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Original Article

Influence of seasons, different plant parts, and plant growth stages on saponin quantity and distribution in *Bacopa monnieri*

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Abstract

Brahmi or *Bacopa monnieri* (L.) Wettst. is becoming popular as a food supplement due to its enhancing effect on memory and intellect. Previous studies showed that a group of saponins are active compounds in this plant. However, until now little evidence has been obtained to indicate whether saponins are consistently present throughout the plant growth stages or the compounds are affected by the seasons. In order to answer those questions, we cultivated Brahmi under the net house in three seasons. Influence of plant growth stages on saponin quantity and distribution was also investigated. In each season, treatments were plant ages with different plant parts having a factorial completely randomized design with 3 replications. Five saponins, i.e. bacoside A_3, bacopaside II, bacopaside X, bacopasaponin C and bacopaside I, were analyzed using HPLC and reported as total saponins.

The results showed that total saponin contents in Brahmi were the highest in rainy season while the weight yield of Brahmi was the highest in summer. Ages of Brahmi (1-4 months) slightly affected total saponin content. High level of total saponins (1.91±0.48% w/w) was detected at the shoot of Brahmi. These findings indicate that the saponin quantity is affected by seasons and the distribution of the saponins is different in each part of the plant. This information will be beneficial to the production of Brahmi for both household and industry.

Keywords: Brahmi, developmental stage, HPLC, saponins, seasonal variation

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1. Introduction

Bacopa monnieri (L.) Wettst. or Brahmi is a medicinal plant belonging to Scrophulariaceae family. The leaf and flower-bearing stems are 10-30 cm long and arise from creeping stems that form roots at the nodes. The leaves are ovate-oblong, sessile, opposite, obtuse apex approximately 2x1 cm with entire margin. Flowers are pale blue, purple or white, solitary on long pedicles in the leaf axils. The corolla is 5-lobed. The fruit is an up-to-5 mm capsule which develops in the persistent calyx. It grows in marshy land and can be found throughout the United States, Australia and Asia (Al-Saadi and Al-Mousawi, 1984).

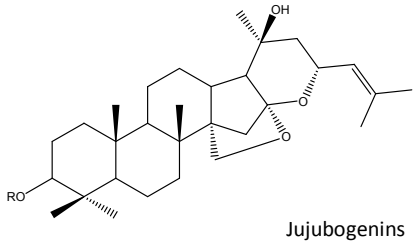
Brahmi has been used in Ayurvedic medicine to improve memory and intellect (Singh and Dhawan, 1997). Presently, several studies have been shown that Brahmi provides biological effects especially for a therapeutic potential in treatment or prevention of neurological diseases and improvement of cognitive processes (Singh and Dhawan, 1997; Stough *et al.*, 1997; Vohora *et al.*, 2000; Das *et al.*, 2002; Sairam *et al.*, 2002; Russo *et al.*, 2003; Russo and Borrelli, 2005; Limpeanchob *et al.*, 2008; Uabundit *et al.*, 2010). The active components reported to be responsible for the cognitive enhancement are triterpenoid saponins, classified as pseudojujubogenin and jujubogenin glycosides (Figure 1).

At the present time, Brahmi can be found as an ingredient in food supplements, teas and cosmetic products. The raw materials of Brahmi are in great demand by these industries. Nevertheless, little evidence is available from studies on cultivation, collection and quality control of Brahmi (Mathur *et al.*, 2000; Ganjewala *et al.*, 2001). Some studies showed that different seasons could affect the accumulation of bacoside A, a mixture of saponin components (Mathur *et al.*, 2000; Ganjewala *et al.*, 2001). In order to determine the appropriate time and plant part for collecting Brahmi, three different factors including seasons, plant parts, and plant ages were investigated in this study. The levels of five major saponin glycosides, i.e. bacoside A_3, bacopaside II, bacopaside X, bacopasaponin C and bacopaside I, were evaluated using high performance liquid chromatography (HPLC) (Phrompittayarat *et al.*, 2007).

