Materials and Methods
4. MATERIALS AND METHODS

The present work entitled on "Molecular Identification of Methicillin-Resistant *Staphylococcus aureus* through the amplification of *mecA* gene and Anti-MRSA activity of *Acorus calamus* (L.), *Tridax procumbens* (L.) & *Pisum sativum* (L.)" have done in the Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram.

4.1. Collection of Clinical Samples

A total of 126 clinical samples (Wound swab, Pus and Sputum) were collected using sterile cotton swabs and sterile containers (from Hi-Media, Mumbai) from different age groups of individuals including in-patients and out-patients from the private and Government hospitals in Namakkal District, Tamil Nadu, India. The collected samples were immediately transferred to the laboratory and processed.

4.2. Identification of *Staphylococcus aureus* (James and Natalie, 1998)

The collected samples were plated on different media, namely Nutrient agar medium, Mannitol salt agar medium, Blood agar medium, MacConkey agar medium, DNAse Agar medium, MeReSa agar medium and HiCrome MeReSa agar medium and incubated at 37°C for 12 - 24 hours. The incubated agar media plates were studied for Morphological characteristics, Staining reaction, Biochemical characteristics, Confirmation of Methicillin Resistance, Antibacterial activity by using standard antibiotics and Plant drugs.

4.2.1. Nutrient Agar Medium

The samples were inoculated in Nutrient agar medium and incubated for 18-24 hours at 37°C, and observed the results.
Media Composition/Liter

Peptone - 5.0g
NaCl - 5.0g
Yeast Extract - 2.0g
Beef Extract - 1.0g
Agar - 15g
pH - $7.4 \pm 0.2$ at 25°C

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15 lbs pressure at 121°C for 15 minutes. Pour into sterile Petri dishes)

4.2.2. Mannitol Salt Agar Medium

Mannitol Salt Agar (MSA) is a selective and differential medium. The high concentration of salt (7.5%) selects for members of the genus *Staphylococcus*, since they can tolerate high saline levels. The collected samples were plated on Mannitol salt agar medium and incubated for 24 hours at 37°C. After the incubation, the results were noted.

Media Composition/Liter

NaCl - 75.0g
D-Mannitol - 10.0g
Pancreatic Digest of Caesin - 5.0g
Peptic Digest of Animal Tissue - 5.0g
Beef Extract - 1.0g
Phenol Red - 0.025g
Agar - 15.0g
pH - $7.4 \pm 0.2$ at 25°C

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and
Autoclave it at 15lbs pressure at 121°C for 15 minutes. Pour into sterile Petri dishes.

4.2.3. MacConkey Agar Medium

A colony from nutrient agar medium was inoculated with MacConkey agar medium and incubated for 18-24 hours at 37°C. After the incubation, the results could be observed.

Media Composition/Liter

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>20.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0</td>
</tr>
<tr>
<td>Bile Salts</td>
<td>5.0</td>
</tr>
<tr>
<td>Neutral Red</td>
<td>0.007</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.4± 0.2</td>
</tr>
</tbody>
</table>

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15lbs pressure at 121°C for 15 minutes. Pour into sterile Petri dishes)

4.2.4. Blood Agar Medium

The suspected colony of *Staphylococcus aureus* from Mannitol Salt Agar medium was streaked on blood agar medium and incubated at 37°C for 24 - 48 hours to observe the haemolytic colonies.

Media Composition/Liter

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef Extract</td>
<td>10.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>
Sheep Blood - 50.0ml (Defibrinated)

pH - 7.3± 0.2 at 25°C

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15 lbs pressure at 121°C for 15 minutes. Cool to 45°C-50°C. Aseptically add 50.0mL of sterile, Defibrinated sheep blood. Mix thoroughly and pour into sterile Petri dishes)

4.2.5. DNAse Agar Medium

A colony from nutrient agar medium was streaked on the middle portion of Deoxyribonuclease agar medium as a straight line and incubated for 24 hours at 37°C. After the incubation, the results should be observed by adding few drops of 1 mol/l% hydrochloric acid on the straight colony. The positive results should be the presence of clearing zone around the colony within 5 minutes.

