Research in Pharmaceutical Sciences, February 2020; 15(1): 76-86
Received: September 2019
Accepted: February 2020

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Anti-proliferative and cytotoxic effect of Iranian snake *(Vipera raddei kurdistanica)* venom on human breast cancer cells *via* reactive oxygen species-mediated apoptosis

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Abstract

Background and purpose: Breast cancer is the most commonly occurring cancer in women around the world. Despite new advances in cancer therapy, breast cancer remains a disease with high morbidity and mortality. Snake venom is a poisonous mixture of different molecules, such as carbohydrates, nucleosides, amino acids, lipids, proteins, and peptides. Previous studies demonstrated that some snake venoms showed *in vitro* anti-cancer effects. In this study, the effects of the Iranian snake (*Vipera raddei kurdistanica)* venom on breast cancer cells were investigated.
Experimental approach: The effect of increasing concentrations of snake venom on breast cell viability was assessed by trypan blue, MTT, and lactate dehydrogenase measurements. Apoptosis was detected and quantified by fluorescent staining and DNA fragmentation assay. The expression level of some apoptotic-related genes was investigated using real-time polymerase chain reaction (RT-PCR). The Western blotting method was also used to detect the protein expression profiles in the cells.
Findings / Results: After treatment for 24, 48, 72, and 96 h, the cell viability was significantly reduced in a time- and dose-dependent manner ($P < 0.05$). The venom effect on normal breast cells was significantly smaller than cancer cells ($P > 0.05$). Apoptosis was significantly increased ($P < 0.05$). The RT-PCR and western blot data confirmed the increase of apoptosis in cells treated with venom.
Conclusion and implications: These data suggested that the *vipera raddei kurdistanica* venom had a cytotoxic property *via* activation of apoptosis in breast cancer cells.

Keywords: Apoptosis; Breast cancer; Cell culture; *Vipera raddei kurdistanica*; Snake venom.

INTRODUCTION

Cancer is the second major threat to public health behind cardiovascular disease (1). Breast cancer, the most common cancer among women, accounts for 29% of new cancer diagnoses and 15% of cancer deaths (2). According to the world health organization (WHO), the incidence and death rates of breast cancer are predicted to increase in the future (3). Current strategies for treating breast cancer include surgery, radiation therapy, chemotherapy, hormone therapy, and targeted therapy (4). But most of these methods have undesirable side effects by the non-specific targeting of cells and damage to healthy normal cells (5). Moreover, the development of drug resistance is one of the most important obstacles to effective cancer treatment (6).

Snake venoms which are secreted by the snake in the predation or defending against threats, present a great toxicity potential that make them attractive for anti-cancer research.

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362. 278717

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It is a complex mixture of enzymes, peptides, carbohydrates, minerals, and proteins of low molecular mass with specific chemical and biological activities. Some studies have shown the potentials of snake venoms in the treatment of cancers. Phospholipase A2, L-amino-acid oxidase, metalloprotease, disintegrin and other peptides from snake venom are cytotoxic to cancer cells (7). *Vipera raddei kurdistanica* (*Montivipera raddei*) is a venomous viper species found in Armenia, Turkey, Iran, Azerbaijan, and Iraq (8,9). The objective of this research was to investigate the anticancer properties of *Vipera raddei kurdistanica* venom on breast cancer cells.

MATERIAL AND METHODS

Cell lines, reagents, and snake venom

For this exprimental *in vitro* study, two breast cancer cell lines (MCF-7 and MDA-MB-231) and normal human mammary epithelial cell line (MCF-10a) were obtained from the National Cell Bank of Iran. Trypsin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), acridine orange (AO), ethidium bromide (EB), dimethyl sulfoxide (DMSO), tween-20, propidium iodide (PI), JC-10, dichloro-dihydro-fluorescein diacetate (DCFH-DA), non-fat dry milk, and diphenylamine were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). NP-40 lysis buffer, antibodies, and enhanced chemiluminescence (ECL) western blotting substrate were purchased from AbCam (Cambridge, UK). Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum (FBS) were procured from Gibco (USA). Lyophilized venom was supplied by Razi Vaccine and Serum Research Institute. Cells were treated with 0, 0.62, 1.25, 2.5, 5, and 10 µg/mL of venom in cell culture media.

Cell culture

Breast cells were cultured in monolayer in RPMI 1640 medium, supplemented with 10% FBS and 1% anti-biotics (penicillin/streptomycin; 100 U/mL). The cells were maintained in a 37 °C humidified incubator equipped with 5% CO2 (10).

Viability and cytotoxicity assay

Cells were incubated with various concentration of venom for 24, 48, 72, and 96 h. MTT assay and trypan blue staining were performed as described previously (11,12). The cytotoxicity of venom was quantified using lactate dehydrogenases (LDH) activity assay in cells medium. After treatment, 100 µL of medium from each sample was transferred to new 96 well plates. LDH activity was measured using cytotoxicity detection kit (Roche, Germany) according to the manufacturer's procedure (13).

