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Snake venom, bee venom and their components exert an anti-cancer effect by triggering apoptosis and cell cycle arrest in prostate cancer

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Original
Article

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ABSTRACT

Background: Snake venom (SV), bee venom (BV) and their bioactive components are unique sources for cancer therapy development. The present study evaluated the anticancer potential of SV, BV and their major components (SV phospholipase A2 (svPLA2), melittin (MEL) and BV phospholipase A2 (bvPLA2)) against human prostate adenocarcinoma (PC3).

Materials and methods: Cytotoxicity was conducted using MTT biochemical assay. Genotoxicity was performed using real time PCR for detection of pro-apoptotic and anti-apoptotic genes as well as the biomarker genes for prostate cancer. Cell arrest accumulation was highlighted using flowcytometry.

Results: MTT assay showed that treatment with SV and BV and their major components resulted in cellular morphological changes and significant cytotoxic effects in PC3. Furthermore, our results indicate that the svPLA2 gives much lower cytotoxic effect than the crude SV in PC3 cells in the highest tested concentration of 100 µg/ml. On the other hand, the major components of BV (bvPLA2 and MEL) showed more potent efficacy on PC3 cells than the crude BV. Interestingly, we showed that SV, svPLA2, BV, bvPLA2 and MEL suppressed the mRNA expression of the anti-apoptotic protein Bcl2, while increased the mRNA expression of the pro-apoptotic protein Bax. Moreover, they decreased the overexpressed prostate tumor marker genes. The cell cycle analysis showed that SV and svPLA2 arrested the cell cycle at G0/G1 phase, while BV, bvPLA2 and MEL arrested cell cycle at G2/M phase.

Conclusion: Our work demonstrated that SV, BV and their major components inhibit prostate cancer possibly via triggering apoptosis and cell cycle arrest.

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Key Words: Apoptosis, bee venom, cell cycle, prostate cancer, snake venom.

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1. INTRODUCTION

Prostate cancer is the most common cancer, as well as the second leading cause of cancer-associated mortality in men worldwide^[1]. Although the use of chemotherapeutics in cancer therapy remains the predominant option for clinical control, the development of resistance and associated severe side effects are common and critical problems^[2]. Therefore, there is an urgent need to develop novel safe and effective therapeutic agents to fight this disease. Recently, several diary compounds have been at the forefront research of new therapies against prostate cancer.

Apoptosis is the process of programmed cell death which has a critical role in anti-cancer effects of chemotherapeutics^[3]. Evidence shows that the failure of cells to undergo apoptosis has been associated with development of resistance to cancer therapy^[4,5]. Therefore,

therapeutic agents that target the apoptotic pathway, including pro-apoptotic proteins activators or inhibitors of anti-apoptotic proteins, may lead to the regression of cancer cells and an improved prognosis of refractory disease^[6].

The biodiversity of venoms made them a unique source from which new therapeutic agents may be developed. Bee venom (BV) contains a variety of different peptides including melittin (MEL), phospholipase A2 (bvPLA2), apamin, adolapin and mast cell-degranulating peptide^[7]. BV has been used as a traditional medicine to treat a variety of conditions, such as back pain, rheumatism, cancerous tumors, and skin diseases by its antibacterial, antiviral, and anti-inflammatory effects^[8]. Several studies have revealed that BV and its components; MEL and bvPLA2, have anti-proliferative effect on various cancer cells such as prostate^[9], ovarian^[10], liver^[11], cervical^[12] and renal^[13] cancer cells through induction of apoptosis.

Snake venom (SV) is a complex mixture of enzymes, peptides, carbohydrates, minerals and proteins of low molecular mass with specific chemical and biological activities^[14]. Components of SVs can be used in the treatment of cancer, arthritis, thrombosis, multiple sclerosis, pain, neuromuscular disorders, blood and cardiovascular disorders, infections and inflammatory diseases^[15]. SV components including phospholipase A2 (svPLA2) basically inhibit cell proliferation and promote cell death by increasing or decreasing the expression of proteins that control cell cycle, inducing cancer cell apoptosis thereby controlling tumor size and cytotoxicity^[16]. However, the comparison of the anticancer activities of crude SV, BV and their major components in prostate cancer cell lines has not been reported yet. Therefore, the aim of the present study was to evaluate the anticancer activities of the crude SV, BV and their major components (svPLA2, melittin and bvPLA2) against human prostate adenocarcinoma (PC3) and to explore the effects of these tested compounds on the prostate adenocarcinoma biomarker genes; namely prostate-specific antigen (PSA) and prostate cancer antigen 3 (PCA3), and the expression levels of the apoptotic genes as well as on the cell cycle of the tested tumor cells.

