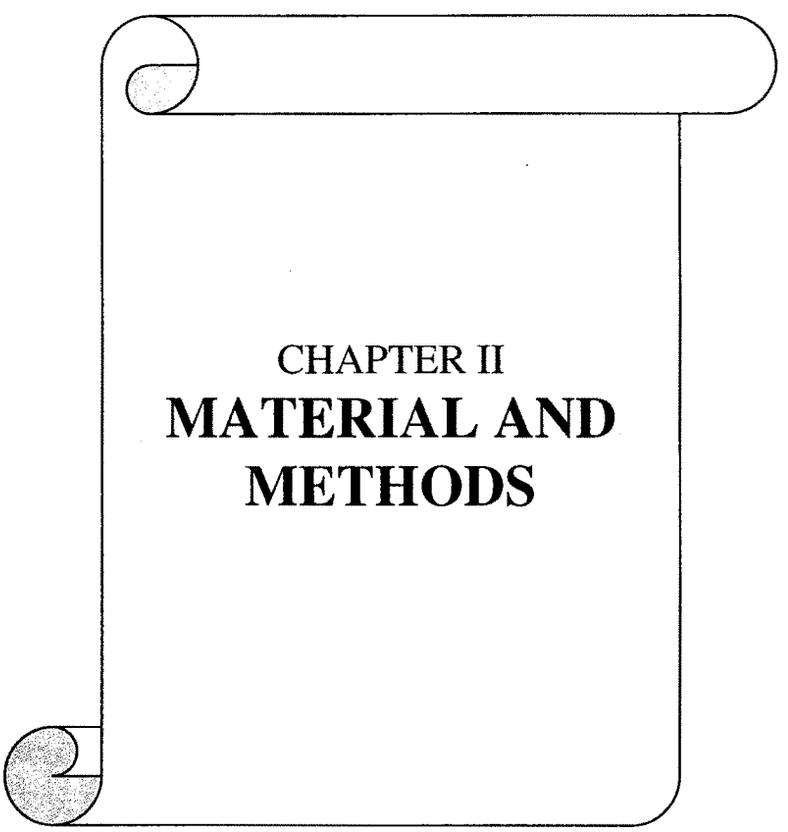


CHAPTER II

MATERIAL AND METHODS

CHAPTER - II

MATERIAL AND METHODS

1) MATERIAL :-

A) Selection of Animal :-

The animal selected for the present study is *Viviparus bengalensis* (Lamarck). The adult fresh water prosobranch snails, *Viviparus bengalensis were collected from Rankala Talav , Kolhapur (Maharashtra).*

a) Classification of animal :

Phylum - Mollusca

Class - Gastropoda

Sub Class - Prosobranchia

Order - Mesobranchia

Family - Viviparidae

Genus - ***Viviparus***

Species - ***bengalensis*** (Lamarck)

The *Viviparus bengalensis* is locally, readily available species through out the year. They show high biotic potential and stands captivity well. They are rapidly reproducing animals and having high ability to disperse, so population increases very fastly.

Several of aquatic snails attain the status of pests calling for immediate control. The snail, *Viviparus bengalensis* causes extensive damage to paddy roots. Ultimately leading to poor quality yield, as productivity is decreased.It also commonly eats the vegetation available in their habitat. Several organic, inorganic, synthetic and natural compounds have been used for control of snails (Bhide, 1998; Gupta and Bakre, 1996; Pereira and Pereira, 1975; Moreton and Gardner, 1976).

Due to its easy availability and well known pest in the paddy farming , animal is selected for the present investigaton.

B) Selection of organ :-

For present study, the selected organ is gill, since gills are highly specialized organ with number of vital functions i.e. respiration, osmoregulation etc. It offers a suitable material for studies of the effects of the toxic substances because toxicants directly enters the body of snail mainly through the gill surface. It is sensitive bio-indicator of water pollution. Because of the synthetic molluscicides the physicochemical properties of water are changed, which further affect the oxygen consumption, metabolism and ultimately physiology of the snail. During those type of stresses to survive they pay or spend a lot of energy to face the molluscides.