Media Composition/Liter

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptose</td>
<td>20.0g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0g</td>
</tr>
<tr>
<td>Deoxyribonucleic Acid</td>
<td>2.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0g</td>
</tr>
<tr>
<td>pH</td>
<td>7.3± 0.2 at 25°C</td>
</tr>
</tbody>
</table>

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15 lbs pressure at 121°C for 15 minutes. Pour into sterile Petri dishes)

4.2.6. Gram Staining

The isolated organism was subjected to Gram staining. The Gram-positive bacteria appear as violet in colour and Gram-negative bacteria appear as pink in colour.
Staining Composition

**Crystal Violet**

Solution A

- Crystal Violet: 2.0g (90% dye content)
- Ethyl Alcohol: 20.0ml (95%)

Solution B

- Ammonium Oxalate: 0.8g
- Distilled Water: 80.0ml

(Note: Mix Solutions A and B)

**Gram's Iodine**

- Iodine: 1.0g
- Potassium Iodide: 2.0g
- Distilled Water: 300.0ml

**Ethyl Alcohol (95%)**

- Ethyl Alcohol (100%): 95.0ml
- Distilled Water: 5.0ml

**Safranin**

- Safranin O: 0.25ml
- Ethyl Alcohol (95%): 10.0ml
- Distilled Water: 100.0ml

4.2.7. Catalase Test

The isolated organism was inoculated in nutrient agar slant by means of Zig - Zag Streak. All the tubes were incubated at 37°C for 24 hours. Four drops of 3% hydrogen peroxide is allowed to flow over the entire surface of the slant. The results were noted for the presence or absence of bubble formation.

**Composition**

- Hydrogen Peroxide: 3% (H₂O₂)
4.2.8. Coagulase Test

0.5 ml of diluted citrated plasma in a small tube was incubated with heavy saline suspension of the organisms and was incubated at 37°C for 1-4 hours. It was examined for every 15 minutes for the formation of the coagulum compared with control and the results were observed.

4.2.9. Carbohydrate Fermentation Test

The carbohydrate fermentation tests were done based upon the acid and gas production (fermentation) by the organism in the provide medium. The provided sugar medium was Glucose, Lactose, Maltose, Sucrose and Mannitol.

The medium was composed of peptone water and 0.5% of the respective sugar. Durham's tubes were placed in the tubes containing sugar solution and were inoculated with the specimen.

The results were interpreted based on the production of acid and gas in the media. Production of acid was noted by the change of colour in the medium from blue to yellow and gas production by uplift of the Durham's tube.

Composition

<table>
<thead>
<tr>
<th>Composition</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10.0g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0g</td>
</tr>
<tr>
<td>Respective Sugar (each)</td>
<td>5.0g</td>
</tr>
<tr>
<td>Bromothymol Blue</td>
<td>0.03g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.1</td>
</tr>
</tbody>
</table>

(Carbohydrates are sterilized through filtration)

4.2.10. Indole Test

The colony from the Nutrient agar slant was inoculated into peptone
broth and incubated at 37°C for over night. To observe the results, 0.5 ml of Kovac's reagent was added to the side wall of the test tube slowly. The formation of red colour ring shown that the positive result.

**Composition**

<table>
<thead>
<tr>
<th>Peptone Water</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

Kovac's Indole Reagent

- Paradimethylamino benzaldehyde: 2.0g
- Iso amyl alcohol: 30ml
- Concentrated Hcl: 10ml

**4.2.11. Methyl Red Test**

The colony from the nutrient agar slant was inoculated into glucose phosphate broth and incubated at 37°C for over night, and this reaction was done to test the production mixed acid during fermentation. To observe results, add 0.04% methyl red (0.5ml), appearance of red colour was positive result.

