Cytotoxic, Apoptotic and Anti-proliferative Effects of the Crude Scorpion Venom
1 Cytoxicity assays

1.1 Determination of cell viability (MTT reduction)

1.1.1 Effect of *A. crassicauda* venom on cell viability

The crude venom of *A. crassicauda* decreased viability of SH-SY5Y and MCF-7 cells in a dose-dependent manner (Fig.3.1). The number of viable cells of SH-SY5Y after exposure with 10, 20, 50, 100 or 200 µg/ml of venom decreased to 85.9, 76.1, 68.8, 64.6 and 54%, respectively. Cell viability of MCF-7 after treatment with 10, 20, 50, 100 or 200 µg/ml of venom reduced to 90.2, 85.5, 77.6, 65.2 and 56.8 %, respectively.

Cell viability of the two cell lines at 10µg/ml was not significant as compared to control, however at 20µg/ml, cell viability was significant but between the two doses (10 and 20µg/ml), the difference was not significant. Similarly, although higher doses of scorpion venom (50, 100 or 200 µg/ml) inhibited growth of cells significantly as compared to control, the cell viability of the two cell lines at 200µg/ml was not significant as compared to 100µg/ml. The IC$_{50}$ value for SH-SY5Y was calculated to be 207.7µg/ml and that for MCF-7 was 269µg/ml.

![Fig.3.1: Cytotoxicity of *A. crassicauda* venom on SH-SY5Y and MCF-7 cells by MTT assay. Each data represents the mean ± SEM of three independent experiments. Significances indicated are shown in comparison to control. N.S (non significant), *p<0.05, **p<0.01, ***p<0.001](image-url)
1.1.2 Effect of *B. saulcyi* venom on cell viability

Venom inhibited viability of SH-SY5Y and MCF-7 cells at a concentration of 10-200 μg/ml in a dose-dependent manner. This effect was more pronounced in SH-SY5Y than the MCF-7.

The numbers of viable cells of SH-SY5Y and MCF-7 at the lowest dose (10 μg/ml) after 24 hr incubation was decreased by 9.53% and 4.67% respectively and rate of inhibition at highest dose (200 μg/ml) was elevated up to 59% and 43% respectively. Inhibited cell viability of SH-SY5Y at 20 μg/ml was significant (p<0.05) as compared to control, but insignificant as compared to lower dose. Venom decreased growth of both the cell lines at concentration of 50μg/ml and more.

Effects of the different concentrations of the venom on the growth of cells are presented in graphs (Fig.3.2).

The IC₅₀ value of *B. saulcyi* venom for SH-SY5Y was calculated to be 133.5μg/ml and that for MCF-7 was 272.1μg/ml.

![Fig.3.2: Cytotoxicity of *B.saulcyi* venom on SH-SY5Y and MCF-7 cells by MTT assay. Each data represents the mean ± SEM of three independent experiments. Significances indicated are shown in comparison to control. N.S. (non significant), *p<0.05, ***p<0.001.](image)
1.1.3 Effect of *O. doriae* venom on cell viability

Venom of *O. doriae* decreased cell viability of SH-SY5Y and MCF-7 cells in a dose dependant manner (Fig.3.3). At a dose of 20μg/ml and above, cell viability decreased significantly. While treatment with 10μg did not affect the cell viability (90.75%) but at 20, 50, 100 and 200μg/ml, viability decreased to 75.53 %, 55.52%, 37.85% and 14.30% respectively.

At a dose of level 10, 20, 50,100 and 200 μg/ml of *O. doriae* venom, viability of MCF-7 decreased to 86.8, 80.6, 67.7, 62.2 and 39.9% respectively. While 10μg/ml venom affected cell viability significantly (p<0.01), but a dose of 20 μg or more, decreased the cell viability to a greater significant level (p<0.001).

The IC$_{50}$ value of *O. doriae* venom for SH-SY5Y was calculated to be 62.18μg/ml and that for MCF-7 was 118.8 μg/ml.

![Fig.3.3: Cytotoxicity of *O. doriae* venom on SH-SY5Y and MCF-7 cells by MTT assay. Each data represents the mean ± SEM of three independent experiments. Significances indicated are shown in comparison to control. N.S. (non significant), ** p<0.01, ***p<0.001.](image)
1.2 Lactate dehydrogenase assay (LDH)

1.2.1 Effect of *A. crassicauda* venom on the release of lactate dehydrogenase

After determining cell viability, lactate dehydrogenase release was evaluated 24 hr latter to confirm cell death. Release of lactate dehydrogenase enzyme was increased in treated SH-SY5Y and MCF-7 with venom of *A. crassicauda* in a dose-response manner (Fig.3.4). Release of LDH in treated SH-SY5Y cells with 10, 20, 50 and 100 μg/ml of scorpion venom increased to 24.55±1, 26.86±2.17, 41±3.56 and 53.4±3.31 % respectively as compared to control (22.4±1.17). In treated MCF-7 cells with 10, 20, 50 and 100 μg/ml of venom, LDH release increased to 24.8±0.8, 26 ±1.5, 30.31±1.2 and 46.82±2.7 % respectively as compared to control(22.93±0.6). The effect on two cell lines was insignificant at 10 and 20 μg/ml but significant at a dose of 50 and 100 as compared to control. Therefore, these two doses were selected for further studies.

