

Anti-proliferative Effects of *Androctonus amoreuxi* Scorpion and *Cerastes cerastes* Snake Venoms on Human Prostate Cancer Cells

SHORT
COMMUNICATION

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The present study evaluated the effects of *Androctonus amoreuxi* scorpion venom, *Cerastes cerastes* snake venom and their mixture on prostate cancer cells (PC3). An MTT assay was used to determine the anti-proliferative effect of the venoms, while quantitative real time PCR was used to evaluate the expression of apoptosis-related genes (*Bax* and *Bcl-2*). Furthermore, colorimetric assays were used to measure the levels of malondialdehyde (MDA) and antioxidant enzymes. Our results show that the venoms significantly reduced PC3 cell viability in a dose-dependent manner. On the other hand, these venoms significantly decreased *Bcl-2* gene expression. Additionally, *C. cerastes* venom significantly reduced *Bax* gene expression, while *A. amoreuxi* venom and a mixture of *A. amoreuxi* & *C. cerastes* venoms did not alter *Bax* expression. Consequently, these venoms significantly increased the *Bax/Bcl-2* ratio and the oxidative stress biomarker MDA. Furthermore, these venoms also increased the activity levels of the antioxidant enzymes, catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase. Overall, the venoms have cytotoxic and anti-proliferative effects on PC3 cells.

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Key Words: Venoms, Apoptosis, *Bcl-2* family proteins, *Bax/Bcl-2* ratio, Oxidative stress

INTRODUCTION

Scorpion venom is a natural product that is a complex mixture of molecules and plays an important role in defense and prey capture. Some scorpion venoms show cytotoxic effects against a wide range of cancer cell lines. Indian black scorpion (*Heterometrus bengalensis*) venom has anti-proliferative, cytotoxic, and apoptogenic activities against human leukemic cell lines U937 and K562.¹ *Rhopalurus junceus* scorpion venom induces apoptosis in HeLa cells and necrosis in A549 cells, which is mainly via modulation of apoptosis gene expression.²

Another class of natural product is snake venom toxin, which has been reported to possess cytotoxic effects against cancer cell lines. A heat stable protein toxin (drCT-1) from Indian *Daboia russelli russelli* venom has cytotoxic and apoptotic properties

against human leukemic cells (U937 and K562), and it significantly decreases Ehrlich ascites carcinoma cell viability.³ Venom extracted from the *Walterinnesia aegyptia* snake in combination with silica nanoparticles down-regulates the expression of the *Bcl-2* gene, enhances the activation of caspase-3, and induces apoptosis in human breast carcinoma cell lines (MDA-MB-231 and MCF-7).⁴

Apoptosis is regulated by various protein families, including the *Bcl-2* family and the caspase family. Anti-apoptotic *Bcl-2* family genes (e.g., *Bcl-2*, *Bcl-XL*, and *Mcl-1*) and pro-apoptotic (e.g., *Bax* and *Bak*) genes are demonstrated to be important proteins functioning in the apoptotic process or clinical cancer therapy.⁵ The *Bcl-2* family plays an important role in the activation of caspases and dominates the intrinsic pathways of apoptosis. Additionally, agents that cause oxidative stress are considered as

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mediators of apoptosis. Some studies have reported that the exposure to toxins/venoms increases the levels of oxidative markers.^{6,7} Consequently, understanding the changes occurring in the expression of apoptosis-related genes and oxidative stress biomarkers and antioxidant enzyme activity levels is important for understanding the mechanisms of apoptosis in cancer cell lines.

The present study evaluated the effects of scorpion venom from *Androctonus amoreuxi*, snake venom from *Cerastes cerastes*, and a mix of the venoms from *A. amoreuxi* & *C. cerastes* on cell viability. In addition, we measured apoptosis-related genes (*Bax* and *Bcl-2*), the *Bax/Bcl-2* ratio, malondialdehyde (MDA) levels, and the changes in antioxidant enzyme activities.

MATERIALS AND METHODS

1. Tumor cell line

The human prostate cancer cell line (PC3) was provided by VACSERA (Giza Governorate, Egypt). The cells were maintained at 37°C in a humidified incubator with 5% CO₂ and RPMI 1640 media with L-glutamine (Lonza, Verviers, Belgium), which was supplemented with 10% heat inactivated FBS (Biowest, Nuaille, France).

2. Venom source

Scorpion venom from *A. amoreuxi* and snake venom from *C. cerastes* were obtained from VACSERA (Egypt).

