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Quantitative Determination of the Major Saponin Mixture Bacoside A in *Bacopa monnieri* by HPLC

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Bacoside A, the putative bioactive component of the Indian medicinal plant *Bacopa monnieri*, was found to be a mixture of saponins with bacoside A_3 (1), bacopaside II (2), jujubogenin isomer of bacopasaponin C (3) and bacopasaponin C (4) as major constituents. An HPLC method together with an optimised extraction procedure was developed for the estimation of 1–4 in *B. monnieri* to enable standardisation of the latter. Concentration ranges of the analytes in samples of *B. monnieri* collected from different regions of India were 0.14–0.85% (w/w) (1), 0.12–0.69% (2), 0.05–0.72% (3) and 0.05–0.44% (4). The importance of using bacoside A, with known concentrations of 1–4, as a reference standard for the routine analysis of *B. monnieri* is highlighted. Two common flavonoids, luteolin and apigenin, were present in all samples of *B. monnieri*.

Keywords: Quantitative HPLC; bacoside A; bacoside A_3; bacopasaponin C; bacopaside II; jujubogenin; pseudojujubogenin; *Bacopa monnieri*.

INTRODUCTION

Bacopa monnieri (Linn.) Pennel (Scrophulariaceae), commonly known as brahmi, is an important drug in Ayurvedic medicine for the improvement of intelligence, memory and revitalisation of sensory organs (Sivarajan and Balachandran, 1994). Alcoholic/hydroalcoholic extracts of the whole plant have been shown to possess nootropic activity (Singh and Dhawan 1997; Das *et al.*, 2002). The major chemical constituents isolated and characterised from the alcoholic extract are dammarane-type triterpenoid saponins with jujubogenin and pseudojujubogenin as the aglycones (Fig. 1), including bacosides A_1–A_3 (Jain and Kulshreshtha, 1993; Rastogi *et al.*, 1994; Rastogi and Kulshreshtha, 1999), bacopasaponins A-G (Garai *et al.*, 1996a, b; Mahato *et al.*, 2000; Hou *et al.*, 2002) and bacopasides I-V (Chakarvarty *et al.*, 2002, 2003).

The nootropic activity of the extract has been attributed to the presence of two saponins, namely bacoside A and bacoside B, of which the former is the more important (Singh *et al.*, 1988; Dhawan and Singh, 1996; Singh and Dhawan, 1997). Several pharmacological, clinical, analytical and agronomical studies concerning *B. monnieri* have recently been published (for a review see Deepak and Amit, 2004) with reports on the content of bacoside A. Many products derived from *B. monnieri* are available on the international market with labels that claim a specific content of bacosides A and B. Surprisingly, however, the chemical identities of these two saponins have still not been established using modern spectroscopic methods. Consequently there is an ambiguity in the quality control of *B. monnieri* based on the content of bacosides. In this context, the need to establish the chemical identities of bacosides A and B has been highlighted (Deepak and Amit, 2004).

Preliminary studies with ^{13}C-NMR indicated that bacoside A was a mixture of saponins in accordance with an earlier report (Kawai and Shibata, 1978), and not a single chemical entity as had been proposed (Chatterji *et al.*, 1965). In the present study, we have isolated the four major individual saponins of bacoside A and describe an HPLC procedure for the quantitative determination of these saponins in *B. monnieri* for standardisation and quality control purposes.

EXPERIMENTAL

Plant material. Fresh plants of *B. monnieri* were collected from Bangalore, India during April 2003 and air dried. The identity of the plant material was confirmed by the National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, and a voucher specimen (BM/PC/0403) is available at the Department of Agronomy, Natural Remedies Pvt Ltd, Bangalore, India. Samples of *B. monnieri* were also collected in the wild from different regions of India (Table 1) and air-dried. Further dried samples of *B. monnieri* were procured from commercial suppliers (Table 1). Specimen samples obtained from all of the sources mentioned are preserved in the author's laboratory.

