Journal of Evidence-Based Integrative Medicine
Volume 23: 1-8

DOI: 10.1177/2156587217751796
journals.sagepub.com/home/cam
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Scorpion Venom Causes Apoptosis by Increasing Reactive Oxygen Species and Cell Cycle Arrest in MDA-MB-231 and HCT-8 Cancer Cell Lines

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Abstract

Objectives. The objective of this study was to examine the effect of scorpion venoms on cancer cell progression, apoptosis, and cell cycle arrest. Scorpion venoms are known to possess numerous bioactive compounds that act against cancer progression by inducing apoptosis. In this study, we have taken the venoms from the following 2 species of scorpion—*Androctonus crassicauda* and *Leiurus quinquestriatus*—and tested the anticancer properties of the venom against breast and colorectal cancer cell lines. *Methods.* Milking of scorpion venom and culturing the breast and colorectal cancer cell lines were done according to the standard procedure. The venom cytotoxicity was assessed by MTT methods, and the cellular and nuclear changes were studied with phase contrast and propidium iodide staining, respectively. The cell cycle arrest and accumulation of reactive oxygen species were analyzed on a Muse cell analyzer. *Results.* The venoms exerted cytotoxic effects on breast and colorectal cell lines in a dose- and time-dependent manner. Enhanced apoptotic cells, increase in reactive oxygen species, and cell cycle arrest were observed after challenging these cell lines with scorpion venoms. *Conclusions.* Scorpion venom induces apoptosis in breast and colorectal cell lines as reflected by the changes in the cell morphology and cell cycle studies. Furthermore, a high percentage of total reactive oxygen species as well as apoptotic cells also contribute to cell death as observed after venom treatments. To the best of authors' knowledge, this is the first scientific evidence demonstrating the induction of apoptosis and cell cycle arrest by these species of scorpion venoms.

Keywords

MTT assay, G0/G1 phase, colorectal, breast cancer cell lines, cell cycle assay

Received March 14, 2017. Received revised May 10, 2017. Accepted for publication May 26, 2017.

Cancer is the leading cause of death in all developed and developing countries. In 2016, about 1.7 million new cancer cases and approximately half a million cancer deaths were projected to occur in the United States.[1] Breast cancer is the most common type of cancer among women,[2,3] and colorectal cancer is the second most common type of cancer in the Kingdom of Saudi Arabia.[4] Both of these cancer types have increased significantly in the Kingdom within the past few decades.[2,5] There are several methods of treatment modalities that have been used for cancer treatment such as surgery, radiotherapy, chemotherapy, hormone replacement therapy, and immunotherapy.[6] These types of treatment may elicit adverse effects on normal cells, leading to various toxic side effects such as neurotoxicity, hepatotoxicity, and nephrotoxicity.[7] To circumvent these drawbacks, researchers are now leaning toward alternate treatment strategies. Since ancient times the natural products extracted from plants and animals have been used for the treatment of diseases throughout the world.[8] Usages of plant parts for treatment are common but not used within the major population of the Kingdom. Moreover, products from animals and their parts such as blood, hooves, spines, urine, milk, and venoms are being used for the treatment of various diseases.[9,10]

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Venoms are commonly known for their negative effects and cause severe health problems for human; venoms carry a variety of toxins that can cause neurotoxicity, nephrotoxicity, cytotoxicity, respiratory arrest, dermatitis, allergic reactions, hemorrhage, and disseminated intravascular coagulation.[11] However, despite their negative effects, these venoms are a rich source of pharmacologically active compounds.[12] Some venomous arthropods like snakes, bees, scorpion, wasps, ants, spiders, and caterpillars produce pharmacologically active molecules, which also possesses anticancer activity.[13] Scorpion venom contains several bioactive compounds such as mucopolysaccharides, free amino acids, phospholipases, hyaluronidases, amines, and nucleotides.[14] Keeping in view the abundance of biologically active molecules that may also act against cancer, we undertook this study to test anticancer activity of scorpion venoms in breast and colorectal cancer cell lines. Additionally, in support of our current study several literature sources also reported the role of scorpion venom on cancer cells.[15] In this study, we have investigated the anticancer properties of scorpion venom obtained from *Androctonus crassicaud*a and *Leiurus quinquestriatus*. *Androctonus crassicaud*a is commonly known as the Arabian fat-tailed scorpion and this formidable scorpion can inject potentially lethal venom. The species is mainly found in Egypt and the Middle East region including the Arabian Peninsula, Iran, Iraq, Israel, and Turkey.[16] *Leiurus quinquestriatus* is known as the Palestine yellow scorpion. This particular species are found throughout the Middle East, Turkey, Iran, Oman, and Yemen.[17] Both these scorpions belong to the family of Buthidae, a family that is known to have medically important and beneficial venoms.[18] The present study is the extension of our previous published work where we used the venom from *Androctonus bicolor* and determined the cytotoxic effect of the venom on breast and colorectal cancer cell lines.[19]

