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# Effect of Heteroscorpion Venom on Mcf Cell Lines

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**Abstract-** Scorpion and its organs have been used to cure epilepsy, rheumatism, and male impotency since medieval times. Scorpion venom which contains different compounds like enzyme and non-enzyme proteins, ions, free amino acids, and other organic inorganic substances have been reported to posses antiproliferative, cytotoxic, apoptogenic, and immunosuppressive properties. We report the cytotoxic and antiproliferative effects of scorpion venom in human MCF-7 cells. After exposure of cells to medium containing varying concentrations of venom (10, 20, 30, 50, and 75 µM/ml), cell viability decreased 62%, 58%, 49%, 41% and 29% respectively, after 24 hrs. Cells expressed morphological changes like swelling, inhibition of neurite outgrowth, irregular shape, aggregation, rupture of membrane, and release of cytosolic contents after treatment with venom. The cytotoxicity of heteroscorpion venom against MCF (Michigan Cancer Foundation) cancer cell lines was investigated through standard MTT assay and Comet assay. The Comet assay method for measuring deoxyribonucleic acid (DNA) strand breaks, the Comet DNA damage determined under microscopic analysis. heteroscorpion venom showed moderate toxicity on MCF respectively. Interestingly, venom exhibited remarkable cytotoxicity results with IC50 of 28.53µM on MCF cell lines which are comparable to the infamous anti-cancer drug, MCF cells were treated with 50 µM of heteroscorpion venom and incubated at 37 °c for 24 hrs along with a set of control cells to determined Comet DNA damage where respectively in order of tail length 63µm, and head length 41 µm. Our results show cytotoxic and antiproliferative potential of scorpion venom in human MCF-7 cells. These properties make scorpion venom a valuable therapeutic agent in cancer research.

Keywords: Heteroscorpion venom, MCF (Michigan Cancer Foundation), (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), deoxyribonucleic acid (DNA).

## I. INTRODUCTION

Some scorpion venoms inhibit the growth of various types of cancers. Only a few toxins have been found to be the responsible for these anticancer effects and exert their action by three different mechanisms.

- Blocking a specific ion channel [1]
- Inhibiting cancer cells invasion binding to a specific site (different from an ion channel) on the plasmatic membrane [2] and
- Activating intracellular pathways inducing apoptosis [3]

Breast cancer is one of the most common malignancies worldwide. Although several novel therapeutic methods have been utilized, curative effects in advanced breast cancer remains poor. Developing new drugs against breast cancer will be essentials for improving therapeutic intervention and prognosis for this malignancy. Scorpion venoms are complex mixtures which may contain over two hundred different peptidic compounds [4]. These compounds act directly on a broad spectrum of cell membrane ion channels [5], and indirectly by activating cellular metabolic pathways probably via secondary messengers [6] or by electrostatic interaction with the cell membrane [7]. In recent years there have been many studies [8, 9, 3] aimed at identifying scorpion peptides with antineoplastic activity. Scorpion venoms and toxins isolated from Leiurus quinquiestratus, Buthus martensii and Heterometrus bengalensis have been found to kill specifically human glioma and leukemic cells [10,11,3]. Chlorotoxin, a peptide isolated from L. quinquiestratus, specifically binds to glioma cells and prevents their proliferation [2]. Recent advances in nanotechnology have led to the development of biocompatible nanoparticles for in vivo molecular imaging and for targeted therapy using chlorotoxin [8]. The existence of a chlorotoxin- like peptide has been reported in the venom of martensii [11]. Also, bengalin a peptide of 72 kDa isolated from the venom of H. bengalensis, has antiproliferative and apoptogenic activities against human leukemic cells [3]. In this work, we studied the action of Tityus discrepans scorpion venom and two purified peptidic components on SKBR3 human breast cancer cells and on non-malignant MA104 cell line. Two purified peptides, called here neopladine 1 and 2, were found to be active on SKBR3 human breast carcinoma cells and had negligible effects on MA104 cells. This work demonstrated that neopladine 1 and 2, induce apoptosis on SKBR3 cells via the activation of Fas signaling by induction of FasL expression. It was suggested that this multifunctional nanoparticle system may find potential application in cancer diagnosis and treatment [12].

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**Aim and Objective of study**

- Investigate the antitumor activity of Heteroscorpion venom showing different levels as well as different mechanisms of their action using MTT assay.
- Sensitivity and specificity in predicting and detection of DNA damage by using comet assay

## II. METHODOLOGY

***Materials and Methods***

***A. Media preparation for MCF-7 cell culture***

1. Selection of MCF-7 Cell and culture of MCF-7 cell lines
2. Collection of heteroscorpion venom
3. Protein estimation using bradford method
4. Cells treatment
5. Cell viability assay
6. MTT assay
7. Comet assay
8. RNA Extraction

***B. Equipments Required***

Laminar air flow, Water bath, Refrigerator, $CO_2$ Incubator, Centrifuge, Haemocyto meter, Freezer, ELISA reader, Hot air oven, Electrophoresis, Bright field transmission light microscope, Autoclave, Weigh balance, Micro pipette, Pasteur pipette, and, Measuring cylinder

***C. Materials Required***

Filters, Syringe, Test tube stand, Distil water, T25 and T75 Culture Flasks, 15ml and 50ml Centrifuge tubes, Parafilm, Ependorf, Cover slips, Vial, 96 well platem, and, Glass Slides

