a). Literature survey

Literature survey on different aspects of this study has been carried out in various institutions. The literature includes ancient as well as modern literature.

b) Interview with traditional healers, local inhabitants:

The Malabar region of Kerala was explored during the ethno botanical survey. Survey was conducted in the Malabar region of Kerala from March 2008 – June 2011. Information was collected through investigations and by interviewing the local people during various field trips. Semi-structured interviews with the aid of a questionnaire were used to obtain data including local names of plants, plant parts used in medicinal preparation, therapeutic effects, diseases treated, and method of preparation, method of administration, dosages, and duration of treatment. Interviews were conducted individually with traditional medical practitioners (TMPs). Specific questions based upon Performa designed by Jain and Goel (1995) were used to collect information and the resultant information was recorded in the ethno botanical field book.

**PROFORMA FOR FIELD WORK**
(Jain and Goel, 1995 with modifications)

<table>
<thead>
<tr>
<th>Place</th>
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<tr>
<td>Date</td>
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<tr>
<td>Serial name</td>
<td>:</td>
</tr>
<tr>
<td>Informers Name</td>
<td>:</td>
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<tr>
<td>Tribe/Local people</td>
<td>:</td>
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<tr>
<td>Age</td>
<td>:</td>
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<td>Sex</td>
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</table>

Experience in treating Skin diseases :

<table>
<thead>
<tr>
<th>Name of disease</th>
<th>Name of plant (local name)</th>
<th>Mode of administration</th>
<th>Method of preparation</th>
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**FIELD DATA SHEET**
c) Field Survey and authentication:

Various forest localities, agricultural farms, tribal villages have been visited regularly for the collection of plant specimens. Plants with their local name, botanical name, family, part of plant used and ethno botanical uses are recorded. The plants were collected, pressed, and later identified using Gamble’s flora (Gamble., 1935). Local names were provided in their own language by the traditional physicians. During field
visits, digital images of plants have been taken to show habit, habitat, flower, fruit, and other pre-diagnostic characters. From this survey, 113 plant species were identified which belongs to 103 genera.

d) Herbarium preparation

All specimens collected from various districts have been prepared as per standard methods given by Jain and Rao (1978) and Bridson and Forman (1999). Collected plants are identified with the help of standard floras and manuals.

3.1.2 PRELIMINARY ASSESSMENT

In the present study for preliminary assessment for local Health tradition, experience of local traditional healers and clinical evidence of Ayurveda were used. This involved documentation of health practices, desk research for finding out and compiling scientific data on supportive phytochemical reference and reports of clinical evidence or pharmacological findings of these practices. The safe and efficacious ethnomedical practices were sorted out. This will help to promote the assessed practices, and information to the incomplete practices and discard the distorted practices. The data collected from 62 physicians (from 7 districts) were tabulated.

3.1.3. QUANTITATIVE ANALYSIS OF DATA

3.1.3.1 Use value (UV) Use value’ is the positive correlation between the importance value of a plant (measured by the number of informants for a particular plant) and the number of uses cited by the informants (Byg and Baslev, 2001). The use value (UV), a quantitative method that demonstrates the relative importance of a species known locally, was calculated according to the following modified Phillips and Gentry (1993b) formula: $UV = \frac{\Sigma U_i}{n}$ (Albuquerque et al., 2006). Where ‘$U_i$’ is the number of uses to treat the different types of skin diseases mentioned by each informant for a given species; ‘$n$’ is the total number of informants.

3.1.3.2 Informants consensus factor (ICF)

The informant consensus factor (ICF) was used to identify the ethno pharmacological importance of the collected plant species (Heinrich et al., 2009). For
the data analysis, informant consensus factor (ICF) was employed to find out the homogeneity in the information given by the informants. All the citations were placed in to 16 different types of skin ailment categories. The total number of use citations and total number of plants claimed for each category is calculated (Table.3). ICF values will be low (near 0), if plants are chosen randomly or if informants do not exchange information about their use. A low value indicates that the informants disagree on the taxa to be used in the treatment within a category of illness. Values will be high (near or more than 1), if there is a well defined selection criterion in the community and/or if information is exchanged between informants or high value (close to 1.0) indicates that relatively few taxa are used by a large proportion of the informants. The ICF is calculated by the following formula (Gazzaneo et al., 2005).

\[ \text{ICF} = \frac{N_{ur} - N_t}{N_{ur} - 1}. \]

Where ‘Nur’ is the Number of use citations in each category of skin disease and ‘Nt’ is the number of species used.

