Materials and Methods
4. MATERIALS AND METHODS

4.1 COLLECTION OF SAMPLES

126 wound isolates were isolated from in and around the hospitals in Erode (I & II), Salem (III) and Coimbatore (IV) District hospitals in Tamilnadu, India during March 2004 to August 2004. No duplicate isolates from a single patient were included. The distribution and sources of all isolates from each hospital were given in Table I and Plate1.

4.2 SCREENING OF *STAPHYLOCOCCUS AUREUS*

All the isolates were inoculated into Mannitol Salt Agar (MSA) a selective media for the isolation of *Staphylococcus aureus*. The MSA plates were incubated at 37°C for 24 hrs. After incubation period the phenotypic characterization of the organisms were studied.

4.3 BIOCHEMICAL TESTS (Barrow and Feltham, 1993)

Isolates were identified according to routine cultural properties. Gram staining, motility, biochemical tests (Catalase, Oxidase, Indole, Methyl Red, Voges Proskauer, Citrate Utilization, Urease and Triple Sugar Iron Agar) and sugar utilization.

4.4 COAGULASE AGGLUTINATION TEST (Baird, 1996)

Slide coagulase tests of all 126 isolates were performed by emulsifying few pure colonies of Staphylococci from Mannitol salt agar on undiluted rabbit plasma. The tube coagulase tests were performed by diluting the plasma in freshly prepared normal saline
Three to four pure colonies were emulsified in 1 ml of diluted plasma and the tube were incubated at 37° C. Results were observed at 1 to 4 hrs.

4.5 LIPASE ACTIVITY TESTS

- Lipovitellin Salt Mannitol agar (Merlino et al., 1996)
- Baird Parker agar (Capita et al., 2001)
- Vogel Johnson agar base (Klapes and Vesley, 1986)

The different strains of *Staphylococcus aureus* were inoculated on above mentioned media. The plates were kept for incubation at 37°C for 24 hrs. After incubation the results were observed.

4.6 ANTIBIOTIC SENSITIVITY TEST (Bauer et al., 1966)

The isolates were tested for their sensitivity against the antibiotics Amikacin (30mcg), Cefazolin (30mcg), Ceftazidime (30mcg), Ceftizoxime (30mcg), Cephoxitin (30mcg), Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Clindamycin (2mcg), Co-Trimoxazole (Trimethoprim/Sulphamethoxazole) (1.25/23.75mcg), Erythromycin (15mcg), Gentamicin (10mcg), Kanamycin (30mcg), Methicillin (5mcg), Nalidixic acid (30mcg), Netillin (30mcg), Norfloxacin (10mcg), Ofloxacin (5mcg), Oxacillin (1mcg), Penicillin G (10 units), Rifampicin (5mcg), Tetracycline (30mcg), Vancomycin (30mcg) and Moxalactam (30mcg) by the disc diffusion method.

- The cultures were enriched in sterile nutrient broth for 6-8 hrs at 37°C.
- The cultures were aseptically swabbed on the surface of sterile Mueller Hinton Agar (MHA) plates using sterile cotton swabs.
The antibiotic discs were aseptically placed over the seeded MHA plates sufficiently separated from each other to avoid overlapping of the inhibition zones.

The plates were incubated at 37°C for 24 hrs and the diameter of the inhibition zone was measured in mm.

All the media used in this experiment were obtained from Hi-media Laboratories Ltd., Mumbai, India.

4.7 MINIMUM INHIBITION CONCENTRATION (MIC) – MICRODILUTION
(Kumarasamy et al., 2002)

Microtitre plate wells from each column in row 1 were marked and 100μl of antibiotic stock (Ciprofloxacin 1mg/ml, Oxacillin 0.2mg/ml, Vancomycin 6mg/ml) and blank without antibiotic solution was added.

50μl of saline was added to rows 2-11. Two fold serial dilutions were performed by transferring 50μl of solution from row 1 to row 2, using a multichannel pipette. This was repeated down the row 2 to row 12.

40μl of double strength nutrient broth and 10μl of different bacterial solution was added to all the wells in separate column, so the final concentrations of the inoculum in all the wells were 5x10^5 cfu/ml.

To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37°C overnight.

The bacterial growth was determined after addition of 40μl of P-iodonitrotetrazolium violet (0.2mg/ml).

