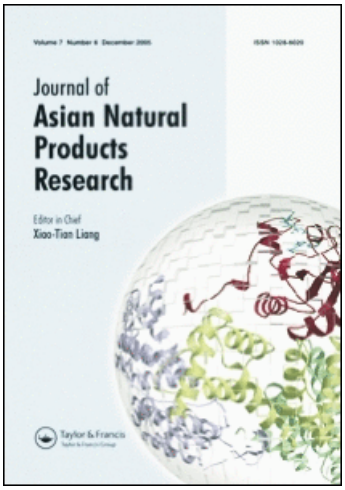

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Terminoside A, a new triterpene glycoside from the bark of *Terminalia arjuna* inhibits nitric oxide production in murine macrophages

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TERMINOSIDE A, A NEW TRITERPENE GLYCOSIDE FROM THE BARK OF *TERMINALIA ARJUNA* INHIBITS NITRIC OXIDE PRODUCTION IN MURINE MACROPHAGES

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Terminoside A (**1**), a new oleanane-type triterpene was isolated from the acetone fraction of the ethanolic extract of stem bark of *Terminalia arjuna*. The structure was established as olean-1α,3β,22β-triol-12-en-28-oic acid-3β-D-glucopyranoside. On the basis of spectral data and chemical reactions, terminoside A, potently inhibited nitric oxide (NO) production and decreased inducible nitric oxide synthase (iNOS) levels in lipopolysaccharide-stimulated macrophages.

Keywords: *Terminalia arjuna*; Triterpene glycoside; Terminoside A; Nitric oxide

INTRODUCTION

Stem bark of *Terminalia arjuna* (Roxb.) Wight & Arnot. (Combretaceae) has been used for centuries in India's native Ayurvedic system of medicine as an excellent cardiotonic [1]. Various pharmacological studies have deciphered the bark to be effective against angina pectoris, congestive heart failure, artherosclerosis and myocardial necrosis [2,3]. In view of promising value of *T. arjuna* bark as a cardioprotectant, extensive research has been carried out on it and a number of terpenoid saponins (arjunic acid, arjunolic acid, arjungenin, arjunglycosides), flavonoids (arjunone, arjunolone, luteolin), gallic acid, ellagic acid and phytosterol have been isolated from the bark [4] but the issue of active principles and their mechanism of action remain to be elucidated.

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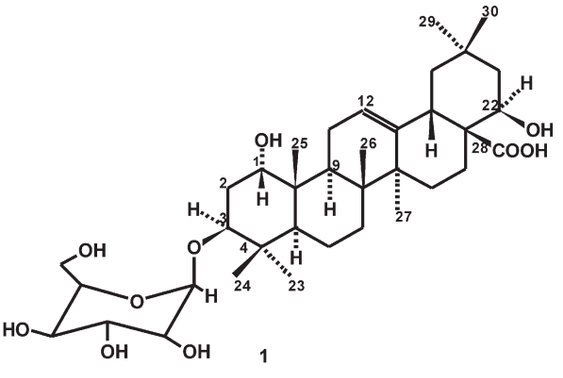
Elevated levels of nitric oxide (NO) generated by the inducible isoform of NO synthase (iNOS) are implicated in the pathophysiology of a number of cardiovascular ailments like heart failure, atherosclerosis, ischaemic cardiomyophathy and myocardial necrosis [5,6]. In the present study we report the isolation and characterization a new triterpene glycoside, namely terminoside A (**1**), from the stem bark of *T. arjuna*. Compound **1** potently decreases NO production and iNOS levels in lipopolysaccharide (LPS) stimulated rat peritoneal macrophages, a commonly employed model system generating NO *via* iNOS [7]. Inhibition of iNOS generated NO by a constituent of *T. arjuna* bark may, at least partially, account for the basis of its cardioprotective activities.



1

RESULTS AND DISCUSSION

The acetone fraction of ethanolic extract from the bark of *T. arjuna* was chromatographed successively on silica gel (60–120 mesh). The flash column chromatography eluted with $CHCl_3$–MeOH (96:4) afforded terminoside A (**1**).

