The effect of flavonoids on the *in vitro* activity of antibiotics against *Staphylococcus aureus*

By

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Thesis submitted in Fulfillment of the Requirements for the Degree Magister Scientiae

MSc

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November 2012

KEYWORDS

Staphylococcus aureus

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Multidrug resistance

Flavonoids

Antimicrobial susceptibility testing

Combination studies

Synergistic ratios

Efflux pumps

Efflux pump genes

Polymerase Chain Reaction (PCR)

ABSTRACT

Staphylococcus aureus is a Gram-positive coccus belonging to the Stapylococcaeae family. *S. aureus* causes a wide range of infections that range from skin infections to life-threatening infections such as pneumonia and endocarditis and is the major cause of hospital and community-acquired infections. Despite antibiotics being available for the treatment of *S. aureus* infections, resistance to a number of antibiotics has developed over the years due to their improper and continuous use. *S. aureus* develops resistance to various drugs via different mechanisms, one of which is the extrusion of the antibiotics through efflux pumps that play a role in its acquisition of multidrug resistance. The ability of methicillin-resistant *S. aureus* to develop resistance to a variety of antibiotics is causing global concern as treatment options are being limited. Various antimicrobial studies carried out on purified plant-based flavonoids have shown that flavonoids enhance the antibacterial effect of antibiotics.

This study analysed antibacterial effects of the antibiotics; tetracycline, ampicillin, methicillin and vancomycin and three flavonoids; chrysin, naringenin and 7-hydroxyflavone, against methicillin-sensitive ATCC 25923 (MSSA) and methicillin-resistant ATCC 33591 (MRSA) *S. aureus* strains, using the Kirby-Bauer disk diffusion and microtitre microdilution assays. In the Kirby- Bauer assay, the antibiotics demonstrated inhibitory effects on the growth of MSSA ATCC 25923. However MRSA ATCC 33591 was only susceptible to vancomycin, with minimal inhibition zones observed with ampicillin. The flavonoids did not enhance or reduce the antibacterial activities of the antibiotics as the zones of inhibition sizes remained unchanged in the combination studies. Microtitre assay results revealed that naringenin enhanced the antibacterial activities of the antibiotics tetracycline and ampicillin, against MSSA ATCC 25923 and MRSA 33591. This was evident as calculated synergistic ratios by the Abbot formula showed that naringenin had an additive effect. The presence of

the efflux pump genes in MSSA ATCC 25923 and MRSA ATCC 33591 was compared using polymerase chain reaction (PCR). The *mepA* and *gyrA* genes were identified in both strains whereas *sepA* was identified in MRSA ATCC 33591. The presence of efflux pump genes in both MSSA ATCC 25923 and MRSA ATCC 33591 also confirmed that the presence or absence of the genes may contribute to antibiotic resistance. The presence of *sepA* in the MRSA and not the MSSA confirmed that this gene plays a role in conferring drug resistance.

DECLARATION

I, Tiza Lucy Ng'uni, declare that the thesis "**The effect of flavonoids on the *in vitro* activity of antibiotics against *Staphylococcus aureus***" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Tiza Lucy Ng'uni

Date.....

Signed.....

DEDICATION

This thesis is dedicated to my dad, **Brig. Gen. Skinner Ng'uni**, who passed away on 11th May, 2012. Words cannot describe the pain losing you has caused. Even though you are gone, you will always be loved and always be in our hearts. May Your Soul Rest In Eternal Peace.

ACKNOWLEDGEMENTS

- I would firstly like to **thank my God**, for through Him all things are possible. I would not have been able to do all this without Him.

- I would like to thank my supervisor, **Professor BC Fielding**. Thank you so much for believing in me, supporting, encouraging and guiding me. You always go out of your way to ensure that your students are taken care of and for that I am truly grateful. You are the best supervisor one can ever ask for.

- I would also like to thank my co-supervisor, **Professor JA Klaasen**. Thank you for your encouragement and guidance. I truly appreciate the time and effort you put into helping me.

- To my **virology lab colleagues**, thank you for always being there and offering helping hands each time I needed you. I will be forever grateful.

- **To my family**, my mum **Mrs Catherine Ng'uni**, baby girl and siblings. No words can describe how much your support, love, encouragement, comfort and time means to me. Mum, you and dad contributed to making me the person that I am today. I love you all so much and I am truly grateful. I would also like to thank my friends, near and far, for all your support.

- I would like to thank the **National Research Foundation (NRF)** and the **Department of Medical Biosciences**, for the financial support and working space.

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LIST OF ABBREVIATIONS

µg/ml	microgram per milliliter
ABC	ATP-binding cassette transporter
ATCC 25923	Methicillin-sensitive/susceptible *S.aureus*
ATCC 33591	Methicillin-resistant *S. aureus*
ATCC	American Type Culture Collection
Bp	Base pairs
CA-MRSA	Community-acquired/associated MRSA
C_{exp}	Expected efficacy
CLSI	Clinical and Laboratory Standard Institute
C_{obs}	Observed efficacy
CoNS	Coagulase-negative staphylococci
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic acid,

EPs	Efflux pumps family
Fw primer	Forward primer
Go Taq	DNA Taq polymerase
HA-MRSA	Hospital-acquired/associated MRSA
LA-MRSA	Livestock -acquired/associated MRSA
MATE	Multidrug and toxin compound extrusion
MDR	Multidrug resistance
MFS	Major Facilitator super family
Mg/ml	Milligram per milliliter
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
Mm	Millimeter
mM	Millimolar
MRSA	Methicillin-resistant
MSA	Mannitol salt agar
MSSA	Methicillin-sensitive/susceptible
OD	Optical density
PBP	Penicillin-binding protein
PCR	Polymerase Chain Reaction

pmol/µl	Pica mol per microliter
PVL	Panton- Valentine Leukocidin
QRDR	Quinolone resistance determining region
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division family
RT	Reverse Transcription
Rv primer	Reverse primer
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcal cassette chromosome
SMR	Small multidrug resistance transporter
SMZ	Sulfamethoxazole
SR	Synergy Ratios
SSSS	Staphylococcal scaled-skin syndrome
TBE	Tris Borate EDTA
TE	Tris EDTA
TMP	Trimethoprim
TSA	Tryptone soy agar
TSB	Tryptone soy broth

TSST	Toxic shock syndrome toxin
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistant *S. aureus*

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CHAPTER ONE

Literature Review

1.1. INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus that belongs to the Staphylococcaeae family. It was discovered by a surgeon, in pus from surgical abscesses in 1880 in Aberdeen, Scotland by Alexander Ogston (Ogston 1984). It appears as "grape-like clusters" under the microscope and is the major cause of staphylococcal infections. *S. aureus* forms part of the normal flora found on the skin and in the nose and it has been established that 20% of the human population are long-term carriers (Lyon and Skurray 1987; Kluytmans, van Belkum et al. 1997; Tsiodras, Gold et al. 2001). When grown on solid media, it produces colonies with a golden-yellow appearance (Hsueh, Chen et al. 2002; Hsueh, Liu et al. 2002; Azeez-Akande 2010) from which its name - "aureus" meaning "golden" in Latin is derived. *S. aureus* contains a carotenoid pigment called staphyloxanthin which is responsible for the antioxidant properties of the bacterium. Staphyloxanthin enables it to escape death by reactive oxygen species and is responsible for the golden color seen in colonies (Clauditz, Resch et al. 2006).

1.1.1. Infection

Infection with *S. aureus* causes a wide variety of illnesses which include boils, abscesses, pimples, and impetigo scalded skin syndrome which affect the skin. It also infects different parts of the body and cause illnesses such as pneumonia, gastroenteritis, chest pain, bacteremia, endocarditis, osteomyelitis and septicemia (Bernards, Frenay et al. 1998; Richards, Edwards et al. 1999a; Perez-Vazquez, Vindel et al. 2009). It is clear that *S. aureus* can infect different parts of the body causing life-threatening conditions and is the leading cause of both hospital and community-acquired infections (Kuehnert, Hill et al. 2005; Shittu, Udo et al. 2009).

Several risk factors may predispose some people to infection with *S. aureus*. Those at risk include diabetics, young children, elderly people, people that share rooms such as those in boarding schools, prisons, soldiers in basic training, injection drug users, patients with skin infections, and patients using catheters (Becker, Friedrich et al. 2003; Kuehnert, Hill et al. 2005; Moran and Talan 2005; Mulvey, MacDougall et al. 2005). Other risk factors include hospitals where people may share beds, health care personnel and patients, people with lowered immune systems as in the case of HIV/AIDS, cancer and transplant patients, and prior or unwarranted antimicrobial therapy (Fey, Said-Salim et al. 2003; Bratu, Eramo et al. 2005; Kuehnert, Hill et al. 2005).

1.1.2. Diagnosis and identification

Guidelines have been put in place to help govern the proper and efficient identification and laboratory diagnosis of *S. aureus*. A number of tests have been set up that easily identify *S. aureus* and differentiate it from other bacteria including other staphylococcal species (Brown, Edwards et al. 2005). The test that can be used to differentiate *S. aureus* from other staphylococcus species is the coagulase (cell-bound and free coagulase) test, which is considered the standard test for routine identification of *S. aureus* (Brown, Edwards et al. 2005). *S. aureus* is able to produce an enzyme known as coagulase (clumping factor) that is able to form clots (clots plasma) (Wichelhaus, Kern et al. 1999). This test is useful as most of the other staphylococcal species are coagulase-negative, and are termed coagulase-negative staphylococci (CoNS) (Brown, Edwards et al. 2005). *S. aureus* also produces the enzyme deoxyribonuclease (DNase), which is able to denature DNA (as seen when DNase plates are used in biochemical tests). However, confirmation tests should be done when positive results are obtained as CoNS are also capable of producing the

DNase enzyme (Barry, Lachica et al. 1973). The lipase test (with a yellow color and rancid odor smell) and phosphatase test (with a pink color) can also be done (Cookson 1997). Other tests that are used to identify *S. aureus* include the latex agglutination tests that detect various surface antigens including protein A and/or clumping factors. The only problem with these tests is that the specificity may be compromised due to cross-reactivity with CoNS and the fact that some *S. aureus* strains may not produce the clamping factor or protein A (Kaur, Talwar et al. 1988; Blake and Metcalfe 2001; van Griethuysen, Bes et al. 2001).

Molecular-based tests have also been used to identify *S. aureus* and involve the use of Polymerase Chain Reaction (PCR). This test is specific in that it utilizes primers that have been designed to be species-specific and amplify specific target sites (Towner, Talbot et al. 1998; Kearns, Seiders et al. 1999; Mason, Blevins et al. 2001; Grisold, Leitner et al. 2002). A real-time PCR kit, that has been commercially made, has also been employed in the identification of *S. aureus*, which detects specific sequences within the internal transcribed spacer (ITS) *S. aureus* region (Levi and Towner 2004; Harbarth, Hawkey et al. 2011).

Blood cultures have also been used in the diagnosis of *S. aureus* infections, especially when dealing with deep-tissue infections such as endocarditis and bacteremia (Dark, Dean et al. 2009; Stefani 2009). However, blood culture results take time and should be coupled with other identification and diagnostic techniques especially when dealing with critically ill patients (Harbarth, Garbino et al. 2003). Solid agar media has also been used to identify *S. aureus* due to its cluster-forming ability and its ability to ferment carbohydrates, and produces white-deep yellow colonies. This is as a result of indicators, inhibitory substances including antibiotics such as methicillin and oxacillin, that are added to the media and play a role in differentiating and selecting *S. aureus* stains including those that are methicillin-resistant (Blanc, Wenger et al. 2003; Monsen, Olofsson et al. 2003; Safdar, Narans et al. 2003; Perry, Davies et al. 2004). Agar media, such as mannitol salt agar (MSA), a selective

media containing 7- 9% NaCl, has been used. It allows the growth of *S. aureus* and results in the production of golden-yellow colonies as a result of mannitol fermentation (Safdar, Narans et al. 2003; Malhotra-Kumar, Abrahantes et al. 2010).

1.2. VIRULENCE FACTORS

Staphylococcus aureus has the ability to evade the immune system through the synthesis of various virulence factors including exotoxins (Archer 1998; Novick 2003) and hydrolytic enzymes (Lowy 1998; Vojtov, Ross et al. 2002), which are involved in causing infection (Archer 1998; Novick 2003). Some of these virulence factors include leukocidins, proteases, coagulase, hemolysins, enterotoxins, exfoliative toxins and immune-modulating factors (Tait-Kamradt, Clancy et al. 1997; Manders 1998; Rajaraman, Jack et al. 2000; Rooijakkers, van Kessel et al. 2005), which are produced via a number of multifarious regulation pathways (Bronner, Monteil et al. 2004). However, it has been shown that virulence factor expression patterns of *S. aureus* differ during host infection from when it is grown on media, implying that the regulation of these virulence factors is complex due to cellular immune factors and nutrient conditions (Oogai, Matsuo et al. 2011).

1.2.1. Toxins

S. aureus is able to secrete various exotoxins and enterotoxins, depending on the strain, and can be categorized into three groups, namely superantigens, exfoliative toxins, and other toxins.

1.2.1.1. Superantigens

This group induces toxic shock syndrome (TSS) and includes the toxin TSST-1, which usually causes TSS linked to tampon use in women. This toxin is extremely virulent and causes serious *S. aureus* infections (Lucet, Herrmann et al. 1990; Musser, Schlievert et al. 1990; De Boer, Kum et al. 1999). Hypotension, shock, erythematous rash, multi-organ failure, fevers, and skin desquamation is characteristic of such toxins. This group also includes food poisoning associated with staphylococcal enterotoxins, which manifests as vomiting and diarrhea 1-6 hours after *S. aureus* ingestion (Fey, Said-Salim et al. 2003; Mulvey, MacDougall et al. 2005). Enterotoxins are also believed to function as T cell superantigens, and are frequently expressed by *S. aureus* clinical isolates and code for mobile genetic elements (Vandenesch, Naimi et al. 2003). These are responsible for binding to the antigen-presenting cells surfaces via the Major Histocompatibility Complex (MHC) II causing functional association with the T helper cells facilitated by the surface T cell receptors (TCR) (Llewelyn and Cohen 2002; Proft and Fraser 2003).

1.2.1.2. Exfoliative toxins

These types of toxins, which include epidermolytic toxins A and B, are associated with staphylococcal scaled-skin syndrome (SSSS) and are frequent in infants and young children and also seen in hospital nurseries. The peeling of the skin is caused by the protease activity of these toxins as seen in SSSS (Lina, Gillet et al. 1997).

1.2.1.3. Other toxins

Most toxins produced by staphylococci are able to form pores in the host cytoplasmic membrane thereby causing cellular damage (Lazarevic, Beaume et al. 2011). Examples of such toxins are the α (alpha), β (beta), γ (gamma) and δ (delta) hemolysins including bicomponent toxins, which have been shown to cause cellular damage by affecting the cell membrane (Jonsson, Lindberg et al. 1985; O'Callaghan, Callegan et al. 1997; Haslinger, Strangfeld et al. 2003). Severe necrotinizing pneumonia in children can be associated with a bicomponent toxin called Panton- Valentine Leukocidin (PVL) (Fey, Said-Salim et al. 2003; Mulvey, MacDougall et al. 2005). PVL is also responsible for lysing white blood cells and causes severe tissue damage that lead to chronic intermittent infections (Akpaka, Monecke et al. 2011) . It is commonly found in CA-MRSA isolates, and is a pore-forming toxin that targets neutrophils (Prevost, Cribier et al. 1995; Dufour, Gillet et al. 2002).

1.2.2. Protein A

This protein is anchored to the staphylococcal peptidoglycan pentaglycine bridges (chains of five glycine residues) by transpeptidaseSortase A (Schneewind, Fowler et al. 1995). Protein A binds to the Fc region of an antibody and is an IgG-binding protein. Studies done on Protein A show that it contributes to the virulence of the bacteria and is considered to be a B cell superantigen which is also known as staphylococcal protein A (SpA) (Silverman and Goodyear 2006). SpA has been shown to interact with various host proteins and is a major anti-opsonic factor (Kim, Thammavongsa et al. 2012). Studies showed that *S. aureus* required it to bind to the Fc region of the antibody and that mutations to the genes coding for Protein A resulted in lowered virulence of *S. aureus* (Patel, Nowlan et al. 1987). Various recombinant forms of Protein A have been used to bind and purify a variety of antibodies by

immuno-affinity and chromatography. The study of transpeptidases, like sortases, is done in the hope of coming up with new antibiotics to target methicillin-resistant *Staphylococcus aureus* (MRSA) infections (Zhu, Lu et al. 2008).

1.2.3. Role of staphyloxanthin in virulence

Staphylococcus aureus produces staphyloxanthin, an orange-red carotenoid, which gives it its characteristic "golden-yellow" color and also plays a role in its virulence (Wieland, Feil et al. 1994; Pelz, Wieland et al. 2005). The antioxidant properties of *S. aureus*, as a result of staphyloxanthin, enable the microbe to evade death by reactive oxygen species (Clauditz, Resch et al. 2006; Liu, Liu et al. 2008). The effect of staphyloxanthin was assessed by comparing a normal strain of *S. aureus* to one that had been modified to lack the pigment (Duthie and Lorenz 1952). It was noted that the normal strain was more likely to be able to survive incubation with an oxidizing chemical than the mutant strain. The effect of the two strains was also compared by inoculating them on wounds on mice. The pigmented strains caused persistent abscesses while those of the unpigmented ones healed quickly (Liu, Liu et al. 2008).

Evidence of the role of staphyloxanthin in enabling *S. aureus* to survive immune attacks is seen in the various tests. The susceptibility of the bacterium to antibiotics can be improved by designing drugs that can inhibit the production of this pigment (Liu, Essex et al. 2005). The biosynthesis of staphyloxanthin and human cholesterol have similarities in their pathways and thus a drug that was developed to lower cholesterol was shown to block *S. aureus* pigmentation as well as disease progression in a mouse infection model (Liu, Liu et al. 2008). A study carried out on the effect of flavones on staphyloxanthin showed a

reduction in its production, thereby enabling the vulnerability of *S. aureus* to hydrogen peroxide (Lee, Park et al. 2012).

1.3. TREATMENT

Penicillin was usually the drug of choice in the treatment of *S. aureus* infection following its discovery, but resistance to penicillin had been reported in many countries following its introduction as early as 1942. In 1944, Kirby reported penicillinase-producing strains (Kirby 1944). Oxacillin or flucloxacillin have been used in first-line therapy and are penicillinase-resistant beta-lactam antibiotics. These drugs are given in combination with gentamicin to treat serious infections such as endocarditis (Korzeniowski and Sande 1982; Bayer, Bolger et al. 1998) . However, the use of gentamicin poses some controversy as its use can lead to kidney damage (Cosgrove, Vigliani et al. 2009). Antimicrobial susceptibility testing, site of infection and severity determine the duration and type of treatment given (Bamberger and Boyd 2005). Drugs such as intravenous nafcillin, oxacillin (Bactocill) and oral dicloxacillin (Dynapen) have been administered to patients not allergic to penicillin (Bamberger and Boyd 2005). Cephalosporins such as cephalexin (Keflex) and intravenous cefazolin (Ancef) have also been given as substitutes.

Vancomycin is considered to be the best treatment option in MRSA infections. However, it has been shown that the use of vancomycin has a down side in that; its absorption in the gastrointestinal tract is rather poor, has slow bactericidal activity and also has numerous side effects (Levine, Fromm et al. 1991; Gould 2008; Rasmussen, Fowler et al. 2011). Vancomycin is administered intravenously due to the low absorption experienced when administered orally. Nonetheless, treatment failure has been reported and some studies

have actually shown that treatment with β-lactams was more beneficial compared to treatment with vancomycin (Chang, Peacock et al. 2003; Stryjewski, Szczech et al. 2007).

Teicoplanin, a glycopeptide, is another drug that has been used to treat *S. aureus* infections and is administered intravenously or intramuscularly. A number of studies have revealed that its effectiveness is similar to that of vancomycin (Rolston, Nguyen et al. 1994; Yalaz, Cetin et al. 2004). Other drugs such as tigecycline, Linezolid (Zyvox), daptomycin (Cubicin) and telavancin have also been used in treating *S. aureus* infections. Tigecycline is used in the treatment of complex skin infections and has bacteriostatic properties (Rasmussen, Fowler et al. 2011). Linezolid is administered orally or intravenously and has been used to treat pneumonia and skin and soft tissue infections as a result *S. aureus* infection (Lin, Zhang et al. 2008; Tascini, Gemignani et al. 2009) and possesses bacteriostactic capabilities (Bamberger and Boyd 2005). Daptomycin has shown bactericidal properties in "*in vitro*" studies and is used to treat complicated skin and soft tissue infections (Arbeit, Maki et al. 2004). Daptomycin is administered intravenously (Bamberger and Boyd 2005). The FDA approved the use of telavancin for the treatment of skin and skin-structure infections (Rasmussen, Fowler et al. 2011).

Table 1.1. Treatment options for *Staphylococcus aureus* infections

Type of infection	Antibiotic choice	Alternate antibiotic choices	Length of therapy
Simple, uncomplicated skin infections			Five to seven days
MSSA	Cephalexin (Keflex), dicloxacillin (dynapen)	Clindamycin (Cleocin)	
MRSA	Clindamycin, trimethoprim/sulfamethoxazole (Bactrim, Septra), linezolid (Zyvox)	–	
Complex skin and soft-tissue infections			Two to four weeks (varies)
MSSA	Nafcillin	Cefazolin (Ancef), clindamycin	
MRSA	Vancomycin (vancocin)	Linezolid, daptomycin (Cubicin)	
Bacteremia			Two to four weeks (varies)
MSSA	Nafcillin	Cetazolin, vancomycin	
MRSA	Vancomycin	Linezolid, daptomycin	
Catheter-related infections			Two weeks if no infective endocarditis
MSSA	Nafcillin	Cefazolin, vancomycin	
MRSA	Vancomycin	Linezolid, daptomycin	
Osteomyelitis			Four to six weeks
MSSA	Nafcillin, cefazolin	Clindamycin, quinolone plus rifampin (Rifadin)	
MRSA	Vancomycin	Linezolid, daptomycin	
Pneumonia			10 to 14 days
MSSA	Nafcillin	Vancomycin, Clindamycin	
MRSA	Vancomycin, Linezolid	–	

MSSA-methicillin-sensitive *S. aureus*, MRSA-methicillin-resistant *S. aureus* (Lina, Quaglia et al. 1999; Bamberger and Boyd 2005)

1.4. METHICILLIN- RESISTANT *Staphylococcus aureus* (MRSA)

Methicillin-resistant *Staphylococcus aureus* (MRSA) was initially identified in hospitals. The first isolation of MRSA was done in the UK in 1961 (Jevons 1961). Since then, the rates of methicillin resistance have escalated causing increasing concern. Following the introduction methicillin in the 1960s, resistance to methicillin was detected in strains of *Staphylococcus aureus* (Grundmann, Aires-de-Sousa et al. 2006; Sakoulas and Moellering 2008). Despite MRSA being first identified in hospitals, hospital prevention and control measures alone are not sufficient to fight MRSA infections and other measure have to be put in place.

MRSA is considered to be the main cause of staphylococcal infections causing extensive deep tissue (arthritis, osteomyelitis and renal and breast abscesses) and skin and soft tissue (carbuncles and furuncles) infections (McCaig, McDonald et al. 2006; Daniyan, Galadima et al. 2011) which can be life-threatening. MRSA is responsible for causing both hospital-acquired/associated (HA-MRSA) and community-acquired (CA-MRSA) infections. Most of the CA-MRSA infections are confined to the skin and soft tissue and treatment is usually effective. Nonetheless, they are capable of causing illnesses much more severe than those caused by HA-MRSA (Deleo, Otto et al. 2010). The toxins carried by CA-MRSA, such as PVL and PSM, are thought to be responsible for the increased virulence and severe illnesses such as sepsis, toxic shock syndrome and necrotinizing pneumonia. However, it is unknown why the same strain of CA-MRSA can cause treatable skin infections as well as severe infections that can lead to death.

MRSA infection has shown to have spread over the last four decades with an increase in the global trend, causing increased and rigorous morbidity and mortality rates in the healthcare facilities worldwide (Kuehnert, Hill et al. 2005) A number of risk factors play a

role in MRSA infection and these include autoimmune diseases, immunosuppressive therapy, surgery, extended hospital stay, prior or undue antimicrobial therapy and being previously hospitalized (Kuehnert, Hill et al. 2005). MRSA-associated infections pose a challenge in the hospitals due to the fact this organism has become resistant to a number of antibiotics, thereby causing problems.

More effective measures have to be put in place to try and prevent and control MRSA infections (Maranan, Moreira et al. 1997; Bratu, Eramo et al. 2005), which tend to put a toll on the medical and socio-economic costs. Studies carried out show that human infection is potentially brought about as a result of domestic animals (Weese, Caldwell et al. 2006; Weese, Dick et al. 2006; Weese, Rousseau et al. 2006).

1.4.1. Global prevalence and incidence of MRSA

As earlier described, MRSA strains were initially identified in the UK in 1961, shortly after which they developed resistance to the introduction of methicillin (Jevons, 1961). Since then, MRSA epidemics and endemics have been reported worldwide (Ayliffe 1997; Chambers 2001a; Fridkin, Hageman et al. 2003). This has led to the assumption that *S. aureus* epidemiology is changing (Boyce 1998; Herold, Immergluck et al. 1998). MRSA infections are causing problems for hospitals and healthcare facilities as well as numerous communities worldwide (Bratu, Eramo et al. 2005; Kuehnert, Hill et al. 2005; Mulvey, MacDougall et al. 2005). Various reports from different countries around the world indicated that there was an increase in the prevalence of MRSA infection and population risk. Data obtained from North America as well as the Centers for Disease Control and Prevention (CDC) in the United States of America showed that the incidence of MRSA has increased over the years (Hughes 1987; Garner, Jarvis et al. 1988; Horan, Culver et al. 1988; Mulvey,

MacDougall et al. 2005; Azeez-Akande 2010). Additional studies carried out at different hospitals in the US revealed that the prevalence of MRSA rose from 6% in 1998 to 50% in 2002 (Azeez-Akande 2010).

