MATERIALS AND METHODS
Chapter III

MATERIALS AND METHODS

3.1 Materials

Four medicinal plants viz., *Indigofera aspalathoides* Vahl ex DC., *Indigofera viscosa* Lam. *Tephrosia spinosa* (L. f) Pers., and *Tephrosia villosa* (L.) Pers. were collected from different localities in Tirunelveli District. All the voucher specimens of the collections are deposited at the Herbarium of Survey of Medicinal Plant Unit, CCRAS (Siddha), Govt. Siddha Medical College Campus, Palayamkottai.

The taxonomic features collected from the species have been confirmed with the Flora of the Presidency of Madras (Gamble, 1915-1936) and The Flora of Tamil Nadu Carnatic (Mathew, 1983-1988). All the details pertaining to these four plants are given below.

3.1(a) Botanical name : *Indigofera aspalathoides* Vahl ex DC.

<table>
<thead>
<tr>
<th>Family</th>
<th>Fabaceae</th>
</tr>
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<tbody>
<tr>
<td>Voucher number</td>
<td>7010</td>
</tr>
<tr>
<td>Location of plant</td>
<td>Courtallum hills, Tirunelveli and Wolf Hill, Sivanthipatti – a small hillock 7 Km south east of Tirunelveli.</td>
</tr>
<tr>
<td>Altitude</td>
<td>70m MSL</td>
</tr>
<tr>
<td>Regional names</td>
<td>Tamil : Shivanar vembu</td>
</tr>
<tr>
<td></td>
<td>Sanskrit : Sivanimba</td>
</tr>
<tr>
<td></td>
<td>Malayalam : Manali</td>
</tr>
<tr>
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<td>Telugu : Mil</td>
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28
<table>
<thead>
<tr>
<th>(b)</th>
<th>Botanical name</th>
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<tr>
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<td>Indigofera colutea (Burm.f.) Merr. Galega colutea Burm.f.</td>
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<table>
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<td>4189</td>
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<td>Altitude</td>
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<td></td>
<td>Regional names</td>
<td>:</td>
<td>Tamil : Mull Kolinjii</td>
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<tr>
<td></td>
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<td>:</td>
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</tbody>
</table>
Synonyms :  
Cracca villosa L 

Tephrosia hirta Buch. Ham, 

T. incana Graham ex Wight & Arn. 

T. arnigentea Pers. 

Regional names :  
Telugu : Nagurenpali 

Tamil : Poonai kolinjii 

Uriya : Setohdothiya 

3.2 Methods 

Pharmacognostic Studies 

Taxonomical, anatomical and analytical characters were studied in the four medicinal plants Indigofera aspalathoides Vahl ex DC., Indigofera viscosa Lam. Tephrosia spinosa (L. f) Pers., and Tephrosia villosa (L.) Pers. 

3.2.1 Macroscopic (Taxonomic) Studies 

The plants were collected from December 2004 to June 2005 and preserved. The Morphological and Taxonomical observations were made by using a student microscope and the characters were described in technical terms. 

3.2.2 Anatomical Studies 

Collection of specimens 

The plants specimens for the proposed study were collected from Tirunelveli District. Care was taken to select healthy plants with normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Farmalin-5ml + Acetic acid-5ml + 70% Ethyl alcohol-90ml). After 24 hours of fixing, the specimens were dehydrated with graded series of tertiary – Butyl alcohol as per the
schedule given by Sass, 1940. Infiltration of the specimens was carried out by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 mm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with Toluidine blue as per the method published by O’Brien et al. (1964), since Toluidine blue is a polychromatic stain. The staining results were remarkably good; and some cytochemical reactions were also recorded. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with Safranin and Fast-green and KI (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (section taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in glycerine medium after staining. Different cell component were studied and measured.

Photomicrographs

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Labphoto 2 microscopic unit. For normal observations bright field was used. For the
study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifications of the figures are indicated by the Scale-bars. Descriptive terms of the anatomical features are as given in the standard Anatomy books (Easu, 1964).