![Figure 3.4: LDH released in the culture of SH-SY5Y and MCF-7 cells after 24 hr incubation with *A. crassicauda* venom was used as an index of toxicity generated by the toxin. Scorpion venom induced in vitro release of LDH, a cytosolic enzyme in a dose dependent manner in SH-SY5Y and MCF-7 cells, 24 hr after incubation. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *p<0.05, ** p<0.01 and *** p<0.001](image-url)
1.2.2 Effect of *B. saulcyi* venom on release of lactate dehydrogenase

Scorpion venom of *B. saulcyi* increased release of LDH enzyme in a dose-response effect on SH-SY5Y and MCF-7 cells after 24 hr incubation (Fig.3.5). LDH release in SH-SY5Y was higher than the MCF-7, indicating that *B. saulcyi* venom is more toxic to the human neuroblastoma cell.

Release of LDH in treated SH-SY5Y cells with 10, 20, 50 and 100 μg/ml of scorpion venom up regulated to 27.9±1.12, 29.37±4.53, 37±3.22 and 50.4±4.5 % respectively as compared to control (24.5±2.05). In treated MCF-7 cells with 10, 20, 50 and 100 μg/ml of venom, release of LDH elevated to 22.42±2.91, 24.77±1.8, 35.31±3.1 and 45.8±3.06 % respectively as compared to control (22.13±1.7). This cytotoxic effect on the two cell lines was insignificant at 10 and 20μg/ml of doses but markedly significant effect at 50 and 100μg/ml was seen. Therefore, these two doses were selected for further studies.

![Total LDH release](image)

**Fig.3.5:** LDH released in the culture of SH-SY5Y and MCF-7 cells after 24 hr incubation with venom of *B. saulcyi*. LDH was used as an index of toxicity. Scorpion venom induced in vitro release of LDH, a cytosolic enzyme in a dose dependent manner in SH-SY5Y and MCF-7 cells, 24 h after incubation. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *p<0.05, ** p<0.01 and *** p<0.001
1.2.3 Effect of *O. doriae* venom on release of lactate dehydrogenase

Release of lactate dehydrogenase enzyme increased in treated SH-SY5Y and MCF-7 with venom of *O. doriae* in a dose-response manner (Fig.3.6). In control cells of SH-SY5Y and MCF-7, LDH level was 23.25±0.7 and 15.38±0.78 % respectively. In SH-Y5Y cells, increase of LDH level at 10 and 20μg/ml was not significant as compared to control but at higher dose of 50 and 100μg/ml, it was significant. Release of LDH in treated SH-SY5Y cells with dose of 10, 20, 50 and 100 μg/ml of *O. doriae* venom was elevated to 24.95±1.2, 27.9±2.9, 48.84±07 and 64.48±1.64 respectively. In treated MCF-7 cells with 10, 20, 50 and 100 μg/ml of *O. doriae* venom, release of LDH increased to 19.74±0.66, 21.52±0.56, 29.89±0.51 and 35.05±0.98 % respectively. In MCF-7 increase of LDH was not significant at 20 μg/ml as compared to 10 μg/ml but at two higher doses (50 and 100 μg/ml), LDH increased significantly (p<0.01). Therefore these two doses were considered as optimal dose to carry out all other analysis.

![Fig.3.6: LDH released in the culture of SH-SY5Y and MCF-7 cells after 24 hr incubation with venom of *O. doriae* was used as an index of toxicity generated by the toxin. Scorpion venom induced in vitro release of LDH, a cytosolic enzyme in a dose dependent manner in SH-SY5Y and MCF-7 cells, 24 h after incubation. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *p<0.05, ** p<0.01 and *** p<0.001](image-url)
1.3 Cell morphology after incubating the cell lines with the venoms

Effects of venoms of three species of scorpions (*A. crassicauda, B. saulcyi and O. doriae*) on the morphology of SH-SY5Y and MCF-7 cells were monitored for 24 hr after exposure with two doses of each venom (50 and 100μg/ml) as shown in Fig.3.7-3.9. The SH-SY5Y and MCF-7 cells after treatment with scorpion’s venoms demonstrated similar morphological changes. The untreated cells of MCF-7 dispersed homogeneously and switched to polygonal shape with distinct boundaries and slightly granulated contents after overnight incubation at 37°C and 5% CO₂. The untreated cells of SH-SY5Y cells presented latter changes with clear neurite out growth.

![Fig.3.7: Effects of venom of *A. crassicauda* on the morphological alterations of MCF-7 and SH-SY5Y. Cells were monitored for 24 h at two different doses of scorpion venom. Control MCF-7 cells (A) and SH-SY5Y (B) dispersed homogeneously with distinct boundaries after overnight incubation. After exposure of cells with 50 μg/ml of scorpion venom, MCF-7 cells (C) presented with abnormalities in shape, aggregation, indistinct edges, swelling, rupture of membrane and releasing of cellular contents (black arrows). At this concentration of scorpion venom, SH-SY5Y cells (D) showed above mentioned alterations and destruction of neurite out growth (white arrows). The MCF-7 (E) and SH-SY5Y (F) presented acute changes and more cellular swelling after treatment with 100μg/ml of scorpion venom as compared to 50 μg/ml. Original pics are 20x.](image-url)
The MCF-7 cells after treatment with 50μg/ml of the three venoms demonstrated abnormalities in shape, aggregation, indistinct edges, swelling, rapture of membrane and release of cellular contents. At this concentration, SH-SY5Y cells also showed above mentioned alterations and destruction of neurite out growth. Treatment of cells with 100μg/ml of venoms showed similar but acute changes and more cellular swelling with increased cellular adhesiveness in both of the cell lines.