MATERIALS AND METHODS

Chemicals

RPMI 1640 medium, L-glutamine, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO) and other cell culture materials were all purchased from Fisher Scientific Cell Culture (Houston, TX, USA). Fine chemicals and reagents, unless otherwise specified, were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Culture cells and venoms

Human prostate adenocarcinoma cells; PC3 cells (ATCC number: HTB-22), were obtained from The Egyptian Holding Company for Production of Vaccines and Sera and Drugs (VACSERA)-Egypt. PC3 cells were cultured in RPMI medium supplemented with 10 % FBS, 1 % penicillin/streptomycin and 2 mM L-glutamine. The cell cultures were maintained at 37 °C and 95 % humidified atmosphere with 5 % CO₂. SV Naja haje (Egyptian Cobra) and BV from *Apis mellifera* (honey bee) lyophilized powder were purchased from VACSERA. svPLA2 from Naja haje (Egyptian Cobra), bvPLA2 and MEL from *Apis mellifera* (honey bee) lyophilized powder were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Cytotoxicity assay

Cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were plated at concentration of 2×10⁵ cells/ml in the 96-well tissue culture plate with 100 µL of RPMI medium. The cells were treated with different concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) of SV, BV, svPLA2, bvPLA2, MEL and doxorubicin (DOX) for 24 h at 37°C

to determine their IC₅₀s (the concentration of the drug required to produce 50 % inhibition of cell growth). For each sample, three wells were used for each concentration. After each incubation time, 50 µL MTT salt (5 mg/ml) were added to each well in the dark condition and incubation continued for 4 h. Then, the supernatants were discarded and the formazan precipitates were solubilized by addition of DMSO (50 µl) to each well. The optical densities (ODs) were measured using ELISA plate reader (ELx-800, Biotek, USA) at wavelength 570 nm. Cell viability percent was calculated as (OD of treated cells / OD of untreated cells) × 100. The IC₅₀ value of each venom was calculated using MasterPlex QT Quantitation Software (MiraiBio, Alameda, CA). Morphological changes of treated PC3 cells with each venom were examined 24 h post treatment using an inverted phase contrast microscope and finally morphological events were compared with untreated cells.

Real-time PCR analysis

Total RNA was extracted from control and treated PC3 cells using the GeneJET RNA purification kit (Fermentus, UK) and the purity of the obtained RNA was confirmed spectrophotometrically by OD 260/280 nm. First-strand cDNA was synthesized with 1 µg of total RNA using an RT-PCR kit (#K1621, Fermentas, Waltham, MA, USA) in accordance with the manufacturer's instructions. To access the mRNA expression of Bax, Bcl-2, PSA and PCA3 genes, RT-PCR was performed using QuantiTect SYBR Green PCR kits (Qiagen, Germany). The primer sequences used are described in Table 1. Real-time PCR mixture consisted of 12.5 µL 2x SYBR Green PCR Master Mix, 1 µL of each primer (10 pmol/µL), 2 µL cDNA and 8.5 µL RNase-free water in a total volume of 25 µL. The thermal cycler protocol consisted of an initial enzyme activation at of 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. Melting curves were done after real-time PCR to validate the specific amplification of single products of interest. The relative expression of the target gene was obtained using the comparative 2^{-ΔΔCT}^[17]. All values were normalized to that of β-actin and presented as fold-change.

