

## Pentacyclic Triterpenes in *Euphorbia microsciadia* with Their T-cell Proliferation Activity

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### Abstract

The ethyl acetate partition of dried methanolic extract of aerial parts of *Euphorbia microsciadia* (Euphorbiaceae) afforded three pentacyclic triterpenes, betulinic acid (1) from lupane type, oleanolic acid (2) from oleanane type and ursolic acid (3) from ursane type triterpenes that are reported for the first time in this plant. These three compounds were structurally compared through their mass fragmentation pattern, nuclear magnetic resonance (NMR) data and their biologic immunomodulatory effects. The structures of the isolated compounds were elucidated by <sup>13</sup>C- and <sup>1</sup>H-NMR as well as 2D-NMR, IR and by the aid of mass fragmentation pattern and comparing with the literature. After running T-Cell proliferation assay, oleanolic acid stimulated proliferation of T-Cells at lower concentration 0.5 µg/mL, while betulinic acid and ursolic acid showed inhibitory activity against T-Cell proliferation with IC<sub>50</sub>-value > 50 µg/mL and 3.01 ± 0.47 µg/mL, respectively.

**Keywords:** *Euphorbia microsciadia*; Oleanane, ursane and lupane pentacyclic triterpenes; Triterpene mass fragmentation pattern; T-Cell proliferation assay.

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### Introduction

Euphorbiaceae is one of the largest families of the phylum Anthophyta. In this family, the largest genus is *Euphorbia* which comprises well over 2000 species in tropical and temperate zones of Asia and other parts of the world. In Iran, 70 species are reported out of which 17 are endemic (1). In traditional medicine, *Euphorbia*

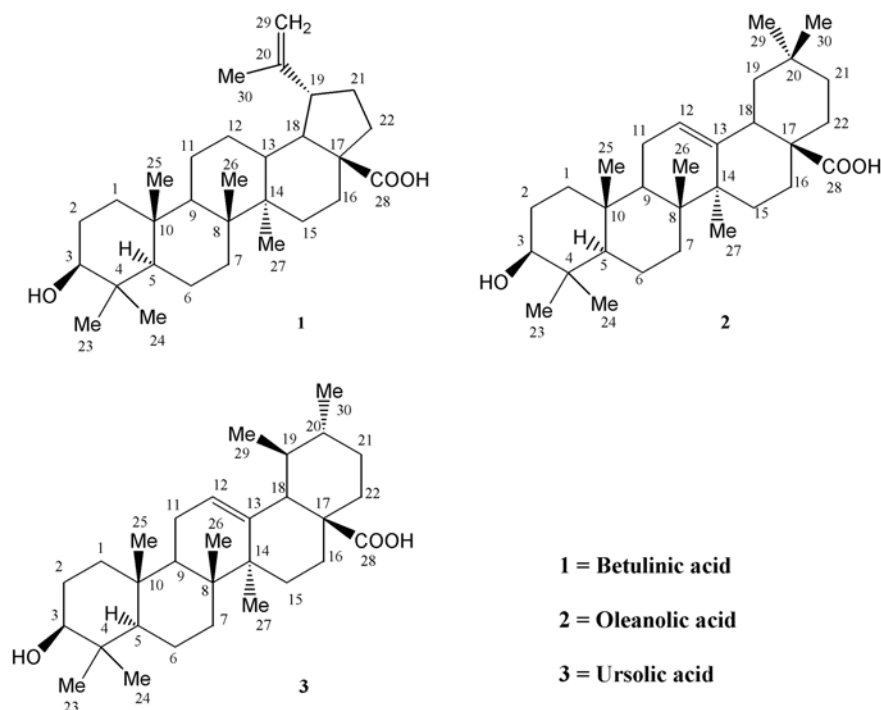
was used as the treatment of intestinal parasites, gonorrhoea, wart cures and also in the treatment of skin diseases (2). However, multidisciplinary biological screening tests carried out in recent years have shown that some of them were useful as anti-tumors, pesticides and antiviruses.

