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Note

A new naturally acetylated triterpene saponin from *Nigella sativa*

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ABSTRACT

A new glycosylated triterpene **1** was identified as 3-*O*-[β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl]-11-methoxy-16-hydroxy-17-acetoxy hederagenin from an ethanolic extract of seeds of *Nigella sativa* Linn. Identification of the naturally acetylated saponin was based on chemical and spectroscopic analyses including FABMS, ^{1}H, ^{13}C, and 2D NMR and DEPT. The saponin was a penta hydroxy pentacyclic triterpene, in which one hydroxyl group was acetylated and other one was methylated naturally.

The genus *Nigella* comprises 20 species (Ranunculaceae), native to South Europe, North Africa and South West Asia. Its species are reported to be medicinal plants in the scientific literature as well as in folklore and horticulture, and their medicinal values are well documented.[1,2] The seeds of *Nigella sativa* Linn. are also known as black cumin or kalongi in Hindi. They have been used for medicinal purposes as a natural remedy for a number of illness and conditions that include bronchial asthma, cough, rheumatism, hypertension, diabetes, inflammation, eczema, fever, tumor, and influenza.[3–6] Carminitive carminative, diuretic, lactogouge, and vermifuge properties have been attributed to a variety of active phytoconstituents in seeds and its oil.[7–11] Various bioactive compounds have been isolated such as alkaloids, steroids, cycloartenol, fatty acids, sugars, flavonoids of trigillin quercetin-3-glucoside, and an isobenzofuranone derivative.[12–23] A bioactive principle α-hederin was isolated from the seeds, and was reported to have in vivo anti-tumor activity.[9]

The present study deals with the isolation and structural elucidation of a new triterpene glycoside3-*O*-[β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl]-11-methoxy-16-hydroxy-17-acetoxy hederagenin **1** from ethanolic extracts of the seeds. The ethanolic extracts were subjected to normal phase silica gel column chromatography using hexane followed by benzene–methanol step gradient to give four fractions. Fraction II containing saponin **1** was eluted from the column with 20:80 benzene–EtOAc. This fraction was further subjected to preparative paper chromatography, using H_2O, 15% AcOH, and BAW (4:1:5 *n*-BuOH–AcOH–H_2O upper phase) to give compound **1**. Then, it was purified using preparative TLC (silica gel, 65:45:12 $CHCl_3$–MeOH–H_2O), and finally recrystallized from methanol–acetone. It was recognized to be a triterpene glycoside from its positive reactions with the Molish and Salkowaski test.[24] Acid hydrolysis (1 M HCl, 6 h, 100 °C) of compound **1** afforded hederagenin, glucose, xylose, and rhamnose that were identified by co-chromatography with authentic samples. Its IR spectrum exhibited absorptions at *ν*(KBr) 3850–3550 (OH), 2921 (CH), 2868 (C═C), 1660–1640 (C═O to adjacent OH), and 1150–1054 cm^{-1} (*O*-glycosidic linkage). The positive-ion FABMS spectrum of compound **1** showed a molecular ion peak [M+Na+H]$^+$at *m/z* 996, corresponding to $C_{49}H_{80}O_{19}$. Both the ^{1}H and ^{13}C NMR spectra depicted data (Table 1) typical of a triterpene of either oleanane or ursane skeleton containing a double bond between C-12 and C-13 and five hydroxyl groups, in which one is found methylated and a second is acetylated.[25] The appearance of double doublet at *δ* 3.2(H-18) by ^{1}H NMR and a low field value of C-13 olefinic carbon at *δ* 150.0 in the ^{13}C NMR spectrum suggested that it was an oleanane derivative rather than ursane, and thus the aglycon was a β-amyrine, oleanane-Δ^{12} type.[26,27] A fragment ion at *m/z* 493 in its FABMS was due to [M−$OCOCH_3$], suggesting the presence of an acetyloxy group in this new genin.[28] The structure was further supported by the characteristic retro-Diels–Alder cleavage Δ ^{12}-pentacyclic triterpene skeleton leading to *m/z* 308 $[C_{18}H_{28}O_4]^+$ and *m/z* 223 $[C_{14}H_{23}O_2]^+$, thus indicating that three hydroxyl groups were situated at C-3, C-17, and C-23 positions, whilst another hydroxyl

