3. MATERIALS AND METHODS

3.1 STUDY AREA

The village Manalmedu is located 15 km north of Mayiladuthurai town. It is situated in Mayiladuthurai Taluk of Nagapattinam District in the state of Tamilnadu (Fig. 2). Most of the study area is occupied by agricultural wetlands, which are used for paddy and sugarcane cultivation. The main water source for irrigation is the river Cauvery. The entire study area is known for its large-scale agricultural operations, which include the cultivation of paddy, sugar cane, banana, groundnut, pulses and other cereals.

This area is gifted with a small population of small mammals, reptiles and amphibians. Mammals like Mongoose (*Herpestes edwardsi*), Jackal (*Canis aurens*), Flying fox (*Pteropus giganteus*), Indian field mouse (*Mus boodiga*), Three striped palm squirrel (*Funambulus palmarum*), Mole Rat (*Bandicota indica*) and Herpeto fauna like Brooki gecko (*Hemidactylus brooki*), Horse gecko (*Hemidactylus frenatus*) and the Garden lizard (*Calotes versicolor*) are found here.

The members of the amphibian family like the Indian toad (*Bufo melanostictus*), the Skipper frog (*Rana cyanophlyctis*), Green
frog (*Rana hexadactyla*), Bull Frog (*Rana tigrina*) and the common toad (*Bufo melanostictus*) are extensively found. The green frogs for the experiments were collected from this study area.

### 3.1.1 Questionnaire survey method

The agricultural land extent, agricultural pattern, pesticides used by the farmers and amphibian mortality due to application of pesticides in the field were collected using a questionnaire (Appendix I).

### 3.2 PROCUREMENT AND REARING OF EXPERIMENTAL ANIMALS:

Adult, healthy green frogs (*Rana hexadactyla*) ranging in weight from 75 to 80 gm were collected from ponds and canals of this village Manalmedu using a sweep net or hand picked. Immediately after the collection the animals were acclimatized to the prevailing laboratory conditions to groups (10 animals in a group) in tanks (90 X 50 cm) under normal day/night cycle, at 23 to 25°C. Considering the amphibian habit, 30 cm of water level was maintained in the above said tanks and also with a slanting bottom surface, so that the frogs can also come to water area of the tanks. The frogs were fed with live earthworms and grasshoppers, as food at regular intervals. The frogs were divided
into two different groups and kept in two different aquarium tanks. One group was maintained as the control and the other for experimental study. Both the control and experimental frogs were kept on complete starvation for 48 hours prior to the commencement of the experiments. This provision was made to nullify the effects of feeding on the physiology and biochemistry and also to reduce the quantity of excretory products in the tank.

### 3.3 ROGOR (DIMETHOATE) - STRUCTURE AND PROPERTIES

- **Common name**: Dimethoate
- **Chemical class**: Organophosphorus
- **Chemical name**: O, O-dimethyl S-[(N-methyl carbamoyl)methyl]phosphorodithioate
- **Empirical formula**: C₁₅H₂₌O₃NPS₂
- **Structural formula**: 
  
  \[
  \begin{array}{c}
  \text{CH}_3O \\
  \text{S} \\
  \text{P} - \text{S} - \text{CH}_2 - \text{CONH} - \text{CH}_3 \\
  \text{CH}_3O
  \end{array}
  \]
- **Molecular weight**: 229
- **Melting point**: Pure-50°C  
  Technical – slightly lower
- **Vapour pressure**: 10⁻³ Hg room temperature
- **Solubility (g/litre at room temperature)**:  
  - Water: - 25  
  - Olive oil: - 15  
  - Petroleum: - 1
Stability
Hydrolysed aquous alkali
K=6.8 min\(^{-1}\) moles\(^{-1}\) at 25°C relatively stable in
natural and weekly acidic solutions

Uses
Systemic and contact insecticides or
fungicides

Compatibility
: Compatible with most insecticides and
fungicides

Phytotoxicity
: No phytotoxicity reported at the recommended
doses

Formulations available with
rallies
: 30 per cent EC

Manufacture
Rallis
India Limited
Ralli House
21, D-Sukhaduala Marg
Mumbai 400 001.

