Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 66 (2005) 2719–2728
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Triterpenoid glycosides from *Bacopa monnieri* ☆

Chillara Sivaramakrishna [a], Chirravuri V. Rao [a], Golakoti Trimurtulu [a], Mulabagal Vanisree [b], Gottumukkala V. Subbaraju [a,*]

[a] *Laila Impex Research Center, Unit I, Phase III, Jawahar Autonagar, Vijayawada 520 007, India*
[b] *Department of Applied Chemistry, Chaoyang University of Technology, Taichung-41301, Taiwan*

Received 20 May 2005; received in revised form 20 September 2005
Available online 15 November 2005

Abstract

Two triterpenoid glycosides have been isolated along with 10 known saponins from *Bacopa monnieri*. Structures of the compounds have been elucidated as 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] jujubogenin (**1**) and 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (**2**) by high resolution NMR spectral data and chemical correlations. Further, the chemical compositions of bacosides A and B have been delineated.

Keywords: *Bacopa monnieri*; Scrophulariaceae; Bacoside A; Bacoside B; Jujubogenin; Pseudojujubogenin; Triterpenoid saponin

1. Introduction

Bacopa monnieri (Linn.) Pennel [Syn: *Herpestis monnieri* (Linn) Kunth; *Monniera cuncifolia* Michx (Family: Scrophulariaceae)], also referred as Brahmi or Jalbrahmi and water hyssop, has been used for centuries in Ayurvedic system of medicine as a brain tonic, memory enhancer, revitaliser of sensory organs, anti-anxiety, cardio-tonic, diuretic, antidepressant and anticonvulsant agent (Chopra et al., 1969 and Monograph, 2004). Antiinflammatory, analgesic, antipyretic properties have also been recorded (Jain et al., 1994) for *Bacopa* extracts. Several clinical studies confirmed the beneficial actions of *B. monnieri* (Russo and Borrelli, 2005). These pharmacological actions are mainly attributed to the saponin compounds present in the alcoholic extract of the plant. The initial chemical investigations described the occurrence of two saponins, designated as bacosides A and B (Chatterjee et al., 1963, 1965 and Basu et al., 1967). However, the subsequent studies have revealed that bacosides A and B are not single chemical entities as suggested, but they are found to be mixtures of saponins (Kawai and Shibata, 1978). The major chemical constituents isolated and characterized from *Bacopa* are dammarane type of triterpenoid saponins with jujubogenin or pseudojujubogenin moieties as aglycone units.

Several pharmacological (Singh et al., 1988, 1997) and clinical studies (Nathan et al., 2001 and Stough et al., 2001) on the extracts of *B. monnieri* standardized to the bacosides A and B have been published. *B. monnieri* extracts are widely available with label claims on the content of bacosides A and B in the international nutraceutical market. The HPTLC and UV analytical methods (Gupta et al., 1998; Shrikumar et al., 2004; Pal and Sarin, 1992) used for the standardization of bacosides A and B are rather vague and found to be highly prone to deviation from the true concentrations. For example, a commercial sample of *B. monnieri* that was standardized to 50% bacosides A and B by UV method, in fact exhibited less than 20% of the total *Bacopa* saponins, when estimated by HPLC against pure authentic samples (Ganzera et al., 2004).

☆ Laila Communication # 38.

* Corresponding author. Tel.: + 91 866 2546216; fax: +91 866 2541303.
E-mail address: subbarajugottumukkala@hotmail.com (G.V. Subbaraju).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.09.016

The composition of bacoside A has been established very recently as a mixture of four triglycosidic saponins, Bacoside A_3, Bacopaside II, 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin and Bacopasaponin C (Deepak et al., 2005). No further literature reports seem to be available on the chemical composition of bacoside B and total *Bacopa* saponin fraction, nor does a process available for producing standardized *Bacopa* fraction, whose total saponin concentration is more than 50% by HPLC method of analysis. The potential usefulness of *Bacopa* has been an incentive to investigate these aspects. The authors attempted a thorough reinvestigation of *B. monnieri* and successfully isolated 12 pure triterpenoid saponins, including two previously unreported diglycosidic saponins. The present communication reports the isolation and characterization of two new saponins (Figs. 1 and 2) and 10 known compounds from *B. monnieri*. The present study also describes an analytical procedure for the qualitative and quantitative assessment of the chemical compositions of bacosides A and B. Establishing chemical identities of *Bacopa* saponin fractions by identifying total spectrum of pure bacopa sapanins and chemical standardization of these fractions will greatly help to reduce afore-mentioned drawbacks and improve scientific validation of pharmacological and clinical data (Deepak and Amit, 2004).

2. Results and discussion

The hydroalcohol extract was subjected to column chromatography over silica gel using mixtures of $CHCl_3$, MeOH and H_2O. The fractions containing identical saponins were combined and rechromatographed over normal phase silica and/or reversed phase polymeric gel to yield 12 pure saponin compounds (Fig. 2). All the compounds responded positively to the froth test for saponins (Simes et al., 1959) and Libermann–Burchard test for triterpenes (Brieskorn and Herrrig, 1959) and Molisch test for sugars (Furniss et al., 1978). Ten of these saponins were found to be known and identified as 3-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] jujubogenin (bacopaside IV, Chakravarty et al., 2003), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside V, Chakravarty et al., 2003), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-O-{α-L-arabinofuranosyl-(1 → 2)}-*O*-(β-D-glucopyranosyl)] jujubogenin (bacoside A_3, **3**, Rastogi et al., 1994), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)}-β-D-glucopyranosyl] pseudojujubogenin (bacopaside II, **4**, Chakravarty et al., 2001), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin (Higuchi et al., 1984), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl] pseudojujubogenin (Bacopasaponin C, Garai

