

## Mitochondrial and caspase pathways are involved in the induction of apoptosis by nardosinen in MCF-7 breast cancer cell line

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### Abstract

Natural products isolated from plants provide a valuable source for expansion of new anticancer drugs. Nardosinen (4,9-dihydroxy-nardosin-6-en) is a natural sesquiterpene extracted from *Juniperus foetidissima*. Recently, we have reported the cytotoxic effects of nardosinen in various cancer cells. The aim of the current study was to investigate the anticancer features of nardosinen as well as its possible molecular mechanisms of the nardosinen cytotoxic effect on breast tumor cells. MTT assay showed that nardosinen notably inhibited cell proliferation in a dose-dependent manner in MCF-7 breast cancer cells. The growth inhibitory effect of nardosinen was associated with the induction of cell apoptosis, activation of caspase-6, increase of reactive oxygen species (ROS), and loss of mitochondrial membrane potentials ( $\Delta\Psi_m$ ). Western blot assay following treatment with nardosinen showed that the expression levels of the Bax were significantly up-regulated and the expression levels of the Bcl-2 were significantly down-regulated. Our results finally exhibited that nardosinen induces apoptosis in breast cancer cells via the mitochondrial and caspase pathways.

**Keywords:** *Juniperus foetidissima*; Apoptosis; Breast cancer; MCF-7; Sesquiterpene

### INTRODUCTION

Breast cancer is one of the most common cancers and the leading cause of cancer-related death among women (1). About 1,685,210 new breast cancer cases and about 595,690 death from this disease in the United States has been estimated in 2016 (2). To date, there are several conventional methods for breast cancer treatment such as surgery (3), radiotherapy (4), chemotherapy (5) and hormone therapy (6), however, most often they lead to serious side effects (7,8). Recently, attentions are focused on herbal compounds as the natural sources of the anti-cancer drugs (9). *Juniperus foetidissima* is a tree that belongs to the Cupressaceae family (10). To date, antioxidant and antitumorogenic effects of *J. foetidissima* extract have been confirmed by several studies. For instance, Sadeghi-aliabadi, *et al.* indicated that ethanol extracts of the plant have cytotoxic effects on three tumor cell lines

of HeLa, KB, and MDA-MB-468 (11). In addition, Lesjak, *et al.* have shown that methanol extracts and essential oils of the leaves and seed cones of *J. foetidissima* species have antioxidant and anti-inflammatory properties (12). Moreover, previous study has shown the pharmacological potential of sesquiterpens in the induction of apoptosis in tumorigenic cells and cancers. It has been shown that nardosinen markedly suppresses reactive oxygen species (ROS) generation, pro-inflammatory protein production, and cancer cell proliferation accompanied by apoptosis (13). It has been shown that sesquiterpenes induce robust apoptosis in primary human acute myeloid leukemia (AML) cells and blast crisis chronic myeloid leukemia (CML) cells, however, it has no significant effects on normal hematopoietic cells (14,15).

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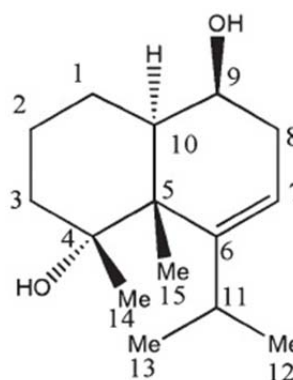
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Moreover, Zhang, *et al.* exhibited that parthenolide which is a sesquiterpene has pivotal roles in reducing of intracellular thiols and calcium causing cell death in human colorectal cancer cells (16). Apoptosis is a programmed cell death and a highly arranged way to a demolition of tumorigenic and unusual cells and plays a basic role in tissue maintenance and homeostasis (17). Proteolytic enzymes such as caspases are important effector molecules in apoptosis that their activation or inactivation is important in the treatment of cancer (18). It has been shown that plant extracts can cause cell death via induction of cell apoptosis through caspase activation (19). A study showed that britannin, a sesquiterpene lactone, suppresses cell growth and induces apoptosis through caspase activation and the mitochondrial pathways in MCF-7 and MDA-MB-231 human breast cancer cells (20). In our previous study, we showed that sesquiterpene nardosinen from *J. foetidissima* has the anti-proliferative effect (21). However, the mechanism and apoptotic pathway of nardosinen in breast cancer cell lines have not been elucidated. This study was performed to examine biochemical pathways inducing apoptosis against human breast cancer in MCF-7 cell line.