Isolation of bacoside A. The activity-guided isolation of bacoside A was carried out using the brine shrimp lethality assay to direct the fractionation of a methanolic

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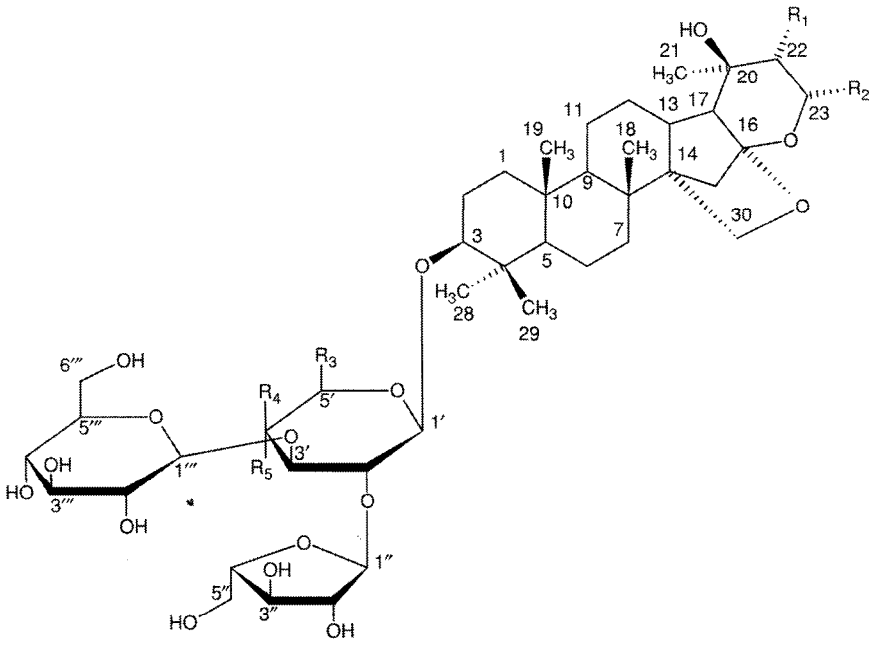

	R_1	R_2	R_3	R_4	R_5	Aglycones
1	H	$CH{=}C(CH_3)_2$	CH_2OH	H	OH	jujubogenin
2	$CH{=}C(CH_3)_2$	H	CH_2OH	H	OH	pseudojujubogenin
3	H	$CH{=}C(CH_3)_2$	H	OH	H	jujubogenin
4	$CH{=}C(CH_3)_2$	H	H	OH	H	pseudojujubogenin

Figure 1. Structures of the major saponins of bacoside A.

extract of *B. monnieri* as previously reported (D'Souza *et al.*, 2002). Coarse powdered whole plants of *B. monnieri* (1 kg) were refluxed for 2 h with methanol (3 × 4 L). The combined methanol extract was concentrated under vacuum and dried at 70°C to yield 140 g of extract. A portion (50 g) of this extract was loaded onto a silica gel column (1 kg; 60–120 mesh) and eluted with chloroform:methanol mixtures containing increasing

Table 1. Concentrations of 1–4 in bacoside A and in various samples of *B. monnieri*

Sample	Quantity of **1–4** as determined by HPLC[a] (%w/w)				Total **1–4** (% w/w)
	1	**2**	**3**	**4**	
Bacoside A					
Isolated	22.1 (3.3)	19.8 (2.8)	29.4 (1.3)	18.0 (0.6)	89.3
Reference standard[b]	22.7 (3.1)	21.4 (2.2)	24.9 (4.5)	18.2 (3.6)	87.2
Field collected samples of *B. monnieri* from					
Bangalore (April 2003)	0.363 (0.7)	0.230 (3.1)	0.291 (1.9)	0.102 (2.2)	0.986
Kolar (March 2003)	0.441 (1.8)	0.427 (1.9)	0.111 (2.1)	0.110 (0.9)	1.089
Chitradurga (March 2003)	0.504 (1.9)	0.594 (1.3)	0.113 (2.4)	0.101 (0.8)	1.312
Cudlur (August 2002)	0.324 (1.1)	0.292 (1.7)	0.126 (2.4)	0.046 (3.2)	0.788
Jammu (August 2003)	0.139 (0.7)	0.183 (1.0)	0.149 (1.5)	0.053 (1.5)	0.524
Lucknow (August 2003)	0.441 (4.7)	0.459 (1.5)	0.142 (0.7)	0.099 (0.2)	1.141
Cheruthurthy (June 2003)	0.162 (4.8)	0.364 (1.3)	0.052 (5.2)	0.047 (4.7)	0.625
Ahmedabad (August 2003)	0.289 (1.1)	0.489 (0.7)	0.081 (1.9)	0.087 (0.6)	0.946
Commercial samples of *B. monnieri* from					
Kolkata 1	0.649 (0.9)	0.688 (1.9)	0.720 (0.6)	0.436 (1.7)	2.493
Kolkata 2	0.847 (4.0)	0.598 (5.3)	0.714 (1.8)	0.382 (1.4)	2.541
Delhi	0.604 (3.0)	0.197 (1.9)	0.473 (1.8)	0.130 (4.4)	1.404
Vijayawada	0.248 (0.4)	0.119 (1.0)	0.235 (0.8)	0.089 (0.7)	0.691
Indore	0.467 (2.3)	0.187 (4.0)	0.388 (1.2)	0.139 (1.8)	1.181
Bangalore sample of *B. monnieri*					
Leaves	0.649 (3.2)	0.378 (3.0)	0.543 (3.2)	0.210 (3.9)	1.780
Stems	0.139 (3.7)	0.115 (4.8)	0.203 (4.9)	0.051 (6.3)	0.508

