CHAPTER -III

MATERIALS AND METHOD:

3.1 PHARMACOGNOSTIC AND PHYTOCHEMICAL STUDY

3.1.1 Collection and Authentication of Specimen

The plant of *Tecomella undulata* (Sm.) Seem was collected from the garden of H. N. G. University, Patan District, Gujarat (India). On the north side of the Gujarat state at 23°41’ to 23°55’ North Longitude and 71°31’ to 72°20’ east Longitude. The plant material were identified and authenticated by Dr. R.S. Patel associate professor of Maninagar science college Ahmedabad.

3.1.2 Drying of plant material

The leaves and stem of *Tecomella undulata* (Sm.) Seem after collection were first washed and cleaned with water and subjected to dry under shade for about 15 weeks. Shade dried and they were then powdered using a mortar pestle for reduction of size. It was passed going through mesh 40 and the fine powder was collected and stored in air tight container to protect from light and moisture for further analysis.

3.1.3 Pharmacognostical study

Macroscopic and microscopic study of both stem and leaf of *Tecomella undulata* prescribed in method (Khandelwal et. al. 1996 and Kokate C. et. al. 2005, 2008)

Macroscopic study
The fresh leaves and stem of plant were taken for macroscopic organoleptic evaluation like size, shape, colour, surfaces, appearance, fracture, texture, odour and taste.

**Microscopic study**

The thin transverse sections of fresh stem and leaf of *Tecomella undulata* were taken and placed in watch glass containing chloral hydrate solution and boil for few minutes until they are transparent then stained with the mixture of phloroglucinol and HCl.

**Powder Microscopy**

A pinch of powder of both stem and leaf treated with various chemical reagents like chloral hydrate solution, phloroglucinol, HCl, glycerin and iodine analyze to various characteristics.

### 3.1.4 Determination of physicochemical parameters

(Indian Pharmacopeia, 1996 and Kokate CK, 2008)

The physical-chemical parameters, the total ash, water-soluble, acid insoluble ash, loss on drying and extractive values such as the crude soluble in petroleum ether, chloroform soluble, methanol soluble, ethyl acetate soluble, acetone soluble and water-soluble extract should be calculated on the basis values specified in the Indian pharmacopoeia and WHO Guidelines.

**Determination of Ash Values**

**Total ash value**
5g fine powder of stem and leaf of *Tecomella undulata* was taken in crucible and to burn to ashes at temperature increase up to 450 °C and cooled at room temperature then to measured weight. The total ash was measured in percentage.

\[
\text{Ash\%} = \frac{\text{Loss in weight}}{W} \times 100
\]

**Acid-insoluble ash**

The ash + 25 ml 2N HCl boiled for five min. The remaining insoluble matter was collected, wash off with water and hot water cooled, and weighed. Acid insoluble ash was measured in percentage.

\[
\text{Ash\%} = \frac{\text{Loss in weight}}{W} \times 100
\]

**Water-soluble ash**

Take 5g ash and added 25 ml D/W boiled for 5 min insoluble material, collects less paper filter, washed in hot water is turned on 15 minutes is not higher than 450 °C. Calculated the water-soluble ash from drug powder.

\[
\text{Ash\%} = \frac{\text{Loss in weight}}{W} \times 100
\]

**Loss on Drying**

5gm of stem and leaf powder of *Tecomella undulata* was collected and not down the initial weight. The stem and leaf powder were heated at 105 °C in hot air oven and to measured weight. This procedure was to repeat again and again till a
constant weight was obtained. After drying was completed tarred glass bottle was keep for cooling at room temperature in desiccators.

\[
\text{Loss on drying (\%)} = \frac{\text{Loss in weight}}{W} \times 100
\]

**Extractive Values**

The extractives of various solvent of dried sample were evaluated by prescribed method given in Indian Pharmacopia, 1996.

**Organic solvent soluble extractive value:**

Weight 5g of shade-dried stem and leaf powdered of *Tecomella undulata* was mixed with 100 ml of organic solvent was a closed vessel, stirring frequently, the first 6 hours and 18 hours, and then allowed standing. After this process it was filter and filtrate was dry at 105°C then measured weight in Percentage.