- ***Media Preparation***

***A. Reagents required***

1. DMEM
2. Antibiotic and antimycotic (pen-strap, nystatin)
3. Sodium pyruvate
4. 10% FBS (Fetal bovine serum)

**Protocol**

1. DMEM was taken, (DMEM A variation of MEM, called Dulbecco's modified Eagle's medium (DMEM), (Dulbecco/Vogt modified Eagle's minimal essential medium), contains approximately four times as much of the vitamins and amino acids present in the original formula and two to four times as much glucose. Additionally, it contains iron and phenol red. DMEM is further divide into high glucose type (4500g/L glucose) and low-glucose type (1000g/L glucose). High-glucose DMEM is suitable for some tumor cells with faster growth speed and difficult attachment, as it is beneficial to retain and grow in one place.)
2. 10% FBS (Fetal bovine serum) was taken heat inactive at 55 °C for 30 minute in water bath
3. The medium was sterilized immediately by filtering through a sterile membrane filter with porosity of 0.22 micron or less, using positive pressure rather than vacuum to minimize the loss of carbon dioxide.
4. Sterilized medium was Storage at 2 to 8 °C.

**Medium preparation for mcf-7 cells**

1. 500ml DMEM was taken and 1% sodium pyruvate was added
2. From the 500ml medium 50ml medium was removed.
3. 50ml (100ml/1000ml) FBS (Fetal bovine serum) was added (Rich variety of proteins in fetal bovine serum maintains cultured cells in a medium in which they can survive, grow, and divide).
4. 5ml (10ml/1000ml) of antibiotics and antimycotic were added to the medium antibiotics and antimycotic were used to prevent contamination in cell culture.
5. Then the medium was ready to use.

- **Collection of MCF-7 Cells**

MCF-7 strains commercially obtained from NCCS (National Centre for Cell Science) Pune, lndia.

MCF-7 is a human breast cancer cell line that was first isolated in 1970 from the malignant adenocarcinoma breast tissue of a 69-year old woman. MCF-7 is the acronym of Michigan Cancer Foundation - 7, referring to the institute in Detroit where the cell line was established. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium.

These include the ability for MCF-7 cells to process estrogen via estrogen receptors. MCF-7 cells are also sensitive to cytokeratin. When grown in vitro, the cell line is capable of forming domes and the epithelial like cells grow in monolayer.

Culture of MCF-7 cell lines

**Reagents required**

1. 70% ethanol
2. PBS (Phosphate-buffered saline)
3. Trypsin-EDTA

**Reagents Preparation**

**Phosphate-buffered saline [1000 ml]**

Sodium chloride (137mM) 8.0gm; Potassium Chloride (2.7mM) 0.2 gm; Potassium Dihydrogen Phosphate (1.4mM) 0.25gm; Disoduim and Hydrogen Phosphate (4.3mM) 1.44 gm; dissolved in 800 ml of Double-distilled water, adjusted pH to 7.4 and made up to 1000 ml.

**70% ethanol [1000 ml]**

100% ethanol was taken measured 700ml it dissolved 300ml distilled water and made upto 1000ml

**Trypsin-EDTA**

Small beaker was taken Added 10 ml of10X PBS, Added 0.1 ml 0.5 M EDTA final concentration should be 0.5 mM EDTA, Added 0.1 g of D-glucose, Added 0.5 ml of Penicillin/

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Streptomycin, added 10 ml 2.5% Trypsin, added 0.3 ml Phenol Red (0.5%), pH with NaOH to 7.35, made up volume with m-water to 100 ml. Sterile filter through Millipore apparatus (0.22 um), aliquot into sterile 15 ml blue-capped sterile tubes, flame tube and cap. Place all but one aliquot in –20°C freezer dilute one aliquot 1:5 in 1X PBS to make the final solution (0.05%trypins/EDTA). Aliquot and freeze at –20°C. When needed, thaw one tube out in the TC water bath (37 C), and stored at 4 C after use. Good for ~ 1 month. 0.1% Trypsin (100 ml).

**Protocol**

**Revival of cells**

1. The cryopreserved vial was taken containing MCF-7 cells.
2. The cryopreserved vial was placed immediately in a 37 °C water bath to thaw cells.
3. The vial was wiped with 70% ethanol before opening cryopreserved vial Ethanol is used in cell culture wipes and in most common antibacterial hand sanitizer gels at a concentration of about 62% v/v as an antiseptic.
4. Vials contents was transfer to 15ml centrifugation tube under sterile condition with culture medium.
5. The medium was centrifuged at 1300 -1500 rpm (fixed angle microlitre rotor, 24 × 1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 3 minutes, and then the supernatant was discarded.
6. The new T 25/ T 75 flask was taken and fresh culture medium was added.
7. After the centrifugation fresh culture medium was added to 15 ml tube and mixed well by pipetting.
8. Then cell mixture was transferred to T 25/ T 75 flask and mixed well by pipetting. And examine the flask under the microscope.
9. Then culture was incubated at 37 °C in 5% co2 incubator till the confluence of cells obtained (80% to 90%).

