Examination of the antibacterial activities of some semi-synthetic chalcone-derivatives alone and in combination with polymyxin B

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PhD **May 2013**

Examination of the antibacterial activities of some semi-synthetic chalcone-derivatives alone and in combination with polymyxin B

A thesis submitted in partial fulfilment of the requirement of Robert Gordon University for the degree of Doctor of Philosophy

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May 2013

Declaration

This thesis has been composed by myself and has not been submitted in any previous application for the award of any degree. The body of work recorded in herein was absolutely carried out by me. All verbatim extracts have been distinguished by quotation marks and sources of information were either referenced or acknowledged.

Erere O. Medu.

Abstract

In view of the increasing global challenge of bacterial resistance, there exists an urgent need for the rationale development of antibacterial compounds with either novel or multiple mechanisms of action. Two chalcone-derivatives, F1 and F23, demonstrated MICs within the range of 16 to >512 µg/ml against two plant pathogens (*P. caratovoram* and *C. michiganensis* subsp. *michiganensis*) as well as important clinical bacterial species. Both compounds displayed an MIC of 32 µg/ml against quinolone-resistant *S. aureus*. Whilst possessing weak activities individually, each semi-synthetic agent displayed notable synergistic action with polymyxin B against *S. aureus*, *C. violaceum*, *E. coli* and *Ps. aeruginosa*, thereby recording FICs within the range of <0.093 to 2 that indicated the existence of synergism in some instance. These chalcone compounds applied with polymyxin B displayed a notable FIC_{index} of <0.093 against the Neisseriaceae *C. violaceum*, and a potential noteworthy capacity to extend the spectrum of activity of the latter antibiotic to include Gram-positive *S. aureus* species. F1 inhibited staphylococcal replication in broth and the combination of either of both chalcone-derivatives with polymyxin B instituted a metabolic blockage in *S. aureus* and other bacterial species as determined through a modified MTT reduction assay. The combined agents inflicted major disruptions to the *S. aureus* cytoplasmic membrane bilayer as evidenced by the release of intracellular potassium as well as the influx of Sytox Green fluorescent stain. Notable levels of cell membrane potential dissipation, leakage of intracellular potassium ions and blockage of reducing enzymes activities occurred within the first 30 minutes, well in advance of significant loss in cell viability that was recorded usually after 4 – 8 hours, suggesting these activities were prerequisites to cell death. In erythrocyte lysis assay, the synergistic combinations of 128 µg/ml of either of both chalcone derivatives with 128 µg/ml polymyxin B displayed the lowest degree of haemolysis, followed by that occurring with 32 µg/ml of the chalcone-derivatives combined with 256 µg/ml of the polypeptide antibiotic. In conclusion, further structure activity modifications aimed at improving the aqueous solubility of these chalcone-derivatives as well as the antibacterial activity recorded for certain combination concentrations of polymyxin B with either of these semi-synthetic agents may be required before considerations are made for the possibility for potential external formulations. Such preparations may include

antiseptic creams, lotions, ointments, as well as aerosols that can be applied with nebulizers in targeted delivery for such cases like cystic fibrosis.

Acknowledgements

Obviously, this fascinating but sometimes daunting as well as iconic journey is now coming to an end. It required a clear vision, defined purpose, sound mind and an unswerving focus.

During these years, I have met and worked with several people who must be thanked for their invaluable support, help and encouragement. I wish to thank the Robert Gordon University, Institute of Social Welfare and Health Research, School of Pharmacy and Life Sciences as well as their staff for granting me access to their facilities.

I am deeply grateful to my principal supervisor Dr. Andrew James Lamb as well as to my alternate supervisors, Drs. Kerr H. Matthews and Di Salvo Alberto. Upon my request, Andrew offered me the opportunity to work in this project, showing a consistently immense as well as an unsurpassable commitment, enthusiasm and guidance over the years. His extensive knowledge in the field of study, invaluable advices as well as the demonstrated willingness to meticulously and critically read through this dissertation is much appreciated. Furthermore, I wish to thank the collaborators in this project including Dr. Richard Brown, Juliet Wilcox and Sherif B. Abdel Ghani of the School of Chemistry, University of Southampton, as well as Ziddan H. Ziddan and Huessein M. Ali of the Plant Protection Department and Agricultural Biochemistry Department respectively, both within the Faculty of Agriculture, Ain Shams University, Egypt.

I am thankful for the firm and unbiased role, as well as the comments of Dr. Jeanette Robertson during my transfer to PhD. process. They have indeed helped tremendously.

I also wish to thank late Ms. Vivienne Hamilton, Mrs. Moira Innes, Mrs, Angela Ross, Mrs. Margaret Brown, Drs. Noelle O'Driscoll, Olga Labovitiadi and Tina Lowes for the cordial working relationship we shared in the laboratories, especially PA27. Upon Andrew's request, Noelle was supportive when I needed to setup the first protocol for MIC determination using the micro titre plate reader. Thanks to Dr. Susanne Boyle for the group tutorial on the use GraphPad prism software and to Dr. Tina Lowes for the recent tutorial on cDNA synthesis and PCR. Many thanks to Dr. Colin Thompson for providing guidance on the use of Perkin Elmer LS Luminescence Spectrometer and to Dr. Stuart Cruickshank for instructions on the use of the fluorescent microscopes. I also wish to appreciate

Mrs. Moira Middleton for her kindness in the chemistry laboratory as well as both Mr. Raymond Reid and Mrs. Maureen Byres for their assistance on the use of the flame emission spectrophotometer. Mr. Brian Dejonckheere was always willing to assist in issues related to computer hard and softwares, even when approached along the corridors. I really appreciate. I also acknowledge my office mates including Dr. Vibhu Paudyal, Dr. Maxwell Dapar, Chidinma Ibie, Oke Ndu, Caroline Macleod and Donald Philips. Thanks to Omo and Aakash, two of my contemporaries who are based in the Garthdee and St. Andrew's campuses respectively.

The most special thanks are to my friends and family members. The unexpected and sudden transition of my mum, Mrs. Anna Medu, 6 months into the commencement of this project and at a time we thought we needed her most as a family made me really sad. She had encouraged me to move on with the opportunity when it arose and I miss her dearly and daily, but pray her soul finds solace in the LORD's bosom; for death is such a terrible short-changer. My appreciations to my wife, Tejiri, for her prayers. I am also thankful for our children Ano and Rode, especially the succour and humour they provide. I also appreciate my siblings (especially Igho and Orho), for their encouragements and courage during our most trying period. I am most especially thankful for all the support provided by my parents (in-law), Mr. and Mrs. James Omeru, all through this period. They are exceptionally wonderful people. Finally, I wish to acknowledge Mr. and Mrs. Esse Agbasi, Mrs. Awopetu, Dr. and Mrs. Jonathan Alao (MD), Dr. Uvie Mafuru (MD), Dr. A.O Oyedele, Professor Onawunmi, Professor Wilson Erhun, Dr. Andrew Onosode and my beloved dad, Mr. Nevice U Medu.

“... your main problem is that you do not think” – Professor Adebayor Lamikanra.

“Truly, thoughts are things, and powerful things at that, when they are mixed with definiteness of purpose, persistence and a burning desire for their translation into riches, or other material objects.” - Napoleon Hill’s ‘think and grow rich’.

“I am yet to know a man who stumbled into success” – Napoleon Hill.

“Let not your heart be troubled .... In my Father’s house are many mansions (rooms and opportunities). For if it were not so, I would have told you.” – Jesus of Nazareth.

In memory of my dear and caring mum,

Mrs. Anna B. Medu – Nee Orogodo Oyibo

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Abbreviations

A	Absorbance
ATP	Adenosine triphosphate
ATCC	American type culture collection
BZK	Benzalkonium chloride
cAMPs	Cationic antimicrobial peptides
cfu/ml	Colony forming units per millilitre
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
FDA	Food and Drug Administration
FMN	Flavin mononucleotide
FAD	Flavin adenine dinucleotide
K^+	Potassium ions
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MTT	C,N-diphenyl-N-4,5-dimethyl thiazol 2 yl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NAD(P)H	Nicotinamide dinucleotide phosphate
NCTC	National collection of type culture
ND	Not determined
OD	Optical density
RNA	Ribonucleic acid
RGU	Robert Gordon University
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SDH	succinate dehydrogenase
UK	United Kingdom
USA	United States of America
XTT	2, 3-bis [2-methoxy-4-nitro-5-sulphophophenyl] -2*H*-tetrazolium-5-carboxanilide
WHO	World Health Organisation

Chapter 1

1.0 Introduction

1.1 Common causes of Infection

The WHO 2008 health statistics report on infectious, communicable and parasitic diseases confirms that such ailments remain the leading cause of morbidity and mortality globally (Mathers, Fat and Boerma 2008). The disease conditions include diarrhoea, HIV/AIDs, tuberculosis, pneumonia, endocarditis, urinary tract infections, malaria, trypanosomiasis and leishmaniasis (Goossens et al. 2005, Angulo et al. 2009). Diverse species are responsible for infectious diseases of bacterial origin and often include species of common pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. Infections are often deep-sited within tissues and as such require treatment with antibacterial compounds that are bactericidal in action but also show an excellent safety profile towards uninfected host tissues (Liu et al. 2011). Such attributes enable the use of long term dosage regiments in order to access deep sites of infection, eradicate the pathogen, while at the same time cause minimal adverse effects for the host. Examples of such compounds are ceftriazone, vancomycin, teicoplanin, ceftaroline and telavancin, all of which have been used extensively in the past with positive outcomes (Liu et al. 2011).

1.2 Common pathogenic bacterial species

1.2.1 *Staphylococcus aureus*

1.2.1.1 Pathogenicity of *S. aureus*

More than 100 years ago, *S. aureus* was identified as an important pathogenic bacterial species (Lowy 1998). This bacterium was first recovered from surgical abscess in 1880 and has hitherto been isolated from diverse clinical conditions. *S. aureus* can cause minor skin infections, inflammations, septicaemia, respiratory tract and lung infections, endocarditis as well as chronic bone infections when in contact with these sites, under conditions that promote cell

survival and replication (Liu et al. 2011, Sheagren 1984, Smith et al. 1999, Tenover and Moellering 2007, Hersh et al. 2008).

1.2.1.2 Phenotypes of resistant *S. aureus* and impact on antibiotic therapy

The evolution, acquisition and dissemination of antibiotic resistance in *S. aureus* have been accompanied by changing disease pathology, epidemiology and prognosis (Liu et al. 2011, Bishop and Howden 2007). *S. aureus* species initially developed resistance to penicillin and subsequently to methicillin (Liu et al. 2011, Rivera and Boucher 2011). Methicillin-resistant *S. aureus* (MRSA) is now pandemic and has become a major cause of hospital acquired infections globally (Enright et al. 2002). More than 19,000 MRSA-related mortalities per annum are reported in the U.S.A alone and 70 % of all strains of *S. aureus* in hospitals are methicillin-resistant (Leung et al. 2011). Subsequent emergence of community acquired MRSA with genes expressing increased virulence has also been reported (Vandenesch et al. 2003, Charlebois et al. 2004, Chambers and DeLeo 2009).
Teicoplanin and vancomycin are glycopeptides that are reserved for MRSA infections because of these drugs effectiveness and the need to preserve their clinical utility by reducing the chances for the development of resistance against these agents by pathogens (Liu et al. 2011, Enright et al. 2002). But the clinical use of these antibiotics has been accompanied by the emergence of glycopeptide intermediate- (GISA/VISA) and heterogeneous- resistant *S. aureus* (h.VISA) since about 1993 (Appelbaum 2007). MRSA strains with reduced sensitivity to teicoplanin were first reported in 1995 (Mainardi et al. 1995) and was followed in 1997 by the isolation of *S. aureus* strains in Japan with reduced sensitivity and heterogeneous resistance to vancomycin (Hiramatsu et al. 1997). These two incidences increased the volume of international medical debate on the impact of evolving bacterial resistance upon the future prospects of antibiotic therapy (Hiramatsu et al. 1997). Currently, controversy still exists in the medical community on the future role of vancomycin in the treatment of serious infections caused by methicillin-resistant *S. aureus* (Howden et al. 2010).

1.2.1.3 Cell wall structure and staphylococcal resistance toantimicrobial compounds

The cell wall of Gram-positive bacteria consists of an inner cytoplasmic membrane and a thick outer peptidoglycan (murein) layer with teichoic and lipoteichoic acids, as well as cell wall proteins (figure 1.1) (Navarre and Schneewind 1999, Heilmann and Götz 2010).

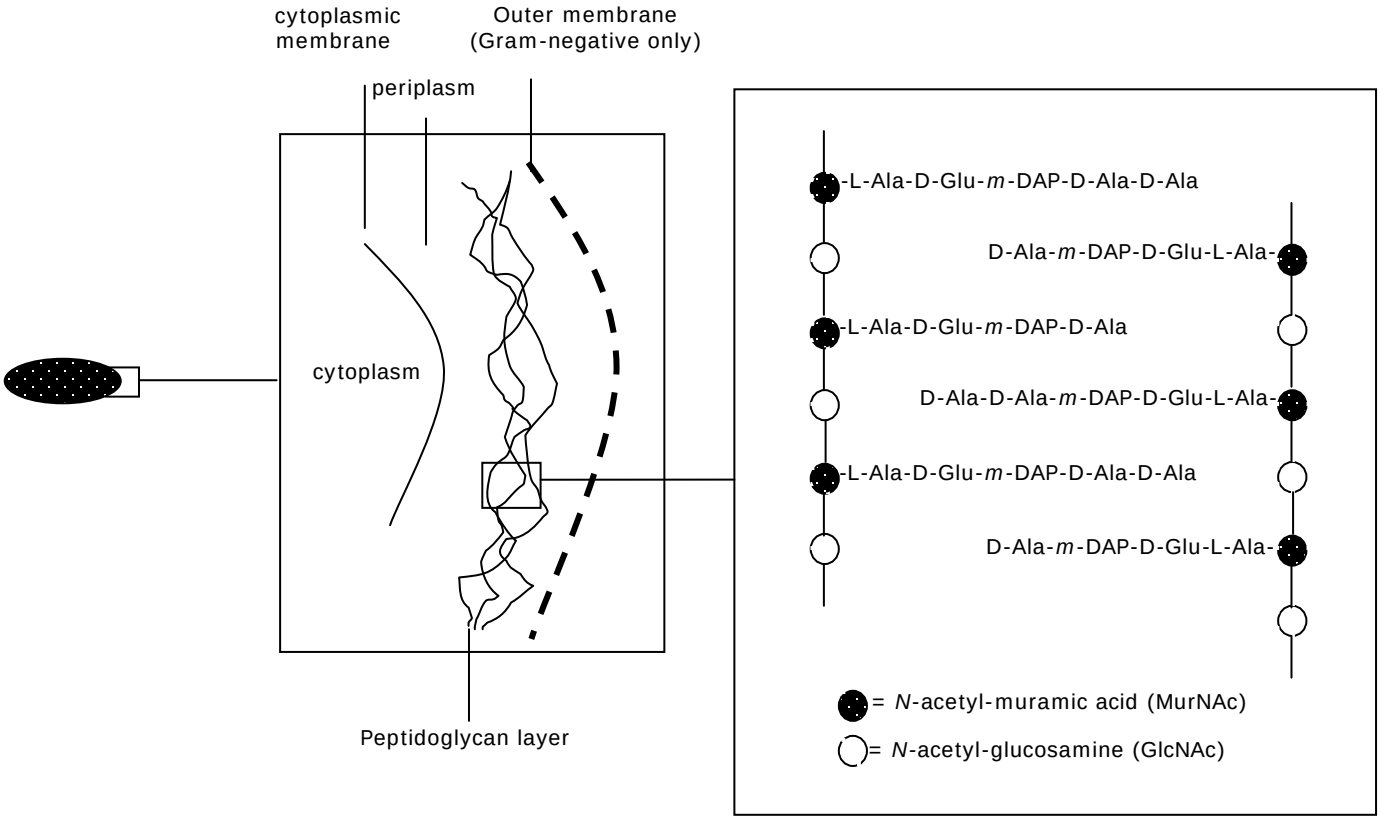


Figure 1.1: Diagrammatic representation of bacterial cell wall and peptidoglycan (Bugg and Walsh 1992).

Peptidoglycan is a semi-flexible exoskeleton required to withstand the internal cytoplasmic turgor and osmotic pressure, as well as to function like elastic scaffolding that gives shape to the cell (Vollmer and Höltje 2004, Lorian 2005). This structure consists of alternating units of uridine diphosphate-*N*-acetylglucoseamine and uridine diphosphate-*N*-acetylmuramic acid that are cross-linked with short peptide chains (Paradis-Bleau et al. 2008), thereby forming a mesh-like polymeric structure that surrounds the cytoplasmic membrane (Vollmer 2007, Vollmer and Born 2009). The continuously cross-linked mesh forms a polyionic and amphoteric network (Lorian 2005). This network is relatively coarse and porous with an exclusion limit of up to 100 000

Da that enables antimicrobial compounds (usually 300 to 700 Da in size) to readily diffuse through this layer (Lorian 2005).

Peptidoglycan is an essential component for bacterial viability, growth and replication (Goehring and Beckwith 2005) with a conserved composition for different strains of *S. aureus* (Tomasz 2006). The peptidoglycan layer from the same strains consists of identical patterns of linear oligo (*GlcNac-MurNAc*) glycan strands when examined with high performance liquid chromatography, suggesting that cell wall composition may be strain-specific (Tomasz 2006). This may partly explain why different strains of *S. aureus* express different sensitivity to distinct antimicrobial compounds (Howden et al. 2010).

The bacterial cell wall also contains teichoic acids, long chain polymers made of either ribitol or glycerol residues with phosphodiester links and uronic acids. These acids are bound to the cell wall as lipoteichoic acid with one end of their chain anchored to either phospholipids in the cytoplasmic membrane or attached to *N*-acetylmuramyl residues in the peptidoglycan layer. In both instances, the free ends of the teichoic acids protrude at the staphylococcal surface (Bertsche 2009). The protruding teichoic acids are linked together by hydrophilic polysaccharides (various hexose stereoisomers and linkages), that can absorb water and transform the layer into a gel known as either glycocalyx, slime, alginate or capsule (Lorian 2005, Bertsche 2009). Teichoic acids in the capsule contribute to staphylococcal-host cells attachment (Heilmann and Götz 2010, Greenberg, Fischer and Joiner 1996). For instance, there is reduced nasal colonisation, reduced binding to endothelial cells and attenuated virulence in staphylococcal strains with reduced cell wall teichoic acids and in strains with gene-mediated annihilation of teichoic acids (Weidenmaier et al. 2005). The mechanism by which *S. aureus* develops resistance to β-lactams and glycopeptides is associated with mutational modifications leading to inactivation of penicillin-binding proteins (Howden et al. 2010) and increased thickness of the cell wall (Sieradzki, Pinho and Tomasz 1999). Both factors reduce the capacity of antibiotics to access and interact with their primary cell wall targets. Resistant bacterial species are thought to have developed peptidoglycans with altered composition, allowing for a reduced fraction of highly cross-linked muropeptide alongside a proportionate increase in the occurrence of muropeptide monomers and dimers (Sieradzki, Pinho and Tomasz 1999). Organisms with such altered peptidoglycan structures can express reduced susceptibility to antibiotics whose

primary target is the impairment of cell wall biosynthesis and may continue to replicate in the presence of these compounds.

1.2.1.4 Genes and enzymes that regulate staphylococcal wall biosynthesis as potential targets for novel antibacterial compounds

An array of genes is known to code for precursors in the biosynthesis of staphylococcal cell wall components (De Lencastre et al. 1999). During cell wall formation (figure 1.2), these genes therefore code for the stepwise synthesis of the pentaglycine bridge that gets attached to the lysine residue of the stem peptide (Tomasz 2006, KOPP et al. 1996). Such genes include *femX*, *femA*, *femB* and *femC* (Berger-Bächi and Rohrer 2002). The gene *femX*, is also referred to as *fmhB* and codes for enzymes responsible for interpeptide formation as well as the addition of the 1st glycine interbridge residue to the stem peptide. As would be expected, the inactivation of this gene leads to cell death (Rohrer et al. 1999). The genes *femA* and *femB* code for proteins that are responsible for interpeptide formation, addition of the 2nd, 3rd, 4th and 5th glycine to the stem peptide and their inactivation either abolishes or reduces methicillin resistance (Berger-Bächi and Rohrer 2002). The *femC* codes for glutamine synthetase repression and the inactivation of this gene reduces the amidation of glutamate of the stem peptide as well as the cell resistance to methicillin (Berger-Bächi and Rohrer 2002, Figueiredo et al. 2012). These genes are therefore essential for bacterial survival (Hübscher et al. 2007) and could be possible targets for the design of novel antibacterial compounds (KOPP et al. 1996). MurF enzyme (figure 1.2) catalyses the formation of a peptide bond between D-alanine-D-Alanine (D-Ala-D-Ala) and the cell wall precursor, uridine 5'-diphosphoryl *N*-acetylmuramoyl-L-alanyl-D-glutamyl-meso-diaminopimelic acid (UDP-MurNAc-Ala-Glu-meso-A2pm) accompanied by the hydrolysis of ATP to ADP and inorganic phosphate to produce UDP-*N*-acetylmuramylpentapeptide.

A potential antibiotic, MurFp1, in the form of a phage display-derived inhibitor for this essential cell wall biosynthesis enzyme (MurF) (figure 1.2) has been developed (Paradis-Bleau et al. 2008). MurFp1 enforces a blockage of the bacterial wall synthesis by interfering with the utilisation of MurF amide ligase enzyme (Paradis-Bleau et al. 2008).

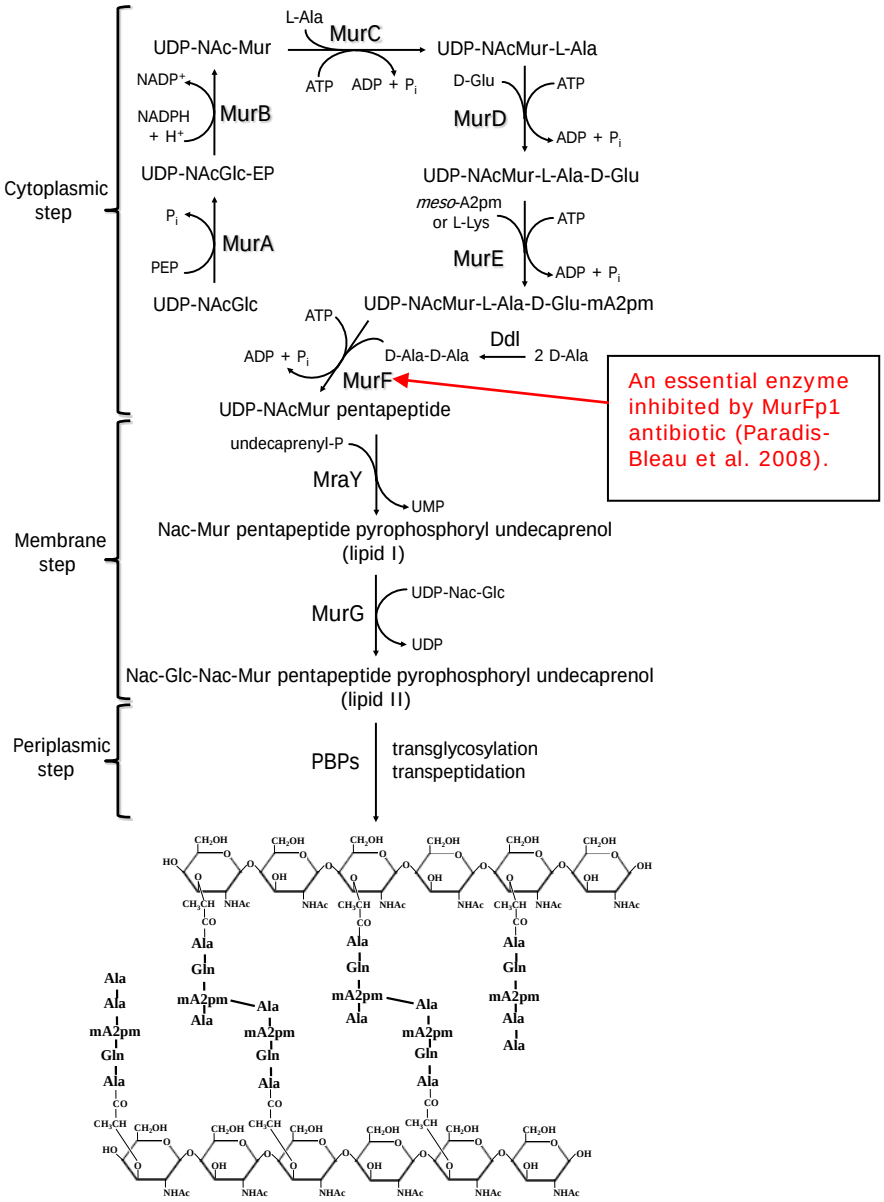


Figure 1.2: Diagrammatic representation of bacterial cell wall biosynthesis involving the cytoplasmic, membrane as well as periplasmic segments of the pathway. The formed peptidoglycan consists of alternating units of uridine diphosphate-*N*-acetylglucosamine and uridine diphosphate-*N*-acetylmuramic acid that are cross-linked with short peptide chains. Adapted from Paradis-Bleau et al. 2008.