3.2.3 Analytical studies

For analytical studies, air dried leaf, stem, root powders and the extracts of the powdered plant material in various solvents were examined under ordinary light and in ultra violet light (UV 365 nm). The fluorescence characters were determined according to the methods of Chase and Pratt (1949). Physio-chemical characters such as total ash, acid insoluble ash, water soluble ash, sulphated ash, moisture content, alcohol soluble extractive values and water soluble extractive values were determined by employing standard methods of analysis as described in Pharmacopoeia of India (Anonymous, 1996). The percentages of extractive values in various solvents were also determined and presented.

3.2.3. (A) Fluorescence analysis

The air dried and powdered plant material and the extracts of the powder in various solvents viz., petroleum ether, benzene, chloroform, methanol and water were examined under ordinary light and ultra violet light (UV 365 nm). These powders were also treated with various chemical reagents viz., acetone, ethyl alcohol, 1N aqueous NaOH, 1N HCl, 50% H2SO4 and 50% HNO3, and the changes in colour were recorded. These fluorescence characters were determined according to the methods of Chase and Pratt (1949).
3.2.3. (B) **Determination of total ash**

Two grams of accurately weighed plant sample was taken in a previously weighed silica dish and ignited carefully not exceeding dull red heat until the ash was free from carbon. It was then cooled and then weighed. The total percentage of ash was calculated with reference to the air dried plant sample.

3.2.3. (C) **Determination of acid insoluble ash**

The ash obtained above was boiled for five minutes with 25 ml of dilute hydrochloric acid. Insoluble matter was collected on an ashless filter paper (Whatman 41), washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air dried plant sample.

3.2.3. (D) **Determination of water soluble ash**

A known weight of ash was boiled for five minutes with 25 ml of distilled water, filtered through ashless filter paper (Whatman 41). The insoluble matter was collected on the ashless filter paper, washed with hot water, and ignited to constant weight at a low temperature. The weight of insoluble matter from the weight of the ash was subtracted; the difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried plant sample.

3.2.3. (E) **Determination of sulphated ash**

Two grams of the air dried, powdered sample was taken in a nickel crucible and moistened with concentrated sulphuric acid. It was ignited gently and moistened again with concentrated sulphuric acid and then reignited. The crucible was cooled and weighed. The percentage of sulphated ash was calculated with reference to the air dried samples.
3.2.3. (F) Determination of moisture content

Two grams of the fresh sample was weighed in a pre weighed silica dish. It was dried in the oven at 105°C and weighed at intervals of one hour until two successive constant weights were obtained. The loss of weight was recorded as moisture content.

3.2.3. (G) Determination of alcohol soluble extractive value

Five grams of the air dried, macerated powdered plant sample was soaked with 100 ml of alcohol in a closed flask for twenty four hours, shaken frequently during first six hours and allowed to stand for eighteen hours. It was filtered rapidly taking precautions against loss of alcohol. 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish. Again it was dried at 105°C and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air dried sample.

3.2.3. (H) Determination of water soluble extractive value

Five grams of the air dried, macerated powdered plant sample was soaked with 100 ml of chloroform water in a closed flask for twenty four hours. Shaken frequently during first six hours, it was allowed to stand for eighteen hours. Then it was filtered rapidly taking precautions against loss of chloroform. 25 ml of the filtrate was evaporated to dryness in a tared flat bottomed shallow dish. Again it was dried at 105°C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried sample.

3.2.3. (I) Determination of extractive values (Successive extraction)

Ten grams of the air dried and coarsely powdered plant material was taken in a soxhelt apparatus and successively extracted with petroleum ether, benzene, chloroform,
and methanol till the extracts became colourless. Each time before extracting with the next solvent, the powdered material was dried in a hot – air oven below 50°C to remove the solvents. Each extract was concentrated by distilling off the solvent in a water bath. The extracts obtained with each solvent were weighed and the percentage extractive values were calculated with reference to the air dried sample.