**Water soluble extractive value:**

Weight 5g of dried stem and leaf powdered of *Tecomella undulata* was mixed with 100 ml of water was a closed vessel, stirring frequently, the first 6 hours and 18 hours, and then allowed standing. After this process it was filter and filtrate was dry at 105°C then measured weight in Percentage. Other extracts were found with same procedure.

**3.1.5 Preliminary Phytochemical Screening**

The methods used to detection for various phytochemicals present in leaf and stem powder of *T. undulata* like alkaloids, glycosides, saponnins, sterols, phenolic compound, tannins, quinones, anthroquinones, triterpinoids, protein, carbohydrates etc. The qualitative tests for phytoconstituents present in were *T. undulata* were
carried out by prescribed method (Harborne, 1998; Evans and Trease, 2002; Kokate et al., 2008; Patter et. al., 2011).

A. Test for Alkaloids

The stem and leaf extracts of *Tecomella undulata* was converting into base using NH$_4$ and added chloroform for converting into acidic form with dilute HCl.

1. Mayer’s reagent test

In this test used Potassium Mercuric Iodine solution. 1ml of the filtrates was added with 1ml of Mayer solution gave cream color ppt.

2. Dragendorff’s reagent test

In this test used Potassium Bismuth Iodide. To give treatment to acid layer with Dragendorff solution. Show reddish brown ppt.

3. Wagner’s reagent test

To give treatment to filtrate with Wagner solution show reddish brown ppt.

4. Hager’s reagent test

To give treatment to filtrate with Hager solution gave yellow ppt.

B. Test for Glycosides

Extracts were treated for Raymond’s test, Legal’s test, Baljet’s test and Modified Borntrager's test for the detection of glycosides.

1. Raymond’s test

Treat the extract with dinitro- benzene in hot methanolic alkali, violet colour is produced.

2. Legal’s test
Treat the extract with alkaline sodium nitroprusside solution after dissolved in pyridine, blood red color is formed.

3. Baljet’s test
Treat the filtrate with picric acid formation of yellow to orange colour is formed.

4. Modified Borntrager’s test
Add few drop of FeCl₃ solution and boil for 5 min then add the benzene solvent in equal amount extracts. The benzene layer dissolved in ammonia solution (half of its volume). Develop cherry red colour.

C. Test for Cardiac Glycosides
The cardiac glycosides present in plants Tecomella were detected by Kedde’s test and Keller killiani test.

1. Kedde’s test
The stem and leaf powdered of Tecomella undulata were extracted using chloroform and dryness to it by evaporation. Add 90% of alcohol then adds 2 drops of Kedde’s reagent. Develop purple color.

2. Keller killiani test [test for Deoxy sugars]
The stem and leaf powdered of Tecomella undulata were extracted using chloroform and dryness to it by evaporation. Add few drops of FeCl₃. Then carefully 0.5ml of concentrated H₂SO₄ was added. The blue color is produced.

D. Test for Saponin
The presence of saponin in plants was identifying by Forth test and Hemolysis test.

1. Froth Test
Place 2ml solution of drug mixed with water and shaking for 15minutes, stable foam is formed.
2. Hemolysis test

Take 2 ml of 18% NaCl solution in both test tubes. In one test tube only added 2 ml d/w and in other test tube added 2 ml of 1% extracts then added few drops of blood in both test tube and mixed. Show the hemolysis reaction using microscope on slide.

E. Test for Tannins & Phenolic Compounds

The presence of tannins and phenolic compounds was estimating by Gelatin test, FeCl₃ test, Vanillin Hydrochloride test and Lead acetate test.

1. Gelatin test

Add 1% gelatin solution containing 10% NaCl in to test solution. Develop white ppt.

2. FeCl₃ test

Addition of 5% iron(III) chloride solution in to filtrates, blackish blue colour is produced.

3. Vanillin Hydrochloride test

A few drops of vanillin hydrochloride reagent added in test solution formed pinkish red color.

4. Lead acetate test

Add 10% lead acetate solution in test solution formed yellow or white ppt.

F. Test for Flavonoids

1. Shinoda test

Add Magnesium ribbon in the filtrates and then add HCl drop wise. Developed pink or red color.

2. Zinc Hydrochloride reduction test
Add Zn dust and strong HCl in the test solution develop red color after some times.

3. Alkaline reagent test

Add NaOH solution in the test solution. The produced yellow color was disappeared after adding mild HCl.

