Dynamic drug combination methods for suppressing bacterial antibiotics resistance adaptation

Bhone, Myint Kyaw

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Dynamic Drug Combination Methods For Suppressing Bacterial Antibiotics Resistance Adaptation

Bhone Myint Kyaw

School of Mechanical and Aerospace Engineering

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Abstract

Staphylococcus aureus (*S. aureus*) is a common pathogen that can cause high mortality and morbidity. Every year, many people die from *S. aureus* related infections. Methicillin resistant *S. aureus* (MRSA) strain is typically responsible for severe and complicated infections in human beings. For this reason it is also considered as one of the “Superbug”. It is resistant to many drugs and the glycopeptide vancomycin is the only available antibiotic that is active against MRSA infections. However, recent reports indicate that vancomycin intermediate-resistant MRSA strains are emerging worldwide. Therefore, there is a concern that MRSA would gradually become fully-resistant to vancomycin in the near future and newer treatment strategies or compounds that can effectively cure *S. aureus* related infections should be well underway before our last antibiotic runs out.

Plants have been naturally used since the ancient times as medicine and still many plant compounds can be found in the treatment of major ailments such as malaria and cancer. Due to multi-drug resistance problems in clinical settings and the need for newer compounds, there is great interest in phytochemical research because of their hypothesized and proven antimicrobial activities. Combinations of synergistic phytochemicals and antibiotics are assumed to be a new option to solve the drug resistance problem. Their combination can increase the efficacy and the killing rate and the chance of resistance development can be delayed. In this study, the effects of using conventional antibiotics in combination with phytochemicals were studied by using checker board methods and time-kill assays using 12 *S. aureus* strains. Studies suggested that tannic acid was synergistic with fusidic acid, minocycline, cefotaxime and rifampicin and additive with ofloxacin and vancomycin. Quercetin showed synergism with minocycline, fusidic acid and rifampicin.

In a particular treatment option, the stability of the compounds (drugs or phytochemicals) is important as it selects resistance development in bacteria. The stability and the effectiveness of conventional antibiotics or phytochemicals were studied by inducing single-step adaptive resistant development in MRSA. The duration it took to get phenotypic mutant strains for a particular drug/phytochemical

concentration was compared individually or in combination. It was found that conventional antibiotics under study were less stable than phytochemicals and combination of synergistic phytochemicals together with antibiotics could delay resistance formation in MRSA. The effectiveness of using fixed sub-Minimal Inhibitor Concentration (MIC) of phytochemicals in combination with synergistic step-wise increasing concentration of antibiotics were observed in this study. It was found that giving sub-MIC concentrations of tannic acid, quercetin and gallic acid ethyl ester together with fusidic or rifampicin could overcome or delay the occurrence of adaptive resistance in MRSA strains.

Combination therapies using two or more synergistic antibiotics have been used before and their effectiveness in resistance prevention is still questionable because of the stability and the dosing pattern of the compounds used. In this study, with the consideration of the effectiveness of phytochemicals in combination with synergistic antibiotics in resistance prevention or delaying effects on partner synergistic antibiotic, a new noble dynamic drugs dosing combination hypothesis was introduced and tested with 4 MRSA strains for a duration of 10 days and 30 days to understand short-term and long-term effects. Giving more than 20% (0.2 MIC) of tannic acid or quercetin together with synergistic fusidic acid was found to prevent adaptive or genetic mutations in MRSA strains.

Phytochemicals are assumed to have multi-targeted actions. Many authors proposed possible mechanisms of action of phytochemicals and much research is done on individual proposed mechanisms. Possible mechanisms of action of synergistic phytochemicals under study were investigated by using the Ethidium Bromide Accumulation Assay. Tannic acid, quercetin and gallic acid ethyl ester exhibited efflux pump inhibiting activities against four MRSA strains. The effects of phytochemicals on MRSA mutant strains revealed that phytochemicals under study have the ability to lower severe mutant strains into less severe mutant strains by bringing down the MICs. This work opens up the research into the novel strategies but using currently available drugs and synergistic phytochemicals instead of depending on new drugs. This is particularly important because developing new drugs for the prevention of resistance adaptation of multi-drug resistant bacteria like MRSA is extremely time consuming, expensive and comes with a high failure rate.

List of Symbols Abbreviation

MRSA = Methicillin resistant *Staphylococcal aureus*

MSSA = Methicillin sensitive *Staphylococcal aureus*

S. aureus = *Staphylococcus aureus*

MIC = Minimal Inhibitory Concentration

PAP = Porpulation analysis profile

EtBr = Ethidium Bromide

DNA = Deoxyribose nucleic acid

RNA = Ribose nucleic acid

PCR = Polymerase chain reaction

SEM = Scanning Electron Microscopy

CFU = Colony forming units

FA = Fusidic acid

GA = Gallic acid ethyl ester

Rifam = Rifampicin

Mino = Minocycline

Cefo = Cefotaxime

Van = Vancomycin

TA = Tannic acid

GA = Gallic acid ethyl ester

Quer = Quercetin

Eug = Eugenol

Mth = Menthol

CA = Caffeic acid

AUC = Area under curve

ICU = Intensive care unit

PVL- Panton Valentine Leukocidin

EF-G = Elongation factor-G

t-RNA = transfer RNA

m-RNA = messenger RNA

PBPs = Penicillin binding proteins

NAM = N-acetylmuramic acid

NAG = N-acetylglucosamine

ATCC = American type cell culture

BSAC = The British Society for Antimicrobial Chemotherapy

DI = deionized (DI)

CLSI = Clinical Laboratory Standard Institute

IS = Iso-Sensitest

BaCl₂ = Barium Chloride

H₂SO₄ = Sulphuric acid

FIC = Fractional Inhibitory Concentration (FIC)

FICI = Fractional inhibitory concentration index

SYN = Synergism

ANT = Antagonism

MPC = Mutant Prevention Concentration

DDC = dynamic drug combination

DDCR = Dynamic drug concentration responses

RFF = The relative final fluorescence

PK/PD = Pharmacokinetics and pharmacodynamics

LD = Lethal dose

CDs = Codons

OD₆₀₀ = Optical density at 600 nm

PBS = Phosphate-buffered saline

RFF = The relative final fluorescence

EPI = Efflux pump inhibitor

MDR = Multi-drug resistance

HIV = Human Immunodeficiency Virus

List of Publications

1. **Bhone Myint Kyaw**, Arora, Shuchi, Win, Khaing New Win, Chu Sing Lim. *Prevention of Emergence of Fusidic acid and Rifampicin Resistance in Staphylococcus aureus using Phytochemicals* , African Journal of Microbiology Research 5 (22) , pp. 3684-3692.
2. **Bhone Myint Kyaw**, Arora, Shuchi, Chu Sing Lim. *Bactericidal antibiotic-phytochemical combinations against methicillin resistant Staphylococcus aureus*, Brazilian Journal of Microbiology. 2012, pp.938-945
3. **Bhone Myint Kyaw**, Ravi Champakalakshmi, Sakharkar, Meena K, Chu Sing Lim, Kishore R. Sakharkar. Biodegradation of Low Density Polythene (LDPE) by *Pseudomonas* Species. Indian Journal of Microbiology 52 (3), pp. 411-419
4. **Bhone Myint Kyaw**, Arora, Shuchi, Chu Sing Lim. *Anti-pseudomonal and anti-biofilm activity of tannic acid combinations with Antibiotics*, International Journal of Integrated Biology, 11 (3), p. 110-116.
5. **Bhone Myint Kyaw**, Arora Shu Chi, Wim Mar Soe, Jayaraman Premkumar., Chu Sing Lim. “*Dynamic Drug Combination Response on Pathogenic Mutations of Staphylococcus aureus*”. Proceedings of 2011 International Conference on Biomedical and Engineering and Technology (ICBET 2011), Volume 11, p-169-173, June 19, KL, Malaysia (Conference Paper).
6. **Bhone Myint Kyaw**, Chu Sing Lim, Zhou Wei. “Tannic acid as Phytochemical Potentiator for Antibiotic Resistance Adaptation”. Proceedings of 2013 International Conference on Biomedical and Engineering and Technology (ICBET 2013), *Article in Press*, May 19-20, Copenhagen, Denmark (Conference Paper).

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Chapter 1: Introduction

1.1 Background

Nowadays, *Staphylococcus aureus* (*S. aureus*) is a major pathogen in our community, particularly harmful to burn and hospitalized patients. They are also major causes of nosocomial (hospital-acquired) infections. Every year, nearly 2 million people suffer from nosocomial infections and many have died of nosocomial infections caused by *S. aureus* infections, especially methicillin-resistant *S. aureus* (MRSA) infection. The patients with open wounds, invasive devices, burns and mal-immune systems are high risk persons and most burn patients die not because of the disease itself but because of MRSA related infections. They are resistant to most antibiotics such as penicillins (methicillin, dicloxacillin, nafcillin and oxacillin), vancomycin and cephalosporins. Multi-drug resistance is defined by having resistance to 3 or more drugs [1]. Thus, it is necessary to have a clear understanding of why those strains become multi-drug resistant, how resistance evolves and how to overcome them.

Bacterial pathogens may intrinsically be resistant to more than one class of antimicrobial agent, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other organisms (horizontal evolution) [2]. Acquisition of new genetic material by antimicrobial-susceptible bacteria from resistant strains of bacteria may occur through conjugation, transformation, or transduction. In the case of vertical mutation, acquired resistance occurs due to chromosomal mutations. Moreover, massive use of antibiotics or empirical treatment creates selective adaptation, causing the emergence of resistance strains [3].

There are many mechanisms of resistance that bacteria can counterattack against invading antibiotics. Scientifically, accepted mechanisms of mutations are (i) intrinsic nature of resistance to one or more drug, (ii) antibacterial efflux pumps, (iii) altered site of action or targets, (iv) decreased drug uptake, and (v) enzymatic modification or inactivation [4].

Resistance of pathogenic bacteria to current antibiotics has become a major public problem [5] and critical attention is needed for those kinds of bacteria. Discovery of penicillin as a therapeutic agent in 1942 won the battle between humans and micro-organisms and marked a historical milestone [6]. However, inappropriate use of antibiotics by doctors or incompletion to take the full complete dose by the patients has created an evolution of resistant strains and new strategies to combat these are paramount [7].

Agricultural use of antibiotics also contributes a critical concern in antibiotic resistance nowadays [8]. Antibiotics have long been routinely used not only for the treatment of infections, but also as a means of getting animals to market faster by growth promotion [9]. Moreover, they are being used in the prevention or treatment of diseases in livestock and poultry [10]. This produces environmental exposures in a variety of ways, which select for resistant microbes and microbial genes. Most of the antibiotics such as penicillins, sulfonamides, bacitracin, erythromycin and lincomycin are being used in agriculture and nowadays these antibiotics are facing resistance problems in clinical settings [11].

Currently, there are only a limited number of antibiotics to fight against multi-drug resistant bacteria and most of the new drug discovery programs are also under investigation. Glycopeptide vancomycin is the only available antibiotic that is effective for MRSA infections. However, vancomycin-intermediate resistant strains have emerged worldwide recently and there is a worry that MRSA will become fully resistant to vancomycin and there will be no drug to treat MRSA infections. Therefore, it is necessary to consider a new alternative compound or methods/strategies to treat MRSA infections.

Many reports suggest that the use of drug combinations against multi-drug resistant bacterial pathogens has better efficacy compared to monotherapy [6, 12]. The use of western antibiotics, however, has encountered adaptive resistance over time, even in combinations [13]. This further limits the use of antibiotics in combinations, especially to overcome concerns of resistance. Identifying methods and strategies to prevent or delay the development of resistance in MRSA has, therefore, become the cornerstone of antimicrobial drug research against resistant strains of *S. aureus*.

Alternative compounds and secondary metabolites derived from plants or insects offer a rich source as antimicrobial agents [14].

Plants are a rich source of useful secondary metabolites that forms the plant defence mechanism against pathogenic invaders [14, 15]. These include tannins, flavonoids, alkaloids, terpenoids and polyphenols. They have effective antibacterial properties against both Gram positive and Gram negative bacteria [14, 16-18]. Since phytochemicals have higher minimum inhibitory concentrations (MIC) (100-5000 $\mu\text{g/ml}$) than antibiotics (0.031-512 $\mu\text{g/ml}$), they oftentimes cannot be used in monotherapy as sole agents. On the other hand, phytochemicals are known to modulate or modify resistance mechanisms in bacteria [17, 19]. Therefore, their potential use in combinations with antibiotics can help to potentiate the activity of the western drugs, resulting in increased efficacy.

By using combination therapies of antibiotics and phytochemicals, the occurrence of adaptive mutation and the chances of rapid evolution of mutation will likely to be reduced. Therefore, the effects of the synergistic phytochemicals in combination with conventional antibiotics against the emergence of adaptive resistance in MRSA strains were observed in this study. The dynamic dosing combination hypothesis was introduced and tested with different MRSA strains. The possible mechanism of drug combination was tested and proposed.

1.2 Objectives

With the above considerations, the project objectives are as follows:

- To investigate the efficacy of phytochemicals against multi-drug resistance bacteria such as *S. aureus* and to determine the possible drug interactions between the western antibiotics and eastern compounds (phytochemicals)
- To understand the adaptive mutation pattern in multi-drug resistant pathogens and the effects of using antibiotic-phytochemical combinations on it
- To study the novel hypothesis to introduce “dynamic dosing combinational strategies” on known antibiotic-resistance adapting pathogens
- To observe the type of mutation at the molecular level to know its pattern and predict its impact (type of mutation) and to understand the mechanism of action of phytochemicals

1.3 Scope

This project aimed to study the role of phytochemicals in combination with western drugs to treat effectively against multi-drug resistant bacteria like methicillin resistant *S. aureus* (MRSA). The aim was to understand the role of using antibiotic-phytochemical combinations in the prevention of adaptive mutation in MRSA. Additionally, it was also tried to understand a novel dynamic drug-phytochemical combination response hypothesis on pathogenic mutation of MRSA strains and the possible mechanism of actions of phytochemicals used in this study.

Chapter 2 : Literature Review

2.1 *Staphylococcus aureus*

2.1.1 Morphology

Staphylococcus aureus (*S. aureus*) is a golden yellow colored sphere on macroscopic examination and is also known as ‘golden cluster seed’, ‘seed gold’ or ‘golden staph’. On microscopic examination, it is a Gram positive facultative anaerobic coccus that appears in short chains, in pairs, or grape-like clusters. It is completely a spherical cell approximately 1 micrometer in diameter [20].

Figure 2.1 *Staphylococcus aureus* (A) gram stain under compound microscope, (B) under Scanning Electron Microscope, (C) and (D) colonies grown on the Manitol-Salt Agar and Iso-Sensitest Agar [21-23]. (Figures were adopted with permission)

2.1.2 Growth Characteristics

S. aureus is a mesophilic bacteria and optimum growth temperature of 35 °C. However, it can also grow at temperatures between 6.5 °C and 46 °C. The optimum pH for growth is between pH 7 to 7.5 and it can maintain growth between pH 4.2 to 9.3. It can remain viable for several months on agar plates kept at 4 °C. It can withstand heat at 60 °C for about 30 min [24, 25].

2.1.3 Incidence

S. aureus is one of the most common causes of life-threatening bacterial infections. Every year in the United States, about 400,000 hospitalized patients are infected by it. Approximately 100,000 of these patients die from complications due to related infections. More than 8,000 people die every month in the United State due to *S. aureus* infection [26]. *S. aureus* is now global problem and poses significant challenges to the health care community as it evolves rapidly to mutant ones, becoming also resistant to most of the antibiotics currently in use.

Within several decades, methicillin resistant *S. aureus* (MRSA) infections have become rampant [27]. The Center for Disease Control (USA) has been monitoring MRSA cases inside the United State for more than 30 years and their records show that MRSA infections involve 2% of all staphylococcal infections in 1974. That number rose to 22 % by 1995 and 65% by 2008. Today, this figure continues to rise with a limited medical arsenal to combat them. A study conducted in 2007 by the American Medical Association showed that 46 out of every 1000 hospitalized patients in the United States have a MRSA infection. Of these, approximately 32 patients per 100,000 have become serious cases [28].

MRSA infections have now become a global problem. According to the report, Canada, Germany, Greece and Spain reported almost no MRSA infections in 2003. In Greece, 48.6 % of all *Staphylococcal* infections are now due to MRSA, whilst the respective figures for Germany and Spain are 27.2 % and 23.5 %. These figures continue to be on the rise to date. United Kingdom reported about the incidence of

MRSA infections today, tripling between 1997 and 2004. The United Kingdom reported that the incidence of MRSA infections tripled between 1997 and 2004 [29].

2.1.4 Genomic Content

There are eleven genome projects. Among them, 9 projects are already finished and 2 are still underway. The genome is about 2.8 mega base pair long with 2600 open reading frames, comprising 84.5% of the genome. It has 33% of G+C content, 25K base pairs in length with 28.7% G+C content of single plasmid. Phages, plasmids, pathogenic island and *Staphylococcus* cassette chromosome encode for virulence factors, and transposon encoded for increased resistance for antibiotics, where a transposon inserted into a conjugative plasmid also encodes resistance for disinfectants. MRSA is resistant to the methicillin antibiotic and expressed a modified penicillin-binding protein encoded by the *mecA* gene [30].

2.1.5 Methicillin Resistant *Staphylococcus aureus* (MRSA)

MRSA strains are totally or partially resistant to methicillin, oxacilline and penicillin groups because of beta-lactamase production. Typically, MRSA strains are the most rapidly evolving strains and easily adapt resistance to most of the antibiotics currently in use and are a major cause of treatment failure nowadays. In our community, it is supposed that one in three individuals is colonized or a carrier of *S. aureus* and one in ten carries MRSA. For most of the conditions, *S. aureus* is nontoxic and lives peacefully with the body [31]. However, when the barrier breaks down such as with abrasion and cuts, the bacteria enters into the body and can cause serious infections. MRSA secretes various toxins which destroy the host cells causing damage to body or organs. In the blood stream, toxins can attack the white blood cells, break apart red blood cells and cause blood clots, as well as attack the top layer of the skin and thus harm the whole body [26, 32-34].

Figure 2.2 Electron micrograph of *S. aureus* secreting powerful toxins that destroyed host cells in a variety of ways, thus deserving the name “superbug” (Adopted with the permission from Lucent Books [35])

2.1.6 *S. aureus* Capsules

There is a great interest in *S. aureus* capsules as capsular polysaccharides play a role in its antibiotic resistant mechanisms. As the capsule is a strong protective layer, most of the antibiotics find difficulty in penetrating inside the bacteria. The capsule is used to enhance the virulence of *S. aureus* by impeding the phagocytosis mechanism of the host [36, 37]. Therefore, researchers have emphasized on the biochemical structure of *S. aureus* capsules to understand details about its constituents. The biochemical structure of serotypes 1, 2, 5, and 8 capsular polysaccharides have been determined and all serotypes contain hexoaminouronic acids. Trisaccharide repeating units of serotype 5 and 8 are very similar and they are commonly found in clinical isolates of *S. aureus*. They share the same sugar composition and differ only in the linkages between the amino sugars and the position of O-acetylation [38].

2.1.7 Role of *S. aureus* Capsules in Virulence

The virulence and pathogenicity of *S. aureus* depends on types of *S. aureus* capsules. Serotype 5 is the most virulent type compared to others. The mucoid type capsules have more phagocytic virulence factors that mask the C3b (cytokines) particles on the bacterial cell wall, thus preventing recognition of bacterial cells by receptors on the

phagocystic cells. The masking effect of the capsule is very critical in the prevention of an attack by phagocystic cells [38].

2.1.8 Clinical Consequences

S. aureus normally inhabits the surface of skin and on the anterior nares (nose). When the barrier breaks down, infection occurs as the organism can access through the damaged skin or mucus membrane. *S. aureus* can infect the skin, the respiratory, digestive, urinary, and reproductive tracts, and can cause serious infections of heart and bones [39].

Figure 2.3 Common sites of infection and diseases that *S. aureus* causes (Adapted with the permission from Freeman-Cook, 2006 [26])

2.1.9 High Risk Factors for MRSA Infection

There are a number of risk factors that have greatly impacted on the chances of increasing the rate of MRSA infections. The most common risk factors are listed as follows:

- (1) Aged over 65 years
- (2) Immunocompromised person (HIV, patient with chemotherapy, etc.)
- (3) Chronic debilitating patients (Diabetes, Cancer, etc.)
- (4) Frequent or previous hospitalizations
- (5) Prolonged hospital stay
- (6) Patient with invasive medical devices (e.g. Catheters, drainage tubes, etc.)
- (7) Open wounds –traumatic or operative wounds
- (8) Burn patients
- (9) Hospitalization in area where MRSA is endemic
- (10) In-patients in surgical and neonatal ICUs
- (11) Severe underlying conditions or illness [40-44].

As it is often a nosocomial infection, common risk factors are related with exposure to hospital settings. Uncommon risk factors are associated with prolonged or repeated antibiotic therapy for a particular illness, contact with animals (dogs and cats) and minor abrasions [45, 46].

2.1.10 Pathogenesis

S. aureus secretes diverse toxins and causes damage to the host. *S. aureus* toxins can be classified into three main groups and each toxin has its specific role. Enterotoxins (pyogenic or pus forming superantigens) are single chain (25-30kD) proteins which can cause massive proliferation of T cells with subsequent production and release of cytokines leading to acute systemic disease called toxic shock syndrome. These enterotoxins can also cause food poisoning [47, 48]. Exfoliative toxins have protease activities causing the cleavage and lysis of intracellular attachment in stratum granulosum of the epidermal skin leading to very severe disease, especially in infants and young children, characterized by the peeling of skin called staphylococcal scalded skin syndrome [49, 50].

Other groups of toxins act on the cell membrane and are also called cytolytic toxins, membrane damaging toxins or hemolysins. Alpha-toxin is a major hemolysin expressed by human strains of *S. aureus*. This toxin causes hemolysis and

disseminated intravascular coagulation. Beta-toxin hemolysin is expressed by animal strains of *S. aureus* and only by less than 20% of human isolates. This toxin has sphingomyelinase C enzyme activities and causes hydrolysis of cell membrane phospholipids [51]. Delta-toxin is expressed by most human strains of *S. aureus* and they have detergent like actions. However, these toxins are 10,000 to 100,000 times less toxic than the alpha toxin [26].

Staphylococcal leukocidins (Luk) are leukolytic toxins forming cation-selective transmembrane pores in leukocytes and macrophages. Lukocidines are water soluble and consists of two main subunits called S class (LukE, LukS, LukS-PV, LukS-R) and F class (LukD, LukF, LukF-R, LukF-PV, LukM) [52, 53]. Bicomponent toxin-Panton Valentine Leukocidin (LukS-PV) [35] is related to severe pneumonia in children and is secreted by various staphylococcal strains responsible for dermonecrosis [54], skin infections, necrotic pneumonia and septicemia [55]. Bownik *et al.* also reported that LukE/LukD and its LukE subunit significantly reduced respiratory burst activity of phagocytic cells (neutrophils and macrophages) thereby impairing host immune system [56]. The development of resistance to antibiotic in *S. aureus* and development of MRSA strains are associated with the genes encoded for these toxins [6].

2.1.11 Modes of Infection

Person to person contact is the main mode of infection. The skin and mucous membranes are usually potential protective barriers for infection. However, if these protective barriers are broken down (due to damage of skin in trauma, damage of mucosa in some viral infection), the organism invades into the underlying tissues and bloodstream leading to cause infection [57]. Airborne transmission is rare in MRSA. *S. aureus* colonizes most commonly on the anterior nares and can also colonize along the respiratory tract, urinary tracts and on open wounds, IV catheters, etc [58]. Nosocomial infection is achieved via contact with common patient beds, utilities and common floor. Athletes can transmit via skin contact with each other and can also become infected through minor abrasions or damaged skin [27]. Most of the time, *S. aureus* infection shows no symptoms until the organism gets entrance into the body or the person's immune status becomes compromised.

2.2 Plants as Antimicrobial Agents

Use of plant products as sources of antimicrobial agents has been inherited for about 60,000 years [14]. With the advent of the antibiotics in 1942, the use of plant products as antimicrobials has declined. However, in the late 1990s, the usage of plant antimicrobials as supplementary and alternative medicines has been growing exponentially again because of the increased incidence of antibiotic resistance and adverse drug reactions [59]. In addition, more discoveries in the biochemistry and biotechnology arenas have created great opportunities to advance the insight into the areas of antimicrobial plant products [60, 61]. Traditionally, plants provide a potential source of hope for novel drug compounds [62].

The plants herbal mixtures make large contributions to human health care and well-being not only in developing countries, but also in developed countries as well. Because of their popularity as remedies for many infectious diseases and as important components in the health care system, there are frequent searches for the substance with antimicrobial activity in plants [63].

Plants are rich in various kinds of useful secondary metabolites that serve as plant defense mechanism against insects, microorganism and herbivores. Tannins, alkaloids, flavonoids and terpenoids plant groups have been found in vitro to have antimicrobial activities [64]. Several useful antimicrobial plant products and their chemical structure are summarized in Table 2.1 and Figure 2.4 [14].

Table 2.1 Major classes of antimicrobials compounds from plants [14]

Class	Subclass	Examples	Mechanism
Phenolics	Simple phenols	Catechol,	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavonoids Flavone	Chrysin	Bind to adhesins Complex with cell wall
		Abyssinone	Inactivate enzymes Inhibit HIV reverse transcriptase
	Flavonols	Totarol	?
	Tannins	Ellagitannin	Bind to proteins: -bind to adhesins -enzyme inhibition -substrate deprivation -complex with cell wall -membrane disruption -metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or DNA
Lectins and polypeptides		Mannose-specific agglutinin Fabatin	Block viral fusion or adsorption From disulphide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	?

Figure 2.4 Structures of common antimicrobials chemicals [14]

Table 2.2 Phytochemicals used in this study

Name	Chemical Structure	Major Compound	Source of compound
Tannic acid		Polyphenol	Tera pods, gallnuts, tree barks [65]
Quercetin		Flavonoids	Fruits, vegetables and grains
Gallic acid ethyl ester		Phenolics	Grapes, gallnuts, tea leaves, oak
Eugenol		Essential Oils	Clove oil
Menthone		Essential Oils	<i>Pennyroyal</i> , <i>Peppermint</i> and <i>Pelargonium</i> essential oils
Caffeic acid		Terpenoids, Polyphenols	Most of the plants, stem, barks and leaves

2.2.1 Tannic Acid

Tannic acid is the specific commercial form of tannins, water soluble polyphenols which have anticancer effects [65], antioxidant [66], antifungal and antibacterial activities [14]. Tannic acid is present in many foods including tea, cocoa beans, grapes, strawberry and persimmon [67]. The antibacterial properties of tannic acid much depend on the hydroxyl groups of polyphenols and phenolic groups. Tannic acid is also active against food borne pathogens, such as *Bacteroids fragilis*, *Clostridium perfringens*, *Escherichia coli* and *Enterobacter cloacae* [67]. It is also effective against fish borne pathogens like *Cytophaga columnaris*, *Bacillus columnaris*, *Chondrococcus columnaris*, *Flexibacter columnaris* and *Flavobacterium columnare* [68]. Moreover, it has been found to have antibacterial action against multidrug resistant bacteria such as *S. aureus* [69].

Mechanism of Action

There are proposed mechanisms of action of tannic acid [14] even though detailed mechanisms of how it works are still unknown. Antibacterial effects of tannic acid can be achieved by enzyme inhibition [14, 70, 71], binding to specific proteins [14, 72], binding to adhesions, complexing with cell walls, substrate deprivation, membrane deprivation and metal ion complexation [14].

2.2.2 Gallic Acid Ethyl Ester

Gallic acid ethyl ester (3, 4, 5-Trihydroxybenzoic acid) is a natural water soluble phenolic acid present in grapes, gallnuts, tea leaves, oak bark and many other medicinal and edible plants. It is a colorless crystalline organic acid having both hydroxyl groups and a carboxylic acid group in the same molecule so that it can form an ester by a reaction with each other. Gallic acid esters have potent antioxidant, antiangiogenic and anticarcinogenic activities in vitro [73-75]. It also has antifungal and antiviral activities [76].

Mechanism of Action

The effect of gallic acid ethyl ester is thought to be achieved by acting on bacterial cell wall [14].

2.2.3 Quercetin

Quercetin is a flavonoid widely present in fruits, vegetables and grains. Found especially in apples and the outermost layers of onions where the highest concentration of quercetin is contained. It has anticancer effects, antibacterial effects, free radical scavenging effects, antiviral effects including against HIV viral infections [77]. Antibacterial effects of quercetin mainly depend on its concentration. At higher concentrations, quercetin shows antibacterial activity against *Bacillus subtilis*, *Micrococcus luteus*, *S. aureus*, *S. epidermidis*, *Aspergillus flavus* and *Aspergillus parasiticus*.

Mechanism of Action

Quercetin competitively binds to the bacterial DNA gyrase [78]. Therefore, it is contraindicated to be used with fluoroquinolones where it competes at the binding site.