3.3 Phytochemical analysis

The following phytochemical analyses were done on the four medicinal plants *Indigofera aspalathoides* Vahl ex DC., *Indigofera viscosa* Lam., *Tephrosia spinosa* (L. f) Pers and *Tephrosia villosa* (L.) Pers.

3.3.1 Preliminary phytochemical analysis

The air dried powdered plant material was taken in a Soxhlet apparatus and successively extracted with petroleum ether, benzene, chloroform, and methanol till the extracts became colourless. Each time before extracting with the next solvent, the powdered material was dried in a hot – air oven below 50°C to remove the solvents. Water extracts were separately prepared. The extracts thus obtained from each plant were then subjected to qualitative tests for the identification of various plant constituents by the methods described by Brindha et al., 1981 (Table: 3.1). This preliminary phytochemical screening is a qualitative chemical evaluation which indicates spectrum of chemical constituents in the plant drug.
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Test solution + a few drops of CHCl(_3) + 3-4 drops of acetic anhydride and one drop of concentrated H(_2)SO(_4).</td>
<td>Purple colour changing to blue or green</td>
<td>Presence of steroids</td>
</tr>
<tr>
<td>2</td>
<td>Test solution + piece of tin + 3 drops of thionyl chloride</td>
<td>Violet or purple colour</td>
<td>Presence of triterpenoids</td>
</tr>
<tr>
<td>3</td>
<td>Test solution + 2 ml of Fehling's reagent + 3 ml of H(_2)O and boil</td>
<td>Red orange colour</td>
<td>Presence of reducing sugars</td>
</tr>
<tr>
<td>4</td>
<td>Test solution + very small quantity of anthrone + few drops of concentrated H(_2)SO(_4) and heat</td>
<td>Green to purple colour</td>
<td>Presence of sugars</td>
</tr>
<tr>
<td>5</td>
<td>Test solution taken with 2N HCl. Aqueous layer formed, decanted and to which are added one or two drops of Mayer's reagent.</td>
<td>White turbidity or precipitate</td>
<td>Presence of alkaloids</td>
</tr>
<tr>
<td>6</td>
<td>Test solution in alcohol + one drop of neutral ferric chloride</td>
<td>Intense colour</td>
<td>Presence of phenolic compounds</td>
</tr>
<tr>
<td>7</td>
<td>Test solution in alcohol + a bit of magnesium and one or two drops of concentrated HCl and heat</td>
<td>Red or orange red colour</td>
<td>Presence of flavonoids</td>
</tr>
<tr>
<td>8</td>
<td>Test solution in alcohol + Ehrlich reagent and a few drops of concentrated HCl</td>
<td>Pink colour</td>
<td>Presence of catechins</td>
</tr>
<tr>
<td>9</td>
<td>Test solution + H(_2)O and shake</td>
<td>Foamy lather</td>
<td>Presence of saponins</td>
</tr>
<tr>
<td>10</td>
<td>Test solution + H(_2)O + lead acetate</td>
<td>White precipitate</td>
<td>Presence of tannins</td>
</tr>
<tr>
<td>11</td>
<td>Test solution + magnesium acetate solution</td>
<td>Pink colour</td>
<td>Presence of anthroquinones</td>
</tr>
<tr>
<td>12</td>
<td>Test solution + 1% Ninhydrin in alcohol</td>
<td>Blue or violet colour</td>
<td>Presence of amino acids</td>
</tr>
</tbody>
</table>
3.3.2 Quantitative Estimations

Carbohydrates

Principle

Carbohydrates are dehydrated by concentrated sulphuric acid to form furfural. Furfural condenses with anthrone to form a blue coloured complex, which is measured colorimetrically.

Reagents

Anthrone (0.2%) in concentrated H$_2$SO$_4$, perchloric acid, ethanol and D-glucose.