Apoptosis assay

In order to detect of the live tumor cells, early and late apoptotic cells, and necrotic cells, Ao/Eb double staining was done as described previously (14). Also, DNA fragmentation was quantified by TUNEL assay using an *in situ* cell death detection kit, AP (Roche, Germany) according to the manufacturer's instructions (15). The percentage of fragmented DNA after 48 h incubation also was calculated using diphenylamine assay as described by Cohen and Duke (16) and measurement the optical density of samples at 600 nm by a spectrophotometer.

Determination of reactive oxygen species and reduced glutathione

Intracellular reactive oxygen species (ROS) levels were measured and quantified by DCFH-DA. Briefly, cells were treated for 48 h and incubated with DCFH-DA. DCFH-DA was deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7'-dichlorofluorescein (DCF). Fluorescence emission intensity was measured using a microtiter plate reader (16). Intracellular glutathione (GSH) levels were measured using GSH assay kit (Sigma-Aldrich, USA) and according to manufacturer instructions (16).

Measurement of mitochondrial membrane potential and cytochrome C release

JC-10 dye was used to measure mitochondrial membrane potential (MMP) level. JC-10 dye forms aggregates in intact mitochondrial membrane and emitting red fluorescence. When the membrane potential is decreased by mitichondrial damage, the monomeric JC-10 is formed in the cytosol, which emits green fluorescence. Briefly, breast cells were stained with JC-10 and the fluorescence intensity of each sample was monitored using a fluorescence microplate reader at excitation/emission of 490/525 and 540/590 nm. To measure the concentration of cytochrome C in the cytosol, the cytochrome C releasing apoptosis assay kit (Abcam, USA) and human cytochrome C Quantikine ELISA kit (R&D Systems, UK) were used in accordance with their manufacturers' instructions. Finally, absorption was measured at 450 nm using a microplate reader (16).

Real-time polymerase chain reaction

The effect of venom on the expression level of *BAX* (a pro-apoptotic regulator) and *BCL-2* (an anti-apoptotic regulator) genes were estimated using RT-PCR as described earlier (17). All the primers (Table 1) were designed using GeneRunner software, cheeked in NCBI Primer Blast and purchased from the Pishgam Company (Tehran, I.R. Iran). For PCR amplification, following steps were undertaken; an initial step at 50 °C for 15 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 45 sec.

Western blot

The cells were treated with venom for 48 h and then lysed using NP-40 lysis buffer. The total protein concentration was determined using Lowry's method, For immunoblotting, 40 µg protein from each sample was subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred onto a nitrocellulose membrane. The nitrocellulose membrane was blocked with 1% non-fat dry milk in a mixture of TBS and tween-20, and incubated with the primary antibodies against *BAX*, *BCL-2,* and *β-ACTIN*. The membrane was incubated with secondary antibody mouse anti-rabbit IgG conjugated with horseradish peroxidase. After incubation the blotted proteins were visualized with ECL (18).

Statistical analysis

All of the experiments were repeated at least three times independently. The data are presented as mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA) and Tukey's test with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) software, and differences were considered nonsignificant when $P > 0.05$.