In cell wall biosynthesis, Pbp1, Pbp2 and Pbp3 are high molecular weight penicillin binding proteins that carry out both transglycosidase and transpeptidase functions. In other words, they are essential enzymes for the formation of β-1, 4-glycosidase bonds between *N*-acetyl glucosamine (NAG) and *N*-acetylmuramic acid (NAMA), as well as for the peptide bond linkage of the penultimate D-alanine to a glycine receptor on the interbridge, in actively

dividing staphylococcal bacterial species (Maranan et al. 1997, Pinho and Errington 2005). Pbp2a encoded by *mecA* is responsible for *S. aureus* resistance to methicillin (Pinho and Errington 2005).

1.2.2 *Pseudomonas aeruginosa*

1.2.2.1 The Pathogenicity of *Ps. aeruginosa*

Ps. aeruginosa is an ubiquitous and opportunistic Gram-negative bacterium with capacity to either cause or contribute to infections in animal hosts (Bais et al. 2006, Weir 2008). *Ps. aeruginosa* is the main cause of lung infections in patients with cystic fibrosis (Smith et al. 1996) and is also a leading cause of chronic lung infections (Maciá et al. 2005), nosocomial infections that include pneumonia (Rello et al. 1996, Gales et al. 2001) and septic shock (Kurahashi et al. 1999). The virulence of this bacterium contributes to the aetiology and pathogenesis of physical injuries and wounds in burn patients (Lyczak, Cannon and Pier 2000b, Lyczak, Cannon and Pier 2000a, Estahbanati, Kashani and Ghanaatpisheh 2002). *Ps. aeruginosa* is a major cause of secondary infection in immune compromised patients and nosocomial infections caused by other pathogens (Hill et al. 2005, Rowe and Clancy 2006, Bonomo and Szabo 2006). Primary infections caused by *Ps. aeruginosa* and disease conditions associated with this organism are difficult to treat because of its inherent and adaptive resistance to antimicrobial compounds (Hirakata et al. 2002).

1.2.2.2 Mechanisms of *Ps. aeruginosa* resistance to antimicrobial agents

An effective antimicrobial compound must be able to penetrate the bacterial envelope, interact with the target site in sufficiently high concentration and for a long enough duration. In Gram-negative bacteria (such as *E. coli*, *Ps. aeruginosa*, and *Chromobacterium violaceum*), the degree of permeation of hydrophilic antimicrobial agents can be restricted by the lipopolysaccharide layer of the outer cell membrane and the underlying phospholipids (Savjani, Gajjar and Savjani 2009); whilst the translocation of hydrophobic antibiotics is deregulated by over-expression of outer membrane proteins (Cloete 2003). *Ps. aeruginosa* is intrinsically resistant to many antimicrobial compounds such as macrolides, rifampicin and aminoglycosides because of its intact and highly effective outer

membrane barrier (Cloete 2003, Hancock 1997a, Normark and Normark 2002), thereby causing infections with a high rate of morbidity and mortality (Emerson et al. 2002, Kang et al. 2003).

In addition, this organism has an array of effective multi-drug resistant efflux pumps that include MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Hirakata et al. 2002, Lomovskaya et al. 2001a) which are particularly effective against the fluoroquinolones, whose mechanism of uptake is through facilitated diffusion (Lewis 1999). However, both MexAB-OprM and MexXY-OprM are broad specific multi-drug resistant efflux systems, which can extrude a wide range of antibiotics, including hydrophobic as well as amphiphilic antimicrobial compounds (Hancock 1997a, Normark and Normark 2002). The over expression of MexCD-OprJ and MexEF-OprN promotes acquisition of multi-drug resistance through mutational hyper expression of efflux mechanisms (Li, Nikaido and Poole 1995, Gotoh et al. 1998, Köhler et al. 1999). The *mexR* and *nfxB* genes in *Ps. aeruginosa* act as repressors for MexAB-OprM and MexCD-OprJ efflux pumps respectively. Mutation in these genes can lead to over expression of these pumps with consequent resistance to antimicrobial substrates to which this organism was previously susceptible (Evans, Adewoye and Poole 2001). Similarly, mutations of these genes can lead to over expression of chromosomal AmpC β-lactamase causing increased resistance to all β-lactam antibiotics, except carbapenems (Quale et al. 2006, Livermore and Woodford 2006).

Although the outer membrane was previously thought to slow the permeation of hydrophobic substrates, findings from other studies show that the efflux systems play a more prominent role in the extrusion of hydrophobic antimicrobial compounds (Hancock 1997a). Mutant bacterial species defective in specific efflux pathways, but without any outer membrane barrier alterations, are considerably more susceptible to hydrophobic antibacterial agents than are species with effective efflux systems but with altered outer barriers or those possessing effective efflux systems with intact outer membrane barriers (Hancock 1997a).

Hydrophilic antimicrobials permeate the outer membrane through the activated porins, which are integral proteins located in the outer membrane (Bayley and Jayasinghe 2004). A strain of *Ps. aeruginosa* that lacks OprD, an outer membrane porin selective for certain carbon sources, demonstrates resistance to imipenem (Ochs et al. 1999, Tamber, Ochs and Hancock 2006), a reserve antibiotic for penicillinase- and β-lactamase- producing bacteria (Rolinson and

Geddes 2007). The absence of a 35 kDa outer membrane protein, suggested as the port of entry for isothiazolone, is responsible for *Ps. aeruginosa* resistance to this agent (Huang and Hancock 1993, Wolter et al. 2004).

1.2.3 Other bacterial pathogens

Chromobacterium violaceum is an organism usually found in mesophilic environments (soils and water with temperature within the range of 20 to 45 ^{o}C, suitable for bacterial species growth), predominantly in the tropics (Ke et al. 2012). This bacterium belongs to the same family Neisseriaceae along with *Neisseria meningitidis* and *Neisseria gonorrhoeae*; the causative organisms for meningitis and gonorrhoeae respectively (Gillis and Logan 2005, Zinger-Yosovich et al. 2006). *C. violaceum* demonstrates the same antimicrobial susceptibility pattern as *Neisseria* species (to polycationic antimicrobial peptides), although the former bacterium is a saprophyte that thrives harmlessly upon their host (Tzeng et al. 2005), and a safer organism to work with in the laboratory. The isolation of a new strain of *N. gonorrhoeae* that is untreatable with almost all available antibiotics (Bolan, Sparling and Wasserheit 2012) provides further rationale justification for the need for continuous development of new potentially effective antimicrobial agents, as well as synergistic combinations.

Pectobacterium caratovoram (formerly *Erwinia carotovora*) a is a Gram-negative, non-sporing facultative anaerobe characterised by the production of large amounts of extracellular pectic enzymes applied in the degradation of plant cell walls, especially in potatoes (Wegener and Jansen 2007). These enzymes include pectinase, cellulase, proteases and xylanases. *P. caratovoram* is the primary cause of tuber decay in store and black leg (stem root) in the field (Czajkowski et al. 2011). *P. caratovoram* is opportunistic with pathogenicity principally in potatoes that have compromised immunity (Pérombelon 2002). This organism takes advantage of the interplay between the host's weakened immunity, exposure to adverse weather conditions and environmental elements, microbial competition and genetic makeup of the cell strain, such as the phage-selected lipopolysaccharide mutants with increased bacterial virulence (Pérombelon 2002, Evans et al. 2010).

Clavibacter michiganensis subsp. *michiganensis* is a Gram-positive, aerobic bacterium with corniform morphology. *Cl. michiganensis* is responsible for

tomato canker and therefore regarded as a quarantine bacterium within the European Union (Smith, Hennessy and Stead 2001, Van der Wolf et al. 2005, Hukkanen et al. 2005). This phytopathogenic bacterium secretes proteases employed in the degradation of the polysaccharides in plant cell wall (Gartemann et al. 2003).

1.3 Types of bacterial resistance

1.3.1 Bacterial resistance to antibiotics due to mutation and active efflux mechanisms

Various mechanisms exist, including adaptive resistance (sometimes known as tolerance) in bacteria, which are triggered by either environmental factors or the presence of antimicrobial compounds through selective pressure (Albrich, Monnet and Harbarth 2004, Alanis 2005). This form of resistance can be both transient and non-inheritable, in which case, the affected bacterial populations revert to their normal level of sensitivity to compounds upon the removal of the triggering agent (Langsrud, Sundheim and Borgmann-Strahsen 2003, Fernandez et al. 2010, Fernández, Breidenstein and Hancock 2011). In addition, bacteria can demonstrate the expression of either acquired or intrinsic resistance to antimicrobial compounds (Normark and Normark 2002). Intrinsic resistance is encoded and expressed as part of the genetic make-up of the wild type bacterial species or strain (Fernández, Breidenstein and Hancock 2011). Intrinsic bacterial resistance is inheritable, stable and independent of exposure to environmental factors. Each bacterium in the population is resistant to the compound without the need for any additional genetic alterations (Normark and Normark 2002). For instance, mycoplasma is resistant to β-lactam antibacterial compounds due to the absence of peptidoglycan and a conventional bacterial cell wall (Lamoth and Greub 2010). On the other hand, acquired bacterial resistance can occur either by mutation (point mutation, deletion, insertion, inversion and duplication) within the genome or through horizontal transfer of resistant genes (Normark and Normark 2002). The hydrolytic action of β-lactamase to β-lactams and the structural modification induced by aminoglycosidase to aminoglycosides are classified as acquired resistance (Normark and Normark 2002). Mutated porins can express reduced up-take of antibacterial compounds (Livermore, Winstanley

and Shannon 2001). Mutations at ribosomal protein level (either methylation or mutation of ribosomal rRNA) and mutation in penicillin-binding proteins can cause the modification and consequent inactivation of the drug's target site, leading to reduced antibiotic binding and the development of resistance (Davies 1996, Connell et al. 2003a, Guardabassi and Courvalin 2006). Bacterial horizontal acquisition of *mecA* genes leads to expression of resistance to methicillin (Charlebois et al. 2004, Diep et al. 2008) and plasmid coded quinolone-resistant (Qnr) proteins confer resistance to fluoroquinolones in Gram-negative species (Jacoby 2005, Robicsek, Jacoby and Hooper 2006). Once the extra chromosomal element (plasmids, transposons or integrons) that harbours the resistant genes have been taken up by the actively dividing cells, the acquired resistance becomes inheritable, stable, and can be expressed regardless of exposure to environmental factors (such as changes in pH and the presence of particulate matter), as is also the case with intrinsic resistance (Lewis 2008). The expression of up-regulated efflux causes increased dislodgement and removal of the antibacterial compounds through actively re-oriented and aligned tripartite channels that consist of a transporter in the inner membrane, a periplasmic linker protein and a channel protein in the outer membrane of Gram-negative species (Normark and Normark 2002).

1.3.2 Biofilm induced resistance to antimicrobial agents

Structurally, biofilms are arranged such that they consist of an external matrix made of polysaccharides, proteins and nucleic acid (Moscoso, García and López 2006, Flemming and Wingender 2010). This matrix acts as both a scaffolding that supports and strengthens the indigenous bacterial cells and as a protective barrier that inhibits the penetration of foreign noxious agents. The consequent diminished penetrating capacity of antibacterial compound reduces antibiotic access to bacterial target sites of action thereby causing either sub-optimal efficacy or therapeutic failure (Fernández, Breidenstein and Hancock 2011, Lees et al. 2009). The sub-lethal doses to which bacterial cells are exposed can promote the development of resistant bacterial strains through selective pressure that stimulates mutation. Within bacterial biofilms, concentration gradients occur in the distribution of metabolic substrates and products (Wimpenny and Kinniment 1995). Cells are differentially exposed to nutrients and oxygen (Singh,

Paul and Jain 2006). Consequently, biofilms consist of cells in different metabolic and growth phases, such as either slow-growing/stationary or actively dividing cells. Even biofilms formed from a homogenous species exhibit heterogeneous growth phases within the population (Molin et al. 2000). Cells that are embedded deep within the matrix can exist in near anaerobic conditions and adopt slow growth modes due to oxygen and nutrient deprivation; an adaptation that is in contrast to the cells nearer the matrix surface. Cells in different zones of a biofilm will therefore express different sensitivity patterns to various antimicrobial compounds, depending on the drug's mechanism of action (Ito et al. 2009).

In the process of aggregating to form a biofilm, bacterial cells express phenotypic traits that differ from their planktonic counterparts (Varnam and Evans 2000, Stoodley et al. 2002, Hall-Stoodley, Costerton and Stoodley 2004). A major problem associated with bacterial biofilms in infectious diseases is the emergence of bacterial resistance to antimicrobial compounds and the immune system. Bacterial biofilms can demonstrate resistance due to reduced permeability of antimicrobial compounds (Mah and O'Toole 2001, Stewart 2002, Burmølle et al. 2010, Burmølle et al. 2010), while nutrient and oxygen distribution gradients that lead to heterogeneous growth phases within the matrix (Wimpenny and Kinniment 1995) can cause adaptive stress responses (Molin et al. 2000). These adaptive responses together with the over expression of efflux pumps, target mutations and existence of persister cells (which take longer to get killed), can lead to the resistance of biofilms to antimicrobial agents (Stewart 2002). This often results in treatment complications as biofilms can be a 1000-fold more resistant to antibiotics than their planktonic counterparts (Hoyle and Costerton 1991). For instance, urinary tract infections associated with the use of catheters caused by multi-resistant *S. epidermidis* and *Ps. aeruginosa* are more difficult to treat due to the formation of biofilms on the surfaces of the prosthetic devices (Taylor, Prosser and Cleeland 1988, Obst et al. 1989).

1.3.3 Effect of biofilms on bacterial susceptibility to β-lactam antibiotics

Biofilms that consist of actively growing homogenous cells are more susceptible to applied antibacterial compounds rather than those with heterogeneous growth phases of constituent bacteria population (Xu, McFeters and Stewart 2000).

Bacteria in the stationary growth zones of a biofilm are strategically positioned to survive antimicrobial challenges (Gilbert and Brown 1995). For β-lactam antibiotics to be effective, it is required that bacterial cells are in an actively dividing mode (Rothstein et al. 2005). Consequently, these compounds are unable to eradicate dormant, nutrient- and oxygen-deprived cells in bacterial biofilms. Cells in the stationary growth phase in *E. coli* biofilms have been shown to be insensitive to ampicillin but sensitive to ofloxacin and kanamycin (Ito et al. 2009), probably because of the slower pace of cell wall synthesis during this phase.

The therapeutic application of ampicillin leads to the emergence of a spared population that acts as an inoculum after the cessation of antibiotic treatment, causing a relapse of infection and re-occurrence of clinical symptoms (Gilbert et al. 2002). In addition to the biofilm matrix forming a penetration barrier, should the native cells be β-lactamase secreting, the enzyme will accumulate within the biofilm causing a large fraction of administered β-lactam antibiotics to be broken down, even before being able to access their primary target site (Anderl, Franklin and Stewart 2000, Smith 2005). Hence, biofilms of *K. pneumonia* from β-lactamase negative strains are more sensitive to ampicillin than biofilms from β-lactamase-producing strains (Anderl, Franklin and Stewart 2000) due to the absence of drug-inactivation in the former instance.

1.4 Influence of biofilms upon bacterial susceptibility to other antimicrobial compounds

Metabolically active cells in *Ps. aeruginosa* biofilms are able to develop insensitivity to the polymyxins through activation of a lipopolysaccharide-modification operon and MexAB-OprM efflux pump (Pamp et al. 2008). Polymyxins are normally bactericidal to cells displaying either dormant or slow growth phase in the biofilms. In contrast, ciprofloxacin and tetracycline exercise bactericidal activity only against the metabolically active cells in biofilms of the same bacterial species. Consequently, it has been suggested that either two or more antibacterial compounds should be used as a combination therapy, which includes a polymyxin, in cystic fibrosis patients with pulmonary infections that consist of biofilms from multi-resistant *Ps. aeruginosa* (Chernish and Aaron 2003). Cells exposed to lower oxygen concentrations in *Ps. aeruginosa* and *E.*

coli biofilms demonstrate decreased sensitivity to vancomycin, tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol and tetracycline (Ito et al. 2009, Evans et al. 1991). The distribution of oxygen along a gradient also modulates the antibacterial action of the aminoglycosides (Stewart 2002). Bacterial cells exposed to anaerobic conditions in biofilms are differentially protected from the action of various antimicrobial agents even when capable of fermentative growth (Davey and O'toole 2000, Shirtliff, Mader and Camper 2002).

1.5 Mechanism of action, emergence and dissemination of bacterial resistance to antimicrobial compounds

Antibiotics are chemical entities (penicillin, streptomycin) either produced by or derived from fungi, bacteria and other micro-organisms and plants, possessing the capacity to either kill or inhibit the growth of other pathogenic organisms (Singh and Pelaez 2008, James, Vignesh and Muthukumar 2012). Currently, synthetic and semi-synthetic compounds with antimicrobial actions are also commonly referred to as antibiotics (Giuliani, Pirri and Nicoletto 2007, Harvey 2008).

1.5.1 Mechanism of action of antibacterial compounds

The primary mechanism of action for the major classes of antibacterial compounds in clinical use is summarised in the table 1.1. Different classes of antibiotics can interfere with essential cell maintenance processes including the inhibition of cell wall synthesis by the β-lactam antibiotics (Paradis-Bleau et al. 2008, Silver 2003, Hao et al. 2012), blockage of protein synthesis by the tetracyclines (Hilliard et al. 1999, Connell et al. 2003a, Zapun, Contreras-Martel and Vernet 2008) and the inhibition of the activities of vital metabolic pathways such as folic acid synthesis reported with the sulphonamides (Bermingham and Derrick 2002). The interference with DNA replication by the fluoroquinolones has been extensively studied (Drlica and Malik 2003, Owens and Ambrose 2005). Meanwhile, the disruption of cell membrane integrity by colistin and polymyxin B (Fernandez et al. 2010, Fernandez et al. 2010, Zavascki et al. 2007) is another recognised mechanism of antibacterial action.

Class of antibacterial compound	Mechanism of action
Fluoroquinolone (ciprofloxacin)	Inhibition of DNA synthesis
Aminoglycoside (gentamicin), tetracycline, ketolide, macrolide, chloramphenicol, lincosamide	Inhibition of protein synthesis
Rifampin	Inhibition of RNA synthesis
Trimethoprim, sulphonamide	Inhibition of folic acid synthesis
Penicillin, daptomycin, cephalosporin, carbapenem, vancomycin	Inhibition of cell wall synthesis
Polymyxins	Disruption of cell membrane integrity

Table 1.1 Major antibacterial compounds in clinical use and their primary mechanism of action (Cottarel and Wierzbowski 2007).

1.5.2 Bacterial resistance to antibacterial compounds

The incidence of pathogenic bacterial resistance to antibacterial compounds is increasing globally (Silbergeld, Graham and Price 2008). The indiscriminate and wide-spread use of antimicrobial compounds (β-lactams, aminoglycosides, tetracyclines, fluoroquinolones, etc.) in the agro-allied industry and inappropriate dosing regiments in humans has led to the emergence and dissemination of bacterial strains resistant to a wide array of antimicrobial compounds (Levy and Marshall 2004). Major classes of antibacterial compounds to which bacteria have developed resistance include the β-lactams, carbapenems, aminoglycosides, tetracyclines and fluoroquinolones.

1.5.2.1 β-lactams

The β-lactams (penicillins, carbapenems, cephalosporins, and monobactams) are bactericidal compounds that prevent the formation of bacterial cell walls through

inhibition of peptidoglycan bio-synthesis in actively dividing cells. These compounds impede the formation of cross-linkages upon the linear *N*-acetyl glucosamine (NAG) and *N*-acetylmuramic acid (NAMA) strands (transglycosylation) during peptidoglycan biosynthesis; a critical step essential for bacterial survival (Silver 2003). Inappropriate use of β-lactam antibiotics led to the emergence and spread of penicillin-resistant and later on, methicillin-resistant *S. aureus* (MRSA) (Levy and Marshall 2004, Goldmann et al. 1996, O'Brien 2002). Data collected between 1992 and 2004 show that more than 50 % of pathogens identified in intensive care units within this same period were MRSA (Zhang, Rozek and Hancock 2001b, Cardo et al. 2004). The emergence of bacterial strains that are resistant to β-lactam antibiotics can occur through induced synthesis of β-lactamase enzymes (Bonomo and Rice 1999). This enzyme has the capacity to break the β-lactam ring thereby rendering these antibiotics ineffective (Fernández, Breidenstein and Hancock 2011). In addition, alteration in penicillin-binding proteins (PBP) in the cell wall of MRSA that was previously mentioned is an alternative mechanism of resistance to this class of antibiotics (Sieradzki, Pinho and Tomasz 1999). Penicillin binding proteins are the binding sites for β-lactam antibacterial compounds. Bacterial strains with altered binding proteins have been proposed to exhibit resistance to β-lactam antibiotics due to diminished binding capacity for β-lactam antibiotics (Zapun, Contreras-Martel and Vernet 2008).

Carbapenems (imipenem, meropenem, ertapenem, doripenem, etc.) belong to a sub-class of broad spectrum β-lactam antibacterial compounds with a peculiar backbone structure that renders them highly resistant to β-lactamase enzymes (Fonseca 2011). Carbapenems are the choice antibacterial compounds in instances of bacterial infections caused by resistant *E. coli* and *Klebsiella pneumoniae* (Johnson 2008). Bacterial resistance to carbapenems due to acquired carbapenemases emerged in 2000 and has been disseminated globally since then (Corbella et al. 2000, Rolain, Parola and Cornaglia 2010). There are different carbapenemases including enzymes from class A, B (metallo-beta lactamases (MBL)) and D (serine carbapenemases) (Miriagou et al. 2010). The New Delhi metallo-beta-lactamase 1 (NDM-I) is a novel type of MBL (Struelens et al. 2010) disseminated by plasmids encoding carbapenem-resistant metallo-beta-lactamase (PCM) and bacterial strains carrying this genetic element were previously named NDM-1 (Rai et al. 2011). This extra genetic segment encodes

for bacterial resistance to a broad range of β-lactam antibiotics (Moriarty, Elborn and Tunney 2005). The antibiotic resistance activity mediated by this plasmid-borne gene upon diverse and unrelated species compromises their susceptibility to carbapenems, thereby disqualifying this antibiotic as the reserved antibiotic of choice in instances of bacterial resistance to β-lactams (Johnson 2008). The plasmid encoding carbapenem-resistant metallo-beta-lactamase gene was first recovered from a *Klebsiella pneumoniae* strain isolated from a Swedish patient of Indian origin in 2009 (Rolain, Parola and Cornaglia 2010). The following year, the strain had become prevalent in the United States, Asia, United Kingdom, Israel and southern Europe (Wilson and Chen 2012). The rapid global dissemination of this plasmid encoding carbapenem-resistant metallo-beta-lactamase gene in enterobacteriaceae, via horizontal gene transfer, exemplifies the speed with which resistant bacterial strains can emerge and be disseminated due to globalisation and wide-spread travel (Jacqueline et al. 2005, Jacqueline et al. 2005).

1.5.2.2 Aminoglycosides

Aminoglycosides (such as gentamicin, amikacin, kanamycin and neomycin) consist of hydrophilic amino sugar residues bound to a central six-member aminocyclitol ring by glycosidic bonds that function as polycations (Vicens and Westhof 2002). Their polycationic nature facilitates binding to the polyanionic 16S rRNA of the bacterial ribosome (Knowles et al. 2002), thereby blocking bacterial synthesis of proteins. Plasmids such as pXZL34 can encode diminished membrane permeability and up-regulation of membrane efflux pumps (OprM) for the extrusion of aminoglycosides, thereby expressing resistance to these compounds (Westbrock-Wadman et al. 1999). This mechanism of resistance also prevails for β-lactams and tetracyclines (Walsh 2000, Li and Nikaido 2004). Additionally, in enterococci, the aminoglycoside-inactivating enzyme aminoglycoside 2' phosphodiesterase is another mechanism for the expression of bacterial resistance to aminoglycoside antibiotics (Takahashi et al. 2002).

1.5.2.3 Tetracyclines

Tetracyclines (such as tetracycline, doxycycline and minocycline) are broad spectrum antibacterial compounds that consist of a tetracene nucleus with four

fused rings (Lorian 2005). They demonstrate activity against Gram-positive and Gram–negative bacteria, chlamydia, mycoplasmas, rickettsia and some protozoan parasites (Chopra and Roberts 2001). Tetracyclines inhibit protein synthesis through disruption of codon-anticodon interaction between tRNA and mRNA thereby preventing the binding of amino acyl tRNA to the ribosomal acceptor A site. Bacterial mechanisms of resistance against the tetracyclines include the use of efflux-based systems, enzymatic inactivation and ribosomal protection strategies (Chopra and Roberts 2001, Poole 2000, Trieber and Taylor 2002, Trieber and Taylor 2002). However, the up-regulation of outer as well as the cytoplasmic membrane efflux pumps is the primary mechanism by which bacteria express resistance to tetracycline antibiotics (Kumar and Schweizer 2005). Resistance mediated through the use of efflux proteins has been extensively studied leading to the identification of over 300 genes that belong to the major facilitator super-family (MFS) (De Rossi et al. 2002). Bacterial resistance to tetracycline through ribosomal protection was demonstrated by the isolation of Tet(O) and Tet(M) ribosomal protection proteins (RPPs) from *Campylobacter jejuni* and *Streptococcus* species respectively (Aminov et al. 2004, Alfredson and Korolik 2007). In the presence of both proteins, aminoacyl tRNA binding to the A site, normally inhibited by tetracycline, is protected and the drug is released and removed thereby freeing the ribosomes from the antibiotic's inhibitory effect. This action enables aminoacyl-tRNA to continue to bind to the A site for on-going protein synthesis (Burdett 1996, Connell et al. 2003b). Other mechanisms of resistance are the expression of enzymatic degradation of tetracyclines in *Bacteriodes* species and rRNA mutations in *Propionibacterium acnes* and *Helicobacter pylori* (Trieber and Taylor 2002, Ross et al. 1998, Gerrits et al. 2002).

