Unusual ingenoids from Euphorbia erythradenia Bioss. with pro-apoptotic effects

ARTICLE *in* FITOTERAPIA · AUGUST 2013

Impact Factor: 2.35 · DOI: 10.1016/j.fitote.2013.08.016 · Source: PubMed

7 AUTHORS, INCLUDING:

Seyed Mohammad Zarei
Shahid Beheshti University of Medical…
5 PUBLICATIONS **11** CITATIONS
SEE PROFILE

Mustafa Ghanadian
Isfahan University of Medical Sciences
43 PUBLICATIONS **150** CITATIONS
SEE PROFILE

Farzad Kobarfard
Shahid Beheshti University of Medical…
65 PUBLICATIONS **341** CITATIONS
SEE PROFILE

Mahmoud Aghaei
Isfahan University of Medical Sciences
27 PUBLICATIONS **217** CITATIONS
SEE PROFILE

Fitoterapia 91 (2013) 87–94

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Unusual ingenoids from *Euphorbia erythradenia* Bioss. with pro-apoptotic effects

Seyed Mohammad Zarei [a], Abdul Majid Ayatollahi [a], Mustafa Ghanadian [b,*], Farzad kobarfard [c], Mahmoud Aghaei [d], M. Iqbal Choudhary [e], Faranak Fallahian [f]

[a] Phytochemistry Research Center and School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Islamic Republic of Iran
[b] Department of Pharmacognosy, Isfahan Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[c] Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshtee University of Medical Sciences, Tehran, Islamic Republic of Iran
[d] Department of Clinical Biochemistry, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran
[e] Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan
[f] Cellular and Molecular Research Center, Qom University of Medical Sciences, Qom, Islamic Republic of Iran

ARTICLE INFO

Article history:
Received 16 March 2013
Accepted in revised form 17 August 2013
Available online xxxx

Keywords:
Euphorbia erythradenia
Cytotoxicity activity
Ingenane
Tetrahydroingenol
Flora of Iran

ABSTRACT

Dichloromethane-acetone extract of *Euphorbia erythradenia* Bioss., a spurge endemic to the Iran, afforded four novel tetrahydroingenol diterpenes, one new myrsinane type diterpene, and two known triterpenes. Tetrahydroingenoids are novel compounds not only for the double bond reduction but also for their unique hydroxylation pattern at C-11 and C-13. The structures of these compounds were elucidated by spectroscopic methods, and especially 2D NMR measurements. The biological effects of the compounds were done by the MTT and Annexin V-FITC & PI staining assays on different cancer cell lines. The results obtained on EJ-138, CAOV-4, and OVCAR-3 cell lines suggested that tetrahydroingenanes inhibit cell proliferation through apoptosis in cancer cell lines. In conclusion, the new pattern of hydrogenation and hydroxylation of these compounds compared to other ingenoids, along with their apoptosis inducing properties on cancer cells, makes them of great interest for more investigation.

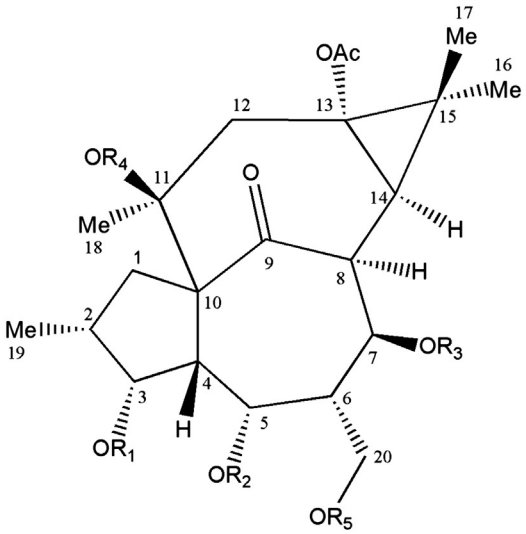
1. Introduction

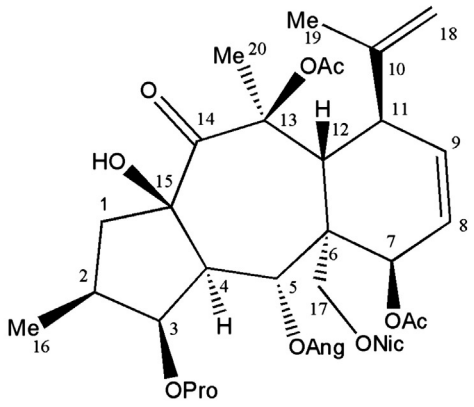
Spurges are characterized by their unique profile of macrocyclic diterpenoids, often occurring as polyesters of the main polyol core (ingol, lathyrol, phorbol, myrsinol, and ingenol) [1]. Recent studies have shown cytotoxic, modulability of multidrug resistance, antivirus and immunomodulatory activities attributed to these macrocyclic diterpenes [1–4]. As part of a project aimed at finding antitumor compounds from diterpenoid polyesters, *Euphorbia erythradenia*, which previously antitumor properties of its metanolic extract were poorly reported [5], was selected for phytochemical analysis. *E. erythradenia* is a rare localised endemic in the Iranian plateau and is most closely related with *Euphorbia gedrosiaca*, which is distributed in central and SE Iran [6]. We now report the isolation and the structure elucidation of novel diterpenes, four of them belongs to the ingenane class with unusual hydrogenation and hydroxylation status (1–4) and one to the myrsinane class (5), along with the known triterpenoids (Fig. 1).

Ingenane type compounds are highly oxygenated tetracyclic diterpene esters that share an ingenol core showing antileukemic or anti-HIV activities as well as promising chemotherapeutic effects for treatment of skin cancer and topical therapy for actinic keratosis and superficial basal cell carcinoma [7,8]. Among them, ingenol-3-angelate (Picato) extracted from *Euphorbia peplus* has been approved by the U.S. Food and Drug Administration (FDA) for the topical treatment of actinic keratosis [8]. Although the exact mechanism of action of this compound is not completely clear, studies have attributed to its ability to activate protein kinase C [8,9].

* Corresponding author. Tel.: +98 3117922553.
E-mail addresses: Ghannadian@gmail.com, ghanadian@pharm.mui.ac.ir (M. Ghanadian).

0367-326X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.fitote.2013.08.016





1 R1= But R2= Ang R3= H R4= H R5= Nic
2 R1= Pro R2= Ang R3= Ac R4= H R5= Nic
3 R1= Pro R2= Ac R3= Bz R4= H R5= Nic
4 R1= But R2= Ang R3= H R4= Nic R5= H

5

Fig. 1. Isolated diterpenes from *Euphorbia erythradenia*.

2. Experimental

2.1. General

The NMR spectra were recorded on a Bruker Avance AV 400, using $CDCl_3$ as solvent, and HRESI-MS spectrometer on Waters Q-TOF Micro YA019 mass spectrometer. Optical rotations were taken using a P-2000 Digital Polarimeter (Jasco Co., Japan). HPLC was carried out on waters 515 using a Pack-Sil column (250 × 20 mm i.d.) packed with 5 μm silica (YMC Co. Ltd., Kyoto, Japan) and hexane:ethyl acetate as mobile phase. Chromatographic materials were silica gel (Merck Co., Germany), 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.