4. Aluminium test

Treat the extract is with 1% aluminium solution, yellow color is produced flavonoids are present.

G. Test for Anthroquinone

The test material was heated with 10% FeCl$_3$ solution and 1ml of conc. HCl. The aqueous layer was separated and shaken with diethyl ether and further shake it with strong ammonia, pink or deep red color is formed.

H. Test for Proteins & Amino Acids

1. Millons test

Add 2ml of Millons reagent with test solution and heated briik red color is produced.

2. Xanthprotic test

Taken the stem and leaf extracts of $T. undulata$ in test tube then added 1ml of concentrated nitric acid. This test tube was boiled for 1 minute. Precipitate is formed after adding liquid ammonia.

3. Biuret test

3 ml of stem and leaf extracts of $T. undulata$ was taken in test tube and added 40% sodium hydroxide and few drops 1% copper sulphate solution was added. Develop violet color.
4. **Ninhydrin test**

The test solution boiled for few minutes with 0.2% solution of Ninhydrin produced blue colour.

I. **Test for Steroids & Triterpenoids**

Libermann- Buchard reaction and Salkowski test were performed to assess the presence of sterols and triterpenoids.

1. **Libermann- Buchard test**

Taken 1 ml of extracts, added 1 ml chloroform and 2-3 ml of acetic anhydride then mixed. In this mixture added 1-2 drops of strong H$_2$SO$_4$ two layers is formed, develop green layer shows presence of steroids and red color shows presence of triterpenoids.

2. **Salkowski test**

Add strong H$_2$SO$_4$ in the filtrate shaken and standing, the lower layer was red colour when a steroid present and develop yellow colour when a triterpenoids present.

J. **Test for Carbohydrates**

Dissolved few amount of stem and leaf extracts of *T. undulata* in little amount of distilled water then separately filtered and filtrates were used for other tests of carbohydrates.

1. **Molisch’s test**

Filtrates with Molisch solution taken in test tube then 0.2ml of con. H$_2$SO$_4$ carefully added the sides of test tube. At the junction violet ring was formed.

2. **Benedict’s test**

2 ml Benedict’s solution was added into filtrate containing test tube and boiled in water bath. Develop Green reddish brown precipitate.
3. Fehling’s test

Dilute hydrochloric acid used to hydrolyze the filtrates and alkali reagent used for neutralization and heated with equal amount of Fehling’s A and B reagents. Develop green to yellow or red ppt.

4. Barfoed’s test

Add a few drops of Barfoed’s reagent (70gm copper acetate monohydrate and 9ml glacial acetic acid in water)to test solution , boiled on water bath, gives orange red.

5. Tollen’s phloroglucinol test

2ml test solution is treated with 2.5ml of concentrated HCl and 0.5% phloroglucinol, heated from yellow to red color produced.

Quantitative Phytochemical Determination

1. Total phenolic content determination

Folin -ciocalteu reagent method was used to determination of total phenolic content.

- Take 1ml of different plant extracts (leaves and stem) of *Tecomella undulata*
- Add 0.5 ml 1N Folin –ciocalteu reagent.
- Allow standing for 10 min.
- 4 ml Na₂CO₃ solution added.
- The reaction mixtures were incubated for 30 min.
- The total phenolic content was measure at 750 nm.
Gallic acid solutions used as standard and was expressed in terms of mg/g phenolic content.

2. Determination of total Alkaloids content

- Take 1 ml different plant extracts *Tecomella undulata*
- Add 5 ml phosphate Buffer.
- Then add 5 ml of bromocresol green solution
- The mixture was shaken with 4 ml chloroform.
- After that the reaction mixtures is collected in volumetric flask (10 ml) and make final volume 10 ml with chloroform.
- The total alkaloid content was measure at 470 nm.
- Atropine solutions used as standard and expressed in terms of mg/g alkaloid content.

3. Total Tannin content Determination

Folin - Ciocalteu method was used for the determination of tannins using tannic acid as a standard.

- Take 1 ml of plant extract is collected in volumetric flask (10 ml)
- 4.0 ml D/W was added.
- Then add 0.4 ml of 1N Folin- ciocalteu reagent.
- Then add 2 ml of saturated Na₂CO₃ solution.
- Make final volume 10 ml with distilled water.
- Measured OD against the blank at 700 nm with spectrophotometer.
- The results were expressed in mg /g of plant extract.
4. Total Flavanoid content Determination

Aluminium chloride method was used for determination of total flavanoids content. Quercetin was used as standard for determination of total flavonoids content.