2.2.4 Eugenol

Eugenol is the principal chemical component of clove oil from *Eugenia aromaticia*, well known for its analgesic, local anesthetic, anti-inflammatory, cardiovascular and antibacterial effects [14, 79, 80]. It belongs to the class of essential oil that is approved by the Food and Drug Administration for use for in humans. Eugenol is active against *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni*, *Samonella enterica*, *S. aureus*, *Lactobacillus sakei* and *Helicobacter pylori* [79].

Mechanism of Action

Antibacterial action of eugenol is achieved mainly by disrupting the cytoplasmic membrane of the cell wall [81].

2.2.5 Menthone

Menthone is a naturally occurring organic compound with the molecular formula of C₁₀H₁₈O. I-menthone is the most available form of menthone. It has anti-fungal effects against *Fusarium verticilloides* and *Fusarium proliferatum* [82]. Menthone can potentiate the influx of tamoxifen into cancer cell thus by acting as a penetrating enhancer [83].

2.2.6 Caffeic Acid

Caffeic acid is a hydroxycinnamic acid, which is a naturally occurring compound consisting of acrylic and phenolic functional groups. It has antioxidant, anti-carcinogenic and antibacterial effects [84]. Moreover, it is known to have antimitogenic, anti-inflammatory and immunomodulatory properties as well [85]. Caffeic acid is a strong and selective matrix metalloproteinase-9 and transcription inhibitor and has been reported for its action to inhibit liver cancer cell metastasis [86].

2.3 Western Drugs

Table 2.3 Antibiotics and their Mechanism of Actions

Function	Antibiotic	Class
Inhibition of bacterial cell wall synthesis	Penicillin	Bactericidal
	Cephalosporin	Bactericidal
	Glycopeptides	Bactericidal
	Carbapenems	Bactericidal
	Monobactams	Bactericidal [87, 88]
Inhibition of bacterial protein synthesis	Aminoglycoside	Bactericidal
	Tetracycline	Bacteriostatic
	Chloramphenicol	Bacteriostatic
	Clindamycin	Bacteriostatic
	Streptogramins	Bacteriostatic
	Fusidic acid	Bacteriostatic
	Macrolides	Bacteriostatic/ Bactericidal [89-91]
Inhibition of nucleic acid synthesis	Fluoroquinolones	Bactericidal [92]
	Rifampicin	Bactericidal [93]
Inhibition of folic acid synthesis	Sulphonamides	Bacteriostatic
	Trimethoprim	Bacteriostatic
	Pyrimethamine	Bacteriostatic [94, 95]

Table 2.4 Antibiotics used in this study

Name	Chemical Structure	Class	Mechanism of actions
Fusidic acid		Isolated from the fungus <i>Fusidium coccineum</i>	Bacterial protein synthesis inhibitor [89]
Minocycline		A tetracycline analog	Bacterial protein synthesis inhibitor [96]
Cefotaxime		Cephalosporin	Bacterial cell wall synthesis [97]
Ofloxacin		Quinolones	Blocking bacterial DNA synthesis [98]

Table 2.4 Antibiotics used in this study (continued)

Rifampicin		Semi-synthetic antibiotic produced from <i>Streptomyces mediterrane</i>	Inhibits DNA dependent RNA polymerase [99, 100]
Vancomycin		Glycopeptide antibiotic	Inhibition of cell wall synthesis [101]

2.3.1 Fusidic Acid

Fusidic acid is a bacteriostatic steroid antibiotic that is produced from the fungus *Fusidium coccineum*. It is a bacterial protein synthesis inhibitor and effective mainly against gram positive bacteria such as *Staphylococcus*, *Corynebacterium* and *Clostridium* species. It is clinically important for the treatment of MRSA but it should be used in combination with other antibiotics because of its low genetic barrier to resistance. Fusidic acid is not useful for other gram negative bacteria, but is active against *Neisseria*, *Legionella*, *Moraxella* and *Bacteroides* [102].

Mechanism of Action

Fusidic acid works by interfering with protein synthesis by inhibiting the elongation factor G (EF-G) [89]. Fusidic acid resistance occurs when it is used alone, so it should be used in combination with other antimicrobial agents. The mechanism of resistance to fusidic acid was studied in *S. aureus* and it was mainly due to the occurrence of a

single step chromosomal mutation in the *fus A* gene that encodes for the elongation factor G (EF-G) [103]. This point mutation results in the alteration of EF-G making fusidic acid no longer able to bind to the EF-G. The less common mechanism is associated with plasmid mediated decreased cell wall and membrane permeability [104].

2.3.2 Minocycline

Minocycline is the most lipid soluble and most active tetracycline antibiotic. It acts like doxycycline which is also a long-acting tetracycline. Minocycline's effects are related to the inhibition of protein synthesis. Although minocycline's broader spectrum of activity, compared to other members of the group, includes activity against *Neisseria meningitidis*, its use in prophylaxis is no longer recommended because of side effects (dizziness and vertigo). Current research is being carried out to examine the possible neuroprotective effects of minocycline against the progression of Huntington's disease, an inherited neurodegenerative disorder. The neuroprotective action of minocycline may include its inhibitory effect on 5-lipoxygenase, an inflammatory enzyme associated with brain aging [105].

Mechanism of Action

Minocycline passes directly through the lipid bilayer or passively diffuses through porin channels in the bacterial membrane. Minocycline binds to the 30S ribosomal subunit, preventing the binding of tRNA to the mRNA-ribosome complex and subsequently interfering with protein synthesis [106].

2.3.3 Cefotaxime

Cefotaxime is a third-generation cephalosporin antibiotic. It has broad spectrum activity against both Gram positive and Gram negative bacteria.

Mechanism of action

The bactericidal activity of cefotaxime results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs). Cefotaxime shows high affinity for penicillin-binding proteins in the cell wall including PBP 1b and PBP III [107].

2.3.4 Rifampicin

Rifampicin is an inhibitor of the essential bacterial enzyme DNA-dependent RNA polymerase and has been used for the treatment of mycobacterial and other bacterial infections and colonization. RNA polymerase appears to be the sole target of rifampicin action. Core RNA polymerase is composed of three subunits, β , β' , and α . Core polymerase combines with one of several σ subunits to enable specific binding to promoters and initiators of transcription. Rifampicin forms a 1:1 complex with RNA polymerase and blocks the initiation of transcription [108].

Resistance to rifampicin occurs by mutations in the *rpoB* gene that encode amino acid changes in the β subunit of *E. coli* RNA polymerase. These mutations are clustered in three highly conserved regions or *rpoB* in the mid-portion of the gene. These regions appear to be involved in the polymerase anti-termination process. Resistance to rifampicin has been associated with mutations in similar regions of the *rpoB* genes of *S. aureus* [30]. Therefore, rifampicin resistance in *S. aureus* is mainly due to mutation in *rpoB* genes.

2.3.5 Ofloxacin

Ofloxacin is a synthetic fluoroquinolone (fluoroquinolones) antibacterial agent that inhibits the super coiling activity of bacterial DNA gyrase and topoisomerase II and IV [109]. Ofloxacin is bactericidal and its mode of action depends on the blocking of bacterial DNA replication by binding to DNA gyrase, which allows the untwisting required for DNA replication. Ofloxacin has 100 times higher affinity for bacterial

DNA gyrase than for mammalian. Ofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria [110] .

Mechanism of Resistance

There are three proposed mechanism of resistance for fluoroquinolone: (i) mutations in the *norA* gene, which encodes a membrane-associated active efflux pump; (ii) mutations in *gyrA* and *gyrB* genes, which encode DNA gyrase; and (iii) mutations in the *griA* and *griB* genes, which encodes DNA topoisomerase IV [109].

2.3.6 Vancomycin

Vancomycin is a glycopeptides-derived antibiotic produced by fermentation by the Actinobacteria species *Amycolatopsis orientalis*.

Vancomycin is active against methicillin resistant (beta-lactam-resistant) *S. aureus* and also is the only available drug that is still effective against MRSA infections. Moreover, it is also effective against most of the strains such as *Listeria monocytogenes*, *Streptococcus pyogenes*, penicillin-resistant *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Actinomyces* species and *Lactobacillus* species [110].

The combination of vancomycin and an aminoglycoside has synergistic action in vitro against many strains of *S. aureus*, *Streptococcus bovis*, enterococci, and the viridans group streptococci [111].

Mechanism of Resistance

There are three major types of resistance in vancomycin, depending on the Van A, B and C phenotype. Van A resistant gene confer the commonest one and Van B and Van C are less significant [110].

Mechanism of Action

The bactericidal action of vancomycin results primarily from the inhibition of cell-wall biosynthesis. Specifically, vancomycin prevents the incorporation of N-acetylmuramic acid (NAM) - and N-acetylglucosamine (NAG)-peptide subunits from being incorporated into the peptidoglycan matrix; which forms the major structural component of Gram-positive cell walls [112].

2.4 Drug Interactions

2.4.1 Drug Synergism

Drug synergism is a combination of drugs to make a effect greater than the effect resulting from the separate individual drugs. It is the most basic reason that physicians use combination therapies to treat multi-drug resistant infections and to provide optimum therapeutic effects on the patients. Generally, there are four main mechanisms for drug synergism [113, 114]:

- Serial synergism, i.e. sequentially inhibiting the common metabolic pathway (e.g Trimethoprim & Sulfamethoxazole in the folate pathway)
- Inhibition of bacterial protective enzymes (e.g. Beta-lactamase enzyme) or efflux mechanism
- Use of cell wall active agents (e.g. combination of Mecillinam and Ampicillin in Amidinocillin)
- Enhancing the uptake of other antimicrobials by using cell wall active agents (e.g Penicillin plus Streptomycin)

Figure 2.5 Illustration of synergistic action between the two drugs [115]

2.4.2 Drug Antagonism

Drug antagonism is where the effect resulting from the combination of the drugs is less than the effect resulting from the separate individual drugs. It has a significant impact on therapeutic failures in the polymicrobial strategies.

Figure 2.6 Illustration of the effects of drug-drug antagonism [115]

2.4.3 Drug Indifference

Drug indifference is the mechanism in which there is no obvious synergistic or antagonistic action between the two drugs. In other words, the effect resulting from the combination of the drugs is not significantly different from the effect resulting from the separate individual drugs.

Figure 2.7 Indifferent effects between the two drugs [115]

2.5 Drug Resistance

Drug resistance means that a formerly effective drug dose is no longer effective in curing a disease or improving a patient's symptoms. It is related to the abilities of microorganism to withstand the antibiotic effects. Resistance arises mainly via natural

selection. Microorganisms have a significant ability to evolve and adapt (about 1 in 10^{-8} microorganisms in a colony has one or more mutations that make it resistant to the drug) [116]. If the microorganism is resistant to more than one drug, it is termed as multidrug resistance. There are four main mechanisms for evolution of antibiotic resistance in pathological bacteria [117-121]. They are namely:

1. Enzymatic inactivation or modification
2. Decreased drug uptake
3. Altered target site and
4. Bypass pathways

Figure 2.8 Mechanisms of antibacterial resistance and spread. Resistance mechanism (left) and mechanisms of spread of antibacterial resistance (right) (Adapted with the permission from Levy and Marshall, 2004 [122])

(1) Enzymatic Inactivation or Modification

Microorganisms have the ability to induce the genes that encode for the production of new enzymes to degrade the drug. These genes are often acquired on plasmids and other mobile genetic materials. The resistance to β -lactam antibiotics by Gram-negative and positive bacteria are attributed by production of the enzymes called β -lactamase. These enzymes catalyze the hydrolysis of the amide bond of β -lactam rings. Aminoglycoside resistance in Gram-negative bacteria is mediated by the enzymatic modification of the antibiotics molecules [123].

(2) Decreased Drug Uptake

This is another important mechanism in which the resistant bacteria protect the antibiotic action by inhibiting the entry of drug into the cell or by pumping the drug out of the cell by expression of efflux pump proteins [124]. Usually, the efflux pumps of resistance strains are non-drug specific proteins and have the ability to recognize and pump out a wide range of structurally and chemically unrelated compounds in an energy dependent manner. Genes for these efflux pump proteins are found in many bacteria as either chromosomally encoded or plasmid encoded. In Gram-negative bacteria, there is outer membrane in addition to cell wall layer which act as permeability barriers and these barriers are responsible for the decreased entry of drug into the cell [125].

(3) Altered Target Site

Alterations and chemical modifications in the drug target lead to the reduced affinity of the drug to its binding site and subsequent activities of the drug [126]. These target alteration mechanism can be the major cause of resistance to single or multiple drugs because different drugs can act on the same target. Vancomycin resistance in *Staphylococcus* and *Streptococcus* species is mediated by the creation of the altered target peptidoglycan which has a 1000-fold lower affinity for vancomycin. Similarly, enterococci develops vancomycin resistance by replacing the terminal alanine with a lactate, which has a greatly reduced affinity for vancomycin [127, 128].

(4) Bypass Pathways

This is a resistance mechanism for the drug in which it blocks metabolite production by enzyme inhibition. Bacteria could bypass the effect of the drug by inducing a new pathway for the production of the same metabolite. This mechanism is a variant of the altered target mechanism of resistance. In methicillin resistant *S. aureus*, there is a production of alternative penicillin binding protein (PBP 2a) in addition to normal penicillin binding proteins. The alternative one is not inhibited by the antibiotics, such as flucloxacillin, and the cell can continue to synthesize the peptidoglycan and bypass the effects of the drug [129, 130].

2.6 Drug Combination Therapy

Within a few decades, antibiotic resistance problems have become a major issue to the public. Drug resistant strains are emerging worldwide and they are resistant to most of the antibiotics currently available. Rapid increases in the drug resistance rate and the development of new antibiotics are unbalanced clinically. Therefore, a new approach to treat multi-drug resistant bacteria is considered as an alternative option. Combination therapies of using two or more antibiotics have achieved good therapeutic outcomes [6, 131, 132].

The discovery of β -lactam antibiotics in the 1940s saved millions of lives in the early years. However, inappropriate use of β -lactam antibiotics by medical staff and in compliance by the patients resulted the emergence of drug resistance. Nowadays, penicillin alone is not effective for the treatment of multi-drug resistant bacteria [133]. Drug combination therapies of using synergistic drugs are also an alternative option and proven better efficacies clinically. β -lactam antibiotics used together with a β -lactamase inhibitor, clavulanate (Brand name: Augmentin, marked by GlaxoSmithKline) has shown better therapeutic outcomes and has been widely used in the market. Another synergistic drug used on the market is the combination of the trimethoprim and sulfamethoxazole, marked as Bactrim[®] (Roche) and Septra[®] (King Pharmaceuticals). Both compounds are folic acid biosynthesis inhibitors and each of them inhibits a specific step in the nucleotide biosynthesis pathway [134, 135].

Combination therapy can be roughly classified by four principle modes of action by which compounds can enhance the activity of each other (Figure 2. 21).

- (1) A second compound (an adjuvant) prevents the degradation or modification of the primary drug (an antibiotic).
- (2) A second compound (an adjuvant) allows the accumulation and retention of the primary drug (an antibiotic) by inhibiting efflux pumps.
- (3) A second compound (an adjuvant) inhibits the intrinsic repair pathway or tolerance mechanism of cells to the primary drug (an antibiotic).
- (4) A second compound is itself an antibiotic that targets a similar or different pathway that is inhibited by the first antibiotic drug [6].

Figure 2.9 Scheme of combination therapy. The antibiotics are shown in blue and purple. The red arrows indicate the cellular processes that result in reduced or loss of antibiotic activity. These processes include: (i) degradation or modification of the drug; (ii) the repair or intrinsic resistance pathway; (iii) the efflux pumps; and (iv) is the combination of the two antibiotics (Adopted with the permission from Cottarel and Jamey, 2007 [6]).

In clinical settings, drug combinations are preferred to decrease the emergence of resistant strains, to expand the antimicrobial spectrum, to minimize the dose related toxicity and to minimize drug resistance. Collarel and Jamey reported that using drug combinations has the potential to slow down the emergence of resistance strains in addition to their synergistic effect [6]. The underlying concept is that the two drugs might act on multiple targets to slow down resistance formation. However, in spite of using two or more synergistic drugs, resistant strains are still emerging and new methods or compounds are necessary in clinical settings [136-138].

Chapter 3 : Experimental

3.1 Microorganisms and Media

Twelve *Staphylococcus aureus* strains were used in this study. MRSA ATCC 43300 and methicillin-sensitive *S. aureus* (MSSA) ATCC 29213 were purchased from ATCC and ten MRSA clinical strains were collected from National University Hospital. The microorganisms were grown at 37 °C in iso-sensitest (IS) broth (Oxoid Ltd., Basingstoke, Hampshire, England) with aeration.

All antibiotics used were purchased from Sigma-Aldrich (Singapore) and gallic acid ethyl ester (assay value 99%) was purchased from Acros Organics (Singapore). Tannic acid (assay value 98%), quercetin (assay value 98%), eugenol (assay value 99%), menthone (assay value 90%) and caffeic acid (assay value $\geq 98\%$) were purchased from Sigma-Aldrich (Singapore).

Stock solutions for antibiotics and phytochemicals were freshly prepared and used. Controls for solvents were also tested for antimicrobial action. Fusidic acid (assay value 98%), minocycline (assay value 98%), cefotaxime (assay value 96%), rifampicin (assay value 97%), ofloxacin (assay value 98%) and vancomycin (assay value 90%) were used in this study.

3.2 Material Preparation

3.2.1 Preparation of Growth Media

For the nutrient media to grow bacteria, both liquid and solid were used to carry out experiments with *S. aureus*. Dehydrated powdered mixture in the form of IS broth and agar were purchased from Oxid, Biomedica. The components and product details of the media powders are given in Appendix D. This media is recommended by the British Society for Antimicrobial Chemotherapy (BSAC) for antibiotic susceptibility testing on *Staphylococcus* species.

Antibiotic-free broth and agar: For IS broth preparation, 23.4 g of IS broth powder was dissolved in 1 litre of DI water with vigorous shaking followed by autoclaving at 121 °C for 20 min. The sterilized broth was kept in a water bath at 37 °C with the lid closed tightly, until its temperature was cooled down to room temperature. The cooled media was dispensed aseptically into clean test tubes in the biological safety cabinet Class II (Gelman, BH Class II series) and stored at 4 °C for not more than two weeks.

IS Agar media was prepared by dissolving 31.4 g of IS agar powder in 1 litre of deionized (DI) water with vigorous shaking followed by autoclaving at 121 °C for 20 min. The media was then allowed to cool down to a temperature of 50 °C and poured into 94 mm diameter petri-dishes up to a height of 2-3 mm (or 20 ml liquid agar in each plate). The plates were allowed to dry in the biosafety cabinet for 15 min and stored at 4 °C for not more than three weeks. Before use, the plates were allowed to open dry in the biosafety cabinet for 15 min or until the condensation over the plates disappeared.

Antibiotic containing agar: Antibiotic containing agar plates were made by dissolving the IS agar powder in DI water (31.4 g/L). After autoclaving and cooling to a temperature approximately 45-50 °C or cool enough to be hand held for 10 sec, the antibiotic solution was added in the desired concentration prepared using the antibiotic stock (explained in the next sections). The bottle was then gently swirled and inverted for 5-10 sec and immediately poured into 94 mm petri-dishes previously named and dated. The plates were allowed to dry in the biosafety cabinet for 15 min, sealed and stored at 4 °C for not more than three weeks. The whole process was carried out aseptically in the biosafety cabinet.

3.2.2 Bacterial Strains and Preparation of Stocks

Reference bacterial strains were used in this study to eliminate the need for characterization of the strains. MRSA ATCC 43300 and MSSA 29213 were reused for the experiments. Both strains are recommended by Clinical Laboratory Standard Institute (CLSI) for quality control. Another 10 clinical *S. aureus* strains collected from National University Hospital (Singapore) were used for experimental procedures. The bacteria were kept suspended in separate vials containing Iso-

Sensitest (IS) broth containing 40 % glycerol (v/v) and stored at -80 °C. The bacterial stocks were prepared on IS agar plates. A sterile wire loop was swirled in the vials maintained at -80 °C and streaked across a fresh IS agar plate followed by incubation at 37 °C for 24 hr. Subsequently, the plates with MSSA, MRSA and clinical stocks were sealed and stored at 4 °C for up to four weeks and refreshed from time to time.

3.2.3 Preparation of Antibiotic Stock Solution

Antibiotic stock solution was usually prepared with the final concentration of 10,000 µg/ml or 10 times the concentration of the highest concentration being tested. The stock solution was then diluted into a working solution of appropriate concentration. Most of the antimicrobial agents were dissolved in sterile water. Sterile distilled water was thus used for serial dilution for testing. However, phytochemicals tested were not dissolved in sterile distilled water. In those cases, the minimal amount of recommended solvent was needed to dissolve these agents in a drop wise manner and they were further diluted the desired concentration with sterile distilled water. In this study, gallic acid ethyl ester was dissolved in ethanol as solvent and then further diluted into desired concentrations with sterile distilled water.

In preparation of antibiotic stock solution, it is essential to ensure the potency of the antimicrobial agents because the antibiotic powder obtained from the manufacturers is not always 100 % pure. The potency of the antimicrobial agent can be determined by using the following formula:

$$\text{Potency} = \text{Purity} \times \text{Active Fraction} \times (1 - \text{Water Content}) \quad (3.1)$$

The amount of antimicrobial agents required for the preparation of antibiotic stock solution can be determined by following formula:

$$\text{Weight (mg)} = \frac{[\text{Volume (ml)} \times \text{Desired concentration} (\frac{\mu\text{g}}{\text{ml}})]}{\text{Potency} (\frac{\mu\text{g}}{\text{mg}})} \quad (3.2)$$

The working solution was then prepared by diluting the stock solution using the following formula:

$$M_1 V_1 = M_2 V_2 \quad (3.3)$$

where M_1 = initial concentration of stock solution;

V_1 = amount needed from the stock solution to be diluted in working solution;

M_2 = desired concentration of working solution; and

V_2 = final diluent amount

The stock solutions were prepared in small vials and then stored at -20 °C. In this study; the antimicrobial stock solutions were prepared according to Table 3.1.

Table 3.1 List of solvents and diluents used

Drugs	Solvents	Diluents
Fusidic acid	Sterile Distilled Water	Sterile Distilled Water
Minocycline	0.1M NaOH/Sterile Distilled Water	Sterile Distilled Water
Cefotaxime	0.1M NaOH	Sterile Distilled Water
Rifampicin	0.1M NaOH/Sterile Distilled Water	Sterile Distilled Water
Ofloxacin	0.1M NaOH	Sterile Distilled Water
Vancomycin	0.1M NaOH	Sterile Distilled Water
Tannic acid	Ethanol (Molecular Grade II)	Sterile Distilled Water
Gallic acid ethyl ester	Ethanol (Molecular Grade II)	Sterile Distilled Water
Quercetin	Ethanol (Molecular Grade II)	Sterile Distilled Water
Eugenol	Ethanol (Molecular Grade II)	Sterile Distilled Water
Menthone	Ethanol (Molecular Grade II)	Sterile Distilled Water
Caffeic acid	Ethanol (Molecular Grade II)	Sterile Distilled Water

3.2.4 Preparation of Bacterial Inoculum

For *S. aureus* species, both BSAC and CLSI recommend the direct colony suspension method to prepare the inoculum. Therefore, the direct colony suspension method was used in this study.

Bacterial inoculum or working bacterial culture was prepared from the stock solution stored at -80 °C fridge at the start of experiment to maintain the purity of the strain. Detailed procedures are described as follows:

- (1) 3 to 5 colonies of microorganisms were isolated from the overnight cultured bacteria isolate by applying a sterile loop and transferring it to 5 ml of sterile IS broth.
- (2) The IS broth was incubated in 37 °C for 1.5 to 2 hr until the turbidity of the broth was the same as 0.5 McFarland standard (1.5×10^8 CFU/ml). McFarland standard is the particulate suspension of barium sulphate and was prepared by adding 0.5 ml of 0.048 M BaCl₂ (1.17 % w/v BaCl₂·H₂O) to 99.5 ml of 0.18 M H₂SO₄ (1% v/v) followed by constant stirring.
- (3) The suspension was further diluted with IS broth to make 1:100 dilutions (1.5×10^6 CFU/ml).

3.3 Experimental Procedures

3.3.1 Procedures of Testing Minimal Inhibitory Concentration (MIC)

MIC determination was carried out according to the method described by Andrews [139]. Here, that MIC(s) of antibiotics are gold standards in all types of susceptibility tests and are even used to confirm unusual resistance where other methods of susceptibility fail to provide a conclusive answer. The MIC is defined as the minimum concentration of an antimicrobial agent required to inhibit visible growth of a test organism after 18-24 hr of incubation at 37 °C. It can be determined by both agar dilution and broth dilution methods.

In the current study, the MICs were determined by broth micro-dilution method [140]. The method was carried out on sterile 96-well plates (Greiner Bio One, Singapore). The antimicrobial working solutions of desired concentrations were prepared from the stocks as explained earlier. In one of the rows of the titer plate, a dilution range of the antimicrobial agent of interest was prepared. For western antibiotics, the range was from 0.0125 to 256 µg/mL, in 2-fold dilutions, and for plant extracts (phytochemicals), this range was from 8 to 8192 µg/mL, also in 2-fold dilutions. Subsequently, the bacterial inoculum size of 10^6 CFU/ml was used to determine MIC. The tests were performed in triplicate rows for each organism and antimicrobial agent for consistency of results. Briefly, 50 µL of IS broth was dispensed in each well of a row (12 wells), followed by 50 µL of antibiotic working solution in the first two wells of the row. From the second well onwards, 50 µL of solution in the well was

diluted into the next well and so on, so that each successive well had half the concentration compared to the previous well and a dilution range was achieved. 50 μ L of prepared inoculum was then added to each well. The same process was repeated for the subsequent rows and triplicate tests. The plate was covered with the lip and incubated at 37 °C for 18-24 hr. Control wells were also prepared in wells of empty rows.

A sterility control with 10 μ L IS broth and a growth control with drug-free bacterial inoculum was prepared. Control for solvents (NaOH and ethanol) used in the preparation of antimicrobial solution were also tested for antimicrobial effects. The results were read as the MIC value at the concentration in which no visible growth was observed as seen against light or measured by spectrometer plate reader (OD < 0.05 at 600 nm, MicroQuant Plate Reader, Biotek). The MIC of *S. aureus* 29213 was used as the control.

3.3.2 Checkerboard Assay

The checker board titration assay is one of the most frequently used techniques to assess drug interactions. Testing was performed by utilizing 96 well micro-titer plate which is the same as the one used in MIC determination. MICs were determined for each drug by broth micro-dilution according to standards of the Clinical Laboratory Standardization Institute (CLSI) [15, 141]. Each combination assay was tested three times for statistical averaging. Fractional Inhibitory Concentration (FIC) index is used to interpret drug interactions. Details about FIC indices and interpretations are as follows:

FIC index of ≤ 0.5 = Synergism

FIC index of > 0.5 and ≤ 1 = Additive effect

FIC index of > 1 and ≤ 2 = Indifference effect

FIC index of > 4 = Antagonism effect [15, 141, 142].

The FIC indices for all combinations were calculated using the formula below:

The FIC indices for a drug in a given well was derived by dividing the drug concentration in the given well by the control MIC of the test organism to that drug.

$$FIC_A = \text{MIC of Drug A in combination} / \text{MIC of drug A alone} \quad (3.4)$$

$$FIC_B = \text{MIC of Drug B in combination} / \text{MIC of drug B alone} \quad (3.5)$$

The FIC index for a well was the sum of the FICs for each of the drugs present in the well:

$$FIC_{index} = FIC_A + FIC_B \quad (3.6)$$

Table 3.2 Example of broth micro-dilution checkerboard panel

	A 0.5	A 1	A 2	A 4	A 8	A 16	A 32	A 64	A 128	A 256	A 512
B 2	A0.5 + B2	A1 + B2	A2+ B2	A4+ B2	A8+ B2	A16+ B2	A32+ B2	A64+ B2	A128+ B2	A256+ B2	A512+ B2
B 4	A0.5 + B4	A1 + B4	A2+ B4	A4+ B4	A8+ B4	A16+ B4	A32+ B4	A64+ B4	A128+ B4	A256+ B4	A512+ B4
B 8	A0.5 + B8	A1 + B8	A2+ B8	A4+ B8	A8+ B8	A16+ B8	A32+ B8	A64+ B8	A128+ B8	A256+ B8	A512+ B8
B 16	A0.5 + B16	A1 + B16	A2+ B16	A4+ B16	A8+ B16	A16+ B16	A32+ B16	A64+ B16	A128+ B16	A256+ B16	A512+ B16
B 32	A0.5 + B32	A1 + B32	A2+ B32	A4+ B32	A8+ B32	A16+ B32	A32+ B32	A64+ B32	A128+ B32	A256+ B32	A512+ B32
B 64	0.5 + B64	A1 + B64	A2+ B64	A4+ B64	A8+ B64	A16+ B64	A32+ B64	A64+ B64	A128+ B64	A256+ B64	A512+ B64
B 128	A0.5 + B128	A1 + B128	A2+ B128	A4+ B128	A8+ B128	A16+ B128	A32+ B128	A64+ B128	A128+ B128	A256+ B128	A512+ B128

DRUG A

DRUG B		1/8 MIC	¼ MIC	½ MIC	MIC	2 X MIC
	1/8 MIC	SYN (1/8 MIC A+ 1/8 MIC B)				ANT (2 MIC A+ 1/8 MIC B)
	¼ MIC		SYN (1/4 MIC A+ 1/4 MIC B)			ANT (2 MIC A+ 1/4 MIC B)
	½ MIC			SYN (1/2 MIC A+ 1/2 MIC B)		ANT (2 MIC A+ 1/2 MIC B)
	MIC					ANT (2 MIC A+ MIC B)
	2 X MIC	ANT (1/8 MIC A+ 2 MIC B)	ANT (1/4 MIC A+ 2 MIC B)	ANT (1/2 MIC A+ 2 MIC B)	ANT (MIC A+ 2 MIC B)	

Figure 3.1 Illustration of the checkerboard method. The concentrations in the shaded boxes are tested. Concentrations designated as SYN and ANT are tested for synergism and antagonism, respectively [115]

3.3.3 Time-kill Curves

Bactericidal activity of each antimicrobial agent was determined according to the CLSI guideline protocol. Viable cells were counted by performing serial dilutions and removing aliquots at different time intervals. Antibiotics and phytochemicals were tested at one-quarter the MIC for each isolate and the concentrations were incrementally increased until a maximum concentration of 4×MIC was reached. At zero time, the final inoculum was determined and samples were taken at 0, 4, 8, 12 and 24 hr of incubation at 37 °C. All experiments were performed in triplicate.

