Chapter III

Materials and Methods
3.1 Chemical Composition, Phyto-chemical Screening and Toxicity Evaluation of *P. roxburghii*:

Different stages of *P. roxburghii* pods were collected from a plant grown at Iroisemba near the Central Agricultural University campus and classified them into 4 groups based on the mean thickness of the pod measured at the site of seeds as tender (6mm and below), immature (6.1 to 10mm), mature (more than 10mm) and seeds (when the pulp could not be used further). All the pods were scrapped to remove the outer green peel. The margin of the pods were removed neatly with the help of a knife and cut into two sizes. One portion, approximately 2 mm in size was used for the determination of moisture, acidity and vitamin C. The other portion about 4/5mm in size was air dried and kept in the oven at 60±5°C for 48 hours. The moisture free samples were then grounded in the form of powder by using a Remi grinder and subsequently sieved (1 mm). The powder samples were collected and kept for various qualitative and quantitative analyses. Similar procedures were followed in case of pulp (pod minus seeds in the mature stage), seeds (whole), cotyledons (Dehulled) and testa (seed coat).

3.1.1. Moisture: Moisture content was determined by keeping 2 g of the finely cut fresh pod at 105°C in an oven up to constant weight. The loss in weight after drying gave the moisture content.

3.1.2. Acidity (Jayaraman, 1988):

3.1.2.1: Reagents:

A. 0.1 N NaOH accurately standardized against 0.1 N oxalic acid.

B. 1% (w/v) phenolphthalein in 95% ethanol.

3.1.2.2. Procedure: 10 g of the finely cut fresh pod after scraping the outer green peel was pulverized in a mortar with pestle. Filtered and volume made up to 100 ml with distilled water. 25 ml aliquot was titrated against 0.1 N NaOH using phenolphthalein indicator and reported as per cent acidity.

3.1.3. Ascorbic acid (Sadasivam and Theymoli, 1987): Ascorbic acid reduces the 2, 6-dichlorophenol indophenol dye to a colourless leuco-base. The ascorbic acid gets oxidized to dehydroascorbic acid. The dye is a blue coloured compound, which turns pink at the end point. Oxalic acid is used as the titrating medium.
3.1.3.1. Reagents:

A. 4% (w/v) Oxalic acid solution

B. 2,6-dichlorophenol indophenol dye solution: weigh 42 mg sodium bicarbonate into a small volume of distilled water. Dissolve 52 mg 2,6-dichlorophenol indophenol in it and make up to 200 ml with distilled water.

C. Ascorbic acid Stock Standard Solution: 100 mg ascorbic acid was dissolved in 100 ml of 4% oxalic acid solution (1 mg/ml).

D: Ascorbic acid Working Standard: 10 ml of the stock solution was diluted to 100 ml 4% oxalic acid. The concentration of working standard was 100 mg/ml

3.1.3.2. Sample extraction: 5 g of the freshly cut pod was extracted with a small volume of 4% oxalic acid and crushed in a glass mortar with pestle. The extract was centrifuged and supernatant was made up to 100 ml with 4% oxalic acid.

3.1.3.3. Procedure: 5 ml of the working standard solution was pipetted into a 100 ml beaker. 10 ml of 4% oxalic acid was added and titrated against the dye. End point is the appearance of pink colour which persists for few minutes ($V_1$ ml). 5 ml of the extract was transferred into a 100 ml beaker. 10 ml of 4% oxalic acid was added and titrated against the dye until appearance of a slight pink colour that persists for some minutes ($V_2$ ml). The ascorbic acid was calculated as per the following formula and reported in mg/100 g edible portion.

3.1.3.4. Calculation: Amount of ascorbic acid mg/100 g sample =

$$\frac{0.5 \text{ mg} \times V_2 \times 100 \times 100}{V_1 \times 5 \times \text{Weight of the sample (g)}}$$

3.1.4. Crude fat/oil (Chopra and Kanwar, 1980): 2 g of the powder was placed in a soxhlet apparatus and extracted with pet. ether (b.p. 60-80) for 5 hours. The extract was filtered and transferred into a previously weighed beaker. The solvent was evaporated on a water bath (100°C). The last traces of moisture were removed by keeping it in an oven maintained at 100°C (±5) for 3 hours. The beaker was removed and allowed to cool inside a desiccator and reweighed. The increase in weight gave the crude fat/oil and reported in percentage using the following formula
Oil % in the sample = Weight of oil (g) x 100/Weight of sample (g)

3.1.5. Crude fibre (Chopra and Kanwar, 1980): Crude fiber was determined by acid and alkaline digestion method following the procedures of Chopra and Kanwar (1980)

3.1.5.1. Reagents:
   A. 1.25% H₂SO₄ (v/v)
   B. 1.25% NaOH (w/v)
   C. 1% HNO₃

3.1.5.2. Procedure: 2.0 g moisture and fat free sample was taken in a 1000 ml beaker and added 100 ml 1.25% H₂SO₄ to it. It was boiled for half an hour and filtered through a muslin cloth (45 threads per inch). Washed with water until the residue was acid free and then transferred the same into the beaker. Again 100 ml of 1.25% NaOH was added and boiled for 30 minutes. It was then filtered and washed with hot water and 1% HNO₃ alternately in a crucible and dried to a constant weight at 100°C. The residue was ignited to get ash and loss of weight due to ignition gave the crude fibre.

3.1.6. Crude carbohydrates (Müller and Tabin 1980): The crude carbohydrate content was calculated by difference following the method of Müller and Tabin (1980) and calculated as per the following relation:

Crude carbohydrate (%) = 100 - [Crude protein (%) + crude lipid (%) + crude fibre (%) + Ash (%)]

3.1.7. Crude protein (Humphries, 1956): Crude protein in the samples was determined by estimating the total nitrogen (Kjeldahl method) in a Kel-Plus Supra-LX nitrogen auto analyzer following the procedure described by Humphries (1956).

3.1.7.1. Reagents:
   A. Conc. H₂SO₄
   B. Salt Mixer (K₂SO₄: CuSO₄·5H₂O: metallic selenium: 50:10:1)
   C. 4% Boric acid (w/v).
D. Mix indicator: 0.3 g bromocresol green and 0.2 g methyl red in 400 ml of 90% ethanol. The indicator colour will change from red in acid solution to blue in alkaline solution.

E. 40% NaOH (w/v).

F. 0.1 N HCl (standardized)

3.1.7.2. Procedure: 0.5 g of the powdered sample was transferred into the digestion tube. 10 ml of conc. H_2SO_4 and 5 g of digestion mixer were added to the sample. The digestion tubes were heated in the digestion block between 360-410°C or until the sample turns colourless or light green. In the distillation unit, the ammonium radicals are converted to ammonia under excess alkali condition (40% NaOH) after neutralizing the acid in the digested sample. The digested samples were heated by passing steam and the ammonia liberated due to the addition of alkali was dissolved in 20 ml of 4% boric acid which was titrated against the standard 0.1 N HCl. Similarly a blank without the sample was also prepared. The per cent nitrogen thus obtained was multiplied by 6.25 to get crude protein.

3.1.7.3. Calculation: % Nitrogen =

\[
\frac{\text{sample titer} - \text{blank titer} \times \text{Normality of HCl} \times 14 \times 100}{\text{Sample weight (g) } \times 1000}
\]

3.1.8. Ash % (Total minerals): 1 g of the powder sample was placed in a crucible and ignited in an electric heater with a covering device till nearly to a constant weight which gave the weight of ash and was reported in percentage.

3.1.9. Gross energy (Osborne and Voogt 1978): The gross energy estimation was based on Osborne and Voogt (1978) following the relation-

Gross Energy KJ/100g DM =

\[
[\text{Crude protein(%) } \times 4] + [\text{Crude lipid(%) } \times 9] + [\text{crude carbohydrate (%) } \times 4]
\]

3.2. Physico-Chemical Characteristics of the Oil:

3.2.1. Normality of the oil (Jayaraman, 1988): The conventional titration against standard NaOH solution was used. A slight modification was needed because of the insolubility of the fatty acids in water. For this purpose, 1 ml of the oil was suspended in 50% aqueous ethanol solution (already neutralized to phenolphthalein end point) and titrated against 0.1 N NaOH
using phenolphthalein indicator. The end point indicates by the appearance of a faint but permanent pink colour. Normality of the oil is calculated by applying the following formula:

\[ V_1N_1 = V_2N_2 \]

Where \( V_1 \) = volume of alkali used
\( N_1 \) = normality of the alkali used
\( V_2 \) = volume of oil used and
\( N_2 \) = normality of the unknown oil.