3.3.2. (A) Sugars and starch (Mc Creedy et al., 1950)

Powdered sample (100 mg) was homogenized in a mortar. 100 ml of 80% ethanol was added to the homogenized mass and filtered. The filtrate was centrifuged at 3000 rpm for about five minutes. 1 ml of aliquot was taken in a corning test tube and 4 ml of anthrone reagent was added. The contents were kept in a boiling water bath for five minutes and allowed to cool. The absorbance of blue colour developed, was read in a spectrophotometer at 630 nanometer (nm).

3.3.2. (B) Starch

The residue after removal of total sugars was repeatedly washed with 80% ethanol to remove the last traces of soluble sugars. Distilled water was then added to the residue and kept in a boiling water bath for fifteen minutes and treated with 9.2 N Perchloric acid (HClO$_4$) with occasional stirring and centrifuged. After collecting the supernatant, the residue was again treated with 4.6 N HClO$_4$. After fifteen minutes of incubation, it was centrifuged and the supernatant was collected. The pooled supernatants were made up to appropriate volume depending on the starch content of the sample. Then the starch
content was determined by 0.2% anthrone in concentrated H₂SO₄ as that of sugar described above.

The sugar content of the sample was determined in terms of mg glucose equivalents based on a standard curve.

3.3.2. (C) Amino acids (Moore and Stein, 1948)

Principle

Ninhydrin, a powerful oxidizing agent reacts with amino acid to give a purple colour. The intensity of the colour is proportional to the concentration of amino acid.

Reagents

0.2 M Sodium acetate buffer (pH 5.5), ninhydrin reagent 0.2% in methyl cellosolve, ethanol and glycine.

Procedure

100 mg of powdered sample was ground well in a mortar with 10 ml of 80% ethanol. The extract was centrifuged at 3000 rpm for ten minutes. The supernatant was taken. To 1 ml of aliquot, 3 ml of distilled water and 1 ml of ninhydrin reagents were added and heated for fifteen minutes in a boiling water bath. The colour intensity at 550 nm was measured in a spectrophotometer. The amount of free amino acids is expressed as glycine equivalents per gram dry weight.

3.3.2. (D) Lipids (Blight and Dyer, 1959)

Reagents

Chloroform: Methanol (2:1)
Procedure

200 mg of the powdered sample was ground in a mortar with 10 ml of distilled water. The pulp was transferred to a 250 ml conical flask and 30 ml of chloroform:methanol (2:1) mixture was added and mixed well. The resulting solution was centrifuged at 5000 rpm for 10 minutes. The clear lower layer of chloroform containing all the lipids was collected in a previously weighed beaker and evaporated. When the solution was free of organic solvents, the weight was determined again. The difference is the amount of the lipids.

3.3.2. (E) Protein (Lowry et al. 1951)

Principle

Protein forms a colored complex with Folin Phenol reagent. The color is formed due to the reduction of phosphomolybdate by tyrosine and tryptophan present in protein. The absorbance was read out at 650 nm with spectrophotometer (Pharma Spec.).

Reagents

0.2M phosphate buffer (pH 7.2),

10% ice cold TCA, 0.2 N NaOH,

A) Alkaline sodium carbonate (2 g Na₂CO₃ dissolved in 100 ml of 0.1N NaOH),

B) CuSO₄ Sodium potassium tartarate (500 mg CuSO₄ dissolved in 100 ml of 1% Sodium potassium tartarate)

Alkaline copper reagent prepared freshly by mixing 50 ml of A and 1 ml of B.

Folin phenol reagent.
Procedure

Extraction

100 mg of fresh leaves was taken and homogenized with a little amount of 0.2 M phosphate buffer (pH 7.2). The homogenate was filtered through a three layered muslin cloth and was centrifuged at 1000 rpm for 10 minutes. The supernatant was taken and an equal amount of 10% ice cold trichloro acetic acid (TCA) was added to it and left for 30 minutes at 4° C. The precipitated protein was centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded and the pellet was used for protein estimation.

