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New triterpene glycosides of *Clematis montana* and their cytotoxic activities

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Abstract : Two new triterpene glycosides designated as montanosides 1 and 2 were isolated from methanolic extract of *Clematis montana*. Their structures have been elucidated as 3-*O*-β-D-glucopyranosyl-oleanolic acid-28-*O*-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-xylopyranoside and 3-*O*-β-D-glucopyranosyl-oleanolic acid-28-*O*-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)]-β-D-xylopyranoside, respectively by NMR and MS data. Cytotoxic activity of the compounds were evaluated against various squamous carcinoma cells (HSC-2) and human gingival fibroblast cells (HGF). Both of the compounds showed potent cytotoxic activity against the test cells.

Keywords : *Clematis montana*, Ranunculaceae, cytotoxic activity, triterpene glycosides.

Introduction

Clematis montana (Ranunculaceae), a woody climber is distributed in temperate Himalaya up to an altitude of 4000 m[1]. Leaves of the plant are used in skin diseases and the seeds have purging properties[2]. Literature search revealed that the leaves have been investigated[3,4] but no work has been reported so far on the steam of this plant. This paper describes the isolation and characterization of two new oleanolic based glycosides from the methanolic extract of the stems of *Clematis montana* and determination of cytotoxic activity for both the compounds.

Results and discussion

Column chromatography of the saponin mixture of the stems of *Clematis montana* gave two new glycosides, designated montanoside **1** and **2**.

Montanoside **1** : Obtained as a white powder $[\alpha]_D$–4.8 (MeOH) and exhibited a $[M-H]^-$ peak at *m/z* 1057 in the neg. FAB-MS. The ^1H NMR spectrum of **1** displayed seven tertiary methyls at δ 0.89, 0.89, 0.92, 1.08, 1.08, 1.16 and 1.25, an olefinic proton and δ 5.43 (1H, br s) and four anomeric protons at δ 4.76 (1H, d, *J* 6.1 Hz), 5.12 (1H, d, *J* 7.9 Hz), 6.12 (1H, br s) and 6.24 (1H, d, *J* 7.9 Hz). The ^{13}C NMR spectrum exhibited the presence of 54 carbon atoms among which the peaks at 122.6 (d) and 144.3 (s) suggested that the aglycone could be oleanolic acid[5]. The ^{13}C NMR spectrum revealed that presence of six quaternary carbon signals at δ 30.8, 37.0, 39.5, 39.9, 42.1 and 47.0, a set of olefinic carbon signals at δ 122.9 and 144.1, one ester carbonyl signal at δ 176.4 and four anomeric carbon signals at δ 95.7, 101.7, 104.9 and 106.3. These spectral data indicated that **1** was the 3,28-bisdesmoside of oleanolic acid [**3**][6] having four monosaccharide units. On acid hydrolysis, **1** afforded oleanolic acid, D-glucose, D-xylose and L-rhamnose[7]. The ^1H and ^{13}C NMR spectrum of the sugar moiety were assigned by the ^1H-^1H, COSY and HMQC techniques in the HMBC experiment, long range correlation were observed between the anomeric proton of 2,4-disubstituted xylosyl moiety and the C-28 (δ 176.4) of aglycone, the proton [δ 5.12 (d, *J* 7.9 Hz) H-1′] of terminal glucosyl moiety and the C-4 (δ 79.4) of 2,4-disubstituted xylosyl moiety, the anomeric proton (δ 6.12, br s) of terminal rhamnosyl moiety and the C-2 (δ 76.4) of 2,4 disubstituted xylosyl moiety and the anomeric proton [δ 6.24 (d, *J* 7.9 Hz) H-1″] of terminal glucosyl moiety and the C-3 (δ 88.7) of aglycone. Based on the above evidences, the **1** was characterized as 3-*O*-β-D-glucopyranosyl-oleanolic acid-28-*O*-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-xylopyranoside.

