Cycloartane Triterpenoids from *Euphorbia Macrosteagia* with their Cytotoxicity against MDA-MB48 and MCF-7 Cancer Cell Lines

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Abstract

The dried plant was extracted with dichloromethane and after defatting with hexane, transferred repeatedly on silica columns using dichloromethane-hexane and ethyl acetate-hexane as mobile phases. Finally the fractions were purified by high performance liquid chromatography using a Pack-Sil column and hexane: Ethyl acetate as mobile phase. The structures of the isolated compounds included: cycloart-25-ene-3β, 24-diol (1), cycloart-23(Z)-ene-3β, 25-diol (2), cycloart-23(E)-ene-3β, 25-diol (3), and 24-methylene-cycloart-3β-ol (4) were elucidated by \(^13\)C- and \(^1\)H-NMR as well as IR and by the aid of mass fragmentation pattern and comparing with the literature. The biological effects of the compounds were done by the MTT assay on two different cancer cell lines including MDA-MB48 and MCF-7. Among these compounds, cycloart-23(E)-ene-3β,25-diol (3) was the most active compound on MDA-MB468 cell line \((\text{LD}_{50} = 2.05 \mu\text{g}\text{mL}^{-1})\) and cycloart-23(Z)-ene-3β, 25-diol (2) was the most active compound on MCF-7 cell line \((\text{LD}_{50} = 5.4 \mu\text{g}\text{mL}^{-1})\).

Keywords: *Euphorbia macrosteagia*; Cycloartane; Cytotoxicity; MDA-MB468; MCF-7.

Introduction

The incidence of cancer in human populations and the increasing need for anti-cancer drugs on the one hand and discovery of effective anti-cancer drugs, such as taxol, vincristin and vinblastin from plants. *E. macrosteagia* as one of the endemic plants to Iran is the subject of this investigation. *Euphorbia macrosteagia* (Persian wood spurge), belongs to the family Euphorbiaceae distributed mostly in central and west parts of Iran. Persian wood spurge is similar to the wood spurge (*Euphorbia amygdaloides*) and a rare species native of semi-moist woods from south-eastern Europe through Asia Minor. In the Iranian traditional medicine, latex is used to treat warts. Despite their toxicity, the uses of *Euphorbia* species in traditional medicine in many parts of the world have a long history. They are used to treat inflammations and tumours \((1)\). Previous investigation on the cytotoxicity assessment of *E. macrosteagia* \((2)\), has showed \(\text{LD}_{50}\) values of 200, 425, and 390...
water fall with elevation of 2130 m A.S.L. located in Yasooj, a city of Kohkilouyeh Va Boyer Ahmad province at Iran. It was identified by Department of Biology, Faculty of Science at University of Isfahan and a voucher specimen (#3340) was deposited in the herbarium of the Isfahan University (Iran).

Extraction and isolation

The air-dried plant material (2 Kg) was macerated with chloroform (20 L×3) at room temperature for 5 days. Filtration and in vacuo concentration resulted in a green gum (110 g), which was subjected on silica gel CC (hexane/dichloromethane, 0→100) to several fractions: Fr 1-Fr 5. Inferred from TLC and 1H-NMR, fraction Fr.1 and Fr.2 contained alkanes and fats, Fr.3 containing beta-sitosterol and fraction Fr. 4 and Fr.5 triterpenes. Fr.4 and Fr.5 were chromatographed on another normal column (hexane/acetone, 0→20). Finally triterpenes was further purified on HPLC using YMC-Pak-Sil column (250 × 20 mm) and hexane:ethylacetate (80:20) as mobile phase to

μgmL−1 for dichloromethane, ethyl acetate and acetone fractions, respectively while other fractions, remarked as noncytotoxic. Therefore, based on previous studies on cytotoxicity effects of E. macrostegia and its fractions, the authors decided to investigate phytochemical contents of the dichloromethane extract of this plant as the most active fraction.

