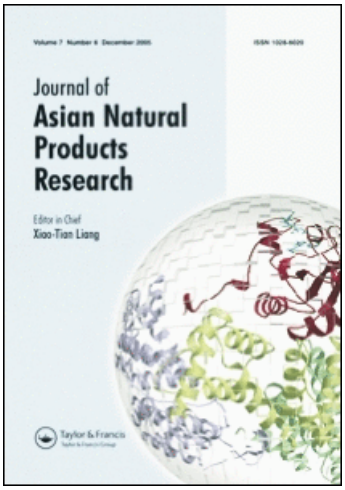

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New myrsinane-type diterpenoids from *Euphorbia aellenii* Rech. f. with their immunomodulatory activity

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Two new 14-desoxo-10, 18-dihydromyrinsol diterpenoids (**1** and **2**) were isolated and characterized from the cytotoxic chloroform fraction of *Euphorbia aellenii* Rech. f. (Euphorbiaceae). The structures of the new compounds were elucidated by spectroscopic methods and their immunomodulatory properties were evaluated by T-cell proliferation and phagocyte chemiluminescence assays.

Keywords: *Euphorbia aellenii*; 10,18-dihydromyrinsol diterpenes; myrsinane; immunomodulatory effect

1. Introduction

Euphorbiaceae is one of the largest families of the phylum Anthophyta. In this family, the largest genus is *Euphorbia*, comprising over 2000 species in tropical and temperate zones of Asia and other parts of the world [1]. In Iran, 70 species are reported, 17 of which are endemic. Among them, *Euphorbia aellenii,* known as 'shirsag' in Iranian ethnopharmacology [2], has been used as a strong laxative, for the treatment of gout, back pain and as a paste on sores [3], and is more probably related to specific types of diterpenes. Myrsinanes are one of the polyester diterpenoids responsible for many pharmacological effects in *euphorbia* such as peripheral analgesic effect which is comparable to standard analgesic drugs pharmacological effects such as, urease inhibitory activity, anti-tumor and anti-HIV properties [4]. Therefore, using NMR-guided fractionation, we isolated two new diterpenoids related to myrsinane (Figure 1) with a typical bond between C-10 and C-18, which are very rare in nature, and were found before only in *Euphorbia prolifera* from China. The immunomodulatory effect of compound **1** was investigated on the oxidative burst activity of whole blood phagocytes and proliferation of human peripheral blood lymphocytes (PBL). Results suggest that *E. aellenii* from Iranian-rich flora could be a new source of 10,18-dihydromyrinsols in making semi-synthetic derivatives for the development of new drugs used for the treatment of inflammatory diseases.

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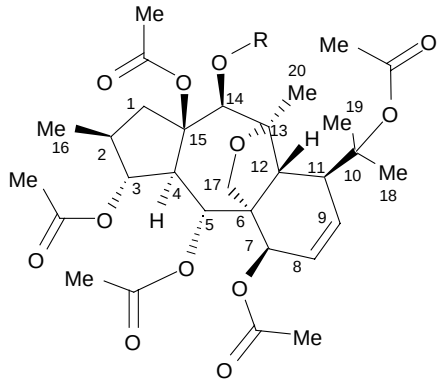

1 R=Benzoylgroup
2 R=Butanoylgroup

Figure 1. The structures of compounds **1** and **2**.

