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Cell proliferation and apoptosis are two counterparts that share the responsibility of maintaining the normal tissue homeostasis [Wang, 2004]. Cancer has been referred to as failure of the mechanisms that control the growth and division of cells. Cancer remains the leading disease with highest mortality in the world.

Chemo- and radiotherapies available as conventional options of cancer therapy at present act by inducing apoptosis or inhibit the proliferation of neoplastic cells [Gerl and Vaux, 2005]. However, these therapies are limited by unwanted side-effects such as damage to healthy tissues [Dyer, 1999], also the resistance developed by numerous tumors to these therapies [Benjamin et al., 1998].

Researchers have been studying alternative forms of cancer therapy by using potential biological molecules to target neoplastic tumours [Dyer, 1999]. It is widely recognized that the induction of apoptosis and inhibition of proliferation in tumor cells are common factors in the biological response of to cancer and key mechanisms to a variety of therapeutic manoeuvres [Dowsett et al., 1999; Zhai et al., 2010].

Recent investigations in vitro as well as in vivo have demonstrated that scorpion venom has the ability to induce apoptosis and inhibit DNA synthesis in a variety of cells [Soroceanu et al., 1998; Omran, 2003a; Wang and Ji, 2005; Das Gupta et al., 2007]. Several studies have reported that scorpion venoms possess numerous peptides with anticancer activity. These peptides act by various mechanisms such as the interaction with ion channels, and changing the membrane permeability of the excitable non-excitable cells [Caliskan et al., 2009; Gomes et al., 2010; Gao et al., 2008a; Ouadid-Ahidouch et al., 2004; Wang and Ji, 2005; Petricevich, 2010]. It is believed that these properties of venoms can be employed as therapeutic tools for the diagnosis as well as treatment of various cancers [Upadhyay, 2010; Petricevich, 2010].
Advanced methods of fractionation, chromatography and peptide sequencing have made it possible to characterize the components of scorpion venoms [Petricevich, 2010].

The work proposed in this thesis was intended to identify the anti-proliferative and apoptotic effects of scorpion venoms and their characterization. The overall aim of the study was to elucidate the cytotoxic effect of the crude venom and peptide fractions of the venom collected from *Odontobuthus doriae*, *Buthotus saulcyi*, and *Androctonus crassicauda* in SH-SY5Y and MCF-7 cells. SH-SY5Y and MCF-7 cells lines were selected because of certain properties. SH-SY5Y is a neuroblastoma cell line that expresses various ion channels and has been used earlier for similar studies on the development of anti cancer drugs [Erika et al., 2010] and studies on the neurotoxicity effects [Li et al., 2009]. MCF-7 is a human breast cancer cell that was has also been used as a good system for the assessment of cytotoxicity and anti tumor activity of animal toxins [Ouadid-Ahidouch et al., 2004; Ip et al., 2008].

Briefly, the objectives of this study were as follows:

1. To study the cytotoxic, apoptotic and anti-proliferative effects of the scorpion venom collected from *Odontobuthus doriae*, *Buthotus saulcyi*, and *Androctonus crassicauda*

2. To study the electrophorotic and chromatographic pattern of the venom, and proteomic analysis

3. To isolate the biologically active peptide fractions from the venom, and study its cytotoxic, apoptotic and anti-proliferative effects *in vitro*, and determination of IC50 and LD$_{50}$

4. Amino acid sequencing analysis of the peptides

Scorpions were collected from different regions of Iran under the permission of Ministry of Health, Govt. of Iran, and kept individually in glass containers. Venom was extracted by mild electrical stimulation (20V, 500mA) of telsons and solubilized in sterile double distilled water, centrifugation at 8000×g for 15 min at 4°C, and the supernatant was
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immediately lyophilized and stored at -20°C until further use. The venom was reconstituted in serum-free DMEM without phenol red and protein was estimated by Bradford method [Bradford, 1976] and kept in 4°C. To expose cells, the reconstituted scorpion venom was disinfected with 1.5% antibiotic-antimycotic solution prior to experimentation.

