CHAPTER 3

MATERIALS AND METHODS

In the present study initially 20 plant species were selected based on the information collected from literature and through field observation. About 10 species were collected from polluted and the same number of 10 species from unpolluted natural areas of Visakhapatnam district, Andhra Pradesh, India. Whole plants were screened for their metal elements and out of them five plants from each area are selected for qualitative and quantitative phytochemical analysis, antimicrobial and tissue culture studies. The collected materials were washed thoroughly under running tap water and finally with sterile distilled water and then the materials were air dried on a sterile blotter under shade to constant weight for a period of 45 days. The selected plant specimens were identified with the help of flora of the presidency of Madras by Gamble (1915 - 1936) and flora of Visakhapatnam by Subba Rao (1977), flora of Srikakulam by Sheshagiri and Sreeramulu (1983). The collected plant specimens were also identified with the Herbarium available in the Department of Botany, Andhra University, Visakhapatnam. Herbarium specimens are deposited with St. Andrew’s College Herbarium (SACH No. 132, 283,287,320,333,356,362,428,449). The selected plant materials (plates 1-2) are given with brief description.
3.1.1 *Abutilon indicum* Linn.

Common in all Districts, especially in the hills. An erect woody herb.

3.1.2 *Achyranthes Aspera* Linn.

In the plains along the roadsides and in waste places.

3.1.3 *Gomphrene celosioides* Mart Beitr.

It is widely distributed and adopted to a wide range of climatic conditions.

3.1.4 *Azadirachta indica* A. Juss

Dry forests of the Deccan and Carnatic; elsewhere largely planted and often found run wild. A very important and useful deciduous tree with a hard close grained wood resembling Mohagany. The bark, leaves, flowers, seeds and the oil they give are all used in for food, medicinal and other purposes.

Table 1a: Plants used in the study

<table>
<thead>
<tr>
<th>NO</th>
<th>NAME OF THE PLANT</th>
<th>FAMILY</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Abutilon indicum</em> Linn</td>
<td>Malvaceae</td>
</tr>
<tr>
<td>2</td>
<td><em>Achyranthes aspera</em> Linn</td>
<td>Amaranthaceae</td>
</tr>
<tr>
<td>3</td>
<td><em>Gomphrena celosioides</em> Mart-Beitr</td>
<td>Amaranthaceae</td>
</tr>
<tr>
<td>4</td>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td>Meliaceae</td>
</tr>
<tr>
<td>5</td>
<td><em>Ocimum Sanctum</em> Linn.</td>
<td>Labiatae</td>
</tr>
</tbody>
</table>
3.1.5 *Ocimum sanctum* Linn

Found in the Plains, cultivated in pots or on pedestals at all Hindu houses and in temples and frequently found run wild. Sacred Basil. An erect much branched softly pubescent under shrub, with red or purple small flowers. As the most sacred of plants to Hindus it is carefully looked after and when large enough to form wood, it is also used medicinally.

3.2. Methodology

**Metal analysis**

3.2.1. Reagents and samples

All chemicals were of analytical reagent grade and were supplied by Merck® (Darmstad, Germany). Concentrated HNO$_3$ (65%) was used for acid digestions. This high purity Nitric acid was used to minimize the procedural blank values. Ultra pure water was prepared by passing doubly deionized water from a milliQ system (Millipore®, USA) and was used throughout the analysis. Two certified reference materials (NIST 164Oa and NIST 1643e) were used for calibrating the system as well for checking the accuracy of the data (Balaram and Gnaneswara Rao 2003).

3.2.2. Instrumentation

A Perkin Elmer SCIEX model Elan® DRC II ICP-MS (Ontario, Canada) at CSIR-National Geophysical Research Institute, Hyderabad, was used throughout for
trace elements analysis of plant and soil samples. The sample introduction system consisted of a standard Meinhard® nebulizer with a cyclonic spray chamber. Instrumental and data acquisition parameters are as listed below.

