RESEARCH ARTICLE

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The Bacillus Calmette-Guérin Derived Purified Protein (PPD) Potentiates *In-Vitro* Anti-cancer Activity of *Cerastes cerastes* Snake Venom in Colon and Prostate Cancer Cells

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Abstract: Prostate and colon cancer represent a major health problem worldwide. In the present study, we evaluated the anti-cancer properties and cytotoxicity of *Cerastes-cerastes* (CC) snake venom on colon (Caco-2) and prostate (PC-3) cancer cells after their pretreatment with variable concentrations of Bacillus Calmette-Guérin (BCG) derived purified protein derivative (PPD). We monitoned the cell cycle arrest profile and specific cellular apoptosis markers (i.e. pro- and anti-apoptotic genes P53, Bax and Bcl-2 in CC- and BCG/PPD-pretreated cells using real time PCR. The cytotoxicity was determined by using MTT assay. Our data show that 24 h-treatment of cancer cells with CC venom induced a concentration-dependent cytotoxicity with IC_{50} values of 60 (Caco-2 cells) and 81 (PC-3 cells) µg/ml. Interestingly, addition of BCG/PPD at 25 and 50 µg/ml markedly increased the CC venom-induced toxicity on cancer cells, with IC_{50} values of 1.04 and 0.59 µg/ml for Caco-2 (up to 102-fold increase) or 2.78 and 0.70 µg/ml for PC-3 cells (up to 116-fold increase). By analyzing the cell cycle arrest and related gene expression pattern, the main phase of cell cycle arrest was found to be G2/M in both cell lines. An S-phase arrest was also observed in PPD pretreated colon Caco-2 cell line to a greater extent than that observed in cells only treated with CC venom. Up regulation of pro-apoptotic and down regulation of anti-apoptotic genes in PPD pretreated cells were significantly enhanced as compared to cells treated with CC venom alone. In this study, we suggest that PPD -via its synergistic action with the CC venom- might be used as an enhancer of the anti-cancer properties of CC venom.

INTRODUCTION

Cancer is one of the major public health problems worldwide. As an example, a total of 1,638,910 new cancer cases and 577,190 deaths from cancer were estimated in year 2012. [1] Cancer is characterized by genetic alterations which give rise to changes in expression, activation or localization of cell regulatory proteins, affecting the signaling pathways that alter their responses to regulatory stimuli and allow the uncontrolled cell growth. This highlights the urgent need for alternative therapeutic options for managing of cancer. The chemo-, radio-, immuno- and gene therapies the current regimens in cancer management. [2] Radiotherapy represents an important part of cancer care and contributes to almost 40% of the successful treatments, with the aim to control proliferation of cancer cells. [3] However, the challenge of radiotherapy to manage cancer is to enhance the selective radiation of cancer cells while minimizing effects on surrounding normal cells. Although chemotherapy remains a predominant option, one of the main challenges is that patients often do not respond or, eventually, develop resistance after initial treatment. [4] This has led to the large studies on anticancer drugs from natural resources. Snake venoms have been always regarded as natural sources of potent candidate drugs as they show some particularly wide spectra of biological activities. Among the pharmacological properties attributed to animal venoms (and their derivatives) are the anti-proliferative [5] and antimicrobial activities. [6] The venom can induce apoptosis in mammalian endothelial cells via production of hydrogen peroxide at high concentration. [7] The tumor suppressor protein -p53- is one of the proteins which play an important role in activation of the intrinsic pathway of cell apoptosis. A better understanding of the apoptosis process should facilitate the emergence of novel targeted therapies that could induce death in cancer cells through either activation of 'death' receptors on the cell surface or through a series of intracellular events (intrinsic pathway) thus stimulating apoptosis. [8] It was observed that p53 was implicated in the transcriptional activation of Bax gene that is involved in apoptosis, but also in the change of Bcl-2/Bax gene expression ratio, highlighting that the relationship between these pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins is one of the key factors affecting the fate of cancer cells. [9] In this context, investigating novel targeted apoptotic activators in cancer cells would represent a new strategy in cancer treatment. [10] BCG can act as an adjuvant in provoking the innate immunity against microbes responsible for different diseases, such as malaria, diphtheria and pertussis, [11, 12] or by co-injecting BCG with vaccines against infectious agents such as *schistosoma japonicum* [13] or *Leishmania spp.* [14] In addition, BCG has proved to be the most effective immunotherapy of non-muscle invasive bladder cancer [15, 16]. BCG is also used for *in-situ* therapy of carcinoma, as well as to prevent superficial bladder cancer recurrence after transurethral resection. [17] In the present study, we evaluated *in-vitro* the anticancer potential of the Egyptian *Cerastes-cerastes* snake venom against Caco-2 and PC-3 cancer cells. The cell cycle

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profile and the pro-apoptotic and anti-apoptotic gene expression levels were investigated. In addition, we assessed the role of BCG/PPD in potentiating the anticancer activity of CC venom.

