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A triterpenoid saponin possessing antileishmanial activity from the leaves of *Careya arborea*

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Abstract

Bioguided-fractionation of the methanol extract of the leaves of *Careya arborea* led to isolation of a triterpenoid saponin, designated arborenin, and characterized as 3-*O*-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-2α,3β-dihydroxy-taraxast-20-en-28-oic acid (**1**), together with desacylescin III (**2**). The structures were determined on the basis of extensive 2D NMR spectroscopic analysis. The saponin showed in vitro antileishmanial activity against *Leishmania donovani* (strain AG 83).

Keywords: *Careya arborea*; Triterpenoid saponin; Arborenin; Desacylescin III barringtogenol C; Barringtogenol D; 2D NMR

1. Introduction

Careya arborea is a handsome deciduous tree, widely distributed throughout the greater part of India. The different parts of the plant enjoy considerable reputation in Indian medicine as astringent and tonic, and are used as antipyretic and antipruritic in eruptive fevers (Phondke, 2000). Previous phytochemical investigations on the leaves and seeds of the plant were limited to the isolation of few sterols and rearranged triterpenes (Row and Sastry, 1964; Das and Mahato, 1982). In continuation of our work on chemical studies on naturally occurring bioactive saponins (Sahu et al., 2002) we report herein the isolation and characterization of a novel triterpenoid saponin, designated as arborenin, with antileishmanial activity, from the methanolic extract of the leaves of this medicinal plant, along with a known triterpenoid glycoside, desacylescin III.

2. Results and discussion

The *n*-BuOH soluble fraction obtained from defatted MeOH extract of *C. arborea* leaves showed moderate in vitro antileishmanial activity against *Leishmania donovani* (strain AG 83). Bioguided fractionation of the active fractions eluted with MeOH–H_2O mixture from the Diaion HP-20 chromatography led to isolation of two triterpenoid saponins.

Arborenin (**1**) has the molecular formula $C_{42}H_{68}O_{14}$ as determined from its high resolution positive ion FABMS (*m*/*z* 819.4457 $[M + Na]^+$). Of the 42 carbon signals displayed in the ^{13}C NMR spectrum, 12 could be assigned to the carbohydrate moiety (10 methine and 2 methylene signals), whereas the aglycone accounted for seven singlets, eight doublets, eight triplets and seven quartets. The ^1H NMR spectrum displayed seven methyl signals, of which six were singlets (δ 0.87, 1.03, 1.04, 1.18, 1.31, 1.73) and one doublet (δ 1.11, $J = 6.6$ Hz). Additional signals observed include those ascribed to an olefinic proton (δ 5.48, *t*, $J = 7.8$ Hz), two oxy methine protons (δ 3.24, *d*, $J = 9.4$ Hz; 4.04, *m*) and two anomeric protons (δ 4.93, *d*, $J = 7.6$ Hz; 5.49, *d*, $J = 7.6$ Hz). These data suggested that

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the aglycone part of **1** was likely to be a pentacyclic triterpene with two hydroxyl groups and a trisubstituted double bond. Comparison of ^{13}C NMR data (Table 1) with those of known triterpenes (Dai et al., 2001; Reynolds et al., 1986) suggested that **1** belongs to the taraxastane series. The ^{1}H-^{1}H COSY spectrum of **1** showed a coupling interaction between the signals of δ 3.24 and δ 4.04, which were assigned to two hydroxyl groups at C-3 and C-2 positions. The HETCOR spectrum showed that the corresponding carbon signals were at δ 95.8 and 66.9, respectively. The assignment of hydroxyl groups to C-2 and C-3 was further confirmed from the HMBC correlations (Fig. 1) between the signal for C-2 and those of H-1, H-3 and H-23; similarly, the signal for C-3 showed correlation with those for H-1, H-2, H-23 and H-24. Both the hydroxyl groups at C-2 and C-3 must be equatorial as evident from the large coupling constant ($J = 9.4$ Hz) for H-3. Furthermore, the H-2 signal showed cross peaks in the NOESY spectrum with H-24 (δ 1.18), H-25 (δ 0.87), and H-1β (δ 2.37) signals, while that of H-3 was correlated with signals for H-1α (δ 1.12) and H-5 (δ 0.82). The ^{13}C signal at δ 178.2, assigned to a carboxyl group, showed HMBC cross peaks with H-18, H-16 and H-22 signals, justifying its assignment to C-28. The HMBC correlations of the signals for C-20 with H-19, H-22, H-29 and H-30 signals, and C-21 with H-19, H-22 and H-30 signals suggested the position of the double bond in the triterpene core (Fig. 1). The relative orientations of the remaining protons of **1** were established from coupling patterns and cross peaks generated from the phase sensitive NOESY spectrum. For example, the NOESY connectivities for H-5α with H-9 and H-1α, and for H-27 with H-18 and H-15α showed the spatial proximity between these protons. Using COSY, HOHAHA, HMBC and NOESY information, assignments for α and β protons of other carbons were also made. From the foregoing evidences it was concluded that the triterpene core of **1** was 2α,3β-dihydroxy-taraxast-20-en-28-oic acid.

