MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Study area:-

The present investigation was carried out to test the impact of sewage water irrigation on popular mulberry varieties Viz., V-1, Anantha and S-36 in the mulberry garden at Sri Krishnadevaraya University, Anantapur of Andhra Pradesh.

3.1.1 Garden selected for the present investigation:-

Sample plot – 1 Garden irrigated with bore well water
Sample plot – 2 Garden irrigated with bore well and sewage water
Sample plot – 3 Garden irrigated with sewage water

3.2 Soil parameters:-

a) pH
b) Electric Conductivity.
c) Organic Carbon.
d) Nitrate nitrogen by phenol disulphonic acid method.
e) Inorganic phosphorus by Stannous Chloride Colorimetric method.
f) Potassium by Flame photometric method.
g) Calcium by titrometric method.
h) Magnesium by titrometric method

3.3 Soil Sampling :-

a.. Based on the soil type, elevation and appearance, the land was
divided into 3 sub-plots.

b. The number of soil samples selected from 3 sub-plots depends on the size of the sub-plots.

c. Soil samples were collected only after 2 days of sewage/bore well irrigated i.e. the soil should not be wet. One square feet land between two plants in Irrigated channel should be selected randomly for collection of samples.

d. Samples were collected from three levels, i.e. from surface of about of area 30 x 30 cms, at sub-surface level of 0-30 cm and ground level of 30-60 cm depth.

e. A ‘V’ shaped pit of 30 cm depth was dug with a spade or suitable appliance at the sampling plots and about 250-500 gms sample was collected. Finally for ground soil, the sample pit was dug in ‘V’ shape at a depth of 30-60 cms.

f. Out of the sample collected at different levels, only three composite samples were to be prepared for testing.

g. Therefore samples of each level should be thoroughly mixed separately.

h. Before mixing pebbles and debris from the sample was removed.

i. Then the sample was spread and divided, and remaining two quarters are mixed thoroughly. This process is continued till the sample was reduced to about 100-200 gm.

J. Then these samples are dried in the shades.

k. Samples of each are packed separately in polythene/cotton bags and closed.

l. Each bag was tied with a label containing following information.
   1. Sample number.
   2. Date of collection of sample.
3 Colour of soil (red / black)
4 Type of land (Un irrigated/ irrigated/ water- logged)
5 Sewage or bore well irrigated.
6 Details of manures and fertilizers applied

3.4 PROCEDURE

3.4.1 Estimation of pH

The pH of the soil was measured by using Digital pH meter (Genie).

Procedure:- 25 ml distilled water was added to 10 grams of soil sample in a 50 ml breaker in the ratio 2.5:1. The mixture was stirred with a glass rod, after necessary calibration of instrument. The suspension was stirred well just before electrode was immersed.

3.4.2 Determination of Electric Conductivity (EC) by using EC meter (Jackon, 1973).

Procedure:- 20 gram soil sample was taken in a 100 ml beaker and 50ml of distilled water was added to it. The mixture was stirred immediately for 30 minutes and the suspension was allowed to settle for one hour. The suspension was filtered to get clear solution. The EC in supernatant was measured using EC Bridge, after necessary calibration of the instrument.

3.4.3 Determination of Organic Carbon (O.C) by Walky and Black Wet Oxidation Method.

18
Procedure:-- 0.5 grams of soil sample was taken and add 10ml of 1N potassium dichromate(K2 CR2O7) solution was added in a 500ml conical flask. The solution was thoroughly mixed and kept unstirred for 30 minutes. 200ml distilled water,10ml(H3po4) Orthophosphoric acid or 3 grams of sodium fluoride (NaF) powder was added and the flask was shake vigorously. 10 drops of Diphenyl amine indicator was added to it. Then flasks was filtered against 0.5N Ferrous Ammonium Sulphate (FAS) solution, till the colour changes from Violet to bright Green and the volume of ferrous ammonium sulphate consumed was noted down. Concurrently a Blank without soils was titrated. Percentage of O.C in the soil was calculated by using the formula.

Calculation :-

\[
O.C\% = \frac{(BTV- STV) \times N \text{ of FAS} \times 0.003 \times 100}{\text{Weight of sample}}
\]

Where,

\(BTV\) = Volume of 0.5 N FAS solution consumed during Blank titration.

