MATERIALS & METHODS
CHAPTER - III
MATERIALS AND METHODS

3.1. Materials

The different species of medicinal plants selected for the present phytochemical studies are *Tylophora subramanii* Henry, *Caralluma umbellata* (Roxb.) Haworth and *Caralluma adscendens* (Roxb.) Haw. var. *attenuata* (Wight) Grav. and Mayuranathan. Mature and healthy plants were collected from nature at different specific locations after the rainy season (February, March and April). The different specimens were identified referring to the Flora of Presidency of Madras (Gamble, 1915-1936); Flora of Tamil Nadu Carnatic (Mathew, 1983); Fascicles of Flora of India by BSI (Jagtap and Singh, 1999) and Bulletin, Madras Government Museum (Gravely and Mayuranathan, 1931). Voucher specimens were documented in the herbarium of St. Xavier's College (Autonomous), Palayamkottai (XCH), Tamil Nadu, India.

The specimens were shade-dried at room temperature (18-20°C) for a period of 3 weeks to 8 weeks depending on the water content. The succulent *Caralluma* species were kept in the desiccators containing calcium in order to reduce the moisture content and to avoid decomposition by microorganisms. The completely dried materials were separately powdered by means of wareing blender. Then the powdered materials were stored in polythene bags having labels. Estimations, phytochemical tests, pharmacognostic studies and chromatographic studies were carried out using the powders. The quantitative estimations were done in triplicates and the mean value was taken.
All the details pertaining to these three plants are given below.

### 3.1.1. Plant Materials

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Family</th>
<th>Voucher Number</th>
<th>Collected locality</th>
<th>Altitude</th>
<th>Synonyms</th>
<th>Tamil Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Tylophora subramanii Henry</td>
<td>Asclepiadaceae</td>
<td>XCH-28063</td>
<td>Tirunelveli district, Kuthiraivetti.</td>
<td>1000m</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>(b) Caralluma umbellata (Roxb.) Haworth</td>
<td>Asclepiadaceae</td>
<td>XCH-28062</td>
<td>Dindigul district, Kamalapuram</td>
<td>Plains</td>
<td></td>
<td>Oddan kilivan</td>
</tr>
</tbody>
</table>

**Synonyms**

- Boucerosia umbellata (Roxb.) Wight & Aru.
- Boucerosia umbellata var. campanulata Hook.F.
- Caralluma campanulata (Wight) N.E.Br.
- Boucerosia campanulata Wight
- Caralluma lasiantha (Wight) N.E.Br.
- Boucerosia lasiantha Wight

**Tamil Name**

- Oddan kilivan
- Kalmulaiyaan
- Sirunkalli
- Yerumai
- Kannimulliyan
3.1.2. Procurement of chemicals and solvents

The chemicals and solvents used throughout the investigation were of analytical grade.

3.1.2.1. Purification of solvents

The solvents such as benzene, carbon tetrachloride, chloroform, ethyl acetate, and ethanol were used to extract and isolate active principles from the chosen plants. These solvents were purified before use by the method reported in the literature (Riddick and Burger, 1970; Vogel, 1989; Willey, 1963).

Benzene was washed thrice with concentrated sulphuric acid 15% of its volume, twice with water and once with sodium carbonate solution (10%). Finally, it was dried over anhydrous calcium chloride and pure benzene was distilled at 80°C. Carbon tetrachloride was washed with water and dried over anhydrous calcium chloride and its pure form was distilled at 76°C. Commercially available chloroform was dried over anhydrous calcium chloride for twenty four hours and it was distilled at 61°C. Ethyl acetate was shaken with anhydrous potassium carbonate, filtered and
distilled. Ethyl acetate was distilled and collected at 77°C. Ethyl alcohol was dried with anhydrous potassium carbonate, filtered and distilled at 78°C. 70-325 mesh silica gel was obtained commercially. Precoated silica gel 60F254 TLC (E-Merck) plates were used. Pancal D was used as spraying reagent for TLC spot detection.

3.1.2.2. Preparation of Pancal D reagent

2.5 g of ammonium hepta molybdate and 1.5g of ceric sulphate were taken together and dissolved in 50ml of concentrated sulphuric acid. The mixture was cooled to 0°C and made up to 500 ml with distilled water. A yellow solution was obtained.

