Journal of
Cancer Science & Therapy

Shebl al., J Cancer Sci Ther 2012, 4.10
DOI: 10.4172/1948-5956.1000161

Research Article Open Access

Cerastes cerastes and *Vipera lebetina* Snake Venoms Apoptotic – Stimulating Activity to Human Breast Cancer Cells and Related Gene Modulation

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Abstract

Apoptosis occurs normally during development and aging as a homeostatic mechanism to maintain cell populations. Dysregulation of apoptosis can disrupt the equilibrium between cell growth and cell death leading to the development of cancer. Thus, the investigation of new biological apoptotic activators could play an important role in cancer therapy. In the present study, *Cerastes cerastes* and *Vipera lebetina* snake venoms were evaluated for their ability to activate apoptosis in cancer cells where test venoms exhibited a concentration and time dependent cytotoxic effect on breast cancer (MCF-7) cells. Typical apoptotic morphological features were demonstrated in venom treated cells detected via transmission electron microscope. In addition, flow cytometric analysis showed an increase in the percentage of apoptotic cells post 24 h treatment relative to venom concentrations. At the molecular level, test venoms induced apoptosis were mediated by up regulation of pro-apoptotic genes (p53 & Bax) and down regulation of anti-apoptotic gene (Bcl-2) in MCF-7 cells, indicating that these venoms could serve as apoptotic stimulators, presenting a novel and potential therapeutic strategy for cancer treatment.

Keywords: *Cerastes cerastes; Vipera lebetina*; Breast cancer cells; p53 gene; Bax gene; Bcl-2 gene

Introduction

Despite significant progress in cancer treatment, cancer remains one of the leading causes of death worldwide. Current conventional cancer therapies such as radio and chemotherapies exert their therapeutic effect by indirectly promoting apoptosis as they induce cellular DNA damage followed by stimulating apoptosis through the intrinsic pathway but they cannot differentiate between malignant and normal cell types [1].

Snake venoms are mixture of numerous proteins and peptides. Several studies have demonstrated the potential of some bioactive compounds from snake venoms as cytotoxic, anti-tumor and apoptosis-inducing agents in different cancer cell lines as well as in some *in vivo* models [2-5]. Components of snake venoms that have already been reported to induce cytotoxicity and apoptosis in cancer cells included atroporin and kaotree isolated from *Crotalus atrox* and *Naja n. kaouthia,* contortrostatin from *Agkistrodon contortrix* and cardiotoxin III from *Naja naja atra* venom [6-9].

Tumor suppressor protein; p53 is one of many proteins that play an important role in activation of the intrinsic pathway of apoptosis. Consequently, mutation of p53 gene, which is observed in as many as half of all human cancers, renders tumor cells resistant to conventional radio and chemotherapies [10]. Understanding of apoptosis has provided the basis for novel targeted therapies that could induce death in both responsive and resistant cancer cells through activation of death receptors on the cell surface or through a series of intracellular events (intrinsic pathway) thus stimulating apoptosis [11].

It was observed that the activity of p53 was implicated in the transcriptional activation of Bax gene that was involved in apoptosis, and also in the alteration of Bcl-2/Bax gene expression ratio, thereby indicating that the relation between these pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins was one of the important factors deciding the fate of cancer cells [12]. In this context, the investigation of novel targeted apoptotic activators in cancer cells would present a new strategy in cancer therapy [13,14]. Accordingly, the present study aimed to evaluate the apoptotic stimulating activity of selected snake venom models and related effect on pro-apoptotic and anti-apoptotic gene expression.

Methods

Cytotoxicity

Cytotoxic effect of *Cerastes cerastes* (*C.cerastes*) and *Vipera lebetina* (*V.lebetina*) snake venoms, kindly supplied from VACSERA Sera Plant-Egypt, was evaluated to breast cancer cell line [MCF-7 cells, ATCC: HTB-22] at 24 and 48 h interval using MTT assay, where test venoms were dissolved in cell culture media (Biowhittaker-Belgium) to contain 10.24 µg/ml and sterile filtered using 0.22 µm syringe filter (Millipore-USA) [15].

