MATERIALS AND METHODS

Different species of *Datura* i.e. *Datura inoxia*, *Datura ferox*, *Datura metel* and *Datura stramonium* were collected from the various localities of Marathwada. They were collected in the polythene bags and brought to the laboratory. They were sorted into root, stem, leaf, seeds and fruit coat. Apart from these sorted plant parts, some pieces were preserved for the anatomical work and remaining plant material were used for preparation of powder. The plant parts were dried in natural condition and after that it was dried in the oven at $60^\circ$C up to the constant weight. After the complete drying the plant parts were cut into small pieces and ground into fine powder, and it stored in the sealed container. The fine powder of the plant parts were used for the chemical analysis.

The preserved plant material as well as the fresh plant parts were taken for the study of anatomy in detail. The plant parts such as root, stem, leaf and petiole were selected for the anatomical study, for the anatomical study the sections were taken by using hand sectioning, the fine sections were selected and observed under microscope. Then the fine section were stained with double staining method, and permanent slides prepared for the detail study of anatomy with the help of microscope, the microphotographs were taken.

The plant powder were used for various qualitative and quantitative analysis were done. In qualitative analysis alkaloids, tannin, saponins, amino acids etc were analysed. In quantitative analysis total ash, acid soluble ash, acid insoluble ash, water soluble ash, water insoluble ash, calcium, phosphorus, sodium, potassium, nitrogen, crude protein, crude fat, crude fiber, gross energy, total sugar, reducing sugar, non reducing sugar, amino acids, amino nitrogen, phenol, tannin, alkaloids.

In addition to this the attempts were made to find out the chemical nature of the methanol extract of the various plant parts by the IR and GC-MS. The IR and GC-MS were done in the SATC, Department of Chemistry, University of Pune, for the chemical analysis in the extract by GC-MS technique.
The antifungal and antibacterial activity were observed from the aqueous extract of the plant parts of the *Datura*.

The *Datura* plant is having high medicinal properties, it is given detail in the chapter no. III.

**CHEMICAL ANALYSIS**

A) **Quantitative analysis**

**Nitrogen (N)**

The dry sample is digested with concentrated sulphuric acid (H\textsubscript{2}SO\textsubscript{4}) in the presence of catalyst. During the digestion, nitrogenous compounds are converted to ammonium sulphate ((NH\textsubscript{4})\textsubscript{2} SO\textsubscript{4}). It is then made strongly alkaline with sodium hydroxide (NaOH). The released ammonia (NH\textsubscript{3}) is distilled into boric acid (H\textsubscript{3}BO\textsubscript{3}) solution. The ammonium tetraborate formed is then titrated against 0.035 N hydrochloric acid for the determination of nitrogen (N).

**Reagents**

1) Concentrated H\textsubscript{2}SO\textsubscript{4}, AR grade, sp. gr. 1.85
2) Catalyst : A mixture of copper sulphate (CuSO\textsubscript{4}), potassium sulphate (K\textsubscript{2}SO\textsubscript{4}) and Selenium dioxide (SeO\textsubscript{2}) in a ratio 1: 9: 0.02.
3) NaOH solution (40%): Dissolve 400g NaOH in 1000 ml of distilled water.
4) Mixed indicator: Dissolve 300 mg bromocresol green and 200 mg methyl red in 95% ethyl alcohol. Make the volume to 500 ml with 95% ethyl alcohol.
5) Boric acid solution : 2% H\textsubscript{3}BO\textsubscript{3} solution is made by dissolving 10 g H\textsubscript{3}BO\textsubscript{3} in 480 ml of glass distilled water. To this about 5 ml mixed indicator is added and the volume is made to 500 ml.
6) 0.035 N HCl : 25 ml of concentrated HCl is taken in a volumetric flask and diluted upto 500 ml with distilled water. This serves as a stock solution. To determine normality of this solution, 1 g ammonium tetraborate ((NH\textsubscript{4})\textsubscript{3} BO\textsubscript{3}) is taken in 50 ml conical flask. To it 10 ml glass distilled water and 2-3 drops of mixed indicator are added. This is
titrated with the stock solution of HCl to calculate the normality using following equation:

\[
\text{Normality of HCl} = \frac{1000}{\text{titration value (ml)} \times 190.72}
\]

After determining the normality of stock solution, 0.035 N HCl is prepared by appropriate dilution.

**Procedure:**

1) **Digestion:** Transfer carefully, accurately weighed 300 mg of dry plant material in a Kjeldahls flask. Add a pinch of catalyst with the help of spatula. Slowly add 7.5 ml concentrated sulphuric acid (H\(_2\)SO\(_4\)). Heat the flasks gently on a digestion stand until the fumes of H\(_2\)SO\(_4\) are freely evolved. Increase heat until acid boils vigorously and digest till the mixture is clear, i.e. apple green in colour or colourless. During digestion care must be taken to avoid particles of indigested carbon sticking on the sides of the tube. Cool the contents of flask and use for the distillation. For this purpose the digested material is made upto a volume of 50 ml in volumetric flask with distilled water.

