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An enzyme-linked immunosorbant assay using polyclonal antibodies against bacopaside I

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Abstract

Bacopa monnieri (L.) Wettst. (Brahmi) is a medicinal plant used as a memory enhancer in Ayurvedic medicines. Its active components are triterpenoid glycosides namely pseudojujubogenin and jujubogenin glycosides. In order to analyze these saponin glycosides, an enzyme-linked immunosorbant assay (ELISA) was developed using polyclonal antibodies against bacopaside I, one of the pseudojujubogenin glycosides found in the plant. Bacopaside I was conjugated with a bovine albumin serum (BSA) to prepare an immunogen. The bacopaside I–BSA conjugate was immunized to a rabbit for producing polyclonal antibodies (PAbs). The results showed that the antibodies were raised specifically against pseudojujubogenin glycosides. An ELISA using anti-bacopaside I PAbs was performed in the range of 1.95–62.5 ng mL^{-1} of bacopaside I and the limit of detection was 0.1 ng mL^{-1}. The method was validated and the applicability of the ELISA for analyzing saponin glycosides from Brahmi was demonstrated.

Keywords: Bacopaside I; *Bacopa monnieri*; Enzyme-linked immunosorbant assay (ELISA); Pseudojujubogenin glycosides; Polyclonal antibodies

1. Introduction

Bacopa monnieri (L.) Wettst. (Brahmi) is a member of Scrophulariaceae family which has been used in Ayurvedic medicine to improve memory and intellect [1]. Recently, there have been several studies on biological effects of this plant, especially for a therapeutic potential in treatment or prevention of neurological diseases and improvement of cognitive processes [1–7]. Triterpenoid saponins, classified as pseudojujubogenin and jujubogenin glycosides (Fig. 1) were reported to be responsible for the cognitive enhancing activity in this plant [1–7]. The methods for determination of these compounds in Brahmi were high performance thin-layer chromatography (HPTLC) [8], ultraviolet–vis spectroscopy (UV) [9] and more recently, high performance liquid chromatography (HPLC) [10–12]. In this study, we improved the sensitivity and speed of the analysis of the saponins in Brahmi by using immunoassay technique. The polyclonal antibodies (PAbs) against the major pseudojujubogenin glycosides, bacopaside I [13] were produced and used in enzyme-linked immunosorbant assay (ELISA) for determination of pseudojujobogenin glycosides in Brahmi. This method has not been reported elsewhere before. The method development, validation and application are demonstrated in this paper.

2. Experimental

2.1. Chemicals

Bacopaside I was purchased from ChromaDex Inc., CA, USA. Bovine serum albumin (BSA) and human serum albumin (HSA) were provided from Fluka Biochemika Stein-

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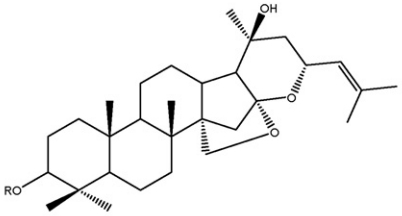
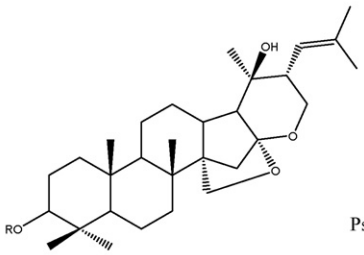


Jujubogenins

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



Pseudojujubogenins

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

Fig. 1. Structures of saponin glycosides from Brahmi.

heim, Switzerland. Peroxidase-labeled IgG was obtained from MP Biomedicals, Illkirch, France. Freund's complete and incomplete adjuvants were purchased from Sigma Steinheim, Germany. 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid was purchased from Wako, Osaka, Japan. bacoside A_3, bacopaside II, bacopaside IV-V, bacopasaponin C isomer and bacopasaponin C were gifts from Professor I. Khan, the National Center for Natural Products Research, MS, USA. The other standard references for cross-reactivity studies were obtained from Department of Medicinal Plant Breeding, Kyushu University, Fukuoka, Japan.