Published data from other countries has also shown an increase in the prevalence of MRSA infections. In hospital wards in France, for instance, a study carried out revealed that the prevalence of MRSA was 33% - 62% (in relation to *S. aureus* isolates) (Mangeney, Bakkouch et al. 1995). Studies carried out in Taiwan indicated a rise in MRSA prevalence from 1981-1986 (Hsueh, Chen et al. 2002; Hsueh, Liu et al. 2002). Other studies carried out in Japan (Lotus, Imamura et al. 1995) and the Republic of Korea (Woojoo and Seunchill 1999) have demonstrated MRSA prevalence of 54% and 70% respectively. However, reports on MRSA prevalence in Africa have been very few. Moreover, in Sudan, a study conducted at a Khartoum hospital revealed a prevalence of 11% (Musa, Shears et al. 1999). Studies carried out between 1996 and 1997 in Nigeria, Cameroon, Kenya and Algeria showed MRSA prevalence rates of 21% – 30% (Nigerian, Cameroon and Kenya) while Algeria had a lower rate of less than 10% (Kesah, Ben Redjeb et al. 2003).

Other studies have also reported MRSA incidence in Ghana (Odonkor, Newman et al. 2012), Ethiopia (Geyid and Lemeneh 1991), Kenya (Omari, Malonza et al. 1997), Nigeria (Rotimi, Orebamjo et al. 1987; Okesola, Oni et al. 1999), Senegal (Sow, Wade et al. 1998), South Africa and Sudan (van den Ende and Rotter 1986; Peddie, Donald et al. 1988; Gardee and Kirby 1993; Musa, Shears et al. 1999). Additional studies need to be carried out in order to establish the epidemiological distribution of methicillin-resistant *Staphylococcus aureus* in other African countries. This will give a detailed outlook of MRSA infection and prevalence and help in its prevention and control.

1.4.2. Hospital-acquired MRSA (HA-MRSA)

MRSA infections were originally detected and identified in hospitals.. Nevertheless, it has since been detected in the community as well as livestock with a high global prevalence (Stefani, Chung et al. 2012). Various epidemiological studies that show the prevalence rates of MRSA have been carried out. Despite these studies being different in terms of study design and population samples, the highest rates (>50%) have been reported in North and South America, Asia and Malta. Intermediate rates were reported in China, Australia, Africa and some European countries e.g. Portugal (49%), Greece (40%), Italy (37%) and Romania (34%). Low prevalence rates were reported in other European countries including The Netherlands and Scandinavia (Grundmann, Aanensen et al. 2010; Mejia, Zurita et al. 2010; Song, Hsueh et al. 2011).

The prevalence of HA-MRSA has also been reported to have reduced in a number of other European countries including Austria, France, Ireland, and the UK. However, other European countries have had rather constant HA-MRSA prevalence rates (Grundmann, Aanensen et al. 2010; Mejia, Zurita et al. 2010; Song, Hsueh et al. 2011). Furthermore, when the percentage of MRSA in relation to HA *S. aureus* infections was determined, extremely high rates were reported in Sri Lanka (86.5%), South Korea (77.6%), Vietnam (74.1%), Taiwan (65.0%), Thailand (570.%) and Hong Kong (56.8%). India and The Philippines (22.6 and 38.1% respectively) had much lower rates (Song, Hsueh et al. 2011).

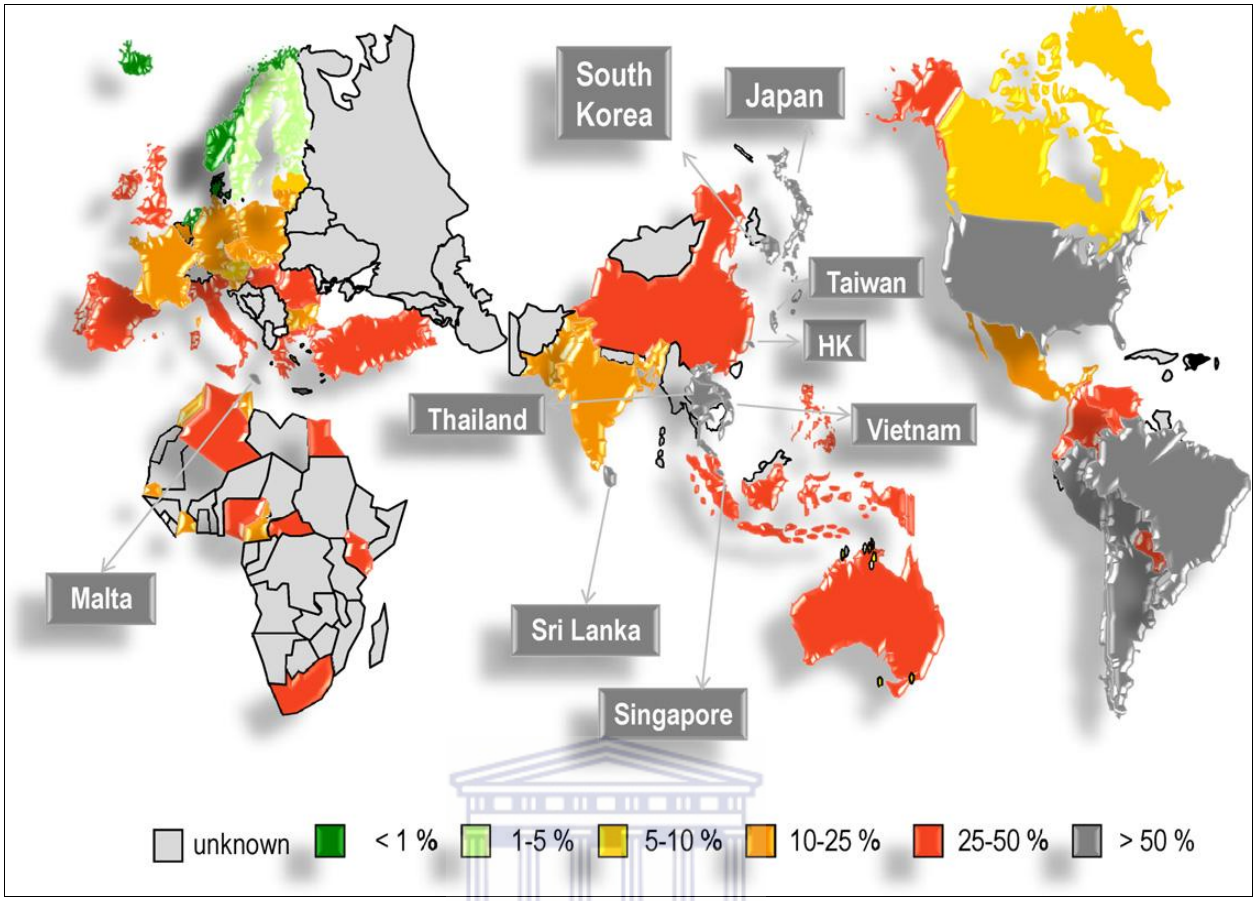

Figure 1.1. Global prevalence of hospital-acquired methicillin-resistant *Staphylococcus aureus*.
Source. (Ito, Ma et al. 2004; Grundmann, Aanensen et al. 2010; Mejia, Zurita et al. 2010; Song, Hsueh et al. 2011)

1.4.3. Community-acquired MRSA

Earlier studies had reported that people with known risk factors (such as contact with health-care facilities and having previous antimicrobial therapy) were prone to infection with community isolates of MRSA. However, recent studies have reported that colonization and transmission was seen in populations that lacked these risk factors. Another study recently carried out in children attending day-care centers revealed that 3% and 24% of the children in the respective centers were colonized with MRSA (Adcock, Pastor et al. 1998) with these isolates being susceptible to a number of antibiotics which were different to the multi-drug resistant hospital isolates. A prospective, population-based study carried out in San Francisco,

CA, in 2004-2005 (Liu, Liu et al. 2008) demonstrated an incidence ten-fold higher in CA-MRSA compared to HA-MRSA (316 vs. 31 per 100 000 population) (Liu, Liu et al. 2008).

After infection with CA-MRSA strains caused the deaths of four children from rural Minnesota and North Dakota in 1999, the problem was brought to national attention (CDC, 1999). These children did not have the risk factors commonly associated with CA-MRSA infection, and the infections were caused by strains that were susceptible to numerous antibiotics, except beta-lactams. These reports provide convincing evidence that MRSA strains have spread to the community and are very important outpatient pathogens (Chambers 2001b).

Latest studies have revealed that CA-MRSA is spreading to the healthcare settings especially in the US, as well as other countries (Otter and French 2011). Epidemiological studies carried out have shown that CA-MRSA infection is high among high-risk groups such as poor homeless young adults, soldiers, children, prison inmates, homosexual males, intravenous drug users, athletes, as well as those in day-care centers (Groom, Wolsey et al. 2001; Weber 2005; Diep, Chambers et al. 2008; Deleo, Otto et al. 2010). Countries that have maintained low MRSA rates are finding the spread of CA-MRSA a challenge. This poses an enormous threat to public health and the community at large – especially with CA-MRSA infection incidence rates increasing among healthy people.

1.4.4. Livestock-associated MRSA (LA-MRSA)

In humans, LA-MRSA was initially discovered in 2003 from a reservoir belonging to the CC398 lineage found in pigs and cattle and has also been found in poultry (Van Cleef, Broens et al. 2010; van Cleef, Monnet et al. 2011). The MRSA CC398 clone was found to have the ability to be transmitted from animals to humans. This new clone is, however,

frequently asymptomatic in livestock animals. Infections associated with MRSA CC398 have been reported in Europe, Asia and the US (Monecke, Coombs et al. 2011). Current evidence has shown, however, that the spread of LA-MRSA into hospitals and/or nursing homes in Europe is not significant. Moreover, the spread of LA-MRSA appears to be dependent on the region as well as the intensity of pig farming, and studies have shown that human infections are likely caused by domestic animals (Weese, Dick et al. 2006). In Europe, at present, invasive LA-MRSA infections including nosocomial spread are unusual (Grundmann, Aanensen et al. 2010). Studies carried out in 51 Dutch hospitals demonstrated that transmission of MRSA CC398 between hospitalized patients was 72% less likely to occur than other MRSA strains (Wassenberg, Bootsma et al. 2011). Even though MRSA had spread from hospitals to nursing homes, studies carried out in 32 German nursing homes revealed that there was no indication of the presence of CA-MRSA or LA-MRSA strains (Pfingsten-Wurzburg, Pieper et al. 2011).

The carriage of LA-MRSA in Europe is believed to be common in people who frequently come in contact with livestock; though illnesses linked to it are quite rare (van den Broek, van Cleef et al. 2009). In The Netherlands, the risk factors associated with LA-MRSA include working in direct contact with animals on farms (Kock, Harlizius et al. 2009; van Cleef, Verkade et al. 2010; Graveland, Duim et al. 2011) as well as working with live pigs in slaughterhouses (Van Cleef, Broens et al. 2010). Over the past few years, various cases of infections such as endocarditis, pneumonia, and necrotizing fasciitis have been reported despite the fact that MRSA CC398 is frequently associated with asymptomatic colonization (Ekkelenkamp, Sekkat et al. 2006; van Rijen, Van Keulen et al. 2008; Mammina, Cala et al. 2010; Soavi, Stellini et al. 2010).

1.4.5. Antimicrobial resistance of MRSA

Antimicrobial agents have been used to treat various bacterial infections since their discovery, and it was believed that they would contribute to the purge of these infections. Nevertheless, diseases considered to have been eradicated or controlled are returning as a result of the organisms' ability to acquire resistance to these antibiotics (Levy and Marshall 2004). The emergence of multi-drug resistant bacteria worldwide is preventing existing drugs from being effective, thus inhibiting the proper and efficient treatment of these infections and thereby creating both medical and socio-economic expenses (Maranan, Moreira et al. 1997; Carbon 1999; Bratu, Eramo et al. 2005).

Since these bacteria developed resistance to old antibiotics rapidly, there was, and still is, a need for the development of new antimicrobial agents. However, resistance to these new antimicrobial agents is rapid and widespread; indicating that this would be the trend even with the new families of antimicrobial agents, thereby limiting their life expectancy (Coates, Hu et al. 2002) Methicillin-resistant staphylococci are among the bacteria that have developed resistance to a number of antimicrobial agents (Neu 1992; Norrby, Nord et al. 2005). *S. aureus* is of great concern because it of its virulence, its ability to cause a wide range of life-threatening infections and its ability to acclimatize to various environmental conditions (Lowy 1998; Kennedy, Otto et al. 2008; Kim, Thammavongsa et al. 2012). Despite effective antimicrobials being available, *S. aureus* causes a mortality rate of about 20-40% (Mylotte and McDermott 1987a; Mylotte, McDermott et al. 1987b). *S. aureus* is currently the number one cause of nosocomial and community-acquired infections (Grundmann, Aires-de-Sousa et al. 2006; Deurenberg, Vink et al. 2007; van Loo, Huijsdens et al. 2007).

1.4.5.1. Mechanism of antibiotic resistance

Antimicrobial resistance results from three main strategies which include enzymatic inactivation of the drug (Davies 1994), target site modification (Spratt 1994), and drug extrusion by efflux (Lomovskaya and Bostian 2006). During target site modification, chemical modifications in the antibiotic target site decrease the affinity of the antibiotic to its binding site (Lambert 2005). In enzymatic inactivation, hydrolytic and transferase enzymes render the antibiotic inactive by degrading the antibiotic, as well as modifying it through acetylation, adenylation or phosphorylation (Over, Gur et al. 2001). Efflux pump proteins coded for by efflux pump genes are responsible for the extrusion of antibiotics, thus allowing the pathogens to avoid antimicrobial effect (Lomovskaya and Bostian 2006).

It has been established that *S. aureus* developed resistance to methicillin via the mec operon, situated on the staphylococcal cassette chromosome mec (SCCmec). The SCC is a large mobile genetic element that varies in size and genetic composition among different strains of MRSA (Katayama, Ito et al. 2000; Hiramatsu, Cui et al. 2001). Resistance is facilitated by the *mecA* gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') and has a reduced affinity for binding β-lactams including penicillins, cephalosporins, and carbapenems (Weems 2001). These allow for resistance to all β-lactam antibiotics as well as other non-beta-lactam antibiotics including erythromycin, clindamycin, gentamicin and ciprofloxacin, due to the mecA complex that possesses insertion sites for plasmids and transposons which enable the development of resistance to these other classes of antibiotics. Thus hindering their clinical use during MRSA infections (Chambers 2001b)

Mutations of the *mecA* gene lead to modifications of the penicillin-binding protein 2a (PBP-2a) product, which consequently result in drug resistance. This modifications mechanism eventually results in the organism being resistant to β-lactams including additional antibiotics that pose the same target site (Stevens, Dotter et al. 2004). Antibiotic resistance resulting from modification of the PBP-2a protein is believed to be as a result a number of factors which include (i) PBP being over-produced, (ii) the bacteria possessing a different PBP with reduced affinity, (iii) recombination of susceptible PBPs with other resistant varieties or (iv) the occurrence of specific point mutations within PBPs that eventually lead to the decrease of their affinity for β-lactams (Hackbarth, Kocagoz et al. 1995; Chambers 1997; Dauwalder, Lina et al. 2008).

Table 1.2. Mechanism of *S. aureus* resistance to antimicrobial agents

Antibiotic	Resistance gene(s)	Gene product(s)	Mechanism(s) of resistance	Location(s)
β-Lactams	1) *blaz*	1) β-Lactamase	1) Enzymatic hydrolysis of β-Lactam nucleus	1) Pl:Tn
	2) *mecA*	2) PBP2a	Reduced affinity for PBP	2) C:SCCmec
Glycopeptides	1) Unknown	1) Altered petidoglycan	1) Trapping of vancomycin in the cell wall	1) C
	2)	2) D-Ala-D-Lac	2) Synthesis of dipeptide with reduced affinity for vancomycin	2) Pl:Tn
Quinolones	1) *parC*	1) ParC(GrlA) component of topoisomerase IV	1,2) Mutations in the QRDR region, reducing affinity of enzyme-DNA complex for quinolones	1)C
	2) *gyrA* or *gyrB*	2) GyrA or GyrB components of gyrase		2) C
Aminoglycosides (e g gentamycin)	Aminoglycoside-modifying enzymes (e.g *aac*, *aph*)	Acetyltransferase, phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides	Pl, Pl:Tn
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: *sulA*	1) Dihydropteroate synthase	1) Overproduction of *p*-aminobenzoic acid by enzyme	1) C
	2) TMP: *dfrB*	2) Dihydrofolate reductase (DHFR)	2) Reduced affinity for DHFR	2)C
Oxazolidinones	*Rrn*	23S rRNA	Mutations in domain V of 23S rRNA component of the 50S ribosome. Interferes with ribosomal binding	C
Quinupristin-dalfopristin (Q-D)	1) Q:*ermA*, *ermB*, *ermC*	1) Ribosomal methylases	1) Reduced binding to the 23S ribosomal subunit	1)Pl, C
	2) D:*vat*, *vatB*	2) Acetyltransferases	2) Enzymatic modification of dalfopristin	2)Pl

Examples of various antibiotic resistance mechanisms employed by *S. aureus*. (Lyon and Skurray 1987; Allignet, Aubert et al. 1996; Lina, Quaglia et al. 1999; Tsiodras, Gold et al. 2001). Pl, plasmid; C. chromosome; Tn, transposon; QRDR, quinolone resistance-determining region.

Table 1.3. MRSA classification based on type of SCCmec present

SCCmec type	Source	Resistance	Genome size (kb)	Ribotype
I	Hospital	Methicillin	34.3	Conserved
II	Hospital	Multi-drug	53.0	Conserved
III	Hospital	Multi-drug	66.9	Conserved
IV	Community	Methicillin	21 – 24	Variable

Source: (Corkill, Anson et al. 2004; Francois, Renzi et al. 2004; Ito, Ma et al. 2004)

1.4.5.2. Methicillin resistance

Methicillin was introduced in 1961 but methicillin-resistance by some isolates followed shortly after its introduction (Jevons 1961). This has resulted in the limitation of therapeutic agents against infections caused by methicillin-resistant isolates (Cosgrove, Sakoulas et al. 2003). The *mecA* gene is responsible for methicillin resistance (Katayama, Ito et al. 2000). Methicillin-sensitive *Staphylococcus aureus* (MSSA) can become methicillin-resistant by acquiring the *mecA* gene, which is the methicillin resistance determinant (Hiramatsu, Cui et al. 2001). The mortality rate from severe MRSA infection has been found to be as high as 10% - 34% (Tumbarello, de Gaetano Donati et al. 2002).

1.4.5.3. β-lactam resistance

β-lactams include broad-spectrum penicillins, cephalosporins, monobactams and carbapenems. The mechanism of antimicrobial resistance by a large number of bacteria is through the degradation of the antibiotics by means of chromosomal-or-plasmid encoded β-lactamases (Bush, Calmon et al. 1995). With regard to carbapenems, mutations can occur that result in the loss of the porin OprD, the main route of uptake (Trias and Nikaido 1990).

Resistance to beta- lactam antibiotics is believed to occur in two ways; (i) by the production of an enzyme beta- lactamase, which subsequently causes an inactivation of the beta- lactam antibiotics and (ii) by the expression of transpeptidases, which are resistant to antibiotic activity. *mecA*, which codes for the penicillin-binding protein PBP2a, is responsible for the antibiotic resistance exhibited by *S. aureus* (Archer and Climo 2001; Finan, Archer et al. 2001; McKinney, Sharma et al. 2001).

Penicillin was introduced in the early 1940s. However, penicillin-resistant staphylococcal strains were detected in both hospitals and the community shortly after (Rammelkamp and Maxon 1942). As the late 1960s approached, over 80% of both hospital-acquired and community-acquired staphylococcal isolates were penicillin-resistant. This trend of resistance, initially appearing in hospitals and then extending to the community, is a pattern now seen even when new antimicrobial agents are introduced and resistance develops (Chambers 2001a).

1.4.5.4. Tetracycline resistance

Tetracycline has been widely used in the treatment of both Gram-negative and Gram-positive bacterial infections, as well as mycolasma, rickettsiae, and protozoan parasites (Chopra, Lacey et al. 1974; Chopra 1975; Markham and Neyfakh 2001). Apart from its therapeutic use in humans and animals, tetracycline has been erratically used in animal husbandry for growth promotion, resulting in extensive tetracycline resistance in both Gram-negative and Gram-positive bacteria (including *S. aureus*). Tetracycline resistance is achieved by two main mechanisms which are active efflux by tetracycline-specific pumps/transporters (Tet-pumps), most of which belong to the MF family (Markham and Neyfakh 2001), and

ribosomal protection (Schnappinger and Hillen 1996). Tet-pumps are generally spread among Gram-negative and Gram-positive bacteria (Roberts 1994).

1.4.5.5. Aminoglycoside resistance

The mode of action of aminoglycosides is the inhibition of protein synthesis by binding to the bacterial ribosomes, thereby resulting in their death. This action is mediated via protonated amine and/or hydroxyl interactions with the ribosomal RNA of the bacterial 30S ribosomal subunit (Lomovskaya and Watkins 2001a). However, the need to identify other compounds that are capable of resisting modification by aminoglycoside modifying enzymes is essential in order to improve the action of these therapeutic agents (Miller, Sabatelli et al. 1997).

Aminoglycoside resistance is believed to be as a result of two main mechanisms which are: specific enzymatic inactivation by modifying enzymes as well as resistance impermeability, which are considered to be the most common mechanisms (Lomovskaya and Watkins 2001a). Ribosomal mutations and active efflux of the drug out of the bacteria also contribute to aminoglycoside resistance (Macmaster, Zelinskaya et al. 2010). These enzymes exert their function by covalently attaching either a phosphate, nucleotide or acetyl moiety to either the amine and/or the alcohol key functional group of the antibiotic. This causes a reduction in the ribosomal binding affinity due to the change in charge. ANT (4') IA aminoglycoside adenylytransferase 4' IA is one of the aminoglycoside modifying enzymes found in *S. aureus* (Porter, Green et al. 2010; Revuelta, Corzana et al. 2010). It attaches an adenyl moiety to 4'hydroxyl group of a number of aminoglycosides such as kamamycin and gentamicin. In Staphylococcal species; it has been found that these bacteria tend to posses

numerous aminoglycoside modifying enzymes that lead to multiple genotypes and complex phenotypes (Miller, Sabatelli et al. 1997).

Aminoglycosides were some of the very few antimicrobial agents that were not affected by the active extrusion brought about by multidrug resistance pumps (MDR). Unfortunately, however, that is not currently the case (Moore, DeShazer et al. 1999).

1.4.5.6. Quinolone resistance

When first introduced in the 1980s, the intended use of fluoroquinolones was to treat Gram-negative bacterial infections, but due to their ability to treat Gram-positive bacterial infections, they had also been used in the treatment of infections brought about by staphylococcal species (Gootz and Brighty 1996). However, quinolone-resistance rapidly emerged especially among methicillin-resistant strains, thus decreasing their use in treatment of staphylococcal infections (Hopper 2002).

Fluoroquinolones exert their antimicrobial effect by inhibiting the activity of type II topoisomerases, DNA gyrase (encoded by the *gyrA* and *gyrB* genes) and DNA topoisomerase IV [encoded by the *parC* (*grlA* in *S. aureus*) and *parE* (*grlB* in *S. aureus*) genes] (Yoon, Lee et al. 2010). DNA gyrase is responsible for the introduction of negative superhelical twists into bacterial DNA, which is essential for the instigation of DNA replication, as well as aid the binding of initiation proteins (Hopper 2002). Topoisomerase IV is essential in ensuring the separation of daughter cells after DNA replication by removing the interlinking chromosomes (Hopper 2002).

Resistance to fluoroquinolones is achieved by chromosomal mutations of topoisomerase IV or DNA gyrase including the action of a multidrug efflux pump (Ng,

Trucksis et al. 1996; Hopper 2002). Alterations in amino acid sequences in vital regions of the enzyme-DNA complex (quinolone resistance determining region [QRDR]) consequently result in the reduction of quinolone affinity for both targets thus conferring resistance (Ng, Trucksis et al. 1996; Hopper 2002). Both single and additional mutations in amino acid sequence play a role fluoroquinolone resistance. Nonetheless, additional and more complex amino acid mutations are essential for fluoroquinolone-resistance to occur (Ng, Trucksis et al. 1996; Hopper 2002).

1.4.5.7. Vancomycin resistance

Following the increase in methicillin resistant *Staphylococcus aureus* (MRSA) infections, the use of vancomycin to treat these infections subsequently increased, thereby influencing the emergence of vancomycin-resistant staphylococci (Kirst, Thompson et al. 1998). The first report of vancomycin-resistant staphylococci was from a clinical isolate of a *Staphylococcus hemolyticus* strain (Schwalbe, Stapleton et al. 1987) and this was later followed by reports of vancomycin-intermediate-resistant *S. aureus* (VISA), in 1997 in Japan (Hiramatsu, Aritaka et al. 1997). More cases of VISA have further been reported in other countries (Hiramatsu, Hanaki et al. 1997; Smith, Pearson et al. 1999). Reports of cases of vancomycin-resistant *S. aureus* infections raise a tremendous amount of concern as this demonstrates complete bacterial resistance to vancomycin as well as an unusual mode of distribution (Hageman, Pegues et al. 2001; Ward, Johnson et al. 2001). It has been established that VISA result from *S. aureus* parent strains that are susceptible to vancomycin due to persistent infection (Sieradzki, Roberts et al. 1999; Smith, Pearson et al. 1999; Moore, Perdreau-Remington et al. 2003). Intermediate resistance to vancomycin is thought to occur

through sequential point mutations in major staphylococcal regulatory genes (Mwangi, Wu et al. 2007; Cui, Neoh et al. 2009; Cui, Li et al. 2009).

