MATERIALS AND METHODS
PLANT MATERIAL:

The chosen plants taken for experimental studies were *Cassia tora* L. (Oriya - Chakunda) and *Bajitania purpurea*, L. (Oriya - Barada). *Cassia tora* L. is a common road side weed and an annual herb belongs to the sub-family "Caesalpiniaceae" of the family Leguminosae under the order Rosales. It is also being used for medicinal purposes (Chopra et. al. - 1956). It is 1-5 feet high with branched tap root. Stem erect woody cylindrical, branched. Leaf petiolate, alternate compound.
with 8-12 pairs of pinna, with a pulvinus at leaf base, leaf-lets opposite, hairy, ovate, acute and out shaped, glands before lowest smaller pair. The leaves are deep area in young stages and become brown with advancement of age. Inflorescence recem in pairs on short axillary stalks. Flowers pedicillate, bracteate, zygomorphic complete, hermaphrodite, hypogynous and pentamerous. Sepals - 5, polysepalaus, imbricate, petals - 5 polypetalaes - yellow - obovate, clawed, ascending impriacute stamen - 10, lower - 7 rarely equal, 3 upper minute and doorsifixed (Staminodes), polyandrous, basifixed and ditrecaes. Carpel - 1, ovory superior, cerilocular plantation marginal. Fruit pods, pods are slender 6-12 long, completely seported with many oblongs seeds. Flowering is seen in winter (i.e. mostly in the month of September and October). The important difference in the morphology of Cassia tora, L. collected from plane area of Cuttack city that the leaf is provided with gland between the lowest smaller pairs while that
leaf of the plant collected from hilly area of Phulbani District to provided with two such glands.

*Baninia purpurea* L. (Oriya - Barada, Bengali - Devakanchan) belongs to the sub-family Caesalpineae and family Leguminosae is also well known for its medicinal value. Plant is a medium size tree but not frequently flowers. Leaf is 5-7" long in size, nerved, very deeply lobed, often oblong, lobes with angular tips. Leaves split from 1/3 of the way down often to near the base, glabrous. Flowers are orange, purple in colour, terminal, pedicilate, racemes with acutely five angled flower bud. Pod is 6-12" by 0.75-1" narrow below, nearly always some what broadening upwards, flat, dehiscent when ripe, with coriaceous twisted valves. Bark is dark-brown. The tree is hardy and brown in colour, blaze with pink outer layer, then pale yellow, rapidly darkening and gradually turns white but on wood again yellowish.
Leaves are eaten as vegetables mostly by tribals and local people during summer. Because during summer (from mid March till mid June) new branches which develops from tree bears young, juvenile, pale green colour leaves which are soft textured in nature.

Phulbani district of Orissa is tribal belt which is situated between $84^\circ-07'-28''$ longitude and $20^\circ-37'-22.5''$ latitude in the world map. As it is the temperate region of Orissa so here all the seasons in a year has got an equal impact on the environmental status of Phulbani district. It has three main seasons i.e. Summer, Winter and Rainy season. Summer season starts from March and continues till the end of June.

At the approach of July heavy rain fall starts and prevails up to the month of mid or far-end of October. Lastly the Winter climate pare its path into this hilly region or environment from the last part of October upto the month of February.
SAMPLING METHOD:

The leaves of *Cassia tora* L. and *Bauhinia purpurea* L. were collected from the different branches of respective plant within the 7th to 15th day of every month. The leaves in each case was selected for the study were as far as possible from same area of a particular plant. Simultaneously the healthy condition of the leaves were choosen for experimental purposes.

METHODS OF QUANTITATIVE ANALYSES OF ORGANIC INGREDIENTS:

1. **EXTRACTION & QUANTITATIVE ESTIMATION OF CHLOROPHYLL**

The extraction of chlorophyll was done as per the method adopted by Nadler et. al. (1972). The leaf samples were cut into small pieces and homogenized with cold 80% alkaline acetone (8 volumes of acetone : 2 volumes of 0.05 M potassium carbonate) in a glass mortar and
pestle. The slurry was centrifuged at 4000-5000 r.p.m. several times by resuspending the residue with additional amount of acetone till the residue was free of chlorophyll (green pigments). All the supernatants were combined, made up to volume and the absorbancy was taken at 645 and 663 nm in a Carl Zeiss Spectro colorimeter. Total chlorophyll was calculated using the equation adopted by Arnon (1949), Arnon's formula i.e \((20.2 \times \text{OD at 645 nm}) + (8.02 \times \text{OD at 663 nm})\) (Arnon 1949)

2. **QUANTITATIVE ESTIMATION OF CARBOHYDRATES:**

The total soluble sugar (Carbohydrate) was estimated by the method adopted by Yemm and Willis (1954). The leaves were ground in 70% ethanol and centrifuged at 5000 x g for 10 mins. From the supernatant, 0.1 ml. of alcoholic extract was taken, 3 ml. of freshly prepared anthrone reagent (150 mg. anthrone in 100 ml. of 72% conc. \(\text{H}_2\text{SO}_4\)) was added and heated at 100° C
for 10 mins. Over a water bath. It was cooled and O.D. was taken at 620 nm by using UV-visible double beam spectrophotometer. Calculation was done from the predetermined calibration curve of synthetic sugar.

