Biochemical Characterization and Cytotoxicity of *Heterometrus xanthopus* (Scorpion) Venom

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Biochemical Characterization and Cytotoxicity of *Heterometrus xanthopus* (Scorpion) Venom

A thesis submitted in partial fulfillment of the requirements for the degree of

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IN

PARASITOLOGY

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Declaration

I hereby declare that the work accomplished in this thesis is the result of my own research carried out in Parasitology lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad. This thesis has neither published previously nor does it contain any material from the published resources that can be considered as the violation of the international copy right law.

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Internal Examiner: _____

(Supervisor)

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بسم الله الرحمن الرحيم

DEDICATION

I would like to dedicate this effort to my respected and loving parents. Anything good that has come to my life has been because of their prayers, love and guidance.

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List of abbreviations

BSA	Bovine serum albumin
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
TFA	*Trifluoroacetic acid*
ACN	Acetonitrile
FPLC	Fast protein liquid chromatography
SDS- page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
HCEC	Human corneal endothelial cell
MCF-7	Michigan Cancer Foundation-7
SEC	Size-exclusion chromatography
kDa	Kilodalton
MW	Molecular weight
TOMO	Tomoxifin
NC	Negative controle
PC	Positive controle
ANOVA	Analysis of variance
BGM	Buffalo green monkey
LDH	Lactase dehydrogenase
RT	Retention times
TFA	Trifuoroacetic acid
ACN	Acitonitrile

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Muhammad Qasim

ABSTRACT

Scorpion venoms are the sea of bioactive peptide catalogue that offer promising molecules that may give rise to the revelation and development of novel drugs. *Heterometrus xanthopus* is the most venomous animal, produces potent venoms that aggravate harsh symptoms in envenomated prey. The present investigation, the peptide profile of venom was studied by electrophoretic methods, size-exclusion (FPLC) and reverse phase chromatography. Cytotoxicity of venom was assessed on human corneal endothelial cell line (HCEC) and breast cancer cell line (MCF-7) by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Reverse phase High performance liquid chromatography (RP-HPLC) of crude venom helped us to see the complexity present in venom. A number of peaks isolated from venom on different retention time with absorbance. The size exclusion chromatography- Fast protein liquid chromatography (SEC-FPLC) and SDS-page analysis elucidated that the *H. xanthopus* venom contain high molecular weight peptides ranging from 139.3 to 44.77 kDa as well as low molecular weight peptides ranging from 11.37 to 8.64 kDa. Biomodal mass distribution peaking between molecular weights of 1 to 20 kDa (57%) and 61 kDa to 80 (17%). This statement confirmed the presence of low molecular weight peptides in abundance. *H. xanthopus* venom had cytotoxic effect on MCF-7 cells and HCEC cell, among all fractions, fraction 7 that contain low molecular weight peptides have great cytotoxic effect, which may explain by presence of low molecular weight toxins in scorpion's venoms. The present work represented the first peptidomic characterization of *H. xanthopus* venom. Contemplate the molecular weight-function relationship of already identified venom peptides, future bioactivity studies may lead to the discovery of potassium and chloride ion channel inhibitors as well as antimicrobial peptides from *H. xanthopus* venom.

INTRODUCTION

The scorpion belongs to Phylum Arthopoda: class Arachinida and order Scropionida (Dunlop & Braddy, 2001). The order Scorpionida includes 2,100 species, 190 genera and 24 families (Gomes & Gomes, 2015). Scorpions are predators of insects. The scorpions possess two venom glands linked to telson sting which is located on the last segment of body. Venom producing gland is imperative for their survival, help feeding and self-defense. The vesicles present in telson are venom producing and storage factory (Marcussi *et al*., 2011). The morphological, physiological and biochemical studies illustrated that scorpions are well adapted to every environment due to their unique characteristics. Scorpions live in every corner of the world except Antarctica. They are found commonly in tropical, semi-arid zones and sub-tropical (Stockmann, 2015). Scorpions are commonly supposed to live in deserts, but indeed, they found in many habitats like intertidal zones, Montane pine forest, rain forests and caves. Scorpions have been established habitat under snow-covered rocks in Himalayas of Asia at height of 12,000 feet (Gouge & Olson, 2011).

Scorpions are nocturnal animals live in crevices, hiding under wood, stones and in debris during the day time (KovAfeftc, 1995). Scorpions are classified into three groups on the basis of habitat (i) burrowing (Psammophilous or Pelophilous) (ii) rock dwelling (Lithophilous) and arboreal (Tikider & Bastwade, 1983). *Buthus* and *Heterometrus* belong to burrowing scorpion category. They live in small pit like burrows (Tilak, 1970).

1.2: Genus *Heterometrus*

The genus *Heterometrus* belongs to family scorpionidae and sub family scorpioninae. *Heterometrus* species are found in all over the India, Pakistan, Burma, Borneo, Sri Lanka, Philippines and Africa. 33 species of *Heterometrus* are existed mainly in tropical and sub-tropical regions of southeastern Asia, including Laos, Thailand, Vietnam, India, Sri Lanka, Nepal and China. *Heterometrus scaber* and *H. fulvipes* are found in the southern part of India, whereas, *H. bengalensis* and *H. swammerdami* are found in the eastern part of India (Gomes & Gomes, 2015).

1.3: Scorpion venom

Scorpion venom is a good source of protein and toxins with physiological and pharmacological activities; it shows activity against targeted-specific inflection of various ion channel functions. Scorpion venom provides a rich source of peptidyle neurotoxins (Ali *et al*., 2016). Venom of scorpion is a cocktail of different peptides, free amines, nucleotides, lipids and other many bioactive compounds (Petricevich *et al*., 2013). The scorpion venom also contain other molecules such as mucopolysaccarides, phospholipids, hyluranidases, protease inhibitors, other molecules such as low molecular weight peptides e. g. serotonin and histamine. It is also mixture of inorganic salts, neurotoxic peptides (Muller, 1993; Martin-Eauclaire & Couraud, 1995). All these compounds are essential for scorpion defense and capture preys compared to snake venom and spider venom, scorpion venom shows a little bit low enzymatic activity, because snake venom has 95% protein contents but, scorpion venom contain salts and lipoproteins (Gwee *et al*., 2002). There are two main groups of scorpion venom: the disulfide-bridge peptides, it target membrane bound ion channels, other is non-disulfide-bridge peptides it is relatively small group of peptides in scorpion venom which have different toxin properties (Fig.1) (Almaaytah & Albalas, 2014).

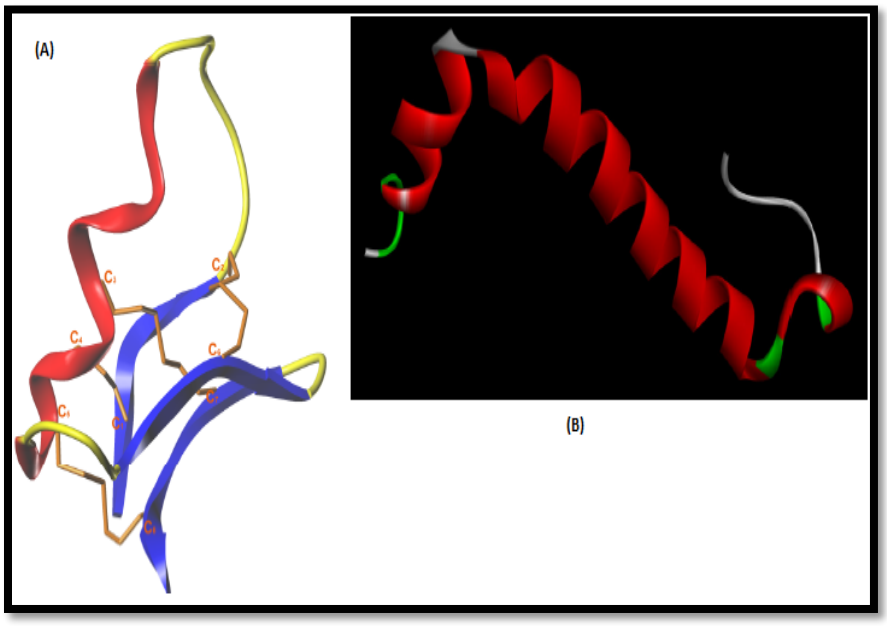

Figure 1: (A) Chlorotoxin with disulfide bonds (B) Mauriporin without sulfide bond (Almaaytah *et al*., 2014; Dardevet *et al*., 2015)

Disulfide-bridge-peptides do contain three to four disulfide-bridges it is further classified in four families on the basis of their interaction with ion channels. The membrane bounded ion channels targeted by disulfide–bridge–peptides families include Na^+, K^+, Ca^+ and Cl^- channels (Possani et al., 1999). The chemical composition of scorpion venom, potency, protein contents and toxicity varies from species to species (Dyason *et al*., 2002; Borges *et al*., 2006). Na+ channel scorpion toxins are peptides of 60±76 amino acid residues in length, with four disulfide bridges strongly bounded (Gordon *et al*., 1998). Toxins specific for Na+ channel from scorpion show toxicity on channel gating mechanism further divided into to two groups: α- and β-toxins (Jover *et al.*, 1980; Gordon *et al*., 1998). Neurotoxins from scorpion venom are classified into α-toxins and β-toxins. The α-toxins of scorpion further classified into three different pharmacological subfamilies these are classical α-mammal toxins, insect α-toxins, α-like toxins, these are divided on the basis of their activities against mammals and insects. The K+ channels are about to 31-39 amino acid residues and bounded by three to four disulfide bonds. K+ toxins are blockers of channels; the way of their action is to bound extracellular face of the channel and obstructs the passage of ions (Olamendi-portugal *et al.*, 1996; Garcia *et al*., 1997). Chlorotoxins are so specific for Cl^- channels, by binding of this toxin to Cl^- channel it block the passage of Cl^- ions, that toxin has 36 amino acid residues and bounded by four disulfide bridges (Debin *et al.*, 1993; Lippens *et al*., 1995). Whereas, Ca^{+2} channels are specific scorpion toxins are highly conserved, dense core formed by α-helix and two to three strands of β-sheet structural motifs, uphold by disulfide bridges (Kobayashi *et al*., 1991).

