physiological characteristics which exerted a substantial impact on the medicinal compounds in herbs (Hemm et al., 2004; Briskin and Gawienowski, 2001; Kurata et al., 1997).

DPPH Assay (1, 1-Diphenyl-2-picryl-hydrazyl)

The results of the DPPH scavenging assay showed that all the extracts were able to reduce the stable DPPH radical to yellow-coloured diphenylpicrylhydrazine. The extracts from *L. pumila* grown under 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ showed a significant effect in inhibiting DPPH, reaching up to 60.28% in *L. pumila* var. *alata* leaves, 57.58% in *L. pumila* var. *pumila* and 53.63% in *L. pumila* var. *lanceolata* leaves at 400 $\mu\text{g/mL}$ (Table 5). The IC_{50} values for BHT, α -tocopherol and the leaves of three varieties of *L. pumila* var. *alata*, *pumila* and *lanceolata* exposed to light intensity at 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ were 78.75, 36.03, 325.67, 360.96 and 371.37 $\mu\text{g/mL}$, respectively. Antioxidant activities were higher in the leaves compared to stems and roots under 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

With increasing TF and TP content, the free radical scavenging power increased in all three varieties of *Labisia pumila*. Antioxidant activities increased in samples from all parts of the plant with increasing light intensity. The roles of TF and TP for scavenging of free radicals in all 3 varieties of *L. pumila* Benth are shown in Figs 3 and 4.

Ferric reducing antioxidant potential (FRAP)

Similar to the DPPH results, the reductive potential of *L. pumila* Benth increased with light intensity (Table 6). *L. pumila* leaves of all three varieties appeared to be active in the reduction of Fe^{3+} , indicating antioxidant activity. The ferric reducing power activity of *Labisia pumila* leaves was

Table 4. Concentration of different flavonoid compounds in the leaves of three varieties of *Labisia pumila* Benth. under different light intensities.

Light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Variety	Flavonoid contents ($\mu\text{g/g}$ dry sample)				
		Kaempferol	Myricetin	Naringin	Quercetin	Rutin
310	<i>Alata</i>	$195.7 \pm 0.16^{\text{d}}$	$97.5 \pm 0.11^{\text{b}}$	$149.3 \pm 0.25^{\text{b}}$	ND	ND
	<i>Pumila</i>	$255.1 \pm 0.24^{\text{b}}$	$53.5 \pm 0.06^{\text{c}}$	$86.9 \pm 0.33^{\text{e}}$	$125.7 \pm 0.05^{\text{b}}$	$38.4 \pm 0.28^{\text{b}}$
	<i>Lanceolat</i>	$142.5 \pm 0.35^{\text{f}}$	$43.5 \pm 0.03^{\text{f}}$	$82.6 \pm 0.04^{\text{f}}$	$64.9 \pm 0.12^{\text{d}}$	ND
630	<i>Alata</i>	$201.2 \pm 0.07^{\text{c}}$	$117.8 \pm 0.19^{\text{a}}$	$172.3 \pm 0.02^{\text{a}}$	ND	ND
	<i>Pumila</i>	$293.9 \pm 0.13^{\text{a}}$	$92.1 \pm 0.07^{\text{c}}$	$125.4 \pm 0.15^{\text{c}}$	$159 \pm 0.22^{\text{a}}$	$56.1 \pm 0.19^{\text{a}}$
	<i>Lanceolat</i>	$176.5 \pm 0.05^{\text{e}}$	$55 \pm 0.01^{\text{d}}$	$94.7 \pm 0.25^{\text{d}}$	$88.2 \pm 0.14^{\text{c}}$	ND

ND: not detected. All analyses were mean of triplicate measurements \pm standard deviation.

Means not sharing a common letter were significantly different at $p \leq 0.05$.