Estimation

2 ml of 0.2N NaOH was added to the residue and shaken well. Aliquots in duplicates were prepared each with 0.5 ml from the source and were made up to 4 ml using distilled water. To this 5.5 ml of alkaline copper reagent was added and shaken well and allowed to stand at room temperature for 10 minutes. Then 0.5 ml of Folin phenol reagent was added rapidly and mixed well. The absorbance was read out at 650 nm after 30 minutes using spectrophotometer. Protein content was calculated by referring to standard curve of Bovine Serum Albumin (BSA) and expressed as mg/g/fresh weight.

3.3.2. (F) Phenolic compounds (Bray and Thorpe, 1954)

Principle

The widest variety of more polar phenolic compounds can readily be oxidized to form a blue coloured complex with a strong oxidizing agent phosphomolybdate present in Folin-Ciocalteu reagent at a higher pH. The intensity of the colour developed is measured in a spectrophotometer.
Reagents

Extraction mixture, n-butanol: acetic acid: water (6:1:2), Folin – Ciocalteu reagent, 20% sodium carbonate.

Procedure

100 mg of the powdered sample was taken and homogenized with the extraction mixture. 1 ml of the extract was taken and 1 ml of Folin-Ciocalteu reagent, followed by 2 ml of 20% sodium carbonate was added. The content was shaken well, heated in a boiling water bath exactly for one minute, and cooled in running water. The blue coloured complex was diluted with appropriate volume of distilled water and the absorbance was read at 630 nm in a spectrophotometer.

3.3.2. (G) Tannin (Burade et al. 2005)

Principle:

Tannin reduces phosphotungungsto-molybolic acid in alkaline condition to produce a highly coloured blue solution. The intensity of colour is proportional to tannins.

Reagents

Folin Denis reagent, Sodium carbonate solution

Methodology

0.5 gram of powdered material was taken and transferred to a conical flask containing 75 ml water. The flask was gently heated and boiled for 30 min. The extract was centrifuged at 2000 rpm for 20 minutes and the supernatant was collected and made up the volume. 1 ml of sample extract transferred to a 100 ml volumetric flask containing 75 ml water. 5 ml Folin Denis reagent, 10 ml of sodium carbonate solution were added
and diluted to 100 ml with distilled water. It was mixed well, and the absorbance was read at 700 nm after 30 minutes. A blank was prepared with water instead of the sample.

3.3.3 Gas Chromatography-Mass Spectrometry

Mature and healthy plant was collected and dried at room temperature (25 - 30°C), for about two weeks. The dried plant materials such as leaf, stem and root were ground to powder.

Soxhlet extraction

About 60 g dried sample was refluxed with 250 ml of the selected solvent for 5 hour on a steam bath. The extract was collected and concentrated.

Procedure

The GC - MS analyses were carried out in a Shimadzu GC – MS - QP 2010 gas chromatograph fitted with a DB1 (methylphenylsiloxane, 30 m × 0.25 mm i.d.) capillary column. Carrier gas, helium with a flow rate of 0.7 mL/min; column oven temperature 70°C, 5 min in 180°C, 180-260°C at 3°C/min, 5 min in 260°C, 260-280°C at 0.2°C/min, and finally 5 min in 280°C; injector temperature, 280°C detector temperature, 290°C, Volume injected, 1 µL of TMS ether derivatives in n-hexane (2%); Split ratio, 3:0. The MS operating parameters were as follows: ionization potential 70 eV; ion source temperature 200°C; quadrupole 100°C, Solvent delay 6.0 min , scan speed 2000 amu/s and scan range 30-600 amu, eV voltage 3000 volts.

