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Original Article

Sesquiterpene lactones from shoot culture of *Artemisia aucheri* with cytotoxicity against prostate and breast cancer cells

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Abstract

Plant tissue culture is used to grow plant cells, tissues, or organs under sterile and determined conditions on culture media. It is alternative to traditional vegetative propagation, and is applied as an effective technology for the production of valuable secondary metabolites. The *Artemisia aucheri* (*A. aucheri*) was obtained from shoot culture grown on MS (Murashige and Skoog 1962) medium. Shade-dried aerial parts of *in vitro* grown *A. aucheri* (50 g) were extracted with dichloromethane-acetone (90:10). The extract was submitted for isolation to sephadex gel chromatography and preparative thin layer chromatography, which resulted in identification of one known eudesmanolide named artemin or 2,5-dihydroxy-12, 6-eudesmanolide-4(15)-en for the first time in this plant. In cell cytotoxicity test, artemin showed cytotoxic activity against DU-145,LNCaP prostate cancer, and MCF-7 breast cancer cells with IC_{50} values of 82.2 ± 5.6, 89.1 ± 6.3 and 111.5 ± 6.7 µM , respectively. Artemin was more active against prostate cancer cells with approximately same cytotoxicity against LNCaP androstane dependent cells and DU 145 which is androstane independent.

Keywords: *Artemisia aucheri;* Breast cancer; Cytotoxicity; Eudesmanolide; Prostate cancer; Sesquiterpene lactone.

INTRODUCTION

Plant cell and tissue cultures, as a unique technology for the production of valuable secondary metabolites, have been used as alternative method to traditional vegetative propagation for production of valuable phytochemicals such as pharmaceuticals, food additives, flavors, fragrances, nutraceuticals, and industrially important biochemicals. In tissue culture the same biological plant with the same genome is used but the controlled criteria and composition of nutrient culture media may affect the variety and quantity of secondary metabolite production in comparison with intact plants. Therefore, study of plant *in vitro* cultures under different controlled and reproducible protocols could lead to production of plant secondary metabolites with a higher content than those in whole plants (1).

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Artemisia aucheri (*A. aucheri*) BOISS with Persian name: 'Derman-e-Koohi' belongs to Asteraceae family and is endemic to Iran. About 500 species of *Artemisia* have been found from which 34 species grow in Iran (2). Ecological distribution of *A. aucheri* is limited mostly to mountainous landscapes with higher slope, sandy soils with annual precipitation of 300-450 mm (3). Morphological features of this plant consist of perennial, suffrutescent, stem numerous and erect, indumentum white-tomentose, leaves pinnate or bipinnate, capitula arranged in a panicle-congested, calathium sessile and ovate, phyllaries imbricate and multiseriate florets 3-4 (2).

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In recent pharmacological studies, ethanolic extract of *A. aucheri* induced regression of aorta atherosclerotic lesions in hypercholesterolemia rabbits (4). In a study by Yang *et al*. verbenone analogues were isolated from *A. aucheri* and showed acaricidal activity against dermatophagoides spp. and *Tyrophagus putrescentiae* (5). In addition, antileishmanial effects of this plant were evaluated by Sharif *et al*. (6). In recent years, a research has shown cytotoxic properties of *A. aucheri* on different human carcinoma cell lines including MCF-7 (human breast cancer cells), SKNMC (human neuroblastoma) and A2780 (human ovarian cancer cells) (7). Verbenone, camphor, 1,8-cineole, transverbenol, chrysanthenone, mesitylene, α-pinene, acyclic monoterpenes, and monoterpene hydroperoxides are bioactive compounds which were reported from its essential oil (8). Rustaiyan and coworkers reported six highly oxygenated geraniol derivatives from *A. aucheri* aerial parts (9). The paper in hand, aimed to isolate and characterize sesquiterpene lactones in shoot cultured from *A. aucheri* in addition to cytotoxic evaluation of isolated compounds against prostate and breast cancer cells.

