CHAPTER-II

Material and Methods

II MATERIAL AND METHODS

1. MATERIAL

A) Choice and Site of Collection of Plant :

The indigenous plant, Sapindus laurifolius (Vahl) selected for the present study belongs to the family Sapindaceae. Generally to be met with about villages in the Konkan southwards to North Kanara Ghats and along Western Ghat in evergreen monsoon forests, Talbot. It is often cultivated in Bengal and in South and North India. It is locally known as Ritha. It is a medium sized to large tree with white flowers. Generally, it and its bark is used as expectorant and demulcent, decoction of its bark is also given to cattle suffering from worm-infested ul cers. Its bark along with its fruits used as fish poison (Agarwal and Ghosh, 1985; Agarwal, 1986). The fruits are also used as detergent and as substitute for soap.

The healthy fruits of this plant were collected from the hills of Panhala of Western Ghat, 15 Km. away from Kolhapur city (Maharashtra) and also from a village, Zarapi(Dist.Sindhudurg) in Konkan near Sawantwadi (Maharashtra).

B) Classification of the Plant :

Division	-	Angeospermae
Class	-	Dicotyledonae
Sub-class	-	Polypetalae
Series	-	Thalamiflorae
Order	-	Sapindales
Family	-	Sapindaceae
Genus	-	Sapindus (Tournef.)
Species	-	S.laurifolius (Vahl.).

C) Morphology of the Plant :

A handsome tree which occurs throughout peninsular India. Leaves abruptly pinnate, leaflets subopposite, 2-3 pairs, 3-7 by 1-4 in, lanceolate or elliptic-lanceolate, acute or acuminate, entire, glabrous above, more or less pubescent beneath, base acute; main nerves about 8-12 pairs; petioles 1/8 in. long, pubescent. Flowers dingy white, in terminal rusty-pubescent panicles, the males numerous, the bisexual flowers few, sepals 5, round-ovate, ciliolate, fulvous pubescent outside, glabrous within, 1/6 in long. Petals 4-5, shortly clawed, narrower than the sepals, lanceolate, villous outside and more or less so within, usually furnished with 2 villous scales attached at each side of the petal about half way up. Disk concave with a fleshy hirsute margin. Stamens 8; filaments villous; anthers oblong, apiculate. Ovary densely hairy. Fruit fleshy, 2-(usually 3-) lobed, clothed with fulvous hairs when young, glabrous and wrinkled when ripe, with 1 seed in each lobe. Seeds blackish, smooth, about the size of a large pea, very hard.

D) Selection of the Fish :

Tilapia mossambica (Peters), a mouth breeding fresh water chichilid fish, which found in rivers, ponds and lakes surrounding Kolhapur city, was selected for the present study. It is easily available and for the reasons mentioned in the analysis of the problem in the introduction chapter, this species of Tilapia was selected.

E) Classification of Fish :

Grade	-	Pisces
Class	-	Osteichthyes
Order	-	Cypriniformes
Sub order	-	Chichilids
Genus	-	Tilapia
Species	-	T. mossambica (Peters)

2. METHODS

The following four types of methods were employed for the present study.The first two types are concerned with the extraction, isolation, characterization and analysis of the natural piscicide, present in the fruits of Sapindus laurifolius (Vahl) and are also concerned with the formulation of the active principle of this plant. The third type is concerned with the analysis of different parameters of water quality. Whereas fourth type is concerned with application of plant piscicide to observe lethal effects, behavioural changes and mucosubstances secretion in different target organs of the fish.

These four types of methods are as follows :

1) Solvent Extraction and TLC separation methods.
2) Phytochemical Analytical Methods.
3) Water quality analysing methods.
4) Physiological response analysing methods.

A) Solvent Extraction and TLC Separation Methods

i) Solvent Extraction Methods :

The dry fruits of Sapindus laurifolius (Vahl) were shade dried and were powdered (40 mesh powder). The dry powder was extracted continuously with

five solvents, petroleum ether, benzene, ethyl acetate, chloroform and ethanol, using a soxhlet apparatus. The extraction was continued till the each solvent was free from any soluble material. Afterwards the each extract was concentrated, evaporated and crystallized by routine chemical extraction and purification methods. The extract obtained were labeled as PE, BE, EAE, CE and EE respectively. These extracts were stored at low temperatures (0^{o} to $2^{o}C$) in order to maintain the potency of the extracts and to avoid contamination till further use.

ii) TLC Separation Method :

TLC was carried out on plates coated with silica gel G. The plates were activated prior to use by heating at $110^{o}C$ for 1 hr. The thickness of silica gel was 0.35 mm. The solvent system was chloroform : methanol (85:15). The chromatograph was developed after exposing in iodine chamber. Immediately after developing the Spot of the chromatograph, Photographs were taken and then these spots were eluted for further analysis.