MCF-7 cells precultured 96-well plates (Nunc-USA) were treated with descending double fold serially diluted venoms at 37°C for the required time interval. Negative cell control was included. Residual living cells were treated with 25 µl of MTT dye (0.5 mg/ml) (Sigma-Aldrich-USA) at 37°C for 4 h. MTT was discarded. Plates were PBS washed three times. Dimethyl sulfoxide (BDH-England) was added as 50 µl/well. Plates were shaked for 30 min to dissolve the produced

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Received August 10, 2012; **Accepted** September 12, 2012; **Published** September 14, 2012

Citation: Shebl RI, Mohamed AF, Ali AE, Amin MA (2012) *Cerastes cerastes* and *Vipera lebetina* Snake Venoms Apoptotic – Stimulating Activity to Human Breast Cancer Cells and Related Gene Modulation. J Cancer Sci Ther 4: 317-323. doi:10.4172/1948-5956.1000161

intracellular blue MTT-Formazan complex. Optical densities (O.Ds) were measured at 570 nm using an ELISA plate reader (Dynatech-England). Data were reported for three independent experiments as mean ± SD [16].

Viability percentage was calculated as follows according to [17].

$$\text{Cellviability\%} = \frac{\text{O.Doftreatedcells}}{\text{O.Dofuntreatedcells}} \times 100$$

Statistical significance between treated and untreated cells was determined using one way ANOVA. Differences at P values less than 0.05 were considered significant.

IC_{50} of test venoms were determined using prism program.

Morphological changes

Morphological changes detected 24 h post MCF-7 cells treatment with different concentrations of test venoms were investigated using inverted microscope (Hund-Germany) [18].

Apoptosis detection using flow cytometry

Quantitative detection of apoptosis induced by snake venom was assessed using annexin-V apoptosis detection kit (Trevigen-USA). MCF-7 cells treated with *C.cerastes* (0.75, 1.5, 3 µg/ml) and *V.lebetina* (1.25, 2.5, 5 µg/ml) venoms as well as untreated cells were washed with 500 µl cold PBS (1X) and collected by centrifugation. Each sample was incubated in the dark for 15 min with 100 µl annexin-V incubation reagent consisting of: 10 µl binding buffer (10 X), 10 µl propidium iodide, 1 µl annexin V-FITC and 79 µl de-ionized distilled water at room temperature. Samples were treated with 400 µl binding buffer (1 X) and processed using flow cytometer (Beckman Coulter Epics XL–USA) within 1 h for maximal signal. All tests were repeated in triplicates.

Transmission Electron Microscope [TEM]

MCF-7 cells were treated with IC_{50} of test venoms. 24 h post treatment cells were collected, fixed with 2.5% PBS buffered gluteraldehyde (TAAB-UK) for 6 h at 4°C, PBS washed, fixed in 1% osmium tetroxide (TAAB-UK) in PBS for 1 h at 4°C and rewashed again using PBS. Each cell pellet was dehydrated in ascending graded concentrations of cold ethanol solutions, immersed in propylene oxide (ICI-UK) and embedded in equal volumes of epoxy/resin (Sigma-Aldrich-USA) mixture. Ultra-thin sections of cell pellet were cut then stained with uranyl acetate and lead citrate (Sigma-Aldrich-USA) and observed under transmission electron microscope (Jeol-Japan) [19].

RNA extraction

RNA was extracted from MCF-7 cells treated with *C.cerastes* (0.75, 1.5, 3 µg/ml) and *V.lebetina* (1.25, 2.5, 5 µg/ml) venoms in addition to untreated control cells post 24 and 48 h treatment according to manufacturer's protocol using SV total RNA isolation system (Promega-Germany). Cells were collected and PBS (ice-cold, sterile) washed twice. RNA lysis buffer (175 µl) and RNA dilution buffer (350 µl) were added to cell pellet, mixed by inversion and heated for 3 min at 70°C. Cells were centrifuged at 14000 rpm for 10 min. The clear lysate was transferred to clean tube and 200 µl of 95% ethanol were added. The mixture was transferred to spin basket assembly and centrifuged for 1 min. RNA wash solution was added as 600 µl, centrifuged for 1 min followed by 50 µl of DNase incubation mix [40 µl Yellow Core Buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl DNase I enzyme] and incubated at room temperature for 15 min. Reaction was stopped by adding 200 µl of DNase stop solution followed by centrifugation for 1 min. Each spin basket was first washed with 600 µl of RNA wash solution, centrifuged for 1 min followed by second wash with 250 µl of RNA wash solution and centrifuged for 2 min to ensure removal of RNA impurities. Finally RNA was eluted using 100 µl of nuclease free water. Extracted RNA was stored at – 70°C.

