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Novel Strategies for Wound Treatment

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ABSTRACT

Conventional wound care involves topical applications of antibiotics, oral antibiotics and silver containing bandages. Traditional wound care merely treats wound infections while allowing body's immune system to take charge of the natural healing process. It is common during the treatment of wounds to encounter persistence of drug resistant infections caused by presence of biofilms and delay of wound healing caused by antimicrobial agents applied to treat wounds. As wound healing is a complex process, wound care cannot be a single dimensional entity. Modern approach proposed in this study of wound care entails 3 main factors into consideration: possible invasion of multidrug resistant bacteria, biofilms formation associated with the wounds and physiological stages of wound repair.

Despite the rise of multidrug resistant bacteria, production of new antibiotics has been declined in the recent years. The urgent need to combat the multidrug resistant organisms caused a resurgence of interest in phytochemicals because of their ubiquitous and antimicrobial nature. Phytochemicals that act synergistically with antibiotics to kill bacteria are especially the center of interest as they can reduce toxicity and delay resistance development. In this study, the antimicrobial effects of phytochemicals in combination with the conventional antibiotics were investigated by employing checkerboard assay and time kill assay using 6 strains of *Staphylococcus aureus*. Ethyl gallate was found to be synergistic with mupirocin, fusidic acid, tetracycline, cefoxitin and indifferent with vancomycin.

Mechanism of action of phytochemicals can be postulated depending on their behavior of interaction with antibiotics. Ethyl gallate's synergistic interaction with protein synthesis inhibitors and its indifference with a cell wall synthesis inhibitor raised an interest in its

possible mechanism of action on cell wall. Scanning electron microscopy and atomic force microscopy observation of bacteria cells after treatment with ethyl gallate further confirmed its action on cell wall. Computational docking studies were performed with lipoteichoic acid synthase enzyme where the results revealed that ethyl gallate was inhibiting the cell wall synthesis by inhibition of polymerization of peptidoglycan. Furthermore, the cytotoxicity assays also ensured that the phytochemicals under study were not toxic to human peripheral blood mononuclear cells.

Infected wounds plagued with biofilms of bacteria are recalcitrant to treatment with antimicrobials. Moreover, dissemination of biofilm associated bacteria can result in fatal complications. Eradication of bacteria in biofilms needs thousand fold concentrations of antibiotics which narrow the therapeutic index of antimicrobials. The biofilm inhibition potential of phytochemicals and antibiotics were studied and compared as single agents and in combinations. Synergistic phytochemical-antibiotic combinations were found to have anti-biofilm activities investigated by microtiter plate assay and scanning electron microscopy.

Staphylococcus aureus which is a common casual organism of wound infection is infamous for its drug resistance properties. When antibiotics are used in therapeutic regimes, it is important to take mutant selection window into consideration. Mutant selection window and mutant prevention concentrations for each of the phytochemicals and antibiotics in focus were studied and compared. Mutant strains of *Staphylococcus aureus* were studied and the duration taken for each antimicrobial was noted along with the mutation frequency of bacteria under treatment. The presence of ethyl gallate was found to close the mutant selection window, decrease mutant prevention concentration and reduce mutation frequency while prolonging the duration of resistance development.

Chitosan and alginate are well known biocompatible natural polymers and have potential to form polyelectrolyte membrane in solutions. Employing this concept a novel natural polymeric polyelectrolyte membrane was synthesized and synergistic combination of fusidic acid and ethyl gallate were incorporated as a model drug combination. Effects of polyelectrolyte membrane on various aspects of wound healing process were determined. Drug eluting polyelectrolyte membrane developed in this study was able to help the wound healing activity by allowing gaseous exchange, retaining moisture in the wound, enabling slow release of drug combination, and assisting the proliferation as well as the metabolic activity of fibroblasts

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Peer Review Journals

1. **SOE W.M**, LIM, C.S, SAKHARKAR KR, SAK.ARKAR M.K & CHIAN, K.S. 2013. Drug Loaded Chitosan Alginate membrane. *Wound Repair and Regeneration*. (Under Review)
2. **SOE, W. M.,** GIRIDHARAN, M., LIN, R. T. P., SAKHARKAR, M. K. & SAKHARKAR, K. R. 2010. Effect of combinations of antibiotics and gallates on biofilm formation in *Staphylococcus aureus*. *Letters in Drug Design and Discovery,* 7**,** 160-164.
3. **SOE, W. M.,** LIN, R., LIM, C.S, SAKHARKAR, K. R. & SAKHARKAR, M. K. 2010. In vitro drug interactions of gallates with antibiotics in *Staphylococcus aureus*. *Frontiers in bioscience (Elite edition),* 2**,** 668-672.
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LIST OF SYMBOLS AND ABBREVIATIONS

AAP	Accumulation – associated protein
Arg	Arginine
Asp	Aspartic acid
Bap	Biofilm associated protein
Bmr	Bacterial multidrug resistant gene
CA	Membrane which contains only Chitosan and alginate
CAP	Membrane which contains chitosan, alginate and propylene glycol
CAPE	Membrane which contains chitosan, alginate, propylene glycol and ethyl gallate
CAPEF	Membrane which contains chitosan, alginate, propylene glycol, ethyl gallate and fusidic acid
CEFO	Cefoxitin
CFU	Colony forming units
CYP28	Gene family of cytochrome P450
CYP2C9	Cytochrome P450 2C9
DAG	Diacylglycerol

DMMB	Dimethyl methylene blue
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Eap	Gene encoding extracellular adherence protein
ECG	Epicatechin gallate
EF G	Elongation factor G
EG	Ethyl gallate
Emp	Extracellular matrix protein-binding protein
EPI	Efflux pump inhibitor
EPS	Extracellular polymeric substance
FDA	Food and drug administration
FTIR	Fourier transform infrared spectroscopy
Fus A gene	Gene responsible for fusidic acid resistance
FUSI	Fusidic acid
Glu	Glutamic acid
GtaB	UTP: alpha-glucose 1-phosphate uridyltransferase
His	Histidine

HIV/AIDS Human immunodeficiency virus/acquired immune deficiency syndrome

HL-60 Leukemia cells of promyelocytic leukemia cell line

LTA Lipoteichoic acid

LtaS Lipoteichoic acid synthase

LtaS Lipoteichoic acid synthase

MDR Multidrug resistance

MIC Minimum inhibitory concentration

MRSA Methicillin resistant *Staphylococcus aureus*

MSSA Methicillin sensitive *Staphylococcus aureus*

MTT (3-(4, 5-dimethyltiazol-2-yl)-2, 5-diphenyltetrazolium bromide

MUPI Mupirocin

NADH Nicotinamide adenine dinucleotide

NADPH Nicotinamide adenine dinucleotide phosphate

NAG N-acetyl glucosamine

NAMA N-acetylmuramic acid

NorA Gene responsible for quinolone resistance

OD Optical density

OD_C	Cut off value of optical density
PABA	Para amino benzoic acid
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PDB	Protein databank
PG	Propylene glycol
PgcA	Alpha-phosphoglucomutase
PIA	Polysaccharide intracellular adhesin
PVL	Panton-Valentine Leukocidin toxin
QUER	Quercetin
RAM	Random - Access Memory
RMSD	Root mean square deviation
RPMI	Roswell Park Memorial Institute medium
RUT	Rutin
S.D	Standard Deviation
Ser	Serine
TB	Tuberculosis

TCP	Tissue culture plate
Tet (O) gene	Gene responsible for tetracycline resistance
Tet (Q) gene	Gene responsible for tetracycline resistance
Tet (X) gene	Gene responsible for tetracycline resistance
TETRA	Tetracycline
Thr	Threonine
tRNA	Transfer RNA
Trp	Tryptophan
TSB	Tryptic soy broth
Tyr	Tyrosine
VANCO	Vancomycin
XTT assay	Cytotoxicity assay which uses water-soluble XTT (2, 3-bis-(2- methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) Reagent
YpfP	Processive diacylglycerol glucosyltransferase

1. INTRODUCTION

1.1 *Research Motivation*

Nowadays, major concern in the treatment of wound healing is the development of multi-drug resistant microorganisms due to inappropriate and indiscriminate use of antibiotics. Multidrug- resistant bacteria can cause infections refractory to the treatment with conventional antibiotics. Among the infections caused by drug-resistant bacteria, bacterial skin infections are of major concern because skin serves as a natural barrier of the body (Kupper and Fuhlbrigge, 2004). Many different types of pathogens are responsible for skin infections. Among these bacteria, Methicillin-resistant *Staphylococcus aureus* (MRSA) is now emerging as a global health concern because of its prevalence in hospital wards. It also has the propensity to form a reservoir in the nasal cavities of healthy people resulting in multiple outbreaks in hospitals (Collins et al., 2010). In recent years, there has been significant development of resistance in the *Staphylococcus aureus* to almost all of the antibiotics designed to use against it (Enright et al., 2002). The logical solution to multidrug resistant problem would be the increased production of novel drugs by pharmaceutical companies. Instead, the pharmaceutical industry has seen the stagnation of the antibiotic development in the past three decades. Spellberg *et al.* reported that FDA approval of new antibacterial agents decreased by 56% over the past 20 years (1998–2002 vs.1983–1987), highlighting the decline in the development of new antibiotics regardless of the need (Spellberg et al., 2004). Despite enormous cost and duration it takes for producing new antibiotics, many antibiotics are rendered ineffective by adaptive resistance development of bacteria. Therefore, there is a need for alternative

strategies, which allows the utilization of existing antibiotics against *Staphylococcus aureus*. One of the options is the use of combination therapies. Hypothetically, combination therapies expand antimicrobial spectrum and delays drug resistance (Lorian, 2005). Moreover, combinations potentiate the killing effect of antimicrobials with the lower dose which in turn reduce the dose related toxicity (Schwalbe et al., 2007). Treatment regimens of cancer chemotherapies, combination drug treatment for tuberculosis and combination drug treatment for HIV are developed to take full advantage of the benefits of drug combination therapies. While many combination regimes use synthetic antibiotics, natural compounds are now opening up a vast frontier for new treatment regimens as combinatory agents. Natural compounds especially the plant products in general have non-selective antimicrobial activity against both gram-positive and gram-negative organisms (Samy and Gopalakrishnakone, 2008). However, phytochemicals have long been regarded as the pharmaceutical market that has been underutilized by the industry (Borris, 1996). Therefore, it is worthwhile to investigate behavior of the phytochemicals as potential agents for the combination therapies.

Skin is the largest organ in the body. It is constantly fighting off invading pathogens. Therefore, the integrity of skin plays a very important role in the body. Functions of skin can be compromised by situations such as burns and wounds. Many therapeutic approaches ranging from the use of topical and systemic antimicrobial agents to drug impregnated bandages have been employed to treat wound infections. However, using antimicrobial agents in the treatment of wounds poses a controversy as topical agents are considered to favor drug resistance. At the same time, the presence of antimicrobials is found to delay wound healing process due to its indiscriminate

toxicity towards fibroblast cells of the skin (Lee and Moon, 2003). Therefore, the need of a novel antimicrobial agent which can delay the drug resistance while at the same time can promote wound healing inspires this project to investigate use of phytochemicals in treatment of wound infection.

To meet the research objective, first part of the research was focused on investigation of phytochemicals as potential combinatory agents to be used with conventional synthetic antimicrobials. Possible drug interactions (indifference, synergism, antagonism) between the antibiotics and the phytochemicals were studied and compared with single agents. Computational approach was attempted to hypothesize the mechanism of action of phytochemicals. After the ideal combination was established, cytotoxic level of one of the phytochemicals was studied by using the human peripheral blood mononuclear cells. The research was expanded to investigate biofilm inhibiting potential of the antimicrobials under study using *Staphylococcus aureus* as a model organism. Effect of these combinations on the resistance development of *Staphylococcus aureus* was further explored. Final part of the research was focused on synthesizing a novel drug eluting membrane made of natural polymeric materials. Polyelectrolyte membrane composed of natural polymers, chitosan and alginate, was synthesized and the synergistic drug combination was incorporated. Effects of the drug eluting membrane on different aspects of wound healing process were determined. The outcome of the research was focused on the development of a bioactive and biocompatible drug eluting membrane that would serve as a delivery medium for the antibiotic-phytochemical combination to the wound.

1.2 *Research Objectives*

The main objectives of this research are –

1. To investigate the antimicrobial phytochemical that can synergize with synthetic antibiotic for treatment of wound infections
2. To investigate the effect of phytochemicals on bacterial cells and mammalians to uncover the mechanism of action of phytochemicals
3. To identify the combination which inhibits the biofilm formation
4. To identify the combinations that can inhibit the resistance development of *Staphylococcus aureus* bacteria which commonly infect wounds
5. To synthesize a novel polymeric membrane containing the synergistic drug combination

1.3 *Organization of the Thesis*

The thesis consists of 9 chapters. Main contents of these chapters are as follows: Chapter 1 presents brief introduction of the research work describing overall and specific objectives, and background motivation of the research. Current literature related to the research work along with the brief introduction of *Staphylococcus aureus* bacteria, conventional antibiotics, phytochemicals, combination therapy, the insights into the drug resistance, new strategies to combat drug resistance, and further insights in development of drug eluting natural polymeric polyelectrolyte membrane are discussed in Chapter 2. Chapter 3 is focused on the investigation of antimicrobial activities of phytochemicals and antibiotics as well as antibiotic-phytochemical interactions. In Chapter 4**,** effect of ethyl gallate on bacterial cells and human peripheral blood mononuclear cells is discussed in nanoscale experiments and

computational methods. Furthermore, effects of combinations of antibiotics and gallates on biofilm formation in 6 *Staphylococcus aureus* strains are discussed in Chapter 5. Chapter 6 discusses the role of ethyl gallate on evolution of drug resistance using *Staphylococcus aureus* as a model organism. In Chapter 7, the synthesis of novel natural polymeric polyelectrolyte membrane is discussed in details followed by the investigations to detect the effect of membrane on different stages of wound healing process. Major findings and conclusion of the present research are discussed in Chapter 8. Finally, recommendations of the future studies are provided in Chapter 9.

2. LITERATURE REVIEW

2.1 *Phytochemical- antibiotic interactions in drug combinations*

2.1.1 *Introduction*

Staphylococcus aureus is a commensal of the skin and one of the most common pathogens that can cause wound infections. Mainly known as Methicillin-resistant *Staphylococcus aureus* or MRSA due to its resistance to antibiotic methicillin, it is also resistant to variety of antibiotics present in the pharmaceutical industry. In many of the wound infections, *Staphylococcus aureus* is found in colonies of biofilms. Common consequence of the presence of MRSA infections in the wound is chronicity and septicemic potential, depending on the immune status of the host. As skin is a natural barrier which protects body against infections and extreme temperature, impairment of skin protections means loss of a significant portion of immune system. Therefore, wounds such as burns, cuts and open injuries need prompt treatment. With antibiotics becoming increasingly ineffective due to the emergence of multidrug resistant bacteria, plant extracts are progressively explored for their antimicrobial activities (Cowan, 1999). Major classes of phytochemicals such as flavones, flavonols, glycosides, phenolic acids, tannins, alkaloids, and essential oils are extensively investigated for their antimicrobial activity. While many of them are found to be weak antimicrobials as single agents, they are also found to be able to potentiate the action of synthetic antimicrobials which provides hope that they have potential for use in combination therapy (Lewis and Ausubel, 2006).

Combination therapy which is also known as polypharmacy is a regime consisting of two or more therapeutic agents together. The ultimate aim of combination therapy is to improve therapeutic efficacy of the treatment. Combination therapies are widely used in treatment of bacterial diseases such as tuberculosis, viral infection such as HIV/AIDS, protozoa infections such as malaria and in routine chemotherapeutic management of cancer (Cottarel and Wierzbowski, 2007). While therapeutic efficacy is the intended effect combination therapies, side-effects due to drug interactions are so common that it is critical to study drug-drug interactions before combinations are prescribed. Even though there are multiple reports of drug-drug interactions between synthetic antibiotics, phytochemical-antibiotic interaction studies are not as prevalent. Given the nature of wounds' susceptibility to various infections, it is critical to study effect of phytochemical-antibiotic interactions on one of the most common pathogens of wounds, *Staphylococcus aureus*.

2.1.2 *Staphylococcus aureus*

Staphylococcus aureus is a spherical shaped, gram- positive bacterium which usually appears as golden colored clusters in gross appearance. It is a facultative anaerobe. Having the ability to survive both in the presence and absence of oxygen renders the versatility to the *Staphylococcus aureus* bacteria to prey on various types of wounds. *Staphylococci* are perfectly spherical cells about 1μm in diameter growing in grape like clusters as demonstrated in Figure 1. It is a gram positive bacterium and the cell wall is composed of interconnecting layers of peptidoglycans in which teichoic acid and lipoteichoic acids are embedded. The presence of thick layer of peptidoglycan enables it to retain the crystal violet stain, giving *Staphylococcus aureus* an appearance of violet color on gram stain as shown in Figure 2, giving rise to the name, a gram positive bacterium.

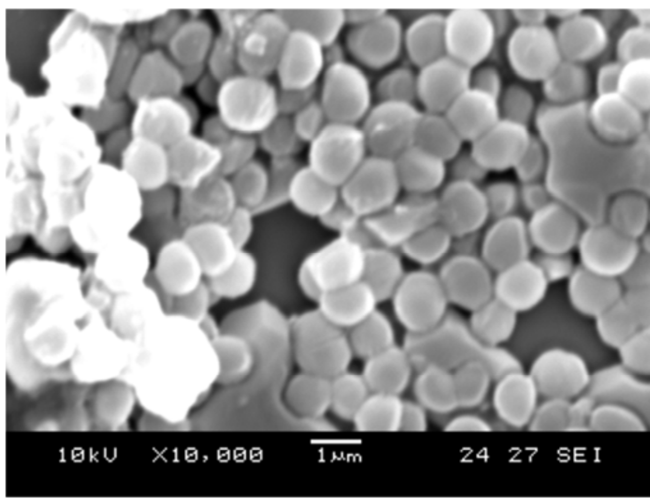


Figure 1: Scanning electron micrograph of *Staphylococcus aureus*

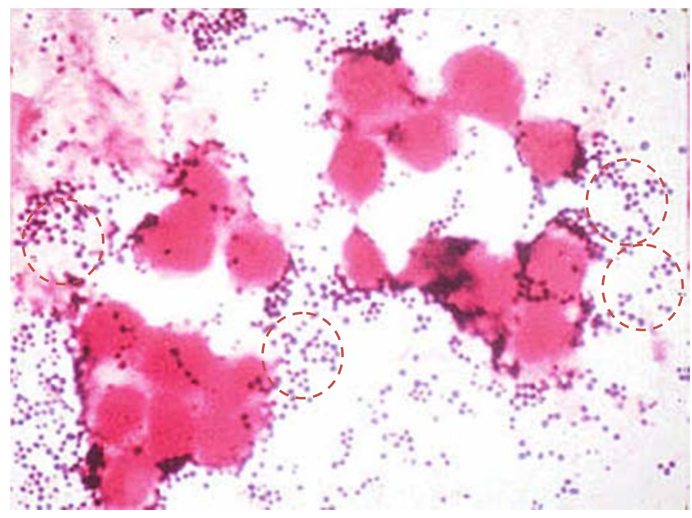


Figure 2: *Staphylococcus aureus* seen as clusters of purple cocci on gram stain

2.1.3 *Reservoir and pathogenesis*

Staphylococcus aureus is commonly found in anterior nasal cavity and on mucous membranes of nearly 30 % of general population. It can also harbor on inanimate objects such as gloves and lab coats of the hospital personnel. This prevalent nature contributes to various outbreaks of MRSA in hospitals around the world, making MRSA one of the most dangerous nosocomial pathogens (Khalik, Nov 20, 2007). *Staphylococcus aureus* characteristically produce different kinds of toxins that can interfere with host defense mechanism causing specific clinical syndromes. Many *Staphylococcal* enzymes such as coagulase, staphylokinase, protease and deoxyrionuclease are associated with invasiveness of bacteria into the host tissue and evasiveness of bacteria from the host immune system.

2.1.4 *Pathogenesis of Staphylococcus aureus*

Staphylococcus aureus infection frequently occurs as a result of inoculation into the open wound or via upper airway after the virus infection compromised the integrity of respiratory mucosal defense. *Staphylococcus aureus* initiate invasion of tissues with the up regulation of virulence genes. Peptidoglycan and lipoproteins present in the cell wall are also thought to be associated with host recognition of bacterial cells (Wardenburg *et al*., 2006, Weidenmaier and Peschel, 2008). *Staphylococcus aureus* is capable of surviving inside macrophages. *Staphylococcus aureus* is able to deploy large number of antioxidant enzymes such as catalases and superoxide dismutase to counteract reactive oxygen species, proteases and lysozymes produced by neutrophils (Kraus and Peschel, 2008, Veldkamp and Van Strijp, 2009). *Staphylococcus aureus* can reside in the host for whole life as they can also interfere with the normal function of B cells produced from spleen. Other virulence mechanisms such as persistence on plastics and resistance to antibiotic by formation of microcolonies called biofilms are also of clinical significance in understanding pathogenic mechanisms of the bacteria (Liu, 2009).

2.1.5 *Manifestation of Staphylococcus aureus infection*

Staphylococcus aureus infections can manifest on all parts of the body as shown in Figure 3. Major systemic manifestations such as pneumonia and septicemia can occur when infection occurs in the lungs and spread into the blood stream. Other systemic manifestations such as osteomyelitis and endocarditis can also occur in association with septicemia. Common reservoir of *Staphylococcus aureus* is the skin. When the integrity of skin is compromised, clinically important *Staphylococcal* infections such

as cellulitis, impetigo and *Staphylococcal* Scalded Skin Syndrome occur on skin. *Staphylococcus aureus* is one of the most common pathogens found in the wounds and burns (Brandt et al., 1997). *Staphylococcal* infections can also cause multifocal superficial abscesses even after the extensive wounds have recovered (Pruitt Jr et al., 1998).

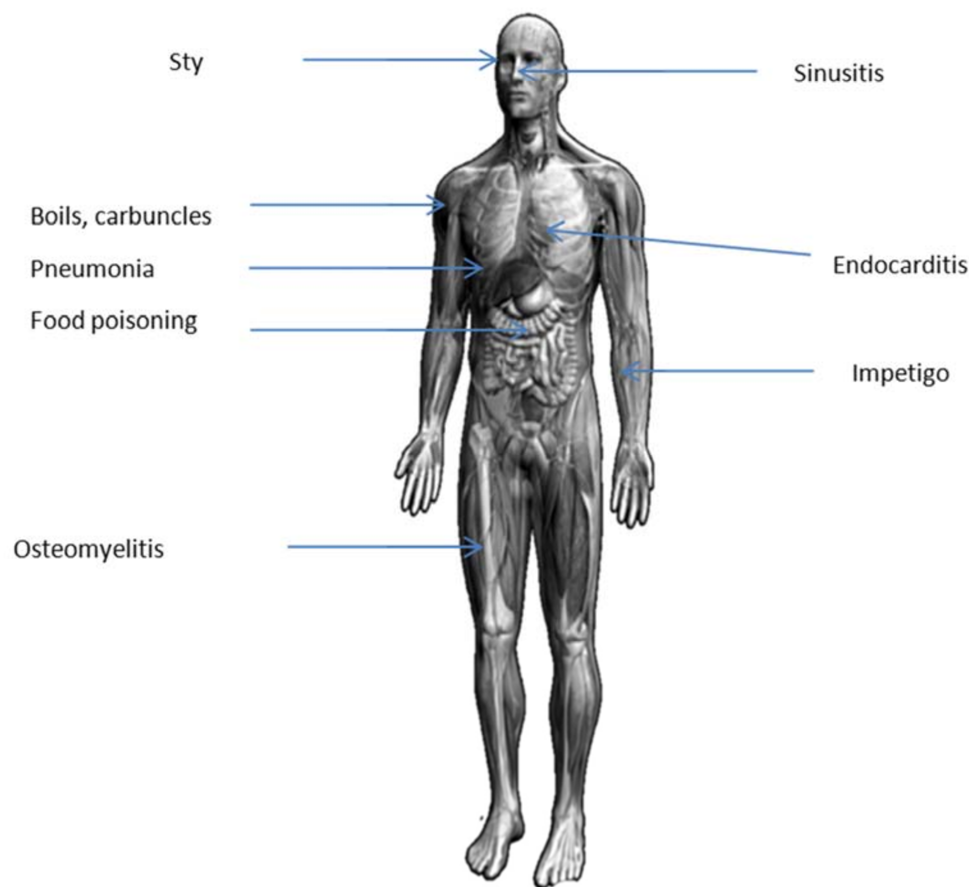


Figure 3: Staphylococcal infections on the body

2.2 Treatment of *Staphylococcus aureus* infection

Staphylococcus aureus is named a 'Superbug' because of its resistance to multiple antibiotics (Foster, 2004). *Staphylococcus aureus* has become a growing concern for physicians because of its high incidence, morbidity, and antimicrobial resistance. Methicillin-resistant *Staphylococcus aureus* appears 1 year after methicillin is introduced. Since then, beta- lactam antibiotics, penicillin, cephalosporins, oxacillin, vancomycin, fusidic acid, mupirocin and linezolid were introduced for the treatment of *Staphylococcus aureus*. However, the bacteria always manage to develop resistance to almost every antibiotic used against its organism. Therefore, American family physician association stated that antimicrobial therapy of *Staphylococcus aureus* should be guided by the susceptibility profile of the pathogen (Bamberger and Boyd, 2005). Beta-lactamase producing strains are treated with Penicillin group of antibiotics such as dicloxicillin, oxacillin, and cephalosporins such as cephalexin or cefazolin. Patients who are allergic to penicillin are treated with vancomycin by intravenous route. Complicated skin, soft tissue infections, and pneumonia are treated with newer group of antibiotics such as linezolid and daptomycin. Community acquired MRSA is commonly treated by fluoroquinolones, trimethoprim/ sulfamethoxazole, tetracycline or clindamycin (Bamberger and Boyd, 2005). For *Staphylococcal* non-complicated skin infections and burn infections, fusidic acid and mupirocin are given as topical agents. Still now *Staphylococcus aureus* is a major contributor to lengthy hospital stays and mortality rates.

2.2.1 *Antibiotics*

Antibiotics are chemotherapeutic agents that inhibit or abolish the growth of micro-organisms such as bacteria, fungi or protozoa. First widely used antibiotic was penicillin which was produced from the fungi known as Penicillium (Clardy et al., 2009). With advancements in organic chemistry, many more antibiotics are obtained by chemical synthesis and isolated from other living organisms. Narrow spectrum antibiotics are specifically effective for certain chosen bacteria while the broad spectrum antibiotics can cover wide variety of bacteria. Antimicrobial agents can be categorized as either bactericidal or bacteriostatic. Bactericidal antibiotics can kill the organism while the bacteriostatic antibiotics can only inhibit the growth or deter the development of bacteria. Mechanism of bacteriostatic antimicrobials mainly focuses on interfering the bacterial protein production, DNA replication and cellular metabolism. Bacteriostatic agents have to work together with the immune system of body to remove microorganisms. Bacteriostatic antimicrobials include tetracyclines, spectinomycin, sulfonamides, trimethoprim, chloramphenicol, macrolides and lincosamides. Bactericidal agents are able to eradicate organisms without the need of the immune system to participate in the process. Mechanism of bactericidal agents are directed against the cell walls, production of reduced intermediates within susceptible bacterial cell walls, bacterial cell membranes, bacterial DNA-dependent RNA polymerase, bacterial DNA gyrase and against bacterial 30S ribosomes. Bactericidal agents include beta-lactam antibiotics (penicillins and cephalosporins), glycopeptides antibiotics, rifampins, aminoglycoside and quinolones. The mechanisms of actions of antibiotics are expressed in details in Figure 4 and the summaries of bactericidal and bacteriostatic actions of antibiotics are given in Table 1.

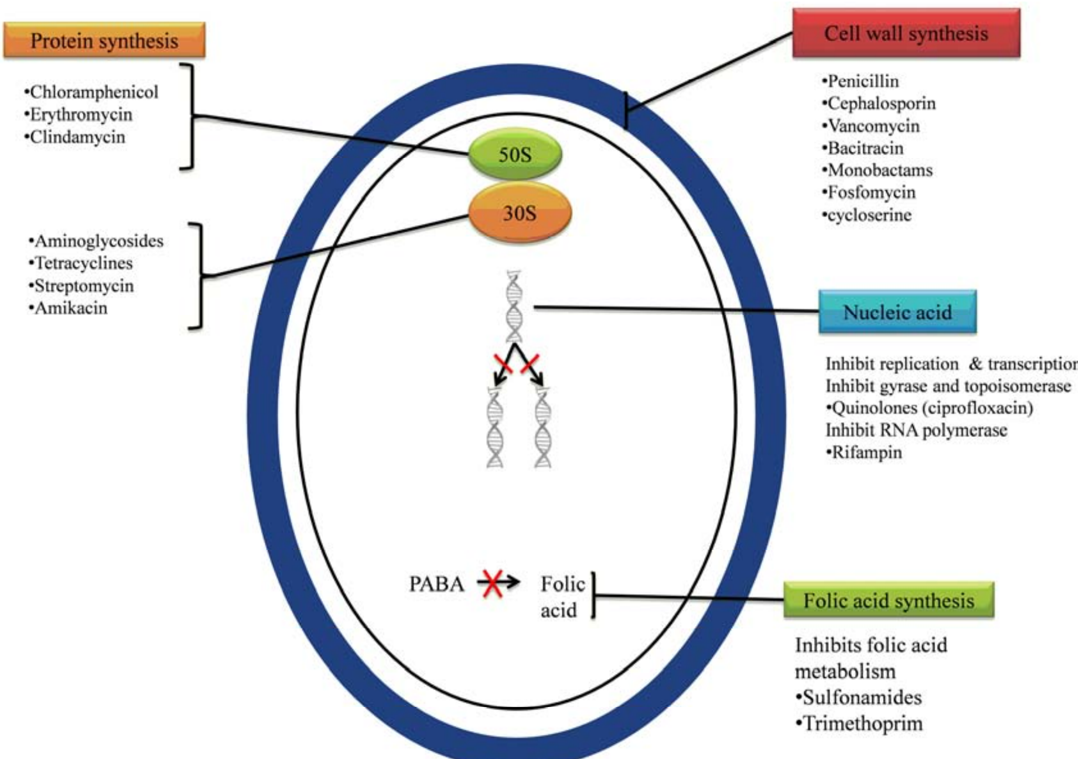


Figure 4: Mechanism of action of antibiotics

Table 1: Antibiotics and their mechanism of action

Mechanism of action	Antimicrobial agents	Activities
Inhibition of bacterial cell wall synthesis	Penicillin	Bactericidal
	Cephalosporin	Bactericidal
	Glycopeptides	Bactericidal
	Carbapenems	Bactericidal
	Monobactams	Bactericidal
Inhibition of bacterial protein synthesis	Aminoglycosides	Bactericidal
	Tetracyclines	Bacteriostatic
	Chloramphenicol	Bacteriostatic
	Clindamycin	Bacteriostatic
	Streptogramins	Bacteriostatic
	Fusidic Acid	Bacteriostatic
	Macrolides	Bactericidal
Inhibition of nucleic acid synthesis	Fluroquinolones	Bactericidal
	Rifampicin	Bactericidal
Inhibition of folic acid synthesis	Sulphonamides	Bacteriostatic
	Trimethoprim	Bacteriostatic
	Pyrimethamine	Bacteriostatic

2.2.2 *Antimicrobial Plant products*

Plants have traditionally been used as the natural healing agents since ancient times before the discovery of microbes. With the advent of synthetic antibiotics, the use of plant products as antimicrobials has declined. Plants possess over 100,000 small molecule compounds most of which have antimicrobial activity (Lewis and Ausubel, 2006). Plant antimicrobials are found to have weaker antimicrobial effect when compared with synthetic antibiotics needing orders of magnitude concentration more than their synthetic counterparts. It can be explained by the fact that the plants use different chemical strategy for the control of microbial infections. Plants, like other multicellular organisms, do not produce potent antibiotics but instead rely on their sophisticated immune systems to thwart invading pathogens (Tegos et al., 2002). Even though there are numerous plant products in use to fight off variety of diseases ranging from bacterial and viral infections to cancer, little is known about the mechanism of action of those phytochemicals. Nonetheless, the advent in biochemistry and pharmaceutical industry has opened up more opportunities and insights into antimicrobial plant products that have long been used generations ago. Another major reason is the ability of plant antimicrobials to resist multidrug resistant problem while there is an upsurge of resistant bacteria resulting from the indiscriminate and injudicial use of antibiotics (Cowan, 1999). Eloff *et al.* reported that 14–28% of higher plant species are used medicinally, of which 74% of pharmacologically active plant derived components were discovered after plant ethnomedical evaluations (Eloff, 1998). Phytochemicals can be mainly differentiated into phenols, phenolic acids, flavones, flavonols, tannins and alkaloids as shown in table 2. In alkaloid group, berberine is one of the most well investigated phytochemicals concerning its efflux pump inhibition

(Gibbons et al., 2004) and anti-staphylococcal properties (Gibbons, 2004). Berberine exerts its antimicrobial effect by intercalating into the bacterial cell wall. Phenolics are the most abundant group of phytochemicals including flavonoids, coumarins, flavones, flavonols, phenolic acids, quinone and simple phenols. Many of the phenolic compounds are found to be cell wall synthesis inhibitors with the exception of abyssinone which acts inside the cell by inhibiting protein synthesis (Aḥmad et al., 2006). Monoterpenoids such as citral and menthone produce antimicrobial activity by disrupting the membrane of bacteria cells. There are many more compounds from plant origin that have antimicrobial actions against multidrug resistant bacteria, biofilms and efflux pumps (Simoes et al., 2009). An alkaloid, reserpine, is reported to have Efflux pump inhibitor (EPI) activity against Bmr efflux pump, which mediates tetracycline efflux in *Bacillus subtilis*. It can also enhance the activity of norfloxacin against *Staphylococcus aureus* by inhibiting NorA. Rhein is reported to kill not only the gram positive but also the gram negative bacteria. Rhein is also found to be a multidrug resistance inhibitor enhancer, having orders of magnitude lower minimum inhibitory concentration when combined with MDR-inhibitor than acting alone. Plumbagin, resveratrol, gossypol, coumestrol, and berberine also show similar properties of enhancing MDR-inhibitors (Tegos et al., 2002). Structure of phytochemicals with MDR-inhibitor enhancing activities are given in Figure 5.

Table 2: Major classes of phytochemicals (Aḥmad et al., 2006)

Examples	Subclass	Class
Cell wall synthesis inhibitors		
Berberine	Isoquinolin	Alkaloid
Chrysin	Flavonoid	Phenolics
Aspigenin	Flavone	Phenolics
Galangin	Flavonol	Phenolics
Epicatechin	Phenolic acid	Phenolics
Citral	Monoterpenoids	Terpenoids
Protein Synthesis inhibitor		
Abyssinone	Flavone	Phenolics
Multiple actions		
Catechol	Simple phenols	Phenolics
Ellagitannin	Tannins	Phenolics
Quinone	Hypericin	Phenolics

*This table was adapted with permission from work of Ahmad *et al.*: Modern phytomedicine; Turning Medicinal Plants Into Drugs

Figure 5: MDR inhibitor enhancer plant antimicrobials

*The figure is presented with permission from Stavri *et al.* (Stavri et al., 2007)

2.2.3 *Combination therapy*

Antimicrobial combination therapies are designed based on the awareness that many diseases have multiple etiologic agents manifesting multiple organ systems. It is also a well-known fact that pharmaceutical combinations are more effective at eradicating diseases than single agents as proven by the successful use of combination of anticancer chemotherapies, anti TB (Albanna and Menzies, 2011) and anti-retroviral therapies for treatment of HIV/AIDS (Torella et al., 2010). In these instances, drug combination therapies are used to provide broad-spectrum empiric coverage in treatment of patients whose causal organism is resistant to the conventional dose of

single antimicrobials, or more susceptible to the combination therapies. These combination therapies are expected to provide concerted action not only focusing on one target but also on many different targets on the organisms (Tegos et al., 2011, Zhang et al., 2011). Drug combinations can be given either as a combination of antimicrobials or a combination of an antimicrobial with another agent that interferes with the elimination or inhibits the metabolism of the antimicrobial (Lorian, 2005). Combination regimes have many therapeutic benefits and detailed mechanisms of benefit are explained as follows.

2.2.3.1 Decreased emergence of resistant strains

Antibiotics are used in combinations to decrease or delay the resistance emergence *in vivo*. When antibiotics with different mechanisms are used together, bacteria need simultaneous development of two or more resistance mechanisms to resist antibiotic pressure. Thus, the probability of resistance development is found to be lower in combinations than in single agents (Chait et al., 2007, Chait et al., 2010). For instance, fusidic acid needs to be given together with rifampicin because of its chemical instability creating a favorable condition for resistant development (Chopra, 1976a). Oxacillin needs to be combined with rifampicin so that the *Staphylococcus aureus* population resistance to oxacillin is reduced by the addition of rifampicin (Traczewski et al., 1983).

2.2.3.2 Reduced dose-related toxicity as a result of the reduced dosage

Several important antimicrobials such as chloramphenicol, aminoglycosides, sulfonamides and 5-fluoroytosines have dose-related toxicities. Therefore, by using another agent which enables those compounds to exert antimicrobial effect with low concentrations would ensure successful and safe clinical outcome. Flucytosine is commonly used in combination with amphotericin B for the treatment of cryptococcal meningitis in non-HIV-infected patients so that the dose- related amphotericinB nephrotoxicity is reduced (Acar, 2000). To lower the dose- related toxicity of anti-TB drugs, isoniazid, rifampicin, ethambutol and pyrazinamide, are given as a combined forms.

2.2.3.3 Polymicrobial infection

Antimicrobial combinations are useful for the treatment of mixed infections because it is necessary to target each of several major pathogens in order to accomplish an effective treatment. In the human body, many etiological agents can co-exist in the pathogenesis and many major microorganisms can be involved in one manifestation of a clinical symptom. Many combinatory regimes are employed for treatment in polymicrobial infections such as peritonitis. Cephalosporins or aminoglycosides which have antibacterial action on gram negative bacteria are given together with clindamycin which is active against anaerobes to inhibit fatal late manifestation of abscess formation (Louie et al., 1977). However, with the advent of broad-spectrum antibiotics such as carbapenams, the rational for use of combinatory regimes to fight polymicrobial become a topic of debate among the clinicians.

2.2.4 *Drug interactions in the combinations*

One of the direct consequences of using antimicrobial combination is that of interaction between the drug molecules. Generally, there are three main types of drug interactions; indifference, synergism and antagonism (Lorian, 2005).

2.2.4.1 Drug indifference

Drug indifference is reported when there is no obvious synergism or antagonism between two drugs. The resulting effect of the combination of the drug is not different from the effect resulted from the individual drugs given separately. Indifference which is also called autonomy is based on the hypothesis that only one metabolic pathway can be growth rate-limiting for an organism at a time (King et al., 1981). Based on this observation, indifference suggests that the combined effect of drugs is not the result of interaction with each another and is simply the effect of the more active drug alone (King and Krogstad, 1983).

2.2.4.2 Drug Synergism

Synergy is defined as two or more agents working together to produce a result not obtainable by any of the agents independently. In other words, it is a positive interaction of two antimicrobials resulting in the effect that is greater than the expected effect of two antimicrobials combined (Lorian, 2005). It is the most fundamental reason that the physicians have utilized combination therapies to combat multi-drug resistant infections and to give optimum therapeutic result to the patients. For example, ampicillin and gentamicin are usually given together for the treatment of enterococcal endocarditis. Moreover, trimethoprim which inhibits tetrachydrofolic

acid synthesis by inhibiting dihydrofolate reductase and sulfamethoxazole which inhibits dihydroteroate synthetase to block dihydropteroic acid being formed which is a precursor for dihydrofolic acid are given together as they act synergistically in inhibition of bacterial folic acid biosynthesis.

2.2.4.2.1 Antimicrobial interactions that result in Synergism

Antibacterial synergism occurs when there is a sequential inhibition of biochemical pathway demonstrated by sequential inhibition of folic acid biosynthesis pathway by trimethoprim and sulfamethoxazole. Beta-lactamase inhibitors potentiate the effect of beta-lactam antibiotics by inhibition of protective bacterial enzymes (e.g. beta-lactamases). Giving cell wall active agents such as penicillin can also enhance the uptake of protein synthesis inhibitors such as streptomycin as cell wall active agents compromise the integrity of cell wall to enhance the entry protein synthesis inhibitors into the cell. There are other antimicrobial combinations that are synergistic in way of unique or unclear mechanisms (e.g. quinupristin/dalfopristin, Sulfamethoxazole and colistin) (Lorian, 2005, Pillai et al., 2005).

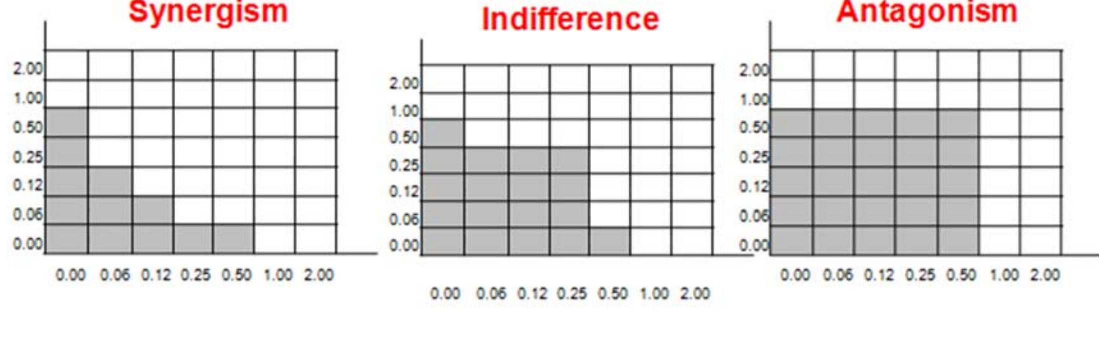
2.2.4.3 Drug Antagonism

Drug antagonism occurs when the therapeutic effect of combination is less than the effect obtained from the individual drugs given alone. Drug antagonism is responsible for many therapeutic failures and more researches are needed in this area (Richard Schwalbe, 2007). Drug antagonism occurs when bacteriostatic antibiotics are given together with beta-lactam antibiotics as reported in treatment regime involving both penicillin and chlortetracycline. Competitive inhibition of 50S ribosomal action can occur in combination regimes of erythromycin and clindamycin leading to the

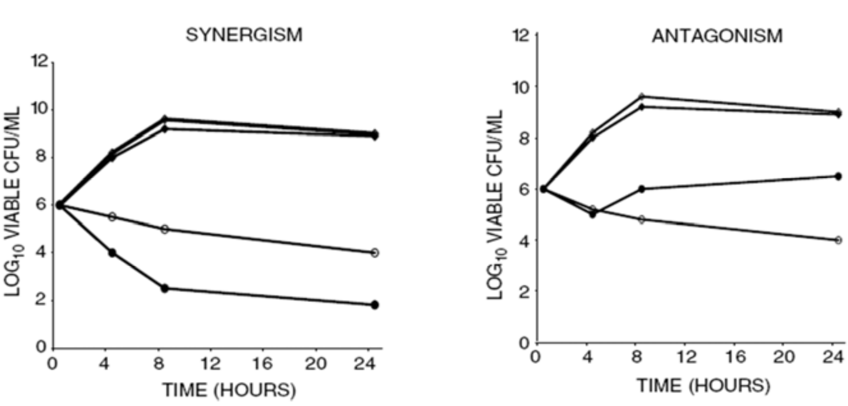
therapeutic failure. When bacteriostatic agents such as linezolid is given together with bactericidal agents such as gentamycin, bactericidal agents are found to be rendered incapacitated due to lack of multiplication of bacterial cells (Lorian, 2005).

2.2.4.4 Antimicrobial interaction testing methods

When antimicrobial combinations are tested with an intent to improve the therapeutic efficacy, it is important to obtain accurate results by employing appropriate methods. There are many different methods that are widely used in many microbiology laboratories, many of them having their own unique advantages and disadvantages. The choice of antimicrobial combination testing methods depends on the type of the organism, availability of facilities and expected accuracy of the test. Most widely used antimicrobial combination testing methods are checkerboard method, disk diffusion method, time killing curves methods, paper strip diffusion method and kinetic spectrophotometry (Richard Schwalbe, 2007, Cappelletty and Rybak, 1996, MacKay et al., 2000). Drug interactions can be explained by isobolograms, as shown or time killing curves as shown in Figure 6.



