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1 Chemicals, antibodies and reagents

DMEM-F12 (Gibco, USA), DMEM (Sigma–Aldrich, USA), Trypsin-EDTA (Sigma–Aldrich, USA), Fetal bovine serum (Gibco, USA), Penicillin-Streptomycin Solution (Sigma–Aldrich, USA), Antibiotic-antimycotic (Invitrogen, USA), PBS (Gibco, USA), Phenol red (Sigma–Aldrich, USA), Trypan blue (Sigma–Aldrich, USA), DMSO (Dimethylsulfoxide)(Sigma–Aldrich, USA), In vitro Toxicology Assay kit: Lactic Dehydrogenase based (Sigma–Aldrich, USA), Caspase-3/CPP32 Colorimetric assay kit (Biovision, USA), JC-1[5, 5’, 6, 6’-tetrachloro-1, 1’, 3, 3’-tetraethylbenzimidazolylcarbocyanine iodide] (Sigma–Aldrich, USA), Triton X-100 (Sigma–Aldrich, USA), BrdU [5-Bromo-2’-Deoxyuridine] (Sigma–Aldrich, USA), DAB [Diaminobenzidine] (Sigma–Aldrich, USA), primary monoclonal anti bromodeoxyuridine (BrdU) antibody (Sigma–Aldrich, USA), NGS (Normal goat serum) (Gibco, USA), Anti-mouse IgG (Fab specific)-peroxidase antibody produced in goat (Sigma–Aldrich, USA), Fixative (Sigma–Aldrich, USA), Harris modified Hematoxylin (Sigma–Aldrich, USA), Agarose (Sigma–Aldrich, USA), Griess reagent (Sigma–Aldrich, USA), PBS [pH 7.4] (Gibco, USA), High-quality Trypsin (BioChemicals, USA), Acrylamide (Sigma–Aldrich, USA), Ampholyte (Fluka, USA), Syringe filter (Sigma– Aldrich, USA), U Bottom 96-wells plate (Sigma–Aldrich, USA), U Bottom 24-wells plate (Sigma–Aldrich, USA). All the other chemicals and reagents were of analytical grade and purchased locally.

2 Collection of scorpion, the milking and storage of venom

2.1 Collection and identification of scorpions

For this study, live scorpions belonging to *Androctonus crassicauda* (Olivier, 1807) and *Buthotus saulcyi* (Vachon, 1949) were collected from Bagh Malek city of Khozestan province, and *Odontobuthus doriae* (Thorell, 1876) were captured from Baghersher city of Tehran province in Iran (Fig. 2.1).

*O. doriae* is an excavator scorpion and usually rests in underground habitat [Ferzanpaee, 1988]. *A. crassicauda* and *B. saulcyi* are undigging scorpions [Ferzanpaee, 1988] and usually seen under stones, dead leaves and holes in the walls. These are nocturnal animals and at nights of suitable seasons (summer and spring) will come out from their shelter to capture prey and reproduce.

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Scorpion fluoresces under UV light. Long wave ultraviolet light is reflected as visible light in the green range [Cloudsley-Thompson and Constantinou, 1983]. This characteristic was used to capture scorpions during the night using ultra violet light (395 nm) [Lowe et al., 2003].

*A. crassicauda* and *B. saulcyi* were captured by searching their probable shelters in day (Fig.2.2) and using ultra violet black light at night [Cloudsley-Thompson and Constantinou, 1983].

The *O. doriae* scorpions were captured after recognition of their shelters through finding of external holes, filling of nests with water and excavation to find out animals (Fig.2.3). Captured scorpions were separately transferred to lab by using dishes or paper envelops (Fig.2.4).

The scorpions were identified by systematic identification keys [Dehghani and Valaie, 2004] and were individually kept in glass containers and suitable condition (25°C, 60-70% humidity and 12 hr length of day) [Zargan et al., 2001, Zargan et al., 2002, Zargan et al., 2003] for more than one year (Fig.2.5). The scorpions were fed with live housefly larva, flour beetle larva, and locust every 10-15 day.
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*Fig. 2.2:* A kind of habitat of *A. crassicauda* and *B. saulcyi* scorpions- Iran (arrow)

*Fig. 2.3:* Capture of excavator scorpions: A, Recognition of habitat of excavator scorpions, B, External holes of excavator scorpion nest, C, Filling of scorpion nests with water and exploration of scorpion shelter, D&E, excavation to find out of the scorpion.
Fig. 2.4: Transfer of scorpions by dish (A) or paper envelop (B)

Fig. 2.5: Housing the captured scorpion and feeding on live insect. A, Cage for housing the live scorpions, B, housefly larva, C, larva of flour beetle

Fig. 2.6: Milking venom from *O. doriae* by electrical simulation
2.2 Milking the venom
Crude venom was obtained from the live scorpion monthly by mild electrical simulation (20V, 500mA) of telson (Fig.2.6). Afterward, the venom was solublized in sterile double distilled water, and centrifugation at 10,000 rpm for 15 min at 4°C to separate and settle down the mucous material. The supernatant was immediately freeze dried and stored at in deep freezer at -20°C until further use [Borges et al., 2006].

