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A novel naphthanol glycoside from *Terminalia arjuna* with antioxidant and nitric oxide inhibitory activities

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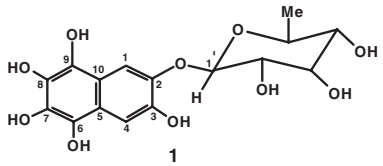
A novel naphthanol glycoside, arjunaphthanoloside (**1**), was isolated from the stem bark of *Terminalia arjuna* and its structure was established as 2,3,6,7,8,9-hexahydroxynaphthalene-2-*O*-α-L(−)-rhamnoside by means of spectroscopic and chemical methods. Compound **1** showed potent antioxidant activity and inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated rat peritoneal macrophages.

1. Introduction

The stem bark of *Terminalia arjuna* (Roxb.) Wight & Arnot. (Combretaceae) is a valuable cardioprotective remedy used for centuries in India's native Ayurvedic system of medicine [1]. The bark is effective against heart failure, angina pectoris, ischaemic cardiomyopathy, coronary artery disease and atherosclerosis [1, 2]. Considering the promising value of the bark as a cardioprotectant a lot of research work has been carried out and a number of bioactive constituents have been isolated [3], but the mechanism of cardioprotective activity of the bark is not clearly understood.

Numerous reports advocate the involvement of reactive oxygen species (ROS) and reactive nitrogen intermediate (RNI) in the pathophysiology of cardiovascular disorders [4–7]. For instance, oxidative stress has been found to decrease vasomotor functions, is directly cytotoxic to endothelial cells or cause oxidation of plasma low density lipoprotein (LDL) increasing their propensity to be taken up by macrophages, a vital factor implicated in molecular pathogensis of atherosclerosis [4, 5]. On the other hand, nitric oxide (NO) generated by the inducible isoform of NO synthase (iNOS) is implicated in heart failure associated with myocardial infarction and also plays an important role in the pathogenesis of atherosclerosis and ischaemic cardiomyophathy [6, 7]. Scavenging of ROS and/or RNI and/or inhibition in their generation may, therefore, afford protection against many cardiovascular disorders. In the present report we have isolated a novel naphthanol glycoside designated as arjunaphthanoloside (**1**) from the acetone fraction of ethanolic extract of stem bark of *T. arjuna.* Compound **1** possesses potent antioxidant activity and also suppresses NO production in LPS stimulated rat peritoneal macrophages, a model system generating NO via iNOS. Our findings could help to identity the mechanism of cardioprotective activities of *T. arjuna* bark.



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2. Investigations, results and discussion

Arjunaphthanoloside **(1)** was isolated by column chromatography of the acetone fraction of the ethanolic extract of the stem bark of *T. arjuna* as a pale yellow compound, which gave characteristic colored reactions of phenols on TLC, a blue-purple coloration in UV fluorescence (366 nm) after spraying with 5% $FeCl_3$ and 1% vanilline-H_2SO_4 reagent followed by heating at 100 °C for 5–10 min. Its IR spectrum showed absorption bands due to hydroxyl groups (3500, 3350 cm^{-1}), an aromatic moiety (1655, 1561 cm^{-1}), and a glycosidic linkage (1072 cm^{-1}). The UV spectrum of **1** revealed absorption maxima at 316 and 394 nm (MeOH), suggesting the presence of naphthalene [8]. The molecular formula of **1** was determined to be $C_{16}H_{18}O_{10}$ on the basis of HREIMS and it was also supported by DEPT and ^{13}C NMR spectra. The EIMS of **1** showed the molecular ion peak at m/z 370, with a prominent fragment ion at m/z 222 corresponding to the loss of a glycosyl unit and other important fragment ions at m/z 252 $[M\text{-}C_4H_6O_4]^+$ and 157 $[M\text{-}C_{10}H_{13}O_5]^+$ showed that the compound **1** consisting the polyhydroxyl group in naphthalene position. The ^1H NMR spectrum of **1** in DMSO-d_6 indicated the presence of two one-proton downfield singlets at δ 7.71 and 7.51 assigned to para-coupled H-1 and H-4, respectively. A one-proton doublet at δ 4.87 (J = 5.49 Hz) was ascribed to the H-1′ anomeric proton. A three-proton upfield doublet at δ 1.12 (J = 6.12 Hz) was accounted to the H-6′ methyl group of the rhamnose moiety. Three one-proton double doublets at δ 4.03 (J = 4.05, 5.49 Hz), 3.83 (J = 4.05, 3.46 Hz) and 3.55 (J = 3.46, 5.49 Hz) were associated with H-2′, H-3′, H-4′ hydroxymethine protons, respectively. The H-5′ carbinol proton appeared at δ 4.63 (J = 5.49 Hz), suggesting the presence of one deoxyhexose unit in the molecule. The

