Chapter-III

MATERIALS AND METHODS
TEST ORGANISM

To be able to understand and study the effects of pesticides on aquatic organisms, it is important to choose a representative organism possessing the necessary characteristics with which such exposure studies can be conducted. Fish are representative of the largest and most diverse group of vertebrates on the earth. They represent most of the trophic levels, are found in all but the most polluted surface water bodies and are therefore are the most suitable test organisms for these studies (Powers, 1989). There are a few criteria to which a test organism should ideally adhere to. The organism should be a representative of an ecological, commercial and recreational important group; occupy a position within a food chain leading to man or other important species; widely available and abundant; amenable to laboratory testing; easily maintained; genetically stable; and adequate background data on the organism should be available; and also be able to show sensitivity to the toxicant (USEPA, 1976). For the purpose of this study, *Labeo rohita* was chosen as the representative fish species to be studied.

Classification:

<table>
<thead>
<tr>
<th>Taxonomic Rank</th>
<th>Taxon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Chordata</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Vertebrata</td>
</tr>
<tr>
<td>Division</td>
<td>Gnathostomata</td>
</tr>
<tr>
<td>Superclass</td>
<td>Pisces</td>
</tr>
<tr>
<td>Class</td>
<td>Osteichthyes</td>
</tr>
<tr>
<td>Sub-Class</td>
<td>Actinopterygii</td>
</tr>
<tr>
<td>Super order</td>
<td>Teleostei</td>
</tr>
<tr>
<td>Order</td>
<td>Labeo</td>
</tr>
<tr>
<td>Species</td>
<td>rohita</td>
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</tbody>
</table>
Labeo rohita belongs to the family Cyprinidae, and is commonly known as 'Rohu'. It is the most valuable edible fish found all over India. The head of it is prominent with pointed snout. It grows very quickly to a length of 45cms (about 0.7kg wt) in the first year and maximal growth reaches up to a length of 91cm in three years (Wealth of India, Fish and Fisheries, 1962). It attains sexual maturity when it is around two years old.

**Procurement and maintenance of animals**

Fish weighing 12 ± 2gm were collected from the local fish farm in Anantapur, Andhra Pradesh and were housed in constant aerated 100L glass aquaria containing dechlorinated tap water at 27±2°C under a photoperiod of 12:12hrs light and dark cycles in groups of 10 animals per tank. The fish were fed daily with groundnut cake and acclimated to laboratory conditions for minimum period of ten days. The temperature of water in the aquaria was 28 ± 2°C and the same was maintained throughout the course of the investigation.

**Test Compound-Cypermethrin**

**Technical name:** (RS-a-cyano-3-phenoxybenzyl, IRS, cis, trans-3-(2, 2-dichlorovinyl)-2, 2-dimethylcyclopropanecarboxylate),

**Structure:**

![Structure of Cypermethrin](image)

**Cypermethrin stock solution:** The cypermethrin used for these studies was reported as 99% pure (Sigma, St. Louis, Mo, USA) and the stock solution was prepared by dissolving
20mg in 500μl of absolute alcohol and stored at 4°C. The daily requirement was taken from this solution.

**Experimental design:** *Labeo rohita* fingerlings (n=6) weighted approximately 12±2gm were exposed to three different concentrations (1, 5 and 10μg/L) of technical grade cypermethrin for a period of 96hr in 5L full glass aquaria with constant aeration. Simultaneously control (untreated) and solvent control (treated with solvent alone) animals were maintained in separate tanks. About 50% of the water in the aquaria was changed daily, however the concentration of cypermethrin and absolute alcohol at the desired levels were maintained by the fresh additions accordingly. All the experimental groups (i.e. control, solvent control and treated) were performed in duplicates. During the experimental period the fish were not fed.

**Sample Collection:** At the end of the experiment fish were anesthetised by keeping them in euthanasia solution for a few seconds followed by removal of gills and liver by opening the viscera. The tissues were immediately freeze dried in liquid nitrogen and stored at -20°C until further analysis.

**Sample Preparation:** The frozen gill and liver samples were homogenate in 2.4M sucrose solution with the help of mortar and pestle and it allowed to centrifuge at 10,000rpm for 10min at 4°C. Then the supernatants were collected and stored in -20°C until for further analysis.

**Preparation of euthanasia solution:**

100mg of (tricaine) methanesulfonate salt (MS-222; Sigma) was dissolved in one liter of tank reservoir water.
Estimation of Proteins:

The concentration of proteins was estimated according to Lowry et al., (1951). The protein sample solution (5ul) was taken into a test tube. To this 5ml of alkaline solution was added. Then 0.5ml of diluted folin-Ciocalteu's reagent (1:1) was added rapidly, mixed and allowed for incubation for 30 minutes at room temperature in dark. The colour developed was read at 750nm against reagent blank in a spectrophotometer (UV-Vis 8500, Tech-Comp, Hong Kong made). Protein content was calculated from a curve prepared from bovine serum albumin and expressed as mg/gm fresh weight.