- 1ml of various plant extracts were collected in volumetric flask (10ml).
- 4 ml water was added and allows standing for 5 min.
- Then add 0.5 ml 10% AlCl$_3$.
- 0.5 ml of 5 % CH$_3$COONa (NaOAc) was added and allows standing for 5 min.
- Add 2ml of 1 M NaOH in test solution and make 10 ml final volume with D/W.
- Measured the OD at 415 nm against a blank with an UV spectrophotometer.
- The content was expressed in mg/g dry weight of extracts.
5. Estimation of Chlorophyll Content by Arnon (1949)

Chlorophyll is a pigment present in plants as primary constituents and absorbs light from the sun and plays a vital role in the photosynthesis process.

**Extraction of Chlorophyll Content**

- Take 100 mg of frozen plant (leaf and stem) tissue.
- Homogenize with 80% chilled acetone (10ml).
- Centrifuge at 5,000 rpm for 10 min.
  - Collect supernatant A in vial.
  - Residue + 10ml 80% acetone.
  - Centrifuge at 5,000 rpm for 10 min.
  - Collect supernatant B in vial.
  - Discarded residue.
- Mix supernatant A and B.
- Calibrate spectrophotometer against acetone at 645 and 663 nm.
- Measure absorbance at 645 and 663 nm.

Calculate total chlorophyll content.
Chl. a (mg/g fresh wt.) = (12.7 × A_{663}) - (2.69 × A_{645}) × V/1000×W

Chl. b (mg/g fresh wt.) = (22.9 × A_{645}) - (4.68 × A_{663}) × V/1000×W

Total Chl. = Chl. a + Chl. B

Where
V = volume of the sample
W = weight of fresh tissue

3.1.6 Qualitative Analysis of Phytochemicals by TLC and HPTLC

Screening of the phytochemicals of the stem and leaf extracts was conducted through the use of Thin Layer Chromatography (TLC).

**Preparation of standard solution**

Dissolve 2mg of lapachol in 25ml of chloroform in a volumetric flask. Concentration of standard solution was (80µg/l).

**Procedure**

Aliquots of 15 µl of the chloroform extracts of the stem and 15 µl of the methanolic extracts of the leaves and 10 µl standard solution of lapachol was applied on a precoated silica gel 60 F_{254} TLC plate of 2mm thickness. The TLC plate was placed in a developing chamber and allowed to develop in a mobile phase consisting of toluene: chloroform: formic acid: methanol (6:0.5:0.2:1.5). Once developed, the TLC plate was removed and sprayed with vanillin sulphuric acid reagent, heat the plate at 70° C. and then viewed under UV light (Camag) of wavelength 254 nm and 356 nm.

After spraying with vanillin sulphuric (Dissolve 1gm of vanillin in 100ml of ethanol and 5ml of concentrated sulphuric acid) reagent different colour bands were
observed and corresponding Rf values are determined. Calculated Rf value of each spot as;

\[
Rf = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent (cm)}}
\]

3.2 ANTIMICROBIAL ACTIVITY

The plant extracts (stem and leaf) of *Tecomella undulata* were tested for their antimicrobial activity.

3.2.1 Extracts Preparation

The leaves and stem of *Tecomella undulata* (Sm.) Seem after collection were first washed and cleaned with water and subjected to dry under shade for about 15 weeks. The dried material crushed and made coarse powder with using mortar pestle for size reduction and stored in air tight container to protect from light and moisture for further analysis.

The dried stem and leaf powder were extracted using a different polarity solvent to petroleum and methyl alcohol, acetone, ethyl acetate and water by using shaker for 72 hrs and filtered the mixtures with Whatman No. 1 filter paper. Then collected these filtrates individually, concentrated by evaporating the solvents and used for antimicrobial activity.