\(STV\) = Volume of 0.5N FAS solution Consumed during sample titration.

\[
O.C\% = \frac{(BTV- STV) \times 0.5 \times 0.003 \times 100}{0.5}
\]
3.4.4 Determination of Nitrate-nitrogen of the soil by Phenolphthalein Disulphonic acid method:

Procedure: - 0 to 5ml working standard was pipetted in a Borosilicate evaporating basins (to give range from 0 to 0.05 mg No⁻³-N) an aliquot soil extract was pipetted into an evaporating basin.

From that points standards and samples were treated in the same way. Brought to dryness on a steam bath, When cool, 2 ml of Phenol disulphonic acid was added and stirred rapidly to bring the residue and acid into contact quickly. after 10 minutes, about 20 ml of water and 1+1 Ammonium hydroxide was added until the pH between 10-11 which can be identified by the appearance of yellow coloration of solution. Filtered though 9 cm Whatman No.541 filter paper into a 50ml Volumetric flask, made up to volume and mixed well. The optical density was measured at 410 nm or with a blue filter, using water as a reference. A calibration curve was prepared from the standards and that was used to determine mg NO₃⁻ Nitrogen the sample aliquot. Blank determination was carried out in the same way and subtracted where necessary.

Calculation:
If \( C = \) mg NO₃⁻-N obtained from the graph then for soil extract,

\[
C(\text{mg}) \times \text{extractant volume (ml)} \times 10^2
\]

\[
\text{Extractable NO₃⁻-N} = \frac{\text{Aliquot(ml)} \times \text{sample Wt (grams)}}{}\]

20
3.4.5 Estimation of Phosphorous of by soil by Calorimetric procedure.

Procedure: The acid and alkaline extract was mixed thoroughly in a 500ml volumetric flask and diluted to the volume, and immediately filtered though Whatman No. 44 filter paper. This exercise was to be done prior to analysis. The combined extract was acidic and prompt analysis will minimize risk of hydrolytic convertion of organic to inorganic phosphorus. A suitable aliquot of filtrate was pipetted in to 50ml volumetric flask to give a range from 0 - 0.03mg P. It was important to include one aliquot of combined acid and alkaline extracts, comparable to aliquot taken for the sample and treat the standard and samples in the same way. The sample or standard was diluted until it fills about two thirds of flask. 2ml of Ammonium molybdate reagent was added and mixed. 2ml of Stannous Chloride reagent was added and mixed, diluted to the final volume and leave for 30 minutes. The colour was read at 700nm with a blue filter using water as reference. A calibration curve was prepared from the standard, and used to determine mg P in the sample aliquot. The blank determination was carried out in the same way and subtracted where necessary.

Calculation:

If C=Mg P obtained from the sample then,

\[
C \text{ (mg)} \times \text{combined volume (ml)}
\]

Inorganic P (%) = \[
\frac{\text{10x aliquot ml x sample wt. (g)}}{\text{combined volume (ml)}}
\]
3.4.6 Determination of Potassium of soil by Flame Photometric method:

Principle: Trace amount of Potassium can be determined in either a direct reading or internal standard type of flame photometer at wavelength of 766.5 nm. The sample was sprayed into a gas flame and excitation was carried out under carefully controlled and reproducible conditions. The desired spectral line was isolated by the use.

Apparatus:
1 Flame photometer either direct reading or internal standard type.
2 Glass wares: Rinse all glass wares with 1:15 HNO₃ followed by several portions of de-ionized distilled water.

Reagents: To minimize potassium pick up store all solutions in plastic bottles. Use small containers to reduce amount of dry elements that may be picked up from bottle walls when the solution was poured. Shake each container thoroughly to wash accumulated salts from walls before pouring.

Stock Potassium solution: Dissolve 1.907gm KCl dried at 110°C and dilute to 1000ml with distilled water.

Intermediate Potassium solution: Dilute 10ml of stock Potassium solution with distilled water to 100 ml. 100ml = 100 pg K. Use this solution to prepare calibration curve in Potassium range or 1 – 100mg/ liter

Standard potassium solution:
Dilute 10 ml intermediate Potassium solution with decionised distilled water to 100 ml, 1.00ml = 10pg. K Use this solution to prepare calibration curve in Potassium range of 0.1 to 1.0mg/ liter.
Preparation of Soil extract by mixed acid digestion:

50mg of soil was taken in a beaker to this 1ml of perchloric acid (HClO₃), 2ml Nitric acid (HNO₃), 0.5ml H₂SO₄ added. After 10 min some amount of water was added to the beaker and filter into 50ml volumetric flask. The final volume was made. The readings are to be taken at 768nm.