C) Selection of Molluscicides (Plant toxins) :-

Three plants selected for the present study are *Clerodendron inerme, Vitex negundo* and *Azadirachta indica.*

The molluscicides selected for study are :

a) Leaf extracts of *Clerodendron inerme* (Geartn).

b) Leaf extracts of *Vitex negundo* (Linnaeus).

c) Neem oil of *Azadirachta indica* (A. juss).

As these plants content the molluscicidal properties they are selected for the present study.

a) *Clerodendron inerme* (Geartn).

In the present study leaves of *Clerodendron inerme* plant were used because it has reported to contain pesticidal activity(Kirtikar and Basu,1987). The genus *Clerodendron* includes overs 450 species of tropical region. *Clerodenadron* species are land scaping plants valued for their unique flowers and an uncertain medicinal qualities. *Clerodendron inerme*, are cultivated to naturalise around the garden. It is used currently as a popular ground cover in coastal gardens of the Hawaiian Islands. *C. inerme* has attractive folliages. It is used as landscaping plant. (Forest Starr, Kim Starr and Lloyd Loope, 2003).

Alcoholic leaf extract of *Clerodendrone inerme* has tested on snail, *Viviparus bengalensis* (Kirtikar and Basu,1987; Grainage and Ahmed, 1985).

i) Classification ;

The systematic position of plant is as follows:

Division - Angiospermae

Class - Dicotyledonae

Order - Lamiales

Family - Verbenacae

Genus - ***Clerodendron***

Species - ***inerme (Geartn)***

ii) **Morphology ;**

The *cleroderdron inerme* is commonly called as Glowry Bower genus. Its vernacular name is Vanjai or Lanjai. A straggling much branched shrub; some time scandent, bark pale brown, branches twiggy. Leaves opposite, elliptic or obovate, obtuse, glabrous or nearly so, base acute, petiole long. Flowers in axillary pedunculate cymes 3 - 9 (commonly 3) flowered, peduncles long, slender, bract minute, linear or subulate calyx. 5 mm long enlarged in fruit, glabrous / puberulous, leathery. Corolla white, tube long. slender, reaching 2.5 cm long, glabrous outside, very hairy inside, lobes subequal, oblong or obtuse with slightly crenellate margins filaments very long, hairy at the base, ovary glabrous, stigma acutely 2 lobed, Drupe pyriform.

iii) **Distribution** :– Throughout India near the Sea, Ceylon.

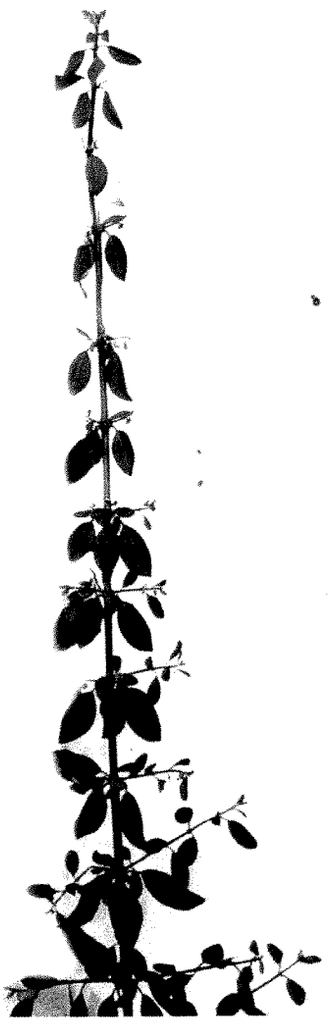
iv) **Medicinal Importance :–** The juice of the leaves and root is considered alternative in scrofulous and venereal affections, the dose being a tablespoonful with / without a little caster oil.

The dried leaves are used for the same purpose and a poultice of the leaves is used to resolve buboes, a bath prepared with them is used in Mania, while the root boiled in oil affords a liniment useful in rheumatism.

In Bombay, the Plant has a great reputation as a febrifuge; the juice of the leaves is used in doses of half an ounce. The medicinal properties of *Clerodendron inerme* closely resemble those of chiretta. The dried leaves

has been found to be quite as efficient as the juice of the fresh plant, they should be dried in the shade to preserve their aroma and may be administered in decoction with aromatics or powdered and made into pills. A tincture has also been found to be an efficient preparation. The leaves smeared with oil are health over the fire applied to recent wounds.

v) **Chemical properties** :- it is having secondary metabolites – hispidulin, scutellarein, apegenin, clerodin, tries 3β-ol , ethyl cholesta.



