GROWTH AND PHYTOCHEMICAL RESPONSES OF *Andrographis paniculata* AS INFLUENCED BY DIFFERENT SHADE LEVELS AND PRUNINGS

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ABSTRACT

Studies on growth and phytochemical responses to light intensities and cultural practices are useful measurements to determine the favorable habitat conditions for the cultivation of medicinal plants. The objective of this study was to determine the effect of shade level and pruning frequency to optimise high dry herbage yield per hectare of *Andrographis paniculata* with high phytochemical content. A factorial experiment was arranged in split plot design with three replications. The shade levels of 0, 20%, 30% and 50% were the main plots while number of pruning namely none, once and twice were the sub plots. Both factors of shade levels and pruning showed significant interaction effect on total leaf area, shoot fresh weight and shoot dry weight. Root fresh weight and dry weight were not influenced by both factors. Shoot fresh weight and shoot dry weight showed significant increase with the increase in shade levels. The lowest shoot dry weight was recorded for plant grown under full sunlight. Pruning frequency significantly produced higher shoot fresh weight by 18.6% and shoot dry weight by 15.4% compared to unpruned plant. There was a significant interaction effect on total phenolic content, total flavonoid and antioxidant content based on FRAP scavenging assay. It was observed that an increase in shade levels and pruning resulted in decrease of total phenolic and total flavonoid content. *A. paniculata* grown under shade and that had undergone pruning once showed higher biomass production.

Keywords: *Andrographis paniculata*, light intensity, method of pruning, biomass production, phytochemical content

INTRODUCTION

Andrographis paniculata is one of the most important medicinal plants grown worldwide. It is known as an annual herb and shrubs plant known as 'hempedu bumi' or king of bitter from the family Acanthaceae (Kumar et al. 2012) which is distributed throughout tropical Asian countries. *A. paniculata* propagates abundantly in India, Pakistan, Sri Lanka, Mauritius, Indonesia and China (Swamy et al. 2012). It grows in a variety of habitats such as plains, hillsides, coastlines, farms and other wastelands. It is one of major herbs that has been proven to possess many pharmacological activities including anticancer and immunostimulatory (Kumar et al. 2012), anti-inflammatory and anti-malarial (Mishra et al. 2007). The suitable growth conditions required to plant *A. paniculata* are those in moderate tropical and subtropical climates with an annual rainfall of 1500 to 2000 mm per year (Zaharah et al. 2001). *A. paniculata* is a shade tolerant species and commonly found in the forest shaded areas and undergrowth (Valdiani et al. 2012).

Light is essential in plant growth and development and interconnected with three main factors namely quality, intensity and duration of light (Thohirah et al. 2009). Light intensity or light quantity refers to the total amount of light that plants received. Different plants have optimum requirements and both deficient and excessive light intensities are injurious. Deficient light intensities tend to reduce plant growth, development and yield (Bareja 2011). However, excessive light intensity will result in photosynthesis inhibition as it has destructive effects on photosynthetic pigments (Kumar et al. 2012). Light intensity was also reported to influence the concentration of secondary metabolites of plants. Phenolic biosynthesis requires light or is improved by light while flavonoid synthesis is totally light

ISSN No 1985-0484

dependent and its biosynthesis rate is related to light intensity. However, different plants had a different response to light intensity alteration and the resulting total phenolic and total flavonoid production (Ghasemzadeh et al. 2010). According to Ghasemzadeh et al. (2010), different light intensities had a direct effect on antioxidant activities in plants with increasing total phenolic content. Total phenolic and flavonoid components for plant grown under shade are increased due to lower temperature.