2.2. Plant material

Aerial flowering parts of *E. erythradenia* Boiss. f. (Euphorbiaceae) were collected in September 2010 from populations growing in Gharbalbiz neighboring Mehriz City, Yazd province (Iran). Plant material was identified by Ali Mirhosseini, plant taxonomist and a voucher specimen (nos. 1947) deposited in Natural Resources Research Center, Yazd, Iran.

2.3. Extraction and isolation

The shade-dried aerial parts of plant material (4 kg) were macerated five days with dichloromethane:acetone (2:1, 24 L × 3), at room temperature. Filtration and in vacuo evaporation of the solvent resulted in a green gum (173 g), which was charged over a cake of 20% paraffin impregnated silica-gel (200 g) packed into a sintered Buchner funnel (150 ∗ 90 mm) using MeOH:H2O as eluant. Washing with MeOH:H_2O (6:4) removed the diterpenoid rich fraction (30 g light brown filtrate) and the cake was next washed with MeOH:H_2O (9:1) to remove apolar constituents. The diterpenoid rich fraction was separated on silica gel CC (hexane/EtOAc, 0 → 60) to afford six fractions: $Fr._{1–6}$ inspection of the NMR showed typical signals of diterpenes in $Fr._5$ eluted with hexane/EtOAc (80:20) and was purified on silica gel CC (hexane/acetone, 0 → 30) to eight subfractions: $Fr._{5a}$–$Fr._{5i}$. Fractions $Fr._{5d}$–$Fr._{5i}$ were subjected to more purification on HPLC using YMC-Pak-Sil column (250 × 20 mm) and hexane/EtOAc (80:20) as mobile phase to yield compound 1 ($Fr._{5D5}$), compound 2 ($Fr._{5E3}$), compound 3 ($Fr._{5G}$), compound 4 ($Fr._{5Fb}$), and compound 5 ($Fr._{5Fa}$) as five new macrocyclic polyester diterpenes (Fig. 1) and two known triterpenes namely cycloartanol ($Fr._{5D1}$) and oleanolic acid ($Fr._{5i}$).

2.3.1. 13-acetyl-3-butanoyl-5-angeloyl-20-nicotinyl-1,2,6,7-tetrahydroingenol (1)

Colorless oil. Colorless amorphous oil, $[\alpha]_{D:}$ −53.3 (c 0.37, CHCl3); UV ($CHCl_3$) λ_{max}: 293 nm. IR (KBr) υ_{max}: 3485, 2962, 2935, 2875, 1724, 1591, 1462, 1379, 1267, 1178, 1113, 1049, 1024, 995, 735, and 717 cm^{-1}. ^1H NMR ($CDCl_3$, 400 MHz, *J* in Hz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 1. HRESI-MS *m/z* 684.3411 (calcd. for $C_{37}H_{49}NO_{11} + H^+$, 684.3378, Δ 4.8 ppm). Positive ESI-MS *m/z* 684 [M + H], 668, 624 [M-Acet], 606, 567, 524[624-Ang], 506, 436 [524-But], 418, 313 [436-Nic], 295, 255.

2.3.2. 7,13-diacetyl-5-angeloyl-20-nicotinyl-3-propionyl-1,2,6,7-tetrahydroingenol (2)

Colorless oil. $[\alpha]_{D:}$ −11.0 (c 0.73, CHCl3); UV ($CHCl_3$) λ_{max}: 299.4 nm. IR (KBr) υ_{max}: 3500, 3059, 2939, 2879, 1712,

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of compounds **1** and **5** in $CDCl_3$.

Pos	δ_H, mult., *J* in Hz	δ_C	Pos	δ_H, mult., *J* in Hz	δ_C
1a	3.16 dd (8.0, 13.6)	42.7	*1a*	3.38 dd (14.8, 8.4)	43.6
1b	1.56 dd (7.4, 14.6)		*1b*	1.74 dd (14.8, 11.6)	
2	1.88 m	37.3	*2*	2.11 m	37.6
3	5.32 dd (3.2, 3.8)	78.6	*3*	5.22 dd (3.4, 3.4)	77.8
4	2.41 dd (3.8, 11.2)	49.3	*4*	2.51 dd (11.8, 3.4)	53.4
5	6.28 dd (1.3, 11.2)	70.4	*5*	6.30 d (11.6)	70
6	2.20*	23.8	*6*	–	47.5
7	3.88 dd (1.3, 5.2)	65.9	*7*	4.81 d (6.4)	68
8	3.40 dd (1.3, 6.4)	34.1	*8*	6.11 ddd (2.0, 6.0, 9.6)	122.3
9	–	204.2	*9*	5.83 dd (4.6, 9.6)	136.8
10	–	48.6	*10*	–	149.7
11	–	86	*11*	3.48 ddd (1.6, 4.4, 9.2)	45.9
12a	1.94 d (16)	24.1	*12*	3.58 d(8)	43.1
12b	2.22*		*13*	–	80.6
13	–	83.8	*14*	–	202.1
14	0.76*	19.6	*15*	–	89.8
15	–	18.4	*16*	0.87 d (6.8)	14
16	1.08 s	29.6	*17a*	4.50 d (12.0)	63.1
17	1.14 s	15.1	*17b*	4.25 d (12.0)	
18	1.73 s	25.2	*18a*	4.98 s	112.3
19	0.89 s	14.1	*18b*	4.88 bs	
20_a	4.95 dd (1.3, 11.6)	64.1	*19*	1.82 s	20.5
20_b	4.52 dd (1.3, 11.6)		*20*	1.67 s	23.4
3-0-But	–	173.1	3-0-Pro	–	173.7
2′	2.23 t (7.6)	35.6	2′	2.35 q (7.6)	27.8
3′	1.46 m	18.1	3′	1.15 t (7.6)	8.9
4′	0.91 t	13.7	5-0-Ang	–	167.4
5-0-Ang	–	168.5	2′	–	128
2′	–	127.4	3′	6.54 bq (7.0)	138.1
3′	6.66 q (6.8)	140	4′	1.47 bd (7.0)	14.2
4′	1.38 d (6.8)	14.2	5′	1.61 s	12.1
5′	1.51 s	11.7	7-0-AC	–	168.9
13-0-AC	–	170.5	2′	2.14 s	21.1
2′	2.13 s	21.3	13-0-AC	–	169.7
20-0-Nic	–	164.8	2′	2.21 s	21.4
2′	9.04 d (1.6)	150.6	17-0-Nic	–	166
3′	–	125.7	2′	9.09 d (1.6)	150.9
4′	8.12 dt (1.6, 8.0)	136.7	3′	–	126.4
5′	7.38 dd (4.8, 8)	123.3	4′	8.11 dt (8.0, 1.6)	136.9
6′	8.79 dd (1.6, 4.8)	153.6	5′	7.42 dd (8.0, 4.8)	123.5
			6′	8.81 dd (4.8, 1.6)	153.9

*Overlapped with other signals.