Table: Scavenging of DPPH and $O_2^{\bullet-}$ radicals and inhibition of LDL oxidation by 1[a]

Group	DPPH	$O_2^{\bullet-}$	LDL Oxidation
	% Inhibition		
1 (μM)			
25	26.47 ± 1.78	35.29 ± 1.96	36.39 ± 0.83
50	55.39 ± 0.93	63.75 ± 3.01	74.73 ± 3.49
100	82.81 ± 2.97	91.04 ± 2.61	86.42 ± 2.93
BHT (50 μM)	18.65 ± 2.31	28.02 ± 0.74	52.19 ± 4.39

[a] Results are expressed as% inhibition. Each value represents mean ± S.E. (n = 5).

sugar was determined to be rhamnoside on the basis of ^{1}H and ^{13}C NMR data of compound **1**. Acid hydrolysis of **1** with 2 N HCl gave rhamnose, which was identified by GC, HPLC and HPTLC techniques, comparing with the authentic sugar sample. The absolute configuration was determined as L-rhamnose by GC-MS of their trimethylsilylate (−)-butylglycosides [9]. The ^{13}C NMR and DEPT spectra of **1** showed 16 resonance signals including eight quaternary, seven methine and one-methyl carbons. The ^{13}C NMR showed the six phenolic quaternary carbon signals resonated at δ 148.5 (C-2), 159.22 (C-3), 154.13 (C-6), 147.36 (C-7), 140.54 (C-8) and 140.08 (C-9), respectively and other carbons signals at δ 113.29 (C-1), 113.23 (C-4), 136.52 (C-5), and 116.29 (C-10). These signals were further authenticated by aglycone of **1**. The ^{13}C NMR spectrum showing the characteristic signals belonging to the sugar moiety included one anomeric peak (C-1′ at δ 100.07), four hydroxy methine peaks (C-2′, C-3′, C-4′, C-5′ at δ 69.76, 72.60, 72.20, 69.30 respectively) and one aliphatic methyl carbon peak at (C-6′ at δ 17.57) [10]. The connectivities among the protons on the naphthalene ring (H-1 and H-4) were revealed by the COSY and HMQC spectra. The HMBC spectrum provided the substitution pattern of the naphthalene core and led to the connectivity between the sugar moiety and aglycones on the basis of the cross peak between C-2 (δ 148.5) and H-1′ (δ 4.87). The presence of 2-O-glycosidic linkage was identified by the attendant downfield shift at δ 148.5 where as aglycone moiety at δ 135.3 for C-2. The α-anomeric configuration for the rhamnose was determined by its C-5 (δ 69.3) data, and its coupling constant. On the basis of these spectral evidences the structure of **1** was determined to be 2,3,6,7,8,9-hexahydroxynaphthalene-2-*O*-α-L-rhamnoside.

Compound **1** showed potent antioxidant activity determined in terms of radical scavenging and inhibition of LDL oxidation. Butylated hydroxy toluene (BHT) was used as a positive control in these studies. Compound **1** effectively and dose dependently scavenged DPPH and superoxide radicals ($O_2^{\bullet-}$) with the IC_{50} values of 38.5 μM and 31 μM, respectively. 50 μM of **1** scavenged DPPH radicals by 55.39% and $O_2^{\bullet-}$ by 63.75%, which were 2.96 and 2.27 folds respectively of DPPH and $O_2^{\bullet-}$ scavenged by the reference antioxidant: BHT at the equal concentration (Table). Having found **1** to be a potent free radical scavenger, its effect on inhibition of LDL oxidation, a consequence of free radical chain reaction was determined. **1** dose dependently inhibited Cu-induced LDL oxidation with an IC_{50} value of 29.0 μM. 100 μM of **1** caused 86.42% inhibition in LDL oxidation and was 1.65 − fold more potent than BHT (Table). Since oxidative stress and its consequences, especially LDL oxidation plays an important role in the pathomechanism of atherosclerosis and coronary artery disease, the scavenging of free radicals and inhibition of LDL oxidation by a constituent of *T. arjuna* may, at least partially, be implicated in its cardioprotective activities.