3. EXTRACTION & QUANTITATIVE ESTIMATION OF TOTAL SUGAR

The total sugar was extracted following the method adopted by Kar & Mishra (1976) and was estimated by Anthrone reagent method (Yoshida et. al. - 1972).

The leaf sample were cut into small pieces and were homogenized with 80% ethanol in a glass mortar and pestle. The homogenate was boiled in a water bath for 5 minutes, cooled and centrifuged at 4000-6000 r.p.m. The residue was suspended in additional amount of 80% ethanol, boiled, cooled & centrifuged at 4000-6000 r.p.m. All the ethanolic extracts were combined, made up to volume & aliquot was taken for the estimation of total
sugar. The extracts were however evaporated to dryness, and lipids & pigments were removed by washing the evaporated extract with ether.

0.5 ml. of alcoholic extract was taken in a test tube to which 0.5 ml. of distilled water was added followed by 5 ml. of anthrone reagent. The test tube was heated for 10-15 mins. For preparing the blank reading a test tube was taken into which 1 ml. of distilled water and 5 ml. of Anthrone reagent was added excepting the plant extract. It was then heated upto 15 mins. The O.D. of the solution was taken at 630 mm. against the blank reading.

**PREPARATION OF ANTHRONE REAGENT:**

200 mg of Anthrone was taken in 100 ml. of Conc H₂SO₄. After dissolved it was put in ice bath.
STANDARD CURVE:

A standard curve was drawn by taking known strength of D-glucose and from the curve, the quantity of total sugar could be calculated.

4. EXTRACTION & QUANTITATIVE ESTIMATION OF REDUCING SUGAR:

Reducing sugar was estimated by Nelson's copper reagent method (Nelson - 1944) using the above ethanolic extract.

Copper reagent A and B were prepared and they were mixed in a proportion of 25:1. The mixed solution was the copper reagent.

COPPER REAGENT - A:

25 gms of anhydrous Na$_2$CO$_3$, 25 gms of Na-K-tartrate
(Rochelle salt), 20 gms of NaHCC\textsubscript{3} and 200 mg of anhydrous Na\textsubscript{2}SO\textsubscript{4} were dissolved in about 800 ml. of distilled water and then diluted with distilled water to make the volume 1 liter. The temperature of the solution should not be allowed to fall below 20\textdegree C.

**COPPER REAGENT - B:**

15 gms of CuSO\textsubscript{4}, 5H\textsubscript{2}O were dissolved in 100 ml. of distilled water, followed by 1 - 2 drops of Conc H\textsubscript{2}SO\textsubscript{4}.

**ARSENO - MOLYBDATE REAGENT:**

25 gms of Ammonium molybdate was dissolved in 400 ml. of distilled water and to that 21 ml. of Conc H\textsubscript{2}SO\textsubscript{4} was added with constant stirring, 3 gms of sodium biarsenate (NaH ASO\textsubscript{4}) were dissolved in 250 ml. of distilled water. Then both the solution were mixed
and placed in an incubator at 37° C for about 24 hours. The reagent was stored in a glass stoppered brown (coloured) bottle for use.

1 ml. of plant extract was taken in each test tube & to it 1 ml. of copper reagent was added. Then 1 ml. of Arsenomolybdate solution was added followed by 7 ml. of distilled water.

The test tubes were heated upto 20 min. O.D. was taken at 650 nm against the blank reading. The standard curve was done from the known strength of D-glucose solution and from it the quantity of reducing sugar was calculated.

5. **QUANTITATIVE ESTIMATION OF STARCH:**

200 mg. of dried leaf powder was placed in a 50 ml. centrifuge tube for each sample 25 ml. of 80%
ethanol was added and tubes were heated over water bath at 80-85° C for 10 minutes. Then the samples were centrifuged at 2000 g. for 15 minutes and supernatants were collected into a 50 ml. beaker. This type of extraction was repeated thrice and stored for determination of soluble sugars. The residue, contained the starch.

Then 5 ml. of water was added to the residue, followed by 6.5 ml. of 52% PCA and was allowed to stand for 5 minutes. Again 20 ml. of water was added followed by centrifuge at 2000 r.p.m. Supernatant were collected in a 100 ml. volumetric flask. Then 5 ml. of water was added to the residue and extraction was repeated with PCA. The contents were transferred quantitatively into volumetric flask and volume was made upto 100 ml. with water and was filtered through Whatman No.42 filter paper. Dilution was done to the aliquot of the filtrate to a known Volume and sugar was analysed with anthrone.
reagent. Then the sugar content was calculated in terms of glucose equivalent and the conversion factor of 0.9 was used to convert the values of glucose to starch. (As per method adopted by McCready et al. - 1950)

6. **QUANTITATIVE ESTIMATION CF LIPIDS**:

Fifty grams of fresh material was homogenized with a mixture of 300 ml. chloroform and 150 ml. methanol. The mixture was filtered through a porous glass filter and the residue was the homogenized with the same volume of solvent. The filtrates were combined and transferred to a graduated cylinder, and 0.2 volume of 0.6% aqueous NaCl was added. The solution was gently mixed to avoid the formation of an emulsion. The aqueous phase and the interface were removed. The aqueous phase may still contain some polar lipids and was back washed with chloroform to recover the polar lipids. The Nonlipid contaminants were removed from the organic phase by
partial evaporation of solvent in a rotary evaporator, followed by extraction with hexane chloroform (3:1). After stirring, the mixture was allowed to settled and the supernatant was decanted from the solid, nonlipid contaminants.

