Comparison of Various Extraction Methods of *Bacopa monnieri*

Watoo Phrompittayarat[a,b], Waraporn Putalun[c], Hiroyuki Tanaka[d], Kanchalee Jetiyanon[e]
Sakchai Wittaya-areekul[f] and Kornkanok Ingkaninan[a,b,*]

[a]Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
[b]Cosmetics and Natural Products Research Center, Naresuan University, Phitsanulok 65000, Thailand.
[c]Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.
[d]Department of Medicinal Plant Breeding, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan.
[e]Department of Agricultural Sciences, Faculty of Agriculture Natural Resources and Development, Naresuan University, Phitsanulok 65000, Thailand.
[f]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand.
*Corresponding author. E-mail address: k_ingkaninan@yahoo.com (K. Ingkaninan)
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Abstract

Bacopa monnieri (L.) Wettst. (Brahmi) has been used in Ayurvedic medicine as nervine tonic for promoting mental health and improving memory brain function. Tetracyclic triterpenoid saponins were reported to be the responsible molecules for cognition enhancement. In order to optimize the extraction methods of Brahmi for commercial purpose, nine different methods were compared. Five major saponins *i.e.* bacoside A_3, bacopaside II, bacopasaponin C isomer, bacopasaponin C and bacopaside I in the extracts were determined using HPLC technique and calculated as total amounts of saponins. Among nine methods, the highest yield (27.89±0.48 %) was found in the extract obtained from maceration of the plant material in methanol at room temperature for 3 days. However, the extract containing the highest amount of total saponins (19.28± 0.12 %) was obtained from percolation with ethanol after the plant material was soaked in water.

Keywords: *Bacopa monnieri*; Triterpenoid saponins; Extraction method; High performance liquid chromatography

Introduction

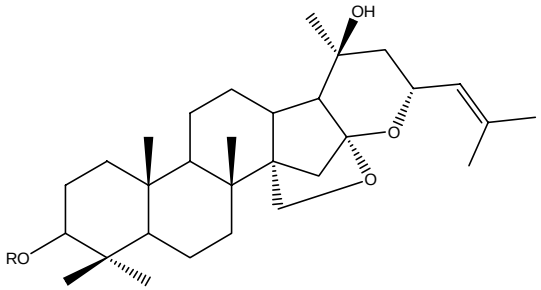
Bacopa monnieri (L.) Wettst. (Brahmi) is a medicinal plant used for enhancing memory and improving function of the brain in Ayurvedic medicines (Sivarajan & Balachandran, 1994). Recently, many studies have revealed its pharmacological roles as cognition-enhancer (Das et al., 2002; Singh & Dhawan, 1997; Stough et al., 2001; Sumathi et al., 2002), antidepressant (Sairam et al., 2002), antioxidant (Pawar et al., 2001; Russo et al., 2003), antiulcerogenic agent (Sairam et al., 2001), and calcium antagonist (Dar & Channa, 1999). Dammarane-type triterpenoid saponins, classified as pseudojujubogenin and jujubogenin glycosides were reported to be responsible for the cognition enhancing activity of this plant (Das et al., 2002; Singh & Dhawan, 1997; Stough et al., 2001; Sumathi et al., 2002). Various extraction methods were used. However, no one has compared the quality for quantitative yields of these extraction methods. In this study, nine extraction methods of Brahmi were compared. The amount of five major saponins *i.e.* bacoside A_3, bacopaside II, bacopasaponin C isomer, bacopasaponin C and bacopaside I (Figure 1) in each extract were determined as total saponins using HPLC method. The method with the highest percentage of total saponins could be practically used for commercial application.

Materials and Methods

Chemicals

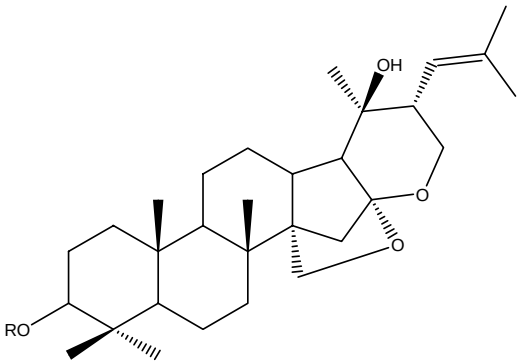
Acetonitrile and methanol, both HPLC grade, were purchased from Labscan Asia Co. Ltd. (Thailand). Orthophosphoric acid (AR grade) was from BDH (England). The reference standards

i.e. bacoside A_3, bacopaside II, bacopasaponin C isomer, and bacopasaponin C were obtained as a gift from Professor I. Khan, the National Center for Natural Products Research, University of Mississippi, MS, USA. Bacopaside I was purchased from Chromadex (CA, USA).