Calculations:

a) For reference to calibration curve

\[ \text{Mg K/lit - mg K/it. in portion } \times D \]

b) For bracketing approach

\[ \text{mg K/lit = } \frac{(B - A)(s - a)}{(b - a)} + A \times D \]

Where

B = mg K/lit, in upper bracketing standard.
A = mg K/lit, in lower bracketing standard.
b = Emission intensity of upper bracketing standard.
a = Emission intensity of lower bracketing standard.
s = Emission intensity of sample.
D = Dilution ratio.

3.4.7 Estimation of Calcium of soils by EDTA Titrimetric method.

Procedure:-

Prepare the sample solution as follows:
50mg of soil was taken in a 50 ml beaker. To this 1ml perchloric acid, 2ml Nitric acid and 0.5 ml conc. Sulphric acid were added. After 10 minutes some amount of water was added to it and filtered into a 50ml volumetric flask and final volume was made by obtain a reference point by mixing about 5ml MnOH with indicator (about 0.1 gram Murexide or 5 drops of Calcon or 5 drops of glyoxal) and dilute to about 100ml with water. Pipette out 5ml of the sample solution into another titration flask. Then add about 100ml H2O, 5ml NaOH and indicator (about 0.1 gram Murexide 5 drops of calcon) These samples were again titrate with EDTA solution until the colour matches that of the reference end point. Black determination was carried out in the same way and subtracted wherever necessary.

**Calculation:**

If 1ml EDTA solution are required for titration to soils

\[
\text{Ca} \% = \frac{T \text{ (ml)} \times \text{solution volume (ml)}}{102 \times \text{ aliquot (ml)} \times \text{sample of wt (grams)}} \times 103
\]

### 3.4.8 Magnesium estimation of soil by EDTA titrometric Method

**Procedure:**

Prepare the sample solution as follows.
50 mg of soil was taken in a 50 ml beaker. To this 1 ml of Perchloric acid, 2 ml Nitric acid and 0.5 ml concentrated Sulphuric acid was added. After 10 minutes some amount of water was added to it and filtered into a 50 ml Volumetric flask and final volume was made by obtaining a reference point by mixing about 5 ml NaOH with indicator (about 0.1 gram Murexide or 5 drops of Calcon or 5 drops of glyoxal) and diluted to about 100 ml with distilled water. Pipette up to 5 ml of the sample solution into another Titration flask, then about 100 ml H2O, 5 ml NaOH and indicator about 0.1 gram Murexide 5 drops of calcon added, titrated with EDTA solution until the colour matches to that of the reference end point. Blank determination was done in the same way and subtracted where necessary.

**Calculation:**

If T ml EDTA solution was required for titration for soils

\[
\frac{T \text{ (ml)} \times \text{solution volume (ml)}}{Mg/100 \text{ gm}} = \frac{10^3}{10^2 \times \text{aliquot (ml)} \times \text{sample of wt (grams)}}
\]

4. **Water Parameters**

4.1.1 **Sampling of water:**

Water samples were collected in a clean and dry two liter polytene can. The cans were rinsed with water sample 2 to 3 times.
before the sampling of water was finally made. In case of bore well water the machine was kept on for half an hour.

### 4.1.2 Preservation of water sample

The samples were transported to the laboratories in ice box with least disturbance. The samples were stored in the refrigerator at 4°C. The analysis of Physiochemical properties of water samples collected was fixed or preservation was made within 48 hours of collection as prescribed in standard methods.

### 4.2 Water parameters

4.2.1 pH.
4.2.2 E C
4.2.3 Nitrate Nitrogen by Pheno disulphonic acid method.
4.2.4 Phosphorus by Venado Molybdo phosphoric acid Calorimetric method.
4.2.5 Potassium by Flame photometric method.
4.2.6 Carbonates.
4.2.7 Bicarbonates.
4.2.8 Calcium.
4.2.9 Magnesium.
4.2.10 Total Hardness.
4.2.11 Free Carbon dioxide.
4.2.12 Dissolved oxygen.
4.2.13 Sulphate.
4.2.14 Chloride.
4.2.1 Estimation of water pH:

The pH of water was measured by using Digital pH meter (Genie)

Procedure: The given water samples was taken in a 50ml beaker. The pH was read after necessary calibration of instrument.

4.2.2 Estimation of Electric Conductivity of water using Electric Conductivity meter.

About 30-40 ml of water samples was taken after thorough shaking. It was filtered to obtain a sample. The electric conductivity was measured using EC bridge, after necessary calibration of the instrument.

