CHAPTER - III
MATERIALS AND METHODS

Study area

The latitude, longitudes and altitude of the study area was found with the aid of the topographical map of Tamil Nadu (Number 58:1/11) procured from the Regional Office of the Survey of India at Chennai. The study area was found with the help of transparent OHP sheet and graph sheet. The scale given in the topographical map was used to calculate total area in which the study was done.

Calculation of total area

The physical boundary was decided on the basis of the ability to reach by human access during the study. This physical boundary was traced over a transparent plastic sheet by placing over the topographical map. The boundary was marked using a permanent marker. The traced boundary was then placed on the graph sheet and full, half and quarter squares were counted and the entire half and quarter squares were rounded up to the full squares. The total number of squares was calculated by following the scale given in the topographical map (1:50,000) i.e., 1 cm in map is equal to 0.5 km in ground truth.

Mapping of the study area

Mapping is an integral component of biodiversity inventorying studies. Maps depict the physical features and boundaries, and help to visualize vast spatial distribution of the information on a small sheet of paper.
Various kinds of maps such as the Toposheets, Revenue maps, Satellite imageries and village maps were used to classify the landscape elements of the study area. The Toposheets are quite useful for locating various types of forests, streams and rivers. Toposheets (or topographical maps) are prepared on the basis of intensive ground survey by the Survey of India. It gives a good picture of the physical features such as fresh water bodies, altitudes, contours, roads, vegetation pattern, etc.

**Field maps**

The field maps of Malaiyur village were prepared by pooling the data from the following maps.

- Revenue maps,
- Taluk maps,
- Toposheets, and
- Satellite imagery (open source)

The field map carried information about village boundaries, latitudes and longitudes. The study site has both natural and manmade ecosystems. In the beginning an intensive field survey was made to record different landscape elements and then the ground truths were incorporated in to the field map of Malaiyur with the help of the survey of India Toposheet.

**Satellite map**

The use of remote sensing for studying natural resources of various regions has practically revolutionized environmental studies. Satellite orbiting in the sky overhead has been provided reliable, repeated, observations on the earthscape ever
since the Landsat satellite went up in the year 1972. The remote sensing satellites received signals from light reflected and emitted from earth’s surface on many different intervals of wavelengths. The signal received is recorded as digital information, it can be converted in to an image on the computer monitor or a photographic print by assigning separate colors to each band. Since the colors assigned are different from the original, these images are called false color composites. False colour composite imagery available in open source (Google Earth) were used in the present study for the classification of the landscape elements by supervised classification method of GIS 9.3 ESRI.

A 117.5 sq. km study area in Pachamalai, spanned in Tiruchirappalli and Salem district was classified using Google Earth satellite imagery. In the satellite imagery of the study area, the following landscape element types have been determined: dry deciduous forest, dry evergreen, scrub jungle, riparian vegetation, cultivated land and grazing land (dominated mostly by grasses).

Identification of vegetation types

Different types of landscape elements in the study site were identified and mapped with the help of a survey of India toposheets. This landscape map was compared with a hard copy of false color composite of satellite imagery and correspondence was established between ground truth and imagery. Certain ground control points which can be reliably located on the topographic maps on the satellite image were identified and ground truthed. All this information lead to generation of supervised classification of the landscape.
Land holding information, Cropping patterns and other agricultural data were studied with the help of village revenue information. Landscape map was extrapolated with information obtained on earlier status of the landscape from topographic map, satellite imagery, and village maps, and also from interviews with the local people to construct the ecological elements of the landscape viz. dry evergreen, deciduous forest, riparian, scrub jungle, cultivated land and grazing land (dominated mostly by grasses) (Gadgil, 2001). In the Forest management scenario, the study site spanned into three major reserved forests of the Pachamalai hills. The study site was divided based on the human approach in terms of human settlements. A total of nine human settlements namely, Valasakalpatti village (cultivated field and scube forest), Edappadi village (scube forest riparian vegetation, dry evergreen and deciduous), Melpalthankarai village (cultivated field), Keezhpalthankarai village (cultivated field), Naripadi village (cultivated field), Sinnakarattur village (cultivated and scrub forest), Uthambium village (scrub jungle, dry evergreen, deciduous, grazing land and cultivated field), Murungappatti village (cultivated field) and Manmalai village (scrub forest, dry evergreen, deciduous, grassland and cultivated field). The vegetation types of each of the above locations were treated as different landscape elements. A total of 45 sampling points were selected in these areas for the vegetation analysis. Vegetation type, landscape element (Gadgil, 2001) of each location was decided based on the existing method of forest classification. Though the sampling sites spanned in a same location their disturbance level, altitude, and formation was considered in further dividing them into landscape elements.
Inventory of species Diversity