1.5.2.4 Fluoroquinolones

The fluoroquinolones include nalidixic acid, ciprofloxacin and sparfloxacin and are synthetic bactericidal compounds that inhibit either or both topoisomerases II (DNA gyrase) and IV (Hooper 2000). DNA gyrase catalyses the introduction of negative superhelical twists in circular DNA of actively growing cells (Drlica and Malik 2003, Drlica and Hooper 2003). This is an important step for bacterial replication and survival as separation of DNA strands must precede DNA

transcription and replication, which results in excessive positive super-coiling of the DNA in front at the point of separation. The introduction of negative super-coiling by DNA gyrase prevents this excessive positive twisting (Lorian 2005). The wide-spread misuse of fluoroquinolones has also been accompanied by the emergence and dissemination of strains of bacterial species resistant to this class of antibiotic (Levy 2002), especially because of their direct inhibitory action against DNA synthesis (Hooper 2001). Fluoroquinolones are currently ineffective in most cases of shigellosis, cholera and camphylobacteriosis due to bacterial resistance (Sack and World Health Organization 2001). Quinolone resistant *S. aureus* (QRSA) is associated with chromosomal mutations either in genes encoding for subunits of the drug's target enzymes (DNA gyrase and topoisomerase IV), diffusion channels in the outer membrane, or multi-drug resistant efflux systems (Hilliard et al. 1999).

1.5.2.5 Newer antibacterial compounds in clinical use

Newer clinically effective antibacterial compounds should target either novel or multiple sites as their mechanisms of action in order to address the challenges associated with the rapidity with which resistant strains emerge to newly introduced antibiotics (Chopra et al. 1997). This will increase the clinical life-span and utility of such newly developed antibacterial agents (Bais et al. 2006), since bacterial species will require longer time to develop resistance mechanisms against either novel or multiple patterns of assault. Some recently developed antimicrobial agents in clinical use are as follow.

1.5.2.5.1 Telavancin, oritavancin and vancomycin

Telavancin is available for use clinically as an alternative glycopeptide for vancomycin-resistant bacterial species (Aksoy and Unal 2008). This new antibacterial agent demonstrates a pronounced depolarisation of membrane potential, disrupts cell membrane integrity and inhibits the biosynthesis of lipid II, a precursor required for the production of peptidoglycan during bacterial cell wall formation (Rashid, Weintraub and Nord 2011, Moellering Jr 2011). Likewise, oritavancin (a lipoglycopeptide) is also effective against vancomycin-resistant organisms (Guskey and Tsuji 2010). Oritavancin's mechanism of action is by the sequestration of lipid II, through which the peptidoglycan repeat unit is

covalently linked to a C_{55}-lipid transporter (Kim et al. 2008). This sequestration leads to inhibition of cell wall synthesis (Kim et al. 2008). In addition, oritavancin has the capacity to collapse membrane potential, increase membrane permeability and eventually induces loss of membrane integrity (Domenech et al. 2009). This agent has also been shown to inhibit RNA synthesis (Zhanel et al. 2010) and is active against slow growing cells as well as staphylococcal biofilms challenged *in vitro* (Belley et al. 2009). Oritavancin demonstrates a rapid bactericidal activity greater than that of vancomycin (Kim et al. 2008). A notable bactericidal action can be achieved after 15 minutes in the former agent; unlike the latter antibiotic that requires about 24 hours with activity restricted to actively dividing cells (McKay et al. 2009a).

Vancomycin also possesses a narrow therapeutic window that discourages routine use especially because inaccurate dosing often results in sub-optimal concentrations of the drug and a loss of clinical effectiveness (Hall et al. 2009). On the other hand, patients receiving more than 50 mg/kg body weight vancomycin have been increasingly diagnosed with severe nephrotoxicity (Lodise et al. 2008, Hazlewood et al. 2010). Connected to this concern, is the fact that treatment of MRSA infections with sub-optimal vancomycin concentrations leads to the emergence of *S. aureus* strains with intermediate sensitivity to vancomycin, typically characterised by their thickened cell walls (Hiramatsu 2001). The presence of these strains is associated with the failure of vancomycin therapy in MRSA infections, as much of the administered dose are entrapped within the enlarged cell wall and consequently denied access to their molecular target sites (Hiramatsu et al. 1997, Soriano et al. 2008).

1.5.2.5.2 Linezolid

Linezolid is an oxazolidinone bacteriostatic antibacterial compound developed for Gram-positive organisms that inhibits protein synthesis in susceptible pathogens (Stefani et al. 2010). Linezolid is also effective *in vitro* against both methicillin-resistant *S. aureus* and vancomycin-resistant enterococci. Linezolid retains FDA approval for specific infections caused by vancomycin-resistant *E. faecium*, *S. aureus*, *S. pneumoniae*, *S. pyogenes* and *S. agalactiae* (Stefani et al. 2010, McNeil et al. 2000). Such infections include nosocomial and community-acquired pneumonia, uncomplicated to complicated skin and soft tissue infections as well

as diabetic foot infections devoid of supine-state induced ulcers and oesteomyelitis (Falagas, Siempos and Vardakas 2008). However, despite the limitations of vancomycin therapy previously mentioned, linezolid is not a significantly more effective than vancomycin for the treatment of Gram-positive infections and both compounds have comparable resistance profiles (Falagas, Siempos and Vardakas 2008). A combination of linezolid with sub-inhibitory doses of imipenem has been applied for the improved efficacy against methicillin-resistant *S. aureus* (Jacqueline et al. 2005).

1.5.2.5.3 Daptomycin

Daptomycin is a cyclic lipopeptide with rapid bactericidal activity against both methicillin- and vancomycin-sensitive as well as resistant staphylococci and enterococci (Castanheira, Jones and Sader 2008). Daptomycin's rapid bactericidal activity against Gram-positive bacteria is associated with the lipopeptide's capacity to competitively bind staphylococcal membrane-bound calcium ions and be inserted into the cytoplasmic membrane after structural modifications thereby leading to membrane-pore formation (Jung et al. 2004a). In addition, daptomycin also kills bacteria via depolarisation of cytoplasmic membrane potential, inhibition of amino acid transport and blockade of peptidoglycan cross-linkages in cell wall formation of actively dividing cells (Allen, Alborn Jr and Hobbs Jr 1991).

Daptomycin is approved for skin and soft tissue infections, bacteraemia and right-sided endocarditis caused by methicillin-resistant *S. aureus* and other sensitive Gram-positive pathogens except vancomycin-resistant pathogens (Arbeit et al. 2004, Fowler Jr et al. 2006). Vancomycin-intermediate strains of *S. aureus* demonstrate diminished sensitivity to daptomycin because of the entrapment of daptomycin within the thickened cell wall and the consequent reduced availability of the antibiotic at the target sites (Wootton, MacGowan and Walsh 2006). Also, daptomycin is not recommended for pulmonary infections (in community-acquired pneumonia) because of inactivation by pulmonary surfactant (Silverman et al. 2005). In this instance, ceftriazone is a preferred option (Pertel et al. 2008).

1.5.2.5.4 Telithromycin

The ketolide, telithromycin is a semi-synthetic derivative of macrolide antibiotics with amplified binding capacity to bacterial ribosomes (Lonks and Goldmann 2005). Ketolides bind simultaneously to domains II and V of the 23S rRNA in bacterial ribosomes, unlike macrolides that are only bound to domain V (Jenkins, Brown and Farrell 2008). Hence, telithromycin demonstrates *in vitro* activity against macrolide-resistant and multi-drug resistant isolates of *S. pneumoniae* and other common respiratory tract pathogens (Lonks and Goldmann 2005, Jenkins, Brown and Farrell 2008). Other relatively new antibacterial compounds include tigecycline, which belongs to same class as tetracycline (Peterson et al. 2006); moxifloxacin and gemifloxacin are ciprofloxacin (Pletz et al. 2004); whilst ertapenem is a once daily parenteral β-lactam carbapenem (Vetter et al. 2002, Shah and Isaacs 2003) and cefditoren is a streptogramin (Spangler, Jacobs and Appelbaum 1996).

1.5.2.5.5 Polymyxins

Polymyxins (figure 1.3) are effective against *Acinetobacter* species, *Pseudomonas aeruginosa*, *Klebsiella* species and *Enterobacter* species (Bonomo and Szabo 2006, Falagas, Kasiakou and Saravolatz 2005).

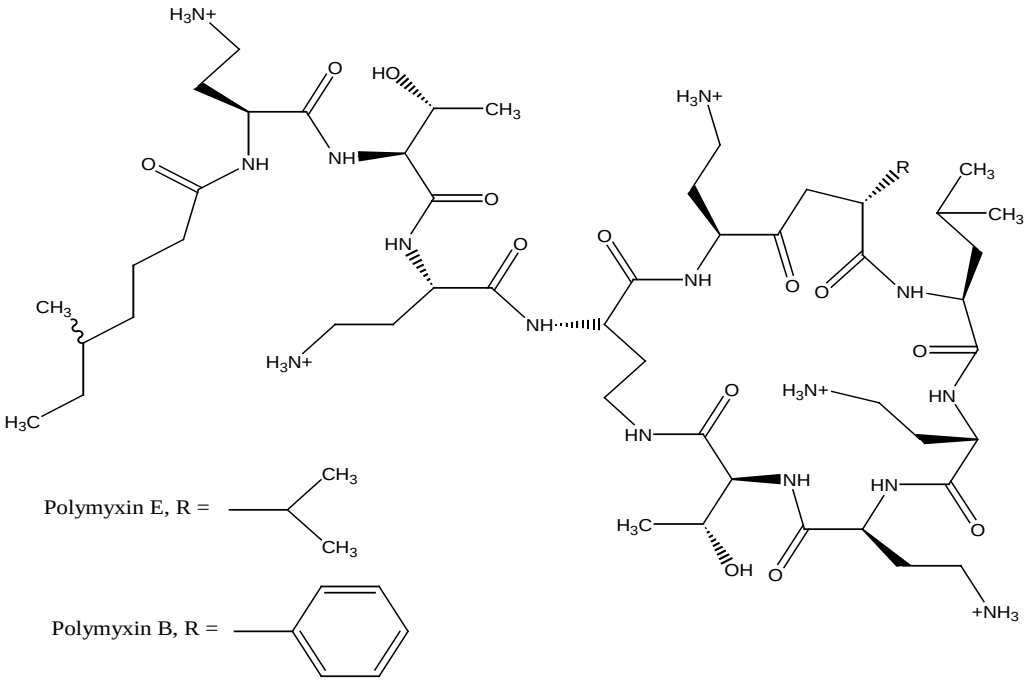


Figure 1.3: Chemical structures of polymyxin B and colistin (polymyxin E), showing five positive charges. Adapted from Tsubery et al. 2000.

These peptide antibiotics are clinically ineffective against most Gram-positive bacterial species at tolerable therapeutic doses, most probably due to the absence of their primary target - the outer membrane lipopolysaccharide (LPS) structure (Zhang, Rozek and Hancock 2001a, Hancock and Rozek 2002a, Vaara et al. 2008, Martti 2010). The polymyxins primarily target the LPS of the outer membrane structure of Gram-negative bacterial species (Hancock and Sahl 2006). These compounds competitively displace Mg^{2+} and Ca^{2+} from the anionic LPS surface (Thompkins 2010), leading to some degree of disturbances and formation of blebs (multiple protrusions) on the membrane bilayer surface (Hancock 1997c). Permeability of the cell envelope is increased, permitting the self-promoted uptake of these polypeptide antibiotics (Hancock 1997b), leakage of intracellular potassium (O'Driscoll 2011), loss of osmotic integrity and cell death (Falagas, Kasiakou and Saravolatz 2005, Hancock and Sahl 2006, O'Driscoll 2011, Wu and Hancock 1999).

1.5.2.5.5.1 Adverse effects of polymyxins

Polymyxin B and colistin interfere with the membrane integrity of the linings of the nephron, thereby causing nephrotoxicity, an adverse side effect of polymyxin B and colistin treatment (Bonner 2011). Polymyxins can bind to megalin in the apical membrane of the nephron's proximal tubules, as well as to isolated brush border membranes, with higher affinity than other antibiotics such as gentamicin (Vaara et al. 2008). Furthermore, extensive re-absorption due to continually administered high (intravenous) doses can lead to the accumulation of polymyxins in the tubular cells of the nephron, thereby causing dose-related nephrotoxicity (Vaara and Vaara 2010, Zavascki et al. 2008). There is therefore a strong rationale to investigate other measures whereby the safer clinical use of the polymyxins can be achieved, such as the application of these compounds at lower concentrations through synergistic combinations with other established antibiotics.

1.5.3 The re-assessment and renewed clinical application of polymyxin B and colistin

The clinical use of polymyxins (figure 1.3) as antimicrobial therapeutic agents was abandoned in the 1970s because of concerns relating to their dose-

dependent adverse effects of nephrotoxicity and neurotoxicity (Falagas, Kasiakou and Saravolatz 2005). However, due to the dearth in discovery and development of new effective as well as clinically useful antimicrobial agents, these concerns were re-evaluated. It was noted that these adverse events and the issues surrounding such concerns were valid but seemingly over-stated. Polymyxins were therefore re-introduced in 2002 as an antibiotic, reserved for conditions involving multi-resistant Gram-negative bacterial species (Hirsch and Tam 2010). Currently most multi-resistant Gram-negative bacterial strains are commonly susceptible to polymyxin B and colistin (polymyxin E) (Zavascki et al. 2007, Falagas, Kasiakou and Saravolatz 2005). However, if both antibiotics are not used appropriately, such susceptible bacterial species could mutate into new strains that are resistant to these compounds due to selective pressure. Clinicians must therefore be urged to discourage the use of polymyxin B and colistin as monotherapeutic agents, especially when applied as an antibiotic of last resort (Vaara and Vaara 2010, Hirsch and Tam 2010).

1.5.3.1 Development of modified polymyxins

In view of the toxicity concerns previously raised about colistin and polymyxin B, attempts are being made to develop newer polymyxins with improved safety profile and bactericidal action against multi-resistant Gram-negative organisms (Martti 2010). Newer polymyxins are being developed from polypeptide derivatives that carry only three cationic charges instead of the conventional five (figure 1.3) (Tsubery et al. 2000, Martti 2010). As a result of this structure-activity manipulation, some recently developed CAPs such as NAB739 and NAB7061, demonstrate a reduction in their capacity to get bound to the acidic phospholipid sites of the brush-border membrane in the proximal tubular cells of the kidneys (Vaara and Vaara 2010), leading to reduced likelihood for the occurrence of their dose-related nephrotoxicity adverse event. Some of these new compounds such as NAB739 have direct antibacterial activity whilst others such as NAB7061 demonstrate the capacity to sensitize bacteria to the effects of other antibacterial compounds (Vaara et al. 2012).

1.6 Flavonoids as a potential source of antimicrobial compounds

Flavonoids are polyphenolic organic compounds each possessing about 15 carbon atoms and usually 2 benzene rings joined by a linear 3 carbon atoms (figures 1.4 and 1.5).

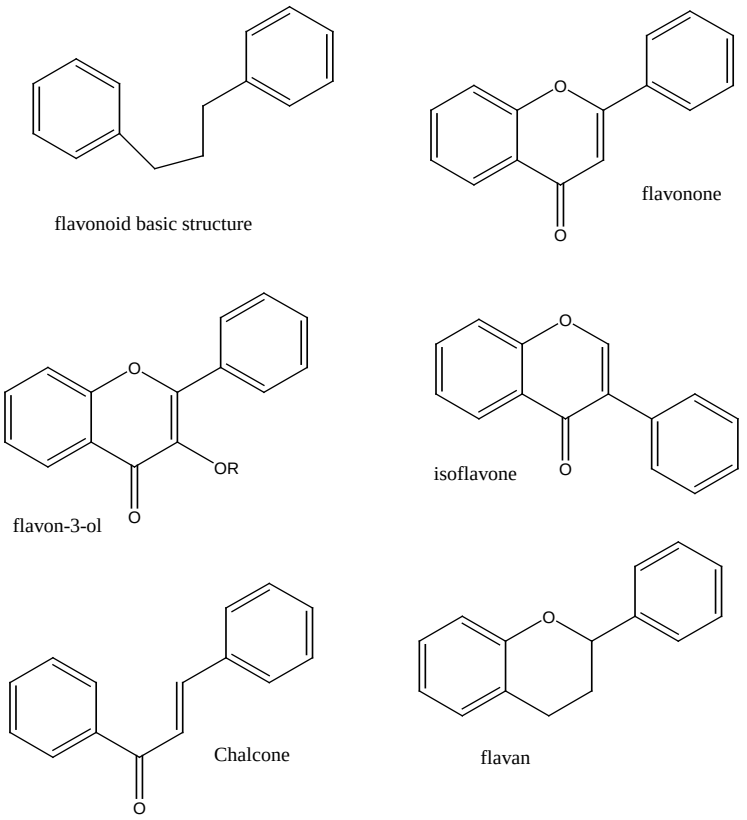


Figure 1.4: Basic structures of flavonoids; adapted from Cushnie and Lamb 2011.

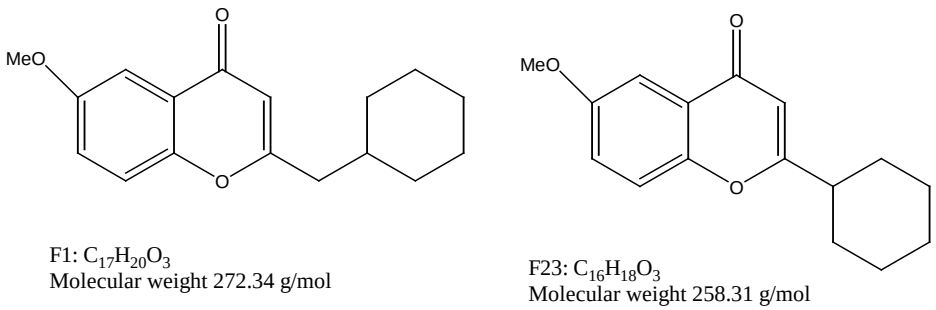


Figure 1.5: Diagrams of the chemical structures of [2-(cyclohexymethyl)-6-methoxy-4*H*-chromen-one] (F1) and 2-cyclohexyl-6-methoxy-4*H*-chromen-4-one (F23); adapted from Abdel Ghani et al. 2008 and Ghani et al. 2012.

These polyphenolic compounds are characteristically present in higher plants, especially in angiosperms where they function as flowering pigments. Flavonoids

have demonstrated a wide range of activities including antileishmanial (Wong et al. 2007), antitrypanosomial (VA 2010), cancer chemo-preventive (Heo, Sohn and Au 2001) and antibacterial (Cushnie and Lamb 2005a) potentials. Flavonoids from *Glycyrrhiza glabra* extract demonstrate antibacterial activity against methicillin-resistant *S. aureus* (Fukai et al. 2002b) and *Helicobacter pylori* (Fukai et al. 2002a). *In vitro* susceptibility studies of the antibacterial activities of flavonoids and their semi-synthetic analogues suggest that they may have potential as lead compounds for the development of novel antimicrobial agents (Fukai et al. 2002b). The flavonol galangin demonstrates bactericidal action against species of *S. aureus* through cell aggregation (Cushnie et al. 2007) and also induces cytoplasmic damage that leads to the leakage of intracellular potassium (Cushnie and Lamb 2005b).

The 2 semi-synthetic chalcone-derivatives (F1 and F23) (figure 1.5), are flavonoid compounds produced by the micro-wave assisted closure of the heterocyclic ring (Abdel Ghani et al. 2008) and both were shown to demonstrate weak antibacterial as well as antifungal activities (Ghani et al. 2012). Beside investigations covering their potentials for direct antimicrobial activity, flavonoids of natural origin, their synthetic and semi-synthetic counterparts have been assessed for synergistic actions with established antibiotic as well as the suppression of bacterial virulence (Cushnie and Lamb 2011). (-)-Epicatechin gallate reduces the MICs of the β-lactam antibiotic, oxacillin, against species of MRSA by as much as 512-fold (Hamilton-Miller and Shah 2000); but like other flavon-3-ols, demonstrates susceptibility to bacterial esterases, thereby diminishing its chances as a candidate for further development for potential useful clinical applications (Hamilton-Miller and Shah 2000, Stapleton et al. 2004, Stapleton et al. 2004). However, (-)-epicatechin gallate displays broad-spectrum inhibitory activity against the growth of both Gram-positive as well as Gram-negative bacterial species and has therefore been considered for possible applications in the control of common oral infections such as dental carries and periodontal disease (Taylor, Hamilton-Miller and Stapleton 2005, Shah, Stapleton and Taylor 2008). Interestingly, a semi-synthetic analogue of (-)-epicatechin gallate with improved stability against esterification as well as hydrolysis due to amide substitution (for an ester linkage) demonstrates a similar degree of previously recorded synergistic action with oxacillin (Anderson et al. 2005) and

will therefore be more likely considered for further development for potential clinical applications.

1.7 Combinational antimicrobial therapy

Combination antimicrobial therapy involves the simultaneous application of compounds with different molecular mechanisms of action and target sites within the bacterial pathogen (Silver and Bostian 1993). Hence, an effective combination therapy leads to either additive or synergistic activity (Lorian 2005). Such effective combinations of compounds also leads to a reduction in the required dose for each of the 'synergistic' or 'additive' compounds, which in turn leads to a reduction in the potential for the occurrence of dose-related adverse events by each compound (Rybak and McGrath 1996). A reduction in the potential for toxicity would enhance the chances for the clinical development of such new compounds combination, which have already been found to be effective (Owens and Ambrose 2005). This has been demonstrated for the combination of either streptomycin or dihidrostreptomycin with rifampicin, which is clinically effective for the treatment of brucellosis especially in the tropics (Asif 2012, Dooley et al. 2012). In addition, with the application of combinational antibacterial therapy, it becomes more difficult for the treated pathogen to evolve into mutant strains that are resistant to multiple compounds (Lomovskaya et al. 2001a, Xilin and Drlica 2002). The simultaneous antibacterial activity at diverse molecular sites hinders the organism from recuperating and prolongs the time required for bacterial species to develop resistant strains against the antibacterial compounds used, especially through mutation (Levin and Harris 1975). It is recommended that certain antibiotics such as isoniazid should not be administered alone for the treatment of bacterial infections because bacterial resistance against such compounds can emerge very rapidly (Zhao and Drlica 2001). Also, infectious diseases caused by hetero-pathogens are better treated with combinational antibacterial therapy considering that different molecular targets are affected simultaneously (Levin and Harris 1975).

Finally, in emergency situations that do not permit time for the conduct of bacterial susceptibility testing, the combination of antibacterial compounds is often applied to achieve an effective broad-spectrum and empirical management of life-threatening infectious disease conditions. Overall, an effective antibiotic

combination therapy may ultimately lead to a reduction in the time required for a complete therapeutic regimen, an improvement in patient compliance and a more effective therapeutic outcome (Rello et al. 1997, Niederman 2003, Dellit et al. 2007).

1.7.1 Combination of antibacterial compounds in clinical use

Some antibiotic combination therapies have achieved therapeutic success already, such as the combined application of either streptomycin or dihidrostreptomycin with rifampicin, described above (Dooley et al. 2012). In addition, co-amoxiclav consists of a novel combination of clavulanic acid with amoxicillin, an amino penicillin with improved oral bioavailability and half-life (Cottarel and Wierzbowski 2007, Gisbert et al. 2006). Herein, the β-lactamase enzyme in resistant Gram-positive bacteria is irreversibly bound to clavulanic acid thereby preventing the enzyme from degrading the β-lactam ring and enhancing the capacity of amoxicillin to inhibit cell wall formation (Craig and Ebert 1992, Navarro 2005). Following a similar mechanism of action as the one detailed above, ampicillin sodium has been successfully combined with salbutam sodium, another β-lactamase inhibitor, to achieve improved activity against β-lactamse secreting Gram-positive species, such as in the treatment of *Stenotrophomonas maltophilia* and *Acinetobacter baumannii* (Miller, Ratnam and Payne 2001).

Synercid is a commercial product containing types A and B streptogramin bacteriostatic compounds, dalfopristin and quinupristin as a 70:30 bactericidal combination respectively (Manfredi and Sabbatani 2010). Although both compounds are individually bacteriostatic in action (Vouillamoz et al. 2000), the combined product synercid is predominantly bactericidal against Gram-positive multi-resistant organisms including vancomycin-resistant and multi-resistant *Enterococcus faecium*, *S. epidermidis* and *S. aureus* (McNeil et al. 2000, Karageorgopoulos and Falagas 2009). The FDA approved the combined streptogramins for complicated skin and soft tissue infections caused by methicillin-susceptible *S. aureus*, *Streptococcus pyogenes* as well as infections caused by vancomycin-resistant *E. faecium*. In *S. aureus*, resistance to quinupristin and dalfopristin combinations seems to be due to a mutation in the L22 ribosomal protein (Malbruny et al. 2002).