3.1.3.3. Fidelity level (FL)

The fidelity level is useful for identifying the inhabitants ‘most preferred species in use for treating certain ailments (Alexiades and Sheldon 1996). FL values vary from 1.0% to 100%. Generally, a FL of 100% for a specific plant indicates that all of the use-reports mentioned the same method for using the plant for treatment (Srithi et al. 2009). The fidelity level (FL), the percentage of informants claiming the use of a certain plants for the same major purpose, was calculated according to the following formula (Alexiades and Sheldon 1996).

\[ \text{FL} \% = \frac{N_p}{N} \times 100. \]

‘Np’ is the number of informants who independently suggested the use of a plant species for a particular skin disease (Usemention); ‘N’ is the total number of informants who mentioned the same plant for any skin diseases.

On the basis of Quantitative analysis five plants have been taken for further anatomical, phytochemical and anti-microbial studies.

3.2. PHARMACOGNOSTIC STUDIES
3.2.1 Plants selected for the study

(1)  *Aristolochia indica*  Family: Aristolochiaceae  
(2)  *Bischofia javanica*  Family: Euphorbiaceae  
(3)  *Derris scandens*  Family: Fabaceae  
(4)  *Myristica fragrans*  Family: Myristicaceae  
(5)  *Vitex negundo*  Family: Lamiaceae  

The materials collected from wild for Taxonomical studies, micromorphological studies, anatomical and chemical studies. The collected materials were subjected to taxonomic identification with the help of various floras. Authenticated herbarium sheets were prepared and deposited in the CMPR Herbarium.

3.2.2 Plant materials for Micro-morphological studies

The selected parts of genuine plant sources were subjected to micromorphological studies to record the identity and variation between plants.

3.2.3. Plant material for Histological and histochemical studies

Plant materials were collected in bulk quantity and one portion was preserved for anatomical and histochemical studies and the remaining portions were shade dried, powdered (20 mm mesh size) and sealed in an air tight bottle for the estimation of physicho-chemical parameters, preliminary phytochemical investigation, and antimicrobial activity.

3.2.4 Fixatives for Anatomical studies

Combination of Formalin, Acetic acid and Alcohol were widely used for fixation in plant microtechnique. This fluid is ideal for anatomical studies because it is stable and has got hardening action, rapid penetration and material can be stored for long time.

FAA- standard proportion (Johanson, 1940).

70% Ethyl alcohol - 90 ml  
Acetic acid - 5 ml
Formalin - 5 ml

3.2.5 Stains and reagents for Anatomical studies

- Saffranin: Dissolved 1gm saffranin in 100 ml distilled water
- Fast green: Dissolved 1gm Fast green in 100 ml clove oil

3.2.6 Stains for histochemical studies

- Ferric chloride S: Dissolved 5 g of ferric chloride in 100 ml of water.
- Glycerol S: Mixed equal amounts of glycerol and water.
- Hydrochloric acid: a suitable commercially available reagent.
- Iodine S: Dissolved 2.6 g of iodine and 3 g of potassium iodide in sufficient water to produce 100 ml.
- Nitric acid S: Concentrated nitric acid.
- Phloroglucinol: dissolved 1 g of phloroglucinol in 100 ml of ethanol.
- Potassium hydroxide: dissolved 56 g of Potassium hydroxide in 1000 ml of water
- Ruthenium red S: In 10 ml of 10% lead acetate solution dissolved 0.008 g of ruthenium red.
- Sudan red S: Dissolved 0.5 g of sudan red in 100 ml of glacial acetic acid AR
3.2.7. Material for the raw drug powder studies

Microscopic studies of the powdered drug of useful part were carried out with proper staining.

