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Active Fractions of Dichloromethane Extract of *Artemisia Aucheri* Inhibit Proliferation of Human Breast Cancer MCF-7 Cells via Induction of Apoptosis

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The antiproliferative effect of dichloromethane extract of *Artemisia aucheri* (*A. aucheri*) has been demonstrated previously on human cancerous cell lines. In the current study, further fractionation was carried out on the dichloromethane extract of *A. aucheri* and their cytotoxic effects were evaluated on three human cancer cell lines; SKNMC, MCF-7, and A2780. Cell viability was determined by MTT assay and activation of caspases was evaluated by spectrophotometry. Quantitative real time RT-PCR was used to evaluate the genes expression. Detection of DNA fragmentation was carried out by flow cytometry. The obtained results showed that fractions 5 and 7 (F5 and F7) have a potent cytotoxic effect, especially against MCF-7 cells. F5 and F7 also induced apoptosis through the DNA fragmentation and mitochondrial membrane potential (MMP) disruption in MCF-7 cells. The caspase-3, 9 enzyme activities were also increased after exposure to F5 and F7. Moreover, caspase-8 activity increased significantly after exposure to F7 but not F5. The level of mRNA expressions of Bax and Smac/DIABLO was increased after exposure to both fractions. A detectable decrease was also observed in the mRNA expression of Bcl-2 after exposure to F7. No change was observed in the level of mRNA expressions of tumor suppressor P53 after exposure to F7. Therefore, the cell cycle arrest and apoptosis induced by F7 could probably be mediated through a p53-independent mechanism. Taken together, these observations demonstrated that the cytotoxic effect of F5 and F7 on MCF-7 cells is likely exerted via apoptotic cell death.

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Introduction

Todays, the use of natural products as potential cytotoxic agents has entered cancer studies and numerous attempts have been made to introduce bioactive plant-derived products to medicine [1]. *Artemisia aucheri* Boiss. (*Asteraceae*) with the Persian name of "Dermaneye koohi" is among the species wildly grow in Iran [2]. Investigations on the volatile oils from the aerial parts of *A. aucheri* have resulted in the identification of some oxygenated monoterpenoids like camphor [3], verbenone [4], linalool [5, 6], 1,8- cineol [7], α- thujone [8], and borneol [9] as its major constituents. Rostayan *et al.* also reported the presence of secondary metabolites including six highly oxygenated geraniol derivatives in this species [10]. Some investigations were done to examine the *in vitro* antifungal [5, 11, 12], antimicrobial [13], and acaricidal [14] activities of the volatile oil and crude extract of *A. aucheri*. The in *vitro* and *in vivo* anti-leishmanial activities of *A.aucheri* have also been previously demonstrated [15-17]. Besides, scientists have reported a significant antimalarial activity for dichloromethane extract of the species in a cell-free β-hematin formation assay [18], and *in vivo* effectiveness of ethanolic extract on the chloroquine-sensitive strain of Plasmodium berghei [19]. In addition to the reported pharmacological properties, a previous *in vitro* experiment, conducted in our laboratory, demonstrated that semipolar fractions obtained from petroleum ether extract of *A. aucheri* possess cytotoxic and apoptotic effects against SKNMC, human neuroblastoma cell line [20]. Further purification of that fraction eventually led to isolation of two tetrahydrofuran- type sesquiterpenoids which were proposed to be responsible for the cytotoxic and apoptotic effects of *A.aucheri* [21].

In a previous study, we evaluated the cytotoxic effect of the different extracts of *A. aucheri* on three human cancer cell lines (SKNMC, MCF-7, and A2780) and showed that dichloromethane extract of *A. aucheri* had high potent cytotoxic effects on human cancerous cell lines [22]. In continuation of our previous study, further fractionation on dichloromethane extract was carried and cytotoxic effects of fractions were evaluated on human cancer cell lines. Also, more details and methods were applied to explore the molecular mechanism in which the cytotoxic effect of the potent fractions is mediated on the most sensitive cell line.

Materials and methods

Chemicals

Silica gel 60 (0.040–0.063 mm) was purchased from Merck, and all the solvents used for extraction and fractionation from Caledon and Scharlau. 3-(4,5-dimethylthiazol-2yl)-2,5 – diphenyltetrazoliumbromide (MTT), rhodamine 123, and caspases activity detection kit were procured from SigmaAldrich (St Louis, MO,USA). Cell culture medium, penicillin–streptomycin solution, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). RNA isolation kit with high purity was purchased from Roche (Mannheim, Germany). Real time polymerase chain reaction (RT-PCR) kit was supplied by Invitrogen (Carlsbad CA). BCA protein assay kit obtained from Pierce (Pierce, Bonn, Germany).

Plant material

Aerial parts of *A. aucheri* were collected from Chahar-Bagh region (Golestan province, Iran) in September 2011. Sample was authenticated by Mr. S. A. Hosseini (Agricultural and Natural Resources Research Center of Golestan Province, Gorgan, Iran) by comparing it to deposited voucher specimen (No. 2383).