[a] Mean values ($n = 3$): RSD values given in parentheses.
[b] From Chromadex (lot no. 01-02010-101).

proportions of methanol. The fractions eluted with chloroform:methanol 85:15 were combined and concentrated under vacuum (7 g). This fraction was subjected to flash chromatography on silica gel (100–200 mesh) and the fractions eluted with chloroform:methanol 85:15 were combined, concentrated under vacuum and crystallised from 70% methanol in water to obtain bacoside A (2.5 g).

Chromatography protocols. TLC was performed on layers of silica gel $60F_{254}$ (Merck, Darmstadt, Germany) using toluene:ethyl acetate:methanol:glacial acetic acid (3:4:3:1) (system S-1) or butanol:ethyl acetate:water (5:1:4, v/v/v; upper layer; system S-2). Layers were sprayed with 20% sulphuric acid in methanol and heated at 120°C for 5 min. The HPLC system consisted of a Shimadzu (Kyoto, Japan) model LC-8A pump, an SPD-M10ADVP photodiode array detector (PAD) and an FRC-10A fraction collector. Analytical separations were carried out on a Shim-pack (Shimadza, Kyoto, Japan) PREP-ODS (H) kit column (250 × 4.6 mm i.d.; 5 μm), protected with a Phenomenex (Torrance, CA, USA) C_{18} guard column, using a gradient of acetonitrile (A) and water containing 0.05% (v/v) orthophosphoric acid (B) as the mobile phase; the elution programme was 0–25 min from 30:70 (A:B) to 40:60, and 25–35 min from 40:60 to 60:40; the flow rate was 1.5 mL/min and detection was at 205 nm. Preparative separations were carried out on a Shim-pack PREP-ODS (H) KIT C_{18} semi-preparative column (250 × 20 mm i.d.; 5 μm), containing the same quality of stationary phase as the analytical column, using acetonitrile:water (32.5:67.5) as the mobile phase; the flow rate was 25 mL/min and detection was at 205 nm. Following the injection of aliquots (200 mg) of bacoside, the effluent containing the solute corresponding to the observed peaks was collected by means of a fraction collector (20 mL fractions). The collected fractions were concentrated under vacuum and their purities checked by analytical HPLC. The fractions corresponding to each peak showing >95% purity (by area normalisation) were combined and subjected to ^{13}C-NMR spectroscopy. Four compounds (**1–4**) were isolated. The ^{13}C-NMR spectra (proton decoupled) of the samples were measured using a Bruker (Zurich, Switzerland) AMX400 NMR spectrometer.

Quantitative analysis. In order to determine the detector linearity, different concentrations, in the range 50 μg/mL to 6.4 mg/mL, of each of the isolated components **1–4** of bacoside A were prepared in methanol, and aliquots (20 μL) of each concentration were analysed in triplicate by HPLC ($n = 3$). Calibration curves were constructed by plotting the amount injected (μg) against the corresponding peak areas (mAbs) and the regression equations were calculated.