Fig.3.8: Effects of venom of O. doriae on the morphological alterations of MCF-7 and SH-SY5Y. Cells were monitored for 24 h at two different doses of scorpion venom. Control MCF-7 cells (A) and SH-SY5Y (B) dispersed homogeneously with distinct edges after overnight incubation. After exposure of cells with 50 μg/ml of scorpion venom, MCF-7 cells (C) presented with abnormalities in shape, aggregation, indistinct edges, swelling, rupture of membrane and releasing of cellular contents (black arrows). At this concentration, SH-SY5Y cells (D) showed above mentioned alterations and destruction of neurite out growth. The MCF-7 (E) and SH-SY5Y (F) presented acute changes and more cellular swelling (white arrows) after treatment with 100μg/ml of scorpion venom as compared to 50 μg/ml. Original pics are 20x.
Fig. 3.9: The morphology and microscopic characteristics of human neuroblastoma SH-SY5Y and MCF-7 cells were monitored during 24 h after exposure with venom of *B. saulcyi*. The round shaped SH-SY5Y (A1) and MCF-7(A2) cells switched to polygonal form with granulated contents, homogenous and distinct edges after overnight incubation at 37°C and 5% CO₂ as shown in B1 and B2 respectively. The polygonal cells of SH-SY5Y presented destruction of neurite outgrowth (white arrows), swelling, rupture of membrane and release of cellular contents (black arrows) after treated with 50 ng/ml of venom (C1&D1). MCF-7 cells exhibited variety of cellular changes such as shrinkage, plasma membrane blebbing, swelling (black arrows), rupture of membrane and release of cytosolic contents (white arrows) after exposure with 50 µg/ml of venom (C2&D2). After treatment with 100 µg/ml of venom, SH-SY5Y (E1&F1) and MCF-7(E2&F2) exhibited acute changes as compared to 50 µg/ml. Original pics are 20x.
2 Effect of the crude venoms on the biochemical markers of oxidative stress

2.1 Reactive nitrogen species

2.1.1 Effect of *A. crassicauda* venom on reactive nitrogen species
Venom of *A. crassicauda* increased the level of nitric oxide in SH-SY5Y and MCF-7 cells in a dose-response effect (Fig.3.10). In SH-SY5Y cell, 50 and 100 μg/ml of scorpion venom significantly increased the nitrite level from 5.79±0.46 in control to 24.31±0.7 and 38.46±1.64 μM/ml respectively. In MCF-7 cells, nitrite level in control was 2.34±1 μM/ml whereas in cells treated with 50 and 100 μg/ml of scorpion venom, 3.25±0.7 and 4.52±0.9 μM/ml respectively. In this cell line, increment of NO at dose of 50 μg/ml of scorpion venom was insignificant as compared to control.

2.1.2 Effect of *B. saulcyi* venom on reactive nitrogen species
Production of Nitric oxide was increased in treated cells with 50 and 100 μg/ml of *B. saulcyi* venom. In SH-SY5Y, venom increased production of NO significantly in a dose-response effect in comparison to controls (Fig.3.10). In this cell, 50 μg/ml dose of venom elevated the nitrite content from 5.79±0.46 μM/ml in control to 8.21±0.5 μM/ml, whereas 100 μg/ml showed 10.96±0.39 μM/ml. NO production enhanced in MCF-7 cells after exposure with venom but this elevation was not significant at 50 μg/ml dose of venom as compared to control. In this cell line, nitrite level in control was 2.34±1 μM/ml whereas after exposure with 50 and 100 μg/ml of venom, it raised to 3.55±0.31 and 5.24±0.2 μM/ml respectively.

2.1.3 Effect of *O. doriae* venom on reactive nitrogen species
Venom of *O. doriae* enhanced production of nitric oxide in SH-SY5Y and MCF-7 cells in a dose-response effect (Fig.3.10). In SH-SY5Y cell, 50 and 100 μg/ml of scorpion venom significantly increased the nitrite level from 5.79±0.46 in control to 12.33±0.69 and 22.19±0.98 μM/ml respectively. In MCF-7 cells, nitrite level in control was 2.34±1 μM/ml whereas in cells treated with 50 and 100 μg/ml of scorpion venom, 8.32 ±0.08 and 9.38±0.5 μM/ml respectively.
Fig. 3.10: Nitric oxide production was determined by measuring nitrite in culture medium of control and treated cells with 50 and 100 µg/ml of venoms. Concentration of NO was determined by comparison of absorbance value to the nitrite standard curve and expressed as µM/ml. A1, treated cells with 50 µg/ml of *A. crassicauda* venom. A2, treated cells with 100 µg/ml of *A. crassicauda* venom. B1, treated cells with 50 µg/ml of *B. saulcyi* venom. B2, treated cells with 100 µg/ml of *B. saulcyi* venom. O1, treated cells with 50 µg/ml of *O. doriae* venom. O2, treated cells with 100 µg/ml of *O. doriae* venom. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *(p<0.05), ** (p<0.01), *** (p<0.001)

### 2.2 Reduced glutathione (GSH)

Treatment of SH-SY5Y and MCF-7 cells for 24 hr with the two selected doses of venoms resulted in decrease in glutathione level in a dose dependent manner (Fig. 3.11). Depletion in glutathione level in treated MCF-7 was more than SH-SY5Y cells. GSH in control cells of SH-SY5Y and MCF-7 was 0.428±0.007 and 0.329±0.005 µgm GSH/mg protein respectively. In treated cells of SH-SY5Y with 50µg/ml of *A. crassicauda*, *B. saulcyi* and *O. doriae* venoms, GSH decreased to 0.398±0.005, 0.402±0.013 and 0.399±0.01 µgm GSH/mg protein whereas in MCF-7, decrement was 0.120±0.004, 0.121±0.004 and 0.134±0.015µgm GSH/mg protein respectively.

