CHAPTER-II

Material and Methods

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1. MATERIAL

A) Choice and site of collection of plant :

The plant, Acacia concinna is selected for the present investigation due to its easy and rich availability in the Western Ghat. It is known to contain toxin and it has some biocidal properties (Sawant, 1974).

This plant A. concinna (DC) belongs to the family Leguminosae. Generally to be met with about in Deccan, Caranatic area along Western Ghat in evergreen monsoon forests, some plants are also observed in Andhra Pradesh, Assam and Bihar. It is locally known as 'Shikekai' (Sawant, 1974).

It is stout, very prickly climbing shrub, powder of leaves of this plant used for liver disorders, lice killing and also for cattle poisoning. The powder of seeds has also medicinal value and used to induce vomitting, skin diseases and at the time of parturition. Pods are used as detergents for washing hair, woolen and silk fabrics.

The healthy fruits of this plant were collected from Dapoli, the area in front of agricultural college and from 'Walmiki hills' of Western Ghat, 40 kilometers away from Karad town (Maharashtra).

B) Classification of the plant :

Division	-	Angiospermae.
Class	-	Dicotyledonae.
Sub-class	-	Polypetalae.
Series	-	Calyciflorae.
Order	-	Rosales.
Family	-	Leguminosae.
Sub-family	-	Mimosae.
Genus	-	Acacia.
Species	-	Concinna.
Example	-	Acacia concinna (Decaisne)

C) Morphology of plant :

Distribution and Habit : It is a stout, very prickly, woody climbing shrub which occurs in West coast, Andhra Pradesh and Bihar.

Stem : Surface of stem is hairy.

Leaf : Bipinnate compound, alternate or opposite. Stipulate.

Leaflets are opposite, sessile, shape-oblong, margin-entire, apex - mucronate, leafbase - rounded base, insertion - remal, surface - smooth, Texture - soft, ptyxis - plane. Venation - unicostate reticulate. Leaflets are small more than twenty pairs in each leaf. Each leaf is as long as 3 to 5 cms, while leaflet is 0.5 to 1.0 cms. long and 0.2 to 0.3 cms. wide.

Inflorescence - Globose heads.

Flowering period - March to July.

Flowers - Bractate, regular, bisexual complete, perigynous, pentamerous, sessile and small.

Calyx - Five sepals, gamosepalous, shape - campanulate, aestivation - valvate, petaloid pink colour.

Corolla - Five petals, more or less united, tubular, valvate, yellowish colour

Androecium - Many stamens free, filaments longer than petals, yellow in colour, exserted, anthers bithecous, minute, dehiscense longitudinal-introse.

Gynoecium - Monocarpellary superior ovary, one locule, one ovule on marginal placenta, stigma small terminal, style long filiform, ovary sessile, green hairy, stigma minute.

Fruit - Legume (pod). 7-12 x 2-2$\frac{1}{2}$ cm. long and wide, green when young, becomes red-pink after maturity. Although legumes are generally dehiscent, this fruit is schizocarp.

D) Selection of the fish :

Tilapia mossambica (Peters), a mouth breeding fresh water, chichlid fish, which found in rivers, ponds and lakes around the Kolhapur city, was selected for present study. 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 the fish :

Grade	-	Pisces.
Class	-	Osteichthyes.
Order	-	Cypriniformes.
Sub-order	-	Chichlids.

Genus	-	Tilapia
Species	-	mossambica
Example	-	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 the A. concinna (DC) 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 where as the 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 :

A) Solvent extraction method.

B) Phytochemical analytical methods.

C) Water quality analysing methods.

D) Physiological response analysing methods.

A) Solvent extraction method :

The fruits of A. concinna 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 Soxhlet's apparatus. The extraction was continued to boil till each solvent was free from any soluble material, afterwards each extract was concentrated, evaporated and crystallized by routine chemical extraction and

purification methods. The extracts obtained in petroleum ether, benzene, ethyl acetate, chloroform and ethanol were labelled as E_1, E_2, E_3, E_4 and E_5 respectively. These extracts were stored in desiccator at low temperatures (0^0 to 2^0C) in order to maintain the potency of the extracts and to avoid contamination till further use.

