II. MATERIAL & METHODS

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A) MATERIAL

1) Plant Material :

i) Botanical considerations of plant L. eriocephalus :

Lasiosiphon eriocephalus Decaisne (Marathi Rametha or Rami or Datpadi).

This is a much branched shrub or small tree growing in the Western Ghats of India. The healthy twigs of this plant were collected from the hills of Panhala, 15 Km. away from the Kolhapur, in the month of October, 1987.

ii) Classification of L. eriocephalus :

Division	-	Angiosperms
Class	-	Dicotyledons
Series	-	Apetalae
Order	-	Daphanales
Family	-	Thymeleaceae
Genus	-	Lasiosiphon
Species	-	eriocephalus
Example	-	Lasiosiphon eriocephalus

iii) Biological properties :

Leaves : The tree has many branches with mottled barks. The leaves are subsessile, oblong lanceolate acute, apiculate, glaucous and glaborous above, glaborous or silky beneath, base acute.

Flowers : Petioles are very short, yellow or yellowish white, sessile with dense terminal head, surrounded at the base by an involucre of large imbricate eliptic oblong acute, silky-long lobes. Lobes are obtuse flat enclosed in the perianth.

iv) Chemical properties :

This tree causes powerful burning of eyes, nostrils and face when contacted. It is also powerful vesicant. The hexane extract of the roots was found to be lethal to cats at 0.5 mg/kg. A biscoumarin lasiosiphalin was isolated from this plant but does not appear to be toxic principle. The toxic constituents are probably diterpenoids of the daphnetoxin group (Viswanathan and Joshi, 1983).

The leaves are the most toxic part, however, bark and stem have also toxic principles in them. The ethanol extract of the leaves have been shown to be lethal to the fish T. mossambica at very low concentration (Harold, 1987).

2) Animal material :

i) Collection of fish :

The undesirable variety of fish. S. mossambica were selected as experimental animals. The selected fishes were 2 to 3 months old and 35 to 50 gm in weight. They were collected from Kapiltirth tank, Kolhapur in the month of December, 1987.

ii) Classification of fish :

Grade - Pisces

Class - Osteichthyes

Order - Cypriniformes

Sub-order - Chichilids

Genus - Sarotherodon

Species - mossambica

iii) Organs selected for study :

The important target organs including liver, kidney, gill and brain were selected for the present investigation.

B) METHODS

1) Method of piscicide extraction :

The leaves of L. eriocephalus were shed dried and powdered. The powder was taken in Soxhlate's apparatus and extracted with adequate amount of ethyl alcohol. The soxhalation was run for 9-10 hours. Thus, the extract was obtained and then concentrated at controlled temperature (38 - 40°C) in ovan. Dried compound was always stored at low temperature in freeze (0° to 4°C) in order to maintain the potency and to avoid the contamination.

2) Experimental procedure :

The fishes were collected from nature and were acclimatized to the laboratory conditions at 20 ± 2°C in aquaria for 3 weeks. All the aquaria were thoroughly cleaned every week with disinfecants, air dried and then fishes were transferred in aquaria having water. They were fed on alternate days with dry food 'fish feed'.

Feeding was stopped before 24 hrs of the commencement of the test. The fishes were not fed during the test period. Six different concentrations of ethanol extract were prepared containing 20, 40, 80, 120, 160 and 200 ppm. Twenty fishes were exposed at a time to a desired concentration in separate aquarium. A control of twenty fishes was also maintained for each experiment. Ten fishes were removed from each experimental and controlled group at the end of specific time interval and their length and weights were determined.

The fish were sacrificed for assessing their haematological parameters as per the standard methods (Brown, 1976) and for estimation of alkaline phosphatase activity in the target organs like liver, kidney, gill and brain. The blood samples and various tissues of the selected organs were taken at different hours as shown in the following table :

Concentration of ethanol extract (ppm)	Time when blood and tissues were taken (at 12 hrs interval)
20	96
40	84
80	72
120	60
160	48
200	36

The blood samples were collected through the caudal vein of the fish. The different haematological parameters including R.B.C. number/mm^3

W.B.C. number/mm^3, clotting time in minutes and percentage of haemoglobin were estimated at different hours as mentioned above. Similarly, various tissues were processed for histoenzymological detection and biochemical assay of alkaline phosphatase activity during intoxication due to piscicide in the ethanol extract of the leaves of L. eriocephalus.