(A)



Symbols: ◇, growth control; ◆, agent A; ○, agent B; ●, agent A+B. (Adapted and reprinted with permission from Eliopoulos, G.M. and Moellering, R.C., Jr. Antimicrobial combinations, in *Antibiotics in Laboratory Medicine*, Lorain, V., Ed., Williams Wilkins, Baltimore, 1996, figure 9.6, p. 340.)

(B)

Figure 6: **A: Isobologram of drug interactions B: Time killing Curves** [48]

2.2.5 *Plants antimicrobials as combination agents*

Plants antimicrobials have been increasingly explored as potential agents for combination therapy with antibiotics in recent years. This synergy research is expanding because of the therapeutic superiority of many herbal drug extracts over single constituents of conventional antibiotics. The efficacy of these plant extracts used for centuries was verified in many cases by clinical studies. Even though many

synergistic combinations were reported for combination between phytochemicals and antibiotics, the mechanisms underlying these synergy effects remained vague (Hemalswarya and Doble, 2006). Investigation of mechanisms behind the synergism between phytochemicals and antibiotics is a challenging task because phytochemicals are made up of complex mixtures of major compounds (Borris, 1996, Cowan, 1999, Jayaprakasha et al., 2003, Samy and Gopalakrishnakone, 2008, Zhang et al., 2009). Nonetheless, it is a worthwhile frontier to explore because of the enormous advantage that would bring to the scientific and industrial research and applications. Current literature reports that many phytochemical compounds possess MDR pump inhibition activity (Gibbons et al., 2004), inhibition of beta- lactamase activity or production (Gibbons, 2004), anti-R-plasmid activity, synergy with antibiotics, target virulence and pathogenicity of bacteria and gene transfer mechanisms (Cowan, 1999, Stapleton et al., 2004). Therefore, this study would focus on interaction of phytochemicals with synthetic antibiotics to obtain optimal effect of the antibiotics.

2.3 *Computational methods in study of antimicrobial mechanisms*

Understanding drugs and their modes of action poses a fundamental challenge in drug discovery and clinical medicine. Elucidating new drug targets for existing drugs or identifying new drugs become key strategies to meet the challenge. Moreover, vast number of antimicrobials that are available in the market act by disruption of interaction between proteins and their respective ligands (Wagner and Ulrich-Merzenich, 2009). Systematic identification of protein-drug interaction networks is crucial in order to correlate complex modes of drug action to clinical indications. Moreover, it is a well-known fact that the physiological functions of a protein

molecule are inextricably linked to its three-dimensional (3D) structure (Kirchmair et al., 2012).

With advancements in computational methodologies and structural determination methods such as X- ray crystallography, number of proteins with known 3-dimensional structures has escalated in recent years (Kitchen et al., 2004). Hence, once a molecular model is available, the identification and study of its putative ligand-binding sites can be proceeded with ease. Molecular "docking" may then be performed *in silico* to predict the modes of interaction between the ligand and the target. Many target proteins have been selected for therapeutic agents by using computational methods starting from hit identification (rapid screening of large databases of potential drugs *in silico* to identify potential ligands for protein target of interest) to lead optimization (optimizing of the orientation of ligand-protein binding mode to design more potent and selective analogues of the compound) (Reddy et al., 2007). The process of bringing new compounds into therapeutic usage requires many important steps. Developments in computational methods allow less costly and time saving *in silico* approach of high throughput target identification screening procedure. After screening, drug like compounds are sought out with their respective targets for in vitro experiments. *In vitro* studies can be performed with sub-cellular targets such as enzymes, receptors and genes, or with whole organisms such as bacteria or viruses. The process can continue to *in vivo* studies with animal models with ultimate aim of bringing the drug to patients. The process of drug discovery is depicted in the following diagram [Figure. 7].

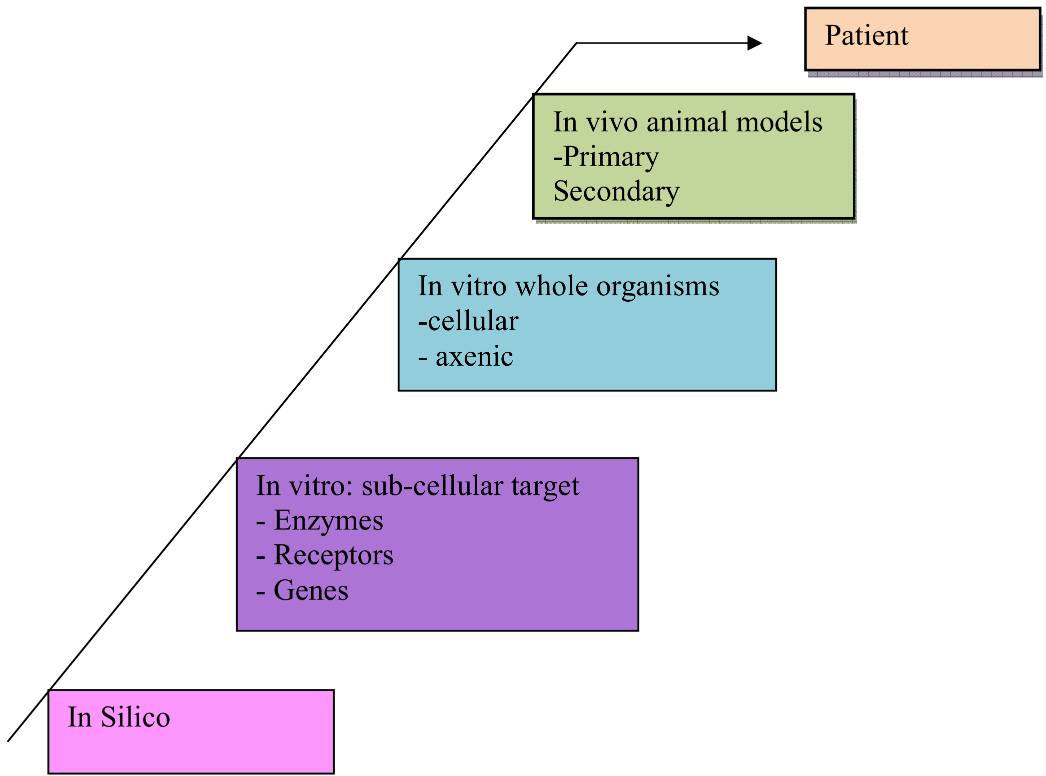


Figure 7: Drug Development process

2.3.1 *Molecular Docking*

Molecular docking is a process in which a ligand is docked to the active site of the target protein by using automated computer algorithm. Docking process involves determination of orientation of compound, conformational geometry and scoring, in order to achieve accurate structural modeling and correct prediction of activity (Halperin et al., 2002). Ligand-protein docking becomes one of the major steps in the drug development as many compounds can be docked into the protein active site for computational analysis. Even though docking process does not take bioavailability, toxicity, and other physiological processes in body into consideration, docking

simulations are able to simulate the active site-ligand interactions with the results that are comparable to biochemical assays (Young, 2009). In every docking program, search algorithm and scoring algorithm are main key components. While search function is responsible for optimum orientation in which the ligand can be docked to protein, scoring function is responsible to determine whether the orientation chosen by the search algorithms are energetically favorable. Scoring function is also responsible for computation of binding energy resulted from the interaction between ligand and protein.

2.3.2 *Search Algorithms*

As computational docking is a process in which two molecules are fitted together, one of the challenging tasks is numerating the number of ways in which those molecules can be put together. Speed and effectiveness in covering relevant conformational space are two important elements of the search procedure (Halperin et al., 2002). Search strategies can be grid approaches in which a potential field is created and evaluated numerically on a rectangular grid in the active site. If the grid is properly constructed, the grid-based algorithm is able to give the exact same result as a full force field method which is much more time consuming than grid-based system. Monte Carlo Algorithm also known as Metropolis Monte Carlo search is built around a random number generator (Kitchen et al., 2004a, Dias and de Azevedo, 2008). Position, orientation, and conformation are chosen randomly after the ligand is positioned at random spaces around the protein. Monte Carlo algorithm samples the space thoroughly as random displacement and rotation applied are followed by energy evaluation of the position to compare with previous energy (Kitchen et al., 2004b). Therefore, Monte Carlo algorithm takes a long time to implement. Tabu algorithm is a

modified version of Monte Carlo search. Even though Tabu search produce random positions like Monte Carlo search, it keeps tracks of positions that have already been sampled so as to avoid re-computing positions. Therefore, Tabu search needs only few iterations and thus, runs on faster time in contrast to Monte Carlo search. Another search algorithm called simulated annealing is similar to Tabu algorithm as it also keeps track of the sample position calculations. It is a metaheuristic approach which uses information about the positions that have already been sampled. Sampling is especially thoroughly done in places of low energies and less thoroughly in places of high energy. Genetic algorithm is different from other strategies as they are modelled on genetic traits. Calculation with this algorithm begins with the creation of population made up of random particular ligand positions. Successive generations are produced by keeping the best fit positions for optimization. This method is accurate and quick if the parameters are set up appropriately.

2.3.3 *Scoring system*

Scoring system is a quantification of how well the ligand fits the active site and it is the most important part of a docking program. There are different types of scoring systems based on two main criteria: a geometric match and energetic match. Geometric match (Huang et al., 2010) prevents overlapping between atoms of a receptor and a ligand. It detects maximum shape compatibility to ensure that there is no large cavity at the interface. Energetic match is based on good hydrogen bonding, good charge complementarity, polarity and low free energy.

Protein-ligand docking uses three basic types of scoring functions (force-field, empirical, knowledge-based) and the consensus scoring technique (Huang et al.,

2010). In **force filed scoring system**, scoring functions are based on physical atomic interactions, including Van der Waals (VDW) interactions, electrostatic interactions, and bond stretching/bending/torsional forces. Even though a need to consider how to treat the solvent pose a major challenge for force-field scoring system, some systems solve the problem by including a solvation term in scoring function energy equation. **Empirical scoring functions** estimate the binding energy of a complex on a set of weighted energy terms (Dias and de Azevedo, 2008). Therefore, empirical scoring functions are much faster in binding score calculations when compared with force filed scoring. **Knowledge based scoring function** can also be referred to as statistical-potential based scoring functions. In knowledge based scoring system, atomic structures are experimentally predetermined to produce structural information from which the energy potentials are derived. These scoring functions rank complex structure candidates so that only the most highly ranked models are adopted. In consensus scoring system, compounds and their poses are tested within a docking simulation based on acceptable results as predicted by multiple scoring functions (Oda et al., 2005). Scoring functions for protein ligand docking are shown in the following figure [Figure 8].

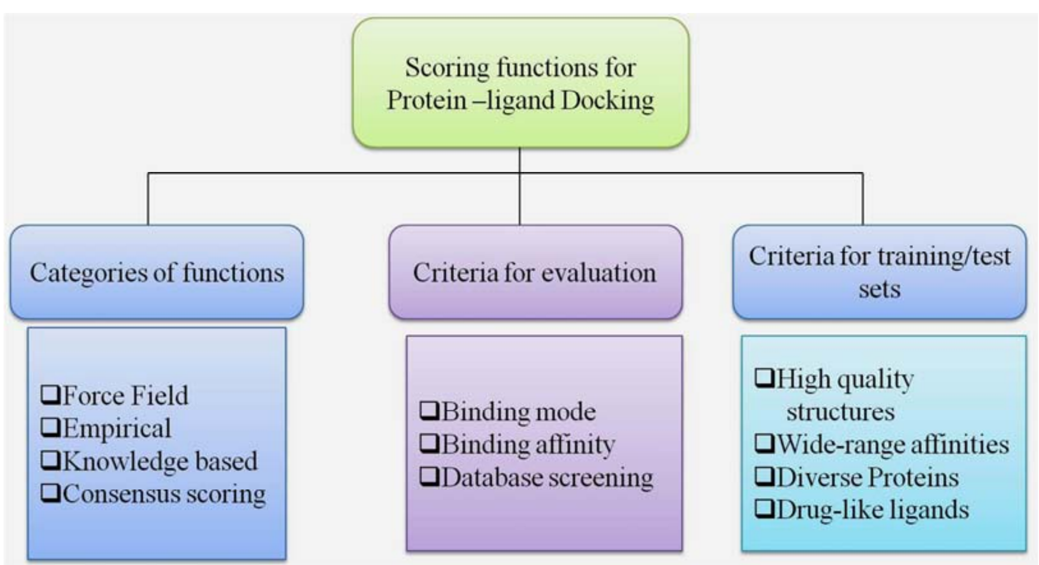


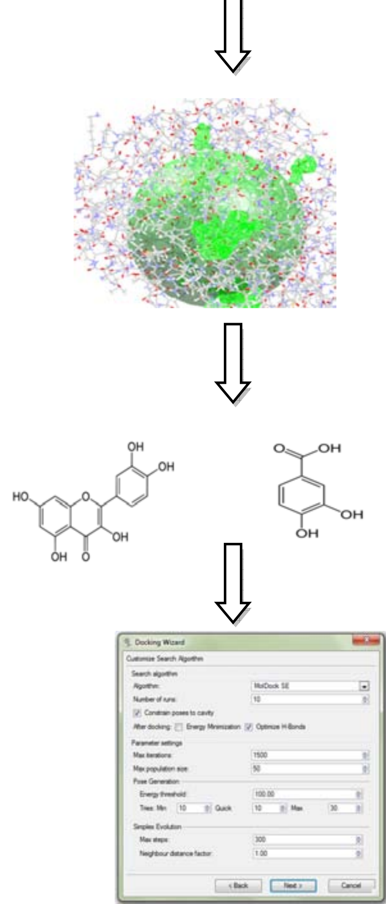
Figure 8: Scoring functions for protein ligand docking

2.3.4 *Analysis of the results*

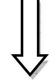
After the docking program is run, poses are generated. With each pose there is an associated binding energy with which the ligand is bonded with the active site of the protein. A few of the best binding energies need to be selected to compare the inhibition properties of ligand with the lowest energy binding orientation being regarded as the best binding mode.

Preparation of protein structure

- Remove exterior water molecules
- Add hydrogen molecules
- Correct bonds and atom types if necessary
- Optimize



Set docking parameters



Run docking
- Individual calculations
- Batch calculations

Figure 9: Illustration of docking process

2.4 *Biofilms*

Biofilm is a cluster of multilayered cells which is enclosed in a matrix of self-enveloped extracellular polysaccharides (EPS) as shown in Figure 10. The adherence of these microorganisms to biotic as well as abiotic surfaces protects them from host immune system and antimicrobial drugs (O'Gara and Humphreys, 2001). Biofilms are distinguished by surface attachment, structural heterogeneity, genetic diversity, complex community interactions, and extracellular matrix of polymeric substances (Aparna and Yadav, 2008). Bacterial biofilms play major role in antibiotic resistance because of their phenotypic and genotypic heterogenicity, and ability to produce EPS (Costerton et al., 1999). Biofilms cause poor antibiotic penetration, nutrient limitation, slow growth, and adaptive stress responses against antibiotics. Moreover, biofilms that are refractory to current treatment are often found to be composed of multi-layered defense system formed by persistor cells. In *Staphylococcus aureus* bacteria, Biofilm formation is controlled by expression of polysaccharide intracellular adhesin (PIA) which is a gene product of icaADBC (Krukowski et al., 2008).

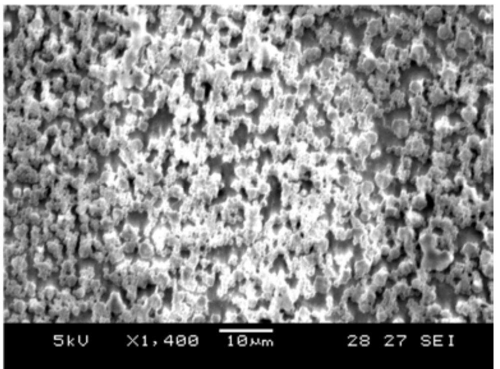


Figure 10: Scanning Electron Micrograph of MRSA Biofilm

2.4.1 *Biofilm formation process*

Biofilm formation is one of the most well-known defense mechanism of bacteria. Many pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are able to form biofilms when they encounter unfavorable environmental condition or stress exerted by the antibiotics (Costerton et al., 2003, Stewart, 2002, Stewart and Costerton, 2001, Lewis, 2001, Donlan, 2001, Raad et al., 1995). *Staphylococcus aureus* produce biofilm forming substance called polysaccharide intercellular adhesin (PIA) which causes intracellular adhesins to help bacteria adhere to the surface. It is further supported by a protein called Staphylococcus binding fibronectin. *Staphylococcus aureus* secretes proteins that are capable of interacting with various cells and extracellular matrix such as Map, (Kreikemeyer et al., 2002) Eap, and Emp (Johnson et al., 2008). *Staphylococcus aureus* can also produce other biofilm associated proteins such as Bap, and accumulation – associated protein (AAP) (Kuusela, 1978, Ryden et al., 1983, Proctor et al., 1982, Gotz, 2002).

2.4.1.1 Biofilm development

Biofilm development is comprised of five stages:

i. Initial attachment
ii. Irreversible attachment
iii. Maturation I
iv. Maturation II
v. Dispersion

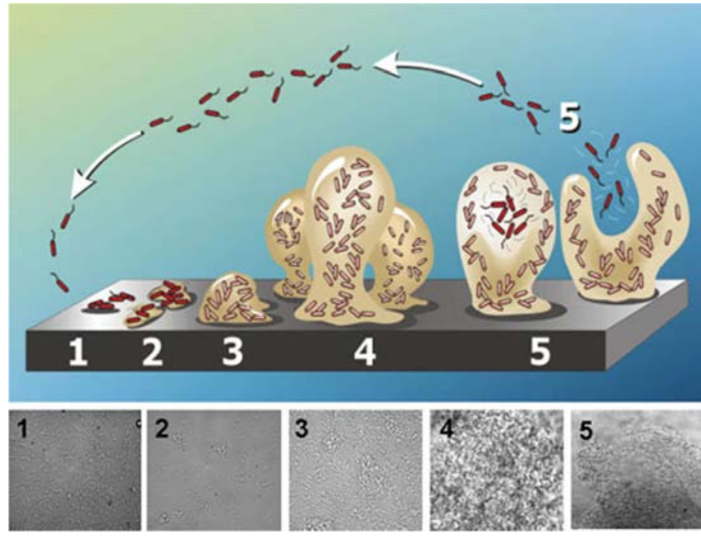


Figure 11: Five stages of biofilm formation

As shown in the diagram, biofilm formation starts with the attachment of bacteria to the surface. Adhesion can occur on any biotic or abiotic surface. Adhesion to abiotic surface is mainly mediated by non-specific interactions such as hydrophobic attraction, while adhesion to a living surface is mediated by specific molecular docking mechanisms (Dunne Jr, 2002). *Staphylococcus aureus* has a capacity to attach surfaces of many indwelling or implanted medical devices by interacting directly with human matrix protein that is covered on the device. Even though initial cell-surface contact is reversible, irreversible attachment to the surface occurs with the aid of EPS, the extracellular polysaccharide substances. Micro-colonies of bacterial cells are formed by division of cells and growth of the population. Mature biofilm cells possess channels that penetrate into the deeper layers of biofilm to carry out nutrient transport (Periasamy et al., 2012). Bacteria in the biofilm stay dormant unless physical detachment occur due to shear forces or release of planktonic cells caused by programmed set of events triggered by quorum sensing molecules. Biofilm detachment has important clinical implications because of the dispersion of bacterial cells, leading to formation of niches in new sites.

2.4.2 *Antibiotic resistance of biofilms*

Bacteria in a system of biofilms are resistant to most antibiotics. Even in individuals with competent innate and adaptive immune responses, biofilm-based infections are rarely resolved. According to Steward *et al* (Stewart and William Costerton, 2001), there are three hypothesis associated with antibiotics being rendered ineffective by the biofilms. First, multiple layers of bacteria along with secreted EPS cause of slow or incomplete penetration of the antibiotic into the biofilm. Therefore, concentration of administered antibiotics has to be increased high enough to kill biofilm bacteria (Bagge et al., 2000). Second, chemical microenvironment within the biofilms is altered. Slimy layer covering the biofilm creates a medium devoid of oxygen which enables the bacteria to exist in the dormant state so that bactericidal antibiotics have little effect (Nichols et al., 1988). Third, biofilms promote the formation of self-protective phenotypes by the subpopulation of micro-organisms (Das et al., 1998). *In vivo* experiments show that antibiotics are able to suppress symptoms of infection by killing free-floating bacteria shed from the attached population, but fail to eradicate those bacterial cells still embedded in the biofilm. Therefore, when antimicrobial therapy stops, the biofilm acts as a nisus for recurrence of infection.

Biofilm formation can be eradicated effectively with individual antibiotic or combination of the antibiotics. Antibiotics such as rifampin, tigecycline, daptomycin, N-acetylysteine (in combination with tigecycline) can be used for eradication of biofilms. Minocycline, daptomycin, and tigecycline are also found to be more efficacious than Linezolid (Costerton et al., 2003, Stewart, 2002, Stewart and Costerton, 2001, Lewis, 2001, Donlan, 2001, Raad et al., 1995).

2.4.3 *Effect of Biofilms on wound healing*

Wound environments are known to support biofilms. Serralta *et al* reported that wounds are capable of hosting biofilms and in turn, biofilms can have significant effect on wound inflammation, infection and wound healing (Serralta, 2001). As *Staphylococcus aureus* is a commensal in the skin and nose, it is one of the earliest contaminants into the wound that can adhere, proliferate, and synthesize EPS to start the formation of biofilm. In this biofilm community, the host immune system is at war with intractable microbial community. For immune system to win the war there is often a need of help from topical antimicrobial agents. Therefore, it is important that the antimicrobials delivered to the wounds are able to inhibit the biofilm formation.

Varieties of natural compounds are reported to have biofilm inhibiting activities**.** Natural compounds such as Epigallocatechin gallate have been found to inhibit biofilm formation of *Staphylococcus aureus* (Stapleton et al., 2007). Honey is also found to have biofilm inhibitory effect on *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Alandejani et al., 2009). Medicinal herb called *Andrographis paniculata*, the Creat, is also found to have inhibitory effect on quorum sensing activity in the biofilm formation of *Pseudomonas aeruginosa* (Jiang et al., 2009, Kumar et al., 2011).

2.5 ***Drug resistance***

Drug resistance is the reduction in the effectiveness of a drug in curing a disease or in improving a patient's symptoms. This term is extended to 'multi-drug resistant' if the organism is resistant to more than one drug (Bauer et al., 1966). Increased use of variety of antimicrobials and the clinical introduction of numerous closely related compounds in 1980s and 1990s are clearly associated with the emergence and dissemination of resistant strains (Pournaras et al., 2009, Udou, 1998, Shlaes et al., 1993). Compared to humans, bacteria multiply and evolve more rapidly, allowing 1 million or more opportunities to mutate only in 24 hour period. Therefore, mutations that provide selective advantage in a given environment are rapidly propagated among the bacteria present in same colonies (Boerlin and Reid-Smith, 2008). Moreover, cost-containment requirements in modern hospitals have led to increased patient-to-staff ratios, which have been associated with reduced adherence to strict infection control measures (Pittet et al., 2000). In the past decades, many attempts have been made to understand the mechanism and development of resistance of *Staphylococcus aureus* to many antibiotics (Foster, 2004, Lindsay and Holden, 2004). Bacteria may be intrinsically resistant to antimicrobial products. It may also acquire resistance by *de novo* mutation or *via* the acquisition of resistance genes from other microorganisms (Fajardo et al., 2008).

2.5.1 *Intrinsic resistance*

Some bacteria are protected from specific antibiotics due to their inherent structural and functional characteristics. This innate insensitivity also known as intrinsic resistance is usually discovered before the drug is reached to clinical usage (Fajardo et al., 2008). Intrinsic resistance may occur due to lack of affinity of the drug for the

bacterial target, inaccessibility of the drug into bacterial cells, being actively pumped out of bacterial cells or inactivated by variety of enzymes. Studying intrinsic resistance of different species can predict potential problems that may emerge under selective pressure (Lorian, 2005). Many instances of intrinsic resistance are: *Pseudomonas aeruginosa*'s resistance against sulfonamides, trimethoprim, tetracycline and chloramphenicol because of the lack of uptake of antibiotics to achieve effective intracellular concentration, and metronidazole resistance in many aerobic bacteria because they lack the ability to anaerobically reduce the drug to its active form. This intrinsic resistance is especially common in synthetic antibiotics as many of them are produced from living organism such as bacterial and fungal species (Sheldon Jr, 2005).

2.5.2 *Acquired resistance*

Many instances of drug resistance that are commonly seen and well researched are caused by acquired resistance. Acquired resistance involves mutations or horizontal gene transfer via transformation, transduction or conjugation as shown in Figure 12. Acquired resistance development mechanisms can be categorized into following four mechanisms (Cloete, 2003, Livermore, 1995, Ghuysen, 1991, Gold and Moellering Jr, 1996, Neu, 1992). First, drug inactivation or modification can be brought about by enzymatic deactivation antibiotics such as Penicillin-G in many penicillin-resistant bacteria through the production of beta-lactamases. Second, some bacteria alter target sites so that antibiotics cannot exert their effect. Alteration of target sites such as penicillin binding proteins (PBP) can be found in MRSA and many other penicillin-resistant bacteria. Third, some bacteria are capable of altering their metabolic pathways so that the antibiotics acting on that particular pathway are ineffective.

Fourth, many bacteria acquire mechanism to reduce accumulation of drug inside the cells by decreasing drug permeability or by increasing formation of efflux pumps. The resistance mechanisms are summarized in the following figure [Figure 13].

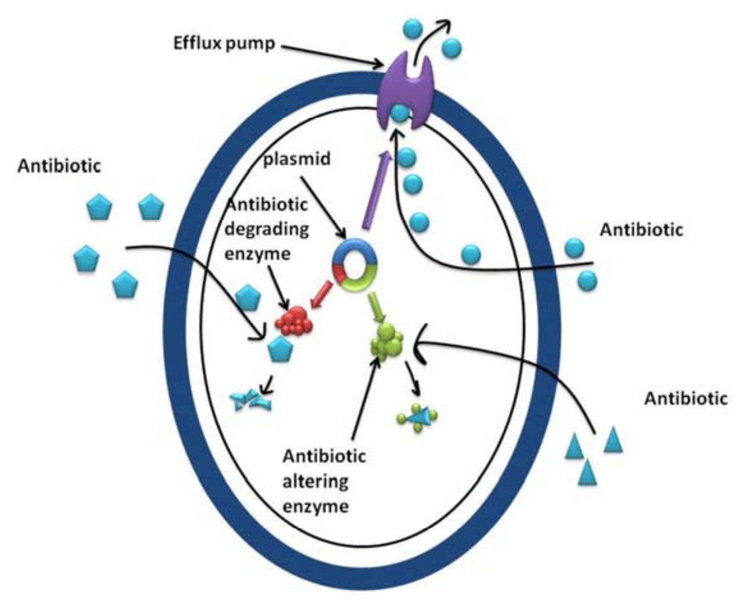


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Figure 12: Mechanism of drug resistance

2.5.3 *Mutant selection window and mutant prevention concentration*

When antibiotics are used in treatment regimes, it is imperative that the concentration of drug taken needs to be consistent to maintain the concentration of antibiotic that is exposed to the bacteria. Many studies have indicated that fluctuating the concentration of exposure or lowering the concentration of exposure will enhance the growth of mutant population (Gullberg et al., 2011, Kohanski et al., 2010). For many forms of resistance, two events must occur: resistant mutants must be generated, and the resulting mutants must be selectively enriched in the bacterial population (Zhao and

Drlica, 2001). There is a range in antibiotic concentration that can enrich resistant development called mutant selection window (MSW) as shown in Figure 13. MSW ranges from minimum inhibitory concentration (MIC) which is the minimum amount of antibiotic concentration to kill 10^5 CFU/ml of bacteria to mutant prevention concentration (MPC), a concentration at which the formation of single-step mutants is inhibited (Drlica, 2003). By definition, cell growth in the presence of antibiotic concentrations greater than the MPC requires an organism to develop two or more spontaneous chromosomal point mutations (Drlica, 2003). The mutant prevention concentration (MPC) is a novel concept that has been employed in evaluating an antibiotic's ability to minimize or limit the development of resistant organisms (Canton and Morosini, 2011). Thus, the MPC has been defined as the MIC of the least susceptible single-step mutant. Therefore, resistance is expected to develop rarely above MPC. In medical laboratory practice, MPC is approximated experimentally as the lowest concentration that allows no colony growth when more than 10^{10} cells are inoculated on drug containing agar plates. Within the context of the mutant selection window hypothesis, the window would be closed if MIC = MPC, to eliminate the concentration range that would enrich the formation of mutants. Ng *et al* (Ng et al., 1996) and Pan *et al* (Pan et al., 1996) pointed out that an antimicrobial agent that inhibits two different targets with equal efficacy would require a cell to acquire two concurrent mutations for growth and thus would result in the recovery of few resistant mutants. Moreover, dual targeting compounds offer many of the advantages of combination therapy without the problems associated with pharmacokinetic mismatches and increased adverse events associated with the use of two agents (Drlica, 2003). Therefore, research of drugs that have more than one target in bacteria can be deemed as one of the solutions to looming problem of drug resistance.

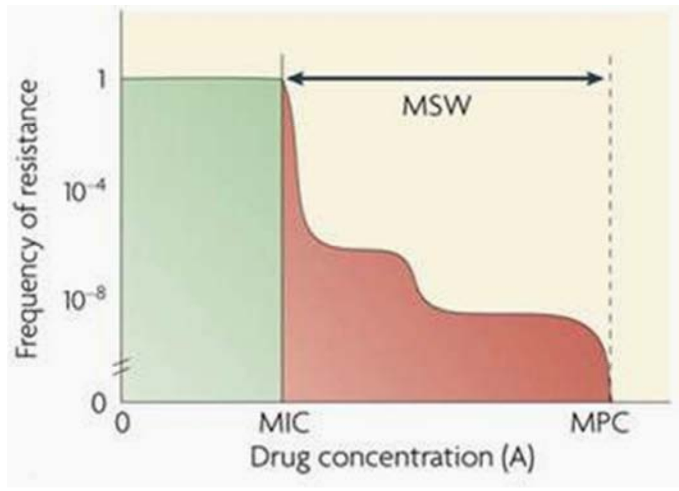


Figure 13: Mutant selection window

2.6 *Wound and wound management*

Skin is the largest organ in the body which covers approximately 2 m^2 of the body. It weighs about 8 pounds in a normal adult person (Habif, 2004). It acts as a waterproof insulating shield that guards the body against hazardous chemicals and multitude of microorganisms that are ubiquitous in the environment. Skin functions more than just protecting the body. Its functions also include regulation of heat and evaporation, resistance to water, and sensation of external stimuli (Pruitt Jr et al., 1998). Traumatic loss of the skin tissue such as burn, diabetic ulcers and pressure sores lead to impairment of skin functions along with fluid loss, hypothermia, infections and creation of locally immunocompromised regions.

2.6.1 *Wound Healing*

Wounds occur when there is a break in continuity of the surface of a skin with the loss of anatomical structure and function, resulting either from the physical damage or due to underlying medical condition (Guo and Dipietro, 2010). Wound healing is a complex mechanism with the involvement of the interaction between cells, and extracellular matrix molecules. There are four stages of wound healing (Guo and Dipietro, 2010; Hunt, 2000). In **hemostatic phase**, bleeding activates a complex interaction between the clotting factors, and platelets together with the formation of fibrin network to form a clot which later transformed into scab to protect the wound. In **inflammatory phase**, which commences within a few minutes to 24 hours after injury, protein rich exudates are released from the wound along with the inflammatory mediators such as histamine and serotonin in order to allow vasodilatation to enable macrophages to initiate phagocytosis. In **migratory phase**, epithelial cells and fibroblasts migrate towards the injured area to replace the lost tissue. Starting from day 3, proliferating fibroblasts and basal cells are nourished by in-growth of capillaries and lymphatic vessels. Further epithelialization takes place until the whole wound area is covered by the proliferating fibroblasts. **Maturation/remodeling phase** occurs after the formation of granulation tissue with wound contraction to strengthen newly formed epithelium. Variety of factors including oxygenation, infection, venous sufficiency, age, immune status of a person and nutrition can affect wound healing.

The following local factors are also important in wound healing process:

1. Hemostasis
2. Humidity and moisture of wound environment
3. Proliferation of fibroblasts
4. Water vapor transmission rate
5. Infection

2.6.2 *Drug delivery to the wounds*

Drug delivery is the method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals (Kumar, 2008). When drugs are administered, majority of the administered drug is lost due to the drug being taken up by unintended tissue, or removed from the body by excretion mechanisms or destroyed before the arrival to the target tissue (Moses et al., 2003). Therefore, it is a challenging task to design a method to deliver the drug with minimal toxicity and maximal intended effect. There are various methods of delivering a drug to the target tissue in the human body. Most common methods include the delivery of drug through mouth (per oral), skin (topical), nasal, buccal, sublingual, vaginal, ocular, and rectal and other mucosa (trans- mucosal), and systemic circulation (intravenous), intramuscular, subcutaneous routes. The delivery of a drug depends on the property of the drug and defenses or barriers in our body system (Kim et al., 2009).

2.6.3 *Topical antimicrobials*

Topical antimicrobials are antimicrobials that are delivered to the wound via normal or broken skin to kill or control the growth of microorganism in wounds (Liptak, 1997). Even though many wound treatment regimens utilize systemic antibiotics, there has

been increase in an interest in the use of topical antimicrobial dressing. Topical antimicrobials have certain advantages over the systemic antimicrobials. Topical applications can avoid deadly post antibiotic diarrhea from *Clostridium difficile* (Chin, 2006, Brown and Zitelli, 1995, Queen et al., 1987, Cuzzell, Cuzzell, 1997). Topical agents can be advantageous to the patients who have compromised arterial flow and chronic edema which are limiting the healing process of the wounds. It can also give local effect to the superficial local wounds especially burns and ulcers with minimal systemic side effects.

2.6.4 *Wound dressings and wound dressing materials*

Wound dressing is an adjunct used to promote healing by preventing further harm to the wound. Dressings are designed to be in direct contact with the wound with many expected characteristics that would help the wound to heal. Wound dressings are expected to be able to stop the bleeding and start the clotting process. Furthermore, they are also expected to absorb the exudates such as blood and plasma, reduce pain, help wound debridement, protect the wound from infection and promote healing (Cuzzell, Cuzzell, 1997).

Wound dressing materials can be classified into passive dressing, interactive dressings and bioactive dressings (Queen et al., 1987). Traditional wound dressings are made of cotton wool or cellulose while synthetic materials are made of polyamide and rayon polyesters which usually keep the wound dry and free of infection. However, in recent years, new insights into wound healing bring out the importance of keeping moist environment for optimum healing (Harding, 2012, Kouraba, 2012). As a result, modern dressings are made with significant emphasis on exudate management. Many

of the modern dressing materials can be classified into groups depending on the material that they are made of. **Hydrocolloid dressings** (e.g. carboxymethylcellulose) pectin and gelatin are designed mainly for exudate management, water vapor permeability, ease in removability and some antibacterial properties. As alginate dressings such as Sorban™ and Tegagen™ are highly absorbent, they are used in highly exuding wounds (Boateng et al., 2008). Calcium alginate dressings are particularly useful for early wound management because of the hemostatic ability of calcium ions, the gelation property due to the hydrophilic interaction with body fluids and antibacterial properties (Wang, 1986). **Hydrogel dressings** such as those made from poly (methacrylates) or polyvinylpyrrolidine are swellable but insoluble. As those hydrogels has pre-existing 70-80% of water, they are not suitable for highly exuding wounds. However, they are ideal dressings which keep the wound moist, clean, non- adherent and regulate the temperature of the wound by providing cooling environment. Dressings which are made from the biomaterials that can actively take part in the wound healing process are called **bioactive wound dressings** (Stashak et al., 2004, Kim et al., 2008a). They include either tissue engineered or natural products such as collagen, chitosan and alginate which biodegradable, biocompatible and able to take part in the formation of tissue matrix. For the treatment of infection prone wound such as diabetic ulcers and burn wounds, it is preferable for the topical drugs to be delivered directly to the wound. With this intent, medicated wound dressings to deliver antimicrobials or desired drug product into the wound are designed. In some instances, other wound healing promoters such as fibroblast growth factor, platelet growth facts are incorporated into the dressings.

2.7 ***Problem Statement***

Effective treatment of the wound needs many factors to be taken into consideration such as immune status of the patient, location and type of the wound, and type of the dressing which is well-suited to the condition of the wound. It is a well-known fact that wounds are susceptible to infections, many of which are capable of producing biofilms and develop drug resistance to antimicrobials used for treatment. Even though it is now increasingly difficult to use existing antibiotics to treat wound infections due to drug resistance, the production of newer drugs has also been declined. Starting from the 1930s, development of antibiotics groups increased until the 1970s. However, starting from 1970s there is a decline in the development of antibiotics (Paccaud, 2012). There were only 2-3 antibiotics produced in the past 10 years as shown in the timeline which depicts antibiotic production and development of resistance [Figure.15]. From the timeline, it is clearly seen that the pharmaceutical companies are fighting a losing battle because the antibiotics are rendered ineffective soon after they are introduced (Walsh, 2003). Moreover, motivation to develop new antibiotics is reduced as antibiotics yield low financial return in contrast to the drugs used in chronic diseases (Morel and Mossialos, 2010, Butler and Cooper, 2011). This slow pace and reduction in the momentum of antibiotic developments is worrying physicians and public health specialists (Morel and Mossialos, 2010). Even though rational prescribing of antibiotics to maintain the effectiveness has been practiced increasingly, there is still a need of novel antibiotics to combat the resistance problem. Therefore, this study attempts at studying possible use of phytochemicals to alleviate the problem of drug resistance.

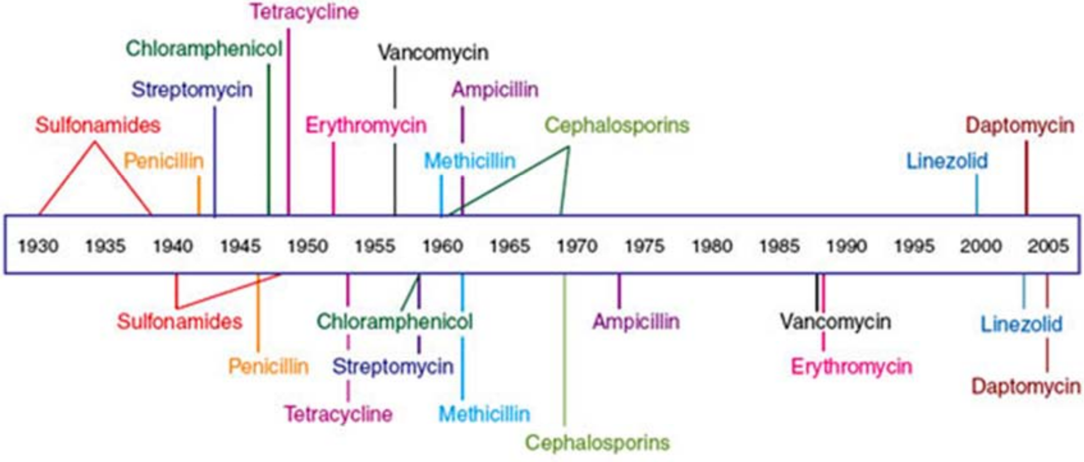


Figure 14: Antibiotic deployment and resistance development (Clatworthy et al., 2007)

Drug resistance problem is not only looming in the systemic treatment of infections but also in the wound infections. Wounds also create a special ground for developing drug resistance because of the type of treatment with the use of topical antibiotics (Del Rosso and Kim, 2009). Wound treatment is especially important as they are susceptible to superadded infections as a result of the exposed skin. Inadequate treatment of wounds infections could ultimately result in fatal septicemia as well as increase problems with drug resistance (Douglas et al., 2004, Sharp, 2009, Chin, 2006, Brown and Zitelli, 1995). Therefore, any treatment strategy of the wound would need to take biofilm formation into consideration.

According to the World Health Organization, more than 80% of the world's population residing in Asia and Africa relies on traditional herbal medicine for their primary healthcare (WHO fact sheet, 2008). Traditionally, many of cuts, bruises and wounds were treated with locally available leaves crushed and pounded into the wounds. These traditional healing practices shed lights that phytochemicals can be potential drugs

targeting the wound infections to promote of wound healing. However, apart from researches to utilize honey as wound healing agent, there is not much literature on using phytochemicals to treat wound infections. Therefore, the limited research about phytochemicals' influence on healing wounds has inspired this project to explore the use of phytochemicals in a novel strategy wound treatment.

Variety of wound dressing materials were used for the treatment of the wounds since ancient times including mud, milk, plants as well as cobwebs, leaves and honey (Queen et al., 1987). Modern wound dressing materials include gauzes impregnated with antibiotics or other agents that would help the wound healing process such as films, gels, foams, hydrocolloids, alginates, hydrogels and polysaccharide pastes, granules and beads (Stashak et al., 2004, Morin and Tomaselli, 2007, Cuzzell). Some recent studies show that inclusion of antibiotic in wound dressing can prevent the superadded infections and other nosocomial infections occurred to burn patients in comparison with other patients who have not been given antibiotic ointments (Rode et al., 1989). While antibiotics are able to kill the bacteria, they may sometimes delay the process of wound healing because of their cytotoxicity toward the proliferating fibroblasts as investigations into cytotoxicity of chlorhexidine indicates (Boyce et al., 1995). The relative value of antimicrobial dressings can only be determined by balancing their antimicrobial and cytoxicity characteristics (Fleming, 1919). Fleming *et al* (Fleming, 1919) also stated that anything that is bactericidal may well be tissuecidal. Many commercial wound dressing materials that incorporate antiseptics face with one major drawback, i.e., the problem in supporting fibroblast activity in the proliferation phase. Therefore, this study aims to improve the biological activity of wound dressings in all aspects of wound healing process, i.e., optimizing the local

environment of the wound, kill bacteria, inhibit biofilm formation, reduce resistance associated with topical use of antibiotics and promote fibroblast activity.

3. PHYTOCHEMICAL -ANTIBIOTIC COMBINATIONS

3.1 *Introduction*

In this chapter, antimicrobial activity of synthetic antibiotics of different classes - (protein synthesis inhibitors- mupirocin, fusidic acid, tetracycline; cell wall synthesis inhibitors - vancomycin, cefoxitin) and phytochemicals (ethyl gallate, epicatechin gallate, rutin, quercetin and protocatechuic acid) were studied. Further on, the interaction of phytochemicals and antibiotics were assessed by determining fractional inhibitory concentration indices (FICI) calculated from checkerboard method of combinatory testing. Time dependent killing kinetics of antimicrobials as single agents and as combinatory agents were assessed and compared by using time-kill assay.

3.1.1 *Bacterial strains*

4 Methicillin-resistant strains (MRSA ATCC43300, MRSA C1, MRSA C2, MRSA C3), 2 methicillin sensitive strains (MSSAC1, MSSA C2) were used in this study. Bacteria stock solutions were stored at -80°C in 15% glycerol broth before the study. The growth media used were Iso Sensitest broth and Iso Sensitest agar (ISB, Biomedia).

3.1.2 *Synthetic antibiotics*

3.1.2.1 Tetracycline

Tetracycline is a broad- spectrum bacteriostatic antibiotic that is produced from the *Streptomyces* genus of *Actinobacteria*. It inhibits protein synthesis by preventing the binding of aminoacyl tRNA to 30 S subunit of bacterial ribosome. Even though tetracycline is a protein synthesis inhibitor, it doesn't affect eukaryotic protein synthesis as eukaryotic cells lack the uptake mechanism for tetracycline into the cells (Gossen and Bujard, 1992). Tetracycline hydrochloride used in this study is soluble in water. Besides being mainly indicated for *rickettsial* and *chlamydial* infections, it is also effective in the treatment of staphylococcal soft tissue and skin infections (Miller, 2008). Chemical structure of tetracycline is shown in the following figure [Figure 15].