Table 1: The primer sequences used for real-time polymerase chain reaction:

Gene	Primer sequences
Bax	F: 5'-ATGGACGGGTCCGGGGAGCA-3' R: 5'-CCCAGTTGAAGTTGCCGTCA-3'
Bcl-2	F: 5'-GTGAACTGGGGGAGGATGT-3 R: 5'-GGAGAAATCAAACAGAGGCC-3'
PSA	F: 5'-GACCACCTGCTACGCCTCA-3' R: 5'-GGAGGTCCACACTGAAGTTTC-3'
PCA3	F: 5'-AAAGGAAGCAGAGATCCCCTG-3' R: 5'-GGGGAGGCTCATCGAT-3'
GAPDH	F: 5'-CAAGGTCATCCATGACAACCTTG-3' R: 5'-GTCCACCACCTGTTGCTGTA G-3'

Cell cycle analysis using flow cytometry

PC3 cells precultured in 25 cm² cell culture flasks were treated with an IC₅₀ of test venom dissolved in RPMI-1640 medium, for 24h. Only adherent cells were harvested, washed with cold PBS, and fixed gently with 70 % ethanol. After ensuring fixation, the cells maintained at temperature of 4°C overnight and then incubated with PBS containing 40 µg/ml PI, 0.1 mg/ml RNase and 0.1 % Triton X-100 in a dark room at 37°C for 30 min. Cell cycle analysis was measured by a flow cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm.

Statistical analysis

The data are presented as mean ± SD from three different experiments and comparisons between means were carried out using one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. The level of significance was fixed at $P < 0.05$ for all statistical tests using SPSS Program.

RESULTS

The cytotoxicity of SV, svPLA2, BV, bvPLA2, MEL and DOX on PC3 cells

To evaluate the effects of SV, svPLA2, BV, bvPLA2, MEL and DOX on the viability of PC3 cells, MTT assay was carried out. PC3 cells were treated with serial concentrations (0.01, 0.1, 1, 10 and 100 µg/ml) of the test products for 24 hours. As shown in (Figure 1), PC3 cell viability was significantly decreased in a dose-dependent manner by all treatments to 2.08 %, 15.59 %, 1.82 %, 1.63 %, 1.17 %, and 17.62 % at 100 µg/ml SV, svPLA2, BV, bvPLA2, MEL and DOX, respectively. The IC₅₀ values for SV, svPLA2, BV, bvPLA2, MEL and DOX were 0.06, 0.84, 0.09, 0.15, 0.11, and 0.42 µg/ml, respectively. The tested venoms and their components were almost efficient as DOX, a currently used anti-cancer drug.

Regarding the morphological changes, it was noticed that the control cells exhibited polygonal shape with distinct boundaries and homogenous or slightly granulated cellular contents. On the other hand, cells incubated with test products at IC₅₀ concentration showed a number of morphological abnormalities after 24 h of treatment. Treated cells started to show gradual cell shrinkage, cell rounding, detaching from the surface of tissue culture flasks and floating in the tissue culture medium, and finally they showed obvious cell swelling and rupture (Figure 2).

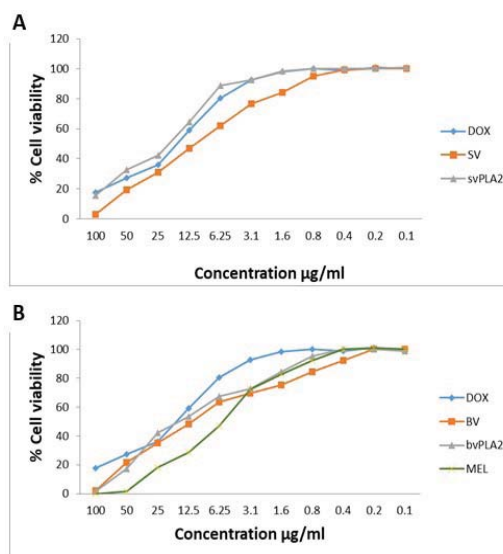


Figure 1: Drug The cytotoxicity of (A) DOX, SV and sv-PLA2 and (B) DOX, BV, bvPLA2 and MEL on PC3 cells. Cells were seeded in 96-well tissue culture plates and treated after 24 h with different concentrations of SV, BV, svPLA2, bvPLA2, MEL and DOX. Percent of cell viability was calculated in relation to the untreated control using MTT colorimetric assay method.