Optimisation of extraction procedure. Powdered material (9 g) of sample (Kolkata 1; see Table 1) was refluxed five times with 100 mL of methanol each time for 20 min. Each extract was filtered through Filtroll 201 (SRS Scientific, Bangalore, India) filter paper and concentrated to dryness under vacuum. The residues were re-dissolved and diluted in methanol to 25, 10 and 5 mL (using volumetric flasks), and 2.5 and 1.25 mL (using micropipettes: Gilson, Villiers le Bel, France) for the first to the fifth sequential extract, respectively. Each diluted sample was filtered through a nylon filter and subjected to HPLC (20 μL aliquot; $n = 3$).

Analysis of samples. The powdered *B. monnieri* sample (1 g) was extracted with methanol by reflux (5 × 20 mL; 15 min each). The five extracts were combined and concentrated to dryness under vacuum, dissolved in methanol (5 mL) and analysed (20 μL aliquot) by HPLC in triplicate. No further sample purification was found to be necessary.

Recovery studies. By employing a stock solution (10 mg/mL), bacoside A containing known quantities of **1–4** (Table 1) was added to 1 g samples of *B. monnieri* (Kolkata 1). In each case the spiked sample was subjected to the extraction procedure as described above and then analysed by HPLC (20 μl) in triplicate.

RESULTS AND DISCUSSION

Bacoside A was isolated from the methanol extract of *B. monnieri* as a major component in a yield of 0.7% w/w from the raw material on dry weight basis. Initially its identity was ascertained by TLC (single spot; R_f 0.44) in solvent system S-1 as described in the Indian Herbal Pharmacopoeia (Anonymous, 2002) and by comparison with the reference standard (Chromadex, Santa Ana, CA, USA).

The ^{13}C-NMR spectrum of bacoside A [δ 16.4, 16.6, 18.3, 18.5, 18.9, 21.7, 25.6, 26.1, 26.8, 27.2, 27.8, 28.6, 30.1, 36.1, 36.9, 37.1, 37.2, 37.5, 38.8, 39.7, 39.9, 45.5, 46.2, 51.3, 53.0, 53.5, 53.8, 54.0, 56.2, 62.1, 62.4, 62.5, 62.7, 65.9, 66.1, 68.5, 68.6, 70.3, 71.5, 71.6, 71.9, 75.3, 75.5, 77.1, 77.8, 77.9, 78.1, 78.5, 79.1, 83.6, 83.8, 84.9, 88.7, 89.1, 104.7, 105.0, 105.1, 105.7, 109.9, 110.3, 110.6, 124.1, 127.1, 133.0, 134.2 (C_5D_5N, 75 MHz)] exhibited carbon signals, typical for saponins, ascribed to both jujubogenin and pseudo-jujubogenin aglycones (Fig. 1) along with sugar carbon signals that indicated that bacoside A was a mixture of saponins. Analytical HPLC of bacoside A gave four major peaks (Fig. 2) and the compounds corresponding to each peak were subsequently isolated by preparative HPLC. The isolated compounds (**1–4**) were characterized as bacoside A_3 (**1**), bacopaside II (**2**), jujubogenin isomer of bacopasaponin C (**3**) and bacopasaponin C (**4**) by comparison of the ^{13}C-NMR spectral data of the isolated compounds with those in the literature values (Rastogi *et al.*, 1994; Garai *et al.*, 1996a; Li *et al.*, 1999; Chakravarty *et al.*, 2002).

Thus bacoside A, crystallised during purification from methanol extract, was found to be a mixture of saponins with compounds **1–4** as major constituents. Since *B. monnieri* is standardised and assessed mainly on the content of bacoside A, an HPLC procedure (with UV detection) was developed for the quantitative determination of **1–4**. Linearity of detection at 205 nm for all four identified components of bacoside A was observed in the range of 2–64 μg (injected amount) with coefficients of determination (r^2) > 0.999. Relative standard deviation (RSD) values were <4.0 for all concentrations injected ($n = 3$).

