# Antimicrobial Activity of a Natural Product from *Callistemon rigidus* and its Mechanism of Action against *Staphylococcus aureus*

***A thesis submitted in fulfillment***

***of the requirement for the award of the degree of***

**DOCTOR OF PHILOSOPHY**

**in**

**BIOTECHNOLOGY**

**by**

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# CERTIFICATE

Certified that the thesis "Antimicrobial activity of a natural product from *Callistemon rigidus* and its mechanism of action against *Staphylococcus aureus*" submitted by Ms. Charu Gomber, in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is a record of candidate's own independent and original research work carried out by herself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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# CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled "Antimicrobial activity of a natural product from *Callistemon rigidus* and its mechanism of action against *Staphylococcus aureus*" in fulfillment of the requirement for the award of the Degree of Doctor of Philosophy at the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period from January 2006 to February 2010, under the supervision of Dr. Sanjai Saxena, Assistant Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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# LIST OF PUBLICATIONS

## Publications Related to Thesis Work

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4. Sanjai Saxena & **Charu Gomber** (2010): Surmounting antimicrobial resistance in the millennium superbug: *Staphylococcus aureus.* ***Central European Journal of Medicine***, 5 (1): 12- 29.
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## Book Chapter

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3. **Charu Gomber** and Sanjai Saxena (2006) Overcoming multidrug resistance *Staphylococcus aureus* by two bioactive fractions from *Callistemon rigidus* R. Br. Presented at the 47th Annual Conference of The Association of Microbiologists of India - Microbiology: The Challenges Ahead, 6- 8th Dec, 2006, Barkatullah University, Bhopal.
4. **Charu Gomber** and Sanjai Saxena (2006) *Callistemon rigidus* as a new plant providing potent phytochemicals in designing new antimicrobial drug and cosmeceutical formulation. Presented in International Conference and Expo on Botanical Products, 25- 27 March, 2006, University of Rajasthan, Jaipur.

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2. Sanjai Saxena, **Charu Gomber** & Shivani Tayal (2007): Anticandidal activity of the phylloplane fungal isolates of the weed *Lantana camara*. ***Proceedings of the National Academy of Sciences***, ***India***, 77 (B), IV, 409- 413.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| +ve HAP | Gram Positive Hospital Acquired Pneumonia |
| 2D- NMR | 2D- Nuclear Magnetic Resonance Spectroscopy |
| 4CL | 4-Coumarate: Coenzyme A Ligase |
| 5-MHC-D | 5- Methoxyhydnocarpin- D |
| AAC | Acetyl Transferase |
| ABC | ATP-Binding Cassette |
| ABF | Alkaloidal Bioactive Fraction |
| ADR | Antimicrobial Drug Resistance |
| AIIMS | All India Institute of Medical Sciences |
| ANT | Adenylate Transferase |
| AST | Antimicrobial Susceptibility Testing |
| AWD | Agar Well Diffusion |
| C4H | Cinnamate-4 –hydroxylase |
| CA-MRSA | Community Acquired MRSA |
| CCAP | Community Associated Pneumonia |
| CDC | Centre for Disease Control |
| CFU | Colony Forming Unit |
| CHS | Chalcone Synthase |
| cIAIs | Complicated Intra-Abdominal Infections |
| CLSI | Clinical and Laboratory Standards Institute |
| CoPS | Coagulase Positive Staphylcocci |
| CoNS | Coagulase Negative Staphylcocci |
| cSSTIs | Complicated Skin and Soft Tissue Infections |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| DDW | Double Distilled Water |
| DMSO | Dimethyl Sulphoxide |
| DNA | Deoxyribose Nucleic Acid |
| dNTP | Deoxynucleotide Triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| EMRSA | Epidemic-MRSA |
| ESI | Electro Spray Ionization |
| ET | Exfoliatin Toxin |
| EtBr | Ethidium Bromide |
| EUCAST | European Committee for Antimicrobial Susceptibility Testing |
| FAME | Fatty Acid Modifying Enzyme |
| GI | Gastro Intestinal |
| GISA | Glycopeptide Intermediate *S. aureus* |
| GMC | Government Medical College |
| GMMO’s | Genetic Modified Microbes |
| HA-MRSA | Hospital Acquired MRSA |
| HCl | Hydrochloric Acid |

| | |
|---|---|
| HDL | High Density Lipids |
| hhC | 8- hydroxydihydrochelerythrine |
| hhS | 8 –hydroxydihydrosanguinarine |
| HIV | Human Immunodeficiency Virus |
| HMR | High Mupirocin Resistance |
| HPLC | High Performance Liquid Chromatography |
| hVISA | hetero Vancomycin Intermediate *S. aureus* |
| IC | Inhibitory Concentration |
| ICUs | Intensive Care Units |
| IMTECH | Institute of Microbial Technology |
| INR | Indian Rupees |
| INT | 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride |
| IV | Intravenous |
| KB | Kirby Bauer |
| KDa | Kilodaltons |
| MARSA | Multi- antibiotic Resistant *Staphylococcus aureus* |
| MATE | Multidrug and Toxic Efflux |
| MBC | Minimum Bactericidal Concentration |
| MDR | Multi Drug Resistance |
| MF | Major Facilitator |
| MH | Muller Hinton |
| MIC | Minimum Inhibitory Concentration |
| MLST | Multilocus Sequence Typing |
| MRSA | Methicillin Resistant *Staphylococcus aureus* |
| MS | Mass Spectrometry |
| MSSA | Methicillin Sensitive *Staphylococcus aureus* |
| MTCC | Microbial Type Culture Collection |
| MTT | 3-(4,5-Dimethyl-2-thiazolyl- 5-diphenyl-2H)-tetrazolium bromide |
| NA | Nutrient Agar |
| NaCl | Sodium Chloride |
| $NaH_2PO_4$ | Sodium Dihydrogen Phosphate |
| NaOH | Sodium Hydroxide |
| NCEs | New Chemical Entities |
| PAL | Phenylalanine Ammonia Lyase |
| PBP | Penicillin Binding Protein |
| PCR | Polymerase Chain Reaction |
| PDA | Potato Dextrose Agar |
| PFGE | Pulsed Field Gel Electrophoresis |
| PTLC | Preparative Thin Layer Chromatography |
| QD | Quinupristin- Dulfopristin |
| QRDR | Quinolone Resistance-Determining Region |
| QSAR | Quantitative Structure Activity Relationship |
| R&D | Research & Development |

| | |
|---|---|
| RMAs | Resistance Modifying Agents |
| RNase | Ribonuclease |
| RND | Resistance-Nodulation-Division |
| RTIs | Respiratory Tract Infections |
| SARs | Structure Activity Relationships |
| SCC | Staphylococcal Cassette Chromosome |
| SDS | Sodium Dodecyl Sulphate |
| SE | Staphylococcal Enterotoxins |
| SEM | Scanning Electron Microscopy |
| SMR | Small Multidrug Resistance |
| SOD | Superoxide Dismutase |
| SSTIs | Skin & Soft Tissue Infections |
| TCIRD | Thapar Centre for Industrial Research & Development |
| TE | Tris EDTA |
| TLC | Thin Layer Chromatography |
| TSST | Toxic Shock Syndrome Toxin |
| TTC | 2, 3, 5-Triphenyl Tetrazolium Chloride |
| UK | United Kingdom |
| USA | United States of America |
| USFDA | United States Food and Drug Administration |
| uSSTIs | Urinary Skin and Soft Tissue Infections |
| UV | Ultra Voilet |
| VAP | Ventilator Associated Pneumonia |
| VRSA | Vancomycin Resistant *S. aureus* |
| WHO | World Health Organization |
| XOD | Xanthine Oxidase |
| XRD | X-ray Diffraction |

# LIST OF SYMBOLS

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| µg | Microgram |
| µg/disk | Microgram per disk |
| µl/min | Microlitre per minute |
| µl/ml | Microlitre per millilitre |
| µM | Micromolar |
| g | Gram |
| g/l | Gram per litre |
| h | Hours |
| Kv | Kilovolt |
| $Lh^{-1}$ | Litre per hour |
| M | Molar |
| mg | Milligram |
| mg/l | Milligrams per litre |
| mg/ml | Milligrams per millilitre |
| min | Minute |
| ml | Millilitre |
| mM | Milli Molar |
| mm | Millimeter |
| ng | Nanogram |
| nm | Nanometer |
| OD | Optical density |
| $R^2$ | Correlation Coefficient |
| rpm | Revolution per minute |
| s | Second |
| U/µl | Units per microlitre |
| U/mg | Units per milligram |
| v | Volt |
| v/v | volume by volume |
| w/v | weight by volume |
| µg/g | Microgram per gram |
| µg/ml | Microgram per millilitre |
| µl | Microlitre |

Dedicated to my Family….

# TABLE OF CONTENTS

- *Executive Summary*.....i
- *List of Figures*.....ii- iv
- *List of Tables*.....v

## 1. INTRODUCTION

1.1 Infectious diseases: Global Health Threat.....01
1.2 Antimicrobial Drug Resistance.....02-03
1.3 *Staphylococcus aureus*: Biology.....03-05
1.4 Evolution of Multidrug Resistant *Staphylococcus aureus*.....05-08
1.5 MRSA: The Indian Scenario..... 08-11
1.6 The need of new anti-MRSA drugs.....11-15
1.7 Microbial Natural Products in Antibacterial Drug Discovery.....16-19
1.8 Plant Based Molecules as anti-MRSA drugs and resistance modifying agents .....20-26

## 2. PRESENT APPROACH

2.1 Present approach.....27
2.2 Renaissance of natural products: drug discovery programs.....27-30
2.3 Hypothesis- selection of *Callistemon rigidus*.....30-31

## 3. REVIEW OF LITERATURE

3.1 Antimicrobial Drug Resistance: A global quandary.....32
3.2 *Staphylococcus aureus*: Pathophysiology.....32-33
3.3 Spectrum of *S.aureus* infections.....33-34
3.4 Virulence determinants of *S. aureus*.....34-36
3.5 Multiple drug resistance in *S. aureus* .....36-39
3.6 Therapeutic power of plants.....39-43
3.7 Anti- staphylococcal phytochemicals.....44
3.8 Antimicrobial agents present in Myrtaceae.....45-47
3.9 General phytochemical procedures.....47-53
3.10 Biological assays.....53-57
3.11 Analytical techniques for the characterization of bioactive compounds.....57-58
3.12 Mode of Action.....58-60

## 4. MATERIALS AND METHODS

4.1 Culture Repository.....61-63
4.2 Antibiogram of test panel: Kirby-Bauer disk assay.....63
4.3 *mec A* characterization of the test isolates.....63-65
4.4 Collection of plant material.....65-66
4.5 Preparation of crude leaf extract.....66
4.6 Evaluation of bioactivity of crude extract.....66-69
4.7 Phytochemical analysis of crude extract.....69-70
4.8 Isolation of crude alkaloidal extract.....70-71
4.9 Modification of procedure- Microwave assisted extraction of the crude Alkaloid Bioactive Fraction.....71

4.10 Evaluation of bioactivity of crude ABF……………………………………………………………………71-72
4.11 Evaluation of bioactivity of microwave extracted ABF…………………………………………………72
4.12 Standardization of TLC fractionation of the Alkaloidal Bioactive Fraction……………………72-75
4.13 Mass extraction of alkaloids- Preparative thin layer chromatography…………………………75-76
4.14 Evaluation of bioactivity of TLC fractionated alkaloids…………………………………………………76
4.15 Mode of action of CSS6 and CSS8: Cellular studies……………………………………………………76-77
4.16 Mode of action of CSS6 and CSS8: Biochemical studies……………………………………………77-80
4.17 Tentative nature of leads……………………………………………………………………………………80

## 5. RESULTS

5.1 Culture Repository…………………………………………………………………………………………81-82
5.2 Antibiogram as per KB disc assay…………………………………………………………………………81-96
5.3 Biochemical and *mec* A/type 2 *ccr* characterization of the test isolates…………………….97-98
5.4 Growth curve of test isolates……………………………………………………………………………..98-100
5.5 AWD Assay of crude methanolic extract…………………………………………………………………100-102
5.6 MIC of crude methanol extract by *in vitro* microbroth dilution assay……………………….102-104
5.7 Phytochemical analysis of the crude extract…………………………………………………………..105
5.8 Mass extraction and fractionation of the crude methanolic extract…………………………105-106
5.9 Microwave assisted extraction of the Alkaloidal Bioactive Fractions (ABF)………………107
5.10 MIC and $IC_{50}$ of ABF by *in vitro* microbroth dilution assay………………………………….....108-109
5.11 TLC fractionation of ABF……………………………………………………………………………………..109
5.12 MIC of individual fractions on selected isolates…………………………………………………..109-112
5.13 MIC and kill kinetics of CSS1, CSS6 and CSS8 against pure selected isolates…………….113-122
5.14 Structural changes based on SEM analysis……………………………………………………………123-125
5.15 Biochemical mode of action of CSS6 and CSS8……………………………………………………..126-135
5.16 Efficacy of CSS6 and CSS8 with commercial antibiotics……………………………………………131
5.17 Tentative nature of leads……………………………………………………………………………………136-139

## 6. DISCUSSION

6.1 Culture Repository for antimicrobial screening………………………………………………………140-141
6.2 Antibiogram by KB Disc assay..........................................................................141-142
6.3 Biochemical characterization of the test panel staphylococci....................................142-144
6.4 Growth curve of studies of staphylococcal isolates..................................................144-145
6.5 Agar Well Diffusion (AWD) Assay of crude methanolic extract...................................145-146
6.6 *In vitro* microbroth dilution assay of crude methanolic extract for MIC determination..........................................................................................................146-147
6.7 Phytochemical analysis of crude methanolic extract.................................................147-148
6.8 Fractionation of the crude methanol extract...............................................................148-150
6.9 Microwave assisted extraction of alkaloid bioactive fraction ......................................150
6.10 MIC and $IC_{50}$ of ABF by in vitro microbroth dilution assay............................................151
6.11 TLC fractionation of ABF...........................................................................................151-153
6.12 MIC of individual fractions on selected isolates...........................................................153
6.13 MIC and kill kinetics of CSS1, CSS6 and CSS8 against extended test panel....................154
6.14 Structural changes in bacteria: Mechanism of antibacterial activity.............................155
6.15 Biochemical mode of action of CSS6 and CSS8...........................................................155-158
6.16 Efficacy of CSS6 and CSS8 with commercial antibiotics................................................159
6.17 Tentative nature of leads- CSS6 and CSS8……………………………………………………………159-161

**7. CONCLUSION**........................................................................................................162-163

**8. BIBLIOGRAPHY**........................................................................................................164-206

**9. APPENDIX**........................................................................................................207-208

# EXECUTIVE SUMMARY

The present work was oriented to evaluate the anti-staphylococcal potential of methanolic extract of leaves of *Callistemon rigidus R.Br.* The test panel comprised of 34 clinical and 4 standard isolates of *S. aureus*. Crude methanolic extract of *C. rigidus* exhibited a MIC in range of 5-40 µg/ml against the test panel isolates by *in vitro* microbroth dilution assay. Bioactive fraction from the methanol extract of leaves was isolated by bioassay guided liquid- liquid fractionation protocol. The bioactive fraction tested positive only for alkaloids phytochemically and hence designated as ABF. The ABF induced 3-log reduction in colony counts in the test panel isolates at 3.125 µg/ml concentration only. The yield of the ABF was improved by 4% by using microwave oven assisted treatment of the pulverized leaves of *C. rigidus.* Further TLC fractionation of the ABF yielded ten alkaloidal fractions designated as CSS1-CSS10.

CSS1-CSS10 were evaluated individually against a limited panel of test isolates to determine their MIC by using *in vitro* microbroth assay based on visual dye reduction method. CSS1, CSS6 and CSS8 exhibited potential to be taken up further for their antimicrobial properties based on their MIC, $MIC_{50}$ and $MIC_{90}$ values. The MIC values of CSS1, CSS6 and CSS8 are 26.69 µg/ml, 16.8 µg/ml and 23.78 µg/ml. Further CSS1, CSS6, and CSS8 were tested against the extended panel of test isolates and gave a MIC of 26.69 µg/ml, 7.93 µg/ml, and 22.45 µg/ml respectively. The time kill kinetics revealed that CSS1 was bacteriostatic while CSS6 and CSS8 were bactericidal as well as bacteriostatic. SEM studies indicated that at MIC values CSS6 and CSS8 induced cell lysis, cell shrinkage and irregular shapes. Expression of enzymes viz lipase, protease and SOD which are the virulence factors in *S. aureus* were found to be inhibited at the MIC and sub-MIC levels of CSS6 and CSS8 thereby causing susceptibility. Hence CSS6 and CSS8 were multi-targeting.

The phytochemical analysis indicated that CSS6 and CSS8 are alkaloidal in nature. The elemental composition of CSS6 and CSS8 were C-62.32%, H-7.34%, N-3.05%, O-27.24% and C-64.22%, H- 7.89%, N-5.54%, O-22.34% respectively. The CSS6 exhibited ESI-MS ions at m/z 803.2 and m/z 413.5 while CSS8 exhibited only m/z of 493.5. As structural elucidation was not contemplated in the present research proposal it is warranted that detailed spectroscopic studies are required for arriving to the structures of CSS6 and CSS8 for their possible exploitation in anti-staphylococcal drug development.

## LIST OF FIGURES


| S.No. | Title | Page No. |
|---|---|---|
| 1.1 | Timeline of antibiotic drug discovery | 1 |
| 1.2 | Global burden of infectious diseases (WHO, 2004) | 2 |
| 1.3 | Broad mechanism of antibacterial resistance | 3 |
| 1.4 | *S. aureus* SEM micrograph | 3 |
| 1.5 | Prevalence of MRSA in India | 9 |
| 1.6 | Chemical structure of systemic anti-staphylococcal agents under development | 14 |
| 1.7 | Trend of new antibacterial drug approvals by USFDA | 13 |
| 1.8 | Current trends in drug discovery | 25 |
| 1.9 | Plant natural products with potential for anti-infective drug development | 26 |
| 3.1 | Virulence determinants of *S. aureus* | 35 |
| 3.2 | First natural products used as drugs | 40 |
| 3.3 | Current drugs developed from terrestrial plants | 42 |
| 4.1 | Microplate template for the growth curve of microorganisms | 62 |
| 4.2 | Petri dish template for AWD assay | 67 |
| 4.3 | Microtitre plate template for *in vitro* microbroth dilution assay for determination of MIC of crude extract | 68 |
| 4.4 | Procedure to isolate Alkaloid Bioactive Fraction from crude methanol extract of leaves of *Callistemon rigidus* | 73 |
| 4.5 | Microwave assisted extraction procedure of Alkaloid Bioactive Fraction from leaves of *Callistemon rigidus* | 74 |
| 5.1 | Distribution of staphylcococcal isolates in the test panel | 81 |
| 5.2a | Antibiogram by Kirby Bauer disk assay | 85 |
| 5.2b | Percentage resistance of pus isolates against the test antibiotics | 86 |
| 5.2c | Percentage resistance of burn isolates against the test antibiotics | 86 |
| 5.2d | Dendrogram and antibiogram of pus isolates against the test antibiotics | 87 |
| 5.2e | Dendrogram and antibiogram of burn isolates against the test antibiotics | 88 |
| 5.2f | Percentage resistance of urine isolates against the test antibiotics | 89 |
| 5.2g | Percentage resistance of vaginal isolates against the test antibiotics | 89 |
| 5.2h | Dendrogram and antibiogram of urine and vaginal isolates against the test antibiotics | 90 |
| 5.2i | Percentage resistance of blood isolates against the test antibiotics | 91 |
| 5.2j | Percentage resistance of wound isolates against the test antibiotics | 91 |
| 5.2k | Percentage resistance of catheter isolates against the test antibiotics | 92 |
| 5.2l | Percentage resistance of standard isolates against the test antibiotics | 92 |
| 5.2m | Dendrogram and antibiogram of blood, wound and catheter isolates against the test antibiotics | 93 |
| 5.2n | Dendrogram and antibiogram of standard isolates against the test antibiotics | 94 |
| 5.2q | Dendrogram and antibiogram of unclassified isolates against the test antibiotics | 95 |
| 5.2o | Percentage resistance of eye and ear isolates against the test antibiotics | 96 |
| 5.2p | Percentage resistances of unclassified isolates against the test antibiotics | 96 |
| 5.3(a) | Screening of staphylococcal isolates for the presence of *mec A* gene. | 97 |

| | | |
|---|---|---|
| 5.3(b) | Screening of staphylococcal isolates for the presence of type 2*ccr* as a marker for community existence | 98 |
| 5.4 | Generation time of the staphylococcal isolates comprising the antibacterial test panel | 100 |
| 5.5 | Maximum inhibition zone in the test panel isolates at a concentration of 66 g of crude methanol extract in the well during *in vitro* agar well diffusion assay. | 101 |
| 5.6 | Agar well diffusion assay of the crude methanol extract against staphylococcal isolates exhibiting the inhibition zone formation at different concentration of crude methanol extract | 101 |
| 5.7 | Dose response relationship between crude methanol extract and inhibition zones of the test microorganisms | 102 |
| 5.8 | *In vitro* microbroth dilution assay of crude methanol extract | 102 |
| 5.9 | Group wise $MIC_{50}$ and $MIC_{90}$ of crude methanol extract by *in vitro* microbroth dilution assay. | 103 |
| 5.10 | Phytochemical test of crude methanol extract for alkaloids and flavonoids | 105 |
| 5.11 | Isolate group wise distribution of $IC_{50}$ concentration of the ABF (a) pus isolates (b) urine and vaginal isolates (c) blood, wound and catheter isolates (d) burn isolates (e) ear, eye and unclassified (f) standard isolates | 110- 111 |
| 5.12.1 | Kill Kinetics of CSS1 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates | 117- 118 |
| 5.12.2 | Kill Kinetics of CSS6 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates | 119- 120 |
| 5.12.3 | Kill Kinetics of CSS8 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates | 121- 122 |
| 5.13 (A) | Scanning Electron Micrograph of *Staphylococcus aureus* | 124 |
| 5.13 (B) | SEM of Sau G10 after exposure to MIC of CSS6 at 12h indicated deformity in the shape of the cells, cell shrinkage and formation of amorphous syncytium | 124 |
| 5.13 (C) | Cell shrinkage, deformity in division , appearance of appendages apart from rupturing of the cells of Sau G10 after exposure to MIC of CSS6 at 20h | 124 |
| 5.13 (D) | Formation of irregular shape, crenations and characteristic dumble structure in Sau G10 due to exposure of MIC of CSS6 at 20h | 124 |
| 5.13 (E&F) | Cell shrinkage and formation of an amorphous syncytium by MIC of CSS 8 and CSS6 respectively at 20 hours exposure to Sau G25. | 124 |
| 5.13 (G&H) | Scanning Electron Micrograph of *Staphylococcus aureus* exhibiting the formation of triad apart from amorphous syncytium formation and shrinked cells of Sau G10 by CSS8 after 12h exposure at MIC | 125 |
| 5.13 (I&J) | Shrinkage of cells and reduction in size is marked with appearance of surface appendages, improper separation and bridge formation between the cells | 125 |
| 5.13 (K&L) | Deformation of cells, formation of bridges causing dumble formation, appearance of surface appendages and irregular septum formation. | 125 |
| 5.14 | Screening of microbial test panel for production of extracellular lipase | 127 |
| 5.15 | Screening of microbial test panel for production of extracellular protease | 127 |

| | | |
|---|---|---|
| 5.16 | Screening of microbial test panel for production of intracellular SOD | 128 |
| 5.17 | Effect of CSS6 on extracellular expression of SOD | 132 |
| 5.18 | Effect of CSS8 on extracellular expression of SOD | 132 |
| 5.19 | Effect of CSS6 on intracellular expression of SOD | 133 |
| 5.20 | Effect of CSS8 on intracellular expression of SOD | 133 |
| 5.21 | Effect of CSS6 on protease expression | 134 |
| 5.22 | Effect of CSS8 on protease expression | 134 |
| 5.23 | Effect of CSS6 on lipase expression | 135 |
| 5.24 | Effect of CSS8 on lipase expression | 135 |
| 5.25 | Dragendroff's test of the compounds CSS1, CSS6 and CSS8. | 136 |
| 5.26 | ESI MS of CSS6 | 137 |
| 5.27 | MS/ MS of m/z 803.8 of CSS6 | 138 |
| 5.28 | MS/MS of m/z 413.5 of CSS6 | 138 |
| 5.29 | ESI MS of CSS8 | 139 |
| 5.30 | MS/ MS fragmentation of m/z 493.5 of CSS8 | 139 |

## LIST OF TABLES


| S.No. | Title | Page No. |
|---|---|---|
| 1.1 | Systemic anti-staphylococcal agents under different phases of development | 15 |
| 1.2 | Microbial secondary metabolites developed into antimicrobials | 18- 19 |
| 3.1 | Anti- staphylococcal plant natural products | 46 |
| 4.1 | Concentrations of the crude methanol extract evaluated by Agar Well Diffusion (AWD) Assay | 67 |
| 4.2 | TLC standardization for fractionation of Alkaloid Bioactive Fraction | 75 |
| 5.1 | Culture collection | 82 |
| 5.2 | Biochemical characterization of the test panel isolates | 99 |
| 5.3 | MIC of crude methanol extract of *Callistemon rigidus* leaves by *in vitro* microbroth dilution assay | 104 |
| 5.4 | Phytochemical test of crude methanol extract | 105 |
| 5.5 | CFU reduction by ABF from methanol extract by *in vitro* microbroth dilution assay | 106 |
| 5.6 | ANOVA (one-way) and Dunnett's multiple comparison tests to assess CFU reduction by ABF at different concentration in isolate groups. | 107 |
| 5.7 | MIC of microwave extracted Alkaloid Bioactive Fraction by *in vitro* microbroth dilution assay | 108 |
| 5.8 | Average MIC and $IC_{50}$ of ABF by *in vitro* microbroth dilution assay | 109 |
| 5.9 | Characteristics and yield of TLC separated fractions of the ABF | 112 |
| 5.10 | Visual MIC determination of ABF fractions by *in vitro* microbroth dilution assay | 112 |
| 5.11 | Comparative efficacies of CSS1, CSS6 and CSS8 based MIC, $MIC_{50}$ and $MIC_{90}$ values on limited test panel | 113 |
| 5.12 | Comparative efficacies of CSS1, CSS6 and CSS8 based MIC, $MIC_{50}$ and $MIC_{90}$ values on extended test panel | 113 |
| 5.13 | Comparative kill kinetics of CSS1, CSS6 and CSS8 against selective clinical and standard staphylococcal isolates | 116 |
| 5.14 | Average expression of SOD, protease and lipase by different groups of test panel staphylococci | 128 |
| 5.15 | Comparison of the standard antibiotics and the leads CSS6 and CSS8 for anti-staphylococcal activity | 131 |
| 5.16 | Phytochemical nature of the bioactive leads | 136 |

# Chapter 1
# INTRODUCTION

# 1.0 INTRODUCTION

## 1.1 Infectious Diseases: Global Health Threat

The discovery of miracle drug penicillin by Sir Alexander Fleming marked the beginning of "Golden Era of Antibiotics" which prevailed for more than 50 years with a belief among clinicians that infectious diseases could be controlled and eventually mastered. However their optimism was thwarted by the emergence of resistance to multiple antibiotics among deadly pathogens like *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. Today the newly emerging and re-emerging infections are responsible for 1,70,000 deaths in America alone every year. More than 13 million deaths annually of which a large proportion comprising of children, adolescents are due to infectious diseases thereby making them second leading cause of mortality after cardiovascular diseases (Spellberg *et al.,* 2004; WHO, 2005).

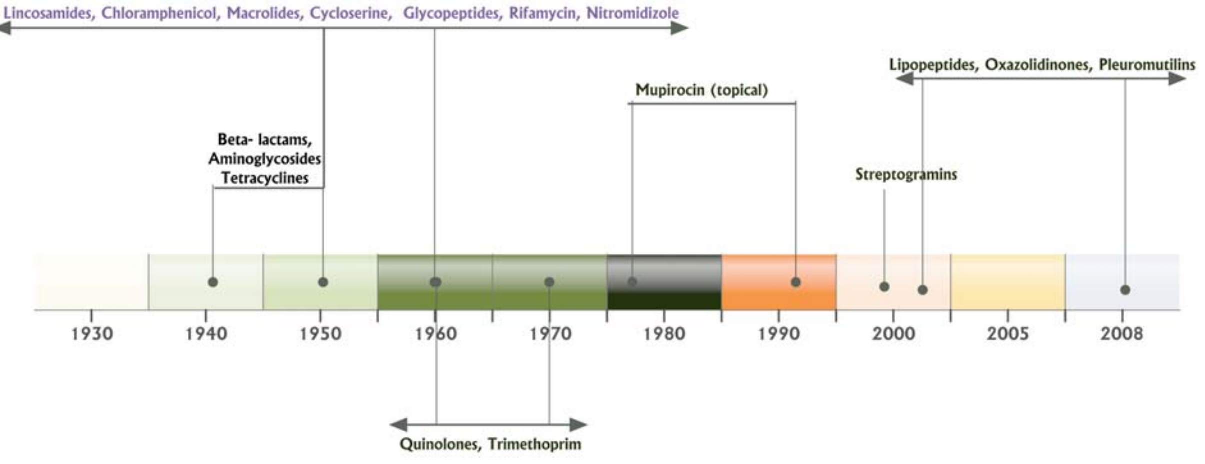



**Fig. 1.1 Timeline of antibiotic drug discovery**

The quest to discover new antimicrobials persists amongst clinical microbiologists and pharmacologists due to continuous evolution of resistance mechanisms in bacteria (Fig. 1.1). In developing countries infectious diseases are the third leading causes of mortality. Of these lower respiratory tract infections (RTIs ) contribute to around 81% global mortality followed by HIV, diarrheal diseases, tuberculosis, malaria and other infections (Fig. 1.2) (WHO, 2004).

## 1.2 Antimicrobial Drug Resistance (ADR)

ADR is a natural phenomenon which refers to the ability of microorganisms to resist the action of antibacterial drugs. It is an ancient defense tactic aiding the microbes to develop highly nuanced and carefully regulated responses to inhibit the action of myriad antibiotics. The introduction of an antibiotic into an environment results in the death of most, if not all, of the resident susceptible strains. Conversely at times, resistant survivors are able to propagate and overcome the antibiotic action. Antibiotic resistance epitomizes the Darwin's theory of natural selection. A selective process (presence of antibiotics in the environment) leads to proliferation of resistant organisms thus allowing organisms with novel mutations or newly acquired characteristics to survive and proliferate (Lewis *et al.,* 2002). Further the phenomenon is overstated by factors such as widespread and inappropriate use of antibiotics, extensive use of these agents as growth enhancers in animal feed and poor regulatory control on commercialization and use of drugs (Kapil, 2005; Swartz, 1997; Tomasz, 1994). Over the last decade almost every bacteria has become stronger and adapted to both, environment and antibiotics. These antibiotic resistant bacteria can rapidly spread to family members and coworkers threatening the community with a new infectious strain which is more difficult as well as highly expensive to treat.

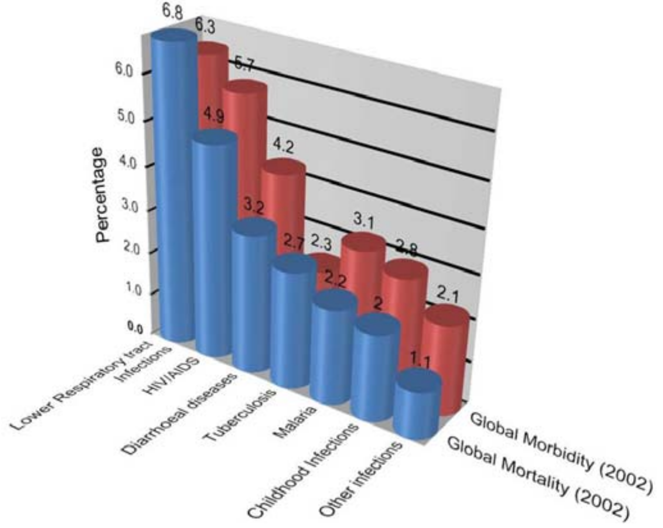



**Fig1.2 Global Burden of Infectious Diseases (WHO, 2004)**

Resistance genes are generally carried on plasmids and help bacteria to survive against environmental stress. At times the resistance genes may also occur on the bacterial

chromosome, the larger DNA molecule that stores the genes needed for the reproduction and routine maintenance of a bacterial cell. Selective pressure of anti-infective agents induce mutations in bacterial genomes (plasmid/ chromosome) that favour reduced susceptibility or pick up genes through horizontal gene transfer by conjugation, transformation or transduction. In case of transformation and transduction, the gene will survive and provide protection from an antibiotic only if integrated stably into a plasmid or chromosome (Levy, 1998). These mutations/ resistance genes assist the bacteria to overcome antibacterial action by various biochemical pathways (Fig. 1.3). These include (i) decreased intracellular accumulation of the antibiotic by an alteration of outer membrane permeability resulting in diminished transport across the inner membrane; (ii) active efflux of the antibiotic outside the cell due to expression of an active efflux pump; (iii) alteration of the target by mutation or enzymatic modification; (iv) enzymatic degradation of the drug; (v) enzymatic modification of the drug and (vi) bypass of the drug target. The coexistence of several of these mechanisms in the same host can lead to multidrug resistance (MDR).

**Fig. 1.3 Broad mechanism of antibacterial resistance**

### 1.3 *Staphylococcus aureus*: Biology

*Staphylococcus aureus* are gram positive (0.7-1.2μm), non-motile, non-spore forming, and facultative anaerobic spherical bacteria that occur in microscopic clusters resembling grapes (Fig. 1.4). They are catalase positive, hardy microbes; withstand heat at 60°C for 30 minutes, highly salt, lipid tolerant and remain viable for months on agar

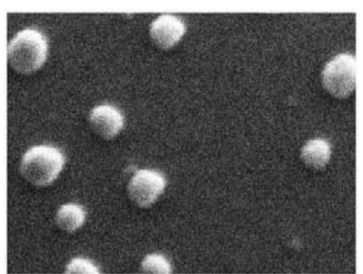


**Fig1.4 *S.aureus*: SEM micrograph**

plates stored at 4°C. They are extracellular, pyogenic (pus eliciting), tissue invasive microbes, first observed by Koch in 1878 in human pus. Sir Alexander Ogston (Ogston, 1883) recognized them as a major human pathogen causing wound suppuration and coined the term *Staphylococcus.* However, the final name *Staphylococcus aureus* by Rosenbach (1884) is based on the golden colour of the colonies.

**Disease Burden:** *Staphylococcus aureus* causes a multitude of diseases ranging from mild and requiring no treatment to severe and potentially fatal. It is a common pathogen in skin and soft tissue infections (SSTIs) viz. boil, impetigo contagiosa, ecthyma, carbuncle, furuncle, cellulitis and abscess (Moran *et al.,* 2005; Peacock *et al.,* 2001). Failure of the immune system to contain the infection at the focal site results in blood stream infections commonly referred to as bacteraemia. Bacteraemia caused by *S. aureus* is by far the most common life-threatening manifestation with an overall mortality rate of around 30% (Karchmer, 2000; Steinberg *et al.,* 1996; Wenzel and Edmond, 2001). Serious infections include breast infections, pneumonia, osteomyelitis, ocular infections, endocarditis and meningitis (Fowler *et al.,* 2003; Jensen *et al.,* 1993; Mermel *et al.,* 2001; Zimmerli *et al.,* 1998).

Humans are natural reservoirs of *S. aureus* and their asymptomatic colonization occurs in the moist squamous epithelium of anterior nares (Chambers, 2001; Moss *et al.,* 1948; Peacock *et al.,* 2001) and occasionally on the skin, nails, hair, axillae, perineum and vagina. Nasal carriage perhaps is a major risk factor for *S .aureus* infections (Kiser *et al.,* 1999; Kluytmans *et al.,* 1997) with the cell wall associated lipotechoic acid serving as the core factor behind nasal carriage (Weidenmaier *et al.,* 2004). People prone to *S. aureus* infections include newborns; drug users using injections; breast-feeding women and patients with skin disorders, surgical incisions or chronic disorders. Immunocompromised patients with implants and prostheses are at a higher risk of contracting staphylococcal infection. Intravenous catheters often are

contaminated with staphylococci, allowing the bacteria to enter the bloodstream thus causing bacteraemia (Mermel *et al.,* 2001).

### 1.4 Evolution of Multidrug Resistant *Staphylococcus aureus*

Prior to the antibiotic era, the mortality of patients infected with pathogenic *S. aureus* exceeded over 80% (Waldvogel, 2000), and 70% developed metastatic infections (Skinner and Keefer, 1941). The discovery of penicillin in the 1940s immediately improved this prognosis, with over 94% of strains exhibiting susceptibility (Medeiros, 1997). However, within a very short span *S. aureus* displayed signs of its remarkable ability to evolve and grow stronger upon exposure to penicillin. By 1942, even before penicillin was available for all doctors to prescribe, penicillin resistant strains emerged first in the hospitals in Australia and eventually in the community (Isbister *et al.,* 1954; Kirby, 1944; Rammelkamp and Maxon 1942). The strain rapidly spread across other continents with a speed and virulence reminiscent of influenza pandemic (Williams, 1959). The resistant bacteria produced enzymes called β-lactamases, which destroyed not only penicillin but also many more modern antibiotics of the β-lactam class such as ampicillin and amoxicillin thus making the strain resistant to multiple antibiotics. The strain was termed the 80/81 strain based on its bacteriophage susceptibility pattern. Methicillin, a penicillase resistant β-lactam of penicillin class was introduced in 1959 (Beecham) but *S. aureus* rapidly acquired resistance leading to emergence of Methicillin resistant *S. aureus* (MRSA). The very first report of MRSA came from a British Hospital (Jevons, 1961).

**The first MRSA**: The rapid spread of the 80/81 strain demanded new antibiotics to treat *S. aureus* infections. Methicillin (semi-synthetic penicillin), resistant to breakdown by β-lactamases was introduced in 1959. Only two years later, however, the first case of MRSA was reported from the UK (Enright *et al.,* 2002; Jevons, 1961). Although MRSA strains have been known for many years, they were rare in 1960s, sporadic in 1970s, epidemic in the 1980s and widespread and endemic in hospitals since the 1990s (Dunford, 1997).

**Global spread of MRSA:** Initially the spread of methicillin resistance remained limited; the resistance being expressed only under conditions of low temperature and high salt concentrations which were very different than the conditions that prevailed at the site of infection.

Microbiologists were confident of the long term efficacy of methicillin and its congeners as successful anti-staphylococcal agents however, reports of multidrug resistant MRSA emerged from Switzerland, Denmark, Australia and India (Pal and Ray, 1964; Parker and Hewitt, 1970; Roundtree and Beard 1968). After initial spread during 1960's, the prevalence of MRSA decreased in the late 1970 and early 1980s, apparently through a combination of a better infection control and increased use of gentamicin (Ayliffe, 1997). During mid 1980's, gentamicin resistant MRSA strains reemerged as colonist rather than pathogen resulting in renewed concern and debates regarding the significance of this strain. In Australia, an epidemic of multidrug resistant (MDR) MRSA strain was reported in the state of Victoria, which after 1982 caused outbreaks in hospitals in UK (Pavillard *et al.,* 1982). Till mid 1990's the proportion of MRSA infections remained limited after which cases of MRSA bacteraemia rose dramatically. This increase coincided with the emergence of two particular epidemic MRSA (EMRSA) strains: EMRSA-15 and EMRSA-16. By 2002, 57.1% of *S. aureus* were MRSA. Today every 5 in 100 people carry MRSA on their skin making them extremely susceptible to infection through open wounds, injections, catheters and intra-venous (IV) sites. A steadily increasing trend has been observed in European nations with MRSA levels around 40% in the UK, Ireland, Greece, Italy, Malta and Portugal; 19% in Germany and 11% in Austria (Smith and Coast, 2002).

MRSA was restricted to hospitals for the first three decades after becoming a super pathogen and was responsible for a variety of nosocomial infections. This was known as Hospital acquired MRSA (HA-MRSA) (Levine *et al.,* 1982; Saravolatz *et al.,* 1982). However, community

strains of MRSA started emerging when long term health care patients interacted with the healthy or susceptible people from community which included the healthcare workers. This unexpected epidemiological move was observed in early 1990's when MRSA strains began to appear in the community among people who had none of the usual risk factors for such infections (de Lancastre, 2007). In recent years, outbreaks of MRSA have been frequently reported outside hospital settings, including athletic teams, military recruits and prison inmates (Campbell *et al.,* 2004; CDC, 2003a, b). These findings suggest that MRSA and other forms of multidrug resistant *S. aureus* pose a major health problem in healthcare settings which are no longer confined to ICUs.

**The burden of MRSA:** The mortality due to MRSA infections have also increased from 51 to 1652 between 1993-2006 in UK while in the USA there was an increase from 11000 to 17000 deaths and in some parts of north America it has outnumbered the AIDS patient (Klevens *et al.,* 2007). MRSA infections today represent nearly 60% of nosocomial *S. aureus* isolates detected in hospital intensive care units (Kronemyer, 2004) accounting for 12% of all bacteraemia, and the pathogen has been implicated in surgical wound infection (28%) and skin infections (21%). The standardized incidence rate of invasive MRSA in US in 2005 was 31.8 per 100,000 persons (Klevens *et al.,* 2007). This is in contrast to the rates for *Streptococcus pneumoniae* or *Haemophilus influenzae*, that ranged from 14.0 per 100 000 to less than 1 per 100 000 respectively, largely due to success of vaccination programmes (CDC, 2002; Rosenstein *et al.,* 2001; Whitney *et al.,* 2006). Antibiotic treatment costs between 3,000 and 5,000 Indian rupees (INR) per day with prolonged hospital stay. Hospital stays due to MRSA infections have increased 10 folds since 1995 in US. This further puts an enormous economic burden with an estimated cost of INR 700,000 compared with INR 3, 80,000 for all other stays and the length of hospitalization being more than double- 10 days for MRSA infection versus 4-6 days for all other stays.

**Mechanism of methicillin resistance in *S. aureus*:** High-level resistance to methicillin in *S. aureus* strains is conferred by the horizontally acquired *mec A* gene encoding an alternative penicillin binding protein (PBP) viz. PBP 2a/ PBP2α- a 78KDa protein intrinsically insensitive to methicillin and all other β-lactams including oxacillin, cephalosporins, cefamycins and carbepenums (Beck *et al.,* 1986; Foster, 2004; Wielders *et al.,* 2002). PBPs are enzymes located on the extracellular surface of the cytoplasmic membrane responsible for the glycosyltransferase and transpeptidation reactions which are imperative steps in the formation of linear glycan chains and cross-linkage of the peptidoglycan chain respectively (van Heijenoort, 2001). Methicillin blocks the transpeptidase activity of the PBP2 enzyme without inactivating the transglycosylase activity leading to altered cell wall synthesis and thus bacterial cell death. However due to its low affinity to the β-lactams the PBP2a compliments the transpeptidase activity of PBP2 and the cell wall made by the combined catalysis of PBP2-PBP2a is only slightly different from that made by PBP2 in the absence of β-lactams (Leski and Tomasz, 2005) thus conferring resistance to the organism. Transcription of PBP2a proteins is controlled by the MecR-MecI-MecA regulatory system, which is a part of a mobile genetic element- a unique molecular vector called the staphylococcal cassette chromosome *mec* (SCC*mec*) (Katayama *et al.,* 2000). This island may also contain additional genes encoding resistance to other antibiotics.

### 1.5 MRSA: The Indian Scenario

The significance of MRSA as a major refractory pathogen currently having a status of top three dreaded infectious microorganisms of the world remains undisputed (Nordmann *et al.,* 2007). The number of cases involving community associated (CA)-MRSA has drastically increased worldwide (Skiest *et al.,* 2007) and in some countries it has become an epidemic (Zetola *et al.,* 2008).

Several surveys in India indicate a regional variation in MRSA epidemiology. According to the National Staphylococcal Phage Typing Centre, New Delhi, there is an increase in the occurrence of MRSA strains from 9.83% in 1992 to 45.44% in 1998 (Mehndiratta *et al.,* 2001) with an average rate of HA-MRSA being 32% (Mehta *et al.,* 1996). Conflicting reports of MRSA prevalence of 38.44% and 54.8% among *S. aureus* isolates have been reported in same hospital (Anupurba *et al.,* 2003; Tiwari *et al.,* 2008). Surveillance studies by various research groups across northern India revealed a usual rate of methicillin resistance to be 39 % among *S. aureus* strains; with the maximum resistance being reported from Uttar Pradesh (48.51%) (Anupurba *et al.,* 2003; Prasad *et al.,* 2000; Tiwari *et al.,* 2008) followed by Chandigarh (35.44%) (Bansal *et al.,* 2008; Zahoor *et al.,* 2006), New Delhi (34.12%) (Dhawan *et al.,* 2004; Goyal *et al.,* 2002; Mohanty *et al.,* 2004; Saxena *et al.,* 2003; Tyagi *et al.,* 2008; Vidhani *et al.,* 2001) and Punjab (10.5%) (Aggarwal *et al.,* 2001). Similar studies by researchers demonstrated methicillin resistance rates of 35% among *S. aureus* strains from hospitals across different states of south India. Maximum resistance was reported from Tamil Nadu (39.89%) (Angel *et al.,*2008; Kownhar *et al.,*2008; Rajaduraipandi *et al.,* 2006; Rallapalli *et al.,* 2008) with Kanchipuram, exhibiting methicillin resistance as high as 72.4% amongst clinical strains collected from medical laboratories across the town (Prakash *et al.,* 2007) followed by Karnataka (27.93%) (Arakere *et al.,* 2005; Aravind *et al.,* 2000; Bhat *et al.,* 2008; Chawla *et al.,* 2008; Krishna *et al.,* 2004) and Pondicherry (20.9%) (Mathanraj *et al.,* 2009; Srinivasan *et al.,* 2006). Further studies across hospitals in Mumbai (Mathur *et al.,* 2007; Mehta *et al.,* 1998; Patil *et al.,* 2006, Sachdev *et al.,* 2003), Surat (Mulla *et al.,* 2007) and Nagpur (Takhniwale *et al.,* 2002) demonstrated lower rate of methicillin

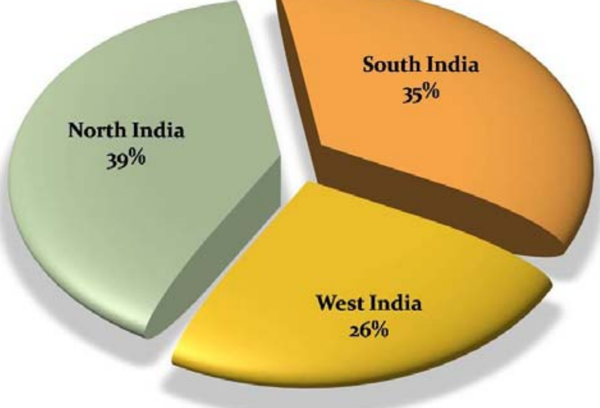



**Fig 1.5 Prevalence of MRSA in India**

resistance (26%) in the western part of the country. Limited data is available from hospitals in central and eastern India. Verma *et al.,* (2000) reported a rapid increase in the prevalence of MRSA from 12% in 1992 to 80.89% in 1999 at a tertiary care centre at Indore in central India. In addition a study at tertiary care hospital in Assam- north eastern India revealed 23.6% HA-MRSA strains (Majumder *et al.,* 2001).

Overall monitoring studies from 2000-2008 by researchers across diverse parts of the country revealed maximum methicillin resistance among strains in north India followed by south India and west India (Fig. 1.5). MRSA strains reported across all studies were multiple drug resistant exhibiting 100% resistance to penicillin and high level of resistance to cephalosporins, ciprofloxacin, amikacin, rifampicin, tetracycline, erythromycin etc. On the other hand all the strains were sensitive to vancomycin and linezolid. Molecular typing of the strains using PFGE/MLST and *spa* typing revealed that the strains were related to Hungarian and Brazilian MRSA clones (Arakere *et al.,* 2005) undoubtedly portraying the intercontinental spread of MRSA. Alarming reports of CA-MRSA have also surfaced in the last few years. One study has reported the prevalence of CA-MRSA in 46.1% patients (Krishna *et al.,* 2004). Variation in the conflicting prevalence data of MRSA can be attributed to time, place, and clonal expansion of the isolates accompanied by the drug pressure in the community.

More recently the reports of vancomycin resistance in *S. aureus* have emerged (Assadullah *et al.,* 2003; Saha *et al.,* 2008; Tiwari and Sen, 2006) and necessitate the need of surveillance's and monitoring the spread of MRSA through network of sentinel hospitals distributed by geographic location and size as is practiced in developed countries (Diekema *et al.,* 2001).

The spread of MRSA by direct or indirect person to person contact and the increasing prevalence rates of MRSA make’s the present therapeutic scenario worrisome and emphasizes

the need of local and national surveillance programs that can assist in designing appropriate control measures for the emergence and spread of this deadly superbug.

### 1.6 Need of New anti-MRSA drugs

Today a serious situation is being faced by clinicians, paramedics and healthcare workers worldwide wherein the MRSA has become refractory practically towards every drug class being administered during chronic, resistant staphylococcal infections. MIC creep is a term assigned to population MIC shift over a period of time against a particular antibiotic. The reduced susceptibility of MRSA towards glycopeptide drugs vancomycin and teicoplanin (Hiramatsu *et al.,* 1997) which eventually led to appearance of vancomycin resistant *S. aureus* (VRSA) (Hiramatsu *et al.*, 2002) could be attributed to MIC creep beyond susceptible range.

The failure of treatment of MRSA infections remains common despite rare or infrequent appearance of GISA or VRSA. This is due to increased MICs of vancomycin MRSA infections despite being susceptible. This is also attributed to hetero-resistance towards vancomycin. The detection of hVISA in clinical isolates of *S. aureus* is insurmountable by standard diagnostic methods in clinics as it employs the use of very low inoculum levels as well as the incubation period is also very short to detect growing Vancomycin sub-populations (Liu and Chambers, 2003). Apart from vancomycin, linezolid, daptomycin, and tigecycline are approved by the USFDA for the treatment of MRSA strains, however clinical failure of daptomycin has been reported in MRSA infections exhibiting a MIC of ≥2 mg/ml (Hayden *et al.,* 2005). Linezolid, the first oxazolidinones class of drug was introduced in the year 2000. The first linezolid resistant *S. aureus* was reported within a year of its introduction from patient receiving oral linezolid treatment of peritoneal dialysis associated peritonitis (Tsiodras *et al.,* 2001). The linzeolid resistant MRSA was subsequently reported from a patient who received an intravenous and then oral linezolid therapy for the treatment of empynema for 21 days (Wilson *et al.,* 2003).

Tigecycline has been found to be effective in the management of cSSTIs and cIAIs caused by MRSA (Peterson, 2008).

Mupirocin was largely used as a topical intervention for the disinfection of healthcare workers and patients to restrict the movement of MRSA from the clinical environment to the community. This drug was introduced in 1985 in UK after being discovered and developed by Beecham. Mupirocin initially exhibited good control towards MRSA but after 2 years of introduction MRSA started getting refractory behavior. However, there exist variable reports of resistance of MRSA to mupirocin depending upon the extent of the use of the drugs (Orrett, 2008). Other strategies of checking the spread of MRSA include 2% chlorhexidine digluconate bath to the patients who are MRSA carriers to reduce skin carriage and mupirocin cream to prevent nasal carriage (Gemmell *et al.,* 2006). However, this strategy would not be effective on all patients (Garvey *et al.,* 2007; Simor *et al.,* 2007) and hence newer interventions are sought. It has recently been evaluated that a combination of ranalexin, an anti-microbial peptide from *Rana catesbeiana* and lysostaphin, a highly specific anti-staphylococcal endopeptidase inhibits the growth of MSSA and MRSA in a synergistic manner and could also be used as a topical intervention to combat refractory MRSA skin and soft tissue infections. It was found that in combination, ranalexin with lysostaphin reduces the number of viable MRSA *in vitro* and on human skin *ex vivo* to greater extents than either compound individually. This combination kills bacteria in suspension within 5 min, is stable for at least 5 days and remains effective in low pH and high salt conditions and in the presence of gelling agents (Desbois *et al.,* 2009). Thus these newer interventions could be helpful in restricting the movement of refractory MRSA clones from hospitals to community as well as to other geographic area. The need of newer drugs is ever growing and persistent despite disinfection protocols and prescribing policies in hospital formulary are being stringently adhered to in overcoming the drug resistant chronic MRSA infections.

Some major new drugs like ceftobiprole, telavacin, amadacycline (Table 1.1) are in different phases of development for the treatment of resistant MRSA infections. However all these new drugs being developed are extension of previously known antibiotic classes and are more susceptible to encounter resistance by MRSA despite rational drug designing has been carried out using QSAR (Fig. 1.6). Despite addition of these new agents to treat MRSA infections, it is clearly indicated that since the current drug classes have been rendered susceptible to MRSA the lifetime of efficacy of these new chemical entities is limited as there are chances of encountering cross resistance. Floroquinolones were introduced in 1962; followed by oxazolidinone-linezolid in 2000 (Walsh 2003a, b) and a lipopeptide- daptomycin in 2003 (Spellberg *et al.,* 2004) and newer classes of antibiotics having new targets sites of action than previously known are extremely essential to overcome MRSA infections. Similarly exploring chemistries which could act at more than one target could further enhance MRSA susceptibility.

The number of antibacterial drugs approved by US Food and Drug Administration (USFDA) has decreased uniformly over the years (Fig. 1.7). This is attributed to less expertise of people in antimicrobial drug discovery and development as well as higher R&D costs in anti-microbial drug development as compared to drugs used (Goozner, 2004; Reichert, 2003) in metabolic diseases /disorders. However, smaller R&D companies have taken efficient course of developing new antimicrobials which are eventually transferred to pharmaceutical majors on milestone payment basis till clinical trials and market introduction.

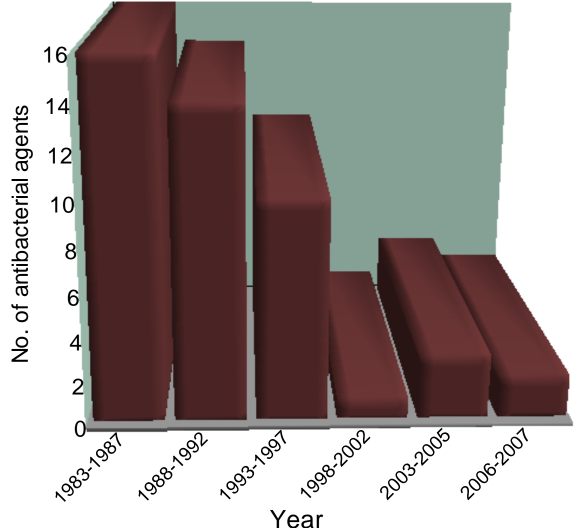



**Fig. 1.7: Trend of new antibacterial drug approvals by USFDA**

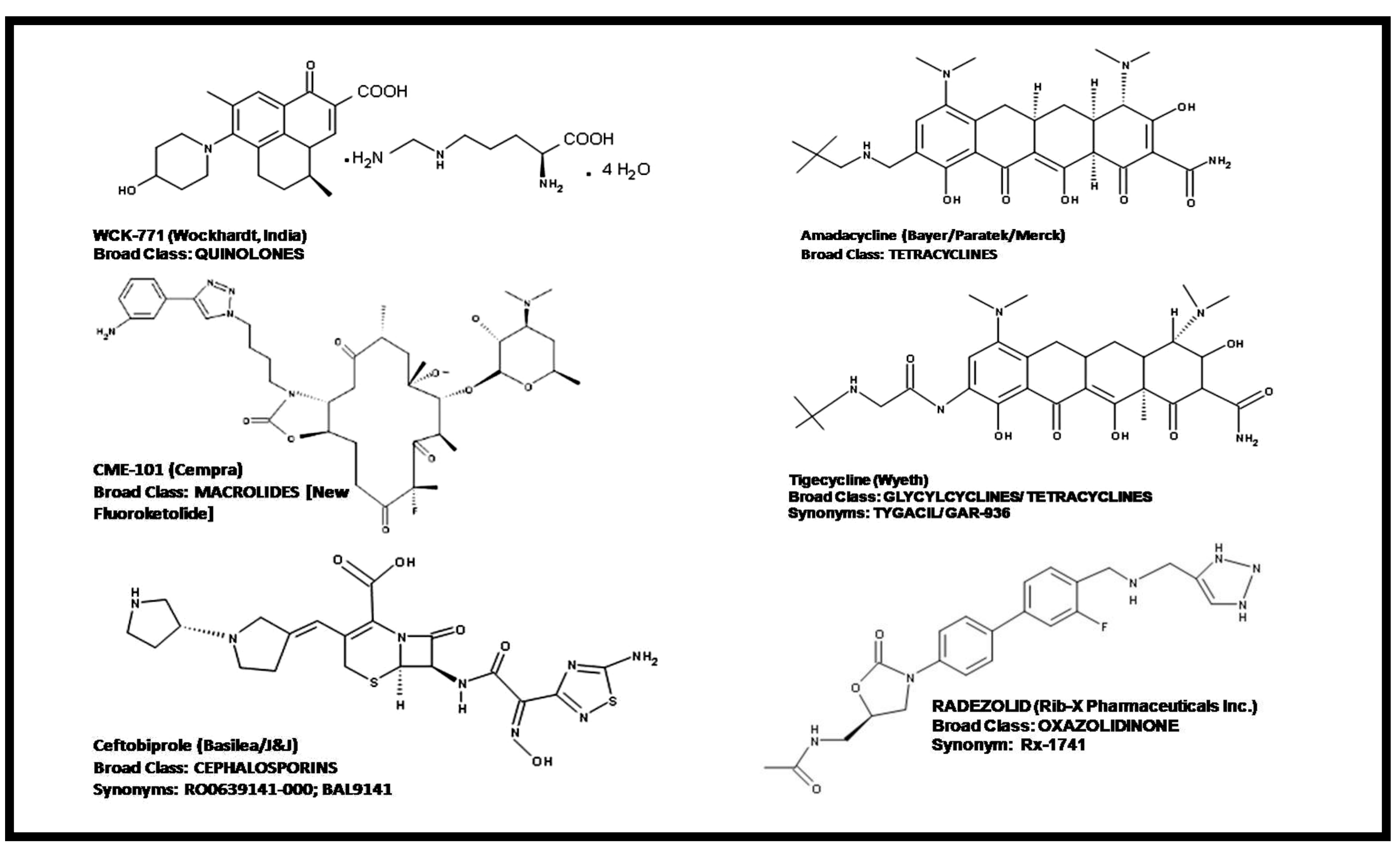



**Fig.1.6: Chemical Structure of Systemic Anti-Staphylococcal agents under Development**

## TABLE 1.1: SYSTEMIC ANTI-STAPHYLOCOCCAL AGENTS UNDER DIFFERENT PHASES OF DEVELOPMENT

| COMPOUND, FORMULATION | COMPANY | ANTIBIOTIC CLASS | DEVELOPMENT PHASE | MAJOR INDICATIONS |
|---|---|---|---|---|
| **Telavancin, i.v[1].** | Theravance/ Astellas | GLYCOPEPTIDE | [2]NDA submitted (USFDA) | CSSTI, +ve HAP and VAP |
| **Ceftobiprole, i.v.** | Basilea Pharmaceuticals , J&J | CEPHALOSPORIN | NDA submitted (USFDA) | CSSTI, +ve HAP and CAP (Phase III completed) |
| **Amandacycline, i.v./oral** | Bayer, Paratek, Merck | TETRACYCLINE DERIVATIVE | [3]Phase II | cSSTI, Diabetic Foot Infections, CAP |
| **Radezolid(Rx-1741), i.v./oral** | Rib-X Pharmaceuticals Inc. | OXAZOLIDINONE | Phase II completed (Oral) | CAP,uSSTI |
| **NXL-103 , oral** | Novexel | STREPTOGRAMINS (Lipopristin/Floprostin) | Phase II | CAP |
| **WCK-771, i.v.** | Wockhardt (Oral prodrug WCK-2349) | QUINOLONE | Phase II, Nadifloxacin approved as topical agent | Gram positive Infections |
| **CEM-101, oral** | Cempra Pharmaceuticals | MACROLIDE | Phase I completed | CAP |
| **Tigecycline, i.v.** | Weyth | TETRACYCLINE DERIVATIVE | Commercialized | Gram positive Infections |

CAP- Community Associated Pneumonia ; uSSTI- Urinary Skin and Soft tissue infections; + ve HAP- gram positive Hospital Acquired Pneumonia; VAP- Ventilator Associated Pneumonia ; cSSTI- Complicated Soft and Skin Tissue Infections [1]*i.v.- intra-venous , oral route of delivery;* [2]*new drug application filed at US Food and Drug Administration;* [3]*Phase1- testing in healthy volunteers to determine pharmacokinetics and safety; Phase-2 testing in a small number of patients to determine safety and efficacy; Phase 3- testing in a large number of patients to determine efficacy and safety.*

### 1.7 Microbial natural products in antibacterial drug discovery

Microbes are considered to be a good starting point for discovery of antibacterial drugs after the serendipitous discovery of penicillin. To date microbes have been the most prolific sources of structurally diverse secondary metabolites which have been maximally exploited as antimicrobial drugs. All antibacterial classes i.e. β-lactams (penicillins, cephalosporins, carbapenams and monobactams), aminoglycosides (amikacin, neomycin and kanamycin), tetracyclines, macrolides (erythromycin) and glycopeptides (vancomycin) have been discovered from microbial sources (Table 1.2). Microbially produced antimicrobial drugs have been responsible for enhancing the life expectancy from 47 in early 1960s to 75 years in 2000. Microbial natural products thus have remained the most successful guideposts to antibacterial drug discovery (Von Nussbaum *et al.,* 2006).