Time-kill curves were plotted as log₁₀ CFU/ml over 24 hr. Synergism was defined as a decreased colony count of ≥100 CFU/ml or bactericidal activity was increased ≥ 3 log₁₀ CFU/ml at 24 hr for the combination compared with the count obtained for the most active single agent. Antagonism was defined as an increase in colony count of ≥ 100 CFU/ml or bactericidal activity was decreased ≤ 3 log₁₀ CFU/ml at 24 hr [15, 141, 143].

3.3.4 Population Analysis Profiling (PAP)

The PAP test was performed to determine the heterogeneity or homogeneity of a bacterial population in terms of antibiotic resistance, since MIC value only described the apparent antibiotic resistance. PAP tests also provide insights about homogeneous or heterogeneous reduced susceptibilities of a bacterial isolate. The method was adopted and modified from Wootton *et al.* in which they used to determine heterogeneity of MRSA strains for reduced susceptibility to vancomycin [144].

The antibiotic of interest for which resistance needed to be established required that procedure be modified accordingly. Inoculum of isolates, in which resistance to the antibiotic of interest was to be determined, were prepared having 10³-10⁴ CFU/ml. 10 µl of this inoculum was plated on IS agar plates containing antibiotic having the following concentrations; 0, 0.0625, 0.125, 0.25, 0.5, 1 and 2 times its MIC. The plates were incubated at 37 °C for 48-72 hr. The colonies were counted and plotted as CFU/ml (log₁₀CFU/ml) and relative percentage growth (compared to growth on drug-free agar) against the concentration of the antibiotic. The area under the curve (AUC)

was calculated using Sigma Plot Software (2001) for the graphs of viable counts versus antibiotic concentration for each strain and compared with the control strain, by dividing the AUC of the test strain by AUC of the control strain [144, 145].

The values close to 1 (ratios from 0.9 to 1.2) suggested similarity to the control strain with little or no heterogeneous resistance. Higher ratio values (>1.2) suggested heterogeneous populations and further tests to confirm resistant phenotypes were carried out by selecting resistant mutants on agar plates with higher concentration of the antibiotic ($\geq 2\times$ MIC).

3.3.5 Mutation Frequency and Mutant Prevention Concentration

The mutation frequency is defined as the number of mutant bacteria in a culture. In the experiments with *S. aureus* under selective pressure of antibiotics and antibiotic combinations for extended time periods (discussed in later sections), the mutation frequency was determined and mutants were selected with the antibiotic of interest at $2\times$ and $4\times$ MIC. The frequency was then used to establish if the antibiotic combination was able to modulate the occurrence of mutational events compared to single antibiotic (fusidic acid) in long term drug exposure.

This was also followed by molecular analysis of the mutants. The experimental procedures have been adopted from Drugeon *et al.* and Khan *et al.* [146, 147], and are briefly described in this section. A bacterial suspension of the strain in which the mutation frequency is to be determined was prepared containing 10^8 - 10^9 CFU/ml. 100 μ l of this suspension was plated onto agar medium containing the antibiotic of interest at concentrations equal to $2\times$ and $4\times$ MIC (MIC of the parent strain). The frequency of antibiotic resistant mutants was calculated by dividing the total number of colonies counted after 48 hr of incubation at 37 °C on the drug containing plate by the total number of CFU plated initially.

Mutation prevention concentration (MPC) was determined for the parent strain (*S. aureus* ATCC 43300). It is defined as the concentration (μ g/ml) of an antimicrobial agent which prevents the emergence of resistant mutants in a bacterial population selected at concentrations higher than the MIC of antibacterial agent [148]. A

suspension of parent strain was prepared containing 10^8 - 10^9 CFU/ml. 100 μ l of this suspension was plated in duplicate onto IS agar containing the antibiotic at concentrations equal to 2 $\times$, 4 $\times$, 8 $\times$, 16 $\times$ and 32 $\times$ MIC. The plates were incubated for 72 hr at 37 °C with aeration. The MPC was read as the concentration at which there were no visible colonies observed after 72 hr. The mutation selection window was then determined with minimum concentration equal to the MIC and maximum concentration equal to the MPC. MPC and the mutation selection window are important measurement of antibiotic potency together with MIC that give insights into its ability to restrict the emergence of resistant mutants upon long term treatment with it.

It has been highlighted by Khan *et al.* that for it to be a clinically useful measurement, the MPC must be lower than the maximum concentration of drug in serum (C_{max}) or at the site of infection [147]. When combined with other pharmacokinetic parameters it can provide useful information on the ability of the antimicrobial agent to select resistant mutants *in vivo*. Hence, if the MPC of an antibiotic can be achieved *in vivo* then the development of resistance is less likely and treatment is more likely to be successful.

3.3.6 Resistance Studies

3.3.6.1 Procedure for Study of Development of Drug Resistance in MRSA

MRSA drug resistance was evaluated based on the fact that insufficient drug concentration will give rise to drug resistance [129]. MRSA drug resistance was tested with a single drug alone or in combinations until mutants were grown and the duration to get resistant mutants to be appeared at particular drug concentrations is compared. Details procedure carried out for the MRSA drug resistant is mentioned as follows:

- (1) The working solutions for the antibiotics and bacteria were prepared to get desired concentrations.
- (2) Test tubes were taken for the exposure of bacteria with different concentrations of antibiotics.

- (3) In the 1st test tube, IS broth 9 ml was mixed with 1 ml of prepared bacterial suspension (1:100 dilutions i.e. 1.5×10^6 CFU/ml) as the positive control test tube.
- (4) In the 2nd test tube, IS broth 10 ml alone was added as negative control or sterile control test tube.
- (5) In the 3rd test tube, “x” ml of calculated amount of 1/8 MIC concentration of antibiotic was mixed with calculated amount of IS broth and 1 ml of bacterial suspension.
- (6) Then, these three test tubes were stored in a 37 °C incubator for 24 hr.
- (7) After 24 hr, 0.01 ml was taken from each test tube and was directly sub-cultured on normal IS agar plate. 0.01 ml of antibiotic exposed bacterial solution was also sub-cultured on 1/8 MIC agar plate.
- (8) Then all cultured plates were incubated in a 37 °C incubator for 24 hr.
- (9) In the study, the positive control agar plate should have bacteria colony growth and free of other bacterial species contamination and negative control agar plate also should be free of the organism.
- (10) When the bacteria grew on 1/8 MIC agar plate, 3-5 bacterial colonies of bacteria colonies were taken from this agar plate and prepared into a bacteria suspension into standard suspension of 1:100 dilutions (1.5×10^6 CFU/ml).
- (11) Then three test tubes of positive control, negative control and test tube containing double concentration of antibiotic solution (i.e. 1/4 MIC) were prepared as above and repeat the procedure.
- (12) If there was no bacterial growth on the MIC agar plate, 3-5 bacterial colonies were taken from the normal agar plate on which antibiotic exposed bacteria were grown. Then the procedure was repeated until there was bacterial growth on the MIC agar plate.
- (13) The procedure was repeated until the development of resistant MRSA appeared.

3.3.6.2 Procedures for Analysis of Spontaneous Resistance in MRSA

For analysis of spontaneous resistance, large inocula [ca. 10^9 colony forming units (CFU)] of the test strains were serially diluted and spread on agar plates with increasing concentration of antibiotics. The plates were then incubated for 48 hr at 37 °C before the colonies were enumerated. The population curves were drawn by

calculating and plotting the number of cells against the concentration of the drug. Colonies isolated on antibiotic agar plates were re-tested for MIC values.

For progressive resistance selection, bacteria were exposed in IS Broth with a stepwise two-fold increasing concentration of antibiotics alone for the total of 10 consecutive days, as described previously [13, 149]. Briefly, a series of tubes containing two-fold increasing concentrations of antibiotics were inoculated with 10^7 CFU/ml (final concentration). Following a 24 hr incubation at 37 °C, 0.1ml samples from the tubes containing the highest antibiotic concentration and still showing turbidity were used to inoculate a new series of tubes containing antibiotic dilutions.

To examine the potential of secondary agents that suppress the emergence of antibiotic resistance, experiments were performed as described above in the presence of $0.25 \times \text{MIC}$ of the phytochemicals (quercetin, tannic acid and gallic acid ethyl ester). Sub-inhibitory concentrations were used to allow bacterial growth on the partner drug. The MIC for each antibiotic was determined after each passage. In addition, the MICs for the partner drugs (phytochemicals) were determined in resistant derivatives selected by exposure to antibiotic alone or in combination. Each experiment was performed twice. The stability of resistant derivatives was accessed by serial passage of the organisms on antibiotic-free medium for 5 consecutive days.

3.3.6.3 Procedures for Testing Dynamic Drug Combination Response on MRSA Mutations

Preparation of Inoculum and Incubation Conditions

Bacterial suspensions from the stock solution stored at -80 °C were spread onto IS agar plates and incubated at 37 °C for 24 hr. Inoculums were prepared in IS broth using 3 to 5 well-formed colonies from the 24 hr culture of *S. aureus* to a concentration of 1.5×10^8 CFU (colony forming units)/ml as per 0.5 McFarland standards [139]. It was further diluted into 1:10 dilution to obtain a concentration of 10^7 CFU/ml (final concentration) to use for the experiment. The test tubes containing inoculums and various drug combinations were incubated at 37 °C on a rotator shaker of 100 rpm overnight (18-24 hr). Then, the samples were taken out for CFUs, MICs

and PAPs analysis. Then, the tubes were centrifuged for 3 min at 13,000 rpm by a using high speed refrigerated centrifuge (KUBOTA 6500, Japan). The supernatants were discarded and the IS broth containing drug combinations were added. Then, the tubes were re-incubated for another overnight.

Preparation of Working Solution

The MICs of fusidic acid, tannic acid and quercetin were 0.03125-0.25 $\mu\text{g/ml}$, 256 $\mu\text{g/ml}$ and 512 $\mu\text{g/ml}$ respectively. Fusidic acid was synergistic with both phytochemicals: tannic acid and quercetin. Their MIC values and synergistic activities were reposted previously [150, 151]. In this experiment, we used this combination pair and tested for a dynamic drug combinatory hypothesis. Since a full MIC is regarded as 100% efficacy, therefore, working solutions were prepared as 0.8 MIC (80%), 0.6 MIC (60%), 0.5 MIC (50%), 0.4 MIC (40%) and 0.2 MIC (20%), respectively, depending on MIC values. Fusidic acid was dissolved in sterile distilled water and tannic acid/quercetin was dissolved in ethanol (molecular grade, Sigma, Singapore). Tannic acid/quercetin was firstly dissolved in ethanol and then diluted with sterile distilled water. All stock and working solutions not used were stored at - 20 °C for repeated use up to two weeks. Detailed procedures for preparation of different working concentrations of fusidic acid, tannic acid and quercetin against MRSA ATCC 43300 strain are as follows:

From the Stock solutions of 4 $\mu\text{g/ml}$ (for fusidic acid), 4096 $\mu\text{g/ml}$ (for tannic acid) and 4096 $\mu\text{g/ml}$ (for quercetin) to get final volumes of 10ml:

Table 3.3 Preparation of different concentrations of working solutions

Drug/ Phytochemical	MIC (%)	Amount of Antibiotics from the Stock solution	Amount of H₂O
Fusidic acid	1 MIC (100%)	0.078 μl	9.92 ml
	0.8 MIC (80%)	0.0625 μl	9.938 ml
	0.6 MIC (60%)	0.0469 μl	9.953 ml
	0.5 MIC (50%)	0.0391 μl	9.961 ml
	0.4 MIC (40%)	0.03125 μl	9.969 ml
	0.2 MIC (20%)	0.0156 μl	9.984 ml
Tannic acid	1 MIC (100%)	0.625 μl	9.375 ml

	0.8 MIC (80%)	0.5 μ l	9.5 ml
	0.6 MIC (60%)	0.375 μ l	9.625 ml
	0.5MIC (0.5%)	0.313 μ l	9.969 ml
	0.4 MIC (40%)	0.25 μ l	9.75 ml
	0.2 MIC (20%)	0.125 μ l	9.975 ml
<i>Quercetin</i>	1 MIC (100%)	1.25 ml	8.75 ml
	0.8 MIC (80%)	1 ml	9 ml
	0.6 MIC (60%)	0.75 μ l	9.25 ml
	0.5MIC (0.5%)	0.625 μ l	9.375 ml
	0.4 MIC (40%)	0.5 μ l	9.5 ml
	0.2 MIC (20%)	0.25 μ l	9.75 ml

The drug response experiments were formulated with starting combination of 80-20%, 60-40%, 50-50%, 40-60% and 20-80%, of MIC of phytochemical and antibiotic established earlier for the parent strain. All combinations added up to 100% drug dosage, that quantitatively equate to MIC of the phytochemical or antibiotic that amounted to a bactericidal response in the time-kill assay.

Drug combination methods were designed to cover all possible combinations of antibiotic and phytochemical such as Dynamic *vs* Static, increasing concentration of antibiotic *vs* decreasing concentration of phytochemical (Fa_Increasing or Ascending methods) and decreasing concentration of Antibiotic *vs* increasing concentration of phytochemical (Fa_Decreasing or Descending methods). All drug combination methods were applied against MRSA ATCC 43300 for the first ten days and then continued for 30 days to understand the short-term and long-term effects of drug combination therapies. The efficacies of the combinations methods were later tested by using three MRSA clinical strains collected from National University Hospital, Singapore for the duration of ten days. The most effective methods resulting from DDCR hypothesis were further validated with another synergistic combination of fusidic acid (antibiotic) and quercetin (phytochemical).

Figures 3.2 to 3.4 illustrated the drug combination strategies used in the study, with different starting concentrations. The combination methods are named as accordingly.

Figure 3.2 The Static and Dynamic drug combination strategies used in the study, with different starting concentrations. The combination methods are named accordingly

Figure 3.3 Drug combination methods used in this study. The methods are named as “Descending Methods (Fa_Decreasing)” as fusidic acid concentration used is reducing with time

Figure 3.4 Drug combination methods used in the study. The methods are named as “Ascending Methods (Fa_Increasing)” as fusidic acid concentration used is increasing with time

3.3.6.4 Procedure for Testing the Effects of Phytochemicals on Reverse Mutations in MRSA

Three clinical strains of MRSA (C1, C2 and C3) collected from National University Hospital, Singapore were used in this study. The MICs were also within an acceptable range of susceptibility (≤ 0.5 $\mu\text{g/ml}$). These strains were exposed to sub-MIC concentrations of fusidic acid for a period of 10 continuous days and their MICs were increased up to 32 times and named as accordingly to the drug concentrations that they were being exposed. For every strain, a total of 5 sub-MIC concentrations: 0.8, 0.6, 0.5, 0.4 and 0.2, of fusidic acid were individually used and named accordingly. For instance, the MRSA clinical strain 1 (C1) was being exposed to 0.8 MIC of fusidic acid alone for a period of ten days and it was named as “C1_80”. MRSA ATCC 43300 strain was used as a control strain.

The adapted bacterial strains showing the increase fusidic acid MICs were grown at 37 °C in IS Broth with aeration together with sub-MIC concentration of phytochemicals. IS broth and agar were used throughout the study and were purchased from Oxoid (Singapore).

The effect of phytochemicals on the three MRSA clinical strains which exhibit high MICs for fusidic acid were tested. A sub-MIC concentration (0.3 MIC) of phytochemicals was used to test their effect on reverse mutation as there was no bactericidal effect at this concentration. The strains were incubated with 0.3 MIC of each phytochemical for 10 continuous days in IS nutrient broth. Every day, 0.01ml of bacterial strains was taken out and tested for MICs.

3.3.7 Real-time Ethidium Bromide (EtBr) Accumulation Assay

The activity of drug combination against potential bacteria efflux system was assessed by the semi-automated EtBr method as previously described by Ramalhete *et al* using a Rotor-Gene 3000TM Thermal Cycler with real-time analysis software (Corbett Research, Sydney, Australia) [152]. Briefly, strains were cultured in IS broth until they reached an optical density at 600 nm (OD_{600}) of 0.6 and were then centrifuged at 13,000 rpm for 3 min. Bacterial pellets were re-suspended and were washed twice in

phosphate-buffered saline (PBS) (pH 7.4) containing glucose to yield a final concentration of 0.4%. The OD₆₀₀ was adjusted with PBS-glucose to 0.6, the suspension was mixed by vortex and 50 µL of the suspension was transferred to 0.2 ml tubes, followed by addition of 45 µL of PBS containing 1 µg/ml of EtBr (Sigma-Aldrich, Singapore).

The tubes were placed in a Rotor-Gene 3000 Thermal Cycler and the amount of fluorescence representing accumulated EtBr by the bacterium was monitored on a real-time basis.

From the real-time data using relative fluorescence (RF) over a 30-min period of EtBr accumulation, the slope of the accumulation-time curve was calculated by linear regression analysis (Microsoft Excel). The greater the difference in the slope between the strains treated with the phytochemical and the strain without any compound was indicative of the activity of the compound to inhibit the efflux of EtBr. The relative final fluorescence (RFF) of the last time point (60 min) of the EtBr accumulation assay was also calculated according to the formula:

$$\text{RFF} = \text{RF}_{\text{treated}} - \text{RF}_{\text{untreated}} \quad (3.7)$$

RF_{treated} is the RF at the last time point of the EtBr retention curve of bacterial strains with the phytochemical and RF_{untreated} is the RF at the last time point of the EtBr retention curve of the strain without any compound [152].

3.3.8 Extraction of Genomic DNA from *S. aureus*

It is important to get a pure template for PCR reactions. As the interested gene is located in the genome, it was extracted by using a standard kit from Qiagen Pty Ltd., with a modified protocol for *S. aureus*. Extraction of genomic DNA was carried out by using a commercial Qiagen kit (Cat. No: 69504). Detail procedures carried out are as follows:

(1) *S. aureus* was cultured in IS agar plate for overnight

- (2) From the overnight culture, select 3-5 well-formed colonies and incubate in 5ml of IS broth at 37 °C overnight and use for extraction of genomic DNA
- (3) Harvest bacterial cells in a microcentrifuge tube by centrifuging for 10 min at 7500 rpm and discard supernatant.
- (4) Resuspend bacterial pellet in 180 µl of enzymatic buffer, consisting of 20 mM tris-Cl, pH 8.0, 2 mM sodium EDTA, 1.2% Triton[®] X-100 and lysozyme 20mg/ml.
- (5) Incubate for at least 30 min at 37 °C
- (6) Add 25 µl proteinase K and 200 µl Buffer AL (without ethanol). Mix by vortexing.
- (7) Incubate at 56 °C for 30 min. If required, incubate for 15 min to inactivate pathogens.
- (8) Add 200 µl ethanol (96-100%) to the sample and mix thoroughly by vortexing
- (9) Pipet the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.
- (10) Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube.
- (11) Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 14,000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube.
- (12) Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 8000 rpm to elute required genomic DNA.
- (13) For maximum DNA yield, it is recommended to repeat elution once as described in Step 12.

3.3.9 Polymerase Chain Reactions (PCR)

All the reagents used for PCR reaction are purchased from Fermentus (Thermo Scientific) and Bio-Rad (Singapore). Hi-fidelity polymerase enzyme (Bio-Rad), which has proof reading activity and has 10 times more specific action than normal Taq polymerase enzyme, was used for PCR reactions. Primers as indicated in Table 3.4 were synthesized based on gene bank sequence reference number AJ237696 and used

in this study. PCRs were initiated with 10 min denaturation at 95 °C, followed by 35 cycles of 20 sec at 95 °C, 20 sec at 55 °C (annealing), and 2.5 min at 72 °C (elongation). Final elongation was set at 72 °C for 10 min. PCR products were visualized by agarose gel electrophoresis and purified by using QIAquick® Gel Extraction Kit (Qiagen, Singapore) prior to sequencing.

Table 3.4 Primers used in PCR to amplified *fusA* gene

Gene Amplifications (nucleotide positions)	Primer	Sequence (5' to 3')
<i>FusA</i> (1159 to 1870) ¹	EF-GF1 EF-GR	ATTCTTCCGTGTGTATTCAGG AGAAATTATTTATAGCGATGC
<i>FusA</i> (1729 to 2155) ¹	EF-GF2 EF-GR	AGAAATTATTTATAGCGATGC TGGTCAATACGGTGATGTTCA

¹ Nucleotide positions are indicated according to GenBank sequence number AJ237696

3.3.10 Gel Electrophoresis

Agarose powder from 1st Base Ltd (Singapore) was used for gel casting. Gel Red nucleic acid staining solution (Biotium, Singapore) was used as it is sensitive, safe and environmentally friendly. Pre-casting method was used. Agarose power (0.8%) together with Gel Red staining solution was placed in microwave oven and placed for 2 to 3 min until the colour of the liquid became clear and translucent. Then, the hot solution was poured into gel tray and allowed to become solid. PCR products stained with loading dye are placed into the loading well of the agarose gel and set the voltage at 110V for 45 min to 1 hr. Then the gel was placed under VU light and visualized.

3.3.11 DNA Purification

DNA purification of the samples from gel electrophoresis was carried out by using the QIAquick® Gel Extraction Kit (Qiagen, Singapore) prior to sequencing. It is necessary to obtain purified DNA that is free of agarose, RNA and nuclease because that will affect sequencing results. Detail procedures are as follows:

(1) Excise the DNA fragments from the agarose gel with a clean and sharp scalpel

- (2) Weight the gel slice in a colourless tube. Add three volume of Buffer QG to 1 volume of gel (100mg ~ 100 μ l)
- (3) Incubate at 50 °C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 min during the incubation.
- (4) After the gel slice has completely dissolved, check the colour of the mixture is still yellow (similar to the colour of Buffer QG without dissolving agarose). If the colour of the mixture is orange or violet, add 10 μ l of 3 M Sodium Acetate, pH 5.0 and mixed. The colour of the mixture will turn into yellow.
- (5) Add 1 gel volume of isopropanol and mix.
- (6) Place a QIAquick spin column in a provided 2 ml collection tube.
- (7) To bind DNA, apply the sample to the QIAquick spin column and centrifuge for 1 min.
- (8) Discard the flow-through and place QIAquick spin column into the same collection tube.
- (9) Add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.
- (10) To wash, add 0.75 ml of Buffer PE to QIAquick spin column and centrifuge for 1 min.
- (11) Discard the flow-through and centrifuge the QIAquick spin column for an additional 1 min at 13,000 rpm.
- (12) Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
- (13) To elute DNA, add 50 μ l of Buffer EB (10mM Tris-Cl, pH 8.5) or H₂O to the centre of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 μ l of elution buffer to the centre of QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

3.3.12 DNA Sequencing

The purified DNA samples were outsourced to 1st Base Pte Ltd (Singapore) for sequencing where the samples were analysed by using the BigDye[®] Terminator v3.1 cycle sequencing kit chemistry which is based on the Sanger Chain Termination method. The output sequences were viewed by Chromas lite software. The nucleotide sequence was then analyzed by Sequence Manipulation Suite 2 [153]. ClustalW2

[154] was used to perform nucleotide alignment. Gene Bank Accession number AJ237696 was used to obtain reference nucleotide sequence for *fusA* gene.

3.3.13 Scanning Electron Microscope Analysis (SEM)

The samples were fixed in 5% glutaraldehyde (5% v/v 0.1M phosphate buffer, pH 7.4) for 3 hr, rinsed twice in 0.1M phosphate buffer, pH 7.4, then post-fixed in a 2% glutaraldehyde - 3% formaldehyde solution (v/v 0.1M phosphate buffer, pH 7.4) for 1 hr. The combination of these aldehydes provided a good quality of fixation as formaldehyde rapidly penetrates tissues stabilizing the structure of biological material whilst glutaraldehyde permanently fixes the sample.

The samples were then rinsed twice with 0.1M phosphate buffer, pH 7.4 before being sequentially dehydrated for 20 min in 70%, 90% (both v/v 0.1M phosphate buffer, pH 7.4) and 100% acetone [155]. Samples were subsequently dried in a 60 °C incubator for SEM analysis. Sample surfaces were sputtered with gold using JEOL AUTO FINE COATER (model- JFC 1600, Japan) prior to SEM analysis. The SEM pictures were taken by scanning electron microscope (JEOL JSM5600LV, JAPAN), applying a voltage of 10 kV.

Chapter 4 : Effects of Phytochemicals in Combination with Antibiotics against MRSA

4.1 Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of infection in hospitals [156]. It has been nicknamed a ‘Superbug’ due to its multi-drug resistance to most of the contemporary antibiotics [124]. Recently, it has also shown resistance to the glycopeptide, vancomycin, which is known to be the last defence antibiotic against the pathogen. Due to its multi-drug resistance patterns and rapid adaptive resistance to various antibiotics, critical attention is necessary to find new ways to combat infections caused by MRSA. At this point, the use of drug combinations rather than single drugs provides better clinical outcomes, as the use of single agent is highly associated with the occurrence of resistance [157].

Antibiotics with different mechanisms of action and that are active against *S. aureus* were chosen for this study. Fusidic acid and minocycline (protein synthesis inhibitor), rifampicin (inhibitor of DNA dependent RNA-polymerase), cefotaxime (third generation cephalosporin, disruption of cell wall), vancomycin (glycopeptides, inhibition of cell wall biosynthesis) and ofloxacin (quinolone, DNA-gyrase inhibitor) were used in combination with six phytochemicals against twelve *S. aureus* strains. The phytochemicals used were, tannic acid (tannin, found in tree bark and leaves), quercetin (flavanoid, found in colored fruits and vegetables), gallic acid ethyl ester (tea catechin, found in most teas), caffeic acid (plant phenol, found in leaves and stems), eugenol and menthone (essential oils). The combinations were assessed by checkerboard assay and the bactericidal synergistic pairs were assessed by time-kill assays.

In this study, the efficacy of different phytochemicals in combination with selected antibiotics was tested against 12 strains of *S. aureus* (ATCC MRSA 43300, ATCC methicillin sensitive *S. aureus* or MSSA 29213 and 10 MRSA clinical strains collected from National University Hospital, Singapore). Out of the six phytochemicals used, tannic acid was synergistic with fusidic acid, minocycline,

cefotaxime and rifampicin against most strains tested and additive with ofloxacin and vancomycin. Quercetin showed synergism with minocycline, fusidic acid and rifampicin against most of the strains. Gallic acid ethyl ester showed additivity against all strains except in combination with vancomycin where it showed an indifferent effect. Eugenol, menthone and caffeic acid showed an indifferent effects with the antibiotics. Interestingly, no antagonism was observed within these interactions. Based on the fractional inhibitory concentration indices, synergistic pairs were further examined by time-kill assays to confirm the accuracy and killing rate of the combinations over time. The two methods concurred with each other with 92% accuracy and the combinatory pairs were effective throughout the 24 hr duration of assay. The study suggests a possible incorporation of effective phytochemicals in combination therapies for MRSA infections.

4.2 Materials and Methods

Detailed methods for sample preparations, minimum inhibitory concentration (MIC), fractional inhibitory concentration index (FICI) and time-kill assay were mentioned in Chapter 3.