2) **Distillation:** This is usually carried out with the Markham's steam distillation apparatus. Heat the steam boiler to produce steam. Keep a 50 ml conical flask, containing 10 ml boric acid solution, at the delivery end of the condenser. Tip of the condenser should be just beneath the surface of H\(_3\)BO\(_3\) solution. Introduce 5 ml of previously diluted, digested sample into the distillation flask through funnel. Close the funnel with ground glass rod. Put 10 ml NaOH solution in the funnel and introduce it slowly into distillation flask. The ammonia formed due to the treatment of NaOH passes along with the steam and is absorbed by H\(_3\)BO\(_3\), at the condenser outlet to form ammonium tetraborate ((NH\(_4\))\(_3\)BO\(_4\)). This results into the change in colour of H\(_3\)BO\(_3\) solution from pink to green. Continue distillation till the
volume becomes to about 20 ml. Titrate the \((\text{NH}_4)_3\text{BO}_4\) with 0.035 N HCl till pink colour reappears and record titration value. Calculate strength of NH₃, in the distillate using equation:

\[
1 \text{ ml } 0.035 \text{ N HCl} = 0.5 \text{ mg of N}
\]

Calculate the amount of N for 50 ml of the sample, which will be equivalent to that present in 300 mg of dry plant material. Compute the N per cent in dry sample and record it as N % of dry matter (DM).

**CRUDE PROTEIN (CP)**

On an average, most of the proteins have 16 % nitrogen in their composition. Thus the amount of N content, when multiplied by 6.25, gives the crude protein (CP) content of the sample.

**CRUDE FAT (CFat)**

The fats present in the plant material are extracted in the solvent consisting of chloroform (CHCl₃) and methanol (CH₃OH). This is done in soxhlet extraction assembly and after complete evaporation of the solvent, the amount of extracted fat is measured.

**Reagents**

1) Solvent: Chloroform + methanol (2:1)

**Procedure:**

Weigh 2 g dry plant material and transfer it into a thimble prepared with Whatman filter paper No. 1. Plug the mouth of thimble with fat free absorbent cotton. Take clean, dry 250 ml receiver flask from the soxhlet assembly and add the solvent to it just to reach the level of the neck. Introduce the thimble with sample into the soxhlet. Assemble the apparatus and place it on heating mantal with temperature controlling device. Fit water condenser at the top of the soxhlet. Extract the fat for 8 hours at 60°C. When the extraction is over, remove the thimble from soxhlet. Assemble the apparatus again and heat to recover most of the solvent from the receiver flask. When the receiver flask contains about 25 ml solvent along with the extracted fat, disconnect the receiver flask. Transfer the solvent in a clean, previously weighed beaker with rinsing for 2 to
3 times. Evaporate the solvent completely and dry it in a hot air oven at 95°C, cool in a dessicator and weigh. Measure the amount of fat, extracted per 2 g of the sample, and calculate the amount of Cfat as percent of dry matter (DM).

**CRUDE FIBRE (CF)**

Crude fibre (CF) is determined as that fraction remaining after digestion with dilute solutions of sulphuric acid (H$_2$SO$_4$) and sodium hydroxide (NaOH) under carefully controlled conditions. The major part of it contain carbohydrates and it is a valuable parameter in deciding the nutritive quality of animal feed.

**Reagents:**

1) 1.25 % H$_2$SO$_4$: Dissolve 5 ml con. H$_2$SO$_4$ in 395 ml distilled water.

2) 2.5% NaOH: Dissolve 5 g NaOH in 100 ml distilled water and make the volume to 200 ml with distilled water.

3) 70% ethyl alcohol.

**Procedure:**

Transfer 2 g defatted sample to a 500 ml spoutless beaker and add 200 ml 1.25% H$_2$SO$_4$ to it. Break up the lumps with the help of glass rod having a rubber policeman. Cover the beaker with a conical flask, half filled with cold water, which serves as water condensor. Boil for 30 minutes and make up any loss in volume during the boiling with hot distilled water. Filter through Whatman filter paper No. 54 by washing the residue several times with hot distilled water. Take out the residue back in the beaker with 100 ml water and to it add 100 ml 2.5% NaOH. Boil for 30 minutes as earlier. Filter through previously weighed Whatman filter paper No. 54. Wash the residue several times with hot water and lastly with 70% alcohol. Dry it over night at 100°C to a constant weight. Cool and weigh. Incinerate the residue along with filter paper in a crucible at 600±20°C for 2 hours in a muffle furnace until all the carbonaceous matter is burnt. Cool the crucible in a desiccator and weigh. Record the loss in weight as crude fibre (CF) and calculate the amount of CF on DM basis.