In this research, three triterpenes of three different common pentacyclic classes were isolated from the aerial parts of *Euphorbia microsciadia* (Euphorbiaceae) which is a perennial plant that grows in some parts of Iran (3); there has been efforts to elucidate them

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**Figure 1.** Pentacyclic triterpenes from *Euphorbia microsciadia* (1-3).

through Nuclear magnetic resonance (NMR) and other spectroscopic methods, especially the mass fragmentation pattern.

### Experimental

#### General

The  $^1\text{H-NMR}$  spectra were recorded on a Bruker Avance AV 300 but  $^{13}\text{C-NMR}$  and 2D-NMR spectra were recorded on a Bruker Avance AV 600 NMR instrument, using  $\text{CDCl}_3$  as solvent. The IR spectra were recorded on a JASCO 302-A spectrophotometer and the mass spectra (EI and HREI-MS) were measured in an electron impact mode on Varian MAT 112 or MAT 312 spectrometers. Fast atom bombardments (FAB) MS were measured on Jeol HX110 mass spectrometer.

#### Chromatographic conditions

HPLC: LC-908 Recycling Preparative HPLC Hitachi company (Japan), Column: Jaigel-2H and 1H columns (600 × 20).

Column chromatography run by Merck, Silica gel, 63-200 $\mu\text{m}$ , flash chromatography on LiChroprep® Si 60 (25-40  $\mu\text{m}$ ), Reverse column chromatography by LiChroprep RP-18, 40-60  $\mu\text{m}$  (Merck) and size exclusion chromatography by Sephadex® LH-20 from Sigma-Aldrich. Thin-layer chromatography (TLC) was performed on precoated silica gel GF-254 plates, 20 × 20 cm, 0.5 mm thick (Merck). The visualization of TLC plates was achieved at 254 and 366 nm and cerium sulphate spray reagent 10%  $\text{H}_2\text{SO}_4$  was used for detection.

#### Plant material

The aerial parts of flowering plant of *Euphorbia microsciadia* Boiss (Euphorbiaceae) were collected in August 2007 from populations growing in Galil-e-Shirvan (near the Turkmenistan border), Northern Khorasan province in Iran. The plant was identified by Mrs. Yasamin Naseh, plant taxonomist (Department of botany, herbaceous sciences research center at Ferdowsi University of Mashhad). A voucher

**Table 1.**  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR data for the pentacyclic triterpenes in *Euphorbia microsciadia*<sup>a</sup>.

C	1	2	3
	$^{13}\text{C}$ - NMR	$^{13}\text{C}$ - NMR	$^{13}\text{C}$ - NMR
1	38.6 t	39.1 t	38.78 t
2	27.0 t	27.9 t	23.4 t
3	78.7 d	79.7 d	79.0 d
4	38.7 s	40.0 s	39.5 s
5	55.3 d	55.9 d	55.2 d
6a	18.2 t	19.1 t	18.2 t
7a	34.2 t	33.4 t	33.0 t
8	40.5 s	40.2 s	39.0 s
9	50.4 d	48.32 d	47.6 d
10	37.0 s	37.8 s	36.6 s
11	20.8 t	24.0 t	23.7 t
12	25.4 t	123.3 d	125.7 d
13	38.2 d	143.3 s	137.9 s
14	42.3 s	42.4 s	41.9 s
15	30.5 t	28.4 t	29.3 t
16	32.1 t	24.1 t	23.3 t
17	56.1 s	46.6 s	47.9 s
18	49.1 d	41.9 d	52.7 d
19	46.9 d	46.6 t	30.6 d
20	150.6 s	30.4 s	30.3 d
21	37.0 t	34.5 t	27.2 t
22	29.6 t	31.3 t	37.0 t
23	27.5 q	28.8 q	23.4 q
24	15.2 q	16.2 q	16.9 q
25	15.8 q	16.0 q	16.9 q
26	16.0 q	17.7 q	15.4 q
27	14.5 q	26.6 q	24.2 q
28	189.1 s	172.0 s	175.9 q
29	106.4 t	33.7 q	21.0 q
30	19.2 q	24.2 q	23.3 q

Chemical shifts are given in ppm

specimen (NO. 2023) of the plant was deposited in the herbarium of the Pharmacognosy department, Faculty of Pharmacy and Pharmaceutical Sciences at Isfahan University of Medical Sciences (Iran).