Figure 15: Chemical structure of tetracycline

3.1.2.2 Fusidic acid

Fusidic acid is a bacteriostatic antibiotic that is produced from the fungus *Fusidium coccineum*. It is a bacterial protein synthesis inhibitor which is effective mainly against the gram positive bacteria such as *Staphylococcus, Corynebacterium* and *Clostridium* (Miller, 2008). Even though it is clinically important for the treatment of MRSA, it is used in combination with other antibiotics because of its low genetic barrier to

resistance (Collignon and Turnidge, 1999). While fusidic acid is not useful for most gram negative bacteria, it is active against *Neisseria, Legionella, Moraxella* and *Bacterioides* (Chopra, 1976b, Collignon and Turnidge, 1999). Fusidic acid inhibits bacterial protein synthesis by disruption of functions associated with elongation factor G (EF-G) (Muhonen et al., 2008). Fusidic acid binds to EF-G and stabilizes the ribosome-EFG complex to prevent the binding of aminoacyl tRNA which ultimately results in inhibition of subsequent protein synthesis (Collignon and Turnidge, 1999). Chemical structure of fusidic acid is shown in the following figure [Figure 16].

Figure 16: Chemical structure of fusidic acid

3.1.2.3 Mupirocin

Mupirocin is a unique antimicrobial mainly used for the decolonization of MRSA in the patient's nasal passages (Eltringham, 1997). Mupirocin (formerly pseudomonic acid A) is a unique antimicrobial which contains a short fatty acid side chain (9-hydroxy-nonanoic acid) linked to a monic acid by an ester linkage (Hill, 1994, Humphreys et al., 2009). Mupirocin inhibits bacterial RNA and protein synthesis by binding to isoleucyl-tRNA synthetase. Mupirocin contains an epoxide side chain

structurally similar to isoleucine. This epoxide side chain competes with the amino acid for the binding site on isoleucyl-tRNA synthetase. As a result, isoleucine is prevented from binding to nascent peptide chain, stopping protein synthesis. Mupirocin is active against many gram-positive and some selective gram-negative bacteria. Mupirocin is bactericidal at a concentration achieved by topical applications. Mupirocin is mainly given as topical application as the systemic absorption is minimal and once it is absorbed, mupirocin is rapidly metabolized into inactive monic acid (Goodman et al., 2008). Chemical structure of mupirocin is shown in the following figure [Figure 17].

Figure 17: Chemical structure of mupirocin

3.1.2.4 Vancomycin

Vancomycin is a complex glycopeptide antibiotic. It is bactericidal to most bacteria except enterococci (Treadwell, 1995, Schwalbe et al., 2007, McEvoy, 2006., Fekety, 1995). Vancomycin is very active on gram-positive bacteria while gram-negative bacilli and mycobacteria are resistant to it (Aronson, 1997). Vancomycin acts by inhibiting the bacterial cell wall synthesis. It inhibits the polymerization or transglycosylase reaction by binding to D-Ala-D-Ala terminus of the cell wall

precursor unit attached to its lipid carrier to block linkage to the glycopeptide polymer which is situated within the cell wall (Goodman et al., 2008). Vancomycin may cause severe side effects such as ototoxicity and nephrotoxicity. Therefore, in clinical practice, vancomycin is reserved as drug of "last resort", being used only after treatment with other antibiotics had failed. Chemical structure of vancomycin is shown as follows [Figure 18].

Figure 18: Chemical structure of vancomycin

3.1.2.5 Cefoxitin

Cefoxitin is a beta-lactam antibiotic belonging to the group of second generation cephalosporin. As a cephalosporin, it is produced from 7- aminocephalosporanic acid (Goodman et al., 2008). Cefoxitin is different from other cephalosporins by the modification of position 7 in beta lactam ring with the methoxy group. This modification allows high degree of stability in the presence of beta-lactamases, penicillinases and cephalosporinases. It is a bactericidal agent. It inhibits bacterial cell wall synthesis by binding to specific penicillin-binding proteins (PBPs). Cefoxitin is active against gram positive bacteria, gram negative bacteria, and anaerobes such as

Bacterioides fragilis. Cefoxitin is usually administered intravenously and it is excreted by kidney. The chemical structure of cefoxitin is shown in the following figure [Figure 19].

Figure 19: Chemical Structure of cefoxitin

3.1.3 *Phytochemicals*

3.1.3.1 Epicatechin Gallate

Epicatechin gallate is a flavonoid extracted from green tea (*Camellia sinensis*). It is known to have antioxidant properties as it inhibits lipid peroxidation process which occurs when phospholipid bilayers are exposed to aqueous oxygen radicals (Terao et al., 1994). It also reported to restore the sensitivity of methicillin-resistant *Staphylococcus aureus* to beta-lactam antibiotics (Shiota et al., 1999). Epicatechin gallate exerts antimicrobial activities by disruption of cell wall (Stapleton et al., 2007). Other well-known properties of epicatechin gallate are antihepatotoxic, antimutagenic, antioxidant, and immune-stimulant properties. It is also a xanthine oxidase inhibitor and a glucosyl-transferase inhibitor. Epicatechin gallate is known to be able to modulate the action of beta-lactam antibiotics (Shah et al., 2008, Stapleton et al., 2004, Stapleton et al., 2007). Chemical structure of epicatechin gallate is shown in the following figure [Figure 20].

Figure 20: Chemical structure of epicatechin gallate

3.1.3.2 Ethyl gallate

Ethyl gallate (3, 4, 5-Trihydroxybenzoic acid) is natural water soluble phenolic acid present in grapes, gallnuts, tea leaves, oak bark and many other medicinal and edible plants. Ethyl gallate is an ethyl ester of gallic acid. It is a colorless crystalline organic acid having both hydroxyl group and a carboxylic acid group in same molecule. Ethyl gallate have potent antioxidant, anti-angiogenic, anti-carcinogenic, antiseptic and pesticide properties. It also has antifungal and antiviral activities. Ethyl gallate is also reported to have antimycobacterial activities (Kubo et al., 2002). Chemical structure of ethyl gallate can be seen as follows [Figure 21].

Figure 21: Chemical structure of ethyl gallate

3.1.3.3 Rutin Hydrate

Rutin is a flavonoid glycoside which is found in buckwheat, asparagus, fruits and flowers of pagoda tree; citrus fruits such as orange, grapefruit, lime; and berries such cranberries and mulberries. Rutin, which is a glycoside between quercetin and disaccharide, bears structural similarity to quercetin with the exception of hydroxyl functional group. Rutin is also known as Vitamin P as it can enhance antioxidant activity of vitamin C (Ghiasi et al., 2010). Rutin exerts antioxidant activity by binding metal Fe^{2+} to block out hydrogen peroxide so that the free radical formation is halted. Rutin is also reported to prevent formation of blood clots by inhibiting platelet aggregation (Jasuja et al., 2012). Chemical structure of rutin is shown in the following figure [Figure. 22].

Figure 22: Chemical structure of rutin

3.1.3.4 Quercetin

Quercetin is a flavonoid usually found in the apple peel, black and green tea, onions, red grapes, tomato and broccoli and many more. Studies also indicated that quercetin is present in tea tree flowers. Quercetin is reported to inhibit bacterial protein synthesis by competitively binding to DNA gyrase which is an action similar to fluorquinolone antibiotics (Smirnova et al., 2012). Quercetin is also able to inhibit liver enzymes involved in drug metabolism such as CYP28, CYP2C9 (Choi et al., 2012). Chemical structure of quercetin is seen as below [Figure 23].

Figure 23: Chemical structure of quercetin

3.1.3.5 Protocatechuic acid

Protocatechuic acid (3, 4-dihydroxybenzoic acid) is a natural phenolic compound found in many edible and medicinal plants such as onion, mushrooms and fruits of acai palm. It can also be found as a metabolite of green tea. Protocatechuic have varying activities against cancer cells. It is also reported to show antigenotoxic effects and tumoricidial effect against HL-60 leukemia cells by inducing apoptosis (Anter et al., 2011, Smirnova et al., 2012). Protocatechuic acid is also found to possess weak

antimicrobial activity against *Pseudomonas aeruginosa* (Sakharkar et al., 2009). Chemical structure of protocatechuic is shown in the following figure [Figure. 24].

Figure 24: Chemical structure of protocatechuic acid

3.2 *Experimental methods*

3.2.1 *Antibiotic- Phytochemical combination studies*

3.2.1.1 Preparation of antibiotic Stock solution

Antibiotic stock solution was made with the concentration of 10,000µg/ml. It was then further diluted at the start of each experiment to obtain working concentration. Potency of the antibiotic was given either by the manufacturer or calculated according to the following formula (Richard Schwalbe, 2007).

$$Potency = Purity \ x \ Active \ fraction \ x \ (1 - water \ content)$$

Equation 1

After the potency was obtained, the weight of the chemical was calculated by using the following formula.

$$Weight\ (mg) = \frac{volume(ml)x\ desired\ concentration\left(\frac{\mu g}{ml}\right)}{antibiotic\ potency\ (\frac{\mu g}{mg})}$$

Equation 2

After the desired stock solution was prepared, working solution was calculated and prepared by using the formula,

$$M_1V_1 = M_2V_2$$

Equation 3

Where,

M_1- the initial concentration of the stock solution

V_1 - the amount of volume needed from the stock solution to be diluted in the final working solution.

M_2 - the desired concentration to be used as working solution,

V_2 - the amount of final diluted solution

3.2.1.2 Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentration was determined by microbroth dilution method which was performed in 96-well microtiter plates with an inoculum of 10^6 CFU/ml (Schwalbe et al., 2007). Iso Sensitest Broth was used in this study. Microbroth dilution assay was done in accordance with the procedure stated by antimicrobial sensitivity testing protocol, which is a modification of CLSI protocol (Schwalbe et al., 2007). All

MIC determinations were done in triplicate. The bacterial culture was incubated at 37°C for 24 hours. MIC was defined as the lowest concentration of antibiotic that could inhibit the visible growth. MIC readings were performed by optical density reading with spectrophotometer at 600nm.

3.2.1.3 Checkerboard Assay

Antibiotic interactions were determined by the checkerboard assay. Checkerboard assay is one of the most frequently used methods to assess drug interactions due to its simple nature (White et al., 1996, Cappelletty and Rybak, 1996, Livermore, 2000). Bacterial concentration used for this analysis was 10^6 CFU/ml. Combinations were performed using 96-well microtiter plates, and incubated at 37°C for 24 hours. MIC of drug combinations of different concentrations was calculated by optical density reading with spectrophotometer at 600nm. Drug interactions (synergy, indifference or antagonism) were measured by determining the fractional inhibitory concentration (FIC) index.

FIC of each antibiotic was calculated by using this formula

$$FIC\ for\ antibiotic\ A = \frac{MIC\ of\ antibiotic\ A\ in\ combination}{MIC\ of\ antibiotic\ A\ alone}$$

Equation 4

$$FIC\ for\ antibiotic\ B = \frac{MIC\ of\ antibiotic\ B\ in\ combination}{MIC\ of\ antibiotic\ B\ alone}$$

Equation 5

Summation of FIC (∑FIC) index for each combination was calculated as follows.

$$\sum FIC = FIC\ of\ antibiotic\ A + FIC\ of\ antibiotic\ B$$

Equation 6

Interpretation:

The synergism, indifference and antagonism were interpreted as follows:

Synergism = ∑FIC is ≤ 0.5

Indifference = ∑FIC is > 0.5 and ≤ 4

Antagonism = ∑FIC is > 4.

3.2.1.4 Time Kill Assay

In time kill method, bacterial strains were grown in the presence and absence of antimicrobial agents both alone and in combination with phytochemicals. Tubes containing Drug A (Antibiotic), Drug B (Phytochemical), Drug A+B, positive control and negative control, were inoculated with 1.5 x 10^6 CFU/ml of bacteria and incubated at 37°C for 24 hours. The cultured broth was taken out at 4 hours, 8 hours and 24 hours for plating and determination of viable counts. The viable colony counting (CFU/ml) was measured at 48 hours after incubation at 37°C. Synergy was defined as ≥ 2 log10 CFU/ml fold decrease by the combination when compared with the most active single agent at any time during the 24 hour experiment. Antagonism was defined as a ≥ 2 log10 CFU/ml fold increase by the combination compared with the most active single agent. Time kill assay was performed as a confirmation, for combinations that show synergism in the checkerboard assay.

3.2.1.5 Statistical Analysis

Each set of minimum inhibitory concentration determination and time kill assays were performed in triplicate. Different antibacterial effect of antibiotics as single agents and antibiotics in combination of phytochemicals were determined by statistical significant testing using one way ANOVA (Analysis of Variance). Post-hoc comparisons with Bonferroni corrections were performed using SPSS software version16.1. Statistical significance was determined at $p<0.05$.

3.3 ***Results***

3.3.1 *Minimum inhibitory concentration*

Antimicrobial activity of phytochemicals and antibiotics were assessed by establishing minimum inhibitory concentrations using microbroth dilution method. It was found that the minimum inhibitory concentrations of antimicrobials were different depending on the strain. MIC of mupirocin (M) was (0.25-4 μg/ml), tetracycline (T) (0.12-1 μg/ml), fusidic acid (F) (0.01-0.03 μg/ml), vancomycin (V) (0.5 μg/ml), cefoxitin (C) (1-2 μg/ml). On the other hand, MICs of phytochemicals were found to be much higher; epicatechin gallate (ECG) (32-128 μg/ml), ethyl gallate (EG) (1024 μg/ml), rutin (R) (1024 μg/ml), quercetin (Q) (1024 μg/ml), protocatechuic acid (4096 μg/ml). MIC values are shown in the following figures [Figure 25-26]. Bacterial viability was also determined 18-24 hours after the treatment with each antimicrobial as shown in Figure 27 and 28. Each bacterial viability determination was done in triplicate and standard deviations were obtained.

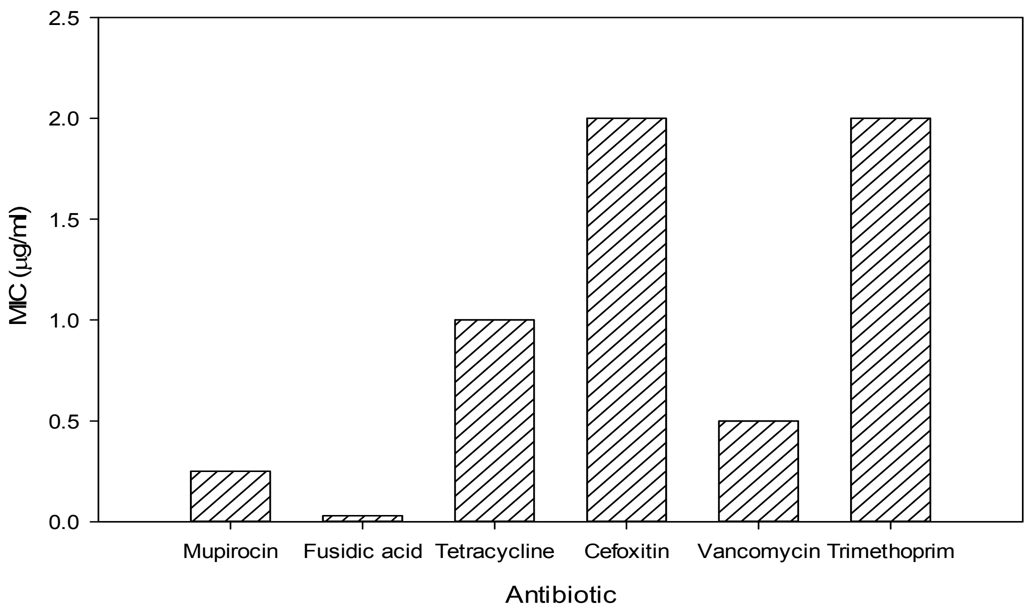


Figure 25: MIC of antibiotics

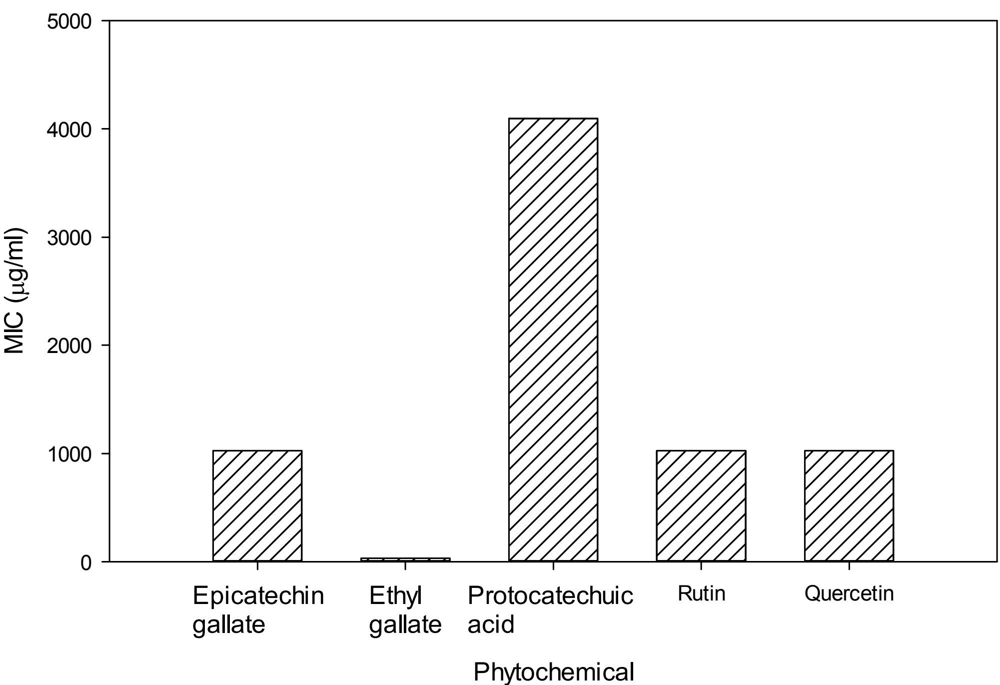


Figure 26: MIC of phytochemicals

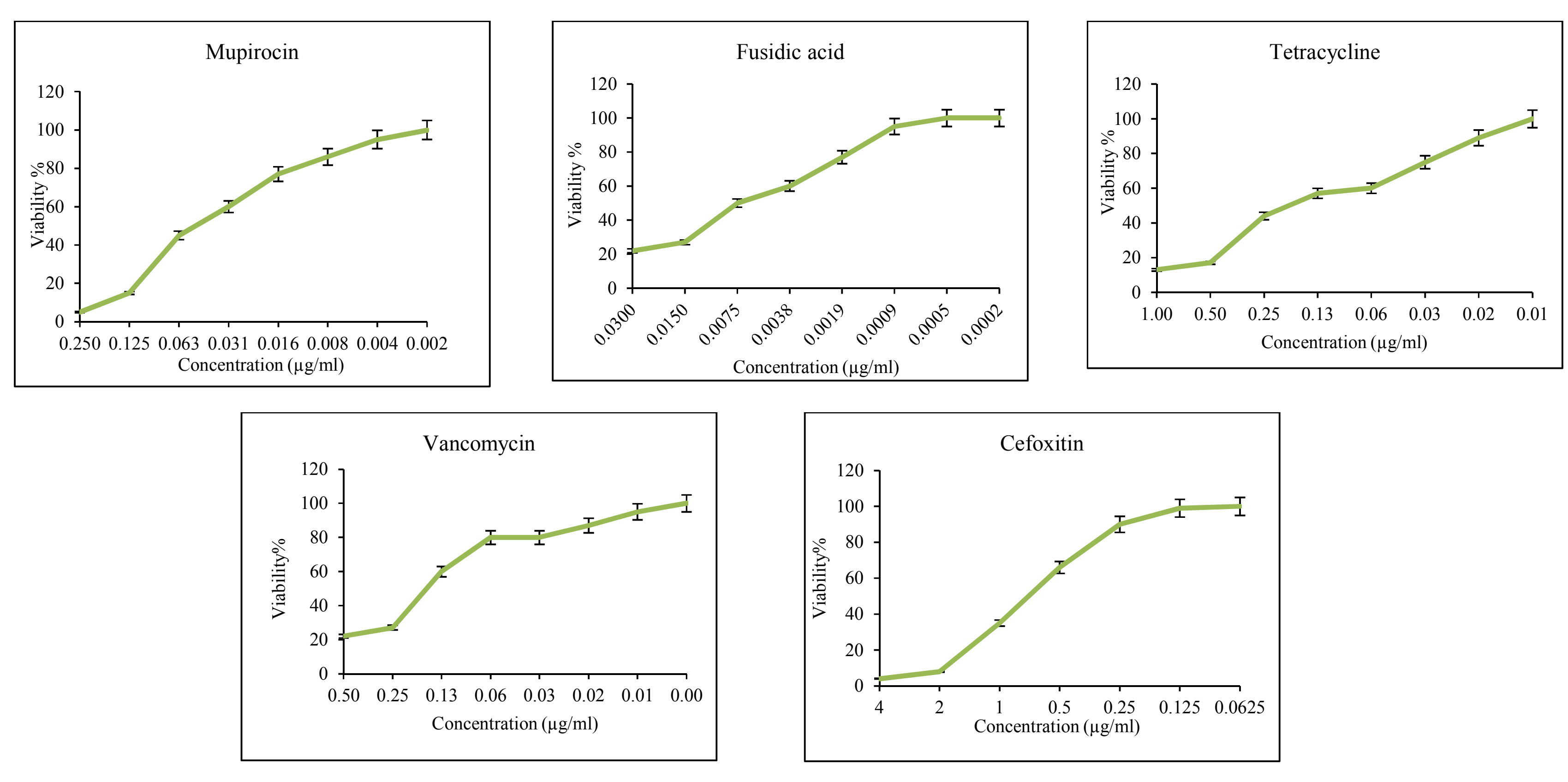


Figure 27: Bacterial viability after treatment with antibiotics

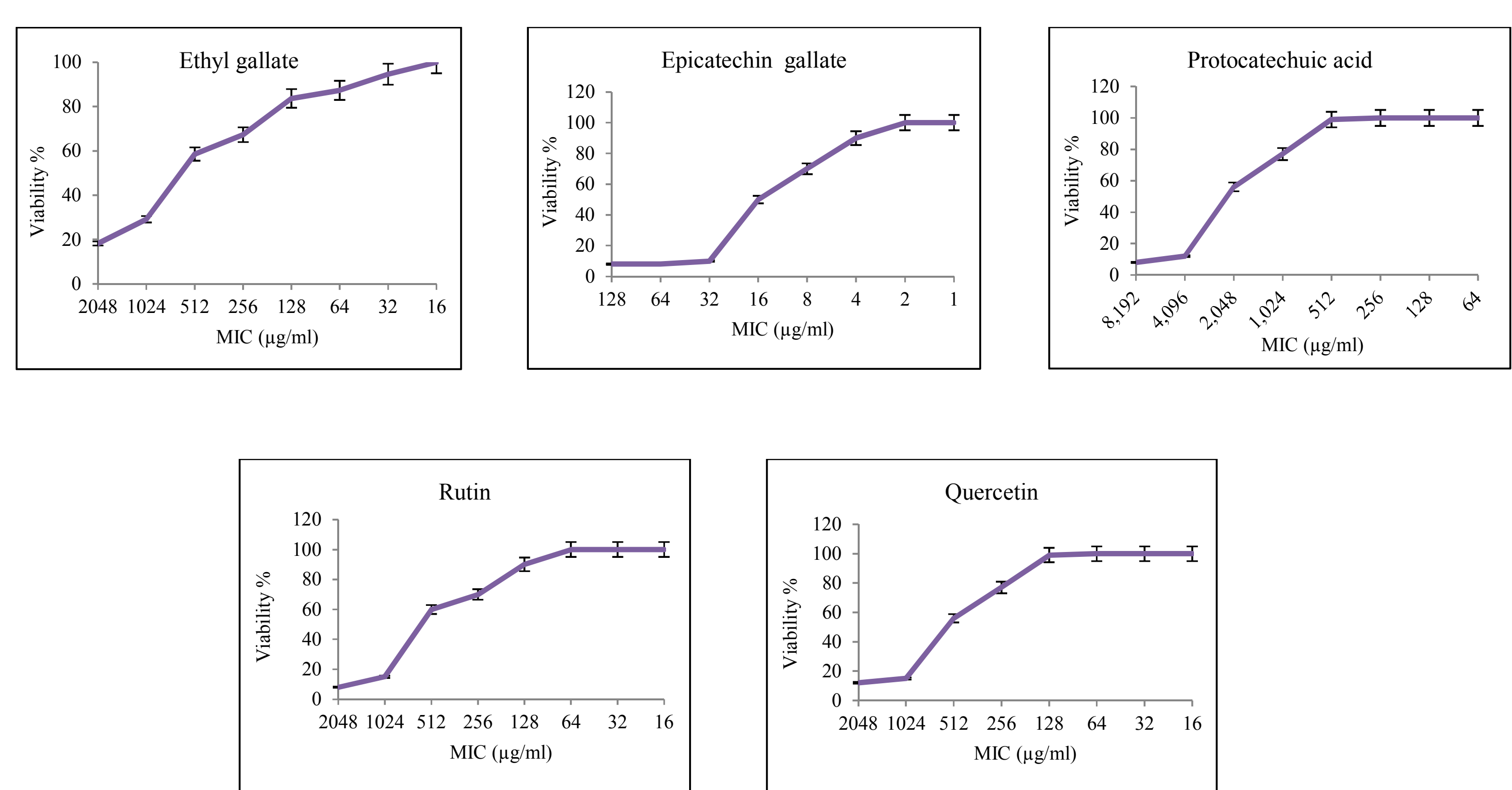


Figure 28: Bacterial viability after treatment with phytochemicals

3.3.2 *Checkerboard combinations with phytochemicals*

Fractional concentration (FIC) was obtained by calculating the fraction of MIC of antimicrobial as a single agent to MIC of antimicrobial in combination with another antimicrobial (i.e. antimicrobial in combination with phytochemical). Fractional inhibitory concentration index (FICI) was obtained by addition of respective FICs. FICI ≤ 0.5 was regarded as synergistic, FICI of $0.5 < \text{FICI} \leq 4$ was regarded as indifferent and FICI > 4 was regarded as antagonistic. FICI calculations showed that ethyl gallate was synergistic with fusidic acid, mupirocin and tetracycline (FICI ≤ 0.5) resulting in four-fold reduction of MIC for all of the *Staphylococcus aureus* bacteria under study. FICI calculations showed that epicatechin gallate showed synergism in combination with tetracycline for all *Staphylococcus aureus* strains. However, ECG showed selective synergism (i.e. depending on the strain of *Staphylococcus aureus*) when it was combined with fusidic acid and mupirocin. Rutin was synergistic to fusidic acid and tetracycline in all of the strains. However, rutin showed strain dependent synergism in combination with mupirocin. All other phytochemical-antibiotic combinations showed indifference ($0.5 < \text{FICI} \leq 4$). FICI and minimum inhibitory concentrations of antimicrobials as single agents and in combinations are shown in details in Table 3: Minimum inhibitory concentrations and FICI of antibiotics as single agents and in combinations As the combination of phytochemicals (rutin, protocatechuic acid, epicatechin gallate and quercetin) except ethyl gallate, showed selective synergism with the antibiotics, time kill studies were performed only for ethyl gallate and antibiotic combinations.

Table 3: Minimum inhibitory concentrations and FICI of antibiotics as single agents and in combinations

MRSA ATCC	AB	Mupirocin (µg/ml)	FICI	Fusidic acid (µg/ml)	FICI	Tetracycline (µg/ml)	FICI	Vancomycin (µg/ml)	FICI	Cefoxitin (µg/ml)	FICI
MRSAATCC											
Phyto:	MIC	0.25		0.01		0.12		0.5		2	
EG	1024	0.06	0.5	0.0025	0.5	0.03	0.5	0.5	1	0.5	0.5
ECG	64	0.25	1	0.01	1	0.03	0.5	0.5	1	2	1
PA	4096	0.25	1	0.01	1	0.12	1	0.5	1	2	1
Rut	1024	0.06	0.5	0.0025	0.5	0.03	0.5	0.5	1	2	1
Quer	1024	0.25	1			0.12	1	0.5	1	2	1
MRSAC1											
Phyto:	MIC	4		0.03		1		0.5		2	
EG	1024	1	0.5	0.0075	0.5	0.25	0.5	0.5	1	0.5	0.5
ECG	128	4	1	0.015	0.75	0.25	0.5	0.5	1	2	1
PA	4096	4	1	0.03	1	1	1	0.5	1	2	1
R	1024	1	0.5	0.06	0.5	0.25	0.5	0.5	1	2	1
Q	1024	4	1	0.03	1	1	1	0.5	1	2	1
MRSAC2											
Phyto:	MIC	4		0.03		1		0.5		2	
EG	1024	1	0.5	0.0075	0.5	0.25	0.5	0.5	1	0.5	0.5
ECG	128	4	1	0.015	0.75	0.25	0.5	0.5	1	2	1
PA	4096	4	1	0.03	1	1	1	0.5	1	2	1
R	1024	4	1	0.06	0.5	0.25	0.5	0.5	1	2	1
Q	1024	4	1	0.03	1	1	1	0.5	1	2	1
MRSAC3											
Phyto:	MIC	4		0.01		0.12		0.5		2	
EG	1024	1	0.5	0.0025	0.5	0.03	0.5	0.5	1	0.5	0.5
ECG	64	4	1	0.01	1	0.03	0.5	0.5	1	2	1
PA	4096	4	1	0.01	1	0.12	1	0.5	1	2	1
R	1024	4	1	0.0025	0.5	0.03	0.5	0.5	1	2	1
Q	1024	4	1	0.01	1	0.12	1	0.5	1	2	1
MSSA C1											
Phyto:	MIC	0.25		0.01		0.12		0.5		1	
EG	1024	0.06	0.5	0.0025	0.5	0.03	0.5	0.5	1	0.25	0.5
ECG	64	0.25	1	0.01	1	0.03	0.5	0.5	1	1	1
PA	4096	0.25	1	0.01	1	0.12	1	0.5	1	1	1
R	1024	0.06	0.5	0.0025	0.5	0.03	0.5	0.5	1	1	1
Q	1024	0.25	1	0.01	1	0.12	1	0.5	1	1	1
MSSAC2											
Phyto:	MIC	0.25		0.03		0.25		0.5		2	
EG	1024	0.06	0.5	0.0075	0.5	0.06	0.5	0.5	1	0.5	0.5
ECG	128	0.25	1	0.015	0.75	0.06	0.5	0.5	1	2	1
PA	4096	0.25	1	0.03	1	0.25	1	0.5	1	2	1
R	1024	0.125	0.75	0.06	0.5	0.06	0.5	0.5	1	2	1
Q	1024	0.25	1	0.03	1	0.25	1	0.5	1	2	1

* synergism

3.3.3 *Time kill studies with Ethyl gallate*

After phytochemical-antibiotic interactions were assessed *in vitro* using checkerboard method, time-kill assays were performed. While basic information about combination potential had been obtained using checkerboard method, the time-kill studies were performed in order to obtain more dynamic description of antimicrobial activity and interactions depending on time (White et al., 1996, Cappelletty and Rybak, 1996). Time kill studies were performed using 4MRSA strains and 2 MSSA strains. Synergy was defined as ≥ 2 log10 CFU/ml fold decrease by the combination when compared with the most active single agent at any time during the 24 hour experiment. Antagonism was defined as a ≥ 2 log10 CFU/ml fold increase by the combination compared with the most active single agent. It was observed that ¼ MIC of EG (256 µg/ml) caused four-fold reduction of MIC in four of the antibiotics; fusidic acid (Protein synthesis inhibitor), mupirocin (Isoleucyl t-RNA synthetase inhibitor), tetracycline (30S ribosome inhibitor) and cefoxitin (cell wall synthesis inhibitor). This reduction was observed in all *Staphylococcus aureus* strains under investigation. However, addition of ¼ MIC of EG (256 µg/ml) expressed indifferent effect in combination with vancomycin in all the bacterial stains. It is noted that all the combinations have shown significant reduction in the bacterial count when it is compared with the single agents ($p<0.05$). Further statistical significance testing of the combinations proved that synergistic combinations are equally effective. Moreover, the combinatory action of phytochemical-antibiotic combinations significantly peaked at 8 hours and lasted for 24 hours. All the synergistic combinations tested by time-kill method confirmed that EG can prolong and potentiate the bactericidal activity of tetracycline mupirocin, fusidic acid and cefoxitin. Difference of colony forming unit

at 0 hr, 4 hr, 8 hr, and 24 hr for all *Staphylococcus aureus* strains were calculated and shown in details in Figure 29-34.

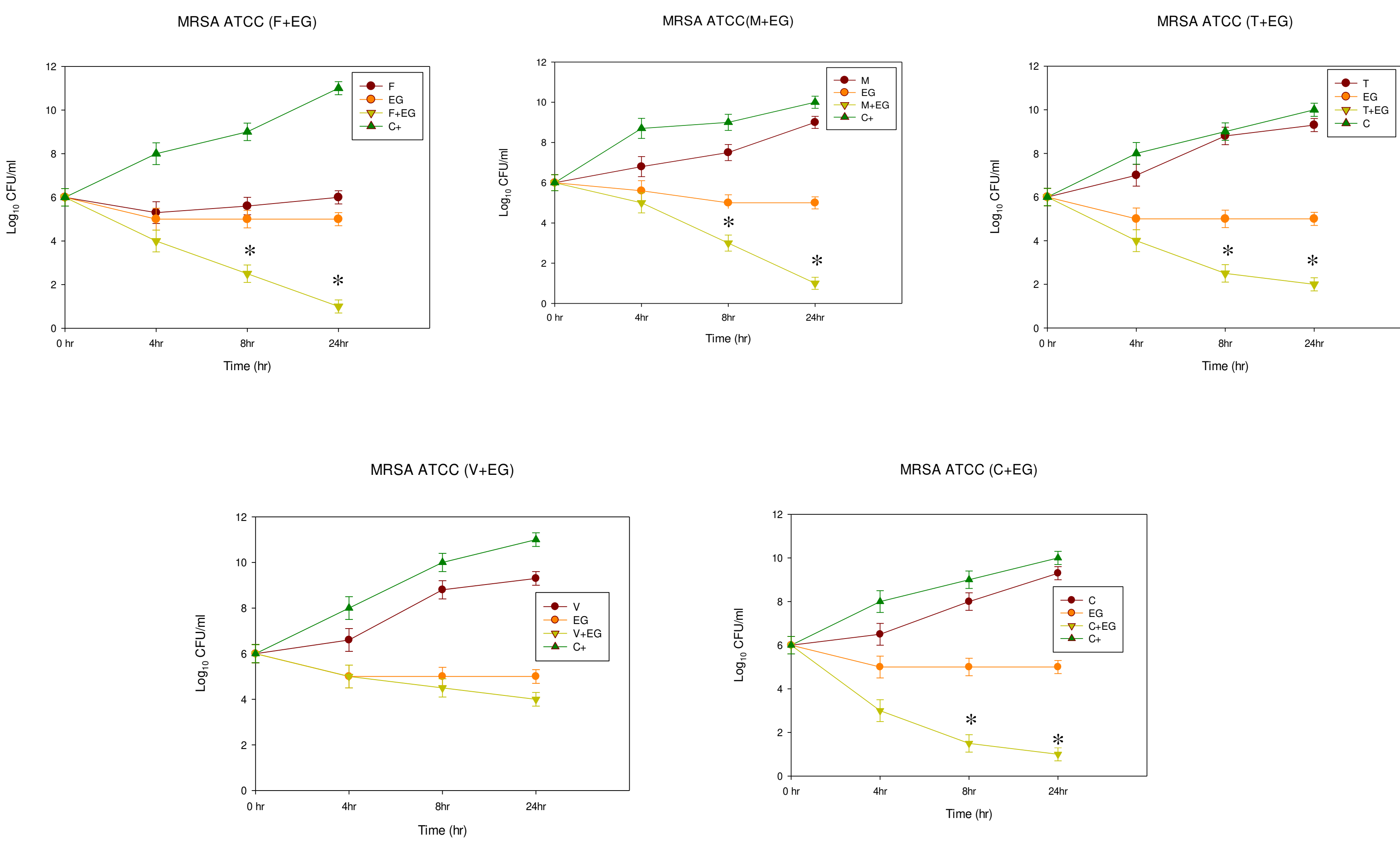


Figure 29: Time kill graphs of combinations tested on MRSA ATCC * F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control*; *: p<0.05

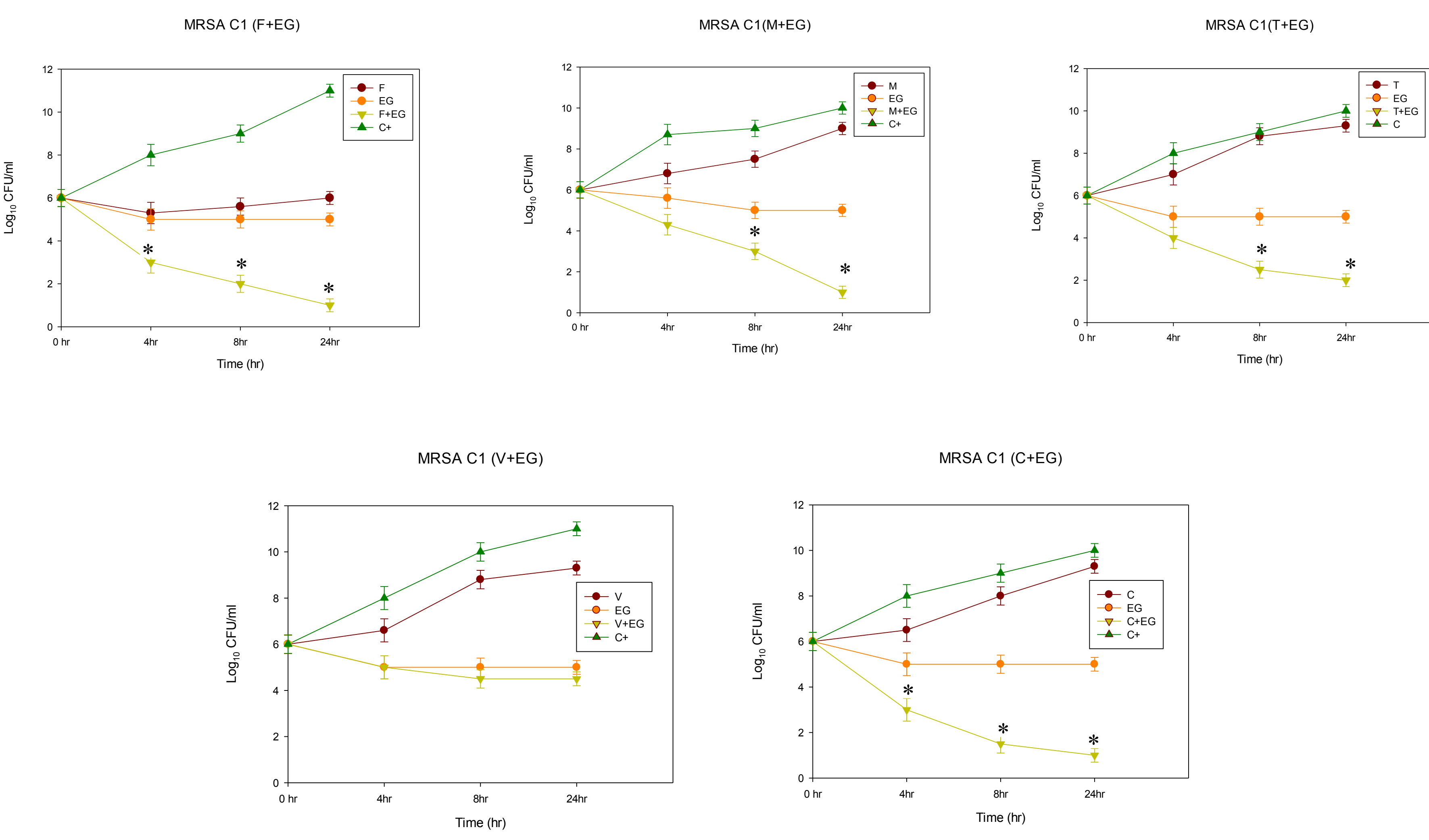


Figure 30: Time kill graphs of combinations tested on MRSA C1 * F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control*; *: $p<0.05$

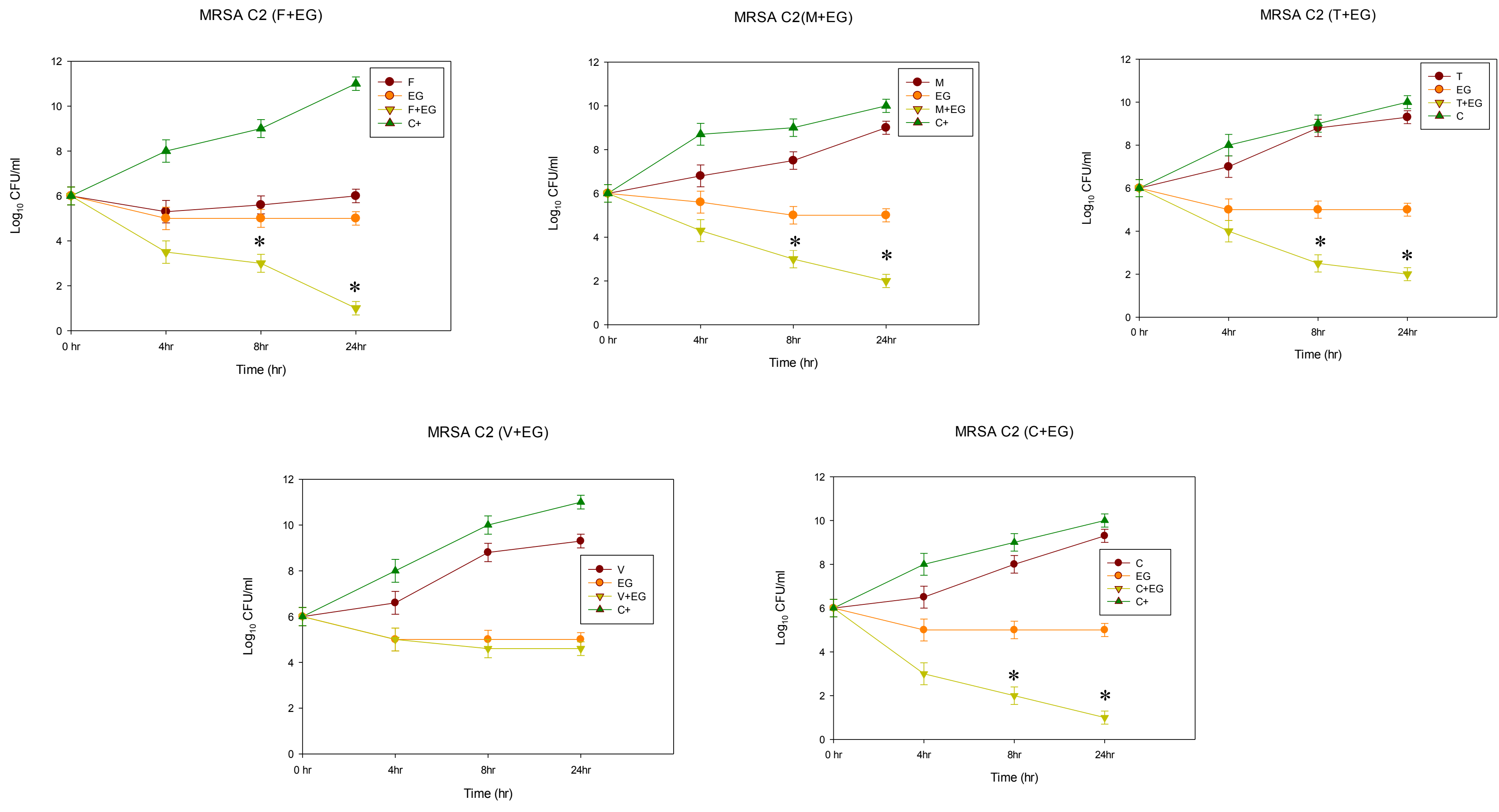


Figure 31: Time kill graphs of combinations tested on MRSA C2 * F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control*;