Field visits were organized in every season for overcoming the identification problem by collecting the specimen in their flowering stages. Random enumeration of the species was made. The new unidentified specimens alone were preserved as herbarium collection and identified later by referring specimens at Southern Regional Centre, Botanical Survey of India, Coimbatore and Rapinat Herbarium and Centre for Molecular Systematics, Tiruchirappalli, Tamil Nadu. The random enumeration was carried out. The taxonomic identification was made following Bentham and Hookers system of classification as reported by Gamble (1935), Mathew (1982, 1983, 1988, 1996, 1998) and Pallithanam (2001).

Vegetation analysis

The study was classified into different landscape elements. Transect with quadrat method was followed for sampling vegetation. Transects were laid along straight lines. The length of transect was kept constant as 450 m transect, two or three smaller parallel transects were laid in smaller landscape elements. In a 450 m transect 9 quadrates of 10 m × 10 m in alternating sequence were laid with an inter quadrat distance of 40 m. The corner trees of transect and quadrat were marked with their respective numbers using paint to facilitate repeated future sampling along the same transect. Trees were sampled in 10 m × 10 m. shrub layers were sampled in 5 m × 5 m - sub-quadrat within the main quadrat. Herb layer was sampled in two separate sub - quadrat of 1 m × 1 m within 5 m × 5 m shrub quadrats.

In the multiple stage sampling method, the major sampling unit is square in shape and 10 m × 10 m in size. Minor sampling units of 5 m × 5 m and 1 m × 1 m
were arranged inside the major sampling unit in a cluster. To begin with, the four corners of each major plot was marked with painted iron pegs. Additional marks were placed at every 2 m point on the outer boundary of the plot (Diagram-1).

**Diagram-1**

*Schematic representation of the ecological sampling through transect and quadrat method*

The samples were counted for their presence and/or absence and abundance in each of the sample. The quadrat data of all the transects were pooled together in one sample. Species abundance matrix was generated; the diversity and similarity indices were calculated using Microsoft Excel Program.

**Diversity indices**

The alpha scale diversity (the level of diversity as calculated index in each sample) was calculated in each of the transect and its quadrats. The Shannon and
Weiners Diversity Index was calculated by using the following formula (Shannon and Weiner, 1963; Whittaker, 1975; Gadgil, 2001; Ghate et al., 2001):

\[ H' = -\sum p_i \ln p_i \]

where \( p_i \) = Relative abundance of the species

\( \Sigma p_i \) = Sum of all the relative abundance of all the species encountered in the sample

**Soil sample collection**

Soil samples were collected randomly from three permanent places in every three study site at monthly intervals from January 2007 to December 2007. The soil samples were collected by coring method from 0 to 10 cm and 10 to 20 cm soil layers. The soil samples from the core was homogenized thoroughly. The composite soil sample was used for detailed analysis.

Efforts were made to take the samples in such a way that it is representative of the site. Only one to ten gram of soil was used for each chemical analysis.

**Sampling procedure**

A map was prepared of the area to be covered in a survey covering different landscape elements. The geographic coordinates of samples was entered on the map, designated by alphabets A, B, C etc. Each area is traversed separately. A slice of the plough-layer is cut at intervals of 15 to 20 steps or according to the area of the landscape element. Generally 10 to 20 spots were taken for one composite sample.
A V-shaped cut is made with a spade to remove 1 to 2 cm slice of soil. The sample was collected on the blade of the spade and put in a clean polythene bag. In this way samples were collected from all the spots marked for one sampling unit. In case of hard soil, samples were taken with the help of augur from the plough depth and collected in the polythene bag.

Soil from the bag was poured on a piece of clean paper or cloth and mixed thoroughly. The soil was spread evenly and divided it into 4 quarters. Two opposite quarters were rejected and the rest of the soil again mixed. The process was repeated till left with about half kg of the soil, collect it and put in a clean cloth bag. Each bag was properly marked to identify the sample.

The bags used for sampling were always being clean and free from any contamination. If the same bag was to be used for second time, turned it inside out and remove the soil particles. The details of the sample were written in the information sheet. A copy of this information sheet was put in the bag and the mouth of the bag was carefully tied.