Experimental

General experimental procedures

The NMR spectra were recorded on a Bruker Avance AV 400, using CDCl3 as solvent. HPLC was carried out on a waters 515 using a YMC-Pack-Sil column (250 × 20 mm i.d.) and hexane:EtOAc as mobile phase. Chromatographic materials were silica gel (Merck Co., Germany). Thin layer chromatography detection was achieved by spraying the silica gel plates with cerium sulfate in 10% aq.H2SO4, followed by heating.

Plant material

Plant material was collected from Margoon
yield compounds 1-4.

**Cycloart-25-ene-3β,24-diol (1)**  
White crystals; MW(g/mol): 442; yield: 0.0010% ; \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta_{\text{H}} 4.95, 4.86 \text{ (each 1H, brs, H-26)}, 4.03 \text{ (H, t, } J = 5.8 \text{ Hz, H-24)}, 3.30 \text{ (H, dd, } J = 4.4, 10.8 \text{ Hz, H-3)}, 1.73 \text{ (3H, s, H-27)}, 0.99 \text{ (3H, s, H-30)}, 0.98 \text{ (3H, s, H-18)}, 0.91 \text{ (3H, s, H-28)}, 0.90 \text{ (3H, d, } J = 6.4 \text{ Hz, H-21)}, 0.83 \text{ (3H, s, H-29)}, 0.57, 0.36 \text{ (each } 1\text{H, d, } J = 4.0 \text{ Hz, H-19a, b}); \(^{13}\)C-NMR data: see Table 1; EIMS \(m/z\): 442 (5), 427 (5), 424 (12), 409 (17), 381 (8), 355 (2), 315 (7), 302 (21), 297 (8), 203 (28), 175 (59), 43 (100).

**Cycloart-23Z-ene-3β,25-diol (2)**  
White crystals; MW(g/mol): 442; yield: 0.0004% ; \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta_{\text{H}} 4.96 \text{ (1H, m, H-23)}, 4.94 \text{ (1H, brs, H-24)}, 3.22 \text{ (1H, dd, } J = 4.4, 11.2 \text{ Hz, H-3)}, 1.27 \text{ (3H, s, H-26)}, 1.26 \text{ (3H, s, H-27)}, 0.90 \text{ (2× 3H, s, H-18, H-29)}, 0.81 \text{ (3H, s, H-30)}, 0.79 \text{ (3H, d, } J = 6.4 \text{ Hz, H-21)}, 0.74 \text{ (3H, s, H-28)}, 0.49, 0.25 \text{ (each } 1\text{H, d, } J = 4.4 \text{ Hz, H-19a, b}); \(^{13}\)C-NMR data: see Table 1; EIMS \(m/z\): 442 (3), 427 (5), 424 (12), 409 (17), 383 (3), 363 (5), 357 (3), 326 (16), 315 (6), 302 (30), 297 (9), 269 (7), 175 (52), 43 (100).

**Cycloart-23E-ene-3β,25-diol (3)**  
White crystals; MW(g/mol): 442; yield: 0.0015% ; \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta_{\text{H}} 5.72 \text{ (1H, ddd, } J = 15.6, 8.4, 6.0 \text{ Hz, H-23)}, 5.54 \text{ (1H, d, } J = 15.6 \text{ Hz, H-24)}, 3.30 \text{ (1H, dd, } J = 4.4, 10.8 \text{ Hz, H-3)}, 1.37 \text{ (2× 3H, s, H-26, H-27)}, 1.03 \text{ (3H, s, H-29)}, 0.99 \text{ (3H, s, H-18)}, 0.91 \text{ (3H, s, H-30)}, 0.89 \text{ (3H, d, } J = 4.6 \text{ Hz, H-21)}, 0.83 \text{ (3H, s, H-28)}, 0.58, 0.36 \text{ (each } 1\text{H, d, } J = 4.0 \text{ Hz, H-19a, b}); \(^{13}\)C-NMR data: see Table 1; EIMS \(m/z\): 442 (3), 427 (6), 425 (15), 409 (5), 383 (3), 363 (5), 357 (3), 326 (16), 315 (6), 302 (13), 300 (30), 297 (9), 269 (7), 175 (52), 43 (100).