The combination of trimethoprim and sulphamethoxazole provides a synergistic bactericidal product that inhibits the folic acid metabolism in two independent and specific steps of the folic acid biosynthesis pathway (Kavatha et al. 2003). Trimethoprim competitively inhibits dihydrofolate reductase and blocks the conversion of dihydrofolic acid to tetrahydrofolic acid (Kalan and Wright 2011). The accumulation of dihydrofolate reductase leads to the inhibition of the synthesis of polyglutamylfolates by folylpoly- γ-glutamate synthase (Kesavan et al. 2001, Liani et al. 2002). Simultaneously, sulphamethoxazole can stand in as a surrogate substrate for dihydropteroate synthase, resulting in a compound that cannot be further metabolised to dihydrofolate (Kalan and Wright 2011). The combination of trimethoprim and sulphamethoxazole has been a viable option for the treatment of MRSA (Grim et al. 2005). However, allergy to sulphonamides could sometimes result in toxic epidermal necrolysis and has been noted as a major concern (Glasser and Burroughs 2003, Lee et al. 2004).

In the management and treatment of tuberculosis, the causative organism, *Mycobacterium tuberculosis*, is treated with a combination of isoniazid, rifampicin and pyrazinamide or ethambutol simultaneously for approximately 6 months (Ginsberg and Spigelman 2007). Rifampicin inhibits DNA-dependent RNA polymerase, preventing protein synthesis whilst the other 3 antibacterial compounds disrupt *M. tuberculosis* membrane and wall through direct as well as indirect mechanisms (Cottarel and Wierzbowski 2007). There are reports of the emergence of *M. tuberculosis* strains resistant to this standard combinational therapy which has heightened the concerns for the development and introduction of new compounds into the regime (Pletz et al. 2004). Sparfloxacin and moxifloxacin have been considered as antibacterial compounds in the antibiotic combinational treatment and management of tuberculosis, especially in HIV/AIDS patients because of their long half-lives (Pletz et al. 2004, Grosset 1992). These examples demonstrate that the combination of different antibacterial compounds could be effective *in vivo*.

1.7.2 Combination of antibiotics with agents that are devoid of antibacterial actions themselves

The adjuvant (clavulanic acid) is devoid of significant antibacterial activity but prevents the degradation and modification of the antibacterial compound

(amoxicillin) (Cottarel and Wierzbowski 2007). The combination of the latter antibiotic with the former agent therefore yields a synergistic antibacterial action (Vree, Dammers and Exler 2003). In another instance, an adjuvant may enhance the accumulation and retention of the antibacterial compound by inhibiting the efflux of the latter. For instance, MPex Pharmaceuticals have developed various combinational antibacterial compounds, one of which involves the combined application of a non-antimicrobial compound, MC-207,110 with known antibiotics thereby producing new effective treatment combinations. MC-207,110 is a repressor for bacterial outer membrane efflux pumps, such as MexAB-OprM, MexCD-OprJ and MexEF-OprN in *Ps. aeruginosa* and their homologue AcrAB-TolC in *E. coli* (Lomovskaya et al. 2001a, Lomovskaya and Bostian 2006, Poole and Lomovskaya 2006). In the combination, MC-207,110 potentiates the antibacterial activity of laevofloxacin against *Ps. aeruginosa*, by preventing the efflux of laevofloxacin from the periplasmic space (Kriengkauykiat et al. 2005). This combination reduces the modal MIC of laevofloxacin against *Ps. aeruginosa* strains (with over expressed efflux pumps and mutants with *gyrA* and *parC* genes, that confer resistance to laevofloxacin) by 32- to 64-fold (Lomovskaya et al. 2001a). In addition, the intrinsic resistance of *Ps. aeruginosa* towards laevofloxacin is decreased 8-fold and the frequency of emergence of new resistant strains to fluoroquinolones is repressed (Lomovskaya et al. 2001a, Lomovskaya and Bostian 2006).

1.7.3 Combination of antibiotics with cationic peptides

Polymyxins are amphipatic compounds with hydrophobic regions at their amino terminus (Giacometti et al. 2000). The polymyxin-like peptide, ranalexin, enhances the entry of several hydrophobic substrates into both Gram–positive and Gram–negative bacterial cells (Giacometti et al. 2000). The application of a hydrophilic cationic antimicrobial peptide in combination with a hydrophilic fluorescent compound increased the outer membrane porin diameter and influx of the hydrophobic fluorescent probe (Delcour 2009). Either ranalexin or polymyxins can synergistically promote enhanced antimicrobial actions of lipophilic and amphiphilic agents like rifampin, macrolides, fusidic acid and novobiocin against *Ps. aeruginosa* (Hancock 1997c, Giacometti et al. 2000, Vaara and Porro 1996). The combined application of ranalexin with polymyxins yields

synergistic antimicrobial activity (Giacometti et al. 2000). This is understandable considering that both compounds exhibit similar mechanisms of interaction towards the phospholipids present on the cell membrane thereby increasing bacterial membrane permeability and disrupting osmotic integrity (Hancock 1997b, Giacometti et al. 1999, Giacometti et al. 2000, Giacometti et al. 2000, Vaara and Porro 1996).

1.8 Biological redox reactions

1.8.1 The electron transport chain and respiratory process in prokaryotic cells

The electron transport chain couples the electron transfer between a donor (such as either nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide ($FADH_2$), etc. and an acceptor such as oxygen (O_2), for the transfer of H^+ ions (protons) across bacterial membranes (Krebs et al. 1999). This proton transport leads to the build-up of an electrochemical gradient (potential difference) which is utilised in the generation of chemical energy in the form of adenosine triphosphate (ATP) (Kröger et al. 1992). This process defines the cellular mechanism for extracting energy from redox reactions, such as the oxidation of sugars and fats during respiration and extraction of energy from sunlight in photosynthesis (Kröger et al. 1992, Berridge and Tan 1998). Bacterial respiratory systems are similar in many respects to those present in mitochondria of higher organisms, such as eukaryotes or plants. For instance, both are membrane-bound, with similar respiratory carriers and capacity to conserve energy to be used either for ATP synthesis or to drive mechanical work (Kröger et al. 1992). In the mitochondria of multi-cellular organisms, the reduction of oxygen to water, NAD^+ to NADH and succinate to fumarate (as shown in figure 1.6) generates the electrochemical proton gradient required for these processes. Also, bacteria have electron transport chains similar to that demonstrated in plant chloroplasts through photosynthesis (Bolton and Hall 1979, Kramer and Crofts 2004, Boghossian et al. 2011). Therefore, in all domains of life, the electron transport chain oxidises NADH and succinate produced from the citric acid cycle and thereby provides energy required to power ATP synthase. ATP synthase facilitates the provision of energy in the form of ATP - the currency

expended for the performance of all cellular reactions. Finally, bacteria can use NADH and succinate as electron donors in the presence of an organic matter as energy source. These are inducible enzymes, selected from the bacterial DNA library and are synthesized only when needed for growth (Guerin et al. 1982, Muffler et al. 2002).

1.8.2 Generating the electrochemical gradient and the electron transport chain

An electrochemical potential gradient required to initiate biological redox reactions and it is usually generated in the cell membrane. Translocation of 4 protons across the cell membrane occurs during the oxidation of every molecule of NADH, thereby building up an electrochemical potential used for the production of ATP. NADH binds to dehydrogenase to form NADH dehydrogenase complex (complex I). The transferred electrons are received by the isoalloxazine ring of FMN, which is identical to flavin adenine dinucleotide (FAD) (Berridge et al. 1996). Electrons are then transferred again through a second prosthetic group of NADH dehydrogenase through a series of Fe-S clusters and finally to co-enzyme Q (CoQ) (ubiquinone), as shown in figure 1.6.

$$NADH + H^+ + CoQ + 4H^+_{in} \rightarrow NAD^+ + CoQH_2 + 4H^+_{out}$$

$$FAD + succinate \rightarrow Fumarate + FADH_2$$

$$FAD + 2H^+ + 2e \leftrightarrow FADH_2$$

Figure 1.6: Building up the electrochemical gradient that initiates the process for ATP generation.

Ubiquinone (CoQ) accepts 2 electrons and is reduced to ubiquinol ($CoQH_2$), as a result. This final transfer leads to expulsion of $4H^+$ (Berridge, Herst and Tan 2005a).

1.8.3 MTT reduction assay

An assay involving C,N-diphenyl-N′-4,5-dimethyl thiazol 2 yl tetrazolium bromide salt (MTT) salt (figure 1.7) reduction to formazan by the normal physiological processes in bacterial species can be used to evaluate the potential capacity of

antimicrobial agents to cause a blockage to cell metabolism, as a potential mechanism of action.

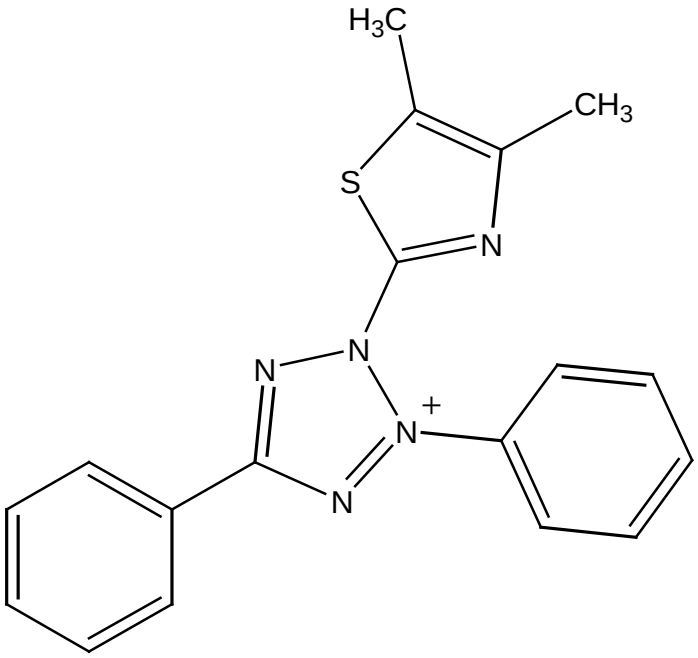


Figure 1.7 Schematic diagram of C,N-diphenyl-N-4,5-dimethyl thiazol 2 yl tetrazolium bromide (MTT).

Bacteria (Wang et al. 2010a), fungi (Bernas and Dobrucki 2002), plant (Bruggisser et al. 2002) as well as mammalian (Morgan 1997) cells have the capacity to reduce this salt into insoluble formazan crystals (Morgan 1997). Substances that can cause the cessation of this reaction in the midst of viable organisms may be seen as capable of blocking the metabolism of such cells, an event that likely precedes their death. A similar assay has been used to examine the susceptibility of *Mycobacterium tuberculosis* strains to rifampicin (Abate, Mshana and Miorner 1998).

1.8.4 Enzymes responsible for biological reduction reactions

The biological breakdown of various tetrazolium salts, including 2, 3-bis [2-methoxy-4-nitro-5-sulphophophenyl] -2H-tetrazolium-5-carboxanilide (XTT), MTS and MTT, is achieved through the activities of various cell reducing enzymes (Berridge, Herst and Tan 2005a). For mammalian cells, data gleaned from fractionation studies suggest the reduced pyridine nucleotide cofactor NADH, is responsible for most MTT reduction (Berridge, Herst and Tan 2005a). Other enzymes include succinate dehydrogenase (Wang et al. 2010b). The reduction of

MTT salt can occur in the mitochondria, endosome, lysosome, plasma membrane as well as cytoplasm (Bernas and Dobrucki 2002).

1.8.4.1 NADH dehydrogenase, Cytochrome Q, C and ATP synthase

Nicotinamide adenine dinucleotide (NADH) dehydrogenase is a metabolic enzyme also referred to as either "NADH:quinolone reductase" or "complex I". This cofactor, alongside "complex II" are important enzymes in reduction activities in cell physiology (figure 1.8).

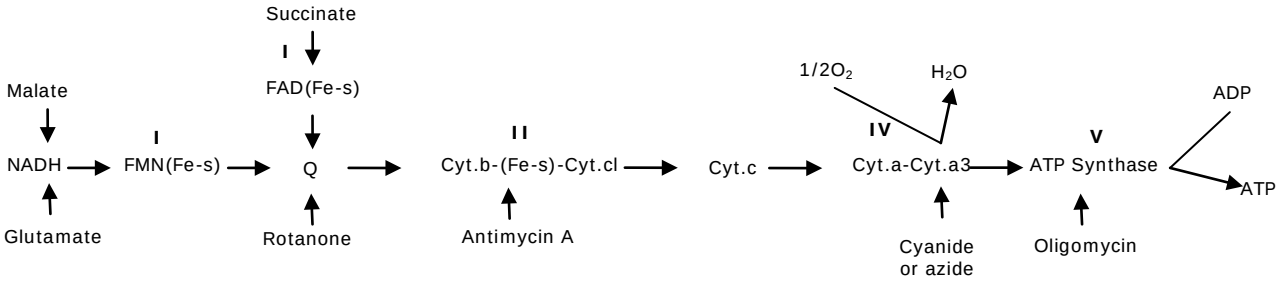


Figure 1.8: Diagrammatic representation of the ATP generation pathway in bacteria, showing the important steps, I to V.

The actions of these enzymes are required for the biological reduction reactions and the reduction of dimethyl thiazolyl diphenyl tetazolium salt (MTT) *in vivo* (Stoward, Campbell and Al-Sarraj 1982, Bigliardi et al. 1994).

In eukaryotic cells, complex I is located in the inner mitochondrial membrane catalysing the transfer of electrons from NADH to coenzyme Q (CoQ) as the rate-limiting enzyme required for oxidative phosphorylation (electron transport chain) in the mitochondria (Stoward, Campbell and Al-Sarraj 1982). NADH dehydrogenase is the largest and most complex enzyme of all the electron transport chain enzymes (Janssen et al. 2007) containing 45 independent polypeptide chains in mammals; 7 of which are encoded by the mitochondrial genome. The functionally most important subunit of this complex contains flavin prosthetic groups (i.e. flavin mononucleotides) and 8 Fe-S clusters (Aydin and Altunbasak 2006, Carroll et al. 2006). Complex I is L-shaped, consisting of a long membrane embedded domain with approximately 60 helixes and a hydrophilic peripheral domain containing all the known redox centres as well as binding sites for NADH. This peripheral hydrophilic centre has been crystallized from *Thermus thermophilus* and the disfunction of this centre is associated with aging and many degenerative diseases in humans (Sazanov and Hinchliffe 2006, Beal

1995). Other additional enzymes required for oxidative phosphorylation are complexes III, IV and V (ATP synthase) (Liu et al. 1997).

1.8.4.2 Succinate dehydrogenase and other enzymes

The reduction of MTT by the normal physiological processes has also been associated with a flavin-containing enzyme, succinate dehydrogenase (SDH) (Abe and Saito 1998, Huet et al. 2005). Succinate is an effective substrate for MTT reduction, especially in the presence of mitochondrial fractions (Berridge et al. 1996). But, there are other non-mitochondrial enzymes required for cellular MTT reduction such as nicotinamide dinucleotide phosphate (NAD(P)H)-dependent oxido-reductases like NQOI, cytochrome P450, non-mitochondrial dehydrogenases and flavin oxidases (Berridge and Tan 1998, Berridge, Herst and Tan 2005a, Liu et al. 1997). In addition, non-mitochondrial pyridine nucleotide-dependent enzymes and other terminal electron-acceptors, some of which need the assistance of intermediate electron acceptors, are also involved in the reduction of MTT (Vistica et al. 1991, Abate, Mshana and Miorner 1998, Wang et al. 2010a) However, the intracellular rate of MTT reduction correlates with intracellular NAD(P)H concentration (Berridge, Herst and Tan 2005a). In addition, sub-cellular fractionation studies have shown that NADH and NAD(P)H are more efficient in promoting the reduction of MTT than succinate (Liu et al. 1997, Berridge and Tan 1993). Such studies have also shown that reduced pyrimidine nucleotides (>90%) and succinate (<10%) account for the non-mitochondrial reduction of intracellular MTT (Berridge, Herst and Tan 2005a). This finding contradicts previously published opinion that succinate dehydrogenase is responsible for most cellular MTT reduction which led to the pseudonym “succinate dehydrogenase inhibition” assay (Van Noorden and Butcher 1989). Nonetheless, in eukaryotic cells, 77 % of the <10% MTT reduction credited to succinate dehydrogenase occurs within the mitochondria, under the auspices of complex II (Berridge and Tan 1993, Christmas and Turrens 2000). But the fact that the succinate dehydrogenase inhibitor, thenoyltrifluoracetone (TTFA), demonstrates a negligible effect upon intracellular reduction of MTT strengthens the argument that NAD(P)H is the major electron donor in MTT reduction (Berridge and Tan 1998, Berridge and Tan 1993a, Berridge, Horsfield and Tan 1995). Other oxido-reductase enzymes such as

intracellular dehydrogenases, oxidases and peroxidases are also capable of catalysing electron transfers from an electron donor to an acceptor tetrazolium salt (Berridge, Herst and Tan 2005a).

1.8.5 Tetrazolium salts reduction in eukaryotic and prokaryotic cells

Eukaryotic cell viability has been studied by the regulation of their cellular metabolic activities using the MTT assay. The water soluble yellow C,N-diphenyl-N′-4,5-dimethyl thiazol 2 yl tetrazolium bromide (MTT) salt is reduced to water-insoluble purple formazan crystals by the dehydrogenase system, (complex I), of viable cells (Carmichael et al. 1987). The crystals can be dissolved in an organic solvent like DMSO and quantitatively analysed using spectrophotometry. Usually, the concentration of formazan is directly proportional to the density of metabolically active cells in the culture (Gabrielson et al. 2002, Tunney et al. 2004).

The mono-tetrazolium salt, MTT is especially suitable for end-point determination assays because of its capacity to form water-insoluble formazan crystals. One main application of cellular tetrazolium dye reduction is the assessment of cell proliferation on the basis that the dye reduction is a function of the density of viable cells in the exponential growth phase (Cook and Mitchell 1989). Cell growth conditions, cell source and type of dye must be defined (Cook and Mitchell 1989). In other words, for both eukaryotic and bacterial cell proliferation/metabolic assay, the experiments are influenced by species, density of inoculum, growth phase and growth conditions. Therefore, in the presence of growth modifying compounds (like noxious or antibacterial compounds), especially those to which the organisms are sensitive, tetrazolium salt reduction will be influenced by the sensitivity of cells to the foreign substance. Such substances, depending on the onset and sub-cellular site of action, may affect cell respiration, metabolism, replication and density. In turn, this will have an effect on the rate of reduction of MTT salt to formazan.

The intracellular reduction of tetrazolium salts is not restricted to the mitochondria in eukaryotes as components of the cytoplasm, non-mitochondrial membranes, endosomal as well as lysosomal organelles and plasma membranes are all involved in the reduction of MTT salt to formazan crystals (Berridge, Herst and Tan 2005a). Reduction of the salt also occurs at the cells' external surface

and periphery of the plasma membrane through trans-plasma membrane electron transport (Berridge, Herst and Tan 2005a).

1.8.6 Sub-cellular sites for MTT reduction

MTT has a positively charged quaternary tetrazole ring core (figure 1.7) that contains four nitrogen atoms surrounded by three phenyl moieties. Reduction disrupts the tetrazoline ring leading to colour transformation from yellow to purple and the generation of formazan crystals (Berridge et al. 1996, Berridge and Tan 2000, Bruggisser et al. 2002). The net positive charge on the tetrazole ring facilitates uptake of MTT across the plasma membrane of viable cells through the membrane potential (¥m -30 to -60 mV, negative inside) and if not reduced in the cytoplasm, also crosses the mitochondrial inner membrane (¥m -150 to -170 mV, negative inside) in eukaryotic cells (Berridge and Tan 1998, Berridge and Tan 1993, Berridge and Tan 2000, Berridge, Herst and Tan 2005b). MTT alongside rhodamine B has been applied in the determination of mitochondrial membrane potential (Reungpatthanaphong et al. 2003). Altogether, intracellular NAD(P)H from the Krebb's cycle and electron transport chain in mitochondria, NADH from ubiquinone cycle within the plasma membrane and various substrates that are freely dispersed within the cytoplasm reduce the salt to water-insoluble formazan crystals.

1.8.6.1 Evidence for the sub-cellular localisation of MTT reduction

One of the findings that revealed the non-mitochondrial and extra-cellular reduction of tetrazolium salts was the development of a new generation of water soluble tetrazolium salts with their prototype as WST-I. WST-I bears a net negatively charged disulphonated inner salt containing iodine residue. This water-soluble salt can be reduced extracellularly to soluble formazan by electron transport across the plasma membrane of actively dividing cells (Herst et al. 2004). The net negative charge exists because of its possession of two negatively charged sulphonated groups and one positively charged phenyl ring. This arrangement generates an inner salt, with a net negative charge that excludes it from uptake by plasma membranes (Herst et al. 2004). Therefore, the net positive charge on the MTT salt and the plasma membrane potential appear to be the main factors influencing the cellular up-take and sites of

reduction (Wang et al. 2010a, Murphy 2007). Further on, WST-I is reduced extracellularly, most likely by electron transport across plasma membrane from intracellular NADH to WST-I with 1-methoxy-5-methyl-phenazinium methyl sulphate (mPMS) as an intermediate (Herst et al. 2004). Hence, contrary to previous beliefs, WST-I is not reduced by succinate dehydrogenase in the mitochondria of metabolically active cells (Berridge, Herst and Tan). And, unlike many other newer generation water-soluble salts, WST-1 can also be reduced extracellularly in the absence of an intermediate electron acceptor (Berridge, Herst and Tan). Finally, evidence obtained from confocal imaging has shown that MTT-formazan crystals are found outside the mitochondria, in the cytoplasm and around the plasma membrane in viable cells with intact membrane integrity (Bernas and Dobrucki 2002, Bernas and Dobrucki 2000). In fact, epi-fluorescence microscopy studies have shown that sub-cellular reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a fluorescent cyano-tetrazolium salt similar to MTT and also bearing a net positive charge, occurs slowly in the periphery of plasma membrane in HepG2 cells (Berridge, Herst and Tan 2005b, Bernas and Dobrucki 1999, Roslev and King 1993, Smith and McFeters 1997).

1.8.6.2 Contrary opinions on the sub-cellular sites for MTT reduction

Liu *et. al.* published in 1997 that MTT is membrane-impermeable when it was incorporated into large unilamella liposomes and consequently suggested that the salt is taken up across the plasma membrane through endocytosis (Bruggisser et al. 2002, Larm, Cheung and Beart 1997). However, this view has been discredited by three valid and superior arguments. Firstly, synthetic liposomes are completely devoid of membrane potential and are therefore incomparable to plasma membranes of viable cells. Secondly, the suggestion that MTT readily undergoes cellular up-take and gets reduced intracellularly has been supported by imaging studies using HepG2 cells (Bernas and Dobrucki 2002, Bernas and Dobrucki 2000). And thirdly, MTT in combination with rhodamine B has been applied to quantify mitochondrial membrane potential wherein the formazan crystals formed within the mitochondria acted as a fluorescence quencher for rhodamine inside this organelle (Reungpatthanaphong et al. 2003).

1.9 Aims and objectives of the study

In summary, the prevailing global incidence of bacterial resistance makes it rational for newer, more effective antibiotics and strategies for combating these organisms to be developed. The mechanisms of action of either agents with promise or newly found effective combinations of compounds, should be elucidated at the early stages of development with a view to giving priority to compounds that belong to classes other than the ones to which currently used antibiotics belong and possessing either novel or multiple mechanisms of action.
The aims and objectives of this study include:

1. To screen for *in vitro* antibacterial activity from a series of semi-synthetic flavones.
2. To examine the antibacterial mode of action of these semi-synthetic flavones.
3. To investigate the existence of either additive or synergistic antibiotic action between these semi-synthetic flavones and known antibiotics, including polymyxin B; using a chequerboard as well as time-kinetic viability assays for possibility of extending the antimicrobial spectrum and efficacy of the decapeptide agent against Gram-positive bacterial species. The organisms examined include *S. aureus* species, *C. violaceum*, *E. coli* and *Ps. aeruginosa*.
4. To elucidate the potential mechanisms of action for the effective combined application of the chalcone-derivatives with polymyxin B using a modified bacterial MTT-reduction assay and an evaluation of the impact of the combined antibacterial activity upon cytoplasmic membrane integrity through measurement of the leakage of intracellular potassium ions. Visual examination of the capacity of the combined agents to induce changes in the fluorescence intensity of applied stains in *S. aureus* over a time-course was also undertaken using microscopy.
5. Finally, an attempt will be made to examine the *in vitro* haemolytic effect of the chalcone-derivatives with polymyxin B. The capacity of the effective combinations to disrupt the plasma membrane will be examined in order to have an insight into the utility of potential future formulations, especially in the form of external applications.

