CHAPTER 4

MATERIALS AND METHODS

4.1 COLLECTION OF PLANT MATERIALS AND IDENTIFICATION

The plants materials *Aegle marmelos*, *Solanum nigrum* and *Cassia fistula* were purchased from local Ayurvedic medicinal shop Chennai, and they were identified and authenticated by the Chief Botanist, Tamil Nadu Aromatic Medicinal Plants Corporation Limited (TAMPCOL), Arignar Anna Siddha Medical College and Hospital Campus, Chennai, Tamil Nadu, India.

*Aegle marmelos*

Latin name : *Aegle marmelos*

Family : Rutaceae

English name : Holy fruit

Sanskrit : Vilvah, Sivadrumah

Tamil : Vilvam

**Habitat:** It grows all over India, Andaman, and Nicobar Islands. And dry forests throughout the plains of India.
Aegle marmelos full view

Aegle marmelos tree bearing fruit

Figure 1 Aegle marmelos
Solanum nigrum

Latin name : Solanum nigrum
Family : Solanaceae
English name : Black nightshade
Sanskrit : Kakamachi
Tamil : Manitakali

Habitat: It is found throughout the plains and waste places in India

Solanum nigrum a full view plant

Solanum nigrum plant with fruit

Figure 2 Solanum nigrum
**Cassia fistula**

- **Latin name**: *Cassia fistula*
- **Family**: Caesalpinioidea
- **English name**: Indian labrum
- **Sanskrit**: Aragvadha
- **Tamil**: Konnei

**Habitat:** It is found throughout the plains and waste places in India.
4.2 ETHANOLIC EXTRACTION OF PLANT MATERIALS

- The plant materials were dried in shade, and powdered in a mechanical grinder.
- The powder of *Aegle marmelos, Solanum nigrum* and *Cassia fistula* plant materials was initially defatted with petroleum benzene (60-80°C) followed by 1000 ml of ethanol by using a Soxhlet extractor for 72 hrs at a temp not exceeding the boiling point of the solvent [Lin et al., 1999].
- The extract was filtered using Whatman filter paper (No. 1) and then concentrated in vacuum and dried at 45°C for ethanol removal, and the extracts were kept in sterile bottles under refrigerated conditions until use.
- The dry weight of the plant extracts was obtained by the solvent evaporation and used to determine concentration in mg/ml. The extract thus obtained was directly used in the assay of antimicrobial activity.

4.3 SELECTION OF ANTIBIOTICS

Broad spectrum antibiotics, *Penicillin* and *Tetracyclin* were used as control drugs.

4.4 SELECTION OF BACTERIAL STRAINS

Microorganisms *Escherichia coli, Pseudomonas aeruginosa Staphylococcus aureus,* and *Bacillus subtilis* pure slant cultures were obtained from National Collection of Industrial Microorganism (NCIM) Pune, India.
**Escherichia coli**

Domain : Bacteria  
Phylum : Proteobacteria  
Class : Gammaproteobacteria  
Order : Enterobacteriales  
Family : Enterobacteriaceae  
Genus : *Escherichia*  
Species : *coli*

*Figure 4* *Escherichia coli* NCIM: 2065
**Pseudomonas aeruginosa**

Domain : Bacteria  
Phylum : Proteobacteria  
Class : Gammaproteobacteria  
Order : Pseudomonadales  
Family : Pseudomonadaceae  
Genus : *Pseudomonas*  
Species : *aeruginosa*

*Figure 5 Pseudomonas aeruginosa NCIM: 2200*
Staphylococcus aureus

Domain : Bacteria
Phylum : Firmicutes
Class : Bacilli
Order : Bacillales
Family : Staphylococcaceae
Genus : Staphylococcus
Species : aureus

Figure 6 Staphylococcus aureus NCIM: 2079
Bacillus subtilis

Domain : Bacteria
Phylum : Firmicutes
Class : Bacilli
Order : Bacillales
Family : Bacillaceae
Genus : Bacillus
Species : subtilis

Figure 7 Bacillus subtilis NCIM: 3079
4.5 DETERMINATION OF ANTIMICROBIAL ACTIVITY

Antimicrobial activity was performed by standard method, disc diffusion on agar and the MIC was calculated using dilution method.