## MATERIALS AND METHODS

### Materials

Roswell Park Memorial Institute medium (RPMI-1640), trypsin-EDTA, phosphate-buffered saline (PBS), penicillin, and streptomycin were obtained from Gibco (BRL, Eggenstein, Germany). Caspase-3 and caspase-6 colorimetric assay kits were obtained from BioVision (Milpitas, CA USA). Mouse monoclonal anti-Bcl-2 and anti-Bax antibodies and horseradish peroxidase (HRP)-conjugated anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent ROS detection kit was obtained from Marker Gene Technologies (MGT, Inc, USA). Annexin-V fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-di-phenyltetrazolium-bromide (MTT), dimethyl sulfoxide (DMSO) and other reagents were provided from Sigma-Aldrich (Poole, Dorset, UK).



**Fig. 1.** Chemical structure of 4, 9(α)-dihydroxy-nardosin-6-en.

### Cell culture

The human breast cancer cell lines MCF-7 were supplied by National Cell Bank of Iran (NCBI). The cells were cultured in RPMI-1640, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Cells were incubated at 37° C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### Isolation and purification of nardosinen

Isolation and purification of nardosinen were carried out as described previously (21). Chemical structure of nardosinen has been shown in Fig. 1.

### Cell viability assay with MTT reduction

The effects of different concentration of nardosinen on the living cells were evaluated using the MTT assay. Cell viability was determined using the MTT assay, as described previously (22). Briefly, MCF-7 human breast cancer cells were cultured at  $5 \times 10^3$  cells in each well of a 96-well plate. After overnight incubation to allow cell attachment, the RPMI 1640 in each well was replaced with media containing various concentrations of nardosinen (0, 1, 10, 20, 40, 80 and 100 µM) and incubated for 48 h. At the end of incubation 20 µL of MTT (5 mg/mL in PBS) solution was added to each well and incubation continued further for 4 additional h at 37 °C to evaluate the intensity of cytotoxicity caused by nardosinen. Active mitochondria in live cells reduce MTT to crystalline purple blue formazan. The

supernatants were aspirated carefully, and then 200  $\mu$ L of DMSO was added to each well to dissolve the insoluble formazan. The plates were incubated in dark for an additional 10 min and the absorbance values were read using a Microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Stock solutions of compounds were prepared in dimethyl sulfoxide, and the solvent was added to the control cultures in all experiments. The final concentration of vehicle (dimethyl sulfoxide) was 0.1%. Data were collected from several experiments, and the percentage of cell growth inhibition was determined by comparison with the dimethyl sulfoxide-treated control cells. The number of live cells was measured as a percentage using the formula: (mean OD of treated cells/mean OD of control cells)  $\times$  100

#### ***Quantitative analysis of apoptotic cells by annexin-V/propidium iodide staining***

To determine apoptosis of breast cancer cell lines, we employed annexin-V FITC/PI apoptosis detection kit according to the manufacturer's protocol as described previously (23). Cells were plated at a density of  $3 \times 10^5$  per well in a six-well plate and incubated with or without various concentration of the tested compound (1, 10, 40, 80 and 100  $\mu$ M) for 48 h. The treated and untreated cells were harvested and washed twice with phosphate buffered saline (PBS). The cell pellets were re-suspended in 500  $\mu$ L of binding buffer. 5  $\mu$ L of annexin-V- FITC and 5  $\mu$ L of PI were added to the cell suspension. The staining samples were incubated for 15 min at room temperature in dark condition. Stained samples were analyzed by FACSCalibur flow cytometer (USA). The analysis was performed by Flow Jo software version 7.6.1 (Tristar, CA, USA).