MATERIALS AND METHODS

Human colorectal carcinoma Caco-2 (ATCC-HTB-37)and Human Prostate carcinoma, PC-3 (ATCC-CRL-1435) cell lines, *Cerastes-cerastes* snake venom and Bacillus Calmette-Guérin derived purified protein derivative(BCG/PPD) with a protein content of 760 µg/ml, were kindly supplied from cell culture, Helwan Animal House and BCG departments (VACSERA-Egypt), respectively.

Preparation of *Cerastes-cerastes* Snake Venom and BCG/PPD

Cerastes-cerastes snake venom was extracted by mild electrical stimulation (20 V, 500 mA) and diluted in sterile double-distilled water. After centrifugation at 8,000g for 15 min at a temperature of 4°C (Jouan KI 22, France), the supernatant was immediately lyophilized and stored at a temperature of -20°C until used. [18] *Cerastes-cerastes* snake venom was reconstituted in serum and phenol red-free medium and protein content of BCG/PPD was estimated using the Bradford method, as described. [19]

Cytotoxicity (MTT Assay)

Human colorectal (Caco-2) and prostate (PC-3) cancer cells were propagated in 75 cm^2 cell culture flasks using RPMI-1640 medium (Gibco-USA) supplemented with 10% (v/v) fetal bovine serum (Gibco-USA) and incubated in 5% (v/v) CO_2 incubator at a temperature of 37°C. Confluent cells were detached using 0.25% (w/v) trypsin solution and 0.05% (v/v) ethylene diaminetetraacetic acid (Gibco-USA) for 5 min. Cells were plated at a concentration of 2 x 10^5 cells/ml in 96-well cell culture plates and incubated at a temperature of 37°C for 24 hours to achieve confluency. The medium was decanted and fresh medium containing various concentrations of CC venom and BCG/PPD was added for cytotoxicity determination using colorimetric MTT reduction assay. [20] Dead cells were washed out using phosphate-buffered-saline (PBS) and 50 µl of MTT stock solution (5 mg/ml) were added to each well. After 4 h incubation period, the supernatants were discarded and the formazan precipitates were solubilized by addition of 50 µl per well of dimethyl sulfoxide (DMSO) or 0.4% (v/v) acidified isopropanol. Plates were incubated in the dark for 30 min at a temperature of 37°C and absorbance was determined at a wavelength of 570 nm using micro plate reader (Biotek ELX -800, USA). The cell viability percentage was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD of treated wells x 100}}{\text{OD of control wells}}$$

The cell viability (%) was blotted against the tested venom concentrations. Statistical significance between treated and untreated cells was determined using one way ANOVA. Differences at P values less than 0.05 were considered significant. The IC_{50} values of test venoms were determined using Masterplex-2010 software program. The effect of BCG/PPD as anticancer synergetic agent was examined by evaluating the cytotoxic potential of *C. cerastes* snake venom in Caco-2 and PC-3 cells pretreated with 25 and 50 µg BCG/PPD for 24 h. Morphological alterations of cells were analyzed using an inverted microscope (Nikon-Japan).

Cell Cycle Analysis

Caco-2 and PC-3 cells pre-cultured in 25 cm^2 cell culture flasks were treated with an IC_{50} of test venom dissolved in RPMI-1640 medium, for 24h. For cell cycle analyses, the cells were harvested and fixed gently with 70% (v/v) ethanol in PBS, maintained at a temperature of 4°C overnight and then re-suspended in PBS containing 40 µg/ml PI and 0.1 mg/ml RNase and 0.1% (v/v) Triton X-100 in a dark room. After 30 min at 37°C, the cells were analyzed using a flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle and sub-G1 group were determined and analyzed, as described previously. [21]