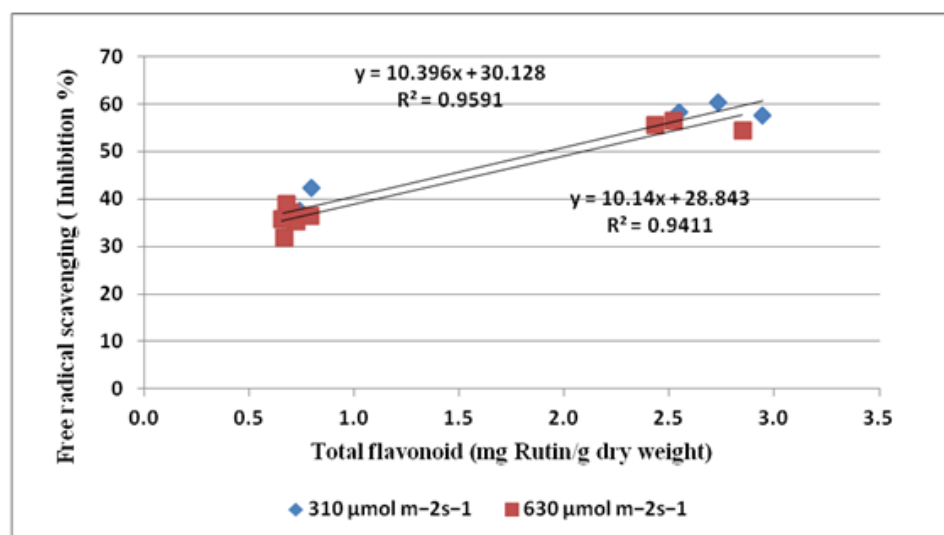


Fig 4. Relationship between total flavonoids and total antioxidant activities of *Labisia pumila* under different light intensities (310 and $630 \mu\text{mol m}^{-2}\text{s}^{-1}$).

higher than the stems and roots, but the values were lower than all the standards (BHT, ascorbic acid (vitamin C) and α -tocopherol). The reductive potential of leaf extracts under $630 \mu\text{mol m}^{-2}\text{s}^{-1}$ in all 3 varieties and standards at concentration of $400 \mu\text{g/ml}$ were as follows: vitamin C > BHT > α -tocopherol > *alata* leaf > *pumila* leaf > *lanceolata* leaf with respective values of 99.58%, 99.18%, 96.17%, 57.15%, 55.68% and 53.19% under $630 \mu\text{mol m}^{-2}\text{s}^{-1}$. Significant differences ($p \leq 0.05$) in ferric reduction were observed between different light intensities, varieties and different parts of the plant (Table 4). The result demonstrated a positive relationship between total flavonoids and total antioxidant activities of *L. pumila* Benth under different light intensity (Figs 3 and 4). Transition metals like iron are potentially pro-oxidative and the capability of an antioxidant to maintain the transition metal in its reduced state is an effective means of preventing lipid oxidation (Halliwell and Gutteridge, 1990). The ferric reducing antioxidant power (FRAP) assay developed by Benzie and Strain (1996) is a commonly used assay to evaluate the antioxidant potential of fruits and vegetables. FRAP assay determines the ferric reducing ability of the samples at low pH, forming a concentrated blue color as the ferric tripyridyltriazine (Fe^{3+} -TPTZ) compound is reduced to the ferrous (Fe^{2+}) form. Prior et al. (2005) suggested that these results may vary depending on the time scale that the ferric reducing ability was allowed to occur. Thaipong et al. (2006) recommended that a reduction in iron could be an indicator of antioxidant activity.

Similarly, the cupric reducing antioxidant capacity (CUPRAC) assay is a colorimetric method of measuring the ability of a substance to keep potentially pro-oxidative copper in its reduced state (Apak et al., 2008).

Materials and methods

Plant materials

Three varieties of *L. pumila* i.e. var. *alata* (Stone 6030 (KLU)), *pumila* (Stone 7233 (KLU)), and *lanceolata* (Stone 8385 (KLU)) were used in the study. Uniform 4-week-old seedlings with 5-6 leaves were selected and transferred into pots which were filled with a mixture of burnt rice husk and coco peat (ratio 1:1) (Jaafar et al., 2010). The plants were grown under two levels of shade using tildenet material (30% and 70% shade) at the glasshouse complex of University Putra Malaysia (UPM). The average light intensity passing through each shading treatment as determined using a portable light meter (Spectrum Handheld Quantum meter, USA) were 630 and $310 \mu\text{mol m}^{-2}\text{s}^{-1}$ of photosynthetically active radiation (PAR), respectively. The experiment consisted of a factorial arrangement of treatments (light and variety) in a randomized complete block design with 3 replications. Plants were harvested three months after planting. The leaves, stems and roots in all three varieties were cleaned, separated, and freeze dried for further analysis.

Table 5. DPPH scavenging activities of the methanolic extracts (400 µg/mL) from different plant parts of three varieties of *Labisia pumila*. under different light intensities.