Resistance to glycopeptides is mediated via the acquisition of the *vanA* gene. This gene codes for an enzyme that produces an alternative peptidoglycan to which vancomycin will not bind (Showsh, De Boever et al. 2001). It has been suggested that alterations in peptidoglycan biosynthesis appears to play a role in lowering vancomycin susceptibility (Hiramatsu, Hanaki et al. 1997). A change in peptidoglycan biosynthesis also leads to the reduction of cross-linkages formed between peptidoglycan strands, consequently leading to more D-Ala-D-Ala residues being revealed (Hanaki, Kuwahara-Arai et al. 1998a; Hanaki, Labischinski et al. 1998b; Hanaki, Labischinski et al. 1998c). The distorted cross-linking is as a result of a reduction in the levels of L-glutamine available for amidation of D-glutamate in the pentapeptide bridge (Walsh and Howe 2002). The increase in the amount of D-Ala-D-Ala residues results in the binding and trapping of vancomycin which further prevents drug molecules from reaching their target sites, thus rendering the bacteria vancomycin-resistant (Sieradzki, Roberts et al. 1999; Avison, Bennett et al. 2002).

Table 1.4. Antibiotic resistance rates of MRSA

Antimicrobial agent	Rate of resistance (%)
Erythromycin	90 - 95
Gentamycin	75 -93
Fluoroquinolones	30 - 90
Clindamycin	75 - 83
Ketolides	82 - 98
Tetracycline	18 - 82
Trimethoprim/sulfamethoxazole	16 - 65
Quinupristin/dalfopristin	4 - 31
Fusidic acid	5 - 10
Vancomycin	0 - 5
Oxazolidinones (e.g Linezolid)	0 - 1
Tigecycline	0
Daptomycin	0

Antibiotic resistance may be unpredictable in developing countries and rate may be lower in some cases.
Source: (Hsueh, Chen et al. 2002; Hsueh, Liu et al. 2002; Rello and Diaz 2003; Azeez-Akande 2010)

1.5. PREVENTION AND CONTROL

A wide range of MRSA infections happen in hospitals and healthcare facilities. Transfer of MRSA is frequent between healthcare providers and patients, mainly because some healthcare providers do not follow the laid-down procedures for proper hand washing (Tacconelli 2008). It is important for healthcare personnel to practice good hygiene in the healthcare facilities. Employing basic hand washing techniques is relevant in the effective prevention of transmission of *S. aureus* as it may be passed on from the healthcare provider to the patients and vice-versa. The spread is usually through human-to-human contact. The

use of gloves and plastic aprons and the use of a private room can play a role in the prevention of infection as this reduces the risk of skin-on-skin contact (Boyce 2004; Boyce, Havill et al. 2004). Once the bacterium is introduced into the bloodstream, it can lead to a number of life-threatening infections (Lowy 1998).

Ethanol has shown to be effective against MRSA and it can also be used with ammonium to increase the effectiveness. Nosocomial infections can be prevented by routine and terminal cleaning. CO_2 NAV- CO_2 alcohol vapor, a non-flammable that does not damage metals or plastics, can also be used. Antiseptic washes and shampoos can be used for patients carrying the resistant strain. Antibiotic ointments can also be used in reducing MRSA colonization (Loeb, Main et al. 2003).

1.6. EFFLUX PUMPS AND EFFLUX PUMP GENES

Efflux pumps are transport proteins responsible for the extrusion of toxic substances, including nearly all classes of clinically-important antibiotics from inside the bacterial cells into the external environment, and are present in both antibiotic-sensitive and antibiotic-resistant gram-negative and Gram-positive bacteria (Piddock 2006a). Efflux simply means the action a cell employs to pump out solutes (Van Bambeke, Balzi et al. 2000). Efflux pump genes and proteins can be found in both bacteria that are antibiotic-susceptible and those that are resistant to the antibiotic. Nevertheless, it is possible for a strain that is susceptible to an antibiotic to become resistant due to the overproduction and subsequent over-expression of an efflux pump (Levy and Marshall 2004; Stavri, Piddock et al. 2007).

There are two mechanisms that enable an efflux mutant to become resistant to antibiotics and these are: (i) the efflux pump protein expression is increased or (ii) Substitution(s) of an amino acid in the protein may enable it to be more capable to export the

solute (Piddock 2006a). This enables the bacterium to become less susceptible to the antimicrobial agents as their intracellular concentration is reduced (Wang, Dzink-Fox et al. 2001; Webber and Piddock 2001; Adewoye, Sutherland et al. 2002). Efflux pumps are capable of transporting a variety of compounds which include antibiotics of different classes, or can be specific to a particular compound, therefore leading to multiple drug resistance (MDR). This type of resistance is dependent on the species, drug and infection. The efflux pumps are encoded by genes. When expression of these genes is increased, it enables the bacterium to become even more resistant (Oethinger, Kern et al. 1998; Oethinger, Podglajen et al. 1998; Oethinger, Kern et al. 2000)

1.6.1. Types of efflux pumps

Efflux pumps belong to five superfamilies and are categorized into two distinct groups which are: primary transporters (that use the hydrolysis of ATP) and secondary transporters (that use protons or sodium ions for energy) (Lomovskaya and Watkins 2001b; Stavri, Piddock et al. 2007). The five major families include MF (major facilitator), MATE (multidrug and toxic efflux), RND (resistance-nodulation-cell division), SMR (small multidrug resistance) and ABC (ATP binding caste) (Paulsen, Brown et al. 1996; Lomovskaya and Watkins 2001b; Kaur 2002; Lynch 2006).

1.6.1.1. Primary transporters

Given the fact that these transporters use ATP as their energy source, they are also known as ATP binding cassette (ABC) transporters. These proteins can be found in both eukaryotes and prokaryotes and are found almost everywhere in membrane systems (Chang 2003). An example of an ABC transporter is P-glycoprotein 1 (P-gp, MDR1) found in

humans, which has been frequently studied. This is responsible for the resistance exhibited to the cytotoxic compounds used in cancer chemotherapy (Lage 2003). Despite the lack of evidence linking ABC transporters to MDR in human or animal pathogens so far, it is believed that ABC are present in the genomes of pathogenic bacteria and probably may confer resistance similar to that of P-glycoprotein 1 (Piddock 2006a; Mahamoud, Chevalier et al. 2007).

These transporters consist of two hydrophobic transmembrane domains and two cytoplasmic domains that are involved in ATP binding. The ABC transporters found in bacteria are highly specific for substrates such as amino acids, sugars, metallic cations, vitamins, organo-iron complexes including antibiotics (Higgins 2001; Chang 2003).

1.6.1.2. Secondary transporters

This group of transporters includes the major facilitator superfamily (MFS), resistance-nodulation-cell division (RND), small multidrug resistance (SMR) superfamily, drug/metabolite transporter (DMT) superfamily, multidrug and toxic compound extrusion (MATE) and superfamily of the multidrug/oligodaccharidyl-lipid/polysaccharide flippase (MOP) (Levy 2002; Piddock 2006a; Stavri, Piddock et al. 2007). The pumps belonging to the MFS family, to which the efflux pump NorA belongs, have been studied in detail. (Piddock 2006a).

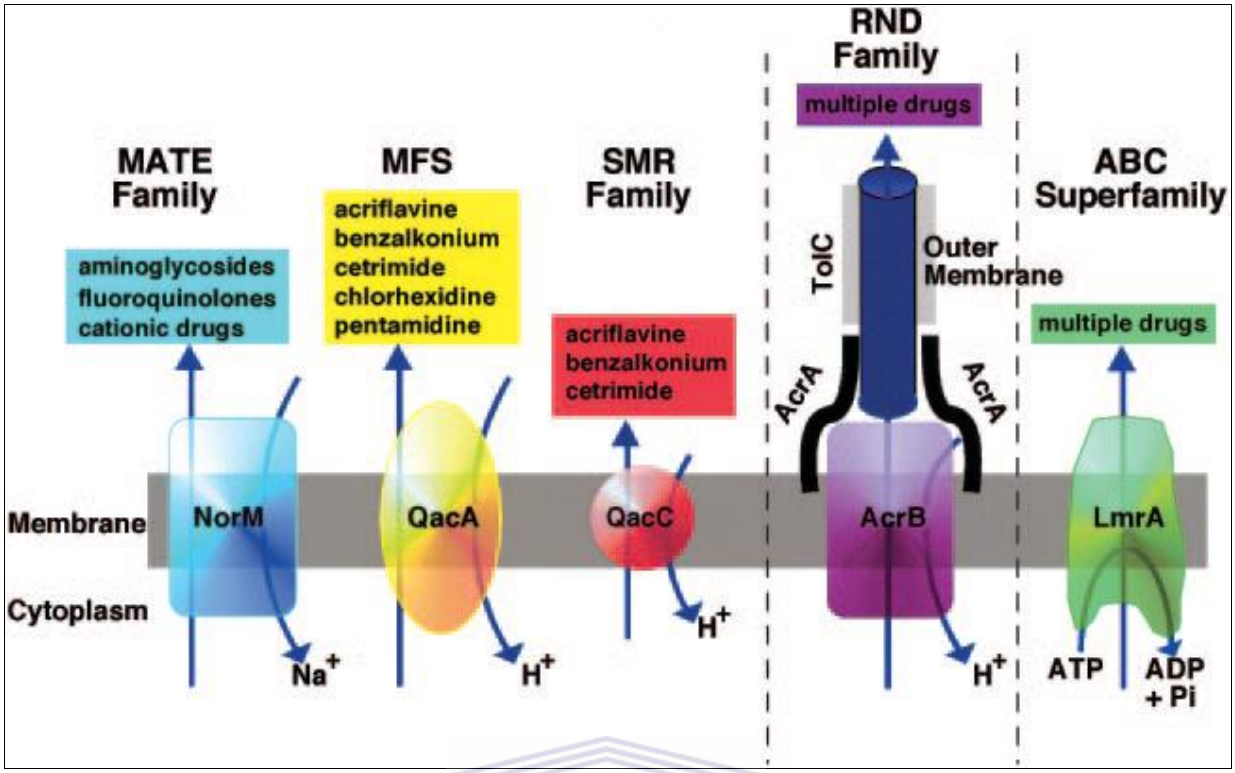

Figure 1.2. Diagrammatic representation of the five classes of bacterial efflux pumps. Diagram also showing the inner membrane (e.g AcrB) and outer membrane (e.g TolC , an outer membrane protein channel) accessory and transport proteins including a periplamic accessory protein (e.g AcrA): ABC: ATB-binding cassettes, MFS: major facilitator superfamily, RND: resistance-nodulation-division, SMR: small multidrug resistance, MATE: multidrug and toxic compound extrusion (Piddock 2006a).

1.6.2. Staphylococcus aureus Efflux pumps and Efflux pump genes

It has been established that numerous genes code for efflux pumps and a portion of these are believed to be MDR (Saier and Paulsen 2001). It has also been shown that genome size plays a role in the number of efflux pump genes. This is evident in the large genomes that contain large numbers of efflux pump genes (Paulsen, Chen et al. 2001).

The NorA efflux pump is chromosomally encoded and is present in *S. aureus*. It belongs to the major facilitator superfamily (MFS) and possesses 12 transmembrane-spanning regions (Yoshida, Bogaki et al. 1990). NorA depends on the proton motive force for its action and it is associated with resistance to drugs such as fluoroquinolone, ciprofloxacin

and ethidium (Poole 2000). Ten alleged proteins within the *S. aureus* genome that share the sequence homology with NorA have been identified (Kaatz, Seo et al. 2000).

NorA can be found in both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Some of the other MDR efflux pumps that have been studied are QacA and QacB, which are MFS pumps encoded on plasmids. Another chromosomally-encoded MFS pump is MdeA. MepA is a multi-drug and toxin extrusion (MATE) family MDR pump that is also chromosomally encoded (Paulsen, Brown et al. 1996; Kaatz, McAleese et al. 2005). Some genes have recently been described in *S. aureus* and these include *norB*, *norC* and *sdrM*, which are all MFS pumps genes (Truong-Bolduc, Dunman et al. 2005; Yamada, Hideka et al. 2006). The *sepA* gene, another gene that has been described, codes for a transporter, which has some similarities to the SMR (staphylococcal multi-resistance) protein family (Narui, Noguchi et al. 2002). Other *S. aureus* efflux pump genes include chromosomally encoded genes (*norA*, *mepA*, *mdeA* and *sepA*) and plasmid-encoded genes (*qacA/B*, *smr*, *qacG*, *qacJ* and *qacH*) (Kumar and Varela 2012). *S aureus* also possesses efflux pump genes *grlA/grlB* and *gyrA/gyrB*, which encode topoisomerase IV and DNA gyrase respectively (Ferrero, Cameron et al. 1995; Ng, Trucksis et al. 1996; Takahata, Yonezawa et al. 1996).

1.6.3. Role of MDR pumps in antibiotic drug resistance

The pumps are responsible for the export of substances from the bacterial cell, thereby allowing it to survive the hostile environment. The efflux pumps play a role in the secretion of intracellular metabolites and have been thought to contain components that play a role in pathogenicity (Lomovskaya and Watkins 2001b). It has also been shown through tissue

culture studies that components of RDN (resistant nodulation division) efflux pumps are important for invasion, adherence, and/or colonization of the host cell.

The incidence of the over-expression of efflux pumps in clinical isolates should be determined so that the clinical significance of the MDR conferred by specific efflux pumps can be established. The measurement of over-expression can be done in two ways: (i) by measuring the expression of RNA (ii) by measuring protein expression using Western blotting. The measurement of protein expression is commonly used as it is easily available (Piddock 2006; Piddock 2006a).

Despite extensive studies done on the mechanism involved in the regulation of the efflux pumps in laboratory-derived mutants, the actual mechanisms responsible for the increased efflux in clinical isolates have been shown to fall into four groups: (i) mutations on the local repressor gene (ii) mutations in a global regulatory gene (iii) mutations in the promoter region of the transporter gene and (iv) insertion elements upstream of the transporter gene (Piddock 2006; Piddock 2006a).

1.6.4. Efflux pump inhibitors

Efflux pump inhibitors (EPI) are used in order to counteract the resistance that is exhibited by the efflux pumps. These inhibitors are made in the hope that they may improve the efficacy of antibiotics (Lomovskaya and Watkins 2001a). The expected role for the EPI is to decrease the intrinsic bacterial resistance to antibiotics; reverse the acquired resistance associated with efflux pump overexpression; and reduce the frequency of the emergence of resistant mutant strains (Pages, Masi et al. 2005; Mahamoud, Chevalier et al. 2007). Carbonylcyanide*m*-chlorophenylhydrazone (CCCP), valinomycin and dinitrophenol (DNP) are some of the EPIs used to completely stop the efflux of different molecules. These affect

the bacterial membrane energy levels and are proton motive force inhibitors (Mallea, Chevalier et al. 1998; Thota, Reddy et al. 2010). Other inhibitors of the proton motive force are omeprazole, verapramil and reserpine (Neyfakh, Borsch et al. 1993).

1.7. ANTIMICROBIAL ACTIVITIES OF FLAVONOIDS

Over the years, antibiotics have been used for the treatment of various infections and diseases. However, due to their increased and improper use, a number of bacteria have developed resistance to quite a large number of them (Levy and Marshall 2004; Usha, Jose et al. 2010). *Staphylococcus aureus*, as described earlier, is one the bacteria that has developed resistance to numerous antibiotics. This is causing tremendous global problems in the healthcare sector and in the community at large (Gilbert and McBain 2003; Bratu, Eramo et al. 2005; Costa, Ntokou et al. 2010).It has been observed that bacteria rapidly develop resistance to new classes of antibiotics and the rate at which resistance develops is higher than the rate at which these new classes of antibiotics are manufactured., This shows that multi-drug resistance may develop even to new families of these antimicrobial agents. This situation further prevents the effectiveness of these drugs. It is for this reason that alternative methods that may give rise to combating these micro-organisms and managing these diseases should be explored (Coates, Hu et al. 2002). Natural products have been studied and used over the years as therapeutic agents, and have provided an important alternative route for the treatment of bacterial diseases (Payne, Gwynn et al. 2007). The use of plants is due to the fact that they can be lucidly selected for antimicrobial testing according to ethnomedicinal use (Cos, Vlietinck et al. 2006).

Flavonoids are an example of plant products that have been studied and used by physicians and lay healers to treat various infections (Havsteen 1983). They are a group of

heterocyclic organic compounds widely distributed in plants and plant-related products and add colour, flavour, and processing characteristics to several foods (fruits and vegetables) and drinks (tea, wine) (Yang, Kotani et al. 2001; Havsteen 2002; Cushnie and Lamb 2011). Flavonoid compounds have a fundamental structural feature that consists of a 2-phenyl-benzo[α]pyrane or flavane nucleus comprising two benzene rings (A and B) linked via a heterocyclic pyrane ring (Brown 1980). More than 400 flavonoid derivatives have been recognised to possess various health benefits such as anti-inflammatory (Gabor 1986; Middleton, Kandaswami et al. 2000) , anti-viral (Vandeputte, Kiendrebeogo et al. 2010) and anti-cancer (Elangovan, Sekar et al. 1994; Middleton, Kandaswami et al. 2000; Yang, Kotani et al. 2001); properties. Some of the other uses and applications of flavonoids include modern agents (Zhang, Cao et al. 2008) and adjuncts (Stapleton, Shah et al. 2007) for the treatment of bacterial infections; drugs for treating toxin-mediated disease (Choi, Yahiro et al. 2007); anti-virulence therapies (Vandeputte, Kiendrebeogo et al. 2010); and capture molecules for removing endotoxin from pharmaceutical preparations (Delehanty, Johnson et al. 2007).

The pharmacological activities of flavonoids are believed to be mainly due to their ability to inhibit certain enzymes, their ability to search for oxygen free radicals, as well as their ability to chelate iron (Cushnie and Lamb 2005). The number and arrangement of their phenolic groups is believed to be the reason for their antioxidant properties as well as their ability to inhibit enzymatic activities (Havsteen 1983). Fourteen classes of flavonoids have been identified and these are differentiated according to their chemical nature including the position of substituents on the A, B and C rings (Hendrich 2006) as shown in figure 1.3.

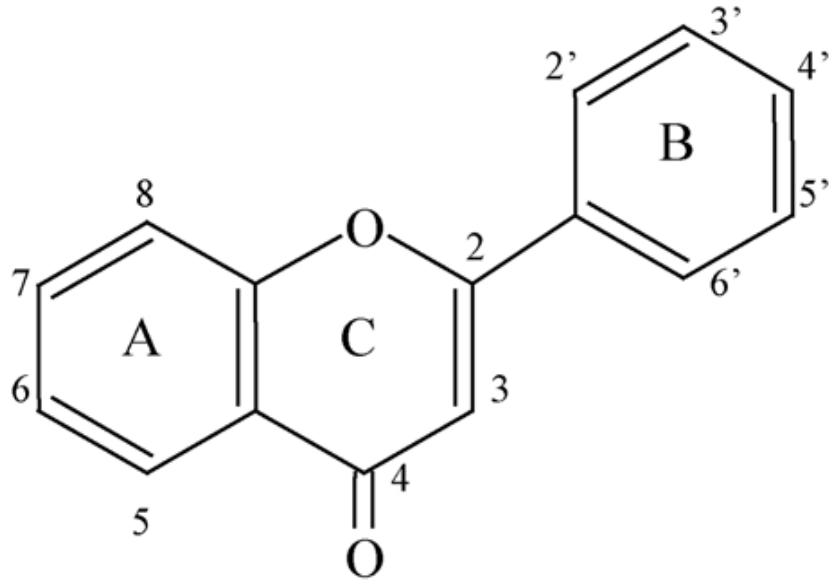

Figure 1.3. The skeleton structure of flavones (a class of flavonoids), including ring names and position numbers (Cushnie and Lamb 2005).

Over the years, the antibacterial activities of flavonoids have been studied and earlier research (1987-2004) has shown that they are attributed to three mechanisms (Cushnie and Lamb 2005) which are: damage to the cytoplasmic membrane, (caused by perforation (Ikigai, Nakae et al. 1993) and/or reduction in membrane fluidity (Tsuchiya and Iinuma 2000)), inhibition of nucleic acid synthesis (Mori, Nishino et al. 1987) caused by topoisomerase inhibition (Bernard, Sable et al. 1997; Plaper, Golob et al. 2003) and inhibition of energy metabolism caused by NADH-cytochrome *c* reductase inhibition (Haraguchi, Tanimoto et al. 1998). During the period 2005-2010, supplementary confirmation has been presented that supports each of the outlined mechanisms. Evidence has been presented that supports two new mechanisms which are: inhibition of cell wall synthesis [cause by D-alanine-D-alanine ligase inhibition (Wu, Kong et al. 2008)] as well as inhibition of cell membrane synthesis [caused by inhibition of FabG (Zhang and Rock 2004; Li, Zhang et al. 2006; Zhang, Kong et al. 2008), FabI (Zhang, Kong et al. 2008), Rv0636 (Brown, Papaemmanouil et al. 2007) or KAS III (Jeong, Lee et al. 2009).

Since 2005, various studies that have outlined the antibacterial activities of flavonoids have been carried out. These have facilitated advances in their understanding and development, which have consequently enabled more effective and efficient ways of moving forward in these studies (Cushnie and Lamb 2011). Numerous structural features that improve the antibacterial properties of flavonoids have been identified in current medicinal chemistry studies (Cushnie and Lamb 2011).These studies enable the optimisation of flavonoid activity as well as understanding their mechanism of action (Cushnie and Lamb 2011).

The antibacterial activities of flavonoids have paved the way for the study of synergism with various antimicrobial agents. Studies have shown that flavonoids and other plant products play a role in enhancing the efficacy of antimicrobial drugs (some of which have shown to be ineffective to some bacterial agents) (Hemaiswarya, Kruthiventi et al. 2008; Rosato, Vitali et al. 2008; Rosato, Vitali et al. 2009). Recent studies have also shown that some of these plant products/compounds efficiently inhibit the efflux pumps involved in antibiotic resistance (Schmitz, Fluit et al. 1998; Smith, Kaatz et al. 2007). Other studies have also shown that flavonoids have the capacity to reduce the production of virulence factors such as staphyloxanthin and α-hemolysin (Lee, Park et al. 2012). It is for this reason that more studies should be carried out in order to fully understand flavonoid activity, as well as increase their use as therapeutic agents to help alleviate the problems brought about as a result of drug resistance.

1.8. AIMS OF THE STUDY

The first aim of this study was to determine the minimum inhibitory concentrations (MICs) of four antibiotics (tetracycline, ampicillin, methicillin and vancomycin) and three flavonoids (chrysin, naringenin and 7-hydroxyflavone), as well as in combination against methicillin-sensitive ATCC 25923 (MSSA) and methicillin-resistant ATCC 33591 (MRSA) *S. aureus* strains, using Kirby-Bauer (disk diffusion) and microtitre (microdilution) assays. Synergistic ratios would be calculated for the microtitre assay combination results to assess whether any synergistic or additive effects could be identified due to the presence of the flavonoids. The second aim was to compare the presence of three efflux pump genes (*mepA*, *sepA* and *gyrA*, including the house-keeping gene *16S*) in the MSSA ATCC 25923 and MRSA ATCC 33591 strains using polymerase chain reaction (PCR).

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CHAPTER TWO

Effect of flavonoids on the *in vitro* antibacterial activities of antibiotics against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus*

2.1. ABSTRACT

Staphylococcus aureus is the leading cause of both hospital- and community-acquired infections worldwide. Despite the availability of antimicrobial agents, *S. aureus* has managed to develop resistance to a substantial range of antimicrobial agents, thus limiting its therapeutic options; as seen in the case of methicillin-resistant *S. aureus* (MRSA). It is for this reason that alternative treatment options have been and are still being studied to alleviate the problems caused by the high prevalence and widespread infection rates of MRSA. Plant products provide a few such alternative therapies that are being studied for their distinct antibacterial characteristics and are generating a tremendous amount of interest. Flavonoids are a group of heterocyclic organic compounds present in photosynthesising cells and plant-related products, studied for their various antibacterial properties.

This study investigated the antibacterial effects of four antibiotics (namely tetracycline, ampicillin, methicillin and vancomycin) and three flavonoids (chrysin, naringenin and 7-hydroxyflavone) using the Kirby-Bauer disk diffusion and microtitre susceptibility tests. The aim was to determine the minimum inhibitory concentrations (MICs) of the antibiotics and flavonoids as well as to assess their effect individually and in combinations against a methicillin-sensitive MSSA ATCC 25923 and methicillin-resistant MRSA ATCC 33591 *S. aureus* strains.

The disk diffusion results showed that all four antibiotics inhibited the bacterial growth of MSSA ATCC 25923, while only ampicillin and vancomycin had inhibitory effects on the MRSA ATCC 33591 strain. None of the three flavonoids inhibited the bacterial growth of both MSSA ATCC 25923 and MRSA ATCC 33591. Flavonoid and antibiotic combinations did not enhance the inhibitory effect on the bacterial growth, as the sizes of the inhibition zones remained similar to those of the antibiotic controls. In the microtitre assay,

naringenin proved to enhance the antimicrobial activities of tetracycline and ampicillin against both MSSA ATCC 25923 and MRSA ATCC 33591 strains, albeit at sub-inhibitory concentrations that were ineffective against the two *S. aureus* strains. Synergystic ratio calculations also revealed that naringerin has additive effects when combined with the antibiotics in the microtitre assay. Future studies should consider the effects of naringerin on the multidrug resistance efflux pump genes of MSSA and MRSA.