3.4 HISTOLOGICAL METHODS

The kidney, liver and pancreatic tissues were carefully
removed by dissection after the frogs were anesthetized. Neutral
Formalin (10 per cent) was used as fixative as recommended by
Pearse (1968). Paraffin embedding was carried out and 4 \(\mu\) to 6 \(\mu\)
thick sections were used for staining with Heiden-Hains-
haematoxylin-eosin stain.
### 3.5 TOXICOLOGICAL METHODS

#### 3.5.1 Screening Test

The test procedure adopted to measure the acute lethal dose is termed as preliminary screening test. The object of the test is to obtain approximate identification of the dose level of pesticide likely to be hazardous to this species of frog. The preliminary tests were conducted to get the approximate range of the dose of the pesticide which would be covered in the full-scale test.

#### 3.5.2 Range Finding Test

Ten frogs were kept in five polypropylene jars, each jar containing two frogs with one litre of water. The range of pesticide given was between 39 mg/l to 59 mg/l. Frogs of each jar were maintained for 120 hours, behavioural changes of frogs were recorded at regular intervals.

#### 3.5.3 Full Scale Test

After completing the range finding test, full scale tests were conducted, the percentage mortality of the experimental frogs with reference to time and pesticide concentration were recorded. The LC$_{50}$ of this pesticide was estimated using the software Toxi. Biol. The frogs lying at the bottom or floating ventrally were
considered as dead and were removed immediately. The least concentration at which 100 per cent mortality was observed up to 120 hours was considered 120 hours lethal dose (LC\textsubscript{100}) and 100 percent survival observed up to 120 hours was considered the sub lethal dose (LC\textsubscript{0}). The concentration at which there was only 50 percent survival at 120 hours was considered as LC\textsubscript{50}.

### 3.6 PREPARATION OF SERUM

Approximately 1 ml. of freshly drawn blood was placed in a small test tube; frozen for 15 minutes at room temperature, centrifuged at 2500 rpm for 10 minutes and decanted to get clear serum.

### 3.7 BLOOD PARAMETERS AND METHODS EMPLOYED

The following blood parameters were investigated using the methods or apparatus given against each.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Blood parameters</th>
<th>Method/Instrument</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total RBC Count</td>
<td>Haemocytometer</td>
</tr>
<tr>
<td>2</td>
<td>Total WBC Count</td>
<td>Haemocytometer</td>
</tr>
<tr>
<td>3</td>
<td>Haemoglobin Content</td>
<td>Haemometer</td>
</tr>
<tr>
<td>4</td>
<td>Blood Glucose</td>
<td>Dubois et al. (1956)</td>
</tr>
<tr>
<td>5</td>
<td>Blood Urea</td>
<td>Rajagopal &amp; Ramakrishnan (1983)</td>
</tr>
<tr>
<td>6</td>
<td>Serum Uric acid</td>
<td>Caraway (1955)</td>
</tr>
<tr>
<td>7</td>
<td>Serum Protein</td>
<td>Biuret Method (Rajagopal &amp; Ramakrishnan, 1983)</td>
</tr>
</tbody>
</table>
3.8 HAEMATOLOGICAL METHODS

Blood samples were collected from the test animals with the help of a hypodermic heparinized glass syringe. Blood was drawn from the ventricle of the heart (Farrar and Frye, 1979) and it was immediately transferred to a plastic container already rinsed with the anti-coagulant EDTA (Ethylene Diamine Tetra Acetic acid).

3.8.1 Red Blood Corpuscles count (RBC)

The RBC cells were counted using a Haemocytometer and a Neubauer counting chamber. The RBC pipette was rinsed with sodium citrate and the blood was sucked up to 0.5 mark. Then Hayem’s diluting fluid was sucked up to 101 mark. The fluid in the pipette was thoroughly mixed. A drop of diluted blood was applied to the counting chamber and a cover slip was placed. The slide was kept for 5 minutes for settling down of corpuscles. Erythrocytes were counted in 5 small squares (4 corners and one central chamber).
**Calculation:**

Total number of Erythrocytes counted in 80 small squares = N

Number of cells in one smaller square = (16x5) \( N/80 \)

Area of one smaller square = \( 1/20 \times 1/20 \) sq. mm. = \( 1/400 \) sq. mm.

