CHAPTER-II
Chapter- II

Study Site

Manipur, a tiny hill-state in the North-eastern part of India situated between latitude of 25°51' N and 25°41'N and longitude 93°42' E and 97°47' E with numerous hill ranges is drained by two river system - Barak, Brahmaputra System of Assam and the Chindwin Irrawadi River system of Burma. The Chindwin Irrawady River system consists of Imphal River and its tributaries viz. Irl River, Thoubal River etc. The Imphal River which is 960 km long originates from the Tiki lok 6.6 km from Changoubung, a Naga village of Senapati district., Manipur at a height of 2400m (MSL) and it runs from north to south through the heart of Imphal Valley. Among the tributaries, Irl River is the most important. The Irl River originates from 2.5 km away from Lakamei, a Naga village in Senapati district, Manipur at a height of 2400 km (MSL) and flows over the unstable hilly tract. It has a number of main tributaries such as the Ithai Lok, Thang Lok, Ekou Lok, Leimakhong, Ichai Lok etc. The Irl and its main tributaries and rills constitute the Irl River system. The part of the land surface, the rainwater of which is drained out by the Irl river system is called the Irl catchment or drainage basin. This drainage basin has an area of 1,285 sq. km. The Irl River discharges its water into the Imphal River at Lilong. The Irl River is a tributary of Manipur River which is called Imphal River in its upper part before its confluence with Khuga River. The Irl River basin lies between the latitudes of 24°70'N-25°30'N and the longitudes 93°55'E-94°20'E and the length from its head to the point of its confluence with the Imphal River is 144.5km. This master stream after crossing the southern hill deposits its water into the Myitha River, a tributary of Chindwin River in Myanmar and becomes the part and parcel of the Irrawaddy River system and ultimately drains into the Andaman Sea. Since time immemorial, the Manipur River
serves the people of Manipur in the districts of Ukhrul, Senapati, Imphal East and Thoubal. This river forms the main sources for drinking water, household purposes and for various agricultural practices. The sampling site selected for the investigation are the intake point of various Water supply scheme of PHED, Manipur. Eight different sites were selected for sampling in the two Rivers. Location of the study site is shown in the map (Fig. 1).

In the Imphal River, the following sites are selected.

**Site I (Kiyamgei)**

The site is from the downstream of Imphal River. At this site, the river water is used for washing clothes, taking bath and other household purposes. Pebbles were found at this site.

**Site II (Mahabali)**

The site selected here, is near the Mahabali Mandir in Imphal. People use the river water for washing clothes, taking bath and other household purposes. People of different community are inhabited here. The distance between site I and II is 5.55 km. Pebbles were found at this site.

**Site III (Khuman Lampak)**

Khuman Lampak is one of the sampling sites. This site is mostly used for washing clothes by washer men. People also take bath here. The distance between site II and III is 5.37 km. Small rocks and pebbles were found at this site.

**Site IV (Koirengei)**

It is the fourth sampling site. The site is totally different from the other three sites. The River water was used for cleaning utensil, bathing, washing clothes, but the
waste are not discharge. Less human disturbance was seen. The distance between the site III and IV is 12.32 km. Rocks were in the river beds.

For Irl River, four different sampling sites were selected.

**Site I (Top Dusara)**

The site Top Dusara was the first site taken for investigation from Irl River. The River Water is used for drinking, washing clothes, bathing. No dumping of waste was seen at this site. Fishing by local people was sometime observed. Small rocks and pebbles were found at this site.

**Site II (Angom Leikai)**

Angom Leikai is the site II for sampling. In this site no dumping of waste was seen. Bathing, washing of clothes was rarely seen. People used River water for drinking and household purpose. Pebbles were found at the river bed.

**Site III (Sawombung)**

The third sampling site was Sawombung. Among the four different sites of Irl River, this site was observed as the busiest site. At this site people used river water for washing clothes, bathing, and collecting snails. No wastes were dump at this site. Rocks and Pebbles were found at the river beds.

