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Evaluation of Anticancer Potentials of Bee Free Venom and Chitosan Nano-Conjugated One: *In Vitro* Study

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Abstract

Limited efficacy of the current first-line treatment for cancer cells leads to further development of efficient strategies. Recently, much attention has been given to nanoparticle-based drug delivery systems loaded with dual drugs to improve current disease therapies by overcoming toxicity. In the present study, we explore an approach to conjugate chitosan nanoparticles (CsNPs) with bee venom (BV) of *Apis mellifera*, then detect the enhancement of BV anticancer activity and related toxicity and anticancer potential. Chitosan nanoparticles were prepared by the ionotropic gelation method of chitosan (CS) cations with sodium tripolyphosphate (TPP) anions. Cytotoxicity of the nanoparticles against hepatocellular carcinoma (HEPG2) and Prostate cancer (PC3) was evaluated using MTT assay. Data recorded revealed that chitosan nanocapsulated venom enhanced the toxic effect of bee venom, and flow cytometry analysis showed appreciable arrested cell cycle in the G2/M for HePG2 and PC3. Also down regulation and up regulation of Bcl2 and Bax was detected respectively in tested cell lines post treatment with the venom, compared with the control groups of the cancer cells respectively.

Key words: *Apis mellifera*, venom , Anticancer , Nanoconjugation , BCL-2, Bax , cell cycle

INTRODUCTION

Bee venom (BV) contains a variety of different peptides, including melittin (a major component of BV), apamin, adolapin, and mast cell degranulating peptide (**Park *et al.,* 2004**). Bee venom has been used as an oriental medicine for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and pain (**Son *et al.,* 2007 and Park *et al.,* 2007**). Furthermore, it has anti-cancer activity (**Liu *et al.,* 2002**). Cancer is a globally prevalent disease that is considered to be the one of the most common cause of death in economically developing countries (**Jemal *et al.,* 2011 and Hidetomo *et al*., 2013**). The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most cases (conventional dosage forms), only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biochemical properties, therefore developing a drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects *in vivo* is a challenging task. Nowadays, nanotechnology is found in a wide range of applications in the pharmaceutical industry (**Gupta, 2006**). Due to new advances in nanotechnology, it is now possible to produce drug nanoparticles that can be utilized in a variety of innovative ways (**Jain, 2008**). Both synthetic and natural polymers were studied aiming to produce nanoparticles (**Tiyaboonchai, 2003**). However, among the variety of polymers that were used for drug-loaded nanoparticles, chitosan has received great attention in both the medical and pharmaceutical fields (**Shahbazi *et al.,* 2008**). Chitosan also acts as a penetration enhancer by opening the tight epithelial junctions; therefore, it is particularly exploited in protein and vaccine delivery (**Van der Lubben *et al.,* 2001a, Van der Lubben *et al.,* 2001b**). Chitosan nanoparticles have been widely investigated for the delivery of polypeptides such as insulin (**Avadi *et al* 2010**), tetanus toxoid (**Vila *et al* 2004**), diphtheria Toxoid (**Rezaei Mokarram &Alonso 2006**), snake venom (**Mohammadpour dounighi *et al* 2015**) and proteins (**Xu & Du 2003**). The aim of the present study was to compare between the anticancer effect of the free and chitosan nanocapsulated bee venom

Materials and Methods

Bee venom extraction

Bee venom collection was carried out in the department of Agriculture, Plant Protection Research Institute, Agriculture Research Center, Dokki, Giza, during the 2014 &2015 summer season. Venom was collected from the honeybee (*Apis mellifera* L.) workers by the electric shock device (VC-6F model from Apitronic Services, 9611 No. 4 Road, Richmond, B.C., Canada). The electrical impulses stimulated the bee workers to sting through latex or sheet placed on a glass plate. The glass plate was carefully transported to the laboratory and left to allow drying of the bee venom at room temperature, then dry venom was scraped using sharp scraper (**Fakhim-Zadeh, 1998**). The fresh bee venom was packed up in dark glass jars and stored in a cool and dry place.