3.2.2. Acid value of the oil (Core and Pearson, 1962): The free fatty acids in the oil were estimated by titrating it against KOH in the presence of phenolphthalein indicator. The acid value (or number) is defined as the mg KOH required to neutralize the free fatty acids present in 1 g of the sample.

3.2.2.1. Reagents:
A. 1% (w/v) phenolphthalein in 95% ethanol.
B. 0.1 N KOH
C. Neutral solvent: 25 ml ether and 25 ml 95% ethanol is mixed with 1 ml of phenolphthalein solution and neutralized with 0.1N KOH.

3.2.2.2. Procedure: 1 g of the fat extracted from \( P. \) roxburghii seeds was dissolved in 50 ml of neutral solvent in a 250 ml conical flask. 1 ml of phenolphthalein indicator was added. The solution was shacked and titrated against 0.1 N KOH until a faint pink colour appeared which persists for about 15 seconds.

3.2.2.3. Calculation: Acid value (mg KOH/g sample)

\[ \text{Acid value} = \frac{\text{Titre Value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the Sample (g)}} \]

3.2.3. Saponification value of the oil (AOAC, 1975): When oil or fat is heated with KOH (or alkali), it is saponified and releases fatty acids and glycerol. Each molecule of fat/oil uses 3 molecules of KOH for saponification. Saponification value is the number of mg of KOH required to saponify the fatty acids resulting from the complete hydrolysis of 1 g of fat or oil.

3.2.3.1. Reagents:
A. 0.5 N HCl
B. Alcoholic KOH: 40 g KOH in 1 litre of ethanol (w/v)

C. 1% phenolphthalein in 95% ethanol.

3.2.3.2. **Procedure:** 1 g of the fat extracted from *P. roxburghii* seeds was added in 50 ml of alcoholic KOH and refluxed for about 1 hour. Cooled to room temperature. 1 ml of phenolphthalein indicator was added and titrated against 0.5N HCl until the pink colour just disappears. A blank was also run with the same procedure but without the sample.

3.2.3.3. **Calculation:** Saponification value

\[
\text{Saponification value} = \frac{28.05 \times \text{(titre value of blank} - \text{titre value of sample)}}{\text{Weight of Sample (g)}}
\]

3.2.4. **Iodine value of the oil** (AOAC, 1975): The oils contain both saturated and unsaturated fatty acids. Iodine gets incorporated into the fatty acid chain wherever the double bond exist. Hence, the measure of iodine absorbed by oil gives the degree of unsaturation. Iodine value is defined as the number of grams of iodine absorbed by 100 g of the oil.

3.2.4.1. **Reagents:**

A. Hanus Iodine Solution: 13.6 g of iodine dissolved in 825 ml glacial acetic acid by heating and cool to room temperature. 25 ml of this solution was titrated against 0.1N sodium thiosulphate. Subsequently, another solution of 200 ml glacial acetic acid and 3 ml of bromine was prepared. To 5 ml of this solution, 15% KI solution was added and titrated against 0.1 N sodium thiosulphate. Bromine value was calculated so as to double the halogen content of the remaining 800 ml of the above iodine solution as follows:

\[
X = \frac{B}{C},
\]

where \(X\) = ml of bromine solution required to double the halogen content,

\(B = 800 \times \text{thiosulphate equivalent of 1 ml of iodine solution}\) and

\(C = \text{thiosulphate equivalent of 1 ml of bromine solution}\).

B. 15% KI solution (w/v).

C. 0.1% Sodium thiosulphate solution (w/v)
D. 1% Starch (w/v).

3.2.4.2. Procedure: 1 g of the fat extracted from *P. roxburghii* seeds was dissolved in 10 ml of chloroform in an iodine flask. 25 ml of the Hanus solution was added slowly. Mixed and allowed to stand for 30 minutes in the dark with occasional stirring. 10 ml of 15% KI was added and shook thoroughly. The stopper and the sides of the flask were washed down with 100 ml of boiled and cool water. It was titrated against 0.1N sodium thiosulphate until the yellow solution turns almost colourless. After this, a few drops of starch indicator was added and titrated until the blue colour completely disappears. A blank solution was also run with the same procedure but without the sample.

3.2.4.3. Calculation: Iodine value = \[
\frac{(B-S) \times N \times 12.69}{\text{Weight of Sample (g)}}
\]
Where B = ml of thiosulphate for blank
S = ml of thiosulphate for sample
N = normality of thiosulphate solution

3.3. Determination of Important Biochemical Constituents:

3.3.1. Total soluble sugars (Morris, 1948): 100 mg powdered sample was extracted with a small volume of 80% ethanol in a pestle and mortar for three times. The extracts were pooled and centrifuged at 5000 x g for 10 min. The volume of the supernatant was made up to 20 ml with 80% ethanol and transferred into batches of 5 ml cryo-vials and stored at 4°C until use. 1 ml of the extract from the cryo vial was evaporated to almost dryness and dissolved in 5 ml distilled water. The extract was used for the determination of total soluble sugar using anthrone reagent. The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses and hexuronic acids. Carbohydrates are dehydrated by conc. H_2SO_4 to form furfural. Furfural condenses with anthrone (10-keto, 9-10-dihydroanthracene) to form a blue green coloured complex which is measured at 630 nm.

3.3.1.1. Reagents:

A. Anthrone reagent: It was prepared by dissolving 200 mg of anthrone in 100 ml of ice cold 95% H_2SO_4 just before use.
B. Standard glucose: 100 mg analytical grade glucose was dissolved in 100 ml dd water. 10 ml of this solution diluted to 100 ml dd water and stored in the refrigerator with a few drops of toluene on it (100 μg/ml).

3.3.1.2. Procedure: 0.1 ml of the extract was transferred in a test tube and made up to 1 ml with dd water. 4 ml of cold anthrone reagent was added quickly while keeping the tubes inside ice cold water. Mix thoroughly. Similarly standard glucose solutions ranging from 20 μg to 100 μg were transferred into different test tubes and subjected to the same procedure. The tubes were heated for 8 min in a boiling water bath. Cool rapidly and the absorbance was taken at 630 nm in a double beam UV-Visual spectrophotometer against a blank solution.

3.3.1.3. Calculation: Amount of carbohydrate (%) =

\[
\frac{C \times \text{Total vol. of extract} \times \text{dilution factor} \times 100}{\text{Aliquot (ml)} \times \text{Wt. of sample (mg)}}
\]

Where C = concentration (mg) from the standard curve.

3.3.2. Starch (Hedge and Hofreiter, 1962): The residue left in the tube at 3.3.1 was dried in an oven at 50°C for 24 hrs and used for the estimation of starch using anthrone reagent. The residue was extracted with perchloric acid. In hot acidic medium starch is hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone.

3.3.2.1. Reagents:

A. Anthrone reagent: Same as in 3.3.1.1A.

B. Standard glucose solution: Same as in 3.3.1.1B.

C. 52% Perchloric acid.

3.3.2.2. Procedure: 5 ml of dd water and 6.5 ml of 52% perchloric acid were added to the residue and kept inside a refrigerator for 20 min. The tubes were centrifuged for 10 min at 5000 x g and the supernatant preserved. The extraction was repeated with the same procedure and preserved the supernatant. The supernatants were pooled and made up to 100 ml. 0.1 ml of the extract was transferred in a test tube and subjected to the same procedure as in 3.3.1.2.
3.3.2.3. Calculation: Same as in 3.3.1.3. The value obtained from the above relation is multiplied by 0.9 to arrive at the starch content.

3.3.3. Free amino acids (Moore and Stein, 1948): The extract used for the determination of total soluble sugar (3.3.1) was used for the estimation of free amino acids. Ninhydrin (triketohydrindenehydrate), a powerful oxidizing agent reacts with α-amino acid between 4 and 8 pH and decarboxylates to give an intensely bluish-purple coloured compound which is measured at 570 nm. The amino acids proline and hydroxyproline gives a yellow colour.

3.3.3.1. Extraction: 100mg powdered sample was extracted with a small volume of 80% ethanol in a pestle and mortar for three times. The extracts were pooled and centrifuged at 10,000 rpm for 10 minutes. 1 ml of the ethanol extract was evaporated to almost dryness and dissolved in 5 ml distilled water.

3.3.3.2. Reagents:

A. 0.2 M Citrate buffer, pH 5.0.

B. Ninhydrin reagent: 0.8 g of stannous chloride (SnCl2.2H2O) was dissolved in 500 ml of 0.2 M citrate buffer (pH 5.0). To this solution, 20 g ninhydrin (analytical grade) was added in 500 ml of methyl cellosolve (2-methoxyethanol). It was prepared on the day of experiment.