Reagents

1. Solution A

4gm of NaOH and 20gm of Na₂CO₃ were dissolved and made to 100ml with distilled water.

2. Solution B

175mg of CuSO₄.7H₂O and 250mg of sodium potassium tartarate were dissolved and made to 20ml with distilled water. Solution A and B were mixed and the volume was made up to 1000ml with distilled water.

3. Folin-Ciocalteu’s reagent:

The commercial reagent (Merck, India) was diluted with the equal volume of distilled water on the day of use.
Seperation of Proteins by Electrophoresis technique:

Linear Discontinuous SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of proteins on SDS-PAGE was carried out by the method described by Laemmli (1970).

A. Preparation of slab gel (Broviga, India)

1. The glass plate-sandwich was prepared by using clean glass plates and spacers of 1.0mm thickness and sealed by applying grease.

2. The appropriate resolving gel mixture was prepared as follows.

Recipe for 12% resolving gel

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>3.3ml</td>
</tr>
<tr>
<td>30% Acrylamide mix</td>
<td>4.0ml</td>
</tr>
<tr>
<td>1.5M Tris buffer (pH-8.8)</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005ml</td>
</tr>
</tbody>
</table>

3. The TEMED was added lastly to the resolving gel, gently swirled and then poured between the glass plates leaving sufficient space at the top for stacking gel.

4. After polymerization of resolving gel, the stacking gel was poured and kept undisturbed till polymerization.

5. The comb was carefully placed 0.5cm above the resolving gel.

The stacking gel (5%) was prepared as follows.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2.7ml</td>
</tr>
<tr>
<td>30% Acrylamide mix</td>
<td>0.5ml</td>
</tr>
<tr>
<td>0.5M Tris buffer (pH-6.8)</td>
<td>0.3ml</td>
</tr>
</tbody>
</table>

32
10% SDS 0.04ml
10% APS 0.04ml
TEMED 0.005ml

6. The comb was removed carefully and washed wells with the reservoir buffer.
7. The gel was fixed on to the electrophoresis tank.
8. The slab-gel was pre-runned for 5min at low voltage of current using power pack (Applex, France).

Sample Preparation and loading:

1. The known amount of protein was dissolved in sample buffer to a final concentration of 5mg/ml.
2. The samples were incubated in boiling water bath for 3 min and loaded on the gel slots using micropipette along with standard molecular weight protein marker (Genei, Bangalore) ranging from 14.3 to 97.4 kDa were electrophoresed.
3. The samples were spin to remove undissolved materials, if any, before loading.
4. The electrophoresis was carried at 50V initially stacking gel. Once the sample entered into resolving gel the voltage was increased to 120V.
5. Once the tracking dye reached the bottom of the gel, the power supply unit was stopped and the gel was removed carefully and placed it in staining solution.

Detection of proteins by Coomassie Brilliant Blue (CBB) staining:

1. The gel was placed in 5 volumes of CBB staining solution for overnight.
2. The gel was washed with the distilled water and destained using destaining solution till the back ground is clear and appearance of sharp bands (Sambrook et
al., 1996). The electrophoregrams were photographed and analyzed using gel
documentation system (Vilber Lourmat Gel Documentation System, France).

**Reagents:**

1. **30% Acrylamide mix (w.v)**
   
   29gm of acrylamide and 1gm of bisacrylamide was dissolved in 50ml of distilled
   water and made volume to 100ml.

2. **Sodium Lauryl Sulfate (SDS)10% (w/v)**
   
   1gm of SDS was dissolved in double distilled water and made volume to 10ml.

3. **Ammonium persulphate (APS) 10% (w/v)**
   
   100mg of APS was dissolved with distilled water and made volume to 1000ul, this
   was made fresh just before use.

4. **TEMED (N,N,N',N'-tetramethylethylenediamine):** It is used as supplied (Merck)

5. **Stacking gel buffer: 0.5M Tris buffer (pH 6.8)**

6. **Resolving gel buffer (or) Tank Buffer:**
   
   Tris glycine buffer (pH 8.3): 3gm of Tris, 14.4g of glycine and 1gm of SDS was
dissolved in 800ml of distilled water and volume was made up to 1000ml with
distilled water.

   All the solutions were prepared carefully, filtered through watman No.1 filter
   paper.

**Sample buffer:**

Sample buffer was prepared as below.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Tris buffer (pH 8.8)</td>
<td>3.7ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.8ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>
2% β-mercaptoethanol  2.0ul
0.002% Bromophenol blue  2.0ul
Bromophenol blue was used as tracking dye.

Coomassie Brilliant Blue (CBB) R-250 solution (Staining solution)

0.25gm of CBB R-250 (Merck) was dissolved in 45ml of methanol, 45ml of distilled water and 10ml of glacial acetic acid.

Destaining solution:

It was prepared with 40ml of methanol, 45ml of distilled water and 15ml of glacial acetic acid.

Immunoblot Analysis (Semi-dry blot method):

Western blotting:

The proteins separated on SDS-PAGE were transferred onto a solid support PVDF (Polyvinylidene fluoride, Bio-Rad) membrane using semi-dry electroblotting apparatus.

