Chapter 3

MATERIAL AND METHODS

3.1. Material

3.1.1. Chemicals and reagents

Various chemicals and reagents for biochemical test, pH measurement and preparation of plant extracts were used. The chemicals used during the experiment were purchased from Hi Media Laboratories, India; Qualigens Fine Chemicals, India; Sisco Research Laboratories (SRL), India; Merck, India.

Chemicals used included: ethanol, acetone, amyl alcohol, glycerol, DMSO (Dimethyl sulphoxide), sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), hydrogen peroxide (H₂O₂), barium chloride, ammonium oxalate, potassium iodide, H₂SO₄, potassium hydroxide (KOH), tetramethyl-p-phenylenediamine dihydrochloride, p-dimethyl aminobenzaldehyde, α-napthol, various dyes viz., crystal violet, methylene blue, iodine, safranine, methyl red, etc.

3.1.2. Media

During the lab work, different types of media were used for culturing bacteria and for performing biochemical tests. The media were purchased from Hi Media Laboratories, India. Their name, composition and purpose are given below.
3.1.2.1. Nutrient agar medium

**Composition**

- Peptone: 5.0 g
- Beef extract: 3.0 g
- Sodium chloride: 8.0 g
- Agar: 15.0 g
- Distilled water: 1000 ml
- pH: 7.0

**Purpose** - General purpose media used for the maintenance of microbial growth.

3.1.2.2. MacConkey agar medium

**Composition**

- Peptone: 20.0 g
- Sodium chloride: 8.0 g
- Bile salt: 1.5 g
- Lactose: 10.0 g
- Neutral red: 10.0 ml
- Solution (1% aqueous)
  - Crystal violet: 0.001 g
  - Agar: 15.0 g
  - Distilled water: 1000 ml
  - pH: 7.1

**Purpose** - Identifying lactose fermenters.
3.1.2.3. Eosin methylene blue agar medium (EMB)

**Composition**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Eosin Y</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>0.065 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**Purpose** - For selective growth of *E. coli*.

3.1.2.4. Mannitol salt agar medium

**Composition**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>
**Purpose**- Identifying *Staphylococcus aureus*.

### 3.1.2.5. Carbohydrate fermentation broth

**Composition**

- Peptone 10.0 g
- Carbohydrate 5.0 g
  (Glucose, sucrose, lactose)
- Sodium chloride 5.0 g
- Phenol red .018 g
- Distilled water 1000 ml
- pH 7.3

**Purpose**- Identifying sugar fermenters.

### 3.1.2.6. Tryptone broth

**Composition**

- Tryptone 10.0 g
- Sodium chloride 5.0 g
- Calcium chloride 1.0 g
- Distilled water 1000 ml

**Purpose**- Indole test.
3.1.2.7. Glucose phosphate broth

**Composition**

Peptone 7.0 g  
K$_2$HPO$_4$ 5.0 g  
Dextrose 5.0 g  
Distilled water 1000 ml  

**Purpose** - Methyl red and Voges- prauesker test

3.1.2.8. Simmon’s citrate agar

**Composition**

NH$_4$H$_2$PO$_4$ 1.0 g  
K$_2$HPO$_4$ 1.0 g  
Sodium chloride 5.0 g  
Sodium citrate 2.0 g  
Magnesium sulphate 0.2 g  
Bromothymol blue .08 g  
Agar 15.0 g  
Distilled water 1000 ml  

**pH** 6.9  

**Purpose** - Citrate utilization test.
3.1.3. Glasswares and plasticwares

All Glasswares and plasticwares used during the experiment were purchased from Borosil, India; Tarson, India respectively. Glasswares and plasticwares used included: Sample containers, plastic vials, petri plates, petri plate stand, flasks, glass spreader, beakers, micropipettes, test tubes, culture bottles.

3.1.4. Antibiotics

All antibiotics used during the study were purchased from HiMedia, India. The antibiotic discs and their concentrations were: amikacin (AK, 30µg), cefixime (SF, 5µg), ceftazidime (FG, 30 µg), ciprofloxacin (RC, 5 µg), netilmicin (NT, 30 µg), nitrofurantoin (FD, 300 µg), norfloxacin (NX, 10 µg), ofloxacin (ZN, 5 µg).