C. Diluent solvent: Prepared by mixing equal volume of dd water and n-propanol.

D. Stock standard leucine solution: It was prepared by dissolving 50 mg of leucine in 50 ml dd water.

E. Working standard leucine solution: 10 ml of stock solution was diluted to 100 ml with dd water (100 µg /ml).

3.3.3.3. Procedure: 1 ml of the sample was pipetted in a test tube and 1 ml of ninhydrin solution was added to it and mixed thoroughly. The tubes were heated for 20 min in a boiling water bath. 5 ml of the diluent solvent was added to the mixture while still inside the water bath. After 20 min, the tubes were cooled under running tap water and read the absorbance of the purple colour against a reagent blank (prepared by taking 1 ml of 80% ethanol instead of extract) in a UV-Visible spectrophotometer at 570 nm. Total free amino acid was calculated using standard
curve prepared from leucine by pipetting out 0.1 to 1.0 ml of working standard solution.

3.4. Storage Protein Fractionation and Estimation:

3.4.1. Protein fractionation and estimation (Thimmaia, 2006; Lowry et al., 1951): Studies on the solubility fractionation and characterization of seed proteins were done following the method described by Thimmaia (2006). The concentration of the different fraction of protein was estimated following the procedure of Lowry et al., (1951). The proteins are soluble in different solvents thereby enabling extraction of the different protein fractions.

3.4.1.1. Reagents:

A. 4% NaCl (w.v)

B. 60% Isopropanol (v/v) and

C. 0.4% NaOH (w/v)

D. Solution A: 2% Sodium carbonate (anhydrous) in 0.1 N NaOH (w/v)

E. Solution B: 0.5% Copper sulphate (CuSO4.5H2O) in 1% Sodium potassium tartrate (w/v).

F. Solution C: 50 ml of solution A with 1 ml of solution B mixed just prior to use.

G. Folin-Ciocalteu reagent (FCR).

H. Standard protein stock solution: 50 mg bovine serum albumin (BSA) in 50 ml dd water.

I. Working standard solution: 10 ml of the stock solution diluted to 50 ml with dd water (200 µg protein/ml)

3.4.1.2. Procedure: 1 g seed powder was extracted for 1 hr with the following solvents in the ratio of 1:10 (w/v) using a magnetic stirrer.

A. Water to extract albumins

B. 4% NaCl solution for globulins

C. 60% Isopropanol for prolamines and

D. 0.4% NaOH for glutelins.
After each extraction, the slurry was centrifuged at 5000 x g for 10 min. The supernatant collected and residue used for the subsequent extraction. 0.2 ml of the different extracts transferred into test tubes and vol. made up to 1 ml with dd water. A tube with 1 ml of water served as the blank. 5 ml of solution C was added and incubated at room temperature for 10 min. 0.5 ml of FCR was added and mixed well and incubated at room temperature in dark for 30 min. After 30 min, the absorbance was read at 660 nm in a UV-Visual spectrophotometer against the blank. The amount of different fraction of proteins were calculated from the standard curve drawn with 40 µg to 200 µg/ml of BSA.

3.5. Phenolics:

3.5.1. Total soluble phenols (Bray and Thorpe, 1954): Total soluble phenols were estimated from the water extract used for determination of soluble sugar at 3.3.1. Phenols react with an oxidizing agent, phosphomolybdate in Folin-Ciocalteu reagent under alkaline conditions and result in the formation of a blue coloured complex, the molybdenum blue which is measured at 650 nm in a UV-Visual spectrophotometer.

3.5.1.1. Extraction: Same as in 3.3.3.1.

3.5.1.2. Reagents:

A. Folin-Ciocalteu reagent: Commercially available. Diluted 1:1 with water.

B. 20% Na₂CO₃ (w/v).

C. Catechol standard stock solution: Prepared by dissolving 100 mg in 100 ml dd water.

D. Catechol working standard solution: 10 ml of the stock solution diluted to 100 ml with dd water (100 µg/ml).

3.5.1.3. Procedure: 0.5 ml of the sample extract was transferred into a test tube and vol. made up to 3.0 ml with dd water. 0.5 ml of Folin-Ciocalteu reagent was added. After 3 min, 2.0 ml of 20% Na₂CO₃ was added and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one min, cooled and measured the absorbance at 650 nm against a reagent blank. Total phenols were calculated using a standard curve of catechol and expressed as mg phenols/100 g material.
3.5.2. **Ortho-dihydric phenols** (Mahadevan and Sridhar, 1986): Ortho-dihydric phenols are highly reactive and toxic to pathogens and their enzymes. They were estimated from the same extract used for estimation of soluble phenols above by Arnow’s reagent. Arnow’s reagent specifically reacts with orthodihydric phenols and produces a pink coloured complex which is measured at 515 nm in a UV-Visual spectrophotometer.

**3.5.2.1. Extraction:** Same as above.

**3.5.2.2. Reagents:**

A. 0.05 N HCl

B. 1.0 N NaOH

C. Arnow’s reagent: Prepared by dissolving 10 g of sodium nitrite (NaNO₂) and 10 g of sodium molybdate (NaMoO₄) in 100 ml of water and was stored in a brown bottle.

D. Catechol standard stock solution: Prepared as in 3.5.1.2C

E. Catechol working standard solution: Prepared as in 3.5.1.2D

**3.5.2.3. Procedure:** 0.5 ml of the sample extract was transferred in a test tube and made up to 1.0 ml with dd water. 1.0 ml of 0.05 N HCl, 1.0 ml of Arnow’s reagent, 8.0 ml of water and 2.0 ml of 1.0 N NaOH were added. It was mixed thoroughly. The absorbance of the pink colour was measured at 515 nm. The amount of ortho-dihydric phenols present in the sample was calculated using the standard curve prepared from catechol (3.5.1.3.) and expressed as mg/100 g material.

3.5.3. **Bound phenols** (Chattopadhyay and Samadar, 1989): Occurrence of esterified phenols in bound form with cell components is known. The bound phenols may be liberated by treatment of plant tissues with NaOH at room temperature. The alkali extract contains the released phenols which are measured at 290 nm in a UV-Visual spectrophotometer using the Folin-Ciocalteu reagent.

**3.5.3.1. Extraction:** 100 mg of powder sample was ground with 4 ml of SDS solution in a mortar with pestle. The contents were transferred in a tube and centrifuged at 3000 x g for 5 min. The supernatant was discarded. The residue washed successively once with 5 ml of SDS solution, twice with 5 ml
of water, twice with 5 ml of ethanol and twice with 5 ml of diethyl ether. The supernatants were discarded and the residue was dried and preserved.

3.5.3.1. Reagents:
   A. Absolute Ethanol
   B. Diethyl ether
   C. 0.5 M NaOH
   D. 3% Sodium lauryl sulphate solution (SDS:w/v).
   E. 20% NaCO₃ (w/v)
   F. Folin-Ciocalteu reagent (commercially available).
   G. Catechol standard: As in 3.5.1.2C and D.

3.5.3.2. Procedure: 3 ml of 0.5 M NaOH was added to the above dry residue and kept at room temperature overnight. Next day, the mixture was centrifuged and the supernatant was saved. 0.5 of the supernatant was transferred into a tube and subjected to the same procedure as in 3.5.1.3.

3.5.4. Anthocyanin estimation (Swain and Hillis, 1959): Anthocyanins are the most important and widespread groups of colouring materials in plants. It is estimated from the extract at 3.5.1. The alcohol extract of the sample was treated with HCl in aqueous methanol followed by anthocyanin reagent. The colour intensity was measured at 525 nm in a UV-Visual spectrophotometer.

3.5.4.1. Extraction: 100mg powdered sample was extracted with a small volume of 80% ethanol in a pestle and mortar for three times. The extracts were pooled and centrifuged at 10,000 rpm for 10 minutes.

3.5.4.2. Reagents:
   A. 0.5 N HCl in 85% methanol.
   B. Anthocyanin reagent: 1 ml of 30% H₂O₂ with mixed 9 ml of methanolic HCl.

3.5.4.3. Procedure: 1 ml of the alcohol extract was transferred into a test tube. 3 ml of aqueous methanolic HCl and 1 ml of anthocyanin reagent were added. The blank
tube was prepared in the same manner by adding 1 ml of aqueous methanolic HCl solution instead of anthocyanin reagent. All the tubes were kept in the dark for 15 min and measured the absorbance at 525 nm against the blank. The amount of anthocyanins was calculated from the relation that 10 µg of cyanin hydrochloride in methanol-HCl gives absorbance of 0.405 in a 1.0 cm cell at 525 nm.