#### Extraction and isolation

The dried plant (4.5 Kg) was extracted with MeOH (18 L) at room temperature for four days by three times and the resulting extract was concentrated to a 400 g dark green gum. This

gum was partitioned into *n*-heptane and aqueous MeOH (80%). The defatted MeOH extract was evaporated and dissolved in H<sub>2</sub>O. The aqueous extract was partitioned into H<sub>2</sub>O and ethyl acetate (EtOAc). Thereafter, EtOAc part was subjected on RP-18 column chromatography eluting with aqueous methanol 30%, 60% and 100% respectively. The fraction obtained on elution with H<sub>2</sub>O : MeOH (40 : 60) transferred on another column using Hexane/EtOAc (0→50%). Later, the Hexane : EtOAc (80 : 20) part was separated from the rich chlorophyll content and pigments by size exclusion chromatography on sephadex (LH-20) using Dichloromethane (DCM) / MeOH (1 : 2). Finally, fractions which showed violet spots on TLC with cerium sulphate reagent were selected and purified on Recycling HPLC Jaigel-2H and 1H columns with chloroform as the mobile phase to yield three pure compounds (1-3) that were crystallized in DCM : MEOH (1 : 3).

#### T-Cell proliferation assay

Fresh venous blood from a normal healthy volunteer was collected in heparinized container and mixed with equal volume of RPMI-1640 incomplete media containing L-glutamine (Mediatech Inc., Herndon, VA, U.S.A.), then layered onto lymphocyte separation medium (LSM) (from MP Biomedicals, Inc., France) and centrifuged at 400×g for 20 min at 25°C. The mononuclear cell buffy coat was removed and cells were washed with incomplete RPMI-1640 and centrifuged for 10 min at 4°C and 300×g. The peripheral blood mononuclear cells (PBMNCs) were resuspended in supplemented RPMI-1640 at 2.5×10<sup>6</sup> Cells/mL containing 10% fetal bovine serum (FBS) from PAA laboratories GmbH (Pasching, Austria). In a 96-well round-bottomed microtiter plate, 50 μL of cell suspension plus 50 μL of phytohemagglutinin (PHA) with a final dilution of 5 μg/mL, 50 μL supplemented RPMI-1640 and 50 μL of test compounds in a final concentration of 0.5, 5 and 50 μg/mL were added. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 72 h. Further incubation for 18 h after the addition of thymidine [<sup>3</sup>H] (Amersham, Buckinghamshire, UK) was done and cells were harvested using cell harvester (Inotech Dottikon, Switzerland) and the incorporation was measured by a liquid