Table 1
^{13}C NMR chemical shifts[a] of **1**, **3** and **5** in pyridine-d_5

Carbon no.	**1**	**3**	**5**		**1**
1	47.8 *t*	48.2 *t*	39.1 *t*	Glucose-1	
2	66.9 *d*	68.9 *d*	28.1 *t*	1′	104.6 *d*
3	95.8 *d*	83.8 *d*	78.1 *d*	2′	82.5 *d*
4	41.2 *s*	39.9 *s*	39.4 *s*	3′	78.6 *d*
5	55.8 *d*	56.1 *d*	55.7 *d*	4′	71.3 *d*
6	18.5 *t*	18.7 *t*	18.8 *t*	5′	78.4 *d*
7	34.6 *t*	34.3 *t*	32.5 *t*	6′	62.4 *t*
8	41.0 *s*	40.9 *s*	40.3 *s*	Glucose-2	
9	51.0 *d*	50.9 *d*	47.3 *d*	1″	105.7 *d*
10	38.0 *s*	38.7 *s*	37.3 *s*	2″	76.7 *d*
11	22.1 *t*	22.3 *t*	23.9 *t*	3″	78.2 *d*
12	29.6 *t*	25.4 *t*	122.6 *d*	4″	72.0 *d*
13	39.4 *d*	43.2 *d*	144.4 *s*	5″	78.3 *d*
14	42.3 *s*	41.4 *s*	41.1 *s*	6″	63.0 *t*
15	27.9 *t*	27.7 *t*	30.8 *t*		
16	33.7 *t*	28.1 *t*	76.6 *d*		
17	49.1 *s*	42.2 *s*	49.7 *s*		
18	49.4 *d*	48.4 *d*	41.1 *d*		
19	37.9 *d*	42.4 *d*	41.0 *t*		
20	143.1 *s*	83.9 *s*	38.1 *s*		
21	117.9 *d*	27.3 *t*	90.7 *d*		
22	38.5 *t*	32.3 *t*	76.4 *d*		
23	28.3 *q*	29.2 *q*	28.8 *q*		
24	17.7 *q*	17.5 *q*	16.6 *q*		
25	17.6 *q*	17.8 *q*	15.5 *q*		
26	16.4 *q*	15.9 *q*	17.2 *q*		
27	15.0 *q*	14.4 *q*	29.0 *q*		
28	178.2 *s*	176.7 *s*	61.0 *t*		
29	23.7 *q*	18.6 *q*	29.8 *q*		
30	22.2 *q*	24.1 *q*	28.8 *q*		

[a] Assignments based on ^{13}C, DEPT, HSQC, HMBC experiments and multiplicities as determined by DEPT.

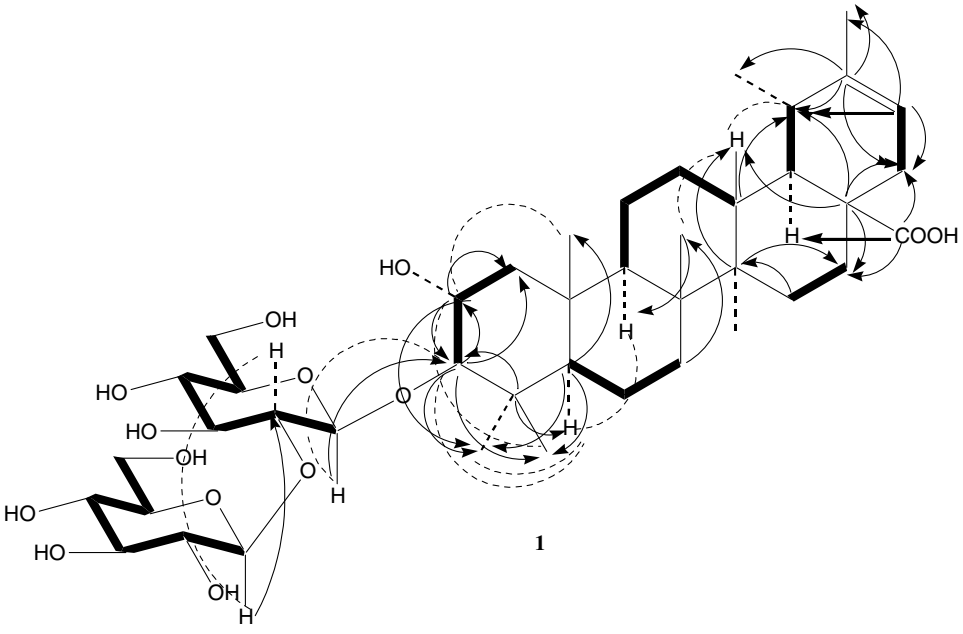


Fig. 1. COSY (▬) HMBC (→) NOESY (-----) correlations of **1**.