Reverse transcription and polymerase chain reactions

Extracted RNA was reverse transcripted to cDNA using RevertAid first strand cDNA synthesis kit (Fermentas–Lithuania) where extracted RNA (1 µg), random hexamer primer (1 µl) and DEPC-treated water (to 12 µl) were incubated at 65°C for 5 min. Reaction buffer (4 µl, 5 X), 1 µl ribolock RNase inhibitor (20 U/µl), 2 µl dNTP Mix (10 mM) and 1 µl reverse transcriptase (200 U/µl) were added and incubated at 25°C for 5 min followed by thermal treatment at 42°C for 60 min. Reaction was terminated by heating at 70°C for 5 min. The cDNA products were stored at -70°C till use. Verification of cDNA synthesis from extracted RNA was carried out using GAPDH specific internal control primers. The expression of pro-apoptotic genes (p53 & Bax) and anti-apoptotic gene (Bcl-2) was carried out using newly synthesized cDNA as templates for PCR. Twenty five µl dream Taq green master mix (Fermentas–Lithuania), 4 µl cDNA, 2 µl forward primer, 2 µl reverse primer and 17 µl nuclease free water were pre-denaturated at 94°C for 3 min. Amplification was performed (35 cycles); each cycle consisting of denaturation at 94°C for 30 sec, annealing at 58°C (GAPDH), 57°C (p53), 58°C (Bax) and 55°C (Bcl-2) for 30 sec followed by extension at 72°C for 45 sec. Reactions were terminated by heating at 72°C for 5 min. Non-reverse transcribed RNA was also included to confirm the absence of genomic DNA. PCR product (10 µl) was loaded on 1% agarose gel and visualized using UV transillumiator after staining with ethidium bromide. Polymerase chain reactions were carried out in triplicates followed by denstiometric analysis of bands intensities using gel documentation system. Data representing mRNA expression levels of p53 and Bax/Bcl-2 were calculated as mean of band intensities ± SD and plotted against test venoms concentrations and time intervals. Statistical significance was carried out using one way ANOVA [20]. Differences at P values less than 0.05 were considered statistically significant.

Primer sequences and the PCR product size were described in Table 1.

Results

Cytotoxicity

Data recorded revealed that test venoms showed cytotoxicity to breast cancer (MCF-7) cell line in a dose dependent manner. Recorded

Gene	Primer sequences (5' -3')	Product size (bp)	Reference
P53	F: TCA GAT CCT AGC GTC GAG CCC	438	[42]
	R: GGG TGT GGA ATC AAC CCA CAG		
Bax	F: ATG GAC GGG TCC GGG GAG CA	322	[43]
	R: CCC AGT TGA AGT TGC CGT CA		
Bcl-2	F: GTG AAC TGG GGG AGG ATT GT	216	[43]
	R: GGA GAA ATC AAA CAG AGG CC		
GAPDH	F: CAA GGT CAT CCA TGA CAA CTT TG	496	
	R: GTC CAC CAC CCT GTT GCT GTA G		

Table 1: Primer sequences of apoptosis related genes and internal control.

IC_{50} of *C.cerastes* was 1.5 µg/ml, while IC_{50} of *V.lebetina* was 2.5 µg/ml. It was observed that *C.cerastes* showed statistically significant higher cytotoxicity to MCF-7 cells than *V. lebetina* venom at P<0.05. In the mean time, venoms cytotoxicity to MCF-7 cells post 24 and 48 h treatment exhibited time and dose dependent profile (Figure 1).

Morphological changes

Untreated MCF-7 cells were homogeneously distributed in the culture field showing a polygonal shape with distinct boundaries and homogenous cellular contents (Figures 2A,2E). On the other hand, various morphological abnormalities were recorded 24 h post venoms treatment. At the lowest concentrations, cells lost their characteristic appearance, became rounded and detached out of the culture surface, while other cells retained their normal morphological appearance (Figures 2B,2F). Increasing venoms concentrations resulted in increased cellular irregularities and larger areas devoid of cells (Figures 2C,2G). At the highest concentrations cells showed obvious deterioration and deformation with severe shrinkage and condensation of their cellular contents (Figures 2D,2H).