2. Methods

2.1 Chemicals

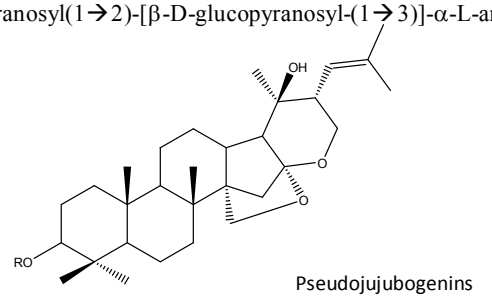
Acetonitrile and methanol (HPLC grade) were purchased from Labscan Asia Co. Ltd. (Thailand). Orthophosphoric acid (AR grade) was from BDH Chemical, England. The saponin glycoside reference standards, bacoside A_3, bacopaside II, bacopaside X, bacopasaponin C were obtained from the National Center for Natural Products Research, MS, USA. Bacopaside I was purchased from ChromaDex, CA, USA. Their chemical structures are shown in Figure 1.



Jujubogenins

R

Bacoside A_3 α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopaside X α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl



Pseudojujubogenins

R

Bacopasaponin C α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl
Bacopaside I α-L-arabinofuranosyl(1→2)-[6-*O*-sulfonyl-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl
Bacopaside II α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl

Figure 1. Structures of saponin glycosides from Brahmi

2.2 Plant materials

Brahmi was collected from Petchaburi Province, Thailand, and identified by Professor Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen was kept at the PBM Herbarium, Mahidol University, Bangkok, Thailand. The vegetative parts were transplanted into a container filled with farm soil and placed under the net house at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Thailand.

2.3 Net-house experiments

Experiments were conducted in the rainy season (July-October 2005), winter season (November 2005-February 2006) and summer season (March-June 2006) under the net house. The experimental design in each season was factorial completely randomized design with 3 replications. Treatments were plant age comprising 1, 2, 3 and 4 months old. The experimental unit was a cement pot 55 cm diam. and 40 cm height filled with farm soil.

The cultivation of Brahmi was modified from the report of Mathur *et al.* (2000) and the traditional method. Shoots of Brahmi cut at approximately 15 cm length were planted at 5 spots in the pot filled with soil. The water level in the pot was 1-2 cm above the soil. At each plant age, six different parts including shoots (aerial parts collected at 10 cm from the apex), lower parts (the rest of the aerial parts below "the shoot"), upper stems (stems collected at 10 cm from the apex), lower stems (stems collected below 10 cm from the apex), leaves and roots were harvested for further investigation.

Each harvested sample was weighed, cut into small pieces, and dried at 50°C in a hot air oven for 8 h. The dried samples were ground and kept at -20°C until analysis of total saponins.

2.4 Soil characterization

Soil was collected at 15-30 cm under the ground from a farm in Tambon Tapo, Amphur Muang, Phitsanulok, Thailand, in three seasons. Some chemical and physical properties such as pH, organic matter, total nitrogen phosphorus potassium (NPK) and texture of the soil samples were analyzed at Department of Natural Resources and Environmental Science, Faculty of Agriculture, Natural Resource and Environment, Naresuan University.

2.5 Determination of saponins using HPLC

Each dried sample (0.5 g) was soaked in 5 ml of water for 1 h and then pressed to discard water before sonication with 95% ethanol (6 ml/g dried plant, 10 min) 3 times. The ethanolic extracts were filtrated and adjusted to 10 ml. Each sample was done in triplicate.

The saponins in Brahmi were determined using the HPLC method previously reported (Phrompittayarat *et al.*, 2007). The separation was performed using a Shimadzu HPLC system equipped with a SPD-M10AVP photodiode array detector (PDA), an LC-10ATVP pump (Shimadzu, Japan) and a Rheodyne injector with 20 µl loop. A Luna RP-18 column (150x4.6 mm, 5µm particle size) was used together with a Phenomenex RP-18 guard column (Torrance, CA, USA). The mobile phase consisted of 0.2% phosphoric acid and acetonitrile (65:35 v/v). The pH of the mobile phase was adjusted to 3.0 with 5 M NaOH. The flow rate was 1.0 ml/min. The total run time was 20 min. All peaks were integrated at 205 nm. They were initially assigned by comparing retention times with standards and confirmed with characteristic spectra obtained from the PDA. The purity of the peak was also confirmed by the PDA.