The concentrated extract is injected into the GC/MS instrument (Hewlett Packard 5890 GC/MS with Mass Selective Detector with an HP-1 glass capillary column). The sample is volatilized at the injection port and eluted through a capillary column under increasing temperature. As the sample moves through the column, various components
are separated due to their affinity for the stationary phase of the column and can be identified by retention time (the time it takes for a compound to pass through the column and gas chromatograph system). Each chemical component in a sample has a distinct retention time measured in minutes, shown in a peak on a graph which measures abundance on the ordinate against retention time on the abscissa. The integrated peak is correlated to the concentration of the chemical. A mass selective detector breaks up each chromatographic component into fragment ions, which are shown by their abundance, with each ion represented as a vertical line in increasing molecular weight. The height of each line corresponds to the abundance of that ion. The resulting mass spectrum is unique to that chemical. This mass spectrum forms a “fingerprint” that can identify the compound by a computer search of mass spectra. A computer search of the mass spectra corresponding to all the chromatographic peaks for a sample should yield a statistical match for nicotine at a 12.9 min retention time value if they were present two modes of GC/MS were possible with this instrumental method. First, there is a “Scan” mode which looks at all the constituents of a sample, listing whatever chemical components are present.

**Compound Identification**

Components of the methnolic extracts were identified by comparison of their mass spectra and retention indices with those published in the literature and contained in the NIST '98 MS computer library (Wiley).

**3.4. Antibacterial activity assay**

**A. Preparation of plant extract**

Extracts were made from air dried samples. 60 g of the leaf, stem, and root (powdered test materials) were separately extracted successively with of 400 ml of
petroleum ether (60 - 80°C), benzene, chloroform and methanol. This sequence of solvents allows for leaching all compounds based on their polarity. The individual fractions were collected and concentrated to obtain crude extracts. Water extract was prepared separately. For the purpose of experimental use, each extract sample was dissolved in respective solvent so as to get 1/10 solution.

B. Microbial strains

The antibacterial activity was tested against ten randomly selected bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Salmonella typhii*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus vulgaris* and *Bacillus subtilis*. The selected bacterial strains were obtained from Department of Microbiology, Sri. Paramakalyani College, Alwarkuruchi, Tirunelveli District.

C. Culture media and inoculum

The media used for antibacterial tests were Muller Hinton (MH) agar, (Hi-media Laboratories Pvt. Ltd, India). Each organism was maintained in a respective culture medium and was recovered for testing by sub culturing on a fresh media. An inoculum of each bacterial strain was transferred in 10 ml of M.H. agar broth and incubated over night at 37°C.

D. Preparation of sterile antibiotic discs

Antibacterial activity was assayed by filter paper disc diffusion method. Whatman No. 1 filter paper of 6 mm diameter was used. These discs were sterilized before use. The extracts of the medicinal plants were added to the sterile disc. Each sterile disc was incorporated individually with 200 – 500 μl of extract of the medicinal plants using
micropipette. Precautions were taken to prevent the flow of the solvent extract from the outer surface of the disc. The condensed extracts were applied to the disc.

E. Antibacterial assay

Antibacterial assay was demonstrated by a modification of the method described by Lennette (1985). 0.5 ml of the dilute microbial culture was spread on sterile Muller Hinton Agar plates. The presoaked and dried discs were placed on the seeded plates and gently pressed down to assure contact.

Streptomycin 10 mg/ml was used as positive control and the respective solvents which were used to dissolve the crude extracts served as negative control. The plates were incubated at room temperature for 24 hrs. After the incubation period the inhibition zone around the discs were measured and recorded. Three replicates for each concentration were maintained.

3.5 Tissue Culture

Plant materials

Tissue culture were studied in the four medicinal plants *Indigofera aspalathoides* Vahl ex DC., *Indigofera viscosa* Lam. *Tephrosia spinosa* (L. f) Pers., and *Tephrosia villosa* (L.) Pers.