ICP-MS Instrumental and Data Acquisition Parameters

3.2.3. Instrumental Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>RF Power</td>
<td>1100 W</td>
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<tr>
<td>Argon Gas Flow</td>
<td>0.84 L/min</td>
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<tr>
<td>Nebulizer</td>
<td>1.20 L/min</td>
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<tr>
<td>Auxiliary</td>
<td>15 L/min</td>
</tr>
<tr>
<td>Plasma</td>
<td>7.75 Sample Uptake Rate</td>
</tr>
<tr>
<td>Lens Voltage</td>
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Data Acquisition Parameters

Quantitative Mode

Measuring Mode Peak Hopping

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</thead>
<tbody>
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<td>Point per Peak</td>
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</tr>
<tr>
<td>Number of Sweeps</td>
<td>50</td>
</tr>
<tr>
<td>Dwell Time</td>
<td>50 ms</td>
</tr>
<tr>
<td>Integration Time</td>
<td>2500 ms</td>
</tr>
<tr>
<td>Replicates</td>
<td>3</td>
</tr>
<tr>
<td>Internal Standard</td>
<td>103Rh</td>
</tr>
</tbody>
</table>

3.3.4. Materials

A hot plate with digital temperature controller with a maximum temp of 250°C was used for digestion. Teflon® beakers thoroughly cleaned, soaked in 1:1 HNO₃ for 6 hours and thoroughly rinsed with milliQ water were used for digestion. Thoroughly acid cleaned 100ml and 250ml standard flasks were used for volume make up. Whatman filter paper no.42 was used for filtration purposes.
3.3.5. Determination of Trace Metals in Soil

About 0.05-g soil samples were weighed and taken in a clean PTFE Teflon® beaker. Each sample was moistened with a few drops of water. Then 10mL of the acid mixture containing a 7:3:1 ratio of HF, HNO₃, and HClO₄ was added to each beaker and the sample swirled until completely moistened. The beakers were covered with lids and the samples left standing overnight after adding 5mL of $^{103}$Rh (1μg/ml) as an internal standard. The next day, the beakers were heated on a hot plate at 220°C for about 1 hour, after which the lids were removed, the contents evaporated to near dryness. The evaporation process was repeated after adding 5 mL of the above acid mixture in each case. Finally, the residue was dissolved by gently heating in 20mL of 1:1 HNO₃. Clear solutions were obtained for all samples. After cooling to room temperature, the volume was made up to 250ml, and these final solutions were stored in polyethylene bottles. The concentrations of different metals in these solutions were analyzed by ICP-MS. International geochemical standards SO-1 and SO-2 was used for calibration as well as to check the accuracy and precision.

3.3.6. Determination of Trace Metals in Plant

Open acid digestion method was followed for the determination of metal concentration, wherein representative samples of dried plant tissues (approximately (~0.5g) were taken in Teflon® beakers and 30ml conc. HNO₃ were added in each. They were heated on hot plate (~100°C) for two hours keeping the lids. At boiling stage, about 4-5ml H₂O₂ was added drop-wise and heated further and the volume was reduced to
about 10ml. During this entire process all organic material gets oxidized and the inorganic contents are extracted into the solution. To this 5 ml of 1μg/g Rh solution was added to act as an internal standard and the solution was transferred to 250ml of volumetric flask and diluted to 250 ml with Milli-Q® water. The solution was analyzed by ICP-MS for trace elements.

3.4. Phytochemical screening

3.4.1. Solvent Extraction of Plant Material:

The completely shade dried plant material were ground into fine powder using electric blender. The powdered plant material was subjected to successive solvent extraction taking from nonpolar to polar solvents like hexane, chloroform and methanol, 60gms of powdered plant material was subjected to soxhlet extraction for 8 hrs with 300ml of the various solvents. Each time before employing the solvent of higher polarity marc was dried. The extracts obtained were later kept for evaporation to remove the excessive solvents and brought to complete dryness over a water bath to yield the crude extracts. These extracts were collected, labeled and stored at 4°C for further study

3.4.2. Qualitative analysis of phytochemicals:

The extract was tested for the presence of bioactive compounds by using following standard methods given by Brindha et al., (1981):
3.4.2.1. Test for alkaloids

0.5gm of extract was dissolved in 10ml of dilute HCl (0.1N) and filtered. The filtrate was used to test the presence of alkaloids.

