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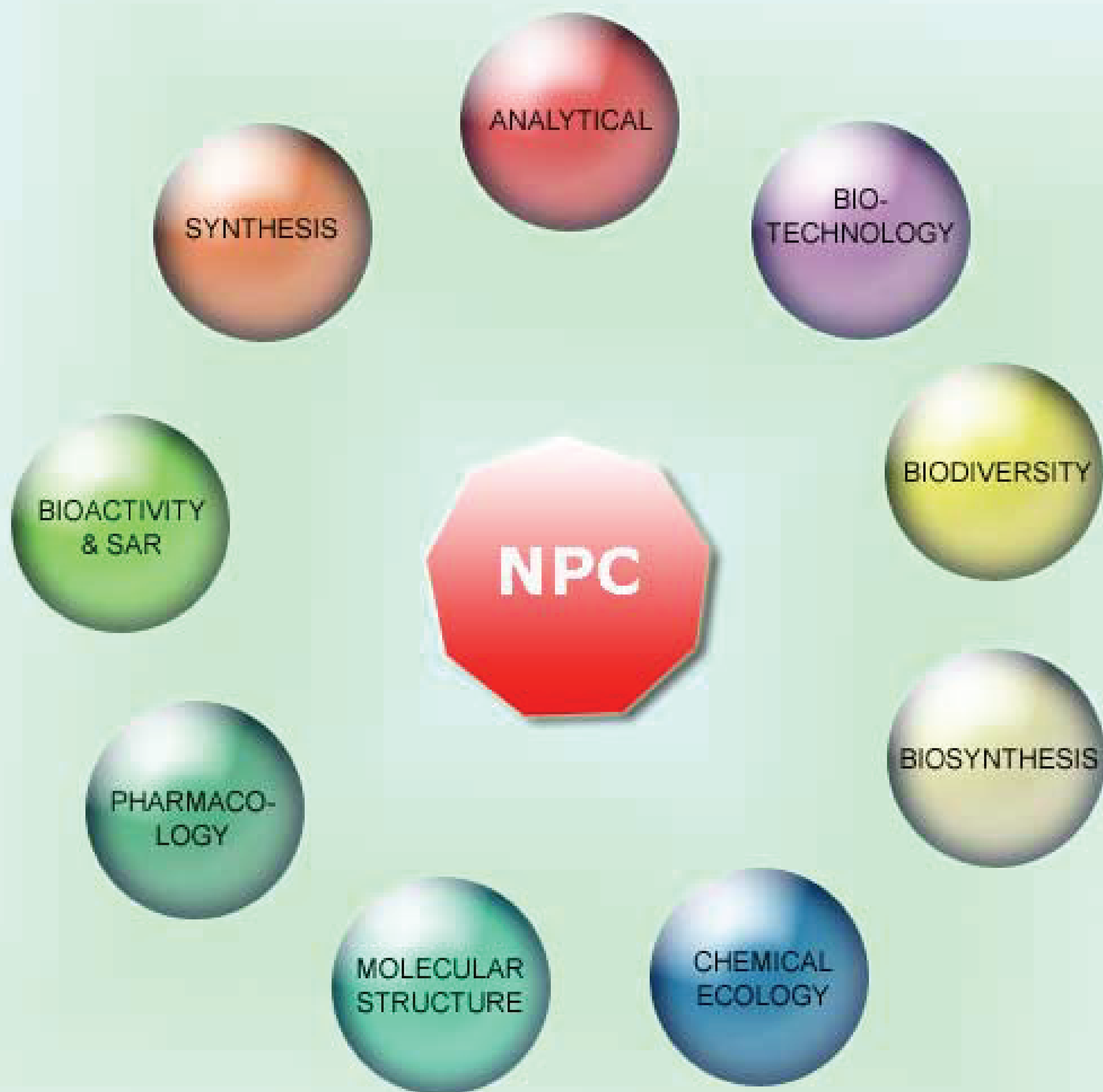

This Issue is Dedicated to
Professor Masimo Curini
On the Occasion of his 70th Birthday

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A New Triterpene Glucoside from *Genista numidica*

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A new oleanolic acid triterpene glucoside, 3-*O*-β-D-glucopyranosyl-3β,21β,28-trihydroxy-olean-12-en-27-oic acid (**1**), has been isolated together with twelve known compounds from the chloroform and ethyl acetate extracts of *Genista numidica* Spach (Fabaceae) aerial parts. The structures were elucidated by spectroscopic and spectrometric analyses, mainly 1D-, 2D-NMR and MS data, and comparison with the literature. The antiproliferative activity of isolates was investigated on Jurkat, HeLa, and MCF7 cell lines. The most active triterpene, 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28,29-tetraol, showed activity in all cell lines. Further studies revealed that this compound induced in HeLa cells a cytostatic response.