SH-SY5Y and MCF-7 cells were obtained from national facility for animal tissue and cell culture (NCCS), Pune, India. Cells were propagated in 75 ml plastic flasks in DMEM-F12 and DMEM medium, respectively. Media were supplemented with 10% heat inactivated fetal bovine serum, 10µl/ml penicillin-streptomycin solution or 1% antibiotic-antimycotic. Cells were incubated at 37°C, 5% CO₂ and humidified atmosphere and medium was replaced three times a week. For enumeration, 100µl of cells concentration were stained with trypan blue (0.2 %) and cells were counted using a haemocytometer.

The cytotoxicity of the crude venom and the isolated fractions of the venom were estimated using MTT reduction and LDH release. Oxidative stress was analyzed by assessment of the reactive nitrogen species, level of reduced glutathione and catalase.

Apoptotic effect was studied by determining mitochondrial depolarization, caspase-3 activity and DNA fragmentation (DNA gel electrophoresis and In situ TUNEL assay). The cell cycle arrest of scorpion venom was estimated using immunocytochemistry (BrdU incorporation).

Electrophoresis patterns of venoms of three species of scorpions was studied on Tricine-SDS-PAGE, native and denatured isoelectric focusing (IEF), and two-dimensional electrophoresis.

Determination of chromatographic patterns and separation of fractions were carried out using semi preparative HPLC equipped with a UV detector and a C18 reverse column.
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Amino acid sequencing analyses of selected peptides was done using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF/MS).

The LD₉₀ of crude venoms and selected fractions was determined in the Swiss albino strain of mice, and calculated using arithmetic method (Karber and adapted by Alui and Nwude [1982]) and the graphic method (Miller and Tainter [1944]).

Results of the study were as follows:

The crude venom decreased viability of SH-SY5Y and MCF-7 cells in a dose-dependent manner. The IC₉₀ of A. crassicauda, B. sauleyi and O. doriae venom in SH-SY5Y cells was 207.7, 133.5 and 62.18 µg/ml, respectively, and the same in MCF-7 cells was 269, 272.1 and 118.8 µg/ml, respectively.

Venoms decreased the number of nuclei undergoing DNA synthesis in neuroblastoma and breast cancer cells. The results of this study suggest that venom of A. crassicauda, B. sauleyi and O. doriae arrest DNA synthesis and induce apoptosis in human neuroblastoma and breast cancer cells through induction of various pro-apoptotic mediators such as the nitric oxide and caspase-3. Nitric oxide induced apoptotic effect through depolarization of mitochondrial membrane, triggering mitochondrial permeability transition and liberation of apoptogenic factors in the cytoplasm, causing an increase in caspase-3 activity. Caspase-3 induced DNA fragmentation in cells is hallmark of apoptosis.

These results suggest that crude venoms arrest DNA synthesis and induce apoptosis in human neuroblastoma and breast cancer cells through dependent or independent nitric oxide mediated pathways.

Tricine SDS-PAGE profile of the crude venom of A. crassicauda revealed seven major bands with molecular weights ranging from 6.6-109 kDa. Crude venom of B. sauleyi also
showed seven major bands (molecular weight ranging from 4.1 - 104), and electrophoresis of *O. doriae* venom showed nine protein bands (ranging from 3.9 to 205 kDa).

Subjecting samples of crude venom (10µl contained 15-20µg proteins) to native and denatured IEF and stained with silver staining resulted in different band profile of isolated proteins. In the native IEF, most of the proteins were aggregated in limited place of strip as compared to denatured IEF gel. The second dimensional electrophoresis which was performed by running the sample of native IEF strips demonstrated poor electrophoretical pattern. Therefore, for the purpose of these investigations, the denatured strip was used for further studies.

2D-PAGE profile of the crude venom of *A. crassicauda* revealed minimum 109 protein spots in pl (isoelectric point) range of 4 to 9 and molecular weight from 6.6-205 kDa. Similarly, the electrophoretic pattern of second dimension of crude venoms of *B. sauleyi* and *O. doriae* exhibited at least 96 and 84 protein spots, respectively, with pl between 4-9 and molecular weight ranging from 3.6 - 205 kDa.

The single-step separation of different fractions from crude venoms of *A. crassicauda* by HPLC resulted in isolation of 22 fractions, marked as F1-F22. Fourteen fractions were obtained from crude venom of *B. sauleyi* that were marked as F1-F14. Crude venom of *O. doriae* also yielded 14 fractions, marked F1 to F14.

