MATERIALS & METHODS
Chapter 2
MATERIALS AND METHODS

(I) TISSUE CULTURE WORK

This study was undertaken with the objective of developing a protocol for high frequency shoot regeneration and complete plant development of *Acacia catechu*, *Cassia fistula* and *Bauhinia purpurea* that could be used for further experiments.

2.1 Plant Collection

The Plant samples of *A. catechu*, *C. fistula* and *B. purpurea* used in this study were taken from, Gujarat Environment Education and Research Foundation, Gandinagar with the assistance of local plant keepers and authenticated by officials of Gir foundation.

2.2 Plant Materials

Mature seeds and nodal explants of *A. catechu*, *C. fistula* and *B. purpurea* were collected from field grown mature plants. They were used as experimental plant materials for *in vitro* shoot regeneration. For callus induction, leaves and immature cotyledons (from green pods) were collected from the respective plant and washed under running tap water followed by sterilized distilled water.

2.3 Chemicals

Most of the chemicals for culture medium were procured from Hi-media and Growth regulators were obtained from Sigma chemicals.

2.4 Glasswares

Borosil glass culture tubes (150 x 25 or 100 x 25 ), wide mouth 100 ml, 250 ml
and 500 ml conical flasks, 90 mm glass petri dish were routinely used for tissue culture work.

### 2.5 Nutrient Media

MS (Murashige and Shoog, 1962) was used for regeneration studies. The constituents of these media are given in Table 1

**Table 1 The constituents of MS media**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amount as Formulated (mg/l)</th>
<th>Amount as stocks (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macronutrients (Stock I)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1,650</td>
<td>33,000</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1,900</td>
<td>38,000</td>
</tr>
<tr>
<td>CACl₂.2H₂O</td>
<td>440</td>
<td>8,800</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370</td>
<td>7,400</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>3,400</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NAH₂PO₄.H₂O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Macronutrients (Stock II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>166</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>1,240</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>22.3</td>
<td>4,460</td>
</tr>
<tr>
<td>MnSO₄.H₂O</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>8.6</td>
<td>1,720</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25</td>
<td>50</td>
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</table>
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<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄ · 5H₂O</td>
<td>0.025</td>
<td>5</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>0.025</td>
<td>5</td>
</tr>
<tr>
<td>Iron (Stock III)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>27.8</td>
<td>5,560</td>
</tr>
<tr>
<td>Na₂·EDTA, 2H₂O</td>
<td>37.3</td>
<td>7,460</td>
</tr>
</tbody>
</table>

Vitamins & amino acids (Stock IV)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inositol</td>
<td>100</td>
<td>20,000</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>Phridoxine, HC1</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>Thiamine, HC1</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>400</td>
</tr>
<tr>
<td>Sucrose (g/l)</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

Constituents have been grouped as used in different stocks.

The basal media were supplemented with different combinations of growth regulators as per the requirements of various experiments.

2.6 Growth Regulators

The growth regulator stocks used in different experiments

<table>
<thead>
<tr>
<th>Growth regulators</th>
<th>Abbreviation</th>
<th>Dissolved in</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4–dichlorophenoxyacetic</td>
<td>2,4-D</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>Indole – 3 acetic acid</td>
<td>IAA</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>Indole – 3 – butyric acid</td>
<td>IBA</td>
<td>1N NaOH</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Chemical</th>
<th>Abbreviation</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Naphthaleneacetic acid</td>
<td>NAA</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>6-Benzylaminopurine</td>
<td>BAP</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>Kinetin</td>
<td>Kn</td>
<td>1N NaOH</td>
</tr>
<tr>
<td>(6-Furfurylaminopurine)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dissolved in minimum amount of the solvent to obtain a clear solution.
Final volume was made up with double distilled water.

2.7 Preparation of Stock Solutions

For MS media, four stock solutions viz. Stock I, II, III and IV were prepared as given in Table 3.1. FeSO$_4$.7H$_2$O and Na$_2$EDTA.2H$_2$O were weighted separately in the required quantities and each was dissolved separately in 100 ml distilled water, Na$_2$EDTA solution was warmed slightly. The two solutions were mixed, pH was adjusted to 5.5 and the final volume was made up to 250 ml.