B) Phytochemical analytical methods :

1) Ultraviolet spectrophotometric method :

The extracts E_1, E_2, E_3, E_4 and E_5 dissolved in CCl_4 solvent separately. The UV spectra of each extract were recorded on a Shimadzu-UV-240 spectrophotometer.

The dissolved samples were transferred to a silica cell. The cell is so made that the beam of light passes through a 1 cm thickness of solution. A matched cell containing pure solvent was also prepared. The cell was then placed in an appropriate place in the spectrophotometer. This was so arranged that out of two beams one of ultraviolet light was passed through the solution of sample and another through the pure solvent. The intensities of the transmitted beams were then compared over the whole wave length range of the instrument. The intensity of absorption peak λ max was measured, from this intensity, the type of chromophore in the sample could be decided and from that chromophore's mol. wt. could be calculated.

2) Nuclear magnetic response (NMR) spectrophotometric method :

The extracts E_1, E_2, E_3 and E_4 were dissolved in CCl_4 solvent separately, whereas extract E_5 was dissolved in TFA (trifloro acetic acid) solvent

The NMR spectrum of each solvent was recorded on Perkin-Elmer model, R-32 spectrometer(U.S.A.) using 5% solutions prepared in carbon tetrachloride (CCl_4) and TFA.

Each nucleus has it's own spin. This spin possesses the magnetic movement such nuclei when placed in the magentic field, rotate slowly. By changing field strength different peaks of different groups were obtained. These groups were identified by comparing these spectra of samples with the spectrum of standard sample like T.M.S. | $Si(CH_4)_4$ |.

3. Infrared (IR) spectrophotometric method :

The IR spectra of E_1, E_2, E_3 and E_4 extract were recorded on Perkin-Elmer Model 783, (U.S.A.) by using mulling agent Nujol (a mixture of paraffinic hydrocarbons). IR spectra of E_5 extract was recorded by preparing mixture of this extract with KBr.

The energy of molecular vibrations corresponds to IR region of electromagnetic spectrum. Molecular vibration may be detected and measured in an infrared spectrum. The useful vibrations have the narrower range of 2.5 to 16 μm. The position of bands in spectrum is measured in μm. The functional groups have vibrational frequencies. Hence functional groups can be identified by their characteristic vibrational frequencies, which is useful for assigning a compound of which class is present in the sample.

C) Water quality analysing methods :

1) 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 intervals.

2) Detection of dissolved oxygen :

Water sample wastaken in 250 ml. capacity dark bottle and was treated with 1 ml magnesium sulphate solution by a pipette, with it's tip well below the water. This was followed by 1 ml. alkaline iodide solution in the same manner. The bottle was stoppered, shook well and allowed to settle down.

Afterward 2 ml. (or more) H_2SO_4 solution 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 gms/lit) unitl 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

V = Volume of sodium thiosulphate.

3) Determination of hardness of water :

100 ml of sample was taken into a conical flask, to which about three drops of phenolphthalein indicator was added.

This was titrated with 0.02 N sulphuric acid from the burette till the pink colour disappeared and amount of acid used during titration was recorded. Then two to three drops of methyl orange indicator were 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 amount of acid used was also recorded. The total hardness of water in terms of $CaCO_3$ was calculated by using the formula -

$$\text{Total Hardness as ppm } CaCO_3 = T - 2P \times 10$$

where, P = ml of acid used for titration using phenolphthalein indicator
and
T = ml of acid used for total titration (using phenolphthalein plus methyl orange indicators)

4) Recording the temperature :

The temperature of water used in the experiments was recorded from time to time and as far as possible the experiments were performed at nearly constant temperature ($22 \pm 0.5^{o}C$).

D) Physiological response analysing methods :

1) Experimental procedure for physiological study :

The experiments were carried out in a rectangular glass 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 water. Feeding was stopped before 24 hrs of the commencement of the test and the fishes were also not fed during test period.

2) Methods to record % survival and % mortality of fish :

The extract (E_5) was dissolved into an appropriate solvent and desired concentrations were prepared for bioassays.

The lethal effect of plant toxin (E_5) on the fish was assessed in terms of percent lethality from zero hr to 96 hrs and were represented as LC_0, LC_{50} and LC_{100} representing no effect, 50% mortality and 100% mortality respectively.

3) Estimation of LC_{50} value :

The equation of line of regression of Y on X i.e. $Y = a + bX$, is used for the estimation of LC_{50} value of extract E_5 for fish T. mossambica.