The three fractions F5, F7 and F17 that obtained from the crude venoms of *O. doriae*, *B. sauleyi* and *A. crassicauda* scorpions, respectively, were found to be more cytotoxic than the crude venoms and other isolated fractions.

The results of this study suggest that three isolated fractions from *A. crassicauda* (fraction 17), *B. sauleyi* (fraction 7) and *O. doriae* (fraction 5) inhibited cell growth in human neuroblastoma and breast cancer cells through its anti-proliferative action on cell
cycle, induced apoptosis mediated by depolarization of mitochondrial membrane, caspase-3 and DNA fragmentation which are established hallmark event in apoptosis.

The LD$_{50}$ of crude venom of *A. crassicauda* in mice (*Swiss albino*) was found to be 0.27 mg/kg whereas for *B. saульyi* and *O. doriae*, it was 1.725 and 0.14 mg/kg, respectively.

The median lethal dose of F17 of *A. crassicauda* was found to be 0.1mg/kg whereas for F7 of *B. saульyi*, it was 1.0 mg/kg. The LD$_{50}$ of F5 of *O. doriae* was calculated to be 0.42 mg/kg.

SDS-Page of three fractions F5, F7 and F17 revealed 5, 3, and 2 protein bands, respectively. The two protein bands, each from fraction F5 and F7, and one protein band from fraction F17, marked as P1 to P5, were selected for amino acid sequencing.

Amino acid sequencing analyses of selected peptides using a matrix-assisted laser desorption MALDI-TOF/MS led us to 5 new short chain peptides. The majority of hits comprised proteins expected to be found anti apoptotic, antimicrobial and antitumor proteins.

The toxicity of scorpion venom has been reported to be due to small molecular weight toxins [Chagot et al., 2005]. Most of these toxins are structurally related disulphide-rich short proteins (23–75 amino acid residues long) and have high affinity to ion channels such as Na$^+$ and K$^+$ ion-channels [Possani and Rodriguez, 2006]. It is believed that this property of venom can be employed for the diagnosis and therapy of various cancers [Upadhyay, 2010]. The LD$_{50}$ studies on various fractions suggest that F5 which makes the peptide a better candidate for developing chemotherapeutic agents.

The antitumor toxins isolated from scorpion venoms have a variety of molecular weight (e.g., Chlorotoxin: 3.95 kDa [Silva et al., 2010], bengalin: of 72 kDa [Das Gupta et al., 2009]). The peptides with small molecular weights (mainly 3-8 kDa) are easy to
synthesize, and may be a good candidate for anticancer therapy. For instance, TM601 is a synthetic anti-neoplastic polypeptide with sequence derived from the chlorotoxin peptide with 36-amino-acid residues (3.95 kDa), isolated from scorpion Leiurus quinquestriatus [Silva et al., 2010].

In this study, we have been able to successfully demonstrate the anti-proliferative and apoptotic activity of the crude venoms and five purified peptides isolated from the crude venom of three different species of scorpions in Iran in SH-SY5Y and MCF-7 cells.

The proteomics analysis of the isolated peptides further revealed that the peptides have considerable homology with other proteins reported in cell differentiation and apoptosis. Of particular importance is the 88% similarity of P3 with Chrysophsin2, which is important from the evolutionary point of view. This peptide, chrysophsin, has been characterized from the gills of the red sea bream, and support the geological and morphological evidences suggesting that some of the oldest scorpion fossils were wholly or partially aquatic [Pocock 1901; Kjellesvig-Waering, 1986; Dunlop, 1999]. The result further provides evidence of mechanistic convergent evolution. Gasparini et al. [2004] have reported that the scorpion and sea anemone toxins block Kv1 channels.

In this study, we found lot of similarity among various isolated peptide fractions and key proteins controlling various fundamental cellular pathways such as apoptosis. We do assume that a comparison of various peptides isolated from the crude venoms would provide an insight into the functioning of these proteins, and at the same time would help us identifying a better peptide with desired effects in cancer.

We do assume that the studies on the purified peptides in animal models may provide lead to development of new anticancer peptides. Five identified peptides from the crude venoms in this thesis are short chain peptides (P2, P3 and P4) with Molecular Weight ranging from 2.9 to 4.2 kDa. This is an added advantage because smaller peptides are easy to synthesize or clone.
References