The stock solutions of growth regulators (Grs) were prepared as listed in Table 3.2. Stocks I, II and III were autoclaved in glass bottles and stored at 4°C whereas stock IV and GR stock solutions were prepared in sterile distilled water and stored at – 20°C.

2.8 Medium Preparation

1. Stocks I, II, III and IV and GR stock solutions were pipetted out in the required volumes in a beaker.
2. The required amount of sucrose and casein hydrolysate was weighted and added to the stocks. Distilled water was added to make half of the final volume of the medium.
3. The solution was mixed thoroughly on a magnetic stirrer and pH was adjusted to 5.8 using 1N NaOH or HCl as per requirement.
4. Final volume was made up with distilled water. In case of agar medium, the required amount of agar was added and melted by heating.
5. Medium was dispensed in suitable culture vessels; 30 ml medium was
poured in each 100 ml conical flask, while 15 ml medium was pipetted into each 150 x 25 mm culture tube, and the flasks/tubes were stoppered with cotton plugs (non-absorbent cotton wrapped in cheese cloth) or polypropylene caps.

6. The culture tubes/flasks were covered with brown paper or aluminum foil and
7. autoclaved at 15 psi (1.06 kg/cm²) for 20 min at 120°C.
8. Thermolabile compounds and antibiotics, viz. zeatin, kanamycin, hygromycin, were filter sterilized, using filter sterilization assembly equipped with 0.22 μm pore cellulose nitrate membrane filters. The filters – sterilized solutions were added to autoclaved media cooled to ~45°C and mixed thoroughly. Media were then dispensed into autoclaved flasks/culture tubes under aseptic conditions and allowed to gel.

2.9 Sterilization

2.9.1 Sterilization of Glassware and Instruments

1. The glassware were wrapped in aluminum foil or brown paper and steam sterilized in an autoclave at 1.06 kg/cm² and 120°C for 20 min.
2. Instruments used for handling of explants/cultures, such as forceps, scalpels, needles etc. were sterilized by dipping them in 95% ethanol followed by flaming.

2.9.2 Surface Sterilization of Plant Materials

Mature Seeds
Healthy intact (seed coat crack-free) seeds were selected and surface sterilized as follows. Seeds were washed with 1% Tween 80 for 5 min, given 30 sec rinse with 70% ethanol, treated with 0.1% HgCl₂ for 7 min and rinsed 5 to 6 times with sterile distilled water. The seeds were then soaked in sterile distilled water overnight.
**Immature Pods and Leaves**

Green pods and leaves were collected from the field, washed thoroughly in running tap water and then treated for 10 min with 1% Tween 80, followed by a 15 min treatment with 1.5% sodium hypochlorite (4% v/v). They were then washed 4-5 times with sterile distilled water. Immature seeds were dissected out of the pods and leaves were dissected in small pieces were treated for 5 minutes with 0.5% sodium hypochlorite. They were then washed 4-5 times with sterile distilled water.

**2.10 Regeneration in Axenic Cultures**

**Shoot Regeneration from Intact Seeds**

Seed coats of the overnight soaked sterilized seeds were removed using fine corseps and the seeds were kept on solidified MS medium containing different GR combinations.

**Shoot Regeneration from Cotyledonary Nodes**

1. Mature seeds devoid of seedcoat were germinated on MS medium supplemented with different GRs.
2. After two to three weeks, cotyledons were removed and the shoots were scraped off from the cotyledonary nodes (Cns).
3. The CN was now excised and kept on MS medium supplemented with different GR combinations.

**2.11 Callus Induction from Imature Pods and Leaves**

Immature pods and leaves from young twigs were used as explants for callus induction. They were cultured on solid MS medium containing various concentrations of 2,4-D (0.5 to 2 mg/l)
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2.12 Culture Conditions

Cultures were incubated in a culture room under 16 h light and 8 h dark photoperiod at constant temperature of 18 ± 2°C or 24 ± 2°C, the light intensity being 60 μmoles/m²/sec provided by white fluorescent light supplemented with 100 W incandescent bulbs. Relative humidity in the culture room was 78 ± 4%. For callus culture the explants were incubated in diffused light of 1500 lux at 25 ± 2°C. Callus cultures were harvested at day 30 of cultivation and dried at 60°C.