#### ***Western blot analysis***

The Bcl-2 and Bax proteins contents were detected by western blot analysis as described previously (24,25). In brief, cells were lysed 2 h at 4  $^{\circ}$ C in buffer that contains 20 mM hydroxymethyl aminomethane HCL (Tris-HCl) (pH 7.5), 0.5% nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM b-glycerol 3-phosphate, and 0.5%

protease inhibitor cocktail and disrupted by centrifugation at 1400 rpm for 10 min in 4  $^{\circ}$ C. Protein concentration was diagnosed by the Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of proteins (30–50  $\mu$ g) were applied to SDS polyacrylamide gel for electrophoresis and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature then incubated with mouse monoclonal antibodies over night at 4  $^{\circ}$ C. The membranes were washed 3 times with PBS-Tween 0.5% after incubating with required secondary antibodies for 1 h at room temperature and then the membrane was rewashed 3 times. Signals were visualized by enhanced chemiluminescence (ECL) detection reagent (Amersham Corp. Arlington Heights, IL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for normalization.

#### ***Caspase assay***

Assessment of caspase 6 was conducted using the caspase colorimetric assay kit on the basis of manufacturer's instruction. Briefly, cells were cultured overnight and treated with different concentration of nardosinen. Cells collected and lysed in lysis buffer for 10 min on ice, then centrifuged at 10,000 g for 1 min. After centrifugation, the supernatants were incubated with a caspase-3 substrate in reaction buffer. Samples were incubated in 96-well flat bottom micro plates at 37  $^{\circ}$ C for 1 h. The amount of released p nitroaniline (pNA) was measured using a Microplate reader (Bio-Rad Hercules, California, USA) at 405 nm wavelength.

#### ***Mitochondrial membrane potential ( $\Delta\Psi_m$ ) analysis***

Membrane potentials ( $\Delta\Psi_m$ ) is an important parameter of the mitochondrial function and was estimated by using a lipophilic cationic JC-1 probe. JC-1 enters mitochondria and changed its fluorescent properties based on the accumulation of probe.

Mitochondrial depolarization leads to decrease in aggregation of JC-1 and an

increase in green fluorescent JC-1 monomers (26). MCF-7 was cultured in black clear-bottom 96-well plates. After incubation with different concentrations of nardosinen (1, 10, 40, 80 and 100  $\mu\text{M}$ ) for 48 h, cells were loaded with JC-1 by replacing the culture medium with 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) buffer (40 mM, pH 7.4) containing 4.5 g/L glucose (high glucose medium) or 1.5 g/L glucose (low-glucose medium), 0.65% NaCl, and 2.5 mM JC-1 for 30 min at 37 °C, then washed once with HEPES buffer. Fluorescence was determined by using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments) that allows for these sequential measurements of each well at two excitation/emission wavelength pairs, 490/540 and 540/590 nm. Changes in the ratio between the measured red (590 nm) and green (540 nm) fluorescence intensities indicate changes in mitochondrial membrane potential.

#### Measurement of intracellular reactive oxygen species

Intracellular changes in ROS was measured using the Marker Gene live cell fluorescent ROS detection kit. Briefly, cells were harvested to a density of  $25 \times 10^3$  per well in a 96-well plate and treated with different concentrations of nardosinen (1, 10, 40, 80 and 100  $\mu\text{M}$ ) for 48 h. After incubation, cells were further treated with 20  $\mu\text{M}$  2',7'-

dichlorofluorescein diacetate in Hanks' balanced salt solution (HBSS) at 37 °C for 30 min in the dark. Subsequently, cells were harvested and washed with PBS and analyzed for dichlorofluorescein (DCF) fluorescence by a synergy HT Multi-Mode Micro plate Reader (BioTek instruments).

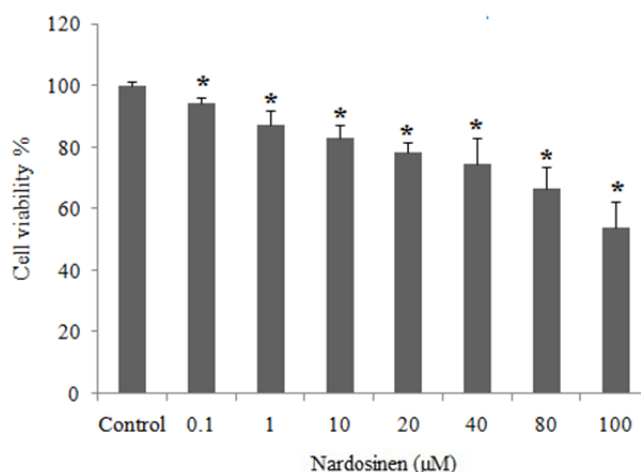
#### Statistical analysis

Non-parametric one-way analysis of variance (ANOVA) with the Dennett's test was used to analyse the data, using software Graph pad Prism. Each experiment was performed in triplicate independently.  $P < 0.05$  was considered as significant difference. Results are expressed as means  $\pm$  SD.

