Chapter II
MATERIALS AND METHODS

A) Literature survey:
A critical survey of concerned literature has been made on Genus *Uraria* of the world in general and India in particular, in the beginning of the proposed work during 2005 and then updated in consecutive years. Detailed information on *Uraria* in Maharashtra, their distribution, status, diagnostic features etc. was collected through referring systematic account of the Genus in various districts, state and National Floras and research publications. A critical note on each species was prepared by consulting literature and herbarium at Botanical Survey of India, Pune and Herbarium Department of Botany, Dr.Babasaheb Ambedkar Marathwada University, Aurangabad.

B) Field Work:
On the basis of Literature and herbarium information, several field trips (more than 100) of short and long duration were undertaken to various localities in Maharashtra as well as to some parts of Kerala & Karnataka. Intensive trips were organized to collect rare species from their localities of occurrence. Attempts have been made to study phenology of all the species collected through extensive field studies. Critical field observations on each member collected have been made in the field itself. All the species collected have been processed for herbarium and deposited in the Herbarium of the Department. The members have been photographed in their natural habitats with Nikon film and Digital Cameras. Pickled material viz. stem, leaves flowers and fruits have been preserved in the laboratory in 70 % alcohol or FAA for further detailed studies.
<table>
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<tr>
<th>Sr.No.</th>
<th>Plant Name</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Altitude</th>
<th>Collect. Date</th>
<th>Field No.</th>
<th>Locality</th>
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C) Taxonomic study:

1. Study of general morphology i.e. life form of collected species.

During filed visits, observations were made on life form, distribution, present field status, ecology, phenology, variations of species in their natural habitat. Different kinds of life forms such as herbs, shrubs, woody, have been studied. The *Uraria* collected in the field were identified, studied and the specimens were processed for herbarium by using usual methodology (Foreman, 1992). Detailed distributional records, field observations and notes were made on the species in their localities of occurrence.

2. Study of Floral morphology: including vexilum, keel and karina

Fresh and prickled plant materials and flowers collected were studied in the laboratory under the stereomicroscope ZEISS (Stemi SV 11 Apo) and photographed with digital camera Nikon coolpix 4500. The dissected floral parts have also been studied under light microscope and simple dissecting microscopes, stereomicroscope and photographed. Careful observations on flowers and floral peculiarities, anomalies were made and noted. In all collected species the nature of flowers, Vexilum keel, & carina has been studied.

COMPARATIVE MORPHOLOGY

The 03 taxa of *Uraria* Desv. were collected from different localities of Maharashtra, state of India. (Table No. 01). The plant parts root, stem, leaves, flowers, pod were collected using secateurs and cutlass. The plant specimens collected were pressed using a plant press, using blotting paper and news paper. The plant press was tightened using the straps. The objectives of pressing plant is to extract moisture in the shortest period of time, while preserving the morphological integrity of the plant and to yield material that can be mounted on herbarium sheet for long time storage.

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The specimens were pressed immediately upon collection for best quality specimens. The dried specimens were poisoned to prevent fungal and insect attack using a mixture containing 5 L Methylate spirit, 4-6 drops of phenol and 200 ml of mercuric (II) chloride. Some of the specimens were poisoned by dipping whole plant into plastic tray containing the poison and other were poisoned by using a brush on both the Adaxial and Abaxial side (Bridson and Forman 1992).

Identification and authenticication of the species were done at Herbarium of Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (Acronym BAMU). The comparative morphological studies were based on 33 morphological parameters (Table no. 02). The samples were collected from the medium sized authentically identified plant species from different localities of Maharashtra. The roots, stems and leaves were removed carefully by cutter pricking without damaging the plants. The materials were collected in polythene bags and brought to the laboratory within 2-5 hours. Some parts were preserved in 70% alcohol for their dermatology and anatomical work, vessels etc. These were initially dried in shade and later in oven at 60°C till constant weight, then made into fine powder and stored in sealed plastic container for further analysis.

The morphological characters of the plant species selected were studied in detail and herbarium sheets are preserved in the Herbarium of Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Fresh and dried parts were studied morphologically in the field as well as in the laboratory regarding their colour, texture etc. Various characters of plants like anatomy, dermatology, leaf constants, were studied. Procedures for these studies are given below.
ANATOMY OF ROOT, STEM, LEAF AND PETIOLE: -

The anatomical characters of the roots, stems, leaves and petiole were studied with the help of free hand transverse sections taken with blades. Sections were dehydrated with different alcohol grades and stained with safranin and light green. These permanent preparations were observed under microscope and photographed.

DERMATOLOGY OF LEAVES:

i) Trichomes:

Trichomes are outgrowths of epidermal cells. (Roy, 2006). For studies of trichomes following procedure was adopted-

1. Scrap the trichomes from leaf surfaces with the help of razor.
2. Stain trichomes in safranin and mount in glycerin on a slide.
3. Observe slide under microscope and mention the type of trichome.
4. Take the dimensions with the help of ocular micrometer.
5. Draw the diagrams with the help of camera lucida at stage level.
6. Determine the observations for upper and lower epidermis separately.

ii) Stomata:

Stomata are microscopic pores on the epidermal surface of aerial parts of higher plants formed by a pairs of specialized epidermal cell termed guard cells, which control opening and closing of the pore by changing their turgidity and thus regulates the gaseous exchange between plants and environment. (Roy, 2006).
For studying stomata following procedure was adopted-

1. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide and mount in glycerin water.

2. Arrange a camera lucida and drawing board for making the drawings to scale.

3. Place the slide with epidermal peel on the stage. Trace the epidermal cells and stomata.

4. Mention the type of stoma and occurrence of stoma (amphistomatic / epistomatic / hypostomatic).

5. Measure the length of stoma, dimension of guard cell and dimension of subsidiary cell with the help of ocular micrometer.

6. Determine the values for upper and lower epidermis separately.

7. Record the result for ten fields and calculate the average number of stomata per square mm.

**DETERMINATION OF STOMATAL NUMBER:**

**Definition:** It is average number of stomata per square mm of the leaf.

**Procedure:**

1. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide and mount in glycerin water.

2. Draw a diagram with camera lucida by drawing a square of 1 mm with help of stage micrometer.

3. Place the slide with epidermal peel on the stage.
4. Count the stomata present in the area of 1 mm square. Include the cell if at least half of its area comes within the square.

5. Record the result for each of the ten fields and calculate the average number of stomata per square mm.

**DETERMINATION OF STOMATAL INDEX:**

**Definition:** Stomatal index is the percentage, which the number of stomata forms to the total number of cells, each stoma being calculated as one cell.

Stomatal index can be calculated by using following equation.

$$ I = \frac{S}{E + S} \times 100 $$

$I=$ Stomatal index, $S=$ No. of stomata per unit area, $E=$ No. of epidermal cells in the same unit area.

**Procedure:**

1. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide, mount in glycerin water and observe under microscope.

2. Draw the diagram with help of camera lucida by drawing square of 1 mm by stage micrometer.

3. Count the number of stomata, also the number of epidermal cells in each field.

4. Calculate the stomatal index using above formula.

5. Determine the values for upper and lower epidermis separately.
6. Record the result for each of the ten fields and calculate the average stomatal index for both epidermises separately.

**DETERMINATION OF VEIN-ISLET NUMBER:-**

**Definition:** A vein-islet is the small area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islets per square millimeter of a leaf surface. It is determined by counting the number of vein-islets in an area of one square millimeter of the central part of leaf between the midrib and margin.

**Procedure:**

1. Clear a piece of leaf with 10% KOH solution.

2. Draw a square of 1 mm by stage micrometer.

3. Place the slide with cleared piece of leaf on the stage.

4. Trace the veins, which are included within the square with the help of camera lucida and photographed also.

5. Count the number of vein-islets in the square millimeter. Where the islets are intersected by the square, include those on two adjacent sides and exclude those islets on other sides.

6. Record the result for each of the ten fields and calculate the average number of vein-islets in an area of one square millimeter.
DETERMINATION OF VEIN TERMINATION NUMBER:

Definition: Veinlet termination number is defined as the number of Veinlet terminations per square millimeter of leaf surface midway between the mid rib and margin.