3.2.2 Selection of bacterial and fungal strains

For this study *B. subtilis, S. aureus, E. coli, P. aeruginosa* bacterial and *C. albicanse* fungal strain were selected. The organisms were inoculated in to N-broth in order to determine their viability and maintain on N-agar slant at 4°C.
3.2.3 Agar Disc Diffusion Method

The antimicrobial activity was selected plant extract was done by an agar disc diffusion method by using pacific bacterial and fungal test organism was used for antimicrobial activity from the different stem and leaf extracts of *Tecomella undulata* (Langfeild *et al.*, 1995; Ncube *et al.*, 2008). N-agar (Appendix 2) for bacterial strain and Sabouraud’s agar (Appendix 4) for fungal strain were used.

After solidification of Nutrient agar medium, test organisms were spread on Petriplate under aseptic conditions. To made 5mm disc on the both type of agar medium using sterile cup borer and different plant extracts were poured.

As a negative control used standard antibiotic solution like Streptomycin (for Bacterial strain) and Nystatin (for Fungal strain) used as a positive control.

All the plates were put for incubation at 37°C (fungal plates at 28°C) for overnight. Without adding the cultures of organisms used as a negative control under sterile conditions.

Measure the zone of inhibition for shown the antimicrobial activity from the respective plant extracts and antibiotic. Three times the whole experiment was faults and the mean values were tabulated.

3.3 MICROPROPAGATION the advanced technique

The advanced technique has been use to study the be effective growth of plant in a minimal required medium. Micropropagation is an efficient technique for multiplication of the endangered species and *ex situ* conservation of plant biodiversity. It is the rapid propagation method to produce microbiologically sterile plants in limited space under controlled and aseptic conditions. In this study micropropagation was carried out using leaf segment and internodal segment of *Tecomella undulata*. 
3.3.1 Source of Explants

The field grown plant *Tecomella undulata* was selected for the source of explants in the present investigation. Leaf and internodal segments were used as explants for micropropagation. Explants used for study were obtained from plants growing in the H.N.G. University botanical garden, Patan (Gujarat).

3.3.2 Explants Sterilization

The sterilization is the crucial step for explants preparation. The explants were first washed off by tap water for removal of external dust and then deep in Tween-20 for 10 minutes for surface sterilization, after that they were treated with a fungicide 1% bavistin and shake well for 30 minutes in case of leaf and for 1hrs in case of internodal segments, washed with sterilized distilled water.

Thereafter they were taken in Petri plate and sterilized by NaOCl for 5 min, followed by 0.1% HgCl₂ for 2-5 min, then after in 70 % ethanol for 2-3 min. These were subsequently cleaned 4-5 times washed by sterilized double distilled water under aseptic conditions for removal of traces of sterilant.

3.3.3 Culture Medium (Appendix-1.)

The medium used for cultivation this MS medium having the essential nutrients containing 3% sucrose and 8% agar is used for the present study. The nutrient medium consisting of macroelements, microelements inorganic salts, organic constituents and vitamins. The MS medium was augmented with different PGRs including BAP, KIN, 2, 4-D, NAA and IAA with concentrations ranging from 1.0-3.0 mg/l.

3.3.4 The other regulators that Plant Growth Promoting Regulators (PGRs)
Besides the basal components, some other additives were also included in the medium whenever necessary. These were growth substances, auxins like NAA (a-naphthalene acetic acid), IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2, 4- D (2, 4-dichlorophenoxyacetic acid) and cytokinins like BAP (6-benzylaminopurine) and KIN (6-furfurylaminopurine) and were supplemented in the basal medium separately as well as in different combinations. For the entire study the PGRs concentration was used in mg/l (milligram per liter).

3.3.5 Sterilization of Culture Medium

After the preparations for the medium, medium dose 50 ml of sterile bottles after culture at 121 °C 15 psi 15 - 30 minutes, and were transferred to the media storage room for 48 hrs to check the appearance of any contamination in the medium.

3.3.6 Sterilization of Equipments and Glassware’s

Ultra violate lamp was put in LAF (laminar air flow) for remove contamination which is on for half an hour. The table of laminar air flow was cleaned with 70% ethanol for remove contamination. All the sterilization process does before inoculation. During inoculation hand also must cleaned with 70% ethanol.

Flame was used for sterilization of forceps and scalpels during each inoculation. After sterilization the explants were cultured on MS medium containing various combination or alone plant growth hormones.

