

Cytotoxic effect of *Drimia maritima* bulb extract and induction of mitochondrial apoptotic signaling in human breast cancer cells, MCF-7 and MDA-MB-468

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Background: *Drimia maritima* (*D. maritima*) is a plant belonging to the family Asparagaceae, which has been used for the treatment of several ailments including cancer around the world. To our knowledge, there is no comprehensive study about the molecular mechanisms of anticancer activity of this plant, yet.

Materials and methods: In the current study, cell viability, apoptosis induction, ROS production, mitochondrial apoptotic pathway, and ER stress mediators have been evaluated in breast cancer cells, MCF7, and MDA-MB-468 treated with *D. maritima*.

Results: Significant cytotoxic effects were observed in MCF-7 and MDA-MB-468 cells after exposure to *D. maritima*. Apoptosis induction was determined using Annexin-V-FITC and propidium iodide staining. Furthermore, an increase of ROS, loss of mitochondrial membrane potential, the release of cytochrome c, activation of caspases, and elevation in the Bax/Bcl-2 ratio was determined. *D. maritima* dose-dependently increased the mRNA expression of ER stress markers such as *CHOP*, *ATF-4*, *GADD34*, and *TRIB3* in MCF-7, and MDA-MB-468 cells.

Conclusion: These data suggest that *D. maritima* induces apoptosis in human breast cancer cells via the mitochondrial-mediated pathway. In addition, endoplasmic reticulum stress seems to be involved in *D. maritima*-induced cell death.

Keywords: *Drimia maritima*, ROS, apoptosis, mitochondria, breast cancer, ER stress

Introduction

Breast cancer is one of the most common cancer in females. Despite advances in medical treatments and appropriate therapies, the mortality in this malignancy is still high.¹ One of the major concerns in conventional therapeutic approaches is the risk of undesired and severe side effects arising from the targeting of both normal and cancer cells.² Natural products with anticancer therapeutic potential have gained great attention due to their favorable safety and efficacy profiles. The discovery of plant-derived substances with therapeutic potential usually starts from a pharmacological investigation of plant extracts as the source of secondary metabolites.³

The Endoplasmic Reticulum (ER) serves many functions including protein synthesis and folding. A great variety of stress signals such as hypoxia, oxidative stress, and Ca²⁺ overload can cause disturbance of ER homeostasis and subsequent induction of unfolded protein response (UPR). The UPR is an adaptive stress response pathway that is mediated by activation of three transmembrane proteins: inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). However,

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under certain circumstances that UPR fails to restore ER homeostasis, apoptotic signaling pathways will be triggered via several different mechanisms.⁴⁻⁶ The process of programmed cell death, or apoptosis, is the most potent defense in multicellular organisms against cancer; therefore, finding natural agents targeting signaling apoptosis pathways may be an effective method for cancer therapy and prevention.

Drimia maritima (*D. maritima*), plant belonging to the family Asparagaceae have been applied from the ancient time for the treatment of different diseases including cough and respiratory ailments, cardiac failure, jaundice, skin problems, and gastric disorders.⁷ Furthermore, *D. maritima* has been used in Iranian Traditional Medicine (ITM) considering cancer and cancer-related disorders.⁸ Phytochemical analysis has revealed the presence of cardiac glycosides, phenolic compounds, phytosterols, and other phytochemical constituents in this plants.⁷⁻⁹ A literature survey indicates that no comprehensive studies on the anticancer activities of *D. maritima* have been provided yet. Accordingly, the present study attempts to evaluate the anticancer properties of *D. maritima* and its underlying molecular mechanisms in two human breast cancer cells, MCF-7 and MDA-MB-468.

Materials and methods

Reagents and chemicals

RPMI 1640, Fetal Bovine Serum (FBS), Trypsin-EDTA, Penicillin-Streptomycin, and MTT were obtained from Thermo Fisher Scientific (Waltham, MA, USA). AnnexinV-FITC apoptosis detection kit and Caspase-6 and -9 colorimetric assay kits were bought from Biovision (Mountain View, CA, USA). Fluorescent Reactive Oxygen Species detection kit was obtained from Marker Gene Technologies (Eugene, OR, USA). The antibodies against Bax, Bcl-2, and cytochrome c were bought from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). JC-1 was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Plant material and preparation of the extract

D. maritima bulbs were collected from Kohgiluyeh va Boyer Ahmad Province, Iran (2015). The scientific name was authenticated by Dr Hamid Moazzeni Zehan, Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen (TMRC 3722) was kept for future reference. The quality control assessment of *D. maritima* was conducted according to British Pharmacopoeia in triplicate and acid-insoluble ash and ethanol value were examined.¹⁰

For preparing the methanol extract, 10 mg of powdered shade-dried *D. maritima* bulbs were macerated with methanol (1:10) three times. The solvent was refreshed every 24 hours and the filtrates were combined and evaporated to dryness under reduced pressure in a rotatory evaporator. The extract was then dissolved in dimethyl sulfoxide (DMSO) (Sigma), sterilized by filtration, and subsequently diluted to appropriate working concentration. The solvent was added to the control cultures in all experiments. The final concentration of DMSO was not more than 0.1%.