3) Haematological Methods :

The total number of erythrocyte (TRBC) and total number of leucocytes (TWBC) in blood were estimated by making a count of them in diluting blood employing an instrument haemocytometer. The haemocytometer or counting chamber is a microscope slide upon which is ruled a series of small squares of known size. When a coverslip is placed on this slide, a small chamber is created into which diluted blood can be spread. The standard depth of the chamber is 1/10 mm. since the dilution factor of blood, the area and depth of the chamber are all known, it requires only a simple conversion to express the blood count in terms of numbers of cells per cubic millimeter of undiluted blood.

The TRBC and TWBC counts in the present investigation were taken in improved Neubauer's haemocytometer. The improved Neubauer's haemocytometer has a deep H-shaped groove. Each central platform bears one counting chamber, each counting chamber is formed by several straight perpendicular and horizontal lines enclosing squares of various measurements. The over all area on the scale is 9 mm^2, 3 mm on each side. When white blood cells are considered, the cells found on 4 mm^2 are counted. The four large corner squares (each of which is 1 mm^2 and is divided into 16 smaller squares) are used for this purpose. When the

number of red cells is estimated, the central area is used. The central square millimeter is divided into 25 smaller squares. Each square measures 1/5 x 1/5 = 1/25 mm^2. Each of these squares is further divided into 16 smallest squares making a total of 400 divisions. Each with an area of 1/400 mm^2.

The haemocytometer includes two graduated pipettes in which dilution of blood is done. Dilution pipette for red cells has a capillary stem of a uniform bore with a well ground conical tip. Just above the stem is a bulb. The capacity of the capillary stem is designated arbitrarily as 'one' while the capacity of bulb is 100 times that of the stem. The junction between the stem and the bulb is marked and labelled as '1'. The capillary stem is divided into 10 equal parts from the tip to the mark '1' and the fifth division is again heavily marked and labelled as '0.5'. Just above the bulb there is mark of 101. The bulb contains red bead helps mixing the blood with diluting fluid and allows easy identification of the pipette at distance. The pipette has a white glossy surface opposite the graduation marks. This facilitates reading the pipette. The other end of the pipette is fitted with a soft and light rubber tubbing and mouth piece. A dilution pipette for white cells has graduation 0.5, 1 and 11 and white bead in the bulb.

i) R.B.C. count (TRBC) :

Requirements :

Haemocytometer, microscope, cotton plug etc.

Hayem's diluting fluid :

NaCl	-	0.65 gram
Na_2SO_4	-	5 gram
$HgCl_2$	-	0.5 gram
Distilled water	-	200 ml.

Procedure :

Haemocytometer was kept ready, the counting chamber was examined under the microscope and confirmed that it was clean. The microscope was adjusted so that the ruled lines were seen clearly. The blood was obtained by cutting the caudal vein of fish, by taking all the necessary precautions. The blood was drawn upto 0.5 mark of the diluting pipette.

Then diluting fluid was taken in watch glass and sucked accurately upto the 101 mark. This process yielded 1 : 200 dilution of the red blood cells. The pipette was held horizontally and removed the rubber tubbing It was rolled between the palms to ensure thorough mixing.

Charging the chamber :

The coverglass was placed resting over the flanges on either side of the depressed platform. At least three drops of diluted blood were blowed out. The pipette was held at an angle of about 45^{o} and touched it in the centre of the depressed platform and edge of the coverglass. Because of the capillary action the fluid ran in the capillary space to fill it. When drop was too big and was formed too rapidly it came over in the moat and over the coverglass. In case of such an accident the

process was repeated. After charging the chamber correctly, the fluid was allowed to settle for a period of three minutes on the stage of microscope. Now the slide was fixed on the stage with clips, adjusted the light, mirror and diaphragm so as to give the best viewing field. The focus was adjusted at the central one sq. mm. ruled area under the low power. It was changed over to high power focus and adjusted the high till the cells and the rulling were seen correctly. The cells were counted. While counting the number of erythrocytes in chamber, those touching to lower and right hand lines were discarded but all those touching the upper and left hand lines were included.

Calculations :

Let 'N' be the number of cells counted in 80 small squares.

The number of cells in one small square is N/80

Area of one small square is 1/400 sq. mm.