1.4: Therapeutics potential of scorpion venom

The animals that produce venom thought to be notorious completely give negative results in accidental contact with humans from one side they cause minor signs and symptoms, for example allergic reactions and dermatitis, or very severe symptoms while on other hand, they have also useful toxins. Scorpion venoms and their extracts are useful in treatment of variety of nervous system diseases such as epilepsy, apoplexy pains, sudden Spasm, cardiovascular and cerebrovascular diseases, inflammation, hepatopathy and facial paralysis for more than 1000 years since the Sung Dynasty. Scorpion venom has antitumor activity that is helpful in treatment of different kinds of cancers as well as it also has antimicrobial agents (Zhou *et al*., 1989).

Despite all efforts made by developed countries to treat cancer, it is still a major cause of death. Surgery, chemotherapy and radiotherapy make available insufficient protection. The treatment of cancer by these techniques can harm normal cell to along with cancer cells as well as too much expensive. The cancer treatment from natural product has been practiced over a century. Scorpion toxins have very strong anticancer activities it kill cancerous cells (Mamelak & Jacoby, 2007). The florescent toxins derived from scorpion venom when injected to cancerous tissue helps to indicate the boundary of tumor (Lyon *et al*.,2002). Scientists developed anticancer drugs from proteins and peptides originating from venomous animals like scorpions. These proteins may bind selectively to cancer cell membrane to stop proliferation and may stop the metastasis of these cells. The protein and peptides isolated from venomous animals divided on the basis of their binding mechanism: ion channel toxins from scorpion, which effect on cancer cells by changing the physiology of cancer cells through blockage of specific ion channels (Jäger *et al*., 2004). Chlorotoxin from scorpion venom binds to cancerous cell membrane and block the Cl^- ion channel leads to death of cancer cells (Deshane *et al*., 2003).

Scorion venom also contain large amount of low molecular weight peptides (Possani *et al*., 1999). Venom constituents from different sources of venomous animals have also potential antimicrobial activities (Conde *et al*., 2000). *H. xanthopus* (Scorpion) is one of the most noxious Arthropods. The venom of *Heterometrus* genus contain Substances L, Toxin-HB, HsTX1, kappa-Hefutoxin 1, heteroscorpin 1, Hp 1090, HmTx and HsAp and HsAp have been discovered (Gomes & Gomes, 2015). Its venom has variety of antimicrobial substances like hadrurin, scorpine, Pandinin 1, and Pandinin 2 that are capable to efficiently kill MDR (Multiple drug resistance) pathogens (Ahmed *et al*., 2012).

The first recognized substances from venom gland of scorpion *Mesobuthus eupeus* are Meucin-24 and Meucin-25. Which are selectively kill *Plasmodium falciparum* and restrain the development of *Plasmodium berghie*, these are malarial parasites, and surprisingly they have no cytotoxic effect on normal mammalian cells. These two venom-derived proteins are used for the development of anti-malarial drugs (Gao *et al*., 2010). In recent times, natural antimicrobial peptides are focusing more attention as biological compounds and be good cut-out to develop

beneficial agents, including antiviral agents against an array of viruses (Li *et al*., 2014; Zhang *et al*., 2013).

1.5: Scorpion venom toxicity to normal cells

Among scorpions, 25 species are dangerous to humans (de Roodt *et al.*, 2003). Hence, in tropical countries scorpion envenomation is serious health problem (Dehesa-Dávila & Possani, 1994). The biological effects of scorpion stings are mostly due to the occurrence of low-molecular-weight proteins in the venom that apply powerful effects on excitable cells (Jalali *et al*, 2007). Membrane channel blockers are known to control certain cellular behavior in the metastatic cascade (Laniado *et al*., 2001). *Heterometrus* envenomation tempted many changes in the metabolic profile of prey that might worsen the pathophysiological conditions of patients. This effect is due to the direct or indirect acts of venom constituents. On injection of venom it releases of mediators is also involved in this process (Babu *et al*., 1971). The scorpion venom acts on exposed fibers or on muscles directly or through nerves and neuromuscular intoxication, this activity of venom is attributed to neurotoxins present in venom of scorpions. Scorpion neurotoxins depolarize the excitable membranes results in increase in the sodium permeability of resulting membrane, thereby reducing and amount of sodium inactivation (Katz, 1966). The scorpion venom also effect respiratory system (Ismail *et al*., 1973; Freire-Maia *et al*.,1973) and homeostasis mechanism of body (Yarom *et al*., 1970).

1.6: Purification and characterization of venom

Several methods have been employed for the purification of venom protein for the structural studies. The crude venom of scorpion is extracted in water and centrifuged. It is purified by gel filtration column and recycling on Sephadex G-50 (Couraud *et al*., 1982; Hagag *et al*., 1983). Modern techniques like spectrophotometry, chromatography, SDS-Page and bioinformatics tools have been using for isolation, purification and characterization peptides present in scorpion venom to study structure and functional relationships intensely (Shao *et al*., 2007).

1.7: Role of scorpion venom in treatment of cancer

Cancer is derived from Latin word stands for crab due to the resemblance of cancerous cells with crab having claws reaching out of the surrounding tissues (Fallis, 2013). Cancer is not

a single disease rather it is a group of diseases described by the uncontrolled growth and increase of abnormal cells that may cause death. The treatments of cancer are dealing in many ways including surgery, radiation, chemotherapy, hormone therapy, immune therapy and targeted therapy (Siegel *et al*., 2016).

In the current era, cancer is a very challenging issue as more and more peoples are suffered from different kinds of cancer either, due to various kinds of conditions such as pollution and radiations, e.g. ultra violet rays and X-rays. Common malignancies are now treating with these kinds of therapies like Lung, breast, colorectal, pancreatic cancer some other kinds such as lymphoma, Leukemia and myeloma.

The mechanisms of action and toxicities of targeted therapies differ from those of traditional cytotoxic chemotherapy and generally give better results than traditional chemotherapy, some adverse effects of targeted therapy observed also, such as acne forms rash, cardiac dysfunction, thrombosis, hypertension, and proteinuria and many other deformities (Gerber, 2008).

Now a days scorpions venom peptides have attracted attention as substitute for chemotherpeupeutic agents that may overcome the limitations of current drugs, providing specific cytotoxicity for cancer cells with capabilty to aviod multidrug-resistance mechanism, addictive effects in combination therapy and safety (Tong-ngam *et al.*, 2014).

Scorpion venom peptides have an immense variety of biological activities, immune-modulatory activities and pharmacological functions. Many peptides derived from different species of scorpion venom showed cytotoxicity against malignant cells and tumors (Wang & Ji, 2005; Gupta *et al*., 2007; Fu *et al*., 2012). Crude scorpion venom and some purified proteins can damage cancer propagation and hold cell cycle (Gomes *et al*., 2010; Heinen & da Veiga, 2011). Studies showed that the different elements of scorpion venom can induce anti-proliferative effects on eight human solid cell lines which are: Human liver carcinoma cell lines (HepG2 and SMMC-7721), a human non-small cell lung cancer cell line (A549), human osteosarcoma cell lines (MG63, U2OS, and KHOS), a human colon cancer cell line (HCT-116) and a human cervical cancer cell line (HeLa) also, they bring cell cycle arrest, apoptosis and DNA damage (Zhang *et al*., 2013). Cells list has been shown that the venom of *Odentobuthus doriae* induces

swelling. Inhibition of neurite extension, break the membrane and expulsion of cytosolic substances in the human neuroblastoma cells. The scorpion venom also increases platelet accumulation and significantly increases lactase dehydrogenase (LDH) level in treated cells as compared to normal cancerous cells. So, that induces more apoptogenic and cytotoxic effects.

Two basic components are designed to cure cancer; one is stoppage of DNA replication and another induction of cell death. Scorpion venom encourages DNA fragmentation, which might be caused by acute necrosis that raises caspase- 3 sharply (Gadwalkar *et al*., 2006). The scorpion venom component III (SVC III) discriminately acts against human leukemia Jurket cell line and THP-I cells. Scorpion venom component III (SVC III) uses its effects by modulating the NF-kb signaling pathway, nuclear factors kB is very vital transcriptional factor and plays important role in proliferation, synthesis of immunocytes, increasing of T and B lymphocytes and in cell apoptosis. Thus, the scorpion venom play important role in vanishing of glioma cells. Cholorotoxin specifically binds to the glioma cells but not with normal neurons, astrocytes and fibroblasts and further, it binds with fluorescent dye to display the tumor lines and makes surgical removal easier (Lyons *et al*., 2002; Veiseh *et al*., 2007; Jacoby *et al*., 2010).

Scorpion venom is also very effectual against cancer. It has cystine knot (cystine rich amino acids) with additional disulfide bond. So, it selectively binds to the brain tumor cells. Many peptides are still an enigma and lot of research is needed to recognize other therapeutically peptides from scorpion venom. From above note, it seems that these bioactive molecules have been produced specifically to cure different types of cancer up to greater extent (Mishal *et al*., 2013).

Aim and Objectives

1. To find active fraction from *H. xanthopus* venom by electrophoretic and chromatographic pattern.
2. To study in-vitro cytotoxic, apoptotic and anti-proliferative effects of isolated fractions.