The leaves of *C.inerme*

b.) ***Vitex negundo* (Linnaeus)** . The *Vitex negundo* is commonly called as nirgundi or katri

i) Classification :

Division - Angiospermae

Class - Dicotyledonae

Order - Lamiales

Family - Verbenaceae

Genus - ***Vitex***

Species - ***negundo (L.)***

ii) Morphology :- It is a large shrub or sometime a small slender tree; bark thin, grey ;branch lets quadrangular, whitish with a fine tomentum foliolate, leaflets lanceolate, acute, the terminal leafiet 5-10 by 1.6-3.2 cm with a petiole 1-1.3 cm long, the lateral leaflets smaller with a very short petiole all glabrous above, covered with fine white tomentum beneath, base acute. Flowers in pedunculate branched tomentose cymes opposite along the quadrangular tormentor's rachis of a long terminal often compound pyramidal panicle. Bract lanceolate, caducous. Calyx 3mm long white tomentose. Corolla 1 cm long bluish purple tormentors outside, ovary glabrous, style glabrous, stigma forked. Drupe less than 6 mm dia, blac when ripe.

iii) Distribution :-

Throughout India, Ceylon, Afghanistan Tropical Africa, Madagascar, China, Phillipines.

iv) Medicinal Importance :-

The plant has pungent, bitter, acrid taste, heating astringent, cephalic, stomachic, antihelmintic, promotes the growth of hair useful in diseases of the eyes, consumption, inflammations. Leucoderma, enlargement of the spleen, bronchitis, asthma, biliousness, painful, teething of children – The root is an antidote to snake – venom (Ayurveda).

The root is considered as tonic, febrifuge and expectorant. The leaves are aromatic, vermifuge and tonic. A decoction of Nirgundi leaves is given with the addition of long pepper in catarrhal fever with heaviness of head and dullness of hearing. A pillow stuffed with the leaves of Nirgundi is placed under the head for relief of headache. The juice of leaves removes worms from the ulcers.

v) Chemical Properties :-

It contains n-tritriacontane, n-nonacosane ,β - sitosterol, P-hydroxy benzoic acid etc.

An oil prepared with the juice of the leaves is applied to sinuses and scrofulous sores.

The leaves are discutient and are useful in dispersing surelling of joints from acute rheumatism and of the testes from suppressed gonorrhoea. In the Konkan, the juice of the leaves with that of Maka (Eclipta alba) and Tulasi (Ocimum sanctum) is extracted and Ajwan seeds are bruised and

steeped in it, and given in doses of six massas for rheumatism. The juice in half tola dose with ghi and black papper is also given, and in splenic enlargement two tolas of the juice with two tolas of cow's urine is given every morning.

The decoction of the leaves are used as a bath by women in India. The people of Mysore are in the habit of treating febrile, catarrhal and rheumatic affections by means of a vapour bath prepared with this plant.

The dried leaves are smoked for the relief of headache and catarrh. The dried fruits acts as a vermifuge.

In Ehina and Malaya, the fruits are given for headache, catarrh and watery eyes.

In Ceylon, the leaves, bark and roots are used for toothache, rheumatism, eye disease; and as a tonic, carminative and vermifuge.

Among the Mundas of Chota Nagpur, an expectorant is prepared from the sap. Some six green branches are warmed over a fire and the sap which flows from their cut extremity is collected in a vessel. This sap is warmed and mixed with clarified butter in which three pounded bulbs of garlic have been fried, The patient drinks a little of this now and then.

The plant is recommended for the treatment of snak-bite (Charaka, Sushruta, Vegbhata, Vrindamadhava, Rasaratnakara) and scorpion – sting (Charaka, Sushruta).

In case of snake-bite, the bruised roots, bark, and leaves are applied to the wounds. The expressed juice of the fresh leaves is poured into the nostrils in stuper and coma, and is given internally. A decoction of the roots and bark is also given internally.