**TOTAL ASH**

The residue after incineration of sample at 550-600°C is known as ash.
For this purpose the sample is subjected to a high temperature upto 600°C and then the ash content is determined. During ignition to such a high temperature all organic compounds decompose and pass off in the form of gases, while the mineral elements remain in the form of ash.

**Procedure:**

Take 2 g oven dry sample in a previously weighed vitrosil silica crucible. Heat it on hot plate for about 30 minutes, till the sample is sufficiently charred and turns black. Replace the lid of the crucible and keep it in muffle furnace. Allow the temperature to raise upto 600°C and keep it constant for 2 hours. Remove the crucible on cooling and transfer directly to desiccator, cool and weigh immediately. Find out the weight of ash, obtained per 2 g of sample, and calculate the ash content as per cent of dry matter (DM).

**WATER SOLUBLE ASH (WSA)**

The ash was boiled for 5 minutes with 25 ml of distilled water. Insoluble matter was collected in ashless filter paper and washed with hot water, ignited and weighed. Weight of the insoluble matter was subtracted from the weight of ash. The difference in weight represents the water soluble ash. Percentage of water soluble ash was calculated with reference to the air dried drug.

**WATER INSOLUBLE ASH**

The percentage of water insoluble ash was calculated by subtracting the value of the percentage of water-soluble ash from that of total ash.

**ACID INSOLUBLE ASH (AIA)**

**Reagents:**

1) 5 N Hydrochloric acid (HCl): Dilute 41.7 ml concentrated HC1 to 100 ml with distilled water.

**Procedure:**

Add 50 ml of 5N HC1 to the ash obtained in crucible as above. Heat the mixture for 30 minutes in hot water bath. Allow to cool and filter through Whatman filter paper No. 42. Wash the filter paper with water until the washings are free from acid. Dry the filter paper along with acid insoluble portion of ash in an oven at 100 °C overnight. Transfer it to desiccator and
weigh. Determine AIA per unit weight of the sample used for ashing and calculate it as per cent of dry matter.

The filtrate obtained during the determination of AIA, is collected and made to the volume upto 100 ml. This acid soluble portion of ash is stored for the determination of the minerals like calcium (Ca) and phosphorus (p).

**ACID SOLUBLE ASH (ASA)**

The percentage of acid soluble ash was calculated by subtracting the value of the percentage of acid insoluble ash from that of total ash.

**NITROGEN FREE EXTRACT (NFE) AND TOTAL CARBOHYDRATES (TC)**

Carbohydrate portion of biological material is made of two parts nitrogen free extract (NFE) and crude fibre (CF). NFE is also known as soluble carbohydrate, which consists of water soluble vitamins, monosaccharides (pentoses and hexoses), oligosaccharides (compound sugars) and polysaccharides (starches). Insoluble carbohydrate or CF contain mainly polysaccharides consisting of hemicellulose and cellulose. The CF content of material gives an indication of bulkiness of a material. These two parameters are calculated by difference. NFE is represented (on DM basis) by a figure obtained when the sum of ash, protein, Cfat and CF of a material is subtracted from 100.

\[ \% \text{ NFE} = 100 - (\% \text{ CP} + \% \text{ Cfat} + \% \text{ CF} + \% \text{ ash}) \]

TC is then determined as either

\[ \% \text{ TC} = \% \text{ NFE} + \% \text{ CF} \]

\[ \% \text{ TC} = 100 - (\% \text{ CP} + \% \text{ Cfat} + \% \text{ ash}) \]

Since the figures are determined by difference, instead directly, it may include cumulative errors of the other determinations.

**CALCIUM (Ca)**

Acid soluble ash fraction of the plant material is used for determination of calcium (Ca). For this purpose the Ca in an aliquat is precipitated as calcium oxalate. The precipitate is then dissolved in acid and the content of oxalate ions determined titrimetrically with potassium permanganate (KMnO$_4$).
Reagents:

1) Methyl red indicator: Dissolve 1 g methyl red in sufficient alcohol to make 1 litre solution.

2) Ammonium oxalate ((COO.NH₂). H₂O) solution: Dissolve 6 g of ammonium oxalate in sufficient distilled water to make 100 ml solution.

3) 2 N sulphuric acid (H₂SO₄): Dilute 5.6 ml concentrated H₂SO₄ (AR grade) to 100 ml with distilled water.

4) 0.01 N Potassium permanganate (KMnO₄): Dissolve 316 mg KMnO₄ in distilled water and dilute it to the volume of 1 litre. Keep the solution in glass stoppered bottle and store in dark.