MATERIALS AND METHODS

2.1: Collection of scorpions

The scorpions (*Heterometrus xanthopus*) were collected from Haripur district, Khyber Pukhtoonkhwah, Pakistan, 33.9960° N, 72.9368° E, temperature: 21.5 °C (Fig. 2). The collection was conducted in month of July from dry locations by bait method. They were collected in plastic jars with proper oxygen supply and brought to parasitology lab, Quaid-i-Azam University Islamabad and was kept in optimized isolated soil chamber (RH= 80%±26ºC). The scorpions were identified by using key (ovařík, 2015). The insects and liver of chicken along with water were provided as food source in the petri plates

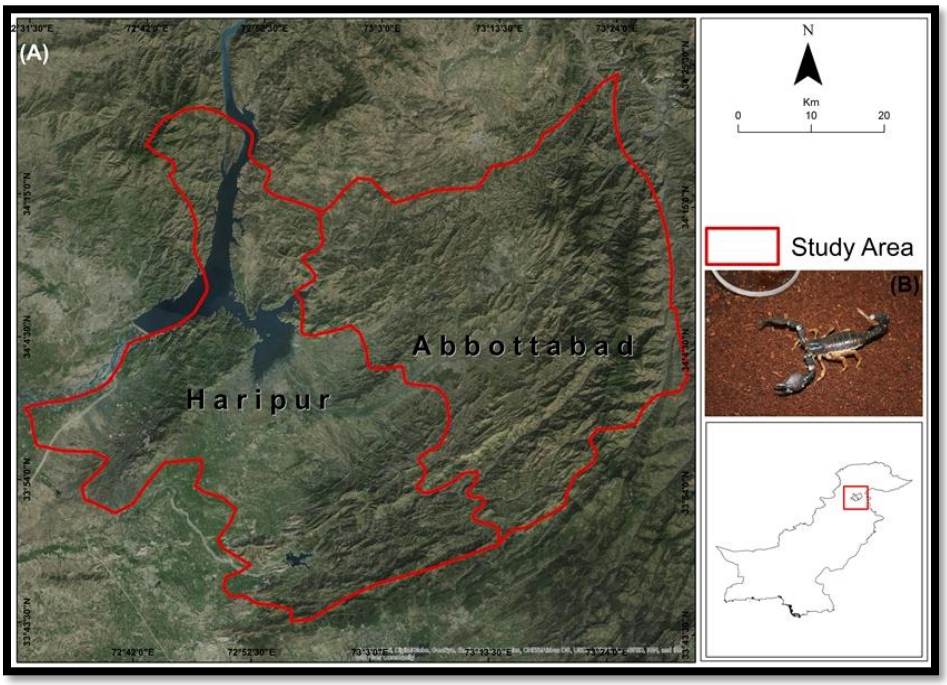

Figure 2: (A) *Heterometrus xanthopus* sampling location (B) scorpion in captivity

2.2: Venom extraction and preservation

The extraction of venom was carried out by electrical stimulation using 12V batteries in tail joints, for electrical conductance concentrated sodium chloride solution was applied in tail joints near telson (Nagaraj *et al*., 2015).

Venom was collected in PCR tubes and stored at -20 °C until use. Venom extraction was repeated after 15 days of previously extraction (Fig. 3). Crude venom was centrifuged for about a minute to settle down some drops of venom stacked at the walls of the PCR tube.

Figure 3: Venom extraction by electrical stimulation

2.4: Protein estimation

The protein contents present in crude venom were obtained using Bovine Serum Albumin (BSA) as standard in 96-well microtiter plate through Bradford assay. The BSA (Merck, Germany) standard concentrations 6.2512, 5025, 0050, 0075.00 and 100.00 mg/ml were used. Protein estimation was done by measuring the absorbance at 595nm for set of samples and BSA, a standard curve was estimated for BSA with the following equation.

$$y=3E+30x3E+30$$

$$R^2 = \#N/A$$ (Bradford, 1976).

2.5: Reverse Phase –High performance Liquid Chromatography (RP HPLC)

Crude venom analysis was performed using areverse-phase HPLC using Nucleosil 7 C18 column (250× 10mm; Macherey-Nagel, Düren, Germany) Assigned to LSPD-M20A UV-VIS detector and LC-20AT pump with a 100µl sample injection loop. Two mobile phases, A (0.1% V/V TFA/H_2O) and B (0.1% V/V TFA/ACN) freshly prepared from HPLC grade solvents were used. Before use mobile phases were degassed via sonication. Lyophilized scorpion venom was dissolved in mobile phase A. Dissolved Sample was centrifuged at 14,000rpm for ten minutes (Ali *et al*., 2016). 100 µl Supernant was injected. A linear gradient (0-100%) of mobile phase B were used at a flow rate of 1ml/min. The peptide absorption was monitored at 214nm.

2.3: Fast-Protein Liquid Chromatography

Chromatographic behavior of scorpion venom (0.5 mg) was also analyzed by fast protein liquid chromatography (FPLC, GE Healthcare, UK) using Size exclusion column (Superdex-75, 10/300 GL) and phosphate saline buffer (50mM Phosphate-HCl buffer, pH 7.5, containing 100mM NaCl and 0.02% NaN_3) as mobile phase. Column was calibrated by purified bovine serum albumin (0.1 mg BSA, Merck Germany) under similar buffering conditions. Lyophilized venom was dissolved in the same buffer, which is then centrifuged at 14,000 rpm for ten minutes, 0.1 ml supernatant was loaded on to the column. The eluent was monitored at 280 nm. The data was analyzed by automated UNICORN 5.0 software (GE Healthcare, UK). The fractions were further analyzed by SDS-PAGE to get better insight of venom composition SEC-FPLC.

2.4: SDS-PAGE Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of whole venom was also carried out under reducing (5 minutes incubation at 100^oC) and Non- reducing conditions (without incubation) in 12.5% polyacrylamide slab gel. 20µg venom was dissolved in 15µl respective reducing (with SDS and β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) sample diluting buffer. Each sample was loaded in separate lane. The markers of known molecular weights were loaded as standard. Gel were run at room temperature at 120 V; run was stopped when dye front was less than 10mm from the base of the slab. Overnight

staining of gel was done by colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/l ammonium sulphate, 1 g/L Coomassie blue G-250) and de-stained in deionized water (Ali *et al.*, 2013).

2.5: Cell viability assay on HCEC (normal) cell line and MCF-7 breast cancer cell line

The cytotoxicity of the *H. xanthopus* on HCEC was determined by MTT assay. Cells (1×10^5Cells/ well) were plated in 96 well culture plate (Falcon 3077, Becton Dickinson Labware, New Jersey), with various known concentrations (1, 2, 3 and 4mg/ml) of fraction obtained by FPLC from *H. xanthopus* venom at 37 °C with continuous supply of 5% CO_2 in SCO6WE SHEL LAB Basic CO_2 Water Jacketed Incubator, 6 (Shel Lab, USA). After 24 hours of incubation cell viability was determined by standard MTT assay (Salahuddin *et al*., 2015). The effect of various fractions on the proliferation of HCEC cells were expressed via percent cell viability. The anti-cancer activity of venom fractions was also tested on MCF-7 by the MTT method. Percent viability of cells was plotted against the different concentrations of fractions of venom.

Percent viability

The linear equation given below was used to calculate percent viability. 100 % viability was taken as negative control. By the formula given below, % viability of test compounds was calculated, comparing it with the negative control.

Percent viability= (A sample–A Blank) / (A control– AB lank)

2.6: Procedure

- Previously cultured cell were collected, and observed under the microscope for confluences and contamination. It was assured that the samples were more than 80% confluent and no signs and indication of bacterial and fungal contamination were observed.
- Firstly, the old cultured media was removed. The cells were then washed with PBS. This was done to separate certain bound serum, magnesium and calcium.
- Then such quantity of trypsin was added so that it can cover the entire surface of the cells. The trypsin covered cells were incubated at 37°C for 5 minutes. Cells were

observed under inverted microscope to check whether they were detached from the flask or not.

- When the cells were completely detached from the flask; almost 10ml of media was poured into the flask. This assembly was then kept in a humidified incubator with 5% CO_2 at 37°C temperature for half an hour. Some damaged cells might be regenerated at this step.
- In the next step, all the cells were collected in a 15ml falcon tube and centrifuged at 55000 rpm for about 5 minutes. This fast centrifugation pelleted out the cells.
- Cells were then counted by hemacytometer and 10 µl cell suspensions was taken from falcon tube for this purpose
- Cell number was adjusted at 2.5 * 105 cells per ml. 96 well plate needs about 10 ml of cell suspension which contains approximately 2.5 * 106 cells per 10 ml.
- 100µl cell suspension was added to each well and each well received about 25000 cells.
- On 96 well micro titer plate (round bottomed), serial dilutions of venom was applied. 7 FPLC fractions, the concentrations were 1, 2, 3 and 4 mg/ml
- Tomoxifin (an anticancer drug) was used as a positive control. The steps given below were done. For negative control only MTT and cells were added
- The mixture was incubated for 24 hours in CO_2 incubator with 5% CO_2 and at 37°C. After one day incubation, 10 µl of MTT solution was added to each of the well.
- Following it, the mixture was again incubated in CO_2 incubator for 3 hours at 37°C.
- Solubillization solution (100 µl) was poured and mixed by pipetting up and down repeatedly to until complete solubillization was observed.
- Finally absorbance was measured at 570 nm

2.7: Statistical analysis

The statistical significance was determined using one-way ANOVA (Analysis of variance) with Tukey's multiple comparison test using Graph prism 5 among fractions and positive control (PC), results with P value less than 0.05 was considered to be significant. The statistically significant data were denoted by stars (***).

RESULTS

3.1: Protein estimation

The concentration of total protein in crude venom was found to be 2mg/5mg (Table 1 and Fig. 4). The concentration of proteins in fraction 1 was 1ug/ul, the concentration of protein in fraction 2 was also same as fraction 1 1ug/ml. All of the fractions contained same concentration of protein except fraction 5 which have slightly more i.e. 1.5ug/ml.

