FemA based drug design for potentiation of β-lactam antibiotics against Methicillin resistant *Staphylococcus aureus*

A thesis submitted in fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

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OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

Submitted By

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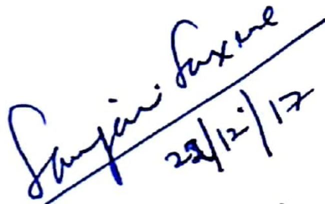
December, 2017

Certificate

It is hereby certified that the thesis ***"FemA* based drug design for potentiation of β-lactam antibiotics against Methicillin resistant *Staphylococcus aureus*"** which is submitted by **Mrs. Divya Singhal (Regd. No.** 900900020**),** in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar Institute of Engineering and Technology (Deemed University), Patiala, Punjab, India, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.



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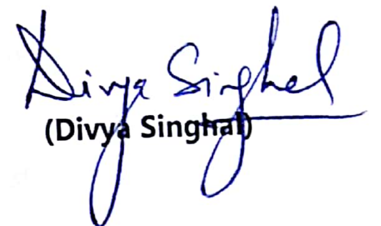
Candidate's Declaration

I hereby declare that the work which is being presented in the thesis ***"FemA* based drug design for potentiation of β-lactam antibiotics against Methicillin resistant *Staphylococcus aureus*"** submitted by me for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is true and original record of my own independent and original research work carried out under the kind supervision of Prof. Sanjai Saxena, Department of Biotechnology, Thapar Institute of Engineering and Technology (Deemed University), Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.



(Divya Singhal)

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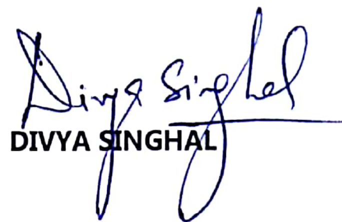

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DIVYA SINGHAL

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Dedicated

to

My Family

Publications

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2. Singhal D. and Saxena, S., (2017): Catechin gallate a promising resistance modifying candidate to potentiate β-lactam antibiotics to overcome resistance in Staphylococcus aureus. *Current Medicine Research and Practice,* 7:224–228.
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Table of Contents

- *List of tables* i-ii
- *List of figures* iii-vi
- *Executive Summary*

Sr. No.	Chapter	Page No.
1.	**Introduction**	**1–9**
2.	**Present approach and Hypothesis**	**10-11**
2.1	Hypothesis	10
2.2	Objective	10-11
3.	**Review of Literature**	**12-31**
3.1	Pathogenicity of *Staphylococcus aureus*	12
3.1.1	Virulence Determinants	12
3.2	Target Sites for Antibiotics	14
3.2.1.	Cell wall Synthesis Inhibitors	14
3.2.2.	Translation Inhibitors	15
3.2.3.	DNA Synthesis Inhibitors	16
3.3	Resistance Mechanism of MRSA	17
3.4	Resistance Modifying Agents (MA's)	18
3.4.1	Acting on modified target site of antimicrobial agents	18
3.4.2	As membrane permeabilizing agents	18
3.4.3	Inhibitors of enzymes that inactivate antibiotics	19
3.4.4	Inhibitors of efflux pumps	19
3.5	Mechanism of Modulating Methicillin Resistance	20
3.6	Computational Methods in Drug Design	21

3.7	Methods of Determining Resistance	25
3.7.1.	Detection of genes	25
3.7.2.	MIC Assay	26
3.7.3.	Checkerboard and FIC Index	28
3.7.4.	Time kill curve	29
3.8	Analytical Techniques	30
3.8.1.	High-Performance Liquid Chromatography (HPLC)	30
3.8.2.	Mass Spectrometry (MS)	31
4	**MATERIALS AND METHODS**	**33-46**
4.1	Structure Databases	33
4.2.	Identifications of Binding Sites	33
4.2.1	SiteHound	33
4.2.2.	QsiteFinder	33
4.2.3.	Pocket Finder	34
4.2.4	CASTp (Computed atlas of surface topography of proteins)	34
4.3	Docking Tools	34
4.3.1	Autodock Vina	34
4.3.2	Dock	35
4.4	*In silico* Interaction and Toxicity Studies	36
4.4.1	Toxicological studies	36
4.4.2.	Lipinski filters	36
4.5	Culture repository	37
4.5.1	Procurement of test isolates	37
4.5.2	Maintenance of cultures	37

4.5.3	Preparation of 0.5 McFarland turbidity standard (CLSI, 1997)	37
4.5.4:	Growth curve studies of test isolate	38
4.6	*mecA* and *femA* characterization of the test isolates	39
4.6.1	Isolation of bacterial genomic DNA	39
4.6.2	PCR for the detection of *mec A and femA*	40
4.7	*In vitro* susceptibility studies of *S. aureus* isolates	41
4.7.1	*In vitro* microbroth dilution assay: determination of MIC	41
4.7.2	Checkerboard Assay and FIC Index	42
4.7.3	Time Kill Assay of Synergistic Formulations	45
4.8	Evaluation of functional Expression of *FemA* protein	45
4.8.1	Peptidoglycan (PGN) cell wall extraction	45
4.8.2	Derivatization of Samples	46
4.8.3	LC-MS Analysis	47
5.	**Results**	**48-87**
5.1	Structure Databases	48
5.2.	Identification of Binding Sites	53
5.2.1.	SiteHound	53
5.2.2.	Q-SiteFinder	54
5.2.3.	PocketFinder	55
5.2.4.	CASTp	56
5.3.	Docking Tools	57
5.3.1	AutoDock Vina	57
5.3.2.	Dock	58
5.4	Interaction and Toxicity studies	59

5.4.2	Toxicological studies	61
5.4.3	Lipinski filters	62
5.5	Test Cultures	63
5.5.1	Growth curve studies of test isolates	63
5.6	*mecA* and *femA* characterization of the test isolates	64
5.7	Evaluation of microbial activity of *S. aureus* isolates	65
5.7.1	*In vitro* microbroth dilution assay: determination of MIC	65
5.7.2	Checkerboard : FIC INDEX Assay	67
5.7.3	Time Kill Curve	76
5.8	Evaluation of *FemA* protein functional Expression	81
5.8.1	nLC-MS	81
6	**Discussion**	**88-99**
7	**Conclusion**	**100**
8	**Bibliography**	**101-129**
9	**Appendix**	**130-139**

List of Table

Table No.	Table Title	Page No.
Materials and Methods		
4.8.2.1	nLC-MS samples with nLC-Ms code and their description	47
Results		
5.1	Summary of Catechin isomers	51-52
5.2	Details of active sites in L-domain as predicted by SiteHound tool	53
5.3	Details of active sites in L-domain as predicted by Q-SiteFinder tool	54
5.4	Details of active sites in L-domain as predicted by PocketFinder tool	55
5.5	Details of active sites in L-domain as predicted by CASTp tool	56
5.6	Results predicted by AutoDock Vina	57
5.7.	Results predicted by Dock	58
5.8	Toxicity results predicted by Osiris	61
5.9	Lipinski results	62
5.10.	MIC of *S. aureus* all three strains w.r.t. antibiotic oxacillin, penicillin and test compound CG	67
5.11	FIC INDEX of *S. aureus* NCTC 6571 with antibiotic oxacillin and test compound CG	69
5.12	FIC INDEX of *S. aureus* MTCC 737 with antibiotic oxacillin and test compound CG	70
5.13	FIC INDEX of *S. aureus* MTCC 96 with antibiotic oxacillin and test compound CG	71
5.14	FIC INDEX of *S. aureus* NCTC 6571 with antibiotic penicillin and test compound CG	72
5.15	FIC INDEX of *S. aureus* MTCC 737 with antibiotic penicillin and test compound CG	73
5.16	FIC INDEX of *S. aureus* MTCC 96 with antibiotic penicillin and test compound CG	74
5. 17.	Summary of FIC Values for all three strains *S. aureus* for antibiotic oxacillin (O) and penicillin (P) with Catechin Gallate (CG)	75

5.18 Comparative kill kinetics of *S. aureus* NCTC 6571 with absence of any antibiotic, in presence of oxacillin, CG and formulation of oxacillin and CG 77

5.19 Comparative kill kinetics of *S. aureus* NCTC 6571 with absence of any antibiotic, in presence of penicillin, CG and formulation of penicillin and CG 77

5.20 Comparative kill kinetics of *S. aureus* MTCC 737 with absence of any antibiotic, in presence of oxacillin, CG and formulation of oxacillin and CG 78

5.21 Comparative kill kinetics of *S. aureus* MTCC 96 with absence of any antibiotic, in presence of oxacillin, CG and formulation of oxacillin and CG 78

5.22 nLC-MS Results Summary (APPENDIX) 135

List of Figures

Fig. No.	Figure Legend	Page No.
Introduction		
1.1	2D Structure of β-lactam antibiotics	2
1.2.	2D Structure of 5th generation of β lactam antibiotics and Texiobactin	5
1.3.	2D structures of resistance modifying agents of β-lactamase producing MRSA	5
1.4.	Schematic representation of an idealized cell-wall peptidoglycan of *S. aureus* and its *fem*-deletion mutants.	7
1.5.	2D structures of resistance modifying agents of β-lactam antibiotics from plants	9
Review of Literature		
3.1.1.1.	Pathogenicity of *S. aureus*, with structural and secreted products	13
3.6.1	Citation percentage of most common docking programs period	23
3.6.2.	Evolution of the number of citations/year for the 7 most cited protein-ligand docking programs over the period 2001-2011.	23
Materials and Methods		
4.1	Docking grid prepared by AutoDock Vina	35
4.2	Config File (APPENDIX)	130
4.3	INSPH File (APPENDIX)	130
4.4	OUTSPH File (APPENDIX)	130-132
4.5.	Showsph.in File (APPENDIX)	132
4.6.	Showbox.in File (APPENDIX)	133
4.7.	Grid.in File (APPENDIX)	133
4.7.1	Microtitre plate template for *in vitro* microbroth dilution assay for determination of MIC	42
4.7.2	Microtitre plate template for checkerboard assay for determination of FIC	44

4.8. Rigid.in File (APPENDIX) 134

Results

5.1 Structure of *S.aureus* in two domains (a) Cartoon View (b) Surface View having L-shaped (white) substrate binding domain 49

5.2 *S. aureus FemA* protein sequence and secondary structure in three domains 50

5.3 Active sites predicted by SiteHound tool 53

5.4 Active sites predicted by Q-SiteFinder tool 54

5.5 Active sites predicted by PocketFinder tool 55

5.6 Active sites predicted by CASTp tool 56

5.7 AutoDock Vina results: *Fem*A protein complex with ligand Catechin Gallate and Catechin 59

5.8 UCSF Dock results: *Fem*A protein complex with ligand Catechin Gallate 59

5.9 Interaction of Catechin Gallate with different residues of *FemA* protein given by LIGPLOT+ tool 60

5.10 Growth Curve of *S. aureus* strains 64

5.11 (a) Screening of staphylococcal isolates for the presence of *mecA* gene with 533 bp amplicon and (b) *femA* gene with 132 bp amplicon as a marker for methicillin resistance 65

5.12 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with antibiotic Oxacillin 66

5.13 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with antibiotic Penicillin 66

5.14 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with test compound CG 66

5.15	Checkerboard result of *S. aureus* NCTC 6571 for five diff. conc. of antibiotic oxacillin with five different concentration of test compound (CG)	69
5.16	MIC of *S. aureus* NCTC 6571 with antibiotic oxacillin and test compound CG from checkerboard method	69
5.17.	Checkerboard result of *S. aureus* MTCC 737 for five diff. conc. of antibiotic oxacillin with five different concentration of test compound (CG)	70
5.18	MIC of *S. aureus* MTCC 737 with antibiotic oxacillin and test compound CG from checkerboard method	70
5.19	Checkerboard result of *S. aureus* MTCC 96 for five diff. conc. of antibiotic oxacillin with five different concentration of test compound (CG)	71
5.20	MIC of *S. aureus* MTCC 96 with antibiotic oxacillin and test compound CG from checkerboard method	71
5.21	Checkerboard result of *S. aureus* NCTC 6571 for five diff. conc. of antibiotic penicillin with five different concentration. of test compound (CG)	72
5.22	MIC of *S. aureus* NCTC 6571 with antibiotic penicillin and test compound CG from checkerboard method	72
5.23	Checkerboard result of *S. aureus* MTCC 737 for five diff. conc. of antibiotic penicillin with five different concentration of test compound (CG)	73
5.24	MIC of *S. aureus* MTCC 737 with antibiotic penicillin and test compound CG from checkerboard method	73
5.25	Checkerboard result of *S. aureus* MTCC 96 for five diff. conc. of antibiotic penicillin with five different concentration of test compound (CG)	74
5.26	MIC of *S. aureus* MTCC 96 with antibiotic penicillin and test compound CG from checkerboard method	74

5.27 Time Kill Curve of *S. aureus* NCTC 6571 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG 79

5.28 Time Kill Curve of *S. aureus* NCTC 6571 alone, with penicillin, withCG and synergistic FIC combination of penicillin and CG 79

5.29 Time Kill Curve of *S. aureus* MTCC 737 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG 80

5.30 Time Kill Curve of *S. aureus* MTCC 96 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG 81

5.31 TIC of *S.aureus* NCTC 6571 82

5.32 TIC of *S.aureus* NCTC 6571 in presence of oxacillin and CG 83

5.33 TIC of *S.aureus* NCTC 6571 in presence of penicillin and CG 83

5.34 TIC of *S. aureus* MTCC 737 84

5.35 TIC of *S. aureus* in presence of oxacillin and CG 84

5.36 TIC of *S. aureus* MTCC 96 85

5.37 TIC of *S.aureus* MTCC 96 in presence of oxacillin and CG 85

5.38 Comparison of Peak area (%) of *S.aureus* NCTC 6571 in absence and presence of antibiotics and test compound formulation 86

5.39 Comparison of Peak area (%) of *S.aureus* MTCC 737 in absence and presence of antibiotic and test compound formulation 86

5.40 Comparison of Peak area (%) of *S.aureus* MTCC 96 in absence and presence of antibiotic and test compound formulation 87

Discussion

6.1. Correlation Matrix of Active Site Prediction Tool Results (APPENDIX) 136

List of Symbols

Sr. No		Symbol
1.	%	Percentage
2.	min	Minute
3.	≥	Equals to or greater than
4.	≤	Equals to or smaller than
5.	°	Degree
6.	°C	Degree celsius
7.	µg	Microgram
8.	µl	Microliter
9.	µm	Micrometer
10.	cm	Centimeter
11.	g	Gram
12.	h	Hour
13	kDa	Kilo Dalton
14	l	Litre
15.	M	Molar
16.	mA	Milliampere
17.	mg	Milligram
18.	R2	Correlation Coefficient
19.	ml	Milliliter
20.	mm	Millimeter
21.	mM	Millimolar
22.	ng	Nanogram
23.	nm	Nnanometer
24.	mAU	Milli Absorbance Unit
25.	rpm	Revolutions per minute
26.	s	Second
27.	U	Unit (activity)
28.	v	Volume
29.	v/v	Volume by volume
30.	~	Approximately
31.	w/v	Weight by volume
32.	µg /ml	Microgram per milligram
33.	µM	Micromolar
34.	U/mg	Units per milligram (Specific activity)
35.	U/ml	Units per milliliter
36.	ng/µl	Nanograms per microliter
37.	bp	Base pair
38.	α	Alpha
39.	β	Beta
40.	γ	Gamma
41.	±	Plus minus
42.	µl/ml	Microlitre per millilitre
43.	µM	Micromolar
44.	g/l	Gram per litre
45.	mg/l	Milligrams per litre

46.	mg/ml	Milligrams per millilitre
47.	OD	Optical density

List of Abbreviations

Sr. No.	Abbreviations	Full Form
1.	+ve HAP	Gram Positive Hospital Acquired Pneumonia
2.	2D- NMR	2D- Nuclear Magnetic Resonance Spectroscopy
3.	AAC	Acetyl Transferase
4.	ABC	ATP-Binding Cassette
5.	ACME	Arginine Catabolic Mobile Element
6.	ADR	Antimicrobial Drug Resistance
7.	AGAR	Australian Group on Antimicrobial Resistance
8.	AST	Antimicrobial Susceptibility Testing
9.	AWD	Agar Well Diffusion
10.	CADD	Computer Aided Drug Design
11.	CA-MRSA	Community Acquired MRSA
12.	CCAP	Community Associated Pneumonia
13.	CDC	Centre for Disease Control
14.	CDDEP	Center for Disease Dynamics, Economics and Policy
15.	CFU	Colony Forming Unit
16.	CG	Catechin Gallate
17.	CLSI	Clinical and Laboratory Standards Institute
18.	cSSTIs	Complicated Skin and Soft Tissue Infections
19.	CTAB	Cetyl Trimethyl Ammonium Bromide
20.	DDW	Double Distilled Water
21.	DHFR	Dihydrofolate Reductase
22.	DHPS	Dihydropteroate Synthase
23.	DMSO	Dimethyl Sulphoxide
24.	DNA	Deoxyribose Nucleic Acid
25.	DNase	Deoxyribonuclease
26.	dNTP	Deoxynucleotide Triphosphate
27.	DSSP	Dictionary of Protein Secondary Structure
28.	EARS- Net	European Antimicrobial Resistance Surveillance Network
29.	EC	Enzyme Commission
30.	ECG	Epicatechin Gallate
31.	EDTA	Ethylenediaminetetraacetic acid
32.	EG	Ethyl Gallate
33.	EGCG	Epigallo Catechin Gallate
34.	EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
35.	EMRSA	Epidemic-MRSA
36.	ESI	Electro Spray Ionization
37.	EtBr	Ethidium Bromide
38.	EUCAST	European Committee for Antimicrobial Susceptibility Testing
39.	FIC	Fractional Inhibitory Concentration
40.	GI	Gastro Intestinal
41.	GISA	Glycopeptide Intermediate *S. aureus*
42.	HA-MRSA	Hospital Acquired MRSA
43.	HBD	Hydrogen Bond Donors
44.	HCl	Hydrochloric Acid
45.	HPLC	High Performance Liquid Chromatography

46.	hVISA	Hetero Vancomycin Intermediate *S. aureus*
47.	IMTECH	Institute of Microbial Technology
48.	INT	2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride
49.	KDa	Kilodaltons
50.	MARSA	Multi- antibiotic Resistant *Staphylococcus aureus*
51.	MATE	Multidrug and Toxic Efflux
52.	MDR	Multi Drug Resistance
53.	MFS	Major Facilitator Superfamily
54.	MH	Muller Hinton
55.	MHA	Muller Hinton Agar
56.	MHB	Muller Hinton Broth
57.	MIC	Minimum Inhibitory Concentration
58.	MR	Molar Refractivity
59.	MRSA	Methicillin Resistant *Staphylococcus aureus*
60.	MS	Mass Spectrometry
61.	MSCRAMMs	Microbial Surface Components Recognizing Adhesive Matrix Molecules
62.	MSSA	Methicillin Sensitive *Staphylococcus aureus*
63.	MTCC	Microbial Type Culture Collection
64.	MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
65.	NA	Nutrient Agar
66.	NaCl	Sodium Chloride
67.	NaH2PO4	Sodium Dihydrogen Phosphate
68.	NaOH	Sodium Hydroxide
69.	NCBI	National Centre of Biotechnology
70.	NCEs	New Chemical Entities
71.	NIH	National Institute of Health, USA
72.	NMR	Nuclear Magnetic Resonance
73.	NTCC	National Collection Culture Type
74.	OD	Optical Density
75.	PBP	Penicillin Binding Protein
76.	PCR	Polymerase Chain Reaction
77.	PHAC	Public Health Agency of Canada
78.	QSAR	Quantitative Structure Activity Relationship
75.	R&D	Research & Development
79.	RNase	Ribonuclease
80.	RTIs	Respiratory Tract Infections
81.	SARs	Structure Activity Relationships
82.	SBDD	Structure Based Drug Design
83.	SBVS	Structure Based Virtual Screening
84.	SCC	Staphylococcal Cassette Chromosome
85.	SDS	Sodium Dodecyl Sulphate
86.	SE	Staphylococcal Enterotoxins
87.	SMR	Small Multi-Drug Resistance Family
88.	SSTIs	Skin and Soft Tissue Infections
89.	TAE	Tris acetate EDTA
90.	TE	Tris EDTA
91.	TFA	Tri Fluororo Acetic Acid
92.	Tris	Tris(hydroxymethyl)aminomethane

93.	TSB	Tryptone Soya Broth
94.	TSS	Toxic Shock Syndrome
95.	TSST	Toxic Shock Syndrome Toxin
96.	TTC	2, 3, 5-Triphenyl Tetrazolium Chloride
97.	UK	United Kingdom
98.	UPLC	Ultra Performance Liquid Chromatography
99.	USA	United States of America
100.	USFDA	United States Food and Drug Administrations
101	uSSTIs	Urinary Skin and Soft Tissue Infections
102	UV	Ultra Voilet
103.	VRSA	Vancomycin Resistant *S. aureus*
104.	WHO	World Health Organization
105.	XRD	X-ray Diffraction

Executive Summary

The present study was oriented to identify the resistance modifying agent (RMA) for β-lactam antibiotics, Penicillin and Oxacillin against Methicillin resistant *Staphylococcus aureus* (MRSA). Initially, identification of RMA's was based on inhibitors of *FemA* protein's structure, which is a novel drug target and has not been exploited so far in antibiotic drug development, using computational tools by virtual library of compounds. Catechin Gallate (CG) was selected as the inhibitor of *FemA* protein from *in silico* studies which comprised of docking, interaction, toxicity and checking of Lipinski violations. Subsequently to identifying CG as a lead molecule from *in silico* studies, its antibacterial property in presence and absence of antibiotics Oxacillin and Penicillin was carried out to arrive to MIC and FIC index of the antibiotic combinations. Further time kill kinetics and mucopolysaccharide content of the test and control organisms was carried out by nLC-MS in the three standard isolates of *S. aureus*.

Three synergistic formulations *viz* formulation of 62.5 µg/ml oxacillin with 7.8 µg/ml of CG, 62.5 µg/ml oxacillin with 31.25 µg/ml of CG and 125 µg/ml of oxacillin with 7.8 µg/ml of CG were found to potentiate oxacillin against three isolates of *S. aureus viz.* NCTC 6571, MTCC 737 and MTCC 96, while one synergistic formulation of 2000 µg/ml penicillin with 7.8 µg/ml of CG was found to be effective against NCTC 6571

Reduction of more than 50% peak area in concentration of muropeptides of test formulations compared to their control, indicates the modulation of functional expression of *FemXAB* proteins by CG the selected inhibitor of *FemA* protein. Subsequently, β-lactam antibiotic efficiency has been increased in MRSA strains. With the use of CG, possibly due to inhibition of FemXAB protein as evident from change in muropepides content. Hence, the present study establishes the potential of Catechin Gallate as potentiator of β-lactam antibiotics against MRSA *in vitro* and warrants extensive studies on clinical isolates and their muropeptides composition.

Chapter 1: Introduction

Introduction

Staphylococcus aureus is gram-positive spherical bacteria existing in microscopic clusters or bunches similar to grapes. It was first recognized by Sir Alexander Ogoston as a major human pathogen causing wound suppuration. *S. aureus* is an opportunistic pathogen despite being an asymptomatic colonizer of skin and anterior nares of healthy individuals. The infections caused by *S. aureus* range from mild superficial skin infections to be extreme like bacteremia, which is potentially fatal. Skin and Soft-tissue infections (SSTI's) generally comprise of boils, impetigo, furuncle, carbuncle, cellulites to abscesses while severe infections comprise of bacteremia, infective endocarditis, osteoarthritis, pleuropulmonary and device-related contaminations

β-lactam antibiotic (Fig. 1.1), Penicillin was first brought into use to treat infections caused by *S. aureus* after its serendipitous discovery by Sir Alexander Fleming (1929). However, due to its widespread and indiscriminate use during the World War II, it soon acquired resistance against Penicillin by production of the enzyme Penicillinase. Even though, Penicillinase resistant version of Penicillin, Methicillin was developed by Beecham (now GlaxoSmithKline, GSK) but *S. aureus* soon acquired resistance against it, and thus methicillin-resistant *S. aureus* (MRSA) emerged, which was reported from a British Hospital. Slowly, MRSA turned out to be sporadic in occurrence in 1970s, epidemic in 1980s and finally becoming endemic and widespread since 1990s. MRSA has two distinct lineages – hospital associated MRSA (HA-MRSA) which is responsible for nosocomial infections and Community associated MRSA (CA-MRSA) which causes serious infections in the community.

MRSA today has acquired, the status of third dreaded pathogen (Saxena and Gomber, 2010), due to its refractory behavior against all antibiotic classes discovered between 1950 to early 2000. This is attributed to its genomic plasticity. MRSA strains are increasing

speedily in several regions and there's a dynamic spread across the world. While HA-MRSA is prevalent to hospitals, the CA-MRSA clones are quickly diffusing through communities in many regions across the world.

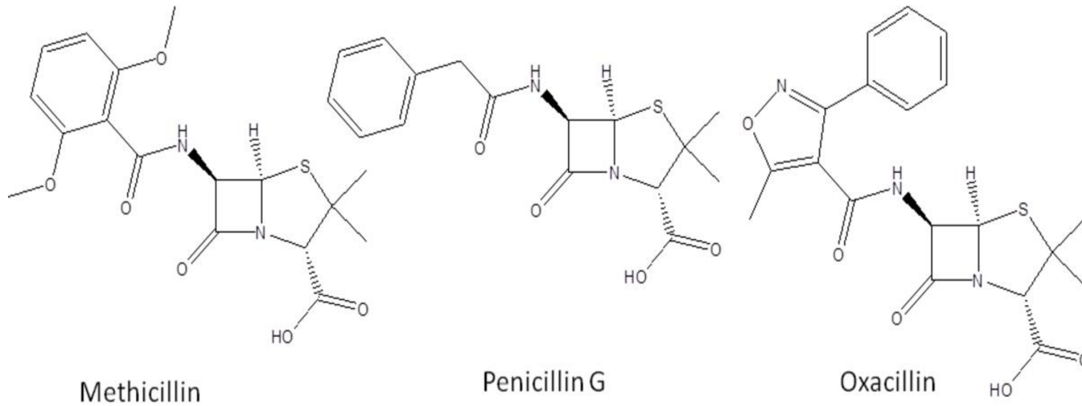

Fig. 1.1 2-D Structure of β-lactam antibiotics

They are also infiltrating the healthcare regions worldwide (Molton *et al*., 2013). High prevalence rates of HA-MRSA, i.e.>50% have been reported in America, Asia and Malta. Intermediate rates (25-50%) of HA-MRSA are reported from countries like China, Australia, Africa, Portugal, Greece, Italy and Romania. Scandinavia and Netherlands have reported the least prevalent rates. HA-MRSA is a prominent nosocomial pathogen in which resistance has already been reported to all the licensed antibiotics (Waters *et al.,* 2017). CA-MRSA has been prevalent in USA with tenfold higher incidence in a study conducted in San Francisco; CA while in western Europe CA-MRSA has remained infrequent. MRSA has been reported to be endemic in India, with incidence ranging from 25% in western part to 50% in South India. Apart from HA-MRSA incidences of CA-MRSA infections are also on the rise in India (Stefania *et al*., 2012; Joshi *et al*., 2013). The antibiotics currently used against *Staphylococci* predominantly target the (i) cell wall or Envelope (ii) ribosome and the translation process (iii) nucleic acids on the transcription machinery. The acquisition of resistance in *Staphylococci* occurs either by horizontal transfer of resistance determinants or through the mutations in

genes. Horizontal acquisition of resistance comprises of resistance determinants such as plasmids, transposons and the Staphylococcal Cassette chromosome (SCC *mec*) carrying the *mecA* gene. While acquisition of resistance of mutation in gene's results in (i) alteration of the drug target rendering the inhibitor ineffective (ii) de-repression of the chromosomally encoded multidrug efflux resistance pumps (iii) and stepwise mutations, which alter the chemical composition and structure of the cell wall thereby reducing the access to the target. However, the global spread of MRSA clones with acquisition of resistance to other drug classes has posed a critical situation for clinicians to treat chronic infections caused by MRSA/ MARSA (multi-antibiotic resistant *Staphylococcus aureus*)(Foster, 2017).

Thus there is a need to develop innovative approaches to develop novel treatment methodologies. Initiatives like GAIN (Generating Antibiotic Incentives Now) act in 2012 by US FDA, initiation of Innovative Medicine Initiative (IMI) as well as New Drugs 4 Bad Bugs (ND4BB) programs in Europe have created networks for carrying out clinical trials of novel antibacterial agents as well as their registration for faster deployment in treating resistant and chronic infections caused by MRSA. Antibiotics such as Vancomycin and Teicoplanin have been in clinical use for years are now getting increasingly resistant after the emergence of resistant clones. Other anti-MRSA agents like Ceftaroline, Ceftobiprole, Dalbavancin, Telavancin has been approved by the regulatory agencies since 2009. However, these antibiotics seem to be arsenal for treatment of critical infective conditions, due to multidrug resistant MRSA (Bal *et al*., 2017; Assis *et al*., 2017).

Developing innovative approaches to re-use existing antibiotics appear as a promising strategy for immediately addressing the escalating antimicrobial resistance crisis. Penicillins and the other β-lactam antibiotics generally target the cell wall formation by which the osmoticum gets disturbed resulting in its death. There are two resistance mechanisms,

which are displayed by the MRSA viz. β-lactamase enzyme and the modified PBP2 expression. It has been noted that MRSA also has *SCCmec* gene, which is to be believed to be horizontally transferred from *S. sciuri*. Vancomycin, a glycopeptide antibiotic was preferred antibiotic drug for MRSA infections, which acted on the D-Ala-D-Ala C-terminal peptide bond physically blocking its recognition and cross-linking, thereby destabilizing the cell wall formation. However, the upsurge of vancomycin resistance was through a mechanism wherein accumulation of muropeptides monomers gathered and sequestered large amount of vancomycin.

Presently, Ceftobiprole and Ceftaroline are the only 5th generation of β-lactam antibiotics belonging to Cephalosporin class, which act as efficient agents against MRSA and VRSA (Fig.1.2). More recently a natural product Texiobactin (Fig. 1.2) has been identified to inhibit *S. aureus* growth by blocking the peptidoglycan synthesis, precursor molecule Lipid II. However, it is still under pre-clinical studies. Hence other strategies of re-deploying current antimicrobials to treat MRSA and VRSA infections comprise of preparation of drug cocktails. In a drug cocktail, antibiotics are combined as their synergistic activity could be used as possible modality to treat MRSA and VRSA infections. Similarly β-lactamase inhibitors have been successfully combined with antibiotics to revert the resistance induced due to expression of β-lactamases revealed by the bacteria (Nikoladis *et al*., 2014; Assis *et al*., 2017).

Clavulanic acid, Tazobactam and Sulbactam (Fig. 1.3) have been formulated with different β-lactam antibiotics to overcome β-lactamase producing MRSA. Augmentin, i.e. Amoxicillin and Clavulanic acid combination has been successful in the treatment of soft tissue and skin infections caused by MRSA. Amoxicillin is a PBP2 inhibitor while Clavulanic acid is a β-lactamase inhibitor. However, with the emergence of mutated forms of β-lactamase TEM-1 have rendered clavulanate less sensitive and therefore, it may not be out of

place to mention that β-lactamases as a target for novel inhibitor development may have a limited future (Assis *et al*., 2017).

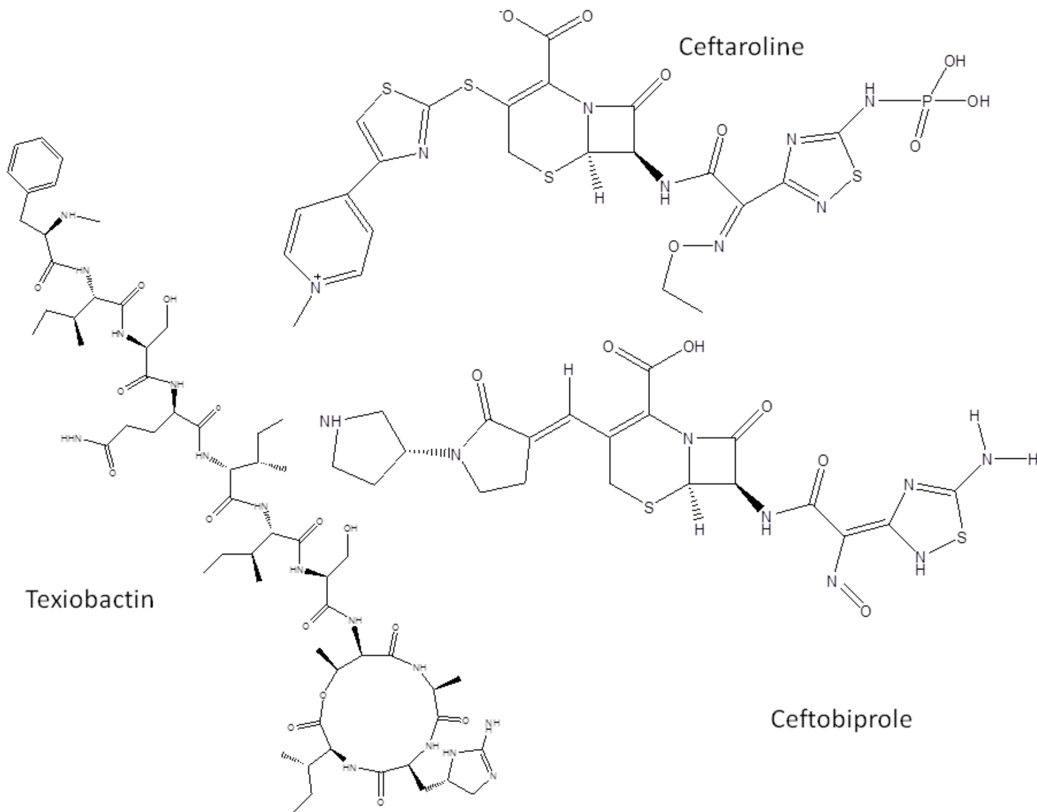

Fig. 1.2. 2-D Structure of 5th generation of β lactam antibiotics and Texiobactin

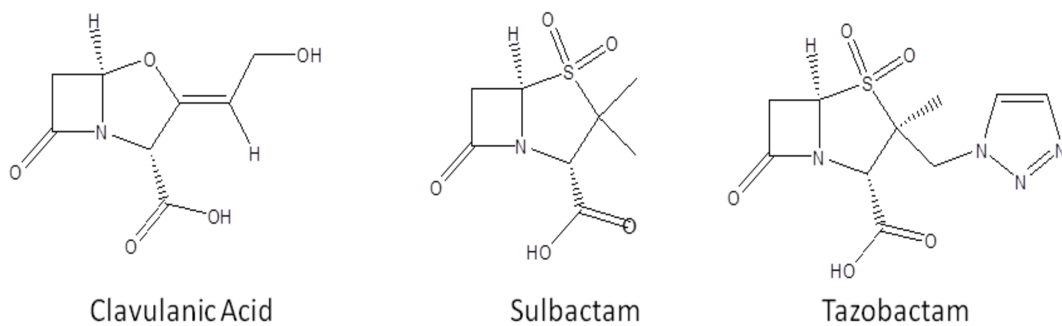

Fig. 1.3. 2-D structures of resistance modifying agents of β-lactamase producing MRSA

Another promising target for reversal/re-potentiation of β-lactam antibiotic is *FemABX* proteins. Structurally Peptidoglycan layer in *S. aureus* is composed of repeating units of N-acetylglucosamine (GlcNAc) and N-Acetylmuramic acid (MurNAc) with stem peptide (L-

Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala) which is attach to the pentaglycine binding segment. The PG lattice formation due to cross-linking occurs by a peptide bond formation between the terminal glycine residue of the bridging segment of one glycan chain and D-Ala [4th amino acid (aa)] of the stem peptide of the adjoining side chain which is generally catalyzed by *FemA* (Fig.1.4). If the pentaglycine bridging segment is ill formed or shortened it leads to failure is cross linking and thus causes loss of methicillin resistance mediated by PBP2a. Consequently, developing inhibitors of this *FemABX* protein is a promising approach to rejuvenate the β-lactam antibiotics. Previously an inhibitor of *S. aureus FemA* has been reported to potentiate the activity of imipenem against MRSA (Sharif *et al*., 2009). 4

Plants have served as novel sources of antimicrobial agents as well as anti-microbial resistance modifying agents. Several plants secondary metabolites or photochemical has been found to re-potentiate the antibiotic against drug-resistant bacteria when co-administered as a synergistic formulation. Catechin gallate (CG), Epicatechin gallate (ECG), Epigallocatechin gallate (EGCG) (Fig. 1.5) from *Camellia sinensis* have been found to synergistically enhance the antimicrobial action of β-lactam, Ofloxacin, Carbapenems and Tetracycline's by mechanisms such as β-lactamase inhibition, PBP2a synthesis inhibition, interaction with peptidoglycan, Ethidium bromide (EtBr) efflux inhibition. Bicalein (Fig. 1.5) from *Scutellaria* species has been found to re-potentiate β-lactam by interaction with the peptidoglycan. Similarly Tellimagrandin, (shown in Fig.1.5) Rugosin from *Rosa Canina;* Corilagin (Fig. 1.5) from *Arctostaphylos uva-ursi* have been found to synergistically re-potentiate β-lactam by inhibition or inactivation of PBP/PBP2a activity. Likewise, Berberine (Fig. 1.5), a hydrophobic alkaloid isolated from *Berberis* species has been reported to additive effect with Ampicillin and synergistic effect with Oxacillin against MRSA (Abreu *et al.,* 2012).

Fig. 1.4. Schematic representation of an idealized cell-wall peptidoglycan of *S. aureus* and its *fem*-deletion mutants. Source (Sharif *et al.*, 2009)

Flavonoid is a class of polyphenolic compounds having 2-phenyl-benzyl-γ-pyrone structure which are universally present throughout the plant kingdom. Due to diverse structural variation of flavonoids in the plant kingdom, they play a multifarious role in the plants, thereby providing protection to them against biotic as well as abiotic stresses

including oxidative stress. As flavonoids play an immense role in protection of plants against infections caused by microorganisms, these may be effectively utilized for treating infections caused by bacteria in humans directly or indirectly as adjuvant of currently used antibiotics to re-potentiate their activities in treating infections caused by the drug-resistant bacteria. Catechins, the most reduced form on the C3 unit in flavonoids compounds extracted from green tea (*Camellia sinensis*) and has been extensively researched due to their antimicrobial activity. Catechins and their derivatives have activities against a wide range of bacteria (Hamilton-Miller *et al*., 1995; Critchfield *et al*., 1996; Cowan, 1999; Pandey and Kumar, 2013). Studies conducted over last two decades indicated that there are various other polyphenolic compounds in green tea and black tea like Catechin, Epicatechin.

ECG, EGCG, CG which shows synergistic activities with aminoglycosides and β-lactam antibiotics against MRSA (Zhao *et al*., 2001; Gibbons *et al*., 2004; Taylor, 2005; Stapleton *et al*., 2006). Among these catechins derivatives, catechins having gallate moiety play an essential role in increasing the susceptibility of β-lactam against MRSA (Shibata *et al*., 2005). Galloylated catechins have been found to inhibit the formation of biofilms of *Staphylococcus*. Therefore, used in food industry to prevent food spoilage and poisoning from *Staphylococcus* strains (Anderson et al., 2011).

As *FemA* protein appears to be a novel target for screening inhibitors for re-potentiating β-lactam antibiotics. In present work we have *in silico* screened a library of catechin derivatives for their potential to inhibit *FemA* and further evaluated the best inhibitor for the reverting the antimicrobial resistance of MRSA towards selected antibiotics.

