Dialiumoside, an Olean-18-ene Triterpenoid from *Dialium excelsum*

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Phytochemical investigation of the stem bark and fruits of *Dialium excelsum* led to the isolation of a new triterpenoid, named dialiumoside (**1**), together with twelve known compounds (**2** – **13**). The structure of the new compound as well as those of the known compounds were established by means of spectroscopic methods and by comparison with previously reported data. Compounds **1** – **13** were tested for their cytotoxic activity against the human cervix carcinoma KB-3-1 cells and the related multi drug-resistant P-gp-expressing KB-V1 cells. Compounds **1** and **13** showed weak biological activity in cytotoxicity assays while other compounds were inactive.

Key words: Dialium excelsum, Dialiumoside, Triterpenoids, Cytotoxicity, KB-3-1 and KB-V1 Cells

Introduction

Dialium excelsum is a small tree that grows in tropical West Africa including Cameroon. Different parts of plants of the genus *Dialium* have been used in several African countries in folk medicine for the treatment of various diseases. In Nigeria, different extracts from *Dialium guineense* are used as a readily available moluscicide in the villages [1, 2]. In Benin and in Cameroon, traditional practitioners use the leaves and fruits of plants of the genus *Dialium* to treat waterborne parasitic diseases, fever and malaria [1, 3]. In West Africa, and particularly in Senegal, the fruits of *Dialium* species are eaten as food [4]. The fruits are known to possess trypsin and chymotrypsin inhibitory properties, and antioxidant activities [5, 6]. A previous phytochemical study of plants of the genus *Dialium* revealed the presence of polyphenols and oleanolic acid glycosides [7]. To the best of our knowledge, no phytochemical or pharmacological study has so far been reported on the species *D. excelsum*. As part of our continuing search for bioactive or new compounds from Cameroonian medicinal plants, we investigated the CH_2Cl_2-MeOH (1 : 1) extracts of the stem bark and fruits of *D. excelsum*. We report herein on the isolation and structure elucidation of a new triterpenoid, dialiumoside (**1**), together with the cytotoxic properties of all compounds isolated.

Results and Discussion

The air-dried, powdered stem bark and fruits of *D. excelsum* were extracted separately with CH_2Cl_2-MeOH (1 : 1). The crude extract from the stem bark was subjected to successive column chromatography over silica gel to yield ten compounds, including the new olean-18-ene triterpenoid named dialiumoside (**1**), taraxerol (**2**) [8], betulinic acid (**3**) [8], ursolic acid (**4**) [8], quinovic acid (**5**) [8], lichexanthone (**6**) [9], docosanoic acid, 22-hydroxy-2,3-dihydroxypropyl ester (**7**) [10], β-sitosterol (**8**) [11], 3-*O*-β-D-glucopyranosyl-β-sitosterol (**9**) [12], and 3-*O*-β-D-glucopyranosyl-quinovic acid (**10**) [13].

The crude CH_2Cl_2-MeOH (1 : 1) extract obtained from the fruits was subjected to repeated column chromatography over silica gel to afford three known compounds: tritriacontan-1-ol (**11**), friedelin (**12**) [8] and luteolin (**13**) [14]. The structures of compounds **1** – **13** were established by means of spectroscopic methods and by comparison of the results with data previously reported for the known compounds.

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data for compound **1** in $CDCl_3$-MeOD.

Position	**1**		**1a**
	δ_C	δ_H (m, J in Hz)	δ_C
1	39.3	1.75 (m)	38.7
2	26.3	1.83 (m)	23.7
3	89.6	3.08 (dd, J = 11.6; 4.4)	81.0
4	39.4	–	37.9
5	56.2	0.75 (m)	55.7
6	18.5	1.52 (m)	18.2
7	35.0	1.46 (m)	34.5
8	41.1	–	40.8
9	51.7	1.29 (m)	51.2
10	37.4	–	37.2
11	21.4	1.57 (m)	21.0
12	25.9	1.64 (m)	26.0
13	41.7	2.26 (m)	41.3
14	42.9	–	42.7
15	29.8	1.27 (m)	29.4
16	33.9	1.38 (m)	33.5
17	47.7	–	48.0
18	137.9	–	136.7
19	132.7	5.11 (s)	133.1
20	32.2	4,75 (br s)	32.1
21	33.9	1.94 (m)	33.5
22	33.9	1.33 (m)	33.5
23	28.1	0.68 (s)	28.0
24	16.2	0.65 (s)	16.5
25	16.0	0.47 (s)	16.0
26	16.7	0.66 (s)	16.7
27	15.0	0.45 (s)	14.9
28	179.8	–	182.8
29	30.2	0.57 (s)	30.3
30	29.1	0.60 (s)	29.1
1′	103.2	4.75 (d, J = 1.3)	–
2′	71.9	3.86 (br s)	–
3′	71.5	3.67 (dd, J = 9.5; 3.2)	–
4′	73.4	3.37 (t, J = 9.5)	–
5′	68.8	3.75 (m)	–
6′	17.3	0.96 (d, J = 6.3)	–