Terminoside A was obtained as colourless crystalline solid having an optical rotation $[\alpha]_D$ +28 (*c* 18 MeOH). The pseudomolecular negative ion peak at *m/z* 650 $[M]^+$ in its FABMS suggested its molecular formula to be $C_{36}H_{58}O_{10}$, which was also supported by ^{13}C NMR and DEPT data. The FT-IR (KBr) spectrum revealed the presence of hydroxyl (3460 cm^{-1}), a glycosidic linkage (1082 cm^{-1}), carboxylic (3435, 1720 cm^{-1}) and olefinic (1665 cm^{-1}). The negative FABMS of **1** exhibited pseudomolecular ions at *m/z* 650 $[M]^+$, and fragment ions appeared at *m/z* 488 $[M+H-Glc]^+$. The EIMS spectroscopy of aglycone **2** exhibited a molecular ion peak at *m/z* 488 $[M]^+$ and other important ion peaks due to a retro-Diels–Alder fragmentation pattern of α-amyrin type at *m/z* 265, 223, which are the characteristic of olean-12-ene derivative compounds [8,9] possessing two hydroxyl groups in rings A/B and one hydroxyl group in ring D or E. Subsequent formation of the ion peaks at *m/z* 247 $[265-H_2O]^+$, 232 $[247-Me]^+$, 219 $[265-HCOOH]^+$, 201 $[219-H_2O]^+$, 186 $[201-Me]^+$, 189 $[219-2\times Me]^+$, 158 $[C_{16,17}-C_{13,18}$ fission$]^+$, 100 $[C_{17,22}-C_{18,19}$ fission$]^+$, 70 $[C_{21,22}-C_{18,19}$ fission$]^+$, 184 $[C_{7,8}-C_{9,10}$ fission$]^+$, 170 $[C_{6,7}-C_{9,10}$ fission$]^+$, 156 $[C_{5,6}-C_{9,10}$ fission$]^+$, 116 $[C_{4,5}-C_{1,10}$ fission$]^+$ and 86 $[C_{2,3}-C_{5,6}$ fission$]^+$ suggested a saturated nature of rings A and B having two hydroxyl groups in ring A at C-1 and C-3 positions, carboxylic group at C-28 and the third hydroxyl group at C-22. The ^{13}C NMR of **1** showed 36 resonances, of which 30 were accounted for by the aglycone moiety **2** and the other six by the sugar moiety. The presence of an olefinic proton at δ5.22 (H-12)

corresponded to the signal at δ122.19 (C-12) in the HMBC NMR spectrum, and seven methyl groups in the ^{1}H NMR spectrum of **1** were consistent with the aglycone being a substituted Δ^{12}-oleanane-type triterpene [10]. The absence of secondary methyl proton signals ruled out an ursane skeleton in **1** [11]. The ^{1}H NMR of C-1α, C-3β, and C-22β oxygenated protons resonated as double doublets at δ3.14 (J = 5.13, 3.30 Hz), 3.62 (J = 9.89, 5.12 Hz) and 3.12 (J = 9.16, 5.13 Hz) respectively. The ^{13}C NMR signals for oxygenated carbon at C-1, C-3, and C-22 resonated at δ75.6, 80.25 and 72.65, respectively. A downfield signal at δ175.74 was assigned to the C-28 carboxylic carbon. With respect to analogous data, the aglycone moiety **2** is an oleanolic acid type skeleton, which suggested that these positions are substituted in **1** (Table I). The hydroxyl position of C-1 & C-22 were determined by the ROESY spectrum which showed the correlation of H-1 with the methyl protons H_3-24 and H_3-25, indicating the hydroxyl group to be at 1α. The double doublet nature of H-22 and the ROESY correlations between H-21 and H-22 and between H-22 and H_3-29 indicated the configuration of 22-OH to be β. An anomeric proton was observed at δ5.08, which corresponded, in turn, to the signal at δ103.5, indicating the presence of a sugar unit. Acid hydrolysis of **1** with 2 M HCl gave aglycone **2** and glucose, which was identified by PC and

TABLE I ^{1}H and ^{13}C NMR spectral data* of compound **1** and aglycone **2** and **1** in DMSO-d_6 (600 MHz)