The following precautions were followed for soil sample collection; sample was not done in unusual area like unevenly fertilized, marshy, old path, old channel, old bunds, area near the tree, site of previous compost piles and other unrepresentative sites. Before putting soil samples in bags, they were examined for cleanliness as well as for strength. Information sheet was clearly written with pencil.
Preparation of soil samples for analysis

Handling of samples

As soon as the samples were brought to the laboratory, they were checked with the accompanying information list. Information regarding samples was entered in a register.

Drying of samples

Samples were dried in enameled trays. Care was taken to maintain the identity of each sample at all stages of preparation. During drying, the trays were numbered. The soils were allowed to dry in the air. Alternatively, the trays were placed in racks in a hot air cabinet whose temperature was maintained at 35° C and relative humidity was maintained from 30 to 60%.

Post drying care

After drying, the samples were taken to the preparation room. Air dried samples were ground with a wooden pestle and mortar so that the soil aggregate were crushed but the soil particles do not break down.

After grinding, the soil was screened through a 2 mm sieve. The practice of passing only a portion of the ground sample through the sieve and discarding the remainder is erroneous. This introduces positive bias in the sample as the rejected part may include soil elements with differential fertility. The entire sample was, therefore, be passed through the sieve except for concretions and pebbles of more than 2 mm. The coarse portion on the sieve was returned to the mortar for further grinding. Sieving and grinding was repeated till all aggregate particles became fine enough to pass the sieve and only pebbles, organic residues and concretions remain
excluded. When the soil is to be analyzed for trace elements, containers made of copper, zinc and brass was avoided during grinding and handling. Sieves of different sizes were obtained in stainless steel. Plastic sieves (tea filter) were used for fine sieving. After the sample was passed through the sieve, it was again homogenized. The soil samples were stored in cardboard boxes in wooden drawers. These boxes were numbered and arranged in rows in the wooden drawers, which were in turn fitted in a cabinet in the soil sample room.

Soil fungus

Analysis of Soil Fungi

For the determination of the soil fungi, the standard plate count was used. It was relatively easy to perform.

Procedure

500 mg of air dried soil sample was serially diluted with a series of sterile distilled water blanks. Upon third dilution, 1:1,000,000 occurred from second and third dilution, measured amounts of the diluted sample was inoculated by spread plate method on potato dextrose agar. After 24 to 48 hours of incubation, plate with 30 to 300 colonies were selected for counting and identification by further pure and subculture.

Soil Sample Analysis

For the preparation of PDA (Potato Dextrose Agar) medium 250 g of potato pieces boiled in water were filtered and filtrate was made up to 1 liter. To this solution 20 g dextrose powder was added followed by heating to a syrupy viscous consistency. Standard solutions of all the samples of 50 ppm concentration were
prepared by dissolving known quantity of compounds in known volume of DMSO (dimethylsulphoxide).

The soil pH is the negative logarithm of the active hydrogen ion (H+) conc. in the soil solution. It is the measure of soil acidity or neutrality. It is a simple and important estimation, since soil pH influences to a great extent the availability of nutrients to crops. It also affects microbial population in soils. Most nutrient elements are available in the pH range of 5.5 to 6.5.

**Reagent Preparation**

- Buffer solutions of pH 4.7 and 9.0
- Calcium chloride solution (0.01M): 1.47 g of CaCl₂.2H₂O was dissolved in one liter of water to obtain 0.01M solution.

**Procedure**

1. The pH meter was calibrated using 2 buffer solutions, one with the buffer with neutral pH (7.0) and the other was chosen based on the range of pH in the soil. The electrode was inserted alternately in the beakers containing 2 buffer solutions and reading was adjusted. The instrument indicating pH as per the buffers was considered ready to test the samples.

2. Ten gram of soil sample was weighed into 100 ml beaker, add 20ml of CaCl₂ solution.

3. The soil was allowed to absorb CaCl₂ solution without stirring, then thoroughly stirred for 10 second using a clean glass rod.

4. The suspension was stirred for 30 minutes and the pH was recorded on the calibrated pH meter.
**Materials & Methods**

**Chapter-III**

**Electrical Conductivity (EC)**

The electrical conductivity (EC) is a measure of the ionic transport in a solution between the anode and cathode. It is a measurement of the dissolved salts obeying the Ohm’s law.

**Reagent**

0.01M Potassium chloride solution: A small quantity of potassium chloride at 60° C for two hours. Weighed 0.7456 g of it and dissolved in distilled water and made the volume upto one liter. This solution gave an electrical conductivity of $1411.8 \times 10^{-3}$ i.e. 1.412 mS/cm at 25°C.