Chapter 2

Materials and Methods

2.1 Chemicals and reagents

2.1.1 Dissolution of semi-synthetic chalcones, and selected antimicrobial compounds

The semi-synthetic chalcone-derivative compounds (supplied by Professor Richard C. D. Brown, University of Southampton, UK.) were used to prepare stock 5-10 mg/ml solutions in neat dimethyl sulfoxide (DMSO) using 1.5 ml centrifuge tubes (Fisher Scientific UK. Ltd., Loughborough, UK.). Aqueous stock solutions of 10 to 50 mg/ml penicillin G, polymyxin B, colistin, gentamicin, benzalkonium chloride, ciprofloxacin and ascorbic acid (all purchased from Sigma-Aldrich, Poole, UK.) were also prepared. A stock solution of acetyl salicylic acid (purchased from Aldrich) was prepared to yield 25 mg/ml in 20 % $^{v}/_{v}$ DMSO. All stock solutions were stored at -20 ^{o}C. On the other hand, ascorbic acid and acetyl salicylic acid solutions were prepared daily when required because of aqueous instability.

2.1.2 Growth media and bacterial diluents

2.1.2.1 General growth media and diluents

Nutrient broth, nutrient agar and Mueller-Hinton broth were purchased from Oxoid Ltd. (Basingstoke, UK.). All media suspensions were prepared following the manufacturer's label instructions and were autoclaved at 121 ^{o}C for 15 minutes by moist heat sterilisation. Agarose was purchased from Fisher Scientific UK. Ltd. and was autoclaved by moist heat as described above. Also, general purpose grade sodium chloride was purchased from Fisher Scientific UK. Ltd. and was used to prepare 0.9 % $^{w}/_{v}$ sodium chloride. Finally, tablets used to prepare phosphate buffer saline (PBS) were purchased from Sigma-Aldrich Company Ltd., Poole, UK.

2.1.3 Miscellaneous chemical compounds and fluorescent dye

Sodium dodecyl sulphate and 20 % $^{v}/_{v}$ chlorhexidine digluconate were purchased from Fisher Scientific UK. Ltd. Dimethyl thiazolyl diphenyl tetrazolium (MTT) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich. An aliquot of 5 mg/ml MTT stock solution in 0.9 % $^{w}/_{v}$ sodium chloride solution was prepared weekly and stored at the same temperature stated in section 2.1.1 above. Ethanol was purchased from Hayman Ltd., Witham, UK. Finally, the intact bacterial membrane-impermeant dye, Sytox Green, 5 mM was purchased from Molecular Probes (Invitrogen Ltd., Paisley, UK.).

2.1.4 Laboratory equipment

All disposable plastic utensils and materials were purchased from Fisher Scientific. Plastic tips for pipettes and 1.5 ml centrifuge tubes were sterilised by moist autoclaving at 134 ^{o}C for 3¼ minutes as a porous load. Separately, all glassware were sterilised by dry heat sterilisation at 160 ^{o}C for 2 hours.

2.2 Bacterial storage and cultivation

2.2.1 Bacterial species and strains

Bacterial strains were *Staphylococcus aureus*, National Collection of Type Cultures (NCTC) 6571 (methicillin-sensitive, MSSA); *Staphylococcus aureus* NCTC 11940 (methicillin-resistant, MRSA); *Staphylococcus aureus*, American Type Culture Collection (ATCC) 1628 (quinolone-resistant, QRSA); *Escherichia coli* NCTC 4174; *Pseudomonas aeruginosa* NCTC 6750 and *Chromobacterium violaceum* CV026 that was received as a gift from Nottingham University, UK. All NCTC strains were obtained from National Collection of Type Cultures (NCTC, Porton Down, Salisbury, UK.) and the ATCC strain was from American Type Culture Collection (Manassas, Virginia, USA.). In addition, the plant pathogens *Pectobacterium caratovoram*, National Collection of Plant Pathogenic Bacteria (NCPPB) 312 and *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 2979 were obtained from the National Collection of Plant Pathogenic Bacteria, Food and Environment Research Agency (Sand Huton, York, UK.).

2.2.2 Bacterial storage

Bacterial stock cultures were stored using Protect Bacterial Preserver beads in cryotubes (Technical Service Consultants Ltd., Lancashire, UK.) at -80 ^{o}C (Upright freezer TS80-140; Life Sciences International, Colchester, UK.).

2.2.3 Bacterial sub-culture

2.2.3.1 Preparation of bacterial master plates

When required, a 10 ml volume of Mueller-Hinton broth (Oxoid Ltd., Basingstoke, England) was inoculated with a bead from the bacterial stock culture obtained from the Protect Bacterial Preserver cryotube. The broth was incubated at 37 ^{o}C for 24 hours in an orbital incubator set at 100 rpm (Sanyo Gallenkamp PLC, Loughborough, UK.). The bacterial culture obtained for each species was streaked on a pre-dried Mueller-Hinton agar plate (Oxoid Ltd., Basingstoke, England) and incubated at 37 ^{o}C for 24 hours. Equivalent 10 ml volumes of Mueller-Hinton broth inoculated with beads from stock cultures of the plant pathogens *Pectobacterium caratovoram* and *Clavibacter michiganensis* were incubated at 25 and 18 ^{o}C respectively in a shaking water bath set at 100 rpm. The bacterial culture obtained from each sample was streaked on a pre-dried Mueller-Hinton agar plate and incubated at 25 ^{o}C for 48 hours. All master plates were stored at 4 ^{o}C and each bacterial species was sub-cultured on a monthly basis.

2.2.3.2 Preparation of bacterial experimental culture

For routine experimental purposes, a single colony from each master plate (except the plant pathogens) was used to inoculate a sterile 50 ml Mueller-Hinton broth. The broth was incubated for 24 hours as described above. Meanwhile, bacterial broth cultures prepared from beads of the plant pathogens, *Pectobacterium caratovoram* and *Clavibacter michiganensis* were incubated in a shaking water bath set at 100 rpm for both organisms and temperatures of 25 and 18 ^{o}C respectively, for 48 hours.

2.3 Preparation of bacterial enumeration graphs

Graphs of optical density plotted against concentration of bacterial cells (colony forming unit per ml (cfu/ml) (shown in appendix 11.2) were prepared by following the viable count dilution method described below.

2.3.1 Preparation of bacterial suspension

Fifteen ml of a 24 hours incubated 50 ml Mueller-Hinton broth culture was centrifuged at 4,000 rpm for 10 minutes in a MSE Chilspin (Fisons, Loughborough, UK.). The supernatant was decanted carefully and the pellet was washed once in 10 ml of 0.9 % $^{w}/_{v}$ sodium chloride by centrifugation at 4,000 rpm for 10 minutes. The pellet was then re-suspended in 10 ml of 0.9 % $^{w}/_{v}$ sodium chloride and diluted to yield a range of bacterial populations with 7 different optical density readings within 0.1 to 0.2 units at OD_{500} (Helios spectrophotometer, Thermo spectronic Corporation, Madison, USA.).

2.3.2 Preparation of agar plates

A batch of fourteen surface dried nutrient agar plates (90 mm diameter) were prepared by keeping them upright with lids open in a sterile cabinet (Horizonzatal Laminar Flow Cabinet, %HLF B/U; Hepaire Manufacturing Ltd., Avon, UK.) at room temperature, for 1 hour. The base of the plates were labelled into four quadrants and labelled for dilutions of 1×10^{-5} to 1×10^{-8}

2.3.3 Dilution of bacterial suspensions and plating out

Each of the seven bacterial densities prepared in section 2.3.1 above was diluted ten-fold serially down to 1×10^{-8} in 0.9 % $^{w}/_{v}$ sodium chloride. Five aliquotes of 20 µl from 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions were carefully pipetted onto each quadrant of the pre-dried agar plate respectively. Duplicate agar plates were prepared for each density. Following absorption of the inoculum into the agar, the plates were incubated for 24 hours at 37 ^{o}C.

2.3.4 Enumeration of viable cells

At the end of the incubation period, all agar plates were examined. The colonies from all 10 volumes (duplicate plates) of the appropriate dilution were counted and the mean value calculated and recorded. However, the quadrants that yielded bacterial colonies in the range of 5 to 50 were preferably used to assess the level of bacterial growth and to calculate the population of cells in the original suspensions. A graph of cell densities (cfu/ml) against corresponding optical densities (at OD_{500}) was plotted for the bacterial suspension. Such enumeration graphs were prepared for each of the bacterial strains employed in this project.

2.4 Preparing bacterial suspension with a defined density

2.4.1 Applying the enumeration graph to estimate bacterial density in a suspension

Following on from section 2.2.3.2 above, 15 ml of a 24 hours culture was centrifuged at 4000 rpm in an MSE Chilspin for 10 minutes. The pellet recovered was washed once by re-centrifuging with 15 ml of 0.9 % $^{w}/_{v}$ sodium chloride as described above, and re-suspended in 5 ml of 0.9 % $^{w}/_{v}$ NaCl solution. Approximately a 100-fold dilution of the suspension was then made with 0.9 % $^{w}/_{v}$ saline as diluent. The density of the bacterial suspension was estimated by adjusting the spectrophotometer (Cecil CE 3021, Cecil instruments Ltd., Milton Technical Centre, Cambridge, UK.) reading to correlate with the desired cell density in a previously prepared enumeration graph of the bacterial species.

2.4.2 Verifying cell density of the bacterial suspension

A ten-fold serial dilution and plating out of the density of the adjusted bacterial suspension were performed as described previously in section 2.3.3. Following on, the mean colony count from the plates were recorded as described in section 2.3.4 and the actual viable population of the test bacterial suspension was calculated as colony forming unit per millilitre (cfu/ml).

2.5 Bacterial susceptibility testing

The screening of some semi-synthetic chalcone-derivatives (flavonoids) for potential antibacterial activity was conducted using a modified agar-test-compound two-fold serial dilution assay. Bacterial susceptibility testing for the chalcone-derivatives and selected compounds was conducted using the micro-titre broth antibiotic two-fold serial dilution assay.

2.5.1 Determination of the MIC of some antimicrobial compounds

The minimum inhibitory concentration (MIC) of the antibacterial compounds was determined against the selected pathogenic bacterial species and strains stated in section 2.2.1 above. The MIC was determined using a micro broth two-fold serial dilution assay adapted from the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2009). A Versamax Microplate Reader (Molecular Devices Ltd., Wokingham, Berkhire, UK.) was optimised to read statically incubated plates, after a round of orbital oscillation, every 30 minutes, for 24 hours at OD_{650} and 37 ^{o}C. A final cell density of 1 x 10^{6} cfu/ml in a final volume of 200 µl Mueller-Hinton broth per well was used.

The stock antibacterial compound solution was subject to two-fold serial dilution to yield a desired range of test concentrations within the wells of a 96-well micro-titre plate (gamma-irradiated; Bibby Sterilin Ltd., Stone, UK.). To achieve this, volumes of 40 µl sterile water were transferred into wells 2 to 8 of rows C, D, E and F. Then, 80 µl of a suitable concentration of the antibiotic under examination was transferred into the first wells of same rows. A two-fold serial dilution was carried out by transferring 40 µl from the first column into the second. The procedure was repeated up to the 8th column of these rows wherein the extra 40 µl volume was discarded. Following on, 60 µl water and 100 µl double strength Mueller-Hinton broth, containing 2 x 10^{6} cfu/ml bacterial suspension, were added to the wells from the 1st to the 8th columns in order to ensure that only the wells that received an even distribution of temperature were engaged. Wells for positive (containing broth and inoculum density of the bacteria) and negative (containing broth devoid of inoculum density bacteria and broth devoid of bacteria containing the test range of antibacterial concentration) controls were included.

The micro-titre plate was then covered with an optically clear and gas impermeable seal (Fisher Scientific) to avoid evaporation and inconsistent well volumes. The plate was incubated at the set conditions described above. The MIC was determined as the lowest concentration of test antibacterial compound that inhibited growth of the bacterial species, under investigation, after incubation at 37 ^{o}C for 24 hours (Andrews 2001). Data reported were obtained from at least three separate assays that gave at least two identical data.

2.5.2 Determination of MIC of compounds with poor aqueous solubility

The MICs of the chalconederivatives against the test bacterial species mentioned in section 2.2.1 above was determined with a micro broth dilution assay. The plate reader was set as described in section 2.5.1.2 above. However, the test chalcone derivatives were subject to two-fold serial dilution to yield a suitable range of test concentration in 2.5 % $^{v}/_{v}$ DMSO within Mueller-Hinton broth. In order to achieve this, volumes of 40 µl 12.5 % $^{v}/_{v}$ DMSO were transferred into wells 2 to 8 of rows C, D, E and F in the micro-titre plate. Then, 80 µl of a suitable concentration of the chalcone derivative dissolved in 12.5 % $^{v}/_{v}$ DMSO was transferred into the first wells of the same rows. A two-fold serial dilution was carried out by transferring 40 µl from the first column into the second. The procedure was repeated up to the 8th column of these rows wherein the extra 40 µl volume was discarded. At this point, 60 µl water and 100 µl double strength Mueller-Hinton broth containing 2 x 10^{6} cfu/ml bacterial suspensions were added to the wells from the 1st to the 8th column of the rows mentioned above. The controls were extended to include a positive solvent (Mueller-Hinton broth with 2.5 % $^{v}/_{v}$ DMSO and inoculum population of the bacterial species) and a negative solvent (Muller-Hinton broth containing 2.5 % $^{v}/_{v}$ DMSO with no inoculum bacterial species) controls respectively. The micro-titre plate was then sealed and incubated at the set conditions previously described in section 2.5.2.1 above. Similarly, the MIC was determined as previously described in the same section above. The reported data were obtained from at least three separate assays that gave at least two identical data.

2.5.3 Determining minimum bactericidal concentration for test compounds

The minimum bactericidal concentration (MBC) for the test antimicrobial compounds against the selected bacterial species was determined by a replica plating procedure using a 96-pin multi-point replicator (Boekel Scientific, Feasterville PA, USA.). Approximately one micro litre inoculum was transferred from each well to the surface of chalcone derivative-free and antibiotic-free nutrient agar in a 13.5 cm diameter plate. The plate was left to sit on the bench top for approximately 3 minutes, to allow time for the agar to absorb the inoculum and then incubated at 37 ^{o}C for 24 hours. Following on, the MBC was determined as the least concentration of the compound under examination that prevented the bacterial growth (Lorian 2005). The reported data were obtained from at least three separate assays that gave at least two identical data.

2.6 Evaluating the mode of action of chalcone-derivatives in combination with other antibiotics

The existence of synergism between the chalcone-derivatives and selected antimicrobial compounds was determined with a chequerboard method. A 96-well micro plate containing chequered concentrations of the compounds being examined in Mueller-Hinton broth was prepared. The protocol for preparing the plate was adapted from Lorian et. al., Antibiotics in Laboratory Medicine (Lorian 2005, Pillai et al. 2005a, Petersen et al. 2006). Bacterial culture and suspension with a defined density were prepared as previously described in sections 2.2.3.2 and 2.4.1 respectively. Dispensing of media, bacterial suspension, diluents and test compounds under examination were conducted with an 8-tip 10 - 100 µl variable multi-channel pipette (Calibra 852, Socorex Isba S.A, Ecublens, Switzerland).

The column 1, wells A to F in the micro-titre plate was taken to be the y-axis and row G, with wells in column 2 to 7 was taken to be x-axis. An 80 µl volume of 12.5 % $^{v}/_{v}$ DMSO was added to the wells in the micro-titre plate from column 1 to 7, rows A to F. Then 80 µl volume of chalcone-derivative solution with a known concentration in 12.5 % $^{v}/_{v}$ DMSO was added to the first two rows of column 1 to 7. As described in section 2.2.7, serial two-fold dilutions were carried out in a decreasing order down the rows A to F, as the y-axis, in order to

obtain a chalcone-derivative concentration equal to either MIC or 2 x MIC in the row A, wells 1 to 7 and the lowest dilution concentration in row F, wells 1 to 7. Similarly, in another micro-titre plate, 30 µl sterile water was introduced into each well in the first 6 columns using rows A to G. Then, 30 µl of aqueous antibiotic solution with known concentration was added to columns 6 and 7. Two-fold serial dilutions were carried out backward, from column 6 to 2 of wells A to G, as described in section 2.5.2.1. A concentration equal to either MIC or 2 x MIC was obtained in column 7 and the lowest dilution concentration in column 2. Aliquots of 20 µl of the serial dilutions of the selected compounds were withdrawn from the 30 µl from the wells of the second micro-titre plate and transferred into the exact replicate well number in the first micro plate containing 80 µl chalcone-derivative solutions. This was followed by the addition of 20 µl volumes of sterile water to wells A to F of column 1, and 80 µl sterile water to wells A to G of column 7 in order to make up the volumes of all the wells to a 100 µl each. Then, 100 µl double strength Mueller-Hinton broth, containing 2 x 10^6 cfu/ml bacterial suspension, was added to each well of the micro-titre plate. Final volume was 200 µl in all the wells and inoculum density was 1 x 10^6 cfu/ml. Wells in row H (1 to 7) and column 8 (A to H) were used for positive and negative controls. The plate was sealed and incubated in the Versmax reader set as previously described in section 2.5.2.1 above.

Chapter 3

Examination of test bacterial species susceptibility to the chalcone-derivatives and selected antimicrobial compounds

3.1 Introduction

Bacterial susceptibility testing is the recommended first step in defining the most suitable antimicrobial agent when dealing with organisms that contribute to an infectious disease (Liu et al. 2011, Clinical and Laboratory Standards Institute 2009). This testing is necessitated by the continuous emergence of resistant bacterial strains to available antimicrobial agents, to which such organisms were previously sensitive (Clinical and Laboratory Standards Institute 2009). The standard test involves culturing an inoculum of the test bacterial isolate individually in the presence of a range of antibiotics for a defined time period. The relative sensitivity of the pathogenic organism to the antimicrobial compounds *in vitro* is then ascertained. Data obtained from these susceptibility assays, along with the classification and mechanism of action of the various antimicrobial compounds, clinical experience and knowledge of local pathogens, as well as the expected potential adverse effects and costs are some factors that influence the choice of drug selection for antibacterial therapy (Rex et al. 1997, Tsuji and Rybak 2006).

The potency of a newly developed antimicrobial compound can also be ascertained by examining the sensitivity and susceptibility of a range of pathogenic organisms to the compound. The antibacterial potency of a series of structurally related semi-synthetic chalcone-derivatives against selected bacterial species was therefore undertaken. The organisms employed were a representation of Gram-positive antibiotic-sensitive and resistant strains of *S. aureus*. Others included *C. michiganensis* and *P. caratovoram* both of which are serious plant pathogens. The Gram-negative species, *E. coli* and *Ps. aeruginosa* were also considered alongside the saprophyte *C. violaceum* (that belongs to the same family of neisserheae with *N. gonorrhoea*) (Zinger-Yosovich et al. 2006). Considering that it is safer to work with a saprophyte and that *C. violaceum* belongs to the same family (Neisseriaceae) as *N. gonorrhoea*, the presumption was that data obtained from assays conducted with this organism may give an

indication of the sensitivity of *N. gonorrhoea* to the chalcone-derivatives. Infections caused by this latter bacterial species was recently declared as being at the verge of being re-classified as an untreatable organism with available antibiotics in the clinics (Bolan, Sparling and Wasserheit 2012).

Unfortunately, these chalcone-derivatives demonstrated poor aqueous solubility and in addition, were only available in very limited quantities. Both factors made it extremely difficult for their preliminary antibacterial properties to be established using either conventional broth or agar dilution assays. We therefore developed a novel method that enabled the use of reduced quantities for the determination of their minimum inhibitory concentrations against a range of test bacteria assayed simultaneously, whilst dealing with the challenges posed by these compounds inherent poor aqueous solubility.

The objectives of the work detailed in this chapter were to screen for *in vitro* antibacterial activity of a series of structurally related family of chalcone-derivatives and some commercial antimicrobial agents against selected strains of human and plant pathogenic bacterial species.

3.2 Materials and methods

3.2.1 Examination of bacterial sensitivity to the chalcone-derivatives using a modified agar dilution assay

The sensitivity of test bacterial species to the chalcone-derivatives was ascertained by a newly developed modified antibiotic-agar micro dilution technique. A 2.4 ml aliquot of 2.56 mg/ml stock chalcone-derivative solution (in 12.5 % $^{v}/_{v}$ DMSO as solvent) and 3.6 ml molten Mueller-Hinton agar were mixed in a McCartney bottle (maintained at 50 - 55 ^{o}C in a water bath) to obtain flavonoid-agar solution in 5 % $^{v}/_{v}$ DMSO. After suitable mixing, 3 ml of the flavonoid-agar solution was withdrawn and transferred to another McCartney bottle containing 3 ml molten agar previously equilibrated with 5 % $^{v}/_{v}$ DMSO and also held at same condition stated above. The content of the second bottle was mixed thoroughly and the process of two-fold serial dilutions was continued to yield flavonoid concentrations in a range from 1.024 mg/ml to 4 µg/ml, in molten agar containing 5 % $^{v}/_{v}$ DMSO.

Then, 300 µl of each dilution was introduced into separate wells (8 wells used for each concentration) of a 96 multi-well flat bottom micro-titre plate (Fisher Scientific, Loughborough, UK.) in a descending concentration order, beginning from the first column. The agar in the micro-titre plate was allowed to set at room temperature and then surface-dried under laminar air flow for 15 minutes. A sterile 96 pin stainless steel multi-point inoculator (140500 Boekel Scientific, Feasterville PA, USA.) was used to carefully transfer a 1 µl volume of the test bacterial suspensions (5 x 10^7 cfu/ml), contained in a replicate micro-titre wells in duplicate, onto the agar surface. Therefore, inoculum density was approximately 5 × 10^4 cfu/ml for each bacterial species. Each bacterial species was applied to duplicate rows of the agar.

The actual bacterial density in each bacterial sample was established by colony count determination (section 2.4.2) in order to verify the actual inoculum density and bacterial purity. Negative controls (sterile agar and sterile agar containing 5 % $^{v}/_{v}$ DMSO); and positive controls (inoculated agar and inoculated agar containing 5 % $^{v}/_{v}$ DMSO) were included (4 columns). The inoculated plates were allowed to stand for 3 minutes to enable the applied bacterial suspensions to soak into the agar. The plates were incubated at 37 ^{o}C for 24 hours. The MIC (in units of µg/ml) was determined as the lowest concentration of test antibacterial compound that inhibited visible growth of the bacterial species under investigation (Andrews 2001). The reported data were obtained from at least three separate assays.

3.2.2 MIC determination for test compounds against test bacterial species

The antibacterial potency of these chalcone-derivatives was verified by determining their minimum inhibitory concentration against the test bacterial species with the antibiotic-broth micro dilution protocol described in section 2.5.2. The minimum inhibitory concentration of some selected antimicrobial compounds was also determined as described in section 2.5.1.

3.2.3 Determination of MBCs for chalcone-derivatives and antimicrobial compounds against test bacterial species

The minimum bactericidal concentration for the semi-synthetic compounds and antimicrobial compounds were determined by the multi-inoculator replica method described in section 2.5.3.

3.3 Results

3.3.1 Screening the series of chalcone-derivatives for antibacterial potency

The sensitivity of the test bacterial species to the chalcone-derivatives was reported in the form of the minimum inhibitory concentration of the compound being examined against the test organism. The MICs for the chalcone-derivatives obtained from a modified antibiotic-agar dilution assay against selected strains of *S. aureus* and an *E. coli* species are reported in table 3.1.

Compound	MIC against bacterial species (µg/ml)			
	MSSA	MRSA	QRSA	*E. coli*
F1	64	128	32	256
F5	128	128	>512	256
F6	>512	128	>512	>512
F7	256	64	>512	128
F9	>512	>512	>512	ND
F12	>512	>512	>512	ND
F17	128	64	>512	128

Table 3.1: Sensitivity of selected test bacterial species to the chalcone-derivatives, reported as MIC of these compounds from an antibiotic-agar dilution assay. The data represent two consistent results from three replicates. MSSA: methicillin-sensitive *S. aureus* NCTC 6571; MRSA: methicillin-resistant *S. aureus* NCTC 11940; QRSA: quinolone-resistant *S. aureus* ATCC 1628; and *E. coli* NCTC 4174. ND: Not determined. Structures and chemical names of these semi-synthetic chalcone-derivatives are provided in appendix 11.1. Solvent was 5 % $^{v}/_{v}$ DMSO and inoculum density was 5 x 10^4 cfu/ml.

The antibacterial activity displayed by these chalcone-derivatives was modest, with the reported MIC values from this modified agar dilution technique within the range of 32 to >512 µg/ml. Data presented in table 3.1 also show QRSA was most sensitive to F1 by displaying an MIC value of 32 µg/ml (as is also displayed in figure 3.1), but was insensitive to the presence of F5, F6, F7, F9, F12 and F17.

All these 6 compounds were considered inactive against this strain of quinolone-resistant *S. aureus* because they displayed MIC values that were >512 µg/ml.