MATERIALS AND METHODS

General procedures

Chromatographic materials were silica gel (Merck Co., Germany), and Sephadex LH-20 (Pharmacia Inc., Piscataway, NJ, USA). Thin layer chromatography (TLC) detection was achieved by spraying the silica gel plates with cerium sulfate in 10% aq. H_2SO_4, followed by heating. The structures of the compounds were elucidated using spectral methods proton nuclear magnetic resonance (^{1}H-NMR), carbon-13, distortionless enhancement by polarization transfer (DEPT), nuclear overhauser effect spectroscopy (NOESY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC), using $CDCl_3$ as solvent. NMR spectra were taken by Bruker 400 (Bruker Co., Germany).

Plant tissue culture

The *A. aucheri* was obtained from stock shoot culture in Plant Stress Center of Excellence in Isfahan University Herbarium center, University of Isfahan, Isfahan, I.R. Iran. Plants were grown on MS medium Murashige and Skoog 1962 (Sigma-Aldrich, Germany) supplemented with 30 g/L sucrose and 8 g/L agar (pH 5.8) in the culture room at 25 ± 1 °C with 16 h photo period under 64 µmol photons/m^{2}s light (10).

Plant tissue culture

Plant tissue culture technique as a rapid and fast method under controlled conditions in cultivation of medicinal and aromatic plant has recently received a lot of attention as an effective method for the production of valuable secondary metabolites. As mentioned in plant material part, we used *A. aucheri* tissue culture samples for this study. Stem sections with one node were cut from parent plant and transferred to jars containing MS medium. After one week, the root appeared on the stem sections and gradually new leaves and buds were also observed. Cultivation period was one month and all plant cultivation steps were kept under sterile conditions. Thus, the obtained plants with this method were grown under *in vitro* condition which is different with plants grown in the field condition (Fig. 1).

Fig. 1. *Artemisia aucheri* plant cultivated with tissue culture technology.

Extraction and isolation

Shade-dried aerial parts of *in vitro* grown *A. aucheri* (50 g) were ground into fine powder. It was macerated in 500 mL dichloromethane-acetone (90:10) for 3 days

for three times at room temperature and away from light. Then sonication was performed for 2 h in a water bath sonicator. The extract was filtered and evaporated under 45 °C and reduced pressure. To remove the lipids and chlorophyll, the residues were subjected to vacuum liquid chromatography (Rp-18, MeOH: H_2O; 70:30) (11). The defatted fraction was concentrated under 40 °C and reduced pressure. The residue was fractionated by sephadex LH 20 column chromatography using methanol (60%), n-hexane (30%), and acetone (10%) system solvents into 72 vials. Based on TLC spot colors visualized by vanillin sulfuric acid, it yielded 2 subfractions possessing deep blue color known for sesquiterpene lactones. Vials 8-23 were added to each other (fraction 1) and applied to preparative layer chromatography silica gel plate (hexane: ethyl acetate; 45:55) to yield compound 1 (2.4 mg). Vials 32-42 (fraction 2) were also subjected to silica gel column preparative TLC using hexane:ethyl acetate 45:55 which yielded compound 2 as pure entity (8.7 mg).

Cell culture

DU-145 as androstane-independent and LNCaP as androstane-dependent prostate cancer cell lines and MCF-7 breast cancer cells were purchased from Pasture institute of Iran, Tehran, I.R. Iran. Subsequently, cells were cultured in RPMI-1640 medium contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in 5% CO2 at 37 °C.

Cell cytotoxicity assay

As previously described (11,12), DU-145, LNCaP, and MCF-7 cancer cell lines in RPMI media were seeded separately in 96-well plates (5×10^3 cells/well) and incubated in 5% CO2 at 37 °C. After 24 h, the media were replaced by fresh media containing serially diluted concentrations of compounds **1**, **2** (1, 10, 40, 80, 100, 150, 250 µM) and incubated for 48 h. Wells with vehicle treated cells, and doxorubicin hydrochloride 1 µM (EbewePharma, Austria) were considered as negative and positive controls, respetively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as much as 5 mg/mL in PBS was added to the wells and incubated for another 4 h. Then supernatants were aspirated out and 150 µL of dimethyl sulfoxide (DMSO) were added to the wells. Well plates were shaken for 10 min and then optical density (OD) values were read at 570 nm in the microplate reader (Bio-Rad, Hercules, CA, USA). Cell viabilities were calculated by the following equation:

$$\text{Cell viability (\%)} = (OD_{compound} - OD_{blank}) / (OD_{negative\ control} - OD_{blank}) \times 100$$