2.3 Preparation of working solution of venom for analysis and investigations
The venom was reconstituted in serum free media without phenol red. The protein content of sample was estimated by Bradford method [Bradford, 1976]. Venom was disinfected with 1.5-2% antibiotic-antimycotic solution (Invitrogen, USA) and kept in 4°C until used.

3 Cell culture
Human neuroblastoma (SH-SY5Y) and breast cancer (MCF-7) cell lines were used throughout the study. The cell lines were purchased from the national facility for animal tissue and cell culture (NCCS), Pune, India.

SH-SY5Y and MCF-7 were cultured in Dulbecco’s modified Eagle’s medium–nutrient mixture F12 ham (DMEM-F12) [Klegeris and McGeer, 2003] and DMEM [Czeczuga-Semeniuk et al., 2004] medium respectively, supplemented with 10% heat inactivated fetal bovine serum, 10μl/ml penicillin-streptomycin solution or 1% antibiotic-antimycotic solution in 25 and 75 ml plastic flasks. Cells were grown at 37°C with 5% CO₂ in a humidified incubator. The medium was changed three times a week.

The cell lines were passaged when the cells reached approximately 75-85% confluence. For passage or using the cells for assay, cultures of SH-SY5Y and MCF-7 were rinsed 2 times with 3 ml PBS (pH 7.4) and exposed with 1-1.5 ml 0.05% Trypsin containing 0.02% EDTA for 2 and 5 min, respectively. Cultures were harvested and pelleted by centrifugation at 1,200 rpm for 5 min. The cells were immediately re-suspended in 2-3ml
fresh medium and monitored for cell number by counting the cells with a hemocytometer using an inverted microscope (Nikon, Japan).

For new passage, $10^4$ round shape cells were usually seeded in new plastic flask, where the round cells switched to polygonal shape after overnight (Fig.2.7).

For treatment with venom, cells were seeded in sterile 12, 24 or 96-well plate with care taken to keep the cell number almost equal in all the wells. Following overnight incubation ($37^\circ\text{C}$ and 5% CO$_2$), medium was aspirated and incubated with various concentration of venom. The seeding density was different in different tests as indicated therein.

**Fig.2.7:** The majority of round shape of SH-SY5Y and MCF-cells after seeding in flask or well plate switched to polygonal shape. A, Round SH-SY5Y cells, B, polygonal shape of SH-SY5Y, C, Round shape of MCF-7, D, Polygonal shape of MCF-7.
4 Assessments of cytotoxicity, apoptotic and anti-proliferative effect of the venom

For the assessment of cytotoxicity, apoptosis and anti-proliferative activity of the venom, both crude venom and the isolated fractions of the venom were used.

4.1 The cytotoxicity assays

4.1.1 Determination of cell viability (MTT reduction)

Cytotoxicity of the crude venom and the isolated fractions was carried out by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction assay [Mosmann, 1983]. This assay was applied for three different set of experiments as given under:

A: Determination of cell viability after exposure of cells with different concentration of crude venom (10, 25, 50, 100 and 200 μg/ml).

B: Determination of cell viability after exposure of cells with (10 μg/ml) isolated fractions of the venom to detect the most toxic fraction.

C: Determination of cell viability following exposure of cells with (5, 10, 15 and 20 μg/ml) selected fractions of venoms to elucidate dose-response and estimation of IC₅₀.

For MTT assay, cells were seeded in 96-well plate at a density of 2×10⁴ cells per well and incubated overnight. Media was removed and cells exposed to medium containing venom for 24 hr. 20μl of MTT stock solution (5mg/ml) was added to each well. This was followed by incubation for 1 hr. When purple colored precipitates were visible under the microscope (Fig.2.8), media was carefully discarded. For solubilization of formazan crystals (MTT formazan), 200μl of DMSO (Dimethylsulfoxide) was added to each well and cells were incubated in dark at room temperature for 2 hr. The color (purple) developed was measured at 570 nm by a microplate reader (Bio-Rad, U.S.A).
4.1.2 Lactate dehydrogenase assay

Release of Lactate dehydrogenase (LDH) was analyzed as a marker for cell membrane integrity [Decker and Lohmann-M., 1988] in order to confirm the results of MTT reduction assay. This assay was carried out for three different set of experiments as given under:

A: Assessment of cytotoxicity after exposure of cells with different concentration of crude venom (10, 25, 50 and 100 µg/ml)

B: Assessment of cytotoxicity after exposure of cells with (10 µg/ml) isolated fractions of venom

C: Assessment of cytotoxicity after exposure of cells with (5, 10, 15 and 20 µg/ml) selected fractions of venom

Cells were seeded in 96-well plate at a density of $2 \times 10^4$ cells/well in culture medium. After overnight incubation, medium was replaced and cells were exposed to venom. Cells were incubated for 24 hr and LDH activity was measured in the cell lysate and
supernatant using *in vitro* toxicology assay kit (Sigma) in accordance with the manufacturer’s instructions. The absorbance was determined at 490 nm using plate reader.

