## APPENDIX – 1

## ESTIMATION OF PROTEIN

### (Lowry *et al.*, 1951)

Protein can be estimated by different methods as described by Lowry and also by estimating the total nitrogen content. No method is 100% sensitive. Hydrolyzing the protein and estimating the amino acids alone will give the exact quantification. The method developed by Lowry *et al*. (1951) is sensitive enough to give a moderately constant value and hence largely followed. Protein content of enzyme extracts is usually determined by this method.

### PRINCIPLE

The blue color developed by reduction of the Phosphomolybdic –Phosphotungstic components in the Folin – Ciocalteau reagent by the Amino acids Tyrosine and Tryptophan present in the Protein and also colour developed by the Biuret Reaction of the Protein with the alkaline Cupric Tartrate are measured in the Lowry's method.

### MATERIALS

- **Reagent A:** 2% Sodium Carbonate in 0.1N Sodium Hydroxide
- **Reagent B:** 0.5% Copper Sulphate (CuSO4.5H2O) IN 1 % Potassium Sodium Tartrate
- **Reagent C:** Alkaline Copper Solution: Mix 50 ml of A and 1 ml of B prior to use
- **Reagent D:** Folin-Ciocalteau reagent (Reagent D): Reflux gently for 10 hours a mixture consisting of 100g sodium tungstate ($Na_2WoO_4.2H_2O$), 25g sodium molybdate ($Na_2MoO_4.2H_2O$), 700ml water, 50 ml of 85% phosphoric acid and 100 ml of concentrated Hydrochloric acid in a 1.5 L flask. Add 150 g Lithium Sulphate, 50 ml water and few drops of Bromine Water. Boil the mixture for 15 minutes without condenser to remove excess Bromine. Cool, dilute to 1litre and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1N NaOH to a Phenolphthalein end - point).
- **Stock Standard Solution:**

  Weigh accurately 50 mg of Bovine Serum Albumin and dissolve in distilled water and make up to 50 ml in a Standard flask.

- **Working Standard Solution:**

  Dilute 2 ml of the Stock solution to 10 ml with distilled water in a Standard flask. One ml of this solution contains 200 µg.

**PROCEDURE**

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard into a series of test tubes.
2. Pipetted out 0.2ml of the sample extract in to another test tubes.
3. Made up the volume to1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Added 5 ml of Reagent C to each tube including the blank. Mix well and allowed to stand for 10 minutes.
5. Then added 0.5 ml of Reagent D, mix well and incubated at room temperature in the dark for 30 minutes.
6. The blue color developed was read at 660 nm.
7. Draw a Standard graph and calculate the amount of the sample present in the extract.

**CALCULATION**

Express the amount in mg/g or 100 g sample.

## APPENDIX-2

## ESTIMATION OF TOTAL CARBOHYDRATE BY ANTHRONE METHOD

**(Hedge and Hofreiter, 1962)**

**PRINCIPLE:**

Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl methyl furfural. This compound forms with anthrone a green colored product with an absorption maximum at 630nm.

**MATERIALS:**

- **2.5N hydrochloric acid:**

  22.7ml of hydrochloric acid was made up to 100ml with distilled water.

- **Anthrone reagent:**

  Dissolved 200mg of anthrone in 100ml of ice cold 95% sulphuric acid.

- **Standard Glucose solution:**

**Stock standard:** 100mg of glucose in 100ml of distilled water.

**Working standard:** 10ml of stock standard diluted to 100ml with distilled water.

**PROCEDURE:**

1. Weighed 100mg of the sample into a boiling tube.
2. Hydrolyzed by keeping it in a boiling water bath for three hours with 5ml of 2.5N Hcl and cool to room temperature.
3. Neuratilised it with solid sodium carbonate until the effervescence ceases.
4. Made up the volume to 100ml and centrifuge.
5. Collected the supernatant and take 0.5 & 1ml aliquots for analysis.
6. Prepared the standard by taking 0.2, 0.4, 0.6, 0.8 & 1ml of the working standard.
7. Make up the volume to 1ml in all the tubes including the sample tubes by adding distilled H2O.
8. Then added 4ml of anthrone reagent.
9. Heated for eight minutes in a boiling water bath.
10. Cooled rapidly & read the green to dark green colour at 630mm.
11. Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
12. From the graph calculated the amount of carbohydrate present in the sample tube.

**CALCULATION**

Amount of Carbohydrate present in 100 mg of the sample = (mg of glucose) (Volume of Test Sample) X 100

## APPENDIX-3

## ESTIMATION OF FREE SUGAR BY ANTHRONE METHOD

**(Hedge and Hofreiter, 1962 with modification)**

**PRINCIPLE**

Free sugars are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to Hydroxymethyl furfural. This compound forms with Anthrone a green colored product with the absorption maximum at 630 nm.