Among the solvents tested, bacoside A was found to be highly soluble (>25 mg/mL) in methanol. Optimisation of the extraction procedure was carried out by

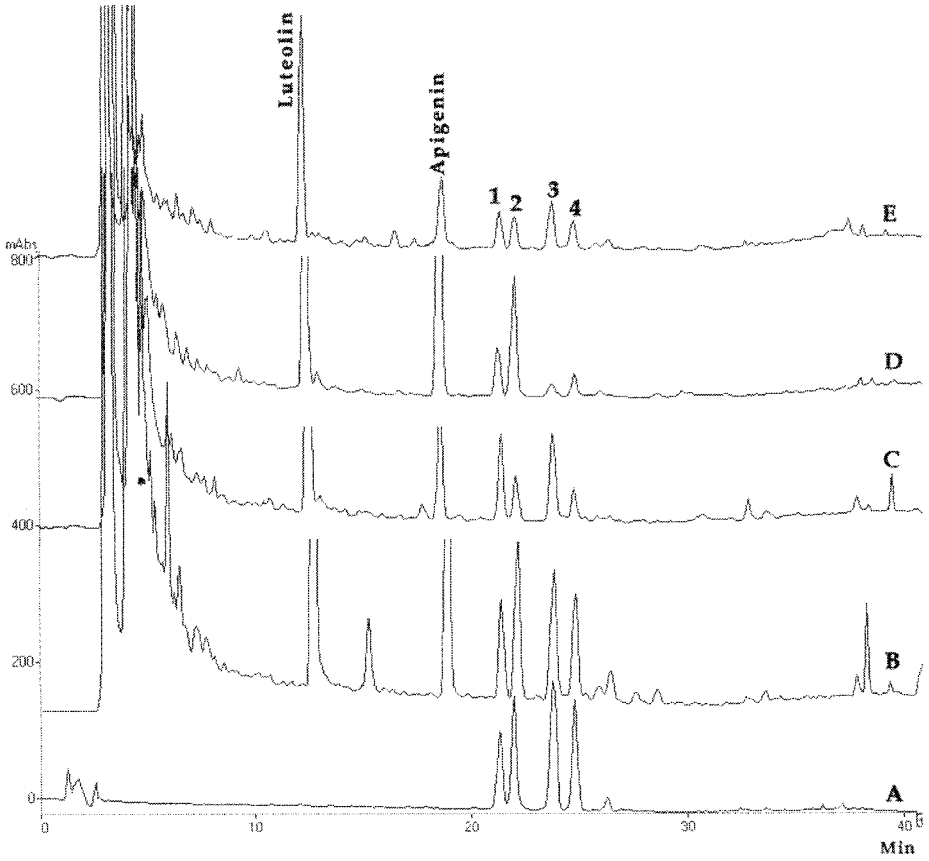

Figure 2. HPLC chromatograms of extracts of samples of *B. monnieri* showing (A) bacoside A (isolated); (B) Kolkata 1 sample; (C) Delhi sample; (D) Ahemadabad Sample; and (E) Vijayawada sample. Key to peak identities: **1**, bacoside A_3; **2**, bacopaside II; **3**, jujubogenin isomer of bacopasaponin C; **4**, bacopasaponin C. (For extraction and chromatographic protocols see Experimental section.)

analysing the contents of **1–4** in each methanol extract of the powdered sample (Table 2). The amount found in a sixth extract was less than 1% of the total quantity of **1–4** recovered from the previous five extractions, and hence the combined sample of five extracts was used for analysis of all the samples used in this study. Recovery studies were performed by adding bacoside A containing known quantities of **1–4** to a sample of *B. monnieri* and subjecting the latter to the extraction procedure and HPLC. Greater than 90% recoveries were observed for **1–4** at two different levels of spiking (Table 3). The contents of **1–4** in the isolated bacoside A were compared with those of a commercial reference standard (Table 1). Thirteen samples of *B. monnieri* collected from different sources (from all major regions of India) were analysed by HPLC. All the samples had similar qualitative HPLC fingerprints in the bacoside A region (Fig. 2) wherein the separation of **1–4** was not affected by any interfering peaks. However, significant quantitative variations were observed in the concentrations of **1–4**, which were in the ranges of 0.14–0.85%, 0.12–0.69%, 0.05–0.72% and 0.05–0.44% (w/w), respectively. Moreover, the concentrations of **1–4** were greater in the leaves compared with the stems (Table 1). The presence of two common flavonoids, namely, luteolin and apigenin, was confirmed in all samples of *B. monnieri* (Fig. 2) by comparison of retention times and on-line UV spectra of the peaks (Greenham *et al.*, 2003) with those of reference standards.