***: $p<0.05$**

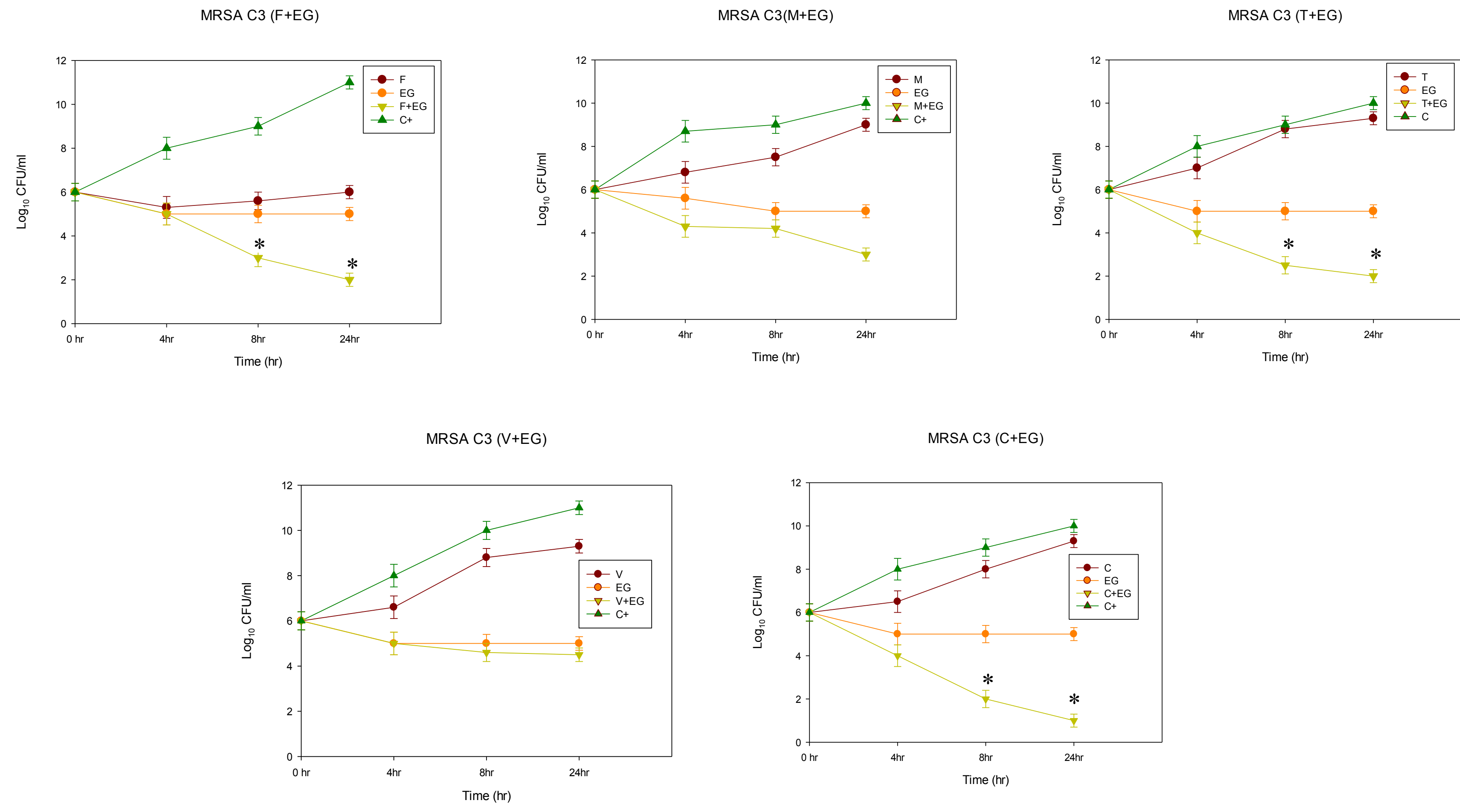


Figure 32: Time kill graphs of combinations tested on MRSA C3 * F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control*;

***: $p<0.05$**

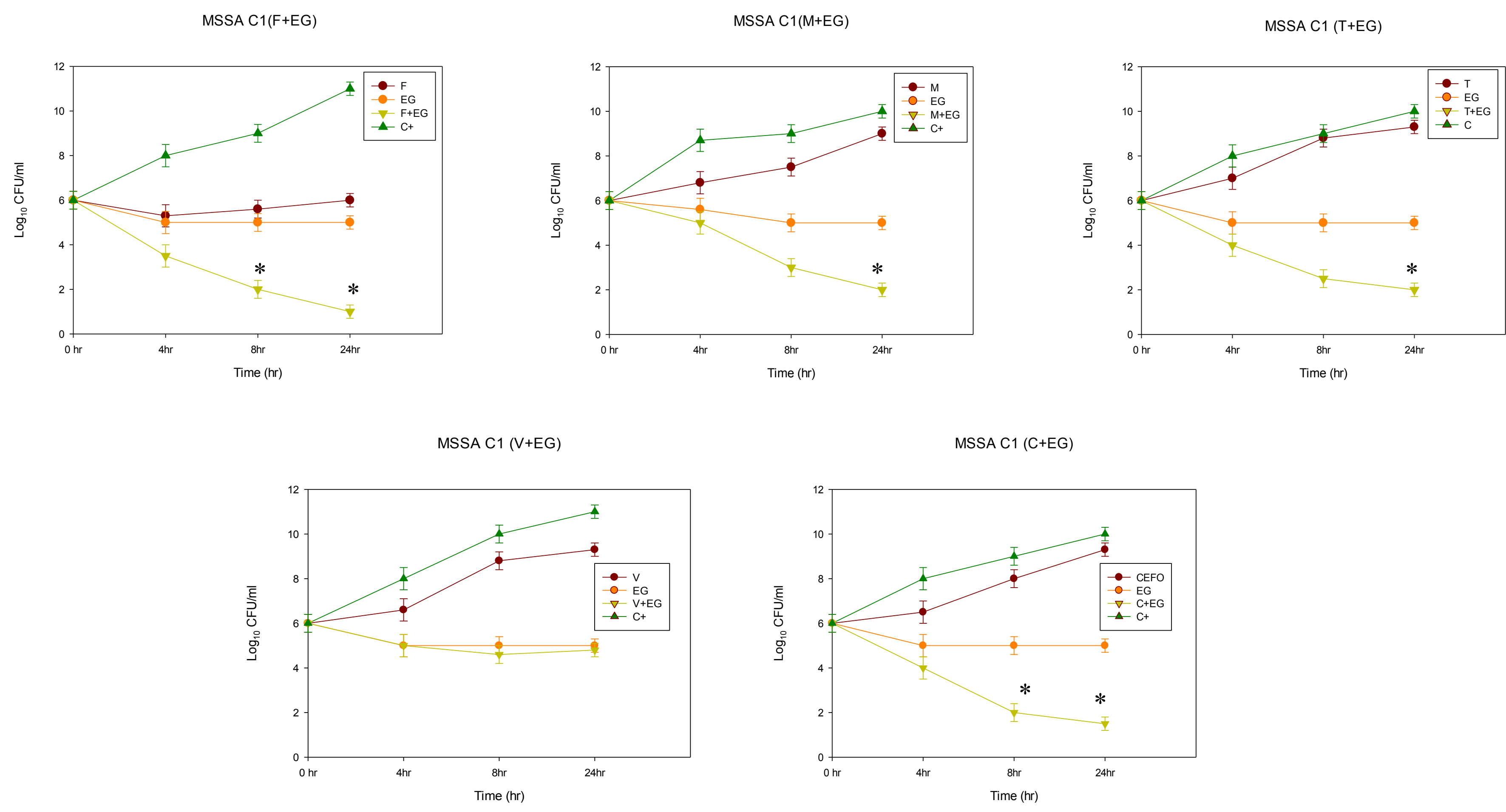


Figure 33: Time kill graphs of combinations tested on MSSA C1 * F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control* *: $p<0.05$

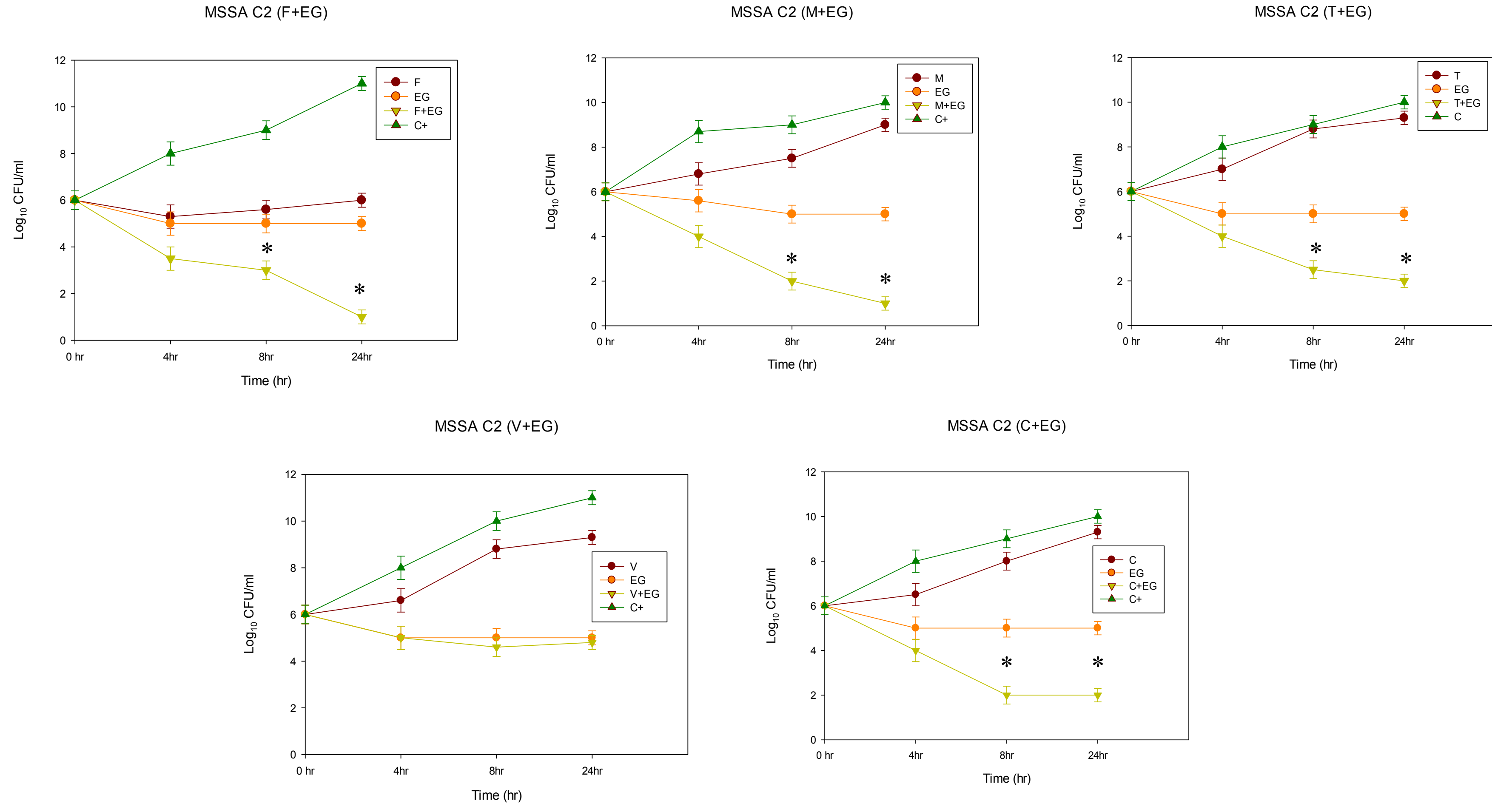


Figure 34: Time kill graphs of combinations tested on MSSA C2* F- fusidic acid, M-mupirocin, T- tetracycline, V-vancomycin, C- cefoxitin, EG-ethyl gallate, C+- positive control*

***: $p<0.05$**

3.4 ***Summary***

This study provided the information about the antimicrobial activity of phytochemicals and synthetic antibiotics. Minimum inhibitory concentrations (MIC) of phytochemicals and antibiotics were established. It was observed that MIC of epicatechin gallate was the lowest (32-128 µg/ml) and MIC of protocatechuic acid (4096 µg/ml) was the highest against *Staphylococcus aureus*. Even though phytochemicals as single agents were found to be weak antimicrobials, they can potentiate the antimicrobial activity of synthetic antibiotics effectively. The findings from this study concurred with the observations reported by Stavri *et al* concerning phytochemicals' ability to potentiate antibiotics as demonstrated by berberine's ability to inhibit efflux pumps in potentiating quinolones (Stavri et al., 2007). Therefore, it could be assumed that ethyl gallate (EG) could also be regarded as a good combination agent despite being a weak antimicrobial by itself. EG was observed to be able to potentiate the activity of the all the protein synthesis inhibitors as well as a beta-lactam antibiotic against both MSSA and MRSA, while ECG selectively potentiated the activity of mupirocin against MSSA. It was also interesting to note that tetracycline was synergistic with both ECG and EG in all *Staphylococcus aureus* strains under study. MIC levels of both of the phytochemicals in this study were lower than the reported toxicity level for these phytochemicals. According to thorough studies of antimicrobial activity of phytochemicals performed by Cowan *et al,* EG was reported to have a relative toxicity of 1.5 and ECG has a relative toxicity of 2 in the toxicity studies graded from 0 being very safe and 3 being very toxic (Cowan, 1999). From this study, EG and ECG were found to have the potential to be used in the routine strategy for treatment of wounds as they have the capability to increase the susceptibility of organism to antibiotics.

In this study, antimicrobial agents having their potential combinatory activity were chosen from different classes of antibiotics. 3 of the compounds (mupirocin, fusidic acid and tetracycline) were commonly used as ointments in treatment of wounds. Even though *Staphylococcus aureus* strains used in this study were susceptible to those antibiotics, there is a potential that the bacteria might develop resistance to those antibiotics soon after the treatment has been started. Therefore, resistance development is commonly associated with topical agents. By investigating the potential combinatory agents for those topical agents, the study attempts to reduce the resistance formation associated with those synthetic antimicrobials.

4. ETHYL GALLATE'S ACTION ON CELLS

4.1 *Introduction*

In this chapter, mechanism of action of ethyl gallate was investigated by observing the effect of sub-MIC concentration of ethyl gallate on *Staphylococcus aureus* bacteria using scanning electron microscope and atomic force microscope. This observation was followed by computational study of ethyl gallate along with other medically important gallates and catechins (epicatechin gallate, epigallocatechin gallate, catechin, epicatechin). Human mononuclear cells were extracted from the peripheral blood and treated with ethyl gallate to estimate the biocompatibility of ethyl gallate at the minimum inhibitory concentration.

4.1.1 *Actions of catechins and gallates*

Gallate compounds have been investigated for their combination actions with other antibiotics in the last decade. Stapleton *et al* observed that epicatechin gallate mediates alterations to the physical nature of the bilayer, and elicits the structural changes to wall's teichoic acid, a labile component of peptidoglycan, resulting in modulation of the cell-surface properties necessary to maintain the beta-lactam-resistant phenotype (Stapleton *et al*., 2004). Kubo *et al.* postulated that ethyl gallate inhibits the respiratory chain of MRSA bacteria (Kubo et al., 2002). Moreover, the surfactant nature allows alkyl gallates to target extra-cytoplasmic regions to produce antimicrobial activity without the need to enter the cell. Thus, cellular pump mechanisms have no effect on ethyl gallate (Hendrich, 2006).

Many studies also report that the presence of galloyl moiety shown, in Figure 35, is a major contributing factor for the antimicrobial activity (Kubo et al., 2002, Retico et al., 1981, Shibata et al., 2005). Shibata *et al.* emphasized the role of alkyl gallates in potentiating beta lactam antibiotics by studying the combinations of alkyl gallates with oxacillin in both MRSA and MSSA strains (Shibata et al., 2005). Epigallocatechin gallate (EGCG), which was structurally similar to epicatechin gallate, can reduce the tolerance of *Staphylococcus aureus* bacteria to high ionic strength and low osmotic pressure in their external atmosphere that can inflict damage of the cell wall. EGCG was also shown to be synergistic with oxacillin by sequential inhibition of peptidoglycan in the cell wall (Hu et al., 2001, Zhao et al., 2001). Even though the mechanisms responsible for the synergism between the combinations are not yet known, the presence of antimicrobial activity and selective synergistic activities with protein synthesis inhibitors and indifference activities with peptidoglycan synthesis inhibitor, indicates that these combinations of antibiotics and phytochemicals improve therapeutic efficacy over single agents. Therefore, further investigations were performed to obtain more insights into mechanism of action of the gallates (Chadwick et al., 1986, MacKay et al., 2000, Shibata et al., 2005).

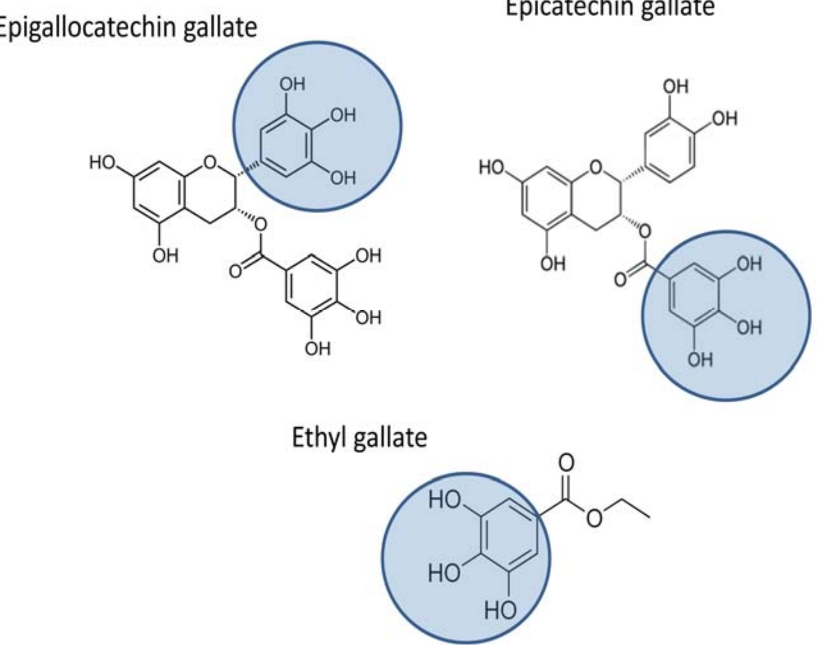


Figure 35: Galloyl moiety of Gallate compounds

4.1.2 *Bacterial cell wall synthesis inhibitors*

Principally, bacterial cell wall is the main stress-bearing and shape maintaining element in the bacteria (Scheffers and Pinho, 2005). Bacterial cell viability largely depends on the integrity of cell wall in both gram positive and gram negative cells. Cell wall biosynthesis pathway was the first to be exploited for antibacterial treatment because of the unique structural properties. Differences in bacterial and mammalian cells wall structures allow the antimicrobials to achieve effective killing without harming the host cells (Goodman et al., 2008). Gram positive cell wall is mainly composed of peptidoglycan. Peptidoglycan is a frame work of glycan chains linking N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAMA). Interspersed between these polymers are teichoic acid and teichuronic acid

molecules. Peptidoglycan biosynthesis pathway consists of different phases: cytoplasmic phase, cytoplasmic membrane phase and cell surface phase. Each phase can be targeted by different group of cell wall acting agents. Bacterial cell wall synthesis and antibiotics targeting the cell wall synthesis are explained in details in the following diagram.

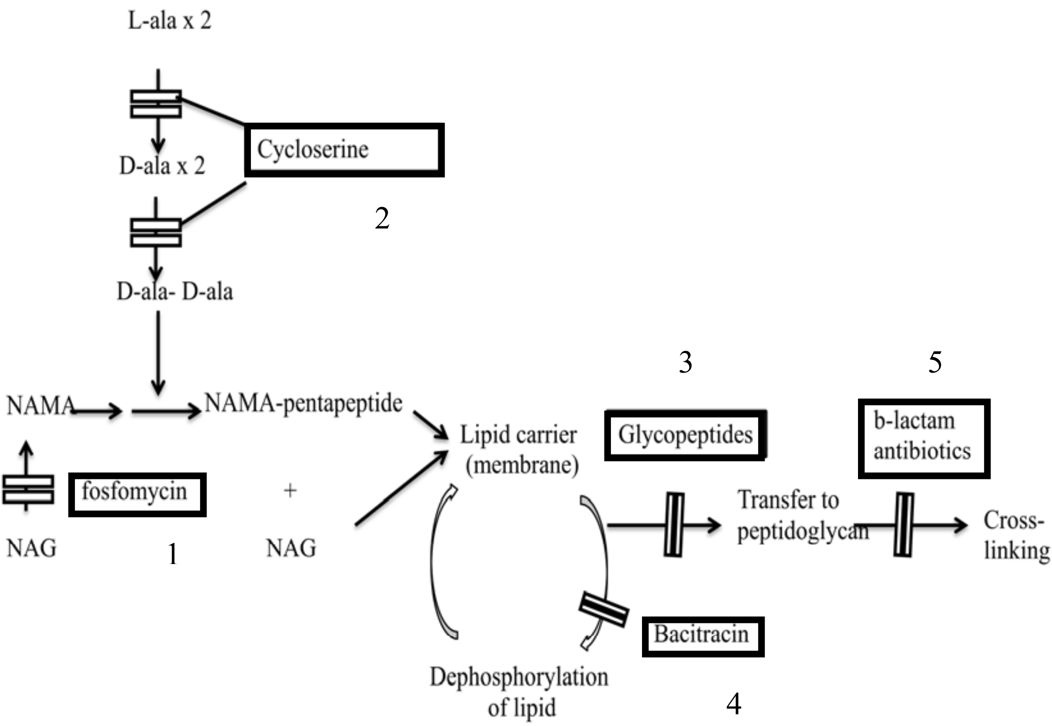


Figure 36: Bacteria cell wall synthesis inhibitors

* 1. Fosfomycin blocks the synthesis of N-acetyl muramic acid (NAMA) from N-acetyl glucose (NAG) by inhibiting action of pyruvyltransferase action. 2. Cycloserine blocks the linkage of D-alanine and D-alanyl D-alanine by inhibiting in two sequential steps; conversion of L-alanine to D-alanine and ligase action on the subsequent step. 3. Glycopeptides inhibit the transglcosylation process to stop translocation of whole unit of NAMA-pentapeptide+NAG 4. Bacitracin inhibits the reycling of phosphate group which is needed to serve as a native carrier for another round of translocation.5. Beta-lactam antibiotics pevent the final step of peptidoglycan synthesis by inhibiting the action of transpeptidase in order to stop the crosslinking of peptide side chains.

4.1.3 *Lipoteichoic acid synthase enzyme*

Lipoteichoic acids (LTA), membrane-anchored molecules, are secondary wall polymers which are found in the cell envelopes of Gram-positive bacteria. Predominant type of lipoteichoic acid contains 1, 3-linked poly (glycerophosphate) chain attached by phosphodiester bond to a glycolipid or phosphatidyl glycolipid which, in the free state, occur as characteristic membrane lipids in gram- positive bacteria (Fischer, 1988). LTA in *Staphylococcus aureus* is anchored to the membrane by the glycolipid diglucosyl-diacylglycerol. After glycolipids are synthesized in the cytoplasm in a process assisted by the proteins PgcA, GtaB and YpfP, they are transferred from the inner leaflet of the membrane to the outer leaflet. Subsequently, the LTA synthase enzyme LtaS polymerizes the polyglycerolphosphate backbone of LTA using a phospholipid phosphatidylglycerol (PG) as donor molecule for the glycerolphosphate repeating units (Kiriukhin et al., 2001, Fedtke et al., 2007). Gründling *et al* (Grundling and Schneewind, 2007) reported that LTA biosynthesis is essential for growth and cell division in *Staphylococcus aureus*. Fedtke *et al.* also reported about the role of LTA in controlling bacterial surface properties and autolysin activity. However, the precise functions of LTAs are still unknown. Nonetheless, the recent discovery of Lipoteichoic acid synthase (LtaS), an enzyme responsible for polymerization of polygylcerolphosphate backbone of LTA, and its exclusive presence in bacteria, has provided hope that lipoteichoic acid synthesis pathway can be a possible target for novel drugs development (Karatsa-Dodgson et al., 2010). The following figure [Figure 37] depicts the proteins involved in lipoteichoic acid in details.

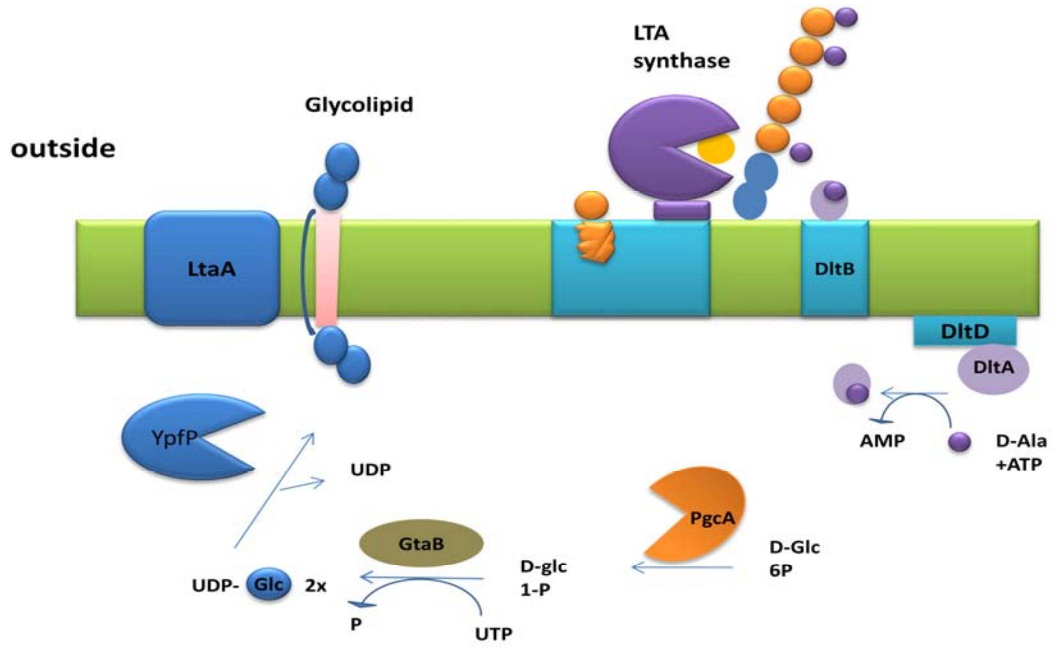


Figure 37: Proteins required for Lipoteichoic acid synthesis

*α–phosphoglucomtase (PgcA) converts D-glucose 6 phosphate (D-glc6P) to D-glucose 1-phosphate (D-glc1P). α-glucose UTP 1-phosphate uridyltransferase (GtaB) helps the formation of UDP-glucose. Diglucosyl-diacylglycerol [Glc_2-DAG] is formed with the help of YpfP, a processing enzyme that transfers glucose to diacyl glycerol using UDP-glucose as a substrate. Lipoteichoic acid protein A (LtaA) helps translocation of the glycolipid from inner leaflet to outer leaflet of the membrane. Subsequently, LTA synthase enzyme (LtaS) helps polymerization of polyglycerolphosphate backbone of LTA using phospholipid phosphatidylglycerol (PG) as a donor molecule. Finally dlt operon (DltA) helps incorporation of cell wall molecules D-ala to lipoteichoic acid.

4.1.4 *Action of Ethyl gallate on human peripheral blood mononuclear cells*

Even though there are numerous compounds with biological activities which have been investigated, many of them are not suitable for therapeutic use because of their toxicity, carcinogenicity and mutagenic properties (Mbwambo et al., 1996). Therefore, there is a need to establish the safety of the compound for usage if the compounds are intended for therapeutic effect. There are various ways to simulate the effect of drug on the cells of

human body. Organ culture system and animal models are among the well-known studies. Even though animal experiments can give good approximation to the human system, these experiments usually involve large number of animal experiments. Moreover, it is humane and more economical that these compounds are tested *in vitro* before they are tested on animals (Alley et al., 1988, Maravelias et al., 2000, Mori, 1993). Organ culture system has its own advantages as the relationship between various tissue components and various cells is maintained in a manner similar to that of the physiological conditions in our body. While the organ culture system can be deemed as more meaningful physiological test method, it has its own disadvantages mainly because of the problem in the procurement and a need of the large amount of living organisms to duplicate their function (Mori, 1993). The difficulty also lies in quantifying the effect of the drug due to the heterogenicity of the cells or variation of size of the tissue.

On the other hand, cell culture methods are proved to be simple, specific and efficient approximation of the cells on the organ systems (Alley et al., 1988, Borenfreund and Puerner, 1985). Therefore, in order to determine the cytotoxicity, *in vitro* cytotoxicity assays need to be performed. Cytotoxicity test is a simple and sensitive measure of the effect of various substances such as drugs, chemicals and other industrial agents for simulation of their behavior when they are in contact with the mammalian cells. It gives good estimation of *in vivo* tests behavior without having to use the animals (Mosmann, 1983). Cytotoxicity assays on cells can sensitively measure cytotoxicity and pharmacological activity of test substances. Moreover, they can quantify cytotoxicity by simulating cell metabolism or intercellular interaction. Therefore, *in vitro* cytotoxicity test can be regarded as a good predictor of risks that occur in a person after being given a

drug. A high cytotoxicity in mammalian cell would be a good predictor of side effects that could have an ill effect on the patient that is administered. In this study, in order to determine the cytotoxicity, human peripheral blood mononuclear cells were extracted and treated with ethyl gallate. Cytotoxicity assay was performed at 24 hours, 48 hours and 72 hours intervals. MTT assay was used as a standard cytotoxicity assay in this study (Skehan et al., 1990, Mosmann, 1983). In MTT assay, cells in the exponential phase of growth were exposed to ethyl gallate. At 24 hours, 48 hours and 72 hours, the drug was removed and allowed to proliferate for two to three population-doubling times to distinguish between the cells that remain viable to continue proliferation and those that cannot proliferate.

4.2 ***Experimental methodology***

4.2.1 *Detection of ethyl gallate's action on bacterial cells*

Morphology of ethyl gallate treated *Staphylococcus aureus* bacteria cells were obtained by scanning electron microscopy and atomic force microscopy. *Staphylococcus aureus* cells were grown to mid-logarithmic phase in IS broth supplemented with 1/2MIC of ethyl gallate and incubated for 8 hours. IS broth that is not supplemented with ethyl gallate was used as a control. 2 ml of each culture was sedimented by using a centrifuge at 3500g. The pellet was washed twice with 2 ml of phosphate buffer saline. Then the cells were re-suspended in 1X phosphate buffer saline (1XPBS). 100 µl of the cell suspension was taken out and inoculated on coverslip and left to air dry for atomic force microscopy. The remaining cells were fixed by the addition of 2.5% glutaraldehyde. The fixation was allowed to continue overnight at 4ºC. After the fixation, the cells were then washed with

1XPBS for 2 times, they were then post- fixed by using 1% glutaraldehyde + 3% formaldehyde solution. The specimen was then subjected to serial dehydration by using 50%, 70%, 90%, 100% ethanol. The cells were then coated with gold and observed under JEOL Scanning Electron Microscope.

4.2.2 *Scanning electron microscopy*

It is important to understand structural and physical properties of bacterial cells if the function of a cell surface is to be known. Many powerful tools that are used to visualize the cell surface such as scanning electron microscopy and transmission electron microscopy require cell manipulation such as fixation before visualization. Scanning electron microscopy is a valuable adjunct to light microscopy for studying gross cell morphology (Afrikian et al., 1973). It uses electron beam instead of light for imaging. Electron guns produce beam of electrons which go through a vertical path along the microscope located in vacuum. Electron beam was guided to reach the specimen by the use of electrical and magnetic fields. X-rays that are emitted from the impact of electron to the specimen are detected and analysed on screen. Thus, it allows observation and characterization of heterogeneous organic and inorganic materials on a nanometer to micrometer scale. When conventional SEM is used, 2 dimensional images can be observed with area ranging from 1cm to 5 microns with 20X to approximately 30,000X spa in spatial resolution of 50 to 100 nm (Afrikian et al., 1973, Fingerman, 2007). Visualization with SEM requires the specimen to be prepared by fixation, dehydration and critical point drying before being coated with gold. Next, the completely dried specimen is placed in vacuum chamber for examination (Firtel and Beveridge, 1995).

4.2.3 *Atomic Force Microscopy*

After performing scanning electron microscopy, the visualization of cell morphology was further explored by using atomic force microscopy. Atomic force microscopy was used because of its ability to produce high spatial resolution and low-distortion images. It also has the ability to map distribution of single molecules and ability to provide true 3-Dimensional images (Trache and Meininger, 2008, Liu and Wang, 2010). The atomic force microscope otherwise known as scanning force microscope is a high-resolution type of scanning probe microscope. Atomic force microscopes are used to measure, capture and manipulate the images at nanoscale. Operating modes of AFM depend on application, which are mainly differentiated into contact mode and non-contact mode as the information is gathered by feeling the surface with a mechanical probe. AFM is also regarded as a useful tool to study the change in cell surface morphology resulting from the treatment with external agents such as enzymes and antibiotics (Dufrene, 2002, Dufrene, 2008, Liu and Wang, 2010). In this study, AFM images were obtained by air-drying the microbial samples as they can provide suitable hardness for scans without significant topographic changes (Eaton et al., 2008, Liu and Wang, 2010). *Staphylococcus aureus* bacteria were harvested after the treatment with 1/2 MIC of antibiotic for 4 hours. They were then air-dried. AFM was carried out with a Veeco Multimode IVa atomic force microscope (Veeco, Santa Barbara, CA), equipped with a j-type scanner (100 x 100 x 5 mm^3 scan range). Bacteria morphology studies were carried out in the tapping mode in air, using silicon cantilevers with a resonant frequency of approximately 150 kHz (MikroMasch, Tallinn, Estonia).

4.2.4 *Action of ethyl gallate on human peripheral blood mononuclear cells*

Human mononuclear cells were extracted from peripheral blood, MTT (3-(4, 5-dimethyltiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma Aldrich) was dissolved in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) at 5 mg/ml. The MTT stock solution was stored in the dark at 4°C and used within a week. RPMI medium and antibiotic solution (penicillin G/streptomycin), Fetal Bovine serum (FCS), Dimethyl sulfoxide (DMSO), Trypsin, Histopaque solution, were obtained from Sigma Aldrich. Experiments were carried out at the republic polytechnic School of Applied science.

4.2.4.1 Extraction of peripheral blood mononuclear cells

Venous blood was collected in preservative- free heparinized tubes. Blood was processed within 2 hours to obtain optimum result. 3 ml of Histopaque was added into 15 ml centrifuge tube. About 3 ml of whole blood was carefully layered onto Histopaque-1077 which was a medium in which the mononuclear cells were separated. The mixture was then centrifuged at 400g for 30 minutes at room temperature. After centrifugation, the middle layer within 0.5 cm of opaque interphase containing monolayer cells was carefully aspirated with Pasteur pipette. The upper layer was discarded. The opaque interface was transferred with a Pasteur pipette into clean conical centrifuge tubes. 10 ml of isotonic phosphate buffered saline solution was added into this tube and mixed by gentle aspiration. The mixture was then centrifuged for 10 minutes. The resulting supernatant was aspirated and discarded. The cell pellet was re-suspended with 5 ml of isotonic phosphate buffered saline solution and mixed by gentle aspiration with pipette. Then the mixture was centrifuged again at 250g for 10 minutes. The washing was repeated and the

supernatant was discarded. The cells are then re-suspended in RPMI medium. The resulting cell number was counted using cell counter.

4.2.4.2 Cytotoxicity assay

Cells were counted and seeded into a 96 well plate at a density of 0.5 x 10^4 cells per well. Cells were treated immediately with the different concentrations of ethyl gallate. The plates were incubated at 37ºC in incubator in the presence of 5% CO_2. At 24 hours, 150µl of culture medium containing MTT was added to each well and re-incubated for 4 hours. The incubated mixture was then centrifuged to precipitate MTT crystals at the bottom of the plate. The supernatant medium was then gently removed and washed gently in 0.01M PBS. 100 µl DMSO was added into each well to solubilize the crystallized formazan product. After incubating at room temperature for 10 to 15 minutes, the wells were examined to ensure crystals had been fully solubilized and the plates were read on a Gen5 plate reader at 570 nm and a reference wavelength of 690 nm. The absorbance readings for 690 nm were subtracted from the 570 nm readings and the results were adjusted by dividing the average by the 0.1 % DMSO control to detect any toxicity that may have occurred in this control treatment set.

4.2.5 *Computational analysis of the mechanism of action of phytochemicals*

Many studies have indicated that computational approaches, such as predicting target interaction networks, predicting HIV cleavage sites in proteins and predicting antimicrobial peptides can provide many useful insights and data which would have been time-consuming and costly if experiments were soley employed (Alexander and Rietschel,

2001). Computational modeling combined with the information derived from the structural bioinformatics tools can provide useful insights for both basic research and drug development. Computational docking operation has been proved to be a useful vehicle for investigating the interaction of a protein receptor with its phytochemical ligand to reveal their binding mechanism. Series of studies exploring binding modes and activities of resveratrol as an inhibitor of cyclooxygenase-2 (Murias et al., 2004), antifolate activity of phytochemicals against *Pseudomonas aeruginosa* (Jayaraman et al., 2011), and prediction of drug-likeness and target activities of natural phenolic compounds on cancer cells (Chen et al., 2012) are all found to utilize computational methods successfully.

4.2.6 *Molecular Docking*

The protein structure for Lipoteichoic acid synthase enzyme (1.60 Å resolution) in complex with (2R)-2, 3-dihydroxypropyl phosphate was retrieved from Protein Data Bank (http://www.pdb.org/pdb/home/home.do) (PDB ID: 2W5T). Model 3D structures of the antibiotics and phytochemicals used in this study were obtained from Pubchem (http://pubchem.ncbi.nlm.nih.gov/). Docking studies were performed by MolDock™ - Molegro Virtual Docker™, utilizing a cavity prediction algorithm. MVD was installed on Windows Vista operating system on an Intel Pentium IV processor with 2GB RAM. Lipoteichoic acid synthase protein structure devoid of water molecules was transferred to the workspace keeping its ligand, glycerol phosphate attached to its active site and kept as a reference ligand after minimizing the energy. Next the protein and ligand molecules were prepared by the addition and optimization of hydrogen molecules.

During the import of 3D structure of the ligands, charges and bond orders were assigned followed by determination of torsional angle for 3D structure. Cavities were identified by using the grid based cavity prediction algorithm. Active sites were identified according to the ligand molecule binding to the protein. A total of 5 cavities were identified and docking was constrained to the cavity containing the active site where the active ligand was bound. The energy between the target protein and ligand were measured using MolDock score. Other important parameters such as number of runs, grid resolution, and cross over rates were then optimized for each run. RMSD threshold was set as 1.00A°. Optimum poses with the highest re-rank and MolDock score were retained in the workspace for detailed evaluation of each pose.

Table 4: List of Phytochemicals

Ligand	CID No	Mol: Formula	Mol: weight	H-bond donor/ acceptor	Structure
Ethyl gallate (EG)	13250	$C_9H_{10}O_5$	198.1727 [g/mol]	3/5	
Epicatechin gallate (ECG)	65056	$C_{22}H_{18}O_{10}$	442.3723 [g/mol]	7/10	
Epigallocatechin gallate (EGCG)	65064	$C_{22}H_{18}O_{11}$	458.3717 [g/mol]	8/11	
Catehin (C)	9064	$C_{15}H_{14}O_6$	290.2680 [g/mol]	5/6	
Epicatechin (EC)	72276	$C_{15}H_{14}O_6$	290.2680 [g/mol]	5/6	

4.3 ***Results***

4.3.1 *Action of ethyl gallate on bacterial cells*

Both scanning electron micrographs and atomic force micrographs were taken 4 hours after the treatment of bacteria cells with 1/2 MIC of ethyl gallate. Control picture [Figure 38(A)] illustrated well- developing *Staphylococcus aureus* cells with their rounded appearance. However, in ethyl gallate treated samples, it could be seen that the bacterial cells have adhered to each other creating a deformed cell mass [Figure 38 (B)]. Some lysed cells could be seen as collapsed cocci. Within the clusters, many cells were found to express different morphology with rougher surface texture when compared with the untreated cells. In Figure 38 (B), bacterial cells in focus were surrounded by the slimy substance forming a protective layer which might be the indication of either intracellular bacterial substance leakage or extracellular polysaccharide production. In Figure 39, many bacterial cells were seen to express inability to maintain their native cocci structure. More interestingly, some of deformed cells were seen to possess pores on their surfaces, as shown in Figure 39 indicated with blue arrow, indicating that ethyl gallate was acting on the cell wall most probably on peptidoglycans. It was found that the while bacterial cells maintain their cocci shaped appearance in the absence of treatment; they lose their capacity to maintain cocci structure in the presence of ethyl gallate. Disfigured *Staphylococcus aureus* cells can be seen clearly in red circle in Figure 39. The replica of the scanning electron microscopy study was also performed using atomic force microscopy (AFM) in same exact conditions of treatment with ethyl gallate and a positive control. Bacterial cells in the absence of ethyl gallate have well-rounded cocci appearance

and they form structured clusters as shown in Figure 40 (A). When the bacterial cells were treated with ethyl gallate, many bacteria seemed to lose their structural integrity in the same manner as found in Figure 39. Bacterial cells were found to collapse and coalesce with each other as depicted in Figure 40 (B and C). In the previous chapter, it was found that ethyl gallate was synergistic to protein synthesis inhibitors while it was indifferent to cell wall synthesis inhibitors, vancomycin. Many literatures indicate that cell walls are the most vulnerable targets for antibiotic action. After the attack of cell wall active antimicrobials, repair of any damage to the cell wall of microbes are mainly accomplished by replication, if the damage is not extensive. This important insight supported by the cell wall morphological observations signifies possible ethyl gallate's action on cell wall peptidoglycan synthesis.

