IV. Material and Methods
Material And Methods

IV. 1. Survey and selection of medicinal plants for this study:
Survey of medicinal plants was done in and around Sangli by visiting various sites and taking the help of traditional practitioners, healers and users. Availability of these plants, the seasons in which they are present in ample and the purpose for which they are used, are taken into consideration. With this view, the following plants were selected for the present study and authenticated by consulting the experts.

1. *Allium sativum*  2. *Aloe vera*
3. *Andropogon citratum*  4. *Azadirachta indica*
5. *Calotropis gigantea*  6. *Cassia auriculata*
7. *Catharanthus roseus*  8. *Cleome viscosa*

In addition, three ayurvedic preparations which are routinely used by the practitioners, have been used during the course of investigation,

1. Triphala - It is a mixture of equal amounts of *Emblica officinalis* (Amla), *Terminalia chebula* (Hirda) and *Terminalia belera* (Behada) having known action in digestive disorders.
2. Face pack - Saroj Pimple pack: It is an ayurvedic mixture with unknown ingredients and unknown anti-microbial activity containing natural medicinal plants and herbs. It claims to demolish pimples and black heads, bringing fairness (Manufacturer: Dr. Sarika Mahendra Rathi, naturopathist, 769/5, “Saroj”, off Bhandarkar road, Pune - 411004).
3. Kofit - Ayurveda bio-enhanced herbal cough formula. It is an ayurvedic syrup with known ingredients, composition and anti-microbial activity

[Plates 1-5]
PLATE 1

Plants under investigation:

a) *Allium sativum*

b) *Aloe vera*

c) *Andropogum citratum*

d) *Azadirachta indica*
PLATE 2

Plants under investigation:

a) Calotropis gigantea  
b) Cassia auriculata  
c) Catharanthus roseus  
d) Cleome viscosa
Plates 3

Plants under investigation:

a) Curcuma longa

b) Eucalyptus globulus

c) Phyllanthus niruri

d) Pongamia pinnata
PLATE 4

Plants under investigation:

a) *Piper betel*

b) *Terminalia arjuna*
PLATE 5
Commercial herbal samples used in the study.

1) Kofit
   i) Front view    ii) Information details

2) Triphala

3) Saroj face pack
   i) Front view   ii) Information Details
IV. 2. Preparation of plant extracts:

Whole plant as well as different parts of the plants were used.

a) Aqueous extract: Known amount of plant material was washed with sterile distilled water and crushed in a grinder. A very fine paste was prepared in a grinder, using a known amount of sterile distilled water. The material was filtered through a muslin cloth and Whatman no. 40 filter paper. The fine powder obtained on drying was used as the sample for investigation. Preparation of aqueous extracts has been detailed in Table 1 (Bambode and Shukla, 1973).