Table 1: Protein concentration in various fractions obtained by SEC-FPLC

No. of fractions	Amount of Sample (ul)	Dye (ul)	Total (ul)	Absorbance (nm)	Concentrations (ug/ul)
1	10	300	310	0.467	1/10
2	10	300	310	0.1461	1/10
3	10	300	310	0.47	1/10
4	10	300	310	0.482	1/10
5	10	300	310	0.542	1.5/10
6	10	300	310	0.465	1/10
7	10	300	310	0.449	1/10

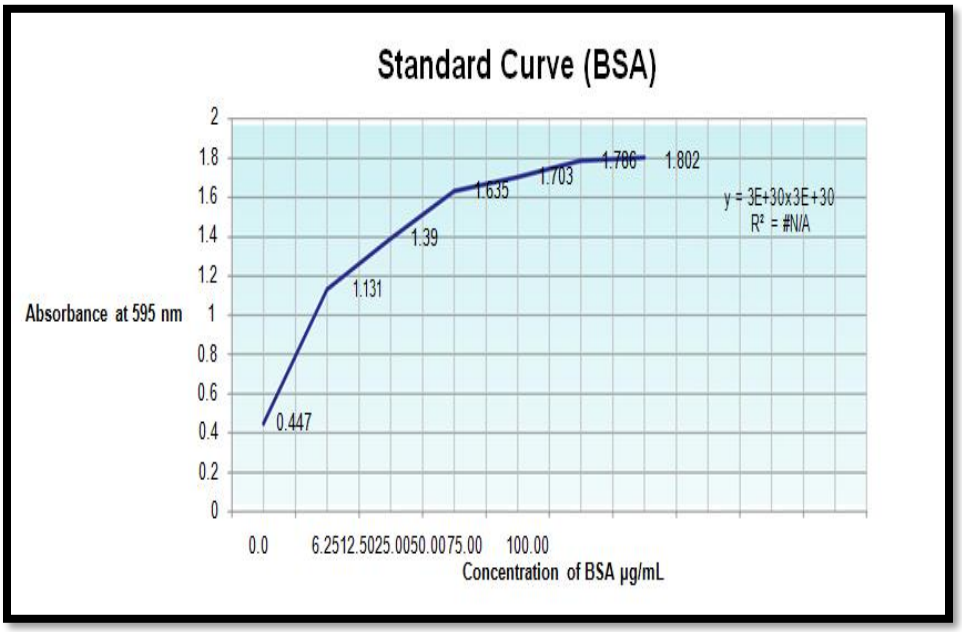

Figure 4: Bradford assay standard curve for determination of protein contents in major fractions, isolated by SEC-FPLC.

3.2: Reverse-Phase Chromatography

Venom profile obtained through HPLC analysis showed enormous peaks corresponding to different peptide fractions. The crude venom was analyzed quantitatively. The venom do comprise some intently associated profiles on the subject of the foremost components and time of elution, the result demonstrated that venom of *H. xanthopus* contain variety of components. But the majority of components eluted in 4 to 40 minutes. Moreover the result also showed the complexity in venom. In first 100 minutes it is giving continuous peaks on different absorbing rates. Venom profile showed a distinct peaks present in the *H. xanthopus* venom on (4, 10, 20, 26, 28, 38, 56, 66, 86 and 100 min) with respect to elution time (Fig.5).

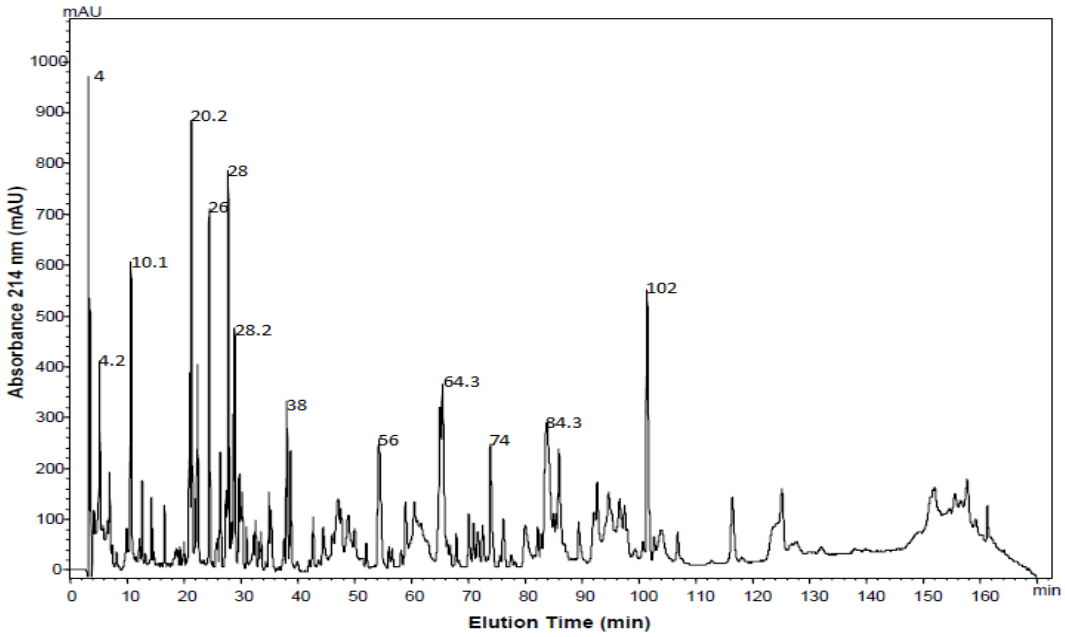

Figure 5: RP-HPLC separation of soluble crude venom

3.3: SDS-PAGE analysis of crude venom

Electrophoretic profile showed the presence of enormous number of peptides in venom. The results revealed that venom of *H. xanthopus* contain both low molecular peptides ranging from 14 kDa to 4 kDa and high molecular weight peptides ranging from 61 kDa to 200 kDa. The venom was analyzed under reduced and non-reduced states. In reduced condition below 4 kDa and above 200 kDa peptides were also detected. In non-reduced state the protein remained in original position, it gave a single piece of band which is 6 kDa in size. A known molecular weight peptides marker was also run with sample that are 200, 116.2, 97.4, 66.2, 45, 31, 21.5, 14.4 and 6.4 kDa respectively (Fig.6)

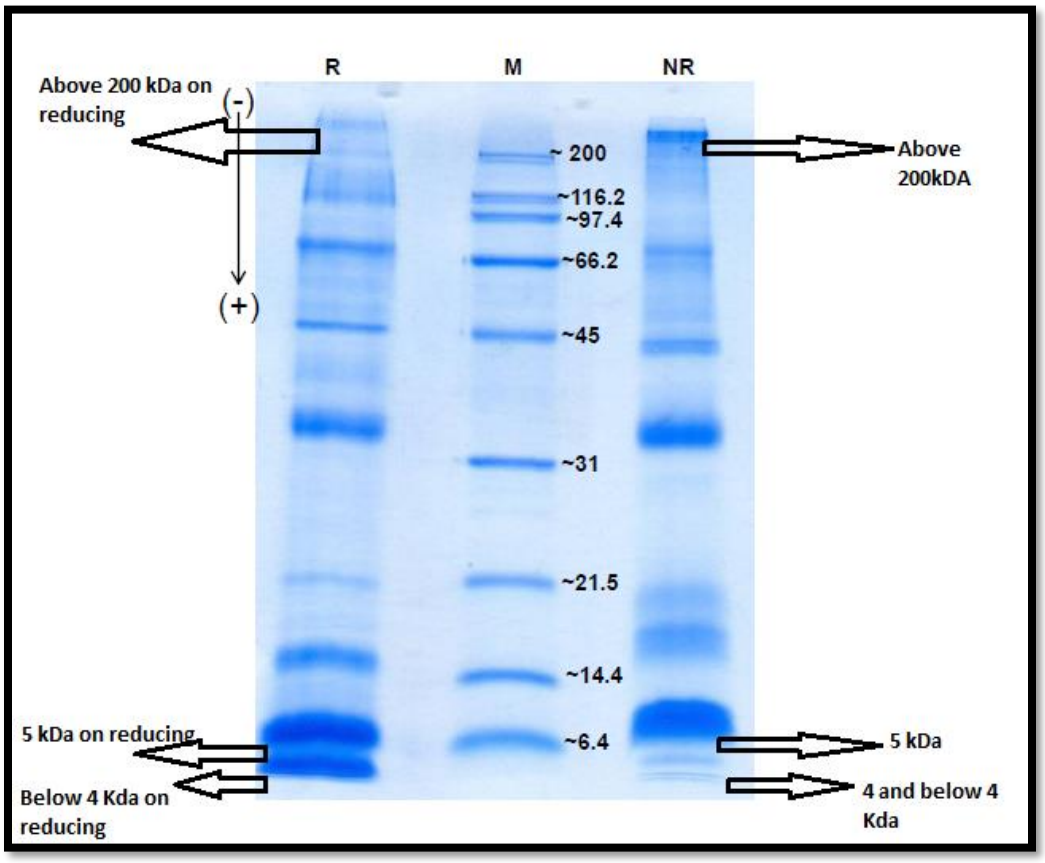

Figure 6: SDS-PAGE analysis of whole venom. Arrows corresponds the peptides.

3.4: Fast-Protein Liquid Chromatography

Seven predominant fractions were obtained from SEC-FPLC run: a fantastically sharp protein peak with a predicted molecular weight of 200 kDa and mixture of numerous peptides peaks in approximately 60 to 8 kDa. Surprisingly, the first peak produced in SEC gave band of 139.3 to 77.44 kDa on SDS-page analysis. Other peaks comparably have low molecular weight peptides ranging from 80 to 40 kDa. The mostly expectedly low molecular weight peptides found in abundance their molecular weight has detected from 16 kDa to 8 kDa (Fig.7).