2.13 Observations

Cultures were observed at weekly intervals to assess growth/regeneration events. Details observations on number of explants forming shoots, number of shoots/explant, quality of shoots; number of roots/shoot, length of root and quality of roots were recorded.

2.14 Statistical Analyses of Data

Analysis of Variance (ANOVA)

The experiments were laid out according to randomised block design (for single factor experiments) or nested design (two factor experiments). Each experiment usually had three to five replicates, the number of cultures per replicate varied and are listed with different experiments. The analysis of variance appropriate for the design was carried out to detect significance of differences among the treatment means (Compton, 1994).

Duncan's Multiple Range Test (DMRT)

The treatment means were compared using DMRT, for which the following procedure was adopted.
1. The treatment means were arranged and ranked in an ascending order.

2. The least significant ranges (LSR) were calculated using the following formula.

\[ \text{LSR} = rp \left( \frac{s^2}{R} \right) \]

where, \( rp \) values are table values of significant studentised ranges for the different distances in the ranks of the two means being compared, \( s^2 \) is the error mean square and \( R \) is the number of replications in the experiments.

3. For comparing any two means, their differences were compared with the LSR appropriate for their rank distance. If the difference was equal to or larger than LSR, the means were declared significantly different from each other and they were assigned different letters as their superscript.
(II) Microbiological Work

This study was undertaken with the objective of testing the antibacterial and antifungal activities of *Acacia catechu*, *Cassia fistula* and *Bauhinia purpurea* leaves and callus extracts that could be used for further experiment.

2.1 Leaves and Callus Collection

The leaves samples of *A. catechu*, *C. fistula* and *B. purpurea* used in this study were taken from National Gir Foundation, Gandinagar Gujarat with the assistance of local plant keepers and authenticated by officials of Gir foundation. Fresh leaves were washed under running tap water followed by sterilized distilled water, shade dried and then powdered with the help of sterilized pestle and mortar. Callus tissues obtained from different hormonal combinations were dried and powdered. The powders were further subjected for different extraction protocols.

2.2 Chemicals

Most of the chemicals used for microbiological work were procured from Hi-media and Sigma chemicals.

2.3 Glasswares

Borosil glass culture tubes (150 x 25 or 100 x 25), wide mouth 100 ml, 250 ml and 500 ml conical flasks, 90 mm glass petri dish were routinely used for microbiological work.

2.4 Preparation of the Extracts

2.4.1 Aqueous Extraction

Shade dried 10 gm powders of leaves were dissolved in 400 ml distilled water till
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one fourth of the extract initially taken was left behind after evaporation. The solution was then filtered using muslin cloth. Filtrate was centrifuged at 5000 rpm for 15 min. The supernatant was again filtered using Whatman Filter No. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles and stored at 4°C until further use.

2.4.2 Organic Solvent Extraction of Leaves and Callus

Shade dried 10 gm powders of leaves and callus were thoroughly mixed with 100 ml organic solvent (viz., methanol, hexane and acetone). The mixture was placed at room temperature for 24 h on shaker with 150 rpm. Solution was filtered through muslin cloth and then re-filtered by passing through whatman filter No. 1. The filtrate thus obtained was concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts for each type of organic solvent were prepared by mixing well the appropriate amount of dried extracts with dimethyl sulphoxide (DMSO) to obtain a final concentration of 100 mg/ ml that was used for evaluation of antibacterial and antifungal activities. Each solution was stored at 4°C after collecting in sterilized bottles until further use.

2.5 Antimicrobial Activity Assays

2.5.1 Microorganisms and Culture Media

2.5.1.1 Microbial Cultures

Five strains of bacteria and yeast were used as test microorganisms. The bacterial strains included:

Gram-positive: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 10707) Gram-negative: *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), The Yeast: *Candida albicans* (ATCC 10231) All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Life Sciences, Gujarat University
and MG Institute of Science. All the cultures were maintained and sub cultured on nutrient agar medium.