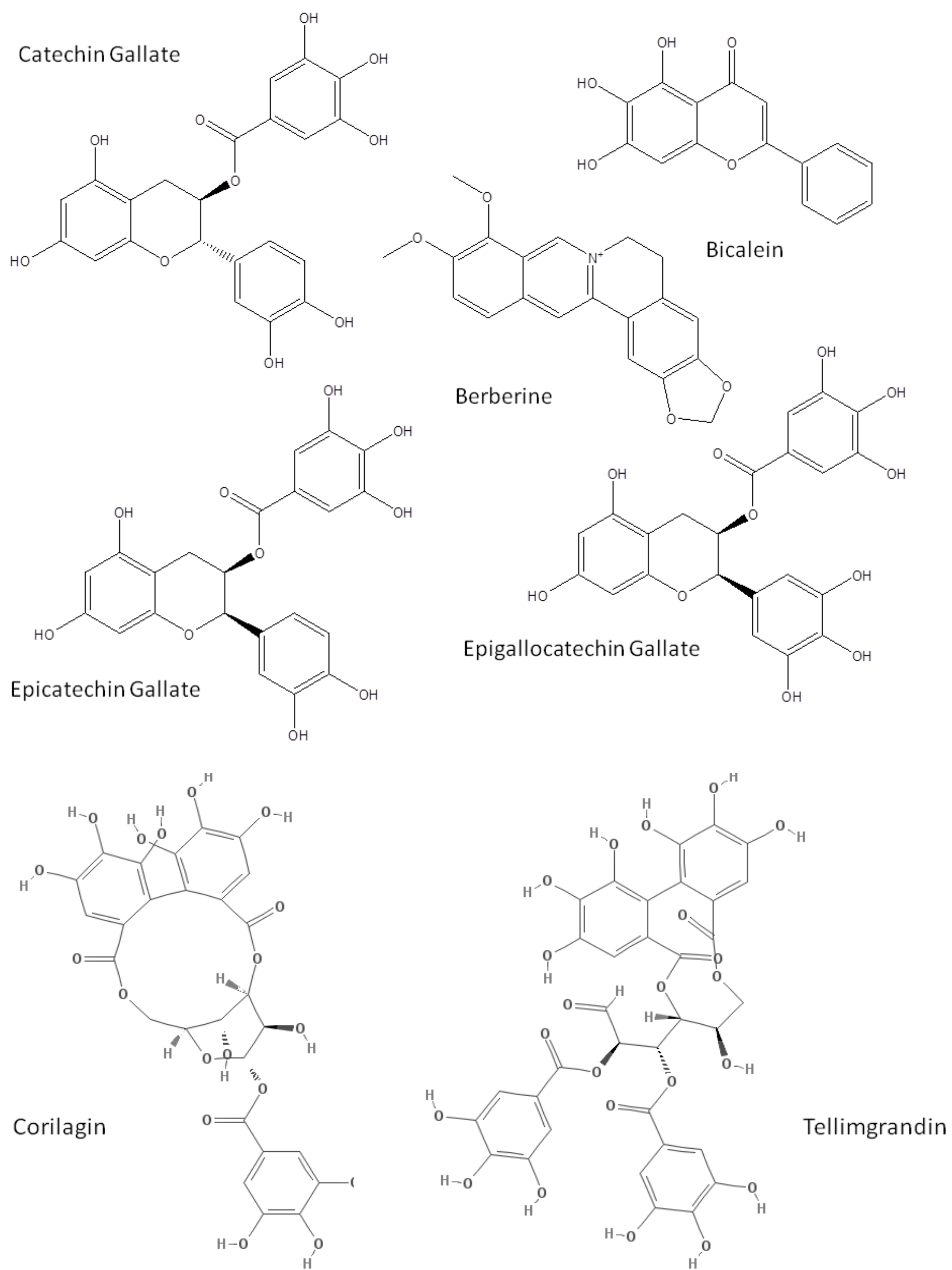

Fig. 1.5. 2-D structures of resistance modifying agents of β-lactam antibiotics from plants

Chapter 2: Present Approach

2.1 Hypothesis

Peptidoglycan or murein is the most important component of gram positive bacteria, which not only provides them shape, but also protects the cell from their own internal turgor pressure. Due to this, targeting at this site can inhibit the synthesis of peptidoglycan, which can cause cell lysis and death of bacteria (Reed *et al.,* 2015; Typas *et al*., 2012). Flavonoids, like Epicatechin gallate and Epigallocatechin gallate, catechin gallate, are the key components of green tea (*Camellia sinensis*) which are reported for alteration in the architecture of *the S. aureus* cell wall (Ando *et al.,* 1999; Stapleton *et al.,* 2007). Despite this, their mechanism of action is not known properly. Therefore, to study the mechanism of action of these flavonoids, in MRSA strains, we have selected the complete library of catechins for virtual screening of an inhibitor against *FemA* protein (Singhal and Saxena, 2015), which is an essential protein for pentaglycl bond formation during the cross-linking of peptide chains, for the elongation of peptidoglycan (Heijenoort, 2001; Maidhof *et al*., 1991; Rohrer *et al*., 1999). Selected inhibitor was further tested experimentally, for its synergistic antibacterial behavior with selected β-lactam antibiotics against standard *S. aureus* cultures.

2.2 Objectives

Based on the above hypothesis, the objectives of the current study are following:

1. Active site prediction of *FemA* using bioinformatics tools.
2. *In silico* identification of inhibitors of *FemA*.
3. *In vitro* assessment of inhibitor against methicillin resistance *Staphylococcus aureus*.
    a) Methicillin resistance determination of *S. aureus* isolates by *mecA* amplification.
    b) MIC determination of Penicillin on a panel of *mecA* and *femA* isolates.

c) Protein Expression of *femA* and *mecA* in the presence of β-lactam and selected inhibitor.

3.1 Pathogenicity of *Staphylococcus aureus*

S. aureus is a most abundant human pathogen, which is responsible for most of the infections. Open wounds, skin, soft tissues (mucosa membrane of nose, vagina, etc.), pharynx, gastrointestinal tracts are the main site of infection (Gordon and Lowy, 2008). Similar to other micro-organisms *Staphylococcus* spread the infections in the host by tissue invasion, toxin production (Chang *et al*., 2003). Abilities of *Staphylococcus* to up-regulate virulence factors and adherence to the host surface are the key factors which are responsible for its persistence in blood stream and its infections (Naber, 2009). Teichoic acid is an essential polymer for the adhesion of virulence factors on the surface of bacteria (Weidenmaier *et al.,* 2004). Despite, of a large number of antibiotics for *Staphylococcus* treatment, another most vital factor is resistance against antibiotics or MRSA (CDDEP, 2015).

3.1.1 Virulence Determinants

Virulence factors of *Staphylococcus* are depending on their functions which are divided into various categories (Gordon and Lowry, 2008);

In attachment: Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are those virulence factors which are responsible for adherence to the host tissue. They bind to clumping factors and various proteins like fibronectin-binding, collagen and bone sialoprotein–binding. Genes responsible are *clfA, clfB, fnbA, fnbB, can, sdr, bbp*. MSCRAMMs are liable for infections of endovascular system, prosthetic-devices, bone and joints. (Patti *et al.,* 1994; Foster *et al.,* 1998; Tung *et al.,* 2000; Menzies *et al.,* 2003).

In Persistence: After adherence to host tissue, it grows and persists in different ways and is also reported to form biofilm (Zago *et al.,* 2015). Along with biofilm production, they form small-colony variants (SCVs), which may contribute to constant and frequent infections in the host. Genes responsible for persistence are *iCa* locus and *hemB* mutations. These kinds of

virulence factors is the main cause of relapsing of infection (Ogawa *et al.,* 1985; Hamill *et al.,* 1986; Kahl *et al.,*1998; Proctor *et al* .,1995; 1998; Donlan *et al.,* 2002; Arrecubieta and Lowy, 2006).

In invading host defense mechanism: Production of the anti-phagocytic capsule (eg. Capsule 5 and 8) is the main defense mechanism of bacteria inside the host. Protein A of bacteria binds to the F_c segment of immunoglobulin and prevents the bacteria from phagocytosis. Leukocidins, CHIPS, Eap, and phenol-soluble modulins are the other virulence factors, which prevent the bacteria inside the host. Genes liable are *cap5* and *8* for capsule formation, *spa* for protein A, *chp* for CHIPS, *lukS-PV* and *lukF-PV* are responsible for leukocidins formation and leukocyte destruction and *psm-a* gene for production of phenol soluble modulins (Riordan *et al.,* 2004; Tzianabos *et al.,* 2004; Foster, 2005; Wang *et al.,* 2007).

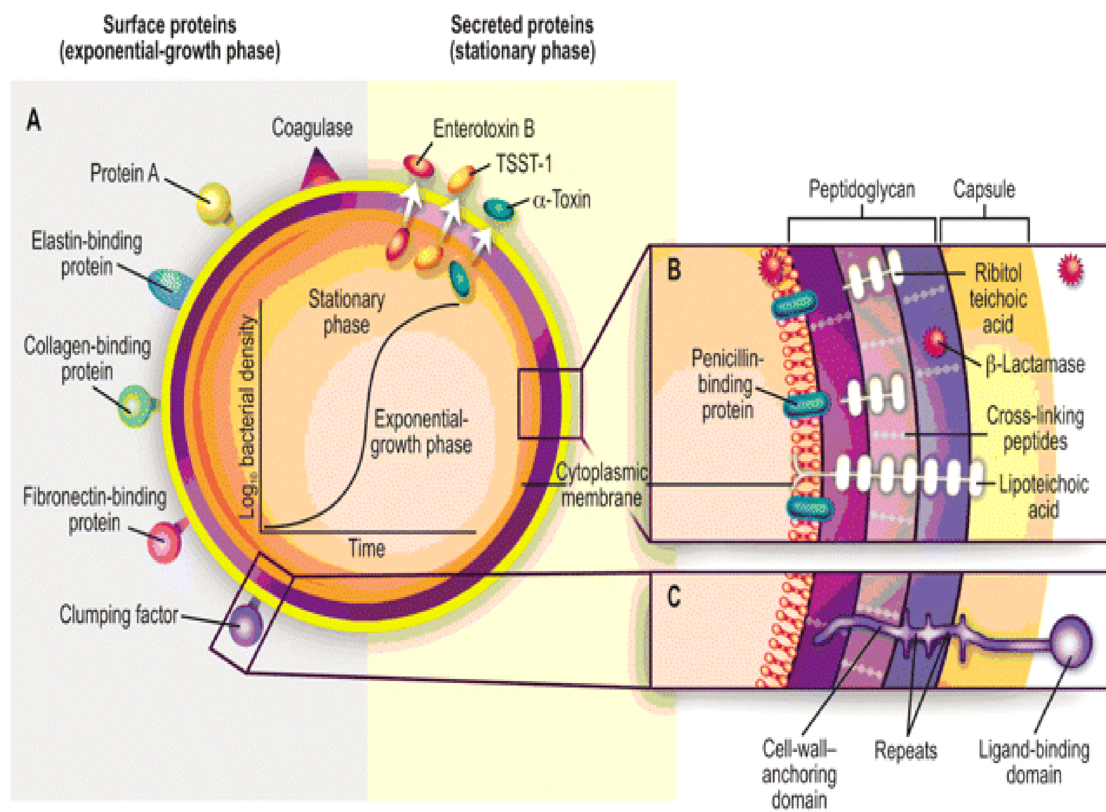

Fig. 3.1.1.1. Pathogenicity of *S. aureus*, with structural and secreted products. (A), Surface and secreted proteins. (B and C), Cross-sections of the cell envelope.TSST-1, toxic shock syndrome toxin 1. This Figure is adapted from [Lowy, 1998].

In tissue invasion: Proteases, lipases, nucleases, phospholipase C, and metalloproteases are those virulence factors, which are liable for destruction of host defense mechanism. Genes liable are *V8, hysA, hla, plc, sepA* (Projan *et al.,* 1997).

In toxin mediated diseases: By interacting with the host coagulation and interaction pathway *Staphylococcus* produces enterotoxins and causes toxic shock syndrome (TSS), and also produces toxins like toxins A and B, A-toxin, is liable for food poisoning. Genes responsible for these toxins are *sea-q, tstH, eta, etb, hla*. (Bhakdi *et al.,* 1991; Timmerman *et al.,* 1993; Heumann *et al.,* 1994; Dinges *et al.,* 2000; Prevost *et al.,* 2003).

Least responsible for virulence: Coagulase, arginine catabolic mobile element (ACME), and bacteriocin are the least responsible virulence factors (Baba *et al*., 2002; Diep *et al.,* 2006).

3.2 Target Sites for Antibiotics

Cell wall Synthesis, Protein Synthesis and DNA Synthesis are the three main target sites in the bacteria, where antibiotics generally interact to induce inhibition (Kohanski *et al.,* 2010; Saxena and Gomber 2010).

3.2.1. Cell wall Synthesis Inhibitors

Cell wall is the outermost and main component of bacterial structure. *Staphylococcus* cell wall or peptidoglycan layer is made up of interactions between sugars and peptide bonds. There are three classes of antibiotics, which target cell wall synthesis. First broad class of antibiotics is β-lactam's which includes all antimicrobial agents having β-lactam structure in their core region. It includes penams or Penicillin derivatives, Cephems or Cephalosporins, Carbapenems and Monobactams. They interfere with cell wall synthesis by providing pseudo-substrate for trans-peptidation reaction, which causes weak bond formation and osmotic imbalance leads to bacterial death (Holten *et al*., 2000) Second class of antibiotics which interfere with cell wall synthesis are glycopeptides (antibiotics having glycolic structure). This group comprises of

Vancomycin, Telavacin, Teicoplanin, Ramoplanin, Bleomycin and Decaplanin. They prevent the addition of new units of sugars and stem peptide by binding with (D-Alal-D-Ala) peptide bond and in this, way inhibit peptidoglycan formation (Pootoolal *et al.,* 2002; Tile, 2013). Third class of antibiotics is Lipopeptides, antimicrobial agents having lipids attached to the peptide bonds (Spellberg *et al.,* 2004). It includes Daptomycin and polymixin B; they interfere with cell wall synthesis by forming complex with the monophosphorylated bactoprenol carrier (Schneider *et al.,* 2009; Tile, 2013; Sakoulas *et al.,* 2016)

3.2.2. Translation Inhibitors

Translation is a process of synthesis of proteins, which includes initiation, elongation and termination step. Various classes of antibiotics interfere with either of these protein synthesis steps by interfering with 50s or 30s subunit of ribosomes, which includes Lincosamides, Oxazolidinones, Aminoglycosides, Macrolides, Tetracyclines, and Chloramphenicol. Lincosamides inhibits protein synthesis by blocking 23s portion of 50s subunit of bacterial ribosomes, which results from early detachment of the peptidyl-tRNA from the ribosome (Tenson, *et al.,* 2003). Lincomycin and clindamycin are two antibiotics of this class. Oxazolidinone's class of antibiotics has been 2-oxazolidinones in their structure. They target initial step of protein synthesis by binding between N-formylmethionyl-tRNA and ribosome (Shinabarger *et al*., 1999). Oxazolidinones are marketed as Linezolid, Posizolid, Tedizolid, and Radezolid. Chloramphenicol is a semi-synthetic class of antibiotics, which binds to the ribosomal 50s subunit and inhibits the peptidyl transferase step of translation [Byarugaba, 2010].

Aminoglycosides class of antibiotics have amino-sugar's in their structure and interfere with protein synthesis by association with 30s ribosomal unit, which disturb protein elongation. Macrolides class of antibiotics having 14-16 membered lactone ring in their

structure. They inhibit protein synthesis by three mechanisms (i) Preventing peptidyltransferases (Gary *et al*., 2009). (ii) Inhibiting ribosomal translation (Drainas *et al*., 1987; Davis *et al.,* 2014). (iii) Early detachment of the peptidyl-tRNA from the ribosome (Tenson *et al*., 2003). Clarithromycin, Erythromycin and Roxrithomycin are 14-membered while Azithromycin is 15-membered macrolide only example. Tetracyclines have the four-ring structure; they target 30s ribosomal unit of bacteria and inhibit protein synthesis by preventing the association of charged aminoacyl-tRNA to the ribosome. Tetracycline, minocycline and doxycycline are antibiotics of this class (Chopra *et al*., 2001; Connell *et al*., 2003).

3.2.3. DNA Synthesis Inhibitors

Some antibiotics targets either bind with DNA or inhibit various steps of DNA synthesis. Fluoroquinolones and Sulphonamide are two classes of antibiotics, which target DNA synthesis. Fluoroquinolones inhibits DNA replication by targeting topoisomerase II and topoisomerase IV, which are required for maintaining the chromosomal topology. In gram-positive bacteria, main target site of fluoroquinolones is a type II DNA topoisomerases (gyrases) that is required for synthesis of bacterial DNA and mRNA.

Various generations of flouroquinolones were launched and withdrawn from the market due to their adverse effects. Fluoroquinolones which have been withdrawn are Temafloxacin (1992), Trovafloxacin (1999) and Gatifloxacin (2006) (Zimmerman *et al*., 1999; Reuters, 1999; Bertino *et al*., 2000; Moseley *et al*., 2013). Sulphonamides are structural derivatives of p-amino benzoic acid, which inhibits DNA synthesis by inhibiting tetrahydrate folic acid i.e. dihydropteroate synthase (DHPS) essential for DNA synthesis. DHPS catalyzes alteration of p-amino benzoic acid to dihydropteroic acid. Trimethoprim is a sulphonamide which has high affinity for dihydrofolate reductase (DHFR) enzyme and consequently,

competitively inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid, which is the last step of the DNA synthesis pathways (Jensen and Lyon, 2009).

3.3 Resistance Mechanism of MRSA

Methicillin resistant *S. aureus* (MRSA) is also called as Oxacillin resistant *S. aureus* (ORSA) or multiple resistant *S. aureus* or superbug, as it carries several resistance genes (Madhavan and Murali, 2011). MRSA was first discovered in 1961 in UK, just after the discovery of methicillin (Jevons, 1961; Enright *et al.,* 2002). This intrinsic resistance is primarily owing to the acquisition of staphylococcal chromosomal cassette (40-60Kb) which carries the *mecA* gene (SCC*mec*). SCCmec elements are highly dissimilar and of 11 types (i to xi) (Peacock and Paterson, 2015). SCCmec types are integrated at the same site (attBscc) which is located at the 3′ end of *orfX* gene of unknown function (Ita *et al.,* 1999). SCC*mecA* gene is the main molecular determinant of resistance, and it spreads by horizontal transfer of gene among susceptible strains.

This *mecA* gene encodes for altered (78kDa) PBP2a or PBP2′. This altered PBP2a decreases the affinity of β-lactam as compared to the native PBPs (Reynolds and Brown, 1985). In addition to this *SCCmecA* gene, it consists of 3 cassette chromosome recombinase genes (*ccrA, ccrB and ccrC*) and 2 mec regulator genes (*mecI and mecR*). *ccr* genes are responsible for mobility and recombinases of Sccmec. *mecR1* gene encodes for *MecR1* protein, which is a membrane-bound signal translocation protein whereas *mecI* encodes for the transcriptional regulators (*mecI*) (Peacock and Paterson, 2015). Selective antibiotics usage creates a pressure, which results into mutations and deletion within *mecI* or promoter/operator regions of *mecA*, resulting repressor inactivation and PBP2a expression (Suzuki *et al.,* 1993; Kobayashi *et al.,* 1998; Stapleton and Taylor, 2002 and Shukla *et al.,* 2004). Along with *mecA* there are other factors which are also responsible for methicillin resistance

viz. *Fem* or auxiliary (*aux*) factor, *mur*F, *fmt*A-C, *sig*B, *hmr* A, *hmr* B, *dlt*, *pbp2*, *cta*A, while factors like *ilm* and global regulators *agr* and *sar* genes have minute effect on resistance (Berger-Bachi, 1994; Chambers, 1997; Fernández *et al.*, 2004).

3.4 Resistance Modifying Agents (RMA's)

RMA's are the new approach to overcome resistance of antibiotics. They are generally used in combination with resistant antibiotics to reverse the resistance. There are mainly four kinds of RMA's depending on their mechanism of action (Abreu *et al.,* 2012).

3.4.1 Acting on modified target site of antimicrobial agents

Target site modification is the most common method of developing the resistance. Tetracycline, β-lactam and glycopeptide are the classes of antibiotics which have developed resistance through this mechanism. Cell wall structural enzymes (PBPs) are the target sites of β-lactams. Production of altered low affinity PBPs i.e. PBP2a by *mecA* gene is responsible for resistance. Modified Cephalosporin's, Carbapenems and Trinem are the β-lactam antibiotics, which are designed against PBP2a. While Tellimagrandin I, Rugosin B from *Rosa canina L*. and Corilagin from *Arctostaphylos uva-ursi are* RMA's against PBP2a.

Similarly, RMA's for glycopeptide antibiotics is Silybin from *Silybum marianum* which are synergist against vancomycin resistant strains. They target the modified peptidoglycan structures of the resistant strains (Foster, 2017; Nikolaidis *et al.,* 2014).

3.4.2 As membrane permeabilizing agents

Outer membrane proteins (OMPs) act as a selective barrier, due to their high lipopolysaccharide content for various hydrophobic solutes. Reduced OMPs permeability due to the modification of OMPs is the cause of resistance in the antibiotics like β-lactam, Carbapenems, Tetracycline, Chloramphenicol, Fuoroquinolones and Sulfonamides. Lipophilic compounds can reverse the resistance of antibiotics by increasing their permeability non-

specifically (Abreu *et al.,* 2012). Example of this class of RMA's is 5-methoxyhydnocarpin D (5-MHC-D) and pheophorbide A, which assist the diffusion of berberine into *S. aureus* (Saxena and Gomber, 2010).

3.4.3 Inhibitors of enzymes that inactivate antibiotics

Self-defense mechanism or internal enzymes produced by the bacteria against the action of antibiotics is another cause of resistance. β-lactamases overproduction is the example of this type of resistance, because it hydrolyze's the Penicillin's and Methicillin. Such antibiotics degrading bacterial enzymes are important target for reversal of resistance.

RMA's for β-lactams against β-lactamases are Clavulanic acid, Sulbactam and Tazobactam. Clavulanic acid has been combined with the antibiotic Amoxicillin as a formulation called Augmentin, while Ticarcillin has been combined with Timentin. Sulbactam has been combined with Ampicillin as Unasyn. Tazobactam is currently combined with Piperacillin as Tazocin (Abreu *et al.,* 2012; Nikolaidis *et al.,* 2014).

3.4.4 Inhibitors of efflux pumps

Efflux pumps are the one of the most important mechanism of antibiotic resistance. Gram-positive bacteria generally have three types of antibiotics efflux transporter families: Major facilitator superfamily (MFS), Small multi-drug resistance family (SMR) and ATP binding cassette family (ABC). *NorA* protein (for fluoroquinolones); *MsrA* protein (for macrolides) are examples of efflux pumps which are responsible for their resistance in *S. aureus*. Developments of inhibitors, which can target efflux pump proteins, are helpful in reversal of resistance. Along with these inhibitors, synthesis of analogs of antibiotics which are not recognizable by efflux pumps can be another good strategy against resistance (Abreu *et al.,* 2012; Foster, 2017). Reserpine is an RMA for *NorA* protein efflux pump) for use with fluoroquinolones such as Moxifloxacin, Ciprofoxacin and Sparfloxacin; and Tet(K) efflux pump

for Tetracyclines. Carnosic acid is RMA for *NorA* protein as well as for Tet(K) efflux pump. 5-MHC-D from *Berberis* plant, Capsaicin and Boronic acid are other RMA's which target *NorA* protein efflux pump and reverse the resistance of Ciprofloxacin (Abreu *et al.,* 2012; Saxena and Gomber, 2010; Assis *et al*., 2017).

3.5 Mechanism of Modulating Methicillin Resistance

PBP2a protein expression is mainly responsible for methicillin resistance, which is encoded by *mecA* gene. Interference in the expression of *mecA* gene and the activity of the PBP2a protein can modulate the resistance mechanism. Biochemical studies have shown that PBP2a has specific substrate requirement. Factors, which affect substrate formation, can also modulate methicillin resistance. Pentaglycine chain and stem peptide formation are the two important substrates for PBP2a (Berger-Bachi and Tschierske, 1998; Stapleton and Taylor, 2002). Pentaglycine chain formation requires *FemABX* protein for sequentially addition of five glycine residues at the position 1,2,3,4 and 5. *FemX* adds 1st glycine residue, *FemA* adds 2nd and 3rd glycine residues, while *FemB* adds 4th and 5th glycine residues. Inactivation of genes coding for Fem proteins was earlier thought to be lethal for bacteria, but mutations restore the cell viability (Berger-Bachi and Tschierske, 1998).

During pentaglycine formation, inhibition of *FemABX* proteins can acts as new drug targets (Labischinski and Johannsen, 1999). Moreover, inhibition of these proteins can also affect the secretion of virulence factors (Ton-That *et al., 1998).* Addition of serine residues in replacement of glycine can also reduce the methicillin resistance. It, furthermore, indicates that glycine residues are essential for PBP2a and by inhibiting the activity of PBP2a protein will not reverse the resistance (Tschierske *et al.,* 1997). Similarly addition of glycine residues in replacement of Alanine-Alanine residues of stem peptide, reduces the methicillin resistance (Jonge *et al.,* 1996), which indicates that normal stem peptide configuration is essential for

PBP2a activity (Stapleton and Taylor, 2002). Inactivation of *femF* or *murE* gene coding for UDP-N-acetylmuramyl tripeptide synthetase (that catalyzes the addition of the L-lysine residue to the UDP-linked muramyl dipeptide cell wall precursor) leads to decrease in resistance due to accumulation of UDP-linked muramyl dipeptide (Ludovice *et al.,* 1998; Gardete *et al*., 2003). It also indicated the requirement of normal stem peptide for resistance. Disturbance of either stem peptide or pentaglycine formation may leads to weak cell wall formation or may make the cell susceptible.

3.6 Computational Methods in Drug Design

In drug discovery process, computational methods are one of the most important techniques which are generally exploited for hit identification as well as lead optimization. These are divided mainly in two categories like **structure based (SB)** and **ligand based (LB)** (Sliwoski *et al*., 2014).

SB drug design (SBDD) methods are based on the structural knowledge of the target site. SBDD approaches include three methods: (1) ***virtual screening*** (screening of ligand in large 3-D databases, corresponding to specific binding site of target protein with the help of docking programs), (2) ***de novo* ligand design** (Construction of new ligand molecules according to binding site of target protein in a stepwise manner) and (3) **optimization of known ligands** (evaluation of proposed analogs of known ligands corresponding to binding sites)(Klebe, 2000; Wang *et al*., 2000; Schneider and Fechner, 2005; Jorgensen,2004*)*. While LB methods use only ligand information, they predict the ligand activity depending on the similarity to the previously known active ligands or construct quantitative structure activity relationship (QSAR). It includes pharmacophore modeling, SAR, QSAR, 3-D QSAR (Guner, 2000; Tropsha, 2010).

Among SBDD approaches, virtual screening or docking is the most commonly used technique. Docking is a molecular modeling technique to predict the best orientation of one small molecule towards the bigger target molecule (DNA, RNA or Protein) in the vicinity of each other, when both molecules combines to form a stable complex. It helps in predicting the bonding strength between these two molecules in terms of binding affinity, binding energy or scoring functions (Sliwoski, *et al*., 2014). Main requisite of SBDD is high-resolution structural data of the target sites (Kitchen 2004). Whereas LBDD approaches are used where there is no structural information available (Guner, 2000; Sliwoski, *et al*., 2014).

Various SBDD tools available are AutoDock (Goodsell *et al.,*1990 and Morris *et al .,* *2009),* DOCK (*Kuntz, et al*., 1982*),* FlexX (Rarey *et al*., 1997), GOLD (Jones *et al*., 1997), LigandFit (Venkatachalam *et al*., 2003), Glide (Friesner *et al*., 2004), FRED (Schulz-Gasch and Stahl, 2003), Surflex (Jain *et al*., 2003) and many more. AutoDock was used for developing the first clinically approved HIV integrase inhibitor (Schames, *et al*., 2004). According, to the ISI Web of Science; AutoDock was the most cited, docking tool among the 22 docking programs between 2001 to 2005 (Fig. 3.6.1(a)) Its citation has increased, from 36% to 48%. (Souse *et al.,* 2006). In the last decade, AutoDock has been reported as most approved and most cited docking tool among all the docking tools (Fig. 3.6.1 (b)). Following the Autodock, Dock, GOLD, Flex and Glide are the popular tools.

According to yearly citations of these tools AutoDock is an again topmost tool with a citation of 500/year (Souse *et al.,* 2013) (Fig. 3.6.2). So far, 60 Docking programs have been discovered in last two decades, out of these programs AutoDock Vina has maintained its top position in all docking studies for prediction of top ranking poses with best scores. While GOLD and MOE-Dock are following the AutoDock in ranking (Pagadala *et al*., 2017).

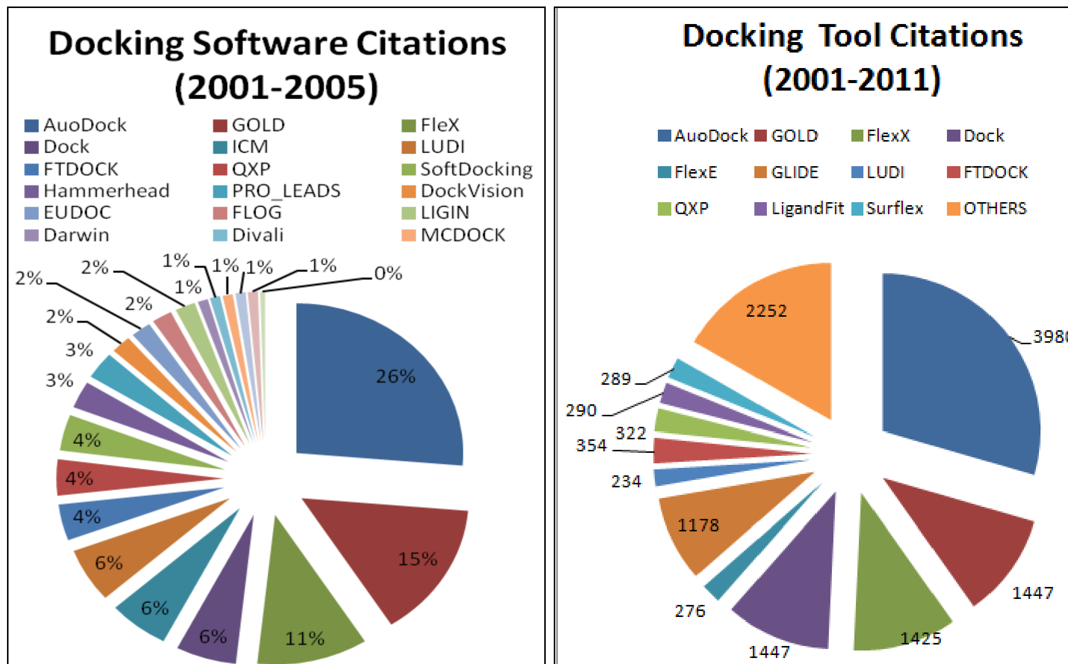

Fig. 3.6.1 Citation percentage of most common docking programs period (a) 2001-2005 (b) 2001-2010

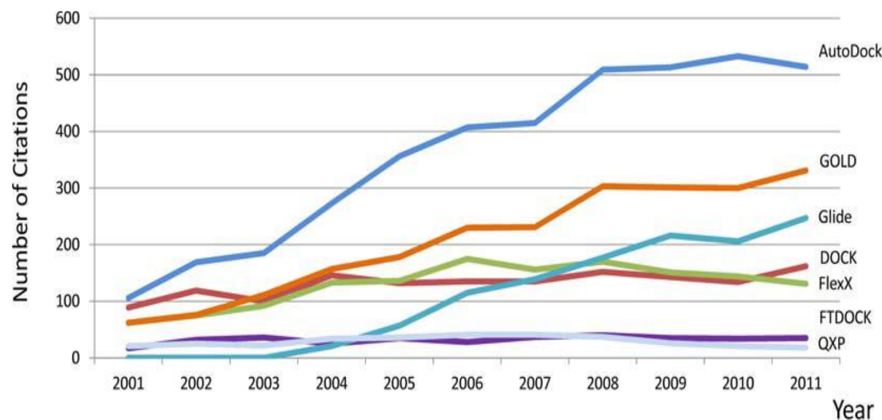

Fig. 3.6.2. Evolution of the number of citations/year for the 7 most cited protein-ligand docking programs over the period 2001-2011.

Almost all the docking tools require target protein and ligand structure file in the 3-D format. Structure proteins files are retrieved from 3-D databases like RCSB-PDB (Berman *et al.*, 2000), SWISS-MODEL (Kiefer *et al.*, 2009) and MODBASE (Pieper *et al.*, 2009) while PubChem (Kim *et al.*, 2015), Zinc (Irwin *et al.*, 2015), LigandExpo (Berman *et al.*, 2000) are examples of small binding structure databases.

Information of binding sites of protein is another essential requirement of docking, after the structure of protein. Binding sites are the small pockets or regions on the tertiary surface of proteins, where ligands bind to form non-covalent interactions, which are specific in nature. Mostly, it is the largest pocket in the protein. Binding site prediction is based on various methods like phylogenetic analysis, identifying structural similarity with proteins of known function and identifying regions on the protein surface with a potential for high binding affinity (Laurie *et al.,* 2006). Binding site prediction has been used in many SBDD projects and has been incorporated into a number of docking tools.

Binding tools which are not incorporated into the docking tools and most of these are freely available are SiteHound (http://sitehound.sanchezlab.org) (Hernandez *et al.*, 2009), Q-SiteFinder (http://www.bioinformatics.leeds.ac.uk/qsitefinder) (Laurie *et al.,* 2005), PocketFinder (http://www.modelling.leeds.ac.uk/pocketfinder/) (Duka, 2013 and Laurie *et al.,* 2005), Computed atlas of surface topography of proteins (CASTp) (http://cast.engr.uic.edu/) (Binkowski *et al*., 2003 and Dundas *et al*., 2006), and many more.

In the discovery of small molecule inhibitors through structure based virtual screening (SBVS) SiteHound server was used for binding site characterization of H1047R PI3K-kinase (Lionta *et al.,* 2014). SiteHound server gives more accurate results in those proteins which bind to ATP, phosphopeptides, and phosphosugars (Ghersi *et al*., 2015). Q-SiteFinder was used to identify ligandable sites in A1-ARF6 active cholera toxin (Gangopadhyay and Datta, 2015). Q-SiteFinder has more accuracy rate than PocketFinder (Laurie and Jackson, 2005).

According to comparative research on 5416 protein-ligand complexes and 9900 Apo forms, for the holo structures, all four softwares viz. SiteFinder, Fpocket, PocketFinder, and SiteMap have identified 95% similar pockets; whereas, in Apo structures; PocketFinder has

shown 96% accuracy. In the absence of correct binding site information, blind docking can be performed.

Poor pharmacokinetics, side effects and toxicity of drugs are major reasons of failure of drugs in clinical and pre-clinical trials, which may result into unnecessary loss of time and money. Bendazac, Bromfenac, Dilavelol, Pemoline, Temafloxacin and Trovafloxacin are examples of such medicines which are withdrawn from market due to their hepatic-toxicity (Waterbeemd and Gifford, 2003). To avoid these failures, *in silico* toxicity prediction tools are used prior to experimental studies. Most of these tools are statistical based on prior knowledge. Examples of currently available toxicity prediction tools are ToxiPred (Gupta *et al.,* 2013), OSIRIS Property Explorer (Mandal *et al*., 2009) and Protox which is an *in silico* rodent oral toxicity prediction tool (Drwal *et al.,* 2014) etc.

3.7 Methods of Determining Resistance

3.7.1. Detection of genes

mecA and *femA* are two most common genes of *S. aureus,* which are generally present in resistant strains. These two genes have retained strong conservation among the methicillin-resistant strains (Vanuffel *et al*., 1999; Murakami *et al*., 1991; Akcam *et al*., 2007; Rostamzad *et al.,* 2016). Several researchers reported different primers of *mecA* and *femA* gene and their different methods of detection of these genes. For *mecA* gene, mainly three sets of forward and reverse primers are reported, which code for 533, 286 and 310bp product, while in case of *femA* gene three, most common primers are reported which code for 509,132 and 686 bp product. First set of primer for *mecA* was complimentary to 1283-1303 (5'AAA ATC GAT GGT AAA GGT TGG C 3') and 1793-1814 bp (5' AGT TCT GCA GTA CCG GAT TTG C 3') codes for 533 bp (Murakami *et al*., 1991; Kobyashi *et al*., 1994; Kampf *et al*., 1999; Japoni *et al*., 2004; Froberg *et al*., 2004; Saiful *et al*., 2006; Rallapali *et al*., 2008; Xiong *et al*., 2008;Mathew *et al*.,

2010; Islam *et al*., 2011. Second set of primer for *mecA* was complimentary to 45813-45833 (5'TGC TAT CCA CCC TCA AAC AGG 3') and 46098-46078 (5' AAC GTT GTA ACC ACC CCA AGA 3') codes for 286 bp product (Kondo *et al*., 2006; Ruppe *et al.,* 2009;Abimanyu *et al.,* 2013).Third set of primers for mecA gene is complimentary to 318-342bp (5'GAT GAA ATG ACT GAA CGT CCG ATA A 3')and 603-627 (5'CCA ATT CCA CAT TGT TTC GGT CTA A 3')which codes for 310 bp (Paule *et al.,* 2005; Kalhor *et al.,* 2012).

First set of primers for *femA* gene is complimentary to 595-614bp (5'AGA CAA ATA GGA GTA ATG AT 3') and 1084-1103 bp (5' AAA TCT AAC ACT GAG TGA TA 3') codes for 509 bp (Kobyashi *et al*., 1994 but has not been used experimentally thereafter. Second set of primers which has been commonly used for *femA* gene detection is complimentary to 1444-1463 bp (5'AAA AAA GCA CAT AAC AAG CG 3') and 1556-1575 (5' GAT AAA GAA GAA ACC AGC AG 3') which codes for 132 bp (Mehrota *et al.,* 2000; Froberg *et al*., 2004; Pelisser *et al.,* 2009; Mathew *et al*., 2010; Duran *et al.,* 2012). Third set of *femA* gene primers is complimentary to (5' CTT ACT TAC TGG CTG TAC CTG 3') and (5' ATG TCG CTT GTT ATG TGC 3') codes for 686 bp product (Akcam *et al*., 2009; Braoias *et al*., 2009; Kitti *et al*., 2011).

3.7.2. Minimal Inhibitory Concentration (MIC) Assay

Broth dilution methods are quantitative methods useful for microorganisms with the variable growth rate. The results are shown in terms of MIC values that are "lowest concentration that resulted in reduction of inoculum viability after a specific incubation time" (Carson *et al.,* 1995). The determination of the MIC involves a semi-quantitative test procedure which provides associate degree estimately to the least concentration of an antimicrobial required to stop microbial growth.

MIC is often determined by semi-automated microtitre method, having 96-wells or 384-wells configurations. Indicators used for determination of end point in broth dilution

methods are 2, 3, 5-triphenyl Tetrazolium chloride (TTC), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT), 2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride (INT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), fluorescein diacetate and resazurin (Chand *et al.,* 1994; Mann and Markham, 1998; Gabrielson *et al*., 2002; Sarker *et al.,* 2007)

In this microplate well assay, the wells which do not exhibit a pinkish/red colouration due to formation of formazen, are interpreted as MIC (Sommers, 1980). Microbroth dilution method has various advantages over the traditional MIC determination method as it uses fewer amounts of media, simple in handling an oversized sample size, and it is more rapid and repeatable (CLSI, 1997).

Antibacterial activity of three Malaysian plants (*Piper sarmentosum, Moringa oleifera, Murraya koenigii*) using microboth dilution method was performed for both gram-positive and gram-negative bacteria (*S. aureus, E. coli, P. aeruginosa and B. subtilis*) (Saad *et al.,* 2014). Comparative study of E-test, disc diffusion and microbroth dilution assay was performed for clinical and reference isolates of *S. aureus, E. coli* and *Pseudomonas* sp. to assess the reliability of *in vitro* susceptibility tests (Saxena and Gomber, 2008).