Procedure:

An aliquat (25 ml) of the acid soluble ash portion is diluted to about 150 ml with distilled water. Few drops of methyl red are added and the mixture is neutralised with ammonia (NH₃) solution till the pink colour changes to yellow. The solution is heated to boiling and 10 ml ammonium oxalate solution is added. The mixture is allowed to boil for a few minutes. Glacial acetic acid is then added till distinctly pink colour reappears. The mixture is then kept aside for 12 to 24 hours at room temperature. When the precipitate of calcium oxalate settles down, it is filtered through Whatman filter paper No. 42. The precipitate is washed several times with water, to make it free from acid. It is then transferred in a small beaker by piercing a hole in the filter paper and by pouring over it about 15 ml 2 N H₂SO₄. This is heated to above 40°C and titrated against 0.01 N KMnO₄ solution until the first drop, which gives the solution a pink colouration persisting for at least 30 seconds.

The amount of Ca is calculated using an equation:

1 ml of KMnO₄ = 0.2004 mg of Ca

The per cent Ca on DM basis is then calculated on the basis of the amount of sample used for preparing / estimation ash, the volume to which acid solution of ash is diluted and the volume of the aliquat taken for the precipitation of calcium.
PHOSPHORUS (P)

The acid soluble portion of ash is diluted and treated with molybdate solution. The phosphomolybdic acid formed is then reduced by the addition of 1, 2, 4 - Aminonephthol sulfonic acid (ANSA) reagent which produces blue colour. The intensity of the colour, which is proportional to the amount of phosphorus present, is measured using colorimeter.

Reagents:

1) 10 N H\textsubscript{2}SO\textsubscript{4}: Carefully add 200 ml concentrated H\textsubscript{2}SO\textsubscript{4} (36 N) to 520 ml of distilled water.

2) Molybdate solution: Dissolve 25 g of ammonium molybdate in 20 ml. of distilled water. Transfer it to a volumetric flask containing 500 ml. of 10 N H\textsubscript{2}SO\textsubscript{4} and bring the final volume to 1 litre using more distilled water. Mix well and store in brown bottle.

3) Aminonaphtholsulfonic acid (ANSA) reagent: (a) 15% sodium bisulphite (NaHSO\textsubscript{3}): Take 30 g reagent grade NaHSO\textsubscript{3} in a beaker. Add 200 ml of distilled water and stir to dissolve, (b) 20% sodium sulphite (Na\textsubscript{2}SO\textsubscript{3}): Dissolve 20 g of reagent grade anhydrous Na\textsubscript{2}SO\textsubscript{3} in distilled water and dilute to 100 ml. Filter if necessary, (c) ANSA reagent: Take 195 ml of 15% NaHSO\textsubscript{3}, solution in a beaker. Add 500 mg of 1, 2, 4 - aminonaphtholsulfonic acid, and mix thoroughly. To this add 5 ml of 20% Na\textsubscript{2}SO\textsubscript{3} followed by thorough mixing. If the solution is not complete, add more Na\textsubscript{2}SO\textsubscript{3}, 1 ml at a time, with shaking but avoid in excess. Transfer this ANSA reagent to a brown-glass bottle and store in cold.

4) Standard phosphorus (P) solution: Dissolve exactly 351 mg pure dry monopotassium phosphate (KH\textsubscript{2}PO\textsubscript{4}) in 500 ml of distilled water and transfer to a 1 litre volumetric flask. Add 10 ml of 10 N H\textsubscript{2}SO\textsubscript{4}, dilute to the mark with water and mix. Five ml of this solution contains 0.4 mg phosphorus.

Procedure:
Take 0.5 ml acid soluble portion of ash in a test tube (the amount of this may be modified depending on the phosphorus content). Dilute it to a volume of 10 ml with distilled water. Simultaneously take a blank containing only 10 ml distilled water. Add 1 ml molybdate solution to each test tube and mix, then add 0.4 ml ANSA reagent and again mix. Allow to stand for 5 minutes and read the optical density (O.D.) at 660 mµ using colorimeter by setting it to zero with the blank.

Establish the O. D. of standard phosphorus solution by preparing a standard graph containing 0 to 1 ml standard phosphorus solutions in series of test tubes. Determine the amount of phosphorus in an aliquat with the help of standard graph and calculate the phosphorus content in the plant sample considering its amount taken for ashing, volume of the acid soluble ash and amount of aliquat used for the reaction.

**GROSS ENERGY (GE)**

The determination of gross energy (GE) of feed and food products is a technique frequently employed in nutritional investigations. A method described below for the determination of GE employ the oxidation of sample with a solution of potassium dicromate (K₂Cr₂O₇) in H₂SO₄. Energy value is obtained by dividing the amount of 1.5 N K₂Cr₂O₇ required to oxidise 1 g of material by a factor depending on the protein content. This technique gives the results in good agreement with those obtained by Bomb calorimetry.

**Reagents**:

1) **1.5 N K₂Cr₂O₇**: Dissolve 73.5 g K₂Cr₂O₇ in distilled water and make the volume to 1 litre.

2) **0.15 N sodium thiosulphate (Na₂S₂O₃)** solution: Dissolve 37.5 g Na₂S₂O₃ in water and dilute it to 1 litre.

3) **Potassium iodide (KI)** solution: 100 g of KI and 32 g of sodium bicarbonate (NaHCO₃) are dissolved in distilled water and diluted to 500 ml.