Jujubogenins

	R
Bacoside A_3 (**1**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopasaponin C isomer (**3**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl



Pseudojujubogenins

	R
Bacopaside II (**2**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl
Bacopasaponin C (**4**)	α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl
Bacopaside I (**5**)	α-L-arabinofuranosyl(1→2)-[6-*O*-sulfonyl-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranosyl

Figure 1. Structures of saponin glycosides from Brahmi.

Plant materials

Brahmi was collected from Phetburi, Thailand in September, 2004. It was identified by Assoc. Prof. Dr. Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen was kept at the PBM Herbarium, Mahidol University, Thailand. The aerial part was collected, cut into small pieces and dried in a hot-air oven at 50 ^{o}C for 12 hrs. The dried plant material was coarsely powdered.

Extraction method

The coarse powder of Brahmi (30 g) was extracted using nine different methods.

Method 1: The dried plant material was macerated in 180 ml of 95% ethanol for 3 days at room temperature and the resulting extract was filtered through filter paper (Whatman no.1). The residue from the filtration was extracted again twice using the same procedure. The filtrates obtained were combined and then evaporated to dryness under reduced pressure.

Method 2: The same procedure was conducted as in the Method 1 except that ethanol was

replaced by methanol as an extraction solvent.

Method 3: The dried plant material was decocted in 300-ml of water at 60 ^{o}C for 3 hrs. Then the extract was filtrated. The filtrate was dried using freeze-dried technique.

Method 4: The dried plant material was soaked in 300-ml water for 24 hrs prior to maceration with 95% ethanol (180 ml). The rest of the extraction procedure was performed as described in the Method 1.

Method 5: The dried plant material was defatted with hexane (180 ml) for 24 hrs prior to maceration with 95% ethanol (180 ml). The rest of the extraction procedure was performed as described in the Method 1.

Method 6: The dried plant material was extracted with 95% ethanol (400-ml) using a Soxhlet apparatus set at 50 ^{o}C for 3 hrs. The extract was filtrated and dried under reduced pressure.

Method 7: The dried plant material was extracted using the same procedure as described in Method 4 except that after the plant material had been soaked with water for 24 hrs; the water was squeezed out of the plant material prior to maceration with 95% ethanol.

Method 8: The dried plant material was soaked in 300-ml water. After 24 hrs, the water was squeezed out of the plant material. The plant material was extracted with 400-ml 95% ethanol using a Soxhlet apparatus set at 50 ^{o}C for 4 hrs. The extract was filtrated and dried under reduced pressure.

Method 9: The dried plant material was soaked in 300-ml water. After 24 hrs, the water was squeezed out of the plant material. The plant material was percolated with circulating 95% ethanol (200 ml) for three rounds. The residue was extracted again twice using the same procedure. The combined extract was filtrated and dried under reduced pressure.

%yield of the extract was calculated using the following equation.

$$\%\text{yield} = W_{\text{crude extract}}/W_{\text{dried plant}} \times 100$$

$W_{\text{crude extract}}$ = weight of crude extract

$W_{\text{dried plant}}$ = weight of dried plant material

Liebermann-Burchard test

The Liebermann-Burchard test for detection of triterpenes was performed as described by Houghton & Raman (1998). The sample extracts were redissolved in acetic anhydride and treated with a few drops of concentrated sulphuric acid. The change of color was observed.

HPLC assay

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, 5 µm 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 the wavelength of 205 nm. They were initially assigned by comparing retention times with the standards and confirmed with characteristic spectra obtained from the PDA. The purity of the peak was also confirmed by the PDA.

Calibration curves of five saponin glycosides, bacoside A_3, bacopaside II, bacopasaponin C isomer, bacopasaponin C, bacopaside I, were prepared based on peak areas of seven concentrations. Linearity was obtained in the concentration range of 500-7.8 µg/ml. The equations and the good to fitness (r^2) are shown in Table 1. All data were processed using Class-VP software (Shimadzu, Japan).