The initial wt. of the empty container = $W_1$
Final wt. of the container containing the lipid = $W_2$

The quantity of lipid present in the tissue = $W_2 - W_1$

7. **QUANTITATIVE ESTIMATION OF TITRATABLE ACIDITY**:

The entire complete leaves were taken separately for analysis, each weighing 5 gms. Freshly gathered entire leaves were dropped immediately in 20 times the volume of hot 70% ethanol. The material was then boiled for 5-10 minutes and ground to a fine paste by mortar and pestle. Three repeated extractions were done for complete recovery. The filtrate was evaporated off over
water bath and the residue was dissolved in 10 ml. of 20% ethanol. To remove the proteins in solution this mixture was centrifused for 30 minutes at 2000 r.p.m. The clear liquid was decanted and double glass distilled water was added to make the volume to 100 ml. Out of this 100 ml. of the extract, half was kept separately for subsequent analyses of soluble fractions of organic compounds i.e. amino acids, sugars, organic acids etc. This method however is the method adopted by Patnaik and Laloraya (1961).

The residue after filtration was taken in separate conical flask suspended with 6 N hydrochloric acid for the analysis of hydrolysate of oligosaccharides, proteins etc. The conical flask with 6 N hydrochloric acid and residue were autoclaved for 1 hour so that the oligosaccharides broke into free sugars, and proteins into aminoacids and amides. It was then filtered and evaporated over water bath till the residue was left...
behind 20% ethanol was added (1 gm / 1 ml. on fresh weight basis) and centrifused at 2000 r.p.m. for 30 minutes to get the clear supernatant liquid. This was kept separately (in a specimen bottle at 0-4°C to avoid microfloral infection) for analysis of hydrolysate products of bound fractions in the residue.

5 ml. of the other half extract was taken each time for titration against freshly prepared N/100 Sodium hydroxide (the strength of Sodium hydroxide was obtained by titrating against the known strength of Oxalic acid). All calculations were based on the amount of alkali used (equivalent of Oxalic acid strength) to neutralise 5 ml. of this aliquot representing 0.25 gm. of fresh wt. of the tissues.

8. QUANTITATIVE ESTIMATION OF ASCORBIC ACID (VITAMIN-C)

During recent years many workers like Ischer-wood and Navis
(1956), Steward et. al. (1954) etc., have estimated the keto-acids present in various plant tissues. The methods have been critically evaluated by Fowden and Webb (1955). The extraction procedure was followed as adopted by Tower, Thompson and Steward (1954) and Patnaik and Das (1973).

The plant material was extracted taking 2 gms. of fresh leaves from each material separately and was ground with 10 ml. of 5% oxalic acid, centrifused and the clear liquid obtained was diluted upto 25 ml.

Taking 6 test tubes as a set, to each tube 2 ml. of 2% Ammonium molybdate solution and 3 ml. of 0.15 (N) sulphuric acic was taken, shaken thoroughly and kept in ice beaker. Then 2 ml. of 1.5 x 10^{-3} (M) Disodium hydrogen phosphate was added and shaken. Leaving a blank to each tube 1 ml., 2 ml., 3 ml., 4 ml., 5 ml. of plant extract was added in a sequence. Then distilled water
was added to make the volume 1.0 ml. The same procedure was adopted in each case.

Then the optical density was taken using the colorimeter at 660 nm. The quantity of Ascorbic acid was calculated from the predetermined curve of synthetic ascorbic acid (See Table - )

9. QUANTITATIVE ESTIMATION OF TOTAL NITROGEN:

1) THEORY

Micro Kjeldahl's method with its various modifications constitute the basis of Nitrogen estimation in plant parts. Briefly the method constitutes the reduction of organic nitrogen to ammonia, ammonia is distilled and ammonium sulphate is formed with dil $\text{H}_2\text{SO}_4$ and it determined by titration with standard alkali.

The oxidation is accompanied by heating the sample in nitrogen free $\text{H}_2\text{SO}_4$, when the nitrogen reacts with
the acid to form (NH₄)₂SO₄ and (NH₄)₂SO₄ in the distillation flask, was made alkaline by the addition of 40% NaOH by supplying stem NH₃ & NH₄OH is absorbed in measured quantity of H₂SO₄ to form (NH₄)SO₄, and excess of H₂SO₄ is titrated against standard alkali.

II) METHOD FOR EXTRACTION:

500 mgs. of fine, dried powder of plant materials were taken for digestion. Dried powders were taken in Kjeldahl's flasks to which 1.5 gms. of triple catalyst and 10 ml. of ConC N₂ free H₂SO₄ were added. Then the flasks were heated in a fume cup-board for about 2 to 2½ hours till clean solution was seen having crystals at the bottom of the flask. Then the flasks were cooled and the contents were transferred quantitatively to 100 ml. measuring flasks and by adding distilled water the volumes was made up to the mark.