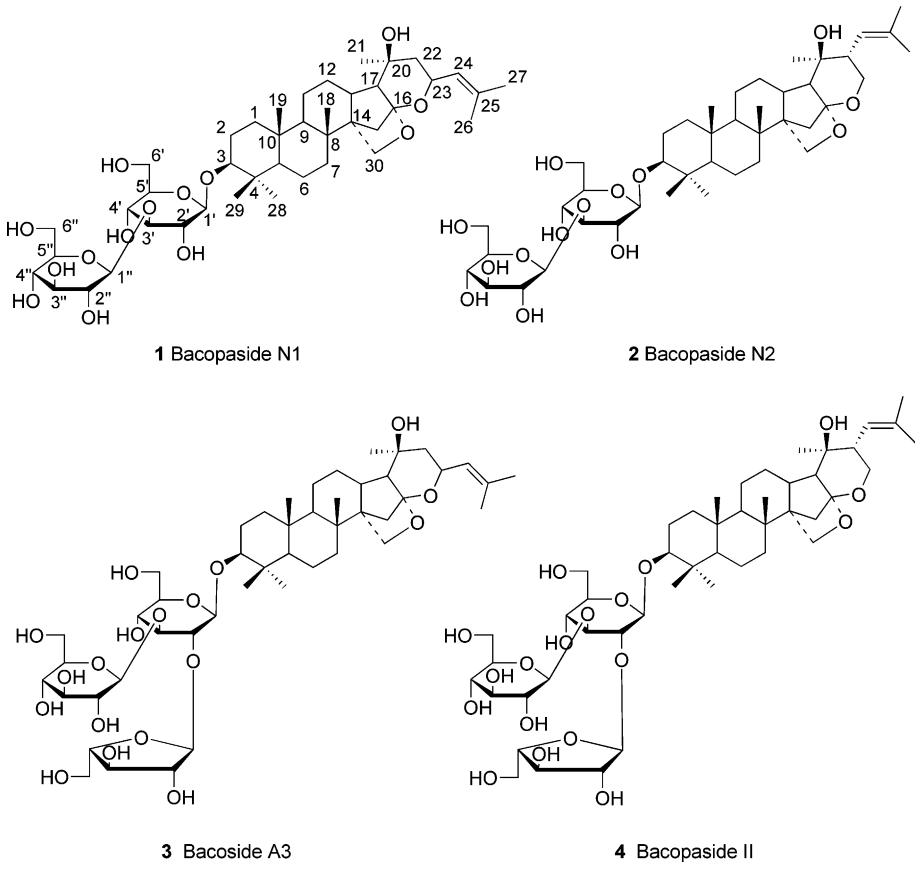
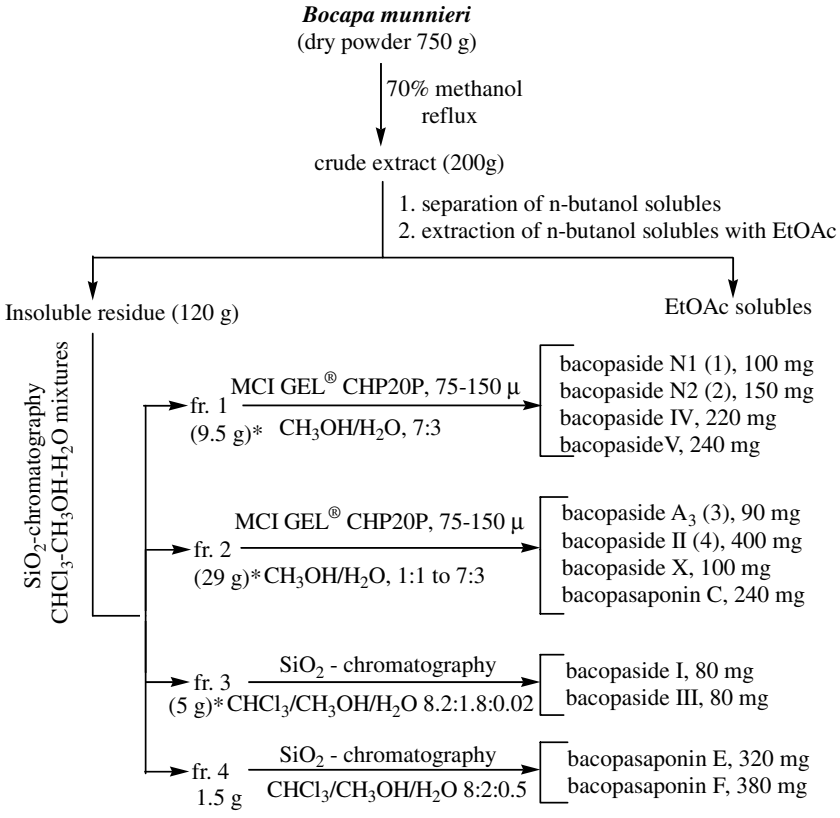


Fig. 1. New saponins (N1 & N2) from *Bacopa monnieri*.



*Only partial quantities 3g, 7g and 1.5g of the fractions 1, 2 and 3 respectively were subjected to further purification

Fig. 2. Isolation of *Bacopa* saponins.

et al., 1996), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{6-*O*-sulphonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside I, Chakravarty et al., 2001), 3-O-[{6-O-sulfonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside III, Hou et al., 2002), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl]-20-O-(α-L-arabinopyranosyl) jujubogenin (bacopasaponin E) and 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-β-D-glucopyranosyl]-20-O-(α-L-arabinopyranosyl) jujubogenin (bacopasaponin F, Mahato et al., 2000) by a comparison of their ^{1}H and ^{13}C NMR, and other spectral data with literature values. The names in the parenthesis are the common names assigned to them by original investigators.

Bacopaside N1 (**1**) was obtained as a white amorphous powder, m.p. 256–260 °C, $[\alpha]_D$: −25.3 ° (CH_3OH, *c* 0.025). Its IR spectrum showed bands at 3430, 2925, 2855 and 1633 cm^{-1}. The strong and broad IR band at 3430 is an indication for the presence of multiple hydroxyl groups in the molecule. Its LC–MS exhibited a peak at *m/z* 819.5 $(M + Na)^+$ corresponding to the molecular weight 796. The molecular ion peak at 795 in the negative ion mode confirms its molecular weight. The molecular weight in conjunction with C,H analysis data led to the assignment of molecular formula as $C_{42}H_{68}O_{14}$. The assignment (Tables 1 and 2) of ^{1}H NMR and ^{13}C NMR data was based

Table 1
^{1}H NMR data of bacopasides N1 (**1**) and N2 (**2**) in pyridine-d_5 (δ in ppm, *J* in Hz)