Apart from the use of microbial natural products as antibacterial(s), they have been responsible for production of other applications in the pharmaceutical industry as immune-suppressants, hypocholesterolemic drugs, anticancer agents, antiparasitic agents and gastrointestinal motor stimulators (motilides) (Demain, 1999; Demain and Sanchez, 2009; Knight *et al.*, 2003). In the agricultural scenario also microbial secondary metabolites have been developed into insecticides, fungicides, nematicides and herbicides (Saxena and Pandey, 2001). Thus the 80 year contribution of microorganisms to medicine and agriculture has been overwhelming. Antibiotic resistance unfortunately has created a dangerous situation and the need of new antibiotics and antibiotic classes is clear. Despite the persisting issue of multidrug resistance in bacteria, screening of microbial biodiversity for new antibacterial compounds has been de-emphasized by large pharmaceutical companies, due to diminishing discoveries of novel molecules and declining productivity apart being a capital intensive exercise. This is basically attributed to lack of access of the microbial diversity from different matrices other than soil, conventional culturing and screening techniques. Microbial natural products have not only been

directly used as antimicrobials or templates for new antimicrobial pharmacophores but have also yielded some important enzyme inhibitors like clavulanic acid which has been responsible for reversion of the β-lactam resistance in the gram positive bacteria as combination drugs like Augmentin®. Some pharmaceutical industries and biotechnology companies have revamped the antimicrobial drug screening programs using advanced genomic and proteomic driven techniques for discovering new antimicrobial natural products by contributing as new class of antibiotics. Of interest is bacterial fatty acid pathway which serves as a target for the development of antimicrobial drugs like platensimycin from *Streptomyces platensis* (Wang *et al.,* 2006). Platensimycin was screened out of 83,000 bacterial culture strains against a *S. aureus* strain in which *fab F/B* were targeted for inhibition. However it was recently concluded by Brinster *et al.,* (2009), that platensimycin exhibited poor antimicrobial effects in the presence of exogenous fatty acids in the test medium or human serum assay indicating a four-fold increase in the MIC. Thus, despite genomics being used it was found that Type II fatty acid synthase is not a suitable target for gram positive drug resistant pathogens. The major limitation of microbial secondary metabolites as drugs today is attributed to limited interference in the bacterial physiology to render it susceptible for a longer duration. The targets currently being exploited remain the same i.e. protein synthesis inhibitors, cell wall synthesis inhibitors, cell membrane agents, DNA topoisomerase inhibitors, RNA synthesis inhibitors and folic acid metabolism inhibitors. There is a need to revalidate the *in vitro* susceptibility test results in animal models essentially or else they could become less efficacious as in the case of platensimycin. Hence natural products from different genomes like plants could provide better structural and functional classes of chemical compounds which have not been explored/ exploited so far.

TABLE 1.2: MICROBIAL SECONDARY METABOLITES DEVELOPED INTO ANTIBACTERIALS

| ANTIBIOTIC CLASS | SOURCE | NAME & CHEMICAL STRUCTURE | CREDITS |
|---|---|---|---|
| *Penicillin's* | *Penicillium notatum* (Fungi) | Penicillin V | Monsanto (1956) USP 2870064 (1959) |
| | Synthetic ß-lactam (ß-lactamase inhibitor) | Meticilin | Beecham Pharmaceuticals (1959) |
| *Cephalosporins* | *Cephalosporium acremonium* (Fungi) | Cephalosporin | G. Brotzu (1948) |
| | 1st generation cephalosporin (Semi-synthetic) | Cephalexin | Eli Lilly and Co.(1970) |
| | 2nd generation cephalosporin (Semi-synthetic) | Cefaclor | Eli Lilly and Co. (1990) |
| | 3rd generation cephalosporin (Semi-synthetic) | Cefdinir | Astellas Pharma Inc. Japan (1991) |
| | 4th generation semi-synthetic cephalosporin | Cefipime | Bristol Meyers Squibb (1994) |
| *Carbapenums* | *Streptomyces cattleya* (Actinomycetes) | Imipenum | Merck & Co. (1985) |
| *Aminoglycosides* | *Streptomyces kanamyceticus* | Kanamycin | H. Umezawa *et al.*, (1957) |

| | Synthetic derivative of Kanamycin | Amikacin | Bristol Meyers Squibb (1976) |
|---|---|---|---|
| *Macrolides* | *Saccharopolyspora erythraea* (Actinomycetes) | Erythromycin | Eli Lilly and Co. (1952) |
| | Synthetic Macrolide | Clarithromycin | Taisho Pharmaceuticals (1970) |
| *Polyketides* | *Streptomyces genus* | Tetracycline | Pfizer(1955) |
| | Synthetic derivative of tetracycline | Doxycycline | Pfizer(1960) |
| *Lincosamides* | *Streptomyces lincolnensis* | Lincomycin | UpJohn & Co. (1962) |
| | Synthetic derivative of Lincomycin | Clindamycin | UpJohn & Co. (1964) |
| *Glycopeptide* | *Amycolatopsis orientalis* | Vancomycin | Eli Lilly and Co. (1958) |
| *Streptogramins* | *Streptomyces pristine spiralis* | Prsitinamycin IIA | May & Baker (1988) |

### 1.8 Plant based molecules as anti-MRSA drugs and resistance modifying agents (RMA's)

Natural products provide an unmatched availability of chemical diversity out of biodiversity which presents huge opportunities for drug development. Practically all ancient systems of medicine like the Unani, Siddha, Ayurveda, Kampo, Traditional Chinese Medicine and traditional western medicine involved the use of medicinal plants. The specialized biochemical capabilities make plants able to synthesize and accumulate a vast array of secondary metabolites in response to various environmental stresses like competition for nutrients, space, allelopathic interactions and pathogenesis (Saxena and Kumar, 2002).

The potential of higher plants as sources for new drugs however is still largely unexplored. Among the estimated 2,50,000 plant species existing worldwide, only a small percentage (10%) has been investigated for their biological chemistry and pharmacological uses (Hamburger and Hostettmann, 1991). Modern pharmacology is highly dependent on plant-based drugs. Natural compounds offer themselves to be directly used as drugs, as precursors for semi synthesis or as templates for the design of synthetic agents. Natural product research is thus an integral component of pharmaceutical research and development programs (Fig. 1.8 a, b). In the area of anti-infectives the use of natural products has been primarily based on microbial secondary metabolites. It has been found that microbial products are being continuously explored from undistrubed ecological niche's using genomic and proteomic based technologies and only a couple of new antibacterial classes (targets) have been explored so far. In pharmacognostic and ethnopharmacological studies the very first activity screen of any plant extract is antimicrobial. Plant extracts are considered antimicrobial if they really produce a MIC of 100 µg and below. Despite a large number of plant extracts and compounds being designated as "antimicrobials" there are hardly any lead extracts or lead compounds that are being pursued for anti-infective drug development.

Plants produce a variety of phytoalexins and phytoanticipins in response to bacterial invasion (Darvill and Albersheim, 1984). Phytoalexins are synthesized *de novo* whereas phytoanticipins are present in pre-infection conditions. The phytoalexin resveratrol is commonly found in food and drinks, like red wine, grapes, and peanuts. Resveratrol in individual studies has proven to have anti-inflammatory properties apart from being proving a potent anti-oxidant which has been ascribed to prevent cancer, coronary heart disease and is anti-diabetic. Antimicrobial and anti-inflammatory activities are desirable features in development of formulations used in alleviating many skin conditions. Resveratrol has been found to possess potential anti-dermatophytic activity against bacteria as well as fungi responsible for human skin infections. However it was more effective on fungal dermatophytic infections over bacterial indicating that a molecule of plant defense could also be used medicinal agent in treatment of human skin infection of different etiologies. All compounds produced as phytoalexins or phytoanticipins may not be essentially "antimicrobial" but could be playing a regulatory role which would be responsible for inducing the resistance in plant. This could be helpful in finding new targets of antimicrobial activity as well as developing new effective drug classes using the natural product template or combinatorial chemistry. Systemic antibiotics from plants have not been developed so far despite the fact that an equally number of plant based anti-cancer drugs like taxol, vincristine and vinblastine have revolutionized the field of anti-cancer chemotherapy. The only plant based compound having a broad spectrum antibacterial activity is pyrithione present in *Polyalthea nemoralis* but is better known as antiseptic agent present in Head and Shoulders shampoo independently discovered by chemists (Han *et al.,* 1981). Pyrithione is a potentially toxic antiseptic. Less toxic and weak antibacterial could also be developed into potential antibiotic therapies by combining agents which could modify the resistance pattern in the invading microbes. These are generally referred to as resistance modifying agents (RMA's).

Plants possess phytochemicals with remarkable resistance modifying abilities. This was uncovered by the discovery of 5'-methoxyhydnocarpin D (5'-MHC-D) and pheophorbide A which potentiate a putative weak antimicrobial alkaloid, berberine (MIC 256 µg/ml) from *Berberis fremontii* (Tegos *et al.*, 2002). MDR pumps present in gram positive bacteria like *S. aureus* have likeliness for cationic amphipathic molecules as substrates. Berberine being cationic amphipathic alkaloid is similar to quaternary ammonium antiseptics in chemical properties and mode of action by intercalating with DNA and accumulating in the cytoplasmic membrane of the bacterium (Jennings and Riddler, 1983). 5'-MHC-D disrupts *Nor A* MDR pump and facilitates the penetration of berberine into the bacterial cell leading to accumulation and cell death. Thus a combination of a weak plant antimicrobial along with a RMA could be used effectively in overcoming the infections caused by MDR bacteria.

5'-MHC-D induced a fourfold decrease in the MIC of norfloxacin against *S. aureus* (Stermitz *et al.*, 2000). Further RMAs could be helpful in uncovering new modes of action of the resistance reversal thereby providing ample opportunities for designing new molecules to which resistance is not known.

The above concept of formulating a resistance inhibitor with a conventional antibiotic is well accepted as exemplified by the use of Augmentin® (Glaxo Smithkline). Augmentin is a combination of amoxicillin (a β-lactam antibiotic) and clavulanic acid, a microbially derived β-lactamase inhibitor specifically used for treating patients having infections due to β-lactamase producing pathogens. The plant based antibacterial and resistance modifying agents can be classified as monoterpenes, diterpenes, sesquiterpenes, triterpenes, phenols, flavanoids, alkaloids, polyketides and sulfur containing chemical moieties.

1,8-cineole is a monoterpene from *Artemisia asiatica* which has a prominent anti-staphylococcal activity with a MIC of 2 µl/ml. It is also a constituent of tea tree oil which has

been widely used as a topical disinfectant agent to check the spread of MRSA. Gauianolide (a sesquiterpene) possesses an excellent anti-MRSA activity with a MIC of 1.95 µg/ml and a moderate toxicity to human colon carcinoma cell line ($IC_{50}$=16 µM). Mansinone F has a superb anti-MRSA activity with a MIC comparable to vancomycin- a widely used anti-MRSA antibiotic.

Novobiocin is microbially produced coumarin which uses DNA gyrase as an antibacterial target. Napthopyrone, a polyketide produced by *Cassia quinquangulata* has a MIC of 3.125 and 6.25 µg/ml against *S. aureus* and MRSA respectively. Cryptolepine, an alkaloid from *Cryptolepis sanguinolenta* has a 7.8 µg/ml MIC whereas mahanine (carbazole alkaloid) has 12.5 µg/ml MIC against standard *S. aureus.* Flavonoids represent the most prolific group of anti-staphylococcal compounds. Apigenin and luteolin are the simplest flavonoids possessing a MIC of 3.9- 62.5 µg/ml (Gibbons, 2004). Epicatechin gallate and sophraflavone G are flavonoids exhibiting antibacterial activity by acting on the cytoplasmic membrane. Pterocarpans are phytoalexins and biosynthetically related to isoflavonoids possessing anti-staphylococcal properties. Isoflavaones and pterocarpans produced in *Sophora flavescens and Erythina zeyheri* possess anti-MRSA properties apart from anti-viral activity which is attributed to neuraminidase inhibition (Ryu *et al.,* 2008). Erythrabassin-II has been found to possess a more potential antibacterial activity as compared to streptomycin (Fig. 1.9a).

Epicatechin gallate affects the PBP2' protein expression when combined with a β- lactam antibiotic thereby reverting methicillin resistance in *S. aureus*. Caffeine has been found to induce reduction in MIC of oxacillin by 128 folds in MRSA. Carnosic acid has been found to potentiate erythromycin activity by 16-32 folds in *S. aureus* expressing MsrA and TetK resistance proteins. Apart from 5-MHC-D, phaeopharbide A, a penta substituted pyridine (2, 6-dimethyl -

4–phenyl–pyridine-3,5-dicarboxylic acid diethyl ester) from *Jatropha elliptica* augments ciprofoxacin and norfloxacin activity against *S. aureus* SA-1199B.

Piperine an alkaloid from *Piper nigrum* and *Piper longum* has also been reported to enhance the accumulation of ciprofloxacin by *S. aureus.* Plant extracts as a whole also have been found to exert antibiotic potentiating activity against the multidrug resistant *S. aureus.* Extract of *Puncia granatum* (Pomegranate) exerted an ethidium bromide uptake in *S. aureus* RN-07044 and simultaneously exhibited synergistic interactions with chloramphenicol, tetracycline, gentamycin and oxiciilin in MRSA and MSSA isolates tested (Braga *et al.,* 2005). *Mezoneuron bethamianum* (Caeselpinaceae) and *Securinega virosa* (Euphorbiaceae) extracts potentiated the activity of flouroquinolone, tetracycline and erythromycin resistant strains of *S. aureus* (Dickson *et al.*, 2006) (Fig 1.9b).

Thus, anti-staphylococcal plant natural products (Fig. 1.9a) appear to be lucrative sources when compared to combinatorial chemistry libraries due to diverse functional group chemistry and chirality which appear to be the essential features for biological activity. Further the use of synergists which could potentiate the resistant drugs for treating MRSA infections (Fig. 1.9b) is also of prime importance as they could be developed into drug formulations thereby rejuvenating the current armamentarium of antimicrobial drugs at the same time uncovering newer mechanism of drug resistance reversal for generating new class of antibacterial compounds using proteomic and genomic tools.

Plant based natural products are alive and well in developing new drugs other than anti-infectives. It can therefore be emphasized that it is pertinent to screen plant based new antimicrobial /anti-staphylococcal agents to not only to revamp the current armamentarium against MDR-MRSA but to find out newer classes of antimicrobials having unique mode of action.

**Fig 1.8. Current trends in drug discovery**

**Fig. 1.8(a) Distribution and evaluation of New Chemical Entities in different therapeutic areas**

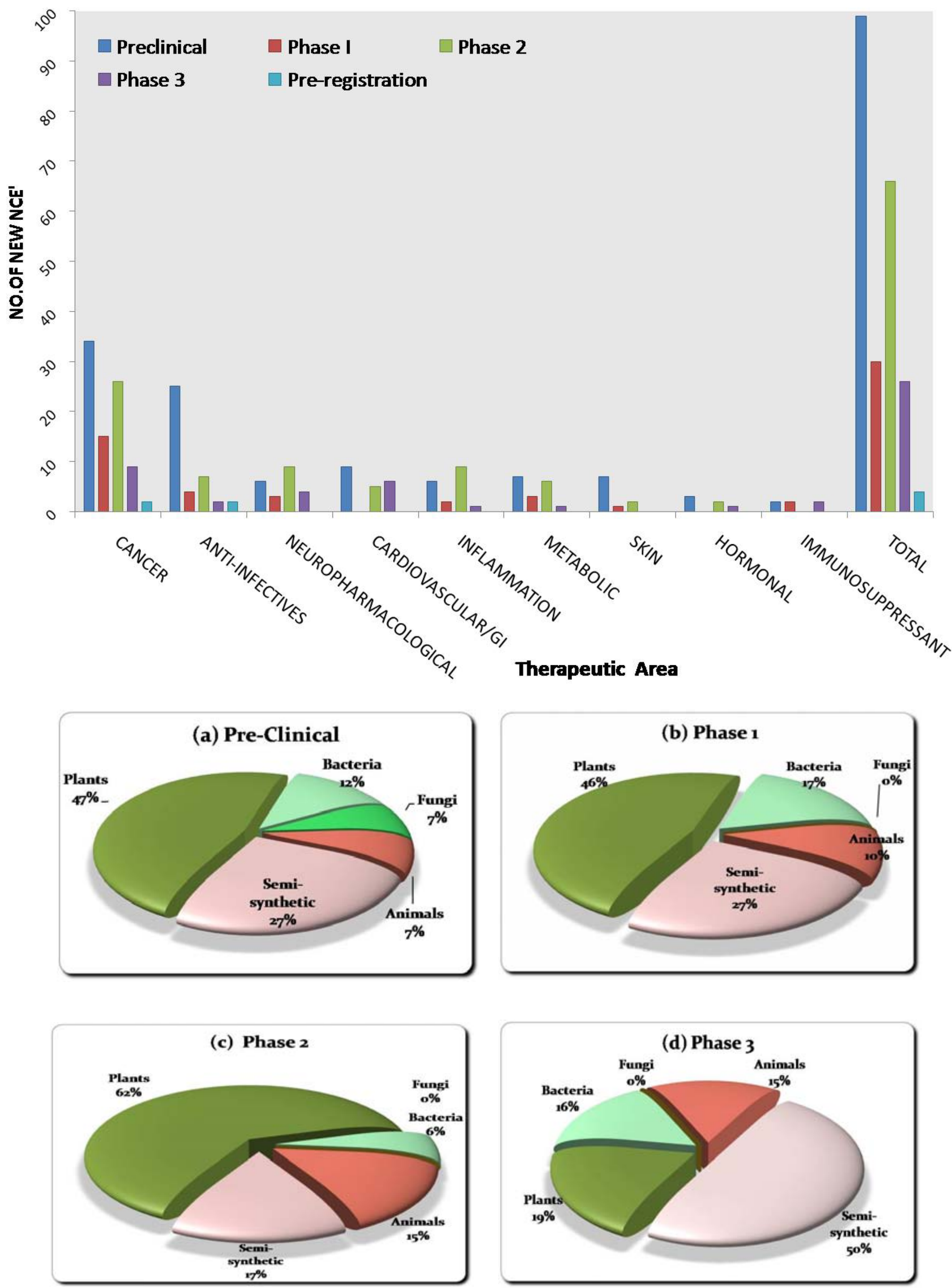



**Fig. 1.8(b) Classification of the sources of New Chemical Entities under evaluation**

*(Source: Pharmaprojects, 2008)*

**Fig.1.9 Plant natural products with potential for anti-infective drug development**

**Fig. 1.9(a) Potential Anti-staphylococcal agents from plants**

Bornyl Coumarate
Source plant: *Piper methysticum*

1,8- Cineole
Source plant: *Artemisia asiatica*

Cryptolepine
Source plant: *Cryptolepis sanguinolenta*

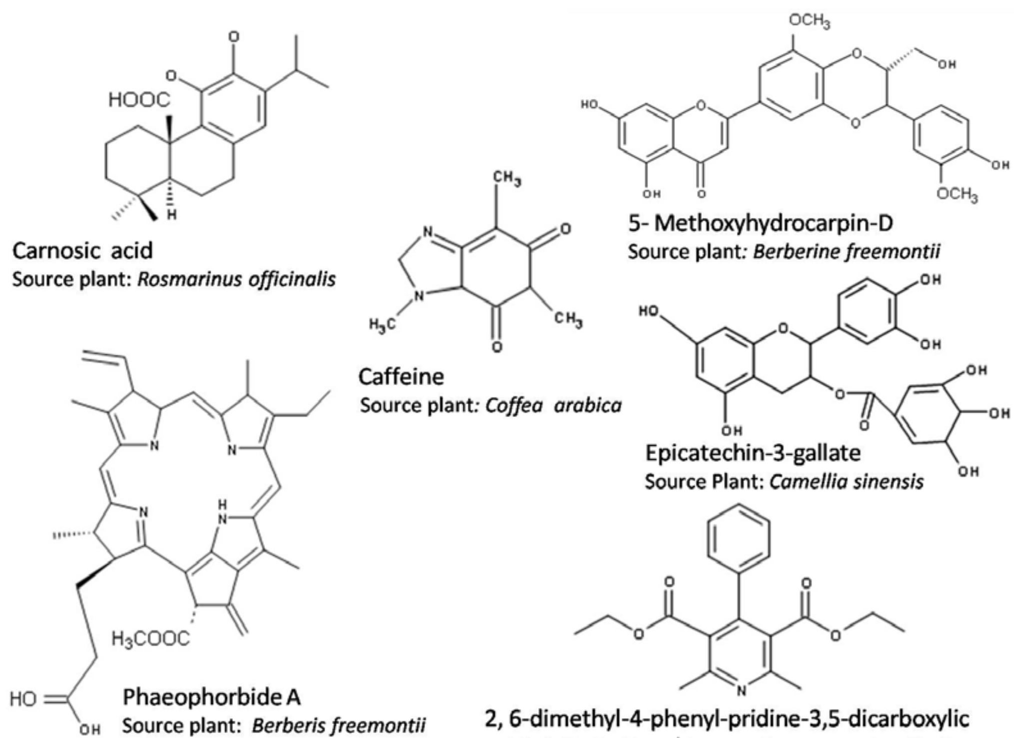
Sophoraflavanone G
Source plant: *Sophora flavescens*

derivative arnicolide C (a guaianolide )
Source plant : *Artemisia gilvescens*

Naphthopyrone
Source plant: *Cassia quinquangulata*

Mansinone F
Source plant: *Ulmus davidiana*

**Fig. 1.9(b) Staphylococcal resistance modifying agents of plant origin**



# Chapter 2
# PRESENT APPROACH

## 2.0 PRESENT APPROACH

Biodiversity plays a significant role and the complex diverse interactions between the organisms lead to production of a wide range of chemicals (compounds) which are produced naturally for survival and competitiveness both at the species as well as at the genus level (Koehn and Carter, 2005).

The nature's combinatorial chemistry has resulted in a million of natural structural chemotypes. Natural products have been a prolific and major source of pharmacophores between 1981-2002 apart from a shift in the discovery paradigm with the advent of genomics, proteomics, combinatorial synthesis and metabolic engineering (Jung, 2006; Newmann *et al.,* 2003).

There has been a disinterest in using plant based compounds for the development by the pharmaceutical industry in early 1990's because of inconsistency in sourcing of authentic plant raw material, followed by the characterization of the extracts, standardization problem due to complex composition of extracts and compounds of interest being isolated in too low quantities thereby numerous repetitions to have appreciable amount of bioactivity as well as characterization. Thus the whole process is time and labor intensive. Synergistic or antagonistic interactions of the components of standardized extracts give misleading results. The other drivers which delineate the pharmaceutical industry from taking plant based natural product could be poor stability, low quantities of pure compound as well as increasing market demand. Hence apart from stability large scale synthesis becomes a hindrance in successful commercial exploitation.

### 2.1 Renaissance of natural products drug discovery programs

New technologies in the area of chemical and biological sciences has revolutionized the science of drug development with a provision of continuous and reliable supply of drugs to the

pharmaceutical industry to meet the growing market demands (Ortholand and Ganesan, 2004; Paterson and Anderson, 2005). Development of efficient bioassays has provided the greatest impetus to the screening of novel natural products wherein they can be very selectively, quickly and economically evaluated. Further apart from primary screening, advances in separation sciences and structure elucidation technologies like HPLC, 2D-NMR, MS and XRD have helped in the straightforward assessment of complex natural products.

Biotechnological advancements have led to the development of inter-generic expression systems for harnessing important chemical molecules produced by plants in fewer quantities to meet the growing demand. Genetically modified microbes (GMMO's) could be used in production of plant phytochemicals to overcome the bottlenecks of large biomass requirement for extraction of the desired phytochemical as well as variability in the raw material with respect to the bioactive compound. GMMO's provide a cost efficient production technology for the production of desired phytobiomolecule under defined conditions, a technology which has already been perfected during production of antibiotics from microbes. A radiant example is genetic modification of *E. coli* and *S. cerevisiae* for the production of precursor of the antimalarial drug artemisinin isolated from *Artemisia annua*.

Complete biosynthetic pathway reconstruction of artemisinin production in bacteria and yeast is being attempted despite partial mimicking has lead to production of two precursors, amorpha- 4, 1, 1-diene and artemisinic acid which provide lucrative alternative for chemical or biotransformation into artemisinin rather than extraction of the plant. Further the conversion of above precursors is a 2 step chemical conversion process with recovery of 30-40% highly pure artemisinin when compared to direct extraction from plant biomass (Zeng *et al.,* 2008).

Plant phenylpropanoid pathway is responsible for the production of an array of metabolites classified as lignins, salicylates, coumarins, hydroxycinnamic amides, pigments, and flavonoids. Natural products of flavonoids class have recently gained a lot of attention due to multifarious activities like antimicrobial, cancer chemopreventive, antioxidant, and anti-asthmatic. These phenylpropanoid and flavonoid biosynthetic enzymes have recently been found to be attractive targets for metabolic engineering, for production of flavonoids with desirable traits economically.

Three artificial gene clusters viz. PAL (phenylalanine ammonia lyase), 4CL (4-coumarate: coenzyme A ligase) and CHS (chalcone synthase) have been cloned in *E. coli* which produced relevant functionally active enzymes that converted phenylalanine to pinocembrin chalcone and tyrosine to naringenin chalcone. The earlier block of expression of active C4H (Cinnamate-4 – hydroxylase) has been overcome by using a 4CL gene from the gram-positive filamentous bacterium *Streptomyces coelicolor* A3 that can activate cinnamic acid to cinnamoyl-CoA, as well as 4-coumaric acid to 4-coumaroyl-CoA thus bypassing the C4H step for the production of pinocembrin chalcone, from phenylalanine via the phenylpropanoid pathway. Thus genetically engineered microbes or bugs can be efficiently used for biosynthesis of pure and bulk production of important medicinal compounds (Hwang *et al*., 2003). More recently, *S. cerevisiae* has become a valuable platform for production of heterologous terpenoids such as the precursors of taxol which was originally isolated from *Taxus brevifolia* (DeJong *et al.,* 2006).

Alkaloids constitute the most diverse group of plant phytochemicals with myriad activities ranging from anti-cancer to anti-microbial. Alkaloids of benzylisoquinoline group consist of 2500 pharmaceutically active chemically defined structures. Aporphine type benzylisoquinoline alkaloids like magnoflorine has been reported to possess HDL (High density lipids) protection under oxidative stress thereby preventing from atherosclerotic disease. It has

also been reported to inhibit human lymphoblastodial cell killing by HIV-1. Recently *E. coli* has been developed which produces (S)-reticuline, a key intermediate in the synthesis of benzylisoquinoline alkaloids from dopamine and cost effective production of alkaloids is thus possible over chemical synthesis (Minami *et al.,* 2008).

Screening of the endophytic fungal diversity of the plants could be another facet of harnessing the plant chemical diversity. Endophytes are microbes which are being hosted by plants without any obvious signature of their existence. It has been hypothesized that these microbes during the course of evolution developed inherent changes in their biosynthetic pathway which became nearly similar to the plant biosynthetic pathway and thus could produce the phytochemicals. *Taxomyces andreane* and *Pestalatiopsis microspora* have already been isolated from *Taxus* sp. having the capacity to produce Taxol, the anti-cancer drug (Strobel *et al.,* 1996). *Camptothecin* is another anti-cancer pentacyclic quinolone drug isolated from the Chinese plant *Camptothecia acuminata* which functions by acting as a DNA topoisomerase- 1 inhibitor. An endophytic *Neurospora crassa* from *Nothopodytes foetida* has been found to produce camptothecin (Rehman *et al.,* 2008). Camptothecin in *Nothopodytes foetida* was found to be present in the roots. Thus this approach opens avenues in exploiting plant medicines through microbial route by using strain improvement methods and optimization of fermentation parameters (Strobel and Daisy, 2003).

Microbial production of plant metabolites has thus rejuvenated the plant natural product screening programs. The quality and quantity of the plant metabolite produced through microbial route is much superior since it is devoid of other interfering moieties.

## 2.2 Hypothesis – selection of *Callistemon rigidus*

*Callistemon rigidus* R. Br is a flowering ornamental plant of the family Myrtaceae. The cut shoots/leaves of *Callistemon rigidus* did not allow rottening of the water contained in the flower

vase (Sanjai Saxena, personal observation) and hence a question of its potential as an antimicrobial was raised. Furthermore the leaves of *Callistemon rigidus* seldom exhibit attack by plant pathogens (Gilman, 1999). This further substantiated the hypothesis. There were only two bibliographical reports on the phytochemical analysis of volatiles present in the dried leaves (Jirovetz *et al.*, 1997, 1998) and no reports existed on fresh leaves. Furthermore no reports existed with reference to its antimicrobial spectrum warranting investigations on its antimicrobial activity.

## 2.3 Objectives

Based on the above hypothesis the **objectives** of the current study are given as under

1. Phytochemical screening of the lead extract.
2. Bioactivity- guided isolation and characterization of the lead compound(s).
3. *In vitro* evaluation of the anti-staphylococcal potential of the lead(s) by agar well diffusion assay, microbroth dilution assay and plate count assay.
4. Determination of efficacy of the isolated lead(s) against commercially available antibiotics and to study their mode of action.

# Chapter 3
# REVIEW OF LITERATURE

## 3.0 REVIEW OF LITERATURE

### 3.1 Antimicrobial Drug Resistance: A Global quandary

Statements like "In the race for supremacy, microbes are sprinting ahead" and "Microbial resistance to treatment could bring the world back to pre-antibiotic age" given by documents of World Health Organization emphasize the seriousness of the most challenging global fight today: the fight against multidrug resistant, human pathogenic microbes. In the last century antibiotics have undoubtedly made a major contribution to improvements in both human and animal health. Despite enormous therapeutic success, bacterial infectious diseases are still among the leading causes of death worldwide and pose an enormous burden in terms of morbidity and mortality.

Humans have been a host for bacteria over billion years during the course of evolution. The antibiotic era which marked the rapid development of new antibacterial agents between 1945-1965 were usually natural products either produced by the microbes that serve as anti-competitive factors enabling the invasion of a strain in an established microbial community (Navaratna *et al.,* 1998; Riley and Wertz, 2002) or were signal molecules having other functions but inducing antibiotic effects at high concentrations (Clardy and Walsh, 2004). Concern is growing that therapeutic options will become increasingly limited if resistance rates continue to rise. *S. aureus* is the best example of evolution of pathogenic bacteria throughout the antibiotic era through its unique adaptability at the genomic level. The development of a resistance mechanism, starting with penicillin and methicillin, until the most recent, linezolid and daptomycin has created an unparalleled warning to chemotherapeutic era lacking an effectual antibiotic against this organism.

### 3.2 *Staphylococcus aureus:* Patho-physiology

*Staphylococcus aureus* (Greek *staphyle* = bunch of grapes, Latin *coccus* = spherical bacterium,

*aureus* = golden), is the most common *Staphylococcus* species causing infections in human. Staphylococci divide in more than one plane and the cells are arranged singly, in pairs, tetrads, grape like irregular clusters or in a biofilm. They are pyogenic (pus eliciting), tissue invasive, extracellular human pathogens, first seen by Koch in 1878 in human pus.

*Staphylococcus aureus* was first recognized as a major human pathogen Sir Alexander Ogston (1880) who implicated its role in wound suppuration and coined the term *Staphylococcus* (Ogston, 1883). The specific name *aureus* was coined with *Staphylococcus* by Rosenbach based on the golden colour of the colonies and hence named *Staphylococcus aureus*.

Humans are a natural reservoir of *S. aureus* and asymptomatic colonization occurs in humans, most frequently in the moist squamous epithelium of anterior nares (Chambers, 2001; Moss *et al.,* 1948; Peacock *et al.,* 2001) and occasionally on the skin, nails, hair, axillae, perineum and vagina. Nasal carriage perhaps is a major risk factor for *S. aureus* infections (Eiff *et al.,* 2001; Kiser *et al.,* 1999; Kluytmans *et al.,* 1997) with the cell wall associated lipotechoic acid serving as the core factor behind nasal carriage (Weidenmaier *et al.,* 2004). Nasal carriage in humans can be distinguished into persistent carriage, intermittent carriage and non-carriage (Eriksen *et al.,* 1995). Approximately 20% of humans are persistently colonized intranasal by a single strain of *S. aureus*. Another 60% of individuals are intermittent nasal carriers of *S. aureus* strains that change with varying frequency. The remaining 20% are classified as persistent non-carriers (Kluytmans *et al.,* 1997). Although *S. aureus* colonization of the nares is asymptomatic, nasal carriage is a risk factor for subsequent infection, particularly in patients who have undergone a surgical intervention, admitted ICU and those who are HIV positive or immunocompromised (Kluytmans *et al.,* 1997).

### 3.3 Spectrum of *S. aureus* infections

*S. aureus* is an opportunistic pathogen infecting practically every part of the human body. It was

initially responsible for hospital acquired infections but now community strains have evolved which are responsible for community epidemics (WHO, 2005). People prone to *S. aureus* infections include newborns; injecting drug users; breast-feeding women; patients with skin disorders; surgical incisions or those suffering from chronic disorders like diabetes and cancer. Immunocompromised patients with implants and prostheses are also at a higher risk of contracting staphylococcal infection. Intravenous catheters are often contaminated with staphylococci thus allowing the bacteria to enter the bloodstream thereby causing bacteraemia (Mermel *et al.,* 2001). *S. aureus* bacteraemia is by far the most common life-threatening manifestation of *S. aureus* infection with an overall mortality rate of around 30% (Karchmer, 2000; Steinberg *et al.,* 1996; Wenzel and Edmond, 2001).

*S. aureus* is also responsible for skin and soft tissue infections (SSTIs) like boil, impetigo contagiosa, ecthyma, carbuncle, furuncle, cellulitis and abscess (Moran *et al.,* 2005; Peacock *et al.,* 2001; Stevens, 1996). Serious infections include breast infections, pneumonia, osteomyelitis, ocular infections, endocarditis and meningitis (Bradley, 2002; Fowler *et al.,* 2003; Jensen *et al.,* 1993; Mermel *et al.,* 2001; Zimmerli *et al.,* 1998). Local and systemic effects of staphylococcal toxins can result in fatal syndromes viz. toxic shock syndrome (TSS), scalded skin syndrome and food borne gastroenteritis. Currently it is the leading cause of bloodstream infections, skin and soft tissue infections, and pneumonia (Pfaller *et al.,* 1998).

### 3.4 Virulence determinants of *S. aureus*

The term 'virulence' refers to microbe's "ability to invade the tissues of the host", or "the potential to cause a disease". The success of *S. aureus* as a pathogen and its ability to cause a range of infections throughout the human body is a consequence of its extensive virulence factors (Fig. 3.1). *S. aureus* carries a large repertoire of virulence factors, including over 40 secreted proteins and enzymes that it uses to establish and maintain infections (Diep *et al.,*

2006). It displays wider variety of virulence factors virtually more than any human pathogen and is a model organism for the study of pathogenesis of infection. Potential virulence factors include: (1) surface proteins (adhesions and fibrinogen binding proteins) that promote attachment of staphylococci to the damaged host tissues; (2) inhibitors of phagocytosis: capsule, Protein A - binds IgG molecules the wrong way thus disrupting phagocytosis; polysaccharides that form a biofilm or slime layer on indwelling medical devices; yellow pigment from carotenoids protect bacterium against free radicals, and catalase breaks down peroxide (phagocytes use free radicals and peroxide to kill bacteria); (3) bound coagulase - "clumping factor" which causes coagulation of plasma and bacteria clumping, and free coagulase, which again triggers plasma coagulation; (4) invasins that promote bacterial spread in tissues: kinases, hyaluronidases (destroy hyaluronic acid in connective tissue), and fatty acid modifying enzyme (FAME); (5) cytotoxins that lyse cell membranes: hemolysins (lysis of erythrocytes), leukotoxin and Panton-Valentine leukocidin-PVL (lysis of leukocytes); (6) Exfoliatin Toxins–ETA and ETB, and superantigen toxins, which may trigger massive non-specific T-cell response: enterotoxins SEA-G and toxic shock syndrome toxin (TSST-1); (7) antibiotic resistance - inherited and acquired.

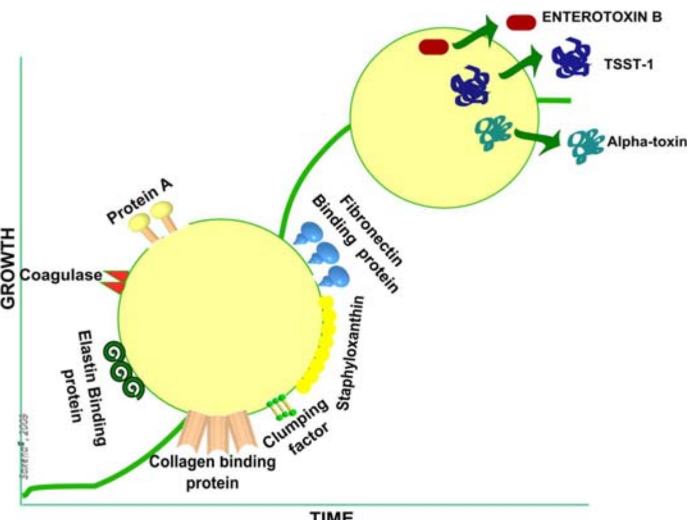



**Fig.3.1. Virulence determinants of *S. aureus***

Some of these virulence factors are known to cause or be associated with specific diseases, for example, toxic shock syndrome toxin (TSST) and toxic shock syndrome; Panton-Valentine leukocidin (PVL) and necrotizing pneumonia and skin diseases (Gillet *et al.,* 2002; Lina

*et al.,* 1999); the exfoliative toxins A and B (ETA and ETB) and scalded skin syndrome, impetigo, skin infections, and topic dermatitis (Capoluongo *et al.,* 2001; Lina *et al.,* 1997); and the family of staphylococcal enterotoxins A and B (SEA and SEB) and food poisoning (Arbuthnott *et al.,* 1990).

### 3.5 Multiple drug resistance in *S. aureus*

MRSA and is variants pose threat of reduced susceptibility to practically all drug classes including the semi-synthetic molecules like the linezolid, quinupristin- dalfopristin besides vancomycin. Antimicrobial drugs broadly have three interfaces of action on the microbe's viz. cell wall synthesis; protein synthesis and nucleic acids (Saxena and Gomber, 2009).

**Cell wall synthesis inhibitors:** Methicillin is a cell wall synthesis inhibitor by presenting itself as pseudo-substrates for the transpeptidases (now penicillin-binding proteins or PBP) that acylated them, thereby causing weak peptide bond formation susceptible to osmotic changes and leading to lyses of the bacterium. Five variants of PBP's have been described in Methicillin sensitive *Staphylococcus aureus* (MSSA). Variant which is responsible for providing the methicillin /β-lactam resistance is PBP2' or PBP2a. PBP2' is a surrogate transpeptidase having low or no binding affinity for β-lactams as compared to other PBP and therefore can express at antibiotic concentrations that are lethal for other PBP, thereby conferring resistance to *S. aureus*. The gene responsible for the expression of PBP2a' is *mecA* which is a part of genomic island designated as staphylococcal cassette chromosome mec (SCCmec) (Fuda *et al.,* 2004; Hartman and Tomasz, 1984; Song *et al*., 1987). Today MRSA is one of the leading causes of bacterial infections representing 60% of the nosocomial *S. aureus* isolates detected in hospital intensive care units (Chambers, 2001; Kronemyer, 2004). Infection with MRSA accounts for 12% of all bacteremias, and the pathogen has been implicated in surgical wound infection (28%) and skin infections (21%).

Apart from β-lactams, glycopeptides class of drugs also interferes with the cell wall biosynthesis by interfering in the transglycosylation and transpeptidation processes to induce drug susceptibility. Vancomycin, a glycopeptide antibiotic induced susceptibility through the process of inhibition of transglycosylation and transpeptidation steps which is achieved by blocking the precursor, D-Alanyl-D-Alanine (D-Ala-D-Ala), by the antibiotic through hydrogen bonding leading to inhibition of UDP-MurNac-pentapeptide formation required for cell wall synthesis. The vancomycin resistance in *S. aureus* is predominantly associated with the *van A* gene which is also induced by teicoplanin.

**Bacterial translation inhibitors:** Translation machinery of eukaryotic and prokaryotic organisms varies significantly and therefore could be exploited in designing drugs which interfere with the bacterial translation. Antibiotic classes viz. macrolides, aminoglycosides, tetracyclines, and oxazolidinones are functioning via protein synthesis inhibition. Macrolides like ethyromycin, clarithromycin are evaded by bacteria by three strategies (a) target site modification by methylation or mutation thereby preventing binding of the antibiotic to the ribosomal target; (b) efflux of the antibiotic and (c) inactivation of the antibiotic. *erm* gene is responsible for resistance of *S. aureus* to erythromycin by expression of *erm* methylase, which dimethylates the single adenine residue in the nascent 23S rRNA, a part of the 50s ribosomal subunit. Aminoglycosides, have been deactivated by MRSA by bringing changes in particular amino groups eventually losing the bonding affinity to 30S ribosomal unit. ANT (adenylate transferase) and AAC (acetyl transferase) are the major enzymes that participate in this mechanism. MRSA acquire tetracycline resistance through either efflux of the drug due to acquisition of *tet K* and *tet L* genes on the plasmid or through ribosomal protection mediated through *tet M* or *tet O* determinants located on the transposon or the chromosome. Efflux pumps are proteins which resist the entry of antibiotics. Efflux proteins act as pumps and export an extensive range of structurally unrelated antibiotics from the cell, resulting in reduced intracellular concentration

of the biocide and thus reduced susceptibility. The prokaryotic kingdom possesses five different kinds of efflux pumps, namely MATE (multidrug and toxic efflux), MF (Major facilitator), SMR (small multidrug resistance), RND (resistance-nodulation-division) and ABC (ATP-binding cassette). The best characterized MDR pumps are MFS proteins Qac A/B and Nor A. Qac A expels approximately 30 chemical compounds belonging to 12 chemical classes that could be broadly classified as toxic mono/bivalent ions, cations, and lipophilic compounds, whereas Nor A is related to fluoroquinolones (Mitchell *et al*., 1998; Walmsley *et al.,* 2003). Two efflux pumps (ABC transporters) *vgaA* and *vgbB* also play important roles in reduced sensitivity to streptogramins (Allignet *et al.,* 1997).

**Mutation or DNA damage**: At times a change in a single base pair (missense or non-sense) mutation also results in development of reduced susceptibility against a drug molecule. A mutated gene encoding for riboprotein L4 of *S. aureus* causes resistance to linezolid. *S. aureus* resists the flouroquinolones class of drugs by altering the chromosome or gene responsible for the expression of topoisomerase IV or DNA gyrase or by inducing a multidrug resistance efflux pump. The first resistance mechanism is alteration of the *gyr* A gene that encodes subunit A of DNA gyrase, i.e. topoisomerase, the target of fluoroquinolones. Another mechanism to attain fluoroquinolone resistance relates to mutation in the *Sma* IA locus of the chromosomal DNA of *S. aureus* (Trucksis *et al.,* 1991). Amino acid substitutions in vital regions of the enzyme-DNA complex commonly referred to as the quinolone resistance-determining region (QRDR) reduces affinity of quinolones for both of its targets (Ng *et al.,* 1996). The reduced susceptibility of MRSA to the Quinupristin- Dalfopristin (QD) combination is due to genes coding for acetyltransferases *vat A*, *vat B* and *vat C* (Allignet *et al.,* 1993).

MRSA were initially susceptible to the quinolones; however, resistance to this class of drugs has developed rapidly such that today more than 80% of MRSA isolates are quinolone-

resistant (Bauernfeind *et al.,* 1994). Mupirocin (a topical antibiotic), is the option for the treatment of these cases. Vancomycin and teicoplanin are the last resort for serious staphylococcal infections (May *et al.,* 1998). The recent emergence of variants of MRSA with high level resistance (HMR) to mupirocin (probably due to the acquisition of a transferable plasmid containing the *ileS-2* gene encoding an additional IRS enzyme) and vancomycin due to the acquisition of the *vanA* gene from enterococci, has created an unparalleled warn of a chemotherapeutic era lacking an effectual antibiotic against this organism (Cosgrove *et al.,* 2004; Upton *et al.,* 2003; Walsh and Howe, 2002).

The fight against the millennium superbug MRSA is still not over as they are responsible for a variety of nosocomial and community based infections all over the world. Strategies like expansion of the known drug classes; rational drug discovery using QSAR are being employed but they do not seem to provide a permanent plausible solution to the problem of resistance. There exists an immense need of antibiotics to overcome the resistant chronic infections caused by MRSA. The current requirement is for less broad spectrum agents and drugs targeted for a specific organism or a disease. Thus there is a need to discover new classes of drugs as they would not avoid resistance but slow down the process of resistance development in the target microorganisms thereby decreasing the chance of intractable infections as encountered currently. The new drugs discovered through combinatorial chemistry and rational drug design, i.e. driven by genomics and identifying novel drug targets, should be conserved and utilized only under chronic and serious infections fatal to life. Thus natural products could be used for providing the new chemical classes as well as templates for anti-staphylococcal products having potential to combat MRSA.

### 3.6 Therapeutic Power of Plants

As discovery and development of cheap, readily accessible therapies to overcome intractable MRSA infections are the need of the hour. Biomatrices like microbes, plants, animals are widely

being explored from different geographic realm of the earth to develop new armamentarium against MDR pathogens. Globally one-third of the population is still deficient in treatment with modern medicine due to economical facets. These mostly rely on the folklore remedies or traditional medicine. Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singly or in combination to treat, diagnose and prevent illness or maintain well- being (WHO, 2003). 80% of the population in Africa, Asia and Latin America use traditional medicine to help meet their primary health care needs (WHO, 2003). Plants have formed the basis of sophisticated traditional medicinal systems viz. Ayurveda, Siddha, Chinese traditional medicine and Unani, in existence since thousands of years (Cragg and Newmann, 2003). The earliest records of use of plants as drugs dating back to the 2600 BC describe the use of approximately 1000 plants including oils of *Cedrus* sp., *Cupressus sempevirens*, *Glycyrrhiza glabra*, *Commiphora* sps and *Papaver somniferum*, all of which are still in use today for the treatment of an array of ailments from coughs and colds to parasitic infections and inflammation. Many traditional herbal medicines contain active principles and the specific pharmacological activity of these can be explored by screening methods employing elaborate bioassays. Traditional/ folklore knowledge in India remains scientifically underexplored apart from its biodiversity. These could provide leads to explore treatment of some difficult diseases including infectious diseases. Ethno-medicinal aspects largely focus from the basis of plant selection of the plants (Verpoorte *et al.*,

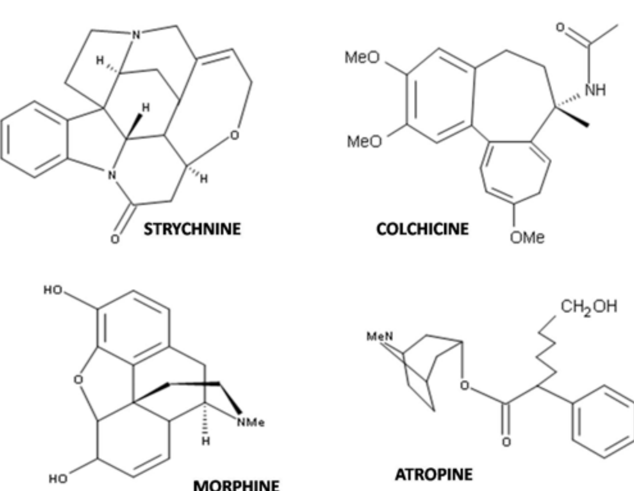



**Fig. 3.2 First natural products used as drugs**

2005). The approach of re-standardization of the traditional medicine in light of the principles of modern experimental medicine is referred to as reverse pharmacology. The idea of using pure compounds as drugs i.e. the science of isolating the active ingredients from plant extracts initiated in early 1800s forms the basis of modern medicinal chemistry and pharmacology. Amongst the first active principles to be isolated were strychnine, morphine, colchicine and atropine (Fig. 3.2).

The first commercial pure natural product, morphine, was produced by Merck in 1826 and the first semi-synthetic pure drug based on a natural product, Aspirin, was launched by Bayer in 1899. Since then plants have provided us New Chemical Entities (NCEs) for the development of drugs against various pharmacological targets viz. cancer, HIV, bacterial and fungal infections, malaria, Alzheimer's disease and pain (Jachak and Saklani, 2007). However, the ethno-medicinal uses form only a fraction of the diversity of estimated 2, 50,000 plant species (angiosperms and gymnosperms). Screening for new drugs involves assessment of biological activities of the plant extracts for confirming the presence of novel phytochemicals assigning the activity to the extract. For the purpose of lead discovery, or for the scientific validation of a traditional medicinal plant or a phyto-pharmaceutical, active principles in complex matrices need to be identified. Therefore, the interfacing of biological and chemical assessment becomes the critical issue. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds. Over the past 10 years, many new biochemical assays in microplate and highthroughput format have been developed to reduce time and enhance efficacy in drug discovery programs using natural products. Improvements in analytical and organic chemistry techniques like methods of extraction, chromatography, spectroscopy have revolutionized the prospects of discovering plant based antibacterials (Shinde and Dhalwal, 2007). Furthermore, molecular biology has become essential to medicinal plant drug discovery as it assists the determination and

implementation of appropriate screening assays directed towards physiologically relevant molecular targets.

The above advances have renewed interests of the pharmaceutical companies in exploiting plants as a major source of drug leads. Examples of recent advances helpful in streamlining drug discovery from natural products include (a) reformation of screening process for natural products (b) improved natural product sourcing (c) advances in organic synthesis methodologies (d) development of combinatorial biosynthesis (e) progress in the field of microbial genomics and (f) improved genomics and proteomics tools helping in target based drug discovery (Newman *et al.,* 2003).

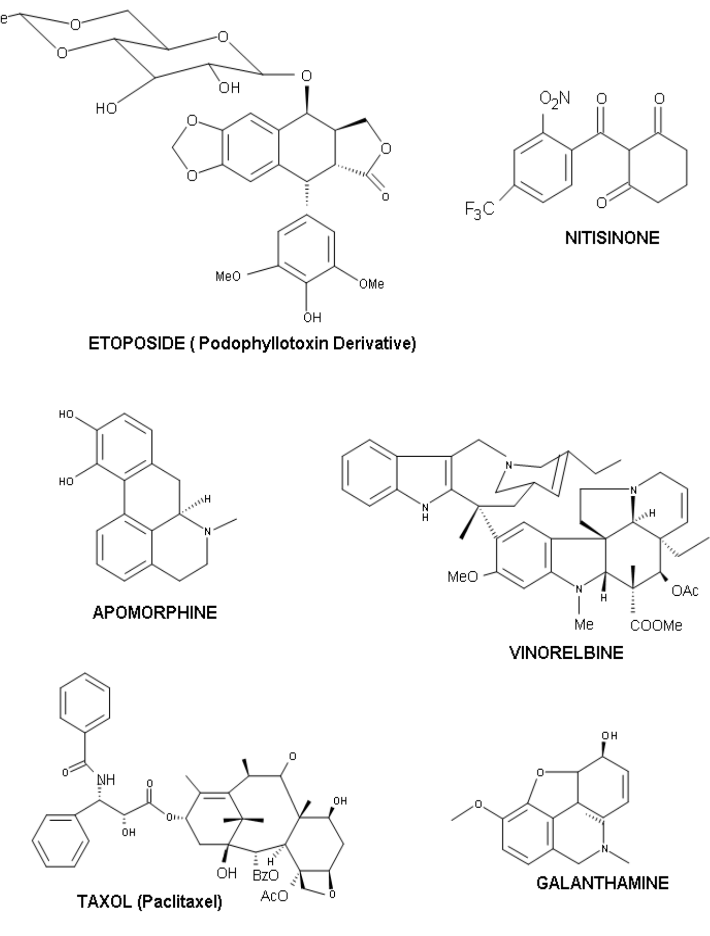



**Fig. 3.3 Current drugs developed from terrestrial plants**

The World Health Organization (WHO) is promoting the use of traditional medicine as a source of less expensive, comprehensive medical care especially in the developing nations. In the United States alone, plant-derived therapeutics represent approximately 25% of the prescription drugs, amounting to a retail value of approximately $15.5 billion. Such herbal medicines are easily available, cheaper, time tested and safer than some of the modern synthetic drugs (Shinde and Dhalwal, 2007). Some recent examples of NCEs discovered or modified from plants (Fig. 3.3) are galanthamine hydrobromide, an Amaryllidaceae alkaloid

obtained from *Galanthus nivalis* which acts as a selective inhibitor of acetylcholinesterase for the possible cure for Alzheimer's disease (Heinrich and Teoh, 2004); apomorphine hydrochloride is a morphine derivative for the treatment of Parkinson's disease (Deleu *et al.,* 2004) and nitisinone is derived from a leptospermone isolated from *Callistemon citrinus* with a potential to successfully cure hereditary tyrosinaemia type 1. Other plant based drugs include arteether from artemisinin isolated *Artemisia annua* which is a potential antimalarial drug, camptothecin, paclitaxel, vinblastine as chemotherapeutic agents for treating cancer (Bagchi *et al*., 1997). Other plant derived anticancer agent is AVE-8062 which is a derivative of combrestatin isolated from *Combretum caffrum*; homoharringtonine, an alkaloid active against hematologic malignancies isolated from *Cephalotaxus harringtonia* undergoing clinical trials (Chin *et al.,* 2006).

### 3.7 Anti-staphylococcal phytochemicals

Screening of plant extracts for anti-staphylococcal potential is one of the most popular approaches to arrive to chemical templates having novel mode of action in overcoming infections caused by resistant staphylococci and its clones. Recently rhodomyrtone, an acylphloroglucinol has been isolated from the *Rhodomyrtus tomentosa* having a MIC equal to 0.5µg/ml which is similar to vancomycin (Saising *et al*., 2008). Higher plants have evolved efficient biochemical defense mechanisms, comprising of a wide variety of secondary compounds including alkaloids, flavonoids, terpenoids and saponins possessing potential to be developed into anti-staphylococcal agents. The other plant acylphloroglucinols which possess effective anti- staphylococcal activity are hyperforin (*Hypericum perforatum*) and myrtucomulone A (*Myrtus communis*). Hyperforin is active against MRSA with a MIC of 1µg/ml (Schempp *et al.*, 1999). 1, 8-cineole is a monoterpene from *Artemisia asiatica* with MIC of 2µl/ml (Kalemba *et al.,* 2002). Sesquiterpenes include guaianolides and mansinone F from *Artemisia gilvescens* (Kawazoe *et al*., 2003) and *Ulmus davidiana* var *japonica* (Shin *et al*., 2000).

Chalcone, 2'(OH)-Chalcone, 2'4'-($OH_2$)-Chalcone's are promising members of the flavonoid class of plant natural products which have therapeutic potential to combat MRSA infections (Alcaraz *et al*., 2002). Aculeatin D and naphthopyrone are polyketide class of anti-staphylococcal agents isolated from *Ammomum aculeatum* and *Cassia quinquangulata* respectively (Heilmann *et al*., 2001; Li *et al*., 2001). Abetic acid, 19-acetoxy-7,15-isopimaradien-3β-ol and 7,15-isopimaradien-19-ol are anti-staphylococcal diterpenoids isolated from *Dauphinia brevilabra* (Dellar *et al*., 1996) and *Aeollanthus rydingianus* respectively (Rijo *et al*., 2009). Cryptolepine (*Cryptolepis sanguinolenta*); chelerythrine (*Zanthoxylum clava-herculis*) are alkaloidal anti-staphylococcal natural products (Cimanga *et al*., 1996; Gibbons *et al*., 2003). Other alkaloidal compounds possessing anti-staphylococcal activity include ramiflorine A and ramiflorine B with a MIC of 25 µg/ml from stem bark of *Aspidosperma ramiflorum;* pendulamine A, pendulamine B and penduline from root extract of *Polyalthia longifolia* var. *pendula* (Faizi *etal.,* 2003). Megistoquinones I and II from bark of *Sarcomelicopa megistophylla* (Fokialakis *et al.,* 2002*)*. The minimal inhibitory concentration of pendulamine A and pendulamine B is in a MIC range of 0.02-20 µg/ml. Antimicrobial alkaloid juliflorine and benzene insoluble alkaloidal fractions from *Prosopis juliflora* were found to be active against staphylococcal skin infections (Ahmad *et al.,* 1995). Conodurine and conoduramine are two antibacterial alkaloids isolated from stem bark of *Tabernaemontana van heurkii*. These alkaloids demonstrated anti-staphylococcal activity at a concentration of 20 µg/ml (Munoz *et al.,* 1994). 4-acetyltropolone has been found to possess anti-staphylococcal activity and has been isolated from *Thujopsis dolabrata* (Morita *et al*., 2002) 8-hydroxydihydrosanguinarine (hhS) and 8- hydroxydihydrochelerythrine (hhC) are benzo(c)-phenanthridine type alkaloids isolated from *Chelidonium majus* possessing anti-staphylococcal activity in clinical isolates in a MIC range of 1.95-62.5 µg/ml (Zuo *et al*., 2008). Thus, different chemical classes of plant natural products have exhibited potential anti-staphylococcal activity but have been seldom exploited as anti-microbial drug molecules (Table 3.1).

### 3.8 Antimicrobial agents present in Myrtaceae

Extracts of plant parts of *Eucalyptus globus; Rhodomrytus tomentosa; Callistemon rigidus; Melaleuca alternifolia; Syzygium aromaticum; Eucalyptus camaldulensis; Psidium guajava; Myrtus communis; Pimenta pseudocaryophyllus* and *Eugenia uniflora* have been found to exhibit potential anti-bacterial activity (Anas *et al*., 2008; Appendino *et al*., 2002; Ayepola and Adeniyi, 2008; dePaula *et al*., 2009; Fadeyit and Akpan, 2006; Gomber and Saxena, 2007; Saxena and Gomber, 2006).

Only a few of these plant extracts have been further studied for isolation of the antibacterial scaffold from them. Antibacterial acylphloroglucinols have been isolated from *Myrtus communis* and have found to be active against *Escherichia coli* and *Staphylococcus aureus*. The compounds have been identified as myrtucommulone A and myrtucommulone B (Rotstein *et al.*, 1974). Rhodomyrtone, another acylphloroglucinol from *Rhodomyrtus tomentosa* has been found to be anti-staphylococcal with MIC of 0.5 µg/ml (Saising *et al*., 2008). *Myrtus communis* also produces semi-myrtucommulone which has antibacterial potential to combat multidrug resistant *Staphylococcus aureus* resistant to tetracyclines, macrolides, and floroquinolones between a MIC of 0.5 µg/ml to 2.5 µg/ml (Appendino *et al*., 2002). Macrocarpels H, I and J possess a MIC range of 0.2-6.25 µg/ml against oral pathogens. These have been isolated from leaves of *Eucalyptus globus* (Osawa *et al*., 1996). Rhodomyrtoxin and ursolic acid-3-p-coumarate have been reported from apricot myrtle (*Pilidiostigma tropicum*) from Northern Queensland, Australia which is hitherto new with respect to its cytotoxic and antibacterial potential. Rhodomyrtoxin exhibits a strong anti-staphylococcal activity with a MIC of 0.28 µM when compared to the positive control gentamicin sulfate with a MIC of 1.07 µM (Setzer *et al*., 2006). Bullaketals A and B from *Lophomyrtus bullata* have been found to possess antibacterial activity of 30 µg/disk against *Bacillus cereus*. These exhibit cytotoxic activity against Mouse leukemia cell lines (Larsen *et al.*, 2005).

## TABLE 3.1 ANTI-STAPHYLOCOCCAL PLANT NATURAL PRODUCTS

| Compound (Class) | MIC (μg/ml) | Plant Name | Family | Reference |
|---|---|---|---|---|
| 1,8- Cineole (Monoterpene) | 2 μl/ml[a] | *Artemisia asiatica* | Compositae | Kalemba *et al.*, 2002 |
| Guaianolides (Sesquiterpene) | 1.95[b] | *Artemisia gilvescens* | Compositae | Kawazoe *et al.*, 2002 |
| Mansinone F (Sesquiterpene) | 0.39-3.3[b] | *Ulmus davidiana var.japonica* | Ulmaceae | Saxena and Gomber, 2009 |
| 4- acetyltropolone (Tropolones) | 1.56[c] | *Thujopsis dolabrata* | Cupressaceae | Morita *et al.*, 2002 |
| Hinokitiol (Tropolones) | 0.3[c] | *Thujopsis dolabrata* | Cupressaceae | Fujii *et al.*, 1995 |
| Cryptolepine ( Alkaloid) | 7.8[a] | *Cryptolepis sanguinolenta* | Periplocaceae | Gibbons *et al.*, 2004 |
| Chelerythrine ( Alkaloid) | 4[a]; 8[b] | *Zanthoxylum clava-herculis* | Rutaceae | Gibbons *et al.*, 2003 |
| Quinquangulin (Polyketides: Naphthopyrone) | 3[a]; 3.125[b] | *Cassia quinquangulata* | Fabaceae | Li *et al.*, 2001 |
| Aculeatin D (Polyketides ; Polyenes) | 0.8[c] | *Amomum aculeatum* | Zingiberaceae | Heilmann *et al.*, 2001 |
| Diallyl tetrasulphide (Sulphur containing compounds) | 0.5[a]; 2[b] | *Allium sativum* | Alliaceae | Tsao and Yin, 2001 |
| Hyperforin (Acylphloroglucinol) | 0.1[a]; 1[b] | *Hypericum perforatum* | Clusiaceae | Gibbons *et al.*, 2002 |
| Myrtucomulone (Acylphloroglucinol) | 0.5-2[a] | *Myrtus communis* | Myrtaceae | Mansouri *et al.*, 2001 |
| Rhodomyrtone Acylphloroglucinol) | 0.5[a] | *Rhodomyrtus tomentosa* | Myrtaceae | Saising *et.al*, 2008 |
| Falcarindiol (Polycetylenic) | 8-32[b] | *Angelica dahurica* | Apiaceae | Lechner *et al.*, 2004 |
| 8- hydroxydihydrosanguinarine ( Alkaloid) | 0.49 to 15.63[b] | *Chelidonium majus* | Papaveraceae | Zuo *et al.*, 2008 |
| 8-hydroxydihydrochelerythrine ( Alkaloid) | 0.49 to 15.63[b] | *Chelidonium majus* | Papaveraceae | Zuo *et al.*, 2008 |
| 19-acetoxy-7,15-isopimaradien3β-ol (Diterpenoids) | 3.9 to 15.62[a] | *Aeollanthus rydingianus* | Lamiaceae | Rijo *et al.*, 2009 |
| 7,15-isopimaradien-19-ol (Diterpenoids) | 3.9 to 15.62[a] | *Aeollanthus rydingianus* | Lamiaceae | Rijo *et al.*, 2009 |

a-MIC against S*. aureus*; b-MIC against MRSA; c-MIC against *Staphylococcus epidermis*

Gallomyrtucommulone B is galloylated aklylphloroglucinol glucosides isolated from *Myrtus communis* having anti-staphylococcal activity with a MIC range of 64-256 μg/ml (Appendino *et al*., 2006). α- and β-amyrin from *Psidium guajava* (Guava) was found to possess good antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* (Sanches *et al*., 2005). Myrtaceae family thus appears to be source to novel adducts of phytochemicals with potential biological activity for development as leads for new drugs.

