

Note

New cytotoxic saponin of *Albizzia lebeck*

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The methanolic extract of the stem bark of *Albizzia lebeck*, a new cytotoxic saponin is isolated and characterized with the help of FABMS, ^{13}C NMR and chemical studies. The isolated compound exhibited potent cytotoxic activity against human aqueous cell carcinoma [HSC-2 and HSC-3].

Keywords: *Albizzia lebeck*, Mimoseae, cytotoxic, saponin, bisglycoside

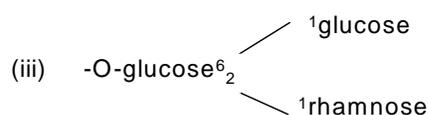
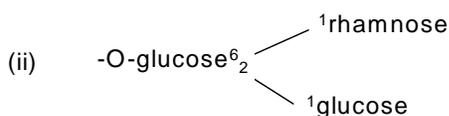
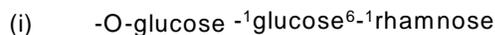
The genus *Albizzia* of the Mimoseae comprises over 150 species. *Albizzia lebeck* (L.) Benth. (Mimoseae) is a tree and reported to have antiseptic, antidysentric and antitubercular activities¹. A survey of literature revealed that different workers²⁻⁵ have isolated e-chinocystic acid, β -sitosterol, friedelon-3-one, γ -sitosterol from the stem bark and seed and saponins from leaves and seeds of *Albizzia lebeck*, but there is no report on isolation of saponins from stem bark. This prompted us to investigate stem bark of this plant for saponins. This paper described the isolation and characterization of a new oleanolic acid based bisglycoside from the methanolic extract of the bark of *Albizzia lebeck* along with cytotoxic activity.

Results and Discussion

Column chromatography of the saponin mixture of the stem bark of *Albizzia lebeck* gave compound **1** (positive to the characteristic test for triterpenic saponin). It also gave sugar [glucose and rhamnose (Co-Pc)] on complete hydrolysis with 7% alcoholic sulphuric acid. Thus, the compound was anticipated to be a triterpenic saponin, which was later confirmed by spectral (^1H NMR, ^{13}C NMR, EIMS and FABMS) and hydrolytic studies. The ^1H NMR spectrum of **1** showed the presence of seven tertiary methyls (δ 0.88, 0.96, 1.07 and 1.27), one secondary methyl (1.78, d, $J = 6.2$ Hz), an olefinic proton (5.44) and four anomeric proton (6.09, d, $J = 8.1$ Hz; 4.92, d, $J = 7.7$ Hz; 6.57, s;

4.97, d, $J = 7.7$ Hz). The EIMS of this compound showed intense peaks at m/z 456 ($\text{C}_{30}\text{H}_{48}\text{O}_3$), 248 and 207, thereby indicating that **1** would be a glycoside of oleanolic acid or ursolic acid⁶. The ^{13}C NMR spectrum exhibited the presence of 54 carbons, among which the peaks at δ 125.6 (d) and 144.3 (s) suggested that the aglycone could be oleanolic acid⁷. Fast atom bombardment mass spectrometry showed a molecular peak at m/z 1112 ($\text{M}+\text{Na}+\text{H}$)⁺, thereby indicating the molecular weight to be 1088 ($\text{C}_{54}\text{H}_{88}\text{O}_{22}$). Thus on the basis of hydrolytic and spectral data **1** was an oleanolic acid tetraglycoside. The sugar moieties consist of one rhamnose (δ 1.78 due to secondary methyl) and three glucoses. Alkaline hydrolysis of **1** gave a product whose ^1H NMR spectrum revealed the presence of three anomeric protons (6.09 d, $J = 8.09$ Hz; 6.57, s; 4.97 d, $J = 7.7$ Hz) along with secondary methyl protons (δ 1.78) were observed, therefore, the product may be oleanolic acid triglycoside. The sugar moieties consisted of one rhamnose (δ 1.78 due to secondary methyl) and two glucoses. Compound **1** was completely hydrolysed with 7% H_2SO_4 in MeOH to afford a colourless product whose ^1H NMR spectrum revealed the presence of one anomeric proton at δ 6.02 (1H, d, $J = 8.1$ Hz) but no secondary methyl protons were observed (rhamnose absent). Therefore, the product was identified as oleanolic acid 28-O- β -D-glucopyranoside. Compound **1** was partially hydrolysed (1% MeOH- H_2SO_4) to lucyoside (3-O- β -D-glycopyranosyl 28-O- β -D-glycopyranoside of oleanolic acid)⁸. The ^{13}C NMR of **1** was very similar to that of lucyoside⁹. However in the ^{13}C NMR spectrum of **1**, some additional signals assignable to sugar moieties attached to the C-3 hydroxyl group were also observed. By subtracting the chemical shifts due to the oleanolic acid substituted at C-28 by a glucose (94.8, 75.8, 75.2, 72.2, 78.2 and 63.1, corresponding to C-1', C-2', C-3', C-4', C-5', and C-6' respectively), the remaining 18 peaks were attributed to three sugars, two glucoses and a rhamnose. The signal at δ 106.9 (d), 101.3 (d) and 105.3 could be assigned to anomeric carbons of a glucose etherified with C-3 hydroxyl of oleanolic acid, rhamnose and an etherified glucose, respectively⁸. The signal at 69.5 (t) indicate that one of the C-6 hydroxyl group of the glucose was etherified⁹.