The minimum inhibitory concentrations (MIC) of the isolates were taken as the lowest concentration of the antibiotic of which the bacterial tested did not show visible growth.
4.8 MINIMUM INHIBITION CONCENTRATION (MIC) – HICOMB TEST
(Tenover et al., 1996)

➢ The MIC of Methicillin were determined by using the HiComb test system (Hi-
media, Mumbai).
➢ The HiComb test was performed with isolates which were mecA positive and
selected Multi Drug Resistant – Methicillin Resistant *Staphylococcus aureus*
(MDR-MRSA).
➢ A HiComb test strip was placed onto a Mueller Hinton Agar plate supplemented
with 2% NaCl.
➢ After incubation at 37°C for 24 hrs, the MIC was read at the point of intersection
between the zone edge and the Hicomb test strip.

4.9 SCREENING OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

The following selective media with appropriate antibiotic supplement was added
and inoculated with the various strains of *Staphylococcus aureus* for the isolation of the
MRSA.

- CHROM agar with oxacillin 4mg/l (Merlino et al., 2000)
- Oxacillin Resistant Screening Agar Base (ORSAB) with Oxacillin 2mg/l (Blanc et
  al., 2003)
- Baird Parker Agar (BPA) with Ciprofloxacin 8mg/l (Brown and Walpole, 2001)
- Mannitol Salt Agar (MSA-4 Ox) with Oxacillin 4mg/l (Lally et al., 1985)
- Blood Agar (BA) with Oxacillin 2mg/l (Brown and Walpole, 2001)
- Mueller Hinton Agar (Ox- MH Agar) with 4% NaCl, Oxacillin 6mg/l (Griethuysen
  et al., 1999)

The isolates of *Staphylococcus aureus* were inoculated on the above mentioned
selective media plates. The plates were kept for incubation at 37°C in an inverted position
for 24 to 48 hrs. After incubation, the plates were observed for growth.
4.10 ISOLATION OF CHROMOSOMAL DNA (Sambrook and Russel, 1989)

- 2 grams of wet packed bacterial cells were suspended in 25ml of saline EDTA solution in a centrifuge tube.
- 1ml of Lysozyme was added to the tube and incubated the mixture at 37°C for 45 min.
- After incubation, to bring complete lyses of cells, 2ml of 25% SDS was added and the mixture was heated at 60°C in a water bath for 10min with stirring.
- The mixture was allowed to cool at room temperature.
- Then 9 ml of sodium per chlorate was added in the mixture were mixed well then to that equal volume of chloroform iso-amylalcohol (equal to total volume of extract mixture) was added.
- The emulsion was centrifuged at 10,000 rpm for 10 min.
- After centrifugation the upper aqueous phase was carefully transferred to a fresh glass beaker and then DNA was precipitated by adding double the volume of 95% ethanol over the aqueous phase.
- Then the two layers were mixed gently with a circular motion of the stirring rod spooled all the fibers of DNA on to rod.
- The precipitated DNA was air dried and suspended in 20μl of TE buffer.
- TAE buffer (20%) was used for both electrophoresis running and agarose gel preparation.
- During the electrophoresis, the samples were loaded with equal volume of staining buffer.
- A marker λ/EcoR1 (Bangalore Genei Pvt. Ltd., Bangalore) was included parallelly on the one lane to visualize the size of the chromosomal DNA.
- Ethidium bromide stained chromosomal DNA bands were visualized using UV reflected light.
4.11 DETECTION OF meca GENE BY POLYMERASE CHAIN REACTION (PCR) (Merlino et al., 2002)

PCR was performed by using Taq polymerase and oligonucleotide primer were meca F primer 1282 (5’ - AAA ATC GAT GGT AAA GGT TGG C-3’) and meca A R primer 1793 (5’ - AGT TCT GCA GTA CCG GAT TTG C-3’) purchased from MWG Biotech Pvt. Ltd., Bangalore, India.

The polymerase chain reaction was cycled in master cycler 5333 Eppendorf version 2.30.33-09 using a reaction mixture of 20µl consisting of Taq polymerase buffer 2 µl, dNTP mixture 2 µl, 1 µl of each primer, DNA sample 1 µl, Taq polymerase enzyme 0.2µl and distilled water 12.8 µl.