3.3.7 Cultural conditions

The cultures were incubated under light intensity (3500- 4000 lux) for 16/8 hrs cycles of light and darkness at 25± 2°C with 55± 5% relative humidity.

3.3.8 Inoculation of explants

Inoculation process was carried in Laminar Air Flow Chamber. The explants after sterilization was cut into pieces (0.5 and 1.0 cm) and inoculated on MS medium
having various concentrations (1.0-3.0 mg/l) cytokinins and auxins individually and as well as with combinations of auxins (0.5mg/l).

3.3.9 The induction of Callus for the selected plant (*T. undulata*)

Callus induction involved inoculation of different explants viz. Leaf and Internode inoculated with different PGRs containing MS medium individually as well as in various combinations. After two weeks callus was formed. Data were recorded in term of number of days required for callus induction, callusing response, visual growth of callus, callus color, texture and the growth of callus (fresh and dry weights) was also measured.

3.3.10 Shoot Regeneration and Multiplication

The developed calli transferred on cultured tube containing various PGRs (1.0-3.0mg/l) having MS medium for multiple shoot development individually as well as in combination. The differentiation of among specific phenomenon like shooting percentage, shooting days, shoots number and length of shoot.

3.3.11 Rooting of regenerated shoots

Developed micro shoots were cultured on different concentrations of NAA, IAA and IBA (0.5-2.0mg/l) containing half-strength MS medium separately. The differentiation of the among specific phenomenon like rooting percentage, rooting days, roots number and length of root.

3.3.12 Hardening of planlets

*In vitro* regenerated plantlets were potted on plastic pots containing equal ratio of sterile sand, soil and farmyard manure (1:1:1), the plantlets were draped with transparent polythene bag to maintain nearly 80% humidity level for first ten- days. After this the plantlets were shifted to green house at 25 ± 2°C under less humidity and natural sun light and finally, they were transplanted in the natural condition.
3.3.13 Statistical analysis

The experiments were conducted had five replicates. The data was represented by mean ± SE. Quantified the all effect of different treatment and determined the level of significance by ANOVA.

3.4 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Here three primers were selected for RAPD analysis. RAPD fingerprints generally use to be a useful to detecting genetic diversity of *in vitro* regenerated plants through plant tissue culture.

3.4.1 Plant material

Mother plant and *in vitro* raised plant was used for RAPD analysis.

3.4.2 Isolation of Genomic DNA from plant tissue (Sequence of primers Appendix-6)

The isolation of DNA was extracted from young leaves of mother plant and *in vitro* developed plant *T. undulata* by modified CTAB protocol (Doyle and Doyle, 1990).

Procedure

- Genomic DNA was isolated from 2 leaf samples of mother plant and *in vitro* developed plant using Chromous genomic DNA isolation kit.
- Genomic DNA was loaded on agarose gel for checking the quality
- Genomic DNA was taken for PCR amplification using Fluorescent labeled RAPD primer (3).
The fluorescent labeled PCR products were run on an ABI 3130 Genetic analyzer.

The Binary output obtained was used to generate phylogenetic relationship between groups.

3.4.3 RAPD analysis

Different 3 primes used for further analysis based on their ability polymorphic amplified products of mother plant and in vitro regenerated plant DNA were mixed with master kit of PCR-RAPD (Williams et al., 1990). After electrophoresis gels were stained with ethidium bromide and visualized in a gel documentation system.

3.5 SOIL ANALYSIS

Physical analysis such as soil pH, soil conductivity, soil texture, water holding capacity (WHC), moisture content and chemical analysis such as chloride, organic carbon (Walkeli-Black method), phosphorus by Olsen’s method (Horta and Torrent, 2007), sulphur and nitrogen (micro-kjeldhal method) of soil were determined by Biochemical test method prescribed by Buurman and klerk, 1996; Singh et al., 1999; Chaturvedi and Sankar, 2006, Jayraman (1981).

Sampling and Processing

It is important to evaluate the characteristic of the soil which provide anchorage and nutrition to the plant, there by influencing the plant growth and development. The top 15cm surface soil was collected from a garden field and the garden soil is spread on tray for removing moisture. Dry soil is store in air tight beg for to check all physical and chemical parameters. (Chaturvedi and Sankar, 2006.)