From the above argument and referring to the ^{13}C NMR chemical shifts of methyl glucosides¹⁰, the three possible structures were assigned for **1** as shown below:



The possibility of structure (i) has been ruled out on the basis of FABMS and ^{13}C NMR spectral studies. The appearance of peaks at m/z 1128 (M+K+H)⁺, 1112 (M+Na+H)⁺, 1111 (M+Na)⁺, 1089 (M+H)⁺, 927 (M+H-162)⁺, 943(M+H-146)⁺, 642 (M+Na+H-2X 162-146)⁺ in the FABMS of **1** confirmed that either (ii) or (iii) may be possible structure of **1**.

Finally the structure (iii) has been assigned to **1** on the basis of glycosidation shifts of the ^{13}C NMR signals (Table I). In the ^{13}C NMR spectrum of **1** the anomeric carbon of rhamnose was seen at δ 101.3 and that of third glucose was at 105.3. By calculating glycosidation shifts it can be assumed that glucose should be the central sugar attached to C-3 group of the aglycone with other sugars, attached to the central sugar at C-2' and C-6' position respectively¹¹. The anomeric carbons resonance of the rhamnose (δ 101.3) seemed to be shifted up field as compared to

that of methyl rhamnopyranoside (δ 102.4). The changes were caused by linkage of C-1' of the rhamnose to C-2' of the central sugar and C-1' of the glucose to C-6' of the central sugar^{12,13}. So (iii) should be the final structure of saponin **1** a new cytotoxic bisglycoside, 3-O- β -D-glucopyranosyl(1-6)-[α -L-rhamnopyranosyl-(1-2)]- β -D-glucopyranosyl, olean-12-ene-28-oic acid-28- β -D-glucopyranoside.

The pure compound **1** (Figure 1) exhibited potent cytotoxic activity against HSC-2 and HSC-3 cells with 50% cell growth inhibition of 4.2 and 3.1 $\mu\text{g}/\text{mL}$ respectively.

Materials and Methods

Plant Material

The plant material was collected from Painula, Tehri Garhwal UK (India) in the month of September. The authentication of plant material was made at the Department of Botany, Garhwal University, SRT Campus Badshahithaul, Tehri Garhwal UK, India. A voucher specimen is available at the herbarium of Botany Department (H-79).

Extraction and Isolation

The air dried and powdered stem bark (2 kg) was defatted with petroleum ether in a Soxhlet. The solvent free stem bark was exhaustively extracted with 90% MeOH until the extract become colourless. The concentrated mass was shaken with CHCl_3 and filtered. The residue was taken up in H_2O and extract with *n*-BuOH (4 \times 300 mL). The BuOH extract concentration under reduced pressure yielded a saponin mixture (59 g) which was chromatographed to afford colourless crystals of **1** (210 mg).