Light Intensities (µmol m ⁻² s ⁻¹)	Extraction Source	Inhibition (%)		
		Alata	Pumila	Lanceolata
310	Leaves	56.55 ± 1.65 ^c	54.30 ± 0.87 ^d	51.70 ± 1.47 ^f
	Stem	36.29 ± 1.33 ^j	35.33 ± 0.92 ^k	30.41 ± 0.63 ⁿ
	Root	38.89 ± 0.97 ^h	36.97 ± 0.49 ^{jk}	33.80 ± 0.77 ^l
630	Leaves	60.28 ± 0.81 ^a	57.58 ± 0.31 ^b	53.63 ± 0.15 ^e
	Stem	37.73 ± 1.17 ⁱ	36.09 ± 0.25 ^{jk}	32.15 ± 0.39 ^m
	Root	42.33 ± 0.55 ^g	37.62 ± 0.67 ⁱ	33.86 ± 0.58 ^l

All analyses were mean of triplicate measurements ± standard deviation. Results expressed in percent of free radical inhibition. Means not sharing a common letter were significantly different at P ≤ 0.05.

Table 6. FRAP activities of the methanolic extracts (400 µg/mL) from different parts of three varieties of *Labisia pumila* Benth. under different light intensities.

Light Intensities (µmol m ⁻² s ⁻¹)	Extraction Source	Total antioxidant (FRAP) activities (%)		
		Alata	Pumila	Lanceolata
310	Leaf	55.23 ± 0.17 ^b	51.72 ± 0.72 ^d	50.96 ± 0.59 ^e
	Stem	25.23 ± 0.28 ⁿ	33.26 ± 1.23 ^j	29.64 ± 0.45 ^m
	Root	35.88 ± 0.15 ^h	35.37 ± 0.33 ^h	31.92 ± 0.29 ^k
630	Leaf	57.15 ± 0.83 ^a	55.68 ± 0.56 ^b	53.19 ± 0.82 ^e
	Stem	35.42 ± 0.62 ^h	32.21 ± 0.97 ^k	30.56 ± 0.35 ^l
	Root	38.09 ± 0.87 ^f	36.87 ± 0.8 ^g	34.32 ± 0.18 ⁱ

All analyses were the mean of triplicate measurements ± standard deviation. Results expressed in percent of antioxidant power. Means not sharing a common letter were significantly different at P ≤ 0.05.

Extraction method

Samples were extracted using methanol as a solvent based on Crozier et al. (1997). Two g samples of freeze-dried leaf, stem and roots were weighed and placed into 100 ml conical flasks, and added with 40 ml of 80% (v/v) methanol. It was followed by an addition of 10 ml of 6 M HCl. The mixture was refluxed for 2 hours at 90 °C, filtered through Whatman No. 1 filter paper (Whatman, England) and the filtrate was evaporated using a vacuum rotary evaporator (Buchii, Switzerland). The dried crude extract was weighed and dissolved in methanol and stored at -20 °C for further experiments.

Determination of total phenolics

Total phenolics content was determined using Folin–Ciocalteu reagent according to the method described in Ismail et al. (2010) and total phenolic content was expressed as mg gallic acid equivalents/g dry matter of plant material.

Determination of total flavonoids

Total flavonoids was determined based on aluminium chloride colorimetric assay described in Ismail et al. (2010). Total flavonoids content of extracts was expressed as mg rutin equivalent/g dry matter of plant material.

Analyses of phenolic and flavonoid compounds by RP-HPLC

Analyses of phenolic and flavonoid compounds in the leaves of all three varieties was quantitatively determined by reversed-phase HPLC based on the method described in Crozier et al. (1997) with some modifications. Phenolic standards used were gallic acid, caffeic acid and pyrogallol. Flavonoid standards used were quercetin, rutin, myricetin, kaempferol and naringin at stock concentrations of 100 µg/ml. An aliquot of sample extracts was loaded on the HPLC equipped with an analytical Intersil ODS-3 column (5

µm, 4.6 x 150 mm, Gl Science Inc). Solvents comprising of de-ionized water (solvent A) and acetonitrile (solvent B) were used. The pH of water was adjusted to 2.5 with trifluoroacetic acid. The phenolic compounds were detected at 280 nm, while flavonoid compounds were detected at 350 nm. The column was equilibrated by 85% solvent A and 15% solvent B. Then the ratio of solvent B was increased to 85% in 50 min followed by a corresponding reduction in solvent A to 15%. This ratio was maintained for 60 min for analysis with a flow rate of 0.6 ml/min.

DPPH radical-scavenging activity

The free radical scavenging activity of the plant extracts was determined using the DPPH assay as described in Gülçin et al. (2004). One ml methanolic extract of each plant sample at different concentrations were mixed with 3 ml 0.1 mM solution of 1,1-diphenyl-2-hydrazil (DPPH) in methanol and incubated for 30 min in the dark. The absorbance of the mixture was read using a visible spectrophotometer (Novaspec II visblespectro) at 517 nm. BHT and α-tocopherol were used as antioxidant standards. Free radical scavenging activity of the sample was calculated according to the following formula: Free radical scavenging activity = [(A₀ - A₁) / A₀] x 100%

Where, A₀ was the absorbance of the control reaction, and A₁ was the absorbance in the presence of the sample.