mRNA Expression Levels of Cell Apoptosis-related Genes

Total RNA was extracted from control and treated Caco-2 and PC-3 cells using the Gene JET RNA Purification kit (Fermantus-UK) according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratio and by gel electrophoresis, respectively. First-strand cDNA was synthesized with 1 µgof total RNA using a Quantitect Reverse Transcription kit (Qiagen, Germany) in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80°C until use for determination of the expression levels of P53, Bax and Bcl-2 genes using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) using QuantiTect SYBR Green PCR kits (Qiagen, Germany) and forward and reverse primers for each gene. The nucleic acid sequences of the primers were as follows: P53(F: 5'-TCA GAT CCT AGC GTC GAG CCC-3' and R: 5'-GGG TGT GGA ATC AAC CCA CAG-3') and Bax(F: 5'-ATG GAC GGG TCC GGG GAG CA-3' and R: 5'-CCC AGT TGA AGT TGC CGT CA-3') as well as anti-apoptotic gene Bcl-2 (F: 5'-GTG AAC TGG GGG AGG ATT GT-3' and R: 5'-GGA GAA ATC AAA CAG AGG CC-3') compared to β-actin as a housekeeping gene (F: 5'-CAA GGT CAT CCA TGA CAA CTT TG-3' and R: 5'-GTC CAC CAC CCT GTT GCT GTA G-3'). 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 . Amplification conditions and cycle counts were a temperature of 95°C for 15 min for the initial activation, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. Melting curves were performed after real-time PCR to demonstrate the specific amplification of single products of interest. A standard curve assay was performed to determine the amplification efficiency of the primers used. Relative fold changes in the

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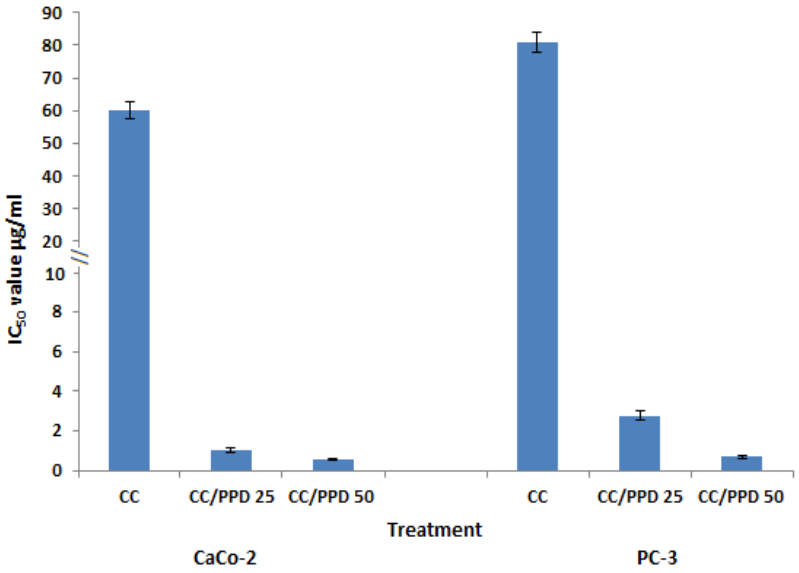

Figure 1: Comparative evaluation of the IC_{50} values of Cerastes-cerastes (CC) snake venom used either alone or post cellular treatment with PPD, as assessed by MTT assay. The data show a marked decrease in CC venom IC_{50} value in cancer cells pretreated with PPD at 25 and 50 µg/ml in both colon (Caco-2) and prostate (PC-3) cancer cell lines. The results are presented as mean ± SD of triplicates

expression of target genes (P53, Bax and Bcl-2) were accomplished using the comparative 2−ΔΔCt method [22] with the β-actin gene as an internal control to normalize the level of target gene expression. ΔΔCT is the difference between the mean ΔCT (treatment group) and mean ΔCT (control group), where ΔCT is the difference between the mean CT gene of interest and the mean CT internal control gene in each sample. Logarithmic transformation was performed on fold change values before being statistically analyzed, using the fold change values of three replicates for each gene measured.

Statistical Analysis

All experiments were carried out in three independent tests. Data were expressed as the mean±standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA). The results were considered statistically significant at probability <0.05.

RESULTS

Cytotoxicity

The cytotoxic effect of test venom was assessed by recording the various morphological changes of cells. Induced cell toxicity was accompanied with morphological changes in the culture fields showing a polygonal shape with distinct boundaries and homogenous cellular contents. On the other hand, a number of morphological abnormalities of cells were observed 24 h post treatment with the CC venom. 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. Increasing venom concentrations resulted in increased cellular irregularities and larger areas devoid of cells. At the highest concentrations, cells showed obvious deterioration and deformation with severe shrinkage and condensation of their cellular contents., The viability % of treated cells was concentration-dependent and the IC_{50} values were similar (P>0.05) for Caco-2 and PC-3 cell lines, with values of 60 and 81 µg/ml respectively. Also, the effect of PPD on cancer cells was evaluated and showed no cytotoxic effect on both cell lines. PPD exhibited a strong potentiating of the cytotoxic effect of *C. cerastes* venom with IC_{50} values of 1.04 µg/ml and 0.59 µg/ml on Caco-2 cells or 2.78 µg/ml and 0.7 µg/ml on PC-3 cells, when pretreated with BCG-PPD at concentrations of 25 and 50 µg/ml, respectively (Figure 1-3).