Antibacterial activity of 33 flavonoids against MRSA was examined by *in vitro* microbroth dilution method. Out of these 33 flavonoids, 11 compounds exhibited MIC range from 32 to 128 µg ml^{-1}, while rest of compounds such as, baicalein, myricetin, datiscetin, quercetagetin, (-)-EGC and (-)-EGCG exhibited bactericidal activity with minimum bactericidal concentrations (MBC) varying from 64 to 128 µg ml^{-1}. Only 3', 4'-dihydroxyflavone showed least MIC values ranged from 2-64 µg ml^{-1} (Alcaraz *et al*., 2000). Antibacterial activity of EGCG derivatives were evaluated for 17 *S. aureus* strains and MIC found to be ≥128 µg ml^{-1}(Matsumoto *et al.,* 2012).

3.7.3. Checkerboard and FIC Index

Checkerboard method is widely used for evaluation of *in vitro* synergy for numerous drugs (Hsieh *et al.*, 1993). In checkerboard study, concentrations tested for each antibiotic are 4-5 two-fold dilutions below and above the estimated MIC. This test is performed in 96-well micro-titer plates. Similar stock concentration of drugs X & Y were diluted along the X-axis & Y-axis respectively, resulting into a unique combination of the two drugs being tested. Two columns of wells are reserved for control sets having the control organisms with no drug and control organism with known MIC for tested antibiotics (Krogstad and Moellering, 1986). MIC observed from checkerboard method among different combinations is used further for calculation of Fractional Inhibitory Concentration (FIC) values (Mandal *et al.*, 2004). FIC indices were calculated using the formula: FIC index = (MIC of drug X in combination/MIC of drug X alone) + (MIC of drug Y in combination/MIC of drug Y alone). In antimicrobial combination, synergy is defined as $\sum FIC \leq 0.5$, partial Synergy ≤ 0.5, Partial synergy > 0.5–1, Indifference >1 to <2 and Antagonism as $\sum FIC \geq 2$ (Schelz *et al.,* 2006; Lee *et al.*, 2010).

To evaluate the synergy among different antibiotics or flavonoids or plant extracts with antibiotics checkerboard method has been used in various studies. Synergy between Phenothiazines and Oxacillin was investigated using checkerboard method for 10 MRSA isolates (Hadji-nejad *et al.,* 2010). Synergy of tetracycline, mupirocin, and fusidic acid with ECG, and Ethyl Gallate (EG) was observed using two MRSA and two MSSA strains of *S. aureus* (Soe *et al.,* 2009). Effect of several terpenes on β-lactam antibiotics such as Penicillins was studied by checkerboard method and FIC index determined against MRSA and *E. coli* strains. Total synergy was observed only with carvon a, terpene with FIC index of 0.0078 while rest of terpenes exhibited indifference or antagonism with MRSA strains (Gallucci *et al.*, 2006). Similarly, according to the comparative study of combination of derivatives of Cg such as ECG

and EGCG with Oxacillin and combination of non-gallolylated catechins such as EC and EGC with Oxacillin in the presence of EC and EGC reported that non-galloylated catechins significantly increased the effect of ECG and EGCG against ORSA (Stapleton *et al.,* 2005).

3.7.4. Time kill curve

Time kill assay is used for studying the rate at which concentration of antibacterial agents such as antibiotics; combination of antibiotics, pure compounds and combination of pure compounds with antibiotics kills a bacterial test isolate. It is also used to study both concentration dependent and time dependent bactericidal activities.

In this assay bacterial isolate is grown in presence and absence of antibacterial agents, then fixed dilutions of 10µl is plated on nutrient agar plate and further incubated at 37°C for 18 h. Viable cells were counted in terms of Colony forming units (CFU) at specific intervals of time. Log of CFU ml^{-1} are plotted graphically on y-axis with time intervals at x-axis.

Decrease of 3 log_{10} (CFU ml^{-1}) folds indicates bactericidal activity (99.9% killing of inoculum) and decrease of ≥2 log_{10} (CFU ml^{-1}) folds indicates synergy, while the increase of ≥2 log_{10} (CFU ml^{-1}) folds represents antagonism (Schwalbe *et al.,* 2007; EUCAST, 2000; Miles and Misra, 1938). Estimation of viable counts at a specific time is an important factor for determining the drug dose in pharmacological studies.

Various antimicrobial agents in their pure form and in combinations have been subjected to time kill studies using *in vitro* microbroth dilution assay to evaluate their efficacy (May *et al*., 2000; Climo *et al*., 2001; Osburne *et al.,* 2006). Plant extracts have also been subjected to time kill studies to examine their efficacy as compared to standard antibiotics or their combinations for therapeutic use (Okemo *et al*., 2001; Lipipun *et al*., 2002; Farooqui *et al*., 2015).

3.8 Analytical Techniques

3.8.1. High-Performance Liquid Chromatography (HPLC)

HPLC is a most widely used analytical technique for separation and purification of various pure and impure compounds from mixture. Separation in HPLC is based on different structure and composition of molecules (Kupiec, 2004). It was introduced by Russian botanist Tswett in 1930 for the separation of plant pigments. Later, in early 1940s, Martin and Synge reintroduced liquid-liquid chromatography for separation of acetyl amino acids (Scott, 2003). HPLC uses high pressure liquid for separation of sample mixture through a column filled with sorbent.

Sorbent of HPLC is generally a granular material of solid particles *viz.* silica. Pressurized liquid is generally the mixture of solvents in different composition, i.e. water, methanol, acetonitrile; this is also referred as mobile phase. Temperature and composition of solvents play a major role in the separation process by affecting the interactions between sorbent and sample. These interactions can be of various types like hydrophobic, ionic, dipole-dipole or combination of them (Gerber *et al.,* 2004).

HPLC plays an important and critical role in the field of pharmaceutical, and food-industry manufacturing, research and analysis, since it is used to carry out qualitative and quantitative analysis of raw ingredients used in the manufacture of drugs. Moreover, the importance of HPLC use in these fields falls under the stringent regulations established by the U.S. Food and Drug Administration (FDA). This obligates all pharmaceutical companies to detect the quality of their products by using the HPLC before allowing them to sell it in the global market (Hassan, 2012). It is also used in disease diagnosis. Urine is a most common source for analyzing drug concentration in the human body.

Similarly, blood serum is used for detection of diseases (Sundstrom *et al*., 2013). HPLC is also used in solving legal matters like detection of doping agents, cocaine, opioids, BZDs, ketamine, drug metabolites; glucuronide conjugates, amphetamines, LSD, cannabis, and pesticides, etc. (Pelander *et al.,* 2003 and Kolmonen *et al.,* 2007). HPLC has also played a significant role in the study of chemical composition of bacterial cell wall**.** Initially, it was first applied for PG analysis by Bernd Glauner and the late Uli Schwarz (Abraham, 1984). *Caulobacter crescentus* was the first gram-negative bacteria in which first PG analysis study was performed by HPLC (Markiewicz *et al*., 1983). Thereafter PG analysis was performed in *E. coli* by similar group (Glauner *et al*., 1988). PG composition was first studied in MRSA isolates, with the help of HPLC by De Jonge group and they identified the functional role of PBP2a in cell wall synthesis (De Jonge and Tomasz, 1993).

3.8.2. Mass Spectrometry (MS)

Separation of compounds in mixture based on their structure and determination of structure of a compound is an essential and a never-ending quest in drug discovery, which can be fulfilled by techniques like mass spectroscopy. It is one of the most indispensable analytical techniques. As the name suggests, mass spectroscopy characterizes each of the components individually by providing information on the mass to charge ratio as well as on the structure of the analytes.

It works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios (Sparkman, 2000). It was originally discovered by Eugen Goldstein in 1886 and later improved by J.J. Thomson, who created mass spectrograph by reducing the pressure (Downard, 2007). Presently, mass spectrometry, in its myriad forms, has become a vital analytical tool in research and development of pharmaceutical industry (Cai *et al.,* 2002). Its application extent in the entire drug discovery

and development process from target identification to lead identification to animal and human testing. As well as detection of peptides and proteins is also an important aspect in laboratory medicine (Hoofnagle, 2010).

First time analytical technique of reversed-phase HPLC with MS was applied in the Gram- negative bacteria for the analysis of muropeptides (Glauner *et al*., 1988). While it was used to analyze the free cell wall stem peptides in *Streptococcus pneumoniae* (Bustos, *et al.*, 1987). HPLC and FABMS techniques was used to study the staphylococcal peptidoglycan generated from purified cell walls and confirmed the identity of pentaglycine chains as a main inter-peptide cross-bridge(De Jonge *et al*., 1993). *Staphylococcus* muropeptide composition and the role of *FemX* protein in addition of first glycine to the pentaglycine inter-peptide was also analyzed by the MS studies (Rohrer *et al*., 1999). Application of MS technique was also used with UPLC in peptidoglycan study of *E. coli* and *S. aureus* (Kuhner *et al.,* 2014).

4.1 Structure Databases

Structural databases used during this study include, both protein and Ligand databases. RCSB protein database www.rcsb.org was used for retrieval of 3-D *FEMA* protein file in the PDB data format (i.e. PDB id 1LRZA and Uniprot id P0A0A5) (Berman *et al.,* 2000; Benson *et al.,* 2002; Kim *et al*., 2012).

PubChem compound database www.ncbi.nlm.nih.gov/PCompound was used to retrieve complete library of catechins as ligands (Bolton *et al.,* 2008). Ligand files were downloaded in the 3-D SDF data format.

4.2. Identifications of Binding Sites

For identification of reliable binding sites in *FemA* protein, following *in silico* active site prediction tools were used:

4.2.1 SiteHound

SiteHound is an online binding site identification tool based on interaction of chemical probe and protein structure. Probe may vary depending on ligand, currently carbon and phosphate probe is available. Modeller, AutoGrid and EasyMIFs are other tools, which are used for identification of binding sites and their structural features for input PDB file. SiteHound is available at http://sitehound.sanchezlab.org (Hernandez *et al*., 2009)

4.2.2. QsiteFinder

It is an energy based-site detection tool, which uses interaction energy among Van der waal probe and protein to identify the energetically favored binding sites. Clustering of these sites is based on the spatial proximity, and then their ranking is based on the total sum of interaction energy of all sites within the cluster. It is online available at http://www.bioinformatics.leeds.ac.uk/qsitefinder (Laurie *et al.,* 2005).

4.2.3. Pocket Finder

Its algorithm is similar to LIgsite algorithm, as written by same author (Hendlich *et al.,* (1997). It measures the degree to which each grid point is hidden inside the protein (i.e. total seven directions, out of which three in the x, y and z direction and remaining four in cubic diagonals). It is available at http://www.modelling.leeds.ac.uk/ pocket finder/ (Duka, 2013 and Laurie *et al.,* 2005)

4.2.4 CASTp (Computed atlas of surface topography of proteins)

It is an online tool for computation of pockets on protein surface and voids buried inside the protein. Its algorithm is based on solvent accessible surface model (Richards' surface), and molecular surface model (Connolly's surface) CASTp web server is freely available World at http://www.cast.engr.uic.edu/ (Binkowski *et al*., 2003 and Dundas *et al*., 2006).

4.3 Docking Tools

4.3.1 Autodock Vina

Autodock Vina is a docking tool of Scripps Research Institute. It is available at http://vina.scripps.edu. It is the latest docking tool. It uses Pdbqt input files both for ligands as well as protein. Before docking there is a need to prepare a PDB or SDF data format files of proteins and ligands. In this study, used 1lrz.pdb target protein file as well as SDF ligand format files which were first converted to the Pdbqt data format, with the help of MGL tool. Along with format conversion, both protein and ligand files needed some more changes such as, deletion of all water molecules, addition of all hydrogen atoms, using no bond order method, and addition of charges.

However, in case of ligands, addition of all hydrogen atoms, including both polar and non-polar ones, addition of Gasteiger charges, merging of all non-polar charges, detection of aromatic carbons and rotatable bonds; settings of torsion forces are required.

Center grid coordinates used were 35.829×63.416 ×94.89 while size used for x, y, z were 76×102×20, respectively (Fig. 4.1). Spacing used was 1.000 at different exhaustiveness values (Trott and Olson, 2009). Parameters used are given in Config file (Fig. 4.2. Appendix) Command used by AutoDock Vina was following: /cygdrive /c/Program\ Files/The\Scripps\Research\Institute/ Vina/ Vina --config conf.txt --log log.txt

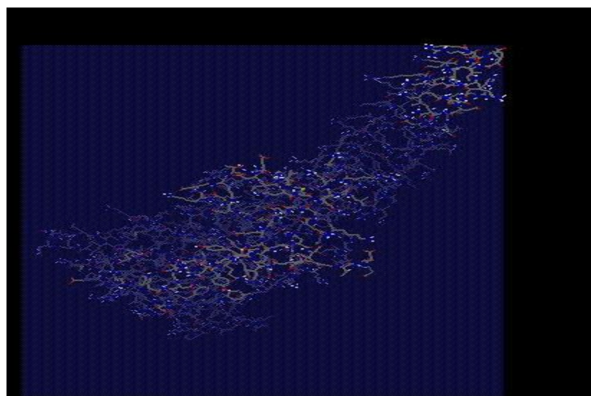

Fig. 4.1 Docking grid prepared by AutoDock Vina

4.3.2 Dock

Dock 6.5, a molecular docking program developed by UCSF Lab which is used in this study. It uses Mol2 data format files for both protein and ligand molecules. Both protein and ligand files were prepared by chimera software. In case of protein preparation, histidine protonation was done by selecting option of 'Unspecified determined by method' of dock prep module of chimera. While in case of ligand file's preparation, hydrogen atoms were added as well as AM1-bcc force field partial charges were assigned.

It has both options for using binding sites; user can define their own coordinates as well as calculates sphere clusters using Sphgen software. In this study, Sphgen was used for calculation of the binding site. It uses DMS software output file (receptor.ms) which consists of molecular surface of receptor, as well as INSPH file were used as input (Fig. 4.3. Appendix).

OUTSPH file generated by Sphgen consists of 78 clusters, out of these; cluster 1 having 111 spheres with their coordinates was used for generation of grid box file for 1LRZA protein (Fig. 4.4. Appendix). For visualization of spheres, first conversion of sphere file to PDB format was performed by show sphere program of dock by using showsph.in file (Fig. 4.5.

Appendix) and then visualization was done by chimera software. For generation of the grid box, two input files are needed showbox.in and grid.in (Fig. 4.6 and Fig. 4.7 Appendix).

Docking was performed with all ligand files using grid spacing of 0.3 A° and input file rigid.in (Fig. 4.8 Appendix). Orientations of docking are ranked on the basis of molecular mechanics-like scoring function i.e. Dock score in case of UCSF Dock (Lang *et al*., 2009).

4.4 *In silico* Interaction and Toxicity Studies

Protein residues forming hydrogen and hydrophobic interaction with the ligand molecule is further calculated by LigPlot+ software (Laskowski and Swindells, 2011)

4.4.1 Toxicological studies

To determine the toxic level of best *FemA* inhibitors, toxicity studies were performed by online tool, OSIRIS property explorer (http://www.organicchemistry.org/prog/peo/) during toxicological analysis various *in silico* tests were performed to detect the risk of mutagenicity, carcinogenicity, teratogenicity, irritants and reproductive effects.

These predictions depend on comparison between the pre-computed sets of structural moieties whose properties are already known with structural moieties of molecules loaded by the user on the server (Sander, 2014).

4.4.2. Lipinski filters

Lipinski filters or Lipinski rule of five distinguishes between drug like and non drug like molecules. It is also known as Pfizer's rule of five. It is a thumb rule to predict the probability of a chemical to be orally active drug in humans. According to this rule, molecular mass of the molecule should be < 500 Dalton, lipophilicity represented by LogP should be < 5, hydrogen bond donors (HBD) should be < 5; hydrogen bond acceptors (HBA) should be < 10 and molar refractivity (MR) should lie between 40-130. Molecule violating more than two rules is

considered as non-drug molecule. Lipinski filtering was performed by Lipinski filter tool of SCFBIO at IITDelhi server (Jayaram).

4.5 Culture repository

4.5.1 Procurement of test isolates

The test cultures of *S. aureus* used in various experimental studies were obtained from IMTECH, Chandigarh.

4.5.2 Maintenance of cultures

Three standard strains of *Staphylococcus aureus* viz. NCTC6571, MTCC737 and MTCC96 were used in this study. The bacterial isolates were preserved in 30% glycerol stock and frozen at -70°C. These were recovered and sub-cultured on Mueller Hinton broth (MHB) and Mueller Hinton Agar (MHA) (HiMedia, India).

Stocks of the cultures were maintained on MHA at 4°C throughout the study. Sodium salts of antibiotics Oxacillin and Penicillin were procured from HiMedia, Mumbai. CG used in the study was obtained from Sigma Aldrich and dissolved in DMSO prior to use.

4.5.3 Preparation of 0.5 McFarland turbidity standard (CLSI, 1997)

0.5 McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility test. It was prepared by addition of 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 the amber-colored bottle to prevent photo-degradation. The shelf life of 0.5 McFarland solutions in the tightly sealed amber-colored bottle is up to six months.

Inoculum Preparation: Culture activation was carried out by streaking it on a MH agar plate, followed by overnight incubation at 37°C. A single colony was pulled out from this plate and

transferred to MHB and incubated at 37°C for 18 h. Subsequently the culture was vortexed vigorously and compared with 0.5 McFarland standards against black stripes on white sheet to adjust visual turbidity. Sterile saline solution was used as diluents to adjust the visual turbidity of bacterial cultures prior to the test.

4.5.4: Growth curve studies of test isolate

Growth is an orderly increase to 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 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 curves studies of control and test isolate were carried out by Miles and Misra's method (Miles *et al*., 1938). Nutrient agar plates which were divided into equal sectors were used. Each sector is labeled with different time interval of 0h, 3h, 6h, 9h, 12h, 15h, 18h, 20h and 24h. Three plates were needed for each strain. Inoculum is serially diluted by hundred time dilution and at every three-hour interval 20 µl inoculum is dropped to the surface of agar plate at the respective intervals from a height of 2.5 cm.

Plates are left upright for dry for 15-20 min after drying inverted plates were incubated for 18–24 hours at 37°C. Each sector is observed for counting of colonies. Following equation was used for the calculation of number of CFU/ml from the original aliquot and calculation of generation time:

CFU per ml = Average number of colonies for a dilution x 100 x dilution factor.

Generation Time= t/ (3.3 log b/B)

Whereas,

t=Time of growth, B= Number of bacteria at the beginning of time interval,

b= Number of bacteria at the end of time interval

4.6 *mecA* and *femA* characterization of the test isolates

4.6.1 Isolation of bacterial genomic DNA

Total DNA was isolated from 2ml overnight grown bacterial culture in MHB, and then taken into microcentifuge tube. Centrifugation of microcentrifuge tubes, was performed for 5 min at 8000 rpm to obtain the bacterial pellet. The bacterial pellet was resuspended in 740 µl of the buffer (pH 8.0) and further 20 µl of (100mg/ml) lysozyme was added and mixture incubated for 5 min at room temperature.

After incubation, 40 µl of 10% Sodium Dodecyl Sulphate (SDS) and 3 µl of 20 mg/ml Proteinase K was added, mixed gently and incubated for 1 hr at 37 °C. Following this, first 100 µl of 5M NaCl and then 100 µl of 10% Cetyl trimethyl ammonium bromide (CTAB)-0.7 M NaCl solution were added and incubated the tubes for 10 min at 65 °C. After the incubation, 3 µl of 10mg/ml RNase was added and incubated at 37 °C for 15 min.

Equivalent to the volume of the microcentrifuge tubes, equal volume of mixture of chloroform and isoamylalcohol in the ratio of 24:1 was added, mixed and centrifuged for 10 min at 10,000 rpm. Upper aqueous phase of these microcentrifuge tubes was transferred to the new microcentrifuge tube and equal volume of phenol: chloroform: isoamyl alcohol mixture in the ratio of 25:24:1 was added, mixed and then centrifugation was repeated, for 10 min at 10,000 rpm.

The upper aqueous phase was then transferred to the new microcentrifuge tube and 0.6 volume of cold isopropanol was added, mixed gently and incubated for 30 min at 4 °C, for maximum DNA precipitation. After the incubation, obtain DNA pellet was collected by centrifugation for 15 min at 12,000 rpm. Washing of DNA was performed with 70% cold

ethanol and finally DNA was resuspend in 30 µl TE buffer and kept overnight at 4°C. Subsequently, DNA was stored at -20°C for future use.

4.6.2 PCR for the detection of *mecA and femA*

Two sets of primers were used for this study. Forward Primer of first pair of primers was derived from the *mecA* gene region, corresponded to the nucleotides 1283-1303 bp (5'AAA ATC GAT GGT AAA GGT TGG C 3') and the reverse primer was complimentary to nucleotide 1793-1814 bp (5' AGT TCT GCA GTA CCG GAT TTG C 3') (Murakami *et al.*,1991; Kobyashi *et al.*, 1994; Kampf *et al.*, 1999; Japoni *et al.*, 2004; Froberg *et al.*, 2004; Saiful *et al.*, 2006; Rallapali *et al.*, 2008; Li *et al.,* 2008; Mathew *et al.,* 2010; Islam *et al.*, 2011).

Forward primer of second set of primer was derived from the region of the *femA* gene, which was corresponded to nucleotides 1444-1463 bp (5'AAA AAA GCA CAT AAC AAG CG 3') and the reverse primer was complimentary to nucleotide 1556-1575 (5' GAT AAA GAA GAA ACC AGC AG 3') (Mehrota *et al.,* 2000; Froberg *et al.*, 2004; Pelisser *et al.,* 2009; Mathew *et al.*, 2010; Duran *et al.,* 2012).

For the first set of primers, polymerase chain reaction comprises of 50 ng 1 µl target DNA, 2 µl of 10X PCR buffer, 1 µl of 10mM dNTP mixture, 2 µl of 25mM $MgCl_{2,}$, 2 µl of 10µM each mecA forward and reverse primer. Final mixture reaction was supplemented with 2U of Taq DNA polymerase and sterile ddH_2o to make the final volume of 20 µl. PCR was carried out in automated thermocycler. After an initial denaturation of 5 min at 95°C, 30 cycles of amplification was carried out as follows : Denaturation at 94 °C for 30 s, Annealing at 55 °C for 30s, Extension at 72 °C for 30 s, followed by additional cycle of extension for 15 min at 72 °C to complete partial polymerizations.

Similarly, for second set of primers, the same amount of target DNA, 5 µl of 10X PCR buffer, 1 µl of 10mM dNTP mixture, 6 µl of 25mM $MgCl_2$, 1 µl of 10µM each *femA* forward and

reverse primer. Final mixture reaction was supplemented with 1U of Taq DNA polymerase and sterile ddh$_2$o to make the final volume of 50 µl.

PCR was carried out in same automated thermocycler. After an initial denaturation of 5 min at 95 °C, 30 cycles of amplification was carried out as follows : Denaturation at 94°C for 5 min, Annealing at 58°C for 2 min, Extension at 72°C for 3 min, followed by additional cycle of extension for 15 min. at 72 °C to complete partial polymerizations. Both amplified products were analyzed using 1.5% gel.

4.7 *In vitro* susceptibility studies of *S. aureus* isolates

4.7.1 *In vitro* microbroth dilution assay: determination of MIC

The minimal inhibitory concentration (MIC) of Penicillin, Oxacillin and CG was determined using *in vitro* microbroth dilution assay using 96 well titer plate's (EUCAST, 2003). Briefly, 16-20 h old culture suspensions of all the test microorganisms were adjusted with 0.5 McFarland solution using sterile physiological saline (0.85% NaCl) to achieve a final bacterial count of approximately 10^5 CFU/ml^{-1}of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 bacterial suspension was then added to 125 µl MHB in control (Ctrl) wells as well as test wells (rows 1-4, 5-8 and 9-12) respectively.

Well, number A1, B1 and C1 was Ctrl set for *S. aureus* NCTC 6571, similarly well number A5, B5 and C5 for *S. aureus* MTCC 737 and A9, B9, C9 for MTCC 96. Test wells for *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 were designated from D1-C4, D5 to C8, D9-C12. D-H wells with row id's 4, 8 and 12 were blanks (Fig. 4.7.1).

The titer-plates were then incubated for 3 h at 37 °C and then 25 µl of eight different concentrations of antibiotic was added in test wells (D1-C4) for *S. aureus* NCTC 6571 , (D5-C8) for *S. aureus* MTCC 737 and (D9-C12) for *S. aureus* MTCC 96 and 25 µl of sterile physiological saline was added in all Ctrl wells. Same conditions and template were used for all antibiotics

(Penicillin/Oxacillin) and the test compound in individual titer-plates. Antibiotic Oxacillin was tested for 500 µg/ml to 3.9 µg/ml concentrations, while Penicillin was tested for 8000-62.5 µg/ml CG was tested for 250-0.975 µg/ml. After 24 hr of incubation 30 µl of 0.02% Triphenyl Tetrazolium Chloride (TTC) was added as indicator in all wells and then MIC was observed. All concentrations were tested in triplicates (EUCAST 2000; EUCAST 2003; Andrews, 2001).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ctrl	C2s1	C5s1	C8s1	Ctrl	C2s2	C5s2	C8s2	Ctrl	C2s3	C5s3	C8s3
B	Ctrl	C3s1	C5s1	C8s1	Ctrl	C3s2	C5s2	C8s2	Ctrl	C3s3	C5s3	C8s3
C	Ctrl	C3s1	C6s1	C8s1	Ctrl	C3s2	C6s2	C8s2	Ctrl	C3s3	C6s3	C8s3
D	C1s1	C3s1	C6s1	Blank	C1s2	C3s2	C6s2	Blank	C1s3	C3s3	C6s3	Blank
E	C1s1	C4s1	C6s1	Blank	C1s2	C4s2	C6s2	Blank	C1s3	C4s3	C6s3	Blank
F	C1s1	C4s1	C7s1	Blank	C1s2	C4s2	C7s2	Blank	C1s3	C4s3	C7s3	Blank
G	C2s1	C4s1	C7s1	Blank	C2s2	C4s2	C7s2	Blank	C2s3	C4s3	C7s3	Blank
H	C2s1	C5s1	C7s1	Blank	C2s2	C5s2	C7s2	Blank	C2s3	C5s3	C7s3	Blank

Fig. 4.7.1: Microtitre plate template for *in vitro* microbroth dilution assay for determination of MIC (Ctrl-Control-uninoculated MH broth, C1-C8-Eight dilution of Concentration (highest to lowest), s1-*S. aureus* NCTC 6571, s2- *S. aureus* MTCC 737, s3- *S. aureus* MTCC 96)

4.7.2 Checkerboard Assay and FIC Index

Checkerboard assay was carried out to evaluate the synergistic potential of CG with β-lactam antibiotics (Penicillin and Oxacillin) in different concentrations for possible use as a formulation. The synergistic activity was further determined by the FIC index (Sato *et al.*, 2004). Five different dilutions 2MIC, MIC, 1/2MIC, 1/4MIC and 1/8MIC of antibiotics and test compound combinations were used in this experiment.

The concentration range of antibiotics for the checkerboard assay was between 1000 µg/ml to 62.5 µg/ml except for Penicillin which exhibited a MIC of 8000 µg/ml and hence tested from 16000 µg/ml to 1000 µg/ml. Similarly, concentration range for CG was 125 µg/ml

to 7.8 µg/ml for *S. aureus* NCTC 6571 and *S. aureus* MTCC 96 while, it was 250 µg/ml to 15.6 µg/ml for *S. aureus* MTCC 737.

Each well was dispensed with 125 µl MHB and 50 µl of culture inoculum having 10^5 CFU/ml. After 3 h of incubation, 12.5 µl of CG of the combination was serially diluted along the well number (A7-G9) in a set of fifteen consecutive wells and 12.5 µl of the second drug (either Oxacillin or Penicillin) was also diluted in a set of three consecutive wells along the well number (A7-B9, B10-C12, D1-E3, E4-F6, and F7-G9) of titer plate and then titer-plates were incubated for 24 h at 37°C under aerobic conditions (Fig. 4.7.2).

Subsequently, addition of 30 µl of TTC as an indicator in each well except blanks and incubated for another 1 h. The resulting combinations of two antibiotics were checked for MIC and complete assay was performed in triplicates (Bajaksouzian *et al*., 1997; Orhan *et al.,* 2005).

Further FIC Index was calculated using following equation: = $FIC_{(A)} + FIC_{(B)}$

Whereas,

$FIC_{(A)}$ = (MIC A in presence of B/MIC A alone)

$FIC_{(B)}$ = (MIC B in the presence of A/MIC B alone).

Correlations between FIC and the effect on the combination of antibacterial agents were indicated: Synergy ≤ 0.5, Partial synergy > 0.5–1, Indifference >1 to <2 and Antagonism ≥ 2 (EUCAST 2000; Lee *et al*., 2010)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ctrl1	Ctrl1	Ctrl1	Ctrl2	Ctrl2	Ctrl2	2MIC TC+ 2MIC (P/O)	2MIC TC+ 2MIC (P/O)	2MIC TC+ 2MIC (P/O)	2MIC TC+ MIC (P/O)	2MIC TC+ MIC (P/O)	2MIC TC+ MIC (P/O)
B	2MIC TC+ 1/2MIC (P/O)	2MIC TC+ 1/2MIC (P/O)	2MIC TC+ 1/2MIC (P/O)	2MIC TC+ 1/4MIC (P/O)	2 MIC TC+ 1/4MIC (P/O)	2MIC TC+ 1/4MIC (P/O)	2 MIC TC+ 1/8MIC (P/O)	2MIC TC+ 1/8MIC (P/O)	2MIC TC+ 1/8MIC (P/O)	MIC TC+ 2MIC (P/O)	MIC TC+ 2MIC (P/O)	MIC TC+ 2MIC (P/O)
C	MIC TC+ MIC (P/O)	MIC TC+ MIC (P/O)	MIC TC+ MIC (P/O)	MIC TC+ 1/2MIC (P/O)	MIC TC+ 1/2MIC (P/O)	MIC TC+ 1/2MIC (P/O)	MIC TC+ 1/4MIC (P/O)	MIC TC+ 1/4MIC (P/O)	MIC TC+ 1/4MIC (P/O)	MIC TC+ 1/8MIC (P/O)	MIC TC+ 1/8MIC (P/O)	MIC TC+ 1/8MIC (P/O)
D	1/2MIC TC + 2MIC (P/O)	1/2MIC TC + 2MIC (P/O)	1/2MIC TC + 2MIC (P/O)	1/2MIC TC + MIC (P/O)	1/2MIC TC + MIC (P/O)	1/2MIC TC +MIC (P/O)	1/2MIC TC+ 1/2MIC (P/O)	1/2MIC TC + 1/2MIC (P/O)	1/2MIC TC+ 1/2MIC (P/O)	1/2MIC TC+ 1/4MIC (P/O)	1/2MIC TC+ 1/4MIC (P/O)	1/2MIC TC+ 1/4MIC (P/O)
E	1/2MIC TC + 1/8MIC (P/O)	1/2MIC TC+ 1/8MIC (P/O)	1/2MIC TC + 1/8MIC (P/O)	1/4MIC TC+ 2MIC (P/O)	1/4MIC TC+ 2MIC (P/O)	1/4MIC TC+ 2MIC (P/O)	1/4MIC TC+ MIC (P/O)	1/4MIC TC+ MIC (P/O)	1/4MIC TC+ MIC (P/O)	1/4MIC TC+ 1/2MIC (P/O)	1/4MIC TC+ 1/2MIC (P/O)	1/4MIC TC+ 1/2MIC (P/O)
F	1/4MIC TC+ 1/4MIC (P/O)	1/4MIC TC+ 1/4MIC (P/O)	1/4MIC TC+ 1/4MIC (P/O)	1/4MIC TC+ 1/8MIC (P/O)	1/4MIC TC+ 1/8MIC (P/O)	1/4MIC TC+ 1/8MIC (P/O)	1/8MIC TC+ 2MIC (P/O)	1/8MIC TC+ 2MIC (P/O)	1/8MIC TC+ 2MIC (P/O)	1/8MIC TC+ MIC (P/O)	1/8MIC TC+ MIC (P/O)	1/MIC TC+ MIC (P/O)
G	1/8MIC TC+1/2 MIC (P/O)	1/8MIC TC+1/2 MIC (P/O)	1/8MIC TC+1/2 MIC (P/O)	1/8MIC TC+ 1/4MIC (P/O)	1/8MIC TC+1 /4MIC (P/O)	1/8MIC TC+ 1/4MIC (P/O)	1/8MIC TC+ 1/8MIC (P/O)	1/8MIC TC+ 1/8MIC (P/O)	1/8MIC TC+1/8 MIC (P/O)	Blank	Blank	Blank
H	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank

Fig. 4.7.2: Microtitre plate template for checkerboard assay for determination of FIC
(Ctrl 1-Control1, Ctrl 2-Control 2, TC-Test Compound, P- Penicillin, O-Oxacillin)

4.7.3 Time Kill Assay of Synergistic Formulations

The time kill assay was used to ascertain the efficacy to kill the bacteria in terms of reduction in the CFU count/ml. It was performed only in the cases exhibiting a synergistic effect of the CG with the antibiotic by the checkerboard method. Miles and Misra plate count method (Miles *et al.,* 1938) was used to ascertain the bacterial count of different treatments. Briefly, the method comprised of withdrawing a 20 µl aliquot form each test sample under aseptic conditions using sterile tips at different time intervals viz. 0, 6, 12, 18 and 24 h.

The aliquots were placed on a MH plate as a single drop and observed for the growth of bacteria as pin head colonies after incubation of 12-18 h at 37°C. These were counted to arrive to CFU/ml of the test sets at different time intervals. Decrease of ≥2-log $_{10}$ CFU count indicates synergy, decrease of ≤ 2-log $_{10}$ CFU count indicates additivity while the increase of ≤ 2-log 10 CFU count indicates antagonism with the combinations compared with control sample having no antibiotics (Barry *et al., 1999;* Peterson *et al*., 2006; Sopirala *et al*., 2010).

4.8 Evaluation of functional Expression of *FemA* protein

4.8.1 Peptidoglycan (PGN) cell wall extraction

2 ml 16-24h old culture suspension with OD $_{578}$ < 10 was taken to obtain 300 ml purified PGN. Suspension grown in the absence of antibiotics was used as control, while suspensions having presence of antibiotics and test compound (CG) in different formulations were taken as test samples. All Control and test samples were taken in a 2 ml microcentrifuge tube and centrifugation was performed for 5 min at 10,000 rpm. The pellets so obtained were resuspended in 1 ml 0.25% SDS solution in 0.1 M Tris/HCl (pH 6.8) and the resulting suspension was boiled for 20 min at 100 °C in a heating block.

Centrifugation of above suspension for 5 min at 10,000 rpm was performed, and then two times washing with 1.5 ml ddH_2O. After washing pellets they were resuspended in 1 ml

ddH_2O in respective microcentrifuge tube. Ultrasonication of all the samples performed for 2-4 min. Subsequently, 500 µl of solution B (15 µg/ml DNase and 60 µg/ml RNase in 0.1 M Tris/HCl, pH 6.8) was added and samples were incubated for 60 min, at 37 °C in a shaker. Subsequently, 500 µl of solution C (50 µg/ml trypsin in ddH_2O) was added and incubated for the additional 60 min under similar conditions.

For the inactivation of enzymes, suspension was boiled for 3 min at 100 °C in a heating block. Centrifugation of samples was performed for 5 min at 10,000 rpm and pellet obtained was washed with 1 ml ddH_2O. To release the WTA (Wall Teichoic Acid), pellet was resuspended in 500 µl of 1 M HCl and incubated for 4 h at 37 °C in a shaker. The suspension obtained was centrifuged for 5 min at 10,000 rpm. Pellet washing was performed with ddH_2O until pH between`5–6 was not achieved for the supernatant. Afterwards, pellet was resuspended in 100–250 µl digestion buffer (12.5 mM sodium dihydrogen-phosphate, pH 5.5) to obtained the OD_{578} of 3.0. Afterwards, 1/10th volume of the digestion buffer of mutanolysin solution (5.000 U/ml of mutanolysin in ddH_2O) was added.

The samples were then incubated for 16hrs at 37°C on a shaking incubator with 150 rpm. Inactivation of mutanolysin was performed by boiling the samples at 100 °C for 3 min, and subsequently the samples were centrifuged for 5 min at 10,000 rpm and extract the supernatant for future use and preserve the samples in -20 °C. (Kuhner *et al.,* 2014).

4.8.2 Derivatization of Samples

Before applying the sample to the nLC system, reduction of MurNAc to NAc-muraminitol is essential. 50 µl of reduction solution (10 mg/ml sodium borohydrate in 0.5 M borax in ddH2O at pH 9.0 was added. Then the samples were incubated at room temperature for 20 min. Reaction was stopped with addition of 10 µl phosphoric acid (98%). The resulting pH must lies between 2 and 3. Then analysis of samples was then carried out by nLC-MS (Table 4.8.2.1).

Table 4.8.2.1 nLC-MS samples with nLC-Ms code and their description

Sr. No.	Code	MS-CODE	Strains
1	6C	DIVYA_A1Z	*S. aureus* NCTC 6571
2	6FCO	DIVYA_B1Z	*S. aureus* NCTC 6571(Ox 62.5 µg/ml+ CG7.8 µg/ml)
3	6FCP	DIVYA_C1Z	*S. aureus* NCTC 6571(P 2000 µg/ml + CG 7.8 µg/ml)
4	7C	DIVYA_D1Z	*S. aureus* MTCC 737
5	7FCO	DIVYA_E1Z	*S. aureus* MTCC 737 (Ox 62.5µg/ml + CG 31.5 µg/ml)
6	9C	DIVYA_F1Z	*S. aureus* MTCC 96
7	9FCO	DIVYA_G1Z	*S. aureus* MTCC 96 (Ox 125 µg/ml + CG 7.8 µg/ml)

4.8.3 LC-MS Analysis

The reconstitution of peptides were performed in 0.1% formic acid in LC-MS grade water and subjected to nLC (Nano-Advance, Bruker, Germany) followed by identification by captive ion source (Bruker Daltonics) spray in Maxis-HD qTOF (Bruker, Germany) mass spectrometer (MS) with high mass accuracy and sensitivity. The peptides were enriched by nano trap column (Acclaim Pep Map, particle size-5µm, pore size-100Å, Thermo Scientific) and separated on an analytical column (KYA Tech, 0.1x150mm, 3µm particle size and 200Å pore size) with a flow rate of 400 nL/min.

Solvent A used was 100% water in 0.1% formic acid, and solvent B used was 100% acetonitrile in 0.1% formic acid. 57 min gradient was used which ranges from 5-95% acetonitrile. The mass spectrometer was operated in data-dependent acquisition mode for MS acquisition. The precursor ion scan MS spectra was carried out at m/z ranges of 400-2200 were acquired in the Q-TOF with resolution R = 75 000.

Chapter 5: Results

5.1 Structure Databases

RCSB-PDB database was used to download the structure format files of *FemA* protein (i.e. 1LRZA) in PDB format. *FemA* (Aminoacyltransferase *FemA*) protein sequence is 426 amino acids long and having molecular weight of 50.03 KDa. It is translated by *femA* gene. Classification databases, SCOP, CATH and INTERPRO have divided *FemA* protein into three domains but with different names.

According to the SCOP classification, first domain (d1lrza1) is made up of all α-helices having long α-hairpin fold and belong to tRNA-binding arm and named as Methicillin resistance protein *FemA* probable tRNA-binding arm (65 residue long). Second (d1lrza2) and third domain(d1lrza3) are made up of Alpha and β-proteins (a+b) having Acyl-CoA N-acyltransferases fold and belong to Acyl-CoA N-acyltransferases family and named as *FemXAB* nonribosomal peptidyltransferases (165 and 170 residue respectively) with [TaxId: 1280](Fig.5.1).