**Procedure**

1. Forty gram soil was taken into 250 ml conical flask, added with 80 ml of distilled water, the flask was stoppered and shacked on reciprocating shaker for one hour. Filtered through Whatman No.1 filter paper. The filtrate was ready for measurement of conductivity.

2. The conductivity electrode was washed with distilled water and rinsed with standard KCl solution.

3. Small amount of KCl solution was poured into a 25 ml beaker and dipped in the electrode solution. Adjust the conductivity meter to read 1.412 mS/cm, corrected to 25°C.

4. The electrode was washed and dipped it in the soil extract.

5. The digital display was recorded. The reading in mS/cm of electrical conductivity.
Organic Carbon Analysis

Loss of weight on ignition can be used as a direct measure of the organic matter contained in the soil. It can also be expressed as the content of organic carbon in the soil. It is generally assumed that on an average organic matter contains about 58% organic carbon. Organic matter/organic carbon can also be estimated by volumetric and colorimetric methods. However, the use of potassium dichromate (K₂Cr₂O₇) involved in these estimations is considered as a limitation because of its hazardous nature. Soil organic matter content can be used as an index of N availability (potential of a soil to supply N to plants) because the content of N in soil organic matter is relatively constant.

Volumetric method (Walkley and Black, 1934)

Reagent Preparation

- Phosphoric acid: 85%
- Sodium fluoride solution: 2%
- Sulphuric acid: 96 % containing 1.25% Ag₂SO₄
- Standard 0.1667M K₂Cr₂O₇: Dissolve 49.04 g of K₂Cr₂O₇ in water and dilute to 1 liter
- Standard 0.5M FeSO₄ solution: Dissolve 140 g Ferrous Sulphate in 800 ml water, add 20 ml concentrated H₂SO₄ and make up the volume to 1 liter.
- Diphenylamine indicator: Dissolve 0.5 g reagent grade diphenylamine in 20 ml water and 100 ml concentrated H₂SO₄.
**Procedure**

1. One gram of the prepared soil sample was weighed in 500 ml conical flask.
2. Ten millilitre of 0.1667M K₂Cr₂O₇ solution and 20 ml concentrated H₂SO₄ containing Ag₂SO₄ were added.
3. Mixed thoroughly and allowed the reaction to complete for 30 minutes.
4. The reaction mixture was diluted with 200 ml water and 10 ml H₃PO₄.
5. Ten milliliters of NaF solution was added and 2 ml of diphenylamine indicator.
6. Titrate the solution with standard 0.5M FeSO₄ solution to a brilliant green colour.
7. A blank without sample is run simultaneously.

**Calculation**

Percent organic Carbon (X) = \frac{10(S - T) \times 0.003}{S} \times \frac{100}{\text{Weight of soil}}

Since one gram of soil is used, this equation simplifies to: \frac{3(S - T)}{S}

where,

S = ml FeSO₄ solution required for blank

T = ml FeSO₄ solution required for soil sample

3 = Eq W of C (weight of C is 12, valency is 4, hence Eq W is 12÷4 = 3.0) 0.003

= weight of C (1 000 ml 0.1667M K₂Cr₂O₇ = 3 g C. Thus, 1 ml 0.1667M K₂Cr₂O₇ = 0.003 g C) Organic Carbon recovery is estimated to be about 77%.
Therefore, actual amount of Organic carbon (Y) will be:

\[
\text{Per cent value of organic carbon obtained} \times \frac{100}{77} \quad \text{(or)}
\]

\[
\text{Percentage value of organic carbon} \times 1.3
\]

\[
\text{Percent Organic matter} = Y \times 1.724
\]

(Organic matter contains 58 % organic carbon, hence 100/58 = 1.724)

*Note:* Published organic C to total organic matter conversion factor for surface soils vary from 1.724 to 2.0. A value of 1.724 is commonly used, although whenever possible the appropriate factor was determined experimentally for each type of soil.

**Available Nitrogen** (Subbia and Asija, 1956)

The mineralizable N is estimated as an index of available nitrogen content and not the total nitrogen content. The easily mineralizable nitrogen was estimated using alkaline KMnO₄, which oxidizes and hydrolyses the organic matter present in the soil.