3.2. Methods
3.2.1. Pharmacognostic Studies
3.2.1.1. Macroscopic observation (Taxonomy)

Fresh plants collected from the wild were brought to the laboratory before desiccation. The morphological and taxonomical characters were carefully examined under stereo microscope (Model: AE-31). The observations were recorded in technical terms.

3.2.1.2. Microscopic observation

Mature and healthy plants with normal plant parts were collected and the required samples of different organs viz., leaf, petiole, young internodes, young and old root and young and old stem were cut and removed from the plant and then fixed in FAA (5ml formaldehyde solution + 5 ml glacial acetic acid + 90 ml 70% ethyl alcohol). 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 tertiary butyl alcohol solution attained super saturation. The specimens were cast into paraffin blocks by the method of Johansen (1940).

3.2.1.3. Sectioning and staining

Paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10 to 12 μm. Dewaxing of the sections was done by customary procedure (Johansen, 1940). The sections were stained with toluidine blue as per the method of O’Brein et al. (1964). Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cytochemical reactions were obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage and blue to the protein bodies. Whenever necessary, the sections were also stained with safranin and fast green.

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections were prepared (sections taken parallel to the surface of the leaf) by clearing of leaf with 5% sodium hydroxide or epidermal peelings by partial maceration employing Jeffrey’s maceration fluid (Sass, 1940) were prepared.

Small segments of lamina were immersed in Jeffrey’s maceration fluid (10% chromic acid and concentrated nitric acid mixed in equal volumes) and kept in thermostat at 60°C for a few hours. Due to partial maceration, the epidermal peeling gets separated from each other. Then the peelings were washed thoroughly, stained with 0.5% aqueous safranin or 0.25% aqueous toluidine blue and mounted in glycerin for microscopic examination.
For the study of venation pattern, leaf bits measuring 1 cm square were boiled in alcohol to remove the chlorophyll and other pigments and then immersed in warm sodium hydroxide (5-10%) for several hours. As the leaf bits became transparent they were washed thoroughly, stained with safranin and mounted in glycerin.

3.2.1.4. Photomicrographs

Microscopic descriptions of the root stem and leaves have been carried out and the anatomical characters are presented as photomicrographs. Microphotographs of different magnifications were taken with Nikon Labphot 2 Microscopic Unit to reveal the anatomical characters. 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 taken from the works of Esau (1979) and Metcalf and Chalk, (1979). Measurement of cells was made with micrometer. For each element, 15–20 measurements were taken and average is presented.

3.3.1. Fluorescence analysis

For analytical studies, air-dried leaf, plant powders and the extracts of the powdered plant material in various solvents such as petroleum ether, chloroform, ethanol and water were examined under ordinary white light and in ultra-violet light (UV 365nm). The fluorescence characters were determined according to the methods of Chase and Pratt (1949). These powders were also treated with various chemical reagents viz., acetone, ethyl alcohol, 1N 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).

3.3.2. Determination of physico-chemical characters

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.

3.3.2.1. 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.

3.3.2.2. 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 No. 41), washed with hot water, ignited and weighed. The percentage of acid insoluble ash was calculated with reference to the air-dried plant sample.

3.3.2.3. 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.

3.3.2.4. Determination of sulphated ash

Two grams of the air-dried and 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.

3.3.2.5. Determination of moisture content

Two grams of the fresh plant 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.

3.3.2.6. Determination of alcohol soluble extractive value

Five grams of the air-dried, macerated and 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 tarred 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.
3.3.2.7. **Determination of water soluble extractive value**

Five grams of the air-dried, macerated and powdered plant sample was soaked with 100ml of water in a closed flask for twenty four hours. It was shaken frequently during first six hours and 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 tarred 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.

3.3.2.8. **Determination of extractive values (Cold percolation parallel extraction)**

Ten grams of the air-dried and coarsely powdered plant materials were taken in a conical flask, extracted (cold percolation parallel extraction method) with petroleum ether, chloroform, ethanol and water and then the solvent has been filtered off. All the extracts were concentrated by solvent evaporation. The extracts obtained with each solvent were weighed and the percentage extractive values were calculated with reference to the air-dried sample (Veeramuthu et al., 2006).