**MATERIALS**

- **Anthrone Reagent**

  Dissolve 200 mg of Anthrone Reagent in 100 ml of 95% ice cold Sulphuric acid.

Prepared fresh before use.

- **Standard Glucose**

  **Stock standard:** Dissolved 100 mg in 100 ml of water.

  **Working standard**: 20 ml of stock diluted to 100 ml with distilled water and stored in

  refrigerator, after adding few drops of Toluene.

**PROCEDURE**

1. Taken 0.2ml of the sample into boiling test tubes.
2. Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard '0' serves as blank.
3. Made up the volume to 1ml in all the tubes including the sample tubes by adding distilled water.
4. Added 4 ml of Anthrone Reagent to all the tubes.
5. Heated for eight minutes in a boiling water bath.
6. Cooled rapidly and read the green to dark green colour at 630 nm.
7. Draw a standard graph by plotting concentration of the standard on the X –axis versus absorbance on the Y–axis.
8. From the graph calculated the amount of free sugar present in the sample tube.

**CALCULATION**

Amount of Free sugar present in 100 mg of the sample = (mg of sugar) (Volume of Test Sample) X 100

## APPENDIX –4

## ASSAY OF SUPEROXIDE DISMUTASE ACTIVITY

## (Misra and Fridovich, 1972)

**PRINCIPLE:**

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically at 600nm.

**REAGENTS:**

1. Potassium phosphate buffer (50 mM, pH 7.4)

2. Methionine (45 µM)

3. Riboflavin (5.3 µM)

4. Nitro Blue Tetrazolium (NBT) (84 µM)

5. Potassium cyanide (20 mM)

**PROCEDURE:**

0.5g of the ripe and unripe fruit pulp was ground separately with 3.0 ml of potassium phosphate buffer. The homogenates were centrifuged at 2000 rpm for 10 minutes and the supernatants were used for the assay. The incubation medium contained, in a final volume of 3.0 ml, 50 mM potassium phosphate buffer (pH 7.4), 45 µM methionine, 5.3 µM riboflavin, 84 µM NBT and 20 mM potassium cyanide. The tubes were placed in an aluminium foil-lined box maintained at 25°C and equipped with 15W fluorescent lamps. After exposure to light for 10 minutes, the reduced NBT was measuredspectrophoto-metrically at 600nm. The maximum reduction was observed in the absence of the enzyme. One unit of enzyme activity was defined as the amount of enzyme giving a 50% inhibition of the reduction of NBT. The values were calculated as units/mg protein.

## APPENDIX – 5

## ASSAY OF POLYPHENOL OXIDASE

**(Esterbauer *et al*., 1977)**

**PRINCIPLE**

Phenol oxidases are copper proteins, which catalyze the aerobic oxidation of certain phenolic substrates to quinines, which are auto-oxidized to dark brown pigments generally known as melanin, which can be estimated spectrophotometrically at 495 nm.

**REAGENTS**

1. Reaction medium - Tris-HCl (50mM, pH 7.2), sorbitol (0.4M), NaCl (10mM)

2. Catechol (0.01M)

3. Phosphate buffer (0.1M, pH 6.5)

**PROCEDURE**

**Preparation of enzyme extract**

The enzyme extract was prepared by macerating 5 g of leaf tissue in 20 ml reaction medium containing tris-HCl. The homogenate was centrifuged at 2000 g for 10 minutes at 4ºC, the supernatant was used for the assay.

**Assay**

Both phosphate buffer (2.5 ml) and catechol solution (0.3 ml) was pipetted out into the experimental cuvette and the spectrophotometer was set at 495 nm. The sample (0.2 ml) was added to the same cuvette and the changes in absorbance were monitored for every 30 seconds up to 5 minutes. One unit of either catechol oxidase / laccase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinine/minute. The activity of PPO can be calculated using the formula,

**Enzyme unit** = K x ($\Delta$A / minute)

where, K for catechol oxidase = 0.272, K for laccase = 0.242

## APPENDIX – 6

## ESTIMATION OF PEROXIDASE

**(Reddy *et al.*, 1995)**

**PRINCIPLE**

In the presence of the hydrogen donor pyrogallol, peroxidase converts $H_2O_2$ to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{Oxidized pyrogallol} + H_2O$$

**REAGENTS**

1. Pyrogallol (0.05M); Phoshphate buffer (pH 6.5) – 630 mg of pyrogallol in 100 ml of 0.1M Phosphate buffer.
2. Hydrogen peroxide (1%)

**PROCEDURE**

One gram of the sample was mascerated with 5 ml (w/v) 0.1 M phosphate buffer (pH 6.5) in a homogeniser. The homogenate was then centrifuged at 300 g for 15 minutes.