**Sub culture or passage MCF-7Cells**

1. The cells were sub cultured by passaging after attaining the confluence of cells to (80% to 90%).
2. The medium was removed from the T 25/ T 75 culture flask using sterile pipette.
3. T 25/ T 75 cells culture flask were washed with PBS (Phosphate-buffered saline) to remove the residual medium and discarded.
4. Trypsin-EDTA was added to cells, sufficiently covering the surface area to detach the cells from the surface of the flask and rinse the cells in trypsin-EDTA by gently swirling the flask.
5. The flask was incubated at 37°C till the cells are detached incubation time will vary depending on cell type.
6. Cells were removed from incubator observed under microscope to see the cell detachment. When the cells appear rounded, tap the flask to detach cells completely.
7. 2ml of fresh medium was added to neutralize trysinization.
8. Detached cells were Pipette out from flask into fresh 15ml centrifugation tube.
9. The medium was centrifuged at 1300 -1500 rpm for 3 minutes, and then the supernatant was discarded.
10. Cell culture medium was added to a new T 25/ T 75 culture flask using sterile pipette and diluted as appropriate into the flask and mixed well with using sterile pipette.
11. Then the flask was incubated at 37 °C in 5% co2 incubator.
12. Fresh culture medium was Replaced every 2 to 3 days.

**Cell Counting**

**Haemocytometer**

Haemocytometer diagram including the 16 corner squares which should be used for counting. The object of the experiment is to determine the concentration of cells in given sample. Aliquot sample containing immobilized cells.

When cells were placed on the chamber and is covered with cover glass capillary action completely fill the chamber with sample looking at the chamber through a microscope, the number of cells in it can be determined by counting.

Different kinds of cells can be counted separately as long as they are visually distinguishable.

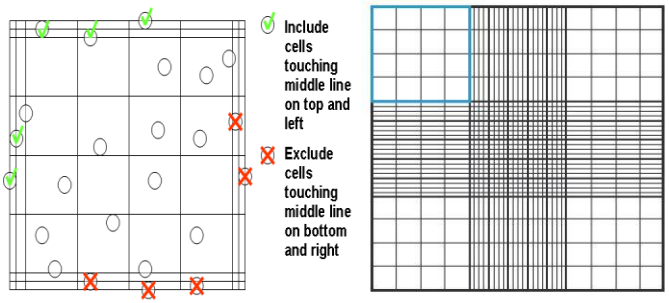


**Figure 1: Haemocytometer**

**Reagents required**

70% ethanol
Trypan blue

**Reagents Preparation**

**70% ethanol [1000 ml]**

100% ethanol was taken measured 700ml it dissolved in 300ml distilled water and made up to 1000ml

**Protocol**

1. Haemocytometer was taken and placed on white paper and Haemocytometer and cover slips were cleaned 70% ethanol.
2. The cells were trypsinized using with trypsin-EDTA and centrifuged it at 1300 -1500 rpm (fixed angle microlitre

rotor, 24×1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 3 minutes, and then the supernatant was discarded.

3. 10µl of culture was added into fresh eppendorf and 10µl of trypan blue was added and mixed well with using sterile pipette.
4. 2-3 drops of culture with trypan blue were placed on heamocytometer. Carefully fill the haemocytometer by gently resting the end of the Gilson tip at the edge of the chambers. Take care not to overfill the chamber. Allow the sample to be drawn out of the pipette by capillary action, the fluid should run to the edges of the grooves only. Re-load the pipette and fill the second chamber if required.
5. The cells were observed under microscope and count the number of cells in this area of 16 squares. When counting, always count only live cells that look healthy (unstained by trypan blue). Count cells that are within the square and any positioned on the right hand or bottom boundary line.
6. Dead cells stained blue with trypan blue can be counted separately for a viability count. Live cells appear colorless and bright (refractile) under phase contrast. While the dead cells stain blue and are non-refractile keep the separate count of viable and non-viable.

**Viability**

They trypan blue is used to stain any dead cells. Cells looking faint or dark blue within the grid being counted are counted as dead cells. To check the viability of the cells required:

Live cell count (not including trypan blue cells)

Total cell count including those stained with trypan blue

**Percentage of viability**= Total viable cells (unstained)/ Total cells (viable+dead) x100

**Viable cells/ml** = average viable cells count per square x dilution factor x $10^4$

**Average viable cells count per square** = total number of viable cells in 4 square x dilution factor x $10^4/4$

**Dilution factor** =total volume (volume of sample + volume diluting liquid)/volume

**Formula for the number of cells per ml**

$$\boldsymbol{concentration} = \frac{\text{Counting of cells}}{4(\text{squares})} * \text{dilution factor} * 10^4$$

$$\text{Number of cells} = \text{concentration (cells/ml)} \times \text{volume of sample (ml)}$$

**Freezing of MCF-7 cells**

Freezing of the MCF-7 cells at -80°C for long term use by cryopreservation method using cryopreservant DMSO (Dimethyl sulfoxide)

**Figure 2: -80°C freezer, Mr.Forsty freezing container**

**Reagents required**

DMSO (Dimethyl sulfoxide)

Fbs 20%

**Protocol**

1. Cells should be growing well or knowing to be in log phase.
2. The cells were trypsinized using with trypsin-EDTA and centrifuged it at 1300 -1500 rpm (fixed angle microlitre rotor, 24×1.5 ml, Biofuge Stratos, Thermo Electron Corporation, Germany) for 3 minutes, and then the supernatant was discarded.
3. Cryomedium was prepared (10%DMSO and 90% culture medium).
4. Tube was taped to disperse the cells. And culture medium was added.
5. Then culture medium was mixed with cryomedium and Placed 1ml aliquot into the cryovial.
6. The vial was putted in -80°C freezing container ((Mr.Forsty 5106 Cryo 1 °C)) for overnight for slow cooling(-1°C/minute cooling rate).
7. Then vial was putted into vapor phase of liquid nitrogen for long term storage.