3.4. **Preliminary phytochemical analysis**

The air-dried and powdered plant materials were taken in different amber coloured bottles, extracted (by cold percolation parallel extraction method) with petroleum ether, chloroform, ethanol and water, and then the solvent was filtered off. The soaking procedure was repeated using the same plant materials for a minimum of four times with fresh solvents. 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). The preliminary phytochemical screening is a qualitative chemical evaluation which indicates spectrum of chemical constituents in the chosen plant.
### 3.4.1. Phytochemical screening

The following phytochemical analyses were carried out using the three chosen plants as per the method of Brindha et al. (1981).

**Preliminary phytochemical screening (Brinda et al., 1981)**

<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 + minimum quantity of CHCl₃ + 3–4 drops of acetic anhydride and one drop of con.H₂SO₄</td>
<td>Purple colour develops changing to blue or green</td>
<td>Presence of steroid</td>
</tr>
<tr>
<td>2</td>
<td>Test solution + Piece of tin + 3 drops of thionyl chloride</td>
<td>Violet or purple colour develops</td>
<td>Presence of triterpene</td>
</tr>
<tr>
<td>3</td>
<td>Test solution + very small quantity of anthrone + a few drops of con H₂SO₄, heated</td>
<td>Green to purple colour develops</td>
<td>Presence of sugar</td>
</tr>
<tr>
<td>4</td>
<td>Test solution + 2ml of equal volume of Fehling's reagents* + 3ml of water + heated in water bath</td>
<td>Red orange colour develops</td>
<td>Presence of reducing sugars</td>
</tr>
<tr>
<td>5</td>
<td>Test solution is taken with 2N HCl. Aqueous layer formed is decanted and to it one or two drops of Mayer's reagent** are added</td>
<td>White turbidity or precipitate develops</td>
<td>Presence of alkaloid</td>
</tr>
<tr>
<td>6</td>
<td>Alcoholic test solution + one drop of ferric chloride</td>
<td>Intense colour develops</td>
<td>Presence of phenolic groups</td>
</tr>
<tr>
<td>7</td>
<td>Alcoholic test solution + a bit of magnesium and one or two drops of con. HCl and heated. Cool under running water</td>
<td>Red or orange red colour develops</td>
<td>Presence of flavones</td>
</tr>
<tr>
<td>8</td>
<td>Test solution + 4–5ml of Millon's reagent</td>
<td>A white precipitate is formed. On warming the precipitate turns brick red or it may be dissolved, giving a red solution</td>
<td>Presence of protein</td>
</tr>
<tr>
<td>9</td>
<td>Alcoholic test solution + Ehrlich reagent and a few drops of con.HCl</td>
<td>Pink colour develops</td>
<td>Presence of catachin</td>
</tr>
<tr>
<td>10</td>
<td>Test solution + water shaken well</td>
<td>Foamy lather develops</td>
<td>Presence of saponin</td>
</tr>
<tr>
<td>11</td>
<td>Test solution + water + lead acetate</td>
<td>White precipitate develops</td>
<td>Presence of tannins</td>
</tr>
<tr>
<td>12</td>
<td>Test solution + magnesium acetate solution</td>
<td>Pink colour develops</td>
<td>Presence of anthraquinone</td>
</tr>
<tr>
<td>13</td>
<td>Test solution + 1% ninhydrin in alcohol</td>
<td>Blue or violet colour develops</td>
<td>Presence of amino acid</td>
</tr>
</tbody>
</table>

1. * Fehling’s reagent: (Equal volume of Fehling’s A and B solutions).
   Fehling’s solution A: 35g of CuSO₄ 5H₂O in 500ml distilled water.
   Fehling’s solution B: 50g of NaOH and 173g of Na-K tartarate (Rochelle salt) in 500ml distilled water.
2. ** Mayer’s reagent: 5g of KI and 1.4 ml of mercuric chloride dissolved in 100ml of distilled water.
3.4.2. Quantitative estimations

3.4.2.1. 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 at 620 nm.

Reagents

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

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

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

Starch (Sadasivam and Manickam, 1996)

The residue, after the 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, kept in a boiling water bath for fifteen minutes, treated with 9.2 N perchloric acid (HClO$_4$) with occasional stirring and centrifuged. After collecting the supernatant liquid, the residue was again treated with 4.6 N HClO$_4$. After fifteen minutes of incubation, it was centrifuged and the supernatant liquid was collected. The pooled supernatant liquids were made up to appropriate volume
depending on the starch content of the sample. Then the starch content was determined by using 0.2% anthrone in concentrated H$_2$SO$_4$ as that of described above for sugar. The sugar and starch content of the sample was determined in terms of mg glucose equivalents based on a standard curve.