In other experiment, 100 µg/ml of venom of *A. crassicauda*, *B. saulcyi* and *O. doriae* decreased GSH level in treated SH-SY5Y cells to 0.391±0.008, 0.399±0.004 and
0.394±0.011 µgm GSH/mg protein, but in MCF-7 glutathione depleted to 0.085±0.006, 0.079±0.007 and 0.084±0.004 µgm GSH/mg protein respectively. Although the reduction of glutathione level in treated MCF-7 with 50 and 100 µg/ml doses of three species of scorpions was significant between two doses, but in SH-SY5Y cells, the decrement in GSH activity between the two doses was not significant.

![Graph showing Estimation of GSH in SH-SY5Y and MCF-7](image)

**Fig.3.11**: Glutathione (GSH) was estimated in cell lysate of control and treated cells with 50 and 100 µg/ml of scorpion venom and expressed as µgm GSH/mg protein using molar extinction coefficient of 13600. A1, treated cells with 50 µg/ml of *A. crassicauda* venom, A2, treated cells with 100 µg/ml of *A. crassicauda* venom, B1, treated cells with 50 µg/ml of *B. saulcyi* venom, B1, treated cells with 100 µg/ml of *B. saulcyi* venom, O1, treated cells with 50 µg/ml of *O. doriae* venom, and O2, treated cells with 100µg/ml of *O. doriae* venom. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *(p<0.05), ** (p<0.01), *** (p<0.001)

2.3 Catalase activity

The two concentration of venom viz 50 µg/ml and 100 µg/ml of *A. crassicauda, B. saulcyi* and *O. doriae* venom decreased the catalase activity of treated SH-SY5Y and MCF-7 as compared to control (Fig.3.12).

The activity level of catalase in control cells of SH-SY5Y and MCF-7 were 58.07±1.4 and 37.69±0.75 µmoles of hydrogen peroxide consumed/min/mg protein respectively. At
50 μg/ml of *A. crassicauda*, *B. saulcyi* and *O. doriae* venoms, the catalasaes activity in SH-SY5Y cells reduced to 55.24±0.63, 55.67±0.95 and 54.77±1.20 whereas at 100 μg/ml of venoms concentration, decrement was 53.47±0.08, 53.34±0.50 and 51.21±0.9 μmoles of hydrogen peroxide consumed/min/mg protein respectively. Although decrement in catalase activity in SH-SY5Y cells between the two doses of *O. doriae* venom was significant (p<0.01) but in other two scorpion venoms, this reduction between two concentration was not significant.

Catalase in treated cells of MCF-7 cells with 50 μg/ml of *A. crassicauda*, *B. saulcyi* and *O. doriae* venoms decreased to 5.87±0.60, 5.76±0.6 and 5.63±0.51, whereas at concentration of 100 μg/ml, decrement was 3.94±0.48, 3.9±0.39 and 4.05±0.28 μmoles of hydrogen peroxide consumed/min/mg protein respectively. In MCF-7 cells the reduction of catalases activity was significant between the two doses of the venoms.

**Fig.3.12**: Catalase activity was estimated in cell lysate of control and treated cells with 50 and 100 μg/ml of scorpion venom and expressed as micro moles of hydrogen peroxide consumed/min/mg protein. A1, treated cells with 50 μg/ml of *A. crassicauda* venom, A2, treated cells with 100 μg/ml of *A. crassicauda* venom, B1, treated cells with 50 μg/ml of *B. saulcyi* venom, B1, treated cells with 100 μg/ml of *B. saulcyi* venom, O1, treated cells with 50 μg/ml of *O. doriae* venom, O2, treated cells with 100 μg/ml of *O. doriae* venom. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control.

N.S (non significant), *(p<0.05), ***(p<0.001)
Cytotoxic, Apoptotic and Anti-Proliferative Effects of the Crude Venoms

CHAPTER 3

3 Apoptosis effects

3.1 Assessment of Mitochondrial Membrane potential

3.1.1 Effect of *A. crassicauda* venom on mitochondrial membrane potential

Venom of *A. crassicauda* inhibited mitochondrial membrane potential in the two cell lines but it was more pronounced in SH-SY5Y cell as compared to MCF-7 (Fig.3.13). In SH-SY-5Y, the ratio of green to red was 0.16±0.01 in control whereas in treated cells (50,100 μg/ml), the ratio was 0.28±0.04 and 0.61±0.024 respectively.

In MCF-7 cells, this ratio in control cells was 0.173±0.006 and in treated cell 0.19±0.009 and 0.23±0.01 respectively. Although inhibition of mitochondrial membrane potential in SH-SH5Y was significant at 50 and 100 μg/ml of scorpion venom as compared to control cells but in MCF-7 cells at 50μg/ml no significant effect was detected.

3.1.2 Effect of *B. saulcyi* venom on mitochondrial membrane potential

Venom of *B. saulcyi* Induced mitochondrial depolarisation in both of types of cells (Fig.3.13). In SH-SY-5Y the ratio of green to red was 0.16±0.01 in control whereas in treated cells viz. 50 and 100μg/ml, it was found to be 0.377±0.01 and 0.383±0.011 respectively.

In MCF-7 cells, this ratio in control cells was 0.173±0.006 and in treated cell with 50 and 100 μg/ml, 0.2±0.005 and 0.42±0.01, respectively. Although collapse of mitochondrial membrane potential in neuroblastoma cell (SH-SH5Y) was significant at 50 and 100 μg/ml doses of venom as compared to control cells but in breast cancer cell (MCF-7) at 50μg/ml no significant effect was noticed.