## 3.9 General Phytochemical Procedures

### 3.9.1 Extraction of Plant Natural Products

Plants are complex matrices, producing a range of secondary metabolites with different functional groups and polarities. The precise mode of extraction of plant material is of paramount importance and depends on the texture and water content of the plant material. Extraction methods, used pharmaceutically, involve separation of medicinally active portions of plant tissues from the inactive/ inert components by using selective organic solvents with appropriate extraction technology. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity (Green, 2004). Various extraction methods include maceration, cold percolation/ steeping, soxhlet extraction, distillation, supercritical fluid extraction and microwave assisted extraction for obtaining the crude extract for initial assessment of biological activity.

**Cold Percolation:** Developed by Kerry Bone, cold percolation still remains the cornerstone of extraction technology. The method allows the processing of the herbs in liquids without the use of heat under defined conditions to conserve the constituents of the whole herb. It entails continuously percolating a suitable solvent through a bed of herbaceous material in the vessel at room temperature for desired time duration that varies from hours, days to weeks depending upon the plant material used, to produce a highly concentrated extract having a large

proportion of the active principles of the herbaceous material. Methanolic cold extracts were prepared from strawberry to isolate the phenolics and anti- cancer agents (Zhang *et al*., 2008); gum of *Jatropha divaricata* was prepared from pulverized stem percolation method for the isolation of two terpenes viz. ent-3β-14-α-hydroxypimara-7,9 (11), 15-triene-12-one and ent-15(13→8) abeo-8-β-(ethyl) pimarene (Denton *et al.*, 2001). Plant parts of *Bryonopsis laciniosa* were extracted by cold percolation and tested against *S. aureus*, *Micrococcus luteus* and *Bacillus cereus* (Ehsan *et al.*, 2009). Extracts of medicinal plants like *Lawsonia inermis*, *Hibiscus rosa-sinensis* and *Tamarindus indica* were prepared by cold percolation method for their antimicrobial potential (Muthu *et al*., 2005). Cold percolation methods for crude extract preparation have been prepared from *Phyllanthus acidus*; *Lawsonia inermis* and *Pimenta pseudocaryophyllus* (de Paula *et al*., 2009; Jagessar *et al*., 2008; Malekzadeh, 1968).

**Microwave Assisted Extraction (MAE):** Microwave-assisted extraction (MAE) has received considerable attention as an alternative to solid-liquid extraction for the extraction of secondary metabolites from plants (Dandekar and Gaikar, 2002). This process is rapid, economical and efficient than traditional methods. Microwaves allow simple, rapid and low solvent consuming processes (Jain 2009; Kiss *et al*., 2000). Microwave heating disrupts the weak hydrogen bonds increasing the penetration of the solvent into the matrix, and thus, facilitates the salvation of the target compounds. Microwaves act directly on water molecule within the cells *in situ*, ensuing a rapid increase in the cell temperature. The resulting pressure due to water vapor of the cell leads to rupture of the cell membranes and cell walls (Gao *et al*., 2004; Lucchesi *et al*., 2004) and generates cavities through which the internal material flows out and the solvent penetrates into the cell easily, thereby increasing the yield of the phytoconstituents (Chemat *et al*., 2005; Dandekar & Gaikar, 2002). Several medicinal moieties like Taxol, Artemisinin, Embein and coumarins have been efficiently extracted from respective medicinal plants using microwaves when compared to conventional processes (Hao *et al.*, 2002; Latha, 2007; Martino *et al*., 2006;

Mattina *et al*., 1997). Piperine is an alkaloid extracted from *Piper nigrum* which is radioprotective, bioenhancer and insecticidal has been efficiently extracted using microwaves (Raman and Gaikar, 2002). Berberine, palmatine and jatrorrhizine have been extracted through microwaves from *Coscinium fenestratum* (Deevanhxay *et al*., 2009).

### 3.9.2 Phytochemical Analysis

Phytochemical analysis involves the assessment for the presence of broad chemical classes of compounds like saponins, glycosides, phenolics, alkaloids and tannins. Further fractionation of the extracts as per the broad class present and biological activity of each class is carried out in a bioassay guided fractionation protocol. The broad class of compounds which exhibits the bioactivity is taken up for the isolation of the bioactive moiety. Further isolation is achieved through thin layer chromatography (TLC) or high performance liquid chromatography (HPLC). Phytochemical screening of the crude extracts of *Urena lobata* indicated the strong presence of phenolics and absence of alkaloids as confirmed by ferric chloride and Dragendroff's reagent respectively (Adewale *et al*., 2007). Methanolic crude extracts of *Acacia nilotica* was fractionated as stronger acids, neutral compounds, bases and weaker acids using methods of Roberts *et al*., (1981) and subsequently resolved by TLC and column chromatography (Raghavendra *et al*., 2006). Similarly *Murraya paniculata* crude leaf extracts was analyzed phytochemically and antibacterial activity of a coumarin was reported against *S. aureus*, *B. cereus* and *C. albicans* (Zachariah *et al.*, 2009). Ethanolic extracts of *Ocimum sanctum*, *Rhododendron setosum*, *Eucalyptus globulus*, *Azadirachta indica* and *Elscholtzia fruticura* were analyzed for the presence or absence of different chemical classes (Chhetri *et al*., 2008). *Caeselpinia pulcherrima* exhibited the presence of tannins and saponins which were exhibiting highest antibacterial activity followed by *Euphorbia hirta* which exhibited the presence of cardiac glycosides and steroids apart from the presence of tannins and saponins. *Casurina equisetifolia* followed *Eurphorbia hirta* in the antibacterial activity and it indicated the presence

of tannins, saponins and flavonoids (Parekh and Chanda, 2007). Thus knowing the broad chemical class as well as bioactivity of these classes is an important aspect in designing a separation protocol for the isolation of the bioactive compounds. Further one step liquid- liquid partitioning of the crude extracts could also be used for the isolation of a particular broad class from the plant material.

### 3.9.3 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a simple, rapid, and affordable technique for the analysis of natural products from different sources. Although it is mainly used for qualitative purposes, combined with other analytical procedures, such as UV/visible spectrophotometry, it can become a reliable and sensitive method for mass purification. Preparative thin layer chromatography (PLC) is an effective and easy means of obtaining small quantities of compounds from complex mixtures, which can be used for different purposes viz. structure determination, by spectroscopic methods or investigation of their biological activity. It has the advantage of simplicity, versatility, high speed, specific sensitivity and simple sample preparation. It is regarded as the most convenient approach to analysis and fractionation of herbal extracts by many pharmacopoeias.

Preparative TLC has been used for isolation of different classes of chemical compounds present plant crude extracts. Methanolic extracts of leaves of *Eucalyptus camadulensis* and *Terminalia catappa* have been separated for the isolation of the anti-bacterial agents (Babayi *et al*., 2004), pyrrolizidine alkaloids have been isolated from crude root extracts using liquid- liquid partitioning and further separation through TLC distinguished by their retention time. The highest retention was of more polar N-oxides followed by tertiary bases exhibiting the medium retention and diesterified N-oxides having the lowest retention (Mroczek *et al*., 2006).

The other examples of use of preparative TLC for isolation of bioactive from plant extracts include separation of ramiflorine A and B from *Aspidosperma ramiflorum* (Tanaka *et*

*al*., 2006), indole alkaloids pyrrazilidine alkaloids from *Brachyglottis hectori* (Bai *et al*., 2006), tazeline-type alkaloids from *Galanthus elwessi* (Sidjimova *et al*., 2003), alkaloids from *Clerodendron paniculatum* and *Psychotria malayana* (Hadi and Bremner 2001), benzylisoquinoline alkaloids from *Anisocycla jollyana* (Kanyinda *et al*., 1995), isoquinolone alkaloids from *Corydalis rutifolia* (Sener *et al*., 1992), cyclopeptide alkaloids from *Zizyphus xylopyra* (Pandey *et al*., 1986). Preparative TLC combined with open-column chromatography remains a straightforward means of purifying natural products, although variants of planar chromatography are also being exploited in natural product isolation (Hostettman *et al*., 1998)

### 3.9.4 Bioautography: Bioassay guided TLC separation.

Planar chromatography is a powerful technique for separating certain classes of compound of biological interest. Bioautography is a method to localize antibacterial activity on a chromatogram. It is an essential technique in natural products chemistry. Without bioassay-guided fractionation it would be an overwhelming task to isolate active compounds from a crude extract or identify the fractions of interest. Plants extracts are complex mixtures of natural chemical substances, which have antimicrobial effect. In order to have any assurance of isolating the active compound(s) it would be necessary to isolate every component of an extract or at the very least all of the major components. All of this would be time consuming and costly. TLC bioautography allows the screening of plant material and subsequent bioassay-guided fractionation and isolation.

Broadly bioautographic methods are classified as (i) agar diffusion (ii) agar overlay (iii) direct TLC bioautographic detection. Direct bioautography is applicable to microorganisms that can grow directly on the TLC plate (Botz *et al.,* 2001; Horvath *et al*., 2002). The agar-overlay technique is a hybrid of the two other methods and is applicable to a broad spectrum of microorganisms. It produces well-defined zones of inhibition and is not sensitive to contamination. Active compounds are transferred from the stationary phase to agar layer

(which contains the microorganism) by a diffusion process. After incubation, the plate is sprayed with a tetrazolium salt (e.g. MTT), which is converted to a formazan dye by the microorganism. Inhibition zones are observed as clear spots against a purple background (Freixa *et al*., 2001; Hostettmann, 1998; Wedge and Nagle, 2000).

Three antibacterial phloroglucinols- hyperbrasilol B, hyperbarsilol C and isohyerbrasilol B have been isolated from petroleum ether extracts of leaves and flowers of *Hypericum brasiliense* using TLC bioautography with *Bacillus subtilis* as the test microorganism (Rocha *et al.*, 1996). Eugenol was isolated from *Ocimum gratissimum* (Nakamura *et al*., 1999); bezophenanthridine alkaloids- dihydrochelerythrin and dihydrosanguanrine from *Bocconia arborea* (Navarro and Delgardo, 1999) using direct bioautography. Direct bioautography was also used in identification of an antifungal compound canthin-6-one from *Zantoxylum usambarense* (Weidong *et al*., 2002), 2-methoxy-1, 4-naphthoquinone (MNQ) from *Impatiens balsamina* (Yang *et al*., 2001). Thymus phenols, rosmarinic and caffeic acids, were detected from some *Thymus* taxa (*Thymus vulgaris*, *Thymus serpyllum*, *Thymus citriodorus* and *Thymus citriodorus*) by TLC (Hovarth *et al*., 2002). Chaaib *et al*., (2003), monitored antibacterial and antioxidant activities of eleven compounds isolated from the dichloromethane extract of *Fagara zanthoxyloides*. Alkaloids, phenols and flavonoids from crude extracts of 15 Indian medicinal plants have been identified as bioactive phytoconstituents possessing antibacterial activity against extended spectrum of β-lactamase producing multidrug resistant bacteria (Ahmad and Aqil, 2006). Bioautography also led to the isolation of a resistance modifying agent piperitone present in essential oils of *Artemisia annua*, *Artemisia dracunculus* and *Eucalyptus globus* which potentiate nitrofurantoin activity against *Enterobacter cloacae* by TLC bioautography (Shahverdi *et al*., 2006). Astilbin or 3-O-α-L-rhamnoside-5,7,3’,4’- tetrahydroxydihydroflavonol was isolated from crude extracts of *Harungana madagascariensis* (Moulari *et al*., 2006). Chelidonium

alkaloids have been tested by TLC bioautography to decipher their antibacterial potential (Sarkozi *et al*., 2007).

## 3.10 Biological Assays

### 3.10.1 *In vitro* Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing is an *in vitro* procedure to evaluate the potency of killing of infectious microbes (bacteria and fungi) of antimicrobial agents in a pathological laboratory. The procedures used can be broadly differentiated into diffusion and dilution techniques. Initially diffusion techniques were developed which was followed by dilution techniques. The diffusion techniques have been generally classified as the PRESCREEN ASSAY and the dilution techniques are SCREENING ASSAYS as they provide quantitative reliable information about the antimicrobial agent with respect to reduction in number of the infectious microorganisms in a defined time period. *In vitro* antimicrobial susceptibility test (AST) is used to determine the efficacy of potential antimicrobials from biological extracts against a panel of microbes and determine their potency in terms of Minimal Inhibitory Concentration (MIC). Standardized *in vitro* tests help in comparing the efficacy of natural products to that of currently used antibiotics (Devienne and Raddi, 2002).

### 3.10.2 Prescreen Assay

These assays comprise diffusion methods like the Kirby Bauer (KB) Disc assay and Agar Well Diffusion Assay. A bioassay, which is applied to a large number of initial samples to determine whether or not they have any bioactivity of the desired type, is referred to as a **PRESCREEN ASSAY**.

#### 3.10.2.1 Kirby Bauer (KB) Disc Assay

Kirby Bauer and his colleagues standardized the filter paper disc diffusion method initially developed by Vincent and Vincent (1944) and further improvised by Bondi *et al*., (1947). The KB disc assay is advancement in methodological standardization and zone diameter interpretation

against a quantitative MIC. This method generally is used for testing the pure compounds against a defined set of microorganisms referred to as the microbial test panel and their activity against the microbe is classified as Sensitive, Intermediate and Resistant based on the zone diameter obtained. These values of inhibition zones which classify the microbe as sensitive, intermediate or resistant to a particular antimicrobial agent are referred to as MIC breakpoints. Currently two standards references by CLSI (Clinical Laboratory Standards Institute) and EUCAST (European Union Community on Antimicrobial Susceptibility testing) are followed globally. Antibiogram is the resistance pattern of a microorganism against a battery of antimicrobial agents tested by KB disc assay.

**3.10.2.2 Agar Well Diffusion (AWD) Assay**

Agar well diffusion assay is an advanced version of the ditch well assay initially designed by Alexander Fleming which uses wells prepared in agar to evaluate the antimicrobial qualities of antiseptic solutions. The technique is popularly referred as Agar Well Diffusion Technique, Radial Diffusion Assay or Cylinder Plate Assay (Lehrer, 1991). The test involves placing the sample of the test material in wells made in agar plates which are further swabbed with the test microorganism. Clear zones referred to as 'zones of inhibition' are formed across the well showing the susceptibility of the test organism to the test material. This assay is a popular screening assay employed by clinical microbiologists and pharmacogonists working in the area of antimicrobial drug discovery from natural resources (Nakamura *et al.,* 1999). Antibacterial activity of acetone, methanol and aqueous extracts of *Scadoxus multiflorus* has been evaluated by AWD assay (Aliero *et al.,* 2008). Antibacterial activities of ethanol and aqueous extracts of leaves of *Lecaniodiscus cupanoides* were evaluated by AWD assay (Oboh *et al*., 2008). Similarly antibacterial studies on extracts of *Flabellaria paniculata* (Abo and Olugbuyiro, 2004), *Polyalthia longifolia* (Ghosh *et al*., 2008), *Mimosa pudica* (Gandhiraja *et al*., 2009), *Ficus racemosa* (Mandal *et al.,* 2000), *Piper guineese*, *Zingiber officinale*, *Aframomum meleguta* (Konning *et al.,* 2004)

were done by AWD assay. Antimicrobial potential of isoflavones from *Iris germanica* (Orhan *et al.,* 2003), essential oils from *Mentha suaveolens* (Qumzil *et al*., 2002) has been done using AWD assay.

AST standard tests can be divided into diffusion and dilution methods. Common diffusion tests include agar well diffusion; agar disk diffusion and bioautography, while dilution methods include broth micro/ macrodilution and time kill studies.

**3.10.3 Microplate Broth Dilution Assay and MIC Determination**

Broth dilution methods are quantitative rapid methods suitable for microorganisms with variable growth rate. The results are expressed in terms of MIC (Minimal Inhibitory Concentration) values, which "the lowest concentration which resulted in maintenance or reduction of inoculum viability after a specific incubation period" (Carson *et al.,* 1995). The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. MIC can be determined by semi-automated microtitre method, having 96 well or 384 well configurations. Indicators such as tetrazolium salts (TTC, MTT), fluorescein diacetate (Chand *et al.,* 1994) and resazurin (Mann and Markham, 1998) can be used for the determination of the end-point. The end-point of all experiments is the observation of no growth in one of the wells, interpreted as the MIC (Sommers, 1980). Microbroth dilution method has several advantages over the traditional MIC determination method as it uses fewer amounts of media, easy in handling a large sample size and is more rapid and repeatable (CLSI, 1997).

Antifungal activity of essential oil of tree tea was compared with ketoconazole, econazole and miconazole using microboth dilution method against *Malassezia* species (Hammer *et al.,* 2000). Essential oils of *Satureja parnassica*, *Santiria trimera*, *Achillea multifida* and *Salvia rignens*, antimicrobial flavonoids from *Dalea scandens* have been evaluated for their MIC using microbroth dilution method (Baser *et al.,* 2002; Martins *et al.,* 2003; Nanayakkara *et*

*al.,* 2002). Some plant extracts as methanol and dichloromethane extracts of seeds of *Prunus padus* and *Prunus spinosa* and ethyl acetate extract of roots of *Dalea scandens* (Kumarasamy *et al.,* 2004; Nanayakkar *et al.,* 2002) having significant activity against methicillin resistant *Staphylococcus aureus* have been determined by microbroth dilution method. Antibacterial activities of arjunic acid, arjungenin and arjunetin from the bark of *Terminalia arjuna* have been evaluated against *S. aureus* and *S. epidermidis* by microbroth dilution assay (Singh *et al*., 2008). Alkanin and shikonin (naphthazarines) and their derivatives from *Arnebia euchroma* were found to possess anti-staphylococcal activity in MIC range of 1.56- 6.25 µg/ml by *in vitro* microplate bioassay. Simvaststin and fluvastatin, the anti-hypercholesterolemic drugs have been tested for their anti-MRSA activity by microplate dilution assay. The MIC of simvastatin was 74.9 µg/ml against MRSA (Jerwood and Cohen, 2008). Hinokol, magnalol and piperitylmagnalol from *Magnolia officinalis* possess anti-MRSA activity in the MIC range of 6.25-25 µg/ml by *in vitro* microtitre based assay (Jr Syu *et al*., 2004). Abyssomicin C, isolated from *Verrucosispora* spp. possess strong anti-staphylococcal activity with the range of 4-13 µg/ml as determined by *in vitro* microplate assay (Reidlinger *et al*., 2004).

Thus, dilution methods have been found to be more appropriate for assaying polar as well as non-polar plant extracts, for the determination of MIC and MBC values. Further dose response curve can also be performed easily and efficiently which helps in determination of $IC_{50}$ and $IC_{90}$ values (Cos *et al*., 2006).

**3.10.4 Time kill studies and Plate count Assay**

Time kill studies are carried out to evaluate the efficiency of the kill induced by pure compounds, combinations of compounds and extracts at their MIC values and simultaneously help in quantitative estimation of the viable cell counts which is an important facet of pharmacokinetics and pharmacodynamics during animal studies for deciding the dose of the drug. Numerous antimicrobial agents in their pure form and as combinations have been

subjected to time kill studies using microbroth dilution assay to evaluate their efficacy (Climo *et al*., 2001; Lopez- Brea *et al*., 1998; Mandal *et al.,* 2004; May *et al*., 2000; Tsao and Yin, 2001; Yin *et al.,* 2002). Plant extracts have also been subjected to time kill studies to assess their efficacy over standard antibiotics and their combinations for their use as herbal drugs (Lipipun *et al*., 2002; Okemo *et al*., 2001; Yin *et al*. 2002). Plate count assay is a quantitative estimation of the time kill induced by a test compound or a combination in terms of Colony Forming Units (CFU's). Appropriate dilution of the aliquots of the test combinations at different time intervals are plated over a nutrient agar plate as a point inoculum within a range of 5μl to 10μl and incubated at 37°C for 18 h. The number of viable colonies are counted and expressed as Cfu's $log_{10}$/ml. Plate count assay provides a clear picture of post antibiotic effect also which is not evident in agar well diffusion assay and microbroth dilution assay (EUCAST, 2000, Miles and Misra, 1938).

Time kill studies of trovafloxacin, penicillin and vancomycin against *Streptomyces oralis* have been assessed using surface plate count as per Miles and MIsra method to arrive to the conclusion that time kill of trovafloxacin was superior to other antibiotics (Rafey, 2001). Rifampin resistance in *Mycobacterium tuberculosis* has also been assessed by viable surface count method (Billington *et al.*, 1999).

## 3.11 Analytical techniques for the characterization of bioactive compounds

### 3.11.1 Mass Spectrometry (MS)

Screening natural compounds for biological activity continues today in the never-ending quest to find new compounds for use as or in drug discovery. Isolating and purifying the new compounds for the active function plays an important role in the pattern. Targeting a promising compound has to be the first step. In this context, dereplication of natural product extracts helps in rapid identification of novel, biologically active metabolites and is an integral part of the drug discovery process. Using high- throughput screening procedures it is possible to integrate the physical and chemical data with the biological activity of well defined sub-fractions of the

crude extract (Cordell and Shin 1999). Next, the structures of the new compounds have to be determined before the structure-activity relationships (SARs) can be analyzed. The mass spectrometry detects the isolated molecules. Mass spectroscopy (MS) is one of the indispensable analytical techniques. As the name implies mass spectroscopy characterizes each of the components individually by providing information on the molecular weight as well as on the structure of the analytes. Today mass spectrometry, in its myriad forms, has become an indispensable analytical tool in bio-pharmaceutical research and development (Cai *et al.* 2002). Its applications span the entire drug discovery and development process from target identification to lead identification to animal and human testing.

### 3.11.2 Elemental Analysis

Elemental analysis experimentally determines the amount (typically a weight percent) of an element in a compound. The most common type of elemental analysis is for carbon, hydrogen, nitrogen and oxygen also referred to as CHNO analysis. This type of analysis is especially useful for organic compounds. The elemental analysis of a compound enables one to determine the empirical formula of the compound. The empirical formula is the formula for a compound that contains the smallest set of integer ratios for the elements in the compound that gives the correct elemental composition by mass. Thus this analysis confirms the tentative fragmentation pattern obtained through mass spectroscopy. Hippuric acid has been found in *Tiliacora acuminata* male plant based on CHN analysis (Selvaraj *et al*., 2009). CHN analyzer has also been used in identification of antioxidant constituent in *Cotinus coggygyria* (Westenburg *et al*., 2000).

## 3.12 Mode of action

From the discovery of penicillin in 1928 and during the four decades after World Wall II, many advances were made in antimicrobial therapy. Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principle mode of action. The common modes of action are interference with the cell membrane and cell wall, interference

with nucleic acids, and enzyme interactions (Hugo and Russell, 1999; Neu, 1992; Tenover, 2006). NADH cytochrome C reductase has been found to be inhibited by licochalcones from *Glycirrhiza* which have a potent antimicrobial activity against gram positive bacteria (Haraguchi *et al*., 1998)

Extensive morphological changes in *E. coli* O157:H7 were observed with oregano oil at MIC concentration indicating bactericidal activity (Burt and Reinders, 2003). *Lactobacillus fermentum* culture supernatant induced antibacterial activity against *S. aureus*, *Salmonella* and *Shigella*. It was attributed to destruction of cell membrane leading to pathogenic cell shrinking or cracking as found in the SEM studies (Klayraung and Okonogi, 2009). Prominent cell damaging activities of methanolic extract of *Ocimum basilicum* was observed in *S. aureus*, *P. aeruginosa* and *L. monocytogenes* in the scanning electron micrographs (Kaya *et al*., 2008). N-alkyl-thio-β-lactams have been found to selectively inhibit MRSA. They possess bacteriostatic activity which is attributed to the modification of coenzyme A into alkyl-Co A with mixed disulphides thereby inhibiting fatty acid biosynthesis. This was further confirmed by scanning electron microscopic studies as there was no change in the natural shape of *S. aureus* unlike the penicillin which caused cell wall damage in MSSA (Revell *et al*., 2007). It has been found that epigallocatechin gallate reduces the tolerance of MRSA to high salt concentration and low pressure in their external environment thereby damaging the bacterial cell wall and potentiating the activity of β-lactam antibiotics (Zhao *et al*., 2001).

*Myrtus communis* extracts have been found to induce their antibacterial effects by induction of free radicals and significantly reducing the expression of SOD and catalase by *S. aureus* (Najar *et al.*, 2009). Flouroquinolones antibiotics norfloxacin, ofloxacin and ciprofloxacin inhibit the DNA gyrase activity which is parallel to their antibacterial activities indicating the mode of their action (Nishimo and Takahata, 1988). *Eleutherine americana* extract have been found to inhibit protease and lipase enzymes in *S. aureus* apart from enterotoxin production in food thereby acting a biopreservative for food (Ifesan and Voravuthikunchai, 2009).

Acyldepsipeptides have been found to possess potent antibacterial activity against gram positive isolates *in vitro* and in animal models. These have been found to inhibit core of bacterial proteases (Osterhelt *et al*., 2005). Amdinocillin has been found to cause leakage of β-lactam antibiotics apart from binding to penicillin binding proteins (Sanders *et al*., 1987). Polyphenols corilagin and tellimagrandin I from *Arctostaphylos uva-ursi* and *Rosa canina* respectively reduced the MIC of β-lactam antibiotics significantly in MRSA. The mechanism of this remarkable potentiation of β-lactam antibiotic by corilagin and tellimagrandin is attributed to decrease in the production of penicillin binding protein (PBP 2') (Shiota *et al*., 2004).

A variety of mechanisms are being explored to overcome resistance in MRSA using natural products as tools.

# Chapter 4
# MATERIALS & METHODS

# 4.0 MATERIALS AND METHODS

## 4.1 Culture repository

### 4.1.1 Procurement of test isolates

The test cultures including control organisms and clinical isolates of *Staphylococcus aureus* obtained from different sources constituted the antimicrobial assay panel to evaluate the efficacy of antimicrobial leads of *Callistemon rigidus* R.Br. The cultures were procured from Department of Microbiology, All India Institute of Medical Sciences (AIIMS), New Delhi; Department of Microbiology, Government Medical College (GMC), Patiala; Lady Harding Medical College, New Delhi and Microbial Type Culture Collection (MTCC); Institute of Microbial Technology (IMTECH), Chandigarh**.** A high protease expressing strain of *S. aureus* (Sau MTB) was gifted by Dr. A. Staffan, Professor, Microbiology & Tumor Biology Centre, Karolinska Institute, Sweden.

The cultures were classified based on their source as pus isolates, urine cultures, blood cultures, vaginal swabs, wound subcultures, burn isolates, catheter tip isolates, ear discharge swabs, eye isolates and miscellaneous isolates.

### 4.1.2 Maintenance of cultures

The cultures were maintained in Tryptone soy broth (HiMedia) with 2% glycerol and stored at 4°C. The cultures were activated prior to every test. For activation, culture from broth was streaked on Muller Hinton (MH) agar (HiMedia) plate and incubated overnight at 37°C. A single colony was picked and transferred in Muller Hinton broth (HiMedia) and incubated for 18- 24 hours at 37°C.

### 4.1.3 Preparation of 0.5 McFarland turbidity standard (CLSI, 1997)

McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test. 0.5 McFarland standard was prepared by adding 0.5 ml of 0.048M $BaCl_2$ (Merck) to 99.5 ml of 0.18M $H_2SO_4$ (Merck) with constant stirring. The optical density of the solution was recorded at

625 nm and should be in the range of 0.08-0.1. The McFarland solution was stored at room temperature in amber colored bottle to prevent photo-degradation. The shelf life of 0.5 McFarland solution in tightly sealed amber colored bottle is up to 6 months.

**Inoculum Preparation:** Activation of cultures was carried out by streaking on a Muller- Hinton (MH) agar plate followed by overnight incubation at 37°C. A single colony was picked from this plate and transferred to MH broth and incubated at 37°C for 18 h. Subsequently the culture was vortexed vigorously and compared with 0.5 McFarland standard against black stripes on white sheet to adjust visual turbidity. Sterile saline or broth solution was used as a diluent to adjust the visual turbidity of bacterial cultures prior to the test.

**4.1.4: Growth curve studies of test isolates**

Growth is an orderly increase in the number of microbial cells and quantity of cellular constituents. A growth curve clearly depicts the rate of microbial cell multiplication with respect to time. Standardization of growth pattern of microorganisms is thus of prime importance in

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **B** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **C** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **D** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **E** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **F** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **G** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |
| **H** | CTRL | SMP01 | SMP02 | SMP03 | SMP04 | SMP05 | SMP06 | SMP07 | SMP08 | SMP09 | SMP10 | BLK |

**Microplate reader protocol template used for growth curve of test microorganisms (CTRL-** Control-uninoculated MH broth; **SMP**- refers to sample and **BLK** –refers to Blank.

**Fig 4.1 Microplate template for the growth curve of microorganisms**

clinical studies wherein time-kill assays are done to determine the reduction in bacterial cell count in presence of lead compound/ antibiotic either by calculating $\log_{10}$ CFU reduction or by a change in the optical density. Growth curve

studies of clinical and control isolates were carried out on a 96-well microtitre plate using BIOTEK® Powerwave 340 Microplate reader.

To each test well 125 μl of MH-broth and 50 μl of the McFarland adjusted test isolate was added. The control well contained only 125 μl of MH-broth. The plate was incubated at 37°C, 120 rpm. Eight replicates readings were taken to arrive to the average optical density readings and the curve was monitored till 24 h when death phase was initiated.

## 4.2 Antibiogram of test panel: Kirby-Bauer Disk Assay (KB Disk Assay)

KB Disk Assay was used to evaluate the susceptibility of test microorganisms against a battery of 37 antibiotics broadly classified in as quinolones, aminoglycosides, penicillin's, macrolides, oxazolidones, cepahlosporins, glycopeptides, tetracyclines, carbapenums etc. 22.5 ml molten MH agar (HiMedia) was poured in 100 mm sterile petri dish to give a mean depth of 4.00 ± 0.5 mm for carrying out the antibacterial resistance profiles. 0.5 McFarland standardized inoculums of 18 h old test microorganisms were swabbed on the 24 h old MH agar plates using sterile cotton swabs.

Antibiotic disks (HiMedia) were placed on the surface using a sterile dispenser and pressed lightly with sterilized forceps so that disk was properly studded into the medium. In each plate four disks were radially placed at almost 30 to 36 mm distance to avoid overlapping of the zones of inhibition. The plates were incubated at 37°C for 18 h. All the tests were performed in triplicates. Zone diameters were measured with Hi Antibiotic Zone Scale- C (HiMedia) through bottom of the plates. Results were reported as clinically "resistant", "intermediate" and "susceptible" strains according to zone diameter recommended by Clinical and Laboratory Standards Institute (CLSI, 2002).

## 4.3 *mec A* characterization of the test isolates

### 4.3.1 Isolation of bacterial genomic DNA

A modified CTAB method was followed for bacterial genomic DNA. Bacterial culture was grown

overnight in MH broth and 2 ml of the culture was transferred into a micro-centrifuge tube and centrifuged at 8000 rpm, 5 min to obtain the bacterial pellet. The pellet was re-suspended in 740 µl of Tris EDTA (TE) buffer (pH 8.0). To this 20 µl of 100 mg/ml lysozyme was added and incubated at room temperature for 5 min, followed by the addition of 40 µl of 10 % SDS and 3 µl of 20 mg/ml proteinase K, gentle mixing and incubation for 1 h at 37°C. Then 100 µl of 5M NaCl was added and mixed thoroughly. After addition of 100 µl of 10% CTAB-0.7M NaCl solution, the tubes were incubated for 10 min at 65°C. Equal volume of chloroform and isoamyl alcohol (24:1) was added, mixed well and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube and an equal volume of phenol, chloroform and isoamyl alcohol (25:24:1) was added, mixed well and centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a new tube and 3µl RNAse (10 mg/ml) was added. The tubes were incubated at room temperature for 5min after which 0.6 volumes of cold isopropanol was added, mixed gently and incubated at 4°C for 30 min for maximum DNA precipitation. The DNA pellet was obtained by centrifugation at 12000 rpm, 15 min. The DNA was washed with 70% cold ethanol and re-suspended in 30 µl TE buffer and kept overnight at 4°C. The DNA was stored at -20°C until further use. The isolated DNA was analyzed using horizontal 0.8% agarose gel electrophoresis.

#### 4.3.2 PCR for the detection of *mec A* (Ruppe *et al.*, 2009)

*mec A* pair of primers derived from the region of the *mec A* gene, were used for the detection of methicillin resistance in the *SCCmec* cassette in the clinical and standard staphylococcal isolates in the test panel. The forward primer corresponded to mA1-5’ TGCTATCCACCCTCAAACAGG3’ and the reverse primer to mA2-5’AACGTTGTAACCACCCCAAGA3’. The final reaction volume for the PCR was 20 µl and comprised of: DNA (from test bacteria)-1 µl; dNTPs (stock 2.5mM each)-4 µl, forward primer (10 µM)-0.5 µl; reverse primer (stock 10 µM)-0.5 µl; 10X PCR buffer-2.5 µl and sterile double distilled water-11 µl. DNA amplification was carried out in thermal cycler

(Mycycler, BIORAD). Initial denaturation was done for 5 min at 95°C. After this 1U of Taq DNA polymerase (0.5 µl of Taq DNA polymerase stock of 5U/ µl) was added. 30 cycles of amplification were then performed as follows: denaturation at 94°C for 60 s, annealing at 57°C of 60 s and DNA extension at 72°C for 120 s, followed by an additional cycle of 4 min at 72°C to complete partial polymerizations. Amplified products were analyzed using horizontal 1.5% agarose gel electrophoresis.

**4.3.3 PCR for the detection of specific methicillin resistance (Rallapalli *et al.,* 2008)**

The second primer set derived from *mec A* region was used for identification of specific methicillin resistance associated with the coagulase gene. The forward primer corresponded to nucleotides 1282 to 1303 (5' AAAATCGATGGTAAAGGTTGGC) and the reverse primer was complementary to nucleotides 1793 to 1814 (5'*AGTTCTGCAGTACCGGATTTGC*). Bacterial genomic DNA (aliquot of 1µl containing 50 ng of genomic DNA) was added to PCR mixture consisting tenfold concentrated reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8·3), with final concentrations of 0.5 mM each dNTP, 2.5 mM $MgCl_2$ and 0.1 µM of each *mec A* primer. This mixture was supplemented with 2U of Taq DNA polymerase. The final reaction volume for PCR was 20 µl.

DNA amplification was carried out in an automated thermocycler (My Cycler, BIORAD). After an initial denaturation step for 5 min at 95°C, 30 cycles of amplification were performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and DNA extension at 72°C for 90 s, followed by an additional cycle of 5 min at 72°C to complete partial polymerizations. Amplified products were analyzed using horizontal 1·5% agarose gel electrophoresis.

## 4.4 Collection of plant material

Leaves of *Callistemon rigidus* were collected from Thapar Centre for Industrial Research and

Development (TCIRD), Thapar Technology Campus, Patiala. A voucher specimen was deposited at the herbarium of the Department of Biotechnology & Environmental Sciences and numbered as #7San03. Healthiness of the leaves was confirmed by culturing them on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates.

## 4.5 Preparation of crude leaf extract

Fresh and healthy leaves (free from any infection) of *Callistemon rigidus* were thoroughly washed under running water for 30 min to remove dirt, air dried followed by complete drying at 37°C in a tray dryer. The dried leaves were ground into a coarse powder using a blender. Cold percolation (steeping) method, using methanol as the solvent was used for extraction to obtain the crude extract. 75 g of dried pulverized leaves were dipped in 150 ml methanol (Schott, Germany) and kept at shaking at 120 rpm, 28°C for 72 h. After extraction the solvent was filtered and evaporated *in vacuo* to obtain the crude extract. The percent yield of the extract was recorded and subsequently washed with chloroform. The chloroform layer was pooled and evaporated *in vacuo*. The extracts were stored at -20°C for evaluation of anti-staphylococcal activity and for mass purification of the lead fractions.

## 4.6 Evaluation of bioactivity of crude extract

### 4.6.1 Agar Well Diffusion Assay (AWD Assay)

Antibacterial activity of the crude extract was evaluated by agar well diffusion assay (Perez *et al.* 1990, Lehrer, 1991). 5 mm wells were cut in 24 h old MH agar (Hi Media) plates (mean depth± 4.00mm). The extract was evaluated at fourteen different concentrations within a concentration range of 0.555-66.66 mg/ml as per European Committee of Antimicrobial Susceptibility Testing (EUCAST, 2000) (Table 4.1)

| Concentration (mg/ ml) | Concentration (μg/ μl) | Concentration (μg/ ml) | Effective extract (in μg) in well after dispensing 30μl of the respective stock |
|---|---|---|---|
| 0.555 | 0.555 | 555 | 16.65 |
| 1.11 | 1.11 | 1110 | 33.33 |
| 2.22 | 2.22 | 2220 | 66.66 |
| 3.33 | 3.33 | 3330 | 99.99 |
| 6.66 | 6.66 | 6667 | 199.8 |
| 13.33 | 13.33 | 13333 | 399.9 |
| 20.0 | 20.0 | 20000 | 600.0 |
| 26.66 | 26.66 | 26667 | 799.8 |
| 33.33 | 33.33 | 33333 | 999.9 |
| 40.0 | 40.0 | 40000 | 1200 |
| 46.66 | 46.66 | 46667 | 1398 |
| 53.33 | 53.33 | 53333 | 1599 |
| 60.0 | 60.0 | 60000 | 1800 |
| 66.66 | 66.66 | 66667 | 1999.8 |

**Table 4.1 Concentrations of the crude methanol extract evaluated by Agar Well Diffusion (AWD) Assay**

30 μl of the test extract in dimethyl sulfoxide (DMSO) was dispensed in the test wells. Solvent blank was included as the control. The wells were sealed with molten MH agar. After 15 min the plate was swabbed with 18-24 h old, 0.5 McFarland adjusted culture of the test isolate. Antibacterial activity was interpreted by determining the width of the clear zone of inhibition (diameter of inhibition zone) formed across the test well calculated by measuring the distance between the edge of the well and the outer edge of the inhibition zone. All the tests were performed in triplicates.

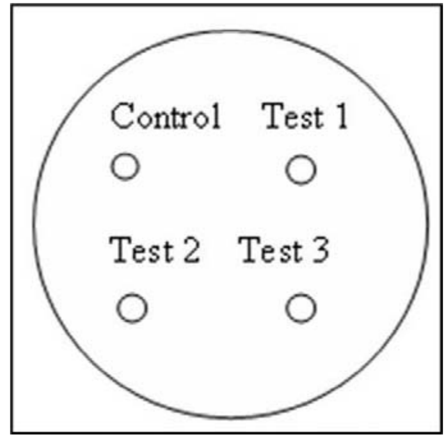



**Fig.4. 2 Petri dish template for AWD Assay**

**4.6.2 *In vitro* microbroth dilution assay: determination of MIC (Jorgensson *et al.*, 1999; EUCAST 2000)**

*In vitro* microbroth dilution method was performed using 96-well microtitre plate to evaluate the MIC of the crude extract against the test panel of clinical and standard staphylococcal

isolates. The turbidity of the test inoculums was visually adjusted by comparing it to a 0.5 McFarland standard using a sterile MH broth. Stock solutions of the crude extract were prepared in the range of 320-0.15625 µg/ml to achieve a final concentration of 80- 0.0195 µg in the test wells.

Two sets of controls were used in the assay. Control $C_1$ comprised of 125 µl MH broth, 50 µl of turbidity adjusted test inoculums and 25 µl of sterile saline. The second control set $C_2$ consisted of 125 µl MH broth, 25 µl of test extract concentrations and 50 µl of MH broth. The test well initially comprised of 125 µl MH broth, 50 µl of turbidity adjusted test inoculums of the test bacteria. $C_1$ (control set 1) was designated by well id A1, A3, A5, A7, A9 and A11. $C_2$ (control set 2) was designated by well id B1, B3, B5, B7, B9 and B11. The test wells were designated from C1 to F2 to C11 to F12. G and H wells with id's2, 4, 6, 8, 10 and 12 were blanks. (Fig.4.3)

| | Isolate 1 | | Isolate 2 | | Isolate 3 | | Isolate 4 | | Isolate 5 | | Isolate 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | C1 | T7 | C1 | T7 | C1 | T7 | C1 | T7 | C1 | T7 | C1 | T7 |
| B | C2 | T8 | C2 | T8 | C2 | T8 | C2 | T8 | C2 | T8 | C2 | T8 |
| C | T1 | T9 | T1 | T9 | T1 | T9 | T1 | T9 | T1 | T9 | T1 | T9 |
| D | T2 | T10 | T2 | T10 | T2 | T10 | T2 | T10 | T2 | T10 | T2 | T10 |
| E | T3 | T11 | T3 | T11 | T3 | T11 | T3 | T11 | T3 | T11 | T3 | T11 |
| F | T4 | T12 | T4 | T12 | T4 | T12 | T4 | T12 | T4 | T12 | T4 | T12 |
| G | T5 | Blk | T5 | Blk | T5 | Blk | T5 | Blk | T5 | Blk | T5 | Blk |
| H | T6 | Blk | T6 | Blk | T6 | Blk | T6 | Blk | T6 | Blk | T6 | Blk |

**Fig 4.3. Microtitre plate template for *in vitro* microbroth dilution assay for determination of MIC of crude extract.**

All the wells except the blanks were initially dispensed with 125 µl MH broth. Subsequently visually adjusted inoculums of the test bacteria were dispensed in the control set 1 and the test wells. It was allowed to incubate at 37°C, 120 rpm and 2.5 h duration to overcome the lag phase as predetermined in the growth phase studies. Subsequently 25 µl of the test extract in graded doses was dispensed in the control set 2 and test wells sequentially to have a serial two fold

dilution of the test extract. These were again incubated at 37°C, 120 rpm for 24 h. Subsequently 10 µl aliquots were withdrawn to determine the viable counts on MH agar medium by modified Miles and Misra method. 20 µl of 0.02% MTT in all wells and incubated for an hour. MIC was noted as the lowest concentration of the test extract which did not yield a visible growth indicated by dye reduction from colourless to pink.

### 4.7 Phytochemical analysis of crude extract

Phytochemical screening of the herbal extract determines the biologically active non- nutritive constituents that contribute to the flavor, colour and other characteristics of the plant part. Examples of these are alkaloids, flavonoids, tannins, saponins, anthraquinones etc. Different tests as mentioned were done to establish the qualitative chemical profile of the methanolic leaf extract of *Callistemon rigidus*.

**4.7.1 Test for alkaloids (Harbone, 1973; Oloyede *et al.,* 2005; Trease and Evans, 1996):** Weighed 0.5 g of the extract. It was defatted it with 5% ethyl ether for 15min. Thereafter the the defatted sample was recovered for 20 min with 5.0 ml of aqueous HCl on a steam bath. Subsequently centrifuged the resulting mixture for 10 min at 3000 rpm to collect the supernatant. To 1.0 ml of the supernatant added a few drops of Marquis Reagent were added. Similarly to another 1.0 ml of the supernatant a few drops of Dragendorff's reagent were added. Turbidity or precipitation with either of these reagents indicated the presence of alkaloids.

**4.7.2 Test for anthraquinones (Trease and Evans, 1996):** To 5.0 g of the crude herbal extract, 10.0 ml of benzene was added. The resulting solution was filtered and to the filtrate 5.0 ml of 10% ammonia solution was added followed by vigorous shaking. Violet color in the ammonical (lower) phase indicates the presence of free hydroxy anthraquinones.

**4.7.3 Test for tannins (Trease and Evans, 1996):** Stirred 5.0 g of the crude extract with 10.0 ml of distilled water. Filtered and added a few drops of ferric chloride reagent to the filtrate. A blue-black precipitate indicates the presence of tannins.

**4.7.4 Test for saponins (Oloyede *et al.,* 2005):** The ability of saponins to produce froth in aqueous solution was used as evidence to the presence of saponins in the extract. 0.5 g of herbal extract was mixed with 5.0 ml of distilled water in a test tube and subjected to vigorous vortexing. The appearance of froth upon slight warming of the vortexed mixture indicated the presence of saponins.

**4.7.5 Test for flavonoids (Aynehchi *et al.,* 1981):** 0.5 g of crude methanol extract was dissolved in 5 ml of water and ethanol mixture (1:1) and centrifuged. The solution was treated with 0.5 ml conc. hydrochloric acid (HCl) and 100 mg of zinc turnings. The presence of flavonoids was indicated by the appearance of pink/ magenta color within two minutes which could be extracted with butanol.

**4.7.6 Test for detection of glycosides, glycolipids:** Prepared the diphenylamine reagent by adding 10 ml of 10% diphenylamine in ethanol, 100 ml HCl and 80 ml glacial acetic acid. This reagent was spayed over the developed TLC chromatogram lightly followed by heating for 30-40 min at 110°C. Appearance of blue spots indicates the presence of glycosides/ glycolipids.

### 4.8 Isolation of crude alkaloidal extract (Hadi and Bremner, 2001)

Based on the results of alkaloid analysis, a protocol was standardized for the extraction of alkaloids from crude methanolic extract (Fig 4.4). The methanolic extract was dipped in 5% acetic acid ($CH_3CO_2H$) for 1.5 h at 120 rpm. The insoluble material was removed by filtration. The aqueous acidic layer was pooled and washed with dichloromethane ($CH_2Cl_2$) so as to remove chlorophylls followed by basification of the aqueous solution and further dichloromethane extraction. Thus organic and aqueous phase factions wee obtained which were then tested for

the antimicrobial activity (refer section 4.11). It was found that the organic phase of dichloromethane fraction of the basified aqueous solution exhibited antimicrobial activity. Further the nature of this fraction was established by phytochemical testing as highlighted in section 4.7.

## 4.9 Modification of procedure- Microwave assisted extraction of the crude alkaloid bioactive fraction (ABF)

Microwave assisted extraction was employed for direct extraction of the alkaloid from leaves by subjecting the dried pulverized leaves to microwaves for cellular destruction initially and the directly extracting by steeping with mild organic acid instead of methanol to avoid interference of the other organic moieties present in the leaves. The final fraction was tested for its antimicrobial activity as well as phytochemically tested using Dragendroff's, Mayer's and Marquis Reagents to confirm the presence of alkaloids (Fig 4.5).

## 4.10 Evaluation of bioactivity of crude ABF

The bioactivity of the crude alkaloid bioactive fraction was evaluated by *in vitro* microbroth dilution method using 96- well microtitre plate and plate count assay.

### 4.10.1 *In vitro* microbroth dilution assay

The *in vitro* microbroth dilution assay of the crude bioactive extract was performed to establish to MIC as well as $MIC_{50}$ and $MIC_{90}$ of the alkaloid bioactive fraction. Stock solution of ABF was prepared and graded doses were evaluated to assess the antimicrobial/anti-staphylococcal potential as per the method discussed previously in section 4.6.2.

### 4.10.2 Plate count assay

The surface plate count assay or Miles and Misra method (Miles and Misra, 1938; Slack and Wheldon, 1978) was used for the estimation of viable count at different test concentrations of the ABF. Briefly 10 µl aliquots were withdrawn using a sterile tip micropipette from the test

samples containing different concentrations of ABF at different time intervals viz. 2, 4, 6, 8, 10, 22, 24h. These were placed as a single drop on MH agar plates divided into number of sectors and observed for the growth of the bacteria as pinhead colonies without the presence of any confluence after incubation of 12-18 h at 37 °C. The concentration responsible for inducing 50% kill of the total viable counts was referred to as $IC_{50}$ during the *in vitro* assay against the test bacteria. The viable count per 10 µl was then extrapolated to viable counts per ml to assess the reduction in viable counts.

## 4.11 Evaluation of bioactivity of microwave extracted ABF

### 4.11.1 Microbroth dilution assay- Determination of $MIC_{50}$ and $MIC_{90}$

The *in vitro* microbroth dilution assay of the microwaves extracted ABF was performed to establish the MIC as well as $MIC_{50}$ and $MIC_{90}$ of the ABF. Stock solution of ABF was prepared and graded doses were evaluated to assess the antimicrobial/anti-staphylococcal potential as per the method discussed previously in section 4.6.2

### 4.11.2 Determination of $IC_{50}$

*In vitro* time kill studies were performed to evaluate the bactericidal/bacteriostatic effect of the ABF against the microbial test panel. Plate count assay as described in section 4.10.2 was performed to determine the $IC_{50}$ of the microwave extracted ABF.

## 4.12 Standardization of TLC fractionation of the Alkaloid Bioactive Fraction

The alkaloid bioactive fraction was separated by TLC plates (Merck Alumina Sheets $GF_{254}$, 5cm X 20cm) using various solvent systems to achieve complete separation of the mixture (Table 4.2) (Svendson and Verpoorte, 1984; Fleiger, 2003; Houghton, 2003; Mroczek, 2003). The fraction was loaded 1 cm above the plate end which was then dipped into the mobile phase in the development chamber. Plates were developed face-down to a distance of 16 cm in horizontal sealed glass chamber (JSGW) after conditioning for 60 min with mobile phase vapour.

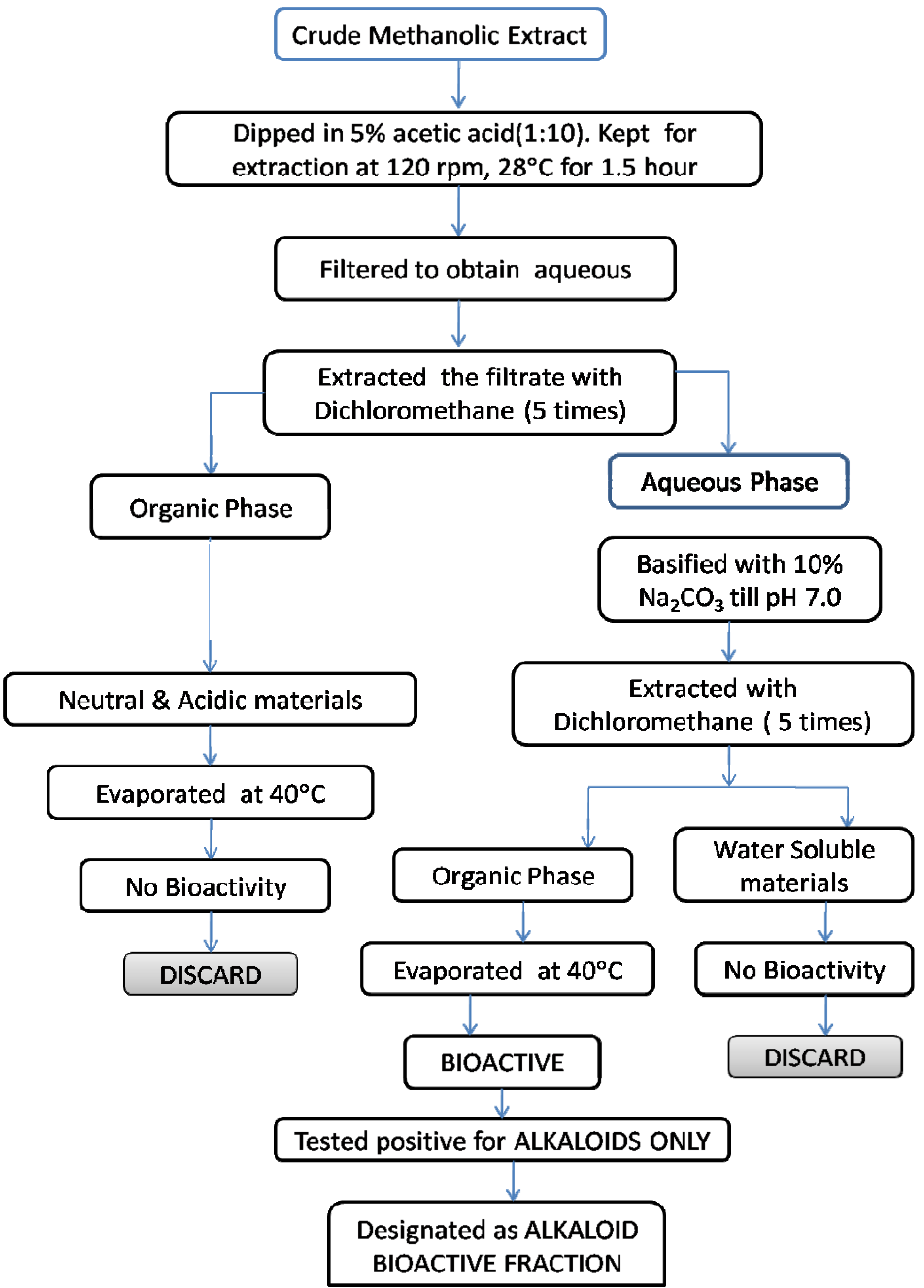



**Fig.4.4 Procedure to isolate Alkaloid Bioactive Fraction from crude methanol extract of leaves of *Callistemon rigidus***

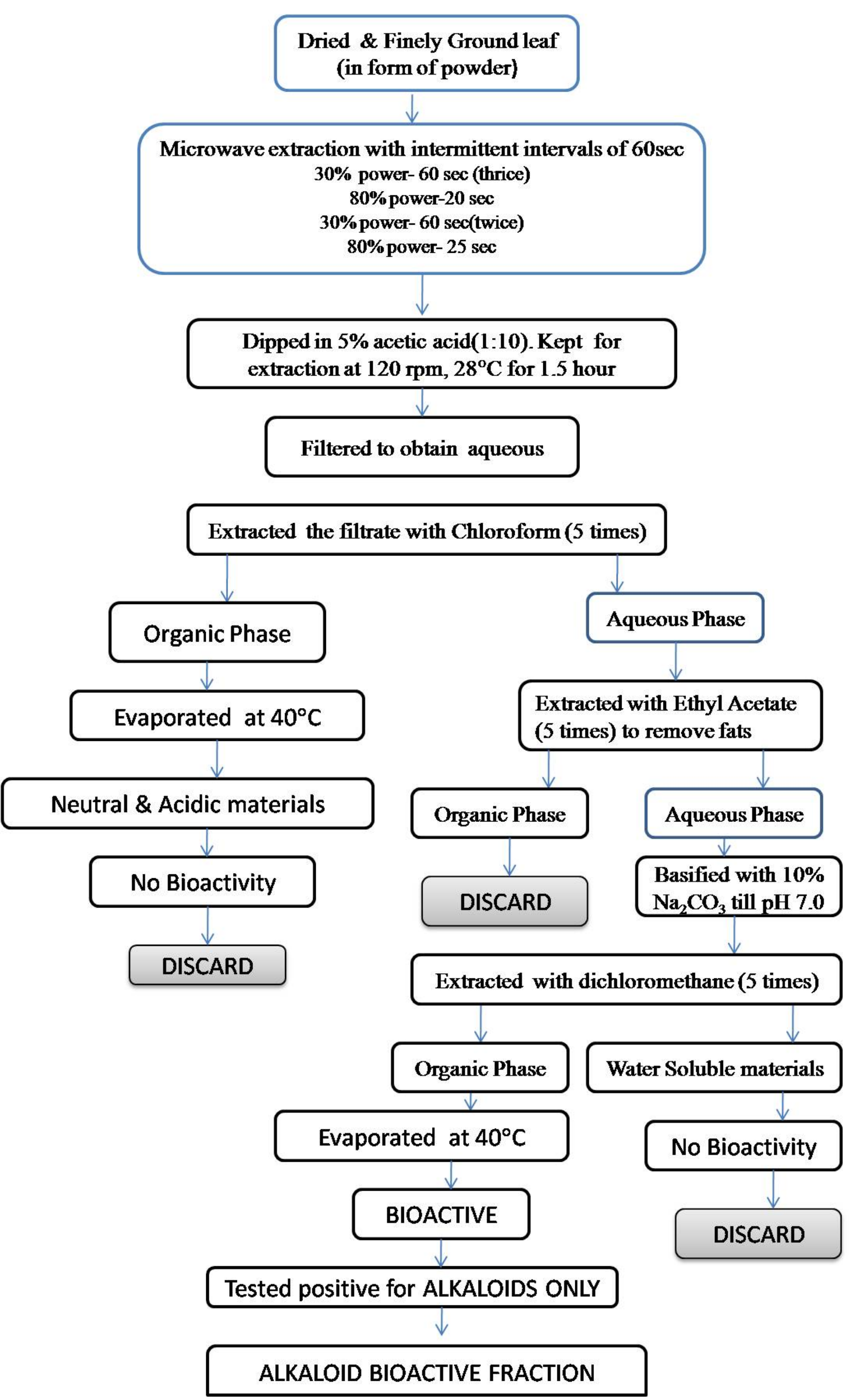



**Fig. 4.5 Microwave assisted extraction procedure of Alkaloid Bioactive Fraction from leaves of *Callistemon rigidus***

After development with the mobile phase, the solvent end run was marked. Then the plates were evaporated to dryness at an ambient temperature of 28°C. Dragendroff reagent (with acetic acid) and iodine vapour were used as the locating reagent to check the separation of the ABF. The separated components were detected as bands/spots and their $R_f$ value was calculated.

| Mobile Phase Code | Solvents Used | Ratio |
|---|---|---|
| S1 | PE: EA: FA | 30:10:1 |
| S2 | BZ: ACE: DEE:10% Ammonia | 4:6:1:0.3 |
| S3 | OLM: 25 % Ammonia | 9:1 |
| S4 | CHL: 25 % Ammonia | 9:1 |
| S5 | CHL: OLM: 25 % Ammonia | 85:15:0.7 |
| S6 | CHL: ACE: DEA | 5:4:1 |
| S7 | CHL: DEA | 9:1 |
| S8 | C.hex: CHL: DEA | 5:4:1 |
| S9 | C.hex: DEA | 9:1 |
| S10 | BZ: EA: DEA | 7: 2: 1 |
| S11 | C.hex: CHL + 0.05% DEA | 7: 3 |
| S12 | DCM: OLM + 1 drop FA | 9: 1 |
| S13 | DCM: OLM + 1 drop Ammonia | 9: 1 |
| S14 | OLM: $NH_4OH$ | 96: 4 |
| S15 | HEX: CHL: DEA | 6: 3: 1 |
| S16 | EA: ISP: Ammonia | 17: 2: 1 |
| S17 | CHL: OLM on Basified Plate | 85: 14 |
| S18 | C.Hex: ISP: 25 % Ammonia | 7: 2: 1 |
| S19 | BZ: EA: DEA | 7: 2: 1 |
| S20 | C.Hex: EA: Ammonia | 17: 2: 1 |
| S18/20 | C.Hex: ISP: EA: Ammonia | 17: 2: 2: 1 |
| S2/18 (a) | C.Hex: BZ: ISP: ACE: DEE: Ammonia | 5:4:2:7:1:1 |
| S2/18 (b) | C.Hex: BZ: ISP: ACE: DEE: Ammonia | 5:4:4:5:1:1 |
| **Abbreviations:** PE- Petroleum Ether; EA-Ethyl acetate; FA-Formic Acid; BZ-Benzene; ACE-Acetone; DEE- Diethylether; OLM-Methanol; CHL- Chloroform; DEA- Diethylamine, C.Hex-Cyclohexane, DCM- Dichloromethane; HEX- Hexane, ISP- Isopropanol. | | |

**Table 4.2 TLC standardization for fractionation of Alkaloid Bioactive Fraction**

### 4.13 Mass extraction of alkaloids- Preparative thin layer chromatography

Mass purification of the alkaloids was carried out by TLC spot/ band elution for its bioactivity against the microbial test panel comprising standard and clinical staphylococci. Briefly the ABF was loaded on a 20cm X 20cm aluminum sheets pre-coated with 0.25 mm layer of silica gel Si

$60GF_{254}$ (Merck) as an even concentrated band. The mobile phase used was cyclohexane: benzene: isopropanol: acetone: diethylether: ammonia (5:4:4:5:1:1). The TLC chamber was properly sealed after pouring the mobile phase for 60 min for conditioning the chamber with the mobile phase vapor at 28°C. The TLC plates were developed face down to a distance of 16cm in the TLC chamber (JSGW). After development the mobile phase was evaporated to dryness. A 5 x 20cm strip was cut from the TLC chromatograms and sprayed with Dragendroff's reagent/ iodine vapour to reconfirm the separated components of the ABF based on their $R_f$ values. These were recovered in methanol and appropriately coded for the evaluation of their bioactivity.

## 4.14 Evaluation of bioactivity of TLC fractionated alkaloids

### 4.14.1 Microbroth dilution assay

Microbroth dilution assay was carried out as described in section 4.6.2 using a limited microbial test panel comprising of five clinical isolates Sau G1, Sau G3, Sau G9, Sau G17 and Sau A4 based on their resistance profile and standard isolate Sau NCTC 6571 to assess the efficacy of the TLC fractions CSS1 to CSS10. The best fractions were selected based on their visual MIC.