**Cycloart-24-en-3β-ol (4)**  
White crystals; MW(g/mol): 440; yield: 0.0005% ; \(^1\)H-NMR (CDCl\(_3\), 400 MHz): \(\delta_{\text{H}} 4.73, 4.71 \text{ (each } 1\text{H, bs, H-31a,b)}, 3.31 \text{ (1H, dd, } J = 4.4, 11.2 \text{ Hz, H-3)}, 1.05 \text{ (3H, d, } J = 6.4 \text{ Hz, H-27)}, 1.04 \text{ (3H, d, } J = 6.8 \text{ Hz, H-26)}, 0.99 \text{ (2× 3H, s, H-18, H-30)}, 0.91 \text{ (3H, s, H-28)}, 0.90 \text{ (3H, d, } J = 9.2 \text{ Hz, H-21)}, 0.83 \text{ (3H, s, H-29)}, 0.57, 0.36 \text{ (each } 1\text{H, d, } J = 4.0 \text{ Hz, H-19a, b}); \(^{13}\)C-NMR data: see Table 1; EIMS \(m/z\): 440 (7), 425 (12), 407 (21), 315 (8), 300 (19), 297 (11), 286 (28), 203 (55), 175 (72), 69 (100).

**Cell culture**  
MCF-7 and MDA-MB468 human breast cancer cell lines 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.

**MTT viability assay**  
Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MCF-7 and MDA-MB468 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 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 (3).

**Statistical analysis**  
All samples were presented as mean ± SD for three measurements. Significance was attributed to p-values (P < 0.05) and the probability values obtained by the student t-test between sample and control data.

**Result and Discussion**  
Compound 1, white crystals, showed the molecular formula of \(C_{30}H_{50}O_2\) based on El-
MS \( m/z \) 442 and number and multiplicity of \(^{13}\)C-NMR spectra. The six-degree of unsaturation and the \(^{13}\)C-NMR data (Table 1), suggested the presence of one double bond and, therefore, a pentacyclic skeleton. EI-MS fragmentation pattern, supported \( m/z \) 355 and 302, typical ions of 4,4’dimethyl 9:19 cycloesterols (4). \(^1\)H-NMR revealed a pair of doublets in the up-field area 0.57, 0.36 (each 1H, d, \( J = 4.0 \) Hz, H-19a, b), characteristic of cycloartane cyclopropane ring (4), one secondary methyl group at 0.90 (3H, d, \( J = 6.4 \) Hz, H-19a, b), and five singlet methyls at \( \delta \) 1.31. Two double doublet protons at \( \delta \) 3.30 (1H, dd, \( J = 4.4, 10.8 \) Hz, H-3) and \( \delta \) 4.03 (1H, t, \( J = 5.8 \) Hz, H-24) revealed presence of two carbinolic protons and a pair of olefinic protons at \( \delta \) 4.95 and 4.86 (each 1H, brs, H-24) showed low coupling constants with at \( \delta \) 4.96 (1H, m, H-23) due to their cis orientation while in compound 3, olefinic pair protons at \( \delta \) 5.72 (1H, ddd, \( J = 15.6, 8.4, 6.0 \) Hz, H-23) and \( \delta \) 5.54 (1H, d, \( J = 15.6 \) Hz, H-24) with large coupling constant (\( J = 15.6 \) Hz) allowed assignment of trans geometry to the \( \Delta^23(24) \). In both compounds, downfield chemical shifts of two singlet methyl protons (Me-26, and Me-27) of the side chain atoms were in accordance with the second hydroxyl group on C-25 at \( \delta \) 128.8. As Ayatollahi and coworkers described EI-MS fragmentation pattern of cycloartanes (4), presence of monounsaturated side chain was also confirmed by the \( m/z \) 315 and 297 in EI-MS. In addition, \( m/z \) 381 together with 355 [M-H\( _2\)O-C\(_{12}\)H\(_9\)]\(^+\) fragments due to the elimination of parts of side chain during a Me Lafferty process, inferred presence of one hydroxyl in side-chain.