4.3 Results

MIC, FIC Index and Time-kill Assay

MIC value for tannic acid was 128 - 512 µg/ml, quercetin was 512 - 1024 µg/ml, gallic acid ethyl ester was 1024 µg/ml, eugenol was 512 - 2048 µg/ml, menthone was 4096 and caffeic acid was 512 - 1024 µg/ml respectively. MIC value for fusidic acid was 0.03125-0.25 µg/ml, cefotaxime was 2-512 µg/ml, ofloxacin was 0.5 - 512 µg/ml, minocycline was 0.25 - 128 µg/ml, vancomycin was 1-4 µg/ml and rifampicin was 0.004 - 0.5 µg/ml respectively, depending on the strains (see Table 4.1 and 4.2). Mostly, for western drugs, clinical strains under investigation have higher MIC values compared with MSSA and MRSA strains. Interesting, phytochemicals showed uniform MICs against all strains tested with variation in one or two dilutions only. That suggests that phytochemicals are effective to all strains under investigation, not depending on clinical or standard ATCC strains. Phytochemicals combined with

different antibiotics were tested against 12 strains of *S. aureus* for their combinatory actions.

Tannic acid was found to have synergism with fusidic acid, cefotaxime, minocycline and rifampicin, while it showed additive effects with vancomycin and ofloxacin. Quercetin was found to have synergism with fusidic acid, minocycline and rifampicin. It showed indifferent effects when combined with vancomycin, cefotaxime and ofloxacin. Gallic acid ethyl ester was additive with all antibiotics except with vancomycin whereas it showed indifferent effects against all strains. Caffeic acid, eugenol and menthone showed indifferent effects when combined with antibiotics.

Table 4.1 MICs and reduction in MICs of western drugs in combination with phytochemicals

Bacteria Strain	Antibiotic	MIC mg L ⁻¹						
		Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (1024 mg L ⁻¹)	Eug (1024 mg L ⁻¹)	Mth (4096 mg L ⁻¹)
MRSA 43300	Fusidic acid	0.03125	0.0039	0.0078	0.016	0.03125	0.03125	0.03125
	Cefotaxime	8	1	8	4	8	8	8
	Minocycline	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Rifampicin	0.016	0.004	0.004	0.008	0.016	0.016	0.016
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	0.5	0.25	0.5	0.25	0.5	0.5	0.5
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
MSSA 29213	Fusidic acid	0.03125	0.0078	0.0078	0.016	0.03125	0.03125	0.03125
	Cefotaxime	2	0.5	2	1	2	2	2
	Minocycline	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Rifampicin	0.004	0.001	0.001	0.002	0.004	0.004	0.004
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	1	0.5	1	0.5	1	1	1

Table 4.1 MICs and reduction in MICs of western drug in combination with phytochemicals (Continued)

Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C1	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	16	4	16	8	16	16	16
	Minocycline	0.5	0.125	0.125	0.25	0.5	0.5	0.5
	Rifampicin	0.008	0.002	0.002	0.004	0.008	0.008	0.008
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	1	0.5	1	0.5	1	1	1
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (128 mg L ⁻¹)	Eug (128 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C2	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	4	1	4	2	4	4	4
	Minocycline	4	1	1	2	4	4	4
	Rifampicin	0.016	0.004	0.004	0.008	0.016	0.016	0.016
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	1	0.5	1	0.5	1	1	1

Table 4.1 MICs and reduction in MICs of western drug in combination with phytochemicals (Continued)

Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (128 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C3	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	4	1	1	2	4	4	4
	Rifampicin	0.016	0.004	0.004	0.008	0.016	0.016	0.016
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	256	128	256	128	256	256	256
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (32 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C4	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	64	16	16	32	64	64	64
	Rifampicin	0.032	0.008	0.008	0.016	0.032	0.032	0.032
	Vancomycin	2	1	2	2	2	2	2
	Ofloxacin	256	128	256	128	256	256	256

Table 4.1 MICs and reduction in MICs of western dugs in combination with phytochemicals (Continued)

Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (32 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C5	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	8	2	2	4	8	8	8
	Rifampicin	0.032	0.008	0.008	0.016	0.032	0.032	0.032
	Vancomycin	4	2	4	4	4	4	4
	Ofloxacin	512	256	512	256	512	512	512
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (32 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C6	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	4	1	1	2	4	4	4
	Rifampicin	0.016	0.004	0.004	0.008	0.016	0.016	0.016
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	128	64	128	64	128	128	128

Table 4.1 MICs and reduction in MICs of western dugs in combination with phytochemicals (Continued)

Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (32 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C7	Fusidic acid	0.5	0.125	0.125	0.25	0.5	0.5	0.5
	Cefotaxime	128	32	128	64	128	128	128
	Minocycline	16	4	4	8	16	16	16
	Rifampicin	0.5	0.125	0.125	0.25	0.5	0.5	0.5
	Vancomycin	2	1	2	2	2	2	2
	Ofloxacin	128	64	128	64	128	128	128
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (32 mg L ⁻¹)	Quer (256 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C8	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	128	32	32	64	128	128	128
	Rifampicin	0.5	0.125	0.125	0.25	0.5	0.5	0.5
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	128	64	128	64	128	128	128

Table 4.1 MICs and reduction in MICs of western dugs in combination with phytochemicals (Continued)

Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (64 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C9	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	128	32	128	64	128	128	128
	Minocycline	64	16	16	32	64	64	64
	Rifampicin	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Vancomycin	1	0.5	1	1	1	1	1
	Ofloxacin	64	32	64	32	64	64	64
Bacteria Strain	Antibiotic	Antibiotic alone	Antibiotic with					
			TA (128 mg L ⁻¹)	Quer (128 mg L ⁻¹)	GA (256 mg L ⁻¹)	CA (256 mg L ⁻¹)	Eug (512 mg L ⁻¹)	Mth (1024 mg L ⁻¹)
C10	Fusidic acid	0.25	0.0625	0.0625	0.125	0.25	0.25	0.25
	Cefotaxime	512	128	512	256	512	512	512
	Minocycline	2	0.5	0.5	1	2	2	2
	Rifampicin	0.5	0.125	0.125	0.25	0.5	0.5	0.5
	Vancomycin	4	2	4	4	4	4	4
	Ofloxacin	256	128	256	128	256	256	256

Table 4.2 FIC indices of combinatory pairs

Antimicrobial Agents	Σ FICI											
	MRSA 43300	MSSA 29312	MRSA C1	MRSA C2	MRSA C3	MRSA C4	MRSA C5	MRSA C6	MRSA C7	MRSA C8	MRSA C9	MRSA C10
TA + FA	0.5 (S)	0.375 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)
TA + Cefo	0.375 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.75 (A)	0.75 (A)	0.5 (S)	0.5 (S)	0.75 (A)	0.5 (S)	0.5 (S)
TA + Mino	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.75 (A)	0.5 (S)	0.5 (S)
TA + Van	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)
TA + Oflox	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)
TA + Rifam	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.75 (A)	0.5 (S)	0.5 (S)	0.5 (S)
Quer + FA	0.5 (S)	0.5 (S)	0.5 (S)	0.375 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.375 (S)
Quer + Cefo	2 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)
Quer + Mino	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.75 (A)	0.5 (S)	0.5 (S)	0.5 (S)	0.5 (S)
Quer + Van	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)
Quer + Oflox	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)
Quer + Rifam	0.375 (S)	0.375 (S)	0.5 (S)	0.5 (S)	0.5 (S)	0.75 (A)	0.5 (S)	0.5 (S)	0.375 (S)	1 (A)	0.5 (S)	0.5 (S)
GA + FA	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)
GA + Cefo	1 (A)	0.75 (A)	1 (A)	0.75 (A)	0.75 (A)	1 (A)	1 (A)	1 (A)	0.75 (A)	1 (A)	0.75 (A)	1 (A)
GA + Mino	0.75 (A)	0.75 (A)	1 (A)	1 (A)	1 (A)	0.75 (A)	0.75 (A)	1 (A)	1 (A)	0.75 (A)	1 (A)	0.75 (A)
GA + Van	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)
GA + Oflox	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	1 (A)	0.75 (A)	0.75 (A)	1 (A)	0.75 (A)	1 (A)	1 (A)
GA + Rifam	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	0.75 (A)	1.5 (I)	1 (A)	0.75 (A)	0.75 (A)
CA + FA	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	1.5 (I)	1.5 (I)
CA + Cefo	2 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)
CA + Mino	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	1.5 (I)	1.5 (I)
CA + Van	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)
CA + Oflox	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)
CA + Rifam	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)
Mth + FA	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)
Mth + Cefo	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)
Mth + Mino	2 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)
Mth + Van	2 (I)	2 (I)	2 (I)	2 (I)	1.5 (I)	2 (I)	1.5 (I)	2 (I)	2 (I)	2 (I)	2 (I)	2 (I)
Mth + Oflox	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	1.5 (I)	2 (I)	1.5 (I)	1.5 (I)	2 (I)	2 (I)	1.5 (I)	1.5 (I)

Table 4.2 FIC indices of combinatory pairs (Continued)

Mth + Rifam	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)
Eug + FA	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)
Eug + Cefo	1.5	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)
Eug + Mino	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)
Eug + Van	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)
Eug + Oflox	2	(I)	2	(I)	1.5	(I)	2	(I)	1.5	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)
Eug + Rifam	1.5	(I)	1.5	(I)	1.5	(I)	2	(I)	1.5	(I)	2	(I)	2	(I)	1.5	(I)	1.5	(I)

S* represents “Synergism”, A represents “Additive” and I represents “Indifferent” effects.

Abbreviations; TA = tannic acid, Quc = quercetin, GA = gallic acid ethyl ester, CA = caffeic acid, Eug = eugenol, Mth = menthone, FA = fusidic acid, Cefo = cefotaxime sodium, Mino = minocycline, Van = vancomycin, Oflox = ofloxacin, Rifam = rifampicin

Figure 4.1 Interactions between antibiotics and phytochemicals. Antibiotics are classified based on their targets and mechanism of action against MRSA strains. FA, fusidic acid; Rifam, rifampicin, Mino; minocycline; Cefo, cefotaxime; Van, vancomycin. Phytochemicals are classified based on their classes. TA, tannic acid; GA, gallic acid ethyl ester; Quer, quercetin, Eug, eugenol; Mth, menthone; CA, caffeic acid. Dark Green Line: Synergistic, Light Green Line: Additive, Red Line: Indifference or no interaction.

Figure 4.2 A-L: Time-kill curves of tannic acid and fusidic acid against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MRSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C4, G = MRSA C5, H = MRSA C6, I = MRSA C7, J = MRSA C8, K = MRSA C9, L = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.3 A-H: Time-kill curves of tannic acid and cefotaxime against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C3, E = MRSA C6, F = MRSA C7, G = MRSA C9, H = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.4 A-K: Time-kill curves of tannic acid and minocycline against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C4, G = MRSA C5, H = MRSA C6, I = MRSA C7, J = MRSA C9, K = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.5 A-K: Time-kill curves of tannic acid and rifampicin against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C4, G = MRSA C5, H = MRSA C6, I = MRSA C8, J = MRSA C9, K = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.6 A-L: Time-kill curves of quercetin and fusidic acid against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C4, G = MRSA C5, H = MRSA C6, I = MRSA C7, J = MRSA C8, K = MRSA C9, L = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.7 A-K: Time-kill curves of quercetin and minocycline against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C4, G = MRSA C5, H = MRSA C7, I = MRSA C8, J = MRSA C9, K = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Figure 4.8 A-J: Time-kill curves of quercetin and rifampicin against *S. aureus* strains observed over 24 hr. (A = MRSA 43300, B = MSSA 29213, C = MRSA C1, D = MRSA C2, E = MRSA C3, F = MRSA C5, G = MRSA C6, H = MRSA C7, I = MRSA C9, J = MRSA C10 and C+ = Positive Control). X-axis shows time in hr.

Table 4.3 Differences between bacterial counts ($\log_{10}$ CFU/ml) of combinations and the most active single agent (antibiotic) used alone

Strains/ Tannic acid	Fusidic Acid (0.25 MIC)				Cefotaxime (0.25 MIC)				Minocycline (0.25 MIC)				Rifampicin (0.25 MIC)			
	4H	8H	12H	24H	4H	8H	12H	24H	4H	8H	12H	24H	4H	8H	12H	24H
MRSA 43300	4	5.9	7.5	7.6	4.1	5	5.5	5.7	4	5.9	7.3	7.3	3.4	4.6	5.8	7
MSSA 29213	3.2	5.5	6.5	5.7	4	5	6.4	6	3.9	4	4.3	5.1	3.7	5.5	6.2	6.5
Clinical Stain C ₁	4.1	4.2	4.47	4.8	3.3	3.9	4.4	5.1	5	5.2	5.1	4.5	3.6	5.3	6.1	5.6
C ₂	4.4	4.7	4.9	4.6	3.3	4.4	4.3	4.5	5	5	5.3	5.1	3.6	4.7	5.5	5.3
C ₃	3.9	4.2	4.4	4.5	4.3	5.1	5.4	5.2	4.6	4.5	4.3	4.8	3.9	5.5	5.9	7.1
C ₄	3.2	3.3	3.5	1.9	-	-	-	-	4.6	4.2	5	4.9	4.5	5.3	5.7	6.2
C ₅	3.7	3.6	3.7	3.9	-	-	-	-	4.8	5.1	5.6	5.5	3.9	4.6	5.5	5.2
C ₆	4.2	5.2	5.7	7.3	4.4	3.9	3.1	1.7	5.2	5.6	5.7	5.5	4	5.6	6.3	7.1
C ₇	4.8	3.9	3.8	4.2	5.1	5.6	5.8	5.9	5.6	5.8	5.7	6.1	-	-	-	-
C ₈	3.7	3.8	3.72	2.2	-	-	-	-	5.2	5.5	5.5	5.4	4.1	4.3	5.4	5.9
C ₉	4.1	4.4	4.46	5	4.3	5.0	5.3	5.2	5.2	5.5	5.8	5.5	2.5	2	2.2	1.9
C ₁₀	5.1	7.1	7.73	7.5	5.1	5.2	5.6	5.3	5.3	5.1	5.8	5.4	4.8	5.6	6.5	6.2
Strains/Quercetin (0.25 MIC)	Fusidic Acid (0.25 MIC)				Minocycline (0.25 MIC)				Rifampicin (0.25 MIC)							
	4H	8H	12H	24H	4H	8H	12H	24H	4H	8H	12H	24H				
MRSA 43300	4.1	4.5	4.6	4.6	4.6	5.1	5.1	4.4	3.3	5.9	6.0	5.8				
MSSA 29213	4.5	5.4	6.4	6.5	3.8	3.8	4.3	4.5	4.0	4.6	4.9	5.9				
Clinical Stain C ₁	5.4	6.1	6.3	6.5	4.5	5.0	4.9	5.4	3.6	4.7	5.9	7				
C ₂	5.9	6.1	7.3	7.4	4.5	4.7	4.5	5.3	4.8	5.3	6	5.6				
C ₃	4.7	5.2	6.1	6.3	4.1	5.2	5.4	5.7	2.2	4.8	4.6	4.2				
C ₄	4.3	4.6	5.1	4.9	2.8	2.7	2	2	3.8	5.9	4.6	5.0				
C ₅	5	5.3	6.5	6.4	4.7	5.2	5.5	5.2	4.4	5.2	5.7	4.0				
C ₆	6	5.9	6.5	6.8	-	-	-	-	5.1	4.5	6.7	5.5				
C ₇	5.5	5.9	6.2	6.2	4.1	4.9	5.1	4.7	3.8	5.1	6.5	5.5				
C ₈	4.8	5.2	5.1	4.6	5	5.2	5.2	5.2	-	-	-	-				
C ₉	4.8	4.6	5.4	5.3	4.7	5.4	5.7	5.6	3.7	4.1	2	0.8				
C ₁₀	5.2	5.3	5.2	5.6	5.1	5.1	5.2	5.1	3.2	4	6.0	5.2				

4.5 Discussion

Combination therapies have been used with an aim of better efficacy and improved treatment option [13]. Combinations of conventional antibiotics and phytochemicals are more recent alternative methods for the treatment of multi-drug resistant bacteria like MRSA. Khan *et al.* [147] reported the potentiating effect of the phytochemical piperine on ciprofloxacin activity against *S. aureus* strains. Similarly, Soe *et al.* [17], found that ethyl gallate addition to fusidic acid and tetracycline in combinations can overcome the resistance in MRSA. These reports illustrate the potentiating effect of phytochemicals on the mechanism of action of antibiotics.

In this study, tannic acid and quercetin showed synergy with most of the antibiotics tested. Both phytochemicals were able to reduce the MIC of the antibiotics from 4 to 8 fold. Figure 4.1 clearly demonstrates the interactions of phytochemicals with antibiotics. Phytochemicals, especially the polyphenol and flavonoid groups, have positive interactions (synergism or additivity) with antibiotics of different classes. This clearly points out the role of phytochemicals together with antibiotics in anti-MRSA therapy. In the time-kill assay these synergistic pairs were able to show high killing rates starting from 4 hr up to 24 hr, with a few exceptions (see Figure 4.2-4.8). The Log₁₀ difference between the most active drug of the combination (antibiotics) and the combination with phytochemicals, clearly suggested suppression of populations that otherwise had a high growth rate with a single agent (see Table 4.3). The high log₁₀ difference was also observed until 24 hr, which demonstrates the potentiating effect of tannic acid and quercetin on the selected antibiotics. However, for some pairs where the FICI suggested synergism, the time-kill assay did not concur with the synergism definition (≥ 3 log₁₀ difference). In total, interpretations of the FICI calculated for all combinations against all strains, the time-kill assay was 92% in concurrence with the checkerboard assay.

Phytochemicals have been shown to have antimicrobial activity against a broad spectrum of organisms [14]. Their multi-targeted approach suggested by many plays a role in reducing the probability of development of resistance [17, 19, 147]. In appropriate combinations and doses they also play a role in increasing the susceptibility of the pathogen to various drugs [158]. In the present study, antibiotics

with different mechanisms of action were used with broad spectrum phytochemicals. Most of the combinations tested showed a positive interaction (synergy and additivity) with none being antagonistic. This clearly suggested that phytochemicals are able to potentiate various antibiotics in suitable combinations. Therefore, phytochemicals can be considered as a potential additive for resistance modulation when used with a suitable antibiotic. In addition to lowering the dose of the antibiotic in combination (to up to 8 folds), the overall efficacy of the treatment is improved. However, detailed analysis of the resistance patterns of the pathogen under consideration is important for incorporation into clinical practice.

In general, phytochemicals are concentrationally weaker than antibiotics. This was also evident in this study, as the MICs of the antibiotics were much lower than those of the phytochemicals. Incorporation of these antibacterial compounds as single agents would require enormously high concentrations for sufficient bioavailability *in vivo*. Therefore, their combinations at sub-MIC levels with more active antibiotics, also at sub-MIC, would be more suitable and realistic in a clinical setting. This would even bring down the side effects caused by each of these antibacterial drugs. Although, for clinical applications, the total toxicity levels of the phytochemicals must be taken into account, including the pharmacokinetics and pharmacodynamics (PK/PD) models of the drug. The acute toxicity levels of tannic acid and quercetin were, $LD_{50} > 120$ mg/kg and $LD_{50} > 159$ mg/kg, respectively, obtained from the manufacturer. These levels are much higher than the MIC obtained for the phytochemicals alone as well as in combination (FIC), suggesting their therapeutic significance.

Combination therapy has been identified as a rational approach to tackle concerns of resistance in MRSA [12]. This is because of its several advantages over monotherapy, including reduction of resistance and total drug intake, thereby reducing side effects [6]. In addition to antibiotic combinations, antibiotics with complementary and alternative medicines such as phytochemicals and insect extracts have also been shown to be effective *in vitro* [15, 17, 159]. The present study also illustrates the use of phytochemicals in useful combination with antibiotics that easily subjects *S. aureus* to adaptive resistance.

Both checkerboard and time-kill assay results demonstrated that tannic acid was able to prolong and potentiate the bactericidal activity of fusidic acid, cefotaxime, minocycline and rifampicin. Similar effect was observed with quercetin in combination with fusidic acid, minocycline and rifampicin. The possible mechanism of actions that how these phytochemical and antibiotic interactions resulted in synergistic or additive effects have to be suggested based on individual drug/phytochemical's mechanism of action. As mentioned in Chapter 2, antibacterial effects of tannic acid can be achieved by enzyme inhibition [14, 70, 71], binding to specific proteins [14, 72], binding to adhesions, complexing with cell walls, substrate deprivation, membrane deprivation and metal ion complexation [14]. Fusidic acid and minocycline both target on bacterial protein synthesis and rifampicin targets on DNA dependent RNA polymerase enzyme. It is most likely that tannic acid acts on bacterial cell wall and thus facilitating the entry of partner drugs (fusidic acid, minocycline and rifampicin) to reach their targets with the cell to give rise synergistic effects. As most of the synergistic drugs have different mechanism of actions [160], it is the best way to consider different mechanism of tannic acid among its proposed mechanisms. Similarly, tannic acid and cefotaxime both act on the bacterial cell wall and the exact target that how they act to give rise synergistic effect is still unknown.

Quercetin acts on bacterial DNA gyrase [78] and its influence negative supercoiling of DNA during replication [161]. Quercetin is synergistic with rifampicin, fusidic acid and minocycline. Rifampicin acts on DNA-dependent RNA polymerase enzyme. Fusidic acid and minocycline inhibit bacterial protein synthesis and quercetin acts on bacterial DNA gyrase enzyme. It is mostly like that these compounds act on bacterial DNA replication and protein synthesis steps and brings together synergistic effects. The exact mechanisms of actions of synergistic pairs are still need to be clarified.

In this study, the synergistic effects could be observed as early as 4 hr post inoculation, with maximum effects observed at 24 hr of incubation. Therefore, phytochemicals (tannic acid and quercetin) in combination with the antibiotics under study were able to provide stable therapeutic outcomes with higher efficacy in terms of killing rate throughout 24 hr. These synergistic therapeutic pairs could be useful in combating *S. aureus* infections in a hospital or community setting.

Chapter 5 : Effects of Antibiotic-Phytochemical Combinatory Methods on MRSA Adaptive Mutation

5.1 Introduction

Evolution of adaptive resistance in *S. aureus* was tested against selected phytochemicals and antibiotics alone and in combination. The minimal inhibitory concentrations (MIC) and the FIC indices of synergistic pairs (as described in Chapter 4) were used in this study. Synergistic pairs were chosen to study their effects on the prevention of mutations. Resistance selections for each compound and combinatory pairs were done by sub culturing the bacterial strain onto the respective phytochemicals or antibiotic at sub-MIC values and observing the adaptive resistance evolution with time. Results obtained by this method showed that combinatory pairs were superior to single compounds in preventing the mutation adaptation in *S. aureus*. Moreover, it was found that using antibiotic-phytochemical combinations (at sub MIC level) could greatly delay the occurrence of mutations in MRSA.

The interaction of phytochemicals combined with antibiotics was discussed in Chapter 4. The synergistic pairs were chosen for further study on the effects of the emergence of resistance development on the *Staphylococcus aureus* MRSA strain 43300 compared with single compounds. According to FIC indices (described in Chapter 4), tannic acid was found to be synergistic with fusidic acid, cefotaxime, minocycline and rifampicin whereas quercetin was found to have synergism with fusidic acid, minocycline and rifampicin. Therefore, the effect of these combinatory pairs on the emergence of resistance development in *S. aureus* was discussed in this Section.

5.2 Materials and Methods

5.2.1 Bacterial strain and media used

Methicillin resistant *S. aureus* ATCC 43300 was used in this study. All antibiotics and phytochemicals used in this study were purchased from Sigma Life Science,

Singapore. Details about inoculation of bacteria and media used are discussed in Chapter 3.

5.2.2 Preparation of inoculums

Detailed procedure for inoculums preparation was as follows:

- (1) MRSA ATCC 43300 was sub-cultured on an IS agar plate.
- (2) Then the agar plate was placed in a 37 °C incubator for overnight.
- (3) 3-5 colonies of microorganism were transferred from this overnight cultured isolate into the test tube containing 5ml of IS broth.
- (4) The test tube was incubated in 37 °C until the turbidity of the broth reached 0.5 McFarland Standard (1.5×10^8 CFU/ml).
- (5) Then the mixture was further diluted with IS broth to make 1: 100 dilutions (1.5×10^6 CFU/ml).

5.2.3 Procedure for study of the development of drug resistance in *S. aureus*

- (1) The working solutions for the antibiotics and bacteria were prepared to get desired concentration.
- (2) 3 test tubes were taken for the exposure of bacteria with different concentrations of antibiotics.
- (3) In the 1st test tube, IS broth 9 ml was mixed with 1 ml of prepared bacterial suspension (1:100 dilutions i.e. 1.5×10^6 CFU/ml) as the positive control test tube.
- (4) In the 2nd test tube, IS broth 10 ml alone was added as a negative control or sterile control test tube.
- (5) In the 3rd test tube, “x” ml of a calculated amount of 1/8 MIC concentration of antibiotic was mixed with a calculated amount of IS broth and 1 ml of bacterial suspension.
- (6) Then, these three test tubes were stored in a 37 °C incubator for 24 hr.
- (7) After 24 hr, 0.01 ml was taken from each test tube and was directly sub-cultured on a normal IS agar plate. 0.01 ml of antibiotic exposed bacterial solution was also sub-cultured on 1/8 MIC agar plate.
- (8) Then all cultured plates were incubated in a 37 °C incubator for 24 hr.

- (9) In the study, the positive control agar plate should have bacterial colony growth and be free of other bacteria species contamination and the negative control agar plate also should be free of the organism.
- (10) When the bacteria grew on 1/8 MIC agar plate, 3-5 colonies of bacteria colonies were taken from this agar plate and prepared as a bacteria suspension into a standard suspension of 1:100 dilution (1.5×10^6 CFU/ml).
- (11) Then three test tubes of positive control, negative control and test tube containing double concentration of antibiotic solution (i.e. 1/4 MIC) were prepared as above and the procedure was repeated.
- (12) If there was no bacterial growth on the MIC agar plate, 3-5 colonies were taken from the normal agar plate on which antibiotic exposed bacteria were grown. Then the procedure was repeated until there was bacterial growth on the MIC agar plate.
- (13) The procedure was repeated until the development of resistant MRSA appeared.

5.3 Results and Discussion

The stability and the effects of the phytochemicals and antibiotics under study were observed by the one-step mutation methods mentioned before. The starting concentration began from sub-MIC concentrations ($0.125 \times \text{MIC}$) of each phytochemical or antibiotic individually or in combination. The duration it took to develop resistant strains between each step was observed and compared.

Table 5.1 Development of resistance to fusidic acid in MRSA

MIC	Duration
1/8 (0.13) MIC	1 day
1/4 (0.25) MIC	2 days
1/2 (0.5) MIC	3 days
1 MIC	4 days
2 MIC	5 days
4 MIC	6 days
8 MIC	7 days
16 MIC	8 days
32 MIC	9 days

Figure 5.1 Development of resistance to fusidic acid in MRSA. Fusidic acid resistance was induced by exposing with sub-MIC concentration (starting from 0.13 MIC) of fusidic acid and observed the duration (days) to get colonies on higher MICs.

Figure 5.1 and Table 5.1 show the resistance evolution of MRSA to western drug fusidic acid. The resistance occurred at the 1 MIC concentrations at the 4th day of experiment. The colonies were also grown on 2 MIC concentrations at the 5th day, 4 MIC at the 6th day, 8 MIC at the 7th day, 16 MIC at the 8th day and 32 MIC at the 9th day of the experiment.

Table 5.2 Development of resistance to rifampicin in MRSA

MIC	Duration
1/8 (0.13) MIC	1 day
1/4 (0.25) MIC	2 days
1/2 (0.5) MIC	3 days
1 MIC	6 days
2 MIC	7 days
4 MIC	9 days
8 MIC	11 days
16 MIC	12 days
32 MIC	14 days

Figure 5.2 Development of resistance to rifampicin in MRSA

Figure 5.2 and Table 5.2 illustrate the resistance evolution of MRSA to the western drug rifampicin. The resistance occurred at the 1 MIC concentrations at the 6th day of experiment. The colonies were also grown on 2 MIC concentrations at the 7th day, 4

MIC at the 9th day, 8 MIC at the 11th day, 16 MIC at the 12th day and 32 MIC at the 14th day of the experiment.

Table 5.3 Development of resistance to minocycline in MRSA

MIC	Duration
1/8 (0.13) MIC	1 day
1/4 (0.25) MIC	2 days
1/2 (0.5) MIC	3 days
1 MIC	7 days
2 MIC	8 days
4 MIC	9 days
8 MIC	12 days
16 MIC	13 days
32 MIC	15 days

Figure 5.3 Development of resistance to minocycline in MRSA

Figure 5.3 and Table 5.3 show the resistance evolution of MRSA to the western drug minocycline. The resistance occurred at the 1 MIC concentrations at the 7th day of experiment. The colonies were also grown on 2 MIC concentrations at the 8th day, 4 MIC at the 9th day, 8 MIC at the 12th day, 16 MIC at the 13th day and 32 MIC at the 15th day of the experiment.