**Composition**

<table>
<thead>
<tr>
<th>Methyl Red Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl Red</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Distilled Water</td>
</tr>
</tbody>
</table>

**4.2.12. Voges-Proskauer Test**

The colony from the nutrient agar slant was inoculated into glucose phosphate broth and incubated at 37°C for over night. To observe results, 0.6ml of 5% α-napthol and 0.2ml of 40% potassium hydroxide were added,
appearance of pinkish red colour was positive result.

Composition

MR-VP Broth (or) Glucose Phosphate Broth
Peptone - 0.5g
Glucose - 0.5g
Dipotassium Hydrogen Phosphate - 0.5g
Distilled Water - 100ml
pH - 7.4 - 7.6

α-Napthol
α-Napthol - 5.0g
Ethyl Alcohol - 100ml

Potassium Hydroxide
Potassium Hydroxide - 40.0g
Distilled Water - 100ml

4.2.13. Citrate Utilization Test

Slant of Simmon’s Citrate agar medium was inoculated with the isolated organism and incubated at 37°C for 24 hours and change of colour from green to blue indicates the positive result.

Composition

Simmon’s Citrate Agar Medium
Magnesium Sulphate - 0.2g
Ammonium Dihydrogen Phosphate - 1.0g
Dipotassium Phosphate - 1.0g
Sodium Citrate - 2.0g
Sodium Chloride - 5.0g
Bromothymol Blue - 0.08g
Agar - 15.0g
Distilled Water - 1000ml
pH - 6.8 - 7.0
(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15 lbs pressure at 121°C for 15 minutes. Pour into sterile test tubes and make into slants)

4.2.14. Triple Sugar Iron (TSI) Agar Test

A composite medium widely used for the triple sugar iron agar test which indicates if a bacterium ferments glucose only (or) lactose and sucrose also with (or) without gas formation besides showing the production of H₂S. The medium was distributed in tubes with a butt and slant. After inoculation, if the slant remains red, it found to be alkaline in nature. The bacteria oxidize amino acids giving amines which are alkaline in nature gives pink colour.

Glucose – 1%; Lactose – 10%; Sucrose – 10%

Acid was produced in-the fermentation of sugars and in the case the butt become yellow colour bubbles indicates the gas production, the production of gas was due to the fermentation of sugars, they produce acid to give yellow colour. In some cases, the blackening of the medium shows formation of H₂S in the TSI.

The triple sugar iron agar medium contains ferric ammonium citrate and sodium thiosulfate. Sodium thiosulfate was the source of sulphur, this produces H₂S. Ferric ammonium citrate reacts with H₂S which results in the formation of ferric sulphide, this result in blackening.

Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>10.0g</td>
</tr>
<tr>
<td>Caesin enzyme hydrolysate</td>
<td>10.0g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0g</td>
</tr>
</tbody>
</table>
Sucrose - 10.0g
Glucose - 1.0g
Ferrous Sulphate - 0.2g
Sodium chloride - 5.0g
Sodium thiosulphate - 0.3g
Phenol Red - 0.024g
Agar - 15.0g
pH - 7.4

(Add components to distilled water and bring volume to 1.0L and Mix thoroughly. Gently heat and bring to boiling. Distribute into flasks and Autoclave it at 15 lbs pressure at 121°C for 15 minutes. Pour into sterile test tubes and make into slants)

4.2.15. Nitrate Reduction Test

Any medium that supports the growth of the organism and contains a 0.1% concentration of KNO₃ is suitable for performing this test. The medium was inoculated with a Loopful of the test organism and incubated at 35°C for 18-24 hours. At the end of incubation, 1ml each of reagent-A (α-naphthalamine) and reagent-B (sulphanilic acid) were added to the test medium. The results were observed for the development of red colour within 30 seconds.