At present, there is no standard farming practice in Malaysia for 'hempedu bumi' to be produced commercially. With no standardised farming practices in place, it is difficult to achieve consistency in output and quality, with supply often not being enough to cope with market demand (Ganesan 2011). Pruning is an important cultural practice that has been shown to enhance both productivity and quality of plant. A study by Yilmaz et al. (2004) on tea showed that pruning had affected the composition and quality of tea leaves. Calatayud et al. (2008) reported that pruned plant has higher capacity to promote the photosynthetic light reaction, a large number of metabolic sinks and a higher turgor pressure compared to unpruned plant. Plants have a functional equilibrium between their above (leaves) and below ground parts (roots). If plants are pruned, the starch reserves in the roots are utilised for shoot growth to maintain equilibrium (Zeing 2003). Furthermore, studies related to the growth and physiology requirements of the species in relation to different light intensities and method of pruning are still lacking (Saravanan et al. 2008). Therefore, the objective of this study was to determine the effect of shade level and pruning frequency to optimise high dry herbage yield per hectare.

MATERIALS AND METHODS

Experimental design

The experiment was carried out at the agricultural farm, University Putra Malaysia (UPM) using a factorial design from June 2014 to September 2014. The treatments were arranged in a split plot design with three replications. The main plots were shade levels while the sub plots were number of pruning. The shade levels used were 0, 20%, 30% and 50% and number of pruning carried out were 0, once and twice. First pruning was done at six week after transplanting (WAT) and second pruning was done at eight WAT. Netting was used to provide shade to the plants. The level of light intensity was calculated based on the light intensity measured at 1200 hr (approximately 1600 to 1700 mmol $m^{-2}s^{-1}$) using a light meter (Li-COR Model LI-189). The data were analysed using analysis of variance (ANOVA) by GLM procedure. A significant difference between means was done by least significant difference (LSD) test ($p < 0.05$).

Planting materials and maintenance

Scarified seeds were sown in petri dish and kept in the seed germinator at 28°C with 70 % relative humidity for 7 to 10 days. The scarification method was conducted as described by Talei et al. (2012). Seedlings were transferred into jiffy pots and placed at the nursery for 1 month. Munchong soil series was used as planting media. The soil was mixed with biochar (15t/ha) and organic fertilizer containing 1.77% of N before transplanting. The amount of organic fertilizer was calculated based on nitrogen element requirement of 210 kg ha^{-1} N. Four week old seedlings were transplanted into polybags (35 cm x 40 cm) and irrigated twice daily. Weeding activity was carried out manually. Pruning was done by cutting the tip of the growth bud (angled at a moderate 35 to 45 degree slant) at the same level of the apical shoot tips for each plant. First pruning was done at six WAT and second pruning was at eight WAT. Whole plants were harvested after 12 WAT prior to the sign of flowering.

Growth analysis

The parameters measured were plant height, total leaf area, number of branches, fresh weight (FW) and dry weight (DW). The plant heights were recorded at 2 weeks interval after transplanting. Plant height was measured from the base of the plant at media surface to the top of the youngest newly expanded leaf using steel meter-ruler (Cornelissen et al. 2003). After harvesting, plants were washed and separated into shoots (leaves and stems) and roots and weighed. Leaf samples were measured for

total leaf area per plant using a leaf area meter (LI-3100C, Li-COR, USA). All samples were oven dried at 50°C for 4 to 5 days until a constant weight was as weighed. Shoots were further analysed for their antioxidant activities and secondary metabolites compounds.

Extraction of total phenolic acids and total flavonoids

Extraction of total phenolic acid and total flavonoid assay was conducted using a modified method of Hakiman and Maziah (2009). A total of 0.5 g dried shoots were ground and placed in 150 mL conical flask. The samples were homogenised with 50 mL distilled water and transferred into covered flask. Then the mixture was centrifuged for 5 min at 14000 rpm (ALC PK110, Italy). The supernatant was collected and used for total phenolic acids and total flavonoids quantification. The extracts were stored at - 40 ° C before analysis.

