Changes in Antioxidant Activity and Active Compounds of *Bacopa monnieri* (L.) Wettst. Over Successive Growth Stages

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ABSTRACT

Brahmi [*Bacopa monnieri* (L.) Wettst.], also called Water Hyssop, is an herbaceous plant that has been used as herbal medicine to improve and restore memory. This research investigated the changes in antioxidant activity and the active compounds during cultivation 12 weeks to find out the appropriate amount of active compound and crop yield. The main indentified active compounds were luteolin and apigenin, bacoside A3, bacopaside II, bacopasaponin X, bacopasaponin C, and bacoside I. The results showed that plant growth varies over its lifetime. Brahmi plants begin flowering 6 weeks after the establishment of new cuttings and flowering increases up to week 12. The antioxidant activity and active compound were similarly changed. Active compounds were produced gradually at week 2, 4, and 6. The highest amount was shown in week 8 and tended to dramatically drop in week 10 and increased in week 12. However plant growth was positively increased with plant ages. This study indicated that antioxidant activity and active compounds were varied with the growth stage. Regarding capacity per plant, the highest content of active compounds was found in week 12. Therefore, this period should be considered for Brahmi harvesting to obtain high active compounds and crop yield.

Keywords: Bacosides, memory, flavonoids, free radical, saponin

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INTRODUCTION

Brahmi [*Bacopa monnieri* (L.) Wettst.] belong to Scrophulariaceae. It is a creeping herb that is commonly found in many parts of Asia, including Thailand. Brahmi has been used as a remedy in Ayurvedic medicine for more than 3,000 years for stimulating memory and mental development (Kashmira and Jagruti, 2010; Shinomol *et al.*, 2011). It requires a warm-moist climate with a temperature range of 30 to 40°C and a relative humidity of 60% to 80% with a good sunshine duration (Niir, 2000; Baruah *et al.*, 2014). The above ground parts of the plant (leaves and stem) contain phytochemical compounds that positively affect the nervous system. These are substances classified as triterpenoid saponins and are called “bacosides”. Several different bacosides in Brahmi have been chemically isolated and described (Bhattacharya *et al.*, 2000). Those that have been reported to have the greatest effect on the human nervous system are bacoside A and bacoside B (Russo and Borrelli, 2005). Bacoside A, a major active principle of *Bacopa monnieri* known for its cognitive effects is a mixture of saponins like bacoside A3, bacopaside II, an isomer of bacopasaponin C and bacopasaponin C (Bansal *et al.*, 2016). Numerous studies have shown bacoside can help stimulate learning, improve and restore memory, protect against brain damage, psychosis, relieve anxiety (Subashri and Koilpillai, 2012), antistress (Chowduri *et al.*, 2002). Most importantly perhaps, bacosides have powerful antioxidant properties (Anbarasi *et al.*, 2005; Sathiyanarayanan *et al.*, 2010; Srivastava *et al.*, 2010; Alam *et al.*, 2012; Subashri and Koilpillai, 2012). Antioxidant effects of Brahmi in memory areas of the rat brain such as the hippocampus, frontal cortex, and striatum have been documented (Bhattacharya *et al.*, 2000; Kapoor *et al.*, 2009).

In 2011, Phrompittayarat *et al.* reported that Brahmi tips and leaves contain the most saponin during the rainy season but Brahmi plants had the highest fresh weight during the hot season. However, Mathur *et al.* (2000) were reported that Brahmi plant collected from northern India showed high vegetative growth and the yield of bacoside-A in monsoon season (June-September). As well as a report of Bansal *et al.* (2016) also found that biomass and bacoside-A were highest during summer (15th June-15th September). Since Brahmi can grow easily in tropical area and season also had an effect to yield an active compound. Therefore, it is important to increase Brahmi production to meet market demand, and especially to be able to produce Brahmi plants that have a high concentration of bacosides. Most of the research that has been done on Brahmi to date has concerned it's medicinal and of pharmaceutical properties, but there is still a lack of practical knowledge about its growth and how much of the various active compounds were produced in Brahmi plants at different stages of growth.

