Chapter III

Material and Methods
MATERIAL AND METHODS

2.1-INSECTICIDE:

Pyrethrin, technical grade, was obtained as a gift from M/S Rallis India Limited. The purity of pyrethrin was 95%.

2.2-HERBICIDE:

Femoxone (2, 4- D), technical grade, was procured from Chemical India Limited, Haryana. The purity of femoxone was 80%.

2.3-EXPERIMENTAL ANIMALS:

Healthy, young albino rats were selected and utilized as control and for experimental work. Albino rats, 75 to 90 days old with mean body weight, 300 gm. were selected as experimental animals. The animals bought from Banaras and were acclimatized, at laboratory conditions. They were fed proper animal food (animal food care, Mumbai) and inspected regularly for any disease or parasitism. These animals were bred and reared in the best proper atmosphere. The rats were separated according to age and weight and brought up in the best healthy and hygienic conditions. The temperature of the cages was maintained 35°C (±1°C) and the humidity was 80% (±5%). Water was provided libitum.
2.4-DETERMINATION OF SUBLETHAL CONCENTRATION OF PYRETHRIN AND FERNOXONE TOXICITY:

For parental application, pyrethrin was dissolved in petroleum ether and further diluted in water while fernoxone was dissolved in distilled water. To calculate LD_{100}, LD_{50} and sub lethal concentration, solutions of different amount of the pyrethrin and fernoxone were prepared in appropriate solvent and given according to the body weight of the animal.

20 albino rats were taken and divided into two groups of 10 rats each of 5 males and 5 females. For determination of LD_{50}, 500 mg pyrethrin/kg body weight in 1 ml. of distilled water was injected intraperitonially to the rats of first group. Similarly, the rats of second group, a solution of 700 mg fernoxone/kg body weight in 1 ml. of distilled water was infused intraperitoneally. None of the so treated rats were survived after 96 hrs. (LD_{100}).

The experiment was repeated with other 20 albino rats divided similarly into two groups and pesticidal doses administered were 250 mg pyrethrin/kg body weight and 350-mg/kg-body weight of fernoxone. Respectively the treated rats were observed for 96 h. and it was noticed that 50% rats of each group died and rest 50% survived (LD_{50}).
Above experiment was repeated on other groups of rat’s consisting of similar number of rats. The pyrethrin and fenoxone doses administered were 200 mg pyrethrin/kg body weight and 250mg fenoxone/kg body weight, separately to two groups of rats. All experimental rats survived for 96 h. (LD₀).

Above experiment was conducted with fresh batch of 20 albino rats divided as in earlier groups. Doses of 150 mg pyrethrin/kg body weight and 220 mg fenoxone/kg body weight were administered intra peritoneally to the experimental rats and were observed up to 6 weeks. All the rats survived and were found active. Thus, these doses i. e. 150mg/kg body weight of pyrethrin and 220mg/kg body weight of fenoxone were found to be sub lethal for the experimental albino rats. The results are presented in table 1 and 2 for the determination of LD₁₀₀, LD₂₀ and sublethal doses.

Table-2.1: Shows toxicity of pyrethrin

<table>
<thead>
<tr>
<th>Pesticide name</th>
<th>Grade with concentration %</th>
<th>Doses (mg/ml)</th>
<th>Duration</th>
<th>Mortality</th>
<th>LC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrethrin</td>
<td>Technical 95%</td>
<td>500</td>
<td>96 h.</td>
<td>All died</td>
<td>LD₁₀₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>96 h.</td>
<td>Half died</td>
<td>LD₅₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200</td>
<td>96 h.</td>
<td>All alive</td>
<td>LD₀</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150</td>
<td>6 weeks</td>
<td>All alive</td>
<td>Sub lethal dose</td>
</tr>
</tbody>
</table>
The aforementioned doses were prepared from freshly prepared stock solution of pyrethrin in ether, which was further diluted with water. Different doses of pyrethrin solution were prepared in 1ml distilled water from the stock solution, which was prepared in ether+water.