b) Acetone extract: Known weight of plant material was washed with sterile distilled water and crushed in a grinder using a known amount of acetone. The mixture was refluxed at 60 °C for one hour. The extract was filtered through muslin cloth and Whatman no. 40 filter paper. Acetone was allowed to evaporate and the powder obtained on drying was used as a sample. Preparation of aqueous extracts has been detailed in Table 2 (Shekhawat and Prasad, 1971).
### Table 1
**Details of preparation of aqueous extracts of plants used during the study**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the plant sample</th>
<th>Plant part used</th>
<th>Weight of plant material</th>
<th>Amount of distilled water added</th>
<th>Dry weight of the aqueous powder extract</th>
<th>Weight of powder extract (x 10^2 gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Allium sativum</em></td>
<td>Bulblets</td>
<td>20 gm</td>
<td>50 ml</td>
<td>0.680 gm</td>
<td>0.0340</td>
</tr>
<tr>
<td>2</td>
<td><em>Aloe vera</em></td>
<td>Leaf pulp</td>
<td>30 gm</td>
<td>50 ml</td>
<td>0.450 gm</td>
<td>0.0150</td>
</tr>
<tr>
<td>3</td>
<td><em>Andropogon cirratum</em></td>
<td>Fresh leaves</td>
<td>20 gm</td>
<td>50 ml</td>
<td>1.250 gm</td>
<td>0.0625</td>
</tr>
<tr>
<td>4</td>
<td><em>Azadirachta indica</em></td>
<td>Fresh leaves</td>
<td>20 gm</td>
<td>50 ml</td>
<td>1.850 gm</td>
<td>0.0925</td>
</tr>
<tr>
<td>5</td>
<td><em>Calotropis gigantea</em></td>
<td>Fresh leaves</td>
<td>60 gm</td>
<td>50 ml</td>
<td>4.800 gm</td>
<td>0.0803</td>
</tr>
<tr>
<td>6</td>
<td><em>Cassia auriculata</em></td>
<td>Fresh leaves</td>
<td>100 gm</td>
<td>250 ml</td>
<td>1.400 gm</td>
<td>0.0140</td>
</tr>
<tr>
<td>7</td>
<td><em>Catharanthus roseus</em></td>
<td>Fresh leaves</td>
<td>80 gm</td>
<td>50 ml</td>
<td>3.100 gm</td>
<td>0.0386</td>
</tr>
<tr>
<td>8</td>
<td><em>Cleome viscosa</em></td>
<td>Fresh leaves</td>
<td>55 gm</td>
<td>50 ml</td>
<td>3.500 gm</td>
<td>0.0636</td>
</tr>
<tr>
<td>9</td>
<td><em>Curcuma longa</em></td>
<td>Rhizome</td>
<td>5 gm</td>
<td>20 ml</td>
<td>0.850 gm</td>
<td>0.1700</td>
</tr>
<tr>
<td>10</td>
<td><em>Eucalyptus globulus</em></td>
<td>Fresh leaves</td>
<td>40 gm</td>
<td>50 ml</td>
<td>1.000 gm</td>
<td>0.0250</td>
</tr>
<tr>
<td>11</td>
<td><em>Phyllanthus niruri</em></td>
<td>Fresh leaves and stem</td>
<td>20 gm</td>
<td>50 ml</td>
<td>1.200 gm</td>
<td>0.0600</td>
</tr>
<tr>
<td>12</td>
<td><em>Piper betel</em></td>
<td>Fresh leaves</td>
<td>30 gm</td>
<td>30 ml</td>
<td>1.500 gm</td>
<td>0.0500</td>
</tr>
<tr>
<td>13</td>
<td><em>Pongamia pinnata</em></td>
<td>Fresh seeds</td>
<td>90 gm</td>
<td>70 ml</td>
<td>27.90 gm</td>
<td>0.3100</td>
</tr>
<tr>
<td>14</td>
<td><em>Terminalia arjuna</em></td>
<td>Bark</td>
<td>50 gm</td>
<td>100 ml</td>
<td>3.700 gm</td>
<td>0.0740</td>
</tr>
<tr>
<td>15</td>
<td>Triphala</td>
<td>Readymade powder</td>
<td>20 gm</td>
<td>50 ml</td>
<td>1.300 gm</td>
<td>0.0675</td>
</tr>
<tr>
<td>16</td>