2. Results and discussion

Compound **1** was assigned the molecular formula $C_{37}H_{46}O_{13}$ based on HR-ESI-MS at *m/z* 737.2592 $[M + K]^+$. The IR spectrum showed a prominent peak of carbonyls (1737 cm^{-1}), C—O functionalities (1058–1245 cm^{-1}), and aromatic absorption (1645, 1610, and 1448 cm^{-1}) without evidence of free hydroxyl group. Five singlet methyl protons (δ_H 2.11, 2.09, 1.97, 1.96, and 2.02), along with sequential loss of 60 mass unit, suggested the presence of five acetate groups [5,6]. In addition, the base ion peak of EI-MS at *m/z* 105 (C_6H_5CO) together with ^{1}H NMR signals at δ_H 7.45 (t, $J = 7.2$), 7.58 (t, $J = 7.2$), and 8.09 (d, $J = 7.2$) implied the presence of one benzene ring [5] in the molecule. Taken together, the 15° of unsaturation and ^{13}C NMR spectrum suggested the presence of one benzoyl ester, five acetates, one olefinic bond, and, therefore, a tetracyclic skeleton. The resonances of the polyol core consisted of four methyls (three tertiary and one secondary), two methylenes, ten methines, and four quaternary carbons, with overall, eight oxygenated carbons. Using the HMBC experiment, we found four oxymethine protons (δ_H 3.42, 4.85, 5.29, and 5.93) were geminal to one benzoyl and three acetate carbonyls. Consequently, two other acetyl groups were placed on quaternary oxygenated carbons (δ_C 85.9 and 89.1). Moreover, the signals of a 3H-doublet at δ_H 0.75 (d, 6.4), three 3H-singlet (δ_H 1.22, 1.52, and 1.62), and two vicinal olefinic protons at δ_H 5.87 (dd, 10.2, 5.4) and 6.15 (dd, 10.2, 6.6) were observed in ^{1}H NMR spectrum. Furthermore, geminal oxymethylene protons at δ_H 3.48 (dd, 8.0, 1.1) and 4.09 (br d, 8.0), together with their long-range coupling constant with H-5 ($J = 1.1$ Hz) and their relatively small geminal *J* value ($J = 8.0$ Hz), were indicative of tetrahydrofuran ring of myrsinane-type skeleton [5]. Therefore, on the basis of the above findings and NMR signals, this compound resembled euphorprolitherin A, a 14-desoxo-10(18) dihydromyrinsol, extracted from *E. prolifera* [7], except for esters and C_2 as tertiary carbon (δ_C 37.8) instead of quaternary oxygenated one. The assignment and the connectivity were determined using DQF-COSY and HMBC spectra indicating two partial structures: (A) CH2—CH(CH3)—CHO—CH—CH—O and (B)—CHO—CH=CH—CH—CH as C_1—C_2 (C_{16})—C_3—C_4—C_5 and —C_7—C_8 =C_9—C_{11}—C_{12}, respectively. In addition, H_{12} and two 3H-singlets (δ_H 1.62 and 1.52) correlated in the HMBC spectrum with 10-CO as well as with C-11. These long-range correlations indicated the existence of a single bond between C-10 and C-18 as well as assigning these two methyls as Me-18 and Me-19 lying on C-10 (Figure 2). Regarding H-4α as reference point, the NOE cross-peaks which were observed between H-4α/H-2, H-7, H-14; H-7/H-17b and H-17a, H-14/Me-20, Me-20/H-11, and H-17a/H-7 supported α-position for these protons, whereas the NOE cross-peaks of Me-16β/H-3, H-3/H-5, and H-5/H-12 confirmed their β-orientations (Figure 3).

Compound **2**, a colorless oil, showed the molecular formula as $C_{34}H_{48}O_{13}$ based on HR-ESI-MS at *m/z* 703.2793 $[M + K]^+$, in accordance with the number and the multiplicity of the ^{13}C NMR spectral data (Table 1). IR spectrum

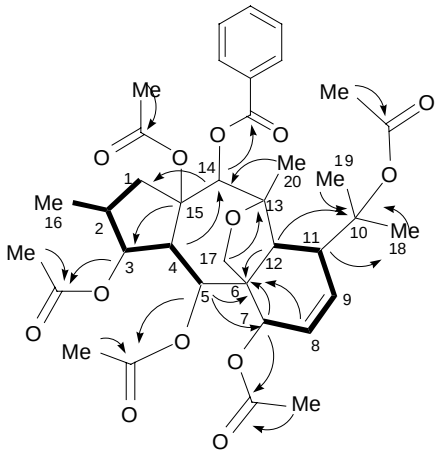

Figure 2. COSY (in bold) and key HMBC correlations (→) of compound **1**.