**Collection of heteroscorpion venom**

Heteroscorpion venom collected from department zoology. Venom was obtained monthly by mild electrical simulation (20 V, 500 mA) and was solubilized in sterile double distilled water. After centrifugation at 8000x g for 15 min at 4°C, supernatant was immediately lyophilized and stored at -20°C until use. The crude venom was dissolved in DMEM-F12 and protein content was determined by Bradford and Biuret method. Venom was disinfected with 1.5% antibiotic–antimycotic solution.

Scorpion venom is a mixture of polypeptides, nucleotides, lipids, mucoproteins, biogenic amines, and other unknown substances. A broad range of bioactive peptides are already purified and characterized from scorpion venoms, with a total number estimated to approach 100 000 different ones, among them only 1% is mostly known.

**Protein estimation using Bradford method**

The protein content in the heteroscorpion venom is measured quantitatively using Bradford method is selected because of its accuracy and feasibility.

**Working principle**

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins (Bradford 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer or microplate reader.

**Reagents:**

BSA (1mg/ml) solution
Commassie blue 1x dye solution
Protein with Unknown concentration

**Reagents preparation**

Bovine serum albumin (BSA) stock solution: BSA stock is prepared by dissolving 100mg of BSA in 100ml of distilled water.

**Bradford reagent**

Dissolved 50 mg of Coomassie Brilliant Blue G-250 in 50 ml of methanol and 100 ml 85% (w/v) phosphoric acid (H3PO4) added. The acid solution mixture added slowly into 850 ml of H2O and let the dye dissolved completely (note: Do not add H2O into the acid solution). Filtered using with whatman paper to remove the precipitates just before use stored in a dark bottle at 4°C.

**Procedure**

1. Taken ten clean test tubes and prepared ten different concentration of standard protein ranging from 100µgm/ml.
2. Added 4ml of Bradford reagent to each of the tube and incubated them at room temperature for 5 min.
3. For blank added 1 ml of distilled water to 4 ml of Bradford reagent. After incubation measure the optical density at 595nm.
4. The standard graph is plotted by taking the concentration on X-axis and absorbance on Y-axis.
5. In the case of heteroscorpion venom we taken the 1 ml of heteroscorpion venom sample and followed the above mentioned procedure.
6. The protein concentration in the heteroscorpion venom is estimated from the standard graph.

## III. CELLS TREATMENT

**Reagents required**

Heteroscorpion scorpion venom

**Protocol**

1. 96 wells plate was taken and seeded cell with culture incubated for overnight in Co2 incubator.
2. After overnight incubation the medium was removed and added fresh medium. Then Heteroscorpion scorpion venom was added to culture at different concentrations (5, 10, 20, 30, 50, and 75 µM/ml).
3. Seeded plate was incubated at 37°C 5% Co2 incubator for different time periods 24hrs 48hrs.

**Cell viability assay (MTT assay)**

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The MTT Reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

**Reagents required**

MTT reagent (0.4mg/ml)
DMSO

**Reagents Preparation**

**MTT reagent**

Prepare 12 mM MTT stock solution by adding 1 mL of sterile PBS to 0.4 mg vial of MTT (Component A). Mix by vortexing or sonication until it dissolves. Occasionally there may be some particulate material that will not dissolved this can be removed by filtration or centrifugation. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light.

**Protocol**

1. Seeded plate was incubated in Co2 incubator for different time periods 24hrs and 48hrs was taken removed medium using sterile pipette.
2. 100µl MTT reagent (0.4mg/ml) was added to each well and Incubated at 37°C for 3-4hrs in Co2 incubator. When purple-colored precipitates were visible under the microscope
3. MTT reagent was removed carefully and added 100µl DMSO to each well incubated for 10-15 minute in Co2 incubator. DMSO solubilize formazan crystals (MTT formazon) the color (purple) was developed (this work done under dark condition).
4. After 10-15 minute absorbance was measured with reader at 570nm (reference absorbance value are at 595nm/450nm)

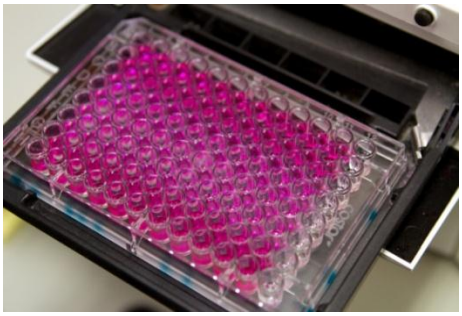

**Figure 3: 96 wells plate with culture**

**Comet assay**

The Comet Assay, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. DNA damage caused by apoptosis and cytotoxicity and be used in the field of genotoxicity testing, human bio monitoring, cellular response to DNA damage, and for screening cancer therapeutics. Swedish researchers Ostling & Johansson developed this technique in 1984. Singh, et al., later modified this technique, in 1988, as the Alkaline Comet Assay. The resulting image that is obtained resembles a "comet" with a distinct head and tail. The head is composed of intact DNA, while the tail consists of damaged (single-strand or double-strand breaks) or broken pieces of DNA. While most of the applications of the Comet Assay have been to study animal eukaryotes, there have been reports of successful application in the study of plant cells. Individual cells are embedded in a thin agarose gel on a microscope slide. All cellular proteins are then removed from the cells by lysing. The DNA is allowed to unwind under alkaline/neutral conditions. Following the unwinding, the DNA undergoes electrophoresis, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus. After staining with a DNA-specific fluorescent dye such as ethidium bromide or propidium iodide, the gel is read for amount of fluorescence in head and tail and length of tail. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. The Comet Assay can be used to detect DNA damage caused by double strand breaks, single strand breaks, alkali labile sites, oxidative base damage, and DNA cross-linking with DNA or protein. The Comet Assay is also used to monitor DNA repair by living cells.