The leaves of *V. negundo*

The leaves of *V. negundo*

c.) ***Azadirachta indica* (A. Juss) :** It is commonly known as Neem, Nimb, Kaduneem / Kadulimb

i)Classification :-

Division - Angiospermae

Class - Dicotyledonae

Order - Germials

Family - Meliaceae

Genus - ***Azadirachta***

Species - ***indica (A. juss)***

ii) Morphology :-

The plant is large sized tree, 15-20 mtr. in height with a clear bole of 7.0 mtr having grayish dark grey tubercled bark; leaves compound, immaripinnate, leaftlets, subopposite, seraate, very oblique at base; flower cream or yellowish white in axillary panicles, staminal tubes conspicuous, cylindric, widening above, 9-10 lobed at the apex; fruits one seeded drupes with woody endocarp greenish yellow when ripe, seeds ellipsid, cotyledons thick, fleshy and oily.

iii) Distribution :-

Throughout India, in deciduous forests, also widely cultivated.

iv) Medicinal Importance :-

The bark is bitter, astringent, acrid, refrigerant, depurative, antiperiodic, vulnerary, demulcent, insecticidal, liver tonic, expectorant, urinary astringent, antihelmintic, pectoral and tonic. It is useful in vitiated conditions of *pitta*, hyperdipsia, leprosy, skin diseases, eczema, leucoderma, pruritus, intermittent and malerial fevers, wounds, ulcers, burning sensation,

tumour, tubercular gland, anoxia, vomiting, dyspepsia, intestinal worms, hepatopathy, cough, bronchitis, urorrhea, diabetes, inflammation, amenorrhoea, lumbago, haemorrhoids, otalgia, syphilis and fatigue.

The leaves are bitter, astringent, acrid, depurative, antiseptic, ophthalmic, anthelminitc, alexeteric, appetizer, insecticidal, demulcent and refrigerant. They are useful in vitiated conditions of ***pitta***, burning sensation, leprosy, skin diseases, leucoderma, pruritus, ophthamopathy, intestinal worm, dyspepsia, ulcers, tuberculosis, boils, eczema and malarial and intermittent fevers.

v) Chemical properties :-

It contains azadiracthin nimbin, nimbinin, nimbidin, myricetin, glycoside – melicinin, faepferol – 3 – glucoside.

Neem oil contains - Nimbin, Nimbinin and Nimbidin out of these nimbidin contains active constituent and contains sulphur (Curr.Sci;1942, 278 and chem..Abstr.1943, 723) nimbosterim are highly pungent essential oil nimbosterol, nimbecetin and fatty acids and present in nim oil (J.Sci,industru, Res.1947, 198.chem.Absm.1947,6021).

Neem oil of *A.indica*

D) Advantage :-

a) They are phytotoxins i.e. natural molluscicides or botanical molluscicides.

b) They are easily available and found locally.

c) They are very cheap.

d) They are more effective.

e) They are biodegradable.

f) Thus they kept aquatic environment safe.

2) METHODS :-

A) Mortality study:

a.) Preparation of molluscicides :

The fresh and clean leaves of *Clerodendron inerme* and *Vitex negundo* were collected from in and around the campus of Shivaji University,Kolhapur and Gad-Mudshingi village, Dist. Kolhapur. Then the leaves were washed properly with running tap water and then after distilled water. The leaves were kept in oven for over night at 50^0c temperature. Then powders were prepared by mortal and pestle. The powdered material were stored at low temperature and used for the further extraction procedure.

Clerodendron inerme and *Vitex negundo* leaf powder(each 150gm) powder were taken and soaked in 1500 ml 95% of ethyl alcohol for 4-6 days. Both the mixtures were stirred continuously by stirrer, after which mixtures were filtered by whattman filter paper. The ethyl alcohol was removed under reduced pressure by using flash or vaccum evapporater to concentrate the material. The crude residue then dissolved in 1 liter acetone and considered as "stock solution". The stock solution (crude) was further diluted into acetone to get desired concentration in ppm 100, 200, 300, 400 and 500 ppm and bioassayed on *Viviparus bengalensis*_ by static bioassay method.