Ferric-reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) of the extracts was determined as described in Yen and Chen (1995). One ml (concentration of 100, 200, 300, and 400 µg/ml) of sample extracts were mixed with 2.5 ml of potassium phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1 g/100 ml). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%) was added to the mixture to stop the reaction. Equal volume of distilled water was added

followed by 0.5 ml ferum chlorate (0.1 g/100 ml) (FeCl₃). The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at 700 nm. Ascorbic acid, BHT and α -tocopherol were used as standard antioxidants.

Statistical analysis

The experiment was factorial based on a Randomized Complete Block with three replications. The data on antioxidant activities, and phenolic and flavonoid compounds were analyzed using the analysis of variance procedure in Statistical Analysis System (SAS) Version 9 (SAS Institute, Cary, NC). The results were expressed as mean \pm standard deviation. Significant differences among means from triplicate analyses ($p \leq 0.05$) were determined by Duncan's Multiple Range Test.

Conclusion

This research indicated the effect of imposing varying levels of greenhouse irradiance on the accumulation and distribution of phenolics and flavonoid compounds as well as their antioxidant activities in the leaves, stems and roots of all three varieties of *L. pumila* (var. *alata*, *pumila* and *lanceolata*). From the result, it was concluded that *L. pumila* plant contained substantial amounts of antioxidants under high irradiance ($630 \mu\text{mol m}^{-2}\text{s}^{-1}$) as reflected by the high total phenolic and flavonoid contents in all varieties. The results also demonstrated strong correlations and direct relationships of total phenolic and flavonoid contents with antioxidant activity under the different light intensities. The manipulation of greenhouse microenvironment seemed able to cause alterations in the types and levels of secondary metabolite (such as phenolic and flavonoid compounds) and modify their partitioning in the different plant parts. Understanding the effects of microenvironments and their interactions with plant species on the accumulation and partitioning of secondary metabolites pose a great challenge to establish an alternative method for biopharmaceutical production of local herbs. It would be indeed a challenge to establish a factory-run system in a multi-tiered controlled environment for greenhouse niche production of targeted key metabolites.

Acknowledgements

The authors would like to thank the Ministry of Higher Education Malaysia, and the Research Management Centre, Universiti Putra Malaysia (UPM) for sponsoring this work under the Research University Grant Scheme No. 91007. Authors also wished to acknowledge the support given by the Faculty of Agriculture, and the Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia for laboratory facilities.

References

Ao C, Li A, Elzaawely AA, Xuan TD, Tawata S (2008) Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. Extract. *Food Control* 19: 940–948
Apak R, Guclu K, Ozyurek M, Celik SE (2008) Mechanism of antioxidant capacity assays and CUPRAC (cupric ion reducing antioxidant capacity) assay. *Microchimica Acta* 160: 413–419