**Reagents Required:**

- Phosphate-buffered saline
- Lysis solution
- 0.75% w/v normal melting point agarose
- 0.5% w/v low melting point agarose
- Electrophoresis buffer
- Neutralization buffer
- Silver staining Reagents, Reagents for Fluorescent staining

**Reagents Preparation**

**Phosphate-buffered saline [1000 ml]**

Sodium chloride (137mM) 8.0gm; Potassium Chloride (2.7mM) 0.2 gm; Potassium Dihydrogen Phosphate (1.4mM) 0.25gm; Disoduim and Hydrogen Phosphate (4.3mM) 1.44 gm; dissolved in 800 ml of Double-distilled water, adjusted pH to 7.4 and made up to 1000 ml.

**Lysis solution**

**Stock solution**

146.1 gm (2.5M) Sodium Chloride, 37.2 gm (100mM) of Disodium EDTA and 1.2 gm (10mM) of Tris were added to 700 ml of double distilled water and stirred till dissolve. Then 12 gm of Sodium Hydroxyl pellets were added to the mixture and stirred once again. When dissolved completely, 10 gm of Sodium Laurylsarcosinate or 1gm of Sodium Dodecylsulphate were added and stirred once again. Adjusted pH to 10 and adjusted the final volume to 890 ml with double-distilled water. Filtered the solution and stored at room temperature.

**Working solution**

1. Taken 108 ml of stock lysis solution and 1.6 ml of 1% Triton X-100 was added.
2. Freeze it in a refrigerator for 40-60 min prior to use. It is recommended to prepare fresh working lysis solution.
3. 0.75% w/v Normal Melting Point Agarose (NMPA)
4. 188 mg of NMPA was added to 25 ml of Phosphate-Buffered Saline (PBS) in a 100-ml beaker. Seale the beaker with aluminum foil and melt NMPA in the microwave oven at low power for 1-2 min.
5. 0.5% w/v low melting point Agarose.
6. 125 mg of Low Melting Point Agarose (LMPA) was added to 25 ml of PBS in a 100-ml conical flask. Seale the mouth of the conical flask with aluminum foil and melt LMPA in microwave oven at low power for 1-2 min.

**Electrophoresis buffer**

**Stock Solution I**

Dissolve 200gm of Sodium Hydroxide (10 N) in 500 ml of double-distilled water.

**Stock Solution II**

Dissolve 14.89gm of Disodium EDTA (200 mM) in 200 ml of double-distilled water and adjusted the pH to 10 with Sodium hydroxide.

**Working solution**

Taken 30 ml of Stock solution I and 5 ml of Stock solution II mixed and adjusted the volume to 1000 ml with cold double-distilled water. Final volume depends upon the capacity of the electrophoresis tank.

**Neutralization buffer**

Dissolve 48.5 gm of Tris (0.4 M) in 800 ml of double-distilled water. Adjusted pH to 7.5 with concentrated Hydrochloric acid and adjusted the final volume to 1000 ml.

Reagents for silver staining

**Fixative solution**

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Dissolve 75gm of Trichloroacetic acid, 25gm Zinc Sulphate and 25gm of glycerol in 400 ml of double-distilled water and stirred for 20-30 min, adjusted final volume to 500 ml.

**Staining solution A**

Dissolve 25gm of Sodium Carbonate in 500 ml of double-distilled water by constant stirring for 20-30 min

**Staining solution B**

Dissolve 100 mg of Ammonium Nitrate, 100 mg of Silver nitrate, 500 mg of Tungstosilicic acid and 250 µl of Formaldehyde in 500 ml of double-distilled water. It is recommended to prepare fresh staining solutions A and B.

**Protocol**

**Preparation of Slides**

**First layer**: Dust free, plain slides were covered with a layer of 140 µl of 0.67% NMPA and allowed to dry for 10 minutes in hot air oven. This layer serves as an anchor for additional layers to prevent the slippage.

**Second layer**: About 110 µl of NMPA was layered as second layer and was immediately covered with a cover slip and was kept at 4°C for 10minutes to allow the agarose to gel.

**Third layer:** 30 µl of blood sample (approx. 1000 to 50,000 cells) was mixed with 110 µl of warm 0.5% LMA and this mixture was layered as third additional layer and gelled at 4oC for 10 minutes.

**Fourth layer:** A fourth additional layer of 110 µl of LMPA was added on top and gelled again in the similar way as mentioned above to sandwich the middle sample layer and to prevent the loss of sample.

**Lysing**

After the fourth layer of gel was set, the slides were treated overnight in freshly prepared chilled lysing buffer solution at 4oC. With this treatment the cell membrane and nuclear membrane were lysed and the majority of proteins were removed to expose the nucleotides.

**Alkali treatment**

The slides were then removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side avoiding spaces and with agarose end facing the anode. The tank was filled carefully with fresh electrophoretic buffer to a level approximately 0.25 cm above the slides. The slides were left in the high pH (pH >13) buffer for 20 min to allow unwinding of DNA and expression of alkali labile sites before electrophoresis.

