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Structure elucidation by NMR spectroscopy of a new acetylated saponin from *Centratherum anthelminticum*

B. K. Mehta,* Darshana Mehta and Amrita Itoriya

School of Studies in Chemistry, Vikram University, Ujjain 456010 (M.P.), India

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Abstract—A new glycosylated triterpene has been isolated from the seeds of *Centratherum anthelminticum*, a medicinally important plant. The structural analysis of its acetylated derivative was performed by ^{1}H, ^{13}C NMR, ^{1}H–^{1}H COSY, HMQC, HMBC and DEPT spectroscopy. The saponin was shown to contain hederagenin and six sugar residues forming two glycosyl chains. The complete structure of the saponin was established as 3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-28-*O*-[β-D-glucuronopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl]-hederagenin.

Keywords: *Centratherum anthelminticum*; Saponin; Acetylated; NMR spectroscopy; Hederagenin

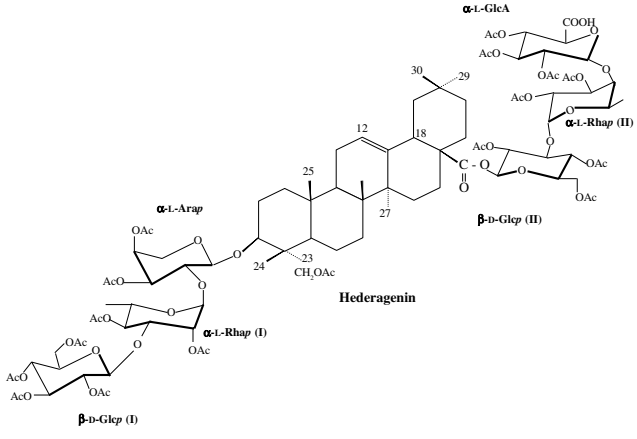
1. Introduction

The plant *C. anthelminticum* (Willd.) Kuntze. (Compositae), commonly known as 'Somraj' and its seeds are known as 'Kalijiri' in Hindi, is distributed throughout in India up to 5500 ft and is reported to be a medicinally important plant.[1] This species has a wide variety of applications in traditional medicine, especially for the treatment of fever, cough, diarrhoea and as a general tonic. It has been reported to possess febrifugal, alterative, anthelminthic, antiphlegmatic, cardiac, diuretic and digestive properties.[2] The different extracts of plant have shown antifertility, antimicrobial, antifilarial and anthelminthic activities.[3–5] In the present study we report on the isolation and characterization of a novel triterpenoid glycoside 3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-28-*O*-[β-D-glucuronopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl]-hederagenin, from the methanolic extract of the seeds.

2. Experimental

2.1. General

NMR spectra were measured on a solution of the acetylated saponin (30 mg) in $CDCl_3$ at ambient temperature. The high resolution 1D and 2D NMR spectra (^{1}H–^{1}H COSY, HMQC and HMBC) and ^{13}C spectral analysis were performed using a JEOL-JNM-500 MHz spectrometer. All chemical shifts (δ) are given in ppm



* Corresponding author. E-mail: bkmehta11@yahoo.com

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and Me_4Si was used as internal standard. The carbon type (CH_3, CH_2, CH) was determined by DEPT experiments. Conventional pulse sequences were used for COSY, HMQC and HMBC. The column chromatography was carried out on silica gel and TLC on silica gel G. Spots were visualized by exposure to iodine vapour or by spraying with H_2SO_4–vanillin solution followed by heating at 105 °C for 5 min. For sugar analysis, paper chromatography was performed using Whatman filter paper No 1 and visualized by aniline hydrogen phthalate as spraying agent.

2.2. Plant material

The seeds (4 Kg) of *C. anthelminticum* were collected from the local medicinal market of Ujjain city and were identified by the authorities at School of Studies in Botany, Vikram University, Ujjain.