Regarding to these findings, and literature data (4), compound 1 identified as cycloart-25-en-3β, 24-diol. It is also found in other Euphorbia species like \( E. aellenii \) (4), \( E. heteradena \) (5) and \( E. sessiliflora \) (6). Compound 2, and 3 showed the molecular formula of C\(_{30}\)H\(_{50}\)O\(_2\) based on positive EI-MS \( m/z \) 442 and in accordance with their number and the multiplicity of \(^{13}\)C-NMR spectra (BB and DEPT). Their \(^1\)H-NMR revealed six tertiary singlet methyls, one secondary methyl group, and a pair of doublets in the up-field area characteristic of cycloartane cyclopropane ring and one carbinolic proton related to 3(β)-OH group. In compound 2, in olefinic pair protons, \( \delta \) 4.94 (1H, brs, H-24) showed low coupling constants with at \( \delta \) 4.96 (1H, m, H-23) due to their cis orientation while in compound 3, olefinic pair protons at \( \delta \) 5.72 (1H, ddd, \( J = 15.6, 8.4, 6.0 \) Hz, H-23) and \( \delta \) 5.54 (1H, d, \( J = 15.6 \) Hz, H-24) with large coupling constant (\( J = 15.6 \) Hz) allowed assignment of trans geometry to the \( \Delta^23(24) \). In both compounds, downfield chemical shifts of two singlet methyl protons (Me-26, and Me-27) of the side chain atoms were in accordance with the second hydroxyl group on C-25 at \( \delta \) 70.8 and 68.2, respectively. Therefore, based on aforementioned data and complete agreements of \(^{13}\)C- and \(^1\)H-NMR with other reported data in literature (7; 8), compound 2 and 3 were identified as cycloart-23Z-en-3β, 25-diol and

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**Table 1.** \(^{13}\)C-NMR chemical shifts of the triterpenoids from *Euphorbia macrostegia.*

| C   | 1  | 2  | 3  | 4  | C   | 1  | 2  | 3  | 4  |
|-----|----|----|----|----|-----|----|----|----|----|
| 1   | 31.9| 32.0| 32.0| 32.0| 16  | 26.5| 26.4| 26.4| 26.5|
| 2   | 30.4| 30.4| 30.4| 30.4| 17  | 52.2| 52.0| 52.1| 52.3|
| 3   | 78.9| 78.8| 78.8| 78.9| 18  | 18.0| 19.3| 18.1| 18.1|
| 4   | 40.5| 40.5| 40.5| 40.5| 19  | 29.9| 30.1| 29.9| 29.9|
| 5   | 47.1| 47.7| 47.1| 47.1| 20  | 35.9| 36.4| 36.3| 36.4|
| 6   | 21.1| 21.1| 21.1| 21.1| 21  | 18.3| 18.3| 18.4| 18.3|
| 7   | 28.1| 28.1| 28.1| 28.2| 22  | 32.0| 39.1| 39.4| 35.0|
| 8   | 48.0| 48.0| 48.0| 48.0| 23  | 31.5| 125.6| 130.8| 31.3|
| 9   | 20.1| 20.0| 20.0| 20.0| 24  | 76.7| 139.3| 134.4| 156.9|
| 10  | 26.1| 26.1| 26.0| 25.8| 25  | 128.8| 70.8| 68.2| 33.8|
| 11  | 26.0| 26.0| 26.0| 26.0| 26  | 11.4| 29.9| 24.4| 22.0|
| 12  | 32.9| 32.8| 32.8| 32.9| 27  | 17.2| 29.9| 24.3| 21.9|
| 13  | 45.3| 45.3| 45.3| 45.3| 28  | 19.3| 19.3| 19.3| 19.3|
| 14  | 48.7| 48.8| 48.8| 48.8| 29  | 14.0| 14.0| 14.0| 14.0|
| 15  | 35.6| 35.6| 35.6| 35.9| 30  | 25.4| 25.5| 25.4| 25.5|
Cycloartane Triterpenoids from Euphorbia Macrostegia with their Cytotoxicity Effects on Two Cancer Cell Lines MDA-MB468 and MCF-7.