Cell Cycle Analysis

Toxicity of *C. cerastes* venom was accompanied by cell cycle arrest in both Caco-2 and PC-3 cell lines. Cell cycle analyses have demonstrated that there was an accumulation of arrested cells in the G2/M phase in both cell lines either after cellular treatment with CC alone or in the case of CC venom and PPD pretreated cells. On in addition, S phase arrest was found to be significantly higher in Caco-2 cells with 16.0% and 19.7 % of cells in the case of CC venom and CC venom-PPD pretreated cells as compared to untreated cells (8.8 %). Caco-2 cells exhibited an increase in the percentage of apoptotic cells as indicated by sub-G1 phase recording 24.4 % and 21.8 %, in addition to elevated sub-G1 phase that was observed in PC-3 cells in the order of 32.7% and 24.8% post CC venom treatment and CC venom-PPD pretreatment, respectively, compared to control cells (Figure 4-5).

mRNA Expression Levels of Apoptosis-related Genes

Our data showed that pretreatment of Caco-2 and PC-3 cancer cells with BCG-PPD potentiates the apoptotic activity of CC venom. The level of expression of pro-apoptotic genes (P53 and Bax) was clearly up-regulated, while the expression level of anti-apoptotic genes was down-regulated (P<0.05) indicating its potential efficacy in directing cancer cells towards programmed death (Figure 6-8).

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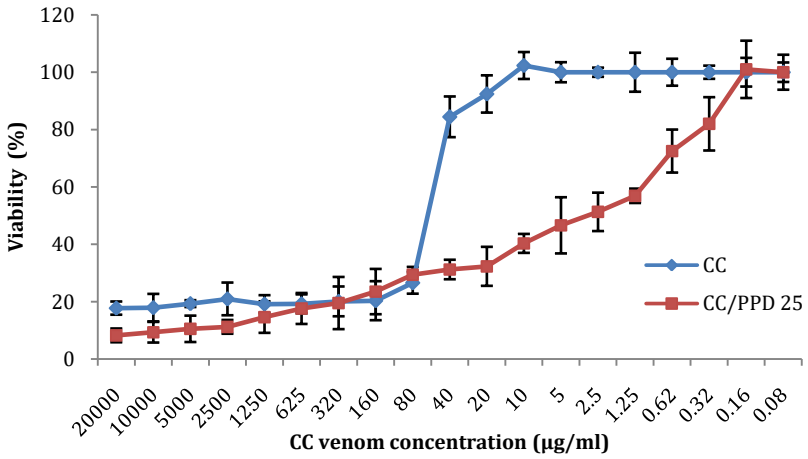

Figure 2: Effect of Cerastes-cerastes (CC)snake venom and CC venom-PPD pre-treatment of prostate PC-3 cancer cells on cell viability (%), as assessed by MTT assay

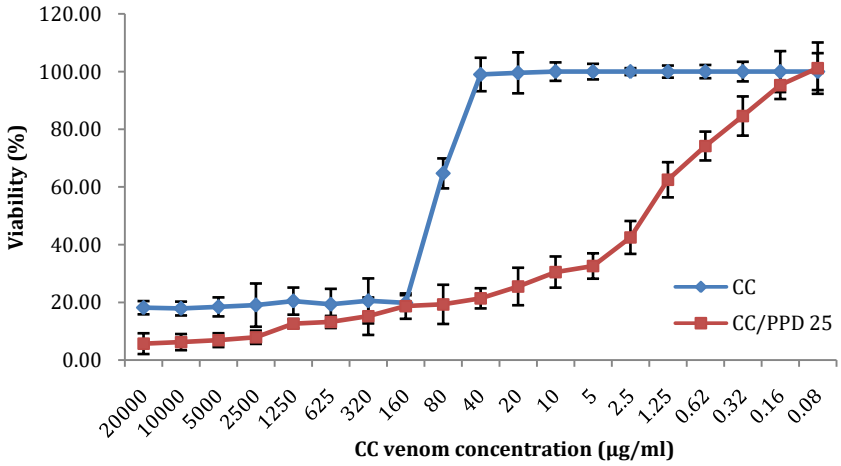

Figure 3: Effect of *Cerastes-cerastes* (CC) snake venom and CC venom-PPD pre-treatment of colon Caco-2 cancer cells on cell viability (%), as assessed by MTT assay