3. In vitro cell viability assay

The effect of the venoms studied (scorpion venom *A. amoreuxi*, snake venom *C. cerastes*, and a mix of *A. amoreuxi* & *C. cerastes* venoms [mixed 1:1]) on PC3 cell viability was determined by an MTT assay.⁸ PC3 cells were seeded in 96-well plates (Costar[®], Corning, Switzerland) at a density of 1×10^4 cells/well and incubated for 24 hours at 37°C in a humidified incubator with 5% CO₂. After incubation, the media was replaced with 100 µL/well of different concentrations of each venom or their mixture (0.39, 1.56, 6.25, 25, 100 µg/mL) dissolved in serum-free medium. After 24 hours of treatment, the medium was replaced with 100 µL/well of the MTT solution (0.5 mg/mL in PBS), followed by incubation for 3 to 4 hours at 37°C. The supernatants were carefully removed, and the formazan crystals were solubilized with dimethyl sulfoxide (150 µL/well). The plates were gently shaken for 15 minutes at 37°C. Absorbance was determined at 570 nm with a microplate reader (Bio Tek, Winooski, VT, USA). The percentage of viability was calculated using the following

formula: Viability (%) = (A_{570} of treated cells / A_{570} of control cells) × 100.

4. RNA isolation and cDNA Synthesis

After treatment of the PC3 cells with the IC₅₀ (median inhibitory concentration) values of the venoms for 24 hours at 37°C in a humidified incubator with 5% CO₂, the cells were harvested. The total RNA was isolated from treated and untreated PC3 cells using an RNeasy mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The concentration and purity of the isolated RNA were detected by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in 20 µL reaction mixture containing 10 µL RNA sample (1 µg), 2 µL 10× RT Buffer, 0.8 µL 25× dNTP Mix (100 mM), 2 µL 10× RT random primers, 1 µL MultiScribe Reverse Transcriptase, 1 µL RNase inhibitor, and 3.2 µL nuclease-free water. Reverse transcription was performed using thermal cycler (Applied Biosystems) with the following temperatures and times: Step 1, 10 minutes at 25°C; Step 2, 120 minutes at 37°C; Step 3, 5 minutes at 85°C; then Step 4, hold at 4°C.

5. Quantitative real-time-PCR

SYBR Green quantitative real-time-PCR (qPCR) was performed on cDNA extracted from treated and untreated PC3 cells. The expression of target genes was quantified with SYBR Green PCR master mix using StepOne real-time PCR system (Applied Biosystems). Each qPCR amplification reaction was performed in 20 µL reaction mixture containing 10 µL Power SYBR Green PCR master mix (2×), 2 µL cDNA sample (100 ng), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), and 6 µL double-distilled water. The cycling conditions started with initial 10 minutes at 95°C, followed by 40 two-step cycles of 15 seconds at 95°C and 1 minute at 60°C. The amplification was checked by melting curve analysis. The relative gene expression was calculated using $2^{-\Delta\Delta C_T}$ method,⁹ where $\Delta\Delta C_T$ values of each sample were calculated from C_T values; $\Delta\Delta C_T = [C_T \text{ target gene} - C_T \text{ GAPDH}]_{(\text{treated sample})} - [C_T \text{ target gene} - C_T \text{ GAPDH}]_{(\text{untreated sample})}$. The primers used in the experiments were as follows:^{10,11} *Bax*: F, 5'-GCTGGACAT TGGACT TCCTC-3' and R, 5'-TCAGCCCATCTTCTCCAGA-3'; *Bcl-2*: F, 5'-TGTTGGATGACTGATACCTGAACC-3' and R, 5'-CAGCCAGGAGAAATCAAACAGAG-3'; *GAPDH* (housekeeping gene): F, 5'-CGTCTGCCATCAACTTTTCG-3' and R, 5'-CGTTTCTCAGGCTCCCTCT-3'. The primers were synthesized by LGC Biosearch technologies (Novato, CA, USA).

6. Biochemical analysis of oxidative stress and antioxidant biomarkers

PC3 cells were treated with the IC_{50} values of the venoms for 24 hours in a humidified atmosphere of 5% CO_2 at $37^\circ C$. After that treatment, the cells (floating and adherent cells) were harvested, washed twice with cold PBS (by centrifugation), and pelleted at $2,000 \times g$ for 5 minutes at $4^\circ C$. The cell lysate was prepared by resuspending the cell pellet in a cold PBS, followed by sonication on ice and then centrifugation at $4,000 \times g$ for 15 minutes at $4^\circ C$. The supernatant was used for assaying.