Preparation of the extract and fractions

The dried-powdered aerial parts (200 g) of *A. Aucheri* were extracted with petroleum ether (PE) (40-60) and dichloromethane (DCM), respectively (sequential maceration with *ca*. 3 × 2 L of each solvent). DCM extract was concentrated using a rotary evaporator at a maximum temperature of 45°C to yield 8.61 g of dried material. The DCM extract (7.00 g) was fractionated by vacuum liquid chromatography (VLC) over silica gel using

mixtures of ethyl acetate (EtOAC) and petroleum ether (PE) (5: 95 to 100:0) to afford 8 fractions (F1-F8). The procedure was followed by using pure acetone and methanol as the eluting solvents to give two other fractions (F9 and F10).

Cell culture

MCF-7 (human breast cell carcinoma), SKNMC (human neuroblastoma cell line), and A2780, (human ovary carcinoma cell line), were obtained from Pasteur Institute (Tehran, Iran). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) with 10% (v/v) fetal bovine serum, 100 Uml^{-1} penicillin, and 100 U ml^{-1} streptomycin. The medium was changed 2-3 days and sub-cultured when the cell population density reached to 70–80% confluence.

MTT cell proliferation assay

Cell proliferation was determined using the 3-(4,5-dim-ethylthiazol-2-yl)-2,5diphenyltetrazoliumbromide) (MTT) assay following the methods described by Freimoser *et al*.,1999. The cells were plated in 96-well cell culture plates at a cell density of 15×10^4 cells/well. After 24 h the spent medium was replaced with fresh medium containing increasing concentrations (0-500 µM) of the different fractions. A negative control of untreated cells was included. The cells were incubated for a further 24h, after which a 0.5 mg/ml MTT solution (10 µl/ well) was added and the cells incubated for an additional 4h. Then, the medium was removed, and the purple formazan crystals were dissolved in DMSO (200 µl/ well). Absorbance was determined on an ELISA plate reader (Biotek, H1 M) with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630).

Morphological studies

The cells were plated in 6-well cell culture plates at a cell density of 7×10^5 cells /well. After 24h, different concentrations of selected fractions were added to the medium. As a negative control, cells were treated with DMSO. Images of the cells were taken after 24 h at a 20 magnification using a Matic microscope equipped with a Leica digital camera.

Cell cycle analysis

The effect of the IC_{50} concentration of the most potent fractions on cell cycle distribution was determined by flow cytometric analysis of the nuclear DNA content, which has been previously described [24]. Briefly, cells were grown exponentially to reach 50–60% confluency and were exposed to the fractions or DMSO, and then incubated for 2 h at 4°C in the dark with 750 µl of a hypotonic buffer (50 mg/ml PI in 0.1% sodium citrate, 100 µg/ml plus 0.1% triton X-100) before flow cytometric analysis using Partec TM cytometry (Germany).

Caspase-3, 8 and 9 activation assay

Caspase-3, 8 and 9 activity assays were carried out using the sigma colorimetric caspase kit. This assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, AcDEVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9) in equal amounts of cell protein. The cells were plated in quadruplicate in 6-well tissue culture plates (700×10^3 cells/well), incubated overnight, and then treated with different concentrations of the selected fractions for 24 h. Next, the cells (7×10^5) were harvested and lysed in 30 µl of the cell lysis buffer included with the kit, and protein concentrations were equalized for each condition. Subsequently, 10 µl of cell lysate was combined with an equal amount of substrate reaction buffer containing caspase-3,8 and 9 colorimetric substrates. This mixture was incubated for 2 h at 37 °C, and the absorbance was then measured at 405 nm using a plate reader (BioTek, H1M).

Analysis of apoptosis–related gene expression

Total RNA was extracted from MCF-7 cells (7 × 10^5) pretreated with IC_{50} concentration of the most potent fractions using high pure isolation kit (Roche, Mannheim, Germany) according to the manufacturer instructions. Quality and quantity of total RNA were assessed using a Nano DropTM 2000 spectrophotometer (USA) and samples stored at -70 °C until the use. The primers used in this study were selected from previously published studies 27, 28.Thermal cycler program was run as follows: 15 min at 50 °C for cDNA synthesis, 10 min at 95 °C followed by 40 cycles of 15s at 95 °C to denature the DNA, and 45 s at 60 °C to anneal and extend the template. Melting curve analysis was performed to ascertain specificity by continuous acquisition from 65 °C–95 °C with a temperature transient rate of 0.1 °C/s. All the reactions were performed in triplicate in a Corbett system (Australia). The value obtained for the target gene expression were normalised to β-actin and analyzed by the relative gene expression -ΔΔCT method where -ΔΔCT= (CT target–CT β-actin) Unknown – (CT target – CT β-actin) Calibrator.

