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Original Article

Persianolide-A, an eudesmanolide-type sesquiterpene lactone from *Artemisia kopetdaghensis*, induces apoptosis by regulating ERK signaling pathways

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Abstract

Background and purpose: Herbal components, particularly sesquiterpenes, are progressively recognized as a crucial resource for developing effective therapeutic agents for breast cancer. In this study, the effect of a sesquiterpene lactone known as 8-O-dihydroxy-11α,13-dihydroeudesma-4(15)-en-12,6α-olide (persianolide-A) was examined in breast cancer cell lines.
Experimental approach: MDA-MB-231 and MCF-7 cancer cells were grown in DMEM solution with 10% FBS. Then, an MTT assay was performed to evaluate cell viability. Apoptosis was detected by annexin-PI staining. A caspase 3/7 activity assay kit was used to assess the activity of caspase-3 and caspase-7. Protein expression of Bcl-2, Bax, and p-ERK1/2 was determined by western blotting.
Findings/Results: This study showed that the IC_{50} values of the persianolide-A for MCF-7 and MDA-MB-468 cells are 34.76 and 54.48 µM, respectively. In addition, persianolide-A showed a significant increase in apoptosis in both MDAMB-231 and MCF-7 breast cancer cell lines. Persianolide-A significantly increased the expression of the pro-apoptotic protein Bax and decreased the expression of the anti-apoptotic protein Bcl-2. Also, presinolide-A treatment led to a substantial increase in caspase activity with a ratio of 3/7 in both MCF-7 and MDA-MB-231 cancer cells. In addition, the study showed that persianolide-A decreased the expression of p-ERK1/2 protein.
Conclusion and implications: The results of this study suggest that persianolide-A, sourced from *Artemisia kopetdaghensis*, induces cell apoptosis in breast cancer cell types. The molecular mechanisms could be implicated in the modulation of the ERK1/2 signaling pathway.

Keywords: Apoptosis; Artemisia kopetdaghensis; Breast neoplasms; ERK1/2; Persianolide-A, Sesquiterpene lactone.

INTRODUCTION

Cancer is a major global health problem, causing one in four deaths (1, 2). Among them, breast cancer is common in women (3). According to the World Health Organization, it ranks fifth in cancer-related deaths worldwide and is the second leading cause of cancer deaths in women (4). Standard treatments for breast cancer encompass chemotherapy, radiotherapy, surgery, and hormone therapy; however, the absence of a conclusive therapeutic target poses a challenge in its treatment (5). Furthermore, it should be noted that the mortality rate is significantly elevated in individuals who receive treatment for the condition, which shows the ineffectiveness of these treatments (6). As a result, there is a rising interest in developing and identifying new potential therapeutic agents against breast cancer (7-9).

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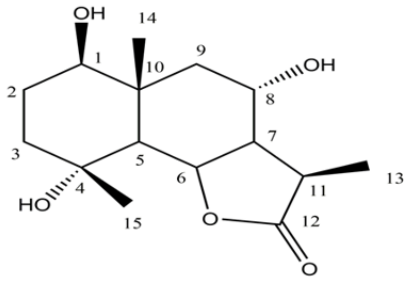

Fig. 1. The structure of persianolide-A (1, 8-O-dihydroxy- 11α,13- dihydroeudesma -4(15)-en-12,6α-olide, isolated from *Artemisia kopetdaghensis* (22).

Herbal components have been recognized recently as a crucial resource for breast cancer therapy (8-10). Flavonoids, phenolic acids, alkaloids, and sesquiterpene lactones (STLs) play an essential role in drug discovery in this field. They work through anticancer signaling pathways, including apoptosis (11-13). Apoptosis is an initial defense mechanism in multicellular organisms to counteract tumor development by eliminating genetically impaired cells (14). STLs encompass a broad and notably varied collection of secondary plant compounds, with their highest prevalence in the Asteraceae family (15). They are categorized into germacranolides, eudesmanolides, eremophilanolides, guaianolides, and pseudoguaianolides (16). Among these compounds, parthenolide (17), artemisinin (18), and thapsigargin (19) have been studied as potential anticancer precursors combating cancer cells. STLs' bioactivities are primarily associated with the stable methylene-lactone ring frequently occurring in their structure (15).