3.5.5. Leuco-anthocyanin (Swain and Hillis, 1959): Leucoanthocyanins are the colourless polymeric matters present in plants. It was estimated from the water extract at 3.2.12 above. The alcohol extract of the plant when treated with leuco-anthocyanin reagent gave a colour complex which was measured at 550 nm in a UV-Visual spectrophotometer.

3.5.5.1. Extraction: Same as in 3.3.3.1.

3.5.5.2. Reagents:

A. Leuco-anthocyanin reagent: 25 ml of 36% HCl diluted to 500 ml with n-butanol.

3.5.5.3. Procedure: 1 ml of extract transferred into a test tube and 10 ml of leuco-anthocyanin reagent was added. The tubes were shaken properly and heated in a water bath at 97±1°C without covering the tubes. After 3 min the tubes were covered with glass stoppers and continued heating for a total period of 40 min. A blank tube was also maintained similarly with the extract but without heating. The tubes were cooled under a running tap and measured the absorbance at 550 nm in a UV-Visual spectrophotometer and expressed the results as $A_{550} \times 1000$.

3.6. Elemental Analysis:

Wet digestion method of Capar et al., (1978) was followed for the analysis of different elements. Na and K were estimated in a systronics-105 flame photometer. Sulphur and phosphorus were estimated in a UV-VIS double beam spectrophotometer following the procedures described by Tandon (1993). Ca, Mg, Zn, Fe Cu, Mn and Co were estimated in a Parkin Elmer atomic absorption spectrophotometer, Analyst AA-200. The air and acetylene flow rate were maintained at 10.0 and 2.5 liters /min.

3.7. Phyto-chemical Screening of P. roxburghii:

10 g powder sample was extracted with ethanol in a Soxhlet apparatus for 7 hours. 10 ml
extract was isolated for flavonoid test. The remaining extract was evaporated to dryness on a water bath and used for alkaloid and saponin test. All the tests were carried out following the methods described by Kapoor et al., (1969) and Das and Bhattacharya (1970).

3.7.1. Test for flavonoids: Flavonoids are plant pigments which include flavones, flavonols, isoflavones, flavonones, catechins, Leuco-anthocyanins, anthocyanins, aurones and chalcones. One of the most useful test is the so-called cyanidin reaction. To 1 ml of the extract, a few drops of conc. HCl and some pieces of magnesium turnings were added. It was warmed up to 60°C. Appearance of pink or magenta colour shows presence of flavonoid.

3.7.2. Test for alkaloid: To a small part of residue, 5 ml of 1% HCl was added and filtered. To the filtrate Dragendorff's (solution containing 0.85 g bismuth sub-nitrate in 10 acetic acid and 40 ml water mixed with a solution of 8.0 g KI in 20 ml water) reagent was added. Appearance of any precipitate or turbidity shows presence of alkaloids. In order to rule out any possibility of a false positive test, a confirmatory test for alkaloids was performed. Small portion of the residue was dissolved in 5 ml of 1% HCl, it was filtered and made distinctly alkaline with 28% ammonium hydroxide (NH₄OH) solution and extracted with an equal volume of chloroform. The chloroform solution was extracted with an equal volume of 1% HCl which was separated and tested with Dragendorff's reagent. Any precipitate or turbidity confirmed the presence of alkaloids.

3.7.3. Test for saponins: To a small part of the residue, a little tap water was added and shook vigorously. Appearance of a honey comb like froth that persists for about 15 minutes shows presence of saponins. A portion was also dissolved in chloroform and filtered. A few drops of conc. H₂SO₄ and 1 ml of acetic anhydride were added to 1 ml of the ice-cool filtrate. The appearance of blue, bluish green or reddish brown colour often accompanied by the formation of a pink ring showed the presence of saponins.

3.7.4. Test for tannins: Tannins were detected by the gelatin salt block test. A small part of the residue, was dissolved in water and tested with gelatin solution (1%), gelatin salt solution (1% gelatin+10%NaCl) and salt solution (10% NaCl). Appearance of a white precipitate with gelatin solution (1%) or gelatin salt solution was taken as a positive test for tannins but if a precipitate was obtained with salt solution alone, the test was treated as negative.

3.7.5. Test for cyanide: To about 2 g of the sample, 5 ml conc. HCl was added. It was fitted with a gas delivery tube with its end dipping in distilled water contained in another tube. Heat the sample and the gas evolved was collected inside water. The test tube containing
water was made alkaline with 0.1 N NaOH. A few drops of freshly prepared ferrous sulphate (FeSO) was added and boiled. 2 drops of ferric chloride (Fe₂SO₃) solution was added and acidified with HCl. Appearance of deep blue or green colour shows the presence of cyanide.

3.8. Toxicity Evaluation in *P. roxburghii*:

The seeds of *P. roxburghii* were cut into small pieces, shade dried ground in Remi grinder and finally passed through a 30 mesh sieve to get them in powder form. Petroleum ether (b.p. 60-80°C) extract of the powder was taken in soxhlet apparatus for 7 hr. The extract was concentrated in a flash evaporator. Benzene was added up to 5% (v/w) of the extract to make a stock solution. The desired dilution was made with distilled water using 0.5% triton x100 as emulsifier in each case. The treatments along with two controls viz. emulsified water and water alone were replicated thrice.

For contact toxicity test, fresh leaves of the Indian bean *Lablab purpureus* (Linn.) were collected from the unsprayed field and washed thoroughly with tap water. After drying, each leaf was dipped into a specific concentration of the extract and dried again under a ceiling fan in a Petri dish (15 cm dia.). Twenty healthy aphids were released into each Petri dish and the aphid mortality was counted after 24, 48, 72 and 96 h of the release.

3.9. Organoleptic Taste:

13 *Parkia roxburghii* cultivars of almost the same age group having different morphological characters were collected from the main market place at Imphal for the evaluation (Photo Plate 3.9.1). Five pods were randomly selected from each cultivar. Observations on physical parameters viz. pod weight, pod length, stalk length, number of seeds per pod and colour were recorded. Chlorophyll and carotinoid in the pods were also worked out as they determined the colour of the pods. This was conducted to see any correlation between flavour and physical characters of the *P. roxburghii* pods.

3.9.1. Chlorophylls and carotinoids (Jayaraman, 1988): The pods were thoroughly washed and blotted dried. The skin of the pods neatly scraped and used for the estimation of Chlorophylls and carotinoids.

3.9.1.1. Reagents:

A. 80% acetone (v/v).
Photo plate 3.9.1: Morphologically different 13 cultivars of Pukka rothoreshi selected for the organoleptic taste
3.9.1.2. **Procedure:** The outer green skin was carefully scrapped with the help of an indigenous apparatus called ‘Yongkhot’. 1 g of the fresh skin was transferred into a test tube and 10 ml acetone was added. Covered with a parafilm and kept inside the refrigerator for 48 hrs. 1 ml of the extract was diluted to 10 ml 80% acetone and its optical density was measured at 3 wavelengths 490, 645 and 663 nm. Using the following equations, the concentrations of the pigments were directly calculated.

\[
\begin{align*}
\text{Total Chlorophyll (g lit)} & = (0.0202) \cdot (\text{OD}_{645}) + (0.00802) \cdot (\text{OD}_{663}) \\
\text{Chlorophyll a (g lit)} & = (0.0127)(\text{OD}_{663}) - (0.00269)(\text{OD}_{645}) \\
\text{Chlorophyll b (g lit)} & = (0.0229)(\text{OD}_{645}) - (0.00468)(\text{OD}_{663}) \\
\text{Carotene (g lit)} & = (\text{OD}_{490}) - (0.114)(\text{OD}_{663}) - (0.638)(\text{OD}_{645})
\end{align*}
\]

3.9.2. **Determination of flavour** (Meitei and Singh 1990): For ascertaining flavour, organoleptic test method described by Meitei and Singh (1990) was adopted. Five apparently healthy persons within the age group of 20-30 were selected from five communities viz; Meitei, Mao-naga, Kuki, Koireng and Muslim. They had been explained about the underlying principles about how the flavor they conceived was to be converted into values/scores. The spread of scores was from 1 to 5 where 1 = very poor, 2 = poor, 3 = moderate, 4 = good and 5 = best. 1 g of the needly scrapped pod was kept on a clean plate. The organoleptic scores thus obtained were directly treated as flavour values.

