CHAPTER 2

MATERIALS AND METHODS

II. MATERIALS AND METHODS

1. MATERIAL

A) Choice and Site of Collection of Plant :

The indigenous plant, Lasiosiphon eriocephalus Dacaisne selected for the present studies is belonging to the family Thymeleaceae. It is locally known as Rametha or Datpadi . Plants, especially healthy twigs were collected from the hills of Panhala, which is located 15 Km away from Kolhapur, from where the mountain Sahyadri and Western Ghat starts. The plants were collected from their natural habitat in the month of December, 1985.

B) Classification of the Plant :

Division	-	Angiosperms
Class	-	Dicotyledons
Series	-	Apetalae
Order	-	Daphnales
Family	-	Thymeleaceae
Genus	-	Lasiosiphon
Species	-	Eriocephalus.

C) Morphology of the Plant :

A much branched shrub, sometimes a small tree with mottled barks The leaves are subsessile, 2.3 by 3/4 - 1 inch oblong lanceolate acute, apiculate, glaucous and glaborous above, glaborous or silky beneath, base

acute; petioles very short, flowers yellow or yellowish white, nearly sessile in erect dense terminal heads 1 - $1^{1}/_{2}$ inch in diameter, surrounded at the base by an involucre of large imbricate elliptic oblong, acute silky - long; lobes 1/10 - 1/8 in long, oblong obtuse flat, with a linear usually long, ellipsoid - oblong pointed enclosed in the perianth.

D) Selection of the Fish :

For the present study Tilapia mossambica Peter's was selected. This is a common fish in the rivers, the reservoirs and the ponds surrounding Kolhapur city. It is easily available and for the reasons mentioned in the analysis of the problem in the introductory chapter, this species of Tilapia was selected.

E) Classification of Fish :

Grade	-	Pisces
Class	-	Osteichthyes
Order	-	Cypriniformes
Sub-order	-	Cichlids
Genus	-	Tilapia
Species	-	Mossambica

2. METHODS

There are four types of methods used for the present investigation. First two types are for extraction and characterization of active principle from the plant and other two types for application of the plant toxin to

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observe behavioural and lethal effects and for demonstration and characterization of different mucosubstances in the different tissues selected for the studies. These four types of methods are as follows :

1) Extraction methods,
2) Methods of Analysis,
3) Experimental Procedures,
4) Histological and Histochemical methods.

A) Extraction Method :

The leaves of Lasiosiphon eriocephalus, Decaisne were collected from the Panhala Ghat region. The shade dried leaves were powdered (40 mesh powder). The dry powder (250 gm) was extracted continuously with three solvents, benzene, chloroform and ethanol, using a Soxhlet apparatus. The extraction was continued till the each solvent was free from any soluble material.

Benzene Extract : The benzene extract was collected and was distilled in vacuo under reduced pressure to get a dry dark green powder. Henceforth the dark green powder will be referred to as Benzene Extract (BE).

Chloroform Extract : The chloroform extract which was greenish yellow in colour was collected and distilled in vacuo under reduced pressure. This would be known as Chloroform Extract (CE).

Ethanol Extract (EE) : was the alcoholic extract which was dark brown in colour.

These compounds were stored at low temperatures (0^{o} to $2^{o}C$) in order to maintain the potency of the extracts and to avoid contamination.

B) Methods of Analysis :

Following phytochemical methods were adopted for the further analysis.

i) Melting points were determined by using paraffin tube method.

ii) UV Spectra were recorded on a Shimadzu Spectrophotometer UV-240.

iii) NMR Spectra were recorded on Perkin-Elmer Model Spectrometer R 32, USA .

iv) IR Spectra were recorded on Perkin Elmer, 783 UK .

v) Atomic Absorption Spectrophotometer, Perkin-Elmer 3030, USA was used.

vi) TLC was carried out on plates coated with Silica gel G. The plates were activated prior to use by heating $110^{o}C$ for 1 hr. The thickness of the silica gel was 35 mm. The chromatograph was developed after exposing in Iodine chamber. The results were recorded and the spots were eluted for further analysis.