Proton	Bacopaside N1	Bacopaside N2
H_3-18	1.06 (*s*)	1.06 (*s*)
H_3-19	0.69 (*s*)	0.69 (*s*)
H_3-21	1.37 (*s*)	1.37 (*s*)
H_3-26	1.67 (*s*)	1.68 (*s*)
H_3-27	1.65 (*s*)	1.60 (*s*)
H_3-28	1.28 (*s*)	1.27 (*s*)
H_3-29	0.97 (*s*)	0.97 (*s*)
H-3	3.31 (*dd*, 9.0 and 4.8)	3.32 (*dd*, 11.0, 4.2)
H-13	2.81 (*m*)	2.82 (*m*)
H-22/H_2-22	1.63 (*m*), 1.73 (*m*)	2.57 (*d*, 10.2)
H-23/H_2-23	5.18 (*t*, 9.0)	3.86 (*d*, 10.5), 4.70 (*d*, 10.7)
H-24	5.50 (brd, 7.8)	5.84 (*d*, 10.1)
H_2-30	4.18 (*m*), 4.27 (*m*)	4.15 (*m*), 4.24
H-1′	4.88 (*d*, 7.2)	4.88 (*d*)
H-2′	4.03 (*m*)	4.04 (*m*)
H-3′	4.20 (*m*)	4.20 (*m*)
H-4′	4.05 (*m*)	4.05 (*m*)
H-5′	3.93 (*m*)	3.93 (*m*)
H_2-6′	4.51 (*m*)	4.51 (*m*)
H-1″	5.29 (*d*, 7.1)	5.29 (*d*, 7.6)
H-2″	4.03 (*m*)	4.03 (*m*)
H-3″	4.23 (*m*)	4.23 (*m*)
H-4″	4.17 (*m*)	4.17 (*m*)
H-5″	4.01 (*m*)	4.01 (*m*)
H_2-6″	4.29 (*m*)	4.30 (*m*)

Table 2
^{13}C NMR data bacopasides N1 (**1**) and N2 (**2**) in pyridine-d_5 (δ in ppm)

Carbon	Bacopaside N1	Bacopaside N2
1	38.66	38.68
2	26.58	26.59
3	88.88	89.02
4	39.64	39.65
5	56.09	56.10
6	18.31	18.32
7	36.08	36.08
8	37.55	37.54
9	53.0	53.02
10	37.25	37.25
11	21.77	21.76
12	28.54	28.62
13	37.16	37.15
14	53.75	53.52
15	36.87	36.94
16	110.56	110.31
17	54.03	51.33
18	18.88	18.85
19	16.26	16.27
20	68.51	71.88
21	30.05	27.20
22	45.48	46.29
23	68.58	66.13
24	127.13	124.18
25	134.10	132.91
26	26.53	26.05
27	18.31	18.46
28	28.01	28.02
29	16.77	16.77
30	65.83	65.87
	3-O–Glc	*3-O–Glc*
1′	106.31	106.34
2′	74.41	74.40
3′	88.99	88.89
4′	69.98	70.00
5′	77.90	77.94
6′	62.71	62.73
	Glc	*Glc*
1″	105.93	105.95
2″	75.47	75.48
3″	78.24	78.26
4″	71.65	71.67
5″	78.65	78.67
6″	62.53	62.56

on COSY, HMQC and HMBC spectra. Comparison of ^1H NMR and ^{13}C NMR spectral data with those of the known compounds, that were isolated and identified as part of the present investigation, suggested that **1** is also a dammarane type of triterpenoid saponin with a jujubogenin or pseudojujubogenin carbon skeleton for the aglycone moiety. The carbon chemical shifts of the triterpenoid unit, especially δ 54.0, 68.5, 30.0, 127.1 and 134.1 assigned to C-17, C-20, C-21, C-24 and C-25, respectively, are in agreement with values appropriate for those of a jujubogenin carbon skeleton. The jujubogenin structure is further confirmed by HMBC correlations from one of the methylene proton on C-22 appearing at δ 1.73 to C-17, C-20, C-21 and C-23 carbons and from a methine proton on C-23 resonating at δ 5.18 to C-24 and C-25.

Of the 42 carbons present in the carbon-13 NMR spectrum, 30 carbons could now be assigned to the triterpene moiety. The presence of 12 remaining deshielded carbons including two signals at δ 105.9 and 106.3, assignable to anomeric carbons of a carbohydrate unit, suggest a diglycosidic moiety with two hexose sugars for the carbohydrate part of the saponin structure. The paper chromatographic analysis of acid hydrolysate for the compound responded positively for the presence of glucose only suggesting that the diglycosidic sugar unit may be composed of two glucose units. The glucopyranose nature of the sugar unit attached directly to the aglycone moiety was confirmed and the type of glycosidic linkage established by the HMBC correlations, from H-3 to the anomeric carbon C-1′, from H-1′ to C-3 and C-5′, from H-4′ to C-3′, C-5′ and C-6′, and from H-3′ to C-2′ and C4′. The HMBC correlations observed from the second anomeric proton H-1″ to C-3′ and C5″, established the glycosidic linkage between the two sugar units as C-1″ → C-3′. Other HMBC correlations, especially from H_2-6″ to C-5″ and C-4″, from H-4″ to C-3″ and from H-3″ to C-2″ confirmed glucopyranose structure of the second sugar unit. These assignments were further confirmed and the configuration of glucose units established by chemical correlation following a semi-synthetic preparation of **1** from a known triglycosidic saponin called bacoside A_3 (**3**) using mild hydrolysis. The hydrolysis product exhibited a peak at m/z 819 $(M + Na)^+$ in the positive LC–MS mode and at 795 in the negative mode indicating the loss of pentose moiety from the starting triglycosidic compound. This selective hydrolysis may has been the result of relative vulnerability of more labile arabinofuranosyl-(1 → 2)-β-D-glucopyranosyl, glycosidic linkage in **3**, compared with two other glycosidic linkages (Martin von, 1995; Greene and Wuts, 1999; Mannich and Siewert, 1942). As the nature of the glycosidic linkage between two glucose units and their stereochemistry in bacoside A_3 is already known, by correlation, the structure of bacopaside N1 can now be assigned as 3-*O*-[β-D-glucopyranosyl(1 → 3)-β-D-glucopyranosyl] jujubogenin (**1**).

