3.1. IN VITRO PROPAGATION STUDIES

3.1.1. Material procurement

The dry pods of *Mudulea sericea* (Willd.) A. Cheval. were collected in the month of November 2001 from Eastern Ghats, Srisailam Reserve forest in Kurnool district of Andhra Pradesh. The healthy pods were selected and shade dried. The seeds were separated and stored in a dry container.

3.1.2. Sterilization of Paraphernalia

All the required glassware used in the preparation of media and other instruments used at the time of carrying out inoculations were sterilized in sequence. First, they were soaked in a chromic-sulphuric acid mixture for several hours and thoroughly washed with Labolene in a high-pressure jet of tap water with brushing followed by several vigorous rinses first with tap water and subsequently with distilled water. The cleaned glassware is dried in hot air oven for 24 hours and stored in dust free cup board. Instruments used for inoculations are also cleaned by the same and after distilled water cleaning they all were autoclaved and then oven dried. This is done before and after each use in order to ensure a microbial free environment.

Contaminated tubes were decontaminated by autoclaving first at 15 lbs for 20 min and then the cleaning procedure is followed as per the other glassware.
3.1.3. Media preparation

The nutrient media adopted in the present investigation were Murashige and Skoog (MS) medium (Murashige and Skoog, 1962); White's medium (White, 1963), B5 medium (Gamborg et al., 1968) and Woody plant medium (WPM) (Lloyd and McCown, 1981). The inorganic nutrients, both macro and micro, vitamins of the above mentioned media are shown in Table 2.

Stock solutions (40x) of micronutrients, vitamins and growth hormones were prepared separately and stored in a refrigerator for 2 months. Iron stock was prepared separately as chelated form as the sodium salt of ferric ethylene diamine tetra acetic acid and stored in amber coloured bottle in a refrigerator. For the preparation of stock solutions of hormones different solvents were used; concentrated HCl for cytokinins (BAP and KN), 1 N NaOH for auxins (IAA, IBA and NAA), ethyl alcohol for 2,4-D, 2,4,5 T and 2,4,5 TP and DMSO for TDZ (Table 3).

The macronutrients were prepared in concentrations of 5x, 10x etc. depending upon the frequency of usage. While preparing macronutrient stocks, in order to avoid the precipitation, calcium (supplied in the form of nitrate or chloride) was prepared separately.

Appropriate quantities of stock solutions were mixed together to prepare required volume of basal medium. Hormonal stocks were added in required concentrations either individually or in various combinations. The carbon source fortified to the media is usually sucrose (20 g/l) and the medium was made up to the final volume with double distilled water. pH of the medium was adjusted between 5.6 to 5.8 using 0.1 N HCl or 0.1 N NaOH. Media were gelled with 0.8g agar (Bacteriological grade) and boiled to dissolve it. 15 ml of this medium was dispensed into 21 x 150mm Borosil test tubes. These test tubes were air-tightened with aluminium foil. These test tubes were sterilized at 15 lb/in² for 15 min at 120°C. The test tubes were incubated vertically for organogenesis and slants were prepared for callus initiation. After cooling the medium was ready for inoculation. The prepared
TABLE 2
Chemical composition of different media used in the present investigation

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount (mg l⁻¹)</th>
<th>MS</th>
<th>B₅</th>
<th>WPM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>370</td>
<td>250</td>
<td>370</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>-</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>-</td>
<td>150</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>2500</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>-</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>440</td>
<td>150</td>
<td>-</td>
<td>96</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>134</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂·2H₂O</td>
<td>-</td>
<td>-</td>
<td>556</td>
<td></td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>-</td>
<td>-</td>
<td>990</td>
<td></td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>3</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>22.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>8.6</td>
<td>2</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>COCl₂·6H₂O</td>
<td>0.025</td>
<td>0.025</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.75</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>27.85</td>
<td>27.80</td>
<td>27.80</td>
<td></td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>37.35</td>
<td>37.30</td>
<td>37.30</td>
<td></td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>Organic supplements</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Myoinosital</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Growth regulator category</td>
<td>Name of the plant growth regulator</td>
<td>Solvent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------</td>
<td>------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auxin</td>
<td>2,4-Dichloro phenoxy acetic acid</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indole-3-Butyric acid</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naphthalene-3-acetic acid</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dicamba</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Picloram</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indole-3-acetic acid</td>
<td>EtOH / 1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,4,5-T</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,4,5-TP</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokinin</td>
<td>Benzyl amino purine</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kinetin</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thidiazuron</td>
<td>DMSO or KOH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gibberellin</td>
<td>GA$_3$</td>
<td>1N NaOH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
medium was used only after '4' days to identify and avoid microbial contamination through media, if any.