2.2. INTRODUCTION

Staphylococcus aureus is the leading cause of serious infections in both hospitals and communities (Lowy 1998). It has been established that *S. aureus* is the most widespread pathogen isolated from bloodstream infections, skin and soft tissue infections, and pneumonia (Bernards, Frenay et al. 1998; Pfaller, Jones et al. 1999; Deresinski 2005). Pneumonia has proven to be the most common disease associated with *S. aureus* infection, causing the highest mortality and morbidity rates in hospitals despite appropriate antimicrobial therapy being administered early (Rubinstein, Kollef et al. 2008). Not only is *S. aureus* responsible for causing pneumonia in hospitals, it is also responsible for causing community-acquired fatal necrotising pneumonia, despite community-acquired infections being mainly associated with the skin (Kollef 2005). *S. aureus* has been shown to be an important causative agent of community-acquired pneumonia in healthy children and adults (Kollef, Shorr et al. 2005)

Despite efforts to combat *S. aureus* infection, it has been able to develop resistance to a variety of antimicrobial agents, leading to complications brought about by the limitation in therapeutic options and in so doing creating a health-care burden (Chuang, Hsiao et al. 2012). This is evident particularly when dealing with methicillin-resistant strains, which count among the most important nosocomial and community-associated pathogens worldwide

(Frazee, Lynn et al. 2005; Kuehnert, Hill et al. 2005; van Loo, Huijsdens et al. 2007; Shittu, Nubel et al. 2009).

Methicillin-resistant *S. aureus* (MRSA) was first isolated in the United Kingdom in 1961 (Jevons 1961) and, since then, methicillin-resistance rates among *S. aureus* strains have increased and account for approximately 50% of all *S. aureus* isolates (Adcock 2002). In 1980, as a result of the increased resistance exhibited by MRSA to a wide range of antimicrobial agents, with the exception of glycopeptides antibiotics, vancomycin usage increased over the years, thereby increasing the chances that *S. aureu*s would develop vancomycin-resistance (Kirst, Thompson et al. 1998). Tetracycline has been used on a large scale in the treatment of Gram-positive and Gram-negative infections and animal husbandry for promoting growth. It is for this reason that resistance to tetracycline has developed (Lomovskaya and Watkins 2001a). *S. aureus* has shown resistance to β-lactams, with the resistance mechanism being mainly due to chromosomal or pasmid encoded β-lactamases (Bush, Calmon et al. 1995). Other drugs to which *S. aureus* has shown resistance include erythromycin, clindamycin, gentamicin, ciprofloxacin, trimethoprim/sulphamexaxole and linezolid (Styers, Sheehan et al. 2006). Resistance of *S. aureus* to various agents is the result of a variety of factors, including cellular changes (which prevent the accumulation of antimicrobial agents), changes in the cell envelope (which decrease the uptake of these agents) or the expression of efflux mechanisms (Poole 2002; Gilbert and McBain 2003; Russell 2003; Piddock 2006a).

The increased ability of *S. aureus* to develop multidrug resistance has limited the options for therapeutic treatment, and that resistance is believed to have been caused by the unsystematic and inappropriate use of the current antimicrobial drugs available (Usha, Jose et al. 2010) which create challenges for overall global health (Stuart and Bonnie 2004; Olayinka, Anthony et al. 2009). However, the rate at which new antibiotics are being

developed is much lower than the rate at which antibiotic resistance is being developed, making it difficult to combat staphylococcal infections (Hancock 2005). It is for this reason that new drugs that exhibit reduced resistance to these pathogens have to be investigated (Sarkar, Kumar et al. 2003; Fischbach and Walsh 2009). However, some diseases that were believed to have been eradicated and controlled re-emerge even after the introduction of new antimicrobial agents, suggesting that even the new antimicrobial agents may not be effective for long because of the high bacterial capacity to develop resistance (Coates, Hu et al. 2002; Levy and Marshall 2004; Gould 2007). As a result of the increased emergence of multidrug resistant microorganisms and the need to combat them, alternative methods have to be explored.

Natural products have shown to be effective against many bacterial diseases (Payne, Gwynn et al. 2007) and increasingly interest is being shown in plant-derived compounds (Rukayadi, Lee et al. 2009; Guzman, Gupta et al. 2010). This is because plants can be selected and used for antibacterial testing (Cos, Vlietinck et al. 2006). Plants contain compounds such as alkaloids, tannis, terpenoids and flavonoids, which have been shown to possess antimicrobial characteristics, thus allowing them to be used as antimicrobial agents (Cowan 1999; Lewis and Ausubel 2006). Flavonoids are a group of heterocyclic organic compounds present in photosynthesising cells and plant-related products such as honey and propolis (Havsteen 1983; Havsteen 2002). They have been identified in vegetables, nuts, fruits, seeds, stems, flowers, wine and tea (Middleton, Kandaswami et al. 2000). Flavonoid compounds have a fundamental structural feature that consists of a 2-phenyl-benzo[α]pyrane or flavane nucleus comprising two benzene rings (A and B) linked via a heterocyclic pyrane ring (Brown 1980). Flavonoids reportedly contain numerous beneficial medicinal properties that include antimicrobial activity, anti-inflammatory activity, enzyme inhibition, oestrogenic activity (Havsteen 1983), anti-allergic activity, antioxidant activity (Middleton, Kandaswami

et al. 2000), vascular and cytotoxic antitumor activities (Harborne and Williams 2000), antivirulence (Vandeputte, Kiendrebeogo et al. 2010). Flavonoids have been used to treat toxin-mediated disease and bacterial infections and it has been shown that when different flavonoids are combined, their bacterial and bacteriostatic actions are enhanced (Fukai, Marumo et al. 2002; Choi, Yahiro et al. 2007) A number of studies carried out have reported interactions between flavonoids and mammalian cells (Harborne and Williams 2000; Middleton, Kandaswami et al. 2000).

The aim of this study was to examine the antimicrobial activities of antibiotics (methicillin, vancomycin, tetracycline and ampicillin) and flavonoids (naringenin, chrysin and 7-hyroxyflavone) on their own, and in combination against methicillin-sensitive (MSSA) ATCC 25923 and methicillin-resistant (MRSA) ATCC 33591 *S. aureus* strains.

2.3. MATERIALS AND METHODS

2.3.1. Bacterial strains and growth conditions

The bacterial isolates used for the study were *S. aureus* strains ATCC 25923 and ATCC 33591, representing methicillin-sensitive and -resistant strains respectively, obtained from the American Type Culture Collection (ATCC). The bacterial strains were cultured on tryptone soy agar (TSA) (Oxoid Ltd, UK) plates and incubated at 37ºC, overnight. A single colony forming unit (CFU) was inoculated into tryptone soy broth (TSB) (Oxoid Ltd, UK) and incubated at 37°C, with shaking for 16-18 hours. 100 µl of the overnight culture was later inoculated into 5ml TSB for 5-8 hours until a turbidity equivalent to 0.5 McFarland standard was reached, (~ 1×10^8 to 2×10^8 CFU/ml).

2.3.2. Antibiotics and flavonoids

The antibiotics and flavonoids used were purchased in powder form from Sigma-Aldrich, USA. The antibiotics included methicillin, vancomycin, tetracycline and ampicillin. The flavonoids used were chrysin, naringenin and 7-hydroxyflavone. All the antibiotics and flavonoids were used in the Kirby-Bauer disk diffusion assay for both the combination studies but only tetracycline and ampicillin were used for the combination studies in the microtitre assay. Naringenin was the only flavonoid that was used in the microtitre assay as chrysin and 7-hydroxyflavone formed suspensions when dissolved in the solvent.

2.3.3. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of the antibiotics and flavonoids were determined as outlined by the Clinical and Laboratory Standards Institute (CLSI 2007) for both the Kirby-Bauer and microtitre assays.

2.3.3.1. Disk diffusion susceptibility test (Kirby-Bauer assay)

The antibiotics and flavonoids were made up and serially diluted in 100% dimethyl sulfoxide (DMSO) to yield concentrations ranging from 1.25 to 50 µg/ml and 0.002 mg/ml to 2 mg/ml for antibiotics and flavonoids, respectively. 40 µl of each antibiotic and flavonoid concentration was then loaded onto Munktell filter disks previously sterilised through autoclaving, in triplicate and placed in the incubator overnight to dry. 40 µl of the solvent (DMSO) was also loaded onto a control disk to assess and confirm that it would not have an effect on the bacterial growth at the maximum concentration used (100%). 100 µl of the MSSA ATCC 25923 and MRSA ATCC 33591 overnight culture, was inoculated in 5ml TSB

and incubated for 5-8 hours at 37ºC with shaking until an optical density at 600 nm ($OD_{600\ nm}$) of 0.6 was reached, exhibiting turbidity equivalent to that of 0.5 McFarland standard. 100 µl of the bacterial culture was then spread onto TSA plates and allowed to dry for between 10-15 minutes to allow any excess moisture to be absorbed before the disks were applied. The disks were then placed onto the agar plates and incubated at 37ºC for 18 hours. Inhibition zones were then measured and recorded.

For the combination studies, concentrations of antibiotics that had zone sizes big enough to show antagonism but small enough to show synergy were used. The antibiotic concentrations chosen for the combination studies were 1.25, 2.5, 5, and 12 µg/ml, for tetracycline, ampicillin, methicillin and vancomycin respectively. These antibiotic concentrations were combined with flavonoid concentrations ranging from 0.002 mg/ml to 2 mg/ml for all three flavonoids used. The antibiotic and flavonoid combination mixtures were then serially diluted and 40 µl loaded onto disks, in triplicate, and allowed to dry by incubating them at 37ºC overnight. Control disks were also included that contained 40 µl of each antibiotic at the chosen concentration as well as a DMSO control disk. 100 µl of MSSA ATCC 25923 and MRSA ATCC 33591 overnight culture was inoculated in 5 ml TSB and incubated for 5-8 hours at 37ºC with shaking until an optical density at 600 nm ($OD_{600\ nm}$) of 0.6 was reached and then spread onto TSA plates. The disks were then placed onto the agar plates and incubated at 37ºC for 18 hours. Inhibition zones were then measured and recorded.

2.3.3.2. Microdilution susceptibility test (Microtitre assay)

Two antibiotics – tetracycline and ampicillin – and the flavonoid naringenin were chosen for the microtitre assay. The bacterial strains were first grown on TSA plates overnight at 37ºC and then inoculated into 5 ml TSB broth and incubated at 37ºC with shaking for 4-6 hours until an optical density at 600 nm ($OD_{600\ nm}$) of 0.6 was reached exhibiting turbidity equivalent to that of 0.5 McFarland standard. This was then added to 15 ml of warm fresh broth to further dilute the bacterial culture. The experiments were carried out in 96 well microtitre plates and the MICs of the antibiotics for this microtitre microdilution method were determined as given by the Clinical and Laboratory Standards Institute (CLSI 2007). The concentrations used ranged from 0.05165 µg/ml to 1 µg/ml for ampicillin and 0.0625 µg/ml - 32 µg/ml for tetracycline and these were made up in two-fold serial dilutions. The concentrations used for naringenin remained the same ranging from 2 mg/ml to 0.002 mg/ml.

The antibiotics were dissolved in 1ml distilled water and naringenin was dissolved in 1 ml of 99 % methanol. It was established that at 5%, methanol did not have an effect on bacterial growth; hence a volume of 10 µl naringenin, dissolved in 99% methanol, was used, which contained methanol at a concentration of 5%. The experimental wells each contained 10 µl naringenin, 20 µl antibiotic, 70 µl fresh TSB and 100 µl of bacterial culture in triplicate for each antibiotic and each bacterial strain. The control wells contained; 10 µl 99% methanol plus 90 µl TSB for the methanol control, 20 µl antibiotic plus 80 µl TSB for the antibiotic control, 10 µl naringenin plus 90 µl TSB for the naringenin control and the bacterial control contained 100 µl TSB. Each control well contained 100 µl of the bacterial strains, and this was done in triplicate and each well had a final volume of 200µl. A TSB control was also

included, containing 100 µl of TSB in triplicate. The plates were then sealed and incubated at 37ºC with shaking for 18 hours and then read at 620nm in a plate reader.

For the combinations studies, two concentrations for each of the antibiotics were chosen, set at 0.03125 and 0.0625 µg/ml for ampicillin, and 0.25 µg/ml and 1 µ/ml, for tetracycline. At these concentrations, the percentage of bacterial growth was high enough to show any synergy that might be present after combining the antibiotic and flavonoid.

Methanol was the solvent of choice for naringenin as it could not dissolve in distilled water. 40 mg of naringenin was weighed and dissolved in 1 ml of 99% methanol. The first three wells contained 140 µl of TSB while the others contained 80 µl of TSB. 20 µl of naringenin was then added to the first three wells and then serially diluted into the remaining wells. This ensured that naringenin was at a concentration ranging from 0.02 mg/ml to 2 mg/ml and methanol at a concentration of 5% that did not affect the bacterial growth. 20 µl of the antibiotics at the different concentrations was then added to the wells followed by 100µl of the two bacterial strains as outlined earlier. Control wells contained; 10 µl methanol plus 90 µl TSB, 20 µl antibiotic plus 80 µl TSB, 10 µl naringenin plus 90 µl TSB all containing 100µl of each of the bacterial strains as outlined earlier. Bacterial controls contained 100 µl of each of the bacterial strains plus 100 µl of TSB. The TSB control contained 100 µl of TSB. These were also all done in triplicate and each well had a final volume of 200 µl. The 96 well microtitre plates were then sealed and incubated at 37ºC with shaking for 18 hours and then read at 620 nm in a plate reader.

2.3.4. Data analysis

The synergy ratios (SR) for the concentrations used in the combination studies were calculated using the Abbott formula $\%C_{exp}=A+B-(AB/100)$. Where, *A* was the control level of the antibiotic, *B* the control level of naringenin and C_{exp} represented the expected efficacy of the mixture (naringenin and antibiotic combination) (Levy, Benderly et al. 1986). After calculating the $\%C_{exp}$ (expected efficacy), the SR was obtained using the formula $SR=C_{obs}/C_{exp}$, with C_{obs} representing the observed efficacy. The ratios obtained after these calculations were made enabled the determination of what synergistic interaction was present in the mixture. If the SR is greater than 1.5, then synergistic interactions are present, if between 0.5-1.5 then there is an additive effect and if the SR is below 0.5, then the effect is antagonistic (Gisi, Binder et al. 1985; Gisi 1996). This enabled the evaluation of the level of interaction/association of naringenin and antibiotics. The results outlined were obtained after all these parameters were taken into account.

2.4. RESULTS

2.4.1. Antimicrobial susceptibility testing

Methicillin, tetracycline, ampicillin and vancomycin were used in the disk diffusion susceptibility test including the flavonoids chrysin, naringenin and 7-hydroxyflavone on their own as well as in combination. For the microtitre assay, tetracycline and ampicillin were the antibiotics of choice and naringenin was the flavonoid used, on their own and in combination. The results for both assays are described below.

2.4.1.1. Disk diffusion susceptibility test (Kirby-Bauer assay)

Table 2.1. Zone Diameter Interpretive (mm) Standards for *Staphylococcus* species.

	Zone diameters to the nearest whole mm		
Antibiotic (Concentration)	**Resistant**	**Intermediate**	**Susceptible/sensitive**
Tetracycline (30µg/ml)	≤14	15-18	≥19
Ampicillin (10µg/ml)	≤28	–	≥29
Methicillin (5µg/ml)	≤9	10-13	≥14
Vancomycin (30µg/ml)	–	–	≥15

Table 2.1 shows the zones of inhibition interpretive standards for interpreting the zone diameters for tetracycline, ampicillin, methicillin and vancomycin, as outlined by the CLSI. This was done in order to establish if the bacteria were resistant, intermediate or sensitive to the antibiotics at the concentrations used.

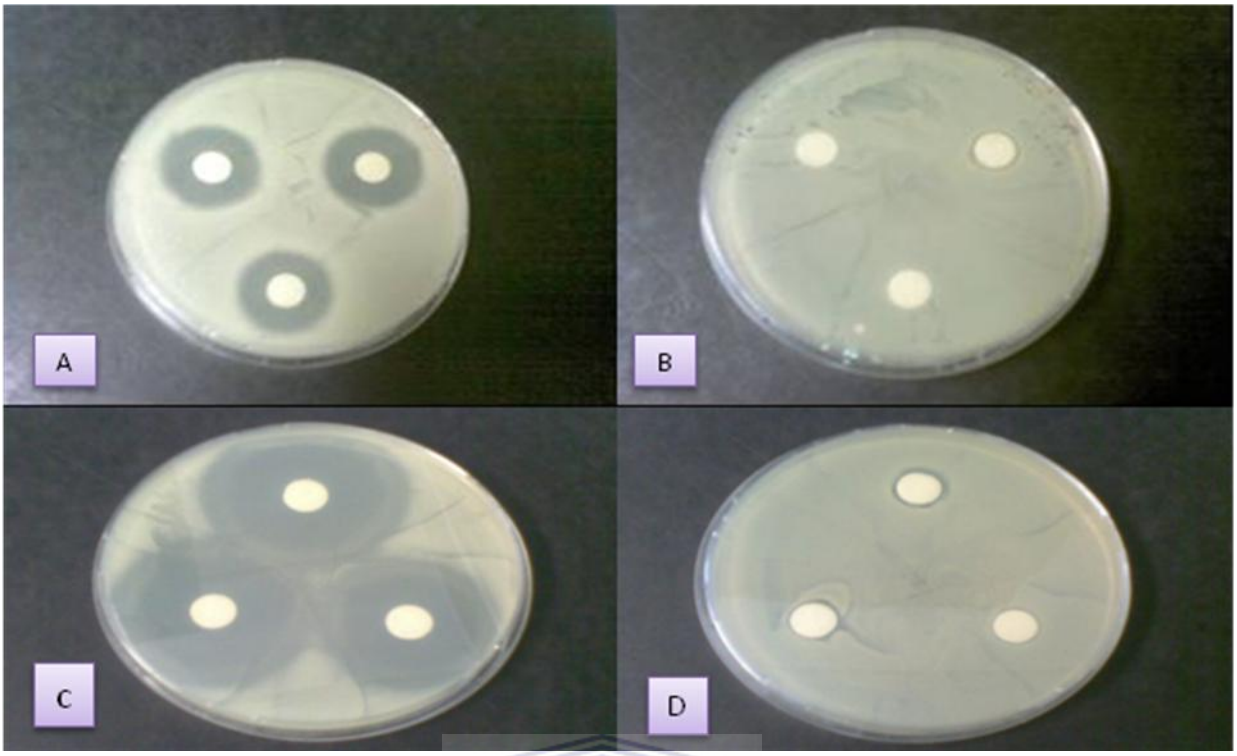

Figure 2.1. TSA plates with disks containing tetracycline and ampicillin at concentrations of 12, 25 and 50 µg/ml against MSSA (ATCC 25923) and MRSA (ATCC 33591) strains. **(A)** tetracycline against MSSA (ATCC 25923) **(B)** tetracycline against MRSA (ATCC 33591) **(C)** ampicillin against MSAA (ATCC 25923) **(D)** ampicillin against MRSA (ATCC 33591).

Figure 2.1 shows TSA plates containing tetracycline and ampicillin at concentrations of 12, 25 and 50 µg/ml. The zones of inhibition at different antibiotic concentrations against MSSA ATCC 25923 and MRSA ATCC 33591 strains are outlined in Table 2.2. Ampicillin had the largest zones of inhibition with corresponding zone sizes of 31 to 46 mm against MSSA ATCC 25923. However, MRSA ATCC 33591was resistant to ampicillin at the same concentrations, with small zones (11 mm) of inhibition observed. MSSA ATCC 25923 was also susceptible to tetracycline at concentrations of 12, 25 and 50 µg/ml with zones of inhibition sizes of 21, 23 and 26 mm respectively. MRSA ATCC 33591 was resistant (0-10 mm) to tetracycline concentrations (1,25-50 µg/ml).

Table 2.2. Inhibition zones (mm) of antibiotics against MSSA ATCC 25923 and MRSA (ATCC 33591) strains.

	Inhibition Zone diameter (mm)			
Concentration (µg/ml)	**Tetracycline**	**Ampicillin**	**Vancomycin**	**Methicillin**
		MSSA (ATCC 25923)		
1.25	15.3 ± 1.2 (I)	10 ± 0 (R)	10 ± 0 0	─ (R)
2.5	16.6 ± 0.5 (I)	19 ± 0.9 (R)	12 ± 0 0	13 ± 1.2 (I)
5	20 ± 0 (S)	31 ± 1.2 (S)	14 ± 0.5 0	19 ± 0.9 (S)
12	21 ± 0.5 (S)	37 ± 0.5 (S)	15 ± 0.9 (S)	24 ± 1.7 (S)
25	23 ± 0 (S)	42 ± 0 (S)	16 ± 0.5 (S)	27 ± 2.1 (S)
50	26 ± 0.9 (S)	46 ± 0.5 (S)	17 ± 1.2 (S)	35 ± 1.9 (S)
		MRSA (ATCC 33591)		
1.25	─ (R)	11 ± 0.5 (R)	10 ± 0 0	─ (R)
2.5	─ (R)	11 ± 0 (R)	12 ± 0.4 0	─ (R)
5	─ (R)	11 ± 0.5 (R)	14 ± 0.4 0	─ (R)
12	─ (R)	11 ± 0 (R)	15 ± 0.2 (S)	─ (R)
25	─ (R)	11 ± 0.5 (R)	17 ± 0.2 (S)	─ (R)
50	10 ± 0 (R)	12 ± 0 (R)	18 ± 0.8 (S)	─ (R)

Diameter of zones of inhibition, including diameter of disk, expressed as means of three replicates; S- susceptible, I- moderate susceptible (intermediate), R- resistant, 0- no given values (as outlined by the CLSI). ─: no zones of inhibition (equivalent to no inhibitory effect). Values are expressed as mean ± standard deviation.

MSSA ATCC 25923 was susceptible to; tetracycline at concentrations of 5 to 50 µg/ml with zones of inhibition of 20 to 26 mm, with concentrations of 1.25 and 2.5 µg/ml showing intermediate results with zones of 15.3 and 16.6 mm respectively; ampicillin at concentrations of 5 to 50 µg/ml with zones of inhibition of 31 to 46 mm; vancomycin at concentrations of 12 to 50 µg/ml with zones of inhibition of 15 to 17 mm and methicillin at concentrations of 5 to 50 µg/ml with zones of inhibition of 19 to 35 mm. MRSA ATCC 33591 was resistant to all antibiotics except vancomycin, which it showed susceptibility to at concentrations of 12 to 50 µg/ml with zones of inhibition of 15 to 18 mm. Different concentrations of chrysin, naringenin and 7-hydroxyflavone including the DMSO control did not show any inhibitory effect on the bacterial growth as no zones of inhibition were observed on the TSA plates (Appendix I). Combinations of chrysin, naringenin and 7-hydroxyflavone with the four antibiotics did not increase or decrease the sizes of the initial inhibition zones of the antibiotics (Appendix I).

2.4.1.2. Microdilution susceptibility test (Microtitre assay)

Table 2.3 represents the MIC interpretive standards for *Staphylococcus* species as outlined by the CLSI.

Table 2.3. MIC (µg/ml) Interpretive Standards for *Staphylococcus* spp.

	MIC (µg/ml)		
Antibiotic	**Susceptible/sensitive**	**Intermediate**	**Resistant**
Tetracycline	≤4	8	≥16
Ampicillin	≤0.25	–	≥0.5

The MIC interpretive standards were used to compare the MICs of tetracycline and ampicillin in the microtitre assay. The MICs of the antibiotics were obtained by serially diluting the antibiotics and ensuring that the ranges were within those outlined by the CLSI. Figures 2.2, 2.3 and 2.4 below, show the bacterial growth of MSSA ATCC 25923 and MRSA ATCC 33591 in different tetracycline, ampicillin and naringenin concentrations. The concentrations of tetracycline and ampicillin chosen for the combination studies with naringenin were those that showed little or no inhibitory effect on the bacterial growth. This enabled any synergistic or additive effect that naringenin would have to be measurable, as any reduction in the bacterial growth rate at these concentrations would definitely have been as a result of naringenin.

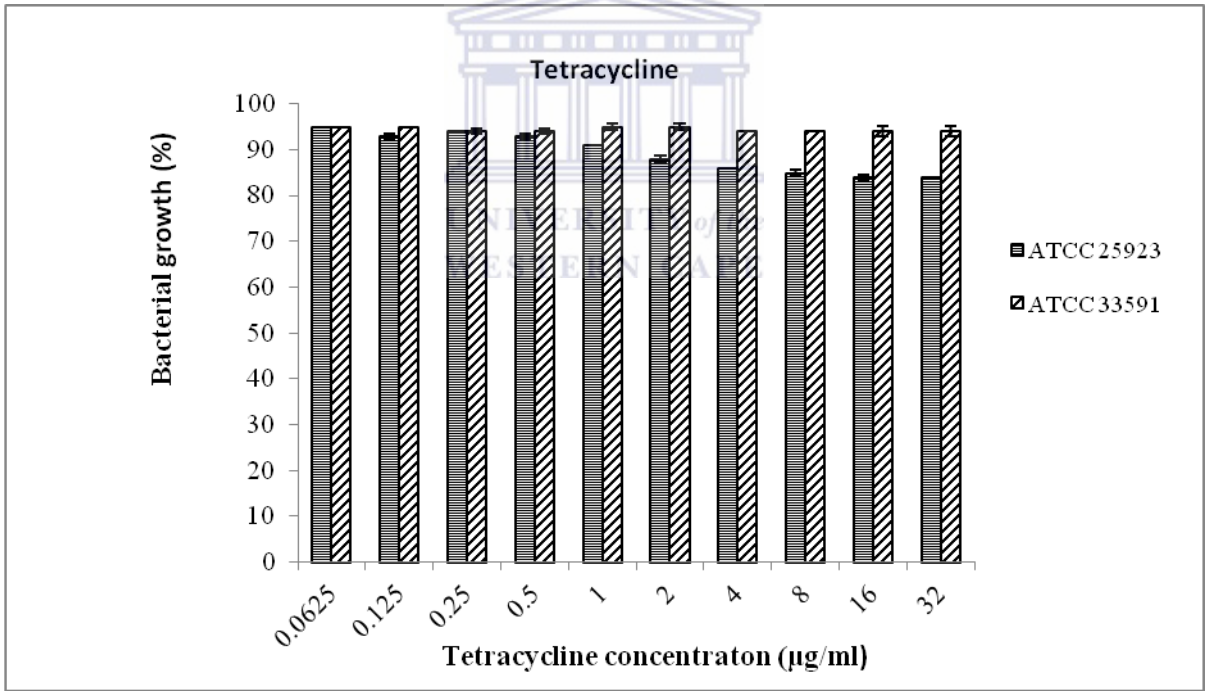

Figure 2.2. Effect of minimum inhibitory concentrations (MICs) of tetracycline on the bacterial growth of MSSA ATCC 25923 and MRSA ATCC 3359. Bacterial growth is expressed as means of three replicates and values are stated as means ± standard deviation.