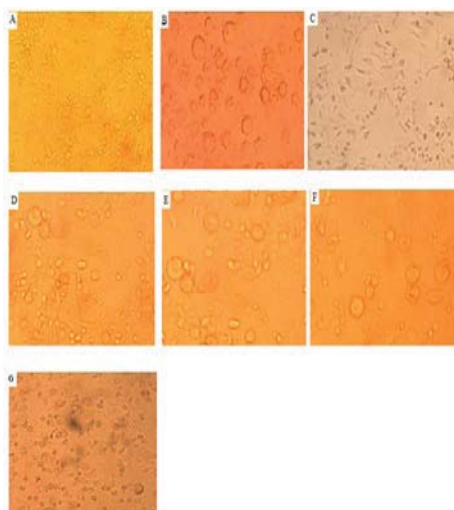


Figure 2: Morphological changes of (A) control untreated PC3 cells and treated PC3 cells for 24 h with IC₅₀ concentration of (B) SV, (C) BV, (D) bv-PLA2, (E) sv-PLA2, (F) MEL and (G) DOX using inverted microscope.

Effect of SV, svPLA2, BV, bvPLA2, MEL and DOX on the expression of apoptosis related genes

As shown in (Figure 3), all treatments showed a trend of up regulation of Bax and down regulation of Bcl2 gene expression levels. It was noticed that 24 h treatment with IC₅₀ concentration of SV, svPLA2, BV, bvPLA2, MEL and DOX increased the pro-apoptotic Bax gene expression to reach approximately 3.9-fold, 3.8-fold, 4.4-fold, 3.7-fold, 6.2-fold and 7.9-fold, respectively as compared to the control value. Regarding the anti-apoptotic Bcl2 gene expression, treatment with SV, svPLA2, BV, bvPLA2, MEL and DOX decreased its expression by 49 %, 33 %, 40 %, 13 %, 72 % and 96 %, respectively.

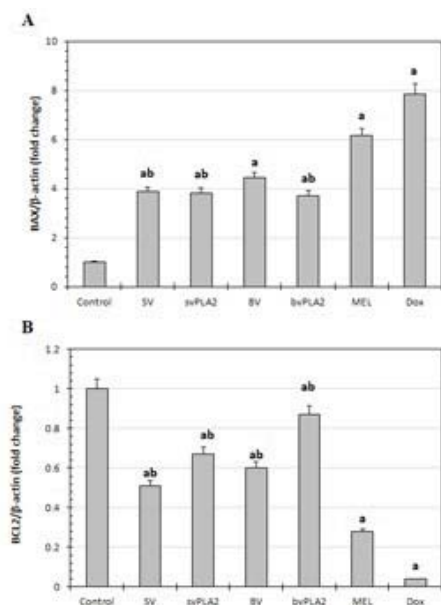


Figure 3: Effect of treatment of PC3 cells for 24 h with IC₅₀ concentrations of SV, svPLA2, BV, bvPLA2, MEL and DOX on mRNA expression level of apoptosis related genes: (A) Bax and (B) Bcl2. Data are expressed as mean \pm SD of three independent experiments. a: Significantly different from control at $P < 0.05$. b: Significantly different from DOX at $P < 0.05$.

Effect of SV, svPLA2, BV, bvPLA2 and MEL on the expression prostate cancer biomarkers (PSA and PCA3)

As illustrated in (Figure 4), incubation of PC3 cells with IC₅₀ concentrations of SV, svPLA2, BV, bvPLA2, MEL and DOX for 24 h reduced PSA gene expression levels by 57 %, 31 %, 70 %, 63 %, 27 %, 31 %, 10 % and 79 %, respectively as compared to the control value. In the meantime, all

treatments decreased PCA3 gene expression levels by 27 %, 31 %, 70 %, 63 %, 27 %, 31 %, 10 % and 79 %, respectively as compared with the control value.