Organic supplements were added to see whether they have any promotory effect on morphogenesis. Different organic supplements such as CH, YE, ME and CM were added individually to the medium and sterilized. But, for adding CM, a separate procedure was followed. The coconut milk was drained in an aseptic environment and deproteinized by heating up to 80-100°C for 10 minutes. The supernatant was separated by filtration and stored frozen at -10°C. This was used whenever required.

3.1.4. Inoculations

Before starting inoculations, the laminar airflow cabinet was sprayed with 70% alcohol and all the required paraphernalia was transferred to it and were exposed to UV light for 30 min. After 30 min airflow was on for 15 min and then only the inoculations were carried out near the spirit lamp. Hands and inoculating area were swabbed with alcohol frequently to minimize the contamination. During inoculations before and after every use the instruments were dipped in 70% alcohol and flamed.

3.1.5. Establishment and maintenance of in vitro cultures from germinated seedlings

Different seedling explants were excised from aseptically grown seedlings and inoculated in different media. The cultures that were established were sub-cultured for every 30 days. Results were noted after the second sub-culture. A label giving details of the experiment, name of the plant, explant, medium, date of inoculation etc., was stuck to each and every test tube. Twenty replicates were maintained and the experiments were repeated thrice. The cultures were incubated at 2000 lux light under cool white, fluorescent tubes. The photo period regime was 16 h light/8 h dark diurnal cycles. The cultures were maintained at around 25 ± 2°C by using air-conditioners.
3.1.6. Experimental protocol

Various seedling explants, such as root, hypocotyl, cotyledons, cotyledonary node, node, internode, leaf and shoot tip explants were excised and cultured on selected medium. The explants showing better regenerative capacity were alone selected and subjected to various concentrations of hormones either alone or in combinations, with different carbon sources, varied concentrations of sucrose, coconut milk etc.

Number of shoots, percentage of response, nature of response and length of the shoots were recorded after 30 days from cotyledonary nodal, nodal and shoot tip explants, that were inoculated on MS, B5, WPM media fortified with 2% sucrose, 2 mg/l BAP. Basing on the response, the results were analysed statistically and the suitable medium was selected depending on the high rate of multiple shoots. Further experiments were conducted with that selected medium only.

3.1.7. Rhizogenesis

To induce rooting MS 1/4 strength, 1/2 strength, full strength, B5 and White's media were employed. Shoots were inoculated on various media fortified with various concentrations of auxins (NAA, IAA and IBA) gelled with 0.6% agar and 0.2% phytagel. The inoculated tubes were kept in dark for 48 hours or bottoms of the tubes were covered with foil to promote rooting.

3.1.8. Acclimatization

After the induction of rooting, the plantlets were removed carefully and the roots were washed thoroughly in running tap water till the traces of medium adhering to the roots was removed. The plantlets were transferred to pots containing sterilized vermiculate (100%); vermiculate, sand and soil mixture (50:25:25 V/V). The potted plants were covered with polythene bags to maintain high humidity during the first 10 days. On 11th day small pores were made to the polythene bag. The plantlets were kept in the culture room for ‘5’ days. Later on the polythene bag was removed and the plantlets were exposed to the room temperatures initially. Gradually the plantlets were
transferred to greenhouse, later on to the soil. MS medium without sucrose and agar was used to irrigate the seedlings during acclimatization.

