Chapter III

Materials and Methods
MATERIALS AND METHODS

3.1. Ethno-Medico-Botanical-Inventory:

Tribal societies are storehouses of accumulated experience and knowledge of indigenous vegetation. The focus of the present study is to register the ethno botanical knowledge possessed by tribal people. They represent the pockets of human gene pool and have distinct habits and habitats with ample knowledge on the medicinal properties of their surrounding plants.

Based on the thorough review of literature available on ethnomedico botanical studies, it is concluded that very scanty literature is added from the Anantapur district. But there is no further studies taken in the study area.

The present research work is focused on the Nigidi forest range based on the careful review. The area is inhabited certain tribes namely, yanadis, Yerukulas, Sugali and some nontribal rural people who were store house of medicobotanical secrets.

These tribes possess reasonably good knowledge on their surrounding medicinal properties of plants used for different purposes. The ethnobotanical information on the drug yielding plants was recorded following the standard procedures adopted by Schults (1960), Jain (1981), Croom (1983), Lipp (1989) and Martin (1995).

Before the commencement of plant exploration trips several interviews were conducted in tribal huts / villages / thandas of representative spots in the study area. Mostly the elder people, preferably gramapedda or peddamanishi, some times old
villagers, labor women were also involved in interviews. Informants were taken into the fields to locate the medicinal plants during field trips. Enquiries were made on the food habits, occupation, health practices, medicine, belief, traditional ceremonies, traditions and customs pertaining to the tribals included in the present study.

The intensive and extensive field explorations were conducted in Nigidi forest range for ethnomedico botanical inventorization. During initial exploration studies, the tribal pockets with rich knowledge on medicinal plants were identified. Simultaneously the floral diversity was also studied carefully. With the tribes who had wisdom of treating, human and veterinary ailments, several interviews were held to get sufficient and correct information in the field. The information regarding to the medicinal plants was obtained from tribes like Yanadi, Yerukola, and Sugali and some non tribal rural people some aged women, shepherd, farmers and Natuvaidyas. The pockets nearer to Gangireddyvari palle, Batrepalle, Kalasamudramu villages were selected and interviews were held several times in different seasons. These ethnic people are closely related to plant diversity, who had intimate attachment to the forest area.

The original information given by the tribals regarding the mode of preparation of crude drugs, dosage of drug, purpose of use, administration etc., was carefully recorded in the field note book.

**Identification plant material**

Provisional identifications were made with the help of Gamble's "Flora of Presidency of Madras" (1915-35) using the field observations. The identifications were later confirmed with the help of the latest monographs, local floras and by comparison with authentic specimens in the S.K. University, Herbarium (SKU), Anantapur. The specimens were deposited in "SKU", an internationally recognised herbariorum by Index herbariorum, New York, MH (Madras Herbarium, Coimbature) and CAL (Central National Herbarium, Kolkata).
3.3. Plant Material

On the basis of scarce distribution and potential therapeutic properties of plant drugs 120 plant samples belonging to 109 genera and 55 families of flowering plants and non flowering plant were selected for preliminary phytochemical studies. Fresh parts of each plant species viz., bark, leaf, root, rhizome, whole plants were collected in bulk quantity during the exploration trips and thoroughly washed with running tap water followed by sterile distilled water, chopped into small fragments and shade dried. The dried samples were ground to coarse powder (each 100g) and stored in polythene containers at room temperature. The samples were used for the chemical analysis to detect the different classes of secondary metabolites.

Preliminary Phytochemical Screening:


Experimental procedure

Preliminary phytochemical screening of plants was done following the standard procedures / tests adopted / pioneered by Amarasingham et al., (1964), Weiffering (1966), Das and Battacharjee (1970), later modified by Chhabra et al., (1984), Gibbs (1974), Santaram (1983), and Harborne (1984). For qualitative detection of the constituents, 60 grams of each sample was extracted with Ether, Alcohol and hot water successively using soxhlet apparatus and the residue was collected after evaporation at reduced pressure at minimum temperature to enable restore the heat sensitive natural compounds. The rational for adopting such a sequential extraction procedure was based on the polarity of solvents that could leach out compounds soluble in that particular solvent (Waltor, 1971). These
concentrated extracts were used for testing the chemical constituents. The method employed for screening is shown schematically in chart (Fig. 37).