3.1.3 Effect of *O. doriae* venom on mitochondrial membrane potential

Venom of *O. doriae* inhibited mitochondrial membrane potential in the SH-SY5Y and MCF-7 cell lines (Fig.3.13). In SH-SY-5Y, the ratio of green to red was 0.16±0.01 in
control whereas in treated cells with 50 and 100 µg/ml of venom, the ratio was 0.225±0.013 and 0.28±0.007, respectively.

The ratio of green/red fluorescence intensity of JC-1 in control MCF-7 cells was 0.173±0.006 and in treated cell with 50 and 100 µg/ml of *O. doriae* venom, increment was 0.212±0.029 and 0.619±0.014 respectively. Therefore mitochondrial membrane potential as a function of green/red fluorescence decreased significantly at the two concentrations of venom viz. 50 µg/ml (p<0.05) and 100 µg/ml (p<0.001).

Inhibition of mitochondrial membrane potential in SH-SY5Y and MCF-7 cells was significant between two concentrations of scorpion venom.

![MPP determination](image)

**Fig 3.13**: Mitochondrial membrane potential was estimated by using JC-1(10 µM) probe in control and sample cells treated with 50 and 100 µg/ml of scorpions venoms for 24 h. Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm). The ratio of green/red fluorescence as a measure of mitochondrial membrane potential was calculated. A1, treated cells with 50 µg/ml of *A. crassicauda* venom, A2, treated cells with 100 µg/ml of *A. crassicauda* venom, B1, treated cells with 50 µg/ml of *B. saulcyi* venom, B1, treated cells with 100 µg/ml of *B. saulcyi* venom, O1, treated cells with 50 µg/ml of *O. doriae* venom, O2, treated cells with 100 µg/ml of *O. doriae* venom. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. N.S (non significant), *(p<0.05), **(p<0.01), *** (p<0.001)
3.2 Measurement of Caspase-3 activity

3.2.1 Effect of *A. crassicauda* venom on caspase-3 activity

Venom of *A. crassicauda* elevated activity of caspase-3 in a dose dependent manner in SH-SY5Y as well as MCF-7 cells (Fig.3.14). In treated SH-SY5Y cells with 50 and 100 μg/ml of scorpion venom, caspase-3 activity significantly increased to 0.090±0.005 and 0.099±0.002 μM pNA liberated/h/ml respectively as compared to 0.049±0.001 μM pNA liberated/h/ml in control. In MCF-7 this activity upregulated from 0.0211±0.001 μM pNA liberated/h/ml in control to 0.045±0.002 and 0.046±0.005 μM pNA liberated/hr/ml in 50 and 100 μg/ml doses respectively. In this cell type, caspase-3 activity in between treated cell was insignificant.

3.2.2 Effect of *B. saulcyi* venom on caspase-3 activity

Venom of *B. saulcyi* enhanced activity of caspase-3 significantly in a dose dependent manner in SH-SY5Y and MCF-7 after 24 hr incubation in comparison to controls (Fig.3.14). At 50 and 100 μg/ml doses of venom the activity of caspase-3 in SH-SY5Y increased to 0.083±0.007 and 0.092±0.003 μM pNA liberated/hr/ml respectively in comparison to 0.049±0.001 μM pNA liberated/hr/ml in control. In MCF-7 this activity raised from 0.0211±0.001 μM pNA liberated/hr/ml in control to 0.065±0.002 and 0.13±0.008 μM pNA liberated/hr/ml in 50 and 100 μg/ml doses respectively.

3.2.3 Effect of *O. doriae* venom on caspase-3 activity

Venom of *O. doriae* increased the activity of caspase-3 in a dose dependent manner in SH-SY5Y and MCF-7 cells (Fig.3.14). In SH-SY5Y the activity of caspase-3 was elevated from 0.049±0.001 μM pNA liberated/hr/ml in control to 0.076±0.004 and 0.129±0.001 μM pNA liberated/hr/ml in 50 and 100 μg/ml doses respectively. In treated MCF-7 cells with 50 and 100 μg/ml of *O. doriae* venom, the activity of caspase-3 increased to 0.033±0.001 and 0.058±0.005 μM pNA liberated/hr/ml respectively in comparison to 0.0211±0.001 μM pNA liberated/hr/ml of control. In both treated cell lines, caspase-3 activity between two concentrations of venom was significant (p<0.01).
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Caspase-3 activity

SH-SY5Y  MCF-7

A1  A2  B1  B2  O1  O2

Control  0.049±0.001  0.090±0.002  0.099±0.002  0.083±0.007  0.092±0.009  0.07±0.004  0.129±0.001

50 µg/ml of A. crassicauda venom

100 µg/ml of A. crassicauda venom

50 µg/ml of B. saulcyi venom

100 µg/ml of B. saulcyi venom

50 µg/ml of O. doriae venom

100 µg/ml of O. doriae venom

Fig. 3.14: Caspase-3 activity in cells after exposure with scorpion venom was assessed by measuring DEVD-pNA hydrolysis in lysates of control and treated cell. The absorbance of cell lysate and buffer was subtracted to calculate the caspase-3 activity. Comparison of the absorbance of pNA from a treated sample with control allows determination of the fold increase in Caspase-3 activity. A1, treated cells with 50 µg/ml of A. crassicauda venom, A2, treated cells with 100 µg/ml of A. crassicauda venom, B1, treated cells with 50 µg/ml of B. saulcyi venom, B2, treated cells with 100 µg/ml of B. saulcyi venom, O1, treated cells with 50 µg/ml of O. doriae venom, O2, treated cells with 100 µg/ml of O. doriae venom. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. *(p<0.05), **(p<0.01), *** (p<0.001)

3.3 Gel electrophoresis DNA fragments

Agarose gel electrophoresis revealed two fragments in treated cells with 50 µg/ml of A. crassicauda venom corresponding to 200-400 bp in SH-SY5Y and 500-700 bp in MCF-7 cells (Fig. 3.15), but at 100µg/ml, scorpion venom did not induce DNA damage in the two types of cells. At the two concentrations viz. 50 and 100 µg/ml, B. saulcyi venom induced fragmentation of DNA in SH-SY5Y and MCF-7.