Cell lines and culture condition

The breast cancer cell lines, MCF-7, MB-MDA-468, and normal fibroblast cell line AGO1522 were purchased from National Cell Bank of Iran (NCBI). The cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin, and incubated at 37°C, 5% CO₂, and 95% humidity.

Evaluation of cell viability by MTT assay

Cytotoxicity of *D. maritima* was determined by the MTT assay, as described previously.¹¹ Cells were seeded in a 96-well plate at a concentration of 5×10³ cells/well and incubated at 37°C overnight. Afterward, cells were treated with *D. maritima* methanolic extract (0.1–500 µg/mL). After 48 hours, 20 µL of 5 mg/mL MTT solution was added to each well and further incubated for 4 hours. Thereafter, the supernatant was gently replaced by 200 µL DMSO and the absorbance values were measured at 570 nm using a microplate reader.

Apoptosis assay by flow cytometry

Apoptosis could be detected by staining the cells with Annexin V-FITC and Propidium iodide (PI) solution followed by flow cytometry analysis.¹¹ In brief, cells were seeded to a density of 5×10⁵ in a six-well plate and treated with *D. maritima* (10, 100, and 500 µg/mL) for 48 hours. Then, cells were washed with cold PBS and re-suspended in the 1x binding buffer containing Annexin V-FITC and PI solution. The stained cells were examined by FACS Calibur flow cytometry (BD Biosciences, San Jose, CA, USA).

Quantitative real-time RT-PCR

The total RNA of the MCF-7 and MDA-MB-468 cells were extracted using Trizol reagent (Thermo Fisher Scientific) and then reverse transcribed into first-strand cDNA using Revert Aid M-MuLV Reverse Transcriptase (Fermentas, Germany). Real-time PCR (qPCR) of cDNA was performed using

Applied Biosystems instrument (ABI 7500 Real-Time PCR System, USA). Relative expression levels of genes were normalized to GAPDH and relative quantification values were determined using the comparative $2^{-\Delta\Delta Ct}$ analysis method.¹² Quantitative RT-PCR was performed using specific primers, which are listed in Table 1.¹²

Western blot analysis

After treatment with *D. maritima* extract, cells were washed with cold PBS and lysed with an appropriate lysis buffer, RIPA (20 mM Tris-HCl, 0.5% Nonidet P-40, 0.5 mM PMSF, 100 mM b-glycerol 3-phosphate, and 0.5% protease inhibitor cocktail). The protein concentration was determined using the Bradford protein assay. Then, SDS-denatured samples were separated on SDS-polyacrylamide gels and then transferred to a PVDF membrane. The membrane was incubated with PBST solution (5% non-fat dry milk in PBS containing 0.1% Tween-20) and then incubation with the monoclonal antibodies against Bax, Bcl-2 and cytochrome c was performed, overnight. After incubation with corresponding secondary antibodies, detection was carried out using Enhanced Chemiluminescence (ECL).¹¹

Caspase activity assay

Colorimetric assay kits were used to detect the activities of caspase-6 and -9 in the MCF-7 and MDA-MB-468 cells.¹³ The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (p-NA) after cleavage from the labeled substrate (LEHD-pNA for caspase-9 and VEID-pNA for caspase-6). In brief, after centrifugation of cell lysates, the supernatant was added to a supplied reaction buffer containing dithiothreitol, and LEHD-pNA and VEID-pNA as substrates and incubated for 1 hour in the dark. The amount of p-NA released was measured at 405 nm using a Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

ROS assay

The MarkerGene™ Live Cell Fluorescent ROS Detection Kit (Marker Gene Technologies) was used to measure ROS generation in MCF-7 and MDA-MB-468 exposed to *D. maritima*.¹⁴

This kit uses a cell-permeable substrate (DCFH-DA) as a fluorogenic marker for ROS detection. Briefly, after incubation with DCFH-DA, cells were harvested and washed with HBSS and analyzed by a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$) using JC-1

Lipophilic cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was used to evaluate $\Delta\Psi_m$.¹¹ JC-1, in healthy cells with high mitochondrial $\Delta\Psi_m$, can enter into mitochondria and form complexes emitting red fluorescence. During the loss of $\Delta\Psi_m$ in apoptotic cells, JC-1 remains in monomeric form with green fluorescence emission. The ratio of red to green fluorescence provides a measure of $\Delta\Psi_m$. Briefly, after treatment with *D. maritima*, the culture medium of the cells was replaced with HEPES buffer containing JC-1 and after 30 minutes, the Red/green fluorescence was measured using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments).