Preparation of chitosan nanoparticles and cross linking bee Venom-loading

Chitosan nanoparticles (CsNPs) were prepared mainly by the ionotropic gelation method of chitosan (CS) cations with sodium tripolyphosphate (TPP) anions according to the procedure reported by **Rocha Soares *et al*., 2012 and Mohammad pourdounighi *et al*., 2015**. Chitosan was prepared at concentration (1mg/ml), the concentration of acetic acid in the aqueous solution was 1.5 folds of chitosan. Sodium tripolyphosphate (TPP) was dissolved in purified water (<1.3 mS/cm) at concentration equal the amount of chitosan (1mg/ml), drops of TPP solution were added to chitosan solution. Nanoparticles were spontaneously formed by the rapid mixing by homognizer (~20000 rpm) for 60 min over an ice bath to allow complete stabilization of the nanosystem until opalescent suspension obtained. The bee venom-loaded nanoparticles were formed by addition of venom at concentration 300 μg/ml to TPP solution prior to the incorporation of chitosan solution. Finally nanoparticles were separated by centrifuge at 11000 rpm and 4 °C for 90 min; lyophilized and stored at 4°C.

Cell culture and in vitro cytotoxicity assay

Hepatocellular carcinoma (HePG2), Prostate cancer (PC3) as cancer cell lines were grown in RPMI-1640 medium and VERO cells clone CCL-81 heteroploid cell line as normal cell line were grown in 199-E medium. (GIBCO-USA). Both media were supplemented with 10% fetal bovine serum (FBS) and 1mM sodium pyruvate, 2mM L-glutamine and antibiotics (penicillin 100 IU/ml, streptomycin 100μg/ml) at 37 °C and 5% CO2. The MTT assay was performed to assess the cytotoxicity of free bee venom and bee venom loaded chitosan nanoparticles against the test cell lines. The cells were 24 hrs precultured in a concentration of a 1×10^4 cells/well in 96 tissue culture plates. Media were replaced by100 μL of the different tested materials. Free an nanocapsulated bee venom was added to the precultured plates and 2 fold serially diluted. Plates were incubated for 24h post treatment with bee venom and 48h post treatment with nanocapsulated bee venom to permit the release of trapped bee venom. Treatment media were removed and 50 μl of MTT stain used as 0.5mg/ml, added to each well and left for 4 h. Afterward, 50 μL DMSO were added to each well and the absorbance was read at 570 nm using LERX-800 Biotek –USA ELISA reader

Apoptosis and cell cycle

In order to investigate the type of cell death induced by the bee venom, flow cytometric analysis was performed. The tested cancer cell lines were treated with 1/2 the IC50 value for 24 h. The treated and untreated cells were trypsinized and pelleted down, by cold centrifugation at 2000 rpm(Jouan-Ki 22 , Franc) for 10 min at 4 °C, and pellets were washed with (PBS) phosphate buffer saline (ADWIA-Egypt) , fixed with ice-cold 70 % drop-wise ethanol that was added drop-wise in stirring condition on vortex, and dropwise added. Fixed cells were kept at +4°C at 4 °C overnight. Finally the pellets suspended in PBS treated with RNase A enzyme for 30 min at 37 °C and stained with propidium iodide 50 μg/ml from stock solution (2.5mg/ml) in 500 μl of PBS solution, incubated in the dark at room temperature for 15 min. The cells were analyzed using flow cytometry (Becton Dickinson, San Jose, CA, USA). Distribution of cell cycle phase of nuclear DNA was determined on fluorescence detector equipped with 488 nm laser light source and 623 nm and pass filter **(Hsu *et al.*, 2007).**

Real Time PCR:

RNA extraction and cDNA synthesis

HePG2 and PC3 cells at a density of 1×10^5 cells/ml were seeded into 25cm2 baby flasks (TPP-Swiss). Cancer cells were treated with the IC_{50} for 24 hour and untreated flask was considered. Affected cells were harvested and rest of cells wee trypsinized and cold centrifuged . Pelted cells were washed with PBS as previous, then transferred to eppendorf tubes. The total RNA was extracted according to total RNA isolation Kit (Promega – Germany) manufacturer's protocols. The purity of RNA samples were assayed by spectrophotometry. High-quality RNAs were stored at −80 °C until use. The cDNA products from RNA were carried out using Revert Aid™ First Strand cDNA synthesis kit based on the manufacturer's protocols. The reaction mixture was performed in thermal cycler (Bio-Rad, Hercules, CA, USA) according to the manufacture protocol.