According to the CLSI MIC interpretive standards the MICs for tetracycline are ≤4 and ≥6 µg/ml for sensitivity and resistance respectively. The tetracycline concentrations used

ranged from 0.0625 to 32 µg/ml (Figure 2.2). The bacterial growth controls for the MSSA ATCC 25923 and MRSA 33591 strains were 95 and 94% respectively. In this study, MSSA ATCC 25923 showed a bacterial growth rate of between 84 to 88% at concentrations of 2 to 32 µg/ml (showing a 7 to 11% reduction in bacterial growth rate when compared to the bacterial control), whereas MRSA ATCC 33591 had a bacterial growth rate of 94 to 95% at the same concentrations. MSSA ATCC 25923 showed a bacterial growth rate decrease from 91% at 1 µg/ml to 84% at 32 µg/ml (the highest concentration used). The MRSA ATCC 33591 strain showed resistance to tetracycline even at the highest concentration used (32 µg/ml). The concentrations chosen for the combination studies were 0.25 and 1 µg/ml which had bacterial growth rates of 94 and 91% respectively, for MSSA ATCC 25923 and bacterial growth rates of 95 and 96% for MRSA ATCC 33591 respectively.

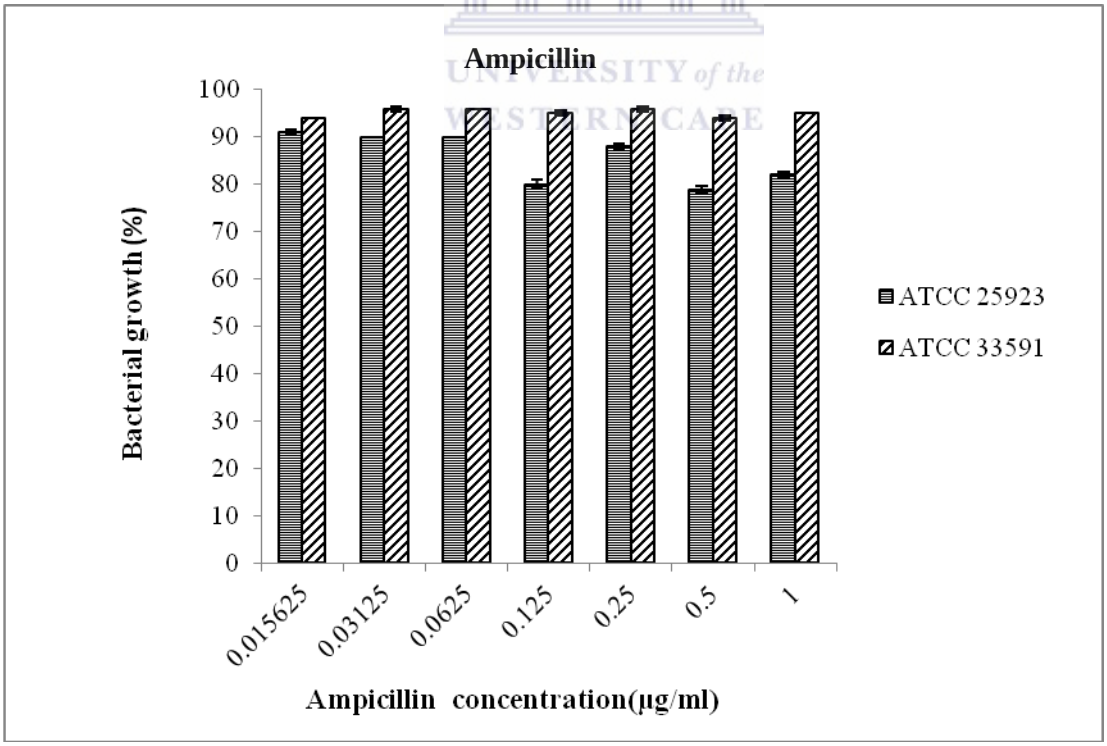

Figure 2.3. Effect of minimum inhibitory concentrations (MICs) of ampicillin on the bacterial growth of MSSA ATCC 25923 and MRSA ATCC 3359. Bacterial growth is expressed as means of three replicates and values are stated as means ± standard deviation.

The MICs for ampicillin are ≤ 0.25 and ≥ 0.5 µg/ml, for sensitivity and resistance, as outlined by the CLSI. The bacterial controls had bacterial growth rates of 95 and 94% for MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively (Figure 2.3). In this study, 0.125 and 0.5 µg/ml ampicillin showed an 80% bacterial growth rate for the MSSA ATCC 25923 strain (showing a 15% reduction in bacterial growth rate) compared to the MRSA ATCC 33591 that showed a bacterial growth rate of about 95% at the same concentrations. At 0.25 and 1 µg/ml ampicillin, the bacterial growth rate was 88 and 82% respectively (showing a 7 to 13% reduction in bacterial growth rate). The bacterial growth rate for the MSSA ATCC 25923 strain decreased from 91% at 0.015625 µg/ml, to 80% at 0.125 µg/ml ampicillin. The MRSA ATCC 33591 strain was resistant to ampicillin at all concentrations used. The ampicillin concentrations chosen for the combination studies, with naringenin, were 0.03125 and 0.0625 µg/ml which both had bacterial growth rates of about 90 and 96%, for MSSA ATCC 25923 and MRSA ATCC 33591 respectively.

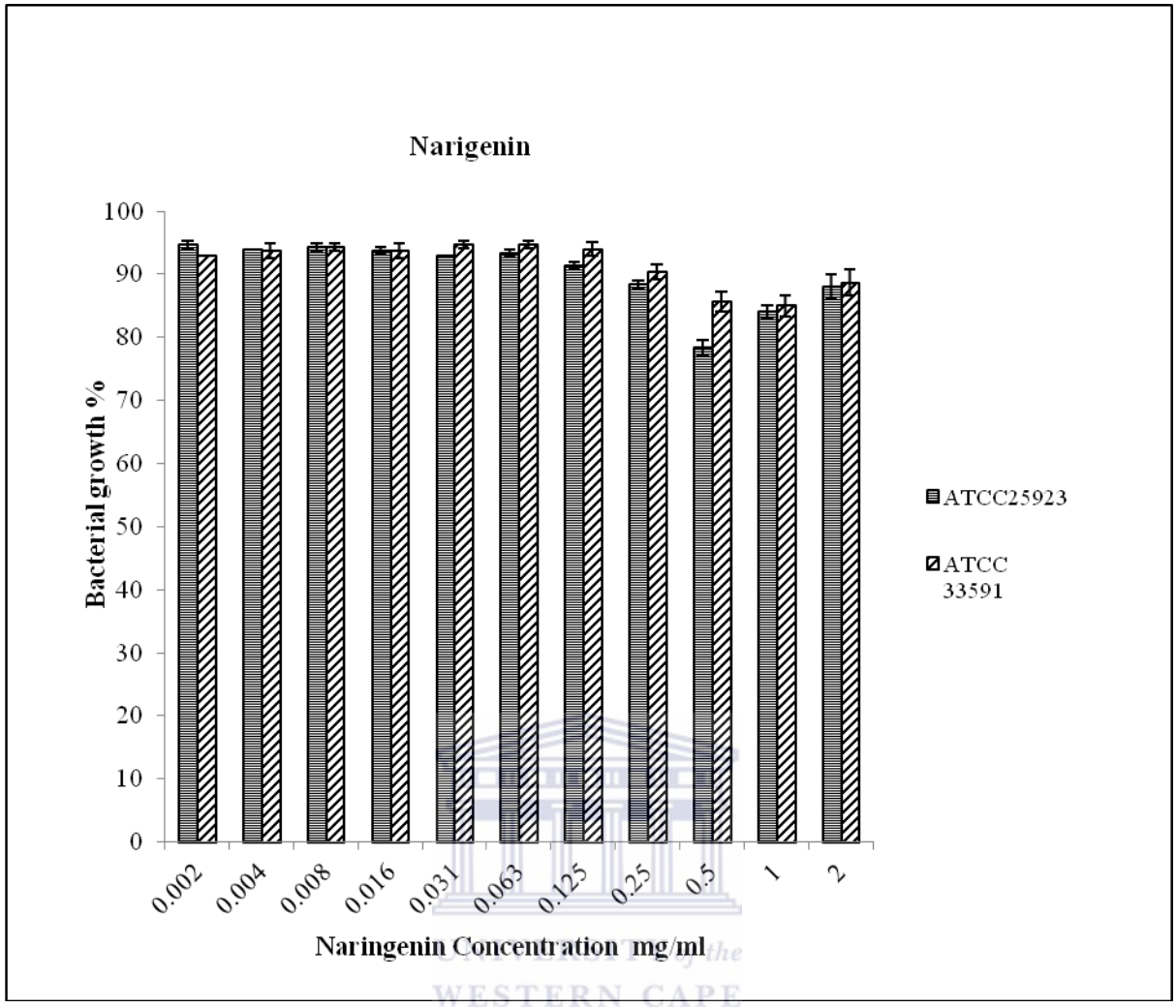

Figure 2.4. Effect of different naringenin concentrations on the bacterial growth of MSSA ATCC 25923 and MRSA ATCC 3359. Bacterial growth is expressed as means of three replicates and values are stated as means ± standard deviation.

Figure 2.4 shows results obtained after different naringenin concentrations were tested against MSSA ATCC 25923 and MRSA ATCC 33591 strains. The bacterial controls had bacterial growth rates of 95 and 94% for MSSA ATCC 25923 and MRSA ATCC 33591 respectively. Naringenin showed minimal inhibitory effect on the bacterial growth rate (which remained unchanged between 90 and 95%) for both strains at concentrations between 0.002 and 0.063 mg/ml naringenin. The bacterial growth rate was then reduced from 94%, at 0.063 mg/ml naringenin, to 88 and 78% at naringenin concentrations of 0.25 and 0.5 mg/ml respectively. 1 and 2 mg/ml naringenin had a bacterial growth rate of 86 and 89%

respectively for the MSSA ATCC 25923 strain. The MRSA ATCC 33591 strain was resistant to naringenin at concentrations of 0.002 to 0.125 mg/ml with bacterial growth rates of 94%. The bacterial growth rate decreased to 90 and 86% at concentrations of 0.25 to 1 mg/ml respectively, with 2 mg/ml showing a bacterial growth rate of 89%. The same range of naringenin concentrations were selected for the combination studies with tetracycline and ampicillin.

Table 2.4. Antibacterial activity of naringenin (0.004 to 2 mg/ml) in combination with tetracycline (1µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591.

	MSSA ATCC 25923			**MRSA ATCC 33591**		
Tetracycline (1 µg/ml) + naringenin (mg/ml) Concentration	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation
0.004	90	0.91	Add	95	0.96	Add
0.008	89	0.90	Add	94	0.95	Add
0.016	89	0.90	Add	95	0.96	Add
0.031	89	0.90	Add	95	0.96	Add
0.063	87	0.88	Add	95	0.96	Add
0.125	**85**	0.86	Add	94	0.95	Add
0.25	**85**	0.86	Add	**88**	0.89	Add
0.5	89	0.90	Add	91	0.92	Add
1	89	0.90	Add	92	0.93	Add
2	90	0.91	Add	91	0.92	Add

Add- Additive effect

As described in Figure 2.2, tetracycline had bacterial growth rates of 91 and 95% for MSSA ATCC 25923 and MRSA ATCC 33591 respectively at a concentration of 1 µg/ml. The bacterial, antibiotic, naringenin and methanol controls had bacterial growth rates of 94,

90, 90 and 93% for MSSA ATCC 25923 and 95, 94, 90 and 94% for MRSA ATCC 33591 respectively. Table 2.4 shows that the combination of tetracycline with naringenin at 0.125 and 0.25 µg/ml against MSSA ATCC 25923, reduced the bacterial growth rate from 91% to 85% (6% reduction in bacterial growth rate) when compared to tetracycline used on its own. Nonetheless, MRSA ATCC 33591 showed a reduction in bacterial growth rate to 88% at 0.25 mg/ml naringenin and tetracycline combination (7% reduction in bacterial growth rate), while tetracycline on its own had a bacterial growth rate of 95% at 1 µg/ml (Figure 2.2). The synergistic calculations confirmed these results between naringenin and tetracycline as showing additive effects, meaning that naringenin did enhance the antibacterial activity of tetracycline.

Table 2.5. Antibacterial activity of naringenin (0.004 to 2 mg/ml) in combination with tetracycline (0.25 µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591 strains.

	MSSA ATCC 25923			MRSA ATCC 33951		
Tetracycline (0.25 µg/ml) + naringenin (mg/ml) Concentration	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation
0.004	92	0.93	Add	95	0.95	Add
0.008	92	0.93	Add	95	0.95	Add
0.016	92	0.93	Add	95	0.95	Add
0.031	92	0.93	Add	95	0.95	Add
0.063	91	0.92	Add	94	0.94	Add
0.125	**88**	0.89	Add	94	0.94	Add
0.25	**84**	0.85	Add	**89**	0.89	Add
0.5	**84**	0.85	Add	**87**	0.87	Add
1	90	0.91	Add	93	0.936	Add
2	90	0.91	Add	92	0.92	Add

Add- Additive effect

As outlined earlier (Figure 2.2), tetracycline had bacterial growth rates of 94% for both MSSA ATCC 25923 and MRSA 33591 at a concentration of 0.25 µg/ml. The bacterial, antibiotic, naringenin and methanol controls had bacterial growth rates of 94, 93, 91 and 93% for MSSA ATCC 25923 and 95, 95, 91 and 95% for MRSA ATCC 33591 respectively. Table 2.5 showed that the combination of 0.25 µg/ml tetracycline with naringenin at 0.125, 0.25 and 0.5 µg/ml against MSSA ATCC 25923 resulted in bacterial growth rates of 88 and 84% respectively (a bacterial growth reduction of 6 to 10%), when compared to tetracycline on its own. However, MRSA ATCC 33591 showed an 87 and 89% bacterial growth rate at naringenin concentrations of 0.25 and 0.5 mg/ml in combination with 0.25 µg/ml tetracycline (5 to 7% reduction in bacterial growth rate), while tetracycline on its own had a bacterial

growth rate of 94% at 0.25 µg/ml. The synergistic calculations confirmed these results between naringenin and tetracycline as showing additive effects, meaning that naringenin did enhance the antibacterial activity of tetracycline.

Table 2.6. Antibacterial activity of naringenin (0.004 to 2 mg/ml) in combination with ampicillin (0.0625µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591 strains.

	MSSA ATCC 25923			**MRSA ATCC 33591**		
Ampicillin (0.0625 µg/ml) + naringenin (mg/ml) Concentration	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation
0.004	86	0.87	Add	95	0.96	Add
0.008	85	0.86	Add	94	0.95	Add
0.016	86	0.87	Add	95	0.96	Add
0.031	82	0.83	Add	95	0.96	Add
0.063	**72**	0.73	Add	95	0.96	Add
0.125	**69**	0.67	Add	93	0.94	Add
0.25	**73**	0.74	Add	**89**	0.90	Add
0.5	83	0.84	Add	91	0.92	Add
1	82	0.83	Add	92	0.93	Add
2	88	0.89	Add	**89**	0.90	Add

Add- Additive effect

Table 2.6 shows the combination of 0.0625 µg/ml ampicillin with 0.004 to 2 mg/ml naringenin. As outlined in Figure 2.3, ampicillin produced bacterial growth rates of 90 and 96%, for MSSA ATCC 25923 and MRSA ATCC 33591 respectively, at a concentration of 0.0625 µg/ml. The bacterial, antibiotic, naringenin and methanol controls had bacterial growth rates of 94, 90, 90 and 93% for MSSA ATCC 25923 and 95, 93, 92 and 94% for

MRSA ATCC 33591 respectively. The study showed that 0.0625 µg/ml ampicillin combined with 0.063, 0.125 and 0.25 mg/ml naringenin yielded bacterial growth rates of 72, 69 and 73% respectively, for the MSSA ATCC 25923 strain, compared to the bacterial growth rate of 90% at 0.0625 µg/ml ampicillin (with a reduction of bacterial growth rate of as high as 21% at 0.125 mg/ml naringenin). The MRSA ATCC 33591 strain had a bacterial growth rate of 89% at 0.25 and 2 mg/ml naringenin combined with 0.0625 µg/ml ampicillin. The synergistic calculations confirmed these results between naringenin and ampicillin as showing additive effects, meaning that naringenin did enhance the antibacterial activity of ampicillin.

Table 2.7. Antibacterial activity of naringenin (0.004 to 2 mg/ml) in combination with ampicillin (0.03125µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591 strains.

	MSSA ATCC 25923			**MRSA ATCC 33591**		
Ampicillin (0.03125 µg/ml) + naringenin (mg/ml) Concentration	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation	Bacterial cell growth (%)	Synergy ratio (SR)	Interpretation
0.004	89	0.90	Add	94	0.94	Add
0.008	90	0.91	Add	94	0.94	Add
0.016	90	0.91	Add	94	0.94	Add
0.031	90	0.91	Add	94	0.94	Add
0.063	90	0.91	Add	94	0.94	Add
0.125	**86**	0.87	Add	92	0.92	Add
0.25	**76**	0.77	Add	92	0.92	Add
0.5	**77**	0.78	Add	**89**	0.89	Add
1	86	0.87	Add	92	0.92	Add
2	86	0.87	Add	93	0.93	Add

Add- Additive effect

Table 2.7 shows the combination of 0.03125 µg/ml ampicillin with 0.004 to 2 mg/ml naringenin. As outlined in Figure 2.3, ampicillin produced bacterial growth rates of 90 and 96% at a concentration of 0.03125 µg/ml. The bacterial, antibiotic, naringenin and methanol controls had bacterial growth rates of 93, 90, 91 and 92% for MSSA ATCC 25923 and 94, 93, 93 and 93% for MRSA ATCC 33591 respectively. The study showed that 0.03125 µg/ml ampicillin combined with 0.125, 0.25 and 0.5 mg/ml naringenin, yielded bacterial growth rates of 86, 76 and 77% respectively, compared to the bacterial growth rate of 90% at 0.03125 µg/ml ampicillin alone. The MRSA ATCC 33591 strain had a bacterial growth rate of 89% at 0.5 mg/ml naringenin combined with 0.0625 µg/ml ampicillin. The calculated synergistic ratios confirmed these results between naringenin and ampicillin as being additive effects, meaning that naringenin did enhance the antibacterial activity of ampicillin.

2.5. DISCUSSION

In the disk diffusion assay, the antibiotics showed remarkable antimicrobial activity with ampicillin producing the largest inhibition zones for MSSA ATCC 25923. Tetracycline had zones that ranged from 10 to 26 mm, vancomycin 10 to 17 mm and methicillin 13 to 35 mm for MSSA ATCC 25923 with the largest zones corresponding to the highest concentration. Vancomycin was the only antibiotic that had similar inhibition zone sizes for both strains showing that MRSA ATCC 33591 was susceptible to it with the greatest effect seen at concentrations of 12, 25 and 50 µg/ml. Tetracycline and methicillin did not have any inhibitory effect on MRSA ATCC 33591 as no inhibition zones were present. Ampicillin had small zones of 12 mm, which was not considered an inhibitory effect. Chrysin, naringenin and 7-hydroxyflavone did not have any inhibitory effect on bacterial growth at any of the

concentrations used as observed by the absence of inhibition zones on the TSA plates for both MRSA ATCC 33591 and MSSA ATCC 25923.

Combination studies with the disk diffusion assay revealed that the flavonoids did not enhance the antibacterial activity of the antibiotics. DMSO used as the solvent for the disk diffusion assay did not have any effect on bacterial growth even at a 100% concentration when loaded onto the disk despite reports that it has some antimicrobial activity (Dayang, Noraziah et al. 2008). It has been shown that flavonoids may diffuse out of the disks, used in the disk diffusion assay, at a low rate thereby affecting their antibacterial activities (Zheng, Tan et al. 1996). This could explain why the flavonoids did not seem to have any inhibitory effect either on their own or in combination with the different antibiotics.

Naringenin was the flavonoid of choice for the microtitre assay. Chrysin and 7-hydroxyflavone were not included as they precipitated and formed suspensions when diluted in the solvent. This precipitation would have affected the level of contact of the bacterial cells with the flavonoid and consequently the antibiotic during combination studies. The precipitation could have resulted in interpreting it as false negative since the precipitation would have been misinterpreted as bacterial growth. The solvent of choice for naringenin was methanol as it dissolved without precipitating. DMSO was not used as a solvent because it was observed that it had some effect on the bacterial growth when used in the microtitre assay. Methanol was used at a 5% concentration to ensure that it did not have any effect on bacterial growth. Distilled water was the solvent used for the antibiotics. MSSA ATCC 25923 was susceptible to both tetracycline and ampicillin at MICs of ≤ 4 µg/ml and ≤ 0.25 µg/ml respectively, as illustrated by the MIC interpretive standards outlined by the CLSI. MRSA ATCC 33591 showed a consistent rate of resistance against the bacterial growth for both antibiotics.

The two concentrations chosen for the combination studies were 0.25 and 1µg/ml for tetracycline and 0.03125 and 0.0625 µg/ml for ampicillin. Bacterial growth at these concentrations was high enough to demonstrate inhibitory effects naringenin would have in the combination studies. This was essential in establishing and classifying the effect as being synergistic, additive or antagonistic. Naringenin was used at concentrations ranging from 0.002 to 2 mg/ml. Naringenin concentrations of 0.125, 0.25 and 0.5 mg/ml enhanced the antibacterial activity of 0.25 and 1 µg/ml tetracycline against MSSA ATCC 25923 and MRSA ATCC 33591. In the case of ampicillin, naringenin concentrations of 0.063, 0.0125, 0.25 and 0.5 µg/ml enhanced antibacterial activity against both strains.

Numerous studies have been carried out that highlight the beneficial properties of flavonoids. However, acylated flavonoids are rather new, therefore, only a limited amount of scientific information is available (Gatto, Falcocchio et al. 2002; Celiz, Audisio et al. 2010; Celiz, Daz et al. 2011). Naringenin is a flavonone mainly found in citrus fruits and possesses phytochemical properties (van Acker, Hulshof et al. 2001; Lee, Lee et al. 2011). It has been shown to inhibit bacterial motility proving that it is capable of interfering with the proton motive force (Cushnie and Lamb 2005). A study on the effect of naringenin on lung metastasis showed that it reduced the incidence and number of lung metastatic colonies (Qin, Jin et al. 2011). Another study also revealed that naringenin showed a higher antibacterial effect compared to other flavones (Nur, Noraziah et al. 2012). Studies have reported that structural changes in the flavonoids aids in the acquisition of antimicrobial activities. The addition of two or three extra hydroxyl groups at positions 7, 2´ and 4´ to 5-hydroxyflavanones and 5-hydroxyisoflavanones inhibited *Streptococcus mutans* and *Streptococcus sobrinus* growth using the disk diffusion assay (Osawa, Yasuda et al. 1992). 3-hydroxy, 5-hydroxy and 7-hydroxyflavones have also been shown to have antioxidant properties (Firuzi, Mladenka et al. 2004; Firuzi, Lacanna et al. 2005). Tsuchiya and

colleagues (1996) demonstrated that 2´,4´- or 2´,6´-dihydroxylation of the B ring and 5,7-dihydroxylation of the A ring in the flavanone structure played a critical role in rendering the anti-MRSA flavonoid ability (Tsuchiya, Sato et al. 1996). Chrysin has numerous beneficial properties such as antioxidant, anti-inflammatory, anti-diabetogenic, anti-hypertensive and axiolytic properties (Tahir and Sultana 2011). Studies have also shown that sub-inhibitory concentrations of chrysin are capable of preventing the production of α-hemolysin (pore-forming toxin responsible for tissue damage) by *S. aureus* (Wang, Qiu et al. 2011).

Antibacterial activities of plant flavonoids against MRSA have generated a lot of interest (Tsuchiya, Shimizu et al. 1985; Xu and Lee 2001; Nanayakkara, Burandt et al. 2002; Nishida and Satoh 2004). Several studies have also demonstrated the synergistic effects of plant extracts with various antibiotics and a subsequent reduction in the MIC of the antibiotics against some resistant bacterial strains including *S. aureus* (Darwish, Aburjai et al. 2002; Stapleton, Shah et al. 2004; Braga, Leite et al. 2005; Fadli, Saad et al. 2012). Combination studies demonstrated the synergistic effect of flavonoids, such as 6, 7-dihydroxyflavone and isoflavanone with antibiotics oxacillin and mupirocin against MRSA (Shirataki, Wakae et al. 2004; Sato, Tanaka et al. 2006). It is believed that the mechanism of antibacterial action may be as a result of the flavonoid being able to reverse bacterial resistance to β-lactam antibiotics (Eumkeb, Siriwong et al. 2012). Synergistic interactions between propolis and tetracycline (Mirzoeva, Grishanin et al. 1997) as well as amipicillin (Stepanovic, Antic et al. 2003) have also been documented. Synergistic interactions between pentacyclic triterpenoids and antibiotics such as methicillin and vancomycin have also been reported (Chung, Navaratnam et al. 2011).