Procedure:
1. Clear a piece of leaf with 10% KOH solution.
2. Draw a square of 1 mm by stage micrometer.
3. Place the slide with cleared piece of leaf on the stage.
4. Trace the veins, which are included within the square with the help of camera Lucida and photographed also.
5. Count the number of vein terminations in the square millimeter.
6. Record the result for each of the ten fields and calculate the average number of vein terminations in an area of one square millimeter.

Maceration of Uraria:

The stem and root vessels were studied by maceration techniques. The pieces of stem and root were boiled in Jeffery’s fluid (Johanson, 1940). The photographs were taken by Sony digital camera. Model Cyber Shot DSC S950. The dimensions of the cells were measured with help of microscope.

SEED GERMINATION:

The seed were collected by the method described by Neergard (1977) from different localities from August to November in 2006-2010 from a population growing in wild forest of Nagzira of Bhandara districts, Gondia (Bodalkasa),
Nanded (Fuggadigutta) and Botanical garden University of Pune. Botanical garden Dr. Babasaheb Ambedkar Marathwada University Aurangabad. The seed were kept in polythene bag in dormant condition and germination was recorded in Department of Botany, Dr. Babasaheb Ambedkar Marathwada University Aurangabad.

**Presoaking:** Presoaking was done described by the method (Airi, *et al.* 2009).

**Procedure:**

1. Take well dried seeds wash thoroughly with distilled water three to four times.

2. Take a petriplate wash with distilled water put a filter paper add some water to wet the filter paper.

3. Keep the clean seeds in petriplate presoaked in water at 12 hours, 24 hours, 36 hours, 48, hours and 72 hours respectively.

4. Keep the petriplate in sunlight for germination and observe the percentage of seed germination. Radical comes out considered as germinated seed.

**Hot Water: 50°C and 60°C**

Hot water treatment given by the treatment described by (Kobmoo *et al.* 1984).

**Procedure:**

1. Take well dried seed wash thoroughly with distilled water three to four times.

2. Take a petriplate washed with distilled water put a filterpaper add some water to wet the filterpaper.
3. Add the seeds in beaker heat the water in beaker for 50 °C and 60°C for 2 hour, 4 hour and 6 hour respectively.

4. Immediately remove the seeds from beaker and keep the seeds on filter paper for germination. Keep the petriplate in sunlight for germination and observe the percentage of seed germination. (Bonam et al. 1974).

**Mechanical Scarification:**

Mechanical scarification is a technique for overcoming the effect of an impermeable seed coat. Mechanical scarification done by rubbing seeds between two pieces of sandpaper (Schmidt 1980), or using a file, a pinor a knife to rupture the seed coat. Seed may also be mixed with coarse sand and shaken vigorously in a jar (Schmidt, 1980). Even a Vice can be used to squeeze seeds along the suture until they crack open. Care must be taken not to injure the embryo. It may be necessary to open a couple of seeds to see where the embryo is located in relation to the micropyle, Keep the seed in petriplate and observe the percentage of seed germination.

**Acid Treatment: Conc. Sulphuric acid, conc. Hydrochloric acid and Nitric acid.**

Procedure:

1. Wash the seeds carefully by distilled water three to four times.

2. Keep the seed in conc Sulphuric acid, Hydrochloric acid, Nitric acid in a different beaker for 5 minute, 10 minute and 15 minute respectively.

3. Immediately remove seeds, wash thoroughly three to four times with distilled water and keep on petriplate.
4. Keep the petriplate in sunlight and observe the percentage of seed germination.

**Treatment of Indol acetic acid, Indol buteric acid, Gibberlic acid, Napthalene acetic acid**

Procedure:

1. Wash the seeds carefully by distilled water three to four times.

2. Keep the seed in Indol acetic acid, Indol buteric acid, Gibberlic acid, Napthalene acetic acid in a different beaker for 2 hour, 4 hour and 6 hour respectively.

3. Immediately remove seeds, wash thoroughly three to four times with distilled water and keep on petriplate.

4. Keep the petriplate in sunlight and observe the percentage of seed germination.

**Treatment of Alkali 2 %KOH, 2%NaOH**

Procedure:

1. Wash the seeds carefully by distilled water three to four times.

2. Keep the seed in 2% KOH and 2% NaOH in a different beaker for 5 min, 10 min and 15 min. respectively.

3. Immediately remove seeds, wash thoroughly three to four times with distilled water and keep on petriplate.

4. Keep the petriplate in sunlight and observe the percentage of seed germination.
2-4 D (Dichlorophenoxyacetic acid)

Procedure:

1. Wash the seeds carefully by distilled water three to four times.

2. Keep the seed in 2-4D (50ppm) and cytokinin in a different beaker for 2 hour, 4 hour and 6 hour respectively.

3. Immediately remove seeds, wash thoroughly three to four times with distilled water and keep on petriplate.

4. Keep the petriplate in sunlight and observe the percentage of seed germination.

CHILLING: (4°C):

Chilling treatment was given by the method described by (Tompsett and Pritchard 1998).

Procedure:

1. Wash the seeds carefully by distilled water three to four time.

2. Keep the seeds in distilled water in beaker for 36 hour, 48 hour and 72 hour respectively.

3. Keep the beaker in freeze at 4°C.

4. Immediately remove seeds, wash thoroughly three to four times with distilled water and keep on petriplate.

5. Keep the petriplate in sunlight and observe the percentage of seed germination.
**Cowdung slurry:**

Effect of cowdung was observed by the method described by (Paul and Hossain 1996).

**Procedure:**

1. Wash the seeds with distilled water two to three times thoroughly.

2. Cowdung was collected from the garden Dept. of Botany Dr. Babasaheb Ambedkar Marathwada University Aurangabad in a petriplate.

3. Well washed seeds were kept in a cowdung for 12 hour, 24 hour and 36 hour respectively.

4. Seeds were removed after completion of time period and kept in petriplate, the petriplate were kept in sunlight.

5. Observe the percentage of seed germination.

**DRY HEAT (80°C):**

Oven or dry heat is not often recommended, and the temperatures required are more suitable to an incubator than a kitchen oven. For this seed coat treatment the seed should be placed in shallow containers in a preheated incubator or oven. Influence of heat was observed by method described by (Herranz *et.al.*1998).

1. Wash the seeds carefully by distilled water three to four time.

2. Keep the seeds in a preheated oven at (80°C) at 2 hours, 4 hours and 6 hours respectively.

3. After the treatment, the seed should be cooled immediately and kept on blotting paper (Emery 1987).
4. Keep the petriplate in sunlight and observe the percentage of seed germination.

**Statistical Analysis:**

Statistical analysis of data was carried out in order to note down the significance of various treatments. Statistical methods for determination of mean, standard deviation, standard error and critical difference between treatments were followed as per Mungikar (1997, 1999, 2003).

**SEEDLING MORPHOLOGY:**

Seedling shows characteristics pattern of development. From seeds to the seedlings many stages are observed. Whether these stages are species specific in genus *Uraria* or not was the main aim of the study. If they are species specific then we can use this as a criterion for species delimitation as the individuals of *Uraria* species are at vegetative stage looking alike and identification can be possible only at flowering and fruiting stage.

The seeds giving out the radical were considered as germinated. Average time required for radical to come out, formation of zone of absorption, cotyledons to come out was calculated in hours from seeds placed over wet blotting paper. Length and breadths of cotyledonary leaves were measured when they are fully grown. Their shapes and apices were noted. On the 21 day of germination, the length of hypocotyls, roots were also measured. Measurements of second pair of leaves were given along with their shapes and apices and time required for their formation. Length of second internodes is also provided.
Seed morphology and testa topography:

Seeds of three species of genus *Uraria* Desv. were collected from different localities from the fields, however, studies are based on seeds collected from many localities. Voucher specimens of seeds and Herbarium sheets are deposited in BAMU Herbarium, Dept of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (MS). All the mature seeds for the present study came from the collection made from Maharashtra, particularly from Thane, Nanded, Gondia, Bhandara and Ratnagiri (Table no.01). The seeds were collected in the fields from dry pods of different plants. The dry matured seeds were cleaned, and examined by stereoscope to show different exomorphic parameters viz, seed length, width, seed index, shape, size, color, surface texture, position of hylum etc. 10 seeds were observed for each species under the study. The stereoscopic measurements were taken with the help of Pico meter in Millimeter (mm) dimensions. For SEM studies, the seeds were dried and fixed to specimen stubs with an adhesive and placed on revolving discs of Joel fine ion sputter (JFC 1600 fine coater). Seeds were uniformly coated with gold. Specimens stubs were then fixed to the specimen holder of Scanning Electron Microscope (Joel JSM 6360A) maintained at accelerating potential voltage of 15 KV, and photomicrographs of Seeds hylum, entire seed, and subhylar region were taken at different magnification in Unit of the Scanning Electron Microscope in Dept of Physics, University of Pune, Pune.