The extracted DNA was amplified for 30 cycles, consisting of

60 seconds at 94°C for Denaturation
30 seconds at 50°C for Annealing
90 seconds at 72°C for Primer extension

The PCR products were electrophoresed on 2% agarose gel prepared in TBE buffer, gel was stained with ethidium bromide and photographed.

4.12 ISOLATION OF PLASMID USING ALKALINE LYSIS METHOD (Niels, 1994)

- 5 ml overnight culture of S. aureus strain was grown in broth containing the required antibiotic marker.
- 1.5 ml of the culture was spin in a microfuge tube at 10,000 rpm for 2 min at 4°C.
- The pellet was suspended with 100 µl solution-I (50 mM Glucose, 20 mM Tris-Cl, pH-8 and 10 mM EDTA).
The suspension was vortexed for 2 min.
> 200 μl of freshly prepared solution-II (0.2N NaOH, 1% SDS) was added, with gentle mix by inverting the tubes.
> The tubes were kept on ice for 5 min.
> Then 150 μl of ice cold solution-III (5M Potassium Acetate, pH 4.6 and Glacial Acetic Acid) was added and mix gently by inverting the tube and kept on the ice for 5 min.
> After incubation the tubes were centrifuged for 10,000 rpm for 5 min at 4°C.
> The supernatant was transferred to a fresh tube then mixed with two volume of 95% ethanol by inverting the tubes.
> The tubes were centrifuged for 10,000 rpm for 5 min at 4°C.
> Pellet was collected and air dried under vacuum.
> It was resuspended with 10 μl of TE buffer for further process.
> 0.7% agarose gel was used for plasmid visualization.

4.13 COLLECTION OF PLANT SEED

Totally Nine seeds: *Elettaria cardamomum, Mangifera indica, Moringa oleifera, Phoenix dactylifera Tamarindus indica, Annona squamosa, Artocarpus heterophyllus, Cucurbita maxima* and *Momordica charantia* (Plate 10) were collected during June 2004 to December 2004 from different part in and around Erode District, Tamilnadu, India. The few seed samples were obtained from local markets. All the seeds were identified and confirmed by the Botanist Dr. R. Murugan, Lecture, PG and Research Department of Botany & Microbiology, Government Arts and Science College for Men, Krishinagiri, Tamilnadu, India. The herbarium was deposited (Herbarium No. SASCMD 01-09) at PG and Research Department of Microbiology, Sengunthar Arts and Science College, Tiruchengode, Tamilnadu, India.
4.14 PREPARATION OF SEED POWDER

The seeds were washed with distilled water, then surface sterilized with 10% sodium hypochlorite solution rinsed with sterile distilled water and air dried at room temperature. The samples were ground into a fine powder.

4.15 DISC DIFFUSION METHOD (Kim et al., 2004)

- Sterile paper discs (6mm, Hi-media, Mumbai) were loaded with 50 μl (30mg/ml) of the extracts dissolved in 10% dimethyl sulfoxide (DMSO) and were left to dry for 12 hrs at 37°C in a sterile room.
- Bacterial suspensions were diluted to match the 0.5 McFarland standard scale (approximately 1.5x10⁸ cfu/ml) and they were further diluted to obtain a final inoculum.
- After Mueller – Hinton agar was poured into petri dishes to give a solid plate and inoculated with 100 μl of suspension containing 1x10⁸ CFU/ml of bacteria, the discs treated with extracts were applied on the medium.
- Paper discs treated with DMSO were used as negative control.
- The plates were then incubated at 37°C for 24 hrs.
- Inhibition zone diameters around each of the disc were measured and recorded at the end of the incubation time.

46 SOLVENT EXTRACTION METHOD (Chessbrough, 2000)

- 200 ml of a solvent was mixed with 50 g each of the powdered plant seed material.
- The mixture were kept for 24 hrs in tightly sealed vessels at room temperature, protected from sunlight and mixed several times with a sterile glass rod.
- This mixture was filtered through whatman no.1 filter paper and the residue, adjusted to the required concentration (50ml of solvent for the residue of 50 g of powdered seed material) with the extraction fluid for further extraction and it was
repeated thrice and a clear colorless supernatant extraction liquid was finally obtained.

- The extracted liquid was subjected to rotary evaporation in order to remove the solvents.
- The semisolid extract produced was kept in a freezer at -80°C overnight and then subjected to freeze drying for 24 hrs at -60°C in 200 ml vacuum.
- Then the extract was stored in an airtight container at 4°C in refrigerator for further use.