Attempted enzymatic hydrolysis of arborenin (**1**) was precluded due to solubility problem. When hydrolysis was carried out with mineral acid it afforded an aglycone (**3**) and only D-glucose as sugar component identified by GLC analysis of the hydrolysate after derivatization. Although most of the ^{13}C NMR signals of **3** showed similarity with that of **1**, differences were noticed for signals attributed to ring E. The presence of a δ-lactone ring was suggested by the appearance of an absorption peak at 1742 cm^{-1} in its IR spectrum and a signal at δ 176.7 in the ^{13}C NMR spectrum. The HMBC spectrum displayed cross peaks between C-28 and H-18 (δ 1.04), H-16 (δ 1.22) and H-22 (δ 1.50) signals, as also between C-20 and H-29 (δ 0.88), H-30 (δ 1.27) and H-19 (δ 1.57) signals, providing confirmation for a δ-lactone ring between C-20 and C-28. It is presumed that the aglycone was rearranged during acid hydrolysis to a six membered lactone. Thus the structure of **3** was deduced as 2α,3β-dihydroxy-taraxastan-28,20β-olide. It is worthy of mention that Das and Mahato (1982) isolated the compound (careyagenolide) from the acid hydrolysis of the ethanolic extract of the leaves of the plant. The NMR spectral data of **3** is presented in Tables 1 and 2.

The interglycosidic and sugar–aglycone linkages as well as signal and structural assignments of the sugars in **1** were established on the basis of the following arguments. The anomeric proton signals (H-1′ and H-1″) appeared as doublets ($J = 7.6$ Hz,) indicating β-configuration ($^4C_1$ conformation) for both the glucopyranosyl units. The observed HMBC connectivities of the signal for H-1″ (δ 5.49) of the terminal glucose with that of C-2′ (δ 82.5) of the inner glucose, and of the signal for H-1′ (δ 4.93) of the inner glucose with that of C-3 (δ 95.8) of the aglycone moiety established the sugar–sugar and sugar–aglycone linkages in **1**. The results of NOESY experiment also supported the proposed linkages. The absolute configurations of the monosaccharides were chosen in keeping with those mostly encountered among the plant glycosides. From the foregoing evidences, the structure of arborenin was elucidated as 3-*O*-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-2α,3β-dihydroxy-taraxast-20-en-28-oic acid (**1**).