The HPLC method was validated for linearity, limit of detection, precision and accuracy. The shoot of Brahmi in month 4 during the summer season was used as a sample for method validation.

The linearity of the method was evaluated in the 7.8 to 200.0 µg/ml range. Six concentration levels of five standard saponins were chosen for generating the calibration curves. Three determinations (n=3) were carried out for each solution. The correlation graphs were constructed by plotting the peak heights obtained versus the injected amounts. The limit of detection (LOD) was determined by serial dilution of the mixture of five standards. The concentration that gave a signal to noise ratio of 3 was regarded as minimal detectable amount recorded.

Intra- and inter-day precisions were determined by the analysis of the samples at concentration of 2 mg/ml and expressed as relative standard deviation (R.S.D) (%). In the intra-day precision experiment, five determinations (n=5) were carried out for each solution within one day. The inter-day precision was measured in triplicate (n=3) for three consecutive days. The accuracy of the method was evaluated by analyzing the mixture prepared by adding 20 µg/ml of standard saponins to the Brahmi extract, contained a known amount of the analyzed saponins.

2.6 Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and the treatment means were separated by using Tukey's honestly test $P \leq 0.05$ using SPSS version 11.0 software.

3. Results and Discussion

3.1 Soil characterization

All soil textures collected from three seasons were loam. The percentages of organic matters were in range of 4.3 to 6.8. The percentage of total nitrogen in rainy season and winter were 0.07 and 0.05, while in summer it was 0.02. The highest amount of available phosphorus (6.8 ppm) was

found in summer and it was 5 times higher than that in rainy season and winter. Available potassium ranged from 42.5 to 57.4 ppm. Soil pH was very mildly acid in the rainy season (6.8) and winter (6.9) but was moderately acid in summer (5.5).

3.2 Effect of seasons on the growth of Brahmi

The environmental condition, temperatures and relative humidity, were recorded in all periods by a data logger. The data were separated into daytime (6.00 AM - 5.99 PM) and night time (6.00 PM to 5.99 AM). The temperatures during the daytime in the rainy season, winter and summer were 32.0-34.8°C, 30.3-36.7°C and 34.9-39.2 °C and during night time were 25.8-26.3°C, 21.5-25.6°C and 26.2-27.2°C, respectively. The relative humidity in the rainy season, winter and summer were 72.9-84.0%, 68.8-70.8% and 68.5-86.8% respectively during the daytime and nearly 100% during night time in all seasons.

The growths of Brahmi at different ages and seasons represented by dried weights of Brahmi are summarized in Figure 2. In the rainy and winter seasons, the weight of Brahmi gradually increased over a 4-month-period. However, the weight dramatically increased in summer resulting in the highest yield compared with two other seasons. At month 4, the average weight of Brahmi grown in summer was 2 and 3 times greater than that in the rainy season and winter, respectively. The results from the present studies also indicate that temperature and humidity may affect Brahmi's growth. Ganjewala *et al.* (2001) and Mathur *et al.* (2000) reported that the lowest growth of Brahmi was found in winter (September to December) which is in agreement to our findings. However, they reported that the highest growth was obtained in the monsoon season (June to September).

3.3 HPLC analyses of saponin glycosides in Brahmi

Five major triterpenoid saponin glycosides in Brahmi were detected by HPLC as shown in Figure 3. Calibration curves were generated by linear regression based on the peak heights. Within the range of saponin concentrations injected (7.8-200.0 µg/ml), linearity was obtained. Detection limit was in the range of 0.20 to 0.78. The percentage intra- and inter-day RSD values of five investigated saponins were lower