Africa can be a source of therapeutically useful botanical antimicrobial compounds. Further studies can be initiated on medicinal plants especially in combination with antimicrobial agents. Medicinal plants and plant compounds have been used in a number of

cultures to treat various infections and it is for this reason that studies are being done to screen them for antimicrobial activities in the hope of administering them in combination with antibiotics (Betoni, Mantovani et al. 2006; Chung, Navaratnam et al. 2011; Stefanovic, Stanojevic et al. 2012). Investigating plant extracts through structural alterations may pave the way for the development of pharmacologically acceptable antimicrobial agents. However, the MICs of plant derived antimicrobial compounds are more elevated than those of fungal or bacterial produced antibiotics and this tends to limit their therapeutic use (Gibbons, Moser et al. 2004). Antibiotic resistance still remains a global problem and the search for solutions still remains a challenge, hence the need to focus on other ways to manage these infections. Further studies on the interactions between flavonoids and antibiotics is one way in which multidrug resistance mechanism of bacteria can be overcome (Braga, Leite et al. 2005; Herath, Mikell et al. 2006; Celiz, Daz et al. 2011; Wang, Qiu et al. 2011).

This study showed that naringenin enhanced the antibacterial activities of tetracycline and ampicillin, in the microtitre assay, at concentrations that initially did not have any effect on bacterial growth. However, naringenin, chrysin and 7-hydroxyflavone did not enhance the antibacterial activities of the antibiotics in the disk diffusion assay, which led to the conclusion that the poor and/or slow diffusion of the flavonoids from the disks may have been a contributing factor. Future prospects will involve using different concentrations and types of flavonoids, in combination with various antibiotics, to challenge *S. aureus* and analyze the effect at a molecular level. The efflux pump genes of *S. aureus* can also be analyzed to assess which ones may be up/down regulated in the presence of these compounds.

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CHAPTER THREE

Comparing the presence of *mepA, sepA* and *gyrA* genes in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* using Polymerase Chain Reaction (PCR)

3.1. ABSTRACT

Efflux pump proteins are expressed in both antibiotic-susceptible and antibiotic-resistant bacteria. Efflux pumps are transport proteins responsible for the extrusion of toxic substances, including a number of antibiotics, from inside the bacterial cell to the external environment. These efflux mechanisms have been widely identified as major and important components in rendering bacteria resistant to many classes of antibiotics. Whereas certain efflux pumps are capable of expelling a number of different antibiotics (referred to as multidrug resistance (MDR) pumps), others are antibiotic-specific. A number of efflux pump genes have been identified in *Staphylococcus aureus* and these have been shown to play a role in conferring resistance to many different antibiotics.

In this study, three efflux pump genes - *mepA*, *sepA* and *gyrA* - were analyzed using Polymerase Chain Reaction (PCR); 16S was included as a positive control. The aim of the study was to determine which of the three genes were present or absent in methicillin-sensitive and methicillin-resistant *S. aureus* strains MSSA ATCC 25923 and MRSA ATCC 33591, respectively. The results showed the presence of the *mepA* and *gyrA* in both MSSA ATCC 25923 and MRSA ATCC 33591 strains. However *sepA* was only present in the MRSA ATCC 33591 strain. The presence of *mepA* and *gyrA* in both strains suggests that possible chromosomal mutations, resulting in their over-expression, may be responsible for their role in drug resistance. The presence of *sepA* MRSA and not MSSA suggested that it plays a role in conferring resistance possibly encoding a drug resistant protein.

3.2. INTRODUCTION

In the last century, a number of measures have been put in place to manage, treat and prevent infectious diseases. Antibiotics, in combination with chemical compounds used as

disinfectants and antiseptics (also called biocides), have been used to eradicate and manage numerous pathogenic bacteria that cause life-threatening conditions (Gilbert and McBain 2003). Alarmingly, due to the increased use of antibiotics and biocides in the treatment of these pathogens, some bacterial strains have developed resistance to one or more classes of these antibiotics and biocides, causing concern worldwide (Gilbert and McBain 2003; Orji, Nworie et al. 2012). Bacteria develop resistance to antibiotics due to three main strategies, which are: drug inactivation by enzymes (Davies 1994), modification of target sites (Spratt 1994) and extrusion of antibiotics and toxic substances by efflux (Nakaido 1994).

Staphylococcus aureus (*S. aureus*) is part of the normal flora found on the skin and mucous membranes of the mouth, nose and ears of 20-30% of the human population (Peacock, de Silva et al. 2001). Globally, this pathogen is considered to be one of the most prevalent and important clinical isolates in humans (Waters, Contente-Cuomo et al. 2011; Chuang, Hsiao et al. 2012). Currently, *S. aureus* is the main pathogen associated with community-acquired infections and the use of traditional hospital control measures alone is inadequate (Deresinski 2005; Stefani, Chung et al. 2012).

Methicillin-resistant *Staphylococcus aureus* (MRSA) was initially identified in the 1960s, commonly associated with hospitals and healthcare facilities. In Africa, one of the first reported cases of MRSA was isolated from clinical samples collected from hospitalized African and Indian children in Durban (Scragg, Appelbaum et al. 1978). Subsequently, between the mid-1980s and early 1990s, there was a sharp increase in the reports of MRSA cases in numerous African countries, including Ethiopia (Geyid and Lemeneh 1991), Kenya (Omari, Malonza et al. 1997), Nigeria (Rotimi, Orebamjo et al. 1987; Okesola, Oni et al. 1999), Senegal (Sow, Wade et al. 1998), South Africa (van den Ende and Rotter 1986; Peddie, Donald et al. 1988; Gardee and Kirby 1993) and Sudan (Musa, Shears et al. 1999). Also, an increase in MRSA prevalence has been reported in other parts of the world,

including several European (Rasmussen, Fowler et al. 2011) and Asian countries such as Taiwan (Hsueh, Chen et al. 2002), Japan (Lotus, Imamura et al. 1995) and the Republic of Korea (Woojoo and Seunchill 1999). MRSA causes a wide range of deep tissue infections such as osteomyelitis, arthritis, endocarditis and renal and breast abscesses (Daniyan, Galadima et al. 2011). It also causes wound, skin and soft tissue infections particularly carbuncles, furuncles and scalded skin syndrome (Richards, Edwards et al. 1999a; Richards, Edwards et al. 1999b; Perez-Vazquez, Vindel et al. 2009).

Methicillin-susceptible/sensitive *S. aureus* (MSSA) usually develop into MRSA by acquiring a large DNA fragment, known as staphylococcal chromosomes cassette *mec* (SCCmec) type IV, and inserting it into their genomes (Hiramatsu, Cui et al. 2001; Rasmussen, Fowler et al. 2011). The SCCmec contains the methicillin resistant determinant gene, *mecA*, which encodes for a modified penicillin-binding protein (PBP2a) with low affinity for β-lactams (Hiramatsu, Cui et al. 2001; Pinho, Filipe et al. 2001; Monecke, Coombs et al. 2011; Sanchini, Campanile et al. 2011); PBP2a is essential, as it plays a role in cell wall synthesis of the bacteria (Shopsin and Kreiswirth 2001). More recently, however, MRSA strains with a modified *mecA* gene (designated $mecA_{LGA251}$) have been isolated in the UK and Denmark among the human and bovine populations. This gene was shown to be only 70% homologous to the *S. aureus mecA* (Garcia-Alvarez, Holden et al. 2011).

Bacteria that have a multidrug-resistant (MDR) phenotype, which are controlled by MDR efflux pumps (EPs), create the most concern in the healthcare sector (Costa, Ntokou et al. 2010). MDR EPs are cytoplasmic membrane transport proteins found in both Gram-positive and Gram-negative bacteria. These pumps are able to expel antimicrobial agents of different classes, including biocides (Morar and Wright 2010); this ability to extrude these various compounds contributes to increased levels of bacterial resistance (Van Bambeke, Balzi et al. 2000; Borges-Walmsley, McKeegan et al. 2003; Poole 2005). MDR EPs were

first discovered in human cancer cells resistant to fluoroquinolones (Rice 2006). Currently, efflux pumps are categorized into five diverse families which are: major facilitator super (MFS) family, ATP-binding cassette (ABC) transporters, small multidrug resistance (SMR) transporters, multidrug and toxic compound extrusion (MATE) family and resistance-nodulation-cell division (RND) family (Kaur 2002; Lynch 2006; Morar and Wright 2010; Cui and Davidson 2011; He, Thorpe et al. 2011; He, Zhang et al. 2011). To date, a number of MDR EPs have been identified in *S. aureus* which are encoded by either chromosomal-borne genes (*norA, norB, norC, mepA, sepA* and *sdrM*), or plasmid-borne genes (*qacA/B, smr, qacG, qacJ and qacH*) (Poole 2005; Poole 2007). Typically, whereas chromosomally-encoded MDR EPs result in reduced susceptibility to both antibiotics and biocides, the plasmid-encoded pumps reduce the susceptibility to biocides only (Poole 2007).

The *gyrA* and *gyrB* genes encode subunits of DNA gyrase, which together with the enzyme topoisomerase IV, are crucial for bacterial replication (Bates, O'Dea et al. 1996). Topoisomerase IV, encoded by *grlA* and *grlB*, is believed to be the principle target site for fluoroquinolone action (Sreedharan, Oram et al. 1990; Hooper and Wolfson 1991; Goswitz, Willard et al. 1992). However, mutations in both the *grl* and *gyr* gene loci are usually responsible for the quinolone resistance exhibited in *S. aureus* isolates (Ferrero, Cameron et al. 1995). These mutations can consequently result in the continued function of these vital bacterial enzymes, even in the presence of normally toxic concentrations of fluoroquinolone (Kaatz and Seo 1995).

The aim of this study was to compare the presence of three EP genes (*mepA*, *sepA* and *gyrA*, including the house-keeping gene *16S*) in methicillin-sensitive ATCC 25923 (MSSA) and methicillin-resistant ATCC 33591 (MRSA) strains using Polymerase Chain Reaction (PCR). Optimization of PCR was done by running primer concentration, magnesium chloride

($MgCl_2$) concentration and annealing temperature profiles. Whereas *mepA* and *gyrA* were identified in both *S. aureus* strains, *sepA* was detected in the MRSA ATCC 33591 strain only.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial strains and growth conditions

The bacterial isolates used for this analysis were MSSA (ATCC 25923) and MRSA (ATCC 3359)1 strains obtained from the American Type Culture Collection (ATCC). The bacterial isolates were cultured on tryptone soy agar (TSA) (Oxoid Ltd, UK) plates and incubated at 37ºC, for 16-18 hours. A single colony forming unit (CFU) was inoculated into tryptone soy broth (TSB) (Oxoid Ltd, UK) and incubated for 5-8 hours, with shaking, at 37°C to a turbidity equivalent to 0.5 McFarland standard (~1×10^8 to 2×10^8 CFU/ml).

3.3.2. Quantification of Total RNA and cDNA concentration

The quantification of total RNA and cDNA was done using the the Quant-iT™ assay system. The working solution was made by diluting the Quant-iT™ reagent, using a 1:200 ratio, in Quant-iT™ buffer. 200 µl of working solution was required for each sample and standard. 199 X n µl of Quant-iT™ buffer was mixed with 1 x n µl of Quant-iT™ reagent (where n represents the number of standards plus number of samples) to a make up the Quant-iT™ working solution. 10 µl of each standard was added to 190 µl of the working solution. Then 180-199 µl of working solution was mixed with 1-20 µl of each sample to make up a final volume of 200 µl (Table 3.1).

Table 3.1. Volumes used in the Qubit® Fluorometer system.

	Standard (Assay tubes)	**Sample** (Assay tubes)
Working solution volume	190 µl	180-199 µl
Volume of **standard**	10 µl	–
Volume of **sample**	–	10-20 µl
Total volume in assay tube	200 µl	200 µl

The tubes were vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. After calibrating the Qubit® fluorometer with known standards, fluorometer sample readings were taken and the concentrations (µg/ml) calculated using the formula:

Concentration of sample = QF value x 200/X

Where:

QF = Qubit® fluorometer reading,

X= number of µl of sample added.

3.3.3. Total RNA extraction

Total RNA was extracted from bacterial isolates MSSA ATCC 25923 and MRSA ATCC 33591 strains using the SV Total RNA Isolation System (Promega) as per the manufacturer's instructions. Bacterial cultures were grown in tryptone soy broth (TSB) overnight with shaking at 37ºC.

The following day, a 1:50 dilution of the culture was made and grown to an OD_{600} of 0.6 (read using a spectrophotometer). 1 ml of culture was then transferred to a 1.5 ml

microcentrifuge tube and centrifuged at room temperature (r.t) for 2 minutes at 14 000 × g (this process was repeated until all the culture was centrifuged). The supernatant was then carefully removed and the pellet left as dry as possible. The pellet was resuspended in 100 µl freshly prepared TE (Trypsin EDTA) [0.05% trypsin (w/v) and 0.53 mM EDTA dissolved in 1X PBS] containing 3mg/ml lysozyme and tapped gently to mix. The resuspended pellet was incubated at room temperature for 3-5 minutes. 175 µl RNA Lysis Buffer [4M GTC (guanidine isothiocyanate), 0.01M Tris (pH 7.5), 0.97% (β-Mercaptoethanol)] was then added and the tube inverted to mix thoroughly. 350 µl RNA Dilution Buffer was added and mixed by inversion. The cleared lysate solution was then transferred to a fresh microcentrifuge tube by pipetting ensuring not to disturb the pelleted debris. 200 µl 95% ethanol was added to the cleared lysate and mixed by pipetting 3-4 times. The mixture was then transferred to the Spin Column Assembly and centrifuged at 14 000 × g for 1 minute at r.t. The liquid in the Collection Tube was discarded and the Spin Basket put back into the Collection Tube.

600 µl RNA Wash Solution [60 mM potassium acetate, 10 mM Tris-HCl (pH 7.5 at 25°C) and 60% ethanol] was added to the Spin Column Assembly and centrifuged at r.t for 1 minute at 14 000 × g. The Collection Tube was emptied as before and placed on a rack. The DNase incubation mix was made by combining 40 µl Yellow Core Buffer [0.0225 M Tris (pH 7.5), 1.125 M NaCl and 0.0025% yellow dye], 5 µl 0.09 M $MnCl_2$ and 5 µl of DNase I enzyme (in this order) in a sterile tube and mixed gently by pipetting. 50 µl of this DNase incubation mix was then applied directly to the membrane inside the Spin Basket ensuring that the solution thoroughly covers the membrane. This was then incubated for 15 minutes at 20-25ºC.

After the incubation period, 200 µl DNase Stop Solution [2 M GTC, 4mM Tris HCl (pH 7.5) and 57% ethanol] was added to the Spin Basket and centrifuged at r.t for 1 minute at

14 000 × g. 600 µl RNA Wash Solution was then added and centrifuged at 14 000 × g for 1 minute at r.t. The Collection Tube was emptied and 250 µl RNA Wash Solution was then added and centrifuged at high speed for 2 minutes. The Spin Basket from the Collection tube was transferred to a 1.5 ml Elution Tube and 100 µl Nuclease-Free Water added to the membrane ensuring that it is completely covered. The Spin Basket Assembly was then placed in the centrifuge and centrifuged at 14 000 × g for 1 minute at r.t. The Spin Basket was then discarded and the Elution Tube containing purified RNA stored at -70ºC.

3.3.4. Reverse Transcription of Total RNA (First strand DNA synthesis)

After total RNA was extracted, first strand cDNA was synthesized using the GoScript™ Reverse Transcription System (Promega). Each component was mixed and briefly centrifuged before use. 4 µl experimental RNA (40 µg/ml) was combined with 1 µl Primer Oligo $(dT)_{15}$ (500 µg/ml, 0.5 µg/reaction). This was then heated to 70ºC in a heating block for 5 minutes and immediately chilled on ice for 5 minutes. The mixture was centrifuged for 10 seconds in a microcentrifuge and stored on ice until reverse transcription mix was added.

The reverse transcription reaction mix was prepared as per manufacturer's instructions (15µl for each cDNA reaction) and components briefly combined on ice in the following order: 4.0 µl GoScript 5X Buffer, 1.5 µl $MgCl_2$ (25 mM), 1.0 µl PCR Nucleotide Mix (10 mM, final concentration 0.5 mM each dNTP), 0.5 µl Recombinant RNasin® Ribonuclease Inhibitor (40 U/µl), 1.0 µl GoScript™ Reverse Transcriptase and 7 µl Nuclease-Free Water. 15 µl of reverse transcription mix and 5µl of RNA and primer mix were combined. Reverse transcription PCR was conducted in a thermal cycler and amplification conditions were as follows: annealing at 25ºC for 5 minutes, extension at 42ºC

for 1 hour, followed by an incubation extension at 72ºC for 15 minutes to inactivate the reverse transcriptase enzyme.

3.3.5. Primer Selection

The primers were selected according to those used by Couto et al (2008) and purchased from Inqaba Biotechnical Industries (Pty) Ltd. Primers chosen had the following parameters: length ranged from 18-25 bases, and the T_m (melting temperature) ranged from 58.35 - 64.52 for the three genes of interest. The primer sets used are described in Table 1.

Table 3.2. Primers used for the amplification of *Staphylococcus aureus* efflux pump genes.

Gene	Primer	Sequence	Size (bp)	Reference
mepA	MepA forward	ATGTTGCTGCTGCTCTGTTC	718	(Couto, Costa et al. 2008)
	MepA reverse	TCAACTGGTCAAACGATCACG		
sepA	SepA forward	GCAGTCGAGCATTTAATGGA	103	(Couto, Costa et al. 2008)
	SepA reverse	ACGTTGTTGCAACTGTGTTAAGA		
gyrA	GyrA forward	TCGTGCATTGCCAGATGTTCG	394	(Couto, Costa et al. 2008)
	GyrA reverse	TCGAGCAGGTAAGACTGACGG		
16S	16S_27 forward	AGAGTTTGATCMTGGCTCAG	492	(Lane 1991)
	16S_519 reverse	GWATTACCGCGGCKGCTG		

3.3.6. Optimization of PCR conditions

Optimization of efflux pump gene amplification was done by changing various parameters in the standard PCR protocol. The conditions changed were primer concentration, $MgCl_2$ concentration and annealing temperature.

3.3.6.1. Optimization of primer concentration

Each primer was used at a concentration that ranged from 10-100 pmol/µl. In this study, it was found that the optimal primer concentration was 10 pmol/µl for all the primer sets used and at this concentration, it was high enough for amplification to take place and low enough to prevent any non-specific binding due to the presence of large amounts of unused primer.

3.3.6.2. Optimization of $MgCl_2$ concentration

In order to determine the optimal $MgCl_2$ concentration for each primer set, different concentrations of $MgCl_2$ were used and incorporated in the standard PCR reaction procedure. The $MgCl_2$ concentrations ranged from 0 to 3mM. The change in $MgCl_2$ volumes was rectified by adding the corresponding amount of nuclease-free water to ensure that the final volume remained the same. After performing the PCR, the products were run on a 1.5% agarose gel for *mepA* and 2% agarose gel for *sepA* in order to select the concentration that yielded the best results.

3.3.6.3. Optimization of annealing temperature

Optimal annealing temperature was determined using temperatures that ranged from 53 to 56°C for the three genes. The annealing temperature used for *mepA* and *sepA* were 53, 54, 55 and 56°C, while annealing temperatures of 54 and 56°C were used for *gyrA*. The annealing temperatures were selected by using temperatures that were 5°C lower than the melting temperatures (T_m) of the primer sets of the different genes. The subsequent annealing temperatures were obtained increasing the temperature in increments of 1°C. Since the lowest T_m for both *sepA* and *mepA* primer sets was 58.35, an initial annealing temperature of 53ºC was used with subsequent 1ºC increments to determine the other annealing temperatures. The initial annealing temperature used for *gyrA* was 54ºC. The optimal annealing temperature for 16S was not determined as it was the house-keeping gene that was used a positive control and produced good results at all temperatures.

3.3.7. PCR conditions used

The three efflux pump genes including the house-keeping gene (used as a positive control) were amplified by PCR with the primers listed in Table 3.2. The reaction mixture, which was made up to a final volume of 25 µl, consisted of: 1 µl of cDNA (1.543 µg/ml), 2.5U (0.5 µl) of *Taq* Polymerase, 5 µl 5X *GoTaq* Flexi buffer, 1 µl (10 pmol) of each primer, 0.5 µl dNTP (10mM) mix and 1.5 mM of $MgCl_2$ (25 mM). The reactions were carried out in a thermocycler with the following amplification conditions: DNA was denatured at 95ºC for 3 minutes, followed by 30 cycles of denaturation at 95ºC for 45 seconds, annealing at 54ºC (for *sepA*) and 56°C (for *mepA* and *gyrA*) for 45 seconds, and extension at 72ºC for 45 seconds, followed by a final extension at 72ºC for 15 minutes. The PCR products were diluted, at a ratio of 1:6, with 6 x Blue/Orange loading dye containing [(0.4% (v/v) orange G,

0.03% (v/v) bromophenol blue, 0.03% (v/v) xylene cyanol FF, 15% (v/v) Ficoll® 400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0)] and run on a 1% (w/v) agarose gel, containing 1 µl Ethidium Bromide, in 1X TBE buffer containing (89 mM tris (hydroxymethyl) aminomethane, 0.089 mM boric acid, 2 mM EDTA (pH 8). Electrophoresis was done at 80 volts and 2 amps for 50 minutes. The amplification products were visualized with a transilluminator under ultraviolet (UV) light and the sizes of the *S. aureus* genes of interest confirmed by comparing their sizes to those of the DNA molecular weight marker.

Table 3.3. PCR conditions used in this study.

Reagent	Negative control (µl)	16S (positive control) (µl)	Genes of interest (µl)
cDNA	0	1	1
dNTP	0.5	0.5	0.5
$MgCl_2$	1.5	1.5	1.5
5X flexi buffer	5	5	5
Forward primer	1	1	1
Reverse primer	1	1	1
Go Taq	0.5	0.5	0.5
Nuclease free Water	15.5	14.5	14.5
Final volume	**25**	**25**	**25**

3.4. RESULTS

3.4.1. Optimization of primer concentration

At primer concentrations of 50 pmol/µl and 100 pmol/µl, high incidence of primer dimers suggested that the primer concentration was too high, resulting in non-specific binding. At a primer concentration of 10 pmol/µl, the gel was had less background/smears. This showed that the incidence of non-specific binding was reduced, thus resulting in sharper bands obtained on the agarose gel (Figures 3.1 to 3.3). Primer dimer incidence was also reduced making this the ideal primer concentration for use in this study.

3.4.2. Optimization of $MgCl_2$ concentration

Magnesium (Mg^{2+}) is an essential cofactor for the Taq polymerase enzyme and it stabilizes dsDNA (double-stranded DNA). Mg^{2+} concentration is important as it controls specificity of the reaction and low Mg^{2+} ion concentrations result in low yields of PCR product or cause failure of the reaction, whereas very high concentrations increase chances of non-specific products and also promote misincorporation. Since the amount of magnesium present in a reaction is very important, magnesium optimization was essential. After $MgCl_2$ profiling (0 to 3 mM) was done for the three genes, the optimal $MgCl_2$ concentration was chosen as being 1.5 mM for *sepA* (Appendix II, Figure 6) and *gyrA* for both the MSSA ATCC 25923 and MRSA ATCC 33591 strains. $MgCl_2$ profiling for *gyrA* was required done because this gene was easily detected using the initial PCR conditions used. However, the optimal $MgCl_2$ concentration for *mepA* differed for the two strains. The optimal concentration for the MSSA ATCC 25923 strain was 1 mM, whereas the MRSA ATCC 33591 strain had an optimal $MgCl_2$ concentration of 1.5 mM (Appendix II, figure 5). At these concentrations, the

bands produced were sharper and the amount smears due non-specific products was reduced compared to the other $MgCl_2$ concentrations.

3.4.3 Optimization of annealing temperature

Annealing temperature plays an important role in the gene amplification process as it ensures that the primers bind to the gene/product of interest. This helps in the prevention of non-specific binding. As described in section 3.2.5.5, the initial annealing temperatures used for optimization were 53°C for *sepA* and *mepA* and 54°C for *gyrA.* It was observed that the product amplification results obtained for *gyrA* were the same even at the annealing temperatures used for *mepA* and *sepA* (Appendix II, Figure 2). Thus annealing temperature optimization was mainly done for *mepA* and *sepA.* Appendix I, Figures 1 to 4, show the results obtained for *mepA* and *sepA* at different annealing temperatures. Annealing temperatures of 54°C (*sepA*) and 56°C (*mepA* and *gyrA*) were chosen as the amplification products obtained had less smears and the bands were sharper on the agarose gel.

Figures 3.1, 3.2 and 3.3 show PCR amplification results obtained after optimization of PCR conditions was done. The conditions used were: primer concentration of 10 pmol/µl, $MgCl_2$ concentration of 1mM, for *mepA* MSSA ATCC 25923, 1.5mM for *sepA, gyrA* and *mepA* for the MRSA ATCC 33591 strain and annealing temperatures of 54°C (*sepA*) and 56°C (*mepA* and *gyrA*).

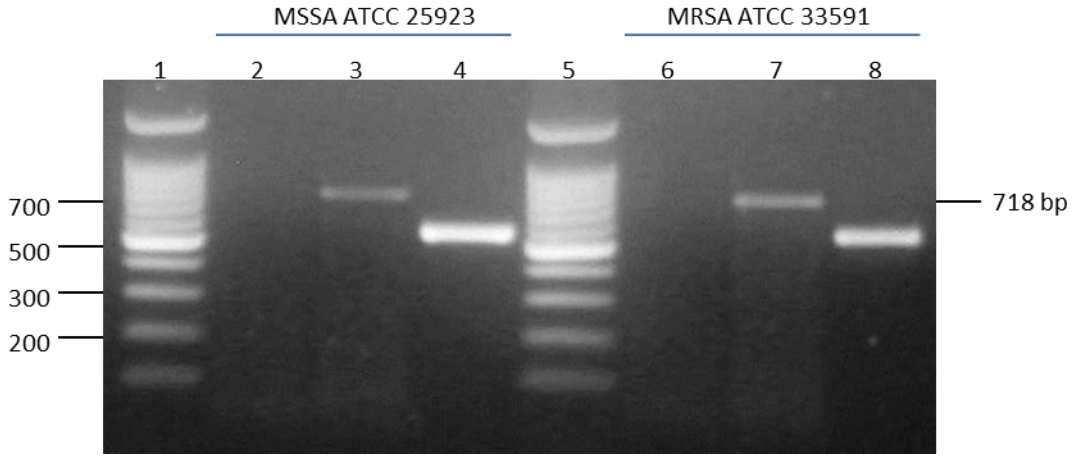

Figure 3.1: 1.5% agarose gel electrophoresis analysis of *mepA* amplified by PCR using gene specific primers. Lane 1 and 5: 100bp DNA molecular weight marker; Lanes 2 and 6: no template PCR control (negative control); Lanes 3 and 7: *mepA* gene amplification; Lanes 4 and 8: *16S* house-keeping gene amplification (internal positive control) for both the MSSA ATCC 25923 and MRSA ATCC 33591.