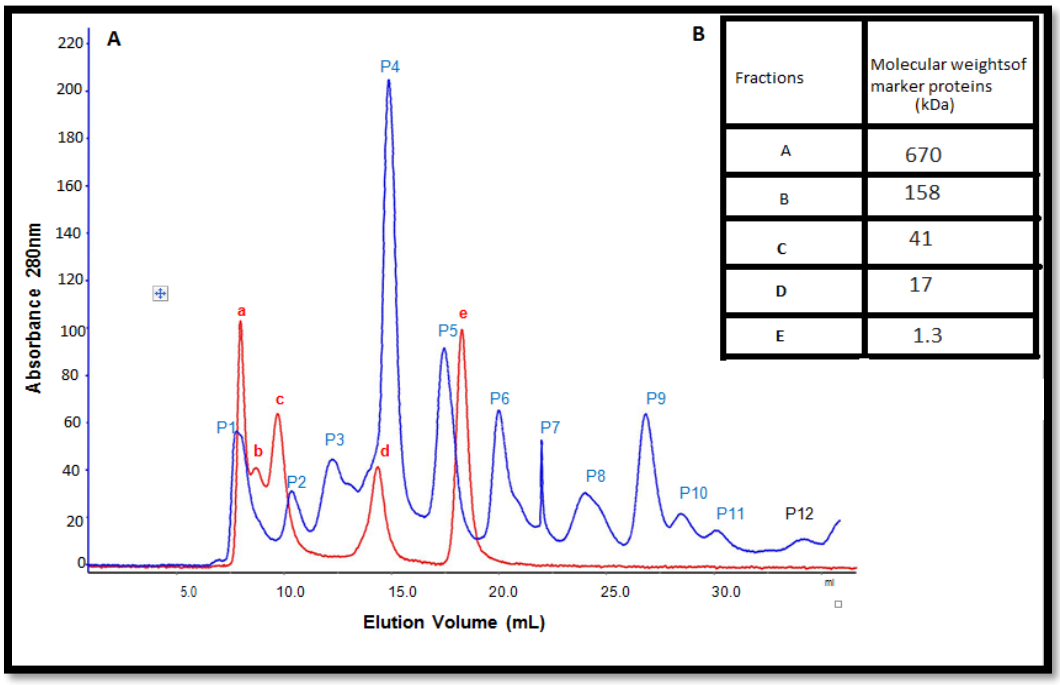

Figure 7:(A) Separation of *H. xanthopus* venom by using a superdex 75 molecular exclusion column on FPLC system.(B) Molecular weight markers

3.5: SDS-PAGE Analysis of fractions

Polyacrylamide SDS-PAGE analysis was performed on seven selected fractions obtained by SEC-FPLC. Each fraction showed particular bands, which indicates molecular weight of peptides. As shown in Figure 2: fraction 1 contains 139.3, 110.2, 90.50, and 77.45. Fraction 2 contains 52.37, 19.70, 16.18, and 9.5. Fraction 3 contains 71.61, 66.22, 19.68, 13.30, and 10.11. Fraction 4 contains 66.22, 13.30, 11.37 and 9.35. Fraction 5 contains 68.80, 44.77, 24.88, 14.33, and 9.35. Fraction 6 contains 41.39, 11.37, 9.72, and 8.64. Fraction 7 contains 9.72and 8.64. The results demonstrated that venom of *H. xanthopus* contain variety of peptides, the proteins of 200 kDa molecular weight also included in venom which is a very large compound in proteins family. Side it was observed that venom contain very low molecular weight peptides ranging from 11 kDa to 8 kDa (Table 2, Fig.8)

Table 2: Molecular weights of peptides obtained by SEC-FPLC. Each peak corresponds to protein bands

Bands	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7
1	139.3	52.37	71.61	66.22	68.80	41.39	9.72
2	110.2	19.70	66.22	13.30	44.77	11.37	8.64
3	90.50	16.18	19.68	11.37	24.88	9.72	
4	77.45	9.5	13.30	9.35	14.33	8.64	
5			10.11		9.35		

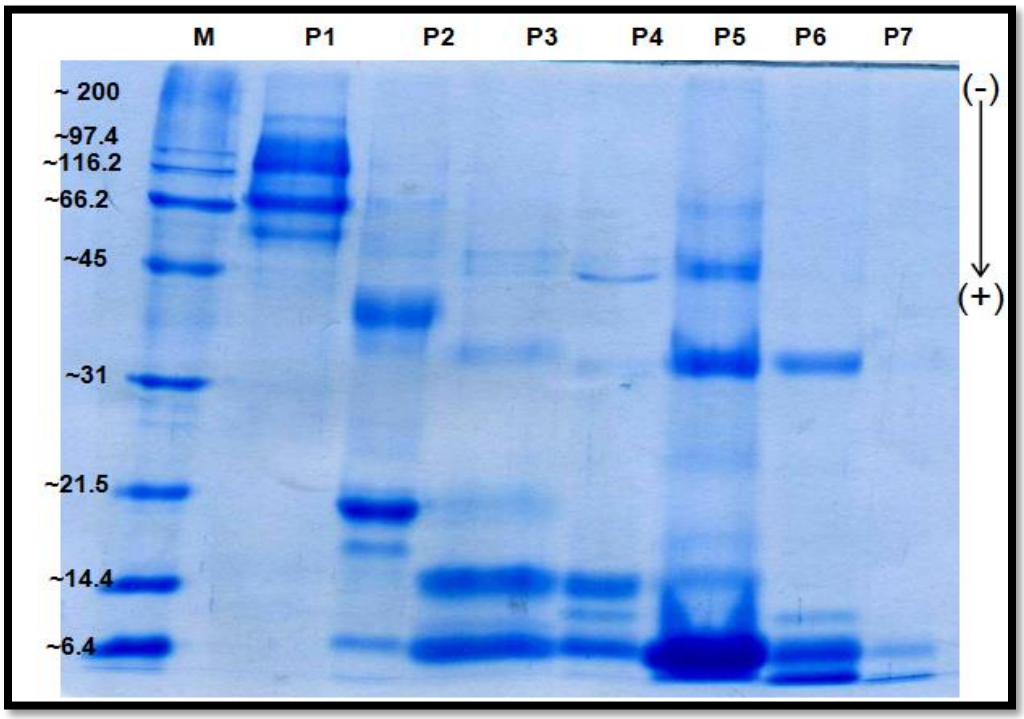

Figure 8: Electrophoresis patterns of *H. xanthopus* venom on SDS-PAGE.

3.6: Determination of Molecular weights of unknown peptides in SDS-page analysis

The molecular weight of marker was determined by making standard curve. The green dots denoted the molecular weight of each band, the first dot traveled 2 cm distance, it indicated the molecular weight 200 kDa, and second dot traveled 7 cm distance which corresponded to 116 kDa band. The third band traveled 10 cm distance which indicated the molecular weight of 97 kDa, the fourth is 45 kDa distance traveled by this is 2.5 cm. fifth dot determined that it contained 31 kDa protein. The sixth dot denoted 21.5 kDa. The seventh band denoted 14.4 kDa. The last one marker is lowest molecular weight 6.4 kDa (Fig.9).

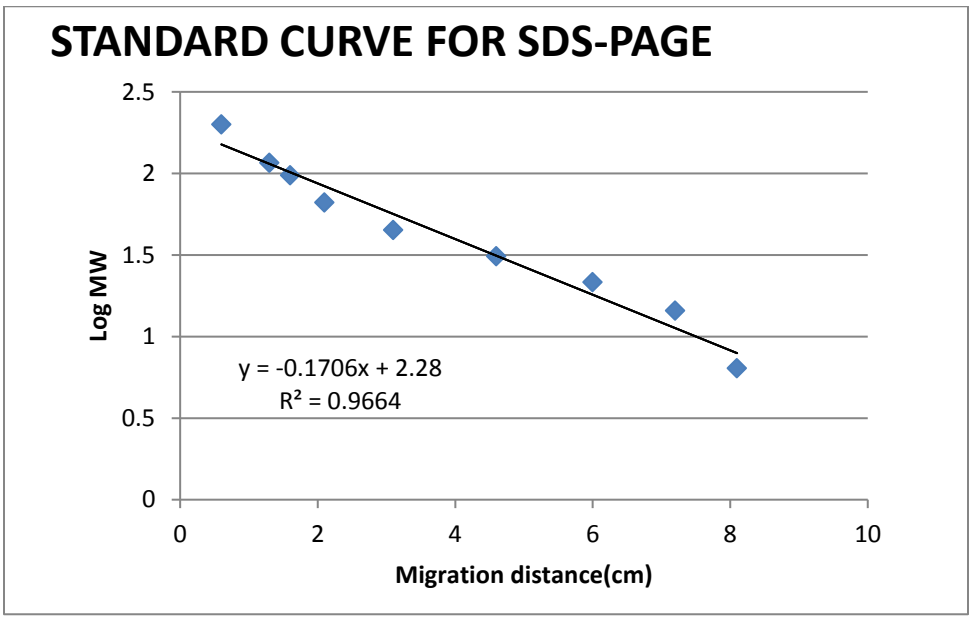

Figure 9: Standard curve for unknown band by using SDS-PAGE

3.7: Molecular weight distribution

The molecular weight distribution of peptides is shown in the Figure10 in the form of histogram. It has represented a bimodal mass distribution for the scorpion venom in which most of the peptides fall in either 6.5 to 7.5 kDa or 9.4 to 20.4 ranges. Peptide mass profile of *H. xanthopus* partially matches this general trend considering that one third of its peptides are in the 60 to 80 kDa ranges.

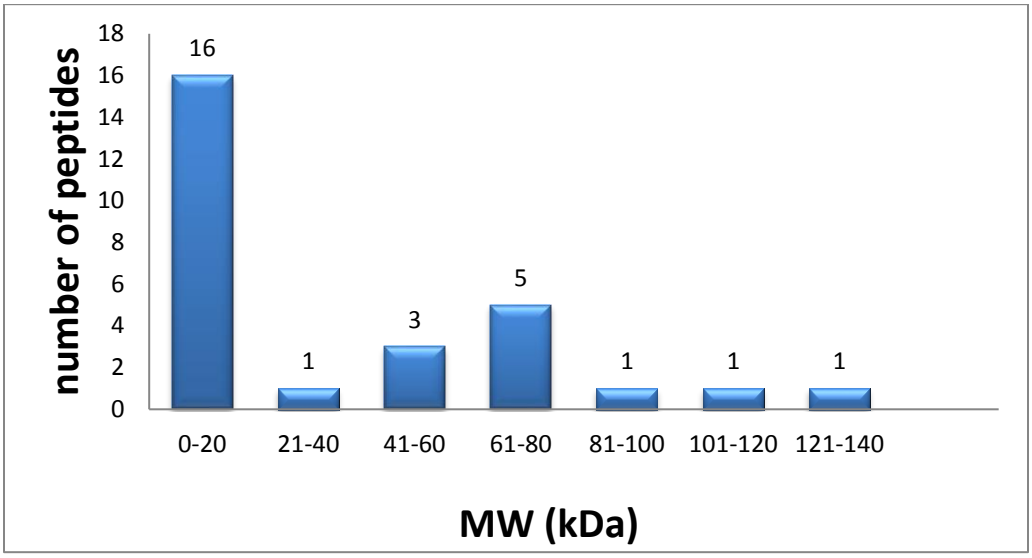

Figure 10: Molecular weight distribution of *H. xanthopus* venom peptides.