Table 5.4 Development of resistance to cefotaxime in MRSA

MIC	Duration
1/8 (0.13) MIC	1 day
1/4 (0.25) MIC	2 days
1/2 (0.5) MIC	3 days
1 MIC	4 days
2 MIC	6 days
4 MIC	7 days
8 MIC	8 days
16 MIC	10 days
32 MIC	11 days

Figure 5.4 Development of resistance to cefotaxime in MRSA

Figure 5.4 and Table 5.4 show the resistance evolution of MRSA to the western drug cefotaxime. The resistance occurred at the 1 MIC concentrations at the 4th day of experiment. The colonies were also grown on 2 MIC concentrations at the 6th day, 4 MIC at the 7th day, 8 MIC at the 8th day, 16 MIC at the 10th day and 32 MIC at the 11th day of the experiment.

Table 5.5 Development of resistance to tannic acid in MRSA

MIC	Durations
1/8 MIC	1 day
1/4 MIC	2 days
1/2 MIC	3 days
1 MIC	17 days

Figure 5.5 Development of resistance to tannic acid in MRSA

Figure 5.5 illustrates the resistance evolution of MRSA to tannic acid. The resistance occurred at the $\frac{1}{8}$ MIC concentrations at the 1st day, $\frac{1}{4}$ MIC at the 2nd day, $\frac{1}{2}$ MIC at the 3rd day and 1 MIC at the 17th day of experiment.

Table 5.6 Development of resistance to quercetin in MRSA

MIC	Durations
1/8 MIC	1 day
1/4 MIC	2 days
1/2 MIC	3 days
1 MIC	14 days

Figure 5.6 Development of resistance of quercetin in MRSA

Figure 5.6 and table 5.6 show the resistance evolution of MRSA to quercetin. The resistance occurred at the $\frac{1}{8}$ MIC concentrations at the 1st day, $\frac{1}{4}$ MIC at the 2nd day, $\frac{1}{2}$ MIC at the 3rd day and 1 MIC at the 14th day of experiment.

Figure 5.7 Resistant development to fusidic acid, cefotaxime and minocycline in combination with tannic acid against MRSA 43300

Figure 5.7 shows the emergence of resistance in MRSA 43300 with the combinatory drug pairs where resistance of (0.13 + 0.13) MIC of tannic acid and fusidic acid resistant mutants appeared on Day 1, (0.25 + 0.25) MIC combination resulted on Day 9, (0.5 + 0.5) MIC combination resulted on Day 25 and (1 + 1) MIC combination was grown on Day 57 respectively.

In the case of the combinatory pair of tannic acid and cefotaxime, (0.13+ 0.13) the MIC of tannic acid and cefotaxime resistant mutants appeared on Day 1, (0.25 + 0.25) MIC combination resulted on Day 9, (0.5 + 0.5) MIC combination resulted on Day 11 and (1 + 1) MIC combination was grown on Day 35 respectively.

Similarly, in the process of resistance development to the tannic acid and minocycline combinatory pair, (0.13+ 0.13) MIC mutants appeared on Day 1, (0.25 + 0.25) MIC mutants resulted on Day 9, (0.5 + 0.5) MIC mutants on Day 17 and (1 + 1) MIC mutants were grown on Day 47 respectively.

Figure 5.8 Resistance development to fusidic acid, cefotaxime and minocycline in combination with tannic acid against MRSA 43300

Figure 5.8 shows the emergence of resistance in MRSA ATCC 43300 with the combinatory drug pairs whereas resistance of (0.13 + 0.13) the MIC combination of tannic acid and rifampicin mutants appeared on Day 1, (0.25 + 0.25) MIC combination resulted on Day 9, (0.5 + 0.5) MIC combination resulted on Day 16 and (1 + 1) MIC combination appeared on Day 34, respectively.

For the combinatory pair of quercetin and minocycline, (0.13 + 0.13) the MIC of tannic acid and cefotaxime resistant mutants appeared on Day 1, (0.25 + 0.25) MIC

combination resulted on Day 9, (0.5 + 0.5) MIC combination resulted on Day 16 and (1 + 1) MIC combination resulted on Day 49 respectively (see table 5.7).

Similarly, as in the combinatory pair of quercetin and fusidic acid, (0.13 + 0.13) the MIC of tannic acid and cefotaxime resistant mutants appeared on Day 1, (0.25 + 0.25) MIC combination resulted on Day 9, (0.5 + 0.5) MIC combination resulted on Day 16 and (1 + 1) MIC combination was grown on Day 42 respectively. The combination of quercetin and rifampicin also showed the same pattern of mutant formation.

Table 5.7 Comparison of the time durations required to obtain resistant phenotypes

Drug or Phytochemical	MIC	Duration to get resistant phenotypes (Days)
<i>Tannic acid</i>	1 MIC	17
<i>Quercetin</i>	1 MIC	14
Fusidic acid	1 MIC	4
Cefotaxime	1 MIC	4
Minocycline	1 MIC	7
Rifampicin	1 MIC	6
<i>Tannic acid</i> + Fusidic acid	1 + 1 MIC	57
<i>Tannic acid</i> + Cefotaxime	1 + 1 MIC	35
<i>Tannic acid</i> + Minocycline	1 + 1 MIC	47
<i>Tannic acid</i> + Rifampicin	1 + 1 MIC	34
<i>Quercetin</i> + Fusidic acid	1 + 1 MIC	42
<i>Quercetin</i> + Minocycline	1 + 1 MIC	49
<i>Quercetin</i> + Rifampicin	1 + 1 MIC	37

According to table 5.7, it was found that resistance development in MRSA to western antibiotics (fusidic acid, rifampicin, minocycline and cefotaxime) were more rapid than resistance development to phytochemicals (tannic acid and quercetin). The combination of phytochemicals and western antibiotics gave valuable results showing a longer duration of resistance development in *S. aureus* compared to those of the antibiotics given alone.

Antibiotic resistance is a critical issue to be solved. Most of the antibiotics have more or less resistance problems in clinical setting and there are only a few antibiotics available to combat MRSA. Vancomycin remains the primary drug used to treat these infections, but its use involves several side effects and resistant strains are emerging

in worldwide [128]. Therefore, it is necessary to develop new compounds or new strategies to fight against MRSA. In this study, it was evident that the antibiotics under study gave rise to 1×MIC resistant strains within one week *in vitro*. These results support the fact that antibiotics resistance can occur rapidly and will lead to treatment failure. However, phytochemicals took at least 2 weeks to develop 1×MIC resistance in strains.

There is another interesting concept presented by many authors for preventing adaptive mutations in bacteria. The MPC or mutant prevention concentration hypothesis uses higher doses of antibiotics than the MIC value to prevent the occurrence of mutations in bacteria [162-164]. However, the basic drawback is that most of the antibiotics are synthesized from bacterial or fungal origin and the stability of the drug is questionable and the bacteria can easily adapt to those antibiotics and will give rise to resistance problems. Another drawback of using MPC is that the side effects occurred by using high doses of antibiotics. Using high concentration of western antibiotics will definitely give rise more or less side effects. Using the synergistic phytochemicals under study can solve this problem as the phytochemicals under study can potentiate the effects of antibiotics and can prolong the duration to develop as resistant strains. That means that the phytochemicals under study can narrow down the mutant selection window of partner antibiotics and using 1×MIC or sub-MIC concentrations of antibiotics in combination with synergistic phytochemicals will have equal efficacy or more of using high doses of antibiotics. As per Table 5.7, the combination of antibiotics and phytochemicals will take at least 34 days to develop 1+1 MIC-resistant mutants. That means that within 34 days, it is safe to use the combination pairs. As most of the treatment schemes are planned to give less than 7 days for short-term therapy and maximum 30 days for long-termed therapy, the durations determined in this study is sufficient to interpret if the combinations of antibiotics and phytochemicals under study have stability for use in both short-term and long-term treatment plans.

Zhao *et al.* also proposed that MPC is a solution to prevent the adaptive mutation in MRSA [165]. In the current study, the MPC for MRSA ATCC 43300 is 0.5 µg/ml for fusidic acid. It is 16 fold higher than the actual MIC. It is certain that by using the MPC of the antibiotic alone will increase the side effects of the drug. Secondly, using

high dosage alone is not a solution for this problem as the bacteria will rapidly adapt and will cause resistance problems. Even well-known synergistic drug combinatory drugs called co-amoxiclav (which is combination of amoxicillin and clavulanic acid) is facing resistance problems because the two drugs have individually more or less resistance problems in clinical settings [166]. This clearly indicates that the MPC concept or using two synergistic antibiotic drugs is not the solution for the MRSA resistance problem and a new concept or a strategy is in need for the MRSA resistance problem.

In this study, the synergistic combinatory pairs (discussed in Chapter 4) were used to understand their role and stability in resistance formation in respect to individual use. Possible mode of interaction of the synergistic pairs are also mentioned in Chapter 4.5. It was found that giving phytochemicals together with antibiotics took at least 34 days to get 1×MIC resistance strains. For the antibiotics alone, it took 4 days for fusidic acid and cefotaxime to get 1×MIC resistant mutants. For rifampicin and minocycline, it took 6 and 7 days respectively to get 1×MIC resistant mutants. It was found that the use of synergistic combination of antibiotic and phytochemical could delay the resistance formation rather than using antibiotics alone. Therefore, combinations should be considered for use in long-term therapy for MRSA infections.

The above finding suggested that using synergistic combinations of antibiotic and phytochemical could decrease the risk of resistance development of *S. aureus in vitro* and can narrow the mutant selection window of partner synergistic antibiotics. Overall, the combinations of synergistic antibiotics and phytochemicals under study were able to prevent or suppress resistance development of MRSA.

Chapter 6 : Effects of Phytochemicals on the Emergence of Fusidic Acid and Rifampicin Resistance in MRSA

6.1 Introduction

In this study, antimicrobial activities of tannic acid, gallic acid ethyl ester and quercetin were determined against five strains of *S. aureus*. Tannic acid and quercetin were found to be synergistic with fusidic acid, and rifampicin, while gallic acid ethyl ester was found to be additive to the before- mentioned antibiotics. Furthermore, the effects of these combinatory pairs on mutation prevention of fusidic acid and rifampicin were studied. It was also investigated for mutant selection of *S. aureus* for fusidic acid and rifampicin resistant *S. aureus* in vitro by combining with $0.25 \times \text{MIC}$ of the phytochemicals (tannic acid, quercetin and gallic acid ethyl ester). Five strains of *S. aureus* (MRSA, MSSA and 3 MRSA clinical strains) were serially exposed in IS broth to two-fold stepwise increasing concentrations of fusidic acid, rifampicin and minocycline alone or in combination with a fixed concentration of $[0.25 \times \text{minimum inhibitory concentration (MIC)}]$ of either of the phytochemicals (tannic acid, quercetin and gallic acid ethyl ester). The fusidic acid/rifampicin MIC were determined after each cycle. Exposure of either fusidic acid or rifampicin alone is found to cause gradual mutant selection for *S. aureus* with an increasing MIC. All phytochemicals tested can prevent or greatly delay fusidic acid and rifampicin resistance. Only the MIC of fusidic acid/rifampicin combined with gallic acid ethyl ester shows slightly increased (2 fold) in MIC starting from 8th cycle. However, when compared with the western drug alone, the phytochemicals can markedly delay or prevent the occurrence of resistance.

6.2 Materials and Methods

6.2.1 Microorganisms and Antimicrobial Agents

A total of five *S. aureus* strains: methicillin resistant *S. aureus* (MRSA) 43300, methicillin sensitive *S. aureus* 29213 and three MRSA clinical strains collected from National University Hospital (NUH), Singapore, were used in this study. The microorganisms were grown at 37 °C in IS Broth (Oxoid Ltd., Basingstoke, Hampshire, England) with aeration. Gallic acid ethyl ester (assay value 99%), Quercetin (assay value 98%) and tannic acid (assay value 98%) and fusidic acid (assay value 98%) and rifampicin (assay value 97%) were used in this study. Stock solutions for antibiotics and phytochemicals were freshly prepared and used. Controls for solvents were also tested for antimicrobial action using appropriate concentrations.

6.2.2 Susceptibility Studies

Minimum inhibitory concentrations (MICs) were determined in IS Broth by the micro broth dilution method according to Clinical and Laboratory Standards Institute guidelines [15]. The MIC was defined as the lowest concentration of drug that inhibits bacterial growth following incubation for 18 hr at 37 °C. The MIC of MSSA ATCC 29213 was used as the control.

6.2.3 Combination Studies (Checker Board Assay)

The checkerboard assay is one of the most frequently used techniques to access drug interactions. Testing was performed utilizing 96-well microtiter plates. MICs were determined for each drug by broth microdilution according to standards of the Clinical Laboratory Standardization Institute (CLSI) [15, 141]. Each combination assay was performed three times. Detailed interpretations for the checker board assay are described in Chapter 3.

6.2.4 Resistance studies

For analysis of spontaneous antibiotic (fusidic acid or rifampicin) resistance, large inocula [ca. 10^9 colony forming units (CFU)/ml] of the test strains were serially diluted and spread on agar plates with increasing concentrations of antibiotics. The plates were then incubated for 48 hr at 37 °C before the colonies were counted. The population curves or population analysis profiles (PAPs) were drawn by calculating and plotting the number of cells against the concentration of the drug [167]. Colonies isolated from antibiotic containing agar plates were re-tested for MIC values.

For progressive resistance selection, bacteria were exposed in IS Broth with stepwise two-fold serial increasing concentration of fusidic acid/rifampicin alone for the total of 10 consecutive days, as described previously [13]. Briefly, series of tubes containing two-fold increasing concentration of antibiotics were inoculated with 10^7 CFU/ml (final concentration). Following 24 hr incubation at 37 °C, 0.1ml samples from the tubes containing the highest antibiotic concentration and still showing turbidity were used to inoculate a new series of tubes containing antibiotic dilutions. To examine the potential of secondary agents (phytochemicals) to suppress the emergence of the antibiotic resistance, experiments were performed as described above in the presence of $0.25 \times \text{MIC}$ of the phytochemicals (tannic acid, quercetin and gallic acid ethyl ester). Sub-inhibitory concentrations were used to allow bacterial growth on the partner drug. The MICs for each antibiotic were determined after each passage. In addition, the MICs for the partner drugs (phytochemicals) were determined in resistant derivatives selected by exposure to antibiotic alone or in combinations. Each experiment was performed twice. The stability of resistant derivatives was assessed by serial passage of the organisms on antibiotic-free medium for 5 consecutive days.

6.3 Results

Antimicrobial susceptibilities of bacterial strains are shown in Table 6.1. The MIC of rifampicin ranged from 0.004 to 0.016 µg/ml and fusidic acid MICs ranged from 0.0313 to 0.25 µg/ml for all vancomycin susceptible strains. Fusidic acid was less active against clinical strains although all strains tested showed susceptibility. Tannic acid, gallic acid ethyl ester and quercetin were found to be effective against *S. aureus* strains. Tannic acid was found to be the most effective agent as its minimal inhibitory concentration (MIC) was lower than gallic acid ethyl ester and quercetin. For each test performed, MICs of MSSA 29213 were within the acceptable range. Details about MICs are listed in table 6.1.

Table 6.1 Minimum inhibitory concentrations (MICs) of fusidic acid (FA), rifampicin (Rifam), vancomycin (Van), tannic acid (TA), gallic acid ethyl ester (GA) and quercetin (Quer) for *S. aureus* strains

Strain	MIC (µg/ml)					
	Van	Rifam	FA	TA	GA	Quer
<i>Staphylococcus aureus</i> MRSA 43300	0.5	0.016	0.0313	256	1024	512
MSSA 29213	0.5	0.004	0.0313	256	1024	512
Clinical strain C1	1	0.008	0.25	256	1024	512
Clinical strain C2	1	0.016	0.25	256	1024	512
Clinical strain C3	1	0.016	0.25	256	1024	512

FA: Fusidic acid, TA: tannic acid, GA: gallic acid ethyl ester, Quer: quercetin

6.3.1 Combination Study

The fractional inhibitory concentration (FIC) indexes used to qualitatively analyse the combinations is shown in Table 6.2. Fusidic acid and rifampicin showed synergism with TA and quercetin, and showed an additive effect with GA against all tested strains.

Table 6.2 FIC indices of fusidic acid and rifampicin against *S. aureus* strains

Antimicrobial Agents	MRSA 43300	MSSA 29213	C1	C2	C3	Remark
Fusidic Acid + Tannic acid	0.5	0.375	0.5	0.5	0.5	Synergism
Fusidic Acid + Quercetin	0.375	0.375	0.5	0.5	0.5	Synergism
Fusidic Acid + Gallic acid	0.75	0.75	1	1	1	Additive
Rifampicin + Tannic acid	0.5	0.5	0.5	0.5	0.5	Synergism
Rifampicin + Quercetin	0.375	0.375	0.5	0.5	0.5	Synergism
Rifampicin + Gallic acid	1	0.75	0.75	1	1	Additive

Figure 6.1 Population analysis profile of Fusidic acid against *S. aureus* strains (MRSA 43300, MSSA 29213 and 3 MRSA clinical strains: C1, C2 and C3)

Figure 6.2 Population analysis profile of Rifampicin against *S. aureus* strains (MRSA 43300, MSSA 29213 and 3 MRSA clinical strains: C1, C2 and C3)

6.3.2 Resistance Studies

Resistance studies were performed with 5 strains of *S. aureus* (MRSA 43300, MSSA 29213 and three MRSA clinical strains).

To search for the presence of spontaneous antibiotics-resistant subpopulation, large bacterial inocula (i.e. 10^9 CFU/ml) were spread on fusidic acid and rifampicin containing agar plates. Population analyses of fusidic acid and rifampicin are shown in Figure 6.1 and 6.2. All *S. aureus* strains tested showed a homogeneous pattern of resistance to fusidic acid and rifampicin and no growth occurred in the presence of rifampicin at ≥ 2 µg/ml and fusidic acid at ≥ 2 µg/ml.

Figure 6.3 Selection of fusidic acid resistant *S. aureus* after serial exposure to fusidic alone or in combination with $0.25 \times$ the minimum inhibitory concentration (MIC) of tannic acid, gallic acid ethyl ester and quercetin

Figure 6.4 Selection of Rifampicin resistant *S. aureus* after serial exposure to fusidic acid alone or in combination with $0.25 \times$ the minimum inhibitory concentration (MIC) of tannic acid, gallic acid ethyl ester and quercetin

The ten cycle exposure of the 5 strains of *S. aureus* to fusidic acid in combination with the three phytochemicals is shown in Figure 6.3. The graphs show resistance selection during cyclic exposure of bacteria to two-fold stepwise increasing concentration of fusidic alone or in combination with a fixed concentration ($0.25 \times \text{MIC}$) of phytochemicals (TA, Quer and GA). Serial exposure to fusidic acid alone resulted in development of resistant strains. In contrast, addition of a fixed concentration of tannic acid, quercetin and gallic acid could greatly delay or prevent the occurrence of resistance, as observed in the graphs.

A similar experiment with serial stepwise drug and drug combination exposure was carried out with rifampicin and the three phytochemicals at a fixed dose ($0.25 \times \text{MIC}$). The graphs are shown in Figure 6.4. Likewise, the serial exposure of rifampicin alone could not prevent the emergence of resistant isolates. As in the case of fusidic acid and combinations, the addition of a fixed concentration of the phytochemicals could greatly delay or prevent the development of resistant species.

Table 6.3 summarizes the MIC changes of the antibiotics at the end of the tenth cycle with and without the addition of phytochemicals.

Table 6.3 Differences between MICs of western drugs alone and in combination with phytochemicals after the 10th continuous cycle

Strain	Original MIC of FA ($\mu\text{g/ml}$)	MIC ($\times$ original MIC) of FA at the 10 th cycle with/without phytochemicals				Original MIC of Rif ($\mu\text{g/ml}$)	MIC ($\times$ original MIC) of Rif at the 10 th cycle with/without phytochemicals			
		TA	Quer	GA	None		TA	Quer	GA	None
MRSA 43300	0.0313	0.0313	0.0313	0.0625	0.5	0.016	0.016	0.032	0.032	0.256
MSSA 29213	0.0313	0.0313	0.0313	0.0625	0.25	0.004	0.004	0.008	0.008	0.032
C1	0.25	0.0313	0.0313	0.0625	0.25	0.008	0.008	0.016	0.016	0.064
C2	0.25	0.0313	0.0313	0.0625	0.5	0.016	0.016	0.032	0.032	0.256
C3	0.25	0.0313	0.0313	0.0625	0.5	0.016	0.016	0.016	0.016	0.128

To elaborate, fusidic acid combined with a fixed concentration ($0.25 \times \text{MIC}$) of tannic acid and quercetin resulted in no change in MIC in all 5 strains tested after 10th cycle, while FA combined with gallic acid ethyl ester resulted in a slightly changed in MIC (maximum 2 fold) starting from the 8th to 10th cycle depending on the strains. The MIC of fusidic acid rose to 0.25 $\mu\text{g/ml}$ ($8 \times \text{MIC}$) in MSSA 29213, 0.5 $\mu\text{g/ml}$ ($10 \times \text{MIC}$) in MRSA 43300, 2 $\mu\text{g/ml}$ ($8 \times \text{MIC}$) in clinical strain C1, 4 $\mu\text{g/ml}$ ($16 \times \text{MIC}$) in

clinical strain C2 and 4 µg/ml (8×MIC) in clinical strain C3. The fusidic acid MIC was not changed in combination with a fixed concentration of tannic acid and quercetin. There was a 2 fold increase in fusidic acid MIC observed in combination with gallic acid ethyl ester in all tested strain.

6.4 Discussion

Combination therapies have been used clinically with the aim of increasing treatment efficacies and decreasing the emergence of drug-resistant mutants. Moreover, there have been various reports that combination therapies using western antibiotics and phytochemicals could delay the emergence of mutations in *S. aureus* strains. Entenza *et al.* reported that emergence of daptomycin resistance could be prevented or greatly delayed by combining a fixed concentration (sub-MIC) of ampicillin in methicillin resistant *S. aureus* and enterococci during mutation studies with stepwise increasing concentration of antibiotics [13]. However, the use of western drugs alone or in combination is facing drug resistance problems and phytochemicals under study might have a new or alternate solution to use as synergistic pairs with the western drugs. Therefore, further research is needed for better compounds that are active against pathogens whilst having less frequency of mutation.

Phytochemical has research become a new area to study because of their antibacterial and antifungal, antioxidant actions and anti-carcinogenic effects depending on the subgroups to which they belong [14]. Tannic acid, gallic acid ethyl ester and quercetin are phytochemicals that have antibacterial effects [16, 18, 66, 67, 69, 77, 168]. However, the mechanisms of action of the phytochemicals under study and on how they work together with antibiotics on the effects of the mutations has not been reported yet. Therefore, the later segment reports a study on how these phytochemicals worked together with antibiotics. The effects of combination pairs on the emergence of resistance in *S. aureus* strains were also investigated herein.

Figure 6.3 demonstrated that with 10 continuous stepwise serial exposure to fusidic acid alone, the MIC was increased up to 8-16 MIC while a combination of fusidic acid with a fixed concentration (0.25×MIC) of phytochemicals (TA, Quer and GA) showed no change or a slightly increased (maximum 2 fold) MIC.

To elaborate, fusidic acid combined with a fixed concentration ($0.25 \times \text{MIC}$) of tannic acid and quercetin resulted in no change in MIC in all of 5 strains tested after the 10th cycle, while FA combined with gallic acid ethyl ester resulted in a slight change in MIC (maximum 2 fold) starting from the 8th to the 10th cycle depending on the strains. The MIC of fusidic acid was increased to 0.25 $\mu\text{g/ml}$ (8 times $\times$ original MIC) in MSSA 29213, 0.5 $\mu\text{g/ml}$ (10 times $\times$ original MIC) in MRSA 43300, 2 $\mu\text{g/ml}$ (8 times $\times$ original MIC) in clinical strain C1, 4 $\mu\text{g/ml}$ (16 times $\times$ original MIC) in clinical strain C2 and 4 $\mu\text{g/ml}$ (8 times $\times$ original MIC) in clinical strain C3. The fusidic acid MIC was not changed in combination with a fixed concentration of tannic acid and quercetin. There was a 2 fold increase in fusidic acid MIC when in combination with gallic acid ethyl ester in all tested strains.

Benjamin *et al.* reported the cut off value of the fusidic acid MIC for resistant and non-resistant strains. Susceptibility was generally defined as an MIC of ≤ 0.25 or ≤ 0.5 $\mu\text{g/ml}$ where resistance as an MIC of ≥ 2 $\mu\text{g/ml}$. According to this definition, the MICs of fusidic acid in MRSA 43300 and MSSA 29213 were within the susceptibility range whereas clinical strains were out of the acceptable range after the 10th day cycle. Although the MICs of MRSA 43300 and MRSA 29213 were within the limit of susceptibility range, their MICs were increased markedly up to 8-16 folds during the experiment using single drugs where the combination with phytochemicals showed no significant changes or only slight changes (maximum 2 fold) in MICs. The results were quite interesting because they showed phytochemicals have a great impact on the prevention of emergence of mutations in the *S. aureus* strains tested.

Moreover, gallic acid ethyl ester at a sub-MIC concentration could greatly delay the occurrence of a mutation in the tested strains while tannic acid and quercetin sub-MIC concentration could prevent the occurrence of mutations. According to the results obtained *in vitro*, it was concluded that phytochemicals have a role in the prevention of mutation and a higher impact on mutant prevention.

One of the mechanisms discussed regarding fusidic acid resistance was about alteration of elongation factor G mediated chromosomally [169, 170], where it was associated with the translocation step in which mRNA was processed via codon 1 to

start another round of polypeptide production [171]. Chopra reported that *S. aureus* resistant mutant selected from fusidic acid containing media had an altered elongation factor [172]. Therefore, fusidic acid resistant mutants could be selected by exposure to sub-MIC concentrations of antibiotic alone and the mechanism was mainly in alteration of the elongation factor. As the organism which had an altered elongation factor had a higher MIC value than normal strains [172], alteration of elongation factor G in *S. aureus* was a key factor in the emergence of resistance. Mason *et al.* also suggested a significant association between high rates of resistance to fusidic acid and the clinical use of topical (local) fusidic acid among MSSA isolates [173].

Another factor discussed regarding fusidic acid resistance mechanisms was the alteration of permeability: that is, plasmid-mediated resistance (acquisition of the resistance gene *fusB* from other microorganisms) to *S. aureus* and inactivation by enzymes such as CAT-I (chloramphenicol acetyltransferase-I) which can inactivate the action of fusidic acid by competitively binding it and sequestering it [170].

The mechanism by which phytochemicals (TA, Quer and GA) can prevent or delay the emergence of resistance is still unclear. But phytochemicals mostly have multi-targeted actions, which could provide a solution for this problem. Tannic acid and quercetin have synergistic action and gallic acid ethyl ester has additive effect with fusidic acid according to the FIC indexes mentioned above, which showed that the two drugs work together against *S. aureus*.

Fusidic acid combined with rifampicin, β -lactams and glycopeptides was also effective against *S. aureus* bacteremia, endocarditis, and osteomyelitis [102, 104]. Benjamin *et al.* also recommended that the rate of resistance to fusidic acid will be low if fusidic acid is appropriately used in combination with other antibiotics [171]. However, the effects of fusidic acid combined with phytochemicals and the use of phytochemicals on the prevention of the emergence of mutations is new in this area.

Figure 6.4 illustrated that the selection of emergence of rifampicin resistance with rifampicin alone and combined with a fixed concentration ($0.25 \times \text{MIC}$) of phytochemicals (TA, Quer and GA). After 10 days of continuous stepwise serial exposure to rifampicin alone, the MIC was increased up to 8-16 MIC, but the

combination of rifampicin with a fixed concentration ($0.25 \times \text{MIC}$) of phytochemicals (TA, Quer and GA) showed no change or a slightly increased (maximum 2 fold) MIC. In brief, rifampicin combined with fixed concentrations ($0.25 \times \text{MIC}$) of tannic acid resulted in no change in MIC in all 5 strains tested after the 10th cycle, while rifampicin combined with quercetin and gallic acid ethyl ester resulted in a slight change MIC (maximum 2 fold) starting from the 9th and the 10th cycle depending on the strains tested.

The rifampicin resistance problem is still unresolved and needs to be explored for newer approaches to overcome it. As the rate of treatment failure with standard regimens for tuberculosis is high and treatment with second-line strategies also has high side effects and less efficacy, newer approaches or methods to prevent rifampicin resistance are necessary [174]. Therefore, there is a need to have a clear understanding of rifampicin resistance patterns nowadays.

Rifampicin is a first line drug in the treatment of tuberculosis. Moreover, rifampicin can also be used in combination with fusidic acid [175], sulfamethoxazole-trimethoprim [157], minocycline [174] and daptomycin [176], against Gram positive bacterial infections especially caused by MRSA. Rifampicin is recommended for use in combination with other drugs since rifampicin monotherapy is definitely associated with the occurrence of mutations and it is contraindicated [157, 171, 174, 175]. Moreover, Neogi *et al.* reported that rifampicin resistance may occur in clinical practise despite the use of combinations with minocycline and fusidic acid [174]. Therefore, there is a need to find new combination methods that are more effective against MRSA to prevent the development of mutations rifampicin resistance.