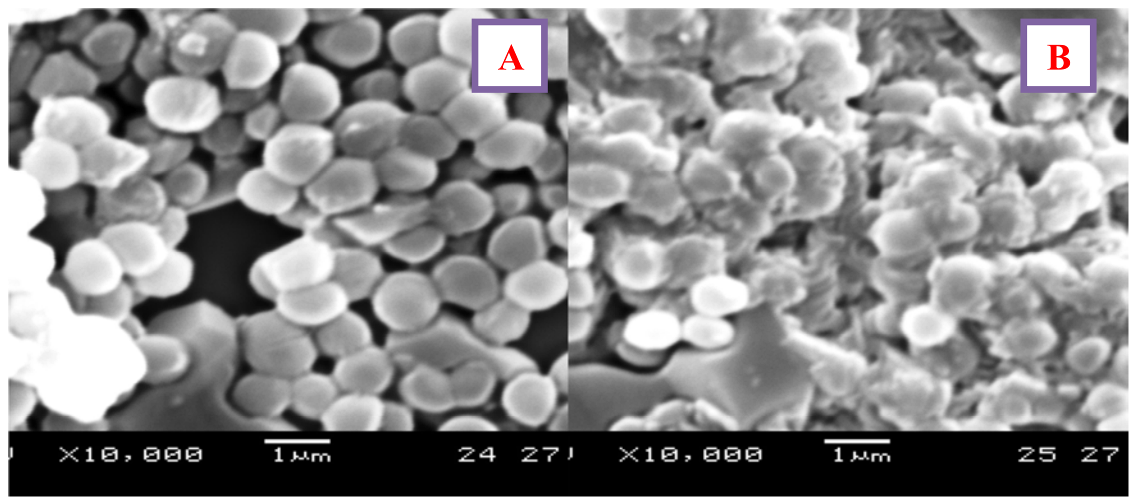


Figure 38: Bacterial cells before (A) and after treatment (B)

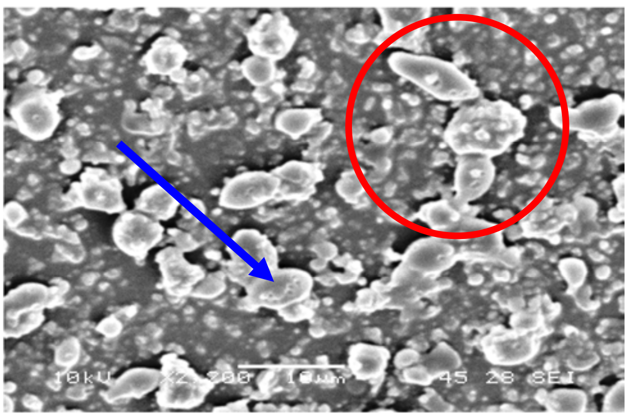
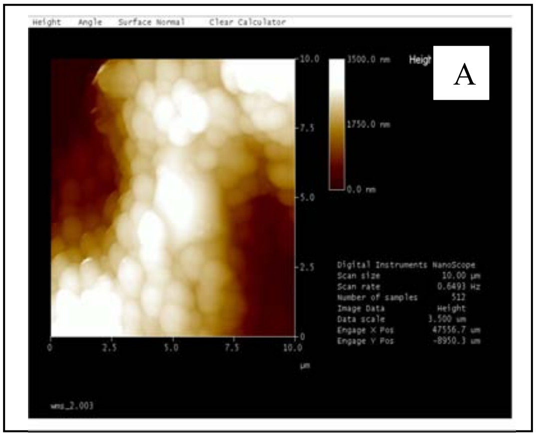
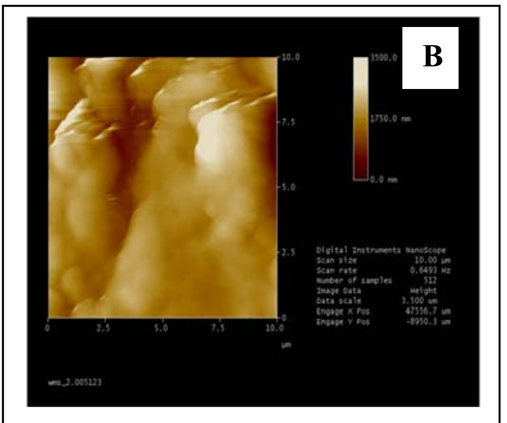


Figure 39: Morphological changes of bacteria cell on scanning electron microscopy

*the blue arrow point to the formation of pores on the cell wall and the red circle shows that cells shapes have been compromised after treatment





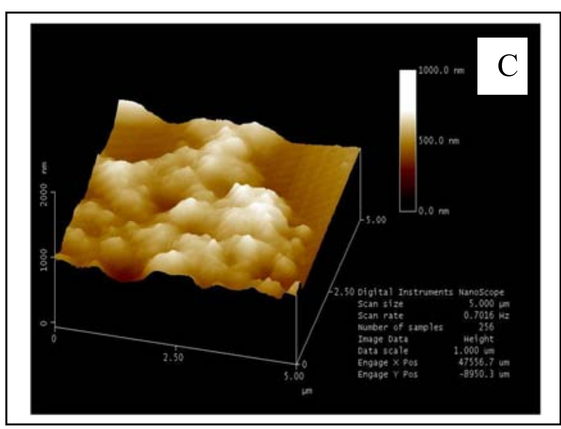


Figure 40: Atomic force microscopy of bacterial cell before (A) and after treatment (B, C)

4.3.2 *Action of Ethyl gallate on human peripheral blood mononuclear cells*

In MTT cytotoxicity assay, the whole population of human peripheral blood mononuclear cells was exposed to ethyl gallate. Viability was determined by measuring the metabolic parameter of NADH/NADPH concentration. Viability of mononuclear cells was calculated by using untreated mononuclear cells as control. The duration of the test was only for 3 days as *in vitro* viability of mononuclear cells started to decline after 3 days. It was found that at concentration of 2048 µg/ml, mononuclear cell viability was less than 50% on D1. Overall viability was deemed to be significantly affected by the presence of ethyl gallate at the concentration of 1024 µg/ml and 2048 µg/ml ($p<0.05$). However, it is interesting to note that viability was restored in D2 and D3 as seen in Figure 41. Despite being able to reach only 80% of the viability, it could be safe to deduce that mononuclear cells were not killed, but rather inactivated temporarily. However, lower concentration (32 µg/ml -512 µg/ml) of phytochemicals showed more promising biocompatibility as the mononuclear cell viability had never fallen even at the start of the treatment, and viability reaching the same level as control at the end of Day 3. Therefore, it could be concluded that ethyl gallate is not cytotoxic to human peripheral mononuclear cells which are important for fighting infections.

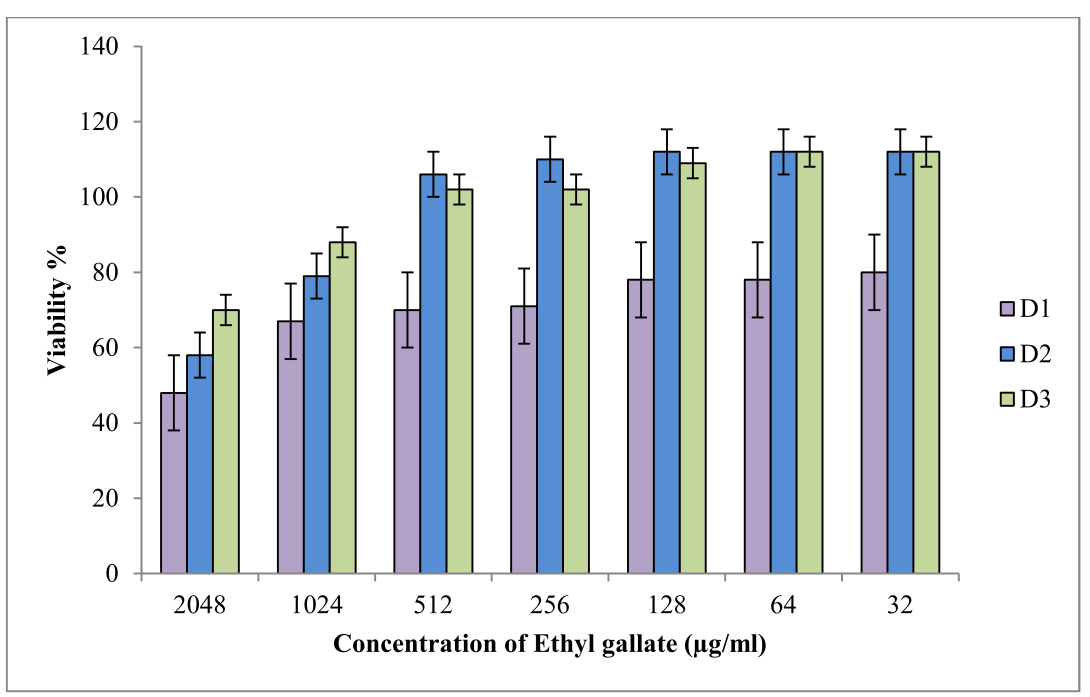


Figure 41: Cytotoxicity testing of Ethyl gallate

4.3.3 *Molecular docking analysis*

Glycerol phosphate docking to Lipoteichoic acid synthase (LtaS): Glycerol phosphate (GP) was the proposed active ligand for the LtaS enzyme. It was bound to the active site in a pocket created by Trp354, Asp349, His347, and Arg356. The cofactor Mn^{2+} was coordinated by Thr300, Glu255, Asp475, and His476. The phosphate structure was further stabilized by hydrogen bonding to Thr300, His416 and Trp354. This structure was interpreted as the pre-substrate hydrolysis state, where the catalytic Threonine residue (Thr300) was ready to attack the phosphate atom and release glycerol-phosphate from a substrate to form a covalent glycerol-phospho-threonine intermediate (Lu et al., 2009). Here, it was important to mention that, GP was coordinated to Mn^{2+} with electrostatic interaction with Oxygen atom on tail end of glycerol phosphate. The hydroxyl groups of

the head were balanced by the hydrogen bonds with Thr354, Asp349, Arg356, and His349.

Phosphatidyl glycerol docking to the active site: Previously, it was reported that the glycerol-phosphate head group of the membrane lipid phosphatidyl glycerol (PG) served as donor for the glycerol-phosphate units, which form the LTA (LipoteichoicAcid) backbone (Xia et al., 2010). Even though direct biochemical evidence was lacking, cleavage of the PG head group and subsequent glycerol phosphate polymerization were presumed to be the reactions catalyzed by LtaS as depletion of this enzyme in *Staphylococcus aureus* led to a complete absence of polyglycerol-phosphate LTA. Moreover, expression of LtaS in a heterologous Gram-negative bacteria host, which naturally lacks LTA, was found to initiate the production of polyglycerol-phosphate polymers (Lu et al., 2009). When phosphatidyl glycerol was docked into the pocket of the active site, the hydroxyl group of phosphate residue was bonded to Thr 300, Trp 354, and His416 as expected.

Phytochemicals docking to the active site cavity of LtaS: The structures of all the phytochemicals used in this study are shown in Table 4. All the phytochemicals used in this study showed similar docking mode in the active site of LtaS. Most of the active site residues interacting with phytochemicals were same as those interacting with the substrate glycerol phosphate (GP). Major interactions with active site residues involving hydrogen bonding and hydrophobic interactions are listed in Table 5. Catechin formed hydrogen bonds with Thr300, His416, Glu255, Thr412 and His253; epicatechin gallate formed hydrogen bonds with Thr300, Ser256, Asp349, Glu297, Arg356, Tyr417; epigallocatechin

gallate formed hydrogen bonds with Thr300, Ser256, Asp349, His347, Arg356, Tyr417; epicatechin formed hydrogen bonds with Thr300, Trp354, Asp349, His347, Arg356, Glu297, Glu255. Catalytic residue Thr300 formed bonds with all of the phytochemicals under investigation in this experiment.

Ethyl gallate docking to the active site: When ethyl gallate was docked to the LtaS, it was observed that ethyl gallate docking occurred in the cavity at the active site, forming hydrogen bond interactions with two of the three hydroxyl groups of the benzene head by binding to Trp354, Asp349, His347, and Arg356. Earlier investigations reported that similar docking mechanism occurs for glycerol phosphate docked to LtaS enzyme (Lu et al., 2009). In this case, ethyl gallate's galloyl group took the place of the phosphate group, thereby preventing the formation and release of glycerol- phospho- threonine intermediate. This interaction prevents the additional steps necessary for the production of polyglycerol-phosphate chains.

In this study, other gallates and catechins showed similar binding modes as the most of the active site residues interacting with glycerol-phosphate ligand as shown in Table 5. Most active residues were Thr300 and Arg356, which were unanimously forming hydrogen bond interactions with all the phytochemicals. Major protein -ligand interactions at specific residues are given in Table 5 and 6 elaborating on ranking based on the Rerank score, MolDock score, interaction energy, H-bond energy, torsions, ligand efficiency1, Ligand Efficiency 3 and protein-ligand affinity.

Table 5: Ligand-protein interactions

Ligand	**Residues involved in hydrogen bonding**
Glycerol phosphate (substrate)	Thr300, Trp354, Asp349, His347, Arg356
Phosphatidyl glycerol	Asp349, Arg356, Trp354, Thr300, His253, Glu255, Thr412
Ethyl gallate	Thr300, Trp354, Asp349, His347, Arg356
Catechin	His416, Glu255, Thr300,Thr412, His253
Epicatechin gallate	Ser256, Asp 349, Glu297, Arg356,Thr300,Tyr417
Epigallocatechin gallate	Ser256, Asp 349, His 347, Arg356,Thr300,Tyr417
Epicatechin	Thr300, Arg356, His347, Asp349, Glu297, Trp354, Glu255

Table 6: Molecular Docking Results

	Mol Dock Score	Re rank Score	Interaction Energy (Kcal/mol)	Torsion	H-bond	Ligand Efficiency (LE1)	Ligand Efficiency (LE3)	Docking Score	Affinity
C	-89.47	-68.21	-104.08	1	-6.08	-4.26	-3.24	-95.39	-18.16
EG	-81.93	-71.06	-90.464	3	-10.71	-5.85	-5.07	-85.69	-20.61
ECG	-124.79	-49.75	-143.59	4	-16.09	-3.90	-1.55	-135.57	-23.57
EGCG	-117.63	-83.074	-146.97	4	-10.05	-3.56	-2.5	-119.45	-17.61
EC	-88.71	-74.81	-112.79	1	-14.44	-4.22	-3.56	-95.99	-22.54

*Torsion - number of rotatable bonds in the pose

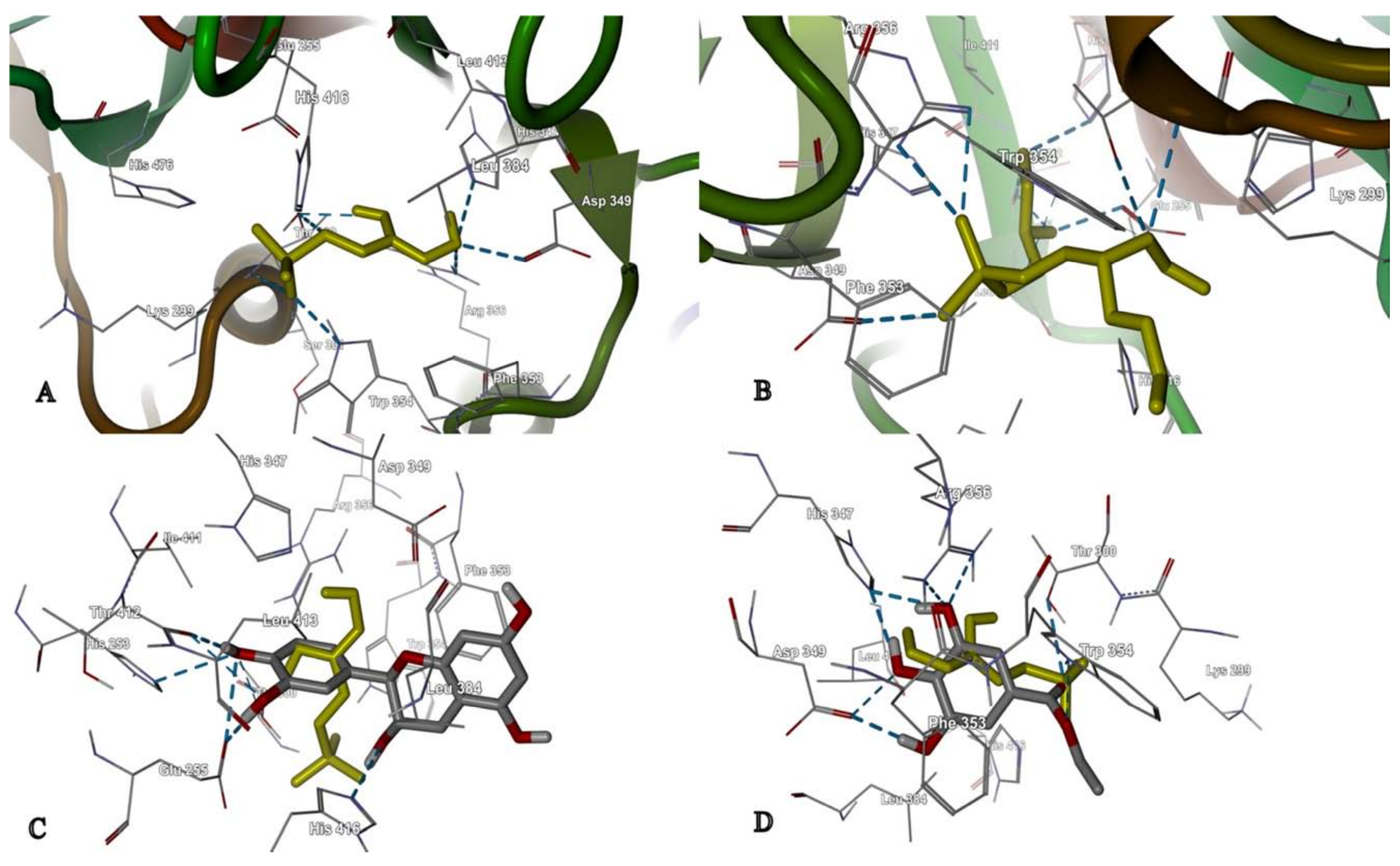


Figure 42: Binding Poses A: Glycerol Phosphate; B: Phosphatidyl glycerol; C: Catechin; D: Ethyl gallate;

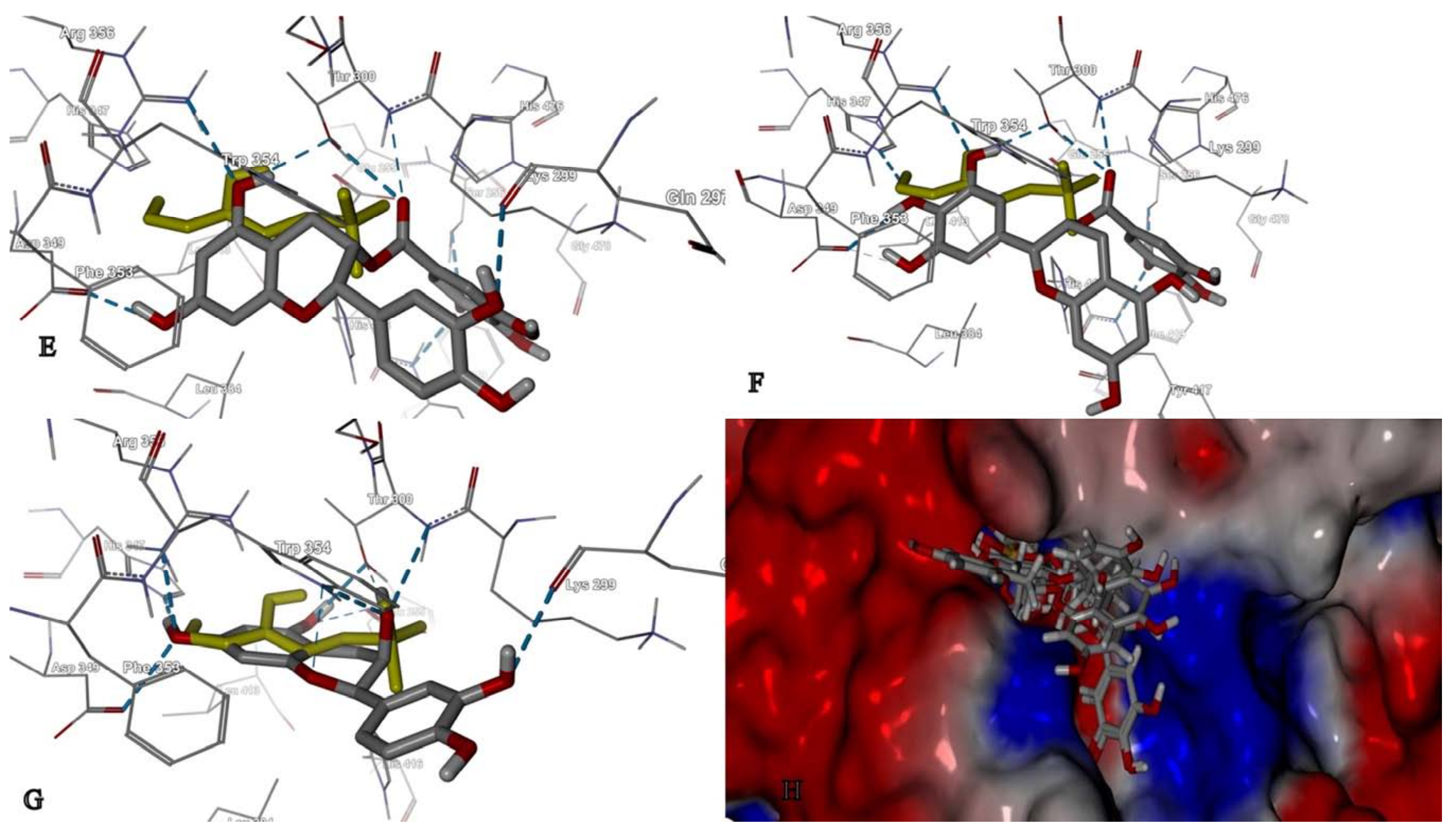


Figure 43: Binding Poses E: Epicatechin gallate; F: Epigallocatechin gallate; G: Epicatechin; H: All Catechins and Gallates

4.4 *Summary*

Despite decades of antimicrobial usage, the association between antibiotics and the development of drug resistance has not been fully delineated. Nonetheless, it was clear that the use of antibiotics to treat bacterial infections causes a continual cycle in which antibiotic treatment leads to the emergence and spread of resistant strains, forcing the use of additional drugs leading to further multi-drug resistance (Ejim et al., 2011). Combination therapy has provided some relief in this direction as combinations of drugs attack the microorganism at several points simultaneously, thereby reducing the risk of resistance (Neuman, 1990).

From the experimental investigation, it was observed that all the combinations of ethyl gallate with the protein synthesis inhibitors (fusidic acid, mupirocin and tetracycline) showed synergism. This suggested that ethyl gallate and protein synthesis inhibitors (fusidic acid, mupirocin and tetracycline) had different sites of inhibition and possibly inhibit different steps in the same pathway or different pathway, thus resulting in synergistic mode of action (Jayaraman et al., 2011). Docking analysis suggested the possible binding of ethyl gallate to LtaS, suggesting the possible mechanism of action of ethyl gallate in relation to cell wall synthesis.

Interestingly, ethyl gallate also showed synergism with the beta-lactam antibiotic, cefoxitin. Previous studies (Shibata et al., 2005) suggested that alkyl gallates were able to potentiate the effect of beta-lactam antibiotics, which were also categorized as cell wall synthesis inhibitors. According to the findings in this study, it is possible that while ethyl gallate inhibits the LtaS, the beta-lactam antibiotics are inhibiting the cross-linking of the

nascent peptidoglycan layer by binding to penicillin binding protein, thereby acting at different sites to prevent cell growth and resulting in synergistic modes of action. In addition, the structure of ethyl gallate was also found to favor its action on the cell wall because of its amphiphilic nature. Molecules of alkyl gallate as shown in the Table 4 consisted three sections: a lipophilic alkyl chain at one end which was connected via an ester linkage to the galloyl group bearing polar hydroxyl group at the other end. The amphiphilic property made the cell membranes of *Staphylococcus aureus* one of the most likely and preferred target sites for the action of alkyl gallate (Zhao et al., 2001a). Thus, the proposed mechanism of action of ethyl gallate from this study was - ethyl gallate is able to bind to the active site of LtaS which is designed to be occupied by the glycerol phosphate moiety of the phosphatidyl glycerol, thus inhibiting the polymerization of the cell wall peptidoglycans.

5. BIOFILM INHIBITION STUDIES

5.1 *Introduction*

In this chapter, biofilm inhibition potential of antibiotics (mupirocin, fusidic acid, and tetracycline), phytochemicals (epicatechin gallate and ethyl gallate), combinations (mupirocin-epicatechin gallate, mupirocin-ethyl gallate, fusidic acid-epicatechin gallate, fusidic acid-ethyl gallate, tetracycline-epicatechin gallate, tetracycline-ethyl gallate) was studied by observation of static biofilms. Quantification of biofilm mass was carried out by using microtiter plate assay. Observations of biofilm formation were performed using scanning electron microscopy. Biofilm forming behaviors were tested with 6 strains of *Staphylococcus aureus*; 4 MRSA strains and 2 MSSA strains.

5.1.1 *Studies of static biofilms*

Biofilms are complex three dimensional structures of functionally heterogeneous organized communities embedded in a hydrated matrix of extracellular polymeric substances (EPS) secreted by bacteria. Biofilm forming behavior is expressed by bacteria in the presence of hostilities to their survival such as nutritional depletion and attack of immune cells of the body (Hall-Stoodley and Stoodley, 2009). These phenotypically different bacteria adhere to any surface they get in contact with. They later form colonies in which the member bacteria are capable of expressing different surface molecules, antibiotic resistance, nutrient utilization and virulence factors (Costerton et al., 1995, Schwank et al., 1998, Aparna and Yadav, 2008). Studies of biofilms provide important

ground for the study of behavior of bacteria as bacteria involved in biofilms coordinate each other by using cell signaling molecules with a process known as quorum sensing. There are several approaches and systems to study earlier stages of biofilm formation (Merritt et al., 2011). Some systems adopt dynamic methods such as the use of steady flow of nutrients and bacteria to create biofilms while some studies adopt static conditions of biofilms by using methods such as microtiter plate assay, air liquid interface assay, and Kadouri Drip-Fed biofilm assay (McLean and Simpson, 2008). Some of the modified microtiter plate assays employ spectrophotometer as well as variety of dyeing reagents for spectrofluorometric quantification. In many of spectrofluorometric assays, one of the most widely used reagents is crystal violet. Many other dyes such as safranin are also used to study the static behavior of bacteria in biofilms *in vitro* (Stepanovi et al., 2007). Many quantification assays need to be supplemented by other biofilm assessment such as visualization of biofilms to assess the mechanism for attached microbes. Preparation of biofilms for microscopic viewing can be established at air-liquid interface on different surface materials. Over the last decades, many types of experiments are performed for *in vitro* quantification of biofilm formation (McLean and Simpson, 2008). The methods ranged from conventional plating (Costerton et al., 1995, Stewart and Costerton, 2001) to several surrogate assays such as crystal violet (CV) assay, Syto9 assay, fluorescein diacetate (FDA) assay, resazurin assay, XTT assay and dimethyl methylene blue (DMMB) assay (Merritt et al., 2011). This study employed crystal violet dye to quantify formation of static biofilms in microtiter plate assay which was further supported by morphological studies of biofilms using scanning electron microscope.

5.2 ***Experimental methodology***

5.2.1 *Preparation of bacteria culture*

Staphylococcus aureus strains under investigation were incubated on Muller-Hinton agar for 24hrs aerobically at 37°C. Three to four well-isolated identical colonies were suspended in 5ml of tryptic soy broth (TSB) supplemented with an additional 1% glucose (Stepanovi et al., 2007) and incubated without shaking for 18-24 hours (Deighton, 2001). The inoculum was adjusted to 0.5 McFarland -standard (approximately 10^{8} CFU ml^{-1}).

5.2.2 *Biofilm culture in microtiter plates*

Bacteria stock culture was centrifuged for 1 min to harvest the bacterial pellet. The bacterial concentration was diluted by using tryptic Soy Broth (TSB) supplemented with 1% glucose to obtain 1/100 of initial concentration. 50µl of the diluted culture was inoculated into the each well of microtiter plates. 150µl of TSB medium supplemented with 1% glucose was also added in the same wells. These wells were marked to serve as positive controls for biofilm formation. The negative control contained only 200µl of TSB supplemented with 1% glucose. The test for biofilm formation, in the presence of different antimicrobial agents and combinations was done by preparing the antimicrobials and phytochemicals at synergistic concentrations obtained by the previous chapter (Chapter 4) making up to 200µl using TSB medium supplemented with 1% glucose. Each experiment was done in flat-bottomed 96 well microtiter plates in triplicate. The inoculated microtiter plates were incubated for 24 hrs. at 37°C under static and aerobic conditions.

5.2.3 *Crystal violet staining*

After incubating in 37ºC incubator for 24 hours, the contents of the wells were removed. The wells were then were washed for three times with 300µl of sterile phosphate buffered saline (1X PBS; pH 7.2) solution to remove planktonic bacteria. The washing was followed by drying the plates at 60°C. 150µl of crystal violet was added to each well and re- incubated at room temperature for 15mins. The stains were then aspirated and excess stains were washed off under running tap water. After the removal of excess stains, 150µl of 95% ethanol was added to the wells to re-solubilize the fixed dye at room temperature for 30mins.

5.2.4 *Quantification of biofilm*

Optical density (OD) of each well was determined using UV Spectrophotometer at 600nm. The cut off value (OD_C) for determination of the presence of biofilms was calculated using the formula: OD_C = OD of the negative control+ (3 X S.D of the negative control). The average OD was then compared with the cut off value. Negative value was taken as an indicator of no biofilm formation while a positive value was regarded as an indicator of biofilm presence. OD_C values were calculated for each microtiter plate separately. Experiments were performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated. Statistical analysis comparing the condition of biofilm formation in the presence and absence of phytochemicals was performed using one way ANOVA with post-hoc comparisons with Bonferroni corrections. Statistical significance was taken at $p<0.05$.

5.2.5 *Morphological study of biofilm using SEM*

As many bacteria prefer aerobic growth, biofilm formation mostly occurs at the interface between the medium and air. Biofilms were grown on 2mm x 20mm glass cover slips and incubated aerobically for 24h at 37ºC. The concentrations and combinations of antimicrobial agents used were the same as that of microtiter plate quantification technique. At the end of 24 hour incubation, cover slips were washed gently using PBS. The biofilm found to be adherent to a coverslip was fixed with 5% glutaraldehyde for 3hrs. The fixing agents were then rinsed for two times using 1XPBS, pH=7.2. Post-fixation treatment was performed using 2% glutaraldehyde - 3% formaldehyde solution for 1 hour. Fixed samples were then subjected to dehydration by gradual introduction with 70%, 90% and 100% acetone prepared in 1X PBS. The samples were then dried overnight before analysis under SEM (Law et al., 2001). The samples containing cover slips were mounted on aluminum stubs using carbon tape and sputter coated with gold and observed in JEOL Scanning Electron Microscopy (SEM).

5.3 ***Results and Discussion***

5.3.1 *Quantification of biofilm formation using microtiter plate method*

Biofilm forming potential of *Staphylococcus aureus* strains under study was established by using tissue culture plate (TCP) assay with TSB medium. All six *Staphylococcus aureus* strains under investigation displayed biofilm positive behavior of varying degrees as shown in Figure 44. Biofilm forming potential of the MRSA strains varied significantly

depending on the type of the strain ($p<0.05$) while biofilm forming potential of MSSA strains are nearly the same.

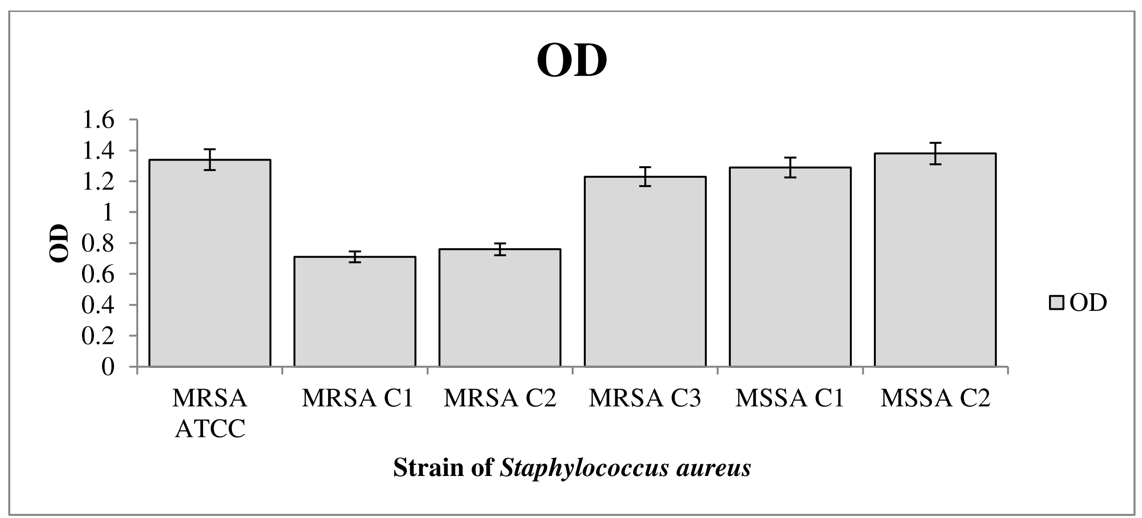


Figure 44: Biofilm forming potential of different strains

Further on, biofilm inhibition potentials of antibiotics in the presence and absence of phytochemicals were investigated. It was observed that synthetic antibiotics (tetracycline, mupirocin, and fusidic acid) were more effective in inhibiting the formation of biofilm as compared to phytochemicals (EG and ECG) given alone at sub- inhibitory concentrations. However, EG and ECG in combination with synthetic antibiotics (tetracycline, mupirocin, and fusidic acid) were significantly more effective than individual drugs in inhibiting the formation of biofilm in all the six strains under investigation. It was observed that the combination of ethyl gallate and mupirocin showed 73-98%, ethyl gallate and fusidic acid showed 80-92%, ethyl gallate and tetracycline showed 85-97%, epicatechin gallate and mupirocin showed 73-96%, epicatechin gallate and fusidic showed 74-96%, and epicatechin gallate and tetracycline showed 75-93% reduction of inhibition in the biofilm

formation as compared with the antibiotics acting alone at sub- inhibitory concentration. Even though it was reported in chapter 4 that ethyl gallate was synergistic with tetracycline, mupirocin and fusidic acid while epicatechin gallate is synergistic only with tetracycline using FIC index, biofilm analysis showed that all the combination of the phytochemicals and the antibiotics including the non-synergistic combinations of epicatechin gallate showed effective inhibition of biofilm in all the strains under investigation [Figure 45-50]. Statistical analysis showed that combination with ethyl gallate consistently proved to be better at inhibiting biofilms than the combinations that use epicatechin gallate (p<0.05). It was also observed that the combination of tetracycline with ethyl gallate, and tetracycline with epicatechin gallate were most effective in inhibiting the biofilm formation (p<0.05).

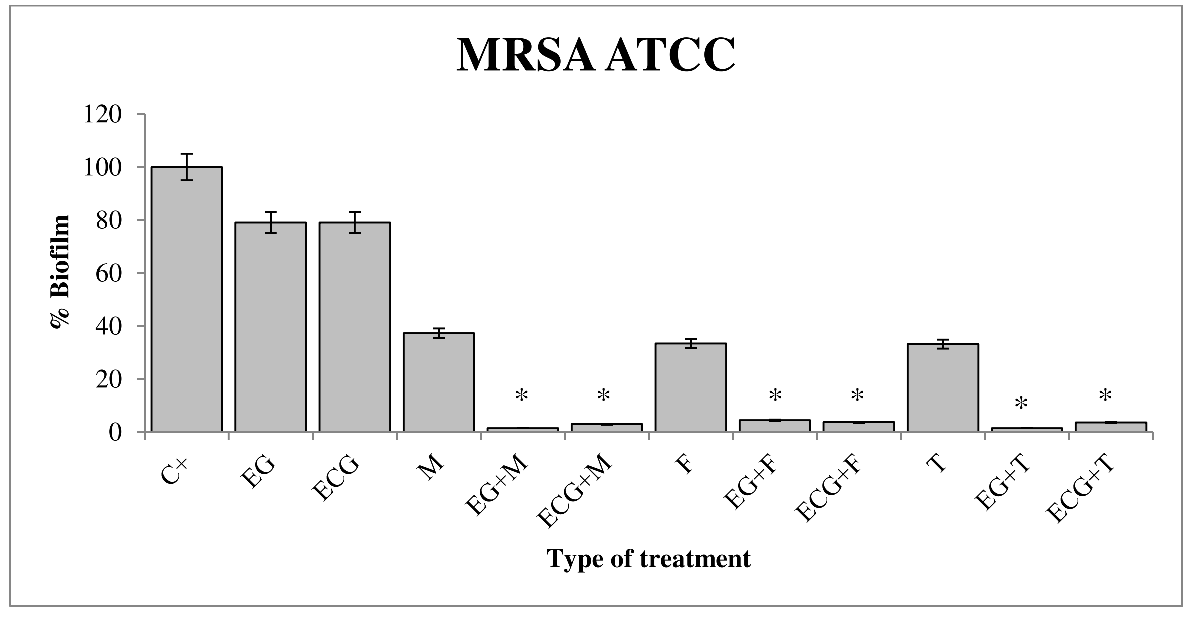


Figure 45: Biofilm inhibition potential of different treatment conditions on MRSA ATCC *: p<0.05

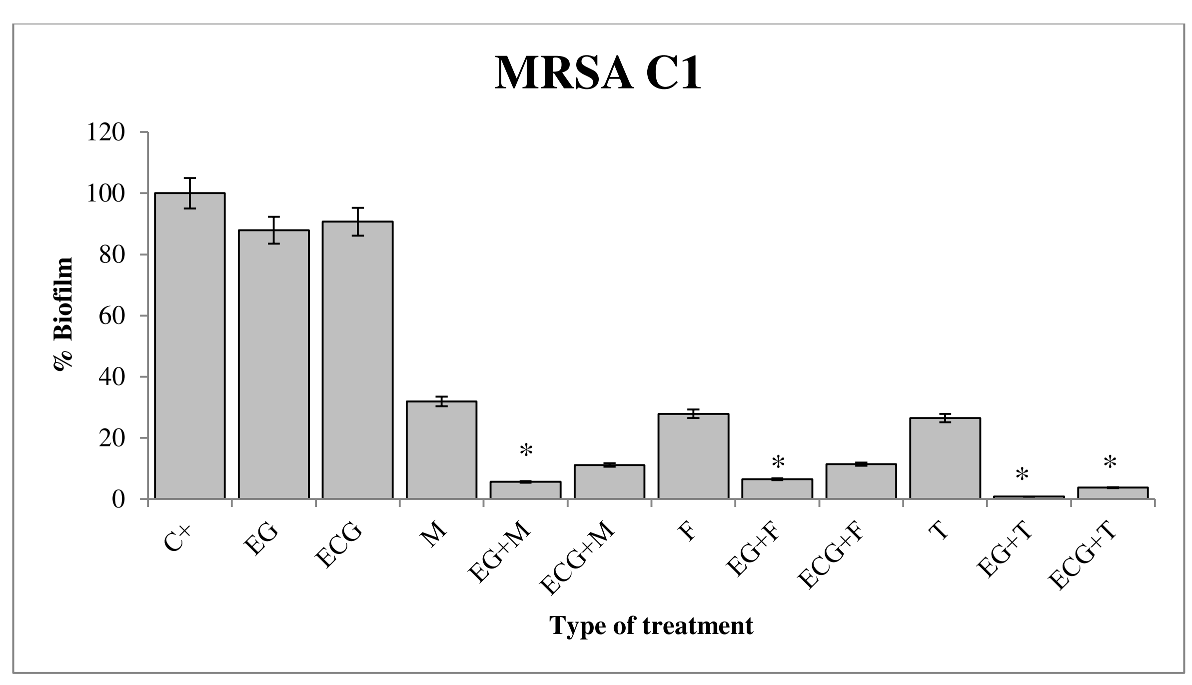


Figure 46: Biofilm inhibition potential of different treatment conditions on MRSA C1*: p<0.05

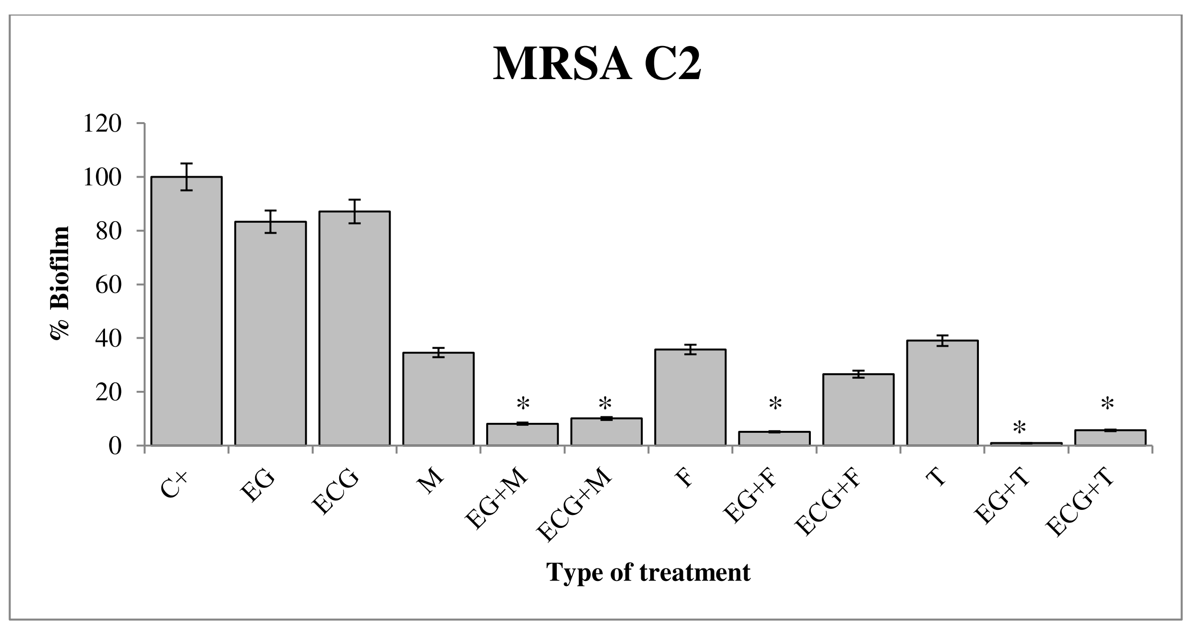


Figure 47: Biofilm inhibition potential of different treatment conditions on MRSA C2*: p<0.05

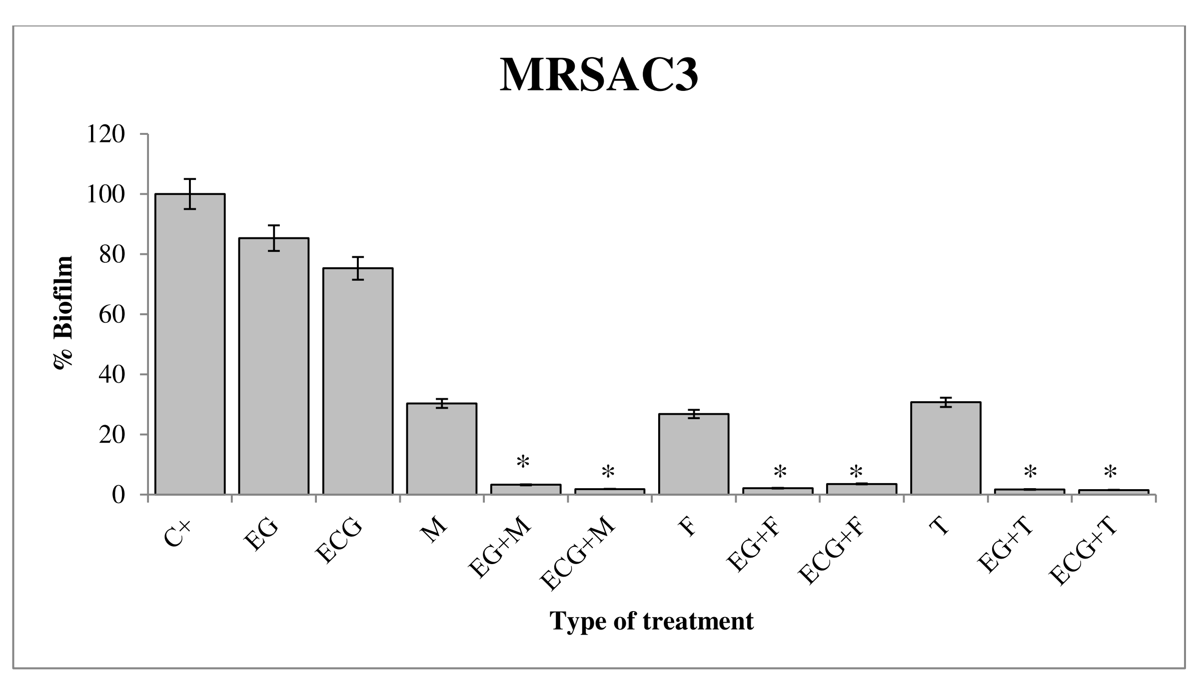


Figure 48: Biofilm inhibition potential of different treatment conditions on MRSAC3*: p<0.05

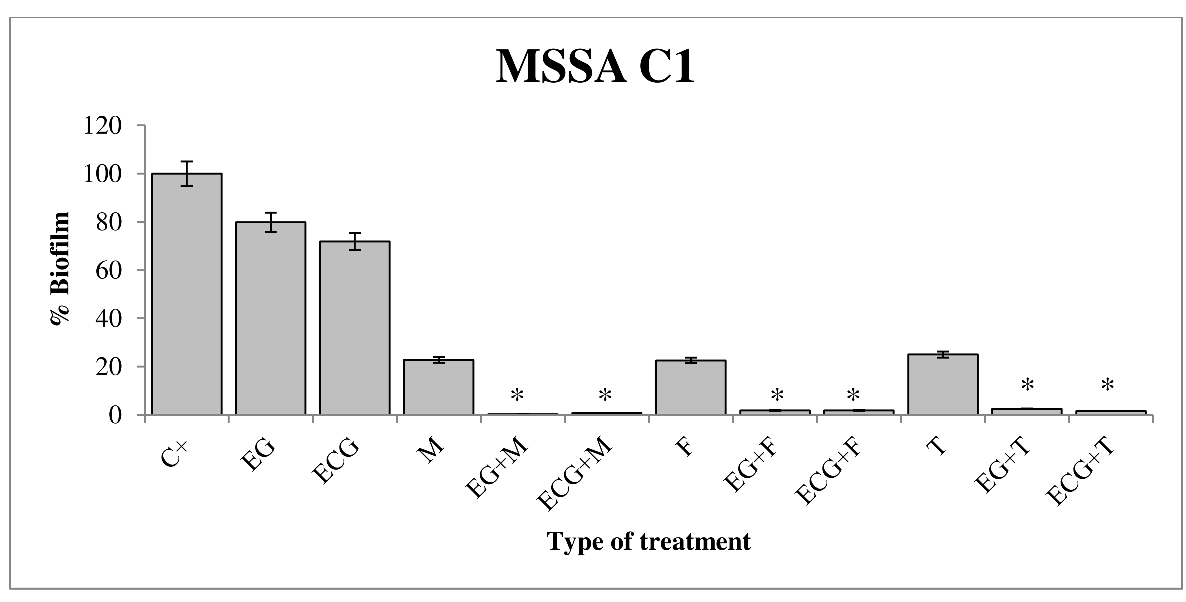


Figure 49: Biofilm inhibition potential of different treatment conditions on MSSAC1*: p<0.05

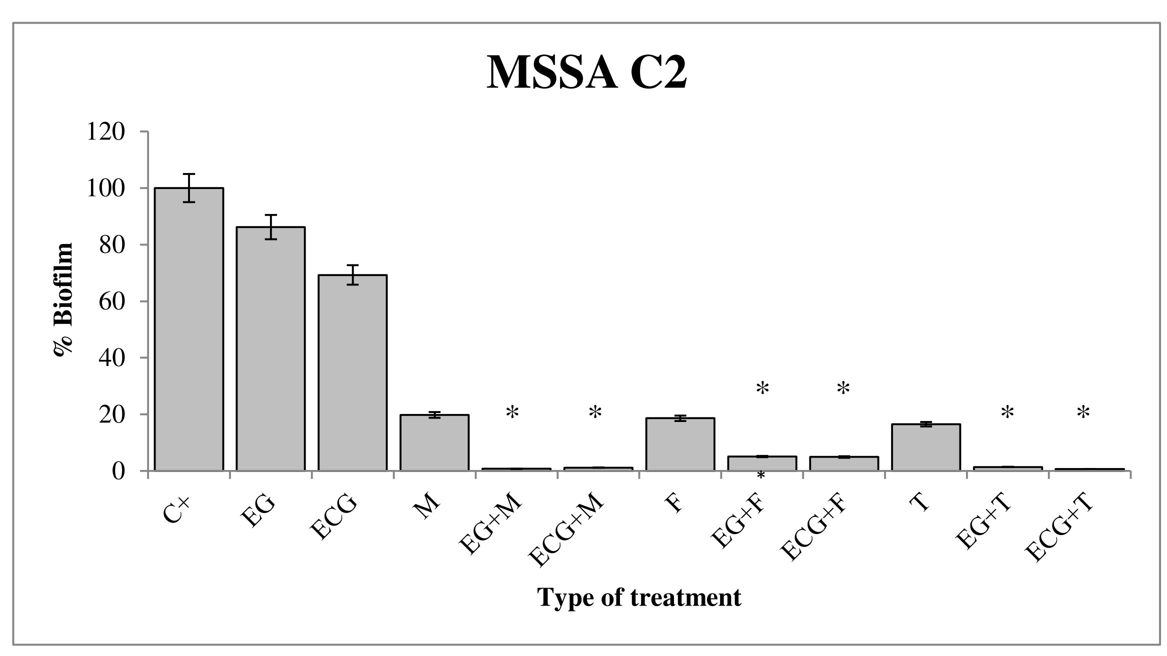


Figure 50: Biofilm inhibition potential of different treatment conditions on MSSA C2; *: p<0.05

5.3.2 *Scanning electron microscopy of biofilms*

After the anti-biofilm activity of phytochemicals alone and in combination with the antibiotics were assessed by the microtiter plate assay, detailed study of the structural and organization of biofilms were assessed by using scanning electron microscopy after growing the bacterial cells at the air-liquid interface. As *Staphylococcus aureus* bacteria is an aerobic bacteria which is also a facultative anaerobe, it can grow well either in the presence or absence of oxygen. Bacteria growth was evidently observed in the center of the cover slips after the planktonic cells were washed off carefully. It was found that the bacteria biofilms were mostly concentrated in the center of the coverslips and they were not easily stripped off by washing. Scanning electron micrographs of all the strains under

Even in the presence of sub- MIC concentrations of phytochemicals and antibiotics, exopolysaccharide formation and cell aggregation were clearly seen as shown in Figure 51-56. However in the presence of combination of phytochemicals and antibiotics, there was no significant formation of exopolysaccharide or biofilms. Moreover, less bacterial cells and less biofilm organization were observed as it was assumed that the planktonic bacteria which were not able to attach to the surface were dropped off during washing after the treatments. The antibiotics under study, fusidic acid, mupirocin and tetracycline were bacteriostatic antibiotics and thus unable to inhibit biofilm formation. However, these antibiotics were rendered bactericidal by the addition of 1/4MIC of epicatechin gallate and ethyl gallate.