[A] Physical analysis
1. Soil pH
2. Soil Conductivity
3. Soil texture
4. WHC
5. Moisture Content

[B] Chemical analysis

6. Chloride
7. Organic carbon
8. Phosphorus
9. Nitrogen
10. Sulphur

[A] Physical analysis

3.5.1 Soil pH

Prepare a soil-water suspension by mixing soil sample in distilled water in 1:5 ratios. Shake the suspension thoroughly and measured pH using a pH meter and pH paper.

3.5.2 Soil Conductivity

Electrical Conductivity (EC) is the ability of a material to transmit an electrical charge. Prepare a soil-water suspension by mixing soil sample in distilled water in 1:5 ratios. Shake the suspension thoroughly and measured EC measured using conductivity meter. The conductivity is expressed in volume, which is mS/cm or µS/cm.

3.5.3 Soil Texture

Soil constitutes unconsolidated mineral particles of different size. Soil texture was analyzed with the use of sieves of different number of meshes.

Procedure
100gm of soil was gently crushed and pass successfully through series of sieves of different mesh size. Different sized mineral particles thus obtained are weighed. Now, based on relative proportion of each fraction, the texture of soil is determined as given in table. Soil texture can also be determined by examining soil samples under hand lens.

As per the international society of soil sciences, the weathered minerals are classified as follows:

<table>
<thead>
<tr>
<th>Gravel</th>
<th>&gt; 2.0mm diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse sand</td>
<td>2.0-0.2mm diameter</td>
</tr>
<tr>
<td>Fine sand</td>
<td>0.2-0.02mm diameter</td>
</tr>
<tr>
<td>Silt</td>
<td>0.02-0.002mm diameter</td>
</tr>
<tr>
<td>Clay</td>
<td>&lt;0.002mm diameter</td>
</tr>
</tbody>
</table>

Based on the relative proportion of different sized mineral particles, soil can be classified into four textural groups.

<table>
<thead>
<tr>
<th>Textural group</th>
<th>Relative proportion of different sized mineral particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy soil</td>
<td>85% sand + 15% clay or silt or both</td>
</tr>
<tr>
<td>Loamy sand</td>
<td>70% sand + 30% clay or silt or both</td>
</tr>
<tr>
<td>Loam</td>
<td>50% sand + 50% clay or silt or both</td>
</tr>
<tr>
<td>Silt</td>
<td>90% silt + 10% sand</td>
</tr>
</tbody>
</table>

3.5.4 Moisture Content

It is generally estimated as percentage moisture content on oven dry weight basis. Weigh the empty crucible (A). Take 100g of soil in the crucible and further
weighted (B). Keep the crucible in oven for 12 hours and then cool down the crucible at room temperature and further weight (C).

**Calculation**

\[
\text{Moisture content} \% = \frac{(B-C) \times 100}{(C-A)}
\]

**3.5.5 Water holding capacity of soil (WHC)**

WHC can be determined by the use of air dried and sieved soil, Weight out the empty crucible then filled with soil and kept in a bigger container filled with water that water enters from beneath. Take it out from water container and allow it to drain the excess water until it stops to come out. Weight the crucible (crucible + soil + water) and note down it. Put it in hot air oven at 105°C for overnight. After it weight the crucible (crucible + dried soil).

**Calculation**

Actual weight of moist soil = total moist weight (A) – weight of crucible (C) = X grams.

Actual dry weight of soil = total dry weight (B) – weight of crucible (C) = Y grams.

\[
\text{Water Holding Capacity} \% = \frac{X - Y}{Y \times 100}
\]

**[B] Chemical analysis**

1. Chloride
2. Organic carbon
3. Phosphorus
4. Nitrogen
5. Sulphur
1. Estimation of Chloride from soil

- Take 50 ml of aliquot from soil extract.
- Add few drops of $K_2CrO_4$ then titrate the solution with 0.02N $AgNO_3$ till the first reddish brown tinge appears.
- Note the titer value.