**Electrophoresis**

Electrophoresis was carried out at room temperature for 40 min at 300 mA, 20V. The current was adjusted to 300 mA (mill amperes) by raising or lowering the buffer level in the tank.

**Neutralization**

After electrophoresis, the slides were flooded 3 times gently with chilled neutralizing solution (Tris pH 7.5) for 5 minutes so as to remove any traces of detergents and alkali which would otherwise interfere with staining then the slides were washed thrice with distilled water and air dried completely.

**Silver staining**

The slides were Silver stained by the method of Ahuja and Saran (1999). Briefly the air-dried slides were immersed in the fixing solution for 10 minutes and washed gently with double distilled water several times. The washed slides were allowed to air dried for about 1 hour before staining. Before staining 68 ml of staining (B) was mixed with 32 ml of staining solution (A) and poured over the dried slides so as to cover the slides uniformly. This step was repeated with a fresh mixture of staining solutions until with grayish colour developed on the slides.

Note: The whole procedure was carried in dim light to minimize artificial DNA damage.

**Evaluation of DNA damage**

For screening the slides a bright field transmission light microscope (LeitzLaborlux) was used. Comet tail length was measured in each case using an ocular micrometer fitted in the eyepiece. Randomly 100 cells were selected from duplicated slides at 400 X magnification. Mean tail length was calculated for each sample for quantification of the DNA damage.

**Calculation**

Length of the Comet tail (µm) = Total length of comet – Head diameter

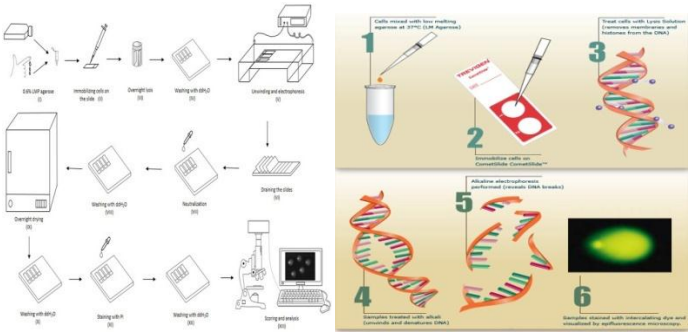


**Figure 4: comet assay**

**RNA Extraction**

**Principle:**

RNA (Ribonucleic acid) is a polymeric substance present in living cells and many viruses, consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine, and uracil, which are bonded to the ribose sugar. RNA is used in all the steps of protein synthesis in all living cells and carries the genetic information for many viruses.

The isolation of RNA with high quality is a crucial step required to perform various molecular biology experiment. TRIzol Reagent is a ready-to-use reagent used for RNA isolation234 from cells and tissues.TRIzol works by maintaining RNA integrity during tissue homogenization, while at the same time disrupting and breaking down cells and cell components. Addition of chloroform, after the centrifugation, separates the solution into aqueous and organic phases. RNA remains only in the aqueous phase.

After transferring the aqueous phase, RNA can be recovered by precipitation with isopropyl alcohol. But the DNA and proteins

can recover by sequential separation after the removal of aqueous phase. Precipitation with ethanol requires DNA from the interphase, and an additional precipitation with isopropyl alcohol requires proteins from the organic phase. Total RNA extracted by TRIzol Reagent is free from the contamination of protein and DNA. This RNA can be used in Northern blot analysis, in vitro translation, poly (A) selection, RNase protection assay, and molecular cloning.

**Reagents Required:**

- Chloroform (without any additives, such as isoamyl alcohol)
- Isopropyl alcohol
- 75% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution

The SDS solution must be prepared using DEPC-treated, autoclaved water. DEPC inactivates the RNases by the covalent modifications of the histidine residues.

**Homogenization**

Growth medium on the cells was discarded and cells were washed with ice cold 1X PBS. The monolayer was then covered with 1 ml of l TRIzol and the cells were lysed and homogenized by repeated pipetting.

**Protocol**

- After reaching the confulency cells were trypsinsied and pelleted out.
- Wash the pellet with PBS solution and centrifuged to pellet out the cells.
- Discard the supernatant and added trizol regent to the pellet depending upon the quantity of the pellet
- Incubate the pellet with Trizol at room temp for 5 mins. Add 200-500µl of choloroform per 1ml of trizol solution.
- Mix it thoroughly and incubated at room temp for 2-3 mins. Centrifuge at 10,000 rpm for 15 min at 4°C.
- After centrifuge three layers are formed.
- Transfer the upper aqueous phase to a fresh 1.5 ml eppendrof. 500µl of isoproponal added for every 1 ml of trizol solution.
- Incubate at room temp for 10 mins and centrifuged at 10,000rpm for 10 mins at 4°C.
- The supernatant discarded and washed the pellet with 75% ethanol.
- Centrifuge at 10,000rpm for 10 mins at 4°C. Discard the supernatant and the pellet was air dry.
- Re-suspend the pellet in 100µl of RNase free water.

## IV. RESULTS AND DISCUSSION

We have determined the effect of heteroscorpion venom on MCF-7 cell lines to evaluate its association with the MCF-7 cancer cells

The results obtained are presented in those sections.

**Collection of MCF-7 Cells**

MCF -7 cells clines were procured from NCCS (National Centre for Cell Science) Pune, India.