Determination of total phenolic compounds

Total phenolic compounds were determined using Folin-Ciocalteu assay according to Hakiman and Maziah (2009). One milliliter extract was added with 1 mL of Folin-Ciocalteu phenol reagent and the mixture was thoroughly mixed. Ten mL of 7% Na_2CO_3 were added after 5 minutes, the mixture was diluted to 25 mL with the addition of 4 mL of distilled water, and the mixture was incubated for 90 minutes at room temperature. Lastly, the absorbance of the mixture was measured using spectrophotometer (UV-3101 PC, PRIM Secomam Europe) at 750 nm. The measurement of total phenolic compounds was repeated in triplicates. The total phenolic acids were expressed as mg gallic acid equivalents (GAE) per g sample by using an equation obtained from gallic acid calibration curve ($y = 1.961 – 0.001x$, $r2= 0.87$).

Determination of total flavonoid

The total flavonoid content was expressed as mg catechin equivalents (CE per 100 g dry weight) and was conducted using Aluminium Chloride Colorimetric method as described by Hakiman and Maziah (2009). One mL of extract was transferred into 10 mL volumetric flask containing 4 mL of distilled water. The following was added into the volumetric flask at specific time ie. 0.3 mL of 5% sodium nitrite ($NaNO_2$) solution at time 0, 0.3 mL of 10% aluminium chloride ($AlCl_3$) at 5 minutes and 2 mL of 1 N sodium hydroxide (NaOH) at 6 minutes. The solution was then immediately diluted with 2.4 mL distilled water and mixed. The absorbance of mixture was measured using spectrophotometer (UV-3101 PC, PRIM Secomam) at 510 nm.

Extraction of antioxidant compounds

Extraction of antioxidant compounds was conducted employing the method modified by Wong et al. (2006). A total of 0.5 g dried shoots were grind and placed in 150 mL conical flask. A total volume of 25 mL of distilled water was added and covered with aluminium foil. The conical flasks containing the samples were placed in orbital shaker for 1 h in the dark at room temperature. Then the samples were filtered using Whatman No. 1 paper. The extracts were stored at - 40 ° C before analysis.

DPPH free radical scavenging assay

The antioxidant capacity of *A. paniculata* extracts was studied through evaluation of the free radical scavenging effect on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The antioxidant activity was determined using methods as described by Wong et al. (2006) with some modifications. A total of 40 µL of extract was added to 3 mL of 0.1 mM methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand at room temperature. After 30 minutes incubation at room temperature, the decrease in absorbance was measured at 515 nm using a spectrophotometer (UV 3101 PC, PRIM Secomam Europe). The absorbance at 515 nm of methanolic DPPH solution without added samples was measured and used as control. The blank mixture of methanolic DPPH (250 µL) solution and 80% methanol was measured as control and used as calibration.

The percentage of DPPH inhibition relative to control was calculated by the following formula:
Percent of inhibition (%) = [(Ac – As)/Ac] x 100
Where Ac = Absorbance of the control; As= Absorbance of the tested sample after 30 minutes.

Ferric reducing antioxidant power

The FRAP assay was performed according to Wong et al. (2006). Stock solutions prepared including 10mM of 2,4,6-tris (1-pyridyl)-5-traizine (TPTZ) solution in 40 mM HCl, 20 mM of ferric chloride ($FeCl_3.6H_2O$) and 300 mM acetate buffer. Acetate buffer was prepared by mixing 3.1 g of sodium acetate and 16 mL of acetic acid and the pH was adjusted to 3.6. The extract (200 µL) of was added to 3 mL of FRAP reagent and the reaction mixture was incubated in a water bath at 37°C for 30 min. Readings of the coloured product were then taken at 593 nm using spectrophotometer (UV 3101 PC, PRIM Secomam, Europe). A higher absorbance reading indicated a higher reducing power. The percent of antioxidant was calculated using the formula, percent of antioxidant (%) = [(A593 of sample-A593 of control)/A593 of sample] x 100.