1651, 1576, 1441, 1379, 1259, 1182, 1140, 1072, 1038, 968, 739 and 704 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz, *J* in Hz): δ_H 3.16 (dd, $J = 8.0, 13.6$, 1H, H-1a), 1.66 (dd, $J = 13.2$, 1H, H-1b), 1.86 (m, 1H, H-2), 5.29 (dd, $J = 3.2, 4.2$, 1H, H-3), 2.35 (dd, $J = 4.2, 11.6$, 1H, H-4), 6.27 (dd, $J = 1.3, 11.6$, 1H, H-5), 2.23 (overlapped, H-6), 4.92 (dd, $J = 1.3, 6.4$, 1H, H-7), 3.56 (dd, $J = 1.3, 6.0$, 1H, H-8), 1.93 (d, $J = 18.0$, 1H, H-12a), 2.18 (overlapped, H-12b), 0.79 (overlapped, H-14), 1.08 (s, 3H, H-16), 0.97 (s, 3H, H-17), 1.75 (s, 3H, H-18), 0.88 (d, $J = 6.4$, 3H, H-19), 4.93 (dd, $J = 1.3, 11.6$, 1H, H-20a), 4.56 (dd, $J = 1.3, 11.6$, 1H, H-20b), 2.30 (q, $J = 7.6$, 2H, Pro-2′), 1.04 (t, $J = 7.6$, 3H, Pro-3′), 6.53 (q, $J = 6.8$, 1H, Ang-3′), 1.27 (d, $J = 6.8$, 3H, Ang-4′), 1.47 (s, 3H, Ang-5′), 2.15 (s, 3H, 7-OAc), 2.13 (s, 3H, 13-OAc), 9.11 (d, $J = 1.6$, 1H, Nic-2′), 8.08 (dt, $J = 1.6, 8.0$, 1H, Nic-4′), 7.38 (dd, $J = 4.8, 8.0$, 1H, Nic-5′), 8.78 (dd, $J = 1.6, 4.8$, 1H, Nic-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 42.8 (t, C-1), 37.2 (d, C-2), 78.2 (d, C-3), 50.4 (d, C-4), 70.4 (d, C-5), 23.8 (d, C-6), 69.1 (d, C-7), 35.1 (d, C-8), 204.3 (s, C-9), 47.9 (s, C-10), 85.6 (s. C-11), 22.2 (t, C-12), 84.1 (s, C-13), 18.9 (d, C-14), 18.4 (s, C-15), 29.4 (q, C-16), 14.8 (q, C-17), 24.9 (q, C-18), 13.9 (q, C-19), 63.9 (t, C-20), 173.7 (s, Pro-C-1′), 27.5 (t, Pro-C-2′), 8.8 (q, Pro-C-3′), 166.2 (s, Ang-C-1′), 128.0 (s, Ang-C-2′), 138.1 (d, Ang-C-3′), 13.9 (q, Ang-C-4′), 11.8 (q, C-5′), 170.1 (s, 7-OAc-C-1′), 21.3 (q, 7-OAc-C-2′), 170.6 (s, 13-OAc-C-1′), 21.3 (q, 13-OAc-C-2′), 164.8 (s, Nic-C-O), 150.8 (d, Nic-C-2′), 125.4 (s, Nic-C-3′), 136.6 (d, Nic-C-4′), 123.3 (d, Nic-C-5′), 153.7 (d, Nic-C-6′); HRESI-MS *m/z* 712.3376 (calcd. for $C_{38}H_{49}NO_{12} + H^+$, 713.3333, Δ 6.1 ppm). Positive ESI-MS *m/z* 712 [M + H], 684, 652 [M-Acet], 610, 592 [652-Acet], 518 [592-Prop], 492, 418 [518-Ang], 369 [492-Nic], 282, 256.

2.3.3. 5,13-diacetyl-7-benzoyl-20-nicotinyl-3-propionyl-1,2,6,7-tetrahydroingenol (3)

Colorless oil. $[\alpha]_{D:}$ −49.2 (c 0.18, CHCl3); UV ($CHCl_3$) λ_{max}: 291.8 nm. IR (KBr) υ_{max}: 3477, 2960, 2937, 2877, 1724, 1591, 1460, 1377, 1267, 1180, 1140, 1113, 1026, 739 and 714 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz, *J* in Hz): δ_H 3.19 (dd, $J = 7.6, 13.6$, 1H, H-1a), 1.68 (dd, $J = 9.2, 13.2$, 1H, H-1b), 1.81 (m, 1H, H-2), 5.40 (dd, $J = 3.2, 3.6$, 1H, H-3), 2.44

(dd, J = 3.6, 11.6, 1H, H-4), 6.27 (dd, J = 1.3, 11.6, 1H, H-5), 2.23 (overlapped, H-6), 4.99 (dd, J = 1.3, 6.4, 1H, H-7), 3.60 (dd, J = 1.3, 6.0, 1H, H-8), 1.93 (d, J = 17.2, 1H, H-12a), 2.26 (overlapped, H-12b), 0.80 (overlapped, H-14), 1.08 (s, 3H, H-16), 0.98 (s, 3H, H-17), 1.78 (s, 3H, H-18), 0.87 (d, J = 6.8, 3H, H-19), 5.06 (dd, J = 1.3, 11.6, 1H, H-20a), 4.57 (dd, J = 1.3, 11.6, 1H, H-20b), 2.26 (q, J = 7.6, 2H, Pro-2′), 0.93 (t, J = 7.6, 3H, Pro-3′), 2.18 (s, 3H, 5-OAc), 7.70 (d, J = 8.0, 2H, Bz-2′, 6′), 7.03 (overlapped, Bz-3′, 5′), 7.16 (t, J = 7.4, 1H, Bz-4′), 2.14 (s, 3H, 13-OAc), 8.84 (d, J = 1.6, 1H, Nic-2′), 7.60 (dt, J = 1.6, 8.8, 1H, Nic-4′), 7.01 (overlapped, Nic-5′), 8.54 (dd, J = 1.6, 4.0, 1H, Nic-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 42.8 (t, C-1), 37.3 (d, C-2), 78.1 (d, C-3), 50.3 (d, C-4), 70.4 (d, C-5), 23.8 (d, C-6), 69.9 (d, C-7), 35.2 (d, C-8), 204.3 (s, C-9), 48.1 (s, C-10), 85.6 (s. C-11), 22.3 (t, C-12), 84.2 (s, C-13), 18.9 (d, C-14), 18.4 (s, C-15), 29.4 (q, C-16), 14.9 (q, C-17), 25.0 (q, C-18), 13.9 (q, C-19), 63.6 (t, C-20), 173.5 (s, Pro-C-1′), 27.5 (t, Pro-C-2′), 8.7 (q, Pro-C-3′), 170.1 (s, 5-OAc-C-1′), 21.3 (q, 5-OAc-C-2′), 165.1 (s, Bz-C-O), 129.2 (s, Bz-C-1′), 129.4 (d, Bz-C-2′, 6′), 128.0 (d, Bz-C-3′, 5′), 132.8 (d, Bz-C-4′), 170.7 (s, 13-OAc-C-1′), 21.3 (q, 13-OAc-C-2′), 165.3 (s, Nic-C-O), 150.6 (d, Nic-C-2′), 125.3 (s, Nic-C-3′), 136.2 (d, Nic-C-4′), 123.1 (d, Nic-C-5′), 153.0 (d, Nic-C-6′); HRESI-MS m/z 734.3187 (calcd. for $C_{40}H_{47}NO_{12} + H^+$, 734.3177, Δ 1.4 ppm). Positive ESI-MS m/z 734 [M + H], 726, 706, 684, 650, 614 [M-2Acet], 540 [614-Prop], 471, 455, 295, 209.

