Chapter 3

EXPERIMENTAL WORK
3. MATERIALS AND METHODS

3.1. Plant material

The stem bark of plant is procured from Triputi region of Andhra Pradesh and was verified by Dr. K. Madhava Chetty, faculty of botany at Sri Venkateswara University, Tripura, Andhra Pradesh. The specimen is assigned an accession no. 6310 and was deposited at the herbarium section of departmental museum for reference.

3.2. Pharmacognostic evaluation

Stem was taken up for organoleptic as well as cellular studies. Coarse powder was used to study powdered histological features, physicochemical parameters and phytochemical nature. For the cellular studies, transverse sections of stem were prepared and stained as per standard procedures (Brain, 1975; Pandya, 2010; Khandelwal, 2008). The powder microscopy of stem bark was carried out according to the procedures mentioned in practical Pharmacognosy by KR Khandelwal (Khandelwal, 2008).

3.3. Physicochemical evaluation

The plant material was subjected to various physicochemical parameters like moisture content, ash values, extractive values, foaming index in agreement with well-established standard methods and procedures (Indian Pharmacopeia, 1996; WHO, 1992). Preliminary phytochemical screening was performed using the standard procedure described by KR Khandelwal in his book practical Pharmacognosy (Khandelwal, 2008).

3.4. Fluorescence analysis

Fine powder of bark material was taken in small quantity on a clean and dry microscopic slide and treated with various chemical reagents uniformly and kept as such for about two minutes. The slide was then inspected in visible light and
under ultraviolet radiations (both at 254 nm and 365 nm) under UV chamber. The colors of fluorescence reflected after reacting with various reagents were observed and recorded.

3.5. **Preparation of extracts**

3.5.1. **Preparation of ethanolic extract**

The ethanolic extract was prepared by the method described by Rosenthaler (1930). Weighed 200 g of powered plant material and was packed in “Soxhlet’s extraction apparatus”, using ethanol as vehicle. It was refluxed till the colorless solvent started returning back to the reservoir. The dilute extract along with solvent is then concentrated by removing the solvent with the help of rotary evaporator. Then the content was transferred to a dry, clean and already weighed petri dish and kept at room temperature till the solvent is removed completely. The petri dish was weighed again to calculate the extractability percentage and finally stored in a dessicator at cool and dry place.

3.5.2. **Preparation of hydro alcoholic extract**

Powdered bark material (200 gms) which is completely dried is extracted with ethanol: water (50:50) by using soxhlet extractor assembly for two days. The extract was strained and filtrate was distilled by using distillation assembly until the ethanol gets removed and then in vacuum under reduced pressure to make it free from solvent. Concentrated extract was then kept in a desiccators to protect from moisture. Resulted extract was weighed and percent extractability was determined on air-dried basis.

3.5.3. **Preparation of aqueous extract**

Powered material (200 g) was taken in flask to which required quantity of distilled water was added. Then flask was kept on heating mental for boiling at 100°C. Heating was done till the contents were reduced to one third of total content. The contents were cooled and filtered through muslin cloth so as to remove the insoluble material in a dry, clean and already weighed petri dish. Then the extract was lyophilized to make it
free of water to prevent the microbial deterioration till further use. The dried extract is weighed to calculate the extractability percentage and finally stored in dessicator at cool and dry place.

3.6. **Phyto-chemical screening**

The previously prepared dried extracts were studied for qualitative detection of phyto-constituents present in the plant material (*Kokate, 1994*). These tests include the treatment of plant extracts (test solution). The results were concluded by viewing the appearance of different colors or by the formation of precipitates.

3.7. **Biological evaluation**

All the three prepared extracts were screened for anti-oxidant activity by following the DPPH assay and ABTS assay. Anti-cancer property by using cell lines with the MTT assay and anti microbial activities are investigated *in vitro*. The extract which showed dose dependent % age inhibition of viable cell count has been selected for further fractionation into different fractions by using different solvents of varying polarity. The different fractions were prepared by shaking the previously prepared extract with respective solvent in separating funnel for 4-5 times repeatedly. The residue obtained after fractionation is then subjected to anti-oxidant activity and anti-cancer activity. The biologically active fraction is then put forward for the separation of bioactive phyto-constituents by chromatography (column).