3.2.8. Material for polarization and fluorescent microscopic studies

Polarization and fluorescent microscopic studies of the useful parts like leaves, stem bark, heart wood were conducted.

3.2.9. EQUIPMENTS USED

- Microtome: Automatic plant microtome MT-3 model were used for taking uniformly thin sections.
- Microscopes:
  1. Normal compound microscope with camera attachment: Trinocular ‘Zeiss’ microscope attached with ‘Canon’ digital camera connected to the computer was used for the observation and transferring microscopic images of the plants studied.
  2. Polarization Microscope: Motic BA400 Polarising microscope attached with motic camera 3000 was used for the polarization studies.

3.3. PHYTOCHEMICAL STUDIES

3.3.1. Materials and reagents for phytochemical studies

Plant materials were shade dried, powdered (20mm mesh size) and sealed in an air tight bottle for the estimation of physicho-chemical parameters, preliminary phytochemical investigation, and antimicrobial activity.

3.3.2. Reagents for phytochemical studies

a) Test for Carbohydrate

Fehling's Test: Plant extract, Fehling's solution A and B

Benedict's test: Plant extract, Benedict's reagent.

Molisch's test: Plant extract, Molisch's reagent.
b) **Test for Anthraquinone glycosides**

Borntrager's Test: Plant extract, H$_2$SO$_4$, benzene, diluted ammonia

c) **Test for Coumarins** : Plant extract, NaOH

d) **Test for Quinone** : Plant extract, Concentrated H$_2$SO$_4$

e) **Test for Steroids**

Salkowski Test : Plant extract, Chloroform, concentrated H$_2$SO$_4$

f) **Test for alkaloids**

Hager's Test : Plant extract, Dilute HCl and Hager's reagent.

Mayer's Test: Plant extract, Dilute HCl, Mayer's reagent

Dragendorff's Test : Plant extract, Dilute HCl and Dragendorff's reagent.

Wagner's reagent test : Plant extract, Dilute HCl and Wagner's reagent

g) **Test for flavonoids** : Plant extract, Lead acetate

h) **Test for Tannins and Phenolic compounds**

FeCl$_3$ solution test: Plant extract, 5% FeCl$_3$ solution.

Lead Acetate Test : Plant extract, Lead acetate

i) **Test for Saponins**

Foam Test : Plant extract, Distilled water

j) **Test for triterpenes** : Plant extract, Concentrated H$_2$SO$_4$

3.3.6. **Assay/ Analytical Methods**

1. **TLC Identity test**

Thin Layer Chromatographic (TLC) studies conducted using pre-coated plates of silica gel 60 F$_{254}$ (E. Merck) of uniform thickness of 0.2mm.
2. High Pressure Liquid Chromatography (HPLC) system

Shimadzu prominence LC-20AD UFLC system equipped with online degasser, autosampler and diode array detector. Waters Spherisorb ODS (2) 5μm, 250 mm x 4.6 mm column was used.

3. High Pressure Thin Layer Chromatography (HPTLC)

HPTLC studies were done using aluminium plates precoated with silica gel GF₂₅₄ 0.2mm (E.Merck), Camag automatic sampler V, Camag automatic developing chamber with humidity controller ADC-2, Camag TLC Densitometric scanner 3, Camag TLC visualizer for photo-documentation and with WINCATS operating software were used.

4. GCMS analysis

Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The chromatographic column for the analysis was done by HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a constant increase of 10°C. The injection was performed in split mode at 250°C.