Measurement of mitochondrial membrane potential

Mitochondriahas been shown to play a key role in the induction of apoptosis [25]. In the current study, rhodamine 123 fluorescent dye, a cell permeable cationic dye, was used for monitoring mitochondrial membrane potential (MMP). Depolarization of MMP during cell apoptosis results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence intensity. At the end of treatment, cells were added with 5 mg/l rhodamine 123 for 30 min at 37 °C. The fluorescence intensity was measured at an excitation wavelength of 488nm and an emission wavelength of 520 nm using a fluorescence microplate reader (BioTek, H1M, and USA).

Determination of Intracellular ROS

Intracellular ROS level was examined using DCF-DA. DCF-DA is a non-fluorescent lipophilic ester that easily crosses the plasma membrane and passes into the cytosol, where the acetate group is rapidly removed by unspecific esterases. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence. The intensity of this fluorescence is generally considered to reflect the level to which ROS are present [23]. After seeding for 24 h, cells were washed with PBS buffer (pH 7.4). The cells treated with selective compounds for 24 h. After washing with PBS, the cells were incubated with 20 µL DCF-DA at 37°C for 30 min. The percentage of DMSO in solution did not exceed from 0.5%. After incubation, cells were lysed with Triton X-100. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (BioTek, H1M, USA).

Statistical analysis

All the data was analysed using one-way ANOVA using Graph Pad Prism software (GraphPad software, San Diego, CA, USA). Differences between the mean± SEM (standard error of the mean) of samples were considered significant at $P < 0.05$. The IC_{50} values were generated from the MTT results using GraphPad Prism software.

Results

Antiproliferative effects of fractions

Results of MTT assay for detection of antiproliferative effects of the fractions are demonstrated in Fig. 1 (a-c). The does-dependent investigations showed that F5 and F7 fractions were the most potent fractions specifically against MCF-7 cells, as demonstrated by their IC_{50} values (9.3 and 15µg/ml, respectively). The IC_{50} of F5 against A2780 and SKNMC were 72.3 and 80 µg/ml, respectively and these values were found 31.7 and 15.6 µg/ml for fraction F7.