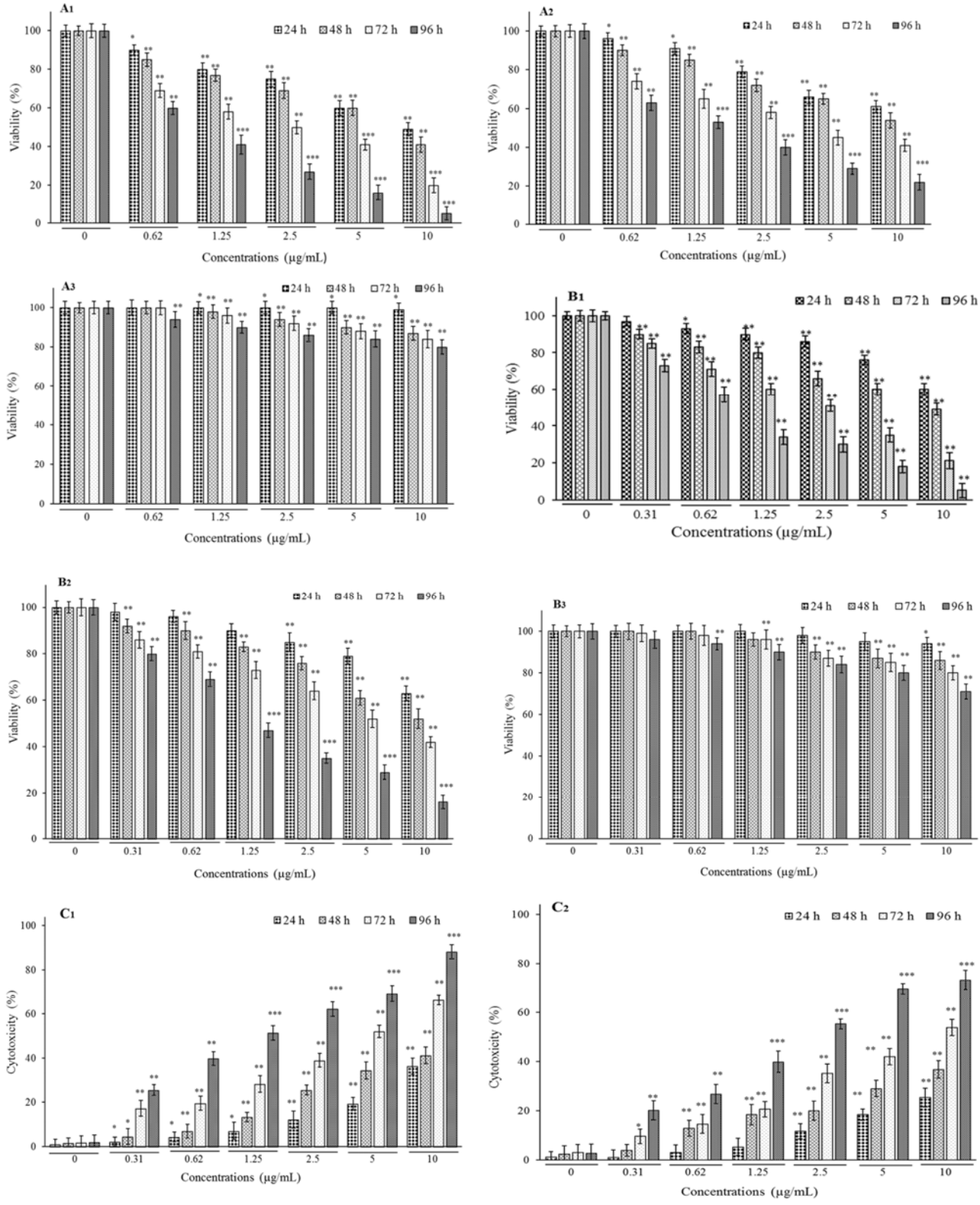
RESULT

Cell viability

The effect of the venom on cell viability was evaluated using the MTT assay, trypan blue staining and LDH measurement (Fig. 1A-C). The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 5 software (GraphPad Software Inc, San Diego, USA). After 24, 48, 72, and 96 h, the IC_{50} values were 18.53, 8.96, 2.14, and 0.98 µg/mL for MCF-7,20.29, 11.01, 5.99, and 1.27 µg/mL for MDA-MB-231, and 243.44, 240.13, 111.67, and 37.09 for MCF-10a.

Table 1. The primer sequences of key genes.

Genes	Forward sequences	Reverse sequences
BAX	5′-CCTGTGCACCAAGGTGCCGGAACT-3′	5′-CCACCCTGGTCTTGGATCCAGCCC-3′
BCL-2	5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′	5′-GGTGCCGGTTCAGGTACTCAGTCA-3′


A1
24 h
48 h
72 h
96 h
Viability (%)
Concentrations (µg/mL)
A2
A3
B1
B2
B3
C1
C2
Cytotoxicity (%)

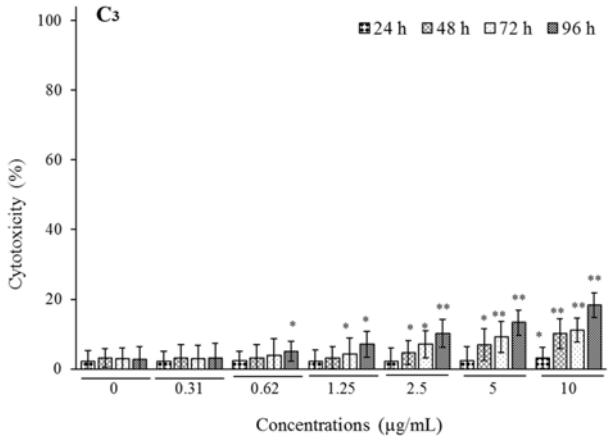


Fig. 1. The effect of venom on cancer and normal breast cells. Cells viability was determined after 24, 48, 72, and 96 h using the (A_1-A_3) MTT assay, (B_1-B_2) trypan blue staining, as described in the methods section, and (C_1-C_2) the cytotoxicity was determined by LDH activity measurement. The indices 1-3 in A-C parts are indicating MCF-7, MDA-MB-231, and MCF-10a cells, respectively. Control wells were treated with equivalent amount of medium without fetal bovine serum. *P < 0.05 and **P < 0.01, and ***P < 0.001 show significant differences compared with control.