Culture medium

Murashige and Skoog (1962) media was supplemented with different growth regulators tried for the *in-vitro* culture. The composition of the media is given in the Table: 3.2. All solutions for the media were prepared in double distilled water.
### Table: Composition of Murashige and Skoog (M.S) Medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Group</th>
<th>Final concentration (mg/l)</th>
<th>Stock solution (gm/500 ml)</th>
<th>Volume of stock solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate</td>
<td>1, 2.</td>
<td>1650.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium nitrate</td>
<td></td>
<td>1900.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>A</td>
<td>370.00</td>
<td>18.5</td>
<td>10</td>
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<tr>
<td>Manganese sulphate</td>
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<tr>
<td>Zinc sulphate</td>
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<td>8.60</td>
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<tr>
<td>Copper sulphate</td>
<td></td>
<td>0.025</td>
<td>0.00125</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>B</td>
<td>440.00</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td></td>
<td>0.83</td>
<td>0.0415</td>
<td></td>
</tr>
<tr>
<td>Cobalt chloride</td>
<td></td>
<td>0.025</td>
<td>0.00125</td>
<td></td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>C</td>
<td>170.00</td>
<td>8.5</td>
<td>10</td>
</tr>
<tr>
<td>Boric acid</td>
<td></td>
<td>6.20</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Disodium molybdate</td>
<td></td>
<td>0.25</td>
<td>0.0125</td>
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<tr>
<td>Ferrous sulphate</td>
<td>13, 14</td>
<td>27.84</td>
<td>0.2784/100ml mixed</td>
<td>1</td>
</tr>
<tr>
<td>Sodium EDTA</td>
<td></td>
<td>37.24</td>
<td>0.3724/100ml</td>
<td></td>
</tr>
<tr>
<td>Thiamin – HCl</td>
<td>D</td>
<td>1.0</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>Niacin</td>
<td></td>
<td>0.5</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine – HCl</td>
<td></td>
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<td>0.025</td>
<td></td>
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<tr>
<td>Glycine</td>
<td></td>
<td>2.0</td>
<td>0.1</td>
<td></td>
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<tr>
<td>Myo-inositol</td>
<td>19</td>
<td>100.00</td>
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</table>
Macroelements

The different macro elements (Ammonium nitrate, Potassium nitrate, Calcium chloride, Magnesium sulphate and Potassium dihydrogen orthophosphate) were weighed separately and dissolved in required quantity of water for the preparation of stock solutions.

Iron source

Iron source for the culture medium consisted of two compounds viz., Ethylene diamine tetra acetic disodium salt and ferrous sulphate. Both the solutions were prepared separately. Na$_2$EDTA (373 mg) was dissolved in 40 ml of nearly boiling water and cooled. Ferrous sulphate (278 mg) was dissolved in 40ml of water. The two freshly prepared solutions were mixed and the final volume was adjusted to 100 ml with water. From this stock solution, 10ml was measured and added for the preparation of one liter medium.

Carbon source and Agar

Sucrose was used as the carbon source in the preparation of all the media. The quantity of sucrose (1-4% w/v) required in each case was weighed out, just after the addition of all other components of the medium. For the solidification of semi solid medium, agar (Hi-Media, India) at 0.5 – 0.8% was used.

Growth regulators

25 mg of cytokinin was dissolved initially in a few ml of NaOH (1N). Auxins were dissolved in one of pure ethyl alcohol and finally the volume was made up to 100 ml by the addition of water. The stock solutions of the micronutrients and growth hormones for the various media were prepared and stored in the refrigerator.
Preparation of the nutrient medium

Required amount of macronutrients and micronutrients were measured from the already prepared stock solutions and mixed with hundred ml of double distilled water. To this solution an appropriate volume of Fe-Na$_2$EDTA solution was added from the respective stock solution. Similarly the organic supplements were also measured and added. Three or four percentage of sucrose was weighed and dissolved whenever needed. The final volume in each case was made up to the required volume by adding water. Thus the basal medium was prepared. To this basal medium, hormones were added and the pH was adjusted to 5.8 by using 0.1N sodium hydroxide (NaOH) or 0.1N hydrochloric acid (HCl). The medium was gelled with agar (Hi - Media, India, 0.5 – 0.8% w/v) whenever needed. The molten media were dispensed in the culture vials. The culture vials containing the medium were then autoclaved at 15 lbs pressure at 120°C for 15 minutes.

Preparation of explants source

Shoot tip and node explants were collected from young shoots of selected plants. Explants were collected from natural populations, free from any visible disease symptoms.