Table 2. Concentrations of 1–4 in sequential methanol extracts of *B. monnieri* (optimisation of extraction procedure)[a]

Extraction	Quantity of extract obtained[b] (mg)	Quantity of **1–4** as determined by HPLC[c] (mg)				Total **1–4** (mg)	Percentage of total **1–4** obtained in five extractions
		1	**2**	**3**	**4**		
First extract	1099.4	27.1 (2.2)	32.9 (0.6)	30.2 (1.9)	17.4 (3.2)	107.6	51.7%
Second extract	619.2	14.0 (3.8)	15.9 (2.3)	15.6 (0.9)	8.9 (2.4)	54.4	26.2%
Third extract	297.6	7.4 (1.1)	7.2 (4.0)	8.0 (5.5)	4.1 (2.5)	26.7	12.8%
Fourth extract	151.8	3.9 (5.9)	3.6 (3.4)	4.2 (4.6)	1.9 (0.9)	13.6	6.5%
Fifth extract	40.7	1.7 (5.5)	1.6 (3.9)	1.7 (2.8)	0.8 (3.8)	5.8	2.8%
Total		54.1	61.2	59.7	33.1	208.1	

[a] See Experimental section for details.
[b] From 9 g of *B. monnieri* (Kolkata 1 sample).
[c] Mean values ($n = 3$); RSD values given in parentheses.

Table 3. Recoveries of 1–4 from samples of *B. monnieri* spiked with bacoside A

Sample	1		2		3		4	
	Quantity determined by HPLC[a] (mg)	Recovery[b] (%)	Quantity determined by HPLC[a] (mg)	Recovery[b] (%)	Quantity determined by HPLC[a] (mg)	Recovery[b] (%)	Quantity determined by HPLC[a] (mg)	Recovery[b] (%)
Non-spiked[c]	6.49 (0.9)		6.88 (1.9)		7.20 (0.6)		4.35 (1.7)	
Spiked[c] with 10 mg of bacoside A that contained[d]								
2.21 mg of **1**	8.62 (0.4)	96.4						
1.98 mg of **2**			8.82 (0.7)	98.0				
2.94 mg of **3**					9.99 (0.8)	94.9		
1.80 mg of **4**							6.12 (1.3)	98.3
Spiked[c] with 20 mg of bacoside A that contained[d]								
4.42 mg of **1**	10.74 (3.3)	96.1						
3.97 mg of **2**			10.89 (3.6)	101.00				
5.88 mg of **3**					12.80 (3.3)	95.2		
3.61 mg of **4**							7.67 (4.0)	92.0

[a] Mean values ($n = 3$); RSD values given in parentheses.
[b] Recovery % = [(estimated quantity in spiked sample—estimated quantity in non-spiked sample)/quantity spiked] × 100.
[c] Kolkata 1 sample, 1 g.
[d] Values taken from Table 1.

The HPLC results also substantiated the existence of bacoside A as a mixture of **1–4** in *B. monnieri* as revealed by the analysis of different samples. It is interesting to note that HPLC-MS coupled anthelmintic assay-guided fractionation of the methanolic extract of *B. monnieri* had earlier resulted in the identification of an active fraction containing four major saponins (Renukappa *et al.*, 1999). In the active fraction, the presence of **1**, **3** and **4** were confirmed while the other saponin remained unidentified.

The quantitative variations in the contents of **1–4** in different samples of *B. monnieri* as observed in our studies need to be examined further with special reference to the biological activity of bacoside A. Extrapolation of the observed quantitative variations indicate that a reference standard of bacoside A, if obtained from different sources, could also exhibit variations in the contents of **1–4**. A report (Pawar *et al.*, 2001) indicating significantly higher potency of **1** as compared with **4** in inhibiting the release of superoxide from polymorphonuclear cells, emphasises the importance of a knowledge of the concentrations of **1–4** in *B. monnieri*. Thus it becomes logical and perhaps necessary to use bacoside A with known percentages of **1–4** or alternatively to use the individual saponins (**1–4**) as reference standards. The HPLC procedure described herein can be utilised for the quantitative determination of **1–4** in *B. monnieri* for standardisation. Previously published procedures based on UV (Pal and Sarin, 1992) and HPTLC (Gupta *et al.*, 1998) do not provide information on the concentration of individual saponins of bacoside A.

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