**Procedure:**
Introduce exactly 50 mg dry sample conical flask of 250 ml capacity. Add 8 ml of 1.5 N K$_2$Cr$_2$O$_7$ followed by 16 ml concentrated H$_2$SO$_4$. Simultaneously prepare a blank for each set. Mix well the contents of the flask and set aside for 90 minutes with intermittent shaking. Dilute the oxidised solution with distilled water, cool and make upto 100 ml.

Withdraw a 10 ml aliquot from each flask and to it add 4 ml of KI solution. Store in dark for 30 minutes, dilute with 20 ml distilled water and titrate the liberated iodine with 0.15 N Na$_2$S$_2$O$_3$ solution using starch as an indicator. The excess dicromate present is calculated from the titration figure and subtracted from blank value to obtain the quantity of 1.5 N K$_2$Cr$_2$O$_7$ used in the oxidation.

Determine the amount of 1.5 N K$_2$Cr$_2$O$_7$ required for oxidation of 1 g sample and calculate the GE in KCal per g of sample using following equation:

$$\text{GE (Kcal/g DM)} = \frac{\text{ml 1.5 N K}_2\text{Cr}_2\text{O}_7 \text{used to oxidise 1 g sample}}{(23.39 - 0.069 P + 0.000226 P^2)}$$

Where P is the crude protein (CP) content in the sample expressed as per cent of dry matter (DM).

**POTASSIUM (K)**

The acid soluble portion of ash was diluted and feed to flame photometer atomizer.

**Chemicals:**

1) 10 mEq/litre (1 mEq/litre = 39 ppm).

Dissolve 0.746 gms of pure dry KC1 in a litre of glass distilled water,

2) 200 mEq/litre (1 mEq/litre - 23 ppm) NSL. Dissolve 11.69 gms of pure dry NaCl in a litre of glass distilled water.

**Procedure:**

Take 1 ml. of acid soluble portion, of ash in a measuring cylinder. Dilute it to a volume of 25 ml with distilled water. Simultaneously feed distilled water to atomizer and adjust the set F.S. control Aspirate the standard mixed solution
1.7/0.8 mEq per litre on Na/K solution and wait at least for 30 sec. Adjust set F.S. Control of Na side for a read out of 170 and that at the K-side for read out of 80. Repeat steps 4, 5, 6 and 7 (Flame photometer manual modi 127) until the reading are stabilized the unit now stands calibrated. The pressure is 0 to 10 mEq/l and power 230 V + 10% 50 Hz to be maintained. Now feed sample solution to the atomizer to get the relative concentration wait at least for 30 sec before taking the next reading.

Establish the reading of standard stock solution of potassium by preparing a standard graph containing 0.01 to 0.08 ml standard potassium solution in series of reading.

Determine the amount of potassium on aliquot with the help of standard graph and calculate the potassium content in plant sample considering its amount taken for ashing volume of the acid soluble ash and amount of aliquot used for the reaction.

**TOTAL CARBOHYDRATES**

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into more simpler sugars. The carbohydrate content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

**Reagents:**

1) 2.5 N HCl
2) Anthrone Reagent: Dissolve 200 mg anthrone in 100 ml of ice cold 95% H$_2$SO$_4$, Prepare fresh before use.
3) Standard Glucose: Stock solution – Dissolve 100 mg glucose in 100 ml distilled water. Working standard – Dilute 10 ml of stock solution to 100 ml with distilled water. Store in refrigerator after adding a few drops of toluene.

**Procedure**

1) Weigh 100 mg of the sample into a boiling tube.
2) Hydrolyse by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cool to room temperature.
3) Neutralise it with solid sodium carbonate until the effervescence ceases.
4) Make up the volume to 100 ml and centrifuge.
5) Collect the supernatant and take 0.5 ml aliquat for analysis.
6) Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. '0' serves as blank.
7) Make up the Volume to 1 ml in all the tubes including the sample tubes by adding distilled water.
8) Then add 4 ml of anthrone reagent.
9) Heat for eight minutes in a boiling water bath.
10) Cool rapidly and read the green to dark green colour at 630 nm.
11) Draw a standard graph by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis.
12) From the graph calculate the amount of carbohydrate present in the sample tube.

**Calculation**

Amount of carbohydrate present in 100 mg of the sample

\[
\text{mg of glucose} = \frac{\text{Calculation}}{\text{Volume of test sample}} \times 100 \text{ (mg/100 mg)}
\]

**CELLULOSE**

Cellulose, a major structural polysaccharide in plants, is the most abundant organic compound in nature, and is composed of glucose units joined together in the form of the repeating units of the disaccharide cellobiose with numerous cross linkages. It is also a major component of cell wall.

**Reagents:**

1) Acetic Nitric Reagent: Mix 150 ml of 80% acetic acid and 15 ml of concentrated nitric acid.
2) Anthrone Reagent: Dissolve 200 mg anthrone in 100 ml concentrated sulphuric acid. Prepare fresh and chill for 2 hours before use.