Statistical analysis

Cell viabilities and IC_{50} values were expressed as mean ± standard deviation (SD) of the mean. Statistical analysis was done using one-way ANOVA with Turkey's post hoc analysis, using SPSS software. The results were considered as significant when $P < 0.05$. IC_{50} values were calculated in Excel worksheets using logarithmic scale scatter (X and Y) charts.

RESULTS

Spectral data of isolated compounds

Dichloromethane-acetone extract of *in vitro* grown *A. aucheri* (50 g) gave 5 g dark green gum. After repeated column chromatography two pure compounds were isolated. These compounds were submitted for ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HSQC, HMBC, NOESY, and mass spectra for elucidation. Spectral data of isolated compounds (Fig. 2) were as follows:

Undetermined sesquiterpene (1, 0.005): amorphous white powder, UV (EtOAc, nm) λmax: 246, 274; IR (NaCl) υ_{max}: 3446, 2935, 2856, 1770, 1672, 1454, 1383, 1248, 1032 cm^{-1}. ^{1}H-NMR ($CDCl_3$, 400 MHz): 4.65 (dd, J = 7.9, 7.9 Hz, H-2), 2.89 (overlapped, H-3b), 1.27 (overlapped, H-3a), 5.00 (bd, J = 10.2 Hz, H-5), 3.55 (dd, J = 11.2 Hz, 4.4, H-6), 1.72 (m, H-7), 2.23 (m, H-8b), 1.25 (overlapped, H-8a), 4.39 (dd, J = 8.8, 8.8 Hz, H-9), 1.27 (overlapped, H-11), 1.25 (d, J = 6.9 Hz, H-13), 5.83 (s, H-14b), 5.67 (s, H-14a), 1.86

(s, H-15). ^{13}C-NMR data ($CDCl_3$, 100 MHz):203.4 (C-1), 83.0 (C-2), 38.3 (C-3), 140.5 (C-4), 124.9 (C-5), 77.7 (C-6), 52.8 (C-7), 41.1 (C-8), 80.6 (C-9), 142.8 (C-10), 29.7 (C-11), 177.9 (C-12), 12.4 (C-13), 123.3 (C-14), 18.5 (C-15) ppm; positive ESI-MS (*m/z*): 281 $(M+H)^+$.

2,5-Dihydroxy-12,6-eudesmanolide-4(15)-en (2, 0.018%): amorphous white powder. ^{1}H-NMR ($CDCl_3$, 400 MHz): 4.17 (dd, *J* = 11.2 Hz, 4.8, H-1), 1.88 (overlapped with H-8b, H-2b), 1.68 (overlapped with H-8a, H-2a), 1.75 (bdd, *J* = 7.8 Hz, 3.0, H-3b), 1.28 (overlapped with H-15, H-3a), 4.26 (d, *J* = 10.4 Hz, H-6), 2.38 (overlapped with H-11, H-7), 1.88 (overlapped with H-2b, H-8b), 1.66 (overlapped with H-2a, H-8a), 2.19 (ddd, *J* = 13.9 Hz, 5.5, 1.6, H-9), 2.36 (overlapped with H-7, H-11), 1.25 (d, *J* = 6.4 Hz, H-13), 0.91 (s, H-14), 4.05 (s, H-15b), 4.00 (s, H-15a). ^{13}C-NMR data ($CDCl_3$, 100 MHz): 29.6 (C-2), 29.9 (C-3), 144.8 (C-4) ,79.2 (C-5), 81.7 (C-6), 71.8 (C-1), 45.4 (C-7), 22.8 (C-8), 30.3 (C-9), 30.1 (C-10) , 41.2 (C-11), 179.2 (C-12), 12.5 (C-13), 13.2 (C-14), 112.3 (C-15) ppm; positive ESI-MS (*m/z*): 267 $(M+H)^+$. Figure 2 indicates the structure of compound 2.