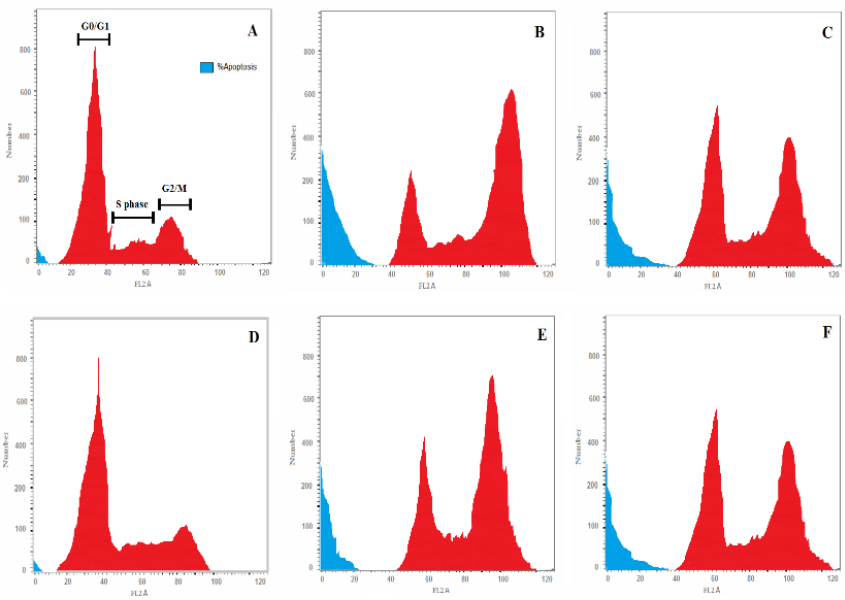

Figure 4: Cell cycle pattern analysis of *Cerastes-cerastes* (CC) snake venom and CC venom-PPD pre-treated cells compared to control cells. The cells were fixed with ethanol, treated with PI and the number of cells in each phase was counted according to the resulting fluorescence detected by flow cytometry analysis. [A], [D]: prostate PC-3 and colon Caco-2 control cells; [B], [E]: PC-3 and Caco-2 cells treated with IC50 of CC venom; [C], [F]: PC-3 and Caco-2 cells pretreated with PPD at 50 µg/ml followed by a treatment with an IC50 value of CC venom, respectively

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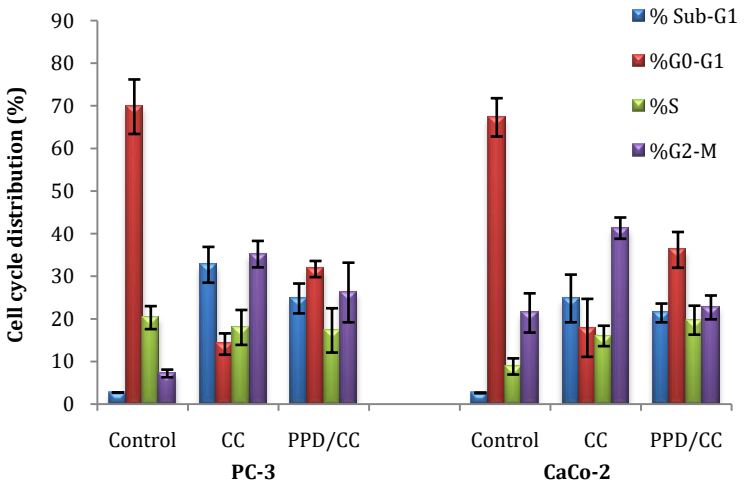

Figure 5: Effect of Cerastes cerastes (CC) snake venom and CC venom-PPD pre-treated cells on the cell cycle progression in prostate (PC-3) and colon (Caco-2) cancer cells. One cell set was treated with IC50 of CC venom whereas the second set was pretreated with 50 µg/ml PPD prior to CC venom treatment. The cell cycle distribution was analyzed by flow cytometry which indicates that cell cycle arrest occurred in the G2/M phase in the case of prostate PC-3 cancer cells and in both S and G2/M phases in the case of colon Caco-2 cancer cells. All the treatments resulted in an increase in the percentage of apoptotic cells as evidenced by the higher number of cells in sub-G1 phase. The analyses were carried out in (independent) triplicates, and the data were expressed as the mean percentage of cells in each phase ± SD