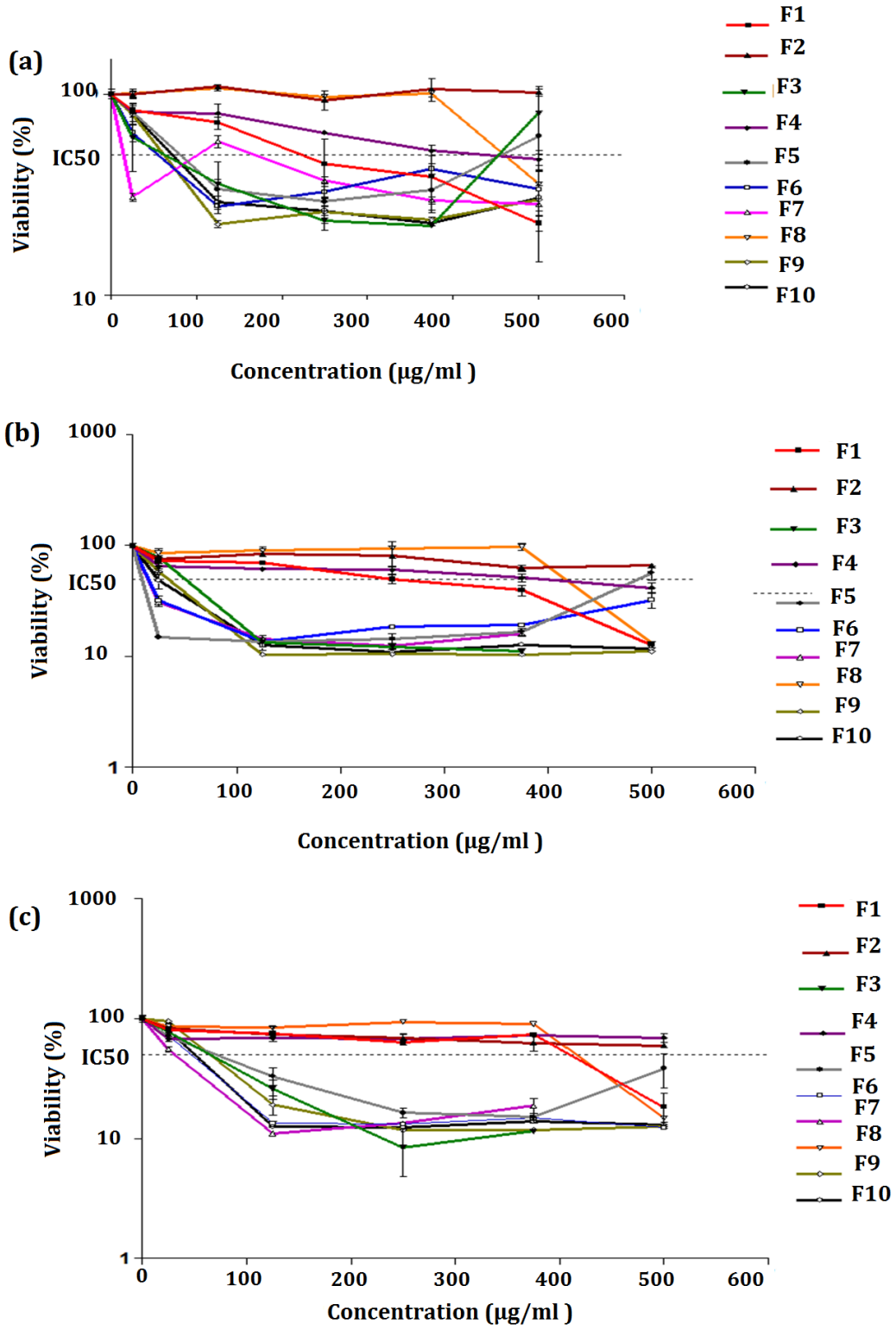

Fig. 1. Cytotoxic effcts of different fractions in (a) SKNMC, (b) MCF-7, and (c) A2780 cell lines.. Data are presented as mean ± S.E.M (n = 3).

These results were supported by an investigation into the effect of F7 and F5 on the morphology of cells in culture. Fig. 2 shows the effects on the morphology of MCF-7 cells treated for 24 h with IC_{50} concentration of F5 and F7 fractions. The cells treated with potent fractions showed signs of growth inhibition, shrinkage, vacuolization and detachment from the culture flask. It must be noted that F1, F2, F4 and F8 did not show any significant cytotoxic effects on any cell line at the concentration up to 400µg/ml. Therefore, in subsequent experiments, F7 and F5 were adopted for identification of mechanisms of action on MCF-7 cell line.

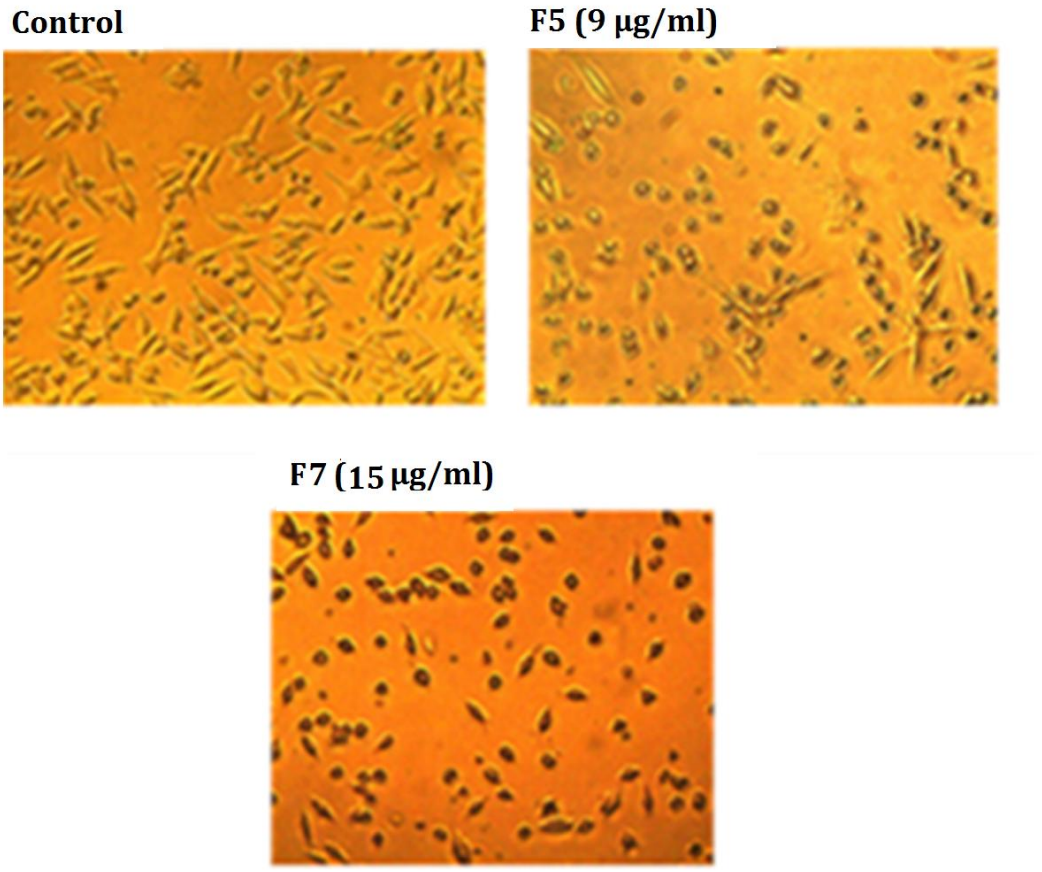

Fig. 2. The photomicrograph presenting morphological changes of MCF-7 cells.

Effect of F5 and F7 fractions on cell cycle analysis

The cellular poptosis following the treatment with two selected fractions of *A. aucheri* was measured by PI staining and flow cytometry, to evaluate the sub-G1 peak resulting from DNA fragmentation. Flow cytometry histograms of the cells treated with F5 (9 µg/ml) and F7 (15 µg/ml)- for 24 h demonstrated an increase in the percentage of cells in SubG1 phase as an indicative of apoptotic cells, in treated but not in control cells (Fig. 3).