2.2. Plant materials

The different parts (top, stem and leaf) of Brahmi were collected from Phitsanulok, Thailand in different seasons (rainy season, winter, and summer). The plants were identified by Associate Professor Dr. Wongsatit Choukul, Faculty of Pharmacy, Mahidol University, Thailand. The voucher specimen (Phrompittayarat001) is kept at the PBM Herbarium, Mahidol University, Bangkok, Thailand. The plant material was cut into small pieces and dried at 50 °C for 10 h. Then, it was coarsely powdered.

2.3. Sample preparation

The 0.5 g dried Brahmi powdered was soaked in 5 mL water for 24 h and then the water was squeezed out of the plant material before sonicated with 95% ethanol (6 mL g^{-1} dried plant, 10 min) for three times. The ethanol extract was filtrated and adjusted to 10 mL.

2.4. Synthesis of antigen conjugates

Bacopaside I–BSA conjugate was synthesized by periodate oxidation method [14]. Bacopaside I (4 mg) in 0.5 mL methanol was added dropwise to 0.5 mL of $NaIO_4$ solution (4 mg mL^{-1}) and then stirred at room temperature for 1 h. After that, BSA (4 mg) in 1 mL carbonate buffer (50 mM, pH 9.6) was added and stirred for 5 h. The reaction mixture was dialyzed in water for five times and then lyophilized. Finally, the bacopaside I–BSA conjugate (6.8 mg) was obtained. The same method was used to synthesize bacopaside I–HSA conjugate.

2.5. Determination of hapten number in bacopaside I–protein conjugates by matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

The hapten numbers of the bacopaside I–BSA and bacopaside I–HSA conjugates were determined by MALDI-TOF-MS as previously described [15–17] (Fig. 2). A small amount (1–10 pmol) of an antigen conjugate was mixed with a 10^3-fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid. The mixture was subjected to the MALDI-TOF-MS (Voyager Elite, Perspective Biosystems,

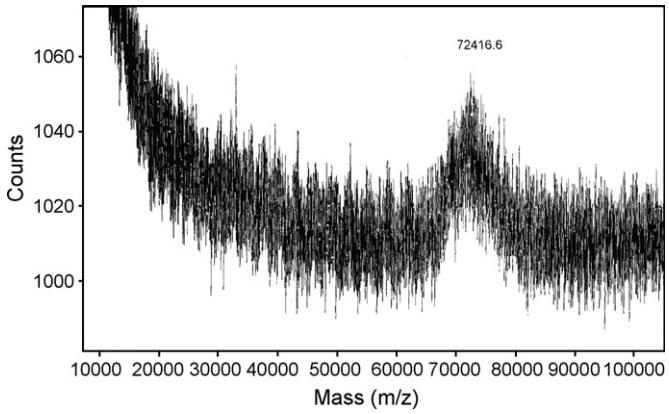


Fig. 2. MALDI-TOF mass spectrum of bacopaside I–BSA conjugate.

Framingham, MA, USA) and irradiated with a nitrogen laser (256 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20 kV potential into a 2.0-m evaluated tube. The data were analyzed using GRAMS/386 software (Galactic Industries, Salem, NH, USA).

2.6. Immunization

A 2 kg female New Zealand white rabbit (National Animal Centre, Mahidol University, Nakhon Pathom, Thailand) was injected subcutaneously with a 1:1 mixture of Freund's complete adjuvant and 100 μg mL^{-1} of bacopaside I–BSA conjugate. After 4 weeks, the rabbit received intramuscular booster injection of the 1:1 emulsion of bacopaside I–BSA conjugate (50 μg) and Freund's incomplete adjuvant. The final immunization was conducted by intravenous injection of 50 μg mL^{-1} of the conjugate. After 3 days, the rabbit was checked for the presence of antibodies using direct ELISA and then the rabbit was bled. The blood was centrifuged at 1500 rpm for 20 min to remove remaining blood cells. Finally, the serum was stored in −80 °C before used.

2.7. Purification of antibody

The PAbs from the serum was purified using a Protein G column [16] (0.46 cm × 11 cm, Amersham Pharmacia Biotech, Uppsala, Sweden). The serum was loaded to the column and then the column was washed with 20 mM phosphate buffer (pH 7.4). After that, bound IgG was eluted with 100 mM citrate buffer (pH 2.7). The eluted IgG was neutralized with 1 M Tris–HCl buffer (pH 9.0) and dialyzed five times with water. Finally, the purified antibodies were lyophilized and kept at −80 °C.