RESULTS AND DISCUSSION

Light is one of the most important environmental factors, as it acts as a source of energy and informational signals that regulate plant growth and development. The results obtained showed that height of the plant increased with increased shade levels (Figure 1). At 12 weeks after transplanting (WAT) the tallest plant was recorded from plant grown under 50% of shade. In this experiment the results obtained suggested that the response of *A. paniculata* to light is similar to many shade avoiding plants growing under different shade levels. In both natural and agricultural plant communities, light might become a limiting resource under high plant density. In such a situation, plants have evolved to either tolerate or avoid shade. In general, under low light intensities, shade tolerant species tend to adapt to a highly conservative utilisation of resources, commonly accompanied by low growth rates, thinner leaves, reduced apical dominance (increased branching) and low elongation response. The shade-avoiding (or sun-loving) species normally tend to adapt their development to favor elongation at the expense of leaf development, and to increase apical dominance (reduced branching), allowing young and growing tissues to escape from shade (Martinez-Garcia et al. 2010). There was also a relatively taller plants observed at 12 WAT when plant had undergone pruning once and twice as compared to non-pruning plant (Figure 2).

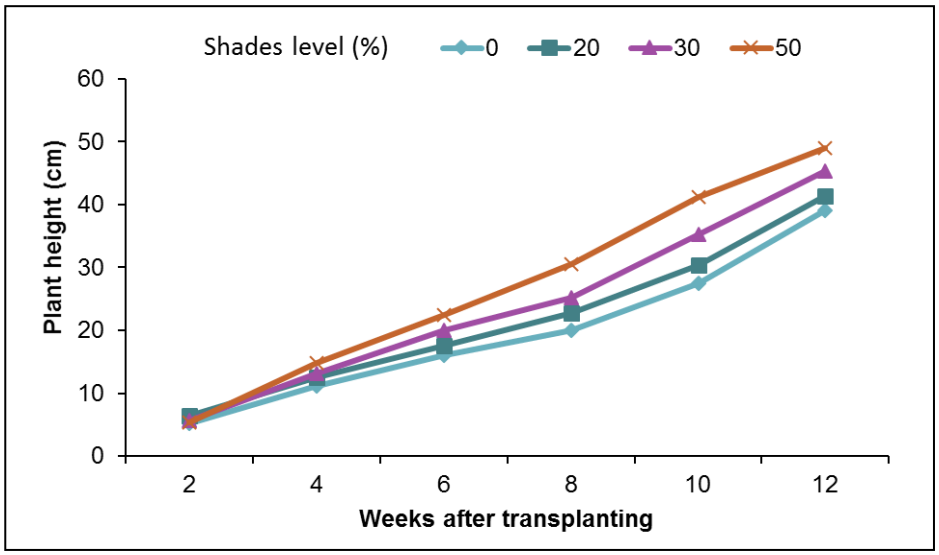

Figure 1. Effects of shades levels on plant height at various plant ages

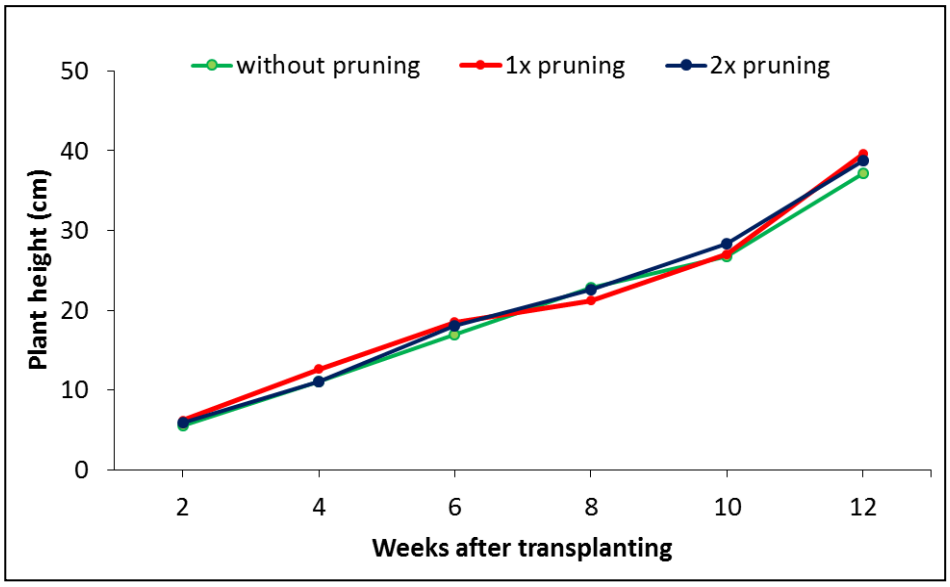

Figure 2. Effects of pruning on plant height at various plant ages

There was a significant interaction effect between shade levels and pruning of *A. paniculata* for total leaf area, shoot fresh weight and shoot dry weight (Table 1). However, no significant interaction on number of branches, root fresh weight and root dry weight was obtained. The fresh and dry shoot weight significantly increased with the increased shade levels. Interestingly, the lowest shoot dry weight was recorded from the plant grown under full sunlight. Similarly, the method of pruning significantly produced higher shoot fresh weight by 18.6% and shoot dry weight by 