Montanoside **2** : It ws obtained as a white powder, $[\alpha]_D^{25}$–4.2° (MeOH) and exhibited a $[M-H]^-$ peak at *m/z* 1219 in neg. FAB-MS. The ^1H NMR spectrum of **2** displayed seven tertiary methyls at δ 0.87, 0.89, 0.89, 1.08, 1.09, 1.16 and 1.25, an olefinic proton at δ 5.42 (1H, br s) and five anomeric proton at δ 4.71 (1H, d, *J* 6.7 Hz), 5.12 (1H, d, *J* 7.9 Hz), 5.12 (1H, d, *J* 7.9 Hz), 6.14 (1H, br s) and 6.13 (1H, d, *J* 7.9 Hz). The above ^1H NMR data of **2** were very similar to those of **1** except for the new additional anomeric proton at δ 5.45. In ^{13}C NMR spectrum of **2**, the signals due to the aglycone moiety were in good agreement with those of **1**, although the

signals due to the sugar moiety were not identical. On acid hydrolysis, **2** afforded oleanolic acid, D-glucose, D-xylose and L-rhamnose[7]. Furthermore a comparative study of the ^{13}C NMR spectrum of **1** to that of **2** indicated the presence of an additional glucosyl unit in **2**, which was linked to the trisaccharide moiety. The ^{1}H and ^{13}C NMR spectrum of **2**, which could be assigned by using 2D-NMR techniques (^{1}H-^{1}H COSY, HMQC and TOCSY) showed that the signals due to the pentasaccharide moiety consisted of three glycopyranosyl moieties, and one xylosyl moiety and one rhamnosyl moiety. In the HBMC experiment, long rang correlation were observed between the anomeric protons [δ 4.71 (d, *J* 6.7 Hz)] of 2,4-disubstituted xylosyl moiety and the C-28 (176.4) of aglycone, the anomeric proton [δ 5.13 (d, *J* 7.9 Hz) H-1′] of terminal glycosyl moiety and the C-4 (δ 80.1) of 2,4-disubstituted xylosyl moiety, the anomeric proton [δ 5.45 (d, *J* 7.9 Hz) H-1‴] of terminal glycosyl moiety and the C-3 (δ 83.2) of 3-substituted rhamnosyl moiety, the anomeric proton [δ 6.14 (br s)] of 3-substituted rhamnosyl moiety and the C-2 (δ 76.2) of 2,6-disubstituted xylosyl moiety and the anomeric proton [δ 6.33 (d, *J* 7.9 Hz) H-1″] of terminal glucosyl moiety and the C-3 (δ 88.7) of aglycone. Based on the above data **2** was elucidated as 3-*O*-β-D-glucopyranosyl-oleanolic acid-28-*O*-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)]-β-D-xylopyranoside.

The isolated compounds **1** and **2** were evaluated for their cytotoxic activity against human sqaumous carcinoma (HSC-2) and human gingival fibroblast cells (HGF)[8]. The compounds **1** and **2** exhibited potent cytotoxicity against HSC-2 cells with respective LD_{50} values of 4.3 μg/ml and 8.4 μg/ml and against HGF cells with LD_{50} values of 18 μg/ml and 43 μg/ml, respectively.