The anti-tumor influence of STLs has been related to regulating different signaling pathways, such as extracellular signal-regulated kinase (ERK) pathways (20). The ERK signaling pathway is a complex network of genes and proteins that transmit signals from the cell's exterior to the nucleus, influencing various cellular processes. Key genes within this pathway include mitogen-activated protein kinase 1 (MAPK1) and MAPK3, which encode the ERK proteins. These genes and their associated proteins are pivotal in controlling cell growth, differentiation, and responses to external stimuli, contributing to the overall regulation of cellular functions. Due to its versatile roles in acquiring malignant phenotypes, obstruction of the ERK pathway in tumor cells is logically expected to cause an anti-proliferative and anti-metastatic (21). A previously conducted phytochemical examination of aerial parts of *Artemisia kopetdaghensis* led to the isolation and identification of eudesmane-type STL, including 11α,13-dihydroeudesman-4(15)-en-olide (Fig. 1) named persianolide-A (22). Few studies to date have focused on the mechanism of action of STLs against breast cancer cells. In our ongoing attempt to discover pharmacologically effective compounds among STLs, persianolide-A was subjected to the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay to examine its cytotoxicity against breast cancer cells. Furthermore, related factors associated with the process of apoptosis, such as Bax, Bcl-2, and p-ERK1/2 expression, were tested by western blotting.

MATERIALS AND METHODS

Dulhocco's modified eagles medium (DMEM) was acquired from Gibco (Rockville, Maryland). Powder of MTT was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Furthermore, dimethyl sulfoxide (DMSO) was obtained from Merck (Munich, Germany). Serum derived from fetal bovine and trypsin-ethylenediaminetetraacetic acid (EDTA) was graciously provided by Bioidea (Tehran, Iran). The annexin/propidium iodide (PI) apoptosis assay kit was purchased from BioLegend (San Diego, California). The caspase 3/7 colorimetric activity assay kit was secured from Kia Zist (Hamedan, Iran). From Santa Cruz Biotechnology (California, USA), antibodies targeting Bax, Bcl-2, and p-ERK1/2 protein were acquired.

Persianolide-A

Our previous study, including the analysis of chemical components in the upper parts of *A. kopetdaghensis*, led to the discovery and identification of persianolide-A (22). This compound belongs to the sesquiterpene lactone group known as eudesmane. Determining its structure relies on a thorough examination of

various spectral data that includes both 1D and 2D nuclear magnetic resonance (NMR) and HRESIMS. Calculations and experimental evaluations using electronic circular dichroism (ECD) spectral data were used to decide on its exact configuration (22).

Cell culture conditions

The human breast cancer cell lines (MDA-MB-231 and MCF-7) were provided by the Pasteur Institute of Iran. The cells were grown in a DMEM solution with a 10% concentration of fetal bovine serum (FBS) and 1% penicillin G/streptomycin antibiotics. They were subsequently placed in a controlled incubator at 37 °C, containing 5% CO_2 and 95% humidity.

Evaluation of viability assay

The MTT assay was conducted as previously described (23). Ten thousand cells were cultured in each well of a 96-well plate. Following, the cells were treated with different concentrations of persianolide-A (1-200 µM) for over 48 h. An MTT stock solution with a 5 mg/mL concentration was prepared. Next, 20 µL of MTT solution was added to each well. After 4 h, DMSO was introduced. Finally, the number of viable cells was determined by measuring the optical density at 570 nm by the microplate reader.