B.) Phytochemical Analytical Methods

i) NMR Spectrophotometric Method :

The NMR spectra of PE, BE, EAE, CE and EE were recorded on Perkin-Elmer Model R-32 spectrometer U.S.A. using 5% solutions prepared in carbon tetrachloride.

ii) IR Spectrophotometric Method :

The IR spectra of PE, BE, EAE, CE and EE were recorded on Perkin-Elmer model 783, U.S.A. by using mulling agent nujol (a mixture of paraffinic hydrocarbons).

iii) UV Spectrophotometric Metho d :

The PE, BE, EÆ, CE and EE obtained in the pure form were dissolved in CCl_4 solvent. The UV spectra of each extract were recorded on a Shimadzu-UV-240, spectrophotometer.

iv) Melting Points :

The melting points were determined by using paraffin tube method of PE, BE, EAE, CE and EE of the fruit powder.

v.) Inorganic ion Determination Method :

Some of the important inorganic ions were detected in each extract by using Atomic Absorption Spectrophotometer, Perkin-Elmer 3030, U.S.A.

C) Water Quality Analysis Methods

i.) Determination of pH :

The pH of water used during toxicological experiments was measured by glass electrode digital pH meter, before and during the experiment at regular time interval.

ii) Determination of D.O. :

A water sample was taken in 250 ml capacity dark bottle and was treated with 1 ml maganous sulphate solution by a pipettle, with its tip well below the surface of water. This was followed by 1 ml alkaline iodide solution in the same manner. The bottle was stoppered, shook well and allowed the precipitate to settle down. Afterwards 1 ml (or more) H_2SO_4 was added and again shook the bottle until the precipitate dissolved and iodine was liberated.

50 ml of this dissolved solution was titrated with standard sodium thiosulphate (6.25 g/lit) until a very pale straw colour formed. Then 0.5 ml of starch was added in the titration flask and titration was continued till the blue colour disappeared. The volume of sodium thiosulphate solution used in the experiment was noted and from the following formula O_2 content of water was calculated

$$\text{Dissolved } O_2 = 5.04 \times V \times N \text{ mg of } O_2/\text{lit.}$$

where,

N = Normality of sodium thiosulphate and

V = Volume of sodium thiosulphate.

iii) Determination of Hardness :

100 ml sample was taken into a conical flask, into which about 3 drops of phenolphthalein indicator was added. This was titrated with 0.02 N sulphuric acid from the burette till the pink colour disappeared and number of ml of acid used in experiment was recorded. Then 2-3 drops of methyl orange indicator was added into the titration flask and again the titration was continued, with 0.02 N sulphuric acid, till yellow colour disappeared and with further addition of acid, the colour changed to red at the end of the titration. This final number of acid used was also recorded. The total hardness of water in terms of $CaCO_3$ was calculated by using formula :

$$\text{Total hardness mg/lit } CaCO_3 = T - 2P \times 10$$

where,

P = ml of acid used for titration using phenolphthlein and

T = The ml of acid used for total titration (using phenolphthlein plus methyl orange indicators)

iv) Recording the Temperature :

The temperature of water used in the experiments was recorded time to time and as far as possible the experiments were performed at nearly constant temperature ($25 \pm 0.5^{o}C$).

D) Physiological Response Analysing Methods

i) Experimental Procedure for Physiological Study :

The experiments were carried out in a rectangular galss container (Acquaria) of 25 liters capacity. The fish were 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.

Five extracts - PE, BE, EAE, CE and EE were dissolved in an appropriate solvent and a desired concentrations were prepared for bio-assays.

ii) LC_0, LC_{50}, LC_{100} :

The lethal effect of the plant toxin on the fish was assessed in terms of per cent lethality zero hours to 72 hours and were represented as LC_0, LC_{50} and LC_{100} representing no effect, fifty per cent mortality and 100 % mortality respectively.

iii) Behavioural Observations :

The behavioural responses due to plant toxin were estimated on the basis of body movements, opercular movements, responses to the external

stimuli such as touch, light, vibration etc., and secretion of mucus and blood clots by the gills and by the body surface. These responses were recorded at the regular time intervals and at different doses of the plant toxin.

iv) Histological and Histochemical Methods :

The small pieces of different organs, liver, gills, kidney, buccal epithelium and intestine of a common fresh water fish T. mossambica 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 extracted from the fruits of Sapindus laurifolius 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 till all the fixative was washed away, dehydration in alcohol grades, 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 :

To observe the normal histology and altered histopathological changes due to the application of plant toxin, routine Haematoxyline Eosine (HE) method was used before observing mucosubstances in the various tissues, selected for the present studies. These observations were compared with the earlier reports on this fish species.

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 muco-substances, 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 Glycoger
(Lillie, 1954; Lison, 1960)

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

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.

) 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 Technique (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.