### Table- 2.2: Shows toxicity of fernoxone

<table>
<thead>
<tr>
<th>Pesticide name</th>
<th>Grade with concentration %</th>
<th>Doses (mg)</th>
<th>Duration</th>
<th>Mortality</th>
<th>LC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fernoxone</td>
<td>Technical 80%</td>
<td>700</td>
<td>96 h.</td>
<td>All died</td>
<td>LD&lt;sub&gt;100&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>96 h.</td>
<td>Half died</td>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>96 h.</td>
<td>All alive</td>
<td>LD&lt;sub&gt;0&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>220</td>
<td>6 weeks</td>
<td>All alive</td>
<td>Sub lethal dose</td>
</tr>
</tbody>
</table>

Preparation of stock solution of fernoxone was dissolved in 1 ml distilled water.

### 2.5-BIOCHEMICAL STUDIES:

To investigate the effect of pyrethrin and fernoxone, healthy rats of both the sexes up to the age of 75-90 days and weighing between 280-300 gm were selected. Rats were divided into many groups, with 10 rats in each group. Pyrethrin and fernoxone both were given parentally, intraperitoneally. For Pyrethrin 10mg/ml and for fernoxone 5mg/ml were
given every day to the rats of different groups. After the injections of the toxicants the rats were watched carefully for any changes in their behaviour and was recorded.

The various biochemical parameters were studied at intervals of 1st day, 5th days, 10th days, 20th days, 40th days, 80th days, 120th days and 150th days. Blood was obtained directly from the heart with the help of heparinized syringes, 3.8% sodium citrate solution was used as an anticoagulant. 0.5ml. anticoagulant was added to 2 ml. of blood. After centrifugation at 5000rpm for 10minutes, serum was collected, marked and refrigerated.

2.5.1-DETERMINATION OF SERUM GLUTAMATE OXALATE TRANSAMINASE:

Method:

SGOT was estimated by UV kinetic method using Span Diagnostic Reagent Kit.

Principle:

\[
\alpha\text{-oxoglutarate} + \text{L-aspartate} = \text{L-glutamine} + \text{Oxaloacetate}
\]

\[
\text{NDH} + \text{NADH}_2 = \text{L-malate} + \text{NAD}
\]
Normal Values:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>25°C</th>
<th>30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Up to 18/U/L</td>
<td>Up to 25/U/L</td>
<td>Up to 37/U/L</td>
</tr>
<tr>
<td>Female rat</td>
<td>Up to 15/U/L</td>
<td>Up to 21/U/L</td>
<td>Up to 31/U/L</td>
</tr>
</tbody>
</table>

Sample material:

Blood serum

Reagent:

<table>
<thead>
<tr>
<th>Buffered substrate</th>
<th>Concentration in test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer (pH-7.5)</td>
<td>80mmol/L</td>
</tr>
<tr>
<td>Aspartate</td>
<td>240mmol/L</td>
</tr>
<tr>
<td>α-oxoglutarate</td>
<td>12.0mmol/L</td>
</tr>
<tr>
<td>MDH</td>
<td>0.42U/ml</td>
</tr>
<tr>
<td>NADH₂</td>
<td>0.18mmol/L</td>
</tr>
</tbody>
</table>

Stability of the reagent:

The reagent 1 and 2 are stable between 2°C to 8°C for 6 months. The reagents 3 and 4 should be prepared fresh for the test from the stock solutions. Reagent 5 should be prepared fresh which is stable between 0°C to 4°C for 5 days.

Procedure:

Wavelength-340nm

Cuvette-1cm light path
Chapter 2 – Material and Methods

Temperture-25°C, 30°C and 37°C

Pipette into cuvette-

Buffered substrate 1.0ml
NADH₂ 0.02ml
MDH 0.01ml
LDH 0.01ml
Mix and all sample 0.2ml
Oxoglutarate 0.04ml

Mix and read initial absorbance. Note readings after 1, 2 and 3 min. Determine the mean absorbance change per min. (A/min)

Calculation:

SGOT: U/L = 1051 X A/min

2.5.2- DETERMINATION OF SERUM GLUTAMATE PYRUVATE TRANSAMINASE:

Method:

Estimation of SGPT was based on UV-kinetic using Span Diagnostic Kit.