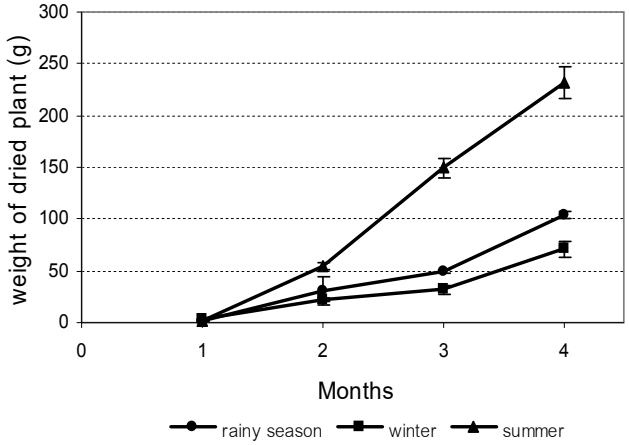


Figure 2. Weight of dried Brahmi materials grown in the pot in different months and seasons. Vertical bars represent standard deviations of 3 replications.

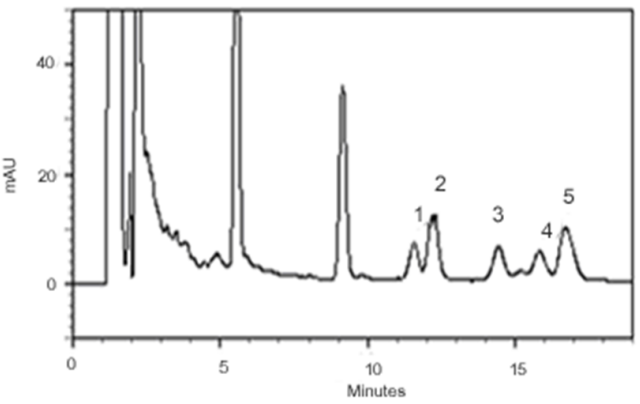


Figure 3. HPLC-chromatograms of Brahmi extract (2 mg/ml) under optimized HPLC conditions (column: Luna C-18, 5 µm, 100 mm×4.6 mm; mobile phase: 0.2% phosphoric acid and acetonitrile (65:35 v/v, pH 3.0); flow rate: 1 ml/min; detection: 205 nm; injected sample volume: 20 µl). The peaks were identified as follows: **1**, bacoside A_3; **2**, bacopaside II; **3**, bacopaside X; **4**, bacopasaponin C; **5**, bacopaside I.

Table 1. Regression equations of calibration curves and detection limits of 5 investigated saponins (n=3) using HPLC assay

compound	regression equation	r^2	LOD(µg/ml)
Bacoside A_3	y=0.00442x - 0.5890	0.9998	0.39
Bacopaside II	y=0.00335x - 0.5275	0.9999	0.20
Bacopaside X	y=0.00533x - 0.4571	0.9999	0.20
Bacopasaponin C	y=0.00398x - 0.3527	0.9999	0.39
Bacopaside I	y=0.01204x - 4.8253	0.9989	0.78

Note: y = the peak area (mAU); x = the concentration of compound (µg/ml)
r^2 = a good of fitness LOD = Limit of detection

Table 2. Intra- and inter-day precision and percent recovery of the investigated saponins under optimized HPLC conditions

Compound	Intra-day RSD (%) (n=5)	Inter-day RSD (%) (n=3)	%Recovery
Bacoside A_3	0.92	1.06	114.06
Bacopaside II	1.34	1.25	112.58
Bacopaside X	1.33	1.54	107.94
Bacopasaponin C	2.13	0.35	110.36
Bacopaside I	1.72	2.30	88.88

Table 3. Percentage of total saponin content of Brahmi in different parts and different seasons

Season/Month	%Average total saponins in Brahmi (SD)		
	Shoot	Lower part	Root
Rainy season			
Month 1	2.57(0.03)	0.72(0.08)	0.56(0.04)
Month 2	2.21(0.20)	0.70(0.01)	0.63(0.04)
Month 3	2.32(0.29)	0.60(0.09)	0.52(0.05)
Month 4	2.03(0.04)	0.68(0.04)	0.68(0.02)
Winter			
Month 1	1.50(0.23)	1.26(0.06)	0.28(0.05)
Month 2	2.30(0.08)	1.52(0.08)	0.24(0.05)
Month 3	1.30(0.12)	1.15(0.25)	0.39(0.07)
Month 4	1.74(0.21)	1.14(0.12)	0.56(0.02)
Summer			
Month 1	1.18(0.20)	0.91(0.05)	0.28(0.02)
Month 2	1.58(0.23)	1.03(0.11)	0.55(0.06)
Month 3	2.53(0.25)	0.89(0.11)	0.65(0.01)
Month 4	1.72(0.04)	0.76(0.11)	0.73(0.05)

than 3 (Table 2). Percent recoveries of investigated saponins ranged from 89 to 114% indicating an adequate accuracy of the method (Ermer and Miller, 2005).