2.3. Isolation and purification of the saponin

The seeds were shade, dried, cleaned and powdered coarsely. The powdered seeds were extracted by *n*-hexane, benzene and methanol serially each for 72–92 h in soxhlet extractor. From the methanol extract, the solvent was removed under reduced pressure by rotary film evaporator to yield a dark brownish residue (150 mg). The dried residue was fractionated on silica gel column, by eluting with different solvent mixtures in their increasing order of polarity. A portion of the elute benzene/EtOAc (1:1) was acetylated with acetic anhydride in the presence of pyridine (48 h; 25 °C; solvent, $CHCl_3$) and chromatographed on a column of silica gel, using a discontinuous gradient from 3:1 benzene/EtOAc to 1:1 benzene:methanol. Fractions 40–50 (2500 mL) benzene/methanol (9:1) afforded the acetylated saponin as pale white, shiny crystals (180 mg), mp 280–285°, $[\alpha]_D$ +15 (*c* 1.6, $CHCl_3$). The following TLC solvent systems were used: for saponin **A**. $CHCl_3$:MeOH:AcOH:H_2O (15:8:3:2); for sapogenin **B**. $CHCl_3$:MeOH (9:1) for monosaccharides **C**. $CHCl_3$:MeOH: H_2O (8:5:1).

2.4. Acid hydrolysis

A solution of saponin (6 mg) in 80 % methanol:benzene (5 mL) was refluxed for 6 h with 4 mL of 1 M HCl. The organic layer was evaporated under reduced pressure. Distilled water was added to reaction mixture and extracted with $CHCl_3$. The TLC analysis of the chloroform layer with **B** solvent system gives aglycon.

2.5. Identification of the sugars of saponin

The water soluble fraction was subjected to silica gel column chromatography, using CH_2Cl_2:MeOH:H_2O (17:6:1) as eluent to afford D-glucose, L-arabinose, L-rhamnose and glucuronic acid. TLC for monosaccharides **C**: R_{fs}: 0.13 (Glc), 0.2 (Ara), 0.29 (Rha), 0.25 (GlcA).

3. Results and discussion

The isolated compound gives positive Salkowaski and Molish's reactions indicating its triterpenoid glycosidic nature.[6] In the ^{13}C NMR spectrum of saponin, the signals attributed to the aglycon (genin) range from δ 10 to 50, except those of C-23, C-3, C-12, C-13 and C-28 (Table 1). The double bond signals at δ 121.99 and 144.0 in the ^{13}C NMR spectrum indicated that the aglycon was an oleanane-Δ^{12} type. The signals between δ 60 and 105 contains resonance for all of the sugar carbons, except those of the methyl groups of the two rhamnose observed at δ 17.1 and 17.2. The comparison of the ^{13}C

Table 1. ^1H and ^{13}C NMR chemical shift data ($CDCl_3$, internal Me_4Si) for the aglycon moiety of the acetylated saponin

Atom	**1**	Ref.[a]	(Difference)
C-1	38.5	37.7	(+0.8)
C-2	25.6	23.0	(+2.6)
C-3	82.7	74.3	(+8.4)
C-4	41.03	40.6	(+0.43)
C-5	47.4	47.7	(−0.3)
C-6	18.3	18.1	(+0.2)
C-7	32.9	32.3	(+0.6)
C-8	38.57	39.3	(−0.73)
C-9	48.1	47.7	(+0.4)
C-10	36.5	36.8	(−0.3)
C-11	22.8	23.0	(−0.2)
C-12	122.4	122.0	(+0.4)
C-13	144.0	143.6	(+0.4)
C-14	41.5	41.6	(−0.1)
C-15	27.9	27.7	(+0.2)
H-3	3.48	4.77	(−1.29)
H-12	5.32	5.28	(+0.04)
H-18	2.8	2.85	(−0.05)
H-23	3.92, 4.04	3.68, 3.85	(+0.24, +0.19)
C-16	23.7	23.4	(+0.3)
C-17	46.3	46.6	(−0.3)
C-18	42.1	41.3	(+0.8)
C-19	45.9	45.8	(+0.1)
C-20	29.8	30.6	(−0.8)
C-21	33.7	33.8	(−0.1)
C-22	32.4	32.3	(+0.1)
C-23	65.2	65.3	(−0.1)
C-24	12.8	13.1	(−0.3)
C-25	16.0	15.8	(+0.20)
C-26	16.9	16.8	(+0.1)
C-27	25.2	25.8	(−0.6)
C-28	175.21	177.8	(−2.6)
C-29	32.9	33.1	(−0.2)
C-30	23.3	23.6	(−0.3)
CH_3-24	0.80	0.89	(−0.9)
CH_3-25	0.96	0.96	(0.0)
CH_3-26	0.73	0.71	(+0.2)
CH_3-27	1.1	1.09	(+0.01)
CH_3-29	0.90	0.91	(−0.01)
CH_3-30	0.90	0.82	(+0.08)