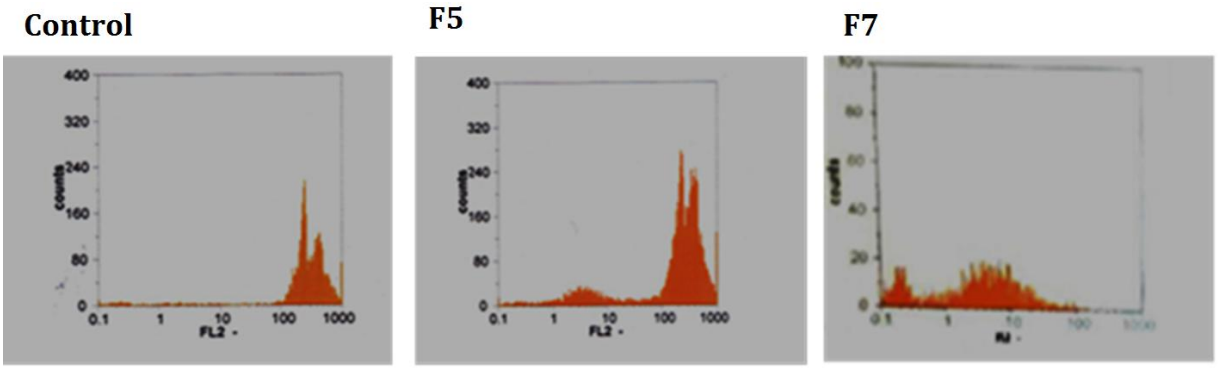

Fig. 3. Flow cytometry histograms of apoptosis assays by PI method in MCF-7 cells. Cells were treated with F5 (9 µg/mL) and F7 (15 µg/ml) of dichloromethane extract of A. aucheri for 24 h.

Effect of the selected fractions on MMP

To evaluate the role of mitochondria in the apoptosis induced by the most potent fractions, rhodamine 123 was performed to monitorthe ability of F5 and F7 to modify the MMP. After 24 h, the signal of rhodamine decreased s approximately 53% and 41%, in the MCF-7 cells exposed to IC_{50} concentration of F5 and F7, respectively (Fig. 4).

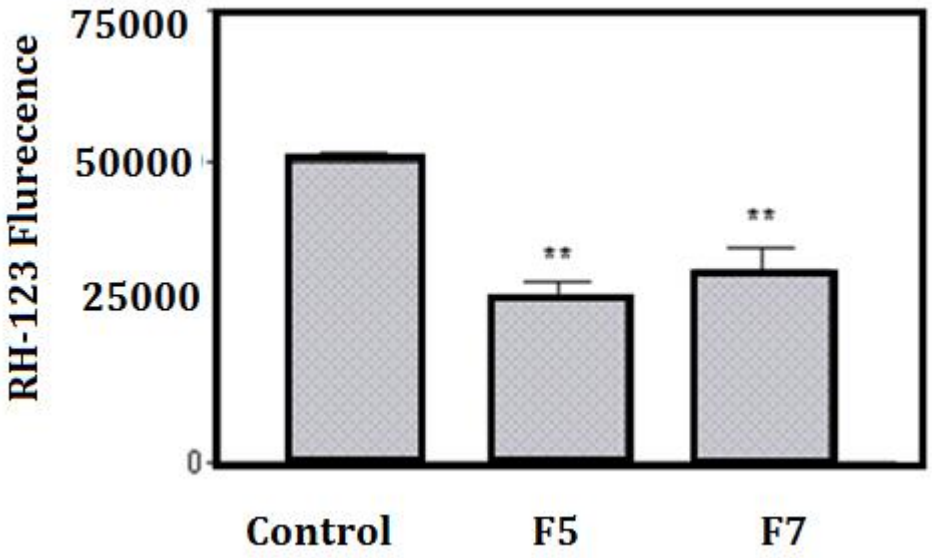

Fig. 4. Effect of fractions 5 and 7 of dichloromethane extract of *A. aucheri* on mitochondrial membrane potential (MMP) collapse as detected by Rhodamine 123. Data are expressed as the mean± SEM of three separate experiments. ** $p<0.01$ vs. control.

Effect s of F5 and F7 fractions on Caspase - 3, - 8, and - 9 Activity

In the current study for improvement of MTT results and also characterizing the mechanism of cell death involved in our experiments, the activity of caspases was evaluated. The obtained results showed that 24 h treatment with IC_{50} concentration of selected fractions increased caspase-3 activation in human breast carcinoma cell lines (Fig. 5). To determine which apoptotic pathway is activated by fractions, we evaluated the activation of caspase-8 and -9, the apical proteases in extrinsic and intrinsic pathways, respectively [29]. Both fractions were able to increase caspase-9 in MCF-7 cells, thus implying that intrinsic pathway is involved in apoptosis induced by both fractions. In contrast to the F7, which increased the activation of caspase-8 as a mediator of extrinsic pathway, F5 did not alter the activation of caspase-8 (Fig. 5).