3) 67 % sulphuric acid

Procedure

1) Add 3 ml acetic/nitric reagent to a known amount (0.5 or 1 gm) of the sample in a test tube and mix in a vortex mixer.
2) Place the tube in a waterbath at 100°C for 30 min,
3) Cool and then centrifuge the contents for 15-20 min.
4) Discard the supernatant.
5) Wash the residue with distilled water.
6) Add 10 ml of 67% sulphuric acid and allow it to stand for 1 hr.
7) Dilute 1 ml of the above solution to 100 ml with water
8) To 1 ml of this diluted solution, add 10 ml of anthrone reagent and mix well
9) Heat the tubes in a boiling water bath for 10min,
10) Cool and measure the colour at 630nm.
11) Set a blank with anthrone reagent and distilled water.
12) Take 100 mg cellulose in a test tube and proceed from step No. 6 for standard. Instead of just taking 1 ml of the diluted solution (Step 7) take a series of volumes (say 0.4 to 2 ml corresponding to 40-200 µg of cellulose) and develop the colour.

Calculation

Draw the standard graph and calculate the amount of cellulose in the sample.

REDUCING SUGAR (RS)

The majority of method for the determination of glucose are based upon the ability of glucose in hot alkaline solution to reduce certain metallic ions of which the cupric and ferric cyanide ions are most commonly used. The following method was used for estimating water-soluble reducing sugars.
Procedure:

Transfer 2ml of the sample extract to a folin-Wu-sugar tube graduated at 25 ml and to other similar tubes add 2 ml of standard sugar solutions containing 0.2 to 0.4 mg of glucose respectively. To each tube add 2 ml of the alkaline copper solution. The surface of mixture must now have reached the constricted part of the tube. Transfer the tubes to rapidly boiling water bath and heat for 8 minutes. Cool in running water without shaking. To each tube add 2 ml of phosphomolybdic acid reagent. After about 1 minute dilute to the mark with water and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue colour is formed in the bulb of the tube. Transfer the solution to suitable container and determine the O.D. at 420 µm, setting the photometer to zero density with a blank obtained by treating 2ml of water with alkaline copper reagent heating etc. Just as in the analysis of the sample filtrate.

Reagents:

1) **Standard sugar solution:**

These standard sugar solution should be in hand (a) a stock solution -1 per cent glucose made up in saturated benzoic acid solution (b) a solution containing 2 mg of sugar in 1 ml (20 ml of stock solution diluted 100 ml with water) (c) solution containing 0.2 and 0.4 of sugar in 2 ml made by dilution of (b) with water. The dilute standards are best made up fresh a couple of times a week, Merck’s highest purified dextrose is satisfactory.

2) **Alkaline Copper Solution:**

Dissolve 40 g of pure anhydrous sodium carbonate in about 400 ml of water and transfer to a litre flask, add 7.5 g of tartaric acid and when the latter has dissolved add 4.5 gm of crystallised copper sulphate. Mix and make up to a volume of 1 litre, if the chemicals used are not pure a sediment of cuprous oxides may form in the course of 1 or 2 weeks.

If this solution happen remove the supernatant reagent with a siphon or filter through a good quality filter paper. The reagent seems to keep indefinitely.

To test for the absence of cuprous copper in the solution the deep blue colour of
the copper should almost completely vanish. In order to forestall all improper use of this reagent attention should be called to the fact that it contains extremely little alkali, 2 ml by filtration (using the falling of the blue copper filtration colour as indicator) requiring only about 1.4 ml of normal acid.

3) **Phosphomolybdic acid solution:**

    To 35 gm of molybdic acid and 5 gm of sodium tungstate add 200 ml of 10 per cent sodium hydroxide and 200 ml of water. Boil vigorously for 20 to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid. Cool, dilute to about 350 ml and add 125 ml of concentrated (85%) phosphoric acid. Dilute to 500 ml.

    For higher values of percentage glucose or with deeper cuvettes carry out the analysis using less filtrate plus water to 2 ml and correct the calculations accordingly.

**TOTAL SUGAR**

    For total sugar 50 ml of the sample extract was acid hydrolised by boiling with 5 ml 1 N HCL cooled and then 5 ml 1 N NaOH added and followed the procedure of Reducing sugar.

**NON REDUCING SUGAR**

    It can be obtained from a simple calculation, it was as follows:

    \[
    \text{\% of Total sugar} - \text{\% of Reducing sugar} = \text{\% of Non Reducing Sugar}
    \]

**TOTAL FREE AMINO ACID**

    The amino acids are colourless ionic compounds that form the basic building block of proteins. Apart from being bound as proteins, amino acids also exist in the free form in many tissues and are known as free amino acids. They are mostly water soluble in nature. Very often in plants during disease conditions the free amino acid composition exhibits a change and hence, the measurement of the total free amino acids gives the physiological and health status of the plant.