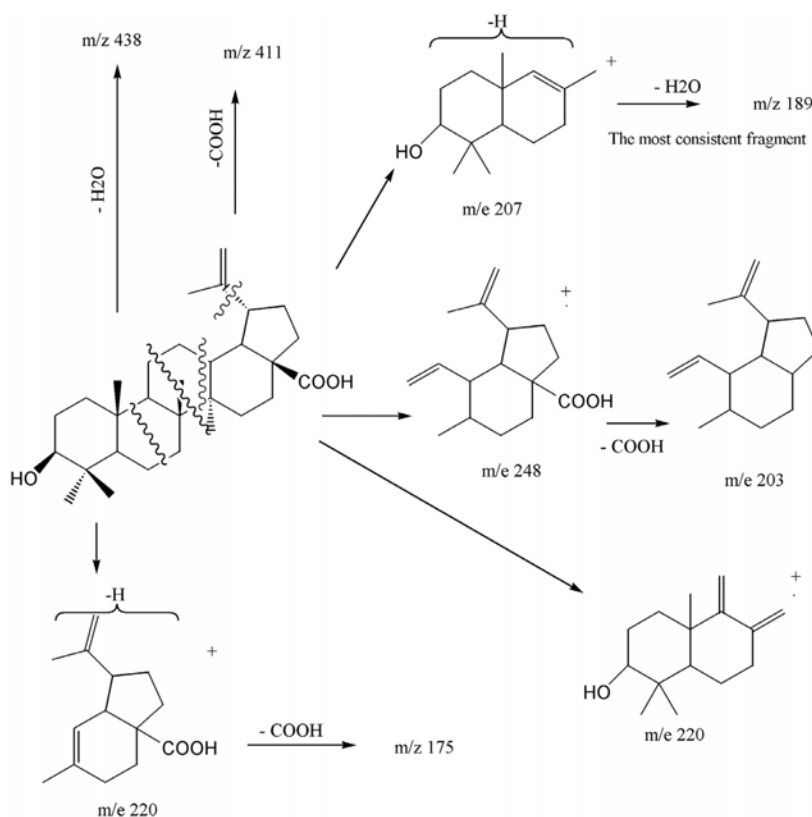


Figure 2. EI-Mass fragmentation pattern of Betulinic acid.

scintillation counter (Beckman coulter, LS 6500, Fullerton, CA, USA) (4).

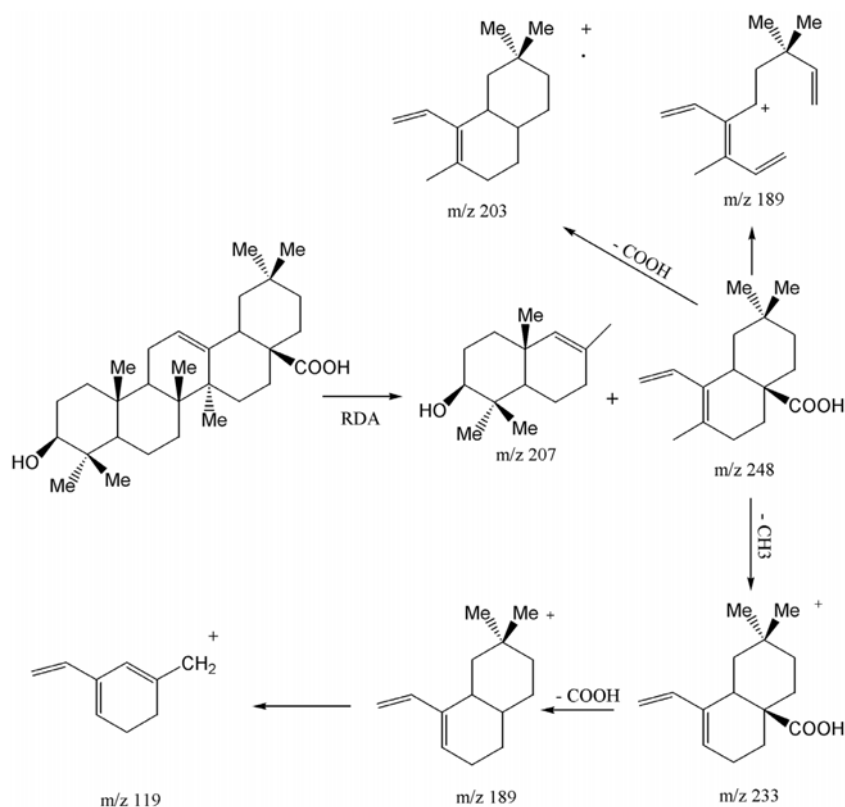
#### Statistical analysis

The  $IC_{50}$  values were calculated using an Excel based program and reported as mean  $\pm$  standard deviation (SD) of the mean. Significance was attributed to p-values ( $p < 0.05$ ) and the probability values obtained by the student t-test between the sample and control data.