Compound **1** was obtained as a colorless powder, $[\alpha]_D^{25} = -17.4$ (c = 0.01, MeOH). It gave a positive response to the Liebermann-Burchard test for terpenes and the Molish test for glycosides. The molecular formula of **1**, $C_{36}H_{58}O_7$, with eight double bond equivalents was determined by HRMS ((+)-ESI), which showed the pseudo-molecular ion peak $[M+Na]^+$ at m/z = 625.4087 (calculatedm/z = 625.4075 for $C_{36}H_{58}O_7Na$). The IR spectrum displayed characteristic absorptions for a hydroxyl group (3391 cm^{-1}), a C=C group (1684 cm^{-1}), and glycosidic C–O bonds (1051 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) showed singlets due to seven CH_3-C groups at δ = 0.45, 0.47, 0.57, 0.60, 0.65, 0.66, and 0.68, and a doublet for one CH_3-CH group at δ = 0.96 (d, J = 6.3 Hz). The additional features of the ^{1}H NMR spectrum were

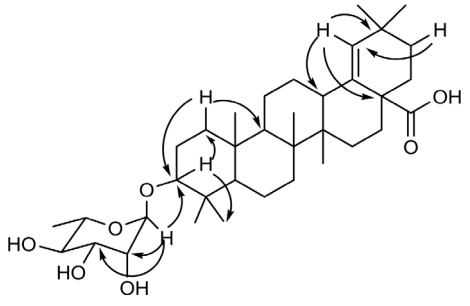

Fig. 1. Selected HMBC correlations for compound **1**.

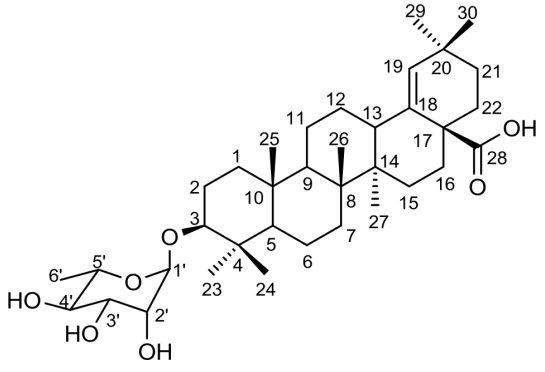

Fig. 2. Structure of compound **1**.

signals of a triterpenoid H-3 oxymethine proton at δ = 3.08 (dd, J = 11.6; 4.4) and that of an olefinic proton at δ = 5.11 (s).

The ^{13}C NMR and DEPT spectra of **1** (Table 1) are in complete agreement with the $C_{36}H_{58}O_7$ molecular formula: they exhibited 36 carbon signals, among them seven quaternary carbon atoms and one carboxyl group (δ = 179.8), ten methine groups including the anomeric carbon of the sugar moiety at δ = 103.2 and one sp^2 olefinic carbon at δ = 132.7, ten methylene groups, and eight methyl groups (δ = 15.0, 16.0, 16.2, 16.7, 17.3, 28.1, 29.1, 30.2). The presence of signals at δ = 132.7 and 137.9 is characteristic of an olean-18-ene triterpenoid skeleton [15]. The position of the Δ^{18} double bond was confirmed by correlations observed in the HMBC spectrum between the singlet at δ = 5.11 (H-19) and the carbons C-13 (δ = 41.7), C-21 (δ = 33.9) and C-17 (δ = 47.7). The appearance of fragment ions at m/z = 191 and 204 in the EI mass spectrum resulting from bond cleavage between carbons C-8/C-14 and C-9/C-11 further confirmed this position [16]. The C-28 position of the carboxyl group was in accord with the chemical shift of the carbon C-17 (δ = 47.7) [15]. For higher plant triterpenoids derived from oxidosqualene cyclization, an oxygenated function has been as-