C) Experimental Procedures :

The experiments were carried out in a rectangular glass container (Acquaria) of 25 litres capacity. The fish was acclimatized to the laboratory conditions for a week in the conditions similar to their natural habitat. Special care was taken to maintain the same DO, pH, temperature and hardness of the water. Feeding was stopped before 24 hrs. of the commencement of the test. The fishes were not fed during the test period.

Three compounds were dissolved in an appropriate solvent and a desired concentrations were prepared for bio-assays.

a) Benzene Extract (BE) :

A desired quantity of the extract was dissolved in benzene.A known quantity of the diluted BE was added to the test container (Acquarium). In the normal (control) test container the same amount of benzene, which was used to dissolve the BE was added. The number of fishes used for the experiment ranged from 10-20. The weight of fish ranged from 25-50 gms and the length from 5-7 cm. The behavioural changes were closely observed for the first two hours. Dissolved oxygen, pH, temperature and hardness of the water in the each container were recorded before and after the expe-riments. The observations were continued and recorded for a period of 24 hours.

b) Chloroform Extract (CE) :

CE was dissolved in chloroform and a desired quantity of the CE was added to the test containers. The experiments were carried out as in BE.

c) Ethanol Extract (EE) :

Since alcoholic extract was water soluble, various concentrations of this compound were prepared by using chlorine free tap water. The earlier procedures for carrying out the experiments for BE and CE were followed.

d) LC_{50} :

The LC_{50} values were calculated by semi-log graphical interpolation method. The LC_{50} values were calculated by plotting the readings on the graph paper, where percent mortality was taken on the y-axis and per cent concentration on x-axis. The interpolation at 50 % mortality thus calculated gave the LC_{50} values (Doudoroff, 1951).

D) Histological and Histochemical Methods :

The small pieces of different organs, liver, gills, kidney and buccal epithelium of a control fish, were dissected out and were fixed in the ice cold calcium acetate formalin (CAF-2 % in 10 % formalin fixative) for 24 hrs. (Spicer et al.,1967). Similarly, these organs were taken from the fish after exposure to a desired concentration of the plant toxin (extract) for a certain period of time or when the fish was in a coma state.

The fixation of the tissues was followed by washing in chilled distilled water and in running tap water, dehydration in alcohol, cleaning in xylene and paraffin embedment. The sections were cut at 5 to 6 µ. The sections were employed for various histological and histochemical staining techniques described below.

a) Histological Method :

For histological observations routine Haematoxyline-Eosine (HE) method was used before observing mucosubstances in the various tissues, selected for the present studies.

b) Histochemical Methods :

Spicer et al. (1967) suggested the histochemical classification of mucosubstances in which the mucins were named by staining site in which they are found and subtyping them as far as possible as neutral mucosubstances, acidic mucosubstances (sulfomucins, sialomucins and hyaluronic acid). The specificity of various methods are described below :

i) NEUTRAL MUCOSUBSTANCES :

A) Periodic Acid - Schiff Reaction (PAS)
(McManus, 1946 Hotchkiss, 1948)

1) After dewaxing and hydrations sections were brought to distilled water.
2) Oxidized with 0.5 % periodic acid for 10 minutes.
3) Washed with distilled water.
4) Treated with Schiff's Reagent for 10 minutes.
5) Rinsed three times (total 6 minutes) with 0.5 % sodium meta-bisulphite.
6) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in canada balsam.

Result : Periodate reactive, hexose containing Mucosubstances stain pink Magenta.

B) Diastase Digestion - PAS Technique for Identification of Glycogen
(Li llie, 1954; Lison, 1960)

1) After dewaxing and hydrations sections were brought to distilled water.

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2) Incubated for one hour at 37°C in the following medium 0.1 % malt diastase in 0.2 M phosphate buffer at pH 6.0.

3) Washed in distilled water.

4) Processed as in I-A for PAS, staining procedure.

Result : Loss of PAS reactivity or reduction in the staining intensity indicates the presence of glycogen.

ii) ACID MUCOSUBSTACES :

A) Alcian Blue (AB) at pH 2.5 (Mowry, 1956)

1) After dewaxing and hydration, sections were brought to distilled water.

2) Rinsed in 3 % acetic acid.

3) Stained with AB (1 % AB in 3 % acetic acid pH 2.5) for 30 minutes.