15.4% compared to non-pruning plant (Table 1). This growth results were also found to occur on pruned roses plants (Calatayud et al. 2008). This is made possible due to the expansion of new shoots that caused a temporary depletion of stored metabolites from older shoots, hence causing a decrease in flower production (Calatayud et al. 2008).

Table 1. Number of branches, total leaf area, shoot fresh weight (FW), shoot dry weight (DW), root fresh weight and root dry weight as affected by shade levels and number of pruning of *A. paniculata*

Factors	No of branches	Total leaf area (cm^2)	Shoot FW (g)	Shoot DW (g)	Root FW (g)	Root DW (g)
Shades levels (S) (%)						
0 (full sunlight)	15.7 a	756.93 c	26.21 d	6.49 d	15.58 b	2.24 b
20	16.1 a	937.30 b	31.18 c	8.60 c	15.19 b	2.37 b
30	16.3 a	1103.32 a	45.60 b	13.24 b	25.81 a	3.84 a
50	16.7 a	1093.94 a	49.11 a	15.52 a	25.23 a	3.85 a
Pruning frequency (P)						
0	15.4 a	826.16 b	34.41 b	10.21 b	20.08 a	3.03 a
1x	16.4 a	1013.41 a	39.83 a	11.40 a	20.22 a	3.14 a
2x	15.7 a	1004.06 a	40.82 a	11.78 a	21.00 a	3.06 a
S*P	ns	*	*	*	ns	ns

Means within column followed by the same letter are not significantly different by LSD, $p \leq 0.05$
ns and * represent non-significant and significant at $p \leq 0.05$, respectively.

There was a significant linear interaction effect between shade levels and pruning frequency on shoot mass, whereby optimum shoot fresh weight was found when plant grown under 50% shade and had

one time pruning (Figs. 3 and 4). According to Morelli and Ruberti (2002), this might be due to elevated levels of auxin in the cells of stems in response to the ratio of red to far-red light wavelengths which is perceived by phytochrome. Moreover, plants grown in a shady condition invest relatively more of the products of photosynthesis and other resources in leaf area in order to maximize light interception which concurrently increase relative growth rate. This morphological response is characteristically shown by a high total leaf area or high specific leaf area which is associated with relatively few, small palisade mesophyll cells per unit area. Therefore, it was not surprising that *A. paniculata* grown under 30 and 50% shade have higher total leaf area compared to under full sunlight.