4.2.3 Estimation of Nitrate-Nitrogen of the soil by Pheno Disulphonic acid method:

Procedure:-

50 ml of given sample was taken in a Borosilicate evaporating basin and brought it to dryness on a steam bath but do not bake. After cooling, add 2ml of phenol disulphonic acid, stir rapidly to bring the residue and acid into contact quickly. left for 10 minutes, about 20ml water and 1+1 Ammonium Hydroxide until the pH between 10-11 which can be identified by the appearance of yellow colouration of solution, filtered through 9cm whatman No.541 filter paper into a 50,ml Volumetric flask. Made up to volume and mixed well. The optical density was measured at 410 nm or with a blue filter, using water as a reference.
Calibration curve was prepared from the standards and used for determination of content $\text{NO}_3^{-2-3}$ $\text{N}$ in the sample aliquot. Blank determination was done in the same way and subtracted where necessary.

**Calculation:**

If $C = \text{mg NO}_3\text{-N obtained from the graph then for water}$

$$\frac{C \text{ (mg)} \times 10^3}{\text{Aliquot (mg)}}$$

**NO}_3^{-1-1}= \text{---------------------------}$$

4.2.4 Estimation of Phosphorous in water by venado Molybdo Phosphoric Colorimetric method.

**Procedure:**

1) **Sample pH adjustment**: sample pH was adjusted by adding 0.05ml (1drop) phenolphthalein colour removal from sample and the red colour was discharged with the 1+1 Hcl before diluting to 100ml.

2) **Colour removal from sample**: Excessive colour was removed in the sample by shaking about 50ml sample with 200mg Activated carbon in an Elenmeyer flask for 50 minutes and filterd to remove Carbon. Check each of carbon for phosphate because some batches produce high reagent Blanks.

3) **Colour development in the sample**: To the 35 ml sample taken in a 50ml volumetric flask, 10 ml venerate Molybdate reagent was
added and diluted to the mark with distilled water. A blank was prepared in which 35ml distilled water was substituted for the sample. After 10 minutes or more, the absorbance of sample was measured at wavelength of 400-490 nm depending on the sensitivity desired. The colour was stable for days and its intensity was unaffected by variation in the temperature.

Unknown phosphorous concentration was determined by using Calibration graph.

**Calculation:**

\[
\frac{\text{Mg P (in 50ml final volume)} \times 1000}{\text{ml of sample}} = \frac{\text{Mg P/ litre}}{}
\]

**4.2.5 Determination of potassium by flame Photometric method**

**principle:** Trace amount of potassium can be determined in either a direct reading or internal standard type of flame photometer at wavelength of 766.5nm. The sample was sprayed into a gas flame and excitation was carried out under carefully controlled and reproducible conditions. The desired spectral line was isolated by the use

**Apparatus:**

1. Flame photometer either direct reading or internal standard type.
2. Glass wares rinse all glass wares with 1:15 Hno3 followed by several portions of deionizer distilled water.

**Reagents:** to minimize potassium pickup store all solutions in plastic bottles. Use small containers to reduce amount of dry elements that
may be picked up from bottle walls when the solution is poured. Shake each container thoroughly to wash accumulated salts from walls before pouring.

**Stock: - Potassium solution:** Dissolve 1.907gm KCL dried at 110° C and dilute to 1000 ml with distilled water.

Intermediate potassium solution: Dilute 10 ml of stock potassium solution with distilled water to 100ml. 1.00ml = 100 µg.K. Use this solution to prepare calibration curve in potassium range of 1-100mg/litre.

**Standard Potassium solution:**

Dilute 10 ml intermediate potassium solution with deionised distilled water to 100ml, 1.00 =10µg.K. Use this solution to prepare calibration curve in potassium range of 0.1 to 1.0 mg/litre.

The readings are to be taken at 768 nm.

**Calculations:**

a) For reference to calibration curve

\[ \text{mg K / lit} = \text{mg K / lit. in portion} \times D. \]

b) For bracketing approach

\[ \frac{(B - A)(s-a)}{(b-a)} + A \times D \]

Where

- B= mg K /lit, in upper bracketing standard.
- A= mg K /lit, in lower bracketing standard.
- a = Emission intensity of upper bracketing standard.
- b= Emission intensity of lower bracketing standard.
4.2.6 **Determination of Carbonate or Phenolphthalein alkalinity of water.**

**Procedure:** To the 50 ml of water sample in 250ml conical flask. 2-3 drops of phenolphthalein indicator was added and titrated against 0.05 N NaOH solution till the appearance persistent pink colour. Then the mg of CaCO$_3$ /litre can be calculated by using formula,

$$\frac{AXN}{50000}$$

Where,

A=ml of titrant.
N=Normality of NaOH.