In this study, we have used the venoms obtained from species of *Androctonus crassicauda* (designated as venom 1) and *Leiurus quinquestriatus* (designated as venom 2). We have demonstrated the dose-dependent cytotoxic property of both the venoms against breast and colorectal cancer cell lines. Additionally, we found enhanced reactive oxygen species generation, higher rate of apoptosis, and cell cycle arrest in both breast and colorectal cancer cell lines, after they were challenged with scorpion venoms. The implication of the study will be to develop a safe mode of target-specific delivery of the venom/toxin using either a nanoparticle approach and/or a liposomal drug delivery strategy. To the best of the authors' knowledge and a literature search, this study is unique in the sense that no one to date has performed a study of this nature on breast and colorectal cancer cell lines using scorpion venoms found in the Kingdom of Saudi Arabia.

Materials and Methods

Collection of Scorpion and Milking of Its Venom

Collection of the scorpions and milking of their venoms was achieved as described by Al-Asmari et al.[19] Prior to beginning the animal work, approval was obtained from the Research Ethics Committee of Prince Sultan Military Medical City. The Research Ethics Committee supervises the animal care units in Prince Sultan Military Medical City, Riyadh, Kingdom of Saudi Arabia.

Cell Culture

Human breast cancer cell line MDA-MB-231 (obtained from breast mammary glands) and colorectal cancer cell line HCT-8 (obtained from the ileocecal adenocarcinoma of a 67-year-old male) were cultured in Dulbecco's Modified Eagle Medium supplemented with antibiotic-anti-mycotic as reported earlier.[20,21]

MTT Colorimetric Assay to Determine the Cytotoxic Effect of the Venoms

The cytotoxic property of venoms were assessed by MMT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay as reported earlier from our laboratory.[19]

Determination of Reactive Oxygen Species

To evaluate the effect of scorpion venoms on reactive oxygen species generation, the assays were performed on the Muse Cell Analyzer (Millipore, USA) according to the manufacturer's protocol and also as described earlier.[22]

Morphological Assessment of the Cells: (1) Phase Contrast Microscopic Studies and (2) Propidium Iodide Staining

All morphological assessment under the influence of venom 1 and venom 2 were performed according to standard procedure as reported recently from our laboratory.[19]

Cell Cycle Analysis

Cell cycle arrest and determination of DNA enrichment in a specific cell cycle phase was achieved on the Muse cell analyzer using the kit from Millipore. All the experimental steps were as described recently from our laboratory.[23]

Statistical Analysis

Student's t test was performed using GraphPad Prism 4.0 software. The mean was reported with standard deviation ($\pm$ SD). Differences were considered to be statistically significant when P values were $\leq .05$.

Results

Cytotoxicity

The foremost objective of this study was to examine the cytotoxicity induced by scorpion venoms and to determine the IC50 values thereof. MTT assay was performed as an indirect measure to determine the viability of cells after venom (venom 1 and venom 2) treatment. The cytotoxic effect was determined in a dose-dependent manner to obtain the appropriate concentration of venom for 50% cell-kill. This dose was the IC50 of