There was a significant interaction for both factors, shade level and number of pruning on total phenolic content, total flavonoid and FRAP scavenging assay. However, there was no significant interaction effect of DPPH assay (Table 2). The highest antioxidant activity by DPPH method was recorded by plant grown under 20% shade, however DPPH scavenging assay was not affected by number of pruning. Total phenolic content showed a negative response toward shade levels whereby increase in shade levels resulted in decreased in total phenolic (Figure 5). A similar response was observed on total flavonoid content towards shade levels (Figure 6). There was a reduction of 16.3% as shade level increased from 0 to 50% for one time pruning.

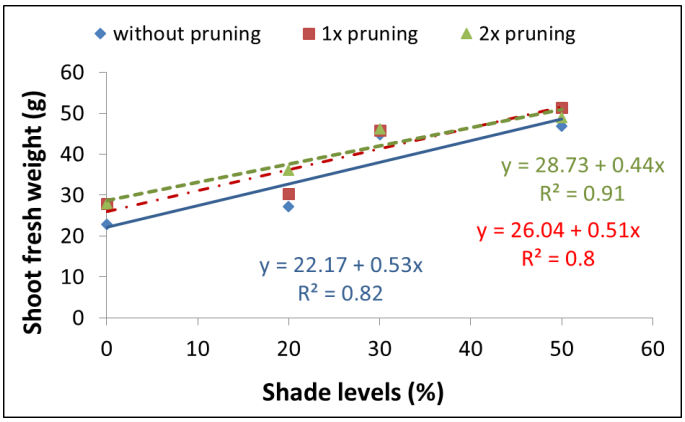

Figure 3. Shoot fresh weight as affected by different shade levels and number of pruning of *A. paniculata.* (LSD at $p \leq 0.05$)

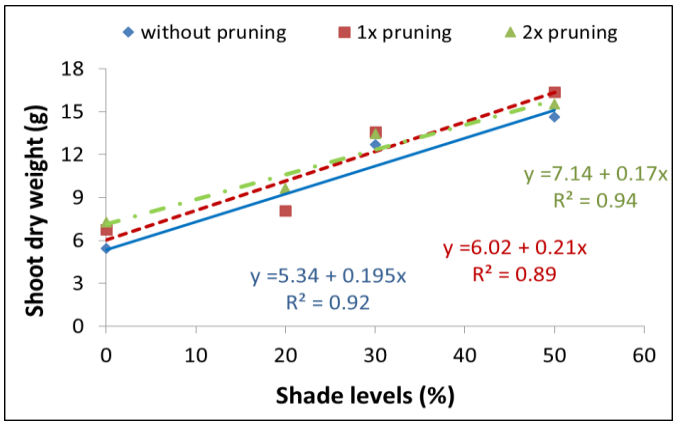

Figure 4. Shoot dry weight as affected by different shade levels and number of pruning of *A. paniculata.* (LSD at $p \leq 0.05$)

Table 2. Total phenolic, total flavonoid and antioxidant activities (DPPH and FRAP assay) as affected by shade levels and number of pruning of *A. paniculata*

Factors	Total phenolic (mg GAE/g DW)	Total flavonoid (mg CE/g DW)	DPPH scavenging assay (%)	FRAP scavenging assay (%)
Shades levels (S) (%)				
0 (full sunlight)	8.63 a	1.40 b	83.6 ab	84.6 a
20	8.56 a	1.46 a	85.2 a	80.7 b
30	7.67 b	1.40 b	81.9 b	79.7 c
50	7.33 c	1.32 c	82.0 b	79.3 c
Pruning frequency (P)				
0	7.85 b	1.32 c	82.5 a	80.7 b
1x	8.09 ab	1.49 a	83.7 a	82.2 a
2x	8.19 a	1.37 b	83.4 a	80.3 b
S*P	*	*	ns	*

Means within column followed by the same letter are not significantly different by LSD, $p \leq 0.05$
ns and * represent non-significant and significant at $p \leq 0.05$, respectively.