**Site IV (Pungdongbam)**

The fourth sampling site was Pungdongbam. No wastes were dump here. The people used the river water for drinking and other household purpose. Washing, bathing inside the river was not observed at the site during the studies. Pebbles were found at the river bed.
Climatic conditions

The climate in Manipur may be broadly classified as three main seasons: Summer season (March to June), Rainy season (July to October) and Winter (November to February). The meteorological data were collected from the ICAR Lamphel, Imphal. Graphically meteorological data of the study period (March’2005 to Feb’07) are illustrated in Fig.2.
Fig. 1: Location Map of Imphal and Irl River, Manipur
Photographic presentation of 8 Study Sites from Imphal and Iril Rivers

Site-1. Kiyamgei (Imphal River)

Site-2. Mahabali (Imphal River)

Site-3. Khuman Lampak (Imphal River)
Site-4. Koirengei (Imphal River)

Site-5. Top Dusara (Iril River)

Site-6. Angom Leikai (Iril River)
Site-7. Sawombung (Iril River)

Site-8. Pungdongbam (Iril River)

Confluence point of Imphal and Iril River at Lilong
Fig. 2: Ombrothermic diagram for the investigation period of March 2005 to February 2007.
Materials and Methods

The present investigation was carried out in two freshwater bodies of river: Imphal and Iril River of Manipur. The study period was from March’05 to February’07. Different method and techniques were adopted for the collection of macrophytes and water samples, for the physico-chemical analysis of water and biochemical analysis of macrophytes. The physico-chemical analysis was done throughout the year on monthly basis. For phytosociological and biochemical studies, the macrophytes were studied from the month of October to February of the study period (i.e. 2005 to 2007). In this river system, the aquatic plants grow luxuriantly during these months when the water level was lower. One of the reasons for selecting these months for investigation was that during summer and rainy seasons the water levels were not constant, so at that period the macrophytes cannot grow in this river system. In Manipur, the rainy season sometimes starts from the month of February, March and ends in the month September. So the most favourable for study of this aquatic plant were winter. As the investigation needs closed observation, these months were selected. For the estimation of biochemical, certain macrophytes that were dominant in the two rivers were chosen. Most of the macrophytes that were chosen for biochemical estimation were macro algae. These macro algae grow luxuriantly during the winter season. As these plants grow directly in the water, the impact of nutrient to the biochemical component can be undertaken for analysis. For phytosociological study, the macrophytes as well as aquatic macro algae were selected.
A. Physico-chemical characteristics

Physico-chemical characteristics analysis was carried out following the methods of APHA (1989) and referring simplified versions given in Trivedy and Goel (1984). Samples were collected from subsurface. Physico-chemical analysis of water for water temperature, $p^H$, Dissolved Oxygen, Free CO$_2$ and Total alkalinity were conducted at the spot soon after collection of the samples. Analyses for the remaining parameters were carried out in the Laboratory.

Water Temperature

The temperature of water was taken with a spatial thermometer which can reach one foot below surface while the graduated mark was above the surface, the value was taken in degree Celsius ($^\circ$C).

Water $p^H$

The $p^H$ value of water was recorded with a $p^H$ meter (portable pen type model), and double electrodes system meter in the laboratory.

Turbidity

When light is passed through a sample having suspended turbidity, so of the light is scattered by the particles. The scattering of the light is generally proportional to the turbidity. The turbidity of a sample is thus measured from the amount of light scattered by the sample taking a reference with standard turbidity suspension. The determination of turbidity is interfered by the presence of debris and other rapidly settles able matter. The true colour in the sample reduces the values of turbidity. The Turbidity is determined by the Nephelometric method with the Nephelometer. The value was expressed in terms of NTU.
Electrical Conductivity

Electrical conductance is the ability of a substance to conduct the electric current. In water, it is the property caused by the presence of various ionic species. Electrical conductivity is generally measured with the help of a conductivity meter having a conductance cell containing electrodes of platinum coated with platinum black or carbon. The unit of electrical conductivity is expressed as $\mu$mhos cm$^{-1}$.