Quantitative real–time PCR

Gene quantitation was performed using the light Cycler FastStar DNA Master SYBR Green I Kit (Rooche Diagnostics). Primers were designed with Gen Bank Primer-Blast program, NCBI. The real-time PCR primers for *BCL2* were as follow: **F**: 5′- TGA AGT CAA CAT GCC TGC CC-3′and **R**: 5′- AAA GCC AGC TTC CCC AAT GA-3′ (amplicon size, 453 bp). The *BAX* real-time PCR primers were **F:** 5′- AGG ATG CGT CCA CCA AGA AG-3′ and **R**:5′- TGT CCA GCC CAT GAT GGT TC-3′ (amplicon size, 266 bp). GAPDH was used as an endogenous control gene for the quantitative reverse transcription– PCR assay. The sequence of the primers for reference gene GAPDH was **F**: 5′- CTC TGC TCC TCC TGT TCG AC-3′ and 5′ **R**: 5′- GCG CCC AAT ACG ACC AAA TC-3′ (amplicon size, 121 bp). All PCRs were performed in total volume of 10 μL, with 6.525 μL of SYBR Green PCR Master Mix, 1 μL cDNA (100 ng), and the final concentration of the primers was 0.2 μL (0.5 μm) and 2.275 µl of RNase-free water. Negative controls (non-template controls) were included in each run. The thermal cycling conditions were as follows: 48 °C for 30 min followed by 40 to 45 cycles at 95°C for 10 s, 69°C for 10 s, and final stage was followed by a melting curve at 72°C.

Analysis of qPCR data

The CT cycle was used to determine the expression level in control cells and cells treated with bee venom. The gene expression level was calculated as described by **Yuan *et al.*, (2006)** using Applied Biosystem Step One ™ Instrument software. The results were expressed as the ratio of reference gene to target gene by using the following formula:

$$\Delta Ct = Ct1 - Ct2$$

Where, Ct is cycle number at the threshold level of log-based fluorescence Ct1 cycle number at the threshold level of log-based fluorescence of target genes Ct2 cycle number at the threshold level of log-based fluorescence of endogenous control genes to determine the relative expression levels, the following formula was used:

$$\Delta\Delta Ct = \Delta Ct\ (treated) - \Delta Ct\ (control).$$

Thus, the expression levels were expressed as n-fold differences relative quantity (RQ) to the calibrator.

Results

cytotoxicity assay

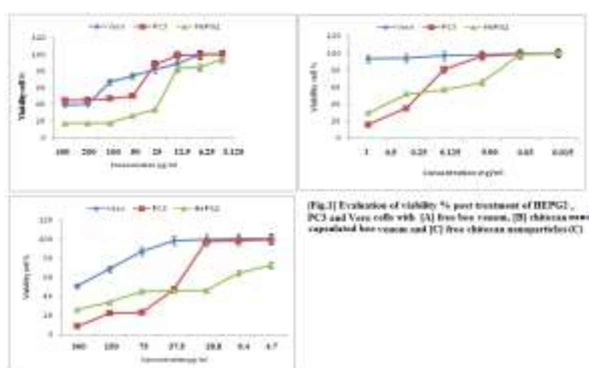
To assess the cytotoxic effect of free and chitosan nanocapsulated bee venom on cell growth of the tested cancer cell lines (HePG2 & PC3) and normal cell line (Vero cells), cell viability were tested by MTT assay