supported the presence of carbonyls (1737 cm^{-1}), olefinic group (1645 and 1610 cm^{-1}), and C—O (1245–1058 cm^{-1}) functions without free hydroxyl group absorption. As inferred from the NMR spectroscopic data and 11° of unsaturation, the structure of **2** differed from **1** only for 14-O-butanoyl ester [δ 175.8, 36.6 (2.65 t, $J = 6.8$ Hz, $H_{2'}$), 19.1 (1.22 m, $H_{3'}$) and 13.8 (0.92 t, $J = 6.8$ Hz, $H_{4'}$)] in **2** instead of 14-O-benzoyl group in **1** [5]. NOESY spectrum and *J*-coupling constants, compared with those of **1**, proposed that **2** had the similar configuration (Figure 3).

2.1 T-cell proliferation assay

The anti-proliferation effect of the test compounds on lymphocytes was determined by measuring the phytohemagglutinin (PHA)-induced T-cell proliferation using radioactive thymidine incorporation [8]. Addition of **1** to PHA-stimulated human peripheral blood lymphocytes (PBL)s in the concentration ranges 0.5, 5, and 50 μg/ml resulted in dose-dependent suppression of T-cell proliferation (p-value > 0.05) by 39 ± 5.0, 68 ± 2.0, and $72 \pm 1.6\%$ respectively, with IC_{50} of 4.48 ± 0.73 μg/ml. Prednisolone as control suppressed the T-cell response potently even at the lowest concentration (0.5 μg/ml).

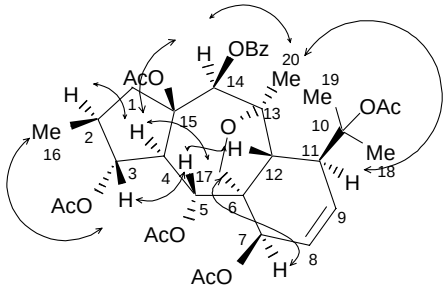

Figure 3. Key NOESY cross-peaks (↔) detected for compound **1**.

2.2 Phagocyte chemiluminescence assay

Phagocytic cells on activation induce release of reactive oxygen free radicals which are then quantified by a luminol-enhanced chemiluminescence assay [9,10]. Results indicate that compound **1** inhibited the zymosan-induced oxidative burst in whole blood phagocytes (up to 50%) at a concentration of less than 0.5 μg/ml. The molecular mechanism causing the immunomodulatory effects of 10,18-dihydromyrisols in stimulated polymorphonuclear cells is still under investigation and it may be mediated by three main mechanisms: cell death, scavenging of reactive oxygen species (ROS), and inhibition of enzymes involved in the signal transduction pathways of the ROS generation process by these cells [9].

3. Experimental

3.1 General experimental procedures

The ^1H NMR spectra were recorded on a Bruker Avance AV 300, and ^{13}C NMR and 2D NMR spectra were recorded on a Bruker Avance AV 600 NMR instrument, using $CDCl_3$ as solvent. Infrared spectra were recorded on a FT-IR-8900 Shimadzu spectrophotometer by casting the sample from the chloroform solution on a NaCl plate; ultraviolet (UV) spectra were recorded on a Hitachi U-3200 spectrophotometer; EI-MS spectra were measured in an electron impact

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of the myrsinol diterpenes[a].