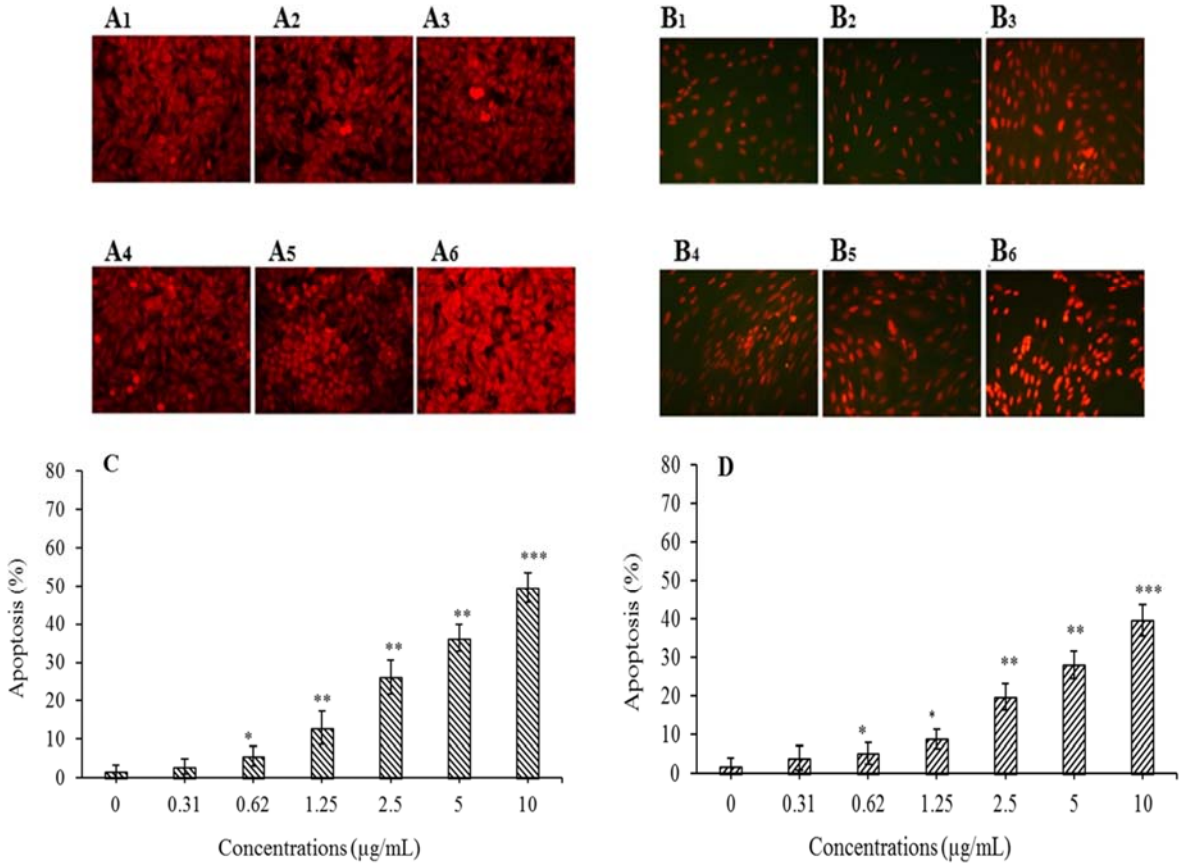


Fig. 2. The effect of venom on apoptosis in (A) MCF-7, and (B) MDA-MB-231 cells was evaluated using TUNEL staining. 1, Control group; 2, in the presence of 0.62; 3, 1.25; 4, 2.5; 5, 5; 6, 10 µg/mL of extract for 48 h. C and D indicate mean percentage of apoptotic MCF-7, and MDA-MB-231 cells, respectively from three independent experiments performed in triplicate. Control cells treated with medium without fetal bovine serum. *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences compared with control.

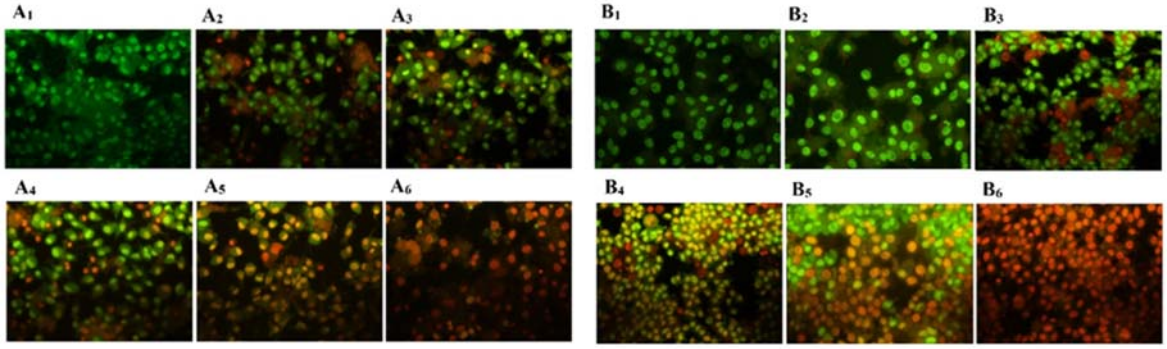


Fig. 3. (A) MCF-7, and (B) MDA-MB-231 cells were stained by AO/EB and observed under fluorescence microscope. 1, Control group; 2, in the presence of 0.62; 3, 1.25; 4, 2.5; 5, 5; and 6, 10 µg/mL of venom for 48 h. Control wells were treated with equivalent amount of medium without fetal bovine serum. Green live cells showed normal morphology with uniform nuclei; yellow early apoptotic cells showed nuclear margination and chromatin condensation. Late orange apoptotic cells showed fragmented chromatin and apoptotic bodies and necrotic cells had solid orange nuclei.