The objective of this study was to investigate the antioxidant activity and biologically active compound over successive growth stages in order to determine the optimal harvest time according to the highest content of active compounds.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Brahmi plants [*Bacopa monnieri* (L.) Wettst.] were provided by the Botany and Weed Science Division, Department of Agriculture. The plants were cultivated in a greenhouse, Bangkok, Thailand (13°51'13.4"N 100°34'09.1"E) from May 2014 to August 2014. The environments during cultivation were recorded. The average temperature was in the range 27°C–34°C and relative humidity was 62%–79%. The above-ground stems were taken, selecting healthy mature side branches used for cuttings. Each cutting of approximately 10 cm in length was planted about 3 cm deep in moist peat moss and the containers were covered with clear plastic to retain moisture for 2 weeks to promote rooting. After that, the cuttings were transferred to 6–inch pots filled with a potting soil mixture of one part baked clay, 2 parts organic fertilizer, and ¼ part sand, with 10 cuttings per pot. The pots were placed on saucers and watered daily (500 ml).

Experimental Design

The experiment followed a completely randomized design (CRD) with 6 treatments (branches harvested from 2–week, 4–week, 6–week, 8–week, 10–week, and 12–week old plants) with 3 repetitions for each treatment, each repetition consisting of 10 branches per pot. Plant growth data were recorded every 2 weeks until week 12.

Plant Growth Analysis

Plants were measured a number of new side branches per plant, by counting the branches that grew from the original cutting. Length of new side branches in centimeters, by randomly selecting 3 new side branches that grew from the original cutting and finding the mean of their lengths once each week. A number of flowers per plant, counted from when the first flower buds appeared, throughout the length of the experiment. Plant fresh weight in grams, by collecting the entire plant (the original cutting and all its new side branches) and weighing on a balance to 3 decimal places. Plant dry weight in grams, by weighing the entire plant following drying in a hot air oven at 60ºC for 2 days.

DPPH Radical Scavenging Activity

DPPH assay is common to use for evaluating antioxidant activity in the plant because of the method is practical, efficient and quick. Therefore, this assay was used for analyzed antioxidant activity in this experiment. One gram of freshly above–ground part was extracted with 10 ml of 95% ethanol and placed in the dark at room temperature for 24 hrs. The extracts were centrifuged at 8,000 rpm for 5 min and collect the supernatant for further analysis.

Antioxidant activity of the plants harvested at each growth stage was measured DPPH radical scavenging activity following the method of Cousins *et al.* (2007) with a modification. A 0.1 mM solution of DPPH (1, 1–diphenyl–2–picrylhydrazyl radical) was prepared by dissolving in distilled ethanol in the dark. A range of dilutions of Brahmi extract was prepared and placed in test tubes with a volume of

0.5 ml Brahmi extract in each tube, to which was added 3 ml DPPH solution. The tubes were shaken to mix and left to stand in the dark for 20 minutes measuring with a UV–spectrophotometer at 517 nm. The absorbance was used to calculate radical scavenging activity using the following formula:

$$DPPH\ scavenging\ activity\ (\%) = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

The percentage of DPPH radical scavenging activity values obtained from the above formula were then plotted on a graph against the concentrations of the different dilutions to find the linear equation and R^2. With the resulting linear equation, the EC_{50} was then found when y = 50. The EC_{50} value, expressed in grams per liter ($g_{FW}L^{-1}$), is the substances' ability to reduce free radicals by half. Thus, the higher a substances' EC_{50}, the less antioxidant activity it exhibits, and the lower a substances' EC_{50}, the greater antioxidant activity it exhibits.