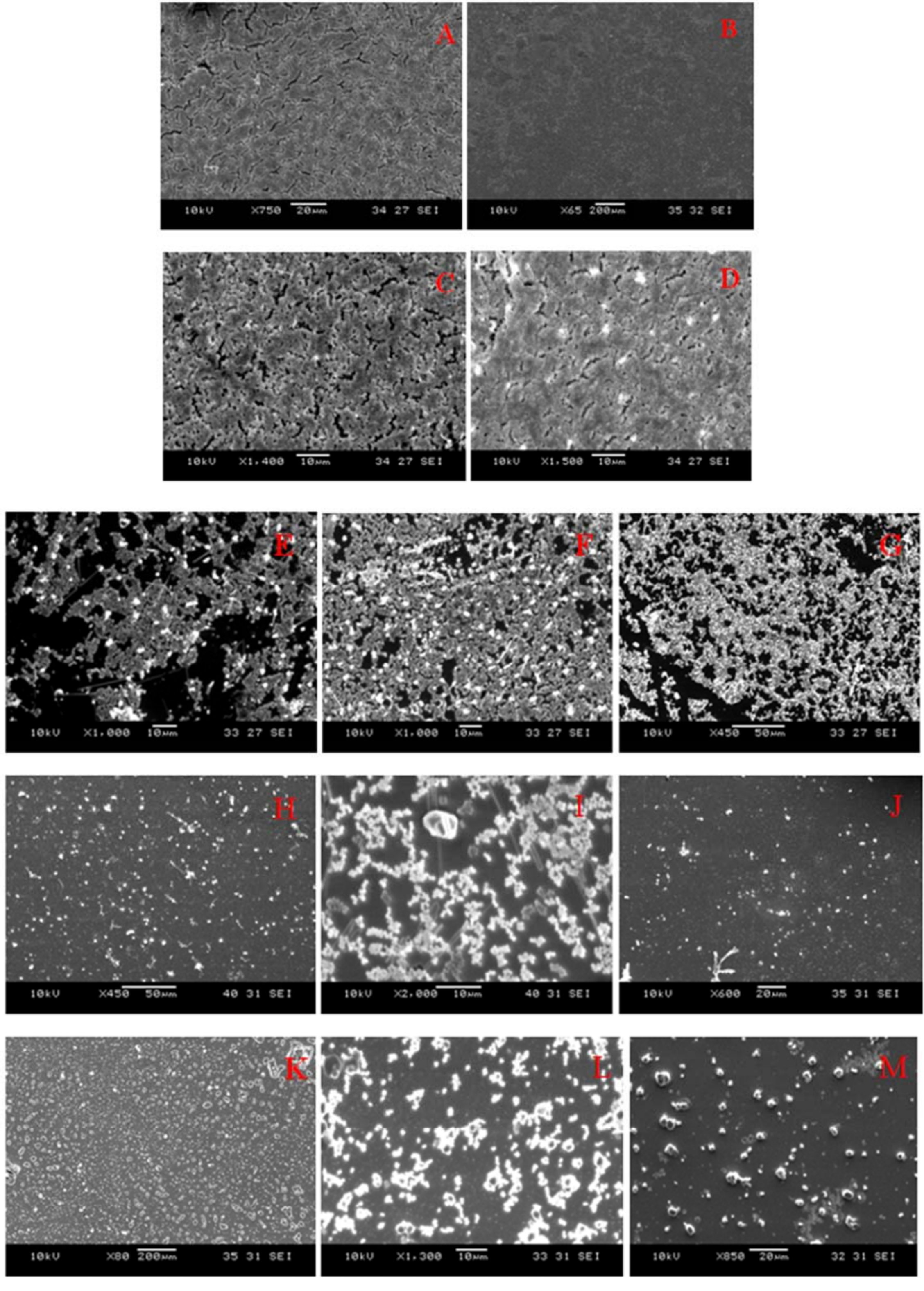


Figure 51: Scanning electron micrographs illustrating changes in biofilm formation by *MRSA* ATCC strain after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

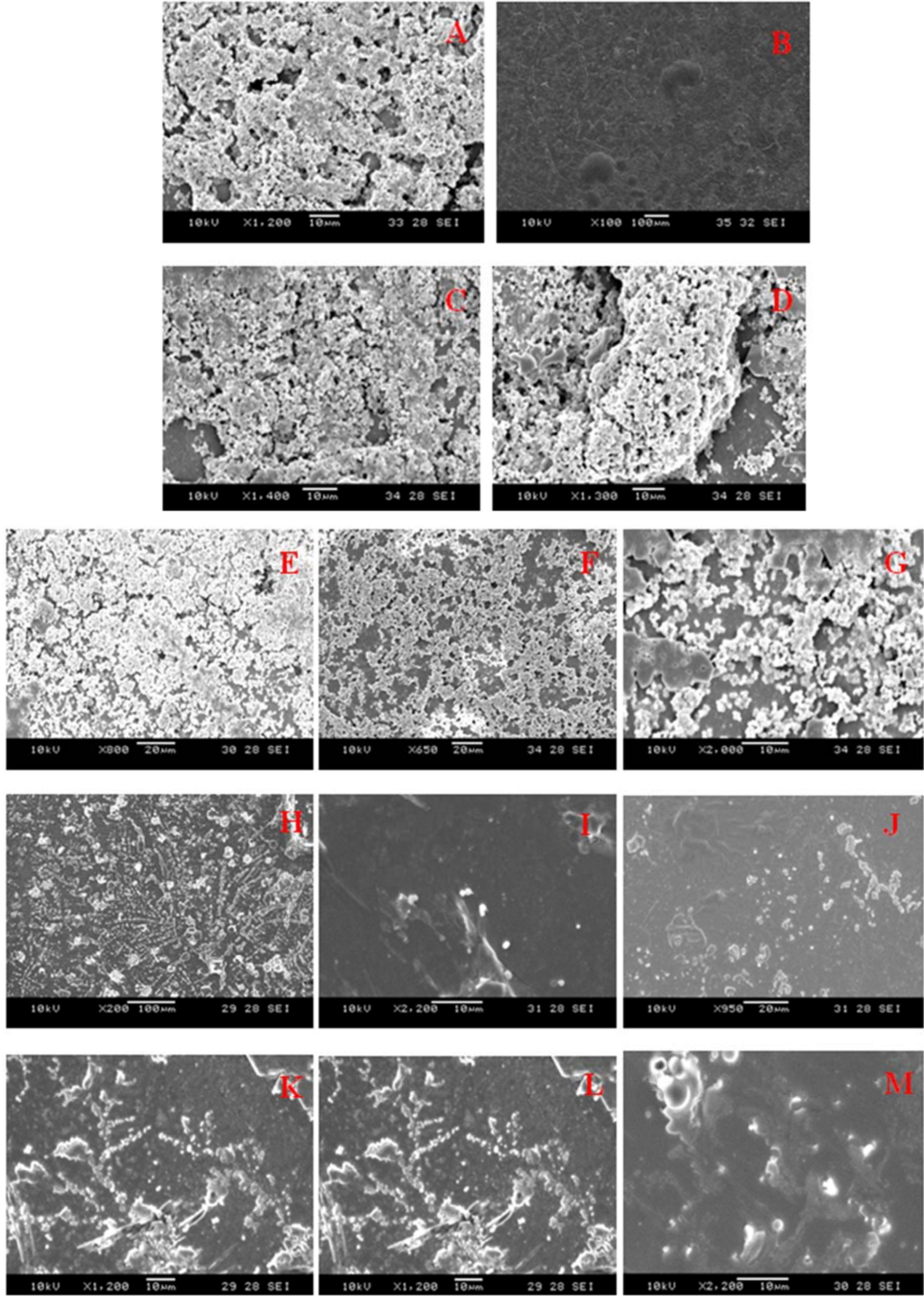


Figure 52: Scanning electron micrographs illustrating changes in biofilm formation by *MRSA* C1 Resistant strain after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

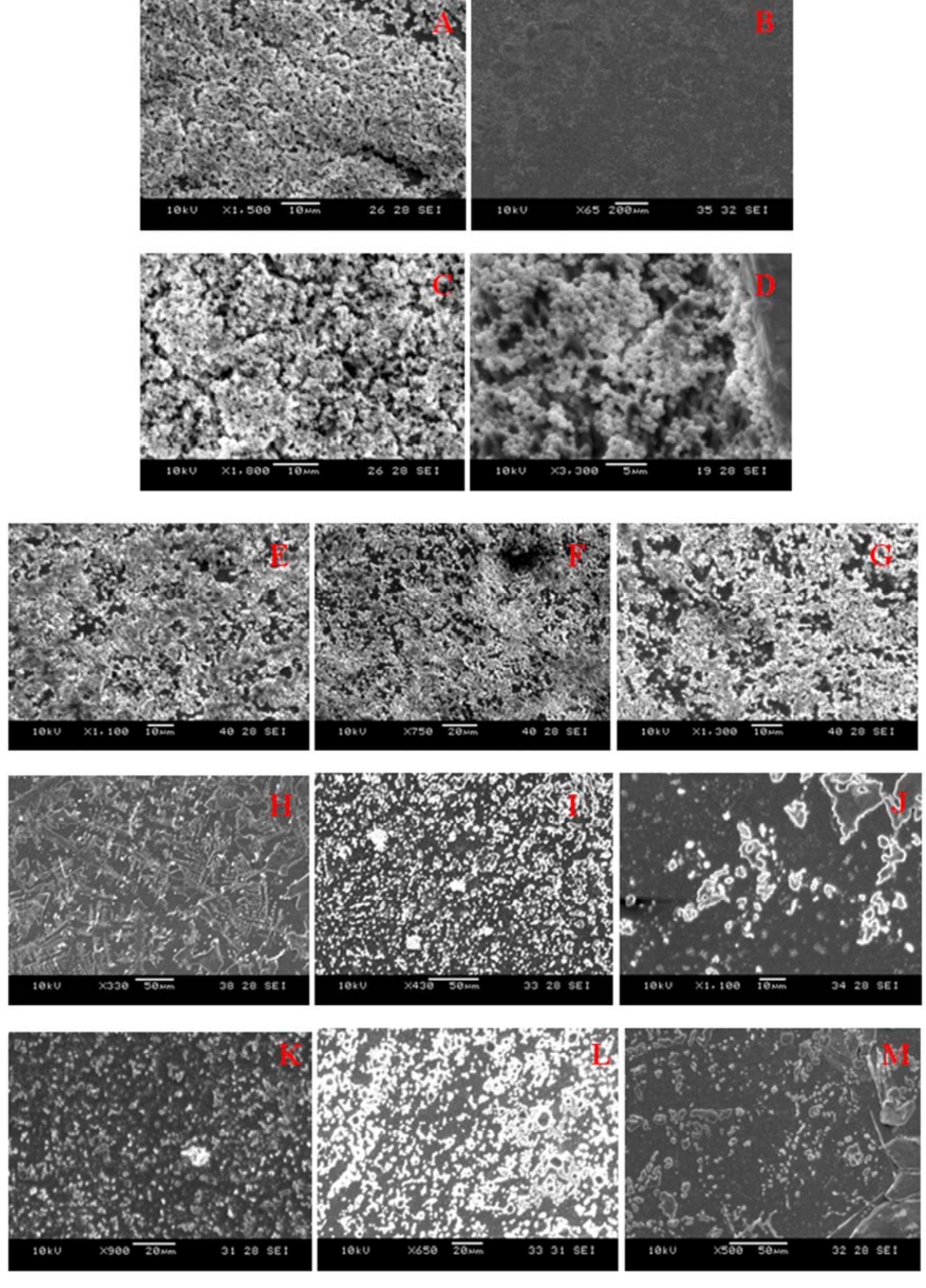


Figure 53: Scanning electron micrographs illustrating changes in biofilm formation by *MRSA* C_2 strain after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

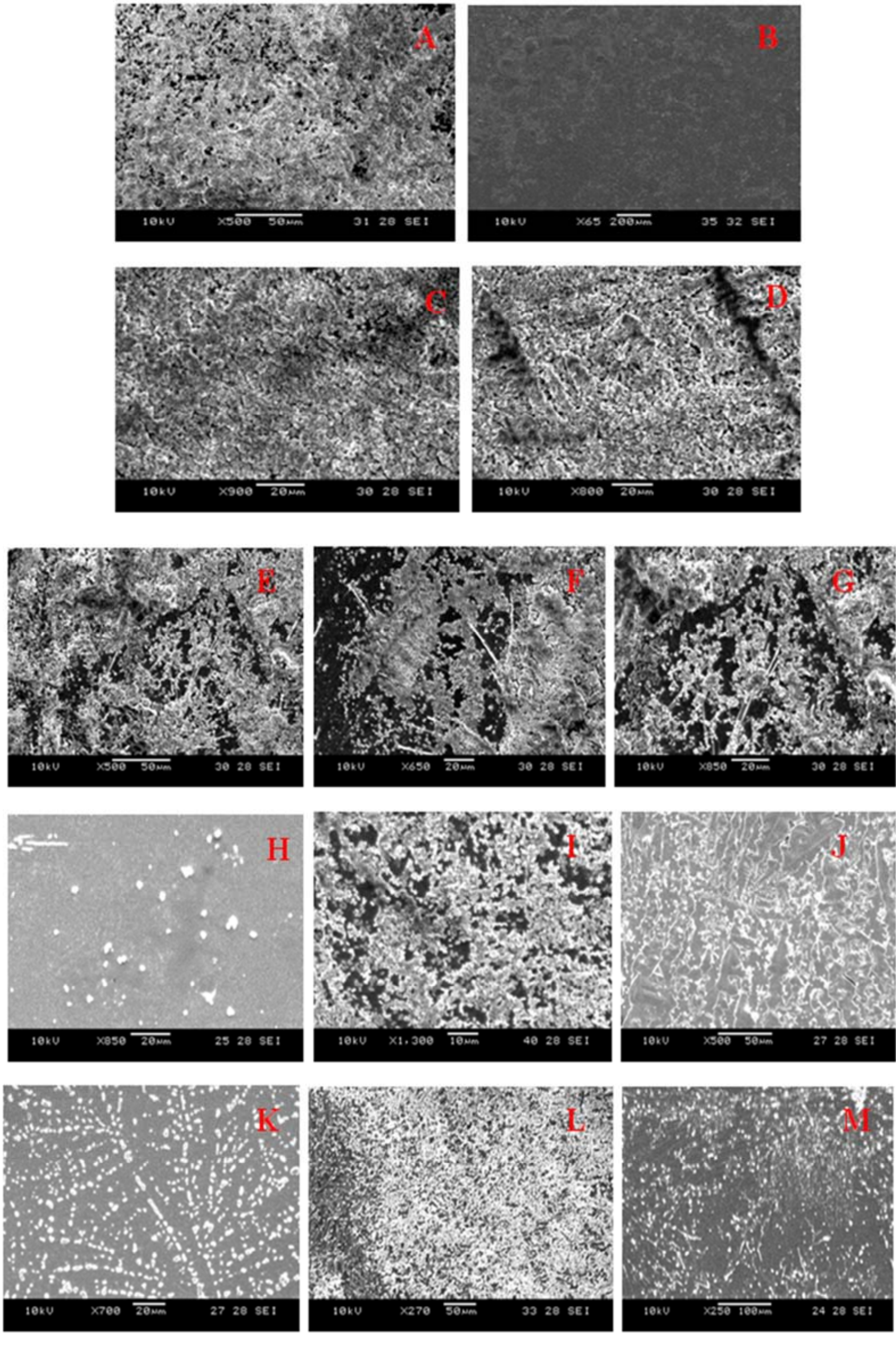


Figure 54: Scanning electron micrographs illustrating changes in biofilm formation by *MRSA* Clinical C_3 after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

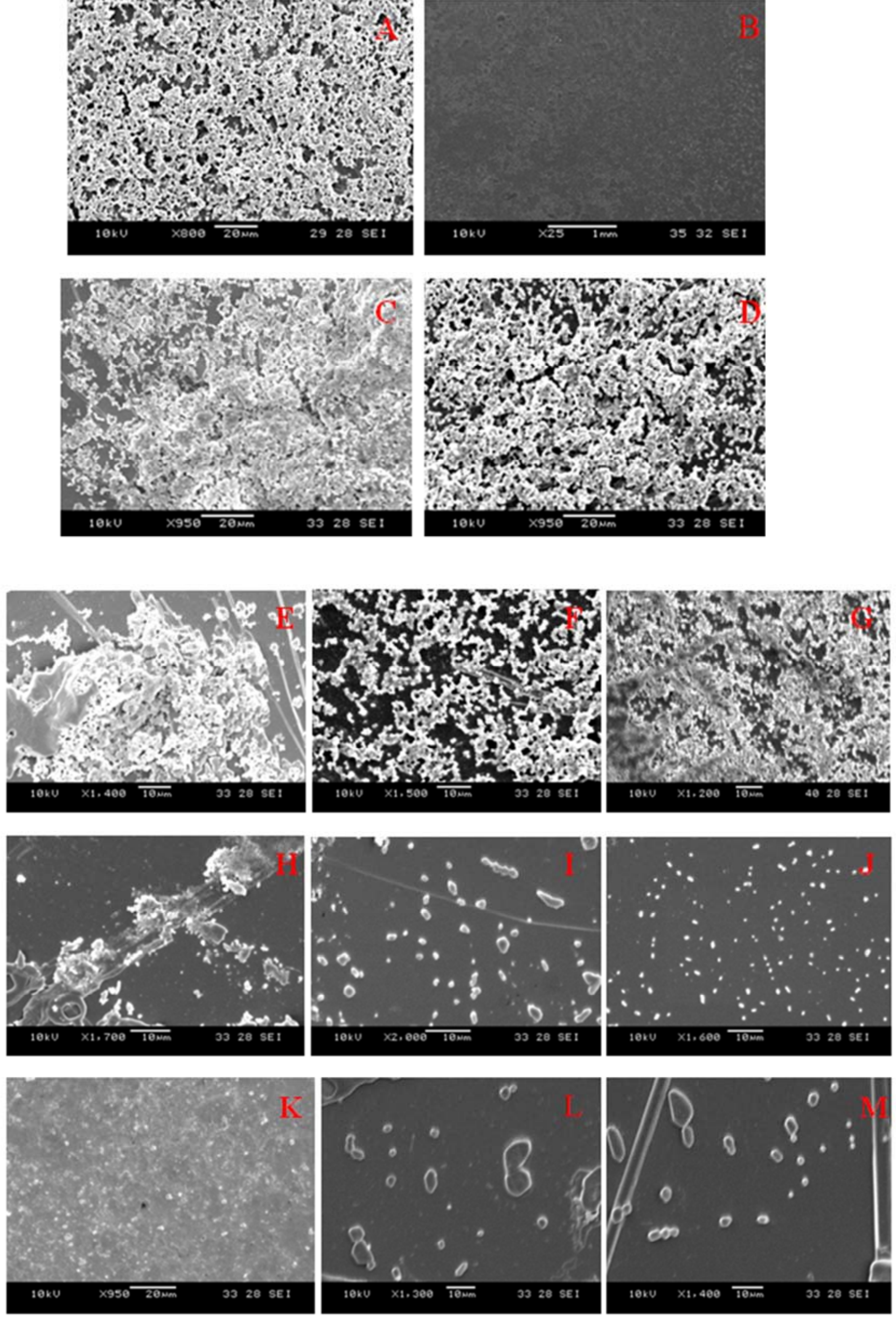


Figure 55: Scanning electron micrographs illustrating changes in biofilm formation by *MSSA* C1 strain after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

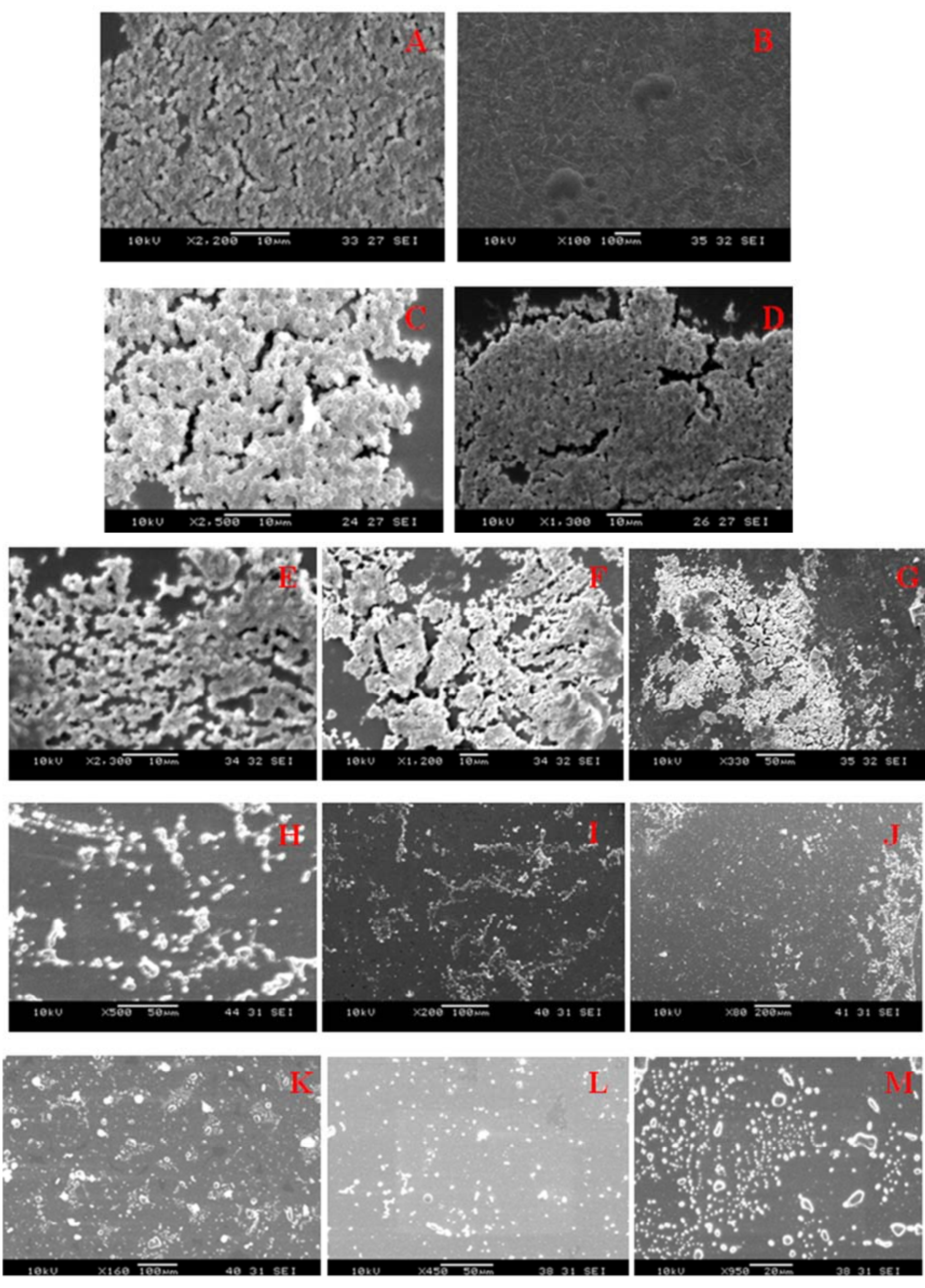


Figure 56: Scanning electron micrographs illustrating changes in biofilm formation by *MSSA* C2 strain after 24h of treatment with A) Control without drugs B) Sterility control C) Ethyl gallate D) Epicatechin gallate E) Mupirocin F) Fusidic acid G) Tetracycline H) Ethyl gallate plus Mupirocin I) Ethyl gallate at plus Fusidic acid J) Ethyl gallate plus Tetracycline; K) Epicatechin gallate plus Mupirocin; L) Epicatechin gallate plus Fusidic acid; M) Epicatechin gallate plus Tetracycline

5.4 *Discussion*

In chronic wounds, biofilms protect its own bacterial colonies by secreting slimy exopolysaccharide substances which form a protective layer so that it is impenetrable for antibiotic and immune cells (Kennedy et al., Gristina, 1994). Many virulent bacteria are able to form biofilms in the wounds especially *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria. In the presence of biofilms, antibiotic concentration needed to reach bacteria is more than 1000 times higher than the concentration to kill planktonic bacteria. In some cases, antibiotics that are specialized for eradicating biofilms are needed to cure biofilm associated wounds. Moreover, genetic and molecular mechanisms of biofilms of *Staphylococcus aureus* are complex because of their capability to adhere to the surface and ability to form self-protective multi-layers (Alandejani et al., 2009, Periasamy et al., 2012). This study emphasized the effect of phytochemicals on static biofilms by the use of microtiter plate assay which was an effective method to assess bacteria's ability for attachment to abiotic surface such as bandages, stents and other synthetic materials over time. In this study, it was observed that the combinations which included EG was able to produce more biofilm inhibition when compared with combinations using ECG ($p<0.05$). Quantification of biofilms by microtiter plate assay was supplemented by visualization of biofilms using scanning electron microscopy. In scanning electron microscopy, bacteria were found to be able to produce exopolysaccharide layers and form well establish biofilm colonies in the presence of individual antimicrobials, both in the cases of antibiotics and phytochemicals. This was made evident by the fact that biofilms are found cover whole of the surface area of background substrate. However, in the bacterial samples that were treated with

combination of antibiotic and phytochemicals, surface coverage by the biofilms as well as exopolysaccharide production are found to be significantly less. This suggests the therapeutic potential of EG and ECG in combination with antibiotics under investigation as biofilm inhibitors. This is the first study of its kind that investigates the combination effect of ethyl gallate and epicatechin gallate with non-beta-lactam antibiotics such as mupirocin, fusidic acid and tetracycline on biofilm. Despite the lack of knowledge for the underlying mechanism relating to the synergistic effect of these combinations and their role in biofilm inhibition, the identified combinations has potential value in treating life-threatening bacterial infections caused by *Staphylococcus aureus*. They also provide a new front line for novel ways of combating drug resistance.

5.5 ***Summary***

In this study, static conditions for bacterial biofilms were used to study anti-biofilm effect of the compounds. Even though there were some limitations as to concerns of nutrient depletion with the growth of bacteria, studying the static films was regarded to give important information about the activity of antimicrobials in attenuating biofilm formation. Many studies reported that bacteria attempt a repair process when their biofilm colonies are being attacked by the antimicrobials or other adverse reagents. Biofilm repair involves synthesis of the precursors within the cytoplasm, translocation of these precursors to the outer portions of the cell wall, and final assembly of the biofilm matrix. Studies have shown that biofilm repair is dependent on a carbohydrate source, an energy source, certain enzymes, and functioning efflux pumps. It would be informative to study the mechanisms behind the inhibition of biofilm formation by epicatechin gallate and

ethyl gallate. Therefore, biofilm inhibition study of ethyl gallate and epicatechin gallate opens up more area of research in mechanism of biofilm inhibition as well as their application in the treatment of wound infections.

6. RESISTANCE EVOLUTION OF *STAPHYLOCOCCUS AUREUS*

6.1 *Introduction*

In this chapter, drug resistant development behavior of *Staphylococcus aureus* ATCC 43300 was studied. 3 synthetic antibiotics (mupirocin, fusidic acid, tetracycline) and a phytochemical (ethyl gallate), combinations (mupirocin- ethyl gallate, fusidic acid- ethyl gallate, tetracycline- ethyl gallate) were studied for resistance development of *Staphylococcus aureus* bacteria. Bacteria were induced for resistance by exposing them with step-wise (two-fold) increase in antibiotic concentration. Resistance studies were analyzed on the basis of mutation frequency, mutant selection window and mutant prevention concentrations.

6.1.1 *Resistance to Mupirocin*

Even though more than 90% of *Staphylococcal* strains are mupirocin susceptible, mupirocin resistance has already been reported in both methicillin resistant and methicillin sensitive *Staphylococcus aureus* (Eltringham, 1997). Low level resistance occurs when MIC of mupirocin is >100 µg/ml. Low level resistance is not clinically significant as the topical applications use concentration of more than 100 µg/ml for local application (Eltringham, 1997). Low-level resistance is reported to be mediated by altered access of binding sites on isoleucyl-tRNA synthetase. High-level resistance is mediated by a transferable plasmid that codes for a modified isoleucyl-tRNA synthetase. High-level

resistance is mediated by a plasmid or chromosomal copy of a gene encoding a "bypass" synthetase that binds mupirocin poorly (Goodman et al., 2008). The gram-negative organism, *Pseudomonas aeruginosa*, is intrinsically resistant to mupirocin as it is acquired from *Pseudomonas fluorsecens* (Lorian, 2005).

6.1.2 *Resistance to tetracycline*

Tetracycline resistance is not only widespread but it is also inducible (Goodman et al., 2008). There are three main mechanisms, shown as following, involved in the development of tetracycline resistance.

6.1.2.1 Limiting the access of tetracycline to the ribosome

Bacteria obtain the mechanism to limit the access of tetracycline to ribosomes by formation of efflux pumps (Levy, 1992). Efflux pumps actively remove tetracycline out of the cell with higher rate than the rate of uptake to keep low intracellular tetracycline concentration so that bacterial protein synthesis can continue unhindered. There are 8 classes of tetracycline efflux genes. Classes A to E efflux genes are found in *Enterobacteriacea* family, *Hemophilus* family, *Moraxella*, *Aeromonas* and *Vibrio*. Class P genes is found only in Clostridium species. Class K and L genes are found in gram-positive cocci and bacilli. Class K is most often found in *Staphylococcus* species (Pournaras et al., 2009, Garau et al., 2009).

6.1.2.2 Ribosomal protection

Ribosomal protection is one of the important resistance mechanisms which enable both gram positive and gram negative organisms to resist tetracycline. Ribosomal protection proteins such as Tet (M), Tet (O) and Tet (Q) are soluble cytoplasmic proteins which are involved in mediation of tetracycline resistance. As they have sequence similarity to elongation factors, they inhibit tetracycline's action by acting as tetracycline resistant elongation factors (Agwuh and MacGowan, 2006, Chait et al., 2007, Chopra and Roberts, 2001, Finch, 2009). These ribosomal proteins also have the ability to free ribosomes by dislodging tetracycline from its ribosomal binding site so that the aminoacyl tRNA can bind to its binding site and protein synthesis can continue unhindered.

6.1.2.3 Production of tetracycline inactivating enzymes

This mechanism involves the cytoplasmic proteins that chemically modify tetracycline by a reaction that takes place in the presence of oxygen and NADPH. The resistance gene for this mechanism is classified as Tet (X) gene but the exact clinical significance of these genes is still not known.

The following figure [Figure 59] illustrates the summary of various tetracycline resistance mechanisms.

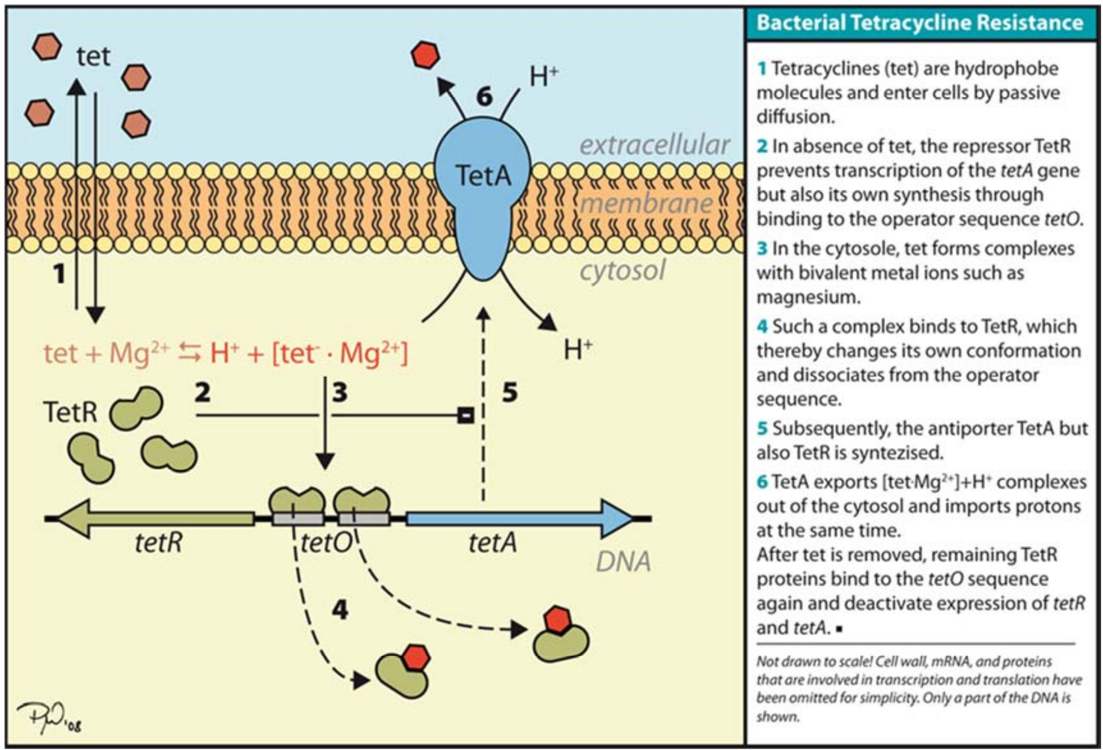


Figure 57: Resistance mechanism to tetracycline (Christian and Wolfgang, 2003)

6.1.3 *Resistance to fusidic acid*

Even though fusidic acid has potent antimicrobial action against *Staphylococcal aureus*, resistance readily occurs when it is used alone (Chopra, 1976). Thus, fusidic acid is used in combination with other antimicrobial agents. The mechanism of resistance to fusidic acid was studied in *Staphylococcus aureus* and it was mainly due to the occurrence of single step chromosomal mutation in fusA gene which encodes for elongation factor G (EF-G). This point mutation results in structural alteration that blocks fusidic acid from binding to EF-G (Mason et al., 2003, Turnidge and Collignon, 1999, Whitby, 1999). Another well-known fusidic acid resistance is caused by plasmid mediated reduction in cell wall and membrane permeability.

6.2 ***Experimental methodology***

6.2.1 *Preparation of inoculums*

Staphylococcus aureus strain ATCC 4330 was cultured on IS agar plate and incubated in 37°C incubator for 24 hrs. 3-5 colonies of microorganism were transferred from this overnight cultured isolate into the test tube containing 5 ml of IS broth where they were incubated to reach the turbidity of 0.5 McFarland standard ($1.5x10^8$ CFU/ml). The bacterial culture was then further diluted with broth to obtain 1.5 x 10^6 CFU/ml.

6.2.2 *Determination of mutation frequency of bacteria*

Mutation frequency is the frequency at which mutation events occur in a given population. It is one of the important factors that influence the appearance and spread of acquired antibiotic resistance. Mutation frequency is a fraction of mutant bacteria to the total bacteria cells in the population (Drlica and Zhao, 2007). Mutation frequency can be elicited by inoculating high concentration of bacteria on different concentrations of antibiotic treated agar plates. Assessing the surviving bacteria after specified period gives the number of mutations that can arise from the population of inoculated bacteria. In this study, mutation frequency of bacteria was assessed in the presence of antibiotics fusidic acid, tetracycline and mupirocin; both as single agents and in combination with ethyl gallate at synergistic concentrations. Bacterial stock solution was prepared at 10^9 CFU/ml by overnight incubation of 3-4 colonies of pre-plated bacteria in broth. 100µl (10^8 CFU) of bacterial stock solution was taken out and inoculated onto agar plates prepared with 2XMIC and 4XMIC of different antibiotics (mupirocin, fusidic acid, tetracycline) and

incubated at 37°C for 48 hours. Plating was done in triplicate. After 48 hours, number of colonies was counted and average number of colonies was calculated. Mutation frequency was calculated by the division of total number of colonies formed by the number of colonies inoculated.

6.2.3 *Development of resistant strains in vivo*

In this study, ATCC strain of *Staphylococcus aureus* bacteria was sequentially induced to develop resistance to synthetic antibiotics (mupirocin, fusidic acid, tetracycline). Bacteria were exposed to stepwise two- fold increment of drug concentrations (antibiotics, phytochemicals and combinations) until they were resistant to 32 X previous MIC. The duration it took for the resistance to arise was assessed. In this case, *Staphylococcus aureus* ATCC 43300 was used for repeatability and reproducibility of the experiment. ATCC strain of *Staphylococcus aureus* is a well- known bacteria which has been studied extensively. Therefore, by choosing the bacteria which is well known, genetic studies should allow the researchers to conduct the study with vast information should the need arise. The stability of resistant genes was assessed by incubation on drug-free agar plates followed by assessment of minimum inhibitory concentration. 4 test tubes containing 1/8MIC of antibiotic, 1/8 MIC of phytochemical, 1/8MIC Antibiotic +1/8MIC of phytochemical, IS broth were inoculated with 1ml of bacterial stock solution containing $1.5x10^6$ CFU/ml of bacteria. They were incubated in 37°C for 18-24 hours. After 24 hours, bacterial solution was taken out. Cells were harvested out by centrifuging at 3500g for 15 minutes. They were diluted to reach 10^8 CFU/ml. 100 µl of bacterial suspension was taken out to be sub-cultured on normal IS agar plate, 1/8MIC antibiotic treated plate,

1/8 MIC ethyl gallate treated plate, and 1/8MIC antibiotic+1/8 MIC ethyl gallate treated plates. Subsequently, all cultured plates were incubated again at 37°C for another 24 hrs. Whenever bacteria growth was observed in plates, 3-5 colonies of bacteria colonies were taken from that agar plate and those bacteria were subjected to the same procedure of treatment for another 24 hours with two-fold increasing concentration of respective antimicrobial. The procedure was repeated until the resistant MRSA appeared. The time taken for each of phytochemical, antibiotics and combinations were plotted and compared. Concurrently, after every passage, new minimum inhibitory concentrations for each batch of resistant bacteria were determined.

6.2.4 *Determination of MIC, MPC and stability of resistant phenotypes*

Furthermore, mutant selection window and mutant prevention concentrations were assessed for each antibiotic under study in the presence and absence of phytochemicals. Mutant formation in sub-population of bacteria is reported to be enriched within the range of concentration called mutant selection window (Drlica, 2003). Within this window, the resistance is selected for the drug concentrations high enough to inhibit the wild-type growth but low enough for some resistance mutants to grow. When a drug is administered with the intention to exert maximum killing to the bacteria, it inhibits the growth of susceptible pathogen while at the same time creates a selective advantage to the resistance lineages which would eventually make the drug ineffective. Even though there are numerous studies that identified the impact of single drugs on the bacterial resistance, there is little information about how the combination of drugs inhibits the bacterial growth because combinations work different from the single agents. It has been reported by

Michel *et al.* (Michel et al., 2008, Michel and Gutmann, 1997) that different drug combinations have significantly different impacts on the size of the window of drug concentrations where resistance is selected for. Therefore, after studying the effect of drug combinations on biofilm formation, the effect of drug combinations on mutant selection window and resistance formation of the *Staphylococcus aureus* were studied. Strains that were resistant to mupirocin, tetracycline and fusidic acid in the presence and absence of ethyl gallate were developed and results were compared. Mutant selection window was taken as the concentration ranging from MIC (minimum inhibitory concentration) to mutant preventing concentration (MPC).