3.1.9. Callus cultures

Different seeding explants like root, hypocotyl, cotyledon, internodal and leaf segments were inoculated on MS medium supplemented with 2 mg/l 2,4-D. High rate of callus induction was observed and recorded from the cotyledonary explants. These cotyledonary explants were further assessed for callus induction using different auxin supplements such as 2,4-D, 2,4,5-T, 2,4,5-TP, picloram, dicamba, IAA, NAA and IBA.

3.1.10. Somatic embryogenesis

Methods to induce somatic embryogenesis from seedling explants were tried by using various concentrations of auxins in combination with BAP (0.1mg/l).

3.1.11. Suspension cultures

Green compact callus initiated from cotyledonary explants on MS medium fortified with dicamba (2 mg/l). Suspension cultures were established from that callus by transferring the callus to MS liquid medium supplemented with 3% sucrose and 2 mg/l dicamba. The cultures were maintained on a gyratory shaker at 130 rpm. After 10 days cell aggregates were observed. Microscopic observations were made after 10 days to find out the size of the clumps that are formed.

3.1.12. In vitro germination studies

Infertile seeds were identified and eliminated by floating method (Anon, 1992). Healthy seeds were collected and used for further experimentation. Germination studies were conducted by using different substrata like pots, culture tubes containing 1/4, 1/2 and full strength semisolid nutrient agar media fortified with various concentrations of BAP, Kn, GA3 etc. Some seeds were subjected to scarification with conc. H2SO4. Scarified seeds were inoculated on different media. Some seeds that were subjected to the scarification were also treated with H2O2. Some seeds were treated with H2O2.
alone. The optimum duration of the treatment with conc. H$_2$SO$_4$ and H$_2$O$_2$ was identified. Normal seeds and the pre-treated seeds were finally sterilized with 0.1% HgCl$_2$ (W/V) for a minute. After each and every treatment the seeds were rinsed thoroughly with sterilized double distilled water at least thrice. These sterilized seeds were germinated on various media (MS, B$_5$, WPM) fortified with various concentrations of hormones (BAP, Kn, GA$_3$) alone or in combinations. The response of the seed and the percentage of germination were recorded and the best suitable medium for germination was identified.

3.1.13. Organogenesis

Healthy seeds of *Mundulea sericea* were thoroughly rinsed with labolene and tap water for 30 min. The seeds were then subjected to scarification with conc. H$_2$SO$_4$ for 4 min to soften the hard seed coat. Then the seeds were treated with H$_2$O$_2$ for 4 min. and washed thoroughly with tap water. The treated seeds were transferred to laminar hood for further experimentation. The seeds were surface sterilized using 0.1% (W/V) HgCl$_2$ for a minute and washed thoroughly with sterilized double distilled water thrice, each wash lasting for 5 minutes. These seeds were germinated on ‘MS’ medium supplemented with various concentrations of cytokinins BAP, Kn and GA$_3$. The inoculated seeds showed emergence of radicle on 3rd day. Seedlings of 45 days were selected for the further *in vitro* studies.

Explants like root, hypocotyl, cotyledonary node, cotyledon node, leaf and shoot tip were cut and cultured on semi-solid medium with their cut ends inserted in the medium. Single explant was inoculated in each tube. For each explant, 20 replicates were maintained. Culture media consists of MS salts fortified with various concentrations of auxins (NAA, IAA, IBA, picloram, dicamba) and cytokinins (BAP, Kn and TDZ) (alone and in combinations) and GA$_3$.

The multiple shoots were isolated and subjected for rooting experiments with various concentrations of auxins (NAA, IAA and IBA).
3.1.14. Acclimatization of micro propagules

The *in vitro* raised micro propagules were cleaned thoroughly with sterile distilled water to remove every trace of agar. These were transferred to plastic pots containing vermiculate, sand and soil (50:25:25). They were kept in green house and acclimatized. The survival rate of the transplanted propagules was calculated.