3.4.2.2. 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 (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 (Pharma Spec-1700). The amount of free amino acids is expressed as glycine equivalents per gram dry weight.

3.4.2.3 Protein (Lowry et al., 1951)

**Principle**

Protein forms a coloured complex with Folin Phenol reagent. The colour is formed due to the reduction of phosphomolybdate by tyrosine and tryptophan present in protein. The absorbance was read at 650 nm using spectrophotometer (Pharma Spec-1700).
Reagents

0.2 M phosphate buffer (pH 7.2), 10% ice cold TCA, 0.2 N NaOH, alkaline sodium carbonate (2 g of Na₂CO₃ dissolved in 100 ml of 0.1 N NaOH), CuSO₄ in sodium potassium tartarate (500mg of CuSO₄ dissolved in 100 ml of 1% sodium potassium tartarate); Alkaline copper reagent was prepared freshly by mixing 50 ml of alkaline sodium carbonate and 1 ml of CuSO₄ sodium potassium tartarate, commercial Folin phenol reagent diluted to 50% using distilled water.

Procedure

Extraction

100 mg of fresh material 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 trichloroacetic 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.

2 ml of 0.2 N 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 4ml using distilled water. To this 5.5 ml of alkaline copper reagent was added, 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 at 650 nm after 30 minutes using spectrophotometer Pharma Spec-1700. Protein content was calculated by referring to standard curve of Bovine Serum Albumin (BSA) and expressed as mg/g/dry weight.
3.4.2.4. 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 (Pharma Spec-1700).

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. One 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.

3.4.2.5 Tannin (Burade et al., 2005)

Principle

Tannin reduces phosphotungsto-molybdic acid in alkaline condition to produce a highly coloured blue solution. The intensity of colour is proportional to the amount of tannins present.

Reagents

Folin Denis reagent, sodium carbonate solution.
Procedure

0.5 g 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 to a known volume. 1 ml of sample extract was transferred to a 100 ml volumetric flask containing 75 ml water. 5 ml Folin Denis reagent and 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.

3.5. Antimicrobial activity assay

3.5.1. Preparation of plant extracts

For the purpose of experimental use each extract sample was dissolved in respective solvent so as to get 1/10 solution.

3.5.2. Microbial strains

The antimicrobial activity was tested against ten randomly selected microbial strains such as Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter aerogenes, Serratia marcescens, Proteus vulgaris, Staphylococcus aureus, Staphylococcus albus, Beta-haemolytic streptococci, Bacillus subtilis and one fungus Candida albicans. The selected microbial strains were obtained from Department of Microbiology, Sri. Paramakalyani College, Alwarkuruchi, Tirunelveli District.

3.5.3. Culture media and Inoculum

The media used for antimicrobial tests were Muller Hinton (MH) agar and Sabouraud Dextrose Agar (SDA). Each organism was maintained in a separate
culture media and was recovered for testing by sub culturing on a fresh media. Inoculums of each bacterial strain were transferred in 10 ml of Muller Hinton agar broth and incubated overnight at 37°C.

3.5.4. Preparation of sterile antibiotic discs

Antimicrobial activity was assayed by filter paper disc diffusion method. Whatman No. 1 filter paper of 5 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. The same procedure has been followed for the fungus using Sabouraud Dextrose Agar.

3.5.5. Antimicrobial assay

Antimicrobial assay was conducted by the method described by Lennette (1985) with some modification. 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.

Gentamicin (10 µg/ml) was used as positive control and the 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.
3.6. High Performance Thin Layer Chromatography (HPTLC)

The following are the various steps involved in the HPTLC:

- Selection of TLC/HPTLC plates and sorbent
- Sample preparation including any clean up and pre-chromatographic derivatization
- Application of sample
- Development (separation)
- Detection including post-chromatographic derivatization
- Quantification
- Documentation

Sample & standard

Selection of chromatographic layer

Layer Pre-washing

Layer pre-conditioning

Application of sample and standard

Chromatographic development

Detection of spots

Scanning and documentation of Chromatoplate
Procedure

The extracts thus obtained from each plant were then subjected to anhydrous sodium sulphate to remove the traces of moisture content. The chloroform layer is concentrated using rotary evaporator under reduced pressure.