### Results and Discussion

Compound 1 was obtained as colorless needles with molecular formula of  $C_{30}H_{50}O$  (calculated: 426.7194) on the basis of positive electron ionization high-resolution mass spectrometry (EI-HRMS) of the molecular ion peak at  $m/z$  456.3587 matched with the number of carbons and hydrogens counted in NMR

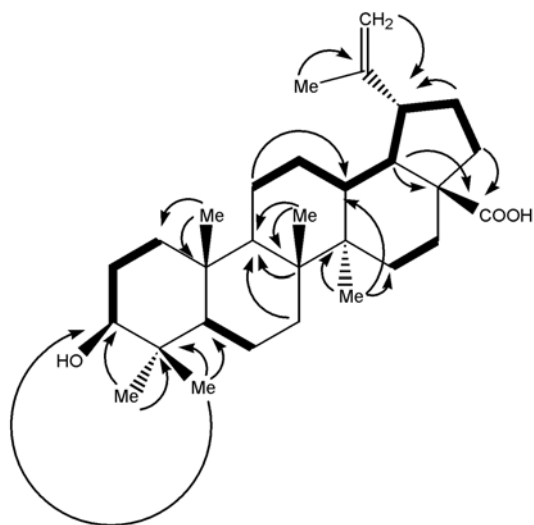
data (Table 1). With regard to the seven degrees of unsaturation,  $^{13}\text{C}$ -NMR and Distortionless Enhancement by Polarization Transfer (DEPT) spectral data, five rings, one acidic carbonyl and one double bond were detected in the molecule. IR spectrum showed a broad peak of hydroxyl group at  $3446\text{ cm}^{-1}$ , prominent carbonyl absorption at  $1685\text{ cm}^{-1}$ , double bonded peaks at  $1645$  and  $1604$ , and C-O functionalities at  $1236$ ,  $1107$  and  $1034\text{ cm}^{-1}$ .  $^1\text{H}$ -NMR revealed six singlet methyls at  $\delta_{\text{H}}$  of 1.67 s, 0.95 s, 0.94 s, 0.91 s, 0.80 s and 0.73 s, a pair of olefinic protons at  $\delta_{\text{H}}$  of 4.71 and 4.58 (each one H, br-s) characteristic of exocyclic methylene group, a carbinolic proton at  $\delta_{\text{H}}$  of 3.17 (dd,  $J_{ax,ax} = 10.8$ ,  $J_{ax,eq} = 5.1\text{ Hz}$ ,  $\text{H}_3$ ) referring to its axial and  $\alpha$  orientation (5),  $\delta_{\text{H}}$  of 2.95 (dt,  $J = 11.1$ , 3 Hz,  $\text{H}_{19}$ ), 2.24 (br-d,  $J = 12.3\text{ Hz}$ ,  $\text{H}_{16a}$ ), 2.16 (br-t,  $J = 10.4\text{ Hz}$ ,  $\text{H}_{13}$ ) and 1.95 (dd,  $J = 11.1, 4.5\text{ Hz}$ ,  $\text{H}_{21a}$ ) which were characteristic for lupane



**Figure 3.** EI-Mass fragmentation pattern of oleanolic acid.

triterpenes (5). Further information about the compound was obtained from typical EI-Mass related to the fragmentation pattern of lupane type triterpenes (Figure 2) through the presence of  $m/z$  438 [M-H<sub>2</sub>O], 411 [M-COOH], 248 [C<sub>16</sub>H<sub>24</sub>O<sub>2</sub>], 203 [248 - COOH], 220 [C<sub>15</sub>H<sub>24</sub>O], 203 [220 - OH], 220 [C<sub>14</sub>H<sub>20</sub>O<sub>2</sub>], 175 [220 - COOH], 207 [C<sub>14</sub>H<sub>23</sub>O], 189 [207 - H<sub>2</sub>O] 205 and 207 [M - C<sub>16</sub>H<sub>27</sub>] characteristic series for betulinic acid (6). <sup>13</sup>C-NMR (BB and DEPT) spectra showed thirty carbons comprised of seven methyls, eleven methylenes six methines and six quaternary carbons. Based on above observations, the spectral data of compound 1 was similar to those previously reported of betulinic acid (5, 6), except for the assignment of five-member ring carbons. Therefore, the authors decided to assign it again by Double-Quantum Filtered Correlation Spectroscopy (DQF-COSY) and Heteronuclear Multiple