Bacopaside N2 (**2**) was also white amorphous powder, m.p. 278–282 °C, $[\alpha]_D$: −25.0° (CH_3OH, *c* 0.0058). Its IR spectrum showed bands at 3435, 2928, 2860 and 1639 cm^{-1}. It exhibited same molecular weight as **1** in LC–MS [m/z 819.5 $(M + Na)^+$ corresponding to 796]. The molecular weight is further supported by a molecular ion peak at 795 in the negative ion mode. The ^1H NMR (Table 1) and ^{13}C NMR (Table 2) spectral data resemble closely with those assigned for **1**, except the signals for C-17, C-20, C-21, C-24 and C-25 and the differences being highly characteristic of those found between jujubogenin and pseudojujubogenin (Chakravarty et al., 2003) isomers. This assignment is further confirmed by crucial HMBC correlations. A methylene proton H-23 at δ 3.85 showed correlation to a methylene carbon at 66.13 in HMQC spectrum and showed correlations to C-16, C-20, C-22 and C-24

carbons in HMBC spectrum indicating a pseudojujubogenin carbon skeleton for **2**. The carbohydrate moiety was found to be same as that in **1** by paper chromatographic analysis of the acid hydrolysate. The compound may thus be presumed as pseudojujubogenin counterpart for the bacopaside N1 (**1**). This assignment is confirmed and the structure is assigned as 3-*O*-[β-D-glucopyranosyl(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (**2**) by its semi-synthetic preparation from bacopaside II (**4**) using the same experimental procedure mentioned previously for the preparation of bacopaside N1 from bacoside A_3. The semi-synthetic product is found to be identical with **2** by TLC, ^{1}H NMR, ^{13}C NMR and LC–MS data.

The isolation of authentic samples for all the detectable triterpenoid saponins in *B. monnieri* extract has given us the opportunity to develop an analytical procedure to study the chemical composition of bacosides A and B. The chemical identity of these *Bacopa* saponin fractions were delineated by a new HPLC method optimized to get good separation among different components in the mixtures. Peaks in the chromatogram were initially assigned by spiking bacosides A and B mixtures, randomly, with individual authentic samples. Using the new analytical method, the chemical compositions of commercial samples of bacoside A and bacoside B were established. Bacoside A was recently established as a mixture of four triglycosidic saponins, bacoside A_3, bacopaside II, bacopaside X and bacopa saponin C (Deepak et al., 2005). Our data for the composition of bacoside A matched with the published data. We have now evaluated the qualitative and quantitative assessment of bacoside B and established that it is composed of four minor diglycosidic saponins, bacopaside N1 (**1**), bacopaside N2 (**2**), bacopaside IV and bacopaside V, which include two new compounds that are presently being reported as a part of this paper. The Fig. 3 depicts a chromatogram for an enriched sample of bacoside B. The validity of the analytical method was verified using several analytical and statistical parameters (Table 3). The linearity of the detector response for the standards varies in the range of 1–25 μg on column and a detection limit of at least 1 μg on column

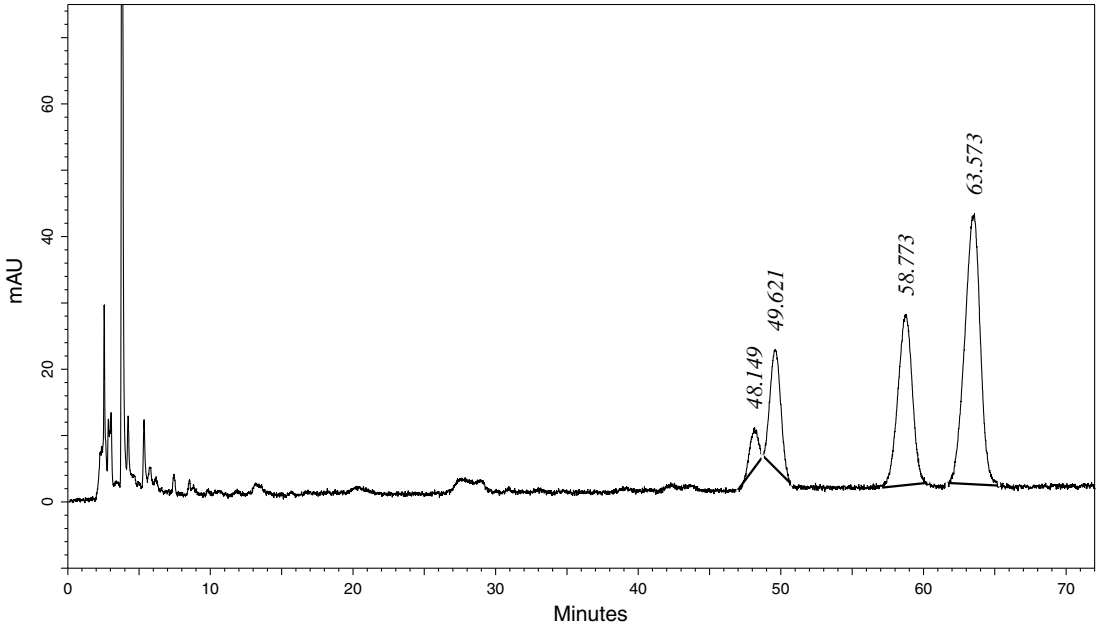


Fig. 3. Chromatogram for enriched bacoside B fraction. Retention time: name (structure #, absolute %) 48.15 min: Bacopaside N1 (**1**, 3.37%), 49.62 min: Bacopaside N2 (**2**, 7.85%), 58.77 min: Bacopaside IV (41.5%), 63.57 min: Bacopaside V (28.2%). Total concentration: 80.92%.

Table 3
Analytical data for the sample and calibration data for standards

Sample mixture			Standards			
Name	R_t (min)	Abs (%)	Range (ng)	Regression curve	R^2	%RSD
Bacopaside N1	48.15	3.37	2000–13,000	$y = 239.34X - 26559$	0.9999	<2
Bacopaside N2	49.62	7.85	2500–25,000	$y = 234.5X + 72501$	0.9999	<2
Bacopaside IV	58.77	41.5	1000–4000	$y = 210.36X - 31940$	0.9987	<2
Bacopaside V	63.57	28.2	1500–5000	$y = 337.32X - 1299$	1	<2

R_t is retention time for the compounds in the mixture; X reflects peak area; Y the amount of compound; R^2 is regression coefficient; RSD is relative standard deviation.

Table 4
Brine shrimp lethality of *Bacopa* saponins and fractions

S. No.	Name of compound	IC50 (μg/mL)
1	*Bacopa* crude extract (15%)	70
2	Bacoside A (55%, ref std)	29
3	Bacoside B (47%, ref std)	65
4	Bacopaside N1	>100
5	Bacopaside N2	>100
6	Bacopaside IV	>100
7	Bacopaside V	>100
8	Bacopaside A3	>100
9	Bacopaside II	32.5
10	Bacopaside X	36.1
11	Bacopasaponin C	3.9
12	Bacopaside I	75
13	Bacopaside III	>100
14	Bacopasaponin E	>100
15	Bacopasaponin F	>100
16	Podophyllotoxin	4.5

was determined. A regression coefficient of at least 0.999 was observed for all the standards. All the standards and samples were injected in triplicate and a maximum relative standard deviation of 2% was observed. The retention times were found to be stable during the whole study period.