<td>Face pack</td>
<td>Readymade powder</td>
<td>10 gm</td>
<td>25 ml</td>
<td>0.550 gm</td>
<td>0.0530</td>
</tr>
<tr>
<td>17</td>
<td>Kofit</td>
<td>Readymade syrup</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 2
**Details of preparation of acetone extracts of plants used during the study**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the plant sample</th>
<th>Plant part used</th>
<th>Weight of plant material</th>
<th>Amount of Acetone added</th>
<th>Dry weight of acetone powder extract</th>
<th>Dry weight of the powder extract (x 10^2 gm %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Allium sativum</em></td>
<td>Bulblets</td>
<td>10 gm</td>
<td>20 ml</td>
<td>0.425 gm</td>
<td>0.0422</td>
</tr>
<tr>
<td>2</td>
<td><em>Aloe vera</em></td>
<td>Leaf pulp</td>
<td>10 gm</td>
<td>20 ml</td>
<td>0.128 gm</td>
<td>0.0128</td>
</tr>
<tr>
<td>3</td>
<td><em>Andropogon cirratum</em></td>
<td>Fresh leaves</td>
<td>10 gm</td>
<td>20 ml</td>
<td>0.520 gm</td>
<td>0.0520</td>
</tr>
<tr>
<td>4</td>
<td><em>Azadirachta indica</em></td>
<td>Fresh leaves</td>
<td>10 gm</td>
<td>27 ml</td>
<td>0.350 gm</td>
<td>0.0350</td>
</tr>
<tr>
<td>5</td>
<td><em>Calotropis gigantea</em></td>
<td>Fresh leaves</td>
<td>40 gm</td>
<td>20 ml</td>
<td>0.0016 gm</td>
<td>0.0004</td>
</tr>
<tr>
<td>6</td>
<td><em>Cassia auriculata</em></td>
<td>Fresh leaves</td>
<td>10 gm</td>
<td>20 ml</td>
<td>0.150 gm</td>
<td>0.0150</td>
</tr>
<tr>
<td>7</td>
<td><em>Catharanthus roseus</em></td>
<td>Fresh leaves</td>
<td>40 gm</td>
<td>20 ml</td>
<td>2.000 gm</td>
<td>0.0050</td>
</tr>
<tr>
<td>8</td>
<td><em>Cleome viscosa</em></td>
<td>Fresh leaves</td>
<td>20 gm</td>
<td>25 ml</td>
<td>0.400 gm</td>
<td>0.0200</td>
</tr>
<tr>
<td>9</td>
<td><em>Cynara longa</em></td>
<td>Rhizome</td>
<td>5 gm</td>
<td>5 ml</td>
<td>2.200 gm</td>
<td>0.4400</td>
</tr>
<tr>
<td>10</td>
<td><em>Eucalyptus globulus</em></td>
<td>Fresh leaves</td>
<td>50 gm</td>
<td>20 ml</td>
<td>2.700 gm</td>
<td>0.0540</td>
</tr>
<tr>
<td>11</td>
<td><em>Phyllanthus niruri</em></td>
<td>Fresh leaves and stem</td>
<td>10 gm</td>
<td>25 ml</td>
<td>0.950 gm</td>
<td>0.0950</td>
</tr>
<tr>
<td>12</td>
<td><em>Piper betel</em></td>
<td>Fresh leaves</td>
<td>30 gm</td>
<td>20 ml</td>
<td>0.900 gm</td>
<td>0.0300</td>
</tr>
<tr>
<td>13</td>
<td><em>Pongamia pinnata</em></td>
<td>Fresh seeds</td>
<td>60 gm</td>
<td>50 ml</td>
<td>4.300 gm</td>
<td>0.0716</td>
</tr>
<tr>
<td>14</td>
<td><em>Terminalia arjuna</em></td>
<td>Bark</td>
<td>40 gm</td>
<td>40 ml</td>
<td>2.000 gm</td>
<td>0.0500</td>
</tr>
<tr>
<td>15</td>
<td>Triphala</td>
<td>Readymade powder</td>
<td>10 gm</td>
<td>20 ml</td>
<td>1.250 gm</td>
<td>0.0125</td>
</tr>
<tr>
<td>16</td>
<td>Face pack</td>
<td>Readymade powder</td>
<td>10 gm</td>
<td>25 ml</td>
<td>0.480 gm</td>
<td>0.0480</td>
</tr>
<tr>
<td>17</td>
<td>Kofit</td>
<td>Readymade syrup</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
IV. 3. Isolation of pathogens:

