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New dammarane triterpenoidal saponins from *Bacopa monniera*

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Two dammarane glycosides have been isolated from aqueous extract of the aerial parts of *Bacopa monniera* Wettst. The chemical structure of one of the compound has been established as 20-*O*-α-L-arabinopyranosyl jujubogenin, a novel compound, on the basis of LC-MS, IR, 1D- and 2D-NMR studies. The other compound has been identified as 3-*O*-α-L-arabinopyranosyl jujubogenin, which is being reported for the first time from *B. monniera.* The compounds have been screened for their immunomodulatory potential using NBT reduction and chemiluminescence assay using PMN cells.

Keywords: Bacoside A_4 and A_5, *Bacopa monniera*, respiratory oxyburst

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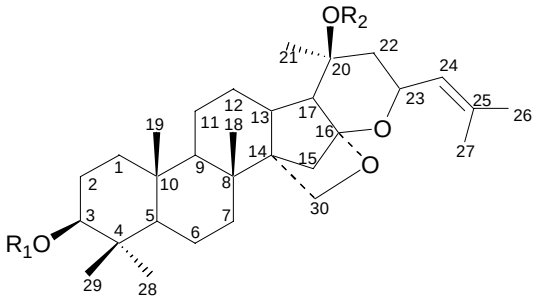
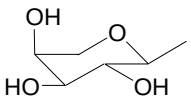
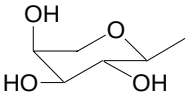
The herb *Bacopa monniera* (Scrophulariaceae) is an important traditional medicine of Indian subcontinent. The standardized extract of the plant is used as a clinical medicine for memory enhancement and intellect improvement. The plant is reported to contain a number of saponins belonging to dammarane triterpenoids with jujubogenin and pseudojujubogenin skeleton[1-7]. The memory related biological effect has been attributed to two complex mixtures of saponins referred as bacoside A and B[8,9]. In an earlier study, it was reported that bacoside A_3, a jujubogenin glycoside acts as a major inhibitor of superoxide release from the polymorphonuclear (PMN) cells[10]. In the same study it was also reported that bacopasaponin C, a pseudojujubogenin glycoside, was comparatively less active as compared to bacoside A_3. To investigate further, the aqueous extract of aerial parts of the plant were studied. The isolation, structure elucidation and bioactivity of two saponins named as bacoside A_4 and A_5 are reported in the current study. Bioactivity of the compounds was tested on NBT reduction and luminol-enhanced chemiluminescence assay.

Results and Discussion

The aqueous extract obtained after 70% methanolic extraction of the aerial parts of *B. monniera* was fractionated as described in experimental section to yield crystalline compounds **1** and **2**. Both the compounds appeared in the aqueous extract even though they are relatively non-polar as compared to other saponins reported from the plant, while none of the relatively polar saponins reported so far appeared in the aqueous extract. Further, both compounds were also present in the ethyl acetate fraction of methanolic extract and the parent methanolic extract confirming that they are not artifacts formed during the isolation procedure. Their appearance in aqueous extract can be



	R_1	R_2
1		H
2	H	

explained by assuming that these molecules might have been entrapped or complexed with some water-soluble matrixes that were liberated in during the aqueous extractions.

Both the compounds gave positive test for the presence of triterpenoidal saponins. Their IR spectrum showed bands at 3435 (polyhydroxy system), 2943 (CH stretching), 1636 and 835 (C=C stretching), 1383, and 1063 cm^{-1} (OH). In ESI-MS spectrum, both showed signals at m/z 604. The fragmentation pattern showed signals at m/z 455 and m/z 473 indicating the presence of dammarane type of skeleton[1] and a signal indicating loss of a pentose moiety. The pentose was identified as L-arabinose during hydrolytic studies.