Table (1) demonstrated the (IC_{50}) values of free and chitosan nanocapsulated bee venom. The (IC_{50}) values of free bee venom were 20 & 49.4µg/ and 16.5 & 36.08 µg/ml in case of Chitosan nanoparticles loaded bee venom HePG2 and PC3, respectively. Nevertheless, treatment with free chitosan nanoparticles (1mg/ml) for 48 h has not inhibitory effect on the viability of normal VERO cells. Although BV alone has inhibitory effect against normal Vero cells and its IC_{50} value was 156.8 µg/ml, while when normal Vero cells treated with bee venom chitosan nanoparticles, IC_{50} value was increased to reach 311µg/ml, this mean that nanoparticles showed a higher anti-proliferative potentials of cells Vero cells. Data

presented in figure (1) illustrated the comparison between the effect of treatment with free and chitosan nanocapsulated bee venom on the different used cell lines. Also, results showed that liver cancer cells more susceptible than prostate cancer in all tested compounds compared to normal Vero cells.

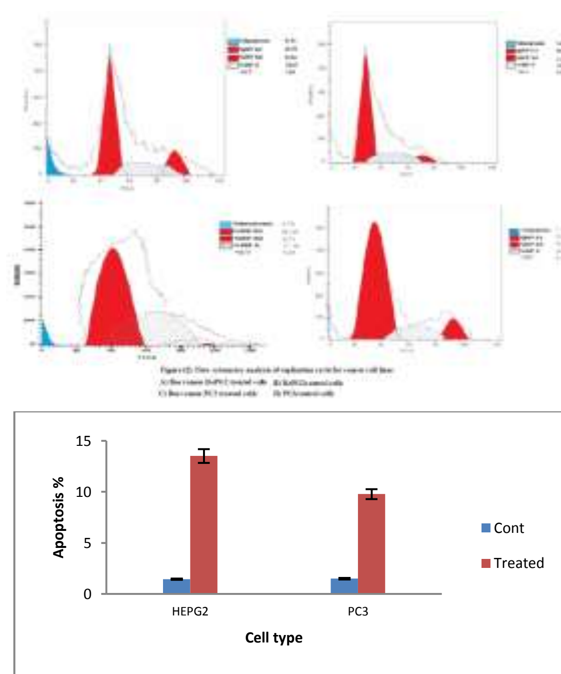
[Table 1] The IC_{50} values of tested free bee venom and chitosan nanoconjugated one on different cancer cell line (HEPG2 &PC3) and normal Vero cell lines

Test materials	Cell lines	IC_{50}
Bee venom	Vero	156.8
	HePG2	20.00
	PC3	49.4
Chitosan nanoparticles	Vero	-
	HePG2	512.00
	PC3	410.2
Chitosan - bee venom nanoconjugated	Vero	311.00
	HePG2	16.55
	PC3	36.08



Apoptosis and cell cycle arrest:

1. Data recorded in [Fig.2] summarized the changes of arrested cell cycle phases in two the types of cancer cell lines (HePG2 and PC3) using flow cytometry 24 h post bee venom treatment. It worthy to mention that the flow cytometric analysis revealed that treatment of HePG2 and PC3 with bee venom induced G2/M arrest phase. HePG2-treated cells showed 47.76 % G1/G0, 26.07 % S, and 12.66% G2/M (Figure 2A), while control cells recorded 68.43% G1/G0, 22.84% S, and 7.29% G2/M (Figure 2B). In case of prostate cancer (PC3) treated cells showed 56.22 % G1/G0, 2.74% S, and 31.26 % G2/M (Figure 2C), while the control cell showed 55.7 % G1/G0, 28.4% S, and 14.4 % G2/M (Figure 2D). These results demonstrated that bee venom induced apoptosis in the two used cancer cell lines. In the mean time apoptotic activity was monitored recording a significantly elevated apoptotic % (P<05) than control and the % of apoptosis was cell type dependent [Fig.3].