Transmission electron microscope

Untreated MCF-7 cells showed the presence of intact cell membrane, normal appearance of nuclear chromatin with even distribution of heterochromatin and euchromatin (Figures 3A,3E). On the other hand 24 h venom treated cells exhibited signs of apoptosis including chromatin condensation (Figures 3B,3F,3H), cytoplasmic vacuoles (Figures 3C,3F,3G), cell membrane blebbing or budding (Figure 3G), nuclear shrinkage (Figures 3C,3G) and loss of the internal organization of the mitochondria with undistinguishable cristae structure (Figures 3D,3H).

Apoptosis detection using flow cytometry

Flow cytometric analysis revealed that test venoms induce apoptosis in venom treated cells compared to untreated cells. The percentage of early and late apoptotic cells was significantly increased at P<0.05 with increasing test venoms concentrations. In addition, flow cytometric data recorded a statistically significant decreased in the percentage of viable cells compared to untreated cells post 24 h treatment (Figure 4).

Effect of *C. cerastes* and *V. lebetina* venoms on apoptosis related genes

C. cerastes and *V. lebetina* were found to selectively stimulate apoptosis in MCF-7 cells where the level of mRNA expression of pro-apoptotic genes (p53 and Bax) was up regulated in 24 and 48 h venom treated cells compared to untreated cells. On the other hand, Bcl-2 (anti-apoptotic gene) was down regulated (Figure 5A). The densitometric analysis of the band intensities verified that *C. cerastes* and *V.lebetina* MCf-7 treated cells resulted in a statistically significant concentration and time dependent increase in p53 and Bax/Bcl-2 mRNA expression levels at P<0.05 thus it could be concluded that test venoms would be able to trigger cancer cells towards apoptosis through regulating the expression of these genes (Figure 5B).

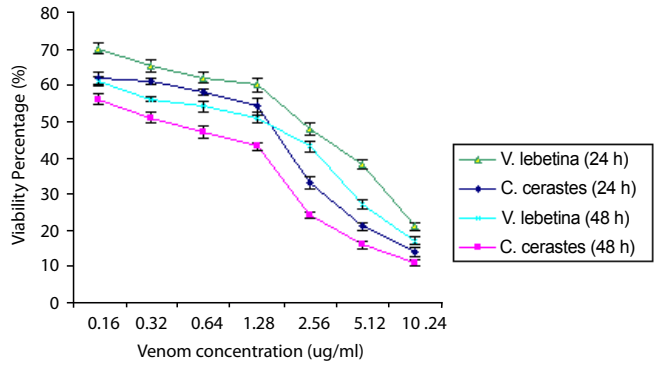


Figure 1: Evaluation of cytotoxic effect of *C.cerastes* and *V.lebetina* snake venoms to MCF-7 cells using MTT assay based on the amount of active lactate dehydrogenase released from residual viable cells showing statistically significant cytotoxic effect of test venoms relatively to time and concentrations at P < 0.05. Error bars represent mean ± SD of 3 independent experiments.

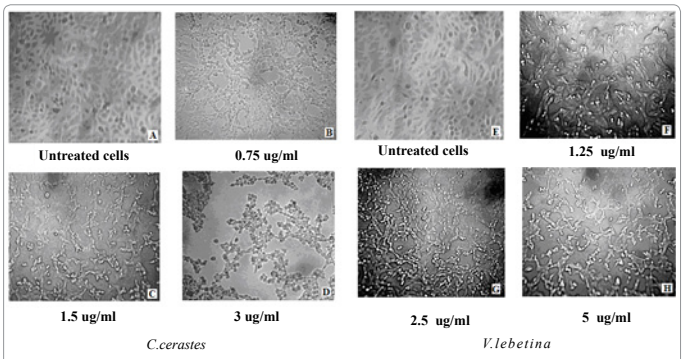
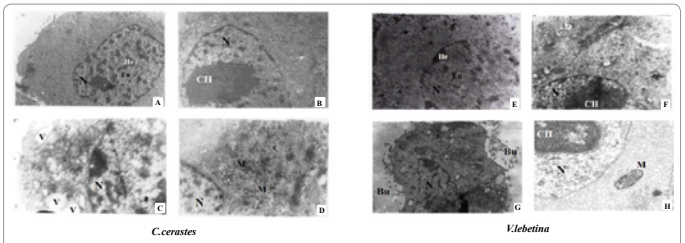