Composition

<table>
<thead>
<tr>
<th>Nitrate Broth</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0g</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>3.0g</td>
</tr>
<tr>
<td>Potassium Nitrate</td>
<td>1.0g</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.8 - 7.2</td>
</tr>
</tbody>
</table>

Sulphanilic Acid

<table>
<thead>
<tr>
<th>Sulphanilic Acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphanilic Acid</td>
<td>0.16g</td>
</tr>
</tbody>
</table>
Glacial Acetic Acid - 5.7ml
Distilled Water - 14.3ml

α-Napthalamine Reagent
Alpha Napthalamine - 0.1g
Glacial Acetic Acid - 5.7ml
Distilled Water - 14.3ml

4.3. Identification of Methicillin - Resistant *Staphylococcus aureus* (MRSA)

A Colony from Nutrient agar medium was streaked on the MeReSa Agar medium and HiCrome MeReSa Agar (Special mediums to isolate Methicillin-Resistant *Staphylococcus aureus* (MRSA) - HiMedia, Mumbai, India) medium and incubated for 18-24 hours at 37°C. After the incubation, the results should be observed for Greenish-blue and bluish-green color colonies on the agar medium respectively.

**Composition**

MeReSa Agar Medium

Casein Enzymic hydrolysate - 10.0ml
Beef Extract - 5.0g
Glycine - 10.0g
Sodium Pyruvate - 10.0g
Lithium Chloride - 5.0g
Mannitol - 10.0g
NaCl - 10.0g
Indicator Mix - 0.13g
Agar - 20.0g
Distilled Water - 500ml
pH - 7.1 ± 0.2 (at 25°C)

(Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 45 - 50°C. Aseptically add sterile
rehydrated contents of 1 vial of MeReSa selective supplement (FD229-HiMedia). Mix well and pour into sterile Petri plates.

**HiCrome MeReSa Agar Medium**

- Casein Enzymic Hydrolysate: 13.0g
- Yeast Extract: 2.5g
- Beef Extract: 2.5g
- Sodium Pyruvate: 5.0g
- NaCl: 40.0g
- Chromogenic Mixture: 5.3g
- Agar: 15.0g
- Distilled water: 500ml
- pH: 7.0 ± 0.2 (at 25°C)

(Suspend 41.65 grams in 500ml distilled water. Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 45 – 50°C. Aseptically add sterile rehydrated contents of 1 vial of MeReSa selective supplement (FD229-HiMedia). Mix well and pour into sterile Petri plates)

### 4.4. Isolation of Genomic DNA from MTCC-96, Methicillin Sensitive and Resistant Staphylococcal Clinical Isolates

The chromosomal DNA were isolated from Methicillin sensitive, resistant Staphylococcal isolates and MTCC-96 (*Staphylococcus aureus* – Sensitive Reference Strain) through Modified DNA extraction for rapid PCR detection of *Staphylococcus aureus* and MRS protocol (Aziz Japoni, *et al.*, 2004) and confirmed through Agarose gel electrophoresis.

#### 4.4.1. Materials

- PBS (Phosphate Buffer Saline)
  - Sodium chloride: 8.0g
  - Potassium chloride: 0.2g
  - Disodium hydrogen orthophosphate: 2.3g
Potassium dihydrogen phosphate - 0.2g
Distilled Water - 1000 ml
pH - 7.2 to 7.4

- SDS (Sodium Dodecyl Sulphate)
  100ml - 10%

- TE Buffer (1000ml)
  Tris HCL - 10mM
  EDTA - 1mM
  pH - 7.6

- Proteinase K
  Lysozyme - 10mg/ml
  Rnase - 40μl

- Phenol: Chloroform (1:1)
- Isopropanol
- Ethanol (70%)

4.4.2 Genomic DNA Isolation Method (Aziz Japoni, et al., 2004)

- The isolated Methicillin sensitive and resistance Staphylococcal isolates and MTCC-96 (Staphylococcus aureus - Sensitive Reference Strain) were grown in Luria Bertani Broth (LB) for 18 – 24 hours at 37°C.