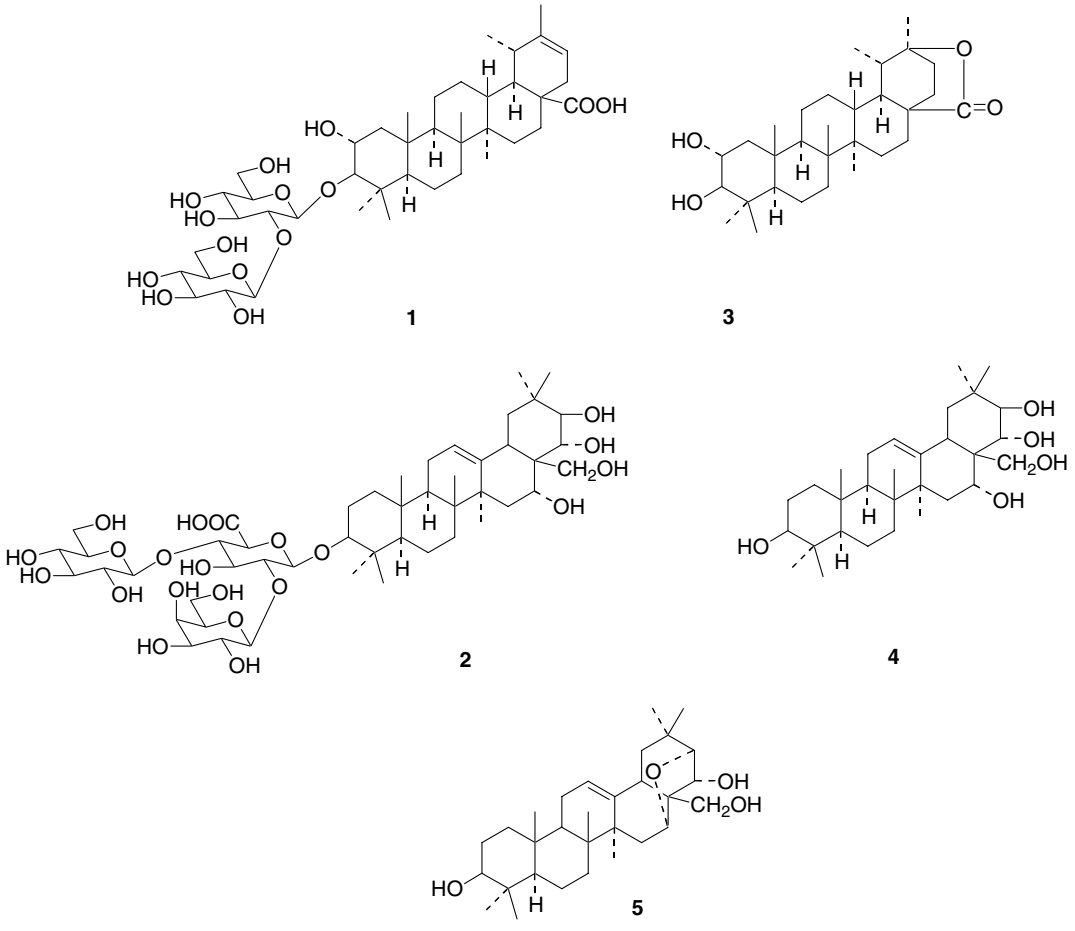
Compound **2** showed in its MALDI-TOF MS the $[M + Na]^+$ ion at *m*/*z* 1013 indicating the molecular weight to be 990. Of the 48 carbon signals displayed in the ^{13}C NMR, 30 were assigned to the aglycone part and 18 to the oligosaccharide moiety. The presence of two sp^2 carbon signals at δ 122.4 (*d*) and 143.7 (*s*), coupled with the information from ^1H NMR (six methyl singlets and a vinyl proton triplet) indicated that the aglycone is likely to possess an olean-12-ene skeleton. Acid hydrolysis of **2** furnished two aglycones, one major and one minor, together with glucuronic acid, glucose and galactose identified by GLC. The major aglycone was identified as barringtogenol C (**4**) by comparing its ^{13}C NMR chemical shifts with those reported in the literature (Pal et al., 1994). The minor product (**5**) had a molecular formula $C_{30}H_{48}O_4$ as determined from ^{13}C NMR data and the observation of a sodiated-molecular ion peak $[M + Na]^+$ at *m*/*z* 495.07 in the positive

Table 2
^1H NMR spectral data of **1** (aglycone part), **3** and **5** (pyridine-d_5)[a], with *J* value in Hertz

Carbon no.	1	3	5
1	1.12 (+), 2.37 (*dd*, 4.6, 12.8)	1.25 (+), 2.36 (*dd*, 4.4, 12.4)	1.02 (*m*), 1.59 (+)
2	4.04 (*m*)	4.13 (*m*)	1.87 (+), 1.87 (+)
3	3.24 (*d*, 9.4)	3.41 (*d*, 9.4)	3.46 (*dd*, 5.5, 10.5)
5	0.82 (*d*, 11.7)	0.96 (*m*)	0.88 (br *d*, 11.9)
6	1.45 (+), 1.29 (+)	1.36 (+), 1.54 (+)	1.41 (*dq*, 3.2, 12.4), 1.59 (+)
7	1.33 (+), 1.33 (+)	1.36(+), 1.36 (+)	1.27 (*m*), 161 (+)
9	1.45 (+)	1.44 (*dd*, 3.0, 12.8)	1.73 (+)
11	1.29 (+), 1.62 (br *d*, 10.5)	1.17 (*m*), 1.57 (+)	1.87 (+), 1.90 (*m*)
12	1.18 (+), 1.79 (+)	1.54 (+), 1.54 (+)	5.35 (*t*-like)
13	2.83 (*t*, 12.5)	1.25 (+)	–
15	1.18 (+), 1.79 (+)	1.10 (*m*), 2.29 (*dt*, 4.3, 13.3)	1.78 (*m*), 1.87 (+)
16	1.53 (*dt*, 2.8, 13.4), 2.32 (*m*)	1.22 (*m*), 2.02 (*m*)	4.92 (br *d*, 3.2)
18	1.29 (+)	1.04 (*dd*, 5.5, 11.7)	2.98 (*dd*, 6.4, 11.9)
19	2.44 (*t*, 6.6)	1.57 (+)	1.73 (+), 1.82 (*m*)
21	5.48 (*d*, 7.8)	1.51 (+), 1.80 (*m*)	3.92 (*s*)
22	2.0 (br *d*, 14.0), 2.64 (*dd*, 7.1, 15.4)	1.50 (+), 1.50 (+)	4.70 (*s*)
23	1.31 (*s*)	1.26 (*s*)	1.24 (*s*)
24	1.18 (*s*)	1.08 (*s*)	1.06 (*s*)
25	0.87 (*s*)	0.92 (*s*)	0.94 (*s*)
26	1.03 (*s*)	0.91 (*s*)	0.90 (*s*)
27	1.04 (*s*)	0.89 (*s*)	1.71 (*s*)
28	–	–	3.88, 4.22 (both *d*, 10.5)
29	1.11 (*d*, 6.6)	0.88 (*d*, 6.9)	1.0 (*s*)
30	1.73 (*s*)	1.25 (*s*)	1.18 (*s*)