### 4.14.2 MIC, $MIC_{50}$ and $MIC_{90}$ determination of the CSS1, CSS6 and CSS8

CSS1, CSS6 and CSS8 were tested against a panel of ten clinical isolates and two standard isolates of *Staphylococcus aureus* to determine the MIC, $MIC_{50}$ and $MIC_{90}$ respectively as per the methods described in section 4.6.2 and 4.10.2

## 4.15 Mode of action of CSS6 and CSS8: Cellular Studies

### 4.15.1 Changes in Cell structure: Scanning Electron Microscopy (Sawer *et al.*, 2005)

Clinical isolates of *Staphylococcus aureus* were treated with CSS6 at MIC and assessed for cellular changes at 12 h and 20 h respectively. The control comprised of untreated cells. The control and treated bacterial samples were centrifuged at 8000 rpm for 5 min and washed with sodium phosphate buffer of pH 7.2. The pellets were obtained and re-suspended in 500µl of primary fixative (composition: 2% paraformaldehyde and 2.5% glutraldehyde in 0.1M sodium

phosphate buffer of pH7.2) at 4°C for 4 h followed by centrifugation at 8000 rpm for 5 min. Subsequently they were washed with sterile distilled water and pellets re-suspended in 500 µl in sterile distilled water. A drop of each suspension was spread as a thin film on small glass piece (1cm x 1cm) and air dried at room temperature for 2 h. An ethanol graded dehydration series (50, 60, 70, 80, 90 and 100%) was used to ensure complete dehydration of the samples. The specimens were then coated with gold in low- pressure argon atmosphere using E-1010 gold ion sputter coating unit. The specimens were subsequently scanned with Hitachi S-3400N scanning electron microscope (Hitachi, Japan) operating at an accelerating voltage of 15.00 Kv and working distance of 10.1 mm.

## 4.16 Mode of action of CSS6 and CSS8: Biochemical studies

### 4.16.1. Assay for extracellular protease (Chavira *et al*., 1984)

A standard curve was prepared using different concentrations of standard protease (Subtilisin A, Type III, from *Bacillus licheniformis*, SIGMA, USA) and the release of peptide fragments bound Azo-dye was spectrophotometrically estimated at 520 nm. One unit of activity was arbitrarily designated as the amount of protein which yields an $A_{520}$ of 0.5.

Extracellular protease activity in the stored supernatant collected at different incubation time was determined by azo-dye impregnated collagenase assay. The microbial test panel was screened for high protease expressing clinical isolates. Sau G3, Sau G5 and Sau G6 were high protease expressing clinical isolates. Briefly 1% of 18- 24 h old cultures of Sau G3, Sau G5, Sau G6 and standard strains Sau MTCC 737 and Sau MTCC 740 were inoculated in MH- broth and incubated initially for 2.5 h in a 96 well Microtitre plate. Then MIC and sub-MIC concentrations of CSS6 and CSS8 were dispensed in the test sets only as standardized during *in vitro* microbroth dilution assay. Aliquots of culture were withdrawn after completion of different incubation

durations viz. 2 h; 4 h; 6 h; 8 h; 10 h and 24 h. These were centrifuged at 12000 rpm, 15 min and the supernatant stored at -20°C till further use.

The reaction solution comprised of 5 mg azocoll (Sigma, USA) suspended in 1 ml of sterile potassium phosphate buffer (pH 7.2) which was maintained at 37°C for an hour. Subsequently 50 µl of the culture supernatant was added followed by a further incubation at 37°C, 65 rpm exactly for 1 h. A control without culture supernatant was run for each set. The reaction was terminated by centrifuging residual azocoll at 12000 rpm for 20 min. O.D. of the resulting supernatant was noted at 520 nm on Microtitre plate reader (Biotek, USA).

#### 4.16.2. Assay for superoxide dismutase (Barriere *et al*., 2001; Barriere *et al*., 2006)

The SOD expression was evaluated from crude extracts of the test panel microorganisms and the highest SOD producing isolates viz. Sau G3, Sau G4, Sau G9 were selected along with standard isolates Sau MTCC 737 and Sau MTCC 740 for assessing the affect of CSS6 and CSS8 on the expression of SOD by staphylococci.

For studying the expression of SOD crude extracts of cells were prepared. The cells were centrifuged at 10,000 rpm for 10 min to prepare a cell pellet. The supernatant was discarded and the cells were washed with phosphate buffered saline (pH 7.0) and pelletized by centrifuging at 10,000 rpm for 10 min. The washed cells were resuspended in 500 µl of the same buffer, 0.1 g of acid washed glass beads (150-212 µm) were added, and the samples were vortexed twice for 5 min with intermittent ice cooling. The extract was centrifuged for 10 min, 8000 rpm at 4°C and the supernatants were collected and kept at -20°C. The SOD activity was measured using a RANSOD kit (Randox Co., Antrim, United Kingdom). 0.05 ml of cell lysate was mixed with 1.7 ml of mixed substrate comprising of 0.05 mM xanthine and 0.025 mM INT. Further 0.25 ml of XOD was added to the above mixture and absorbance was recorded at 505 nm after 30 s (A1) and 3 min (A2). The blank comprised of 0.05 ml buffer and 1.7 ml of mixed

substrate and 0.25 ml of XOD. SOD was determined as number of units of SOD per mg of cellular protein.

#### 4.16.3 Assay for extracellular lipase (Kouker and Jaeger, 1987)

Rhodamine B and olive oil based specific plate assay was used to evaluate the extracellular lipase production among clinical isolates of *S. aureus*. The composition of growth medium (g/l) was nutrient broth-8 g; sodium chloride-4 g; and agar- 10 g. The pH of the medium was adjusted to 7.0 followed by autoclaving at 121°C; 15 psi for 15 min 1 mg/ml stock of rhodamine B (Loba chemie) was prepared in distilled water and sterilized by filtration. To the autoclaved growth medium at 60°C was added olive oil (2.5 % v/v) and rhodamine B stock solution (0.001% w/v) was added with vigorous stirring and emulsified by mixing for 1 min. The growth medium was then allowed to stand for 10 min at 60°C to reduce foaming following which 20 ml of medium was poured into sterile petri dish. A standard curve of lipase was prepared using lipase from *Candida rugosa* (Sigma, USA) in a concentration range of 7-70 Units/ml. The different concentrations were dispensed in 3 mm wells and incubated at 37°C for 16 h initially upto 48 h. The results of halos were recorded by irradiating the plates with UV light at 350 nm under a gel doc XR (BIO RAD). A standard curve was then plotted using log lipase concentration versus halo diameter in mm.

The cell free culture supernatant of high lipase expressing clinical isolates viz. Sau G10, Sau G17, Sau G23, Sau G24, Sau G25 and two standard isolates Sau MTCC 740 and Sau MTCC 737 were collected from control as well as test wells treated with MIC and sub-MIC concentration of CSS6 and CSS8. 10 µl of the cell free culture supernatant of control as well as test sets of the test bacteria were dispensed in 3 mm wells in the rhodamine nutrient agar plate followed by incubation at 37°C for 16 h and 48 h. The reading of zone halos for lipase production was recorded by irradiating the plates with UV light at 350 nm under gel doc XR (BIO RAD).

The plates were incubated for 48 h at 37°C following which lipase producers were identified by irradiating plates with UV light at 350 nm. Agar plates containing trioleoylglycerol and rhodamine B appear opaque and are pink colored. After 16 h of incubation bacterial colonies began to show an orange fluorescence; with continuing incubation time orange fluorescent halos were formed around the colonies of lipase producing strains.

## 4.17 Tentative nature of leads

### 4.17.1. Phytochemical Analysis

Phytochemical testing of the antimicrobial leads CSS6 and CSS8 was done to establish the tentative nature of the leads. Tests for alkaloids, flavonoids, glycosides, tannins, saponins, and anthraquinones were done as per the methods described in section 4.7.

### 4.17.2. Elemental Analysis

The fractions CSS6 and CSS8 were subjected to elemental analysis by applying standard procedures for the detection of carbon, hydrogen, nitrogen and oxygen. The analysis was carried on a Flash EA T112 series- CHNS-O analyzer (Thermo Electron Corporation, Cheshire, UK). The elemental analysis was done at the Centre with Potential for Excellence in Biomedical Sciences, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh.

### 4.17.3 MS-MS Analysis

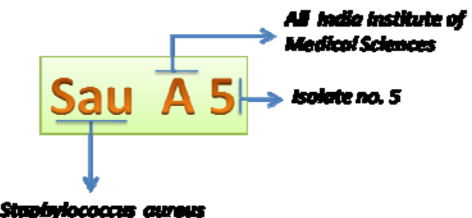
Mass spectrometry using Electrospary ionization (ESI) of fractions CSS6 and CSS8 was done on a micromass Q- Tof Micro mass spectrophotometer (Waters Corporation) to determine the mass of the leads. The instrument conditions used were ; Capillary voltage-2.5 Kv, Cone voltage- 25 v; Source temperature- 80°C; desolvation temperature- 200°C ; desolvation gas- 530Lh$^{-1}$; cone gas 50 L h$^{-1}$, Syringe flow= 5µl/ min. The work was done at Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh. The ESI-MS/MS was carried out in positive ion mode only using the same conditions described above.

# Chapter 5
# RESULTS

## 5.0 RESULTS

### 5.1 Culture repository

A total of 34 clinical isolates and 4 control isolates of *Staphylococcus aureus* were included in the test panel initially to screen the antimicrobial potential of the extracts of *Callistemon rigidus* and to isolate the lead antimicrobial compound(s). The coding of the culture was done as per WHO designation of *Staphylococcus aureus* as "Sau" which was followed by the first capital letter from where the culture was resourced and further the number indicated the isolate number from a particular repository (Table 5.1). The test panel of staphylococci were classified as pus, burn, urine, vaginal, eye, ear, blood, catheter tip, unclassified and standard based on their origin. The test panel was dominated by pus isolates followed by burn isolates and urine and control strains (Fig. 5.1)



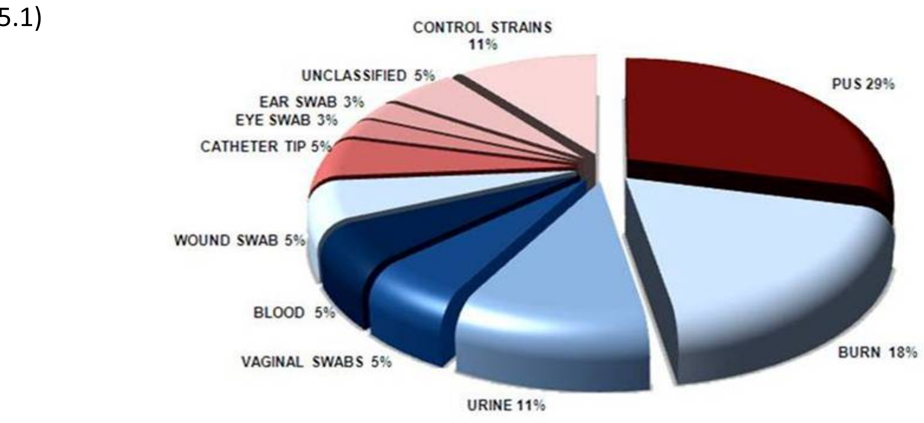




**Fig. 5.1 Distribution of staphylcococcal isolates in the test panel**

### 5.2 Antibiogram as per KB Disc assay

KB disc assay is the most common diagnostic test in clinical pathology to find out antibiotic sensitivity against different infectious microorganisms which is helpful in development of an antibiogram (Fig 5.2 a). As evident from Fig 5.2(b), all pus isolates were resistant to penicillin-G followed by over 90% resistance in ceftizoxime, cefpodoxime, cefixime and erythromycin. The

**Table 5.1 : Culture Collection**

| S.No. | Organism | Culture ID | Source | Repository |
|---|---|---|---|---|
| 1 | *Staphylococcus aureus* | Sau A1 | PUS | AIIMS |
| 2 | *Staphylococcus aureus* | Sau A2 | PUS | AIIMS |
| 3 | *Staphylococcus aureus* | Sau G1 | PUS | GMCP |
| 4 | *Staphylococcus aureus* | Sau G2 | PUS | GMCP |
| 5 | *Staphylococcus aureus* | Sau G3 | PUS | GMCP |
| 6 | *Staphylococcus aureus* | Sau G10 | PUS | GMCP |
| 7 | *Staphylococcus aureus* | Sau G23 | PUS | GMCP |
| 8 | *Staphylococcus aureus* | Sau G24 | PUS | GMCP |
| 9 | *Staphylococcus aureus* | Sau G25 | PUS | GMCP |
| 10 | *Staphylococcus aureus* | Sau G27 | PUS | GMCP |
| 11 | *Staphylococcus aureus* | Sau G28 | PUS | GMCP |
| 12 | *Staphylococcus aureus* | Sau G15 | BURN | GMCP |
| 13 | *Staphylococcus aureus* | Sau G9 | BURN | GMCP |
| 14 | *Staphylococcus aureus* | Sau G16 | BURN | GMCP |
| 15 | *Staphylococcus aureus* | Sau G17 | BURN | GMCP |
| 16 | *Staphylococcus aureus* | Sau G18 | BURN | GMCP |
| 17 | *Staphylococcus aureus* | Sau G19 | BURN | GMCP |
| 18 | *Staphylococcus aureus* | Sau G 26 | BURN | GMCP |
| 19 | *Staphylococcus aureus* | Sau A3 | URINE | AIIMS |
| 20 | *Staphylococcus aureus* | Sau G4 | URINE | GMCP |
| 21 | *Staphylococcus aureus* | Sau G5 | URINE | GMCP |
| 22 | *Staphylococcus aureus* | Sau G11 | URINE | GMCP |
| 23 | *Staphylococcus aureus* | Sau G7 | VAGINAL SWABS | GMCP |
| 24 | *Staphylococcus aureus* | Sau G13 | VAGINAL SWABS | GMCP |
| 25 | *Staphylococcus aureus* | Sau G6 | BLOOD | GMCP |
| 26 | *Staphylococcus aureus* | Sau G12 | BLOOD | GMCP |
| 27 | *Staphylococcus aureus* | Sau A4 | WOUND SWAB | AIIMS |
| 28 | *Staphylococcus aureus* | Sau G14 | WOUND SWAB | GMCP |
| 29 | *Staphylococcus aureus* | Sau G20 | CATHETER TIP | GMCP |
| 30 | *Staphylococcus aureus* | Sau G21 | CATHETER TIP | GMCP |
| 31 | *Staphylococcus aureus* | Sau G22 | EYE SWAB | GMCP |
| 32 | *Staphylococcus aureus* | Sau G8 | EAR SWAB | GMCP |
| 33 | *Staphylococcus aureus* | Sau A5 | UNCLASSIFIED | AIIMS |
| 34 | *Staphylococcus aureus* | Sau MTB | UNCLASSIFIED | KAROLINSKA INSTITUTE* |
| 35 | *Staphylococcus aureus* | Sau LHMC | NCTC6571 | LHMC |
| 36 | *Staphylococcus aureus* | Sau I2 | MTCC 740 | IMTECH |
| 37 | *Staphylococcus aureus* | Sau I3 | MTCC 737 | IMTECH |
| 38 | *Staphylococcus aureus* | Sau I4 | MTCC 902 | IMTECH |

GMCP: - Government Medical College, Patiala.
AIIMS: - All India Institute of Medical Sciences, New Delhi.
LHMC: - Lady Harding Medical College, New Delhi.
MTCC, IMTECH: - Microbial Type Culture Collection, Institute of Microbial Technolog10y, Chandigarh, India
MTB- Microbiology & Tumor Biology Centre, Karolinska Institute, Stockholm, Sweden
NCTC- National Cell Typing Centre (NCTC), UKNCC

isolates were susceptible to methicillin, piperacillin, imipenum and chloramphenicol. The correlation matrix was based on the zone size of the antibiotic against the test isolates and not the resistance pattern for finding out similarity. Imipenum exhibited the largest zone of inhibition for pus isolates followed by co-trimoxazole and trimethoprim (Fig. 5.2d). Among the burn isolates Fig.5.2(c), penicillin-G, cephoxitin and cefixime exhibited 100% resistance followed by 86% resistance in nalidixic acid, moxifloxacin, cefipime, tetracycline and trimethoprim. Here the clustering was done on the basis of similarity of total non-response/ resistance pattern. Co-trimoxazole and trimethoprim were brought into one cluster. Maximum inhibition zone was exhibited by Imipenum (Fig. 5.2e). Penicillin-G, carbenicillin, cephoxitin, ceftazidime, ceftizoxime, cefpodoxime and cefixime exhibited cent percent resistance while methicillin, piperacillin, amikacin, neomycin, ofloxacin, ciprofloxacin, linezolid, imipenum and chloramphenicol exhibited sensitivity to urine isolates (Fig. 5.2 f). Vaginal isolates exhibited susceptibility to amikacin, neomycin, methicillin, piperacillin, clarithromycin, norfloxacin, ofloxacin, ciprofloxacin, pefloxacin, cephalexin, cephalothin, cefaclor, cefprozil, cefuroxime, ceftriaxone, ceftazidime, cefoperazone, cefipime, imipenum, co-trimoxazole and chloramphenicol whereas antibiotics viz. carbenicillin, nalidixic acid, moxifloxacin, cephoxitin, cefpodoxime, cefixime and trimethoprim showed 100% resistance and kanamycin, penicillin-G, erythromycin, sparfloxacin, ceftizoxime, cefdinir, tetracycline, linezolid, and vancomycin exhibited 50% resistance (Fig. 5.2 g).

In the urine and vaginal isolates, cefixime was totally resistant whereas antibiotics from cephalexin to ceforperzone were clustered based on their zone sizes. Trimethoprim, co-trimoxazole and Imipenum induced largest zone of inhibition among the test isolates (Fig. 5.2h). The susceptible antibiotics in blood isolates were neomycin, methicillin, piperacillin, ofloxacin, ciprofloxacin, cephalothin, cefoperazone, linezolid, tetracycline, vancomycin and chloramphenicol while the drugs which showed 100% resistance were penicillin G, cephoxitin

and carbenicillin. Ceftizoxime, cefpodoxime and cefixime exhibited 50% resistance among isolates of the test panel. Rest all other antibiotics exhibited 50% resistance pattern (Fig. 5.2 i). In wound isolates, penicillin-G, carbenicillin and cephoxitin exhibited 100% resistance while 50% resistance among isolates is exhibited by erthyromycin, clarithromycin, nalidixic acid, ceftazidime, ceftizoxime, cefpodoxime, cefixime, cefdinir and cefoperazone (Fig. 5.2 j). Amongst the catheter isolates, out of thirty seven antibiotics tested only 6 were found to be susceptible and these were neomycin, methicillin, piperacillin, linezolid, vancomycin and chloramphenicol. Eleven antibiotics exhibited 50% resistance viz. kanamycin, norfloxacin, ofloxacin, pefloxacin, sparfloxacin, moxifloxacin, cephalexin, cefaclor, cefprozil, tetracycline and imipenum while rest all other antibiotics were resistant (Fig. 5.2 k). Blood, wound and catheter isolates have exhibited highest sensitivity towards linezolid based on their zone size correlation matrix (Fig. 5.2m). Standard isolates displayed 100% resistance to penicillin-G, cephoxitin, cefaclor, cefprozil, cefpodoxime, cefixime and cefdinir while chloramphenicol, linezolid, tetracycline, cephalexin, pefloxacin, ciprofloxacin, ofloxacin, piperacillin, methicillin and neomycin exhibited total susceptibility (Fig. 5.2 l). Erythtomycin/ clarithromycin were clustered in one group followed by clusters of cephalothein, ceftriaxone ; cefophoxitin, cefaclor; norfloxacin, ofloxacin, pefloxacin; neomycin, cefoperazone; cephalexin, chloramphenicol; piperacillin, vancomycin. Imipenum exhibited maximum sensitivity (Fig. 5.2n).

Among eye/ ear isolates, the susceptible antibiotics were amikacin, neomycin, methicillin, piperacillin, norfloxacin, ofloxacin, ciprofloxacin, pefloxacin, cephalexin, cefaclor, cefprozil, ceftriaxone, cefipime, tetracycline, imipenum, co-trimoxazole and chloramphenicol. Eight antibiotics were resistant which include penicillin- G, carbenicillin, erythromycin, clarithromycin, cephalothin, cephoxitin, cefpodoxime and cefixime (Fig. 5.2 o). Amongst the unclassified isolates majority of the antibiotics were susceptible except penicillin-G, carbenicillin, cephoxitin, cefpodoxime, cefixime which exhibited 100% resistance while cefuroxime,

ceftazidime, ceftizoxime, cefdinir, cefipime and trimethoprim exhibited 50% resistance (Fig. 5.2 p). Eye, ear and unclassified isolates have been clubbed according to their similarity indices as a dendrogram in Fig. 5.2(q) along with their antibiotics.

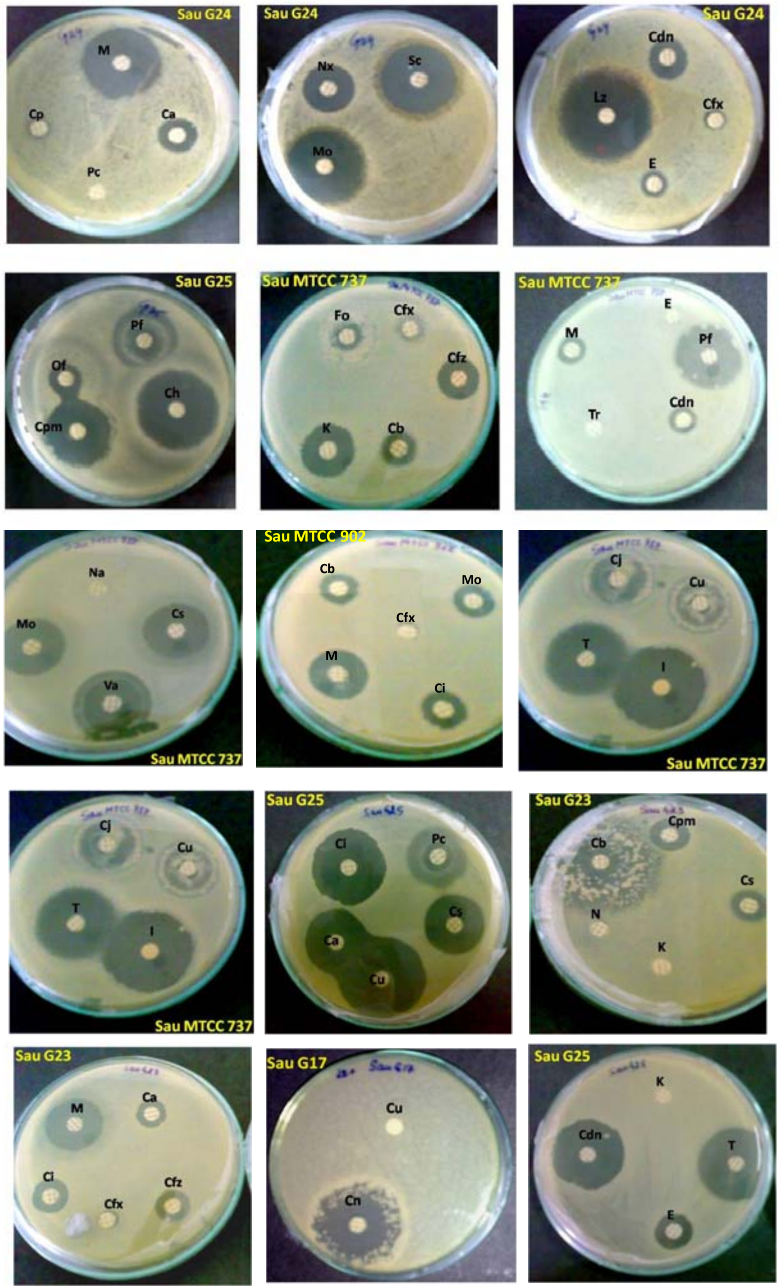



Fig 5.2 (a) Antibiogram by Kirby Bauer Disk Assay.

Codes of the antibiotic discs are as follows: *M-Methicillin; Cp Cephalexin; Pc- Piperacillin; Ca-Ceftazidime; Nx-Norfloxacin; Mo -Moxifloxacin; Sc- Sparfloxacin; Lz-Linezolid; Cdn-Cefdinir; Cfx-Cefixime; E-Erythromycin; Pf-Pefloxacin; Of-Ofloxacin; Cpm-Cefipime; Ch-Cephalothin; Fo-Fosfomycin; Cfz- Cefprozil; K-Kanamycin; Cb-carbenicillin; Tr-Trimethoprim; Na- Nalidixic acid; Cs-Cefoperazone; Va-Vancomycin; Ci-Ceftriaxone; Cj-Cefaclor, Cu-Cefuroxime; T-Tetracycline; I-Imepenum; N-Neomycin; Cn-Cefoxitin*

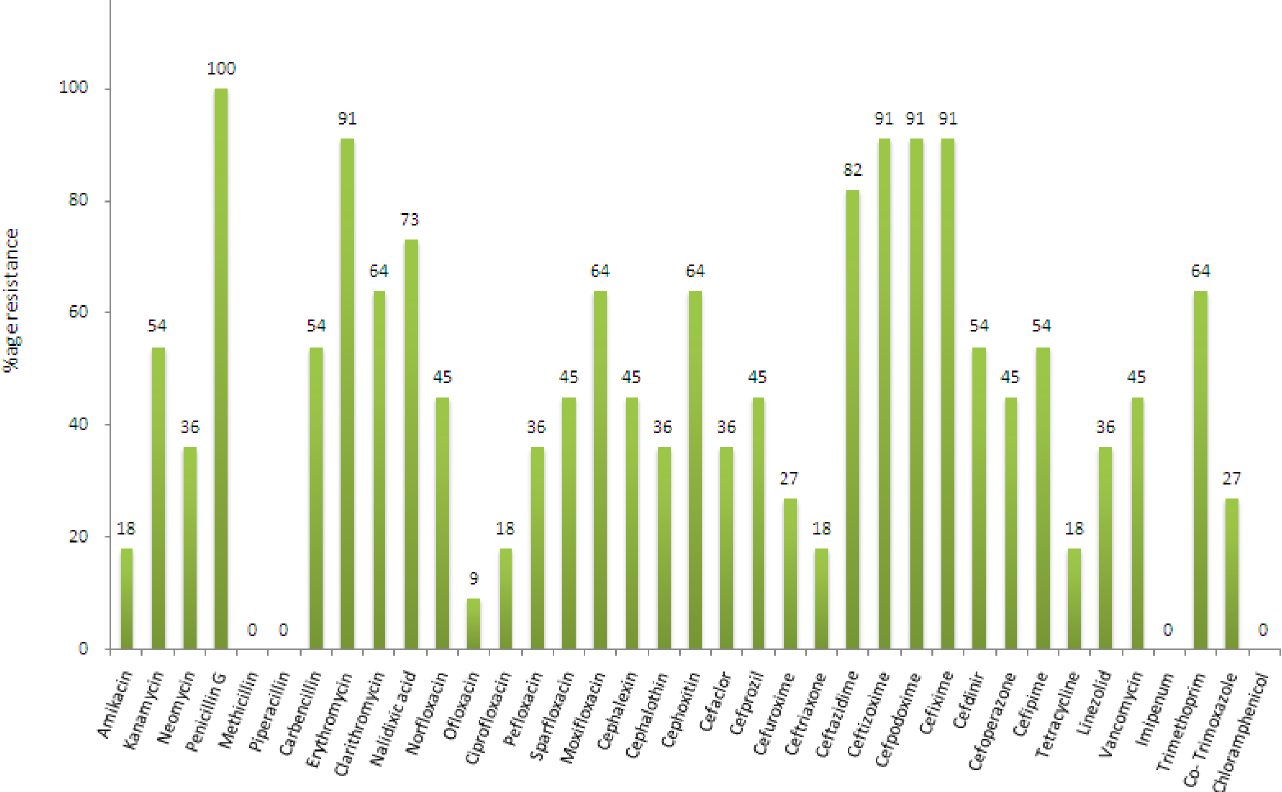



**Fig. 5.2bPercentage resistance of pus isolates against the test antibiotics**

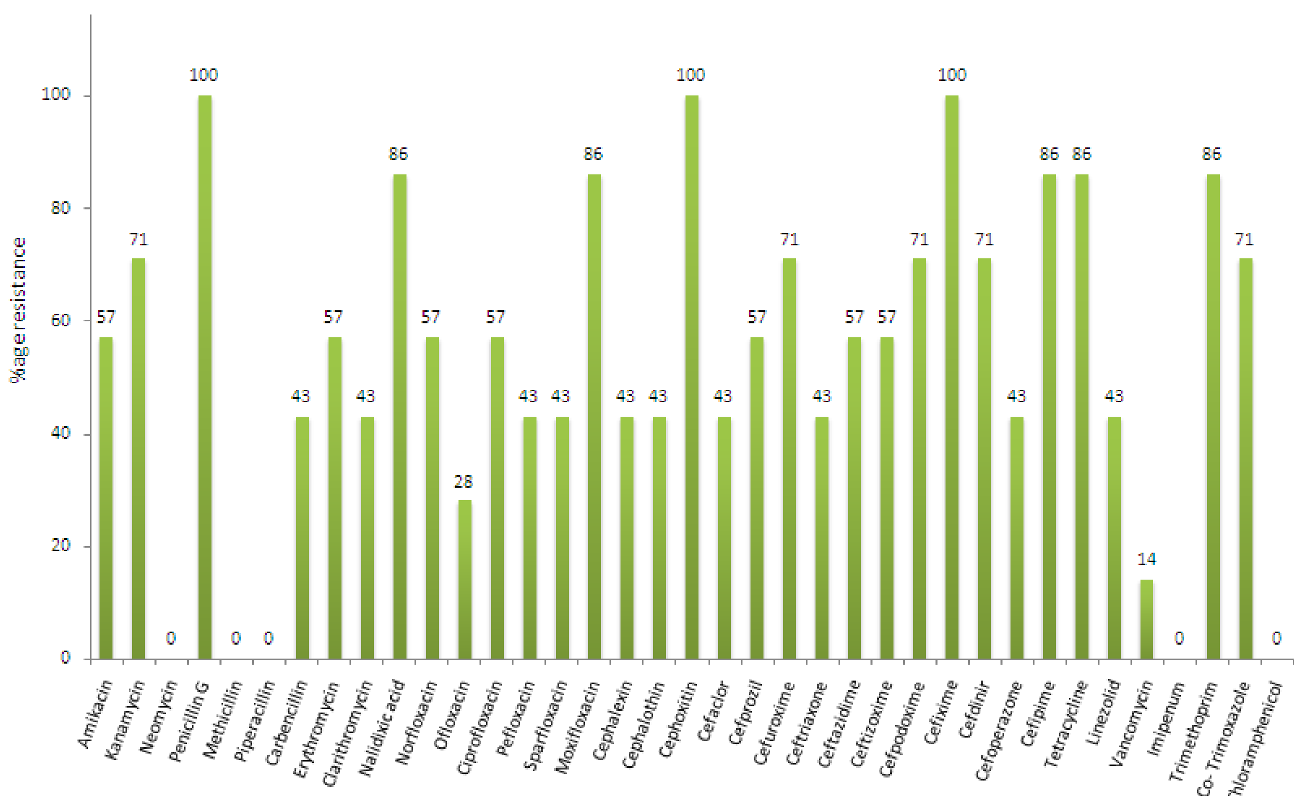
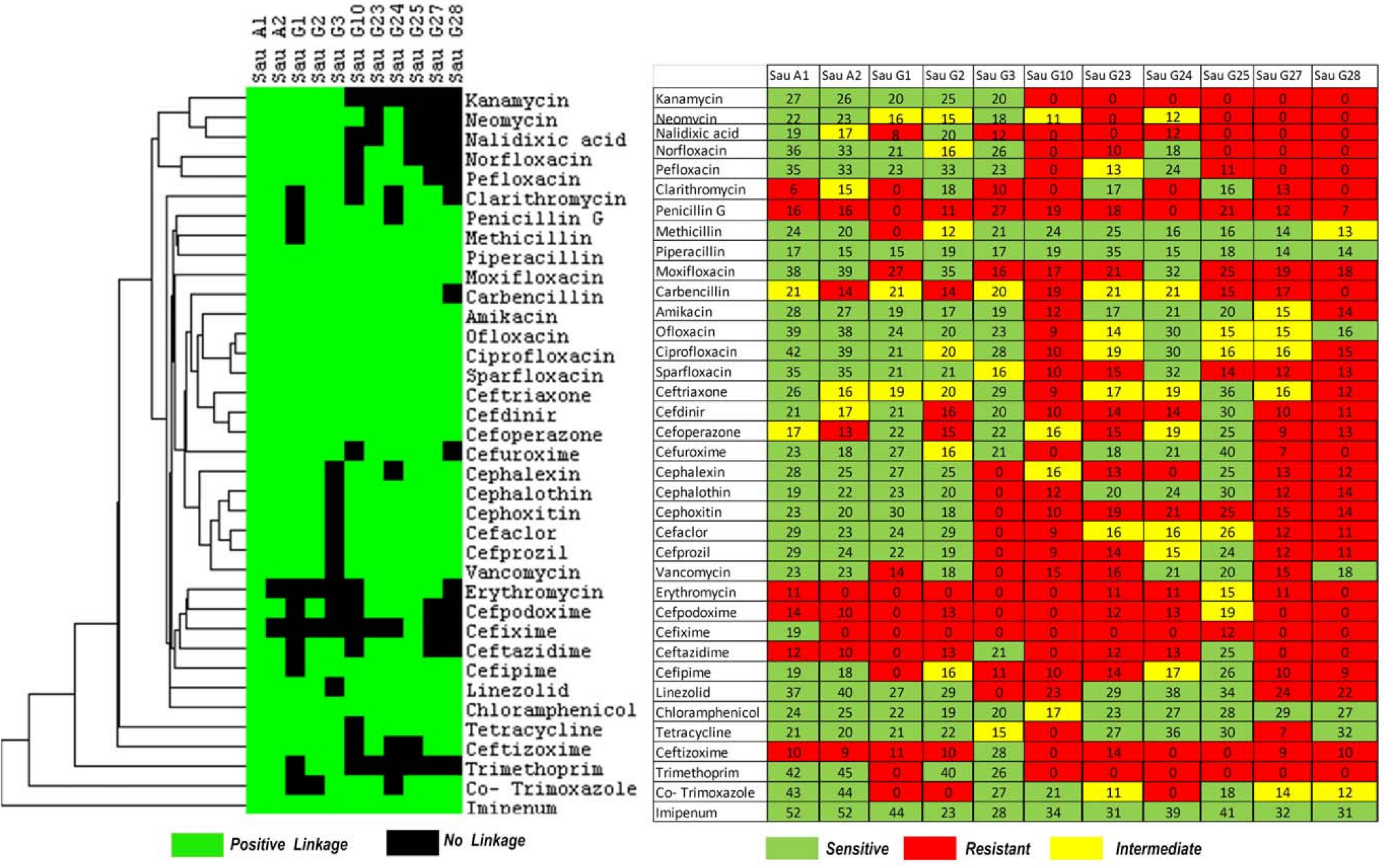



**Fig 5.2c Percentage resistance of burn isolates against the test antibiotics**

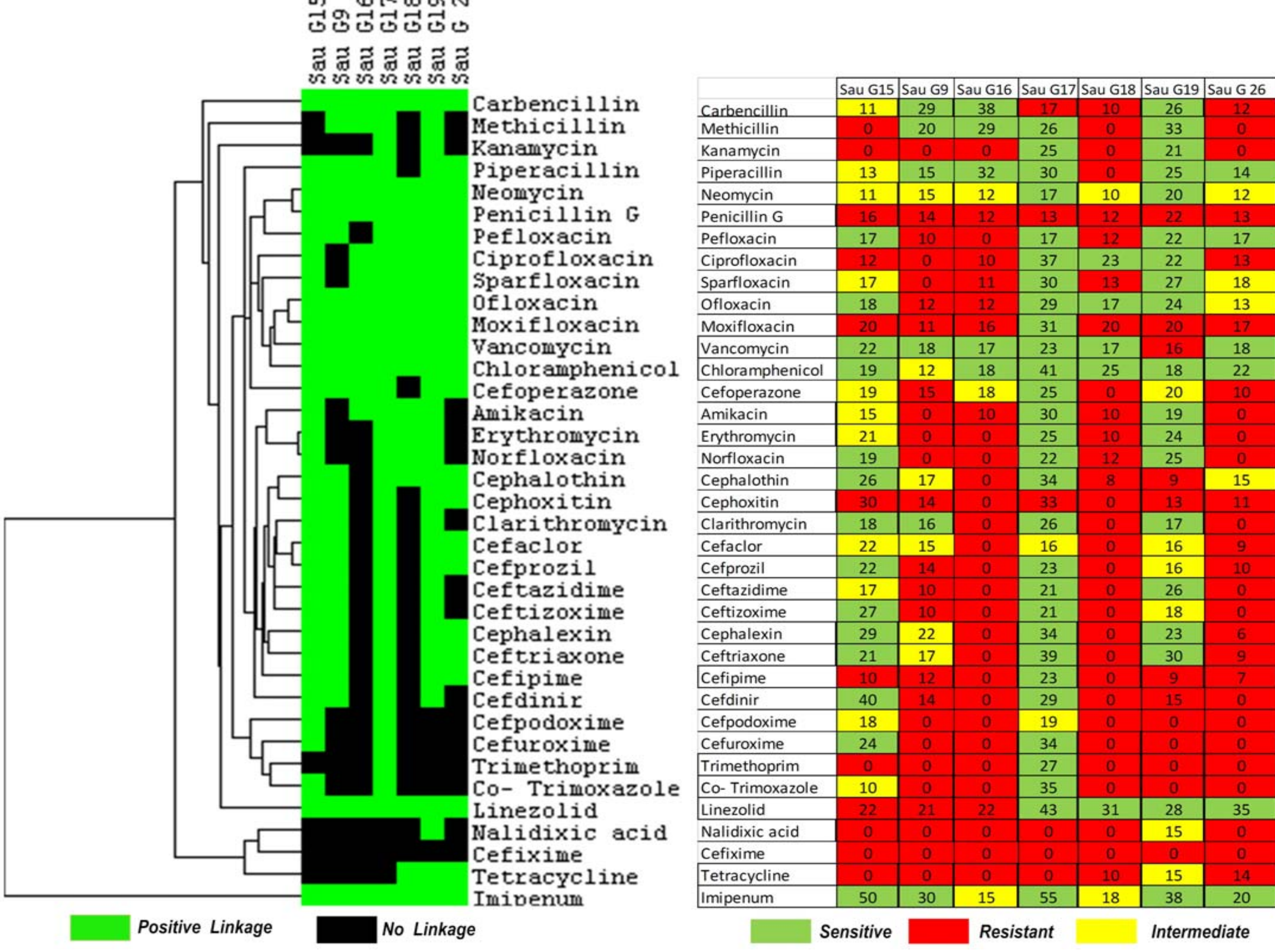
**Fig 5.2d Dendrogram and antibiogram of pus isolates against the test antibiotics**




| | Sau A1 | Sau A2 | Sau G1 | Sau G2 | Sau G3 | Sau G10 | Sau G23 | Sau G24 | Sau G25 | Sau G27 | Sau G28 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kanamycin | 27 | 26 | 20 | 25 | 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| Neomycin | 22 | 23 | 16 | 15 | 18 | 11 | 0 | 12 | 0 | 0 | 0 |
| Nalidixic acid | 19 | 17 | 8 | 20 | 12 | 0 | 0 | 12 | 0 | 0 | 0 |
| Norfloxacin | 36 | 33 | 21 | 16 | 26 | 0 | 10 | 18 | 0 | 0 | 0 |
| Pefloxacin | 35 | 33 | 23 | 33 | 23 | 0 | 13 | 24 | 11 | 0 | 0 |
| Clarithromycin | 6 | 15 | 0 | 18 | 10 | 0 | 17 | 0 | 16 | 13 | 0 |
| Penicillin G | 16 | 16 | 0 | 11 | 27 | 19 | 18 | 0 | 21 | 12 | 7 |
| Methicillin | 24 | 20 | 0 | 12 | 21 | 24 | 25 | 16 | 16 | 14 | 13 |
| Piperacillin | 17 | 15 | 15 | 19 | 17 | 19 | 35 | 15 | 18 | 14 | 14 |
| Moxifloxacin | 38 | 39 | 27 | 35 | 16 | 17 | 21 | 32 | 25 | 19 | 18 |
| Carbencillin | 21 | 14 | 21 | 14 | 20 | 19 | 21 | 21 | 15 | 17 | 0 |
| Amikacin | 28 | 27 | 19 | 17 | 19 | 12 | 17 | 21 | 20 | 15 | 14 |
| Ofloxacin | 39 | 38 | 24 | 20 | 23 | 9 | 14 | 30 | 15 | 15 | 16 |
| Ciprofloxacin | 42 | 39 | 21 | 20 | 28 | 10 | 19 | 30 | 16 | 16 | 15 |
| Sparfloxacin | 35 | 35 | 21 | 21 | 16 | 10 | 15 | 32 | 14 | 12 | 13 |
| Ceftriaxone | 26 | 16 | 19 | 20 | 29 | 9 | 17 | 19 | 36 | 16 | 12 |
| Cefdinir | 21 | 17 | 21 | 16 | 20 | 10 | 14 | 14 | 30 | 10 | 11 |
| Cefoperazone | 17 | 13 | 22 | 15 | 22 | 16 | 15 | 19 | 25 | 9 | 13 |
| Cefuroxime | 23 | 18 | 27 | 16 | 21 | 0 | 18 | 21 | 40 | 7 | 0 |
| Cephalexin | 28 | 25 | 27 | 25 | 0 | 16 | 13 | 0 | 25 | 13 | 12 |
| Cephalothin | 19 | 22 | 23 | 20 | 0 | 12 | 20 | 24 | 30 | 12 | 14 |
| Cephoxitin | 23 | 20 | 30 | 18 | 0 | 10 | 19 | 21 | 25 | 15 | 14 |
| Cefaclor | 29 | 23 | 24 | 29 | 0 | 9 | 16 | 16 | 26 | 12 | 11 |
| Cefprozil | 29 | 24 | 22 | 19 | 0 | 9 | 14 | 15 | 24 | 12 | 11 |
| Vancomycin | 23 | 23 | 14 | 18 | 0 | 15 | 16 | 21 | 20 | 15 | 18 |
| Erythromycin | 11 | 0 | 0 | 0 | 0 | 0 | 11 | 11 | 15 | 11 | 0 |
| Cefpodoxime | 14 | 10 | 0 | 13 | 0 | 0 | 12 | 13 | 19 | 0 | 0 |
| Cefixime | 19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | 0 |
| Ceftazidime | 12 | 10 | 0 | 13 | 21 | 0 | 12 | 13 | 25 | 0 | 0 |
| Cefipime | 19 | 18 | 0 | 16 | 11 | 10 | 14 | 17 | 26 | 10 | 9 |
| Linezolid | 37 | 40 | 27 | 29 | 0 | 23 | 29 | 38 | 34 | 24 | 22 |
| Chloramphenicol | 24 | 25 | 22 | 19 | 20 | 17 | 23 | 27 | 28 | 29 | 27 |
| Tetracycline | 21 | 20 | 21 | 22 | 15 | 0 | 27 | 36 | 30 | 7 | 32 |
| Ceftizoxime | 10 | 9 | 11 | 10 | 28 | 0 | 14 | 0 | 0 | 9 | 10 |
| Trimethoprim | 42 | 45 | 0 | 40 | 26 | 0 | 0 | 0 | 0 | 0 | 0 |
| Co- Trimoxazole | 43 | 44 | 0 | 0 | 27 | 21 | 11 | 0 | 18 | 14 | 12 |
| Imipenum | 52 | 52 | 44 | 23 | 28 | 34 | 31 | 39 | 41 | 32 | 31 |

Sensitive Resistant Intermediate

**Fig 5.2e Dendrogram and antibiogram of burn isolates against the test antibiotics**




| | Sau G15 | Sau G9 | Sau G16 | Sau G17 | Sau G18 | Sau G19 | Sau G 26 |
|---|---|---|---|---|---|---|---|
| Carbencillin | 11 | 29 | 38 | 17 | 10 | 26 | 12 |
| Methicillin | 0 | 20 | 29 | 26 | 0 | 33 | 0 |
| Kanamycin | 0 | 0 | 0 | 25 | 0 | 21 | 0 |
| Piperacillin | 13 | 15 | 32 | 30 | 0 | 25 | 14 |
| Neomycin | 11 | 15 | 12 | 17 | 10 | 20 | 12 |
| Penicillin G | 16 | 14 | 12 | 13 | 12 | 22 | 13 |
| Pefloxacin | 17 | 10 | 0 | 17 | 12 | 22 | 17 |
| Ciprofloxacin | 12 | 0 | 10 | 37 | 23 | 22 | 13 |
| Sparfloxacin | 17 | 0 | 11 | 30 | 13 | 27 | 18 |
| Ofloxacin | 18 | 12 | 12 | 29 | 17 | 24 | 13 |
| Moxifloxacin | 20 | 11 | 16 | 31 | 20 | 20 | 17 |
| Vancomycin | 22 | 18 | 17 | 23 | 17 | 16 | 18 |
| Chloramphenicol | 19 | 12 | 18 | 41 | 25 | 18 | 22 |
| Cefoperazone | 19 | 15 | 18 | 25 | 0 | 20 | 10 |
| Amikacin | 15 | 0 | 10 | 30 | 10 | 19 | 0 |
| Erythromycin | 21 | 0 | 0 | 25 | 10 | 24 | 0 |
| Norfloxacin | 19 | 0 | 0 | 22 | 12 | 25 | 0 |
| Cephalothin | 26 | 17 | 0 | 34 | 8 | 9 | 15 |
| Cephoxitin | 30 | 14 | 0 | 33 | 0 | 13 | 11 |
| Clarithromycin | 18 | 16 | 0 | 26 | 0 | 17 | 0 |
| Cefaclor | 22 | 15 | 0 | 16 | 0 | 16 | 9 |
| Cefprozil | 22 | 14 | 0 | 23 | 0 | 16 | 10 |
| Ceftazidime | 17 | 10 | 0 | 21 | 0 | 26 | 0 |
| Ceftizoxime | 27 | 10 | 0 | 21 | 0 | 18 | 0 |
| Cephalexin | 29 | 22 | 0 | 34 | 0 | 23 | 6 |
| Ceftriaxone | 21 | 17 | 0 | 39 | 0 | 30 | 9 |
| Cefipime | 10 | 12 | 0 | 23 | 0 | 9 | 7 |
| Cefdinir | 40 | 14 | 0 | 29 | 0 | 15 | 0 |
| Cefpodoxime | 18 | 0 | 0 | 19 | 0 | 0 | 0 |
| Cefuroxime | 24 | 0 | 0 | 34 | 0 | 0 | 0 |
| Trimethoprim | 0 | 0 | 0 | 27 | 0 | 0 | 0 |
| Co- Trimoxazole | 10 | 0 | 0 | 35 | 0 | 0 | 0 |
| Linezolid | 22 | 21 | 22 | 43 | 31 | 28 | 35 |
| Nalidixic acid | 0 | 0 | 0 | 0 | 0 | 15 | 0 |
| Cefixime | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tetracycline | 0 | 0 | 0 | 0 | 10 | 15 | 14 |
| Imipenum | 50 | 30 | 15 | 55 | 18 | 38 | 20 |

**Fig 5.2f Percentage resistance of urine isolates against the test antibiotics**

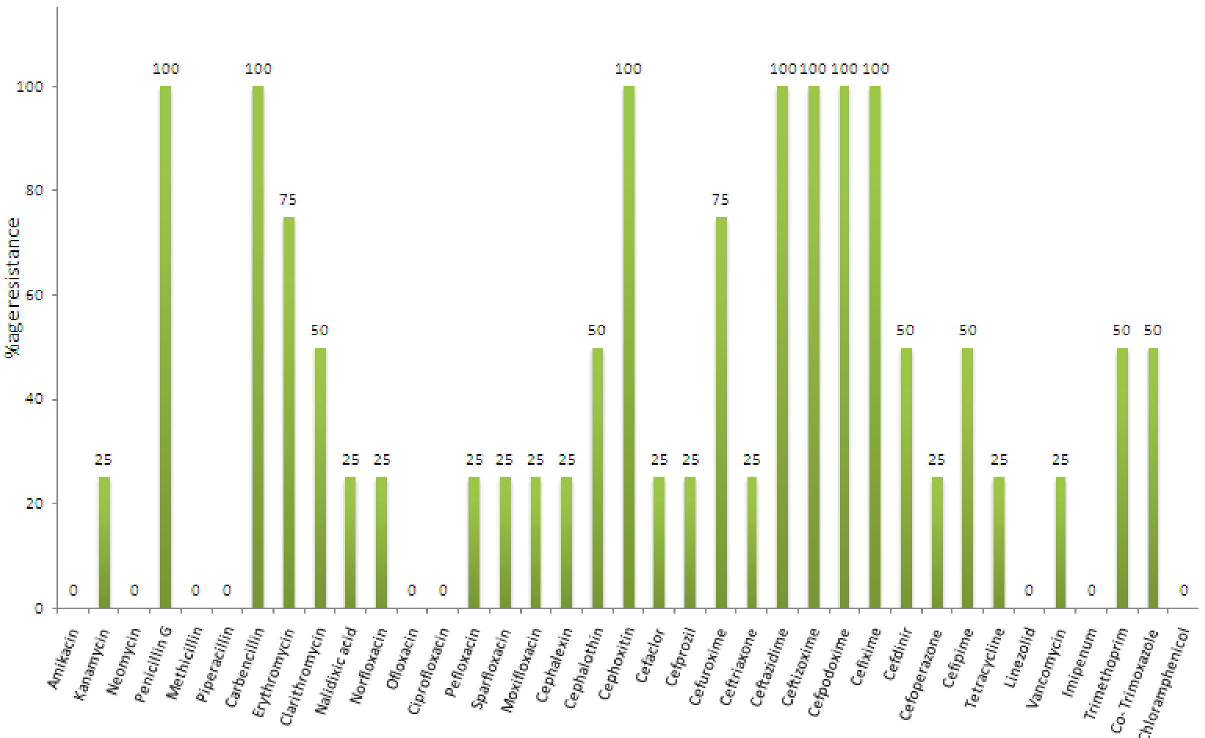



**Fig 5.2g Percentage resistance of vaginal isolates against the test antibiotics**

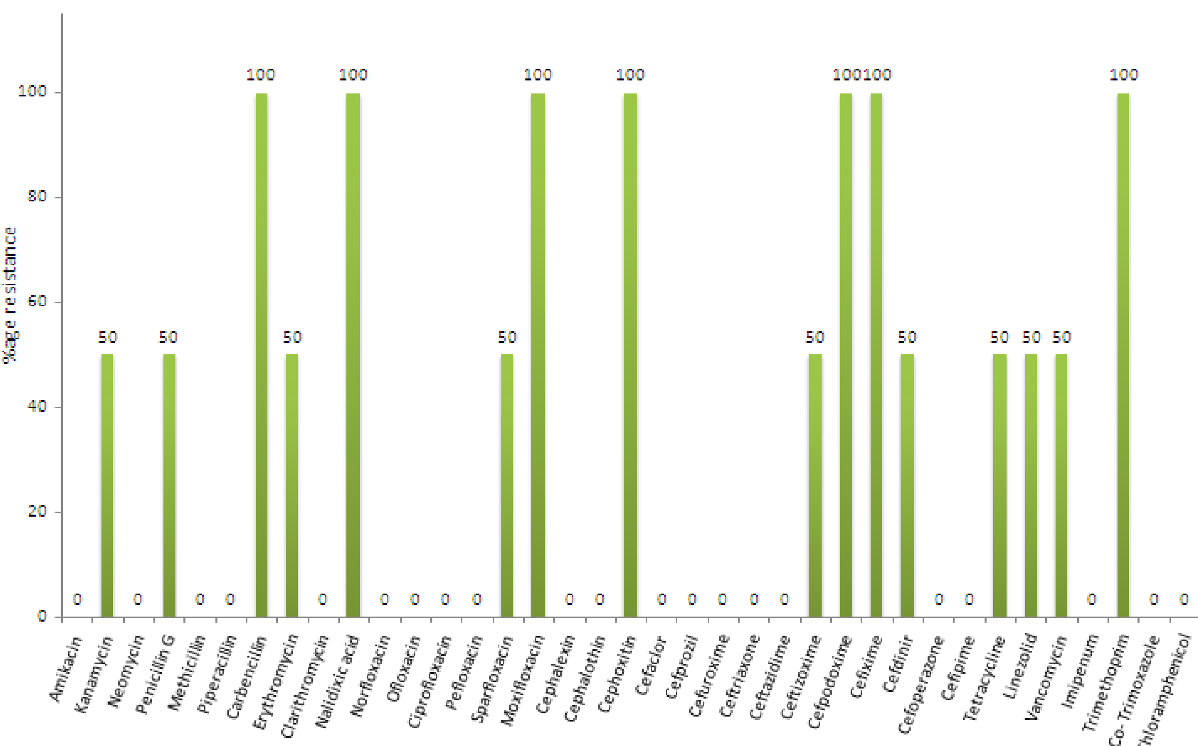
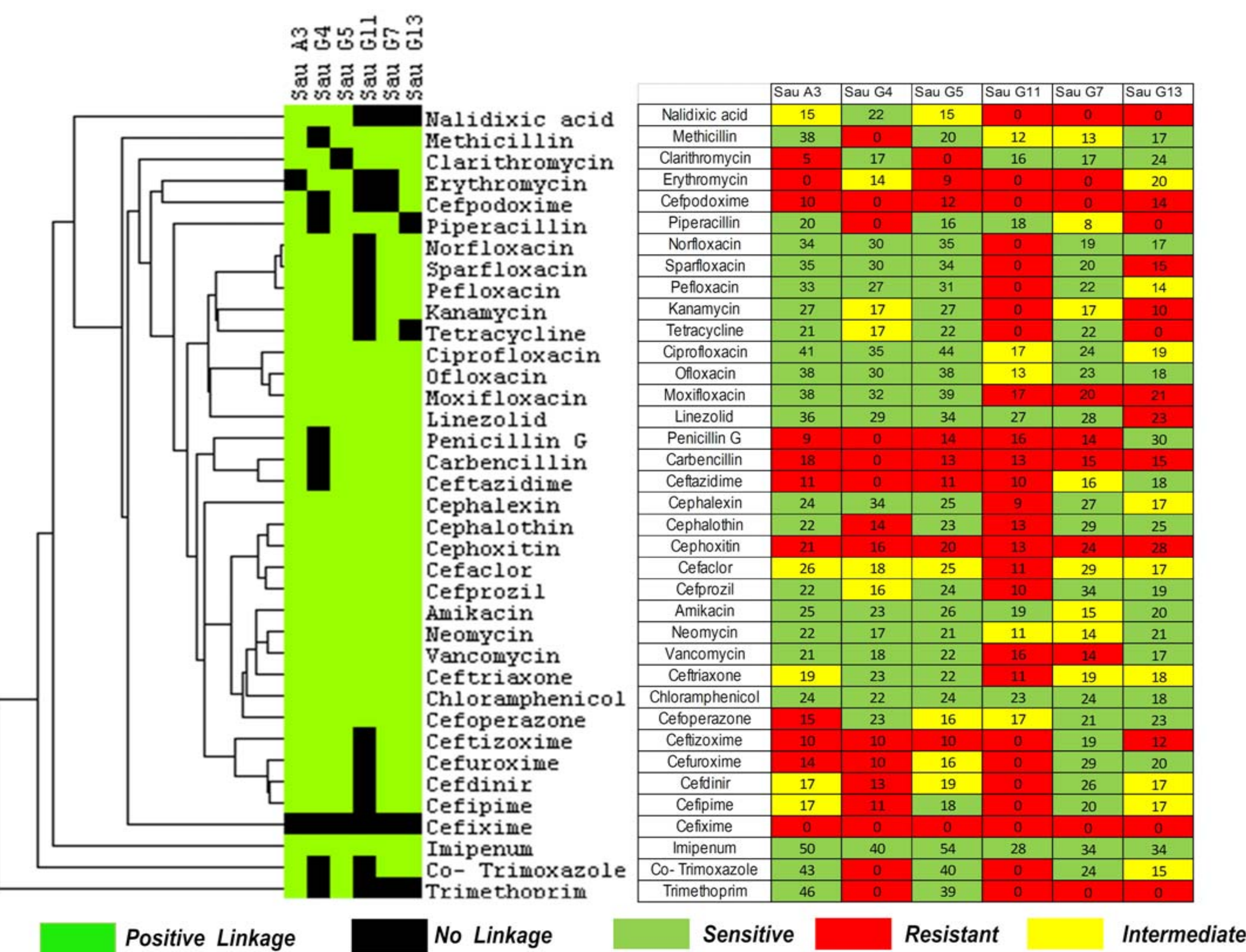


**Fig 5.2h Dendrogram and antibiogram of urine and vaginal isolates against the test antibiotics**



| | Sau A3 | Sau G4 | Sau G5 | Sau G11 | Sau G7 | Sau G13 |
|---|---|---|---|---|---|---|
| Nalidixic acid | 15 | 22 | 15 | 0 | 0 | 0 |
| Methicillin | 38 | 0 | 20 | 12 | 13 | 17 |
| Clarithromycin | 5 | 17 | 0 | 16 | 17 | 24 |
| Erythromycin | 0 | 14 | 9 | 0 | 0 | 20 |
| Cefpodoxime | 10 | 0 | 12 | 0 | 0 | 14 |
| Piperacillin | 20 | 0 | 16 | 18 | 8 | 0 |
| Norfloxacin | 34 | 30 | 35 | 0 | 19 | 17 |
| Sparfloxacin | 35 | 30 | 34 | 0 | 20 | 15 |
| Pefloxacin | 33 | 27 | 31 | 0 | 22 | 14 |
| Kanamycin | 27 | 17 | 27 | 0 | 17 | 10 |
| Tetracycline | 21 | 17 | 22 | 0 | 22 | 0 |
| Ciprofloxacin | 41 | 35 | 44 | 17 | 24 | 19 |
| Ofloxacin | 38 | 30 | 38 | 13 | 23 | 18 |
| Moxifloxacin | 38 | 32 | 39 | 17 | 20 | 21 |
| Linezolid | 36 | 29 | 34 | 27 | 28 | 23 |
| Penicillin G | 9 | 0 | 14 | 16 | 14 | 30 |
| Carbencillin | 18 | 0 | 13 | 13 | 15 | 15 |
| Ceftazidime | 11 | 0 | 11 | 10 | 16 | 18 |
| Cephalexin | 24 | 34 | 25 | 9 | 27 | 17 |
| Cephalothin | 22 | 14 | 23 | 13 | 29 | 25 |
| Cephoxitin | 21 | 16 | 20 | 13 | 24 | 28 |
| Cefaclor | 26 | 18 | 25 | 11 | 29 | 17 |
| Cefprozil | 22 | 16 | 24 | 10 | 34 | 19 |
| Amikacin | 25 | 23 | 26 | 19 | 15 | 20 |
| Neomycin | 22 | 17 | 21 | 11 | 14 | 21 |
| Vancomycin | 21 | 18 | 22 | 16 | 14 | 17 |
| Ceftriaxone | 19 | 23 | 22 | 11 | 19 | 18 |
| Chloramphenicol | 24 | 22 | 24 | 23 | 24 | 18 |
| Cefoperazone | 15 | 23 | 16 | 17 | 21 | 23 |
| Ceftizoxime | 10 | 10 | 10 | 0 | 19 | 12 |
| Cefuroxime | 14 | 10 | 16 | 0 | 29 | 20 |
| Cefdinir | 17 | 13 | 19 | 0 | 26 | 17 |
| Cefipime | 17 | 11 | 18 | 0 | 20 | 17 |
| Cefixime | 0 | 0 | 0 | 0 | 0 | 0 |
| Imipenum | 50 | 40 | 54 | 28 | 34 | 34 |
| Co- Trimoxazole | 43 | 0 | 40 | 0 | 24 | 15 |
| Trimethoprim | 46 | 0 | 39 | 0 | 0 | 0 |