3.8. **Fractionation of the therapeutically active extract**

Hydroalcoholic extract is proved to be acceptable for further studies. For this the extract is treated with various solvents to obtain its fractions with the respective solvent.

\[
\begin{align*}
50:50 \text{ ethanol: water extract} &+ 80 \text{ ml of water} \\
\downarrow \\
\text{Suspended in petroleum ether} \\
\downarrow \\
43
\end{align*}
\]
Shake vigorously

Aqueous layer

Dichloromethane added

And Shaken

Ether layer

Evaporated

Aqueous layer

Dichloromethane layer

Petroleum ether fraction

Ethyl acetate added

Evaporated

Shaken

Ethyl acetate layer

Dichloromethane layer (discarded)

Aqueous layer

n-butanol added

Shaken

Aqueous layer

n-butanol layer

Ethanol added and shaken

Ethanol layer

Fig. 3.1: Flow chart showing the scheme of fractionation
3.9. Anti-oxidant activity

Different *in vitro* antioxidant activity assessment protocols are used to check the antioxidant potential of some plants and phyto-constituents. Moreover these assays are also helpful in studying the involved mechanism of action (*Antolovich et al.*, 2002). The most relevant spectrophotometric methods for carrying out the antioxidant assay of natural products are DPPH, ABTS⁺ (*Heo*, 2008; *Kang*, 2008; *Banerjee*, 2008; *Dairam*, 2008; *Mantle*, 2003) as they are easy and rapid to perform, and involve sensitive and reproducible methods as well. The mechanisms involved in DPPH and ABTS are HAT and SET (*Prior et al.*, 2005).

3.9.1. Assay of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

DPPH is a free radical, stable at room temperature and undergoes absorption at 517 nm. The trapping of DPPH radical forms the basis of antioxidant assay based on electron transfer. DPPH is a dark colored crystalline organic compound which forms violet colour in solution which gets reduced by antioxidant molecules, and the colour of solution turns pale yellow to colourless (*Calliste et al.*, 2001 and *Calliste et al.*, 2010). This change is visible and thus DPPH provides simple and fast method to evaluate antioxidant ability of various compounds/plant extracts which act as free radical scavengers (*Chang et al.*, 2007) by spectrophotometry. Reduction of the DPPH radicals can be supervised spectrophotometrically by the decline in absorbance at 517 nm.

Fig.3.2: Reaction of DPPH radical with other radicals (‘R = ‘H, alkyl radical etc.)
The test substances, ECA, PECA, EWCA, WCA, CA 2.1 and CA2.2 were evaluated for *in vitro* antioxidant activity by ABTS radical assay using concentrations ranging from 1000 μg/ml to 65 μg/ml.

### 3.9.1.1. DPPH solution
0.002 mg of DPPH is dissolved in 100 ml of methanol to get stock solution of DPPH.

### 3.9.1.2. Standard solution
Rutin was used as a standard free radical scavenger and was prepared by liquefying 20 mg of rutin in 20 ml methanol to get 1000 μg/ml stock solution. Required concentrations of 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml were made by serial dilutions technique.

### 3.9.1.3. Test solution
Weigh about 20 mg of the test samples separately and dissolve in 20 ml of methanol each to get 1000 μg/ml solution. Serial considerations were then made to obtain concentration of 25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml.

### 3.9.1.4. DPPH assay
Various concentrations (25 μg/ml, 50 μg/ml, 75 μg/ml and 100 μg/ml.) of compound and standard were prepared separately in methanol in test tubes and labeled them. 2ml of DPPH solution (0.002% in methanol) was mixed with 2ml of different concentrations of compounds and standard separately. The tubes were kept undisturbed in dark for half an hour at room temperature and then by using UV-Visible spectrophotometer the optical density was studied at 517nm. The absorbance of DPPH control was also noted.

\[
\text{DPPH radical scavenging activity} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Test}}}{\text{Abs}_{\text{Control}}} \times 100
\]
Where Abs control is the absorbance of DPPH radical in methanol; Abs test is the absorbance of sample extract. All determinations were performed in triplicate (n = 3).