Evaluation of apoptosis with annexin-PI staining

This research used flow cytometry to evaluate cell apoptosis, using a double staining method with annexin V and fluorescein isothiocyanate (FITC)-labeled PI. This technique was designed to investigate whether the growth-inhibiting effect of persianolide-A was linked to the initiation of apoptotic cell death. In summary, cells were cultured in a 6-well plate at 4×10^5 cells per well. Then, cancer cells were incubated with persianolide-A at concentrations of 34.76 µm for MCF-7 and 54.48 µm for MDA-MB 231 for 48 h. Afterward, the cells were washed twice with a cold phosphate-buffered saline (PBS) solution. Following centrifugation, the cells were suspended in 500 µL of binding buffer at 1×10^6 cells/mL density. Subsequently, 5 µL of annexin V-FITC and PI dye (50 ng/mL, 1 µL) were added and allowed to incubate at room temperature for 10 min. Finally, the stained cells were analyzed by a flow cytometer to ascertain the levels of apoptosis.

Assessment of caspase 3/7 enzymatic activity

The caspase 3/7 colorimetric activity assay kit was employed to evaluate the activity of caspase-3 and caspase-7, following the guidelines provided by the manufacturer. Initially, cells were planted at a concentration of 4×10^5 cells/mL in a 6-well plate and allowed to adhere for 24 h. Then, the cells were exposed to the IC_{50} concentration of persianolide-A (34.76 µM for MCF-7 and 54.48 µM for MDA-MB 231) for 48 h. The cells were then collected and resuspended. Caspase lysis buffer (500 µL) was added and then the cells were incubated at 4 °C for 20 min. For further investigation, the sediment was discarded. Protein levels were assessed using the Bradford method, and consistent protein quantities were utilized for the analysis. Once the protein concentrations were adjusted, 50 µL of each supernatant sample was introduced into a well. Subsequently, 55.5 µL of the working solution (containing 50 µL of caspase buffer, 5 µL of caspase substrate, and 0.5 µL of dithiothreitol) was added to each sample. Afterward, the specimens were placed in an incubator at 37 °C for 90-120 min. After the incubation period, the microplate reader measured absorbance at 405 nm.

Western blotting

The experiment involved seeding 5×10^5 breast cancer cells within every well of six-well plates. Subsequently, the cells were exposed to the STL sample for 48 h, followed by extraction of total protein content using radioimmunoprecipitation assay lysis buffer enhanced with 0.5% protease inhibitor cocktails and 0.5 mM phenylmethylsulfonyl fluoride. For protein quantification, the Bradford assay was employed. Each sample was exposed to 30 µg of proteins and underwent electrophoresis using a 12% sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE). Following that, the proteins were moved onto a membrane made of polyvinylidene fluoride. Particular mouse monoclonal antibodies

targeting Bcl-2, Bax, and p-ERK1/2 were primarily employed for protein detection. We used a secondary antibody to observe the protein bands; we mainly used a goat secondary antibody against mouse IgG linked with horseradish peroxidase and an ECL chemiluminescent substrate.

Statistical analysis

The statistical examination employed a one-way ANOVA followed by the Tukey post hoc assessment. The data is displayed as the mean ± SD. *P*-values < 0.05 were considered statistically significant. The IC_{50} value was calculated utilizing GraphPad Prism 8.2.1 through analysis of the concentration-response curve.

RESULTS

The effect of persianolide-A on cell viability

Cell cytotoxicity following treatment with persianolide-A was evaluated using the MTT test. Figure 2A and B depicts a concentration-dependent reduction of cell viability of MDA-MB-231 and MCF-7 cells treated with persianolide-A. Additionally, these results demonstrated a more pronounced inhibitory effect on MCF-7 cells than MDA-MB231 cells, starting at 1 µM and reaching its peak at 200 µM. On the other hand, the viability of MDA-MB231 cells treated with persianolide-A at 25-200 µM significantly decreased. The IC_{50} values for inhibiting 50% of cell growth were 34.76 µM in MCF-7 cells and 54.48 µM in MDA-MB-231 cells. This observation underscores the compound's heightened selectivity towards MCF-7 cells.