(+, overlapped with other signals).
[a] Assignments based upon COSY, TOCSY, HETCOR, NOESY, DEPT and HMBC.

ion MALDITOF mass spectrum. Comparison of the 1D and 2D NMR spectra of **5** with those of **4** revealed that **5** possesses a similar structure except for the rings D and E. The ^{1}H NMR spectrum showed the signals for seven methyls (δ 0.90, 0.94, 1.00, 1.06, 1.18, 1.24 and 1.71, all *s*), one olefinic proton (δ 5.35, *t*-like), two hydroxy/oxy methylene protons (δ 3.88 and 4.22, $2\times d$, $J = 10.5$ Hz), four hydroxy/oxy methine protons (δ 3.46, *dd*, $J = 5.5$, 10.5 Hz; 3.92, *s*; 4.70, *s*; 4.92, br *d*, $J = 3.2$ Hz). One of the hydroxyl groups could be assigned to C-3 on biogenetic grounds as well as from the HMBC correlation of the proton signal (δ 3.46) with C-4, C-23 and C-24 signals. The presence of another hydroxyl group at C-22 was established from the HMBC correlations between the H-22 signal (δ 4.70) and those of C-21, C-20, C-18, C-17 and C-16. A hydroxy methyl nature of C-28 could be inferred from the observed HMBC cross peaks between signals of 28-H$_2$(δ 3.88, 4.22) and those of C-18, C-16 and C-22. Thus only one more oxygen atom remained to be assigned. The downfield chemical shift attributed to H-16 (δ 4.92) and H-21 (δ 3.92) as well as to the corresponding carbons (Table 1) suggested that both these carbons are oxygenated. Since the molecular formula allowed only one oxygen atom, the presence of an ether linkage between C-16 and C-21 appeared likely. This was confirmed from the HMBC cross peaks observed between the 21-H (δ 4.70) and C-16 (76.6) signals. Dreiding model studies revealed that such a ring formation is likely between C-16$_\alpha$ and C-21$_\alpha$ bonds. The model studies also suggested H-22 to be β-oriented, since the 21-H-22-H dihedral angle is then nearly 90°, which explains the singlet nature of the two proton signals. The α-orientation of 22-OH group is also supported by the NOESY relationship observed between H-22 and H-30 signals. Thus the structure of **5** was established as 16α,21α-epoxy-olean-12-en-3β,22α,28-triol (barringtogenol D). It is noteworthy that barringtogenol D was obtained when barringtogenol C was refluxed with hydrochloric acid (Chakraborti and Barua, 1963; Nakano et al., 1969). It is presumed that barringtogenol D was obtained during acid hydrolysis of **2**. These evidences as well as the ^{13}C NMR chemical shifts (Table 1) of compound **2** suggested its identity with desacylescin III (Yoshikawa et al., 1996).



3. Antileishmanial activity

To determine whether any of the compounds (**1**–**5**) had any effect on the growth of *L. donovani* AG 83, promastigotes (2.5×10^6 cells ml^{-1}) were exposed to various concentrations of the compounds for 7 days. The numbers of live promastigotes were counted by trypan blue exclusion. Compound **1** was found to inhibit the growth in a dose

dependent manner. At a concentration of 15 μg ml^{-1} **1** inhibited the growth by 55% on the 2nd day, 70% growth was inhibited on day 4 and 75% on day 7. The other compounds **2**–**5** virtually had no effect on the survival of *L. donovani* AG 83 promastigotes up to a concentration of 30 μg ml^{-1}. The IC_{50} values for **1** and amphotericin B (positive control) were calculated to be 15 and 0.22 μg ml^{-1} which varied inversely with incubation time.

The protozoan parasite *L. donovani* survives and multiplies within mammalian macrophages. It is, therefore, of interest to test the efficacy of the compounds on intracellular amastigotes. Peritoneal macrophages of BALB/c mice were infected with *L. donovani* AG 83 promastigotes in vitro. Infected macrophages after subsequent washings were incubated with different concentrations of the compounds (**1**–**5**). Similar to its effect on promastigotes, compound **1**, at a concentration of 12.5 μg ml^{-1}, reduced the parasite burden by 57% on day 1 and 77% on day 2. The compounds (**2**–**5**) reduced intracellular parasite burden only marginally, compared with medium or DMSO (Table 3). The IC_{50} of inhibition was calculated to be 12.5 and 0.10 μg ml^{-1} for arborenin (**1**) and amphotericin B, respectively.