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Table 1
^{1}H and ^{13}C NMR chemical shifts for the aglycon moiety **1**[a]

Atom	1		Ref.[25]	
	δ_H	δ_C	δ_C	δ_H
1		37.6	38.8	
2		25.5	25.5	
3	4.10 (1H, dd, *J* 11.5, 4.7)	86.0	83.1	4.13 (1H, m)
4		42.7	43.5	
5		51.0	48.2	
6		18.2	18.2	
7		31.6	32.8	
8		37.5	40.5	
9		48.3	47.1	
10		34.9	36.7	
11	4.12 (1H, dd, *J* 7.5,3.6)	71.2	71.8[28]	4.00 (1H, dd, *J* 8.7, 3.4)[28]
12	5.49 (1H, d, *J* 7.0)	123.2	123.8	5.44 (1H, br s)
13		150.0	142.7	
14		40.4	41.8	
15		27.0	34.7	
16	4.l78 (1H, m)	75.2	67.7	4.71 (1H, br s)
17		75.6	47.1	
18		51.2	40.1	
19		44.2	47.3	
20		31.5	36.1	
21		33.9	81.2	
22		34.8	71.2	
23	4.20 (2H, m)	68.4	64.8	
24	1.09s	14.7	13.6	1.05s
25	0.94s	16.7	16.2	0.91s
26	1.01s	18.2	17.1	1.01s
27	1.28s	27.0	27.4	1.78 s
28	—	181.2	66.4	
29	0.94s	33.9	29.7	1.09 s
30	1.01s	24.4	20.2	1.29s
Other				
	2.10 (s, 3H, Ac)	22.8		
	3.90 (s, 3H, OMe)	52.5		

[a] Pyridine-d_5, ppm, coupling constant in Hz at 300 and 75 MHz.

group was situated at C-16. Further other abundant ions were obtained at *m/z* 391, due to the cleavage of ring E with acetyloxy group and at *m/z* 307, due to the cleavage between ring C and ring D. Thus, position of different groups was justified by mass fragmentation technique.[29] The signal at δ 4.12 (dd, *J* 7.5, 3.6 Hz) was typical of allylic oxymethine proton and was positioned at C-11.[33] The presence of three anomeric protons at δ 5.08 (d, *J* 8.0 Hz), 5.31 (d, *J* 7.8 Hz), and 6.2 (br s), as well as one methyl group at δ 1.56 (d, *J* 6.0 Hz), indicated that the rhamnose unit is attached to the aglycon through α-linkage, and both the glucose and the xylose units are attached through β-linkage. The comparison of the ^{13}C chemical shifts of the aglycon with the literature data identified it as hederagenin, in which C-16 and C-17 positions were hydroxylated and oriented as β-configuration.[25,30] The downfield chemical shift of the C-28 (δ 181.2) indicated the presence of a chelated hydroxyl function at C-16. Carbinolic carbon of C-3

Table 2
HMBC correlation data of compound **1**

δ_H	Long range coupling to δ_C
0.94 (H-25)	34.9 (C-10), 37.6 (C-1), 48.3 (C-9), 51.0 (C-5)
0.94 (H-29)	24.4 (C-30), 31.5 (C-20), 33.9 (C-21), 44.2 (C-19)
1.01 (H-26)	31.6 (C-7), 37.5 (C-8), 40.4 (C-14), 48.3 (C-9)
1.01 (H-30)	31.5 (C-20), 33.9 (C-29), 44.2 (C-19)
1.09 (H-24)	42.7 (C-4), 68.4(C-23), 86.0 (C-3), 51.0 (C-5)
1.28 (H-27)	27.0 (C-15), 37.5 (C-8), 40.4 (C-14), 150 (C-13)