Dissolved Oxygen (DO)

To estimate the concentration of Dissolved Oxygen present in the water samples, Winkler's method was used. The water samples collected in 250ml reagent (Manganous sulphate and Alkaline iodide). The fixed samples were then brought to the laboratory after adding conc. H$_2$SO$_4$ and the samples were titrated against sodium thiosulphate solution using starch as an indicator. The amount of Dissolved Oxygen has been expressed in terms of mgl$^{-1}$.

Calculation

When the whole contents have been titrated

$$\text{Dissolved Oxygen, mgl}^{-1} = \frac{(\text{ml} \times N) \text{ of sodium thiosulphate} \times 8 \times 1000}{V_2 - V}$$

When only a part of the content has been titrated

$$\text{Dissolved Oxygen, mgl}^{-1} = \frac{(\text{ml} \times N) \text{ of sodium thiosulphate} \times 8 \times 1000}{V_2 \left( \frac{V_1 - V}{V_1} \right)}$$

Where, $V_1$ = Volume of sample bottle

$V_2$ = Volume of contents titrated

$V$ = Volume of MnSO$_4$ and KI added (2ml)
Free Carbon dioxide (FCO₂)

Free Carbon dioxide in the water accumulates due to microbial activity and respiration of organisms. This imparts the acidity to water because of the formation of carbonic acids. FCO₂ is determined by titrating the sample using sodium hydroxide solution and phenolphthalein indicator. The amount of dissolved free CO₂ is expressed in mg l⁻¹.

Calculation

\[
\text{FCO}_2, \text{mg} \text{l}^{-1} = \frac{(\text{ml} \times n) \text{of NaOH} \times 1000 \times 44}{\text{ml sample}}
\]

Total Hardness

Hardness is the property of water which prevents the lather formation with soap and increases the boiling point waters. The major cations imparting hardness are calcium and magnesium. The anion responsible for hardness is temporary if it is associated mainly with carbonates and bicarbonates, and permanent, if with sulphates and chlorides. Hardness is determined by the titration method (EDTA-method). The value of total hardness was expressed in mg l⁻¹.

Calculation

\[
\text{Hardness, mg} \text{l}^{-1} \text{as CaCO}_3 = \frac{\text{ml EDTA used} \times 1000}{\text{ml sample}}
\]

Calcium

Calcium is one of the most abundant elements found in the natural water. It is an important ion in imparting the hardness to the waters. At high pH much of its quantities may get precipitated as CaCO₃. Calcium was determined by the titrimetric method (EDTA-method).
Calculation

\[ \text{Calcium mg}^{-1} = \frac{(x) \text{ml EDTA used} \times 400.8}{\text{ml sample}} \]

For estimation of calcium, in 50 ml of sample was added 2 ml of 1N NaOH and 100-200 mg of murexide indicator and pink colour developed. It was titrated with EDTA solution until pink colour changed to purple.

**Magnesium**

Magnesium occurs in all kinds of natural water, but its concentration remains generally lower than the calcium. It is also one of the important cation imparting hardness to the water. The major sources of Magnesium in natural water are various kinds of rocks and sewage besides industrial wastes. Magnesium was calculated from the EDTA calcium and total hardness titrations. The value of Mg was expressed in mg/l.

Calculation

\[ \text{Magnesium, mg}^{-1} = \frac{(y-x) \times 400.8}{\text{ml sample} \times 1.645} \]

Where \( x \) = EDTA used for Ca determination

\( y \) = EDTA used for hardness (Ca-Mg) determination using the same volume of sample as used for Ca

Volume of EDTA used in Hardness determination and calcium determination were noted.

**Chloride**

Chloride occurs naturally in all types of waters. The most important source of chloride in natural waters is from industries, irrigation drainage, discharges of sewage.
For determination of the chloride content in water was done by titrating the water samples with 0.02 N AgNO₃ using K₂CrO₄ (Potassium chromate) as indicator. The value of Chloride was expressed in terms of mg/l⁻¹.