**Fig. 5.2i Percentage resistance of blood isolates against the test antibiotics**

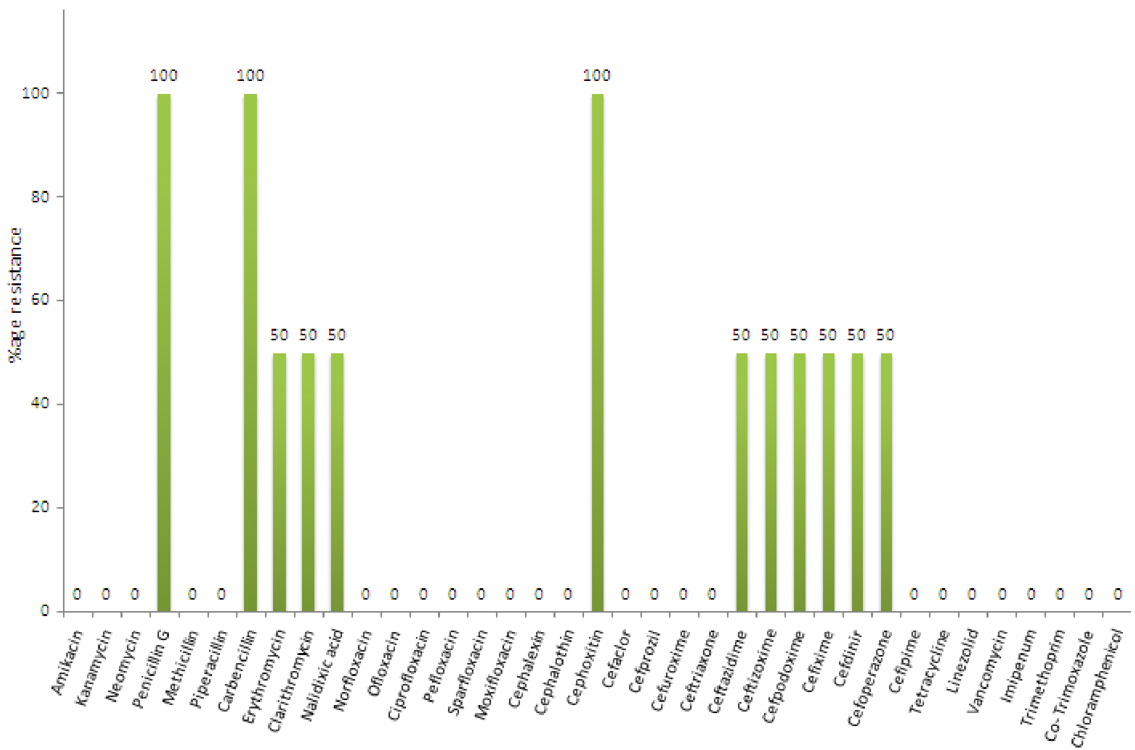
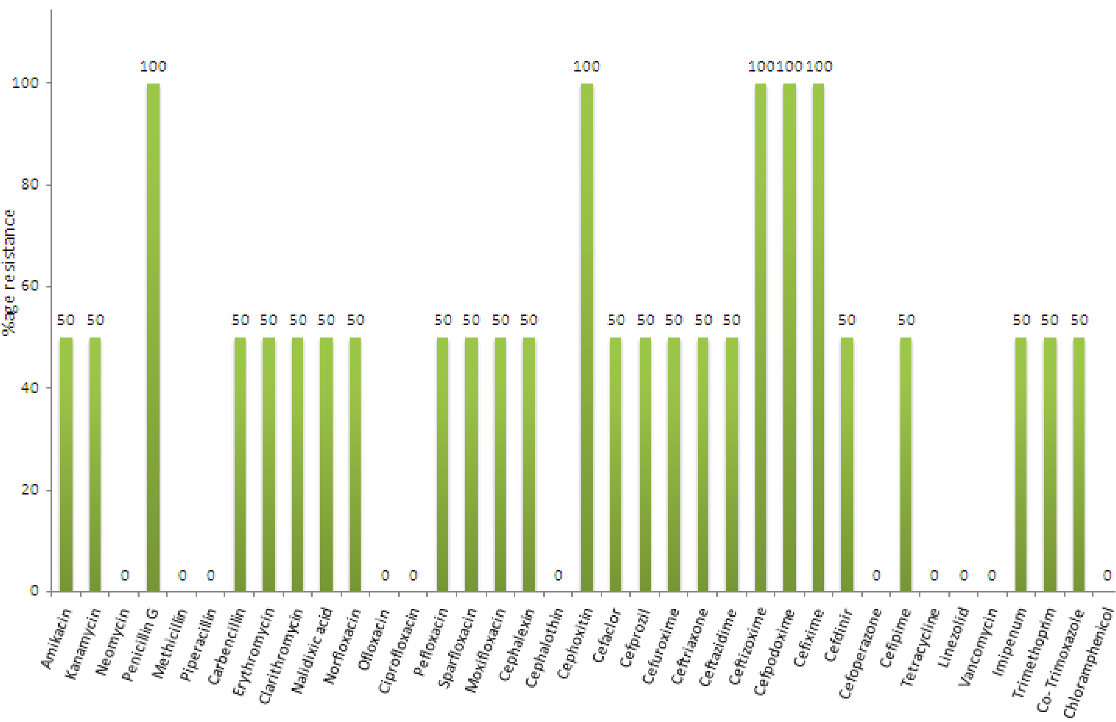



**Fig 5.2j Percentage resistance of wound isolates against the test antibiotics**



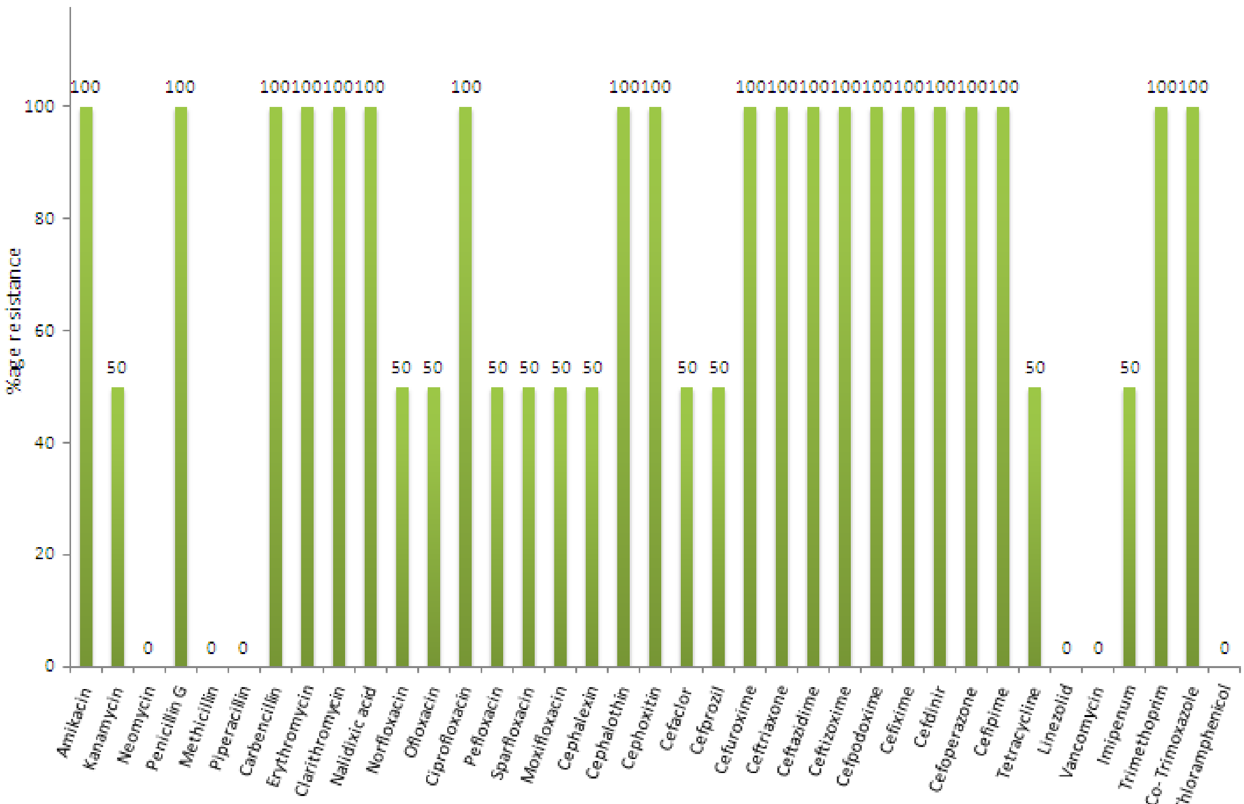



**Fig 5.2 (k) Percentage resistance of catheter isolates against the test antibiotics**

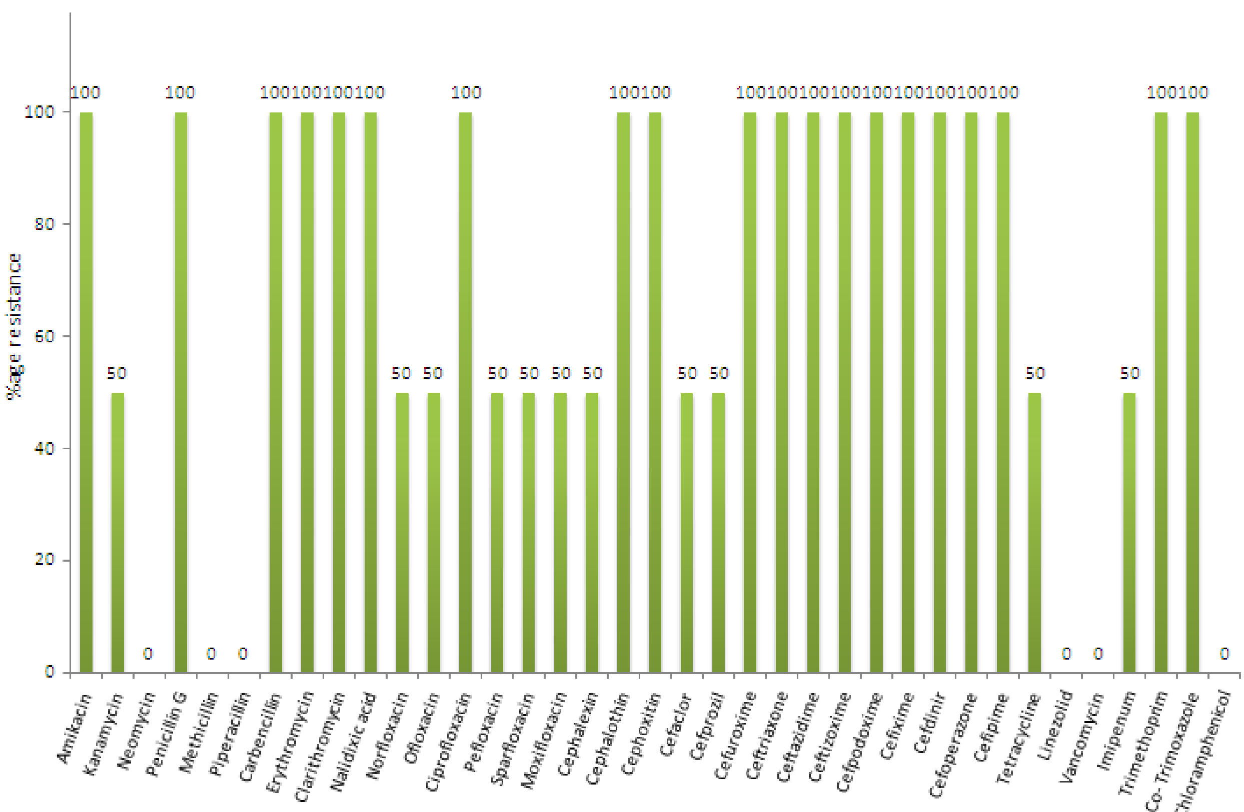
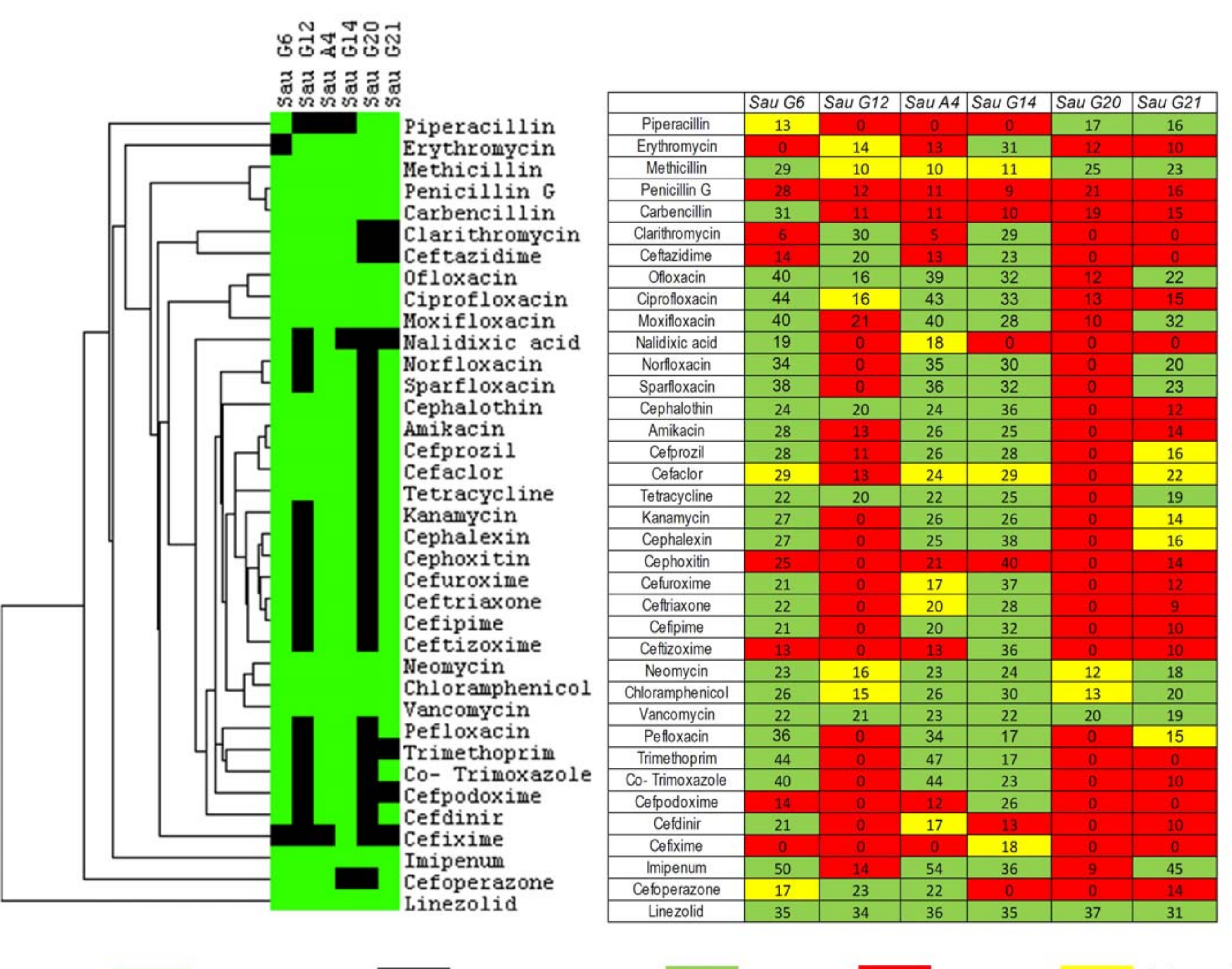


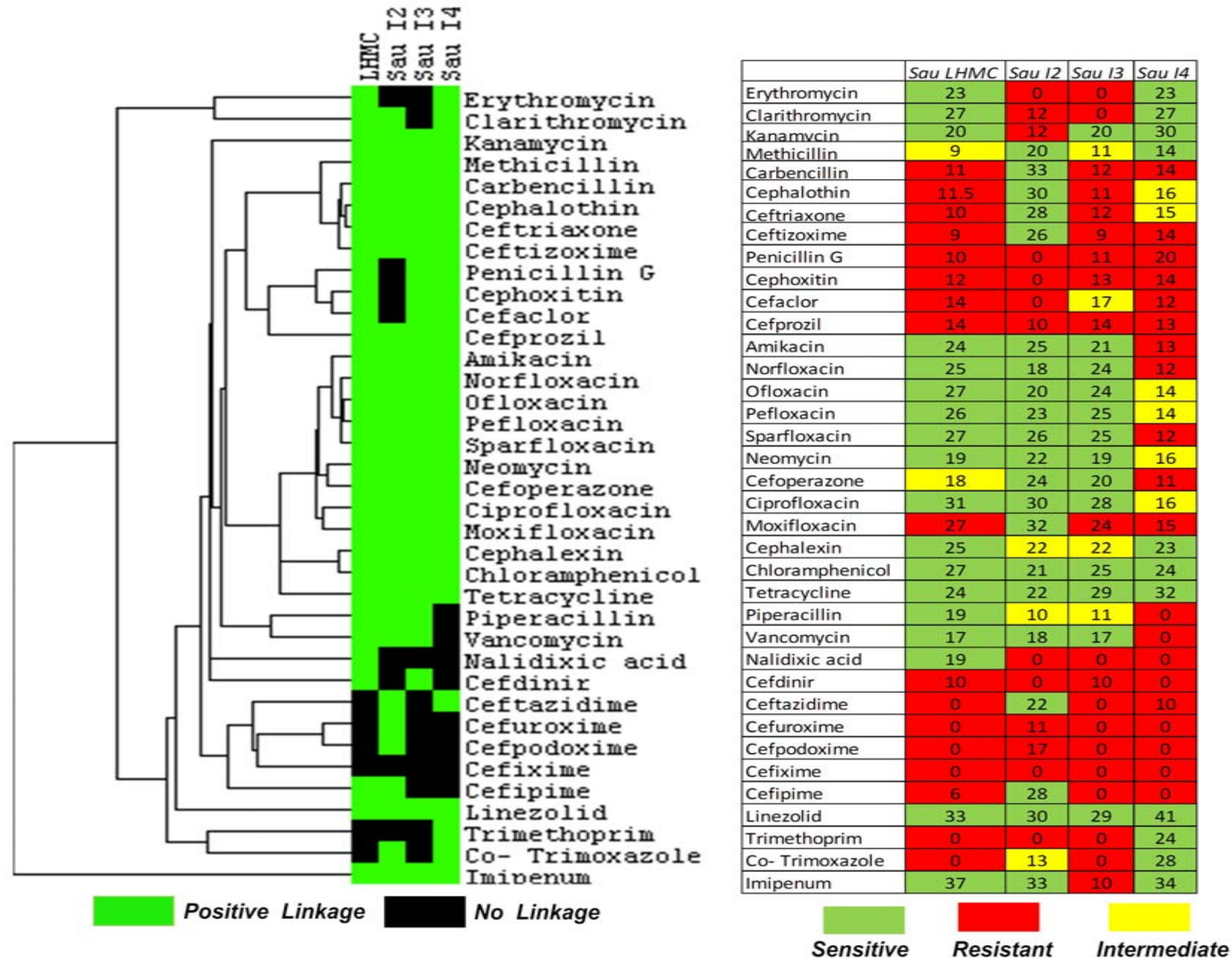

**Fig 5.2l Percentage resistance of standard isolates against the test antibiotics**

**Fig 5.2 (m) Dendrogram and antibiogram of blood, wound and catheter isolates against the test antibiotics**




| | Sau G6 | Sau G12 | Sau A4 | Sau G14 | Sau G20 | Sau G21 |
|---|---|---|---|---|---|---|
| Piperacillin | 13 | 0 | 0 | 0 | 17 | 16 |
| Erythromycin | 0 | 14 | 13 | 31 | 12 | 10 |
| Methicillin | 29 | 10 | 10 | 11 | 25 | 23 |
| Penicillin G | 28 | 12 | 11 | 9 | 21 | 16 |
| Carbencillin | 31 | 11 | 11 | 10 | 19 | 15 |
| Clarithromycin | 6 | 30 | 5 | 29 | 0 | 0 |
| Ceftazidime | 14 | 20 | 13 | 23 | 0 | 0 |
| Ofloxacin | 40 | 16 | 39 | 32 | 12 | 22 |
| Ciprofloxacin | 44 | 16 | 43 | 33 | 13 | 15 |
| Moxifloxacin | 40 | 21 | 40 | 28 | 10 | 32 |
| Nalidixic acid | 19 | 0 | 18 | 0 | 0 | 0 |
| Norfloxacin | 34 | 0 | 35 | 30 | 0 | 20 |
| Sparfloxacin | 38 | 0 | 36 | 32 | 0 | 23 |
| Cephalothin | 24 | 20 | 24 | 36 | 0 | 12 |
| Amikacin | 28 | 13 | 26 | 25 | 0 | 14 |
| Cefprozil | 28 | 11 | 26 | 28 | 0 | 16 |
| Cefaclor | 29 | 13 | 24 | 29 | 0 | 22 |
| Tetracycline | 22 | 20 | 22 | 25 | 0 | 19 |
| Kanamycin | 27 | 0 | 26 | 26 | 0 | 14 |
| Cephalexin | 27 | 0 | 25 | 38 | 0 | 16 |
| Cephoxitin | 25 | 0 | 21 | 40 | 0 | 14 |
| Cefuroxime | 21 | 0 | 17 | 37 | 0 | 12 |
| Ceftriaxone | 22 | 0 | 20 | 28 | 0 | 9 |
| Cefipime | 21 | 0 | 20 | 32 | 0 | 10 |
| Ceftizoxime | 13 | 0 | 13 | 36 | 0 | 10 |
| Neomycin | 23 | 16 | 23 | 24 | 12 | 18 |
| Chloramphenicol | 26 | 15 | 26 | 30 | 13 | 20 |
| Vancomycin | 22 | 21 | 23 | 22 | 20 | 19 |
| Pefloxacin | 36 | 0 | 34 | 17 | 0 | 15 |
| Trimethoprim | 44 | 0 | 47 | 17 | 0 | 0 |
| Co- Trimoxazole | 40 | 0 | 44 | 23 | 0 | 10 |
| Cefpodoxime | 14 | 0 | 12 | 26 | 0 | 0 |
| Cefdinir | 21 | 0 | 17 | 13 | 0 | 10 |
| Cefixime | 0 | 0 | 0 | 18 | 0 | 0 |
| Imipenum | 50 | 14 | 54 | 36 | 9 | 45 |
| Cefoperazone | 17 | 23 | 22 | 0 | 0 | 14 |
| Linezolid | 35 | 34 | 36 | 35 | 37 | 31 |

**Fig 5.2 (n) Dendrogram and antibiogram of standard isolates against the test antibiotics**




| | *Sau LHMC* | *Sau I2* | *Sau I3* | *Sau I4* |
|---|---|---|---|---|
| Erythromycin | 23 | 0 | 0 | 23 |
| Clarithromycin | 27 | 12 | 0 | 27 |
| Kanamycin | 20 | 12 | 20 | 30 |
| Methicillin | 9 | 20 | 11 | 14 |
| Carbencillin | 11 | 33 | 12 | 14 |
| Cephalothin | 11.5 | 30 | 11 | 16 |
| Ceftriaxone | 10 | 28 | 12 | 15 |
| Ceftizoxime | 9 | 26 | 9 | 14 |
| Penicillin G | 10 | 0 | 11 | 20 |
| Cephoxitin | 12 | 0 | 13 | 14 |
| Cefaclor | 14 | 0 | 17 | 12 |
| Cefprozil | 14 | 10 | 14 | 13 |
| Amikacin | 24 | 25 | 21 | 13 |
| Norfloxacin | 25 | 18 | 24 | 12 |
| Ofloxacin | 27 | 20 | 24 | 14 |
| Pefloxacin | 26 | 23 | 25 | 14 |
| Sparfloxacin | 27 | 26 | 25 | 12 |
| Neomycin | 19 | 22 | 19 | 16 |
| Cefoperazone | 18 | 24 | 20 | 11 |
| Ciprofloxacin | 31 | 30 | 28 | 16 |
| Moxifloxacin | 27 | 32 | 24 | 15 |
| Cephalexin | 25 | 22 | 22 | 23 |
| Chloramphenicol | 27 | 21 | 25 | 24 |
| Tetracycline | 24 | 22 | 29 | 32 |
| Piperacillin | 19 | 10 | 11 | 0 |
| Vancomycin | 17 | 18 | 17 | 0 |
| Nalidixic acid | 19 | 0 | 0 | 0 |
| Cefdinir | 10 | 0 | 10 | 0 |
| Ceftazidime | 0 | 22 | 0 | 10 |
| Cefuroxime | 0 | 11 | 0 | 0 |
| Cefpodoxime | 0 | 17 | 0 | 0 |
| Cefixime | 0 | 0 | 0 | 0 |
| Cefipime | 6 | 28 | 0 | 0 |
| Linezolid | 33 | 30 | 29 | 41 |
| Trimethoprim | 0 | 0 | 0 | 24 |
| Co- Trimoxazole | 0 | 13 | 0 | 28 |
| Imipenum | 37 | 33 | 10 | 34 |

**Fig 5.2 (q) Dendrogram and antibiogram of eye, ear and unclassified isolates against the test antibiotics**

| | Sau G22 | Sau G8 | Sau A5 | Sau MTB |
|---|---|---|---|---|
| Sparfloxacin | 0 | 35 | 37 | 24 |
| Clarithromycin | 0 | 0 | 33 | 20 |
| Nalidixic acid | 0 | 16 | 17 | 21 |
| Erythromycin | 0 | 11 | 27 | 17 |
| Vancomycin | 0 | 22 | 26 | 17 |
| Norfloxacin | 15 | 34 | 36 | 25 |
| Pefloxacin | 16 | 31 | 35 | 29 |
| Ciprofloxacin | 16 | 42 | 44 | 28 |
| Ofloxacin | 17 | 35 | 40 | 29 |
| Moxifloxacin | 17 | 40 | 39 | 30 |
| Linezolid | 12 | 37 | 37 | 33 |
| Methicillin | 21 | 24 | 23 | 10 |
| Kanamycin | 11 | 28 | 29 | 19 |
| Cefprozil | 17 | 26 | 29 | 16 |
| Amikacin | 22 | 24 | 27 | 24 |
| Cephalexin | 23 | 23 | 30 | 25 |
| Tetracycline | 19 | 28 | 27 | 24 |
| Neomycin | 17 | 22 | 23 | 18 |
| Penicillin G | 16 | 19 | 23 | 17 |
| Carbencillin | 16 | 18 | 23 | 15 |
| Piperacillin | 20 | 18 | 24 | 22 |
| Ceftriaxone | 17 | 20 | 24 | 21 |
| Cefdinir | 14 | 17 | 20 | 14 |
| Chloramphenicol | 25 | 24 | 36 | 27 |
| Cephoxitin | 20 | 20 | 27 | 18 |
| Cefipime | 18 | 16 | 23 | 10 |
| Cefoperazone | 17 | 13 | 21 | 22 |
| Cefaclor | 28 | 24 | 31 | 19 |
| Cefuroxime | 15 | 8 | 20 | 5 |
| Ceftizoxime | 17 | 7 | 18 | 0 |
| Ceftazidime | 23 | 12 | 17 | 0 |
| Cefpodoxime | 13 | 12 | 13 | 0 |
| Cephalothin | 14 | 0 | 24 | 15 |
| Co- Trimoxazole | 12 | 40 | 29 | 12 |
| Trimethoprim | 0 | 43 | 37 | 0 |
| Cefixime | 0 | 0 | 0 | 0 |
| Imipenum | 54 | 46 | 54 | 31 |

Positive Linkage No Linkage Sensitive Resistant Intermediate

**Fig 5.2 (o) Percentage resistances of eye and ear isolates against the test antibiotics**

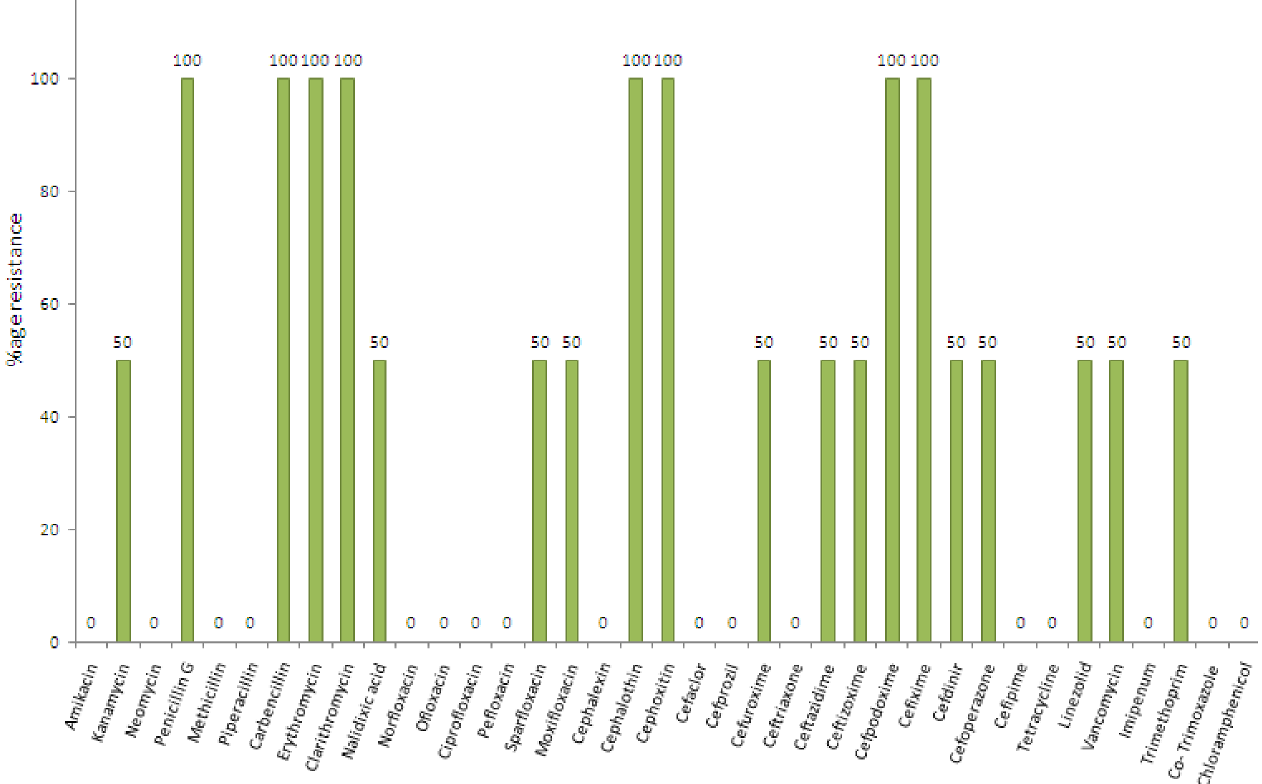
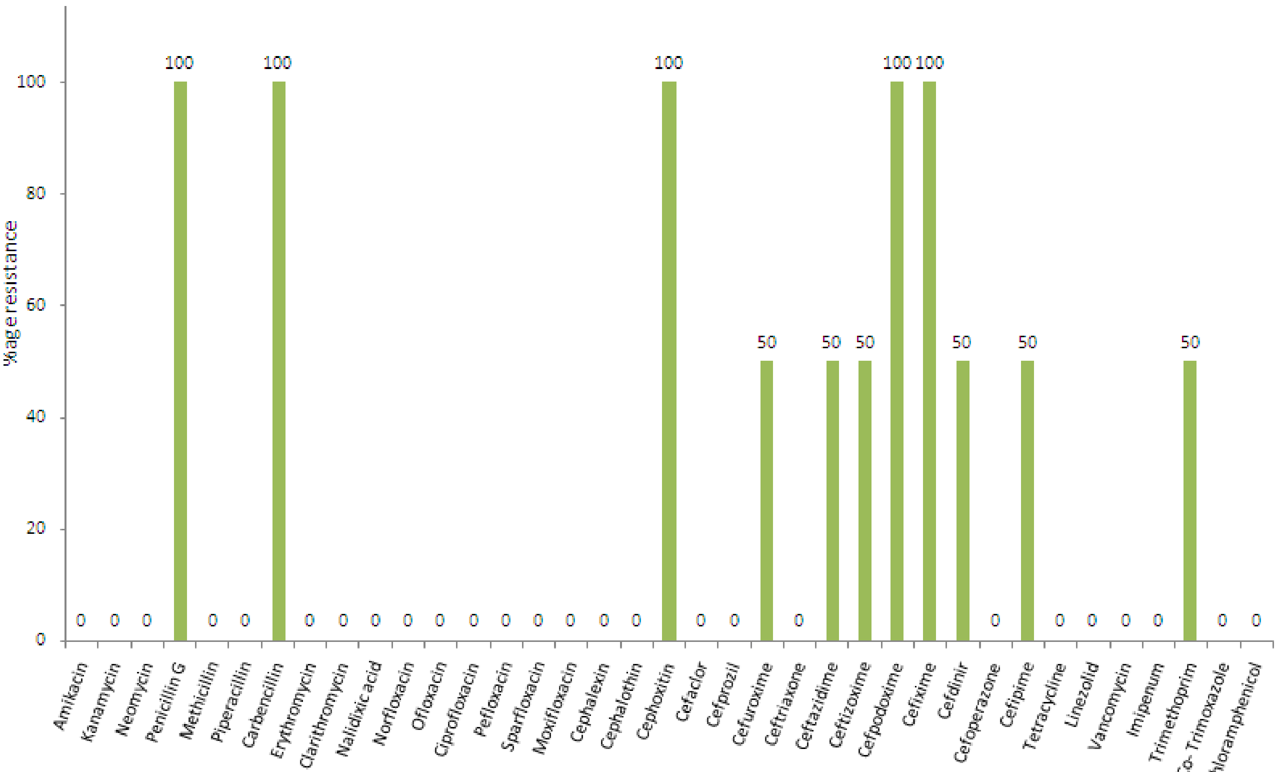


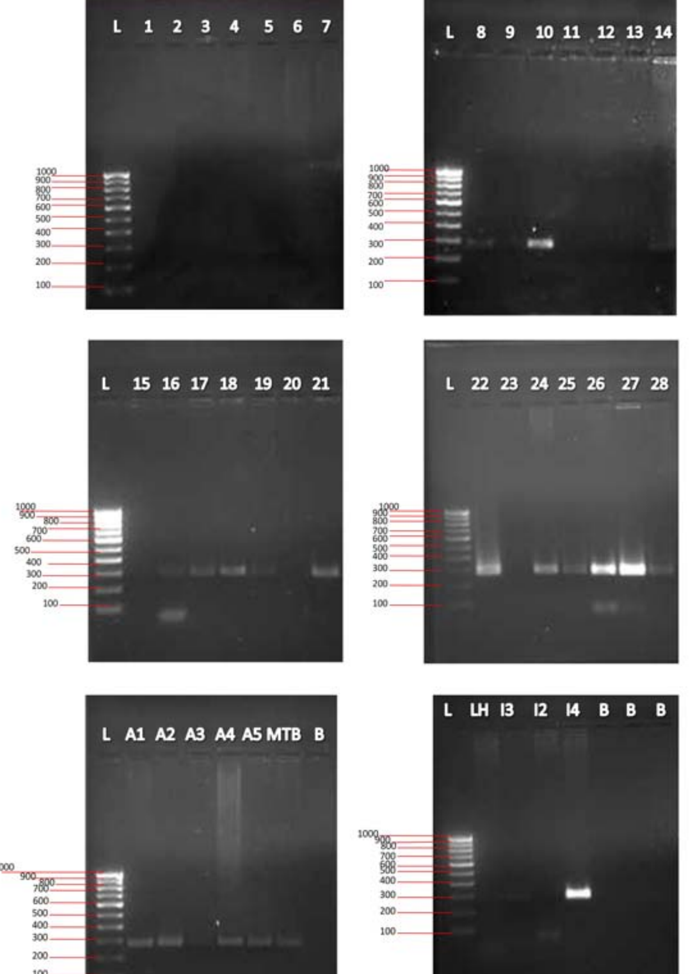

**Fig 5.2 (p) Percentage resistances of unclassified isolates against the test antibiotics**



## 5.3 Biochemical and molecular characterization of the test isolates

The biochemical characterization of the test panel isolates comprised of catalase and coagulase activities. Further they were screened for the presence of *mecA* in *SCCmec* cassette as well as for specific methicillin resistance by PCR amplification. Out of 38 isolates in the test panel, only six isolates viz. Sau G2, Sau G7, Sau G8, Sau G16, Sau G18 and Sau G23 were found to be coagulase negative (Table 5.2). All isolates were catalase positive.

18 isolates were found to be *mecA* positive using primer corresponded to mA1-5' TGCTATCCACCCTCAAACAGG3' and the reverse primer to mA2-5'AACGTTGTAACCACCCCAAGA3'. The amplified product was observed at 286bp- 300bp (Fig 5.3 a). 11 isolates were found to give a positive result with second primer set comprising of specific the forward primer corresponded to nucleotides 1282 to 1303 and the reverse primer complementary to nucleotides 1793 to 1814 of the mec A gene (Fig 5.3b, Table 5.2). 91% of the pus isolates and 71% of burn isolates exhibited methicillin resistance. The urine and vaginal isolates were not *mecA* positive. Sau A1, Sau G24, Sau G27, Sau G18, Sau G26 and Sau MTCC 902 exhibited methicillin resistance by both primer sets used for *mecA*.




← **Fig. 5.3(a)** Screening of staphylococcal isolates for the presence of *mecA* gene.

**Lane coding of gels**

*L- 100bp DNA Ladder; 1- Sau G1; 2- SauG2, 3- SauG3, 4- Sau G4, 5- Sau G5, 6- Sau G6, 7- Sau G7; 8- Sau G8, 9- Sau G9, 10- Sau G10, 11- Sau G11, 12- Sau G12, 13- Sau G13, 14- Sau G14, 15- Sau G15, 16- Sau G16, 17- Sau G17, 18- Sau G18, 19- Sau G19, 20- Sau G20, 21- Sau G21, 22- Sau G22, 23- Sau G23, 24- Sau G24, 25- Sau G25, 26- Sau G26, 27- Sau G27, 28- Sau G28, A1-Sau A1, A2- Sau A2, A3- Sau A3, A4- Sau A4, A5- Sau A5 MTB- Sau MTB, B- Blank, LH- Sau NCTC6571; I3- Sau MTCC 737, I2- Sau MTCC 740 and I4- Sau MTCC902*

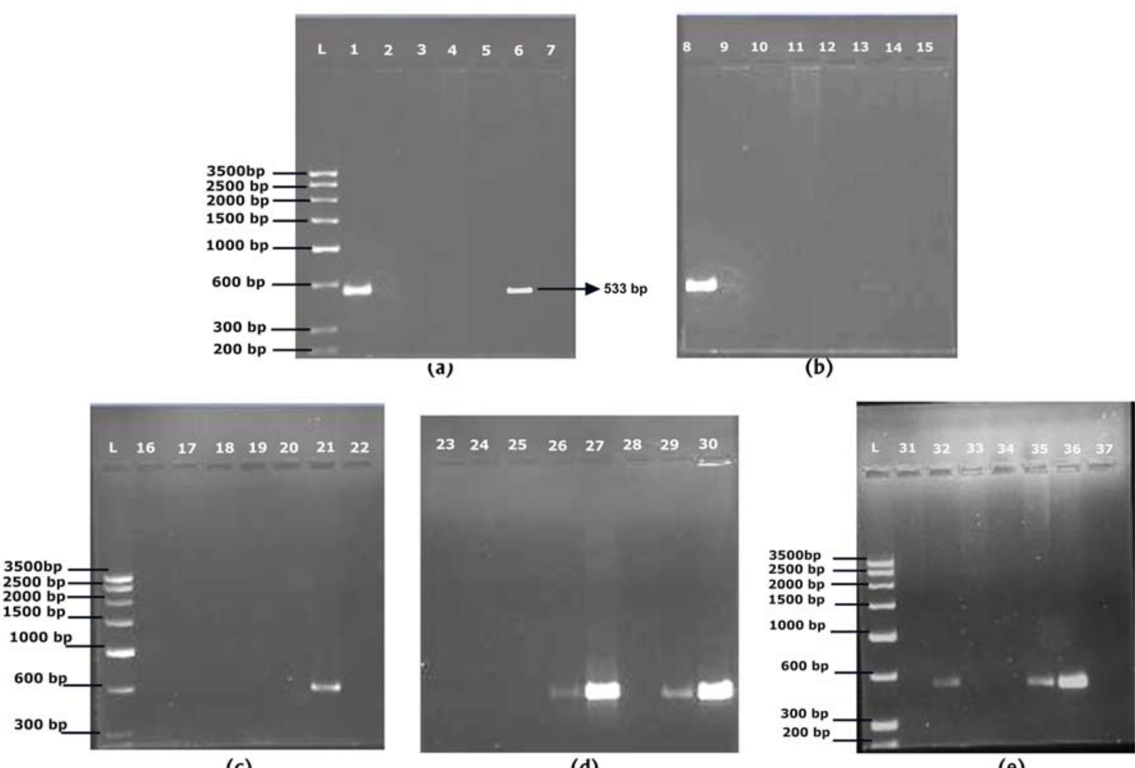



**Fig. 5.3 (b)** Screening of staphylococcal isolates for the presence 533 bp amplicon as a marker for methicillin resistance

**Lane coding of the gels**

*L- DNA ladder, 1-Sau A1, 2-Sau A2, 3-Sau A3, 4-Sau A4, 5-Sau A5, 6-Sau G1, 7-Sau G2, 8- Sau G3, 9-Sau G4, 10- Sau G5, 11- Sau G6, 12- Sau G7, 13- Sau G8, 14- Sau G9, 15- Sau G10, 16- SauG13, 17- SauG 14, 18- Sau G15, 19- SauG16, 20- Sau G17, 21- Sau G18, 22- Sau G19, 23- Sau G20, 24- Sau G21, 25-Sau G22, 26- Sau G23, 27- Sau G24, 28-Sau G 25, 29- Sau G26, 30- Sau G27, 31- Sau G11, 32- Sau G12, 33- Sau NCTC6571, 34- Sau MTCC737, 35- Sau MTCC740, 36- Sau MTCC902, 37- Sau MTB*

39% isolates were methicillin sensitive while 61% were methicillin resistant as they were amplified by either primer set for *mecA* gene amplification which is a constituent of *SCCmec* cassette of *Staphylococcus aureus* for methicillin resistance.

## 5.4 Growth Curves of the test isolates

The growth curves of the clinical isolates were established on a medium throughput system, Power wave 340 (Biotek, USA). The mean lag phase of the cultures ranged between 1.5-2 h, followed by a $\log_{10}$ phase which extended up to 10-12 h and then entered into stationary phase until 16-18 h and this was followed by death phase up to 24 h. The average generation time of isolates was 44 min in the log phase (Fig. 5.4)

| Isolate Code | Group | Catalase | Coagulase | mec A (Ruppe et.al, 2009) Primer Set I* | mecA (Rallapalli et al.,2008) Primer Set II** |
|---|---|---|---|---|---|
| Sau A1 | PUS | + | + | + | + |
| Sau A2 | PUS | + | + | + | - |
| Sau G1 | PUS | + | + | - | + |
| Sau G2 | PUS | + | - | - | - |
| Sau G3 | PUS | + | + | - | + |
| Sau G10 | PUS | + | + | + | - |
| Sau G23 | PUS | + | - | - | + |
| Sau G24 | PUS | + | + | + | + |
| Sau G25 | PUS | + | + | + | - |
| Sau G27 | PUS | + | + | + | + |
| Sau G28 | PUS | + | + | + | - |
| Sau G15 | BURN | + | + | - | - |
| Sau G9 | BURN | + | + | - | - |
| Sau G16 | BURN | + | - | + | - |
| Sau G17 | BURN | + | + | + | - |
| Sau G18 | BURN | + | - | + | + |
| Sau G19 | BURN | + | + | + | - |
| Sau G26 | BURN | + | + | + | + |
| Sau A3 | URINE | + | + | - | - |
| Sau G4 | URINE | + | + | - | - |
| Sau G5 | URINE | + | + | - | - |
| Sau G11 | URINE | + | + | - | - |
| Sau G7 | VAGINAL | + | - | - | - |
| Sau G13 | VAGINAL | + | + | - | - |
| Sau G6 | BLOOD | + | + | - | - |
| Sau G12 | BLOOD | + | + | - | + |
| Sau A4 | WOUND | + | + | + | - |
| Sau G14 | WOUND | + | + | - | - |
| Sau G20 | CATHETER | + | + | - | - |
| Sau G21 | CATHETER | + | + | + | - |
| Sau G22 | EYE | + | + | + | - |
| Sau G8 | EAR | + | - | + | - |
| Sau A5 | UNCLASSIFIED | + | + | + | - |
| Sau MTB | UNCLASSIFIED | + | + | - | - |
| Sau LHMC | NCTC6571 | + | + | - | - |
| Sau I2 | MTCC740 | + | + | - | + |
| Sau I3 | MTCC737 | + | + | - | - |
| Sau I4 | MTCC902 | + | + | + | + |

***Primer Set I***
***mA1-5' TGCTATCCACCCTCAAACAGG3'(Forward)***
***mA2-5'AACGTTGTAACCACCCCAAGA3' (Reverse)***

***** Primer Set II***
***mA1-5' AAAATCGATGGTAAAGGTTGGC(Forward)***
***mA2-5'AGTTCTGCAGTACCGGATTTGC( Reverse)***

**Table 5.2 Biochemical characterization of the test panel isolates**

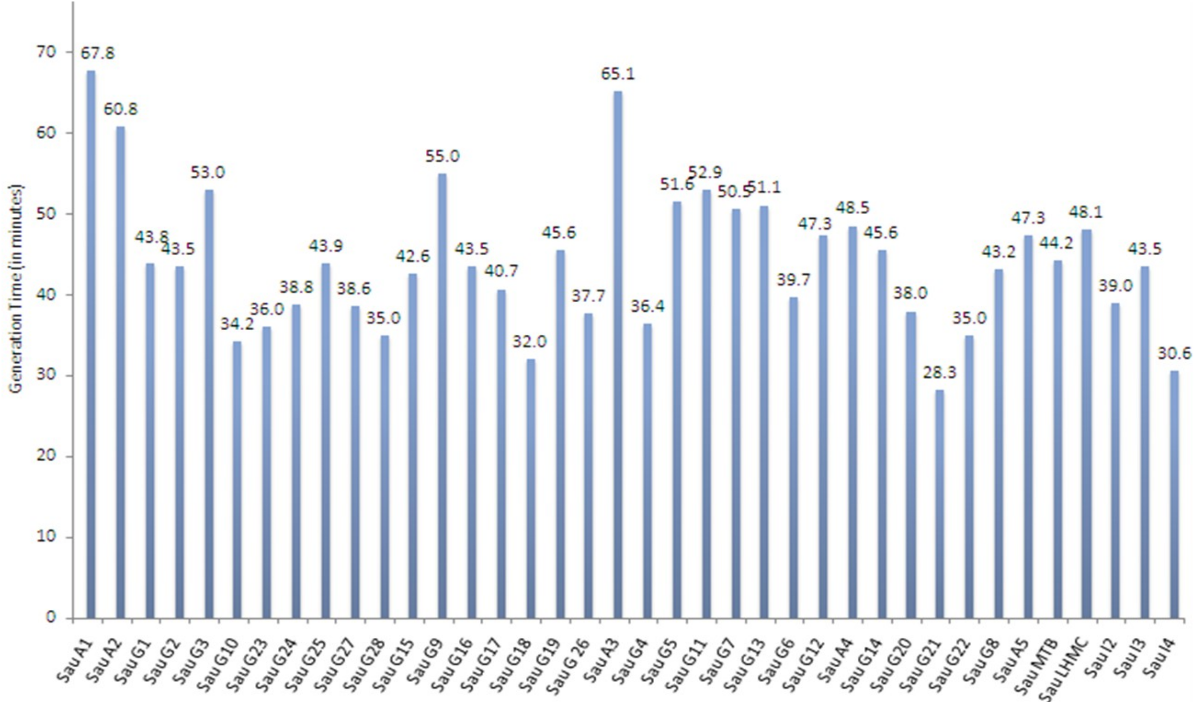



**Fig. 5.4 Generation time of the staphylococcal isolates comprising the antibacterial test panel**

## 5.5 AWD assay of crude methanolic extract

The crude extract against the test isolates tested at a concentration of 66.66 µg induced different inhibition zones suggesting potential antimicrobial activity. The pus isolates exhibited a mean inhibition zone (MIZ) diameter of 12.66±0.57 mm to 24.66±0.57 mm. Only two isolates Sau G3 and Sau G24 exhibited resistance and did not induce any inhibition. The MIZ diameter range in the case of burn isolates ranged between 11.33±0.28 mm to 24.00 mm and none of the isolates were found to be resistant to the crude methanolic extract of the leaves of *Callistemon rigidus*. Among the urine isolates the MIZ ranged between 11.66±0.57 to 19.00 ±0. Blood isolate Sau G6 and Sau G13 a vaginal isolate were found to be resistant to the crude methanolic extract as no zone of inhibition was induced in them in the agar well assay. The MIZ diameter of the susceptible wound and catheter isolates ranged between 12-15 mm. The eye isolate was also not susceptible to the crude methanolic extract while the ear exhibited a MIZ diameter range in between 14.66±0.577 mm. Unclassified isolates were least susceptible and exhibited a MIZ range between 6.66±0.577 - 12.66±0.577 mm while in the standard isolates the MIZ ranged between 10.66±0.577- 20.66±0.28. The Pus isolates were the most susceptible isolates to the

crude methanolic extract (Fig 5.5, 5.6). The test extract exhibited a concentration dependent antibiotic activity when tested with a range of 16.65- 399.9 μg (Fig.5.7).

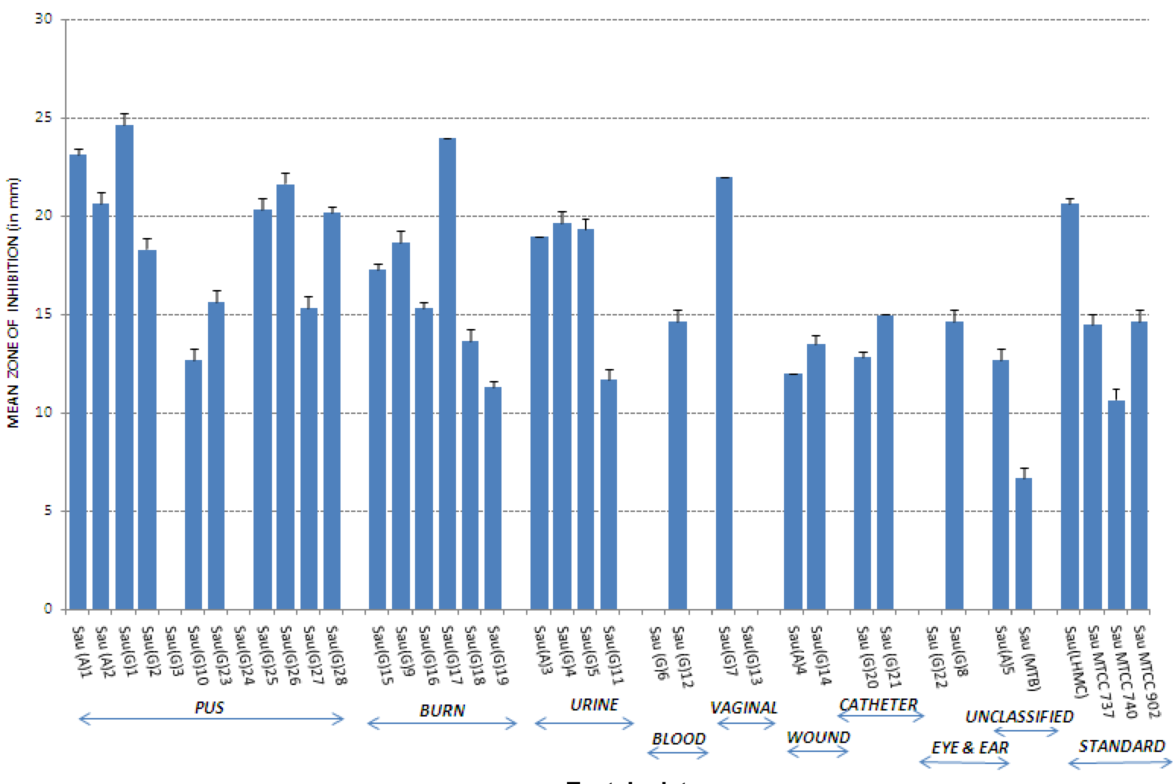
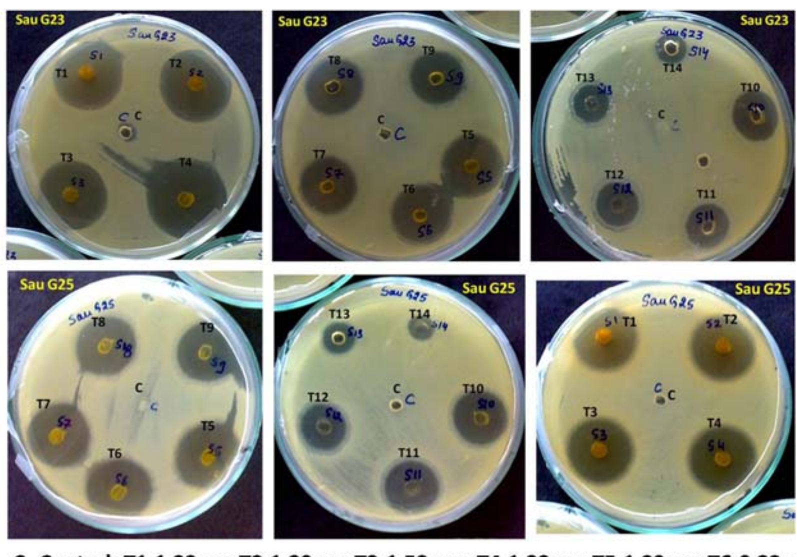



**Fig 5.5 Maximum inhibition zone in the test panel isolates at a concentration of 66.66 μg of crude methanol extract in the well during *in vitro* agar well diffusion assay.**




C- Control; T1-1.99mg; T2-1.80mg; T3-1.59 mg; T4-1.39mg; T5-1.20mg; T6-0.99mg; T7-0.79mg; T8-0.60mg ; T9-0.39 mg; T10-0.19 mg; T11-0.099mg; T12- 66.66 μg; T13- 33.30 μg; T14-16.65 μg

**Fig. 5.6 Agar well diffusion assay of the crude methanol extract against staphylococcal isolates exhibiting the inhibition zone formation at different concentration of crude methanol extract**

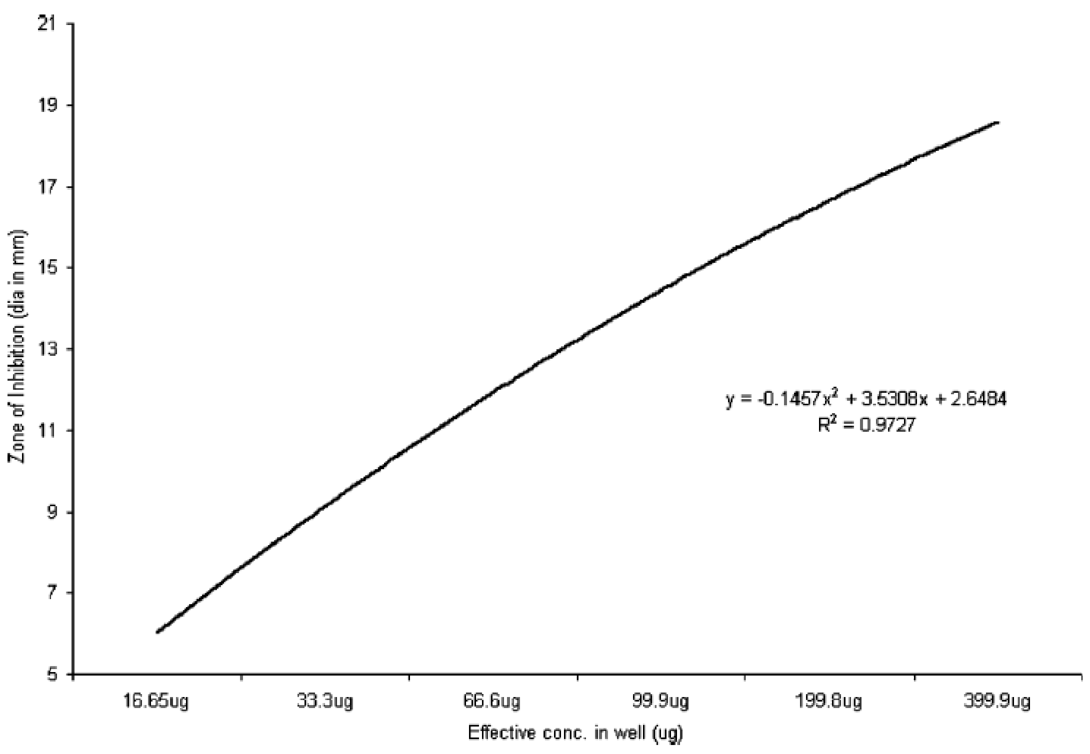



**Fig. 5.7 Dose response relationship between crude methanol extract and inhibition zones of the test microorganisms**

## 5.6 MIC of crude methanol extract by *in vitro* microbroth dilution assay

The overall $MIC_{50}$ and $MIC_{90}$ of the crude methanol extract as assessed by *in vitro* microbroth dilution assay (Fig. 5.8) were 5 µg/ml and 40 µg/ml respectively. In the pus isolates the MIC range was between 1.25-40 µg/ml and only SauG3 exhibited resistance towards the crude methanol extract. In blood, wound and

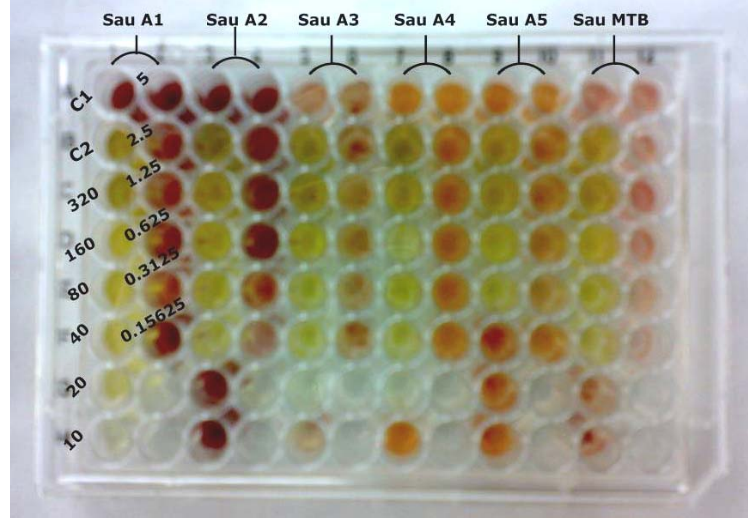



**Fig. 5.8 *In vitro* microbroth dilution assay of crude methanol extract**

**Legends**

C1- Control 1, C2- Control 2, 320-320 µg/ml; 160-160µg/ml; 80-80µg/ml; 40-40µg/ml; 20-20µg/ml; 10-10µg/ml; 5-5 µg/ml; 2.5-2.5 µg/ml; 1.25-1.25 µg/ml; 0.625-0.625µg/ml; 0.3125-0.3125µg/ml; 0.15625-0.15625 µg/ml concentration of crude extract

catheter isolates the MIC range was between 1.25-80 µg/ml. The MIC range of urine and vaginal isolates was between 1.25-20 µg/ml. Burn isolates exhibited a MIC range between 2.5-20 µg/ml. Eye isolate was totally resistant while the ear isolate exhibited a MIC of 5 µg/ml. The unclassified isolates were also exhibited high resistance with a MIC range between 40-80µg/ml. The standard isolates exhibited a MIC range similar to urine and vaginal isolates within a range of 1.25-20 µg/ml (Table 5.3). Group wise highest average MIC, $MIC_{50}$ and $MIC_{90}$ values were of unclassified isolates which was followed by blood, catheter and wound isolates (Fig.5.9). Thus microbroth dilution provided a much clearer susceptibility pattern of test isolates towards the crude methanol extract under *in vitro* conditions.

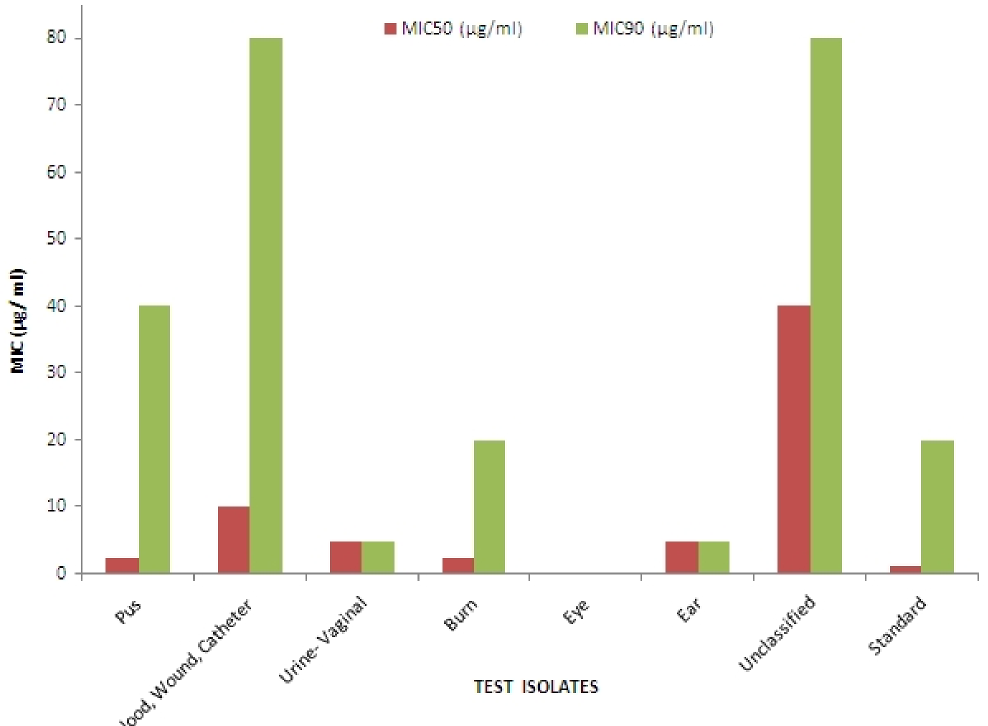



**Fig. 5.9 Group wise $MIC_{50}$ and $MIC_{90}$ of crude methanol extract by *in vitro* microbroth dilution assay.**

| Isolate Groups | Isolate id | MIC (µg/ml) |
|---|---|---|
| PUS | Sau A1 | 10 ± 0.00 |
| | Sau A2 | 40 ± 0.00 |
| | Sau G1 | 1.25 ± 0.00 |
| | Sau G2 | 10 ± 0.00 |
| | SauG 3 | 0 ± 0.00 |
| | Sau G10 | 1.25 ± 0.00 |
| | Sau G23 | 2.5 ± 0.00 |
| | Sau G24 | 40 ± 0.00 |
| | Sau G25 | 1.25 ± 0.00 |
| | Sau G26 | 5 ± 0.00 |
| | Sau G27 | 1.25 ± 0.00 |
| | Sau G28 | 2.5 ± 0.00 |
| BLOOD, WOUND, CATHETER | Sau G6 | 2.5 ± 0.00 |
| | Sau G12 | 1.25 ± 0.00 |
| | Sau A4 | 20 ± 0.00 |
| | Sau G14 | 10 ± 0.00 |
| | Sau G20 | 80 ± 0.00 |
| | Sau G21 | 80 ± 0.00 |
| URINE- VAGINAL | Sau A3 | 20 ± 0.00 |
| | Sau G4 | 5 ± 0.00 |
| | Sau G5 | 5 ± 0.00 |
| | Sau G11 | 2.5 ± 0.00 |
| | Sau G7 | 5 ± 0.00 |
| | Sau G13 | 1.25 ± 0.00 |
| BURN | Sau G15 | 2.5 ± 0.00 |
| | Sau G9 | 10 ± 0.00 |
| | Sau G16 | 2.5 ± 0.00 |
| | Sau G17 | 2.5 ± 0.00 |
| | Sau G18 | 20 ± 0.00 |
| | Sau G19 | 5 ± 0.00 |
| EYE & EAR | Sau (G)22 | 0 ± 0.00 |
| | Sau(G)8 | 5 ± 0.00 |
| UNCLASSIFIED | Sau A5 | 80 ± 0.00 |
| | Sau MTB | 40 ± 0.00 |
| STANDARD | Sau LHMC | 20 ± 0.00 |
| | Sau MTCC 737 | 2.5 ± 0.00 |
| | Sau MTCC 740 | 1.25 ± 0.00 |
| | Sau MTCC 902 | 1.25 ± 0.00 |

**Table 5.3 MIC of crude methanol extract of *Callistemon rigidus* leaves by *in vitro* microbroth dilution assay** (All values given are Mean± SD values of triplicate readings)

## 5.7 Phytochemical analysis of the crude extract

The phytochemical analysis of the crude extract revealed the presence of alkaloid class as the most prominent component in the crude methanol extract followed by flavonoids, tannins, saponins and glycosides (Fig 5.9; Table 5.4). Anthraquinones were absent in the crude methanol extract.