Mass Spectrum of β-sitosterol

System: Agilent 5975 MSD
Injection: 1 µL at 270°C with split ratio 10
Column: 5 m length, 0.25 mm ID, 0.25µ film DB-5MS UI
He column flow rate: 5 ml/min for 9 minutes followed by flow program of 8 ml/min up to 30 ml/min.
GC Oven: 50°C followed by 25°C/min to 330°C and 3.8 min hold for total of 14 min.
EI Source: 12 mA emission, 70 eV electron energy, 56 ml/min He makeup flow.
SMB transfer-line temperature: 270°C for 5 min followed by temperature program of 10 °C/min to 330°C
Mass range: 50-950 amu at about 1.6 Hz scan frequency.
3.4. ANTIMICROBIAL STUDIES

- **Materials and Instruments**
  
  Plant extracts, Mueller Hinton Agar (MHA), cork-borer (0.6cm), antibiotics nitrofurantoin (300mg/disc), chloramphenicol (30mg/disc), cephalxin (30mg/disc) and gentamicin (40mg/ml), dimethyl sulfoxide, cotrimoxazole

- **Autoclave, Incubator**

**Microorganisms: BACTERIA-**

**Gram positive strains**

- *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160) and *Enterococcus faecalis* (ATCC 29212).

**Gram negative organism’s**

- *Escherichia coli* (MTCC 40), *Klebsiella pneumoniae* (MTCC 3384), and isolated pathogenic *Salmonella typhi*.

**ANTIMICROBIAL AGENTS**

**GENTAMICIN - 40mg/ml**

**Preparation of fresh leaf juice**

100gm of fresh tender leaves crushed into paste using sterile thistle and mortar and juice is squeezed through sterile muslin cloth. This process is done at room temperature. Muslin cloth was used to filter the plant residues and the filtrate thus obtained further purified by filtration through Millipore membrane filter of 0.45 μm pore-size (Ronald, 1995). This sterile juice was stored in sterile capped bottles and kept in refrigerator until when required. Fresh tender leaves washed with distilled water and shade dried. The antimicrobial activity of the fresh juice is tested in the following concentrations 25 μl, 50 μl, 75 μl, 100 μl.

**Preparation of alcoholic Extract**

For the preparation of alcoholic extract, the dried materials powdered and 50 gm of powder extracted with Ethanol using soxhlet apparatus. The plant material was
extracted for 12 hrs. The extract was dried to remove the solvent and stored in airtight containers and kept in refrigerator. 1 g of extract is taken and is diluted to 2 ml. Then the antimicrobial activity is tested in the following concentrations 20 µl (10 mg), 40 µl (20 mg), 60 µl (30 mg) and 80 µl (40 mg).

**Evaluation of fresh juice & Ethanol extract for antibacterial activities**

Sensitivity of five different bacterial strains to juice and extract were measured in terms of Zone of inhibition using agar disc diffusion and well diffusion methods of antibacterial screening against two gram positive strains ie. *Staphylococcus aureus* MTCC 3160 & *Bacillus subtilis* MTCC 441, *Enterococcus faecalis* (ATCC29212) and three gram negative strains ie. *Escherichia coli* MTCC 401, *Klebsiella pneumoniae* MTCC 3384 and *Pseudomonas aeruginosa* MTCC741. The inoculum size of the test strain standardized according to the Clinical and Laboratory Standards Institute (CLSI, 2005) guidelines. Susceptibility tests were performed by a modified agar-well diffusion method. All the glass wares and culture media were sterilized by moist heat sterilization. About 25 ml of Muller Hinton Agar (Hi media M173) media was poured in the sterile Petri plates at 40°C. One ml volume of the standard suspension of test bacterial strain was spread evenly on Mueller Hinton Agar (Hi Media M173) plate using a sterile cotton swab and the plates were allowed to dry at room temperature. Each test organism was inoculated in 4 Petri plates. Four wells with the size of 6 mm in each plate were aseptically punched with sterile cork borer (6mm dia). 25, 50,75 and 100 µl of fresh juice were added with micropipette into the four wells . 20,40,60,80 µl Ethanolic extract were applied into the wells for each test organism. Fourth Petriplate was used for positive and negative control experiments. The positive control prepared from Gentamicin (Hi Media, CMS 461) in a concentration 0.5mg/ml ,and 50 µl is added to the wells as positive control. Distilled water 50 µl and Dimethyle Sulphoxide (DMSO) 50 µl used as negative control for juice and extract respectively. The plates were kept on uniform platform for 1-2 hr to diffuse the samples uniformly and then the bacterial cultures were incubated at 37°C in incubator for 24 hrs. Strict aseptic condition was ensured while doing all the procedures by using Laminar Air Flow Chamber. At the end of the incubation period, the diameter of zone of inhibition was measured in milli meter and recorded in tables.
B. METHOD OF STUDY