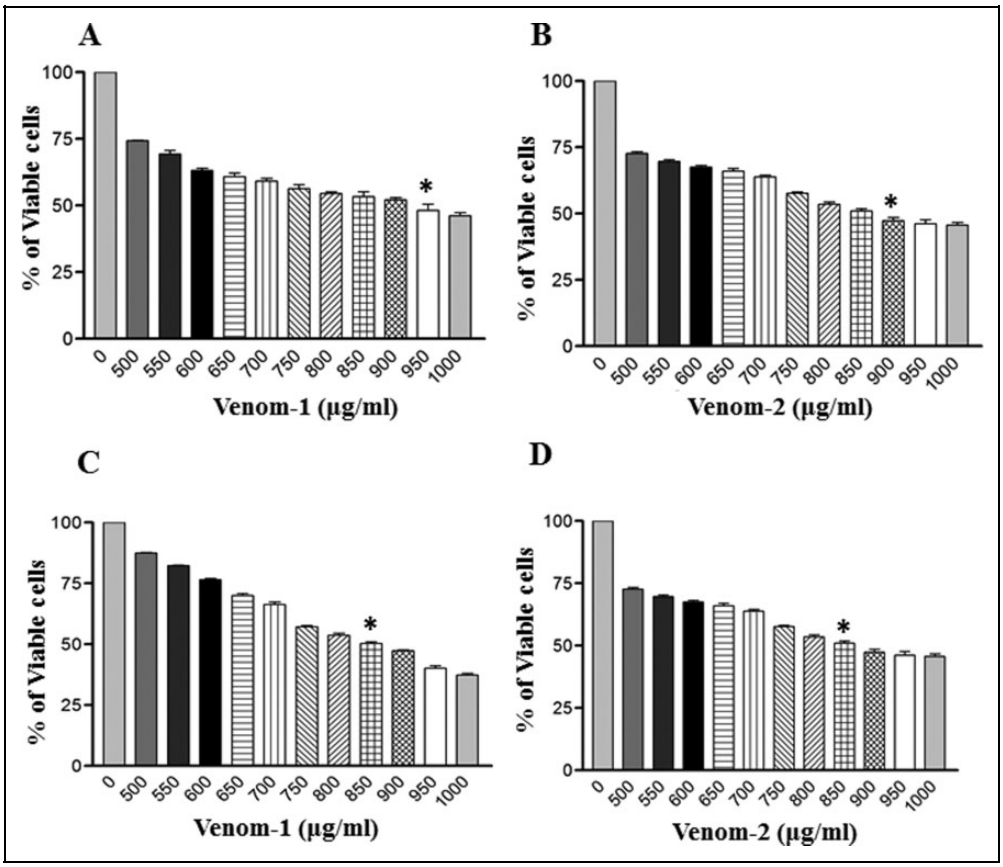

Figure 1. Bar graphs showing the cytotoxic effect and hence IC50 values of scorpion venoms on breast and colorectal cancer cell lines. Columns (A) and (C) show the cell viability pattern after venom 1 treatment for 24 hours on breast and colorectal cancer cell lines, respectively. Columns (B) and (D) represents the effect of venom 2 on the cell lines as mentioned above. An asterisk indicates IC50 values.

that particular venom in a specific cell line. The venoms exhibited cytotoxic effects on MDA-MB-231 and HCT-8 cells in a dose- and time-dependent manner. Percent viability of the MDA-MB-231 and HCT-8 cell lines are shown in Figure 1A to D. The IC50 values for venom 1 in breast and colorectal cancer cell lines were found to be 950 µg/mL and 850 µg/mL, respectively, after 24 hours of treatment. Similarly, the IC50 values for venom 2 at 24-hour treatment were found to be 900 µg/mL and 850 µg/mL, respectively. Furthermore, the IC50 values after 48-hour treatment of breast and colorectal cancer cell lines were found to be 850 µg/mL and752 µg/mL (for venom 1) and 815 µg/mL and 695 µg/mL (for venom 2), respectively. The asterisk on the column indicates the IC50 values. Furthermore, the IC50 values obtained at 24 hours were used throughout the course of this study. (For brevity profiles obtained at 48-hour treatments are not shown.)

Reactive Oxygen Species (ROS) Formation

As shown in Figure 2, a dramatic increase in ROS formation was observed on scorpion venom treatment both in breast (Figure 2A and B) and colorectal (Figure 2C and D) cancer cell lines. An enhancement of about 85% to 90% in ROS generation was observed when compared with the control in both the lines, after challenge with scorpion venoms. In the histogram, M1 and M2 represent the cellular population. All the cells showing positive staining are referred to as ROS (+), while the cells that do not stain with the ROS-specific dye used from the assay kit are referred to as ROS (−). It is evident from the histograms and the bar graphs that numbers of positive cells (ROS positive) is greater in venom-treated cells as compared to the control. Hence, it may be concluded that venom treatment increases the conversion of normal cells into high ROS-generating cells in breast and colorectal cancer cell lines, which ultimately leads to the cell death.