Many beneficial actions of *B. monnieri* have been attributed to the presence of saponin compounds in extracts. *Bacopa* extracts and fractions were found to inhibit Brine shrimp, *Artemia salina* (D'Souza et al., 2002). The Brine shrimp lethality has been reported to be corroborative with the cytotoxicity data determined for 9KB and 9PS cells (McLaughlin et al., 1992 and McLaughlin et al., 1998). *Bacopa* saponin fractions have infact demonstrated cytotoxic activity against sarcoma-180 cancer cells (Elangovan et al., 1995). The Brine shrimp lethality has now been evaluated for various *Bacopa* extracts and pure compounds. The data (Table 4) indicate that the magnitude of inhibition was found to depend on total *Bacopa* saponin concentration. It was also observed that bacoside A fraction was found to be more active than bacoside B. Consistant with this observation, only compounds from bacosides A fraction showed better activity. Bacopasaponin C is more potent of all the saponins and its activity is comparable to that of podophyllotoxin.

3. Experimental

3.1. General

Melting points (uncorrected) are determined on a MEL-Temp melting point apparatus. IR spectra were recorded on a Perkin–Elmer model Spectrum BX, FT-IR instrument using KBr disc. The 2D NMR spectra were recorded on Bruker Varian 600 MHz INOVA instrument and ^{1}H NMR spectra were recorded on Bruker Varian 600 MHz INOVA instrument or Bruker AMX-400 FT NMR spectrometer using d_5-pyridine and ^{13}C NMR spectra are recorded on Bruker AMX-600 FT NMR spectrometer using d_5-pyridine; Mass studies were performed on LC–MS system equipped with Agilent 1100 series LC/MSD detector and 1100 series Agilent HPLC pump. Elemental analysis was carried with a Vario EI Elemantar Instrument. Optical rotation measured with JASCO DIP 370 digital polarimeter. Analytical HPLC studies were done on Shimadzu system PDA system equipped with LC10 ATVP pumps and SPD M10 AVP PDA detector and auto injector, and loaded with Class-VP software using C_{18} Phenomenex Luna (250 × 4.6, 5 μm) column and 0.1% H_3PO_4/CH_3CN (60:40) mobile phase, UV absorbance detector (205 nm); Reversed phase silica gel MCI-Gel CHP 20P (Mitsubishi Chemical Corporation, Japan), 75–150 μm and normal phase silica gel ACME (60–120 mesh) were used for column chromatography. Silica gel precoated plates (Alugram Sil G/UV_{254}) were used for thin layer chromatography using the solvent system EtOAc/MeOH/H_2O (75:15:10) and visualized by immersing the plate in vanillin sulfuric acid reagent followed by heating at 110 °C. The solvents and other chemicals were supplied by S.D. Fine-Chem Limited, Mumbai.

3.2. Plant material

B. monnieri (whole plant) was collected from Aswaraopet, Andhra Pradesh, India, during March 2004 and identified by Dr. K. Hemadri, Taxonomist. A Voucher specimen (No. 7012) is deposited at the herbarium of Laila Impex Research Centre, Vijayawada.

3.3. Extraction and isolation of pure saponins from *B. monnieri*

3.3.1. Purification of individual compounds

The air-dried and powder plant material (750 g) was extracted with 70% MeOH under reflux for 4 h. The plant material was removed by filtration and the extract was concentrated under vacuum. The dark colored residue (200 g) was dissolved in 2 L of *n*-BuOH and washed with water. The *n*-BuOH layer was evaporated and dried under vacuum to obtain a thick paste (164 g). The EtOAc extractives were removed from the thick paste under reflux and the residue (120 g) subjected to column chromatography over silica gel using eluants of increasing polarity starting from $CHCl_3$ to MeOH. The fractions that were eluted with $CHCl_3$/MeOH 8.5:1.5 yielded a mixture of four diglycosidic saponins (9.5 g), the fraction eluted with $CHCl_3$/MeOH 8:2 yielded a mixture of four triglycosidic saponins (29.0 g), the fractions eluted with $CHCl_3$/MeOH/H_2O 8:2:0.03 yielded a mixture of two sulfated saponins (5.0 g) and finally the fraction eluted with $CHCl_3$/MeOH/H_2O 8:2:0.5 yielded a mixture of two tetraglycosidic saponins (1.5 g). The diglycosidic saponins mixture (3.0 g) was found to be a mixture of four components by HPLC analysis. It was subjected to rechromatography over reversed phase polymer resin (MCI GEL® CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) using 7:3 MeOH/H_2O mixture to yield two new compounds, 3-*O*-[β-D-glucopyranosyl-

(1 → 3)-β-D-glucopyranosyl] jujubogenin (bacopaside N1, **1**) and 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (bacopaside N2, **2**) along with two known saponins 3-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] jujubogenin (bacopaside IV) and 3-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside V) in partially pure form. These partially pure compounds were further subjected to rechromatography on reversed phase resin column under similar conditions to obtain **1** (100 mg), **2** (150 mg), bacopaside IV (220 mg) and bacopaside V (240 mg). The triglycosidic saponins mixture (7 g) was subjected to flash chromatography over reversed phase polymer resin (MCI GEL® CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) using solvents of decreasing polarity from 1:1 MeOH/H_2O mixture to 7:3 MeOH/H_2O mixture to afford four known saponins, 3-*O*-[β-D-glucopyranosyl-(1 → 3)-*O*-{α-L-arabinofuranosyl- (1 → 2)}-*O*-(β-D-glucopyranosyl)] jujubogenin (bacoside A_3, **3,** 90 mg), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)}-β-D-glucopyranosyl] pseudojujubogenin (bacopaside II, **4**, 400 mg), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin (bacopaside X, 100 mg), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopasaponin C, 240 mg) as pure compounds and further quantities of these materials as mixtures. The sulfated *Bacopa* saponin fraction (1.5 g) was subjected to rechromatography over silica gel using $CHCl_3$/MeOH/H_2O (8.2:1.8:0.02) to yield two pure saponins, 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{6-*O*-sulphonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside I, 80 mg) and 3-*O*-[{6-*O*-sulfonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside III, 80 mg). The tetraglycosodic fraction was subjected to normal phase silica chromatography followed by further purification on reversed phase polymer resin (MCI GEL® CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan) using solvents of decreasing polarity from 1:1 MeOH/H_2O mixture to 8:2 MeOH/H_2O to afford two known dammarane type jujubogenin bisdesmosides, 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl]-20-*O*-(α-L-arabinopyranosyl) jujubogenin (bacopasaponin E, 320 mg) and 3-*O*-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-β-D-glucopyranosyl]-20-*O*-(α-L-arabinopyranosyl)] jujubogenin (bacopasaponin F, 380 mg).