### 4.1.3 Analysis of cell morphology

The round cells were seeded in 96-well plate at a density of $2 \times 10^4$ cells/well and exposed to 50 and 100 μg/ml of crude venom, and 10 μg/ml of selected fraction. In another set of experiments, round cells were seeded in similar density ($2 \times 10^4$ cells/well) and incubated overnight. Medium was replaced and polygonal cells were exposed to above concentration of venom. Morphological alterations of round and polygonal cells were monitored during 24 hr incubation period at 37°C and 5% CO$_2$ using an inverted microscope (Nikon, Japan).

### 4.2 Apoptosis assays

#### 4.2.1 Effect of oxidative stress

##### 4.2.1.1 Reactive nitrogen species assay

Nitrite production was determined in the supernatant of cultured cells [Ding et al., 1988]. The cells were seeded in 96-well plate at density of $2 \times 10^4$ cells/well. Cells were incubated overnight, thereafter media was discarded and cells were exposed to medium containing crude venom (50, 100 μg/ml) and selected fractions (10μg/ml). After 24 hr, media from each well was transferred to fresh tube. After centrifugation at 500×g for 5 min at 4°C, 100μl of the supernatant was transferred to a fresh 96-well plate, mixed with an equal volume of Griess reagent (0.04 g/ml in PBS, pH 7.4) and incubated at room temperature for 10 min.

The absorbance was measured within 30 min at 540 nm by a microplate reader (Bio-Rad, U.S.A). Nitrite concentration in control and treated cells was calculated using sodium nitrite standard reference curve and expressed as μM/ml.
4.2.1.2 Reduced glutathione (GSH)

Total reduced glutathione (GSH) was measured following the method of Sedlak and Linsay [1968]. Cells were seeded in 12-well plate at a density of $5 \times 10^5$ cells/well in culture medium. After overnight incubation, culture medium was replaced with medium containing 50 and 100 $\mu$g/ml of crude and 10$\mu$g/ml of selected fractions for 24 hr. After harvesting, the cells were washed with PBS (pH 7.4) and pelleted by centrifugation at 5000$\times$g for 5 min at 4°C. The cells were freeze fractured by incubation in -20°C for 30 min and were resuspended in 200$\mu$l chilled cell lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1mM EDTA and 1% Triton X-100 for 30 min at room temperature. Thereafter, cell lysates were sonicated for 10-15 min and centrifuged at 2000$\times$g for 10 min and supernatant was collected. After estimation of protein by Bradford assay [Bradford, 1976], an aliquot of supernatant was deproteinized by adding an equal volume of 10% TCA and was allowed to stand at 4°C for 2 hr. The contents were centrifuged at 500$\times$g for 15 min and supernatant was collected. 20$\mu$l sample was mixed with 75 $\mu$l lysis buffer, 55 $\mu$l Tris buffer (pH 8.5) containing 0.02 M EDTA and 25 $\mu$l DTNB [5, 5’ Dithio Bis (2-N Benzoic acid)]. Absorbance of the chromogen (yellow) was read at 412 nm by a microplate reader [Sajad et al., 2010]. The result was expressed as $\mu$g GSH/mg protein using molar extinction coefficient of 13600.

4.2.1.3 Catalase activity

Catalase activity in the cell lysates was assayed as per Sinha [1972]. Cells were seeded in 12-well plate at a density of $5 \times 10^5$ cells/well in culture medium. After overnight incubation, medium was replaced with another medium containing 50 and 100 $\mu$g/ml of crude and 10$\mu$g/ml of selected fractions for 24 hr. After harvesting, the cells were pelleted by centrifugation at 5000$\times$g for 5 min at 4°C. Thereafter, cells were freeze fractured by incubation in -20°C for 30 min following incubation with 200 $\mu$l lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1mM EDTA and 1% Triton X-100] for 30 min at room temperature. Lysates were sonicated for 10-15 min, centrifuged at 2000$\times$g for 10 min and supernatant was collected. After estimation of protein by Bradford assay
[Bradford, 1976], in an eppendorf tube 5µl of sample/well was mixed with 50µl of lysis buffer, 20µl of distilled water and 25µl of hydrogen peroxide (15%). After shaking, the samples were incubated for 2 min at 37°C and mixed with 100µl of dichromate acid reagent (0.1M potassium dichromate in glacial acetic acid) following heating in a water bath at 100°C for 10-15 min until the color of the sample was changed to greenish/faint greenish. 200µl/well of sample was transferred into flat bottomed 96-well plates and absorbance was read at 570 nm in a plate reader [Sajad et al., 2010]. The results were converted into activity using molar extinction of catalases as 43.6 and express as micro moles of hydrogen peroxide consumed/min/mg protein.