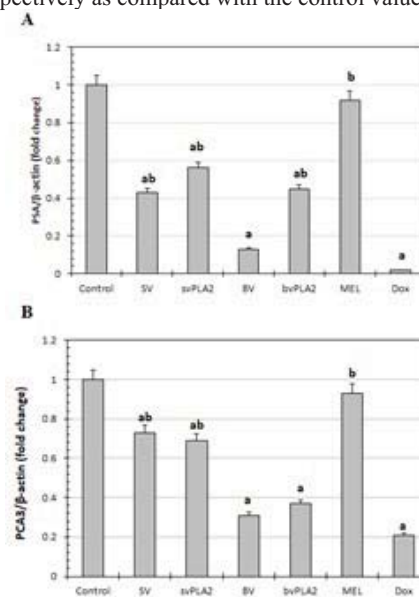


Figure 4: Effect of treatment of PC3 cells for 24 h with IC₅₀ concentrations of SV, svPLA2, BV, bvPLA2, MEL and DOX on mRNA expression level of prostate cancer biomarkers: (A) PSA and (B) PCA3. Data are expressed as mean \pm SD of three independent experiments. a: Significantly different from control at $P < 0.05$. b: Significantly different from DOX at $P < 0.05$.

Cell cycle analysis using flow cytometry

Cell cycle analysis have demonstrated that there was an accumulation of arrested cells in the G2/M phase in PC3 cell lines after treatment with SV, svPLA2, BV, bvPLA2, MEL and DOX. In the meantime, the arrest of treated cells was accompanied by increased apoptotic population compared with that of control cells (Figures 5 and 6). The distribution of the treated cancer cells with tested compounds in different phases of the cell cycle showed that SV and svPLA2 arrested the cell cycle at G0/G1 phase, which represented the DNA content in tumor cells, 24 hours post treatment. The cell cycle arrest at G0/G1 phase prevent the initiation of DNA synthesis and consequently replication of tumor cells and this was indicated and represented by the decreased percentages of S phase in treated cancer cells compared to their controls. BV, bvPLA2 and MEL arrested cell cycle at G2/M phase which represented DNA damage that can be repaired so cells failed to enter mitosis (Figure 7).

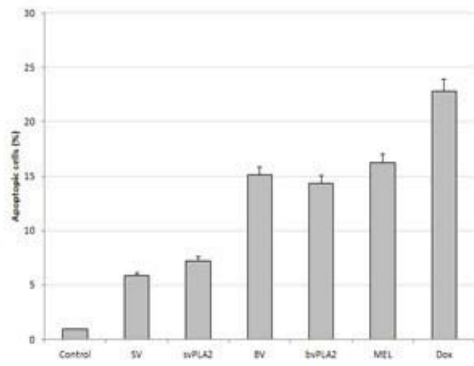


Figure 5: Evaluation of apoptotic profile of human prostate cancer cell line (PC3) post treatment with test products by flowcytometry.

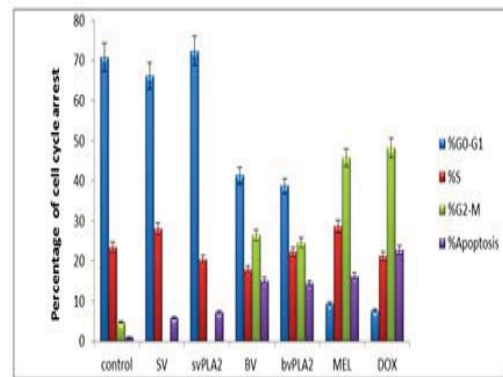


Figure 6: The percent of cell arrest at each of the cell cycle phases of PC3 cells post treatment with test products.