10 ml.of digested material taken in the distilation
flask and 10 ml. of 40% NaOH was added into it and the pinch cock was closed. Previously 10 ml. of N/70 H₂SO₄ was taken in a Conical flask and fitted by stand in such a way that the tip of the silver tube, dipped in the acid. The steam was then allowed to pass through the distillation flask. Ammonia gas was bubbled through the acid in the conical flask where it was absorbed by the acid to form (NH₄)₂SO₄ and water. The operation was carried on till the bubbling was stopped. The content in the conical flask was titrated against N/10 NaOH solution of known strength.

10. **QUANTITATIVE ESTIMATION OF SOLUBLE NITROGEN:**

Two grams of plant material samples were taken in mortar and pastle separately and a fine paste was made in addition of 2% trichloroacetic acid. The whole content was taken in a centrifuse tube and centrifused at 3000 - 4000 r.p.m. for one hour. The clean solution
was decanted off and precipitations were again grinded in the mortar and pestle. It was again centrifused at the same r.p.m. The clean solution was decanted off to the measuring flask (50 ml.). It was kept as per stock solution 10 ml. of stock solution was taken in a digestion flask and digested with 10 ml. of nitrogen free conc. $\text{H}_2\text{SO}_4$ and with 1 gm. of triple catalyst. After digestion the solution was made upto 100 ml. in a measuring flask. Out of this 10 ml. was taken for distillation. The rest of the procedure followed as described for total nitrogen.

11. **QUANTITATIVE ESTIMATION OF AMINO ACIDS**:

Quantitative estimation of amino acids was done by ninhydrin reagent method as adopted by Moor & Stein (1948).

Extraction of leaf of the plant was made by 80%
ethanol as described for total sugar. The volume was made upto 10 ml.

I) **CITRATE BUFFER pH5**:

2.626 gms. of citric acid was dissolved in 25 ml. of IN NaOH solution at pH5.

II) **STANUS CHLORODE SOLUTION**:

32 mg. of stanus chlorode was dissolved in 20 ml. of citrate buffer.

III) **NINHYDRIN SOLUTION**:

200 mg. of Ninhydrin dissolved in 5 ml. of methyl selesolve.

To 0.5 ml. of each plant extract sample 0.5 ml. of distilled water was added. 1 ml. of Ninhydrin reagent (Moore and Sta in 1948) was added. The test tubes were heated to 20 minutes over water bath. They were cooled and their O.D. were taken at 570 nm against the blank reading.
Standard curve was drawn by taking different concentration of known strength of glycine. From it quantitative amount of amino acid of the leaf was calculated.

12. QUANTITATIVE ESTIMATION OF INDIVIDUAL AMINO ACIDS FROM PLANT MATERIALS:

The located amino acid spots developed over the two dimensional chromatogram (described under the heading qualitative detection of amino acids by two dimensional chromatogram). The detected individual spot was cut carefully before spraying with Ninhydrin spraying reagent. The cut piece was taken in a test tube, and was eluted with 80% hot ethanol over a water bath till the amino acid quantitatively entered into the 80% ethanolic situation. That was confirmed by negative ninhydrin test of the cut piece after elution. The individual dissolved amino acid was transferred quantitatively into
a small test tube and 1% Ninhydrin solution was added to make the required volume. However, the colour was developed after heating the mixture over water bath for 10 minutes. After cooling O.D. of it was measured at 550 nm by Spectronic 20. Hence, the quantity of individual amino acids were determined from the pre-determined calibration curve of synthetic amino acid i.e. glycine. (which gives blue violet colour with Ninhydrine). However, the quantity of all the amino acids the materials could be obtained by this method excepting 'Proline' (since it gives yellow colour with Ninhydrin reagent).

13. **QUANTITATIVE ESTIMATION OF PROTEIN**

Protein extraction was made as described by Fletcher and Osborne (1966). The leaves were boiled with 80% ethanol (W/v) in a water bath till all the pigments were extracted. The leaves were then homogenized with cold 10%. Trichloro acetic acid (W/v) and
centrifused. The residue was then washed twice with 10% trichloroacetic acid. The remaining residue was washed successively with absolute ethanol (once), ethanol : chloroform (3:1, v/v, twice - once), ethanol : ether (3:1, v/v, once) and finally with ether (once). The residue was evaporated to dryness. Protein was then solubilized with IN NaOH, then centrifuged and the supernatant was made up to volume. Aliquot from this supernatant was taken after proper dilution for the estimation of protein using Folin - Ciocalteu reagent (Lowry et al. 1951) with bovine serum albumin (Sigma) as the reference standard. 0.4 ml. of plant extract of each part was taken in a test tube. To it 2 ml. of Coomper reagent was added and it was incubated for 10 minutes. Then 0.2 ml. of phenol reagent was added and finally 2.4 ml. of distilled water was added. It was kept for 15 minutes. Then O.D. was measured at 540 nm against the blank reading.

A known strength of Bovine Albumin Serum was taken in different strength as per above excepting the
plant extract. A standard calibration curve was obtained. The quantity of protein present in the material was determined from the standard curve.