The MDA level and antioxidant enzyme (catalase [CAT], superoxide dismutase [SOD], glutathione peroxidase [GPx], glutathione reductase [GR], and glutathione-S-transferase [GST]) activity levels were, according to the manufacturer's instructions, assayed in treated and untreated PC3 cells using readymade kits provided by Biodiagnostic (Cairo, Egypt) and Milton Roy

spectronic 21D UV-Visible spectrophotometer (USA). The total protein was determined in PC3 cells by the Bradford method.¹² The MDA and the antioxidant enzyme activity levels were calculated per mg protein.

7. Statistical analysis

All experiments were independently performed at least three times. The IC_{50} values were determined using GraphPad Prism7 (GraphPad software, La Jolla, CA, USA). The P values were obtained by comparing the control group versus each treatment group using unpaired Student t -test. The differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The present work evaluated the changes that occurred in a human PC3 cells treated with the venoms studied. Many reports

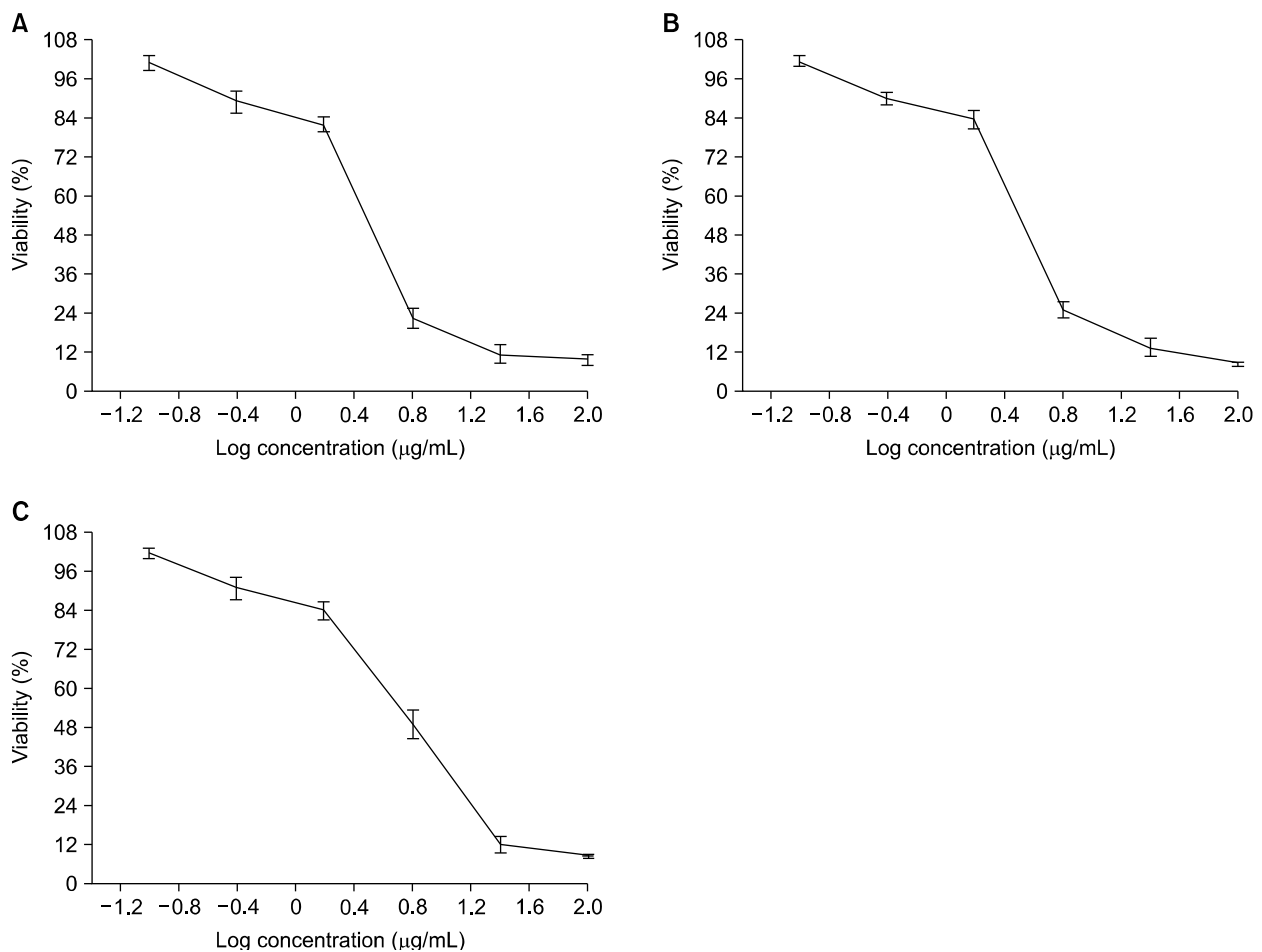


Figure 1. Anti-proliferative effects of (A) Scorpion venom from *Androctonus amoreuxi*. (B) Snake venom from *Cerastes cerastes*. (C) Mix of *A. amoreuxi* & *C. cerastes* venoms on PC3 cells. The cells were exposed to various concentrations (0.39, 1.56, 6.25, 25, 100 µg/mL) of the venoms for 24 hours. The data are expressed as the mean \pm SD.

have demonstrated the cytotoxic, anti-proliferative, and apoptotic effects of different snake and scorpion venoms against different cancer cell lines.^{1,4} This evidence, like our results, supports the potential of these venoms to act against an epithelial cancer cell line. The venom's effect on the viability of PC3 cells was evaluated by an MTT assay. We observed that treatment with these venoms resulted in a dose-dependent decrease in the PC3 cell viability (Fig. 1). The IC₅₀ values for *A. amoreuxi* venom, *C. cerastes* venom, and a mix of *A. amoreuxi* & *C. cerastes* venoms on

PC3 cells were calculated as 3.04, 3.21, and 5.58 µg/mL, respectively, for a 24 hours treatment. The effect of the venom at its IC₅₀ was studied on the expression of apoptosis-related genes (*Bax* and *Bcl-2*). Our results showed that a 24 hours treatment of PC3 cells with these venoms caused a significant down-regulation of *Bcl-2* compared to control cells (*P*value < 0.05; Fig. 2B). These latter results agree with studies that have reported the down-regulating effect of some snake and scorpion venoms on *Bcl-2* gene expression.^{2,13} We also found that *Bax* expression was