4.2.2 Apoptosis effects

4.2.2.1 Assessment of Mitochondrial Membrane potential (ψm)
Mitochondrial membrane potential was measured with specific cationic dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) [Smiley et al., 1991]. Briefly, cells were seeded in 12-well plate at a density of 1×10^6 cells/well and incubated overnight. Medium was replaced and cells were exposed to 50 and 100 µg/ml of crude and 10µg/ml of selected fraction for 24 hr. The harvested cells were incubated in 0.5 ml JC-1 (10µM) for 8 min at room temperature in dark. After centrifugation for 5 min at 500×g and washing twice by PBS (pH 7.4) to remove unincorporated dye, pellets were re-suspended in 2ml PBS (pH 7.4). Red fluorescence (excitation 570 nm, emission 610 nm) and green fluorescence (excitation 490 nm, emission 535 nm) were measured using a spectrofluorimeter (spectrometer LS50B, Perkin Elmer). The mitochondrial membrane potential was estimated as the ratio of green to red fluorescence.

4.2.2.2 Measurement of Caspase-3 activity
Caspase-3 activity was evaluated using a commercially available Caspase-3/CPP32 Colorimetric assay kit (Biovision) according to the manufacturer’s instruction. Briefly, cells were seeded in 12-well plate at a density of 1×10^6 cells/well overnight. Medium was discarded and cells were exposed to different concentrations of crude (50 and 100 µg/ml)
venom or 10μg/ml of selected fraction for 24 hr. After harvesting, cells were washed with PBS (pH 7.4) and pelleted by centrifugation at 5000×g for 5 min at 4°C. The cells were re-suspended in 100μl chilled cell lysis buffer [10mM Tris, 1mM EDTA, and 1% Triton X-100; pH 7.4] and incubated on ice for 20 min. Aliquots were analyzed for protein [Bradford, 1976], and lysate volume equivalent to 50 μg of protein was brought to 100μl with lysis buffer in 96-well plate and mixed with reaction buffer (containing 10mM DTT). All the samples were incubated for 1 hr at 37°C after adding 5μl of 4 mM DEVD-pNA (200μM). The absorbance was read at 405 nm in microplate reader (Bio-Rad U.S.A).

4.2.2.3 DNA fragmentation analysis

4.2.2.3.1 Electrophoresis DNA fragmentation

Cells were seeded at a density of 5×10^4 - 4×10^6 cells/well in 24-well plate and incubated overnight. Thereafter media was carefully decanted and cells were exposed to 500μl medium containing 50 and 100 μg/ml of crude and 10μg/ml of selected fractions. After incubation for 24 hr, the cells were harvested and centrifuged at 500xg for 5 min at 4°C. Pellet was then washed twice with 0.5 ml TE buffer (Tris- EDTA). MCF-7 cells were lysed in 50 μl of chilled DNA lysis buffer for 30 min on ice. Lysis buffer for MCF-7 contained 0.5% Triton X-100, 25 mM EDTA and 25 mM Tris-HCl, pH 7.4 and for SH-SY5Y, 0.5% Triton X-100, 20mM EDTA and 5mM Tris-HCl, pH 8.0.

Extraction of DNA was carried out by adding 50μl of 0.1mg/ml proteinase K, 150mM NaCl and 0.2% (w/v) SDS and incubated at 50°C for 3hr. Nucleic acid was extracted by adding equal volume of solution containing phenol/chloroform/Isoamyl alcohol (25:24:1) [Kweon et al., 2004]. After centrifugation at 1000×g for 5 min at 10°C, supernatant was transferred to a fresh tube. DNA was precipitated by two volumes of cold absolute ethanol (Fig.2.9) and pelleted by centrifugation (20000×g for 30 min at 4°C). Supernatant was carefully discarded by rapidly inverting tubes and DNA was washed twice by ice-cold 70% ethanol. It was then air dried for 5-10 min and mixed in 50 μl TE buffer containing RNase (0.2mg/ml). After incubation at 55°C for 1 hr, DNA was stored at -
20°C until use. The extracted DNA was analyzed by loading 10-20 μg into 1.5% agarose gel containing ethidium bromide (1 μg/ml) by UV transilluminator (Nugen Scientific, India with UV Photo MW version 11.01 for windows).

![Image of DNA fragmentation assay](image.png)

**Fig.2.9**: DNA fragmentation assay. A, Electrophoresis chamber, B, Power supply, C, DNA extracted from the cells, D, The band appeared after loading 10-20 ng of control DNA onto a 1.5% agarose gel containing ethidium bromide

### 4.2.2.3.2 In situ Tunnel assay

Tunnel assay was performed as described previously [Moshiri et al., 2009] after minor modification. Cells were seeded in 24-well plate at a density of $2 \times 10^4$ cells/well in culture medium. After overnight incubation, culture medium was replaced with medium containing 10μg/ml of selected fraction of venom. After incubation for 24 hr, the cells were washed with PBS (pH 7.4) and fixed using 4% paraformaldehyde in PBS for 30 min and rinse twice for 30 sec each time in PBS. Next, cells were incubated in methanol containing 0.3% H$_2$O$_2$, followed by 20 min incubation in terminal transferase buffer. Cells were incubated for 1 hr at room temperature in 300 U/ml terminal transferase enzymes and 10nM, dNTP-BrdU labeling mixture in terminal transferase buffer and then incubated in terminal transferase stop solution for 10 min [Gavrieli et al., 1992].
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Cells were rinsed in PBS (pH 6.0), incubated for 30 min at room temperature in blocking buffer (1.5% NGS, 0.5% BSA and 0.1% Triton x-100) and then incubated overnight at 4°C in rabbit monoclonal anti- BrdU system.