2.3.4. 13-acetyl-5-angeloyl-11-nicotinyl-3-butanoyl-1,2,6,7-tetrahydroingenol (4)

Colorless oil. $[\alpha]_{D:}$ −124 (c 0.09, CHCl3); UV ($CHCl_3$) λ_{max}: 292.6 nm. IR (KBr) υ_{max}: 3492, 2962, 2951, 2875, 1726, 1377, 1282, 1257, 1232, 1136, 1072, 1036 and 735 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz, J in Hz): δ_H 2.68 (dd, J = 10.8, 14.8, 1H, H-1a), 1.50 (dd, J = 7.6, 14.6, 1H, H-1b), 2.43 (m, 1H, H-2), 5.51 (dd, J = 3.2, 4.2, 1H, H-3), 2.95 (dd, J = 4.2, 10.4, 1H, H-4), 5.88 (dd, J = 1.3, 10.4, 1H, H-5), 2.26 (overlapped, H-6), 5.01 (dd, J = 3.2, 6.4, 1H, H-7), 2.26 (dd, J = 1.3, 7.6, 1H, H-8), 1.94 (dt, J = 2.8, 16.4, 1H, H-12a), 2.19 (overlapped, H-12b), 0.85 (overlapped, H-14), 1.10 (s, 3H, H-16), 0.97 (s, 3H, H-17), 1.58 (s, 3H, H-18), 1.34 (dd, J = 1.2, 7.2, 3H, H-19), 5.25 (dd, J = 1.3, 12.4, 1H, H-20a), 4.55 (dd, J = 1.3, 12, 1H, H-20b), 2.18 (t, J = 7.6, 2H, But-2′), 1.49 (m, 2H, But-3′), 0.90 (t, J = 7.6, 3H, But-4′), 6.43 (q, J = 6.8, 1H, Ang-3′), 1.00 (d, J = 6.8, 3H, Ang-4′), 1.55 (s, 3H, Ang-5′), 1.98 (s, 3H, 13-OAc), 9.19 (dd, J = 2.0, 1H, Nic-2′), 8.21 (dt, J = 2.0, 8.0 1H, Nic-4′), 7.42 (dd, J = 4.8, 8.0, 1H, Nic-5′), 8.81 (dd, J = 2.0, 4.8, 1H, Nic-6′); ^{13}C NMR ($CDCl_3$, 100 MHz): δ_C 46.5 (t, C-1), 35.7 (d, C-2), 78.2 (d, C-3), 50.7 (d, C-4), 68.4 (d, C-5), 22.9 (d, C-6), 70.1 (d, C-7), 41.7 (d, C-8), 210.2 (s, C-9), 47.7 (s, C-10), 84.0 (s. C-11), 23.0 (t, C-12), 79.6 (s, C-13), 20.0 (d, C-14), 19.3 (s, C-15), 28.6 (q, C-16), 15.5 (q, C-17), 22.2 (q, C-18), 14.0 (q, C-19), 62.5 (t, C-20), 172.0 (s, But-C-1′), 35.8 (t, But-C-2′), 18.0 (t, But-C-3′), 13.6 (q, But-C-4′), 167.4 (s, Ang-C-1′), 127.7 (s, Ang-C-2′), 138.4 (d, Ang-C-3′), 15.4 (q, Ang-C-4′), 11.9 (q, C-5′), 166.0 (s, Nic-C-O), 150.9 (d, Nic-C-2′), 126.4 (s, Nic-C-3′), 137.0 (d, Nic-C-4′), 123.6 (d, Nic-C-5′), 153.7 (d, Nic-C-6′), 170.1 (s, 13-OAc-C-1′), 21.4 (q, 13-OAc-C-2′); HRESI-MS m/z 684.3401 (calcd. for $C_{37}H_{49}NO_{11} + H^+$, 684.3378, Δ 3.36 ppm). Positive ESI-MS m/z 684 [M + H], 624 [M-Acet], 602, 514, 473, 455.

2.3.5. 13-acetyl-3-butanoyl-5-angeloyl-20-nicotinyl-1,2,6,7-tetrahydroingenol (5)

Colorless oil. $[\alpha]_{D:}$ −6.25 (c 0.24, CHCl3); UV ($CHCl_3$) λ_{max}: 228, and 265 nm. IR (KBr) υ_{max}: 3481, 2960, 2925, 2854, 1732, 1716, 1653, 1591, 1464, 1373, 1259, 1180, 1130, 1082, 1026, 964, 741 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz, J in Hz) and ^{13}C NMR ($CDCl_3$, 100 MHz) see Table 1. HRESI-MS m/z 710.3187 (calcd. for $C_{38}H_{47}NO_{12} + H^+$, 710.31708, Δ 2.3 ppm). Positive ESI-MS m/z 710 [M + H], 650 [M-Acet], 590, 550 [650-Ang], 490 [550-Ac], 471, 457, 367 [490-Nic], 339, 293 [367-Prop], 275.

2.4. MTT viability assay

Human ovarian carcinoma cancer cell line (CAOV-4 and OVCAR-3) and human bladder carcinoma cancer cell line (EJ138) were obtained from Pasteur Institute of Iran. The cell lines were grown adherently in RPMI-1640 media supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2/95% air. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 5 × 10^3 cells/well in 5% CO_2 at 37 °C in RPMI medium (containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin) in 96-well plates. After incubation overnight to allow for cell attachment, the RPMI medium in each well was replaced with by media containing various concentrations of compounds and incubated for 48 h. Afterwards, 20 μl of MTT (5 mg/ml in PBS) was added to each well and the cells were incubated for another 4 h at 37 °C. The supernatants were then aspirated carefully and 200 μl of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for an additional 10 min and the absorbance values were read by the microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells / mean OD of control cells) × 100. The results expressed as percent of control cells which were not treated [10].

2.5. Assay for detection of apoptosis using annexin V/PI staining

Detection of apoptosis was conducted using the annexin VFITC/PI apoptosis detection kit according to manufacturer's protocol. Briefly, cells plated to a density of 3 × 105 per well in six-well plate and incubated with different concentrations of test compounds (**1**–**4**) for 48 h. Floating cells as well as residual attached cells were collected, washed with PBS twice, stained for 15 min at room temperature with annexin-V-FITC and PI, and examined using FACS calibur flow cytometer (USA). Analysis was performed by the software supplied in the instrument. Annexin V binds to phosphatidyl serine that becomes exposed on the plasma membrane of cells undergoing apoptosis. This allows the discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (annexin V positive, PI negative) to late apoptotic cells (annexin V positive, PI positive) [10].

2.6. Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) was performed with the Dunnett's test, using software GraphPad

Prism. Each experiment was carried out in triplicate and repeated three to four times independently. $p < 0.05$ was considered significant. All data are expressed as means ± SD.