Carbon	^{13}*C NMR* **1**	^{1}*H NMR* **1**	^{13}*C agl* **2**	^{1}*H agl* **2**
1	75.6	3.14 dd (5.13, 3.30)	74.9	3.00 dd (5.2, 3.4)
2	27.7	1.60 m, 2.21 m	27.1	1.61 m, 2.25 m
3	80.25	3.62 dd (9.89, 5.12)	70.1	3.52 dd (9.75, 4.9)
4	38.8	–	38.3	–
5	54.87	0.69 dd (6.96, 4.21)	55.2	0.71 dd (8.51, 4.19)
6	18.1	1.45 m, 1.21 m	19.1	1.47 m, 1.23 m
7	32.2	1.21 brs, 1.40 m	32.0	1.25 brs, 1.41 m
8	39.1	–	38.8	–
9	47.26	1.60 dd (5.16, 9.89)	46.9	1.67 dd (5.15, 9.91)
10	37.68	–	38.1	–
11	23.2	1.82 m, 1.69 m	24.0	1.91 m, 1.67 m
12	122.2	5.22 d (8.6)	122.1	5.12 d (8.1)
13	143.21	–	143.76	–
14	41.07	–	42.1	–
15	26.98	1.60 m, 2.21 m	26.4	1.57 m, 2.01 m
16	28.3	1.60 m	28.6	1.61 m
17	45.2	–	46.1	–
18	43.1	2.90 brs	43.4	2.87 brs
19	46.8	0.79 brs	47.1	0.79 brs
20	34.8	–	35.0	–
21	31.75	1.59 d (9.16), 1.52 d (5.31)	32.2	1.62d (5.51)
22	72.65	3.12 dd (9.16, 5.13)	73.0	3.13 dd (9.15, 5.21)
23	28.71	0.88 brs	28.63	0.81 brs
24	16.2	0.62 brs	17.69	0.68 brs
25	16.65	0.86 brs	18.26	0.80 brs
26	17.0	1.27 brs	16.7	1.32 brs
27	24.46	0.91 brs	26.2	0.94 brs
28	175.8	–	175.7	–
29	27.99	0.82 brs	28.13	0.80 brs
30	24.46	0.69 brs	23.26	0.71 brs
3-O-Glc 1				
1′	103.5	5.08 d (6.22)		
2′	74.7	3.41 brs		
3′	77.7	3.10 dd (5.13, 5.86)		
4′	72.36	3.08 dd (5.86, 4.76)		
5′	76.66	3.20 d (4.76)		
6′	60.60	3.44 d (5.86)		

*Coupling constants are given in hertz (Hz) in parentheses.

TABLE II Effect of **1** on LPS-stimulated NO production, iNOS activity and viability of macrophages; each value represents the mean ± SE of **4** independent experiments

			(*% LPS treated control*)	
Group	*Nitrite* (μM)	*% Inhibition in NO*	*iNOS*	*Viability*
Normal	3.15 ± 0.36	–	28.85 ± 1.63	100.0 ± 1.51
LPS-treatment				
Control	34.51 ± 1.59*	0	100.0 ± 3.11*	75.12 ± 3.24*
1 (μM)				
25	27.24 ± 0.51*,†	21.06	86.02 ± 5.81*	79.34 ± 2.01*
50	15.43 ± 1.24*,‡	55.28	61.15 ± 4.43*,‡	84.41 ± 1.42*,¶
75	11.29 ± 0.93*,‡	67.23	43.61 ± 3.92‡,§	86.39 ± 2.83¶,§
100	8.22 ± 1.86‡,**	76.18	37.35 ± 3.61‡,**	93.12 ± 4.12†

*$p < 0.001$ compared to normal, untreated group.
†$p < 0.01$.
‡$p < 0.001$ compared to LPS-treated control.
¶$p < 0.05$.
§$p < 0.01$.
**$p < 0.05$.