Calculation

\[
\text{Chloride, mg/l} = \frac{(ml \times N)_{\text{of AgNO₃}} \times 1000 \times 35.5}{\text{ml sample}}
\]

**Total Alkalinity**

The alkalinity of water is a measure of its capacity to neutralise acids. The alkalinity of natural water is due to the salts of carbonate, bicarbonate, borates, silicates and phosphates along with the hydroxyl ions and strong bases such as carbonates and bicarbonates. For Total alkalinity determination, 100 ml of water sample was titrated against N/50 conc. H₂SO₄ using methyl orange and phenolphthalein as indicator. The value was expressed in terms of mg/l⁻¹.

Calculation:

\[
\text{Total alkalinity as CaCO₃, mg/l} = \frac{(V_1 \text{normality of HCL}) \times 1000 \times 50}{V_2}
\]

Where, \( V_1 \) = vol. of HCL used in ml

\( V_2 \) = vol. of sample in ml

**Potassium**

Potassium is naturally occurring element. However, its concentration remains quite lower than the sodium, calcium and magnesium. It has got a more or less similar chemistry like sodium and remains mostly in solution without undergoing any precipitation. The major source in natural fresh water is weathering of rocks. Potassium was determined by Flame Photometric method using flame photometer (Jackson 1958). The value was expressed as mg/l⁻¹.
Sodium

Sodium is one of the important cation occurring naturally. Domestic sewage is one of the important sources of sodium to the fresh water. Salts of sodium are highly soluble in water. The increased pollution of surface and ground water during the past decade has resulted in a substantial increase in sodium content. Sodium was determined by Flame photometric method (Jackson, 1958) and value was expressed in mgl\(^{-1}\).

Nitrate

Nitrate is the mostly highly oxidised form of nitrogen compounds commonly present in natural waters, because it is the product of the aerobic decomposition of organic nitrogenous matter. Spectrophotometric method (APHA, 1989) was used for estimation of NO\(_3\)-N. The reading was recorded at 410nm and the value was expressed in terms of mgl\(^{-1}\).

Nitrite

Nitrite forms a diazonium salt with sulphanilic acid in acid medium (2.0-2.5pH), which combines with α-naphthylamine hydrochloride and form a pinkish dye. The colour so produced obeys Beer’s law and can be determined colorimetrically by Sulphanilic acid solution Method. The value of estimated Nitrite was expressed in terms of mgl\(^{-1}\).

Total Phosphorus

Phosphorus determination is useful in measuring the water quality since it is an important plant nutrient and may play a role of a limiting factor among all other essential plant nutrients (Dugan, 1972). Over 85% of the total phosphorus usually exists in bound organic organismal (living and dead) form, only inorganic phosphorus
as orthophosphate (PO₄-P) play a dynamic role in aquatic ecosystem (Adoni. 1985). Total Phosphorus was determined by H₂SO₄-HNO₃ method. 2.5ml of the sample was taken in Kjeldahl flask of 100ml. 1ml of H₂SO₄ and 5ml of conc.HNO₃ was added. After digesting the sample up to 1ml, 20 ml of distilled water and 1 drop of phenolphthalein was added and titrated with 5 N NaOH. The solution turns pink. And reading was done at 690 nm on a spectrophotometer. The value was expressed in terms of mgl⁻¹.

Inorganic Phosphorus

Inorganic phosphate was estimated by the stannous chloride method. 50 ml of the filtered clear sample was taken in a clean conical flask. 2ml of ammonium molybdate solution was added. Blue colour appeared was taken within 5 to 12 min after addition of lost reagent. Concentration was found out from the standard curve. Phosphorus determination is useful in measuring the water quality since it is an important plant nutrient and may play a role of a limiting factor among all other essential plant nutrients (Dugan, 1972). The value was expressed in terms of mgl⁻¹.

Organic Phosphorus

Organic Phosphorus is obtained as difference between the Total Phosphorus and Inorganic Phosphorus of the sample. Organic Phosphorus = Total Phosphorus - Inorganic Phosphorus. The value was expressed in terms of mgl⁻¹.