Principle:

GOT catalyses the following reaction:

\[
\text{L-oxoglutarate + Alanine} \rightarrow \text{L-glutamate + Pyruvate + NADH}_2 = \text{Lactate + NAD}
\]

-44-
Normal value:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>25°C</th>
<th>30°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Up to 27/U/L</td>
<td>Up to 29/U/L</td>
<td>Up to 40/U/L</td>
</tr>
<tr>
<td>Female rat</td>
<td>Up to 17/U/L</td>
<td>Up to 22/U/L</td>
<td>Up to 31/U/L</td>
</tr>
</tbody>
</table>

Sample material:

Blood serum

Reagent:

<table>
<thead>
<tr>
<th>Buffered substrate</th>
<th>Concentration in test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer+alanine (pH-7.5)</td>
<td>Tris-100mmol/L and alanine-500mmol/L</td>
</tr>
<tr>
<td>L-oxoglutarate</td>
<td>12.0mmol/L</td>
</tr>
<tr>
<td>LDH</td>
<td>1.2U/ml</td>
</tr>
<tr>
<td>NADH</td>
<td>0.18mmol/L</td>
</tr>
</tbody>
</table>

Stability of the reagent:

The reagent 1 and 2 are stable between 2.8°C for 6 months. The reagent 3 is prepared fresh from the stock solution. Reagent 4 is prepared fresh (stable between 0.4°C for 5 days).

Procedure:

Wavelength-340nm

Cuvette-1 cm light path

Temperature-25°C, 30°C and 37°C

Pipette into cuvette-
Buffered substrate  1.0ml  
NADH  0.02ml  
LDH  0.02ml  
Sample  0.2ml  
L-Oxoglutarate  0.04ml

**Calculation:**

\[ \text{SGPT: U/L} = 1051 \times \text{A/min} \]

**Procedure limitation:**

If the absorbance change exceeds 0.160 O.D./min, specimen was diluted in normal saline and then the test was repeated. For calculations the dilution factor was taken into consideration.

**2.5.3- DETERMINATION OF SERUM ALKALINE PHOSPHATE:**

Alkaline Phosphatase was determined by method using Span Diagnostic Kit.

**Principle:**

Alkaline Phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange-red coloured complex,
which can be measured colorimetrically. The colour intensity is proportional to the enzyme activity.

The reaction can be represented as:

\[
\text{Phenyl Phosphate + Alkaline Phosphatase} \rightarrow \text{Phenol}
\]

\[
\text{pH 10.0}
\]

\[
\text{Phenol + 4 Aminoantyryne Potassium Ferricyanide} \rightarrow \text{Orange coloured complex}
\]

\[
\text{OH}^-
\]

**Reagents:**

Reagent 1: Buffered Substrate, pH 10.0

Reagent 2: Chromogen Reagent

Reagent 3: Phenol Standard, 10mg%

**Preparation of Working Solution:**

One vial of Reagent 1 is reconstituted with 4.5ml of distilled water.

**Procedure:**

Four test tubes were marked as Blank ‘B’, Standard ‘S’, Control ‘C’ and Test ‘T’.

**Blank:**

To 1.0ml working buffered substrate 3.0ml of distilled water was added, mixed well and incubated for 3 minutes at 37° C. Then 2.0ml of Chromogen reagent was added and mixed well. Optical Density was measured against distilled water using a green filter.
Standard:

To 1.0ml working buffered substrate, 3.0ml of distilled water added, mixed well and incubated for 3 minutes at 37°C. To this solution 1.0ml Phenol Standard reagent was added, and the solution was incubated for 15 minutes at 37°C. Then 2.0ml chromogen reagent was mixed with it and optical density was measured.

Control:

To 1.0ml working buffered substrate, 3.0ml distilled water was added, and incubated for 3 minutes at 37°C. To this solution 2.0ml chromogen reagent and 0.1ml serum were added, and the optical density was measured.

Test:

1.0ml working buffered substrate and 3.0ml distilled water were mixed well and incubated for 3 minutes at 37°C. Then 0.1ml serum was added to it, and incubated for 15 minutes at 37°C. 2.0ml of chromogen reagent was mixed in the above solution and optical density was measured against distilled water using a green filter (340 nm).