2.8. Enzyme-linked immunosorbant assay (ELISA)

The reactivity of the PAbs to bacopaside I was determined by direct ELISA. The 96 wells plate (Maxisorb Nunc, Roskilde, Denmark) was coated with 100 μL of 1 μg mL^{-1} bacopaside I–HSA conjugate in carbonate buffer (pH 9.6) and incubated at 37 °C for 1 h. The plate was washed three times with 0.05% Tween 20 phosphate buffer saline (TPBS). After that, the plate was treated with 300 μL of 0.2% gelatin in phosphate buffer saline (GPBS) for 1 h to reduce non-specific binding. Then, the plate was washed three times with TPBS and reacted with 100 μL of the testing antibodies for 1 h. Next, the plate was washed again with TPBS for three times and then the antibody was combined with 100 μL of a solution of a 1:1000 dilution of peroxidase-conjugate goat IgG fraction to rabbit IgG Fc for 1 h. After washing the plate three times with TPBS, 100 μL of substrate solution consisted of 100 mM citrate buffer (pH 4.0) containing 0.003% H_2O_2 and 0.3 mg mL^{-1} of ABTS was added and incubated for 15 min. The plate was measured by using a microplate reader (Packard instrument company, Meriden, CT, USA) at 405 nm.

2.9. Competitive ELISA

The 96 wells plate was coated with 100 μL of bacopaside I–HSA conjugate (1 μg mL^{-1}) in 50 mM carbonate buffer (pH 9.6) and kept at 37 °C for 1 h. After three times washing with TPBS, the plate was treated with 300 μL of GPBS for 1 h to reduce non-specific adsorption. 50 μL of various concentrations of bacopaside I or sample in 20% MeOH were incubated with 50 μL of the PAbs solution for 1 h. The plate was washed three times with TPBS and then the antibody was combined with 100 μL of a solution of a 1:1000 diluted solution of peroxidase-conjugated goat IgG fraction to rabbit IgG Fc and kept for 1 h. After washing the plate three times with TPBS, 100 μL of the aforementioned substrate solution was added and incubated for 15 min. The plate was measured at 405 nm by using a microplate reader.

2.10. Method validation

The immunoassay was validated for sensitivity, precision, specificity and accuracy. The absorbance of nine concentration levels (0.98, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 250 and 1000 ng mL^{-1}) of bacopaside I were measured using the ELISA method developed (Fig. 4). The linearity was obtained at the range of 1.95–62.5 ng mL^{-1}.

The sensitivity expressed as a limit of detection (LOD) was determined from serial dilution of bacopaside I solution. The minimum concentration of bacopaside I that significantly showed higher absorbance than blank was referred as an LOD.

The specificity of the assay was evaluated by cross reactivity of the PAbs with various compounds calculated using the method of Weiler and Zenk [17],

$$\%\ \text{cross reactivity} = \left(\frac{\text{concentration of bacopaside I yeilding, } A/A_0 = 50\%}{\text{concentration of compound under investigation yeilding, } A/A_0 = 50\%} \right) \times 100$$

where A is the absorbance in the presence of the test compound and A_0 is the absorbance in the absence of the test compound.

Reproducibility and precision of the assay were determined by a competitive ELISA. The variations between replicates from well to well (intra-assay) and plate to plate (inter-assay) were determined.

Accuracy was evaluated by analyzing the mixture of various Brahmi extract and the known concentration of bacopaside I.

2.11. HPLC Analysis

The HPLC analysis 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 (150 mm × 4.6 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^{-1}.

3. Results and discussion

3.1. Direct determination of hapten-carrier protein conjugate by MALDI-TOF-MS

Fig. 2 shows the MALDI-TOF-mass spectrum of bacopaside I–BSA conjugate. A broad peak of the conjugate of bacopaside I and BSA appeared approximately at *m/z* 72,417. According to the fact that molecular weights of BSA and bacopaside I were 66,433 and 979, respectively, at least seven molecules of bacopaside I conjugated with the BSA. Generally, 6–20 molecules of hapten conjugated with carrier protein were adequate for immunization. Similar to this experiment, a molecular weight of the bacopaside I–HSA conjugate was detected as 75,594. The hapten number of bacopaside I–HSA conjugate was calculated as 10.