The results indicated cytotoxic effect of the venom on all cell lines tested in a dose- and time-dependent manner. The MCF-7 cells were the most sensitive to the venom as shown by its IC_{50} values. The venom exhibited selective cytotoxicity in breast cells with higher IC_{50} values for normal breast cells. The LDH assay showed that enzyme activity in cell culture medium was increased with increasing concentration of the venom. Cytotoxicity of venom was therefore accomplished with plasma membrane damage.

Apoptosis

The apoptosis induction potential of the venom was explored by TUNEL and AO/EB staining. The results showed that antiproliferative effect occurred by the mechanisms associated with apoptosis. Figure 2 shows the venom activated apoptosis in a concentration-dependent manner. Here again, the MCF-7 cell line was more sensitive to the venom than two other cell lines. This effect was further investigated by AO/EB double staining assay. The morphological changes in the cells such as chromatin condensation and its margination at the nuclear periphery, double-stranded DNA fragmentation, and cellular shrinkage and blebbing are shown in Fig. 3.

For MCF-7 cell line, intact cells (normal morphology) were seen in the control group, whereas early apoptotic cells were found in groups treated with 0.62, 1.25, and 2.5 µg/mL, the late apoptotic cells were observed in cells treated with 5 µg/mL. In 10 µg/mL group, all of the cells were in late stage and some of them were necrotic. In MDA-231-MB, intact cells (normal morphology) were seen in the control and 0.62 µg/mL groups, early apoptotic cells were abundant in the groups treated with 1.25, 2.5, and 5 µg/mL, and in the presence of 10 µg/mL most of the cells were in the late stage and necrotic cells were observed (Fig. 3). These observations suggested that apoptosis was increased in a dose-dependent manner in both cell lines. Also, the calculation of the DNA fragmentation percentage (Fig. 4A) showed that after treatment with 0.62, 1.25, 2.5, 5, and 10 µg/mL for 48 h, DNA fragmentation was increased about 0.92, 2.71, 14.9, 20.24, 34.33, and 49.18%, respectively for MCF-7; and 4.13, 5.81, 7.3, 19.39, 25.03, and 37.62%, respectively for MDA-MB-231 cells.

ROS analysis

The results suggested that ROS production in breast cells was significantly increased by venom treatment (Fig. 4B). Therefore, observed apoptosis in cancer cells were mediated, at least in part, by cellular damage due to excessive generation of ROS.

GSH measurement

GSH levels were decreased by 3, 6, 7, 16, 27, and 34% for MCF-7; and 0, 6, 11, 20, 23, and 29% for MDA-MB-231 after 48 h treatment with 0.62, 1.25, 2.5, 5, and 10 µg/mL of venom, respectively (Fig. 4C).