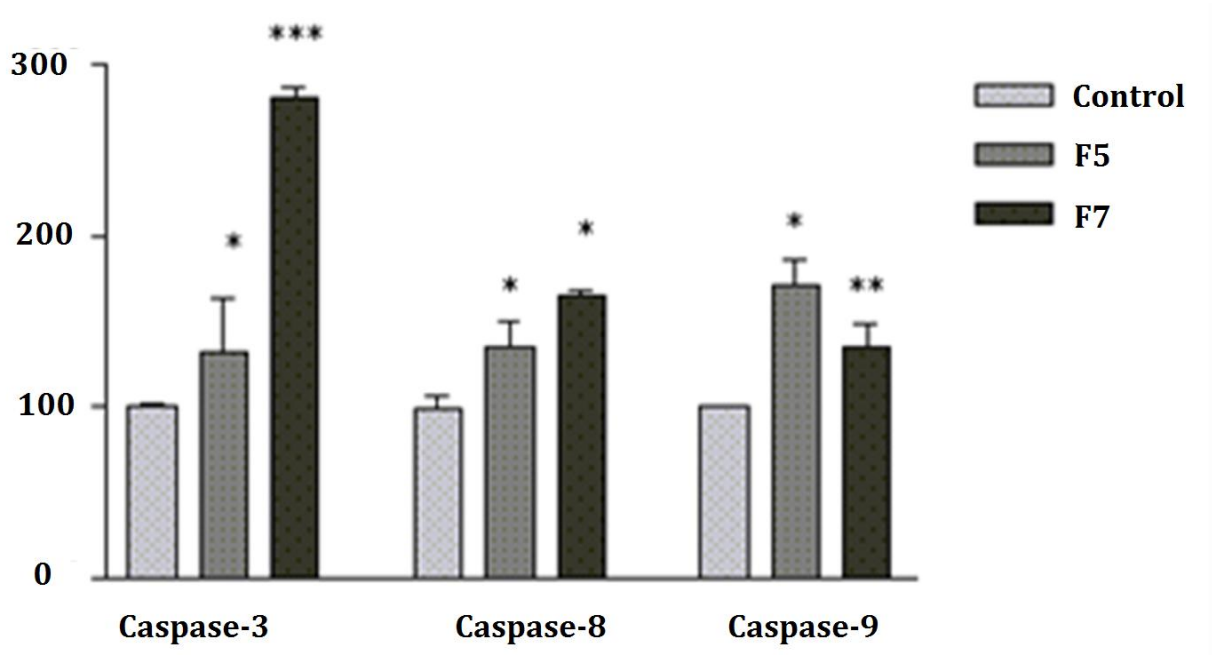

Fig. 5. Involvement of activation of caspases in the induction of apoptosis by potent fractions of dichloromethane extract of *A. aucheri* on MCF-7 human breast cancer cells. Data are presented as mean ± S.E.M. $*P < 0.05$,$**P < 0.01$, versus control.

Determination of the expression levels of apoptosis-regulatory genes

The mRNA expression levels of well-known apoptotic-related genes, Bcl-2, Bax, and p53 were analyzed using one-step RT-real time PCR assay. Fractions 5 and 7 at IC_{50} concentration were used to stimulate the cells over the period of 24 h. Fig. 5-a showed that the expression level of p53 was increased 1.95 folds after 24 h incubation with 9µg/ml of F5. In contrast, we could not observe a significant change in the p53 mRNA after 24 h exposure to F7 (15µg/ml) (Fig. 6). The expression level of the Bcl-2 gene was decreased 0.397 and 0.292 folds when the MCF-7 cells were treated with the IC_{50} concentration of fraction 5 and fraction 7, respectively. Real-time RT-PCR analysis also clearly showed a significant increase of the expression level of Bax after 24 h treatment with F5 and F7.