Active Compound Analysis

Dried Brahmi plants were ground to a fine powder. Twenty–milligram portions of the powder were weighed out and extracted in 1 ml of 70% methanol and sonicated at room temperature for 15 minutes before being filtered through a 0.45 µm pore size syringe filter. The filtered extract was then used for HPLC–UV analysis internal standards from the Bioactive Testing Unit, Pharmacology Department, Naresuan University, to analyze the Brahmi extracts by HPLC, following the method of Phrompittayarat *et al.* (2007).

The HPLC setup was a CMB–20Alite system controller (Shimadzu LC solution) SPD–20A UV/Vis detector, LC–20AT pump with SIL–20A autosampler, using a Phenomenex Luna C18 column (150 x 4.6 mm, 5 µm) equipped with a Phenomenex guard column of type C18 (10 x 4.6 mm, 5 µm). The mobile phase we used was 0.2% phosphoric acid mixed with acetonitrile in the proportion of 65:35 v/v and pH was adjusted to 3 using 5 M NaOH basic solution The substance for analysis (duration 25 minutes) was dispensed in volumes of 20 µl with a flow rate for the mobile phase of 1.0 ml/min, with temperature controlled at 30ºC and measurements were made at 205 nm. The internal standards for 5 types of saponins were prepared at concentrations of 6.25, 12.5, 25, 50, 100 and 120 µg/ml, and internal standards for 2 types of flavonoids were prepared at concentrations of 0.5, 1, 2, 4, 8, 16 and 19.2 µg/ml for making the standard graph for calculating the value of active compounds. The peak height was used for calculation. The amount of active compounds was expressed as grams per 100 g dry weight of plant material (% w/w). Total saponin consisted of the amounts of bacoside A3, bacopaside II, bacopasaponin X, bacopasaponin C, and bacoside I. Total flavonoid consisted of the amounts of luteolin and apigenin, which are believed to be the main compound. The yield of active compounds in Brahmi was reported as milligram of total saponin and total flavonoid per plant as the following equation: $yield\ of\ active\ compound\ mg/plant = [(A/100) \times B] \times 1{,}000$ whereas A = *total saponin/ total flavonoid content* and B = *dried weight*.

RESULTS AND DISCUSSION

Plant Growth and Development

Brahmi plants were grown from cuttings initially sprout new side shoots from the original cutting (Figure 1). Growth parameters such as a number of branches, branch length, number of flowers, fresh weight and dry weight showed significant differences ($P < 0.05$) with positively correlated with increased plant age (Figure 2). New branches were sprouting at a rapidly increasing rate within 2 weeks (5 branches/ plant), but from weeks 4 to week 12 the rate of new branch growth gradually slows to a steady rate and the new stems become straighter (7 branches/ plant) (Figure 2A). The length of new branches, the longer side branches tend to trail along the soil surface rather than sticking up erect. The new branch length at week 2 was 2.78 cm and gradually increased at week 4 and week 6 with 4.85 and 6.38 cm respectively. While, branches length at week 8, 10 and 12 were 9.20, 13.80 and 20.03 cm, respectively, which dramatically higher than the early week (Figure 2B).

Fresh weight and dry weight of Brahmi started a significant difference ($P < 0.05$) at week 10 (Figure 2C and 2D). Fresh weight of Brahmi at week 2, 4, 6 and 8 were 0.50, 1.05, 1.03 and 2.10 g/plant which was not significantly different. However, fresh weight at week 10 was 6.85 g/plant higher than the previous week, but the highest fresh weight of Brahmi was found in week 12, 13.48 g/plant. Changing pattern of dry weight was similar to fresh weight. Dry weight was not significantly different among week 2, 4, 6 and 8, the values were 0.04, 0.11, 0.17 and 0.87 g/plant, respectively. Dry weight at week 10 was 0.42 g/plant higher than in previous weeks. The highest dry weight of Brahmi was found in week12 which showed significantly different from others week with the value 0.87 g/plant. Brahmi plants from cuttings begin to flowering after 6 weeks (Figure 2E), 2 flowers/plant and gradually increased in week 8 (3 flowers/plant). A number of flowers in week 10 and 12 were 5 and 10 flowers/plant which tended to continue flowering.