Similarly, CATH database has classified into three domains, first (1lrza01) and second (1lrza02) domain both are made up of α and β proteins and both belong to aminopeptidase topology and homology while 3rd domain (1lrza03) is made up of only α-proteins and belongs to Methane Monooxygenase Hydroxylase; Chain G, domain one topology and homology.

InterPro database has classified three domains into tRNA-binding arm [IPR010978] *FemABX* peptidyl transferase [IPR003447] Acyl-CoA N-acyltransferase [IPR016181]. In this way sequentially it has three domains Acyl-CoA N-acyltransferase, *FemABX* peptidyl-transferase and tRNA-binding arm while structurally it has two domains *FemXAB* non-ribosomal peptidyl-transferases and Methicillin resistance protein *FemA* probable tRNA-binding arm. *FemA* protein is divided into two domains, domain 1 is divided into two sub-domains 1A

represented in yellow color (residues 1–110, 129–144, and 396–401) and sub-domain 1B, represented in magenta color (residues 145–166, 189–245, and 308–395) with some additional secondary structure elements, which has been shown in green color (residues 111–128, 167–188, and 402–412) and Domain 2, is represented in blue color (residues 246–307)(Fig. 5.1). According to Dictionary of Protein Secondary Structure (DSSP) *FemA* protein, are 42% helical (16 helices made up of 180 residues) and 23% β-sheets (18 strands made up of 100 residues) and rest residues either made turn or bends (Fig 5.1).

PubChem compound database www.ncbi.nlm.nih.gov/PCompound was used to retrieve complete library of catechins as ligands. PubChem database consists of total 116 Ligand files out of which 14 ligands are present only in 2-D structure; there 3-D structures were not present. Further from these 102 ligand files which are present in 3-D SDF data format can be used for docking. Among 102 catechin isomers; only 15 ligand molecules are commercially attainable. Due to non-availability of these 87 catechin isomers chemically they are filtered out further. Details of ligands are given in Table 5.2, which were used throughout this study.

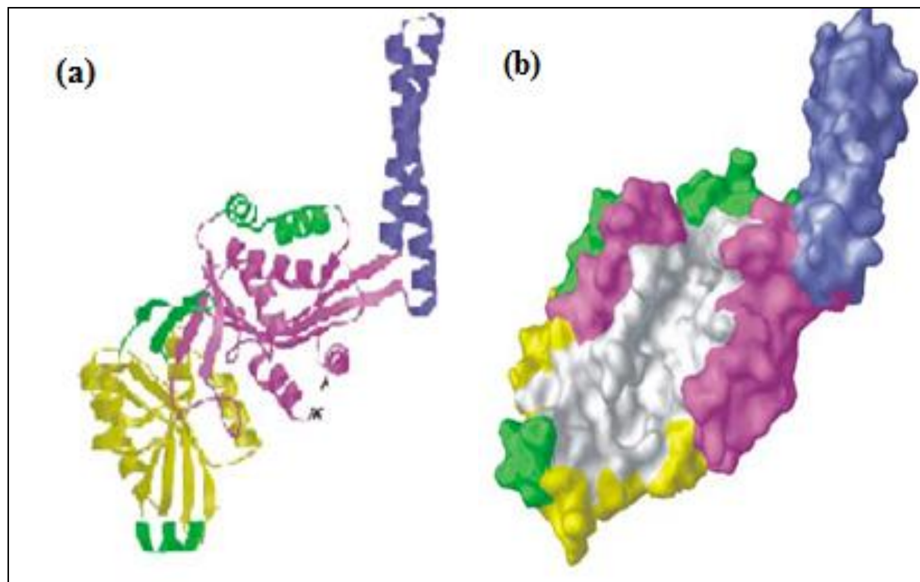

Fig. 5.1 Structure of *S.aureus* in two domains (a) Cartoon View (b) Surface View having L-shaped (white) substrate binding domain

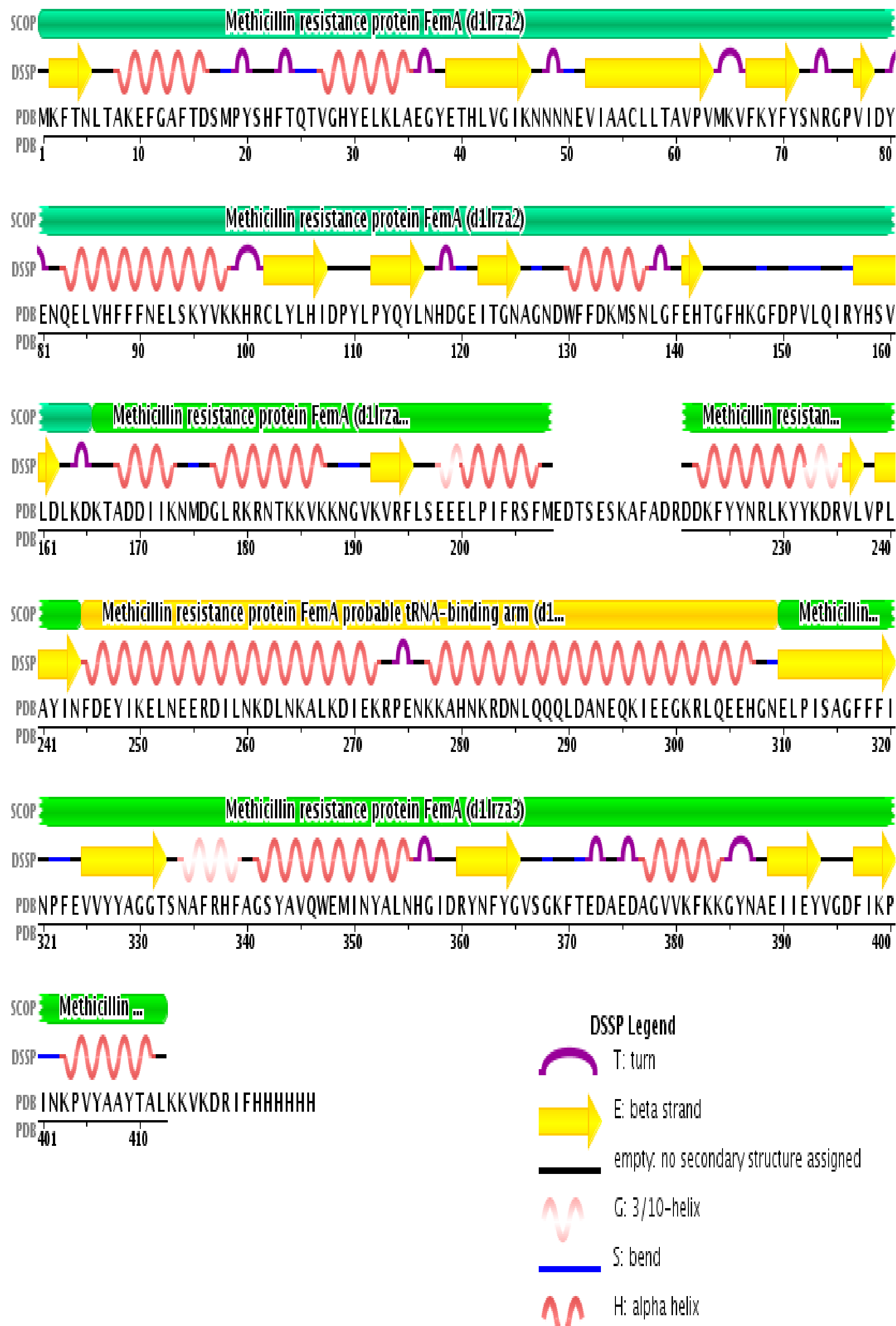

Fig. 5.1 *S. aureus FemA* protein sequence and secondary structure in three domains

Table 5.1 Summary of Catechin isomers

Sr. No.	CID	Name	IUPAC	MW & MF
1	6419835	(-)-Catechin gallate; AC1O4WEY ...	[(2S,3R)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate	MW: 442.372320 g/mol MF: C22H18O10
2	5276454	(-)-Catechin gallate; XEG	[(2R,3S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate	MW: 442.372320 g/mol MF: C22H18O10
3	107905	Epicatechin gallate; (-)-Epicatechin gallate; (-)-Epicatechin-3-gallate ...	[(2R,3R)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate	MW: 442.372320 g/mol MF: C22H18O10
4	182232	(+)-Epicatechin; 35323-91-2; ent-Epicatechin ...	(2S,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: C15H14O6
5	12309507	L-Epicatechin; 490-46-0	(2S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: C15H14O6
6	73160	(-)-Catechin; (-)-Catechol; CATECHIN, ALPHA ...	(2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: C15H14O6
7	289	catechol; pyrocatechol; 1,2-benzenediol ...	benzene-1,2-diol	MW: 110.110640 g/mol MF: C6H6O2
8	122738	Procyanidin B2; Procyanidol B2; Proanthocyanidin B2	(2R,3R)-2-(3,4-dihydroxyphenyl)-8-[(2R,3R,4R)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-3,4-dihydro-2H-chromen-4-yl]-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 578.520240 g/mol MF: C30H26O12
9	9064	CATECHIN; Cianidanol; (+)-Catechin	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-	MW: 290.268060 g/mol MF:

			triol	$C_{15}H_{14}O_6$
10	65064	(-)-Epigallocatechin gallate; EGCG; Epigallocatechin gallate; 989-51-5; Epigallocatechin 3-gallate; Epigallocatechin-3-gallate	[(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate	MW: 458.371720 g/mol MF: $C_{22}H_{18}O_{11}$
11	107957	Catechin hydrate;(+)-Catechin Hydrate	(2R,3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol;hydrate	MW: 308.283340 g/mol MF: $C_{15}H_{16}O_7$
12	367141	(-)-epicatechingallate; Epicatechin gallate; NSC636594; CHEMBL328085;	[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate	MW: 442.372320 g/mol MF: $C_{22}H_{18}O_{10}$
13	1203	DL-Catechin; NSC81746; L-Epicatechin	2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: $C_{15}H_{14}O_6$
14	72276	Epicatechin; (-)-Epicatechin; L-Epicatechin; (-)-Epicatechol	(2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: $C_{15}H_{14}O_6$
15	255538	Epi-Catechol; L-Epicatechin; Epicatechol, (-)-; NSC81161; (.+-.)-Epicatechol; AC1L5SMJ;	(3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol	MW: 290.268060 g/mol MF: $C_{15}H_{14}O_6$

5.2. Identification of Binding Sites

5.2.1. SiteHound

SiteHound tool predicted 10 pockets in the 3-D structure of *FemA* protein, while; only three pockets are found in L-domain (Table 5.2 and Fig. 5.3). It predicted all active site residues of the pocket, energy, volume of pocket and X, Y, Z coordinates of pocket.

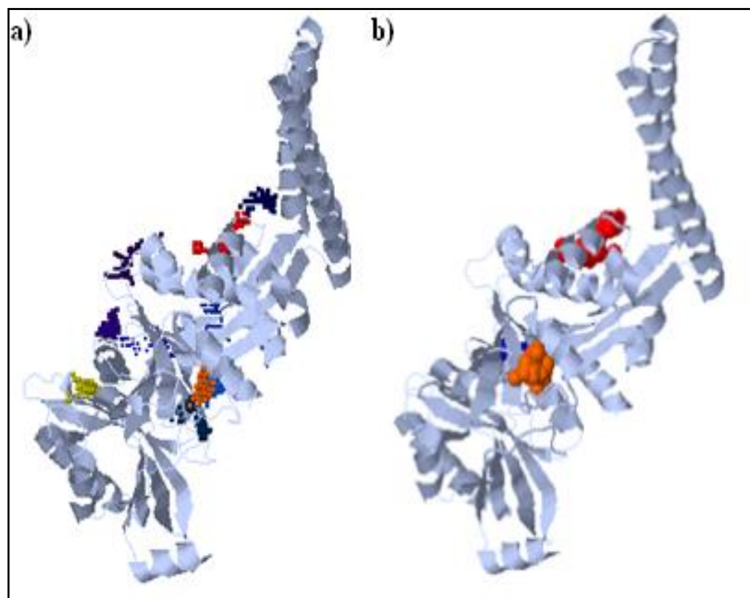

Fig. 5.3 Active sites predicted by SiteHound tool (a) total active sites (b) active sites in L-domain

Table 5.2 Details of active sites in L-domain as predicted by SiteHound tool

Pocket No.	Active site Residues	Energy	Volume	Coordinates
1	186-193, 195,242,251, 344,347,348,351	-775.97	67.00	42.811 80.746 95.313
2	48,119,367-372, 376, 377,380,381,384	-615.26	48.00	47.162 55.021 108.973
7	9,12-13,15-16,23,26-28,31, 231-235,319,321	-499.14	45.00	21.583 65.272 84.445

5.2.2. Q-SiteFinder

Q-SiteFinder tool predicted 10 pockets in the 3-D structure of *FemA* protein, while only five pockets are found in L-domain (Table 5.3 and Fig. 5.4). It predicted all active site residues of the pocket, volume of pocket and minimum and maximum X, Y, Z coordinates of the pocket.

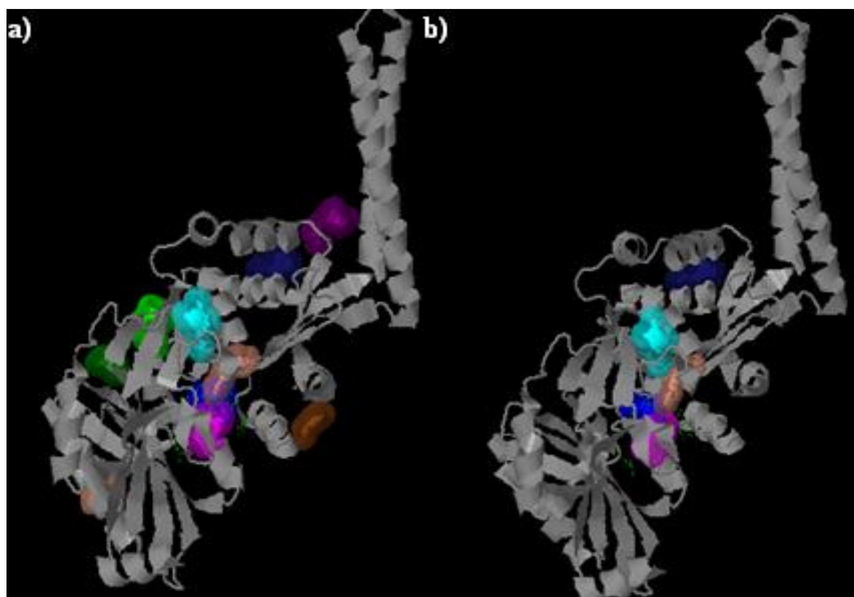

Fig. 5.4 Active sites predicted by Q-SiteFinder tool (a) total active sites (b) active sites in L-domain

Table 5.3 Details of active sites in L-domain as predicted by Q-SiteFinder tool

Pocket No.	Active site residues	Minimum Coordinates	Maximum Coordinates	Site Volume (Cubic Å)
1	118-120	40,48,99	54,64,116	202
4	12,16,23,26-29,31,231-235,321	15,57,75	32,73,91	186
5	183,186-187,191-193,343-345, 347- 348,351-352,355	31,72,89	50,86,103	185
7	22,23-24, 29,33,71,73-74, 108,154,224,228,232,319,328	39,55,69	49,72,82	184
8	149,155,327-330	35,55,69	49,72,82	147

5.2.3. PocketFinder

PocketFinder tool predicted 10 pockets in the 3-D structure of *FemA* protein, while only five pockets are found in L-domain (Table 5.4 and Fig. 5.5). It predicted all active site residues of the pocket, volume of pocket and minimum and maximum X, Y, Z coordinates of the pocket similar to the Q-SiteFinder.

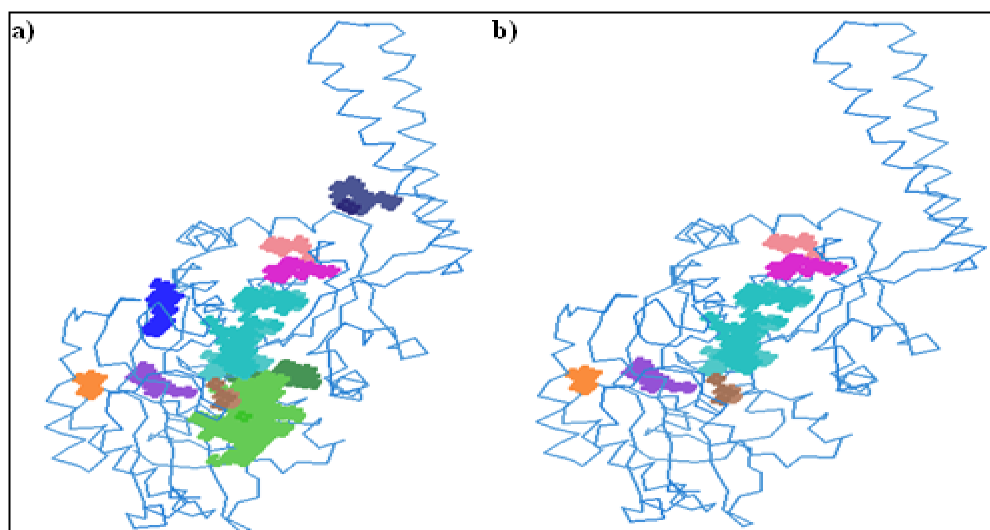

Fig. 5.5 Active sites predicted by PocketFinder tool (a) total active sites (b) active sites in L-domain

Table 5.4 Details of active sites in L-domain as predicted by PocketFinder tool

Pocket No.	Active site residues	Minimum Coordinates	Maximum Coordinates	Site Volume (Cubic Å)
1	74,149,154,158,314-316,327-332, 337,342,345-346	29,49,86	53,72,104	543
6	18-20,74-78,80,109-112	14,48,91	26,60,106	96
7	313,332-333,336-337,340-343	45,63,89	57,75,103	75
8	183,186-187,343-344,347-348	37,71,93	48,83,106	55
9	80-81,111,128,130-131	14,44,101	23,56,111	44
10	150-155	32,44,85	41,54,97	38

5.2.4. CASTp

CASTp tool predicted binding sites based on probe radius. 1.6 Å probe radius was used in this study, and total 38 pockets were predicted in the 3-D structure of *FemA* protein, while only 10 pockets are found in L-domain (Table 5.5 and Fig. 5.6). It predicted all active site residues of the pocket, their area and volume.

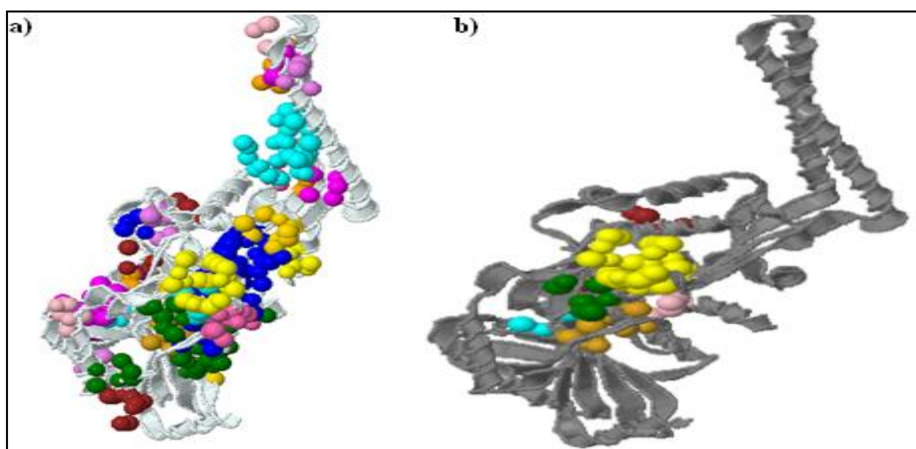

Fig. 5.6 Active sites predicted by CASTp tool (a) total active sites (b) active sites in L-domain

Table 5.5 Details of active sites in L-domain as predicted by CASTp tool

Pocket No.	Active site residues	Area	Volume (Cubic Å)
10	20,76,80,110	41.8	24.1
13	74,154-155,158,364	53.8	33.5
17	348,351,352,355	14.2	10.6
23	367-370,380	50.9	32
25	116,120,147,157,368	32.2	33.4
26	74,108,154,156,395,396	57.8	39.8
31	12,15,16,26,27,235	46.4	41.1
32	15,16,21,22,25,26,321,322	101.2	75.4
33	23,26,28,232,235,321	92.3	68.7
35	118,119,369-372, 374,377,380,381,384	150.7	191.4

5.3.Docking Tools

5.3.1 AutoDock Vina

Table 5.6 Results predicted by AutoDock Vina

Sr.No.	CID	Name	B.A(kcal/mol)
1	6419835	(-)-Catechin gallate; AC1O4WEY	-8.7
2	5276454	(-)-Catechin gallate; XEG	-8.5
3	107905	Epicatechin gallate; (-)-Epicatechin gallate; (-)-Epicatechin-3-gallate	-7.6
4	182232	(+)-Epicatechin; 35323-91-2; ent-Epicatechin	-8.1
5	12309507	L-Epicatechin; 490-46-0	-8.1
6	73160	(-)-Catechin; (-)-Catechol; CATECHIN,	-8.7
7	289	Catechol; pyrocatechol; 1,2-benzenediol	-4.1
8	122738	Procyanidin B2; Procyanidol B2; Proanthocyanidin B2	-8.2
9	9064	CATECHIN; Cianidanol; (+)-Catechin	-8.1
10	65064	(-)-Epigallocatechin gallate; EGCG; Epigallocatechin gallate; Epigallocatechin 3-gallate; Epigallocatechin-3-gallate	-8
11	107957	Catechin hydrate;(+)-Catechin Hydrate	-7.7
12	367141	(-)-epicatechingallate; Epicatechin gallate	-7.4
13	1203	DL-Catechin; NSC81746; L-Epicatechin	-7.3
14	72276	Epicatechin; (-)-Epicatechin; L-Epicatechin; (-)-Epicatechol	-7.3
15	255538	Epi-Catechol; L-Epicatechin; Epicatechol, (-)-; NSC81161; (.+-.)-Epicatechol; AC1L5SMJ;	-7.3

Docking of *FemA* protein with Catechin and Epicatechin analogues have given results in the terms of binding affinity (B.A) in kcal/mol with AutoDock Vina, which ranges from -4.1 to -8.7. Least binding affinity was given by Catechol with Pubmed id 289, and maximum binding affinity was given by Catechin and Catechin Gallate with Pubmed id 73160 and 6419835 respectively (Table 5.6). Best conformation of Catechin Gallate and Catechin predicted by AutoDock Vina software (Figs. 5.7. (a and b) in cartoon view and (c and d) in surface view).

5.3.2. Dock

Table 5.7. Results predicted by Dock

Sr. No.	CID	Name	Grid_ score	Grid _vdw	Grid _es	Int_ eng
1	6419835	(-)-Catechin gallate; AC1O4WEY ...	-45.1953	-39.0488	-6.14644	5.720728
2	5276454	(-)-Catechin gallate; XEG	-44.6425	-39.1664	-5.47615	5.836292
3	107905	Epicatechin gallate; (-)-Epicatechin gallate; (-)-Epicatechin-3-gallate ...	-43.8231	-30.2942	-13.529	6.482648
4	182232	(+)-Epicatechin; 35323-91-2; ent-Epicatechin ...	-37.0576	-30.2171	-6.84049	3.409987
5	12309507	L-Epicatechin; 490-46-0	-34.4991	-28.832	-5.6671	2.239769
6	73160	(-)-Catechin; (-)-Catechol; CATECHIN, ALPHA ...	-34.034	28.39634	-5.63766	3.145205
7	289	catechol; pyrocatechol; 1,2-benzenediol ...	-20.6871	-16.2985	-4.38866	0.055943
8	122738	Procyanidin B2; Procyanidol B2;		Out of Grid		
9	9064	CATECHIN; Cianidanol; (+)-Catechin		Out of Grid		
10	65064	(-)-Epigallocatechin gallate; EGCG; Epigallocatechin gallate; Epigallocatechin 3-gallate;		Out of Grid		
11	107957	Catechin hydrate;(+)-Catechin Hydrate		Out of Grid		
12	367141	(-)-epicatechingallate; Epicatechin gallate;		Out of Grid		
13	1203	DL-Catechin; NSC81746; L-Epicatechin		Out of Grid		
14	72276	Epicatechin; (-)-Epicatechin; L-Epicatechin; (-)-Epicatechol		Out of Grid		
15	255538	Epi-Catechol; L-Epicatechin; Epicatechol, (-)-; NSC81161; (.+-.)-Epicatechol;		Out of Grid		

UCSF Dock gives results in output_scored.mol2 file which contains the geometric coordinates as well as the summary of interaction energies of binding for the docked ligand poses in the order of their grid score. The best pose would have the most favorable grid score. Among the

docked ligands, only seven ligands have shown dock score from range of -45 to -20 (Table 5.7), while rest ligands were out of grid. (-)Catechin Gallate with cid 6419835 has given best dock score of -45.19 similar to the results given by AutoDock Vina tool. Best conformation of the best ligand, i.e. Catechin Gallate has been shown in Fig. 5.8.

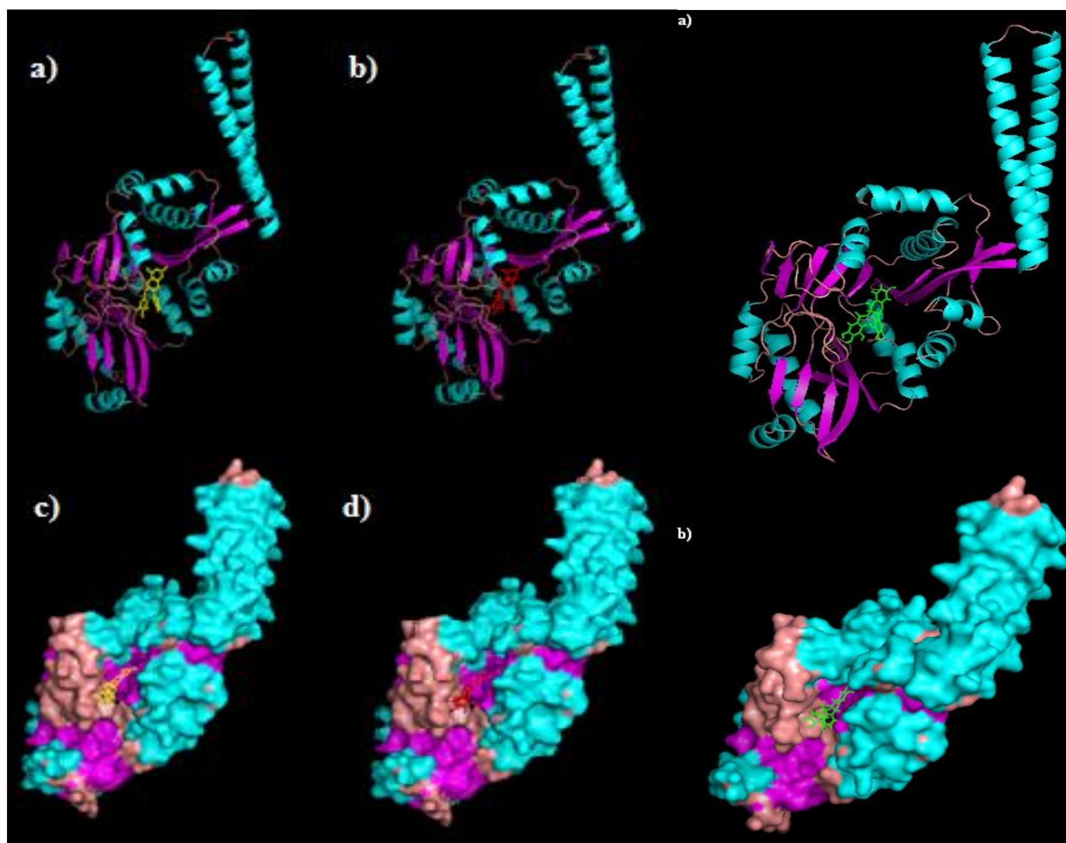

Fig. 5.7 AutoDock Vina results :*Fem*A protein complex with ligand Catechin Gallate and Catechin in cartoon view (a and b respectively). Similarly (c and d)in surface view.

Fig. 5.8 UCSF Dock results: *Fem*A protein complex with ligand Catechin Gallate in (a) cartoon view (b) surface view.

5.4 Interaction and Toxicity studies

Interaction and toxicity profiling of ligands was performed, for the selection of the ligand for *in vitro* studies. All hydrogen and hydrophobic interactions between *FemA* protein and Catechin Gallate models predicted by both software's (Fig. 5.9.) LigPlot+ has predicted four hydrogen bonds with amino acid (Asp 150, Gly330, Phe 363, Lys 383) and two hydrogen

bonds (Gln 154, Lys 383) in AutoDock Vina complex and Dock's complex. Out of these only, Lys 383 is a common hydrogen bond between both complexes.

While nine hydrophobic interactions are found in AutoDock Vina complex with amino acid, Phe 149, Leu 153, Gln 154, Ile 155, Tyr 327, Tyr 328, Ala 329, Tyr 364, Gly 368 and six hydrophobic interactions are found in Dock's complex with amino acid Phe 149, Ile 155, Tyr 327, Tyr 328, Ala 329, Gly 368. Hydrophobic interaction predicted in Dock's complex is common among both complexes.

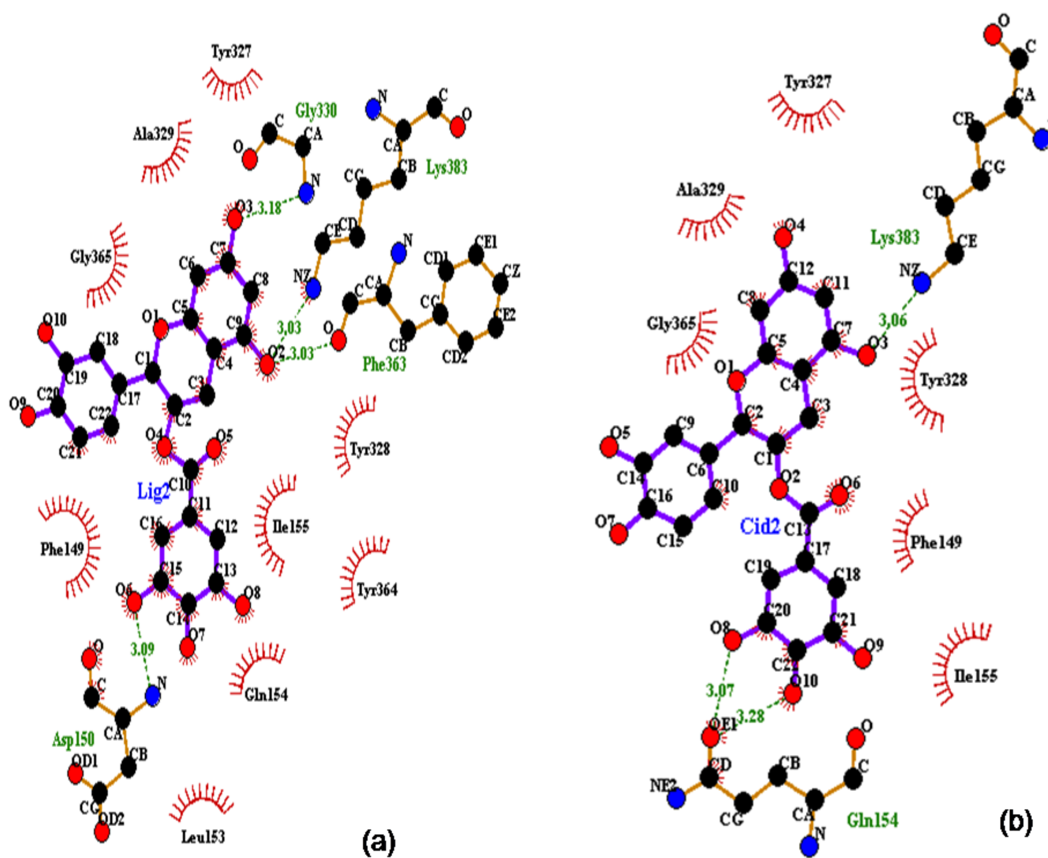

Fig. 5.9 Interaction of Catechin Gallate with different residues of *FemA* protein given by LIGPLOT+ tool (a) AutoDock Vina result file (b) Dock result file.

5.4.2 Toxicological studies

Table 5.8 TOXICITY RESULTS PREDICTED BY OSIRIS

Pubchem CID	Name	Drug likeliness	Mutagenicity risk	Tumorigenicity Risk	Irriating effects	Reproductive Effects	Drug Score
6419835	(-)-Catechin gallate	2.81 (s=0.94)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.74
73160	(-)-Catechin; Catechin L-form	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
5276454	(-)-Catechin gallate;	2.81 (s=0.94)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.74
122738	Procyanidin B2;	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.534
182232	(+)-Epicatechin; 35323-91-2; ent-Epicatechin	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
12309507	L-Epicatechin;	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
9064	CATECHIN; Cianidanol; (+)Catechin;	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
65064	EGCG;(-)-Epigallocatechin gallate	1.57 (s=0.83)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.69
107957	Catechin hydrate; (+)Catechin hydrate	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
107905	Epicatechin gallate; (-)-Epicatechin gallate;	2.81 (s= .94)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.74
367141	(-)-Epicatechin gallate; NSC636594	1.39 (s=0.8)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.68
1203	DL-Catechin; NSC81746; L-Epicatechin	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
72276	Epicatechin; (-)-Epicatechin	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
255538	L-Epicatechin; Epicatechol,	1.92 (s=0.87)	NO (score=1)	NO (score=1)	NO (score=1)	NO (score=1)	0.86
289	catechol; pyrocatechol	"-2.25 (s=0.09)	Yes (score=0.6)	Yes (score=0.6)	Yes (score=0.6)	NO (score=1)	0.112

5.4.3 Lipinski filters

Table 5.9 LIPINSKI RESULTS

Pubchem CID	Name	Mol. Wt.	HBA	HBD	LogP	MR	Number of LV
6419835	(-)-Catechin gallate	442	3	7	3	96.3	1
73160	(-)-Catechin; Catechin L-form	290	1	5	0.52	64.6	0
5276454	(-)-Catechin gallate; (2R,3S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2Hchromen-3-yl 3,4,5-trihydroxybenzoate	442	3	7	3	96.3	1
122738	Procyanidin B2; Procyanidol B2;	578	2	10	0.9	108	2
182232	(+)-Epicatechin; 35323-91-2; ent-Epicatechin	290	1	5	0.52	64.6	0
12309507	L-Epicatechin;	290	1	5	0.52	64.6	0
9064	CATECHIN; Cianidanol; (+)-Catechin;D-catechin;(+) cyanidanol	290	6	5	0.4	NA	0
65064	EGCG;(-)-Epigallocatechin gallate	458	11	8	1.2	NA	2
107957	Catechin hydrate;(+)-Catechin Hydrate	308	7	6	NA	NA	1
107905	Epicatechin gallate; (-)-Epicatechin gallate; (-)-Epicatechin-3-gallate;	442	3	7	3	96.3	1
367141	(-)-Epicatechin gallate; NSC636594	442	10	7	1.5	NA	1
1203	DL-Catechin; NSC81746; L-Epicatechin	290	6	5	0.4	NA	0
72276	Epicatechin; (-)-Epicatechin	290	1	5	0.52	64.6	0
255538	L-Epicatechin; Epicatechol,	290	1	5	0.52	64.6	0
289	catechol;pyrocatechol	110	0	2	1.1	29.8	1

Mol. Wt.:Molecular Weight, HBA:Hydrogen Bond Acceptor,HBD: Hydrogen Bond Donor, MR: Molecular Refractivity, Number of LV: Number of Lipinski's Rule Violation

Table 5.8 indicates the results of toxicological studies, which indicates the toxic or side effects of these chemicals as drug. Toxicity or their side effect is indicated by the score. If the score is less than five for any test that indicates failure of that ligand in that test. As given in Table 5.8, there is only one ligand, which has shown very poor results in toxicity studies, i.e. catechol with cid 289. Seven ligands, i.e. cid (73160, 182232, 12309507, 9064, 1203, 72276, 255538) which are not violating any Lipinski rule of five, which indicates the fitness of physical and chemical properties of these ligands to work as inhibitors (Table 5.9).

While out of rest eight, two, ligands, i.e. (122738, 65064) are violating two Lipinski rules, which indicate that they are unsuitable for the medical purpose but five ligands (6419835, 5276454, 107957, 107905, 367141, 289) which are violating one rule of Lipinski rules can be used. Catechin Gallate with cid 6419835 is falling in this category that's why it can be used for further *in vitro studies.*

5.5 Test Cultures

Standard cultures were used for experimental analysis, *S. aureus* NCTC 6571 was used as control and *S. aureus* MTCC 737 and *S. aureus* MTCC 96 were used as resistant strains.

5.5.1: Growth curve studies of test isolates

Growth curve study was performed by Miles and Misra's method. The mean lag phase of the cultures ranged between 1.5-2 h, followed by a log_{10} phase which extended up to 8-9 h and then entered into stationary phase until 16-18 h, and this was followed by death phase up to 24 h (Fig. 5.10). Generation time observed for *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 were 44.03, 45.30 and 46.91 min respectively. Average generation time of isolates observed was 45.41 min in the log phase.

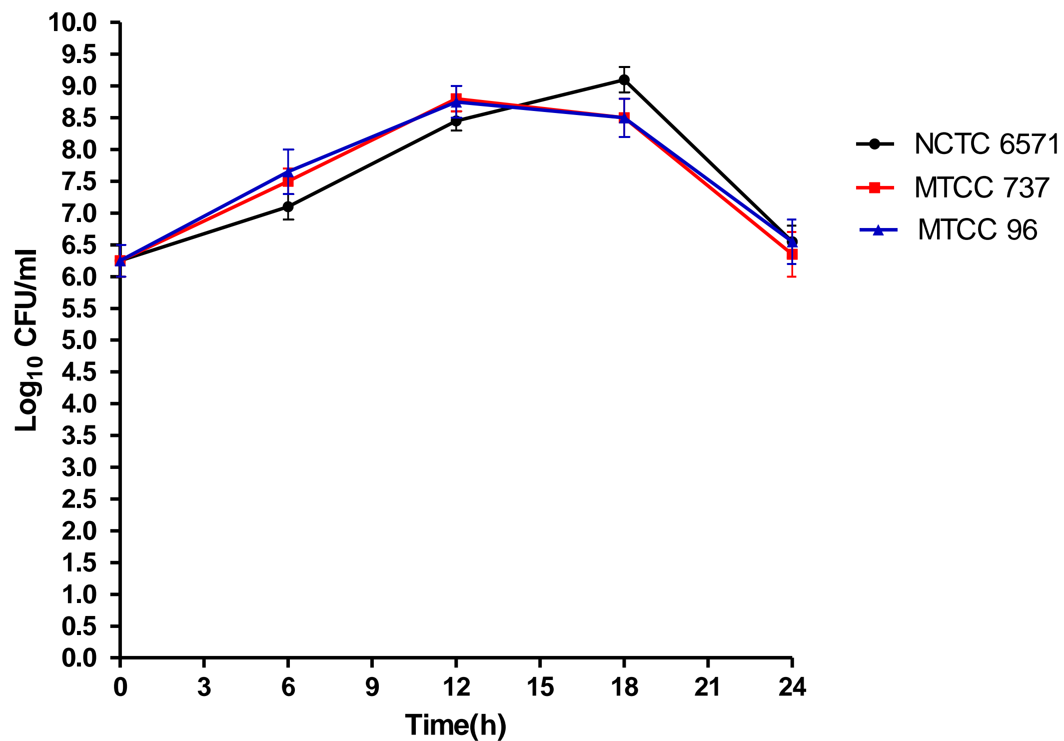

Fig. 5.10 Growth Curve of *S. aureus* strains.
Data represented are mean ± standard deviation of three replicates.

5.6 *mecA* and *femA* characterization of the test isolates

Molecular characterization of all three strains of *S. aureus* was performed for both *mecA* and *femA* gene primers. Two isolate *S. aureus* MTCC 737 and *S. aureus* MTCC 96 both were found to be *mecA* positive using primer corresponded to forward primer mA1-5′AAA ATC GAT GGT AAA GGT TGG C 3', and the reverse primer was complimentary to mA2-5′ AGT TCT GCA GTA CCG GAT TTG C 3′.