Depth of diluted blood in the chamber = \( 1/10 \) mm.

Vol. of diluted blood in 1 smaller square = \( 1/400 \times 1/10 \) = \( 1/4000 \) cu. mm.

No. of cells in 1 cu. mm. of diluted blood = \( N/80 \times 4000 \)

dilution ratio = 1:200

= \( N/80 \times 4000 \times \) dilution ratio

No. of RBC per cu. mm. of undiluted blood = \( N \times 10000 \)/cu.mm.

**3.8.2 White blood corpuscles count (WBC)**

The total WBC count was made with Haemocytometer’s Neubauer counting chamber. WBCs were counted from the control and treated frogs. The blood was drawn up to 0.5 mark in WBC pipette and diluted up to the mark II with Turk’s fluid (Gentian Violet, Glacial acetic acid 3 ml. and distilled water 97 ml.). The pipette was kept horizontally and shaken well for 1 to 2 minutes. Then discarding a few drops, the diluted blood was fed
into the haemocytometer and the cells were allowed to settle for a minute. The leucocytes were counted in four corner squares under the microscope and represented as cells \((10^3)\) per \(mm^3\) of blood.

**Calculation:**

Total number of leucocytes counted in

4 corner squares \(= N\)

Number of cells in one smaller square \(= N / 4\)

Area of one square \(= 1 \text{ sq. mm.}\)

Depth of diluted blood over in the chamber \(= 1 \times 1 \times 1 / 10\)

Volume of fluid in each square \(= 1 / 10 \text{ cu. mm.}\)

Number of cells in 1 cu. mm. of diluted blood \(= N / 4 \times 10\)

(Since the dilution of blood is 1 in 20, the dilution factor is 20).

No. of leucocytes per cu. mm. of diluted blood \(= N / 4 \times 10 \times \text{dilution factor}\)

\(= N / 4 \times 10 \times 20\)

No. of leucocytes per cu. mm. of diluted blood \(= N \times 50 \text{ cu. mm.}\)

**3.8.3 Haemoglobin estimation**

Haemoglobin content of blood was estimated using Haldane’s Haemoglobinometer (Superior, Germany) with permanent coloured glass comparison standards. The haemometer (graduated) tube was first cleaned with distilled water and then
with methylated spirit or 90 per cent alcohol. After drying, it was filled to the level of lowest graduation (2 grams mark) with N/10 HCl solution, using a micropipette, 20cumm of blood was blown into the hydrochloric acid already present in the Haemometer tube. After expelling the blood, the pipette was rinsed with distilled water twice (or) thrice and every time it was expelled into the Haemometer tube. The haemometer tube was placed in the stand and distilled water was added with constant stirring until the colour matched with the colour of the standard. Reading was taken directly from the Haemometer tube exactly 3 minutes after the blood was added to the acid. Haemoglobin was expressed Hb mg/100ml of blood.

3.9 BIOCHEMICAL PARAMETERS

3.9.1 Estimation of blood glucose

The amount of total glucose present in the blood was determined by the method of Dubois et al., (1956). A quantity of 0.1ml of plasma was taken in a test tube and made up to 1 ml. with distilled water. One ml. of 5 per cent phenol solution was added and the contents were mixed thoroughly. The red colour developed was measured in a spectronic 21 at a wavelength of 490 nm. The amount of glucose present was calculated by
comparing its optical density with that of the standard glucose solution.

**Calculations:**

\[
\text{Total amount of glucose} = \frac{\text{O.D. of the Sample}}{\text{O.D. of the Std.}} \times 100
\]

The calculated values were expressed as mg/100ml.

Details of the laboratory procedure adopted in blood glucose estimation is given below:

**Materials used:**

Blood, 5 per cent Phenol, Glucose, Conc. Sulphuric Acid, Distilled water.

**Preparation of reagent:**

5 per cent phenol (5 ml. of phenol with 95 ml. of distilled water), 1 per cent glucose, (1 gram of glucose in 100 ml. of distilled water).

