



## ISOLATION AND CHARACTERIZATION OF TRITERPENES FROM *ZIZYPHUS GLABRATA*

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### ABSTRACT

From the stem bark of *Zizyphus glabrata* a new dammarane-type triterpene, pseudojujubogenin-3-O- $\beta$ -D-glucopyranoside, along with the known ceanothane triterpenes, ceanothic acid, granulolic acid were isolated. The structures of the compounds were fully characterized by detailed NMR investigations including <sup>1</sup>H and <sup>13</sup>C NMR, HSQC, COSY, HMBC and NOESY experiments. In addition, the dammarane glycoside was tested for its potential to inhibit various bacteria and was found to possess significant bactericidal activity. The <sup>1</sup>H, <sup>13</sup>C and full 2D-NMR data on granulolic acid has also been presented. This is the first report on the chemical constituents of the stem bark of *Zizyphus glabrata*.

**Key words:** Triterpene, *Zizyphus glabrata*.

### INTRODUCTION

*Zizyphus glabrata* Heyne (Syn: *Z. Trinervia* Roxb), is a small tree that grows up to 30 ft in height, having olive-brown wood and commonly found in the forests of Peninsular India and Bhutan<sup>1,2</sup>. The leaves and aerial parts of the plant are traditionally used to treat inflammation, to relieve pain, convulsions and viral infections<sup>3</sup>. Plants belonging to the genus *Zizyphus* (Rhamnaceae) have been noted to produce a variety of characteristic secondary metabolites ranging from cyclopeptide alkaloids that possess antibacterial and antifungal activities<sup>4</sup>, and the dammarane class of triterpenes that are reported as sweetness inhibitors<sup>5-7</sup>. The present work describes the isolation and characterization of a new dammarane-type triterpene, pseudojujubogenin-3-O- $\beta$ -D-glucopyranoside along with the ceanothane triterpenes, ceanothic acid, granulolic acid.

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## EXPERIMENTAL

### General experimental procedures

Melting points were measured on a Cipla I-28 digital melting point apparatus and are reported uncorrected. The IR spectra were recorded on a Buck Scientific 500 Infrared spectrophotometer. Silica gel (Acme, 60-120 mesh) for column chromatography and silica gel (Acme 0) was used for preparative thin layer chromatography. Spots on chromatogram were detected under UV light and by spraying with 5% H<sub>2</sub>SO<sub>4</sub> in methanol. The NMR experiments were performed on a Bruker AVANCE DRX-500 spectrometer operating at 500.13 MHz and 125.77 MHz, respectively. Mass spectra were obtained using an Agilent 1100 series LC/MSD in APCI or API-ES mode.

### Plant material

The stem bark of *Zizyphus glabrata* (1.2 kg) were collected at the Khailasa hills, India, in April 2009. The sample was authenticated by Dr. M. Venkaiah, Taxonomist, Botany Department, Andhra University, Visakhapatnam. A voucher specimen (SG/ZGL/03/345) has been deposited at the Herbarium, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam India (Herbarium Code = SKU).

### Extraction and isolation

Powered plant material (900 g) was extracted in a Soxhlet apparatus, successively with hexane, CHCl<sub>3</sub> and MeOH and the extracts were concentrated using a rotary evaporator at a maximum temperature of 45°C. The dark viscous green residue 11 g from the methanol extract was separated over silica gel eluting with different mixtures of petroleum ether-chloroform and chloroform-methanol to give 25 fractions. Fraction 12-18 were combined, purified by repeated preparative TLC that recrystallized from methanol to give **2** (43.0 mg) and **3** (38.0 mg). Fraction 20 was further purified by repeated small column chromatography and recrystallized with chloroform-methanol to give **1** (64.0 mg).