The amplified product was observed at 533bp (Fig. 5.11 a). *S. aureus* NCTC 6571 has shown negative results. While all three strains of *S. aureus* has shown the positive results with *femA* gene primers corresponded to forward primer (5′AAA AAA GCA CAT AAC AAG CG 3'), and the reverse primer was complimentary to 5′ GAT AAA GAA GAA ACC AGC AG 3′. DNA fragment of 132 bp was amplified from DNA of all three strains (Fig. 5.11b).

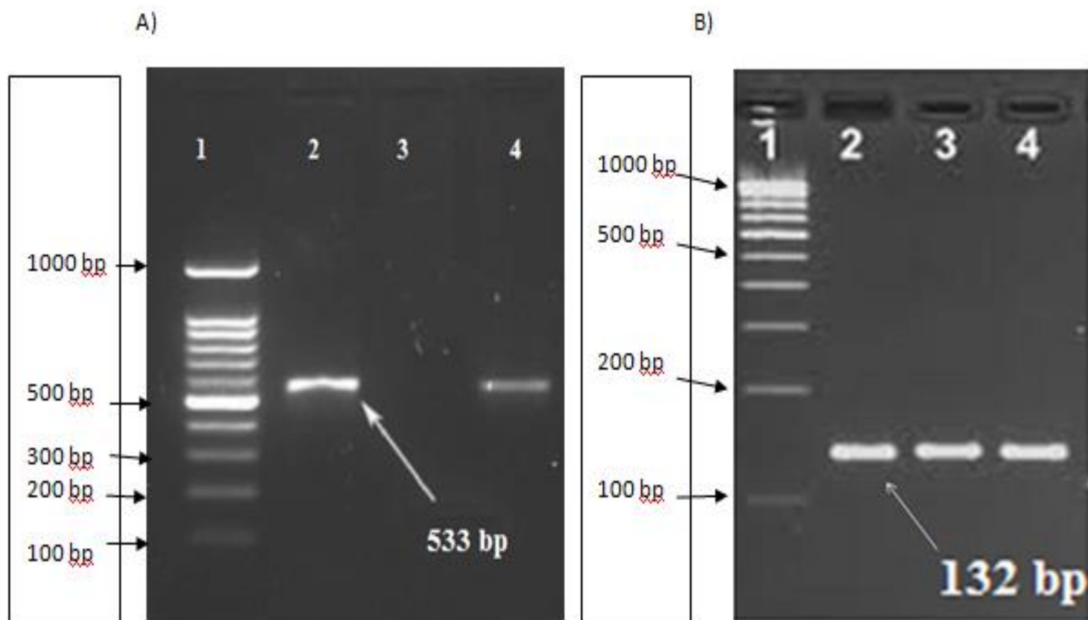

Fig 5.11(a) Screening of staphylococcal isolates for the presence of *mecA* gene with 533 bp amplicon and (b) *femA* gene with 132 bp amplicon as a marker for methicillin resistance

Lane coding of gels
1-Ladder, 2- *S. aureus* MTCC 737, 3- *S. aureus* NCTC 6571, 4- *S. aureus* MTCC 96

5.7 Evaluation of microbial activity of *S. aureus* isolates

5.7.1 *In vitro* microbroth dilution assay: determination of MIC

According to the NCCLS and EUCAST guidelines for microbroth dilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibit the growth within the organism as detected with the naked eye.

MIC observed for antibiotic Oxacillin for all three *S. aureus* isolates is 500 µg/ml (Fig. 5.12) and similarly, for antibiotic Penicillin is 8000 µg/ml for isolates (Fig.5.13). While MIC observed for CG alone is very less as compared to the MIC of β-lactam antibiotics, values observed were 62.5 µg/ml for *S. aureus* NCTC 6571 and *S. aureus* MTCC 96 and 125 µg/ml for *S. aureus* MTCC 737 isolates (Fig. 5.14). All experiments are performed in triplicate. Mean values of MIC with their standard deviation are given in Table 5.10.

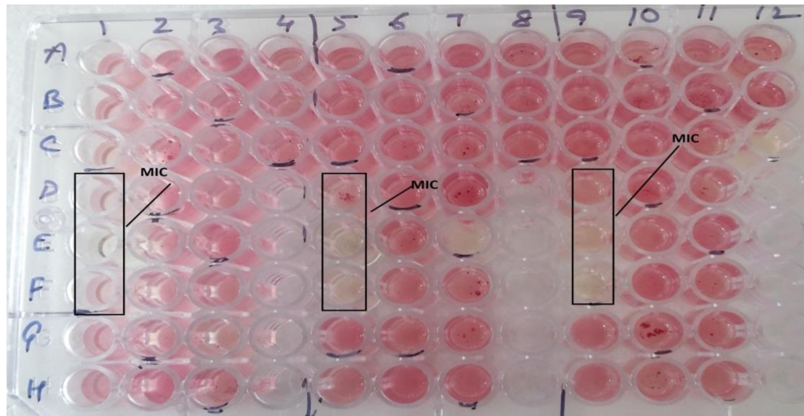

Fig 5.12 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with antibiotic Oxacillin

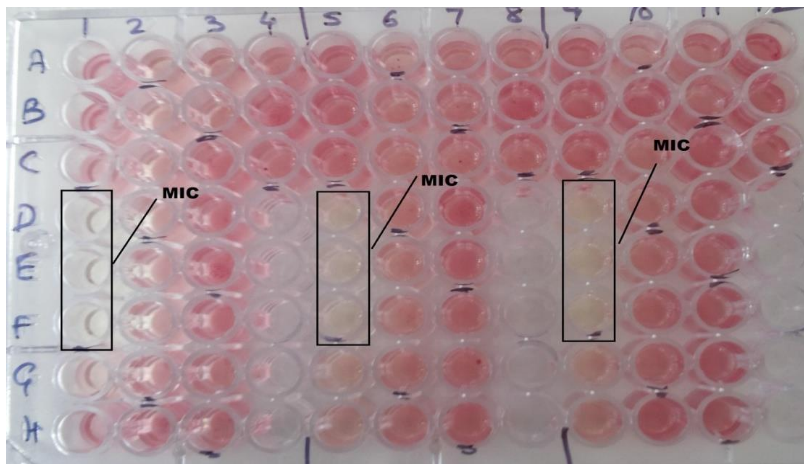

Fig 5.13 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with antibiotic Penicillin

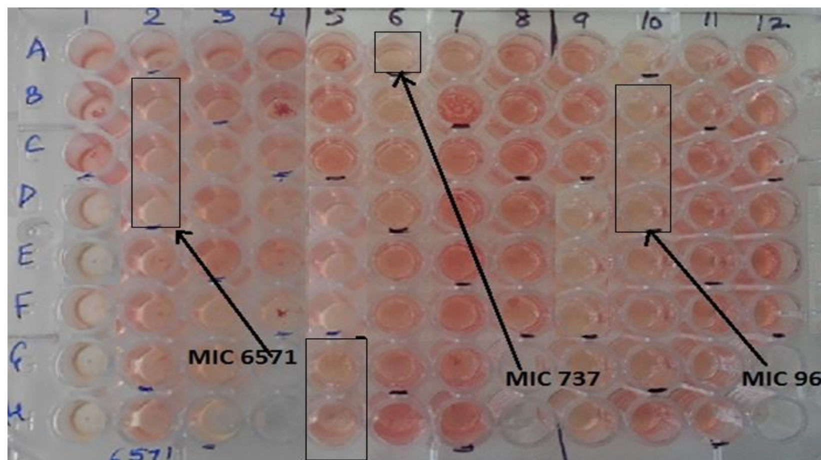

Fig 5.14 Microbroth dilution results of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96 with test compound CG

Table 5.10. MIC of *S. aureus* all three strains w.r.t. antibiotic Oxacillin, Penicillin and test compound CG

***S. aureus* Strain**	**MIC (µg/ml)**		
	Oxacillin	**Penicillin**	**Catechin Gallate**
NCTC 6571	500 ± 0	8000 ± 0	62.5 ± 0
MTCC 737	500 ± 0	8000 ± 0	125 ± 0
MTCC 96	500 ± 0	8000 ± 0	62.5 ± 0

The data presented are mean ± SD of three replicates.

5.7.2 CHECKERBOARD : FIC INDEX Assay

For checkerboard assay and FIC index calculations, antibiotic Oxacillin was tested for 1000-62.5 µg/ml concentration, with 125-7.8 µg/ml concentration of CG for *S. aureus* NCTC 6571 and *S. aureus* MTCC 96, while in case of *S. aureus* MTCC 737, same concentration of Oxacillin was used with 250-15.6 µg/ml concentration of CG in twenty-five combinations for each strain in triplicates. As a result, it was observed that in *S. aureus* NCTC 6571 strain, all twenty-five combinations of Oxacillin with CG have shown no growth in any well, which indicates that 1/8th MIC of CG can reduce the MIC of Oxacillin eight times (Fig. 5.15 and 5.16). Synergy was observed at 62.5 µg/ml concentration of Oxacillin with 15.6-7.8 µg/ml concentration of CG, while with same concentration of Oxacillin, partial synergy, additive and indifference, effect was observed with 31.25, 62.5 and 125 µg/ml concentration of CG (Table 5.11).

Similarly, in case of *S. aureus* MTCC 737 and MTCC 96 1/8th MIC of CG can reduce the MIC of Oxacillin to eight times (Fig. 5.17-5.20). In case of MTCC 737, synergy was observed at 62.5 µg/ml concentration of Oxacillin with 31.25 µg/ml concentration of CG, and 125 µg/ml concentration of Oxacillin with 15.62 µg/ml concentration of CG while with 62.5 µg/ml concentration of Oxacillin, partial synergy, additive and indifference, effect was observed with 62.5, 125 and 250 µg/ml concentration of CG (Table 5.12). In *S. aureus* MTCC 96 strain, synergy was observed at 125 µg/ml concentration of Oxacillin with 15.6-7.8 µg/ml concentration of CG, while with 62.5 µg/ml concentration of Oxacillin, partial synergy and indifference, effect was observed with 31.25, 62.5 and 125 µg/ml concentration of CG (Table

5.13). In comparison to Oxacillin, Penicillin MIC was very high. Therefore, antibiotic Penicillin was tested for 16000-1000 µg/ml concentration, with 125-7.8 µg/ml concentration of CG for *S. aureus* NCTC 6571 and *S. aureus* MTCC 96, while in case of *S. aureus* MTCC 737, similar concentration of Penicillin was used with 250-15.6 µg/ml concentration of CG in twenty-five combinations for each strain in triplicates. Checkerboard results of Penicillin with CG for *S. aureus* NCTC 6571 strain have shown that 1/8th MIC of CG reduced the MIC of Penicillin up to 1/4th (Fig. 5.21 and 5.22). While it is reduced in *S. aureus* MTCC 737 and *S. aureus* MTCC 96 strains where 1/8th MIC of CG can reduce the Penicillin MIC only up to half (Fig. 5.23-5.26).

Synergy was observed only in *S. aureus* NCTC 6571 strain, at 2000 µg/ml concentration of Penicillin with 7.8 µg/ml concentration of CG (Table 5.14). Partial synergy and indifference, were observed at 4000 µg/ml concentration of Penicillin with 15.62 µg/ml concentration of CG or 2000 µg/ml concentration of Penicillin with 31.25 µg/ml concentration of CG and 4000 µg/ml concentration of Penicillin with 62.5 µg/ml concentration of CG or 2000 µg/ml concentration of Penicillin with 125 µg/ml concentration of CG respectively in *S. aureus* NCTC 6571 (Table 5.14). In *S. aureus* MTCC 737, partial synergy, additive and indifference were observed at 4000 µg/ml concentration of Penicillin with 15.62-31.25 µg/ml concentration of CG; 4000 µg/ml concentration of Penicillin with 62.5 µg/ml concentration of CG; 2000 µg/ml concentration of Penicillin with 125 µg/ml concentration of CG and 1000 µg/ml concentration of Penicillin with 250 µg/ml concentration of CG respectively (Table 5.15). Similarly, in *S. aureus* MTCC 96 partial synergy, additive and indifference were observed at 4000 µg/ml concentration of Penicillin with 7.8-15.6 µg/ml concentration of CG; 4000 µg/ml concentration of Penicillin with 31.25 µg/ml concentration of CG and 4000 µg/ml concentration of Penicillin with 62.5 µg/ml concentration of CG or 2000 µg/ml concentration of Penicillin with 125 µg/ml concentration of CG respectively (Table 5.16). Significant FIC

values for synergy were observed between (0.25-0.38) for two formulations of Oxacillin and CG in all three strains, i.e. *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96, whereas just one formulation of Penicillin and CG for *S. aureus* NCTC 6571 (Table 5.17). Based on these results, four the most synergistic FIC index valued formulations were identified; and used for further experimental analysis.

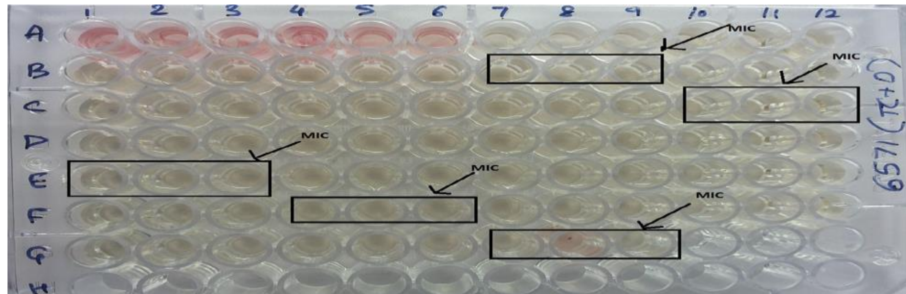

Fig. 5.15 Checkerboard result of *S. aureus* NCTC 6571 for five different concentration of antibiotic Oxacillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	125CG +1000 O	125CG +1000 O	125CG +1000 O	125CG + 500 O	125 CG+ 500 O	125CG + 500 O
B	125CG + 250 O	125CG + 250 O	125CG + 250 O	125CG + 125 O	125CG+ 125 O	125CG + 125 O	125CG + 62.5 O	125CG + 62.5 O	125CG + 62.5 O	62.5 CG + 1000 O	62.5 CG + 1000 O	62.5 CG + 1000 O
C	62.5 CG + 500 O	62.5 CG + 500 O	62.5 CG + 500 O	62.5 CG + 250 O	62.5 CG + 250 O	62.5 CG + 250 O	62.5 CG + 125 O	62.5 CG + 125 O	62.5 CG + 125 O	62.5 CG + 62.5 O	62.5 CG + 62.5 O	62.5 CG + 62.5 O
D	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 250 O	31.25 CG + 250 O	31.25 CG + 250 O	31.25 CG + 125 O	31.25CG + 125 O	31.25 CG + 125 O
E	31.25 CG + 62.5 O	31.25 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG + 1000 O	15.6CG + 1000 O	15.6 CG + 1000 O	15.6 CG + 500 O	15.6 CG + 500 O	15.6CG + 500 O	15.6 CG + 250 O	15.6 CG + 250 O	15.6 CG + 250 O
F	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O	7.8 CG + 1000 O	7.8 CG + 1000 O	7.8 CG + 1000 O	7.8 CG + 500 O	7.8 CG + 500 O	7.8 CG + 500 O
G	7.8 CG + 250 O	7.8 CG + 250 O	7.8 CG + 250 O	7.8 CG + 125 O	7.8 CG + 125 O	7.8 CG + 125 O	7.8 CG + 62.5 O	7.8 CG + 62.5 O	7.8 CG + 62.5 O			
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Fig. 5.16 MIC of *S. aureus* NCTC 6571 with antibiotic Oxacillin and test compound CG from checkerboard method

Table 5.11 FIC INDEX of *S. aureus* NCTC 6571 with antibiotic Oxacillin and test compound CG

	125CG + 62.5 O	62.5 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG + 62.5 O	7.8 CG + 62.5 O
FIC CG	2	1	0.5	0.25	0.125
FIC O	0.125	0.125	0.125	0.125	0.125
FIC I	2.125	1.125	0.625	0.375	0.25
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	PARTIAL SYNERGY	SYNERGY	SYNERGY

CG:Catechin Gallate, O:Oxacillin, I:Index

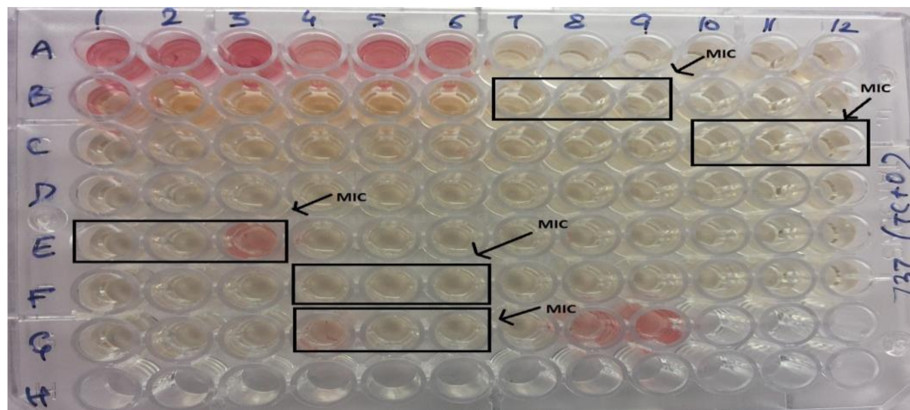

Fig. 5.17 Checkerboard result of *S. aureus* MTCC 737 for five different concentration of antibiotic Oxacillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	250 CG +1000 O	250 CG +1000 O	250 CG +1000 O	250 CG + 500 O	250 CG + 500 O	250 CG + 500 O
B	250 CG + 250 O	250 CG + 250 O	250 CG + 250 O	250 CG + 125 O	250 CG + 125 O	250 CG + 125 O	250 CG + 62.5 O	250 CG + 62.5 O	250 CG + 62.5 O	125 CG +1000 O	125 CG +1000 O	125 CG +1000 O
C	125 CG + 500 O	125 CG + 500 O	125 CG + 500 O	125 CG + 250 O	125 CG + 250 O	125 CG + 250 O	125 CG + 125 O	125CG + 125 O	125 CG + 125 O	125 CG + 62.5 O	125 CG + 62.5 O	125 CG + 62.5 O
D	62.5 CG +1000 O	62.5 CG +1000 O	62.5 CG +1000 O	62.5 CG +500 O	62.5 CG +500 O	62.5 CG + 500 O	62.5 CG + 250 O	62.5G + 250 O	62.5 CG + 250 O	62.5 CG + 125 O	62.5 CG + 125 O	62.5 CG + 125 O
E	62.5 CG + 62.5 O	62.5 CG + 62.5 O	62.5 CG + 62.5 O	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 250 O	31.25 CG + 250 O	31.25 CG + 250 O
F	31.25 CG + 125 O	31.25 CG + 125 O	31.25 CG + 125 O	31.25 CG + 62.5 O	31.25 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG + 1000 O	15.6 CG + 1000 O	15.6 CG + 1000 O	15.6 CG + 500 O	15.6 CG + 500 O	15.6 CG + 500 O
G	15.6 CG + 250 O	15.6 CG + 250 O	15.6 CG + 250 O	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O			
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Fig. 5.18 MIC of *S. aureus* MTCC 737 with antibiotic Oxacillin and test compound CG from checkerboard method

Table 5.12 FIC INDEX of *S. aureus* MTCC 737 with antibiotic Oxacillin and test compound CG

	250 CG + 62.5 O	125 CG + 62.5 O	62.5 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG+125O
FIC CG	2	1	0.5	0.25	0.125
FIC O	0.125	0.125	0.125	0.125	0.25
FIC I	2.125	1.125	0.625	0.375	0.375
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	PARTIAL SYNERGY	SYNERGY	SYNERGY

CG:Catechin Gallate, O:Oxacillin, I:Index

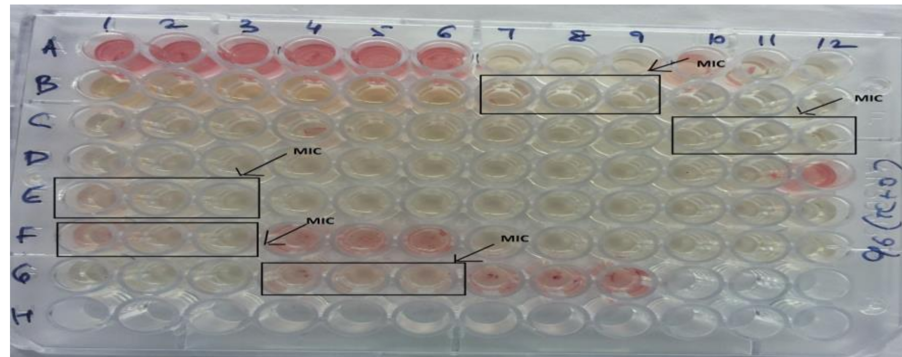

Fig.5.19 Checkerboard result of *S. aureus* MTCC 96 for five different concentration of antibiotic Oxacillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	125CG +1000 O	125CG +1000 O	125CG +1000 O	125CG + 500 O	125CG+ 500 O	125CG + 500 O
B	125CG + 250 O	125CG + 250 O	125CG + 250 O	125CG + 125 O	125CG+ 125 O	125CG + 125 O	125CG + 62.5 O	125CG + 62.5 O	125CG + 62.5 O	62.5 CG + 1000 O	62.5 CG + 1000 O	62.5 CG + 1000 O
C	62.5 CG + 500 O	62.5 CG + 500 O	62.5 CG + 500 O	62.5 CG + 250 O	62.5 CG + 250 O	62.5 CG + 250 O	62.5 CG + 125 O	62.5 CG + 125 O	62.5 CG + 125 O	62.5 CG + 62.5 O	62.5 CG + 62.5 O	62.5 CG + 62.5 O
D	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 1000 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 500 O	31.25 CG + 250 O	31.25 CG + 250 O	31.25 CG + 250 O	31.25 CG + 125 O	31.25CG + 125 O	31.25 CG + 125 O
E	31.25 CG + 62.5 O	31.25 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG + 1000 O	15.6CG + 1000 O	15.6 CG + 1000 O	15.6 CG + 500 O	15.6 CG + 500 O	15.6CG + 500 O	15.6 CG + 250 O	15.6 CG + 250 O	15.6 CG + 250 O
F	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 125 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O	15.6 CG + 62.5 O	7.8 CG + 1000 O	7.8 CG + 1000 O	7.8 CG + 1000 O	7.8 CG + 500 O	7.8CG + 500 O	7.8 CG + 500 O
G	7.8 CG + 250 O	7.8 CG + 250 O	7.8 CG + 250 O	7.8 CG + 125 O	7.8 CG + 125 O	7.8 CG + 125 O	7.8 CG + 62.5 O	7.8 CG + 62.5 O	7.8 CG + 62.5 O			
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Fig. 5.20 MIC of *S. aureus* MTCC 96 with antibiotic Oxacillin and test compound CG from checkerboard method

Table.5.13 FIC INDEX of *S. aureus* MTCC 96 with antibiotic Oxacillin and test compound CG

	125 CG + 62.5 O	62.5 CG + 62.5 O	31.25 CG + 62.5 O	15.6 CG + 125 O	7.8 CG+125 O
FIC CG	2	1	0.5	0.25	0.125
FIC O	0.125	0.125	0.125	0.25	0.25
FIC I	2.125	1.125	0.625	0.5	0.375
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	PARTIAL SYNERGY	SYNERGY	SYNERGY

CG:Catechin Gallate, O:Oxacillin, I:Index

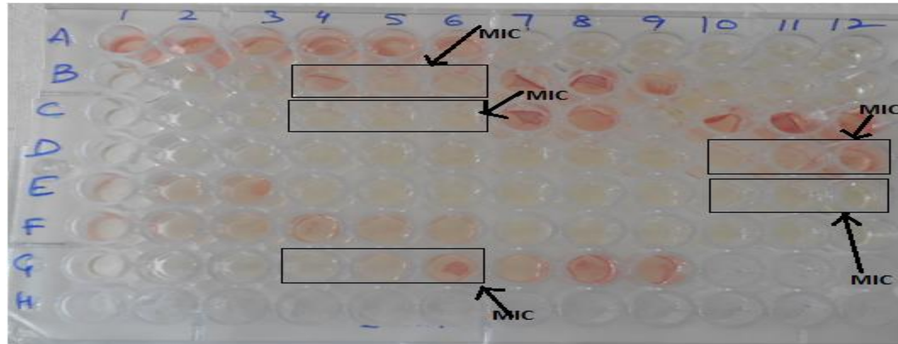

Fig. 5.21 Checkerboard result of *S. aureus* NCTC 6571 for five different concentration of antibiotic Penicillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	125CG +16000 P	125CG +16000 P	125CG +16000 P	125CG + 8000 P	125CG + 8000 P	125CG + 8000 P
B	125CG + 4000 P	125CG + 4000 P	125CG + 4000 P	125CG + 2000 P	125CG + 2000 P	125CG + 2000 P	125CG + 1000 P	125CG + 1000 P	125CG + 1000 P	62.5 CG + 16000 p	62.5 CG + 16000 p	62.5 CG + 16000 p
C	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 2000 P	62.5 CG + 2000 P	62.5 CG + 2000 P	62.5 CG + 1000 P	62.5CG + 1000 P	62.5 CG + 1000 P
D	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 4000 P	31.25 CG + 4000 P	31.25 CG + 4000 P	31.25 CG + 2000 P	31.25 CG + 2000 P	31.25 CG + 2000 P
E	31.25 CG + 1000 P	31.25 CG + 1000 P	31.25 CG + 1000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 8000 P	15.6 CG + 8000 P	15.6 CG + 8000 P	15.6 CG + 4000 P	15.6 CG + 4000 P	15.6 CG + 4000 P
F	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 1000 P	15.6 CG + 1000 P	15.6 CG + 1000 P	7.8 CG + 16000 P	7.8 CG +16000 P	7.8 CG + 16000P	7.8 CG + 8000P	7.8 CG + 8000 P	7.8 CG +8000 P
G	7.8CG /+ 4000 +P	7.8 CG + 4000 P	7.8 CG + 4000 P	7.8 CG + 2000 P	7.8 CG + 2000 P	7.8CG + 2000 P	7.8 CG + 1000 P	7.8 CG + 1000 P	7.8 CG + 1000 P			
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Fig. 5.22 MIC of *S. aureus* NCTC 6571 with antibiotic Penicillin and test compound CG from checkerboard method

Table 5.14 FIC INDEX of *S. aureus* NCTC 6571 with antibiotic Penicillin and test compound CG

	125CG + 2000 P	62.5 CG + 4000 P	31.25 CG + 2000 P	15.6 CG + 4000 P	7.8 CG + 2000 P
FIC CG	2	1	0.5	0.25	0.125
FIC P	0.25	0.5	0.25	0.5	0.25
FIC I	2.25	1.5	0.75	0.75	0.375
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	PARTIAL SYNERGY	PARTIAL SYNERGY	SYNERGY

CG:Catechin Gallate, P:Penicillin, I:Index

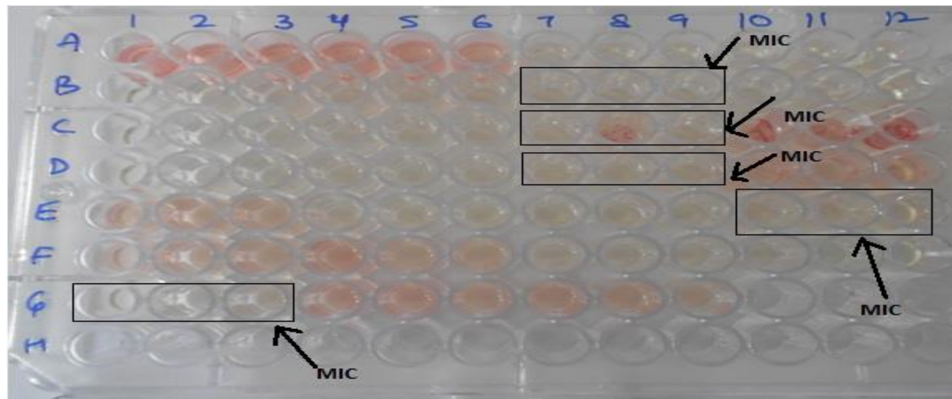

Fig. 5.23 Checkerboard result of *S. aureus* MTCC 737 for five different concentration of antibiotic Penicillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	250CG +16000 P	250CG +16000 P	250C+1600 0 P	250CG + 8000 P	250CG + 8000 P	250CG + 8000 P
B	250CG + 4000 P	250CG + 4000 P	250CG + 4000 P	250CG + 2000 P	250CG + 2000 P	250CG + 2000 P	250CG + 1000 P	250CG + 1000 P	250CG + 1000 P	125 CG + 16000 p	125 CG + 16000 p	125 CG + 16000 p
C	125 CG + 8000 P	125 CG + 8000 P	125 CG + 8000 P	125 CG + 4000 P	125 CG + 4000 P	125 CG + 4000 P	125 CG + 2000 P	125 CG + 2000 P	125 CG + 2000 P	125 CG + 1000 P	125 CG + 1000 P	125 CG + 1000 P
D	62.5 CG + 16000 P	62.5 CG + 16000 P	62.5 CG + 16000 P	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 2000 P	62.5 CG + 2000 P	62.5 CG + 2000 P
E	62.5 CG + 1000 P	62.5 CG + 1000 P	62.5 CG + 1000 P	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 4000 P	31.25 CG + 4000 P	31.25 CG + 4000 P
F	31.25 CG + 2000 P	31.25 CG + 2000 P	31.25 CG + 2000 P	31.25 CG + 1000 P	31.25 CG + 1000 P	31.25 CG + 1000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 8000 P	15.6 CG + 8000 P	15.6 CG + 8000 P
G	15.6 CG + 4000 P	15.6 CG + 4000 P	15.6 CG + 4000 P	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 1000 P	15.6 CG + 1000 P	15.6 CG + 1000 P			
H												

Fig. 5.24 MIC of *S. aureus* MTCC 737 with antibiotic Penicillin and test compound CG from checkerboard method

Table 5.15 FIC INDEX of *S. aureus* MTCC 737 with antibiotic Penicillin and test compound CG

	250 CG + 1000 P	125 CG + 2000 P	62.5 CG+4000 P	31.25 CG + 4000 P	156 CG+4000 P
FIC CG	2	1	0.5	0.25	0.125
FIC P	0.125	0.25	0.5	0.5	0.5
FIC I	2.125	1.25	1	0.75	0.625
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	ADDITIVE	PARTIAL SYNERGY	PARTIAL SYNERGY

CG:Catechin Gallate, P:Penicillin, I:Index

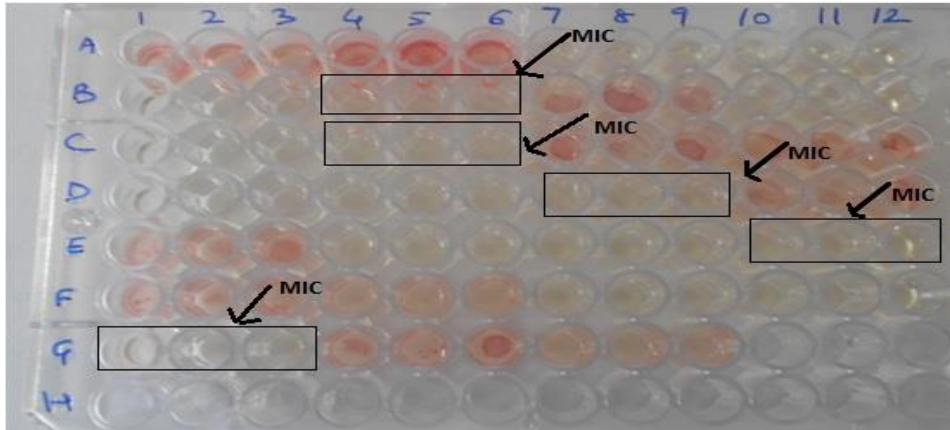

Fig. 5.25 Checkerboard result of *S. aureus* MTCC 96 for five different concentration of antibiotic Penicillin with five different concentration of test compound (CG)

	1	2	3	4	5	6	7	8	9	10	11	12
A	CTRL1	CTRL1	CTRL1	CTRL2	CTRL2	CTRL2	125CG +16000 P	125CG +16000 P	125CG +16000 P	125CG + 8000 P	125CG + 8000 P	125CG + 8000 P
B	125CG + 4000 P	125CG + 4000 P	125CG + 4000 P	125CG + 2000 P	125CG + 2000 P	125CG + 2000 P	125CG + 1000 P	125CG + 1000 P	125CG + 1000 P	62.5 CG + 16000 p	62.5 CG + 16000 p	62.5 CG + 16000 p
C	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 8000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 4000 P	62.5 CG + 2000 P	62.5 CG + 2000 P	62.5 CG + 2000 P	62.5 CG + 1000 P	62.5CG + 1000 P	62.5 CG + 1000 P
D	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 16000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 8000 P	31.25 CG + 4000 P	31.25 CG + 4000 P	31.25 CG + 4000 P	31.25 CG + 2000 P	31.25 CG + 2000 P	31.25 CG + 2000 P
E	31.25 CG + 1000 P	31.25 CG + 1000 P	31.25 CG + 1000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 16000 P	15.6 CG + 8000 P	15.6 CG + 8000 P	15.6 CG + 8000 P	15.6 CG + 4000 P	15.6 CG + 4000 P	15.6 CG + 4000 P
F	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 2000 P	15.6 CG + 1000 P	15.6 CG + 1000 P	15.6 CG + 1000 P	7.8 CG + 16000 P	7.8 CG + 16000 P	7.8 CG + 16000 P	7.8 CG + 8000 P	7.8 CG + 8000 P	7.8 CG + 8000 P
G	7.8CG + 4000 P	7.8 CG + 4000 P	7.8 CG + 4000 P	7.8 CG + 2000 P	7.8 CG + 2000 P	7.8CG + 2000 P	7.8 CG + 1000 P	7.8 CG + 1000 P	7.8 CG + 1000 P			
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Fig. 5.26 MIC of *S. aureus* MTCC 96 with antibiotic Penicillin and test compound CG from checkerboard method

Table 5.16 FIC INDEX of *S. aureus* MTCC 96 with antibiotic Penicillin and test compound CG

	125CG + 2000 P	62.5 CG + 4000 P	31.25 CG + 4000 P	15.6 CG + 4000 P	7.8CG + 4000 P
FIC CG	2	1	0.5	0.25	0.125
FIC P	0.25	0.5	0.5	0.5	0.5
FIC I	2.25	1.5	1	0.75	0.625
SIGNIFICANCE	INDIFFERENCE	INDIFFERENCE	ADDITIVE	PARTIAL SYNERGY	PARTIAL SYNERGY

CG: Catechin Gallate, P:Penicillin, I:Index

Table 5. 17. Summary of FIC Values for all three strains *S. aureus* for antibiotic Oxacillin (O) and Penicillin (P) with Catechin Gallate (CG)

Test isolate	Drug Combi-nation (A/B)	MIC–Drug A (µg/ml)	MIC-Drug B (µg/ml)	Combination MIC (A/B) (µg/ml)	FIC-Drug A	FIC-Drug B	FICI	Interpretation	MIC Reduction (folds)
NCTC 6571	CG / Oxacillin	62.5	500	125/62.5	2	0.125	2.125	Indifference	
				62.5/62.5	1	0.125	1.125	Indifference	
				31.25/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				15.6/62.5	**0.25**	**0.125**	**0.375**	**Synergy**	**8 fold**
				7.8/62.5	**0.125**	**0.125**	**0.25**	***Synergy***	**8 fold**
	CG / Penicillin	62.5	8000	125/2000	2	0.25	2.25	Indifference	
				62.5/4000	1	0.5	1.5	Indifference	
				31.25/2000	0.5	0.25	0.75	Partial synergy	4 fold
				15.6/4000	0.25	0.5	0.75	Partial synergy	2 fold
				7.8/2000	**0.125**	**0.25**	**0.375**	***Synergy***	**4 fold**
MTCC 737	CG / Oxacillin	125	500	250/62.5	2	0.125	2.125	Indifference	
				125/62.5	1	0.125	1.125	Indifference	
				62.5/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				31.25/62.5	**0.25**	**0.125**	**0.375**	***Synergy***	**8 fold**
				15.6/125	**0.125**	**0.25**	**0.375**	**Synergy**	**4 fold**
	CG/ Penicillin	125	8000	250/1000	2	0.125	2.125	Indifference	
				125/2000	1	0.25	1.25	Indifference	
				62.5/4000	0.5	0.5	1	Additive	2 fold
				31.25/4000	0.25	0.5	0.75	Partial synergy	2 fold
				15.6/4000	0.125	0.5	0.625	Partial synergy	2 fold
MTCC 96	CG / Oxacillin	62.5	500	125/62.5	2	0.125	2.125	Indifference	
				62.5/62.5	1	0.125	1.125	Indifference	
				31.25/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				15.6/125	**0.25**	**0.25**	**0.5**	**Synergy**	**4 fold**
				7.8/125	**0.125**	**0.25**	**0.375**	***Synergy***	**4 fold**
	CG/ Penicillin	62.5	8000	125/2000	2	0.25	2.25	Indifference	
				62.5/4000	1	0.5	1.5	Indifference	
				31.25/4000	0.5	0.5	1	Additive	2 fold
				15.6/4000	0.25	0.5	0.75	Partial synergy	2 fold
				7.8/4000	0.125	0.5	0.625	Partial synergy	2 fold

MIC:Minimum Inhibitory Concentration, FIC: Fractional Inhibitory Concentration, O:Oxacillin, P:Penicillin,CG:Catechin Gallate

5.7.3 Time Kill Curve

Synergistic formulations of checkerboard and FIC Index were further tested in time kill assay to see the extent of improvement in the susceptibility of the antibiotics. Time-kill assay results show the decrease in the log_{10} CFU/ml of viable colonies in all tested formulations, as compared to their respective controls as well as, in the absence of either antibiotic or test compound; which indicates the bactericidal activity of the formulation (Fig.5.27-5.30). *S. aureus* NCTC 6571 formulation of 62.5 µg/ml Oxacillin with 7.8 µg/ml of CG, which earlier revealed an 8 folded reduction in MIC of Oxacillin, also revealed 1.7 and approx. 3 log_{10} reductions in CFU at 12h and 18 h respectively. However, it exhibited more than 6 log_{10} reduction in CFU count at 24 h (Table 5.18 and Fig.5.27). Similarly, the formulation of 2000 µg/ml Penicillin with 7.8 µg/ml of CG of *S. aureus* NCTC 6571, which earlier revealed, a 4 fold reduction in MIC of Penicillin, finally revealed 1.8 and 3 log_{10} reductions in CFU at 12h and 18 h. Whereas, this formulation, furthermore, exhibited more than 6 log_{10} reduction in CFU count at 24 h (Table 5.19 and Fig.5.28). Formulation of 62.5 µg/ml of Oxacillin with 31.5 µg/ml of CG has enhanced the susceptibility of *S. aureus* MTCC 737, by exhibiting an 8 folds reduction in MIC of Oxacillin. It also revealed 2 and 2.8 log_{10} reductions in CFU after 12h and 18 h. Further, this formulation induced a 6.7 log_{10} reduction in CFU after 24 h (Table 5.20 and Fig.5.29). Similar log_{10} reductions were found in *S. aureus* MTCC 96, formulation of 125 µg/ml Oxacillin with 7.8 µg/ml of CG, where it exhibited a 1.8, 2.8 and 6.9 log_{10} reductions in CFU after 12h, 18 h and 24 h respectively (Table 5.21 and Fig.5.30). Further, analysis of CFU log_{10} reductions by two-way ANOVA and Bonferroni Posttests of Multiple comparison, indicated that there was a significant reduction at $P<0.001$, in *S.aureus* NCTC 6571, between the control and test formulation of Oxacillin at 12, 18 and 24 h. While there was no significant difference was recorded at 0 and 6h (Table 5.18). Similar results were found among the control and test formulation of Penicillin within the same strain (Table 5.19).