3.9.2. ABTS radical cation scavenging method

Another method to evaluate the antioxidant activity is ABTS cation scavenging method. This method is more flexible as it can be carried out at different pH levels (Brand-William et al., 1995). The assay calculates ABTS+ radical cation formation which takes place by addition of potassium persulfate. The emergence of the blue colored ABTS radical is silenced by antioxidants by electron donation radical scavenging and inhibits the formation of the colored ABTS radical.

3.9.2.1. Outline of the method

The test substances, ECA, PECA, EWCA, WCA, CA 2.1 and CA2.2 were evaluated for in vitro antioxidant activity by ABTS radical assay using varying concentrations from 1000 μg/ml to 62.5 μg/ml.

3.9.2.2. Preparation of test substance for ABTS assay

In 1 ml of methanol, 21 mg of test substance was dissolved to obtain stock of 21 mg/ml concentration. The stock solution was serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 μg/ml).

3.9.2.3. Preparation of standard for ABTS assay

In 0.95 ml of methanol, 10 mg of rutin was solublised to obtain stock of 10.5mg/ml concentration. The stock solution was serially diluted to get lower concentrations (62.5, 125, 250, 500 and 1000 μg/ml).

3.9.2.4. ABTS inhibition assay

To get 2 mM concentration of ABTS was prepared by dissolving 5.48 mg ABTS in 5 ml distilled water and then potassium persulphate (17 mM, 0.03 ml) was added to the above solution. The reaction mixture was kept in dark for overnight at room temperature prior to use and is prepared fresh for each trail. To 0.2 ml of assorted concentrations of the test substances or standards,
1.0 ml of PBS and 0.16 ml of ABTS solution were included to get a final volume of 1.36 ml in eppendorf tubes and mixed using cyclomixer. The same step is repeated for test blank and control blank, instead of ABTS reagent, 0.16 ml of distilled water was taken. After 20 minutes of incubation, 0.1 ml of reaction mixture was pipetted to microtitre plate in triplets for test, control and singlet for test blank and control blank. The absorbance in ELISA reader at 734 nm was measured and the values were record.

3.10. Different cell lines used during study

The biology involved in cancer is studied with human cancer derived cell lines and manifested to be competent in research of genetics approach and characterization. Those cells lines that have been screened at molecular level are successfully used for in vitro studies. A primary culture when shifted to another culture vessel becomes a cell line. Human cancer-derived cell lines are main methods used in vitro for the study of cancer biology, and to test the therapeutic efficacy of anticancer agents (Sharma, 2010). Cell lines have the ability to proliferate and bear genotypic and phenotypic characters.

3.10.1. PC-3

PC3 human prostate cancer cell lines were isolated in 1979 from metastatic prostate tumour in a 62-year-old Caucasian male (Kaighn, 1979). In karyotypic analysis it is clear that PC3 are near-triploid, having 62 chromosomes. Their features are common to neoplastic cells of epithelial origins.

3.10.2. Hep-G2

Hep G2 is an immortalized cell line attained from a liver of 15 years old male Caucasian, with a well differentiated hepatocellular carcinoma. It is suitable for the study of polarized human hepatocytes in lab. Moreover its morphology is epithelial and having 55 chromosome pairs. The cells are adherent, growing as monolayers and in small groups and release some plasma proteins.
3.10.3. MCF-7

MCF-7 is extracted from pleural effusion from a 69-year old female having breast adenocarcinoma. Initially, it contains 85 chromosomes, which has since been reduced to 69 chromosomes. The morphology of cell line is epithelial forming monolayer of dome shape. This cell line retains the typical characters of mammary epithelium.

![Morphology of (a) PC-3 (b) Hep G2 (c) MCF 7 cell lines](image)

3.10.4. **Preliminary in vitro anti cancer activity by using MTT assay**

MTT assay was used to ascertain Cell proliferation using the method described by Heckenkamp *et al.* (1999), with little alterations.