3.1. Survey & taxonomic studies

Interviews were conducted individually with traditional medical practitioners (TMPs). Specific questions based upon Performa designed by Jain and Goel (1995) were used to collect information and the resultant information was recorded in the ethno botanical field book.

For the taxonomic study, the genuine source plant collected from the natural habitat was subjected to taxonomic identification and herbarium preparation. The authenticated samples of herbarium of each drug and useful part were kept in the Herbarium and raw drug museum of AVS- CMPR for further use. The useful part was fixed in Formalin, Acetic acid and Alcohol mixture for further study. The plant material was shade dried and powdered for the Phytochemical, Antioxidant, Antimicrobial and cell line studies.

3.2. PHARMACOGNOSTIC STUDIES

3.2.1. Micro morphological studies

The genuine plant material subjected to micro-morphological studies and recorded the most identifying features of dermal morphology, texture, colour and cut surface. Organoleptic characters like smell, taste and fracture were also studied (Khandelwal, 2008).

3.2.2. Histological studies

Histological studies of the useful part were carried out to find out the type of cells, shape of stem in transection, nature of epidermal hairs, epidermal cell shape, nature of cortex, type of phloem fibres, crystals and cambium, nature of xylem vessel, secondary wood, pith, type of anatomical growth- normal or abnormalities if any. TS & LS were taken using sledge and rotary microtome and double stained with appropriate staining procedure for the preparation of permanent slide. All the anatomical characters were observed under Carl Zeis Axiostar plus microscope with a G3 Canon camera attached to a computer system.
3.2.3. Stains and reagents for General Anatomical studies

1. Safranin: stained lignified, cutinized and suberised structures

2. Fast green: the stains act on non-lignified tissues. It is a good counter stain for safranin.

3.2.4. Histochemical studies

Histochemical characterization of raw drugs were carried out to find the presence of starch, tannin; presence, nature and position of laticifers, resin ducts/ oil ducts; identification of depositions like cutin, lignin, suberin, wax; inclusions like calcium oxalate, calcium phosphate, cystolith etc. using appropriate histochemical techniques.

- **Starch**
  For examine the presence of starch the specimen was stained with Iodine. The blue colour indicated the presence of starch content.

- **Aleurone grains**
  For examining the presence of aleurone grains prepared a specimen in iodine S; aleurone grains get stained yellow.

- **Fixed oil**
  For examining the presence of fixed oil, prepared a specimen in a solution of sudan red; droplet of fixed oil get coloured orange pink.

- **Tannin**
  For examining the presence of tannins, prepared a specimen in ferric chloride S; bluish black or grayish black colouration indicated the presence of tannins.

- **Anthraquinone derivatives**
  For examining the anthraquinone derivatives, prepared a specimen in potassium hydroxide S; anthraquinone give blood red colour.

- **Lignified cells**
For examining the lignified cells or cell walls, the specimen was stained in phloroglucinol and allowed to drying. Added one or two drops of hydrochloric acid. The presence of lignin indicated by the pink or cherry red colour.

3.2.5. Microscopic studies

All the anatomical characters were observed under Carl Zeis Axiostar plus microscope with a G3 Canon camera attached to a computer system.

3.2.6. Raw drug powder studies

For examining the cell structure in powder form, material were powdered and sieved and mounted under glycerol, chloral hydrate and safranin to study the nature and identification of particles.