3.2. Production and characteristic of the polyclonal antibodies against bacopaside I

The bacopaside I–HSA conjugate was used as a coating antigen in the ELISA experiment. The sera obtained from the third immunization showed the presence of antibodies against bacopaside I. The rabbit was then bled and the serum was purified using a Protein G column. The total IgG was obtained and used for further analysis. The reactivity of the various concentrations of PAbs against bacopaside I was tested by using direct ELISA (Fig. 3). The PAbs concentration (1.0 μg mL^{-1}) which showed absorbance at 1.0 was chosen for the competitive ELISA.

3.3. Method validation

The competitive ELISA was validated for sensitivity, specificity, limit of detection, precision and accuracy. The competitive binding was obtained from the antibodies bound to either free bacopaside I or bacopaside I–HSA conjugate adsorbed on a polystyrene microtiter plate. The more free bacopaside I presented, the less antibodies left for bacopaside I–HSA conjugate.

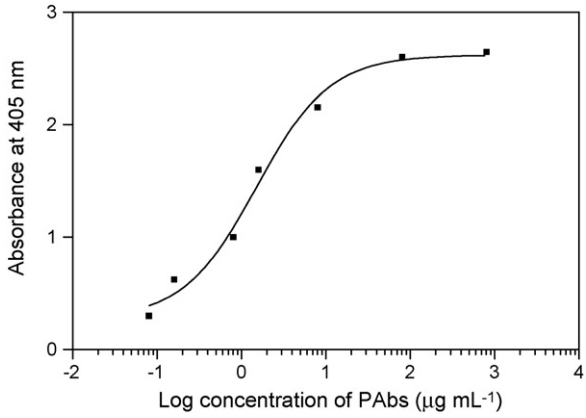


Fig. 3. Reactivities of polyclonal antibodies against bacopaside I.

After washing the plate, the amount of the antibodies bound to bacopaside I–HSA conjugate was measured using secondary antibody (peroxidase-conjugated IgG). The range of the bacopaside I concentration could be detected from 62.5 to 1.95 ng mL^{-1} (Fig. 4). The limit of detection of this assay measuring from the minimum concentration that showed absorbance value more than blank was 0.1 ng mL^{-1}.

The specificity of the assay was determined by the cross reactivity of antibodies with other compounds using the competitive ELISA. The cross-reactivity value was calculated by using the equation developed by Weiler and Zenk [17]. Table 1 shows cross-reactivities of anti-bacopaside I PAbs against various compounds. It is clear that anti-bacopaside I PAbs had high cross-reactivities against pseudojujubogenin glycosides found in Brahmi. It had the same affinity to bacopaside II and bacopasaponin C as to bacopaside I. Moreover, it showed 58% cross reactivity with the pseudojujubogenin glycosides which had one less sugar unit, bacopaside V. These results indicate that the aglycone part is essential for reactivity of the PAbs. This is in agreement with the results that all jujubogenin glycosides gave very low cross-reactivity (0.7–1.8%). Other test compounds did not significantly cross-react with anti-bacopaside I PAbs (Table 1). It is concluded that this method is specific and thus could be applied for the determination of total pseudojujubogenin glycosides.

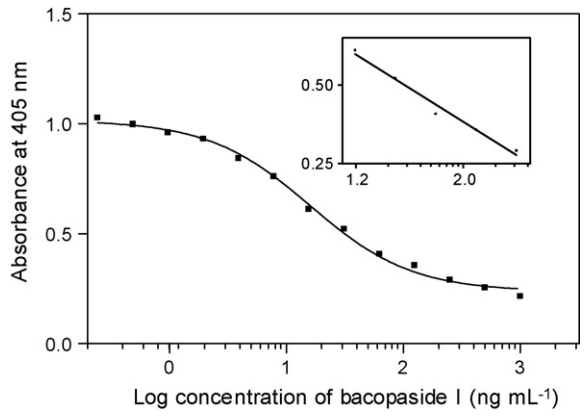


Fig. 4. Dose–response curve of bacopaside I detected by ELISA at 405 nm.