**Procedure:**

A quantity of 0.1 ml of plasma was taken in a test tube and made up to one ml. with distilled water. One ml of 5 per cent phenol solution was added and mixed thoroughly. Five ml of conc. Sulphuric acid was added and kept for 10 minutes. The red colour that developed was measured in a spectronic 21 at a
wavelength of 490nm. The amount of glucose present was calculated by comparing its optical density with that of the standard glucose solution.

3.9.2 Estimation of Blood Urea

Blood was estimated following the procedure of Rajagopal and Ramakrishnan (1983). Urea present in blood was enzymatically converted (by urease) into ammonium carbonate. Then the proteins of blood and the enzyme were precipitated by fresh tungstic acid. The contents were centrifuged. An aliquot of the supernatant containing the ammonium salt was treated with Nessler’s reagent. The procedure was carried out on a blank and a standard. The colours produced were measured at 480 nm and the amount of urea in the blood was estimated by comparing with the optical density (O.D) of the standard urea solution.

Calculations:

5.0 ml of std. supernatant represents 0.05 mg urea.

5.0 ml of blood supernatant represents 0.125 ml blood.

Concentration of Urea in the Sample

\[
\text{O.D. of the sample} = \frac{\text{O.D. of the standard} \times \text{Conc. Std}}{\text{O.D. of the standard}}
\]

Urea in 0.125 ml blood = \frac{\text{O.D. of the sample}}{0.05} \times \text{O.D. of the standard}
Urea in 100ml blood = \frac{\text{O.D. sample}}{\text{O.D. Standard.}} \times 40

The calculated urea values were expressed as mg/100ml.

Details of the laboratory procedure adopted in Blood Urea estimation is given below:

**Materials used:**

Oxalated blood (Potassium oxalate free from NH₃), 10 per cent urease suspension in phosphate buffer. pH 7.0, 10 per cent Sodium tungstate, 2/3 N sulphuric acid, 0.8 per cent urea, stock standard (1ml=8mg), 0.4 per cent urea working standard (1ml = 0.4mg).

**Preparation of reagent 10 per cent Urease suspension in phosphate buffer:**

Ten ml of urease suspension was taken and made up to 100ml. Dissolved pH 7.0 urease tablets in 100 ml of distilled water.

**10 per cent Sodium tungstate:**

Ten grams of sodium tungstate was dissolved in 100ml of distilled water.
**2/3 N Sulphuric acid:**

A quantity of 18.5 ml of con. H₂SO₄ was diluted with 1 liter of distilled water.

**0.8 per cent Urea stock standard:**

Eight hundred mg of urea was dissolved in 100ml water and stored in the cold with a few drops of chloroform.

**0.04 per cent Urea, working standard:**

Fifty ml stock was diluted to 100ml (prepared freshly).

**Nessler’s Reagent: (Double Iodide Solution)**

150 g. of potassium iodide was taken in a flask and 100 mg iodide crystals were added to 100 ml of water. To the same, 150g mercury was also added. The flask was shaken vigorously for about 10 minutes. As the reaction was exothermic, the flask was cooled in running water. Iodine colour was replaced by a greenish tinge. The solution was decanted and the mercury was washed with water.

**Nessler’s Reagent, working solution:**

A quantity of 350 ml. of 10 per cent NaOH was taken into a 1 litre flask, 75ml of 0.8 per cent urea stock was added to double iodide solution and made up to the mark with the distilled water and the contents were mixed well (10 per cent NaOH is to be made from carbonate free 75 per cent NaOH).
Procedure:

Centrifuge tubes (15ml) were taken and marked A, B and C for Standard, Test and Blank respectively and the following procedures were done:

<table>
<thead>
<tr>
<th></th>
<th>A ml</th>
<th>B ml</th>
<th>C ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2.0</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Blood</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>0.04 urea working standard</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Buffered Urease Suspension</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Each tube was mixed gently and incubated in a water bath at 55°C for 15 minutes. Blood urea was transformed into ammonium proteins as well as the enzyme proteins, which are precipitated by the addition of 0.3 ml of 2/3 N H₂SO₄ to each tube. To this mixture, 5.0 ml of water was added and centrifuged, at 2000 r.p.m. for 5 minutes. From each tube, 0.5 ml of solution was transferred into the correspondingly marked dry tubes to which 5.0 ml of water and 2.0 ml of Nessler’s reagent were added. Readings were taken without delay in a photometer at 480 nm after setting the blank at 100 per cent Transmittance. The amount of urea present was calculated by comparing its optical density with that of the standard urea solution.
3.9.3 Estimation of serum uric acid