Experimental Section

The melting point is uncorrected. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on MIDAC, M-Series, and FTIR Instrument. The ^1H NMR were recorded on UNM-GX400 JEOL spectrometer run at 400 MHz and ^{13}C NMR spectra were recorded on the same machine at 100.533 MHz in pyridine- d_5 with TMS as internal standard. FABMS were recorded on JMS-DX300 (JEOL) with a positive mode at an accelerating voltage 2.5KV, gas Xe. Column chromatography silica gel (Merck 60-120 mesh), TLC; Kiesel gel 60G (Merck). The spots on TLC were visualized by spray with 10% alcoholic H_2SO_4 followed by heating. Paper chromatography was

Table I — ^1H NMR Spectral data (δ , ppm) of saponin **1**

| Type of Protons | Values |
|---------------------|---|
| Methyl protons | 0.88 (9H, s), 0.96, 1.07, 1.25, 1.27 (each 3H, s) |
| Rham-methyl protons | 1.06 (3H, d, $J = 6.2$ Hz) |
| H-3 | 3.36 (1H, dd, $J = 4.2$ and 11.5 Hz) |
| H-12 | 5.44 (1H, br-s) |
| H-18 | 3.14 (1H, dd, $J = 9.5$ Hz) |
| Anomeric protons | |
| (a) C'-H (glu) | 6.09 (1H, d, $J = 8.1$ Hz) |
| (b) C'-H (glu) | 4.92 (1H, d, $J = 7.7$ Hz) |
| (c) C'-H (rham) | 6.57 (1H, br-s) |
| (d) C'-H (glu) | 4.97 (1H, d, $J = 7.7$ Hz) |

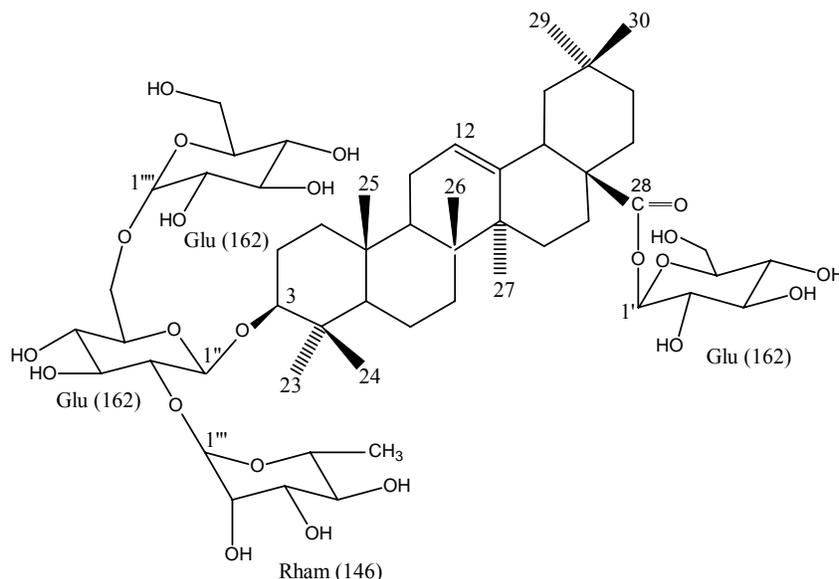


Figure 1

performed on Whatman No.1 paper using the descending mode and aniline hydrogen phthalate as the developer. The following solvent systems were used: (A) CHCl_3 -MeOH (4:1), (B) CHCl_3 -MeOH- H_2O (65:34:10), (C) C_6H_6 - Me_2CO (4:1), (D) EtOAc-pyridine- H_2O (10:4:3) and (E) *n*-BuOH-EtOH- H_2O (5:1:4 v/v).

3-O- β -D-Glucopyranosyl(1-6)-[α -L-rhamnopyranosyl-(1-2)]- β -D-glucopyranosyl olean-12-ene-28-oic acid-28- β -D-glycopyranoside.

Colourless crystals, mp 226-28° (dec), $[\alpha]_D^{25}$ -100 (CHCl_3 -MeOH); I.R. (KBr, cm^{-1}): 3400,1990, ^1H NMR (Pyridine- d_5): [cf Table I], ^{13}C NMR (cf Table II); FABMS: 1128 $[\text{M}+\text{K}+\text{H}]^+$, 1112 $[\text{M}+\text{Na}+\text{H}]^+$, 1111 $[\text{M}+\text{Na}]^+$, 1089 $[\text{M}+\text{H}]^+$, 927 $[\text{M}+\text{H}-162]^+$, 943 $[\text{M}+\text{H}-146]^+$ and 642 $[\text{M}+\text{Na}+\text{H}-2 \times 162-146]^+$. Found C, 59.00; H, 8.10. required C, 59.56; H, 8.08%.

Acidic hydrolysis of 3-O- β -D-glucopyranosyl(1-6)-[α -L-rhamnopyranosyl-(1-2)]- β -D-glucopyranosyl, olean-12-ene-28-oic acid-28- β -D-glycopyranoside.

