CHAPTER 4

MATERIAL AND METHODS
4. Material & Methods

4.1.1 Material and Source

*Melaleuca leucadendron* (Linn.), (Myrtaceae) endemic to western part of Maharashtra, the leaves were collected from Veermata Jijabai Bhonsle Udyan (Victoria Garden) Byculla Mumbai. *Pandanus tectorius* Soland ex Parkinson, the plant leaves were collected from river shore of Fateabad village near Daulatabad fort in Aurangabad Maharashtra, the chemicals used included Standard S 2424 Stigmasterol (Sigma Aldrich), S 3626 Squalene (Sigma Aldrich), β-Sitosterol and Stigmasterol as a gift sample were procured from Dr. S H Ansari Ex Dean Faculty of Pharmacy, Jamia Hamdard University, New Delhi.

Petroleum ether 60-80 (Fisher Scientific India Pvt. Ltd), ethyl acetate (Merck, Mumbai, Pvt. Ltd), methanol (Fisher Scientific India Pvt. Ltd), chloroform (Fisher Scientific India Pvt. Ltd), acetic acid (Merck Mumbai, Pvt. Ltd), toluene (Fisher Scientific India Pvt. Ltd), benzene (Fisher Scientific India Pvt. Ltd) and diethyl ether (Fisher Scientific India Pvt. Ltd) and silica gel (Fisher Scientific India Pvt. Ltd) for column and TLC plates (Silica gel 60 F254, Merck Germany Ltd).

White soft paraffin (Loba Chemei Pvt. Ltd), carbopol 934 (Loba Chemei Pvt. Ltd), carbopol 940 (Loba Chemei Pvt. Ltd), polyethylene glycol 4000 (Loba Chemei Pvt. Ltd), polyethylene glycol 200 (Loba Chemei Pvt. Ltd), triethanolamine (Merck Mumbai Pvt. Ltd), propylene glycol (Merck Mumbai Pvt. Ltd), glycerin (Merck Mumbai Pvt. Ltd), Cetyl alcohol (Loba Chemei Pvt. Ltd), bees wax (Loba Chemei Pvt. Ltd), wool fat (Loba Chemei Pvt. Ltd), sodium lauryl sulphate (Loba Chemei Pvt. Ltd), methyl paraben and propyl paraben (Fisher Scientific India Pvt. Ltd). All other chemicals and reagents used in the research work were of highest purity grade available commercially.
### Table 8  List of Equipment and Instruments

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Name of the equipment and instruments.</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brookfield viscometer</td>
<td>DV-II LV (spindle no.64) Eng.Lab. Inc. USA</td>
</tr>
<tr>
<td>2</td>
<td>Abbe Refractometer</td>
<td>REMI Labs, Devang cooperation, Malad W, Mumbai</td>
</tr>
<tr>
<td>3</td>
<td>Fourier Transform IR Spectrophotometer</td>
<td>FTIR-4100 Jasco Corporation, Japan</td>
</tr>
<tr>
<td>4</td>
<td>Digital Weighing Balance</td>
<td>Eagle, Mumbai India</td>
</tr>
<tr>
<td>5</td>
<td>Digital Magnetic Stirrer</td>
<td>Remi Motors Mumbai India</td>
</tr>
<tr>
<td>6</td>
<td>Digital pH Meter</td>
<td>Equip-Tronics Eq-610</td>
</tr>
<tr>
<td>7</td>
<td>Stability Chamber</td>
<td>TH 200G Thermolab, Mumbai, India</td>
</tr>
<tr>
<td>8</td>
<td>High Performance Thin Layer Chromatography</td>
<td>CAMAG Linomat 5 Hamilton, Bonaduz, Switzerland</td>
</tr>
</tbody>
</table>
4.1.3 Procurement and Authentication of the leaves of *Melaleuca leucadendron* Linn. (Myrtaceae).

The leaves of *Melaleuca leucadendron* (Linn.), (Myrtaceae) were collected from Veermata Jijabai Bhonsle Udyan (Victoria Garden) Byculla Mumbai and authenticated from Herbarium In-charge, Botany department of Dr Babasaheb Ambedkar Marathwada University Aurangabad with accession no 7715 dated 24-02-2009 and the specimen is kept in herbarium of the university. Leaves collected were processed as follows; the fresh leaves were collected and placed in clean dry plastic bags and labeled; some of these leaves were then cut into small pieces to be used for hydrodistillation of the essential oil. The remaining leaves were shade dried and pulverized to coarse powder and passed through 40-60 # sieve. The powdered material was packed in 1kg lots and 5 such packs were packed together and labeled mentioning the name, date and storage conditions. The packs were stored in cool dry place in a ventilated area away from sunlight. Once the packets were opened, any leftover material after use was not returned to the original stock.

4.1.4 Extraction of Essential oil from the leaves of *Melaleuca leucadendron* Linn. (Myrtaceae).

The essential oil obtained from *Melaleuca leucadendron* is hydrodistilled from fresh leaves, by following method:

Fresh leaves of *Melaleuca leucadendron* Linn were collected and chopped into small pieces. The prepared plant material was immediately hydro-distilled by Clevenger apparatus for 3 hours. After cooling, the oil was collected. The yield of essential oil (EO) from hydro distillation of fresh leaves was 0.5-2.5% (v/w). Once condensed, the clear to pale yellow essential oil is separated from the aqueous distillate. The essential oil has been used for almost 100 years in India but is now available worldwide both as neat oil and as an active component in an array of products [14, 15 and 102]. Alternative extraction methods are also available such as the use of
microwave technology have been considered, but none has been utilized on a commercial scale [103, 104, 105] thus only the former technique mentioned here was used.

4.1.5 Procurement and Authentication of the leaves of Pandanus tectorius Soland ex Parkinson (Pandanaceae)

The leaves of Pandanus tectorius Soland ex Parkinson were collected from river shore of Fateabad village near Daulatabad fort in Aurangabad Maharashtra and authenticated from Department of Botany, Dr Babasaheb Ambedkar Marathwada University Aurangabad with accession No 0778. The leaves were then shade dried and pulverized to coarse powder and passed through 40-60 # sieve. The powdered material was packed in 1kg lots and 5 such packs were packed together and labeled mentioning the name, date and storage conditions. The packs were stored in cool dry place in a ventilated area away from sunlight. Once the packets were opened, any leftover material after use was not returned to the original stock.