**Principle**

This is a colorimetric assay that calculate the reduction of 3-(4, 5-dimethyldihiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT (yellow) pierces the cells and reached into the mitochondria where it is reduced to formazan product which is insoluble and dark purple in colour. The cell gets dissolved with an organic solvent and the released solubilised formazan reagent and is determined with the help of
Since reduction of MTT can only take place in metabolically active cells, the level of activity is a measure of the viability of the cells (Mosmann, 1983).

Fig. 3.4: MTT principle showing the reason of change in colour

Cells of Human prostate cell line (PC-3) were harvested by centrifugation at 1000 rpm for 5 min at 2-8°C. Cell pellet was resuspended in growth medium to get 1.5 to 2 × 10^5 cells/ml and 100 µl of cell suspension per well was seeded in 96 well culture plate. The plate was incubated for 24 hours in CO₂ incubator (37°C, 5% CO₂ and 90% relative humidity). The cells were treated with three different concentrations (100µg/ml, 50µg/ml and 25µg/ml) of test material by diluting with growth medium. The plate was incubated for next 24 hours. After that, 100 µl of MTT was added to each well. MTT was formulated by dissolving the 5 mg of MTT in 10 ml of growth medium without FBS. Plate was again incubated for 3-4 hours for the reduction of MTT to formazan. After 3-4 hours, the medium was discarded and 100 ml of DMSO was computed to each well in order to dissolve the formazan crystals. Plates were kept at shaker for 10 seconds and the optical density (OD) was taken at 595nm at multicounty ELISA plate reader. Thus, the cytotoxicity was calculated by using the formula:

\[
\text{Cytotoxicity (\%)} = \frac{\text{OD}_C - \text{OD}_S}{\text{OD}_C} \times 100
\]

5c
Where OD<sub>C</sub> = Optical density of control, OD<sub>S</sub> = Optical density of fraction.

3.10.5. Cytotoxic activity of different fractions

The <i>in vitro</i> cytotoxicity was performed for “Test substances” on Human Breast Cancer (MCF - 7) and Human Liver Cancer (HepG2) cells to find toxic concentration of the test substances.

3.10.5.1. Preparation of test solution

For cytotoxicity studies, 10 mg of all the four test substances were separately dissolved and volume was made up with MEM/DMEM - HG supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by 0.22 μ syringe filtration. To carrying out cytotoxic studies, two fold serial dilutions were prepared from the above stock solution.

3.10.5.2. Cell line and culture medium

Human breast (MCF - 7) and human liver cancer (Hep G2) cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in their respective media viz., MEM/DMEM - HG supplemented with inactivated fetal bovine serum (FBS), penicillin, streptomycin and amphotericin B in a humidified atmosphere. The stock cultures were allowed to grow in culture flasks and experiments were carried out in 96 well plates.
3.10.5.3. Cytotoxicity studies

In both the cell lines, the monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using respective media viz., MEM/DMEM - HG carrying 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was included. A partial monolayer was devised after 24 hrs. The supernatant was removed and monolayer was washed with medium. Different test concentrations of the test substances (100 μl) were added on to the partial monolayer in micro plates which were then incubated at 37±2° C for 3 days in 5% CO₂ atmosphere. The incubated sample were examined microscopically and monitored at an interval of 24 hr. The drug solutions in the wells were removed after 3 days and 50 μl of MTT in PBS was added to each well. After gentle shaking the plates were further kept on incubation for 3 hr at standard conditions. Again, the supernatant was removed from the wells and the plates were gently shaken with the addition of 100 μl of propanol to solubilize the formed formazan in the plates. The absorbance was recorded at a wavelength of 540 nm using microplate reader. The percentage of cell growth inhibition was calculated using the standard formula. The concentration of test substances required to inhibit cell growth by 50% (CTC50) was obtained from the DRC of each cell line.
3.11. **Antimicrobial activity by micro dilution methods**

*Staphylococcus aureus* (ATCC 9144) a Gram +ve, *Pseudomonas aeruginosa* (ATCC 9027) Gram –ve bacteria and *Candida albicans* (ATCC 10231), a fungus were chosen based on their clinical and pharmacological significance (*McCracken WA, 1983*). Antibacterial and antifungal activities of extracts against selected pathogenic strains viz. Gram-positive and negative and pathogenic fungi were explored by micro dilution method. According to the specifications of the Clinical and Laboratory Standards Institute (*Wayne, 2012*) MICs for all of the tested strains were determined.