The effect of persianolide-A on cell apoptosis

Cells apoptosis, exposed to persianolide-A at IC_{50} (34.76 µM for MCF-7 and 54.48 µM for MDA-MB-231) for 48 h, were examined using the flow cytometry. The results demonstrated that persianolide-A effectively induced apoptosis in both MDA-MB-231 (Fig. 3A and B) and MCF-7 (Fig. 3C and D) breast cancer cells. The percentage of apoptosis for the MDA-MB 231 and MCF-7 cells was 52.35% and 49.45%, respectively.

The effect of persianolide-A on activities of caspase 3/7.

To explore the involvement of caspases in initiating apoptosis by persianolide-A, the caspase 3/7 activities in persianolide-A-treated cells were measured using colorimetric assay kits. The outcomes revealed a noteworthy rise in caspase3/7 activity in MDA-MB-231 cells after persianolide-A treatment (Fig. 4A). Similarly, in MCF-7 cells, significant elevation in caspase3/7 activity was observed upon exposure to persianolide-A (Fig. 4B).

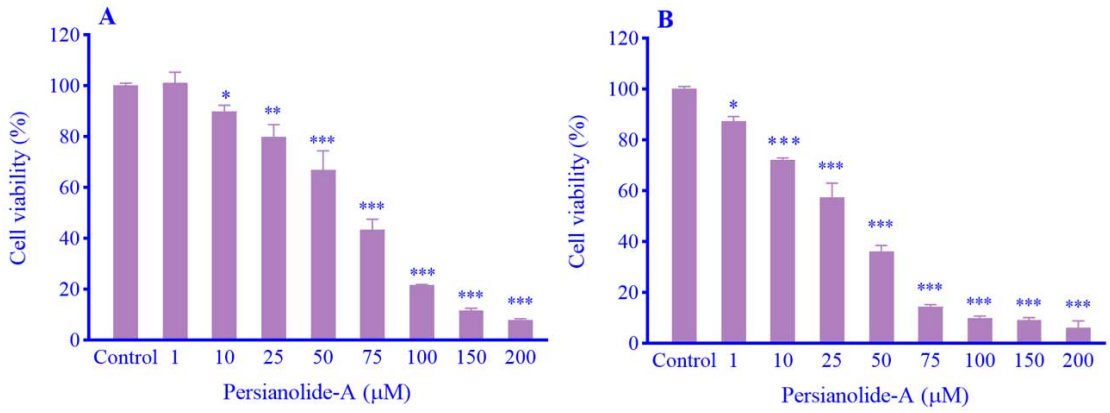

Fig. 2. The effect of persianolide-A on cell viability in breast cancer cells. Various concentrations of persianolide-A were administered to the cells over 48 h, and the viability of the cells was evaluated using the MTT test in (A) MDA-MB-231 and (B) MCF-7 cell lines. As the concentration of persianolide-A increased, there was a notable decrease in the percentage of viable cells. Data are shown as mean ± SD of experiments performed in triplicate. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences in comparison with the control group.