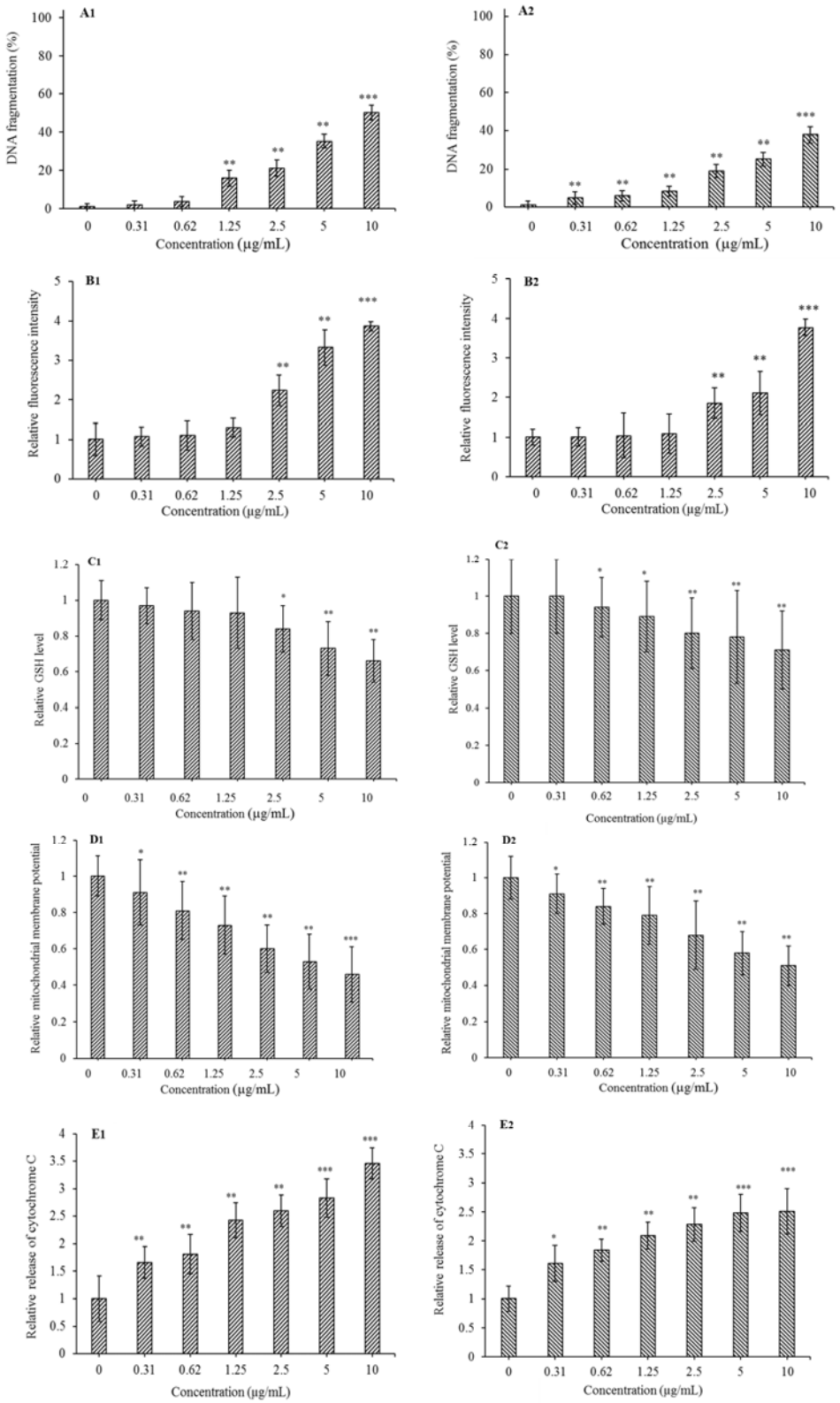


Fig. 4. The effect of venom on (A) DNA integrity, (B) reactive oxygen species generation, (C) GSH level, (D) mitochondrial membrane potential, and (E) cytochrome C release of breast cancer cells. Cells were treated for 48 h. Control wells were treated with equivalent amount of medium without fetal bovine serum. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared with control. The digits 1 and 2 in subscribe in all parts stand for MCF-7, and MDA-MB-231 cells, respectively. GSH, Glutathione.

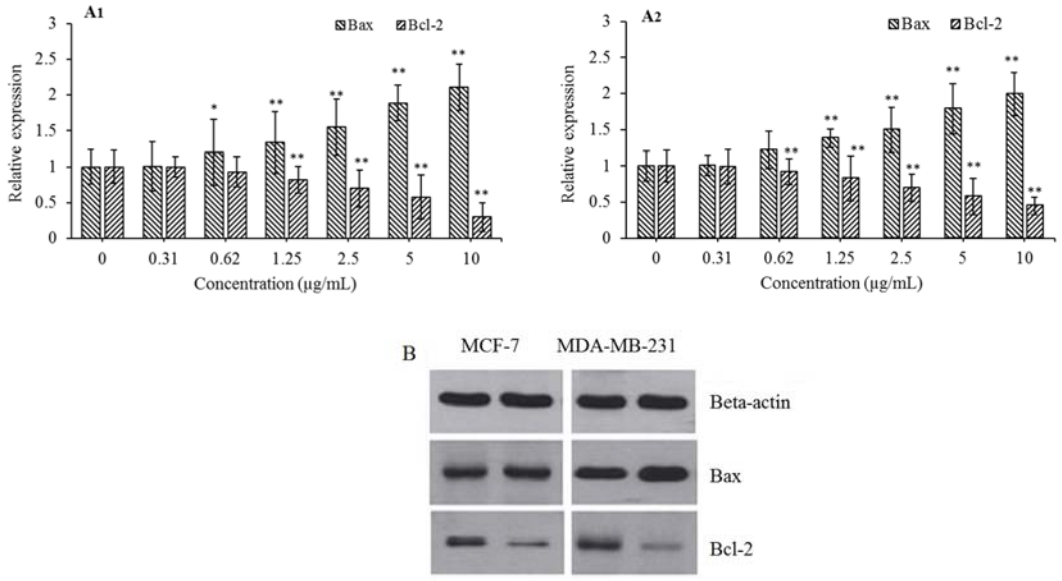


Fig. 5. Expression levels of (A) two apoptotic-related genes and (B) proteins in MCDF-7 (1) and MDA-231-MB (2) breast cancer cells after 48 h treatment. **P* < 0.05 and ***P* < 0.01, indicate significant differences compared with control.

MMP analysis

Oxidative stress induced by ROS, can trigger rapid depolarization of MMP and subsequent impairment of oxidative phosphorylation. As shown in Fig. 4D, the relative MMP was decreased significantly after treatment of cells with venom. Thus, venom depolarized mitochondrial membrane and decreased membrane potential in cells.

Cytochrome C release assay

Cytochrome C release from mitochondria is a critical event in apoptosis. It was increased by venom treatment after 48 h (Fig. 4E), suggesting that induction of apoptosis by venom was originated from mitochondria.

Analysis of genes expression

As shown in Fig. 5, a down-regulation of *BCL-2* and an up-regulation of *BAX* mRNA expression after 48 h treatment were detected. In control, 0.31, 0.62, 1.25, 2.5, 5, and 10 µg/mL groups *BAX* to *BCL-2* ratios were 1.003, 1.2, 1.64, 2.21, 3.26, and 7.03 for MCF-7; and 1.01, 1.32, 1.66, 2.14, 3.08, and 4.42 for MDA-MB231, respectively. Therefore, *BAX*/*BCL-2* ratios were significantly increased for both cell lines.