3.1.5. Biological material

(i) Plant material

Dried fruits of amla, baheda and harad were collected from Shantikunj, Hardwar. The mixture of all the three in equal amount constituted the triphala.

(ii) Bacterial isolates

Bacterial isolates of UTI were isolated from urine samples obtained from patients suffering from UTI. The samples were collected from Subharti Medical College and Lokpriya Nursing Home, Meerut. Among 200 samples collected, 120 belong to female patients and 80 belong to male patients.


3.2. Methods

3.2.1. Collection of samples

The samples were collected in wide mouthed plastic universal containers (350 ml) by clean catch midstream urine method. Before collecting the specimen, the periurethral area was properly cleaned to avoid mild contamination. Once collected, the specimens were transported to the lab (Department of Microbiology, C.C.S. University, Meerut) without any delay and preserved in refrigerator at 4 °C. Microbial counts in a urine sample remain same at 4 °C in refrigerator for as long as 24 h. Besides the organisms isolated, reference strains which were procured from IMTECH, Chandigarh were used as positive control.

3.2.2. Disinfection of inoculation room

Inoculation room was fumigated on alternate days in the evening. Fumigation was done by using crystalline potassium permanganate and formalin. First of all, a thin layer of cotton was laid down in Petri plate and some crystals of potassium permanganate were spread and then formalin was poured over the crystals. Fumes of formaldehyde were emitted from it. The room was closed till next day.

3.2.3. Disinfection of Laminar air flow and inoculation chamber

The laminar air flow and inoculation chamber were cleaned thoroughly with the help of 70% ethanol and cotton wool. The UV light was turned on for at least 15 min. before starting the work.
3.2.4. Preparation of media plates, broth and slants

All the media were prepared by dissolving the contents in distilled water in media preparation bottle and placed on magnetic stirrer for 15 minutes to form a uniform and homogeneous solution and then autoclaved at 15 psi for 15 minutes. The sterilized media was allowed to cool up to 45 °C and approximately 2 ml of the media was poured in sterilized glass petri plates and allowed to solidify. For the preparation of broth, 5 ml of the media is dispensed in 20 ml glass tubes and the opening is closed with cotton plug and covered with aluminum foil and then sterilized by autoclaving at 15 psi for 15 min. The slants of the media were prepared by pouring approximately 10 ml of well homogenized medium in culture tubes and these tubes were autoclaved at 15 psi for 15 min. These tubes were kept in slanting position until they got solidified. The media plates, broths and slants were stored at 4 °C until used.

3.2.5. Preparation of McFarland standard

0.5 ml of 0.048 M BaCl₂ (1.17% w/v BaCl₂.2H₂O) was added to 99.5 M H₂SO₄ (1% w/v) with constant stirring. O.D. of the solution was recorded, which came in the range of .08-0.1 at 625 nm. The standard was distributed into the screw cap tube of the same size and the volume as those growing in the broth culture. The tube was tightly sealed to prevent loss by evaporation. The standard was stored in Amber coloured bottle to prevent it from light at room temperature. The standard was vigorously vortexed to homogenise prior to use (NCCLS, 1997). For standardizing the microbial culture, the turbidity was compared to the McFarland standard (O.D. = 0.5). This was done to ensure that a uniform number of bacteria were always used.
3.2.6. Isolation of pure cultures from urine samples

All specimens received from hospitals may or may not contain urinary tract pathogens. Therefore, they were cultured to test whether the urine sample contained the infectious UTI pathogens. So, the bacterial count was done using plate count method. The serial dilutions of 12-24 h old urine samples were done upto $10^{-6}$ and that dilution was spread on the nutrient agar plate. The plates were incubated overnight and the colony count was performed next day. The samples found positive (microbial count $= or > 10^{5} \text{CFU/ml of urine};$ Dash et al., 2008) were further tested and those found negative were discarded. For enumeration of colony forming units (CFUs), the following formula was applied:

$$\text{CFU per ml} = \text{Volume of sample} \times \text{No. of colonies on plate} \times \text{Dilution factor}$$

The standard protocol for isolation of microorganisms included blending in sterile normal saline (0.85% sodium chloride) and suitable serial dilutions were made of the sample before plating them onto suitable culture media (nutrient agar medium, eosine methylene blue agar, Mac-Conkey agar, mannitol salt agar). The compositions of these media are given in section 3.1.2.1. Pure cultures were made by quadrant streaking method and spread plate method. The plates were then incubated at 37 °C for 24 h.