Morphological Assessment of Post Venom Treatment

The cell lines MDA-MB-231 and HCt-8 were treated with scorpion venoms (venom 1 and venom 2) at their IC50 concentration for 24 hours and 48 hours. Both these lines responded to venom treatment by the induction of apoptosis and necrosis. At the 24-hour treatment time point, MDA-MB-231 cells' apoptosis is greater than 2-fold as compared with the control. Furthermore, the percentage of apoptosis was found to be 36.0%, while the percentage of necrotic cells was 14.7% as compared to MDA-MB-231 control. Additionally, by increasing the venom treatment time for this same cell line up to 48 hours, the extent of apoptosis was raised to 47% as compared to controls (Figure 3A and B). However, we did not

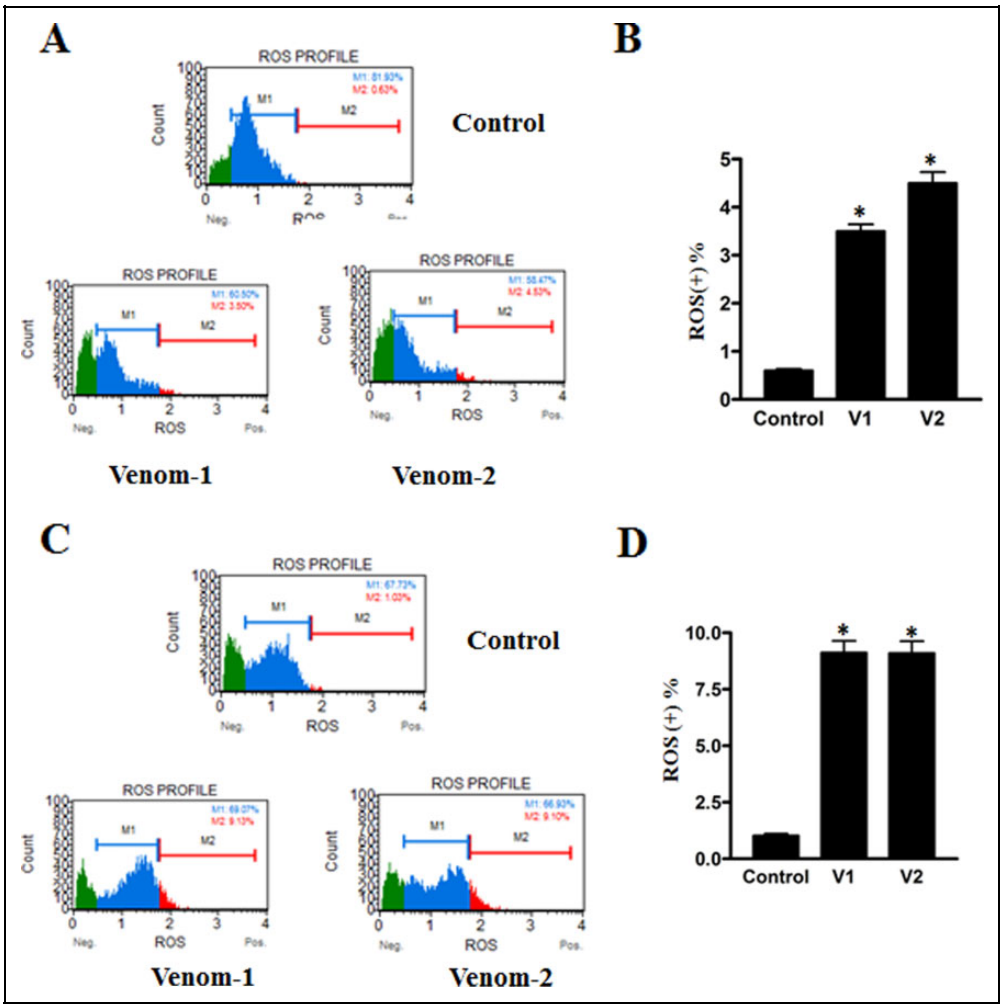

Figure 2. Histogram showing the extent of reactive oxygen species (ROS) generation after scorpion venoms treatment on (A) MDA-MB-213 and (C) HCT-8 cancer cell lines. Bar graphs (B and D) show the quantitative analyses of the ROS generation after venom treatment. A significant increase in ROS formation was observed both in breast and colorectal cancer cell lines, $P < .05$.

notice any appreciable change in the number of necrotic cells in this particular set of experiment. In addition, the extent of apoptosis and necrosis in HCT-8 cell line was found to be 34% higher at 24 hours of treatment, and did not change after 48 hours of treatment when compared with their controls (Figure 3C and D).