BIOCHEMICAL STUDY

Biochemistry is the chemistry (chemical analysis) of plant products. The chemicals present in root, stem and leaf powder are analyzed physically as well as chemically by qualitative and quantitative means. The physical parameters like dry matter and bulk density of powdered stem, root, leaf powder serves as primary data for drug
identification. Estimation, qualitative as well as quantitative, of various chemicals taken together is assumed to produce specific data useful in standardizing a particular drug. The quantitative analysis of elements or chemicals like nitrogen, crude proteins, crude fats, crude fibers, reducing sugars, non reducing sugars, total sugars etc. may fluctuate with the age of the plant, season of collection, hence these values are not considered as criteria. But their use in combination roughly gives the idea about purity of drug. Their values with little variation should be accepted as a base for standardizing a drug. The quantitative analysis of a single chemical or element should not be considered as strict criteria for standardization or evaluation. Similarly presence or absence of a chemical i.e. qualitative analysis of powder will give the criteria to evaluate the drug or to standardize the drug.

In phytochemical investigation the samples of root, stem and leaves powder obtained from 3 species were studied. The parameters used are-

**Physical Evaluation**

1) **Dry Matter (DM)**

Dry matter (DM) was calculated by weighing the sample after drying to a constant weight in an oven at 95 ± 5°C. For this purpose, 100 g of sample was taken in a clean dry pre-weighed tray and kept in oven for 48 hours or more, till constant weight. Weight of the dried sample was reported as percent dry matter (DM).

The dried samples were usually ground to a fine powder and stored in sealed containers for further analysis.
2) Bulk Density

The fine powder of powdered samples was filled in a cube of 1cm x 1cm x 1cm; the heap of powder was removed by a scale to maintain uniformity. The powder was removed from the cube and its weight was taken. That weight was considered as bulk density mg/cm$^3$.

Chemical Analysis

A) Quantitative analysis

Nitrogen (N)

The dry sample is digested with concentrated sulphuric acid ($H_2SO_4$) in the presence of catalyst. During the digestion, nitrogenous compounds are converted to ammonium sulphate (($NH_4$)$_2$ SO$_4$). It is then made strongly alkaline with sodium hydroxide (NaOH). The released ammonia (NH$_3$) is distilled into boric acid (H$_3$BO$_3$) solution. The ammonium tetraborate formed is then titrated against 0.035 N hydrochloric acid for the determination of nitrogen (N).

Reagents

1) Concentrated $H_2SO_4$, AR grade, sp. gr. 1.85.
2) Catalyst: A mixture of copper sulphate (CuSO$_4$), potassium sulphate (K$_2$SO$_4$) and Selenium dioxide (SeO$_2$) 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% \( \text{H}_3\text{BO}_3 \) solution is made by dissolving 10 g \( \text{H}_3\text{BO}_3 \) 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 \( ((\text{NH}_4)_3\text{BO}_3) \) 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 Kjeldahl’s flask. Add a pinch of catalyst with the help of spatula. Slowly add 7.5 ml concentrated sulphuric acid (\( \text{H}_2\text{SO}_4 \)). Heat the flasks gently on a digestion stand until the fumes of \( \text{H}_2\text{SO}_4 \) are freely evolved. Increase heat until acid boils vigorously and digests 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 up to 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 $\text{H}_3\text{BO}_3$ 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 $\text{H}_3\text{BO}_3$, at the condenser outlet to form ammonium tetraborate ($\text{(NH}_4\text{)}_3\text{BO}_4$). This results into the change in colour of $\text{H}_3\text{BO}_3$ solution from pink to green. Continue distillation till the volume becomes to about 20 ml. Titrate the ($\text{(NH}_4\text{)}_3\text{BO}_4$) with 0.035 N HCl till pink colour reappears and record titration value. Calculate strength of NH$_3$, 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 (CF):**

The fats present in the plant material are extracted in the solvent consisting of chloroform ($\text{CHCl}_3$) and methanol ($\text{CH}_3\text{OH}$). 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 desiccators and weigh. Measure the amount of fat, extracted per 2 g of the sample, and calculate the amount of Cf as percent of dry matter (DM).

Crude Fiber (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 contains carbohydrates and it is a valuable parameter in deciding the nutritive quality of animal feed.
Reagents:

1) 1.25 % \(\text{H}_2\text{SO}_4\): Dissolve 5 ml con. \(\text{H}_2\text{SO}_4\) in 395 ml distilled water.
2) 2.5% \(\text{NaOH}\): Dissolve 5 g \(\text{NaOH}\) 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% \(\text{H}_2\text{SO}_4\) 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% \(\text{NaOH}\). 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 desiccators 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 up to 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 rise up to 600°C and keep it constant for 2 hours. Remove the crucible on cooling and transfer directly to desiccators, 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 powder.

**Acid Insoluble Ash (AIA)**

**Reagents:**

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

**Procedure:**

Add 50 ml of 5N HCl 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 up to 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, monosaccharide’s (pentoses and hexoses), oligosaccharides (compound sugars) and polysaccharides (starches). Insoluble carbohydrate or CF contains 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, Cf at and CF of a material is substracted from 100.

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

TC is then determined as either
% TC = % NFE + % CF or

% TC = 100 – (% CP + % Cfat + % 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 (KMnO₄).

**Reagents:**

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

2) Ammonium oxalate ((COO.NH₄). 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 aliquot (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:

$$1 \text{ ml of KMnO}_4 = 0.2004 \text{ mg of Ca}$$

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 H₂SO₄: Carefully add 200 ml concentrated H₂SO₄ (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 H₂SO₄ 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 (NaHSO₃): Take 30 g reagent grade NaHSO₃ in a beaker. Add 200 ml of distilled water and stir to dissolve, (b) 20% sodium sulphite (Na₂SO₃): Dissolve 20 g of reagent grade anhydrous Na₂SO₃ in distilled water and dilute to 100 ml. Filter if necessary, (c) ANSA reagent: Take 195 ml of 15% NaHSO₃, solution in a beaker. Add 500 mg of 1, 2, 4 - aminonaphtholsulfonic acid, and mix thoroughly. To this add 5 ml of 20% Na₂SO₃ followed by thorough mixing. If the solution is not complete, add more Na₂SO₃, 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 (KH₂PO₄) in 500 ml of distilled water and transfer to a 1 litre volumetric flask. Add 10 ml of 10 N H₂SO₄, 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 aliquot 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 aliquot 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 dichromate (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\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} followed by 16 ml concentrated H\textsubscript{2}SO\textsubscript{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 up to 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\textsubscript{2}S\textsubscript{2}O\textsubscript{3} solution using starch as an indicator. The excess dichromate present is calculated from the titration figure and subtracted from blank value to obtain the quantity of 1.5 N K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} used in the oxidation.

Determine the amount of 1.5 N K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{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:

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

Dissolve 0.746 gms of pure dry KC\textsubscript{1} in a litre of glass distilled water,
1) 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 m Eq 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 aliquots 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 simpler sugars. The carbohydrate content can be measured by hydrolysing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

**Reagents:**

- 2.5 N HCl
- Anthrone Reagent: Dissolve 200 mg anthrone in 100 ml of ice cold 95% H₂SO₄, Prepare fresh before use.
- 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 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 aliquot 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

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

**Protein**

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

**Reagents:**

— 2% Sodium Carbonate in 0.1N Sodium Hydroxide (Reagent A)

— 0.5% Copper Sulphate (CuSO₄.5H₂O) in 1% potassium sodium tartrate (Reagent B)

— Alkaline Copper solution: Mix 50 ml of reagent A and 1 ml of regent B prior to use (Reagent C)

— Folin-Ciocalteau Reagent (reagent D) —Reflux gently for 10 hours a mixture consisting of 100g sodium tungstate (Na₂WO₄.2H₂O), 25 g sodium
molybdate (Na$_2$MoO$_4$.2H$_2$O), 700 ml water, 50 ml of 85% phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5 l flask. Add 150 g lithium sulfate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 min without condenser to remove excess bromine. Cool, dilute to 1 L and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1 N NaOH to a phenolphthalein end-point.).