In this study, it was determined that the phytochemicals (tannic acid, quercetin and gallic acid ethyl ester) in combination with rifampicin and fusidic acid could greatly delay or prevent the occurrence of mutations in *S. aureus*. The mechanisms relating to how the phytochemicals prevent the emergence of resistance in *S. aureus* are still unclear. However, one of the reasons why these combinatory pairs are effective against *S. aureus* may be the action of synergistic and additive effects that allows these drugs to work together effectively against *S. aureus* and even can prevent or greatly delay the emergence of mutations.

Our findings suggested that phytochemicals had anti-*Staphylococcal* activities and could prevent or delay the emergence of resistance in combination. Therefore, combination therapy of fusidic acid and rifampicin with tannic acid, quercetin and gallic acid ethyl ester can be potentially used as a drug combination regime for the prevention of emergence of fusidic acid and rifampicin resistance in *S. aureus*.

Chapter 7 : Dynamic Drug Combination Responses on Antibiotic Resistance Mutations of MRSA

7.1 Introduction

Dynamic drug combination treatment is a treatment strategy that has been used for metabolic diseases like cancer and viral infections like HIV infections. In cancer treatments, the doses are varied based on the clinical response of patients. The application of this strategy has shown to achieve better treatment efficacies and clinical outcomes in these conditions [177, 178]. In the case of multi-drug resistant bacteria like *S. aureus*, which causes serious complications and infections, especially in hospitalized patients, dynamic drug treatment has not been tested yet. MRSA has shown resistance to various antibiotic classes across the board [179]. It has been nicknamed a ‘Superbug’ by clinicians [124] because of its rapid adaptive resistance through several mechanisms. Many strategies including antibiotic combination therapy have been applied to combat resistance in MRSA. However, resistant species are still emerging and reported around the world. For instance, many species of vancomycin intermediate resistant MRSA have been reported from various parts of the world [180, 181].

New methods and strategies are critically necessary to formulate to guard against such indiscriminate resistance in *S. aureus*. The use of dynamic drug treatment strategy incorporating resistance modulating agents such as phytochemicals [19], can be an alternative method for preventing adaptive resistance.

In this chapter, the combinatory effects of the phytochemicals (tannic acid and quercetin) and the antibiotic (fusidic acid) against MRSA strains were studied. The synergistic combinatory pairs resulted from Chapter 4 were used to understand dynamic drug combination (DDC) response hypothesis on adaptive resistance of *S. aureus*. Drug combination methods were assigned based on drug proportions prior to their respective MICs (Explained details in later section). Firstly, the effects of using synergistic drug combinatory pair of fusidic acid and tannic acid were studied with different drug combination fashion. The resistance patterns were evaluated by

determining colony forming units (CFUs), minimal inhibitory concentrations (MICs) and population analysis profiles (PAPs) of the strain under drug combination influences continuously for ten days and also thirty days. This was done to understand short term and long term effects and further tested against 3 MRSA clinical strains collected from local hospital to understand the usefulness in clinical settings. The most effective methods resulted from the DDC response hypothesis were further validated with another synergistic combination pair of fusidic acid and quercetin. Based on the results of the experiments, it was found that the effectiveness of dynamic drug concentration method was largely dependent on the starting combination of phytochemical and antibiotic and it could prevent or greatly delay the adaptive mutation of *S. aureus*. Especially, the combination methods with a starting combination with higher concentration of phytochemical (> 20 %) had shown higher degree of resistance reduction from the start compared to higher concentration of antibiotic.

7.2 Materials and Methods

7.2.1 Bacterial strains and media used

Methicillin resistant *S. aureus* ATCC 43300 and 3 MRSA clinical strains collected from the National University Hospital, Singapore, were used in this study. Fusidic acid, tannic acid and quercetin used in this study were purchased from Sigma life Science, Singapore. IS broth and Agar (Biomed, Singapore) were used throughout the study for bacterial growth. Details about materials and media used are discussed in Chapter 3.

7.2.2 Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration of tannic acid against MRSA 43300 was 256 µg/ml, quercetin was 512-1024 µg/ml and fusidic acid was 0.03125-0.25 µg/ml, respectively. MIC determination was carried out according to the method described by Andrews [139]. The MIC for each method was checked daily to understand the adaptive resistant pattern.

7.2.3 Total Population Counts [CFU/ml]

The total population of bacteria under the influence of drug combinations were monitored daily by counting colony forming units [115]. The samples were grown on solid IS agar plates (triplicates) for overnight (18-24 hr) and counted with a colony counter. For each method, SPSS version 21 was used to analyse statistical significance.

7.2.4 Population Analysis Profile (PAP)

PAP of each method was also monitored every 3 days of the experiment. The samples were collected every 72 hr and grown on antibiotic agar plates of different concentration overnight to determine homogeneous or heterogeneous pattern of population. Area under curve (AUC) for the overall period was calculated by using Sigma-Plot software (2001) and the AUC ratios related to the positive control were interpreted to understand the homogeneous or heterogeneous nature of the bacteria population.

7.2.5 Procedure for testing Dynamic Drug Combination Response on MRSA mutations

Detailed procedures for drug/phytochemical preparations and the methods used to test DDC hypothesis were described in Chapter 3.3.6.3.

7.3 Results

The effects of DCC methods on MRSA strains were observed by measuring daily the MICs of each method, total population in terms of colony forming units per milometer and population analysis profiles (PAP) of bacterial growth. The results are shown in Figures 7.1 to 7.12.

Figure 7.1 Colony Forming Units of MRSA 43300 under the influence of different drug-phytochemical combination methods

Figure 7.2 Colony Forming Units of MRSA clinical strain C1 under the influence of different drug-phytochemical combination methods

Figure 7.3 Colony Forming Units of MRSA clinical strain C2 under the influence of different drug-phytochemical combination methods

Figure 7.4 Colony Forming Units of MRSA clinical strain C3 under the influence of different drug-phytochemical combination methods

Figure 7.5 Minimal Inhibitory Concentrations (MICs) of MRSA 43300 strain

Figure 7.6 Minimal Inhibitory Concentrations (MICs) of MRSA clinical strain C1

Figure 7.7 Minimal Inhibitory Concentrations (MICs) of MRSA clinical strain C2

Figure 7.8 Minimal Inhibitory Concentrations (MICs) of MRSA clinical strain C3

Figure 7.9 AUC ratios of the combination methods used against MRSA 43300 strain (AUC Ratio ≤ 1.2 = Homogeneous, >1.2 = Heterogeneous)

Figure 7.10 AUC ratios of the combination methods used against MRSA clinical strain C1 (AUC Ratio ≤ 1.2 = Homogeneous, >1.2 = Heterogeneous)

Figure 7.11 AUC ratios of the combination methods used prior to the positive control against MRSA clinical strain C2 (AUC Ratio ≤ 1.2 = Homogeneous, >1.2 = Heterogeneous)

Figure 7.12 AUC ratios of the combination methods used prior to the positive control against MRSA clinical strain C3 (AUC Ratio ≤ 1.2 = Homogeneous, >1.2 = Heterogeneous)

7.3.1 Population Growth

Colony forming units of MRSA ATCC 43300 strain under the influence of different drug combinations of tannic acid and fusidic acid for the duration of 30 days was illustrated in Figure 7.1. Each combination method has the individual controls for both fusidic acid and tannic acid so as to understand the effect of drug giving in single or in combination doses. It was observed that the effectiveness of the static drug combination was better in the methods containing higher concentrations of phytochemicals such as methods S50 and M10. The population growth difference is observed with $\geq 5\log_{10}$ CFU/ml between these methods and the other three methods: M1, M4 and M7 which contain a higher proportion of fusidic acid except method M7. That means the static combination methods containing higher concentrations of the phytochemical tannic acid have the ability to control bacterial population growth to $\leq 6\log_{10}$ CFU/ml, and thus reducing the chance for occurrence of adaptive mutation rate in MRSA. However, all combination methods were effective ($\geq 3\log_{10}$ CFU/ml difference) as compared with single controls (data not shown).

Fluctuating or Dynamic methods were not effective to control bacterial growth to minimum except those combination methods containing higher proportion of phytochemical tannic acid like Methods M6 and M12.

Both Descending and Ascending methods were effectively used to control the population growth. In Descending methods the overall population growth was increased $> 6\log_{10}$ CFU/ml in the first 5 days and declined to average CFU of $4.56\log_{10}$ CFU/ml at Day 30. The effectiveness of Descending methods was stable throughout the study after Day 5. In Ascending methods, the overall population growth is $\leq 6\log_{10}$ CFU/ml in all methods except methods s3, s7 and s8 because their population growth difference is less than $3\log_{10}$ CFU/ml compared to positive and individual controls. Overall, 85% of the methods used to test Dynamic drug hypothesis is effective ($\geq 3\log_{10}$ CFU/ml difference, P value < 0.05) to control the bacterial growth and thus reduce the chance for the adaptive mutation rate to increase.

The effect of the DDC hypothesis was also tested against MRSA clinical strains (C1, C2 and C3) to understand the effectiveness and usefulness in clinical settings and the

results were shown in Figures 7.2, 7.3 and 7.4. Among Static and Dynamic methods, methods M9, M10, M11 and M12 were found to be the most effective methods (p value < 0.05) having average populations of $4.72 \log_{10}$ CFU/ml in clinical strain C1, $5.12 \log_{10}$ CFU/ml in clinical strain C2 and $4.31 \log_{10}$ CFU/ml in clinical strain C3 respectively. Ascending methods were effective to control population growth (total average CFU $< 8 \log_{10}$ CFU/ml and $\geq 3 \log_{10}$ CFU/ml difference with individual controls) in all 3 clinical strains. Descending methods were also able to control population growth except in methods S3, S4, S5 and S6 in clinical strain C1 and methods S4 and S6 in clinical strain C2 in which total average CFU/ml is over $8 \log_{10}$ CFU/ml. However, the methods showed synergistic effects as their final population is still at least $3 \log_{10}$ CFU/ml difference with individual controls. In general, most of the methods used in Descending and Ascending methods were effective to control population growth in MRSA.

7.3.2 Minimal Inhibitory Concentrations

The minimal inhibitory concentration of the methods used in DDCR hypothesis against MRSA ATCC 43300 was illustrated in Figure 7.5. The highest MIC observed in fluctuating methods is 512 times higher and the lowest MIC is 2-4 times higher than the original MIC. For Static methods, the highest MIC observed is 1024 times and lowest MIC is 2-4 times higher than the normal MIC. In Descending and Ascending methods, the highest MIC observed is 4-16 times and the lowest MIC is 1-2 times higher than the original MIC. It was interesting to observe that the drug combination methods containing higher concentrations of the phytochemical tannic acid can prevent or greatly delay the increased in MIC.

The increase in MICs in Methods like M8, M9, M11 and M12 is 2-4 times higher than the original values within a period of 30 days is quite acceptable when it is compared with the MICs resulting from the Methods M2, M3, M5 and M6 because their MICs at the end of 30 days is 32-512 times than the original MIC values.

All of Descending methods were effective as the MICs at the end of 30 days were 1-4 times higher than the original MICs and, for Ascending methods, MICs increased 1-16 times more than the original value whereas the individual control with 100% fusidic

acid alone gave rise to 1024 times increase in MIC at the end of the 30 days' experiment, meaning that giving fusidic acid alone could definitely give rise to adaptive mutations. Overall, all the methods used in Ascending and Descending methods were effective in preventing or delaying adaptive increase in MICs of tested MRSA ATCC 43300 strain.

MICs of DDC methods on MRSA clinical strains were illustrated in Figures 7.9 to 7.11. In fluctuating methods, it was found that methods M9, M11 and M12 were effective to prevent or delay adaptive increase in MIC in all clinical strains having MICs of ≤ 2 times compared to original MICs.

For static methods, only method M10 is effective to prevent adaptive increase in MIC, having an unaltered MIC after Day 10 experiment. All of the Descending and Ascending methods were effective to prevent or delay adaptive increase in MICs having MICs of ≤ 2 times compared with the original MICs in all MRSA clinical strains.

7.3.3 Population Analysis Profile

Population analysis profiles of the methods used to understand the DDCR hypothesis against MRSA ATCC 43300 was illustrated in Figure 7.9. It was found that 50% of population under the Static combination methods were homogeneously distributed and 45% for fluctuating methods. For descending and ascending methods, the bacteria growth is uniformly distributed 100% and 98% respectively. It was interesting to see uniform bacterial growth under the drug combination of ascending and descending methods. For fusidic acid individual and the 100% control, heterogeneous bacterial growth was observed starting from Day 3. Therefore, fusidic acid alone is not effective to control the bacterial growth.

PAP of the DDC methods against MRSA clinical strains (C1, C2 and C3) were also studied and shown in figures 7.10, 7.11 and 7.12. Homogeneous bacterial growth (AUC ratio ≤ 1.2) was observed within the bacteria population which was under the influence of Ascending and Descending methods in all 3 clinical strains with a few exceptions. Generally the bacterial population under those methods was

homogeneously distributed throughout the study. However, the bacterial growth under dynamic combination methods was heterogeneously distributed except in methods M11 and M12 in clinical strains C1 and C2 and also in methods M8 and M12 in clinical strain C3. In the Static method, heterogeneous bacteria growth was observed in methods M1, M4 and M7 in all 3 clinical strains.

7.4 Discussion

In this study, we tested the novel dynamic drug combination hypothesis on MRSA strains by using synergistic combination of fusidic acid (antibiotic) and tannic acid (phytochemical).

Figure 7.5-8 highlighted the detailed MICs of each method and the control against MRSA ATCC strain and clinical strains. The MICs of fusidic acid were rising with time in most of the methods used in Dynamic methods except Methods M6 and M12. By comparing the MICs of each method, it could be suggested that the phytochemical tannic acid was stable with time by itself or in combination.

Population profiles of the combination methods were also tested and the areas under curve (AUC) ratios of bacterial growth treated with tested combination methods to positive control (bacterial growth without drug influence) were calculated by using Sigma-Plot (2001) software. The results (Figure 7.9-12) showed that resistant strains were growing in a homogeneous pattern except in the methods used in fluctuating and static combination methods in which they revealed increased mutants growth at higher concentration of fusidic acid (heterogeneous pattern). Moreover, individual controls and 100% fusidic acid controls displayed heterogeneous pattern of bacteria growth. Therefore, treatment with fusidic acid as a single therapy should be contraindicated as it will definitely give rise to resistant strains.

The synergistic action of the phytochemicals tannic acid in combination with the antibiotic fusidic acid against MRSA strains was mentioned in Chapter 4. Tannic acid could reduce the MIC of fusidic acid from 1024 folds MIC to 1-2 folds MIC.

We observed the fusidic acid MICs for the control methods were relatively higher than those of the tested methods. Moreover, the methods starting with higher concentrations of tannic acid revealed reduced MIC values compared with the methods starting with higher concentration of fusidic acid. The tannic acid MIC was relatively stable and unchanged even after 10 continuous days' cycle. However, fusidic acid MICs treated with most of the Descending and Ascending methods resulted in unchanged or slightly changed MICs, revealing their effectiveness against MRSA strain.

The differences between the methods were the difference in starting concentrations (different proportions of fusidic acid and tannic acid) and dosage combination pattern. Starting from the second day onwards, dynamic dose proportions showed the same pattern. It was observed that giving a higher proportion of tannic acid gave rise to significant prevention of adaptive mutations with higher efficacy based on the results obtained by unchanged MIC and narrowing of PAPs. Compared with other methods, the combination of tannic acid and fusidic acid with different proportions could also delay mutations to some extent compared with using single compounds.

The influence of DDC methods were also observed against 3 MRSA clinical strains for 10 days. It was found that some of the methods used in Dynamic and Static combinations were effective in terms of controlling the bacteria growth and MICs. However most of the methods used in Ascending and Descending combinations were effective to control the bacteria growth ($\geq 3\log_{10}$ CFU/ml difference with the controls) and MICs (mostly unaltered or a maximum increase of 2 fold).

According to the results achieved from the 30 day experiments with MRSA ATCC 43300 and the 10 day experiment with 3 MRSA clinical strains, the most effective methods were chosen based on bacterial population (final population of ≤ 8 CFU/ml for both 30 days and 10 days experiment) and MIC level (≤ 4 folds increased for 30 days experiment and ≤ 10 days experiment). Method M10 from the Static methods, M9, M11 and M12 from the Dynamic methods, all of the methods from Descending and Ascending methods were found to be effective to control bacterial growth and MICs. Therefore, the effects of these methods on MRSA strains were further validated with another synergistic combinatory pair of fusidic acid and quercetin for the duration of 10 days against 4 MRSA strains. The results are shown in Appendix C. The results

were found to be effective against tested MRSA strains and consistent with the results resulted from using the synergistic pair of fusidic acid and tannic acid.

Therefore, instead of giving the normal dosing scheme, synergistic antibiotics and phytochemicals in combination in a dynamic dosing fashion against MRSA infections can be a new or alternative therapeutic approach to delay or overcome the resistance problem in MRSA strains.

Chapter 8 : Characterization of Resistance Mutants

8.1 Introduction

Fusidic acid is a potent antibiotic that is active against Gram positive bacteria like *S. aureus*. It has a very low MIC against MRSA strains. However, clinical use of fusidic acid has been limited use because of its rapid resistance. There is a worry that fusidic acid resistant strains will spread around the whole world and this antibiotic will be no longer in use.

We have shown that fusidic acid is synergistic with the phytochemical tannic acid and their combination in different proportions could inhibit or prevent increased MIC *in vitro*. However, some of the adapted strains and their control strains show high MICs and the genetic cause for resistance was determined in some of the adapted strains and their controls strain which had high MICs. In this chapter, we firstly analysed mutation frequencies of mutants from the 30 day experiment and then sequenced and analysed the *fusA* gene of fusidic acid resistant strains with their respective primers. 3 out of 12 strains showed point mutations in the *fusA* gene. It was found that 11 point mutations occurred in the mutants: M1, M1.1 and FA100. However, the rest of the mutants didn't have any mutation in the *fusA* gene. Therefore, it was suggested that genetic involvement is not only a single determinant for fusidic acid resistance. Other well-known factors like efflux pumps, overactive enzymes and target adaptation can be involved as fusidic acid resistance determinant factors.

8.2 Materials and Methods

Detailed methods for sample preparations, PCR conditions, DNA extractions and sequencing were described in Chapter 3.

8.3 Results and Discussion

Following the isolation of mutants from the PAP studies, they were passaged on drug-free medium for 5 cycles to assess the stability of hetero-resistance developed to fusidic acid, based on PAP results and MICs. Generally, mutants were capable of undergoing further mutations even when the antibiotic pressure was removed. Therefore, the stability of the resistance was necessary to be assessed by repeated incubation in drug free IS broth and agar then followed by MIC determination.

The mutation frequencies for the isolated strains were evaluated on fusidic acid drugged agar plates having 64 and 128 $\times$ MIC. These are tabulated in Table 8.1 with their respective MICs.

Table 8.1 Mutation frequency of the mutants from 30 days experiment and their control strains

Strains	MIC of the Samples ($\mu\text{g/ml}$) $\times$ 1MIC	Mutation Frequency	
		64 $\times$ MIC	128 $\times$ MIC
MRSA ATCC 43300 (Parent Strain)	1	$< 10^{-9}$	$< 10^{-9}$
FA100 (fusidic acid 100%)	1024	2.2×10^{-7}	1.2×10^{-7}
TA100 (tannic acid 100%)	1	4.1×10^{-8}	$< 10^{-9}$
PC (positive control)	4	5.6×10^{-8}	1.3×10^{-8}
M1.1 (fusidic acid 80% control)	2048	2.4×10^{-7}	1.8×10^{-7}
M3.1 (fusidic acid 80% control)	1024	1.9×10^{-7}	7.5×10^{-8}
M4.1 (fusidic acid 60% control)	512	1.1×10^{-7}	5.1×10^{-8}
M5.1 (fusidic acid 60% control)	1024	1.5×10^{-7}	2.2×10^{-7}
M1 (fusidic acid + tannic acid)	1024	1.8×10^{-7}	5×10^{-8}
M3 (fusidic acid + tannic acid)	512	1.2×10^{-7}	3.8×10^{-8}
M4 (fusidic acid + tannic acid)	128	7×10^{-8}	1.2×10^{-8}
M5 (fusidic acid + tannic acid)	128	5.6×10^{-8}	2.5×10^{-8}
*The strain names are represented as drug, to which it was exposed to for 30 days except Parent strain and the methods which were earlier defined in Chapter 7			

Based on the mutation frequency evaluation, the *S. aureus* strains obtained at the end of 30 days after fusidic acid exposure, showed higher frequency of mutants compared to those exposed to drug-phytochemical combinations.

Single step mutation or point mutations were found within the mutants resulting from methods M1 and its control M1.1. The single step mutations were also noted in FA100 strains.

The substitution as CDs position 1207 of *fusA* gene changed codon 433 of FusA protein sequence from Proline (P) to Threonine (see figure 8.2). Mutations at codon 486 of mutants M1, M1.1 and individual control, FA100 are the first mutations found in this study. There were no previous reports about mutation at codon 433.

Figure 8.2 Multiple sequence alignments of the proteins encoded by the *fusA* gene: the parent strain PC (positive control), Gene Bank sequence and mutants from the methods (M1, M1.1, M3, M3.1, M4, M4.1, M5 and M5.1)

Figure 8.3 Multiple sequence alignments of the nucleotides from the mutants: the parent strain PC (positive control), Gene Bank sequence and mutants from the methods (M1, M1.1, M3, M3.1, M4, M4.1, M5 and M5.1)

Figure 8.4 Multiple sequence alignments of the proteins encoded by *fusA* gene: the parent strain PC (positive control), Gene Bank sequence and mutants from the methods (M1, M1.1, M3, M3.1, M4, M4.1, M5 and M5.1)

Figures 8.3 and 8.4 demonstrated the change in nucleic acids and subsequent amino acid alterations in the *fusA* gene. The change in nucleic acid of Guanosine to Thymine at CDs position 1457 resulted in alteration of protein sequence of Aspartic acid (D) to Tyrosine (Y) in FusA protein encoded by the FA100 mutant. Similarly, the change in Adenine to Cytosine at CDs position 1465 resulted in the alteration of Leucine (L) to Phenylalanine (F) at codon 488 of FusA protein in M1 and M1.1 mutants. The substitution at codon 488 was previously reported by Castanheira *et al* [28]. However, in their study, Leucine (L) was substituted by Lysine (K). The authors also suggested that point mutation of L488K was the only contributing factor that gave rise to an increase in fusidic acid MIC.

The remaining single step mutations observed in FA100 mutants are substitutions of Phenylalanine (F) to Isolucine (I) at CDs 496, Valine (V) to Glycine (G) at CDs 498, Asparagine (N) to Tyrosine (Y) at CDs 501, Serine (S) to Threonine (T) at CDs 515, Serine (S) to Alanine (A) at CDs 516, Alanine (A) to Proline (P) at CDs 547 and Glutamic acid (E) to Lysine (K) at CDs 552.

Scanning Electron Microscope (SEM)

The morphology changes of the mutants were studied with Scanning Electron Microscope (SEM). Figure 8.5 shows the electron micrographs taken by SEM at 6000 times magnifications.

Figure 8.5 Scanning Electron Micrographs of MRSA resistance strains (M1, M3, M4, M5), their control strains (M1.1, M3.1, M4.1, M5.1) and Positive controls (PC1, PC2, PC3)

Elongation factor G (EF-G) is a bacterial protein that is necessary for the translocation step of bacterial protein synthesis [182]. Fusidic acid interacts with a complex of EF-G and the ribosome, in which it inhibits protein synthesis by preventing the release of EF-G-GDP from the ribosome [183]. *fusA* is the gene encoding for EF-G and mutation in the *fusA* gene will result in abnormal functioning of EF-G, thus further inhibiting bacterial protein synthesis [184].

There are two genetic classes of fusidic acid resistance that have been reported previously [165, 166]. The first class of resistance mechanism is associated with mutations in the *fusA* gene and the second class is usually associated with acquisition of the pUB101 plasmid carrying the *fusB* gene which encodes an inducible protein to protect EF-G against the inhibitory action of fusidic acid [185]. In this study, the parent strain used in this study is the standard ATCC strain and the MIC is much lower than the cut off value of 0.5 µg/ml, therefore, it was assumed that there was no plasmid present in the mutants recovered from 30 days combination study. Later it was tested for the presence of the plasmid which contains the *fusB* gene by PCR tests using specific primers. It was found that there was no plasmid inside the mutants recovered from the 30 day study. Therefore, it was focused on the first class of fusidic acid resistance that FA resistance arose from mutation in *fusA* gene.

In this study, single step mutations were observed in the mutants M1, M1.1 and FA100. Mutant 1 was recovered from the 30 day drug combination study of the phytochemical tannic acid and the antibiotic fusidic acid by the Method M1 which contains 80% fusidic acid and 20% tannic acid. Mutant M1.1 was recovered from Method M1.1 which is the 80% fusidic acid control. Mutant FA100 was recovered from the method FA100 which used 100% (1MIC) fusidic acid for the whole 30 days experiment.

In this study, we found 11 point mutation in 3 mutant strains which showed increased fusidic acid MICs. Mutation at CDs 488 was previously reported by Castanheira *et al.* The rest of the mutations were not previously reported even though there were a total of over 30 different amino acid substitutions causing fusidic acid resistance in MRSA [165, 183, 186]. It was interesting to observe the mutation in mutant M1 because it was recovered from the methods contained the synergistic combinatory pairs of tannic acid and fusidic acid. However, the proportion of the phytochemical used in the method is low (only 20%) and it might be one of the facts to consider for the reason for mutation. It can also be assumed that tannic acid at 20% concentration is not enough to inhibit the mutation of fusidic acid.

Mutation found in the mutants M1.1 and FA100 are consistent with high MIC readings and heterogeneous growth in PAP results because they are recovered from the method M1.1 which contained 80% fusidic acid alone and FA100 which contains 100% fusidic acid alone as the controls.

Chopra *et al.* reported that fusidic acid is genetically unstable and single use of fusidic acid will result in genetic mutation and will give rise to mutant strains [172]. Therefore, it was recommended to use in combination with other antibiotics which are active against gram positive bacteria [187]. In this study, it was found that single fusidic acid controls (M1.1 and FA100) gave rise to mutation in the *fusA* gene and thus give rise to an increase in MIC. There are no mutations in the mutants recovered from the methods which contain >20% of phytochemicals in combinations. Therefore, it can be concluded that giving phytochemicals (>20%) in combination with synergistic antibiotic can delay genetic mutation in MRSA.

Morphology changes in relation to gene mutations have been reported previously in MRSA bacterial strains [188], fungal strains like *Candida albicans* [189] and yeast like *Saccharomyces cerevisiae* [190]. Therefore, there is a possibility that the mutants under study might change their morphology. It was revealed that one of the mechanisms for fusidic acid resistance in MRSA strains is by mutation of *fusA* gene. Therefore, the morphology pattern of mutant strains resulted from the DDC hypothesis test was studied with SEM analysis. Figure 8.5 shows scanning electron micrographs of MRSA resistance strains (M1, M3, M4, M5) and their control strains. It was found that mutants that arose from fusidic acid individual controls and the DDC methods (M1 and 1.1) revealed the normal spherical morphology pattern even though there were point mutations in the *fusA* gene.

Chapter 9 : Phytochemical Potentiators for Antibiotic Resistance

Adaptation in MRSA

9.1 Introduction

MRSA has become a well-known pathogen because of its resistance adaptation mechanisms. Among them, efflux pump that pump out incoming antibiotics is one of the accepted mechanisms that confer the increase MIC in MRSA [117, 191]. The overactive efflux pump activity can result from systems that are resistant to a specific class of antibiotics or to a large number of unrelated antimicrobial agents, resulting in a multidrug-resistant phenotype [1, 192].

Much attention has been given in the search for new compounds which have the ability to inhibit or modulate efflux activity of multidrug resistance bacteria when used in combination, thereby increasing the activity of the partner drugs [158]. Some phytochemicals have been reported for their activities to modulate efflux pump activities of multidrug resistant bacteria such as ethyl gallate derived from the plant called *Caesalpinia spinosa* which can potentiate the activities of β -lactam antibiotics. Epicatechin gallate and Epigallocatechin gallate derived from *Camellia sinensis* also have the activity to modify the effects of norfloxacin, imipenem, panipenem and β -lactams [193-195]. Antibacterial action against MRSA and their ability to reduce the MIC of a partner drug when used in combination draws critical attention in phytochemicals research as a new alternative treatment approach for complicated antibacterial therapy [15].

Tannic acid or tannin is a polyphenol which has been reported to have antibacterial activity against MRSA [67]. It has been shown to inhibit or delay the adaptive resistance in MRSA [150]. The synergism with antibiotics against MRSA clinical strains has also been reported previously [196]. The possible efflux inhibiting activity against MRSA was demonstrated by using the universal efflux substrate ethidium bromide in a Rotor Gene 3000TM as described by Ramallete *et al* [152].