1. After separation of proteins by SDS-PAGE, the gel was presoaked in 100% methonal, then washed with deionized water and then soaked in anode solution 2.

2. Five Petri plates were arranged containing anode solution 1, anode solution 2, cathode solution, and methanol (100%) and distilled water. Then filter papers, membrane and gel were arranged in the following manner.

3. Whatman No.1 filter papers (3 numbers) were soaked in anode solution 1, and then placed on anode graphite plate of western blot apparatus.

4. Then another set of whatman No.1 filter papers (6numbers) were soaked in anode solution 2 and placed on filter papers.
5. Then PVDF membrane was placed on filter papers after pre-soaking in anode solution 2.
6. Then gel was placed on the PVDF membrane and care was taken to avoid air bubbles.
7. Lastly, asset of whatman No.1 filters papers (6 numbers) were soaked in cathode solution and placed on gel.
8. The cathode graphite was carefully placed and kept undisturbed. The transfer was started by switching-on the power pack, kept at 60 mA at room temperature for 1 hr (Towbin et al., 1979).

After transfer, the membrane was taken out carefully and stained it with Ponceau S (Sigma) and recorded the molecular weight marker proteins by using pencil on the membrane. Then the membrane was washed three times with 1X TBST buffer for 5 min each.

**Immunodetection:**

After washing with 1x TBST, the membrane was kept in 10ml of blocking solution containing 1% skimmed milk and placed for 30 min on shaking. Then membrane was washed three times with 1X TBST for 5 min each. The membrane was incubated in monoclonal mouse anti-Hsp70 antibody (Affinity Bioreagents, Golden, CO, USA) overnight (dilution 1:10,000, Clone-3a3, dilution in 1xTBST-0.5% BSA).

Again membrane was washed three times with 1x TBST for 5 minutes each followed by the membrane was incubated with horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin was used as a secondary antibody (Sigma, USA) solution (1:1000 ratio to the 10ml of 0.5% BSA) for 1 hr on shaking in room temperature. Finally the membrane was washed 3 time with 1x TBST for 5 minutes each.
Development:

The membrane was placed in 0.05M Na citrate buffer. A pinch of cobalt metal and 100ul H2O2 was added. To this diamino benzidine (DAB) was added and gently agitated till the appearance of clear bands. The reaction was arrested by adding distilled water to the membrane. Images of the blots were scanned, analyzed and recorded using Gel documentation image analysis system (Vilber Lourmat Gel Documentation System, France).

Preparation of reagents

1. 10x TBS Buffer

12.11 g of Tris-Hcl (100mM) and 86 g of Nacl (1500Mm) was dissolved in 800ml of distilled water, pH was adjusted to 7.5 with HCl, and the total volume was made to one liter.

2. Anode solution-1

36.33 g of Tris-HCl (0.3M) was dissolved in 500ml of distilled water, pH was adjusted to 10.4 with HCl, and with 20% methanol (200ml of 100%) the volume was made to one litre.

3. Anode solution -2

3.0275g of Tris-HCl (25mM) was dissolved in 500ml of distilled water, the pH was adjusted to 10.4 with HCl, and with 20% methanol (200ml of 100%) the volume was made to one liter.
4. Cathode solution

1.244g of amino caproic acid (40mM) was dissolved in 100ml of distilled water and the volume was made to one liter.

5. 10xTBS Buffer

12.11 g of Tris- HCl (100Mm) and 96g of Nacl (1500Mm) was dissolved in 500 ml of distilled water and the pH was adjusted to 7.5 with HCl and the volume was made to one litre.

6. 1x TBST Buffer

100ml of 10X TBS buffer, 500 ul of Tween -20 and 900ml of double distilled water were mixed and used.

7. 1% milk powder in TBST buffer (for blocking)

1gm skimmed milk powder and 20 mg NaN 3(sodium azide) were dissolved in 100ml TBST buffer. To avoid precipitation, the solution was centrifuged at 13,000xg at 4°C for 20 min.

8. Preparation of Primary and Secondary antibodies.

0.5% Ovalbumin in TBST buffer

0.5 g ovalbumin and 20 mg Nan3 (sodium azide) were dissolved in 100ml TBST buffer. To avoid precipitation, the solution was centrifuged at 13000xg at 4°C for 20 min.

9. 0.05 M Sodiumcitrate buffer

14.70 g of Sodium citrate (0.05 M) and 10.70gm of citric acid (0.05M) were dissolved in 500ml of distilled water separately and the pH was adjusted to 4.8 by mixing the two solutions.
10. Cobalt metal (sigma)
   It was used as supplied.

11. Hydrogen Peroxide (Merck, India)
   It was used as supplied

12. Diamino benzidine (DAB) (Sigma)
   It was used as supplied

13. Ponceau Stain (Stock):
   10gm of Ponceau S was dissolved in 5% glacial acetic acid solution and is stored in room temperature.

Statistical Analysis:

All the calculations and graphs were generated by using Micro Soft-Office Excel-2003.