Keywords: *Genista numidica*, Fabaceae, Triterpenoid glucoside, Cytotoxic activity.

The genus *Genista* (Fabaceae family) includes about 90 species of shrubs or small trees in the Mediterranean area and in Western Asia, of which 23 grow in Algeria [1]. This genus is known as a good source of active secondary metabolites like essential oils [2-4], saponins [5], quinolizidine alkaloids [6], and flavonoids [7]. A wide range of biological and pharmacological properties has been reported for this genus, such as antimicrobial [8], antioxidant [9], and antiproliferative activities [10]. In the course of our studies on Algerian medicinal plants [11,12], herein the isolation and structural characterization of a new oleanolic acid triterpene glucoside (**1**) along with twelve known compounds, are reported from the chloroform and ethyl acetate extracts of *G. numidica* Spach aerial parts, an endemic trifoliate Algerian species, growing on moist forest floors commonly called Teqtaq. To date only the chemical composition and antimicrobial activity of *G. numidica* essential oil were reported [13], but no phytochemical investigation has never been carried out.

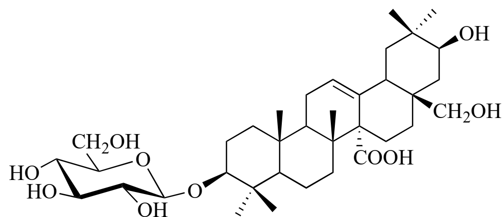

Figure 1: Compound **1**.

Compound **1** (Figure 1) was obtained as an amorphous powder. Its HRESIMS spectrum showed a deprotonated molecular ion peak at *m/z* 649.3926 $[M-H]^-$ allowing the assignment of the molecular formula $C_{36}H_{58}O_{10}$. The ^1H NMR spectrum of **1** (Table 1) showed six tertiary methyl groups at δ_H 0.84, 0.86, 0.89, 0.92, 1.01, and 1.06, one olefinic proton at δ_H 5.56 (br s), one hydroxymethine at δ_H 3.44, and one hydroxymethylene at δ_H 3.50, 3.20 indicating the presence of an oleanane triterpenoid skeleton [5].

Table 1: ^1H (600 MHz) and ^{13}C (150 MHz) NMR data of **1** (*J* in Hz, CD_3OD)[a].

position	δ_H	δ_C
1a	1.68[b]	38.6
1b	1.06 dd (10.8, 4.5)	
2a	1.96[b]	26.3
2b	1.70[b]	
3	3.17 dd (11.7, 3.8)	89.5
4		38.0
5	0.84[b]	55.0
6a	1.54 m	18.0
6b	1.37 m	
7a	1.66[b]	36.5
7b	1.43[b]	
8		40.0
9	2.08 dd (14.4, 4.5)	44.6
10		38.0
11a	1.95[b]	21.9
11b	1.92[b]	
12	5.56 br s	125.5
13		150.0
14		55.0
15a	2.00 ddd (16.0, 14.0, 5.0)	22.4
15b	1.64[b]	
16a	1.70[b]	22.1
16b	1.63[b]	
17		44.0
18	2.37 dd (13.0, 4.0)	46.8
19a	1.68[b]	38.6
19b	1.50[b]	
20		43.7
21	3.44 dd (11.4, 6.0)	73.0
22a	1.47[b]	38.0
22b	1.44[b]	
23	1.06 s	27.0
24	0.86 s	15.6
25	0.89 s	15.6
26	1.01 s	16.0
27		174.0
28a	3.50 d (11.0)	68.9
28b	3.20 d (11.0)	
29	0.92 s	29.0
30	0.84 s	15.6
1'	4.32 d (7.8)	105.9
2'	3.20 dd (9.0, 7.8)	74.2
3'	3.35 t (9.0)	76.8
4'	3.31 t (9.0)	70.0
5'	3.25 m	76.4
6'a	3.85 dd (12.0, 3.5)	61.0
6'b	3.66 dd (12.0, 5.0)	

[a] Chemical shifts in ppm; assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments. [b] overlapped signal.