3. Results and discussion

E. erythradenia was collected in Gharbalbiz neighboring Mehriz City, Yazd province (Iran). Dried aerial parts of flowered plant were extracted with dichloromethane:acetone. After VLC on a cake of 20% paraffin impregnated silica-gel to remove waxes and pigments, fraction eluted with MeOH:H_2O (6:4) was separated on silica gel CC and purified by HPLC on normal phase silica column to afford five new macrocyclic diterpenes (**1**–**5**) which their structure elucidation is outlined here. Compound **1** exhibited a positive HRESI-MS pseudo-molecular ion $[M + H]^+$ at *m/z* 684.3411 (calcd. 684.3378) suggesting its molecular formula as $C_{37}H_{49}NO_{11}$. The IR absorptions indicated carbonyl (1724 cm^{-1}), multiple C–O bonds (1024–1267 cm^{-1}), aromatic bonds (1426–1591 cm^{-1}) and hydroxyl group (3485 cm^{-1}). ^1H NMR signals at δ_H 9.04 (d, $J = 1.6$ Hz), 8.79 (dd, $J = 1.6$, 4.8 Hz), 8.13 (dt, $J = 8.0$, 1.6 Hz), 7.38 (dd, $J = 4.8$, 8 Hz), supported a nicotinyl moiety in the molecule [11]. Singlet methyl proton δ_H 2.13 s with HMBC cross links with esteric carbonyl carbon δ_C 170.5 and loss of 60 *m/z* in the ESI-MS indicated presence of one acetate group [12]. The presence of one butanoyl group was also inferred by NMR signals at δ_C 173.1, 35.6 (δ_H 2.23 t, $J = 7.6$ Hz), 18.1 (δ_H 1.46 m), and 13.7 (δ_H 0.91 t, $J = 7.6$ Hz). In addition, ESI-MS *m/z* at 524 $[M\text{-Ac-}100]^+$, along with NMR signals at δ_C 168.5, 127.4; 140.0 (δ_H 6.63 q, $J = 6.8$ Hz); 14.2 (δ_H 1.38 d, $J = 6.8$ Hz); 11.7 (δ_H 1.51 s) indicated the presence of angeloyl moiety [13]. Taken together, the ^{13}C NMR spectra (BB and DEPT) and the fourteen degrees of unsaturation, supported one acetate, one butanoate, one angelate, one nicotinate, and one ketone function (δ_C 204.2) and therefore, four rings in the main skeleton. Without esters, the remaining twenty carbons of the main diterpene skeleton represented four methyls, three methines, eight methylenes, and five quaternary carbons of which six were oxygenated. The ^1H, ^1H COSY of **1** revealed a coupled spin system beginning from the proton H-1 (δ_H 3.16) to H-14 (δ_H 0.76). Other carbon signals were assigned and connected on the basis of HMBC experiment (Fig. 2). The HMBC cross-peak between the OH groups, δ_C 65.9 (C-7), and 86.0 (C-11) confirmed the location of the OH groups (Fig. 2). The signals at δ_C 78.6 (C-3), 70.4 (C-5), 83.8 (C-13), and 64.1 (C-20) represented as ester bearing carbons. HMBC correlations of H-3, H-5 and H-20 with carbonyl carbons resonated at δ_C 173.1, 168.5 and 164.8, indicated the position of butanoyl, angeloyl and nicotinyl esters at C-3, C-5 and C-20, and therefore acetyl on C-13, respectively. The relative stereochemistry was determined through NOEs and coupling constants. Taking H-4(β) configuration as reference point, NOE cross-peaks of H-4/H-2, and H-20a confirmed their β-orientations. Small coupling constant ($J = 3.2$ Hz) between H-4 and H-3, supported H-3 β-configuration and the large *J* value between H-4 and H-5 ($J = 11.2$ Hz) indicated their *trans*-orientation. In addition, the NOEs between H-5α/H-7, H-6/Me-18, H-14, Me-18/H-8, and H-14/Me-16 supported α-position for these protons (Fig. 3). Compound **2** was assigned the molecular formula $C_{38}H_{49}NO_{12}$ based on positive pseudo-molecular ion $[M + H]^+$ at *m/z* 712.3376 (calcd. 713.3333). NMR spectral data cleared that

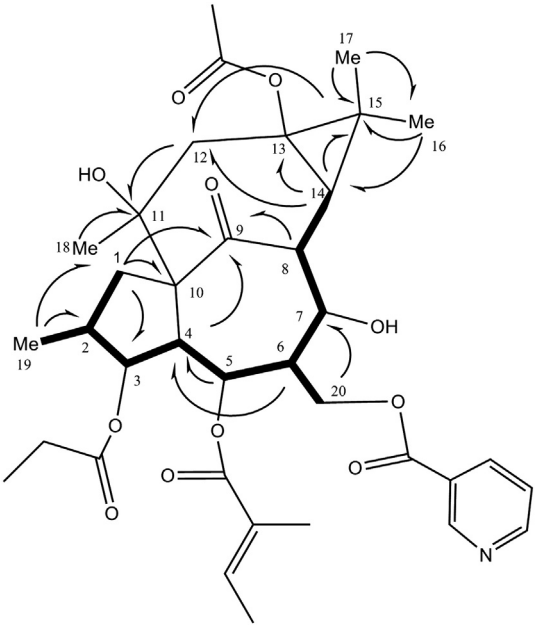


Fig. 2. Key ^1H-^1H COSY (in bold) and HMBC correlations (→) of compound **1**.

compounds **2** and **1** were similar in skeleton differing only in acetyl group [δ_C 170.1 (s, Ac-C-1′), 21.3 (q, Ac-C-2′)] instead of 7-OH moiety and propionyl [173.7 (s, C-1′), 27.5 (t, C-2′), 8.8 (q, C-3′)] [12] instead of 3-O-butanoyl ester. The HRESI-MS of a positive HRESI-MS pseudo-molecular ion $[M + H]^+$ at *m/z* 734.3187 (calcd. 734.3177) supported a molecular composition of compound **3** as $C_{40}H_{47}NO_{12}$. ^{13}C NMR (BB and DEPT) spectral data cleared compounds **2** and **3** which have similar structure, except for the acetyl group [170.1 (s, Ac-C-1′), 21.3 (q, Ac-C-2′)] instead of 5-O-angeloyl moiety and benzoyl group [165.1 (s, Bz-CO), 129.2 (s, Bz-C-1′), 129.4 (d, Bz-C-2′, 6′), 128.0 (d, Bz-C-3′, 5′)] [4] instead of 7-OAc. Compound **4** exhibited HRESI-MS molecular ion $[M + H]^+$ at *m/z* 684.3401 (calcd. 684.3378) suggesting its molecular formula as $C_{37}H_{49}NO_{11}$. Further observation of NMR spectral data cleared compounds **4** and **1** which resemble each other and differ only in the displacement of nicotinyl ester on C-11.