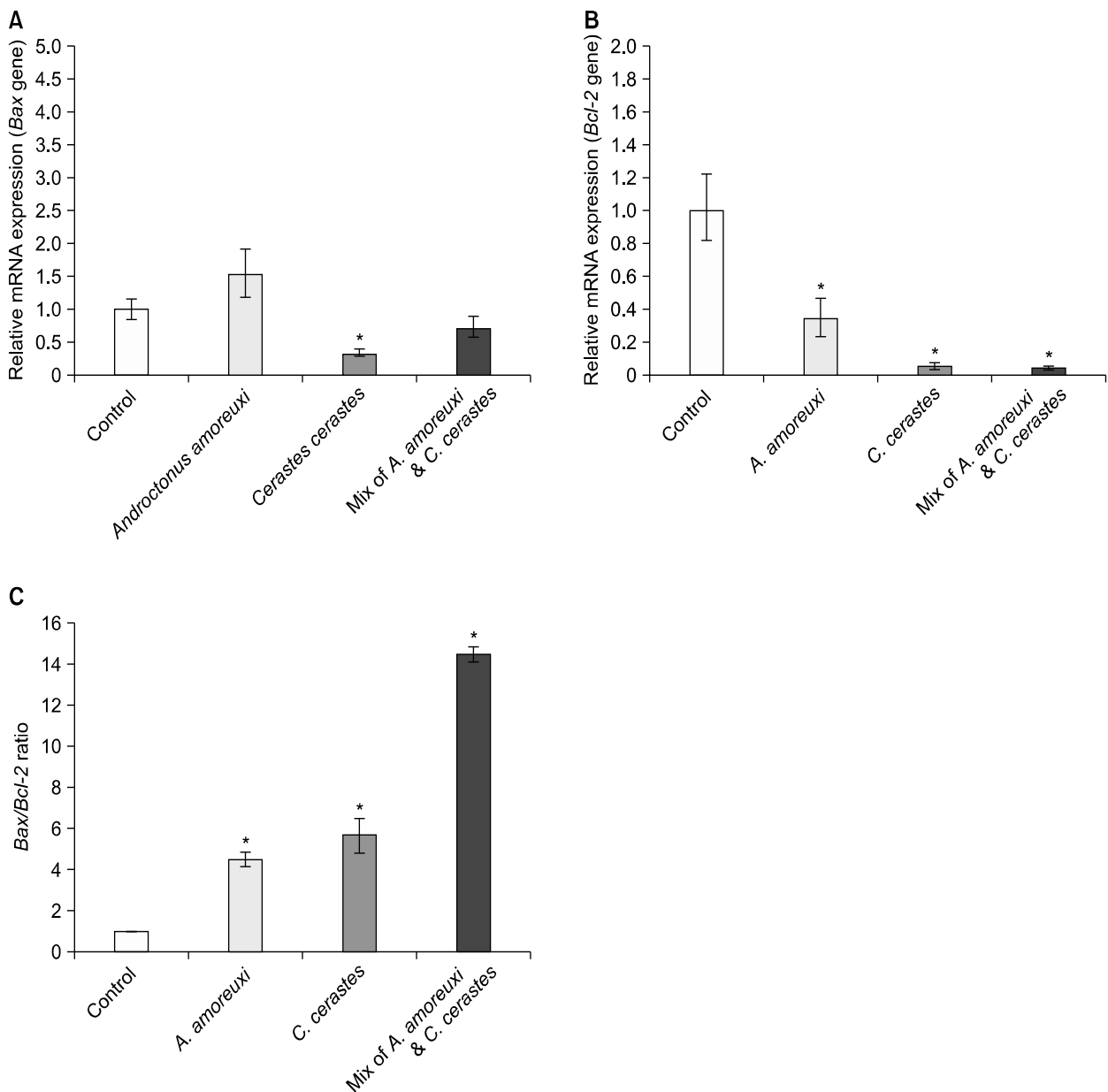


Figure 2. The effects of the venoms studied at the IC₅₀ values on (A) *Bax* gene expression, (B) *Bcl-2* gene expression, and (C) *Bax/Bcl-2* ratio in PC3 cells after 24 hours of treatment. Fig. 2A and 2B represent RQ (relative quantification) values - Error bars are ± RQ_{max}, RQ_{min} - while values of Fig. 2C represent the mean ± SD. *This mark indicates significant difference.

not altered ($P = 0.10$) in the PC3 cells treated with *A. amoreuxi* venom, which agrees with reports that showed a non-significant effect of some scorpion venoms on *Bax*. Díaz-García et al.² reported that *R. junceus* scorpion venom does not alter *Bax* expression and it down-regulates the *Bcl-2* gene in A549 lung carcinoma cells. *Bax* expression was significantly decreased ($P = 0.01$) after *C. cerastes* venom treatment, which is consistent with data that showed the down-regulating effect of some venoms on pro-apoptotic and anti-apoptotic genes. For example, Pang et al.¹⁴ reported the decreases in both *Bax* and *Bcl-2* genes in HCT-8 cancer cells after treatment with *Agkistrodon acutus* snake venom. Díaz-García et al.² reported that *R. junceus* scorpion venom down-regulates both *p53* and *Bcl-2* genes in A549 cell line. On the other hand, *Bax* was not altered ($P = 0.10$) after a mixture of *A. amoreuxi* and *C. cerastes* venoms was applied. Some studies have reported the up-regulation of *Bax* expression after treatment with snake or scorpion venom,^{15,16} which is in contrast with our results. The effect of the venoms studied on *Bax* expression is presented in Figure 2A.