Bond Coherence (HMBC) data (Figure 4). The DQF-COSY spectrum indicated following spin systems of correlated protons: (S<sub>1</sub>) -CH<sub>2</sub>-CH<sub>2</sub>-CHO with  $\delta_H$  0.88 (br-d, J = 10.4, H<sub>1b</sub>); 1.59 (m, H<sub>2a</sub>); 3.17 (dd, J = 10.8, 5.1 Hz, H<sub>3</sub>), (S<sub>2</sub>)-CH-CH<sub>2</sub>- with  $\delta_H$  0.66 (br-d, J = 8.8 Hz, H<sub>5</sub>); 1.46 (m, H<sub>6a</sub>) and (S<sub>3</sub>) CH<sub>2</sub>-CH<sub>2</sub>-CH-CH-CH-CH<sub>2</sub>-CH<sub>2</sub> with  $\delta_H$  1.38 (m, H<sub>11a</sub>), 1.00 (br, H<sub>12b</sub>); 2.16 (br-t, J = 10.4 Hz, H<sub>13</sub>); 1.58 (m, H<sub>18</sub>); 2.98 (dt, J = 11.1, 3 Hz, H<sub>19</sub>); 1.95 (dd, J = 11.1, 4.5 Hz, H<sub>21a</sub>) and 1.17 (dt, H<sub>22b</sub>). Fragment S<sub>1</sub>, due to its attachment to hydroxyl group, proposed to be C<sub>1</sub>-C<sub>2</sub>-C<sub>3</sub>. HMBCs of H<sub>5</sub> with C<sub>1</sub> confirmed S<sub>2</sub> as C<sub>5</sub>-C<sub>6</sub> and HMBCs of  $\delta_H$  2.36 (H<sub>19</sub>) with quaternary exocyclic olefinic carbon  $\delta_C$  150.62 (C<sub>20</sub>), assigned substructure S<sub>3</sub> as C<sub>11</sub>-C<sub>12</sub>-C<sub>13</sub>-C<sub>18</sub>-C<sub>19</sub>-C<sub>21</sub>-C<sub>22</sub> and  $\delta_C$  = 150.6 as C<sub>20</sub>. The position of six singlet methyls, Me<sub>23</sub>, Me<sub>24</sub>, Me<sub>25</sub>, Me<sub>26</sub>, Me<sub>27</sub> and Me<sub>30</sub> bonded to quaternary carbons C<sub>4</sub>, C<sub>8</sub>, C<sub>10</sub>, C<sub>14</sub> and C<sub>20</sub> were determined by their



**Figure 4.** DQF-COSY (in bold) and key (2, 3) J (H→C) HMBC cross-peaks in betulinic acid.

HMBC correlations with substructures  $S_1$ ,  $S_2$  and  $S_3$  as is shown in Figure. 4.