4) Rinsed in 3 % acetic acid.

5) Washed in running water for 5 minutes.

6) Dehydrated, cleared and mounted as usual.

Result : Weakly acidic mucosubstances, hyaluronic acids and sialomucins stain dark blue. Strongly acidic sulfated mucins are stained weakly or not at all.

B) Alcian Blue (AB) at pH 1.0 (Lev and Spicer, 1964)

1) After dewaxing and hydration sections were brought to distilled water.

2) Stained for 30 minutes in 1 % AB in 0.1 N HCl (pH 1.0).

3) Blotted on a puffless filter paper.

4) Dehydrated quickly, cleared and mounted as usual.

Results : Only sulfomucins stains intense blue.

iii) DISTINCTION BETWEEN NEUTRAL AND ACIDIC MUCOSUBSTANCES:

A) AB pH 2.5 - PAS Sequential Staining Technique

(Mowry and Winkler, 1956; Mowry, 1963)

1) After dewaxing and hydration, sections were brought to distilled water.
2) Rinsed briefly in 3 % acetic acid.
3) Staining with 1 % AB in 3 % acetic acid (pH 2.5) for 30'.
4) Rinsed in 3 % acetic acid.
5) Washed in distilled water for 5 minutes.
6) Processed as in I-A for PAS staining technique.

Results : Alcian blue reactive periodate unreactive acid mucosubstances stain blue, alcian blue and PAS reactive mucosubstances stain blue purple and PAS reactive but Alcian blue unreactive mucosubstances colour magenta.

B) AB pH 1.0 - PAS Sequential Staining Technique

(Spicer, 1965; Spicer et al., 1967)

1) After dewaxing and hydration, sections were brought to distilled water.
2) Staining with 1% AB in 0.1 N HCl (pH 1.0) for 30 minutes.
3) Sections were blotted on a puffless filter paper.
4) Processed as in I-A for PAS staining technique.

Result : Only sulfomucins are stained blue or purple blue. NON sulfated and only periodate reactive mucosubstances are stained pink-magneta.

iv) DISTINCTION BETWEEN SULPHOMUCINS AND CARBOXY MUCINS:

A) Aldehyde Fuschsin (AF) :

(Gomori 1950; Halmi and Davies, 1953).

Preparation of AF Crystals :

The crystals of AF were prepared according to the method suggested by Cameron and Steal (1959). To 200 ml boiling distilled water, 1 gm of basic fuschsin was added and solution was allowed to boil for one minute, then cooled and filtered. To the filtrate, 2 ml of conc HCl and 2 ml. of paraldehyde was added. The solution was left stoppered at room temperature. When the solution had lost its reddish colour, usually after 3-4 days, it was filtered and the filterate was discarded. The precipitate was dried on the filter paper at $60^{o}C$.

Procedure :

1) After dewaxing and hydration, sections were brought to distilled water.
2) Rinsed in 70 % alcohol.
3) Stained with AF solution for 30 minutes
4) Rinsed in 70 % alcohol.
5) Dehydrated in 90 % and absolute alcohol cleared and mounted as usual.

Result : Sulfated mucosubstances are stained dark-purple, sialomucins and hyaluronic acids stain light purple some elastic fibers also stain intense purple.

B) Aldehyde Fuschsin - AB (AF-AB pH 2.5), Sequential Staining Techniqu: (Spicer and Meyer, 1960).

1) After dewaxing and hydration, sections were brought to distilled water.
2) Rinsed in 70 % alcohol.
3) Stained in AF staining solution for 30 minutes.
4) Rinsed in 70 % alcohol.
5) Washed in running water for 5 minutes.
6) Rinsed in 3 % acetic acid.
7) Stained with (AB pH 2.5) for 30 minutes.
8) Rinsed in 3 % acetic acid.
9) Washed in Running water for 5 minutes.
10) Dehydrated, cleared and mounted as usual.

Result : Sulfated Mucosubstances stain purple. Non-sulfated mucosubstances like sialic acids and hyaluronic acid stain blue.

TABLE No.1

The list of Piscicidal plants, their botanical names, active principles, effective doses, duration of fish species and the references in the literature.