Ether extract (1) was divided into two portions. The first portion (1.1) was tested for the presence of alkaloids and volatile oils. The second portion (1.2) was saponified with 5ml of 10% KOH on a sand bath and partitioned into ethereal solution (1.2.1) and aqueous alkaline solution (1.2.2). Ethereal solution containing non-saponifiable matter was tested for the presence of carotenoids, steroids and triterpenoids. The aqueous alkaline solution was acidified (pH 3-4) with concentrated Hydrochloric acid and shaken with 15 ml of ether. The ethereal solution (1.2.2.1) containing saponifiable matter was tested for the presence of coumarins, emodins, fatty acids and flavonoids. The aqueous acidic solution (1.2.2.2) was discarded.

Ethanol extract (2) divided into two portions. The first portion (2.1) was tested for the presence of alkaloids, catecholic compounds, dihydro-chalcones, flavones, flavonols, flavonones, gallic-tannins, lignans and reducing compounds. The second portion (2.2) was hydrolyzed with 0.5 ml of 10% HCl by refluxing on a water bath for 30 minutes the contents were cooled and added 15 ml of water. The hydrolysate was shaken with 15ml of ether. The ethereal solution (2.2.1) was tested for the presence of coumarins, anthracene glycosides, flavonoids, steroids and triterpenoids. The aqueous acidic solution (2.2.2) was tested for the presence of anthocyanidins and anthocyanins.

Water extract (3) was divided into two portions. The first portion (3.1) was tested for the presence of alkaloids, polyoses, polyuronoids, reducing compounds and saponins. The second portion (3.2) was worked up in the ethanol extract and tested for the presence of coumarins, anthracene glycosides, flavonoids, steroids and triterpenoids in the ethereal solution (3.2.1) and for anthocyanidins and anthocyanins in the aqueous acidic solution (3.2.2). The presence of anthraquinones, aucubins and iridoids was tested using fresh plant material.
Fig 3. Schematic representation for preliminary phytochemical screening.

1.2
Powdered plant material (15g)

Ether extract 1

Extracted with ether (150ml)

Residue

Ether extract 2

Extracted with ethanol (150ml)

Residue

Ether extract 3

Extracted with hot water (150ml)

Residue discarded

Water extract 3

Ether extract 

Ether extract

Residue

Acidic aqueous solution

Tested for:

Anthocyanidins
Anthocyanins

3.2.2

Tested for:

Coumarins
Anthracene glycosides
Flavonoids
Steroids
Triterpenoids

3.1

Tested for:

Alkaloids
Polyoses
Polyuronoids
Reducing compounds
Saponins

Ether extract

Extracted with hot water (150ml)

Residue

Ether extract

Residue

Acidic aqueous solution

Hydrolysed with HCl (10%) and extracted with ether

3.2

Hydrolysed with HCl (10%)

Alkaloids
Heteroquinoline and extracted with ether

3.2.2

Coumarins
Anthracene glycosides
Flavonoids
Steroids
Triterpenoids

1.2.1

Ethereal solution (Non-saponifiables)

Saponified with KOH (0.5N)

1.2.1

Ether extract

Tested for:

Alkaloids
Volatile oils

1.2

Ether extract

Extracted with ether (150ml)

Residue

1.2.1

Ether extract

Tested for:

Carotenoids
Steroids
Triterpenoids

1.2.2

Ether extract

Tested for:

Alkaloids
Indole/Quinolizidine

Catecholic compounds

2.1

Extracted with ethanol (150ml)

Residue

Ether extract 2

Extracted with ethanol (150ml)

Residue

Ether extract 3

Extracted with hot water (150ml)

Residue
Screening tests for secondary metabolites

1. Alkaloids: The ether, ethanol and water extracts were tested for the presence of alkaloids (Smolenski, et al., 1972). A portion of the ether extract (1.1) was concentrated and residue was digested with 1.5 ml of 2% HCl acid. The resulting acidic solution was divided into three portions. Of these, two portions were tested for alkaloids by adding Mayer’s reagent and Wagner’s reagent respectively, while third served as blank. The formation of a faint turbidity or precipitation and addition of the above reagents indicated the presence of alkaloids. A portion of ethanol extract (2.1) was digested with 1.5 ml of 2% HCl, filtered, neutralized with 10% ammonium hydroxide and extracted with ether. The ether soluble portion was tested for alkaloids as the ether extract. A portion of water extract (3.1) was basified with 10% ammonium hydroxide and extracted with ether. The ether solution was extracted with 10% HCl and the acidic aqueous solution was tested for alkaloids as in the ether extract.