Calculation:

\[
\text{Serum Alkaline Phosphatase} = \frac{\text{O.D. Test} - \text{O.D. Control}}{\text{O.D. Standard} - \text{O.D. Blank}} \times 10
\]
2.5.4 - DETERMINATION OF SERUM LACTATE DEHYDROGENASE:

Lactate Dehydrogenase (LDH) catalyses the conversion of pyruvate to lactate with the oxidation of NADH.

\[ \text{Lactate} + \text{NAD} = \text{Pyruvate} + \text{NADH} \]

Products so formed are coupled with 2,4-Dinitrophenylhydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium, and is measured colorimetrically with a blue filter.

**Reagent:**

The diagnostic kit contained six reagents:

- **Reagent 1:** Buffered Lactate Substrate, pH 10.0
- **Reagent 2 (A):** NAD for Test
- **Reagent 2 (B):** NAD for Graph
- **Reagent 3:** DNPH Colour reagent
- **Reagent 4:** NADH
- **Reagent 5:** Sodium Hydroxide, 4N
- **Reagent 6:** Working Pyruvate Standard, 1mM
Preparation of Working Solution:

For the estimation of LDH Reagent 2(A) and 2(B) were mixed with 0.3ml and 1.4ml of distilled water to prepare solution 1(A) for test and solution 1(B) for graph respectively. Reagent 4 was diluted with 1.2ml of Reagent1 (buffered substrate) and Reagent 5 (sodium hydroxide) was diluted 10 times with distilled water. Serum was diluted 5 times with normal saline. Reagent 1, 3 and 6 were prepared just before use and brought to room temperature before use.

Procedure:

Dry and clean test tubes marked with ‘C’ for control and ‘T’ for test. 1ml of Reagent 1 was mixed in both the tubes, 0.1ml of diluted serum was added in tube ‘T’ and 0.2ml of distilled water was added to tube ‘C’, mixed well and incubated at 37⁰C for 5 minute. 0.2ml of solution 1 (A) was mixed in tube ‘T’, incubated at 37⁰C for 15 minutes. 0.1ml of Reagent 3 was mixed in both the tubes and 0.1ml diluted serum was added in tube ‘C’, and incubated at 37⁰C for 15 minutes. 10ml of solution 1 was added in both the tubes, mixed well by inversion, and allowed to stand at room temperature for 5 minutes. O.D. of test and standard was measured against distilled water on a colorimeter using blue filter. Reagent 6 working pyruvate standard was used for standard graph.
Calculation:

The net O.D. of Test (Tn) was calculated as follows

\[ Tn = \text{O.D. Test} - \text{O.D. Control} \]

Net O.D. of test (Tn) was marked on Y-axis of the standard curve and extrapolated to the corresponding enzyme activity (IU/litre) on X-axis.

2.5.5-EXTRACTION AND ESTIMATION OF GLUCOSE:

A portion of (1ml) serum was taken in two separate small Corning test tubes and to these test tubes, 0.5 ml of 1% H₂SO₄ was added for deproteinization. This protein free filtrate was estimated by Anthrone method for glucose estimation.

Preparation of reagent:

200 mg of anthrone (AR) was dissolved in 100 ml of 95% H₂SO₄ (BDH/AR) in a long neck flask, flask remained immersed in ice cool water, till the reagent is prepared. Reagent was preserved in brown airtight bottle in a refrigerator. Unused reagent was discarded after 7 days.

Glucose standard solutions ranging from 0.001 mg/ml to 0.01 mg/ml prepared from freshly prepared glucose stock solution. To 1 ml of glucose standard solution 0.5 ml of 1% H₂SO₄ and 4 ml of 0.2% anthrone reagent
were added slowly and mixed by careful lateral shaking. The tubes were dipped in chilled water during the mixing of anthrone reagent.

A blank solution was prepared with 1 ml of distilled water + 0.5 ml of H₂SO₄ and 4 ml of 0.2% anthrone reagent. For digestion, tubes were kept immersed in water bath at 80°C for 5 minutes, then tubes were cooled down to room temperature. Percent transmission was recorded at 540nm wavelength on spectronic-20.