3.8: Cytotoxicity assay of *H. xanthopus* venom on HCEC and MCF-7 cell lines

Cytotoxicity of *H. xanthopus* venom was tested on cell lines to confirm its activity. The isolated fractions of different molecular weight by SEC- FPLC were tested on HCEC (normal cell line) and MCF-7 (breast cancer cell line).

3.8.1: Cytotoxicity on HCEC

The fractions isolated from venom of *H. xanthopus* decreased viability of HCEC cells in a dose-dependent manner. We used four concentrations 1mg/ml, 2mg/ml, 3mg/ml and 4 mg/ml of isolated fractions in comparison with Tomoxifin; due to purity of drug we used 250mcg/ml concentration. After analysis the fractions showed cytotoxic effect on cell line. Among all fractions the fraction 7 showed high level of cytotoxic effect as compared to others, Fraction 7 contains low molecular weight peptides ranging from 9.72 to 8.64 kDa. The cytotoxic effects increase with increasing concentration. It kills about 42% cells in culture on 4mg/ml. Other fractions also showed fair toxicity on HCEC cell line. Fraction 6 also kills same quantity of cells. It contains slightly high molecular weight peptides ranging from 41.39 to 8.64 kDa (Table 3 and Fig.11). All the data were analyzed by Graph pad prism showed highly significance statistically the P value of all fractions with PC is less than 0.05 (Table 4)

3.8.2: Percent viability

Percent viability was calculated by linear equation. Negative control was considered as 100 % viability and the test compounds % viability was calculated by comparing it with the negative control.

Table 3: The cytotoxicity of isolated fractions of *H. xanthopus* venom on HCEC

Conc. mg/ml	F1	F2	F3	F4	F5	F6	F7	NC	TOM 250mcg/ml
1	76.7±0.5	77.4±0.6	73.7±0.7	67.3±0.8	64.5±0.6	63.4±0.5	62.5±0.6	100	87.3±2.4
2	74.6±0.3	74.6±0.3	71.2±0.4	64.5±0.7	64.23±0.3	60.6±0.7	60.4±0.4	100	
3	71.2±0.9	71.9±0.9	67.3±0.9	63.9±0.9	61.8±0.4	60.70±0.5	60.0±0.6	100	
4	68.2±0.7	69.4±0.8	64.7±0.8	63.8±0.5	60.9±0.5	58.3±0.5	58.6±0.7	100	

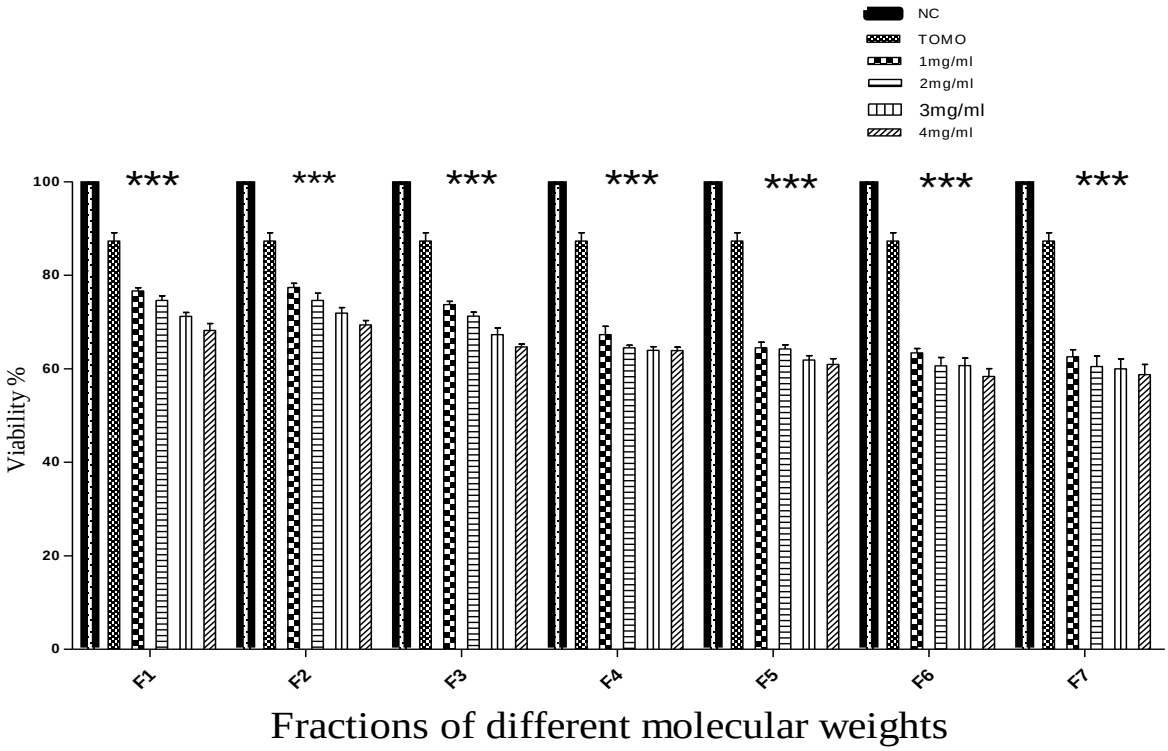

Figure 11: Cytotoxicity assay of *H. xanthopus* venom on HCEC. Stars (***) indicate statistically significant differences at p ≤0.001..

All the fractions were compared to PC: positive control the p values are less than 0.05 hence all the fractions are statistically significant denoted by stars (Table.4).

Table 4: Statistical significance of MTT assay.

Sr.no	**Fractions (Proteins)**	**"Significant? P < 0.05?"**
1	Fraction 1	YES***
2	Fraction 2	YES***
3	Fraction 3	YES***
4	Fraction 4	YES***
5	Fraction 5	YES***
6	Fraction 6	YES***
7	Fraction 7	YES***

3.8.3: Fraction with high cytotoxic effect

Fraction 7 showed maximum cytotoxic effect on HCEC cell (Fig.12), it killed 42 % cells. This fraction contained relatively low molecular weight peptides. NC: negative control; TOMO: Tomoxifin 250mcg/ml positive control; venom (1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml). Error bars represent SD (n = 3). Stars (*, **, ***) denote statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

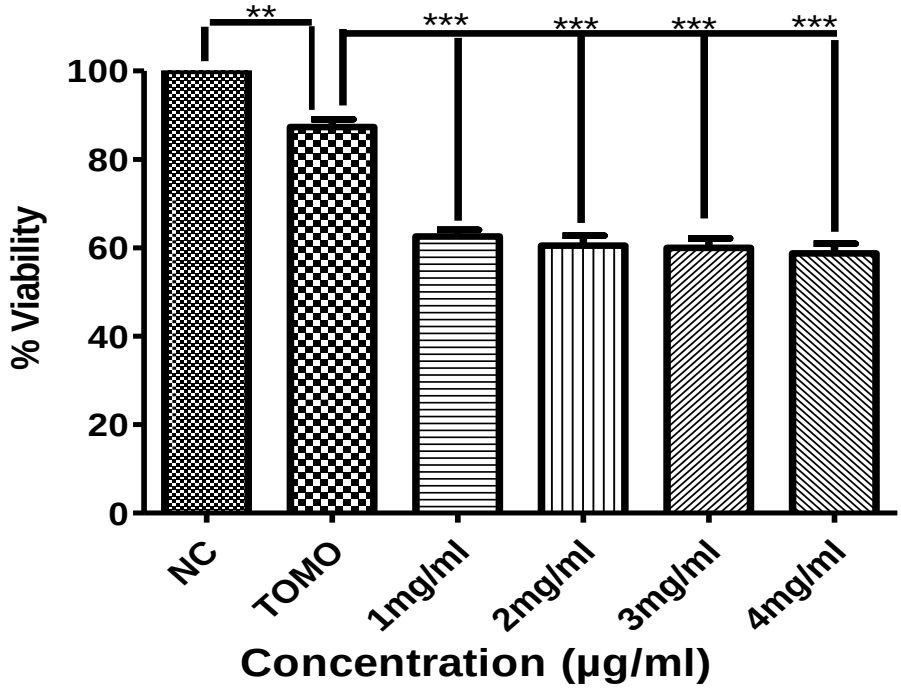

Figure 12: (A) Cytotoxicity assay of *H. xanthopus* venom (Fraction 7) on HCEC cell line.

3.9: Cytotoxicity on MCF-7 cell lines

The results of MTT revealed that among all fractions the fraction 7 showed cytotoxic effect towards MCF-7 cell lines. The fraction 7 contains low molecular weight peptides compared to other fractions. The cytotoxic effect of peptide increased by increasing the concentration about 12% of cells were killed on 4mg/ml. Fraction 4 also showed cytotoxic effect on cells it contained slightly large molecular weight peptides and small too. It also kills 12% of cells on 4mg/ml. the rest of fractions also showed a little bit cytotoxic effect of cells (Fig.13 and Table 5) .