Additionally, the ^{1}H NMR spectrum of **1** showed the presence of one anomeric proton (δ_H 4.32, 1H, d, J = 7.8 Hz). The ^{13}C NMR spectrum (Table 1) confirmed the presence of an oleanane triterpene glycoside with one β-glucopyranose sugar unit. The complete assignments of all protons and carbons were based on the analysis of HSQC, COSY, and HMBC data, indicating the presence of two oxygenated methines at δ_H 3.17/ δ_C 89.5 and δ_H 3.44/ δ_C 73.0, and one primary alcoholic function at δ_H 3.20, 3.50/δ_C 68.9. Furthermore, basing on HMBC correlations between protons at δ_H 3.20 and 3.50 (H_2-28) and δ_C 38.0 (C-22), 44.0 (C-17), and 46.8 (C-18), the primary alcoholic function was assigned at C-28 position. The position of the two hydroxymethines was suggested by HMBC correlations between the two angular methyls at δ_H 0.92 (Me-29) and 0.84 (Me-30) with 73.0 ppm (C-21), which correlated in the HSQC spectrum to the deshielded signal at δ_H 3.44 (1H, dd, J = 11.4, 6.0 Hz, H-21), and by cross peaks between δ_H 1.06 (Me-23) and δ_C 89.5 (C-3) and δ_H 0.86 (Me-24) and δ_C 89.5 (C-3). The chemical shifts of C-14 (δ_C 55.0) and C-7 (δ_C 36.5) indicated that compound **1** had a carboxylic function at C-27 position [**5**]. The location of the glucopyranose moiety was obtained on the basis of the HMBC correlation between the proton signal at δ_H 4.32 (H-1') and C-3 of the aglycon. The configuration of the glucose unit was assigned as D after hydrolysis of **1** with 1 N HCl. The hydrolyzate was trimethylsilylated, and GC retention time of the sugar was compared with that of authentic sample. The analysis of H-3 (dd, J = 11.7, 3.8 Hz) and H-21 (dd, J = 11.4, 6.0 Hz) protons coupling constant showed an axial orientation, indicating therefore the β-configuration of both OH group at C-3 and C-21 [14]. The structure of **1** was therefore elucidated as 3-*O*-β-D-glucopyranosyl-3β,21β,28-trihydroxy-olean-12-en-27-oic acid. Known compounds were identified by NMR and MS data and comparison with literature data as 4-hydroxy-3-methoxy cinnamaldehyde [15], vomifoliol [16], 4-*O*-methylcedrusin [17], syringaresinol [18], quercetin [19], *p*-coumaric acid [20], *p*-hydroxybenzoic acid [21], prunetin [22], 3-methoxyquercetin-7-*O*-β-D-glucopyranoside [23], 3-*O*-β-D-glucopyranosyl-3β,28,29-trihydroxy-olean-12-en-27-oic acid [**5**], 3-*O*-β-D-glucopyranosyl-3β,27,28-trihydroxy-olean-12-en-29-oic acid [**5**], and 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28, 29-tetraol [**5**].

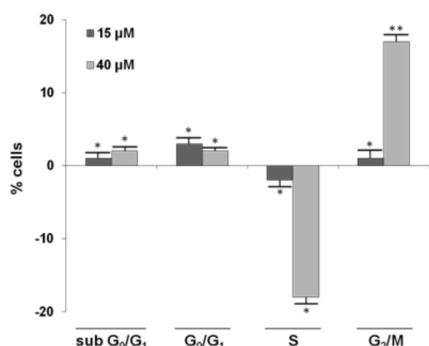

Figure 2: Effect of 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28,29-tetraol on proliferation of HeLa cells. Flow cytometric evaluation of DNA content in HeLa cells exposed for 48 h to compound (15 and 40 µM) or vehicle alone (controls). Data are presented as increase/decrease in percentages of treated cells with a specific DNA content, in respect to control values (control cells, subG0/G1, ≤2%; G0/G1, 62 ± 2.1%; S, 31 ± 1.5%; G2/M, 6 ± 0.3%). All results are mean values ± SD from at least three experiments performed in duplicate (*p< 0.05, **p< 0.001).