3.2. PHYTOCHEMICAL ANALYSIS

The plant material, root, bark, leaves and seeds were collected from Srisailam reserve forest of Kurnool District, Andhra Pradesh, India and identified with the help of Trees of Andhra Pradesh, India (Pullaiah and Sandhya Rani, 1999).

3.2.1. Preliminary phytochemical studies

The plant materials were shade dried and powdered coarsely. 300g of dry powder was extracted with petroleum ether at room temperature using soxhlet apparatus till the liquid was clear. The extracts were then filtered and concentrated under vacuum. The material in the soxhlet apparatus was air dried and then extracted with different solvents viz. Petroleum ether, Benzene, Chloroform, Ethyl acetate, Methanol and Water in order of polarity and subsequently concentrated to get their corresponding residues.

3.2.2. Screening tests for secondary metabolites

**Detection of alkaloids**

The individual extracts were dissolved in chloroform. The solution was extracted with diluted H$_2$SO$_4$ or diluted HCl and acid layer was taken and tested for the presence of alkaloids.

**a. Mayer's test**

To the acidic solution, Mayer's reagent (Potassium mercuric iodide solution) was added. Cream coloured precipitate indicates the presence of alkaloids.
b. Wagner's test

To the acidic solution, Wagner's reagent (Iodine in potassium iodide) was added. Brown precipitate indicates the presence of alkaloids.

c. Hagner's test

To the acidic solution, Hagner's reagent (Potassium bismuth iodide) was added. Reddish brown precipitate indicates the presence of alkaloids.

d. Ammonium Reinckate test

To the acidic solution, ammonium reinckate solution was added. Flocculent pink precipitate indicates the presence of alkaloids.

Detection of Carboxylic acid

One ml. of the various extracts was separately treated with a few ml of sodium bicarbonate solution. Effervescence (due to the liberation of carbon dioxide) indicates the presence of carboxylic acid.

Detection of coumarins

One ml each of various extracts was treated with alcoholic sodium hydroxide. Dark yellow colour shows the presence of coumarins.

Detection of Fixed oils

a. Two ml. each of various extracts were pressed separately between two filter papers. Formation of transparent spot indicates the presence of fixed oils.

b. A few drops of 0.5N alcoholic potassium hydroxide were added to one ml. each of the various extracts with a few drops of phenophthalene as indicator, and the mixture was heated for 1-2 hours. Soap formation shows the presence of fixed oils.

Detection of flavonoids

Five ml. of each extract was separately dissolved in one ml. each of alcohol and then subjected to the following tests.
a. Ferric Chloride test
A few drops of neutral ferric chloride solution were added to one ml. each of the above alcoholic solution. Formation of blackish red colour indicates the presence of flavonoids.

b. Shinoda's test
To one ml. each of alcoholic extract, a small piece of magnesium ribbon or magnesium foil was added, and a few drops of conc. HCl were added; change in colour from red to pink shows the presence of flavonoids.

c. Zinc - HCl reduction test
A pinch of zinc dust and a few drops of concentrated HCl were added to alcoholic extract. Magenta colour indicates the presence of flavonoids.

d. Lead acetate test
To one ml. of alcoholic extract, a few drops of aqueous basic lead acetate solution were added. Reddish brown bulky precipitate indicates the presence of flavonoids. 5 ml. of each extract 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 15% NaCl</th>
<th>Reagent 2 Conc.H₂SO₄</th>
<th>Reagent 3 Mg +HCl hot.</th>
<th>Reagent 4 Sodium amalgum</th>
<th>Flavonoid type</th>
</tr>
</thead>
<tbody>
<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>Flavonones</td>
</tr>
</tbody>
</table>

Detection of Phenols
One ml. each of the various extracts dissolved in alcohol or water was separately treated with a few ml. of neutral ferric chloride solution. Any change in colour indicates the presence of phenols.
Detection of quinones

One ml. each of the various extracts was treated separately with alcoholic potassium hydroxide solution. Quinones gives colouration ranging from red to blue.