Total Solid Content (TS)

The solute content is an important ecological factor in the studies of the aquatic ecosystems. The concentration of the solute content of water was estimated by weighing the residue obtained after evaporating a known quantity of collected water sample in a dish or basin on a hot plate. The value was expressed in mgl⁻¹.
Calculation

\[ \text{Total Solid, mg} \cdot \text{L}^{-1} = \frac{(a-b) \times 1000 \times 1000}{v} \]

where, \( a \) = Final weight of the dish in gm
\( b \) = Initial weight of the dish in gm
\( v \) = Volume of sample evaporated in ml

**Total Dissolved Solids (TDS)**

Total Dissolved Solids denote mainly the various kinds of minerals present in water. TDS do not contain any gas and colloids. These can be determined as the residue left after evaporation of the filtered sample. The value was expressed in terms of mgL\(^{-1}\).

Calculation

\[ \text{TDS, mg} \cdot \text{L}^{-1} = \frac{(a-b) \times 1000 \times 1000}{v} \]

Where \( a \) = Final weight of the dish

**Total Suspended Solid (TSS)**

These solids denote the suspended impurities present in the water in most of the cases they are of organic in nature and pose severe problems of water pollution. Total Suspended Solid was determined by the difference between Total Solid and Total Dissolved Solid. The value was expressed in terms of mgL\(^{-1}\).

**B. Phytosociological Studies**

Phytosociological studies was done through quadrat method (Curtis. 1959; Misra, 1968). Since the assemblage of plants in a community is largely heterogeneous, for the study, two categories of studies were made in the phytosociology viz. subjective or the qualitative and objective or the quantitative analysis.
(a) Qualitative characters

The floristic composition, life-form classification and growth-forms of the different macrophytes were analysed during the investigation period.

Floristic Composition

Survey and sampling were done on periodically during the investigation. The floristic composition of the four study sites of each river were analysed properly. From this composition, the different parameters of the vegetation physiognomy, i.e. Life-forms, growth forms and distribution of the different species were studied.

Life-form

The classifications of the vegetation were classified after Raunkiaer’s life-forms classification as modified by Ellenberg and Mueller-Dombois (1967) and Mueller-Dombois and Ellenberg (1974). Comparisons between the biological spectrums of the study area were analysed with the normal spectrum (Raunkiaer, 1934).

Growth-form

Growth form of the different plant species were classified following the system of classification laid down by Hogeweg and Brenkert (1969).

(b) Quantitative characters

Quadrates of 25×25cm² in dimension, quadrat size predetermined by the species area curve method were randomly laid down randomly (Ambasht, 1970). The vegetational data were quantitatively analysed for Frequency, Density, Abundance and the Importance Value Index (IVI)
Frequency

Frequency denotes the degree of dispersion of the individual species in an area and is usually expressed in terms of percentage occurrence.

\[
\text{Frequency (\%)} = \frac{\text{Number of quadrates in which the species occur}}{\text{Total number of quadrates studied}} \times 100
\]

Density

Density represents the numerical strength of a species where the total number of individuals of each species is divided by the total number of quadrates studied.

\[
\text{Density/Quadrat} = \frac{\text{Total number of individuals of the species in all quadrates}}{\text{Total number of quadrates studied}}
\]

Density/m²

Density has been expressed in terms of plants per sq m (m²) which is obtained by multiplying the value/quadrate by an appropriate factor.

Abundance

Abundance is the study of the number of individuals of different species in the community per unit area of occurrence. The abundance value gives the idea of the distribution pattern of the species along with frequency.

\[
\text{Abundance/Quadrat} = \frac{\text{Total number of individuals of a species}}{\text{Total number of quadrates of occurrence}}
\]

Abundance/m²

Abundance has been calculated in terms of plants per square meter (m²) by multiplying the value/quadrate by an appropriate factor.
Abundance/Frequency (A/F) Ratio

The distribution pattern of the species was measured by calculating the ratio of abundance to frequency (A/F) after Curtis and Cottom, 1956.