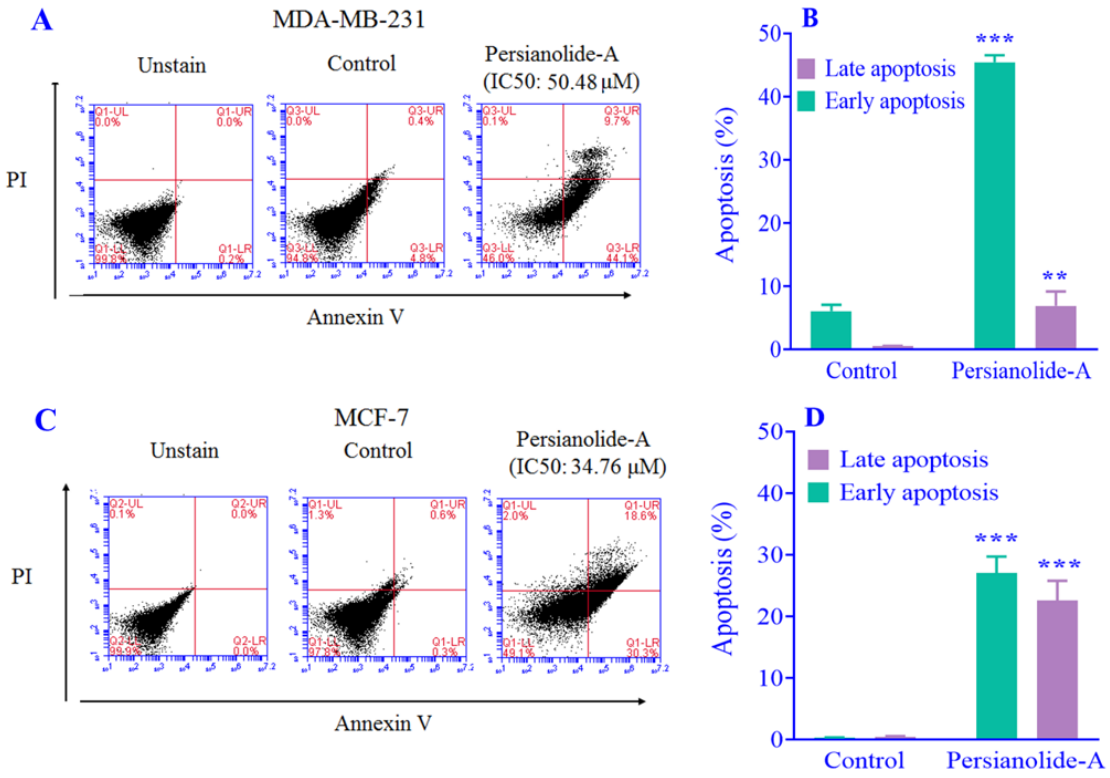

Fig. 3. The effect of persianolide-A on cell apoptosis. MDA-MB-231 and MCF-7 cells were exposed to persianolide-A at IC_{50} values for 48 h (50.48 and 34.76 µM, respectively). Flow cytometry was utilized to evaluate cellular apoptosis. The results demonstrated that persianolide-A induces apoptosis in (A and B) MDA-MB-231 and (C and D) MCF-7 cells. Values represent the mean ± SD, n = 3. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with the respective control group.

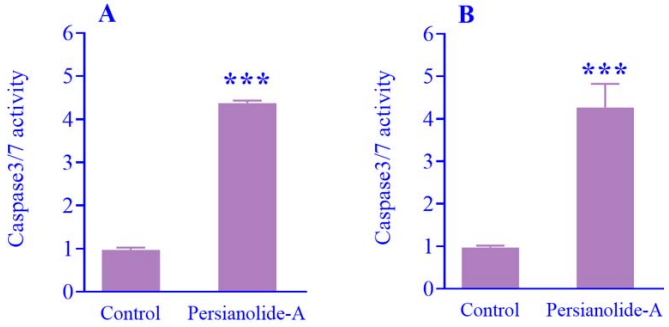

Fig. 4. The effect of persianolide-A on caspase activity 3/7. (A) MDA-MB-231 and (B) MCF-7 cells were treated with persianolide-A at IC_{50} (50.48 and 34.76 µM, respectively). After 48 h, a colorimetric evaluation was performed to ascertain the 3/7 activity ratio. Values represent the mean ± SD, n = 3. $^{***}P < 0.001$ indicates significant differences in comparison with the control group.