4.6 DILUTIONS METHODS

Dilution susceptibility testing methods were used to determine the minimal concentration of antimicrobial compound to inhibit or kill the microorganisms. This can be achieved by dilution of antimicrobial compound in either agar or broth media. Antimicrobials are tested in log₂ serial dilutions (two fold).

4.6.1 Broth Dilution Method

The Broth Dilution Method is a simple procedure for testing a small number of isolates, even single isolate.

Materials required

- Sterile graduated pipettes of 10 ml, 5 ml, and 1 ml.
- Sterile test tubes and test tube racks.
- Overnight broth culture of test and plant extracts.
- Required antibiotics and plant extracts.
- Sterile distilled water-500 ml and freshly prepared nutrient broth medium.
Preparation of microorganisms for experiment

The pure cultures of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis* were sub-cultured in nutrient broth. And the inoculated broth tubes were incubated at 37°C for 24 hours. After completion of incubation period, when growth was observed the tubes were kept into 2-8°C until use.

Growth method

- At least three to five well-isolated colonies, with same morphological characters were selected from the agar plates, of a particular microorganism. Aseptically transfer the culture into the tube containing 5 ml of nutrient broth medium.
- The broth culture is incubated at 35°C for 8 hours
- After incubation period, broth culture becomes turbid

Procedure for dilution method

- Plant extracts and antibiotics (control) where prepared at different concentrations (mg/ml).
- For each plant extracts, one row of 12 sterile test tubes were arranged in racks inside the laminar air flow.
- 4 ml of broth containing mg/ml of one plant extract was prepared in a conical flask the contents were mixed thoroughly with a pipette.
• Then two ml of the prepared mixture was transferred to the first tube of each row.

• Using a fresh pipette, 4 ml of broth was added to the remaining 4 ml in the conical flask and mixed and then 2 ml was transferred to the second tube in each row. Dilutions were prepared for remaining test tubes.

• 2 ml of plant extract free broth was placed to the last tube in the row.

• Each test tube was inoculated with one drop of an overnight broth culture of one test organism. The culture was diluted in such a way, so that diluted test mixture contained $10^6$ organism/ml.

• Test tubes were incubated for 18 hours at 37°C.

• A tube containing 2 ml broth was inoculated with the organism and kept at 4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition.

• Same steps were repeated for another plant extract and other two antibiotics for each bacterial culture.

4.6.2 Disc diffusion Method

Reagents for the disc diffusion test

Mueller-Hinton Agar Medium

• Mueller-Hinton Agar is considered to be the best for routine susceptibility testing of non-fastidious bacteria for the following reasons.
• It shows acceptable batch-to-batch reproducibility for susceptibility testing.
• Medium is transparent, so that the inhibition zone can be visualized clearly.
• It gives satisfactory growth of most non fastidious pathogens.
• A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

**Composition of Mueller-Hinton Agar**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef, infusion form</td>
<td>300gm/ liter</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>17.50gm/ lit</td>
</tr>
<tr>
<td>Starch</td>
<td>1.50gm/ lit</td>
</tr>
<tr>
<td>Agar</td>
<td>17gm/ lit</td>
</tr>
<tr>
<td>pH</td>
<td>7.3 ± 0.2 (at 25°C)</td>
</tr>
</tbody>
</table>

**Preparation of Mueller-Hinton agar**

1. Mueller-Hinton Agar was prepared from a commercially available dehydrated medium (Himedia) according to the manufacturer’s instructions.
2. Immediately after autoclaving, it was allowed to cool in a water bath at 45-50°C. Before using the medium in laminar air flow the outer surface of the conical flask should wiped with cotton using 70% IPA, to avoid cross contamination.
3. Aseptically transfer about 25 to 30 ml of sterile prepared medium into 100mm dia petridishes. The petridish should be flat-bottomed and it should be placed on a level, horizontal surface to give a uniform depth of approximately 4 mm. The agar medium was allowed to cool at room temperature, unless the plate is used in the same day, the prepared should be stored in a refrigerator at 2-8°C.

4. Plates were used within seven days after preparation unless adequate precautions, such as wrapping in plastic or paraflim, have been taken to minimize drying of the agar.

5. A representative sample of each batch of plates was examined for sterility by incubating at 30 to 35°C for 24 hrs or longer.