The ratio of the abundance to frequency (A/F) (Whiteford, 1949) expresses the possible nature of the distribution pattern of the species.

\[
\text{Abundance/Frequency Ratio} = \frac{\text{Abundance of a species}}{\text{Frequency of the same species}}
\]

Importance Value Index (IVI)

The ecological significance of any plant species in a community can be given by Importance Value Index (IVI). The percentage values of relative frequency, relative density and relative dominance are added together and this value out of 300 is taken as the IVI of a species (Curtis, 1959; Misra, 1968). This method is most appropriate for grassland vegetation and for aquatic community, different methods are being used for the determination of IVI of various species by different authors. Billore and Vyas (1982) used the relative values of density, frequency and abundance to determine the IVI of each species. Handoo and Kaul (1982) computed Importance Values as the sum of relative frequency, relative density relative standing crop. Determination of basal area for estimation of the relative dominance of a species in the aquatic community has been found to be very inconvenient and onerous because the aquatic species have different growth forms (free-floating, submerged, emergent forms etc.). Therefore, the determination of basal area of the aquatic plants having different growth forms often poses a great problem. Relative abundance has been used in place of relative dominance to avoid the complicacy. In the present investigation, the value out of 300 has been taken as the IVI for all the species. The relative values of frequency, density, and abundance were determined as follows:
Relative Abundance (R.A. %) = \frac{\text{Abundance of a species}}{\text{Total abundance of all species}} \times 100

Relative Frequency (R.F. %) = \frac{\text{Number occurrence of a species}}{\text{Number of occurrence of all species}} \times 100

Relative Density (R.D. %) = \frac{\text{Number of individuals of a species (in all quadrates)}}{\text{Number of occurrence of all species (in all quadrates)}} \times 100

Relative Abundance (R.A. %) = \frac{\text{Abundance of species}}{\text{Total abundance of all species}} \times 100

**Similarity Index**

The biotic composition of two plant communities is never exactly alike due to differences in inter-specific associations. It can be calculated by formula:

Index of Similarity, \( S = \frac{2C}{A+B} \) (Sorenson, 1984)

Where, \( S \) = Similarity index,
- \( A \) = Number of species in community A
- \( B \) = Number of species in community B
- \( C \) = Number of species common in both the communities.

**Distribution pattern**

The distribution pattern of the species were analysed after Cottom and Curtis (1950).

**Community Co-efficient**

In the present study, it was calculated by the following formula:

Community Co-efficient \( S = \frac{2W}{A+B} \) (Whittaker, 1967)

Where, \( W \) = Sum of the IVI of species occurring at both communities
- \( A \) = Sum of the IVI of species of community A.
- \( B \) = Sum of the IVI of species of community B.
C. Biochemical estimation

The biochemical estimation of the plant samples were conducted during the investigation. The biochemical parameters include the following:

**Estimation of Phosphate buffer soluble proteins**

The phosphate buffer solution protein was estimated by Lowery et al., (1951)

Reagent ‘A’ = 2% sodium carbonate in 0.1N NaOH

Reagent ‘B’ = 0.5% Copper sulphate in 1% sodium potassium tartarate.

Reagent ‘C’ = mix 50ml of reagent B just prior to use.

FUR= the commercial reagent (2N) is diluted with an equal volume of water on the day of use.

50mg of different plant sample (dried) were crushed with 10ml of cold phosphate (pH 7.5; 0.1M) using pestle and mortar. The crushed material was centrifuged at 5000 rpm and the pallet was discarded and supernatant is make upto10ml with the same buffer. From this supernatant, 0.1ml was taken and volume was make upto 1ml with distilled water. 5ml of reagent C is added and incubate at room temperature for 10min. Then 0.5ml of FCR is added and incubated at room temperature in dark for 30 min. The sample developed blue colouration. The standard curve for protein is prepared by a standard protein Bovine Serum Albumin (BSA).