3.10. **Effects of Processing and Cooking Methods in P. roxburghii:**

Pods of different stages and seeds of *P. roxburghii* were collected from Namphalong, a Myanmarese border town in the south east of Manipur, India. Pods after removing the outer green peel were used for the study. In case of seeds, after removing the extraneous material, were divided into two portions, one to be used with seed coat (Whole seed) and another without (dehulled). For dehulling, the hulls (Seed coat) were removed manually from the seeds, soaked overnight for 12 hr (DSS). The materials were weighed and subjected to ordinary and pressure cooking following the method of Saroj and Neelam (1994) with slide modification. In ordinary cooking, the seeds (with seed coat) as well as dehulled and soaked seeds (DSS) placed along with double distilled water (4 times the respective weights, w: v) in a round open beaker and cooked on a heater until they became soft. In case of pressure cooking, both the portions of seeds were pressure cooked (3 lit pressure cooker) at the maximum pressure for 5 min. In this case, the seed to water ratio was 1:3 (w: v). All the
processed/cooked pods/seeds were dried in oven at 45°C to a constant weight. The dried samples were weighed and ground in a Remi grinder, sieved (1mm) and stored at paper bags at room temperature for analytical purposes.

3.10.1. Proximate composition: The processed/cooked materials were blotted with tissue paper and kept overnight in the shade. Moisture content was determined by heating the sample at 65°C in an oven up to constant weight. The loss in weight after drying gave the moisture content. Others were determined as per the procedures described in 3.1.4. to 3.1.9 above.

3.10.2. Determination of total sugar: Total soluble sugar was determined as per the method described at 3.3.1 above.

3.10.3. Flavonoid estimation (Chang et al., 2002 with slight modifications): 50 mg of the powdered sample was transferred into a 50 ml centrifuge tube. 10 ml methanol was added and stirred for 4 hr in a magnetic stirrer. The solvent was evaporated to about 5 ml at room temperature and centrifuged at 10,000 rpm for 15 min. The supernatant was collected and volume made up to 5 ml with methanol.

3.10.3.1. Reagents:

A. Methanol

B. 10% Aluminum chloride (w/v)

C. 1M Potassium acetate.

3.10.3.2. Procedure: 0.1 ml of the extract supernatant was transferred into a test tube. 0.1 ml of aluminum chloride and 0.1 ml of 1M potassium acetate were added and volume made up to 3 ml with dd water. This was allowed to stand for 30 min in room temperature and its absorbance was measured at 415 nm in a UV-Visual spectrophotometer. The calibration curve was prepared using quercetin (10-100µg/ml) in methanol. The concentration of flavonoids was expressed in terms of mg/100g.

3.10.4. Minerals: Calcium(Ca), potassium (K), magnesium (Mg), phosphorus (P) sulphur (S), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu) and cobalt (Co) were determined as per the procedures detailed in 3.6 above.
3.10.5. Enzyme Inhibitors:

3.10.5.1. Trypsin inhibitor (Kakade et al., 1974; Sotelo et. al., 1995): Trypsin inhibitor in the samples was determined as per the method of Kakade et al. (1974) as modified by Sotelo et al. (1995). The trypsin inhibitor activity is measured by inhibiting the activity of trypsin. A synthetic substrate, BAPNA is subjected to hydrolysis by trypsin to produce yellow coloured p-nitroanilide. The degree of inhibition of the yellow colour p-nitroanilide formation by the extract is measured at 410 nm.

3.10.5.1A. Extraction: 1 g powder sample was defatted with 10 volumes (w/v) of water saturated n-butanol for 2 hrs using a magnetic stirrer. It was centrifuged at 5000 x g for 15 min. The supernatant was removed and the residue was dried at room temperature. It was dissolved in 20 volumes of 0.05 M sodium phosphate buffer (pH 7.6) containing 0.15M NaCl and stir with magnetic stirrer for 4 hrs. The contents were centrifuged at 4°C for 15 min at 5000 x g. The supernatant was dialyzed against 0.05M Sodium phosphate buffer in the cold room overnight. The dialysate was centrifuged again at 7000 x g for 20 mins and supernatant preserved at 4°C for analysis of Trypsin and amylase inhibitors.

3.10.5.1B. Reagents:

A. 30% Glacial acetic acid , GAA (v/v)

B. Trypsin (From Bovine pancreas, HiMedia) soln: 6.25 mg was dissolved in 25 ml of 0.001M HCl. 2 ml of this soln diluted to 25 ml for assay.

C. Benzoyl-DL-arginine-paranitroanilide Substrate (Sigma): 80 mg of BAPNA was dissolved in 1 ml of dimethyl sulphoxide (DMSO) and diluted to 200 ml with tris-HCl buffer (pH 8.2)

D. Tris-HCl buffer (pH8.2): 6.0.5 g of tris-hydroxymethyl aminomethane and 2.94 g of CaCl2H2O were dissolved in 900 ml of dd water. pH was adjusted to 8.2 by using dilute HCl and volume made up to 1000 ml.

E. 0.001M HCl: 3.1 ml of concentrated HCl (sp.gr. 1.18) was diluted to a 100 ml volumetric flask. 0.1 ml of this soln was again diluted to 100 ml in a volumetric flask so as to get 0.001M soln.

3.10.5.1C. Procedure: Five aliquots in the range of 0 to 2.0 ml of the sample extract were transferred into different test tubes and vol. made up to 2.0 ml with dd water. 2
ml trypsin solution was added to each tube. To two other tubes, 2ml of sample extract in one tube (Sample Blank) and 2.0 ml of dd water in another tube (Substrate blank) was added. All the tubes were placed in a water bath at 37°C to equilibrate. After 10 min, 5 ml of substrate solution was added to every tube. After exactly 10 min, 1.0 ml of 30% GAA was added to each tube to stop the reaction. 2.0 ml of trypsin solution was added to both the substrate and sample blank. The absorbance (410 nm) of the contents of all the tubes were measured in UV-VIS spectrophotometer, spectrascan-2600 using the substrate blank as reference.

3.10.5.1D. Calculation: Sample (A410)-(Sample blank (A410) x Sample volume/2.0 ml) was plotted vs. sample volume. Trypsin inhibitor activity was calculated from the slope of the linear part of the plot as follows:

Trypsin inhibitor units (TIU)/mg sample

\[= \frac{\text{Slope}}{(0.01 \times \text{sample concentration(mg/ml)}})\]

Where 1 TIU = decrease in A410 by 0.01 in 10 min.

3.10.5.2. Alpha amylase inhibitor activity (Deshpande et al., 1982): α-amylase hydrolyses α-1, 4 linkages of starch molecules in a random manner. The reducing sugars (mainly maltose) produced by the action of α-amylase react with dinitrosalicylic acid. The starch hydrolyzed product concentration under a specified level of α-amylase enzyme with and without inhibitor is used to express the α-amylase inhibitor activity.

3.10.5.2A. Extraction: As in 3.10.5.1A.

3.10.5.2B. Reagents:

A. Starch solution: 1 g soluble starch is dissolved in 100 ml of 0.02M Phosphate buffer (pH 7.0)

B. Dinitrosalicylic acid (DNS) reagent: It is prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 ml of 2N NaOH and 50 ml of dd water. 20 g Rochelle salt was added and shook vigorously. The volume was made up to 100 ml with dd water and stored at 4°C in a brown bottle.

C. α-amylase enzyme solution: 60 mg of α-amylase was dissolved in 200 ml of 0.2M Phosphate buffer (pH 7.0 containing 0.006M NaCl). From this
stock solution, 10 ml was transferred and diluted to 100 ml with the same buffer solution (30μg/ml)

D. Maltose standard solution: 50 mg maltose was dissolved in 50 ml dd water and stored at 4°C (1000μg/ml).

E. 4.5% NaOH: (v/v)

F. 2N NaOH: 8 g NaOH dissolved in approximately 80 ml dd water and standardized against 1N oxalic acid.

G. 0.2M (pH 7.0) Phosphate buffer: 39 ml of 0.2 M monobasic sodium phosphate solution was mixed with 61 ml of 0.2M dibasic sodium phosphate solution and diluted to a total volume of 100 ml.

H. 0.02M, (pH 7.0) Phosphate buffer: 10 ml of the above phosphate buffer was diluted to 100 ml with dd water.

3.10.5.2C. Determination of α-amylase enzyme activity: Before starting the experiment, all the reagents were incubated at 37°C for 15 min or more. Three test tubes were arranged, one as test set, the second as blank and the third as control. To the first tube added 0.5 ml of 1% starch, 0.25 ml of 0.2M phosphate buffer and 0.25 ml of α-amylase enzyme solution. The second tube contained 0.5 ml of 1% starch and 0.5 ml of 0.2M phosphate buffer. The third tube contained 0.5 ml 1% starch, 2 ml DNS reagent, 0.25 ml phosphate buffer and 0.25 ml of α-amylase solution. All the tubes were incubated at 37°C for 3 min. At the end of 3 min, 2 ml DNS reagent was added to the first and the second tubes to stop the reaction. Mixed and transferred into a boiling water bath for 10 min. After cooling under tap water, 10 ml of dd water was added to all the tubes and absorbance recorded at 540 nm in a UV-VIS spectrophotometer, spectrascan-2600 against the second tube. Liberated reducing sugars were expressed as maltose equivalent using the calibration curve. One unit of α-amylase is expressed as mg of maltose produced during 10 min incubation with 1% starch under the specified experimental conditions.