The susceptibilities of MRSA original strain and single step mutants (after 10 passages) to mupirocin, tetracycline and fusidic acid were determined using the microbroth dilution method according to guidelines in antimicrobial susceptibility testing protocols (Schwalbe et al., 2007). The MIC was taken as the lowest drug concentration that could prevent the growth of 10^4 to 10^5 cells.

For MPC determination, *Staphylococcus aureus* bacterial cells were grown to stationary phase, harvested by centrifugation, and re-suspended in the fresh growth medium. Incubation was continued for 5 to 6 h, producing a culture containing 10^{10} to 10^{11} CFU/ml. 100 µl cells were taken out and inoculated onto drug containing agar plates at concentrations of (2MIC, 4MIC, 8MIC, 16MIC, 32MIC). These plates were incubated at 37°C. On 2nd day and 5th days, the plates were taken out and counted for presence of resistant colonies (Zhao et al., 2003). Mutant prevention concentration was taken as the

maximum concentration of antibiotic that express zero growth of bacteria in the agar plates that were inoculated in aforementioned manner.

6.3 ***Results and observations***

6.3.1 *Resistance to Antibiotics*

When bacterial cells were exposed to antibiotics at low concentration (starting from 1/8MIC) and inoculated in the antibiotic containing agar plates that were two-fold higher than the MIC that they were previously exposed to, resistant strains were always found to emerge for propagation. This indicated that even above the concentration that was regarded as minimum inhibitory concentration, some of the bacteria were able to survive the exposure. By 12th passage, bacteria cells were already resistant to 32MIC concentration of antibiotics. All the antibiotics were found to behave in similar manner as can be clearly seen in Figure 58.

6.3.2 *Resistance to ethyl gallate*

The bacterial cells that were harvested from the suspension containing 1/4MIC of drug were regarded as the cells that have already been exposed to 1/4MIC of ethyl gallate. They were then exposed to agar plates containing 1MIC of EG and 2MIC of EG more than the ones they were previously exposed to. As expected, bacterial cells were observed to be readily cultured in 1/4 MIC and 1/2 MIC. However, when the cells that were previously exposed to 1/2 MIC were grown on 1MIC agar plates, no growth was detected and thus cells were needed to be re-exposed to 1MIC for 16 passages. When the bacterial

cells that were resistant to 1MIC were inoculated on 2MIC agar plates, no growth was detected and re-exposure was repeated for 28 passages. This fact indicates that *Staphylococcus aureus* bacteria cannot develop the resistant mechanism readily to ethyl gallate as they did so in the synthetic antibiotics.

6.3.3 *Resistance to combinations*

The resistance development to the combination of phytochemicals and antibiotics were performed with the same protocol as the individual antimicrobial agents. As the bacterial cells were needed to be exposed to sub-MIC concentration of combinations, the exposure concentration was started at 1/8MIC+1/8MIC. In the combination of tetracycline and ethyl gallate, it took 12 days to become resistant to 1/4 MIC+1/4 MIC, 18 days for 1/2 MIC, 42 days for 1 MIC and 56 days for 2MIC. In the combination of fusidic acid and ethyl gallate, it took 4 days to develop resistance to 1/4 MIC +1/4 MIC, 8 days for 1/2 MIC +1/2 MIC, 20 days for 1 MIC +1 MIC and 46 days for 2MIC. In the combination with mupirocin acid and ethyl gallate, it took 4 days for 1/4 MIC +1/4 MIC, 8 days for 1/2 MIC +1/2 MIC, 20 days for 1 MIC +1 MIC and 46 days for 2MIC+2MIC. In all of those combinations, resistance development for the concentration of more than 2MIC+2MIC was not obtained even though the duration of repeated cycles was increased to more than 50 days. When the duration it took for resistant strains to develop for each of the antimicrobials alone and in combinations was compared, it was evident that combinations took longer time to develop resistance in contrast to the synthetic antimicrobials. Moreover, the presence of ethyl gallate was found to significantly affect the resistance development in this study.

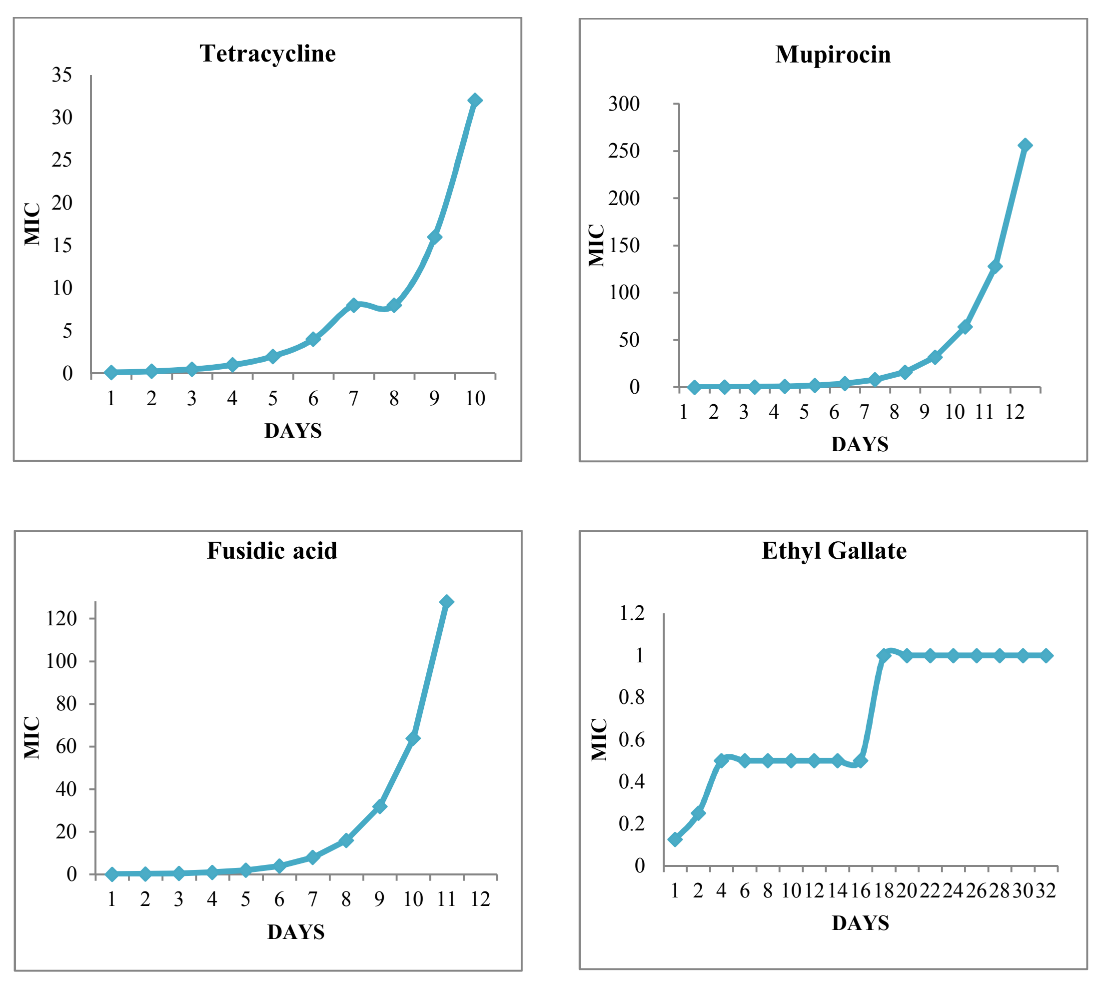
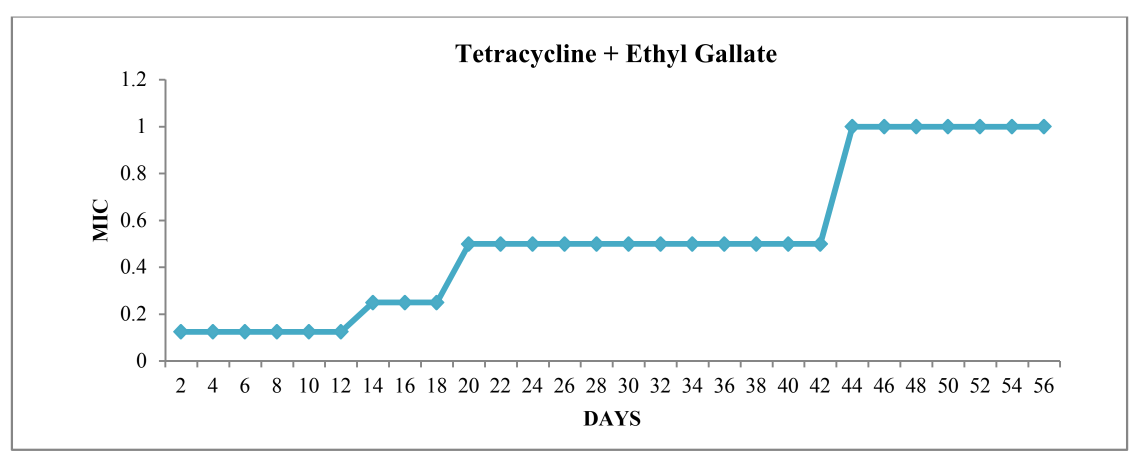
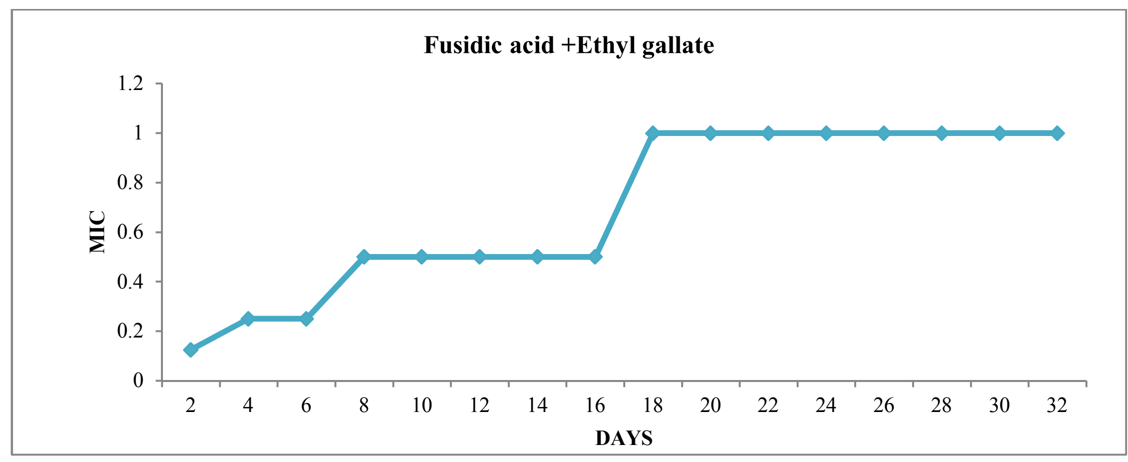


Figure 58: Duration taken for resistance development for single agents





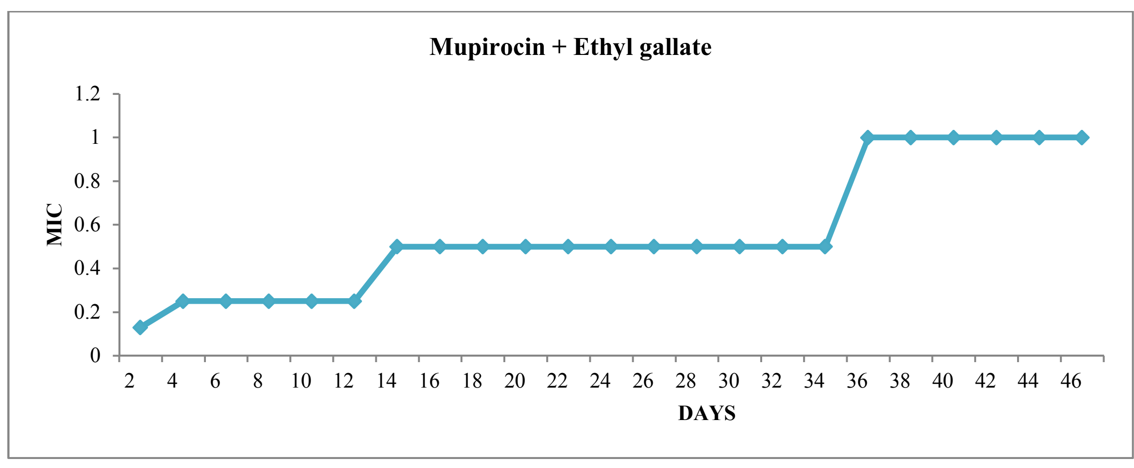


Figure 59: Duration taken for resistance development for combinations

Table 7: Comparison of time taken for development of resistance

Drug	Final MIC for Resistant Bacteria	Duration
Tetracycline	32 MIC	10 passages
Fusidic acid	32 MIC	10 passages
Mupirocin	32MIC	14 passages
Ethyl gallate (EG)	1 MIC	28 passages
Tetracycline + EG	¼+ ¼ MIC	18 passages
Tetracycline + EG	½ + ½ MIC	42 passages
Tetracycline + EG	1 + 1 MIC	56 passages
Fusidic acid + EG	¼+ ¼ MIC	7 passages
Fusidic acid + EG	½ + ½ MIC	16 passages
Fusidic acid + EG	1 +1 MIC	32 passages
Mupirocin + EG	¼+ ¼ MIC	12 passages
Mupirocin + EG	½ + ½ MIC	20 passages
Mupirocin + EG	1 +1 MIC	35 passages

6.3.4 *Mutant selection window determination*

Mutant selection window of mupirocin, tetracycline and fusidic acid were determined in the presence and absence of EG before the resistance development study. MPC was determined after inoculation of bacteria to the serial dilution of agar plates and incubated at 37°C. The colony counts were performed on 2nd and 5th day of incubation. MPC of tetracycline (16µg/ml) was 32 times of its MIC (0.5µg/ml). MPC of fusidic acid (1.92µg/ml) was 64 times of its MIC (0.03µg/ml), and MPC of mupirocin (32 µg/ml) was 32 times of its MIC (1 µg/ml). MIC of ethyl gallate was 1024 µg/ml and MPC of ethyl gallate was 2048 µg/ml. Thus, in comparison to fusidic acid, mupirocin and tetracycline, ethyl gallate showed smaller size of mutant selection window (MSW) for MRSA.

After the development of the single step mutants (within MSW) MRSA resistant to 32 MIC of tetracycline, 32 MIC of mupirocin and 32 MIC of fusidic acid were selected for investigation of MPC and MSW again for resistant strains (MRSA resistant to 32 MIC of tetracycline, mupirocin and fusidic acid, respectively) for tetracycline, mupirocin and fusidic acid. It was observed that all the MPC for mutant strains for tetracycline, mupirocin and fusidic acid were >256 times of its previous MIC. Interestingly, the addition of 1024 (µg/ml) of EG restored the MPC back to previous minimum inhibitory concentration (128 to 0.5µg/ml) in tetracycline, (32 µg/ml to 1 µg/ml) in mupirocin and (7.68 to 0.03µg/ml) in fusidic acid. Therefore, it could be inferred that the mutant selection window of tetracycline, mupirocin and fusidic acid were closed by the addition of 1MIC of ethyl gallate or the resistance developed had been reversed.

Table 8: Mutant selection window of each antibiotic

	MIC (μg/ml)		MPC (μg/ml)	
	EG +	EG -	EG +	EG -
Tetracycline ***pre exposure***	0.125	0.5	0.5	16
Tetracycline ***post exposure***	0.5	64	0.5	>128
Fusidic acid ***pre exposure***	0.0075	0.03	0.03	1.92
Fusidic acid ***post exposure***	0.03	3.84	0.03	>7.68
Mupirocin ***pre exposure***	0.25	1	0.25	32
Mupirocin ***post exposure***	0.25	64	0.25	>128

6.3.5 *Mutation frequency*

Mutation frequency is the number of mutant cells obtained from the inoculation of large number of bacterial cells in gradient of antibiotic containing agar plates. It can be calculated by the division of number of cells surviving the exposure of antibiotic with the number of cells inoculated. Following the isolation of mutant strains for each of the antibiotics, the genetic stability of resistance was assessed by growing the bacterial cells on drug free agar plates so as to remove the drug-pressure. They were then assessed for minimum inhibitory concentrations again by microbroth dilution method. Mutation frequencies of the mutant strains were evaluated by growing them on agar plates that

contains 2MIC and 4MIC of antibiotics. From the study, it was found that *Staphylococcus aureus* shows innate ability of higher selection of mutation frequency in fusidic acid ($4x10^{-5}$) when compared with mupirocin ($3x10^{-6}$) and tetracycline ($5x10^{-5}$). This high mutation frequency explains that resistance is frequently acquired during the treatment with fusidic to MRSA infections as a single agent. Minimum inhibitory concentrations of mutant strains were affected by the presence or absence of ethyl gallate. MIC of final strains increased up to 32-fold when compared to the parent strain in the absence of ethyl gallate. However, in the presence of ethyl gallate, MIC of the final strains was increased up to only 4-fold. It appeared that presence of ethyl gallate affect the mutation frequencies of the bacteria as well. Mutation frequency of final strains in the absence of ethyl gallates increased exponentially with mupirocin (10^{-1}), fusidic acid (10^{-1}), and tetracycline (10^{-1}). However, in the presence of ethyl gallate, the mutation frequency was almost non-existent with $<10^{-9}$ for all of the antibiotics.

Table 9: Mutation Frequency

MRSA 43300	MIC (µg/ml)	Mutation frequency	
		2 x MIC	4 x MIC
Mupirocin (Control)	0.25	$3x\ 10^{-6}$	$<10^{-9}$
Mupirocin (Resistant)	64	10^{-1}	10^{-3}
Mupirocin +Ethyl gallate	1	$2x10^{-9}$	$<10^{-12}$
Fusidic acid (control)	0.03	$4x\ 10^{-5}$	$<10^{-9}$
Fusidic acid (Resistant)	3.84	10^{-1}	10^{-2}
Fusidic acid + Ethyl gallate	0.03	$3x10^{-9}$	$<10^{-12}$
Tetracycline (control)	0.125	$5x10^{-5}$	$<10^{-9}$
Tetracycline (Resistant)	64	10^{-1}	10^{-2}
Tetracycline + Ethyl gallate	0.5	$8x10^{-9}$	$<10^{-12}$

6.4 ***Discussion***

Pharmaceutical industry in the past 20 years has seen reduced antibiotic development. Therefore, with increasing number of drug-resistant bacterial infections, there is a need to develop novel strategies using existing antimicrobials. Studying mutation events in bacteria is a complicated process because it is not a static event. Mutation events in bacteria depend on the wide array of factors influencing the rate and types of mutants that can be selected under the selective pressure of antibiotics. Rate of mutation depend on bacterial stress which was a product of nutritional availability, concentration of antibiotics, inherent ability of some antibiotics to increase the mutability, and ability to produce mutator phenotypes in bacteria. Depending on the specific antibiotic-bacterium interaction at a given antibiotic concentration, either single gene mutations (independent mutations) or several genes (cooperative mutations) are acquired. The results of this study indicated that combination of EG with tetracycline, mupirocin and fusidic acid could delay evolution of resistance to these antibiotics. This was due to the fact that bacteria were presented with more than one target mutation in order to survive in the combination therapeutic medium as the antibiotics and ethyl gallate targeted at different genes to exert their antimicrobial activity. Interestingly, MSW of mupirocin, tetracycline and fusidic acid could be closed by the addition of 1024 µg/ml (1MIC) of ethyl gallate. These results suggest that the addition of ethyl gallate can reduce the resistance selection of the subpopulation enriched by the drugs by being in the mutant selection window.

The present study clearly sheds light on the potential of ethyl gallate as antimicrobial compounds and suggests on the possibility of use of the above synergistic combinations for treating this drug resistant pathogen.

6.5 ***Summary***

To assess the development of mutant evolution in the *Staphylococcus aureus* to the antibiotics under study, resistant strains for mupirocin, tetracycline, fusidic acid and ethyl gallate were developed. When the duration of time it took for the individual drugs to develop resistance and the duration of time it took for the combinations to develop resistance were compared, it was noted that the addition of the ethyl gallate significantly increased the time taken to develop resistance. In other words, it could be suggested that ethyl gallate could hinder the mutation of the antibiotics. After the resistance strains were developed, mutant selection window of the tested strain to the antibiotics of interest (tetracycline, mupirocin, fusidic acid) as well as the phytochemical of interest (ethyl gallate) were investigate further. It was found that the mutant selection window of synthetic antimicrobials (tetracycline, mupirocin, fusidic acid) were much wider than the mutant selection window of the ethyl gallate. As a result, it could be suggested that the concentration range at which the antibiotics were enriched to develop resistance was wider. On the other hand, it was noted that the mutant selection window of ethyl gallate was narrower thus reducing the range of concentration at which the bacteria strain was enriched for resistance. The result also concurred with the longer time it takes to develop resistance in comparison with the antibiotics. Finally, when the antibiotics are combined with ethyl gallate, it indicated the minimum inhibitory concentration actually coincides

with the mutant prevention concentration and thus closing the mutant selection window, thus eliminating the concentration range for resistance development. As resistance development is important in the usage of antibiotics as topical agents, it can be assumed that the usage of ethyl gallate would help hinder the resistance development associated with the usage of topical therapeutic agents.

7. CHITOSAN- ALGINATE POLYELECTROLYTE MEMBRANES

7.1 *Introduction*

Skin is the largest organ in the body. It has many different functions, ranging from protection against multitude of pathogens in the environment, regulation of heat and evaporation, to sensation of external stimuli (Stillman et al., 1980). Traumatic loss of skin tissue in cases such as burn, diabetic ulcers and pressure sores lead to impairment of skin functions along with fluid loss, hypothermia, infections and creation of locally immunocompromised regions. As the regeneration of a damaged skin is a complex mechanism with the involvement of the interaction between cells, and extracellular matrix molecules, creation of the favorable environment for the wound healing is important (Falanga, 2005). In the past decades, many kinds of medicated (antibiotic or antiseptic - cooperated bandages) and non-medicated dressings (conventional bandage without the incorporation of antibiotic or antiseptic) were used in the treatment of wound infections. Recently, there have been reports concerning the advantages of medicated dressings over non medicated ones (Rode et al., 1989). However, medicated wound dressings did not gain much popularity because the antibacterial actions of topical antibiotics in bandages are usually associated with delayed wound healing. Antibiotics or antiseptics present in the dressing have propensity to cause cytotoxic effect on fibroblasts and keratinocytes (Fleming, 1919, Hidalgo and Dominguez, 2001). Thus, there is a need to develop a material which serves as a wound dressing as well as a drug carrier system which is

biocompatible. In the previous chapters, a phytochemical ethyl gallate is found to possess antimicrobial activity, act synergistically with synthetic antibiotics which are commonly used as topical agents such as fusidic acid and mupirocin, hinders resistance development and inhibit biofilm formation which commonly occurs in wounds. Therefore, combination of ethyl gallate and fusidic acid provides a potential topical agent to be used in medicated wound dressings for treatment of wounds.

In this chapter, chitosan and alginate were chosen as natural polymeric materials to formulate polyelectrolyte membranes. Physical properties such as water vapor transmission rate, swelling rate, crosslinking percent, tensile properties, morphological studies by using scanning electron microscopy were performed. MTT assay was performed to explore the biocompatibility of the component of the membrane. Formulations of polyelectrolyte membrane were performed in different polymer ratios.

7.1.1 *Chitosan*

Chitosan, α (1→4) 2-amino-2-deoxy-β-D-glucan, is a biocompatible and biodegradable natural polysaccharide extracted from the shells of the shrimp and crab. It is derived by deacylation of chitin by replacing -NHCO---CH_3 group with acetamide group. Chitosan is used in diverse applications in pharmaceutical industry as antimicrobial agents, slimming medicine, joint strengthening agent and other non- medical applications such as cosmetics, agriculture and water treatments (Kim et al., 2008a). Chitosan is known to have broad-spectrum of antimicrobial activity which is effective against bacteria, fungi, and virus. Chitosan is now being researched widely as a potential drug carrier agent in the wounds and skin infections. Chitosan's potential application as a biogel has been sought

after by studies focusing on developing biocompatible and bioactive polymers. Chitosan is relatively inexpensive, easily applicable, biodegradable, and relatively free of adverse effects on the healing process. Molecular structure of chitosan is depicted in the following figure [Figure 62].

Figure 60: Molecular structure of chitosan

7.1.2 *Alginate*

Alginates are food additives and encapsulating agents commonly used in pharmaceutical products. They are commercially produced from brown seaweed. They are non-repeating copolymers of β-D- mannuronic acid (M) and α-L-guluronic acid (G) which are linked by 1-4 glycosidic bonds (Kim et al., 2008b). Sodium alginate has been used as food additive in ice-creams and dairy products. Alginate has been explored for use in the wound dressing because of it can promote wound healing and reduce blood loss from the wounds. Main advantage of alginate as a wound dressing materials is it can promote healing by maintaining moisture in the wounds. Alginate dressing materials are commonly cross-linked using calcium chloride forming calcium alginate with the formation of insoluble fibrous strands. Moreover, cross-linking with bivalent cation such as Ca^{2+} not only stabilize the mechanical structure but also helps in the formation of thrombin which is

important for hemostasis (Attwood, 1989). Chemical structure of alginate is depicted in the following figure [Figure 63].

Figure 61: Alginic acid

7.1.3 *Chitosan alginate wound dressings*

Alginate, an anionic agent, and Chitosan, a cationic agent, has propensity to form polyelectrolyte complex through ionic interaction with the formation of coacervates. Carboxylate moieties in alginate ionically interact with protonated amines on chitosan to form three- dimensional matrix known as physically cross-linked hydrogel. Chitosan alginate polyelectrolyte membranes have been sought out for the use as a bandage or tissue scaffolds by various groups (Han et al., 2010, Yan et al., 2000, Kim et al., 2008).

7.2 *Experimental methods*

7.2.1 *Materials*

Commercially available chitosan from crab shells of low molecular weight (75% deacetylated), sodium alginate, acetone, acetic acid were purchased from Sigma-Aldrich (Singapore) and ethyl gallate was purchased from Acros chemicals (Germany), Fusidic acid sodium (96%) was purchased from Sigma-Aldrich (Singapore). MTT (3-(4, 5-

dimethyltiazol-2-yl)-2, 5-diphenyltetrazolium bromide) for cell cytotoxicity assay was purchased from Sigma-Aldrich (Singapore).

7.2.2 *Preparation of drug loaded chitosan-alginate polyelectrolyte membrane*

Chitosan was prepared in a solvent (1:1 ratio of 2% acetic acid+2% acetone) in different concentrations ranging from 0.5%, 1%, 1.5%. Even though Chitosan dissolved readily in acetic acid, the reaction between chitosan and alginate with the formation of coacervates was rapid in the absence of the water miscible organic solvents, acetone was added to the solvent as a solvent moderator. Sodium alginate was dissolved in water in concentrations ranging from 0.5%, 1%, 1.5%. Coacervates were formed between chitosan and alginates by drop-wise addition of chitosan solution into the alginate solution with constant swirling action using the magnetic stirrer. The same volumes of chitosan and alginates at the same concentrations, i.e. 1.5% C+1.5% A, 1% C+ 1% A, 0.5% C+0.5% A, were used in this study to form coacervates. The manner of addition of chitosan and alginate in this step was especially important because parameters such as speed of stirring, time of reaction, temperature of the reaction influence the quality of membrane obtained. After the viscous polymeric coacervates were obtained, accurately weighted ethyl gallate and fusidic acid was added. The mixture was left to be mixed for 1 hour. After one hour, fine coacervates were seen to form in the mixture. The resultant viscous coacervates were left to stand for an hour more to remove the air bubbles. The resultant bubble free viscous coacervates were then immersed in 1% $CaCl_2$ for 24 hours at room temperature. Non-cross linked and non-reacted portion of the gels were then removed by washing in sterilized distilled. The

gel was left to dry at room temperature for 24 hours. 3 different concentrations of chitosan and alginate are used (1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A).

7.2.3 *Physical properties of the film*

7.2.3.1 Swelling index (SR%)

Pieces of hydrogel samples (2cmx2cm) were dried at 60 ºC for 12 hours and weighed as (Wa). They were then soaked in pH 7.4 1XPBS at 37 ºC. The samples were taken out and weighed again as (Ws). The swelling ratio was calculated using the equation

$$SR\ \% = \left(\frac{Ws}{Wa}\right) x\ 100$$

Equation 7

Where Ws and Wa were weights of the samples before and after soaking in water (Sung et al., 2010).

7.2.3.2 Determination of percent cross linking

Sample films with dimension 1cmx 1cm were weighed (W_1) before submerging into 1M acetic acid and stirred at room temperature for 48 hours to dissolve non-crosslinked chitosan. The undissolvable hydrogels were filtered and washed with distilled water and acetone to remove non-crosslinked portion. Swollen gels were then dried at 30 ºC for 24 hours. Dried samples were weighed again and taken as (W_2). Percent cross linking was calculated as

$$\text{Percent cross linking} = \left(\frac{W2}{W1}\right) x\ 100$$

Equation 8

Where, W_1 and W_2 were weights of dried samples before and after drying (Sung et al., 2010).

7.2.3.3 Tensile strength of the membranes

Membrane thickness was measured using digital caliper. Mechanical properties of chitosan- alginate membrane were evaluated using an Instron Universal Tensile testing machine with preload 0.5 N to determine the maximum load for each matrix (Sung et al., 2010). Film strips of 6 cm long and 1cm wide samples which are free from air bubbles and physical defects were used in this test. The force and elongation were measured until the films have lost 40% of their tensile strength. Tensile strength was calculated as follows

$$\text{Tensile strength (kg/mm}^2\text{)} = \frac{\text{breaking force (kg)}}{\text{cross sectional area of sample (mm}^2\text{)}}$$

Equation 9

7.2.3.4 Water vapor transmission test

Round piece of a membrane was mounted on the mouth of a cup containing 20 g of $CaCl_2$ and placed in incubator conditioned to have 90% relative humidity at 40ºC. 500 ml of saturated sodium chloride was kept in the incubator to regulate the relative humidity.

Whole cup containing the membrane was weighed before and after putting into the incubator. Water vapor transmission was calculated as follows:

$$\mathrm{WVTR}\left(\frac{\mathrm{g}}{\mathrm{m}^2}/\mathrm{day}\right) = \left(\frac{W_2 - W_1}{S}\right) x\ 24$$

Equation 10

Where, W_1 and W_2 were weights of the whole cup at the first and second hours, respectively. S is the transmitting area of the sample (Sung et al., 2010).

7.2.3.5 Scanning electron microscopy

The morphology of chitosan- alginate polyelectrolyte membrane was observed by scanning electron microscopy by fixing the samples overnight with 2.5% glutaraldehyde in phosphate buffered saline solution (1XPBS) at 4ºC. Samples were rinsed 3 times with PBS buffer for 2 minutes and dehydrated in graded ethanol 70, 80, 90 and 99.99% for 15 minutes each. Then the membranes were mounted on stubs and sputter-coated with gold. They were analyzed using JEOL scanning electron microscope with accelerating voltage 37kV at various magnifications.

7.2.4 *Evaluation of biological properties of the membrane*

7.2.4.1 Disk diffusion test

After ethyl gallate was loaded, prepared films were evaluated for presence of antibacterial activity against *Staphylococcus aureus* by agar plate diffusion method. Zone of growth inhibition were measured. The presence of antimicrobial activity was determined by the measurement of the zone of inhibition formed on the nutrient agar (Schwalbe et al., 2007).

7.2.4.2 Release properties of the membrane

In vitro drug elution tests were performed using paddle method. In short, selected drug loaded film containing 150mg of ethyl gallate was immersed in 200 ml of PBS at 37ºC with gentle shaking. One milliliter aliquots were taken at intervals (1, 2, 3, 4, 24, and 48 hrs.). Presence of ethyl gallate in the sample was analyzed using HPLC using the column (ODS-3V, 250x 4.6mm) using a mixture of acetonitrile and distilled water for mobile phase. Rate of elution of fusidic acid from the solution was detected by UV-Visible spectrometer at a wavelength of 230 nm. The calibration curves were plotted for ethyl gallate and fusidic acid. Finally, the fraction of drugs released from membrane was calculated and plotted.

7.2.4.3 Cytotoxicity testing

The biocompatibility of the membranes was assessed using mouse fibroblast 3T3 cells. . Fibroblast cells were seeded into 96 well plate at a density of 6×10^4 cells per well each of which was prefixed with UV sterilized membrane. The plates were then incubated at 37˚C in the presence of 5% CO_2. On day 1, 2 3, and 4, each plate was taken out to test for cytotoxicity assay using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) reagent. 150 µl of culture medium containing 0.5 mg/ml of MTT was added to each well and re-incubated for 4 hours. The media containing MTT was gently removed and washed in PBS. 200 µl DMSO was added to each well to solubilize the crystallized formazan product. After 10 – 15 min incubation at room temperature, the wells were examined to ensure crystals have been fully solubilized and the plates were read on a spectrophotometer at 570 nm with a reference wavelength of 690 nm.

7.3 ***Results***

7.3.1 *Formulation of wound dressing material*

Ideal wound dressing should be flexible without tearing, possess an ability to control water loss from the wound site to keep the wound moist for optimal healing by retaining fluid exuded from the wound (Boateng et al., 2008). It should also be able to allow gaseous transfer from the wound to the environment, be resistant to bacterial invasions to prevent infections and is biocompatible without delaying wound healing process (Boateng et al., 2008). The goal of the study was to prepare a biocompatible, stable, flexible, and transparent chitosan alginate membrane which could meet the aforementioned requirements of a wound dressing while at the same time able to deliver desired drug to the wound. Chitosan and alginate were used in this study as they were well-known natural polymers with tendency to form polyelectrolytes with potential for wide variety of applications (Kim et al., 2008, Zorzi Bueno and Maria Moraes, 2011). Main advantage of polyelectrolytes coacervates was the water insolubility which allowed them to encapsulate proteins, cells and enzymes in the liquid medium. Moreover, this was the property which was lacking in the individual polymers (McKnight et al., 1988, Yan et al., 2000, Fan et al., 2006). Formation of polyelectrolyte coacervates was an elaborate process which needed meticulous timing of reaction, speed and volume of mixing of the two polymers, and behavior in which calcium chloride was added. Formation of calcium crosslinked membrane was carried out by immersing coacervates of chitosan with alginate in 1% $CaCl_2$ solution. Chitosan was dissolved 2% v/v acetic acid and 2% v/v acetone. As the coacervation reaction between chitosan and alginate were rapid, the rate of reaction was

controlled by the presence of water miscible organic solvent, acetone, in the same concentration as acetic acid premixed with acetone (Yan et al., 2001). Drop-wise addition of chitosan into alginate caused the formation of coacervates separated by polymeric solution which went on for 1 hour until complete reaction took place. After the complete reaction, ethyl gallate and fusidic acid were added in drop-wise manner into the polymeric coacervates. The coacervates were later subjected to cross linking by immersing in 1% calcium chloride solution. The addition of calcium chloride caused the gradual formation of white fibrous strands in the polymeric solution which was left to continue overnight as shown in Figure 62 (A). After unreacted polymers were washed out, thick white crosslinked membrane was obtained as shown in the Figure 62 (B). It was then left to dry at a room temperature in open air. After 8 hours, thin, semi-transparent, flexible polyelectrolyte membrane was obtained for further characterization and studies [Figure 62 (C)]. Scanning electron micrographs of the membrane showed corrugated porous structure which is able to help the gaseous exchange and drug delivery to the wound as shown in Figure 62 (D).

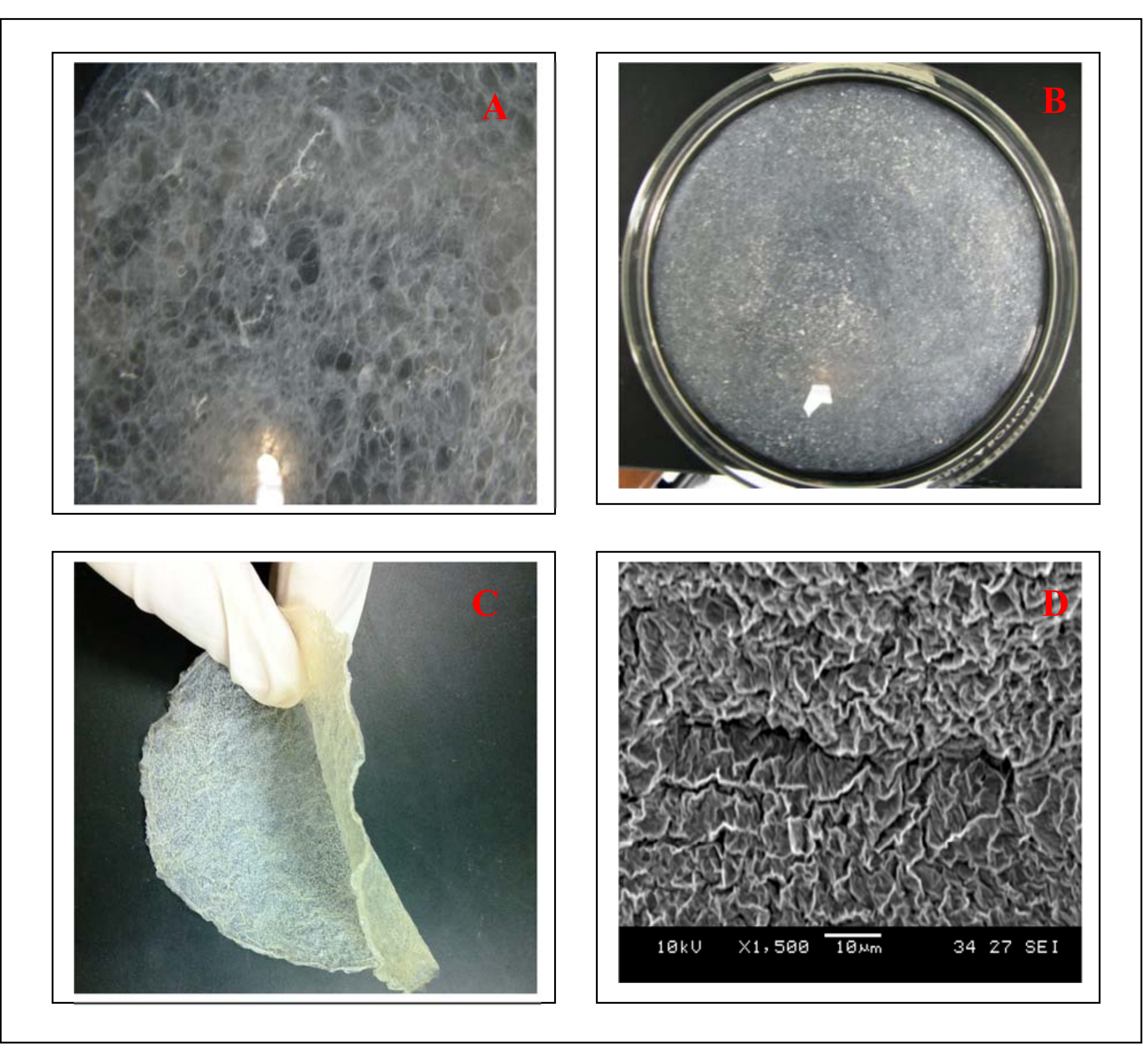


Figure 62: Coacervates of chitosan and alginate (A), thick membrane immersed in CaCl2 (B), dried semi transparent drug eluting CA membrane (C), surface morphology of membrane on SEM (D)

7.3.2 *Water retention test (swelling test)*

Exudates, the liquid produced from the wound after the process of blood clotting called hemostasis, play essential role in all the stages of wound healing. Exudates irrigate the wound continuously and keep it moist to provide the wound with nutrient as well as create a favorable condition for migration and mitosis of epithelial cells. While excess exudates hinder the wound healing process by the increasing proteolytic destruction of wound bed, the lack of it may also cause the surrounding skin to become atrophic, scaly and hyperkeratotic as the delivery of nutrient to the wound area is reduced (Harding, 2012, Kouraba, 2012). Therefore, one of the aims of the polyelectrolyte membrane is based on the property of chitosan and alginate's ability to retain fluid so that the wound bed is kept moist. In this experiment, it was observed that polyelectrolyte membranes could absorb water up to 1600% of their original weight indicating that the polyelectrolyte membrane was able to absorb the exudates produced from the wound maintaining the moisture in the wound to help wound healing process. From the findings, it could be deduced that swelling rate increased with the increase of polymer content. It was also found that the presence of drugs did not affect the swelling rate significantly ($p > 0.05$) as shown in Figure 63.

Figure 63: Swelling rate of different membranes made of different polymer ratios:
1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A *CA- chitosan alginate membrane, CAPE- Ethyl gallate loaded chitosan alginate membrane, CAPEF - ethyl gallate and fusidic acid loaded chitosan- alginate membrane

7.3.3 *Water vapour transmission test*

The optimum wound healing environment requires the gaseous exchange of water vapor and air to be favorably present between the wound and the outside environment. Exudate management involves the permeability to water vapor as the water vapor transmission determines the angiogenesis, epithelialization and fibroblast formation. Even though ideal dressing could control the evaporative water loss from wound at an optimal rate, there is no fixed optimum rate itself (Lamke et al., 1977). However, rate of water vapor transmission for commercial skin dressings were reported to range from 426-2047g/m^2/day (Ruiz-Cardona et al., 1996). As shown in Figure 64, the polyelectrolyte membranes in this study were found to transmit water vapor ranging from ~900g/m2/day

to >1200 g/m2/day. It was noted that the presence of drugs was contributing to the improvement of water vapor transmission across the membrane. Thus, it could be said that the water vapor transmission of drug loaded PEC membrane was comparable to that of commercial skin substitutes.

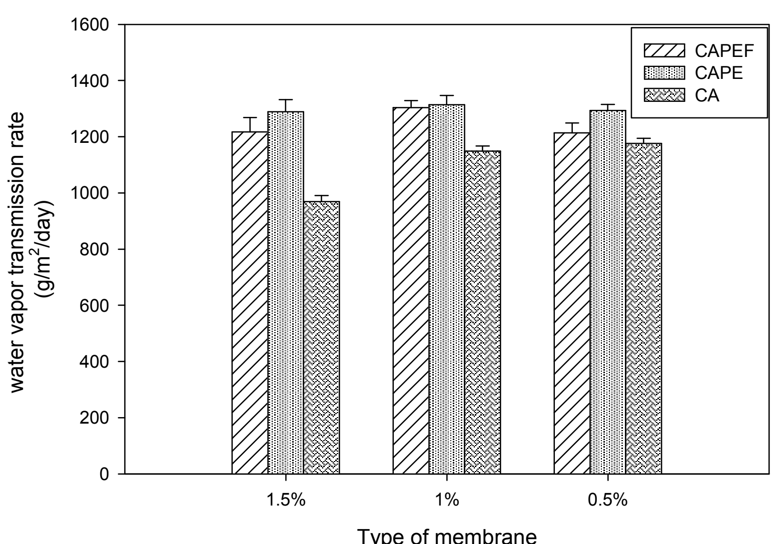


Figure 64: Water vapor transmission rate of membranes made of different polymer ratios: 1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A *CA- chitosan alginate membrane, CAPE- Ethyl gallate loaded chitosan alginate membrane, CAPEF - ethyl gallate and fusidic acid loaded chitosan- alginate membrane

7.3.4 *Crosslinking*

Polyelectrolyte membrane in the study used $CaCl_2$ to crosslink between the sodium alginate and chitosan molecules. The crosslinking behavior is important in the dressing materials because it can influence the water uptake behavior and swellability of the membranes (Akin and Hasirci, 1995, Saarai et al., 2011). In this study, it was found that the percentage of crosslinking increased with increasing polymer content. It was also interesting to note that the higher amount of cross linking was found in the presence of drugs as shown in Figure 65. Thus, it was safe to assume the presence of drugs was acting as rate limiters which could control the timed release of drug present in the membrane.