3.2.7. Identification of UTI isolates

The cultures were identified on the basis of colony morphology, colour of pigmentation, variations in the colony size, microscopic characters, biochemical tests and other standard characters using standard reference books (Collins et al., 1995; MacCarty et al., 2000).
3.2.7.1. Colony morphology

Colony morphology, shape, size, contour, colour, etc. were visualized with the help of hand lens and the characters were recorded.

3.2.7.2. Microscopic characterization

Bacterial colonies were first subjected to Gram’s staining and examined under binocular Stereoscopic (Carl Zeiss) light microscope. For microscopic examination, suitable mounts were prepared using simple staining and Gram staining techniques. The slides were examined under 10X ×100X oil immersion lens and the characters were recorded (Starr et al., 1981).

(i) Simple staining: One loopful of the bacterial colony was transferred in one drop of physiological saline (0.85% sodium chloride) on a clean glass slide. A thin smear was made in approximately 1 cm area followed by air drying and heat fixing. It was stained with methylene blue solution for 1-2 min and then the slide was rinsed with tap water and observed under binocular Stereoscopic (Carl Zeiss, Germany) light microscope. The purpose of simple staining was to determine cell shape, size and arrangement of bacterial cells.

(ii) Gram’s staining: One loopful of the bacterial colony was transferred in one drop of physiological saline (0.85% sodium chloride) on a clean microscopic plain glass slide. A thin smear was made in approximately 1 cm area followed by air drying and heat fixing. It was stained with Gram’s staining reagents (crystal violet solution for 60 sec, Gram’s iodine solution for 60 sec, destainer solution for 10-15 sec. (acetone: alcohol = 25 mL : 75 mL) and counterstained with safranin solution for 60 sec.
(iii) **Motility test:** One drop of the broth culture of isolated strain was placed in the centre of the cover glass and the cavity slide was placed carefully over the cover glass in such a way that the centre of the cavity should face the drop of the broth avoiding air bubbles. The slide was immediately inverted and examined under the 40X objective of binocular Stereoscopic (Carl Zeiss, Germany) light microscope and motility was observed. Relative displacement of microbial cells to each other and debris were considered as motility.

### 3.2.8. Biochemical characterization

The isolated cultures were further identified by following biochemical tests.

#### 3.2.8.1. Catalase test

It is based on the principle that during aerobic respiration, microorganisms produce hydrogen peroxide that is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide into water and oxygen. The reaction is:

$$\text{catalase}$$

$$\text{H}_2\text{O}_2 \quad \rightarrow \quad \text{H}_2\text{O} + \text{O}_2$$

The test was carried out by transferring a colony on microscopic plain glass slide. One drop of 3% hydrogen peroxide (30% commercial solution was diluted to 1:10) was placed and the culture was observed for the production of bubbles for catalase positive isolates.

#### 3.2.8.2. Cytochrome oxidase test

One drop of freshly prepared oxidase reagent (1% tetra methyl-p-phenylene diamine dihydrochloride) was placed on small piece of filter paper. The test culture was placed and rubbed over the area of the
reagent. Change of colour of the reagent by the test organism from grey to blue is indicative of positive isolates.