Table 5: The cytotoxicity of isolated fractions of *H .xanthopus* venom on MCF-7

Conc. mg/ml	F1	F2	F3	F4	F5	F6	F7	NC	PC 250mcg/ml
1	94±0.4	94.2±1.2	97.7±0.9	90.7±0.8	97±1.1	93.5±0.7	89.6±0.8	100	29.3±3
2	96.8±1.2	92.6±1.4	97±1.2	89.6±0.7	96.5±0.7	92±0.8	88.7±1.1		
3	94.8±1.1	91.9±0.9	94±0.6	88.3±1.5	95±0.9	91.7±0.9	88±1.4		
4	94.3±1.4	91±1.2	93.7±1.1	88±1.4	93±1.1	90±0.7	88.6±0.8		

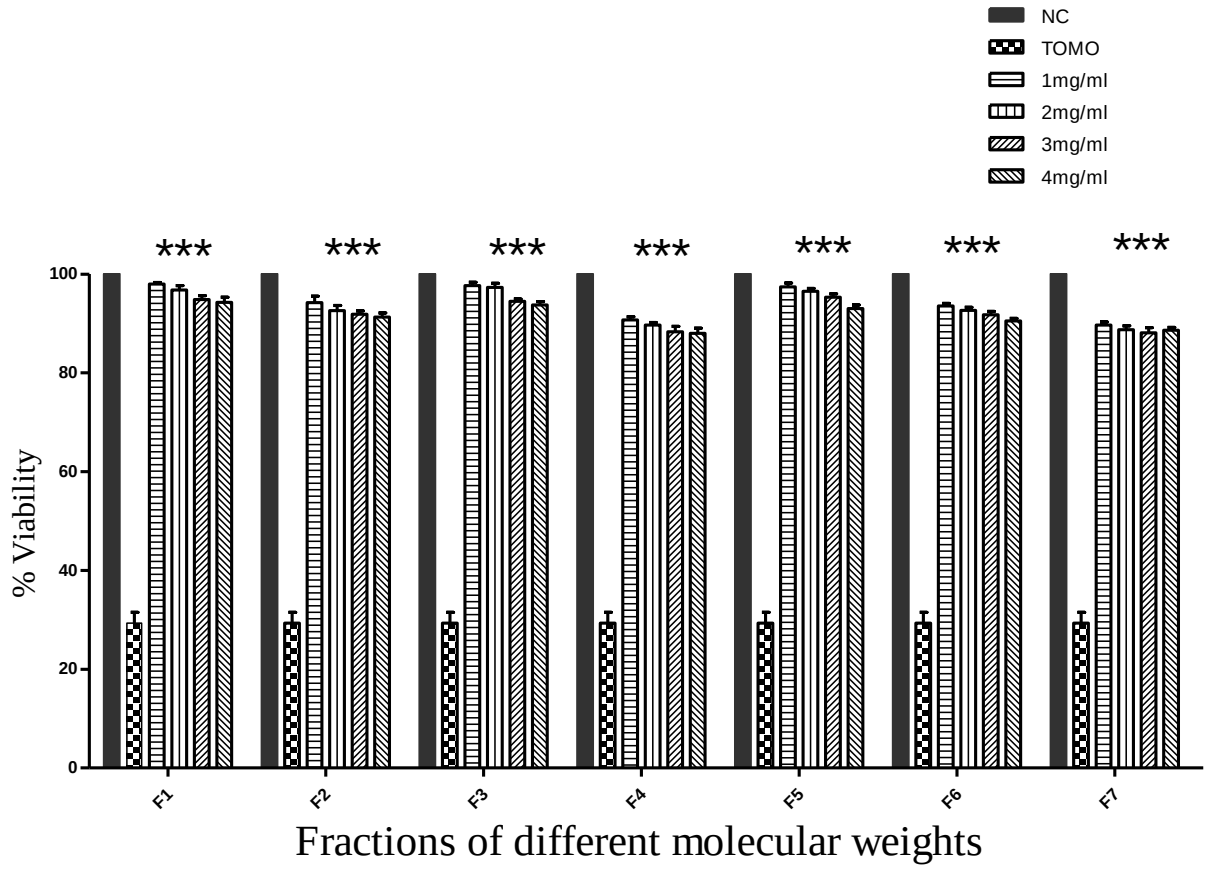

Figure 13: Cytotoxicity assay of *H. xanthopus* venom on MCF-7. Stars (*, **, ***) indicate statistically significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

All the fractions were compared to PC: positive control the p values are less than 0.05 hence all the fractions are statistically significant denoted by stars (Table.6).

Table 6: Statistical significance of MTT assay.

Sr. No	**Fractions (Proteins)**	**"Significant? P < 0.05?"**
1	Fraction 1	YES***
2	Fraction 2	YES***
3	Fraction 3	YES***
4	Fraction 4	YES***
5	Fraction 5	YES***
6	Fraction 6	YES***
7	Fraction 7	YES***

3.9.1: Fraction with high cytotoxic effect

Fraction 7 with maximum cytotoxic effect mostly contains low molecular weight peptides about 12% of cells have killed. NC: negative control; TOMO: Tomoxifin 250mcg/ml positive control; venom (1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml). Error bars represent SD (n = 3). Stars (*, **, ***) denote statistically significant differences at p ≤0.05, p ≤0.01 and p ≤0.001, respectively (Fig.14).

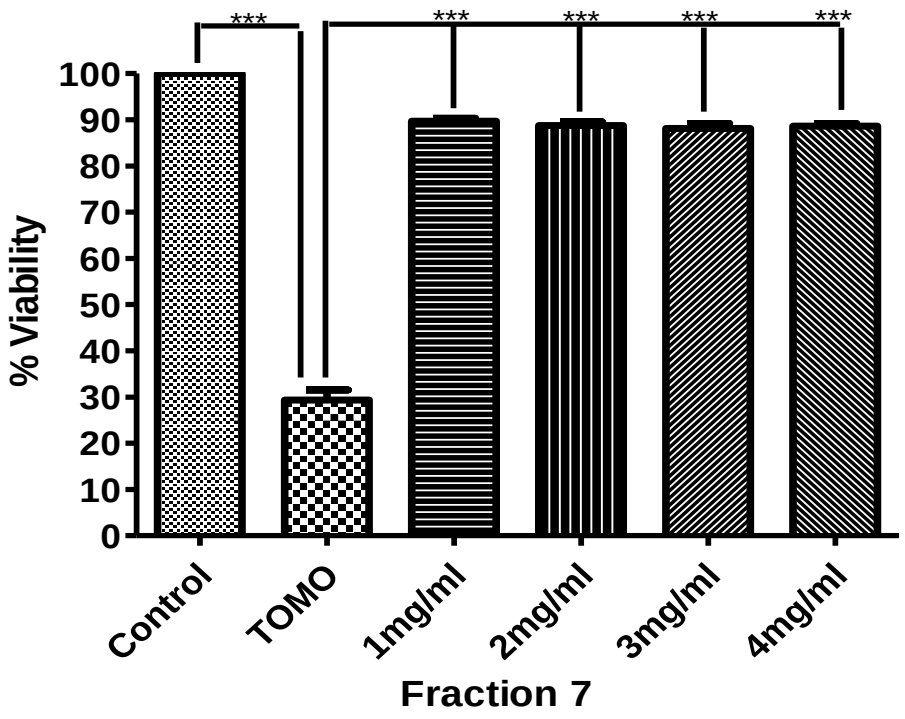

Figure 14: Cytotoxicity of *H. xanthopus* fraction 7 on MCF-7 cell line. NC: negative control; TOMO: Tomoxifin (250mcg/ml): Fraction 7: 1mg/ml, 2mg/ml, 3mg/ml and 4mg/ml.

DISCUSSION

The present study was performed to evaluate the characterization of scorpion venom and to find out it toxicity against cancer cell lines. The species selected was *Heterometrus xanthopus* which is commonly found in Pakistan. Scorpion venom is a mixture of polypeptides, nucleotides, lipids, mucoproteins, biogenic amines, and other unknown materials. The quantities of the produced compounds are variable and can depend on the animal specimen and the range of stings (and subsequently of extractions). Tremendously, scorpion derivatives with enzymatic activities are much less represented (Zhijian *et al*., 2006). Moreover venom consists of different enzymes like, hyaluronidases, phospholipases, sphingomyelinases (Kuhn-Nentwig, 2003). Acetycholinesterases, alkaline phosphatases and proteolytic enzymes (Incesu *et al*., 2005). Neurotoxins peptides are abundant in venom that may contain more than hunderd peptides (Delepierre *et al*, 1999). So far, more than 600 scorpion peptides were demonstrated in the UniProt database (King & Hardy, 2013). Described peptides are categorized as ion-channel toxins which have complex, synergistic neurotoxic effects due to specific ion-channel toxins present in venom. In excitable membranes the low molecular weight peptides which are cysteine rich peptides, directly affect the Na+, K+, Cl^{-}, and Ca2+ ion channels (Debin *et al*., 1993). The blocking activity of certain toxins on the voltage gated channels for impairing the growth of tumors has been studied (Fiske *et al*., 2006; Gómez-Varela *et al.*, 2007).

It is also reported that the venom of many scorpion species contain low molecular weight peptides (less than 10 kDa) (Gomes *et al*., 2010). These low molecular weight peptides distinctly alter the Na+, Ca2+, K+, Cl^{-} ion channels (Possani *et al*., 2000). In another studies showed venom profile with SDS-PAGE analysis stated that both high and low molecular weight proteins are found. Multiple proteins between 10 to 150 kDa were detected with two major bands at 10 and 70 kDa (Erdeş *et al*., 2014). In line with previous study it is stated that SDS-PAGE analysis indicated 10 peptide bands for the venom sample, Seven protein bands of 170, 79.6, 60, 57, 43, 38 and 29 kDa molecular weights were present in the venom of *Mesobuthus tamulus coconsis* (scorpion) (Badhe *et al*., 2007). In another state they showed that the molecular mass distribution of the venom constituents determined in the 80 fractions are isolated, grouped within intervals of

molecular weights, typically 1000 Da aside from each other. Four groups of additives with diverse molecular loads have been determined: 200–1000 Da (8.7%), 1000–5500 Da (47%), 5500–8500 Da (33.7%) and 8500– 44392 Da (10.6%). The majority of peptides have a molecular weight 3000 to 4000 and from 6000 to 7000 (Valdez-Velázquez *et al*., 2013). An additional observation supporting these findings is the results of HPLC separation of scorpion venom of the family Buthidae (Batista *et al*., 2004). In another study reported the first results of a proteome analysis of the venom of the scorpion *Urodacus yaschenkoi* performed by high performance liquid chromatography (HPLC) separation reported 74 fractions of *urodacus yaschenkoi* (Scorpion) by HPLC separation allowing the identification of approximately 274 different molecular masses with molecular weights varying from 287 to 43,437 Da. The most abundant peptides were those from 1 KDa and 4–5 kDa (Luna-Ramírez *et al*., 2013). Venom from the *Rhopalurus junceus* (blue scorpion), fractionated by high performance liquid chromatography (HPLC); the molecular masses of each fraction were determined by mass spectrometry analysis. At least 153 different molecular mass components were identified. Molecular masses varied from 0.466 to 19.755 kDa. The most abundant components presented molecular weights around 4 kDa, , and 7 kDa, , but with small molecular weight differences in addition other peptides with molecular weights on the order of 14 to 19 kDa. Some high molecular weight peptides were also detected such as 45 kDa identified by gel electrophoresis analysis (Rodríguez-Ravelo *et al*., 2013).