[a] Reference compound: methyl ester of hederagenin diacetate.

chemical shifts of the aglycon part with literature data identified it as hederagenin.[7,8] Compared with the corresponding signal in the methyl ester of hederagenin diacetate, the deshielded position of C-3 (δ 82.21) indicates the glycosylation of the OH-3 group,[9] the chemical shift of C-23 remains almost unchanged (δ−0.5). The variation of the C-28 chemical shift (δ−2.6) is attributed to glycosylation of the carbonyl group of hederagenin.

Compared with the ^{1}H NMR spectrum of the methyl ester of hederagenin and hederagenin diacetate,[10] the ^{1}H NMR spectrum of saponin confirms our interpretation. The six singlet resonances at δ 0.73, 0.80, 0.90, 0.90, 0.96 and 1.1 were attributed to the six methyl groups of the triterpene moiety; the resonances at δ 2.8, 3.48 and 5.32 correspond to the H-18, H-3 and H-12 protons, respectively. The shielded position of H-3 (δ 3.48, Table 1) indicated that the OH-3 group is not acetylated, that is, this position is glycosylated. Further, the moderate deviation in the H-23 value of genin in the ^{1}H NMR (δ 3.92, 4.04) from the expected value for 23-CH_2OAc (δ 3.68, 3.85) is attributed to the stereoelectronic effect of the OH-3 sugar chain.

After acetylation of the saponin, the sugar protons showed signals in the δ 3.0–5.5 ranges and their assignment is the basis of the sugar identification process. Increasing length of the sugar chains generated on entangled CHO area, whole analysis was performed by using 2D correlated spectroscopy (COSY). Presence of six sugars were evident from the six anomeric proton signals so observed at δ 4.35, 4.5, 4.68, 4.79, 5.1 and 5.52. In the ^{13}C NMR spectrum the signals at δ 91.5, 97.5, 99.3, 100.4, 101.3 and 103.9 correspond to the six anomeric carbons. The chemical shift values indicate that five of them (δ 97.5, 99.3, 100.4, 101.3, 103.9) are glycosidic and a sixth (δ 91.5) is involved in an ester linkage.

The ring protons of the monosaccharides residues were assigned starting from the anomeric protons by means of a ^{1}H–^{1}H COSY experiment (Table 2) and their respective ^{13}C values from HMQC spectra[11–15] (Table 4). A ^{1}H–^{13}C one bond chemical shift correlation experiment (HMQC) correlated all proton resonances with those of corresponding carbons. On the basis of ^{1}H chemical shift and $J_{H,H}$ coupling constant values[11,12] (Table 3), the ring size, configuration and conformation of six sugar residues were unambiguously determined. The absolute configuration of each sugar was inferred on the basis of its natural occurrence in saponosides. The signal at δ 4.35 was attributed to the anomeric proton of a pentopyranose substituted at O-2 (H-2 at δ 3.97).

The other three spin systems corresponding to three hexoses (H-1 at δ 4.50, 4.68, 5.52) showed them to be in the pyranoid form. There large J values (from 8.0 to 10.0 Hz) indicated that the H-1, H-2, H-3, H-4 and H-5 of these sugars were in axial positions, that is, these sugars are β-D-glucopyranose. A very clear doublet at δ

Table 2. ^{1}H Chemical shift data[a] ($CDCl_3$, internal Me_4Si) for the sugar part of the acetylated saponin

Atom	α-L-Ara *p* (A)	α-L-Rha *p* (R I)	β-D-Glc *p* (G I)	β-D-Glc *p* (G II)	α-L-Rha *p* (R II)	β-D-GlcA (GA)
H-1	4.35	5.1	4.5	5.52	4.79	4.68
H-2	3.97[b]	4.93	4.29	5.11	5.14	4.86
H-3	4.96	5.04[b]	5.07	5.17[b]	5.05	5.45
H-4	5.21	4.15	5.18	4.92	4.03[b]	5.09
H-5ax	3.94	3.83	3.57	3.77	4.12	5.36
H-5eq	3.59					5.68
H-6a			4.44	4.08		
H-6b			3.89	3.42		
CH_3		1.13			1.18	

[a] Assignments may be interchanged within a column.
[b] Nonanomeric protons at the position of interglycosidic linkages.

