CHAPTER – 2
MATERIALS & METHODS

The samples of leaves, inflorescence and fruit for the present investigation were collected from the orchard of Junagadh Agricultural University, Junagadh Gujarat. (India). For studying the developmental studies of fruit from three varieties namely Kesar, Alphonso and Rajapuri two regions were selected Junagadh, Junagadh Agriculture University and Navsari, Navsari Agriculture University.

I. STUDIES CONDUCTED ON THIRTY DIFFERENT VARIETIES

Thirty varieties of *M.indica* growing in Junagadh, Gujarat, are studied for its variation in vegetative and reproductive features of the plant.

Selection of samples

After selecting the typical tree in normal growth and health and of adult bearing age of each variety and in each representative region, the selection of material from such trees was undertaken for detailed examination. The selection of shoots and leaves was made from only the current seasons's growth which had completed growth for the season. The selection was limited to the non-flowering shoots.

The selection of the inflorescence and flowers for study was limited only to the terminal panicles of typical representatives shoots from all over the exposed regions of the tree. Malformed or stunted panicles and those which were produced prematurely or too late in the season were not taken for the study.

For recording fruit description, a dozen typical fruits were selected at random from all exposed sides of the tree. Out of these only one was selected for final detailed description. To record description of the colour, flavor and taste it was necessary to include only such fruits that had attained full maturity on the tree itself. An efficient working basis for the determination of optimum maturity of the material was found to be roughly the distinct appearance of dots on the skin, the minimum exudation of latex in most varieties at the time of picking and the occurrence of characteristic full sound when the fruits were tapped.

The shape of the tree is intimately connected with the pose of the leaf and, therefore, accurate description of the leaves is essential for a complete study.The description of the height is mainly in the relative manner employing such terms as tall, medium and dwarf. These terms may enhance the value of varietal description; their value in classification of varieties may not be high, unless accompanied by quantitative data, which were accordingly collected.

The tree form has been called oval when the tree spread is very much smaller than the height, round when height and spread are almost equal and dome shaped when spread is more marked than the height. Terms used to describe characters adapted from Gongolly *et al*. (1957).

I. MORPHOLOGY

i) Vegetative characters

Morphological studies were carried out by selecting five trees of each variety. Observations were recorded by collecting fifty leaves, five inflorescences and five fruits from each tree. Visual observations like the color of the inflorescence and fruit and the ratio of male to female flowers were made on the field itself. Samples were collected and brought to the laboratory for the quantitative measurement. Length and breadth of leaves were recorded in centimeter (cm) using a stainless steel scale. For studying fruits, the length, diameter and volume was taken. The fresh fruits were washed with running tap water to remove the dust and soil particles. The length and diameter of the fruits were measured in centimeter (cm) using a stainless steel scale and the volume of the fruits were determined by dipping the individual fruit into a glass beaker fully filled with water. The amount of water displaced by the fruit during dipping was collected in the lower vessel/ tray and the amount of water displaced water was considered as a volume of fruit measured in milliliters (ml) (Mazumdar and Majumder 2003)**.**

ii) Floral characters

Shape of the panicle represented one of the most important varietal characteristic. Conical panicle with the length of the main axis markedly greater than the spread is a definite character in some varieties. In other, pyramidal inflorescence with the spread approximating the length forms the distinguishing feature. Size of the panicles is another easily distinguishable character especially if exact dimensions accompany the descriptions. In the present studies, measurements of length of the axis and the spread of the panicle are taken to serve an index of the panicle size.

Hairiness of the panicle has been found to be of considerable diagnostic importance in mango as in most other fruits. To express the varying degrees of hairiness in the mango, the terms found to be adequate were glabarate or sparsely purberulent, moderately puberulent and densely puberulent.

The development of staminodes appears to be a character of sufficient importance even from a causal study of some varieties. Only two groups are deemed sufficient to bring out the varietal differences, viz well developed staminodes.

The above descriptions require to be accompanied by descriptions of relative development of stamens and pistil. Mango varieties differ markedly in regard to the relative size of the stamen which is longer than the pistil, in others, the pistil and stamens are equal, and yet in others, the pistil will be longer than the stamens. The relative position of the stamens and pistil may be parallel or oblique to each other.

iii) Fruit characters

The fruit is held in its normal position, i.e. as it would be while hanging on the tree. The observer faces the fruit in such a way to have the beak and concave surfaces to his left and the convex surface to his right. The stalk end is called the base and the opposite end, the

apex. The left lobe which is generally larger in most varieties is designated as the left or the ventral shoulder and the opposite one as the right or the dorsal shoulder. The size and nature of the stalk which attaches the fruit to the tree as well as the nature of insertion of the stalk to the fruit are given some prominence. The depression that is often present near the point of attachment of the stalk of the fruit is commonly termed as the cavity. The concavity which lies a little above the beak or 'Nak' is known as the sinus. The distance from the point of attachment to the extremity at the distal end is termed as the length of the fruit. The maximum distance between the two shoulders is recorded as the major diameter. The minor diameter is obtained by placing the fruit flat on a smooth surface in the natural position and measuring the depth from the surface on which the fruit rests to the highest point on the fruit.