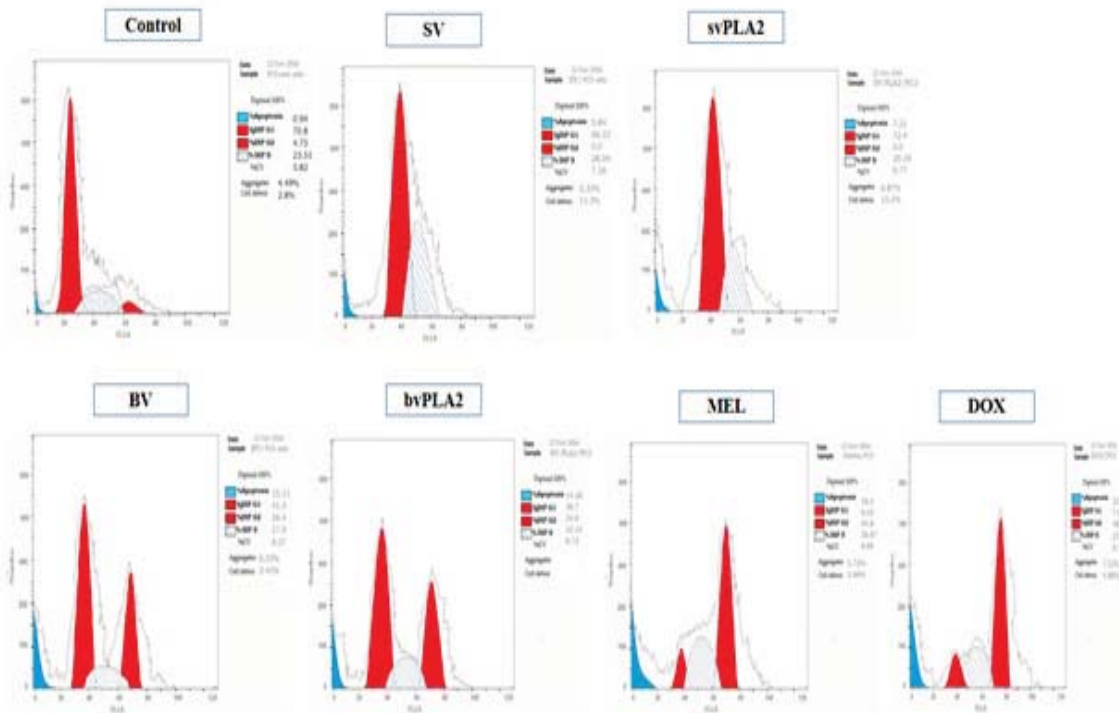


Figure 7: Effects of crude venoms and their components on cell cycle of PC3 cancer cells, post 24 h treatment. Flowcytometric analysis used to illustrate the distribution of G0/G1, S and G2/M phases.

DISCUSSION

Natural compounds are one of the attractive options for treatment of prostate cancer due to the prevalence of this type of cancer in elderly patients and the long latency between pre-malignant lesions and clinically evident cancer^[18]. Although, snake and bee venoms and their components were reported to have potential cytotoxic effects on various types of cancer cells^[16, 19], there is no information on their cytotoxicity as crude venoms compared with their major components. Accordingly, the aim of the present study was to evaluate the effect of crude SV, BV and their major components (svPLA2, bvPLA2 and MEL) on prostate cancer cell line PC3 and to clarify the mechanisms that could be implicated in these effects.

In the present study, MTT assay showed that treatment with SV and BV and their major components (svPLA2, bvPLA2 and MEL) resulted in cellular morphological changes and significant cytotoxic effects in the prostate cancer cell lines PC3. Interestingly, our data showed that the viability of PC3 cells was decreased with increase of SV, BV, svPLA2, bvPLA2 and MEL concentrations in a dose dependent manner. Furthermore, our results indicate that the svPLA2 gives much lower cytotoxic effect than the crude SV in PC3 cells in the highest tested concentration of 100 µg/ml. On the hand, the major components of BV (bvPLA2 and MEL) showed more potent efficacy on PC3 cells than the crude BV.

Our results are in accordance with previous studies which reported that SV can inhibit human prostate cancer cell growth^[19,20]. Interestingly, it has been suggested that cystatin isolated from SV is a potent anti-metastatic agent that contribute to the inhibition of tumor invasion^[21]. In addition, it is well documented that venoms of snakes belonging to the genera *Naja* contain cytotoxins that can readily penetrate into cancer cells and markedly accumulate in lysosomes causing lysosomal leakage and plasma membrane injury^[22]. svPLA2s are low molecular weight enzymes that have different isoforms depending on their geographical source and species of snakes^[23]. svPLA2 displays cytotoxic effects against various tumor cell lines^[16]. It was previously reported that svPLA2s inhibit adhesion and migration of tumor cells^[24, 25].