4.1.6 Preparation of Extracts from the leaves of Pandanus tectorius Soland ex Parkinson (Pandanaceae).

The powdered leaves, 1kg, was extracted successively using Soxhlet extraction apparatus in increasing order of polarity with petroleum ether (PE) yield 7.85 %w/w, ethyl acetate (EA) 7.71 %w/w, methanol (M) 6.7 %w/w and powdered leaves 1kg was directly extracted with methanol to get total methanol extract (M1) 11.8 %w/w. Solvents were evaporated under reduced pressure and dry fractions were weighed and stored in labeled amber glass bottles at 25± 2 °C temperature for further use. [50]

4.1.7 Preparation of Aloe vera gel

Aloe vera gel was extracted from the leaves of aloe vera plant obtained locally at Aurangabad, Maharashtra. A long flat leaf from the aloe vera plant was selected, hand was placed at the base of the leaf and pulled taking care of the spike on aloe vera leaf and the leaf should come out
cleanly and have a white base. The dirt and debris is washed off the leaf if necessary. Using a sharp knife one of the spiked sides of the leaf is peeled, the leaf is then sliced open and the leaf is rendered in two along its length. With the help of a clean spoon the gel is scraped from the leaf into a clean glass container and stored under refrigeration at 4°C.

4.2. Phytochemical Analysis.

4.2.1 Preliminary Pharmacognostic and phytochemical studies of the leaves of *Melaleuca leucadendron* Linn and *Pandanus tectorius* Soland ex Parkinson

(A) The dried and powdered leaves were evaluated for the following pharmacognostic parameters

(i) **Loss on Drying**

About 5 g of powder was accurately weighed, placed in petri dish and dried in hot-air oven at 110°C for four hours. After cooling, it was placed in a dessicator, later the loss in weight was recorded, procedure was repeated till constant weight was obtained.

\[
\text{Loss on Drying} = \frac{\text{Loss in weight}}{W} \times 100
\]

\[W = \text{Weight of the crude drug in grams}\]

(ii) **Ash Value**

About 2g of crude drug powder was accurately weighed in a tarred and previously ignited silica crucible. Incinerated gradually by increasing the heat, not exceeding dull red heat, until free from carbon, cooled and weighed. The percentage of ash was calculated with reference to the air dried drug.

(iii) **Acid Insoluble Ash**

The ash from the above step was boiled for 10 min with 25 ml of dilute hydrochloric acid, and the insoluble matter was collected in a silica crucible (previously ignited and weighed). The percentage of acid-insoluble ash was calculated with reference to the air-dried drug.

(iv) **Water Soluble Ash**

The total ash was boiled for 5 min with 25 ml of water. The insoluble matter was collected in a crucible, washed with hot water, ignited and weighed. The percentage of water soluble ash was calculated with reference to air-dried drug.
(v) Fluorescence Analysis

Many crude drugs show the fluorescence when the sample is exposed to ultraviolet radiation. Evaluation of crude drugs based on fluorescence in daylight is not much used, as it is usually unreliable due to the weakness of the fluorescence effect. Fluorescence lamps are fitted with suitable filters, which eliminate visible radiation from the lamp and transmit ultraviolet radiation of definite wavelength. Several crude drugs show characteristic fluorescence useful for their evaluation.

(B) The essential oil from *Melaleuca leucadendron* Linn and extracts from the dried and powdered leaves of *Pandanus tectorius* Soland ex Parkinson were evaluated to detect the presence or absence of the following phytochemicals:

(i) Tests for phytosterols

The extract was refluxed separately with alcoholic solution of potassium hydroxide till complete saponification. The saponified extract was diluted with water and unsaponifiable matter was extracted with diethyl ether. The ethereal extract was evaporated and the residue (unsaponifiable matter) was subjected to the following test by dissolving the residue in the chloroform.

(a) Salkowski test: To the test extract solution added few drops of conc. sulphuric acid shaken and allowed to stand; lower layer turns red indicating the presence of sterols.

(b) Liebermann-Burchard test: The test solution treated with few drops of acetic anhydride and mixed, conc. sulphuric acid was added from the sides of the test tube, a brown ring at the junction of two layers is formed and the upper layer turns green.

(c) Sulphur test: Sulphur when added in to the test solution, it sinks in it.

(ii) Tests for steroidal glycosides

The solution was prepared by adding extract in water and 1ml of 10% v/v solution of sulphuric acid was added, heated on the water-bath for half an hour and extracted hydrolyzed extract with the chloroform as a solvent, chloroform fraction was separated and concentrated, concentrated fraction was tested for steroids/phytosterols.

(iii) Tests for Triterpinoids

The test extract solution was prepared by dissolving extracts in chloroform.

a) Salkowski test: Few drops of concentrated sulphuric acid were added to the test
solution of the extract, shaken and on standing lower layer turn golden yellow.

b) **Liebermann-Burchard test:** To the test solution of the extract, few drops of acetic anhydride were added and mixed well. 1 ml of concentrated sulphuric acid was added from the sides of the test tube, a red color is produced in the lower layer indicates the presence of triterpenes.

(iv) **Tests for Glycosides**

The test solution was prepared by dissolving extract in alcohol 90% or aqueous alcoholic solution.

(a) **Baljet’s test:** The test solution treated with sodium picrate gives yellow to orange color.

(v) **Tests for Saponins**

The test solution was prepared by dissolving extract in the water.

(a) **Foam test:** Test solution on shaking shows formation of foam, which is stable for 15 min.

(b) **Haemolysis test:** 2 ml of 18% sodium chloride in two test tubes is taken. To one test tube added distilled water and 2 ml test solution to another. Few drops of blood is added to both the test tubes, mixed and observed for haemolysis under microscope.

(vi) **Tests for Carbohydrates**

The test solution was prepared by dissolving test extract with water, Hydrolysed with 2N hydrochloric and subjected to following tests.

(a) **Molisch’s test:** Test solution with few drops of Molisch’s reagent and 2 ml of conc. sulphuric acid added slowly from the sides of the test tube shows a purple ring at the junction of two liquids.

**Barfoed’s test:** Test solution treated with Barfoed’s reagent, boiled on a water-bath, shows brick red colour precipitate.

(b) **Benedict’s test:** The test solution treated with Benedict’s reagent and boiling on water-bath shows reddish brown precipitate.

(c) **Tollen's phloroglucinol test:** 2.5 ml of concentrated HCl and 4 ml of 0.5% phloroglucinol are treated with 1-2 ml test solution. Heat, yellow to red color appears.

**Cobalt-Chloride test:** 3 ml of test solution is treated with 2 ml cobalt chloride. Boil and cool. Add few drops of sodium hydroxide solution. Solution appears greenish
blue (glucose) or purplish (fructose) or upper layer greenish blue and lower layer purplish (mixture of glucose and fructose).