The extracted sample solutions were eluted in thin layer chromatography for separation of compounds. The pre-coated silica gel aluminum plates (Merck, Germany) were cut into 10 cm x 10 cm size. The samples were spotted using applicator LINOMAT IV. The chromatographic tank (10cm x 6cm) was filled with developing solvent system and equilibrated for about 5 minutes. The solvent system such as acetone: hexane (3:7) was used as mobile phase. Thin layer plate was placed gently into the tank and allowed to stand till it reaches about 1cm from the top of the plate; the plate is removed and allowed to air dry. The compounds were detected using UV light (CAMAG TLC SCANNER II).

HPTLC was performed using a CAMAG HPTLC spectrophotometer provided with a scanner II densitometry a Linomat at IV applicator.

HPTLC parameters

<table>
<thead>
<tr>
<th>Instrument used</th>
<th>Camag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plates used</td>
<td>Silica Merck 60F254 Plates.</td>
</tr>
<tr>
<td>Software</td>
<td>Camag</td>
</tr>
<tr>
<td>Applicator</td>
<td>Camag Linomat IV applicator.</td>
</tr>
<tr>
<td>Scanner</td>
<td>Camag TLC scanner II</td>
</tr>
<tr>
<td>Visualizing wavelength</td>
<td>280nm</td>
</tr>
<tr>
<td>Solvent system</td>
<td>acetone: hexane (3:7)</td>
</tr>
<tr>
<td>Volume injected</td>
<td>20μl</td>
</tr>
</tbody>
</table>
3.7. High Performance Liquid Chromatography (HPLC)

Principle

The term HPLC is used to describe a separation method in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase. Due to their differing interactions with the stationary and mobile phases different components of the sample are carried forward at different rates by the moving liquid phase.

Mobile phase : acetonitrile: water (60:40)

Stationary phase : Finely divided solid held inside the column

Uses : It is used to find the amount of a chemical compound present in a mixture.

An isocratic HPLC (Shimadzu HPLC Class VP series) with one LC-10 AT VP, pump (Shimadzu), variable wavelength UV-Visible Detector SPD-10A VP, (Shimadzu), and reverse phase Gemini 5u C18 110A, Phenomenex column (250 × 4.60 mm) was used. The mobile phase components [acetonitrile: water (60:40)] were filtered through 0.2 micron membrane filter before use, and pumped from the solvent reservoir at a flow rate of 0.5 ml/min. The column was maintained at 27 deg. Syringe (Bonaduz Schweiz, Hamilton) was used for injection of respective samples (20 µl).

HPLC Parameters

- Instrument Name - HPLC (Shimadzu)
- Column - C18 ODS, Gemini 5u,110A, Phenomenex
- Mobile phase - Acetonitrile : Water (60:40)
- Detector - SPD-10A VP, (Shimadzu)
- Flow rate - 0.5 ml/min.
- Injection volume - 20 µl
The ethanol crude extracts of the chosen plants were fractionated with column chromatography viz., a silica gel (70/325 mesh) and eluted using mixtures, in their increasing order of polarity, of solvents such as n-hexane: ethyl acetate and n-hexane: ethyl acetate: methanol respectively. The fraction eluted with every 25 ml of solvent mixture was analysed with TLC silica gel glass plate and also with pre-coated silica gel aluminum plates with suitable mobile phase. The different sample single spots were confirmed in UV chamber, isolated into individual fractions and labeled. The isolated individual pure compounds have been characterized using the following spectral techniques.

Ultra-violet spectra were recorded on a Tech Comp UV spectrophotometer using chloroform as the reference. This was used to determine the level of conjugation in the unknown compounds. Infrared spectra were recorded on an IP S 85 Bruker FTIR instrument using KBr pellets. It was used primarily to determine the functional groups present in a molecule. The $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker 500 MHz instrument using Tetramethylsilane as the internal standard. The samples were prepared using CDCl$_3$ and methanol. The NMR gives information about the hydrogen and carbon atoms present in the molecule, in three key aspects such as chemical shift, integration and splitting. Mass spectrum was recorded on a JEOL GCmate instrument and used to determine the molecular formula of the unknown compound.