Note. A trivial name bacopaside X has been assigned to the known saponin, 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin for the sake of convenience.

3.3.2. 3-O-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] jujubogenin (bacopaside N1, 1)

$[\alpha]_D$: −25.3° (CH_3OH, *c* 0.025); Melting point: 256–260 °C; IR (KBr): 3430, 2925, 2855, 1633, 1451, 1380, 1290, 1250, 1078, 1035 cm^{-1}; LC–MS (negative) *m/z* 795 $(M - H)^-$; LC–MS (positive) *m/z* 819 $(M + Na)^+$; Elemental analysis: Found C, 63.78; H, 8.54. $C_{42}H_{68}O_{14}$ requires: C, 63.30; H, 8.60%; ^1H NMR data is given Table 1 and ^{13}C NMR is given in Table 2.

3.3.3. 3-O-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (bacopaside N2, 2)

$[\alpha]_D$: −25.0° (CH_3OH, *c* 0.0058); Melting point: 278–282 °C; IR (KBr): 3435, 2928, 2860, 1639, 1453, 1378, 1288, 1213, 1078, 1035 cm^{-1}; LC–MS (negative) *m/z* 795 $(M - H)^-$; LC–MS (positive) *m/z* 819 $(M + Na)^+$; Elemental analysis: Found C, 63.58; H, 8.46. $C_{42}H_{68}O_{14}$ requires: C, 63.30; H, 8.60%; ^1H NMR data is given Table 1 and ^{13}C NMR is given in Table 2.

3.3.4. 3-O-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] jujubogenin (bacopaside IV)

Melting point: 266–270 °C; IR (KBr): 3429, 2928, 1630, 1453, 1381, 1293, 1077, 1029 cm^{-1}; LC–MS (negative) *m/z* 765 $(M - H)^-$, LC–MS (positive) *m/z* 789 $(M + Na)^+$.

3.3.5. 3-O-[β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside V)

Melting point: 274–278 °C; IR ($CHCl_3$): 3432, 2941, 1625, 1444, 1380, 1287, 1213, 1075, 1030 cm^{-1}; LC–MS (positive) *m/z* 765 $(M - H)^-$, LC–MS (positive) *m/z* 789 $(M + Na)^+$.

3.3.6. 3-O-[β-D-glucopyranosyl-(1 → 3)-O-{α-L-arabinofuranosyl-(1 → 2)}-O-(β-D-glucopyranosyl)] jujubogenin (bacoside A_3, 3)

Melting point: 244–250 °C; IR (KBr): 3406, 2938, 1645, 1451, 1380, 1290, 1216, 1078, 1036 cm^{-1}; LC–MS (negative) *m/z* 927 $(M - H)^-$, LC–MS (positive) *m/z* 951 $(M + Na)^+$.

3.3.7. 3-O-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)}-β-D-glucopyranosyl] pseudojujubogenin (bacopaside II, 4)

Melting point: 251–255 °C; IR (KBr): 3431, 2940, 1638, 1453, 1374, 1287, 1214, 1078, 1034 cm^{-1}; LC–MS (negative) *m/z* 927 $(M - H)^-$, LC–MS (positive) *m/z* 951 $(M + Na)^+$.

3.3.8. 3-O-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin (bacopaside X)

Melting point: 228–233 °C; IR (KBr): 3431, 2927, 2863, 1633, 1458, 1381, 1289, 1217, 1076, 1031 cm^{-1}; LC–MS (negative) *m/z* 897 $(M - H)^-$, LC–MS (positive) *m/z* 921 $(M + Na)^+$.

3.3.9. 3-O-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopasaponin C)

Melting point: 288–290 °C; IR (KBr): 3410, 2942, 1596, 1451, 1385, 1214, 1075, 1028 cm^{-1}; LC–MS (negative) *m/z* 897 $(M - H)^-$, LC–MS (positive) *m/z* 921 $(M + Na)^+$.

3.3.10. 3-O-[α-L-arabinofuranosyl-(1 → 2)-{6-O-sulphonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside I)

Melting point: 260–261 °C; IR (KBr): 3431, 2940, 1637, 1454, 1380, 1258, 1221, 1072, 1013, 938, 790, 585 cm^{-1}; LC–MS (negative) *m/z* 977 $(M - H)^-$, LC–MS (positive) *m/z* 1001 $(M + Na)^+$.

3.3.11. 3-O-[{6-O-sulfonyl-β-D-glucopyranosyl-(1 → 3)}-α-L-arabinopyranosyl] pseudojujubogenin (bacopaside III)

Melting point: 222–224 °C; IR (KBr): 3432, 2932, 1635, 1460, 1383, 1258, 1220, 1074, 1012, 808, 700, 589 cm^{-1}; LC–MS (negative) *m/z* 845 $(M - H)^-$, LC–MS (positive) *m/z* 868 $(M + Na)^+$.

3.3.12. 3-O-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-α-L-arabinopyranosyl]-20-O-(α-L-arabinopyranosyl) jujubogenin (bacopasaponin E)

Melting point: 251–256 °C; IR (KBr): 3408, 2941, 2872, 1640, 1452, 1374, 1291, 1262, 1214, 1079, 1028 cm^{-1}; LC–MS (positive) *m/z* 1053 $(M + Na)^+$, LC–MS (negative) *m/z* 1029 $(M - H)^-$.

3.3.13. 3-O-[β-D-glucopyranosyl-(1 → 3)-{α-L-arabinofuranosyl-(1 → 2)}-β-D-glucopyranosyl]-20-O-(α-L-arabinopyranosyl)] jujubogenin (bacopasaponin F)

Melting point: 255–258 °C; IR (KBr): 3410, 2941, 2874, 1651, 1453, 1374, 1290, 1254, 1243, 1080, 1004 cm^{-1}; LC–MS (positive) *m/z* 1083 $(M + Na)^+$, LC–MS (negative) *m/z* 1059 $(M - H)^-$.