Serum uric acid was estimated by following the method of Caraway (1955). In this method, serum was first deproteinised by tungstic acid. Then the uric acid present in the protein free filtrate was treated with Folin’s uric acid reagent in the presence of sodium carbonate to give a blue colour. The blue colour developed was compared with that of standard uric acid solution by measuring their optical densities in a Spectrornonic 21 at 700 nm, and the amount of uric acid was estimated.

Calculations:

Concentration of sample = \( \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times 0.02 \)

Uric acid in 0.05ml serum = \( \frac{\text{O.D. of sample} \times 0.02}{\text{O.D. of standard} \times 0.5} \times 100 \)

Uric acid in 100ml serum = \( \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times 4 \times \frac{100}{0.05} \)

= mg/100ml

Details of laboratory procedure adopted in serum uric acid estimation:
Materials used:

Serum, 10 per cent sodium tungstate, 2/3 N sulphuric acid, 10 per cent sodium carbonate, Uric acid reagent dilute (Folin & Denis), 20 mg uric acid stock, standard 0.4 mg uric acid working standard.

Preparation of reagents:

10 per cent Sodium tungstate: 10 grams of sodium tungstate, in 100ml of distilled water.

Uric acid reagent (Folin and Denis) Stock:

100 mg sodium tungstate (Na₂WOS₄₂H₂O) was dissolved in 800 ml of distilled water in a round-bottomed flask and 80 ml of 85 per cent phosphoric acid was added. Reflex condenser was attached and gently boiled for 2 hours, cooled to room temperature and diluted to 1 litre with water. This reagent was stored in a brown bottle. The acidity of the reagent is important in the reaction.

Uric acid reagent (Folin and Denis) Dilute:

10 ml of stock was diluted to 100 ml with water. Both stock and dilute reagents were stable.

20mg per cent uric acid stock standard:

100 mg of uric acid was dissolved in 300 ml of 0.77 per cent sodium tertraborate (Na₂B₄O₇) taken in a 500ml standard
flask and 0.9 ml of glacial acetic acid was added and diluted to the mark with water.

**0.4mg per cent Uric acid working standard:**

Dilute 2.0 ml of stock to 100ml with water in a flask (prepared freshly).

**Procedure:**

A quantity of 7.0 ml water was taken in a test tube, 1.0 ml of serum was added followed by 1.0 ml of 10 per cent sodium tungstate and 1.0 ml of 2/3 N H₂SO₄. These were mixed well and left for 5 minutes and filtered. Three test tubes A, B and C were marked as Standard, Test and Blank respectively and the procedures undertaken were as follows:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric acid working standard</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein free filtrate</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
</tr>
</tbody>
</table>

One ml of 10 per cent sodium carbonate was added to each tube followed by 1.0ml of dilute phosphotungstic acid reagent. These were mixed and kept for 30 minutes. Readings were taken in a spectronic 21 at 700 nm. The per cent Transmittance (T) of Standard, Test samples were read and converted into Absorbance.
Serum Uric acid values obtained were expressed as mg/100ml.

### 3.9.4 Estimation of serum proteins

The Biuret method as described by Rajagopal and Ramakrishnan (1983) was used for the analysis of serum proteins. A known quantity of serum (0.5ml) was treated with 28 per cent sodium sulphate to precipitate the globulins. An aliquot of the mixed contents were taken for total proteins and the rest was filtered. Biuret reagent was used to develop a purple colour with the proteins. For the standard, Bovine albumin was used. The colours were measured at 540 nm with a reagent blank. The difference in the values of total proteins/albumin gave the content of globulins. The ratio of albumin/globulins was calculated.

### Calculations:

\[
\text{Concentration of sample} = \frac{\text{O.D. of the sample}}{\text{O.D. of Standard}} \times \text{Conc.of Standard}
\]

\[
\text{Total proteins in 0.15 ml serum} = \frac{\text{O.D. of sample of Total Protein}}{\text{O.D. of Standard}} \times 0.01
\]
Total proteins in 100ml serum = \frac{\text{O.D of sample}}{\text{Total protein}} \times \frac{20}{3} \times \text{O.D. of Standard}

The calculated values were expressed as g/100ml.