(vii) **Tests for Alkaloids**

The test solution was prepared by dissolving extracts in dilute hydrochloric acid.

(a) **Mayer’s test:** Test solution with Mayer’s reagent (potassium mercuric iodide) gives cream colored precipitate.

(b) **Wagner’s test:** Test solution with Wagner’s reagent (iodine-potassium iodide solution) gives reddish brown precipitate.

(c) **Hager’s test:** The acidic solution with Hager’s reagent (saturated picric acid solution) gives yellow precipitate.

(d) **Dragendorff’s test:** The acidic solution with Dragendorff’s reagent (potassium bismuth iodide) shows reddish brown precipitate.

(viii) **Tests for Flavonoids**

The flavonoids are all structurally derived from the parent substance called flavones. The flavonoids, which occur in the form of free, as well as, bound to sugars called glycosides. For this reason, when analyzing flavonoids, it is usually better to examine the flavonoids in hydrolysed plant extracts.

**Preparation of test solution**

To small amount of extract equal volume of 2M hydrochloric acid is added and heated in test tube for 30-40 min at 100°C, allowed to cool, filtered and extracted with ethyl acetate. The ethyl acetate extract is concentrated to dryness, followed the test for flavonoids to ethyl acetate fraction by dissolving the residue with ethyl acetate.

a) **Shinoda test:** Test solution with few fragments of magnesium ribbon and conc. hydrochloric acid shows pink to magenta red colour.

b) **Zn/Hcl reducing test:** Test solution with zinc dust and few drops of hydrochloric acid shows magenta red colour.

ix) **Tests for Proteins**

The extract is dissolved in water.

a) **Millon’s test:** Test solution is treated with Millon’s reagent and heated on a water-bath, protein is stained red on warming.
b) **Xanthoproteic test**: Test solution treated with conc. nitric acid and boiled gives yellow precipitate.

c) **Biuret test**: Test solution with 40% NaoH and copper sulphate solution gives blue color.

**x) Test for Amino acids**

a) **Ninhydrin test (General test)**: Heat 3 ml of test sample and 3 drops of 5% ninhydrin solution in hot water bath 10 min. Purple or bluish color appears.

b) **Test for tyrosine**: Heat 3 ml of test solution and 3 drops Millon’s reagent. Solution shows dark red color.

c) **Test for tryptophan**: 3 ml of test solution and few drops of glyoxallic acid and concentrated sulphuric acid. Reddish violet ring appears at the junction of the two layers.

d) **Test for cysteine**: To 5 ml of test sample add few drops of 40% sodium hydroxide and 10% lead acetate solution. Boil. Black precipitate of lead sulphate is formed.

xi) **Test for Phenols/ Tannins**

The extract is dissolved in 90% alcohol.

a) **Ferric chloride test**: Test solution treated with few drops of ferric chloride solution gives dark color.

b) **Gelatin test**: Test solution treated with gelatin gives white precipitate.

**Xii) Test for Gums**

Test solution is hydrolysed and treated with Benedict’s reagent. Red color is developed. [3, 4, 11, 13, 102 & 106]
4.2.2 Gas Chromatography and Mass Spectroscopic (GC-MS) analysis of the essential oil of *Melaleuca leucadendron* Linn

The essential oil extracted from the leaves of *Melaleuca leucadendron* Linn (EO) was diluted to 1:100 in methanol and injected into Gas Chromatography-Mass Spectrometry apparatus at the condition described below; [29]

- **Instrument model**: Varian Plus/HPCHEM
- **Column**: Fused silica capillary column (30m x 0.25 mm i.d, 0.25 µm Film thickness) Coated with DB-5 (J & W).
- **Column programming**: 40 ºC 2 minute to 170 ºC rate 3 ºC/min.
- **Injector temperature**: 200 ºC
- **Detector temperature**: 230 ºC
- **Carrier gas**: Helium: flow rate 1.0 ml/min at constant pressure of 90kPa
- **Split ratio**: 100:1
- **Accelerating voltage**: 1700 volts
- **Sample size**: 0.5 µl
- **Solvent**: methanol (HPLC grade)

4.2.3 Phytochemical analysis of the extracts of the leaves of *Pandanus tectorius* Soland ex Parkinson (*Pandanaceae*)

4.2.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) was performed for the PE, EA, M, M1 extracts of *Pandanus tectorius* Soland ex Parkinson. The TLC was carried out on silica gel precoated aluminum plates (Merck Silica gel 60 F254) as stationary phase and (petroleum ether : ethyl acetate 70:30 v/v) as mobile phase followed by anisaldehyde-\(\text{H}_2\text{SO}_4\) spray with heating at 100°C for 1 minute. [71]
4.2.3.2 High Performance Thin Layer Chromatography

High Performance Thin-layer Chromatography (HPTLC) was performed for the PE, EA, M, M1 extracts of *Pandanus tectorius Soland ex Parkinson*, using silica gel precoated aluminum plates (Merck Silica gel 60 F254) as stationary phase, and (petroleum ether : ethyl acetate 70:30 v/v) as mobile phase. The developed plates were seen in UV spectrum for visualization at specific wavelength of 254 and 366 nm.

a) Preparation of mobile phase

Mobile phase are prepared by mixing all the solvent petroleum ether: ethyl acetate in the ration of (7:3 v/v) and degassed to remove trapped air.

b) Stock and working standard solution

β sitosterol, stigmasterol and squalene (liquid) were weighed 10 mg/10 ml independently and transferred separately to a 100 ml volumetric flask. The drugs were dissolved in methanol and make up the volume to obtain stock solution of 1000 g/ml. appropriate dilutions of the above mentioned standard stock solution were prepared in 10 ml volumetric flask with methanol. Calibration standards were prepared over the concentration range 100 g/ml. appropriate Microlitre by micro syringe from each standard solution was spotted on the HPTLC plate to obtain final concentration range of 2,4,6,8,10,12 μg/spot.

c) Preparation of sample solution

The 10 mg extracts PE, EA, M and M1 were accurately weighed and transferred to 100 ml volumetric flask and 60 ml methanol was added and ultrasonicated for 15 min at room temperature and diluted up to the mark with methanol. The solution was then filtered through Whatman filter paper number 41 and filtrate was diluted to volume with methanol to get stock solution of concentration of 1000 μg/ml. appropriate dilutions of the above mentioned sample stock solution were prepared in 10 ml volumetric flask with methanol.

d) Chromatographic condition

**Instrument**

CAMAG Linomat 5 "Linomat5_080222" S/N 080222 (1.00.12)