3.12. **Isolation and purification of compounds by column chromatography**

Fraction which showed marked activity is screened further by column chromatography to isolate important phyto-constituents and fractions obtained were monitored on TLC.

3.12.1. **Slurry Formation**

Dried alcoholic extract was taken and dissipated in the least quantity of methanol and then adsorbed on weighed quantity of silica gel, to obtain slurry.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size of silica gel used</td>
<td>100-200 mesh</td>
</tr>
<tr>
<td>Weight of silica gel used (slurry)</td>
<td>90 g</td>
</tr>
<tr>
<td>Weight of extract</td>
<td>30 g</td>
</tr>
<tr>
<td>Diameter of column used</td>
<td>4 cm</td>
</tr>
<tr>
<td>Length of column used</td>
<td>75 cm</td>
</tr>
</tbody>
</table>

3.12.2. **Packing of column**

A glass column was taken and cotton plug was placed at the bottom of the column. Solvent with less polarity (pet. ether) is selected according to gradient elution technique and admixed with required quantity of silica gel and introduced into the column.
Simultaneously, previously prepared slurry of selected dried extract was then charged into the column.

3.12.3. **Elution of the column**

The column was first eluted with petroleum ether followed by gradually increasing the polarity of solvent. Fractions were collected in the volume of 100 ml each and then concentrated and further subjected to TLC.

3.12.4. **Thin layer chromatographic**

The principle involved in TLC is adsorption in which the components a mixture are separated based upon their adsorption on the stationary phase. The stationary phase was coated as a fine sheet adhering to a backing substance above which the solvent system (mobile phase) was enabled to travel by principle of capillary motion. The approach of TLC as analytical tool has found petition in observing and monitoring compound through a separation procedure. Precoated plates are used for TLC technique which is having aluminum foil as backing material. These sheets were cut into desired size and activated. Thin layer Chromatographic studies were carried out by employing a spot of solute and air dried. The plate was then shifted to TLC chamber for proper development of plate by capillary action in the presence of sufficient solvent system. The developed chromatogram provided data about the number of compounds in a mixture. The developed chromatogram was observed in day light, under UV light or with the help of spraying reagent. The spraying reagent used was cerry ammonium sulphate. On the basis of TLC profile, fractions showing same pattern were pooled. The $R_f$ value is the constant and characteristic of the substance which indicates its movement relative to the solvent front in a given chromatographic system (*Furniss, 2007*).
3.13. **Spectral analysis of the isolated compounds**

Phyto-constituents isolated by adsorption technique of chromatography were subjected to TLC using suitable solvent system. After running the plate, it is viewed under short and long UV light and afterwards detecting reagent is used to make the spots more prominent. The $R_f$ value is calculated and compounds were screened by spectroscopic techniques. Spectroscopy techniques serve as a robust tool for the elucidation of unknown compounds. These techniques use the interaction of energy with a sample to perform an analysis, which results into spectrum (the data obtained from spectroscopy).

Different regions of the electromagnetic radiations are involved in interacting with matter. A spectrum is a plot of the intensity of energy detected versus the wavelength of the energy. The recognition of components as qualitative analysis as well as quantitative analysis in a sample (the amount of material) is collected from spectra. The energy is always obtained from electromagnetic waves. The different spectroscopic methods are available for the structure elucidation of unknown components. Spectrometer and spectrograph are the instruments which aided the performance of spectroscopy.