Experimental

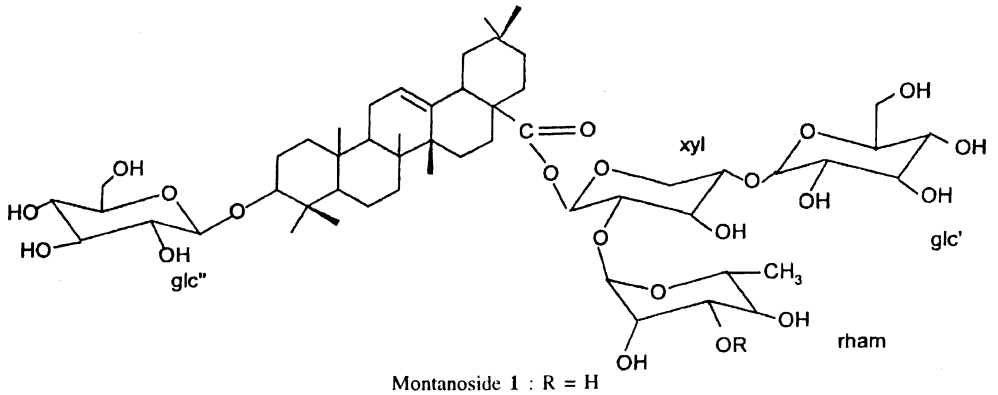
The melting points are uncorrected. Optical rotations were taken on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on MIDAC, M Series and FTIR spectrophotometer. ^{1}H NMR and ^{13}C NMR were measured with a JEOL alpha 500 NMR spectrometer and chemical shift are given on a δ (ppm) scale with tetramethyl silane (TMS) as the internal standard. The FAB-MS was measured with a JEOL DX 303 HF spectrometer and taken in a 3-nitrobenzyl alcohol matrix. Column chromatography was carried out on Kiesel gel (230-40 mesh, Merck), TLC was performed on Kiesel gel 60G (Merck). The spots on TLC were visualized by spray with 10% alcoholic H_2SO_4 followed by heating. Paper chromatography was performed on Whatman No. 1 paper using the descending mode and aniline hydrogen phthalate as the developer. The following solvents 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).

Table 1. ^{13}C NMR data for **1** and **2** (pyridine-d_5)

	1	2	3		1	2
				28-*O*-		
C-1	38.9	38.9	39.9	Glc-1″	106.3	106.6
C-2	26.6	26.6	26.7	Glc-2″	75.4	75.5
C-3	88.7	88.7	88.7	Glc-3″	78.8	78.9
C-4	39.5	39.5	39.6	Glc-4″	71.3	71.3
C-5	56.0	56.0	56.0	Glc-5″	78.7	78.7
C-6	18.5	18.5	18.5	Glc-6″	62.5	62.5
				3-*O*-		
C-7	32.5	32.6	32.5	Xyl-1	101.2	101.4
C-8	39.9	39.9	39.9	Xyl-2	76.2	76.2
C-9	48.1	48.1	48.0	Xyl-3	73.9	74.4
C-10	37.0	37.0	37.0	Xyl-4	79.4	80.1
C-11	23.8	23.8	23.8	Xyl-5	64.4	65.9
C-12	122.9	122.9	122.9	Rha-1	101.7	101.8
C-13	144.1	144.1	144.1	Rha-2	72.2	72.2
C-14	42.1	42.1	42.1	Rha-3	72.5	72.5
C-15	28.2	28.3	28.2	Rha-4	74.1	73.9
C-16	23.4	23.4	23.4	Rha-5	69.8	69.8
C-17	47.0	47.0	47.0	Rha-6	18.6	18.6
C-18	41.1	41.7	41.7	Glc-1′	106.3	106.6
C-19	46.2	46.3	46.2	Glc-2′	75.4	75.5
C-20	30.8	30.8	30.8	Glc-3′	78.8	79.9
C-21	34.0	34.0	34.0	Glc-4′	71.3	71.3
C-22	33.1	33.1	33.1	Glc-5′	78.7	78.7
C-23	28.1	28.1	28.1	Glc-6′	62.5	62.4
C-24	17.0	17.0	16.6	Glc-1‴	106.9	–
C-25	15.6	15.7	15.6	Glc-2‴	75.9	–
C-26	17.5	17.5	17.4	Glc-3‴	78.5	–
C-27	26.1	26.1	26.2	Glc-4‴	71.5	–
C-28	176.4	176.5	180.3	Glc-5‴	78.5	–
C-29	33.1	33.1	33.3	Glc-6‴	62.6	–
C-30	23.7	23.7	23.8			

Plant material :

The plant material was collected from Guptkashi (Rudraprayag, Uttarakhand) in June. The authentification



Montanoside **1** : R = H

Montanoside **2** : R = glc"

of plant material was made at the Department of Botany, Garhwal University Campus, Badshahithaul, Tehri, Garhwal, Uttarakhand.