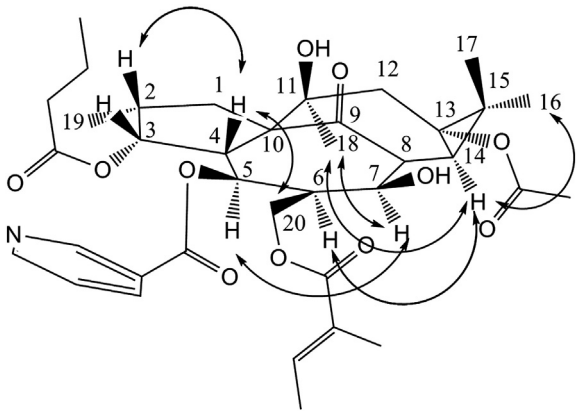


Fig. 3. Key NOESY correlations detected for compound **1**.

The aerial parts of *E. erythradenia* also afforded a new diterpene belonging to the myrsinane type diterpenes (**5**, Fig. 1). Compound **5** was assigned the molecular formula $C_{38}H_{47}O_{12}N$ (calc. 710.31708) based on HRESI-MS *m/z* 710.3187 $[M + H]^+$. Two singlet methyl protons (δ_H 2.21, and 2.14), along with sequential loss of 60 mass unit, suggested presence of two acetate groups. ^{1}H NMR signals at δ_H 9.09 (d, J = 1.6 Hz), 8.81 (dd, J = 4.8, 1.6 Hz), 8.11 (dd, J = 8.0, 1.6 Hz), 7.42 (dd, J = 4.8, 8.0 Hz), the presence of one nicotinyl moiety in the molecule [11]. The presence of one propanoyl and one angeloyl group was also inferred by NMR signals [13]. Taken together, the 15° of unsaturation and ^{13}C NMR suggested presence of one nicotinyl, one propanoyl, one angeloyl, two acetate esters, and two olefinic bonds, and therefore a tricyclic skeleton. The ^{1}H NMR spectrum of **5** showed signals of a pair of olefinic protons at δ_H 5.83 (dd, J = 9.4, 4.6 Hz, H-9), 6.11 (ddd, J = 9.6, 6.0, 2.0 Hz, H-8), external methylene group at δ 4.98 (bs, H-18a), and 4.88 (bs, H-18b), two geminal oxymethylene protons δ 4.25 (d, J = 12.0 Hz, H-17a) and 4.50 (d, J = 12.0 Hz, H-17b), three oxymethine protons δ 5.22 (dd, J = 3.4, 3.4 Hz, H-3), 6.30 (d, 11.6 Hz, H-5), and 4.81 (d, J = 6.4 Hz, H-7). Other protons were observed at δ_H 3.58 (d, J = 8.0 Hz, H-12), 3.48 (dd, J = 9.2, 4.4, 1.6 Hz, H-11), 3.38 (dd, J = 14.8, 8.4 Hz, H-1b), 2.51 (dd, J = 11.8, 3.4 Hz, H-4), and 1.74 (dd, J = 14.8, 11.6 Hz, H-1a), together with three tertiary methyls (δ_H 0.87, 1.82, and 1.67). The resonances in the polyol core, ^{1}H-^{1}H-COSY connectivities of H-1 to H-5, H-7 to H-12, and

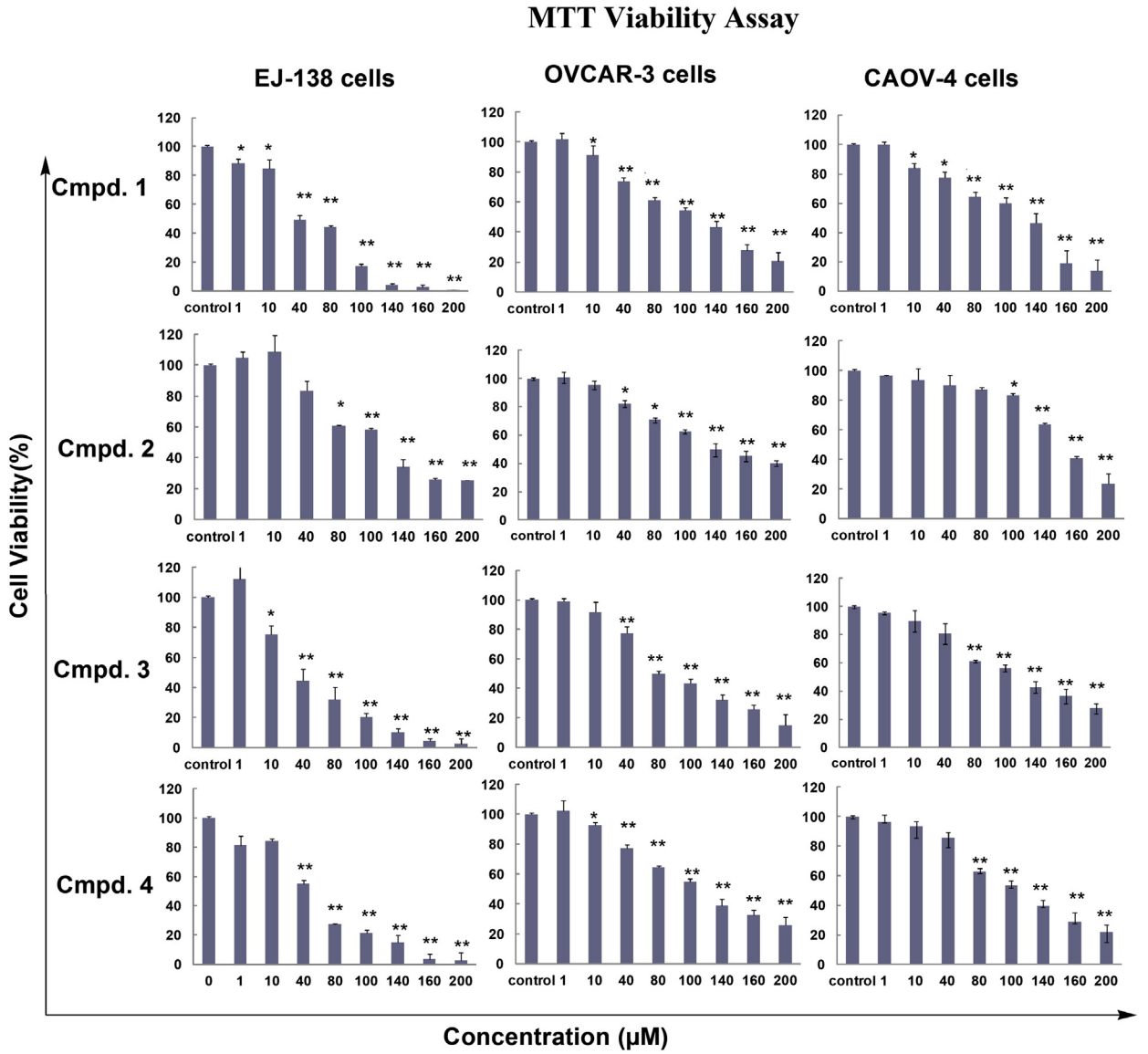


Fig. 4. Cytotoxicity effect of compounds **1**–**4** in ovarian (OVCAR-3, CAOV-4), and bladder (EJ-138) cancer cell lines. Cells were treated with different concentrations of compounds **1**–**4** for 48 hr, and cytotoxicity was assessed by MTT assay. Compounds **1**–**4** reduced cell viability in OVCAR-3, OVCA-4 and EJ-138 cell lines in a dose dependent manner. Results (mean ± SD) were calculated as percent of corresponding control values. *p<0.05 and **p<0.001 are significant. Statistical analysis was performed by ANOVA. Each point represents 4 repeats, each in triplicate.