3.13.1. **UV spectroscopy**

Absorption of light takes place in the UV/Visible part of the spectrum. Absorption of energy radiation in the UV (200-400 nm) and visible (400-700 nm) region of the electromagnetic spectrum takes place on the basis of electronic structure of absorbing atom or molecule. The absorption of electromagnetic radiation corresponds to excitation of electrons in outer orbit resulting in electronic transition. On absorption of energy by a molecule, one of its electrons jumps from LUMO (lower energy) to HOMO (higher energy) molecular orbital. The atoms show rotation and vibration with respect to each other in their new environment and these are recorded as spectra. Peaks in UV spectra seem to be quite broad, often ranging over 20 nm at half-maximal height. UV-Vis spectrum records two parameters viz. $\lambda_{\text{max}}$, which is the wavelength at maximal light absorbance and amount of light absorbed at $\lambda_{\text{max}}$, called absorbance. UV spectroscopy is helpful in governing conjugated organic compounds.
3.13.2. Infrared spectroscopy

IR is well accepted spectroscopic technique for the recognition of wide range of samples (Sibilia, 1996). IR absorption spectrum also known as molecular fingerprint (1200-700 cm\(^{-1}\)), involves the absorption of electromagnetic spectrum regions which is further split into three regions (Denny, 1982):

- Near -infrared (12500-4000 cm\(^{-1}\)) includes overtone bands and mostly valuable in studying food applications.
- Middle-Infrared (4000-200 cm\(^{-1}\)) comprised of absorption bands and helpful for identification of simple structure.
- Far-Infrared (400-30 cm\(^{-1}\)) involves rotational bands and valuable for inorganic molecules.

On absorbing IR radiations (specific frequencies), chemical bonds present in a molecule vibrates due to change in its electrical dipole, hence it is also known as vibrational spectroscopy. These absorptions are resonant frequencies, i.e. transition energy of the vibrating bond is similar to the frequency of the absorbed radiation. The energies are determined by the shape of the molecular potential energy surfaces, the masses of the atoms, and the associated vibronic coupling. Radiation is absorbed by interatomic bonds in organic compounds and thus its structure can be determined. Absorption signals at specific frequencies of IR radiation ("peaks" or "signals") can be correlated to bonds present in the molecule.

3.13.3. Mass spectrometry

Mass spectrometry is a valuable gadget to collect information on the structure of new molecules and supramolecules. Mass spectrometry is emerging as an indispensable study that helps to identify the amount and type of chemicals present in a sample or biomolecule. It involves the conversion of sample to gaseous ions with/without fragmentation, mass-to-charge ratios (m/z). The ions produced by sources are used in mass spectrometry comprise singly charged species resulting from gain or loss of electron by parent molecule. A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio.
(Sparkman, 2000) at given time and represents a slice of array of ion counts. Spectrometry is mainly used to determine molecular weights, and also tells about the quantity of analyte and governs the purity of the sample. Mass spectrometers provide qualitative and quantitative information on the elemental, isotopic, and molecular composition of organic and inorganic samples ranging from simple molecules to complex compounds.

3.13.4. NMR (nuclear magnetic resonance)

Nuclear magnetic resonance spectroscopy has become an indispensable technique for determining chemical structures of natural products. It measures the absorption of electromagnetic radiation in the radio frequency region of roughly 4 MHz to 750 MHz, which corresponds to a wavelength of about 75 m to 0.4 m. The basic principle involved in this technique is resonance. Resonance can be defined as matching of frequency of a system with that of electromagnetic radiation at the strength of magnetic field and it involves flipping of proton from lower energy to higher energy level. NMR is the study of interaction of radio frequency (RF) of the EMR with unpaired nuclear spins in an external magnetic field to extract structural information about the sample under study. This process of absorption of energy and giving a signal in the form of spectrum is called as NMR spectroscopy. The qualitative and quantitative composition of an unknown compound can be obtained from $^1$H- and $^{13}$C-NMR spectra and they are used for the determination of the molecular formula. This spectral technique gives the information about the molecular structure of organic compounds that is different kinds of protons with different chemical, electronic environments and tells about the relative number of protons. A simple spectrum from NMR experiment produces information which is able to provide details about:

- Amounts and types of atoms present in the sample
- Specific environments of atoms within a molecule
- Purity and composition of a sample
- Structural information, including isomerisation