Statistical analysis

Data were analyzed by ANOVA with Dunnett's post-hoc test using SPSS. The IC50 value was determined using GraphPad Prism5 software. Differences were considered statistically significant at $P < 0.05$.

Results

Quality control of the plant material

The quality of the plant material was determined according to British Pharmacopoeia, which demonstrated that *D. maritima* was of good quality and the results were within acceptable limits (Table 2).

D. maritima inhibited cell viability in breast cancer cells

We first characterized the cytotoxic effect of *D. maritima* on MCF-7 and MDA-MB-468 cells using MTT assay.

Table 1 Primer sequences used for quantitative RT-PCR

Gene	Forward primer	Reverse primer
CHOP	CCAGGAAACGAAGAGGAAGA	TCTGACTGGAATCTGGAGAGC
ATF4	ATGGGTTCTCCAGCGACA	GAAAAGGCATCCTCCTTGC
GADD34	CAGAAAGGTGCGCTTCTCC	AAGGCAAGGTCTGGGTGA
TRIB3	GGACATGCACAGCCTGGT	GCTTCTTCTCTCACGGTC
GAPDH	GTCGGTGTGAACGGATTTG	AGGTCAATGAAGGGTTCGT

Table 2 Results of quality control assessment of *Drimia maritima*

	Acid-insoluble ash (%)	Ethanol extractive value (%)
<i>Drimia maritima</i> (present study)	1.29±0.04	74.6±3.4
British Pharmacopoeia limit	1.5<	>68

Note: Data are presented as mean ± SD.

D. maritima significantly inhibited the viability of both MCF-7 and MDA-MB-468 cells in a dose-dependent manner (Figure 1). The most inhibitory effect of *D. maritima* were about 80% and 76% in MCF-7 and MDA-MB-468 cells, respectively, after treatment with 500 µg/mL.

The effective doses of *D. maritima* that inhibited 50% of growth (IC₅₀) of MCF-7 and MDA-MB-468 cells were 20.48±1.17 µg/mL and 25.74±2.05 µg/mL, respectively. Interestingly, the data reveal that *D. maritima* display significantly lower cytotoxicity against AGO1522, a normal human fibroblast cell, with the IC₅₀ values of 43.5±1.73 µg/mL (Figure 1).

D. maritima induced apoptosis cell death in breast cancer cells

To explore whether the *D. maritima* anti-proliferative activity is related to the apoptosis induction, we used flow cytometry to measure the apoptosis rate. Following 48 hours of treatment with *D. maritima* (10, 100, and 500 µg/mL), cells were stained with Annexin V-FITC and PI and then analyzed by flow cytometry. The data of this experiment indicated that the rate of apoptosis (as the sum of early apoptotic cells and late apoptotic cells) increased in MCF-7 and MDA-MB-468 cells to 75.8% and 81.9%, respectively, after treatment with 500 µg/mL *D. maritima* (Figure 2A and B).

D. maritima induced ROS generation in breast cancer cells

Molecules of ROS in low doses are involved in cell cycle progression and proliferation of cancer cells. However, an increase in intracellular ROS can inhibit cancer cell growth and induce apoptosis through oxidative stress-mediated pathways.¹⁵ Our results showed that exposure to *D. maritima* significantly increased the intracellular ROS levels in MCF-7 and MDA-MB-468 cells in a dose-dependent manner (Figure 3).

Mitochondria is involved in the *D. maritima*-mediated apoptosis in breast cancer cells

Mitochondrial dysfunction and subsequently decrease in the level of $\Delta\Psi_m$ is considered as a central event in apoptosis.¹⁶ In order to determine the contribution of mitochondria in the apoptosis induction by *D. maritima*, cells were treated with different concentrations of *D. maritima* (1, 10, 100, and 500 µg/mL) for 48 hours and then incubated with JC-1 dye. Based on our results, as indicated in Figure 4A, *D. maritima* caused loss of $\Delta\Psi_m$ in a dose-dependent manner in MCF-7 and MDA-MB-468 cells.