14. SEPARATION OF CARBOHYDRATES BY DISC PAPER CHROMATOGRAPHY QUALITATIVE ESTIMATION:

I) SOLUBLE SUGARS (MONOSACCHARIDES AND DISACCHARIDES)

The method for the separation and identification of different mono-saccharides and disaccharides were done as per the method described by Patnaik & Laloraya (1962). This method was the modified method of the principles laid by Sarget and Skoog (1960).

Circular paper having 4.2" radius was cut from Whatman filter paper No.1 and was divided equal necessary segments. Marker spots were marked over the chromatogram by means of a drawing pencil. 0.025 ml. of the plant extract was spotted over the marker spot by Shandan's
micropipette. Simultaneously reference spots of synthetic sugar solutions were spotted in other different marked regions of the same chromatogram. While putting the drops over the marker spot care was taken to dry up the spots either by hair dryer or over infra red lamp, so that no spreading off the drops was made over the boundary line of the marker spot. The paper was then run in a previously saturated chamber of the same solvent used for the development of the chromatogram. The required solvent system was N-butanol : acetic acid : water (4:1:5). The chromatogram was allowed to run for 6 hours. The distance move by the solvent front was marked. It was then removed and dried for 24 hours at room temperature (25°C). It was sprayed with Aniline di-phenyl amine phosphate reagent by atomizer. After spraying, followed with drying it was kept in an oven (80 - 90°C) for 20 minutes. The developed spots were identified with respective Rf values of reference synthetic sugar spots. Reagent was sprayed and the sugars
were identified by comparing with the synthetic spots and were tabulated in Table No. XVII, XVIII.

II) CHROMATOGRAPHIC SEPARATION OF OLIGOSACCHARIDES

(QUALITATIVE ESTIMATION):

The extraction obtained for the bound proteins and oligosaccharides were taken here in place of normal extraction and all the other procedures were same as previous. The identified sugars were plotted in Table No. XVII, XVIII.

III) PREPARATION OF SPRAYING REAGENT:

The spraying reagent described by Buchan and Savage (1952) was used here. It consisted of 4% aniline in N-butanol (5 volumes), 4% diphenylamine in N-butanol (5 volumes) and syrupy phosphoric acid (1 volume) mixed together. The precipitated phosphate was filtered out, while the clear filtrate was used as spraying reagent.
15. QUALITATIVE ESTIMATION OF ORGANIC ACIDS:

I) PURIFICATION OF THE EXTRACT FOR THE ESTIMATION OF ORGANIC ACIDS BY ION-EXCHANGE TECHNIQUE:

The above half extract contained various soluble compounds like organic acids, sugars, amino acids, amides and other water soluble compounds. Hence, a further purification of the extract was desired for the estimation of organic acids. This was achieved by ion exchange technique. The method was adopted as per Czapecz (1921) with slight modification.

Zeocart (ion exchange resin) was charged to H⁺ form by soaking in 6N hydrochloric acid for 2 hours, with occasional stirring. After decanting the acid, the resin was washed several times with distilled water. The resin was now ready for use.

Approximately 1 gm. of charged Zeocarb was added
and with occasional stirring it was kept for one hour to bring about complete adsorption of amino acids and sugars. The supernatant contained all the acids. The supernatant was then transferred quantitatively along with the washing and evaporated to dryness. The residue was dissolved in water to give a concentration of 1 ml. per gm. fresh weight of the tissue and then centrifuged for 30 minutes at 2000 r.p.m. The clear liquid was used for qualitative estimation of the organic acids by chromatographic method and their quantitative analysis by other specific methods described below.

To the Zeocarb left after the removal of the acids, 6N formic acid was added and kept for one hour with frequent stirring. It was then filtered. This removed all the absorbed substances and liberated the resin in activated form. The resin after being recharged with 6N hydrochloric acid, was again ready for use.
The effectiveness of the ion exchange technique was tested by

a) Spraying the acid chromatogram with specific reagent for detecting amino acids and sugars indicating their complete removal.

b) The formic acid fraction was evaporated to dryness, dissolved with water and a small fraction was chromatographed for organic acids, amino acids and sugars. This fraction was completely free of organic acids, while amino acids and sugars showed up on the chromatograms.

II) CHROMATOGRAPHIC SEPARATION OF ORGANIC ACIDS:

Since Consdon, Gordon and Martin (1944) first used paper partition chromatography for the separation of complex peptides and amino acids, the technique has been utilised and modified from time to time for the separation of other biological materials.
Now, paper partition chromatography has become the most useful and convenient method of separating the different components of a chemical series, on the filter paper. Like amino acids, the sugars were separated by Partridge (1948) and organic acids by Lugg et. al. (1947), using such technique in different solvent systems.

Organic acids of the leaf of two plants were initially separated by the two dimensional chromatographic technique. Whatman No.1 special chromatographic filter paper of size 28 x 28 cms was cut and a marker point for applying the material to be chromatographed, was spotted at a distance of 3 cm. from the two edges of the filter paper. The sample to be chromatographed (0.025 ml. of the final extract) was applied at the marker spot using Shandon's micropipette. These filter paper sheets were hung in a solvent - saturated chromatographic chamber, with their lower edges dipping in the solvent, ethanol, ammonia and water (90:5:5).
which was used as the first solvent. After about 15 to 20 hours run, the sheets were taken out and dried for 24 hours at room temperature.