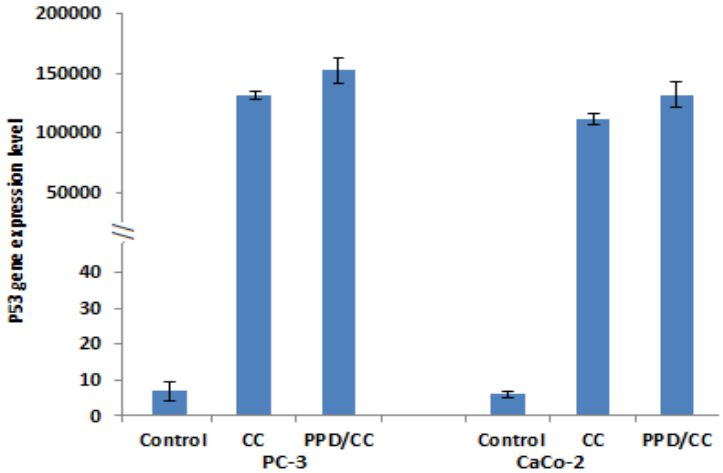

Figure 6: Gene expression level of P53 in prostate PC-3 and colon Caco-2 cancer cells, using real time PCR. The *Cerastes-cerastes* (CC) venom-treated cells exhibited a significant increase in P53 expression level. In the meantime, higher up-regulation in gene express was observed in CC venom-PPD pre-treated cells. The data are expressed as mean ± SD of three independent experiments

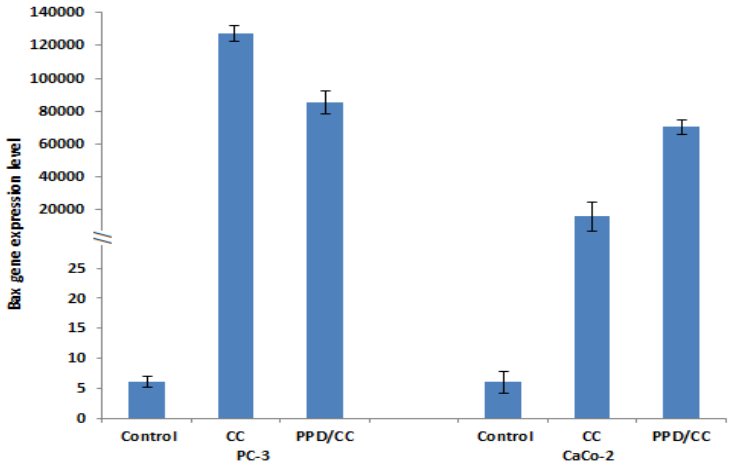

Figure 7: Evaluation of the expression level of pro-apoptotic genes revealed the extensive up-regulation of Bax genes in both prostate PC-3 and colon Caco-2 cancer cell lines either in *Cerastes-cerastes* (CC) snake venom-treated cells or in CC venom-PPD pre-treated cells. The values were considered to be statistically significant at $P<0.05$

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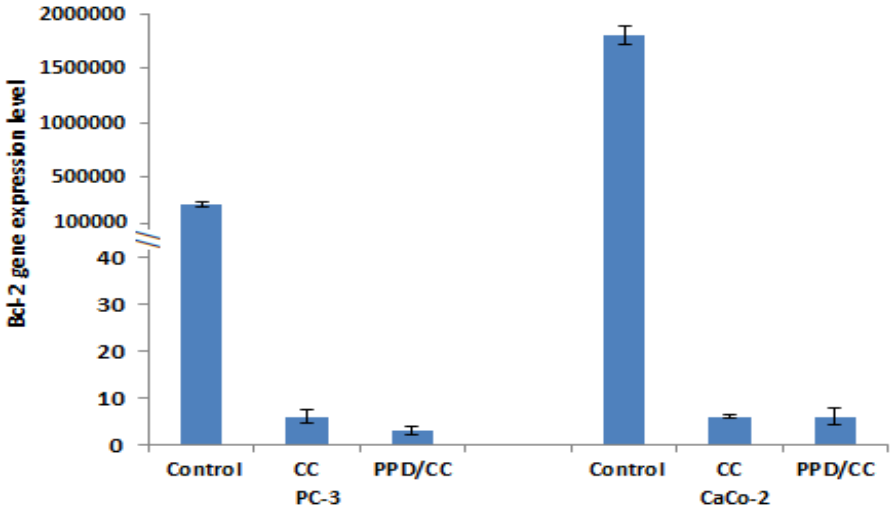