Saponin 1, (25 mg) was hydrolysed with 7% H_2SO_4 in MeOH at 100°C for 4 hr to afford a colourless compound identified as oleanolic acid 28- β -D-glucopyranoside. The filtrate from the hydrolysate was neutralized with Ag_2CO_3 and filtered. The filtrate was concentrated under reduced pressure and the residue tested for the presence of D-glucose and L-

Table II — ^{13}C NMR Spectral data of saponin 1
Chemical Shift (ppm)

| Aglycone | | Sugars | |
|------------|-------|------------|--------------|
| Carbon No. | | Carbon No. | |
| C-1 | 38.9 | g-1' | 94.8 (-10.6) |
| C-2 | 26.6 | g-2' | 79.6 (+4.8) |
| C-3 | 88.9 | g-3' | 75.2 |
| C-4 | 39.5 | g-4' | 71.3 |
| C-5 | 56.0 | g-5' | 78.3 |
| C-6 | 18.6 | g-6' | 69.5 (+7.0) |
| C-7 | 32.3 | g-1'' | 106.9 (+1.5) |
| C-8 | 40.0 | g-2'' | 78.2 (+1.0) |
| C-9 | 48.1 | g-3'' | 75.8 |
| C-10 | 37.1 | g-4'' | 72.2 |
| C-11 | 23.5 | g-5'' | 78.2 |
| C-12 | 112.5 | g-6'' | 63.1 |
| C-13 | 144.3 | r-1''' | 101.3 (-1.1) |
| C-14 | 42.4 | r-2''' | 71.9 |
| C-15 | 28.8 | r-3''' | 72.6 |
| C-16 | 23.8 | r-4''' | 73.9 |
| C-17 | 47.3 | r-5''' | 69.7 |
| C-18 | 42.0 | r-6''' | 18.7 |
| C-19 | 46.5 | g-1'''' | 105.3 |
| C-20 | 30.7 | g-2'''' | 75.2 (+0.4) |
| C-21 | 34.2 | g-3'''' | 78.4 |
| C-22 | 33.3 | g-4'''' | 71.9 |
| C-23 | 28.3 | g-5'''' | 77.7 |
| C-24 | 17.0 | g-6'''' | 62.7 |
| C-25 | 15.7 | | |
| C-26 | 17.5 | | |
| C-27 | 25.9 | | |
| C-28 | 176.5 | | |
| C-29 | 23.5 | | |
| C-30 | 23.8 | | |

g- glucose, r- rhamnose, (+) downfield and (-) up field

rhamnose PC, EtOAc-pyridine-H₂O =10:4:3 *R_f* values 0.23 and 0.42 respectively.

Partial hydrolysis of 3-O-β-D-glucopyranosyl(1-6)-[α-L-rhamnopyranosyl(1-2)]-β-D-glucopyranosyl, olean-12-ene-28-oic acid-28-β-D-glucopyranoside.

Saponin 1, (20 mg) was hydrolysed with 1% MeOH-H₂SO₄ to afford another compound identified as lucyoside⁸.

Alkaline hydrolysis of 3-O-β-D-glucopyranosyl(1-6)-[α-L-rhamnopyranosyl(1-2)]-β-D-glucopyranosyl, olean-12-ene-28-oic acid-28-β-D-glucopyranoside.

Saponin 1, (50 mg) was refluxed for 3 hr with 10% KOH in MeOH (15 mL). The reaction-mixture was poured into H₂O and extracted with *n*-BuOH. The *n*-BuOH layer was purified by repeated preparatory TLC on silica gel (eluent: CHCl₃-MeOH-H₂O=13.7:2, lower layer) to give a product (6 mg) identified as 3-O-β-D-glucopyranosyl(1→6)-[α-L-rhamnopyranosyl(1→2)]-β-D-glucopyranosyl oleanolic acid¹².

Assay for cytotoxic Activity

Cells for trypanized and inoculated at 6×10^3 per each 96 micro well plate [falcon, flat treated polystyrene, Becton Dickinson, San, Jose, CA], and incubated for 24 hr after washing once with PBS. They were treated for 24 hr with out or with test compounds. They were washed once with PBS and incubated for 4 hr with 0.2 mg/mL MTT in DMEM medium supplemented with 10FBS after the medium was removed, the cells were lysed with 0.1mL DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate

using lab systems multis Kan (Biochematic, Helsinki, Finland) connected to a star/DOT matrix printer JL-10. The LD₅₀ value, which reduces the viable cell number by 50% was determined from dose response curve.

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