The antiproliferative activity of triterpene glucosides and lignans was evaluated in Jurkat, HeLa, and MCF7 cancer cell lines. Cells were exposed to increased concentrations of compounds and cell viability was evaluated at 48 h by MTT assay. Half maximal inhibitory concentration (IC_{50}) values, obtained from dose-response curves, showed that only 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28,29-tetraol has an interesting antiproliferative activity with similar potency in all cell lines. In particular, it displayed in Jurkat, HeLa, and MCF-7 cells an IC_{50} value of 37 ± 2.3, 35 ± 1.7 and 50 ± 3.6 µM, respectively. The mechanism underlying its antiproliferative effect was further investigated in HeLa cells. To investigate whether 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28,29-tetraol reduced cells number by affecting cell cycle progression and/or by inducing cell death, HeLa cells were exposed for 48 h at concentrations close to its IC_{50} value, 15 and 40 µM; DNA content was evaluated by flow cytometry analysis of propidium iodide (PI) stained nuclei. As shown in Figure 2, 3-*O*-β-D-glucopyranosyl-olean-12-en-3β,27,28,29-tetraol induced a robust G_2/M block without any significant increase of hypodiploid cells.

Experimental

General experimental procedures: Optical rotations were measured on a Rudolph Research Analytical Autopol IV polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpin GmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D NMR spectra were acquired in CD_3OD (99.95%, Sigma-Aldrich), and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, and HMBC spectra. TLC were performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with Ce $(SO_4)_2/H_2SO_4$ solution. Column chromatography was performed over silica gel (70–220 mesh, Merck); reversed-phase (RP) HPLC separations were conducted on a Shimadzu LC-20AT series pumping system equipped with a Shimadzu RID10A refractive index detector and a Shimadzu injector, using a C_{18} µ-Bondapak column (30 cm x 7.8 mm, 10 µm, Waters–Milford), C_8 Luna PREP column (25 cm x 10 mm, 10 µm, Phenomenex) and mobile phases consisting of MeOH-H_2O mixtures at a flow rate of 2 ml/ min.

Plant material: The aerial parts of *G. numidica* Spach were collected during the flowering stage at Djebel el Ouahech park (Constantine, Algeria) in June 2013. A voucher specimen (n° GN78/06/2013) has been deposited at the Herbarium of the VARENBIOMOL research unit, University of Constantine 1.

Extraction and Isolation: Air-dried aerial parts of *G. numidica* (1.7 Kg) were macerated at room temperature with EtOH–H_2O (8:2, v/v). The extract was concentrated to dryness, and the residue was suspended in H_2O, and partitioned successively with $CHCl_3$, EtOAc and *n*-BuOH, to yield a $CHCl_3$ (2 g), EtOAc (6 g), and *n*-BuOH fraction (15 g), respectively.

The $CHCl_3$ extract (2 g) was subjected to Silica gel column chromatography and eluted with *n*-hexane and a gradient of EtOAc up to 100%, then eluted with $CHCl_3$ and gradient of MeOH (10%, 15%, 20%, 40%, 60%, 100%) to yield 27 fractions. Each fraction was monitored on TLC and similar fractions were combined. Fractions 4 (52 mg) and 5 (40 mg), eluted with *n*-hexane-EtOAc (85:15-82:18), were purified by RP-HPLC (C_{18}) using MeOH-H_2O (45:55) to give 4-hydroxy-3-methoxycinnamaldehyde (3 mg, t_R = 5 min). Fractions 13-15 (186 mg), eluted with *n*-hexane-EtOAc (3:2-1:1), were purified by RP-HPLC (C_8) using MeOH-H_2O (3:7) to give vomifoliol (2 mg, t_R 40 min). Fraction 20 (91 mg), eluted with *n*-hexane-EtOAc (3:7), was purified by RP-HPLC (C_8) using MeOH-H_2O (2:3) to afford 4-*O*-methylcedrusin (2 mg, t_R 45 min) and syringaresinol (1.2 mg, t_R 52 min).