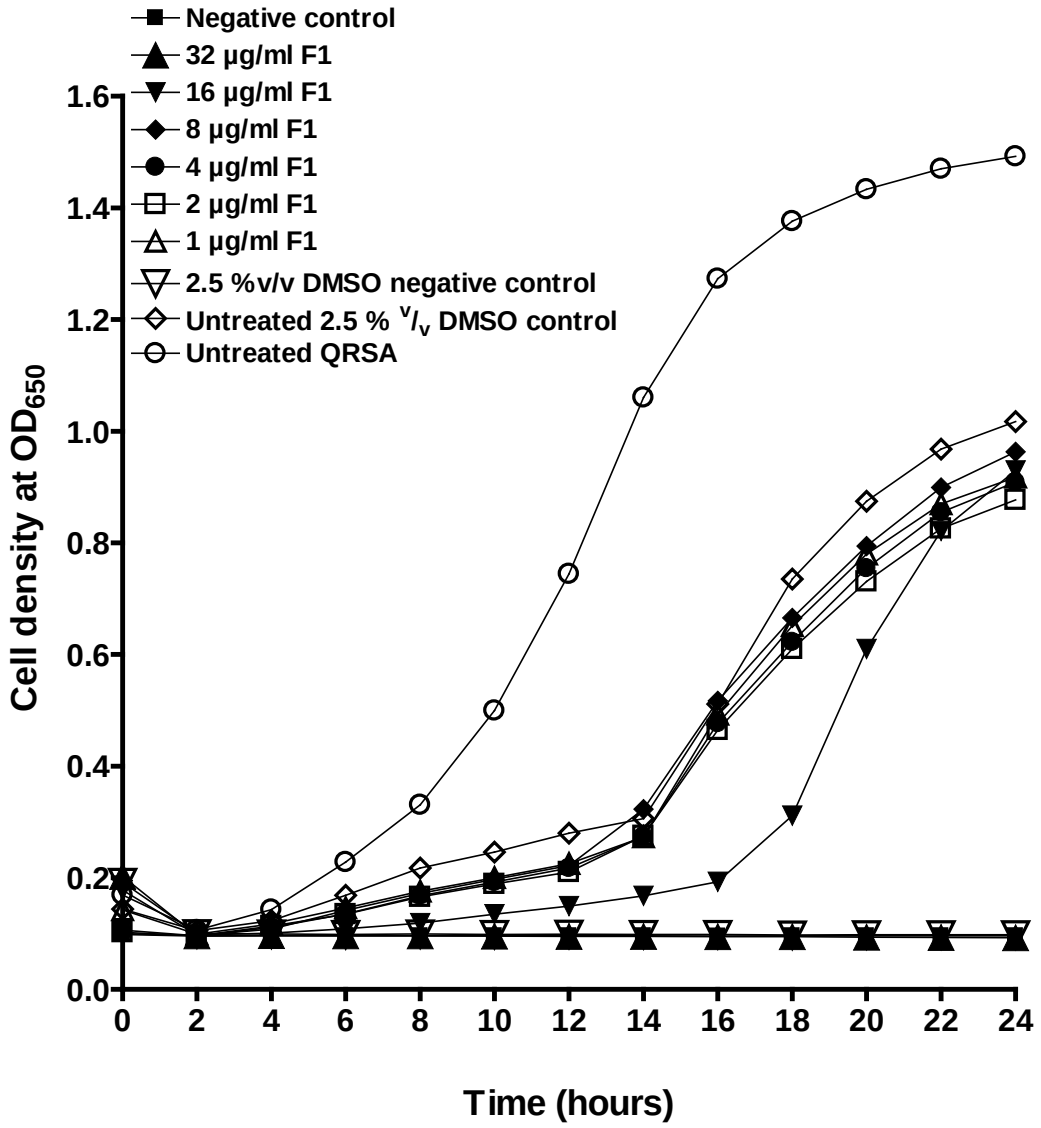


Figure 3.1: MIC of F1 against *S. aureus* ATCC 1628 (QRSA) in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean cell density readings over time were plotted for the outcomes of two experiments with three replicates each.

The data in figure 3.1 also show the pronounced adverse effect of 2.5 % $^{v}/_{v}$ DMSO on the growth kinetic of QRSA cells in the solvent control sample relative to the untreated and DMSO-free populations. This solvent-effect is however overwhelmed by the antibacterial action of 32 µg/ml F1 against this bacterial species. Likewise, F1 showed an MIC value of 64 µg/ml whilst F5 and F17 demonstrated an MIC value of 128 µg/ml against MSSA respectively. MRSA was

most sensitive to F7 and F17 as both compounds displayed an MIC value of 64 µg/ml while F5 and F6 yielded an MIC value of 128 µg/ml against this bacterial species with F1 demonstrating an MIC value of 256 µg/ml. Results presented in table 3.1 also show that F1, F5, F7 and F17 demonstrated antibacterial activity against *E. coli* in the range of 128 - 256 µg/ml. Therefore, compounds F1, F5, F7 and F17 demonstrated only modest broad-spectrum antibacterial activity in the antibiotic-agar micro dilution assay.

3.3.2 Susceptibility of *S. aureus* to the semi-synthetic chalcone-derivatives

The MIC and MBC values for a number of the semi-synthetic chalcone-derivatives obtained from the antibiotic-broth micro dilution assay against MSSA and MRSA are shown in table 3.2.

Compounds	MIC against staphylococcal species (µg/ml)			
	MSSA		MRSA	
	MIC	MBC	MIC	MBC
F2	97.5	›400	400	›400
F6	256	›512	ND	ND
F8	ND	ND	400	›400
F12	128	›512	ND	ND
F13	200	›400	400	›400
F14	ND	ND	400	›400
F15	ND	ND	400	›400
F17	200	›400	400	›400
F21	200	›400	400	›400

Table 3.2: Susceptibility of *S. aureus* NCTC 6571 (MSSA) and *S. aureus* NCTC 11940 (MRSA) to the chalcone-derivatives reported as MIC and MBC values for these compounds obtained from an antibiotic-broth micro dilution assay. The data represents at least 2 consistent readings from 3 replicates. Solvent was 2.5 % $^{v}/_{v}$ DMSO. MSSA: methicillin-sensitive *S. aureus* and MRSA: methicillin-resistant *S. aureus*. ND: Not determined.

F2 and F12 demonstrated modest antibacterial activity with MIC values of 97.5 and 128 µg/ml respectively against MSSA. F13, F17 and F21 demonstrated weak MICs of 200 and 400 µg/ml against MSSA and MRSA respectively. However, approximately 75 % of the reported MBC values are greater than 400 µg/ml and none is less than 90 µg/ml reflecting the general poor antibacterial activity of these chalcone-derivatives. F1 and F23 however demonstrated better activity

(MIC of 64 µg/ml) against MSSA and were therefore further examined against other selected human and plant pathogens, using the antibiotic-broth micro-dilution as well as the minimum bactericidal concentration (MBC) determination assay. The MIC (µg/ml) and MBC values obtained from both assays are presented in table 3.3.

Bacterial species	F1		F23	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
P. caratovoram	16	32	64	64
Cl. michiganensis	16	16	32	32
C. violaceum	>512	512	>512	512
MSSA	64	128	64	128
MRSA	128	512	>1024	1024
QRSA	32	32	32	64
E. coli	256	512	512	>512
Ps. aeruginosa	512	512	256	512

Table 3.3: Susceptibility of test bacterial species to F1 and F23 reported as MIC and MBC values for these compounds obtained from an antibiotic-broth micro dilution and MBC determination assays. The data were 2 consistent readings obtained from 3 replicates. Solvent was 2.5 % $^{v}/_{v}$ DMSO. MSSA: methicillin-sensitive *S. aureus* NCTC 6571; MRSA: methicillin-resistant *S. aureus* NCTC 11940 and QRSA: quinolone-resistant *S. aureus* ATCC 1628.

The MIC and MBC values were in the range of 16 to >1024 µg/ml indicating a range of modest to very weak activity. The plant pathogens *P. caratovoram* and *C. michiganensis* were most sensitive to F1, as this compound demonstrated the same MIC value of 16 µg/ml against both strains. This was followed by MIC values of 32, 64 and 128 µg/ml demonstrated by F1 against QRSA, MSSA and MRSA (figure 3.2) respectively. *C. violaceum*, *Ps. aeruginosa* and *E. coli* displayed lower degrees of sensitivity to F1 with demonstrated MIC values of >512, 512 and 256 µg/ml respectively (table 3.3).

The chalcone compounds (F1 and F23) formed a true solution with neat DMSO, even at very high concentrations, which upon initial aqueous dilution becomes cloudy, forming a milk-white suspension that retains this opaque characteristic even upon the introduction of Mueller-Hinton broth containing bacterial cells. This is evidenced by initial high optical density readings at OD_{650} that seem to support the suggestion that the milk-white suspension is neither translucent nor transparent as reflected by the elevated initial reading of more than 0.4 units

(figure 3.2) at OD_{650} for the sample containing 128 µg/ml concentration of this semi-synthetic agent in 2.5 % $^{v}/_{v}$ of the solvent.

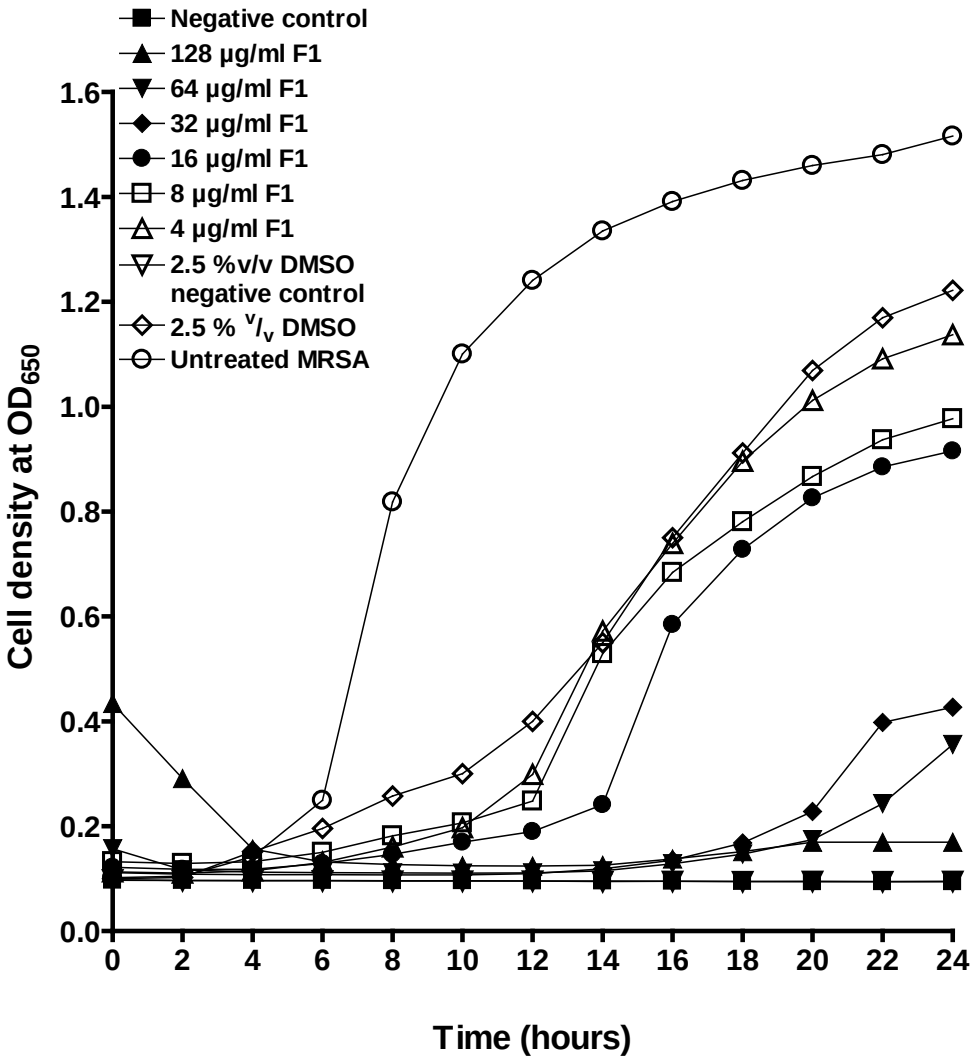


Figure 3.2: MIC of F1 against *S. aureus* 11940 (MRSA) in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean OD readings were plotted against time (hours) for the outcomes of two experiments with three replicates each.

On the other hand, the optical density of the bacterial suspension treated with a two-fold dilution of the above strength was reduced to less than 0.2 unit at the same OD_{650} value whilst a four-fold dilution displayed a value of 0.1 unit.

With the use of a much higher initial F1 concentration of 512 µg/ml in 2.5 % $^{v}/_{v}$ DMSO for the determination of the MIC of this chalcone-derivative against *C. violaceum*, the initial OD reading was seen to be elevated to about 1.5 unit at OD_{650} as demonstrated in the data recorded in figure 3.3.

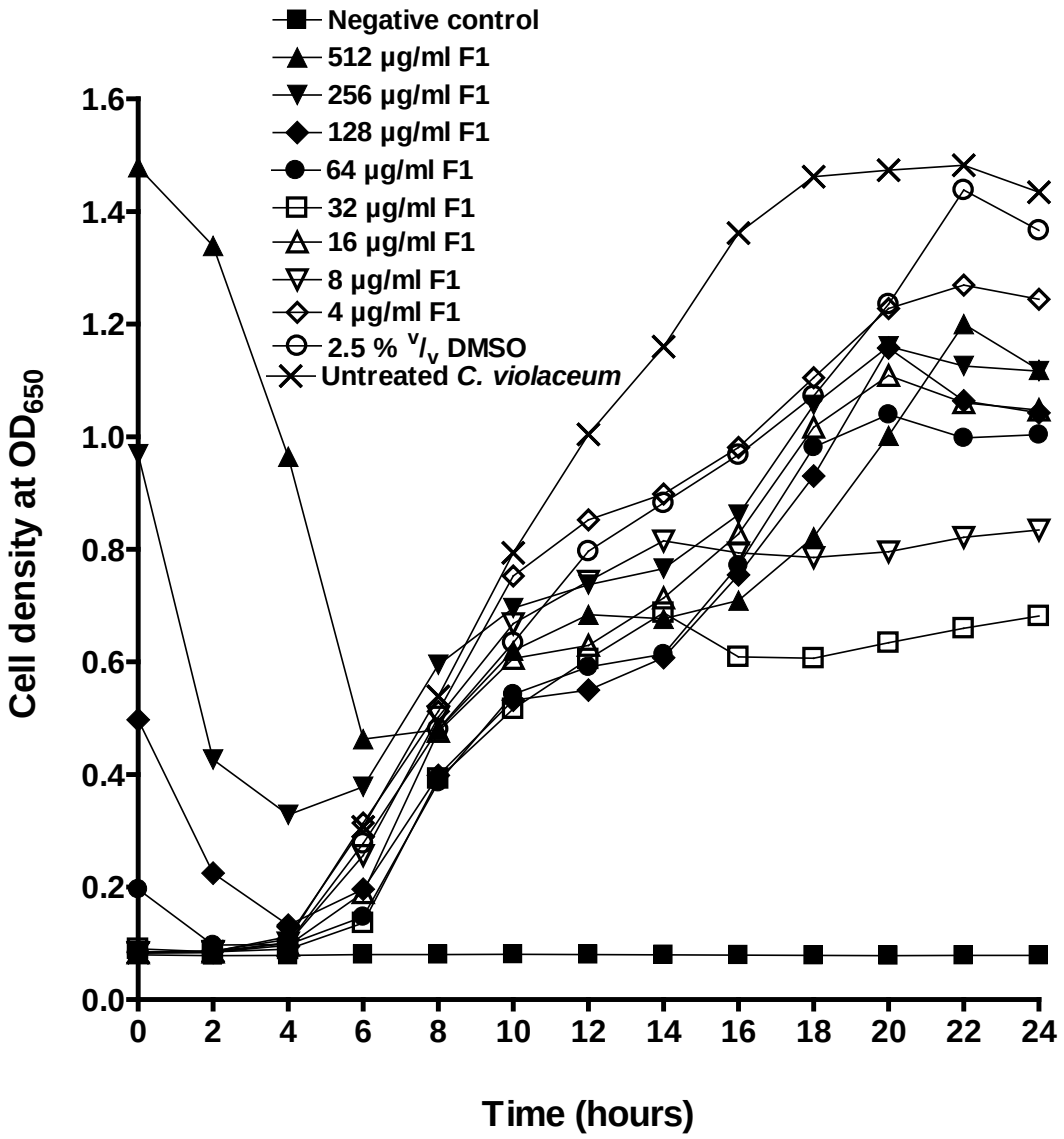


Figure 3.3: MIC of F1 against *C. violaceum* in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean OD readings were plotted against time (hours) for the outcomes of two experiments with three replicates each.

A two and four-fold dilutions reduced these optical density (absorbance) readings to about 1 and 0.5 units respectively at OD_{650} indicating that further dilutions lowered the opacity of this suspension. Hence, although F1 formed a true and lucid solution with neat DMSO at extremely high concentrations (such as 5 to 20 mg/ml), initial aqueous dilutions produced quite opaque suspensions (reflected by the high initial absorbance readings). A reduction in this opacity was obtained as further dilutions were made for the compound such that the lower strengths displayed lower initial absorbance readings due to the attainment of more translucent suspensions by the desired lower F1 concentrations, even in

the presence of Mueller-Hinton broth and bacterial cells, as evidenced by the lower initial optical density readings at OD_{650}. On the other hand, although Mueller-Hinton broth is opaque to the naked eye, control wells containing either this broth alone or with bacterial suspensions did not give high initial optical density readings at OD_{650}. However, the data also show a rapid drop in the elevated optical density readings within the first 2 hours thereby demonstrating the effect of an increase in temperature upon the opacity of suspensions constituted with DMSO and water. The OD_{650} of the cell suspension containing the highest concentration of the semi-synthetic flavonoid in 2.5 % $^{v}/_{v}$ DMSO had dropped from an initial value of 1.5 units at OD650 that was recorded at the on-set of the experiment (and obviously at room temperature as well) to 0.5 within 2 hours upon incubation at 37 ^{o}C. Hence, contrary to a previous opinion, the data presented in the figure 3.3 rather than suggesting either a coalescence or precipitation of the chalcone-derivatives from suspension, actually indicates an incubation of the final suspension at elevated temperature increased the lucidity and translucency of suspensions contained in the wells within 2 hours.

The explanation above is also applicable for the observation made in figure 3.3, wherein a reduction of the initially raised OD_{650} readings in the wells of each bacterial population treated with ≥ 64 µg/ml F1 concentrations was noted within the first 6 hours following an extensive dilution of the suspension containing the chalcone-derivatives in DMSO with water. The effect of extreme dilution coupled with an increase in temperature following incubation at 37 ^{o}C probably led to a decline in the initial opacity recorded for such highly concentrated suspensions within this period. This observation may also be verifying that F1 formed a completely true solution at concentrations lower than 64 µg/ml (in 2.5 % $^{v}/_{v}$ DMSO), as the data presented in figure 3.1 show 1 to 32 µg/ml F1 displaying an initial optical density reading of 0.1 to 0.2 unit at OD_{650}. However, one-step dilution of stock F1 (as well as F23) solutions prepared in 12.5 % $^{v}/_{v}$ DMSO in 1.5 ml centrifuge tubes were stable (and did not coalesce), for more than 6 weeks when stored at -20 ^{o}C. But, in order to avoid the negative effect of repeated thawing upon the potency of the tested chalcone compounds, their stock solutions were prepared with neat DMSO in 1.5 ml centrifuge tubes and stored in the dark at -20 ^{o}C. In most cases (except for the solvent negative control) and as seen in figure 3.3, the initial rapid decline in the opacity readings recorded for wells containing relatively higher concentrations of the chalcone-derivatives were

overtaken at a point by the growth kinetic curves of the suspended cells if the strengths of the compounds were unable to inhibit the bacterial replication.

The MBC values were within the range of 16 and >1024 µg/ml against these test bacterial species; also indicating a range of modest activity to inactivity. F1 demonstrated MBC values of 32, 128 and 512 µg/ml against QRSA, MSSA and MRSA respectively. Although the 512 µg/ml F1 was infective at inhibiting the growth of *C. violaceum* in the data presented in figure 3.3, a 1 µl volume of this suspension sub-cultured onto an antibiotic-free freshly prepared agar after 24 hours and incubated for another 24 hours had no bacterial colony on the plate, unlike samples obtained from all the other wells. This explains how the MBC value of 512 µg/ml for this agent against *C. violaceum* was achieved.

Another observation to note is that the MIC reported for F1 against MSSA and MRSA (64 and 128 µg/ml) from the antibiotic-broth micro dilution assay were one dilution step lower than values reported from the antibiotic-agar micro dilution assay, which were within the range of 128 – 256 µg/ml respectively. This is despite the higher inoculum density of 1×10^6 cfu/ml used in the former assay. This is probably accounted for by the fact that the organisms have greater contact with the test antimicrobial agents in liquid broth media than they do on a solid agar surface. This phenomenon has been previously identified in similar studies (Wiegand, Hilpert and Hancock 2008). The data presented in table 3.3 also show that F23 was modestly active against *C. michiganensis*, *P. caratovoram* and QRSA with MIC values of 32, 64 and 32 µg/ml respectively. This agent may however be considered as being inactive against MRSA because of the very high MIC value of >1024 µg/ml demonstrated against this bacterial species (figure 3.4).

This assertion is also applicable to *C. violaceum* given that this chalcone-derivative likewise displayed a high MIC value of >512 µg/ml against this latter bacterial species (figure 3.5).

However, it can be observed from the data presented in figure 3.4 that the tested concentrations of F23 within the range of 16 to 1024 µg/ml appeared to have inhibited the growth of MRSA for the first 18 hours in comparison to the untreated control and to a degree surpassing the solvent-only control, until these strengths of the agent became apparently overwhelmed by cell replication. But surprisingly, a 1 µl volume of 1024 µg/ml F23 sample sub-cultured onto a freshly prepared antibiotic and chalcone-derivative-free agar had no bacterial colony

after 48 hours unlike those from all other wells containing challenged inoculum populations. This observation is similar to that previously made for *C. violaceum* suspension challenged with 512 µg/ml F1.

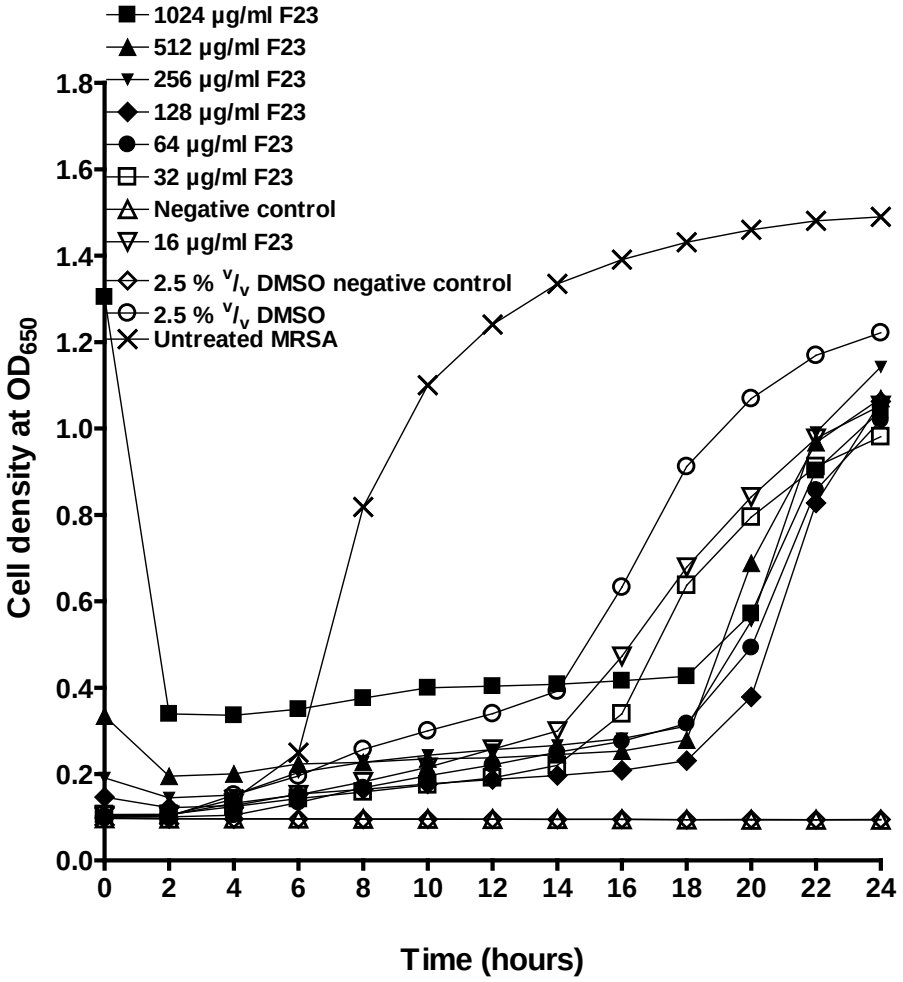


Figure 3.4: MIC of F23 against *S. aureus* 11940 (MRSA) in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean optical density readings over time were plotted for the outcomes of two experiments with three replicates each.

This semi-synthetic agent (F23) was also suggested to be weakly active against *E. coli* and *Ps. aeruginosa* by displaying MIC values of 512 and 256 µg/ml respectively, recorded in table 3.3.

Comparing data presented in table 3.2 to those in 3.3, it can be observed that F23 and F1 demonstrated comparable degrees of antibacterial activity against *C. violaceum* (figures 3.3 and 3.5), and QRSA (figures 3.1 and 3.6) by showing the same MIC values of >512, 64 and 32 µg/ml respectively against these species.