— Protein Solution (Stock Standard)

Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolve it in distilled water and make up to 50 ml in a standard flask.

— Working Standard

Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg proteins.

**Procedure** :

Extraction of Protein from sample

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of the sample and grind well with a mortar and pestle in 5-10 ml of the buffer. Centrifuge and use the supernatant for protein estimation.

**Estimation of Protein** :

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard into a series of test tubes.
2. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank.
4. Add 5 ml of reagent C to each tube including the blank. Mix well and allow standing for 10 min.
5. Then add 0.5 ml of reagent D, mix well and incubate at room temp in the dark for 30 min. Blue colour is developed.
6. Take the readings at 660 nm.
7. Draw a standard graph and calculate the amount of protein in the sample.

**Reducing Sugar (RS)**

The majority of methods 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 2 ml of the powder extract to a folin-Wu-sugar tube graduated at 25 ml and to other similar tubes adds 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 m\(\mu\), 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 bark 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 purify 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 happens 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 foreset all improper use of this reagent attention should be called to the fact that if 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:**

The percentage of non-reducing sugars was calculated by subtracting the value of the percentage of reducing sugars from that of total sugars.

**Extractive Values:**

Different plant species would obviously have different chemical profile. Chemicals present in plant material could be dissolved in different solvent for the purpose of different analysis. Therefore eleven solvents water, alcohol, benzene, petroleum ether, acetone, methanol, chloroform, toluene, propanol, diethyl ether and butanol were selected to determine the soluble substance.

**Determination of Water - Soluble Extractive:**

5 gm of air dried powder, coarsely powdered was macerated with 100 ml of distilled water in a closed flask, for twenty four hours, shaking frequently. Solution was filtered and 25 ml of filtrate was evaporated in a tarred flat bottom shallow
dish, further dried at 100°C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried powder.

**Determination of Methanol-soluble extractive:**

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of methanol in a closed flask for twenty four hours with frequent shaking. It was filtered rapidly, taking precautions against loss of methanol. 25 ml of filtrate was then evaporated in the tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of methanol soluble extractive was calculated with reference to the air dried powder.

**Determination of Alcohol - Soluble Extractive:**

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of alcohol in a closed flask for twenty four hours with frequent shaking. It was filtered rapidly, taking precautions against loss of alcohol. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried powder.

**Determination of Benzene-soluble Extractive:**

5 gm of air dried drug, coarsely powdered was soaked with 100 ml of Benzene in a closed flask for twenty four hours with frequent shaking. It was filtered rapidly, taking precautions against loss of Benzene. 25 ml of filtrate was then evaporated in the tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of Benzene-soluble extractive was calculated with reference to the air dried powder.
Determination of Petroleum Ether - Soluble Extractive:

5 gm of air dried powder, coarsely powered was macerated with 100 ml of petroleum ether in a closed flask for twenty four hours with frequent shaking. It was filtered rapidly, taking precautions against loss of petroleum ether. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of petroleum ether soluble extractive was calculated with reference to the air dried powder.

Determination of chloroform-soluble Extractive:

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of chloroform in a closed flask. For twenty four hours with frequent shaking. It was filtered rapidly, taking precaution against loss of chloroform. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of chloroform soluble extractive was calculated with reference to air dried powder.

Determination of acetone-soluble Extractive:

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of acetone in a closed flask for twenty four hours with frequent shaking. It was filtered rapidly, taking precautions against loss of acetone. 25 ml of filtrate was then evaporated in the tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of acetone-soluble extractive was calculated with reference to the air dried powder.

Determination of toluene-soluble Extractive:

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of toluene in a closed flask. For twenty four hours with frequent shaking. It was
filtered rapidly, taking precaution against loss of toluene. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of toluene soluble extractive was calculated with reference to air dried powder.

**Determination of Propanol-soluble Extractive:**

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of propanol in a closed flask. For twenty four hours with frequent shaking. It was filtered rapidly, taking precaution against loss of propanol. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of propanol soluble extractive was calculated with reference to air dried powder.

**Determination of diethyl ether-soluble Extractive:**

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of diethyl ether in a closed flask. For twenty four hours with frequent shaking. It was filtered rapidly, taking precaution against loss of diethyl ether. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of diethyl ether soluble extractive was calculated with reference to air dried powder.

**Determination of Butanol-soluble Extractive:**

5 gm of air dried powder, coarsely powdered was soaked with 100 ml of butanol in a closed flask. For twenty four hours with frequent shaking. It was filtered rapidly, taking precaution against loss of butanol. 25 ml of filtrate was then evaporated in a tarred flat bottom shallow dish, dried at 100°C and weighed. The percentage of butanol soluble extractive was calculated with reference to air dried powder.
B. QUALITATIVE ANALYSIS

Alkaloids:

Five grams of powdered material was extracted with 50 ml of 5% ammoniacal ethanol for 48 hrs. The extract was concentrated by distillation and the residue was treated with 10 ml of 0.1 N H$_2$SO$_4$. The acid soluble fraction was tested with Mayer's, Wagner's, and Dragendroff's reagents (Paech and Tracey, 1955). A white coloured precipitate denoted the presence of alkaloids (Amarasingham et al., 1964). The preparations of the reagents are as follows:

**Mayer's reagent**: 1.36 g of HgCl$_2$ was dissolved in 60 ml of distilled water and 5 g of KI in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops of this reagent were added, as precipitates of some alkaloids are soluble when the reagent is used in excess.

**Wagner's reagent**: (Potassium Iodide) 1.27 g of I$_2$ and 2 gm of KI were dissolved in 5 ml of water and the solution diluted to 100 ml. It gave brown flocculent precipitates with most of the alkaloids.

**Dragendroff’s reagent**: (Potassium bismuth iodide) 8 g of Bi(NO$_3$)$_3$. 5H$_2$O were dissolved in 20 ml of HNO$_3$ (sp. gr. 1.18) and 27.2 g of KI in 50 ml of water. The two solutions were mixed and allowed to stand when KNO$_3$ crystallised out. The supernatent was decanted off and made up to 100 ml with distilled water.

**Anthraquinone**:

1ml aqueous extract was taken in a test tube. To it 1ml ether solution was added. Test tube was shaken and etherical layer was separated. To it strong ammonia solution was added, shaken kept aside. Lower ammonical layer shows pink red colour if Anthraquinone is present.
**Iridoids:**

The plants were surveyed for iridoids by a simple procedure described by Weiffering (1966) based on the Trim-Hill colour test. Fresh or dry powder material (1 g) was placed in a test-tube with 5 ml of 1% aqueous HCl. After 3-6 hours, 0.1 ml of 0.2% of CuSO$_4$ 5H$_2$O in water and 0.5 ml Conc. HCl was added and when the tube was heated for a short time on a flame, a colour was developed, if iridoids were present (asperuloside, aucubin and monotropein gave blue colour, while herpagide, red-violet; Harborne, 1984).

**Saponins:**

At about 5 g of the powdered leaf material was boiled with 50 ml water for half an hour. This extract was filtered; the filtrate was taken in test-tube after cooling and shaken vigorously (to froth) for a minute or two. The formation of a persistent froth of 1 cm length showed the presence of saponins (Hungund *et al.*, 1971). Foam formation took place even during extraction with aqueous solvents if the concentration of the saponins was more in the plant materials (Harborne, 1984).

**Steroids:**

Steroids are extracted in water and are tested by following tests. The water extract was prepared by boiling 5 g plant material in about 50 ml water.

1) Salkowski reaction: - To the 2 ml water extract, 2ml chloroform and 2 ml conc. H$_2$SO$_4$ was added in a test tube, the mixture was shaken well. The formation of red colour in chloroform layer and greenish yellow fluorescence in acid layer showed the presence of steroids.
2) Libermann – Burched reaction: - To the 2 ml water extract, 2ml chloroform and 1-2 ml acetic anhydride was added. To it 2 drops of Conc. H$_2$SO$_4$ was added from side of test tube. First red, then blue and finally green colour appeared showed the presence of steroids.