The supernatant was used as the enzyme source. All the procedures were carried out at 0-5° C. Three ml of 0.05 M pyrogallol solution and 0.5 to 1 ml enzyme extract was pipette out into a test tube. The spectrophotometer was adjusted to read '0' at 400 nm. To this, 0.5 ml of 1% $H_2O_2$ was added to the test cuvette. The change in absorbance for every 30 seconds upto 3 minutes was recorded.

**CALCULATION**

Change in absorbance / min = X

Weight of the plant material taken = 300 mg

Volume of the extract taken for the assay = 0.02 ml

Change in absorbance for 1.5 ml extract = (X / 0.02) x 1.5 – Y

(i.e) Peroxidase activity in 300 mg plant tissue = Y

Peroxidase activity / g plant tissue = Y x (1000/300) Units

## APPENDIX – 7
## ESTIMATION OF ASCORBIC ACID
## (Roe and Kuether, 1953)

**PRINCIPLE**

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540 nm.

**REAGENTS**

1. 4% TCA
2. 9N $H_2SO_4$
3. 2% 2, 4 - dinitrophenyl hydrazine: 2 g of DNPH was dissolved in 100 ml of 9N $H_2SO_4$
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: 100 mg of ascorbic acid was dissolved in 100 ml of 4% TCA
7. Working standard: 10 ml of the stock solution was diluted to 100 ml with 4% TCA

**PROCEDURE**

About 1 g of the sample was homogenized in 4% TCA up to 10 ml and centrifuged at 2000 rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. It was centrifuged once again and the charcoal residue was removed. The volume of the clear supernatants was noted. 0.5 and1.0 ml aliquots of this supernatant were taken for the assay.

The assay volume was made up 2.0 ml with 4% TCA. 0.2 to 1.0 ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0 ml with 4% TCA. To this, 0.5ml of DNPH reagent was added to all the test tubes, followed by 2 drops of 10% thiourea solution and incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5 ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of $H_2SO_4$. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540 nm. The content of ascorbic acid in the sample was calculated using the standard graph.

## APPENDIX-8
## ESTIMATION OF TOTAL PHENOLS
## (Malick and Singh, 1980)

**PRINCIPLE**

Phenols react with phosphomolybdic acid in Folin - Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650 nm.

**REAGENTS**

1. 80% ethanol
2. Diluted Folin – Ciocalteau reagent
3. 20% Sodium carbonate
4. Stock solution – 100 mg of catechol was made up with 100 ml distilled water
5. Working standard – 10 ml of stock standard was diluted to 100 ml. 1.0 ml of this contains 100 µg of catechol.

**PROCEDURE**

**Preparation of plant extract**

Pre-weighed millet sample (0.5 g) was ground in 5 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected and the residue was re-extracted with 2.5 ml of 80% ethanol. After repeated centrifugation, the supernatants were collected and pooled. The ethanol was evaporated and the residue was dissolved in a known volume of distilled water and used for the estimation of phenol.

**Estimation**

Aliquots (0.2 to 2.0 ml) of the standard catechol solution were made up to 3 ml with distilled water. Folin-Ciocalteau (0.5 ml) reagent was added to each test tube. After 3 minutes, 2.0 ml of 20% sodium carbonate was added to each tubes. After mixing the tubes thoroughly, all the tubes were heated in a boiling water bath for exactly one minute and allowed to cool at room temperature. The blue colour developed was recorded at 650nm against a reagent blank. The concentration of phenols in the sample was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg phenols / g.

## APPENDIX – 9
## ESTIMATION OF FLAVONOIDS
## (Zhishen *et al.*, 1999)

**REAGENTS**

1. 5% sodium nitrite
2. 10% Aluminium chloride
3. 1mM sodium hydroxide
4. Standard solution: 0.011g of catechin dissolved in 100ml of water (110Ug/ml).

**PROCEDURE**

0.1ml of methanolic extracts of plant sample was added to 0.3ml of distilled water. To this 0.03ml of 5% sodium nitrite was added to the tubes and incubated for 5 minutes. To this 1mM sodium hydroxide (0.2ml) was added and made up to 1ml with distilled water. The absorbance reading at 510nm was noted. The final absorbance of each sample was compared with a standard curve made from catechin. From the standard graph, the amount of flavonoids present on the sample was calculated.