Cytotoxicity of the compounds was examined by 18 h ^{51}Cr-release assays with radiolabelled promastigotes and amastigotes of *L. donovani* in the presence of varying concentrations of these compounds. The antileishmanial drug sodium antimony gluconate (SAG) was used for comparison and normal human peripheral blood mononuclear cells (PBMC) were used as a control for nonspecific toxicity. Up to a concentration of 0.1% (v/v), DMSO was found to be nontoxic to human PBMC or *L. donovani* promastigotes and amastigotes. None of the compounds (**1**–**5**) and SAG was found to be toxic to human PBMC up to a concentration of 20 mg l^{-1}. However, compound **1** was found to have significant toxicity on both promastigotes and intracellular amastigotes.

Table 3
In vitro antileishmanial activity of compounds (**1**–**5**) on intracellular amastigotes of *L. donovani* at day 1 of drug treatment[a]

Treatment	Dose	Amastigotes/100 nuclei (mean ± SD)
Medium	–	527.33 ± 31.34
DMSO	0.20%	520.33 ± 10.50
Amphotericin B	0.10 μg ml^{-1}	256.04 ± 14.50[b]
1	12.5 μg ml^{-1}	272.00 ± 17.88[b]
2	30.0 μg ml^{-1}	493.52 ± 10.20
3	30.0 μg ml^{-1}	501.32 ± 14.60
4	30.0 μg ml^{-1}	488.35 ± 19.60
5	30.0 μg ml^{-1}	498.78 ± 25.30

Statistical analyses were performed by one way ANOVA followed by Dunnett's post-hoc test.

[a] Thioglycolate elicited peritoneal macrophages of BALB/c mice were infected in vitro with *L. donovani* amastigotes and incubated with graded concentrations 0.1–30 μg ml^{-1} of **1**–**5** (dissolved in DMSO) for 1 day at 37 °C for the determination of antileishmanial activity on intracellular amastigotes.

[b] $P < 0.01$ verses medium control; mean ± SD of triplicate determination.

4. Experimental

4.1. General procedures

All melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were determined using JASCO 7300FTIR spectrometer. Optical rotations were measured using a JASCO DIP-370 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively using a Jeol ECP-500 spectrometer in C_5D_5N with TMS as internal standard. HRFABMS (positive) was performed on a JEOL MS-700 mass spectrometer. MALDI-TOF MS (positive) was conducted using Perseptive Biosystems Voyager DESTR mass spectrometer. TLC was carried out on silica gel 60 F_{254}, and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. Diaion HP-20 (Mitsubishi Chemicals) and silica gel (silica gel 60, Merck) were used for column chromatography. GLC was performed on a Hewlett–Packard model 5730A instrument using 2% SE-30 on Chromosorb W (60–80 mesh), 3 mm i.d. × 1.5 m, 150 °C column temperature, nitrogen as carrier gas with a flow rate of 15 ml/min.

4.2. Plant material

The plant material was collected from Indian Botanic Garden, Howrah, and identified by Dr. (Ms.) Debjani Basu, Botanist, at Indian Botanic Garden, Howrah, West Bengal, India. A voucher specimen was deposited at the Steroids and Terpenoids Chemistry Department, Indian Institute of Chemical Biology, Kolkata.

4.3. Extraction and isolation

The defatted, air dried powdered leaves of *C. arborea* (1 kg) were extracted successively with MeOH (3x5lt) at ambient temperature. The combined MeOH extract was concentrated, macerated with water, and then partitioned with *n*-BuOH (2 × 1 l). The *n*-BuOH soluble part (20 g) was applied to a column of Diaion HP-20 (400 g) and washed with water followed by 30%, 50%, 80% and 100% of MeOH. Fraction eluted with 80% methanol (2.5 g) was chromatographed over silica gel (70 g). Graded elution was carried out with chloroform followed by various mixtures of $CHCl_3$–MeOH (9:1, 4:1, 7:3, and 3:2). A total of 30 fractions (each 75 ml) were collected and fractions giving similar spots on TLC were combined. Fractions eluted with $CHCl_3$–MeOH (7:3) were combined together and subjected to rechromatography over silica gel (15 g). Fractions (collected in 15 ml lots) eluted with $CHCl_3$–MeOH mixture (3:1) furnished arborenin (65 mg). Further purification of the fraction eluted with $CHCl_3$–MeOH mixture (3:2) over silica gel (10 g) with $CHCl_3$–MeOH mixture (13:7) gave desacylescin III (62 mg).