3) Liebermann’s reaction:- To the 2 ml water extract, 3 ml acetic anhydride was added. The mixture was heated and few drops of Conc. H$_2$SO$_4$ were added. The blue colour appeared showed the presence of steroids.

**Tannins :**

Tannins are extracted in water and are tested by treating them with protein solution when leather precipitates. To the water extract prepared by boiling 5 g plant material in about 50 ml water, 2% freshly prepared gelatin solution was added. The formation of a white (or milky) precipitate showed the presence of tannins in the plant material (Hungund *et. al.*, 1971).

**Flavonoides:**

1) Shinoda test-Stem, leaf and root powder was mixed with few fragments of Magnesium ribbon and to it Concentrated Hydrochloride acid was added drop wise. Pink scarlet color appeared after few minutes, indicating the presence of flavonoids.

2) Alkaline reagent test-Stem, leaf and root powder was mixed with few drops of NaOH solution. An intense yellow color was formed. Yellow color turning hyaline on addition of few drops of diluted acid, indicates the presence of flavonoids.

**Phenolics**

**Ferric Chloride Test-** For the detection Phenolic Compound following tests were studied. Ferric Chloride Test- the extract (50gm) is dissolved in 5 ml of distilled
water. To this few drops of natural 5% ferric chloride solution is added. A dark green color indicates the presence of Phenolic compound.

**Gelatin Tests**-The extract (50gm) is dissolved in 5 ml of distilled water and 2 ml of 10 % sodium chloride solution is added. White ppt indicates the presence of Phenolics compounds.

**Lead Acetate Test**-The extract (50mg) is dissolved in distilled water and to this, 3 ml of 10 % lead acetate solution is added. A bulky white ppt. indicates that the presence of Phenolics compounds.

**Magnesium & Hydrochloric acid reduction Test**- The extract (50 mg) is dissolved in 5 ml of alcohol and few fragment of magnesium ribbon and conc.HCl acid (drop wise) is added. If any pink to crimson color develops it indicates the presence of flavonol glycosides.

**Alkaline reagent Test**- An aqueous solution of the extract is treated with 10 % NH₄OH solution. Yellow fluorescence indicates the presence of flavonoids.

**Detection of Chemicals from Uraria extract by GC-MS technique:**

Powdered samples were studied by the GC-MS (Gas chromatography – Mass spectroscopy) technique, for the study 1gm of sample was taken; it was extracted with methanol in soxhlet apparatus at 60°C. After the complete extraction the extract was evaporated to remove the excess amount of methanol and the concentrated extract was preserved in a specimen tube. Then it was brought to the Central Facility Center, Department of Biochemistry, Shivaji University Kolhapur, for the chemical analysis in the extract by GC-MS technique and the result were taken.
Gel Electrophoresis

Gel electrophoresis is a method that separates macromolecules, either nucleic acids or proteins, on the basis of size, electric charge, and other physical properties. A gel is a colloid in solid form. The term electrophoresis describes the migration of charged particles under the influence of an electric field. Electro refers to the energy of electricity. Phoresis refers to the technique in which molecules are forced across a span of gel, motivated by an electrical current. Activated electrodes at either end of the gel provide the driving force.

Seed protein electrophoresis has been utilized as a powerful tool in solving taxonomic problems and explaining the origin and evolution of a number of wild medicinal plants. (Ahmad and Slinkard 1992, Jha and Ohari 1996, Nath, et. al. 1997, Vladova, et. al. 2001, Ghafoor, et.al. 2002). Taxa of genus *Uraria* often difficult to characterize taxonomically, as it has few distinguishing features amongst the due to morphovariation in species. The aim of present investigation is therefore to study differentiation pattern in seed protein profiles, genetic variation and relationship of 3 taxa of genus *Uraria* Desv. Based on the storage proteins in order to contribute to solve the status of taxa.

Materials:

Chemicals used for electrophoresis were of highest purity available. Broad range proteins standard used were phosphorylase- b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and a-lactalbumin purchased from Genei, Bangalore, India.
Collection of samples:

The dry seeds of *Uraria lagopus, Uraria picta and Uraria rufescens* were collected from different localities of Maharashtra. The specimen was authenticated by the department of Botany, Dr. Babasaheb Ambedkar Marathwada University and the voucher specimen (Table No.1) was deposited for future reference in the Botany Department Herbarium.

Protein profile by electrophoresis:

Polyacrylamide gel electrophoresis (SDS-PAGE)

The Water soluble proteins were analyzed by using the vertical slab Polyacrylamide gel electrophoresis apparatus.

SDS-PAGE:

In SDS-PAGE, Polypeptides were separated according to their molecular weight not by the intrinsic electric charge. Sodium dodecyl sulphate (SDS) is an ionic detergent that denatures proteins by wrapping around the polypeptides backbone. In doing so, the SDS confers a negative charge to the polypeptide in proportion to its length. When proteins were treated with SDS and reducing agent 2ME (2- Mercaptoethanol), the polypeptides become rods of negative charge with equal charge unit per length. Here denaturing discontinuous PAGE system was used as described by (Laemmli, 1970). This system is almost similar to the negative PAGE (Davis, 1964) Except for the presence of SDS.
Preparation of solutions:

1) Stock solution

A) Monomer solution (Acrylamide / bisacrylamide 29.9% T, 0.9% C)

30 gm of acrylamide and 1.04 g of N,N-methylene bisacrylamide were dissolved in distilled water and made up to 100 ml.

B) Resolving gel buffer (1.5 Tris, pH 8.8)

This solution was prepared by dissolving 18.16 gm of Tris buffer in 60 ml of distilled water and stored at 4°C.

C) Stacking gel buffer (1M Tris-HCL, pH 6.8)

12.12 g of this tris was dissolved in 60 ml of distilled water. The pH 6.8 was adjusted with 0.1 N HCL and the final volume was made up to 100 ml with distilled water and stored at 4°C.

D) TEMED (10%) Initiator

It was prepared by adding 100µl TEMED to 900 µl distilled water.

E) Ammonium persulphate (10%)

100 mg of ammonium persulphate was dissolved in 1ml of distilled water. This solution was prepared freshly at the time of use.

F) Electrode buffer (0.25 M Tris, 2M glycine, pH 8.3)

The electrode buffer stock solution was prepared by dissolving 15 g of Tris base and 72 g of glycine in 300 ml of distilled water and then made to the
final volume of 500 ml with distilled water. The pH of solution was adjusted to 8.3. this solution was diluted 10 times at each time of use.

G) Sample buffer (0.08 M Tris-Hcl pH 6.8)

1.6 ml stacking gel buffer was added to 2.5 gm of 87 % glycerol and 0.5 mg of bromophenol blue and mixed thoroughly and the final volume was made upto 10 ml with distilled water. It was stored at 4°C.

H) Fixing solution

57 gm trichloroacetic acid 17 gm sulphosalycyclic acids were dissolved in 150 ml methanol and final volume was made up to 500 ml with distilled water.

I) Staining solution

The staining solution was prepared by dissolving 1.25 gm coomassie brilliant blue R 250 in 230 ml of methanol and 40 ml of acetic acid, the volume was made up to 500 ml with distilled water.

J) Destaining solution

40 ml of acetic acid and 230 ml of ethanol were mixed well and made to the final volume of 500 ml by adding distilled water.

I) Sodium Dodecyl sulphate (10 %)

This was prepared by dissolving 20 g of SDS (Amino detergent) in 150 ml of distilled water and the final volume (300 ml) was adjusted with distilled water.
2) Preparation of resolving gel

12% polyacrylamide gel was prepared by mixing 10 ml monomer solution, 12.5 ml distilled water, 7.5 ml resolving gel buffer, 200 µl APS and 20 µl TEMED. Mixed the solution and immediately this gel solution was poured into gel mould and over layered with saturated n-butanol and allowed to polymerize. This resolving gel was prepared in a Broviga Vertical gel slab apparatus.

After polymerization the butanol was poured off and the top of gel was carefully washed with distilled water and later drained with paper strip.