Executed by Anchrom Test Lab Pvt. Ltd Saturday, January 21, 2012

**Linomat 5 application parameters**

- **Spray gas:** Inert gas
- **Sample solvent type:** Methanol
Dosage speed: 150 nl/s  
Pre dosage volume: 0.2 ul

**Sequence**

Syringe size: 100 μl  
Number of tracks: 15  
Application position Y: 8.0 mm  
Band length: 8.0 mm

Chromatography was performed on a pre-coated silica gel TLC plate. Standard solutions and sample solutions were spotted to the plate as 6 mm wide bands using the Camag 100 μl sample syringe (Hamilton, Bonaduz, Switzerland), with an Camag TLC applicator, Linomat 5 with N2 flow (Camag, Muttenz, Switzerland), at 150 nl/s, positioned 8mm from the bottom and 10 mm from side of the plate. The application parameters were identical for all the analysis performed. The HPTLC plates were developed in a Camag twin trough glass chamber (20 × 10 cm) which was pre-saturated with the mobile phase for 20 min; the length of each run was 80 mm. The TLC runs were performed under laboratory conditions of 25 ± 5°C. The plates were then dried by drier. For quantification, the TLC spots corresponding to sample and standard were quantified at 254 nm and 366 nm using a Camag TLC scanner model-3 equipped with Camag Wincats software (version 2.01.02) and an Hg source, slit width 6 × 0.45 mm, absorption– reflection scan mode and a scanning speed at 20 mm/s. The Wincats software controlling the densitometer produces a calibration plot by linear regression relating standard concentration to the scan area.

### 4.2.3.3 Column Chromatography

The PE extracts were subjected to Column Chromatography (CC) using silica gel for column chromatography (Fisher Scientific 27315) and petroleum ether: ethyl acetate as solvents. Column Chromatography was carried out using silica gel mesh size about (100-200) in column (500 gram capacity). The elution was carried out with petroleum ether (60-80) and gradually increasing the polarity by ethyl acetate, the elutes were collected in 20 ml test tubes and monitored by TLC and fractions with identical TLC pattern were combined. Five fractions were eluted which were labeled as I, II, III, IV and V. Fraction I was light yellow colored liquid, fraction II was a creamy white mixture solid, fraction III was a creamy white colored solid, fraction IV was a brown colored solid and fraction V was a fluorescent green colored solid.
4.2.3.4 Gas Chromatography with High Resolution Mass Spectrometer

Gas Chromatography with High Resolution Mass spectrometer (GC-HRMS) was performed on fractions eluted in Column chromatography. The fractions of Column Chromatography number I, II, III, IV and V were subjected to GC-HRMS. [122-127]

Preparation of test samples

The eluted fractions of column chromatography were dried and 5 mg of each fraction were weighted accurately and transferred in vials and labelled. The samples were provided to SAIF, IIT Powai for further process.

Instrument model Jeol, : AccuTOF GCV : EI, Agilent

Acq. Data Name E56RFC1

Experiment Date Time 01/20/2012 10:44:02

Column Fused silica capillary column (30m x 0.25 mm i.d, 0.25 µm Film thickness) Coated with DB-5 (J & W).

Column programming 40 ºC 2 minute to 170 ºC rate 3 ºC/min.

Injector temperature 200 ºC

Detector temperature 230 ºC

Carrier gas Helium: flow rate 1.0 ml/min at constant pressure of 90kPa

Split ratio 100:1 (100-10-240-8M-10-250-3M-280-HP5-ethyl acetate)

Accelerating voltage 1700 volts

Sample size 0.5 1

Solvent ethyl acetate (HPLC grade)
4.3 Antimicrobial Assay

4.3.1 Screening of antimicrobial potential of the essential oil from *Melaleuca leucadendron* Linn leaves.

(a) Selection of Microorganisms

The microorganisms which cause skin infections were selected for the study. The microorganisms selected include two Gram positive *Staphylococcus aureus* NCIM-2079, *Bacillus subtilis* NCIM-6633, two Gram negative *Pseudomonas aeruginosa* NCIM-2200, *Escherichia coli* NCIM-2065, two fungi *Aspergillus flavus* NCIM-304 & *Candida albicans* NCIM-3471 obtained from Indian type culture collection. [116, 118, 120]

(b) Method of Antimicrobial Assay

The antimicrobial activity of the essential oil (EO) of the plant was determined by the Well-agar plate diffusion method. 20 ml of sterile Nutrient Agar (NA), Potato Dextrose Agar (PDA) and Malt Glucose Yeast and Peptone Agar (MGYP) were aseptically transferred to sterilized petri dishes 9 cm in diameter each at 45°C ±2°C, followed by inoculation of bacteria and fungi strains (0.5). The plates were held for 15-20 minutes at room temperature 25°C ±2°C. The bore where prepared in the petri dishes using borer and sample were added in the bore using micropipette. Then the bacterial culture plates were incubated at 37°C for 24 hours and the fungal culture plates were incubated at 22°C for 48 hours and the diameters of the inhibition zones formed on the NA, PDA and MGYP were evaluated in millimeters. [116, 118-121]

4.3.2 Estimation of Minimum Inhibitory Concentration (MIC) of the essential oil from *Melaleuca leucadendron* Linn leaves.

The standardized 0.5 % polysorbate-80 (sterile) in distilled water was used for the estimation of MIC. The different concentrations of the essential oil were prepared by using two fold serial dilution techniques. The pure essential oil was placed in the 0.5 % polysorbate-80 to form the solutions of different concentrations. The bacterial and fungal cultures were prepared in triplicate and all were incubated. After incubation the MIC was estimated. [116]
4.3.3 Screening of antimicrobial potential of the extracts of the leaves of 
*Pandanus tectorius Soland ex Parkinson (Pandanaceae)*

50 mg of each extract was weighed and dissolved in 50 ml dimethylsulfoxide (DMSO) to get 1mg/ml solution of extracts. The antimicrobial activities of the extracts were studied by the well agar plate diffusion method. Each bacterial strain was cultured overnight on nutrient broth at 37 ºC in an incubator and 20 ml of nutrient agar (NHA), potato dextrose agar (PDA) and MGYP (Malt Glucose Yeast Peptone) agar medium sterilized by autoclave in flask and cooled to 45-50°C, were distributed to sterilized petri dishes with a diameter of 9 cm homogeneously after inoculating cultures (0.5ml) of bacteria and fungi with sterile wire loop. The plates were held for 15-20 minutes at room temperature. The well were prepared (6-7 mm) in each petri dish by using sterile borer and sample were poured (100µl) in the bore using micropipette, solvent DMSO was used as a control in same manner. All plates were kept in refrigerator for diffusion at 4º C for 4 hours, followed by incubation of plates at 37º C for 24 hours. At the end of the 24 hours period, the diameters of the inhibition zones formed on the NHA, PDA and MGYP were evaluated in millimeters.