Apoptotic-related protein expression

The western blot analysis results indicated that after treatment, the expression of pro-apoptotic *BAX* protein was increased in cells compared to control group. On the other hand, the expression of anti-apoptotic *BCL-2* proteins was decreased compared to the control group (Fig. 5).

DISCUSSION

The discovery of new effective agents for cancer therapy is one of the most interesting and challenging issues in the pharmaceutical industry. Most of the chemotherapeutic agents do not distinguish between normal and cancer cells and kill the majority of proliferating cells, so they cause serious side effects such as bone marrow suppression (19). Hence, the development of new anti-cancer agent that is able to distinguish between normal and cancer cells is a great challenge in cancer therapy. The results of the present study demonstrated that *Vipera raddei kurdistanica* venom had a cytotoxic effect on breast cells in a dose- and time-dependent manner. The obtained IC_{50} values demonstrated that MCF-7 were more sensitive to the activity of the venom than MDA-MB-231 cells. The results of MTT, dye exclusion, and LDH assays confirmed the anti-neoplastic potential of the venom against cancer cells compared to the normal breast cells.

A large number of people die every year due to snake venom poisoning. The toxic constituents of venoms vary from species to species; therefore, snakebite showed a variety of life-threatening pathologies. Snake venom, a natural biological resource, contains several components that could be of potential therapeutic value. Use of snake venom in different pathophysiological conditions has been proposed in Ayurveda, homeopathy, and folk medicine. As mentioned earlier, snake venoms consist mainly of proteins and peptides (~95% of its dry weight) with specific chemical and biological activities. While these proteins cause death to humans, they can be used for the treatment of thrombosis, arthritis, cancers, and many other diseases (20). The earliest evidence of anticancer properties of snake venom appeared in the 1930s. Thence, the comprehensive studies about snake venoms showed their potential as a novel anticancer agent. The compounds with antitumor activities isolated from snake venoms include anti-angiogenesis and apoptosis induction agents. Disintegrins including leucurigin, contortrostatin, obtustatin, adinbitor, salmosin are a family of small, non-enzymatic, cysteine-rich proteins with anti-angiogenesis activity isolated from various snake venoms. Leucurogin showed significant anticancer and anti-angiogenesis activities in mice. Contortrostatin inhibits angiogenesis in the primary tumor of human breast cancer in mice. Obtustatin reduced tumor size in mouse model and showed anti-angiogenesis activities. Adinbitor inhibits bFGF-induced proliferation of ECV304 cells, and angiogenesis both *in vivo* and *in vitro*. Salmosin reduces the bovine capillary endothelial cell proliferation and suppresses the growth of both the metastatic and solid tumor in mouse xenografts of lung carcinoma cells (8).

For further verification of anticancer activity of the venom, apoptosis was detected by TUNEL, Ao/Eb, and DNA fragmentation assays. It is clear that DNA fragmentation is a key feature of apoptosis. The results showed an increase in apoptosis in a concentration-dependent manner by the venom. Here again MCF-7 cell line was more sensitive compared to other tested cell lines. In pathological conditions, particularly cancer, cells lose their ability to undergo apoptosis leading to uncontrolled proliferation and, therefore, induction of apoptosis can be a desirable strategy against cancer (21).

Some proteins of snake venom can induce apoptosis. In 1993, it was shown that venoms of some snake activated apoptosis of vascular endothelial cells. L-amino-acid oxidase, from the venom of *Agkistrodon halys, Agkistrodon halys pallas*, *Vipera berus berus*, and *Vipera berus berus* induces apoptosis in some cancer cells. Also, apoptosis can be activated by some disintegrins. A disintegrin purified from the venom of the Indian cobra snake (*Naja naja*), showed anticancer and apoptosis induction activity in MCF-7, A549, and HepG2 cells. VAP and VAP2, two metalloprotease/disintegrin family proteins, induce apoptosis specifically in endothelial cells. Stejnitin induces apoptosis in ECV304 cells (8). Our results showed that intracellular level of ROS was increased by the venom in breast cancer cells in a dose-dependent manner.

Most of the chemotherapeutic agents elevate intracellular level of ROS and alter redox-homeostasis of cancer cells. The distribution of redox balance in the tumor cells induces apoptosis. GSH is considered the most abundant anti-oxidant in cells that protects cell from ROS accumulation and apoptosis. Therefore, reduction of GSH concentration in cell is an indicator of apoptosis (22,23). In the current study, GSH level was significantly decreased in cancer cells. The decrease in GSH and increase in ROS in cells shifted the redox balance in favor of apoptosis.

Data showed that venom induced apoptosis probably through mitochondrial pathway, altered expression of apoptosis regulatory proteins of the *BCL-2* family, increased *BAX*/*BCL-2* ratio, reduced mitochondrial membrane potential, and released cytochrome C from the mitochondria to the cytoplasm.