**Calculation**

\[
\text{mg of Cl}^-/100\text{g of soil} = [(0.02 \times V1) \times (200/V) \times (100/40)] \times 35.5
\]

Where, 0.02 = the normality of $AgNO_3$

\[V1= \text{titer value of } AgNO_3 \text{ (ml)}\]

\[V = \text{the volume of aliquot taken for analysis}\]

\[\text{[1ml of 0.02N AgNO}_3\text{ (0.02M eq. AgNO}_3\text{) }=0.00071gm Cl}^-\]

**Note:** Amount of chloride < 0.02 % indicate non-salinized and > 0.121 % indicates strongly salinized soil.

2. Estimation of Organic carbon from soil

- Take 10 ml of soil extract.
- 10 ml $K_2Cr_2O_7$ reagent and 10 ml $H_2SO_4$ reagent added.
Shake well and allow standing for 30 mins.
Add 5 ml of ortho- phosphoric acid.
Keep it for digestion at 130⁰ C in digestor for 1 hour.
Few drops of ferroin indicator was added.
Titrate with Mohr’s reagent until the color flashes from violet through blue to red pinch.
Measure the ml of reagent utilized.

Calculation

COD (mg/l) = \( \frac{(A-B) \times N \times 8 \times 1000}{\text{ml of sample}} \)

Where,

A = FAS for blank
B = FAS for sample
C = Normality of ferrous ammonium sulfate
8 = Milliequivalent weight of oxygen
FAS = Ferrous ammonium sulfate

3. Estimation of Phosphorus from soil

Take 1 gm of soil. Add a pinch of activated charcoal and 20 ml of 0.5M NaHCO₃. Shake the flask for about 1 hour and filter the suspension through whatman filter paper.
Take various aliquots of sample in ml (soil extract).
- Make the final volume 8.6ml with distilled water.
- Add 1ml ammonium molybdate solution.
- Incubate at room temperature for 10mins.
- Add 0.4ml of ANSA reagents.
- Take O.D. at 660nm.
- Put result in standard curve and find out the concentration.

1. **Estimation of total nitrogen by micro-kjeldhal method from soil**

**Digestion**

- Time required for digestion of sample depends on the type of sample. Soil sample take 7-8hrs. And water sample takes 3hrs.
- For digestion of soil sample add acid and other components as under,
  1gm soil or 10ml water Sample + 12ml H₂SO₄ + 3ml HNO₃ + 5ml H₃PO₄ + pinch of catalyst. Or 3gm soil sample + 18ml H₂SO₄ + 4.5ml HNO₃ + 7.5ml H₃PO₄ + pinch of catalyst. During digestion add 5-6 glass beads to prevent bumping.
- On the micro-Kjeldahl digestion apparatus, Set each heating unit. Continuous to boil until solution become transparent pale green.
- For an additional 30min, turn each heating unit up to its maximum setting and digest then cool it.
- Mix H₂O₂ intermittently.

**Dilution**

After digestion, the sample is diluted up to 50ml with the help of ammonia free distilled water.
Distillation and titration

- Wash the whole assembly with double distilled water and add 5ml digested sample in digestion column of Kjeldahl assembly.
- Add 10ml of saturated NaOH. Fill 25ml boric acid solution in 100ml beaker and put the beaker under condenser. Boil the water containing distillery unit with burner, so as produce the steam which boil the sample in digestion column of Kjeldahl assemble and ammonia gas is produced.
- This ammonia gas is collected in flask which contains boric acid; this will react to produce ammonium borate. End point of this distillation is, Make the double volume of boric acid solution. Run experiment with specific time.
- Remove the flask and then wash the assembly with double distilled water, titrate the mix indicator containing ammonium borate with N/50 H₂SO₄. Here color will change from green to pink indicate end point of titration.

Calculation

\[
\text{NH}_3^- \text{ N mg/l} = \frac{(A-B) \times 280}{\text{Volume of sample}}
\]

Where,

A = ml of titrate for experiment

B = ml of titrate for blank

5. Estimation of Sulphur from soil
**Preparation for standard curve**

- Take aliquots from standard solution.
- Make final volume 10 ml with D/W.
- Add 0.5 ml conditioning reagent.
- Add pinch of BaCl$_2$ and mix vigorously and allow standing for 15 mins.
- Filter with Wattman no. 42 paper.
- Take O.D at 420 nm.
- Prepare Graph

**For sample**

- Take 10g of soil.
- Add 25 ml of extracting solution.
- Shake well for 30 min.
- Add 0.25ml of activated charcoal and continuous shaking for 3 min.
- Filter the soil suspension through whatman filter paper under suction.
- Take aliquots and perform as above.
- Put results in standard curve and find out the concentration.