Depth of diluted blood is 1/10 mm

$\therefore$ Volume of one small square is 1/400 x 1/10 = 1/4000

The cells in 1 c.mm is $\frac{N}{80} \times 4000$

The blood was originally diluted 1 in 200

$\therefore$ Number of cells per c.mm is $\frac{N \times 4000 \times 200}{80}$ that is N x 10,000

ii) White blood cell count : (TWBC) :

Requirements :

Haemocytometer, microscope, cotton plug, etc.

Diluting fluid :

Acetic acid - 2 ml

Gentian violet 1% - 1 ml

Distilled water was added to make the volume upto 100 ml. The glacial acetic acid haemolyses the red cells, while the gentian violet slightly stains the nuclei of white cells.

Procedure :

The apparatus was kept ready as per the R.B.C. counting.

For W.B.C. counting, fresh blood is required so the caudal vein was cut and 0.5 ml blood was drawn in W.B.C. diluting pipette and sucked the diluting fluid upto 11 mark. The pipette was sucked to mix thoroughly. Blood was accurately diluted 1:20 with diluting fluid.

The counting of the white blood cells was done under low power only. Two to three drops of diluted blood were dropped at the inner edges of the slide and the cover glass permited the cells to settle them, counted the W.B.C. in four big squares of the slide. In counting W.B.C., those touching to inner lines on the right and top were included but those touching the line on the left and bottom were discarded.

Calculations :

Let 'N' be the number of W.B.C. counted in 4 large squares.

The number of cells in large square = N/4

Volume of each big square is 1/10 c.mm.

Number of cells in 1 c.mm. = N/4 x 10

Blood is diluted 1 in 20.

Number of W.B.C. per c.mm of undiluted blood is N/4 x 10 x 20 =

$$= \frac{N \times 200}{4} = N \times 50$$

Thus number of W.B.C. per c.mm = N x 50.

iii) Blood smears for morphological observations :

Thin unilayered 10 slides of blood smears of the controlled and experimental fish from every set were prepared and stained with Leishman's stain in a routine manner for morphological observations of blood cells.

iv) Clotting time (CT) :

Capillary tube method :

3-4 capillary tubes about 8 cm x 1.2 cm were prepared. The fish was cleaned and punctured the caudal vein deeply. Then the blood was taken in capillary tubes by capillary action and noted the time. The tubes were placed on a table. At the end of two minutes about 1 cm length of the capillary tube was broken off, at every 10 seconds. The coagulation time was recorded when the fibrin threads of the blood bridged the broken ends of the capillary tube when they separated a distance of 5 mm. The second and third tubes were used to check the observed result.

v) Haemoglobin :

Percentage of haemoglobin was determined by two methods :

1) Sahli's method and 2) Wong's method.

1) Sahli's method :

Requirements :

A haemometer or a haemoglobinometer set contains : A rack with standards fixed in front of a ground glass, a specially graduated tube, a glass rod, a blood pipette with a 20 c.mm mark and a rubber tubbing

(about 1 feet long) with mouth piece to suck the blood.

N/10 Hydrochloric acid.

Procedure :

The graduated tube was placed between the standards in the mark. It was filled with N/10 HCl upto the lowest mark. The sample of blood was obtained and the pipette was filled upto 20 c.mm. mark. The tip was diped in the acid and blowed out gently till all the blood was thrown out at the bottom. The superficial acid was sucked until all the blood in pipette was washed out in the acid. The blood was mixed in the acid with the glass rod.

Waited for sometime (for about 7 to 8 minutes) and then readings were taken. A drop of water was added and mixed again. After adding 2-3 drops of water and mixing, the colour was compared. While comparing, the glass rod was taken out of the solution. The colour was compared while holding the rack against indirect bright but diffused light.

Observations and calculations :

The lowest point of the miniscus was read and recorded. This indicates the gm of haemoglobin per 100 ml of blood or percentage of haemoglobin.

2) Wong's method :

Apparatus :

1) Oswald's micropipette, volumetric flasks (50 ml), test tubes, filter papers, beakers, spectrophotometer.

Chemicals :

Conc. H_2SO_4, 10% sodium tungstate solution, saturated potassium persulfate solution, standard iron solution containing 0.1 mg of ferric iron per ml, 3 N potassium thiosulphate solution.