Agarose Gel electrophoresis revealed a single fragment corresponding to 400-500 bp in SH-SY5Y cells and two bands corresponding to 500-700 bp in MCF-7 cells (Fig. 3.15). In SH-SY5Y, DNA fragmentation (200-300 bp) was observed at a dose of 50µg/ml of O. doriae but at 100µg/ml, venom did not induce DNA damage. In MCF-7 cells, O. doriae venom induced fragmentation of DNA in 200-400 bp regions as compared to the control cells when exposed at the two concentrations (Fig. 3.15).
CHAPTER 3

Fig.3.15: DNA of SH-SY5Y and MCF-7 cells after treatment with venoms of scorpions was extracted and resolved on a 1.5 % agarose gel and visualized with ethedium bromide. A1&A2, DNA fragmentation of SH-SY5Y (A1) and MCF-7(A2) cells after treatment with venom of A. crassicauda. B1&B2, DNA breakage of SH-SY5Y (B1) and MCF-7(B2) cells after exposure with venom of B. saulcyi. O1&O2, DNA laddering in SH-SY5Y (O1) and MCF-7(O2) cells after treatment with venom of O. doriae. C, control cells. M, DNA Marker. 50, treated cells with 50 μg/ml of scorpion venom. 100, treated cells with 100 μg/ml of scorpion venom.

4 Assessment of the anti-proliferative effect by immunocytochemistry

4.1 Antiproliferative effect of A. crassicauda venom

A. crassicauda inhibited cell proliferation of SH-SY5Y as well as MCF-7 (Fig. 3.16-3.17). The number of nuclei undergoing DNA synthesis in SH-SY5Y after exposure with 50 and 100μg/ml of A. crassicauda venom were reduced to 28.9±2.7% and 19.2±0.96% respectively as compared to control (55.6±5.7%). At 50μg/ml, numbers of cells incorporating BrdU in MCF-7 were decreased to 14.19±1.5% as compared to control (23.4±2.4) while 100 μg/ml of venom inhibited incorporation to 7.7±1.8%.
4.2 Antiproliferative effect of *B. saulcyi* venom

*B. saulcyi* inhibited the number of nuclei undergoing DNA synthesis in SH-SY5Y and MCF-7 cells (Fig. 3.16 & 3.18). In SH-SY5Y the number of cells pulsed with BrdU decreased from 56.6±5.7 % in control to 29.04±1.12 % and 12.7±1.3 % at 50 and 100 µg/ml doses respectively. In treated MCF-7 cells with 50 and 100 µg/ml of *B. saulcyi* venom, the number of nuclei undergoing DNA synthesis reduced to 17.9±0.89 and 6.95±0.52 respectively in comparison to 24.03 ±1.6 in control.

4.3 Antiproliferative effect of *O. doriae* venom

*O. doriae* inhibited cell proliferation of SH-SY 5Y as well as MCF-7 (Fig. 3.16 & 3.19). The number of nuclei undergoing DNA synthesis in SH-SY5Y after exposure with 50 and 100µg/ml of *O. doriae* venom were reduced to 8.3±1.58% and 2.6±0.45% respectively as compared to control (55.6±5.7%). At 50 µg/ml, numbers of MCF-7 cells incorporating BrdU was decreased to 12.51±0.96%, while 100 µg/ml of venom inhibited incorporation to 5.39±1.05% as compared to control (24.3±1.6).

![BrdU incorporation](image)