Bcl-2 family proteins have been considered as a main regulator of the mitochondrial apoptotic pathway. The Bcl-2-related proteins either inhibit (particularly Bcl-2) or promote

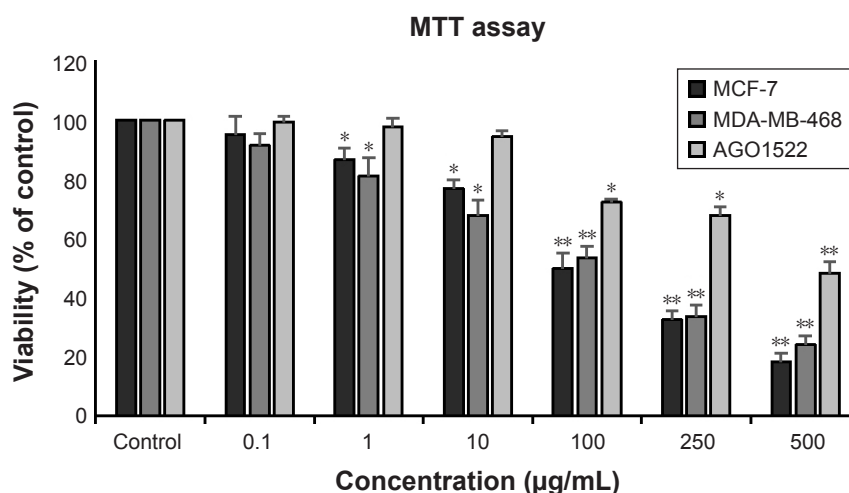


Figure 1 Cytotoxic effect of *D. maritima* against MCF-7 and MDA-MB-468 breast cancer cells, and normal fibroblast cell AGO1522.

Notes: Cells were treated with different concentrations of *D. maritima* (0.1, 1, 10, 100, 250, and 500 µg/mL) and detected by MTT assay after 48 hours. Each value is presented as mean ± SD of three experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated control group.