Table 5.18Comparative kill kinetics of *S. aureus* NCTC 6571 with absence of any antibiotic, in presence of Oxacillin, CG and formulation of Oxacillin and CG

Time(h)	Bacterial Colony Count in Log_{10} CFU/ml			
	Control	Oxacillin	CG	Formulation
0	6 ±0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00
6	6.9±0.06	7 ± 0.10	6.9±0.00	6.7 ± 0.06
12	8.3±0.06	8.3±0.0 0	8.1±0.20	6.6*** ±0.00
18	8.9±0.20	8.5**±0.06	8.5***± 0.06	6*** ± 0.20
24	6.3±0.06	6.6*±0.06	6.5 ± 0.4	0***

Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Bonferroni post test ($p<0.05$) where ***$P<0.001$,**$P<0.01$,*$P<0.05$

Table 5.19 Comparative kill kinetics of *S. aureus* NCTC 6571 with absence of any antibiotic, in presence of Penicillin, CG and formulation of Penicillin and CG

Time(h)	Bacterial Colony Count in Log_{10} CFU/ml			
	Control	Penicillin	CG	Formulation
0	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00
6	6.9 ± 0.06	7 ± 0..06	6.9±0.0	6.8 ± 0.06
12	8.3 ±0. 06	7.3*** ± 0.06	8.1±0.20	6.5*** ± 0.0.1
18	8.9 ± 0.20	8.7±0 .20	8.5***±0.06	5.8*** ± 0.0.2
24	6.3 ± 0.06	6.7*** ± 0.06	6.5 ± 0.4	0***

Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Bonferroni post test ($p<0.05$) where ***$P<0.001$,**$P<0.01$,*$P<0.05$

Table5.20 Comparative kill kinetics of *S. aureus* MTCC 737 with absence of any antibiotic, in presence of Oxacillin, CG and formulation of Oxacillin and CG

Time(h)	Bacterial Colony Count in Log_{10} CFU/ml			
	Control	Oxacillin	CG	Formulation
0	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00
6	7.3 ±0.06	7.2± 0.00	7***± 0.06	6.9*** ± 0.00
12	8.6 ±0.00	8.6± 0.00	8.4***±0.06	6.6*** ± 0..06
18	8.8 ±0.06	8.7±0.06	8.6*** ± 0.10	6*** ± 0.06
24	6.7 ±0.1	6.6± 0.06	6.6* ±0.06	0***

Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Bonferroni post test (p<0.05) where ***P<0.001,**P<0.01,*P<0.05

Table 5.21 Comparative kill kinetics of *S. aureus* MTCC 96 with absence of any antibiotic, in presence of Oxacillin, CG and formulation of Oxacillin and CG

Time(h)	Bacterial Colony Count in Log10 CFU/ml			
	Control	Oxacillin	CG	Formulation
0	6 ± 0.00	6 ± 0.00	6 ± 0.00	6 ± 0.00
6	7.3 ±0.06	7.1*** ± 0.00	7***±0.06	6.8*** ±0.00
12	8.5 ±0.06	8.3*** ± 0.00	8.2*** ± 0.00	6.6*** ±0.06
18	8.8 ±0.00	7.7*** ± 0.06	7.6***± 0.00	6*** ± 0.00
24	6.9 ±0.00	6.8** ± 0.00	6.8± 0.06	0***

Data presented are mean ± standard deviation of three replications. Means with different superscript letters are different by Bonferroni post test (p<0.05) where (***P<0.001,**P<0.01,*P<0.05)

Control and test formulation of *S. aureus* MTCC 737 and *S. aureus* MTCC 96 strains, have shown significant reduction at P<0.001 for 6, 12, 18 and 24 h (Table 5.20 and 5.21).

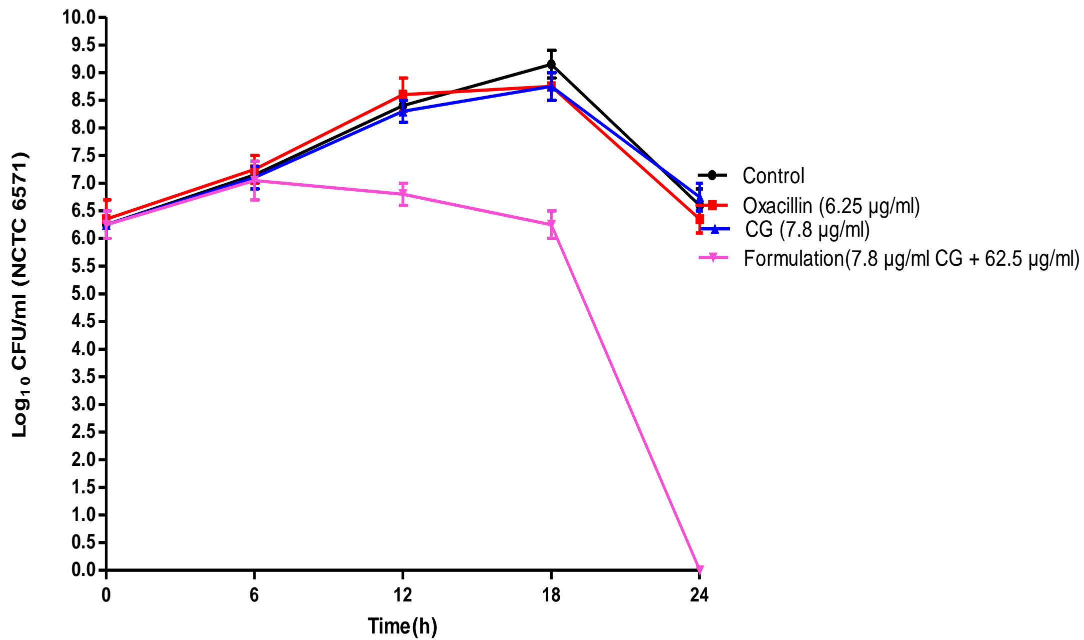

Fig.5.27 Time Kill Curve of *S. aureus* NCTC 6571 alone, with Oxacillin (O), with CG and synergistic FIC combination of Oxacillin and CG

Data represented are mean ± standard deviation of three replicates.

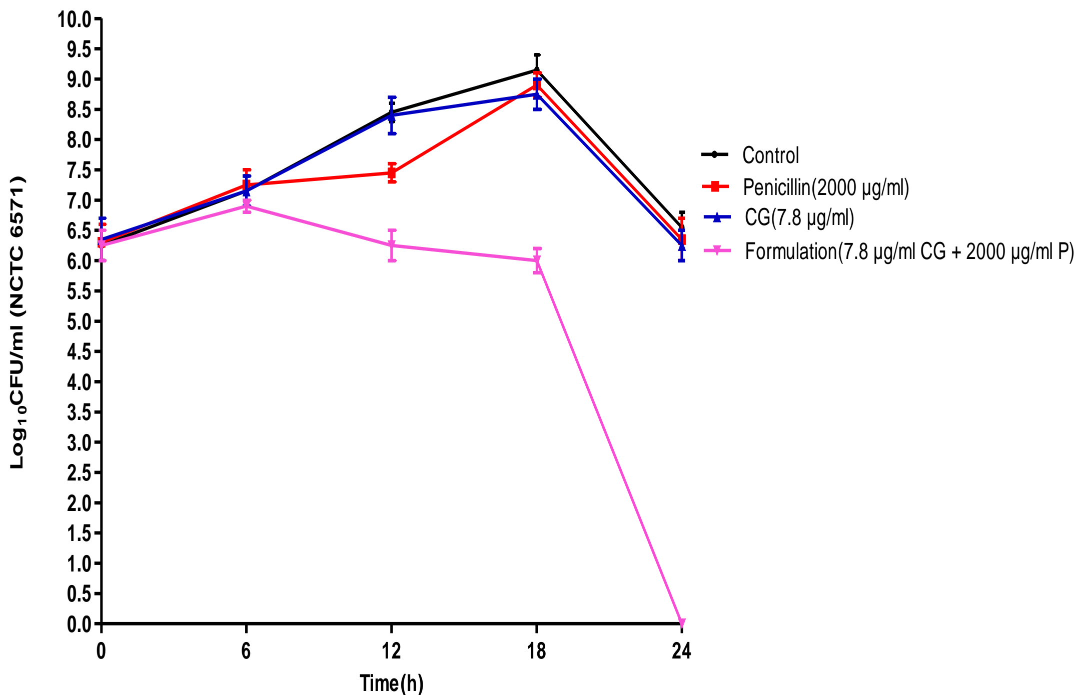

Fig.5.28 Time Kill Curve of *S. aureus* NCTC 6571 alone, with Penicillin (P), with CG and synergistic FIC combination of Penicillin and CG

Data represented are mean ± standard deviation of three replicates.

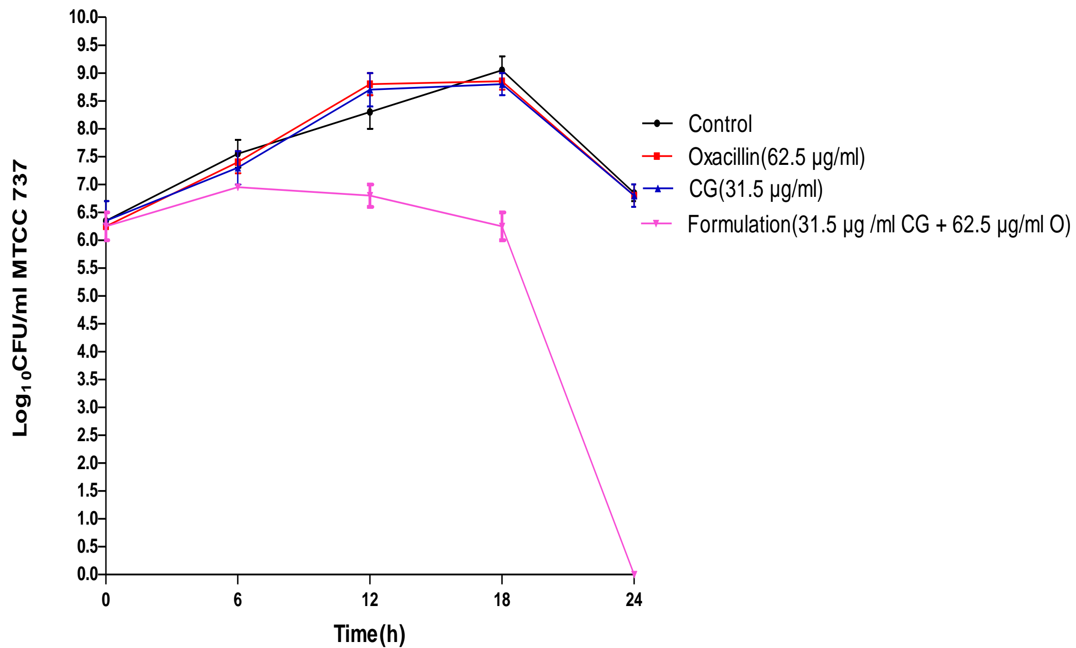

Fig.5.29 Time Kill Curve of *S. aureus* MTCC 737 alone, with Oxacillin, with CG and synergistic FIC combination of Oxacillin and CG.
Data represented are mean ± standard deviation of three replicates.

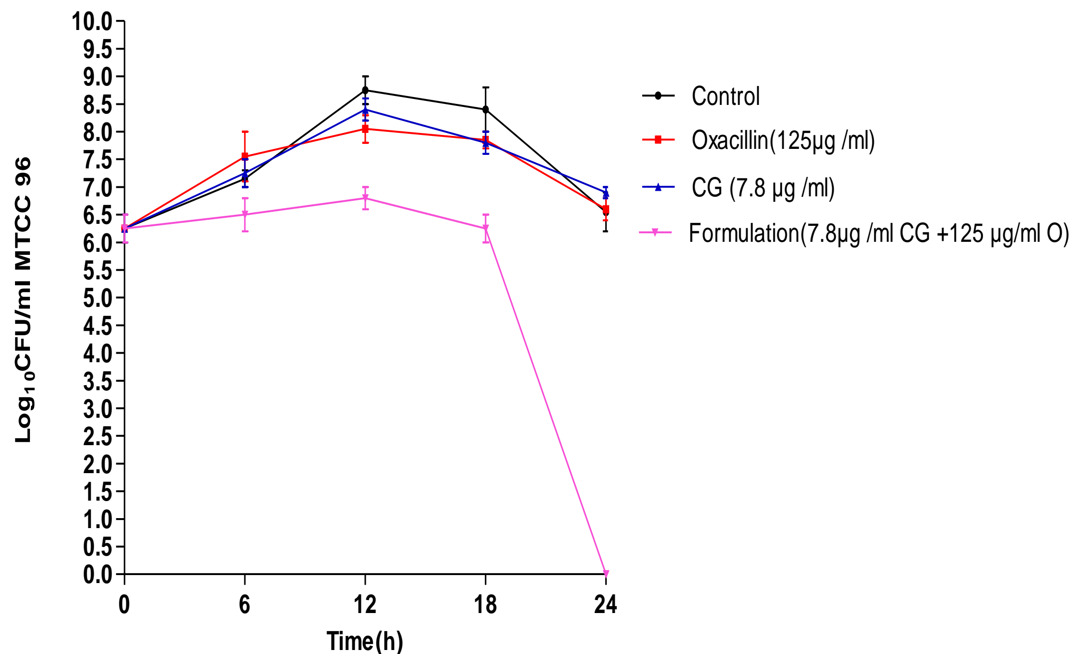

Fig.5.30 Time Kill Curve of *S. aureus* MTCC 96 alone, with Oxacillin, with CG and synergistic FIC combination of Oxacillin (O)and Catechin Gallate (CG)
Data represented are mean ± standard deviation of three replicates.

5.8 Evaluation of *FemA* protein functional Expression

5.8.1 nLC-MS

Seven samples having three controls and four test samples were prepared for nLC-MS analysis. Most synergic and significant test samples proved by FIC index and time kill curve are further analyzed for mass studies. Control samples include muropeptides of *S. aureus* NCTC 6571, *S. aureus* MTCC 737 and *S. aureus* MTCC 96, which are coded as 6C, 7C and 9C. While test samples muropeptides are coded as 6FCO, 7FCO, 9FCO and 6FCP, which are prepared by treating all three *S. aureus* strains with the combination of CG and antibiotic Oxacillin except 6FCP where Oxacillin is replaced by Penicillin (as mentioned previously in section 4.8.2). Expected molecular mass for muropepides of *S. aureus* strains is 1025.503, 1139.544 and 1253.586 in case of monoglycine, triglycine and pentaglycine with sugars and pentapeptide.

Observed Masses with corresponding intensities are shown in Total Ion Chromatogram (TIC) of respective figures (Fig. 5.31-5.37). Relative decrease in percentage of peak area of 6FCO and 6FCP is 43% and 65.3% in monoglycine, 47.4 % and 62.6 % in triglycine, 70% and 85.2% in pentaglycine. As compared to 6C; Percentage of peak area observed for observed mass of 6FCO, and 6FCP has been decreased in all three cases of PGN (Fig. 5.31-5.33). Similarly, in 7FCO and 9FCO percentage of peak area has been decreased from their control 7C and 9C peak area (Fig.5.34-5.37). Relative decrease in percentage of peak area of 7FCO and 9FCO is 97.2% and 78.2% in monoglycine, 80% and 72.9 % in triglycine, 57.5 % and 32.8 % in pentaglycine (Table.5.22 in Appendix)

Further analysis of peak area (in percentage) by two-tailed paired t-test, indicated that there was a significant reduction ($P=0.0485$, $R^2=0.9053$), $P < 0.5$, in *S.aureus* NCTC 6571 between the control and the formulation of 62.5 μg/ml Oxacillin with 7.8 μg/ml of CG. Similar

significance was found among the control and formulation of 2000 µg/ml Penicillin with 7.8 µg/ml of CG in the same strain where (P=0.0135, R^2 =0.9732), P < 0.5 (Fig.5.38). *S. aureus* MTCC 737 and MTCC 96 strains, have shown significant reduction at (R^2 =0.9999 and P<0.0001) for the formulation of 62.5 µg/ml Oxacillin with 31.5 µg/ml of CG and (P=0.0049, R^2 =0.9901), P < 0.01 for the formulation of 125 µg/ml Oxacillin with 7.8 µg/ml of CG respectively (Fig.5.39 and Fig.5.40).

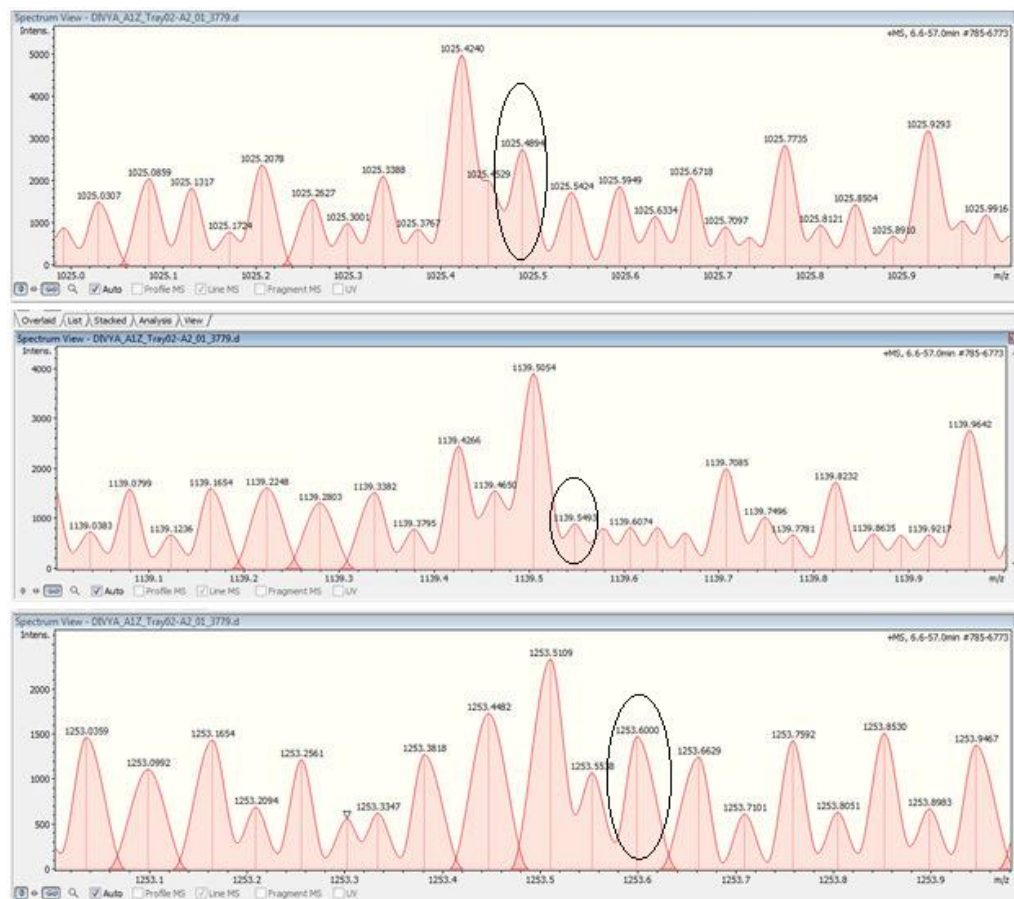

Fig 5.31 TIC of *S.aureus* NCTC 6571

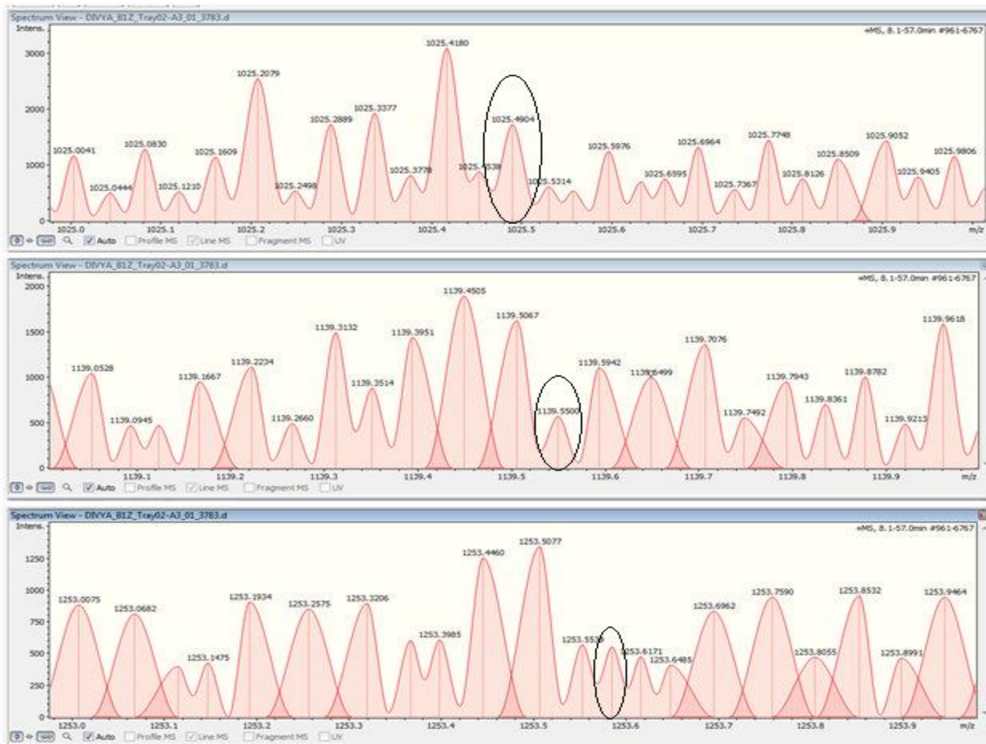

Fig 5.32 TIC of *S.aureus* NCTC 6571 in presence of Oxacillin and CG

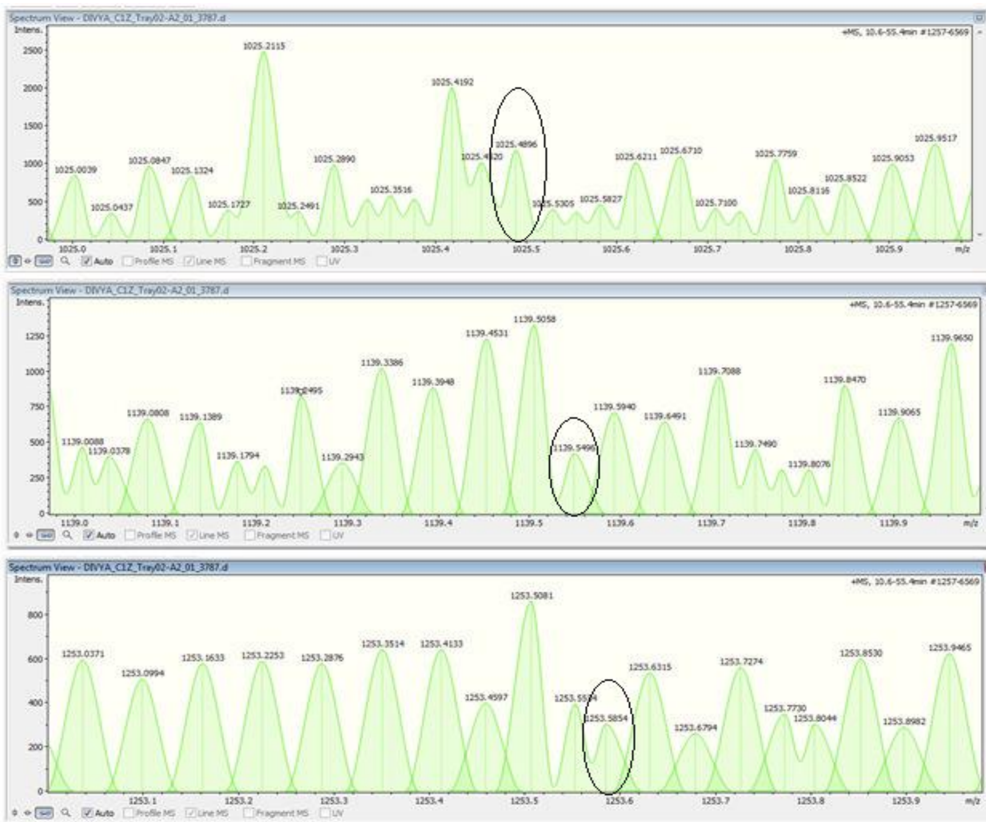

Fig. 5.33 TIC of *S.aureus* NCTC 6571 in presence of Penicillin and CG

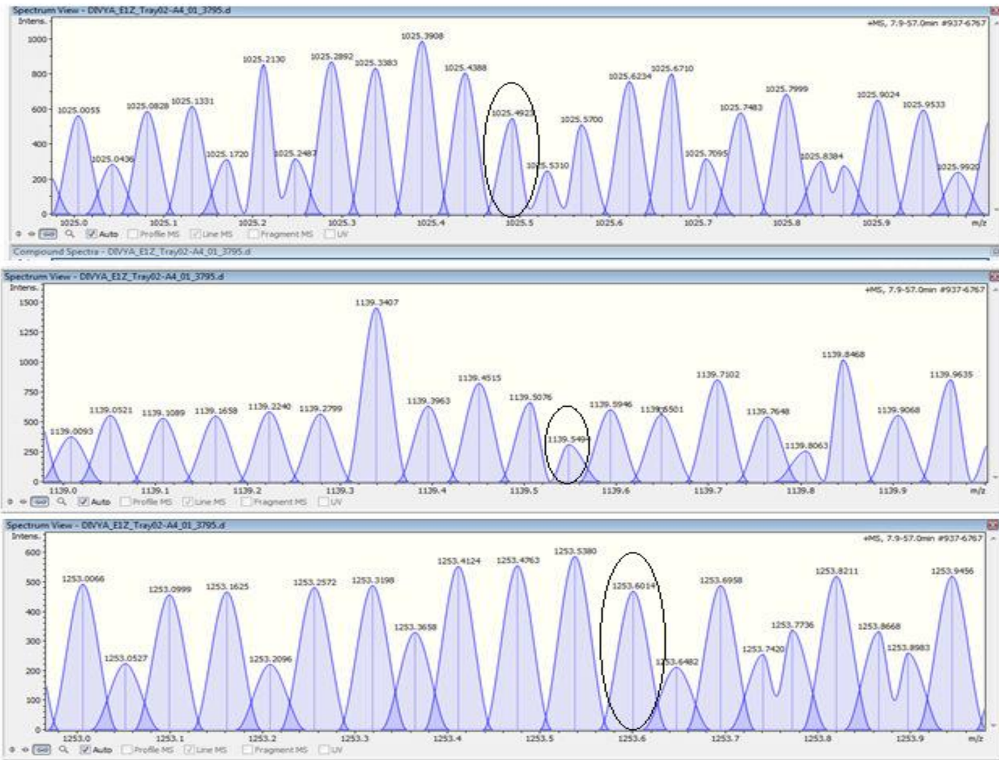

Fig. 5. 34 TIC of *S. aureus* MTCC 737

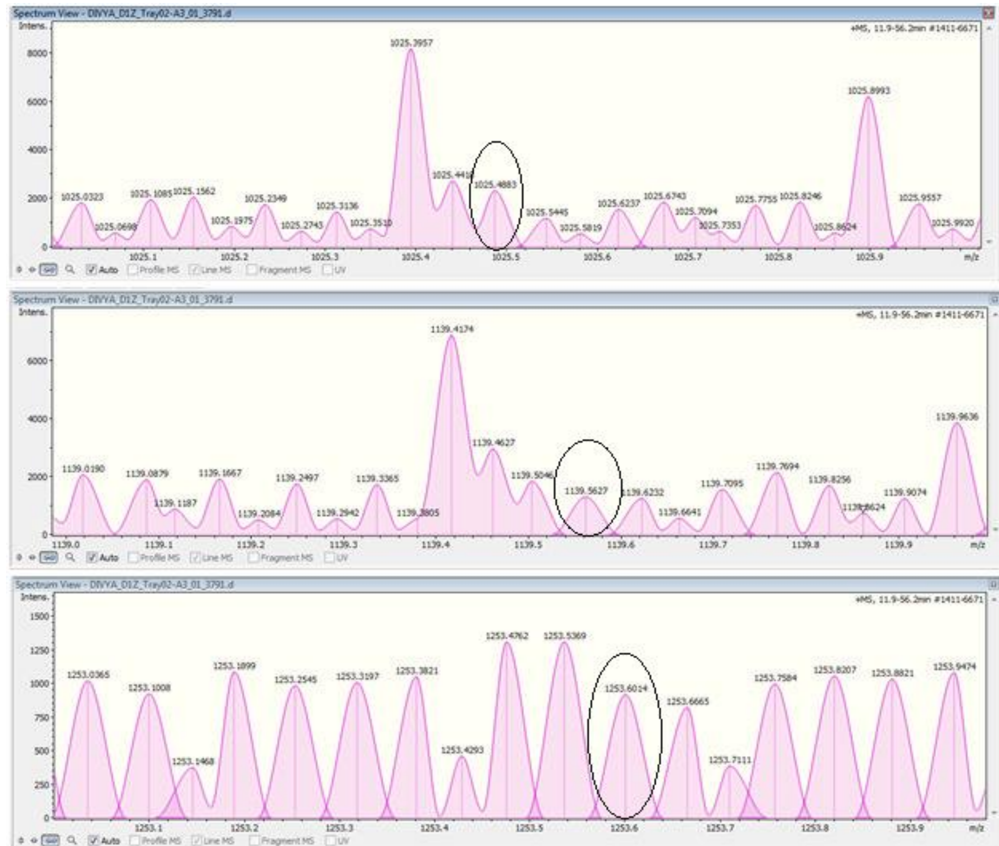

Fig. 5.35 TIC of *S. aureus* MTCC 737 in presence of Oxacillin and CG

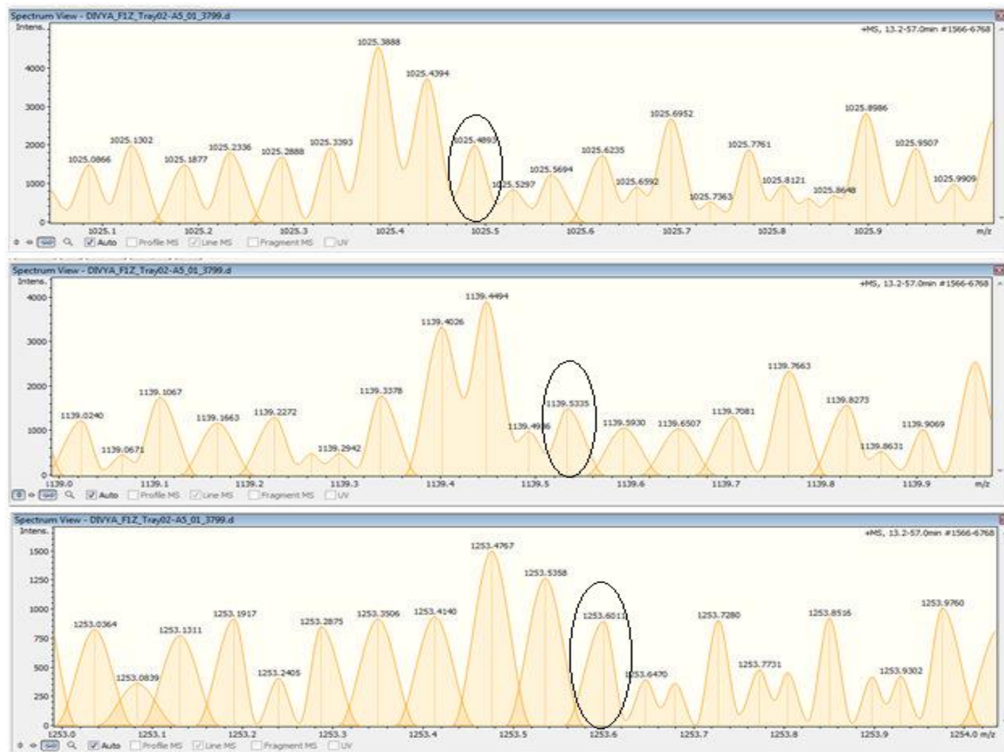

Fig. 5.36 TIC of *S. aureus* MTCC 96

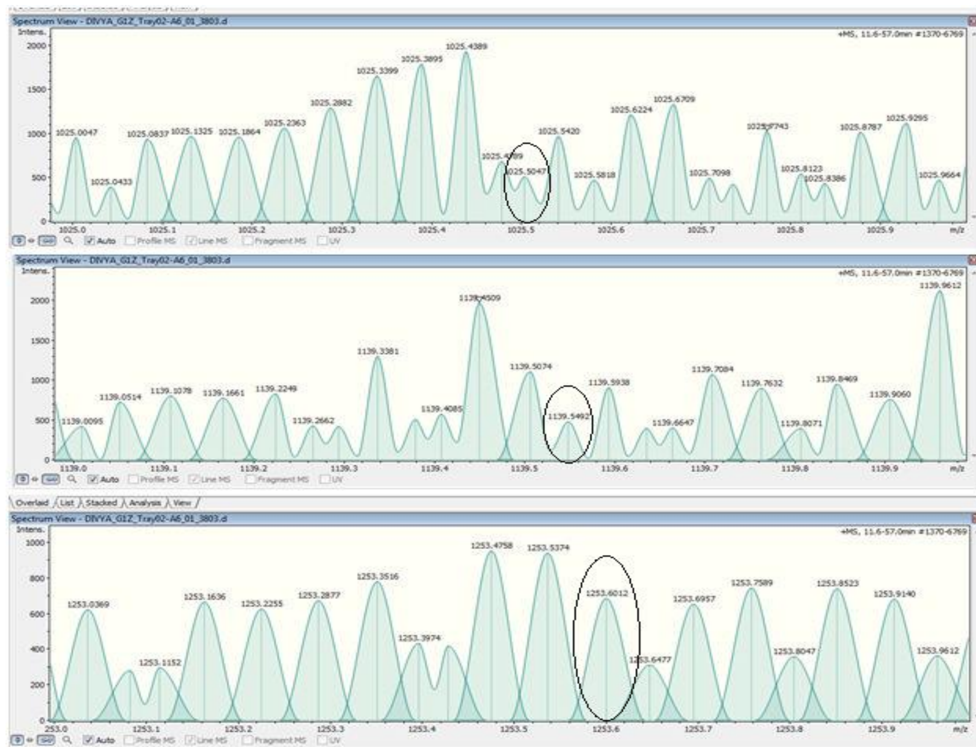

Fig. 5.37 TIC of *S.aureus* MTCC 96 in presence of Oxacillin and CG

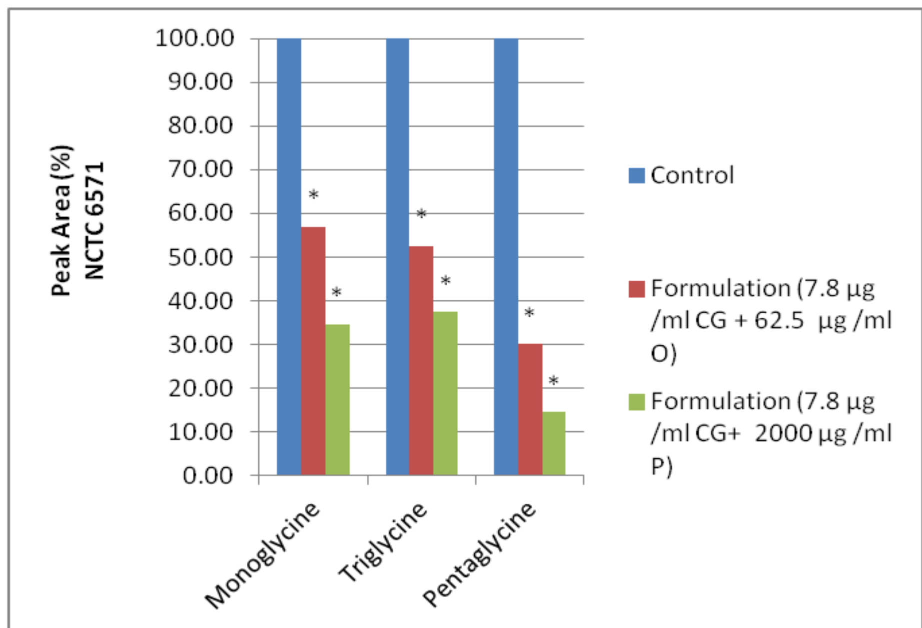

Fig. 5.38 Comparison of Peak area (%) of *S.aureus* NCTC 6571 in absence and presence of antibiotics and test compound formulation

Data indicated significant reduction by two-tailed paired t-test, ($p<0.05$)* refers to $P < 0.5$

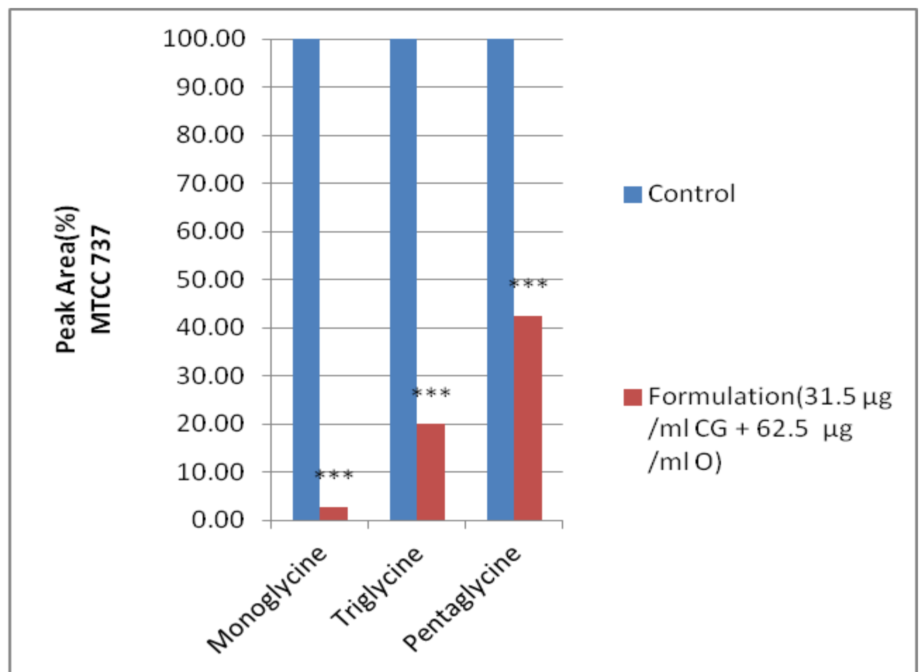

Fig. 5.39 Comparison of Peak area (%) of *S.aureus* MTCC 737 in absence and presence of antibiotic and test compound formulation.

Data indicated significant reduction by two-tailed paired t-test, ($p<0.05$) (*** refers to $P<0.0001$)

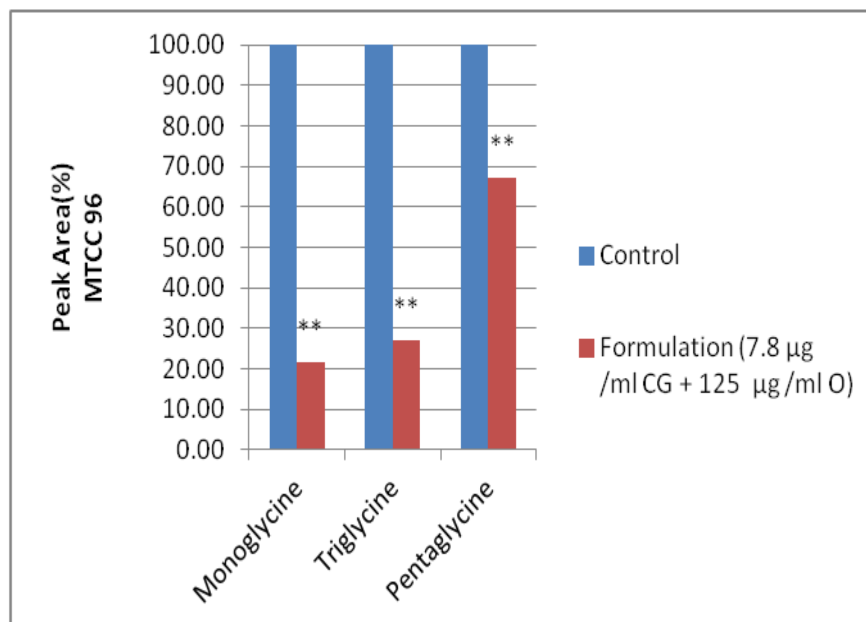

Fig. 5.40 Comparison of Peak area (%) of *S.aureus* MTCC 96 in absence and presence of antibiotic and test compound formulation.