HMBC correlations as is shown in Fig. 1 resembled decipinone, a myrisinane type structure extracted from *Euphorbia decipiens* [14] except for the type and position of the esters. The signals at δ_C 80.6 (s, C-13), 77.8 (d, C-3), 70.0 (d, C-5), 68.0 (d, C-7), and 63.1 (t, C-17) represented the ester bearing carbons. The HMBC and NOESY data indicated the position of the ester groups propanoyl, nicotinyl, angeloyl, and two acetates on C-3, C-17, C-7, C-5, and C-13, respectively.

3.1. MTT viability assay

To evaluate the effects of compounds (**1**–**4**) on the viability of the different cancer cell lines, MTT assays were done. The MTT viability assay of the compounds (**1**–**4**) showed significant cytotoxicity in a dose dependent manner on EJ-138, CAOV-4, and OVCAR-3 human carcinoma cell lines (Fig. 4). Among them compound **3** was the most active with IC50 values of 29.12 ± 1.73, 85.1 ± 2.6, and 110.34 ± 3.71 μM on EJ-138, OVCAR-3, and CAOV-4 cell lines, respectively.

3.2. Apoptosis determination via annexin V/propidium iodide staining

To study whether the tetrahydroingenane-induced cell growth inhibition was related to cell apoptosis, the effect of compounds (**1**–**4**) on cell apoptosis was evaluated. EJ-138, CAOV-4, and OVCAR-3 cells were exposed to the various concentrations (10, 40, 100, 200 μM) of test compounds for 48 h, and analyzed by flow cytometry using FITC-conjugated annexin V (FL1-H) and PI (FL2-H) double staining (Figs. 5, 6).

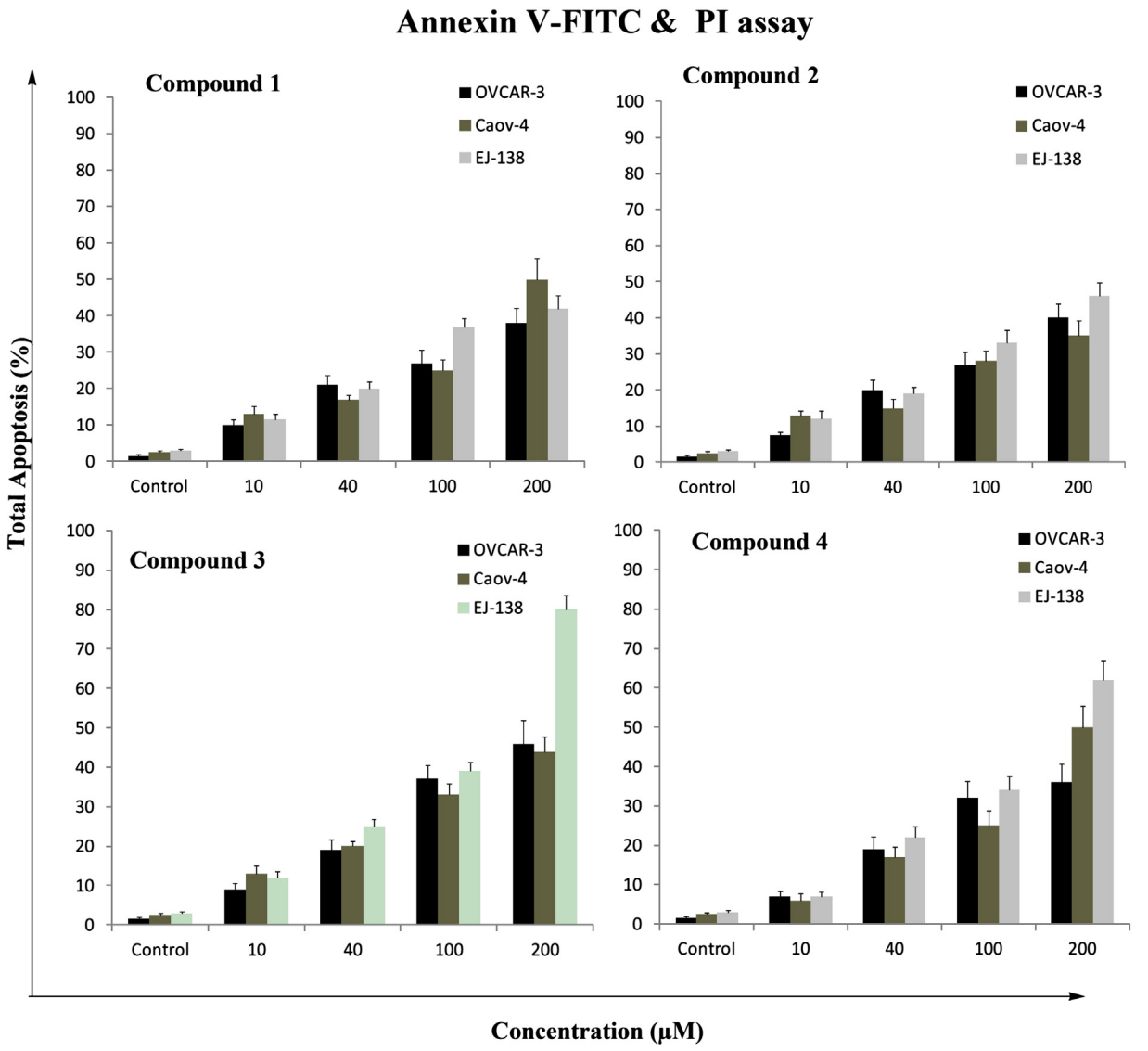


Fig. 5. Apoptotic effect of compounds **1**–**4** in OVCAR-3, CAOV-4, and EJ-138 cancer cells. Carcinoma cells were treated with various concentrations of compounds **1**–**4** for 48 h, stained with annexin-V and propidium iodide (PI) and examined using FACS calibur flow cytometer. Test compounds **1**–**4**, in the concentrations more than 10 μM resulted in a significant increase ($p > 0.05$) in the total apoptosis in a concentration-dependent manner on EJ-138, CAOV-4, and OVCAR-3 cell lines.