The cytochromes are iron containing haemproteins that act as the last link in the chain of aerobic respiration by transferring electrons to oxygen. The test is performed with the dye, as a substitute for oxygen. In the reduced state the dye is colorless and in the presence of cytochrome oxidase and oxygen, it is oxidized, forming blue colour.

\[
\text{Oxidase} \\
\text{Tetra methyl p-phenylene diaminedihydrochloride} \rightarrow \text{indophenol oxide} \\
\text{(blue/blackish purple)}
\]

### 3.2.8.3. Sugar fermentation test

Approximately 5 ml of carbohydrate fermentation broth (section 3.1.2.1) was poured into each test tube and a Durham tube was placed in inverted position in each tube to observe for the production of gas. The tubes were autoclaved at 15 psi for 15 min. After sterilization, the tubes were inoculated with the test isolate and incubated at 37 °C for 24 - 48 h. The tubes were examined for the production of acid and gas in Durham tube. Appearance of yellow colour and the gas bubble in the tube was indicative of acid and gas production, respectively, whereas no change in colour (red) was indicative of negative result.

### 3.2.8.4. IMVIC test

(i) **Indole production test**: The test organism was cultured in a medium, which contains tryptophan. Indole production was detected by Kovac’s reagent, which contains p-dimethylaminobenzaldehyde that reacts
with indole to produce a red coloured compound. Approximately 5 ml tryptone broth (section 3.1.2.1) was poured into each test tube and autoclaved at 15 psi for 15 min. The broth was inoculated with the test organism and incubated at 37 °C for 48 h. 0.5 mL of Kovac’s reagent was added in each tube. Appearance of red coloured ring at the junction of the medium was indicative of positive test.

(ii) Methyl red test: Bacteria ferment glucose to produce a sufficient acidity that gives red colour in the presence of the indicator methyl red. An isolated colony of the test organism was inoculated in 5mL sterile glucose phosphate broth (section 3.1.2.1). The tubes were incubated overnight at 37 °C. Five drops of methyl red solution were added in each tube. An appearance of bright red colour was indicative of positive test while the appearance of yellow colour denotes negative test.

(iii) Voges-Proskauer’s test: The test is based on the ability of certain organisms to produce acetoin when cultured in glucose phosphate broth. Under alkaline conditions and exposure to the air, the acetoin produced from the fermentation of the glucose is converted to diacetyl, which forms a pink coloured compound with α-napthol. Approximately 5 ml of sterile glucose phosphate broth was inoculated with the test organism and the tubes were incubated at 37 °C for 48 h. Twelve drops of Voges Proskauer reagent I (α-napthol) and 2-3 drops of VP reagent II (40% KOH) were added in each test tube. The tubes were then shaken with caps off to expose to oxygen and were allowed to stand for 15-30 min. Development of pink red colour is indicative of positive test while no pink red colour is indicative of negative test.

(iv) Citrate utilization test: The test organism is cultured in a medium containing sodium citrate, ammonium salt and the indicator bromothymol blue. Growth in the medium is shown by a change in colour
of the indicator from light green to blue due to the alkaline reaction following citrate utilization. Slants of the sterile Simmons’s citrate agar (section 3.1.2.1) medium were inoculated with the test organism by streaking. The slants were incubated at 37 °C for 48 h. Change of colour from green to blue is indicative of citrate positive test while no change in colour was indicative of negative test.

**3.2.9. Maintenance of cultures**

Pure cultures of bacteria were maintained on sterile nutrient agar medium at 4 °C for shorter duration of 1 to 2 months. For longer storage, the cultures were stored in skimmed milk at -20 °C in small sterile vials of 2 ml.

**3.2.10 Antibiotic susceptibility testing**

Discs procured from Hi Media were used for sensitivity tests. These are sterile filter paper discs. Each disc is impregnated with the specified amount of antibiotic and dried at low temperature under vacuum.

**Principle:** Antimicrobial susceptibility testing with discs is simple and rapid method and provides a reproducible means of testing bacterial sensitivity to various antibiotic and chemotherapeutic agents. The test is based on the fact that for a given antibiotic, the size of the zone of inhibition is inversely related to the MIC (determined by the dilution method) of the strain being tested when the test condition are held constant (Reeves *et al.*, 1978).