- The grown Staphylococcus sensitive and resistant isolates and MTCC-96 Staphylococcal strain were centrifuged at 8000 rpm for 10 minutes at 4°C and the supernatant was discarded.

- The pellet was suspended with 5ml Phosphate Buffer Saline, shacked for 30 minutes and then centrifuged at 8000rpm for 10 minutes and the supernatant was discarded.
Again the Pellet was suspended with 0.5ml of Phosphate Buffer Saline and the washed bacterial cells were transferred into 1.5ml microfuge tubes and 10µl of Lysozyme was added and incubated at 55°C for 30 minutes.

After the incubation, the tubes were added with 200µl of 10% Sodium Dodecyl Sulphate (SDS) and 5µl of (10mg/ml) Proteinase K and the tubes were incubated in water bath at 33°C for 60 minutes.

The tubes containing suspension was extracted twice with Phenol: Chloroform (1:1) and once with Chloroform (equal volume). Each step of the extraction was performed using a sterile microfuge tube and centrifuged at 12,000 rpm for 5 minutes.

The Staphylococcal DNA containing fraction was recovered from supernatant and precipitated with Isopropanol for overnight at -20°C freezer.

The precipitated DNA was washed with 70% ethanol to separate the protein contamination and then resuspended with 40µl of TE buffer.

4.5. Agarose Gel Electrophoresis of Isolated DNA (Sambrook, et al., 1989)

The visualization of isolated DNA was confirmed by Agarose gel electrophoresis.

4.5.1. Materials

- Electrophoretic Apparatus
- Agarose – 1%
- Gel Loading Buffer (TAE Buffer 50X)
  - Tris – 242 gm
  - Glacial Acetic Acid – 57.1 ml
  - 0.5M EDTA (pH-8) – 100 ml
(Make up the volume to one liter using sterile Distilled water)

- Gel Tracking Dye
  - 1X TAE Buffer - 10ml
  - Glycerol - 3ml
  - Bromothymol Blue - 25 ml

- Ethidium Bromide
  - Stock - 100mg/ml
  - Working Concentration - 0.2μl/ml

**Visualization of DNA from MRSA, MSSA and MTCC-96**

- 1% of Agarose was weighed and mixed with 40ml of 1X TAE Buffer, the slurry was heated in a boiling water bath until the Agarose dissolves the solutions becomes clear.

- The solution was allowed to cool for 50°C and Ethidium Bromide was added (0.2μl/ml)

- The open sides of the open platforms were sealed with cello tapes and placed it on a leveled table.

- The gel solution was poured to the platform and placed the well forming comb in the slot.

- After the solidification, the comb and the sealing tapes were removed and the gel platform was placed on the Electrophoresis tank. The running buffer was poured to cover the gel to a depth of about 1mm.

- The isolated DNA samples were prepared in 1X gel loading buffer. Then the samples were loaded about 20μl into wells using gel loading tips.

- Electrophoresis apparatus was connected with power pack and carried out at 5 V/cm at constant voltage of 50V for 2 hours.

- After running, the apparatus was disconnected and examined the isolated DNA bands through UV-Transilluminator.
4.6. Amplification of mecA gene through Polymerase Chain Reaction
(Sambrook, et al., 1989)

The isolated DNA were amplified through the thermal cycler (TECHNE, UK) using suitable forward and reverse Oligonucleotide primer from Agile Life Science Technologies, Mumbai, India, for the amplification of mecA gene and identified through Agarose gel electrophoresis with suitable marker.