Figure 8: Down-regulation of anti-apoptotic Bcl-2gene expression level in prostate PC-3 and colon Caco-2 cancer cell lines demonstrating the effectiveness of *Cerastes-cerastes* (CC) venom and CC venom-PPD treatments in inducing apoptosis in both cell lines

DISCUSSION

Novel therapeutic strategies for cancer are one of the most important current concerns. Snake venom toxins are particularly attractive to many researchers because of their anticancer potential and relative selectivity, as they showed higher cytotoxicity to tumor cells as compared to normal cells. [23] Snake venom is composed of a mixture of numerous compounds that include proteins, enzymes, carbohydrates, minerals as well as low molecular mass polypeptides with variable biological activities. Thus, they can be potentially used in the management of several disorders such as rheumatoid arthritis, multiple sclerosis, thrombosis, microbial infections and inflammatory diseases, in addition to neurological, cardiovascular and neuromuscular disorders. [24] Along with snake and scorpion venom toxins, bacterial and plant toxins have been proved to exhibit considerable anticancer potential. [25] Local immunotherapy with BCG vaccine demonstrated a superior effectiveness over chemotherapy in the treatment of non-muscle invasive bladder cancer. [26] One of the suggested mechanisms for the antitumor activity of BCG vaccine is that BCG antigens are expressed on the surface of tumor cells by the major histocompatibility complex (MHC) class II molecules and are recognized by CD4-expressing immune cells. The direct activation of CD4-positive cells and/or other cytotoxic cells, as well as the released cytokines, are important determinants in the BCG vaccine-induced anti-tumoral effect on bladder cancer cells. [27] PPD is a purified protein derivative isolated from attenuated *Mycobacterium bovis* known as BCG. It was used for years as an antigen for the diagnosis of tuberculosis infection based on delayed type hypersensitivity. [28] The present study was conducted to evaluate the potential of *C. cerastes* snake venom as anticancer agent against two mostly prevalent cancers in Egypt; colon and prostate cancers. The data obtained showed that the crude venom of *C. cerastes* snake could induce a dose-dependent cytotoxicity and apoptosis in both colon (Caco-2) and prostate (PC-3) cancer cell lines, in agreement with Shebl *et al.*, [10] which reported on the cytotoxic potential of *C. cerastes* venom in breast cancer cells (MCF-7cell line) in a dose- and time-dependent manner. The anticancer activity of *C. cerastes* venom is thought to be related to the presence of phospholipases A2 as two phospholipases A2 isolated from this venom have been shown to possess some significant inhibitory activities on adhesion and migration of IGR39 melanoma and HT1080 fibro-sarcoma cancer cells. [29] In addition, an inhibitory activity on angiogenesis in tumors was also reported. [30] Our results are in concordant with another study based on the *Najanaja oxiana* snake venom. [31] It was reported that the later could induce cytotoxic effects in liver (HepG2), breast (MCF7) and prostate (DU145) cancer cell lines with IC_{50}values of 26.6, 28.9 and 21.2 µg/ml, respectively. The reported values appear to be lower than those obtained in our study presumably due to differences in cell lines and tested snake venom. Morphological examination of cells revealed various features associated with apoptosis, such as cytoplasmic blebbing and chromatin condensation. Flow cytometry analysis showed up to 95% of apoptotic cells at 15 µg/ml venom with a significant activation of caspase-3 at concentrations greater than 2.5 µg/ml. Despite that toxins could induce cell damage, cellular proteins that are associated with controlling the cell cycle and apoptosis are the determinants of cell fate. Cell cycle checkpoints are the pathways that enhance cell death post exposure to toxins and determine the mechanism by which these pathways are regulated. [32] It was noticed that the main cell cycle arrest was at G2/M phase in both cell lines indicating the growth inhibitory effect of tested components. [33] The increased number of cells in sub-G1 phase points out to the greater incidence of apoptotic cells post treatment. [34] Also, the elevated percentage of arrested colon cancer cells occurred during S phase indicating an enhancement of cell death through DNA-damage and biochemical pathways that involve signaling cascades. [35] In addition, it may be advantageous in exhibiting differential toxicity especially that cancer cells have shorter doubling times compared to normal cells [36] and this also might explain in part the observed lower IC_{50} values in colon compared to prostate