The form of the fruit possibly affords the most prominent varietal character and has accordingly been employed largely in the mango classification in the past. Except in sub-normal or malformed fruits, this character seems to be the most valuable under the various diverse environmental conditions. Symmetrical fruits are those which can be divided into two equal halves so as to have the shoulders almost equal and well balanced. When such an imaginary cut is not possible, the fruit is stlyled as asymmetrical. Another group is also met with in which the shoulders are of very unbalanced shape with one markedly higher than the other. The fruits of this group are classed as oblique shaped. The commonest fruit shapes in mango are roundish, ovate, oval, oblong, cordate, reniform, peento etc., with a number of shapes intermediate between these.

The size of the mango is undoubtedly only of relative importance and can only be of practical value if accompanied by accurate data on dimensions, weights, etc. which have therefore been collected and recorded in these studies.There are mainly two types of stalk insertion in mango. The term square is applied when the stalk insertion is in almost vertical plane with the axis of the fruit.Some sort of fleshy protuberance near the insertion of the stalk forming

an extension of the base is found in fruits of certain varieties and this requires to be included in any complete description.

The basal end of the mango is rounded; slightly flattened, obliquely flattened, necked or obliquely rounded.The shoulders differ markedly as regards their size. If they are unequal, mention has to be made as to which of these two shoulders is broader and more prominent. It is also possible to come across varieties wherein one or both the shoulders are absent. Irrespective of their size, the shoulders may be level or one higher than the other.The cavity is absent in some varieties, slightly in others or deep in still other.

The size, shape and prominence of the beak are easily noticeable characteristic features in mango. While it is missing or absent in some varieties, in others it may end in a point, be slight but distinct, slightly prominent, prominent, mammiform, beaked or may be straight, hooked or curved.Color of the fruit with all the various shades and tints affords a useful distinguishing character and is the most difficult for accurate description.

The lenticels are usually subcutaneous. They vary in size between varieties as also in the manner of distribution. In regard to the former, only such relative terms as small, medium and large and with regards to the latter, close, moderately distant or distant are deemed sufficient to bring out the prominent varietal differences.

Similarly relative terminology is employed while describing the thickness of the skin. With regard to the nature of the skin, however, it is found necessary to apply such terms as membranous, leathery or tough and tenacious to feel. Texture of the flesh, its aroma, juiciness and taste are some of the important fruit characters which cannot possibly be missed, but in all of which the terminology has necessarily been rather inexact for lack of any accurate standard. The presence or absence of fiber, the texture and the size of the fibre are also found to be important characters, but here also the description have to be necessarily couched at present in relative terms. To describe the flesh, terms like buttery, meaty, soft and firm are

considered to be adequate provided they are accompanied by relative but sufficiently expressive terms indicative of the flavor as pleasant, aromatic, acid, sub-acid, delightful and piquant.

As in the case of the fruit, the form, size and shape of the seed or stone need to be carefully and completely specified. These are required to be accompanied by weight and dimensions. The texture and position of the fiber seem to afford also some useful information for varietal identification. In some varieties the fibers are confined only to the ventral edge of the seed and do not extend far into the flesh, while in others they are spread all over. Some varieties are characterized by the existence of a few fibers on the ventral edge and short fiber all over the rest of the surface. The fiber may be coarse, stiff or soft. They may be sparse, abundant or intermediate. The veins on the stone, their prominence and their course are too important to be left out. They are usually parallel or forked and may either be slightly depressed, and are rarely either grooved or prominently ridged.

II. MICROMORPHOLOGICAL AND ANATOMICAL STUIDES

i) Leaf micromorphology was carried out to observe stomata and trichomes were conducted. Based on morphological characters, a key to identification has been prepared.