Figure 2: [A-H]: Monitoring of morphological changes in MCF-7 cells post treatment with *C.ceratstes* and *V.lebetina* venoms relatively to concentrations 24 h post treatment [A and E]: Untreated cell control; [B]: cells treated with *C.cerastes* (0.75 µg/ml); [C]: cells treated with *C.cerastes* (1.5 µg/ml); [D]: cells treated with *C.cerastes* (3 µg/ml); [E]: cells treated with *V.lebetina* (1.25 µg/ml); [F]: cells treated with *V.lebetina* (2.5 µg /ml); [G]: cells treated with *V.lebetina* (5 µg /ml) showing distinct cytotoxic effect of test venoms with larger areas of detached cells along with increasing venom concentrations compared to the normal appearance of untreated MCF-7 cells.



N: Nucleus; He: Heterochromatin; Eu: Euchromatin; M: Mitochondria; CH: Chromatin condensation; V and vertical arrows: Vacuoles; Bu: Cell membrane budding.

Figure 3: [A-H]: Morphological features of apoptotic MCF-7 cells post treatment with 50% inhibitory concentration (IC_{50}) of both *C.cerastes* and *V.lebetina* venoms compared to untreated cell control using transmission electron microscope (TEM). [A and E]: Untreated cell control; [B, C, D]: cells treated with *C.cerastes* (1.5 µg/ml); [F, G, H]: cells treated with *V.lebetina* (2.5 µg/ml). Treated cells exhibited characteristic features of apoptosis as chromatin condensation, cytoplasmic vacuoles, cell membrane budding as well as undefined mitochondrial cristae structure.

Discussion

Resistance to chemotherapy is a major problem in treatment of cancer. It often prevents tumor cells from undergoing sufficient levels of programmed cell death; apoptosis, resulting in cancer cell survival and treatment failure [21]. Therapeutic agents that targeted the apoptotic pathway, including pro-apoptotic proteins activators or inhibitors of anti-apoptotic proteins, could overcome the problem of resistance through directing cancer cells to self destruction [22]. Based on our previous finding, the effect of test venoms on different cancer cell lines was investigated revealing variable cytotoxic effect to Hep-2, HepG-

2, Hela and MCF-7 cancer cells (unpublished data). MCF-7 cells were selected as a cancer cell model due to the wide spread of breast cancer compared to other types of cancer cells. In the present study, inhibition of cancer cells proliferation post snake venom treatment was performed by monitoring the activity of lactate dehydrogenase (LDH) using MTT assay. A remarkable cytotoxicity to MCF-7 cells was observed where IC_{50} value of *C.cerastes* was 1.5 µg/ml, while IC_{50} of *V.lebetina* was 2.5 µg/ml. In consistence with other studies the cytotoxic effect of animal venoms on cancer cells were concentration and time dependent [23-25]. Variation in cytotoxic concentration range to cancer cell lines might be regarded to venom variable protein content where it was reported that different degree and nature of anti-carcinogenic property of different snake venoms was attributed to the difference in their constituents [26] as well as the variation in LD50 of test venoms, time post treatment and the difference in cellular reactivity to apoptotic stimulators.

In other studies, snake venoms also demonstrated *in vivo* anticancer activity, where it was reported that Cobra venom apoptotically inhibited the growth of implanted hepatocellular carcinoma cells in mice [27]. In addition, *Echis coloratus* snake venom and *Hydrophis spiralis* sea snake venom had antitumor activity on Erlich ascites carcinoma bearing mice [28,29]. Moreover, it was reported that the cytotoxic activity of *C.cerastes* venom against Ehrlich ascites carcinoma cells in mice might be due to the presence of a cytotoxin rather than to the direct cytolytic effect of the venom [30]. In addition, indian monocellate cobra (*Naja kaouthia*) and Russell's viper (*Vipera russelli*) crude venoms showed cytotoxicity on Ehrlich ascites carcinoma (EAC) cells *in vivo* as well as they exhibited potent cytotoxic and apoptogenic effect on human leukemic cells (U937 & K562) [25].