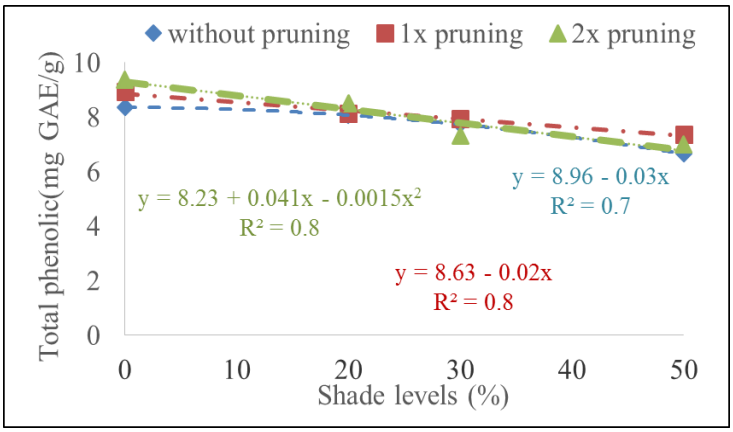

Figure 5. Total phenolic content as affected by different shade levels and number of pruning of *A. paniculata*. (LSD at $p \leq 0.05$)

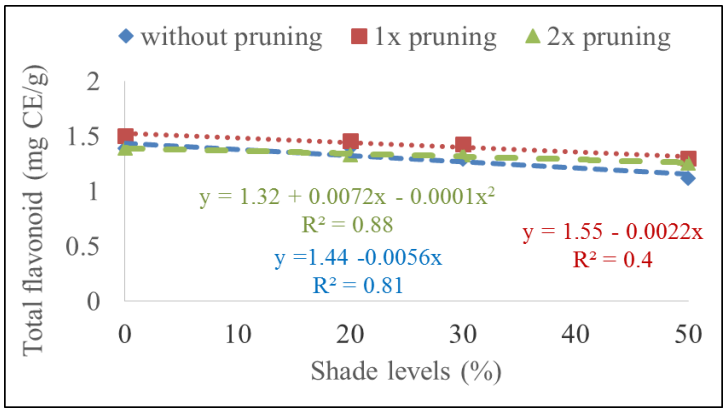

Figure 6. Total flavonoid content as affected by different shade levels and number of pruning of *A. paniculata.* (LSD at $p \leq 0.05$)

Numerous studies have been conducted to identify and evaluate the phytochemical constituents in *A. paniculata* and many other medicinal species. In this study, it seems that different light intensities has a direct effect on antioxidant activities of *A. paniculata* with various values of total phenolic and total flavonoid content. This is because any environmental factor contributed to increase in growth and protein synthesis would cause a decrease in phenolic compounds due to a decrease in phenylalanine available for their synthesis (Similien 2009). This could be observed in this study where the biomass of *A. paniculata* was significantly higher under shade levels while the total phenolic and total flavonoid was significantly lower under this condition. According to Similen (2009), the photo inhibition model associates phenolic production with the plant defense mechanism against oxidative destruction caused by high light intensity. In other words, increasing light intensity would cause an increase in phenolic production by plant. In accordance with results of a study on grape by Mori et al. (2007), increasing light intensities caused increase of anthocyanin degradation in grape skin and also decreased the expression of flavonoid biosynthesis.

CONCLUSION

This study demonstrated that different light intensities and pruning frequency affected the growth of *A. paniculata* growth. There was a significant increase in shoots growth for both shade and pruning. Shoot fresh weight and shoot dry weight showed significant increment with the increasing shade levels. Results also showed that the growth and phytochemical constituents in *A. paniculata* can be altered by growing the crop under different light intensities with pruning. The results also indicated that plant exposed to 50% shade and had at least one pruning can improve biomass production for optimum growth production. Moreover, *A. paniculata* grown under full sunlight and 20% shade could obtain the highest total phenolic content. Besides, the results showed an increasing amount of total phenolic and total flavonoid for pruned plant compared to unpruned plant.

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