The effect of persianolide-A on Bax and Bcl-2 protein expression

To further evidence of cell apoptosis, the presence of Bcl-2 family components, specifically Bax and Bcl-2, in breast cancer cells following exposure to persianolide-A was assessed with the utilization of western blotting. After persianolide-A treatment, a notable decrease in Bcl-2 expression was observed in MDA MB-231 (Fig. 5 A and B) and MCF-7 (Fig. 5 C and D). Additionally, it was observed that the induction of apoptosis by persianolide-A coincided with heightened levels of the proapoptotic Bax expression.

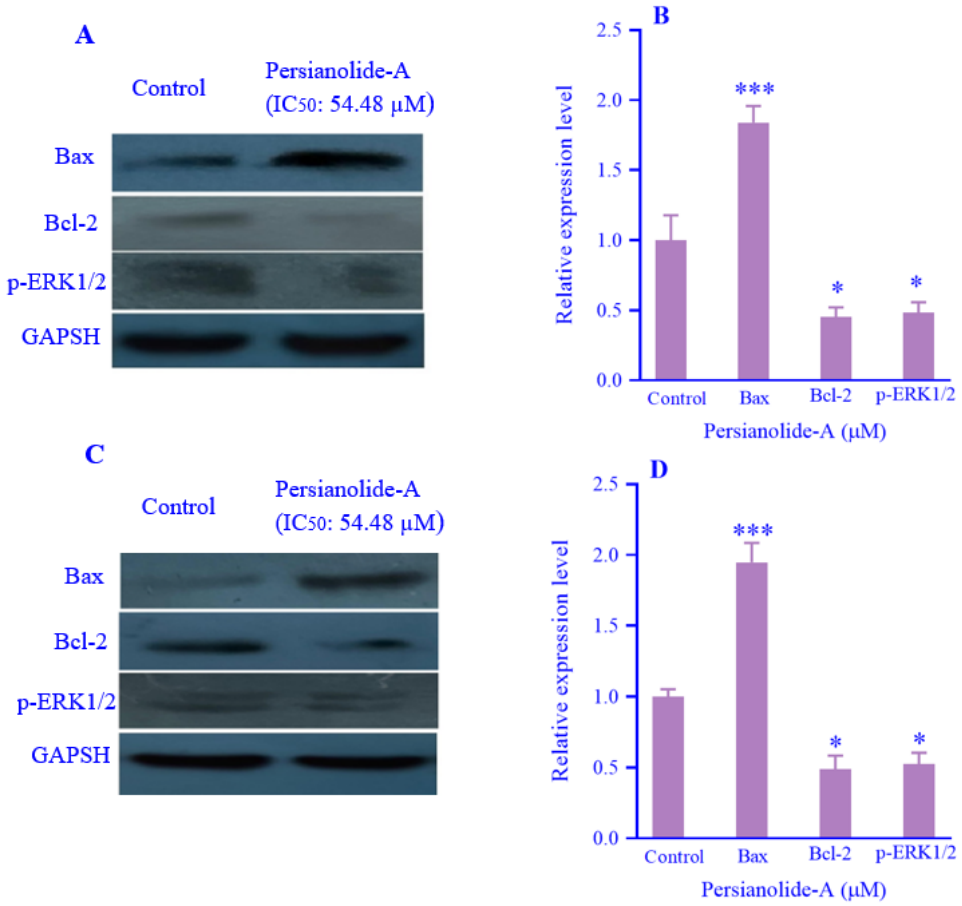

Fig. 5. The effect of persianolide-A on the expression of the proteins Bax, Bcl-2, and p-ERK1/2. (A and B) MDA-MB-231 and (C and D) MCF-7 cells underwent treatment with varying concentrations of persianolide-A for 48 h, following which the protein expressions were evaluated through western blot analysis. Band intensities for Bcl_2, Bax, and ERK1/2 in (B) MDA-MB-231 and (D) MCF-7 were quantified using ImageJ software. To ensure sample equivalency, the loading consistency was verified through comparison with the GAPDH band in untreated cells. Values represent the mean ± SD, n = 3. *$P < 0.05$ and ***$P < 0.001$ indicate significant differences in comparison with the control group.