In addition to this, we have also obtained similar pattern of significantly higher apoptotic and necrotic cells both in breast and colorectal cancer cell lines when treated with venom 2 (Figure 3E-H). In conclusion, scorpion venom when used at their IC50 values introduces significant amount of apoptosis and necrosis in these cell lines, resulting in DNA damage and consequently stopping cancer progression.

Cell Cycle Analysis

Cell cycle stages were identified by use of the Muse cell analyzer (Millipore). As shown in Figure 4A and B, there is a significant enrichment of cells observed in G2/M phase while no significant change was observed in G0/G1 when breast cancer cells were treated with venom 1. For the colorectal cancer cell line (Figure 4C and D), cell enrichment was significantly higher in all 3 phases when compared to control after treatment with venom 1. This implies that a major inhibition of the cell cycle can be achieved on venom 1 treatment in HCT-8 cell line. Furthermore, as shown in Figure 4E to H, a perceptible enrichment in the G2/M phase was observed in both breast and colorectal cancer cell lines when treated with scorpion venom 2. This phenomenon further supports our previous findings of high apoptosis and necrosis in venom-treated cell lines. Therefore, we can postulate that scorpion venom is a noble anticancer agent against breast and colorectal cancer cell lines, which sequestrates cancer cell progression by creating apoptosis, necrosis, and cell cycle arrest.

Discussion

Scorpion venom acts as an anticancer agent due to the presence of various types of bioactive molecules, such as proteins, small peptides, and amine, which inhibits cancer progression.[24] In