3.4 Saponin contents in different parts of Brahmi

The expression of total saponin contents in shoots was significantly higher than that in lower parts and roots in every season ($p<0.05$) (Table 3). Figure 4 demonstrates the percentage of saponin glycosides from different parts of Brahmi collected from month 4 of the summer season as a representative of Brahmi samples. When comparing the total saponin contents in shoots, lower parts, roots, leaves, upper stems, and lower stems, the high percentage of total saponin content in Brahmi was found in leaves and shoots. The other plant parts had total saponin contents only approximately a half of those present in shoot. Even though the leaves contain slightly higher total saponin content than the shoots, for large scale harvesting, collecting shoots would be more

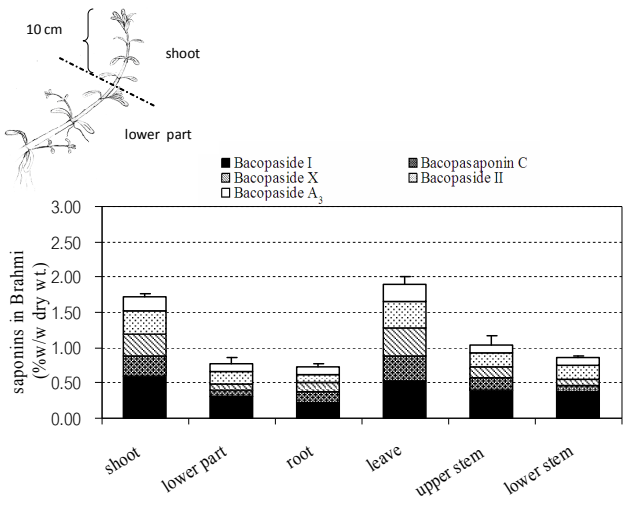


Figure 4. Percentage of saponin glycosides from different parts of Brahmi collected from month 4 of summer season

practical than collecting only the leaves, which may be more time consuming.

3.5 Saponin contents in different growth stages of Brahmi

Among three tested seasons, the highest amount of total saponins in Brahmi was reached at different growth stages (Table 3). In shoot, the highest amount of total saponins in rainy season, summer, and winter was found in Brahmi at the ages of 1 month, 2 months, and 3 months, respectively. Apart from that, the differences of the total saponin content in each month at the same season were mostly not significant. In lower parts, total saponin contents in different ages of Brahmi in the same season were not significantly different. In roots, total saponin contents during the rainy season at all ages of Brahmi were not different while in winter and summer, the highest content was obtained in 4-month-old Brahmi and the lowest content was obtained in 1-month-old Brahmi.

3.6 Saponin contents in Brahmi in different seasons

We found that among three seasons, the highest total saponin contents in shoots and roots of Brahmi were in the rainy season ($p<0.05$). Especially, shoots from the rainy season gave the highest total saponin contents. It might be that high humidity and high temperature in the rainy season are suitable for the production of saponins in Brahmi. This observation was similar to that previously studied by Ganjewala *et al.* (2001) and Mathur *et al.* (2002). They reported that the highest bacoside A content was in Brahmi grown in the monsoon season (June to September). In our study, we also observed that the lower parts had higher saponin content in winter than in summer and the rainy season.

4. Conclusion

The results showed that the highest saponin content was obtained from Brahmi cultivated in rainy season. Shoots have the highest saponin content compared with lower parts and roots. In the same season, saponin contents were not dramatically different among Brahmi in different growth stages (1-4 months). In conclusion, our studies suggest that to obtain a high saponin content of Brahmi for household and industrial use, the shoots should be collected and the harvesting time should start after 1 month of cultivation.

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