Data indicated significant reduction by two-tailed paired t-test, ($p<0.05$) (** refers to $P < 0.01$)

Chapter 6: Discussion

Drug resistance among microbes is the burgeoning problem from last few decades, which is increasing day by day. As reported by global surveillance reports of Australian Group on Antimicrobial Resistance (AGAR) 2013, World Health Organization (WHO) 2014, European Antimicrobial Resistance Surveillance Network (EARS-Net) 2014, Center for Disease Dynamics, Economics and Policy (CDDEP) 2015, Public Health Agency of Canada (PHAC) 2015.The resistance development in *S.aureus* has been reported to be fastest due to the genetic plasticit (Goerke and Wolz, 2004; Kavanagh *et al.,* 2014; Gelband *et al.,* 2015). It is responsible for majority of chronic infections in hospital and community settings (Ray *et al*., 2011). MRSA prevalence rate is up to 50% in most of the countries like US, India, China, Honkong, Singapore, UK, Greece, Northern Ireland, etc. There is an ongoing war between the clinicians and this superbug to develop methods to overcome resistance. Designing of new drugs for novel targets is a very expensive and time-consuming process as they involve screening of the large number of compounds experimentally, multiple validation tests and failure of experiments in pre-clinical and clinical trials. Computational aided drug designing (CADD) is another way out to prevent the screening failure of the large number of compounds, by performing it virtually, which can save time and reduces the cost of drug development (Sliwoski *et al.,* 2014). Identification of modulators for previously discovered drugs with CADD, to treat bacterial infections caused by multiple drugs resistant (MDR) and total drug resistant (TDR) strains can be a better method to overcome resistance.

Natural products and their derivatives have played a significant role in pharmaceutical research. Approximately 40-50% drug's source is natural products or their derivatives (Lahlou, 2013). It can be plant, animal, fungus or other microbes. Classical examples of some medicines from plant sources are Aspirin, Digitoxin, Morphine, Quinine, and Pilocarpine (Newmann and Cragg, 2016). Epibatidine, Cilazapril and Captopril are examples of animal

sources (Cragg and Newman, 2013). Resources of most of the antibiotics are microbial, which includes Penicillin from the filamentous fungus *Penicillium notatum*, Tetracycline from *Streptomyces aureofaciens,* Choramphenicol from *Streptomyces venezuela*. Imipenem, Norcardicin and aztreonam are the example of drugs, which are synthesized from microbial sources (Siddiqui *et al*., 2014). These secondary metabolites have a lot of potential as they are used as anti-inflammatory, anti-cancer, anti-hypertensive, anti-infective, immune-suppressant, as well as in neurological disorders. Along with these, they provide ample opportunities against resistance.

Among all natural product modulators, the maximum well-known and nicely-studied are the flavonoids, which consists of flavonols, flavones, isoflavone, flavanols, flavanolols, flavanones and chalcones. Moreover, every person day by day consumes a tremendous amount of flavonoids from fruits, vegetables, meal supplements, tea and wine, etc. Various studies are conducted worldwide to prove the efficacy of these flavonoids in interfering the mechanism of multiple drug resistance genes by either alone or in combination with other antibiotics.

Flavonoids like 3-4-7-Trimethoxyflavone, 6-Prenylchrysin, Acacetin, Chrysin, Eupatin, Genistein, Fumitremorgin C, Plumbagin is the modulators of ABC transporters, 5-Bromotetrandrine, Abietane diterpene, Agosterol A and derivatives, Alisol B 23-acetate, Amooranin, Baicalein and derivatives, Biochanin, Catechins, Cepharanthine, Curcumin; Grape fruit juice extracts are modulators of P-glycoproteins. Similarly, Cannabinoids, Cepharanthine, Curcumin, *Ginkgo biloba* extract, *Kaempferia parviflora* extracts, Quercetin, Myricetin, are multi drug resistant protein modulators in cancer chemotherapy (Wu *et al*., 2011). *Terminalia arjuna* herbal drug which comprises of tannins, flavonoids, Gallic acid, ellagic acid, phytosterols are effective for *Streptococcus mutants* ATCC-700610; *Streptococcus gallolyticus* ATCC 9809, *S.*

aureus; E. faecalis ATCC-29212 (Vadhana *et al.,* 2015). Other flavonoids from green tea like EGCG and ECG have been reported for their synergistic activity with Norfloxacin, Imipenem, Panipenem and β-lactams against multi drug resistant pathogens (Zhao *et al*., 2001; Gibbons *et al*., 2004; Taylor *et al*., 2005; Stapleton *et al*., 2006). Therefore, in these study catechins and their isomers are explored for their potential as synergist inhibitors of *FemA* protein to potentiates the β-lactam antibiotics against MRSA.

Virtual screening of catechin isomers were performed by AutoDock Vina and Dock tools. Basic requirements of these tools are the 3-D structure of target site and information of the pockets where a ligand can bind and can modulate its function (Kumar, 2013) In the absence of experimental 3-D structure of target, computational structures are designed through homology modeling technique, but in our study X-ray crystallography structure of *FemA* protein was available in RCSB-PDB database with PDB id 1LRZA (Benson *et al.,* 2002). Availability of 3-D structure alone is not the sufficient, next vital factor is the information of an active site where a ligand can bind and inhibit its activity.

In the absence of exact active site location, it can be predicted through various active site prediction tools. Therefore, in this study we have used four most popular active site prediction tools i.e. SiteHound, Q-Sitefinder, PocketFinder and CASTp (Sliwoski *et al*., 2014; Leelananda, and Lindert, 2016). These tools predicted multiple active sites in 1LRZA. To filter these active sites, we have selected the active sites of only L-domain, which is significant for inhibition of the function of this protein (Benson *et al.,* 2002). However, when we correlate these active sites predicted by the four different tools in L-domain, we found no significant correlation in them (Fig. 6.1. Appendix). Therefore, we used Blind Docking for AutoDock Vina Docking tool, where we generated grid box around complete 3-D structure of *FemA* protein and these dimensions were then used for docking. While Dock used biggest pocket predicted,

by its active site prediction tool Sphgen. As shown in result's section, both the tools predicted same ligand (CG with PubChem id 6419835) as best inhibitor or Hit for the target protein.

As the central goal of SB CADD is identification of the compounds that are target specific, bind tightly to the target site, i.e., with massive reduction in free energy and having good ADMET properties to become a drug-like molecule (Sliwoski *et al*., 2014). For hit to lead optimization or to estimate drug-likeness various *in silico* tools are available, which can predict the chemical and metabolic stability, oral bioavailability and minimum toxicity (Young, 2009; Hopkins 2011). Successful applications of these methods are required, to validate the lead *in vitro* and *in vivo* to become a drug (Leelananda, and Lindert, 2016). It was performed by LigPlot+, OSIRIS molecule property explorer and Lipinski Filter tool of SCFBIO in terms of Drug score, which is calculated by combine value of Drug-likeliness, clog P, LogS, molecular weight and four toxicity tests (Mutagenicity Risk, Tumourgenicity risk, irritating and reproductive effects) furthermore, by Lipinski Rule of Five. In our study, CG again qualifies these tests with Drug score of 0.74 and violation of only one Lipinski rule, which shows the potential of CG as drug, therefore, furthermore, *in vitro;* experiments were performed with this ligand molecule (Singhal and Saxena, 2015). Previously various computationally discovered drugs were approved by FDA in clinical studies such as Sorafenib (Nexavar, 1) from Bayer, which is used for treatment of renal cell carcinoma and hepatocellular carcinoma, as well as thyroid cancer; another drug is Ataluren (Translarna) used for treatment of genetic disorder due to nonsense mutation (Newman and Cragg, 2016).

In vitro, testing was performed using standard cultures of *S. aureus,* including NCTC 6571, MTCC 737 and MTCC 96 and standard β-lactam antibiotics of Oxacillin and Penicillin sodium salts from HiMedia, India, while CG sodium salts were purchased from Sigma Aldrich. In the present study, before evaluation of CG on *S. aureus* cultures, molecular typing was

adopted for MRSA genes. MRSA strains have additional (PBP2a), which functions in the presence of β-lactam antibiotics and perform the function of host pbps (Reynolds and Brown, 1985). PBP2a is encoded by *mecA* gene. SCC*mecA* gene is the main molecular determinant of resistance, and it spreads by horizontal transfer of gene among susceptible strains. Selective antibiotics usage created a pressure, which results into mutations and deletions in *mecI* or promoter/operator regions of *mecA*, resulting repressor inactivation and PBP2a expression (Kobayashi *et al*., 1998; Stapleton and Taylor, 2002 and Shukla *et al.,* 2004).

Along with *mecA* there are other essential factors, which are also responsible for methicillin resistance *viz*. *Fem* or auxiliary (*aux*) factor, *mur*F, *fmt*A-C, *sig*B, *hmr* A, *hmr* B, *dlt*, *pbp2*, *cta*A, while factors like *ilm* and global regulators *agr* and *sar* genes have minute effect on resistance (Berger-bachi, 1994; Kobayashi *et al*., 1994; Chambers, 1997). Therefore, in this study, one primer set for *mecA* and another primer for *FemA* were used. The primer set 1 used for *mecA* gene was [5'AAA ATC GAT GGT AAA GGT TGG C 3' (F); 5' AGT TCT GCA GTA CCG GAT TTG C 3'(R)] and the primer set 2 used was [5'AAA AAA GCA CAT AAC AAG CG 3' (F); 5' GAT AAA GAA GAA ACC AGC AG 3'(R)]. In the present study, 2 out of 3 staphylococcal isolates exhibited *mecA* gene amplicon at 533 bp using primer set 1 while all 3 isolates exhibited *femA* gene amplicon at 132 bp. First Primer set, *mecA* gene has been used for phenotypic detection of nosocomial *mecA* positive staphylococcal isolates (De Giusti *et al*., 1999). Second primer set which is of *femA* gene is used for detection of essential source of *FemA* protein, which is requisite for the peptidoglycan biosynthesis (Maidhof *et al*., 1991). Therefore, *FemA* protein is used as a drug target to inhibit the cell wall biosynthesis to potentiate the β-lactam antibiotics.

For *in vitro* validation of the antimicrobial properties of CG various assays were performed such as MIC determination, checkerboard, FIC Index and Time kills kinetics. It can

be precisely achieved if the growth of the microorganism is pre-recorded in terms of lag phase, log phase, stationary phase and the death phase which was achieved by Miles and Misra's method of CFU counting at different time intervals. Some studies have shown that susceptible strain of *S. aureus* had generation time of 29 min while resistant strains had generation time of 40 min (Ender *et al*., 2004). Similarly, in other species generation time of Ciprofloxacin, resistant *S. epidermidis* was reported as 39 min (Gustafsson *et al*., 2003). In our study, we also found average generation time of 45 min, which suggests the resistant behavior of the test organisms

MIC is a standard parameter to define the lowest concentration of an antimicrobial agent, that inhibits the growth of microorganism and that can be visible with the naked eye, after overnight incubation period (Andrews, 2001). It is also used for other susceptibility testing methods. The MIC values of drugs are quantified by using conventional methods, in determination of antimicrobial activities of various β-lactam antibiotics as well as the test compound CG on microorganism.

MIC results have shown all three staphylococcal cultures are resistant to Oxacillin and Penicillin. While, as compared to β-lactam antibiotics, MIC of CG was very less in all three cultures, i.e. 62.5 µg/ml in *S. aureus* NCTC 6571, *S. aureus* MTCC 96 and it was found to be 125 µg/ml in MTCC 737. Whereas MIC for antibiotic Oxacillin and Penicillin, obtained was 500 µg/ml and 8000 µg/ml respectively for all three cultures. Similar MIC was reported for antibiotic Oxacillin in MRSA strains BB568, EMRSA-15 and EMRSA-16 (64 to >512 µg/ml), while MIC reported for various gallic acids, alkyl gallates, and 14 different catechins was very high (64 to >256 mg/l) as compared to our results (Stapleton *et al*, 2004) .Hence, MIC predicts the antibacterial properties of CG in all staphylococcal strains.

Chapter 6: Discussion

As checkerboard method is a standard assay to evaluate the antibacterial behavior of two or more drugs in different combinations in terms of MIC (Hsieh *et al,* 1993; Pemmaraju *et al*., 2013; Stapleon *et al*., 2006; Soe *et al.,* 2009). Generally, two-four fold dilutions of MIC values of drug alone are used in combinations with two-four fold dilutions of another drug. Multiple drugs can be used for analysis. Checkerboard method is one of the best methods to choose the best combination of drugs for antibacterial behavior. 16 *Brucella melitensis* strains from blood cultures were used to investigate the treatment of brucellosis, by checkerboard method for the different combinations of antibiotics such as rifampin with doxycycline, rifampin with trimethoprim-sulfamethoxazole, trimethoprim-sulfamethoxazole with doxycycline, doxycycline with streptomycin and ciprofloxacin with azithromycin (Orhan *et al*., 2005). In *Pseudomonas aeruginosa* multidrug resistant strain's checkerboard method was used to evaluate the effect of combination of antibiotics therapy, where synergistic interaction was found between kanamycin and ampicillin (Jain *et al.,* 2011).

Synergy testing of bacterial strains like *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter cloacae* ATCC 23355 and *S. aureus* ATCC 29213 with various combinations of cefepime or ceftazidime with tobramycin or ciprofloxacin was performed by using time-kill and checkerboard techniques (White *et al.,* 1996). For determination of synergistic effect of emodin with ampicilin or Oxacillin, checkerboard method was used for MRSA and MSSA strains (Lee et al., 2010). Along with combination studies of antibiotics; it is the most essential method of analysis, for evaluation of synergistic behavior of a modulator to a specific antibiotic or combination of antibiotics for improving their effect. Hence similar methods were applied for the validation of antibacterial properties of CG in presence of β-lactam antibiotics.

FIC index is an inhibitory concentration of multiple drug fractions in a combination. It is a numerical value to analyze the outcomes of checkerboard method. It is calculated by summation of FIC of combination drugs, where FIC of each drug is calculated by concentration of that drug in combination, which is further divided by drug MIC (Lee *et al.,* 2010). FIC index ranges from 0 > 2. It is measures in terms of synergy, partial synergy, additive, indifference and antagonism. If it is less than 0.5, it indicates the synergistic effect, if FIC index is within 0.5–0.75, it denotes partial synergy and more than 0.75 to 1 represents additive effect whereas >1–2 represent indifference, and >2 indicate antagonism (Lee *et al*., 2010; Sibanda and Okoh, 2008). FIC of emodin with ampicilin and oxacillin was reported to be 0.37–0.5 and 0.37–0.75, respectively which indicates the synergistic or partial synergistic behavior of emodin against MRSA (Lee *et al.,* 2010).Combinations representing indifference and antagonism can't be used as future drugs. In the present study, we have applied FIC index on checkerboard results, where we found all types of FIC index on different formulations. We have selected only four synergistic formulations having FIC range from 0.25-0.375 were selected for further experimental work.

We found eight-fold reduction in the synergistic formulations of checkerboard and FIC index for all three cultures of *S. aureus* against the antibiotic oxacillin, whereas we found four-fold reduction in MIC of Penicillin only in single strain. No synergistic result was found in formulations of CG with Penicillin in *S. aureus* MTCC 737 and *S. aureus* MTCC 96. Similar studies were reported by Stapleton group; they have tested ECG, EGCG, EC and EGC with Oxacillin in few MRSA strains and found that gallyolated catechins (ECG and EGCG) are more effective to reduce the MIC values of Oxacillin as compared to non-galloylated catechins such as EGC and Epicatechin. Moreover, they also elucidated that EC, and EGC alone had no significance with β-lactams in MRSA strains, but they can potentate the binding of ECG and

EGCG to staphylococcal cells (Shiota *et al*., 1999; Zhao *et al*., 2001; Stapleton *et al.,* 2004; 2006; 2007).

Checkerboard method and time kill kinetics gives reproducible results, in terms of synergy, in the study of multiple drugs (Mackay *et al.,* 2000). Subsequently, synergistic formulation observed from FIC index was further tested by time kill kinetics to observe their synergistic behavior w.r.t. time as compared with the absence of these formulations as control and with the absence of either antibiotic or CG from formulation. Kill kinetic's studies were used to assess the bactericidal and bacteriostatic activity of formulations, by plotting the $\log_{10}$ CFU ml^{-1} w.r.t. time.

Decrease in ≥ $3\log_{10}$ CFU count in original inoculum signifies 99.9% reduction or bactericidal activity in test organism while decrease in < $3\log_{10}$ CFU count signifies bacteriostatic activity (Silva et al., 2011). In the study of interaction of gallates with antibiotics in MRSA and MSSA strains, only ethyl gallate have shown bactericidal activity at 24h with tetracycline antibiotic for MRSA as well as MSSA (Soe *et al.,* 2010). However, in our study, bactericidal activity was observed after 12 h, in all four synergistic formulations, except *S. aureus* MTCC 737 where it was obtained after 18h.

Time kill kinetics data have shown that bactericidal effect is dependent on presence of both antibiotic as well as CG as well as their Concentration and time. Absence of any of these factors will affect the synergy. Thus time kill studies explain the speed of killing of the test microorganisms by preventing the re-growth by antibacterial combinations (Behm *et al*., 2005). Moreover, in this study, there is the observation of no re-growth after 12 h in any synergistic case. All the four synergistic formulation of checkerboard have also shown synergy in time kill studies also (Singhal and Saxena, 2017). Similar results were found in methods used for assessing synergistic antibiotic interactions for isolates of *Burkholderia cepacia*,

Staphylococcus aureus and *Klebsiella pneumonia* ((Mackay *et al.,* 2000). Moreover, we have obtained statistical significance in all formulations of time kill studies at $P<0.001$.

For the further validation, all the three staphylococcal cultures in the absence of any antibacterial agents were used as control sets and all four synergistic combinations of anti-bacterial agents of *in vitro* studies in their respective cultures were used as test sets for the extraction of muropeptides/murein for analysis in nLC-MS. Previous studies on *S. aureus* have shown that peptidoglycan chain of *S. aureus* is made of alternate units of sugars and amino acids. Glycan chain consists of two kinds of NAM and NAG sugar units, which are linked to each other by β-1, 4 linkages. Peptide chain is also named as stem peptide, which is made up of 5 amino acids, as L-Ala-D-Gln-L-Lys-D-Ala-D-Ala. L-Alanine of stem peptide is linked by N terminus to the carboxyl group of muramic acid. Further, cross-linking between these different disaccharide units forms a complex called peptidoglycan or murein or muropeptides. Cross-linking of disaccharide units is due to the formation of pentaglycine bridge between the D-Ala of 4th position of the initial disaccharide with 3rd position L-Lys of another strand. This pentaglycine chain is formed by three FEM proteins, *FEMX* adds first glycine, *FEMA* adds second and third glycine while *FEMB,* which sequentially adds fourth and Fifth glycine (Schleifer and Kandler, 1972; Maidhof, 1991; Chambers, 1997; Rohrer, 1999; Heijenoort, 2001; Benson, 2002; Peacock and Paterson, 2015).

UPLC-MS studies have shown the m/z of 1025.5032 Da and 1253.5861 Da for monoglycine disaccharide and pentaglycine disaccharide respectively (Kuhner *et al.*, 2014). Mostly PGN analysis was performed to know the structural composition of bacteria or to detect the anomaly (Courtin *et al.*, 2006; Packiam *et al.* ,2015). While first extraction method of muropeptides in *S. aureus* was given by De Jonge group, they have separated the peptides from HPLC and observed the m/z of 1026 Da and 1254 Da for monoglycine disaccharide and

pentaglycine disaccharide (De jonge *et al*., 1992). Muropeptides composition of *S. aureus* based on MS and amino acid analyzer was explained as NAG+NAM+Stem Peptide+X, here X can be Gly/Tri Gly/Penta Gly/Ala/tetra peptide and molecular mass of NAG+NAM+ Stem peptide was given as 968.4 Da. Addition of one glycine mass will be equivalent to 1025.46 Da (Rohrer *et al*., 1999). Based on previous studies of PGN analysis in *S.aureus,* in this study we used to validate the effect of CG on β-lactam antibiotics in MRSA strains. Therefore, this study has been designed to compare the disaccharide with monoglycine, triglycine and pentaglycine to study the effect of CG on the functional expression of *FemXAB* proteins.

Similar to above studies, calculated m/z used for this study is 1025.503 Da, 1139.544 Da, and 1253.586 Da for the disaccharide with monoglycine, triglycine and pentaglycine respectively. Generally, most nearby observed m/z values are taken for comparison between control and test sets. Commonly, muropeptides are extracted either by HPC or UPLC and further analyzed by MALDI-TOF or MS (Kuhner et al., 2014; Desmarais et al., 2015). While a different approach was used in *Clostridium difficile,* which include shotgun technique with MS and Byonic software (Bern *et al*., 2017). Instead of using HPLC/UPLC, nLC was used in present study and peak area of specific mass was obtained through MS.

Comparative analysis of peak area of monoglycine disaccharide, triglycine disaccharide and pentaglycine disaccharide of control and test sets have shown more than 50 % reduction in muropeptides of test sets. Muropeptide reduction justifies the CG as RMA for antibiotic Oxacillin and Penicillin for MRSA strains. Moreover, nLC-MS data have shown that, CG is interfering in the functional activity of all the three proteins *FemX, FemA* and *FemB,* it may be due to the formation of CG complex with these proteins. Formation of Fem protein complex with CG may have either modulate the structure of these proteins or modulated the active sites for cell wall biosynthesis substrate. As these three proteins, are homologous to

each other. *FemX* is 36% homologous and 23% identical to *FemA*, while *FemB* is 52% homoloogous and 39% identical to *FemA* (Ehlert *et al.*, 1997). We can say that CG is responsible for the modulation activity of both *FemX* and *FemB* alongwith *FemA*. Hence, this study has shown that, among all catechin isomers which are generally found in green tea, CG is found to be a synergistic RMA for β-lactam antibiotics against MRSA. It should be further used in clinical and pre-clinical studies for reversal of β-lactam resistance.

Conclusion

The present study shows that Catechin Gallate can potentiate the activity of β-lactam antibiotics against MRSA.

1. It is the first study of Catechin Gallate as RMA of β-lactam antibiotics against MRSA.
2. This study is based on both computational as well as experimental results, which holds promising results for pre-clinical studies.
3. Catechin Gallate (7.8 µg/ml conc.) has shown synergistic effect with Oxacillin (62.5 µg/ml conc.) and Penicillin (2000 µg/ml conc.) in NCTC 6571; while it was (31.25 µg/ml) with (62.5 µg/ml conc.) of Oxacillin in MTCC 737 but same conc. Of Catechin Gallate (i.e. 7.8 µg/ml) has shown synergy with (125 µg/ml conc.) of Oxacillin in MTCC 96.
4. Among the Catechin isomers, Catechin Gallate is the best ligand molecule, which can modulate the function of fem factors by inhibiting the function of *FemXAB* proteins.

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Fig4.2.Config File

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receptor = 1LRZ.pdbqt
ligand = ligand.pdbqt
scenter_x = 35.829
center_y = 63.41
center_z = 94.89
size_x = 76
size_y = 102
size_z =  20
num_modes = 9
```

Fig.4.3. INSPH File

```
Receptor.ms
R
X
0.0
4.0
1.4
Receptors.sph
```

Fig.4.4. OUTSPH File

```
Cluster 1 having 111 spheres
ATOM   186  C   SPH   186     33.223  56.485  86.932 TER
ATOM   194  C   SPH   194     32.620  54.643  86.775 TER
ATOM   269  C   SPH   269     33.134  47.900  84.688 TER
ATOM   289  C   SPH   289     35.356  51.919  74.766 TER
ATOM   291  C   SPH   291     36.340  52.947  77.823 TER
ATOM   305  C   SPH   305     30.283  47.287  83.534 TER
ATOM   463  C   SPH   463     28.234  49.420  83.930 TER
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ATOM   565  C   SPH   565     30.333  47.424  83.549 TER
ATOM   567  C   SPH   567     30.955  51.538  86.031 TER
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ATOM  1234  C   SPH  1234     39.280  51.396  88.728 TER
```

```
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ATOM   1268  C   SPH  1268      36.753  53.651  91.271 TER
ATOM   1269  C   SPH  1269      33.894  50.737  84.907 TER
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ATOM   1430  C   SPH  1430      42.119  66.806 113.565 TER
ATOM   1447  C   SPH  1447      45.627  64.517 114.964 TER
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ATOM   1473  C   SPH  1473      50.709  63.301 109.344 TER
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ATOM   1501  C   SPH  1501      54.282  70.920 101.151 TER
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ATOM   1658  C   SPH  1658      43.184  64.416  77.273 TER
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ATOM   1839  C   SPH  1839      30.770  56.443  86.032 TER
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```

```
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ATOM   3067  C   SPH  3067      49.992  63.236  98.845 TER
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ATOM   3127  C   SPH  3127      31.873  64.131 113.569 TER
ATOM   3293  C   SPH  3293      36.013  39.687  83.242 TER
```

Fig.4.5.Showsph.in File

```
Receptors.sph
1
N
selected_spheres.pdb
```

Fig.4.6.Showbox.in File

```
Y
5.0
Receptors.sph
1
Box.pdb
```

Fig.4.7.Grid.in File

```
Compute_grids          yes
Grid_spacing           0.3
Output_molecule        no
Contact_score          no
Energy_score           yes
Energy_cutoff_distance 9999
Atom_model             a
Attractive_exponent    6
Repulsive_exponent     9
Distance_dielectric    yes
Dielectric_factor      4
Bump_filter            yes
Bump_overlap           0.75
Receptor_file          receptor_charged.mol2
Box_file               box.pdb
Vdw_definition_file    /home/pc/AMS536software/dock6/parameaters/vdw_AMBER_parm99.defn
Score_grid_prefix      grid
```

Fig.4.8.Rigid.in File

```
Ligand_outfile_prefix                rigid
Limit_max_ligands                    no
Read_mol_solvation                   no
Write_orientations                   no
Write_conformations                  no
Skip_molecule                        no
Calculate_rmsd                       yes
Use_rmsd_reference_mol               no
Rank_ligands                         no
Num_scored_conformers_written        1
Orient_ligand                        yes
Automated_matching                   yes
Receptor_site_file                   receptorsphere.sph
Max_orientations                     500
Critical_points                      no
Chemical_matching                    no
Use_ligand_spheres                   no
Flexible_ligand                      no
Bump_filter                          no
Score_molecules                      yes
Contact_score_primary                no
Contact_score_secondary              no
Grid_score_primary                   yes
Grid_score_secondary                 no
Grid_score_vdw_scale                 1
Grid_score_es_scale                  1
Grid_score_grid_prefix               grid
Dock3.5_score_secondary              no
Continuous_score_secondary           no
Gbsa_zou_score_secondary             no
Gbsa_hawkins_score_secondary         no
Amber_score_secondary                no
Minimize_ligand                      yes
Simple_max_iterations                1000
Simple_max_cycles                    1
Simplex_score_converge               0.1
Simplex_cycle_converge               1.0
Simplex_trans_step                   1.0
Simplex_rot_step                     0.1
Simplex_tors_step                    10.0
Simplex_final_min_add_internal       no
Simplex_random_seed                  0
Atom_model                           all
Vdw_defn_file                        /home/pc/AS53software/dock6/parameters/vdw_AMBER_parm99.defn
flex_defn_file                       /home/pc/AS53software/dock6/parameters/flex.defn
 flex_drive_file                     /home/pc/AS53software/dock6/parameters/flex_drive.tbl
```

Table.5.22 nLC-MS Results Summary

Strain		NCTC 6571	NCTC 6571	NCTC 6571	MTCC 737	MTCC 737	MTCC 96	MTCC 96
MS Sample		**Control**	**Formulation (7.8 µg /ml CG + 62.5 µg /ml O)**	**Formulation (7.8 µg /ml CG+ 2000 µg /ml P)**	**Control**	**Formulation(31.5 µg /ml CG + 62.5 µg**	**Control**	**Formulation (7.8 µg /ml CG + 125**
Monoglycine Disaccharide	**Observed Mass**	1025.489	1025.49	1025.489	1025.488	1025.492	1025.489	1025.504
	FWHM	0.03	0.03	0.03	0.03	0.02	0.03	0.02
	Height	2733.0	1724.0	1173.0	14100.0	545.0	1985.0	504.0
	Peak Area	90.38	51.49	31.28	421.16	11.63	59.29	12.90
	Peak Area (%)	100	56.98	34.61	100	2.76	100	21.76
Triglycine Disaccharide	**Observed Mass**	1139.549	1139.55	1139.549	1139.563	1139.549	1139.534	1139.549
	FWHM	0.03	0.02	0.02	0.03	0.02	0.03	0.02
	Height	891.0	566.0	420.0	1311.0	311.0	1492.0	475.0
	Peak Area	27.56	14.49	10.30	34.96	6.97	42.97	11.65
	Peak Area (%)	100	1.90	2.67	100	5.02	100	3.69
Pentaglycine Disaccharide	**Observed Mass**	1253.601	1253.586	1253.585	1253.601	1253.601	1253.601	1253.601
	FWHM	0.03	0.02	0.02	0.02	0.02	0.03	0.02
	Height	1470.0	552.00	304.00	919.00	469.00	898.00	686.00
	Peak Area	47.04	14.13	6.81	23.53	10.01	23.95	16.10
	Peak Area (%)	100	3.33	6.91	100	2.35	100	1.49

Fig.6.1.Correlation Matrix of Active Site Prediction Tool Results

Software	Site No.	No. of Residues in specific site	Residue No. in Site	S1	S2	S7	QS1	QS4	QS5	QS7	QS8	PS1	PS6	PS7	PS8	PS9	PS10	CS10	CS13	CS17	CS23	CS25	CS26	CS31	CS32	CS35
				Sitehound			Qsite-finder					Pocket-finder						CASTp								
				S1=15	S2=13	S7=15	QS1=3	QS4=14	QS5=14	QS7=15	QS8=6	PS1=17	PS6=13	PS7=9	PS8=7	PS9=6	PS10=6	CS10=4	CS13=5	CS17=4	CS23=5	CS25=5	CS26=6	CS31=6	CS32=8	CS35=11
				186, 187, 188, 189, 190, 191, 192, 193, 195, 242, 251, 344, 347, 348, 351	48, 119, 367, 368, 369, 370, 371, 372, 376, 377, 380, 381, 384	9, 12, 13, 15, 16, 23, 26, 27, 28, 31, 231, 232, 235, 319, 321	118, 119, 120	12, 16, 23, 26, 27, 28, 29, 31, 231, 232, 233, 234, 235, 321	183, 186, 187, 191, 192, 193, 343, 344, 345, 347, 348, 351, 352, 355	22, 23, 24, 29, 33, 71, 73, 74, 108, 154, 224, 228, 232, 319, 328	149, 155, 327, 328, 329, 330	74, 149, 154, 158, 314, 315, 316, 327, 328, 329, 330, 331, 332, 337, 342, 345, 346	18, 19, 20, 74, 75, 76, 77, 78, 80, 109, 110, 111, 112	313, 332, 333, 336, 337, 340, 341, 342, 343	183, 186, 187, 343, 344, 347, 348	80, 81, 111, 128, 130, 131	150, 151, 152, 153, 154, 155	20, 76, 80, 110	74, 154, 155, 158, 364	348, 351, 352, 355	367, 368, 369, 370, 380	116, 120, 147, 157, 368	74, 108, 154, 156, 395, 396	12, 15, 16, 26, 27, 235	15, 16, 21, 22, 25, 26, 321, 322	118, 119, 369, 370, 371, 372, 374, 377, 380, 381, 384
Sitehound	S1	S1=15	186, 187, 188, 189, 190, 191, 192, 193, 195, 242, 251, 344, 347, 348, 351	1.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00
	S2	S2=13	48, 119, 367, 368, 369, 370, 371, 372, 376, 377, 380, 381, 384		1.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.06	0.00	0.00	0.00	0.38
	S7	S7=15	9, 12, 13, 15, 16, 23, 26, 27, 28, 31, 231, 232, 235, 319, 321			1.00	0.00	0.38	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.17	0.00
Qsite-finder	QS1	QS1=3	118, 119, 120				1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	QS4	QS4=14	12, 16, 23, 26, 27, 28, 29, 31, 231, 232, 233, 234, 235, 321					1.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.27	0.00
	QS5	QS5=14	183, 186, 187, 191, 192, 193, 343, 344, 345, 347, 348, 351, 352, 355						1.00	0.00	0.00	0.00	0.00	0.09	0.67	0.00	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.00	0.00	0.00
	QS7	QS7=15	22, 23, 24, 29, 33, 71, 73, 74, 108, 154, 224, 228, 232, 319, 328							1.00	0.13	0.22	0.09	0.00	0.00	0.00	0.13	0.00	0.05	0.00	0.00	0.00	0.10	0.00	0.11	0.00
	QS8	QS8=6	149, 155, 327, 328, 329, 330								1.00	0.29	0.00	0.00	0.00	0.00	0.17	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pocket-finder	PS1	PS1=17	74, 149, 154, 158, 314, 315, 316, 327, 328, 329, 330, 331, 332, 337, 342, 345, 346									1.00	0.00	0.00	0.00	0.00	0.14	0.00	0.14	0.00	0.00	0.00	0.14	0.00	0.00	0.00
	PS6	PS6=13	18, 19, 20, 74, 75, 76, 77, 78, 80, 109, 110, 111, 112										1.00	0.00	0.00	0.11	0.00	0.47	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00
	PS7	PS7=9	313, 332, 333, 336, 337, 340, 341, 342, 343											1.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	PS8	PS8=7	183, 186, 187, 343, 344, 347, 348												1.00	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00
	PS9	PS9=6	80, 81, 111, 128, 130, 131													1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	PS10	PS10=6	150, 151, 152, 153, 154, 155														1.00	0.00	0.18	0.00	0.00	0.00	0.17	0.00	0.00	0.00
CASTp	CS10	CS10=4	20, 76, 80, 110															1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	CS13	CS13=5	74, 154, 155, 158, 364																1.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00
	CS17	CS17=4	348, 351, 352, 355																	1.00	0.00	0.00	0.00	0.00	0.00	0.00
	CS23	CS23=5	367, 368, 369, 370, 380																		1.00	0.20	0.00	0.00	0.00	0.43
	CS25	CS25=5	116, 120, 147, 157, 368																			1.00	0.00	0.00	0.00	0.00
	CS26	CS26=6	74, 108, 154, 156, 395, 396																				1.00	0.00	0.00	0.00
	CS31	CS31=6	12, 15, 16, 26, 27, 235																					1.00	0.43	0.00
	CS32	CS32=8	15, 16, 21, 22, 25, 26, 321, 322																						1.00	0.00
	CS35	CS35=11	118, 119, 369, 370, 371, 372, 374, 377, 380, 381, 384																							1.00

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.

BUFFERS AND SOLUTIONS

0.5 Mc Farland
BaCl2 (0.048 M) 0.5 ml
H2SO4 (0.18 M) 99.5 ml
Saline

NaCl 0.85 g
DDW 100.0 ml
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

30% Glycerol Stock Solution
Glycerol 30ml
DDW 70ml
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

Digestion Buffer
(12.5mM Sodium dihydrogen Phosphate (PH 5.5))
NaH_2Po_4 2mg
DDW 2ml
Adjust pH 5.5 with H_3Po_4 (98%)
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

0.5 M Borax (Borate Buffer)
Borax 10gm
DDW 100ml
Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min

Reduction Solution
(10 mg/ml Sodium Borohydrate in 0.5 M borax, pH9)
$NaBH_4$ 2mg
Borate Buffer 200 µl

1M HCl
Concentration HCl 1ml
DDW 11ml

70% Ethanol
Absolute Alcohol 70ml
DDW 30ml

0.02% TTC
TTC 20µl
DDW 100ml

MOLECULAR BIOLOGY REAGENTS*

1 M Tris- HCl (pH 8.0)**
Tris- HCl 15.76 g
Sterile DDW 100 ml

0.1M Tris-HCL(pH 6.8)**
1M Tris HCL 10ml
DDW 90ml

0.25% SDS in 0.1M Tris-HCL

SDS	100mg
0.1M Tris-HCL	40ml

0.5 M EDTA (pH 8.0)**

EDTA	18.61g
Sterile DDW	100 ml

Adjust pH to 8.0 using 10 M NaOH

Tris EDTA (1X) **

Tris- HCl	10 mM
EDTA	1 mM

Lysozyme (100mg/ml)

Lysozyme	100 mg
Sterile DDW	1 ml

Proteinase K (20mg/ml)

Proteinase K	20 mg
Sterile DDW	1 ml

RNase A (10mg/ml)

RNase	10 mg
Sterile DDW	1 ml

Trypsin (50mg/ml)

Trypsin	50mg
DDW	1ml

DNase (1mg/ml)

DNase	1mg
DDW	1ml

RNase B (1mg/ml)

RNAse A(10X)	10ml
DDW	90ml

Solution B (15mg/ml DNase, 60mg/ml RNase in 0.1 M Tris-HCl pH 6.8)

DNase	15µl
RNase B	60 µl
0.1M Tris-HCL(pH 6.8)	925µl

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

Screening and Toxicity Analysis of Catechin Isomers Against FemA Protein

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Fem proteins are the essential structural proteins of various gram-positive bacteria. These are of three different types namely FemX (FmhB), FemA and FemB. Only two Fem protein crystallographic structures are available till date, one for FemA in *Staphylococcus aureus* and another for FemX in *Weissella viridescens*is. In this study, computational methods are used to evaluate interaction of FemA protein with catechin and epicatechin analogues. The interaction of FemA protein with catechin and epicatechin analogues are confirmed by binding energy and scores given by Autodock Vina and UCSF Dock docking softwares, which is followed by Lipinski filters and toxicity studies using online Lipinski server of SCFBIO and OSIRIS. Catechin gallate has been found as the best ligand for FemA protein in all aspects and it has outperformed all catechin and epicatechin isomers.

Key words: Fem, virtual screening, gram-positive, bacteria

Methicillin resistance in *Staphylococcus aureus* (MRSA) is a major cause of various nosocomial infections in humans, which are responsible for morbidity and morality. Target site of β-lactam antibiotics predominantly is PBP-2 protein. Production of additional low-affinity PBP-2a or PBP-2' protein by *mecA* chromosomal structural gene is responsible for the resistance of *S. aureus*. Fem genes are the other additional chromosomal genes, which are responsible for methicillin resistance[1]. Fem genes belong to FemXAB family and produces three proteins namely, FemX (fmhB), FemA and FemB. Fem proteins are responsible for sequential building of interpeptide bonds in cell wall of *Staphylococcus aureus.* FemX (fmhB) forms first glycine-lysine bond[2,3], followed by next two bonds by FemA and last two by FemB protein[4]. This penta-glycine chain is essential for integrity of cell wall of *S. aureus.* Inhibition of either of these proteins can acts as a target for new drug development against gram-positive bacteria[5].

In the present work, FemA has been considered as a potential target to identify inhibitors for overcoming resistance to β-lactam antibiotics in *S. aureus.* FemA is the only fem protein of *S. aureus* for which x-ray crystallography structure is available (1LRZA)[5]. It is composed of 426 amino acid residues and has a molecular weight of 50.27kDa.