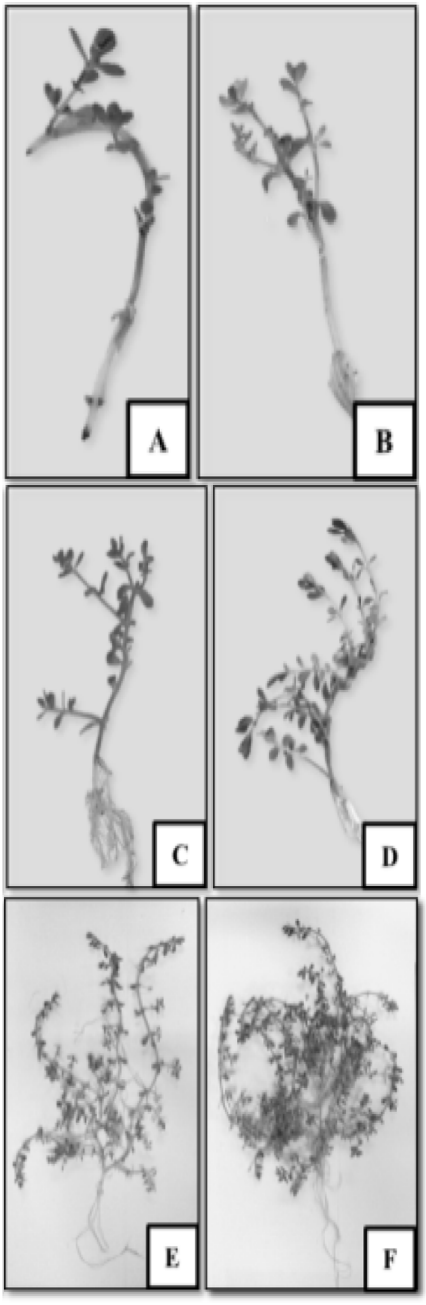

Figure 1 *Bacopa monnieri* characteristic at 2 (A), 4 (B), 6 (C), 8 (D), 10 (E) and 12 (F) weeks