**Materials:**
1) Ninhydrin: Dissolve 0.8gm stannous chloride (SnCl$_2$.2H$_2$O) in 500 ml of 0.2M citrate buffer (pH 5.0). Add this solution to 20 g of ninhydrin in 500 ml of methyl cellosolve (2 methoxyethanol).

2) 0.2M citric buffer pH 5.0.

3) Diluent solvent: Mix equal volume of water and n-propanol and use.

**Procedure:**

**Extraction of Amino acids:**

Weight 500 mg of the plant and grind it in a pestle and mortar with a small quantity of acid-washed sand. To this homogenate, add 5 to 10ml of 80% ethanol. Filter or centrifuge. Save the filtrate or the supernatant. Repeat the extraction twice with the residue and pool all the supernatants. Reduce the volume if needed by the evaporation and use the extract for the qualitative estimation of total free amino acids. If the tissue is tough, use boiling 80% ethanol extraction.

**Estimation:**

1) To 0.1 ml of extract, add 1 ml of ninhydrin solution.

2) Make the volume to 2 ml with distilled water.

3) Heat the tube in a boiling water bath for 20 min.

4) Add 5 ml of the diluents and mix the contents.

5) After 15 min. read the intensity of the purple colour against a reagent blank in a colorimeter at 570 nm. The colour is stable for 1h.

6) Prepare the reagent blank as above by taking 0.1 ml of 80% ethanol instead of the extract.

**Standard:**

Dissolve 50 mg leucine in 50 ml of distilled water in a volumetric flask. Take 10 ml of this stock standard and dilute to 100 ml in another volumetric flask for working standard solution. A series of volume from 0.1 to 1 ml of this standard solution gives a concentration range 10µg to 100µg. proceed as that of the sample and read the colour.

**Result:**
Draw a standard curve using absorbance various concentration. Find out the concentration of the total free amino acids in the sample and express as percent equivalent to leucine.

**AMINO NITROGEN**

Estimate the total free amino acid content and multiply the percentage equivalent of leucine with 14/131 to get the percentage of amino nitrogen.

**TANNINS**

Tannins and tannin-like substances are widespread in nature and are probably present in all plant materials. Those are polyphenolic compounds divided into two main groups—hydrolysable and condensed.

Hydrolysable tannins contain a polyhydric alcohol usually, if not always, glucose esterified with gallic acid or with hexahydroxydiphenic acid. Condensed tannins are mostly flavonols and are probably polymers of flavan 3-ol (catechin) and these cannot be hydrolyzed to simple components.

Among the cereals, sorghum has been found to contain higher amounts of polyphenols. Even though high polyphenol seeds are immune to attack by birds and diseases, they display impaired nutritional quality, lower digestibility and reduction of food consumption.

The tannins are estimated by Folin-Denis Method: This is based on the non-stoichiometric oxidation of the molecules containing a phenolic hydroxyl group.

**Folin-Denis Method**

**Reagents:**

1) Folin-Denis Reagent
   Dissolve 100 g sodium tungstate and 20 g phosphomolybdic acid in 7 ml distilled water in a suitable flask and add 50 ml phosphoric acid. Reflux the mixture for 2 hours and make up to one litre with water. Protect the reagent from exposure to light.

2) Sodium Carbonate Solution
   Dissolve 350g sodium carbonate in one litre of water at 70-80°C. Filter, through glasswool after allowing it to stand overnight.
3) Standard Tannic Acid Solution
   Dissolve 100 mg tannic acid in 100 ml of distilled water.

4) Working Standard Solution
   Dilute 5 ml of the stock solution to 100 ml with distilled water. One ml contains 50µg tannic acid.

**Procedure**

1) Extraction of Tannin: Weigh 0.5g of the powdered material and transfer to a 250 ml conical flask. Add 75 ml water. Heat the flask gently and boil for 30 min. Centrifuge at 2,000 rpm for 20 min and collect the supernatant in 100 ml volumetric flask and make up the volume.

2) Transfer 1 ml of the sample extract to a 100 ml volumetric flask containing 75 ml water.

3) Add 5 ml of Folin-Denis reagent, 10 ml of sodium carbonate solution and dilute to 100 ml with water.

4) Shake well read the absorbance at 700 nm after 30 min.

5) If absorbance is greater than 0.7, make a 1 + 4 dilution of the sample.

6) Prepare a blank with water instead of the sample.

7) Prepare a standard graph by using 0-100 µg tannic acid.

**PHENOLS**

Phenols, the aromatic compounds with hydroxyl groups are widespread in plant kingdom. They occur in all parts of plants. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amount of polyphenols are resistance to bird attack. Phenols include an array of compounds like tannins, flavonols etc. Total phenol estimation can be carried out with the Folin-Ciocalteau reagent.