Stomata and Trichome

For studying stomata and trichome fresh samples were prepared generally from midway between the leaf base and apex of the lamina, according to the modified method of Clark (1960). The leaf fragments were placed in test tubes and treated with 50% Jerffrey's fluid (50% HNO_3 + 50% chromic acid) for 24 hours at 58˚C in oven. They were then removed from the test tube into a petri dish. The epidermal layers both adaxial and abaxial were separated carefully using sharp needles, stained with 1% safranin and mounted in 50% glycerin. The peels were observed under light microscope. Microphotographs were taken on

Olympus (Leica DME) microscope. These photographs were useful for identification and differentiation of epidermal cells on the basis of microscopic features. All the dimensional studies like epidermal cell length and breath, size of guard cell were measured using ocular and stage micrometer.

ii) Vein architecture

These studies were carried out in mature leaf from the terminal part of the branch collected from ten representative plants. The leaves were immersed in 80% ethanol for 48-72 hrs with several changes of solvent in order to remove chlorophyll pigments. The leaf samples were then washed and treated with 3-5% NaOH at 60ºC for 24-36 hrs. The digested leaf tissue was carefully brushed apart to obtain the leaf skeleton. These are further hardened by treating with a saturated chloral hydrate solution for several days, washed, dehydrated and preserved. The major venation pattern was studied with the help dissecting microscope. Venation pattern of minor veins was studied by cutting small bits from the central parts of the leaf skeletons. Photographs were obtained and cluster analysis was done. The terminology of Hickey (1973) is followed for the description of leaf architecture. An identification key was prepared using all studied parameters.

iii) Anatomy of petiole and leaf

Anatomical studies of leaf lamina, petioles and fruits were done by fixing the samples in FAA (formalin (40%): acetic acid: ethyl alcohol (70%) 5:5:90 (v/v)) fixatives for 48 hours. Leaves were cut into pieces, so as the midrib along with lamina regions from the middle part could be fixed. For petiole, leaf lamina and pulvinus base were separated and the middle region was cut for fixing. Fixed samples were dehydrated in a graded series (20, 35, 55, 75, 95 and 100%) of TBA (tertiary-butyl-alcohol) and embedded in paraffin wax (Johansen 1940). Single staining with toluidine blue and double staining with safranin and fast green were done for the cross section. Serial sections of the leaf lamina and petiole for each variety

were observed and micro photographed to visualize the cuticle, resin canals, arrangement of vascular strands, and other anatomical features.

III. STUDY OF MANGIFERIN CONTENT IN LEAVES

Evaluation of mangiferin content in the thirty different varieties was conducted by HPLC. The studies were done for all 30 varieties of leaves. The leaf samples were washed, shade dried for a day and then dried completely in an oven at 38°C. The plant materials were coarsely powdered using a rotary grinder, sieved through BSS mesh 85, and stored in airtight plastic containers to be used for HPLC analysis. Mangiferin standard was procured from SIGMA, Bangalore, India (CAS no. 4773-96-0). HPLC grade acetonitrile and water were procured from Qualigens, Mumbai, India.

Extraction

Fine leaf powder (1 g) of each sample was weighed into an Erlenmeyer flask. Ethyl alcohol (25 ml) was added and the flask subjected to shaking for overnight extraction in an orbital shaker. The extract was filtered using Whatmann No. 41 paper. Solvent was removed under reduced pressure and the residue was reconstituted in HPLC grade water, making the total volume 10 ml in a volumetric flask. Exactly 0.5 ml of this diluted extract was pipette out into a 5 ml volumetric flask and the volume was made up using HPLC grade water, and used directly in HPLC analysis. All samples were filtered through a 0.45 μm Acrodisc syringe filter (Pall Corporation, Mumbai, India) before injection.

Preparation of working standards

Six concentration levels of mangiferin standard 20, 40, 60, 80, 100 and 120 μg/ ml were prepared in HPLC grade water. These working standards were used for linearity study and preparation of the calibration plot for quantification of mangiferin in the samples.

HPLC analysis

HPLC analysis was carried out using a Shimadzu LC20AT series liquid chromatography equipped with a diode-array detector. The analytical column was a Phenomenex Luna C18 (250 x 4.6 lm; 5 μm) with a C18 guard column (Phenomenex, Torrence, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 1.0 ml per min for a total run time of 30 min and the gradient program was as follows: 90% for 5 min, 90% B to 80% B in 16 min, 80% B to 0% B in 9 min and 0% B to 90% B in 5 min. A post run of 10 minutes was set for reconditioning. The injection volume was 20 μl and peaks were monitored at 254 nm. The peaks were identified by congruent retention times and UV spectra when compared with those of the standard. All samples were prepared and analyzed in duplicate.