4.3.4 Estimation of Minimum Inhibitory Concentration (MIC) of the extracts of the leaves of *Pandanus tectorius Soland ex Parkinson (Pandanaceae)*

The standardized 0.5 % polysorbate-80 (sterile) in distilled water was used for the estimation of MIC. The different concentrations of the petroleum ether, ethyl acetate and methanol extracts were prepared by using two fold serial dilution techniques. The pure extracts were placed in the 0.5 % polysorbate-80 to form the solutions of different concentrations. The bacterial and fungal cultures were prepared in triplicate and all were incubated. After incubation the MIC was estimated.

4.3.5 Combined antimicrobial activity of the essential oil (EO) of *Melaleuca leucadendron* leaves along with the extracts PE and M of *Pandanus tectorius*.

To study the combined antimicrobial activity of the essential oil (EO) of *Melaleuca leucadendron* leaves along with the extracts PE and M of *Pandanus tectorius*, antimicrobial activity of EO:PE: M were studies in the ratio of 1:1:1, 2:2:1, 1:2:2 and 5:3:1 respectively. The antimicrobial activity was determined against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Aspergillus flavus*. The antimicrobial activities
of the extracts were studied by the well agar plate diffusion method described in 4.3.3 above. This was followed by the determination of minimum inhibitory concentration described under section 4.3.4.
4.4. Development of Semisolid Formulations

4.4.1 Characterization of Excipients (Preformulation Studies)

The excipients used in formulations were characterized as per USP 2011 NF, Handbook of Pharmaceutical excipients for their physicochemical characteristics such as appearance, solubility, pH, melting point, bulk density, specific gravity and viscosity. [134-135]

4.4.2 Preliminary formulations:

Preliminary semisolid topical applications using various ointment bases taking queue from the ointments reported in literature and gelling systems were prepared by incorporating the most optimum combination of extracts i.e. EO: PE: M in the ratio 2:2:1 as the active medicament. Initially five ointment bases as shown in Table 9 were formulated.

Table 9 Formulation of semisolid topical applications using various ointment bases

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Excipients</th>
<th>F I %w/w</th>
<th>F II %w/w</th>
<th>F III %w/w</th>
<th>F IV %w/w</th>
<th>F V %w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White petrolatum</td>
<td>25</td>
<td></td>
<td></td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Stearyl alcohol</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Propylene glycol</td>
<td>12</td>
<td>10</td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>S.L.S</td>
<td>1</td>
<td>0.60</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Methyl paraben</td>
<td>0.025</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Propyl paraben</td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>PEG 4000</td>
<td>11.2</td>
<td></td>
<td></td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Stearyl alcohol</td>
<td>20.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Glycerin</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Cetyl alcohol</td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>White wax</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>PEG 200</td>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>-------------------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Bees wax</td>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Wool fat</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Extract</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EO: PE: M ( 2:2:1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Purified Water</td>
<td>34</td>
<td>48.4</td>
<td>69</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Modified Hydrophilic Ointment Base (F1)**

**Method:** Melt stearyl alcohol and white petrolatum (steam bath) and warm to about 75°C. Heat the water to 75°C and add the sodium lauryl sulfate, propylene glycol, and methyl & propyl paraben. Add the aqueous phase to the oily phase stir until congealed. Extract is incorporated to the ointment base during the cooling stage with Continuous stirring.

**Canadian formulary base (F II)**

**Method:** PEG 4000 & Stearyl alcohol is melt at 75°C and heat the water to about 75°C add glycerin & surfactant. Then add oily phase to aqueous phase stir until congealed. Extract is incorporated to the ointment base during the cooling stage with Continuous stirring.

**Bellers Base (F III)**

**Method:** Cetyl alcohol and white wax is taken in a beaker. Then melt at 75°C and water is also heated at 75°C to this add propylene glycol and surfactant. Then oily phase is slowly added to aqueous phase & stir until congealed. Extract is incorporated to the ointment base during the cooling stage with Continuous stirring.

**Wool fat ointment (F IV)**

**Method:** Accurately weighed quantities of above Ingredient are taken in a China dish. Bees wax is first melted & then wool fat & white soft paraffin is added to the china dish with continuous stirring. Then drug is incorporated in to the base when it is liquid form, stirring is continues until it solidify.

**PEG Ointment base (F V)**

**Method:** The weighed quantity of PEG 4000 was taken in Glass Beaker and melted. To this melt PEG 200 & Propylene Glycol is added by continuous stirring. The melt was removed from the heating mantle and the stirring was continued until the melt started congealing. Extract is incorporated to the ointment base during the cooling stage with Continuous stirring.
4.4.3 Formulation with PEG bases

From the preliminary formulations studied the PEG ointments were found to be appropriate and thus further studied in the compositions given in Table 10.

**Table 10 Formula for PEG ointment base**

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Ingredient</th>
<th>F V</th>
<th>F VI</th>
<th>F VII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
</tr>
<tr>
<td>1</td>
<td>PEG 4000</td>
<td>37</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>PEG 200</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Propylene Glycol</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>Extract EO: PE: M (2:2:1)</td>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>

**Method:** The weighed quantity of PEG 4000 was taken in Glass Beaker and melted. To this melt PEG 200 & Propylene Glycol is added by continuous stirring. The melt was removed from the heating mantle and the stirring was continued until the melt started congealing. Extracts at the Concentrations of 3%, 5 % and 10 % in the ratio (2:2:1) incorporated to the ointment base during the cooling stage with Continuous stirring.

The PEG Ointment base formulation was evaluated for all the parameters. [87, 93-103, 128-130]

4.4.4 Development of Gel formulations

Semisolid topical applications using various gelling systems were prepared by incorporating the most optimum combination of extracts i.e. EO: PE: M in the ratio 2:2:1 as the active medicament. The gels were initially prepared using various gelling agents as shown in Table 11 below.

**Table 11 Formula for Gel formulation**

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Ingredient</th>
<th>PG1</th>
<th>PG2</th>
<th>PG3</th>
<th>PG4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
</tr>
<tr>
<td>1</td>
<td>Carbopol 934</td>
<td>2</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Carbopol 940</td>
<td></td>
<td></td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Aloe vera gel</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Triethanolamine</td>
<td>1.65</td>
<td>1</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sodium alginate</td>
<td></td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>----------</td>
<td>-------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Glycerin</td>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Calcium Gluconate</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Propylene glycol</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Methyl paraben</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Methyl Hydroxy Benzoate</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Sodium hydroxide (1% w/v)</td>
<td>q.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Extract EO: PE: M (2:2:1)</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Purified water added quantity sufficient for 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**a) Preparation of Simple Gel Carbopol 934 base (PG1):**

**Method:** 95ml of purified water was heated. The carbopol 934 is added in small amounts to the solution using a high speed stirrer and after a smooth dispersion is obtained, the preparation is allowed to stand, permitting entrapped air to separate. Then gelling agent triethanolamine is added drop wise while stirring with a plastic spatula to avoid entrapment the air. The remaining water is then incorporated. The extract was added with stirring.