IV. 3. (i) Study Group A: Isolates from the Microbiology laboratory

Isolation was carried out in the following steps:

- Samples were collected from the Microbiology laboratory.
- Samples were immediately used for further processing. In case of delay, they were preserved by refrigeration (at 10 °C).
- Isolation of the micro-organisms was carried out using corresponding media.
- Identification of the typical isolates was carried out using different biochemical media (Saxena, 1969; Frobisher et al, 1974; Anathanarayan and Paniker, 1986; Dey and Dey, 1998; Gupte, 1998).
- The identified cultures were maintained and preserved on antibiotic assay medium (trypticase soy agar slants) for further study.
- Sensitivity of these isolates to plant extracts and antibiotics was studied using diffusion assay methods.

IV. 3.(ii) Study Group B: Isolates from wound swabs of burns patients

- Sample swabs from wounds of burns patients were collected from the Microbiology laboratory of General Hospital, Sangli.
- The samples were immediately processed. In case of delay, the samples were preserved in a refrigerator (at 10 °C).
- These sample swabs were spread on nutrient agar medium to carry out primary screening of sensitivity of the pathogens in the samples to antimicrobially effective plant extracts and antibiotics, with a control plate.
- Isolation of individual pathogens from these samples was carried out using corresponding media.
- Identification of the typical isolates from the wound swab samples was carried out using appropriate selective and biochemical media (Saxena, 1969; Frobisher et al, 1974; Anathanarayan and Paniker, 1986; Dey and Dey, 1998; Gupte, 1998).
• Isolates were preserved on antibiotic assay medium or trypticase soy agar slants for further study.
• Sensitivity of identified isolates to the effective aqueous plant extracts and antibiotics was checked by diffusion assay methods.

IV. 3. (iii) Study Group C: Standard ATCC cultures
Four standard cultures obtained from Himedia laboratories, Mumbai, were maintained and preserved on antibiotic assay medium slants for further study.
1. *Staphylococcus aureus* ATCC 29213
2. *Enterococcus fecalis* ATCC 29212
3. *Escherichia coli* ATCC 25922
4. *Pseudomonas aeruginosa* ATCC 27853

IV. 4. Detection of anti-microbial activity of the plant extracts against isolates (Study Groups A, B and C)
Agar-cup diffusion assay method (Finegold and Baron, 1986).
• Suspension of young culture of each isolate, in 0.1 ml amount, was spread separately on sterile nutrient agar plates (bacterial culture) or Sabouraud's agar plates (fungal or yeast culture).
• Cups were aseptically made with sterile cork borers (diameter 8 mm).
• Aqueous and acetone extract powders were diluted 1:10 with the help of sterile distilled water.
• 0.1 ml of each extract was aseptically added to corresponding cups and the plates refrigerated for 15 minutes.
• Incubation at appropriate temperatures for prescribed time was carried out.
• Plates were observed for zones of inhibition surrounding the cups.
• Diameters of the inhibition zones were recorded.

IV. 5. Detection of sensitivity of the isolates (Study Groups A, B and C) to antibiotics
Disc diffusion assay method (Bauer et al, 1966)
• Suspension of young culture of each isolate, in 0.1 ml amount, was spread separately on sterile nutrient agar plates (bacterial culture) or Sabouraud's agar plates (fungal or yeast culture).
• Antibiotic discs were placed with gentle pressure, on the surface and the plates refrigerated for 15 minutes.
• Incubation at appropriate temperatures for prescribed time was carried out.
• Plates were observed for zones of inhibition surrounding the discs.
• Diameters of the zones were recorded.

**Antibiotics used**

For sensitivity testing, antibiotic octadiscs number OD - 042 from Himedia and Gram negative Master multidiscs number MD - 002, Micro Master Laboratories, were used to check the response of the study Groups A, B and C isolates.

**IV. 6. Qualitative analysis of the extracts**

Qualitative analysis of the aqueous extracts was carried out with:

(a) A known 1% solution of the standard.

(b) The extract.

1) **Detection of Alkaloids:** (Kokate et al, 2001)

**Mayer's test:** To 1 ml of the test solution, a few drops of Mayer's reagent were added. Development of a cream or grayish white precipitate indicated the presence of alkaloids.

2) **Detection of Fats:** (Singh, 1977)

(i) **Sudan test:** Presence of fats was detected by addition of a few drops of alcoholic Sudan III solution to 1 ml of the test solution. Development of a red colour indicated positive test.