Neem oil from *Azadirachta indica* used for present study is market product Neem Shastra (having emulsifier itself) was obtained from Siemen's Agrineed Industries 298/295 village Angapur (Vandan), Satara (Maharshtra). 1 ml of Neem Shastra was dissolved in 100ml 95% ethyl alcohol; this was a stock solution. This solution further diluted into acetone and desired concentrations were prepared (100 to 500 ppm).

b.) Maintainance of aquarium and animals :

The fresh water, prosobranch snails, *Viviparus bengalensis* were brought to laboratory. Their shells were brushed to remove the mud and fouling biomass. They were kept in five aerated glass aquaria having twenty ltr. capacity which contained dechlorinated water and maintained for laboratory conditions for 6-8 days. twenty snails were kept in each aquarium. The water was changed every day for fresh aeration and removel of wastes. They were provided with green algae, hydrilla like natural vegetation. The dead and unhealthy snails were removed immediately from aquaria during acclimatization period. The normal, healthy and equal sized snails were taken for further toxicological study.

Healthy snails of average size 2.5± 0.5 cms. and weight 3.5 ± 0.5gms, were selected form the stock of laboratory acclimated snails, twenty snails were kept in each of the aquarium having two ltr. capacity. Snails (twenty) were exposed to each plant toxin having concentrations 100,200,300,400 and 500 ppm in a separate aquarium. The control set was also run simaltaneoously.

The number of dead snails in each aquarium was recorded after intoxication period 24,48,72,96 and 120hrs. for the study of mortality. The dead snails were removed immediately. The experiment was repeated for three times.

Per cent mortality and LC_{50} values were calculated.

c.) Calculation of per cent mortality :-

Mortality of the snails was recorded in the from of percentage, which was calculated by the following formula :

$$P = \frac{r}{n} \times 100$$

Where,

P = Per cent mortality,

r = Mortality observed

n = Number of snails exposed in a set

The percentage mortality data with respect to the concentration of plant toxins in ppm was used for calculation of lethal concentrations of plant toxins for different exposure periods. LC_{50} values of the each plant toxin to the snail were calculated by the following methods.

d.) Calculation method of LC_{50} values:-

A table was prepared indicating percentage mortality, empirical probit values of per cent mortality (Y) and concentration of plant toxin (X). From this data another values such as LnX, LnX^2, LnXY, ΣY, ΣLnX, ΣLnX^2 and ΣLnXY were calculated and recorded in the same table but in different columns. By using thier values the LC_{50} values were calculated by the following formula given by Fisher and Yates (1963).

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

Where, values of 'b' and 'a' were calculated by the equations given as below,

$$\text{i)} \quad b = \frac{\Sigma LnXY - \frac{\Sigma LnX . \Sigma Y}{n}}{\Sigma LnX^2 - \frac{(\Sigma LnX)^2}{n}}$$

Where, n = total number of concentrations used.

ii) $a = Y - bLnX$

B) Oxygen consumption study:-

a.) Experimental method:

The snails *Viviparus bengalensis* were exposed to the sublethal concentrations 125.20, 110.80 and 148.90 ppm respectively of phytoxins *C. inerme*, *V. negundo* and *A. indica* (neem oil). Sublethal concentrations was calculated as a 1/3 of LC_{50} value for 24 hrs. of each molluscicide.

The experiment was carried in following sets :

Set I – The O_2 consumption of snails exposed to *Clerodendron inerme,*

Set II – The O_2 consumption of snails exposed to *Vitex negundo* and

Set III – The O_2 consumption of snails exposed to *Azadirachta indica.*

Experiments were carried out separately. The control set was also maintained. After exposure to different concentration of molluscicides for 24,48,72,96 and 120 hrs. (i.e. at intervals of 24 hrs) the four snails were taken out from each of set including the control set and cleaned by fresh non

polluted water. They were placed in an air tight respiratory glass jar for an hour. After one hour snails were taken out of jar. H_2O from this jar was used for analysis of O_2 consumption before introducing the snails and immediately after removed of snails. The D.O. was analysed as per Winkler's method (Welsh and Smith, 1960) The experiments were repeated three times for avoid the errors. The rate of O_2 consumption was measured in terms of ml of O_2/gm body wt /hr/lit.