**b) Preparation of Sodium alginate gel base:**

**Method:** The sodium alginate is welled in a mortar with glycerin, which aids the dispersion. The preservative is dissolved in about 80ml of water with the aid of heat, and is allowed to cool; then the calcium salt is added, which will increases the viscosity of the preparation. The extract was added with stirring. This solution is stirred in a high speed stirrer and the sodium alginate glycerin mixture added slowly while stirring until preparation is homogenous. The preparation should be stored in a tightly sealed container in a wide mouth jar.

**c) Preparation of Modified Carbopol 934 gel base:**

**Method:** About 40 ml of water is taken in a beaker methyl paraben is dissolved in water & propylene glycol is added. Then the solution is stirred at high speed using stirrer. The slowly carbopol 934 is added to the beaker containing above solution. After smooth dispersion is obtained, the preparation is allowed to stand, permitting entrapped air to separate. Then gelling agent triethanolamine added slowly (dropwise) which stirring with plastic spatula to avoid air entrapment. Then pH is adjusted using 1% w/v solution of sodium hydroxide. Then remaining quantity water is incorporated. The extract was added under stirring.
d) Preparation of *Aloe vera*- carbopol 940 gel base:

**Method:** About 40 ml of water is taken in a beaker methyl paraben is dissolved in water & propylene glycol is added. Then the solution is stirred at high speed using stirrer. The slowly carbopol 940 is added to the beaker containing above solution. Then *Aloe vera* gel is incorporated. After smooth dispersion is obtained, the preparation is allowed to stand, permitting entrapped air to separate. Then gelling agent triethanolamine added slowly (dropwise) which stirring with plastic spatula to avoid air entrapment. Then remaining quantity water is incorporated. The extract was added under stirring.

### 4.4.5 *Aloe vera* - Carbopol 940 Gel Formulations

**Table 12 Formula for carbopol 940 gel Formulation**

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Ingredient</th>
<th>PG4</th>
<th>PG4 (I)</th>
<th>PG (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% w/w</td>
<td>% w/w</td>
<td>% w/w</td>
</tr>
<tr>
<td>1</td>
<td>Carbopol 940</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>Triethanolamine</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>Methyl paraben</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>4</td>
<td>Propylene glycol</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td><em>Aloe vera</em> gel</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Extract EO: PE: M (2:2:1)</td>
<td>0.5</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Purified water added quantity sufficient for 100%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Method:** The gel was prepared by adding *Aloe vera* gel to half of the quantity of water and stirred at high speed continuously on stirrer, propylene glycol and methyl paraben were dissolved in remaining proportion of water and added to above solution with continuous stirring at high speed, then extract 0.5%, 1% and 3% in the ratio (2:2:1) was added slowly with stirring, then slowly carbopol 940 was added to the beaker containing above solution. Then triethanolamine added slowly (drop wise) with stirring with plastic spatula to avoid air entrapment.
4.4.6. Evaluation of Semisolid Formulations

All the preliminary semisolid formulations, PEG ointment and gelling systems containing combination of extract i.e. EO: PE: M in the ratio 2:2:1 as the active medicament were evaluated for the following specific parameters.

4.4.6.1 Physical appearance

All the semisolid formulations were checked for color; syneresis and presence of lumps by visual inspection after the formulations have been set in the container, the formulations were further inspected for homogeneity. [132]

4.4.6.2 pH of Formulations

The pH of all formulations was determined by using the digital pH meter at constant temperature. Prior to this pH meter was calibrated using buffer solution of pH 7.0 and 9.2 then the electrode was washed with demineralized water. The electrode was inserted into the sample 10 min prior to taking the readings.

4.4.6.3 Extrudability

It is a useful empirical test to measure the force required to extrude the material from a tube. The formulations were filled in standard caped collapsible tube and sealed. The tube was weighed and recorded. The tube was placed between two glass slides and was clamped. A 500 gm weight was placed over the glass slide and then cap was opened. The amount of cream extruded were collected and weighed. The percent of gel extruded was calculated; and grades were allotted (+++ Excellent, +++Good, ++ Fair, +Poor).

4.4.6.4 Spreadability

One of the criteria for semisolid topical applications is to meet the ideal qualities is that it should possess good spreadability. Spreadability is a term express to denote the extent of area to which the gel readily spreads on application to skin or the affected parts. The therapeutic efficiency of formulation also depends upon its spreading value. Hence, determination of spreadability is very important in evaluating semisolid topical formulation characteristics.

The formulation was placed over the glass plate of 20cm X 5cm. Another glass plate of the same dimension was placed on the top of the gel such that the formulation was sandwiched between the two slides by placing a weight of 100 gram uniformly on the slides. The weight was removed and the excess of the semisolid application was scrapped off. Two slides in position were fixed
to a stand at a 45° angle without the slightest disturbance so that only the lower slide was held firmly by the clamp, allowing the upper slide to slip off freely with the help of 20 gram weight tied to the upper slide. The time taken for the upper slide to separate away from the lower glass plate under the direction of the weight was noted as per ICH guidelines. Experiment was done in triplicate and spreadability was calculated as follows:

\[ S = \frac{M \times L}{T} \]

Where,

- \( S \) = Spreadability
- \( L \) = Length of the glass plate
- \( M \) = Weight tied to the upper plate
- \( T \) = Time taken (sec).