IV. SOIL ANALYSIS AND MINERAL ANALYSIS OF LEAVES

Nitrogen (N):

100 mg of samples were taken in the digestion tube and added 5 ml of conc H2SO4 soultion. The tubes were heated at 400˚C for 3 hrs with the help of KEL Digester and KEL VAC unit. The tubes were allowed to cool at room temperature and the volumes of the extracts were made to 100 ml with distilled water. An aliquout of 5 ml fruit extract, 5 ml of distulled water and 12 ml of 2.5 % NaOH (sodium hydroxide) solution were mixed in the digestion tube. The digestion tube was attached to the KEL PLUS Distillation unit for 9 min and the nitrogen was collected in to a 250 ml conical flask containing 25 ml of dilute H_2SO_4 (sulphuric acid) solution and 7-8 drops of methyl red indicator. This conical flask containing nitrogen was finally tirated with 0.02 N NaOH to determine the amount of total nitrogen content. The amount of nitrogen was calculated using the following formula:

$$\text{Nitrogen content} = \frac{(S-B) \times N \times ME}{W}$$

Where, S – amount of NaOH used for fruit sample

B – amount of NaOH used for standard

N – normality of NaOH used

ME – molecular equivalent of NaOH

W – weight of fruit sample used

Phosphorous (P):

A 500 mg of dsamples were extracted with 5 ml of tri-caid mixture (HNO_3 : H_2SO_4 : $HCLO_4$) (9:4:1) and digested for 3 hrs on a hot plate (SEDKO make) until no residue was left. The final volume made to 100 ml with distilled water and further diluted 5 times using distilled water. An aliquot of 5 ml was taken into a 25 ml volumetric flask, added 5 ml of ammonium vanadomolybdate solution (prepated by solution 'A': 22.5 gm ammonium heptamolybdate in 400 ml distilled water, solution 'B': 1.25 gm ammonium metavandate in 300 ml of hot double distilled water. Solution 'B' was added in a 1 l volumetric flask containing solution 'A' and allowed to cool at room temperature. Slowly 250 ml concentrated nitric acid was added into the prepated mixture and allow to cool to finally make the volume to 1 l with distilled water) and the volume was made to 25 ml with double distilled water. The final absorbance was measured after 10 min using spectrophotometer (SYSTRONICS make) at 470 nm. The amount of phosphorus was calculated using a standard graph prepared from potassium phosphate (100 µg/ ml).

Potassium (K):

Samples of 500 mg were extracted with 5 ml of tri- acid mixture (HNO_3: H_2SO_4: $HCLO_4$) (9:4:1) and allowed to digest fro 3 hrs on a hot plate (SEDKO make) till no residue was left. The final volume was made to 100 ml with distilled water and further diluted 20 times with distilled water. The diluted samples were then analyzed using Flame Photometer (SYSTRONICS make) and the amount of potassium was calculated using standards (1-10 ppm) prepared from potassium phosphate.

Copper (Cu), Maganese (Mn), Zinc (Zn) and Iron (Fe):

Samples of 1 gm were placed in a crucible and placed in the muffle furnace (METCO make) initially at 300˚C for 1 hr and the temperature was slowly increased to 550˚C for 5 hrs or until the ash turned white. The crucibles were cooled and added 2 ml of $HNO_3$to further heat them to dryness. The samples were allowed to cool and dissolved in 50 ml of diluted HCL (1:1). The solutions were filtered using Whatman filter paper and the final volume was made to 100 ml with distilled water. The final filtrates were used for the estimation of element like Cu, Mn, Zn and Fe with the help of Atomic Absorption Spectrophotometer (NOVA make) equipped with WinAAS software (Ver. 3.15). The amount of copper, manganese, zinc and iron were calculated using standars (1-10 ppm).

COMPARATIVE STUDIES CONDUCTED ON THREE DIFFERENT VARIETIES FROM TWO DIFFERENT REGIONS JUNAGADH AND NAVSARI.

Three different varieties of *M.indica* viz., Kesar, Alphonso and Rajapuri gorwing in two different regions of Gujarat, Junagadh and Navsari has been studied for it variation in fruit development.

V. ANATOMICAL DEVELOPMENT OF FRUIT

Fruit for three varieties of two different regions were taken for anatomical studies. In order to procure the same stages of fruit development, bisexual open flowers were tagged. Fruits at every stage of development, starting from the fertilization (visually ovary can be seen bulbous and bigger) i.e. fruit formation till the final ripening stage (total 4 stages).