1.a. Indole alkaloids: Ethanol extract was tested for the presence of indole alkaloids. A few ml of the ethanol extract (2.1) was treated with 1ml of Ehrlich’s reagent (5% p- dimethyl amino benzaldehyde). The development of a violet color indicated the presence of indole alkaloids.

b. Quinolizidine alkaloids: Ethanol extract was tested for the presence of Quinolizidine alkaloids. Dragendorff’s reagent was added to few ml of ethanol extract (2.1). The development of a precipitate indicated the presence of Quinolizidine alkaloids.

2. Anthocyanins and Anthocyanidins: Ethanol and water extract was tested for the presence of anthocyanins and anthocyanidins (Bancroft & Rutzler, 1938). Red colour in acidic aqueous acidic solution (2.2.2 & 3.2.2) of ethanol extract at pH 3-4 indicated the presence of anthocyanins and the change of colour with pH modification (pH 8-9) indicated the presence of anthocyanidins.

3. Anthracene Glycosides: Ethanol and water extracts were tested for the presence of Anthracene Glycosides (Peyer, 1931). Ethereal solutions (2.2.1 & 3.2.1) of ethanol and water extracts were treated with 25% Ammonium hydroxide. The development of red colour indicated the presence of anthracene glycosides.
4. **Anthraquinones:** Fresh plant material was tested for the presence of anthraquinones. The plant material was extracted with 0.5% potassium hydroxide. To the alkaline extract added 1ml of hydrogen peroxide, 1ml of acetic acid and 10f benzene were added. The mixture was treated with an equal amount of dilute ammonia. The development of red colour indicated the presence of anthraquinones.

5. **Aucubins and Iridoids:** Fresh plant material was tested for aucubins and iridoids. The plant material was chopped and treated with five ml of 1% aqueous HCl. After 3-6 hours, the extract was treated with 1ml of Trim-Hill reagent (10ml of Acetic acid, 1ml of 0.2% Copper Sulphate in water and 0.5 ml of concentrated HCl) and heated on water bath. The development of blue colour indicated the presence of aucubins (diterpenoids) while green and red colours indicated other iridoids (mono terpenoids).

6. **Carotenoids:** Ether extract was tested for the presence of carotenoids by Carr-Price's reaction (Goodwin, 1955). Half volume of the ethereal (1.2.1) solution was evaporated and the residue dissolved in antimony chloride followed by the addition of concentrated sulphuric acid. The development of a blue/green color indicated the presence of carotenoids.

7. **Coumarins:** Ether, Ethanol and water extracts were tested for the presence of coumarins (Casparis and Manella, 1944). The ethereal solutions (1.2.2.1, 2.2.1 & 3.2.1) of the three extracts were evaporated and dissolved in water separately. UV florescence (at 254 nm) of these aqueous solutions and the increase in intensity after the addition of 10% ammonium hydroxide indicated the presence of coumarins.

8. **Emodins:** Ether extract was tested for the presence of emodins by Borntrager's reaction (Peyar, 1931). When the alkaline aqueous solution (1.2.2) was red in color, a portion of the ethereal solution (1.2.1) was evaporated and the residue dissolved in benzene followed by the addition of 25% ammonium hydroxide. The development of red color indicated the presence of emodins.

9. **Fatty acids:** Ether extract was tested for the presence of fatty acids (Eckey, 1954). A portion of the ethereal solution (1.2.2.1) was evaporated on a piece of filter paper. The observation of a translucent spot on the filter paper indicated the presence of fatty acids.
10. **Flavonoids:** Ether, Ethanol and water extracts were tested for the presence of flavonoids (Geissman, 1955) by Shinoda’s reaction (Shinoda, 1928). Ethereal solutions (1.2.2.1, 2.2.1 & 3.2.1) of the three extracts were evaporated and the residues dissolved in 50% methanol separately on a sand bath. On the addition of Magnesium powder and concentrated HCl, the development of yellow or red colour indicated the presence of flavonoids.

Ethanol extract (2.1) was tested for the presence of different flavonoids and inferred by their colour reactions with different reagents.