4.4.6.5 Viscosity
Brookefield viscometer (Model-RVTP) was used to measure viscosity at 25° C ±2° C with spindle type-7. The sufficient quantity of gel was filled in wide mouth jar separately. The height of the gel as filled in the jar should sufficiently allow dipping the sample. The RPM of the spindle was adjusted to 2.5 RPM. The viscosities of the ointments were recorded in CPS Centipoise. \[104\]

4.4.7 In-vitro antimicrobial activity of semisolid formulations
The antimicrobial activities of the semisolid formulations were determined by the Well-agar plate diffusion method. 20 ml of sterile Nutrient Agar (NA), Potato Dextrose Agar (PDA) and Malt Glucose Yeast and Peptone Agar (MGYP) were aseptically transferred to sterilized petri dishes 9 cm in diameter each at 45° C ±2° C, followed by inoculation of bacteria and fungi strains (0.5ml). The plates were held for 15-20 minutes at room temperature 25° C ±2° C. The bore where prepared in the petri dishes using borer and formulation sample was added using a sterile syringe aseptically in the well. Then the bacterial culture plates were incubated at 37° C for 24 hours and the fungal culture plates were incubated at 22° C for 48 hours and the diameters of the inhibition zones formed on the NA, PDA and MGYP were evaluated in millimeters. \[116,118-121\]

4.4.8. Estimation of Minimum Inhibitory Concentration (MIC)
The prepared semisolid formulations were tested in a triplicate manner; samples were dispersed in 0.5 % polysorbate-80 to form the dispersions of different concentrations. The bacterial and fungal cultures prepared in triplicate were then incubated at 37° C and 22° C respectively for 48 hours. After incubation the MIC was estimated. \[116,118-121\]
4.5 Optimization of the formulation by factorial design

It is well known that traditional experimentation involves a good deal of efforts and time especially when complex formulations are to be developed. It is desirable to develop an acceptable pharmaceutical formulation in the shortest period of time using minimum number of man-hours and raw materials. In addition to the art of formulation, factorial design is an efficient method of indicating the relative significance of a number of variables and their interactions. Factorial design approach shows interactions between factors that a “one factor at a time” model cannot reveal.

Following are the terms used in factorial:
1. **Factors**: It is a variable, which has to be assigned such as rpm, drug-polymer ratio. The choice of factors to be included in an experiment depends on experimental objective and is predetermined.
2. **Level**: It takes into account the value beyond or below which a batch cannot be made effectively and are preselected high and low values of the variables.
3. **Runs/trials**: They compromise of factorial experiments, which consist of different combinations of all levels of all factors.
4. **Effect of a factor**: Is the change in response caused by varying the levels of the factor.
5. **Interaction**: Lack of additivity is known as interaction; either an antagonist or synergistic effect is observed.

Advantages of factorial design:
1. In absence of interaction, factorial designs have maximum efficiency in estimating main effects.
2. If interaction exists, factorial designs are necessary to reveal and identify interactions.
3. Maximum use of all data since main effects and interactions are calculated from all the data.
4. Factorial designs are orthogonal and all estimated effects and interactions are independent of the effects of other forms. \[133\]
4.5.1 Experimental Design using $3^2$ full Factorial

The formulations containing the active medicament (EO:PE:M in ratio of 2:2:1) in various percent weight by weight were studied and examined for antimicrobial activity, the *aloe vera* gel containing 1% active medicament had better result as compared to other gels and ointment and hence gel was selected for further studies.

The experimental design was done by using $3^2$ factorial, carbopol 940 and *Aloe vera* were selected as the independent variables and studied for experimental runs G1 to G9. The effect of the independent variable on the responses such as appearance, pH, spreadability, viscosity and extrudability were studied.

**Table 13 $3^2$ factorial design**

<table>
<thead>
<tr>
<th>Level</th>
<th>Factor</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$</td>
<td>Carbopol 940</td>
<td>0.6%</td>
<td>0.7%</td>
<td>0.8%</td>
</tr>
<tr>
<td>$X_2$</td>
<td><em>Aloe vera</em> gel</td>
<td>2%</td>
<td>3%</td>
<td>4%</td>
</tr>
</tbody>
</table>

The other formulation ingredients are as used in PG4 (I) formulations were maintained constant.

**Table 14 Experimental Runs using $3^2$ full factorial**

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Run No/Formulation Code</th>
<th>$X_1$</th>
<th>$X_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>G2</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>3</td>
<td>G3</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>4</td>
<td>G4</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>5</td>
<td>G5</td>
<td>+1</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>G6</td>
<td>+1</td>
<td>-1</td>
</tr>
<tr>
<td>7</td>
<td>G7</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>G8</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>G9</td>
<td>-1</td>
<td>+1</td>
</tr>
</tbody>
</table>
4.5.1.1 Multiple regression analysis for $3^2$ factorial design

The responses obtained from $3^2$ factorial batches were subjected to multiple regression analysis. The polynomial equations were determined using the form

$$Y_i = b_0 + b_1 X_1 + b_2 X_2 + b_{11} X_1^2 + b_{22} X_2^2 + b_{12} X_1 X_2 + b_{112} X_1^2 X_2 + b_{121} X_1 X_2^2$$

Where $Y_i$ is the dependent variable, $b_0$ is the arithmetic mean response of the 9 runs, and $b_1$ is the estimated coefficient for the factor $X_1$. The main effects ($X_1$ and $X_2$) represents the average results of changing one factor at a time from its low to high value.

The term $X_1^2$ and $X_2^2$ indicate curve linear relationship. The interaction $X_1 X_2$ shows how the dependent variable changes when two or more factors are simultaneously changed. Microsoft excel was used for multiple regression analysis, statically significant terms were identified using backward elimination method.

The responses studied were $Y_1 =$ Spreadability. $Y_2 =$ Viscosity

4.5.2 Experimental Design using $3^3$ full Factorial

This is a design that consists of three factors, each at three levels. It can be expressed as a $3 \times 3 \times 3 = 3^3$ design. The model for such an experiment is

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + C_k + AC_{ik} + BC_{jk} + ABC_{ijk} + \varepsilon_{ijk}$$

Where each factor is included as a nominal factor rather than as a continuous variable. In such cases, main effects have 2 degrees of freedom, two-factor interactions have $2^2 = 4$ degrees of freedom and $k$-factor interactions have $2^k$ degrees of freedom. The model contains $2 + 2 + 2 + 4 + 4 + 4 + 8 = 26$ degrees of freedom. Note that if there is no replication, the fit is exact and there is no error term (the epsilon term) in the model. In this no replication case, if one assumes that there are no three-factor interactions, then one can use these 8 degrees of freedom for error estimation. In this model we see that $i = 1, 2, 3$, and similarly for $j$ and $k$, making 27 treatments.

To further confirm the appropriate ratio of EO: PE: M in the optimized gel a $3^3$ factorial design was applied to the earlier optimized formulation obtained, by varying EO: PE: M at three levels the resultant 27 formulations were then studied for the antimicrobial activity.
Table 15 3^3 factorial design

<table>
<thead>
<tr>
<th>Factors</th>
<th>Level</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Essential Oil</td>
<td></td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>B Petroleum ether extract</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>C Methanol extract</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

4.5.3 Comparison Antimicrobial efficiency of Best Formulation with Marketed Formulations

Cream and Ointment formulation from market were procured and antimicrobial assay was preformed the result were compared with that of best formulation.