The liberated ammonia was condensed and absorbed in boric acid, which was titrated against standard acid. The method had been widely adopted to get a reliable index of nitrogen availability in soil due to its rapidity and reproducibility. The process of oxidative hydrolysis was, however, a progressive one and thus, a uniform time and heating temperature were allowed for best results. Use of glass beads checks bumping while liquid paraffin checks frothing during heating as is recommended in total N estimation by Kjeldahl method.
Reagents

- **0.32% KMnO₄**: 3.2 g of KMnO₄ was dissolved in water and make up to one liter.
- **2.5% NaOH**: 25 g of sodium hydroxide pellets were dissolved in water and make up to one liter.
- **2% Boric acid**: 20 g of boric acid powder was dissolved in warm water by stirring and diluted to one liter.
- **Mixed Indicator**: 0.066 g of methyl red and 0.099 g of bromocresol green were dissolved in 100 ml of ethyl alcohol. Twenty milliliter of this mixed indicator was added to each liter of 2% boric acid solution.
- **0.1M Potassium Hydrogen Phthalate**: 20.422 g of the salt was dissolved in water and diluted to one liter. This is a primary standard and does not require standardization.
- **0.02M H₂SO₄** was prepared approximately 0.1M H₂SO₄ by adding 5.6 ml of conc. H₂SO₄ to about one liter of distilled water. From this, 0.02 M H₂SO₄ was prepared by diluting a suitable volume (20 ml made to 100 ml) with distilled water. It was against standardized with 0.1M NaOH solution.
- **0.1M NaOH**: 4 gm of NaOH was dissolved in 100 ml distilled water and standardized against potassium hydrogen phthalate.

Procedure

1. Twenty gram of soil sample was taken in 800 ml conical flask.
2. The soil was moistening with about 10 ml of distilled water and the soil was washed down to remove adhering to the neck of the flask.

3. Hundred milliliter of 0.32% of KMnO₄ solution was added.

4. Few glass beads or broken pieces of glass rod was added.

5. 2-3 ml of paraffin liquid was added avoiding contact with the neck of the flask.

6. Twenty milliliter of 2% boric acid containing mixed indicator was measured in a 250 ml conical flask and placed under the receiver tube. The receiver tube was dipped in the boric acid.

7. Tap water was allowed to run through the condenser.

8. Hundred milliliter of 2.5% NaOH solution was added and immediately the rubber stopper was fitted in the alkali trap.

9. The heaters were switched on and distillation was continued until about 100 ml of distillate is collected.

10. The conical flask containing distillate was removed and then the heater was switched off to avoid back suction.

11. The distillate was titrated against 0.02M H₂SO₄ taken in burette until pink colour appeared.

12. A blank was run without soil.

13. The Kjeldahl flask was carefully removed after cooling and drain the contents in the sink.
Chapter-III
Materials & Methods

Calculation

Volume of acid used to neutralize ammonia in the sample = A - B ml

N content in the test sample = (A - B) × 0.56 mg

Percent Nitrogen (A - B) × 0.56 × 5

where,

A = Volume of 0.02M H₂SO₄ used in titration against ammonia absorbed in boric acid.

B = Volume of 0.02M sulphuric acid used in blank titration.

1 ml of 0.02M sulphuric acid = 0.56 mg N (1 000 ml of 1M H₂SO₄ = 14 g Nitrogen). Wt. of soil sample = 20 g. Thus, factor for converting into % Nitrogen = 100/20 = 5

Caution

• All the joints of the Kjeldahl apparatus was checked to prevent any leakage and loss of ammonia.

• Hot Kjeldahl flasks was neither be washed immediately with cold water nor allowed to cool for long to avoid deposits to settle at the bottom which are difficult to remove.

• In case frothing takes place and passes through to the boric acid, such samples were discarded and fresh distillation done.

• Opening ammonia bottles in the laboratory should be strictly prohibited while distillation was on. The titration should be carried out in ammonia free atmosphere.
In case the titration was not to be carried out immediately, the distillate was stored in ammonia free cupboards after tightly stoppering the flasks.

**Available phosphorus**

Two methods are most commonly used for determination of available phosphorus in soils: Bray’s Method No.1 for acidic soils and Olsen’s Method for neutral and alkaline soils.

**Olsen’s method** (Olsen *et al.*, 1954)

**Reagents Preparation**

- Bicarbonate extract: 42 g Sodium bicarbonate was dissolved in one liter of distilled water and pH adjusted to 8.5 by addition of dilute NaOH or HCl. Filtered, if necessary.
- Activated carbon - Darco G 60.
- Molybdate reagent: Same as for the Bray’s Method No. 1.
- Stannous chloride solution: Same as in Bray’s Method No. 1.