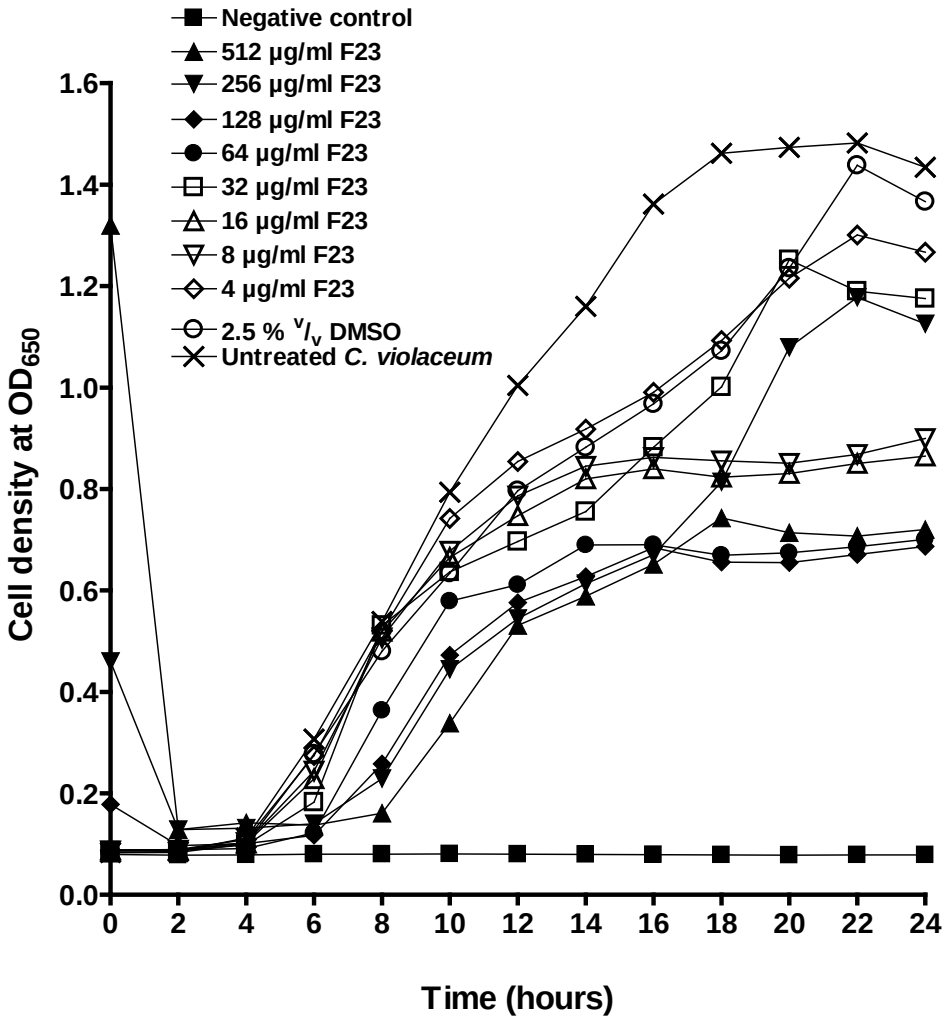


Figure 3.5: MIC of F23 against *C. violaceum* in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean optical density readings over time were plotted for the outcomes of two experiments with three replicates each.

However, MRSA was more than 8 times more sensitive to F1 than F23. Likewise, the plant pathogens *P. caratovoram* and *Cl. michiganensis* were 2 times more sensitive to F1 than F23. These data therefore suggest F1 demonstrated more potent antibacterial activity than F23.

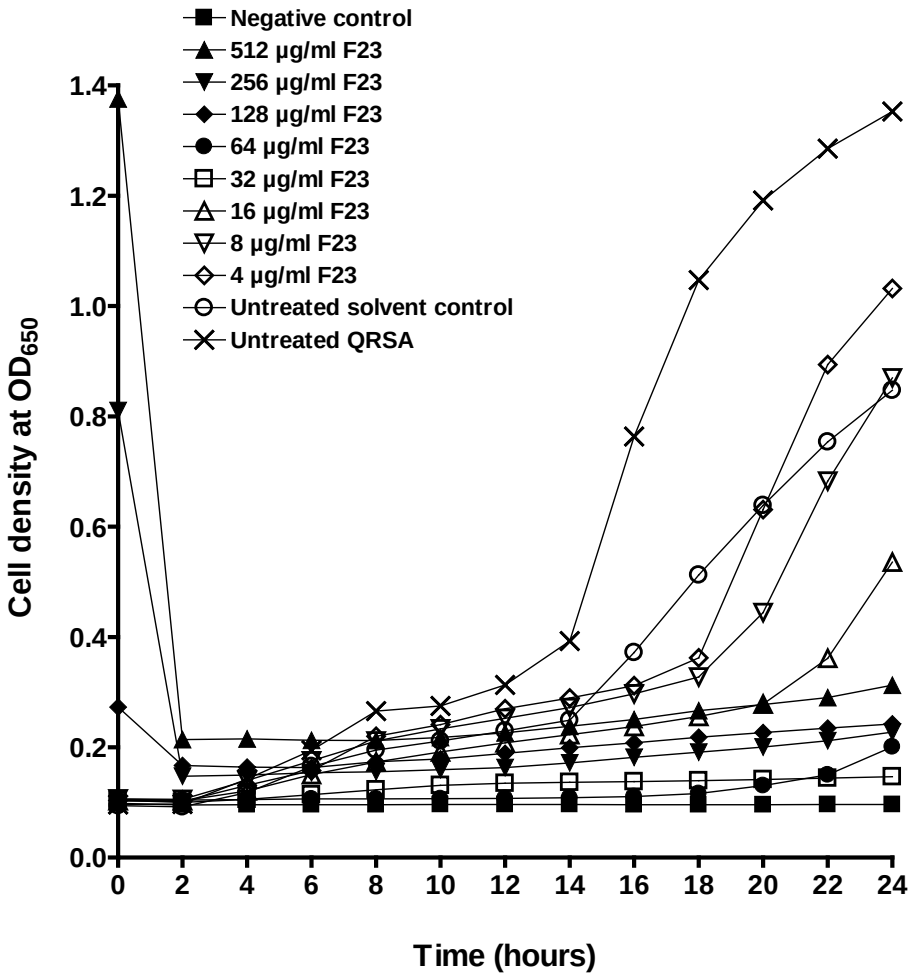


Figure 3.6: MIC of F23 against *S. aureus* 1628 (QRSA) in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean optical density readings over time were plotted for the outcomes of two experiments with three replicates each.

3.3.3 Determining the MIC of selected antimicrobial compounds

In view of the generally weak antibacterial action demonstrated by the chalcone-derivatives against the test bacterial species above, consideration was given for their activity to be assessed in combination with known antimicrobial compounds. There was therefore the prerequisite to determine the susceptibility of these test bacterial strains to these antibiotics. Representative antibiotics with activity targeted at disrupting bacterial membrane integrity, cell wall formation, inhibition of protein synthesis and inhibition of DNA replication were chosen. Examples of such agents are polymyxin B, penicillin, gentamicin and ciprofloxacin respectively (Cottarel and Wierzbowski 2007). The MIC and MBC values obtained from antibiotic-broth micro dilution assay for the selected antimicrobial compounds against the test bacterial species are shown in tables 3.4 and 3.5.

Antimicrobial agents	MSSA		MRSA	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Penicillin G	0.03125	0.03125	ND	ND
Chlorhexidine	1	2	ND	ND
Benzalkonium chloride	1	1	ND	ND
Gentamicin	1.6	1.6	ND	ND
Ciprofloxacin	32	32	1	1
Gramicidin	ND	ND	64	64

Table 3.4: Susceptibility of *S. aureus* NCTC 6571 (MSSA) to selected antimicrobial compounds reported as MIC and MBC values of these compounds. The data were obtained from at least 3 replicates. MSSA: Methicillin-sensitive *S. aureus*.

Bacterial species	Polymyxin B		Colistin	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
MSSA	256	256	140	140
MRSA	128	512	ND	ND
QRSA	256	256	ND	ND
E. coli	0.03125	0.03125	0.28	0.28
Ps. aeruginosa	0.5	1	1.5	1.5
C. violaceum	32	64	ND	ND
Cl. michiganensis	16	32	ND	ND
P. caratovoram	2	2	ND	ND

Table 3.5: Susceptibility of test bacterial species to polymyxin B and colistin (polymyxin E) reported as the MICs and MBCs. ND: Not determined. The data were at least 2 consistent readings from 3 replicates. MSSA: Methicillin-sensitive *S. aureus* NCTC 6571; MRSA: Methicillin-resistant *S. aureus* NCTC 11940 and QRSA: quinolone-resistant *S. aureus* ATCC 1628.

In table 3.4, both chlorhexidine and benzalkonium chloride displayed an MIC value of 1 µg/ml against MSSA whilst ciprofloxacin displayed an MIC value of 32 and 1 µg/ml against MSSA and MRSA respectively. Similarly, an MIC of 64 µg/ml was recorded for gramicidin against MRSA. The lowest recorded MIC as well as MBC value in this study was 0.03125 µg/ml for penicillin G against MSSA in table 3.4 and polymyxin B against *E. coli* as shown in table 3.5 as well as figure 3.7 respectively.

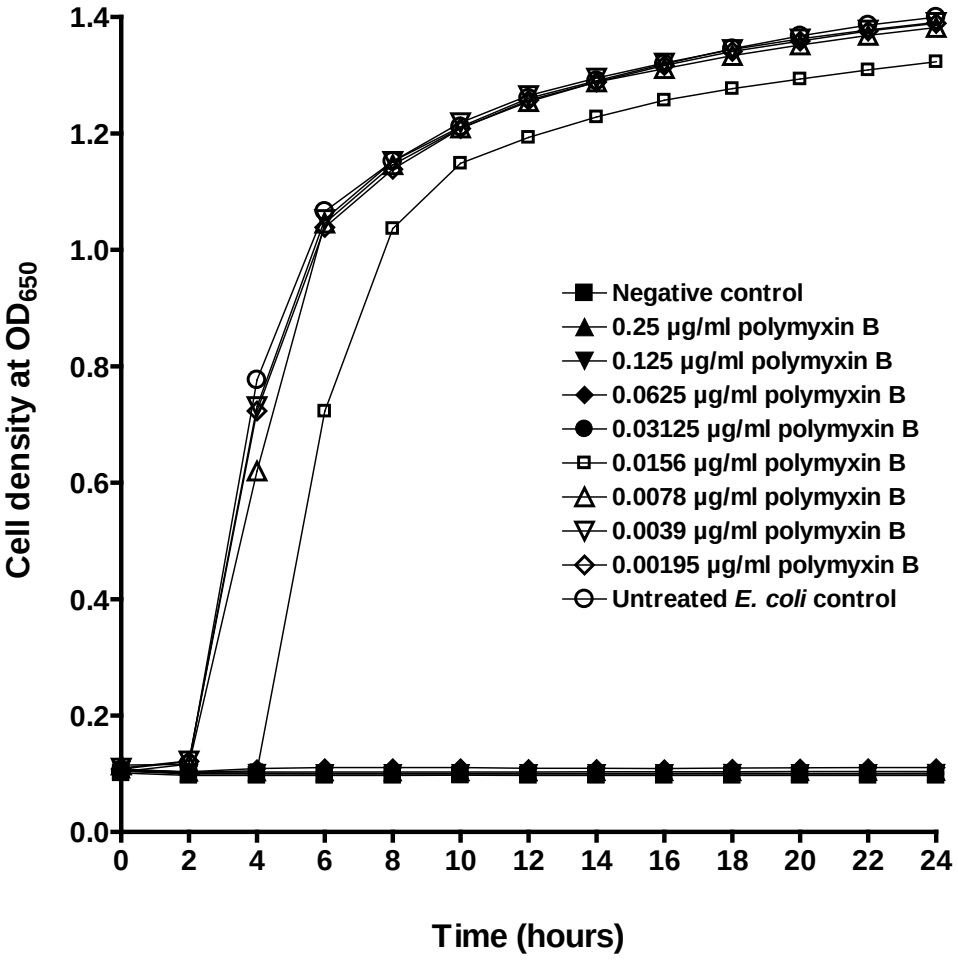


Figure 3.7: MIC of polymyxin B against *E. coli* in Mueller-Hinton broth at 37 ^{o}C. Inoculum density was 1 x 10^{6} cfu/ml. The mean optical densities were plotted against time (hours) for the outcomes of two experiments with three replicates each.

Unlike data presented in figures 3.2, 3.3, 3.4, 3.5 and 3.6 for the activity of the chalcone-derivatives, the initial optical density readings (OD_{650}) for cells treated with polymyxin B in the results reported in figure 3.7 were a much lower value of about 0.1 unit probably because this antibiotic forms a true and lucid solution with water as solvent. As would be expected, polymyxin B demonstrated relatively higher MIC values of 256, 512 and 256 µg/ml against MSSA, MRSA and QRSA respectively, being a Gram-negative specific antibiotic, given that these organisms are Gram-positive. Similarly, the MIC and MBC values of colistin against MSSA were both 140 µg/ml.

3.4 Discussion

All the chalcone-derivative compounds investigated demonstrated poor solubility in aqueous medium. Compounds F1, F2 and F23 formed milk-white suspensions, indicating chemical incompatibility and poor solubility in aqueous medium. The modified antibiotic-agar dilution technique was therefore developed and applied to enable the evaluation of the antibacterial activity of these compounds whilst dealing with the challenge posed by their poor aqueous solubility. In addition, considering that the chalcone-derivatives were supplied in very limited quantities, there was the need to develop an assay that enabled the simultaneous screening of multiple bacterial species with reduced quantities whilst dealing with their poor aqueous solubility. In the assay developed, molten agar-antibiotic mixture in a pre-heated ($\leq 55\ ^{o}C$) static water-bath (to avoid thermal degradation of the test compound) was adjusted to a desired final antibiotic as well as solvent concentration. The suspended chalcone compounds were unable to coalesce out of the agar once the agar became solidified and their presence affected the growth of the bacterial species, if the organism was sensitive to the compound.

The data presented in table 3.1 from the modified antibiotic-agar dilution assay show that F1, F5, F7 and F17 demonstrated activity in the range of modest to very weak broad-spectrum antibacterial action against MSSA, MRSA and *E. coli*. This observation is consistent with findings from our earlier studies which established the poor antibacterial as well as antifungal actions of a series of these chalcone and chalcone-derivatives (Ghani et al. 2012).

Nonetheless, data obtained from the investigations made in this report show that in comparison to the other semi-synthetic agents examined, F1 and F23 were most active against *P. caratovoram*, *Cl. michiganensis*, MSSA and QRSA, with MIC values within the range of 16 to 64 µg/ml. Both compounds had been noted for further antibacterial and antifungal studies (Ghani et al. 2012). Both compounds displayed some degree of antibacterial activity against Gram-negative (*P. caratovoram, C. violaceum*, *E. coli* and *Ps. aeruginosa*) and Gram-positive (*C. michiganensis*, MSSA, MRSA and QRSA) with MIC values in the range of 16 to 512 and 32 to 1024 µg/ml respectively. These compounds did not therefore exhibit preference for any class of bacteria, irrespective of the absence of an outer membrane consisting of an anionic lipopolysaccharide in Gram-

positive bacterial species (Wu and Hancock 1999). This is understandable given that the chemical structures of these chalcone compounds presented in figure 1.5 show the absence of a net charge. The presence of a net positive charge (as seen with the polymyxins in figure 1.3) is likely to have influenced these agents binding affinity for the lipopolysaccharide structure in Gram-negative bacterial species, as with the former polycationic antibiotic (Falagas, Kasiakou and Saravolatz 2005, Jenssen, Hamill and Hancock 2006a, Hale and Hancock 2007), although investigational emphasis is now shifting from pore formation and outer membrane disruption to other alternative intracellular sites, as additional potentential targets of such cationic antimicrobial peptides (Hale and Hancock 2007). It is therefore possible that these chalcone-derivatives can interact with teichoic as well as lipoteichoic acids in the wall of Gram-positive bacterial species. Although the potential mechanisms of action of these chalcone-derivatives are yet to be explored, a similar flavonoid compound (-)-epicatechin demonstrates weak antibacterial action (Anderson et al. 2005, Stapleton et al. 2007). But with some structural activity modifications, (-)-epicatechin gallate (ECg) is more active and is thought to sensitize MRSA to the action of β-lactam antibiotics by mediating alterations to the cytoplasmic membrane, promoting the release of lipoteichoic acid from this bilayer structure and thereby enforcing architectural changes to the cell wall teichoic acid that modulates the wall properties to make them more susceptible to such antibiotics (Stapleton et al. 2007). This action of ECg is also thought to be neither related to the expression of decreased penicillin-binding proteins (PBP) 2a nor to the binding of this flavonoid compound to peptidoglycan. Rather, similar to the antistaphylococcal action of galangin, ECg promotes cell aggregation (Cushnie et al. 2007) and in addition, increases the thickness of cell wall whilst decreasing peptidoglycan cross-linking by 5 – 10 %; which is thought to be of little significant effect upon the overall mechanism of action (Stapleton et al. 2007). Therefore, further structural activity modifications aimed at improving the aqueous solubility and enhancing the antimicrobial potency of both F1 and F23 should be undertaken. This will hopefully lead to a reduction in their MIC values against bacterial species. It is desirable for antibacterial compounds to demonstrate strong activity against pathogenic organisms, with MICs ≤4 to 8 µg/ml against sensitive strains and ≤8 to 16 µg/ml against intermediate and resistant strains of *S. aureus*, as has been noted for vancomycin (Tenover *et. al.* 2007). Antibiotics with MIC

values at sub-microgram levels are more likely to display reduced potential to cause concentration-related adverse effects *in vivo* at therapeutically administered doses (Van Bambeke et al. 2004). The relatively small doses administered will exhibit minimal impact upon other sites that are not the primary target, eliciting insignificant degrees of unwanted effects (Tzeng et al. 2005, Van Bambeke et al. 2004). Unlike the values obtained from this study for the chalcone-derivatives that suggested weak antibacterial activity and verified previously published data for these chalcone-derivatives (Ghani et al. 2012), panduratin A and isobavachalcone display desirably low MIC values of 0.06 to 2.0 and 0.3 to 0.6 µg/ml against species of *S. aureus*, using the same broth micro dilution assay applied in this report, although with a cell density of 5×10^5 and 3.75×10^4 cfu/ml respectively (Mbaveng et al. 2008, Rukayadi et al. 2009).

Comparing data presented in tables 3.1, 3.2 and 3.3, the MIC values for F1 obtained from the antibiotic-broth micro dilution assay were 32, 64, 128 and 256 µg/ml against QRSA, MSSA, MRSA and *E. coli* respectively. The reported MIC values of 32 and 256 µg/ml for F1 against QRSA and *E. coli* are the same from the two different techniques. Hence, the data from the conventional micro broth dilution assay validated the results obtained from the modified antibiotic-agar dilution assay. Meanwhile, the MIC values for F1 from antibiotic-agar dilution assay against the same bacterial species above were 32, 128, 256 and 256 µg/ml respectively. Although the antibiotic-agar micro dilution technique was conducted with a lower inoculum density of 1×10^4 cfu/ml, some of the MIC values from this modified technique were either the same or one to two dilution steps values higher than those obtained from the antibiotic-broth micro dilution assay. This is most probably because there was increased contact and therefore enhanced interaction between the test compound and the challenged bacterial species in the liquid milieu than occurs upon the solid agar surface (Wiegand, Hilpert and Hancock 2008, Mann and Markham 1998). Organisms that were challenged in a planktonic environment have a greater surface area of interaction with the test antimicrobial agent than those placed upon the agar surface and this may explain why the former exhibits lower MIC values, given the greater contact surface area.

Results recorded in table 3.5 show that polymyxin B demonstrated MIC values of 2 and 16 µg/ml against the plant pathogens *P. caratovoram* and *C. michiganensis* respectively. Understandably, the MIC value of polymyxin B

against *P. caratovoram* is much lower than that obtained for *C. michiganensis* because the latter organism is a Gram-positive bacterium devoid of outer membrane, the primary target for polymyxins (Hancock and Chapple 1999). In the same table mentioned above, colistin and polymyxin B were approximately 90 to 8000-fold more active against Gram negative *E. coli* and *Ps. aeruginosa* than Gram-positive MSSA. With an MIC value of 32 µg/ml, polymyxin B demonstrated what may be regarded as a clinically weak activity against *C. violaceum*, a Gram-negative organism, in an antibiotic-broth micro dilution assay. Although this reported activity is much better than the activity of this polypeptide antibiotic against Neisseria species, the availability of other antibiotics that demonstrate far lower MIC values *in-vivo* along with excellent safety profiles makes polymyxin B to be considered as clinically ineffective against *N. gonorrhoea* (Res 2003) and even some subspecies of *N. meningitides* are intrinsically resistant to this antibiotic, with MICs ≥512 µg/ml (Tzeng et al. 2005). Hence, despite this wide margin in the susceptibilities of *N. gonorrhoea*, *N. meningitides* and *C. violaceum* to polymyxin B, the latter organism was employed as a model in this project because it is a saprophyte and the MIC of 32 µg/ml would still be unacceptable clinically. F1 and F23 demonstrated a higher MIC value of 512 µg/ml against *C. violaceum* indicating a much weaker activity. This organism might be thought of as being insensitive to the presence of F1 and F23 and that by speculation, probably so would *N. gonorrhoea*.

The data presented in table 3.3 suggest that both compounds demonstrated weak activity against *P. caratovoram*, MSSA and QRSA and poor activity against *C. violaceum*, *Ps. aeruginosa* and *E. coli*. In addition F23 could be considered inactive to MRSA at concentrations lower than 512 µg/ml. Effective plant antimicrobial compounds are required to show MICs in the range of 100 to 1,000 µg/ml to their target pathogens in practice (Tegos et al. 2002). Therefore the MIC values of 2 and 16 µg/ml reported for polymyxin B against *P. caratovoram* and *Cl. michiganensis* and 32 and 64 µg/ml against *P. caratovoram* by F1 and F23 respectively are noteworthy. It may be worthwhile for the antibacterial activity of F1 and F23 to be evaluated against other phytopathogenic organisms.

3.5 Conclusion

A novel method that permits evaluation of the antimicrobial activities of hydrophobic antibiotics against an array of organisms simultaneously, with a limited quantity of compound was developed and validated. Some data obtained from this modified assay technique and antibiotic-broth dilution assay indicates that F1, F2, F12, F13, F17 and F23 were the most active of the chalcone-derivatives examined, in view of their MIC values against the test bacterial species. F1 and F2 demonstrated the lowest MIC values against the plant pathogens, *P. caratovoram* and *C. michiganensis*, both of which are Gram-positive and Gram-negative respectively. Although continuous collaborations with medicinal chemists on the possible approaches that could be adopted to improve the antimicrobial activities of these chalcone-derivatives are on-going (Abdel Ghani et al. 2008, Ghani et al. 2012), the options of combinational assays must be explored. This may be a viable alternative especially given that a similar compound (ECg) demonstrates synergistic antibacterial activities with β-lactam antibiotics (Stapleton et al. 2004, Stapleton et al. 2007) and an increasing number of flavonoids with direct as well as synergistic actions with antibiotics have been noted (Cushnie and Lamb 2011).

Chapter 4

Examining the antibacterial effect of chalcone-derivatives alone and with antibiotics

4.1 Introduction

Due to the poor output from the developmental process of new antibiotics in the past three decades (Booth and Zemmel 2004), an option with several advantages to combat the continued emergence of resistant pathogenic bacterial strains is the use of combinational therapy. The combination of either two antibiotics or an antibiotic with an adjuvant is considered as a therapeutic alternative strategy with promise because of the potential for an extended spectrum of activity, synergistic action and a longer useful clinical life-span for such antibiotics due to delay in the emergence of resistant bacterial strains(Cottarel and Wierzbowski 2007). Although polymyxins are clinically effective against most Gram-negative multi-resistant pathogens such as *Acinetobacter species*, *Pseudomonas aeruginosa*, *Klebsiella species*, and *Enterobacter species* (Falagas, Kasiakou and Saravolatz 2005), a leading and current opinion is that polymyxins should be applied in combination with other antimicrobial compounds (Kasiakou et al. 2005). It is thought that this approach will enhance the safety and effectiveness of polymyxins as well as increase their useful clinical life-span by reducing the potential for the emergence of resistant bacterial strains. The safety profile of polymyxins applied in combination with other antibiotics is likely to improve due to the use of lower doses to achieve synergy and consequent lower chances for their dose-related nephrotoxicity adverse effect to occur.

The antibacterial activity of the chalcone-derivatives applied in combination with selected antibiotics was undertaken. Attempts to use the agar disc-diffusion method to examine the occurrence of synergism between the chalcone-derivatives and known antimicrobial compounds were unsuccessful due to the poor aqueous solubility of the semi-synthetic chalcone-derivatives. The chequerboard assay is an effective *in vitro* technique frequently applied for investigating the existence of synergism between two or more compounds (Sánchez-Gómez et al. 2011). This assay applies a simple rational technique that

enables the assessment of the antibacterial activity of varying concentrations of chalcone-derivative in combination with varying concentrations of a known antimicrobial compound in a 96-well micro-titre (Lorian 2005). The pattern of distribution of the various test concentrations for the two compounds is shown in figure 4.1.