Cells were washed twice with PBS (pH 7.4) and immediately incubated with 1:200 anti-mouse secondary antibody for 1 hr. Cells were washed two times with PBS (pH 7.4) and peroxide complex was visualized with DAB (3,3'-Diaminobenzidine tetrahydrochloride) and briefly counterstained using hematoxylin (10s).

The brown positive stained cells were photographed with an inverted microscope (Nikon, Japan). For analysis of TUNEL-immunoreactive cells, a person blind to the experimental protocol was employed for counting. Cells were counted using Image J software with cell counting jar (National Institutes of Health, U.S.A). The numbers of TUNEL-labeled cells were averaged from six different photomicrographs in three replicate wells. For data analysis, numbers were expressed as percentage of TUNEL (+) cells per photograph.

4.3 Assessment of the anti-proliferative effect by immunocytochemistry

Cells were seeded in 96-well plate at density 2×10⁴ cells/well and incubated overnight. Cells were exposed to 50 and 100 µg/ml of crude and 10µg/ml of selected fractions. Cells were incubated for 22 h and pulsed with 10µM [Dover and Patel, 1994] 5-bromo-2'-deoxyuridine for 2 hr. Medium was carefully discarded and the cells were subsequently washed twice with PBS (pH 7.4) and dehydrated using ethanol. Optimal fixation of cells was achieved by 4% paraformaldehyde for 30 min and after washing twice with PBS (pH 7.4) containing 1N HCl on ice for 10 min and followed by 2N HCl for 10 min at room temperature. After brief rinsing in PBS (pH 6.0), cells were incubated in methanol containing 0.3% H₂O₂ for 15 min and followed by incubation in blocking buffer (1.5% NGS, 0.5% BSA and 0.1% Triton x-100) for 30 min. Finally cells were incubated with monoclonal anti-BrdU antibody (1:100) in 10% NGS at 4°C overnight. Cells were washed twice with PBS (pH 7.4) and immediately incubated with 1:200 anti-mouse secondary antibodies for 1 hr with constant shaking at room temperature. Cells were
washed two times with PBS (pH 7.4) and peroxide complex was visualized with DAB (3,3'-Diaminobenzidine tetrahydrochloride). Cells were briefly counterstained using Basic Fuschin (0.1%) in ethanol or hematoxylin. After dehydration in ethanol (70%, 80%, 90% and 95%), brown positive stained cells were photographed with an inverted microscope (Nikon, Japan). For analysis of BrdU-immunoreactive cells, a person blind to the experimental protocol was employed for counting. Cells were counted using Image J software with cell counting jar (National Institutes of Health, U.S.A). The numbers of BrdU-labeled cells were averaged from seven different photomicrographs in three replicate wells. For data analysis, numbers were expressed as percentage of BrdU (+) cells per photograph.

4.4 Statistical analysis
All the data presented are mean ±SEM. Analysis between the groups was carried out with one way ANOVA (analysis of variance) with post hoc analysis by Tukey Kramer multiple comparison method. Any variation with p<0.05 was considered to be significant.

5 Determination of LD$_{50}$

The subcutaneous LD$_{50}$ value for crude venoms of *A. crassicausa*, *B. saulcyi* and *O. doriae* and three selected polypeptide fractions was determined in mice (Swiss albino). The lyophilized venom and fractions were dispersed in 0.9% (w/v) sodium chloride solution [Batina et al., 1997], cleared by centrifugation at 10,000 and 3,000 rpm respectively for 10 min at 4°C. An aliquot of the solutions were assayed for total protein by Bradford method [Bradford, 1976]. Different concentration from each sample (Table 2.1) was prepared by adding 0.9% sodium chloride solution.

Five healthy mice, 20±1 g, 6 weeks old [Ozkan et al., 2006] were used for each concentration of venom. Treated and control groups were subcutaneously (Fig.2.10) injected with 100µl of prepared sample and 0.9% sodium chloride solution respectively. Animals of each group were separately kept in a mice cage with free access to water and
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dry mice pellet feeds (Fig.2.10) and were observed for 24 hr for toxicity signs and record of the number of dead mice. The dose that killed 50 percent animals (LD<sub>50</sub>) was calculated graphically by the method of Miller and Tainter [1944] and also Arithmetic method adapted by Alui and Nwude [1982].