Table 1. Equations and good of fitness (r^2) obtained from calibration curves of the analyses of 5 saponin glycosides using HPLC

Component	Equation	r^2
Bacoside A_3 (**1**)	$y = 1.6886(10^{-4})x+0.1058$	0.9998
Bacopaside II (**2**)	$y = 1.3499(10^{-4})x-0.3357$	0.9998
Bacopasaponin C isomer (**3**)	$y = 1.7869(10^{-4})x-1.6754$	0.9999
Bacopasaponin C (**4**)	$y = 1.3479(10^{-4})x-1.8996$	0.9999
Bacopaside I (**5**)	$y = 1.7449(10^{-4})x-1.5552$	0.9999

Results and Discussion

Dried aerial part of Brahmi was used as raw materials for the extraction as described in previous studies (Bhattacharya et al., 2001; Das et al., 2002; Singh & Dhawan, 1997). The advantage of using dried plant materials instead of fresh plant materials for herbal products is that the dried plant materials is easier to store and also has longer shelf life.

To optimize the extraction method for Brahmi, nine different methods were performed. The % yields of the extract and the amounts of total saponins calculated from the five major saponins, including bacoside A_3, bacopaside II, bacopasaponin C isomer, bacopasaponin C and bacopaside I from each method were compared (Table 2). The HPLC method was used for determination of the five saponins present in the extract. A representative HPLC chromatogram of Brahmi extract obtained is shown in Figure 2. In order to keep the extraction process as simple as possible, maceration method was chosen in most experiments. We mainly used ethanol as an extraction solvent as it has been shown in many studies that the biological activity of Brahmi was found in ethanol extracts of the plant (Bhattacharya et al., 2001; Das et al., 2002; Singh & Dhawan, 1997). Also, ethanol is relatively safe and cheap for herbal medicine preparation compared to the toxic methanol or chloroform.

Table 2. Percent yields of crude extracts and total saponins in the extracts obtained from 9 methods

Method	% Yield of extract	% Total saponins in the extract (mean±SD)					
		1	2	3	4	5	Total
1	17.14±0.74	1.17±0.06	1.95±0.10	0.80±0.11	0.34±0.14	1.62±0.50	5.89±0.49
2	27.89±0.48	1.23±0.03	1.97±0.04	0.98±0.02	0.61±0.06	1.82±0.04	6.60±0.12
3	20.22±0.95	0.11±0.00	0.14±0.01	0.07±0.01	0.01±0.00	0.23±0.02	0.56±0.03
4	26.08±1.25	1.14±0.08	2.08±0.13	1.26±0.17	0.67±0.11	2.86±0.36	8.00±0.67
5	16.63±0.87	1.22±0.07	2.14±0.11	0.88±0.11	0.51±0.10	0.90±0.04	5.64±0.43
6	12.26±0.04	1.26±0.20	2.17±0.32	0.96±0.15	0.58±0.10	1.95±0.16	6.91±0.93
7	12.98±0.91	2.50±0.14	4.36±0.25	3.34±0.09	2.16±0.10	6.05±0.28	18.41±0.80
8	11.38±0.78	2.45±0.18	4.13±0.15	2.37±0.04	1.04±0.11	4.54±0.07	14.53±0.46
9	10.09±0.07	3.04±0.02	4.95±0.03	3.32±0.20	1.96±0.05	6.00±0.11	19.28±0.12

Surprisingly, the results showed that methanol gave almost a double yield of the crude extract (27.89±0.48 %) when compared with ethanol (17.14±0.74 %). However, the amounts of total saponins in both extracts were not significantly different (6.60±0.12% and 5.89±0.49%).

Method 3 showed that saponin glycosides in Brahmi could only slightly be extracted by water (0.56±0.03%). The extract also showed negative results with the Liebermann-Burchard test indicating that the triterpenoid saponins in Brahmi could not be extracted with water. Some pre-maceration processes were then tested in order to increase the yields of either the crude extract or the total saponins extracted with ethanol. In Method 4, the plant material was soaked in water for 24 hrs to make the plant soft, swollen and easy for the solvent to penetrate. The wet material was then macerated with ethanol. The results showed that the yield of the crude extract increased to

26.08±1.25 % while the level of the total saponins was higher (8.00±0.67%). In Method 5, the plant material was defatted with hexane prior to maceration with ethanol. Both % yield of the extract and the total saponins (16.63±0.87 and 5.64±0.43, respectively) were not different to that without defatting.