4.4. Arborenin (*1*)

Colourless needles from MeOH, m.p. 310–312 °C (dec); $[\alpha]_D^{21}$ −22.3° (*c* 0.583, MeOH); IR: ν_{max} cm^{-1} (KBr): 3414, 2370, 1628, 1515, 1455, 1377, 1161, 1064 and 660; HRFAB MS: *m/z* observed 819.4457, calc. for $C_{42}H_{68}O_{14}Na$, 819.4507; MALDI-TOF-MS (positive): *m/z* 819.17 $[M + Na]^+$; ^{1}H NMR (C_5D_5N): aglycone part (Table 1); sugar moiety: inner glucose: 4.93 (*d*, *J* = 7.6 Hz, H-1′), 4.29$^+$ (H-2′), 4.26$^+$ (H-3′), 4.14 (*t*, *J* = 7.8 Hz, H-4′), 4.04 (*m*, H-5′), 4.58 (*dd*, *J* = 2.0, 11.6 Hz, H-6′$_a$), 4.29$^+$ (H-6′$_b$); Outer glucose: 5.49 (*d*, *J* = 7.6 Hz, H-1″), 4.14 (*t*, *J* = 7.8 Hz, H-2″), 4.31$^+$ (H-3″), 4.30$^+$ (H-4″), 3.95 (*m*, H-5″), 4.50 (*dd*, *J* = 2.9, 11.5 Hz, H-6″$_a$) and 4.44 (dd, *J* = 4.4, 11.4 Hz, H-6″$_b$), ($^+$overlapped with other proton signals); ^{13}C NMR (C_5D_5N): (Table 2). (Found: C, 63.37; H, 8.54; $C_{42}H_{68}O_{14}$ requires: C, 63.30; H, 8.60%.)

4.5. Acid hydrolysis of *1*

Arborenin (**1**) (25 mg) was hydrolyzed with 2 M HCl in aq. MeOH (10 ml) at 95 °C for 2.5 h on a boiling water-bath, cooled, and 5 ml water was added. Methanol was removed, and the solution was extracted with EtOAc (5 ml × 3). The solvent was washed with water and dried to give a white powder. Purification of the product over silica gel column and subsequent crystallization from MeOH afforded the aglycone (**3**, 11 mg), m.p. 304 °C, $[\alpha]_D^{21}$ +4.76° (*c*, 0.42, MeOH); IR: ν_{max} cm^{-1} (KBr): 3430, 2372, 2336, 1742, 1628, 1552, 1518, 1455, 1379, 1232, 1047, 959 and 614. MALDI-TOF-MS (positive) *m/z* 495.1615 $[M + Na]^+$; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2). (Found: C, 76.29; H, 10.19; $C_{30}H_{48}O_4$ requires: C, 76.23; H, 10.24%.)

The aqueous part of the acid hydrolyzate was neutralized by passing through an anion exchange resin (Amberlite MB-3) column, concentrated and then treated with 1-(trimethylsilyl) imidazole at room temperature for 2.5 h. After excess reagent was decomposed with water, the reaction product was extracted with hexane (3 ml × 3). The TMSi derivative of the monosaccharide obtained after removal of the solvent was identified to be that of D-glucose from GLC analysis.

4.6. Desacylescin III (*2*)

Colourless crystals from CH_3CN–MeOH–water, m.p. 228-230 °C, $[\alpha]_D^{21}$ +14.8° (*c* 0.615, MeOH); MALDI-TOF-MS (positive): *m/z* 1013.29 $[M + Na]^+$. Identical with an authentic sample on the basis of ^{1}H and ^{13}C NMR (C_5D_5N) comparison (Yoshikawa et al., 1996).

4.7. Acid hydrolysis of *2*

A solution of **2** (40 mg) was subjected to acid hydrolysis by the same procedure as described for **1**. Usual work up followed by chromatographic purification on a silica gel column furnished two compounds (**4**, 16 mg) and (**5**, 9 mg). Compound **4** was identified as barringtogenol C on the basis of spectral comparison (Pal et al., 1994). Compound **5** was crystallized from MeOH as fine needles, m.p. 299–302 °C; $[\alpha]_D^{21}$ +10.93° (*c* 1.50, MeOH); IR: ν_{max} cm^{-1} (KBr): 3389, 2370, 2336, 1645, 1462, 1378, 1101, 1039, 784 and 647; MALDI-TOF-MS (positive) *m/z* 495.0778 $[M + Na]^+$; ^{1}H NMR (Table 1); ^{13}C NMR (Table 2). (Found: C, 76.32; H, 10.21; $C_{30}H_{48}O_4$ requires: C, 76.23; H, 10.24%.)

The acid hydrolyzate was worked up following the same procedure as described for **1**. The monosaccharides were identified as D-glucuronic acid, D-glucose and D-galactose by GLC analysis on comparison with authentic samples.