**Colour reactions of flavonoids with different reagents**

<table>
<thead>
<tr>
<th>Reagent 1</th>
<th>Reagent 2</th>
<th>Reagent 3</th>
<th>Reagent 4</th>
<th>Flavonoid type inferred</th>
</tr>
</thead>
<tbody>
<tr>
<td>15% NaCl</td>
<td>Conc. H₂SO₄</td>
<td>Mg + HCl hot</td>
<td>Sodium amalgum</td>
<td></td>
</tr>
<tr>
<td>Pale yellow</td>
<td>Pale yellow</td>
<td>No Change in colour</td>
<td>No change in colour</td>
<td>Dihydro-chalcones</td>
</tr>
<tr>
<td>Yellow</td>
<td>Intense yellow to red</td>
<td>Yellow to red</td>
<td>Red</td>
<td>Flavones</td>
</tr>
<tr>
<td>Yellow to brown by oxidation</td>
<td>Intense yellow</td>
<td>Red to magenta</td>
<td>Yellow to pale red</td>
<td>Flavonols</td>
</tr>
<tr>
<td>Yellow</td>
<td>Yellow</td>
<td>No change in colour</td>
<td>Red</td>
<td>Flavanones</td>
</tr>
</tbody>
</table>

11. **Gallic-tannins and Catecholic compounds:** Ethanol extract (2.1) was tested for the presence of Gallic-tannins and catecholic compounds. 0.5 ml of ethanol extract was diluted with 1ml of water and added 2-3 drops of dilute ferric chloride solution. The development of a blue black colour indicated the presence of gallic-tannins, while a green black colour indicated catecholic compounds.

12. **Lignans:** Ethanol extract was tested for the presence of lignans. 5ml of the extract (2.1) was treated with 1ml of concentrated HCl and 2% furfuraldehyde. The development of red colour indicated the presence of lignans.

13. **Polyoses:** Water extract was tested for presence of polyoses. 2ml of the extract (3.1) was evaporated and the residue was treated with 2-3 drops of concentrated Sulphuric acid followed by 3-4 drops of alcoholic thymol. The development of the red colour indicated the presence of polyoses.
14. Polyuronoids: Water extract was tested for the presence of poly-uronoids. Two ml of the extract (3.1) was admixed with 10ml of alcohol or acetone followed by the addition of 4-5 drops of hematoxylin. The mixture was filtered and the precipitate was washed with alcohol. A violet precipitate indicated the presence of poly-uronoids.

15. Reducing compounds: Ethanol and water extracts were tested for the presence of reducing compounds. 0.5 ml of the extract (3.1) was diluted followed by the addition of 5-8 drops Fehling’s reagent and the mixture was heated. The development of the brick red colour precipitation indicated the presence of reducing compounds.

16. Saponins: Water extract was tested for the presence of saponins (Cambie, et al., 1961) 2 ml of extract (3.1) was shaken for ten seconds and allowed to stand. The formation of a persistent honey-comb like froth indicated the presence of saponins.

17. Steroids and Triterpenoids: Ether, Ethanol and water extracts were tested for the presence of steroids and triterpenoids (Harborne, 1976) by Libermann - Burchard reaction. The ethereal solutions (1.2.1, 2.2.1 & 3.2.1) of the three extracts were evaporated and the residues dissolved in 0.5 ml of acetic anhydride followed by the addition of 0.5 ml of chloroform and 0.5 ml of concentrated hydrochloric acid separately. The development of green colour indicated the presence of sterols while red-violet colour triterpenoids.

18. Volatile oils: Ether extract was tested for the presence of volatile oils. 2ml of the extract (1.1) was evaporated on a porcelain tile, aromatic smell of residue indicated the presence of volatile oils.

The presence of the compounds was recorded by the plus (+) sign for strong reaction and T sign for a weak reaction and “-” for no reaction.

Antimicrobial Assay

The antimicrobial activity was performed by employing the pour plate and disc diffusion methods adopted by Bauer et al., (1966). The suspension of micro organisms
prepared in nutrient broth were inoculated on the nutrient agar in petri dishes at room temperature in sterile condition and mixed thoroughly to ensure uniform growth. This was allowed to stand for 15 minutes so that the medium got solidified. Now prepared crude extract disc was placed carefully over the solidified medium. All the seeded Petri-dishes were incubated at 27 ± 2°C for twenty four hours in case of bacteria and 48 hours for Candidal growth. Positive results were established by the presence of clear zones of inhibition around the disc. The zone of inhibition around each disc was measured and recorded at the end of incubation period.