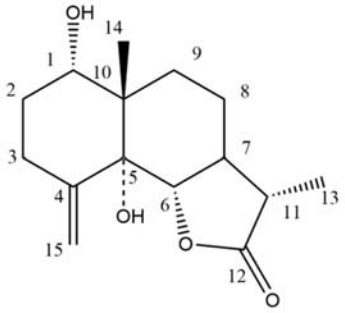

Fig. 2. Structure of compound 2 isolated from *Artemisia aucheri* shoot culture

Cell cytotoxicity assay

Standard MTT assays were done on artemin to test its cytotoxicity against DU-145, LNCaP, and MCF-7 cells (Fig. 3). IC_{50} values (μM) were listed in Table 1.

DISCUSSION

In this research two sesquiterpene lactones (Fig. 1) were isolated from the dichloromethane: acetone (9:1) extract of *A. aucherii* by repeated chromatographic methods.

Compound **1** in NMR spectra showed characteristic signals of a germacranolide sesquiterpene lactone but because of low quantity and consequently weak correlations in HMBC spectra, its structure was not confirmed (13-15). The structure of compound **2** was identified based on its ^{1}H-NMR and ^{13}C-NMR similarities with published article (16). ^{1}H- and ^{13}C-NMR spectra revealed the presence of a fifteen carbons. Further DEPT, HSQC, and HMBC spectra revealed presence of three sp2 signals including one carbonyl carbon (δ_C 179.2, C-12), related to a COO group of an acidic group or a lactone ring, one external methylene group [δ_C 144.8 and 112.3 (δ_H:1H, 5.04, bs, H-15b; 1H, 4.99, bs, H-15a)], two methyls δ_C 12.5 (δ_H: 3H, 1.26, d, *J* = 6.4, Me-13), 13.2 (δ_H: 3H, 0.91, s, Me-14), four methylenes δ_C 29.6 (C-2), 29.9 (C-3), 30.3 (C-9), 22.8 (C-8) and four methines (two oxygenated) δ_C 81.7 (δ_H: 1H, 4.26, d, *J* = 10.4, H-6), 71.8 (δ_H: 1H, 4.17, dd, *J* = 11.2 , 4.8, H-1), 45.4 (δ_H:1H, 2.37, overlapped, H-7), 41.2 (δ_H:1H, 2.38, overlapped, H-11), and two quaternary (one oxygenated) δ_C79.2 (C-5), and 30.1 (C-10) atoms. These data were in full agreement with artemin: 2,5-dihydroxy-12, 6-eudesmanolide-4(15)-en isolated previously from North African *Artemisia* species by Sanz *et al.* (15). However, previously reported ^{1}H and ^{13}C-NMR data were corrected using HSQC and HMBC spectra.

In biological study, artemin showed cytotoxic activities with the IC_{50} values in the range of 80 to 120 μM against prostate and breast cancer cells. In a comparison between cells, it was more active against prostate cancer cells with approximately same cytotoxicity against LNCaP androstane dependent cells and DU 145 which is androstane independent (Fig. 3). These results were in agreement with other studies showed cytotoxic effects of similar sesquiterpene lactones including eudesmanolides against breast and prostate cancer cell lines. Kawasaki *et al.* reported molecular effects of parthenolide a germacranolide sesquiterpene lactone on prostate cancer cells (16).