Details of the laboratory procedure adopted in Serum Protein estimation are:

**Materials used:**

Serum unhaemolysed, 28 per cent sodium sulphite, 0.5 per cent Bovine albumin standard in 0.9 per cent sodium chloride, Biuret reagent dilute.

**Preparation of reagents:**

1. 28 per cent Sodium sulphite: 28 grams of sodium sulphate was dissolved in 100ml of water.
2. 0.5 per cent Bovine albumin in 0.9 per cent sodium chloride:
   i. 500 mg of Bovine albumin was dissolved in 100ml of distilled water,
   ii. 900mg of sodium chloride was dissolved in 100ml water, and the two solutions were added and mixed well.
3. Biuret reagent dilute: Twenty five ml of 3 per cent Cu So₄ solution in 1 litre of 10 per cent potassium hydroxide (KoH) solution.
**Procedure:**

A quantity of 0.5 ml of serum was taken in a test tube and after inverting the tube 3 to 4 times, 9.5ml of 28 per cent sodium sulphite was mixed and 3.0 ml of the resulting solution was immediately transferred into a tube marked ‘TP’ (Total Proteins). Two ml of standard bovine albumin solution (0.01g protein) and 1.0 ml water were taken into a tube marked STD (standard). Three ml of water was taken in a third tube marked. To all the tubes, 5.0 ml of Biuret reagent was added, mixed and incubated for 15 minutes at 37°C. The colour developed was read at 540 nm. The amount of total protein was estimated. The amount of protein present was calculated by comparing its optical density, with that of the standard protein solution and the calculated values were expressed as g/100 ml.

**3.9.5 Estimation of serum cholesterol**

The estimation of serum cholesterol was based on the methods of Zlatkis *et al.*, (1953) and Zak (1959). Serum was treated with ferric chloride acetic acid reagent to precipitate the proteins. The protein free supernatant containing ferric chloride was treated with concentrated sulphuric acid. A reddish purple colour that developed was measured at 560 nm using a suitable standard and a reagent blank. The amount of cholesterol was
measured by comparing with the optical density (O.D) of standard solution.

**Calculations:**

\[
\text{Concentration of sample} = \frac{\text{O.D of Sample}}{\text{O.D of Standard}} \times \text{Conc. Standard}.
\]

\[
\text{Cholesterol in 0.1 ml serum} = \frac{\text{O.D of sample}}{\text{O.D of Standard}} \times 0.2
\]

\[
\text{Cholesterol in 100 ml serum} = \frac{\text{O.D of sample}}{\text{O.D of Standard}} \times 200
\]

The calculated values were expressed as mg/100ml

Details of the laboratory procedure adopted in Serum Cholesterol estimation is given below:

**Materials used:**

Serum, Ferric Chloride – acetic acid reagent (Stock), Cholesterol Standard. (Stock and working), Conc. Sulphuric acid.

**Preparation:**

**Ferric Chloride – acetic acid reagent (stock):**

1g of FeCl₃6H₂O were dissolved in acetic acid, (Analytical grade glacial acid was used, aldehyde free).

**Ferric chloride – acetic acid reagent (working):**
Five ml of stock was diluted to 100ml with acetic acid

**Cholesterol standard (stock):** (0.1 ml = 1.0mg).

100mg cholesterol was dissolved in acetic acid and made up to 100ml in a standard flask with acetic acid and stored in the refrigerator.

**Cholesterol standard (working):** (1.0 ml = 0.04 mg). 4.0 ml stock was diluted to 100ml with working ferric chloride acetic acid reagent. This was stored in the refrigerator.