3) Preparation of stacking gel

In 10% stacking gel solution, 1ml stacking gel buffer, and 7.1 ml distilled water, 1.6 ml monomer solution, 50 µl APS and 10 µl TEMED were added and mixed thoroughly. This solution was quickly poured on top of the polymerized resolving gel. A. comb was placed to form the sample well and the gel was allowed to polymerize. After polymerization the comb was removed carefully. The glass plate with the gel was fixed in the apparatus filled with electrode buffer. Equal protein sample (40 µl) mixed with 2 x loading dye (sample buffer in 1:1 v/v) ratio were loaded in the sample wells. Electrophoresis was run at 150 V for four hours, until the tracking the dye came into the lower buffer tank. The gels were fixed in 10% TCA for 30 minutes. Then the gel were stained with staining solution for 5 hours and washed briefly in distaining solution till the background became clear. The gels were photographed and molecular weight of every band was determined using the gel documentation system. The gene snap software was used for RF values determined.
4) **Application of sample:**

The 10 µl 10 % SDS added to protein sample (90 µl) and mixed with 100 µl loading dye (Containing 1% SDS, 25% glycerol. 0.1 % 2- mercaptoethanol and 100 mg bromophenol blue). This sample was heating in boiling water for 5 minutes and then used for electrophoresis. Electrophoresis was performed foe constant voltage (150 µl) for stained with staining solution for 5 hours and washed briefly in distaining solution till the background became clear. The gel was photographed and molecular weight of every band was determined using gel documentation system. The gene snap softwere was used for RF values determined.

**Material:**

- Casting gel unit for electrophoresis
- Siliconised patsteur pipettes
- Syringses equipped with blunt stab nosed needles
- Vaccum chamber for degrassing gels
- Micropippetes (10-300 µl).

  Stock 30% T: 0.8% C acrylamide monomer

- 1.5 M Tris –HCL buffer, pH 8.8
- 10 (w/v) ammonium persulphate
- Separation gelmixe just prior to use
- 20 ml of acryamide monomer
- 15 ml of Tris HCL Buffer pH 8.8
- 0.6 ml of H₂O
- Stacking gel mixed just prior to use
- 2.66 ml of acrylamide monomer
- 5.0 ml of Tris buffer, pH 8.8
- 0.2 ml of 10% (w/v) SDS
- 12.2 ml of H₂O

**Procedure:**

1) Assemble slab gel unit with the glass sandwich set in the casting mode with 1.5 mm spaces in place.

2) Prepare a separating gel from ingredients listed

3) Add the separating gel to a side arm flask, stopper the flask, and attach to a vacuum pump equipped with a cold trap. Turn on the vacuum and degas the solution for approximately 10 minutes. During this period, gently swirls the solution in the flasks.

4) Turn off the vacuum, open the flask and add 200 µl of ammonium persulphate and 20 µl of TEMED solution.

5) Add a stopper to the flask and degas for an additional 2 minutes while gently swirling the solution to mix the 2 accelerators. Use this solution within a few minutes of mixing, or it will gel in the flask.

6) Transfer the degaseed acrylamide solution to the casting chamber with a Pasteur pipette. Gently fill the center of the glass chamber with the solution
by allowing the solution to run down the side of one of the spacers. Be careful not to introduce air bubbles during this step.

7) Adjust the level of the gel in the chamber by inserting a syringe equipped with a 22 gauge needle into the chamber and removing excess gel.

8) Immediately water layers the gel to prevent formation of curved meniscus. Using second syringe and needle, add approximately 0.5 ml of water to the chamber by placing the tip of the needle, at an angle to a spacer and gently allowing the water to flow down the edge of the spacer and over the gel. Add an additional 0.5 ml of water to the chamber by layering it against the spacer on the opposite side of the chamber. Done approximately, the water will form a layer over the gel, and a clear line of demarcation will observe as the polymerizes.

9) After 30 minutes, the gel should be polymerized. If degassing was insufficient, or the ammonium persulphate not fresh, the polymerization may take an hour or more. When the gel is polymerized, lift the gel in its casting chamber and tilt to decant the water layer.

10) Prepare a stacking gel from the listed ingredients.

11) Degas the stacking gel from the listed ingredients.

12) Add 75 µl of ammonium persulphate and 10 µl of TEMED to the stacking gel and degas for an additional 2 minutes.

13) Add approximately 1 ml of stacking gel to the gel chamber and gently rock back and forth to wash the surface of the separating gel. Pour off the still liquid stacking gel and dispose of properly.
14) Add fresh stacking gel until it nearly fills the chamber, but allow room for the insertion of a Teflon comb used to form sample wells. Carefully insert Teflon comb into chamber. Adjust the volume of the stacking gel as needed to completely fill the spaces in the comb.

15) Allow the gels to polymerize for at 30 minutes prior to use.

**Antioxidant activity**

**Collection of samples**

The roots, stems and leaves of *Uraria lagopus, Uraria picta* and *Uraria rufescens* species were collected from different localities of Maharashtra. The collected specimens were identified with the help of different floras (Naik, 1998 and Yadav and Sardesai, 2002) and the herbarium deposited in BAMU herbarium department of Botany Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (MS) India.

**Preparation of extract**

The each plant materials washed thoroughly by distilled water and kept at room temperature for air drying. After completely dried samples were pulverized into fine powder by mixture grinder. The fine powder samples soaked into methanol (1:10 w/v) and stirred 5 to 10 min by glass rod for proper combination. The mixed samples were kept at 4°C for overnight extraction. The each sample were centrifuged at 6000 rpm for 15 min and supernatants were collected and stored at -20°C for further experiment.
Thin layer chromatography:

**Materials**: Apigenin, Lupiol, Stigmasterol (Sigma Aldrich) TLC (Merck), Methanol, Hexane Chloroform, Ethyl acetate, (Rankem),

**Methods**:

**Collection of samples**:

The stems, leaves and roots of *Uraria lagopus* *Uraria picta* and *Uraria rufescens* were collected from different localities of Maharashtra. The materials were identified with the help of floras and kept in BAMU herbarium Aurangabad. (MS) India. The surfaces of collected materials were washed thoroughly by distilled water and kept for air dry.

**Extraction**: The materials of the plants were pulverized into fine powder by mixture. The fine powders of the samples were soaked in methanol and kept overnight for extraction at 15 °c. The samples were centrifuged at 6000 rpm for 15 minutes. The extraction process repeated until the residues become colorless. The methanol of supernatants was removed by rotary evaporator. The concentrated residues were kept at –20 °c for further experiment

**Fractionations**: The residues of the samples dissolved in 10 % Methanol and the fractionated by Chloroform and Ethyl acetate. Each fractions were concentrated by rotary evaporator and residues dissolved in methanol

**Scanning electron microscopy**:

Seeds of three species of genus *Uraria* were collected from different localities from the fields; however, studies are based on seeds collected from many localities. Voucher specimens of seeds and Herbarium sheets are deposited in
BAMU Herbarium, Dept of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (MS). All the mature seeds for the present study came from the collection made from Maharashtra, particularly from Thane, Nanded, Gondia, Bhandara and Ratnagiri (Table no.02). The seeds were collected in the fields from dry pods of different plants. The dry matured seeds were cleaned, and examined by stereoscope to show different exomorphic parameters viz, seed length, width, seed index, shape, size, color, surface texture, position of hylum etc. 10 seeds were observed for each species under the study. The stereoscopic measurements were taken with the help of Pico meter in Millimeter (mm) dimensions. For SEM studies, the seeds were dried and fixed to specimen stubs with an adhesive and placed on revolving discs of Joel fine coat ion sputter (JFC 1600 fine coater). Seeds were uniformly coated with gold. Specimens stubs were then fixed to the specimen holder of Scanning Electron Microscope (Joel JSM 6360A) maintained at accelerating potential voltage of 15 KV, and photomicrographs of Seeds hylum, entire seed, and subhylar region were taken at different magnification in Unit of the Scanning Electron Microscope in Dept of Physics, University of Pune, Pune.