Compound **1** also inhibited lipopolysaccharide (LPS) stimulated NO generation in rat peritoneal macrophages. The macrophages cultured in the presence of 10 μg/ml LPS for 24 h at 37 °C produced 34.12 nmol nitrite (taken as the index of NO). Presence of **1** during 24 h incubation period significantly decreased NO production without any cytotoxic effect. At the concentrations of 25, 50, 75 and 100 μM, **1** caused 26.83, 35.70, 52.36 and 64.99% inhibition, respectively, in NO production, the IC_{50} was 40 μM.

3. Experimental

3.1. General procedure

UV spectra were determined in spectroscopic grade MeOH on a Perkin Elmer (λ-20) spectrophotometer. IR spectrum was measured on a Bio-rad infracord spectrophotometer using KBr pellets. Optical rotation was measured on a Perkin Elmer model 241. NMR spectra were recorded on Brucker at 600 MHz for ^{1}H and 150.8 MHz for ^{13}C with TMS as internal standard. Complete proton and carbon assignment were based on 1 D (^{1}H, ^{13}C, DEPT) and 2D (HMQC and ^{1}H–^{13}C HMBC) NMR experiments. EIMS were recorded on Joel D-300. The HPLC was carried out on a Shimadzu (LC-10 AT-*VP*) using an ODS column (Waters NOVA-Pak C_{18}, 3.9 × 33 mm). HPTLC was recorded on CAMAG (Linomat-V), using HPTLC plate having silica gel H (5–7 μm). GC-MS analysis carried out with a Shimadzu-QP-2000 mass spectrometer using electron energy of 70 ev and an ionization current of 0.2 mA. TLC was performed on precoated silica gel 60 F_{254} aluminium sheets (0.2 mm, Merck). For column chromatography, normal phase silica gel 60–120 mesh (Merck) was used. Compound was detected by UV fluorescence and spraying with 1% vanilline-H_2SO_4 and 5% $FeCl_3$ reagent followed by heating at 100 °C for 5–10 min.

3.2. Plant material

The bark of *T. arjuna* was collected from Rishikesh, India in November 1998 and authenticated by Dr. M. P. Sharma, a taxonomist in the Department of Botany, Hamdard University, New Delhi, India. A voucher sample No. JH/Phytochem-8/02 is preserved in our laboratory for future reference.

3.3. Extraction and isolation

The air-dried bark of *T. arjuna* (2.5 kg) was exhaustively extracted with ethanol (90%) in a Soxhlet apparatus and then evaporated to dryness in a rotavapour. The residue (270 g) was sequentially refluxed with solvents of increasing polarities, *viz.*, petroleum ether, chloroform and acetone. Petroleum ether and chloroform soluble fractions were found identical on TLC examination but could not be further analyzed because of their meager quantities. The acetone fraction (65 g) was concentrated and chromatographed over silica gel (700 g) column. The column was eluted with a $CHCl_3$−MeOH gradient (85 : 15) and the eluants were collected as 250 ml fractions. Fractions 87–103 (1.2 g) were concentrated and chromatographed through the HPLC using reverse-phase silica gel C_{18} column (ca. 100 g) eluted with MeOH−H_2O (75 : 25). All the fractions were analyzed by TLC ($CHCl_3$−MeOH−CH_3COOH, 85 : 10 : 5) and collectively yielded 45 mg of compound **1**.

3.4. Characterization of the compound

*3.4.1. Arjunaphthanoloside (**1**)*

Elution of column with $CHCl_3$−MeOH (85 : 15) afforded a yellow crystalline solid: M.p. 272–274 °C; $[\alpha]_D^{20}$ − 104.6°, (c 0.69, MeOH); UV (MeOH) λ_{max} (log ε) 316 (5.6), 394 (4.1) nm; IR (KBr) ν_{max} 3500, 3350, 1655, 1561, 1072, 756 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz), δ 7.71 (1 H, s, H-1), 7.51 (1H, H-4), 4.87 (1 H, d, J = 5.49 Hz, H-1′), 4.03 (1H, dd, J = 4.05, 5.49 Hz, H-2′), 3.83 (1 H, dd, J = 4.05, 3.46 Hz, H-3′), 3.55 (1 H, dd, J = 3.46, 5.49 Hz, H-4′), 4.63 (1 H,d, J = 5.49 Hz, H-5′), 1.12 (3 H, d, J = 6.12 Hz, Me-6′); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 113.29 (C-1), 148.5 (C-2), 159.22 (C-3), 113.23 (C-4), 136.52 (C-5), 154.13 (C-6), 147.36 (C-7), 140.54 (C-8), 140.08 (C-9), 116.29 (C-10), 100.07 (C-1′), 69.76 (C-2′), 72.60 (C-3′), 72.20 (C-4′), 69.30 (C-5′), 17.57 (C-6′); ^{13}C NMR of aglycone **1** (DMSO-d_6, 150 MHz) 112.82 (C-1), 135.3 (C-2), 158.33 (C-3), 114.89 (C-4), 136.36 (C-5), 153.30 (C-6), 148.10 (C-7),