Involvement of ERK1/2 in persianolide-A-induced apoptosis

ERK1/2 path is one of the most critical pathways that contribute to the growth and sustenance of breast cancer cells (24). Therefore, we explored whether persianolide-A influences the ERK1/2 signaling pathway in breast cancer cells. Our results indicated that persianolide-A reduced levels of pERK1/2 in both MDA MB-231(Fig. 5 A and B) and MCF-7 (Fig. 5 C and D) cancer cells.

DISCUSSION

Despite progress in diagnosis and treatment, the fatality rate resulting from breast cancer remains high. Therefore, developing new drugs or methods for prevention or treatment are much more desirable (25). Today, active ingredients of natural origin (plant, animal, and inorganic) are isolated or semi-synthesized to discover new drugs due to their abundance, side effects, and drug interactions (26,27). A previously conducted phytochemical investigation of aerial parts of *A. kopetdaghensis* resulted in the isolation and characterization of eudesmane-type STLs including 11α,13-dihydroeudesman-4(15)-en-olide named persianolide-A (22). In our ongoing attempt to discover pharmacologically effective compounds among STLs, persianolide-A was subjected to the MTT assay to evaluate its cytotoxicity against MCF-7 and MDA-MB-231 breast cancer cells.

Furthermore, related factors associated with the sequence of apoptosis such as Bcl-2, Bax, and p-ERK1/2 expression were tested by western blotting. The results of our study unveiled a significant decrease in cell growth, which was concentration-dependent, caused by persianolide-A in MDA-MB 231 and MCF-7 cell lines. The IC_{50} values for these cell lines were 54.48 µM and 34.76 µM, respectively. These results align with prior research efforts. Roy *et al*. previously highlighted that costunolide, an STL extract, achieves 50% hindrance of cell growth in MDA-MB-231 and MCF-7 cell lines at a concentration of 40 µM (25). Furthermore, the study by Liu and colleagues revealed that cacalol as an extraordinary STL compound sourced from the exquisite *Cacalia delphiniifolia* is a potent inhibitor of cell proliferation, effectively impeding the growth of breast cancer cell lines (28). Furthermore, deoxyelephantopin and isodeoxyelephantopin have demonstrated anticancer properties in breast cancer cell lines (29). Other studies confirmed alantolactone (27), gaillardin (13), and britannin (19) as inhibitors of MCF-7 cell growth at concentrations of 35.45, 4.93, and 9.56 µM, respectively. Numerous preceding studies also indicated anti-proliferative effects of the STL family, including gaillardin (13), britannin (19), and eupalinolide O (28); concerning the MDA-MB-468 breast cancer cell line, the IC_{50} values were measured 5.54 µM, 6.81 µM, and 1.04 µM, sequentially.