**Procedure:**

A quantity of 0.2 ml of serum was taken in a 15 ml glass stoppered centrifuge tube and 9.8 ml of working ferric chloride acetic acid reagent was added; the tube was stoppered, mixed and allowed to stand for 15 minutes and centrifuged. Three dry test tubes were marked as A, B and C for Standard, Test and Blank respectively and the tests were proceeded as follows:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working Std. Cholesterol</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein free supernatant of serum</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>Working FeCl₃ Acetic acid reagent</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Con. H₂SO₄</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>
The contents of each tube were mixed well till the colour that developed was uniformly distributed. The tubes were allowed to stand for 30 minutes and then mixed well. The readings were taken in a spectronic 21 with reagent blank at 560 nm. The percent Transmittance was converted into Absorbance. The amount of cholesterol was measured by comparing with the optical density (O.D.) of standard solution.

3.9.6 Estimation of serum phosphorus

Serum phosphorus was estimated by following the method suggested by Gomari (1942). Serum was deproteinised by trichloroacetic acid. The protein free filtrate was treated with the molybdate reagent; when the filtrate was treated with the inorganic phosphate of the filtrate to form phosphomolybdic acid. This was then reduced by metol to give blue coloured oxides of molybdenum. The colour was measured at 680 nm in a spectronic 21 instrument. The amount of phosphorus present was calculated by comparing with standards of known concentrations.

A detailed account of the procedure adopted for phosphorus estimation is given below:
**Calculations:**

Concentration of sample = \( \frac{\text{O.D of sample}}{\text{O.D of Standard}} \) \times \text{Conc. of Standard.}

Inorganic phosphorus in 0.5ml serum = \( \frac{\text{O.D of sample}}{\text{O.D of Standard}} \) \times 0.025

Inorganic phosphorus in 100ml serum = \( \frac{\text{O.D of sample}}{\text{O.D of Standard}} \) \times 5

The calculated values were expressed as mg/100ml.

Details of the laboratory procedure adopted in Serum phosphorus estimation is given below:

**Materials used:**

Serum, 10 per cent Trichloro – acetic acid, Molybdate reagent, Metol solution, Standard phosphate solution

**Preparation of reagents:**

10 per cent trichloroacetic acid: 10g of trichloro acetic acid was dissolved in 100 ml of water.

**Molybdate reagent:**

7.5 g ammonium molybdate was dissolved in 200ml of water. 10ml of Conc. Sulphuric acid was added and made up to 400ml with water.
**Metol Solution:**

(P – diemethyl aminophenol sulphate). 1g of metol was dissolved in 100ml of 3 per cent sodium bisulphate.

**Standard phosphate solution:** 0.2197g KH$_2$PO$_4$ was dissolved in water made up to 1 liter (1.0ml = 0.05mg p) and preserved with a few drops of chloroform.

**Procedure:**

0.5 ml of serum was taken in a 15 ml centrifuge tube and 7.2ml of 10 per cent trichloroacetic acid was mixed well and centrifuged. The three tubes were marked A, B, and C for Standard, Test and Blank respectively. The procedure undertaken was as follows:

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ml</strong></td>
<td><strong>ml</strong></td>
<td><strong>ml</strong></td>
<td></td>
</tr>
<tr>
<td>Std Phosphate</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Protein free supernatant of serum</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>10 per cent trichloro acetic acid</td>
<td>4.5</td>
<td>-</td>
<td>5.0</td>
</tr>
<tr>
<td>Molybdate reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Matol reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The contents of each tube were mixed and kept for 30 minutes. Readings were taken at 680nm. The per cent Transmittance of Standard and Test solutions were read and converted into Absorbance. The amount of phosphorus was
measured by comparing with the optical density (O.D.) of standard solution.

3.9.7 Estimation of Serum Sodium

Serum inorganic ions such as sodium and potassium were estimated in a digital flame photometer (Model – 4.22D) by using the principle of emission spectroscopy (Rajagopal and Ramakrishnan, 1983). The calculated values were expressed as mg/100ml.

3.9.8 Estimation of Serum Potassium

Serum inorganic ions such as sodium and potassium were estimated in a digital flame photometer (Model – 4.22D) by using the principle of emission spectroscopy (Rajagopal and Ramakrishnan, 1983). The calculated values were expressed as mg/100ml.

3.10 STATISTICAL ANALYSIS

To obtain the toxicity curve and the LC50 value regression analysis was performed using Toxi Biol software package and the SPSS (Version 11.0) package conclusions were drawn referring to Sokal and Rohlf (1981).