signed to C-3 (δ = 89.6) [8]. All these data clearly indicate that the aglycon moiety of compound **1** was an olean-18-en-28-oic derivative. This was further confirmed by the fragmentation pattern in the mass spectrum and by comparison of ^{13}C NMR chemical shifts of **1** with those of morolic acid acetate which has identical triterpenoid ABCDE ring moieties [15]. The sugar unit was easily determined as L-rhamnose by analysis of chemical shifts and coupling constant patterns of its proton signals (Table 1) determined in a ^{1}H-^{1}H COSY spectrum. In addition, the set of chemical shifts observed at δ = 103.2, 71.9, 71.5, 73.4, 68.8, and 17.3 (C-1′ to C-6′ respectively) (Table 1) resemble those of the corresponding signals of rhamnose glycoside [17]. The anomeric proton (H-1′) of the rhamnose sugar unit was observed at δ = 4.75 (d, J = 1.3 Hz). The value of the coupling constant suggests the α configuration for the rhamnose. The junction between the aglycon and the sugar moieties was deduced from the correlation observed in the HMBC spectrum between the anomeric proton H-1′ and the carbon C-3 (δ = 89.6) (Fig. 1). Thus, compound **1** is a new triterpenoid that was named dialiumoside with the structure as shown in Fig. 2.

The biological activity of compounds **1** – **13** was determined in cell-based cytotoxicity assays using human cervix carcinoma KB-3-1 cells and the related multi drug-resistant P-gp-expressing KB-V1 cells. P-glycoprotein (P-gp) represents an ATP-dependent efflux system that actively transports structurally diverse xenobiotics to the outside of the cell. For both cell lines, the reduction of resazurin to resorufin was used as readout [18]. Dialiumoside (**1**) and luteolin (**13**) exhibited IC_{50} values of 26.9 (24 – 30 μM 95 % Cl) and 38.5 (27 – 55 μM 95 % Cl) μM against KB-3-1, and of 140.0 (102 – 193 μM 95 % Cl) and 76.4 μM (42 – 140 μM 95 % Cl) against the multi drug-resistant KB-V1 cells. All other tested compounds were inactive against both cell lines. These results show that compounds **1** and **13** exhibit weak bioactivity against both cell lines.

Experimental Section

General

Melting points were determined on a Büchi-540 melting point apparatus. Optical rotations were measured at r. t. in $CHCl_3$ on a Perkin Elmer polarimeter (Model 241). IR spectra were determined on a JASCO Fourier transform IR-420 spectrometer. ^{1}H and ^{13}C NMR spectra were run on a Bruker DRX spectrometer equipped with 5 mm ^{1}H and ^{13}C probes operating at 500 MHz and 125 MHz, respectively, with TMS as internal standard. Silica gel 230 – 400 mesh (Merck) and silica gels (Merck 70 – 230 mesh and 230 – 400) were used for flash and column chromatography, while pre-coated silica gel 60 F_{254} aluminum sheets were used for TLC with different mixtures of ethyl acetate, *n*-hexane and methanol as eluents; spots were visualized under UV lamps (254 and 365 nm) or by heating after spraying with 50 % H_2SO_4 reagent.

Plant material

The stem bark and fruits of *D. excelsum* were collected in February 2010 at Mfou in the Centre Region of the Republic of Cameroon. The plant was identified by Mr. Victor Nana, botanist at the National Herbarium of Cameroon, where a voucher specimen (N 6497/SFR/Cam) is deposited.

Extraction and isolation

The air-dried and powdered stem bark (3.1 kg) and fruits (2.5 kg) of *D. excelsum* were extracted separately in a CH_2Cl_2-MeOH (1 : 1) mixture (10.0 and 9.0 L, respectively) at room temperature within 5 d. The solvents were evaporated under reduced pressure to afford 110 g and 80.5 g of crude extracts, respectively.

100.0 g of the crude extract from the stem bark was subjected to column chromatography over silica gel (230 – 400 mesh, 350 g) and eluted with *n*-hexane, mixtures of *n*-hexane-EtOAc and EtOAc-MeOH of increasing polarities. A total of 112 fractions of 300 mL each were collected and combined on the basis of similar TLC profiles to yield 5 main fractions ($F_{1\text{-}5}$). Fraction F_1 (19.1 g) was subjected to CC on silica gel (Merck, 70 – 230 mesh) and eluted with an *n*-hexane-EtOAc (10 : 0 to 9 : 1) mixture. This resulted in the isolation of lichexanthone (**6**) (60.9 mg), taraxerol (**2**) (50.5 mg) and β-sitosterol (**8**) (15.0 mg). Fraction F_2 (11.3 g) was also subjected to CC on silica gel (Merck, 70 – 230 mesh) and eluted with an *n*-hexane-EtOAc mixture (8.8 : 1.2 to 8.4 : 1.6) to afford betulinic acid (**3**) (30.6 mg) and ursolic acid (**4**) (12.1 mg). Fraction F_3 (15.2 g) was eluted with *n*-hexane-EtOAc mixtures of increasing polarity (8.2 : 1.8 to 6.5 : 3.5) to yield quinovic acid (**5**) (10.0 mg) and 3-*O*-β-D-glucopyranosylquinovic acid (**10**) (12.3 mg). Fraction F_4 (12.3 g) was subjected to repeated column chromatography on silica gel (Merck, 70 – 230 mesh) and eluted with an *n*-hexane-EtOAc mixture (3 : 2 to 1 : 1) to afford docosanoic acid 22-hydroxy-2,3-dihydroxypropyl ester (**7**) (75.5 mg) and dialiumoside (**1**) (15.1 mg). Similar treatment of fraction F_5 (10.0 g) eluted with *n*-hexane-EtOAc solvent systems of increasing polarity (4.5 : 5.5 to 2 : 8) yielded 3-*O*-β-D-glucopyranosyl-β-sitosterol (**9**) (9.2 mg).