Extraction and isolation :

The air dried and powered stems (3 kg) were defatted with petrol in a soxhlet. The solvents free stems 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 extracted with *n*-BuOH (4 × 300 ml). The *n*-BuOH extracts after concentration under reduced pressure yielded a saponin mixture (35 g), which was chromatographed to afford colourless powder of **1** (500 mg) and **2** (310 mg).

Montanoside **1** : A white powder m.p. 252–254° (d), $[\alpha]_D^{25}$–4.8° (*c* 0.90, MeOH). Neg. FAB-MS (*m/z*) : 1057 $[M-H]^-$, 895 [M-H-hexose]$^-$, 733 [M-H-hexose-hexose]$^-$, 587 [M-H-hexose-hexose-deoxyhexose]$^-$, 455 [M-H-hexose-hexose-deoxyhexose-pentose]$^-$. ^1H MNR (pyridine-d_5) : δ 0.87 (3H, s, H_3-25), 0.89 (3H, s, H_3-30), 0.92 (3H, s, H_3-29), 1.08 (3H, s, H_3-24), 1.08 (3H, s, H_3-26), 1.16 (3H, s, H_3-23), 1.25 (3H, s, H_3-27), 3.19 (1H, dd, *J* 11.4, 4.6 Hz, H-3), 5.43 (1H, br s, H-12), xyl-1 to xyl-5, 4.76 (1H, d, *J* 6.1 Hz), 4.47 (1H, overlapped), 4.27 (1H, m), 3.80 (1H, br d, *J* 10.4 Hz), 4.18 (1H, overlapped), rha-1 to rha-6, 6.12 (1H, br s), 4.57 (1H, br s, *J* 9.2, 9.2 Hz), 4.27 (1H, dd, *J* 9.2 Hz), 4.59 (1H, m), 1.63 (3H, d, *J* 6.1 Hz), glc-1′ to glc-6′, 5.12 (1H, d, *J* 7.9 Hz), 4.10 (1H, dd, *J* 7.9, 8.6 Hz), 4.19 (1H, overlapped), 4.21 (1H, overlapped), 3.89 (1H, m), 4.37 (1H, overlapped), 4.48 (1H, overlapped), glc-1″ to glc-6″, 6.24 (1H, d, *J* 7.9 Hz), 4.18 (1H, dd, *J* 7.9, 8.5 Hz), 4.34 (1H, overlapped), 4.37 (1H, overlapped), 4.01 ((1H, m), 4.37 (1H, overlapped), 4.48 (1H, overlapped), ^{13}C NMR (pyridine-d_5) (Table 1).