The ^{1}H NMR spectrum of **1** showed presence of 7 methyls [δ 0.83 (3H), 0.87 (3H), 1.02 (3H), 1.13 (6H), 1.68 (3H) 1.71 (3H)], a doublet at δ 5.15 for the trisubstituted double bond and a doublet at δ 4.26 (*J*=6.34 Hz) for anomeric proton. The ^{13}C NMR (**Table I**), DEPT and HMQC spectrum indicated the presence of 7 methyl, 9 methylene, 11 methine and 8 quaternary carbon atoms. The presence of a methine and quaternary carbon at δ 126.1 and 136.6 shows the presence of a trisubstituted double bond. Further, a methylene at δ 45.2 and a methine at δ 69.6 indicated the presence of a jujubogenin type of skeleton. The HMBC correlation indicated the long-range correlation between the H-21 and C-22, C-17, between H-22 and C-23, between H-23 and C-24, and between H-24 and C-26, C-27 and C-25. The detailed analysis of phase sensitive DQF-COSY indicated the ^{1}H-^{1}H coupling between the H-13 and H-17, between H-22 and H-23, between H-23 and H-24, between H-24 and H-26 and H-27. The ^{1}H-^{1}H COSY, and HMBC data, are shown in **Figure 1**, further confirming the finding. Glycosylation shift was observed at C-3 and the chemical shift values for arabinose indicated its existence in the pyranose form. The position of glycosylation was confirmed by the HMBC correlation between anomeric proton of arabinose and C-3. Based on these observations, the structure of **1** was assigned to be 3-*O*-α-L-arabinopyranosyl jujubogenin. This compound was reported as zizyotin by Mourya *et al.* from *Ziziphus oenoplea*[11]. The authors of the earlier study have not conclusively established the chemical structure as no elaborate spectroscopic data was reported. In the present study, the proposed structure has been confirmed along with the complete chemical and spectroscopic data in its support.

Table I—^{13}C NMR spectral characterization data of **1** and **2** recorded in CD_3OD

Carbon	**1**	**2**
1	39.6	39.6
2	27.0	27.7
3	90.4	79.2
4	40.3	38.3
5	57.2	55.3
6	19.0	19.1
7	37.1	37.4
8	38.4	39.8
9	54.0	53.9
10	38.2	38.3
11	22.4	22.2
12	29.1	28.9
13	37.9	36.8
14	54.5	54.5
15	36.8	36.7
16	111.3	110.9
17	54.2	56.8
18	19.2	19.1
19	16.7	16.6
20	69.4	76.4
21	29.6	25.1
22	45.3	41.7
23	69.6	69.5
24	126.2	126.3
25	136.7	136.1
26	25.8	25.9
27	18.4	18.5
28	28.4	28.5
29	16.8	16.0
30	66.8	66.8
Ara (p)		
1’	106.9	98.8
2’	72.7	72.8
3’	74.2	74.6
4’	69.3	69.8
5’	66.2	66.9

The ^{1}H NMR spectrum of **2** showed presence of 7 methyls [δ 0.76 (3H), 0.86 (3H), 0.95 (3H), 1.16 (3H), 1.21 (3H), 1.68 (3H) and 1.69 (3H)], a doublet at δ 5.13 for trisubstituted double bond and the anomeric proton at δ 4.42 (*J*=6.1 Hz). The ^{13}C NMR (**Table I**) and DEPT were very similar to that of compound **1** with difference in the value for C-3 and C-21. As depicted in **Figure 1**, presence of the jujubogenin skeleton was confirmed by HMBC correlations