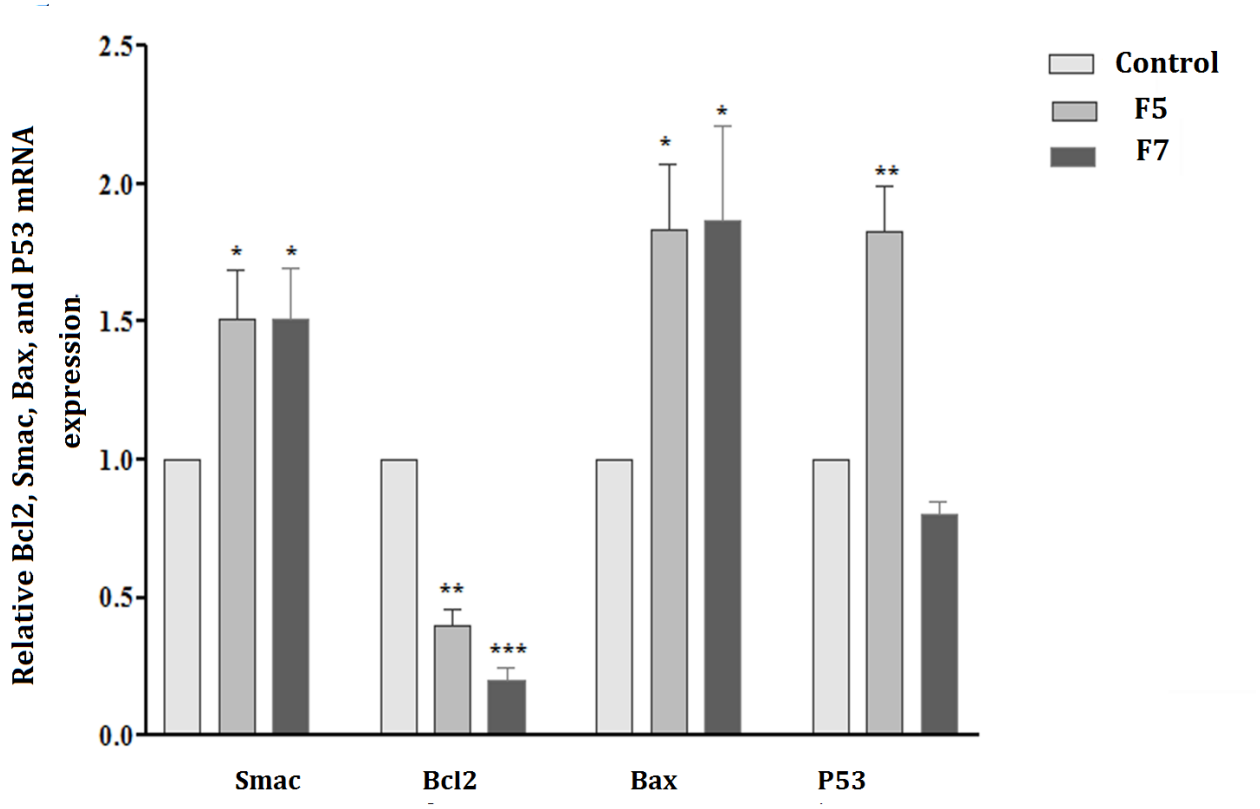

Fig. 6. The effect of F5 and F7 of dichloromethane extract of *A. aucheri* on expression of apoptotic-related genes on MCF-7 human breast cancer cell line. Levels of mRNA are expressed relative to control in the mean ± S.E.M values derived from three independent experiments. ∗$P < 0.05$, ∗∗, $P < 0.01$, and ∗∗∗$P < 0.001$ versus control.

Effects of F5 and F7 fractions on ROS generation

In order to measure oxidative stress induced by active fractions, fluorescent dye DCF-DA was used to measure ROS production. As shown in the Fig. 7, adding F5 (9µg/ml) to MCF7 cells caused a significant increase in ROS levels. It must be noted that treatment with F7 had no effect on intracellular ROS levels in human breast cancer cell line.