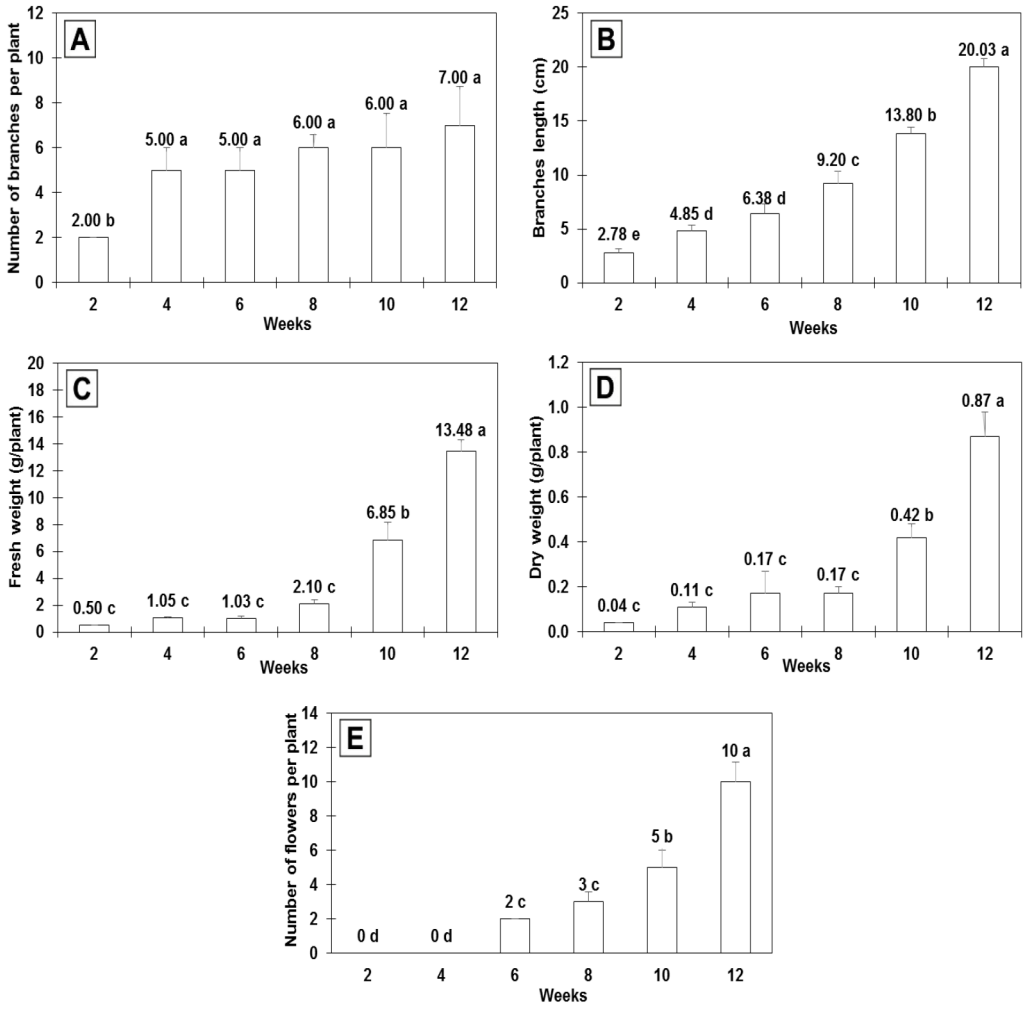

Figure 2 Growth parameters of *Bacopa monnieri* (L.) Wettst: number of branches per plant (A), new branches length (B), fresh weight per plant (C), dry weight per plant (D) and number of flower per plant (E). Mean within each parameter followed by the same letter is not significantly different at $P < 0.05$ on Duncan's New Multiple Range Test

The results revealed that growth of Brahmi was related to the plant age which was in agreement with Phrompiitayarat *et al.* (2011). In the rainy season (July–October) the highest growth was obtained in month 4 which gave the weight of dried plant 2 folds higher than month 3. This plant is a creeping plant which axillary buds on the nodes emerge to form new shoots and elongating into new branches. It is easy to form roots when contacting with the soil and form new branches again. This growth–pattern allows the plant continuous growth.

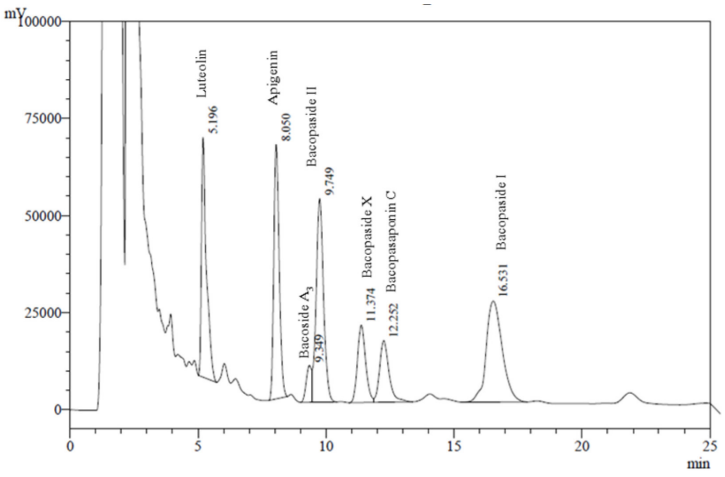

Figure 3 HPLC–chromatogram of Brahmi extract at 8 week–ages, the peak of active compounds as follows: luteolin (RT = 5.196), apigenin (RT = 8.050), Bacoside A3 (RT = 9.349), Bacopaside II (RT = 9.749), Bacopaside X (RT = 11.374), Bacopasaponin C (RT = 12.252), and Bacopaside I (RT = 16.531)