**Fig. 3.16:** Frequency of BrdU positive cells in SH-SY5Y and MCF-7 cells after venom treatment. Effect of scorpion venom on the synthesis of DNA in cells visualized by immunocytochemical detection of thymidine analog BrdU using monoclonal antibody. The data were expressed as the mean±SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. A1, treated cells with 50 µg/ml of *A. crassicauda* venom, A2, treated cells with 100 µg/ml of *A. crassicauda* venom, B1, treated cells with 50 µg/ml of *B. saulcyi* venom, B2, treated cells with 100 µg/ml of *B. saulcyi* venom, O1, treated cells with 50 µg/ml of *O. doriae* venom, O2, treated cells with 100 µg/ml of *O. doriae* venom. * (p<0.5), *** (p<0.001).
Fig. 3.17: Effect of A. crassicauda venom on the synthesis of DNA in SH-SY5Y and MCF-7 cells was visualized by immunocytochemical detection of thymidine analog BrdU using monoclonal antibody. A, Control MCF-7 cells pulsed with BrdU. B, inhibition of the DNA synthesis in treated MCF-7 cells with 50 μg/ml of scorpion venom. C, inhibition of DNA synthesis in MCF-7 cells by dose of 100 μg/ml of scorpion venom. D, control SH-SY5Y cells pulsed with BrdU. E, inhibition of the DNA synthesis in SH-SY5Y cells following treatment with 50 μg/ml of scorpion venom. F, inhibition of DNA synthesis in exposed SH-SY5Y cells with 100 μg/ml of scorpion venom. Arrows point towards BrdU positive cells.
Fig. 3.18: Effect of *B. sauleyi* venom on the synthesis of DNA in SH-SY5Y and MCF-7 cells was visualized by immunocytochemical detection of thymidine analog BrdU using monoclonal antibody. A, Control MCF-7 cells pulsed with BrdU. B, inhibition of the DNA synthesis in treated MCF-7 cells with 50μg/ml of scorpion venom. C, inhibition of DNA synthesis in MCF-7 cells by dose of 100μg/ml of scorpion venom. D, control SH-SY5Y cells pulsed with BrdU. E, inhibition of the DNA synthesis in SH-SY5Y cells following treatment with 50 μg/ml of scorpion venom. F, inhibition of DNA synthesis in exposed SH-SY5Y cells with 100μg/ml of scorpion venom. Arrows point towards BrdU positive cells.
**Fig. 3.19:** Effect of *O. doriae* venom on the synthesis of DNA in SH-SY5Y and MCF-7 cells was visualized by immunocytochemical detection of thymidine analog BrdU using monoclonal antibody. A, Control MCF-7 cells pulsed with BrdU. B, inhibition of the DNA synthesis in treated MCF-7 cells with 50μg/ml of scorpion venom. C, inhibition of DNA synthesis in MCF-7 cells by dose of 100μg/ml of scorpion venom. D, control SH-SY5Y cells pulsed with BrdU. E, inhibition of the DNA synthesis in SH-SY5Y cells following treatment with 50 μg/ml of scorpion venom. F, inhibition of DNA synthesis in exposed SH-SY5Y cells with 100μg/ml of scorpion venom. Arrows point towards BrdU positive cells.
5 Discussion

Venom of some scorpion species can induce cell suicide and arrest DNA synthesis in neoplastic cells [Das Gupta et al., 2007; Gomes et al., 2010]. This is an important property which can be exploited for isolation of compounds of therapeutic importance in cancer research. This study was designed to examine the cytotoxic, and anti-cancer properties of crude venoms isolated from selected species of scorpions, A. crassicauda, B. saulcyi and O. doriae, and elucidate the most plausible pathway followed by these venoms during induction of apoptosis and inhibition of proliferation in human neuroblastoma (SH-SY5Y) and human breast carcinoma (MCF-7) cells.

Venoms of scorpions studied in this study inhibited viability of SH-SY5Y and MCF-7 cells and increased the release of LDH in a dose-dependent manner (Fig.3.4). Results clearly indicate that A. crassicauda, B. saulcyi and O. doriae are more toxic to SH-SY5Y, when compared to MCF-7, which might be due to the presence of effective neurotoxic peptide(s) in these venoms.

Apoptosis results in various cellular changes, ranging from nuclear condensation, cell membrane blebbing and cell shrinkage [Benjamin et al., 1998; Ueda and Shah, 1994]. This study demonstrates that the crude venoms isolated from the three species induced wide range of morphological alterations such as the rupture of membrane, release of cytosolic contents and swelling in MCF-7 and SH-SY5Y cells (Fig.3.7-3.9). In addition, SH-SY5Y showed either shorter or no neurites after treatment. At higher dose level (100μg/ml), both cell lines presented acute swelling and lesser release of apoptotic bodies. These results are in accordance with the earlier findings where bee venom has been reported to induce morphological changes in MCF-7 cells [Ip et al., 2008].

Scorpion venom is a complex mixture of several organic and inorganic compounds [Nisani et al., 2007]. Induction of oxidative stress in treated cells was investigated. The venom was found to reduce the cellular antioxidant level (Fig.3.11-3.12).
This may be attributed to the generation of the reactive oxygen species in the cells. Previous studies showed that free radical generation occurred during scorpion envenomation [Dousset et al., 2005].

Nitric oxide is an important physiological messenger [Durante et al., 1994] and effector molecule with diverse biological actions [Dawson and Dawson, 1995]. Nitric oxide has been demonstrated to affect mitochondrial functioning by degrading the endomembrane system [Finocchietto et al., 2009] and altering the structure of several ion channels [Leonelli et al., 2009]. Moreover, nitrite is a potent reactive intermediate and in presence of superoxide, can form peroxynitrite [Liaudet et al., 2009]. All these probable mechanisms can lead to deleterious consequences on the cellular health.

Induced nitric oxide triggers apoptosis by mitochondrial permeability transition leading to release of apoptogenic factors from mitochondria which can induce chromatin condensation, DNA fragmentation [Hortelano et al., 1997] and caspase activation [Brune et al., 1998]. The primary product of nitric oxide metabolism, nitrite was assayed in SH-SY5Y and MCF-7 cells following venom treatment. As shown in Fig.3.10 crude venom studied in this thesis elevated the production of nitric oxide in treated cells which supports its role in inhibition of neuroblastoma and breast cancer cells.

Nitric oxide has been reported by others to induce apoptosis in neuroblastoma [Moriya et al., 2000; Dhakshinamoorthy et al., 2007] and hippocampal neuronal cells [Sajad et al., 2009]. Moreover, studies demonstrated that induction of inducible nitric oxide synthase plays an essential part in tumor necrosis factor-a-induced apoptosis in MCF-7 [Binder et al., 1999; Mooney et al., 2002].