CONCLUSION

The venom of Iranian snake (*Vipera raddei kurdestanica*) could be beneficial for further development of new chemotherapeutic agents. The present data open a new possible approach in the treatment of breast cancer. Future *in vivo* studies and clinical investigations are warranted.

ACKNOWLEDGMENTS

This work was financially supported by the Vice-Chancellor for Research of Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran under the Grant No. 980455.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest for this study.

AUTHOR'S CONTRIBUTIONS

C. Jalali was responsible for overall supervision. I. Rashidi revised the manuscript. M. Pazhouhi participated in study design, data evaluation, drafting, and statistical analysis. E. Malekara contributed to all experimental work. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

REFERENCES

1. Nagai H, Kim YH. Cancer prevention from the perspective of global cancer burden patterns. J Thorac Dis. 2017;9(3):448-451.
2. Sun YS, Zhao Z, Yang ZN, Xu F, Lu HJ, Zhu ZY, *et al*. Risk factors and preventions of breast cancer. Int J Biol Sci. 2017;13(11):1387-1397.
3. Power EJ, Chin ML, Haq MM. Breast cancer incidence and risk reduction in the Hispanic population. Cureus. 2018;10(2):1-12.
4. Shahali A, Ghanadian M, Jafari SM, Aghaei M. Mitochondrial and caspase pathways are involved in the induction of apoptosis by nardosinen in MCF-7 breast cancer cell line. Res Pharm Sci. 2018;13(1):12-21.
5. Gewirtz DA, Bristol ML, Yalowich JC. Toxicity issues in cancer drug development. Curr Opin Investig Drugs. 2010;11(6):612-614.
6. Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, *et al*. Drug resistance in cancer: an overview. Cancers (Basel). 2014;6(3):1769-1792.
7. Shanbhag VKL. Applications of snake venoms in treatment of cancer. Asian Pac J Trop Biomed. 2015;5(4):275-276.
8. Li L, Huang J, Lin Y. Snake venoms in cancer therapy: past, present and future. Toxins (Basel). 2018;10(9):1-8.
9. Nalbantsoy A, Hempel BF, Petras D, Heiss P, Göçmen B, Iğci N, *et al*. Combined venom profiling and cytotoxicity screening of the *Radde's mountain viper* (*Montivipera raddei*) and *Mount bulgar viper* (*Montivipera bulgardaghica*) with potent cytotoxicity against human A549 lung carcinoma cells. Toxicon. 2017;135:71-83.
10. Khazaei M, Pazhouhi M. Antiproliferative effect of *Trifolium pratens* L. extract in human breast cancer cells. Nutr cancer. 2019;71(1):128-140.
11. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. Appendix 3: Appendix 3B. DOI: 10.1002/0471142735.ima03bs21.
12. Mohamadi A, Aghaei M, Panjehpour M. Estrogen stimulates adenosine receptor expression subtypes in human breast cancer MCF-7 cell line. Res Pharm Sci. 2018;13(1):57-64.
13. Kumar P, Nagarajan A, Uchil PD. Analysis of cell viability by the lactate dehydrogenase assay. Cold Spring Harb Protoc. 2018;2018(6). DOI: 10.1101/pdb.prot095497.
14. Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res. 2015;21:15-20.
15. Khazaei M, Pazhouhi M, Khazaei S. Evaluation of hydro-alcoholic extract of *Trifolium pratens L*. for its anti-cancer potential on U87MG cell line. Cell J. 2018;20(3):412-421.
16. Khazaei M, Pazhouhi M. Temozolomide-mediated apoptotic death is improved by thymoquinone in U87MG cell line. Cancer Invest. 2017;35(4): 225-236.
17. Khazaei M, Pazhouhi M, Khazaei S. Temozolomide and tranilast synergistic antiproliferative effect on human glioblastoma multiforme cell line (U87MG). Med J Islam Repub Iran. 2019;33:39-46.
18. Mahmood T, Yang PC. Western blot: technique, theory, and trouble shooting. N Am J Med Sci. 2012;4(9):429-434.
19. Pan Y, Ma S, Cao K, Zhou S, Zhao A, Li M, *et al*. Therapeutic approaches targeting cancer stem cells. J Cancer Res Ther. 2018;14(7):1469-1475.
20. Pal SK, Gomes A, Dasgupta SC, Gomes A. Snake venom as therapeutic agents: from toxin to

drug development. Indian J Exp Biol. 2002;40(12):1353-1358.
21. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu HY, Lin LT, *et al*. Broad targeting of resistance to apoptosis in cancer. Semin Cancer Biol. 2015;35:S78-S103.
22. Shi MD, Shiao CK, Lee YC, Shih YW. Apigenin, a dietary flavonoid, inhibits proliferation of human bladder cancer T-24 cells via blocking cell cycle progression and inducing apoptosis. Cancer Cell Int. 2015;15:33-44.
23. Juan ME, Wenzel U, Daniel H, Planas JM. Resveratrol induces apoptosis through ROS-dependent mitochondria pathway in HT-29 human colorectal carcinoma cells. J Agric Food Chem. 2008;56:4813-4818.