Table 3. Proton coupling constant (Hz) for the sugar part of the acetylated saponin

	α-L-Ara *p* (A)	α-L-Rha *p* (R I)	β-D-Glc *p* (G I)	β-D-Glc *p* (G II)	α-L-Rha *p* (R II)	β-D-GlcA (GA)
$J_{1,2}$	7.0	1.5	7.7	8.3	1.5	8.6
$J_{2,3}$	9.2	3.5	9.3	9.0	3.7	9.4
$J_{3,4}$	3.4	10.0	9.4	9.3	9.8	9.4
$J_{4,5ax}$	1.3	10.2	9.4	10.0	9.7	10.0
$J_{4,5eq}$	3.7					
$J_{5eq,5ax}$	13.1					
$J_{5,6a}$			2.4	2.6		4.8
$J_{5,6b}$			4.3	5.8		2.3
$J_{6a,6b}$			12.4	11.6		12.4
$J_{5,Me}$		6.2			6.3	

Table 4. ^{13}C Chemical shift data[a] ($CDCl_3$, internal Me_4Si) for the sugar part of the acetylated saponin

Atom	α-L-Ara *p* (A)	α-L-Rha *p* (R I)	β-D-Glc *p* (G I)	β-D-Glc *p* (G II)	α-L-Rha *p* (R II)	β-D-GlcA (GA)
C-1	103.9	97.5	100.4	91.5	99.3	101.3
C-2	72.8[b]	71.5	69.0	70.2	68.7	81.8
C-3	73.3	72.0[b]	72.0	74.0[b]	71.8	75.8
C-4	68.8	74.2	69.5	69.0	74.0[b]	70.2
C-5	63.8	67.8	72.8	73.9	67.0	76.1
C-6		17.1	61.9	61.8	17.2	172

[a] Assignments may be interchanged within a column.
[b] Nonanomeric carbons at the position of interglycosidic linkages.

5.68 attributed to H-6 of one of the hexose characterized it as glucuronic acid. The ^{13}C NMR values so observed at δ 172.6 also supported the proposal.

The chemical shift values of the six sugars indicated that one of the glucose and glucuronic acid (H-1 at δ 4.50 and 4.68), among them were unsubstituted. Moreover, the low field position of the H-1 signal (δ 5.51) for another glucose residue was attributed to a glycosidic linkage between the C-1 of this sugar unit and a carboxylic group (C-28) of the hederagenin. The remaining two sugar residues (H-1 at δ 5.1 and 4.78) were concluded to be 6-deoxyhexopyranose, as also evident from two methyls observed at δ 1.13 and 1.18 confirmed them to be L-rhamnopyranosyl.

The sequences of sugars in saponin were determined by observing long range C–H connectivities from heteronuclear multiple bond correlation spectrum (HMBC).[16] Thus, the correlation observed between C-3 of hederagenin (δ 82.7) and H-1 of arabinose (δ 4.35), between C-2 of arabinose (δ 72.8) and H-1 of rhamnose (δ 5.1), between C-3 of rhamnose (δ 72.0) and H-1 of glucose unit confirmed the sequence linked at C-3 of the aglycon moiety, which was

Glc-(1→3)-Rha-(1→2)-Ara-(1→3)-hederagenin.

The ^{13}C NMR signal due to C-28 of the aglycon moiety at δ 175.21 together with signals relevant to another terminal β-D-glucopyranosyl ester unit (δ 91.5, 70.2, 74.0, 69.0, 73.9, 61.8), indicated esterification of the aglycon carboxyl group with this unit. The cross peaks observed between H-1 of rhamnose (δ 4.79) and C-3 of glucose (δ 74.0), H-1 of glucuronic acid (δ 4.68) and C-4 of rhamnose (δ 74.0) from HMBC confirmed the sequence linked at C-28 of the aglycon moiety, which was

GlcA-(1→4)-Rha-(1→3)-Glc-(1→28)-hederagenin.

Based on the result of the above studies, saponin was assigned the structure 3-*O*-[β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-28-*O*-[β-D-glucuronopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-β-D-glucopyranosyl]-hederagenin.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2004.10.001.

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