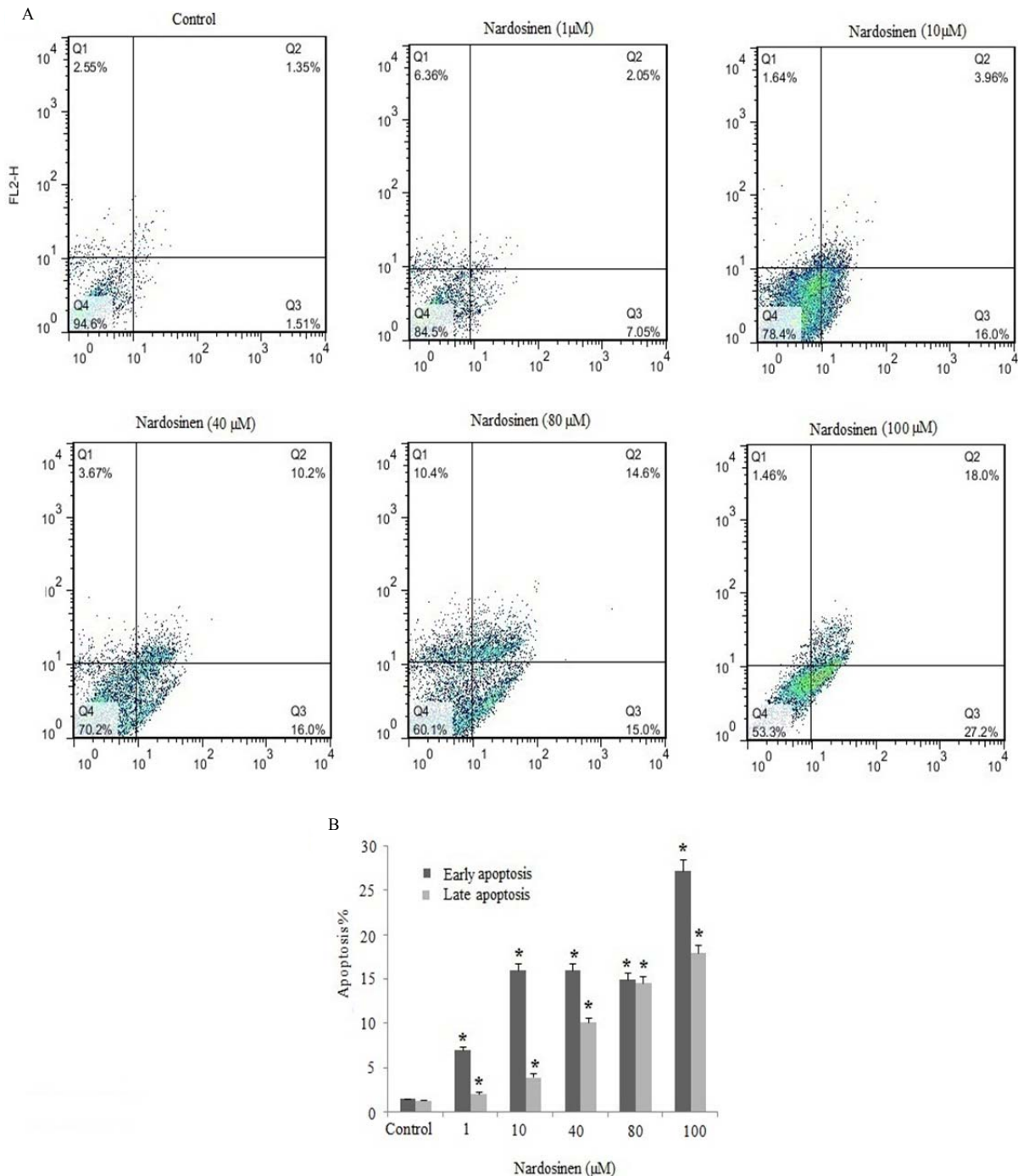
## RESULT

#### Inhibitory effect of nardosinen on breast cancer cells

To clarify cytotoxicity effects of nardosinen on MCF-7 cell lines, MTT assay was designed and performed. MTT assay is based on the measurement of succinate dehydrogenase ability to break tetrazolium dye to its insoluble form, formazan. Thus, this assay can be used as an indicator of assessing cell viability (23). As shown in Fig. 2, nardosinen showed impressive dose-dependent inhibition effect on the viability of MCF-7 cells. A significant decrease was observed in the cell viability at 1–100  $\mu\text{M}$ .



**Fig. 2.** Cytotoxic effect of nardosinen on human breast cancer cell line MCF-7 using MTT assay. Cells were treated with different concentrations of nardosinen for 48 h, each in triplicate and then cytotoxicity was assessed by MTT assay. Nardosinen reduced cell viability in a dose-dependent manner. Results (mean  $\pm$  SD) were calculated as the percent of control values. \* $P < 0.05$  considered significantly different from untreated cells.



**Fig. 3.** Flow cytometry analysis of apoptosis induced by nardosinen in MCF-7. (A) Cells treated with various concentrations of nardosinen for 48 h which was followed by apoptosis detection using annexin-V and propidium iodide using flow cytometry. (B) The percentage of apoptosis after treatment with nardosinen. Results (mean ± SD) were calculated as the percent of control values. \* $P < 0.05$  considered significantly different from untreated cells.

***Nardosinen-induced apoptosis in breast cancer cell lines***

To distinguish the nardosinen inhibitory effect, derivate from its potency to induction of apoptotic cell death, FITC-conjugated annexin-V and PI double staining (detected by flow cytometry) were used as an index to find