[Fig. 3] Evaluation of apoptosis % post cell treatment with bee venom

Anticancer activity:

Assessment of gene expression levels for pre and anti apoptotic Bax and Bcl-2 genes in HePG2 and PC3 cell lines 24 h post bee venom treatment using real time PCR were summarized in [Tab.2], where it was noticed that Bax gene showed an extremely significant up regulation (P<.0001), in the mean time antiapoptotic gene was extremely down regulated (P<0.0001)

Table (2). Effect of bee venom on BCL-2 and Bax genes expression in (HePG2 & PC3) cancer cell lines

Cell lines		Gene expression level IU/ml	
		Bax	BCl-2
HEPG2	Control	2.28	1540.6
	Treated	7042	3.12
PC3	Control	14	255679
	Treated	4871880	127.1

Discussion

It has been previously reported that BV can induce apoptosis in many cancer cell lines namely lung cancer, hepatocellular carcinoma, breast cancer, prostate cancer and ovarian cancer cell lines **(Ip *et al.,* 2008; Park *et al.,* 2011 and Jo *et al.,* 2012)** but the present work is the first to evaluate the effect of chitosan nanocapsulated bee venom on the hepatocellular carcinoma and cancer prostate cancer cell lines. The number of natural product-derived drugs present in the total drug launches was recently analyzed and it was concluded that natural products are still a significant source of new chemotherapeutic agents, especially anticancer **(Newman *et al.* 2003)**. Cancer is becoming a major health problem all over the world. Treatment of cancer involved different clinical protocols, some of them used in combination including surgery, chemotherapy, radiotherapy, gene therapy and some recent immunological approaches **(Omran, 2003)**. Resistance to chemotherapy is a major problem in the treatment of cancer. It is often prevents tumor cells from undergoing sufficient levels of programmed cell death; apoptosis, resulting in cancer cell survival and treatment failure **(Wilson *et al.,* 2009)**. Also, some of synthetic cancer drugs cause non specific killing of cells **(Reddy *et al.,* 2003)**. Hence there is an urgent need for more effective anticancer agents specifically targeted with no resistance developed. Recent advances in nanotechnology have stimulated different applications in biomedicine where nanoparticles are used as drug delivery vehicles allowing rational manipulation of pharmacological profiles of drugs encapsulated in them and hence their concomitant therapeutic indices **(Wang *et al.* 2009).** The current study was designed to prepare chitosan nanoparticles from *Penaeus karatherus* (shrimp exoskeleton) and investigate their ability to inhibit proliferation of cancer cells and related anticancer genetic profile. increasing the related drug anticancer properties as well as reducing their systemic side-effects based on the demonstrated phenomena in cancerous tissues which is the preferably accumulation of macromolecules in tumor tissue due to increased permeability of tumor vascular network in the absence of lymphatic clearing system**.(Sayari *et al.,* 2014; Goodarzi *et al.,* 2014 and Dinarvand *et al.,* 2011).**

The present findings showed that BV inhibited the growth of HEPG2 and PC3 in a concentration and cell type dependent. It was reported that the lower uptake of bee venom is attributed to the resistance experienced by them due to P-gp pump highly expressed on cancer cells (**Hui *et al.* 2008)** which acts as an energy drug efflux pump and leads to decrease in cytotoxic protein accumulation. The nanoparticles conjugation system escapes the Pgp pump as their uptake is mediated by specific endocytic processes **(Sahoo and Labhasetwar 2005)**. Thus, Chitosan nanoparticles-conjugated bee venom preferentially delivered into the nucleus inside the cells for eliciting a better therapeutic effect than native bee venom, where chitosan able to escape the endosomes, offering high potential for nuclear delivery. Molecular entry into the nucleus occurs through the nuclear pore complexes; the efficiency of which is dependent on NP size and the presence of nuclear localization sequence (NLS) **Salma Tammam *et al.,* (2015)**. The antiproliferative and the cytotoxic activities of chitosan nanocapsulated bee venom were supported by MTT assay. The chitosan