3.2.7. Polarization microscopic studies

To locate and distinguish the types of crystals and minerals present in the useful part polarization microscopy was used. The characters were observed under Motic BA 400 polarization microscope.

3.3. PHYTOCHEMICAL STUDIES

3.3.1. Assay/Analytical methods to Identify Chemical constituents

3.3.1.1. Preliminary phytochemical investigation

a) Tests for carbohydrates

Fehling’s Test

1 ml Fehling’s A solution and 1 ml of Fehling’s B solution were mixed and boiled for one minute. Now the equal volume of test solution was added to the above mixture. The solution was heated in boiling water bath for 5-10 minutes. First a yellow, then brick red precipitate was observed.

Benedict’s test

Equal volumes of Benedict’s reagent and test solution were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Solution appeared green showing the presence of reducing sugar.

Molisch’s test
Equal volumes of Molisch’s reagent and test solution were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Appearance of violet or purple colour ring showing the presence of reducing sugar.

b) Tests for Anthraquinone glycosides

Borntrager’s Test

To the 3ml of extract, dil. H$_2$SO$_4$ was added. The solution was then boiled and filtered. The filtrate was cooled and to it equal volume of benzene was added. The solution was shaken well and the organic layer was separated. Equal volume of dilute ammonia solution was added to the organic layer. The ammonia layer turned pink showing the presence of glycosides.

c) Tests for Coumarins

To the 2ml of extract, 10% NaOH was added and shaken well for 5 min showed yellow colour.

d) Tests for Quinone

To the 2ml of extract, conc. H$_2$SO$_4$ was added and shaken well for 5 min showed Red colour.

e) Test for steroids

Salkowski Test

To 2 ml of extract, 2 ml of chloroform and 2 ml of conc. H$_2$SO$_4$ was added. The solution was shaken well. As a result chloroform layer turned red and acid layer showed greenish yellow fluorescence.

f) Tests for alkaloids

Hager’s Test

To the 2-3 ml of filtrate, 1ml of dil. HCl and Hager’s reagent was added and shaken well. Yellow precipitate was formed showing the presence of alkaloids.

Mayer’s Test

To the 2-3 ml of filtrate, 1ml of dil. HCl and Mayer’s reagent was added and
shaken well. Formation of yellow precipitate showed the presence of alkaloids.

**Dragendroff’s Test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Dragendroff’s reagent was added and shaken well. Formation of orange-brown precipitate showed the presence of alkaloids.

**Wagner’s reagent test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Wagner’s reagent was added and shaken well. Formation of redish-brown precipitate showed the presence of alkaloids.

g) Tests for flavonoids

With Lead Acetate; to the small quantity of extract, lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoids.

h) Tests for Tannins and Phenolic compounds

**FeCl₃ Solution Test**

On addition of 5% FeCl₃ solution to the extract, deep blue black colour appeared.

**Lead Acetate Test**

On addition of lead acetate solution to the extract, white precipitate appeared.

i) Test for Saponins

**Foam Test**

To 1ml extract 20ml distilled water was added and shaken well in a measuring cylinder for 15 min. Then 1cm layer of foam was formed.

j) Test for triterpenes

Methanol extract was treated with concentrated sulphuric acid (H₂SO₄). Appearance of reddish brown ring indicated the presence of triterpenes.

Above phytochemicals analysis were carried out using standard procedure (Kokate, 1999; Harborne, 1998, 1973 and Sadashivan and Manickam, 2005).

3.3.1.2. Extraction of the material

1. General method of extraction
In phytochemical evaluation, the first step is extraction of plant material. The selection of extraction method depends on the nature and compounds to be isolated from the plant material. The dried material usually powdered before extraction. Extraction was carried out by cold maceration or by methods involving heating of the drug with suitable solvent.

2. Hot Continuous Extraction (Soxhlet)

In this study, all the extractions were carried out using following method. The finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent in tarred flask is heated, and its vapors condensed in a condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask A. This process was continuous and carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. After the completion of extraction, the solvent distilled off in a water bath and final traces of solvent removed under vacuum at 90°C. Weight of extract obtained was noted.