3.10.5.2D. Preparation of maltose calibration curve: Aliquots of 0.2, 0.4, 0.6, 0.8, 1.0 ml of maltose solution were transferred into different test tubes and volume made up to 1 ml with dd water. To each tube, 2.0 ml of DNS reagent were added and kept in a boiling water bath for 10 min. All the tubes were cooled under tap water. 10 ml
dd water was added and absorbance recorded at 540 nm in a UV-VIS spectrophotometer, spectrascan-2600 against a reagent blank.

3.10.5.2E. Determination of α-amylase inhibitor activity: 0.25 ml of the extract (3.10.2.1.) and 0.25 ml of the α-amylase solution in duplicate were incubated for 15 min at 37°C. To this mixture, 0.5 ml of 1% starch solution was added. A sample blank was also prepared by adding 2 ml of DNS reagent before adding the α-amylase solution. The assay was conducted as described above (3.10.5.2C.). The residual α-amylase activity was calculated and the reduction in the release of reducing sugars due to the addition of the extract was found out. α-amylase inhibitory activity was defined as the number of enzyme units inhibited under the assay conditions and expressed as enzyme units/g.

3.10.6. Toxic Substances:

3.10.6.1. Saponin estimation (Obadoni and Ochuko, 2001 with slide modifications):

3.10.6.1A. Procedure: 10 g of plant sample was dispensed in 200 ml of 20% ethanol. The suspension was heated over a hot water bath maintained at about 50°C and stirred with a magnetic stirrer for 4 hr. The mixture was centrifuged and the residue re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethylether was added and shaken vigorously. The aqueous layer was recovered while the ether layer discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was evaporated to almost dryness on a water bath. The last traces of moisture were removed by drying to an almost constant weight in the oven. The difference in weight represents the saponin content and reported in percentage.

3.10.7. Tannins (Schanderi, 1970): Tannins and tannins like substances are widespread in nature. These are polyphenolic compounds and divided into hydrolysable and condensed Tannins. Hydrolyzable tannins contain a polyhydric alcohol usually, if not always. Condensed tannins are mostly flavonols and theses cannot be hydrolyzed to simple components. Tannin-like compounds reduce phosphotungsto-molybdic acid in alkaline
solution to produce a highly coloured blue solution, the intensity of which is proportional to
the amount of tannins and is measured at 700 nm in a UV-Visual spectrophotometer.

3.10.7.1. Extraction: The water extract prepared at 3.3.3.1 was used for the
estimation of tannins.

3.10.7.2. Reagents:

A. Folin-Denis reagent: prepared by dissolving 100 g sodium tungstate
and 20 g phosphomolybdic acid in 750 ml dd water containing 50 ml
phosphoric acid. The mixture was refluxed for 2 hrs and made up to
1.0 L. The reagent was protected from expose to sunlight.

B. 35% NaCO₃ (w/v)

C. Standard Tannic acid stock solution: 100 mg tannic acid dissolved in
100 ml dd water.

D. Working Standard tannin solution: 5 ml of the stock solution diluted to
100 ml with dd water (50 μg/ml).

3.10.7.3. Procedure: 0.1 ml of the water extract was transferred into a test tube and
vol. made up to 0.5 ml with dd water. 0.5 ml of Folin-Denis reagent and 1.0 ml of
NaCO₃ solution were added and vol. made up to 10 ml with dd water. It was shaken
properly and read the absorbance at 700 nm in a UV-Visual spectrophotometer after
30 min against a blank solution. The amount of tannin was calculated from the
standard curve drawn using 20 to 100 μg/ml tannic acid standard solution and
expressed in terms of mg/100 g material.

3.10.8 Cyanogenic determination (AOAC, 1975): 1 g of the sample was soaked for 4 hours
in distilled water. The suspension was steam-distilled into a dilute NaOH solution. The
distillate was then treated with dilute KI and titrated against AgNO₃ to a faint and permanent
turbidity. The hydrocyanate was calculated taking 1ml of 0.02 AgNO₃ as equivalent to
1.08mg HCN.

3.10.9. Alkaloid determination (Obadoni BO and Ochuko PO, 2001 with modifications as
described by Harbome JB, 1973): 5 g of the defatted sample was transferred into a 250 ml
beaker and 200 ml of 20% acetic acid in ethanol was added and covered to stand for 4 hr.
This was filtered and the extract was concentrated using a water bath to one-quarter of the
original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle down and the precipitate was collected by filtration and weighed. The difference in weight represents alkaloids.

3.10.10. Anti-nutritional Substances:

3.10.10.1 Phytic acid (Wheeler and Ferrel, 1971; Makower, 1970): Extraction of phytic acid and precipitation of phytate from the samples were done by the method of Wheeler and Ferrel, (1971). The iron content of the precipitate and the phytate phosphorous was determined as described by Makower (1970). In this method, the phytate is extracted with trichloroacetic acid (TCA) and precipitated as ferric salt. The iron content of the precipitate is determined colourimetrically and the phytate phosphorous content is calculated from the value assuming a constant 4Fe:6P molecular ratio in the precipitate.

3.10.10.1A. Reagents:

A. 3% TCA (w/v)
B. 3% Sodium sulphate in 3% TCA.
C. 1.5 N NaOH
D. 3.2N HNO₃
E. FeCl₃ Solution: Prepared by dissolving 583 mg in 100 ml 3%TCA
F. 1.5 M Potassium thiocyanate solution(KCSN): Prepared by dissolving 29.15 g KCSN in 200 ml dd water
G. Stock Fe(NO₃)₃ Soln: Prepared by dissolving 0.433 mg in 100 ml dd water (1000 ppm)
H. Fe(NO₃)₃ working soln: 1 ml of stock soln transferred to a 100 ml vol. Flask and make up vol. with dd water (10 ppm/ml).

3.10.10.1B. Procedure: 0.5 g powder sample transferred into a 50 ml centrifuge tube. 15 ml TCA was added and extracted for 30 min with a magnetic stirrer. Centrifuged at 5000 x g for 10 min and 10 ml supernatant transferred into another 50 ml centrifuge tube. 4 ml of FeCl₃ was added quickly and mixed thoroughly. Heated in a boiling water bath for 45 min. If the supernatant is not cleared, 1-2 drops of 3% Sodium sulphate in
3% TCA was added and heating was continued. The tubes were centrifuged at 5000 x g for 15 min and decanted. The residue was washed twice, first with 25 ml of 3% TCA and second with dd water and centrifuged. The residue was preserved and dissolved in 3 ml of 1.5 N NaOH. The volume was raised to around 15 ml with dd water and heated in boiling water bath for 30 min. The content was filtered while hot through a moderately retentive Whatman no. 2 filter paper. The precipitate on the filter paper was washed with 30-40 ml hot dd water and the filtrate was discarded. The precipitate on the filter paper was dissolved with 15 ml hot 3.2 N HNO₃ and the filtrate collected in a 50 ml vol. flask. The filter paper was washed down with several volumes of dd water. Cool to room temperature and volume made up to 50 ml with dd water. 5 ml of the extract was transferred to another 50 ml vol. flask and diluted to around 30 ml with dd water. 10 ml of 1.5 M KCSN was added and volume made up to the mark with dd water. For standards, 5 flask containing 0 to 10 ppm were taken and subjected to the same procedure. Absorbance of all the tubes was taken immediately at 480 nm against the blank.

**3.10.10.1C. Calculation:** Phytate P (mg/100g)

\[
\text{Phytate P (mg/100g)} = \frac{\mu g \text{ Fe(from calibration curve)} \times 0.9}{\text{Wt. of sample in grams}}.
\]

**3.10.10.2. Phenolics:** Total soluble phenols and orthodihydric phenols were determined as per the procedure detailed at 3.5.1 and 3.5.2.