The process of apoptosis entails distinct morphological and biochemical alterations within cells, setting it apart from other forms of cell death. An essential trait signifying apoptotic cell demise is the translocation of phosphatidylserine from the inner cell membrane layer to the outer layer (30). This translocation is detected through annexin/PI dye binding to the negatively charged phosphatidylserines, enabling the quantification of apoptotic cells within sample (31). Our findings indicated that the application of persianolide-A leads to a notable decrease in the ratio of viable cells and an elevation in the initiation of apoptosis. This result showed that persianolide-A significantly initiates cell demise through the apoptotic pathway. This observation aligns with prior studies that demonstrated the strong ability of britannin and gaillardin to the objective is to trigger apoptosis in the breast cancer cell lines, MDA-MB-468 and MCF-7 (13,19). The results indicated that persianolide-A, derived from *A. kopetdaghensis*, inhibited the growth of MDA-MB-231 and MCF-7 cells by inducing apoptosis. To better understand the process of cell apoptosis, we focused on examining the impact of the Bcl-2 family on triggering the apoptotic pathway (32,33). The results revealed that the expression of Bcl-2 as an anti-apoptotic agent was down-regulated; in contrast, the expression of Bax as a pro-apoptotic agent was up-regulated at the same concentrations. Accordingly, in the current study, persianolide-A appeared to function by upregulating the Bax/Bcl-2 ratio in both MDA-MB-468 and MCF-7 cancer cells. The capability of STLs to apoptosis through Bax/Bcl-2 pathways in breast cancer cells has already been widely documented. Studies have demonstrated that STLs including ambrosin, gaillardin, and britannin reduce the Bcl-2 expression while elevating the Bax protein levels, consequently promoting apoptosis in MDA-MB-231 cells (14,23,34). On the other hand, caspases, a class of proteases, with their cleavage and subsequent activation play a crucial role as apoptosis agents (28). To investigate the involvement of caspase family activation in the apoptosis initiated by persianolide-A, the activity of caspases-3/7 was evaluated. The results indicated that treatment of MCF-7 and MDA-MB-231 cells by persianolide-A significantly increases the activity of the caspase-3/7 enzyme. Moreover, persianolide-A triggered apoptosis *via* attenuated survival ERK signaling. The graphical representation (Fig. 6) depicts the hypothesized visual pathway for persianolide A-induced regulated cell death.

Yeo *et al*. showed that the STL β-bisabolene isolated from *Commiphora guidottii*, increases the activity of both caspase-3 and caspase-7 to induce apoptosis in MCF-7 and MDA-MB-231 cancer cells (35).

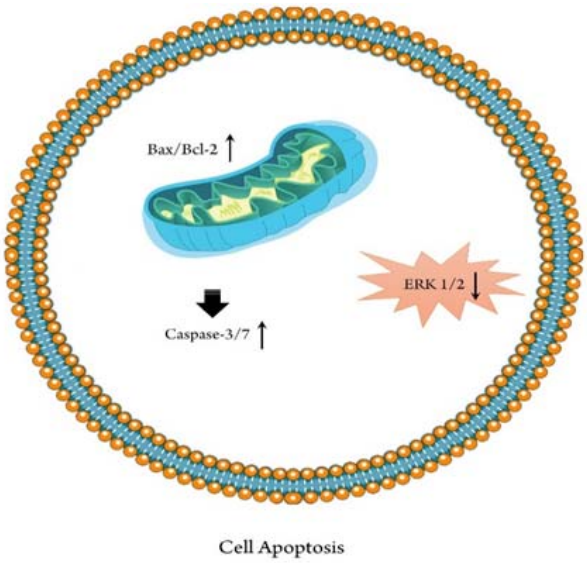

Fig. 6. The graphical representation depicts the hypothesized visual pathway for Persianolide A-induced regulated cell death via intrinsic pathways and attenuated ERK signaling.

ERK is a considerable signaling pathway governing cellular growth, differentiation, and apoptotic processes (36). The results indicated that persianolide-A inhibits the expression of p-ERK protein in MCF-7 and MDA-MB-231 cell lines. In a prior study, the STL dihydroartemisinin, derived from the STL artemisinin, exerts anti-tumor and anti-angiogenic effects by inhibiting the ERK proteins in MDA-MB-231 cells (33). Similarly, the STL costunolide, known for its potential anticancer effects, significantly reduces the p-ERK expression in A431 cells (37).

CONCLUSION

The present study demonstrated that persianolide-A, extracted from *A. kopetdaghensis*, has anticancer effects in human breast cancer cells. The molecular mechanisms could be implicated in the modulation of the ERK1/2 signaling pathway. However, more research is required to determine the molecular mechanism of action of persianolide-A in cancer.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contributions

S.M. Ebrahimi performed the experimental tests and wrote the manuscript; J. Asadi organized the methods; M. Fattahian contributed to the isolation and identification of persianolide-A; S.M. Jafari and M. Ghanadian designed the study. The finalized article was read and approved by all authors.

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