**Material:**

1) 80% Ethanol

2) Folin-Ciocalteau reagent

3) Na₂CO₃ 20%

4) Standard (100 mg catechol in 100 ml water)
   Dilute 10 times for a working standard.
Procedure:

1) Weight exactly 0.5 to 1.0 g of the sample and grind it with a pestle and mortar in 10 time volume of 80% ethanol.
2) Centrifuge the homogenate at 10,000 rpm for 20 min. save the supernatant. Re-extract the residue with five times the volume of 80% ethanol, centrifuge and pool the supernatants.
3) Evaporate the supernatant to dryness.
4) Dissolve the residue in a known volume of distilled water (5ml).
5) Pipette out different aliquots (0.2 to 2ml) into test tubes.
6) Make up the volume in each tube to 3 ml with water.
7) Add 0.5 ml of folin-Ciocalteau reagent.
8) After 3 min, add 2 ml of 20% Na₂CO₃ solution to each tube.
9) Mix thoroughly. Place the tube in a boiling water for exactly one min, cool and measure the absorbance at 650 nm against a reagent blank.
10) Prepare a standard curve using different concentration of catechol.

Calculation:

From the standard curve find out the concentration of phenols in the test sample and express as mg phenols/100g material.

ASSAY FOR TOTAL ALKALOIDS

Each sample was ground to fine powder. To each one gram powder, 0.75ml 25% ammonium hydroxide, 1ml 95% ethyl alcohol and 2 ml of ethyl ether were added. The material was allowed to stand for 12 hrs. and dried.

The dried material was extracted with chloroform for 24 hrs. in a soxhlet apparatus and the extract obtained was evaporated to dryness and the residue was redissolved in chloroform. This solution was allowed to evaporate under infrared heat and the residue was mixed with 2.5 ml 0.1N ethanolic (90%) HCl. The extract, thus obtained was centrifuged, take supernatant and discard the pellet. The solution was evaporate and total alkaloids were weighted after drying at 100°C.

QUALITATIVE ANALYSIS

TANNINS
Tannins are polyphenols of high molecular weight which have the property of combining with protein, forming water insoluble and non-putrescible leather. Based on their reaction with mineral acids two main types of tannins are recognized, the condensed tannins and the hydrolysable tannins. The condensed tannins, which polymerize on hydrolysis, universally occur in Ferns and Gymnosperms and are widespread among the woody angiosperms. In contrast, hydrolysable tannins, which get broken up to simpler units on acid treatment, are limited to dicotyledonous plants and are found in a relatively few families. Tannins are correlated well with other primitive characters and thus the presence of these compounds is considered primitive. Between the two groups, the hydrolysable tannins are advanced. The highly advanced, herbaceous taxa are generally devoid of these compounds.

Condensed tannins or flavolans can be regarded as being formed by the condensation of catechin or gallocatechin molecules and flavon-3, 4-diols to form dimers and higher oligomers with carbon-carbon bonds linking one flavan unit to the next by 4-8 or 6-8 linkage. The name proanthocyanidins is used alternately for condensed tannins because, on treatment with hot acids, some of the carbon-carbon linking bonds are broken and anthocyanidins are released. This property is used for the detection of condensed tannins. Hydrolysable tannins are mostly gallotannins and ellagitannins depending on whether gallic acid or ellagic acid is present esterified with glucose. They yield the corresponding phenolic acid and glucose on hydrolysis.

**SAPONINS**

Saponins are glycosides which form emulsions with water and possess marked haemolytic properties. They possess steroidal or triterpenoid aglycones. The steroidal saponins are common in monocots, while the triterpenoid saponins are found in dicots. Their taxonomic value is less at higher levels of hierarchy although they may be used as useful chemical characters at lower levels.

**IRIDOIDS**
Irodoids are a group of monoterpenoid glycosides present in a number of dicotyledons. The presence of those compounds in a taxon is considered by many (Hegnauer, 1971; Bate-Smith and Swain, 1966; Jensen et al., 1975) to be a valuable phylogenetically significant chemical character.

**ALKALOIDS**

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen containing heterocyclic ring system and high pharmacological activity, often used as a criterion in classification of only those groups of plants which contain them. The presence of various types of alkaloids are used effectively in classifying various taxa (Manske, 1944; Gibbs, 1974; Daniel and Sabnis, 1979).

Alkaloids, as a rule are insoluble in water but soluble in organic solvents. But their salts are soluble in water and insoluble in organic solvents. Alkaloids are normally extracted from plants into weakly acids (1N HCl or 10% acetic acid) or acidic alcoholic solvents and are then precipitated with concentrated ammonia. They are also extracted into any organic solvent after treating plant materials with a base. The bases free the alkaloids and make them soluble in organic solvents. From the organic solvents, the alkaloids are extracted into acidic solutions and tested with specific reagents.

**AMINO ACID (By TLC method)**

Different types of amino acids were identified by the thin layer chromatography (Sadashivam and Manickam, 1992).