TISSUE CULTURE:

Sterilization of Equipments and Glassware’s:

All operations for in vitro culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass materials and chemicals. A horizontal laminar flow cabinet (Envirco Corporation, Foster City, California, USA) with HEPA filter was used. The hood surface was wiped clean with paper towel soaked in 70 % ethanol and sterilized by germicidal ultraviolet light for at least 10 min prior to use. All surgical instruments, glassware and other accessories were sterilized in autoclave at 121 ºC with 15 psi for 30 min and then dried in oven. Surgical instruments like scalpel, forceps, and scissors were sterilized by dipping in 100 % ethyl alcohol and flaming prior to use.
Surface sterilization of Explants

The young healthy explants of *Uraria*. Were collected from botanic garden Dept of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. They were washed first under running tap water (15 - 20 min) to remove surface adhered particles and then with 5% liquid hand wash dettole for 5 min followed by 70% ethanol for 5 Min. The inoculum was rinsed in distilled water (three - four times) and transferred to Laminar air flow cabinet. The inoculum was then surface sterilized by 0.1% (w/v) HgCl$_2$ for 5 minutes. Finally, the explants were washed in sterile distilled water for three - five times to remove the residual HgCl$_2$ and then cut into appropriate sizes for inoculation on to the sterile medium.

The callus induction medium composed of MS containing 3% (w/v) sucrose, 2% (w/v) clarigel with different concentrations of NAA alone or in combination with BAP and Kn for callus induction. The calli were transferred to the fresh medium for further proliferation and maintenance. The well developed calli were selected and subcultured on regeneration media. MS was supplemented with different concentrations of Kn and BAP alone or in combinations with NAA for shoot regeneration. Individual regenerated shoots were excised and used for rooting. Root induction was carried out on full strength of MS supplemented with NAA, IBA and IAA at different concentrations. Medium without plant growth regulators was used as a control. The pH of the medium was adjusted to 5.8 before autoclaving for 15 min at 121ºC. All the cultures were incubated at 25 ± 2ºC with a 16 hr photoperiod (40 μ E/cm$^2$/min/sec) provided by cool white fluorescent tubes. Well developed rooted shoots were removed from the culture vessels, washed gently under running tap water and planted in pots containing 50 % soil and 50 % cockpit (1:1). The plantlets were kept in the greenhouse for acclimation (two -three
weeks) before their subsequent transfer to the field. Humidity was maintained by sprinkling water regularly (Jasrai, et. al. 1999). Plants were gradually exposed to the normal conditions and finally transferred to the Botanical Garden of Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. Each experiment was repeated more than two times. Data were recorded on the percentage of response, number of shoots per explants, number of roots and root length per shoot. Means and standard errors were estimated for each treatment.

**Culture Room**

The explants were incubated in a culture room where the temperature was maintained at 25-26 °C, humidity at 85 % and either under continuous dark or under a photoperiod of 16 h light (25 μ mol s-1m-1) and 8 h dark.

**Preparation of Culture Media**

MS (Murashige and Skoog, 1962) inorganic salts, organic supplements, and vitamins were used as basal media for seed germination, callus induction, callus multiplication, and shoot and root induction. The formulation and composition of MS medium is given.

**PREPARATION OF STOCK SOLUTIONS**

**A. STOCK A: SALTS**

1. Weigh the following reagents:

NH₄NO₂ 35.0 g
KNO₃ 40.0 g
CaCl₂·2H₂O 10.3 g
KH$_2$PO$_4$ 3.5g
H$_3$BO$_3$ 0.1g
MnSO$_4$.H$_2$O 0.4 g
ZnSO$_4$.7H$_2$ O 0.2 g
Kl 0.02g
NaMoO$_4$ 0.004 g
Dissolve them in 200 ml of distilled water.
Keep the solution in a conveniently labeled vial at 4°C.

2. Weigh 5 mg of the following reagents:
CuSO$_4$.5H$_2$O
CoCl$_2$.6H$_2$ O
Dissolve them in 10 ml of distilled water. To 1 ml of the previous solution add 200 ml of distilled water. Keep the solution in a conveniently labeled vial at 400.

**B. STOCK B: MgSO$_4$**

Weigh 3.7 p of MgSO$_4$.7H$_2$O in 100 ml of distilled water.
Keep the solution in a conveniently labeled vial at 4°C.

**C. STOCK C:**

1. Weigh 0.75g of Na$_2$EDTA.
Dissolve while hot in 20 ml of distilled water. Let the solution cool.
2. Weigh 0.55 g of FeSO$_4$.7H$_2$O
Dissolve it in 20 ml of distilled water

3. Mix both solutions and fill up to 100 ml by adding distilled water. Keep the solution in a dark, conveniently labeled vial at 4°C.

**D. STOCK 0: Vitamins**

1. Weigh the following reagents:

   Thyamine HCl 20mg
   Glycine 100mg
   Nicotinic acid 25 mg
   Pyridoxine HCl 25 mg

   Dissolve them in 500 ml of distilled water. Stir well.

   Dispense the solution in 20 ml vials and keep at 0°C.

**MS BASAL SOLUTION** *

For 1 liter of basal medium mix:

   100 ml of Stock Solution A
   10 ml of Stock Solution B
   5 ml of Stock Solution C
   10 ml of Stock Solution D
   100 mg of Inositol

   Make up to 1 liter with distilled water

* Stocks A+B+C+D
PREPARATION OF SOLUTION VGA* (VITAMINS AND GIBBERELLIC ACID)

For 500 ml of VGA mix:

Thyamine HCl 10mg

Glycine 200mg

Nicotinic acid 50mg

Piridoxine HCl 50mg

Gibberellic acid

(Stock 1 000 ppm) 10ml

Make this solution up to 500 ml with distilled water

Distribute this solution in 20 ml vials.

Use 5 ml/l

* This solution was previously called MSA.

PREPARATION OF VITAMIN SOLUTION

For 500 ml of solution mix:

Thyamine HCl 10mg

Glycine 200 mg

Nicotinic acid 50 mg

Piridoxine HCl 50mg

Make it up to 500 ml with distilled water

Distribute this solution in 20 ml vials.

Use 5 ml/l.
PREPARATION OF HORMONE STOCK SOLUTION

GIBBERELLIC ACID (GA3)

Stock solution of gibberellic acid: 1,000 ppm

1. Weigh 0.2 g of gibberellic acid and dissolve well with some alcohol drops.

Add 200 ml of distilled water

2. Keep in a conveniently labeled vial at 0°C.

The gibberellic acid may be sterilized together with the culture medium: however, the loss of some activity is also possible.

One ml of concentrate solution (1,000 ppm) contains 1 ml of gibberellic acid.

NAPTHALEN ACETIC ACID (NAA)

1 Stock solution of NAA 1,000 ppm

Weigh 0.2 g of NAA and dissolve well with some NaOH 1N drops.

2. Add 200 ml of distilled water.

Keep it in a conveniently labeled vial at 0~O.

One ml of stock solution (1,000 ppm) contains 1 mg of NAA.

BENZYL AMINOPURINE (BAP)

Stock solution of BAP: 1,000 ppm

1. Weigh 0.2 g BAP and dissolve well with some drops of NaOH 1N.

Add 200 ml distilled water.
2. Keep in a conveniently labeled vial at 0°C.

BAP may be sterilized together with the culture medium; however, the loss of some activity is also possible.

One ml of stock solution (1,000 ppm) contains 1 mg of BAP

**INDOLE ACETIC ACID (IAA)**

Stock solution of IAA: 1,000 ppm

1. Weigh 0.2 mg of IAA and dissolve well with some alcohol drops. Add 200 ml of distilled water.

2. Keep it in a conveniently labeled vial at 0°C.

Sterilization by filtration is recommended.

One ml stock solution (1,000 ppm) contains 1 mg of IAA.

**KINETINE (Kn)**

Stock solution of KIN: 1,000 ppm

1. Weigh 0.2 g Kn and dissolve well with some drops of NaOH 1N. Add 200 ml of distilled water.

2. Keep in a conveniently labeled vial at 0°C.

Kn may be sterilized together with the culture medium; however, the loss of its activity is also possible.

One ml of the stock solution (1,000 ppm) contains 1 mg of KIN.
2, 4-D

Stock solution of 2, 4-D: 1,000 ppm

1. Weigh 0.2 g of 2, 4-D and dissolve well with some alcohol drops. Add 200 ml of distilled water.

2. Keep in a vial conveniently labeled at 0°C.

2, 4-D may be sterilized together with the culture medium; however, a loss of its activity is also possible.