out apoptotic cells. MCF-7 cells were treated with different concentrations (1, 10, 40, 80 and 100 μM) of nardosinen for 48 h and analyzed by flow cytometry(Fig. 3A). The assay results for control cells were negative for both annexin-V FITC and PI. On the base of annexin-V FITC staining role, considered that

cells with annexin V-FITC-positive and PI-negative are in an early apoptotic stage, while cells with annexin-V FITC-positive and PI-positive are in late apoptotic or necrotic. PI-positive cells were considered as mostly necrotic. The aggregation of early and late apoptotic cells used for distinguish rate of apoptosis. As shown in Fig. 3B, a considerable increase in apoptotic cells that depends on the dose of nardosinen used for the treatment of MCF-7 cells was observed. These results showed an evidence for nardosinen -induced apoptosis in MCF-7cells.

#### ***Nardosinen regulated the expression of apoptotic proteins***

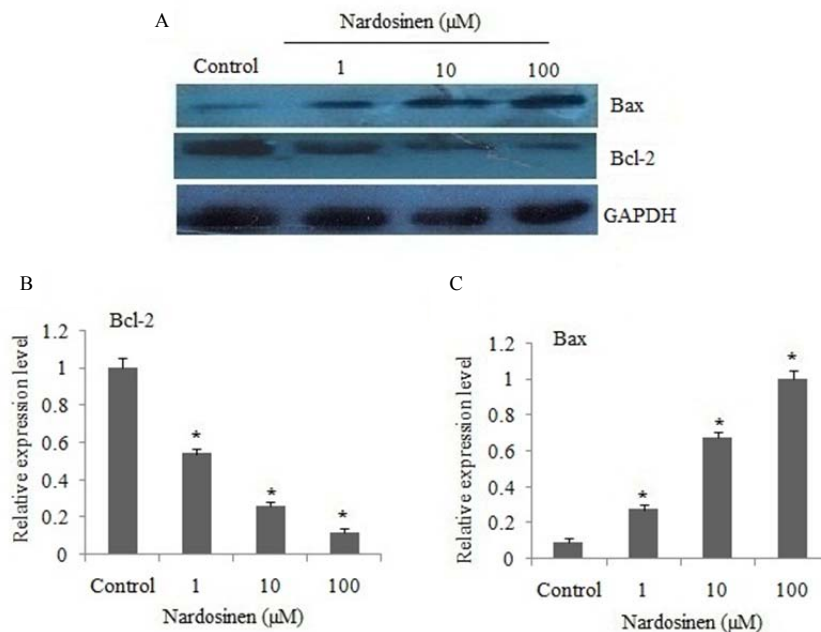
Western blot analysis used to assay Bcl-2 family that plays an important role in triggering apoptosis in cancer cells (Fig.4A). We studied two protein of this family, the Bax which is pro-apoptotic protein and Bcl-2 an anti-apoptotic protein to confirm nardosinen apoptosis induction. The analysis of data received from western blot assay showed that the expression of Bcl-2 was decreased in response to nardosinen treatment, while the expression of Bax protein was increased (Fig. 4B and 4C).