Barros L, Baptista P, Ferreira ICFR (2007) Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays. *Food Chem Toxicol* 45 (2007) 1731–1737
Benzie IFF, Strain JJ (1996) The reducing ability of (FRAP) as measure of antioxidant power: The FRAP assay. *Anal Biochem* 239: 70–76
Bergquist S, Gertsson U, Nordmark LY, Olsson ME (2007) Effects of shade nettings, sowing time and storage on baby spinach flavonoids. *J Sci Food Agric* 87: 2464–2471
Briskin DP, Gawienowski MC (2001) Differential effects of light and nitrogen on production of hypericins and leaf glands in *Hypericum perforatum*. *Plant Physiol* 39: 1075–1081
Brunet JMC, Cetkovic GS, Djilas SM, Tumbas VT, Savatovic SS, Mandic AI, Markov SL, Cvetkovic DD (2009) Radical scavenging and antimicrobial activity of horsetail (*Equisetum arvense* L.) extracts. *Int J Food Sci Tech* 44: 269–278
Chen HG, Yu YG, Zeng OX (2002) Study on extraction of flavonoids and alkaloids from lotus leaf. *Food Sci* 23: 69–71
Crozier A, Jensen E, Lean MEJ, McDonald MS (1997) Quantitative analysis of flavonoids by reversed-phase high-performance liquid chromatography. *J Chromatogr A* 761: 315–321
Ghasemzadeh A, Ghasemzadeh N (2011) Effects of shading on synthesis and accumulation of polyphenolic compounds in ginger (*Zingiber officinale* Roscoe) varieties. *J Med Plant Res* 5: 2435–2442
Ghasemzadeh A, Jaafar HZE, Rahmat A, Wahab P, Halim M (2010) Effect of different light intensities on total phenolics and flavonoids synthesis and antioxidant activities in Young Ginger Varieties (*Zingiber officinale* Roscoe). *Molecules* 11: 3885–3897
Gulcin I, Beydemir IGSS, Elmastaş M, Küfrevioğlu OI (2004) Comparison of antioxidant activity of clove (*Eugenia caryophyllata* Thunb) buds and lavender (*Lavandula stoechas* L.). *Food Chem.* 87: 393–400
Halliwell B, Gutteridge JM (1990) Role of free radicals and catalytic metal ions in human disease: An overview. *Methods Enzymol* 186: 1–85
Hemm MR, Rider SD, Ogas J, Murry DJ, Chapple C (2004) Light induces phenylpropanoid metabolism in Arabidopsis roots. *Plant J* 38: 765–778
Ibrahim MH, Jaafar HZE, Asmah R, Zaharah AR (2012) Involvement of nitrogen on flavonoids, glutathione, anthocyanin, ascorbic acid and antioxidant activities of Malaysian medicinal plant *Labisia pumila* Blume (Kacip Fatimah). *Int J Mol Sci* 13:393–408
Ibrahim MH, Jaafar HZE, Rahmat A, Rahman ZA (2010) The relationship between phenolics and flavonoids production with total non-structural carbohydrate and photosynthetic rate in *Labisia pumila* Benth. under high CO₂ and nitrogen fertilization. *Molecules* 16:162–174
Ismail HI, Chan KW, Mariod AA, Ismail M (2010) Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chem* 119: 643–647
Jaafar HZE, Haris NBM, Rahmat A (2008) Accumulation and partitioning of total phenols in two varieties of *Labisia pumila* Benth. under manipulation of greenhouse irradiance. *Acta Hort* 797: 387–392

- Jaafar HZE, Ibrahim MH, Por LS (2010) Effects of CO₂ enrichment on accumulation of total phenols, flavonoid and chlorophyll content in two varieties of *Labisia pumila* Benth. exposed to different shade levels. In Proceedings of the International Conference on Balanced Nutrient Management for Tropical Agriculture, Kuantan, Pahang, Malaysia, 15–22 February 2010; UPM: Kuala Lumpur, Malaysia. pp. 112–114
- Karimi E, Jaafar HZE, Ahmad S (2011a) Phenolics and flavonoids profiling and antioxidant activity of three varieties of Malaysian indigenous medicinal herb *Labisia pumila* Benth. *J Med Plant Res* 5: 1200–1206
- Karimi E, Jaafar HZE (2011b) Bioactive compounds profiling of three varieties of *Labisia pumila* Benth using HPLC and GC-MS in microwave obtained extracts. *Molecules* 16: 6791–6805.
- Kumari R, Singh S, Agrawal SB (2009) Effects of supplemental ultraviolet-B radiation on growth and physiology of *Acorus calamus* L. (sweet flag). *Acta Biol Cracov Ser Bot* 51: 19–27
- Kurata H, Matsumura S, Furusaki S (1997) Light irradiation causes physiological and metabolic changes for purine alkaloid production by a *Coffea arabica* cell suspension culture. *Plant Sci* 123: 197–203
- Mannfried P (1993) Healing plants. Barron's Educational Series, Inc. NY, 1993
- Oskoueian E, Abdullah N, Zuhainis SW, Omar AR, Ahmad S, Kuan WB, Zolkifli NA, Hendra R, Ho YW (2011) Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Jatropha curcas* Linn. *J Med Plant Res* 5: 49-57
- Prior RL, Wu X, Schaich K (2005) Standardized Methods for the Determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agr Food Chem* 53: 4290-4302
- Ross JA, Kasum CM (2002) Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22: 19–34
- Safaei-ghomi J, Meshkatsadat MH, Asadi M (2010) Analysis of the essential oil of *Salvia brachycalyx* boiss by nano scale injection and antioxidative activity of methanol extract of this plant. *Dig J Nanomater Bios* 5:545 – 549
- Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH (2006) Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity of guava fruit extracts. *J Food Comp Anal* 19: 669-675
- Warren MJ, Bassman J, Fellman JK, Mattinson DS, Eigenbrode S (2003) Ultraviolet-B radiation of *Populus trichocarpata* leaves. *Tree Physiol* 23: 527-535
- Yen GC, Chen HY (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem* 43: 27–32
- Zobel AM, Clarke P (1999) Production of phenolics in seedlings of *Acer saccharum* and *Acer platanoides* in response to UV-A irradiation and heavy metals. *Allelopathy J* 6: 21-34.