**Pseudojubilogenin-3-O-β-D-glucopyranoside (1):** Pale green amorphous powder, m.p. 241-243°C. IR (KBr):  $\nu = 3200, 3640$  (OH), 1465, 1285, 1078, 1012, cm<sup>-1</sup>, <sup>1</sup>H NMR (500.13 MHz, d<sub>5</sub>-pyridine, <sup>13</sup>C NMR (128.77 MHz, d<sub>5</sub>-pyridine) COSY, HMBC and NOESY see Tables 1 and 2 and structure 1. HR-EIMS: $m/z$  (%) = 650 (18) (M<sup>+</sup>). C<sub>36</sub>H<sub>58</sub>O<sub>10</sub> (650.85) : calcd. C 66.43, H 8.98, O 24.58; found C 66.32, H 8.94, O 24.54.

**Ceanothic acid (2):** Colourless needles from Me<sub>2</sub>CO-methanol, m.p. 356-357°C, m.p. 333-335°C, dec.  $[\alpha]^{24} = -51.5^\circ$  (c, 1.01 in CHCl<sub>3</sub>) IR and MS in agreement with the

published data [12],  $^1\text{H}$  NMR (500.13 MHz,  $d_5$ -pyridine)  $\delta$  = 1.09, 1.17, 1.29, 1.41, 1.44, 1.68 (6 x S, 18H, CHMe) 1.71, (m, 1H, 18-H), 3.22 (s, 1H, 1-H), 4.84 (s, 1H, 3-H), 2.23 (d, 1H,  $J$  = 2.8 Hz, 5/9-H), 1.45, 1.54 (m, 2H, 6/7-H), 1.60, (m, 1H, 11-H), 2.11 (d, 1H,  $J$  = 11.4 Hz, 11-H), 1.34, (m, 1H, 12-H), 1.98 (d, 1H,  $J$  = 10.5 Hz, 12-H), 2.79 (dd, 1H,  $J$  = 2.8, 8.5 Hz, 13-H), 1.25, (m, 1H, 15-H), 1.92 (dd, 1H,  $J$  = 2.8, 10.5Hz, 15-H), 1.50, 2.61 (d, 2H,  $J$  = 11.4 Hz, 16 = H), 3.51 (d, 1H,  $J$  = 2.3, Hz, 19-H), 1.50, 2.23 (m, 1H, 21-H), 1.50, 2.23 (m, 1H, 22-H), 4.68, (s, 1H, 30-H (CH<sub>2</sub> = C), 4.87 (d, 1H,  $J$  = 10.0Hz, 30-H (CH<sub>2</sub> = C)).  $^{13}\text{C}$  NMR ( $d_5$ -pyridine)  $\delta$  = 20.7 (24-CH<sub>2</sub>), 15.5, 17.4, 19.2, 20.0, 31.9 (all CHMe), 67.4 (C-1), 85.1 (C-3), 44.2 (C-4), 57.4 (C-5), 19.5 (C-6), 35.1 (C7), 42.5 (C-8), 45.5 (C-9), 50.0 (C-10), 24.6 (C-11), 26.6 (C-12), 39.5 (C-13), 43.9 (C-14), 30.9 (C-15), 33.3 (C-16), 57.0 (C-17), 50.1 (C-18), 48.0 (C-19), 31.7 (C-21), 38.0 (C-22), 110.1 (30CH<sub>2</sub>) 178.4 (2-COOH), 179.3 (28-COOH), NOESY correlations: H-1  $\leftrightarrow$  H-3, H-19  $\leftrightarrow$  H<sub>2</sub>-30, H-5  $\leftrightarrow$  H-3, H<sub>3</sub>-29  $\leftrightarrow$  H<sub>2</sub>-30A/B, H<sub>3</sub>-23  $\leftrightarrow$  H-3, H<sub>3</sub>-24  $\leftrightarrow$  H-3, H-13  $\leftrightarrow$  H<sub>3</sub>-26, H-13  $\leftrightarrow$  H<sub>2</sub>-12 and H<sub>3</sub>-29  $\leftrightarrow$  H-19.