4.6 Skin irritancy study

4.6.1 Primary Dermal Irritation Index (PDII)

Dermal irritation is the production of reversible damage to the skin following the application of a test substance for up to 4 hours. Primary dermal irritation index (PDII) is a method for classifying topical formulations into various categories based on acute toxic reactions observed upon single application of a formulation on skin. Based on the PDII score, the formulation can be graded as irritating or non-irritating.

4.6.2 Selection and maintenance of animals

Healthy young male albino rabbits, weighing 1.5–2 kg at the start of the experiment, were used as the experimental animals in the present study (Institutional Animal Ethics Committee clearance Ref. No. CPCSEA/IAEC/P’cognosy-01/20012-13/78). The animals were housed together in a clean tank which was spacious enough for the free movement of the animals and accommodation to hold drinking water and feed. Room temperature was 25° ±2°C, humidity was 45% ±5% RH with a light period of 13 h (06.00 AM to 19.00 PM). The animals were fed with commercially available standard pellet chow and filtered tap water.
4.6.3 Preparation of animals

Approximately 24 hours before the test, fur was removed by closely clipping the dorsal area of the trunk of the animals. Care was taken to avoid abrading the skin and only animals with healthy, intact skin were used for the study.

4.6.4 Application of the herbal gel

Half a gram of the herbal gel, as the test substance, was applied to an area of approximately 6cm$^2$ of skin and covered with a gauze patch. The patch was loosely held in contact with the skin by means of a suitable semi-occlusive dressing for 4 hours and was then removed. At the end of the exposure period, i.e. 4 hours, residual test substance was removed without altering the existing response or the integrity of the epidermis. Observations were recorded an hour after the removal of the patch. Control animals were prepared in the same manner and 0.5 gram of the gel base, i.e. gel formulated using all the ingredients except the herbal mixture, was applied to the control animals and observations were made similar to the test animals.

Both the control and the test animals were observed every day for any occurrence of skin irritation such as edema or erythma. Per observation of skin, a value between 0 and 4 was recorded where 0 meant no skin erythma and eschar formation and 1, 2, 3 and 4 stood for very slight, well defined, moderate and severe erythma to eschar formation, respectively. It also scored from 0–4, where 0 stood for no edema and 4 stood for severe edema. \[^{[96]}\]

Primary Dermal Irritation Index (PDII) = PDII observed on 12+24+48+72 hrs

4.6.5 Classification system based on PDII

< 0.5: non-irritating, 0.5-2.0: slightly irritating, 2.1-5.0: moderately irritating and >5.0: severely irritating.

4.7 Stability Testing

General Principles

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and to establish a re-test period for the drug substance or a shelf life for the drug product and recommended storage conditions.
Drug Product

The design of the formal stability studies for the drug product should be based on knowledge of the behavior and properties of the drug substance and from stability studies on the drug substance and on experience gained from clinical formulation studies. The likely changes on storage and the rationale for the selection of attributes to be tested in the formal stability studies should be stated.

Stability studies should include testing of those attributes of the drug product that are susceptible to change during storage and are likely to influence quality, safety, and/or efficacy. The testing should cover, as appropriate, the physical, chemical, biological, and microbiological attributes, preservative content (e.g., antioxidant, antimicrobial preservative), and functionality tests (e.g., for a dose delivery system). Analytical procedures should be fully validated and stability indicating. Whether and to what extent replication should be performed will depend on the results of validation studies.

Shelf life acceptance criteria should be derived from consideration of all available stability information. It may be appropriate to have justifiable differences between the shelf life and release acceptance criteria based on the stability evaluation and the changes observed on storage.

Evaluation

The purpose of the stability study is to establish, based on testing a minimum of three batches of the drug substance and evaluating the stability information (including, as appropriate, results of the physical, chemical, biological, and microbiological tests), a re-test period applicable to all future batches of the drug substance manufactured under similar circumstances.

4.7.1 Stability studies of the best formulation selected.

Physical parameters and antimicrobial activity of the best formulation were carried out after stability period of 6 months and one year. The ICH guidelines on stability testing of new drug substances and products were used to assess the gel stability of the selected best formulation.\(^\text{[131]}\)

Samples of the selected best formulation were kept at room temperature and % RH i.e. natural conditions prevalent at Aurangabad, Maharashtra. Separate samples were also kept at 40°C ±2°C with relative humidity 75% ±5 % RH and at 25°C ±2 with relative humidity 60% ±5% RH. These samples were stored for the period of 6 and 12 months in the stability chamber, after the period of 6 months and 12 months evaluation tests were carried out with respect to physical parameters and antimicrobial activity.
4.7.2 Thin Layer Chromatography of best Formulation after stability period of 6 months. 
Thin-layer Chromatography was carried out for best formulation after stability period of six months by suspending 10 mg of gel in 1 ml of methanol using Tween 80 (Polysorbate 80) as suspending agent on silica gel precoated aluminum plates (Merck Silica gel 60 F_{254}) as stationary phase and (petroleum ether: ethyl acetate 70:30 v/v) as mobile phase followed by anisaldehyde-\( \text{H}_2\text{SO}_4 \) spray with heating at 100°C for 1 minute.

4.7.3 High Performance Thin Layer Chromatography of the best formulation after stability period of 6 months and twelve months.

The best formulation was used for HPTLC to confirm the concentration of marker compound stigmasterol in the initial formulation and after a period of 6 months and 12 months. \(^{[122-127]}\)

4.7.3.1 Preparation of mobile phase

Mobile phase are prepared by mixing the solvent petroleum ether: ethyl acetate in the ratio of (7:3 v/v) and degassed to remove trapped air.

4.7.3.2 Stock and working standard solution

Stigmasterol 10 mg and squalene 10ml were weighed independently and transferred separately to a 100 ml volumetric flask. The drugs were dissolved in methanol and make up the volume to obtain stock solution of 1000 \( \mu \text{g/ml} \) appropriate dilutions of the above mentioned standard stock solution were prepared in 10 ml volumetric flask with methanol. Calibration standards were prepared over the concentration range 100 \( \mu \text{g/ml} \) appropriate Microlitre by micro syringe from each standard solution was spotted on the HPTLC plate to obtain final concentration range of 2,4,6,8,10,12 \( \mu \text{g/spot} \).

4.7.3.3 Preparation of sample solution

The accurate quantity of gels 20 mg were dispersed using Tween 80 (Polysorbate 80) as suspending agent in 10 ml methanol to get 2 mg/ml concentration. The appropriate quantities of samples were taken by micro syringe for spot.