The chromatograms were then developed in the second solvent, N-butanol, 6N formic acid and water (10:2:5) for 15 to 16 hours, at right angles to the direction of the first run. The sheets were first dried at room temperature for two days and later at 80° C for 30 to 40 minutes. These were then sprayed uniformly with bromophenol blue (0.04% in 90% alcohol). The organic acids appeared as lemon yellow spots on a pale blue background. The outline of the bands were marked. The organic acids of the extracts were identified by comparing their positions and $R_f$ values with those of reference solutions run on separate sheets simultaneously. However, since all the acids present in the leaves of two plants were found separable an unidimensional ascending chromatogram with N-butanol, formic acid and water
solvent, the later was used for subsequent analyses.

III) IODIDE - IODATE - STARCH REAGENT:

The Iodide - Iodate - Starch reagent adopted by T. Wood (1958) was also tried. It was prepared as follows:

a) Potassium iodide 2%, and Potassium iodate 4% in water - 1 Vol.

b) Starch 2% in water (containing chloroform as preservative) - 1 Vol.

Equal volumes of the two solutions were mixed and the paper was drawn rapidly through the viscous solution and laid on a clear sheet of blotting paper for drying. Acid regions produced dark blue spots at once or within a few minutes. Sensitivity of this method, ranges from 10 to 15 mg. for free acids.
IV) FERRIC CHLORIDE REAGENT:

Ferric chloride reagent was prepared by adding 1 ml. of 2N hydrochloric acid in a 2% solution of anhydrous ferric chloride. It was then diluted five times of it's volume with water. The paper was dipped, drained, blotted and laid flat on a clean blotting paper. The coloured spots were marked immediately as they fade with time.

V) NESSLER'S REAGENT:

Concentrated (B.D.H.) Nessler's reagent was used for the experimental purposes. The chromatographic paper was drawn rapidly through the reagent, otherwise with longer time concentrated alkali would disintegrate it. It was then laid on a sheet of blotting paper. The brown or yellow organic acid spots developed at once.
16. QUALITATIVE ESTIMATION OF KETO ACIDS:

I) EXTRACTION PROCEDURE FROM MATERIALS:

During recent years many workers like Ischer Wood and Niavis (1956), Fowden et al. (1954), Steward et al. (1954) etc., have estimated the keto acids present in various plant tissues. The methods have been critically evaluated by Fowden and Webb (1955). The extraction procedure was followed as adopted by Towers, Thompson and Steward (1954) and later by Patnaik and Das (1973). The procedure was as follows:

The plant parts such as leaves of the three different species each weighing 5 gms. were frozen at 10° C for about 24 hours. The frozen tissues were homogenized with the solvent mixture containing 120 ml. of 80% ethanol, 0.25 ml. of concentrated sulphuric acid and 5 mgs of 2-4 dinitrophenyl hydrazine. After an hour of standing at room temperature it was filtered. The
filtrate was evaporated at room temperature by passing a cold current of air through a hair drier over it.

The residue was extracted first with 20 ml. of ethyl acetate and further reextracted thrice with 5 ml. of aliquots of same solvent (i.e. ethyl acetate). The ethyl acetate extractions were combined and allowed to evaporate to a volume of 2-3 ml. by blowing cold air over it.

The combined ethyl acetate layer (containing the hydrazones and some uncombined hydrazines) were extracted successively with 2 ml. of 10% (W/V) sodium carbonate solution. The final volume was made upto 10 ml. by distilled water. The combined carbonate layers were reextracted thrice with 3 ml. of ethyl acetate to remove excess of free hydrazines. The ethyl acetate fractions were discarded. The carbonate solution was cooled and icecold concentrated hydrochloric acid
was added to neutralise it. The acidified solution was extracted successively four times with 20 ml. of aliquot of ethyl acetate. The extractions were combined and evaporated to dryness by blowing cold air over it. The hydrazones were taken in 20% ethanol to give concentration of 1 ml. / 1 gm. fresh weight basis.

Since keto acids were unstable compounds due to the presence of ketonic group, it was necessary to make them more stable compounds by converting them into their respective keto acid hydrazones by the above process.

II) **SEPARATION OF KETO ACIDS BY CHROMATOGRAPHIC METHOD**

Keto acid hydrazones extracted from the plant materials and synthetic keto acid (after converting to their respective hydrazones) were spotted on Whatman No.1 chromatography filter paper by the micropipette by taking 0.025 ml.
in each case. Several solvent systems were used to separate the hydrazones. In this investigation both ascending and descending chromatographic techniques were adopted. For descending chromatographic technique, the solvent system adopted was N-butanol saturated with ammonia (8:20 W/V). The ascending solvent systems were,

1) Isopropyl alcohol, water and ammonia (40:4:2).
2) Amyl alcohol, ethanol and water (9:1:4).