**Preparation of antibiotic stock solutions**

Powders of two antibiotics Penicillin and Tetracycline (purity 100%) were accurately weighed and dissolved in sterile distilled water to give appropriate dilutions of about 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml to yield the required concentrations. The stocks were aliquot in 5 ml volumes and frozen at -20°C.

**Preparation of plant extracts solutions for the experiment**

The dried plant extracts were weighed and dissolved in sterile distilled water to prepare appropriate dilution to get required concentrations of about 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml. They were kept under refrigerated condition unless they were used for the experiment.
Preparation of dried filter paper discs

- Whatman filter paper (No:1) was used to prepare discs approximately 6 mm in diameter, which are placed in hot air for sterilization.
- After sterilization, the discs were loaded with different concentration of broad spectrum antibiotics like Penicillin and Tetracyclin and prepared plant extract solutions of Aegle marmelos, Solanum nigrum and Cassia fistula of different concentrations and again kept under refrigeration for 24 hrs.

Procedure for performing the Disc Diffusion Test

The antibacterial screening of the extract were carried out by determining the zone of inhibition using disc diffusion method (Sahoo et al., 2006; Rath et al., 1999). All the three plants extracts were tested against four pathogenic bacterial strains of gram positive and gram negative organism by disc diffusion method (Bayer et al., 1986).

Inoculum Preparation

Inoculum of Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis were prepared in nutrient broth medium, same as for broth dilution method and kept for incubation at 35°C for 8 hrs.
Inoculation of Test Plates

- A sterile cotton swab (Hi media, readily prepared sterile swabs) was dipped into the turbid culture suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

- The dried surface of a Mueller-Hinton agar plate was inoculated by swabbing the swab over the entire sterile agar surface. This procedure was repeated by swabbing two more times, rotating the plate approximately 60°C each time to ensure an even distribution of Inoculum. As a final step, the rim of the agar was swabbed.

- The lid may be left aside for 3 to 5 minutes, but not more than 15 minutes, to allow for any excess surface moisture to be absorbed before applying the drug impregnated disks.

Application of Discs to Inoculated Agar Plates

- Previously prepared paper discs were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down firmly to ensure complete contact with the agar surface.

- The discs were placed on the medium suitably apart and the plates were incubated at 5°C for 1 hr to permit good diffusion and then transferred to incubator at 37°C for 24 hrs.
• After completion of 24hrs, the plates were inverted and placed in an incubator set to 37°C for 24 hrs.

4.7 LIVER CANCER

4.7.1 Collection of Animals

Wistar strain male albino rats weighing (100-120g) were obtained from Tamil Nadu Veterinary College, Madhavaram, Chennai, India. They were kept in well ventilated polypropylene cages for 12 hours of light and 12 hours of darkness and they were given commercial pelleted animal feed and water at libitum.

4.7.2 Inducing of Liver Cancer

Fischer 344 male rats fed a choline-methionine deficient diet for from 13 to 24 months developed a 100% incidence of putative preneoplastic hepatocyte nodules and a 51% incidence of hepatocellular carcinoma. The diet contained no added known carcinogen. Analysis of the deficient and supplemented diets revealed no detectable volatile nitrosamines or nitrosamides, nitrite, nitrate or malonaldehyde, <0.9 p.p.b. aflatoxin B₁ and barely detectable levels of Ames positive material with one strain of Salmonella typhimurium. These findings indicate that a dietary deficiency of choline and methionine can be a major rate limiting factor in the development of liver cancer.
4.7.3  Plant Source

_Aegle marmelos, Solanum nigrum and Cassia fistula_ is an important medicinal plant, widely used in the preparation of traditional system of medicine such as Siddha, Ayurveda and Unani. The plant _Aegle marmelos, Solanum nigrum and Cassia fistula_ were purchased from local Ayurvedic medicinal shop Chennai, identified and authenticated by the Chief Botanist, Tamilnadu Aromatic Medicinal Plants Corporation Limited (TAMPCOL), Aringar Anna Siddha Medical College and Hospital Campus, Chennai, India.