(ii) **Solubility test:** A few drops of solvents like alcohol, acetone, chloroform, ether were taken in separate test tubes and a drop of the test sample was added to each of the tubes. After shaking, tubes were allowed to stand for some time. A drop of mixture from each tube was placed on a filter paper and allowed to dry out. Appearance of a clear spot of grease indicated the presence of fats.

3) **Detection of Flavonoids and Tannins:** (Khandelwal, 2001)

**Ferric chloride test:** To 1 ml of the test solution, a few drops of 10% FeCl₃ solution were added. Development of an intense green colour
indicated the presence of flavonoids while development of a dark brownish black colour indicated the presence of tannins.

4) Detection of Glycosides: (Kokate et al, 2001)

Keller Killiani test: To 1 ml of the test solution, glacial acetic acid was added to dissolve the contents. 2 drops of 10% FeCl₃ solution were added and contents transferred to a tube containing 2 ml of conc. H₂SO₄. A separation of two layers was obtained. If the lower layer turned reddish brown and upper layer bluish green, it indicated the presence of glycosides.

5) Detection of Proteins: (Vogel, 1966)

Ninhydrin test: 1 ml of the test sample was heated with 5 drops of concentrated Ninhydrin solution. Appearance of a blue-violet colour indicated the presence of a protein.

6) Detection of Reducing sugars: (Vogel, 1966)

Benedict's test: 5 drops of the test sample were heated with 2 ml of Benedict's reagent. Formation of a red or rust brown colour indicated the presence of a reducing sugar.

7) Detection of Sterols and Triterpenoids: (Khandelwal, 2001)

Salkowaski test: To 2 ml of the test solution, a few drops of conc. H₂SO₄ were added. The tube was shaken and allowed to stand. If lower layer turned red, it indicated the presence of sterols. If it turned yellow, it indicated the presence of triterpenoids.

IV. 7. Determination of Minimum Inhibitory Concentration (MIC)

Determination of MIC was carried out by tube dilution method (Harbourne, 1973).

- The culture used for MIC was decided on the basis of the isolate against which the plant extracts were found to be most effective.
- The isolates under study were diluted at a density adjusted to 0.5 McFarland turbidity with sterile nutrient broth medium. The final inoculum was 5 x 10⁵ CFU / ml of the isolate.
- Negative controls (15 test tubes) with 0.5 ml of sterile nutrient broth medium with 0.01 ml to 0.15 ml of respective plant extracts
(diluted 1:10 with sterile distilled water) were used in the test to compare the turbidity as the criteria of growth.

- **15 sample test tubes containing 0.5 ml of sterile nutrient broth as a diluent were set up for the study. Serially 0.01 ml to 0.15 ml of the extracts (diluted 1:10 with sterile distilled water) were added to these tubes respectively.**

- Each isolate was inoculated in these 15 sample test tubes and incubated at 37°C for 24 hrs.

- The tubes were then examined for the presence of growth considering turbidity as criteria.

- The lowest concentration of the extract in the first sample tube that showed no growth (turbidity) of the test organism was considered to be the MIC / Effective Dose of the organism.

**IV. 8. Qualitative analysis of the effective extracts by High Performance Thin Layer Chromatography (HPTLC)**

HPTLC is a highly modified form of TLC, carried out to verify qualitative and quantitative presence of constituents in herbal extracts under investigation. The speed and efficiency of separation is increased by automation of sample application, chromatogram, detection, accurate and precise in situ quantitation. Stationary phase includes pre-coated silica gels, with very fine particle layer, thinner plates exhibiting 4000 theoretical plates in 3 cm requiring 10 minutes for development (Skoog and Leary, 1992). Small volume samples are used and separation time is reduced by a factor of 10. Locating is done by spraying with a developing reagent, plates are observed under UV light at different wavelengths. Sample is separated into its unique biochemical spectrum represented in a chromatogram, scanned using sophisticated HPTLC scanner, powerful software and a fingerprint is obtained. Each peak represents an active organic constituent. For identification, fingerprint is compared to a previously established reference for that particular extract. It allows processing of many samples in parallel, at low cost (Christian, 2001).
Sample application volumes: To all the five aqueous extracts, taken in 1 ml amount (diluted 1:10 in distilled water), 5 ml of methanol was added, solutions filtered and filtrate used for application.