139.96 (C-8), 140.32 (C-9), 116.50 (C-10), EIMS m/z 370 $[M]^+$; 223 [M-rhamnose]$^+$, 252 $[M\text{-}C_4H_6O_4]^+$, 157 $[M\text{-}C_{10}H_{13}O_5]^+$. HREIMS m/z 369.982 (calcd for $C_{16}H_{18}O_{10}$).

*3.4.2. Acid hydrolysis of **1***

A solution of **1** (15 mg) in 10% HCl-60% EtOH (5 ml) was refluxed in boiling H_2O bath for 6 h. After dilution with H_2O and neutralization with Ag_2CO_3, the solution was extracted with EtOAc. The EtOAc layer was separated from the H_2O layer. Following evaporpation of EtOAc, the EtOAc soluble portion was chromatographed on silica gel (60–120 mesh) (ca 65 g) using $CHCl_3$–MeOH (95 : 5) as eluant to yield aglycone, which was analyzed by IR and NMR. The H_2O layer was concentrated and passed through a NOVA-Pack C_{18} column (Supelco), then separated in several injections by HPLC [HPLC conditions: mobile phase: MeCN–H_2O (3 : 1); flow rate: 0.7 ml/min; detection: refractive index] to afford rhamnose from **1** ($R_t = 11.2$ min, $[\alpha]_D$: + 6.7°) [11].

3.4.3. Molar carbohydrate composition and D^-, L^- configuration

Monosaccharide was analyzed by GC-MS as their trimethyl silylated methyl glycosides obtained from **1** after methanolysis and trimethylsilylation [12]. The configuration of sugar moieties was established by capillary GC-MS of there trimethylsilylated (−)-2-butylglucoside.

3.4.4. DPPH assay

1 ml solution of **1** (in MeOH) was added to 0.5 ml of 0.15 mM DPPH solution (in MeOH). The contents were mixed vigorously and allowed to stand at 20 °C for 30 min. The absorbance was read at 517 nm. The IC_{50} value (the concentration required to scavenge 50% DPPH free radicals) was calculated.

3.4.5. Scavenging of $O_2^{\bullet-}$

The method was adapted from Robak et al. [13]. The reaction mixture (total volume 1 ml) comprising 100 μM xanthine, 60 μM nitroblue tetrazolium, 0.07 U/ml xanthine oxidase was incubated in the presence or absence of **1** at 25 °C for 10 min. Following incubation, the absorbance was immediately read at 560 nm against a blank which did not contain the enzyme.

3.4.6. LDL oxidation

Fasting plasma samples were collected from healthy male adults and LDL was isolated by sequential density gradient ultracentrifugation as previously reported [14]. LDL was dissolved in 0.15 M NaCl, 1 mM EDTA (pH 7.4) for conservation. Just before oxidation reaction, EDTA was removed by dialysis. 75 μg of lipoprotein in a final volume of 1 ml was incubated with 25 μM $CuSO_4$ in the presence or absence of **1** for 6 h at 37 °C. The amount of the conjugated dienes formed was determined spectrophotometrically at 234 nm.

3.4.7. Nitric oxide assay

The assay was performed on thioglycollate-elicited rat peritoneal macrophages prepared according to Li et al. [15]. Adherent monolayers of macrophages (1×10^6 cells/well in 24 well plate) were cultured in phenol red free RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified CO_2 incubator. To determine NO production, macrophages were incubated with 10 μg/ml LPS (*Escherichia coli* 0127 : B8) (Sigma Chem. Co., St. Louis, MO) in the presence of **1** or 0.1% DMSO (control) for 24 h at 37 °C. The amount of nitrite (taken as the index of NO) released in culture medium was determined by griess reagent method. 0.5 ml of culture supernatant was mixed with 0.5 ml of griess reagent (1% sulfanilamide/0.1% naphylethylenediamide dihydrochloride/3% H_3PO_4) and absorbance recorded at 510 nm. Following removal of culture supernatant for nitrite determination, viability of macrophages was determined by MTT assay [16].

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