#### ***Involvement of caspase-6 in apoptosis induced by nardosinen***

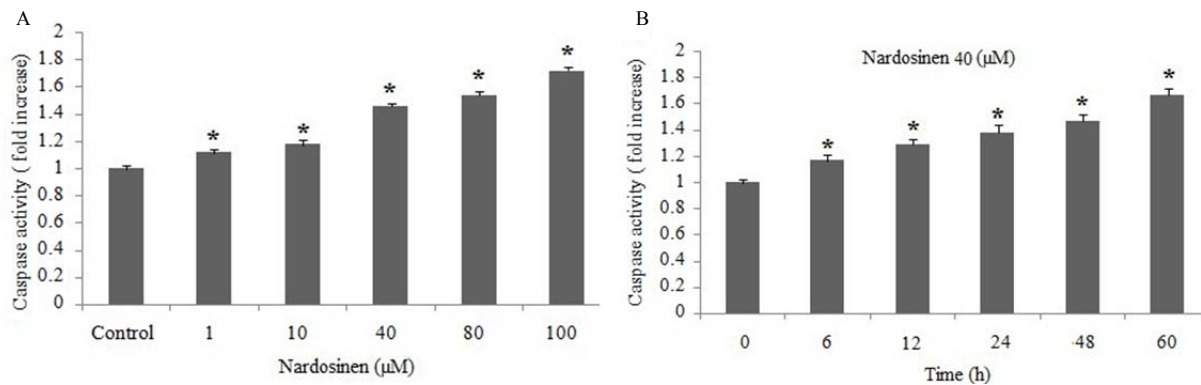
To determine the function of caspase in the induction of apoptosis by nardosinen, we studied the role of caspase-6 by assay kit. As shown in Fig. 5A, nardosinen treatment led to activation of caspase-6 in a concentration-dependent manner ( $P < 0.05$ ). Moreover, as shown in Fig. 5B, nardosinen treatment led to activation of caspase-6 in a time-dependent manner ( $P < 0.05$ ). The results showed that treatment of MCF-7 cells with nardosinen lead to noticeable increase in the activities of caspase-6.

#### ***Role of mitochondrial membrane potential in nardosinen-induced cell apoptosis***

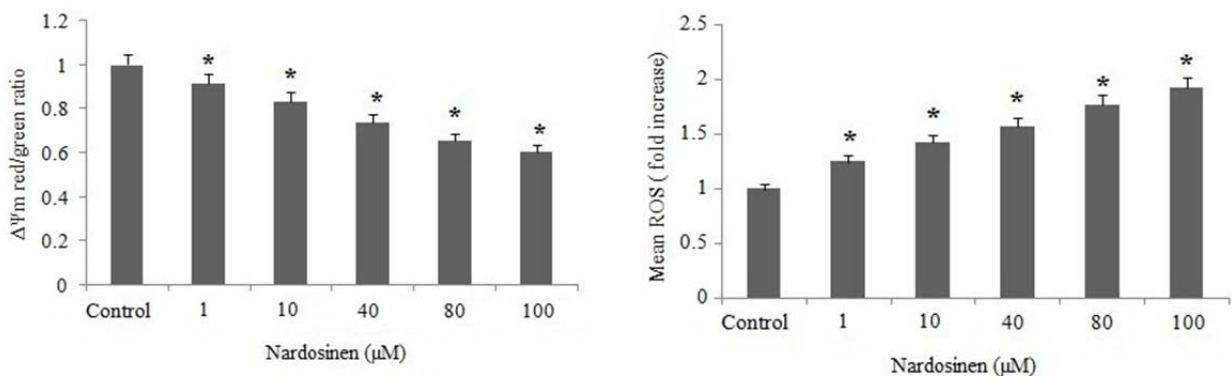
Loss of mitochondrial  $\Delta\Psi_m$  is one of the early biochemical markers of the apoptotic process. To assess whether nardosinen affects the function of mitochondria,  $\Delta\Psi_m$  was measured after treatment of MCF-7 cells with different concentration of nardosinen. JC-1, a cationic dye which indicates potential-dependent accumulation in the mitochondria, was used to determine the depletion in  $\Delta\Psi_m$ . JC-1 emits green light (540 nm) when the  $\Delta\Psi_m$  is lower than 140 mV.