3. Isolation of volatile oil/ essential oil using Clevenger’s apparatus

In this method, the material was completely immersed in water, which was boiled by applying heat i.e., direct fire. The main characteristic of this process is that there is direct contact between boiling water and plant material. When the still is heated by direct fire, adequate precautions are necessary to prevent the charge from overheating. The plant material in the still were agitated as the water boils, otherwise agglomerations of dense material will settle on the bottom and become thermally degraded. From this laboratory trial, the yield of oil from a known weight of the plant material can be determined. During water distillation, all parts of the plant charge must be kept in motion by boiling water; this is possible when the distillation material is charged loosely and remains loose in the boiling water. For this reason only, water distillation possesses one distinct advantage, i.e. it permits processing of finely powdered material or plant parts that, by contact with live steam, would otherwise form lumps through which the steam cannot penetrate.
The hydro distillation usually takes 1-1/2 hours to 7 or more hours, depending up on the plant material and nature of the oil. In general, the oil formed along with the water in the trap is drained at ½ hour, replacing the water after each such removal, until no more oil distils. The resulting oil water mixture is saturated with sodium choride (to salt out oil dissolved in the water, if any) and is collected as pure oil with the use of separating funnel or extracted with pet ether (60\(^{0}\) - 80\(^{0}\)) and filtered to a weighed flask, Na\(_2\)SO\(_4\) (3 to 4 times, the combined either extract, dried over anh. Na\(_2\)SO\(_4\)), washed with pet ether and pet ether is stripped off, using 15-20 cms vigreaux column (to minimize loss of traces of oil due to its slight co-distillation with pet ether). The last traces of solvent (pet ether) are removed by heating on a water bath (~90\(^{0}\)) under reduced pressure (~100mm) for 10 mins and finally briefly (~2-3 mins) at 60\(^{0}\) - 80\(^{0}\), 4-6 mm and the flask is weighed. From the weight of fresh raw material and that of the essential oil obtained, the % yield (W/W fresh weight basis) can be evaluated (Clevenger, 1928).

4. Thin layer Chromatography (TLC) studies

**TLC Identity test**

TLC of alcoholic extract was carried out to find out the number of constituents present in the plant. Chromatography refers to the separation of chemical compounds by partitioning them between a stationary phase and a mobile phase. TLC techniques involved application of sample (dissolved in suitable solvent) at one end of the pre-coated plate, development of the plate in the solvent system in a closed chamber to the specified height and visualization of the plate in natural light, under UV 254 & 366nm or by derivatisation of the plate with suitable reagents. The \(R_f\) values and the colour of the bands were recorded and fingerprint profiles were established. Identification of the chemical marker was done by the comparison of \(R_f\) value, adsorption spectra response to derivatizing reagents etc.

\[
R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}
\]

**High Pressure Thin Layer Chromatography (HPTLC) Analysis**
Chromatographic analyses were performed on silica gel 60 F$_{254}$ TLC plates (20x10 cm; Merck, Darmstadt, Germany). Samples were applied to the plates by means of CAMAG automatic sampler V. Detection and quantification of the developed plate was performed with a CAMAG TLC Scanner 3 at 559 nm. TLC images were documented using CAMAG TLC visualizer. WinCATS as an integrated software was used for the detection as well as for the evaluation of data.

5. GC/MS analysis of essential oil

Gas Chromatography-Mass Spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column. The molecules take different amounts of time, called retention time, to come out of (elute from) the gas chromatograph, and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio. These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame Ionization Detector) detects multiple molecules that happen to take the same amount of time to travel through the column (i.e. have the same retention time) which results in two or more molecules to co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes makes it extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore when an identifying mass spectrum appears at a characteristic
retention time in a GCMS analysis, it typically lends to increased certainty that the analyte of interest is in the sample.