4.2.7 **Determination of Bicarbonate in a given Water sample**

**Procedure:** 50ml of given water sample was taken into a clean conical flask. One or two drops of Methyl orange indicator was added to it and titrated against 0.02 N H2SO4 till the appearance of Orange colour.

The amount of Bicarbonates can be calculated by using formula,
\[ A \times N \times 50000 \]

\[ \text{Mg HCO}_3/\text{Liter} = \frac{\text{ml of sample}}{\text{ml of sample}} \]

Where,

- \( A \) = ml of standard acid used.
- \( N \) = Normality of standard acid.

### 4.2.8 Estimation of Calcium, Magnesium ions and Total hardness of the water.

**Procedure:** To the 50ml of water sample in a conical flask, 1-2 ml of buffer solution was added [Note- The quantity of buffer added will be dependent on the pH of water if the pH of water was more than 8 then add 1ml of buffer. If pH was less then add 2ml of buffer]. A pinch of Erichrome black t indicator was added and titrated against 0.01 EDTA solution.

**Observations:**
The Wine red colour solution turns to purple. Purple colour solution turns to blue. The burette reading from Wine red to purple was used for Ca++ estimation. The burette reading from purple to blue was used for Mg++ estimation. The sum of two readings was used to estimate the total hardness of the given water sample.
Calculations:

Ca++ estimation

\[
\text{Ca}^{++} = \frac{\text{MBR} + N \times \text{EDTA} \times \text{molecular weight of Ca}^{++} \times 1000}{\text{ml of sample}}.
\]

\[
\text{Mg}^{++} = \frac{\text{MBR} + N \times \text{EDTA} \times \text{molecular weight of Mg}^{++} \times 1000}{\text{ml of sample}}.
\]

\[
\text{Total hardness} = \frac{\text{MBR} + N \times \text{EDTA} \times 100 \times 100}{\text{ml of sample}}.
\]

Where,

MBR = Mean Burette Reading.

N = Normality

4.2.9 Determination of Free CO2 present in the given water sample:

**Procedure:** 50ml of water sample was taken in 250ml of conical flask, 2-3 drops of phenolphthalein indicator was added and titrated against 0.05 NaoH solution. The appearance of pink colour indicates the
presence of free CO2. The titration was continued till the disappearance of the pink colour.

Calculation.

\[
\text{AX NX 44000}
\]

\[
\text{Mg CO2 / Liter} = \frac{\text{ml of sample}}{}
\]

4.2.10 Estimation of Dissolved Oxygen

Procedure:-

To the sample collected in 300ml BOD bottle, add 1 ml Manganese sulphate solution followed by 1ml of Alkali iodide azide reagent along the sides of the bottle and the stopper was closed, allowed to stand for 10 mints. Add 1ml of conc. H2SO4 to dissolve ppt re stop and mix by inverting several times until the dissolution was completed. 200ml of this sample was titrated with standard sodium thiosulphate solution of a pale straw colour. Few drops of starch solution were added. Blue colour appears. Titration was continued till the blue colour disappears. End point was noted was equivalent to the amount of mg of dissolved oxygen/liter.

4.2.11. Estimation of sulphate in water by Turbid metric method.

Procedure:-

To the 100ml of sample or suitable aliquot made up to 100ml in a clean dry conical flask, 5ml of conditioning reagent was added and mixed thoroughly
using a magnetic stirrer. While a spoonful of Barium chloride crystals were added and stirred again for one minutes at constant speed.

Immediately after stirring turbidity was measured at 420nm using spectro colorimeter (systonics). A calibration curve was obtained from the solution that range from 0 - 40mg sulphate/ liter which were prepared from standard solution (100mg/liter). This was used to measure the sulphate of water sample.

If \( C = \text{mg S obtained from the graph then the for water} \)

\[
C(\text{mg}) \times 10^3
\]

\[
\text{SO}_4^{2-} \text{-S (mg/lit)} = \frac{\text{------------------------}}{\text{Aliquot (ml)}}
\]

4.2.12 Estimation of chloride by Argentometric method

Procedure:-

50 ml of given water sample was taken in a clean dry conical flask. 1-2 drops of potassium chromate indicator was added to it. The mixture was titrated against 0.0141N AgNO₃ titrant.