No.	Name of Plant	Part of plant used	Active principle	minimum effective dose.ppm.	Time taken for 100% Mortality, Hr.	Fish species used	References
1.	Croton tiglium	Seed powder		3 to 5 ppm			Babu, 1965 & Bhuyan, 1967.
2.	Bassia latifolia	Mahua oil	Saponin (4-6 %)	75 ppm (in pond) 60 ppm (Laboratory)		Cirrhinus mrigala, Puntius ticto, Cyprinus carpio, Colisa fasciata, Channa gachua.	Barrackpore, 1968 and Bhatia, 1970.
3.	Barringtonia acutangula	Seed (Powder)	2 Saponin types*	20 ppm	48 hr.	Wide variety of fish	Barrackpore, 1968
4.	Randia dumetorum	Fruit (Unripe powder)	- ,, -	12 ppm			
5.	Walsura piscidia	Bark (Powder)	- ,, -	10 ppm		Tilapia, Murrels	Chakraworthy, 1972*
6.	Camdtia sipensis	Seed (Tea cake)	Saponin (7-8 %)				Chowdhury, 1968.
7.	Nicotina tobacum	Leaves	Nicotin				Konar, 1969
8.	Millettia pachycarpa	Root (Powder)		2-6 ppm			Bhuyan, 1968

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Table No.1 contd....

No.	Name of Plant	Part of plant used	Active principle	minimum effective dose.ppm.	Time taken for 100% Mortality, Hr.	Fish species used	References
9.	Myrica esculenta Buch-Ham.	Bark		80-100 ppm	12-15 hr		) Ramanujam)) and Ratha)) (1980a,) 1980b,1983))))
10.	Polygonum hydropiper L. var. hydropiper	Leaf		100-125 ppm	10-12 hr		
11.	Potentilla fulgens Wal ex.Lehm	Root		150-200 ppm	8-10 hr		
12.	Xeromphis spinosa (= Randia dumetorum) (Thumb.)Key	Fruit		120-140 ppm	10-12 hr		Ramanujam and Ratha (1983)
13.	Zanthoxylum armatum DC. (= Z.alatum Roxb.)	Fruit (powder)	5 extracts	60 ppm (powder)	8-9 hr	Puntius shalynius, Heteropneustes fossilis (Bloch)	Ramanujam and Ratha (1980a)

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Table No.1 contd...

No.	Name of Plant	Part of plant used	Active principle	Minimum effective dose.ppm.	Time taken for 100% Mortality, Hr.	Fish species used	References
14	Acorus calamus	Root Rhizome, Leaves, Fruits		200 ppm	48 hr	Puntius ticto Ham., Barilium bendelisis Ham., Mystus vittatus Bloch, Nemacheilus rupecola M.Clelland, N.botes Ham. Heteropneustus fossilis Bloch, Channa punctatus Bloch.	Virdi (1982)
15.	Sapindus mukorussi	Root bark, Stem bark leaves, Green twig, Fruit pericarp, Seed, endosperm		200 ppm. (Except pericarp)	48 hr. Root bark, Stem bark, Fruit pericarp		
16.	Xeromphis spinosa (Thumb.) Keay.	Root bark, Stem bark, leaves, fruit.		200 ppm	48 hr. Root bark, Fruit pulp, Seeds.		
17.	Pimelea sp. (Thymelaeceae) P.ligastrina P.linifolia			Daphnane orthoesters 1)Simplex macrin 2)Simplex linimacrin 3)Factor P3 pimelar 4)Linimacrin C			Tyler et al., 1985.

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Table No.1 contd..

No.	Name of Plant	Part of plant used	Active principle	Minimum effective dose.ppm.	Time taken for 100% Mortality, Hr.	Fish species used	References
18.	Euphorbiaceae (Critonoideae Euphorbiaceae) Thymelaeaceae (Daphen,Gnidia, Lasiosiphon,Pimelea) Sunaptolepis, Acquilaria, Daphnopsis.		Tigliane, Daphnane Ingenane			Killi fish - Oryzias latipes	Hirota et.al., 1980. Thorne,1968.
19.	Gymnodinium breeve					Mullet - Mugil cephalus	Kim et al., 1974.