**3.2.10.1 Procedure**

Antimicrobial susceptibility testing was performed on all 100 UTI isolates against 8 antibiotics by following the CLSL disc diffusion method. Sterile Mueller Hinton agar was poured into plates kept on level surface. The
depth of the medium was approximately 4 mm. After solidification of the medium, plates were dried for 30 minutes in an incubator (37 °C) to remove excess moisture from surface. 4-5 similar colonies from a pure culture were selected and transferred into tube containing nutrient broth. Broth culture was incubated at 37 °C for 24 h to obtain turbidity equivalent of 0.5 McFarland standard. Sterile cotton swab was dipped into the culture inoculums and was further used to spread the agar surface of the plate. One disc at a time was taken with the help of flamed forcep and was carefully placed on the surface of the medium. The plates were kept in the incubator at 37 °C for 24 h. At the end of incubation, the diameters of the zones of inhibition were measured in mm using a ruler and compared to the zone diameter interpretive standards (Performance standards for antimicrobial susceptibility testing: 2006). The standard zone of inhibition for the antibiotics used against the isolated bacteria in present study is presented in table 3.1.

3.2.10.2. NCCLS definitions for susceptible, intermediate and resistant

(i) Susceptible or sensitive indicates that the standard dose of antibiotic should be appropriate for treating the patient infected with the strain tested. If the organism is susceptible to a specific antibiotic, there will be no growth around the disc containing the antibiotic. Thus, a “zone of inhibition” can be observed and measured to determine the susceptibility to an antibiotic for that particular organism.

(ii) Intermediate can also be referred to as moderately resistant or moderately susceptible. This indicates that the strain may be inhibited by larger doses of the agent.

(iii) Resistant indicates that an infection caused by the isolate tested is unlikely to respond to treatment with that antimicrobial agent. An
organism that is classified as “resistant” to an antimicrobial agent can cause an infection that fails to respond to therapy with the recommended dose of the antimicrobial agent (“treatment failure”) (Table 3.1).

**Table 3.1.** Standard zone of inhibition (mm) of various antibiotics against bacteria in present study according to National Committee for Clinical Standards (Bauer et al., 1966; CLSL, Jan., 2011).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of antibiotic</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>1</td>
<td>Cefixime</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Netilmicin</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Ciprofloxacin</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Ceftazidime</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Norfloxacin</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>Amikacin</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>Nitrofurantoin</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>Ofloxacin</td>
<td>12</td>
</tr>
</tbody>
</table>

**3.2.11. Collection and processing of herbal samples**

A total of 3 herbal samples and their combination (Table 3.2 and Fig 3.1) were selected and used for the experimental study to determine their antimicrobial activity against UTI isolates. These herbals were collected from the Brahmavarchas Research Centre and Shantikunj, Haridwar.
Table 3.2. Ethno botanical and phytochemical data of medicinal plants selected for antimicrobial activity in present study.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Botanical name</th>
<th>Family</th>
<th>Common name</th>
<th>Parts used</th>
<th>Known phytoconstituents of parts used</th>
<th>Traditional uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Emblica officinalis</em></td>
<td>Euphorbiaceae</td>
<td>Amla</td>
<td>Fruit</td>
<td>Vitamin C, Phosphatides, Tannins, Chebulinic acids (Chopra et al., 1992; Harbone and Baxter, 1995)</td>
<td>Acrid, cooling, refrigerant diuretic, used in diarrhea, dysentery, anaemia, jaundice and cough</td>
</tr>
<tr>
<td>2.</td>
<td><em>Terminalia chebula</em></td>
<td>Combretaceae</td>
<td>Harad</td>
<td>Fruit</td>
<td>Chebulinic acid, tannic acid 20-40%, Anthroquinone, chebulagic acid, corlagin (Harborne and Baxter, 1995)</td>
<td>Laxative, ulcers, used in carious teeth, piles</td>
</tr>
<tr>
<td>3.</td>
<td><em>Terminalia bellerica</em></td>
<td>Combretaceae</td>
<td>Bahera</td>
<td>Fruit</td>
<td>17% tannins, triterpenoid (Chopra et al., 1992)</td>
<td>Antipyretic, leprosy, diarrhea</td>
</tr>
</tbody>
</table>

The fruits were washed with clean water and allowed to air dry. This was done to reduce the microbial load of the plant material during handling and transportation.