nanocapsulated bee venom inhibited the growth of HePG2 and PC3 cells in concentration- dependent manner, while Vero cell showed somewhat a higher resistance to nanoparticles cytotoxic effect recording an IC $_{50}$ in the order of 311µg/ml if compared with IC_{50} value against the two cancer cell lines; 16.55 &36.08 µg/ml for HePG2 and PC3 respectively. These findings reveal that venom loaded chitosan nanoparticles preferentially act on the cancer cells. The competency of drug-loaded nanoparticles in inhibiting the growth of cancer cells was also observed by **Bhowmik and Saha (2013)** who found that the gold nanoparticles (GNPs) conjugated with a protein toxin from the Indian cobra *Naja kaouthia* venom (NKCT1) on leukemic cells (U937 and K562) were more profoundly than native NKCT1 and **Luo *et al.,* 2009; Yang *et al.*, 2009** who have used N succinyl chitosan nanoparticles and lipid nanoparticles on K562 cells for better antitumor effect.There are two main strategies by which chemotherapeutic agents are capable of exerting their effect: [1] is by induction of apoptosis and [2] is by cell cycle inhibition. Many of the compounds under study as antitumor agents act at multiple steps in the cell cycle, and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of the target cells **(Shapiro and Harper 1999).** The present study indicated that the bee venom at concentration 10 µg/ml for HePG2 cell line and 25 µg/ml for PC3 cell line was able to cause cell cycle arrest and it was capable of inducing apoptosis, where bee venom treatment induced G2/M arrest in HePG2 and **PC3** bee venom induced cell cycle arrest in G2/M that may be attributed to venom pathway / mechanism of action relatively to cell line. The above mentioned results agree with **Siu-Wan *et al.,* (2008a)** who demonstrated that bee venom induced G0/G1 arrest and sub-G1 group (apoptosis) on human cervical cancer (Ca Ski cells) and **Siu-Wan *et al.,* (2008b)** who found that bee venom induced cell cycle arrest at G0/G1phase in human breast cancer MCF7 cells with and promoting the S-phase fraction and increased the proportion of cells in the sub-G1 group (apoptosis). Two of the major genes responsible for regulating the mitochondrial apoptosis pathway are antiapoptic Bcl-2 and proapoptic Bax genes (**Tong *et al,.* 2004**). Quantitative real time polymerase chain reaction (qPCR) has become a commonly used method for precise determination of gene expression **(Canales *et al*., 2006)**. In the present study, BV treatment resulted. Bcl-2 down regulation accompanied with up regulation of Bax gene. Bcl-2 inhibits most types of apoptotic cell death, implying a common mechanism of lethality. Bcl-2 is localized to intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticula, and nuclear membranes. Bcl-2 protected cells from H_2O_2- and menadione-induced oxidative deaths. Two model systems of apoptosis showed no increment in generation of endogenous peroxides continued at an inherent rate that was unaltered by Bcl-2. Following an apoptotic signal, cells sustained progressive lipid peroxidation. Overexpression of Bcl-2 functioned to suppress lipid peroxidation completely. And they propose a model in which Bcl-2 regulates an antioxidant pathway at sites of free radical generation. Members of the Bcl-2 family proteins are characterized by their ability to form a complex combination of heterodimers with Bax and homodimers with itself (**Reed, 1997**). Therefore, the ratio of Bax to Bcl-2 is thought to determine the susceptibility of cells to apoptosis (**Korsmeyer, 1999).** The present findings are similar to that of **Siu-Wan *et al.,* (2008a)** who demonstrated that bee venom can induce apoptosis in human cervical cancer (Ca Ski cells) through an increasing in the gene levels of p53 and Bax, but a decreasing in the level of Bcl-2. **Also, Jo *et al*., (2012)** investigated inhibition of cell growth by bee venom in the human ovarian cancer (SKOV3 and PA-1) cells and found that expression of DR downstream pro-apoptotic proteins including caspase-3, 8, and Bax was concomitantly increased, but the expression of Bcl-2 was inhibited by treatment with bee venom. In addition, **Zheng *et al.,* (2015)** found that bee venom suppressed the expression of anti-apoptotic proteins like Bcl-2, while it increased the expression of pro-apoptotic proteins such as Bax, caspase-3, caspase-8 and caspase-9 which are regulated by NF-κB in colon cancer (HCT116). Finally it can be concluded that bee venom can enhance the apoptotic potential of both liver and prostate cancer cell lines

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