*3.4. Conversion of bacoside A_3 (**3**) to bacopaside N1 (**1**)*

A sample of bacoside A_3 (20 mg) in MeOH (8 mL) was treated with 2 N HCl (1.2 mL) and stirred at reflux temperature for 30 min. The reaction mixture was poured into ice water and extracted with *n*-BuOH. The organic layer was washed with water and the solvent evaporated. The residue was subjected to column chromatography over silica using $CHCl_3$/MeOH mixtures. The fractions were monitored by TLC and those containing the saponin were combined and evaporated to obtain a residue (12 mg) that was found to be identical with bacopaside N1 (**1**) by melting point, TLC, HPLC, LC–MS and ^1H NMR data.

*3.5. Conversion of bacoside II (**4**) to bacopaside N2 (**2**)*

A small sample of bacoside II (**4**, 20 mg) was converted to bacopaside N2 (**2**, 10 mg) using the same procedure described for the preparation of bacopaside N1 (**1**). The semi-synthetic material is identical in all respects with the natural compound bacopaside N2.

3.6. Enrichment of bacosides A and B

The residue of *Bacopa* saponin mixture as obtained in procedure 3.3.1, is subjected to silica column chromatography using solvents with a slow gradient of increasing polarity starting from $CHCl_3$. The fractions eluted with $CHCl_3$/MeOH 9:1 were monitored and the identical fractions combined to yield a mixture that was enriched upto 81% of total diglycosidic saponins, 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] jujubogenin (bacopaside N1, **1**), 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (bacopaside N2, **2**), bacopaside IV and bacopaside V. The fractions that were eluted with $CHCl_3$/MeOH (8.5:1.5) were similarly monitored and combined and evaporated to yield a mixture of triglycosidic saponins, bacoside A_3, (**3**), bacopaside II (**4**), 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabinopyranosyl] jujubogenin (bacopaside X) and bacopasaponin C at a total overall concentration of upto 99% by HPLC analysis.

3.7. Method for the estimation of chemical compositions of bacosides A and B

HPLC experiments were performed on Shimadzu system equipped with LC10 ATVP pumps and SPD M10 AVP PDA detector and auto injector. For all separations a Phenomenex Luna C 18 column (4.6 × 250 mm, 5 μm particle size) was used. All the experiments were done in isocratic elution using a mixture of 0.05 M Na_2SO_4 (pH 2.3) and CH_3CN (68.5:31.5) as mobile phase. The flow rate was adjusted to 1 mL/min and the column temperature maintained at 30 °C. The detector wavelength was adjusted to 205 nm. The run time for each separation was 75 min. The size of sample injection was 20 μL, and the solvent for injection was 1:1, 0.05 M Na_2SO_4 (pH 2.3)/CH_3CN. The in-house reference standards were used for estimation. Peaks in the chromatogram for total *Bacopa* saponin fraction, were initially assigned by spiking the experimental sample with individual standard compounds. Standard solution was achieved by weighing accurately about 5.0 mg of each sample of in-house reference standard in to a 10-mL volumetric flask and dissolved and made-up the volume with the injection solvent. Similarly, sample preparation was done by weighing accurately about 15.0 mg of experimental sample in to a 10-mL volumetric flask, dissolved and made-up the volume with solvent. Both standard and sample solutions were filtered through Waters Millipore, 0.45 μm membrane filter before injecting. Absolute concentration of individual saponins were calculated by the formula, percentage of individual component = peak area of the component in the sample × concentration of the standard × purity of standard/peak area of standard × concentration of sample. The total absolute concentration of bacosides A and B fractions were obtained by the addition of individual concentrations of all the components in the enriched fraction together.

Bacoside A. (Triglycosidic fraction): Bacoside A_3 (**3**): 29.45%; Bacopaside II (**4**): 15.81%; 3-*O*-[α-L-arabinofuranosyl-(1 → 2)-{β-D-glucopyranosyl-(1 → 3)-}-α-L-arabino-

pyraosyl]jujubogenin (bacopaside X): 33.23%; Bacopasaponin C: 21.38%.

Total saponin concentration: (98.87%)

Bacoside B. (Diglycosidic fraction, Fig. 3): 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopy-ranosyl] jujubogenin (bacopaside N1, **1**): 3.37%; 3-*O*-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl] pseudojujubogenin (bacopaside N2, **2**): 7.85%; bacopaside IV: 41.5%; bacopaside V: 28.2%.

Total saponin concentration: 80.92

3.8. Determination of brine shrimp lethality

The brine shrimp (*A. salina*) cysts were hatched in a cone shaped vessel (1 L) filled with sterile artificial sea water (prepared using sea salt 38 g/L and adjusted to pH 8.5 using 1 N NaOH) under constant aeration. After 48 h, 10 active nauplii were drawn through a glass capillary and placed in each vile containing 4.5 mL sterile artificial sea water and treated with known concentrations of test substances and the volume was finally made upto 5 mL using sterile artificial sea water and maintained at 37 °C for 24 h under the light of incandescent lamps. The viability/mortality was obtained by counting the surviving larvae. Percentage lethality was calculated by comparing mean values of control and test sets of three tubes each. LC_{50} values were obtained from the graph plotted micro molar concentration against percent lethality. The inhibitory concentrations for *Bacopa* extracts and pure saponins were summarized in Table 4.

Acknowledgements

The authors thank Spectrometric Instrumentation Facility of Indian Institute of Science (IISc) in Bangalore for NMR spectral data and Sri G. Gangaraju and Mr Rama Raju, the Chairman and Director, respectively, of Laila Impex, Vijayawada for their encouragement.

References

Basu, N., Rastogi, R.P., Dhar, M.L., 1967. Chemical examination of *Bacopa monnieri* Wettest Part III – bacoside B. Indian J. Chem. 5, 84–86.

Brieskorn, C.H., Herrrig, H., 1959. The chemical activity of color reactions according to Liebermann-Burchard in sterols and triterpenes as well as in their esters. Mitt Dtsch Pharm Ges Pharm Ges DDR 29, 485–496.

Chakravarty, A.K., Sarkar, T., Masuda, K., Shiojima, K., Nakane, T., Kawahara, N., 2001. Bacopaside I and II: two pseudojujubogenins glycosides from *Bacopa monnieri*. Phytochemistry 58, 553–556.

Chakravarty, A.K., Garai, S., Masuda, K., Nakane, T., Kawahara, N., 2003. Bacopasides III–V: three new triterpenoid glycosides from *Bacopa monnieri*. Chem. Pharm. Bull. 51, 215–217.