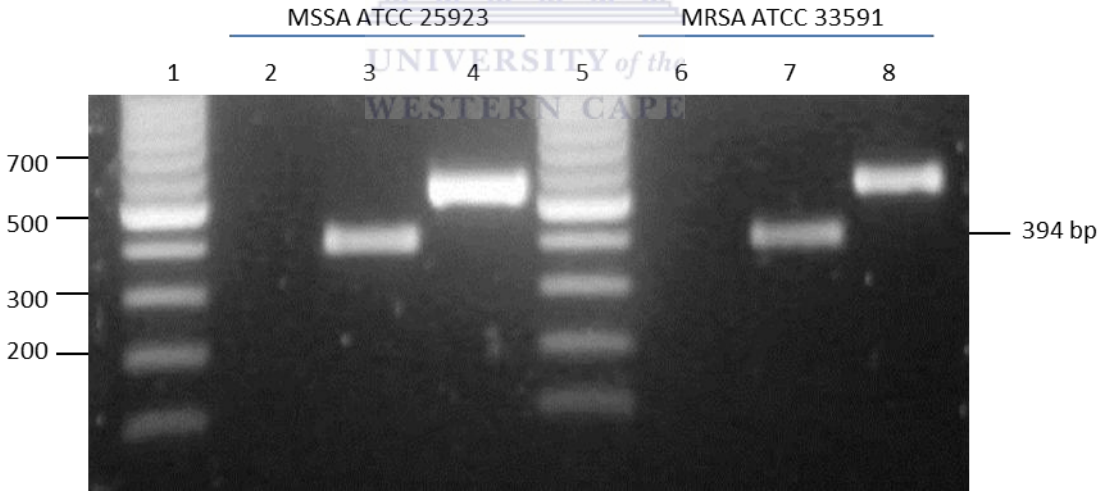

Figure 3.2: 1.5% agarose gel electrophoresis analysis of *gyrA* amplified by PCR using gene specific primers. Lane 1 and 5: 100bp DNA molecular weight marker; Lanes 2 and 6: no template PCR control (negative control); Lanes 3 and 7: *gyrA* gene amplification; Lanes 4 and 8: *16S* house-keeping gene amplification (internal positive control) for both the MSSA ATCC 25923 and MRSA (ATCC 33591).

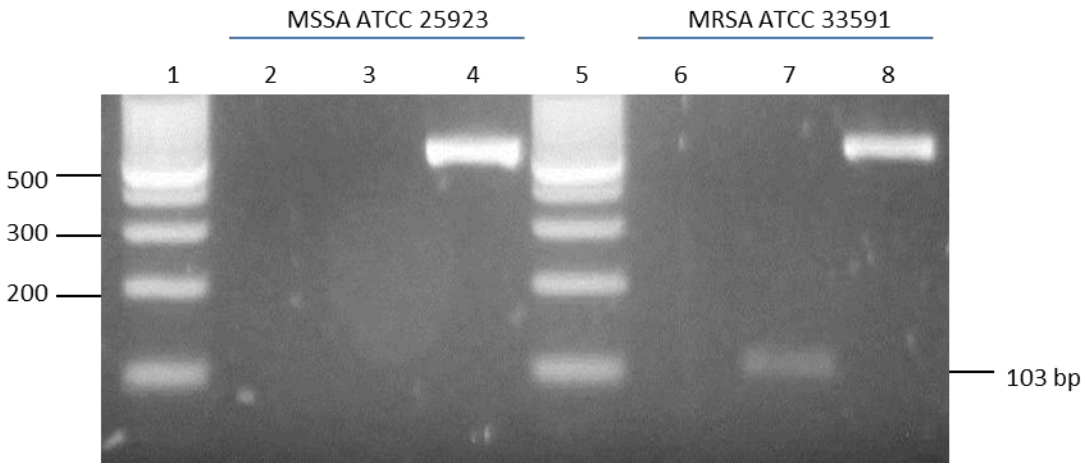

Figure 3.3: 2% agarose gel electrophoresis analysis of sepA amplified by PCR using gene specific primers. Lane 1 and 5: 100bp DNA molecular weight marker; Lanes 2 and 6: no template PCR control (negative control); Lanes 3 and 7: sepA gene amplification; Lanes 4 and 8: 16S house-keeping gene amplification (internal positive control) for both the MSSA (ATCC 25923) and MRSA (ATCC 33591).

3.4.4. Gel electrophoresis and visualization of amplification products

As described earlier, the percentage of agarose gel is essential in ensuring that the PCR products, as well as the DNA molecular marker, separate properly and produce bands that are clear and easy to compare. The agarose gel percentages that were eventually chosen were 1.5% agarose gel, for *mepA* and *gyrA* and 2% for *sepA*. At these percentages, the bands produced were clear and easy to compare and the DNA molecular marker had also separated in a way that made it easy to interpret. A 100bp DNA molecular weight marker was run on every gel to verify the expected molecular weight of the amplification products. PCR products were loaded onto Ethidium Bromide-stained 1.5% and 2% agarose gels amplified at annealing temperatures of 54°C and 56°C for both MSSA ATCC 25923 and MRSA ATCC 33591 strains. The gels were then visualized under UV light in a transilluminator.

Table 3.4. Presence of *mepA*, *gyrA* and *sepA* in MSSA and MRSA strains on *S. aureus*

	MSSA ATCC 25923	**MRSA ATCC33591**
mepA	+	+
gyrA	+	+
sepA	-	+

+ detected by PCR

- not detected by PCR

3.5. DISCUSSION

In this study, PCR was used to identify and compare the presence of efflux pump genes in MSSA ATCC 25923 and MRSA ATCC 33591. Optimization of the PCR was essential in ensuring that only products of interest were amplified for easy and effective comparison. This was done by changing a various parameters in the reactions such as primer concentration, magnesium chloride concentration and annealing temperature. The determined optimal primer concentration was 10pmol/µl, as no non-specific amplicons and no primer dimers were observed on the agarose gels. After performing a magnesium chloride profile for each of the PCR conditions using the optimal 10pmol/µl primer concentration, it was observed that the *mepA* gene in the MSSA ATCC 25923 strain, was detected at a $MgCl_2$ concentration of 1mM; the other genes were detected at $MgCl_2$ concentrations of 1.5mM for both MSSA ATCC 25923 and MRSA ATCC 33591 strains. A 2% agarose gel was used for visualization of the *sepA* gene and a 1.5% agarose gel was used for *mepA* and *gyrA* genes. The difference in the agarose gel percentage ensured proper separation and visualization of PCR amplicons of different sizes.

The absence of the *sepA* gene in the MSSA ATCC 25923 strain under optimal PCR conditions, suggests that this efflux pump gene could play a role in conferring resistance tothis strain. However, a study carried out by Couto et al. (2008) demonstrated the presence of *sepA* in the ATCC 25923 strain. The discrepancies in results could be attributed to the differences in growth conditions and media used as well as the kit employed for total RNA extraction (Rneasy Mini Kit QIAGEN) which could have had an effect on the results obtained (Couto, Costa et al. 2008). Various studies have been carried out that focus on the activity of efflux pumps and their ability to confer resistance to bacteria such as *S. aureus*. A previous study carried out by Narui et al (2002) demonstrated that the *sepA* gene plays a role in the resistance of *S. aureus* to antiseptics. The study also showed that potential mutations that may arise lead to the over-expression of this gene, thereby causing an increase in the level of resistance (Narui, Noguchi et al. 2002). Other studies have reported that *mepA* confers resistance to numerous compounds and antibiotics such as biocides, a variety of dyes as well as fluoroquinolones (Kaatz, McAleese et al. 2005; McAleese, Petersen et al. 2005). It has been shown that *mepA* expression is regulated by MepR (a transmembrane regulator) which is capable of sensing toxic compounds thereby facilitating the expression of *mepA* leading to drug resistance (Kumaraswami, Schuman et al. 2009).

In addition, numerous earlier studies have shown that NorA is responsible for quinolone resistance, which has been shown to arise even in the absence of mutations in topoisomerases (Kaatz, Seo et al. 1993; Kaatz and Seo 1995). *S. aureus* was shown to acquire resistance to glycopeptides, such as vancomycin, by attaining the *vanA* gene from *Enterococcus faecalis* (Weigel, Clewell et al. 2003). The *qacA* gene, found in *S. aureus*, has been shown to confer multidrug resistance to various dyes such as ethidium bromide, antiseptics, disinfectants, benzalkonium chloride and chlohexidine (Littlejohn, Paulsen et al.

1992; Leelaporn, Paulsen et al. 1994). Taken together, data clearly show that antibiotic resistance in *S. aureus* is a complex process, involving different genes and mechanisms.

Multidrug transporters, which are encoded by a number of genes, have also been identified in a number of other bacteria such as *Bacillus subtilis*, where four different transporter genes have been identified, namely *Bmr*, *Blt* (Neyfakh, Bidnenko et al. 1991; Ahmed, Lyass et al. 1995), *Bmr3* (Ohki and Murata 1997) and *EbrAB* (Jack, Storms et al. 2000; Masaoka, Ueno et al. 2000); the proteins encoded by these genes able to extrude drugs from bacterial cells. *Streptococcus pneumonia* has also been shown to possess chromosomally-encoded efflux pump genes *mefA* or *mefE*. Here, these MF family EPs play a role in macrolide resistance by reducing the internal concentration inside the bacterial cell (Sutcliffe, Tait-Kamradt et al. 1996; Tait-Kamradt, Clancy et al. 1997).

Bacteria are able to acclimatize to different unfavourable environments, such as the presence of antibiotics, as a result of their ability to regulate their gene expression processes by either horizontal transfer of genetic material or through numerous modifications to their genetic sequences (Voss, Loeffen et al. 2005). EP proteins contribute to multidrug resistance through their ability to reduce the concentration of the antibiotics by means of antibiotic efflux (Li and Nikaido 2009; Villagra, Fuentes et al. 2012). Antibiotic resistance, as a result of EP proteins, can be reduced by growing bacteria in cultures containing nonphosphotransferase system (PTS) sugars. The latter results in the over-production of other genes which, in turn, increases the level of competition with the antibiotic efflux system, thereby reducing antibiotic resistance (Villagra, Fuentes et al. 2012).

The virulence factors employed by a number of bacteria are not fully understood at the molecular level (Kumar and Varela 2012). The studies of the different mechanisms, by which bacteria are able to cause multidrug resistance, facilitate the development of more

effective antibiotics for the treatment of infections (Lewis 1994; Lewis 2001)**.** It is believed that the unsupervised and uncontrolled intake of antibiotics is the reason for the development of drug resistance, but drug resistance has also been reported in areas where no antibiotics have been used (Wright 2007). For example, a recent study carried out by Zhang and colleagues (2011) demonstrated the development of antibiotic resistant bacteria during the early stage of infant development (Zhang, Kinkelaar et al. 2011).

Multi-drug efflux pumps found in bacteria play a major role in conferring multi-drug resistance (Tennent, Lyon et al. 1989). Studying and understanding the mechanisms by which pathogenic bacteria become resistant is important due to the serious health and social implications they present (Kumar and Schweizer 2005; Gootz 2010). It is for this reason that understanding the ways to inhibit the action of these multi-drug efflux pumps, are vital (Lewis 2001; Lomovskaya and Bostian 2006).

Over-expression of efflux pumps can be induced by the constant presence of antibiotics or substrates (Teran, Felipe et al. 2003). Efflux pump proteins also become resistant to antibiotics through amino acid substitution in their protein structure. This enables them to be more efficient at extruding substances and, just like efflux pump over-expression, results in reduced intracellular antibiotic concentrations. As a result, this renders the bacteria less susceptible to that compound (Levy 2002; Li and Nikaido 2004; Poole 2005; Piddock 2006; Stavri, Piddock et al. 2007). A wide variety of compounds and substrates have previously been tested in order to assess their ability to hamper the activity of *S. aureus* EPs. This is done in the hope of finding specific inhibitors of these pumps (Kaatz, Seo et al. 1993; Neyfakh, Borsch et al. 1993; Kaatz, Moudgal et al. 2003; Kristiansen, Leandro et al. 2003; Kristiansen, Leandro et al. 2006). In this study, the presence of three efflux pump genes (the *mepA, gyrA and sepA*) in *S. aureus* was studied. Results showed that both *mepA* and *gyrA* were present in both methicillin-sensitive, as well as methicillin-resistant, *S. aureus* strains.

EP gene *sepA* was only present in the methicillin-resistant *S. aureus* strain, suggesting that it plays a role in conferring resistance to *S. aureus*.

Future prospects include performing quantitative Real-Time PCR (RT-PCR) to quantify the efflux pump genes as well as investigate the effect of the selected compounds on them.

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CHAPTER FOUR

Summary

Staphylococcus aureus is a Gram-positive coccus first discovered in 1880 in Aberdeen, Scotland, by a surgeon by the name of Alexander Ogston (Ogston 1984). It is found on the skin, nose and mouth of approximately 20-30% of humans (Jack 2000). It causes a wide range of hospital- and community-acquired infections such as deep tissue and skin infections (Jack, Storms et al. 2000; Daniyan, Galadima et al. 2011). *S. aureus* causes these diseases because it is able to produce a number of virulence factors, including toxins and haemolysing enzymes (Tait-Kamradt, Clancy et al. 1997; Rajaraman, Jack et al. 2000). Methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in 1960, not long after the introduction of methicillin (Lewis 2001; Chacon, Estruga et al. 2012). Not only is *S. aureus* resistant to methicillin, but it has also developed resistance to a number of other antibiotics, thereby limiting its treatment options (Taylor, Blakely et al. 2008). *S. aureus* is able to build resistance to a wide range of antibiotics through the acquisition of multidrug-resistant (MDR) phenotypes, which is associated with increased rates of mortality and morbidity (D'Costa, Griffiths et al. 2007; Wright 2007; Wright 2007).

The ability of bacteria, including *S. aureus*, to develop resistance to numerous drugs has led to some urgency and need to explore other treatment options (Cheng, Gosewehr et al. 1996; Lomovskaya and Bostian 2006). Plant compounds have been shown to possess distinctive antimicrobial activities and are able to enhance the antibacterial activity of various drugs (Cushnie and Lamb 2006). Flavonoids are plant products that have a wide distribution in plants and are being studied for their wide range of medicinal properties, which include possible anti-inflammatory (Serizawa, Osawa et al. 1992), antimicrobial (Tsuchiya, Sato et al. 1996), and antioxidant (Chung and Ng 2012) properties. Various studies have been conducted that outline the antimicrobial activities of flavonoids, including their additive or synergistic effects (Barua, Nazeran et al. 2005; Nazeran, Chatlapalli et al. 2005; Stefanovic, Stanojevic et al. 2012).

S. aureus contains a number of multidrug-resistant efflux pumps that play a role in conferring multidrug resistance against numerous antibiotics (Tennent, Lyon et al. 1989; Narui, Noguchi et al. 2002). These efflux pumps are encoded by various chromosomal and plasmid genes such as *mepA, norA, sepA and qacA/B* (Kumar and Varela 2012). Efflux pumps enable the bacteria to become resistant to antimicrobial agents by reducing their intracellular concentration. It has also been established that the slow rate at which efflux takes place allows these bacteria to alter the antibiotic target sites (Lewis 2001). It is for this reason that developing a suite of new antimicrobial agents to target these efflux mechanisms may prove to be beneficial if effective treatments are to be found. (Griffith, Corcoran et al. 2006).

The first aim of this study was to determine the minimum inhibitory concentrations (MICs) of four antibiotics (tetracycline, ampicillin, methicillin and vancomycin) including three flavonoids (chrysin, naringenin and 7-hydroxyflavone), alone and in combination against methicillin-sensitive ATCC 25923 (MSSA) and methicillin-resistant ATCC 33591 (MRSA) *S. aureus* strains, using Kirby-Bauer disk diffusion and microtitre microdilution assays. Synergistic ratios were calculated for the microtitre assay combination results to assess wheter any synergistic or additive effects could be identified due to the presence of the flavonoids. The second aim was to compare the presence of three efflux pump genes (*mepA*, *sepA* and *gyrA*, including the house-keeping gene 16S) in the MSSA ATCC 25923 and MRSA ATCC 33591 strains using polymerase chain reaction (PCR).

For the Kirby-Bauer disk diffusion assay, various concentrations of antibiotics tetracycline, ampicillin, methicillin and vancomycin produced inhibitory effects on the MSSA ATCC 25923 strain that ranged from susceptible to resistant when compared to the MIC interpretive standards for *Staphylococcus* species as outlined by the Clinical and Laboratory Standards Institute (CLSI 2007). The MRSA ATCC 33591 strain was only

susceptible to vancomycin. Flavonoids did not show any inhibitory effect on bacterial growth in the disk diffusion assay and did not enhance or reduce inhibitory activity of the antibiotics in the combination studies. This lack of antibacterial activity exhibited by the flavonoids could be attributed to the inability of the flavonoids to effectively diffuse out of the disks (Zheng, Tan et al. 1996). However, in the microtitre assay, naringenin did enhance the antibacterial activity of tetracycline and ampicillin, as could be observed by the reduction in the bacterial growth rates. This was evident as calculated synergistic ratios by the Abbot formula showed that naringenin had an additive effect. However, concentrations of tetracycline and ampicillin used in the combination studies were those that showed no inhibitory effect on bacterial growth.. Chrysin and 7-hydroxyflavone were not used in the microdilution assay as they formed precipitates when diluted in the solvent, which would have led to unreliable results.

Since naringenin did show an additive effect when combined with tetracycline and ampicillin in the microtitre assay, future work, therefore, is to explore the effect of these flavonoid and antibiotic combinations on the expression and overall activity of these efflux pumps. This is important as these pumps have previously been implicated in multidrug resistance. It has been shown that, in addition to the direct and synergistic antibacterial activities of flavonoids, they are also capable of hindering numerous bacterial virulence factors such as enzymes, toxins and signal receptors, and can also inhibit the working of efflux pumps (Brusco and Nazeran 2005; Cushnie and Lamb 2011). Efflux pump proteins coded for by efflux pump genes are responsible for the extrusion of antibiotics thus allowing the pathogens to avoid antimicrobial effects (Lomovskaya and Bostian 2006). Recent studies have also shown that some flavonoids efficiently inhibit the efflux pumps involved in antibiotic resistance in *S. aureus* (Smith, Kaatz et al. 2007)

The presence of efflux pump genes in MSSA ATCC 25923 and MRSA ATCC 33591 was compared using PCR. However, a lot of optimisation needed to be done in order to amplify these specific genes. Primer concentration and magnesium chloride concentration, as well as annealing temperature, all had to be optimised for the efflux pump genes to be properly amplified and visualised on the agarose gel. Efflux pump genes *mepA* and *gyrA* were seen in both strains, whereas *sepA* was only observed in the MRSA ATCC 33591 strain. The presence of efflux pump genes in both MSSA ATCC 25923 and MRSA ATCC 33591 confirms that these genes are present in both sensitive and resistant strains, and that their over-expression may lead to the bacteria's ability to develop drug resistance. It is therefore recommended that future studies involve challenging the bacteria with various concentrations of antibiotic and naringerin combinations established in this study, to assess the effect that this would have on the efflux pump genes. These studies can facilitate the determination of which genes are up/down regulated. The function and role of these efflux pump genes can also be analyzed by carrying out studies that can knock out the genes in resistant strain and determine if the absence would enable the bacteria become susceptible to the various antibiotics and flavonoids.

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APPENDICES

APPENDIX I

Kirby-Bauer assay results for flavonoids and antibiotic combinations

Table 1. Inhibition zones (mm) of flavonoids (0.004 – 2 mg/ml) in combination with tetracycline (1.25 µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591.

Inhibition zones (mm)													
MSSA ATCC 25923							MRSA ATCC 33591						
Flavonoid Concentration (mg/ml)	**Chr**	**Nar**	**7-hy**	**Tet + Chr**	**Tet + Nar**	**Tet + 7-hy**	**Flavonoid Concentration (mg/ml)**	**Chr**	**Nar**	**7-hy**	**Tet + Chr**	**Tet + Nar**	**Tet + 7-hy**
0.004	10	10	10	15.3	15.3	15.3	**0.004**	—	—	—	—	—	—
0.008	10	10	10	15.3	15.3	15.3	**0.008**	—	—	—	—	—	—
0.016	10	10	10	15.3	15.3	15.3	**0.016**	—	—	—	—	—	—
0.031	10	10	10	15.3	15.3	15.3	**0.031**	—	—	—	—	—	—
0.063	10	10	10	15.3	15.3	15.3	**0.063**	—	—	—	—	—	—
0.125	10	10	10	15.3	15.3	15.3	**0.125**	—	—	—	—	—	—
0.25	10	10	10	15.3	15.3	15.3	**0.25**	—	—	—	—	—	—
0.5	10	10	10	15.3	15.3	15.3	**0.5**	—	—	—	—	—	—
1	10	10.5	10	15.3	15.3	15.3	**1**	—	—	—	—	—	—
2	10	11	10	15.3	15.3	15.3	**2**	—	—	—	—	—	—

Chr= chrysin; Nar= naringenin; 7-hy= 7-hydroxyflavone; Tet= tetracycline; —= no zones of inhibition.

Table 2. Inhibition zones (mm) of flavonoids (0.004 – 2 mg/ml) in combination with ampicillin (2.5 µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591.

Inhibition zones (mm)													
MSSA ATCC 25923							MRSA ATCC 33591						
Flavonoid Concentration (mg/ml)	**Chr**	**Nar**	**7-hy**	**Amp + Chr**	**Amp+ Nar**	**Amp 7-hy**	**Flavonoid Concentration (mg/ml)**	**Chr**	**Nar**	**7-hy**	**Amp + Chr**	**Amp + Nar**	**Amp 7-hy**
0.004	10	10	10	19	19	19	**0.004**	—	—	—	11	11	11
0.008	10	10	10	19	19	19	**0.008**	—	—	—	11	11	11
0.016	10	10	10	19	19	19	**0.016**	—	—	—	11	11	11
0.031	10	10	10	19	19	19	**0.031**	—	—	—	11	11	11
0.063	10	10	10	19	19	19	**0.063**	—	—	—	11	11	11
0.125	10	10	10	19	19	19	**0.125**	—	—	—	11	11	11
0.25	10	10	10	19	19	19	**0.25**	—	—	—	11	11	11
0.5	10	10	10	19	19	19	**0.5**	—	—	—	11	11	11
1	10	10.5	10	19	19	19	**1**	—	—	—	11	11	11
2	10	11	10	19	19	19	**2**	—	—	—	11	11	11

Chr= chrysin; Nar= naringenin; 7-hy= 7-hydroxyflavone; Amp= ampicillin; —= no zones of inhibition.

Table 3. Inhibition zones (mm) of flavonoids (0.004 – 2 mg/ml) in combination with methicillin (5 µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591.

Inhibition zones (mm)													
MSSA ATCC 25923							MRSA ATCC 33591						
Flavonoid Concentration (mg/ml)	**Chr**	**Nar**	**7-hy**	**Met + Chr**	**Met + Nar**	**Met + 7-hy**	**Flavonoid Concentration (mg/ml)**	**Chr**	**Nar**	**7-hy**	**Met + Chr**	**Met + Nar**	**Met + 7-hy**
0.004	10	10	10	19	19	19	**0.004**	—	—	—	—	—	—
0.008	10	10	10	19	19	19	**0.008**	—	—	—	—	—	—
0.016	10	10	10	19	19	19	**0.016**	—	—	—	—	—	—
0.031	10	10	10	19	19	19	**0.031**	—	—	—	—	—	—
0.063	10	10	10	19	19	19	**0.063**	—	—	—	—	—	—
0.125	10	10	10	19	19	19	**0.125**	—	—	—	—	—	—
0.25	10	10	10	19	19	19	**0.25**	—	—	—	—	—	—
0.5	10	10	10	19	19	19	**0.5**	—	—	—	—	—	—
1	10	10.5	10	19	19	19	**1**	—	—	—	—	—	—
2	10	11	10	19	19	19	**2**	—	—	—	—	—	—

Chr= chrysin; Nar= naringenin; 7-hy= 7-hydroxyflavone; Met= methicillin; —= no zones of inhibition.

Table 4. Inhibition zones (mm) of flavonoids (0.004 – 2 mg/ml) in combination with vancomycin (5 µg/ml) against MSSA ATCC 25923 and MRSA ATCC 33591.