In consistence with the present study, it was found that test venoms induced cell growth arrest through chromatin condensation as well as cytoplasmic and mitochondrial alteration thus triggering cells towards programmed cell death [31,32]. The induction of apoptosis was further confirmed using flow cytometric analysis where during early stages of apoptosis the plasma membrane losses asymmetry causing phosphatidylserine (PS) to be translocated from the cytoplasmic face to the outer surface of the plasma membrane. Binding of Annexin-V to the exposed PS could detect early apoptotic cells (Annexin-V positive). During late apoptosis cell membrane lost its selective permeability, allowing propidium iodide to enter the cells where it binds to DNA (Annexin-V and propidium iodide positive) thus late apoptotic and/or necrotic cells could also be detected. On the other hand, viable cells remained unstained [33].

It has long been recognized that tumor suppressor gene, p53, was induced by DNA damage. The resulting increase in p53 level lead either to the induction of cell cycle arrest or apoptosis. Thus, p53 activation contributed to suppression of tumor growth [34]. The Bcl-2 family proteins, including Bcl-2 and Bax, contribute to the regulation of apoptosis. In particular, anti-apoptotic members of the Bcl-2 family, such as Bcl-2, act to prevent or delay cell death, whereas the pro-apoptotic Bax promotes apoptosis [35]. Regarding Bax/Bcl-2 ratio, at both the mRNA and protein levels, many studies reported that the increase in this ratio favors apoptosis [36,37].

At the molecular level and in consistence with the present study, induction of apoptosis was illustrated by up-regulation of both p53 and Bax genes and down regulation of Bcl-2 gene. Other studies revealed that *V. lebetina* snake venom inhibits the growth of human prostate cancer cells by induction of apoptosis through inhibition of nuclear factor KB (NF-KB), this toxin increased the expression of pro-apoptotic genes (p53, Bax, caspase-3, and caspase-9), but down-regulated antiapoptotic (Bcl-2) gene [38]. Consistent with the induction of apoptosis at molecular levels, the level of reactive oxygen species (ROS) was increased and mitochondrial membrane potential