### 2.5.1.2 Culture Media

Mueller-Hinton agar (MHA), Sabouraud dextrose agar (SDA) and Nutrient agar (NA) were used for microbiological studies. The constituents of these media are given in tables 2-4.

#### Table 2 The Constituents of MHA Medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amount as formulated (g/1000ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>1000</td>
</tr>
<tr>
<td>MHA</td>
<td>38</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

#### Table 3 The Constituents of SDA Medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amount as formulated (g/1000ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>1000</td>
</tr>
<tr>
<td>Glucose</td>
<td>20</td>
</tr>
<tr>
<td>Peptone</td>
<td>10</td>
</tr>
<tr>
<td>Agar</td>
<td>20</td>
</tr>
</tbody>
</table>

#### Table 4 The Constituents of NA Medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Amount as formulated (g/1000 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>1000</td>
</tr>
</tbody>
</table>
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### 2.6 Inoculum Preparation

Mueller Hinton broth and Sabouraud dextrose were applied for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in Mueller Hinton broth at 37°C for 18 h and adjusted to a final density of $10^6$ CFU/ml by diluting fresh cultures and comparing with McFarland density. *C. albicans* was aseptically inoculated on Petri dishes containing autoclaved, cooled and settled SDA medium. The Petri dishes were incubated at 31°C for 48 h to give white round colonies against a yellowish background. These were aseptically sub cultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. One ml yeast suspension in normal saline was added to 74 ml of sterile medium, kept at 45°C to give concentration of $2 \times 10^7$ cells/ml.

### 2.7 Antibacterial Assay

*In vitro* antibacterial activities of all aqueous and organic extracts of different plants were determined by standard agar well diffusion assay. Petri dishes (100 mm) containing 25 ml of Mueller Hinton Agar (MHA) were seeded with 100 μl inoculum of bacterial strain (Inoculum size was adjusted so as to deliver a final inoculum of approximately $10^6$ CFU/ml). Media was allowed to solidify and then individual Petri dishes were marked for the bacteria inoculated. Wells of 6 mm diameter were cut into solidified agar media with the help of sterilized cup-borer. 100 μl of each extract was poured in the respective well and the plates were incubated at 37°C for overnight. DMSO and sterilized distilled water were used as negative control while tetracycline antibiotic (one unit strength) was used as positive control. The experiment was performed in triplicate under strict aseptic conditions and the antibacterial activity of each extract was expressed in terms of

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>3.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
</tbody>
</table>
the mean of diameter of zone of inhibition (in mm) produced by the respective extract at the end of incubation period.

2.7.1 Minimum Inhibitory Concentration (MIC)

Plant extracts prepared in methanol were taken to determine MICs by standard two-fold microbroth dilution methodology.

2.7.1.1 MIC for the Bacteria

The antibacterial activity of the extracts was examined by determining the MIC in accordance with Clinical and Laboratory Standard Institute (CLSI) methodology (CLSI, 2005). All tests were carried out in Mueller Hinton broth supplemented with dimethyl sulphoxide, DMSO at a final concentration of 10% (v/v) to enhance their solubility. The extracts were dissolved in MHB. Test strains were suspended in MHB to give a final density of 5x10^5 cfu ml\(^{-1}\) and these were confirmed by viable counts. Dilutions ranging from 100 to 2000 mg/ml of the extracts were prepared in tubes including one growth control, MHB+ DMSO 10% (v/v) and one sterility control MHB + DMSO 10% (v/v + test extracts). The MIC values were determined from visual examinations as being the lowest concentration of the extracts with no bacterial growth. Plates were incubated under normal atmospheric conditions at 37°C for 24 h for bacteria.