Calculation:

\[
\text{Mg Chloride ions/liter} = \frac{A - B \times N \times 35450}{\text{ml of sample.}}
\]
Where,

A = ml titration for sample.
B = ml titration for Blank.
N = Normality of Ag NO₃.

5 Bio chemical parameters - Leaf Estimation.

5.1 Leaf collection

5 fully grown mulberry plants were taken from 5 different places randomly. The tender medium and coarse leaves were separated from each branch. Form each branch equal number of tender medium and coarse leaves collected for estimation.

5.2 Leaf parameters

5.2.1 Determination of moisture.
5.2.2 Determination of chlorophyll.
5.2.3 Determination of sugar by anthrone method.
5.2.4 Determination of protein by FCR method.

5.2.1 Determination of moisture of mulberry leaves.

Procedure: The initial weight of 5 mulberry leaves of given variety was determined. Then the leaves are oven dried for 30 min. The weight of these dried leaves was taken. Then the moisture of leaves can be determined by using formula
Initial weight –final weight

% of moisture loss = \[\frac{\text{Initial weight} - \text{final weight}}{\text{Initial weight}}\] \times 100

5.2.2 Determination of chlorophyll of mulberry leaves.

Procedure:

(1) 1 gm of finely cut and well mixed representative sample of leaf was taken in a clean mortar.

(2) The tissue was grind with the addition of 20 ml of 80% acetone.

(3) Centrifuged (5000 rpm for 5 minutes) & transferred the supernatant to a 100 ml volumetric flask.

(4) The exercise 2 and 3 were repeated for the residue with 20 ml of 80% acetone. Continue the procedure until the residue remains colorless.

Mortar & pestle were washed with thoroughly with 80% acetone and collected the clean washing in the volumetric flask.

(5) Final volume made up to 100 ml with 80% acetone.

(6) Absorbance of the solution of read at 645 and 663 nm against the solvent (80% acetone).

Calculation:

The amount of chlorophyll present in the extract was calculated by using the following formula.
\[ \text{mg chlorophyll a/gm tissue} = 12.7 \times (A_{663}) - 2.69 \times (A_{645}) \times V \times \frac{1}{1000 \times W} \]

\[ \text{mg chlorophyll b/gm tissue} = 22.9 \times (A_{645}) - 4.68 \times (A_{663}) \times V \times \frac{1}{1000 \times W} \]

And

\[ \text{mg total chlorophyll/gms tissue} = 20.2 \times (A_{645}) + 8.02 \times (A_{663}) \times V \times \frac{1}{1000 \times W} \]

Where,

\( A \) = Absorbance at specific wavelength.

\( V \) = Final volume of chlorophyll extract in 80% Acetone.

\( W \) = Fresh weight of tissue extracted.

Note: The amount of tissue taken for extraction may be varied, accordingly amount 80% Acetone used may be altered. So that the final extract has a volume based on 10mg plant material extracted in 1ml of Acetone.

### 5.2.3 Estimation of sugar of mulberry leaves by Anthrone method (Morries, 1948).

**Procedure:** The standard solution of 0.2, 0.4, 0.8&1ml and leaf extract of 0.2ml was pipetted in to a series of test tubes. Distilled water was added to each test tube to make volume to 1ml. In another test tube 1ml of distilled water was taken to serve as Blank. 5ml of
Anthrone reagent was added to each tube. All the test tubes were cooled to room temperature & optical density was measured at 620nm against Blank. By plotting the graph of concentration v/s optical density, the sugar in the mulberry leaf extract was determined μg/ml.

**Calculation:**

\[
\text{Concentration of the sample} = \frac{\text{O.D. of the sample} \times \text{Conc. of the standard}}{\text{O.D of the standard}}
\]

5.2.4 Estimation of Protein of mulberry leaves by Folin Ciocalteau method. [Lowry et al., 1951].

**Procedure:**

Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1ml and made up the volume to 1ml by adding distilled water. 5ml of alkaline Copper reagents was added it in all the tubes. Contents were mixed thoroughly and allowed to stand at room temperature for 20 minutes. 0.5ml of folin-Ciocalteau reagent was added rapidly with immediate mixing. Absorbance was read after 30 minutes at 660nm. Plot the graph of conc. of protein vs. O.D and calculate unknown sample from the graph.

**Calculation:**

\[
\text{Concentration of the sample} = \frac{\text{O.D of the sample} \times \text{Con. of the standard}}{\text{OD of the standard}}
\]