Androgen-independent prostate cancer cells, such as PC3 cells, show an increase in *Bcl-2*.^{17,18} The down-regulating effect caused by the venoms on *Bcl-2* is noteworthy since *Bcl-2* is a highly conserved member of the *Bcl-2* family, and it constitutes an important regulator of apoptosis. *Bcl-2* inhibits and protects cells from apoptosis by blocking cytochrome *c* release from mitochondria and preventing the activation of the caspase 3 dependent pathway.¹⁹ In contrast, the attenuation of *Bcl-2* may also prove favorable in certain clinical settings to enhance alternative modes of cell death. This is because *Bcl-2* is not only a mediator of apoptosis but is also involved in programmed necrosis.²⁰ *Bax* and *Bcl-2* genes express membrane-bound pore-forming proteins that interact through heterodimerization, and *Bax* binds to *Bcl-2* to counteract its function. The *Bax/Bcl-2* ratio appears more important than the individual *Bax* or *Bcl-2*

level in determining a cell's vulnerability to apoptosis. High *Bax/Bcl-2* ratios lead to greater apoptotic activity.^{21,22} Our results showed that the *Bax/Bcl-2* ratio was significantly increased (P value range from 0.0004 to 0.02; Fig. 2C) after the treatment with the venoms at their IC_{50} concentrations. This was because of the significant down-regulation of *Bcl-2* gene expression, which agrees with studies showing an increase in the *Bax/Bcl-2* ratio after snake or scorpion venom application.^{2,23} The increase in the *Bax/Bcl-2* ratio indicates the involvement of mitochondria-mediated apoptosis after venom treatment since the *Bax/Bcl-2* ratio is recognized as a key factor for apoptosis in cell by regulating cytochrome *c* release from the mitochondria to the cytosol.²⁴