5.5 g of the EtOAc extract was subjected to silica gel column chromatography and eluted with CH_2Cl_2 and a gradient of acetone up to 100%, and then followed by increasing concentrations of

MeOH (between 10% and 80%) to yield 35 fractions. Fraction 8 (60 mg) gave a yellow precipitate that was washed with $CHCl_3$ to give quercetin (8 mg). Fraction 10 (108 mg) was purified by RP-HPLC (C_8) using MeOH-H_2O (3:7) to give *p*-coumaric acid (2.7 mg, t_R 18 min) and *p*-hydroxybenzoic acid (2.3 mg, t_R 23 min). Fraction 12 (146 mg) contained a major product as yellow precipitate which was washed with $CHCl_3$ to give prunetin (4 mg). Fraction 16 (430 mg) was purified by RP-HPLC (C_{18}) using MeOH-H_2O (2:3) to give 3-methoxyquercetin-7-*O*-β-D-glucopyranoside (1.8 mg, t_R 40 min). Fraction 18 (339 mg) was purified by RP-HPLC (C_{18}) using MeOH-H_2O (57:43) to give compound **1** (1.2 mg, t_R 25 min), 3-*O*-β-D-glucopyranosyl-3β,28,29-trihydroxy-olean-12-en-27-oic acid (3 mg, t_R 32 min), 3-*O*-β-D-glucopyranosyl-3β,27,28-trihydroxy-olean-12-en-29-oic acid (1.5 mg, t_R 39 min) and 3-*O*-β-D-glucopyranosyl-olean-12-ene-3β,27,28,29-tetraol (2 mg, t_R 44 min).

3-*O*-β-D-glucopyranosyl-3β,21β,28-trihydroxy-olean-12-en-27-oic acid (1)
$[\alpha]_D$: + 32.0 (*c* 0.1, MeOH).
^{1}H and ^{13}C NMR: Table 1.
ESI-MS: *m/z* 649 $[M-H]^-$, 605 $[M-H-44]^-$, 425 $[M-H-44-18-162]^-$.
HRESIMS: *m/z* 649.3926 $[M-H]^-$ (calculated for $C_{36}H_{57}O_{10}$, 649.3952).

Acid hydrolysis of compound 1: A solution of compound **1** (2.0 mg) in 1 N HCl (1 mL) was stirred at 80°C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)-imidazole and pyridine (0.2 mL), and the solution was stirred at 60°C for 5 min. After drying the solution, the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm x 25 m). Temperatures of both the injector and detector was 200°C. A temperature gradient system was used for the oven, starting at 100°C for 1 min and increasing up to 180°C at a rate of 5°C/min. Peak of the hydrolysate was detected by comparison with retention times of authentic samples of D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Cell cultures: Jurkat, HeLa, and MCF-7 cell lines were obtained from the American Type Cell Culture (ATCC). Cells were maintained in DMEM (HeLa and MCF-7) or RPMI 1640 (Jurkat), supplemented with 10% fetal bovine serum, 100 mg/L streptomycin and penicillin 100 IU/mL at 37°C in a humidified atmosphere of 5% CO_2. To ensure logarithmic growth, cells were subcultured every two days. Stock solutions (50 mM) of isolates in DMSO were stored in the dark at 4°C. Appropriate dilutions were prepared in culture medium immediately prior to use. In all experiments, the final concentration of DMSO did not exceed 0.15% (v/v).

Cell viability and cell cycle: The number of viable cells was quantified by MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) assay. Absorption at 550 nm was assessed using a microplate reader (LabSystems, Vienna, VA, USA). In some experiments cell viability was also checked by Trypan Blue exclusion assay using a Bürker counting chamber. IC_{50} values were calculated from cell viability dose–response curves and compared to etoposide, used as positive control. Each experimental condition was tested once in quadruplicate.

Cell cycle was evaluated by PI staining of permeabilized cells according to the available protocol and flow cytometry (BD FACSCalibur flow cytometer, Becton Dickinson) [24]. Data from 5000 events per sample were collected. The percentages of the elements in the hypodiploid region and in G_0/G_1, S and G_2/M phases of the cell cycle were calculated using the CellQuest or MODFIT software, respectively.

Statistical analysis: Data reported in Figure 2 are the mean values ± SD from at least three experiments, performed in duplicate, showing similar results. Differences between treatment groups were analyzed by Student's t-test. Differences were considered significant when $p < 0.05$.

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