**Granulosic acid (3):** Colourless flakes from methanol, m.p. 237-239°C,  $-\alpha]^{24}_{\text{D}}$  = -51.5°C, (1.01 in CHCl<sub>3</sub>).  $^1\text{H}$  NMR (500.13 MHz,  $d_5$ -pyridine),  $^{13}\text{C}$  NMR (125.77 MHz,  $d_5$ -pyridine), COSY, HMBC and NOESY, see Table 3 and structure. 3 HR-EIMS : m.z (%) = 502 (20) (M<sup>+</sup>). -C<sub>30</sub>H<sub>46</sub>O<sub>6</sub> (502.32) : calcd. C 71.68, H 9.22, O 19.10; found C 71.64, H 9.16, O 19.06.

## RESULTS AND DISCUSSION

The stem bark of *Zizyphus glabrata* were extracted successively with hexane, chloroform and methanol that on concentration afforded three dark viscous semisolids. The methanolic residue was separated by silica gel column chromatography to furnish a new dammarane type triterpene glycoside together with the known ceanothane triterpenes, ceanthic acid, granulosic acid.

**Compound 1** was the major isolate obtained in this investigation as pale green amorphous powder, m.p. 241-243°C. It gave a positive for Liebermann Burchard test for triterpenes and Molisch test for sugars. The IR spectrum indicated the presence of a tertiary hydroxyl at 3460 cm<sup>-1</sup> and the absence of a conjugated system in the molecule. The high resolution mass spectrum showed a molecular ion peak at m/z 650.85[M]<sup>+</sup>, supporting the molecular formula of C<sub>36</sub>H<sub>58</sub>O<sub>10</sub> for **1**, deduced from the mass spectrum in conjunction with the  $^{13}\text{C}$  NMR spectrum. The NMR spectrum (Table 1) exhibited signals for 36 carbons: nine methylene [two of them bearing oxygen atoms ( $\delta$  = 66.2 and 68.9)], seven methines [one oxymethine ( $\delta$  = 89.0)], seven methyl carbons, an anomeric carbon  $\delta$  = 107.2 bound to  $\delta$  = 4.97 (1H, *d*, 7.7) according to the HSQC spectrum. Comparison of the NMR data for **1**

(Table 1) with the COSY 45° spectrum, revealed the sugar (pyranose form) to be glucose. The coupling constant of the anomeric proton i.e.  $\delta = 4.97$  (1H, *d*, 7.7) indicated  $\beta$ -configuration of glucopyranosyl moiety. A  $^1\text{H}$  double doublet at  $\delta = 3.38$  ( $J = 4.7, 11.5$  Hz) characteristic for H-3  $\alpha$  having a sugar linked at C-3 was supported by  $^2J$  HMBC correlations with the anomeric carbon 107.2 (G-1), and the geminal methyls [28.4 (C-28), 16.6 (C-29)] located at C-4, NOE correlation between H-3 of the genin and G-1 of the glucose confirmed the attachment of the sugar at position C-3 of the aglycone. These signals resembled a dammarane type triterpene having a single sugar unit in the A ring at 3-O- $\beta$ -position and a free tertiary hydroxyl group<sup>8-11</sup>.

The spectrum also revealed an olefinic methine,  $\delta = 5.42$  ( $\delta=127.2$ ) along with signals typical to that of an isobutenyl side chain. The COSY 45° spectrum revealed that the methyls  $\delta=1.64$  and  $1.72$  and resonances at  $\delta = 25.9$  and  $18.7$  ascribed to C-26 and C-27 were coupled to the unsaturated methine at  $\delta = 5.42$  and were assignable to H-24. The placement of the side chain at C-22 was accomplished through the HMBC experiment. The olefinic methine resonating at  $\delta = 5.42$  (H-24) showed a  $^2J$  correlation with the carbon  $\delta = 46.9$  (C-22) and 3  $J$  long-range couplings with the carbons  $\delta = 68.9$  (C-23),  $25.9$  (C-26),  $18.9$  (C-27) supporting that the side chain was located at C-22 of the three oxygen functions in 1, one was assigned to a tertiary hydroxyl group  $\delta = 69.1$  (C-20) while the two other oxygen's were directly involved in ethers of a ketal group  $\delta = 5.03$  and  $4.28$  (*d*,  $J = 8.6$  Hz, H-30) with resonances  $\delta = 68.9$  and  $66.2$  assignable to  $sp^3$  carbons C-23 and C-30 respectively. The relative stereochemistry at C-3/5/28 and C-18/19 were confirmed by means of the NOESY spectrum. The H-3  $\alpha$  proton showed strong NOE interactions with H<sub>3</sub>-28 resonance and H-5 methine suggesting that they were  $\alpha$ -oriented and the H-24 olefinic methine showed two interactions with the angular methyls H<sub>3</sub>-18 and H<sub>3</sub>-19 establishing  $\beta$ -orientation of the methyls.