4.17 SOXHLET EXTRACTION METHOD (LaMora et al., 1999)

- 200 ml of a solvent was mixed with 50 g each of the powdered plant seed material.
- The mixture was kept to soxhlet apparatus for 4 hrs for 60°C.
- The extracted liquid was subjected to rotary evaporation in order to remove the solvents.
- The semisolid extract produced was kept in a freezer at -80°C overnight and then subjected to freeze drying for 24 hrs at -60°C in 200 ml vacuum.
- Then the extract was stored in an airtight container at 4°C in refrigerator for further use.

4.18 CHECKER BOARD ASSAY (Kumarasamy et al., 2002)

- Microtitre plates wells from each column in row 1 were marked and 100 μl of extract (The test extract was dissolved in 10% DMSO to obtain 30mg/ml stock solution) was added.
- 50 μl of sterile normal saline was added to rows 2-11. Two fold serial dilutions were performed by transferring 50 μl of solution from row 1 to row 2, using a multi-channel pipette. It was repeated down the row 2 to row12.
40 μl of double strength nutrient broth and 10μl of bacterial solution were added to all the wells, so the final concentration of inoculum in all the wells was 5x10^5 cfu/ml.

To prevent dehydration, the plates were covered with a plastic cover and then incubated at 37°C overnight.

The bacterial growth was determined after addition of 40 μl of P-iodonitrotetrazolium violet (0.2 mg/ml).

The minimum inhibitory concentrations (MIC) of isolates were taken as the lowest concentration of the antibiotic of which the bacterial tested did not show visible growths.

4.19 TIME KILLING ASSAY (Zampini et al., 2005)

A standardized suspension of Gram positive Methicillin Resistant *Staphylococcus aureus* (5x10^5 cfu/mL) was added into Mueller Hinton broth containing the seed extracts (30mg/ml).

The mixtures were then incubated at 37°C for 24 hrs at 200 rpm.

A seed extract free control was included.

Viable counts were performed at 0, 2, 4, 6 and 24 hrs after the addition of seed extract, following serial dilution in phosphate-buffered saline pH 7.3.

Bacteria were counted after 24 - 48 hrs incubation at 37°C.

The crude extracts were considered to be bactericidal at the lowest concentration which reduced the original inoculum by ≥3 log_{10} cfu/mL (99.9% reduction in bacterial population) in 4 hrs.

4.20 PREPARATION OF S-9 MIXTURE (Kaur et al., 2002)

Mammalian liver (S-9) extract was purchased from Medox Biotech India Pvt Ltd, Chennai, India. The S-9 mixture was prepared with 10% S-9, 4mM NADPH, 4mM NADH, 5mM glucose-6- phosphate, 8mM MgCl₂, 33mM KCl and 100M Sodium phosphate buffer (pH 7.4).
4.21 MUTATION ASSAY (Araki et al., 1984)

- The preincubation mutation method was carried out in both the presence and absence of the S-9 mixture in order to detect indirect and direct mutagenesis, respectively.
- The two standard test strains, *Salmonella typhimurium* TA98 and TA100 (MTCC 1251 & 1252) were used.
- The seed crude extracts were reconstituted in 10% dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and 30 mg/ml.
- The mixture of seed extract (0.1ml) with 0.5 ml of S-9 mixture or 0.1M phosphate buffer (pH 7.4) for without S-9 and 0.1 ml of the test strain of bacteria was incubated at 30°C for 30 min.
- This was then rapidly mixed with 2 ml of molten top agar containing amino acid histidine (0.1mol), biotin (0.5M) and poured rapidly into a 30 ml Vogel-Bonner minimal agar plate and incubated at 37°C for 48 hrs.
- Positive control were included (aminoflourene (AF2) and 2 amino anthracene (2-AA) in each experiment by using DMSO alone instead of the extract.
- After incubation, revertant (mutant) colonies were counted and compared to the number of colonies formed in unexposed cultures.
- An extract was considered mutagenic when the mean number of revertants was at least double that found in the solvent control culture.
- The negative control/blank was water and the solvent control was 10% DMSO.