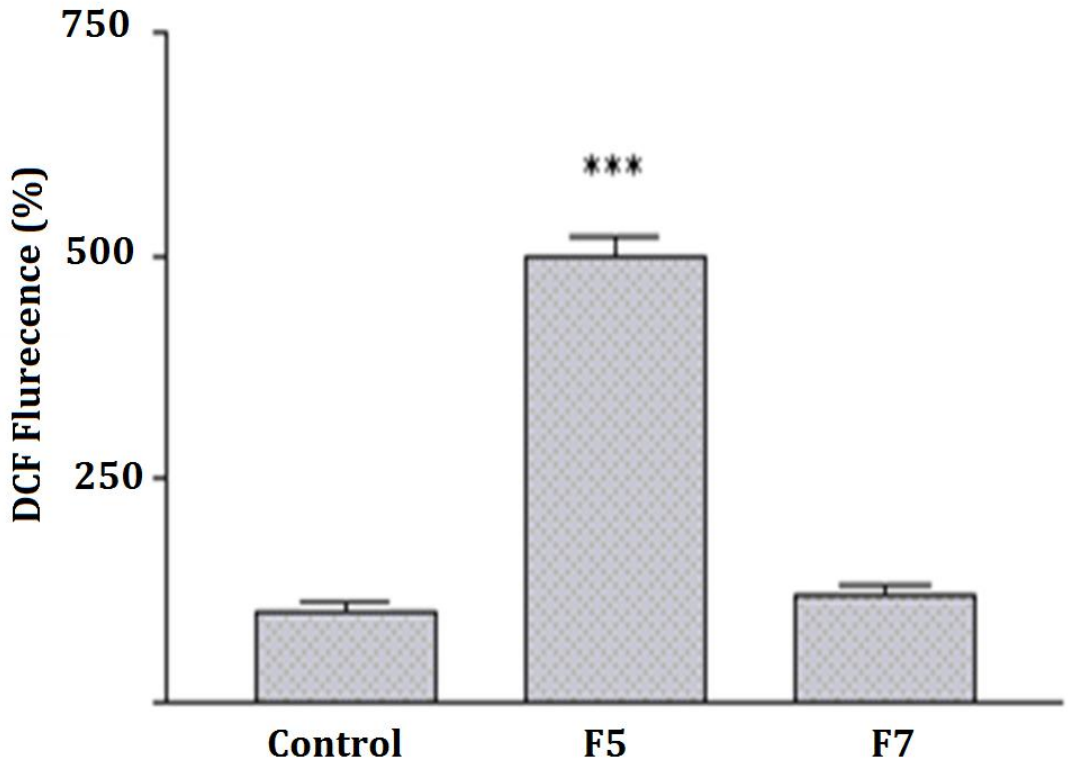

Fig. 7. The effect of active fractions of *A .aucheri* on ROS generation in MCF7 cell line. Data are expressed as the mean ± S.E.M of three separate experiments (n = 4).***p < 0.001 vs. Control

Discussion

Due to the increasing research focus on plants with cancer chemoprevention and antimetastatic potential, this study was designed to evaluate the cytotoxic activitiy of the fractions from dichloromethane extract of *A. aucheri* in three human carcinoma cell lines [1]. The results revealed that some fractions of this plant are potentcytotoxic agents against human cancer cell lines, especially MCF-7 cells. Among the fractions, the highest cytotoxic activity was observed in F5 followed by F7 and F6.

Apoptosis, or programmed cell death, is a highly selected process that plays an important role in cancer treatment. Since Apoptosis-induction capacity, rather than necrosis induction, has been accepted as a mode of action of the antitumor drug [20,21], considerable efforts are being directed toward the development of potential medicines that induce apoptosis in tumor cells [21]. Therefore, in the present study, the mode of cell death induced by F5 and F7 was investigated through examining well-characterized apoptosis markers in MCF-7 cell line. It found that fractions F5 and F7 induced apoptosis in MCF-7 cells, as verified by DNA fragmentation and accumulation of cells in the sub-G1 apoptotic phase. To further-undrestand the mechanisms controlling apoptosis induced by F5 and F7, we examined the expression of some critical genes involved in apoptosis. The proteins of the Bcl-2 family play an essential role in apoptosis and are considered as a target for anticancer therapy [24]. The Bcl-2 protein exhibits an anti-apoptotic effect while Bax is a pro-apoptotic protein of the Bcl-2 family [25]. In the present study, a decrease in the expression of Bcl-2 and an increase in the expression of Bax were observed after treating MCF-7 cell line with F5 and F7. P53 is a key tumor suppressor gene that

has a critical function in apoptosis [25]. Our finding demonstrated that treatment with F5 increased the mRNA expression of P53 while F7 had no effect on the expression level of P53. Therefore, the cell cycle arrest and apoptosis induced by F7 is probably mediated through a p53-independent mechanism. A number of previous studies have shown that apoptosis can be induced in a p53-independent manner [26]. Moreover, it has been well documented that activation of caspase cascade is critical in the initiation of apoptosis in numerous biological systems [27]. In the present study, it was found that fractuion F5 induces apoptosis probably via an intrinsic pathway, because no significant increase in caspase-8 activity was detected in MCF-7 cells. The generation of intracellular ROS was usually associated with cell apoptosis. To investigate this relationship, we examined the levels of ROS in MCF-7 cells treated with active fractions of *A. aucheri*. Intracellular ROS levels were found to be significantly increased and were higher in the F5-treated than untreated cells, indicating that F5 induced apoptotic cell death, which requires the generation of ROS. Previously some studies have been done on the different species of the genus *Artemisia*. Artemisinin, as a well-known secondary metabolite in the genus *Artemisia*, has the ability to induce apoptosis in the pancreatic cell line via the generation of ROS and triggering the intrinsic pathway of cell death [28]. It has been shown that dihydro-artemisinin can inhibit proliferation and induce apoptosis via caspase-3 dependent mitochondrial death pathway in ASTC-a-1 cells [29]. Apoptosis induction has also been observed from other isolated sesquiterpenoids from the genus *Artemisia* [30, 31]. Isolated eudesmane-type sesquiterpenoids from the genus *Artemisia* have shown the cytotoxic effect on various cell lines [32], which is attributed to induction of apoptosis via a mitochondrial and caspase-dependent manner [33, 34]. According to the reported apoptosis induction effects of geraniol [34, 35] and the presence of eudesmanolides and highly oxygenated geraniol derivatives in *A. aucheri* [10], results of the present study can be fairly justified.

Conclusion

Overall, in the present study, we found that F5 and F7 are potent cytotoxic fractions, especially against MCF-7 cell line. More detailed studies showed that these fractions are able to induce apoptosis in MCF-7 cell line. Furthermore, a phytochemical study on active fractions should be performed to recognize the pure component(s) responsible for the cytotoxic activity of the selective fractions.

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Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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