Mature stage- Pedicel and lenticels observed. Ripened stage- fruit fully mature with yellow colour. One month interval was considered for differentiating each stage. For the first stage, fertilized ovary as a whole was fixed, in second and third stage a cut (1mm) was made penetrating till the endocarp along with epicarp and mesocarp region. Fourth stage, pieces containing epicarp and mesocarp were taken for fixing, as by this time endocarp becomes stony. Fixed samples were dehydrated in a graded series (20, 35, 55, 75, 95 and 100%) of

TBA (tertiary-butyl-alcohol) and embedded in paraffin wax (Johansen 1940). Single staining with toluidine blue and double staining with safranin and fast green were done for the cross section. Serial sections of fruits were observed and microphotographed to visualize the development of cuticle, lenticels, resin canals, epicarp, mesocarp and endocarp were done.

VI. BIOCHEMICAL STUDIES

Fruits of three varieties, Kesar, Alphonso and Rajapuri from two different regions of Gujarat (Junagadh and Navsari) were selected. Third and fourth stages were examined for the detailed biochemical studies.

i) pH and Total Acidity

For recording the pH and total acidity of fruits, 5 g of fruit pulp tissue was homogenized with 50 ml of distilled water and allowed to stand for 30 min. The filtrate was collected and centrifuged at 3000 rpm for 10 min. The pH values of the supernatant were determined using a digital pH meter (M-TRONICS).

The total titrable acidity was measured by homogenizing 5 g of fruit pulp tissue (till pulp turns uniform paste) in distilled water and boiled for 1 hr, cooled and made up to 2.5 ml. A 10 ml aliquot, 75 ml of boiling distilled water and few drops of 1% phenopthalein indicator indicator were mixed together. The solution was titrated against 0.1 N NaOH (sodium hydroxide) solutions to give a pink colour (Berwal *et al*. 2004). Total acidity was expressed in terms of percentage (%).

$$\text{Total acidity (\%)} = V_2 \times \frac{V}{V_1} \times \frac{N \times EW}{100} \times \frac{100}{W}$$

Where, W = weight of sample

V = Volume of extract

V_1 = Aliquot taken for estimation

N = Normality of alkali

V_2 = Volume of 0.1 NaOH used

EW = Equivalent weight of acid

ii) Total sugars

A 100 mg of sample was hydrolyzed with 5 ml of 2.5 N HCl (hydrochloric acid) in a boiling water bath for 3 hrs and was later cooled at room temperature. The solution was neutralized with addition of sodium carbonate powder till the effervescence ceased. The final volume was made up to 100 ml and centrifuged (Remi make) ar 5000 rpm for 10 min. An aliquot of 0.5 ml of distilled water and 4 ml of anthrone reagent were taken and was heated for 8min in a boiling water bath. The solution was cool rapidly by placing the tubes in running tap water and the green to dark green colour was measured at 630 nm in spectrophotometer (SYSTRONICS make) (Sadasivam and Manickam 1992). The amount of total soluble sugars was calculated using a standard graph prepared from glucose (100 µg/ ml).

iii) Reducing Sugars:

Fresh fruit sample of 100 mg was extracted twice with hot 80% alcohol. The supernatant was collected and evaporated on hot water bath. The dried sugar crystals obtained were then dissolved in 10 ml of distilled water. An aliquot of 200 µg of alcohol-free extract, 3ml with distilled water and 3 ml and 3 ml of dinitrosalicyclic acid (DNS) reagent was added and the tubes were placed in boiling water bath for 5 min. As the colour developed 1 ml of 40 % sodium potassium tartrate solution was added and mixed well. The tubes were rapidly cooled under running tap water and the absorbance was measured using a spectrophotometer (SYSTRONICS make) at 510 nm (Miller 1972). The amount of reducing the sugars present was calculated using a standard graph prepared from glucose (100 µg/ ml).

iv) Non-reducing Sugars:

A 100 mg of the fruit sample was extracted with hot 80 % alcohol twice. The supernatant was allowed to evaporate on a hot water bath and the dried crystals were dissolved in 10 ml if

distilled water. An aliquot of 1 ml of extract was added 1 ml of H_2SO_4 (sulphuric acid) solution and hydrolyzed by heating at 50˚C for 30 min on a hot plate. The tubes were allowed to cool and added 1 or 2 drops of methyl red indicator. The solution was neutralized by adding 1 N NaOH (sodium hydroxide) drop wise using a pipette. Later 3.0 ml if dinitrosalicyclic acid (DNS) reagent was added to the tube and was placed in boiling water bath for 5 min. As the colour developed 1 ml of 40 % sodium potassium tartrate solution was added and mixed when still warm. The tubes were cooled under running tap water and the absorbance was measured using a spectrophotometer (SYSTRONICS make) at 510 nm (Thimmaiah, 1999). The amount of non-reducing sugars was calculated using a standard graph prepared from glucose (100 µg/ ml).