Inhibition zones (mm)													
MSSA ATCC 25923							MRSA ATCC 33591						
Flavonoid Concentration (mg/ml)	**Chr**	**Nar**	**7-hy**	**Van + Chr**	**Van + Nar**	**Van + 7-hy**	**Flavonoid Concentration (mg/ml)**	**Chr**	**Nar**	**7-hy**	**Van + Chr**	**Van + Nar**	**Van + 7-hy**
0.004	10	10	10	14	14	14	**0.004**	—	—	—	14	14	14
0.008	10	10	10	14	14	14	**0.008**	—	—	—	14	14	14
0.016	10	10	10	14	14	14	**0.016**	—	—	—	14	14	14
0.031	10	10	10	14	14	14	**0.031**	—	—	—	14	14	14
0.063	10	10	10	14	14	14	**0.063**	—	—	—	14	14	14
0.125	10	10	10	14	14	14	**0.125**	—	—	—	14	14	14
0.25	10	10	10	14	14	14	**0.25**	—	—	—	14	14	14
0.5	10	10	10	14	14	14	**0.5**	—	—	—	14	14	14
1	10	10.5	10	14	14	14	**1**	—	—	—	14	14	14
2	10	11	10	14	14	14	**2**	—	—	—	14	14	14

Chr= chrysin; Nar= naringenin; 7-hy= 7-hydroxyflavone; Van= vancomycin; —= no zones of inhibition.

APPENDIX II

PCR Optimization results

Annealing temperature of 53°C

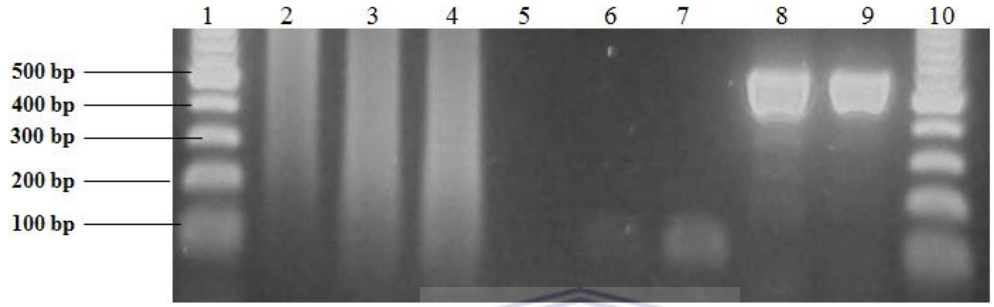


Figure 1. 1% agarose gel analysis of *mepA a*nd *sepA* amplified using gene specific primers at annealing temperature of **53°C** and primer concentration of 10 pmol/µl. Lanes 1 and 10: 100bp DNA molecular weight marker; Lanes 2-4- *mepA* gene amplification- no template PCR control (negative control), for MSSA ATCC 25923 and MRSSA (ATCC 33591) strains respectively; Lanes 5-7: *sepA* gene amplification- no template PCR conrol (negative control), MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively; Lanes 8 and 9: *16S*, house-keeping gene amplification (internal positive control) for both MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively.

Annealing temperature of 54°C

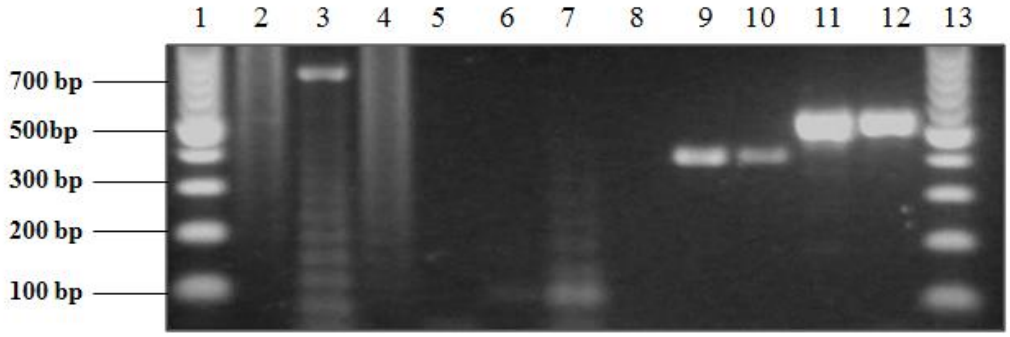


Figure 1. 1% agarose gel analysis of *mepA a*nd *sepA* amplified using gene specific primers at annealing temperature of **54°C** and primer concentration of 10 pmol/µl. Lanes 1 and 13: 100bp DNA molecular weight marker; Lanes 2-4: *mepA* gene amplification- no template PCR control (negative control), MSSA ATCC 25923 and MRSSA (ATCC 33591) strains respectively; Lanes 5-7: *sepA* gene amplification- no template PCR conrol (negative control), for MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively; Lanes 8: 10- *gyrA* gene amplification- no template PCR control (negative control), MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively; Lanes 11 and 12: *16S*, house-keeping gene amplification (internal positive control) for both MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively.

Annealing temperature of 55°C

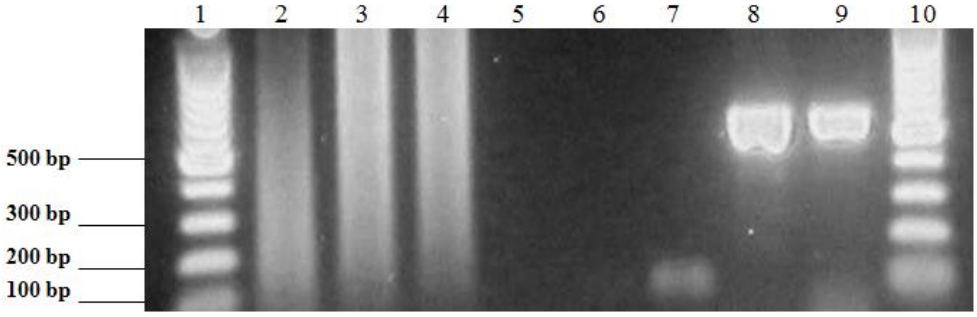


Figure 1. 1% agarose gel analysis of *mepA a*nd *sepA* amplified using gene specific primers at annealing temperature of **55°C** and primer concentration of 10 pmol/µl. Lanes 1 and 10: 100bp DNA molecular weight marker; Lanes 2-4: *mepA* gene amplification- no template PCR control (negative control), for MSSA ATCC 25923 and MRSSA (ATCC 33591) strains respectively; Lanes 5-7: *sepA* gene amplification- no template PCR conrol (negative control), for MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively; Lanes 8 and 9: *16S*, house-keeping gene amplification (internal positive control) for both MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively.

Annealing temperature of 56°C

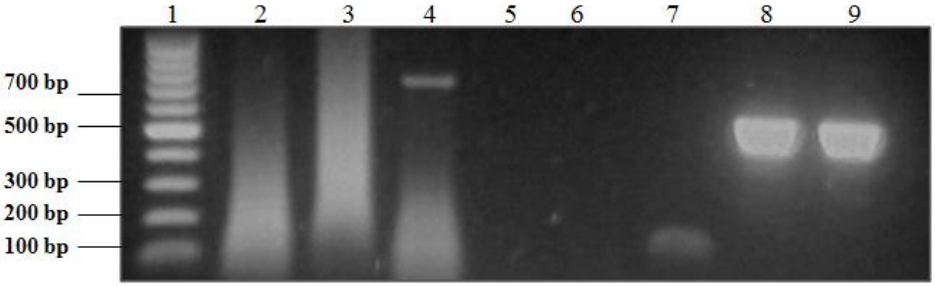


Figure 1. 1% agarose gel analysis of *mepA a*nd *sepA* amplified using gene specific primers at annealing temperature of **56°C** and primer concentration of 10 pmol/µl. Lane 1: 100bp DNA molecular weight marker; Lanes 2-4: *mepA* gene amplification- no template PCR control (negative control), for MSSA ATCC 25923 and MRSSA (ATCC 33591) strains respectively; Lanes 5-7: *sepA* gene amplification- no template PCR conrol (negative control), for MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively; Lanes 8 and 9: *16S*, house-keeping gene amplification (internal positive control) for both MSSA ATCC 25923 and MRSA ATCC 33591 strains respectively.

Magnesium Chloride concentration mepA

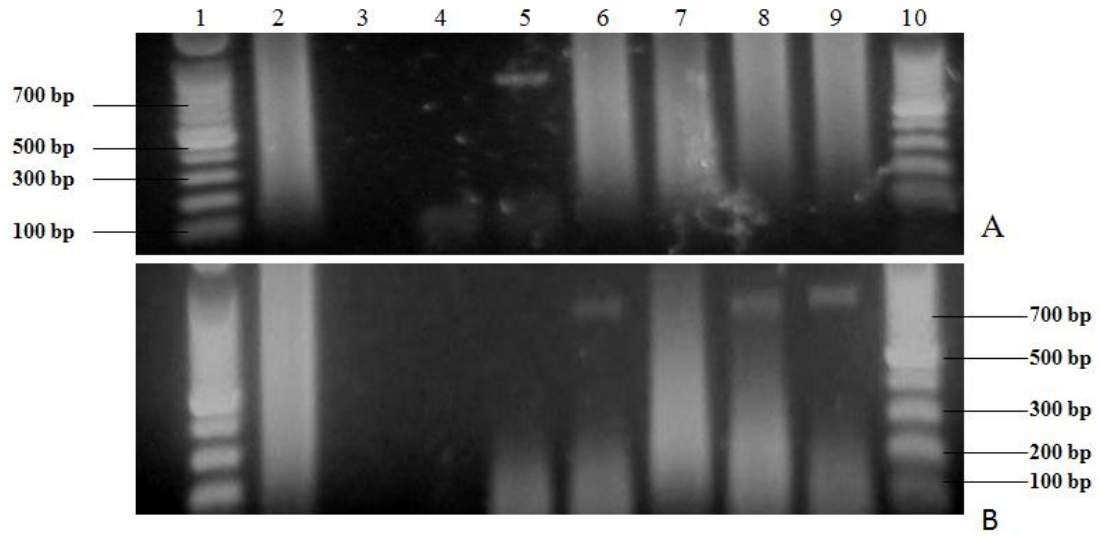


Figure 5. 1.5% agarose gel elecrophoresis of *mepA* magnesium chloride concentration optimization amplified using gene specific primers. Lanes 1 and 10: 100bp DNA molecular weight marker; Lane 2- no template PCR control (negative control); Lanes 3-9: magnesium chloride at concentrations of 0, 0.5, 1, 1.5, 2, 2.5 and 3mM respectively. **(A)** MSSA ATCC 25923 and **(B)** MRSA ATCC 33591 strains.

Magnesium Chloride concentration sepA

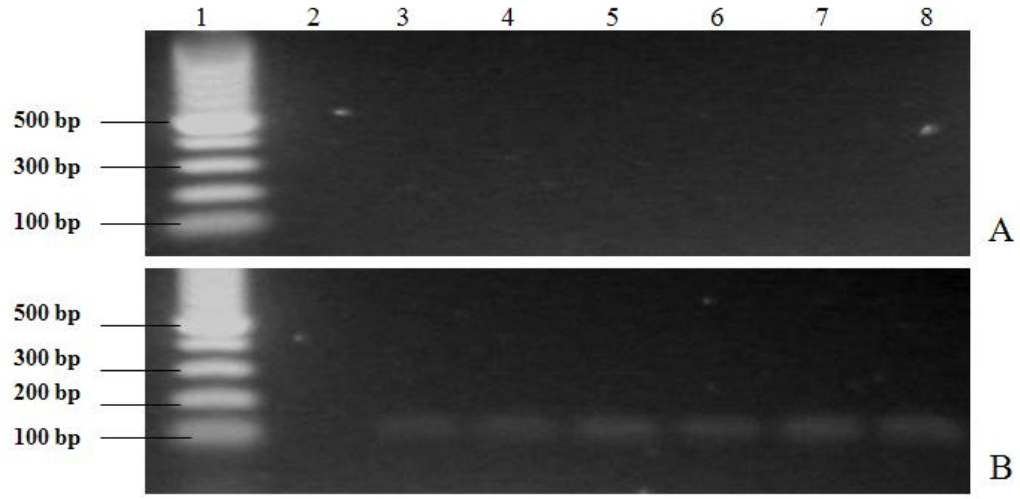
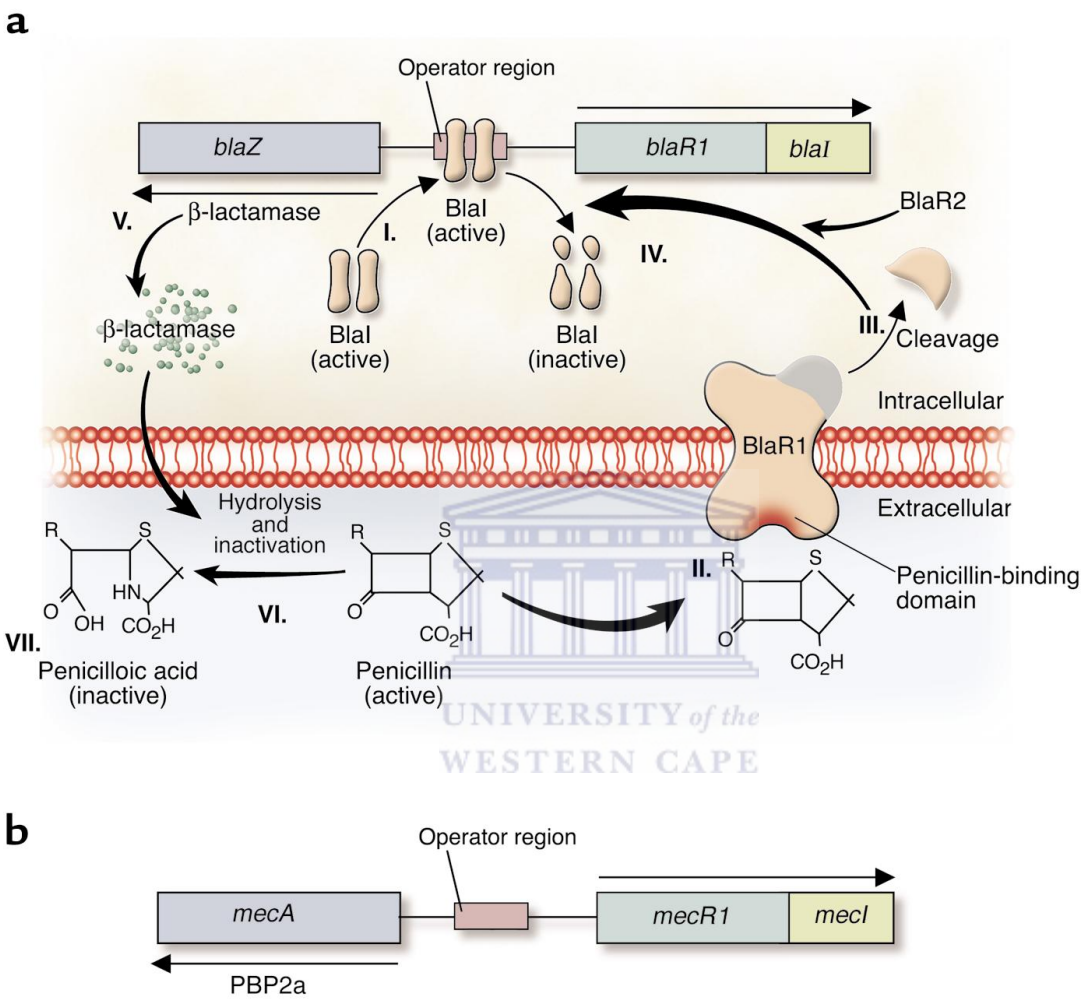


Figure 6. 2% agarose gel elecrophoresis of *sepA* magnesium chloride concentration optimization amplified using gene specific primers. Lane 1: 100bp DNA molecular weight marker; Lanes 2-8: magnesium chloride at concentrations of 0, 0.5, 1, 1.5, 2, 2.5 and 3mM respectively. **(A)** MSSA ATCC 25923 and (**B)** MRSA ATCC 33591.

Appendix III

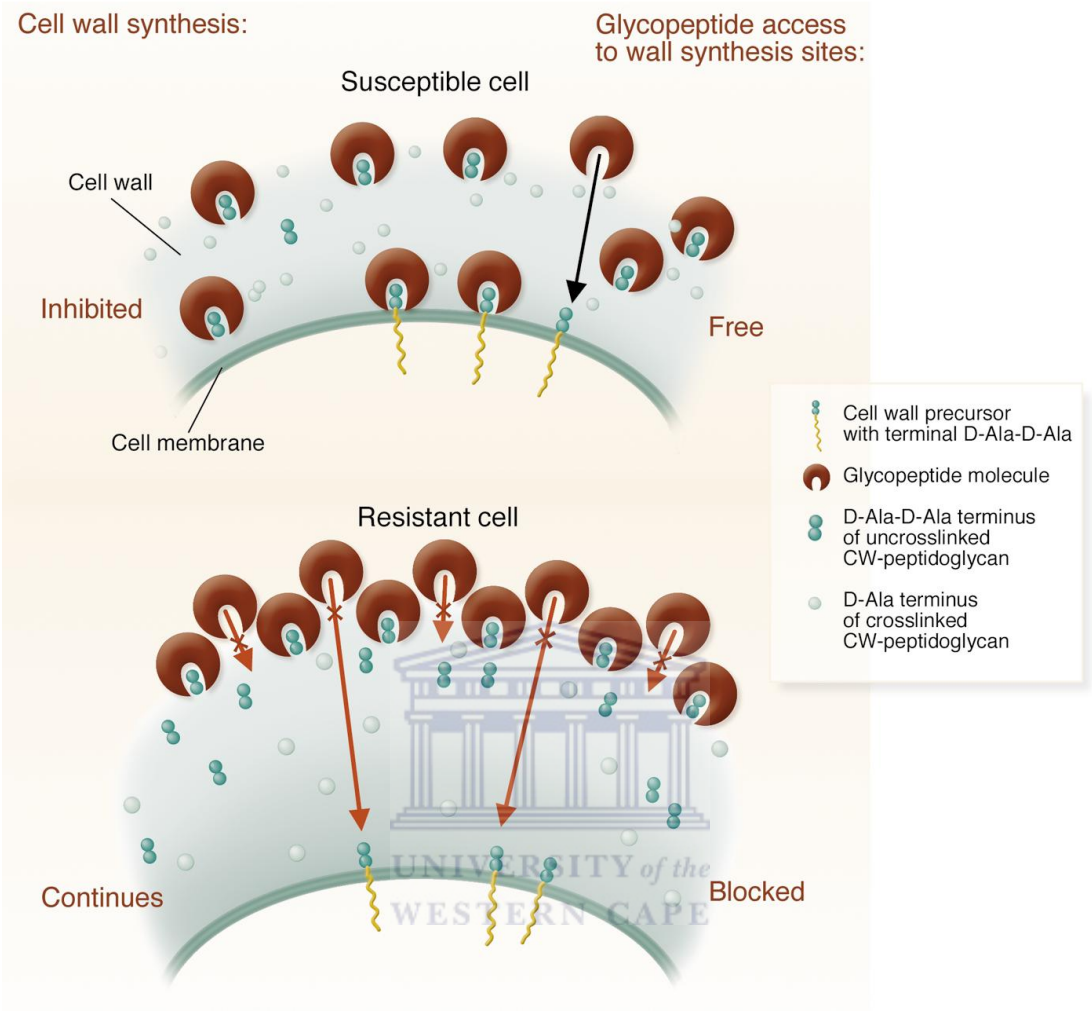
Summary of antibiotic resistance

Resistance to β-lactams and methicillin



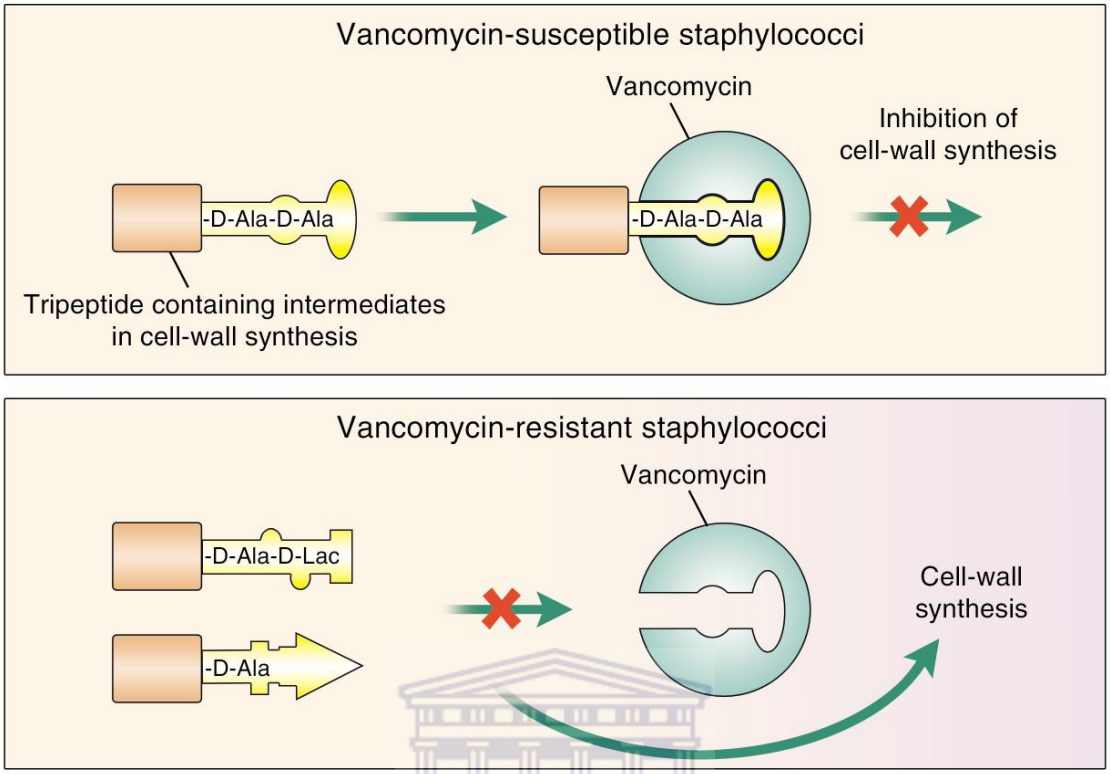
(**a**) Induction of staphylococcal β-lactamase synthesis in the presence of the β-lactam antibiotic penicillin. I. The DNA-binding protein BlaI binds to the operator region, thus repressing RNA transcription from both *blaZ* and *blaR1- blaI*. In the absence of penicillin, β-lactamase is expressed at low levels. II. Binding of penicillin to the transmembrane sensor-transducer BlaR1 stimulates BlaR1 autocatalytic activation. III–IV. Active BlaR1 either directly or indirectly (via a second protein, BlaR2) cleaves BlaI into inactive fragments, allowing transcription of both *blaZ* and *blaR1-blaI* to commence. V–VII. β-Lactamase, the extracellular enzyme encoded by *blaZ* (V), hydrolyzes the β-lactam ring of penicillin (VI), thereby rendering it inactive (VII). (**b**) Mechanism of *S. aureus* resistance to methicillin. Synthesis of PBP2a proceeds in a fashion similar to that described for β-lactamase. Exposure of MecR1 to a β-lactam antibiotic induces MecR1 synthesis. MecR1 inactivates MecI, allowing synthesis of PBP2a. MecI and BlaI have coregulatory effects on the expression of PBP2a and β-lactamase.

Resistance to vancomycin



Mechanisms of *S. aureus* resistance to vancomycin: VISA strains. VISA strains appear to be selected from isolates that are heterogeneously resistant to vancomycin. These VISA strains synthesize additional quantities of peptidoglycan with an increased number of D-Ala-DAla residues that bind vancomycin, preventing the molecule from getting to its bacterial target.

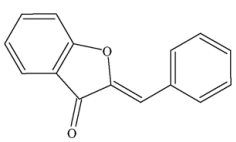
Resistance to vancomycin



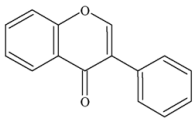
Mechanisms of *S. aureus* resistance to vancomycin: VRSA strains. VRSA strains are resistant to Vancomycin because of the acquisition of the *vanA* operon from an enterococcus that allows synthesis of a cell wall precursor that ends in D-Ala-D-Lac dipeptide rather than D-Ala-D-Ala. The new dipeptide has dramatically reduced affinity for vancomycin. In the presence of vancomycin, the novel cell wall precursor is synthesized, allowing continued peptidoglycan assembly.

Appendix IV

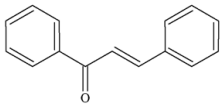
Summary of the structures of flavonoids



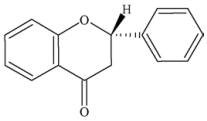
Aurone



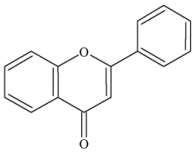
Isoflavone



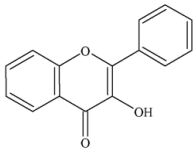
Chalcone



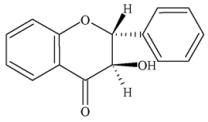
Flavanone



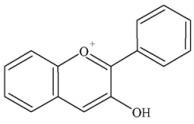
Flavone



Flavonol



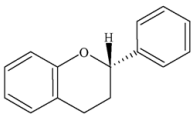
Flavanon-3-ol (also known as 3-hydroxyflavanone or dihydroflavonol)



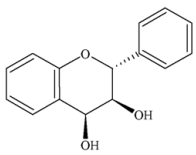
Anthocyanidin

Flavan-3-ol (also known as catechin)

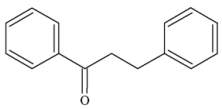
Proanthocyanidin (also known as flavolan or condensed tannin)



Flavan



Flavan-3,4-diol (also known as leucoanthocyanidin)



Dihydrochalcone

The skeleton structures of the main classes of flavonoids: aurones , isoflavones (e.g. Sophoraisoflavone A), chalcones (e.g. Licochalcone A), flavanones (e.g. Naringenin), flavones (e.g. Chrysin), flavonols (e.g. Galangin), flavanon-3-ols (e.g. Dihydrofisetin), anthocyanidins , flavan-3-ols (e.g. Epigallocatechin), proanthocyanidins (occur as dimers, trimers, tetramers and pentamers; *R*=0, 1, 2 or 3 flavan-3-ol structures), flavans (e.g. 6,4'-Dichloroflavan) flavan-3,4-diols (e.g. Leucocyanidin) and dihydrochalcones.