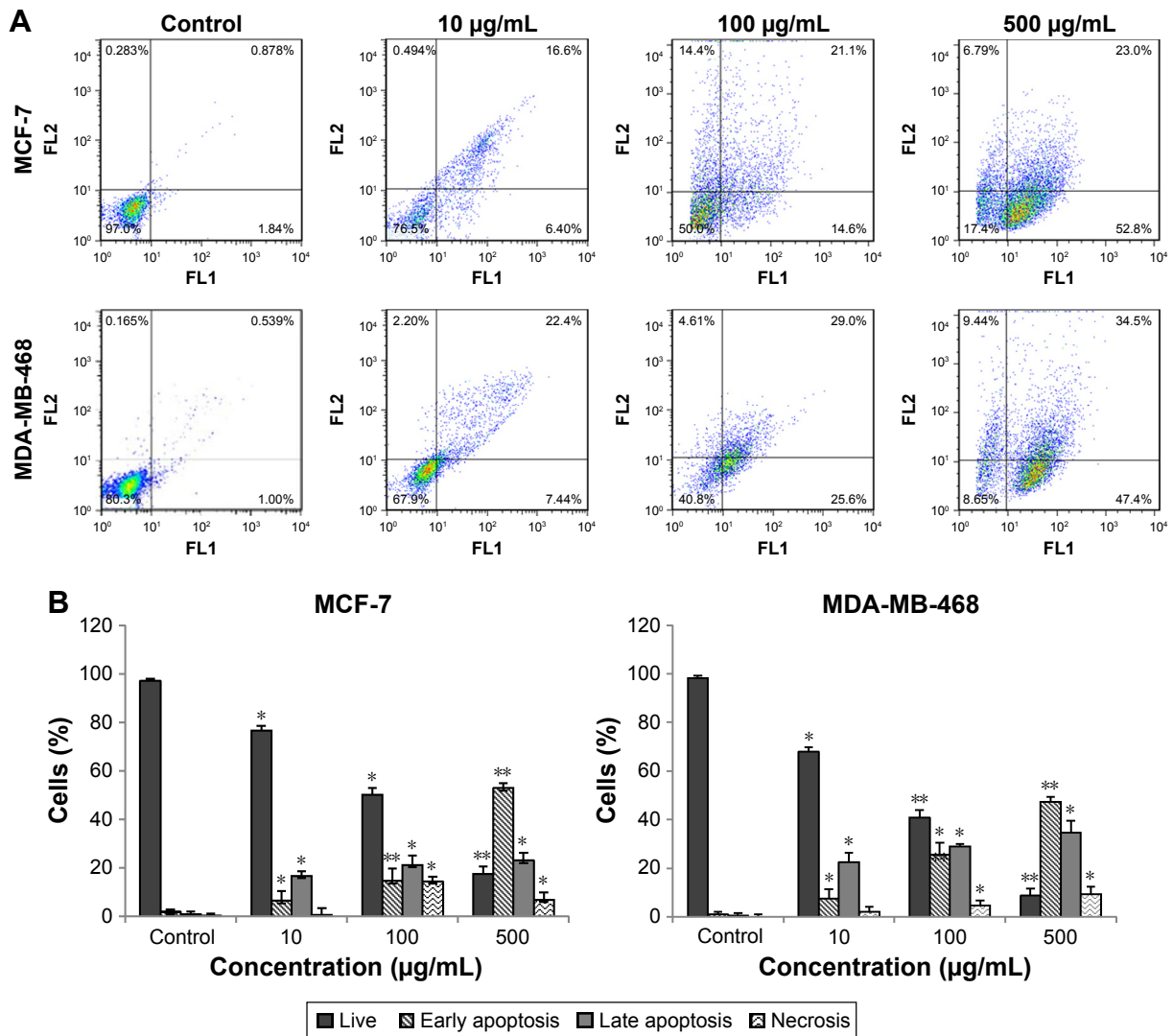


Figure 2 Detection of apoptosis using Annexin V-FITC/PI staining and flow cytometry in MCF-7 and MDA-MB-468 cells after 48 hours treatment with indicated doses of *D. maritima* (A, B). **Note:** Significant differences between control group vs each treated cell line are indicated by * $P < 0.05$ and ** $P < 0.01$.

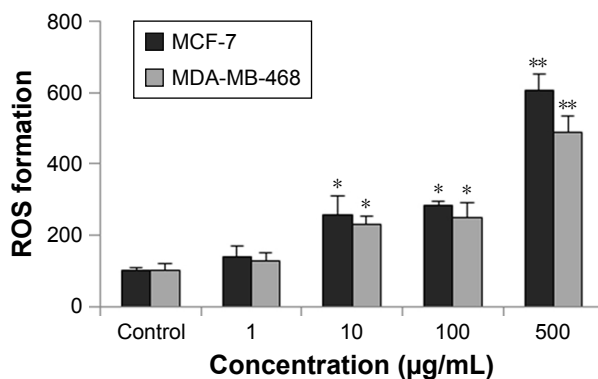


Figure 3 Effects of *D. maritima* on reactive oxygen species (ROS) generation in MCF-7 and MDA-MB-468 cell lines. **Notes:** After treatment with different concentrations of *D. maritima* for 48 hours, cells were loaded with 2',7'-dichlorofluorescein diacetate and fluorescence was measured by Microplate Reader. Results (mean \pm SD) were calculated as a percent of corresponding control values. * $P < 0.05$; ** $P < 0.01$ are significant.

apoptosis (particularly Bax), and the balance between these proteins determines the cellular fate.¹⁷ As shown in Figure 4B and C, expression of proapoptotic protein Bax was significantly increased, whereas the antiapoptotic Bcl-2 protein was decreased after treatment with *D. maritima*. It has been suggested that the increased Bax/Bcl-2 ratio can be recognized as a key factor for the apoptotic process by regulating the release of cytochrome c from mitochondria and activation of the intrinsic apoptotic pathway.¹⁸

Consistently, our results indicated that the treatment with *D. maritima* promoted the release of cytochrome c into the cytosol (Figure 4B and C). These results, together, suggest that *D. maritima* triggers mitochondria-mediated apoptotic pathway through the increase in Bax/Bcl-2 ratio