Procedure :

Preparation of unknown :

With the help of Oswald's micropipette, 0.5 ml well mixed oxalated whole blood was accurately transferred to 50 ml volumetric flask. And then 2 ml of iron free concentrated H_2SO_4 was added and mixed by whirling one or two minutes, then 2 ml of saturated potassium persulfate was added and diluted to 25 ml with water. To this 2 ml of 10% sodium tungstate solution was added. Mixed and cooled to room temperature under the tap, and diluted to volume with water. Then stoppered and mixed the solution by inversion. The solution was filtered through a dry paper and filtrate was collected in dry flask.

Preparation of standard :

Standard solution was prepared in a second 50 ml volumetric flask. 25 ml water was added in the flask, followed by 2 ml of concentrated sulfuric acid, 2 ml of saturated potassium persulfate solution and 2.5 ml of standard iron solution (containing 0.1 mg of ferric iron per ml). Then cooled to room temperature and diluted upto the mark.

Preparation of blank :

Blank solution was prepared similar to standard solution but addition of standard iron solution was omitted.

In three separate test tubes, 20 ml of unknown, standard and blank solutions were taken, respectively. To each test tube 0.5 ml of saturated persulfate solution was added. Then 2 ml of 3 N potassium thiocyanate solution was added in each tube. The solutions were mixed and readings were taken within the next 30 minutes, by setting the photometer to 100% transmitance with the blank solution at 480 mμ.

Calculations :

Per cent transmitance of unknown and standard solutions were recorded and percentage of haemoglobin was calculated by using following formula :

$$\frac{\text{\% Transmitance of standard}}{\text{\% Transmitance of Unknown}} \times \frac{\text{Amount of Standard}}{\text{Amount of Unknown}} \times \frac{100}{3.4}$$

= % Haemoglobin in blood.

4) Enzymological Procedures for Alkalline Phosphatase :

i) Biochemical assay :

The activity of the enzyme was determined according to the method described by Linhardt and Walter (1965).

Preparation of sample :

The homogenization of tissues was carried out in glass morter and pestle which were previously washed, rinsed in distilled water, dried and kept in the ice box of a refrigerator nearly for 3 hours. Such a homogenization has advantages, because there is no loss of enzyme activity due to low temperature or the temperature increase due to friction of the mortor and pestle does not exceed beyond $12^{o}C$ at the end of homogenization.

Reagents :

Alkaline buffer substrate solution : 0.05 M glycine buffer, 5.5 x 10^{-3} M p-nitrophenyl phosphate, pH 10.5

375 mg glycin, 10 mg $MgCl_2.6H_2O$ and 165 mg-p-nitrophenyl phosphate (Sigma) were dissolved in 42 ml 0.1 N NaOH and diluted to 100 ml with doubly distilled water stored in freeze.

0.02 N NaOH.

Enzyme reaction :

To each test tube 1.0 ml of substrate buffer was added and kept for equilibration at 37°C for 10 minutes. Then 0.1 ml of sample was added. The tubes were shaken and kept for incubation for 30 minutes at 37°C. After incubation the reaction was terminated by adding 0.02 N NaOH. A control tube contained 1.0 ml substrate. After the addition of the NaOH to the control tube, 0.1 ml sample was added (All the tubes were centrifused) and the readings were taken against control at 405 mμ

Calculations :

Alkaline phosphatase activity was directly measured by using formula suggested by Linnhardt and Walter (1965) for tissue samples.

$$\frac{\text{O.D. x 11.82 x dilution}}{\text{0.1 x weight of tissue in gm}}$$

= Alkaline phosphatase activity in paranitrophenol μ mol/g.

(ii) Histoenzymological Method :

Substituted naphthol method for alkaline phosphatase :

(Burstone, 1962)

Tissue : The fresh frozen cryostat sections of liver, kidney and gill were taken and fixed in cold acetone for 5 minutes before incubation. Some sections were also fixed in formal-calcium for 3 to 4 hours at $0^{o}C$ and used.

Preparation of incubation medium : 10 mg naphthol AS-MX phosphate was dissolved in 0.25 ml dimethyl formamide. This was diluted with 25 ml distilled water and to this 25 ml tris buffer (0.2 M, pH 8.4 to 8.6) was added. Just before the use 30 mg fast red TR was added and the solution was filtered.

Procedure : i) Sections were incubated for 15 min to 2 hr at room temp.

ii) Washed in water for 5 minutes.

iii) Counterstained with Mayer's hematoxylin.

iv) Washed in water for 5 minutes.

v) Mounted with glycerine jelly.

Result : The red azodye indicated the alkaline phosphatase activity and the nuclei were black.