Surface sterilization of explants

The explants such as nodes and shoot tips were collected from mature plant and excised into a size of less than one centimeter. They were washed well in running tap water to remove the soil and sand particle and also to reduce the microbial load on the surface of the explants. After washing the explants, they were treated in running tap water, continued in a few drops of Labolene detergent or Tween 20 for 5 minutes and rinsed in distilled water. Then the explants were taken to the culture room and the rest of the sterilization process was carried out inside the laminar flow hood. All the explants were then treated with 70% ethanol for 30 seconds and washed four times with sterile
distilled water. Thereafter, the explants were immersed in 0.1% (w/v) Mercuric chloride for 1-2 minutes. Finally, they were thoroughly washed in distilled water four times, before being inoculated onto the culture medium.

**Inoculation of explants**

Inoculation of the explants on the medium was carried out in the laminar flow chamber. The working area in the laminar flow was swabbed with ethyl alcohol (70%) to ensure absolute sterile conditions. Instruments required for inoculation such as scissors, scalpels, surgical blades, forceps and empty petridishes were previously autoclaved and kept immersed in 70% ethyl alcohol in a jar. The surface sterilized explants were transferred into sterile petridishes and excised to appropriate size and then inoculated.

**Shoot regenerative potential from node and shoot tip explants**

Regenerative potential of the node and shoot tip culture was assessed. M.S media supplemented with various concentrations and combinations of cytokinins and auxins were used. Cytokinins used in this experiment were BAP and Kinetin (mg/l). Auxins used were IAA and NAA (0.5 – 3.0 mg/l). BAP, NAA and IAA were used in combinations. Cultures were maintained under cool white fluorescent light (80μ E m⁻² S⁻¹) at 25°C with 16 hours photoperiod.

**Effect of plant growth regulators on induction of callus**

Effect of plant growth regulators on induction of callus from various parts of plant was studied. Node and shoot tip were used as explants. These explants were collected from seedlings and surface sterilized well before inoculating them into the M.S medium fortified with various concentrations and combinations of plant growth regulators. Plant growth regulator 2, 4 - dichlorophenoxy acetic acid (2, 4 D) and 2, 4, 5 - trichlorophenoxy acetic acid (2, 4, 5 T) (0.5- 3.0 mg/l) were used. All types of explants were kept horizontally on the medium for callus induction. Cultures were kept in the
culture room, where controlled environment was maintained for 4 weeks. Percentage of explants showing callusing response, rate of callus proliferation and nature of the callus, which includes the color and texture of the callus were taken into account to measure the impact of plant growth regulators on the ability of the various explants to induce callus. The callus induced from the nodal explants were transferred to M.S medium supplemented with different concentrations of BAP (0.2, 0.5 mg/l), IAA (0.2, 0.5 mg/l) and combination of BAP (0.5 mg/l) and IAA (0.2 mg/l) to initiate proliferation and regeneration of shoots.

**Rooting of shootlets**

Well developed shoots were separated and single shoots were transferred to half strength M.S media for rooting. The plant growth regulators, auxins (NAA and IBA) were used for rooting purposes. Auxin concentrations from 0.2 – 1.0 mg/l were incorporated into the medium and the pH of the medium was adjusted to 5.5 – 5.8 using 0.1N Sodium hydroxide (NaOH) or 0.1N Hydrochloric acid (HCl). Cultures were incubated in the dark for 2 weeks, after which they were exposed to light 12 – 16 h/day for another 2 weeks. Percentage of rooting response, number of roots per shoot and root length were measured in various concentrations and combinations.

**Hardening and field transfer**

The rooted plants were thoroughly washed with running tap water to remove the adhering agar and planted in 3.5 cm root trainers filled with 1:1 sterile soil and vermiculite sterilized mixture. The transplanted plants were then transferred under 30±2°C and 60±5% relative humidity (RH) for 30 days in a glass house for acclimatization, prior to transfer to the net house for further establishment and growth. Only well established 25 – 30 cm tall plants were transferred to the field.