Detection of resins

a. One ml. each of various extracts were treated with few drops of acetic anhydride solution followed by one ml. of concentrated $\text{H}_2\text{SO}_4$. Resins give colourations ranging from orange to Yellow.

b. One ml. each of various extracts were diluted with water. Formation of bulk black precipitate indicates the presence of resins.

Detection of saponins

One ml. each of various extracts was separately mixed with 20 ml. of distilled water and then agitated in a graduated cylinder for 15 minutes. Foam formation indicates the presence of saponins.

Detection of steroids

Various extracts were dissolved in 5 ml of chloroform separately and were subjected to the following tests.

a. Salkowski test

One ml of concentrated Sulphuric acid was added to the above solution and allowed to stand for 5 minutes after shaking. Lower layer turning into golden yellow colour indicates the presence of steroids.

b. Liebermann Burchard test

To one ml each of the chloroform treated extracts, a few drops of acetic anhydride, one ml. of concentrated $\text{H}_2\text{SO}_4$ were added from the sides of the test tube and allowed to stand for 5 minutes. Formation of brown ring at the junction of two layers and the upper layer turning green indicates the presence of steroids.
c. Noller's test

One ml. each of the extract treated with chloroform was treated with a bit of tin foil and 0.5ml. of thionyl chloride, heated gently if necessary. Pink colour shows the presence of steroids.

Detection of tannins

Five ml. each of various extracts was dissolved in minimum amount of water separately filtered and the filtrate was then subjected to the following tests.

a. Ferric chloride test

To the filtrate a few drops of ferric chloride solution were added. A blackish precipitate indicates the presence of tannins.

b. Gelatin test:

To the filtrate, gelatin (Gelatin dissolves in warm water immediately) solution was added. Formation of white precipitate indicates the presence of tannins.

c. Lead acetate test

To the filtrate a few drops of aqueous basic lead acetate solution were added. Reddish brown bulky precipitate indicates the presence of tannins.

Detection of Xanthoproteins

One ml. each of the various extracts were treated separately with few drops of concentrated HNO₃ and NH₃ solution. Formation of reddish orange precipitate indicates the presence of xanthoproteins.

Detection of glycosides

5ml. each of various extracts were hydrolysed separately with 5ml. each of concentrated HCl and boiled for few hours on a water bath and hydrolysates were subjected to the following tests.

a. Legals test

To 1ml of each hydrolysate 1ml of pyridine and a few drops of sodium nitropruside solution were added and made alkaline with NaOH.
b. Borntrager's test

To 1 ml each of hydrolysate, 1ml of chloroform was added and the chloroform layer was separated. To this, an equal quantity of dilute NH₃ solution was added. Change in colour indicates the presence of glycosides.

Lignans

5ml of each extract was treated with 1ml of concentrated HCl and 2% furfuraldehyde. The development of red colour indicates the presence of lignans.

Aucubins and iridoids

Fresh plant material was tested for aucubins and iridoids. The plant material was chopped and treated with 5ml of 1% aqueous HCl. After 3-6 hours the extract was treated with 1ml of Trim Hill reagent (10 ml. 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 indicates the presence of aucubins (diterpenoids) while green and red colour indicates other iridoids and monoterpenoids.

Polyoses

2 ml of each extract 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 indicates the presence of polyoses.

Triterpenoids

The extracts were tested for the triterpenoids (Harborne, 1976) by Libermann-Burchard reaction. The extracts were dissolved in 0.5ml of acetic anhydride followed by the addition of 0.5ml of chloroform and 0.5ml of concentrated hydrochloric acid separately. Development of the red-violet colour indicates triterpenoids.

Reducing compounds: 0.5ml of the extracts were diluted followed by the addition of 5-8 drops of Fehling's reagents and the mixture was heated.
The development of the brick red colour precipitation indicates the presence of reducing compounds.

The presence of the compounds was recorded by indicating '-' for the absence, Tr for Trace, '+' - low, '++'-high and '+++'-very high concentrations.