**Estimation of Carbohydrates (sugar)**

50 mg of dry powered plant samples were weighed and extracted with 5ml of 80% ethanol by using pestle and mortar and centrifuged for 10 min at 1000rpm. The supernatant was evaporated in the petridish until it was free from alcohol. The volume thus obtained was make upto 5ml with distilled water. This aqueous solution was taken for analysis of total soluble sugar, reducing and non-reducing sugars. From the
extract of the samples, the amount of different types of sugars present was determined by using a standard curve prepared from glucose. A reagent blank without the extract was run for every analysis done here.

(a) Estimation of Total Sugar

The content of Total Sugar in the extracts of the sample was determined by anthrone reagent (Dubois et al., 1951). To 0.1ml of the extract of above carbohydrates estimation was make upto 1ml with DW and kept in ice bath. 4ml of freshly prepared anthrone reagent with conc.H_{2}SO_{4} is added and water bath for 10min boiling then allowed to cooled at room temp. The blue green colouration appears and its absorbance was taken at 620 nm.

(b) Estimation of Reducing Sugar

Nelson’s modification of Somogy’s Method (Nelson 1944) was followed for the estimation of Reducing Sugar. The volume was make upto 1ml with distilled water to 0.1ml of the extract. 1ml of Somogy’s reagent (96 ml of reagent A +4 ml of reagent B) was added to the above solution. The mixture was incubated at 100°C for 10min in water bath. After cooling the contents, 1ml of arsenomolybdate (reagent A+ reagent B) is added. The blue- green colour developed and O.D. was taken at 620 nm by spectrophotometer. The values were calculated by a standard curve prepared from glucose.

Estimation of Amino acid

The total free amino acid was estimated by the Yemm and Cocking (1955) method. 50mg of dry samples were homogenised with 10ml of 80% ethanol by pestle and mortar. After, it was centrifuged at 5000rpm for 10min. The volume of the supernatant was make upto 10ml. From this, 2ml of extract is added with 2ml
ninyhydrin (2%: w/v, prepared in isopropanol) and 2ml acetate buffer pH 5.5. The mixture is water bathed for 20 minutes and developed violet colour. Then, the mixture is cooled at room temperature. And the volume is make upto 10 ml with aqueous isopropanol (50%w/v). The O.D of the sample was taken at the wavelength 570nm by the spectrophotometer. The values were calculated by a standard curve prepared from glycine.

**Estimation of Chlorophyll**

Estimation of Total Chlorophyll (T-Chl), Chlorophyll a (Chl-a), Chlorophyll b (Chl-b) are determined by the Arnon’s (1949) method.

**(a)Total chlorophyll**

For the estimation of Total-Chlorophyll, 500mg of fresh plants were crushed with little amount of 90% acetone with the help of a mortar and pestle. The extract was centrifuged at 5000rpm for 10min. The supernatant were taken separately for further studies. The pallet was re-crushed with 90% acetone and centrifuged until it turned white in colour. The volume of the supernatant obtained was make upto 20ml with the 90% acetone. The extract was kept in dark and the absorbance was taken at the wavelength (\(\lambda\)) viz., 470nm, 645nm and 663nm by using spectrophotometer.

The absorbance was incorporated in the formula to calculate the concentration of Chlorophyll.

\[
\text{mg Total Chl/g tissue} = [20.2(D_{645}) + 8.02(D_{663})] 	imes [v/100 	imes w]
\]
(b) Estimation of Chlorophyll ‘a’

In continuation of the above the supernatant was used for the estimation of Chl ‘a’ by Arnon’s formula

\[ \text{Mg Chl ‘a’/g tissue} = [12.7(D_{663}) - 2.69(D_{645})] \times \frac{v}{1000\times W} \]

(c) Estimation of Chlorophyll ‘b’

The same supernatant was taken for the estimation of Chl ‘b’. By Arnon’s formula

\[ \text{Mg Chl ‘b’/g tissue} = [22.9(D_{645} - 4.68(D_{663})] \times \frac{v}{1000\times W} \]