**3.11. Agro-climatic Zones and Quality Parameters in Parkia roxburghii:**

Pods having different morphological characters at varied maturity stages were collected randomly from time to time from the specific sites (Figure 1.2.1) of each agro-climatic region. They had been classified into four groups based on the mean thickness of the pod measured at the site of seeds as – Tender (up to 6 mm and below), Immature (6.1 to 10 mm), Mature (more than 10 mm) and seeds (When the pulp can’t be used further). In case of mature stage, 5 pods were randomly selected from each genotype. All the pods were scrapped to remove the outer green peel. The margin of the pods were removed neatly with the help of a knife and cut into two sizes. One portion, approximately 2 mm in size was used for the determination of moisture, acidity and vitamin C. The other portion about 4/5mm in size was air dried and kept in the oven at 60±5°C for 48 hours. The moisture free samples were then grounded in the form of powder by using a Remi grinder and subsequently sieved
(1 mm). The powder samples were collected and kept for various qualitative and quantitative analyses. Others were the same as in the case of chemical composition described at 3.1.


Divergence studies in *P. roxburghii* comprised of 24 cultivars collected from different agro-climatic zones of Manipur. For the purpose, 3 (three) *Parkia* growing sites located in each zone were identified (Figure 1.2.1). The flow-chart indicating the sites of collection within each agro-climatic zone is shown at figure 3.12.1. Two types of physically different, matured *P. roxburghii* pods available in different agro-climatic zones were collected and the samples were code numbered preceded by PRB standing for *Parkia roxburghii*. Pods were randomly selected from each genotype and photographed (Photo plates-3.12.1 and 3.12.2). Morphological characters of five randomly selected pods in each cultivar were studied. The characters studied were based on the description given by Hopkins (1994) with some minor modifications. Details of the morphological characters were presented in Table 3.12.1. After the study, pods were dried in the shade and seeds removed. Five seeds from each cultivar were randomly selected, photographed and their morphological characters studied (Photo plate-3.12.3 and 3.12.4).

*Parkia roxburghii* is a highly polymorphic species regarding the pattern of variation in the morphological characters. According to Sneath and Sokal (1963,1973) detailed morphological analyses are necessary to assess whether taxonomically significant variation is present in intricate species complex, but the theoretical basis and design of such analysis are not straight forward. However, one of the most potent techniques to measure the genetic diversity is the Mahalanobis's $D^2$ statistic. The main advantages of $D^2$ analysis are:

1. This technique helps in selection of genetically divergent parents for their exploitation in hybridization programme.

2. $D^2$ statistic measures the degree of diversification and determines the relative proportion of each component character to the total divergence.

3. To measure the forces of differentiation at two levels, that is, intra-cluster and inter-cluster levels.

4. It provides reliable estimates of genetic divergence and a large number of germplasm lines can be evaluated at a time for genetic diversity by this technique.
Figure 3.12.1: Flow-chart showing sites of collection of germplasms of *Parkia roxburghii* G. Don cultivars from different Agro-climatic Zones of Manipur.
Photo Plate-3.12.1: Cultivars collected from Sub-tropical plain zone and Sub-tropical hill zone of Manipur.
Photo Plate-3.12.2: Cultivars collected from Mild tropical hill zone and Temperate sub-alpine zone of Manipur.
Photo Plate-3.12.3: Seeds of the cultivars collected from Sub-tropical plain zone and Sub-tropical hill zone of Manipur.
Photo Plate-3.12.4: Seeds of the cultivars collected from Mild tropical hill zone and Temperate sub-alpine zone of Manipur.
3.12.1. Statistical/Biometrical Procedure for \( D^2 \) Analysis:

3.12.1.1. Analysis of variance: The experimental data were analyzed statistically by the method of analysis of variance for single factor experiments (Gomez and Gomez, 1987). The significance of the calculated variance ratio was compared with the table value of ‘F’ distribution. In order to test the significance of mean difference between cultivars, the following statistics were computed.

3.12.1.1A. Standard error of mean difference (S. Ed)

\[
S. Ed = \sqrt{2 \times \text{EMS}/r}
\]

Where, EMS = error mean sum of squares,
\[ r \] = number of replications.

3.12.1.1B. Critical difference (C.D.)

\[
C.D. = S. Ed \times t \text{ for error degrees of freedom at 5\% or 1\% of probability.}
\]

3.12.1.1C. Co-efficient of variation (C.V.): In order to judge the precision of the experiment, the C.V. was calculated as under:

\[
C.V. (\%) = \sqrt{\frac{\text{EMS} \times 100}{\bar{X}}}
\]

\[ \bar{X} \] = the general mean

3.12.1.2. Genotypic and phenotypic variances: Genotypic variance \( (\sigma^2g) \) was estimated according to the formula:

\[
\sigma^2 g = \frac{\text{Msg} - \text{Mse}}{r}
\]

Where, \( \sigma^2g \) = genotypic variance
\[ \text{Msg} = \text{Mean square due to genotype} \]
\[ \text{Mse} = \text{Mean square due to error} \]
\[ r = \text{Number of replications} \]
Phenotypic variance ($\sigma^2_p$) was calculated separately for each character as follows:

$$\sigma^2_p = \sigma^2_g + \sigma^2_e \quad \text{where, } \sigma^2_e = \text{Mse}$$

### 3.12.1.3. Genotypic and Phenotypic Co-efficients of Variation:

Genotypic co-efficient of variation (GCV) was defined as the ratio of the square root of genotypic variance ($\sigma^2_g$) to the population mean ($\bar{X}$) expressed in percentage. It was estimated according to the formula suggested by Burton (1951).

$$\text{GCV} = \frac{\sqrt{\sigma^2_g}}{\bar{X}} \times 100 \quad \text{or} \quad (\sigma_g/\bar{X}) \times 100$$

Where, $\bar{X}$ = grand mean of all genotypes

$\sigma_g$ = Genotypic standard deviation

$\sigma^2_g$ = Genotypic variance

Phenotypic co-efficient of variation (PCV) is defined as the ratio of the square root of phenotypic variance ($\sigma^2_p$) to the population mean ($\bar{X}$) expressed in percentage.

$$\text{PCV} = \frac{\sqrt{\sigma^2_p}}{\bar{X}} \times 100 \quad \text{or} \quad (\sigma_p/\bar{X}) \times 100$$

Where, $\sigma_p$ = Phenotypic standard deviation

$\sigma^2_p$ = Phenotypic variance

### 3.12.1.4. Estimation of Heritability ($h^2$) in Broad Sense:

Heritability is the ratio of genotypic variance to the phenotypic variance. Based on the method of Lush (1940), heritability in broad sense was calculated as follows:

$$h^2_b = \frac{\sigma^2_g}{\sigma^2_p} \times 100 \quad \text{or} \quad \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_e} \times 100$$

Where, $\sigma^2_p$ = Phenotypic variance

$\sigma^2_g$ = Genotypic variance
3.12.1.5. Genetic Advance:

The genetic advance (G.A.) is the difference between the mean of the progeny of selected individuals (\( \bar{X}_p \)) and the base population (\( \bar{X}_0 \)).

Thus, G.A. = \( \bar{X}_p - \bar{X}_0 \)

It was calculated by the formula suggested by Johnson et al., (1955).

\[
G.A. = Kh^2 \sigma_p
\]

Where, 
- \( K \) = Selection differential
- \( H_b^2 \) = Heritability in broad sense
- \( \sigma_p \) = Phenotypic standard deviation

G.A. was also expressed as the percentage of mean as follows:

\[
G.A. \text{ as } % \text{ of mean} = \frac{G.A.}{\bar{X}} \times 100
\]

Where, 
- G.A. = Genetic advance
- \( \bar{X} \) = Grand mean

3.12.1.6. Correlation of Characters:

The correlation co-efficient (r) was computed as:

\[
r(X_1, X_2) = \frac{Cov(X_1, X_2)}{\sqrt{Var(X_1) \times Var(X_2)}}
\]

Where, 
- \( r(X_1, X_2) \) = Correlation between \( X_1 \) and \( X_2 \)
- \( Cov(X_1, X_2) \) = Covariance between \( X_1 \) and \( X_2 \)
- \( Var(X_1) \) = Variance of \( X_1 \)
- \( Var(X_2) \) = Variance of \( X_2 \)
Estimates of genotypic and phenotypic covariance components between two traits \((\sigma gX_1X_2)\) respectively were derived as follows:

\[
\sigma gX_1X_2 = \frac{MSPT - PSPE}{r} \\
\sigma eX_1X_2 = MSPE \\
\sigma pX_1X_2 = \sigma gX_1X_2 + \sigma eX_1X_2
\]

Where, \(\sigma gX_1X_2\) = Genotypic covariance between \(X_1\) and \(X_2\)

\(\sigma eX_1X_2\) = Error covariance between \(X_1\) and \(X_2\)

\(\sigma pX_1X_2\) = Phenotypic covariance between \(X_1\) and \(X_2\)

\(MSPT\) = Mean sum of product due to treatment

\(MSPE\) = mean sum of product due to error and

\(r\) = number of replications.