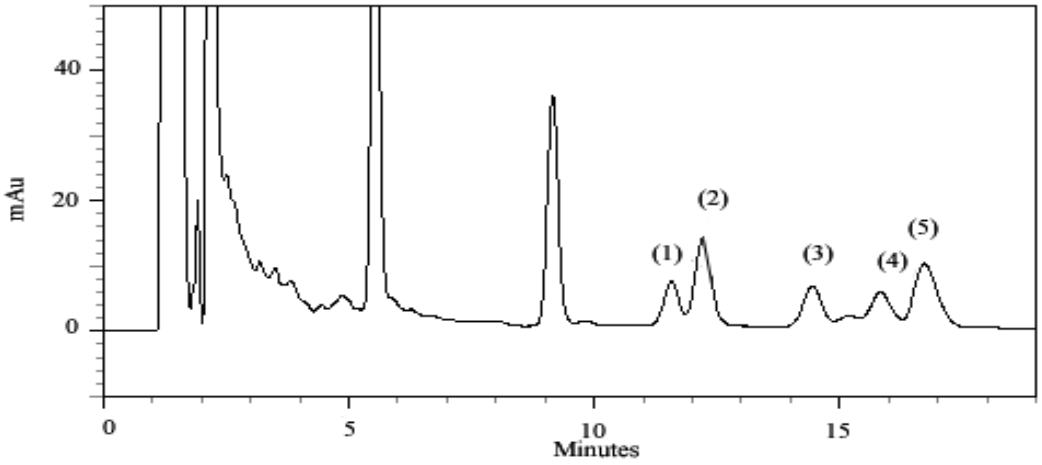


Figure 2. HPLC chromatogram of Brahmi extract obtained from Method 9.The peaks were identified as follows: 1, bacoside A_3; 2, bacopaside II; 3, bacopasaponin C isomer; 4, bacopasaponin C; 5, bacopaside I.

The continuous extraction using high temperature to accelerate extraction process was conducted in Method 6. when the plant material was extracted for 3 hrs in a Soxhlet apparatus, the yield of 12.26±0.04 % was obtained. Again, the amount of total saponins was improved in comparison to Method 1 to 6.91±0.93 %. In Method 7, we followed Method 4 by soaked plant material in water for 24 hrs, but water was squeezed out of the plant material before maceration with ethanol. Although the yield of the extract obtained was not high (12.98±0.91 %), the amount of total saponins obtained was about two time higher than that from the previous methods (18.41±0.80%). While in Method 7, the whole extraction procedure took 9 days, it took only 3 days for the extraction in Method 8 using the Soxhlet apparatus. However, the amount of the total saponins was found to be 14.53±0.46% which was lower than that from Method 7. Therefore, in Method 9, a percolation method was used in stead of a maceration in Method 7. The yield of the extract obtained was not high (10.09±0.07%), but the total amount of saponins was in the same level as that from Method 7 which was the highest among all other methods (19.28±0.12%). Moreover, in comparison to Method 7, the time used for extraction was shorter and high temperature was not needed. Thus, we conclude that among the nine methods studied, Method 9 was the most practical and efficient extraction method for Brahmi. It could be easily up-scaled for commercial purpose.

The preparation of saponin enriched extract has already been patented (Kahol et al., 2003). However, it needed at least 5 steps for extraction *i.e.* defatting, discoloration, extraction, precipitation and partition. More than three kinds of solvents were used. This method gave 28% of bacoside A and 1.95% of yield based on the dry weight. Although the percentage of bacoside A in the extract described in this method was higher than total saponins from Method 9, the total yield of the extract was much less. The cost-effectiveness of the extraction method of Brahmi for each purpose should be considered.

Conclusion

Among 9 extraction methods studied, the highest yield of total saponins (19.28±0.12 %) was obtained from Method 9. In this procedure, Brahmi was soaked with water for 24 hrs.Thereafter the water was squeezed out and the pre-wetted plant material was percolated with ethanol. The method has proven to be practical for commercial proposes.

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