The antimicrobial activity of the extracts of crud drugs were compared with certain standard antibiotics viz., Ampicillin, Tetracycline, Kanamycin and Vankomycin 30 mg/disc respectively. The standard discs were obtained from Hi-media, Bombay. The sterile filter paper discs immersed in respective solvents and distilled water were also prepared as above and used as control to understand the inhibitory effect of solvents on microbial growth.

**Micro Organisms Used**

The following microorganisms (all human pathogenic) were used as test organisms for antimicrobial activity of the crude extracts. The all listed organisms were obtained from the Microbial Type Culture Collection Centre, Institute of Microbial Technology (IMTECH), Chandigarh, India and maintained on their respective media (as per guidelines of IMTECH) in slants at 4°C. **Gram-positive Microbial strains**

1. *Bacillus cereus* (MTCC-1429)
2. *Staphylococcus aureus* (MTCC-737)

**Gram-negative strains**:

1. *Pseudomonas aeruginosa* (MTCC - 1688)
2. *Klebsiella pneumoniae* (MTCC - 109)

**Fungi:**

1. *Candida tropicalis* (MTCC-187)
2. *Aspergillus niger*

**Preparation of media**

Nutrient agar medium (NA) for Microbial cultures (g/l) :

- Beef extract : 5
- Peptone : 5
- Sodium chloride : 3
- Agar-Agar : 20
- Distilled water : 1000
- PH : 7.0 - 7.2

All the media were sterilized in autoclave at 151b / 120 for 15 minutes. The glassware was sterilized in hot air oven at 180°C for two hours. Approximately 20ml of this medium was added to each 90mm sterile petridish. Slants were used to maintain the pure culture to enable the required amount of micro organisms for assay. The master cultures were maintained carefully in sterile condition during sub culture.

**Preparation filter paper disc for Antimicrobial assay**

The concentrated extracts obtained in solvent extraction were used to study the antimicrobial assay. The extracts were concentrated at low temperature and residue used for testing antimicrobial activity.

Whatmann No.1 filter paper discs of 6mm diameter were prepared and sterilized in autoclave in a clean air tight and dry petriplate. The known amount of residue was taken in one ml of respective solvent and the discs in one ml. of respective solvent and the discs were immersed in the extract, thoroughly shaken and kept all night. Later saturated filter paper discs were carefully taken out and dried on the laminar airflow dias. These discs which were immersed in extracts were taken and used for antimicrobial assay.
Calculation of Minimum Inhibitory Concentration (MIC):

The extracts showing significant zones of inhibition were used for determining minimum inhibition concentration (MIC) values using the standard method (Russel & Farr, 1977). The extracts were diluted in appropriate amounts of acetone and a broad range of concentrated solutions were prepared. These extracts used for antimicrobial assay. The extract of least concentration which is showing antimicrobial activity is determined as minimum inhibition concentration (MIC).

Chemical characterization of Essential Oils

Isolation of essential oils

The dried plant material was pounded and subjected to extraction in Soxhlet apparatus and the hexane extract is obtained. The extract is evaporated at room temperature. The concentrated extract is rich in essential oil was collected and subjected to Gas chromatographic studies.

Gas chromatographic studies

The different components in the essential oils were characterized using Gas chromatographic studies. The essential oil was dissolved in n-Hexane and subjected to gas chromatography. The sample was injected at 290°C on OV17, SS packed silica gel column (2m x 3.21 mm) mesh range 80-100, weighing 32% at maximum temperature of 350°C. The flow gas in Nitrogen with split ratio of 1:30 and septum sweep was held constant at 10ml/min. on NVCON made gas chromatogram.
Calculation of Kovats Retention indexes ($R_j$)

The oils were separately spiked with a standard mixture of n-alkane series ($C_9$ - $C_{28}$) and then analyzed by GLC under above-mentioned conditions. Retention indices were directly obtained by application of Kovats procedure (Kovats, 1965).

Identification of Chemical Constituents

Identification and quantification of individual components were accomplished with the help of various computer interpretative techniques as well as individual interpretation of spectral data and the literature. The chemical composition of the studied samples were analyzed and enumerated in following chapters.