**Fig. 4.** Effects of nardosinen on Bcl-2 and Bax expressions in human MCF-7 cells. (A) The cells were incubated with various concentrations of nardosinen and then Bcl-2 and Bax expressions were analyzed using western blots by the use of the corresponding specific antibodies. (B) The expression levels of BCL-2 proteins were decreased after treatment with nardosinen. (C) The expression levels of Bax proteins were increased after treatment with nardosinen. Equal amounts of loadings were confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band as an internal control. Results (mean  $\pm$  SD) were calculated as the percent of control values. \* $P < 0.05$  considered significantly different from untreated cells.



**Fig. 5.** The effects of adenosine on caspase-6 activity in human MCF-7 cells. The activity of caspase-6 increased in a concentration (A) and time (B) dependent manner after treatment with nardosinen. Results (mean  $\pm$  SD) were calculated as the percent of control values. \* $P < 0.05$  considered as statically significantly different from untreated cells.



**Fig. 6.** Effects of nardosinen on mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) in human MCF-7 cells. After incubation of cells with various dose of nardosinen for 48 h, cells were loaded with JC-1 dye and the potential-dependent accumulation in the mitochondria directly evaluated. Results (mean  $\pm$  SD) were calculated as the percent of control values. \* $P < 0.05$  considered as statically significantly different from untreated cells.

**Fig. 7.** Intracellular reactive oxygen species (ROS) level in nardosinen treated MCF-7 cells. ROS levels were monitored using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. The fluorescence intensity was measured on a varian-spectrofluorometer by excitation and emission wavelengths of 485 and 530 nm, respectively. Results (mean  $\pm$  SD) were calculated as the percent of control values. \* $P < 0.05$  considered as statically significantly different from untreated cells.

At higher membrane potential JC-1 emit red light (590 nm). The loss of mitochondrial membrane potential is followed by a red-to-green shift. As shown in Fig. 6, a significant loss of  $\Delta\Psi_m$  occurred after incubation of MCF-7 cells with different concentration of nardosinen and the depletion of mitochondrial  $\Delta\Psi_m$  increased in a dose-dependent manner ( $P < 0.05$ ). The results exhibited that a noticeable loss of  $\Delta\Psi_m$  occurred after treatment with nardosinen and the depletion of mitochondrial  $\Delta\Psi_m$  decreased in a dose-dependent manner.

#### **Effect of nardosinen on ROS in MCF-7 cell line**

ROS play an important role in apoptosis induction under both physiologic and pathologic conditions. We examined the ROS

level after treatment by nardosinen. As shown in Fig. 7, the level of ROS in MCF-7 cells were 1, 1.25, 1.42, 1.57, 1.77 and 1.92 after 48 h exposure to 0, 1, 10, 40, 80 and 100,  $\mu\text{M}$  of nardosinen, respectively. These findings showed that amount of ROS in nardosinen-treated cells were significantly higher in comparison with control cells in a dose-dependent manner ( $P < 0.05$ ). The result showed that nardosinen treatment, dose-dependently, increased ROS production in MCF-7 cells.