Compound 2 was obtained as white powder with the positive EI-HRMS of the molecular ion peak at  $m/z$  456.3578 indicative of molecular formula of  $C_{30}H_{48}O_3$  (calcd. 456.3603) that was in accordance with the number of carbons and hydrogens counted in NMR data (Table 1). The seven degree of unsaturation,  $^{13}C$ -NMR and DEPT spectral data (Table 1), suggested five rings plus one acidic carbonyl and an olefinic group in the molecule. The IR spectrum confirmed presence of hydroxyl group ( $3437\text{ cm}^{-1}$ ), carbonyl group ( $1695\text{ cm}^{-1}$ ) olefinic group  $C=C$  ( $653, 771\text{ cm}^{-1}$ ) and CO functionalities ( $1053, 1034$  and  $1016\text{ cm}^{-1}$ ). Mass spectrum showed prominent peaks at  $m/z$  248 [ $C_{16}H_{24}O_2$ ] $^+$  and 207 [ $C_{14}H_{23}O$ ] $^+$  retro-Diels-Alder (RDA) fragments characteristic for  $\Delta^{12}$ -amyrine series with COOH group (6), 203 [ $C_{15}H_{23}$ ] $^+$  due to the loss of COOH from  $m/z$  248 along with other fragments at  $m/z$  438 [ $M - H_2O$ ] $^+$ , 410 [ $M - HCOOH$ ] $^+$ , 392 [ $410 - H_2O$ ] $^+$ , 189 [ $C_{14}H_{21}$ ] $^+$ , 175 [ $C_{13}H_{19}$ ] $^+$ , 133 [ $C_{10}H_{13}$ ] $^+$ , 119 [ $C_9H_{11}$ ] $^+$  and 69 [ $C_5H_9$ ] $^+$  in Figure 4. The  $^{13}C$ -NMR and DEPT spectra confirmed presence of thirty carbon consisted of eight quaternary, five tertiary, ten secondary carbons and seven methyls.  $^1H$ -NMR showed signals

for an olefinic proton at  $\delta_H$  of 5.26 (t,  $J = 3\text{ Hz}$ ), a carbinolic proton at  $\delta_H$  of 3.20 (dd,  $J_{ax,ax} = 11.5, J_{ax,eq} = 4\text{ Hz}$ ) suggesting it's axial and  $\alpha$  orientation and  $\delta_H$  of 2.81 (dd,  $J = 13.5, 3.5\text{ Hz}$ ) along with seven singlet methyls at  $\delta_H$  of 1.23, 1.11, 0.97, 0.91, 0.89, 0.88 and 0.77. All the above data of compound 2 identified it as oleanolic acid (7).

Compound 3 was obtained as white powder with the positive EI-HRMS of the molecular ion peak at  $m/z$  456.3622 indicative of molecular formula of  $C_{30}H_{48}O_3$  (calcd. 456.3603) that was in accordance with the number of carbons and hydrogens counted in NMR data (Table 1). The seven degrees of unsaturation,  $^{13}C$ -NMR and DEPT spectral data (Table 1), suggested five rings plus one acidic carbonyl and one olefinic group in the molecule. The IR spectrum confirmed the presence of hydroxyl group ( $3442\text{ cm}^{-1}$ ) and carbonyl group ( $1710\text{-}1690\text{ cm}^{-1}$ ). Mass spectrum showed peaks at  $m/z$  248 as a strong peak and  $m/z$  207 in smaller extent as characteristic RDA fragments characteristic for 12-13 double bond  $\alpha$ - or  $\beta$ -amyrine series with COOH group (6) that was confirmed by  $m/z$  203 [ $248 - COOH$ ] as a prominent peak with other fragments at  $m/z$  438 [ $M - H_2O$ ] $^+$ , 410 [ $M - HCOOH$ ] $^+$ , 189 [ $C_{14}H_{21}$ ] $^+$ , 133, 119 and 55.  $^1H$ -NMR spectrum showed an olefinic proton at  $\delta_H$  of 5.27 (t,  $J = 3.3\text{ Hz}$ ), a proton geminal to hydroxyl group at  $\delta_H$  of 3.19 (dd,  $J_{ax,ax} = 10.8, J_{ax,eq} = 5.1\text{ Hz}$ ) inferring its  $\alpha$ - and axial orientation  $\delta_H$  of 2.81 (br-d,  $J = 9\text{ Hz}$ ) five singlet methyls at  $\delta_H$  of 1.23, 1.12, 0.97, 0.90 and 0.76 (H-23, 27, 26, 24 and 25), two doublet methyls at  $\delta_H$  of 0.84 (d,  $J = 6.6\text{ Hz}$ , Me $_{30}$ ) and 0.79 (d,  $J = 6.9\text{ Hz}$ , Me $_{29}$ ) which were characteristics for ursane skeleton (5). The  $^{13}C$ -NMR and DEPT spectra confirmed the presence of thirty carbon consisted of seven quaternary, seven tertiary, nine secondary carbons and seven methyls that were in accordance with ursolic acid reported in the literature (8).