The sheet of paper was developed for about 20-30 hours at room temperature (30° C). Then the paper was dried for 12-15 hours at room temperature. The keto acid hydrazone spots were identified on the basis of their Rf values. But the best method that could be achieved to identify various keto acids in the absence of facilities for hydrogenation, was the colour of the spots obtained by spraying with 4% ethanolic sodium hydroxide solution (4 gms. of sodium hydroxide in 100 ml. of 10% ethanol.)
The colour produced with alkali by different keto acid hydrazones were noted down immediately after spraying with ethanolic sodium hydroxide solution, since they tend to fade away quickly. The colour developed for different keto acid hydrazone spots under ultraviolet lamp was also being noted. The Rf values of different keto acids present in the leaf of two plants during rainy and Summer seasons and the synthetic keto acids were tabulated (Table plate - XXI, XXII). The corresponding spots were identified by comparing with the Rf. values of synthetic hydrazones using different solvent systems as mentioned above, which was done by Patneik (1962) in her Ph.D. work.

Two solvent systems viz. (i) ethyl acetate, acetic acid and water (3:1:2) (ii) ether, formic acid and water (5:2:1) were used for the separation of ascorbic acid and it's related compounds.
Two sets of papers were developed in the above two solvent systems for 25-30 hours at room temperature (30°C). The developed keto acid hydrazone spots on one set of chromatogram in the above mentioned two solvent systems were identified under UV lamp (on the basis of their Rf. values and colour). The other set of two papers run in two different solvent systems were developed by keeping the papers in the ammoniacal silver nitrate solution and it was prepared as follows:

10 ml. of 0.2N silver nitrate was added to 10 ml. of 10% sodium hydroxide solution and then concentrated ammonium hydroxide was added drop by drop till the precipitate of silver oxide was dissolved.

The immediate development of yellowish and brown spots indicate the presence of ascorbic acid and its related compounds (See Table - XXI, XXII).
QUALITATIVE ESTIMATION OF AMINO ACIDS:

I) EXTRACTION OF AMINO ACIDS FROM PLANT TISSUES.

The extraction procedure was followed as described by Ranjan & Laloraya (1960) which has been further developed by Patnaik and Laloraya (1963). 10 gms. of leaves were taken separately for analysis. To these freshly gathered materials 70% hot ethanol was added immediately. The materials were then boiled separately over baths for 5-10 minutes and was grinded into a fine paste. To this paste 5-10 ml. of ethanol was added and again filtered. This filtrate was kept separately. This was done for about three times for complete recovery of the soluble amino acids from the materials. The successive filtrate was added to the original filtrate. (After filtration the residue was suspended in 6N Hcl in conical flask for further analysis of protein bound amino acids). The filtrate was taken in a basin and evaporated to dryness over water bath. The final residue
thus obtained after evaporation was dissolved in 5-10 ml. of 20% ethanol. To remove the extra proteins possibly present in solution, it was centrifuged for 30 minutes at 2000 r.p.m. The supernatant liquid was decanted off and kept separately for analysis for soluble amino acids. The residue which was previously suspended in 6N. HCl was autoclaved for 30 minutes at 18 lbs, pressure & then filtered. The residue left was rejected and the filtrate was evaporated to dryness in a basin over water bath. After complete evaporation, the residue was dissolved with 10 ml. of 20% ethanol and then centrifuged at 2000 r.p.m. The supernatant was kept for protein bound amino acids' analysis.

II) SEPARATION OF SOLUBLE AMINO ACIDS BY TWO DIMENTIONAL CHROMATOGRAPHIC METHOD.

The alcoholic soluble extract was made to volume (1 gm./ 1 ml. on fresh weight basis) in distilled water. The analysis of soluble amino acids was done by two
The separation of Amino acids and amides by two dimentional ascending chromatography was achieved as described by Ranjan and Leloraya (1960). The method may be precisely described as follows. 0.2 ml. of the extract was applied to the marker spot of 36 x 36 cms. Whatman No.1 special chromatographic filter paper. Phenol saturated with 0.5% (W/V) Ammonia was used as the first solvent. At 20-25° C it took 14-18 hours for the solvent to reach the extreme edge of the filter paper. The solvent front was marked. The paper was taken out and dried at room temperature (25° C) for 24-28 hours to remove the phenol. The second solvent, consisting of N-butanol : acetic acid : water (4:1:5). The dried sheet was run at right angle to the direction of the first run. The time required by the second solvent to reach the extreme edge of the paper was 12-16 hours. The chromatogram was removed and dried for 12-14 hours.
at 25°C. Then the dried filter paper was sprayed with the Ninhydrin reagent.

III) SEPARATION OF PROTEIN BOUND AMINO ACIDS.

Protein bound amino acids were assayed by hydrolyzing the alcohol in soluble residue with 6N HCl at 15 lbs pressure for 30 minutes. The hydrolysate dissolved to 1 ml. of distilled water per 1 gm. of fresh weight of the tissue as mentioned in case of soluble amino acids. 0.02 ml. of the extract was applied to the marker spot of 36 x 36 cms. Then developed the chromatogram as described in case of soluble amino acids separation by using two solvents. Then the papers were dried and sprayed with Ninhydrin and the amino acids spots were identified.

IV) PREPARATION OF THE SPRAYING REAGENT AND ITS APPLICATION.

0.1 (W/V) Ninhydrin prepared in N-butanol was
used as the spraying reagent for detection of the amino acid and amide spots. Precaution to be made that the location reagent was homogenously sprayed on the developed chromatogram. The filter paper sheet was allowed to dry at 25° C and then heated at 90° C in an oven for 10-20 minutes. Later they were exposed to atmosphere to absorb moisture resulting in further intensification of the spots.