Oil preparation

Adopting the Pharmacopoeia of India (1966) method, dried bark coarse powder was subjected to hydrodistillation for 8 hours.

3.2.3. Qualitative chemical analysis

Gas Chromatographic - Mass Spectrometric analysis (GC-MS) of essential oil of the stem bark

Gas chromatography is generally used as an analytical tool to separate small quantities of compounds. Acquisition method is GCEIMS (Gas Chromatographic Electron Impact Mass Spectroscopy). The process of gas chromatography is carried out in a specially designed instrument, called a gas chromatograph. Gas chromatograph instrument – Agilent 6890 with 5973N MSD (USA) and a fused silica capillary column with 30m length and 0.25 mm. Internal diameter, coated with polysilphenylene – siloxane 0.25μm. thickness was employed for analysis. The column is located in an insulated oven with adjustable temperature controls. Chromatogram was produced by holding the oven temperature at 50°C initially for 2min, then programmed from 50°C to 280°C at ramp temperature 10°C/min. Helium was used as carrier gas at a linear flow rate of 30mm/s, measured at 250°C with split ratio of 10:1 and the septum sweep was held constant at 1.0ml/min. Total run time was adjusted to 15 minutes. Quantification and retention time, with a spectra physics SP 4290 Integrator, were determined.

Steps in a GC Analysis

Preparing the Instrument

The injection port, the detector and the oven is allowed to come to thermal equilibrium and a slow flow of carrier gas is started well in advance of
instrument use. Gas flow temperature adjusted properly and the recorder is switched on.

**Injecting the Sample**

1.0µL sample dissolved in methanol is injected using micro hypodermic syringe through the silicone rubber septum into the vaporizing chamber.

**Obtaining the Chromatogram**

Column type, temperature of the column, the flow rate of the carrier gas etc. are recorded.

**Calibrating Gas Chromatograms**

There are two ways to calibrate a gas chromatogram to identify the components.

1. To run a chromatogram of a known pure sample immediately before or after the chromatogram of the unknown sample.

2. Sample that has been subjected to chromatogram is mixed with known pure sample, and the chromatogram of this mixed sample is compared with the original chromatogram. If the size of the existing peaks increased, the compound giving rise to the peak may be identical with the known. If the addition of known compound results in a new peak, then, that, the known compound is not present in the sample.

**Quantitative Analysis by GC**

The vapour of a sample is carried through the column by the carrier gas. It continuously condenses and revaporises. The amount of the time the compound stays condensed depends on its volatility and hence its boiling points. Once a compound condenses it can dissolve in the high boiling liquid. The time it remains dissolved depends on its solubility in the liquid phase, thus, a non polar compound with a high vapour pressure moves along a non-polar column at a fairly rapid rate, a less volatile compound moves more slowly. Low boiling (volatile) components will travel faster through the column than will high boiling components. Polar compounds move more slowly. Raise in the column temperature speeds up all the compounds in a mixture.
The time taken for a particular component to pass through the column is called the compound's retention time (RT). The RT is a function of the physical properties of the compound, the rate of gas flow, the temperature, the liquid phase, and the length and diameter of the column. RT is measured from the point of injection of the sample to the top of the compound peak, and is usually reported in minutes. Determining the actual identity of a sample can be done by quantitative and qualitative analysis. Quantitative GC analysis shows the number of components in a sample and their retention time and approximate boiling points. In qualitative analysis, the area under a peak in a gas chromatogram is directly proportional to the amount of compound in the sample. Peak areas and not peak heights are used for quantitative calculations.

**Calculations of Kovats Retention Indices (IR)**

The oils were separately spiked with a standard mixture of a homologous n-alkane series (C₉ - C₂₅) and then analyzed by GC under the above mentioned conditions. Retention indices were directly obtained by applying Kovats procedure (Kovats, 1965; Jennings and Shibamoto, 1980).