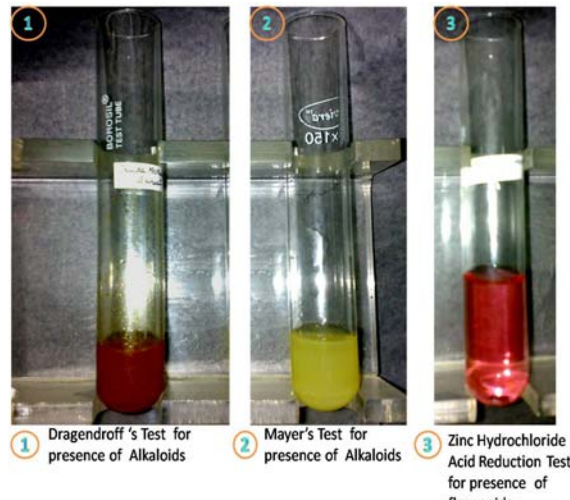



**Fig. 5.10 Phytochemical test of crude methanol extract for alkaloids and flavonoids**

| Class of compounds | Tests | Result |
|---|---|---|
| Alkaloids | Dragendroff's; Mayers reagent | +++ve |
| Anthraquinones | Benzene- Ammonia | -ve |
| Tannins | Ferric chloride reagent | +ve |
| Saponins | Frothing | +ve |
| Flavonoids | Zinc Hcl reduction | +ve |
| Glycosides/ Glycolipids | Diphenylamine reagent | +ve |

**Table 5.4 Phytochemical test of crude methanol extract**

## 5.8 Mass extraction and fractionation of the crude methanolic extract

Bioactivity guided fractionation method of acidified crude methanolic extract was carried out to remove the interfering compounds like chlorophylls and other pigments to obtain aqueous and organic components after extraction by dichloromethane. Bioactivity of the aqueous layer as well as organic layer was tested by *in vitro* microbroth dilution assay using clinical and standard test microorganisms. The organic layer comprised of neutral and acidic components of the extract which did not possess any activity and hence was discarded. Further the neutralized

aqueous layer was re-extracted with dichloromethane to give aqueous and organic layers which were retested for the bioactivity by *in vitro* microbroth dilution assay. It was found that the bioactivity resided in the organic layer and no activity was encountered in the aqueous layer. Hence the aqueous layer was discarded. The MIC of the bioactive organic layer by *in vitro* microbroth dilution assay was found to be 3.125`µg/ml. No significant change was observed in CFU in the control and test sets except at 3.125 µg/ml concentration and thus the concentration response curve could not be established (Table 5.5). Further analysis of $\log_{10}$ CFU reduction by one way ANOVA and Dunnett's Multiple comparison test indicated that there was a significant reduction at $P<0.05$ between the control and ABF concentration of 3.125`µg/ml while in other concentrations no significant difference was recorded in all isolate groups (Table 5.6). Hence this crude organic bioactive layer was taken up for further fractionation to obtain lead fractions. This extract gave a positive test for alkaloids by Dragendorff's and Mayer's reagents. Consequently it was designated as the alkaloid bioactive fraction (ABF). The yield of ABF by this process was 0.025% w/w of leaves.

| Isolate groups | Control | Concentration of ABF tested for bacterial colony reduction in $Log_{10}$ CFU $ml^{-1}$ | | | |
|---|---|---|---|---|---|
| | | 3.125µg/ml | 6.25 µg/ml | 12.5 µg/ml | 25 µg/ml |
| PUS | 8.932 ± 2.86 | 5.97 ± 0.65 | 9.16 ± 2.98 | 8.49 ± 2.83 | 8.97 ± 3.06 |
| BURN | 10.90 ± 0.86 | 6.66 ± 0.21 | 10.05 ± 2.32 | 9.75 ± 1.68 | 10.39 ± 1.91 |
| URINE | 10.59 ± 2.78 | 6.53 ± 0.19 | 11.00 ± 2.80 | 10.05 ± 0.19 | 11.61 ± 1.88 |
| BLOOD | 11.55 ± 0.30 | 6.62 ± 0.36 | 11.93 ± 1.73 | 10.59 ± 0.93 | 11.92 ± 0.93 |
| VAGINAL | 11.79 ± 0.35 | 6.40 ± 0.06 | 11.78 ± 0.12 | 10.10 ± 1.28 | 11.85 ± 0.10 |
| WOUND | 11.06 ± 1.28 | 6.49 ± 0.18 | 11.10 ± 1.26 | 10.70 ±1.28 | 11.85 ± 0.10 |
| CATHETER TIPS | 10.95 ± 1.53 | 6.47 ± 0.10 | 11.24 ± 1.45 | 10.44 ± 1.32 | 10.80 ± 1.25 |
| EYE & EAR | 11.40 ± 1.16 | 6.34 ± 0.03 | 12.06 ± 1.35 | 12.06 ± 0.03 | 11.89 ± 0.50 |
| UNCLASSIFIED | 7.92 ± 4.33 | 5.73 ± 0.9 | 7.99 ± 4.48 | 7.85 ± 4.20 | 8.08 ± 4.24 |
| STANDARD | 5.91 ± 0.00 | 5.23 ± 0.00 | 5.49 ± 0.00 | 5.35 ± 0.00 | 5.21 ± 0.00 |

*All values are means± SD of triplicate readings obtained at 24 hours*

**Table 5.5 CFU reduction by ABF from methanol extract by *in vitro* microbroth dilution assay**

## 5.9 Microwave assisted extraction of Alkaloidal Bioactive Fractions (ABF)

In order to improve the yield of the bioactive fractions, microwave treatment of pulverized leaves of *Callistemon rigidus* was done and the yield of the ABF was 0.1% w/w as compared to the previous procedure.

| Isolate groups | One way ANOVA (level of significance) | Dunnett's Multiple Comparison Test | | | | | Inference |
|---|---|---|---|---|---|---|---|
| | | Treatments | Mean Difference | q | p value | 95% CI of difference | |
| PUS | P<0.05 (***) | Control vs. 3.125µg/ml | 3.30 | 6.16 | P<0.01 | 1.90 to 4.69 | S |
| | | Control vs. 6.25µg/ml | -0.242 | 0.45 | P>0.05 | -1.64 to 1.15 | NS |
| | | Control vs. 12.5µg/ml | 0.429 | 0.80 | P>0.05 | -0.967 to 1.83 | NS |
| | | Control vs. 25µg/ml | -0.080 | 0.15 | P>0.05 | -1.48 to 1.32 | NS |
| BURN | P<0.05 (***) | Control vs. 3.125µg/ml | 4.33 | 7.88 | P<0.01 | 2.902 to 5.773 | S |
| | | Control vs. 6.25µg/ml | 0.652 | 1.18 | P>0.05 | -0.783 to 2.88 | NS |
| | | Control vs. 12.5µg/ml | 1.051 | 1.91 | P>0.05 | -0.384 to 2.486 | NS |
| | | Control vs. 25µg/ml | 0.394 | 0.72 | P>0.05 | -1.041 to 1.830 | NS |
| URINE | P<0.05 (***) | Control vs. 3.125µg/ml | 4.305 | 5.83 | P<0.01 | 2.304 to 6.306 | S |
| | | Control vs. 6.25µg/ml | -0.429 | 0.57 | P>0.05 | -2.424 to 1.578 | NS |
| | | Control vs. 12.5µg/ml | 0.625 | 0.84 | P>0.05 | -1.376 to 1.626 | NS |
| | | Control vs. 25µg/ml | -0.865 | 1.17 | P>0.05 | -2.866 to 1.136 | NS |
| BLOOD | P<0.05 (***) | Control vs. 3.125µg/ml | 4.932 | 7.67 | P<0.01 | 2.989 to 6.875 | S |
| | | Control vs. 6.25µg/ml | -0.420 | 0.65 | P>0.05 | -2.364 to 1.523 | NS |
| | | Control vs. 12.5µg/ml | 0.946 | 1.47 | P>0.05 | -0.996 to 2.890 | NS |
| | | Control vs. 25µg/ml | 0.118 | 0.18 | P>0.05 | -1.825 to 2.062 | NS |
| VAGINAL | P<0.05 (***) | Control vs. 3.125µg/ml | 5.382 | 22.47 | P<0.01 | 4.659 to 6.106 | S |
| | | Control vs. 6.25µg/ml | 0.005 | 0.00 | P>0.05 | -0.717 to 0.728 | NS |
| | | Control vs. 12.5µg/ml | 1.669 | 1.54 | P>0.05 | -0.354 to 1.093 | NS |
| | | Control vs. 25µg/ml | 0.275 | 1.15 | P>0.05 | -0.448 to 0.998 | NS |
| WOUND | P<0.05 (***) | Control vs. 3.125µg/ml | 4.594 | 10.26 | P<0.01 | 3.242 to 5.946 | S |
| | | Control vs. 6.25µg/ml | -0.031 | 0.07 | P>0.05 | -1.384 to 1.320 | NS |
| | | Control vs. 12.5µg/ml | 0.353 | 0.78 | P>0.05 | -0.998 to 1.706 | NS |
| | | Control vs. 25µg/ml | -0.774 | 1.73 | P>0.05 | -2.127 to 0.5774 | NS |
| CATHETER TIP | P<0.05 (***) | Control vs. 3.125µg/ml | 4.523 | 10.93 | P<0.01 | 3.272 to 5.722 | S |
| | | Control vs. 6.25µg/ml | -0.279 | 0.68 | P>0.05 | -1.529 to 0.9703 | NS |
| | | Control vs. 12.5µg/ml | 0.294 | 0.71 | P>0.05 | -0.954 to 1.544 | NS |
| | | Control vs. 25µg/ml | 0.096 | 0.23 | P>0.05 | -1.153 to 1.347 | NS |
| EYE &EAR | P<0.05 (***) | Control vs. 3.125µg/ml | 5.078 | 13.57 | P<0.01 | 3.948 to 6.209 | S |
| | | Control vs. 6.25µg/ml | -0.670 | 1.79 | P>0.05 | -1.801 to 0.468 | NS |
| | | Control vs. 12.5µg/ml | -0.649 | 1.73 | P>0.05 | -1.776 to 0.484 | NS |
| | | Control vs. 25µg/ml | -0.473 | 1.27 | P>0.05 | -1.604 to 0.657 | NS |
| UNCLASSIFIED | P<0.05 (*) | Control vs. 3.125µg/ml | 2.875 | 3.91 | P<0.05 | 0.655 to 5.096 | S |
| | | Control vs. 6.25µg/ml | -0.094 | 0.13 | P>0.05 | -2.315 to 2.126 | NS |
| | | Control vs. 12.5µg/ml | 0.084 | 0.11 | P>0.05 | -2.316 to 2.305 | NS |
| | | Control vs. 25µg/ml | -0.141 | 0.19 | P>0.05 | -2.361 to 2.080 | NS |
| STANDARD | P<0.05 (**) | Control vs. 3.125µg/ml | 2.015 | 7.10 | P<0.01 | 1.158 to 2.871 | S |
| | | Control vs. 6.25µg/ml | 0.422 | 1.49 | P>0.05 | -0.434 to 1.279 | NS |
| | | Control vs. 12.5µg/ml | 0.559 | 1.97 | P>0.05 | -0.297 to 1.416 | NS |
| | | Control vs. 25µg/ml | 0.704 | 2.49 | P>0.05 | -0.151 to1.56 | NS |

**Table 5.6 ANOVA (one-way) and Dunnett's multiple comparison tests to assess CFU reduction by ABF at different concentration in isolate groups.**

## 5.10 MIC and IC $_{50}$ of ABF by *in vitro* microbroth dilution assay

The ABF was found to possess an $MIC_{50}$ and $MIC_{90}$ of 80 µg/ml and 320 µg/ml respectively, against the test panel isolates respectively. The MIC range for pus isolates ranged between 80-640 µg/ml. In burn isolates the MIC range was between 20-640 µg/ml while in urine isolates he MIC range was similar to pus isolates. Vaginal isolates exhibited an average MIC of 80 µg/ml while the blood isolates exhibited average MIC of 200 µg/ml. Wound isolates were most susceptible with a MIC range of 10-80 µg/ml. Catheter tip isolates exhibited an average MIC of 90 µg/ml while eye isolate exhibited a MIC of 320 µg/ml. The ear isolate was sensitive at a MIC of 40 µg/ml. Unclassified isolates exhibited the highest MIC with 640 µg/ml and standard isolates exhibited a MIC range between 1.25-160 µg/ml.

| Isolate group | Pus isolates | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ISOLATE | Sau A1 | Sau A2 | Sau G1 | Sau G2 | Sau G3 | Sau G10 | Sau G23 | Sau G24 | Sau G25 | Sau G27 | Sau G28 |
| MIC (µg/ml) | 320 ± 0 | 640 ± 0 | 320 ± 0 | 80 ± 0 | 80 ± 0 | 160 ± 0 | 80 ± 0 | 640 ± 0 | 160 ± 0 | 80 ± 0 | 80 ± 0 |

| Isolate group | Burn Isolates | | | | | | |
|---|---|---|---|---|---|---|---|
| ISOLATE | Sau G15 | Sau G9 | Sau G16 | Sau G17 | Sau G18 | Sau G19 | Sau G 26 |
| MIC (µg/ml) | 160 ± 0 | 640 ± 0 | 320 ± 0 | 320 ± 0 | 320 ± 0 | 20 ± 0 | 160 ± 0 |

| Isolate group | Urine Isolates | | | | Vaginal Isolates | | Blood isolates | |
|---|---|---|---|---|---|---|---|---|
| ISOLATE | Sau A3 | Sau G4 | Sau G5 | Sau G11 | Sau G7 | Sau G13 | Sau G6 | Sau G12 |
| MIC (µg/ml) | 640 ± 0 | 80 ± 0 | 80 ± 0 | 320 ± 0 | 80 ± 0 | 80 ± 0 | 80 ± 0 | 320 ± 0 |

| Isolate group | Wound isolate | | Catheter Tip | | Eye and Ear | |
|---|---|---|---|---|---|---|
| ISOLATE | Sau A4 | Sau G14 | Sau G20 | Sau G21 | Sau G22 | Sau G8 |
| MIC (µg/ml) | 10 ± 0 | 80 ± 0 | 160 ± 0 | 20 ± 0 | 320 ± 0 | 40 ± 0 |

| Isolate group | Unclassified Isolates | | Standard Isolates | | | |
|---|---|---|---|---|---|---|
| ISOLATE | Sau A5 | Sau MTB | Sau LHMC | Sau MTCC 740 | Sau MTCC 737 | Sau MTCC 902 |
| MIC (µg/ml) | 640 ± 0 | 640 ± 0 | 80 ± 0 | 80 ± 0 | 1.25 ± 0 | 160 ± 0 |

**Table 5.7 MIC of microwave extracted Alkaloid Bioactive Fraction by *in vitro* microbroth dilution assay**

The $IC_{50}$ (inhibitory concentration) or the concentration of the compound which reduces the colony count of microorganism by 50% was also calculated to assess potential of the compound. The $IC_{50}$ ranged between 1.67±0.44 to 202.36±0.22 µg/ml (Fig 5.11 a). The average $IC_{50}$ for the pus isolates was 46.78±3.19 µg/ml. Among the urine isolates the $IC_{50}$ ranged between 18.73 ±0.55 to 129.91± 0.14 µg/ml (Fig 5.11 b) with an average of 70.2± 3.03µg/ml.

The blood and vaginal isolates exhibited an average $IC_{50}$ of 96.29± 3.37 µg/ml and 86.72± 3.03 µg/ml respectively (Fig 5.11c). Wound isolates possessed an average $IC_{50}$ of 56.13± 3.33 µg/ml and catheter tip isolates exhibited average $IC_{50}$ of 47.38± 2.15 µg/ml (Fig.5.11c). The burn isolates have $IC_{50}$ in the range of 10.8±0.52 µg/ml to 224.156±0.26 µg/ml with an average of 32.16±2.41µg/ml (Fig.5.11d). The unclassified isolates exhibited average $IC_{50}$ in the range of 40.5±0.314 µg/ml (Fig. 5.11e) while the standard cultures had $IC_{50}$ in the range of 5.15±0.94 µg/ml to 193.02±0.33 µg/ml (Fig.5.11f). The average $IC_{50}$ of the standard culture was between 29.93±4.32 µg/ml.

| ISOLATE GROUPS | MIC ± SD | $IC_{50}$ ± SD |
|---|---|---|
| Pus | 170.4 ± 2.24 | 46.78 ± 3.19 |
| Burn | 183.79 ± 2.80 | 32.16 ± 2.41 |
| Urine | 146.72 ± 2.55 | 70.2 ± 3.03 |
| Vaginal | 67.77 ± 1.13 | 86.72 ± 3.03 |
| Blood | 73.36 ± 1.09 | 96.29 ± 3.37 |
| Wound | 103.74 ± 5.28 | 56.13 ± 3.33 |
| Catheter | 134.54 ± 3.87 | 47.38 ± 2.15 |
| Eye and Ear | 95.14 ± 2.87 | 29.05 ± 1.87 |
| Unclassified | 89.8 ± 7.12 | 27.76 ± 9.13 |
| Standard | 33.64 ± 6.18 | 29.93 ± 4.32 |

**Table 5.8 Average MIC and $IC_{50}$ of ABF by *in vitro* microbroth dilution assay**

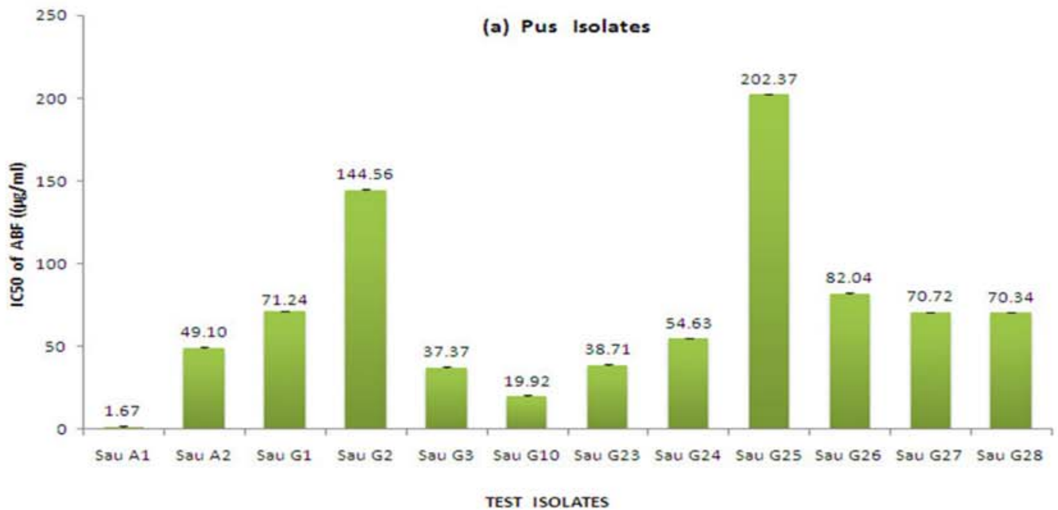
## 5.11 TLC fractionation of ABF

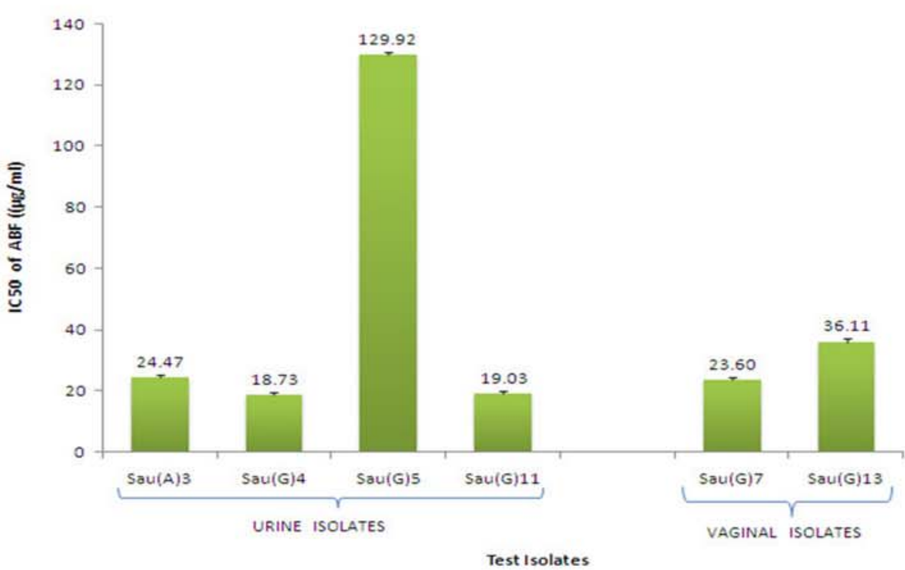
The mobile phase optimized for separation of the ABF comprised of Chloroform, Hexane, Benzene, Isopropanol, Acetone, Diethyl ether and Ammonia in a ratio of 5:4:4:5:1:1. The mobile phase yielded 10 different fractions from the ABF which were designated from CSS1 to CSS10. 250 mg of ABF was thus fractionated of which the highest yield of 14.4% was of CSS4 which was closely followed by CSS6 and CSS8 with 12.08% and 10.08% respectively. The yield of CSS10 was least (Table 5.9)

## 5.12 MIC of individual fractions on selected isolates

The efficacy of the fractions CSS1-CSS10 obtained from visual MIC method was carried out against selected isolates SauG1, SauG3, SauG9, SauG17, SauA4 and NCTC 6571 based on their resistance profiles. CSS1, CSS6 and CSS8 were selected as the potential fractions as they

**Fig. 5.11 Isolate group wise distribution of $IC_{50}$ concentration of the ABF (a) Pus isolates (b) Urine and Vaginal Isolates (c) Blood, Wound and Catheter isolates**





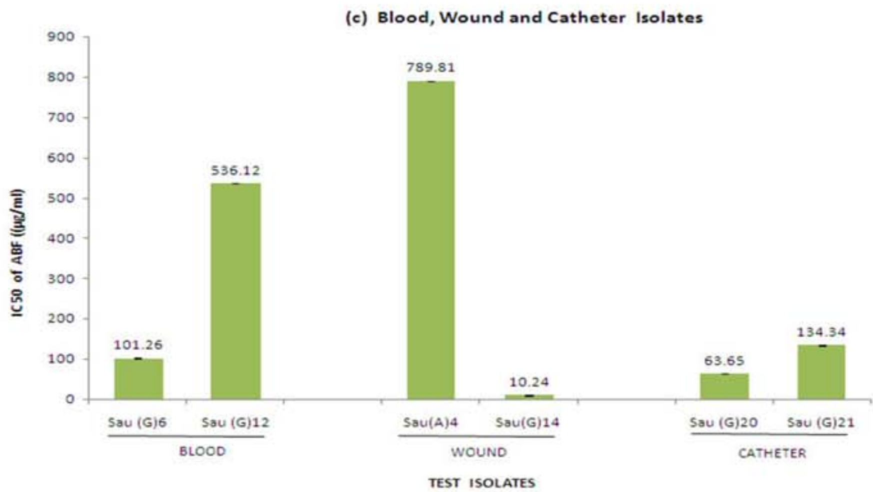
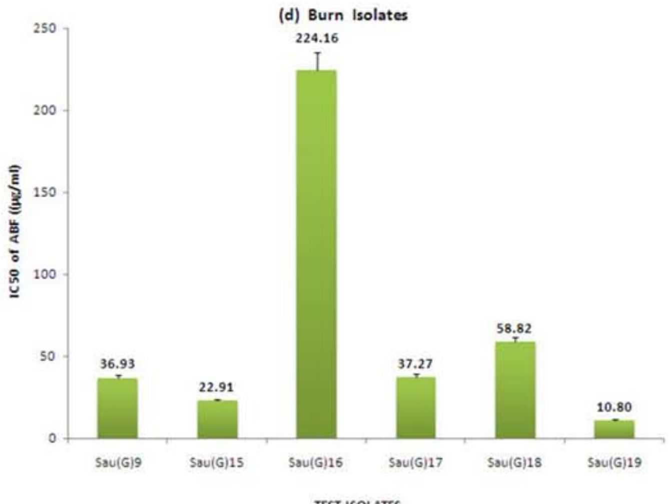
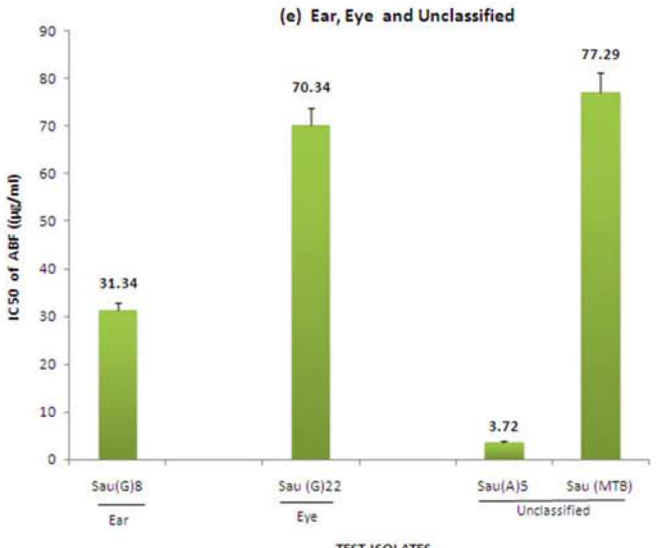
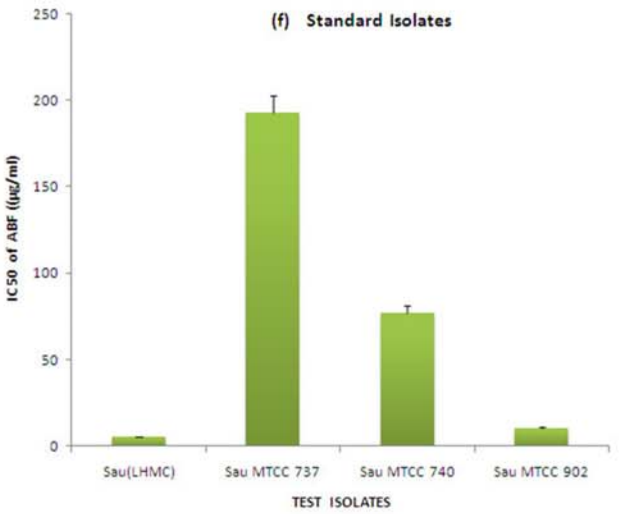



**Fig. 5.11 Isolate group wise distribution of $IC_{50}$ concentration of the ABF (d) Burn isolates (e) Ear, Eye and Unclassified (f) Standard isolates**



exhibited a MIC range of 0.078125-320 µg/ml, 0.3125-640 µg/ml and 2.5-160 µg/ml respectively (Table 5.10)

**Table 5.9 Characteristics and yield of TLC separated fractions of the ABF**

| Fraction ID | Fraction Rf | Physical Appearance | Weight obtained (per 250mg ABF) | Solubility | % Yield (per 250gm leaves) | % Yield (per 250mg ABF ) |
|---|---|---|---|---|---|---|
| **CSS1** | 0.27 | Pink powdery | 21 mg | Methanol | 0.0084% | 8.4% |
| **CSS2** | 0.32 | Cream powdery | 23 mg | Methanol | 0.0092% | 9.2% |
| **CSS3** | 0.37 | Dirty white, slightly sticky | 22.7 mg | Methanol | 0.0091% | 9.1% |
| **CSS4** | 0.44 | Cream needle like | 36 mg | Methanol | 0.0144% | 14.4% |
| **CSS5** | 0.49 | Cream needle like | 14 mg | Methanol | 0.0056% | 5.6% |
| **CSS6** | 0.6 | Pale Colored Powdery | 30.2 mg | Ethyl Acetate/ Chloroform | 0.01208% | 12.08% |
| **CSS7** | 0.73 | Off-White Powdery | 10.1 mg | Ethyl Acetate/ Chloroform | 0.004% | 4.04% |
| **CSS8** | 0.83 | Brown, sticky | 25.2 mg | Chloroform | 0.01% | 10.08% |
| **CSS9** | 0.87 | Brown, sticky | 9.2 mg | Chloroform | 0.0037% | 3.68% |
| **CSS10** | 0.66 | Cream powdery | 11.4 mg | Ethyl Acetate/ Chloroform | 0.0046% | 4.56% |

**Table 5.10 Visual MIC determination of ABF fractions by *in vitro* microbroth dilution assay on limited test panel**

| Test Isolates | Resistance type | MIC values* (µg/ml) of TLC fractions obtained from Alkaloidal Bioactive Fraction (ABF) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *CSS1* | *CSS2* | *CSS3* | *CSS4* | *CSS5* | *CSS6* | *CSS7* | *CSS8* | *CSS9* | *CSS10* |
| Sau G1 | VISA | 0.078± 0.0 | 640± 0.0 | 320± 0.0 | 0.078± 0.0 | 640± 0.0 | 640± 0.0 | 320± 0.0 | 160± 0.0 | 640± 0.0 | 640± 0.0 |
| Sau G3 | MRSA, VRSA | 160± 0.0 | 640± 0.0 | 40± 0.0 | 640± 0.0 | 640± 0.0 | 40± 0.0 | 640± 0.0 | 2.5± 0.0 | 640± 0.0 | 640± 0.0 |
| Sau G9 | MRSA | 40± 0.0 | 640± 0.0 | 320± 0.0 | 640± 0.0 | 640± 0.0 | 5± 0.0 | 640± 0.0 | 160± 0.0 | 640± 0.0 | 640± 0.0 |
| Sau G17 | VISA | 20± 0.0 | 40± 0.0 | 0.625± 0.0 | 40± 0.0 | 640± 0.0 | 20± 0.0 | 2.5± 0.0 | 160± 0.0 | 640± 0.0 | 1.25± 0.0 |
| Sau A4 | MSSA | 0.078± 0.0 | 640± 0.0 | 640± 0.0 | 640± 0.0 | 640± 0.0 | 640± 0.0 | 5± 0.0 | 10± 0.0 | 640± 0.0 | 640± 0.0 |
| Sau NCTC 6571 | AST standard | 320± 0.0 | 640± 0.0 | 320± 0.0 | 640± 0.0 | 2.5± 0.0 | 0.31± 0.0 | 160± 0.0 | 2.5± 0.0 | 640± 0.0 | 640± 0.0 |

**All values are Mean± SD of triplicate readings*

VISA- vancomycin intermediate *S.aureus;* MRSA- Methicillin resistant *S.aureus*; VRSA- Vancomycin resistant *S. aureus*; MSSA- Methicillin Sensitive *S. aureus;* AST- Antimicrobial Susceptibility Testing

## 5.13 MIC and Kill kinetics of CSS1, CSS6 and CSS8 against panel of selected isolates.

CSS1, CSS 6 and CSS8 were selected from the TLC fractions based on their MIC values against the resistant isolates. Their MIC by *in vitro* broth dilution method using endpoint method was obtained against extended spectrum of test isolates to arrive to their MIC, $MIC_{50}$ and $MIC_{90}$ values (Table 5.11). Further kill kinetics of each fraction revealed the level of $Log_{10}$ reduction at a particular time during the exponential phase.

Time kill kinetics of CSS1, CSS6 and CSS8 were performed using a panel of 10 clinically relevant staphylococcal isolates and two standard isolates in order to assess their survival pattern/resistance. The extended test panel gave a higher antibacterial efficacy to CSS6 compared to CSS1 and CSS8 (Table 5.12). A bacteriostatic activity is defined as a maintenance of or a reduction of less than 99.9% (<3 log10) of the total count of CFU/ml in the original inoculums.

| Lead Fractions | Mean ± SD * (µg/ml) | $MIC_{50}$ (µg/ml) | $MIC_{90}$ (µg/ml) |
|---|---|---|---|
| CSS 1 | 26.69 ± 8.86 | 40 | 320 |
| CSS6 | 16.81 ± 8.17 | 40 | 160 |
| CSS 8 | 23.78 ± 8.61 | 40 | 160 |

* Geometric Mean ± GSD

**Table 5.11 Comparative efficacies of CSS1, CSS6 and CSS8 based MIC, $MIC_{50}$ and $MIC_{90}$ values on limited test panel**

| Lead fractions | Mean± SD (µg/ml) | $MIC_{50}$ (µg/ml) | $MIC_{90}$ (µg/ml) |
|---|---|---|---|
| CSS1 | 26.69 ± 10.9 | 40 | 320 |
| CSS 6 | 7.93 ± 9.8 | 20 | 160 |
| CSS8 | 22.45± 12.6 | 10 | 160 |

* Geometric Mean ± GSD

**Table 5.12 Comparative efficacies of CSS1, CSS6 and CSS8 based MIC, $MIC_{50}$ and $MIC_{90}$ values on extended test**

### *5.13.1 Time kill kinetics of CSS1 against extended test panel*

All isolates exhibited a bacteriostatic activity at MIC of CSS1. A 0.46 $log_{10}$ CFU/ml to 1.78 $log_{10}$ CFU/ml observed at 3h to 24 h respectively in Sau G3 (Fig.5.12.1a). A viable count reduction of 4.7 $log_{10}$ CFU/ml and 3.04 $log_{10}$ CFU/ml were found at 20h and 24h in Sau G4 (Fig. 5.12.1b). Similarly a 3.11 $log_{10}$ CFU/ml, 3.88 $log_{10}$ CFU/ml and 4.32 $log_{10}$ CFU/ml reductions in viable

counts were recorded at 12 h, 20 h and 24 h respectively in Sau G5 when compared to control (Fig.5.12.1c). Sau G6 also exhibited a bacteriostatic activity with 3.8 $log_{10}$ CFU/ml and 3.96 $log_{10}$ CFU/ml at 20 h and 24 h respectively (Fig.5.12.1d). Sau G9 exhibited 0.93 $log_{10}$ CFU/ml to 2.09 $log_{10}$ CFU/ml at 3 h and 24 h respectively when compared to the control (Fig 5.12.1e).

Sau G10 exhibited a highly significant reduction in the viable counts from 3 h to 24 h. 5.3$log_{10}$ CFU/ml and 3.41$log_{10}$ CFU/ml reductions were observed at 3 h and 24 h respectively (Fig.5.12.1f). Sau G17 exhibited 0.35 $log_{10}$ CFU/ml and 2.32 $log_{10}$ CFU/ml at 3 h and24 h respectively (Fig.5.12.1g). In Sau G23, 3.07 $log_{10}$ CFU/ml reductions was observed at 6 h followed by 3.77 $log_{10}$ CFU/ml reduction at 20 h and 4.38 $log_{10}$ CFU/ml reduction at 24 h (Fig.5.12.1h). Sau G24 was found to exhibit 1.48 $log_{10}$ CFU/ml reductions at 3hr and 2.80 $log_{10}$ CFU/ml reduction at 24 h thereby exhibiting a bacteriostatic activity (fig.5.12.1i). 3.36 $log_{10}$ CFU/ml reduction in viable counts was observed at 3h and 4.96 $log_{10}$ CFU/ml reduction at 24 h in Sau G25 when compared to control (Fig. 5.12.1j).

***5.13.2 Time kill kinetics of CSS6 against extended test panel***

MIC concentration of CSS6 was bactericidal towards five test microorganisms viz. Sau G4, SauG5, Sau G23, Sau G25 and Sau MTCC 737 in the extended test panel. 4.127 $log_{10}$ CFU/ml and 8.028 $log_{10}$ CFU/ml reductions were observed at 20 h and 24 h in Sau G4 (Fig.5.12.2b). In Sau G5, 3.27$log_{10}$ CFU/ml, 3.08 $log_{10}$ CFU/ml and 5.928 $log_{10}$ CFU/ml reductions in viable counts were observed at 12 h, 20 h, and 24 h respectively (Fig. 5.12.2c). Sau G23 showed a reduction in viable count of 3.55 $log_{10}$ CFU/ml at 12 h; 7.18 $log_{10}$ CFU/ml at 20 h and 9.22 $log_{10}$ CFU/ml at 24 h. MIC of CSS6 was found to be bactericidal for Sau G23 (Fig. 5.12.2h). Sau G25 exhibited a viable count reduction of 3.68 $log_{10}$ CFU/ml respectively at 6 h, 3.98 $log_{10}$ CFU/ml and 8.86 $log_{10}$ CFU/ml at 20 h and 24 h respectively (Fig. 5.12.2j)

In Sau G3, 7.9 $log_{10}$ CFU/ml and 3.73 $log_{10}$ CFU/ml reduction in viable counts was observed at 20 h and 24 h when compared to the control (Fig.5.12.2a). In Sau G6 at 20 h a 4.99 $log_{10}$ CFU/ml reduction was observed while 24 h exhibited a 3.96 $log_{10}$ CFU/ml reduction in the viable counts (Fig. 5.12.2d). 4.8 $log_{10}$ and 7 $log_{10}$ reductions were observed in Sau G9 at 20 h and 24 h respectively when compared to the control (Fig. 5.12.2e). Sau G10 exhibited a drastic reduction at 6h by 5.11 $log_{10}$ CFU/ml and 3.11 $log_{10}$ CFU/ml at 24 h (Fig.5.12.2f).

Sau G24 exhibited reduction in viable counts by 3.41 $log_{10}$ CFU/ml at 6 h, 4.93 $log_{10}$ CFU/ml at 20 h and 7.92 $log_{10}$ CFU/ml at 24 h (Fig.5.12.2i). Sau MTCC737 a standard isolate exhibited a very high reduction in viable counts ranging between 5.24 to 8.67 $log_{10}$ CFU/ml from 12 to 24 h (Fig 5.12.2k). However the second standard culture Sau MTCC740 exhibited a refractory behavior at 20 h by exhibiting a viable count reduction of 2.5 $log_{10}$ CFU/ml only (Fig.5.12.2l).

***5.13.3 Time kill kinetics of CSS8 against extended test panel***

CSS8 exhibited a bactericidal activity only against four isolates of the test panel viz., Sau G5, Sau G10, Sau G24 and Sau G25 at MIC. Sau G6 and Sau G17 in the test panel exhibited a refractory behavior to MIC of CSS8 (Fig.5.12.3d, g). Sau G3 exhibited 8.46 $log_{10}$ CFU/ml reduction at 20 h and 3.62 $log_{10}$ CFU/ml reduction at 24 h (Fig. 5.12.3a). Similarly Sau G4 also exhibited 4.28 $log_{10}$ CFU/ml and 3.71$log_{10}$ CFU/ml reduction at 20 h and 24 h respectively (Fig.5.12.3b). 5.33 $log_{10}$ CFU/ml and 6.66 $log_{10}$ CFU/ml reduction was found in Sau G5 viable counts when compared to control at 20 and 24 h respectively (Fig.5.12.3c). Sau G9 induced a reduction of 6.44 $log_{10}$ CFU/ml at 24 h only. In Sau G10 a 5.2 $log_{10}$ CFU/ml in viable counts occurred at 3h (Fig.5.12.3e) and 7.87 $log_{10}$ CFU/ml reductions at 24 h. Sau G23 exhibited 4.20 $log_{10}$ CFU/ml reduction initially at 3 h with a resistance to CSS8 at 20 h by exhibiting 2.65 $log_{10}$ CFU/ml followed by 3.24 $log_{10}$ CFU/ml reduction at 24h (Fig.5.12.3h). Viable count reduction at 12 h was found to be 5.09 $log_{10}$ CFU/ml followed by 6.14 $log_{10}$ CFU/ml at 20h and 9.51 $log_{10}$ CFU/ml at 24h in Sau G24

when compared to control (Fig.5.12.3i). In Sau G25 the viable count reduction at 3 h was 3.51 $log_{10}$ CFU/ml, 4.85 $log_{10}$ CFU/ml at 12 h, and 9.69 $log_{10}$ CFU/ml after 24 h as compared to the control (fig.5.12.3j).

Among the standard isolates Sau MTCC740 and Sau MTCC737; 5.45 $log_{10}$ CFU/ml and 5.73 $log_{10}$ CFU/ml reduction were observed at 24 h (Fig. 5.12.3k. 3l).

Thus time kill kinetics indicated that CSS1 was a purely bacteriostatic in action while CSS6 has higher number of isolates exhibiting bactericidal effect when compared to CSS8 (Table 5.13).

**Table 5.13 Comparative kill kinetics of CSS1, CSS6 and CSS8 against selective clinical and standard staphylococcal isolates**

| Test Isolates | SOURCE (Resistance type) | 6h, 12h, 24h count reduction {$log_{10}$ Cfu/ml}* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CSS1 | | | CSS6 | | | CSS 8 | | |
| | | *6h* | *12h* | *24h* | *6h* | *12h* | *24h* | *6h* | *12h* | *24h* |
| Sau G3 | Pus (MRSA, VRSA) | 0.24 | 1.06 | 1.78 | 0.27 | 1 | 3.7 | 1.32 | 1.37 | 3.62 |
| SauG4[a] | Urine (MRSA, MARSA) | 2.27 | 3.66 | 3.04 | 2.27 | 3.36 | 6.03 | 0.85 | 2.51 | 3.77 |
| SauG5[a,b] | Urine (MARSA) | 1.29 | 3.11 | 4.32 | 1.29 | 3.27 | 5.29 | 1.7 | 2.32 | 6.63 |
| Sau G6 | Blood (MSSA) | 1.61 | 3.87 | 3.41 | 0.81 | 2.86 | 3.96 | 0.83 | 1.22 | 2.14 |
| Sau G9 | Burn Swabs (MRSA) | 0.21 | 0.83 | 2.09 | 0.88 | 0.81 | 7 | 1.38 | 1.59 | 6.44 |
| Sau G10[b] | Pus (MRSA, MARSA) | 5.11 | 3.87 | 3.41 | 5.11 | 2.85 | 4.11 | 5.79 | 5.8 | 7.88 |
| Sau G17 | Burn (VISA) | 0.43 | 1.89 | 2.32 | 1.38 | 2.28 | 2.28 | 1.83 | 2.06 | 2.43 |
| Sau G23[a] | Pus (MRSA, VISA) | 3.07 | 3.17 | 4.38 | 2.77 | 3.55 | 7.21 | 3.47 | 3.08 | 3.24 |
| Sau G24[b] | Pus (MRSA, VRSA) | 1.27 | 2.21 | 2.8 | 3.41 | 3.67 | 4.9 | 2.2 | 5.09 | 8.51 |
| Sau G25[a,b] | Pus (MRSA,VRSA) | 3.36 | 3.07 | 4.9 | 3.05 | 3.18 | 8.86 | 4.6 | 4.06 | 7.64 |
| Sau MTCC737[a] | AST standard | 0.29 | 1.71 | 2.94 | 2.72 | 5.24 | 6.67 | 1.49 | 1.79 | 5.45 |
| Sau MTCC740 | AST standard | 0.41 | 1.9 | 3.06 | 2.05 | 1.1 | 5.96 | 1.83 | 2.52 | 5.73 |

* Kill curves performed at MIC concentration
[a] Bactericidal activity of CSS6
[b] Bactericidal activity of CSS8

**Fig. 5.12.1 Kill kinetics of CSS1 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G3 , pus isolate (b) Sau G4, urine isolate (c) Sau G5, urine Isolate (d) Sau G6, blood isolate (e) Sau G9, burn isolate (f) Sau G10, pus isolate**

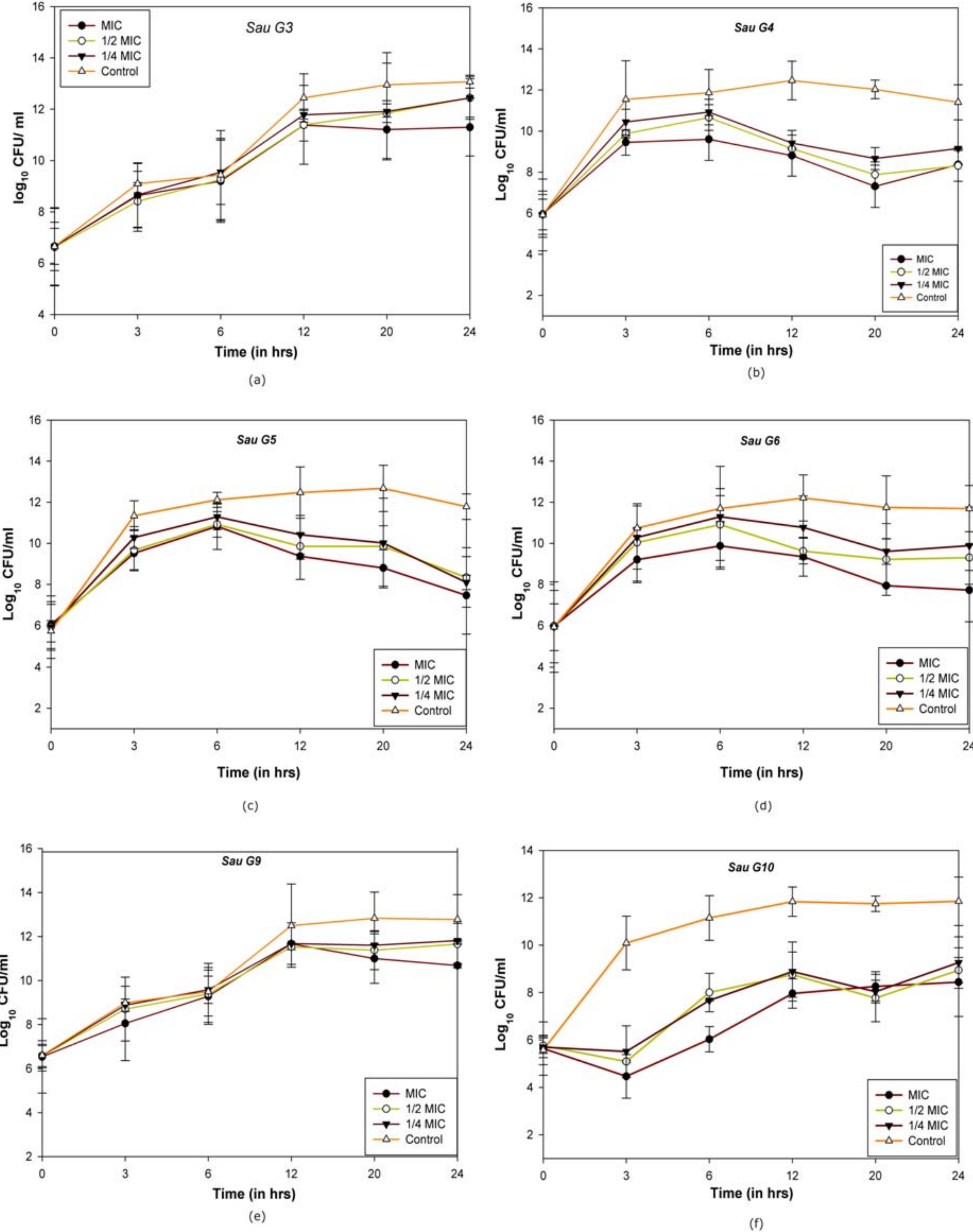


**Fig. 5.12.1 Kill kinetics of CSS1 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G17, burn isolate (b) Sau G23, pus isolate (c) Sau G24, pus isolate (d) Sau G25, pus isolate (e) Sau MTCC 737, standard isolate (f) Sau MTCC 740, standard isolate**

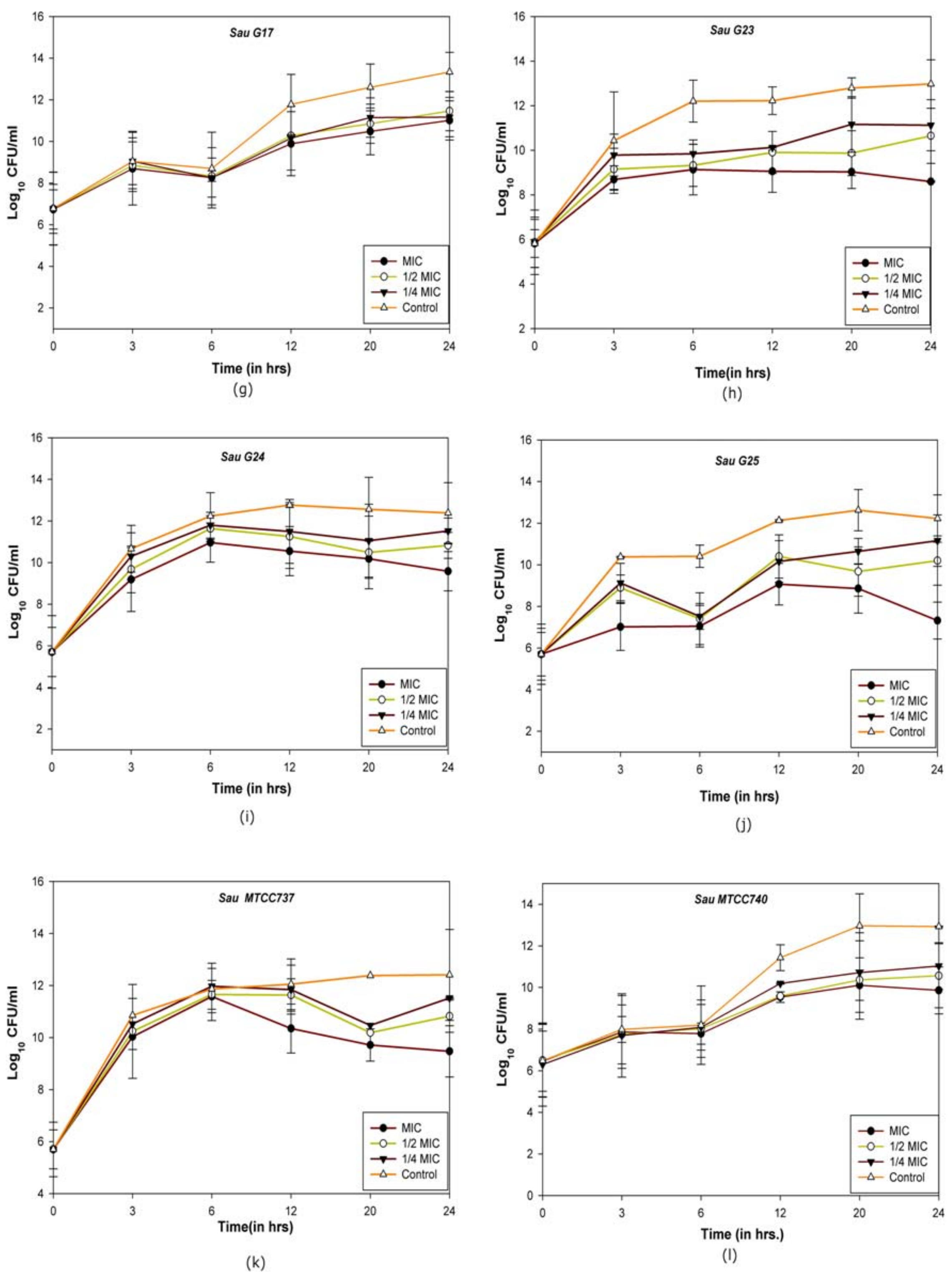


**Fig. 5.12.2 Kill kinetics of CSS6 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G3 , pus isolate (b) Sau G4, urine isolate (c) Sau G5, urine isolate (d) Sau G6, blood isolate (e) Sau G9, burn isolate (f) Sau G10, pus isolate**

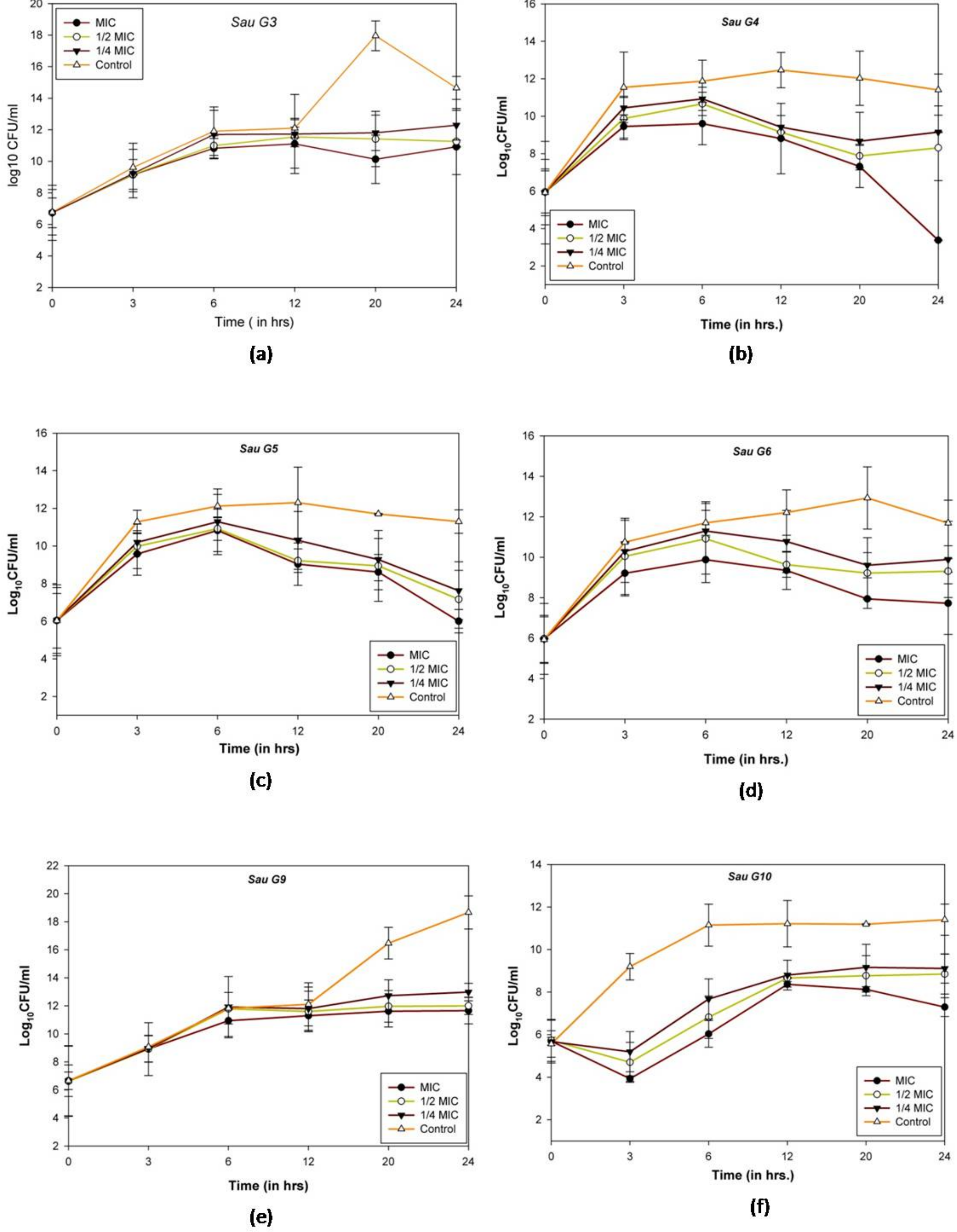


**Fig. 5.12.2 Kill kinetics of CSS1 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G17, burn isolate (b) Sau G23, pus isolate (c) Sau G24, pus isolate (d) Sau G25, pus isolate (e) Sau MTCC 737, standard isolate (f) Sau MTCC 740, standard isolate**

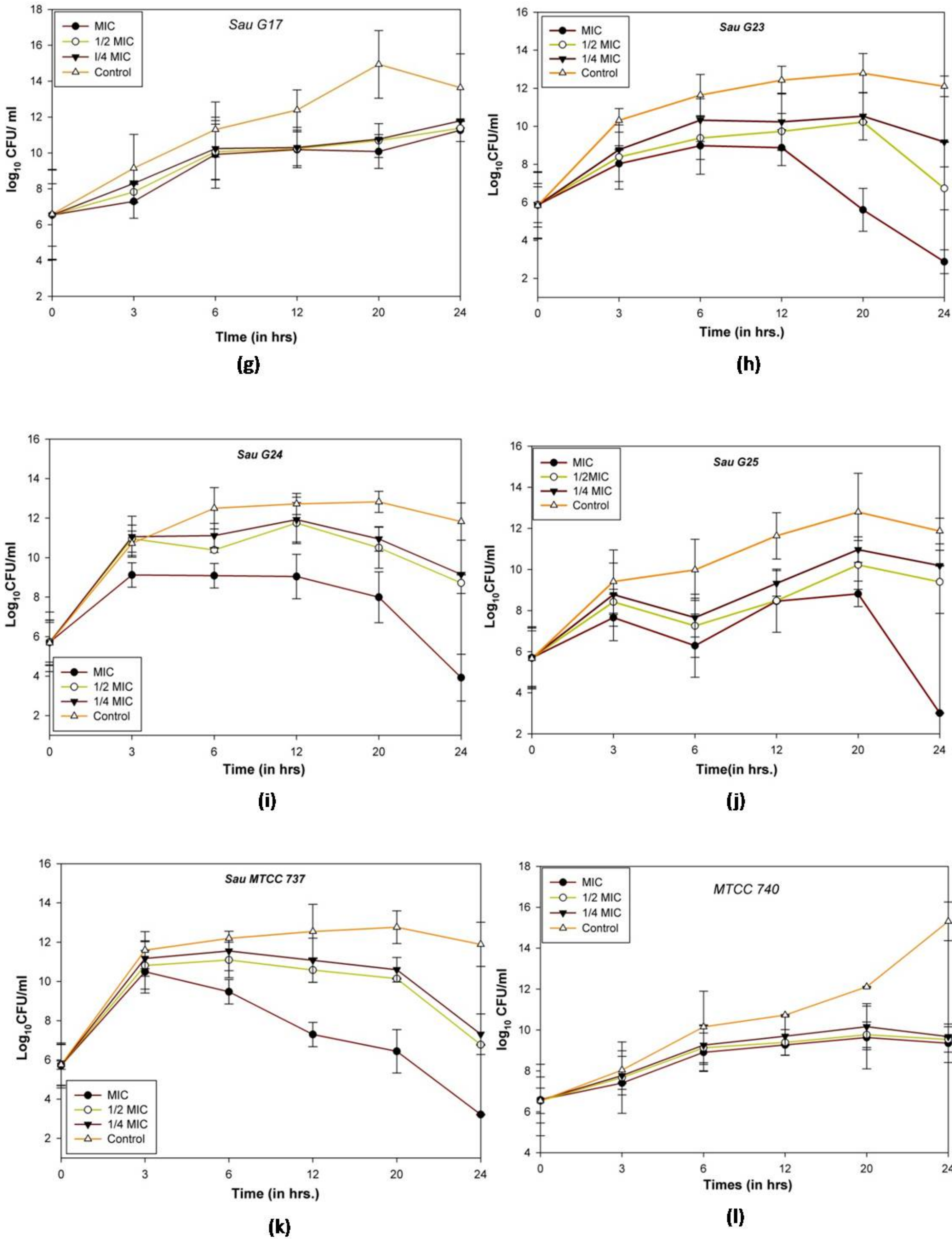


**Fig. 5.12.3 Kill kinetics of CSS 8 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G3 , pus isolate (b) Sau G4, urine isolate (c) Sau G5, urine isolate (d) Sau G6, blood isolate (e) Sau G9, burn isolate (f) Sau G10, pus isolate**

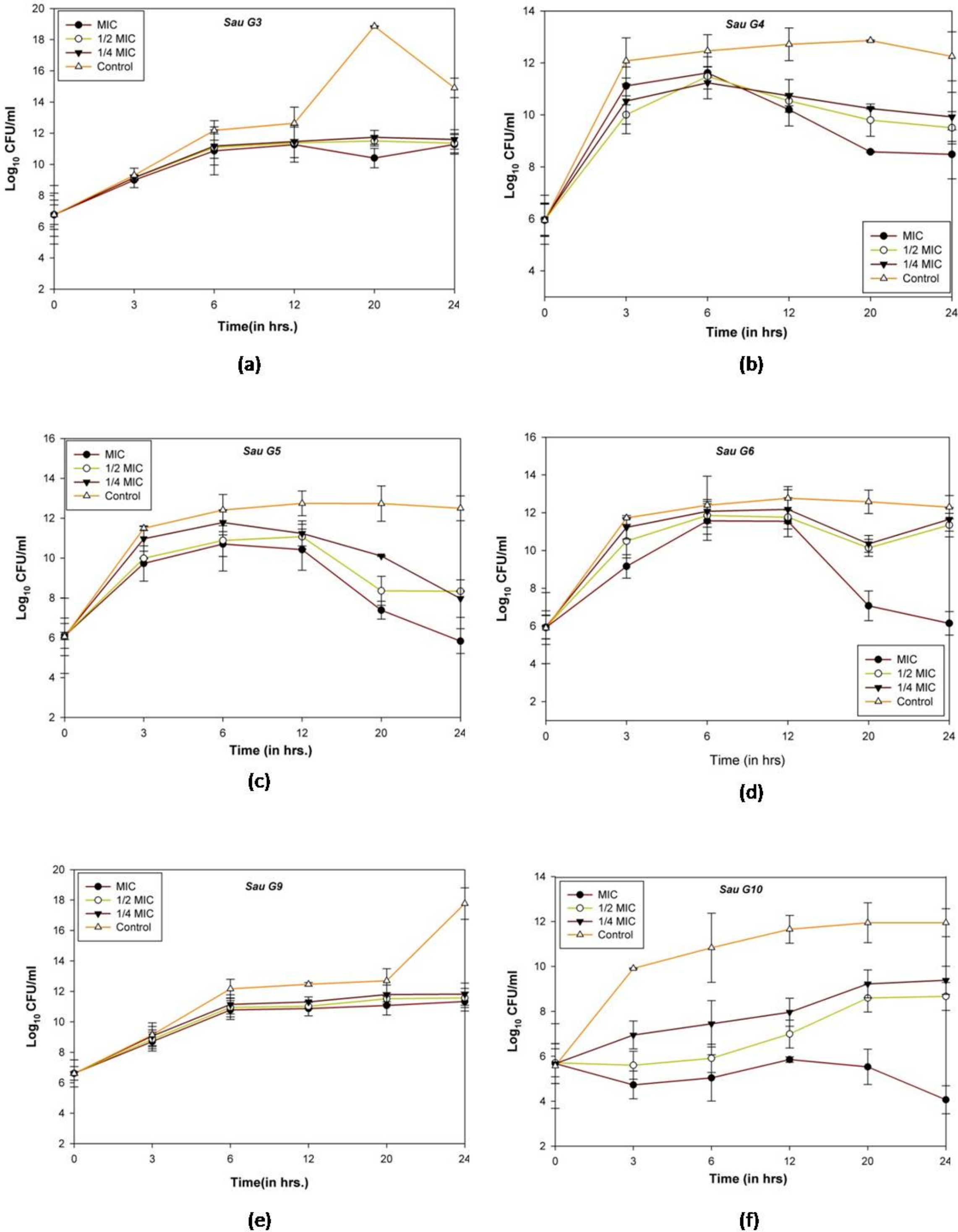
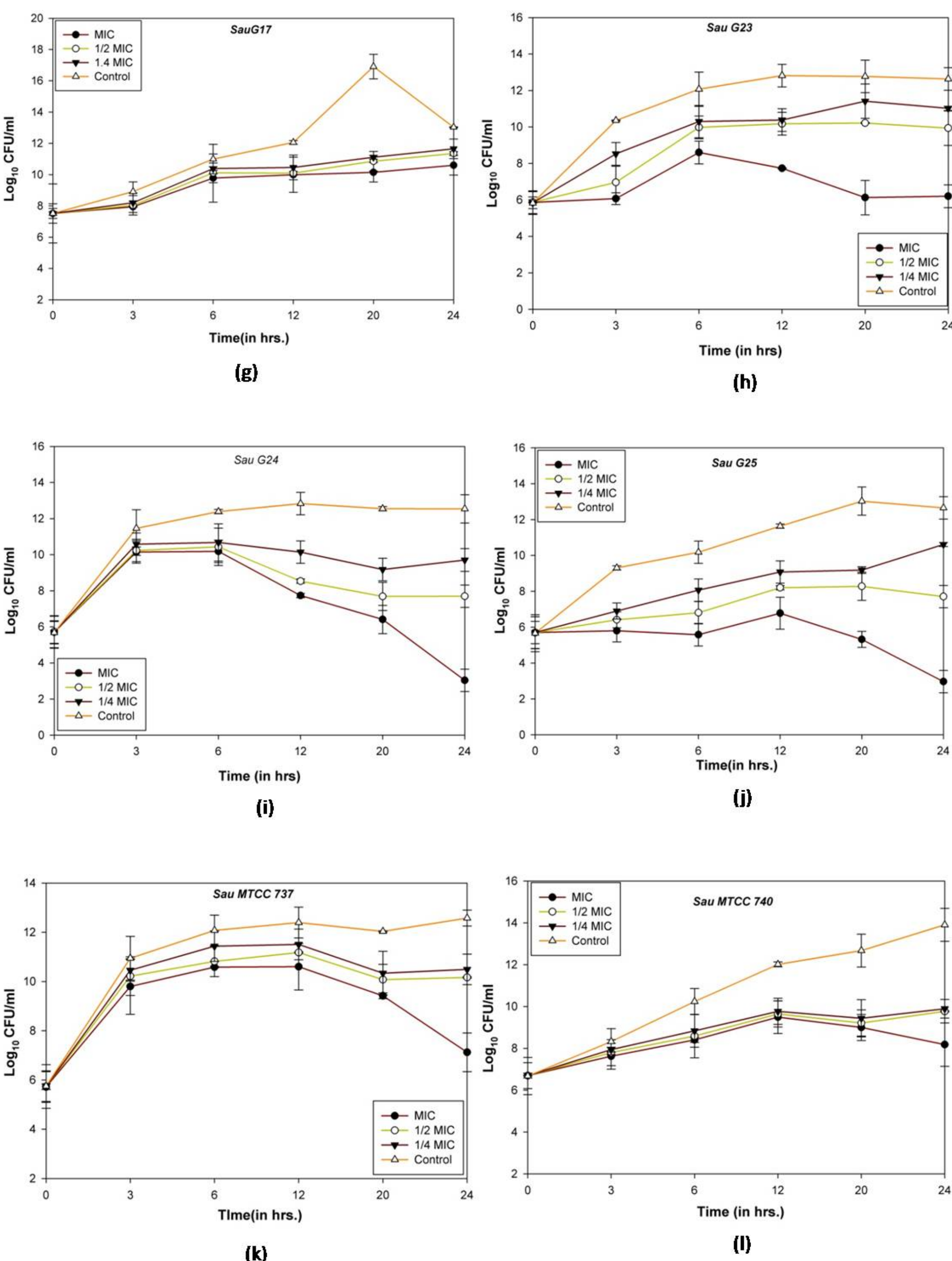


**Fig. 5.12.3 Kill kinetics of CSS 8 at MIC, ½ MIC and ¼ MIC against selective clinical and standard isolates (a) Sau G17, burn isolate (b) Sau G23, pus isolate (c) Sau G24, pus isolate (d) Sau G25, pus isolate (e) Sau MTCC 737, standard isolate (f) Sau MTCC 740, standard isolate**



### 5.14 Structural changes based on SEM analysis

Scanning electron microscopy offers a unique opportunity to observe the surface morphology and structural changes in the bacteria treated with antimicrobial agents as compared to normal cells. The normal staphylococcal cells are generally smooth and rounded but essentially not spherical as observe in the SEM photomicrograph (Fig. 5.13 A). The bacteria treated with CSS6 and CSS8 exhibited varied cell morphology apart from rupturing and complete lysis of the test isolates. After exposure to CSS6 at MIC concentration the bacteria exhibited rupturing of the cells accompanied by cellular deformation at 12 h. The size of the bacterial cell was also grossly altered exhibiting protuberance as well as shrinking of cellular size which is associated with cell death or cell leakage presumably due to damage in the cell wall (Fig. 5.13 B). At 20 h the CSS6 induced crenation in the cellular structure, roughening of outer surface by boss-like protuberances. Apart from the above features the CSS6 treated cells of Sau G10 appeared to be an amorphous syncytium at 20 h (Fig. 5.13 C). Characteristic dumble shaped appearances were also observed frequently apart from irregular division and collapse of bacteria and at the internal septum of the cells indicating suppression of cellular division (Fig. 5.13 D).