Our recorded data is supported by previous studies which demonstrated that BV, bvPLA2 and MEL inhibit cell proliferation in prostate cancer cells in a concentration and time-dependent manner^[9, 11, 26]. MEL is the main active pharmacological component of BV that disturbs the integrity of membrane phospholipid bilayers by pore formation together with the leakage of atomic ions and molecules and the enhancement of permeability that eventually leads to cell lysis^[27]. bvPLA2 enzymes can influence cytotoxicity of tumor cells by various mechanisms including hydrolysis and digestion of cell membrane components and disruption of the lipid bilayers integrity^[28].

Apoptosis is the process of programmed cell death that plays important roles in anti-cancer effects in various types

of cancer^[29]. Apoptosis occurs via two main pathways, the extrinsic and the intrinsic pathway^[30]. Both pathways lead to the activation of caspases, which induce cell death^[31]. The regulation of apoptotic intrinsic pathway occurs through members of the Bcl2 family proteins that can either be pro-apoptotic or anti-apoptotic^[32]. Bcl2 is an anti-apoptotic protein that prevents cell death and apoptosis whereas Bax is a pro-apoptotic protein that stimulates apoptosis^[33]. Bcl2 blocks while Bax induces the release of apoptogenic factors, which in turn stimulate initiator caspases leading to activation of the executioner caspase-3^[32]. Herein, we showed that SV, svPLA2, BV, bvPLA2 and MEL suppressed the mRNA expression of the anti-apoptotic protein Bcl2, while increased the mRNA expression of the pro-apoptotic protein Bax. Thus, SV, BV and their components may induce an alteration of the expression of apoptosis regulatory proteins to provide an advantageous circumstance for the cancer cells to reach death status. These data are in harmony with a study reporting that crude cobra SV could aid in suppressing human breast and liver carcinoma progression via increasing the expression of Bax and decreasing the expression of Bcl2^[34]. In addition, svPLA2 showed their antitumor effect upon variety of tumor cells via induction of apoptosis^[35]. Similarly, BV, bvPLA2 and MEL are reported to induce apoptosis through a decrease in Bcl2 and an increase in Bax expression^[34, 36-38].

Screening using PSA is characterized by low specificity for prostate cancer^[3]. PSA levels may be elevated in benign conditions such as benign prostatic hyperplasia or prostatitis^[39]. On the other hand, PCA3 is a genetic marker with high specificity for prostate cancer because it is typically overexpressed by cancer cells^[39, 40]. The data of the present study showed that SV, svPLA2, BV, bvPLA2, MEL reduced PSA and PCA3 gene expression. To our knowledge the current study is the first to investigate the possibility of using the tested venoms or their components as anticancer agents for prostate cancer through decreasing the overexpressed prostate tumor marker genes PSA and PCA3.

Cell cycle is a pathway that enhances cell death post exposure to toxins and determines the mechanism by which these pathways are regulated, thus cell cycle arrest is targeted as an intervention against cancer^[41]. In our study, the cell cycle analysis showed that SV and svPLA2 arrested the cell cycle at G0/G1 phase compared to control cells, 24 hours post treatment. The increased number of cells in G0/G1 phase point out to the greater incidence of apoptotic cells post treatment and prevention of the initiation of DNA synthesis and consequently replication of tumor cells^[42]. Previous studies showed that SV arrested the cell cycle at G0/G1 phase in colon and prostate cancer cell lines^[37] and in breast and liver cell lines^[34]. On the other hand, BV, bvPLA2 and MEL arrested cell cycle at G2/M phase. In line with our results, BV and MEL were shown to arrest the cell cycle at G2/M phase in breast and liver cell lines^[34].

In conclusion, our work demonstrated that SV, BV and their major components (svPLA2, bvPLA2, MEL) inhibit prostate cancer possibly via triggering apoptosis and cell cycle arrest. Future studies will help estimate the potential of SV, BV and their major components as anticancer agents for the prevention of human prostate cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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