4.22 BIOAUTOGRAPHY (Rohalison et al., 1991)

- Precoated silica gel plates were used (Silicagel 60 F 254, Merck) for thin layer chromatography.
- 5 μl of crude seed extracts were loaded on the TLC plates. They were developed in duplicates in selected solvent systems for each of the seed extracts.
It was allowed to air dried for overnight, each of the plates were placed in a humid chamber and overlaid with 10 ml molten nutrient agar seeded with 0.2 ml of each of Methicillin resistant *Staphylococcus aureus* (MRSA).

Adequate humidity was maintained by placing moist cotton buds at the corners of the plates. The overlaid plates were left for 30 min after which they were incubated at 37°C for 24 hrs and sprayed with a 1 mg/ml triphenyl tetrazolium chloride solution (TTC).

Plates were incubated at 35°C for 3 hrs in the dark for color development.

Growth inhibition areas were compared with the $R_f$ of the related spotted on the TLC plate revealed with different reagents.

### 4.23 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

- HPTLC analysis was carried out using automatic TLC sampler CAMAG linomat IV equipped with CAMAG TLC evaluator software.
- 5 μl of seed extracts (1mg/ml) were spotted on preparative TLC plate (Silicagel 60 F$_{254}$ plates, Merck), which were developed with appropriate solvents.
- Slit dimension 5mmx5mm scanning speed 4.0mm/s, mobile phase hexane: ethyl acetate: acetic acid (7:3:1) was employed.
- Densitometric scanning was performed on CAMAG TLC scanner-3 in the absorbance reflection mode at 254 nm.

### 4.24 COLUMN CHROMATOGRAPHY

- Air dried and pulverized mango seed kernel (300g) was soaked in ethanol for overnight and repeated this processor for thrice.
- The solvent was removed by rotary evaporator and the extract was dried.
- The ethanol extract was to yield 18 g of brown residue.
- 15 g of crude extract was used for column (40mm x 1000mm) chromatography. Silica gel (mesh 60-120) was used as column packing material.
• The column was eluted using a series of solvent systems: 100 % dichloromethane (DCM) and followed Isopropyl alcohol (IPA)/ ethyl acetate (EAC) (2:8, 4:6, 6:4, 8:2, 10:0) finally 100% ethanol. Each series solvent was added 500 ml and fraction was collected 55 ml up to 61th fraction.
• Collected fractions were applied on TLC plate using methanol: ethyl acetate: water: acetic acid (3: 6: 0.5: 0.5) and visualized using UV 254 and 366 nm.
• Similar Rf value fractions were mixed and dried using rotary evaporator.
• Then evaluated the antimicrobial activity of isolated compounds.

4.25 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Fractionation of the *Mangifera indica* extract was performed by HPLC to identify active compounds. A isocratic HPLC (Shimadzu HPLC Class VP series) with one LC-10 AT VP, pump (Shimadzu), variable wavelength UV-Visible Detector SPD-10A VP, (Shimadzu), and reverse phase gemini 5u C18 110A, Phenomenex column (250 X 4.60mm) was used. The mobile phase components acetonitrile:water:aceticacid (60:40:0.5) were filtered through 0.2 micron membrane filter before use, and pumped from the solvent reservoir at a flow rate of 0.5ml/min, which yielded column backup, pressure of 180-20 0 kgf/cm2. The column was maintained at 27°C. 20µl of *Mangifera indica* seed fractions were injected using syringe (Bonaduz schweiz, Hamilton).

4.26 NUCLEAR MAGNETIC RESONANCE (NMR)

$^1$H and $^{13}$C NMR experiments were performed on a Bruker advance DPX300 spectrometer operating at 300 and 75 MHz respectively. Chemical shift values ($\delta$) were reported in parts per million (ppm) relative to appropriate internal solvent standard and coupling constants are given in hertz.
4.27 MASS SPECTROSCOPY (MS)

Molecular mass was determined by a gas chromatography/mass spectrometry-SHIMADZU GC-17-A QP 5000, under the following conditions. Column DP-5, oven temperature 70°C, initial holding time 1 min, rate of increase 10°C/min, final temperature 250°C, final holding time 5 min, column temperature 70°C, injector temperature 250°C, interface temperature 300°C, MS-40-4000 and detector 1.2kv, library search of spectra for elucidation of molecular structures of compounds detected by MS. WILLY 139 library was used for this purpose.

4.28 INFRARED SPECTRUM (IR)

Infrared Spectrum (IR) spectra were recorded on a NICOLET 360 FT-IR spectrophotometer.