The changes that occurred in oxidative stress were tested in PC3 cells after venom treatment. Generally, oxidative stress is due to the imbalance between the antioxidants and pro-oxidants in favor of the oxidants.²⁵ Venom/toxin-induced oxidative stress results in increased levels of oxidative markers,^{6,7} and it sometimes causes malfunctioning of vital organs through membrane destruction, enzyme release, and protein loss.²⁶ Lipid peroxidation was measured by the MDA level, which is a biomarker of oxidative stress and cellular damage, that is evoked by stressors. A 24 hours treatment of PC3 cells with the IC_{50} of the venoms caused a significant increase in MDA in the cell lysate compared to the control cells (Table 1). This agrees with reports that showed an elevation of lipid peroxidation levels after treatment with various types of venoms,^{27,28} and increased lipoperoxidation may result in membrane damage. In the defense against oxidative stress, the cellular antioxidant enzyme system plays an important role. This system includes CAT, SOD, GPx, GR, and GST (the antioxidant enzymes studied). CAT converts H_2O_2 to H_2O , SOD catalyzes the dismutation of the superoxide radical anion, GPx catalyzes GSH (the reduced form of glutathione) oxidation to oxidized glutathione (GSSG) at the expense of

Table 1. Effect of the venoms at the IC_{50} value on the activity of the antioxidant enzymes and MDA level in PC3 cells

Variable/mg protein	Control cell	PC3 cells treated with		
		<i>Androctonus amoreuxi</i>	<i>Cerastes cerastes</i>	Mix of <i>A. amoreuxi</i> & <i>C. cerastes</i> venoms
CAT (mU)	0.84 ± 0.10	1.49 ± 0.12 (0.02)	2.90 ± 0.33 (0.04)	4.00 ± 0.27 (0.003)
SOD (U)	168.75 ± 0.81	232.20 ± 9.01 (0.03)	385.75 ± 32.78 (0.03)	638.24 ± 41.81 (0.01)
GPx (mU)	4.60 ± 0.31	7.64 ± 0.48 (0.02)	14.05 ± 0.49 (< 0.001)	13.03 ± 0.38 (0.0002)
GR (mU)	17.82 ± 0.43	27.73 ± 0.79 (0.003)	47.29 ± 4.01 (0.03)	86.27 ± 7.34 (0.02)
GST (mU)	3.09 ± 0.10	5.22 ± 0.26 (< 0.01)	8.97 ± 0.29 (0.004)	9.68 ± 0.24 (0.0002)
MDA level (nmol)	4.43 ± 0.20	6.77 ± 0.49 (0.04)	14.17 ± 0.90 (0.01)	16.17 ± 1.13 (0.01)

Values are present as mean ± SE (P value). PC3 cells were treated with the venoms for 24 hours. MDA, malondialdehyde; PC3, prostate cancer cell line; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase, GST, glutathione-S-transferase.

hydrogen peroxide or other organic peroxides. GR recycles GSSG back to GSH using NADPH, and GST catalyzes the conjugation of GSH to xenobiotic substrates for detoxification of nucleic acids. The induction of antioxidant enzymes is necessary for the host defense to protect the cell against oxidative stress. Antioxidant enzymes are working simultaneously to prevent the formation of highly cytotoxic hydroxyl radicals. The antioxidant defense system in PC3 cells was tested in response to venom. Our results show that a 24 hours treatment of PC3 cells with the IC₅₀ of these venoms caused a significant increase in the activity of the antioxidant enzymes in the cell lysate compared to the control cells (Table 1). This agrees with reports that showed an increase in lipid peroxidation level and antioxidant enzymes when using venom as a treatment.^{28,29} In addition, da Silva et al.⁷ reported increased activities of CAT and GST in venom-injected experimental animals, which also agrees with our results. However, in contrast to our results, other studies showed decreases in some antioxidant enzymes in case of treatment with venoms.³⁰

In conclusion, the venoms studied have cytotoxic and anti-proliferative activities against PC3 cells.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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