Elevated nitrite level can damage the biomembrane structure including the mitochondrial membranes, and facilitate the formation of permeability transition pore [Vieira and Kroemer, 2007], causing the leakage of pro-apoptotic factors such as the cytochrome-c in the cytoplasm [Caroppi et al., 2009]. Mitochondrial permeability transition (MPT) has been identified as a central point for cellular death by the opening of protein pores, which
allows cytosolic and matrix solutes up to 1.5 kDa to equilibrate across inner membrane. This can induce early events in apoptotic cascade [Lemasters et al., 2002; Kinsey et al., 2007].

In order to evaluate the mitochondrial involvement in the venom induced rise in nitrite level, a cationic dye JC-1 with dual fluorescence properties was used. Scorpion venom was found to induce depolarization in mitochondria of SH-SY5Y and MCF-7 cells in a dose-dependent manner. These results were in close agreement with increment of nitric oxide production in treated cells. This study support the earlier finding where bee and scorpion venoms have been reported to induce apoptosis in breast and neuroblastoma cells respectively by inducing the generation of reactive oxygen species and causing dysfunction of the mitochondrial membrane potential [Ip et al., 2008].

Caspases are a family of proteinases that mediate cell death and caspase-3 is one of the critical enzymes of apoptotic pathway [Miura et al., 2004]. Caspase-3 is an intracellular cysteine protease that exists as a pro-enzyme, becoming activated during the cascade of events associated with apoptosis [Zaric et al., 2010]. The presence of caspase-3 in cells of different lineages suggests that caspase-3 is a key enzyme required for the execution of apoptosis [Wimmer et al., 2004]. This study reports that the venom of scorpions enhanced the activity of caspase-3 in SH-SY5Y and MCF-7 which was clearly marked after 24 hr of treatment by direct estimation of its activity. Previous studies also showed NO dependant apoptosis of HeLa [Gao et al., 2005], MCF-7 cells [Benjamin et al., 1998] and neuronal cells [Dhakshinamoorthy et al., 2007] with increased caspase-3 activity and loss of mitochondrial membrane potential [Moriya et al., 2000].

During apoptosis, in majority of cells, endonucleases produce double-stranded breaks generating DNA fragments [Khodarev et al., 1998] which is considered a hallmark of apoptosis.

Results of agarose gel electrophoresis presented two fragments in treated SH-SY5Y and MCF-7 cells exposed with 50μg/ml of *A. crassicauda*, but exposure at 100μg/ml did not
induce DNA damage in either of the two cell lines. However, at the two dose levels B. saulcyi venom induced DNA fragmentation in the two cell lines. In case of O. doriae, 50μg/ml of venom induced DNA fragmentation in both cell lines, but with 100μg/ml only MCF-7 cells expressed DNA fragments whereas SH-SY5Y cells didn’t (Fig. 3.15). This can probably be due to the acute necrosis which may elevate caspase-3 sharply [Cole and Perez-Polo, 2004] without inducing DNA fragmentation. The morphological analysis confirmed this. It revealed severely swollen cells with lesser apoptotic bodies following incubation with 100 μg/ml venom. The venoms at lower dose seem to inhibit growth of cells by triggering apoptosis but at higher dose, viability of cell decreased due to induction of acute necrosis.

Inhibition of cell proliferation is considered as a basic parameter in cancer studies [Van Heusden et al., 1997]. A range of techniques have been evolved to quantify this process [Boulton et al., 1995; Van Heusden et al., 1997]. To confirm that venom can inhibit DNA synthesis in proliferating breast cancer cells, BrdU incorporation was checked with immunocytochemistry.

BrdU is a halogenated derivative of thymidine that is incorporated into DNA only during S-phase of the cell cycle [Van Heusden et al., 1997] and used for assessment of cell proliferation. Scorpion venoms decreased the number of nuclei undergoing DNA synthesis in neuroblastoma and breast cancer cells (Fig.3.16-3.19). In earlier studies also, scorpion venom has been reported to hamper proliferation of cells such as the prostate cancer cells [Zhang et al., 2009], and human leukemia cells [Das Gupta et al., 2007]. Bee venom can also inhibit proliferation by arresting the S-phase in MCF-7 cells as shown elsewhere [Ip et al., 2008].

In conclusion, results of this study suggest that venom of A. crassicauda, B.saulcyi and O. doriae arrest DNA synthesis and induce apoptosis in human neuroblastoma and breast cancer cells through induction of various pro-apoptotic mediators such as the nitric oxide and caspase-3. Nitric oxide induced apoptotic effect through depolarization of mitochondrial membrane, triggering mitochondrial permeability transition and liberation
of apoptogenic factors in the cytoplasm, causing an increase in caspase-3 activity. Caspase-3 induced DNA fragmentation in cells is hallmark of apoptosis (Fig.3-20). The results of these studies highlight the apoptogenic and cell growth arresting properties of the venom of *A. crassicauda, B. saulcyi and O. doriae*. The study implication in identifying selected peptides that might selectively induce death in cancerous cells.

![Schematic diagram depicting the probable mechanism of action of the crude venom in SH-SY5Y and MCF-7 cells. It might act by inducing the reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Nitric oxide depolarizes the mitochondria, and can lead to the formation of permeability transition pore and leakage of pro-apoptotic factors, resulting in increased caspase-3 activity. Together ROS and RNI can lead to the fragmentation of nuclear DNA and trigger apoptosis thereof.](image_url)

**Fig.3.20:** Schematic diagram depicting the probable mechanism of action of the crude venom in SH-SY5Y and MCF-7 cells. It might act by inducing the reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Nitric oxide depolarizes the mitochondria, and can lead to the formation of permeability transition pore and leakage of pro-apoptotic factors, resulting in increased caspase-3 activity. Together ROS and RNI can lead to the fragmentation of nuclear DNA and trigger apoptosis thereof.