## **DISCUSSION**

Breast cancer is considered as one of the most important causes of mortality among

women that needs more attention for finding new methods of effective treatment (27). Numerous studies have revealed that herbal compounds are more safe and effective than other anticancer treatments (28,29). Nowadays there is a huge tendency amongst researchers to find out the best compounds from herbs. Our prior study exhibited that the nardosinen, for the first time separated from *J. foetidissima*, has inhibitory effects on various cancer cell growth (21). Based on these findings, the present study was designed to examine the anticancer features of nardosinen as well as its possible mechanism of action on the MCF-7 breast cancer cell line. In the current study, the MTT assay confirmed the cytotoxic potency of nardosinen in treated MCF-7 cancer cells. A previous study reported the antiproliferative effect of nardosinen in bladder and ovarian cancer cell cells (21). There are many factors such as cell organelles and molecular pathways that play the pivotal role in this process (17). These features and factors can be used to reliably identify death by apoptosis (30). It has been generally accepted that during the early stages of apoptosis, phosphatidylserine (PS) externalized from the inner to the outer layer of the plasma membrane which generally used to recognize apoptotic cells. Annexin-V FITC is a technique to detect apoptosis by targeting for binding to negatively charged PS at the outer plasma membrane (31). With regard to this fact, the MCF-7 cells stained with annexin V/PI showed that nardosinen increases the rate of apoptotic cells. Several studies have shown the apoptotic effects of another sesquiterpene in various cancer cell lines (20,32).

To confirm the cell death mechanism of nardosinen, we used western blot analysis. By doing this test we find out changes in expression of Bax and Bcl-2 after incubation of MCF-7 cells with nardosinen. It has shown that the pro-apoptotic Bax protein constructs a pore on the mitochondrial membrane that leads to changes in mitochondrial membrane potential. The Bcl-2 protein is an anti-apoptotic molecule, which raises in its expression causes to delay in disrupt of organelles surviving cells from apoptosis (33). Western blot analysis showed that nardosinen treatment dose-dependently, led to decrease in

expression of Bcl-2 and increased expression of Bax. Therefore, this data indicated nardosinen induces apoptosis with Bax/Bcl-2 pathways.

Caspases are a family of protease enzymes playing essential roles in programmed cell death. These proteases activate and affect in executioner way of apoptosis (34). MCF-7 cells do not express the caspase-3 protein due to a 47-base-pair deletion within exon 3 of the CASP-3 gene (20). Caspase-6 categorized as effector caspases that are responsible for some related proteolytic cleavage to cell death (35). To determine the caspase activation during the apoptosis induced by nardosinen, we perused the level of caspase activation by prepared assay kit. The results exhibited the considerable increase in the level of caspase-6 activity in treated MCF-7 cells by nardosinen. This data indicated a critical role for nardosinen-induced apoptosis in MCF-7 cancer cells.

Recent studies showed the importance of ROS in the induction of apoptosis and the importance of stimulate producing ROS in conventional anticancer treatment (36,37). ROS have destructive actions on DNA, proteins and the integrity of plasma membrane that can lead to programmed cell death (37). Base on this observation of increased intracellular ROS production at mitochondrial membrane site that causes disruption of  $\Delta\Psi_m$  and subsequent events can gain useful result about depolarization of  $\Delta\Psi_m$  as a feature of apoptosis. To find out the influence of ROS on apoptosis induced by nardosinen, we assessed the ROS formation and  $\Delta\Psi_m$  in MCF-7 cells. According to this experiment, a remarkable rate of ROS produced in the treated MCF-7 cell by nardosinen to compare with control cells. We find out that nardosinen via ROS production cause debilitation of  $\Delta\Psi_m$  and subsequent induction of apoptosis. A number of previous studies have shown that sesquiterpene compounds induce apoptosis through mitochondrial-dependent ROS production (38,39).

## CONCLUSION

Our results revealed that nardosinen inhibited cell proliferation in MCF-7 human



breast cancer cell line. This data also indicated that nardosinen triggered the mitochondrion-mediated apoptosis pathway through the production of ROS leading to an increase in Bax/Bcl-2 ratio then a decrease in mitochondrial membrane potential, leading to an increase in the protein expression of caspase-6. In fact, this result illustrated that nardosinen can be used as an herbal compound in cancer chemotherapy. Despite these results, further studies are needed to clarify the precise nardosinen mechanism of action and to evaluate its anticancer activities *in vivo*.

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