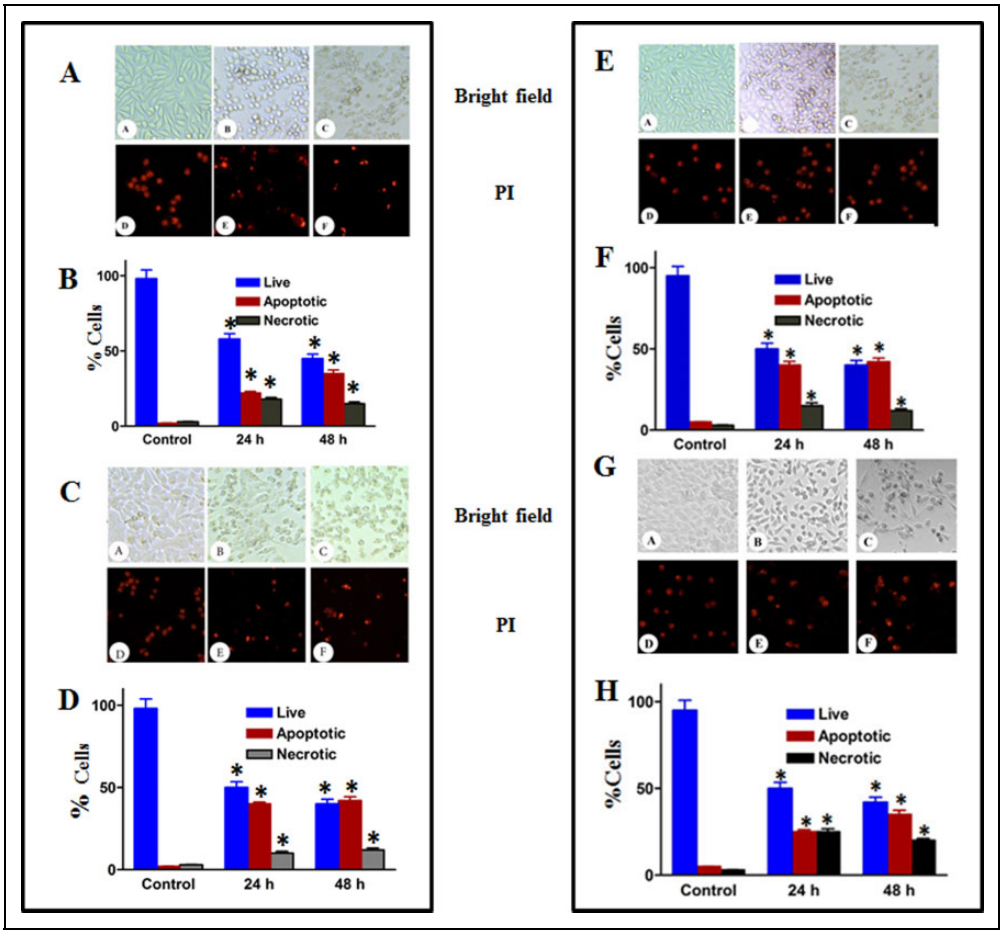

Figure 3. Phase contrast and propidium iodide (PI) staining: (A and C) MDA-MB-231 (breast) and HCT-8 (colorectal) cancer cell lines treated with venom 1 for 24 hours and 48 hours, respectively. Phase contrast field (upper panel) and PI staining (lower panel) (B and D) bar graphs represent the number of viable, apoptotic, and necrotic cells after venom treatment. Statistically significant ($P \leq .05$).

this study, we have identified anticancer properties of the venoms obtained from 2 commonly found species of scorpions: *Androctonus crassicauda* and *Leiurus quinquestriatus.* The effectiveness of the venoms as anticancer agents was tested against MDA-MB-231 (breast) and HCT-8 (colorectal) cancer cell lines. We have shown the cytotoxic effect of the venoms against both these cell lines. In addition, ROS generation, an increased number of highly apoptotic cells, and cell cycle arrest were also observed in the venom-treated breast and colorectal cancer cell lines. Modulating these important cancer survivals parameters using scorpion venoms is a positive approach toward inhibiting cancer progression. It has been well documented that scorpion venoms are very effect tools for the treatment of cancer by inducting apoptosis and inhibiting cell cycle progression.[25-27]

The induction of cytotoxic effect is a key mechanism capable of inhibiting the uncontrolled cell growth that is a hallmark of cancer development. In order to establish an appropriate dose of venom, we measure the cytotoxicity and IC50 values of these venoms on breast and colorectal cancer cell lines. The IC50 values of MDA-MB-231 and HCT-8 cell lines for venoms 1 and 2 are shown in Figure 1. The IC50 values that has been described in the Results section are closely related to the previously reported values, where these values are 700 µg/mL and 890 µg/mL for breast and colorectal cancer cell lines, respectively.[28] Therefore, based on these findings and published work, conclusion can be drawn that scorpion venom possesses cytotoxic property against breast and colorectal cancer cell lines. The other milestone was to analyze the molecular events involved in cellular progression in the absence or in the presence of scorpion venoms. One of the striking parameter was to evaluate the generation of ROS in venom-treated cell lines.

Treatment of cancer cells with venom creates a stressful environment. Once treated with scorpion venoms, cells undergo further elevation of cytotoxicity. These stressful events in the tumor cells results in triggering of the generation of free radicals and hence reactive oxygen species formation begins.[29] To elucidate this mechanism in breast and colorectal cancer cell lines, we determine the extent of ROS generation under the influence of scorpion venoms on these cancer cell lines (Figure 2). A dramatic increase in the ROS-positive cells was detected in venom-treated cell lines when stained with the specific dye for ROS and monitored on the Muse cell analyzer. Our results are in strong agreement with a mechanism