**Procedure**

1. Preparation of the Standard Curve: 0.1916 g of pure dry KH2PO4 in 1 liter of distilled water. This solution contained 0.10 mg P₂O₅/ml. This was preserved as a stock standard solution. 10 ml of this solution was taken and dilute it to 1 liter with distilled water. This solution contained 1 µg P₂O₅/ml (0.001 mg P₂O₅/ml). 1, 2, 4, 6 and 10 ml of this solution was taken in separate 25 ml flasks. To each, 5 ml of the extractant solution was added, 5 ml of the molybdate reagent and diluted with distilled water to about 20 ml. One ml dilute SnCl₂ solution was added, shaken and dilute to the 25 ml. After 10
minutes, the blue colour of the solution was read on the spectrophotometer at 660 nm wavelength. The absorbance was read against µg P$_2$O$_5$.

2. Extraction: 50 ml of the bicarbonate extractant was added to 100 ml conical flask containing 2.5 g soil sample. One gram activated carbon was added. Shaken for 30 minutes on the mechanical shaker and filtered.

3. Development of Colour: 5 ml of the filtered soil extract was aspirated with a bulb pipette in a 25 ml measuring flask; 5 ml of the molybdate reagent was dispensed with an automatic pipette, diluted to about 20 ml with distilled water, shaken and 1 ml of the dilute SnCl$_2$ solution was added with a bulb pipette. Fill-up to the 25 ml and shaken thoroughly. The blue colour was read after 10 minutes on the spectrophotometer at 660 nm wavelength after setting the instrument to zero with the blank prepared similarly but without the soil.

**Calculation**

\[
P (\text{kg/ha}) = \frac{A}{1000000} \times \frac{50}{5} \times \frac{2000000}{5} = 4A
\]

where,

- Weight of the soil taken = 5 g
- Volume of the extract = 50 ml
- Volume of the extract taken for estimation = 5 ml
- Volume made for estimation (dilution = 5 times) = 25 ml
- Amount of P observed in the sample on the standard curve = A (µg).
- Wt. of 1 ha of soil upto a depth of 22 cm is taken as 2 million kg.

As an example, the standard curve prepared by Motsara and Roy.
Caution

In spite of all precautions, intensity of blue colour changes slightly with every batch of molybdate reagent. It is imperative to check standard curve every day by using 2 or 3 dilutions of the standard phosphate solution. If the standard curve does not tally, draw a new standard curve with fresh molybdate reagent.

Available Potassium - Flame photometric method (Toth and Prince, 1949)

Potassium present in the soil was extracted with neutral ammonium acetate of 1 molarity. This is considered as plant available K in the soils. It was estimated with the help of flame photometer. This is a well-accepted method.

Reagent Preparation

- Molar neutral ammonium acetate solution: 77 g of ammonium acetate (NH₄C₂H₃O₂) was dissolved in 1 liter of water. The pH with a pH meter. If not neutral, either ammonium hydroxide or acetic acid was added to neutralize it to pH 7.0.
- Standard potassium solution: Dissolve 1.908 g pure KCl in 1 liter of distilled water. This solution contains 1 mg K per ml. Take 100 ml of this solution and dilute to 1 liter with ammonium acetate solution. This gives 0.1 mg K per ml as stock solution.
- Working potassium standard solutions: Take 0, 5, 10, 15 and 20 ml of the stock solution separately and dilute each to 100 ml with the M ammonium acetate solution. These solutions contain 0, 5, 10, 15 and 20 µg K per ml, respectively.
**Chapter III**  
**Materials & Methods**

**Procedure**

1. **Preparation of the Standard Curve:** The flame photometer was set up by atomizing 0 and 20 µg K per ml solutions alternatively to 0 and 100 reading. Intermediate working standard was atomized readings were recorded. These readings were plotted against the respective potassium contents and connect the points with a straight line to obtain a standard curve.

2. **Extraction:** 25 ml of the ammonium acetate extractant was added to conical flask fixed in a wooden rack containing 5 g soil sample. Shaken for 5 minutes and filter.

3. Potash in the filtrate was determined with the flame photometer.

**Calculation**

\[ K (\text{kg} / \text{ha}) = A \times \frac{25}{5} \times \frac{2000000}{1000000} = 10A \]

where,

\[ A = \text{content of K (µg) in the sample, as read from the standard curve:} \]

Weight of 1 ha of soil up to a plough depth of 22 cm is approx. 2 million kg.