Table 3
^{1}H and ^{13}C NMR chemical shifts (pyridine-d_5, ppm, coupling constant in Hz) for the sugars at C-3 of the saponin **1**[a]

Atom	β-D-*Glc*		α-L-*Rha*		β-D-*Xyl*	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.08 (d, *J* 8.0)	105.0	6.2 (br s)	102.0	5.31 (d, *J* 7.8)	107.0
2	4.20[a]	75.9	4.94[a]	72.4	4.09[a]	76.4
3	4.23[a]	78.8	4.75[a]	83.3	4.16[a]	78.3
4	4.38[a]	79.4	4.59 t 9.5	72.8	4.18[a]	70.9
5	3.88[a]	78.2	4.73[a]	69.2	3.73[a]	67.2
					4.23[a]	
6	4.06[a]	61.5	1.56 (d, *J* 6.0)	19.2		
	4.17[a]					

[a] Signal pattern was unclear due to overlapping.

deshielded at δ 86.0, which was attributed that alcohol group was involved in glucosylation, the chemical shift of C-23 (δ 68.4) and C-28 (δ 181.2) remains almost unchanged, suggesting monodesmosidic nature of hederagenin.[31] Further moderate deviation from the expected value for C-23 is attributed due to the stereoelectronic effect of the compound **1**.[32]

The DEPT experiment showed the presence of nine methyl groups, eleven methylene, and twenty methine groups. The remaining carbon resonances were attributed to nine quaternary carbon atoms. In the HMBC spectrum, a singlet at δ 2.12 showed correlation with δ 181.2 and δ 75.6, which revealed that hydroxyl group was acetylated at C-17, rather than having a carboxylic group[30] (Table 2) and a secondary alcohol at C-16 was confirmed by observing the signal at 75.2 ppm. The signal of methoxyl protons at δ 3.90 (s, 3H) which showed a cross peak with C-11 (δ 71.2), confirmed that a methyl group was present at C-11. The anomeric proton of the glycopyranosyl unit (H-1′, δ 5.08) showed a correlation with C-3 (δ 86.0), and the rhamnopyranosyl (H-1″, δ 6.2) showed a cross peak with C-4′ of glucose (δ 79.4), which is characteristic for C-4′ in a 4 substituted glucopyranosyl unit.[34,35] The terminal sugar unit was found to be xylose in the pyranose form (H-1‴, δ 5.31) showed a cross peak with C-3″ of rhamnose (δ 83.3) unit. The rhamnose and xylose units at C-4′ of glucose were found to be linked as (1→3) confirmed the sequences of sugars at C-3 of the aglycon moiety, which was: xylopyranosyl (1→3)-rhamnopyranosyl(1→4)-glucopyranosyl(1→3)-hederagenin.

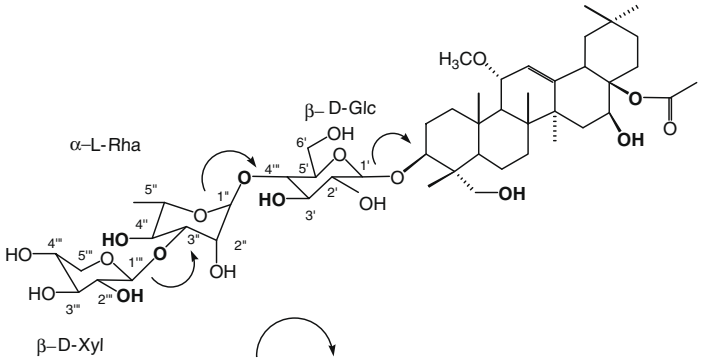

Figure 1. Most significant HMBC correlation.

The ring protons of the monosaccharide residues were assigned by their spin pattern analysis (Table 3), ^1H–^1H COSY, and HMQC experiments. The three sugar moieties were identified as rhamnose unit, inner glucose unit, and terminal xylose unit in the pyranose form by comparing their chemical shift values in the ^{13}C NMR spectrum with the reference data.[35,36] Upon complete acid hydrolysis, compound **1** yielded rhamnose, glucose, and xylose along with the aglycon.