**Mass Spectrometry**

Mass spectral analysis were run by EI (Electron Impact Ionization) at 70 ev by MSD (Mass Selective Detector). Mass spectrometry is a technique of separating the ions in accordance with their masses. Mass spectrometer separates the individual atoms or molecules on the basis of the difference in their masses. In this instrument, ions are separated on the basis of mass to charge ratio. Mass spectrometer records the masses of ions and the spectrum that is produced is called mass spectrum. Mass spectrum gives the information about various masses produced and their relative abundance. The exact molecular formula and molecular weight can be obtained from the mass spectrum. By means of mass spectrometry, it is possible to determine the structure of the organic and inorganic compounds along with their functional groups, and hence this technique is applied for the qualitative and quantitative analysis of the chemical compound.
Basic Principles of Mass Spectrometer

1. Formation of ions by the Electron bombardment of the substance.
2. Acceleration and separation of ions under the combined action fo the electric and magnetic fields in vacuo; and
3. Recording of mass spectrum

Mass spectrometer generally consists of

1. The inlet system (or sample handling system)
2. The ion source (or ionization chamber). Ion source temperature is maintained at 230°C
3. The electrostatic accelerating system.
4. The ion separator (or ion analyzer) and,
5. The ion collector (or the detector and readout system)

A high vacuum is maintained throughout the spectrometer from the inlet unit to the detection unit, otherwise there will be a loss of ions from the sample because of collision with other molecules.

Ions produced from the gaseous sample by electron bombardment are accelerated and then separated by deflection in a magnetic field according to their masses and charges. Ions generate current called ion-current at the collector. The ion current is proportional to the relative abundance of the molecular ion and the positively charged fragment ions. The plot of relative abundance on the ordinate against m/e on the abscissae is called mass spectrum.

In the mass spectrum, the parent peak is the peak of highest mass number (except for the isotope peaks). This parent peak gives the exact molecular weight of the sample from which the molecular formula can be inferred. Since, the intensity i.e., the height of the parent peak is proportional to the concentration of the ion appearing in the peaks, the parent peak in the spectrum is therefore, often less intense or quite small i.e. very small in height. Molecules having π electron system produce a more stable parent ion, hence, a bigger parent peak, than a molecule having σ bonds.
Mass spectrum of a compound is a record of relative abundance of the positively charged ions, against their m/e values. Mass spectrum contains a series of peaks and each peak corresponds to a set of ions of particular m/e value. The largest or more intense peak in the spectrum is called a base peak.

**Components identification and quantification**

Identification and quantification of the individual components were accomplished with the aid of various computer interpretative techniques as well as by individual interpretation of the spectra. The computer techniques include a comparison of unknown spectra with a collection of authentic spectra, and plotting of relative intensities of significant masses of components, mass chromatograms to structural isomers of compounds or to detect some minor components and the components hidden by other components in a completely separated gas chromatographic peak.

The intensities of the different peaks in mass spectrum of a mixture of compounds are compared with that of the pure compounds to get the concentration of each component i.e., compound of the mixture and thus, the composition of a mixture can be determined quantitatively.

The different oil constituents were identified by matching their spectral and retention indices data with those reported in the authentic spectra. (Stenhagan et al., 1974; Masada, 1976; Adam, 1989) and also by Library search DATABASE WILEY 275-L.

**3.3. ANTIMICROBIAL STUDIES**

Antimicrobial studies of crude extracts and essential oil were carried out by comparing their inhibitory effects individually on active cultures of different microbes. The test microbes employed were obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India and National Chemical Laboratory (NCL), Poone, India.
3.3.1 Plant material

Plant material like bark, leaf and seeds were collected from Nallamalai forests of Kurnool district, Andhra Pradesh and shade dried. The dried material was ground to coarse powder and stored in an airtight container at room temperature for further use.

3.3.2 Preparation of various crude extracts

300gms of various plant materials were extracted with different polar solvents with increasing polarity with the help of soxhlet apparatus. The extracts were filtered and concentrated under vacuum.