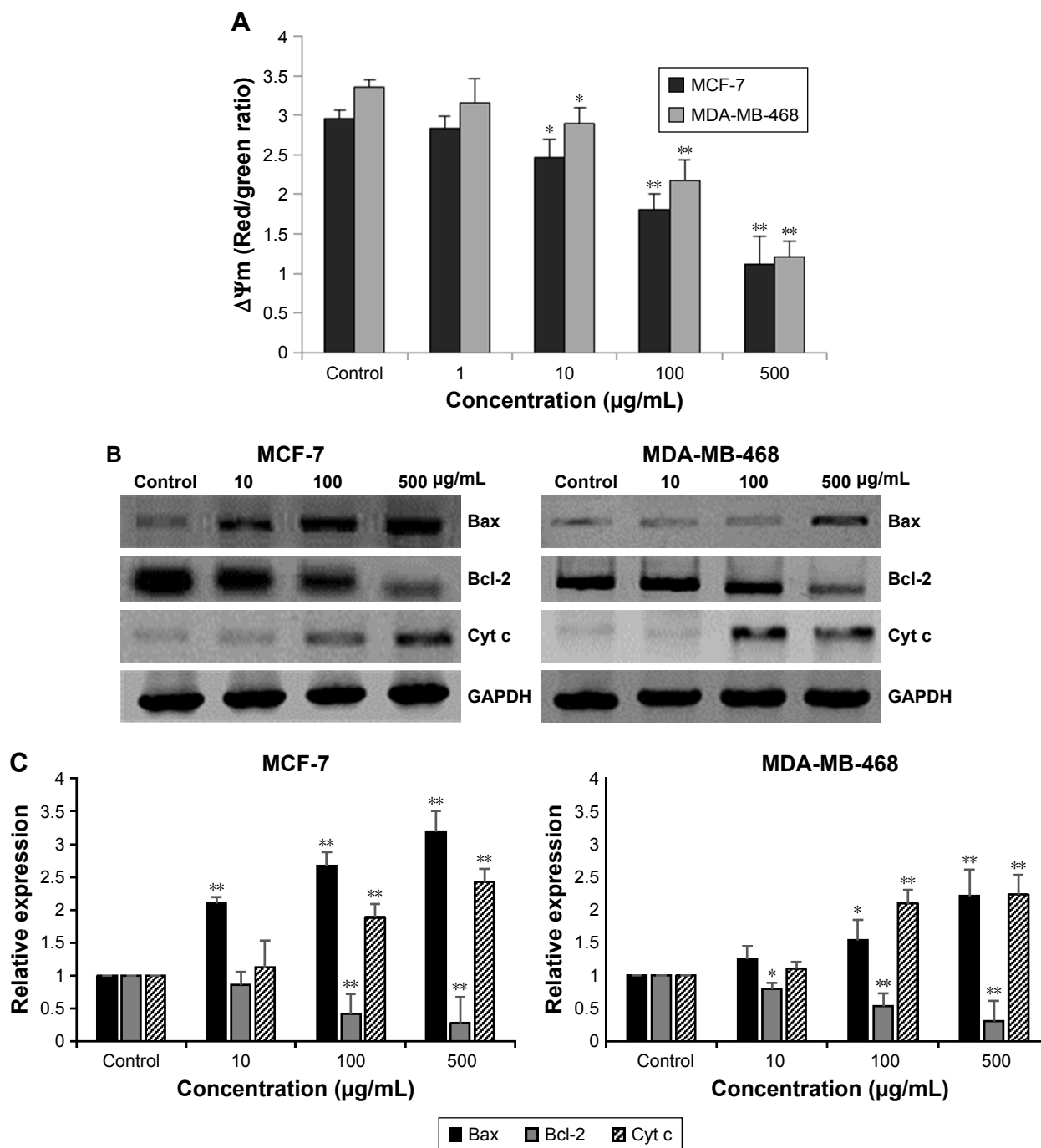


Figure 4 The role of mitochondria in the induced apoptosis by *D. maritima*.

Notes: The effect of *D. maritima* on mitochondrial transmembrane potential ($\Delta\Psi_m$) in MCF-7 and MDA-MB-468 cells (A), after 48 hours treatment with *D. maritima*. The expression of apoptosis-related proteins in MCF-7 and MDA-MB-468 cells (B), after treatment with *D. maritima* for 48 hours. Protein quantification was carried out using ImageJ software (C). Protein levels were normalized to the GAPDH level and are shown relative to the untreated control cells. Data were obtained from at least three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs control.

and subsequent disturbance of mitochondrial outer membrane permeabilization (MOMP).

Involvement of caspase-6 and -9 in *D. maritima*-induced apoptosis

During the mitochondrial apoptotic pathway, the release of cytochrome c from mitochondria leads to activation of

Apaf-1, which then cleaves procaspase-9 into the active form. Active caspase-9, as an initiating caspase, cleaves, and activates downstream executioner caspases such as caspase-3, -6, and -7. As shown in Figure 5A and B, *D. maritima* increased significantly the activities of caspase-6 and -9 in a dose-dependent manner in MCF-7 and MDA-MB-468 cells. MCF-7 cells do not express the caspase-3 protein due to a

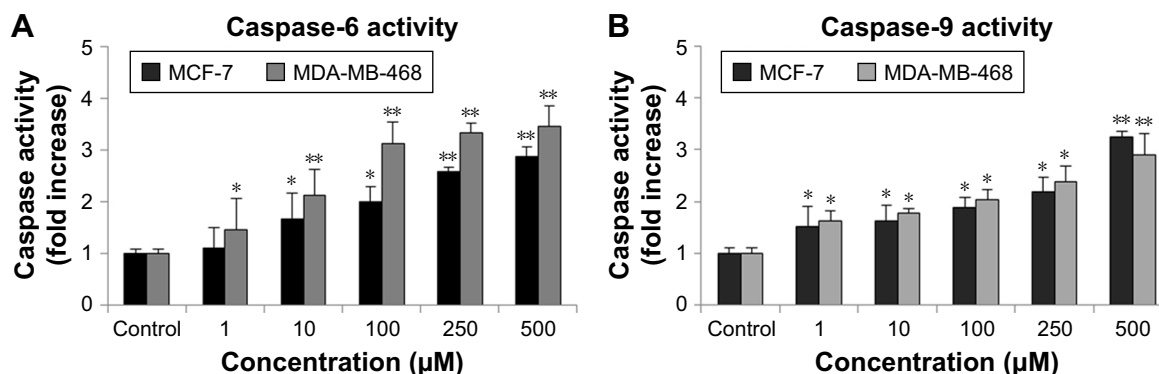


Figure 5 Colorimetric assay of caspase-6 (A) and -9 (B) activation after 48 hours of treatment with various concentrations of *D. maritima* in MCF-7 and MDA-MB-468 cells. * $P < 0.05$ and ** $P < 0.01$ compared with the untreated control group.