Some key HMBC correlations (Table 2) observed were between the methyls ( $\delta = 1.34$  and  $0.74$ ) that exhibited  $^3J$  coupling between themselves indicating their geminal nature and  $^2J$  coupling with the oxymethine C-3 and the methine C-5, while the methyl at  $\delta = 1.44$  showed  $^2J$  correlation with the methine at C-22. The angular methyl, H<sub>3</sub>-18 ( $\delta = 1.02$ ) showed  $^2J$  correlation to the methylene at C-7 and the quaternary carbon at C-14 and  $^3J$  couplings with the quaternary carbon C-10. On the basis of the above spectral data, compound 1 was identified as psedujubogenin-3-O- $\beta$ -D-glucopyranoside, a new natural product.  $^1\text{H}$  and  $^{13}\text{C}$  NMR resonances were assigned using COSY, HMBC and NOESY spectra and are presented in Tables 1 and 2 and on structure 1.

**Table 1:  $^1\text{H}$ ,  $^{13}\text{C}$  NMR and COSY spectral data for dammarane triterpene glycoside 1**

Position	$\delta\text{H}$	$\delta\text{C}$	COSY	Position	$\delta\text{H}$	$\delta\text{H}$	COSY*
1	a) 0.81 (m, 1H) b) 1.49 (m, 1H)	39.0	H-1b, H-11a H-1a, H-2b	16		110.6	
2	a) 1.77-1.90 (m, 1H) b) 2.29 (m, 1H)	26.9	H-3 $\alpha$ H-1b	17 $\alpha$	1.72 (m, 1H)	53.9	H-13 $\beta$
3 $\alpha$	3.38 (dd, 1H, 4.7, 11.5)	89.0	H-2a, H-2b	18 $\beta$	1.02 (s, 3H)	19.0	H-7
4		37.6 <sup>a</sup>		19 $\beta$	1.02 (s, 3H)	17.1	H-7
5 $\alpha$	0.70 (m, 1H)	56.3	H-28 $\alpha$	20		69.1	
6	1.37 (m, 2H)	18.5	H-15b	21	1.44 (s, 3H)	29.8	H-13 $\beta$
7	1.54 (m, 2H)	36.2	H-18, H-19	22	2.16 (m, 1H)	46.9	
8		37.4		23	5.03 (m, 2H)	68.9	H-15a, H-24
9	0.81 (m, 1H)	53.2		24	5.42 (d, 1H, 8.0)	127.2	H-26, H-27
10		37.4 <sup>a</sup>		25		135.4	
11	a) 1.37 (m, 1H) b) 1.49 (m, 1H)	21.8	H-17 $\alpha$ H-11a, H-17 $\alpha$	26	1.64 (s, 3H)	25.9	
12	a) 1.77-1.90 (m, 1H) b) 1.97 (m, 1H)	28.7	H-11a	27	1.72 (m, 3H)	18.7	H-23
13 $\beta$	2.72 (m, 1H)	38.6		28 $\alpha$	1.34 (m, 3H)	28.4	H-5 $\alpha$
14		53.5		29 $\beta$	0.74 (s, 3H)	16.6	H-1b
15	a) 1.77-1.90 (m, 1H) b) 2.20 (d, 1H, 8.3)	39.9	H-13 $\beta$	30	4.28 (d, 2H, 8.6)	66.2	
$\beta$ -D-Glucose							
G-1	4.97 (d, 1H, 7.7)	107.2	G-2	G-4	4.20 (dd, 1H, 8.6, 9.1)	72.2	
G-2	4.07 (t, 1H, 8.7)	76.0	G-5, G-6b	G-5	4.25 (t, 1H, 8.6)	79.0	