**MINERAL ESTIMATION:**

The leaves from three different seasons were collected and dried. Then the dried leaves were powdered. One gram from each of these powdered leaf samples were taken for analysis and the mineral analysis was done by wet-oxidation of plant tissue method (Jackson - 1973). For quantitative analysis, flame emission photometer (Carlzeiss Germany) and atomic absorption spectrophotometer (Varian Techtron model AA - 1100) were used.
The estimation of minerals from the plant sample (leaf) is done by M.L. Jackson's Method (1967). It is done by two steps. I - Digestion, II - Estimation.

1) **DIGESTION:**

One gm. of dry wt. of the sample was powdered and put in the conical flask. To the flask 15 c.c. of Conc. Nitric Acid was added followed by one c.c. of Perchloric acid and left over night. The next day, Conical flask was put on hot plate for digestion and heated slowly. Brown fume came for some time & is followed by white fumes indicating complete digestion.

White residue was left in the conical flask. It was dissolved in a little amount of distilled water and poured into a 25 c.c. measuring flask. The volume in the measuring flask was made up to 25 c.c. by adding distilled water. Then it is filtered through whatman No.1 filter paper and made ready for estimation.
For preparing blank solution, a conical flask was taken. To it 15 c.c. of Conc. Nitric acid was added followed by one c.c. of perchloric acid without plant sample. It was heated on hot plate. Then its volume was made 25 c.c. filtered and the filtrate was collected in Blank Test tube.

II) ESTIMATION:

The estimation was made by taking O.D. in Atomic Absorption Spectrophotometer (Variant Techtron Model 1945, Australia).

For Zn, the O.D. is taken against blank reading at the wavelength of 213.9 nm.

For Copper (Cu) O.D. was taken against blank at 324.7 nm.

For Manganese (Mn) O.D. was taken against Blank at 279.5 nm.
For Iron (Fe), the filtrate was diluted 5 times and O.D. was taken against blank at 248.3 nm.

For Magnesium (Mg), the sample was diluted to 25 times and O.D. was taken against blank at 202.5 nm.

For Calcium (Ca), 4 c.c. of the above sample was taken. To it one c.c. of Etonium nitrate Sn(NO₃)₂ was added. The O.D. was taken again blank at 422.7 nm.

For Potassium (K), the estimation was made by using flame photometer (Emission Spectro Technique). The O.D. was taken at 766.5 nm.

For Phosphorous (P) 2 ml. of the sample aliquot was taken in 50 ml. measuring flask. To it 2 drops of 2 - 4 dinitrophenol was added. Then few drops of sodium carbonate (Na₂CO₃) was added till yellowish colour was discharged by adding few drops of Conc. sulphuric
acid. Then 2 c.c. of sulphate Molybdate was added and cooled. Blue colouration came. The O.D. was taken against blank in Spectro Photometre (Spectronic - 20 U.S.A.) at 660 nm.

From the O.Ds the amount of different minerals were calculated and its percentage were estimated.
MIXTURE OF SYNTHETIC AMINO ACIDS

PHENOL SATURATED WITH AMMONIA

BUTANOL : ACETIC ACID : WATER (4:5:1)

[Plate - 11]
SYNTHETIC AMINO ACIDS CALIBRATION

1. Leucine
2. Isoleucine
3. Tryptophane
4. Arginine
5. Histidine & Lysine
6. Hydroxyprotein
7. Alanine, ( Yellowish brown )
8. L - glutamic acid
10. & loa - Glycine & Serine
11. Brown (?)
12. Yellow (?)
13. xGlutamic acid
14. x
15. Cystine
16. Valine
17. Phenyl analine.
CASSIA TORAL.

- **CHLOROPHYLL vs MONTH.**
- **TOTAL SUGAR CONTENTS vs MONTH.**
- **REDUCING SUGAR CONTENTS vs Month.**
- **NON-REDUCING SUGAR CONTENTS vs Month.**
\( \text{IN BAUMINIA PURPUREA L.} \)

Ref.  0-0 → CHLOROPHYLL VS. MONTH.
     0-0 → TOTAL SUGAR CONTENT VS. MONTH.
     \( \Delta \Delta \) → REDUCING SUGAR CONTENT VS. MONTH.
     \( \blacklozenge \blacklozenge \) → NON-REDUCING SUGAR CONTENT VS. MONTH.

(Fig - 2)
CASSIA TORA • L

REF 1 - ○ ○ → CARBOHYDRATE CONTENT.
         □ □ → STARCH CONTENT.

(Fig - 3)
REF: O CARBOHYDRATE CONTENT OF B. PURPUREA.
VS MONTH.
@ STARCH CONTENT OF B. PURPUREA-L.
VS MONTH.

(Fig-4)
Fig. 5

Ref: 6-6 Ascorbic Acid vs Month for C. Tora L.
6-1 Ascorbic Acid vs Month for B. Purpurea L.
PROTEIN CONTENT OF C. TORAL. VS MONTH.

PROTEIN CONTENT OF B. PURPUREAL. VS MONTH.

AMINO ACID CONTENT OF C. TORAL. VS MONTH.

AMINO ACID CONTENT OF B. PURPUREAL. VS MONTH.

(Fig. 6)