b.) Analysis of D.O. by Winkler's method:

i.) Principle – O_2 in the pre-dechlorinated H_2O is estimated by using modified Winkler's method (Welsh and Smitch, 1960). This method is based on the fact that if maganous chloride is added to a known volume of water containing D.O., a portion will be converted into mangnic hydroxide. This is dissolved in a non oxidizing acid, such as HCl and made to react with potassium iodide so that an equivalent quantity of iodine is then liberated when starch solution is added, it reacts with liberated iodine and produce blue color due to adsorption, titrate this solution against a standard solution of sodium thiosulphate, the colour disappears due to binding of iodine as sodium iodide.

$$4M_nCl_2 + 8NaOH \longrightarrow 4M_n(OH)_2 + 8NaCl$$

$$4M_n(OH)_2 + 2H_2O + O_2 \longrightarrow 4M_n(OH)_3$$

$$4M_n(OH)_3 + 4KI + 12\,HCl \longrightarrow 4M_nCl_2 + 4KCl + 12H_2O + 2I_2$$

$$4Na_2S_2O_3 + 2I_2 \longrightarrow 2NaI + 2Na_2S_4O_6$$

ii.) **Requirements-**

Glass respiratory jar with capacity of two ltr.,beakers, wt. box, rough balance , pipette, burette, reagent bottle (320ml), conical flask, dropper measuring cylinder, rubber tube, blotting paper etc.

iii.) **Chemicals –**

Maganous chloride, sodium hydroxide, potassium iodide, sodium thiosulphate, starch concentrated hydrochloric acid.

iv.) **Procedure –**

Among the exposed twenty snails, four snails were taken for the experiment of average length of each snail was about 2.5 ± 0.5 cm. and average weight of about 3.5 ± 0.5 gms. in respiratory jar. Cork was fitted, then water was added to jar through inlet. After filling the jar H_2O addition was stopped. After waiting for 10 minutes for coming snails out or protruding out, both inlet and outlet was pinched off and time was counted upto one hour.

After completion of one hour water was taken from outlet of respiratory jar to stopper bottle having volume of 320 ml, for further experiment. Fill stopper bottle completely, then close it immediately without any air bubbles, add one ml of Winkler's 'A'. solution into the reagent bottle with the help of pipette. Shake the bottle well and then add 1 ml Winkler's 'B'. solution. Again shake the bottle and keep it aside for the precipitate to settle down. The precipitation is generally brownish in color. Then add concentrated. HCl dropwise and dissolve the precipetate completely. Take 100 ml of this clear solution in a conical flask and add few

drops of 1% starch solution which will act as an indicator. After adding starch solution the colour turns to blue . Titrate this solution against N/80 sodium thiosulphate till the blue colour disappears. Repeat the same experiment by taking fresh water. Experiment was done for control and treated snails at intervals of 24 hours from 24 hr to 120 hrs.

v.) Calculation –

I.) Formula to calculate D.O. :-

$$\text{Volume of dissolved oxygen ml per liter} = \frac{(ml \times N) \times 8 \times 1000}{V_2 \left\{ \frac{V_1 - V}{V_1} \right\}}$$

V – Volume of M_nSo_4 and KI solution

V_1 – Volume of sample of stopped bottle (320 ml)

V_2 – Volume of part of content titrate

N – Normality of sodium thiosulphate used for titration = 0.025 N

II.) Formula to calculate O_2 consumption:-

O_2 consumed by the snails in 1 hour = O_2 present in tap water – O_2 present in the jar water after 1 hour.

$$\text{Oxygen consumed by snails in ml / liter / gm. body wt./hr.} = \frac{\text{Oxygen consumed by the snail in 1 hour}}{\text{Weight of snails in gram.}}$$

C.) BIOCHEMICAL PARAMETERS :

The snails, V. bengalensis were exposed to the sublethal concentrations 125.20, 110.80 and 148.90 respectively of molluscicides from plants *C. inerme, V. negundo* and *A.indica.*

The snails were exposed for different exposure periods such as 24, 48,72, 96 and 120 hrs. The control set was also maintained. Then these exposed fishes were taken for the study of biochemical parameters.