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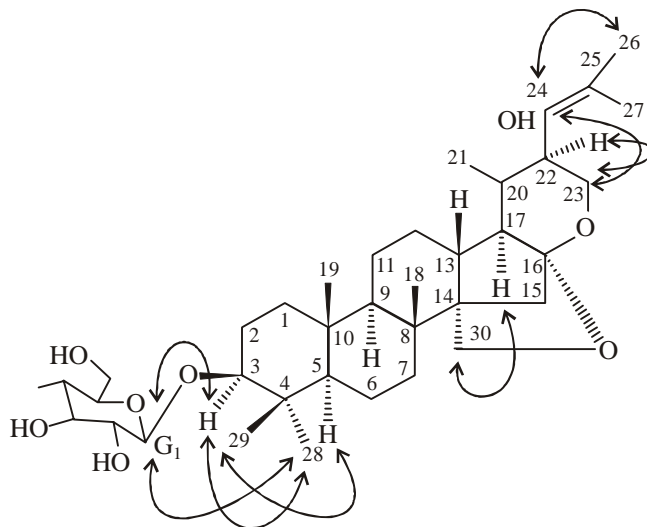
Position	$\delta^{\text{H}}$	$\delta^{\text{C}}$	COSY	Position	$\delta^{\text{H}}$	$\delta^{\text{H}}$	COSY*
G-3	4.02 (m, 1H)	78.6	G-4	G-6	a) 4.63 (dd,1H,2.4,11.7)	63.4	G-6b
					b) 4.42 (dd,1H,5.5,11.7)		

\* Assignments were confirmed by 2D NMR experiments (HSQC, HMBC and 2D-NOESY);

<sup>a</sup> signals are interchangeable, coupling constants 'J' in Hertz.

**Table 2: Key HMBC correlations observed for the dammarane triterpene glycoside 1**

Position	$^2J$	$^3J$
H-1	26.9 (C-2)	
H-2	89.0 (C-3)	
H-3	107.2 (G-1), 28.4 (C-28), 16.6 (C-29)	
H-5		89.0 (C-3)
H-12	38.6 (C-13)	53.9
H-13	53.5 (C-14)	(C-17)
H-18	36.2 (C-7), 53.5 (C-14)	69.1 (C-20)
H-19	56.3 (C-5)	37.6 (C-10)
H-21	46.9 (C-22)	38.6 (C-4), 21.8 (C-11)
H-22	53.9(C-17), 69.1 (C-20)	
H-23	110.6 (C-16)	127.2 (C-24)
H-24	46.9 (C-22)	68.9 (C-23), 25.9 (C-26)
		18.9 (C-27)
H-26/27	127.2 (C-24)	
H-28	89.0 (C-3), 56.3 (C-5)	16.6 (C-29)
H-30	53.5 (C-14)	38.6 (C-13)
G-1	89.0 (C-3)	78.6 (G-3)
G-3	78.6 (G-4)	107.2 (G-1), 79.0 (G-5)
G-4	79.0 (G-5)	63.4 (G-6)



**Fig. 1: Important NOESY interactions of 1**

**Compound 2 :** Ceanothic acid was characterized by analysis of NMR spectra and comparison with the published data<sup>12-14</sup>. The dammaranetype triterpene glycoside is the major compound in *Zizyphus xylopyra*. Jujubogenin glycosides, jujuboside A, C and lotoside I, II have been reported from *Zizyphus lotus*<sup>15</sup> however, this is the first report of a pseudojujubogenin glycoside isolated from the genus, *Zizyphus*. The ceanothane triterpene, 3-O-protocatechuoylceanothic acid has been reported from *Zizyphus jujuba*<sup>16</sup>. The isolation of the dammarane – type glycoside from a plant of the *Zizyphus* genus is not surprising, but it is remarkable to note that *Zizyphus xylopyra* produces both dammarane and the ceanothane class of terpenoids. Compound 1 was tested for its potential to inhibit various bacteria by established methods<sup>17</sup>. It inhibited the growth of *Bacillus pumilus*, *Staphylococcus aureus*, *Escherichia coli* and *Proteus vulgaris* with MICs being 51.2, 102.2, 12.8, 25.6  $\mu\text{g/mL}$ , respectively. The dammarane and ceanothane terpenoids have been reported to possess potent anti-inflammatory activity<sup>18</sup>, the biological activities of the three compounds are of interest and are presently taken up for investigation.