3.12.1.7. D^2 Analysis:

Computation of \(D^2\) statistic and canonical analysis were carried out with the help of computer at the college of agriculture, CAU, Imphal as per SPAR-2 programme of Indian Agricultural Statistical Research Institute (IASRI), Pusa, New Delhi-12.

3.12.1.7A. Mahalanobis’ \(D^2\) - statistic: Mahalanobis’ (1936) defined the distance between two populations as:

\[
D^2 = \sum_{i=1}^{p} \sum_{j=1}^{p} W_{ij} d_i d_j \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (1)
\]

Where, \(W_{ij}\) is the \((ij)^{th}\) element in the inverse of estimated within population variance co-variance matrix, \(p\) is the number of characters involved, \(d_i\) and \(d_j\) are the differences in the means of two populations for the \(i^{th}\) and \(j^{th}\) characters. If ‘\(t\)’ is the number of populations, the total number ‘\(n\)’ of the pairs of population is \(t \times (t-1)/2\).
Since the numerical evaluation of $D^2$ using equation (1), would involve the inversion of a $p \times p$ matrix and the summation of $p^2$ products, a simpler method of using transformed uncorrelated variables was followed. The transformation of correlated variables to uncorrelated ones was done by using pivotal condensation method. The coefficients for the transformation were obtained by dividing the first row of the reduced matrix by the square root of the corresponding pivotal condensation elements (only the absolute values of these were considered). By using this method (Rao, 1952), the $D^2$ value was obtained as a sum of squares of $p$ values of the uncorrelated variables. Thus,

$$D^2 = \sum_{i=1}^{p} f_i^2$$

Where, $f_i$ is the difference between Yi means of the transformed uncorrelated variables.

### 3.12.1.7B. Test of Significance of multiple measurements:

From the estimates of variance and co-variance, using 'V' (stat.) which in turn utilizes Wilk's criteria, a simultaneous test of differences between mean values of a number of correlated variables was done (Rao, 1948).

Using pivotal condensation method, the determinants of error and error + variety matrix was calculated.

\[
V(\text{stat.}) = -m \log_{e} \Lambda \\
= -n - \frac{p+q+1}{2} \times \log_{e} \Lambda
\]

Where $m = -n - \frac{p+q+1}{2}$

$p$ = Number of characters
$q$ = Degrees of freedom for varieties
$n$ = Degrees of freedom for error + varieties

\[\log_{e} \Lambda = 2.3026 \log_{10} \Lambda\]
\[ \Lambda = \frac{|W|}{|S|} \]

When \( |W| \) = Determinant of error matrix and
\( |S| \) = Determinant of error + variety matrix.

\( V \) (stat.) is distributed as \( \chi^2 \) with \( p, q \) degrees of freedom. The tabulated value of \( \chi^2 \) for \( p, q \) degrees of freedom was compared with the calculated value of \( V \) (stat.) for testing the significance.

For calculating \( \chi^2 \) values when \( n > 100 \), the following formula was used:

\[ \chi^2 = Z = \sqrt{2\chi^2 - 2n - 1} \]

Where, \( X \) is a normal deviate corresponding to a probability level (single tail) and \( Z \)
\[ = \sqrt{2\chi^2 - 2n - 1} \]
may be used as normal deviate with unit variance.

3.12.1.7C. Test of significance of \( D^2 \) values: The \( D^2 \) values obtained for a pair of population was taken as the calculated value of \( \chi^2 \) and was tested against the tabulated value of \( \chi^2 \) for \( p \) degrees of freedom, where \( p \) is the number of variables.

3.12.1.7D. Contribution of individual character towards divergence: The contribution of individual character towards divergence was estimated as the percentage of the rank total of individual character from the rank total of all the characters.

3.12.1.7E. Grouping of Varieties into Various Clusters by Tocher’s method:

Arranging the populations in order of increasing magnitude of \( D^2 \) value for each individual population, various clusters were formed following the method suggested by Tocher (Rao, 1952) as follows:

Two populations having smallest distance from each other were considered first to which a third population having smallest average \( D^2 \) value from the first two
populations was added. Then the nearest fourth population was added and so on. At certain stage, when it was felt that after adding a particular population, there was abrupt increase in the average $D^2$, the population was not added in that cluster. Generally, this level should be approximately near to the maximum $D^2$ value between any two populations in the first row of the table where $D^2$ values are arranged in increasing order of magnitude. Similarly, a second cluster was formed. Thus, the process was continued till all the populations are included into one or other cluster.

3.12.1.7F. Average intra-cluster distance: Average intra-cluster distance was estimated as:

$$\sum_{i=1}^{n} \frac{D^2_i}{n}$$

Where,

$$\sum_{i=1}^{n} D^2_i$$

is the sum of distance between all possible combinations of the populations included in the cluster, ‘n’ is the total number of all possible combinations.

3.12.1.7G. Average inter-cluster distance: Clusters were taken one by one and their distance from other clusters was calculated. The distance between two clusters is the sum of $D^2$ values between the numbers of one cluster to each of the number of other cluster divided by the product of number of genotypes in both the clusters under consideration. Average inter-cluster distance was estimated as:

$$\sum \frac{D^2}{n_in_j}$$

Where $\sum D^2$ is the sum of distance between the populations of $i^{th}$ and $j^{th}$ clusters. $n_i$ and $n_j$ are the number of populations in the $i^{th}$ and $j^{th}$ clusters.

3.12.2. Canonical Analysis:

Graphical representation of genetic divergence of varieties through canonical analysis following Rao (1952) and Singh and Chaudhary (1985) was used in the present study. The procedure of calculation was as under:
The sum of squares and sum of products were computed from transformed mean value \( Y \) for each character and character combinations were presented in the matrix \( A_{p \times p} \) from matrix \( A \), \( A^p \) is calculated where \( p \) was the number of characters.

### 3.12.2.1. Calculation of First Standardized Vector (\( V_1 \)) and Canonical Root (\( \lambda_1 \)):

The first approximation trial vector was calculated after obtaining column totals of matrix \( A^p \) and dividing each of the column totals by the highest quantity among them. The second approximation trial vector was again calculated by multiplying each column of \( A^p \) matrix by the first approximation trial vector to obtain another column totals and dividing each value of column totals by the highest numerical value among them. Similarly, the third, fourth and so on approximation trial vectors were calculated by multiplying \( A^p \) matrix by the preceding approximation trial vectors, obtaining column totals and dividing each of them by the highest numerical value among them until the calculated approximation trial vectors were similar to that of earlier ones. Such an approximation trial vector was considered as the first vector. The first vector was standardized by dividing them by the corrected sum of squares of these vector elements to obtain standardized vector (\( V_1 \)).

The first canonical root (\( \lambda_1 \)) was calculated as the \( p^{th} \) root of the highest column total in the last approximation.

### 3.12.2.2. Calculation of Second Standardized Vector (\( V_2 \)) and Canonical roots (\( \lambda_2 \)):

The original \( A^p \) was transformed and represented by \( B^p \). Each \( (i,j)^{th} \) element of \( B^p \) was calculated as:

\[
(i,j)^{th} \text{ element } \frac{A^p_{ij} \cdot \lambda_i^p \cdot x}{\lambda_j^p \cdot x_{ji} \cdot \lambda_j^p \cdot x_{ij}}
\]

Where, \( \lambda_i^p \) is the highest column total in the last approximation.

For calculation of second standardized vector (\( V_2 \)) the same procedure which was required to calculate the first standardized vectors (\( V_1 \)) was followed. The second
canonical root ($\lambda_2$) was calculated as the $p^{th}$ root of the highest column total in the last approximation of the second vector.

3.12.2.3. Estimation of ‘Z’ Values:

‘Z’ values were estimated for plotting various populations in two dimensional graph. ‘Z’ values were estimated as:

$$ Z_i = YV_i $$

Where $Y$ is the matrix of the mean transformed values and $V_i$ is the transpose of the $i^{th}$ vector.

3.12.2.4. Contribution of Vectors Towards Divergence:

Total contribution of all vectors = sum of diagonal elements of A vectors.

$$ \text{Per cent contribution of } \lambda = \frac{2}{\text{Total contribution of vectors}} \times 100 $$

3.12.2.5. Two Dimensional Graphical Representation:

If the total contribution of $\lambda_1$ and $\lambda_2$ exceeded 70% of the total contribution of vectors, two dimensional graphical representation using $Z_1$ and $Z_2$ values were adequate (Vairavan et al., 1973).