Change in the Active Compounds

Antioxidant activity was reported as EC_{50} value which is the ability of the extract to reduce free radicals by half. A lower EC_{50} value indicates higher antioxidant quality. The changing pattern of antioxidant activity of Brahmi has fluctuated which may relate to the growth stage. This change was different from a growth pattern that growing varied with plant age. Vegetative growth stage of Brahmi after cutting taken 4 weeks considered on the number of flowers of these weeks which have not emerged yet (Figure 2E). Antioxidant activity at week 2 and week 4 showed EC_{50} values 8.06 and 7.14 $g_{FW}L^{-1}$ respectively which significantly lower ($P < 0.01$) than antioxidant activity from week 6 and week 8. EC_{50} values were 4.75 and 4.60 $g_{FW}L^{-1}$ respectively (Table 1). According to these weeks, Brahmi has entered the reproductive stage which started from week 6 (2 flowers emerged). However, antioxidant activity decreased at week 10 (EC_{50} = 7.42 $g_{FW}L^{-1}$) thereafter increased at week 12 (EC_{50} = 4.47 $g_{FW}L^{-1}$) (Table1). This result indicated that Brahmi extract taking from young cutting had a relatively low antioxidant quality but after the plants enter to reproductive stage, the antioxidant activity of the extract grew stronger with a fluctuation in some week may due to the source-sink relationship in the plant.

Table 1 Antioxidant activity from the extract of a freshly above-ground part and flavonoid: luteolin and apigenin of *Bacopa monnieri* (L.) Wettst. harvesting at different ages between May 2014 and August 2014

Plant age (weeks)	EC_{50} (g_{FW} L^{-1})	Luteolin (% w/w)	Apigenin (% w/w)	Bacoside A_3 (% w/w)
2	8.06 ± 1.03^{b}	0.02 ± 0.01^{d}	0.04 ± 0.00^{cd}	0.06 ± 0.01^{c}
4	7.14 ± 0.60^{b}	0.05 ± 0.01^{c}	0.05 ± 0.01^{cd}	0.11 ± 0.02^{b}
6	4.75 ± 0.47^{a}	0.02 ± 0.00^{d}	0.01 ± 0.00^{d}	0.12 ± 0.00^{b}
8	4.60 ± 0.46^{a}	0.15 ± 0.00^{a}	0.21 ± 0.04^{a}	0.15 ± 0.02^{a}
10	7.42 ± 1.32^{b}	0.04 ± 0.01^{c}	0.09 ± 0.03^{b}	0.09 ± 0.01^{b}
12	4.47 ± 0.38^{a}	0.08 ± 0.01^{b}	0.24 ± 0.01^{a}	0.09 ± 0.02^{b}

Note: Mean within each column followed by the same letter is not significantly different at $P < 0.01$ based on Duncan's New Multiple Range Test

Table 2 Saponin glycosides content of *Bacopa monnieri* (L.) Wettst. extracts from plants of different ages analyzed between May 2014 and August 2014

Plant age (weeks)	Bacopasid II (% w/w)	Bacopaside X (% w/w)	Bacopasaponin C (% w/w)	Bacopaside I (% w/w)
2	0.43 ± 0.10^{b}	0.14 ± 0.02^{c}	0.12 ± 0.01^{c}	0.40 ± 0.07^{c}
4	0.46 ± 0.08^{b}	0.29 ± 0.05^{b}	0.24 ± 0.04^{b}	0.51 ± 0.08^{bc}
6	0.50 ± 0.03^{ab}	0.39 ± 0.01^{a}	0.36 ± 0.01^{a}	0.65 ± 0.01^{ab}
8	0.62 ± 0.07^{ab}	0.41 ± 0.09^{a}	0.35 ± 0.06^{a}	0.75 ± 0.11^{a}
10	0.46 ± 0.04^{b}	0.20 ± 0.03^{bc}	0.18 ± 0.01^{bc}	0.51 ± 0.06^{bc}
12	0.54 ± 0.12^{ab}	0.26 ± 0.07^{b}	0.22 ± 0.07^{b}	0.61 ± 0.14^{ab}