Montanoside **2** : A white powder [m.p. 258–260° (d), $[\alpha]_D^{25}$–4.2° (*c* 0.85, MeOH), Neg. FAB-MS (*m/z*) : 1219 $[M-H]^-$, 1057 [M-H-hexose]$^-$, 895 [M-H-hexose-hexose]$^-$, 733 [M-H-hexose-hexose-hexose]$^-$, 587 [M-H-hexose-hexose-hexose-deoxyhexose]$^-$, 455 [M-H-hexose-hexose-hexose-deoxyhexose-pentose]$^-$. ^1H MNR (pyridine-d_5) : δ 0.87 (3H, s, H_3-25), 0.90 (3H, s, H_3-30), 0.92 (3H, s, H_3-29), 1.10 (3H, s, H_3-26), 1.15 (3H, s, H_3-24), 1.27 (3H, s, H_3-27), 1.33 (3H, s, H_3-23), 3.25 (1H, br d, *J* 15.8 Hz, H-3), 5.44 (1H, br s, H-12), xyl-1 to xyl-5, 4.71 (1H, d, *J* 6.7 Hz), 4.42 (1H, dd, *J* 6.7, 8.6 Hz), 4.18 (1H, overlapped), 4.23 (1H, m), 3.74 (1H, br d, *J* 11.0 Hz), 4.41 (1H, overlapped), rha-1 to rha-6, 6.14 (1H, br s), 4.97 (1H, br s), 4.81 (1H, dd, *J* 3.7, 9.2 Hz), 4.48 (1H, overlapped), 4.64 (1H, m), glc-1′ to glc-6′, 5.13 (1H, d, *J* 7.9 Hz), 4.03 (1H, dd, *J* 7.9, 9.2 Hz), 4.22 (1H overlapped), 4.21 (1H, overlapped), 3.90 (1H, m), 4.36 (1H, overlapped), 4.50 (1H, overlapped), glc-1″ to glc-6″, 6.33 (1H, d, *J* 7.9 Hz), 4.22 (1H, overlapped), 4.29 (1H, dd, *J* 9.2, 9.2 Hz), 4.36 (1H, overlapped), 4.03 (1H, m), 4.42 (1H, overlapped), 4.48 (1H, overlapped), glc-1‴ to glc-6‴, 5.45 (1H, d, *J* 7.9 Hz), 3.09 (1H, dd, *J* 7.9, 8.5 Hz), 4.22 (1H, overlapped), 4.21 (1H, overlapped), 3.97 (1H, m), 3.39 (1H, overlapped), 4.50 (1H, overlapped), ^{13}C NMR (pyridine-d_5) (Table 1).

Sugar analysis of **1** *and* **2** : A solution of each compound (**1** and **2**) (2 mg) in 2 *N* HCl-dioxane (1 : 1, 2 ml) was heated at 100 °C for 1 h. The reaction mixture was diluted with H_2O and evaporated to remove dioxane through a SEP-PAK-C_{18}. The filtrate from the hydrolysate was neutralized with Ag_2CO_3 and filtered. The filtrate (sugar solution) was analysed by TLC [CH_3CN-MeOH-H_2O (6 : 4 : 1), (**1**) : glusoe, R_f 0.29; rhamnose, R_f 0.54; xylose, R_f 0.40; (**2**) : rhamnose, R_f 0.54; xylose, R_f 0.40; glucose, R_f 0.29].

Alkaline hydrolysis : Compounds **1** and **2** (25 mg each) were separately refluxed with 10% KOH in MeOH (20 ml) for 4 h to afford a prosapogenin, identified as 3-*O*-β-D-glucopyranosyl-oleanolic acid[9]. The filtrate from hydrolysate was neutralized with Ag_2CO_3 and filtered. The filtrate (sugar solution) was analysed by TLC [CH_3CN : MeOH : H_2O (6 : 4 : 1)] for the presence of D-glucose, L-rhamnose and D-xylose (R_f values 0.29, 0.54 and 0.40 respectively). Acid hydrolysis of prosapogenin (3-*O*-β-D-glucopyranosyl-oleanolic acid)[9].

The prosapogenin (10 mg) was hydrolysed with 2 *M* HCl-dioxane (1 : 1, 25 ml) on a boiling water bath for 3 h to afford an aglycone (crystallized from MeOH as colourless needles, m.p. 306–308 °C). The aglycone was identified as oleanoic acid **3**, since the ^1H NMR and ^{13}C NMR data of this compound were in perfect matching with reported data of oleanolic acid[5]. Finally the identity was confirmed by direct comparison (m.m.p., co-TLC and superimposable IR) with an authentic sample.

Assay for cytotoxic activity : Cells were trypsinized and incubated at 6×10^3 per each of microwellplate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA), and incubated for 24 h. After washing once with PBS, they were treated for 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 mg/ml MTT in DMES medium supplemented with 10% FBS. After the medium was removed, the cells were lysel with 0.1 ml DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystem Multiskan (Biochromic, Helsinki, Finaland) connected to a Stav/DOT Matrixprintev JL-10. The LD_{50} value, which reduces the viable cell number by 50% was determined from the dose response curve.

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