	1		**2**	
No.	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)	δ_C
1a	1.78 dd(15.0, 12.6)	45.2	1.78 dd(14.8,12.4)	45.2
1b	2.45 dd(15.0, 7.8)	–	2.46 dd(14.8,7.6)	–
2	1.96 m[b]	37.8	1.95 m[b]	37.8
3	5.29 t(3.3)	76.8	5.28 br	76.9
4	3.42 dd(10.8, 3.3)	49.7	3.41dd(11.2,3.2)	49.5
5	5.93 dd(10.8, 1.1)	68.7	5.93 br d(11.2)	68.6
6	–	53.6	–	53.5
7	4.85 d(6.6)	63.0	4.84 d(6.8)	63.0
8	6.15 dd(10.2, 6.6)	125.7	6.15 dd(8.9,7.4)	125.7
9	5.87 dd(10.2, 5.4)	129.9	5.88 dd(8.9,5.6)	129.8
10	–	85.9	–	85.9
11	3.14 br	44.6	3.14 br	44.6
12	3.24 d(3.6)	37.1	3.24 d(3.2)	37.0
13	–	90.0	–	89.9
14	5.90 s	73.2	5.89 s	72.3
15	–	89.1	–	88.8
16	0.75 d(6.4)	13.9	0.75 d(6.4)	13.9
17a	3.48 dd(8, 1.1)	69.8	3.48 br d(8.8)	69.8
17b	4.09 br d(8.0)	–	4.10 br d(8.8)	–
18	1.62 s	25.2	1.62 s	25.2
19	1.52 s	20.9	1.52 s	20.9
20	1.22 s	24.7	1.22 s	24.7
3-OAc	–	171.2	–	175.8
	2.09 s	22.4	2.09 s	22.3
5-OAc	–	169.4	–	169.4
	2.02 s	21.1	2.01 s	21.1
7-OAC	–	170.6	–	170.6
	1.96 s	21.1	1.96 s	21.1
10-OAC	–	170.8	–	170.8
	2.11 s	22.6	2.11 s	22.5
14-OR	–	165.6	–	175.8
	–	129.4	2.65 t(6.8)	36.6
	8.09 d(7.2)	130.0	1.22 m	19.1
	7.45 t(7.2)	128.7	0.92 t(6.8)	13.8
	7.58 t(7.2)	133.5	–	–
15-OAC	–	168.7	–	168.6
	1.97 s	21.0	1.96 s	21.0

Notes: [a] Assignments aided by the ^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY experiments.
[b] Overlapped with other signals.

mode on a Varian MAT 112 or MAT 312 spectrometer. Fast atom bombardments (FAB) MS were measured on a Jeol HX110 mass spectrometer. Recycling preparative HPLC was carried out on a LC-908 Hitachi Company equipped with UV and refractive index detectors using a YMC-Pack-Sil column (250 × 20 mm i.d.) and monitored at 260 nm. Chromatographic materials were silica gel (25–40 μm; LiChroprep® Si 60) and Sephadex LH-20 (Pharmacia, Inc., Piscataway, NJ, USA). Thin layer chromatography detection was achieved by spraying the silica gel plates with cerium sulfate in 10% aq.H_2SO_4, followed by heating.

3.2 Plant material

Aerial flowering parts of *E. aellenii* Rich. F. (Euphorbiaceae) were collected from plant

populations growing in Galil-e-Shirvan (Iran) and identified by Dr Yasamin Naseh, Herbaceous Sciences Research Center at the Ferdowsi University of Mashhad. A herbarium specimen bearing No. 2024 is preserved in the herbarium of the Faculty of Pharmacy, Isfahan University of Medical Sciences (Iran).

3.3 Extraction and isolation

Air-dried powdered plant (7 kg) was macerated for four days with methanol (20 l × 3) at room temperature, concentrated *in vacuo* (500 g), and then partitioned between aq. methanol and *n*-hexane. The defatted extract was concentrated, dissolved in water, and extracted sequentially with $CHCl_3$ (243 g), EtOAc (167 g), and *n*-BuOH (166 g). The obtained fractions ($Fr._1$–$Fr._4$) were compared *in vitro* for their cytotoxic activities against brine-shrimp eggs [11]. The most active fraction ($Fr._1$, 240 g) was subjected to column chromatography (CC, SiO_2; Hexane–$CHCl_3$, 0 → 100) to afford seven fractions: $Fr._{1a}$–$Fr._{1f}$. NMR analysis showed that $Fr._{1a}$ and $Fr._{1b}$ contained oils and fatty acids, $Fr._{1c}$ triterpenes, and $Fr._{1d}$–$Fr._{1e}$ diterpenes. Therefore, $Fr._{1e}$ was chromatographed on silica gel (Hexane–EtOAc, 0 → 50) to render several fractions: $Fr._{1e1}$–$Fr._{1e7}$. Then, $Fr._{1e6}$, containing mixtures of diterpenes and chlorophyll, was further separated by Sephadex (dichloromethane–MeOH, 2:1) followed by RP18 CC (MeOH–Water, 20:80 → 70:30) to give $Fr._{1e61}$–$Fr._{1e63}$. Finally, $Fr._{1e61}$ as well as $Fr._{1e62}$ was purified by recycling HPLC (Hexane–EtOAc, 70:30) to afford **1** (10 mg, t_R 150 min) and **2** (4 mg, t_R 120 min), separately.