47-base-pair deletion within exon 3 of the *CASP-3* gene,¹⁹ and so we decided to measure the activity of caspase-6, as an executioner caspase in MCF-7 and MDA-MB-468 cells.

D. maritima seem to induce ER stress signaling pathway

To further elucidate the molecular mechanisms underlying *D. maritima*-induced apoptosis, we investigated the effects of *D. maritima* on UPR genes. The mRNA expression levels of ER stress-associated genes such as *ATF4* (Activating Transcription Factor 4), *CHOP* (C/EBP Homologous Protein), *GADD34* (Growth Arrest and DNA damage-inducible 34), and *TRIB3* (tribbles-related protein 3) were evaluated by real-time PCR. *ATF4* is a transcription factor regulating a variety of responses including antioxidant gene expression, amino acid synthesizing enzymes, and proapoptotic machinery.²⁰ *CHOP*, whose induction strongly depends on *ATF4*, is considered as a central molecule in ER stress-induced apoptosis. Studies show that numerous target genes including *GADD34* and *TRIB3* are contributed in *CHOP*-mediated apoptosis.^{21,22}

As shown in Figure 6A and B, after treatment with *D. maritima*, mRNA expression of *CHOP*, *ATF-4*, *GADD34*, and *TRIB3* genes increased in a dose-dependent manner compared to untreated cells. In MCF-7 cells, after exposure to *D. maritima* 100 µg/mL, the expression levels of *CHOP*, *ATF-4*, *GADD34*, and *TRIB3* genes showed an increase of 41 ± 1.25 , 12.88 ± 3.65 , 7.9 ± 1.9 , and 13.4 ± 5.15 , respectively. *CHOP*, *ATF-4*, *GADD34*, and *TRIB3* genes showed an increase of 35.41 ± 4.12 , 16.5 ± 2.39 , 11.35 ± 2.01 , and 11.33 ± 6.15 , respectively, in MDA-MB-468 cells. These results suggest that UPR activation in response to *D. maritima* treatment could promote apoptosis through the *CHOP*-*ATF-4* pathway in breast cancer cells.

Discussion

Targeting apoptotic death pathways has been considered as the most effective therapeutic strategy in all cancer types. In the current study, experiments using MTT assay revealed that methanolic extract of *D. maritima*, a traditionally used medicinal plant, exerts anti-proliferative activity against MCF-7

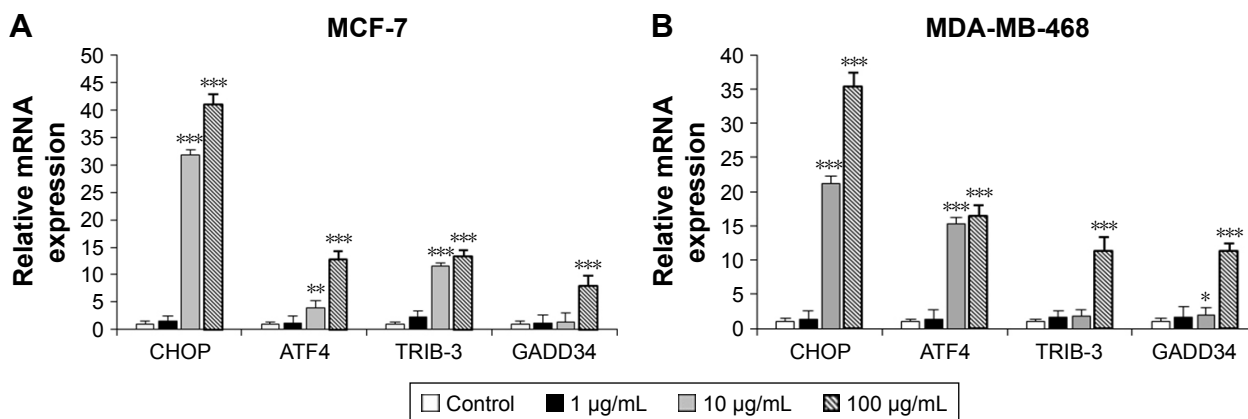


Figure 6 Real-time PCR analysis of mRNA expression levels of *CHOP*, *ATF-4*, *TRIB-3*, and *GADD34* in MCF-7 (A) and MDA-MB-468 (B) cells after treatment with 1, 10, and 100 µg/mL of *D. maritima* for 48 hours.