3.4.2.2. Mayer’s test

Mayer’s reagent: 1.358gm of mercuric chloride in 60ml of water and 5gm of KI in 10ml water. Mix 2 solutions and dilute to 100ml water. Filtrate was treated with Mayer’s reagent. Formation of yellow cream precipitate indicates the presence of alkaloids.

3.4.2.3. Terpenoids

4ml of filtrate (0.5gm of extract + 10ml of chloroform) + concentrated sulphuric acid 3ml was added to form a layer; reddish brown colouration interface indicates the presence of terpenoids.

3.4.2.4. Saponins

Frothing test: (0.5 ml filtrate +5ml distilled water); frothing persistence indicated presence of saponins.

3.4.2.5. Tannins

To the extract 1% of gelatin solution contains sodium chloride was added. White precipitate indicates the presence of tannins.
3.4.2.6. Phenols

The extract was treated with 3 to 4 drops of 1% FeCl₃ solution. Formation of bluish black colour indicates phenols.

3.4.2.7. Flavonoids

The extract was treated with few drops of 10% NaOH solution. Formation of intense yellow colour, which disappears with addition of dilute acid indicates the presence of flavonoids.

3.4.3 Quantitative analysis of phytochemicals:

a. Determination of total Flavonoids content using Swain and Hillis method (1953):

Preparation of standard and test solutions:

The plant extract (50mg) were dissolved separately in 50ml of methanol. These solutions were serially diluted with methanol to get lower dilutions. Phloroglucinol (50mg) was dissolved in 50ml distilled water. It was serially diluted with water to get lower dilutions.

b. Protocol for Flavonoids content estimation:

0.2ml of extract was taken in a test tube and final volume was adjusted to 2.0ml with distilled water. To this 4.0ml of vanillin reagent was added rapidly. Exactly after 15min absorbance was recorded at 500nm against blank. The unknown was read from the standard curve using different concentrations of Phloroglucinol.
c. Protocol for total phenols content estimation:

Total natural phenolic of plant extracts was determined using Folin-Ciocalteu assay (Singleton and Rossi, 1965) with minor modifications. Test tube containing either 500µl of either standard solutions of gallic acid (50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125µg/ml) or crude extracts (diluted 400-fold with distilled deionized water) was prepared. 500µl of 10% Folin-Ciocalteu’s phenol reagent (in DDW) was added into each test tube and mixed. After 20min, 350µl of 1M Na2Co3 solution was added into the mixture. After incubation for 20min at room temperature, the absorbance was determined at 750nm against the parallely prepared blank (500ul of DDW +500µl of 10% FC reagent + 350µl of 1M Na2Co3 solution). The results are expressed as gallic acid equivalents from the standard curve.

3.5. ANTIMICROBIAL ACTIVITY

3.5.1. Invitro antimicrobial assay:

The antimicrobial activity of the Hexane, chloroform and methanol extracts of each sample was evaluated by using well diffusion method or cup plate method which is the most widely used type for identifying the antimicrobial activity, which exploit diffusion of antimicrobial compounds through agar media to demonstrate inhibition of bacteria and fungi. The crude extracts of different plant parts were subjected to antimicrobial assay using well plate method.
3.5.2. Collection of Microbial Cultures:

Based on common diseases in human beings 7 pathogenic bacteria species and one fungal species were selected to perform the antimicrobial action of test samples. Name of the culture is listed in Table 1b. All the cultures were collected from TRIMS, Visakhapatnam, Andhra Pradesh.

3.5.3. Media used for Antimicrobial Assay

For bioassay studies the media used is Mueller-Hinton agar. The addition of the agar to the medium creates a solid matrix and by avoiding any significant mixing, the culture is good for inoculating microbes on surface of the medium as required for isolation of pure cultures.