Table 2.1: Groups of mice for determination of LD50 and subcutaneous injected dose of crudes and selected fractions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration of sample (µg/g weight of mouse)</th>
<th>Control</th>
<th>Treated groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Crude venom</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. crassicauda</td>
<td></td>
<td>0</td>
<td>0.100</td>
</tr>
<tr>
<td>B. saulcyi</td>
<td></td>
<td>0</td>
<td>0.250</td>
</tr>
<tr>
<td>O. doriae</td>
<td></td>
<td>0</td>
<td>0.025</td>
</tr>
<tr>
<td>Selected fractions</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F17 of A. crassicauda</td>
<td></td>
<td>0</td>
<td>0.050</td>
</tr>
<tr>
<td>F7 of B. saulcyi</td>
<td></td>
<td>0</td>
<td>0.125</td>
</tr>
<tr>
<td>F5 of O. doriae</td>
<td></td>
<td>0</td>
<td>0.100</td>
</tr>
</tbody>
</table>

Fig.2.10: Determination of LD<sub>50</sub> of crude venom of A. crassicauda, B. saulcyi and O. doriae and their selected fractions in Swiss albino mice. A, The Swiss albino strain of mouse, B, Treated and control groups were subcutaneously injected with 100µl of toxin sample and 0.9% sodium chloride solution respectively. Mice of each group were separately kept in a mice cage with free access to water and dry mice pellet feeds.
6 Proteomics of scorpion venom and their fractions

6.1 Electrophoresis

6.1.1 SDS-PAGE
Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) purified fractions of scorpion venom in reducing condition was carried out according to the method of Laemmli [1970] to determine the protein profile [Caliskan et al, 2009].

For electrophoresis, freeze dried venom and fractions were solubilized in deionized water and the remaining insoluble material, if any, was removed by spinning at 14,000×g for 10 min at 4°C [Caliskan et al., 2006]. Protein concentration was assessed by the Bradford method, using bovine serum albumin as standard [Bradford, 1976]. Different aliquot of samples were stored at -20°C until use.

Polyacrylamide gel electrophoresis was performed in 15% separating [Ucar and Tafi, 2003, Badhe et al., 2007] and in 4% stacking gels. In each run 10μl of sample containing (15-20μg protein) was diluted in 1:1 ratio with the sample buffer which contained 6% of 1 M Tris-HCl pH 6.80, 0.1% Bromophenol Blue, 2% SDS, 50% glycerol, and 5% of 2-mercaptoethanol. After heating at 95°C for 4 min [Ucar and Tafi, 2003], 20 μl of sample and protein molecular weight marker (3.6-205 kDa) were vortexed, cleared of any precipitates and loaded into the wells. Electrophoretic separations were performed at a constant voltage of 120 V at room temperature until the blue dye front reached the bottom of gel [Caliskan et al, 2009].

After electrophoresis, gel slabs were stained in Coomassie brilliant blue and silver staining to allow visualization of the protein bands. Molecular weight of each band was estimated according to method of Hames and Rickwood [1990] by plotting log molecular weight versus relative mobility (Rf=distance migrated by protein/distance migrated by dye) for a group of protein molecular weight marker.
6.1.2 Tricine-SDS-PAGE

Tricine-SDS-PAGE was performed for protein profiling of the crude venom in 1D and 2D according to method of Schagger and von Jagow [1987] modified by Lesse et al. [1990]. Tricine-SDS-PAGE was carried out in 12% separating and 4% stacking gel and electrophoretic separation was conducted in discontinuous buffer system (cathode buffer: 0.1 M Tris, 0.1 M Tricine, 0.1 % SDS at pH 8.25 and anode buffer: 0.2 M Tris- HCL at pH. 8.9) [Alpagut K. and Koç, 2006].

Samples (10 μl contain 15-20 μg protein) were prepared by mixing an equivalent volume of tricine sample buffer (12% SDS, 6% mercaptoethanol, 30% glycerol, 0.05% Coomassie blue G-250, 150 mM Tris-HCl pH 7.0) [Schagger, 2006] and denatured by heating at 95°C for 5 min.

The samples and protein molecular weight standard (3.6-205 kDa) after vortex were loaded into the wells. Electrophoresis was maintained at constant current 15 mA until the samples migrated through the stacking gel at 30 mA for the remainder of the run and until the tracking dye reached the bottom of separating gel. The gels were stained with silver staining. Then photographs of the gels were taken and molecular weights of the sample bands were estimated according to method of Hames and Rickwood [1990].

6.1.3 Two dimensional electrophoresis (2-DE)

Proteins profile of scorpions venoms were elucidated by Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) according to the method of O Farrell [1975] that separated the proteins according to net charge (isoelectric points or pl) by isoelectric focusing (IEF) in the first dimension, and size by SDS or Tricine SDS-PAGE in the second dimension. In this study, separation of proteins in first dimension was evaluated by using ampholytes and placing the native and denatured venom in a pH gradient generated by an electric field.
6.1.3.1 Native isoelectric focusing (Native IEF)

Isoelectric focusing of the venoms in native condition was carried out according to the reported procedure [Bollag et al., 1996] with some modification. Samples (10 µl contain 15-20 µg proteins) were prepared by mixing an equivalent volume of sample buffer (60% glycerol, 0.66% ampholyte pH 3-10, 3.34% ampholyte pH 6-8, 36% deionized water). The selected native IEF gels [containing 16.6% gel monomer (30% acrylamide, 1% bis- acrylamide), 2% ampholyte pH 3-10, 0.4% ampholyte pH 6-8, 20 µl TEMED and 25 µl of 10% APS] [Giulian et al., 1984] were performed in slab mini-gel apparatus with 1.0 mm thick spacers. The polymerized gels were attached to inner cooling core and prepared sample after centrifugation at 10000 x g were loaded into wells. The gels were conducted in discontinuous buffer system (cathode buffer: 20 mM NaOH and anode buffer: 10 mM H₃PO₄). Electrophoresis was maintained at constant voltage of 200 V for 1.5 hr and 400V for next 1.5 hr at room temperature.