HPTLC by comparison with an authentic sugar sample. The anomeric proton resonance at δ5.08 correlated with the glucosyl H-6′ signal at δ3.44 in the TOCSY experiment [12], which indicated that this sugar unit is glucose. In the HMBC spectrum, the anomeric proton signal at δ5.08 also correlated with the signal at δ80.25 (C-3), which suggested that the glucose unit was connected to the C-3 hydroxyl group. The presence of a 3-O-glycosidic linkage was identified by the attendant downfield shift at δ80.25 for C-3. The connectivity of the glucose unit and the stereochemistry at the C-3 position was confirmed by a ROESY experiment [13], where a correlation was observed between the glycosyl anomeric proton (δ5.08) and 3α-H at δ3.62. Moreover, the ^{1}H NMR coupling constant of H-3 ($J = 9.89, 5.12$ Hz) confirmed that the stereochemistry of C-3 was the α-H position [14]. On the basis of these spectral evidences, compound **1** was characterized as olean-1α,3β,22β-triol-12-en-28-oic acid-3β-D-glucopyranoside.

In LPS-activated macrophage culture system, **1** showed a dose-dependent inhibition in NO synthesis (Table II). The IC_{50} value for NO inhibition was 35 μM. During a 24 h incubation period with **1**, the viability of macrophages did not decrease, indicating that the decrease in NO production was not a consequence of any cytotoxicity of **1**. To elucidate the mechanism responsible for NO inhibition by **1**, its effect on enzymatic activity and the levels of iNOS in macrophages were determined. Enzymatic activity assays to which increasing concentrations (10–200 μM) of **1** were added did not show any statistically significant inhibition in iNOS activity as compared to the control. In contrast, 0.1 mM N^G-monomethyl-L-arginine (L-NMMA), a known inhibitor of iNOS activity, produced 75.69% inhibition in iNOS activity. This suggested that **1** suppresses NO generation in macrophages by ameliorating the levels of iNOS rather than its enzymatic activity. This aspect was analyzed by determining iNOS activity in cytosolic preparations from macrophages cultured in presence of LPS and different concentrations of **1** for 24 h at 37°C and 5% CO_2. A dose-dependent amelioration was obtained (Table II). Thus **1** inhibits NO production by inhibiting the synthesis of iNOS rather than its enzyme activity.

EXPERIMENTAL

IR spectra were recorded on a Perkin Elmer-377 infracord spectrophotometer using KBr pellets. ^{1}H, ^{13}C NMR, APT, ^{1}H-^{1}H-COSY, HMBC, NOESY, ROESY and TOCSY spectra

were recorded on a Bruker 600 MHz spectrometer with TMS as internal standard. FAB-MS spectra were scanned on a JMS-PX 303 mass spectrometer and EI-MS spectra were recorded on a Joel D-300 mass instrument. Optical rotation was measured on a JASCO DIP-SL Polarimeter. HPTLC was carried out on a Camag using Linomat-5, in HPTLC plate silica gel H (5–7 μm). Spots were detected by spraying aniline phthalate reagent, followed by heating.

Plant Material

The bark of *T. arjuna* was collected from Rishikesh, U.P, India, in October 1998. The specimen was identified by Dr M.P. Sharma (Taxonomist), at the Department of Botany, Jamia Hamdard. A voucher specimen has been deposited in the herbarium of the Phytochemistry Research Laboratory, Jamia Hamdard.

Extraction and Isolation

The air-dried and pulverized bark part (2.5 kg) was exhaustively extracted with 95% ethanol at 80°C in a Soxhlet apparatus for five days. The combined extracts were evaporated to dryness under reduced pressure to yield a dried ethanol extract (270 g). The residue was sequentially refluxed with solvents of increasing polarity, *viz.*, light petroleum, benzene, and acetone. The light petroleum and benzene soluble fractions were found to be identical on TLC examination but could not be further analyzed due to meager quantities. The acetone fraction (65 g) was concentrated, loaded into a flash column with (60–120) mesh silica gel and eluted with different solvents of increasing polarity. Elution of the column with $CHCl_3$–MeOH (94:6), (fractions 20–42) furnished a mixture of three compounds (1.5 g), which was rechromatographed and the column eluted with $CHCl_3$–MeOH (96:4) to yield a colourless crystalline compound (**1**, 110 mg).