**Compound 3** was obtained as colourless flakes, m.p. 237-239<sup>0</sup>C. The HR – EI mass spectrum showed a molecular ion peak at  $m/z$  503.69  $[\text{M}]^+$  that correspondent to the molecular formula  $\text{C}_{30}\text{H}_{46}\text{O}_6$ . The <sup>13</sup>C NMR (Table 1) displayed 30 carbon resonances, while the HSQC experiment confirmed that 22 out of the 30 carbons were directly attached to protons. The *J* modulated <sup>13</sup>C experiment revealed the presence of five methyls, ten methylenes, seven methines and eight quaternary carbons that included resonances for two carboxylic functions  $\delta = 178.0$  and  $179.2$ . The <sup>1</sup>H NMR (Table 1) showed five tertiary

methyl singlets, a hydroxymethyl group  $\delta = 3.70, 4.64$  (d,  $J = 9.0$  Hz), an oxymethine  $\delta = 4.96$  and a saturated methane  $\delta = 3.26$  and a series of multiplets from  $\delta = 1.49$  to  $1.59$ . These features revealed the backbone of **3** as a ceanothic acid derivative. This presumption was further confirmed when the  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts of the ceanothane triterpenes **2** and **3** were found to be almost superimposable<sup>12,13</sup>. However, there were minor changes for signals in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR of the A and B rings of the ceanothane skeleton which were also observed in the COSY and the HMBC experiments. The remaining assignments of the  $^1\text{H}$  resonances in **3** were made by comparison with those published for **3a** in the literature. The  $^{13}\text{C}$  assignments were based on the HMBC experiments using  $^2J$  and  $^3J$  correlations while the  $^1J$  C-H interactions observed in the HSQC spectrum allowed unambiguous assignments of the hydroxymethyl and methylene protons in **3**. The relative stereochemistry at H-1/3/9/13 and H<sub>3</sub>-23/25/26/27 and the carboxylic group at H-2/28 were finally determined by 2D NOESY experiments as shown on **3**. Accordingly, the structure of **3** was determined as 24-Hydroxyceanothic acid, known as granulolic acid. Although dimethyl granulosate **3a** has been previously reported from the heartwood of *Colubrina granulosa*<sup>12</sup> and also from *Paliurus ramosissimus*<sup>13</sup>, the present study constitutes the first isolation of **3** as a pure natural product and is reported as a rare ceanothane triterpene. In addition to its  $^1\text{H}$  NMR data,  $^{13}\text{C}$  and full 2D-NMR spectral data has been presented for the first time (see Table 3 and Structure 3)

**Table 3:  $^1\text{H}$ ,  $^{13}\text{C}$  NMR, HMBC, COSY, and NOESY spectra data for the ceanothane triterpene **3****

H/C	$\delta\text{H}$	$\delta\text{C}$	HMBC	COSY	NOESY
1 $\beta$	3.26 (s,1H)	66.8	C-2, C-3, C-5 C-10, C-25		H-25 $\beta$
2-COOH		178.0			
3 $\alpha$	4.96 (s, 1H)	86.2	C-2, C-10, C-23		
4		48.9			
5	2.31 (m,1H)	57.6	C-4, C-6		
6a/b	1.49 (m,2H)	18.8	C-5, C-10		
7	a) 1.49 (m,1H) b) 1.41 (m,1H)	35.5	C-4, C-5, C-24		
8		42.4			