Chatterjee, N., Rastogi, R.P., Dhar, M.L., 1963. Chemical examination of *Bacopa munniera* Wettst.: Part I – Isolation of chemical constituents. Indian J. Chem. 1, 212–215.

Chatterjee, N., Rastogi, R.P., Dhar, M.L., 1965. Chemical examination of *Bacopa munniera* Wettst.: Part II – The constitution of Bacoside A. Indian J. Chem. 3, 24–29.

Chopra, R.N., Chopra, I.C., Verma, B.S., 1969. Glossary of Indian Medicinal Plants. Council of Scientific and Industrial Research (CSIR), New Delhi.

Deepak, M., Amit, A., 2004. The need for establishing identities of bacoside A and B, the putative major bioactive saponins of Indian medicinal plant *Bacopa monnieri*. Phytomedicine 11, 264–268.

Deepak, M., Sangli, G.K., Arun, P.C., Amit, A., 2005. Quantitative determination of the major saponin mixture bacoside A in *Bacopa munnieri* by HPLC. Phytochem. Anal. 16 (1), 24–29.

D'Souza, P., Deepak, M., Rani, P., Kadamboor, S., Mathew, A., Chandrashekar, A.P., Agarwal, A., 2002. Brine shrimp lethality assay of *Bacopa monnieri*. Phytother. Res. 16, 197–198.

Elangovan, V., Govindasamy, S., Ramamoorty, N., Balasubramaanian, K., 1995. In vitro studies on the anticancer activity of *Bacopa munneri*. Fitoterapia 66, 211–215.

Furniss, B.S., Hannaford, A.J., Rogers, V., Smith, P.W.G., Tatchell, A.R. (Eds.), 1978. Vogel's text book of practical organic chemistry, fourth ed. Longman Group Ltd., London, p. 1078.

Ganzera, M., Gampenrieder, J., Pawar, R.S., Khan, I.A., Stuppner, H., 2004. Separation of the major triterpenoid saponins in *Bacopa monnieri* by high-performance liquid chromatography. Anal. Chim. Acta 516, 149–154.

Garai, S., Mahato, S.B., Ohtani, K., Yamasaki, K., 1996. Dammarane type triterpenoid saponins from *Bacopa monnieri*. Phytochemistry 42, 815–820.

Greene, T.W., Wuts, P.G.M. (Eds.), 1999. Protective groups in organic synthesis. Wiley, USA, p. 176.

Gupta, A.P., Mathur, S., Gupta, M.M., Kumar, S., 1998. Effect of the method of drying on the bacoside A content of the harvested *Bacopa monnieri* shoots revealed using a high performance thin layer chromatography method. J. Med. Aromat. Plants 20, 1052–1055.

Higuchi, R., Kubota, S., Komori, T., Kawasaki, T., Pandey, V.B., Singh, J.P., Shah, A.H., 1984. Triterpenoid saponins from the bark of *Zizyphus joazeiro*. Phytochemistry 23, 2597–2600.

Hou, C.C., Lin, S.J., Cheng, J.T., Hsu, F.L., 2002. Bacopaside III, bacopasaponin G and bacopasides A, B, C, from *Bacopa monnieri*. J. Nat. Prod 65, 1759–1763.

Jain, P., Khanna, N.K., Trehan, T., Pendse, V.K., Godhwani, J.L., 1994. Antiinflammatory effects of an Ayurvedic preparation, Brahmi Rasayan in rodents. Indian J. Exp. Biol. 32, 633–636.

Kawai, K.I., Shibata, S., 1978. Pseudojujubogenin, a new sapogenin from *Bacopa monnieri*. Phytochemistry 17, 287–289.

Mahato, S.B., Garai, S., Chakravarty, A.K., 2000. Bacopasaponins E and F: two jujubogenin bisdesmosides from *Bacopa monnieri*. Phytochemistry 53, 711–714.

Mannich, C., Siewert, G., 1942. Ber. Dt. Chem. Ges. 75, 737.

Martin von, P., 1995. Ein neuer Zugang zu 2′-*O*-alkylribonucleosiden und Eigenschaften deren oligonucleotide. Helv. Chim. Acta 78, 486–504.

McLaughlin, J.L., Chang, C.J., Smith, D.L., 1992. Simple bench top bioassay for the discovery of plant antitumor compounds. Am. Chem. Soc. Sympos. Ser. 534, 114.

McLaughlin, J.L., Rogers, L.L., Anderson, J.E., 1998. The use of biological assays to evaluate botanicals. Drug Inform. J. 32, 513–524.

Monograph, 2004. *Bacopa monniera*. Alternative Medicine Review 9, 79–85.

Nathan, P.J., Clarke, J., Lloyd, J., Huchison, C.W., Downey, L., Sough, C., 2001. The acute effects of an extract of *Bacopa monnieri* on cognitive function in healthy normal subjects. Hum. Psychopharmacol. 16, 345–351.

Pal, R., Sarin, J.P.S., 1992. Quantitative determination of Bacosides by UV spectrometry. Indian J. Pharm. Sci. 54, 17–18.

Rastogi, S., Pal, R., Kulshreshtha, D.K., 1994. Bacoside A_3-A triterpenoid saponin from *Bacopa monnieri*. Phytochemistry 36, 133–137.

Russo, A., Borrelli, F., 2005. *Bacopa munniera*, a reputed nootropic plant: an overview. Phytomedicine 12, 305–317.

Singh, H.K., Rastogi, R.P., Srimal, R.C., Dhawan, B.N., 1988. Effect of Bacosides A & B on Avoidance responses in Rats. Phytother. Res. 2, 70–75.

Singh, H.K., Dhawan, B.N., 1997. Neuropsychopharmacological effects of the Ayurvedic Nootropic *Bacopa monnieri* Linn. Indian J. Pharmacol. 29, S359–S365.

Shrikumar, S., Sandeep, S., Ravi, T.K., Umamaheswari, M., 2004. A HPTLC determination and fingerprinting of bacoside A in *Bacopa monnieri* and its formulation. Indian J. Pharm. Sci. 66, 132–135.

Simes, J.J.H., Tracey, J.G., Webb, L.J., Duston, W.J., 1959. Australian phytochemical survey III. Saponins in Eastern Australian flowering plants. Bulletin No. 281, CSIRO, Melbourne, Australia.

Stough, C., Lloyd, J., Clarke, J., Downey, L.A., Hutchison, C.W., Rodgers, T., Nathan, P.J., 2001. The chronic effects of an extract of *Bacopa monnieri* on cognitive function in healthy human subjects. Psychopharmacology 156, 481–484.