4.5.1. Primers for mecA Gene

<table>
<thead>
<tr>
<th>Primer</th>
<th>Seq</th>
<th>G+C content range</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA (F)</td>
<td>5_GGGATCATAGCGTCATTATTC_3</td>
<td>42.90%</td>
</tr>
<tr>
<td>mecA (R)</td>
<td>5_AACGATTGTGACAGATAGCC_3</td>
<td>45%</td>
</tr>
</tbody>
</table>

4.5.2. PCR Reaction Mixture

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile Deionized water</td>
<td>33 µl</td>
</tr>
<tr>
<td>5X Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer I</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer II</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>Total Volume</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
Each PCR reaction mix was prepared in the separate PCR tubes with each DNA templates from experimental samples.

4.5.3. PCR Cyclic Parameters

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>94°C</td>
<td>2 Min</td>
<td>Initial Denaturation</td>
</tr>
<tr>
<td>2.</td>
<td>94°C</td>
<td>30 Sec</td>
<td>Cycle Denaturation</td>
</tr>
<tr>
<td>3.</td>
<td>49°C</td>
<td>1 Min</td>
<td>Annealing</td>
</tr>
<tr>
<td>4.</td>
<td>72°C</td>
<td>1 minute</td>
<td>Extension</td>
</tr>
</tbody>
</table>

Repeat the step 2,3 and 4 for 35 Cycles

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>72°C</td>
<td>7 minutes</td>
<td>Final Extension</td>
</tr>
<tr>
<td>6.</td>
<td>4°C</td>
<td>For Long Time</td>
<td>Storing</td>
</tr>
</tbody>
</table>

Visualization of Amplified DNA

- 1.2% of Agarose was weighed and mixed with 40ml of 1X TAE Buffer, the slurry was heated in a boiling water bath until the Agarose dissolves the solutions becomes clear.

- The solution was allowed to cool for 50°C and Ethidium Bromide was added (0.2µl/ml)

- The open sides of the open platforms were sealed with cellotapes and placed it on a leveled table.

- The gel solution was poured to the platform and placed the well forming comb in the slot.

- After the solidification, the comb and the sealing tapes were removed and the gel platform was placed on the Electrophoresis tank. The running buffer was poured to cover the gel to a depth of about 1mm.

- The isolated DNA samples were prepared in 1X gel loading buffer.
15-20µl of each sample was loaded on the wells together with lanes containing 100bp ladder DNA marker and the Electrophoresis apparatus was connected with power pack and carried out at 5 V/cm at constant voltage of 50V for 2 hours.

After running, the apparatus was disconnected and examined the isolated DNA bands through UV-Transilluminator.

4.6. Antibacterial activity of Antibiotics (Harold, 1998)

Antibacterial activity using the selected antibiotics was determined by Agar Disc Diffusion Method using Muller-Hinton agar medium. The inoculation of all agar plates with MTCC-96, Methicillin-Sensitive and Resistant Staphylococcal isolates as follows

a) Sterile cotton swab was dipped in to a well mixed Nutrient broth; (containing bacterial cultures incubated in shaker for eight hours at 37°C) excess inoculum was removed by pressing the swab against the inner wall of the culture tube.

b) The entire agar plates were swabbed horizontally, vertically and outer edge of the plate to ensured heavy growth over the entire surface. All the culture plates were allowed to dry for about five minutes.

After the inoculation, the different antibiotic discs were placed on the medium using sterile forceps. Then the plates were incubated at 37°C for 18-24 hours. After the incubation, clear zone of inhibition around the disc was measured and the results were noted.

Following concentrations of antibiotic discs were used for present study

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>30 µg/disc</td>
</tr>
<tr>
<td>Cephotaxime</td>
<td>30 µg/disc</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Concentration</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30 μg/disc</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 μg/disc</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 μg/disc</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Methicillin</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Neomycin</td>
<td>30 μg/disc</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>5 μg/disc</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10 Units/disc</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>30 μg/disc</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10 μg/disc</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 μg/disc</td>
</tr>
</tbody>
</table>

4.7. Collection of Plant Sources and Preparation of Plant Extracts

- The Plant specimens were included as the rhizomes of *Acorus calamus*, Whole shrub of *Tridax procumbens* and the seeds of *Pisum sativum* and these were collected from the surroundings of Namakkal District, Tamil Nadu, India. The collected plant species were identified and confirmed (Gamble, 1962).