The effect of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester on Methicillin Resistant *Staphylococcus aureus* (MRSA) (three clinical strains) was studied individually and in combination with the western antibiotic fusidic acid. The combinatory drugs were synergistic or additive to each other and the combined effect of the two drugs could delay or prevent the occurrence of adaptive mutations (Increased MICs) in MRSA. The possible mechanism might be conferred by the phytochemicals under investigation which could potentiate the effect of fusidic acid and/or acts as an alternative target that results in lysis of bacteria.

In this study, it was found that the mutants resulting from the continuous exposure to sub-MIC concentrations of fusidic acid revealed an over active efflux pump mechanism as measured by the ethidium bromide (EtBr) accumulation assay. However, the parent strains have no efflux pump activity. Therefore, it can be concluded that the strains which normally don't have efflux activity can result in over activity of efflux pump if they are being exposed to sub-MIC concentrations of western antibiotics (fusidic acid). Efflux modulating activity of tannic acid was tested against the strains with over active efflux pump activity. It was noticed that EtBr accumulation was increased over time when the strains were being exposed to sub-MIC concentrations (0.3 and 0.6 MIC) of phytochemicals suggesting they play a role in efflux modulating activity against MRSA strains that results in a decrease in MIC of the partner drug and thus, preventing its adaptive mutation.

9.2 Materials and Methods

9.2.1 Bacterial strains

Three clinical strains of MRSA collected from the National University Hospital, Singapore were used in this study. The strains initially exhibited no efflux activity based on the EtBr accumulating assay and their MICs were also within an acceptable range (≤ 0.5 $\mu\text{g/ml}$). These strains were exposed to sub-MIC concentrations of fusidic acid for a period of 10 continuous days and their MICs increased up to 32 times and named accordingly to the drug concentrations that they were being exposed. For every strain, a total of 5 sub-MIC concentrations: 0.8, 0.6, 0.5, 0.4 and 0.2, of fusidic acid were used and named accordingly. For example, the MRSA clinical strain C1 that

was being exposed with 0.8 MIC of fusidic acid alone for a period of ten days was named as C1_80.

9.2.2 Growth condition

The bacterial strains were grown at 37 °C in IS Broth with aeration. IS broth and Agar were used throughout the study and were purchased from Oxoid (Singapore).

9.2.3 Determination of minimum inhibitory concentrations (MICs)

The MICs of the compounds used in this study were previously reported [139]. The MICs of the fusidic acid and tannic acid were 0.25 µg/ml and 256 µg/ml respectively for all strains as determined by the microplate microbroth dilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [15].

9.2.4 Real-time ethidium bromide accumulation assay

The activity of drug combinations against potential bacterial efflux systems was assessed by the semi-automated EtBr method as previously described by Ramalhete *et al* using a Rotor-Gene 3000TM Thermal Cycler with real-time analysis software (Corbett Research, Sydney, Australia) [152]. Briefly, strains were cultured in IS broth until they reached an optical density at 600 nm (OD₆₀₀) of 0.6 and were then centrifuged at 13,000 rpm for 3 min. Bacterial pellets were re-suspended and were washed twice in phosphate-buffered saline (PBS) (pH 7.4) containing glucose to yield a final concentration of 0.4%. The OD₆₀₀ was adjusted with PBS-glucose to 0.6, the suspension was mixed by vortex and 50 µL of the suspension was transferred to 0.2 ml tubes, followed by addition of 45 µL of PBS containing 1 µg/ml of EtBr (Sigma-Aldrich, Singapore).

The tubes were placed in a Rotor-Gene 3000 Thermal Cycler and the amount of fluorescence representing accumulated EtBr by the bacterium was monitored on a real-time basis.

From the real-time data using relative fluorescence (RF) over a 30-min period of EtBr accumulation, the slope of the accumulation-time curve was calculated by linear regression analysis (Microsoft Excel). The greater the difference in the slope between the strains treated with the phytochemical and the strain without any compound was indicative of the activity of the compound to inhibit the efflux of EtBr. The relative final fluorescence (RFF) of the last time point (60 min) of the EtBr accumulation assay was also calculated according to the formula mentioned in Chapter 3.7.

9.3 Results

To determine efflux-modulating activity, it is necessary to use a concentration of any potential efflux pump inhibitor (EPI) that is below its bactericidal concentration [197]. Selection of this concentration was dependent on the MIC of the individual drugs. Sub-MIC concentrations of 0.3 and 0.6 MIC values are chosen to test against EPI activity of MRSA. As shown in Table 9.1, the MICs of the original strains are 0.25 µg/ml. However, after being exposed to sub MIC concentrations, the MIC increased up to 32 times higher than the original strain. As shown in Table 9.1, tannic acid has unique MIC values against all 3 MRSA clinical strains and also against their adapted strains that were being exposed to sub-MIC fusidic acid concentrations. It was noticed that 0.3 and 0.6 MIC of tannic acid has no significant bactericidal values against tested bacterial strains and therefore it was used in the EtBr accumulation assay.

Table 9.1 Minimum inhibitory concentrations (MICs) fusidic acid and tannic acid against parent MRSA clinical strains

Strains	Fusidic Acid MIC × 1x	Tannic Acid MIC (µg/ml)	EtBr (30mis)	EtBr(30mins) P value
C1_Ori	1x (0.25 µg/ml)	256	36.2457	
C1_80	32x	256	0.7834	0.025
C1_60	16x	256	0.7161	0.025
C1_50	16x	256	0.6597	0.025
C1_40	16x	256	0.8057	0.025
C1_20	16x	256	0.9804	0.025
C2_Ori	1x (0.25 µg/ml)	256	21.2270	
C2_80	32x	256	1.3630	0.025
C2_60	16x	256	1.3931	0.025
C2_50	16x	256	1.1804	0.025
C2_40	32x	256	1.2948	0.025

C2_20	16x	256	0.6555	0.025
C3_Ori	1x (0.25 µg/ml)	256	21.5881	
C3_80	32x	256	0.5807	0.025
C3_60	32x	256	2.1850	0.025
C3_50	16x	256	1.6849	0.025
C3_40	16x	256	1.0849	0.025
C3_20	16x	256	1.4894	0.025

Table 9.2 EtBr accumulation assay of MRSA clinical strains

Strains	^a TA 0.3MIC	^a TA 0.6 MIC	^a Parent Strain	RFF (30 mins)			^d RFF (60 mins)	
				^b TA 0.3 MIC	^b TA 0.6 MIC	^c Parent Strain	TA 0.3MIC	TA 0.6MIC
C1_Ori			0.73			33.7557		
C1_80	0.17	0.50		5.0516	14.4629		12.36648	32.5205
C1_60	0.22	0.65		7.1689	15.9955		13.02547	35.1715
C1_50	0.24	0.47		6.4241	13.2404		15.11194	27.6403
C1_40	0.21	0.44		5.9042	13.5708		14.14972	33.1397
C1_20	0.22	0.51		5.7208	15.5274		16.41243	35.0759
C2_Ori			0.57			20.4270		
C2_80	0.18	0.40		4.9728	10.6995		17.21774	28.2127
C2_60	0.18	0.22		4.4383	5.4000		14.02616	28.0528
C2_50	0.20	0.48		4.9738	12.9460		16.18441	31.1137
C2_40	0.15	0.37		3.8846	9.8390		19.33627	34.1521
C2_20	0.19	0.55		4.8293	14.9557		16.1628	32.1715
C3_Ori			0.81			23.9643		
C3_80	0.23	0.58		6.6033	15.8728		19.17639	32.1738
C3_60	0.26	0.40		5.2063	10.1692		17.17538	30.1828
C3_50	0.21	0.50		5.2435	13.5258		21.19573	36.2111
C3_40	0.28	0.60		6.5533	16.0622		18.22613	31.2391
C3_20	0.33	0.50		7.2871	13.3567		18.24931	37.2561

TA, tannic acid

^a Slope of accumulation of EtBr by parent strain (without treatment with any compound) and the efflux exhibiting adapted strains (treated with 0.3 and 0.6 MIC of tannic acid)

^b Relative final fluorescence (RFF) = $RF_{\text{treated}} - RF_{\text{untreated}}$, where RF_{treated} is the relative final fluorescence (RF) at the last time point of the EtBr retention curve (at 30 mins) in the presence of sub-MIC concentrations (0.3 and 0.6 MIC) of tannic acid and $RF_{\text{untreated}}$ is the RF at the last time point of the EtBr retention curve of the untreated control (without the compound)

^c Relative final fluorescence of parent strains at the last time point of the EtBr accumulation curve

^d Relative final fluorescence (RFF) = $RF_{\text{treated}} - RF_{\text{untreated}}$, where RF_{treated} is the relative final fluorescence (RF) at the last time point of the EtBr retention curve (at 60 mins) in the presence of sub-MIC concentration of tannic acid and $RF_{\text{untreated}}$ is the RF at the last time point of the EtBr retention curve of the untreated control (without tannic acid)

9.3.1 Determination of efflux action by EtBr accumulation Assay

Accumulation of EtBr by the MRSA 3 clinical strains and their adapted strains were investigated with the methods described by Ramalhete *et al* [152]. It was noticed that

the accumulation was higher in parent MRSA clinical strains compared with the adapted strains, suggesting that no overactive efflux activities take places. However, the prominent reduction in EtBr levels (overall less than 1) was noticed over a period of 30 minutes in adapted strains, suggesting the over active efflux activities as EtBr is a universal efflux substrate.

9.3.2 Effect of tannic acid on efflux exhibiting strains

Efflux pump inhibiting activity of tannic acid at two sub-MIC concentrations (0.3 and 0.6 MICs) was evaluated against 3 MRSA clinical strains and their adapted strains which showed increased activity of efflux pumps in the EtBr accumulation assay. The strains were exposed to sub-MIC concentrations of fusidic acid alone for 10 continuous days, resulting in an increase in MIC of fusidic acid up to 32 times compared to the parent strain at Day 1. The possible mechanism behind this was evaluated by using already established methods prescribed by Ramalhete *et al* [152].

An example of the results obtained from the semi-automated method, representing accumulation of EtBr by the MRSA strain C1, is shown in Figure 9.1. The efflux inhibiting activity of tannic acid was concentration dependent as the accumulation of EtBr is increased with the addition of 0.6 MIC of tannic acid compared to the results from 0.3 MIC of the compound. The similar results were found when the relative final fluorescence was calculated. The activity of Tannic acid on EtBr accumulation and its RFF was summarized in Table 9.2.

Figure 9.1 Effect of Tannic acid on EtBr accumulation Assay against MRSA C1 strain

9.4 Discussion

The search for new antibacterial compounds has become popular with the spread of multi-drug resistant bacteria and less antibiotics are effective to counteract those bacteria including multidrug resistant *S. aureus* [14]. Phytochemicals have been proposed as alternative drugs because of their antibacterial and anti-cancer effects. Phytochemicals have been reported as modulating or modifying agents when they are given together with other drugs [198]. Ramanhete *et al.* reported that phytochemicals have the action of reversing cancer cells resistance to anti-cancer drugs [152]. Lewis *et al* stated that phytochemicals can even reverse or reduce antibiotic resistance in MDR bacterial strains [199]. Moreover, the side effects occurring the use phytochemicals are considerably less since they are derived from the plants [15]. All these reports are supporting phytochemicals as new therapeutic agents to fight complex disease like MRSA infections.

Tannic acid is a commercial form of tannin and it has been reported for antibacterial activity especially against *S. aureus* [67, 69]. The antibacterial action and its potentiating activity against MRSA strains were previously reported [196]. The proposed possible mechanism of tannic acid that achieves antibacterial effect is mainly by targeting of the bacterial cell wall [14] such as by complexing with a cell

wall protein, membrane disruption, metal ion complexation and binding to adhesins [71, 200]. However, the specific mechanism of how tannic acid works together with antibiotics is still needed to be determined.

Efflux inhibiting activity of tannic acid, quercetin and gallic acid ethyl ester was tested against three MRSA clinical strains which show efflux activities. Table 9.1 shows the MICs of MRSA clinical strains before and after exposure to sub-MIC concentrations of fusidic acid alone. Fusidic acid's MICs increased up to 32 times than the parent strains over a period of ten incubation days. Tannic acid MICs were also checked with both parent strains and adapted clinical strains and found to be equal in MIC. The sub-MIC adaptation of fusidic acid has no effect on tannic acid MIC at all.

The EtBr accumulation assay was tested for both adapted strains and parent strains. It was found that the adapted strains have the ability to pump out the incoming EtBr and the accumulation inside the bacteria is generally low (relative fluorescence index ≤ 1) as compared with parent strains which show increased accumulation (relative fluorescence index: 21-36).

There possible mechanism behind this phenomenon might be the bacteria might have already encoded the efflux expressing proteins that are silent until the bacteria is getting sufficient antibiotic pressure, meaning that the bacteria can rapidly adapt to sub-MIC concentrations of antibiotic. Alternately, it can be assumed that existing efflux proteins are expressed at a higher level, either through induction of expression or mutation in a regulator that acts as a repressor or controls expression.

Efflux modulating activity of phytochemicals on MRSA strains was also investigated. Sub-MIC concentrations (0.3 and 0.6 MIC) of phytochemicals were chosen and tested against MRSA strains which showed over active efflux activity. Figure 9.1 demonstrates EtBr accumulation vs a time curve of MRSA clinical strain C1 with the addition of tannic acid. The accumulation was increased with the addition of sub-MIC concentrations of tannic acid, especially at 0.6 MIC level. The accumulation was in a concentration dependent manner. We also observed the similar results in the tests with quercetin and gallic acid ethyl ester (see Appendix B).

One of the factors that enhances the effect of phytochemicals on MRSA is that it is a gram positive bacterium that is lacking an outer membrane [201]. Therefore, it is much easier to bind cell wall proteins which exhibit efflux activity.

In this study, it was found that the addition of sub-MIC concentrations of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester could inhibit or modulate the overall activity of efflux pumps in MRSA strains resulting in increased accumulation of EtBr. Therefore, the phytochemicals under study can be used as potential efflux pump inhibitor against MRSA strains especially against the resistant strains that arose from sub-MIC exposure to antibiotics.

Chapter 10 : Phytochemicals as the New Therapeutic Agents to Reverse Adaptive Mutation in MRSA

10.1 Introduction

MRSA has become a well-known clinical problem because of its adaptive resistance mechanisms against most of the antimicrobial agents currently used [124]. Only a few antibiotics are effective against MRSA infections and much attention has been given to the search of new compounds which have the ability to inhibit or modulate adaptive mechanism of bacteria when used alone or in combination.

Phytochemicals have been reported for their activities to modulate multidrug resistant bacteria like MRSA. Phytochemicals like ethyl gallate which were derived from the plant called *Caesalpinia spinosa*, can potentiate the activities of β -lactams antibiotics. Epicatechin gallate and Epigallocatechin gallate derived from *Camellia sinensis* have the activity to potentiate the effects of norfloxacin, imipenem, panipenem and β -lactams [193-195]. Anti-MRSA action and their ability to reduce the MIC of a partner drug when used in combination draw critical attention in phytochemical research as a new alternative treatment approach for complicated and multi-drug resistant antibacterial therapy [15].

Tannic acid and gallic acid ethyl ester are phenolic acids which have antibacterial activity against MRSA [67]. They have been shown to inhibit or delay the adaptive resistance in MRSA [150]. The synergism with antibiotics against MRSA clinical strains has also been reported previously [196].

Quercetin is a flavonoid and is widely present in fruits, vegetables and grains. It was found especially in apples and the outermost layers of onions where the highest concentration of quercetin is contained. It has anticancer effects, antibacterial effects, free radical scavenging effects and antiviral effects including against HIV viral infections.

The reversal of mutations in cancer cells by phytochemicals draws attention to phytochemical research. The studies by Lewis reported that the compounds isolated

from plants had the ability to reduce and even reverse the adaptive resistance (increased in MICs) of multi-drug resistant bacterial strains into normal strains [199]. This finding is crucial to focus on antibiotic resistance in multidrug resistance strains whether antibiotic resistance could be reverse by using phytochemicals.

In this study, the effectiveness of phytochemicals on adaptive reverse mutation was tested with MRSA mutant strains adapted from the exposure of sub-MIC concentration of fusidic acid alone. The potency of strains was confirmed by checking daily MICs of the strains after passing in nutrient broth without any compound for 5 continuous days. The adapted strains were then tested for the effects of phytochemicals on them. It was found that the phytochemicals under observation were able to reverse the adaptive mutation of fusidic acid in MRSA strains by reducing the MICs of resistance strains up to 8 fold, suggesting their roles in reverse adaptive mutation.

10.2 Materials and Methods

Detailed explanations about materials and methods used in this chapter are mentioned in the Chapter 3.

10.2.1 Bacterial strains

Three clinical strains of MRSA collected from National University Hospital, Singapore were used in this study. The MICs were also within an acceptable range of susceptibility ($\leq 0.5 \mu\text{g/ml}$). These strains were exposed to sub-MIC concentrations of fusidic acid for a period of 10 continuous days and their MICs increased up to 32 times and named accordingly to the drug concentrations that they were being exposed. For every strain, a total of 5 sub-MIC concentrations: 0.8, 0.6, 0.5, 0.4 and 0.2, of fusidic acid were individually used to expose the MRSA strains and named accordingly. For example, the MRSA clinical strain C1 that was being exposed to 0.8 MIC of fusidic acid alone for a period of ten days was named as “C1_80”. MRSA ATCC 43300 strain was used as a control strain.

10.2.2 Growth conditions

The adapted bacterial strains showing increased fusidic acid MICs were grown at 37 °C in IS Broth with aeration together with sub-MIC concentration of phytochemicals. IS broth and agar were used throughout the study and were purchased from Oxoid (Singapore).

The effects of phytochemicals on the three MRSA clinical strains which exhibit high MIC for fusidic acid were tested. A sub-MIC concentration (0.3 MIC) of phytochemicals was used to test their effect on reverse mutation as there was no bactericidal effect at this concentration. The strains were incubated with 0.3 MIC of each phytochemical for 10 continuous days in IS nutrient broth. Every day, 0.01ml of bacterial strains was taken out and tested for MICs.

10.2.3 Determination of minimum inhibitory concentrations (MICs)

The MIC was defined as the lowest concentration of drug that inhibited bacterial growth following incubation for 18-24 hr at 37 °C [139]. In this experiment, MIC was tested by the microplate microbroth dilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [15] and also by using solid media (agar plate method) as recommended by Schwalbe *et al* [202]. The MICs of the strains were repeated 3 times. Inoculum size was adjusted with 1.5×10^8 McFarland standard and then diluted to 10^6 CFU/ml.

10.3 Results

The MICs for fusidic acid were 0.03125-0.25 $\mu\text{g/ml}$ and the MICs of the phytochemicals varied from 256 -1024 $\mu\text{g/ml}$ respectively for all strains. After 10 days continuous exposure to sub-MIC concentrations of fusidic acid, the MIC of fusidic acid in the strains increased up to 32 times more than normal values depending on the concentration they were being exposed to (see Figure 10.1). The resulting MICs were confirmed again after passing in IS broth for five continuous days to relieve the culture from antibiotic pressure.

Figure 10.1 MICs of individual controls against MRSA ATCC 43300 and clinical strains

Table 10.1 MIC of fusidic acid (FA) before and after incubation with 0.3×MIC of phytochemicals: tannic acid, quercetin and gallic acid ethyl ester

Strains	FA MIC	FA MIC with		
		0.3 MIC TA	0.3 MIC Quer	0.3 MIC GA
C1_80	32×	16×	16×	32×
C1_60	16×	8×	8×	16×
C1_50	16×	8×	8×	16×
C1_40	16×	4×	8×	8×
C1_20	16×	4×	8×	8×
C2_80	32×	16×	16×	16×
C2_60	16×	8×	8×	8×
C2_50	16×	4×	4×	8×
C2_40	32×	4×	8×	16×
C2_20	16×	2×	8×	8×
C3_80	32×	16×	16×	16×
C3_60	32×	16×	16×	16×
C3_50	16×	8×	8×	8×
C3_40	16×	8×	8×	8×
C3_20	16×	4×	8×	8×
MR43_80	16×	4×	8×	8×
MR43-60	16×	4×	8×	8×
MR43_50	32×	8×	4×	8×
MR43_40	16×	2×	4×	4×
MR43_20	16×	4×	2×	8×

The effect of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester on MRSA strains was tested to understand their effects in addition to antibacterial effects. 0.3 MIC of phytochemicals was chosen to avoid antibacterial effect. The adapted strains which exhibited high MICs for fusidic acid were exposed to 0.3×MIC of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester individually for 10 continuous days and their MICs were checked daily. It was noticed that phytochemicals under investigation were able to modulate resistant strains into less resistant strains as the MICs for fusidic acid was reduced by 2 fold.

10.4 Discussion

Phytochemicals have drawn interest for their antibacterial effect and the decline in antibiotic susceptibility nowadays draws critical attention to phytochemical research. They normally come from different sources and have different chemical structures

apart from bacterial and fungi based antibiotics which are able to be recognized by bacteria easily.

The phytochemicals under investigation have antibacterial action against MRSA strains. Their synergistic and additive actions with antibiotics including fusidic acid were reported previously [196]. Adding a sub-MIC concentration of the phytochemical tannic acid, quercetin and gallic acid ethyl ester together with step-wise increasing concentrations of the antibiotic fusidic acid and rifampin have proven effective and have prevented the adaptive mutation of the partner drug [150]. One of the assumptions behind this effect is that the phytochemical itself has anti-MRSA action and also has synergistic action with the partner antibiotics and reduce the population of bacteria effectively by reducing the chance for adaptive mutation. Cowen reported that phytochemicals have more than one action in achieving both anti-MRSA action and preventing mutation against antibiotics [14, 19].

In Figure 10.1, it was shown that giving a sub-MIC concentration of fusidic acid resulted in the occurrence of resistant strains (high MIC) depending on the sub-MIC concentration. Fusidic acid MICs were increased up to 32 times more than normal values within a period of 10 days. Chen *et al.* mentioned that fusidic acid is genetically unstable and a single use of fusidic acid should be contraindicated [203]. Fusidic acid was recommended to be used in combination with rifampicin as both antibiotics are synergistic to each other and effective against Gram positive bacteria like MRSA. However, both antibiotics are facing resistance problems and the combined use of these two antibiotics is not so effective and practical because rifampicin usage in clinical practice is limited and it is used only for tuberculosis infection [204].

Full-bloom rifampicin resistance will lead to failure of the anti-TB treatment scheme. New phytochemicals which are totally different in origin and structure and synergistic with fusidic acid will help to use fusidic acid in clinical practice effectively. Moreover, the use of fusidic acid in combination with phytochemicals will overcome the side effects that the patients suffered as the concentration used for the synergistic pair is only one quarter of normal MIC concentration and phytochemicals can be potential therapeutic compound for MRSA infections. The phytochemicals under

investigation are able to inhibit overall activity of efflux pumps in MRSA as tested by the ethidium bromide accumulation assay (data not shown) and this will help to reduce MICs. Bactericidal effects of each phytochemical were achieved through each specific mechanism. Tannic acid binds to proteins [72], by thus achieving enzyme inhibition, membrane disruption, metal ion complexation, substrate deprivation and binding to adhesins [71, 200, 205]. Quercetin binds to the bacterial DNA gyrase enzyme and inhibits protein synthesis [78].

Table 10.1 demonstrates the MICs of adapted strains before and after incubation with 0.3 MIC of tannic acid, quercetin and gallic acid ethyl ester. It was found that sub-MIC concentrations of phytochemicals were able to reduce or reverse the MIC of fusidic acid in MRSA stains. The strains showed increased sensitivity to fusidic acid (lowered MICs) after incubation with 0.3 MIC of phytochemicals (see table 10.1). It was clear that phytochemicals have potentiation actions other than bactericidal actions.

Chapter 11 : Conclusions and Further Recommendations

11.1 Effects of Antibiotic-Phytochemical combinations on MRSA

With an aim to find out the possible interactions between antibiotics and phytochemicals that have been reported as anti-staphylococcal activities, checkerboard assays were performed. The synergistic pairs were later counterchecked by using time-kill assays to understand the killing rate with time and to confirm the results from checkerboard method. It was found that the phytochemicals tannic acid (polyphenol), gallic acid ethyl ester (polyphenol) and quercetin (flavonoid) have interactions (synergistic or additive) with the antibiotics under study against MRSA standard ATCC and clinical strains. Phytochemicals under study can reduce the MIC of partner antibiotic up to 1/8 MIC in combination and therefore potentiating the effects of antibiotics. The phytochemical tannic acid has synergistic activity with fusidic acid (protein synthesis inhibitor), cefotaxime (cell wall synthesis inhibitor), minocycline (protein synthesis inhibitor) and rifampicin (DNA dependent RNA polymerase inhibitor). Quercetin has synergistic action with the antibiotic fusidic acid, minocycline and rifampicin. Gallic acid ethyl ester has additive action with all of the antibiotics under study. The rest of the phytochemicals under study (caffeic acid, menthone and eugenol) have no interactions with tested antibiotics.

11.2 Effects of Antibiotic-Phytochemical combination on MRSA resistance

11.2.1 Effects of same doses of antibiotic and phytochemicals on MRSA resistance

The effects of antibiotic-phytochemical combination pairs on adaptive resistance in MRSA were tested. The stability or effectiveness of phytochemicals or antibiotic was observed alone or in combinations. It was noticed that the antibiotics under study developed 1×MIC resistant mutants within 7 days and the phytochemical tannic acid took 17 days and quercetin took 14 days to develop 1×MIC resistant mutants in MRSA ATCC 43300 strain. In the case of the combinations, it took more than 30 days to get (1+1 MIC) resistant mutants. It was found that giving antibiotics alone

will definitely give rise to mutant strains. However, the addition of synergistic phytochemicals together with antibiotic will delay the occurrence of adaptive mutations in MRSA. The potentiating effects of phytochemicals bring down the chance of antibiotic resistance in MRSA.

11.2.2 Effects of fixed concentration of phytochemicals on antibiotics resistance in MRSA

To understand the effects of giving a fixed sub-MIC concentration of phytochemicals on adaptive mutations of MRSA, 0.25 MICs of the synergistic phytochemical tannic acid, quercetin and gallic acid ethyl ester was added to stepwise increasing concentrations of the antibiotics fusidic acid and rifampicin against MRSA strains. It was noticed that giving fixed sub-MIC concentrations of phytochemicals under study could delay or inhibit the occurrence of adaptive increases in MICs of partner antibiotics. The resistance modifying and preventing effects of phytochemicals together with synergistic antibiotics could be a new treatment option for the treatment of multi-drug resistance bacteria like MRSA.

11.2.3 Effects of the dynamic drug combination hypothesis on MRSA resistance

The dynamic drug combination hypothesis was tested by using synergistic pair of tannic acid and fusidic acid against MRSA ATCC 43300 strains for the duration of 10 days and 30 days to understand short term and long term effects of the combination methods used in the DDC hypothesis. DDC hypothesis was also tested against clinical strains collected from a local hospital to understand the effectiveness of the hypothesis in clinical settings. The best combinatory pairs were later tested with another synergistic pair of the phytochemical quercetin and fusidic acid. It was found that giving a phytochemical concentration of $\geq 20\%$ in combination with a synergistic antibiotic could delay or prevent the occurrence of adaptive mutations in Descending and Ascending methods. Therefore, the proposed novel dynamic drug combination hypothesis was found to be effective to overcome resistance problems in multi-drug resistant bacteria like MRSA.

11.3 Ethidium Bromide Accumulation Assay

The possible efflux pump inhibiting action of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester were investigated by using Ethidium Bromide Assay. As EtBr is a universal efflux pump inhibitor, the presence of potential Efflux Pump Inhibitor (EPI) will increase the accumulation of EtBr inside the bacteria. It was found that phytochemicals under study has EPI activity as the accumulation of EtBr inside the bacterium was increased with the addition of sub-MIC concentration of phytochemicals. It was also noted that the accumulation depends on the concentration of phytochemicals added and it can be concluded that one of the mechanisms that phytochemicals under study achieved synergistic or additive actions with antibiotics is efflux pump inhibiting activity.

11.4 Effects of phytochemicals on reversal of adaptive resistance in MRSA

The effect of the synergistic phytochemicals on reversal of adaptive MRSA resistance was observed with the MRSA ATCC strain and clinical strains. A sub-MIC concentration (0.3 MIC) of phytochemicals was used to avoid anti-bacterial effects and to understand adaptive action of phytochemicals. MRSA resistant strains that arose from a sub-MIC concentration of the antibiotic fusidic acid was used to understand the reversal effect of phytochemicals. It was found that synergistic phytochemicals under study could reverse the mutant strains into less severe mutant strains as the MICs of mutants strains were reduced with the addition of $0.25 \times \text{MIC}$ of the phytochemicals tannic acid, quercetin and gallic acid ethyl ester.