The fruits are dried in a hot air oven at 45 °C till constant weight is achieved. The dried fruits were grounded in milling machine (Inalsa, Mixer grinder) to obtain the fine dry powder. The powder was weighed using single pan electronic weighing balance (Ohaus model). Equal amount of the three herbal powders were mixed to form triphala.
Fig. 3.1. Fruits of selected herbal plants. **A**- Ripe fruits of *Emblica officinalis*; **B**- dried fruits of *Emblica officinalis*; **C**- ripe fruits of *Terminalia bellerica*; **D**- dried fruits of *Terminalia bellerica*; **E**- ripe fruits of *Terminalia chebula*; **F**- dried fruits of *Terminalia chebula*.
3.2.11.1. Preparation of herbal extracts

The solvents used for extraction were distilled water and ethanol. The herbal extracts were prepared at the rate of 1g/5mL of the solvent in a 250 mL Erlenmeyer flasks. The flasks were closed with cotton plug and aluminum foil.

The herbal powder was soaked in the desired solvent for 48 h at room temperature with intermittent shaking. The mixture was centrifuged at 3500 × g for 20 min and finally filtered through Whatmann filter paper No.1. (Azoro, 2000). The pellet was discarded and the supernatant was collected and concentrated under reduced pressure in a rotary vacuum evaporator (Buchi Type) until semisolid substance was obtained. This was dried inside the crucible under a controlled temperature (45 °C) to obtain solid powder (Jonathan and Fasidi, 2003). The process of extraction was repeated until it was reduced to half of its original weight.

The powder was weighed and reconstituted in 500 mg/mL dimethyl sulfoxide (DMSO) and stored in the refrigerator at 4 °C for testing antimicrobial sensitivity. Before their antimicrobial susceptibility testing, the extract was exposed to UV rays for 24 h and checked for sterility by streaking on NAM.

3.2.11.2. Screening of herbal extracts for antibacterial activity

The antibacterial activity of herbal extracts in different solvents was determined by agar well diffusion method (Okeke et al., 2001). In this method, pure isolate of each bacterium was subcultured in nutrient broth at 37 °C for 24 h. Exactly 0.2 mL of overnight cultures of each organism was dispensed into 20 mL of sterile nutrient broth and incubated for 3-5 h to standardize the culture at 10⁶ CFU/mL (Collins et al., 1995)
The standardized inoculum (10^6 CFU/mL) of each test bacterium was spread with the help of sterile spreader on to a sterile Muller-Hinton agar (MHA) plate (Hi Media) to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer of diameter 6.0 mm was used to bore wells in the agar plates. Subsequently, 50-µL volume of the test extract prepared in different solvents was poured in a well into MHA cultures. Sterile DMSO served as a negative control. For each test species and for each test substance, three independent replicates were used. The plates were allowed to stand for 1 h or more for diffusion to take place and then incubated at 37 °C for 24 h. The zone of inhibition was recorded to the nearest size in mm (Norrel and Messely, 1997). Only extracts and solvent exhibiting apparent and maximum zone of inhibition were chosen for further studies.

3.2.11.3. Determination of MIC of screened herbal extracts

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that completely inhibited the growth (ignoring faint haze or a single colony) for 24 h (Thongson et al., 2004). The MIC for the crude extract was determined only for the 50% ethanol extracts of the test substance(s) by test method agar-well diffusion techniques.

In agar-well diffusion technique, a two-fold serial dilution of the test extracts was prepared by first reconstituting it in DMSO, then diluting it in sterile DMSO only, to achieve a decreasing concentration range of 100 mg/mL to 6.25 mg/mL. 50-µL volume of each dilution was added aseptically into Mueller Hinton agar plates that were already seeded with the standardized inoculums (10^6 CFU/mL) of the test bacterial cells. Sterile DMSO without herbal extract served as negative control. All the experiments were set in triplicate. The test plates were incubated at 37 °C
for 24 h. The lowest concentration of each extract showing a clear zone of inhibition was considered as the MIC.