A known standard solution was taken every time with unknown samples. Readings were calibrated on pre-prepared glucose standard curve.

2.5.6-EXTRACTION AND ESTIMATION OF CHOLESTEROL:

The measured volume of blood was mixed with chloroform: ether (3:1v/v) and lower chloroform layer was collected from the separating funnel. 5ml. chloroform was added to the tube to the dissolve the cholesterol contents. Colour developing reagent, acetic anhydride and concentrated sulfuric acid mixture (10:2v/v) was then freshly prepared in cold, 1ml of which was added to tube. After the incubation of 30 minutes when the optimum colour was developed, the photodensity was recorded on “Spectronic-20” at 620nm. The quantity of cholesterol was estimated by calibrating from a standard curve prepared earlier from known concentration of cholesterol, in the each estimation a known concentration of cholesterol was always taken.
2.6-STATISTICAL ANALYSIS:

Results for serum enzymes, glucose and cholesterol activities in rats have been presented as Mean±SD (Standard deviation) or as Mean±SEM (Standard error of mean). The variation present in a set of data was analysed using one-way analysis of variance (ANOVA) by least significant difference at 99.5 (0.05), 99.9% (p<0.001) confidence level (Ghosh, 1984; Alafonso, 1996).

Mean-

\[ \bar{X} = \frac{1}{n} \sum_{i=1}^{n} X_i \]

\[ \bar{X} = \text{Arithmetic mean} \]

\[ n = \text{Number of Observation} \]

\[ X_i = \text{Values of Observation} \]

Standard Deviation-

\[ \sigma = \sqrt{\frac{\sum d^2}{n}} \]

\[ \sigma = \text{Standard Deviation} \]

\[ d = \text{Deviation} \]

\[ n = \text{Number of observation} \]

Standard Error-

\[ \text{S.E. of } \bar{X} = \frac{S}{\sqrt{N}} \]

\[ \bar{X} = \text{Arithmetic mean} \]

\[ S = \text{Standard deviation} \]

\[ N = \text{Number of observation} \]
2.7-HISTOLOGICAL STUDY:

2.7.1-Tissue fixation and fixatives:

For the histopathological studies of brain, thyroid, adrenal and pancreas, the tissues were fixed in Carnoy’s fixatives for 24hrs (Pearse, 1985).

Carnoy’s Fluid-

Absolute alcohol-60ml.

Chloroform-30ml.

Glacial acetic acid-10ml.

Carnoy’s fluid penetrates very rapidly and gives excellent nuclear fixation with preservation of glycogen and Nissl substance. Little cytoplasmic material is left after fixation in Carnoy’s fluid.

After fixation, the material was thoroughly washed with 70% alcohol and dehydrated in different grades of alcohol 70%, 90% and two changes of absolute alcohol. For clearing the material xylene is used. After that material is kept in the jelly (xylene + paraffin wax in 1:1). After the material is transferred to molten paraffin wax and three changes were given to the material. It is embedded in the wax and Paraffin blocks were prepared. Sections cut at 6μ with the help of microtome.
2.7.2-STAINING TECHNIQUES:

Sections stained with double staining techniques. In double staining techniques *hematoxylin* and *eosin* were used. Haematoxylin is a nuclear stain and eosin is a cytoplasmic stain (Pearse, 1985).

**Ethyl eosin-**

Make up as a 0.5% solution in 95% alcohol. The eosin has very much the same shade and shows the conventional contrast to hematoxylin.

**Hematoxylin-**

**Requirement** - Hematoxylin-25g

Absolute alcohol-150ml.

Ammonium alum (Aluminum or Ammonium sulphate)-220g

Glycrine-600ml.

Methanol-600ml.

Distilled water-2400ml.

**Method-**

For the preparation of hematoxylin, we make two solutions. In first solution we mix 220g of ammonium alum in 2400ml. distilled water. For second solution 25g of hematoxylin is dissolved in 150ml. of absolute alcohol. Now mix these two solutions well by constant stirring. Then put the solution in a bottle without lid at a place where sunlight and air is maximum. After one week 600ml. of glycrine and 600ml. of methanol is mix in this.