Flavanoids like epicatechin gallate and epigallocatechin gallate, which are constituents of green tea have been reported to change the architecture of the cell wall of *S. aureus*[6,7]. Moreso, analogs of these compounds *viz*. cis and trans forms of catechin and epicatechin also exist as naturally occurring flavanoids. Generally the catechin comprise of three aromatic rings. The first two rings are benzene rings with hydroxyl groups while the third is dihydropyran heterocycle with hydroxyl group at carbon 3. As C_2 and C_3 are chiral, these structure exhibit (fig. 1) stereoisomerism. Scientists have synthesized various analogues by substitution of these hydroxyl groups with other alkyl groups or other chemical moiety. For example by addition of gallate group at C3 catechin gallate, epicatechin gallate, EGCG, has been formed, which are stored in various

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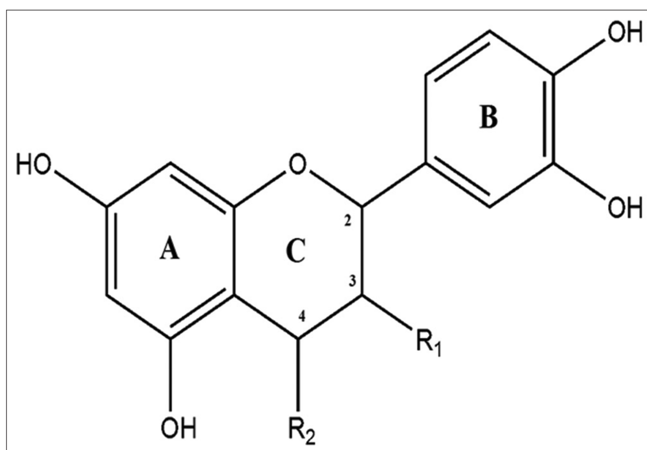

Fig. 1: Structure of catechin
Structure of catechin showing two benzene rings A and B with two hydroxyl groups each and a dihydropyran heterocycle ring C with two R groups (viz. R1 and R2) at C3 and C4 position, except catechol where rings B and C are missing. Catechin gallate is R1 is -H and R2- is 3,4,5-trihydroxybenzoyl group and procyanidin B2 contains R1 is -OH and R2 is catechinyl group.

structure databases like Zinc[8] and Pubchem[9]. Thus in this study, all synthetic and semi-synthetic analogs of catechins have been evaluated as inhibitor to assess their potential to bind with FemA protein.

MATERIALS AND METHODS

Ligand and protein preparation:
FemA protein having 1lrz PDB id was retrieved from RCSB-Protein data bank (www.rcsb.org). Downloaded 1lrz structure was prepared for docking in mol2 and pdbqt format files by deleting all hetero atoms and water molecules by chimera[10] and MGL[11] tools, respectively. Pdb format file of 1lrz having no hydrogen atom was also prepared by Chimera. This file was used as input for generation of ms format file by DMS tool. Similarly, ms format file was used to generate sph format file by sphgen tool.

Ligand structures were retrieved from Pubchem database. Pubchem database consists of 15 catechin and epicatechin analogues, which are available in chemical form. All these molecules were downloaded in sdf format. Similar to protein all ligands were prepared in mol2, pdbqt and pdb format.

Docking studies:
It was carried out by two tools, Autodock Vina and UCSF dock. Autodock Vina[12] uses pdbqt input files both for ligands as well as protein. Center grid coordinates used in this study, for Autodock Vina were 35.829×63.416×94.89 while size used for x, y, z were 76×102×20, respectively. Spacing used in Autodock Vina was 1.000 at different exhaustiveness values. While, in case of UCSF Dock 6.5[13] mol2 files were used and moreover instead of using user defined center coordinates, different sphere clusters were calculated by sphgen software. Sphgen software used dms software output file, which consists of molecular surface of receptor. Total 78 clusters were calculated, out of these, cluster 1 having 111 spheres with their coordinates was used for generation of grid and box files for 1lrz protein. Docking was performed with all ligand files using grid spacing of 0.3 A°.

Rigid docking was performed by both the softwares. The docking orientations are ranked based on a molecular mechanics-like scoring function known as Dock score in case of UCSF Dock, binding energy in case of Autodock Vina. Protein residues forming hydrogen and hydrophobic interactions with ligand molecule is further calculated by Ligplot+ software[14].

Toxicological studies:
To determine the toxic level of best FemA inhibitors, toxicity studies was performed by online server, OSIRIS property explorer[15] (http://www.organicchemistry.org/prog/peo/) during toxicological analysis various *in silico* tests were performed to detect the risk of mutagenicity, carcinogenicity, teratogenicity, irritants and reproductive effects. These predictions depend on comparison between precomputed set of structural moieties whose properties are already known with structural moieties of molecules loaded by user on server.

Lipinski filters:
Lipinski filters or Lipinski rule of five distinguishes between drug like and nondrug like molecules. It is also known as Pfizer's rule of Five. It is a thumb rule to predict the probability of a chemical to be orally active drug in humans. According to this rule molecular mass of molecule should be less than 500 Dalton, lipophilicity represented by LogP should be less than 5, hydrogen bond donors (HBD) should be less than 5, hydrogen bond acceptors (HBA) should be less than 10 and molar refractivity (MR) should be lies between 40-130. Molecule failing in more than 2 rules is considered as nondrug molecule. Lipinski filtering was performed by Lipinski filter tool of SCFBIO at IITDelhi server[16].

RESULTS AND DISCUSSION

Docking of FemA protein with catechin and epicatechin analogues have exhibited 100% results with Autodock Vina while 50% with UCSF DOCK. Ligands were ranked on the basis of binding energy and grid score given by Autodock Vina and UCSF Dock 6.5, respectively. As shown in Table 1 both docking tools has predicted (-)catechin gallate with cid 6419835 as best inhibitor having best binding affinity of -8.7 kcal/mol with Autodock Vina and best dock score of -45.19 with UCSF Dock among all 15 analogues. Best conformations of best ligands predicted by both the softwares are shown in fig. 2a and b.

FemA protein structure consists of one helical arm and one globular domain. Globular domain is further divided into two sub-domains and some secondary structure elements. Helical arm is formed of residues 246-307 and in case of globular domain residues 1–110, 129–144, 396–401 forms first sub-globular domain, and residues 145–166, 189–245and 308–395 forms second sub-globular domain and rest 111–128, 167–188, and 402–412 residues are part of secondary structure elements[5]. Globular domain is similar to HAT domain of *Tetrahymena* GCN5. Globular domain of FemA protein consists of five additional structural elements that are not found in the structure of GCN5. Two β strands that extends from first sub-domain of globular domain and two α helices that lies on the top of second sub-domain of globular domain and one more C-terminal α helix. HAT domain of *Tetrahymena* GCN5 binds with two substrates: Coenzyme A and Peptide[17]. Similar pockets also exist in both sub-domains of globular domain of FemA protein. These pockets form a deep L-shaped channel, which is a suitable site of binding for FemA protein in *S. aureus*[5]. Helical domain of FemA protein is similar to seryl-tRNA synthetases in bacteria[18,19]. Function of helical domain in FemA and seryl-tRNA synthetases is just to holding of charged amino acid t-RNA molecule during addition of glycine in penta-glycine chain[20-23]. Therefore it is not available for inhibitors, only pockets in L-shaped channel of sub-domains of globular protein is available for inhibitors (fig. 2e).

As shown in fig. 2c and d according to both the softwares catechin gallate is binding exactly between the active sites in L-shaped channel of first sub-globular domain of FemA protein. Best ligand conformation predicted by both the docking tools were further used to form complex with protein and these were further used with Ligplot+ to calculate hydrogen and hydrophobic interactions with protein residues. As shown in fig. 3a, according to conformation given by Autodock Vina catechin gallate form hydrogen bonds with GLY 330, LYS 383, PHE 363, ASP 150 residues of FemA protein with distance of 3.18, 3.03, 3.03 and 3.09, respectively (shown in green color), while residues like GLY 365, ALA 329, TYR 327, TYR 328, ILE 155, TYR 364, GLN 154, LEU 153, PHE 149 forms hydrophobic interactions. Similarly, UCSF Dock predicted conformation forms hydrogen bond with

TABLE 1: DOCKING RESULTS OF AUTODOCK VINA AND UCSF DOCK

CID of Pubchem	Name	Grid_score	Binding affinity (kcal/mol)	Dock rank	Vina rank
6419835	(-)-catechin gallate	−45.195	−8.7	1	1
73160	(-)-catechin; catechin L-form	−34.034	−8.7	6	2
5276454	(-)-catechin gallate; (2R,3S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3, 4-dihydro-2Hchromen-3-yl 3,4,5-trihydroxybenzoate; XEG	−44.642	−8.5	2	3
122738	Procyanidin B2; Procyanidol B2	Out of grid	−8.2	Nil	4
182232	(+)-epicatechin; 35323-91-2; ent-epicatechin	−37.057	−8.1	4	5
12309507	L-epicatechin	−34.499	−8.1	5	6
9064	Catechin; cianidanol; (+)-Catechin; D-caechin;(+) cyanidanol	Out of grid	−8.1	Nil	7
65064	(-)-epigallocatechin gallate	Out of grid	−8	Nil	8
107957	Catechin hydrate;(+)-catechin hydrate	Out of grid	−7.7	Nil	9
107905	Epicatechin gallate; (-)-epicatechin gallate; (-)-epicatechin-3-gallate	−43.823	−7.6	3	10
367141	(-)-epicatechin gallate; NSC636594	Out of grid	−7.4	Nil	11
1203	DL-catechin; NSC81746; L-epicatechin	Out of grid	−7.3	Nil	12
72276	Epicatechin; (-)-epicatechin	Out of grid	−7.3	Nil	13
255538	L-Epicatechin; epicatechol	Out of grid	−7.3	Nil	14
289	Catechol; pyrocatechol	−20.687	−4.1	7	15

CID: Compound identifier

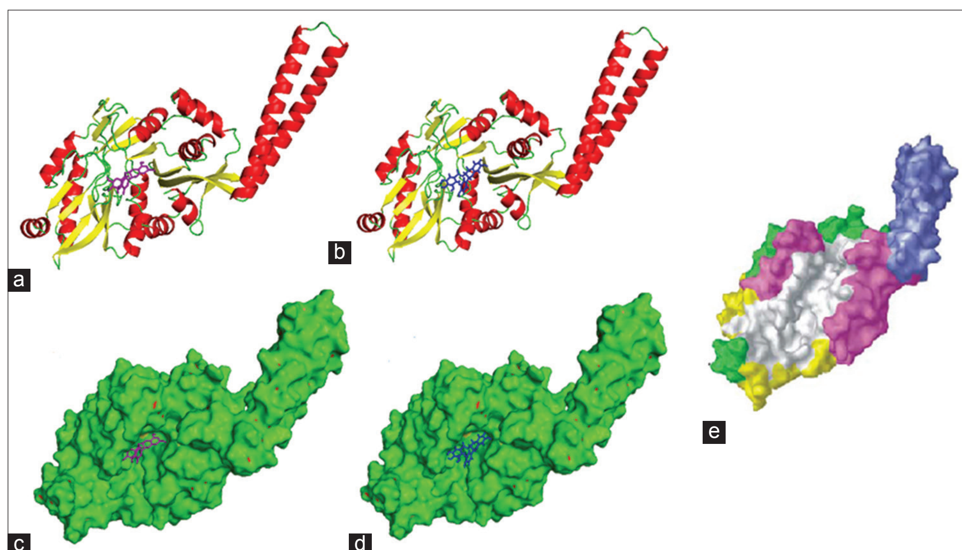

Fig. 2: Docking results of Catechin Gallate with FemA protein.
(a) Autodock Vina result in cartoon view (b) UCSF Dock result in cartoon view (c) Autodock Vina result in surface view (d) UCSF Dock result in surface view (e) active site of FemA protein in L-shaped white color domain in surface view.

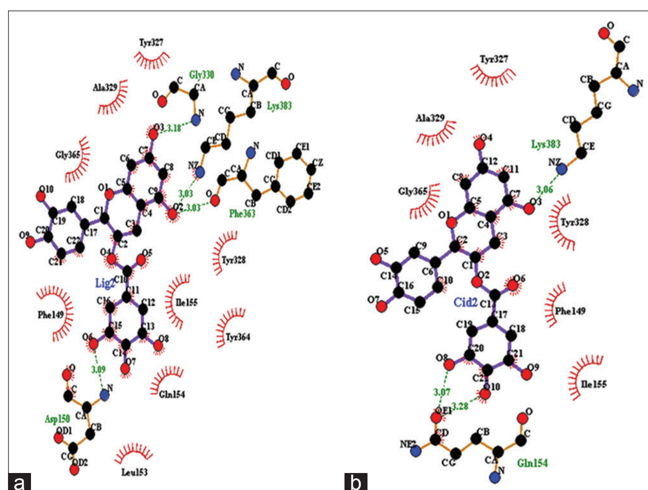

Fig. 3: Interaction of Catechin Gallate with different residues of FemA protein given by LIGPLOT tool.
(a) Autodock vina result file (b) UCSF Dock result file.

LYS 383 and GLN 154 with bond distance 3.06, 3.07 and 3.28, respectively, while there are six residues, which forms hydrophobic interactions, residues are GLY 365, ALA 329, TYR 327, TYR 328, PHE 149 and ILE 155 (fig. 3b). Both softwares predicted catechin gallate as most efficient ligand for FemA protein as compared to other catechin and epicatechin derivatives or flavanoids and have shown LYS 383 residue of FemA protein as a common residue, which forms hydrogen bond and all 6 hydrophobic interacting residues, which were predicted by UCSF Dock were also predicted in Autodock Vina result. It appears that LYS 383 is the most essential residue of FemA protein for interaction with catechin gallate.

Toxicity analysis was performed by OSIRIS tool. According to OSIRIS if score for any category is 1, then it indicates no risk, if it lies between 0.6-0.8 then the chemical may have medium risk but if score is less than 0.6 it is considered as high risk chemical. As shown in Table 2 all the analogues except catechol (s=0.6), have score equal to one for all four risk categories *viz.*-mutagenicity, tumorigenicity, irritation and reproductive effects. Similarly as predicted by other two categories of OSIRIS drug likeness and drug score, which determine whether particular molecule is similar to the known drugs, same molecule catechol was discarded due to poor scores and rest all the molecules are non-hazardous as well as similar to good drugs. Therefore, except catechol; rest 14 molecules are safe and can be used as future drugs. In toxicity analysis catechin gallate has passed all the tests, therefore it may be a ray of hope for various health problems caused by gram-positive bacteria.

As shown in Table 3, two molecules have violated two Lipinski rules, while 6 molecules have violated one rule and rest seven have not violated any Lipinski rule. All the violations are shown in red color and summarized in last category under number of Lipinski value (LV). Pyrocyanidin B2 and EGCG have high chances of failure in preclinical and clinical trials, while rest 13 molecules seems to have very less or no chances of failure.

Ligand molecule catechin gallate Pubchem cid 6419835 has been predicted as a potential inhibitor

TABLE 2: TOXICITY RESULTS PREDICTED BY OSIRIS

CID of Pubchem	Name	Drug likeliness	MR	TR	IE	RE	Drug score
6419835	(-)-catechin gallate	2.81 (score=0.94)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.74
73160	(-)-catechin; catechin L-form	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
5276454	(-)-catechin gallate	2.81 (score=0.94)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.74
122738	Procyanidin B2	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.534
182232	(+)-epicatechin; 35323-91-2; ent-epicatechin	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
12309507	L-epicatechin;	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
9064	Catechin; cianidanol; (+) catechin; (+) cyanidanol	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
65064	(-)-epigallocatechin gallate	1.57 (score=0.83)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.69
107957	Catechin hydrate; (+) catechin hydrate	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
107905	Epicatechin gallate; (-)-epicatechin gallate	2.81 (score=0.94)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.74
367141	(-)-epicatechin gallate; NSC636594	1.39 (score=0.8)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.68
1203	DL-catechin; NSC81746; L-epicatechin	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
72276	Epicatechin; (-)-epicatechin	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
255538	L-epicatechin; epicatechol	1.92 (score=0.87)	No (score=1)	No (score=1)	No (score=1)	No (score=1)	0.86
289	Catechol; pyrocatechol	−2.25 (score=0.09)	Yes (score=0.6)	Yes (score=0.6)	Yes (score=0.6)	No (score=1)	0.112

CID: Compound identifier, MR: mutagenicity risk, TR: tumorigenicity risk, IE: irriating effects, RE: reproductive effects

TABLE 3: LIPINSKI RESULTS

CID of Pubchem	Name	Molecular weight	HBA	HBD	LogP	MR	Number of LV
6419835	(-)-catechin gallate	442	3	7	3	96.3	1
73160	(-)-catechin; catechin L-form	290	1	5	0.52	64.6	0
5276454	(-)-catechin gallate; (2R,3S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3, 4-dihydro-2Hchromen-3-yl 3,4,5-trihydroxybenzoate	442	3	7	3	96.3	1
122738	Procyanidin B2; procyanidol B2;	578	2	10	0.9	108	2
182232	(+)-epicatechin; 35323-91-2; ent-epicatechin	290	1	5	0.52	64.6	0
12309507	L-epicatechin	290	1	5	0.52	64.6	0
9064	Catechin; cianidanol; (+)-catechin; D-catechin;(+) cyanidanol	290	6	5	0.4	NA	0
65064	(-)-epigallocatechin gallate	458	11	8	1.2	NA	2
107957	Catechin hydrate; (+)-catechin hydrate	308	7	6	NA	NA	1
107905	Epicatechin gallate; (-)-epicatechin gallate; (-)-epicatechin-3-gallate	442	3	7	3	96.3	1
367141	(-)-epicatechin gallate; NSC636594	442	10	7	1.5	NA	1
1203	DL-catechin; NSC81746; L-epicatechin	290	6	5	0.4	NA	0
72276	Epicatechin; (-)-epicatechin	290	1	5	0.52	64.6	0
255538	L-epicatechin; epicatechol	290	1	5	0.52	64.6	0
289	Catechol; pyrocatechol	110	0	2	1.1	29.8	1

CID: Compound identifier, HBA: hydrogen bond acceptors, HBD: hydrogen bond donors, MR: molar refractivity, LV: Lipinski value, NA: not available

of FemA protein; by two best tools in terms of binding affinity and docking scores and has also shown nontoxic behavior in toxicity tests and also follows Lipinski rule of five. Therefore, if it can be analysed in *in vitro* studies and chances of failure in preclinical and clinical trials seem to be very less and it can be a ray of hope for the treatment of various *S. aureus* infections.

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There are no conflicts of interest.

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Research article

Catechin gallate a promising resistance modifying candidate to potentiate β-lactam antibiotics to overcome resistance in *Staphylococcus aureus*

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ABSTRACT

Drug discovery is a cost intensive and time consuming approach therefore contingent alternatives are being explored to combat drug resistant bacteria which cause chronic infections. Resistance modifying agents offer plausible alternatives in reviving the current antimicrobial drugs thereby making them effective to be used in clinical settings. We for the first time report the promising activity of (−)-Catechin gallate selected using *in silico* studies with *FemA* protein of *Staphylococcus aureus*. The MIC of the test isolates of *S. aureus viz.* NCTC 6571, MTCC737 and MTCC 96 by visual method was 500 μg and 8000 μg for Oxacillin and Penicillin respectively. (−)-Catechin gallate induced an 8-fold reduction in the MIC of Oxacillin in *S. aureus* NCTC 6571 and MTCC 737 at concentration of 7.8 μg/ml and 31.25 μg/ml respectively. A 4-fold reduction, in MIC of Penicillin and Oxacillin at concentration of 7.8 μg/ml of (−)-Catechin gallate in NCTC 6571 and MTCC 96 respectively. These synergistic combinations in time kill assay exhibited a reduction between 2 and 6 $\log_{10}$ in the CFU of the test microbes thereby suggesting (−)-Catechin gallate to be a promising candidate for further evaluation as a resistance modifying agent to overcome resistance to first generation of β-lactam antibiotics.
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1. Introduction

Discovery of Penicillin in the twentieth century revolutionized the field of medicine and saved countless lives by combating bacterial infections.[1] However, just after four years of its introduction, *Staphylococcus aureus* expressed resistance to Penicillin by production of a β-lactamase enzyme. An analogue of Penicillin, Methicillin/Oxacillin was developed to overcome the β-lactamase resistant *S. aureus*. However with the widespread use of oxacillin/methicillin, antibiotic resistant strains emerged which were referred as Methicillin Resistant/Oxacillin Resistant *Staphylococcus aureus* (MRSA/ORSA).[2]

Antibiotic resistance today is a burgeoning phenomenon due to indiscriminate and inappropriate use of antibiotics which is encountered by the clinicians across the globe. Chronic infections caused by these superbugs are a major cause of mortality and morbidity in the community as well as hospitals.[3] The currently described strains of *S. aureus* exhibit resistance to different classes of antibiotics comprising of all β-lactams, macrolides, aminoglycosides, tetracyclines, rifampicin, quinolones and glycopeptide like vancomycin.[4] *S. aureus* shows extreme genetic plasticity and hence, it is currently developing resistance towards all known classes of antibiotics. However some strains are becoming multi-antibiotic resistant strains thereby posing a greater difficulty and threat as infections caused by them are not retractable/chronic and prove fatal.

Discovery and development of new antibiotics is a time and cost intensive proposition. Therefore one of the best strategies to control bacterial resistance is to extend the life of currently used antibiotics by associating them with modulators of resistance.[5] Resistance modifying agents (RMA's) like Clavulanic acid have been successfully formulated with β-lactam antibiotic Amoxicillin for overcoming resistance to *S. aureus* isolates expressing serine β-lactamases.[6] However, β-lactam resistance mediated through PBP2a in MRSA is not reverted by Clavulanic acid.[7] Hence, there is a need to explore novel RMA's targeting bacterial growth pathways and resistance mechanisms.

FemA is an essential protein which plays a prominent role in formation of interpeptide bonds in the pentaglycine linkages which are essential for the integrity of the cell wall of *S. aureus*.[8] Thus, inhibition of *FemA* could serve as a new site of action for

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potentiating the action of β-lactam antibiotics. Flavonoids like Epicatechin gallate and Epigallocatechin gallate which are constituents of green tea that have been reported to change the architecture of cell wall of *S. aureus*[9] and we have reported that (−)-Catechin gallate (CG) exhibits the best inhibition potential of *FemA* protein as compared to Epicatechin gallate and catechin analogues during *in silico* screening of Catechin library from PubChem database as inhibitors of *FemA*.[10] Thus, the present study was proposed to evaluate the role of (−)-Catechin gallate as a possible synergist of β-lactam antibiotics Penicillin and Oxacillin.

2. Materials and methods

2.1. Bacterial strains and CG

Three standard strains of *Staphylococcus aureus viz.* NCTC6571, MTCC737 and MTCC96 were used in the present study. The bacterial isolates were stored in 30% glycerol stock and frozen at −70 °C. These were recovered and sub-cultured on Mueller Hinton broth (MHB) and Mueller Hinton Agar (MHA) (HiMedia, India). Stocks of the cultures were maintained on MHA at 4 °C throughout the study. Sodium salts of antibiotics Oxacillin and Penicillin were procured from HiMedia, Mumbai. CG used in the study was obtained from Sigma Aldrich and dissolved in DMSO prior to use.

2.2. Antimicrobial activity by *in vitro* microbroth dilution method

The minimal inhibitory concentration (MIC) of Penicillin, Oxacillin and CG were determined using *in vitro* microbroth dilution assay using a 96 well titer Plate.[11] Briefly 16–20 h old culture suspensions of all the test microorganisms were adjusted with 0.5 McFarland solution using sterile physiological saline (0.85% NaCl) to achieve a final bacterial count of approximately 10^5 CFU/ml. 50 μl of this bacterial suspension was then added to 125 μl MHB in control cells as well as test cells. The titer-plates were then incubated at 37 °C for 3 h and then antibiotics (Penicillin/Oxacillin) and the test compound were added to have an effective concentration between 500 μg/ml and 3.9 μg/ml per well. After 24 h of incubation 30 μl of 0.02% Triphenyl Tetrazolium Chloride (TTC) was added as indicator in all wells and then MIC was observed. All concentrations were tested in triplicates.[11]

2.3. Checkerboard assay and FIC index

Checkerboard assay was carried out to evaluate the synergistic potential of CG with Penicillin and Oxacillin in different concentrations for possible use as a formulation. The synergistic activity was determined by the FIC index.[12] Five different dilutions 2MIC, MIC, 1/2MIC, 1/4MIC & 1/8MIC of antibiotics and test compound

Table 1
MIC, FIC and FICI of Penicillin and Oxacillin with Catechin gallate (Cg) against standard cultures of *Staphylococcus aureus*.

Test isolate	Drug Combination (A/B)	MIC – Drug A (μg/ml)	MIC – Drug B (μg/ml)	Combination MIC (A/B) (μg/ml)	FIC-Drug A	FIC-Drug B	FICI	Interpretation	MIC reduction (folds)
NCTC 6571	CG/Oxacillin	62.5	500	125/62.5	2	0.125	2.125	Indifference	
				62.5/62.5	1	0.125	1.125	Indifference	
				31.25/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				15.6/62.5	0.25	0.125	0.375	Synergy	8 fold
				7.8/62.5	**0.125**	**0.125**	**0.25**	***Synergy***	**8 fold**
	CG/Penicillin	62.5	8000	125/2000	2	0.25	2.25	Indifference	
				62.5/4000	1	0.5	1.5	Indifference	
				31.25/2000	0.5	0.25	0.75	Partial synergy	4 fold
				15.6/4000	0.25	0.5	0.75	Partial synergy	2 fold
				7.8/2000	**0.125**	**0.25**	**0.375**	**Synergy**	**4 fold**
MTCC 737	CG/Oxacillin	125	500	250/62.5	2	0.125	2.125	Indifference	
				125/62.5	1	0.125	1.125	Indifference	
				62.5/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				31.25/62.5	**0.25**	**0.125**	**0.375**	**Synergy**	**8 fold**
				15.6/125	0.125	0.25	0.375	***Synergy***	4 fold
	CG/Penicillin	125	8000	250/1000	2	0.125	2.125	Indifference	
				125/2000	1	0.25	1.25	Indifference	
				62.5/4000	0.5	0.5	1	Additive	2 fold
				31.25/4000	0.25	0.5	0.75	Partial synergy	2 fold
				15.6/4000	0.125	0.5	0.625	Partial synergy	2 fold
MTCC 96	CG/Oxacillin	62.5	500	125/62.5	2	0.125	2.125	Indifference	
				62.5/62.5	1	0.125	1.125	Indifference	
				31.25/62.5	0.5	0.125	0.625	Partial synergy	8 fold
				15.6/125	0.25	0.25	0.5	Synergy	4 fold
				7.8/125	**0.125**	**0.25**	**0.375**	***Synergy***	**4 fold**
	CG/Penicillin	62.5	8000	125/2000	2	0.25	2.25	Indifference	
				62.5/4000	1	0.5	1.5	Indifference	
				31.25/4000	0.5	0.5	1	Additive	2 fold
				15.6/4000	0.25	0.5	0.75	Partial synergy	2 fold
				7.8/4000	0.125	0.5	0.625	Partial synergy	2 fold

Bold values indicate the best synergistic combination.

combinations were used for this experiment. The concentration range of antibiotics for the checkerboard assay was between 1000 μg/ml and 62.5 μg/ml except for Penicillin against *S. aureus* NCTC 6571 which exhibited a MIC of 8000 μg/ml and hence tested between 16000 μg/ml and 1000 μg/ml. The antibiotics were dispensed horizontally in a concentration gradient across the rows of the microtiter plate while the test compound (RMA) was dispensed vertically using a two-fold dilution method between the concentration of 250 μg/ml and 7.8 μg/ml. Each well was dispensed with 125 μl MH broth and 50 μl of culture inoculum having 10^5 CFU/ml. The combinations of antibiotics in the wells were dispensed after 3 h of incubation. All the combinations were tested in triplicates.[13,14] Further FIC Index was calculated using following equation: $\sum$ FIC = FIC (A) + FIC (B) where FIC (A) = (MIC A in presence of B/MIC A alone) and FIC (B) = (MIC B in the presence of A/MIC B alone). Correlation between FIC and the effect of the combination of antibacterial agents was indicated: Synergy ≤ 0.5, Partial synergy > 0.5–1, Indifference >1 to <2 and Antagonism ≥ 2.[15]

2.4. Time kill assay

The time kill assay was used to ascertain the efficacy of kill of the bacteria in terms of reduction in the CFU's. It was performed only in the cases exhibiting a synergistic or additive effect of the RMA with the antibiotic by the checkerboard method. Miles and Misra plate count method[16] was used to ascertain the bacterial count of different treatments. Briefly, the method comprised of withdrawing a 20 μl aliquot form each test sample under aseptic conditions using sterile tips at different time intervals *viz.* 0, 6, 12, 18 and 24 h. The aliquots were placed on a MH plate as a single drop and observed for the growth of bacteria as pin head colonies after incubation of 12–18 h at 37 °C. These were counted to arrive to CFU/ml of the test sets at different time intervals.

3. Results

3.1. Antimicrobial susceptibility by *in vitro* microbroth dilution method

The Minimal inhibitory concentration as per CLSI/EUCAST guidelines using *in vitro* microbroth dilution assay is defined as the lowest concentration of antibiotic that completely inhibits the growth of the microorganism by the naked eye. In the present study all the three model test microorganism's exhibited a MIC of 500 μg/ml for Oxacillin. In case of Penicillin, *S. aureus* NCTC 6571, *S. aureus* MTCC737 and *S. aureus* MTCC 96 exhibited a MIC of 8000 μg/ml. Catechin gallate exhibited a MIC of 62.5 μg/ml for *S. aureus* NCTC 6571 and MTCC 96 while for MTCC 737 the MIC was 125 μg/ml (Table 1).

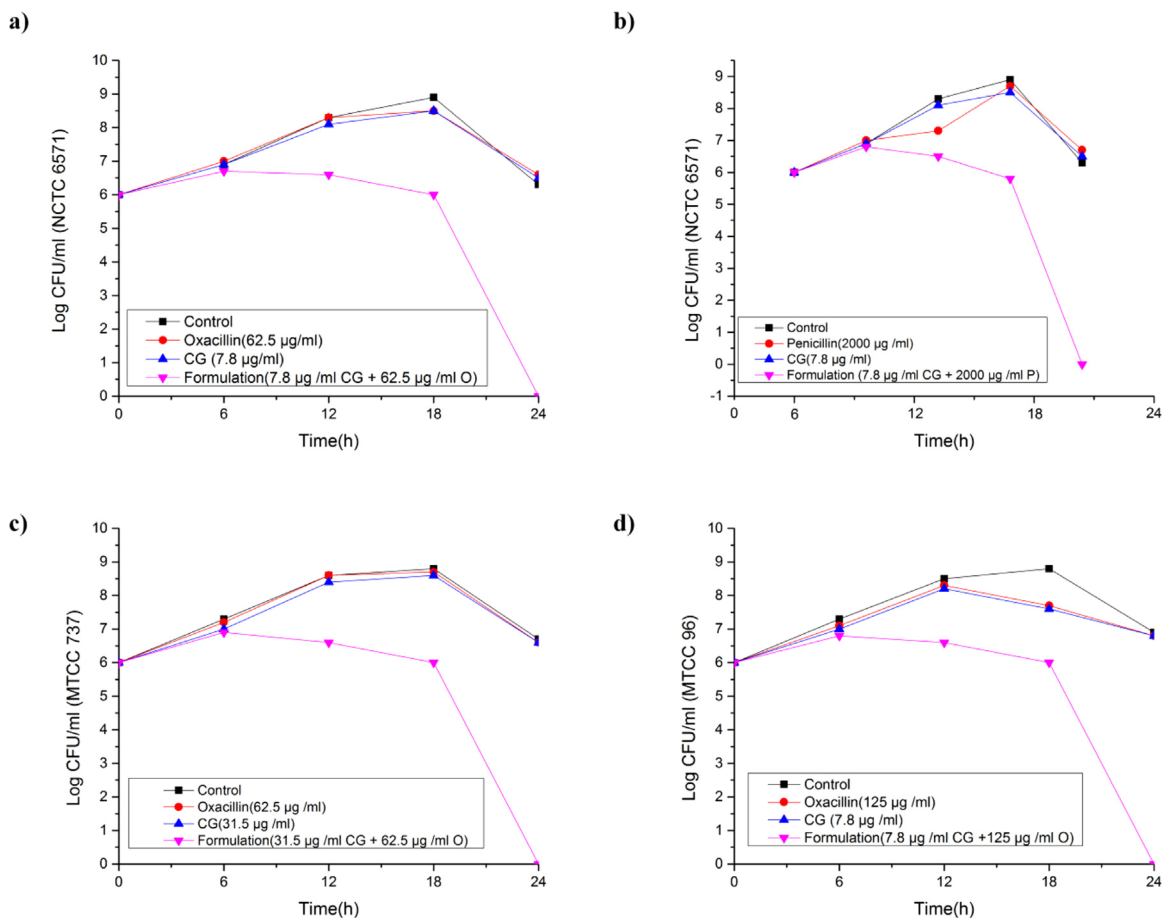

Fig. 1. Time Kill Kinetics of (a) *S. aureus* NCTC 6571 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG (7.8 μg/ml/62.5 μg/ml) (b) *S. aureus* NCTC 6571 alone, with penicillin, with CG and synergistic FIC combination of penicillin and CG (7.8/2000 μg/ml) (c) *S. aureus* MTCC 737 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG (31.25/62.5 μg/ml) (d) *S. aureus* MTCC 96 alone, with oxacillin, with CG and synergistic FIC combination of oxacillin and CG (7.8/125 μg/ml).

3.2. Checkerboard assay and FIC index

Based on the checkerboard assay and FICI, it was found that (−)-Catechin gallate gave synergistic effect in Oxacillin as well as Penicillin when tested against *S. aureus* NCTC 6571, while *S. aureus* MTCC 737 and MTCC 96 exhibited enhanced susceptibility only in the case of synergistic combinations between (−)-Catechin gallate and oxacillin. Penicillin susceptibility was not increased in the case of MTCC 737 and MTCC96. (−)- Catechin gallate at a concentration of 7.8 μg/ml enhanced the susceptibility of Oxacillin and Penicillin by reducing the MIC by 8 folds and 4 folds respectively (Table 1). Similarly the MIC of Oxacillin was reduced by 8 folds and 4 folds against MTCC 737 and MTCC96 (Table 1). However Penicillin only exhibited partial synergy or indifference when tested in different combination with oxacillin in case of MTCC 737 and MTCC96.

3.3. Time kill assay of synergistic formulations

The time kill assay of synergistic formulations was carried out to see the extent of improvement in the susceptibility of the antibiotic by measuring the reduction in viable counts of the test bacteria. The combination of 7.8 μg/ml and 62.5 μg/ml of (−)-Catechin gallate and oxacillin respectively in NCTC 6571 exhibited an 8 fold reduction in MIC of oxacillin, which exhibited a 2-$\log_{10}$ reduction in CFU at 12 h and 3-$\log_{10}$ reduction at 18 h. However after 24 h it exhibited a 6-$\log_{10}$ reduction in CFU count (Fig. 1a). The synergistic combination of (−)-Catechin gallate (7.8 μg/ml) and penicillin (2000 μg/ml) enhanced the susceptibility of *S. aureus* NCTC 6571 to penicillin by reducing the MIC by 4 folds. Further this combination induced a 2-Log_{10} reduction in CFU after 12 h and. 3-Log_{10} reduction by 18 h and finally exhibited a 6.5-Log_{10} reduction after 24 h (Fig. 1b). However in case of *S. aureus* MTCC 737, 2 Log_{10} reduction in CFU was observed after 12 h when compared to the control. This further reduced to 3.2 $\log_{10}$ CFU counts after 18 h and by 24 h it exhibited a 6.7 $\log_{10}$ reduction in CFU count (Fig. 1c). While *S. aureus* MTCC96 exhibited a 2.1 $\log_{10}$ reduction after 12 h approx. 3 $\log_{10}$ reduction in CFU count after 18 h and 6.9 $\log_{10}$ CFU reduction after 24 h (Fig. 1d).

4. Discussion

Antimicrobial drug resistance is a burgeoning problem due to the genomic plasticity of pathogenic microbes, in particular *Staphylococcus aureus* which rapidly develops mechanisms to evade the currently used armamentarium of antibiotics. Today *S. aureus*, more specifically MRSA (methicillin resistant *Staphylococcus aureus*) has been considered among the dreaded bacterial pathogens responsible for chronic infections in the hospital and community settings.[4] Thus, there is an ongoing war between the clinicians and these superbugs to develop methods to overcome their resistance. Moreover finding a novel antimicrobial drug target and developing new antimicrobial molecules altogether is an expensive and time consuming process. Natural products have been a savior as they provide ample opportunities by interfering with these mechanisms which drug resistant bacteria develop to evade the antibiotics. Thus in the present study we explored the potential role of Catechin gallate (CG) as a possible synergist to re-potentiate the first generation β-lactam antibiotics like Penicillin and Oxacillin using standard cultures of *Staphylococcus aureus*. The selection was based on our previous work wherein we screened the molecules which could inhibit the *FemA* protein of *S. aureus*, which plays a critical role in formation of interglycine linkages during the bacterial cell wall formation apart from being implicated to be highly expressed in MRSA and its multidrug variants.[8,10]

Epicatechin and Epicatechin gallate have been found to abrogate β-lactam resistance in MRSA by different mechanisms, predominantly affecting the cell wall architecture of *S. aureus* by getting intercalated.[17,18] Majority of the studies carried till date has been focused on Epicatechin and gallates of Epicatechin as main flavonoids involved in drug resistance reversal. However, only few studies have been undertaken on the antimicrobial or drug resistance reversal properties of Catechin. Catechin hydrate has been previously reported to inhibit urease enzyme in *Staphylococcus saprophyticus*, hence it was evaluated for its antimicrobial/resistance modifying potential. However, it was proved that (+)-catechin was not an effective RMA for β- lactam antibiotics to overcome *S. aureus* though it effectively reversed the resistance of MRSA to Clindamycin and Erythromycin.[19]

5. Conclusion

Based on our previous docking studies we found (−)-Catechin Gallate as the best inhibitor of *FemA* protein. In this study it was also observed that (−)-Catechin gallate was effective in reducing the MIC of Oxacillin in a synergistic manner in the three standard *S. aureus* isolates tested. Significant reduction of 6 $\log_{10}$ was observed in CFU count between 12 and 24 h as compared to control. Hence the present study holds a promise in further evaluating (−) Catechin gallate as a prospective candidate for overcoming resistance to first and second generation β-lactam antibiotics on more number of clinical isolates for its possible use as a resistance modifying agent. The *in vitro* MIC of the antibiotics as well as (−) Catechin Gallate can further be extrapolated by dose calculations which requires the establishing of the C_{max} value which is essentially the highest concentration in plasma and generally is the calculated a product of MIC $\times 2^{\text{(doseinterval/half-life)}}$. Further the dose can be calculated by using the formula (C_{max}. V_d)/F where V_d refers to the volume of distribution of the drug and F is the systematic availability. This would be helpful in carrying out the pre-clinical studies.

Conflicts of interest

The authors have none to declare.

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Comparative Analysis of Active Site Prediction Tools for *FemA* Protein

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Fem proteins are the essential proteins of peptidoglycan layer of gram-positive bacteria. They are responsible for crosslinking of peptide chains of different monomer units by forming a pentaglycineinterpeptide bridge by using glycyl t-RNA as Gly donor. These peptidyltransferases are of three kinds namely *FemA, FemB* and *FemX. FemX*uses lipid II exclusively as acceptor for the first Gly residue. While addition of second & third Gly was catalyzed by *FemA* and fourth, fifth Gly by *FemB*, and both enzymes were specific for lipid II-Gly(1) and lipid II-Gly(3) as acceptors. Crystallographic structure of only *FemA* protein is available till date. Identification of active site is essential for inhibiting the function of protein. In this study various computational tools were used to calculate the active site of *FemA* protein. But no good correlation was found among these computational tools.

Keywords: Fem, gram-positive, active site

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