4.7.4  Preparation of Plant Materials

1 kg of shade dried coarsely powdered plant material(aerial parts) was charged in aspiration bottle and allowed to soak in double distilled water for 48 hrs at room temperature. The plant extract was filtered and concentrated on a water bath and dried in a vacuum. The above mentioned plant material was used for the further experiments.

4.7.5  Experimental Design

The wistar strain male albino rats were divided into 6 groups with 6 animals in each group and were given dose regimen as given below.

Group 1 : Normal control animals (saline-0.9%)

Group 2 : Choline-methionine deficient diet induced liver cancer

Group 3 : Animals treated with paclitaxel at a dose of 20 mg/kg. b.w. for 30 days.
Group 4: Aegle marmelos, Solanum nigrum and Cassia fistula alone treated at a dose of 500mg/kg b.w. for 30 days.

Group 5: Liver cancer bearing rats treated with Aegle marmelos, Solanum nigrum and Cassia fistula extract at a dose of 500mg/kg b.w.

Group 6: Liver cancer bearing rats treated with Aegle marmelos, Solanum nigrum and Cassia fistula in combination with Paclitaxel at a dose of 500mg/kg b.w.

4.7.6 Collection of Samples

The animals were sacrificed by cervical decapitation at the end of the experimental period and blood was collected to separate the serum for carrying out various analyses. The vital organs such as lungs and kidney were dissected out and known weight of lung and kidney tissues were homogenized in 0.1M phosphate buffer (pH-7.0 – 7.4) at 4°C.

4.8 ESTIMATION OF NUCLEIC ACIDS

4.8.1 Extraction of Nucleic acids (Schneider, 1957)

Known amount of the tissue were homogenized in 5.0 ml of ice cold distill water using a Potter-Elvenjem homogenizer with a Teflon pestle, 5.0 ml of 5% trichloroacetic acid was added to the homogenate and this was kept in ice for 30 minutes to allow complete precipitation of protein. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% trichloroacetic acid. Then it was treated with 95% ethanol to remove lipids. The final precipitate was heated at 90°C for 15 minutes when facilitated the quantitative separation of nucleic acids from protein. The
supernatant after centrifugation was used for the estimation of DNA and RNA.

4.8.2 Deoxynucleic acid (DNA) (Burton, 1956)

Reagents

Diphenylamine reagent, 1.5 g of diphenylamine was dissolved in 100 ml of redistilled acetic acid 1.5 ml of concentrated sulphuric acid was added. The reagent was stored at 4°C in dark before use. 0.1 ml of aqueous acetaldehyde (0.16%) was mixed with every 20 ml of the reagent.

Stock Standard

Highly polymerized calf-thymus DNA was dissolved in 5mM Sodium hydroxide to give a concentration of 0.4 mg/ml

Working standard

This was prepared by mixing 2.0 ml of stock solution with an equal volume of 1N perchloric acid was heated at 70°C for 15 minutes.

Procedure

A known volume of the Nucleic acid extract was made up to 3.0 ml with 1 N perchloric acid. This was mixed with 2.0 ml of diphenylamine reagent. A reagent blank and standards were also carried out. This was kept in a boiling water bath for 10 minutes and the blue colour developed was read at 640nm in a spectrophotometer. The valves are expressed as mg/g wet tissue.
4.8.3  Ribonucleic acid (RNA) (Rawat et al., 1977)

Reagent

Oricinol – Ferric chloride reagent, 1 g of Oricinol was dissolved in 100 ml of concentrated hydrochloric acid containing 0.5 mg of ferric chloride, this reagent was prepared freshly.

Standard

This was prepared by dissolving 2.0 mg of Yeast RNA in 600 ml of 5% trichloroacetic acid.

Procedure

Aliquot of Nucleic acid extract were made up to 2.0 ml with 5% trichloroacetic acid. To this 3.0 ml of Oricinol-ferric chloride reagent was added and mixed well. The tubes were heated in a boiling water bath for 20 minutes. Reagents blank and standards were also treated in the same way. The tubes were cooled and the color developed was measured at 640nm using a spectrophotometer. The values are expressed as mg/g wet tissue.