After focusing, a strip of gel 0.5 cm wide was cut into 0.5 cm slices. The slices were incubated in 1 ml 10 mM KCl for about 30 min. The pH accuracy of native gel was estimated by reading pH of KCl solution (Fig.2.11). A strip of each sample with 0.5 cm wide was cut from the gel and stained with silver staining to allow visualization of the protein bands. The other strips were subsequently used to estimation of proteins quantity of venom using Tricine SDS-page.

6.1.3.2 Denatured isoelectric focusing (Denatured IEF)

Isoelectric focusing analyses of venoms in denatured conditions were done according to the reported procedure [Bollag et al., 1996] with some modification. The sample (10 µl contain 15-20 µg proteins) were heated in 95°C for 5 min and were mixed with an equivalent volume of denaturing loading sample buffer (8 M Urea, 0.04 Bromophenol, 1% 2-Mercaptoethanol %0.4, 2% Triton -100, ampholyte pH 3-10 and 2% Ampholyte 6-8) and incubated for at least 1 hr for full denaturation and solubilization. The selected denatured IEF gel [contain 16.6% gel monomer (30% acrylamide, 1% bis- acrylamide), Urea 50%(W/V), 2% ampholyte pH 3-10, 0.4% ampholyte pH 6-8, 20 µl, 20 µl TEMED
and 25 μl of 10% APS) were performed in slab mini-gel apparatus with 1.0 mm thick spacers. The polymerized gels were attached to inner cooling core and were conducted in discontinuous buffer system (cathode buffer: 20mM NaOH and anode buffer: 10mM H₃PO₄).

The prepared sample were loaded into wells after pre-running of gel at constant voltage of 150 V for 10 min and at 200 V for 15 min. Electrophoresis of samples was started at constant voltage of 150 V for 30 min and 200V for next 150 min. After focusing, a strip of gel 0.5 cm wide was cut into 0.5 cm slices. The slices were incubated in 1ml 10mM KCl for about 30 min. The pH accuracy of native gel was estimated by reading pH of KCl solution (Fig.2.11). A strip of each sample with 0.5 cm wide was cut from the gel and stained with silver staining to allow visualization of the protein bands. The other strips were subsequently used to estimation of proteins quantity of scorpion venom using Tricine SDS-page.

6.1.3.3 2-DE and loading IEF gel on to a second dimension slab

The loading of IEF gel on to a second dimension slab was carried out according reporter method [Bollag et al., 1996] with some modification.

The second dimension was carried by using Tricine-SDS-PAGE (12% separating, 4% stacking gels and 1mm thickness). The IEF strip gels were incubated in equilibration buffer [5% 2–Mercaptoethanol, 6.25% 1M Tris-HCl (pH 6.8), 2.3% SDS, 10% Glycerol and a few grains of Bromophenol blue (1%)] for 30 min with gentle agitation, followed by immersion in cathode buffer for a few seconds. The strip was gently placed on a wet Whatman-1 filter paper for 10 min to remove excess equilibration buffer and then was placed on a cassette (3 to 10mm below the top).

Separating acrylamide solution was poured into assembled gel plates through side of cassette and followed by pouring of the stacking gel (2 mm under IEF gel) after polymerization of separating gel. The IEF strip was overloaded with 1-2 ml of agarose solution (%0.5 agarose in cathode buffer) to fill of the top of stacking gel and making a well in one side of strip for loading of standard marker (Fig.2.12). The protein molecular...
weight marker (3.6 - 205 kDa) was loaded into the well and electrophoresis was started with 5mA for 1 hr, then 10mA for 1hr, followed by 15-18mA until the tracking dye reached to about 0.5 cm from the bottom of gel. Gel was removed from cassettes and marked at the upper corner nearest the pointed end of the IEF gel to identify the acidic end of the first-dimension separation. The gels were stained with silver staining to visualize protein spots and estimation of protein quality of scorpion venom.

**Fig. 2.11**: pH estimation of IEF Gels. The slices of IEF strip were incubated in 1ml 10mM KCl for about 30 min and pH accuracy of native and denatured strips were estimated by reading pH of KCl solution.

**Fig.2.12**: Two-dimensional gel electrophoresis and loading an IEF gel on to a second dimension slab
6.1.4 Staining of electrophoresis gels

6.1.4.1 Coomassie blue staining
To visualize the protein bands, the gels of SDS page were stained in 0.1% Coomassie brilliant blue R-250 prepared in 40% methanol and 10% acetic acid for 1 hr. The gels were subsequently destained by soaking in a solution of 10% acetic acid 40% methanol, 50% deionized water [Ucar and Tafi, 2003].