75.0 g of crude extract from the fruits were subjected to column chromatography over silica gel (0.06 – 0.2 nm,

300 g). 140 fractions of 300 mL each were collected by gradient elution using *n*-hexane, *n*-hexane-EtOAc and EtOAc-MeOH solvent systems. Fractions were collected and combined on the basis of similar TLC to yield 3 main fractions (F'_{1-3}). Fraction F'_1 (12.2 g) was subjected to CC on silica gel (Merck, 70 – 230 mesh) and eluted with an*n*-hexane-EtOAc mixture (10 : 0 to 9 : 1) to afford tritriacontan-1-ol (**11**) (15.1 mg). Fraction F'_2 (8.0 g) was subjected to CC on silica gel (Merck, 70 – 230 mesh) and eluted with an *n*-hexane-EtOAc mixture (8.2 : 1.8 to 6 : 4) to yield friedelin (**12**) (30.7 mg). Fraction F'_3 (15.6 g) afforded luteolin (**13**) (60.8 mg) by column chromatography separation on silica gel (Merck, 70 – 230 mesh) using *n*-hexane-EtOAc mixtures (5.5 : 4.5 to 4.5 : 5.5).

Cytotoxicity assays

Cytotoxic activity screening of the isolates was done as described in previous reports [18]. The KB-3-1 and KB-V1 cells were cultivated as a monolayer in DMEM (Dulbecco's modified Eagle medium) with glucose (4.5 $g\,L^{-1}$), L-glutamine, sodium pyruvate and phenol red, supplemented with 10 % (KB-3-1) and 15 % (KB-V1) foetal bovine serum (FBS). 50 $\mu g\,mL^{-1}$ gentamycin was added for the KB-V1 cells. The cells were maintained at 37 °C and 5.3 % CO_2-humidified air. KB-V1 cells were continuously selected during cultivation with vinblastine sulfate (150 nM). On the day before the test, the cells (70 % confluence) were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.05 %; 0.02 % in DPBS) and placed in sterile 96-well plates in a density of 10 000 cells in 100 μL medium per well. The dilution series of the compounds were prepared from stock solutions in DMSO of concentrations of 1 mM or 10 mM. The stock solutions were diluted with culture medium (15 % FBS [KB-V1]; 10 % FBS [KB-3-1]) at least 50 times. Some culture medium was added to the wells to adjust the volume of the wells to the wanted dilution factor. The dilution prepared from stock solution was added to the wells. Each concentration was tested in six replicates. Dilution series were prepared by pipetting liquid from well to well. The control contained the same concentration of DMSO as the first dilution. After incubation for 72 h at 37 °C and 5.3 % CO_2-humidified air, 30 μL of an aqueous resazurin solution (175 μM) was added to each well. The cells were incubated at the same conditions for 6 h. Subsequently, the fluorescence was measured. The excitation was effected at a wavelength of 530 nm, whereas the emission was recorded at a wavelength of 588 nm. The IC_{50} values were calculated as a sigmoidal dose response curve using GRAPHPAD PRISM 4.03. The IC_{50} values equal the drug concentrations, at which vitality is 50 %.

*Dialiumoside (**1**)*

Colorless powder, m. p. 281.5 – 283.4 °C. – $[\alpha]_D^{25} = -17.4$ (c = 0.01, MeOH). – IR (KBr, cm^{-1}): ν_{max} = 3391 (OH), 1684 (C=C), 1051 (C–O). – ^{1}H NMR ($CDCl_3$-MeOD, 500 MHz) and ^{13}C NMR ($CDCl_3$-MeOD, 125 MHz) spectroscopic data, see Table 1. – MS (EI, 70 eV): m/z (%) = 558 $[M–CO_2]^+$ (5), 439 (8), 395 (10), 204 (13), 191 (33), 189 (23), 163 (100). – HRMS ((+)-ESI): m/z = 625.4087 (calcd. 625.4075 for $C_{36}H_{58}O_7Na$, $[M+Na]^+$).

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