3.5.4. Preparation of Culture:

A loop full of clinically tested pre-cultures was reconstituted in sterile peptone water to produce a suspension of microbial cells.
Table 1b: List showing microorganism used in the study

<table>
<thead>
<tr>
<th>Organism</th>
<th>Morphology</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>Gram Positive large endospore former</td>
<td>Food Poisoning</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Gram Positive Rod-shaped, motile, peritrichous flagella and aerobic.</td>
<td>Food Poisoning, Allergic or Hyper sensitive Reaction, Dermatitis and respiratory distress.</td>
</tr>
<tr>
<td>Enterococcus</td>
<td>Gram Positive Lactic Acid Bacteria of the phylum Firmicutes.</td>
<td>Urinary tract infections, bacteremia, bacterial endocarditis and meningitis</td>
</tr>
<tr>
<td>Klesbsiella pneumonia</td>
<td>Gram-Negative bacilli Shorter and thicker rods about 1-2mm encapsulated - thick caps.</td>
<td>Pneumonia, Nasocomial Infection, Pyogenic infection, septicemia and (UTI).</td>
</tr>
<tr>
<td>Shigella boydii (serogroup C)</td>
<td>Gram-Negative bacilli Short, rods measuring from 0.5m to 1.3 m, non-volatile, non spore forming and non-capsulated.</td>
<td>Bacillary Dysentery</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Gram Negative, Straight rod measuring 1.3 x 0.4 to 0.7 mm, motile capsulated.</td>
<td>Urinary tract infections, Diarrhea, pyogenic infections, septicemia.</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Diploid fungus that grows both as yeast and filamentous cell, Chlamydosporous.</td>
<td>Chronic mucocutaneous, Ocular candidiasis, pulmonary candidiasis, urinary tract candidiasis, meningitis, Asthma and gastritis.</td>
</tr>
</tbody>
</table>

Composition of Mueller-Hinton Agar Medium:

Beef, infusion – 300 gm/lt.

Casein – 17.5 gm/lt.

Agar – 17.0 gm/lt.

Starch – 1.5 gm/lt.
3.5.5. Preparation of Media and Plates for Agar Diffusion Method:

To prepare media, for each organism it requires 20 plates of MTT agar for 500 ml of distilled water. 19.5 gm of MH agar was weighed and dissolved in a conical flask. Then it was autoclaved at 15 lbs pressure at 121°C for 20 minutes. After sterilization media was aseptically distributed into Petri plates and allowed to solidify.

After solidification, using a sterile cotton swab each microbial culture was spread uniformly on to the surface of the agar plates. The most widely used type of identifying antimicrobial activity is the diffusion method which exploits diffusion of antimicrobial compounds through the agar media to demonstrate inhibition of bacteria.

The assay was performed by using well-plate method. After inoculation of culture into each Petri plate, a well borer of 5 mm diameter was properly sterilized by flame and used to make 6 uniform wells in each Petri plate. These wells are labeled based on the microbes and plant extract used. To determine the potential of plant extracts they were diluted up to 100 mg/ml of DMSO solution. And from three solvent extract dilution 20 µl was introduced into wells respectively and allowed to diffuse for 45 min. The plates were incubated at 37°C for 24 hours. After proper incubation, the zone of inhibitions was measured with a ruler. Results were noted and presented.