For present biochemical estimation study, gills of *Viviparus bengalensis* was taken as experimental organ. Major biochemical parameters were studied by different methods.

a.) Glycogen estimation by Anthrone method.

b.) Proteins estimation by Micro – Kjeldhal's method.

c.) Lipids estimation b Soxhlet method.

a.) Estimation of glycogen by Anthron Method (Caroll *et. al;* 1956) :-

i) Principle :-

The sulphuric acid in the anthrone reagent hydrolyses the glycogen into glucose and then dehydrates it into furfurals. This compound reacts with anthrone to produce a complex coloured product, the intensity of which is proportional to the amount of glucose present in the glycogen (Caroll *et. al.;* 1956).

ii) Procedure :-

50 mg of gill tissue was taken in digestion flask, then 3 ml of 30 % KOH was added and digested the solution in boiling water bath for 25-30 min. Then 25ml saturated sodium sulphate was added then preciptation was prepared by adding 1-2ml, 95% ethanol. By shaking vigorously, mixture was again heated and kept the mixture for cooling then it was centrifuged.

Remaining ethanol was evaporated by heating the tubes in boiling water bath. The precipitation was dissolved by distilled water and reprecipitated again by two ml, 95% ethanol, centrifuged it, decanted alcoholic supernatant liquid was taken in 50 ml volumetric flask, dilution was made upto the mark.

5 ml was taken from diluted solution, simultaneously, standard solution i.e. 5 ml glycogen solution and 5 ml distilled water was taken in two separate test tubes and used as standard and as a blank, respectively.

All three tubes were kept in cold water bath (10-15°C) and added 10 ml Anthron reagent. Again tubes were cooled and readings were taken at 620 nm setting the colorimeter to 100% transmittance

b.) Estimation of proteins by Micro – Kjeldhal's Method (Nagbhushnam et al.; 1975) :-

i) Principle -

Total protein in the gill tissue was determined by Micro-Kjeldhal's method using sulphuric and phosphoric acid mixture for digestion then Nesselerization.

ii) Procedure –

Total proteins in gills were estimated by Micro – Kjeldhal's Method using direct Nesselerization method by making the appropriate correction for non protein nitrogen (NPN).

50 mg gill tissues was taken in Micro – Kjeldhal's flask then added one ml 1:1 H_2SO_4, flask was heated. Mixture was digested till extra water was evaporated and flask filled with white fumes. Mouth of flask was covered by watch glass. Solution was boiled for 3 minutes. After boiling mixture was cooled. 0.5 ml saturated potassium per sulphate solution was

added dropwise and again mixture was heated till it became watery or colourless. Then the mixture was cooled and transferred to protein tubes and diluted it upto 35 mark by distilled water.

Similarly blank solution was prepared instead of standard solution, distilled water was used, further additions were same as standard.

When three tubes were ready, 15ml Nesseler's reagent was added in each protein tube with shaking by inversion. After few minutes maximum colour was developed. Adjusting colorimeter at 480 – 540 nm to 100% transmittance or zero optical density by blank, readings were taken.

c.) Estimation of Lipids by Soxhlet's Method (Folch *et al.*;1975):-

i) Principle -

Fat is extracted by ether-alcohol mixture with the help of Soxhlet's apparatus, where a limited amount of solvent , with the help of siphon , extract the fat.

iii) Procedure-

Proper arrangement of Soxhlet's apparatus was made, then tissue was oven dried to obtain powder form . The powered tissue taken in Whatman filter paper No.1 and was put in Middle chamber of Soxhlet's Apprattus. Then solvent solution was poured in it such that pouch of whattman filter paper containing tissue gets deeped completely. It was kept for overnight so that all the lipids get dissolved in the solution. Then solvent was again poured in the middle chamber so that all solution flowed to the below flask. Lamp of 100 watt bulb was adjusted in such a way that maximum heat was supplied to the flask. The solution containing dissolved lipid gets evaporated and flower towards condensed. The condensed vapours liquified and again flows down into the flask. Such a cycle was repeated for 4 to 5 times. Removed the flask and boiled in water bath so that all the

solvent solution gets evaporated. Thus we got direct lipid settled at the bottom of that flask. Then total lipid contents were estimated by using formula.

Mg of lipid = wt of round bottom flask after experiment – wt. of round bottom flask before experiment.