**Preparation of standard curves for Zinc**

**Reagent preparation**

- Standard Zinc Solution: 1.0 g of pure zinc metal was taken in a beaker. 20 ml HCl (1:1) was added. Kept for few hours allowing the metal to dissolve completely. The solution was transferred to 1 liter to a volumetric flask. The volume was made up with distilled water. This is 1000 µg/ml zinc solution. For preparation of standard curve, 1000 µg/ml solution was referred as solution A.
1 ml of standard A to 100 ml was diluted to get 10 µg/ml solution to be designated as standard B.

- Distilled water of pH 2.5 ± 0.5: 1 ml of 10% sulphuric acid was diluted to one liter with glass-distilled or mineralized water and pH was adjusted to 2.5 with a pH meter using 10% H₂SO₄ or NaOH. This solution is called acidified water.

- Working Zn standard solutions: 1, 2, 4, 6, 8 and 10 ml of standard B solution were pipette out in 50 ml volumetric flask and made with DTPA solution to obtain 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 µg/ml zinc. The flasks were shaken well. Fresh standards were prepared every time when a fresh lot of acidified water is prepared.

**Procedure**

1. Flaming the solutions: The standards were atomized on atomic absorption spectrophotometer at a wave-length of 213.8 nm (Zn line of the instrument).

2. A standard curve of known concentrations of zinc solution was prepared by plotting the absorbance values on Y-axis against their respective zinc concentration on X-axis.

**Precautions**

- Weighing was done on an electronic balance.

- All the glass apparatus to be used were washed first with dilute hydrochloric acid (1:4) and then with distilled water.

- The pipette was rinsed with the same solution to be measured.

- The outer surface of the pipette should be wiped with filter paper after use.
Materials & Methods

After using the pipette, place them on a clean dry filter paper in order to prevent contamination.

**Preparation of standard curves for Copper**

**Reagents**

- Standard copper solution: 1 g of pure copper wire was weighed on a clean watch glass and transferred it to one liter flask. 30 ml of HNO₃ (1:1) and made up the mark. Stoppered flasks were shaken well. This is 1 000 µg/ml Cu solution and should be stored in a clean bottle for further use. 1 ml of 1000 µg/ml solution of copper was diluted to 100 ml to get 10 µg/ml standard copper solutions.

- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Prepared as same as that done for Zn.

- Working Cu standard solutions: 2, 3, 4, 5, 6 and 7 ml of 10 µg/ml standard Cu solution were pipette out in 50 ml volumetric flasks and made the volume with DTPA solution to get 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 µg/ml copper. Stoppered flasks were shaken well. Fresh standards were prepared every fortnight.

**Procedure**

1. The standards on an atomic absorption spectrophotometer were flamed at a wavelength of 324.8 nm (Cu line of the instrument).

2. The standard curve was drawn with the known concentration of copper on X-axis by plotting against absorbance value on Y-axis.
Preparation of standard curves for Iron

Reagents

- Standard iron solution: 1 g pure iron wire was put in a beaker and approximately 30 ml of 6M HCl was added and boiled. Transferred it to one liter volumetric flask through the funnel giving several washings to the beaker and funnel with distilled water. Make the volume up to the mark. Stoppered flasks were shaken well. This is 1000 µg/ml iron solution.

- Glass-distilled or demineralized acidified water of pH 2.5 ± 0.5: Prepared as same as that done for Zn.

- Working Fe standard solutions: 10 ml of iron stock solution in 100 ml volumetric flask and diluted to volume with DTPA solution. This was 100 µg/ml iron solution. 2, 4, 8, 12 and 16 ml of 100 µg/ml solution and diluted each to 100 ml to obtain 2, 3, 8, 12 and 16 µg/ml of Fe solution.

Procedure

1. The standards were flamed on an atomic absorption spectrophotometer at a wavelength of 248.3 nm (Fe line of the instrument).

2. The standard curve with the known concentration of copper was plotted on X-axis against absorbance value on Y-axis.

Preparation of standard curves for Manganese

Reagents

- Standard Mn solution: 3.0751 g of AR grade manganese sulphate (MnSO₄·H₂O) was weighed on a clean watch glass and transferred one liter flask through the
funnel giving several washings to watch glass and funnel with acidified water and make the volume up to the mark. This solution will be 1 000 µg/ml Mn. A secondary dilution of 5 ml to 100 ml with acidified water gives a 50 µg/ml solution.