#### *T-Cell proliferation assay*

The antiproliferative action was tested against peripheral blood T-lymphocytes (PBLs). Addition of betulinic acid (1) and ursolic acid (3) to PHA, stimulated PBLs in the concentration ranges of 0.5, 5 and 50  $\mu\text{g/mL}$

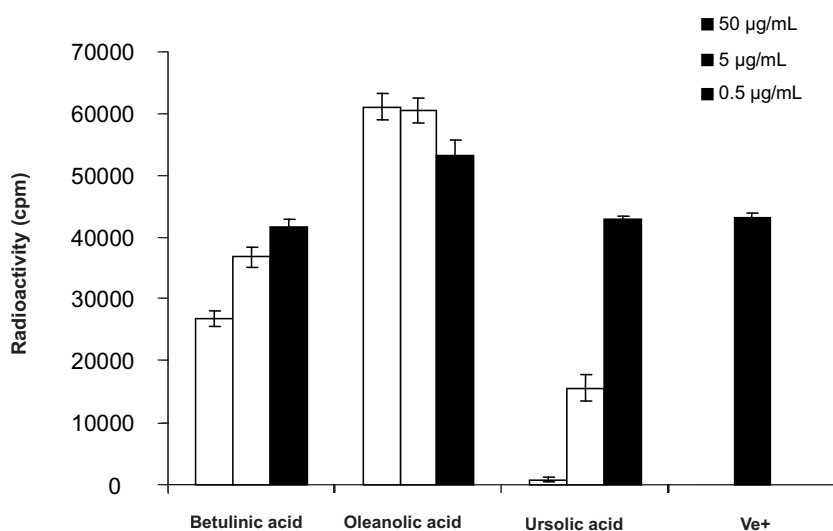


Figure 5. T-Cell Proliferation assay of pentacyclic triterpenes in *Euphorbia microsciadia*.

resulted in suppression of T-cell proliferation with  $IC_{50}$  value  $> 50 \mu\text{g/mL}$  and  $3.01 \pm 0.47 \mu\text{g/mL}$ , respectively, while, oleanolic acid (2) stimulated lymphocyte proliferations even at the low concentration of  $0.5 \mu\text{g/mL}$  by  $24.54\% \pm 5.51$ .

### Conclusion

A large number of pentacyclic triterpenes have been investigated for anti-inflammatory properties. Among these are ursolic acid and the lupane-type triterpenes like betulinic acid. Both of these compounds have been tested in a number of *in-vitro* and *in-vivo* model systems (9-13). Betulinic acid possess moderate inhibitory activity at relatively high concentrations on *in-vitro* proliferation assay using PBLs which was in agreement of other published data (12). Ursolic acid was found to potently inhibit T-cell proliferations while the oleanolic acid showed dose-relative stimulatory effect on lymphocyte proliferation assay agreed with another study by Anamika Khajuria (9) which showed dose dependent immunostimulatory effect on *in-vivo* immune functions in mice. Comparing the results, oleanolic acid and ursolic acid with the same structure differ only in E ring, showing different activities (one inhibitory

another stimulatory) on PBL proliferations indicating that the E ring (and not A) could be responsible for these changes. On the other hand, ursolic acid was found to potently inhibit T-cell proliferations more than betulinic acid indicating that the activity of six-member E ring is more than its five member analogue in lupane types. Therefore, according to above findings, the combination of E ring size as well as C-19, C-20 and C-28 positions could be responsible for the differences in biological effects in pentacyclic triterpenes analogues. Hence, making derivatives by modification on these positions like C-28 amino-derivatives, changing the C-20 alkene in betulinic acid derivatives, changing the nucleophilicity and the strength of hydrogen bonding capability and/or acidity at position C-28 are suggested to improve their immunomodulatory activities.

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