In this study, we investigated the cytotoxicity effects of cycloartane triterpenoids isolated from Euphorbia Macrostegia on two cancer cell lines, MDA-MB468 and MCF-7. The compounds were identified as cycloart-23E-ene-3β, 25-diol (1), cycloart-23(Z)-ene-3β,25-diol (2), cycloart-23(E)-ene-3β,25-diol (3), and 24-methylene-cycloart-3β-ol (4). These compounds were isolated from different Euphorbia species and were found to have significant cytotoxicity activities.

Using MTT assay on two different cancer cell lines, MDA-MB468 and MCF-7, the biological effects of these compounds were evaluated. The cytotoxicity activities of these compounds were determined at different concentrations (0.1, 1, 10, 50, 100, and 200 μg/mL). The results showed that cycloart-23E-ene-3β,25-diol (1) was the most active compound on MDA-MB468 cell line, with an LD$_{50}$ value of 102.3 μg/mL. Cycloart-23(Z)-ene-3β,25-diol (2) was the most active compound on MCF-7 cell line, with an LD$_{50}$ value of 34.0 μg/mL.

The potent cytotoxicity observed by compound 2 and 3 with double bound on C-23 suggested that the cytotoxicity activities of these compounds are related to the position of the olefinic or the hydroxyl group on side chain. By the literature, cycloartanes isolated from other spurge species like Euphorbia rigida (9), E. humifusa (11), and E. aellenii (12) also showed potent cytotoxicity.

The biological effects of these compounds were further analyzed using one-way ANOVA to compare their cytotoxicity against control cells which were not treated (set to 100%). The results showed that the cytotoxicity activities of these compounds were statistically significant (P < 0.05, **P < 0.01). The potent cytotoxicity observed by these compounds could be due to their structural characteristics that allow them to interact with key cellular processes, leading to cell death.

Figure 2. Cytotoxicity effects of the cycloartanes (1-4) in Euphorbia macrostegia on two cancer cell lines MDA-MB468 and MCF-7. In this panel the cytotoxicity tests were presented on two different cancer cell lines including MDA-MB468 and MCF-7 in the presence of different concentrations of cycloart-23E-ene-3β,25-diol (1), cycloart-23(Z)-ene-3β,25-diol (2), cycloart-23(E)-ene-3β,25-diol (3), and 24-methylene-cycloart-3β-ol (4), and control cells which were not treated (set to 100%). For statistical significance one-way ANOVA was used to analyze the differences between each sample and control (*P < 0.05, **P < 0.01).
from *Euphorbia* species showed also apoptosis induction on mouse lymphoma cells (14). Cycloart-25-en-3(β), 24-diol and 24-methylene-cycloartan-3(β)-ol (compound 1 and 4) presented antiproliferated activity on human peripheral blood lymphocytes (4). Cycloartanes were also reported for other biological activities like immunomodulatory effects like positive effect on Th1 cytokine release (IL-2 and IFN-γ), and suppression on Th2 cytokine production (IL-4) (15), inhibition of 11β-hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2) (IL-4) (15), inhibition of 11β-hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2) as a strategy for reducing glucocorticoid action on insulin resistance in type 2 diabetes mellitus and metabolic syndrome (16,17), or stimulating GLP-1 amide secretion in streptozotocin-nicotinamide induced diabetic Sprague Dawley rats (18). Therefore, interesting properties of cycloartanes, especially their antiproliferative effects, candidate them as investigational lead compounds in cancer research.

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