*S. aureus* isolates viz. Sau G25 and Sau G10 were ruptured into an amorphous syncytial matrix at MIC of CSS8 and CSS6 respectively at 20 h (Fig. 5.13 E and F). Formation of a triad indicating irregular division, and severe reduction in cell size was observed in Sau G10 at MIC of CSS8 at 12 h (Fig. 5.13 G and H). Shrinking in the cell size, appearance of surface appendages and bridge like structures between the cells were observed in the case of Sau G25, treated with CSS8 MIC at 12 h. Deformation in the cells (doughnut shape), reduction in cell size, arrested division of the cells along the septum apart from bridge like structures being formed giving a characteristic dumble shape was observed in the cells of Sau G25 treated with MIC of CSS8 at 20 h (Fig 5.13 K and L).

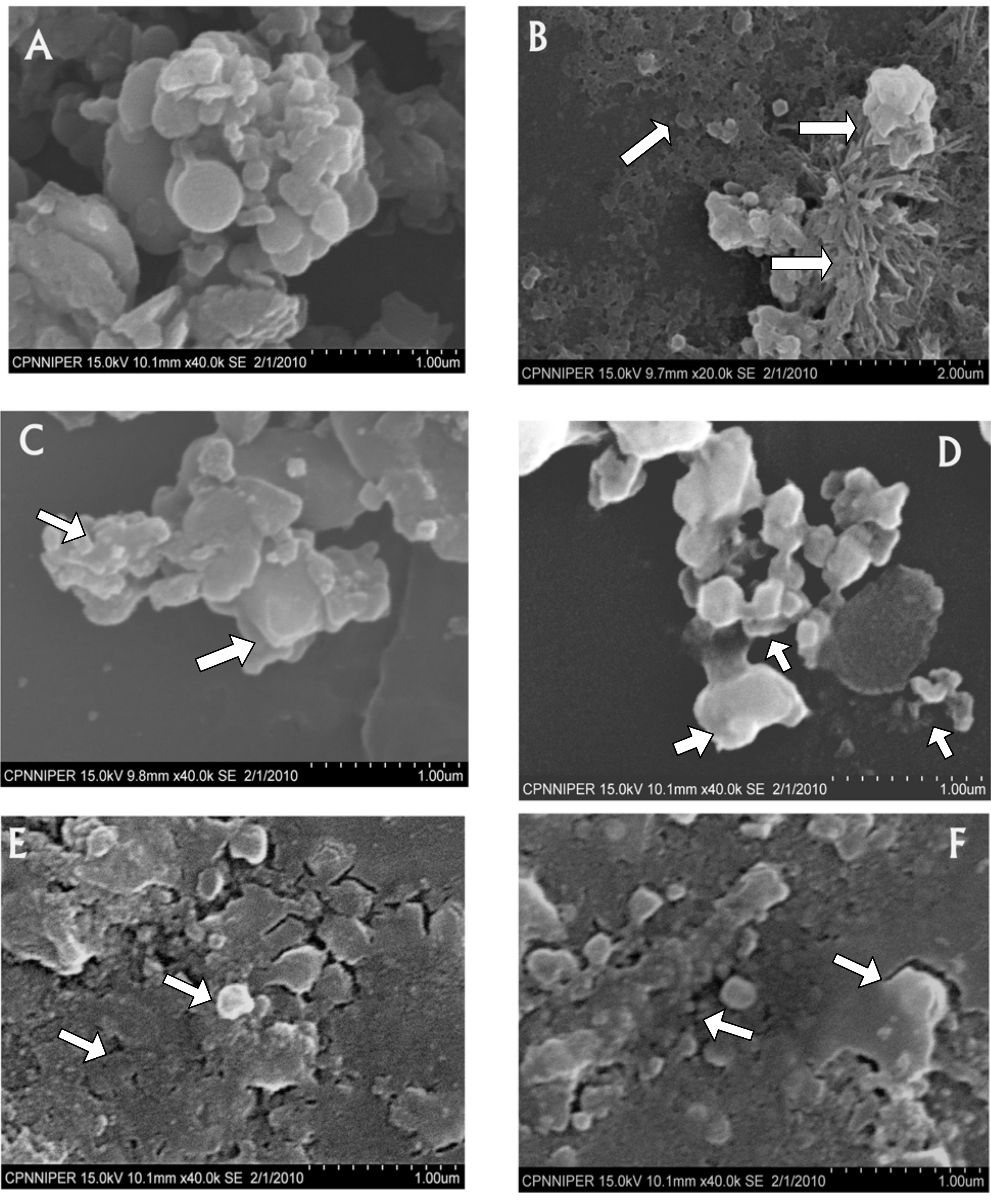



**Fig 5.13 (A). Scanning Electron Micrograph of *Staphylococcus aureus* (B) SEM of Sau G10 after exposure to MIC of CSS6 at 12h indicated deformity in the shape of the cells, cell shrinkage and formation of amorphous syncytium (C) Cell shrinkage, deformity in division , appearance of appendages apart from rupturing of the cells of Sau G10 after exposure to MIC of CSS6 at 20h (D) Formation of irregular shape, crenations and characteristic dumble structure in Sau G10 due to exposure of MIC of CSS6 at 20h (E&F) Cell shrinkage and formation of an amorphous syncytium by MIC of CSS 8 and CSS 6 respectively at 20 hours exposure to Sau G25.**

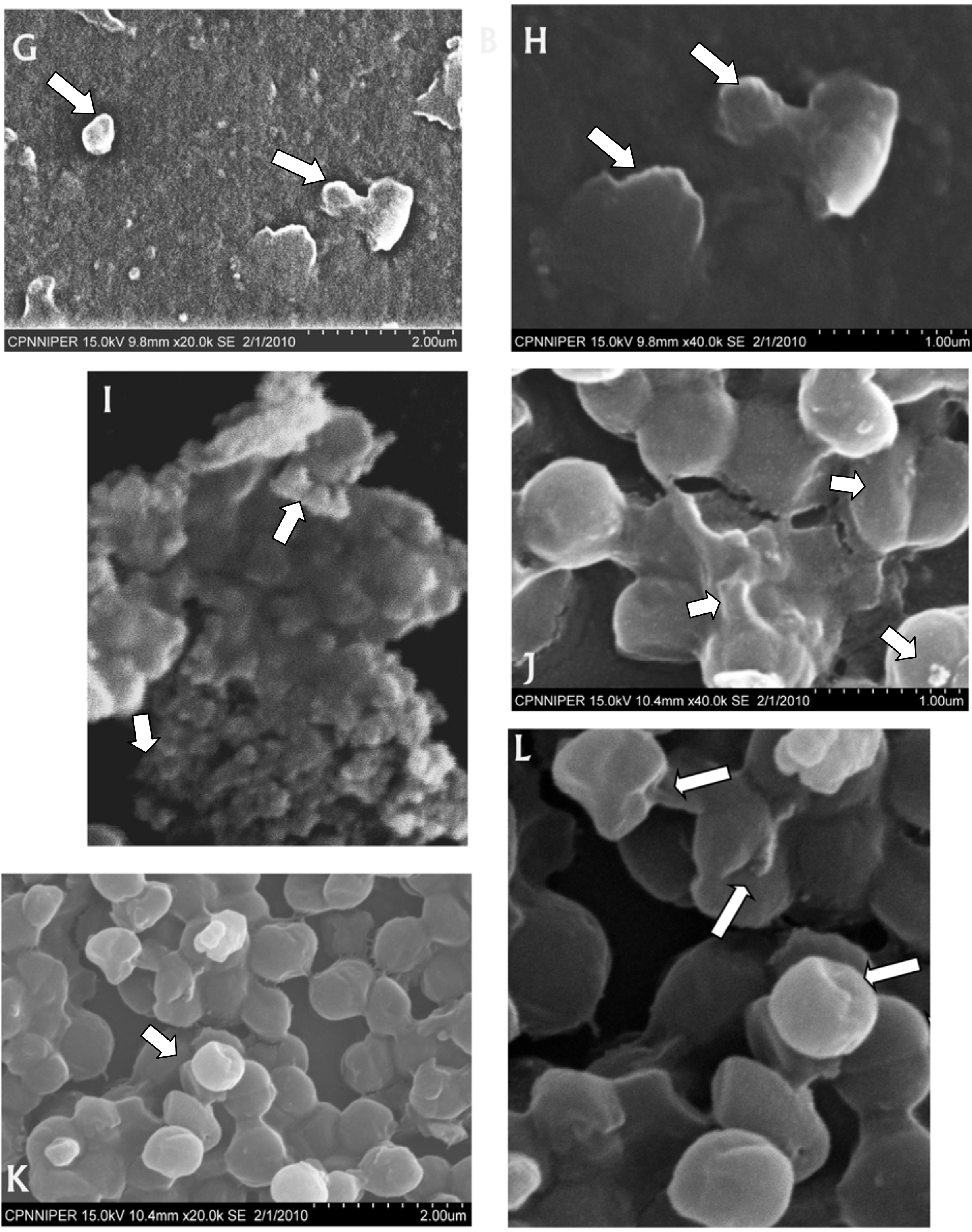



**Fig 5.13 (G&H) Scanning Electron Micrograph of *Staphylococcus aureus* exhibiting the formation of triad apart from amorphous syncytium formation and shrinked cells of Sau G10 by CSS8 after 12h exposure at MIC, (I & J) Shrinkage of cells and reduction in size is marked with appearance of surface appendages, improper separation and bridge formation between the cells ( K&L) Deformation of cells, formation of bridges causing dumble formation, appearance of surface appendages and irregular septum formation**.

## 5.15 Biochemical mode of action of CSS6 and CSS8

All isolates were subjected to screening for production of extracellular virulence associated factors like the Superoxide dismutase, Lipase and Protease in the Staphylococcal isolates. Highest expression of SOD was found in Sau G9, Sau G3, Sau G18 and Sau G6 (Fig. 5.16) amongst the clinical staphylococci in the microbial test panel. Amongst isolate groups burn pathogens exhibited a highest SOD expression followed by eye and ear and blood wound and catheter isolates. The pus isolates exhibited the least SOD expression (Table 5.14). The protease expression was highest in Sau G5, Sau G6 followed by Sau G20, Sau G21, Sau G22 and Sau G18. Sau G19 exhibited the least protease production amongst the clinical isolates (Fig.5.15). The blood wound and catheter isolates exhibited the highest protease expression among the test isolate groups followed by eye and ear isolates and pus isolates (Table 5.14). Lipase production was highest in Sau G25, followed by Sau G23, Sau G24, Sau G27, Sau G16 and Sau G18 (Fig.5.14). Highest lipase production was observed in unclassified isolates followed by eye and ear, and subsequently pus isolates. The least lipase expression was found in standard isolates (Table 5.14). Further based on the expression profiles highest SOD, lipase and protease expressing isolates along with standard isolates were selected to find out the effect of CSS6 and CSS8 at MIC and sub- MIC levels.

### 5.15.1 Effect of CSS6 and CSS8 on expression of SOD

***Extracellular SOD expression***

The effect of CSS6 and CSS8 on extracellular SOD were observed at the MIC and Sub- MIC (½, ¼ MIC) concentrations in mid log phase around 12 h to assess their interaction with the virulence factors. Sau G3 and Sau G9 were the highest SOD producers among the clinical isolates and Sau MTCC 740 and Sau MTCC 737 were used as standard isolates.

It was observed that in CSS6 in Sau G3 inhibited the expression of extracellular SOD at ½ MIC and ¼ MIC when compared to the control. A similar trend was observed in Sau G9 where

**Fig. 5.14 Screening of microbial test panel for production of extracellular lipase**

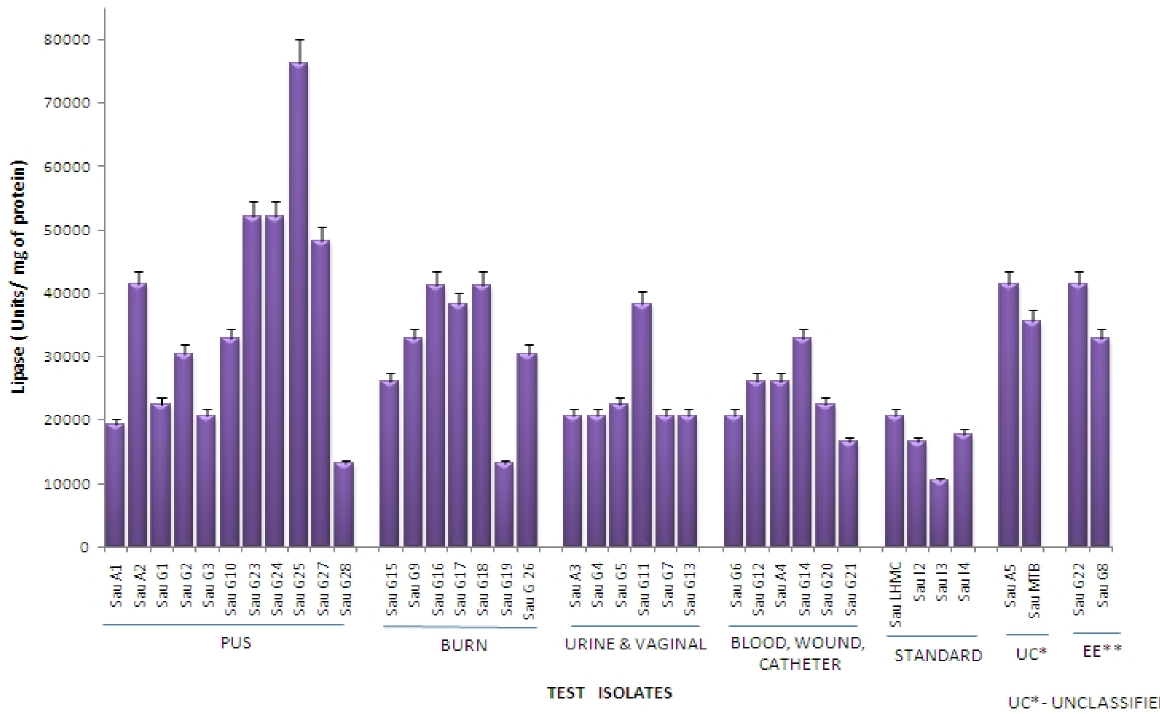



**Fig. 5.15 Screening of microbial test panel for production of extracellular protease**

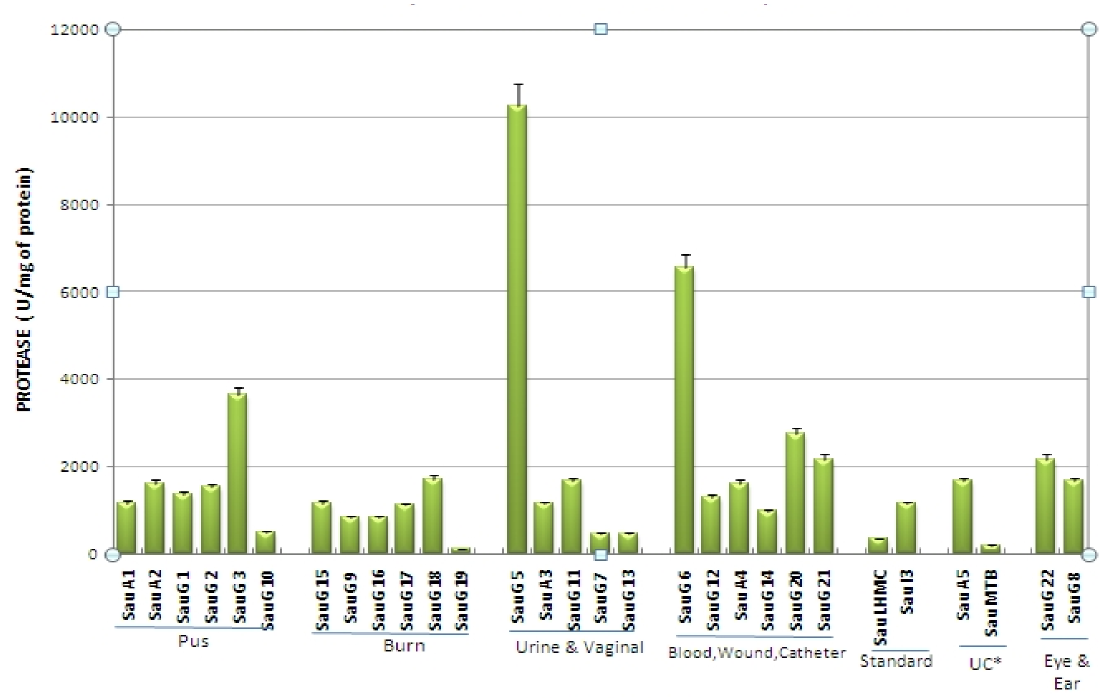


**Fig. 5.16 Screening of microbial test panel for production of intracellular SOD**

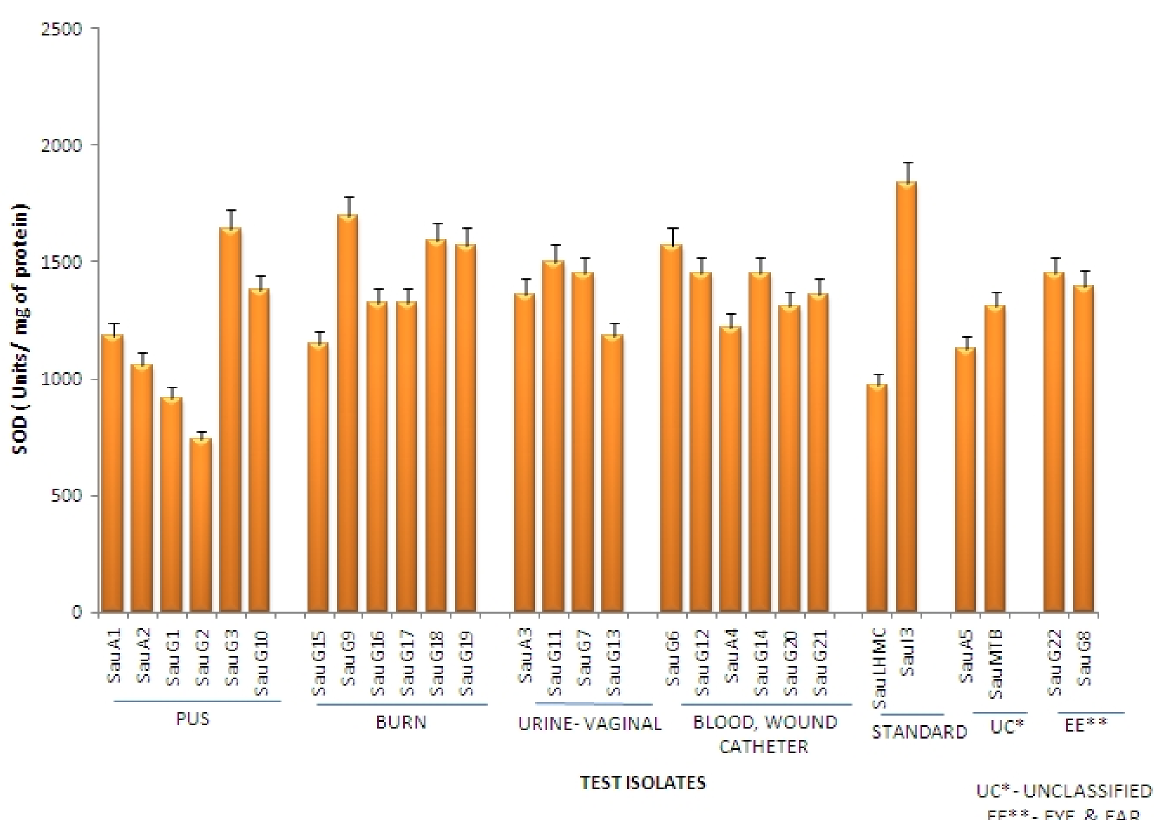



**Table 5.14 Average expression of SOD, protease and lipase by different groups of test panel staphylococci**

| Isolate Groups | Expression of Enzymes by Test isolates | | |
|---|---|---|---|
| | SOD<br>( Units/ mg of Protein) | Protease<br>( Units/ mg of Protein) | Lipase<br>( Units/ mg of Protein) |
| PUS | 1143.6 ± 1.33 | 1390.38± 1.89 | 32872.58 ± 1.70 |
| BURN | 1428.4 ± 1.15 | 759.38 ± 2.52 | 30111.93 ± 1.70 |
| URINE- VAGINAL | 1366.9 ± 1.11 | 800.20 ± 1.91 | 23272 ± 1.49 |
| BLOOD, WOUND, CATHETER | 1387 ± 1.09 | 2073.77 ± 1.95 | 24182.46 ± 1.26 |
| UNCLASSIFIED | 1334.6 ± 1.31 | 575.63 ± 4.46 | 38326.62 ± 1.11 |
| EYE AND EAR | 12142 ± 1.22 | 1901.05 ± 1.20 | 36883.62 ± 1.17 |
| STANDARD | 1420.9 ± 1.05 | 635.54 ± 2.28 | 15855.07 ± 1.34 |

there was a considerable reduction in the extracellular SOD expression at MIC and ½ MIC only. At ¼ MIC the expression of extracellular SOD was similar to the control set. In standard isolates the CSS6 induced a higher SOD expression in the test set as compared to control at MIC, ½ MIC and ¼ MIC at 12 h. (Fig 5.17)

At 12 h, CSS8 also induced a reduction in extracellular SOD production in Sau G3 and Sau G9 when compared to the control sets. In Sau MTCC 737, the MIC of CSS8 abruptly enhanced the SOD expression at 12 h as compared to control at MIC, ½ MIC while at ¼ MIC it was comparably lower but significantly higher than the control (Fig 5.18).

***Intracellular SOD expression***

In clinical isolates, Sau G3 and Sau G9, the intracellular SOD expression was significantly higher at MIC, ½ MIC and ¼ MIC of CSS6 at 12 h. In Sau G9 the expression of intracellular SOD was highest at ¼ MIC. Among the standard isolates, the intracellular SOD expression was higher at MIC concentration in both Sau MTCC 737 and Sau MTCC 740. Compared to MIC, the SOD expression was less but significantly higher than the control set at ½ MIC and ¼ MIC in both standard isolates (Fig 5.19).

In CSS8, there was a significant enhancement intracellular SOD expression was observed at 12 h at the MIC level only and in the sub- MIC level i.e ½ MIC and ¼ MIC the intracellular production was much lower to the control set in Sau G3. In Sau G9, a non- significant change in SOD expression was observed at MIC and significant inhibition at the sub-MIC levels. In Sau MTCC 737 there was an upward trend in higher SOD production as compared to control from MIC till ½ MIC while in Sau MTCC 740, a significant decrease in SOD was observed at MIC and significant enhancement at ¼ MIC (Fig. 5.20).

### 5.15.2 Effect of CSS6 and CSS8 on expression of protease

Protease expression was also assessed at 12 h in high protease producing strains Sau G5 and Sau G6 along with the standard isolates – Sau MTCC 737 and Sau MTCC 740.

A marked decrease was recorded at MIC, ½ MIC and ¼ MIC of CSS6 in Sau G5 as compared to control indicating protease inhibition to be a possible mechanism for inducing susceptibility. In Sau G6 also a significant reduction in protease expression at 12 h occurred in the MIC and ½ MIC concentrations. However at ¼ MIC the protease was not significantly inhibited. In standard isolates also the maximum inhibition was observed at MIC followed by ½ MIC and ¼ MIC (Fig. 5.21).

Marginal decrease in protease production was induced at 12 h in Sau G5 whereas no change was observed at ½ MIC level of CSS8. However a significant reduction was observed at ¼ MIC. In Sau G6, a significant reduction was observed at MIC, ½ MIC and ¼ MIC levels. Similarly a significant decrease in protease production was observed in MTCC 737 and MTCC 740 while the expression of protease was higher or similar to control at ¼ MIC indicating a concentration dependent inhibition (Fig. 5.22).

### 5.15.3 Effect of CSS6 and CSS8 on expression of lipase

Effect of CSS6 and CSS8 on lipase production was observed in the best lipase producing clinical isolates- Sau G25, Sau G24 and Sau G23 apart from the standard isolates- Sau MTCC737 and Sau MTCC 740. In Sau G23 a non-significant reduction in lipase level was observed at MIC of CSS6 while no change in lipase production occurred at the sub-MIC level i.e. ½ MIC and ¼ MIC when compared to control. In Sau G24, a significant reduction occurred in lipase expression at all levels and the same trend was observed in Sau G25. In Sau MTCC 737 a significant education in lipase expression was observed at MIC, ½ MIC and ¼ MIC. However in Sau MTCC 740 there was enhanced lipase production at ½ MIC compared to control (Fig.5.33).

CSS8 at MIC reduced the lipase production but did not induce significant inhibition in lipase production at sub-MIC concentration in Sau G23. Similarly in Sau G24 there was a significant lipase reduction at MIC, ½ MIC and ¼ MIC levels. The same trend was observed in Sau G25 where the maximum lipase inhibition occurred at MIC while at sub-MIC the lipase expression was same. In Sau MTCC 737 there was a significant lipase inhibition at all the three concentration of CSS8 at 12 h while lipase over expression was recorded at all concentration of CSS8 in Sau MTCC 740 (Fig. 5.24).

## 5.16 Efficacy of CSS6 and CSS8 with commercial antibiotics

The compounds CSS6 and CSS have better activity than penicillins, cephalosporins, quniolones when their MIC, $MIC_{50}$ and $MIC_{90}$ values were compared using the same antimicrobial test panel by *in vitro* microbroth dilution assay. The MIC of CSS6 was 7.93 µg/ml while for CSS8 it was 22.45 µg/m. The $MIC_{50}/MIC_{90}$ values of CSS6 was 20/160 µg/ml and for CSS8 it was 10/160 µg/ml. However they are possessing higher MIC value than the vancomycin and chloramphenicol. Further these compounds are alkaloids which have not been exploited so far as antimicrobials directly but have been used as antibiotic potentiators or resistance modifiers (Table 5.15).

Efficacy of the leads when compared to Standard Antibiotics

| Antibiotic | Antibiotic Class | Stock Preparation | | MIC | $MIC_{50}$ | $MIC_{90}$ |
|---|---|---|---|---|---|---|
| | | Solvent | Diluents | µg/ml | µg/ml | µg/ml |
| Penicillin G | Penicillins | Water | Water | 1280 | >1280 | >1280 |
| Oxacillin | Penicillins | Water | Water | 235.4 | 80 | 1280 |
| Cephalexine | 1st Generation Cephalosporin | Water | Water | 103.1 | 80 | 320 |
| Cefuroxime | 2nd Generation Cephalosporin | Water | Water | 196.7 | 80 | 640 |
| Cefaclor | 2nd Generation Cephalosporin | Water | Water | 383.5 | 40 | >1280 |
| Ceftazidime | 3rd Generation Cephalosporin | Saturated $NaHCO_3$ solution | Water | 441.6 | 320 | >1280 |
| Cephotaxime | 3rd Generation Cephalosporin | Water | Water | 1120 | 640 | >1280 |
| Cefixime | 3rd Generation Cephalosporin | Water | Water | 478.3 | 320 | >1280 |
| Cefepime | 4th Generation Cephalosporin | Saturated $NaHCO_3$ solution | Water | 115 | 80 | 640 |
| Ciprofloxacin | Quinolones | Water | Water | 96.6 | 80 | 160 |
| Vancomycin | Glycopeptide | Water | Water | 4.4 | 2.5 | 10 |
| Chloramphenicol | Miscellaneous | Ethanol | Water | 3.02 | 2.5 | 5 |
| CSS 6 | Alkaloid | 50% DMSO | Water | 7.93 | 20 | 160 |
| CSS 8 | Alkaloid | 50% DMSO | Water | 22.45 | 10 | 160 |

**Table 5.15 Comparison of the standard antibiotics and the leads CSS6 and CSS8 for their anti-staphylococcal activity**

Fig, 5.17 Effect of CSS6 on extracellular expression of SOD

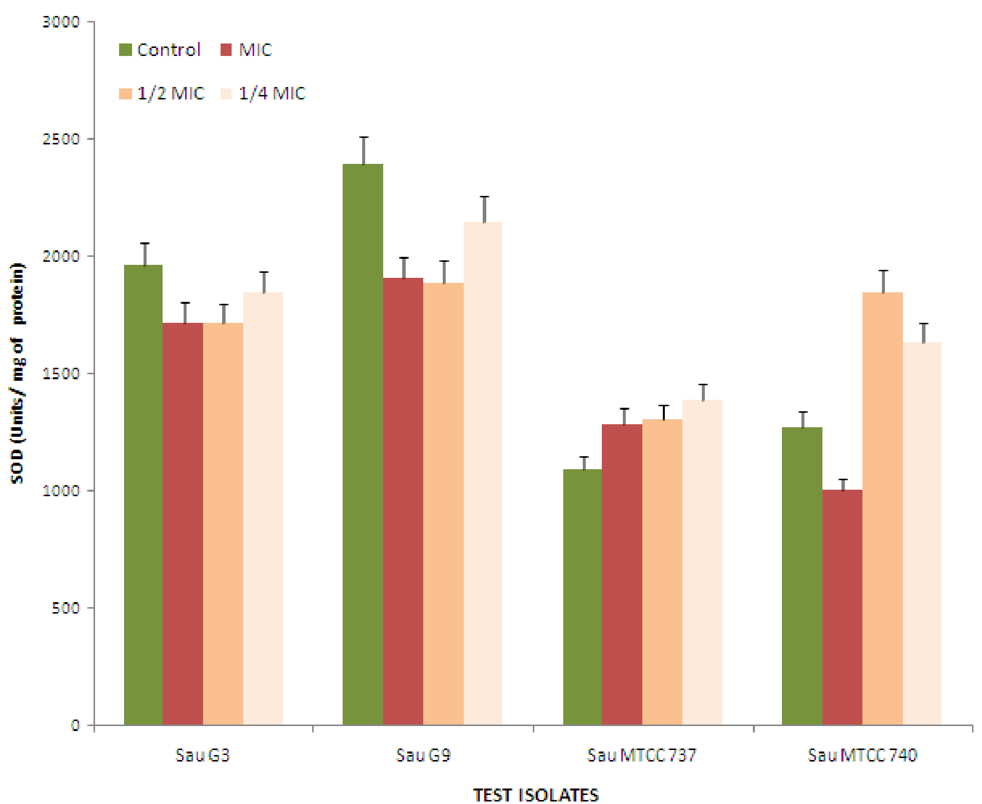
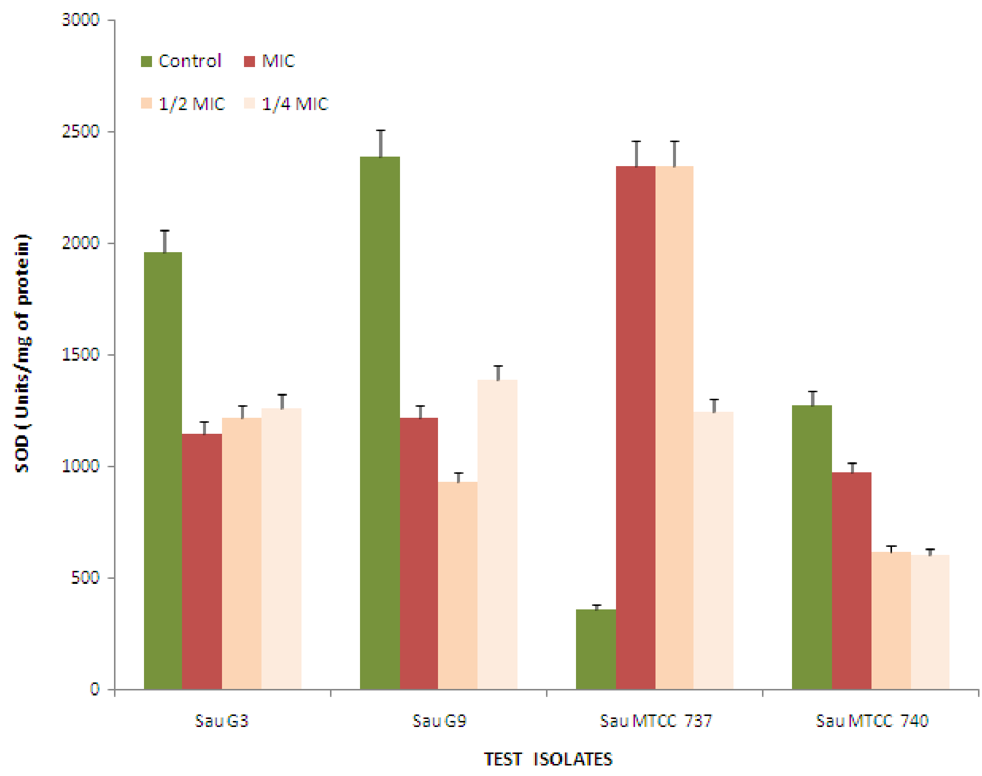



Fig. 5.18 Effect of CSS8 on extracellular expression of SOD



**Fig, 5.19 Effect of CSS6 intracellular expression of SOD**

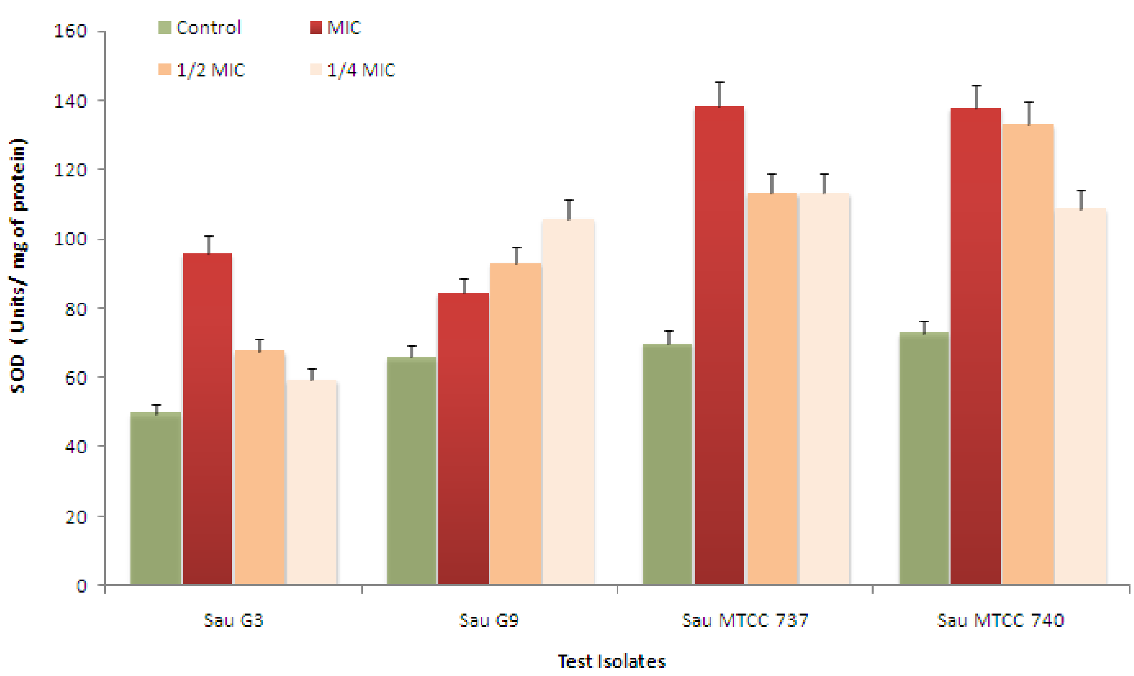



**Fig 5.20 Effect of CSS8 on intracellular expression of SOD**

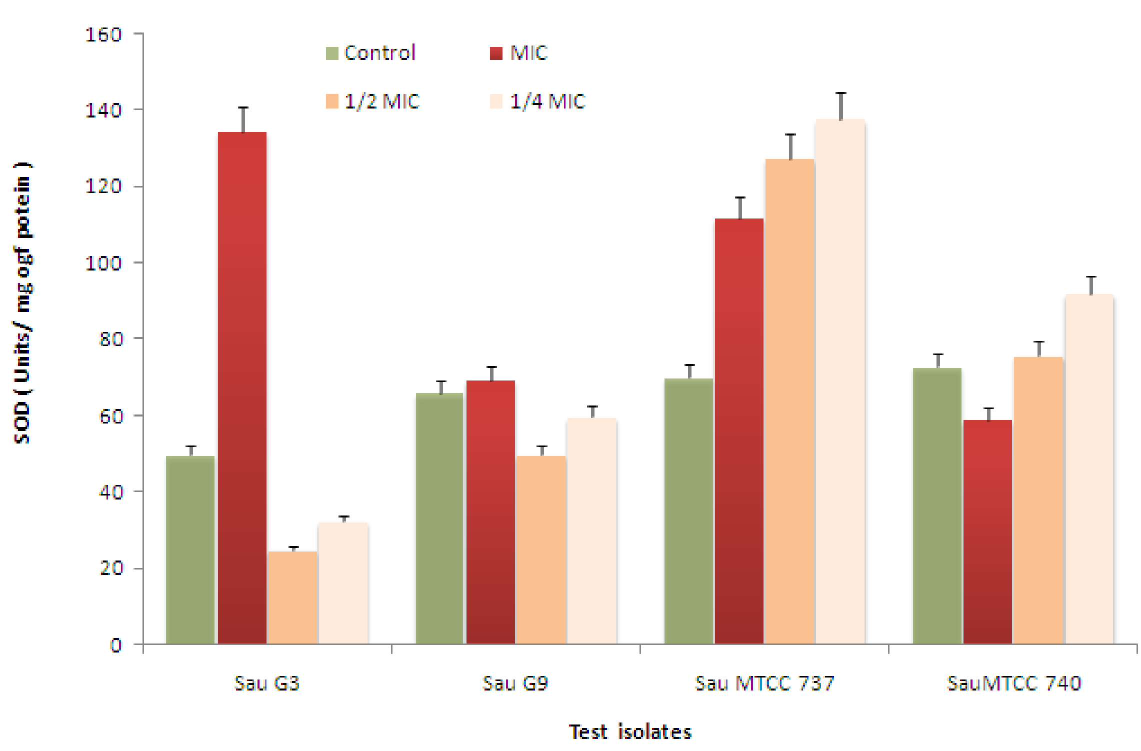


Fig 5.21 Effect of CSS6 on protease expression

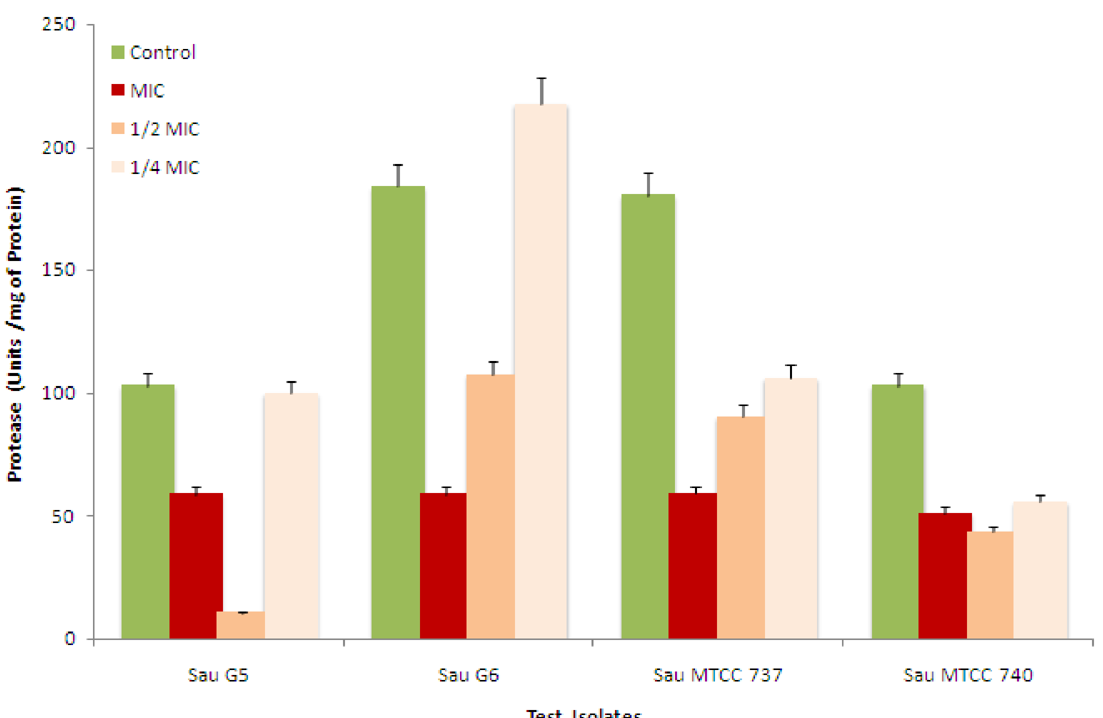



Fig. 5.22 Effect of CSS8 on protease expression

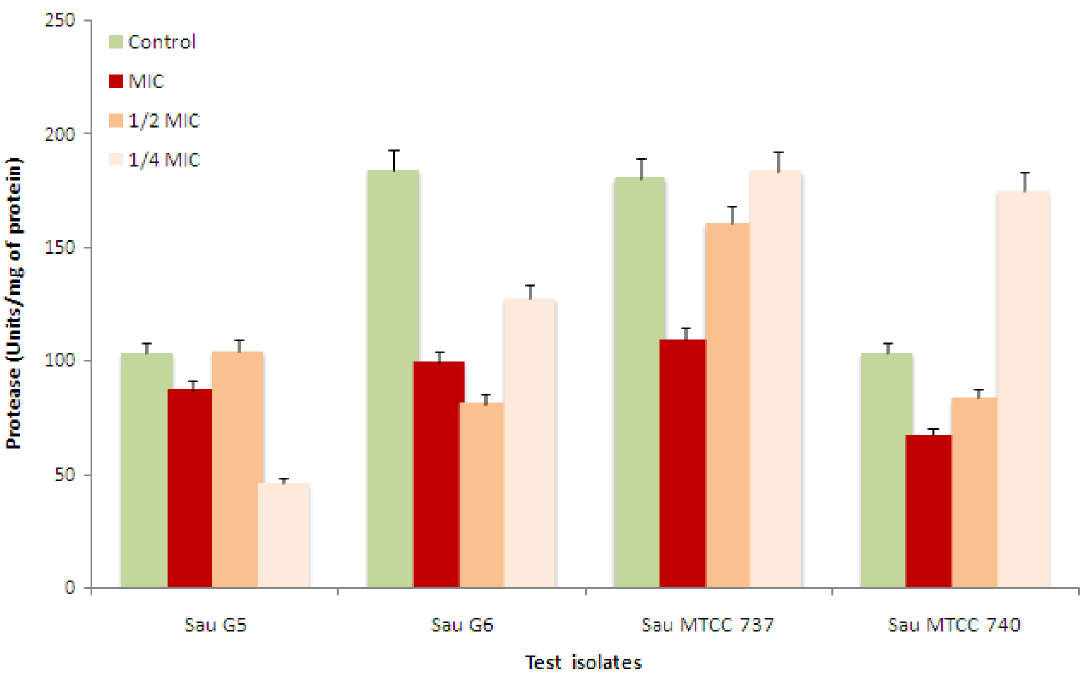


**Fig 5.23 Effect of CSS6 on lipase expression**

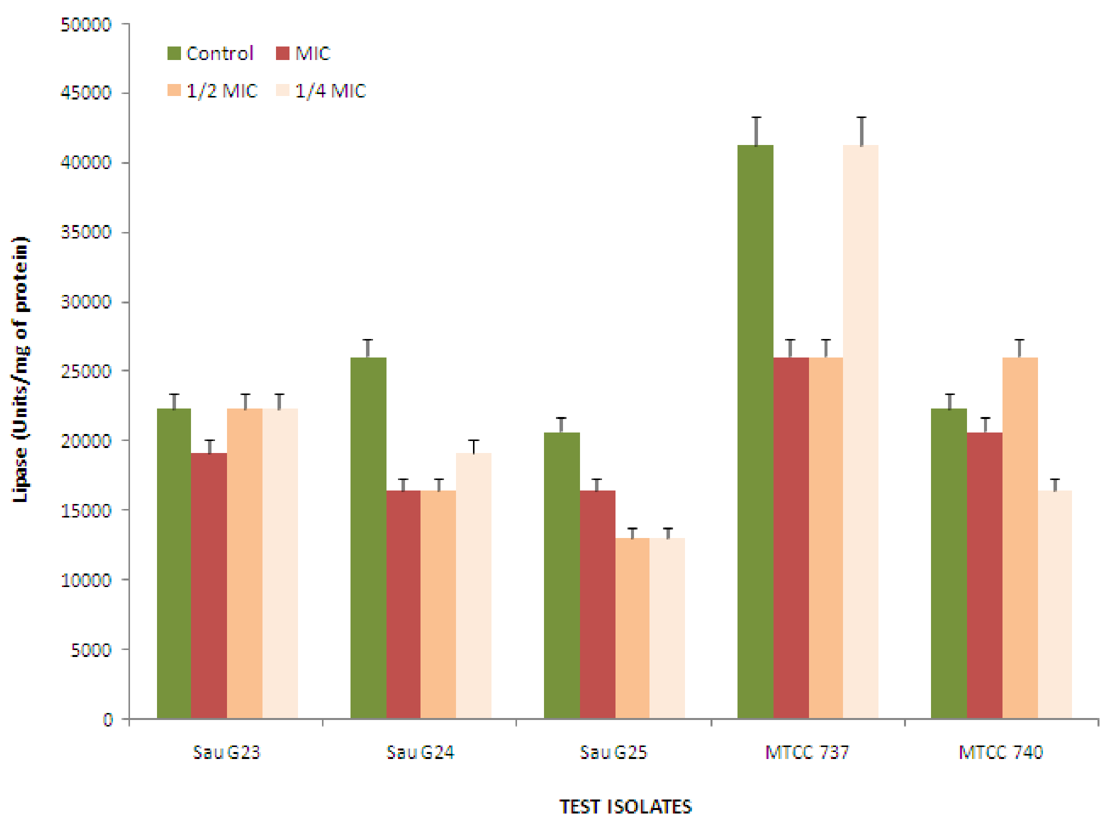
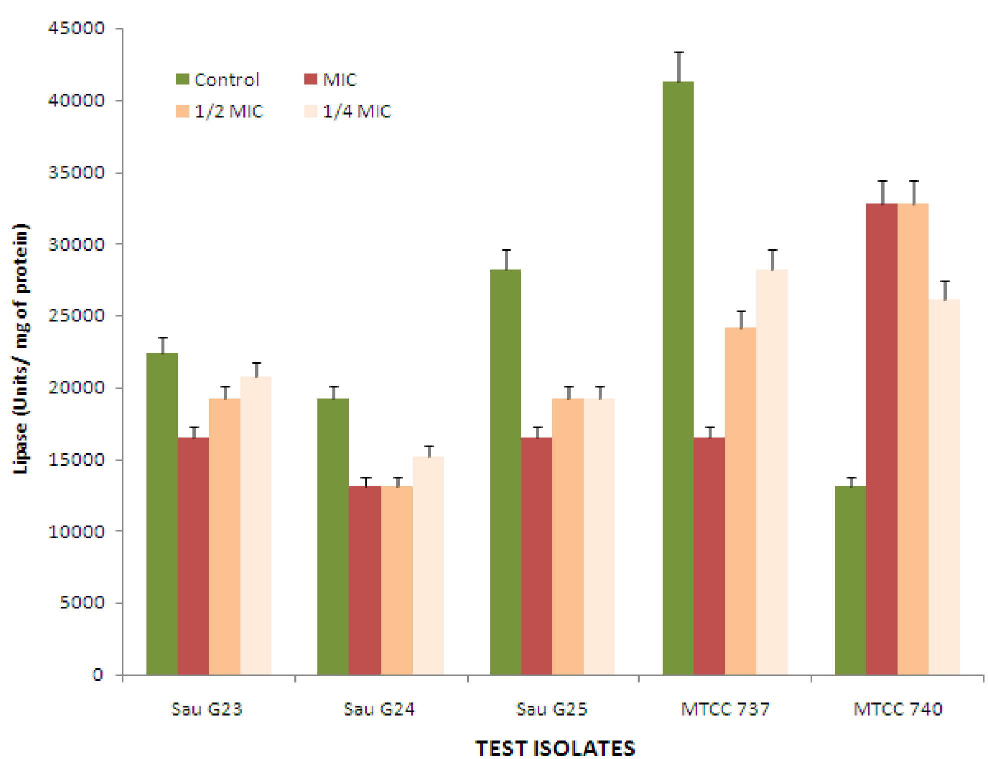



**Fig 5.24 Effect of CSS8 on lipase expression**

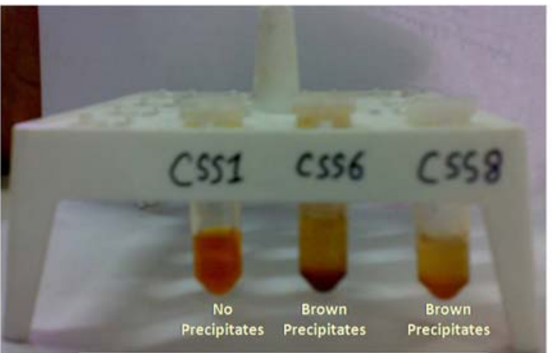


## 5.17 Tentative nature of the leads

### 5.17.1 Phytochemical testing

Phytochemical of the bioactive leads CSS6 and CSS8 revealed that the compounds were testing positive with only with dragendroff's reagent which is a test for identification of alkaloids (Table 5.16; Fig.5.25).

Hence the alkaloid nature of pure compounds CSS6 and CSS8 was confirmed.




Dragendorff's Test for Alkaloids

**Comments**
**Brown precipitates in Dragendorff's Test indicate presence of Alkaloids**
**No precipitation indicates absence of alkaloids**

**Fig. 5.25 Dragendroff's Test of the compounds CSS1, CSS6 and CSS8.**

| Phytochemical test of CSS Fractions | | | |
|---|---|---|---|
| **Test name** | **CSS6** | **CSS8** | **Inference** |
| Dragendorff's Test | +ve | +ve | Alkaloids present |
| Zinc Hydrochloric Acid Reduction Test | -ve | -ve | Flavonoids absent |
| Diphenylamine test | -ve | -ve | Glycosides absent |
| Ferric Chloride reagent test | -ve | -ve | Tannins Absent |
| Frothing Test | -ve | -ve | Saponins Absent |
| Benzene Ammonia test | -ve | -ve | Anthraquinones absent |

**Table 5.16 Phytochemical nature of the bioactive leads**

### 5.17.2. Elemental analysis

The elemental Composition of CSS6 and CSS8 was ascertained in percentage of Carbon (C), Hydrogen (H), Nitrogen (N) and Oxygen (O) using a CHNO analyzer . The elemental composition of CSS6 and CSS 8 was as follows:

**Elemental Composition of CSS6**

Oxygen--- 27.246%

Carbon--- 62.326%

Hydrogen--- 7.341%

Nitrogen--- 3.051%

**Elemental Composition of CSS8**

Oxygen --- 22.347 %

Carbon--- 64.222 %

Hydrogen---7.891%

Nitrogen---5.540%

Thus it could be interpreted clearly from elemental analysis that the compound CSS6 and CSS8 are organic compounds possessing nitrogen which further substantiates the Dragendorff's test

indicating the presence of alkaloids.

**5.17.3 Mass Spectroscopic analysis**

Electrospary ionization MS (ESIMS) of CSS6 gave molecular peaks $(M+H)^+$ at m/z 803.8, m/z 413.4, m/z 360.4, m/z 301.2, m/z 269.1, m/z 187.1 and m/z 105.0. Further molecular peaks at m/z of 803.8 and 413.4 were subjected to MS/MS analysis. The molecular peak at m/z of 803.8 gave fragmentation only at m/z 413.5 while the molecular peak at m/z 413.4 gave fragments at m/z 301.5, m/z 189.4 and m/z 171.4.

Electrospary ionization MS of CSS8 gave molecular peak $(M+H)^+$ at m/z 493.5, m/z 491.1, m/z 413.4, m/z 409.1, m/z 360.5, m/z 351.1, m/z 301.2, m/z 269.1, m/z 245, m/z 231.3, m/z 187.1, m/z 116.1, m/z 105.0. Further fragmentation of the molecular peak at m/z 493.5 gave a fragmentation peaks at m/z 329.5, m/z247.3, m/z 187.4, m/z 105.4.