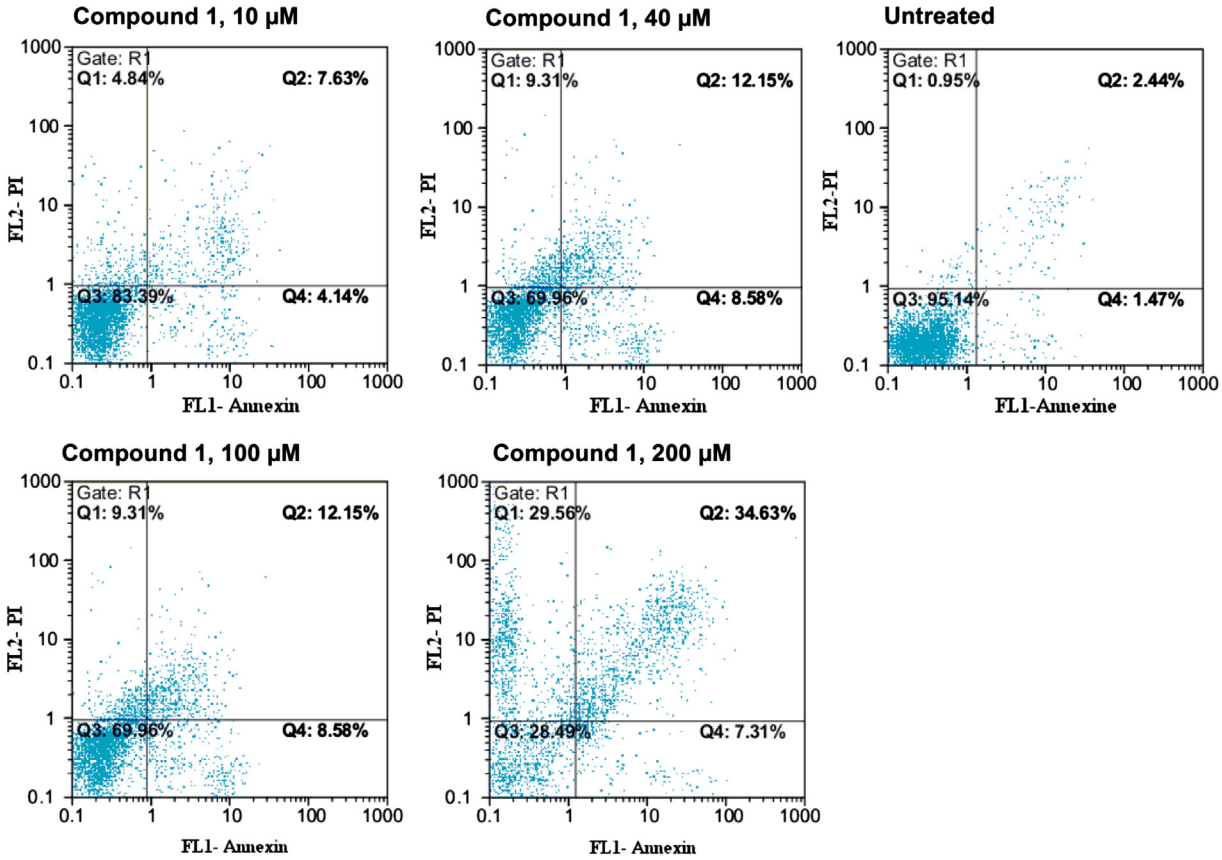


Fig. 6. Flow cytometric analysis of compound **1** on EJ-138 apoptotic cells. Cells were treated with various concentrations of 10, 40, 100, 200 μM for 48 h with annexin-V and propidium iodide (PI) and examined using FACS calibur flow cytometer.

It showed a significant increase in the percentage of late (annexin V positive, PI positive) apoptosis in a concentration-dependent manner ($p < 0.01$). Test compounds **1–4** in the concentrations more than 10 μM resulted in a significant increase ($p > 0.05$) in the total apoptosis (both early and late apoptosis) on EJ-138, CaOV-4, and OVCAR-3 cell lines and suggested that tetrahydroingenanes inhibit cell proliferation through cell apoptosis in cancer cell lines.

Acknowledgment

This paper is part of the thesis of Syed Muhammed Zarei submitted for the fulfillment of the degree of Doctor of Philosophy in Pharmacognosy in Faculty of Pharmacy and Pharmaceutical Sciences, Shahid Beshti University of Medical Sciences, Tehran, Islamic Republic of Iran.

References

[1] Shokoohinia Y, Chianese G, Zolfaghari B, Sajjadi S-E, Appendino G, Taglialatela-Scafati O. Macrocyclic diterpenoids from the Iranian plant *Euphorbia bungei* Boiss. Fitoterapia 2011;82:317–22.
[2] Appendino G, Jakupovic S, Tron CG, Jakupovic J, Milon V, Ballero M. Macrocyclic diterpenoids from *Euphorbia semiperfoliata*. J Nat Prod 1998;61:749–56.
[3] Liang QL, Dai CC, Jiang JH, Tang YP, Duan JA. A new cytotoxic casbane diterpene from *Euphorbia pekinensis*. Fitoterapia 2009;80:514–6.
[4] Ayatollahi AM, Ghanadian M, Mesaik MA, Mohamed Abdella O, Afsharypuor S, Kobarfard F, et al. New myrsinane-type diterpenoids from *Euphorbia aellenii* Rech. f. with their immunomodulatory activity. J Asian Nat Prod Res 2010;12:1020–5.
[5] Izadjoo M. Antitumor properties of metanolic extracts of four species of Euphorbia using potato disk method. PhD diss. Kerman, Iran: School of Medicine, Kerman University of Medical Sciences; 2012.
[6] Pahlevani AH, Maroofi H, Joharchi MR. Notes on six endemic or rare species of Euphorbia subg. Esula (Euphorbiaceae) in Iran. Willdenowia-Annals of the Botanic Garden and Botanical Museum Berlin-Dahlem, 41; 2011. p. 267–76.
[7] Lu Z, Yang M, Zhang J, Chen G, Huang H, Guan S, et al. Ingenane diterpenoids from *Euphorbia esula*. Phytochemistry 2008;69:812–9.
[8] Lebwohl M, Swanson N, Anderson LL, Melgaard A, Xu Z, Berman B. Ingenol mebutate gel for actinic keratosis. N Engl J Med 2012;336:1010–9.
[9] Kedei N, Lundberg DJ, Toth A, Welburn P, Garfield SH, Blumberg PM. Characterization of the interaction of ingenol 3-angelate with protein kinase C. Cancer Res 2004;64:3243–55.
[10] Shirali S, Aghaei M, Shabani M, Fathi M, Sohrabi M, Moeinifard M. Adenosine induces cell cycle arrest and apoptosis via cyclinD1/Cdk4 and Bcl-2/Bax pathways in human ovarian cancer cell line OVCAR-3. Tumour Biol 2013;34:1085–95.
[11] Ayatollahi AM, Ghanadian M, Afsharypuor S, Choudhary MI, Kobarfard F, Rahmati M. Two new lathyrane type diterpenoids from *Euphorbia aellenii*. Fitoterapia 2010;81:891–3.
[12] Ghanadian SM, Ayatollahi AM, Afsharypuor S, Javanmard SH, Dana N. New mirsinane-type diterpenes from *Euphorbia microsciadia* Boiss. with inhibitory effect on VEGF-induced angiogenesis. J Nat Med 2013;67:327–32.
[13] Corea G, Fattorusso C, Fattorusso E, Lanzotti V. Amygdaloidins A–L, twelve new 13 α-OH jatrophane diterpenes from *Euphorbia amygdaloides* L. Tetrahedron 2005;61:4485–94.
[14] Jassbi AR, Fukushi Y, Tahara S. Determination of absolute configuration of decipinone, a diterpenoid ester with a myrsinane-type carbon skeleton, by NMR spectroscopy. Helv Chim Acta 2002;85:1706–13.