Note: % w/w = $g_{DW}/100\ g_{DW}$
Mean within each column followed by the same letter is not significantly different at $P < 0.01$ based on Duncan's New Multiple Range Test

Seven active compounds, 2 flavonoids (luteolin and apigenin) and 5 saponin glycosides (Bacoside A_3, Bacopaside II, Bacopaside X, Bacopasaponin C, and Bacopaside I) from dried Brahmi extraction were determined by HPLC as shown in Figure 3. The data from the chromatograms and peak heights were used to draw a linear equation and find R^2, which was used to calculate the amounts of flavonoids and saponin glycosides in the samples. The changing pattern of active compounds from Brahmi extract at different ages was similar to the pattern of the antioxidant activity. The data showed that the values of active compounds in Brahmi plants differed depending on the week

at which they were harvested, but in general the values of active compounds tended to increase as the plants grew up until a maximum amount and then began to drop. In particular, Brahmi plants harvested at age 8 weeks tended to have the highest content of all the active compounds (Luteolin = 0.15 %w/w, Apigenin = 0.21 %w/w, Bacoside A_3 = 0.15 %w/w, Bacopaside II = 0.62 %w/w, Bacopaside X = 0.41 %w/w, Bacopasaponin C = 0.35 %w/w and Bacopaside I = 0.75 %w/w) (Table 1 and 2)

However, when considered on the yield of active compounds such as a total flavonoid and total saponin per plant, the results revealed that the total amount of active compounds were increased according to the plant age (Figure 4A and 4B). The active compound yields were gradually increased during week 2 to week 10 but dramatically increased thereafter in week12 (fully growth) which presented the highest value of total flavonoid and total saponin with 2.86 mg/plant and 14.66 mg/plant respectively. The values were greater than week 10 almost 5.4 times in total flavonoids and 2.5 times in total saponin.

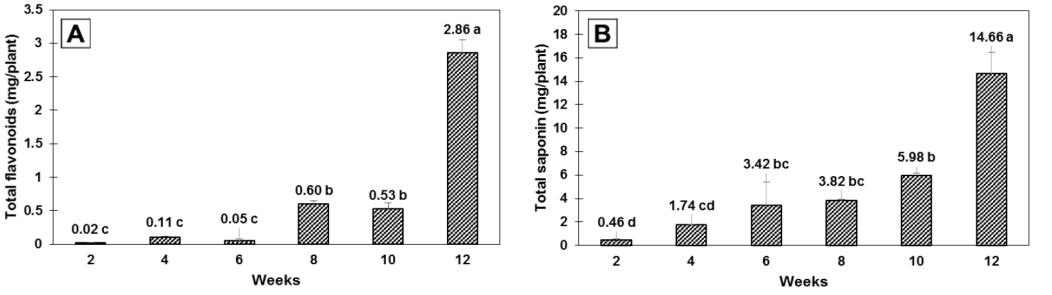

Figure 4 Yield of active compounds per plant from Brahmi at different ages: Total flavonoid contents (A) and total saponin contents (B). Mean within each parameter followed by the same letter is not significantly different at $P < 0.01$ on Duncan's New Multiple Range Test