3.3.3 Preparation of discs for antimicrobial assay

Different concentrations of crude extracts were dissolved in acetone and used for the biological assay. To the crude extracts (1ml) 6mm sterilized Whatmann No. 1 filter paper discs were added and soaked for 24 hours. Later, the filter paper discs were taken out carefully and dried on the laminar hood. Completely dried discs were used for testing antimicrobial activity. A control disc was always maintained.

3.3.4 Microorganisms used

Following human pathogenic and non-pathogenic microorganisms were used as test organisms for antimicrobial activity of the crude extracts.

**Gram positive**

- *Micrococcus roseus* - (MTCC 2522)
- *Bacillus cereus* - (MTCC 1429)
- *Staphylococcus aureus* - (MTCC 737)
- *Micrococcus luteus* - (MTCC 1541)
- *Bacillus megaterium* - (ATCC 2187)

**Gram negative**

- *Pseudomonas aeruginosa* - (MTCC 1688)
- *Escherichia coli* - (MTCC 1687)
- *Klebsiella pneumoniae* - (MTCC 109)
Fungal species

*Candida tropicalis* - (MTCC 187)
*Aspergillus niger* - (MTCC 1344)

3.3.5. Preparation of growth media

Specific growth media were used to culture the microorganisms. The media were sterilized in an autoclave at 15lbs/in² at 120°C for 15 minutes. The glassware used was sterilized in hot air oven at 180°C for two hours. Approximately 20ml. of the required media was poured into each 90mm sterile petridish. Slants were prepared to maintain the pure cultures to enable the required amount of microorganisms for assay. Master cultures were maintained carefully in sterile condition during subculture.

Composition of media

The following specific media were used to culture the microorganisms.

**Nutrient agar medium (NA) for bacterial cultures (g/l)**

- Beef extract : 5
- Peptone : 3
- Sodium chloride : 5
- Agar Agar : 20
- Double distilled water : 1000ml
- pH : 7.0 - 7.2

**Nutrient broth medium (NA) for bacterial cultures (g/l)**

- Beef extract : 5
- Peptone : 3
- Sodium chloride : 5
- Double distilled water : 1000ml.
- pH : 7.0 - 7.2.

**Sabouraud's medium for fungal cultures(g/l)**

- Peptone : 10
- Dextrose : 20
Agar : 20
Double distilled water : 1000ml.
pH : 6

Medium for *Micrococcus roseus* (g/l)

- Casein peptone, tryptic digest : 10
- Yeast extract : 5
- Glucose : 5
- Agar : 15
- NaCl : 5
- Double distilled water : 1000ml.
pH : 7.2-7.4.

Medium for *Micrococcus luteus* (NA) (g/l)

- Beef extract : 1
- Yeast extract : 2
- Peptone : 5
- Agar : 15
- NaCl : 5
- Double distilled water : 1000ml.
pH : 7.2-7.4.

3.3.6. Antimicrobial assay

Antimicrobial activity was performed by using pour plate and disc diffusion, streaking methods adopted by Bauer *et al.* (1966). In pour plate method, the suspension of micro organisms were prepared in the nutrient broth and inoculated in the nutrient agar in petri dishes at room temperature in sterile condition. They were mixed thoroughly to ensure the uniform growth, and allowed to stand for 15 minutes to solidify the medium. After solidification of the medium, the pre-prepared crude extract discs were placed carefully over the solidified medium. All the inoculated petridishes were incubated inverted at 27 ± 2°C for 24 hours for bacteria, and 48-72 hours in case of fungal species. Positive results were established by the presence of clear zones of inhibition around the discs.
3.3.7. Calculation of Minimum Inhibition Concentrations (MIC)

Russel's (1977) procedure was followed for calculating the minimum inhibition concentration. The assessment of anti microbial activity was based on the measurement of diameter of inhibition (growth free) zone formed around the disc and three trials were conducted for each concentration.

Discs containing antibiotics like ampicillin, tetracycline, vancomycin 30mg/disc respectively served as standard controls, obtained from Himedia, 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 the microbial growth.