3.5.6. Minimum inhibitory concentration (MIC) assays.

Based on the literature medicinal plants were identified to have potent antimicrobial activity and minimum inhibitory concentration (MIC) of the extracts were determined according to Elizabeth et al. (2001). A final concentration of 0.5(v/v) Tween-
20(Sigma) was used to enhance crude extract solubility. A series of two fold dilution of each extract, ranging from 0.2 to 150mg/ml, was prepared. After sterilization, the medium was inoculated with 3µl aliquots of culture containing approximately 10^5 CFU/ml of each organism of 24 hours slant culture in aseptic condition and transferred into sterile 6 inch diameter Petri dishes and allowed to set at room temperature for about 10 minutes and then kept in a refrigerator for 30 minutes. After the media solidified a number 3-cup borer (6mm) diameter was properly sterilized by flaming and used to make three to five uniform cups/wells in each petri dish. A drop of molten nutrient agar was used to seal the base of each cup. Different plant crude extracts ranging from 0.2 to 100mg/ml were added to the wells of each petri dish and the control plates without plant extract. Inhibition of organism growth in the plates containing test crude extracts was judged by comparison with growth in blank control plates. The MICs were determined as the lowest concentration of extracts inhibiting visible growth of each organism on the agar plate. Similarly the MICs of methanolic extracts were determined against all other microorganisms.

3. 5.7. Determination of shelf life or stability of the activity

The stability of the bioactive compounds in both solubilized and dry states were measured. 20mg/ml of each was prepared in DMSO and 20 mg of dry samples in small vials were kept at room temperature and were tested for antibacterial activity against *Bacillus cereus* (the most sensitive of all active extracts) at 30 days intervals up to 18 months. All of the active extracts were well stable at room temperature in both dry state
and DMSO up to 18 months and did not show any reduction in activity against *Bacillus cereus*, as compared to the activities of the starting day.

### 3.5.8. Percent of activity

Percent activity of each extract was calculated as 100 X no. of susceptible microbial strains to a specific extract ÷ total no. of tested microbial strains. This term is expressed as % B for percent activity against bacteria, % F for percent activity against fungi and % T for percent total activity against both Bacteria and Fungi.

### 3.5.9. Microbial susceptibility index

Microbial susceptibility index (MSI) is calculated as 100 X no. of extracts effective against each microbial strain ÷ no. of total samples. MSI is expressed as % value and is used to compare the relative susceptibilities among the bacterial and fungal strains. MSI ranges from zero (resistant to all samples) to 100 (susceptible to all samples).

### 3.5.10. Average percent of microbial susceptibility:

Average percent of microbial susceptibility (APMS) is calculated as sum of % activities (%B, %F or %T) ÷ number of total samples. APMS represents overall susceptibility of each group of microbial strain.
3.6. CYTOTOXIC STUDIES

3.6.1. Cell culture

Human cancer cell lines (HepG2) used in this study were procured from National Centre for Cell Science, Pune. All cells were grown in Minimal essential medium (MEM, GIBCO) supplemented with 4.5 g/L glucose, antibiotics (BenzylPencillin – 50units/mL, Streptomycin -50 µg/ml and Amphotericin –B -50 µg/ml), 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37°C in 5% CO₂ incubator.

3.6.2. XTT assay

The biochemical procedure is based on the activity of mitochondrial enzymes which are inactivated shortly after cell death. This method was found to be very efficient in assessing the viability of cells. A colorimetric method based on the tetrazolium salt, XTT, was first described by P.A. Scudiero in 1988. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5x10³ cells/well in growth medium and cultured at 37°C in 5% CO₂ to adhere. After 24hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5,25,50,100,200 µg/ml) in triplicates to achieve a final volume of 100 µl and then cultured for 48 hr. The compound was prepared as 1.0 mg/ml concentration stock solutions in DMSO. Culture medium and solvent are used as controls. Each well then received 50 µl of fresh XTT (0.9mg/ml in RPMI along with XTT activator reagent) followed by incubation for 2hr at 37°C. At the end of the incubation shacked the 96
micro well plate for 15sec. The Optical Density (OD) of the culture plate was read at a wavelength of 490 nm (reference absorbance at a wavelength of 630 nm) on an ELISA reader, Anthos 2020 spectrophotometer.

- **% cell survival:** \(100 - \{\frac{(At-Ab)}{(Ac-Ab)}\} \times 100\)

  Whereas, \(At\) = Absorbance of test
  \(Ab\) = Absorbance of blank
  \(Ac\) = Absorbance of control

- **% cell inhibition:** \(100 - \%\ cell\ survival\)