Table 1
Cross reactivities (CRs) of the PAbs against some naturally occurring compounds

Compound	Classification	CRs (%)
Bacopaside I	Triterpenoid saponins (pseudojujubogenin glycosides)	100.00
Bacopaside II	Triterpenoid saponins (pseudojujubogenin glycosides)	99.84 ± 0.31
Bacopasaponin C	Triterpenoid saponins (pseudojujubogenin glycosides)	102.68 ± 17.16
Bacopaside V	Triterpenoid saponins (pseudojujubogenin glycosides)	58.38 ± 5.62
Bacoside A_3	Triterpenoid saponins (jujubogenin glycosides)	0.77 ± 0.29
Bacopasaponin C isomer	Triterpenoid saponins (jujubogenin glycosides)	1.75 ± 1.12
Bacopaside IV	Triterpenoid saponins (jujubogenin glycosides)	0.83 ± 0.66
Ginsenoside Rg1	Triterpenoid saponins	<0.01
Ginsenoside Rb1	Triterpenoid saponins	<0.01
Glycyrrhizin	Triterpenoid saponins	<0.01
Saikosaponina	Triterpenoid saponins	<0.01
Quilaja saponin	Triterpenoid saponins	<0.01
α-Amyrin	Triterpenoids	<0.01
Diosgenin	Steroids	<0.01
Prednisolone	Steroids	<0.01
Digitonin	Cardiac glycosides	<0.01
Caffeic acid	Purine alkaloids	<0.01
Solanine	Steroidal alkaloids	<0.01
Swertiamarin	Iridoid glycosides	<0.01
Geniposide	Iridoid glycosides	<0.01
Sennoside A	Anthraquinones	<0.01
Aesculetin	Coumarins	<0.01
Griseofulvin	Coumarins	<0.01
Hesperidin	Flavonoids	<0.01
Camphor	Ketones	<0.01
Cinnamic acid	Phenyl propanes	<0.01

Intra-assay and inter-assay precisions were studied. Intra-assay precision was evaluated by the variation of the measurement of bacopaside I from well to well ($n = 5$) and inter-assay precision was obtained from the variation from different plates ($n = 3$) expressed as R.S.D. (%). Intra-assay R.S.D. was less than 2% while inter-assay R.S.D. was in the range of 1–7% (Table 2). The factors that affect the results might be the variations of bacopaside I–HSA conjugate, reagents, temperature and incubation time. Therefore, it is necessary to calibrate the analysis with serial dilutions of the standard bacopaside I in every experiment.

The accuracy of this competitive ELISA was evaluated by the analyses of bacopaside I in the Brahmi extract to which the known amount of bacopaside I was added. The percentages of recovery in the range of 90–96% with low variations (2.46–3.15% R.S.D.) were obtained (Table 3). This result suggested that the competitive ELISA using PAbs against bacopaside I could be used for accurate determination of total pseudojujubogenin glycosides from Brahmi.

3.4. Correlation of pseudojujubogenin glycosides contents in Brahmi samples measured by ELISA and HPLC

Triterpenoid saponins, classified as pseudojujubogenin and jujubogenin glycosides, were reported to be responsible for the cognitive enhancing activity of Brahmi [1–7]. The quantitative analysis of these components in Brahmi is a necessary step in a quality control procedure. Table 4 shows the results from the quantitative analysis of total pseudojujubogenin glycosides in different parts of Brahmi harvested in different seasons using HPLC and ELISA methods. The results from the two methods were compared. Both showed that the leaf of Brahmi gave the highest yield of total pseudojujubogenin glycosides comparing to top and stem. The satisfactory correlation expressed as a coefficient of determination (r^2) of 0.992 was obtained (Fig. 5). It is noted that the competitive ELISA was at least 2500 times more sensitive than HPLC assay calculating from the LODs of ELISA (0.1 ng mL^{-1}) and HPLC assays (0.31 μg mL^{-1}). However, the advantage of HPLC method is that individual saponin glycosides