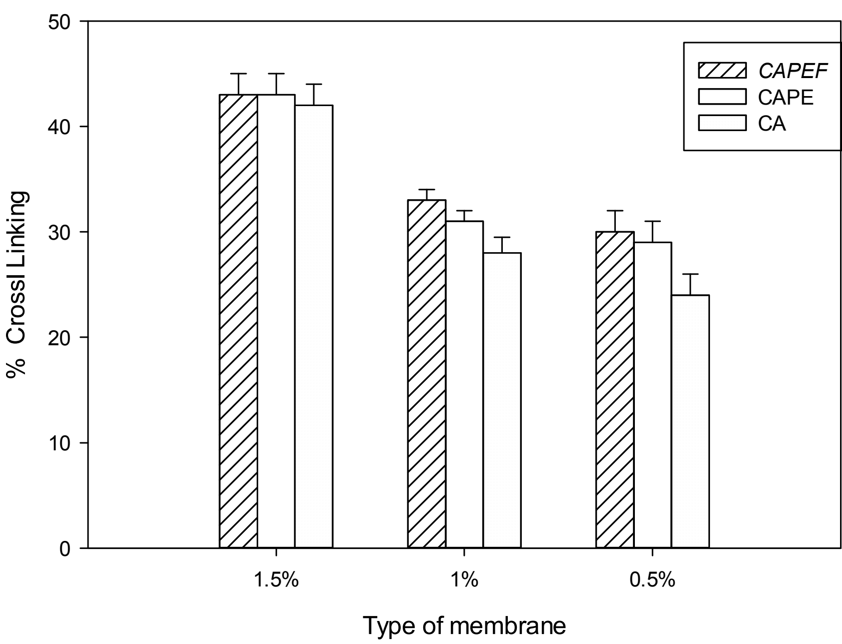


Figure 65: Crosslinking of different membranes made of different polymer ratios: 1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A *CA- chitosan alginate membrane, CAPE- Ethyl gallate loaded chitosan alginate membrane, CAPEF - ethyl gallate and fusidic acid loaded chitosan- alginate membrane

7.3.5 *Antimicrobial testing*

Recently, there have been increases in the active management of the wounds with the use of pharmaceutical agents, to assist in the process of naturally occurring wound healing mechanisms (Yu et al., 2006). In this study disk diffusion test was employed to detect the presence of antimicrobial activity. It indicated that there was a clear zone formed around the membranes as shown in Figure 66. Diameters of zones of inhibitions are plotted on the graph and compared in Figure 66 (a), with the diameters ranging from 9mm in the non-drug loaded membranes to 26 mm in the drug loaded membranes. It could also be noted that combinatory drug loaded chitosan alginate membranes had more antimicrobial activity when compared to ethyl gallate drug loaded ones. Diameters of zones of inhibitions are compared in Figure 66 (b).

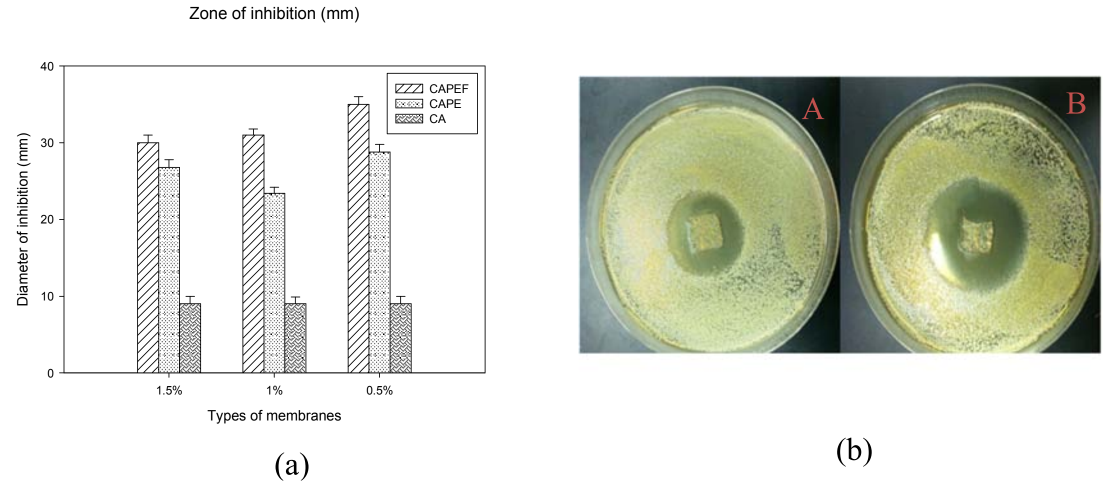


Figure 66: (a) Antibacterial properties of membranes made of different polymer ratios: 1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A *CAPE- chitosan alginate membrane, CAPEF- Ethyl gallate & fusidic acid loaded chitosan- alginate membrane [Zone of Inhibition (mm)]; (b) Disk diffusion test of bacterial inoculated agar plates treated with A: Ethyl gallate loaded Chitosan-Alginate membrane B: Ethyl gallate and fusidic acid loaded Chitosan-Alginate membrane

7.3.6 *Rate of release of antimicrobials from the membrane*

To verify the presence of antimicrobials in the membrane, drug elution tests were performed by taking eluted drug samples at different intervals; 1hr, 2hr, 3hr, 4hr, 24hr, and 48hr. To detect the presence of fusidic acid, UV-Vis spectrometer was used to measure the spectrum of eluents at 230nm which is characteristic wavelength of fusidic acid. Calibration curves were constructed using samples with known serial dilution of known concentration as shown in Figure 67. At 1 hr, 60% of fusidic acid present in the membrane was found to be released. At 2 hour, 78% of fusidic acid was found to be released. Starting from 3 hour, it was found that ~100% of the fusidic acid present in the membrane was found to be released as shown in Figure 67. To detect the presence of ethyl gallate, eluted specimens were tested with HPLC. Calibration curves were constructed with the known concentration of ethyl gallate as shown in Figure 68. It was found that the 68% of the total amount present in the membrane was eluted in the first hour. At the end of 4th hour, ~ 97% of drugs contained in the membrane were already eluted as shown in Figure 68. It was found that fusidic acid eluted faster than ethyl gallate. It could be due to the fact that fusidic acid was more water soluble as opposed to ethyl gallate which was not readily soluble in water

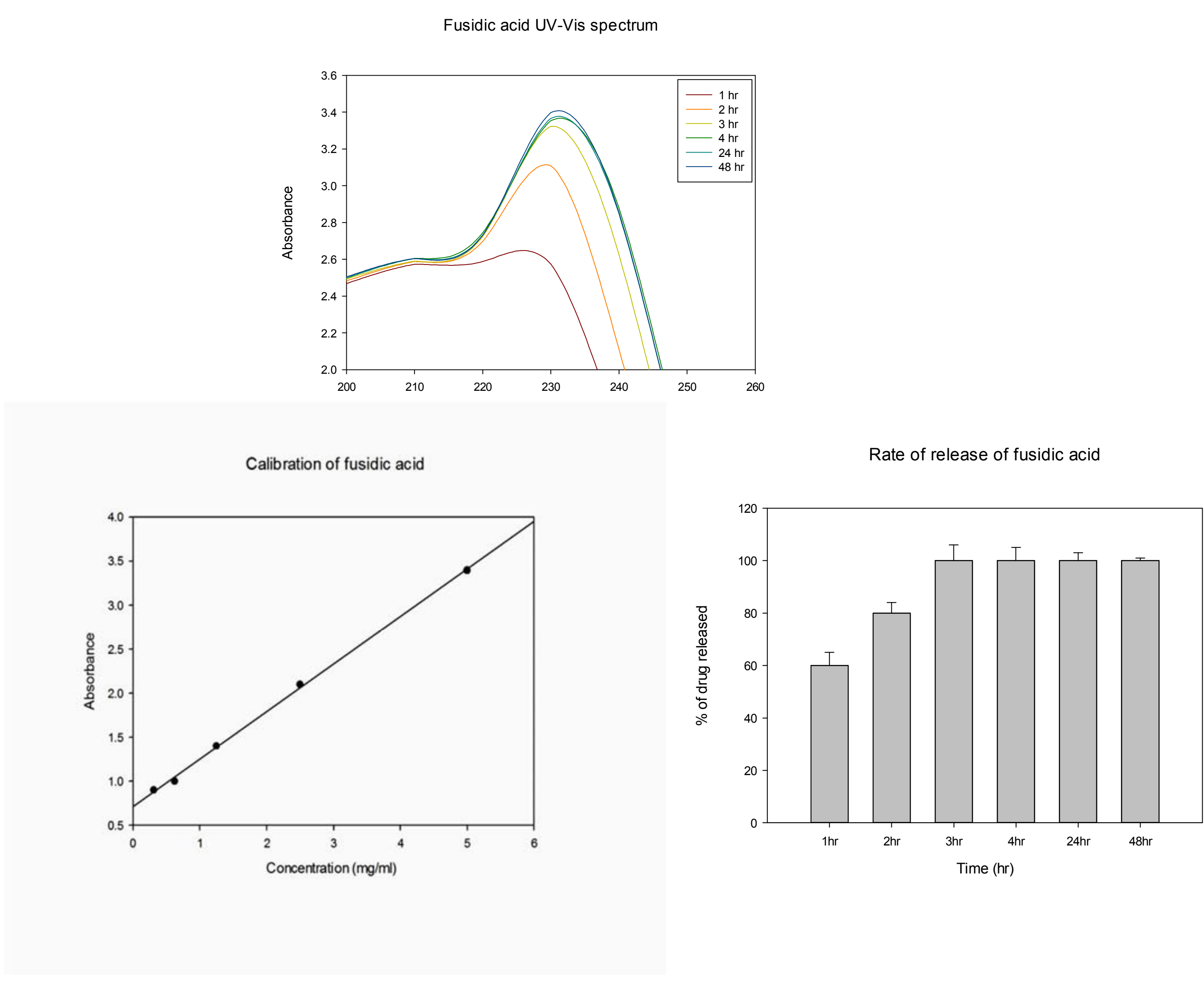


Figure 67: Release of fusidic acid from membrane by UV-Vis spectrometry measurement

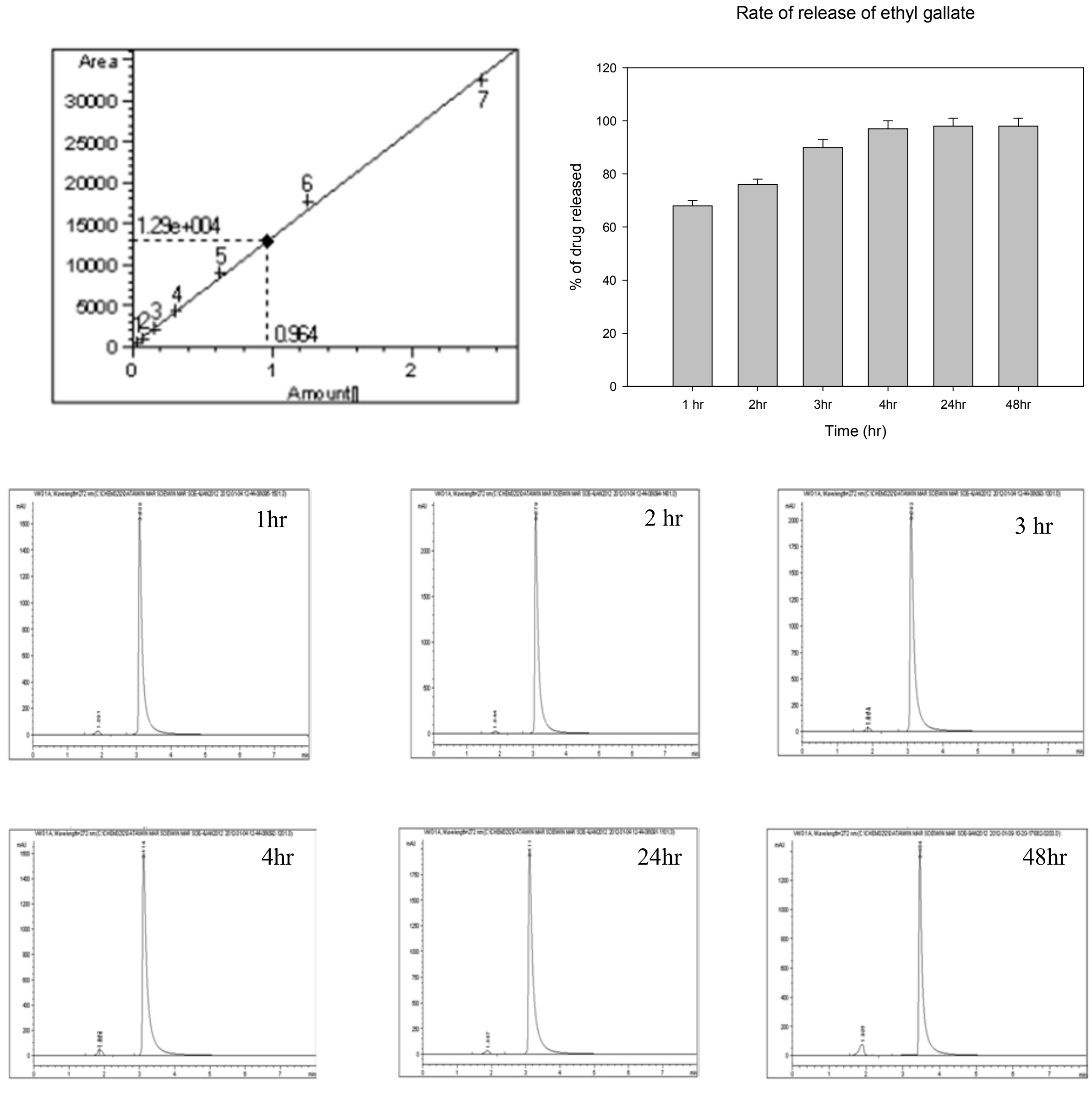


Figure 68: Release of Ethyl gallate from membrane by HPLC analysis

7.3.7 *Biocompatibility of drug loaded chitosan- alginate membranes*

As a rule, any material that comes in contact with the open wound which has potential to disseminate into the blood stream should be biocompatible, i.e., it should not elute any product material which can compromise the wound healing activity. Cell culture models present with inexhaustible source of experimental material as well as a great approximation about how the material would be responded by the cells encountered *in vivo* (Hemalswarya and Doble, 2006, Hidalgo and Dominguez, 2001, Mori, 1993). Thus, cytotoxicity experiments were carried out to assess the biocompatibility using mouse fibroblast cells (3T3). To determine the metabolic activity of viable fibroblast cells, MTT assay was carried out in which the yellow MTT reagent ((3-(4, 5 dimethyl thiazol-2yl)-2, 5- diphenyltetrazolium) was reduced to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of viable cells. MTT assays showed higher viability of membranes devoid of antimicrobials in all 4 days under study. It also showed that cells need to adjust to the contact with the membranes as the observation in all 4 days indicated the gradual increase in the cell count. When drug-loaded CA membrane (CAPEF) was seeded with the fibroblast cells, lower viability was seen when compared with CA membrane with the viable count of cells are seen as 60% on D1. However, the cells made gradual recovery as the viable count were restored to >85% which is in contrast with the latex rubber which showed only 30% viability at the end of D4 and commercial silver impregnated bandage which showed only 20% of fibroblast growth. Fibroblast viability of different membranes from D1-D4 was plotted on graphs as shown in Figure 71. Confocal microscopy and scanning electron microscopy confirmed that the

viable fibroblast cells retain their morphology while they were growing on the membrane as demonstrated in Figure 70 and 71.

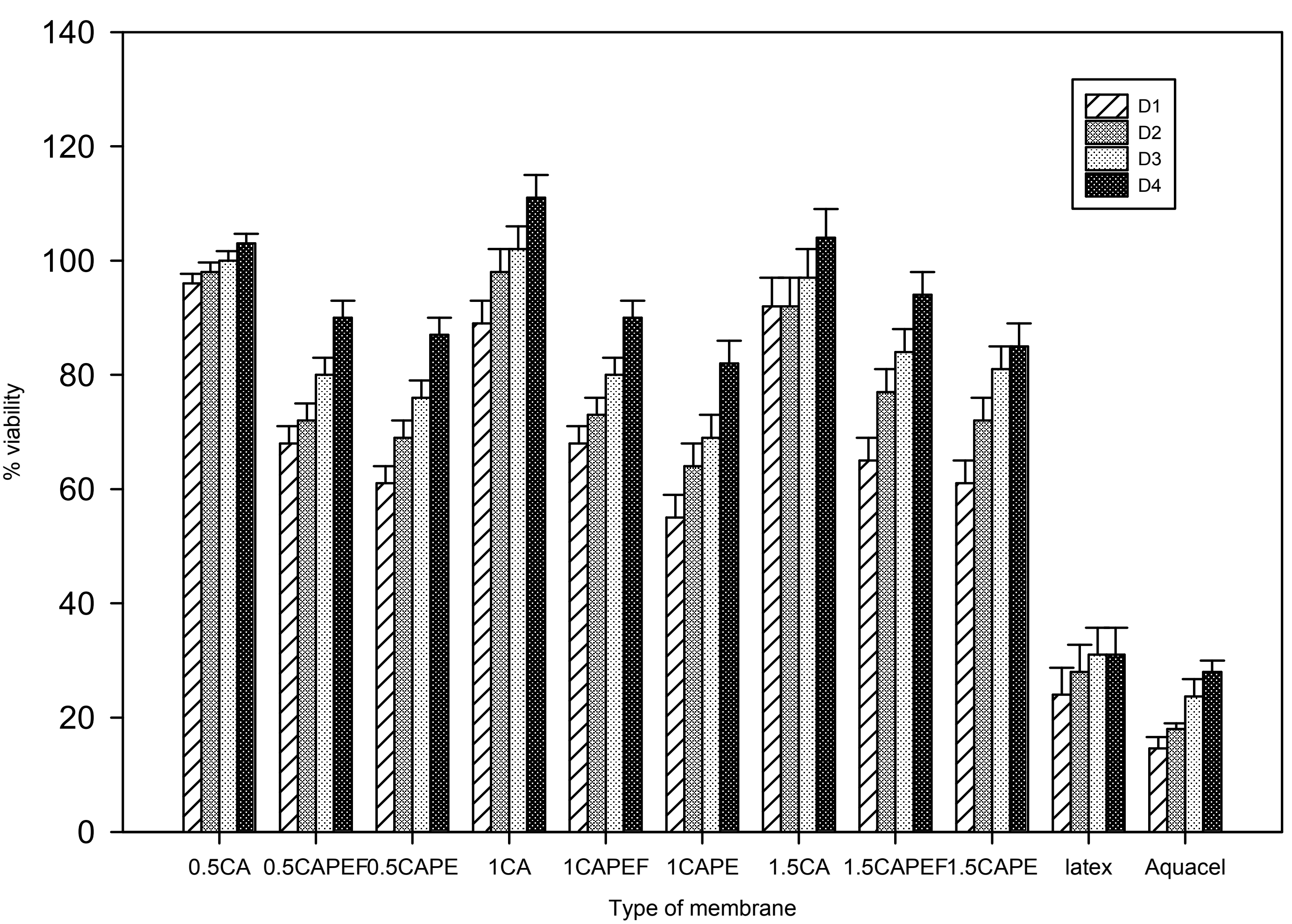


Figure 69: Fibroblast viability of membranes made of different polymer ratios on Day1-D1, Day2-D2, Day3-D3, Day4-D4: 1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A *CA- chitosan alginate membrane, CAPE- Ethyl gallate loaded chitosan alginate membrane, CAPEF - ethyl gallate and fusidic acid loaded chitosan- alginate membrane

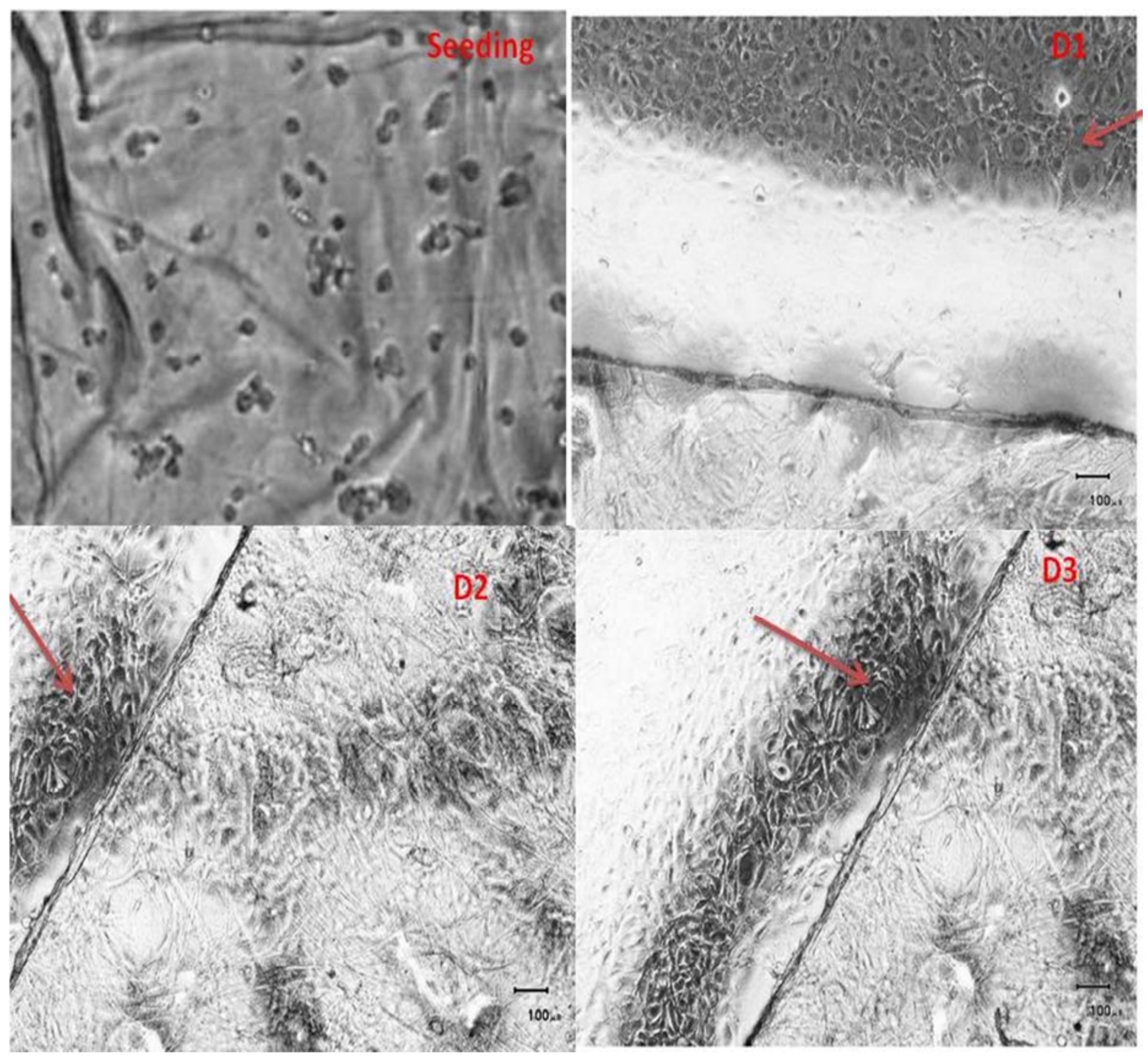


Figure 70: Confocal micrographs of Fibroblasts grown on Drug Loaded Chitosan-alginate membrane showing growth of fibroblasts in red arrows in the vicinity of membrane.

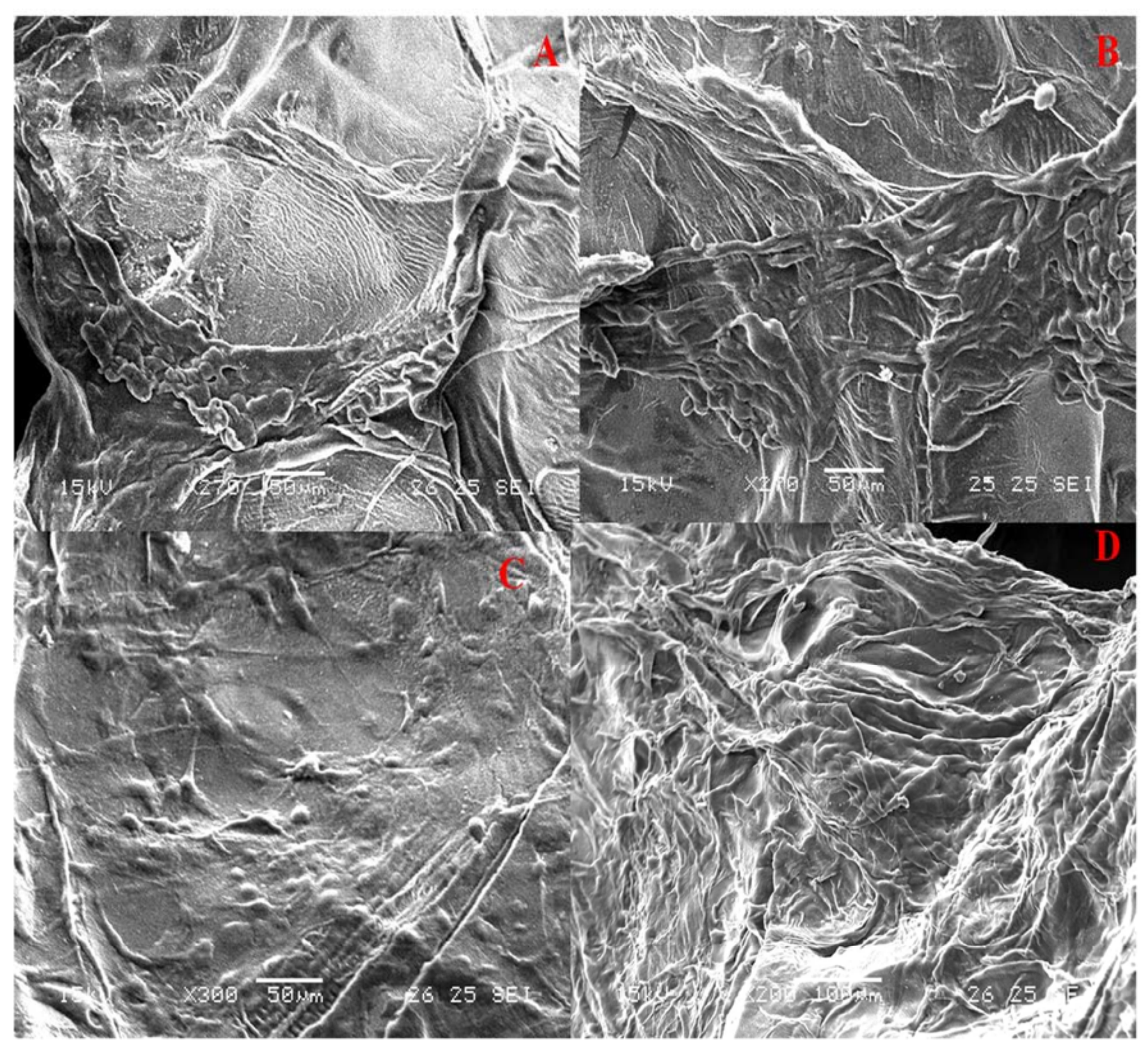


Figure 71: Scanning electron Micrograph of Fibroblasts grown on Drug Loaded Chitosan-alginate membrane on different days after seeding (A-D1,B- D2,C- D3, D- close examination of morphology of fibroblast)

7.3.8 *Tensile property of the membrane*

The tensile strength of the skin is 6-40GPa (Steinstraesser et al., 2010). The drug loaded CA membrane had tensile strength between 1.26 to 6.0 MPa while the non-drug loaded CA membranes had tensile strength ranging from 2.08 to 9.13 MPa. It could be deduced that presence of drugs lowered the maximum loading of membranes as can be seen in Figure 72 ($p<0.05$). It could also been seen that the amount of polymers greatly influence maximum loading that the membranes can endure before breaking.

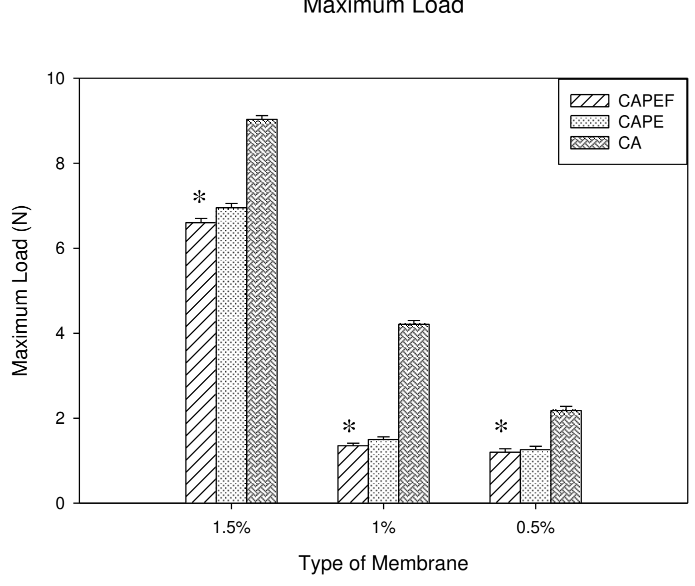


Figure 72: Maximum load of membranes made of different polymer ratios: 1.5%C+1.5%A, 1%C+1%A, 0.5%C+0.5%A CAPE- Ethyl gallate loaded chitosan alginate membrane , CAPEF - ethyl gallate and fusidic acid loaded chitosan- alginate membrane; *p<0.05

7.4 ***Discussion***

The preparation of drug loaded chitosan- alginate polyelectrolyte membrane was described herein. Chitosan and alginate were selected as hydrophobic and hydrophilic polymers and combination of ethyl gallate and fusidic acid was used as antimicrobial drug to be eluted from the membrane. The effects of the membrane on different aspects of wound healing were tested. The chitosan-alginate membrane synthesized in this study was expected to help in all stages of wound healing. The membrane showed that it can maintain the moist environment of the wound by having the swellability rate of 1600% of its original weight. Swelling property described in this study was also found to be polymer dependent, allowing flexibility to customize polymer composition according to the need of the wound. Membranes were observed to be able to allow the gaseous exchange between the wound and the environment preventing anaerobic bacteria proliferation. The membranes were found to be biocompatible in addition to having antibacterial properties. Presence of drugs was not found to compromise the mechanical properties of the membrane. Combinatory drug loaded membranes were found to be able to support the growth of fibroblasts on the membranes which could help in the wound healing unlike the control materials which were the commercial antimicrobial loaded drug eluting bandages.

7.5 *Summary*

This study utilized chitosan and alginate because they are inexpensive polymer materials that are abundant in nature. Fusidic acid and ethyl gallate combination was used in this study as a model drug because the combination of fusidic acid and ethyl gallate were found to be synergistic with the ability to inhibit biofilm formation effectively which commonly occurs in the wounds. The drug combination was also found to be able to inhibit resistance development which was also a threatening factor when wounds are infected with *Staphylococcus aureus*. Moreover, cytotoxic levels of ethyl gallate were also established in Chapter 4. According to the findings of this study, it is safe to conclude that using combinatory drug loaded chitosan- alginate membrane can be a new strategy for wound treatment as the membrane itself can enhance wound healing by improving the wound environment, while the phytochemical-antibiotic combination present in the membrane was found to be able to inhibit bacterial invasion, reduce drug resistance and deter biofilm formation.

8. CONCLUSIONS

8.1 *Phytochemical-antibiotic combinations*

Antimicrobial activity of different classes of antibiotics (protein synthesis inhibitors- mupirocin, fusidic acid, tetracycline, cell wall synthesis inhibitor- vancomycin, cefoxitin), phytochemicals (epicatechin gallate, ethyl gallate, rutin, protocatechuic acid, quercetin) were assessed by microbroth dilution method using 6 different strains of *Staphylococcus aureus*. Antimicrobial susceptibility testing showed that synthetic antibiotics have much lower minimum inhibitory concentrations while phytochemicals have much higher minimum inhibitory concentrations. This finding indicates that phytochemicals have weaker antimicrobial activities as individual agents. Minimum inhibitory concentration assessment was followed by checkerboard method to investigate phytochemical-antibiotic combination interactions for determination of fractional inhibitory concentration index by calculating FIC of each combination. It was found that ethyl gallate showed synergism with all the protein synthesis inhibitors (tetracycline, fusidic acid mupirocin) while it was indifferent with cell wall synthesis inhibitor (vancomycin). However, it was synergistic to cefoxitin which was also a cell wall synthesis inhibitor. The findings from checkerboard analysis were further supported by the time-dependent killing studies carried out with synergistic combinations. It was found that the synergistic combinations were able to express $2\log_{10}$ CFU/ ml reduction of bacteria at any time during 24 hour of treatment when it was compared with individual agents. It was also found that combination of phytochemical and antibiotics exert significantly more bactericidal activity than antibiotics acting alone ($p<0.05$). Therefore, combination of phytochemical and

antibiotics were regarded as prime antimicrobial candidates to be used in treatment of wound infections.

8.2 *Effect of ethyl gallate on bacterial cells and mammalian cells*

Mechanisms of actions of gallates were explored after establishing the fact that they have different interaction with the different group of antibiotics. Extensive literature review indicates that gallate compounds may have multiple targets in bacteria cells. In this study, observing bacterial cells after treatment with sub MIC concentration of antibiotic for 4 hours by scanning electron microscopy and atomic force microscopy indicated that ethyl gallate acts on cell wall. Further on, binding activity of ethyl gallate to lipoteichoic acid synthase enzyme by computational modeling pointed out that, ethyl gallate may target lipoteichoic acid synthase inhibiting the polymerization of lipoteichoic acid which is an important cell wall component. Even though ethyl gallate was found to kill the bacterial cells at a concentration of 1024 μg/ml, it was found to be non-cytotoxic to the human peripheral blood mononuclear cells at that bactericidal concentration as supported by the cytotoxicity test which showed the recovery of metabolic activity of mononuclear cells reaching to ~85% viability on 3rd day after the treatment with ethyl gallate. The synergistic concentration with antibiotics which is 256 μg/ml was found to be more biocompatible with mononuclear cells as it was found to regain ~ 100% metabolic activity on 3rd day. The fact that ethyl gallate is non-cytotoxic to human peripheral blood mononuclear cells despite it being bactericidal against *Staphylococcus aureus* provide positive indication that ethyl gallate can be used in the treatment of wound infections plagued by *Staphylococcus aureus*.

8.3 ***Biofilm inhibition potential of gallates***

Biofilm inhibition potential of ethyl gallate and epicatechin gallate as single agents and as combinatory agents with tetracycline, mupirocin, and fusidic acid were assessed in this section. Biofilm inhibition was quantified by two methods, microtiter plate assay and scanning electron microscopy. Microtiter plate assay stained by crystal violet was performed to quantify biofilm mass formed in the presence and absence of antibiotics alone and in combination with phytochemicals. All the *Staphylococcus aureus* strains were found to be biofilm producing strains. Fusidic acid, ethyl gallate and tetracycline were not able to inhibit the biofilm formation at sub-MIC (1/4MIC) concentrations. When phytochemicals and antibiotics were combined, total biofilm formation was seen to reduce by 73-97% when compared with the control strain. Statistical analysis verified that combination of antibiotics and phytochemicals exert better biofilm inhibition activity than antibiotics as single agents ($p<0.05$). Visualization with scanning electron microscopy revealed that biofilms formation was significantly reduced in all of the antibiotic-phytochemical combinations. Scanning electron micrographs of the biofilms formed by the control strains showed full formation of slime layer surrounding the bacterial colonies while the absence of slime layers were noted in the bacteria treated with the combination. Therefore, it could be assumed that exopolysaccharide slime layer formation of biofilm bacteria was inhibited by synergistic combinations. Thus, ethyl gallate as a combinatory agent is reported in this study to inhibit biofilm formation, the consequence of which is one of the significant morbidities manifesting the wounds.

8.4 *Effects of gallates on resistance evolution of Staphylococcus aureus*

In chapter 6, effect of gallates on resistance evolution of *Staphylococcus aureus* was explored for 3 reasons. First, *Staphylococcus aureus* is notorious for prompt resistance development for most of the antimicrobials used against them. Second, *Staphylococcus aureus* bacteria are notorious bacteria which usually infect the wounds causing biofilms which are main contributors of antibiotic resistance. Third, antimicrobials that are considered for use in the topical treatment of wounds, especially fusidic acid, are prone to develop resistance due to its genetic instability. This susceptibility to resistance is hindering the widespread use of fusidic acid despite its specificity to *Staphylococcus aureus* bacteria and high sensitivity. To assess the development of mutant evolution in the *Staphylococcus aureus* to the antibiotics under study, resistance strains were developed in vivo for mupirocin, tetracycline, fusidic acid and ethyl gallate. When the amount of time it took for the individual drugs to develop resistance and the amount of time it took for the combinations to develop resistance were compared, it was noted that the addition of the ethyl gallate significantly increased the time taken to develop resistance. In other word, it could be suggested that ethyl gallate can hinder the mutation of bacteria to resist antibiotics. After the resistance strains were developed, the mutant selection window of the tested strain for the antibiotics of interest (tetracycline, mupirocin, fusidic acid) as well as the phytochemical of interest (ethyl gallate) were investigated, it was found that the mutant selection window of synthetic antimicrobials (tetracycline, mupirocin, fusidic acid) were much wider than the mutant selection window of the ethyl gallate. As a result, it can be suggested that the concentration range at which the antibiotics were enriched to develop resistance was wider. It concurred with the results that were found that antibiotics

took shorter time to develop resistance. On the other hand, it was found that the mutant selection window of ethyl gallate was narrower thus reducing the range of concentration at which the bacteria strain was enriched for resistance. The result also agreed with the longer time ethyl gallate took to develop resistance in comparison with the antibiotics. Finally, when the antibiotics were combined with ethyl gallate, it indicated that the minimum inhibitory concentration actually coincided with the mutant prevention concentration thus, closing the mutant selection window by eliminating the concentration range for resistance development. As resistance development is very important in the usage of antibiotics as topical agents, it could be assumed that the usage of ethyl gallate would help hinder the resistance development associated with the usage of topical antibiotic agents.

8.5 *Development of chitosan- alginate membrane*

As a novel strategy to provide optimum treatment to the wounds, biocompatible and bioactive natural polymeric drug eluting membrane composed of chitosan and alginate was proposed which was expected to promote the wound healing while inhibiting bacterial growth and biofilm formation. Chitosan- alginate membrane was developed based on the polyelectrolyte property of polycationic compound chitosan and polyanionic compound alginate, to take advantage of their propensity to form coacervates when mixed together. These coacervates were used to act as carriers of the combination of fusidic acid and ethyl gallate while a polyelectrolyte membrane was cast by immersion in 1% calcium chloride solution. Polyelectrolyte membranes were composed with different polymeric ratios (0.5% C+0.5% A, 1%C+1%A, 1.5%C+1.5%A) to study the effect of polymeric

content on the physical properties of the membrane. The resultant washed, dried membranes were tested for physical, chemical and biological activities. From the gross appearance, the membrane was a white, semi-transparent, flexible, and thin membrane. Tensile testing subjected to the membrane using 5kN pre-loading with Instron Tensile tester reported presence of antimicrobials could have effects on the tensile properties of the membrane. The drug loaded CA membrane had tensile strength between 1.26 to 6.0 MPa while the non-drug loaded CA membranes had tensile strength ranging from 2.08 to 9.13 MPa. It was also found that physical properties of drug loaded chitosan alginate membrane were able to support physiological process of wound healing while the presence of calcium ions inhibit the bleeding by initiating a intrinsic clotting mechanism. Water vapor transmission test indicated that the membrane could allow water vapor exchange by about ~900g/m^2/d to >1200 g/m^2/day which was comparable to the property of commercial skin substitutes which allows ~2047g/m^2/day of water vapor to exchange with immediate environment of the wound. Polyelectrolyte membranes were found to absorb water up to 1600% of their original weight. CA membranes were cross-linked to each other (28% to 43%) which contributed to water insolubility and swelling properties of the membranes. Higher polymer content resulted in more amount of crosslinking and also more amount of swelling. The fact that the polymeric membrane were found to swell up to 1600% of their original weight indicated that those membranes can effectively perform the exudate management while at the same time contributing to the painless removal of bandage which eliminates the process of further damaging the wound at the removal, a problem generally encountered with ordinary wound dressing materials. Disk diffusion testing of the membranes indicated that they were capable of eluting

antimicrobials present in the membranes to kill bacterial cells. HPLC of the membrane eluents which determined the presence of antimicrobials in the eluents indicated that the 99% of the drugs present in the membrane was fully eluted by 4th hour of immersion in the PBS solution. Cytotoxicity assays indicated that the chitosan alginate membrane designed in this study produced less cytotoxic effects than the commercial agent by retaining 80% metabolic activity of fibroblasts by 4th day of the treatment. Moreover, scanning electron microscopy of the fibroblasts adhering to the membrane showed that the fibroblasts maintain their normal morphology and adherent properties with the exposure to the membrane.

9. FUTURE RECOMMENDATION

9.1 *Phytochemicals as antimicrobial agents*

This study sheds light on antimicrobial activity of phytochemicals and their ability to potentiate the antimicrobial effect of synthetic antibiotics. While the study was able to single out the antimicrobial effect of gallate compounds, there are many phytochemical agents that have the potential to be explored as antimicrobial agents. Minimum inhibitory concentration of phytochemicals in this study was found to be much higher than the synthetic counterparts explored in this study. Even though the compounds under study have proven to be non-cytotoxic at the concentration at which they kill the bacteria, the ideal scenario would be to modify the structure of phytochemical compounds so that they can exert their antimicrobial effect at lower concentrations. In this study, cytotoxicity testing in this study was performed on the system comprised of culture medium (pH about 7.4, at 37ºC for 24 hours). Limitations of this system present itself in the possibility that phytochemicals in this study could be metabolized by the cells, or oxidized and inactivated by the culture conditions in the first 12-24 hours so that they cannot exert cytotoxic effect to comparatively slow growing cells such as eukaryotes as opposed to fast growing cells such as bacteria. Therefore, it would be advantageous to perform a lymphocyte proliferation assay to observe the effect of ethyl gallate on the lymphocytes stimulated with Phytoheamagglutinin (PHA) by measuring [3H] thymidine incorporation into DNA. This control experiment will be expected to show light on prospective differential effects of ethyl gallate on fast growing (prokaryotic bacteria) and slow growing (eukaryotic cells) systems.

9.2 *Molecular study of mutant strains*

In this study, mutant strains to mupirocin, fusidic acid and tetracyclines were gradually induced to the previously susceptible *Staphylococcus aureus* ATCC strain. They were then treated with combination of antibiotics and phytochemicals to see whether presence of ethyl gallate revert the resistance. After indications that presence of ethyl gallate can restore the susceptibility of antibiotics, it would be interesting to know that how the susceptibility can be restored in the organisms. Depending on the common resistance mechanism of antibiotics under study, molecular studies of resistant gene behavior in the presence of phytochemical can be expected to provide useful insights which will be imperative for drug development. Moreover, studies with the efflux pump activities by comparing the expression of efflux pumps in the presence and absence of phytochemicals can indicate possible efflux pump inhibition studies of phytochemicals.

9.3 *Chitosan- alginate coacervates for drug delivery*

Considering the biocompatible nature of chitosan and alginate, the coacervates can be employed as nanostructured carriers that allow the delivery of drug combinations in various modes such as topically, orally and systemically. As the complex coacervate formation occurs at the interface between chitosan and alginate solution, microcapsules composed of liquid alginate core is formed with the subsequent treatment of calcium chloride. Because of the cationic nature of chitosan and anionic nature of alginate, it can be speculated that the release of drugs from the microcapsules can be pH dependent but can be adjusted by varying the concentration of chitosan and alginate. Moreover, influence of the polymer content has been found to be important in rate of release of drugs

from the membrane, by applying the same principle, drug loaded nanoparticles can be produced and tested for the rate of release using different ratio of combination of the polymers.

9.4 *In vivo studies of drug eluting chitosan-alginate membrane*

The present study reports that polyelectrolyte membrane synthesized here is biocompatible and non -cytotoxic. *In vivo* studies will give further insights into the activity of drug eluents in the physiological environment involving the interaction with living cells and active body immune system. Fibroblast cells isolated from rat skin will be used to assess the cytotoxicity of the polyelectrolyte membrane. It will be followed by the induction of full- thickness transcutaneous dermal wounds to rat models. Macroscopic analysis as well as the histological analysis can be performed to evaluate the applicability of polyelectrolyte membranes for dermal burns. Studying *in vivo* efficacy of bioactive natural polymeric membrane will be expected to enhance the strategy for wound treatment and bring this novel treatment strategy one step closer to clinical application.

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