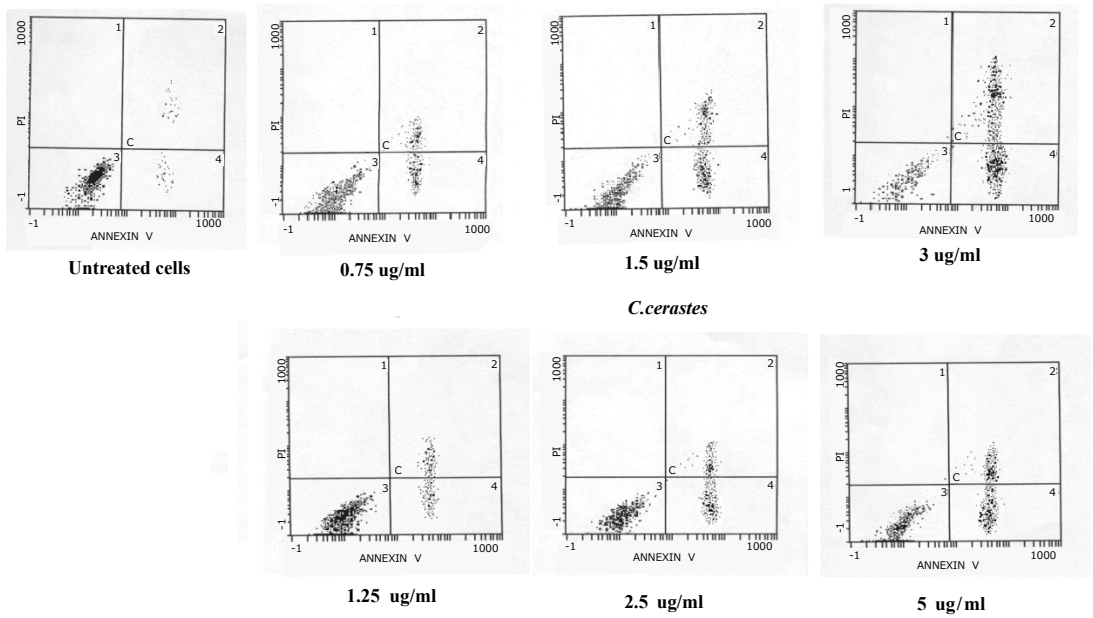


Figure 4: Flow cytometric analysis post 24 h treatment of MCF-7 cells with different concentrations of *C.cerastes* and *V.lebetina* venoms compared to untreated cells control using combined annexin-V and propidium iodide dyes. Data was presented in two-dimensional dot plots, where the regions of early, late apoptotic and viable cells were separated depending on fluorescence intensity. The number of early and late apoptotic cells significantly increased relatively to test venoms concentrations, whereas the number of viable cells was significantly decreased at $P<0.05$. C2: late apoptotic; C3: viable cells; C4: early apoptotic.

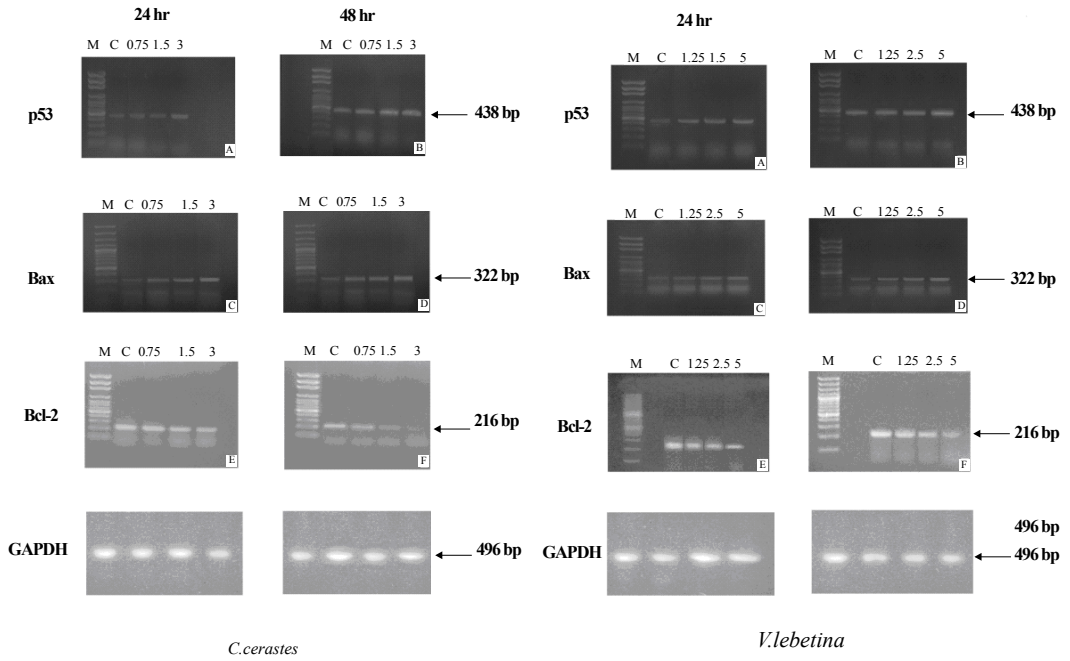


Figure 5a: Alteration of mRNA expression of apoptosis related genes in MCF-7 cells post 24 and 48 h treatment with different concentrations of *C.cerastes* (0.75, 1.5 & 3 µg/ml) and *V.lebetina* (1.25, 2.5 & 5 µg/ml) venoms demonstrating up regulation of p53 and Bax genes and down regulation of BCl-2 expression levels compared to negative cell control. M: Marker; C: control untreated cells.