**Culture of MCF-7 cell lines**

The MCF-7 cell lines were grown in DMEM supplemented with 10 % FBS, 1 % antibiotic antimycotic solution. Cells were incubated at 37 °C with 5 % CO2. Media was replaced after every 2-3 days.

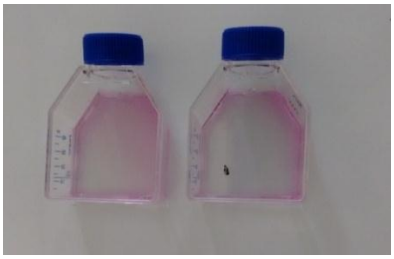


**Figure 5: Representative pictures T-25 Culture flasks with MCF-7 Cells**

After culture of MCF-7 cells were observed under microscope

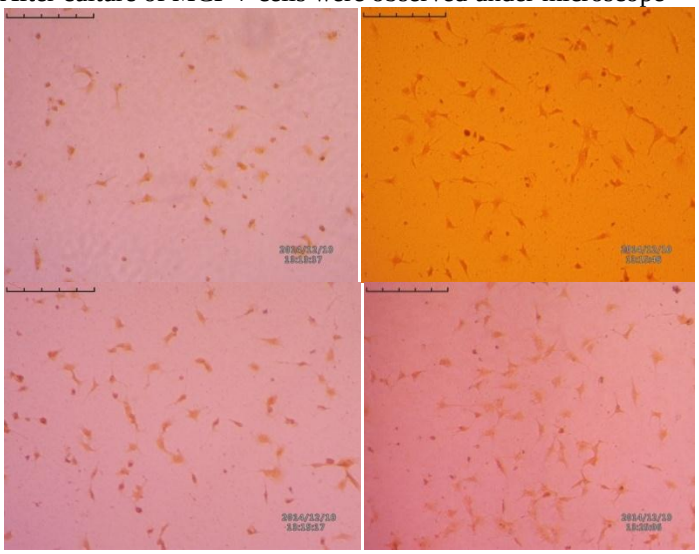


**Figure 6: Representative pictures MCF-7 cells under microscope after 18, 24, 36, 48 hours (left →right)**

**Sub culture or passage MCF-7Cells**

After reaching 80 % confluency, cells were trypsinized with 0.25% trypsin-EDTA. After the complete detachment, cells were seeded in sterile T-25 flasks with split ratio of 1:3.

**Cell viability assay**

The cytotoxicity Heteroscorpion Venom evaluated on MCF Cancer cell lines. Cells were exposed for 24 and 48 hrs to Heteroscorpion venom using the MTT assay. Cell lines growing exponentially were added to 96 well plates at a density of 4 x 104 per well after counting on hemocytometer. Stock solution 100 mM of Heteroscorpion venom was prepared by dissolving it in DMSO and diluted with DMEM media to the required concentration. Concentrations of 1-100 µM were added to wells ensuring equal volumes across the plates. Cell

number/proliferation was measured after 24 hrs and 48 hrs of incubation using standard MTT assay. They exhibited dose-dependent growth inhibitory effect against the tested cell lines and IC50 values were calculated.

Venom decreased cell viability in a dose-dependent manner. At a dose of 10 µM /ml and above, cell viability decreased significantly. While treatment with 5 µM did not affect the cell viability but at 10, 20, 30, 50, 75 µM/ml viability decreased, 62%, 58%, 49%, 41% and 29% respectively.

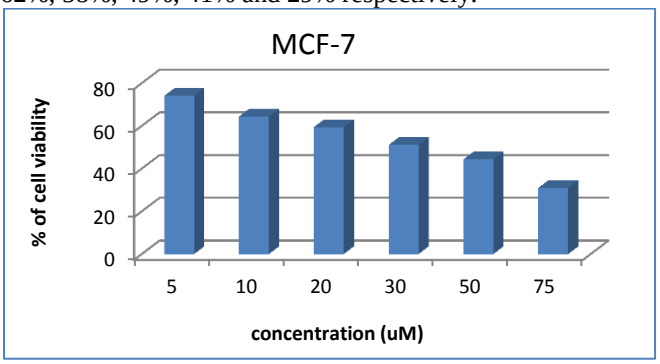



**Figure 7: Representative graph effect of different concentration of heteroscorpion venom on MCF**

MTT reduction in MCF cells after 24 h exposure with varying concentrations of Heteroscorpion venom. Cell viability was expressed as the proportion of absorbance values normalized to the control group after subtracting the blank absorbance from samples and the control. The data are expressed as the mean ± SEM of three independent experiments.

**$IC_{50}$ (µM) values of heteroscorpion venom**

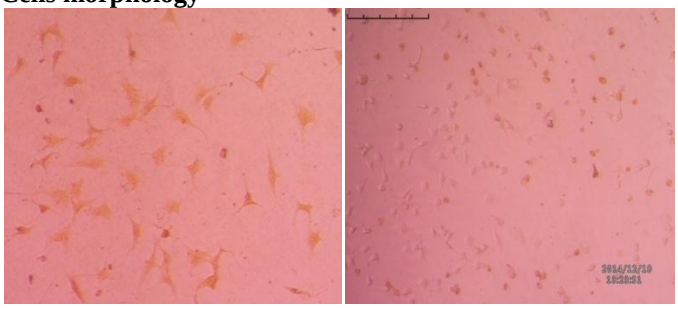
The half maximal inhibitory concentration ($IC_{50}$) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. IC50 represents the concentration of venom that is required for 50% inhibition in vitro. The IC50 value of heteroscorpion venom is given in below table.