Fresh leaves hydro distilled in a Clevenger-type apparatus for 4 hours. At the end of distillation the oils were measured, collected, dried with anhydrous Na₂SO₄, and transferred to 5 ml glass ampules that filled to the top and kept at a temperature of −10°C for analysis. GC-MS analysis was performed on Agilent 5975C VL MSD with triple axis detector, using a HP-5-MS (Agilent) fused silica capillary column (30 m × 0.25 mm; 0.25 μm film thickness composed of 5% phenylmethyl polysiloxane) and temperature programmed as above. The carrier gas was He at a flow rate of 1.0 mL/min and the split mode at a ratio of 1:20. The injection port was set at 220°C. Quadrupole MS operating parameters were at interface temperature 240°C; electron impact ionization at 70 eV with scan mass range of 50-500 m/z at a sampling rate of 1.0 scan/s. Compounds were identified by computer search using digital libraries of mass spectral data [National Institute of Standards and Technology, 2011] and by comparison of their retention indices and authentic mass spectra [Adams, 2007].
3.4. **ANTIMICROBIAL STUDIES**

100g of the plant sample (leaf) was successfully extracted using Soxhlet apparatus with ethanol as solvent. After two days of extraction the solvent was evaporated off using rotary evaporator till dryness and the residue obtained was used for the studies. The extract was dissolved in Dimethyl sulfoxide (DMSO) and used antibacterial screening at the concentration of 1g/5ml. The water extract was prepared by weighing 100mg powder of stem bark by boil with 300ml of distilled water in a water bath for 24 hours, filtered and evaporated. The extract obtained was dissolved in sterile distilled water at a concentration of 1g/5ml and used for antibacterial screening.

**Preparation of inocula:** Several colonies were transferred to sterile peptone water (5 ml) from the sub cultured organism. The suspensions were mixed for 15 seconds to ensure homogeneity and subsequently diluted to match the turbidity of a 0.5 McFarland standard (*i.e.* OD = 0.12–0.15 at k = 530 nm, corresponding to 1–5 x 10^6 CFU/ml).

**Antimicrobial screening:** The antimicrobial assay was performed by two methods viz. agar disc diffusion method (Bauer *et al*., 1966) and agar well diffusion method (Perez *et al*., 1990). Mueller Hinton Agar (MHA) was prepared in plates as the media for test bacteria. The bacterial inoculum (0.5 Mac Farlands standard) was spread evenly on the surface of the MHA plates using a sterilized cotton swab. For agar disc diffusion method, sterile filter paper discs (6mm) were saturated with different concentrations of the test compound, allowed to dry and introduced on the upper layer of the seeded agar plate. For agar well diffusion method, a well was prepared in the plates with the help of a cork-borer (0.6 cm). 100 μl of the test compound was introduced into the well. The plates were incubated overnight at 37 °C. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. Sterile distilled water served as negative control. The result was obtained by measuring the zone diameter. The experiment was done thrice and the mean values are presented. The results were compared with the standard antibiotics nitrofurantoin (300μg/disc), chloram-phenicol (30μg/disc), cephalxin (30μg/disc) and gentamicin (10μg/disc).

**Agar well diffusion method**
Sterile cotton swab on to the standardized suspension and then rotated and compressed against the wall of the test tube so as to expel the excess fluid. Inoculated the surface of the MHA plate with the swab to ensure that the growth is uniform and confluent. Using a sterile cork borer, wells of 6mm diameter were made on agar surfaces. Wells are made equidistantly on each plate, wells are loaded with 5mg, 10mg, 20mg, 30mg, 40mg, 60mg, 80mg and 100mg (corresponding to 3g/5ml) of the plant extract and cotrimoxazole and gentamicin antibiotic discs used as positive control and 10% dimethyl sulfoxide as negative control. Plates were incubated at 37°C for 24 hours in an incubator and examined for zone of growth inhibition expressed in millimeter.

3.5. STATISTICAL ANALYSIS

The data were statistically analysed for accuracy.

3.6. PREPARATION OF THESIS

Compilation of all data available from the studies carried out with necessary photographs for the preparation of final report.

4. RESULT