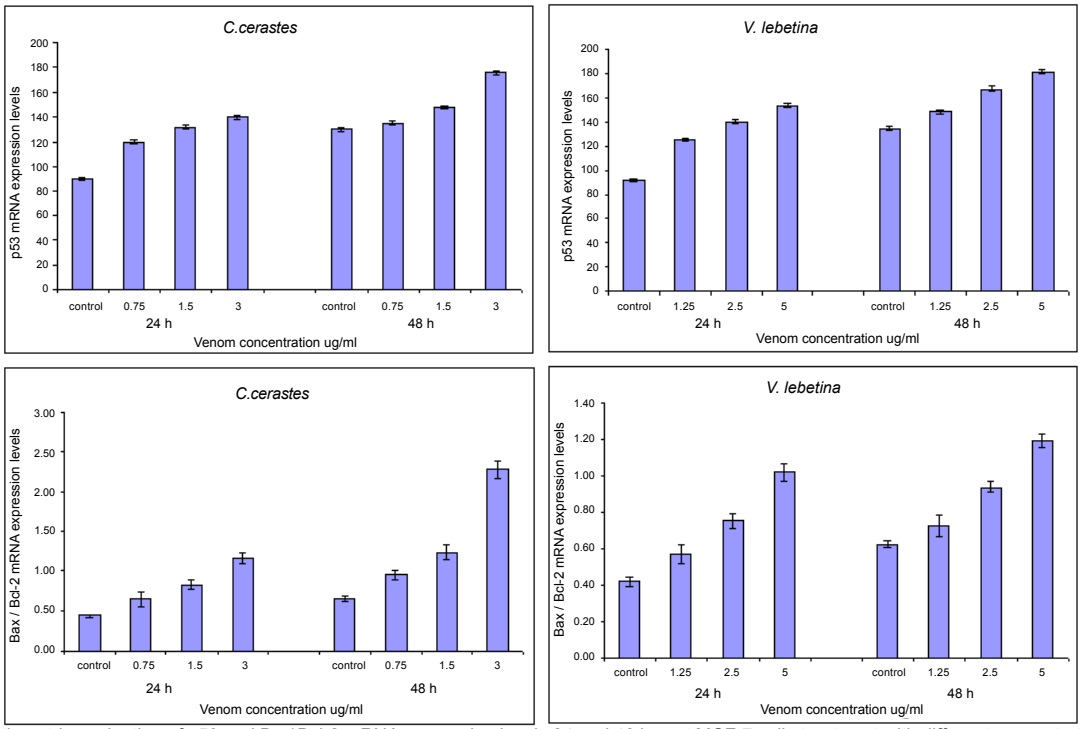


Figure 5b: Denstiometric evaluation of p53 and Bax\Bcl-2 mRNA expression levels 24 and 48 h post MCF-7 cells treatment with different concentrations of *C.cerastes* (0.75, 1.5 & 3 µg/ml) and *V.lebetina* (1.25, 2.5 & 5 µg/ml) venoms revealing statistically significant increase in p53 as well as in Bax\Bcl-2 mRNA expression levels compared to their levels in untreated cells at P<0.5. Error bars represent mean ± SD of triplicates.

(MMP) was also disrupted in *V.lebetina* treated neuroblastoma cells [39]. Various mechanisms have been suggested to elucidate snake venom-induced apoptosis, where it was reported that cardiotoxin III (CTX III), a basic polypeptide isolated from *Naja naja atra* venom, induced apoptosis in human leukemia (K562) cells through mitochondrial mediated pathway in which, venom treated cells lost

the mitochondrial membrane potential, released cytochrome c from mitochondria into the cytosol resulting in activation of caspase-9 and caspase-3. Apoptotic cell death was also accompanied by up-regulation of both Bax and endonuclease G (Endo G) and down regulation of Bcl-xl [8,9]. Besides, CTX III induced apoptosis in human colorectal cancer (Colo-205) cells through mitochondrial and caspase dependent pathway as well as increasing the Bax/Bcl-2 ratio in Colo-205 cells [40]. In another study, CTX III suppressed MCF-7 cells proliferation and induced apoptosis through up regulation of Bax (pro-apoptotic gene) and down regulation of Bcl-XL, Bcl-2 and XIAP (anti-apoptotic genes) [41].

The snake venoms anticancer activities proving progressive potentials, thus further studies are substantial to purify and assess the active antitumor components. It is also recommended that active proteins should be submitted to proteomic studies and used as a prototype for future synthesis of new anticancer drugs depending on activation of the intrinsic pathway of apoptosis targeting cancer cells to self destruction.

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