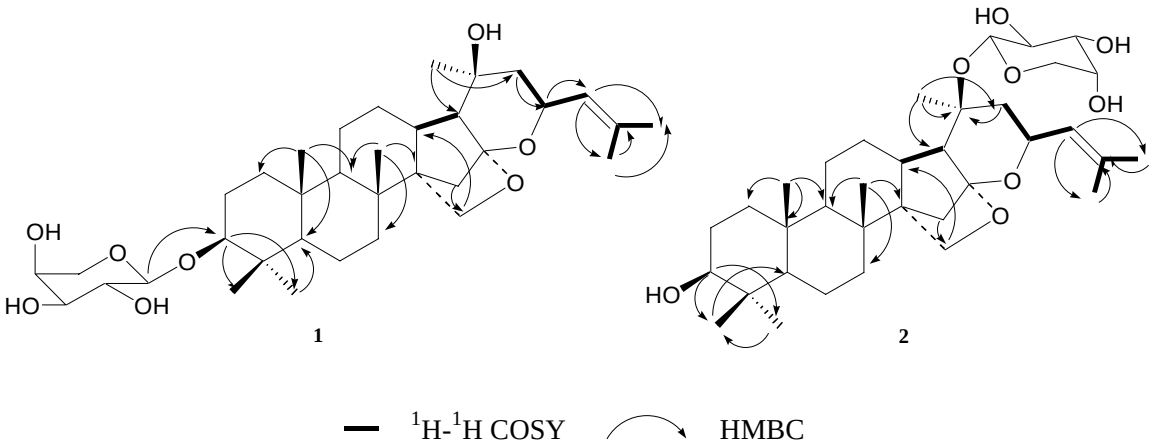


Figure 1—Key ^{1}H-^{1}H COSY and HMBC correlations

between H-21 and C-17, C-20, C-22, between H-22 and C-20, between H-24 and C-26, C-27 and DQF-COSY indicating the coupling between the H-13 and H-17, between H-22 and H-23, between H-23 and H-24, between H-24 and H-26 and H-27. The glycosylation shift was observed at and around C-20 appearing at δ 76.4 while the value of C-3 appeared at δ 79.2 for free hydroxyl functionality. The chemical shift value for anomeric carbon appeared at δ 98.8 as compared to δ 106.9 for **1**. Both values match with literature[6]. The above observations confirm the attachment of arabinose at C-20. Thus, **2** was assigned the structure as 20-*O*-α-L-arabinopyranosyl jujubogenin.

Compounds **1** and **2** were tested for their effect on superoxide release from human PMN cells in the NBT reduction assay. No significant activity was observed as compared to the activity exhibited by bacoside A_3 and standards *viz.* ascorbic acid and quercetin[10] (**Table II**). The arabinose at either C-3 or C-20 has no significant effect on the said bioactivity. It can be postulated that either the type of sugar, their position and/or number of sugars in glycosidic linkage contributes to the activity. But in the chemiluminescence assay, a small amount of activity was observed for both the compounds but **2** was found to be more active than **1** (**Table III**). The study indicates that it is primarily bacoside A_3, one of the major saponins of *B. monniera*, and not compounds **1** and **2**, that is responsible for inhibitory action on the process of respiratory oxyburst.

Experimental Section

ESI-MS (positive) was recorded by direct infusion on Finnigan Matt LCQ. ^{1}H NMR was recorded at 300 MHz and ^{13}C NMR at 90 MHz in CD_3OD, using TMS as internal standard on Bruker DPX 300 NMR spectrometer. IR on KBr disc was recorded on Impact 410 Nicolet IR spectrometer. HPLC was run on Waters Nova-pak C18 3.9×150 mm column using acetonitrile:water:methanol (60:30:10) as the mobile phase at a flow rate of 0.5 mL min^{-1} employing UV-detection at 215 nm. TLC was run on Merck Keiselgel F_{254} precoated plates. For saponin the chromatogram was developed using chloroform: methanol (90:10) as the mobile phase and visualized by spraying with anisaldehyde-H_2SO_4 reagent and further heating at 110°C. For sugars the chromatogram was developed using acetone: chloroform:water (85:10:5) as the mobile phase and visualized by spraying with Molisch reagent and further heating at 110°C.

Plant material

The aerial parts of *B. monniera* were collected from the NIPER medicinal plant garden and shade dried. Plant was identified by the corresponding author. A voucher specimen of this crop is preserved in the herbarium (NPH-entry number: 103) of Department of Natural Products (NIPER).