2.7.1.2 MIC for the Yeasts

The antifungal activity of the extracts was examined by determining the MIC in accordance with CLSI methodology (CLSI, 2008) using Yeast Nitrogen Base Glucose (YNBG) medium supplemented with DMSO at a final concentration of 10% (v/v). The extracts were dissolved in YNBG medium. Yeast strains were cultured for 24-48 h at 35°C on SDA and then suspended in 4 ml of sterile distilled water by adjusting to 1 McFarland using a nephelometer to give a final inoculum concentration of 1.5 ± 1.0 x10^3 cfu ml\(^{-1}\). Dilutions ranging from 100 mg/ml to
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2000 mg/ml of the extracts were prepared in the tubes including one growth control, YNBG + DMSO 10% (v/v), and one sterility control YNBG 10% (v/v + test extracts). A 100 μl suspension of each of Candida strains in YNBG was added to individual wells and incubated at 35°C for 48 h. The MICs of the extracts were defined as the lowest concentration that inhibited more than 80% of visible fungal growth. The final concentration of dimethyl sulphoxide in the assays did not interfere with the bacterial and candidal proliferation.

2.8 Culture Conditions

Plates and tubes were incubated for 24 h at 37°C.

2.9 Observations

Cultures were observed at weekly intervals to assess growth events and details of observations on zone of inhibition, MICs were recorded.

2.10 Statistical Analyses of Data

**Analysis of Variance (ANOVA)**

The experiments were laid out according to randomised block design (for single factor experiments) or nested design (two factor experiments). In each zone of inhibition experiments usually had three replicates and the mean of three replicates was noticed. The significance of the obtained results was checked by one way ANOVA. Standard deviation (SD) was calculated by using Microsoft Excel 2007.

(III) Bioassay

The plant ingredients were extracted from the plants by suitable organic solvent and the extract were separated, purified and identified by column chromatography,
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TLC and GC-MS analysis. The bioassay of the extracts were carried out as described elsewhere (Roy et al., 2010).

2.1 Chemicals

Most of the chemicals and reagents used for TLC and column chromatography were of analytical purity and solvents were of HPLC grade (Merck).

2.2 Glasswares

Silica gel (100-200 mesh), Glass capillary (0.5 mm) and the glass column (30 mm x 300 mm) was used for column chromatography. And TLC plate (a silica gel G plate, 30 mm x 100 mm) was used for TLC operation.

2.3 Collection of Plant Materials

The plant samples of A. catechu, C. fistula and B. purpurea used in this study were taken from National Gir Foundation, Gandinagar (Gujarat). Fresh plant materials were washed under running tap water followed by distilled and sterilized water, air-dried at room temperature (22°C) for 3 weeks and then processed.

2.4 Extraction

The dried leaves (250 gms) were powdered and exaustly macerated with methanol for 1 week in a shaker. The collected extracts were filtered and evaporated under vacuum. The residues were dried and weighed.

Thin Layer Chromatography (TLC)

TLC technique was employed as a means of assessing quality and purity of the plants extracts. The $R_f$ value of a compound determined under identical conditions is characteristic and can be used as an aid to its identity.
2.5.1 Preparation of Chromatographic Plates

The chromatographic plates of size 20 x 20 x 0.4 cm were cleaned with chromic acid, followed by rinsing under tap water and finally with distilled water. The plates were dried in hot air oven at 100ºC. A uniform suspension of silica gel-H was prepared by dispersing one part of adsorbent in 2.5 parts of distilled water using a glass mortar and pestle. A stahl type applicator was cleaned thoroughly and adjusted to get an adsorbent layer of 0.3 mm thickness. The suspension was then poured into the reservoir of the applicator. Care was taken to eliminate air bubbles in the slurry. Then reservoir was inverted and the spreader pulled over the plates uniformly and smoothly. The applicator was removed and the plates were allowed to dry at room temperature and activated at 120ºC for 60 minutes in hot air oven. The plates were kept in dessicator or in oven prior to use.

2.5.2 Solvent Systems Used

TLC of the extracts were made by using different solvents and mixed solvents. Methanol, chloroform, toluene, petroleum ether and ethyl acetate singly and in combination were used as developing agents.

2.5.3 Saturation of Chamber

A sheet of filter paper was laid so as to cover three sides of the TLC chamber from inside and was soaked into the solvent system prior to running of the chromatogram. It was insured that the paper was fully wet and struck to the walls of the chamber before introducing the plates. The chamber was left undisturbed before introducing the plates for half an hour, so that the saturation of chamber with solvent was completed.
2.5.4 Development of Chromatogram

The loaded plates were then placed vertically in the chamber with the bottom edge immersing in developing medium. After the solvent front moved up to a distance of about 18 cm, the plate was taken out, solvent front was marked and the plate was dried in hot air oven at 60ºC.