4.9  ESTIMATION OF GLUCONEOGENIC ENZYMES

4.9.1  Glucose-6-phosphatase (Glucose-6-phosphate: Phosphohydrolase) (King, 1965a)

Reagents

- Citrate buffer : 0.1m, pH 6.5
- Substrate : Glucose -6- phosphate 0.01m
- Trichloroacetic acid : 10%
- Ammonium molybdate
Procedure

The incubation mixture in a total volume of 1.0 ml contained 0.3ml of buffer, 0.5ml substrate and 0.2ml of the enzyme sample. Incubation was carried out at 37°C for 60 minutes. The reaction was arrested by the addition of 1 ml of trichloroacetic acid and centrifuged. The phosphorus content of the supernatant was estimated by the method of (Fiske and Subbarow, 1925). The enzyme activity was expressed as n moles of inorganic phosphorus liberated/mg protein/min

4.9.2 Fructose 1, 6 diphosphate (Fructose -1, 6 phosphate phosphohydrolase, (Gancedo and Gancedo, 1971)

Regents

Tris-Hcl buffer : 0.1 m, pH 7.0
Substrate, Fructose 1,6 diphosphate : 0.05m
Mg cl₂ : 0.1m
Kcl : 0.1m
EDTA : 0.001m
TCA : 10%
Ammonium molybdate

Procedure

The assay medium in a final volume of 2ml containing 1.2 ml of buffer, 0.1 ml of substrate, 0.5 ml of Magnesium chloride, 0.1ml of Potassium chloride, 0.25 ml of EDTA and 0.1 ml of enzyme. The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by the addition of 1
ml of trichloroacetic acid. The suspension was centrifuged and the phosphorus content of the supernatant was estimated by the method of (Fiske and Subbarone, 1925). The enzyme activity was expressed as moles of inorganic phosphorus released /mg protein/min.

4.10 STATISTICAL ANALYSIS

Data are presented as the mean± standard deviation. The one way analysis of variants (ANOVA) was done and the F ration was compared to detect the significant changes between the groups. The tukey’s multiple comparison method was used to compare the means of different groups and the significant was denoted by ‘P’ values. All these analysis were carried out in personnel computer using statistical package for social science version 7.5

4.11 IDENTIFICATION OF ORGANIC COMPOUNDS IN THE PLANT MATERIALS

4.11.1 Sample Preparations

The plant parts of Aegle marmelos, Solanum nigrum and Cassia fistula was washed thoroughly with running tap water and they were rinsed with distill water and each plant material were chopped into small pieces. The aerial parts of the Aegle marmelos, Solanum nigrum and Cassia fistula were sun dried about 30°C at open areas with active ventilation until they attained constant weight.

The sun dried aerial parts Aegle marmelos, Solanum nigrum and Cassis fistula plants material were powdered in a mechanical drier and the
plant materials were stored in air tight container respectively and kept in the refrigerator at temperature below 4°C to maintain the freshness of sample and preserving the loss of volatile compounds present in the sample, for extraction procedure follow the above mentioned procedure (4.1.2).

4.11.2 Test for Phytochemical analysis

The extracts were analyzed for the presence of alkaloids, terpenoids, reducing sugars, Saponins, tannis, Carbonyls, Flavonoids, Phlobatannis and steriods (Adetuyi et al., 2001; Trease and Evans, 1989; Sofowora, 1982).

Test for Alkaloids

Weigh about 0.2 gm of each plant extract in separate test tubes and warmed with 2% Sulphuric acid for 2 minutes. And it was filtered in separate test tube and few drops of Dragencloffs reagent were added and observed for the presence of orange red precipitates for the presence of alkaloids.

Test for Cardiac glycoside (Keller-Killani Test)

Weigh about 0.5 gm of each plant extract in a separate test tube with 2 ml of glacial acetic acid containing a drop of ferric chloride solution. This was under layered with 1 ml of concentrated tetra oxo sulphate (VI) acid. And observe for brown ring formation at the interface (Finar, 1983).
Test for Terpenoids

Weigh about 0.5 g each plant extract in separate test tubes with 2 ml of chloroform. And add concentrated Sulphuric acid carefully to form a layer. And observe for presence of reddish brown colour interface was formed to show positive results for the presence of terpenoids.

Test for reducing sugars

Take three test tubes separately for each plant material and add 2 ml of each crude plant extract in each separate test tube with 5 ml of Distill water and filtered. The filtrate was boiled with 3-4 drops of Fehling’s solution A and B for 2 minutes. Observe for orange red precipitate indicates the presence of reducing sugars.