The results which obtained from the spectral studies and extensive review of the related literature[25,28] confirmed the new structure of compound **1** as 3-*O*-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl]-11-methoxy-16-hydroxy-17-acetoxy hederagenin (Fig. 1).

1. Experimental

1.1. General

NMR spectra were measured on a solution of the saponin (30 mg) in pyridine-d_5. The high resolution 1D and 2D NMR spectra (^1H–^1H COSY, HMQC and HMBC) and ^{13}C NMR spectral analysis were performed using a JEOL-JNM-300 and 75 MHz spectrometer. All chemical shifts (δ) are given in ppm, and Me_4Si was used as an internal standard. The protonation of carbon nature (CH_3, CH_2, and CH) was determined by DEPT (90,135) experiments. Conventional pulse sequences were used for COSY, HMQC, and HMBC.

1.2. Plant material

The seeds of *N. sativa* Linn. were collected from the local medicinal market of Ujjain city and were authenticated by IEMPS, Vikram University, Ujjain.

1.3. Investigation of the saponin constituents

1.3.1. Isolation of 3-O-[β-D-xylopyranosyl(1→3)-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl]-11-methoxy-16-hydroxy-17-acetoxy- hederagenin (1)

Ten Kilograms of the shade-dried, cleaned, and coarse-powdered seeds were extracted with *n*-hexane, benzene, benzene–acetone, and ethanol serially each for 72–85 h in a soxhlet extractor. Ethanol extract was concentrated to dryness under reduced pressure by rotary film evaporator to afford a dark brown syrupy residue (450 mg). The dried sample was fractionated on a normal phase silica gel column chromatography (1000 cm × 25 cm), eluting with hexane, followed by benzene–MeOH mixtures of increasing ratio of MeOH gave four fractions. Fraction II (50% benzene–MeOH) was subjected to rechromatography on silica gel, using a discontinuous gradient from 3:1 benzene–EtOAc to 1:1 benzene–methanol. The fractions were monitored and isolated by paper chromatography, using H_2O, 15% AcOH, and BAW (4:1:5 *n*-BuOH–AcOH–H_2O upper phase) to give **1**. Then, it was purified using TLC (silica gel, 65:45:12 $CHCl_3$–MeOH–H_2O), and finally recrystallized from acetone and methanol. Fractions 40–50 (2500 mL) of 2:8 benzene–EtOAc afforded brown a gummy amorphous powder (80 mg): mp 295 °C; $[\alpha]_D$ +21 (*c* 1.8, pyridine); IR 3850:3550 (OH), 2921 (CH), 2868 (C═C), 1660:1640 (C═O to adjacent OH) and 1150:1054 cm^{-1} (*O*-glycoside linkage), and FABMS: *m/z* 996 $[M+Na+H]^+$, 864 $[(M+Na+H)-Xyl]^+$, 718 $[(M+Na+H)-(Xyl+Rha)]^+$, 556 $[(M+Na+H)-(Xyl+Rha+Glc)]^+$; for ^1H and ^{13}C data see Table 1.

2.3.2. Acid hydrolysis

A solution of saponin **1**(5 mg) in 80% methanol–benzene (5 mL) was refluxed for 6 h with 4 mL of 1 M HCl, under Ar atmosphere. After cooling, the organic layer was evaporated under reduced pressure. Distilled water was added to reaction mixture and extracted with $CHCl_3$.

1.3.3. Identification of the sugars of saponin

The water soluble fraction was neutralized with Ag_2CO_3, and sugars in the aqueous solution were identified by co-chromatography with authentic samples using TLC and solvent system (Kieselgel, eluting solvent, 4:1:1 *n*-BuOH–AcOH–H_2O). The chromatograms were sprayed with aniline hydrogen phthalate reagent, and their data revealed the presence of Glc (R_f 0.10), Rha (R_f 0.12), and Xyl (R_f 0.15) units.

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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.2008.10.004.

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