2.5.5 Location of Spots

The position of the spots was identified first by observing visible spots in any or colour was intensified under the condition of iodine vapour after drying. According to the results, a suitable eluent for separation was selected out.

2.6 Column Chromatography Separation

A chromatographic column was a long glass tube having 100 cm length and 12 cm breath and packed with particles of stationary phase Silica gel (0.063-0.200 mm). Wet-process method was adopted to pack the column. Silica gel acted as a sorbent and mixture of Petroleum ether and ethyl acetate in ratio 80:20 & Toluene and ethyl acetate in ratio 80:20 one by one were the eluents. At the beginning, silica gel was added to the eluent. It was stirred and slowly poured into the column until the bed layer was solid. The temperature of the column was 25°C. The petcock was opened to decrease eluent level until its liquid surface and silica gel level were equal. Then the sample solution was poured slowly into the column along its wall. Finally, the eluent was added to the column, keeping the sorbent covered by the eluent. For both the mixtures five samples are collected. The separated compounds were collected in numbered test tubes and identified by TLC. The same tubes containing the compound were combined for condensing on a rotary evaporator.

The flow speed was an important factor in above column chromatography separation because if the speed were too fast, too much organic solvent would be wasted. Furthermore, the boundary of the colour band was not distinct and
separation was seriously affected. Conversely if it were too slow, the time needed to separate the samples will increase considerably and its economic benefits decrease.

2.7 Calculation of Rf values of Spots Appeared in TLC

When solvent front passed more than half of the TLC plate, it was marked and the plates were taken out and allowed to dry. The plates were then placed in iodine chamber and Rf (Ratio of flow) values were calculated as follows:

\[ R_f = \frac{\text{Distance travelled by the component molecules from the starting point}}{\text{Distance travelled by the solvent front from the starting point}} \]

2.8 GC-MS Analysis

For GC-MS analysis, a Hewlett-Packard-5890-II (Global Medical Instrumentation) gas chromatograph, equipped with a flame-ionization detector (FID) and coupled with an electronic integrator was used. Quantitative data were obtained by electronic integration of the FID-area data, without response factor correction. The gas chromatograph was fitted with a fused-silica capillary column (30 m x 0.25 mm i.d., film thickness 0.25 μm). Helium was used as the carrier gas at 35.6 K Pa pressure with a flow rate of 1ml/min; split ratio of 20.0; injection volume 1μl; injection temperature, 280°C at a rate of 9°C/min; the final temperature of 280°C was held for 5 min. GC-MS analyses were performed on a Hewlett-Packard-5890 gas chromatograph (controlled with the HP Chemstation software), equipped with a 5972 mass-selective detector. The mass spectrometer operated in EI mode at 70 Ev, using a scanning speed of 1.5s over the range 40-300 amu and an ion source temperature of 180°C was used to determine the mass of the unknown compounds.

2.8.1 GC-MS Sample

100 gm of the respective sample was dissolved in 1ml of the solvent. This mixture was sonicated for 15 mins and 1μl aliquot of the greenish clear solution was
directly injected. Samples were introduced to the column via an inlet. This inlet was typically injection through a septum. Once in the inlet, the heated chamber acts to volatilize (vaporise) the sample. In a split system, a constant flow of carrier gas moves through the inlet. A portion of the carrier gas flow acts to transport the sample into the column. Another portion of the carrier gas flow gets directed to purge the inlet of any sample following injection (septum purge). Yet another portion of the flow was directed through the split vent in a set ratio known as the split ratio.

2.8.2 Components Identification

The components of the fractions were identified by comparison of their EI mass spectra to those of Wiley-275 K.L GC-MS computer Library and confirmed by comparison of their Kovats retention index with those of authentic compounds or with the corresponding data published in the literature. The identity of the mass spectra above 95% was needed for the identification of compounds.