Before this, there were few reports on how much of the various pharmacologically active compounds were produced in Brahmi plants at different stages of growth. The results of this experiment showed that substances with antioxidant activity found in Brahmi, namely, luteolin, apigenin, bacoside A3, bacopaside II, bacopaside X, bacopasaponin C, and bacopaside I, were present in increasing amounts as the plants grew in the early stages, then when the plants reached maturity and fully flowered (8 weeks after establishment of new cuttings), they contained the maximum amount of these active compounds, and they had the highest antioxidant activity. After that, the content of the active compounds in Brahmi plants trended to decrease. This indicates that the plants biosynthesize the most active compounds when they are at the flowering stage. This is compatible with the general recommendation that for herbs that are collected for their leaves or the whole plant, it is advisable to harvest them when they have achieved their maximum growth, which is when they flower (Steven and James, 2000). When most plants have completed their vegetative growth stage and begin to produce flowers and fruit that is usually the time when they have a high rate of photosynthesis. At that stage, they synthesize and accumulate the most sugars and carbohydrates to store in the stem and to transport from the site of synthesis in the leaves to where it is needed for developing flowers, fruits, and seeds (Li *et al.*, 2015).

The active compounds are secondary metabolites that are synthesized from the primary metabolites arising from photosynthesis. Luteolin and apigenin are polyphenols, luteolin is one of the most potent DPPH radical scavengers beyond flavones

(Yokozawa *et al.*, 1998). Bacoside A3, bacopaside II, bacopaside X, bacopasaponin C, and bacopaside I are classified as saponins, triterpenoids, and alkaloids (Niir, 2000). The precursors for their synthesis are pentose phosphate phosphoenolpyruvate 3 phosphoglycerate pyruvate and acetyl Co A. The alkaloids are synthesized from amino acids (Walters, 2011). So, all the building blocks for making Brahmi's active compounds are primary metabolites that come from photosynthesis. At the stage when the plant has completed its maximum vegetative growth then it will synthesize enough primary metabolites to convert some to the secondary metabolites that make the plant valuable.

Nevertheless, once the plant begins to flower and greater numbers of flower buds start to develop, the rate of photosynthesis will start to drop as the carbohydrates are transported from the source in the leaves to the sink in the developing flowers. Also, as the plant continues to grow, the length of the side branches increases and the whole plant's fresh weight increases. This could mean that there would be insufficient amounts of primary metabolites available for the plant to synthesize more secondary metabolites. The demands of increasing reproductive and vegetative growth could explain why the antioxidant activity and amount of active compounds in samples from cuttings harvested at week 10 were in most cases less than those harvested at week 8. These data showed that the antioxidant activity and active compound content of plants harvested at week 12 were generally higher than those from week 10, but still not as high as at week 8, even though the number of flowers was greatest at week 12. This might be because many new side branches were growing at that stage. Brahmi plants harvested at week 12 had the highest fresh weight and dry weight. All the new leaves on the new side branches were photosynthesis sites that could help the plant produce more metabolites. It is indicative of a balance between source and sink.

Many studies suggest that harvesting medicinal plant should consider the proper growth stage. And the results of this study also supported that recommendation. Brahmi highly synthesized highly active compounds content at week 8. However, even Brahmi can harvest at that stage but the production yield was not high enough which may affect on commercialize yield. According to Brahmi is a creeping plant which forms roots easily when contact with the soil. This growth pattern allows for Brahmi continuous growth. The results also revealed that active compound varied with plant age. Therefore harvesting Brahmi for industrial should consider high production volume coupled with high active compound content. With this regard, harvesting Brahmi at week 12 has been recommended.

CONCLUSION

The growth and content of active compounds in Brahmi plants during 12 weeks which found that active compounds were changed related to the growth stage. The highest amount of active compounds obtained from the plant that harvested in week 8 were observed and it was during flowering initiation thereafter the amount of active compound was dropped. Even though Brahmi plants tend to synthesize the most active compounds at week 8 but if considerate on the yield of active compounds per plant the larger size will provide the greatest amount of active compound as well. Therefore the production Brahmi for herbal raw materials could harvest at 12 weeks after cultivation in order to high active compounds and crop yield.

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