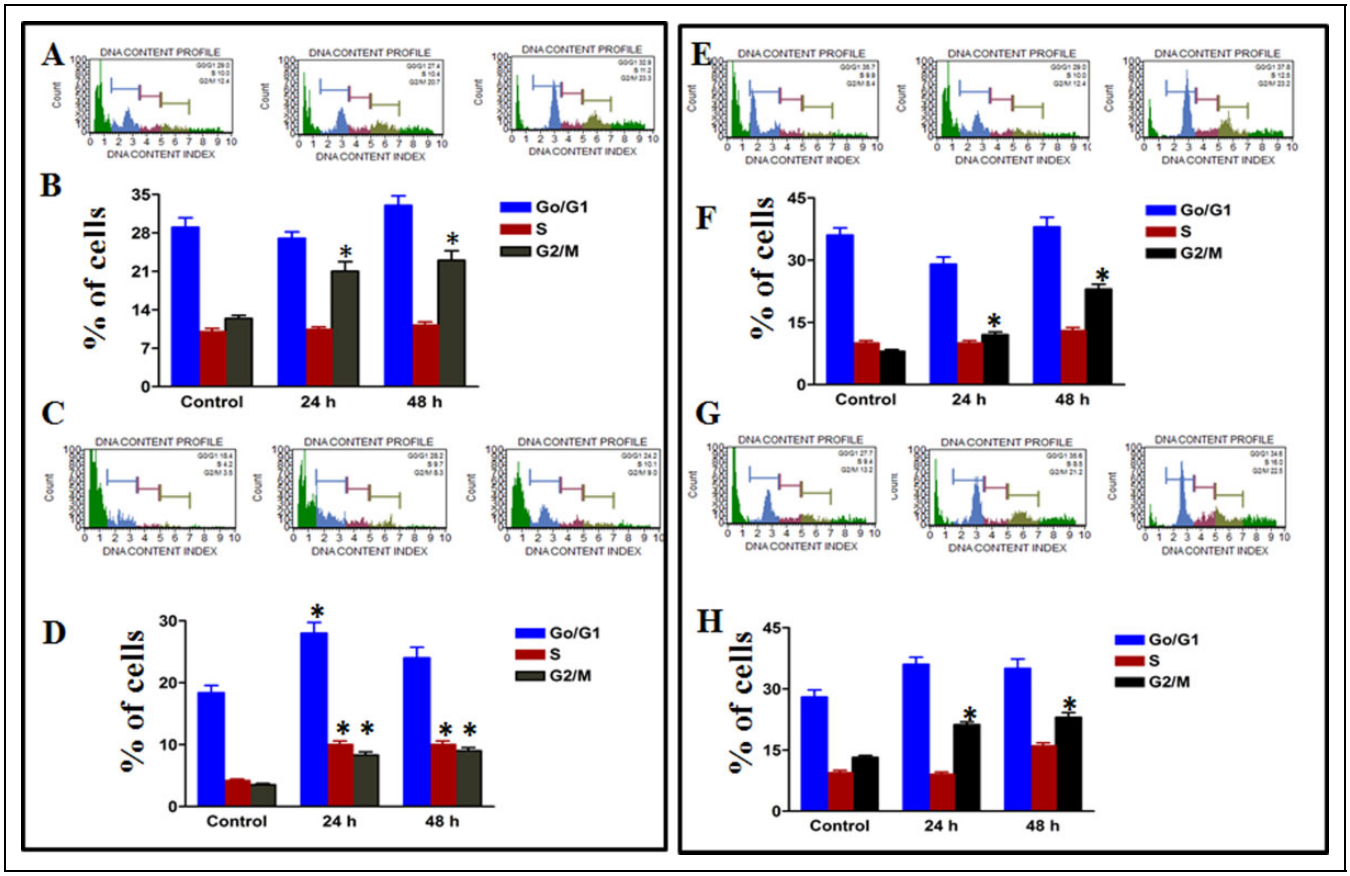

Figure 4. Histogram (left panel) showing cell cycle arrest after venom 1 treatment in (A and C) breast and colorectal cancer cell lines, respectively. Bar graphs (B and D) show the quantitative analyses of the histogram described above. Right panel shows the cell cycle arrest after venom 2 treatment breast and colorectal cancer cell lines (E and G), respectively. Bar graphs (F and H) show the quantitative analyses of the histogram described above. Asterisks mark, statistically significant ($P \leq .05$).

suggesting that hypoxia generates free radicals including ROS in the cancer cells, which ultimately modulates angiogenesis.[30] The next important question is how ROS inhibits cancer cells growth and progression. It is important to note that ROS is a well-known player in the induction of DNA damage and also in altering the drug sensitivity of many cancer types.[31,32] In addition, ROS is also known to disrupt normal protein function, which leads to cell death.[33]

Apoptosis, another hallmark of cancer modulation, is a well-established phenomenon. To examine the effect of scorpion venom on elevated ROS and apoptosis, we performed this assay. As shown in Figure 3, a perceptible increase in apoptotic as well as necrotic cells was detected. The increase in the percentage of apoptotic cells was as high as 40% on venom treatment. In support, stress-related apoptosis is well documented in the literature, including the high apoptotic rate in equine spermatozoa.[34]

Several cellular and architectural alterations take place during the course of programmed cell death (apoptosis) including severe DNA damage as previously described.[19,35] The extent of DNA damage in this study on venom treatment was evaluated by staining the cells with propidium iodide to assess the DNA damage and to analyze the nuclear disruption. As shown in Figure 3, chromatin fragmentation, marginalization, and nuclear swelling were observed vividly on venom treatment in both MDA-MB-231 and HCT-8 cancer cell lines. The extent of necrosis was found to be higher in the HCT-8 cell line, while a higher number of apoptotic cells were detected in MDA-MB-231 cancer cells. These differential findings of the cellular events could be due to the different genetic makeup of the different cancer types. Numerous literatures reveal the role of scorpion venoms inducing apoptosis in various cancer types.[19,27,28,36,37] Interestingly, the species of scorpion venoms that we have chosen for this study has not been previously tested at cellular and molecular levels. Therefore, our study of these venoms against breast and colorectal cancer is unique to date. Furthermore, there is a close association between apoptosis and cell cycle progression.[38]