Test for Saponins

Weigh about 0.2 gm of each plant material in separate test tube and add 5 ml of distill water and then heat to boil. Observe for frothing (appearance of creamy mass of small bubbles) shows the presence of Saponin

Test for Tannis

Small quantity of each plant extract was mixed with water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. And observe for dark green solutions indicates the presence of tannis
Test for Carbonyl

Take 2 ml of each plant extract in separate test tubes and add few drops 2,4, di nitro phenyl hydrazine solution and shaken. And observe for the presence of yellow crystals immediately for the presence of an aldehyde.

Test for Flavonoids

To each plant extract weigh about 0.2 gm in separate test tubes and dissolved diluted Sodium hydroxide and add diluted Hydrochloride. And observe for yellow solutions that turns colorless indicates the presence of flavonoids

Test for Phlobatanin

To each plant extract weigh about 0.5 gm in separate test tubes and dissolved with distill water and filtered. The filtrate was boiled with 2% Hydrochloric acid solution. Observe for red precipitate shows the presence of Phlobatanin

Test for Steroids

To each plant extract add 2 ml of acetic anhydride and add 0.5 gm of methanol extract of each sample with 2 ml of Sulphuric acid .Observe for the color change from violet to blue or green in samples indicating the presence of steroids
4.12 TEST FOR IDENTIFICATION OF FUNCTIONAL GROUP IN PLANT EXTRACT BY (FTIR)

Instrument Used : FTIR (Fourier transformer
                 Infrared spectrophotometer)

Make : Shimadzu

Principle

Organic compounds absorb electromagnetic radiation in the infrared region of the spectrum which produces an increased vibration of bonds either by stretching or bending. Various types of stretching and bending vibrations occur at quantized frequencies.

An infrared spectrum is a graph of percentage of transmittance or percentage of absorbance of infrared radiation versus either decreasing frequency or increasing wavelength. It shows the % of transmittance versus frequency expressed in wave numbers as reciprocal centimeters (cm⁻¹) or the % of transmittance versus wavelength in microns (µ) or micrometers (µm).

General introduction

The normal infrared spectral region extends from 4000 cm⁻¹ to 667 cm⁻¹ and results from vibrational and rotational transitions. This region is most important for organic chemists. The infrared region from 4000 cm⁻¹ to 1400 cm⁻¹ is called functional group region and this exhibits absorption bands corresponding to a number of functional groups.
Sample preparation for Infrared Spectrophotometer

This was done using Infrared Spectrophotometer of Shimazdu Corporation and the extracts were scanned accordance with ASTM 1252-98. A drop of each plant extract (for extraction follow step No 4.1.2) Aegle marmelos was placed on the Sodium chloride cell to obtain the thin layer. Then the cell was placed in the FTIR compartment and scanned through IR region for about 25 scans and chromatogram was obtained. The same procedure was followed for Solanum nigrum and Cassia fistula and chromatogram was obtained.

4.13 TEST FOR IDENTIFICATION OF ORGANIC COMPOUND USING GAS CHROMATOGRAPHY

Principle

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between two non-miscible phases in which mobile phase is a carrier gas moving through (or) passing the stationary phase contained in a column. It is applicable to substance or their derivatives are volatized under the temperature employed. GC is based on mechanism of absorption, mass distribution (or) size exclusion.

Sample preparation

The crude plant extract (for extraction procedure follow the above mentioned steps) material with solvent was analyzed by Shimadzu Gas
chromatography (GC) systems equipped with flame ionization detector (FID) using capillary column. The nitrogen is used as a carrier gas at flow rate of 25 ml/ min at 600 kpa. The injector and detector temperature was maintained at 250°C. Injection was performed in split less mode and the Volume of sample used was 1 µl.

An oven temperature programme with total run time of about 30 min was used. The column temperature after an initial isothermal period of 2 min at 60°C was increased to 200°C at a rate of 5°C/min, and maintained this temperature for 1 min, and finally the temperature increased to 250°C at the rate 6°C/min, and maintained at this temperature for complete analysis. 5µl of extracted plant material was drawn by using 5µl syringe and injected into the port of injector of gas chromatography and chromatogram was obtained.