3.3.1 14-Desoxo-3β,5α,7β,10,15β-O-pentaacetyl-14α-O-benzoyl-10,18-dihydromyrinsol (**1**)

Colorless oil, UV ($CHCl_3$) λ_{max} (log ε): 237 (4.54), 276 (4.21) nm; IR (KBr, $CHCl_3$) ν_{max} (cm^{-1}): 2985, 1737, 1645, 1610, 1448, 1245, 1122, 1097, 1058, 756; ^1H and ^{13}C NMR spectral data: see Table 1; EI-MS: *m/z* 578, 518, 336, 295, 253, 173, 133, 105(100), 101, 77; FAB-MS (pos.): *m/z* 579[M-2HOAc]$^+$, 519[579-HOAc]$^+$, 459[519-HOAc]$^+$, 391 and 338; HR-ESI-MS: *m/z* 737.2592 $[M + K]^+$ (calcd for $C_{37}H_{46}O_{13}K$, 737.2575).

3.3.2 14-Desoxo-3α, 5α, 7β, 10, 15β-O-pentaacetyl-14β-O-butanoyl-10,18-dihydromyrinsol (**2**)

Colorless oil; UV ($CHCl_3$) λ_{max} (log ε): 223 (4.55), 265 (4.38) nm; IR (KBr, $CHCl_3$) ν_{max} (cm^{-1}): 2985, 1737, 1645, 1610, 1448, 1245, 1122, 1097, 1058, 756 cm^{-1}; ^1H and ^{13}C NMR spectral data ($CDCl_3$): see Table 1. FAB-MS (pos.): *m/z* 577 [M-HOBu + 1]$^+$, 517 [M-HOAc]$^+$, 457 [M-HOAc]$^+$, 397; HR-ESI-MS: *m/z* 703.2793 $[M + K]^+$ (calcd for $C_{34}H_{48}O_{13}K$, 703.2732).

3.4 T-cell proliferation assay

PBL were incubated with different concentrations of the test compounds (0.5, 5, and 50 μg/ml in duplicates) in supplemented RPMI-1640 along with PHA at 37°C in CO_2 environment for 72 h. Further incubation for 18 h after the addition of thymidine [3H] (Amersham, Buckinghamshire, UK) was done and cells were harvested using cell harvester (Inotech Dottikon, Switzerland). Finally, proliferation level was determined by the radioactivity count as counts per minute reading recorded from the Beta-scintillation counter [8].

3.5 Phagocyte chemiluminescence assay

Formation of the reactive oxidants in whole blood during the oxidative burst was measured by the Luminol-enhanced

chemiluminescence assay procedure in triplicate tests [9,10]. In brief, whole blood diluted in modified Hank's solution was incubated with different concentrations of compound **1** (50, 25, and 5 μg/ml), positive, negative control, and blank for 30 min. Zymosan (Sigma Chemical Co, St Louis, MO, USA) 100 μl (20 mg/ml), followed by 100 μl (7×10^5 M) luminol (Sigma Chemical Co., St Louis, MO, USA) was added to make a final volume of 0.25 ml except for negative and blank wells. The phagocytosis kinetic with luminometer (Labsystems Luminoskan RS, Helsinki, Finland) was monitored for 50 min in the repeated scan mode, the peak and total integral chemiluminescence reading were reported as relative chemiluminescent light units (RLU).

3.6 Statistical analysis

All data are reported as mean ± SD of the mean and the IC_{50} values were calculated using the Excel-based program.

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