v) Starch:

For the estimation of total starch, 100 mg of sample was homogenized in hot 80% alcohol and centrifuged at 5000 rpm for 10 min. The residue was retained and repeatedly washed with hot 80 % alcohol washings did not give colour with anthrone reagent. The residue was finally dried over a hot water bath and added 5 ml of water and 6.5 ml of 52 % Perchloric acid. The solution mixtures were allowed to extract at 0˚C for 20 min, centrifuged (REMI) and the supernatant was saved. The extraction was repeated by addition of fresh Perchloric acid and again centrifuged (REMI) to pool the supernatant and the final volume made up to 100 ml with distilled water. An aliquot of 0.1 ml was taken and the volume was made up to 1.0 ml by adding distilled water, another 4.0 ml of anthrone reagent was added and boiled for 8 min in a boiling water bath. The samples were cooled rapidly under running tap water and the intensity of green to dark green colour was measured at 630nm using spectrophotometer (SYSTRONICS make) (Devi 2001). The total glucose content was calculated using a standard graph prepared from glucose (100 µg/ ml) and the value obtained was multiplied by a factor 0.9 to arrive at the starch content.

vi) Proteins:

Protein content in fruit was measured by extracting 0.5 g of fresh fruit samples in sodium phosphate buffer (pH 6.8) and centrifuged (REMI) at 5000 rpm for 10 min. After centrifuged, collect the supernatant. An aliquot of 0.2 ml of sample extract was taken in a test tube and the volume was made up to 1.0 ml with distilled water. In the sample reaction mixture 5 ml of solution C (mixed 50 ml of solution 'A' with 1 ml of solution 'B'; solution 'A' containing 2% sodium carbonate in 0.1 N sodium hydroxide; solution 'B' containing 0.5% copper sulphate in 1% sodium potassium tartrate) was added and incubated in a dark chamber for further 30 min at room temperature. The absorbance was measured at 660 nm against blank on spectrophotometer (SYSTRONICS make) (Lowry *et al.* 1951). The amount of protein was calculated using a standard graph prepared from bovine serum albumin (200 µg/ ml).

vii) Phenols:

For the estimation of phenols from the fruits, 0.5 g of the sample were extracted with 10 ml of 80 % ethanol and centrifuged (M-TRONICS) at 10,000 rpm for 20 min. The supernatant was collected and the residue was re-extracted with another 5 ml of 80% ethanol, centrifuged and the supernatant was pooled. The supernatant was evaporated to dryness on a hot water bath and the residue dissolved in 5.0 ml of distilled water. In a tube an aliquot of 0.2 ml, 2.8 ml distilled water, 0.5 ml of Folin-Ciocalteu reagent (commercially available Folin Ciocalteu's reagent diluted with equal volumes of water) were mixed together and after 3 min, 2 ml of 20% Na_2Co_3 (sodium carbonate) solution was added, mixed and kept in a boiling water bath for exactly one minute. The tubes were further cooled under running tap water and the absorbance was measured using spectrophotometer (SYSTRONICS make) at 650 nm against blank (Thimmaiah 1999). The amount of phenolic substance was calculated using a standard graph prepared from catechol (100 µg/ ml).

viii) Dietry Fiber

The standard method for the measurement of total dietary fibre was followed, given by the Association of Official Analytical Chemists (2000 method 985.29). Duplicate samples of dried foods, fat extracted if containing >10% fat, undergo sequential enzymatic digestion by heat stable a-amylase, protease, and amyloglucosidase to remove starch and protein. For TDF determination, the enzyme digestates are treated with alcohol to precipitate SDF before filtering. TDF residues are washed with alcohol and acetone, then dried and weighed. For IDF determination, the enzyme digestates are filtered, residues remaining in crucible are washed with warm water, dried and weighed. For SDF determination, combined filtrate and water washings are precipitated with 4 volumes of 95% ethanol. All the dried DF residues are corrected for protein, ash and blank for final calculation of SDF and IDF values.