Extraction and isolation

The powdered material was defatted and then extracted with 70% MeOH (10×2.5 L) by maceration. The exhausted plant material was further extracted with de-ionised water, and the extract was freeze-dried. 100 g of aqueous extract was dissolved in 250 mL of deionised water and extracted with *n*-butanol. The *n*-butanol extract (5 g) was detannified by lead acetate treatment. The detannified fraction was then

Table II— NBT reduction assay

Compd	Concentration in μg/mL				
	10	25	50	100	200
1	ND	-1.15± 6.3	ND	2.76±4.5	1.56±12.86
2	ND	-3.30±5.7	ND	6.07±3.09	7.12±4.3
Bacoside A_3	45.5±5.5	83±5.26	91.66±5.52	91.66±5.52	ND
Quercetin	ND	4.6± 1.2	12.1±1.9	37.5+3.5	83.6±5.2
Ascorbic acid	24.0±0.3	61.63±1.4	69.16±0.4	75.15±2.2	78.50±2.7

All values are the percent inhibition of NBT reduction expressed as mean ± SEM; n=3, concentrations in μg/mL, ND: not done at this concentration.

Table III—Luminol-enhanced chemiluminescence assay

Compd	Concentration in μg/mL			
	12.5	25	100	200
1	ND	31.3±6.6	40.1±5.7	56.7±4.8
2	ND	44.9±1.7	81.9±.02	88.6±1.9
Bacoside A_3	53.2±2.4	83.2±3.3	99.8±0.2	99.9±0

All values are expressed as mean ± SEM of percent inhibition of luminescence; n=3, concentrations in μg/mL, ND: not done at this concentration.

subjected to column chromatography over silica gel, eluting with ethyl acetate:methanol:water (90:5:5). One of the non-polar fractions on repeated column purification over silica gel with chloroform:methanol (95:5) gave a mixture of crystalline solids. Two crystalline solids were isolated by column chromatography over silica gel using chloroform:water (95:5) as the mobile phase. Compounds **1** (15 mg) and **2** (20 mg) had R_t of 4.0 and 3.25 min in the HPLC and R_f of 0.44 and 0.33 on the TLC, respectively.

Acid hydrolysis

Compound **1** and **2,** 5 mg each were refluxed with 1N HCl for 1 h, freed of acid and extracted with $CHCl_3$. The sugars in aqueous hydrolysate were identified by comparative TLC.

Bacoside A_4 **1** was obtained as colorless needles from MeOH. IR (KBr): 3435, 2943, 1636, 1447, 1383, 1290, 1063, 1001 cm^{-1}; ^1H NMR (CD_3OD): δ 3.13 (dd, *J*= 4.3 and 7.2, H-3), 0.73 (bd, *J*=9.6, H-5), 2.47 (br m, H-13), 2.06 and 1.15 (d both *J*=8.5, H-15), 1.13 (s, 6H, H-18 and H-21), 0.87 (s, 3H, H-19), 0.83 (s, 3H, H-21), 4.67 (m, H-23), 5.15 (d, *J*=7.9, H-24), 1.71 (s, 3H, H-26), 1.68 (s, 3H, H=27), 1.02 (s, 3H, H-28), 0.83 (s, 3H, H-29), 3.97 (dd, *J*=7.0 and 18.6 H-30), 4.26 (d, *J*=6.34, ara H-1).

Bacoside A_5 **2** was obtained as colorless needles from MeOH. IR (KBr): 3444, 2930, 1636, 1448, 1386, 1289, 1067, 1006 cm^{-1}; ^1H NMR (CD_3OD): δ 3.12 (dd, *J*= 6.1 and 9.8, H-3), 0.69 (br d, *J*=9.4, H-5), 2.7 (br m, H-13), 2.20 and 1.1 (d both *J*=8.7, H-15), 1.07 (br d *J*=6.1 H-17), 1.16 (s, H-18), 0.86 (s, H-19), 1.21 (s, H-21), 4.9 (m, H-23), 5.13 (d, *J*=8.49, H-24), 1.69 (s, H-26), 1.68 (s, H-27), 0.95 (s, H-28), 0.76 (s, H-29), 3.98 (dd, *J*=7.2 and 26.3, H-30), 4.42 (d, *J*=6.1, ara H-1).

Biological assay

(i) The nitroblue tetrazolium (NBT) reduction assay was performed as per the modified method reported earlier[10].

(ii) The luminol-enhanced chemiluminescence assay was performed and standardized according to the reported method[12]. The percent inhibition of luminescence was calculated using the formula,

% inhibition of luminescence = [(Control – Sample)/Control] × 100.

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