One ml of the stock solution (1 000 ppm) contains 1 mg of 2, 4-D.

PREPARATION OF CALCIUM HYPOCHLORITE

1. Weigh 50 g of calcium hypochlorite Dissolve it in 1,000 ml of distilled water (50/0).

2. Shake it for 3 to 4 hours and let it rest B to 8 hours, or over night.

3. Filter the solution by using a filter paper and maintain it hermetically closed in a flask in a safe place.

4. Use 50 ml of solution and add 50 ml of distilled water

PREPARATION OF SOLUTIONS FOR pH ADJUSTMENT

Solution to bring down the pH - Hydrochloric acid (HCL) I N

1. Pour 91.4 ml of distilled water into a beaker (Use a mask and gloves to protect you from the acid vapors).

2 With a pipette take out 8.8 ml of hydrochloric acid (commercial concentrate, 36. 5-38.00/o).
**WARNING:** Do not breathe when taking out the acid. Use a rubber-bulb pipette.

3. Homogenize and keep in a broad-mouth vial, closed and at room temperature.

**Solution to bring up the pH** Potassium hydroxide (KOH) 1 N

1. Place 50 ml of distilled water in a beaker

2. Add 5.6 g of KOH and dissolve well.

3. Bring to 100 ml with distilled water. Keep it in a closed broad-mouth vial at room temperature.

**Uses:** According to pH of the medium, add the solutions drop by drop until the required pH is reached.

**In Vitro Culture Techniques**

**Micropropagation Technology**

Micropropagation technology is being widely utilized commercially in the ornamentals industry and in other plant production organization. This propagation method was widely used after the discovery of plant growth regulators, auxins and cytokinins. The discovery of auxin (IAA) and cytokinin (kinetin) created the great opportunities for *in vitro* propagation of higher plants (Pierik, 1997). Some *Phyllanthus* species had been reported to be propagated by in vitro culture techniques. (Rajasubramaniam and Saradhi, 1997) had successfully induced 14 – 16 shoots from each shoot tip for *Phyllanthus fraternus* by using B5 medium supplemented with 10-5 μM BAP. (Catapan, *et. al.* 2000) reported that an average of 21 - 23 shoots could be induced from each nodal segment of *Phyllanthus caroliniensis* using MS medium supplemented with either 5.0 μM BA, 1.25 – 5.0 μM
kinetin or 2.5 – 5.0 µM 2iP. However, 16 - 20 shoots were formed from each nodal segment of *Phyllanthus urinaria* with the presence of 1.0 mg/L kinetin in B5 medium. Lee and Chan (2004) reported that multiple shoots could be produced from the nodal segments of *Orthosiphon stamineus* using MS + 0.5 mg/L BA. Most of the plant cultures could be subcultured once they were established. In fact, subculturing often becomes imperative to maintain the culture or to increase its volume (George and Sherrington, 1984). For example, repeated subculturing of the *in vitro* individual shoot of *Spilanthes acmella* in the proliferation medium could increase the formation of multiple shoot by three folds (Ang and Chan, 2003). There are five basic stages for successful micropropagation of plantlets. The first stage, the preparative stage or stated as phase zero, involved the correct pretreatment of the starting plant material so as to ensure they are disease free as far as possible. The second phase is the establishment of clean starting tissue for aseptic growth and development. It involves a sterilization protocol for producing aseptic tissues. These aseptic tissues will be used for the next stage of shoot multiplication which can be carried out in a number of ways. Generally plant growth regulators are used for shoot multiplication. The shoots obtained in phase two will be used for root induction at the third phase either *in vitro* or *in vivo*. Finally, at phase four, the *in vitro* plantlets are acclimatized for better survival when transferred to greenhouse conditions or to the soil (Pierik, 1997).

### 2.2.1.2 Plant Growth Regulators

The most usual groups of plant growth regulators (PGR) used in tissue culture research are the auxins and cytokinins. The amount of PGR in the culture medium was critical in controlling the growth and morphogenesis of the plant tissues (Skoog and Miller, 1957). Generally a high concentration of auxin and a low concentration of cytokinin supplemented into in the medium could promote cell
proliferation with the formation of callus. On the other hand, low auxin and high cytokinin concentration in the medium resulted in the induction of shoot morphogenesis. Auxin alone or with the presence of a very low concentration of cytokinin was important in the induction of root primordia (Pierik, 1997). There are a number of naturally occurring auxins, however, most of these are not generally available for routine use. Because of their stability, synthetic auxins are extensively employed. The most commonly used are 2,4-dichlorophenoxyacetic acid (2,4-D), 1-napthaleneacetic acid (NAA) and indole-3-butyric acid (IBA). In some chemical compounds which are not strictly auxins, such as dicamba (3,6-dichloro-o-anisic acid) or picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid), have been used as auxin to substitute IBA.

Both of these compounds are herbicides when used at higher concentration (Davies, 1987). They are found to occur naturally in many plants including olive and tobacco (Epstein, et al. 1989). In many instances, addition of any one of these auxins to a basal medium may be enough to initiate and sustain callus growth. However since there may be different sites of action or target molecules, it can be helpful to use more than one auxin simultaneously or achieving the correct balance of the auxin and cytokinin especially when the tissue is recalcitrant (George and Sherrington, 1984). According to Murthy et al. (1998), recalcitrance could be mitigated by the application of other potent synthetic plant growth regulators such as thidiazuron (N-phenyl-n-1,2,3-thidiazol-5-yl urea). Tissue culture of monocotyledons, particularly cereal grains and palms, had been achieved in some cases through the use of rather high levels of synthetic auxins like 2,4-D. High levels of auxin could act as herbicides but cell proliferation in the absence of exogenous cytokinin was frequently achieved, Morphogenesis such as the formation of somatic embryos or adventitious organs from callus tissues was
observed when the auxin was removed or lowered in the culture medium (Krikorian, et. al. 1987). Cytokinins of adenine derivatives are characterized by the ability to induce cell division in tissue cultures usually in the presence of auxin. The most common type of cytokinin found in plants is zeatin. Cytokinins also occurs as ribosides and ribotides. In tissue culture and crown gall culture, cytokinins promote shoot initiation. Lee and Chan (2004) reported that multiple shoots could be produced from the nodal segments of *Orthosiphon Staminous* using MS + 0.5 mg/L BA. In moss, cytokinins induce bud formation. Kinetin, the prototype molecule for the synthetic adenylic cytokinins and zeatin which is about 10 times more potent and generally considered the prototype of the naturally occurring cytokinins, are widely used in tissue culture. Dihydrozeatin, also naturally occurring, is not widely used compared to kinetin or zeatin (N6-triangole2-isopentenyl adenine) (Davies, 1987). Benson (2000) reported that TDZ (1-phenyl-3-(1, 2, 3-thia-diazol-5-yl urea) could display both auxin- and cytokinin type activities and this was most likely due to it having both phenyl and thidiazol groups. Adenine was occasionally added to tissue culture media and acted as a weak cytokinin by promoting shoot formation (Beyl, 2000, 2005). Gibberelic acid (GA3), the end-product of GA metabolism in *Gibberella fujikuroi*, has been commercially available for many years. Its application to dwarf or rosette plants, dormant buds, or dormant seeds can result in dramatic and diverse effects on growth. GA3 can also stimulate the production of numerous enzymes notably alpha-amylase in germinating cereal grains. For fruit setting and growth, this can be induced by exogenous applications in some fruit (e.g. grapes). GA3 can also induce maleness of dioecious flowers (Metzger, 1987). In tissue culture, GA3 was used for inflorescence proliferation to bypass juvenility and maintain the adult phase as most of the perennial plants usually passed through a long juvenile phase of vegetative development before flowering. (Lin, et al. 2004) reported that
ginseng buds were cultured on B5 medium supplemented with 1 mg/l BA and 1 mg/l gibberellic acid to develop new inflorescences for somatic embryogenesis. The regenerated plantlet from the embryogenic callus was found to have a juvenile phase and grew normally. (Ohlsson and Berglund, 2001) found that gibberellic acid could enhance anthocyanin content in the cell culture of periwinkle. This indicated that GA$_3$ could also enhance the metabolic activity within pathway that lead to stress related secondary metabolites and anthocyanin biosynthesis.