- Glass-distilled or de-mineralized acidified water of pH 2.5 ± 0.2: Prepared as same as that for Zn.
- Working Mn standard solutions: Standard curve was prepared by taking lower concentrations of Mn in the range of 0-10 µg/ml. Take 1, 2, 4, 6 and 8 ml of 50 µg/ml solution and made to the volume with DTPA solution to 50 ml to obtain 1, 2, 4, 6 and 8 µg/ml working standards.

**Procedure**

1. The standards were flamed on an atomic absorption spectrophotometer at a wavelength of 279.5 nm (Mn line of the instrument).

2. The standard curve was prepared with the known concentration of Mn on X-axis by plotting against absorbance value on Y-axis.

**Plant-animal interaction**

Plant Pollinator interaction, butterflies are ubiquitous and commonly identifiable insect pollinator. Butterflies were used as a model for pollinator organisms.

**Butterfly sampling**

Belt transects: To find butterfly interaction with plant species, the transect walk method was followed (Pollard and Yates 1993). Sampling was carried out in two rounds, one between January and March and the second between April and
May. Twenty-five transects measuring 300 m each, were randomly marked for sampling. Each transect was surveyed four times, twice in each round. All butterflies seen within twenty metres on either side of the transect were recorded. Transects were walked between 10:00 hrs and 13:00 hrs which corresponds to the peak activity period for most butterfly species. The sampling duration for each transect lasted between 45 to 60 minutes. Most butterflies were identified to the species level using Kunte (2000). Individual butterflies that could not be identified on flight, were caught using a butterfly net, identified and released. However, some butterflies were recorded as unidentified.

**Anthropogenic Interactions**

Anthropogenic interaction with various landscape elements were studied using the following methods.

**Interviews**

Interview was a two-way communication process between a minimum of two persons namely a respondent and an interviewer. In the present study, interview was made between various user groups and farm hold peoples. Recording was done by exact verbs. Non-verbal responses like smile, nodding head, change in tone, eye contact were personally noted down. Such non-verbal signals have lot of hidden meaning which cannot be recorded without proper interception. To overcome this, probing process was adapted.

**Probing**

It was the process of designing follow-up questions instantaneously and asking in such a way that respondent stimulated to respond verbally (Rao, 1996).
Chapter III

Materials & Methods

The anthropogenic interactions were found to be dependency of forest for food, fodder, shelter, medicine and the uses of forest minor products.

Antimicrobial studies

The healthy leaves of some ethno medicinally valued plant materials were collected from above stated study areas and stored in ice box. The leaves were taken to the lab and dried in room condition then powdered by using electric blender.

Preparation of plant extract

Ten grams of powder form of leaves were extracted with 100 ml of distilled water. They were kept for seven days at room temperature (31°C) for complete extraction. The extracts were filtered through Whatman no.1 filter paper. This extract was collected and kept in refrigerator to be used for further process.

Continuous hot extraction using Soxhlet apparatus

Further purification and concentration of leaf sample is done by using soxhlet apparatus. The apparatus is used for the extraction on coarse drug powder placed in a thimble made of filter paper is inserted into the wide tube of the extractor. The solvent which was taken in the flask and heated, the vapours arose from the solvent get in to the condenser through a side tube and the liquid condensed from the vapours drips into the thimble. The solvent liquid level slowly rises and during this period, the dried leaf materials get extracted of their soluble constituents. When the level of the liquid reaches the top of the siphon, it gets siphoned into flask. The suction effect of the siphoning assists permeation of the solvent through the drug. Again a portion of the solvent from the solution vapourised leaving the constituents in the flask itself and the process mentioned...
above was repeated. The same process was repeated two times. The active constituents were solubilized and extracted with water.

**Assay of antibacterial activity**

The nutrient broth was prepared for bacterial inoculums preparation and then identified bacterial colonies were inoculated into the broth culture and used for antibacteria activity.

**Kirby bauer agar well diffusion assay**

The nutrient agar medium was prepared and sterilized by autoclaving at 121°C in 15 lbs pressure for 15 minutes then aseptically poured into the sterile petriplates and allowed to solidify. The bacterial broth culture was swabbed on each petriplates using sterile buds. Then wells were made by well cutter. The organic solvent extracts of leaves were added to each well aseptically. This procedure was repeated for each Petri plates then incubated at 37°C for 24 hrs. After incubation the plates were observed for the zone of inhibition.

**Photo documentation**

Photo documentation was done by using Canon Paxer Shot A 550, with the 4.5 megapikcel and photo plates were made with the Software Adobe 7.0.