- All the plant sources were washed with running tap water and then finally washed with distilled water to remove the dirt.

- The plant parts were dried under shade for seven days then they were kept in hot air oven for four to six hours at 50°C to remove excess moisture.

- The dried plants were separately crushed softly to make powder form using mixer grinder.

- Those crushed powder was loaded in to the clean dry soxhlet
apparatus tightly using soft metal rod.

➢ Then the apparatus was run to get plant extract with different solvents. The solvents were selected by increasing polarity and those were included as Acetone, Chloroform, Isopropanol and Ethanol.

➢ And the apparatus with solvents was run until to get clear solvents in the side tube. The time was measured to get clear solvent in the side tube. Now all the solvents had different extracts with active ingredients.

➢ Then the extracts with different solvents were evaporated using rotary vacuum evaporator to remove the solvents.

4.7.1. Characteristics Features of Plant Extracts

The appearance and amount of the each extracts were observed and measured using electronic balance. A loop full of each different plant extracts were streaked on sterile nutrient agar plates to check the presence of any microbes.

4.7.2. Preparation of Discs using Plant Extracts

➢ The observing capacity of 10mm sterile disc (HIMEDIA) was selected ranges from 6μl to 45 μl.

➢ For the preparation of stock solution, 1mg of each different crude extract was dissolved in 1.6 ml of DMSO.

➢ From these stock, 8μl, 16μl, 32μl and 40μl was added on the sterile discs to get 50μg, 100μg, 150μg, 200μg, and 250μg respectively of plant extracts. Then these prepared discs were used for Anti-MRSA activity in individual plant extracts.

➢ From these stock, 0.5ml of each different plant extracts was mixed with same quantity in other stocks of different plant extracts. Then the
disks were prepared same as above. Then these prepared discs were used for Anti-MRSA activity in combination of two plants.

4.8. Antibacterial activity of Plant extracts

Antibacterial activity using plant extracts were determined by agar disc diffusion method using Muller-Hinton agar medium. The inoculation of all agar plates with MRSA as follows

a) Sterile cotton swab was dipped in to a well mixed Nutrient broth, (containing bacterial cultures incubated in shaker for eight hours at 37° C) excess inoculums was removed by pressing the swab against the inner wall of the culture tube.

b) The entire agar plates were swabbed horizontally, vertically and outer edge of the plate to ensured heavy growth over the entire surface. All the culture plates were allowed to dry for about five minutes.

c) Then the prepared discs with compounds were placed on upper layer of the inoculated plates using sterile forceps. The equal distance was maintained between each disc. The disc was gently pressed down aseptically with the help of alcohol flamed forceps. (Disc should not press into the agar).

All the plates were incubated for 24 hours at 37°C. Then the presence of zone of inhibition could be measured on the plates.

4.8.1. Residual Effects of Solvents and DMSO

To find the residual effects of solvents and DMSO, 10ml of each solvents and DMSO were evaporated separately until to get 1ml of residue. Then the residues were added as small drops using micropipette on the sterile disc by keeping the disks on the hot plate at 50°C to remove excess residues. Then the discs were kept in Muller-Hinton Agar medium plates swapped with over night broth culture of MRSA. One empty sterile disk was kept to check weather it possess any inhibitory activity.
4.9. Determination of MIC and MBC for Individual and Mixed Plant Extracts (Soetan, et al., 2006)

- For the determinations of Minimal Inhibitory Concentrations (MIC) of plant extracts, 8 sterile capped tubes of 7x1.3 cm were arranged in a row. (12 rows contain 4 different individual solvent extracts of 3 plants).

- 1000mg of different plant extract was diluted in 10ml of 0.1% peptone water to obtain a working solution of 100mg/ml. Serial dilution of the working solution was made. 5µl of 1/100th overnight broth culture of each organism was delivered into each tube and incubated for 18 – 24hrs at 37°C.