The lethal effect of plant toxin was assessed in terms of per cent mortality from zero to 96 hours. The LC_{50} values for different periods, were calculated by using number of intoxicated fishes and respective concentration in ppm. The number of intoxicated fishes is a dependent value, therefore, denoted by 'Y' and concentration in ppm is an independent value, therefore, it is denoted by 'X'.

The value of 'b' is calculated by the equation

$$b = \frac{\Sigma Ln\ XY - \frac{\Sigma Ln\ X.\ \Sigma Y}{n}}{\Sigma\ Ln\ X^2 - \frac{(\Sigma\ Ln\ X)^2}{n}}$$

where 'n' = number of concentrations.

Value of 'a' is calculated by the equation

$$a = \bar{Y} - b\ Ln\ \bar{X}$$

The table showing different values of a and b was prepared. Then by using these values of 'a' and 'b', the LC_{50} values for different periods were calculated by the equation -

$$LC_{50} = \frac{5 - a}{b}$$

The regression equation between natural log concentrations in ppm (Ln X) and the probit mortality (Y) was derived statistically, using the formula $Y = a + bX$ (LC_{50} value was also estimated by plotting the graph of the equation of line of regression of 'Y' on 'X' i.e. $y = a + bX$).

4) Methods to record behavioural responses of fish :

The behavioural responses to the fruit extract (E_5) were estimated by using the following assets -

a) Movements of the fish.

b) Opercular movements.

c) Responses to the external stimuli like touch, light and vibrations etc.

d) Equilibrium of fish.

e) Physiological changes like formation of blood clot by gills, change in the colour of gills and secretion of mucus by the body surface and gills.

5) Histological and histochemical methods :

The small pieces of different organs including buccal mass, gill, liver, kidney and intestine of a freshly dissected common fresh water chichlid fish T. mossambica 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 from the fruits of A. concinna) 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 washed away, dehydration in alcohol grades, cleaning in xylene and paraffin embedment. The sections were cut at 5 to 6 µm and employed for various histological and histochemical 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 (H-E) 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 :

A) Neutral mucosubstances :

1) Periodic acid - Schiff's reaction (PAS) :

(McManus, 1946; Hotchkiss, 1948)

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

ii) Oxidized with 0.5% periodic acid for 10 minutes.

iii) Washed with distilled water.

iv) Treated with Schiff's reagent for 10 minutes.

v) Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in canada balsm/D.P.X.

Result : Periodate reactive, hexose containing mucosubstances stain pink magenta.

2) Phenylhydrazine- PAS :

(Spicer, 1965; Spicer et al., 1966; 1967).

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

ii) Oxidized with 0.5% periodic acid for 10 minutes.

iii) Followed by treatment with 5% phenylhydrazine for 30 minutes.

iv) Washed with distilled water.

v) Immersed in Schiff's reagent for 10 minutes.

vi) Rinsed three times (total 6 minutes) with 0.5% sodium meta-bi-sulphate.

vii) Washed, dehydrated, cleared routinely and mounted in canada balsm/D.P.X.

Result : Periodate reactive acid mucosubstances are selectively stained. Periodate engendered dialdehydes are blocked.

3) Diastase digestion - PAS techniques for identification of glycogen:

(Lillie, 1954; Lison, 1960).

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

ii) Incubated for 1 hour at $37^{o}C$ in the following medium.

0.1% malt diastase in 0.2 M phosphate buffer at pH 6.0.

iii) Washed in distilled water.

iv) Processed as in A-1 for PAS staining procedures.

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

B) Acid mucosubstances :

1) Alcian blue (AB) at pH 2.5 :

(Mowry, 1956)

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

ii) Rinsed in 3% acetic acid.

iii) Stained with AB (1% AB in 8% acetic acid pH 2.5) for 30 minutes.

iv) Rinsed in 3% acetic acid.

v) Washed in running water for 5 minutes.

vi) Dehydrated, cleared and mounted as usual.

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

2) Alcian blue (AB) at pH 1 :

(Lev and Spicer, 1964)

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

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

iii) Blotted on a puffless filter paper.

iv) Dehydrated quickly, cleared and mounted in canada balsm/D.P.X.

Result : Only sulfomucins stain intense blue.