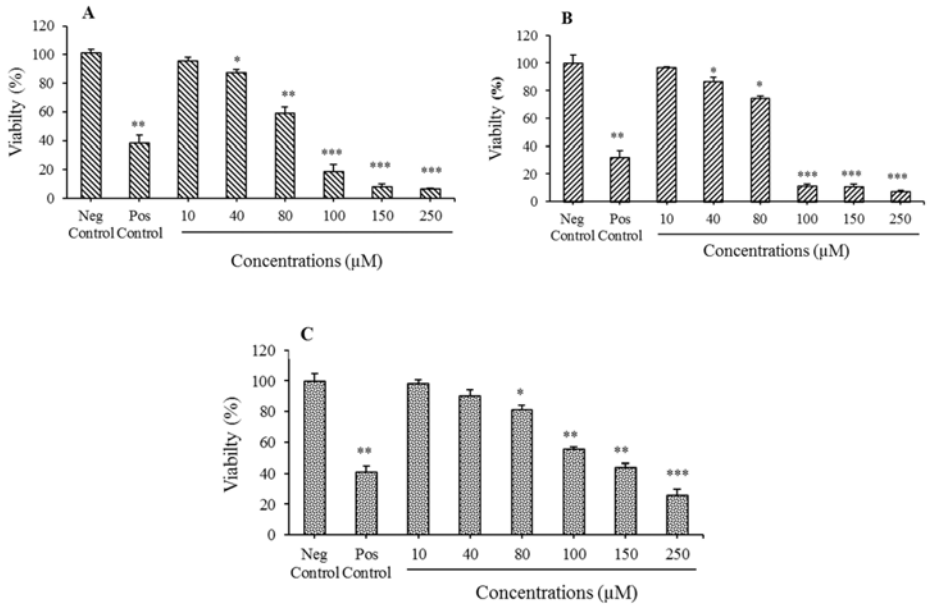

Fig. 3. Cytotoxicity effect of artemin against (A) DU-145, (B) LNCaP, and (C) MCF-7 cells cancer cells. Cells were treated with different concentrations of tested compound (1, 10, 40, 80, 100, 150, and 250 µM) in three replicates. Doxorubicin (1 µM) and vehicle were used as positive and negative controls, respectively. Significant differences are shown compared with negative control (**P* < 0.5; ***P* < 0.01; ****P* < 0.001).

Table 1. IC_{50} values (µM) of artemin isolated from shoot culture of *Artemisia aucheri* against DU-145, LNCaP, and MCF-7 cells. Data are presented as mean ± SD, n = 3

Compounds	IC_{50} values (µM)		
	DU-145 cells	**LNCaP cells**	**MCF-7 cells**
Artemin	82.2 ± 5.6	89.1 ± 6.3	111.5 ± 6.7
Doxorubicin	0.22 ± 0.15	0.33 ± 0.09	0.45 ± 0.08

Arucanolide, another sesquiterpene lactone with germacranolides structure showed potent cytotoxicity against human tumor cell lines, HL60 and SW480 cells (17). In a study on eudesmanolides sesquiterpenes, Negrín *et al.* showed apoptotic properties of the eudesmanolide tanapsin against human tumor cells through a mechanism dependent on reactive oxygen species (18). In another study, eudesmanolide sesquiterpenes isolated from flowers of *Tanacetum vulgare* showed cytotoxic activity *in vitro* against A549 (human lung carcinoma epithelial-like) and V79379A (Chinese hamster lung fibroblast-like) cells (19). Eudesmanolide derivatives from the flowers of *Inula japonica* possessed potent antiproliferative activities against MCF-7 and MDA-MB-231 human breast cancer cells (20).

The limitation of this research was preparation of tissue culture in large amount in laboratory scale which was difficult and led to low quantity of starting plant material (50 g) and restriction for more isolation and characterization. This prevented us from isolation of other sesquiterpene lactones identified preliminary in H-NMR spectra and in TLC profile of subfractions. However, in comparison with other phytochemical studies performed on wild type of this plant, artemin was isolated for the first time from this plant. Quantitative analysis on both wild plant and shoot cultured plant are required to have a good judge about efficiency of this method for production of artemin in higher quantity.

CONCLUSION

Phytochemical analysis of *A. aucheri* shoot culture resulted in identification of one known eudesmanolide named artemin or 2,5-dihydroxy-12,6-eudesmanolide-4(15)-en for

the first time in this plant. In MTT cytotoxicity test, it showed moderate toxicity against prostate (LNCaP, and DU 145) and breast (MCF-7) cancer cells.

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