xi) HPTLC studies

a. Sugars

Fruit samples of 1 gm each were extracted with 10 ml of 95% alcohol, centrifuged, to collect the supernatant. The supernatant were finally concentrated on boiling water bath and finally dissolved in 3 ml of 95% ethanol for profiling sugars. Pre-coated TLC plates (Silica gel $60F_{254}$ aluminium sheets) of 10x10 cm size were pre-washed with methanol and used as a stationary phase. 20µl of concentrated fruit extracts were loaded on the pre-coated TLC plates in ofrm of bands (8 mm) using Linomate 4 (CAMAG make) equipped with WinCATS software (Ver. 1.2). A mobile phase containing n-butanol: ethanol: water (4:1:5 v/v) was placed in TLC chamber (CAMAG make) and allowed to saturated for 30 min. The TLC plate were then placed in the TLC chamber and the samples were allowed to migration a distance of 75 mm. Photometric measurements were performed at 610 nm with CAMAG TLC scanner 3 using Win CATS software (Ver. 1.2) to calculate the different sugars present at their respective concentrations. Sugars such glucose, fructose and sucrose were used as standards.

b. Amino acids

The fruit samples of 1 gm were initially weighed and extracted with 10 ml of 95% alcohol, centrifuged and supernatant was collected.The supernatant were finally concentrated on boiling water bath and finally dissolved in 3 ml of 95% ethanol for the profiling of amino acids. Pre-coated TLC plates (Silica gel $60F_{254}$ aluminium sheets) of 10x10 cm size were pre-washed with methanol and used as a stationary phase. A 20µl of concentrated fruit extracts were loaded on pre-coated TLC plates in form of bands (8mm) using Linomate 4 (CAMAG make) equipped with WinCATS software (ver. 1.2). a mobile phase contained n-Butanol:Acetic acid:Water(8:2:2 v/v) was placed in TLC chamber (CAMAG make) and allowed to saturate for 30 min. the TLC plates were then placed in the TLC chamber (CAMAG make) and the samples were allowed to migrate a distance of 75 mm. Photometric measurements were performed at 365 nm with CAMAG TLC scanner 3 using Win CATS software (Ver. 1.2) to calculate the different amino acids present at their respective concentrations. Amino acids such as cysteic acid, proline, hydroxyproline, aspartic acid, serine, glycine, threionine, alanine, glutamic acid, valine, methionine, isoleucine, tyrosine, leucine, phenylalanine and tryptophan were used as standards.

ix) Enzyme studies

a. Amylase:

The fresh fruit samples (1 gm) were extracted in 10 ml of ice cold 10mM $CaCl_2$ (Calcium Chloride) solution at room temperature for 3 hrs and then centrifuged (REMI make) at 10,000 rpm at 4˚C for 30 min and the supernatant was collected. For amylase assay, 1ml of starch solution and 1 ml of enzyme extract was taken and incubated at room temperature. After 15 min the reaction was stopped by adding 2 ml of DNS reagent (dinitrosalicyclic acid reagent) and the reaction mixture was mixed was heated on a boiling water bath for 5 min. Sodium potassium tartrate solution (1 ml of 40% solution) was added to the reaction mixture and cooled the tubes under running tap water. The final volume was made up to 10ml with

distilled water and the absorbance was read using a spectrophotometer (SYSTRONICS make) at 560 nm (Devi 2001). The protein content in the enzyme extract was measured by using the method of Lowry *et al.* (1951). The specific activity of enzyme is expressed as mg maltose/ min/ mg protein.

b. Invertase:

For the analysis of Invertase, 5 gm of fruit sample was homogenized in chilled mortar and pestle placed in an ice bath with pre-cooled 20% glycerol. The samples were filtered through several layers of cheesecloth and the volume was made up to 100 ml with 20 % glycerol. In the test tube 5 ml of enzyme solution, 10 ml of buffer and 5 ml of sucrose solution was added and incubated at room temperature for 24 hrs. The assay of invertase was carried out by taking an aliquot of 1 ml from the reaction mixture and to it 1, l of DNS reagent (dinitrosalicyclic acid reagent) was added to stop the reaction. The tubes were then allowed to boil in boiling water bath for 5 min and 1 ml of 40% sodium potassium tartrate solution was added and mixed were still warm. The tubes were cooled under running tap water and the absorbance was measured at 510 nm using blank with the help of spectrophotometer (SYSTRONICS make) (Devi 2001). The protein content in t6he enzyme extract was measured by using the method of Lowry *et al.* (1951). The specific activity of enzyme is expressed as mg glucose/ min/ mg protein.

c. Catalase:

The assay of catalase was carried out by homogenizing 10 gm of sample in 0.1 M phosphate buffer (pH 7.0) in a pre-cooled mortar and pestle and centrifuged (REMI make) at 15,000 rpm for 30 min at 4˚C. an aliquot of 1ml of the enzyme extract, 3 ml of phosphate buffer and 2 ml of H_2O_2 was incubated at 20˚C for 1 min. The reaction was stopped after 1 min by adding10 ml of 0.7 N H_2SO_4 and the reaction mixture was tirated against 0.01 N $KMnO_4$ (potassium permanganate) to find out the residual H_2O_2 until a faint pink colour persisted for

at least 15 sec (Thimmaiah 1999). The protein content in the enzyme was measured by using the method of of Lowry *et al.* (1951). The specific activity of enzyme is expressed as units/ min/ mg protein.

d. Peroxidase:

1g of fruit sample was homogenized in 10 ml of 0.1 M phosphate buffer (pH 6.0) in a chilled mortar and pestle, strained through two folds of muslin cloth and centrifuged (REMI make) the homogenate at 16,000 rpm for 20 min at 4˚C. an aliquoat of 1 ml of O-dianisidine, 0.5 ml of H_2O_2, 1 ml phosphate buffer and 2.4ml distilled water were taken and incubated at 30˚C. The reaction was started by adding 0.2 ml of enzyme extract and after 5 min the reaction was stopped by adding 1 ml of 2 N H_2SO_4 (sulphuric acid) and the absorbance was read using spectrophotometer (SYSTRONICS make) at 430 nm (Thimmaiah 1999). The protein content in the enzyme extract was measured by using the method of Lowry *et al.* (1951). Enzyme specific activity is expressed as units/min/mg protein.

e. Cellulase:

The measurement of cellulose assay was performed by extracting 5 gm of sample with 10 ml of 100 mM sodium acetate buffer (pH 6.0) which contained 0.2% sodium dithionite and 1% PVP (MW 44,000) for 1 min and was allowed to centrifuge (REMI) at 10,000 rpm for 20 min. The supernatants were collected and the residue was re-suspended in 2 volumes of 1 M sodium acetate buffer (pH 6.0) containing 6 % NaCl (sodium chloride) and the pH was adjusted to 8.2 with 2 n NaOH (sodium hydroxide). The assay mixture was incubated overnight at 4˚C, centrifuged (REMI make) and filtered using Whatman filter paper. A 2 ml of 1 % CMC (carboxymethycellulose) was incubated and 1 ml of 100 mM sodium acetate buffer (pH 5.0) was added at room temperature. The reaction was initiated by adding 1 ml enzyme extract and aliquots of 0.5 ml were withdrawn at an interval of 6 or 12 hrs and the reducing sugars were estimated using DNS (dinitrosalicyclic acid reagent) method

(Sadashivam and Manickam 1992). The specific acitivity of enzyme is expressed as glucose/min/mg protein.

f. Polygalactouronase:

Fresh fruit samples of 5 g were extracted with 13 ml of tris- HCl buffer (pH 10) and centrifuged (REMI make) at 10,000 rpm for 20 min, the pellets were incubated for 1 hr in 5 ml of extraction buffer and centrifuged (REMI make) at 15,000 rpm for 30 min. an aliquot of 0.1 ml of NH_4Cl (ammonium chloride) and 1 ml of polygalacturonic acid were taken at room temperature and the reaction was initiated by addition of 0.1 ml of enzyme extract and incubated further for 30 min at room temperature. The reaction was further terminated by addition of 0.3 ml of 5 % TCA and centrifuged (REMI make) at 2000 rpm for 30 min and supernatant was collected. Estimation of reducing sugars formed during reaction was estimated by DNS (dinitrosalicyclic acid reagent) method (Sadashivam and Manickam 1992). The protein content in the enzyme extract was measured by using the method of Lowry *et al.* (1951). The specific activity of enzyme is expressed as mg glucose/min/mg protein.

g. Pectinmethylesterase:

Fruit samples of 5 g each was extracted with 15 ml of ice cold 8.8 % NaCl (sodium chloride) solution and centrifuged (REMI make) at 15,000 rpm at 4˚C for 10 min and the supernatants were collected. In a cuvette 2 ml of pure pectin, 0.15 ml of bromothymol blue and 0.83 ml of water was mixed and incubated at 25˚C. The absorbance of the mixture was measured at 620 nm against water blank. The reaction was started by addition of 100 µl of enzyme solytion and the rate of decrease was measured after 1 min using spectrophotometer (SYSTRONICS make) at 620 nm (Sadasivam and Manickam 1992). The protein content in the enzyme extract was measured by using the method of Lowry *et al.* (1951). The specific activity of enzyme PME is expressed as A_{620}/ min/ mg protein.