In this study, we have observed a similar phenomenon of cell cycle arrest in venom-treated MDA-MB-231 and HCT-8 cancer cell lines (Figure 4). We observed the enrichment of G2/M in the breast cancer cell line when treated with venoms 1 and 2 for 24 and 48 hours. The colorectal cancer cell lines showed a different pattern of cell cycle progression when treated with scorpion venoms 1 and 2. All 3 phases of the cell cycle were enriched in venom 1–treated cells, while venom 2 showed

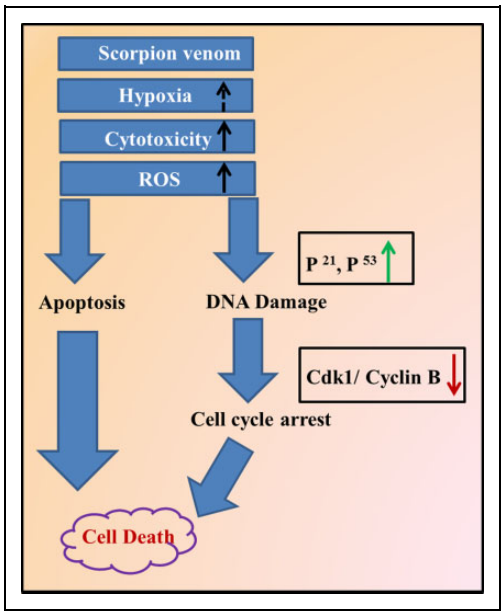

Figure 5. Schema showing the probable mechanism of action of scorpion venom against breast and colorectal cancer cell lines.

selectively for G2/M enrichment only. This variability could also be explained by the genetic makeup of the cells in addition to the efficacy of venoms against the specific cancer cell types. Our results are in good agreement with the previously published work where it has been reported that defects occurs in one or more cell cycle check points depending on the type of the cancer.[39,40] This cell cycle aberration leads to drug resistance, invasion, and cancer metastasis.[25,41] The probable molecular mechanism involved in cancer cell death on venom treatment has been illustrated in the form of a schema (Figure 5).

In summary, the scorpion venom used in this study demonstrated strong potential to inhibit cell cycle progression at different stages in MDA-MB-231 and HCT-8 cancer cell lines. Therefore, it may be postulated that scorpion venom can be used as an alternate tool for the therapy of aggressive colorectal and breast cancer patients.

Conclusion

In this study, we have shown a positive role and great potential for scorpion venoms obtained from 2 different species, namely, *Androctonus crassicauda* and *Leiurus quinquestriatus*, to act as anticancer agents against breast and colorectal cancer cell lines. The venoms induce cytotoxicity, increase the reactive oxygen species, and also enhanced the number of apoptotic cells in these cell lines. Furthermore, a cell cycle halt was also vividly apparent in venom-treated breast and colorectal cancer cells lines. To the best of our knowledge, this is the first study of this kind to describe the successful inhibition of many cancer-related phenotypes using these scorpion venoms.

The implication of this study would be to utilize scorpion venom as an effective and alternate tool of therapy toward more specific treatments for aggressive forms of breast and colorectal cancers.

Acknowledgments

The authors would like to thank the Research Center of Prince Sultan Military Medical City Hospital and King Abdulaziz City for Science and Technology (KACST) for providing the necessary facilities and financial support. Thanks are due to Dr J. C. Lang, the Ohio State University, Columbus, OH, for proofreading of the article.

Author Contributions

ARA, RA, and MI conceived the idea; RA and MI conduct the experiments; MI and RA analyzed the data; RA and MI wrote the article; and MI critically read the article and supervised the study.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Research Center of Prince Sultan Military Medical City Hospital and King Abdulaziz City for Science and Technology.

Ethical Approval

Approval was obtained from the Research Ethics Committee of Prince Sultan Military Medical City, Riyadh, KSA: 1a/2013.

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