**Table 1: IC50 (µM) values of heteroscorpion venom**

| Cell line | $IC_{50}$(µM) |
|---|---|
| MCF | 28.53 |

This data was expressed as the three independent experiments

**Cells morphology**



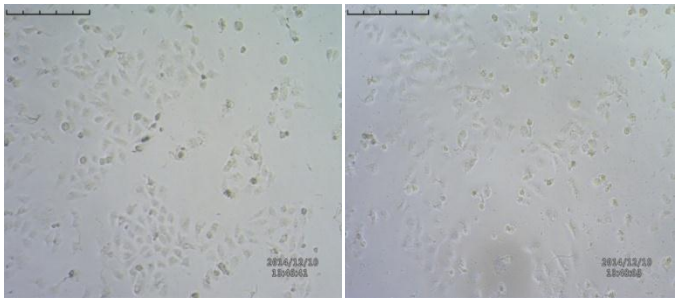


**Figure 8: Cells were exposed for 24 and 48 hrs using the MTT assay after treatment 1 min, 18, 24, and 48 hours (left →right)**

Microscopic characteristics (x200) of cultured MCF-7 cells 24 h after exposure with 20, 30, 50 and 75µM/ml of heteroscorpion venom. A polygonal shape cells after seeding in 96-wells plate. Incubated polygonal cells with 30 µM /ml of venom show loss of neurite out growth, aggregation and swelling. Incubated polygonal cells with 50, 75 µM /ml of venom expressed similar intense changes. The polygonal shaped cells changed to round form with granulated contents, homogeneous and distinct edges after overnight incubation. Polygonal shaped cells after incubation with 30µM/ml of venom inhibited neurite out growth, irregular shape, rupturing of cell membrane, releasing of cellular contents and swelling. Incubated polygonal cells exposed to 75µM/ml of venom exhibited acute similar changes.

**Comet assay**

Comet assay was carried out to check whether Heteroscorpion venom can affect the DNA damage in cells MCF cells were treated with 50µM Heteroscorpion venom and incubated at 37 °C for 24 hrs along with a set of control cells. After the incubation, 400 µl of each cell suspension were collected and washed with PBS. 40 µl of each cell suspension were mixed with 200 µl of 1% low melting point Agarose and spread onto microscope slides precoated with 1% normal melting point Agarose. Glass cover slips were placed on the drops of Agarose, which were allowed to set at 4oC. Then the cover slips were removed and the cells embedded in Agarose were lysed for 16 h by immersion in 2.5 M NaCl, 0.1 M Na2EDTA, 0.01 M Tris base, pH 10.5, 1% Triton X- 100 and 10% DMSO at 4 C (lysis solution). The slides were then placed in a horizontal gel electrophoresis tank and the DNA was allowed to unwind for 40 min in freshly prepared alkaline electrophoresis solution (0.03 M NaOH and 2 mM Na2EDTA, pH > 13). Electrophoresis was carried out in the alkaline solution for 30 min. The slides were washed in chilled neutralization buffer for 10 min to neutralize the excess alkali. The gels were air-dried for 30 min and were stained with silver nitrate .The gels were destained with chilled distilled water, air-dried and scored for comets by microscopic analysis. Comets on each gel were scored and the average tail length measure.

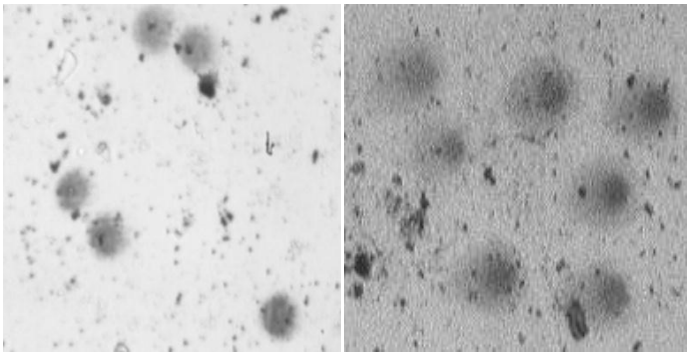


**Figure 9: Representative comet images showing different levels of damage in visual scoring, Control cells MCF (Score 0 (undamaged DNA)), Damaged DNA (various degrees of damage from minor) (left →right)**

The Comet Assay, also called single cell gel electrophoresis (SCGE), is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. DNA damage caused by apoptosis and cytotoxicity and be used in the field of genotoxicity testing, human biomonitoring, cellular response to DNA damage, and for screening cancer therapeutics.
This was data of the comet DNA damage

**Table 2: Comet DNA damage**

| Cell lines | tail length (um) | head length |
|---|---|---|
| MCF | 63 | 41 |

The head is composed of intact DNA, while the tail consists of damaged (single-strand or double-strand breaks) or broken pieces of DNA. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. This data was expressed as the three independent experiments

## V. CONCLUSION

An overview of the heteroscorpion venom which has shown promising results had been discussed. Venom Offer a platform for the design of novel therapeutic compounds. Activity of the venom can be increased by a dose-dependent manner. It seems that opportunities exist to develop venom based drug candidates in the discovery and development of novel therapeutic agents. The encouraging results of preclinical and clinical studies with venom form the basis for further investigations towards the development of venom for better therapeutic profile. Although, venom has some side effects, they are successfully being used in cancer therapy and several other therapies. Therefore there is a need for new approaches that are required to circumvent these drawbacks and pave a way for potent drug therapies.

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