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cancer cells. On the other hand, the P53 gene products are pro-apoptotic stimulators which play an important role in arresting cell growth and activating apoptotic pathways that induce programmed cell death. Members of the Bcl-2 family proteins are important mediators that are correlated with mitochondrial outer membrane permeabilization that are accompanied by apoptosis. These proteins include anti-apoptotic (Bcl-2) as well as pro-apoptotic (Bax) proteins. [37] BCL-2 is located in the outer mitochondrial membrane and plays an essential role in promoting the survival of cells and inhibiting the effects of pro-apoptotic proteins. [38] In contrast, Bax is a pro-apoptotic member of Bcl-2 family and its expression is regulated by the tumor suppressor gene P53. Expression of Bax gene enhances the opening of the mitochondrial voltage-dependent anion channel resulting in loss of membrane potential with subsequent release of cytochrome C. [39] Consistent with the current study, it was reported that *C. cerastes* venom alone could trigger cancer cells towards apoptosis. The *C. cerastes* venom induced an up-regulation of P53 gene and increases the Bax/BCl-2 ratio post 24 and 48 hr treatment in breast cancer (MCF-7) cells. [10] The extensive up regulation that was reported in level of expression of pro-apoptotic genes (P 53 and Bax) in the cancer cells that were pretreated with BCG/PPD highlighted the efficacy of this protein in potentiating the anti-cancer properties of *C. cerastes* venom. In the same context, down regulation of the anti-apoptotic gene (BCl-2) also supports these findings.

Cyclin-dependent kinases (Cdks) are a family of protein kinases that play an important role in regulating the cell cycle. The Cdks exhibits some low kinase activity but this activity increases when it binds to a regulatory protein called cyclin forming cyclin-Cdk complex. This complex phosphorylates a variety of target proteins resulting in cell cycle progression. [40] It has been reported that activated p53 causes a G1 arrest through stimulating p21 expression with a subsequent inhibition of cyclin D/Cyclin dependent kinase. P53 induces a G2 arrest most probably by decreasing the transcription and synthesis of cyclin B1 in addition to blocking DNA replication and thus inhibiting entry into S-phase. [41] In order to justify the correlation between apoptosis induction and Cdk1 inhibition, it was found that surviving is one of the main targets of Cdk1 where the loss of phosphorylation of surviving could lead to decomposition of survivin-caspase-9 complex which subsequently stimulates caspase-9 mediated apoptosis of cells. However, phosphorylation of Bcl-2 is a marker of mitosis, but not related to the regulation of apoptosis. [42] The study by Iinuma *et al*., [43] demonstrated that the use of PPD as a non-specific immunotherapy against human cancer could represent an effective adjuvant therapy against some tumors. It was reported that tumor markers were reduced in 73 % of patients who received PPD by intravenous route. It was also suggested that PPD could sensitize T lymphocytes to release lymphokines which subsequently destruct cancer cells. [43] Although the role of PPD as anticancer is still unclear, there may be a relation between the PPD and mitochondrial membrane permeability and the level of released cytochrome C; also, PPD and cyclin kinase activity inhibition may be associated, Indeed, it is well known that BCG as an anticancer was explained by the stimulation of TH1 and TH2 pathways in a concentration dependent manner, in addition to up-regulation of the expression of p21 in human transitional carcinoma cell lines. The transactivation of p21 in response to BCG occurs through an immediate early, p53-independent pathway. The finding of increased p21, together with the observation that BCG induces cell cycle arrest at the G1/S interface, supports a role for this protein in the biologic response to BCG. [44, 45]

CONCLUSION

Herein, we examined the anti-cancer potentials well as cytotoxicity of *C. Cerastes* snake venom on colon and prostate cancer cell lines (Caco-2 and PC-3) after their pretreatment with different concentrations of BCG/PPD. Their effects on cell cycle arrest and mRNA expression levels of reference apoptosis markers (P53, Bax and Bcl-2) were assessed. A 24 hr treatment of cancer cells with CC venom induced some potent (concentration-dependent) cytotoxic effects. Interestingly, addition of BCG/PPD markedly potentiates CC venom-induced toxicity towards these cells. The main phase of cell cycle arrest was G2/M in both cancer cell lines, with a minor S-phase arrest also observed in PPD pretreated colon cells.

Finally, the cytotoxicity, pattern of cell cycle arrest and apoptotic stimulating activity of CC venom-treated and CC venom-PPD pretreated cancer cells highlight the synergistic role of PPD in potentiating the anticancer activity of *C. cerastes* snake venom. The interplay between PPD concentration, cytochrome C level and cyclin kinase should be further studied to get a better insight into role and mode of action of PPD.

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