6.1.4.2 Silver staining
Silver staining [Switzer et al., 1979] of gel electrophoresis was done according to the reported procedure [Celis et al., 2006] with some modification.

After electrophoresis, Tricine SDS page, IEF and 2DE gels were fixed with prefixation solution (12% trichloroacetic acid) and fixation solution (50% ethanol or methanol, 12% acetic acid and 0.05% commercial formalin (35% formaldehyde) for 1 and 3 hr respectively. After three times washing the gel with 50% ethanol or methanol (in deionized water) for 20 min to remove fixative solutions and remaining detergent, the gel was incubated in Sensitizing solution [0.02% (w/v) sodium thiosulfate (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}) in water] for 1-2 min. Then, gel was washed with deionized water for 1 min and stained by incubation and shaking in staining solution (0.2% (w/v) silver nitrate (AgNO\textsubscript{3}), 0.075% formaldehyde) for 20-30 min.

Gel was washed with deionized water for 1 min and incubated in developing solution [6% (w/v) sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}), 0.0004% (w/v) sodium thiosulfate (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}), 0.05% formaldehyde] and Terminating solution (50% ethanol and 12% acetic acid) for 2-5 min and 10 min respectively. Gel was stored in drying solution (10% ethanol) at room temperature.

6.2 Isolation of the scorpion venom fractions by chromatography
The lyophilized crude scorpion venom was dissolved in 0.1% aqueous trifluoroacetic acid (TFA) containing 5% (v/v) acetonitrile [Corzo et al., 2001] and the insoluble material and
mucous debris was removed by centrifugation at 15000 RPM [Ali et al., 2001] for 20 min [Corzo et al., 2000] at 4°C. The supernatant was filtered through 0.45μm syringe filter, prior to fractionation [Corzo et al., 2001]. The separation of fractions was carried out on reversed-phase semi-preparative high-performance liquid chromatography (HPLC) where the separation was achieved according to the hydrophobicity of the components in the venom. The HPLC system used was CECIL 1100 equipped with a UV Detector using Preparative C18 [SUPELCOSIL™ PLC-18(59185), 25cmx 21.2mm, 12μm] (Fig.2.13).

Fig. 2.13: Isolation and lyophilization of venom fractions. A, Separation of fractions was carried out on reversed-phase semi-preparative HPLC (CECIL 1100). B, Isolated fractions were vacuum dried using lyophilizer.

The column was equilibrated for 15 min in 0.1% TFA, and eluted with a linear gradient program from solution A (0.1% TFA in grade water) to 60% solution B (0.1% TFA in HPLC grade acetonitrile) [Ucar and Tafi, 2003; Soudani et al., 2005] in 60 min at 25°C [Ucar and Tafi, 2003]. The prepared solutions of mobile phase (solutions A and B) were used after filtration through 0.45μm membrane filter and sonication in one hr. The flow rate was 4ml/min and eluent was monitored at absorbance of 220 nm. The fractions corresponding to the peaks which appeared were collected manually and were vacuum dried using lyophilisateur (Fig.2.13). The retention times for each fractions peak were indicated.
6.3 Amino acid sequencing and MALDI-TOF/MS analysis

6.3.1 Excision and de-staining of gel piece

Excision and de-staining of gel piece stained with coomassie dye was carried out according to the procedure earlier reported [Tani et al., 2000; Aulak et al., 2001; Hilton et al., 2001; Lim et al., 2002]. The protein band of selected fraction was excised from 1-D gels and cut into 1x1 - 2x2 mm pieces. The pieces were placed into receiver tube, mixed twice with 200 μl de-staining solution (25 mM ammonium bicarbonate in 50% acetonitrile) and incubated at 37°C for 30 min with constant shaking. De-staining solution was discarded and gel pieces were shrunk by adding 50 μl of acetonitrile followed by 15 min incubation at room temperature. Acetonitrile was carefully removed and gel pieces were air-dried for 5-10 min at room temperature. 10 μl of prepared trypsin solution [1μg/0.1 ml solution of high-quality trypsin (TPCK Treated- BioChemicals) in ultrapure water] was added into tube containing the shrunken gel pieces and incubated at room temperature for 15 min to allow gel pieces to swell and absorb the trypsin solution. 25 μl of digestion buffer was added to the tube and sample was incubated at 37°C for 4 hr with constant shaking. The digestion mixture was carefully transferred into a clean tube.

6.3.2 Amino acid sequencing

MALDI-TOF/MS analysis was performed on an Autoflex mass spectrometer (Applied Biosystems, Tokyo, Japan) using positive acceleration in either linear or reflector mode. Time-to-mass conversion in linear mode was achieved by external calibration using bradykinin (m/z 1061.2), bovine pancreatic b-insulin (m/z 3496.6) and bovine pancreatic insulin (m/z 5734.5) (Sigma) as standards or a peptide mixture as internal calibrant in reflector mode. All experiments were performed using a-cyano-4-hydroxycinnamic acid (Aldrich) as the matrix.