C) Distinction between neutral and acidic mucosubstances :

1) AB pH 2.5 - PAS sequential staining technique :

(Mowry and Winkler, 1956; Mowery, 1963).

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

ii) Rinsed briefly in 3% acetic acid.

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

iv) Rinsed in 3% acetic acid.

v) Washed in distilled water for 5 minutes.

vii) Processed as in A-1 for PAS staining technique.

Result : 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 muco-substances colour magenta.

2) AB pH 1.0 - PAS sequential staining technique :

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

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

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

iii) Sections were blotted on a puffless filter paper.

iv) Processed as in A-1 for PAS staining technique.

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

D) Distinction between sulfomucins and carboxymucins :

1) Aldehyde fuchsin (AF) :

(Gomori, 1950; Halmi and Devis, 1953)

Preparation on AF crystals :

The crystals of AF were prepared according to the method suggested by Cameron and Steel (1959). To 200 ml boiling distilled water, 1 gm of basic fuchsin 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 filtrate was discarded. The precipitate was dried on the filter paper at 60^{o}C.

Staining solution : The staining solution was prepared by dissolving 0.5 gm of dry crystals in 70% alcohol.

Procedure :

i) After dewaxing and hydration, sections were brought to disdtilled water.

ii) Rinsed in 70% alcohol.

iii) Stained with AF staining solution for 30 minutes.

iv) Rinsed with 70% alcohol.

v) Dehydrated in 90% and absolute alcohol, cleared and mounted in D.P.X.

2) Aldehyde fuschin - AB pH 2.5 (AF-AB pH 2.5) sequential staining technique :

(Spicer and Mayer, 1960) :

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

ii) Rinsed in 70% alcohol.

iii) Stained in AF staining solution for 30 minutes.

iv) Rinsed in 70% alcohol.

v) Washed in running water for 5 minutes.

vi) Rinsed in 3% acetic acid.

vii) Stained with AB (pH 2.5) for 40 minutes.

viii) Rinsed in 3% acetic acid.

ix) Washed in running water for 5 minutes.

x) Dehydrated, cleared and mounted as usual.

Result : Sulfated mucosubstances stain purple, non-sulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

A bird's eye view of various histochemical techniques employed in the present investigation along with the chemical reactions involved in the staining and the histochemical interpretations of the staining reactions with the literature, is given in the Table No.2.

TABLE NO. 2

A Summary of histochemical techniques

No.	Histochemical method	Chemical reactions involved	Histochemical result	References
1	Periodic acid Schiff's reaction (PAS)	Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of colored complexes with Schiff's reagent	All polysaccharides and muco-substances colour pink to magenta	Mc Manus (1946), Hotchkiss (1948).
2	Diastase digestion-PAS	Hydrolyses and removes glycogen	Loss of PAS reactivity in sites containing glycogen	Lillie (1954), Lison (1960).
3	Alcian blue pH 1.0	Probably formation of alcian blue complexes with sulfate groups	Weakly and strongly acidic sulfomucins are selectively stained	Lev and Spicer (1964)
4	Alcian blue pH 2.5	Probably formation of alcian blue complexes with carboxyls, sulfate groups	Only carboxymucins and weak sulfomucins are stained blue	Mowry (1956)
5	AB pH 2.5-PAS	Addition of results by single methods	Alcian blue reactive periodate unreactive acid mucosubstances stain blue. Alcian blue and PAS-reactive substances colour purple-blue. Neutral mucosubs-tances colour pink-magenta	Howry and Winkler (1956), Mowry (1963)
6	AB pH 1.0-PAS	Addition of results by single methods	Sulfomucins stain blue or blue-purple. Neutral and non-sulfated periodate reactive mucosubstan-ces stain pink-magenta	Spicer (1965); Spicer et al., (1967b)
7	Aldehyde Fuchsin (AF)	Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups	Sulfated mucosubstances stain dark purple, Sialomucins and hyaluronic acid colour light purple	Gomori (1950); Halmi and Davies (1953)
8	AF - AB pH 2.5	Formation of salt complexes between cationic staining entity and sulfate and sdulfate and carboxyl groups	Sulfomucins stain purple or blue-purple. Sialomucins and other non sulfated acidic mucosubstances stain blue	Spicer and Meyer (1960)