4.8. Parasite culture and growth conditions

L. donovani strain AG 83 was originally obtained from an Indian kala-azar patient (Ghosh et al., 1983) and maintained in golden hamsters. Amastigotes were isolated from spleens of *L. donovani* infected golden hamsters as described (Jaffe et al., 1984). The spleen was rinsed in ice-cold phosphate buffered saline (PBS), glucose (55 mM)/EDTA (2 mM), lightly homogenized, macroscopic particles were allowed to settle. The turbid suspension was decanted, centrifuged at 100*g* for 10 min at 4 °C. The amastigote enriched suspension was centrifuged at 800*g* for 10 min. The pellet was suspended in 45% Percoll (8.0 ml), and finally 25% Percoll (4.0 ml) was layered over the amastigote suspension and further centrifuged at 5000*g* for 1 h. The band containing amastigotes was taken and washed with PBS (3×) and finally resuspended in Medium-199 (Gibco Laboratories, New York, NY, USA), supplemented with 20% FBS. Promastigotes were obtained by transforming amastigotes and were maintained in vitro in Medium-199 supplemented with 8% FBS.

4.9. Isolation of normal human peripheral blood mononuclear cells

PBMC were separated from heparinized whole blood of normal donors by Ficoll–Hypaque density gradient centrifugation as described (Boyum, 1968).

4.10. In vitro growth of L. donovani promastigotes in the presence of compounds (*1*–*5*)

Promastigotes (1×10^6) were incubated with or without various concentrations of the compounds or standard antileishmanial drugs in Medium-199 (1.0 ml) supplemented with 8% FBS at 22 °C. Growth of promastigotes was monitored by counting the number of motile promastigotes microscopically.

4.11. In vitro infection of BALB/c mice peritonial macrophages

Thioglycolate-elicited peritoneal exudate was used as the source of macrophages for better recovery and easier isolation. Approximately 2.5×10^5 macrophages were allowed to adhere to glass coverslips (20×25 mm) in Rose-Well Park Memorial Institute (RPMI)–1640 medium (Gibco Laboratories) supplemented with 10% FBS, and cultured for 5–7 days at 37 °C in 5% CO_2 before in vitro infection with *L. donovani*. Stationary phase *L. donovani* promastigotes (5.0×10^6) were added to each coverslip and incubated for 6 h at 37 °C in CO_2.

4.12. Antileishmanial activity of compounds (1–5) on L. donovani infected BALB/c mice macrophages in vitro

Cover slips were washed (3×) with 10% FBS-supplemented RPMI-1640 to remove uningested parasites and incubated for 2 days in the presence or absence of graded concentrations of compounds and amphotericin B (Sigma Chemical Co., USA). Infected macrophage cultures were washed with FBS, fixed with pre-chilled methanol, stained with Giemsa, and examined microscopically under oil immersion. At least 400 target macrophages were examined for each coverslip. Antileishmanial activity was determined by calculating the number of amastigotes per 100 macrophages.

4.13. Cytotoxicity assays

In vitro grown promastigotes, freshly purified amastigotes, or normal human PBMC (2×10^6 of each) were washed and resuspended in Medium-199 (1.0 ml) containing 8% FBS. Fifty microcuries of sodium chromate-^{51}Cr ($Na_2{}^{51}CrO_4$, BARC, Bombay, India) were added to promastigotes or purified amastigotes and incubated for 6 h at 22 °C. Normal human PBMC were incubated with $Na_2{}^{51}CrO_4$ for 2 h at 37 °C with gentle shaking at 15 min interval. Radiolabelled promastigotes, amastigotes, or normal human PBMC were washed (4×) and resuspended in Medium-199 supplemented with 8% FBS at a concentration of 1.0×10^6 ml^{-1}. Various concentrations (10, 5, 2.5 mg ml^{-1}) each of compounds (**1**–**5**) or sodium antimony gluconate were added to radiolabelled promastigotes or amastigotes (0.5×10^6) in a total volume of 1.0 ml in 5.0 ml tubes, and to radiolabelled normal human PBMC (0.2×10^5) in a total volume of 0.2 ml in microtitre plates. After 18 h of incubation at 37 °C, promastigotes or amastigotes were centrifuged, 0.5 ml aliquots of cell-free supernatants were collected and counted in a gamma counter. For normal human PBMC, 0.1 ml of supernantants were collected from each well without disturbing the cell pellet. The percentage ^{51}Cr release was calculated. The spontaneous release was that obtained from promastigotes, amastigotes, or normal human PBMC incubated with the medium alone, the experimental release was that obtained from these cultures incubated with compounds (**1**–**5**) or sodium antimony gluconate, and the total release was that obtained from these cultures incubated with 1 N HCl. In 18 h assays, the spontaneous release never exceeded 30% of the total release from either form of the parasite or normal human PBMC.

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