Based on the above findings, the following recommendations can be made for future studies:

1. *In vitro* results strongly suggested that the Dynamic Drug Combination hypothesis (DDC) was effective to prevent or delay MRSA adaptive resistance. Therefore, *in vivo* studies of the DDC hypothesis are proposed to understand the efficacy along with pharmacodynamics/pharmacokinetic profiles and potential side effects. There are two methods to test *in vivo* experiment. At first, it is aimed to create diseased animal model with MRSA

mutant strains and observe the effects of proposed DDC methods on it. Finally, autopsy can be done on diseased model to understand the cause of death/ or major organ damage. This method is aimed to test the efficacies of the combinatory pairs and to understand the side effects. Secondly, it is aimed to test toxicity level. The lethal dose can be test with single drug/phytochemical and also in combination.

2. As the phytochemicals under study might have more than one mechanism of action, it is proposed to study details about other possible mechanisms of action. In this study, it was found that phytochemicals under study have efflux pump inhibiting activity on MRSA adaptive resistance strains. As there are more than one type of efflux pumps, the activity of individual efflux pump should be analyze with standard strains which already processes specific efflux pump. Therefore, further research should be done to understand the interactions between adaptive resistance and overactive efflux pump activities. Moreover, genomic sequencing of all the mutant strains should be perform to understand strains adaptation.
3. General mathematical equations can be computed based on DDC hypothesis to understand easy input of drug and phytochemical concentrations with time to get effective treatment outcome.

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Appendix A

Table A-1: MICs of combination methods and their controls against MRSA ATCC 43300

Duration (Days)		D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
	REMARK																
M1	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
M1_MIC		1	1	4	4	4	4	4	8	8	16	16	64	64	64	128	256
M1.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
M1.1_MIC		1	1	4	4	8	8	16	16	16	16	16	128	128	128	256	256
M1.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
M1.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
M2	Test	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40
M2_MIC		1	1	2	2	4	4	2	4	8	8	8	8	8	8	16	16
M2.1	Control	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60
M2.1_MIC		1	1	1	1	2	8	8	8	8	16	16	64	64	128	128	128
M2.2	Control	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40
M2.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M3	Test	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80
M3_MIC		1	1	1	2	2	4	2	4	4	4	8	8	8	8	64	64
M3.1	Control	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20
M3.1_MIC		1	1	1	2	2	4	8	8	16	16	16	32	16	32	64	64
M3.2	Control	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80
M3.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M4	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
M4_MIC		1	1	2	2	4	4	4	8	8	8	8	8	8	8	8	16
M4.1	Control	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
M4.1_MIC		1	1	2	2	2	4	8	8	16	16	16	16	16	32	32	32
M4.2	Control	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
M4.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M5	Test	FA60-TA40	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
M5_MIC		1	1	2	2	4	2	4	4	8	8	8	8	8	8	16	64
M5.1	Control	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80
M5.1_MIC		1	1	4	4	4	8	8	16	16	16	16	16	16	16	32	32
M5.2	Control	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20
M5.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M6	Test	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80
M6_MIC		1	1	2	2	2	2	2	2	2	2	4	4	4	4	4	8
M6.1	Control	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20
M6.1_MIC		1	1	2	4	4	8	8	16	16	16	16	16	16	16	16	64
M6.2	Control	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80
M6.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S50	Test	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

		TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S50_MIC		1	1	2	1	2	2	2	4	2	2	4	4	4	4	8	8
S50.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S50.1_MIC		1	2	4	2	8	16	16	8	16	32	32	32	64	64	64	128
S50.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S50.2_MIC		1	1	1	1	1	1	1	1	2	1	1	2	1	1	1	1
M7	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
M7_MIC		1	1	1	2	2	2	2	2	2	4	4	4	4	4	4	4
M7.1	Control	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
M7.1-MIC		1	1	4	4	4	8	8	16	16	16	16	16	16	16	16	32
M7.2	Control	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
M7.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M8	Test	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20
M8_MIC		1	1	2	2	2	2	2	2	2	2	2	2	2	2	4	4
M8.1	Control	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80
M8.1_MIC		1	1	2	4	4	8	8	16	16	16	16	16	16	16	16	16
M8.2	Control	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20
M8.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M9	Test	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80
M9_MIC		1	1	1	2	2	2	2	2	2	2	2	2	2	2	4	2
M9.1	Control	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20
M9.1_MIC		1	1	2	2	4	8	8	16	16	16	16	32	32	64	32	32
M9.2	Control	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80
M9.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M10	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
M10_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2
M10.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
M10.1_MIC		1	1	1	2	2	4	4	8	8	16	16	16	16	16	32	32
M10.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
M10.2_MIC		1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	2
M11	Test	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40
M11_MIC		1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
M11.1	Control	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60
M11.1_MIC		1	1	2	4	4	8	8	32	32	32	16	16	16	16	32	64
M11.2	Control	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40
M11.2_MIC		1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	2
M12	Test	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20
M12_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2
M12.1	Control	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80
M12.1_MIC		1	1	2	2	4	4	8	8	16	16	16	16	16	16	16	32

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

M12.2	Control	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20
M12.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
FA100	Control	1	1	2	2	2	4	4	8	16	16	16	64	64	64	128	64
TA100	Control	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1
PC	Control	1	1	1	1	1	1	1	1	1	2	2	2	2	2	1	1

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

Duration (Days)		D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29	D30
	REMARK															
M1	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
M1 MIC		256	256	512	512	512	512	512	1024	1024	1024	1024	1024	1024	1024	1024
M1.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
M1.1 MIC		512	512	512	1024	1024	512	512	1024	1024	1024	1024	1024	1024	2048	2048
M1.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
M1.2 MIC		2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M2	Test	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20	FA60-TA40	FA80-TA20
M2 MIC		16	16	32	32	128	128	64	64	128	32	128	64	64	128	128
M2.1	Control	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80
M2.1 MIC		128	128	256	256	128	256	256	256	256	256	256	256	256	512	512
M2.2	Control	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20
M2.2 MIC		1	1	1	1	1	2	2	1	1	2	2	1	1	2	2
M3	Test	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20
M3 MIC		64	128	64	64	128	128	128	256	256	128	128	256	512	512	512
M3.1	Control	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80
M3.1 MIC		128	256	128	256	256	256	512	512	512	512	1024	1024	1024	1024	1024
M3.2	Control	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20
M3.2 MIC		1	1	1	2	1	2	2	1	1	1	1	2	2	2	2
M4	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
M4 MIC		16	32	16	128	64	64	64	128	128	128	128	128	128	128	128
M4.1	Control	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
M4.1 MIC		64	64	64	128	128	128	128	256	256	256	256	512	512	512	512
M4.2	Control	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
M4.2 MIC		1	1	1	1	1	1	1	1	2	1	2	1	1	2	2
M5	Test	FA60-TA40	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
M5 MIC		16	32	32	64	64	64	64	128	128	64	128	128	128	128	128
M5.1	Control	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60	FA80	FA60
M5.1 MIC		64	64	128	256	256	256	512	512	512	256	512	1024	1024	1024	1024
M5.2	Control	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40	TA20	TA40
M5.2 MIC		1	1	1	1	1	1	1	2	2	1	1	1	2	2	2
M6	Test	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40
M6 MIC		8	8	8	16	16	16	8	16	16	16	32	32	32	32	32
M6.1	Control	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60
M6.1 MIC		64	32	32	64	64	64	128	128	128	64	64	128	128	128	128
M6.2	Control	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40
M6.2 MIC		1	1	1	1	2	1	1	1	2	2	1	1	2	2	2
S50	Test	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-	FA50-

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

		TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S50_MIC		4	8	8	16	16	8	8	16	16	16	16	16	16	16	16
S50.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S50.1_MIC		64	128	256	128	256	256	512	256	512	256	512	256	512	512	512
S50.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S50.2_MIC		1	2	1	2	1	1	1	1	2	1	1	1	1	1	1
M7	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
M7_MIC		4	2	2	4	4	2	4	4	4	4	4	4	4	4	4
M7.1	Control	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
M7.1-MIC		64	32	32	64	64	32	64	64	64	64	64	128	128	128	128
M7.2	Control	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
M7.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M8	Test	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60	FA80-TA20	FA40-TA60
M8_MIC		2	2	4	4	8	8	4	4	4	2	2	2	4	4	4
M8.1	Control	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40	FA80	FA40
M8.1_MIC		32	32	32	32	32	16	16	32	32	32	64	64	64	64	64
M8.2	Control	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60	TA20	TA60
M8.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M9	Test	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60	FA20-TA80	FA40-TA60
M9_MIC		4	2	4	4	2	2	2	4	4	8	4	4	4	8	4
M9.1	Control	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40	FA20	FA40
M9.1_MIC		64	64	64	64	64	64	64	64	64	32	64	64	64	64	64
M9.2	Control	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60	TA80	TA60
M9.2_MIC		1	2	1	1	2	1	2	1	1	1	1	1	1	1	1
M10	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
M10_MIC		4	4	2	2	1	2	4	4	4	2	4	4	2	2	2
M10.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
M10.1_MIC		32	16	16	32	32	32	32	32	32	64	64	128	64	64	64
M10.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
M10.2_MIC		1	1	1	2	1	1	1	1	1	1	2	1	1	1	1
M11	Test	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80	FA60-TA40	FA20-TA80
M11_MIC		1	2	2	2	2	2	2	4	4	4	4	4	4	4	2
M11.1	Control	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20	FA60	FA20
M11.1_MIC		64	128	64	64	64	128	128	128	128	64	64	128	128	128	128
M11.2	Control	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80	TA40	TA80
M11.2_MIC		2	1	1	1	1	2	2	2	2	2	2	1	1	1	1
M12	Test	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80	FA80-TA20	FA20-TA80
M12_MIC		1	1	2	1	4	4	2	2	2	2	2	2	2	2	2
M12.1	Control	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20	FA80	FA20
M12.1_MIC		64	64	32	32	64	64	128	128	64	64	128	128	128	128	64

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

M12.2	Control	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80	TA20	TA80
M12.2 MIC		1	2	1	1	2	2	2	2	1	1	1	1	1	1	1
FA100	Control	128	256	512	512	256	256	512	1024	1024	512	512	1024	512	512	1024
TA100	Control	2	2	2	2	2	1	1	1	1	2	2	1	1	1	1
PC	Control	1	1	2	2	2	2	2	2	2	4	4	4	4	2	4

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

Duration (Days)		D0	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15
	REMARK																
S1 (Decreasing)	Test	FA80-TA20	FA60-TA40	FA40-TA60	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S1 MIC		1	2	2	4	2	2	4	4	2	2	1	1	1	2	1	2
S1.1	Control	FA80	FA60	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S1.1 MIC		1	2	4	8	4	8	16	16	32	32	16	16	32	16	64	64
S1.2	Control	TA20	TA40	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S1.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S2	Test	FA80-TA20	FA80-TA20	FA60-TA40	FA60-TA40	FA40-TA60	FA40-TA60	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S2 MIC		1	1	2	1	2	2	1	2	2	4	2	2	2	4	2	1
S2.1	Control	FA80	FA80	FA60	FA60	FA40	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S2.1 MIC		1	2	2	4	2	4	8	8	16	16	32	32	16	32	64	64
S2.2	Control	TA20	TA20	TA40	TA40	TA60	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S2.2 MIC		1	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1
S3	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA40-TA60	FA40-TA60	FA40-TA60	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S3 MIC		1	2	2	4	2	2	4	8	2	4	2	2	4	2	2	4
S3.1	Control	FA80	FA80	FA80	FA60	FA60	FA60	FA40	FA40	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S3.1 MIC		1	2	2	4	8	4	8	16	16	8	8	16	16	32	32	16
S3.2	Control	TA20	TA20	TA20	TA40	TA40	TA40	TA60	TA60	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S3.2 MIC		1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
S4	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S4 MIC		1	2	4	4	8	4	8	8	4	4	2	4	4	8	4	2
S4.1	Control	FA80	FA80	FA80	FA60	FA60	FA60	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S4.1 MIC		1	2	4	4	8	4	16	8	8	16	64	64	32	64	64	128
S4.2	Control	TA20	TA20	TA20	TA40	TA40	TA40	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S4.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S5	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S5 MIC		1	2	2	4	2	1	1	2	2	2	4	4	2	4	4	2
S5.1	Control	FA80	FA80	FA80	FA80	FA60	FA60	FA60	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S5.1 MIC		1	2	2	4	2	8	4	4	16	16	32	32	16	16	32	64
S5.2	Control	TA20	TA20	TA20	TA20	TA40	TA40	TA40	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S5.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S6	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
S6 MIC		1	2	4	8	4	8	16	8	8	4	4	2	4	4	2	4
S6.1	Control	FA80	FA80	FA80	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S6.1 MIC		1	2	4	4	2	4	8	8	16	16	8	16	32	32	16	32
S6.2	Control	TA20	TA20	TA20	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S6.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S7	Test	FA60-	FA40-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-	FA20-

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

		TA40	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S7_MIC		1	1	1	2	1	1	2	2	2	2	1	1	2	2	2	1
S7.1	Control	FA60	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S7.1-MIC		1	2	4	2	4	8	16	16	8	16	16	32	32	64	64	64
S7.2	Control	TA60	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S7.2_MIC		1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1
S8	Test	FA60-TA40	FA60-TA40	FA40-TA60	FA40-TA60	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S8_MIC		1	2	4	2	2	1	1	2	1	1	1	1	1	1	1	1
S8.1	Control	FA60	FA60	FA40	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S8.1_MIC		1	2	4	2	4	8	4	8	4	16	16	32	32	16	16	32
S8.2	Control	TA40	TA40	TA60	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S8.2_MIC		1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1
S9	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S9_MIC		1	1	2	1	2	4	2	2	2	4	4	2	2	1	4	1
S9.1	Control	FA60	FA60	FA60	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S9.1_MIC		1	4	2	2	2	4	4	8	4	8	8	16	16	32	32	16
S9.2	Control	TA40	TA40	TA40	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S9.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S10	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S10_MIC		1	4	2	4	2	2	1	4	4	2	2	2	2	4	4	2
S10.1	Control	FA60	FA60	FA60	FA60	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S10.1_MIC		1	2	2	4	2	4	8	4	8	16	16	32	32	16	16	32
S10.2	Control	TA40	TA40	TA40	TA40	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S10.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S1(Increasing)	Test	FA20-TA80	FA40-TA60	FA60-TA40	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S1_MIC		1	1	2	1	2	2	4	2	4	4	2	4	8	4	4	2
S1.1	Control	FA20	FA40	FA60	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S1.1_MIC		1	2	8	8	16	8	16	16	16	32	32	16	16	32	32	32
S1.2	Control	TA80	TA60	TA40	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S1.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S2	Test	FA20-TA80	FA20-TA80	FA40-TA60	FA40-TA60	FA60-TA40	FA60-TA40	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S2_MIC		1	1	1	2	4	2	2	4	2	2	2	4	4	8	4	4
S2.1	Control	FA20	FA20	FA40	FA40	FA60	FA60	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S2.1_MIC																	
S2.2	Control	TA80	TA80	TA60	TA60	TA40	TA40	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S2.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S3	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA40-TA60	FA40-TA60	FA40-TA60	FA60-TA40	FA60-TA40	FA60-TA40	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S3_MIC		1	1	1	2	1	2	2	4	2	4	4	8	4	8	8	4
S3.1	Control	FA20	FA20	FA20	FA40	FA40	FA40	FA60	FA60	FA60	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S3.1_MIC		1	2	8	4	8	4	16	16	8	8	16	16	32	16	16	32

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

S3.2	Control	TA80	TA80	TA80	TA60	TA60	TA60	TA40	TA40	TA40	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S3.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S4	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA40-TA60	FA40-TA60	FA40-TA60	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
S4_MIC		1	1	1	1	2	2	1	1	4	2	1	2	2	2	4	2
S4.1	Control	FA20	FA20	FA20	FA40	FA40	FA40	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S4.1_MIC		1	2	2	4	2	8	4	4	8	16	16	32	32	16	16	32
S4.2	Control	TA80	TA80	TA80	TA60	TA60	TA60	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S4.2_MIC		1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1
S5	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA40-TA60	FA40-TA60	FA40-TA60	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S5_MIC		1	1	1	2	1	1	1	2	2	1	1	1	1	2	2	1
S5.1	Control	FA20	FA20	FA20	FA20	FA40	FA40	FA40	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S5.1_MIC		1	2	4	4	2	8	8	16	16	32	32	16	16	32	32	16
S5.2	Control	TA80	TA80	TA80	TA80	TA60	TA60	TA60	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S5.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S6	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S6_MIC		1	1	1	2	1	1	1	2	1	2	2	4	2	2	1	1
S6.1	Control	FA20	FA20	FA20	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S6.1_MIC		1	2	2	4	2	4	8	8	16	16	16	32	32	16	16	32
S6.2	Control	TA80	TA80	TA80	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S6.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S7	Test	FA60-TA40	FA40-TA60	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S7_MIC		1	1	2	1	2	2	4	1	2	2	4	4	2	4	4	4
S7.1	Control	FA60	FA40	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S7.1_MIC		1	4	4	8	4	16	16	8	16	16	32	32	32	16	32	32
S7.2	Control	TA40	TA60	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S7.2_MIC		1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1
S8	Test	FA40-TA60	FA40-TA60	FA60-TA40	FA60-TA40	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S8_MIC		1	1	1	2	1	2	4	4	2	2	4	4	2	4	4	4
S8.1	Control	FA40	FA40	FA60	FA60	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S8.1_MIC		1	2	2	4	4	8	4	4	8	8	16	16	8	16	16	32
S8.2	Control	TA60	TA60	TA40	TA40	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S8.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S9	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
S9_MIC		1	1	1	1	1	2	1	1	2	2	1	1	2	2	2	2
S9.1	Control	FA40	FA49	FA40	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S9.1_MIC		1	2	2	4	4	4	8	8	16	16	32	32	16	32	64	64
S9.2	Control	TA60	TA60	TA60	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S9.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S10	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

S10_MIC		1	1	1	1	1	2	1	2	1	1	1	1	1	2	1	1
S10.1	Control	FA40	FA40	FA40	FA40	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S10.1_MIC		1	2	2	4	4	8	4	16	16	32	16	32	64	64	32	64
S10.2	Control	TA60	TA60	TA60	TA60	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S10.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
Duration (Days)		D16	D17	D18	D19	D20	D21	D22	D23	D24	D25	D26	D27	D28	D29	D30	
	REMARK																
S1	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S1_MIC		2	1	1	1	2	1	1	2	2	2	2	4	2	2	2	
S1.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S1.1_MIC		128	128	256	128	128	256	128	128	128	256	256	256	512	256	256	
S1.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S1.2_MIC		1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	
S2	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S2_MIC		2	2	4	4	2	4	4	2	2	2	4	2	2	2	2	
S2.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S2.1_MIC		32	64	64	128	128	64	128	128	256	256	128	256	256	256	256	
S2.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S2.2_MIC		1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	
S3	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S3_MIC		4	1	2	4	4	4	2	4	4	2	2	2	4	2	4	
S3.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S3.1_MIC		16	32	64	64	32	32	64	64	128	128	256	128	256	256	256	
S3.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S3.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
S4	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S4_MIC		2	2	4	2	4	1	4	4	4	2	4	4	4	4	4	
S4.1	Control	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S4.1_MIC		128	256	128	64	128	256	256	128	256	512	256	256	256	256	256	
S4.2	Control	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S4.2_MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
S5	Test	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S5_MIC		2	4	2	4	4	1	2	4	2	2	4	4	4	4	4	
S5.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S5.1_MIC		64	32	32	64	64	32	64	128	128	256	128	256	256	256	256	
S5.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S5.2_MIC		1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	
S6	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

S6 MIC		8	4	4	4	2	2	4	4	4	2	4	4	8	4	4
S6.1	Control	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S6.1 MIC		64	32	32	64	64	128	128	64	128	256	128	128	128	256	256
S6.2	Control	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S6.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S7	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S7 MIC		1	2	2	2	1	1	1	1	1	2	1	1	1	2	1
S7.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S7.1-MIC		32	32	64	64	128	128	64	64	128	128	64	64	128	256	256
S7.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S7.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S8	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S8 MIC		2	2	1	2	2	2	2	2	2	2	1	1	1	2	2
S8.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S8.1 MIC		32	16	16	32	32	64	64	64	128	128	128	256	256	256	256
S8.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S8.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S9	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S9 MIC		2	2	2	4	1	1	2	4	4	1	2	2	4	4	4
S9.1	Control	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S9.1 MIC		16	16	32	32	16	16	32	32	64	64	128	128	256	256	128
S9.2	Control	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S9.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S10	Test	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S10 MIC		2	1	2	2	4	2	1	4	2	1	2	2	2	2	2
S10.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S10.1 MIC		64	64	128	128	256	256	128	128	256	256	128	256	256	256	256
S10.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S10.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
S1(Increasing)	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S1 MIC		4	4	4	2	2	4	8	4	8	8	4	8	8	16	16
S1.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S1.1 MIC		64	64	32	32	64	128	128	128	128	64	128	256	256	128	256
S1.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S1.2 MIC		1	1	1	1	2	2	1	1	1	1	2	1	1	1	1
S2	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S2 MIC		2	2	4	8	8	4	8	8	4	4	8	8	16	8	16
S2.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S2.1 MIC		128	128	64	64	128	128	256	256	128	128	256	256	512	256	256
S2.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20

Table A-1: MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

S2.2 MIC		1	1	1	1	1	1	1	1	2	2	1	1	1	2	1
S3	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S3 MIC		2	4	8	8	4	4	4	8	8	8	8	8	8	8	8
S3.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S3.1 MIC		32	64	64	128	128	64	64	128	64	64	128	256	256	128	256
S3.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S3.2 MIC		1	1	1	1	1	1	1	1	1	1	1	2	1	1	1
S4	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
S4 MIC		4	4	2	2	2	2	1	2	2	2	4	2	2	2	4
S4.1	Control	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S4.1 MIC		64	64	64	64	128	128	64	128	128	64	128	256	256	256	256
S4.2	Control	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S4.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
S5	Test	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50	FA50-TA50
S5 MIC		1	2	2	1	1	1	4	2	1	2	2	2	4	4	4
S5.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S5.1 MIC		16	32	64	64	32	32	64	64	128	128	128	64	128	128	256
S5.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S5.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S6	Test	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60	FA40-TA60
S6 MIC		4	2	1	4	1	2	2	2	2	1	1	2	2	2	1
S6.1	Control	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40	FA40
S6.1 MIC		64	64	128	128	64	128	128	256	256	128	256	256	256	256	256
S6.2	Control	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60	TA60
S6.2 MIC		1	1	1	1	1	1	1	1	2	1	1	1	1	1	1
S7	Test	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80	FA20-TA80
S7 MIC		8	4	4	8	8	8	4	8	8	8	8	8	8	8	8
S7.1	Control	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20	FA20
S7.1 MIC		64	64	128	64	128	128	128	256	128	128	256	256	256	256	256
S7.2	Control	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80	TA80
S7.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S8	Test	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20	FA80-TA20
S8 MIC		4	4	4	8	4	4	8	8	8	4	8	8	8	8	8
S8.1	Control	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80	FA80
S8.1 MIC		32	16	32	32	32	64	64	64	128	128	256	256	256	512	256
S8.2	Control	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20	TA20
S8.2 MIC		1	1	1	1	1	2	2	1	1	1	2	1	1	1	1
S9	Test	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40	FA60-TA40
S9 MIC		4	2	2	1	2	2	1	1	1	1	2	2	2	2	2

MICs of different combination methods and their controls against MRSA ATCC 43300 (Continued)

S9.1	Control	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60	FA60
S9.1 MIC		128	64	64	128	128	128	256	256	128	256	256	128	256	256	256
S9.2	Control	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40	TA40
S9.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
S10	Test	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50	FA50- TA50
S10 MIC		2	1	1	1	1	1	1	1	1	2	1	1	1	1	1
S10.1	Control	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50	FA50
S10.1 MIC		128	128	128	256	256	256	128	256	256	128	256	256	256	256	256
S10.2	Control	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50	TA50
S10.2 MIC		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Figure A-1: Multiple sequence alignment of *fusA* genes of MRSA strain treated with different methods, GeneBank and the individual controls

Figure A-2: Multiple sequence alignment of *fusA* genes of MRSA strain treated with different methods, GeneBank and the individual controls

Figure A-3: Multiple sequence alignment of *fusA* genes of MRSA strain treated with different methods, GeneBank and the individual controls

Figure A-4: Multiple sequence alignment of *fusA* genes of MRSA strain treated with different methods, GeneBank and the individual controls

Figure A-5: Multiple sequence alignment of *fusA* proteins of MRSA strain treated with different methods, GeneBank and the individual controls

Appendix B

Figure B-1: Effects of tannic acid on Ethidium Bromide Accumulation Assay against MRSA clinical strains

Figure B-2: Effects of Quercetin on Ethidium Bromide Accumulation Assay against MRSA clinical strains

Figure B-3: Effects of gallic acid ethyl ester on Ethidium Bromide Accumulation Assay against MRSA clinical strains

Appendix C

Figure C-1: Colony Forming Units of MRSA 43300 under influence of different combination method

Figure C-2: Colony Forming Units of MRSA Clinical strain C1 under influence of different combination method

Figure C-3: Colony Forming Units of MRSA Clinical strain C2 under influence of different combination method

Figure C-4: Colony Forming Units of MRSA Clinical strain C3 under influence of different combination method

Figure C-5: Minimal Inhibitory Concentrations (MICs) of the combination methods against MRSA 43300 strain

Figure C-6: Minimal Inhibitory Concentrations (MICs) of the combination methods against MRSA Clinical Strain C1

Figure C-7: Minimal Inhibitory Concentrations (MICs) of the combination methods against MRSA Clinical strain C2

Figure C-8: Minimal Inhibitory Concentrations (MICs) of the combination methods against MRSA Clinical Strain C3

Figure C-9: Area under curve (AUC) ratios of the combination methods used prior to Positive Control against methicillin resistant *S. aureus* 43300 (AUC Ratio ≤ 1.2 = Homogeneous, > 1.2 = Heterogeneous)

Figure C-10: Area under curve (AUC) ratios of the combination methods used prior to Positive Control against methicillin resistant *S. aureus* Clinical strain C1 (AUC Ratio ≤ 1.2 = Homogeneous, > 1.2 = Heterogeneous)

Figure C-11: Area under curve (AUC) ratios of the combination methods used prior to Positive Control against methicillin resistant *S. aureus* Clinical strain C2 (AUC Ratio ≤ 1.2 = Homogeneous, > 1.2 = Heterogeneous)

Figure C-12: Area under curve (AUC) ratios of the combination methods used prior to Positive Control against methicillin resistant *S. aureus* Clinical strain C3 (AUC Ratio ≤ 1.2 = Homogeneous, > 1.2 = Heterogeneous)

Appendix D

Iso-Sensitest Broth

Oxid Product Code: CM0473¹

Typical Formula*	gm/litre
Hydrolysed casein	11.0
Peptones	3.0
Glucose	2.0
Sodium chloride	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine hydrochloride	0.02
L-Tryptophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
pH 7.4 ± 0.2 @ 25°C	
*Adjusted as required to meet performance standards	

Directions

Add 23.4g to 1 liter of distilled water. Mix well and heat to dissolve. Distribute into tubes or bottles. Sterilize by autoclaving at 121 °C for 15 min.

Description

Oxid Iso-Sensitest™ Broth has been produced in parallel with Iso-Sensitest Agar CM0471. The broth has an identical nutrient formulation without the specially purified agar. Where studies on antimicrobial susceptibilities are to be made in both broth and agar, this will be found to be of particular value. Details of the function of

¹ Source: <http://www.oxid.com/>

the medium and the methodology used for antimicrobial susceptibility tests are discussed in the Section, Susceptibility Testing.

Storage conditions and Shelf life

Store the dehydrated medium at 10-30 °C and use before the expiry date on the label.

Appearance

Dehydrated medium: Straw colored, free-flowing powder

Prepared medium: Pale straw colored solution

Iso-Sensitest Agar

Oxid Product Code: CM0471²

Typical Formula*	gm/litre
Hydrolysed casein	11.0
Peptones	3.0
Glucose	2.0
Sodium chloride	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine hydrochloride	0.02
L-Tryptophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
Agar	8.0
pH 7.4 ± 0.2 @ 25°C	
*Adjusted as required to meet performance standards	

² Source: <http://www.oxid.com/>

Directions

Suspend 31.4g in 1 liter of distilled water and bring to the boil to dissolve the agar. Sterilize by autoclaving at 121 °C for 15 min.

Description

Oxoid Iso-Sensitest™ Agar CM0471 was developed specifically for antimicrobial susceptibility tests. Its formulation was carefully constructed to give a reproducible, semi-defined medium in which the undefined components were kept to a minimal level. However, it allows the growth of the great majority of micro-organisms without further supplementation.

Storage conditions and Shelf Life

Store the dehydrated medium at 10-30 °C and use before the expiry date on the label.

Store the prepared medium at 2-8 °C and use as freshly as possible.

Appearance

Dehydrated medium: Straw colored, free-flowing powder

Prepared medium: Straw colored gel

Table D-1: Single letter database codes for 20 amino acids

Full Name	Abbreviation (3 letters)	Abbreviation (1 letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V