Terminoside A (**1**) is a colourless crystalline solid, $[\alpha]_D + 28°$ (*c* 1.8, MeOH), 110 mg (0.053%) yield, R_f 0.3 ($CHCl_3$–MeOH, 9:1), mp 223–225°C, UV λ_{max} (MeOH) 203 nm; IR (KBr) 3460, 3435, 1720, 1665, 1082, 855 cm^{-1}; ^1H NMR & ^{13}C NMR (DMSO) of compound **1** (see Table I); FABMS of **1**: *m/z* 650 $[M]^+$, 488 $[M+H-Glc]^+$; ^1H NMR and ^{13}C NMR ($CDCl_3$) aglycone **2** (see Table I); EIMS (aglycone **2**) *m/z* 488 $[M]^+$, 265, 223, 247 $[264-H_2O]^+$, 232, 219 $[264-COOH]^+$, 201 $[219-H_2O]^+$, 189, 186 $[201-Me]^+$, 184, 170, 158, 156, 116, 100, 84, 70.

Acid Hydrolysis of **1**. Terminoside A (**1**), (25 mg) was heated with 2 M HCl (5 ml) on a steam bath for 6 h. The reaction mixture was extracted with $CHCl_3$ (3 × 5 ml), the organic layer was washed with water (2 × 10 ml), dried (Na_2SO_4) and evaporated to obtain aglycone **2** (15 mg), mp 188–189°C. The aqueous layer obtained after removal of the aglycone **2** was concentrated under reduced pressure and subjected to Whatman paper chromatography and HPTLC precoated plate followed by development with the solvent system n-BuOH–C_6H_6–Py–H_2O (5:1:3:3). Comparison was performed with an authentic sample of glucose. The sugar was visualized as a brown spot after spraying with aniline phthalate reagent, followed by heating at 110°C for 15 min.

NO Assay. All assays were performed on thioglycollate-elicited rat peritoneal macrophages prepared according to Li *et al.* [15]. Macrophages were cultured in phenol red free RPMI-1640 supplemented with 10% fetal calf serum and 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For the NO assay, macrophages (1×10^6 cells per well in 24 well-plate) were cultured with 10 μg ml^{-1} LPS in both the presence and absence (control) of **1** for 24 h at 37°C in a humidified CO_2 incubator. The amount of nitrite (taken as the index of NO) released in the culture supernatant was determined by the griess reagent method [16]. The culture supernatant (0.5 ml) was mixed with 0.5 ml of griess reagent and the absorbance

recorded at 510 nm. Following removal of culture supernatant for nitrite determination, the viability of macrophages was determined by an MTT assay [17].

iNOS Assay. The iNOS activity was determined in macrophage cytosol. To prepare the cytosol, approximately 5×10^7 macrophages cultured in presence of 10 μg ml^{-1} of LPS for 24 h at 37°C (5% CO_2) were disrupted by 3–4 freeze–thaw cycles in 50 mM Tris–HCl containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 μM pepstatin A, 2 μM leupeptin and 0.1% 2-mercaptoethanol. The lysate was centrifuged at 15000*g* for 30 min; the supernatant was the cytosol. iNOS activity was then determined in cytosol by conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline [17]. The assay mixture consisting of 1 mM NADPH, 10 μM FAD, 1 mM dithiothreitol, 100 μM tetrahydrobiopterin, 10 μM L-arginine, 0.3 μCi L-[^{3}H]arginine and 100 μl cytosol was incubated in both the presence and absence (control) of different concentrations of **1** for 60 min at 37°C. The reaction was terminated by addition of 2 ml of a stop buffer (30 mM HEPES, 5 mM EDTA, pH 5.5). L-[^{3}H]Citrulline was separated from L-[^{3}H]arginine on a Dowex 50 W-X-4 cation exchange column (sodium form) and quantified by scintillation counting. The above assay assessed the effect of **1** on iNOS activity. To determine the effect of **1** on the levels of iNOS in macrophages, macrophages were cultured with 10 μg ml^{-1} LPS in the presence of **1** for 24 h at 37°C (5% CO_2) and the activity of iNOS determined in a cytosolic preparation of these cells as described above but in the absence of **1**.

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