Notes: *GAPDH* was used as an internal control. All data were expressed as mean \pm SD of three experiments (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs untreated control group).

and MDA-MB-468 cells. Moreover, *D. maritima*-treated cells revealed a dose-dependent increase in the percentage of apoptotic cells. To investigate apoptosis induction, double staining with Annexin V-FITC and PI to measure membrane phosphatidylserine (PS) exposure was carried out. Exposure of PS on the external side of the plasma membrane is the key feature of the early apoptosis. Annexin V commonly is used to detect apoptotic cells because of its high binding affinity to PS. PI, a red fluorescent intercalating dye, was employed as a DNA stain to evaluate dead cells.

An active role for mitochondria in intrinsic apoptosis signaling pathway is well established. Several mitochondrial proteins, residing in the intermembrane space of mitochondria, in response to the variety of stimuli are released into the cytosol and activate apoptotic pathways.²³ The Bcl-2 family proteins have a key role in regulating the MOMP and release of mitochondrial proteins into the cytosol. It was shown that the increased Bax to Bcl-2 ratio could disrupt MOMP that could lead to decreased $\Delta\Psi_m$ and release of apoptogenic proteins such as cytochrome c into the cytosol, where it triggered the execution of apoptosis by inducing caspases activation.^{23,24} Consistently, our results show that after treatment with *D. maritima*, the expression of Bax was significantly increased, whereas the Bcl-2 expression was downregulated. Furthermore, *D. maritima* treatment decreased $\Delta\Psi_m$ and triggered the release of cytochrome c into the cytosol. The release of cytochrome c through its effects on Apaf-1 leads to the activation of initiator caspase-9. Active caspase-9 triggers the cleavage and subsequent activation of downstream executioner caspases such as caspase-3, -6, and -7.²⁵ Our findings have shown a clear increase in the caspase-9 and -6 activity in MCF-7 and MDA-MB-468 cells after exposure to *D. maritima*. Therefore, we speculated that *D. maritima* could induce the mitochondrial pathway through the increase of Bax to Bcl-2 ratio, the release of cytochrome c, and caspase cascade activation.

Many anticancer agents exert their inhibitory effects through the generation of ROS and promotion of oxidative stress-induced cancer cell death.²⁶ In our study, we found that the levels of intracellular ROS to be significantly elevated by *D. maritima* treatment in MCF-7 and MDA-MB-468 cells. Increased ROS levels also can induce ER stress response, which in the case of prolonged ER stress could initiate apoptotic death signals.²⁷ During ER stress, the role of PERK-eIF2 α -ATF4 branch of the UPR is essential in the upregulation of *CHOP*, one of the main molecule involved in the ER stress-mediated apoptosis. Numerous target genes including *GADD34*, *TRIB3*, and *BCL-2* have been identified in the *CHOP*-induced apoptotic pathway.⁶ *GADD34* promotes

dephosphorylation of eIF2 α which leads to the translational suppression and subsequent induction of apoptosis pathway.^{28,29} It has also been demonstrated that up-regulation of *TRIB3*, another target gene induced by *CHOP*, has a critical role in ER stress-induced cell death via the ATF4-*CHOP* pathway.³⁰ It has been suggested that the most characterized mechanism of cell death induced by *CHOP* is the downregulation of Bcl-2 and upregulation of Bax proteins.³¹ In the present study, the effect of *D. maritima* on ER stress was investigated by focusing on the ATF4-*CHOP* pathway. We found that *D. maritima* dose-dependently increased the mRNA expression of *ATF-4*, *CHOP*, *GADD34*, and *TRIB3* in MCF-7 and MDA-MB-468 cells. In addition, as mentioned earlier, the Western blot analysis indicated Bcl-2 upregulation and Bax downregulation in cells treated with *D. maritima*.

Conclusion

Collectively, our results support the hypothesis that the mitochondrial pathway and ER stress are involved in apoptosis induction by *D. maritima* in breast cancer cells. To the best of our knowledge, this is the first comprehensive study showing the promising cytotoxic effects of *D. maritima* against breast cancer cells. Consequently, *D. maritima* extract can be evaluated further for potential anticancer properties and isolation of bioactive phytochemicals.

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Author contributions

Dr Hamzeloo-Moghadam contributed to interpretation of data and preparing the manuscript; Dr Aghaei participated in designing and performing experiments; Dr Abdolmohammadi participated in data analysis; Dr Khalaj conducted the preparation of *D. maritima* extracts; Dr Fallahian contributed to analysis and interpretation of data and preparing the manuscript. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflict of interest in this work.

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