Table 2
Intra- and inter-assay precisions of the bacopaside I analysis by ELISA using PAbs against bacopaside I

Concentration level (ng mL^{-1})	Intra-assay R.S.D. (%) ($n = 5$)	Inter-assay R.S.D. (%) ($n = 3$)
7.81	0.89	7.05
15.62	1.19	2.79
31.25	1.50	2.79
62.50	1.80	1.15

Table 3
Recoveries of the investigated saponins from Brahmi extract spiked with various concentrations of bacopaside I ($n = 3$)

Concentration level of bacopaside I added (ng mL^{-1})	Recovery (%)	R.S.D. (%)
15.62	96.12	3.15
31.25	94.09	2.46
62.50	90.19	3.06

Table 4
Total psuedojujubogenin glycosides in Brahmi determined using ELISA and HPLC ($n = 3$)

Part of Brahmi	Harvesting season	% total psuedojujubogenin glycosides in Brahmi ± S.D.	
		ELISA	HPLC
Top[a]	Rainy season	1.35 ± 0.29	1.39 ± 0.04
	Winter	1.25 ± 0.49	1.21 ± 0.14
Stem	Rainy season	0.57 ± 0.39	0.54 ± 0.04
	Winter	0.58 ± 0.13	0.64 ± .007
	Summer	0.75 ± 0.26	0.76 ± 0.11
Leaf	Rainy season	2.61 ± 0.33	2.80 ± 0.21
	Summer	1.34 ± 0.16	1.52 ± 0.08

[a] The part containing stem and leaves that is cut at 10 cm from the top of Brahmi.

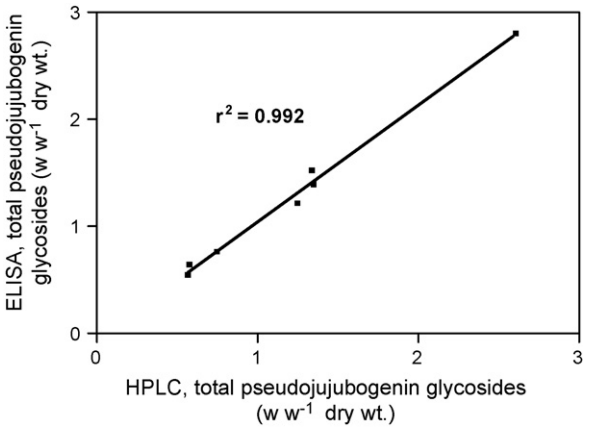


Fig. 5. Correlation between the total psuedojujubogenin glycosides from ELISA and HPLC assays.

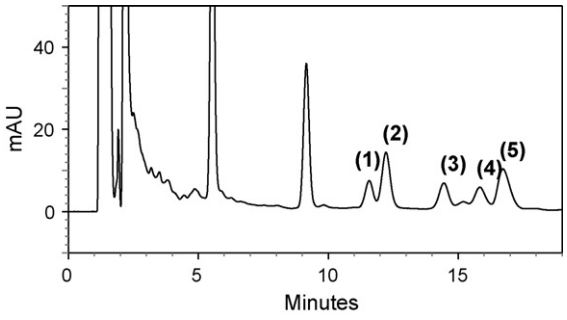


Fig. 6. HPLC chromatogram of Brahmi extract. The peaks are identified as follows: (1) bacoside A_3; (2) bacopaside II; (3) bacopasaponin C isomer; (4) bacopasaponin C; (5) bacopaside I.

in Brahmi could be qualitatively and quantitatively determined separately (Fig. 6).

4. Conclusion

This validated ELISA method using PAb against bacopaside I could be applied for analyses of active ingredients in Brahmi products distributed worldwide. The advantages of the ELISA are its rapidity, sensitivity and simplicity. It is, therefore, suitable method for screening large number of samples which required only small amount of materials and reagents.

Acknowledgements

The grant from National Research Council of Thailand is gratefully acknowledged. The authors thank Naresuan University for a Ph.D. scholarship of the first author. Special thanks for Assoc. Prof. Dr. Wongsatit Chawkul for the identification of Brahmi. Professor I. Khan was acknowledged for giving saponin standard references for this study.

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