- The different solvent extracts of three plants were mixed in 1:1 ratio and above steps were repeated for combined effect of two plants. The sensitivity at the highest dilution of each row was read of were opalescence rather than turbidity was noticed.

- Determination of MBC, 100µl of culture was transferred from each tube and sub-cultured in fresh Nutrient agar medium. After incubation at 37°C for 24hrs, the least concentration shown no visible growth and observed their MBC.

4.10. Preliminary Phytochemical Screening (Harborne, 1973)

Various extracts collected from the plants were tested for identification of its active chemical constituents

4.10.1. Test for Alkaloids

To the small quantity of the test solution, a few drops of Dilute Hcl was added and filtered. The filtered may be tested carefully with various alkaloidal reagents such as,
Mayor's reagent - Creamy Precipitate
Dragondroff's reagent - Orange Brown Precipitate
Hager's reagent - Yellow Precipitate
Wager's reagent - Reddish Brown Precipitate

4.10.2. Test for Proteins and Aminoacids

Small quantities of test solution was dissolved in little quantity of water and treated with following reagents.

Millon's Reagent

Appearance of red colour shows the presence of proteins and free amino acids.

Ninhydrin Reagent

Appearance of purple colour shows the presence of proteins and free amino acids.

Biuret Test

Equal volume of 5% of sodium hydroxide and 1% solution of Copper Sulphate were added. Appearance of pink colour shows the presence of proteins and free Aminoacids.

4.10.3. Test for Anthraquinone glycosides

Borntrager's test

The small quantity of the test solution was boiled with diluted sulfuric acid and filtered. Ether was added to the filtrate and shaken well. The separated organic layer was added with ammonia. The layer became pink to red. It indicates the presence of Anthraquinone glycosides.
4.10.4. Test for Flavonoids

To the small quantity of the test solution, add aqueous sodium hydroxide solution, appearance of blue to violet colour indicates the presence of anthocyanins, yellow colour indicates the presence of flavones, yellow to orange indicates the presence of flavonoids.

To the small quantity of the test solution, add concentrated sulphuric acid appearance of yellowish orange colour indicates the presence of anthocyanins, yellow to orange colour indicates the presence of the flavones and orange to crimson colour indicates the presence of the flavonoids.

Shimoda's test

The small quantity of the test solution is dissolved in alcohol, to the piece of magnesium followed by concentrated Hcl drop was added and heated. Appearance of magenta colour shows the presence of Flavonoids.

4.10.5. Test for Tannin and Phenolic Compounds

The small quantity of the test solution was taken separately in water and tested for the presence of Phenolic compounds and tannins with following reagents such as

- Diluted Ferric Chloride solution (5%) — Violet Colour
- 1% Solution Gelatin containing 10% Nacl — White Precipitate
- 10% Lead Acetate solution — White Precipitate

4.10.6. Test for Carbohydrates

The small quantity of test solution was dissolved in 5ml of distilled water and filtered. The filtrate was subjected for carbohydrates.
Molisch’ test

The filtrate was treated with 2 – 3 drops of 1% alcoholic alpha napthol, and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of purple colour ring indicates the presence of carbohydrates.

Fehling’s test

The filtrate was treated with 1ml of Fehling’s solution and heated. Orange precipitate was obtained shows the presence of carbohydrates.

4.10.7. Test for Saponins

The small quantity of test solution was diluted with 20 ml of distilled water and it is agitated on a graduated cylinder for 15 minutes. The presence of saponins was indicated by the formation of 1cm layer of foam.

4.10.8. Test for Phytosterol

Liebermann Burchard test

The small quantity of test solution was dissolved in a few drops of dry acetic acid; 3 ml of acetic anhydrate was added followed by few drops of concentrated sulfuric acid. Appearance of bluish green colour indicates the presence of Phytosterol.