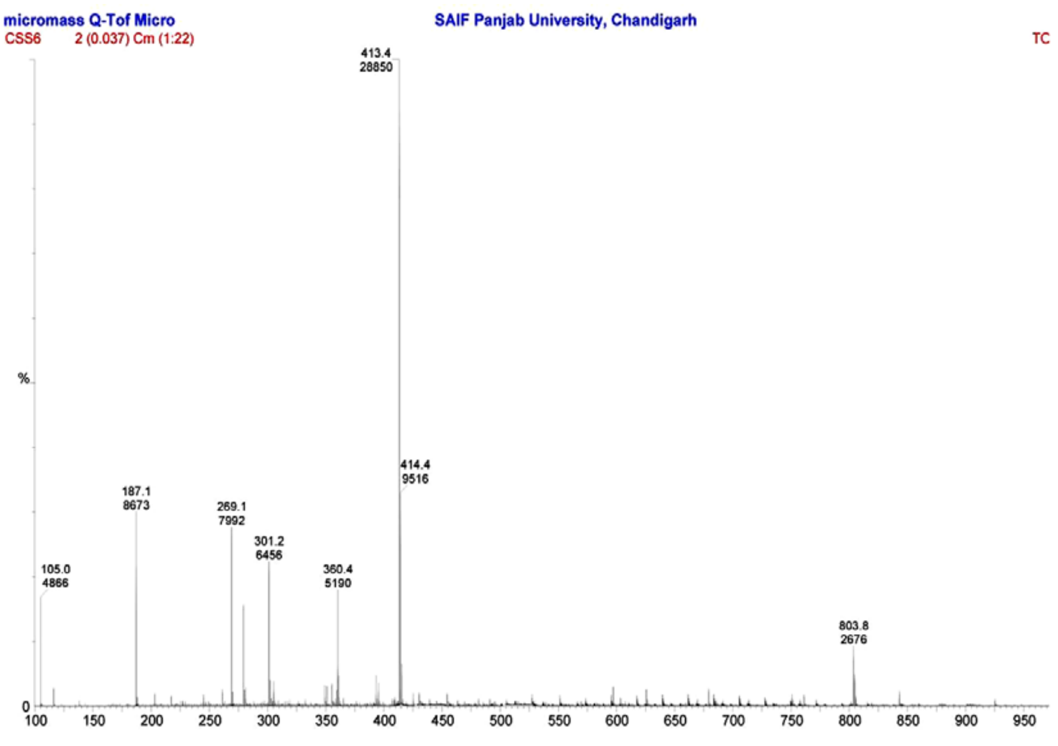



**Fig. 5.26 ESI MS of CSS6**

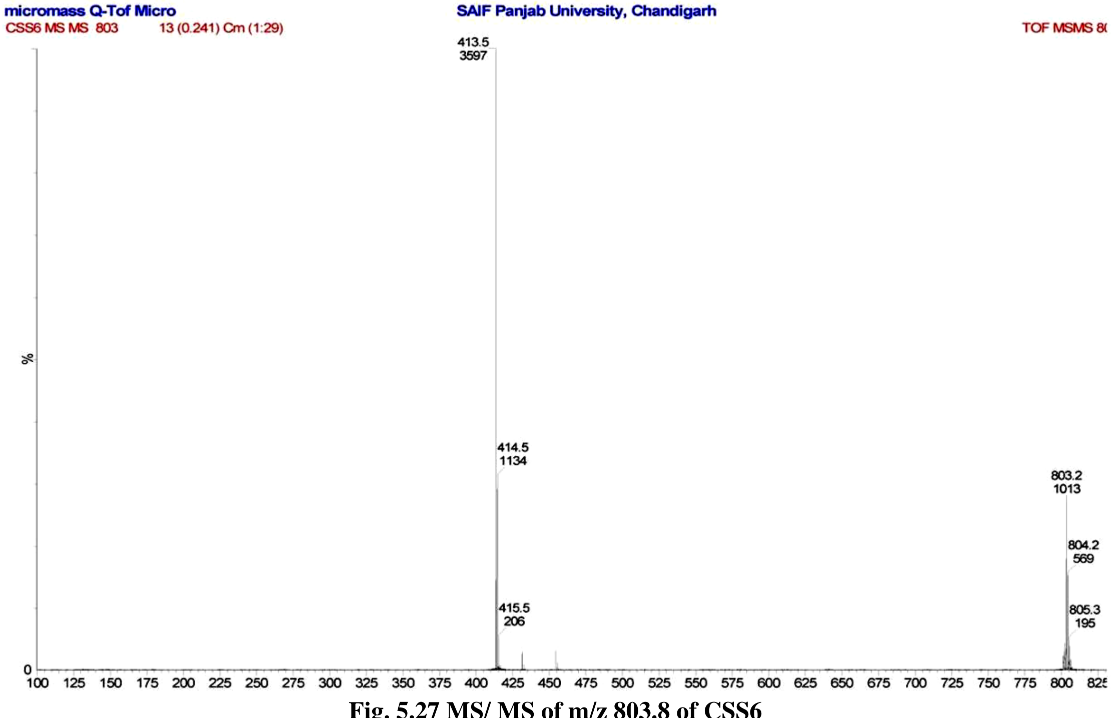



Fig. 5.27 MS/ MS of m/z 803.8 of CSS6

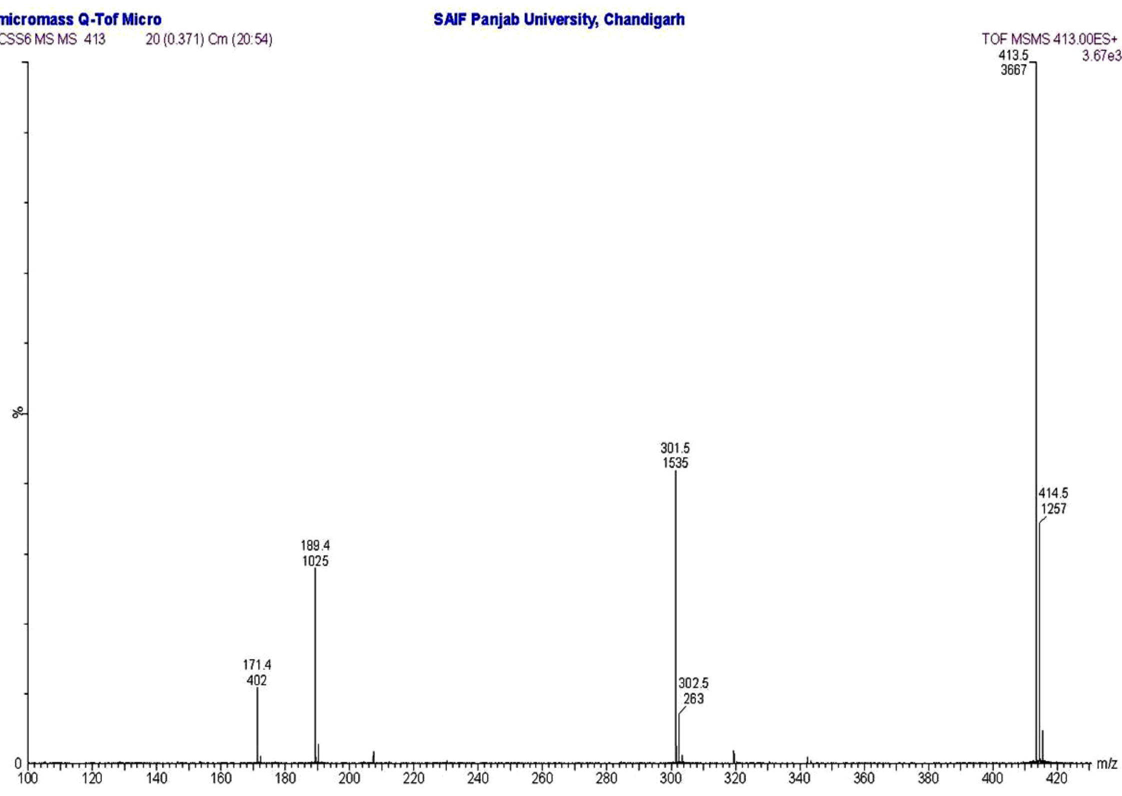



Fig. 5.28 MS/MS of m/z 413.5 of CSS6

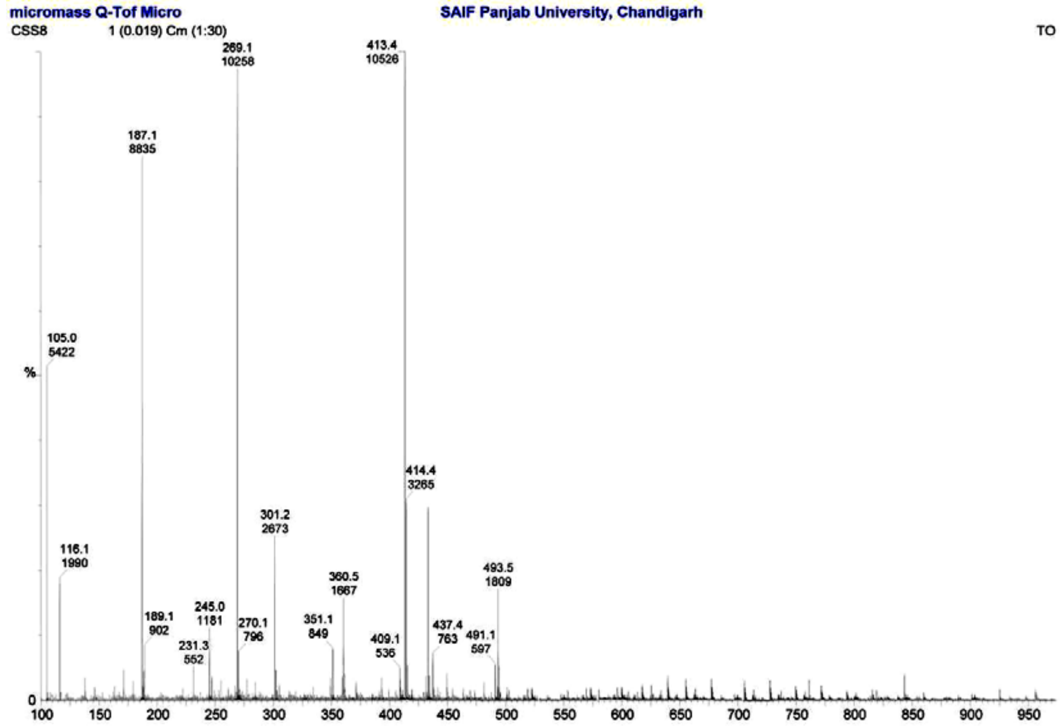



**Fig. 5.29 ESI MS of CSS8**

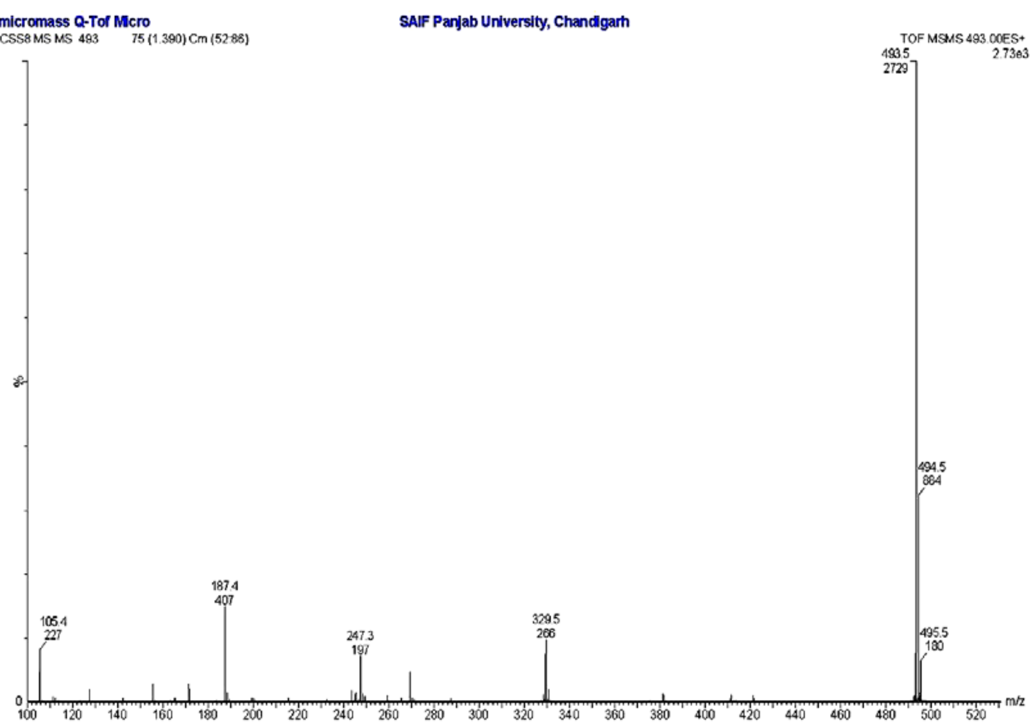



**Fig. 5.30 MS/ MS fragmentation of m/z 493.5 of CSS 8**

# Chapter 6
# DISCUSSION

# 6.0 DISCUSSION

## 6.1 Culture repository for antimicrobial screening

Antibiotic drug resistance has lead to a situation wherein current armamentarium of antimicrobial drugs has become ineffective towards the pathogenic microorganisms. This has led to the emergence of chronic infectious diseases both in hospital as well as community settings. MRSA is a global threat since it has become refractory to current armamentarium of antimicrobial drugs (Saxena and Gomber, 2010). MRSA isolates become multidrug resistant when encounter resistance to more than five classes of antibiotics and are referred to as Multiantibiotic Resistant *Staphylococcus aureus* or MARSA. Diseases caused by MRSA/ MARSA are chronic, difficult to treat and at times prove to be fatal if improperly treated. Apart from causing serious epidemic and endemic infections MRSA are disseminated from hospitals through colonized patients or healthcare workers. The emphasis today is preventing acquisition of multiantibiotic resistance and disease transmission by MRSA isolates prevalent in hospitals and communities. Hence there is an immense need to develop new therapeutic interventions which can overcome MRSA and its resistant clones.

During antimicrobial screening programs both laboratory control strains and clinical isolates are included in the microbial test panel to evaluate the efficacy of the drug or test sample which could be a herbal extract or natural product. The information provided by the clinical or etiological isolate has much relevance since it can be helpful in treating the patient. As *S. aureus* / MRSA is an opportunistic pathogen it has been isolated from different infection sites such as blood, urine, heart, skin, soft tissues like vagina, kidneys, catheter tip and burns where it produces different set of etiological conditions. 99 clinical isolates of MRSA and one standard strain ATCC25923 have been tested with *Nigella sativa* ethanol extract (Hannan *et al*., 2008). Extracts of chinese medicinal plants have also been tested for their antimicrobial potential against 9 clinical isolates of MRSA along with the standard ATCC stain 25923 (Zuo *et*

*al*., 2008b). Clinical MRSA isolates have also been tested for the potential of *Chelidonium majus* aerial part extracts (Zuo *et al*., 2008a). Similarly 149 isolates of *S. aureus* of which 64 were coagulase positive were isolated and tested to assess the role of rhodomyrtone as a natural antibiotic to combat skin related staphylococcal infections (Saising *et al.*, 2008). Anti- MRSA potential of prenylflavones and pacific propolis was tested against 15 clinical MRSA isolates (Raghukumar *et al*., 2010). Extracts of *Psidium guajava* leaves were found to possess anti-staphylococcal activity when tested against multidrug resistant clinical isolates of *S. aureus* of Malabar region of Kerala (Anas *et al*., 2008).

In the present study we also tested the extracts and compounds present in the *Callistemon rigidus* leaves using 34 clinical isolates and 4 standard isolates viz. Sau NCTC 6571; Sau MTCC 737 (Sau NCTC 7447; Sau ATCC 6538P); Sau MTCC 740 (Sau NCTC 6571, Sau ATCC 9144), Sau MTCC 902 (Sau NCTC 8530; Sau ATCC 12598).

### 6.2 Antibiogram by KB disc assay

CLSI (Clinical and Laboratory Standards Institute, previously NCCLS) defines antibiogram as an overall profile of antimicrobial susceptibility results of a microbial species to a battery of antimicrobial agents. It is generally used for monitoring the resistance trends of a population of microbial species in hospitals as well as in the community (Rossney *et al*., 1994). KB disc assay for antibiogram revealed that the maximum resistance in penicillin was 97%, followed by cefixime with 95%. Cephoxitin, cefpodoxime exhibited a resistance of 89% while ceftizoxime exhibited 76% resistance. Carbenicillin, erythromycin and ceftizidime exhibited 68% resistance. Nalidixic acid and trimethoprim exhibited 66% resistance while cefdinir exhibited 60% resistance amongst all clinical isolates in the test panel. 99% resistance to penicillin and 63% resistance to cephalexin, erythromycin and cephotaxime has been reported from the susceptibility pattern of MRSA isolated from major southern districts of Tamil Nadu, India (Rajaduraipandi *et al*., 2006). Antibiogram of 10 MRSA isolates from Bangladesh have been reported to possess 100%

resistance for penicillin, oxacillin, amoxycillin, cloxacillin. However MRSA isolates were sensitive to ciprofloxacin and vancomycin (Islam *et al*., 2008). 16% isolates of the total cultures in the test panel appeared to be MRSA positive on the basis of KB disc assay. Similarly 14% methicillin resistance has been reported by KB disc assay from Kasturba Medical College Hospital, Manipal (Karnataka), India (Shobha *et al*., 2005). 23% MRSA have been reported from urine, high vaginal swab, abscess aspirate, and catheter tip samples from hospitals and primary care centers at Manchester (Brown and Ngeno, 2007).

Dendrogram analysis provides similarity indices based on zone size obtained by KB Disc assay used for antibiogram preparation (Blanc *et al.*, 1994). Isolates sharing common susceptibility pattern against particular antibiotic are grouped into one cluster. This method of reporting allows to directly understanding the epidemiological maps of the susceptibility of particular group of isolates (Giacca *et al*., 1987). Euclidean distance of antibiotic zone sizes of different antibiotics between organisms were used for generating a similarity matrix of test isolates in different groups based on their isolation source viz. pus, urine-vaginal, burn, blood-wound- catheter, eye- ear- unclassified and standard isolates. Cluster analysis has been used to evaluate the sensitivity of 44 antibiotics to pathogens isolated from 183 women with genital inflammatory disease (Ala and Kon, 2007).

### 6.3 Biochemical characterization of the test panel staphylococci

Staphylococci can be broadly classified in two groups- coagulase positive and coagulase negative. Coagulase is a protein produced by several *microorganisms*, which enables the conversion of fibrinogen to fibrin thereby clotting the blood. Coagulase reacts with prothrombin in the blood thereby forming staphylothrombin complex which enables the enzyme protease to convert fibrinogen to fibrin. This results in clotting of the blood. Those *Staphylococcus* which posses coagulase protein are called coagulase positive are designated as CoPS while in which this protein is absent are coagulase negative and designated as CoNS. Coagulase positive as well

as coagulase negative staphylococci are responsible for a variety of infections. In the present study we found that 16% isolates of the panel were coagulase negative while the rest were coagulase positive. Fule *et al.,* (1996) reported that 80.88% of a total of 204 staphylococci were coagulase positive while rest were coagulase negative. 80.3% coagulase positive and 19.3% coagulase negative type have been reported from 320 isolates of *Staphylococcus aureus* by Deepak *et al.,* (1999). 39% coagulase positive isolates are methicillin resistant which is in agreement to Mulla *et al*., (2007). The catalase test differentiates between groups of microorganisms on the basis of catalase production. This test is consistently positive for staphylococci and negative for streptococci. Polymorphonucelar neutrophils are generally produce hydrogen peroxide which is lethal for the ingested *Staphylococcus*. Catalase is a critical component for maintaining viability during long-term starvation which is important for the nosocomial transmission of *S. aureus* or MRSA (Watson *et al*., 1998). The enzyme catalase produced by *S. aureus* converts the hydrogen peroxide to water and oxygen they by protecting it (Mandell, 1975). All isolates in the test panel were catalase positive.

***Molecular typing of Staphylococcal isolates***

Methicillin resistant *Staphylococcus aureus* (MRSA) appeared in 1961 and has become the most prevalent human pathogen responsible for an array of nosocomial and community associated infections. MRSA produces specific penicillin binding proteins PBP2’ (PBP2a) that leads to resistance in β-lactam antibiotics. PBP2’ is encoded by *mecA* gene carried by large mobile genetic element *staphylococcal cassette* chromosome *(*SCC*mec)* which is integrated at the 3’ end of *OrfX* on the chromosome of MRSA strains. The PBP2’ gene is absent in methicillin sensitive or susceptible strains.

Selection of primers for amplification of the *mecA* gene could have significant impact on the accuracy of the test results. The position and G+C content of the primers chosen for the amplification of the *mecA* gene may be critical to the success. Hence in the present study two

primer sets were used for amplification of the *mecA* gene. The primer set I [5'TGCTATCCACCCTCAAACAGG3' (F); 5'AACGTTGTAACCACCCCAAGA 3' (R)] and the primer set II [5'AAAATCGATGGTAAAGGTTGGC3' (F); 5'AGTTCTGCAGTACCGGATTTGC 3'(R)] has been used for typing the current isolates. In the present study 18 out of 38 staphylococcal isolates exhibited *mec A* amplicon at 286-300 bp using this primer set I. Primer set I has also been used for methicillin resistance typing of asian isolates of *Staphylococcus aureus* (Chongtrakool *et al*., 2006). It has also been used to analyze the borderline strains of MRSA (Hiramatsu *et al*., 1992). Primer set II amplified the *mecA* gene amplicon at 533 bp in 11 out of 38 staphylococcal isolates. 6 out of 11 isolates amplified by Primer set II were common with Primer I. Only five isolates were unique which were amplified by the primer set II. The primer has been used for phenotypic detection of nosocomial *mecA* positive CoNS in the neonates (De Giusti *et al*., 1999). The primer II has also been used with *coag* gene in a multiplex PCR (Rallapalli *et al*., 2008). The relevance of more than two primer sets is important since the *mecA* gene is not consistently expressed and certain auxiliary genes may participate in the expression of these genes (de Lencastre and Tomasz, 1994).

### 6.4 Growth curves of clinical staphylococcal isolates

Currently a variety of methods are being used to evaluate the affect of antimicrobial agents on the test microorganisms. This can be accurately achieved if the growth of the microorganism can be recorded in terms of lag phase, exponential or the log phase, stationary phase and the death phase. The growth of a microorganism is generally recorded in terms of absorbance or turbidity change for addressing the variability in number of the microbe in different phases of growth. This is very important parameter to understand the kinetics of the antimicrobial agent which generally is not understood in the CLSI based agar dilution where the MIC values of drugs are quantified by using conventional methods in determination of antimicrobial activities of various drugs on microorganisms. The turbidimetric growth curves of the microorganisms in the

presence of increasing concentrations of anti-microbial agents are obtained and MIC values of drugs can be determined in a shorter period. The results can be detected during exposure. Thus, using kinetic procedures, the effects of antibiotics on target microorganisms can be determined at any desired time-point of the incubation period (Kaya *et al*., 2009). Kinetic measurements of the bacteriostatic, bactericidal and bacteriolytic activities of antimicrobial agents can be performed by bioluminescence, fluorescence, and optical density based on real-time assay with the multi-detection microplate reader (Lehtinen *et al*., 2006). Changes in growth rate and generation time have been found in the presence and absence of SCC*mec* cassette in oxacillin sensitive and oxacillin resistant strains of *S. aureus.* The oxacillin resistant strain had a generation time of 40 min whereas oxacillin susceptible stain of *S. aureus* had generation time of 29 min (Ender *et al*., 2004). Ciprofloxacin resistant *S. epidermidis* had a generation time of 38 to 39 min (Gustafsson *et al*., 2003). In our case since majority of our stains are MRSA the average generation time is 44 min in the log phase which is justified. Thus growth rate determination and generation time is an important parameter for assessment of antimicrobial activity in clinical isolates.

### 6.5 Agar Well Diffusion (AWD) Assay of crude methanolic extract

AWD is a popular prescreen employed by clinical microbiologists, pharmacognosists, and phytochemists working on antimicrobial drug development from plants (Nakamura *et al*., 1999; Alves *et al*., 2000). Antimicrobial activity of numerous plants has been evaluated using agar well diffusion assay (Hoffmann *et al*., 2004; Guven *et al*., 2005; Okoli and Iroegbu, 2005). AWD assay of the leaves of *Bryophyllum pinnatum* and *Kalanchoe crenata* was carried out to assess the anti-microbial activity of the extract in the range of 512 mg/ml to 4 mg/ml (Akinsulire *et al*., 2007). Bark extracts of *Bridelia ferruginea* (Euphorbiaceae) have also been evaluated by agar well diffusion assay for its antibacterial activity against nosocomial strains of *S. aureus*, *S. pyogenes*, *C. albicans* and *E. coli*. The inhibition zones were in the range of 15-36mm (Irobi *et al*.,

1994). Ethanolic extract of *Aloe barbadensis* has a inhibition zone of 20.67±0.67 against clinical staphylococcal isolates (Pandey and Mishra, 2010). Methanolic extract of *Psidium guajava* leaves (Myrtaceae) exhibited an inhibition zone of 15.75±3.5 mm against clinical isolates of multidrug resistant S. aureus (Anas *et al*., 2008). Our results also indicate that an active concentration of 66 µg induced inhibition zones of 19.33±4.87 mm against standard and clinical isolates of *Staphylococcus aureus*. The current work is the first scientific documentation of the antimicrobial potential of *Callistemon rigidus* leaves against multidrug-resistant bacteria (Saxena and Gomber, 2006).

### 6.6 *In vitro* microbroth dilution assay of Crude methanolic extract for MIC determination

Minimum inhibitory concentration**s** (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001). MIC is used as a gold standard parameter for evaluating the susceptibility of microorganisms to antimicrobials and generally used to evaluate the performance of all other methods of susceptibility testing.

Antibacterial activity of the alcohol extract of stem bark of *Picralima nitida* was tested against *Staphylococcus aureus* ATCC 12600 (Nkere and Iroegbu, 2005), where crude extract had a MIC range of 25-50 mg/ml. The extract inhibited only 50% of the isolates. Methanolic extract of peels of grapefruit (*Citrus paradica*) inhibited *Staphylococcus aureus* at 3 mg/ml. Based on these results further studies were initiated and pure antibacterial compound naringin exhibiting a MIC of 1.5 mg/ml was isolated. Study by Ajali and Chukwurah, (2004) demonstrated potential activity of ethanol extract of roots of *Securidaca longipedunculata* against *Staphylococcus aureus* as compared to the positive controls chloramphenicol, penicillin-G and nystatin evaluated in the study. Methanolic extract of *Myrtus communis* also a Myrtaceae family

member exhibits a MIC of 1 mg/ml which is very high compared to our extract (Mansouri *et al*., 2001).

Similarly evaluation of anti-staphylococcal activity of the aqueous and acetone extracts of the bark of *Syzygium jambos* (Myrtaceae) gave a MIC range of 500-1000 mg/l as compared to 0.25-64 mg/l for the positive controls ampicillin and erythromycin. *Zataria multiflora* methanolic extract had a MIC of 3.046±0.797 mg/ml against clinical isolates of CA-MRSA (Rahman *et al*., 2007). *Catharanthus roseus* extracts evaluated by *in vitro* microbroth dilution method using a 96-well microtitre plate using MH broth in concentration of 8- 4096 µg/ml. The methanolic extract of the leaves gave a MIC of 512 µg/ml (Goyal *et al*., 2008). Ethanol extract of *Hyptis martusii* by *in vitro* microbroth dilution assay against clinical MRSA isolates was between 128-512 µg/ml (Coutinho *et al*., 2008).

The methanolic crude extract of leaves of *Callistemon rigidus* in the present study exhibited a MIC in the range of 1.25-80 µg/ml by *in vitro* microbroth dilution assay which was much lower than the extracts that were tested for antibacterial or anti-staphylococcal activity. *In vitro* microbroth dilution assay (dye reduction method) has been found to be the most sensitive method for MIC determination due to the enzymatic reduction of the dye to formazan which occurs only in the viable organisms (Saxena and Gomber, 2008).

### 6.7 Phytochemical analysis of crude methanol extract

In the present study alkaloid content was found to be in high concentration than other constituent’s viz. tannins, saponins, flavonoids, glycosides/ glycolipids. Anthraquinones were totally absent from the crude methanolic extract. Methanolic bark extract of *Betula utilis* exhibited the presence of carbohydrates, alkaloids, and glycosides only. Alkaloids are secondary metabolites which play a defensive role inside the plants. It is therefore imperative to ascertain that the antibacterial activity is contributed by alkaloids or not (Kumaraswamy *et al*., 2008).

Alkaloids have been found to be moderately present in the methanol extract of *Croton zambesicus* (Reuben *et al.,* 2008). *Lagerstroemia indica* leaf extract possess a high concentration of alkaloids apart from tannins, cardiac glycosides, saponins, triterpenes and anthraquinones (Niranjan and Sudarshana, 2010). Ethanol extract of *Eucalyptus globus* has indicated the presence of tannins, Anthraquinones, saponins, flavonoids, cardiac glycosides, alkaloids and steroids and also exhibited antibacterial activity against *S. aureus* (Ibrahim *et al*., 2007). Ethanolic extract of *Psidium guajava* leaf extracts exhibited the presence of alkaloids, flavonoids, tannins and terpenoids (Belemtougri *et al.*, 2006). It is generally attributed that tannins, flavonoids, saponins essentially contribute to antimicrobial activity (Filipowics et al, 2003; Heionen 2007; Wyatt *et al*., 2005). However in *Plinia edulis* (Myrtaceae) has been characterized and found to possess flavonoids, tannins and terpenoids. Despite the presence of these compounds no antibacterial or antifungal activity was recorded when tested against *E. coli*/ *S. aureus* or *Aspergillus niger* or *Candida albicans* respectively (Ishikawa *et al*., 2008). Hence it was imperative to adopt a bioassay guided protocol for fractionation of the crude methanol extract of *Callistemon rigidus* leaves and then characterize the nature of the compound by phytochemical tests which were exhibiting the bioactivity.

### 6.8 Fractionation of the crude methanol extract

Phytochemically maximum alkaloid content was found in the crude methanolic extract of *Callistemon rigidus* in the present study. Further tannins, saponins and flavonoids were also present in the extract in lower contents. Generally tannins, saponins and flavonoids are responsible for antibacterial, antifungal and anti-oxidant activities. However in *Plinia edulis* (myrtaceae family) it has been found that the tannins, saponins, flavonoids and terpenoids did not induce any antibacterial or antifungal action. As *C. rigidus* is also a member of myrtaceae family, it was hypothesized that there is a need to assess the selective activity of the constituents and alkaloids were selected being maximum in content in the crude extract as

assessed qualitatively rather than adopting TLC bioautography. Hence a protocol was designed based on the available literature along with the antibacterial activity of the fractions to isolate the alkaloidal moieties and assess their antimicrobial spectrum against the test panel. For assessing the bioactivity of the fractions, agar well diffusion assays were adopted against a panel of five isolates comprising of one clinical and four standard cultures *of S. aureus*.

Hadi and Brenmner, (2001) have isolated over 100 plant species for antimicrobial properties which represented 49 families and 80 genera of which 23% were tested positive for alkaloids. Their method of alkaloid isolation also involved the use of 5% acetic acid and filtration of the extract followed by separation of aqueous and non-aqueous phase. However we modified the protocol by fractionating the pH adjusted extract with dichloromethane so as to remove chlorophylls and other plant pigments from the extract. The organic phase was found to provide neutral and acidic materials which were found to be non- bioactive whereas the aqueous phase was bioactive. Basification was done with $Na_2CO_3$ to bring the pH to 7.0 instead of pH 10.0 to retain the bioactivity (antimicrobial activity). Further this was extracted with dichloromethane to yield aqueous and organic fractions. The bioactivity resided in the organic fraction. The organic fraction tested positive with Dragendorff's reagent for the presence of alkaloids.

Alkaloids from leaves of *Vallesia glabra* have been isolated using the acid base process (Zeches *et al*., 1995). Methanol extracts of leaves of *Morinda tomentosa* and *Nauclea cordifolia* were extracted by acid base process to isolate alkaloids which tested positive with Dragendorff's reagent and Mayer's reagent. These alkaloids also exhibited to possess antimicrobial activity against *Staphylococcus aureus* which is in agreement with our protocol of isolation of alkaloids and their bioactivity testing (Santiarworn *et al*., 2005).

The *in vitro* microbroth dilution assay of the crude alkaloidal mixture obtained from the methanolic extract of *C. rigidus* leaves was found to be inhibitory only at 3.125 µg/ml. It was observed that the other concentrations viz. 6.25 µg/ml, 12.5 µg/ml and 25 µg/ml were not

inhibitory and did not induce reduction of the bacterial colony counts when compared with control. The inhibitory concentration, 3.125 μg/ml was found to reduce the bacterial colony counts by 3-log reduction when compared with the control set. This observation was unique as majority of the plant extracts and pure compound give a dose response curve and bioactivity at 3.125 μg/ml concentration could probably be attributed to fractional inhibition between the different alkaloids present in the mixture. Voluminous literature on the synergistic and inhibitory affects of alkaloids exists but there is no literature on the combination of alkaloids for antibacterial activity either from the same plant or different plants. Only one patent exist wherein nasal irrigation solutions have been developed using a combination of different alkaloids (Rajamani Tigunait, US Patent application no. 20090035391)

### 6.9 Microwave assisted extraction of Alkaloidal Bioactive Fraction (ABF)

A 4% increase in yield of ABF was found by direct extraction of pulverized leaves of *Callistemon rigidus* over methanolic leaf extract by microwave oven assisted extraction. Microwave assisted extraction of natural compounds to extract glycosides, alkaloids, carotenoids, terpenes and essential oil has been reviewed by Kaufmann and Christen (2002). Microwave assisted extraction of alkaloids has been isolated from *Rhizoma coptidis* (Sun and Liu, 2008). Flavonoids from mulberry leaves have been extracted by microwave assisted extraction (Lei *et al*., 2009). Alkaloid content has been evaluated in *Captis chinenis* (Kamath *et al*., 2009). Camptothecin, an anticancer alkaloid from *Nothapodytes foetida* have been recovered maximally using microwave extraction thereby saving extraction times (Fulzele and Satdive, 2005) which is in agreement with our procedure to enhance the yield of directly treating he pulverized leaves with microwave radiation and subsequently using the acid base process for alkaloid bioactive fraction extraction.

### 6.10 MIC and $IC_{50}$ of ABF by *in vitro* microbroth dilution assay

Minimal Inhibitory concentration and $IC_{50}$ values of any compound or extract provides valuable information on its antimicrobial potential. The MIC refers to minimal inhibitory concentration of an antibiotic which will inhibit the growth of isolated microorganism. $IC_{50}$ is the concentration of the drug that is required to inhibit the growth by 50% under *in vitro* conditions. The MIC of alkaloidal extract from *C. rigidus* was found to inhibit the growth of test panel staphylococci at a concentration of 128.5±3.63 µg/ml while the $IC_{50}$ value was 45.37±3.55 µg/ml. *In vitro* microbroth dilution assay of crude alkaloid extract from leaves of *Acalypha wilkesiana* gave a MIC of 0.4 µg/ml against two isolates of MRSA whereas in our study the value was much higher since the number of isolates in the test panel were 38 (Ezekiel *et al*., 2009). Further total alkaloidal fraction of *Holarrhena floribunda* stem bark extracts by *in vitro* broth micro dilution assay was 96.25±36.6 µg/ml. The $IC_{50}$ value was 46.3±14.7 µg/ml when test panel was of *Bacillus* spp. (Patrice *et al*., 2007). Thus there was a need to fractionate the crude alkaloidal mixture to arrive to leads of the antimicrobial alkaloidal components.

### 6.11 TLC fractionation of ABF

Preparative thin layer chromatography (PTLC) is an effective and easy means of obtaining small quantities of compounds from natural mixtures, which can then be used for spectroscopic determination of chemical structure or investigation of the biological activity (Jozwiak and Waksmundzka – Hajnos, 2007).

Several examples exist wherein PTLC has been used for the isolation of alkaloids from the plant material. This includes isolation of quaternary alkaloids from *Chelidonium majus* (Gołkiewicz and Gadzikowska, 1999), lycopodium alkaloids (Ma *et al*., 1998), protoberberine alkaloids from *Fissistigma balansae* (Chia *et al*., 1998), anthranille alkaloids from *Ticorea longiflora* (Toro *et al.*, 1997), benzylisoquinoline alkaloids from *Anisocycla jollyana* (Kaniyada et al, 1995), alkaloids from *Strychnos icaja* (Frederich *et al*., 2000), pyrrolizidine alkaloids from

*Heliotropium crassifolium* (Farsam *et al*., 2000), alkaloids from *Hernandia nymphaeifolia* (Chen *et al*., 2000). The most important aspect of optimization of PTLC is of course optimization of the chromatographic system. The best chromatographic system depends on the chemical properties of the compounds being separated and the combination of mobile phase solvents. The mobile phase should consist of volatile solvents possessing low viscosity and can be easily removed. For basic compounds it is usually necessary to use aqueous ammonia or short-chain amines as additives. The adsorbent should not react irreversibly with the components being separated. The stationary phases used in PTLC are similar to those applied in analytical TLC, although cost is also important. Normal-phase systems are preferred for preparative purposes.

Crude alkaloids from *Cephalotaxus harringtonia* have been separated using dichloromethane- methanol (9:1) to give 12 different alkaloidal fractions between $R_f$ of 0.15 to 0.84 (Powell, 1971). Six alkaloids have been resolved from crude alkaloidal mixture of *Holarrhena curtisii* using chloroform in basic atmosphere with $R_f$ between 0.28- 0.66 (Cannon *et al.*, 1980). Crude phenolic alkaloids from *Lycopodium selago* have been purified using preparative TLC and provided 262 mg huperzine A when 1.77 g of crude extract was fractionated (Ayer *et al.,* 1989). The yield of Huperzine A was 14%. We also report the yield of individual alkaloid major fractions between 10.08%- 14.4% for alkaloids obtained from crude alkaloidal extract of *C. rigidus* leaves.

Proper resolutions were not obtained by different mobile phases tested. The mobile phase S9 (9:1- cyclohexane: diethylamine) afforded 9 bands with close $R_f$, similarly S16 (17:2:1- ethyl acetate: isopropanol: ammonia) afforded 11 close orange bands when sprayed with Dragendroff's reagent followed by S18 (7:2:1– cyclohexane: isopropanol: 25% ammonia) where 10 orange bands were observed with better resolution but six of them were close and their $R_f$ ranged between 0.37 to 0.58. The mobile phase S20 (17:2:1- cyclohexane: ethyl acetate: ammonia) resolved the crude alkaloid extract into 8 orange bands with very close $R_f$. The solvent

systems S2, S16 and S20 were the fastest moving and took 25 min. for the chromatogram development while S18 took 75 min. Based on these systems solvent system S2/18b was designed which comprised of cyclohexane: benzene: isopropanol: acetone: diethyl ether and ammonia in a ratio of 5:4:4:5:1:1, and gave the best separation of the crude alkaloid mixture yielding 10 fractions with $R_f$ between 0.27 to 0.87 which appeared as orange bands when sprayed with Dragendorff's reagent.

### 6.12 MIC of individual fractions on selected isolates

Alkaloids possess variable antibacterial activities despite being isolated from the same source material and hence they have to be quantified in the terms of MIC. In vitro microbroth dilution assay (using dye reduction) is a method of choice for the assessment of antibacterial potential of the alkaloids. A panel of selected microorganisms comprising of MRSA, MSSA, VISA, VRSA and Standard isolate was used to assess the anti-staphylococcal potential of the isolated alkaloids CSS1-CSS10 from the ABF. Only three fractions CSS1, CSS6 and CSS8 have exhibited potential for further studies as anti- staphylococcal leads. CSS6 was the most potential followed by CSS8 and CSS1 based on their means (geometric). All other fractions exhibited a very high MIC i.e > 500 µg/ml against the isolates of the test panel. Schizogyne and isoschizogaline, indoline alkaloids isolated from *Schizozygia coffaeoides* (apocyanceae) exhibited a MIC of >500 µg/ml and 125 µg/ml respectively for *S. aureus* by *in vitro* microboth dilution assay (Kariba *et al*., 2002). Similarly four pure alkaloids viz hhS, hhC, hS and hC gave a MIC in range of 0.49-7.8 µg/ml, 0.98-15.63 µg/ml; 93.8-750 µg/ml and 375-1500 µg/ml respectively against 20 MRSA isolates. Our results exhibited higher MIC values because of less diversity of the test isolates wherein the potential is only indicative and is not the complete profile (Zuo *et al*., 2008). Similarly fraction III, IV and V out of total five alkaloidal fractions from stem bark of *Aspidosperma ramilflorum* were found to possess a MIC of 250 µg/ml, 15.6 µg/ml and 31.25 µg/ml respectively against staphylococcal isolates (Tanaka *et al*., 2006).

### 6.13 MIC and kill kinetics of CSS1, CSS6 and CSS8 against extended test panel

The kill kinetics of the fractions CSS1, CSS6 and CSS8 were studied to assess their bactericidal and bacteriostatic potential. An extended spectrum of test panel comprising of 10 clinical isolates and 2 standard cultures were used to assess the time related efficacy of killing by CSS1, CSS6 and CSS8 at MIC, ½ MIC and ¼ MIC. Sub-MIC concentrations were tested to assess the post antibiotic effect which would be helpful in understanding the chances of resistance development to the lead fractions.

Bactericidal activity is generally defined when there is a 3 log reduction in the original inoculums by the test antibiotic at any particular instance of the growth curve of the microorganism (Cortez *et al*., 2007). The CSS1 fraction was a bacteriostatic fraction as it could not bring down the CFU count below $10^6$ cells /ml from where the growth was initiated. At sub-MIC concentrations exhibited a post- antibiotic effect in majority of the microbes tested.

CSS6 was found to be selectively bactericidal in Sau G4, Sau G23, Sau G24, Sau G25 and Sau MTCC 737. The bactericidal effect was observed at 20 h and 24 h. At the sub-MIC concentrations a bacteriostatic activity was being observed indicating the role of concentration in bactericidal action.

CSS8 was selectively bactericidal against Sau G5, Sau G10, Sau G24, Sau while exhibited bacteriostatic activity against rest isolates. The bactericidal effect was observed at 20 and 24 h.

Thus time kill studies could help in determining comparative efficacy between the anti-staphylococcal leads CSS1, CSS6 and CSS8. CSS1 was purely bacteriostatic in nature while CSS6 and CSS8 were bactericidal in nature. However the bactericidal activity was more in CSS6 when compared to CSS8. Thus time kill studies provide information on how fast the antibacterial agents can kill certain bacteria and at the same time prevent their regrowth (Behm *et al*., 2005).

### 6.14 Structural changes in bacteria: Mechanism of anti-bacterial activity

To understand the mode of action of CSS6 and CSS8 at MIC, changes in morphology in clinical isolates of *S. aureus* were explored by scanning electron microscopy at 12 h and 24 h. In the present study the reduction in bacterial viable counts were addressed in terms of the lysis of bacterial cells thereby forming an amorphous syncytium, irregular division of cells resulting in formation of dumble shapes and triads and changes in size i.e shrinkage of cells. Daptomycin induced boss like protuberances in the cells after 4 h and 24 h in *S. aureus* Further erroneous cleavage or septation sites were also observed apart from production of defective cell wall material (Wale *et al*., 1989).

Penicillin's have been involved in triad formation in *Staphylococcus* whereas fusidic acid was responsible for the lysis of the cells. Lincomycin caused the lesion formation and doughnut appearance was observed due to erythromycin. Marked changes in size i.e extremely large or small were observed in novobiocin treated cells (Greenwood and O'Grady, 1972).

Cryptolepine, an alkaloid from *Cryptolepis sanguinolenta* has been found to exhibit both bactericidal and bacteriostatic activity against *S. aureus*. Formation of doughnut shape and collapse of cells have been observed in the treated cells apart from shrinkage in cell size. These features are in agreement with our studies on CSS6 and CSS8.

However there are only limited studies exploring the role of natural antibacterial agents /compounds in particular alkaloids in inducing morphological changes in *Staphylococcus aureus* through scanning electron microscopy.

### 6.15 Biochemical mode of action of CSS6 and CSS8

The success of *S. aureus* as a human pathogen is the result of the wide variety of virulence factors that the organism possesses and the immense genome plasticity that assists it in adapting to an array of available chemotherapeutic agents (Saxena and Gomber, 2010). The pathogenicity of the organism is due to the coordinated action of almost 40 different virulence

factors including surface adhesive proteins that promote colonization of host tissue; invasions that promote the bacterial spread; surface factors that help overcome phagocytic engulfment; intracellular enzymes that help overcome oxidative stress and a core of secreted proteins responsible for colonization and infection. Biochemical characterization of the clinical isolates is an important facet in their inclusion in test platform for antimicrobial drug screening. Natural product libraries could be screened for compounds inhibiting the production of these enzymes.

**6.15.1 Expression of superoxide dismutase (SOD)**

*S. aureus* expresses SOD (superoxide dismutase) in response to reactive oxygen species (ROS) such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and the highly reactive hydroxyl radical ($OH^·$). Toxic ROS constitutes a major component of host defense mechanism to which protection is provided by enzymes like SODs and catalases (Cabiscol *et al.*, 2000). By converting $O_2^-$ to $H_2O_2$, SODs protect the bacterial cell against direct damage. Further it also protects against indirect damage due to $O_2^-$ by preventing their interaction with other HOCl (produced during the interaction of $H_2O_2$ with phagocyte derived peroxidases) to form highly reactive ·OH (Clements *et al*., 1999; Karavalos *et al*., 2003; Becerra *et al*., 2006).

*Staphylococcus aureus* produces three types of superoxide dismutases encoded by two genes *sodA* and *sodM* (Valderas and Hart, 2001). Two of the products of the genes i.e. SodA and SodM are homodimers and both utilize Mn as a cofactor (Valderas and Hart, 2001; Karavalos *et al.*, 2003). The third type is a hybrid SOD consisting of subunits of SodA and SodM. *In vitro* role of SOD's in virulence has been investigated in several bacterial species. Virulence is largely dependent upon the ability of *S. aureus* to overcome ROS via expression of higher levels of SOD (Kanafani and Martin, 1985).

In the present study the sub-inhibitory concentration of CSS6 induced inhibition of extracellular SOD in highly SOD expressing isolates Sau G3 and Sau G9. In the standard isolates the expression of SOD was enhanced over the control set indicating oxidative stress induction by

CSS6. A similar trend was indicated by CSS8 on extracellular SOD expression. Intracellular SOD was higher at MIC and Sub- MIC of CSS6 in Sau G3 and Sau G9 which may be attributed to the differential SOD gene expression and is warranted for further studies to understand the exact site of action. Higher intracellular SOD expression in control organisms indicated oxidative stress of CSS6.

In CSS8 the intracellular expression of SOD was observed at MIC but at sub-MIC there was inhibition in the expression in Sau G3 and Sau G9 whereas in the standard isolates the higher SOD expression was observed. Decrease in SOD expression has also been found in the sub MIC of total flavonoids, ethyl acetate, chloroform and methanol extracts of *Myrtus communis* (Myrtaceae) compared to control sets ( Najar *et al*., 2009). Ciprofloxacin also induced oxidative stress in sensitive strains of *S. aureus* while resistant strains did not exhibit any effect (Albesa *et al*., 2004).

**6.15.2 Expression of protease**

Extracellular staphylococcal proteases are a class of proteolytic enzymes which are instrumental in establishment and dissemination of *S. aureus* by breaking tissue proteins like collagen, myoglobin and fibrins. They form a complex interactive network of components with pleiotropic roles in the pathogenesis (Travis *et al*., 1995). A pronounced step in staphylococcal infection for the bacterial dissemination and uptake of nutrients is destruction of host tissue by synergistic action of extracellular proteases along with other enzymes and toxins (Shaw *et al*., 2004). There are different classes of extracellular staphylococcal proteases with respect to tissue organization. Aureolysin is for modulation of immunogenic reactions, staphopain A and B help in tissue invasion, ulceration and sepsis while V8 protease is involved in interference with host defence by inactivation of plasma serpins and immunoglobulin degradation. A significant reduction in protease expression was induced in presence of MIC and sub-inhibitory concentration of CSS6 and CSS8 in high protease expressing isolate Sau G5 as well as in the

standard isolates. Clindamycin and Linezolid are staphylococcal protein synthesis inhibitors like CSS6 and CSS8 (Coyle, 2003). Protease inhibitors have also been recently isolated from *Bauhinia* spp. and also possess antimicrobial activity (Olivia *et al*., 2009). *Eleutherine americana* ethanol extract has been observed to inhibit staphylococcal protease production. Further enterotoxins produced by *S.aureus* were also inhibited at sub-MIC and MIC levels. Thus *Eleutherine americana* extract exhibit a novel application as food additive to combat food borne *S. aureus* (Ifesan and Voravuthikunchai, 2009).

**6.15.3 Expression of Lipase**

Expression of lipase by *S. aureus* in response to the presence of triglycerides and sterols as substrates is primarily responsible for bacteremia (Troller and Bozeman, 1970). Pathogenic *S. aureus* isolates commonly scavenge host sterols for cellular functions like cell wall synthesis necessary for survival. The bulk of bacterial cell membrane so formed is by scavenging the host cholesterol. Lipases along with other proteins help bacteria degrade the host components thereby playing a role in virulence. Staphylococcal lipases are known to possess broad specificity for triglyceride molecules such as ester bond to oleic acid, palmitic acid and stearic acid, which are enriched in human sebum.

Extracellular lipases were being inhibited in high lipase expressing strains indicating that the microbe was being disarmed by rendering its capacity to metabolize the lipids and triglycerides which are essential for causing infections like bacteremia. Both CSS6 and CSS8 have been found to inhibit extracellular lipase enzyme in clinical high lipase expressing isolates of *S. aureus*. Vinaxanthone from *Penicillium* species has been found to be a bacterial enoyl-ACP reductase inhibitor to combat MRSA (Zheng *et al*., 2009) Platencin from *Streptomyces platensis* has been found to be a novel fatty acid inhibitor to combat MRSA (Wang *et al*., 2007). However reports of natural products from plant origin as lipase inhibitors are limited.

### 6.16 Efficacy of CSS6 and CSS8 with commercial antibiotics

The leads CSS6 and CSS8 isolated from leaves of *Callistemon rigidus* were found to be more effective than penicillin's, cephalosporins, and quinlones using a microbial test panel comprising of 10 clinical isolates and two standard cultures of *Staphylococcus aureus.* Even vancomycin at 4 µg/ml is considered resistant based on the kill kinetics data which is the MIC here. Only chloramphenicol was found effective but is not a drug in the current armamentarium of antimicrobials. The present study indicated that lead compounds CSS6 and CSS8 have best parameters in terms of MIC, $MIC_{50}$ and $MIC_{90}$ to combat the multidrug resistant clinical isolates of *S. aureus* after chloramphenicol. Megistoquinone I, megistoquinone II and acronylidine exhibited a MIC of 2350 µg/ml, 750 µg/ml and 20,000 µg/ml which was very high when compared to standard antibiotics netilmicin, amoxycillin and clavulanic acid (Fokialakis *et al.,* 2002). 5',7'-dimethyl-6'-hydroxy-3α-amine-β-yne sitosterol from *Datura metel* leaves exhibited a MIC of 25000µg/ml against *S. aureus* (Okwu and Igara, 2009) as compared to 7.93 and 22.45µg/ml of CSS6 and CSS8 respectively. Reserpine, atropine, berberine, cinchonine, colchicine, ephedrine, harmaline, papaverine, piperine, quinine, scopolamine, strychnine and theobromine were evaluated against a panel of six drug resistant isolates of *Staphylococcus aureus* for their antimicrobial potential. Except berberine which had a MIC of 125 µg/ml all other alkaloids had a MIC > 250 µg/ml (Mohtar *et al*., 2009).

### 6.17 Tentative nature of leads –CSS6 and CSS8

Tentative nature of leads was established by phytochemical testing, elemental analysis and MS/MS spectrum for finding out the tentative masses of CSS6 and CSS8. Phytochemical testing by Dragendorff's reagent confirmed the presence of alkaloid whereas other test for flavonoids, glycosides, tannins, saponins and anthraquinones were absent with both CSS6 and CSS8 indicating their purity being alkaloid only. The European Pharmacopeia uses Dragendorff's colour test for the identification of alkaloids apart from the Mayer's test. 1, 2–methylenedioxy-

8-hydroxy-5-methoxy-oxoaporphine tested positive with Dragendorff's reagent on TLC plate (Xie *et al.*, 1999). Quinolinone alkaloids from the leaves of *Casimiroa edulis* have been isolated and confirmed to be alkaloids by using Dragendorff's reagent (Khaleel, 2002).

Elemental analysis is an analytical method to determine the composition of organic compound qualitatively as well as quantitatively. Qualitatively it indicates the type of elements present in the compound whereas in quantitative terms it provides the percentage of the element present in the organic compound. For organic chemists, elemental analysis always refers to CHNX analysis which is the determination of the percentage weights of carbon, hydrogen, nitrogen, and heteroatom's (X) (halogens, sulfur) of a sample. Elemental analysis gave composition in terms of percentages of CHN and O for CSS6 as well as CSS8. Generally elemental analysis indicates the presence of nitrogen content which serves as a marker for being nitrogen containing compound which could either be an alkaloid or anthraquinone. The presence of anthraquinones is being ruled out in our case since the chemical test was neither positive in the crude methanolic extract, nor in the bioactive fraction and neither in the TLC separated fractions of the ABF. It tested positive with Dragendorff's and Mayer's reagents at all the three stages indicating a strong presence of alkaloidal moiety. Elemental analysis of aporphine alkaloid, N-6/C-7 oxalyl-fused 1, 2, 9, 10-tetramethoxy 6α, 7-didehydroaporphine from stem bark of *Phoebe lanceolata* was found to be C- 67.24%, H- 4.84%, N- 3.56 and O- 24.46% (Semwal *et al*., 2008). Elemental composition of cephalotaxime was C- 68.55%, H- 6.727%, N- 4.44% and O- 20.29% (Powell *et al*., 1972) is similar to the results of elemental analysis of CSS6 and CSS8 indicating them to be alkaloids.

ESI-MS and MS/MS analysis is generally carried out to assess the mass and fragmentation pattern of the leads. This helps in structure elucidation along with other spectroscopic data i.e. IR and NMR. The ESI-MS of vinblastine has a precursor ion ($M+H^+$) m/z of 811 (Verma *et al*., 2007) which is quite near to 803.8 in CSS6 isolated from *Callistemon rigidus*.

The major fragmentation by MS/MS of vinblastine gave 8 fragment ions but only one fragment ion of 413.5 was observed in CSS6. They also used direct extraction procedure for alkaloid recovery from *Catharanthus roseus*. ESI-MS of CSS8 was found at m/z 493 ($M+H^+$) which is quiet near to m/z ($M+H^+$) of gracilamine, a dinitrogenous alkaloid isolated form *Galanthus gracilis* (Unver and Kaya, 2005). Hemsleyasine C has a m/z of 437.27 and is a C-19 diterpenoid alkaloid isolated from *Aconitum hemsleyanium* var. *circinacum* (Gao *et al*., 2007).

Thus based on ESI-MS/MS, elemental analysis and phytochemical data, further detailed spectroscopic analysis is warranted to elucidate the structures of lead molecules CSS6 and CSS8 which has not been contemplated in the present research proposal.

# Chapter 7
# CONCLUSION

# 7. CONCLUSION

The present study was oriented to assess the crude methanolic extract of *Callistemon rigidus* leaves for its anti-staphylococcal activity against 34 clinical and 4 standard strains. Further phytochemical screening of the methanolic extract revealed the presence of high concentrations of alkaloidal moiety qualitatively and a procedure was therefore standardized to isolate the bioactive fraction initially from the crude methanolic extract and finally from dried pulverized leaves of *C. rigidus.* The bioactive moiety tested positive for alkaloids and hence was designated as Alkaloid Bioactive Fraction (ABF).

The MIC of ABF was found to be 3.125 µg/ml and hence further fractionation by TLC was carried out to fractionate the ABF. 10 alkaloid fractions were isolated which were designated as CSS1- CSS10. Based on MIC values CSS1, CSS6 and CSS8 were selected having a MIC of 26.69 µg/ml, 16.81 µg/ml and 23.37 µg/ml respectively against a limited panel of isolates. Further MIC and kill kinetics of CSS1, CSS6 and CSS8 was carried out using *in vitro* microbroth dilution assay and plate count assay to assess their efficacy and antimicrobial behavior against an extended panel comprising 10 clinical isolates and 2 standard isolates. CSS1, CSS6 and CSS8 revealed an average MIC of 26.69 µg/ml, 7.93 µg/ml and 22.45 µg/ml respectively against the test panel. Further evaluation by kill kinetics of these fractions divulged that CSS1 was bacteriostatic while CSS6 and CSS8 exhibited both bacteriostatic and bactericidal action. Tentative mode of their antimicrobial activity was assessed and it was found that they were responsible for morphological changes in the test organisms which included cell lysis, cell shrinkage and irregular shapes. Expression of virulence factors (enzymes) was biochemically evaluated among high SOD, protease and lipase producers and indicated inhibition in expression thereby suggesting multiple targeting of CSS6 and CSS8. The phytochemical analysis, elemental analysis and ESI-MS/MS revealed m/z quite similar to alkaloids as evident from m/z of vinblastine, gracilamine and hemsleyasine.

As structural elucidation was not contemplated in the present research proposal, it is warranted to undertake the detailed structural elucidation by analytical methods for developing CSS6 and CSS8 into commercial anti-staphylococcal antibiotics.

# Chapter 8
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## APPENDIX

### MEDIA COMPOSITION

#### Muller Hinton Agar

| Ingredients | Quantity (g/l) |
|---|---|
| Beef infusion | 300.0 |
| Casein acid hydrolysate | 17.5 |
| Starch | 1.5 |
| Agar | 17.0 |

Final pH at 25°C (7.3±0.2)
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

#### Muller Hinton Broth

| Ingredients | Quantity (g/l) |
|---|---|
| Beef infusion | 300.0 |
| Casein acid hydrolysate | 17.5 |
| Starch | 1.5 |

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

#### Nutrient Agar

| Ingredients | Quantity (g/l) |
|---|---|
| Peptic digest of animal tissue | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| NaCl | 5.0 |
| Agar | 15.0 |

Final pH at 25°C (7.4±0.2)
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

#### Tryptone Soy Broth

| Ingredients | Quantity (g/l) |
|---|---|
| Casein enzymic hydrolysate | 15.0 |
| Papaic digest of soyabean meal | 5.0 |
| Sodium chloride | 5.0 |

Final pH at 25° C (7.3±0.2)
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

#### Potato Dextrose Agar

| Ingredients | Quantity (g/l) |
|---|---|
| Infusion from potatos | 200.0 |
| Dextrose | 20.0 |
| Agar | 15.0 |

Final pH at 25° C (5.6±0.2)
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min.

### BUFFERS AND SOLUTIONS

#### Sodium Phosphate Buffer (0.1M)

| | |
|---|---|
| NaOH (0.2M) | 35.0 ml |
| $NaH_2PO_4$ (0.02M) | 50.0 ml |
| DDW | 15.0 ml |

Final pH at 25°C (7.2)

#### Potassium Phosphate Buffer

| | |
|---|---|
| KOH (0.2M) | 35.0 ml |
| $KH_2PO_4$ (0.02M) | 50.0 ml |
| DDW | 15.0 ml |

Final pH at 25°C (7.2)

#### Phosphate Buffered Saline

| | |
|---|---|
| Potassium Phosphate (monobasic) 1M | 15.6 ml |
| Potassium Phosphate (dibasic) 1M | 34.4 ml |
| Sodium Chloride 5M | 30.0 ml |
| DDW | 920.0 ml |

#### 0.5 Mc Farland

| | |
|---|---|
| $BaCl_2$ (0.048 M) | 0.5 ml |
| $H_2SO_4$ (0.18 M) | 99.5 ml |

#### Saline

| | |
|---|---|
| NaCl | 0.85 g |
| DDW | 100.0 ml |

#### Dragendorff's Reagent

| | |
|---|---|
| Dragendorff Reagent | 1.0ml |
| Glacial acetic acid | 2.0ml |
| Water | 10.0ml |

#### Marquis Reagent

| | |
|---|---|
| Conc. $H_2SO_4$ | 9 parts |
| Formaldehyde | 1 part |

## MOLECULAR BIOLOGY REAGENTS*

**Tris- HCl 1 M (pH 8.0)** **

| | |
|---|---|
| Tris- HCl | 15.76 g |
| Sterile DDW | 100 ml |

**0.5 M EDTA (pH 8.0)** **

| | |
|---|---|
| EDTA | 18.612 g |
| Sterile DDW | 100 ml |

Adjust pH to 8.0 using 10 M NaOH

**Tris EDTA (1X)** **

| | |
|---|---|
| Tris- HCl | 10 mM |
| EDTA | 1 mM |

**Lysozyme**

| | |
|---|---|
| Lysozyme | 100 mg |
| Sterile DDW | 1 ml |

**Proteinase K**

| | |
|---|---|
| Proteinase K | 20 mg |
| Sterile DDW | 1 ml |

**RNase A**

| | |
|---|---|
| RNase | 10 mg |
| Sterile DDW | 1 ml |

**10% SDS** **

| | |
|---|---|
| SDS | 10 g |
| Sterile DDW | 100 ml |

**10% CTAB** **

| | |
|---|---|
| CTAB | 10 g |
| Sterile DDW | 100 ml |

**10% CTAB- 0.7M NaCl** **

| | |
|---|---|
| CTAB | 10 g |
| NaCl | 4.1 g |
| Sterile DDW | 100 ml |

Dissolve 4.1 g NaCl in 80 ml water and slowly add 10 g CTAB (Cetyl trimethyl ammonium bromide) while heating and stirring. Adjust final volume to 100 ml.

**5M NaCl** **

| | |
|---|---|
| NaCl | 29.22 g |
| Sterile DDW | 100 ml |

**Chloroform: Isoamyl Alcohol (24: 1)**

| | |
|---|---|
| Chloroform | 24.0 ml |
| Isoamyl Alcohol | 1.0 ml |

**Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1)**

| | |
|---|---|
| Phenol | 25.0 ml |
| Chloroform | 24.0 ml |
| Isoamyl Alcohol | 1.0 ml |

**TAE Buffer (50X)** **

| | |
|---|---|
| Tris base (2M) | 242.0 g/l |
| Glacial acetic acid (1M) | 57.1 ml |
| EDTA (0.5M), pH 8.0 | 100 ml |
| Sterile DDW | 842.9 ml |

**TAE Buffer (1X)**

| | |
|---|---|
| TAE Buffer (50X) | 2 ml |
| Sterile DDW | 98 ml |

**Ethidium Bromide (EtBr)**

| | |
|---|---|
| EtBr | 10.0 mg |
| Sterile DDW | 1.0 ml |

**6X Loading Buffer**

| | |
|---|---|
| Bromophenol blue | 0.025g |
| Glycerol | 3.0 ml |
| Sterile DDW | 7.0 ml |

**0.8% Agarose**

| | |
|---|---|
| Agarose | 0.8 g |
| TAE buffer (1X) | 100 ml |

**1.5% Agarose**

| | |
|---|---|
| Agarose | 1.5 g |
| TAE buffer (1X) | 100 ml |

*All reagents were molecular biology grade*

** *Reagents were sterilized by autoclaving at 121°C, 15 lbs for 20 min.*