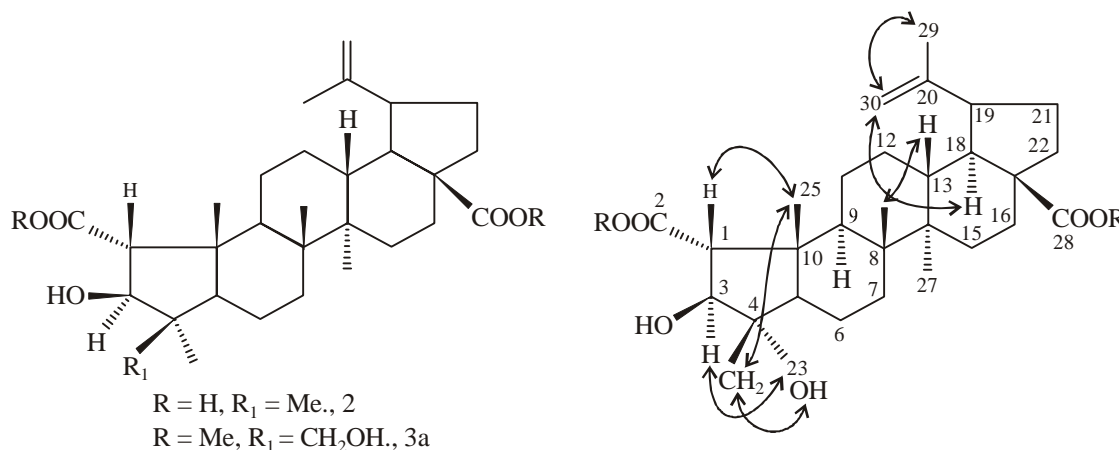
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H/C	$\delta^{\text{H}}$	$\delta^{\text{C}}$	HMBC	COSY	NOESY
9	2.18 (m,1H)	45.7	C-6, C-8, C-10 C-11, C-25		H-1 $\beta$ , H-27 $\alpha$
10		50.4			
11	a) 1.59 (m,1H) b) 2.10 (m,1H)	24.7	C-13	H-25 $\beta$	
12	a) 1.98 (m,1H) b) 1.33 (m,1H)	26.6	C-14	H-26 $\beta$	
13 $\beta$	2.78 (m,1H)	39.5	C-8, C-18		
14		43.9			
15	a) 1.22 (m,1H) b) 1.90 (m,1H)	30.9			
16	a) 1.49 (m,1H) b) 2.60 (m,1H)	33.3	C-8, C-17, C-18, C-20 C-28	H-21, H-29	H-16b
17			57.0		
18	1.69 (m,1H)	50.1			H-30
19 $\alpha$	3.51 (m,1H)	48.0	C-30	H-21, H-22, H-29	
20		151.6			
21	a) 2.23 (m,1H) b) 1.49 (m,1H)	31.7	C-18, C-28	H-21b, H-22	H-19, H-21b, H-21a
22	a) 2.23 (m,1H) b) 1.55 (m,1H)	37.9	C-28		
23 $\alpha$	1.81 (s,3H)	26.1	C-3, C-17, C-24		H-3 $\alpha$ , 24-OH
24 $\beta$	4.64 (d,2H,9.0)	67.0	C-23		24-OH
24-OH	3.70 (d,1H,9.0)		C-4, C-23	H-24	
25 $\beta$	1.47 (s,3H)	19.4	C-9		H-24 $\beta$
26 $\beta$	1.13 (s,3H)	17.4	C-7, C-8		H-13 $\beta$

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H/C	$\delta^{\text{H}}$	$\delta^{\text{C}}$	HMBC	COSY	NOESY
27 $\alpha$	1.06 (s,3H)	15.4	C-13,C-14, C-15		H-6, H-7
28-COOH		179.2			
29	1.67 (s,3H)	20.1	C-19,C-20, C-20, C-30		H-18,H-30a
30	a) 4.68 (d,1H, 13.6)	110.0	C-12, C-19, C-20, C-27	H-29, H-30b	H-19
	b) 4.87 (s,1H)	C-19, C-29			



**Fig. 2: Important NOESY interactions of 3**

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