The present studies showed that *H. xanthopus* venom also have complex type of chromatographic pattern. In RP-HPLC Main well defined peaks present in the *H. xanthopus* crude venom on (4, 10, 20, 26, 28, 38, 56, 66, 86 and 100 min) with respect to elution time. It was quantitative study of venom showed, different peaks shows different peptides with their different molecular weights. The results revealed from SDS-page of crude venom that *H. xanthopus* crude venom contain low molecular weight peptides ranging from 3 kDa to 21 kDa. It also contains high molecular weight peptides ranging from 30 kDa to 200 kDa and above 200.

Peptidomics approach followed in this work has some differences whilst in comparison to normally used crude venom characterization research. In the previous works, crude venom is commonly separated by using RPC that produces more peaks. Since our focus was on peptide

components of the venom, we preferred to use Size exclusion chromatography (SEC) to separate the peptide fraction of the venom (1–10 kDa) from low- (<1 kDa) and high-molecular-weight components (>10 kDa) as suggested by (Vassilevski *et al*., 2010). In another studies stated that the three scorpion species showed different FPLC chromatographic patterns on the basis of number of peaks and their height and location or retention times (RT). The molecular weights of various fractions of the venom from *Androctonus crassicauda* and *Androctonus bicolor* ranged from <5 to 43 kDa whereas for *Leiurus quinquestriatus* venom the molecular weights ranged between <5 and 61 kDa (Al Asmari *et al*., 2012). FPLC have been used for isolation and purification of specific peptides and proteins from the venoms of different scorpion species (Wang *et al*., 2011). In accordance with another study some of toxins with their molecular weight e.g, Birtoxin 6.543 kDa, Dertoxin 6.643 kDa, Ikitoxin, 6.615 kDa Bestoxin 6.603 kDa, Alpha toxin, 7.214 kDa Altitoxin, 6.599 kDa, Parabutoxins, 4.096-4.28 kDa were identified (Inceoglu *et al*., 2003).

The current study showed FPLC chromatography, the column was previously calibrated by known protein markers labeled as 670, 158, 41, 17, and 1.3 kDa, respectively. Six Fractions was separated on basis of their molecular weight , further confirmed by SDS-page analysis as described fraction 1 contain four bands indicating high molecular weight proteins these are 139.3, 110.2, 90.50, 77.45 kDa. Fraction 2 also contain four major bands which are 52.37, 19.70, 16.18, 9.5 kDa. Fraction 3 contain five bands which are 71.61, 66.22, 19.68, 13.30, 10.11 kDa. Fraction 4 contained four bands 66.22, 13.30, 11.37, 9.35 kDa. Fraction 5 contains five bands 68.80, 44.77, 24.88, 14.33, 9.35 kDa. Fraction 6 contain four bands of low molecular weight peptides these are 41.39, 11.37, 9.72, 8.64 kDa. Fraction 7 contain only two bands of very low molecular weight peptides which are 9.72, 8.64 kDa. Above results revealed that venom is pool of different kind of proteins and peptides each band represents a class of proteins. It contains both high and low molecular weight peptides. It has been previously shown that experienced of numerous most cancer cells lines to scorpion venoms induces important changes in cell morphology, dearth of cell, mobile contraction and demise by means of both apoptosis and necrosis (Zargan *et al*., 2011).

In line with previous study a novel protein Bengalin (72kDa) isolated from venom of *H. bengalensis* responsible apoptogenic activities against human leukemic cells U937. The event was characterized by membrane blebbing, chromatin condensation and DNA fragmentation (Gupta *et al*., 2007). This property is characteristic of chlorotoxin (3.8 kDa); a peptide extracted from the venom of *Leiurus quinquestriatus hebraeus*, which specifically binds to chloride-gated channels that are strongly involved in cancer cells mobility mechanism. This commentary is reliable with preceding studies wherein venoms of different scorpions together with, *Androctonus crassicauda* and *Centruroides limpidus limpidus* additionally induced selective and differential cytotoxicity towards extraordinary human malignant cell lines (Caliskan *et al*., 2013).

According to another studies epithelial cancer cells illustrated remarkable cell viability reduction, with medium cytotoxic concentration (IC50) ranging from 0.6-1mg/ml, in a concentration-dependent manner (Díaz-García *et al*., 2013). In another studies shows that the venom of *Odontobuthus doriae* decreased cell viability in a dose dependent manner at a dose of 10, 20, 50, 100 and 200 µg/ml of venom; cell viability decreased to 86.8%, 80.6%, 67.7, 62.2 and 39.9 respectively (Zargan *et al*., 2011). In accordance with another study the MCF-7 was the more susceptible cell line to venom of scorpion.

The scorpion venom can stop the growth and proliferation of MCF-7 cells. The extract of scorpion venom induced on apoptosis thru Caspase-3 up-regulation while Bcl-2 down-regulation in MCF-7 cells (Li *et al.*, 2014). Another study revealed that a serine proteinase-like protein named BMK-CBP, isolated from Chinese red scorpion (*Buthus marthinsii Karsch*) induced apoptosis in MCF-7 cell line (Gao et al., 2008). Another study stated that *in- vivo* studies on naturally developed breast tumor in mice also showed the suppressive effect of ICD-85 (Venom derived peptide) on tumor growth (Zare Mirakabadi *et al*, 2008).

The present study demonstrated that *H. xanthopus* venom fairly reduce the proliferation in MCF-7 in a dose- depended manner. Fraction 7 with most cytotoxic effect about 12% cells has been killed, it contain low molecular weight peptides ranging from 9 kDa to 6 kDa these peptides depicts the toxins name Birtoxin, chlorotoxins (Inceoglu *et al*., 2003). They have also same molecular weights and their function to bind ion-channel and block the function of cell. After F7 the fraction good activity is fraction 6 which contain slightly high molecular weight peptides

ranging from 41.6 kDa to 8.64 kDa. F3 also have cytotoxic effect on viability of cells. Bengalin (72 kDa) is a toxin isolated from *H. bangalinsis* has profound anticancer effect. Supporting the previous statement the L. quinquestriatus venom inhibited the growth of breast cancer cells (MCF-7) (Omran, 2003).

In another studies shows cytotoxic effect on normal cell lines too, the results revealed that venom of *Leiurus quinquestriatus* showed anti-proliferative effect on 293T and C2C12 (Omran, 2003). In accordance with previous studies the scorpions *Odonto buthusdoriae* showed cytotoxic effect on 1321N1 glioma like cell line (Salarian *et al*., 2012). In another studies shows the cytotoxic effect of scorpion venom in vitro, demonstrated that African green monkey kidney (Vero) and Buffalo green monkey kidney (BGM) are sensitive to *Leiurus quinquestriatus* venom, indicating cytotoxicity (Sherif *et al*., 2000).

The seven fractions of the scorpion *H. xanthopus* were tested on HCEC growth. The present results, however, revealed that the fraction 7 is the most cytotoxic fraction among all at higher concentration 4mg/ml, it decreases cell significantly, and fraction 7 contain low molecular weight peptides. The fraction 6 also induces almost same cytotoxic effect on cells, this fraction contain slightly high molecular weight peptides.

The present study indicated that the venom contains cytotoxic factors or inhibitory factors that may counteract cell growth. Furthermore, these cells are sensitive to this venom. HCEC cells shows a decrease in growth, in dose depended manner, but fraction 7 which contain low molecular weight peptides. A myotoxic effect of scorpion venom on the myocardium cells was reported (Gueron *et al*., 1992). Pharmacological and biochemical studies of scorpion venom resulted in enormous polypeptide toxins are isolated and characterized, it contains long chain toxins with 60-70 amino acids directly effect on Na+ channel (Wang & Ji, 2005). In accordance to previous studies, scorpion *Androctonus australis* hector venom checked for cytotoxicity on Renal proximal Tubule cells in vitro the result indicated that it induced apoptotic effect on cells (Saidani *et al*., 2016).

4.1: Conclusion

The present investigation is first elucidation of bioactive molecules present in *H. xanthopus* scorpion venom that used peptidomics methods (SEC, SDS-page and RP-HPLC). We showed that SEC-FPLC and SDS-page analysis is a practical peptidomics tool with high resolution for venom research. Initially we separated the whole venom body in seven different fractions these fractions were analyzed separately by electrophoretic method (SDS-page analysis). A total of 28 venom peptides have been identified in which both the high and low molecular weight peptides existed. Low molecular weight peptides make up most part of venom, detected 57% of within 1 to 20 kDa whereas high molecular peptides noticed 17% within 61 to 80 kDa. *H. xanthopus* venom has a great cytotoxic effect on HCEC and MCF-7 cell lines. This quality may attributed the presence of Sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-) channel toxins in venom they have strong anti-proliferative effects. Future studies on *H. xanthopus* venom may lead to discovery of novel potassium and chloride channel blocker, it may use against many other cancer cell lines. It has many essential peptides may use against bacteria, parasites and viruses. The cytotoxicity on normal cell lines shows that it has some toxins, which may lead to production of antivenin.

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