a) General analysis was done using:
Instrument: CAMAG TLC Scanner 3 "Scanner 3_070408" S/N 070408 (1.14.21),
Stationary phase: TLC Al sheet Silica gel 60 F254 precoated MERCK Cat. No. 1.05554 of size 10 x 10 cm
Mobile phase: Toluene : Ethyl acetate : Formic acid (100%) = 7 : 2 : 0.2
AI - 20 µl, PP - 10 µl, CL - 30 µl, EG - 20 µl, PN - 30 µl
Each sample was applied on a precoated layer 10 mm from the bottom edge, band length 8 mm, distance between the tracks 15 mm, distance from the sides 15 mm
Developing distance: 80 mm
Measurement mode: UV Absorbance / Reflectance
Wave-lengths: 200 nm, 254 nm, 366 nm
Developing reagent / Derivatizing agent: 5% Methanolic sulphuric acid.

b) Flavonoid analysis was done using:
Instrument: CAMAG TLC Scanner 3 "Scanner 3_070408" S/N 070408 (1.14.21),
Stationary phase: TLC Al sheet Silica gel 60 F254 precoated MERCK Cat. No. 1.05554 of size 10 x 10 cm
Mobile phase: Chloroform : Acetone : Formic acid = 7 : 5 : 0.4
Sample application volume: 5 µl of each sample was applied on precoated layer 10 mm from the bottom edge, band length 8 mm, distance between the tracks 17.5 mm, distance from the sides 15 mm
Chamber saturation: 0 min
Developing distance: 80 mm
Measurement mode: UV Absorbance / Reflectance
Development chamber: Camag Twin Trough Chamber 10 X 10 cm with S. S. lid.
Analysis of components was done on the basis of $R_f$ Retention factor values (Gangwar and Kumar, 2006). All the peaks were identified by referring to "Plant drug analysis, A thin layer chromatography, Atlas" by Wagner and Bladt (1996).

**IV. 9. Separation of the most effective plant extract into its components by Thin Layer Chromatography (TLC)**

TLC was carried out to verify qualitative presence of constituents in herbal extracts under investigation. Stationary phase included pre-coated silica gels, about 5 mm thick. 0.1 ml samples (1:10 dilutions in distilled water) were used. Solvent system (Butanol : Acetic acid : Water = 40 : 10 : 50) was used for separation. Locating was done by spraying with a developing reagent (0.5% ninhydrin). Sample was separated in the form of coloured spots on the plate (Ahluwalia and Aggarwal, 2000). For further study and identification, the developed spot areas were scraped, dissolved in acetone, filtered and dried powder obtained in the filtrate used.

**IV. 10. Sensitivity study of the TLC separated components**

Sensitivity study of the components in the effective plant extract separated by TLC was carried out against the isolates from wound infection in burns patients by agar cup diffusion assay method.

**IV. 11. Analysis of antimicrobially active components of the extract by Infra-red (IR) spectroscopy**

Analysis of the antimicrobially effective plant extract and TLC detected components of the plant extract was done by IR spectroscopy (Jasco FTIR – 410 – sr. no. AO 11960585) to know its active chemical groups. The groups were identified using standard references, according to their wave numbers and % transmission (Kemp, 1996).

**IV. 12. Analysis of the TLC detected antimicrobially active components by Gas Chromatography Mass Spectrometry (GCMS):**

Analysis of the antimicrobially effective TLC detected components of the plant extract was done by GCMS QP 2010 to know the structures of its
ingredients. Results were obtained directly in the form of GCMS data and graphs.

IV. 13. Interviews of practitioners in Ayurvedic medicine:
Senior Ayurvedic practitioners in Sangli were interviewed with the help of a questionnaire (Annexure VI) with regards to:

1. the age group of patients attending OPD:
2. common complaints of patients
3. normally prescribed ayurvedic drugs and their herbal components
4. patients' views about Allopathic and Ayurvedic treatments
5. patients' response to the duration of treatment
6. doctors' views about the recent trends in practice