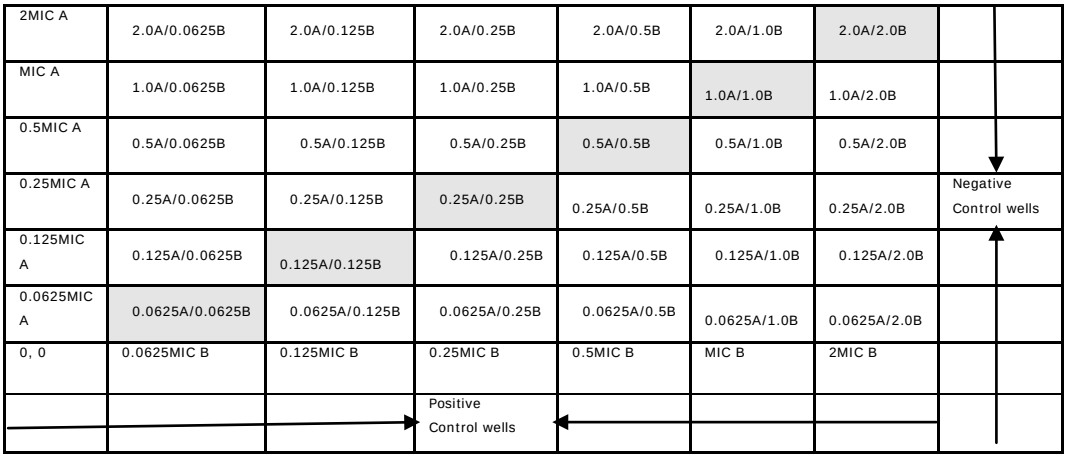


Figure 4.1 Sample of a chequerboard technique showing serial dilutions of two drugs performed with concentrations proportional to MICs of the compounds against the test bacterial species.

A single growth-inhibitory end-point for the challenged inoculum population may then be obtained at any given point, representing the combined synergistic concentration of both compounds (Lorian 2005, Lewis et al. 2002, Roling et al. 2002a). The occurrence of either synergistic or additive action between both compounds can be traced along the shaded 45^{o} axis, although it could occur in wells containing concentrations of compounds that are located well off this line (Pillai et al. 2005a).

The selected antibacterial agents chosen for inclusion in the investigation of potentiated action when applied with the chalcone-derivatives were ciprofloxacin, gentamicin, polymyxin B and penicillin, which all have distinct primary mechanisms of action (table 1.1) (Walsh 2003, Hancock 2005, Bradley and Jones 2006). Ciprofloxacin inhibits DNA replication, gentamicin blocks ribosomal protein synthesis, polymyxins disrupt membrane integrity and penicillin inhibits peptidoglycan synthesis in actively dividing bacterial cells (Cottarel and Wierzbowski 2007). This report will focus only on the data obtained for assays conducted with polymyxin B and ciprofloxacin. Although polymyxins are only clinically useful against Gram-negative organisms (Res 2003), the test bacterial species were selected to include Gram-positive bacterial species (*S. aureus* 6571 (MSSA), *S. aureus* 11940 (MRSA) and *S. aureus* 1628 (QRSA), representing

different strains of antibiotic-sensitive and resistant *S. aureus* species) in an attempt to potentially extend the spectrum of activity of polymyxins. Bearing in mind that polymyxin B is clinically effective against Gram-negative bacterial species (Fernandez et al. 2010), a concomitant activity against these representative Gram-positive species by the combination of this latter antibiotic with any of these semi-synthetic flavonoids may attract further consideration for formulation of a potential antibacterial preparation with a view to eliciting a broader spectrum of action.

Overall antibacterial effect can be rated as either antagonistic, indifferent, additive or synergistic. When the MIC value of either of two compounds in a combination was lower than half the MIC value of any of the individual compounds applied against a test bacterial species, it was suggestive of the existence of either an additive or synergistic relationship between the two compounds (Petersen et al. 2006). The fractional inhibitory concentration (FIC) for the combination doses can be calculated by dividing the MIC value for each of the compounds in the combination by the MIC value obtained for each agent when applied alone and adding the two quotients as shown in the formulas below:

FIC of compound A (FIC_A) = (MIC_A in combination)/(MIC_A alone)

FIC of compound B (FIC_B) = (MIC_B in combination)/(MIC_B alone)

The $FIC_{index} = FIC_A + FIC_B$

Where: FIC_A, MIC_A, FIC_B and MIC_B are FICs and MICs for antibiotic A and B respectively (Chen et al. 2004). The FIC indices were used to characterize the overall antibacterial activity between the chalcone-derivatives and the established antibiotics applied together, as follows: Synergy, $FIC_{index} \leq 0.5$; additive, $0.5 < FIC_{index} < 1$; indifference, $1 < FIC_{index} \leq 4$ and antagonism, $FIC_{index} > 4$ (Chen et al. 2004, Eliopoulos and Moellering 1996). Therefore, the objective for this aspect of the project was the examination of the overall antibacterial effect of the chalcone-derivatives F1 and F23 when each was applied in combination with selected antibiotics against some test bacterial pathogens.

4.2 Materials and Methods

4.2.1 Evaluation of the effect of the chalcone-derivatives in combination with selected antibiotics

The examination of the antibacterial activity of the chalcone-derivatives each applied in combination with selected antibiotics was undertaken *in vitro* in a standard chequerboard assay, as previously described in section 2.6.

4.3 Results

4.3.1 The antibacterial action of F1 applied in combination with either polymyxin B or ciprofloxacin

The data obtained from the assay were interpreted as described above, permitting the determination of the FICs for the effective combination concentrations of either polymyxin B or ciprofloxacin with the semi-synthetic agents in table 4.1.

Bacterial species	MIC (µg/ml)			MBC (µg/ml) of F1 with polymyxin B	FIC_{index}	Interpretation
	F1	Polymyxin B	F1 with polymyxin B			
MSSA	64	1024	16/256	16/256	0.5	synergism
MRSA	128	128	16 /32	16 /32	0.375	synergism
QRSA	32	256	8/32	8/32	0.375	synergism
E. coli	256	0.03125	256/0.03125	512/0.03125	2	indifferent
C. violaceum	>512	32	16/2	32/2	<0.093	synergism
Ps.aeruginosa	256	0.5	16/0.25	64/0.25	0.53125	additive

Table 4.1: The data ($FIC_{indexes}$) obtained from a chequerboard assay for F1 applied in combination with polymyxin B against test bacterial species. The data were at least 2 consistent readings from 3 replicates conducted on separate days. MIC: minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MSSA: *S. aureus* 6571; MRSA: Methicillin-resistant *S. aureus* 11940 and QRSA: quinolone-resistant *S. aureus* 1628. Note that the MIC of polymyxin B applied alone against MSSA had changed from the previously reported 256 to 1024 µg/ml. Inoculum density was 1 x 10^6 cfu/ml.

The data presented in table 4.1 demonstrate that in the *in vitro* combinations, the MICs of F1 and polymyxin B against *S. aureus* 6571 (MSSA) were synergistically reduced from 64 and 1024 (when applied separately) to 16 and 256 µg/ml (when applied in combination) respectively, thereby yielding a FIC_{index} of 0.5. As shown in

both table 4.1 and figure 4.2, the combination also synergistically reduced the MICs of both compounds against *S. aureus* 11940 (MRSA) from 128 and 128 to 16 and 32 µg/ml respectively, with a FIC_{index} of 0.375.

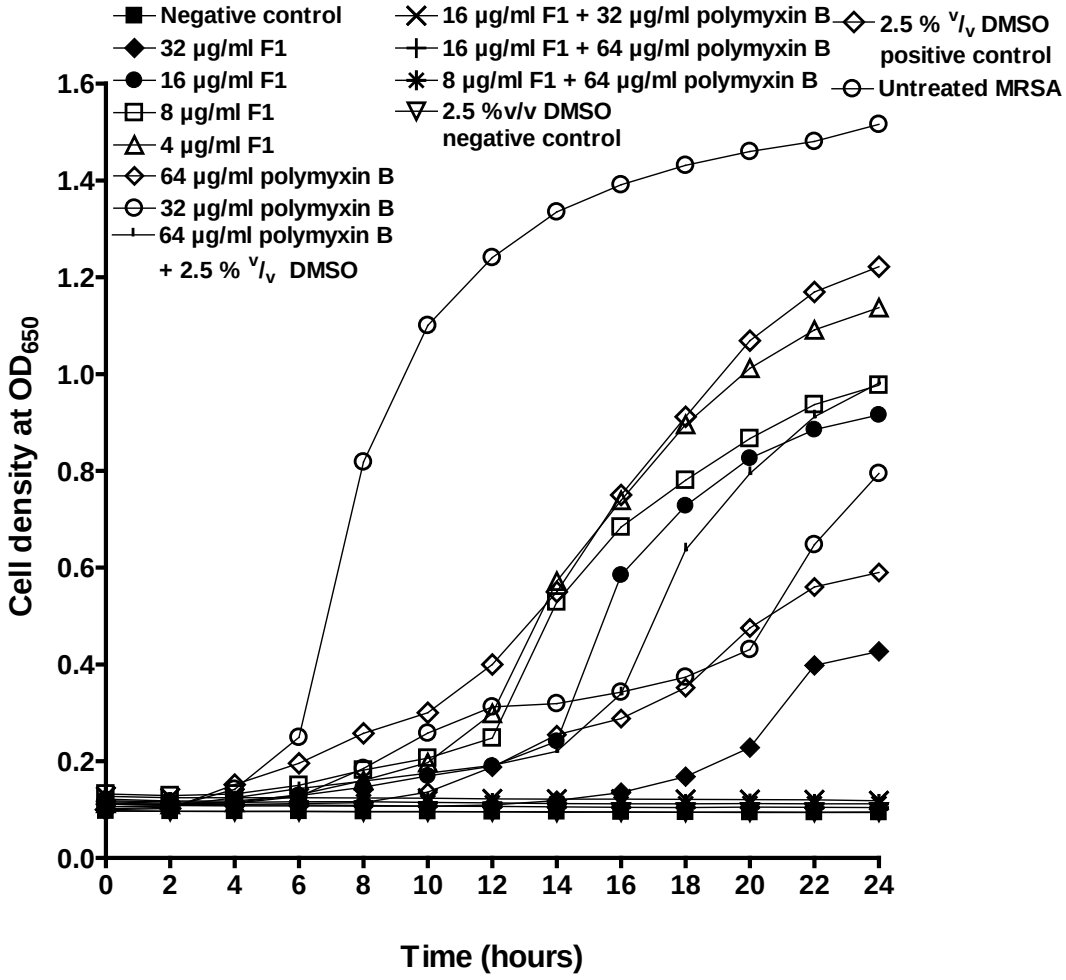


Figure 4.2: Data from chequerboard assays for 16 µg/ml F1 applied with either 32 or 64 µg/ml polymyxin B and 8 µg/ml F1 combined with 64 µg/ml polymyxin B against MRSA. The mean cell densities values at OD_{650} were plotted against time (hours) for the outcomes of two experiments repeated on separate days.

The data presented in figure 4.2 further show that the combinations of 64 µg/ml polymyxin B with either 8 or 16 µg/ml F1 were also synergistic against MRSA, giving an indication that several different combinations of these agents can display bactericidal actions at lower concentrations than would either of the compounds applied separately. The inability of the combination of 64 µg/ml polymyxin B with 2.5 % $^{v}/_{v}$ DMSO, used as a control, to inhibit the growth of the organism suggests the activity reported for the synergistic combinations of F1 with polymyxin B cannot be attributed to the presence of the solvent.

For MSSA, the MICs of both compounds were reduced from 64 and 1024 µg/ml to 16 and 256 µg/ml under a combined application as shown in figure 4.2b.

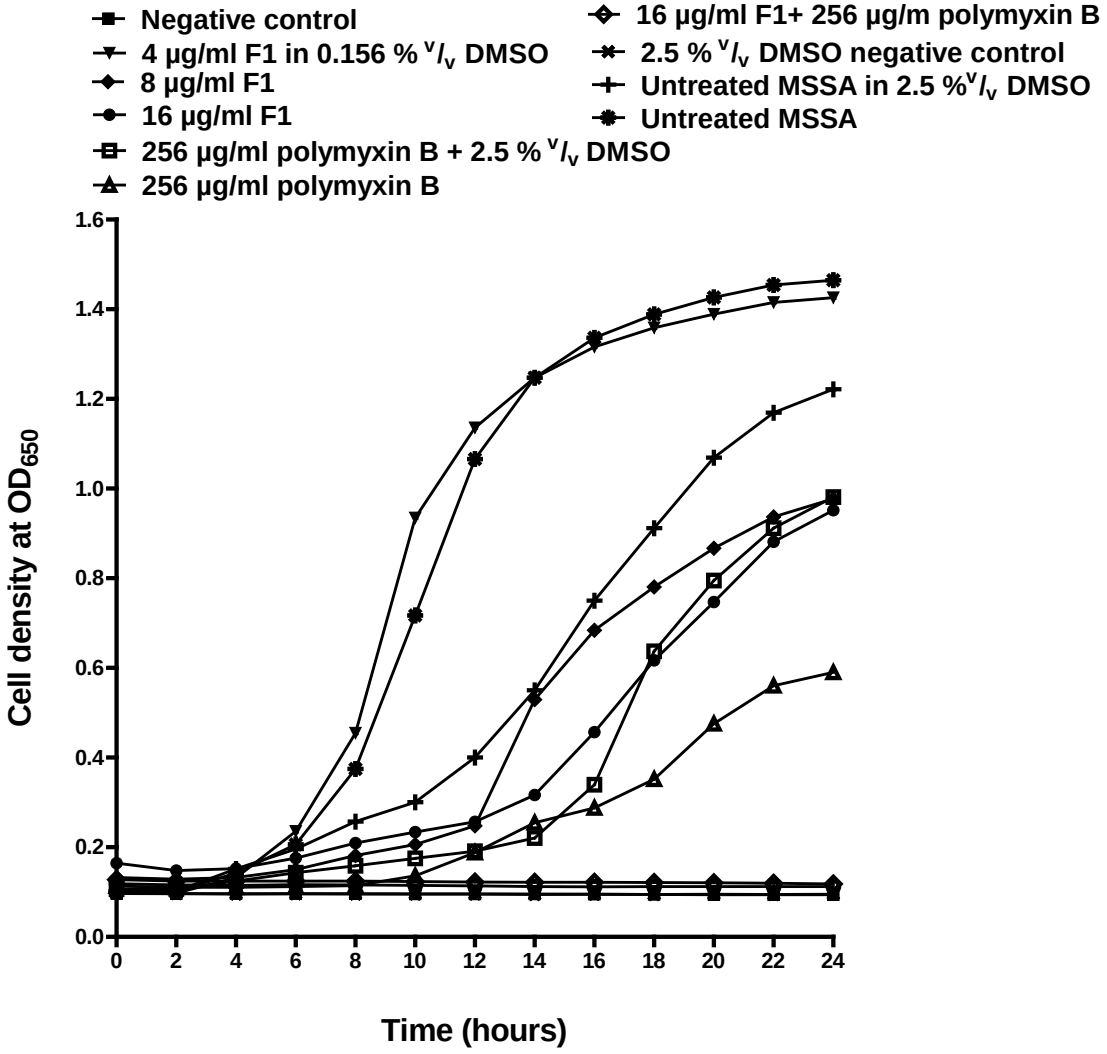


Figure 4.2b: Data from chequerboard assays for 16 µg/ml F1 applied with 256 µg/ml polymyxin B against MSSA. The mean cell densities values at OD_{650} were plotted against time (hours) for the outcomes of two experiments repeated on separate days.

Although the 3 concentrations of 4, 8 and 16 µg/ml of F1 demonstrated increasing activity against MSSA, none of them was bactericidal unlike the combination of the latter strength with polymyxin B. With the quinolone-resistant *S. aureus* 1628 (QRSA), the MICs of both compounds were synergistically reduced from 32 and 256 to 8 and 32 µg/ml respectively (figure 4.3), yielding the same FIC_{index} value of 0.375 as shown in the table 4.1.

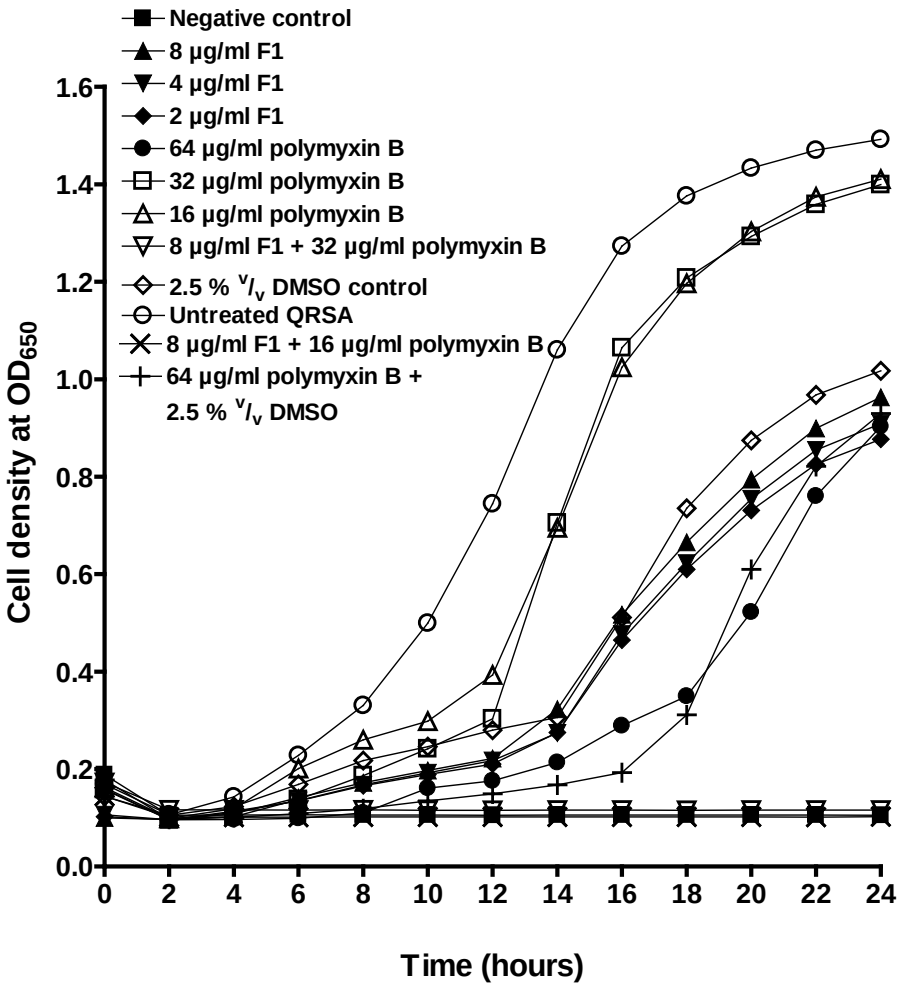


Figure 4.3: Data from chequerboard assays for 8 µg/ml F1 applied with either 32 or 16 µg/ml polymyxin B against QRSA. The mean OD_{650} values were plotted against time (hours) for the outcomes of two experiments repeated on separate days.

The results presented in figure 4.3 corroborates with data in table 4.1 which showed that the application of 8 µg/ml F1 with 16 µg/ml polymyxin B was also synergistic against the challenged inoculum and yielded an FIC_{index} of 0.3125. The two consecutive previous figures show that both MRSA and QRSA cells expressed a some levels of sensitivity to the presence of DMSO as solvent relative to all other bacterial species examined in this study. As previously noted in figures 3.1 and 3.6, the result presented in figure 4.3 also clearly shows the growth kinetic of QRSA suspension treated with only 2.5 $^{v}/_{v}$ DMSO as solvent control were adversely affected in comparison to the untreated positive control cells. The solvent control cells exhibited a longer lag time and a slower growth rate that peaked at an OD_{650} of 1 unit. Whilst the growth kinetic of the cells treated with sub-inhibitory concentrations of 8 and 16 µg/ml polymyxin B but

devoid of DMSO were faster in onset, exhibiting optical density readings of <1.4 unit, the untreated solvent-free control cells demonstrated the fastest onset and rate of growth that peaked at about 1.5 unit at OD_{650}. These polymyxin-treated cells had a growth kinetic curve that was higher in rate and extent than those treated with the chalcone-derivatives containing DMSO as solvent and therefore displayed curves that were separated from those obtained from either the latter or DMSO solvent control samples. However, the degree of the bactericidal action of the combination of F1 with polymyxin B makes this solvent effect insignificant. In addition, cells treated with 64 µg/ml polymyxin B in the presence of 2.5 % $^{v}/_{v}$ DMSO demonstrated uninhibited growth suggesting the augmentative antibacterial action recorded for the 2 synergistic combinations of F1 and polymyxin B against QRSA above may not be attributable to the presence of the solvent.

With *E. coli*, both F1 and polymyxin B were indifferent in their combined antibacterial action with a FIC_{index} of 2, as both retained their original MIC values of 256 and 0.03125 µg/ml when applied in combination. Of note, with the *in vitro* combined application of both compounds against *C. violaceum*, the MICs of both compounds were reduced from 512 and 32 to 16 and 2 µg/ml respectively as shown in figure 4.4.

This combination demonstrated considerable antibacterial synergism, with a FIC_{index} of 0.094, in this instance. This FIC_{index} gave an indication that *C. violaceum* was the most susceptible organism to the combination of F1 with polymyxin B amongst the test bacterial species. The data presented in the figure suggests an extension of the endpoint time from 24 to 48 hours may have been more revealing as the effective combination strengths began to become overwhelmed by cell growth after 20 hours of incubation. To corroborate this point, results presented in table 4.1 also show that the combination of 32 µg/ml F1 with 2 µg/ml polymyxin B, which were higher than the former mentioned strengths, were recorded as the bactericidal concentration against *C. violaceum*, thereby verifying the observation being reported for the action of 16 µg/ml F1 with 2 µg/ml polymyxin B in figure 4.4. As was previously shown in figure 3.5, the impact of 2.5 % $^{v}/_{v}$ DMSO on *C. violaceum* was comparatively minimal in that the solvent treated cells were seen to demonstrate a rate and an extent of growth kinetic that is comparable to the untreated population. In figure 4.4b, F1 applied in combination with polymyxin B demonstrated an additive action against

Ps. aeruginosa with a FIC_{index} of 0.53. The MICs of F1 and polymyxin B against this bacterial species was reduced from 256 and 0.5 µg/ml (when applied alone) to 16 and 0.25 µg/ml (when applied in combination) respectively.

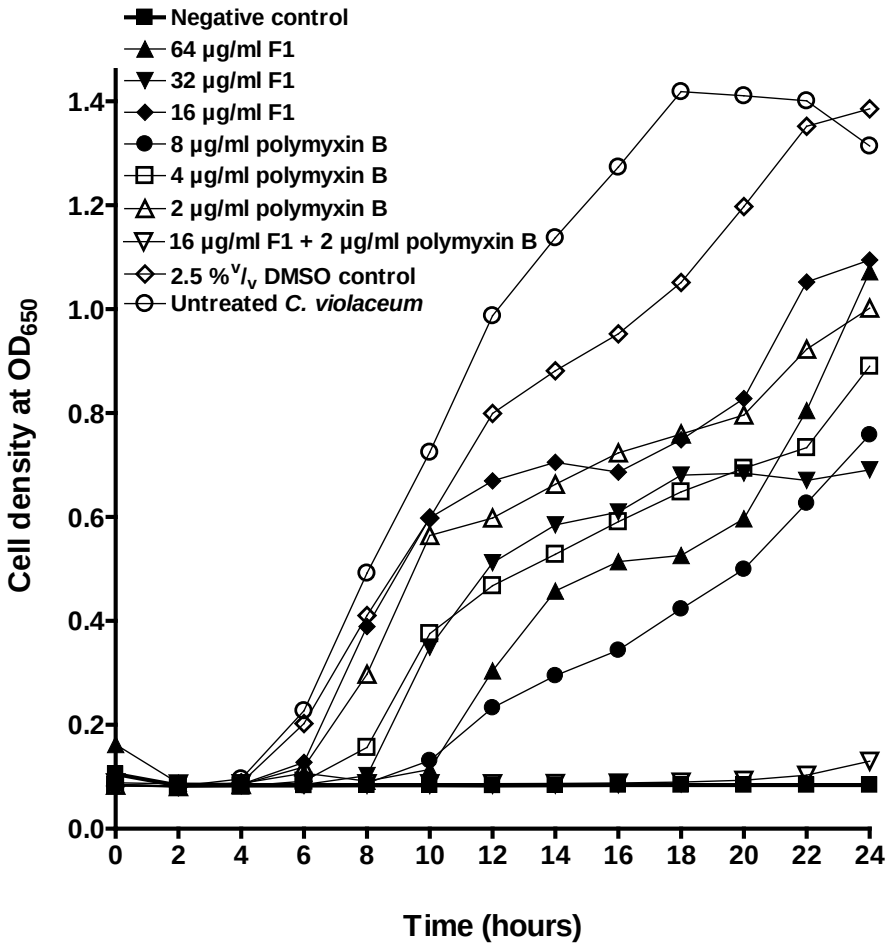


Figure 4.4: Data from chequerboard assays for 16 µg/ml F1 applied with 2 µg/ml polymyxin B against *C. violaceum*. The mean cell densities at OD_{650} from two experiments repeated on separate days were plotted against time (hours).

Therefore F1 and polymyxin B demonstrated a 16 and a 2-fold reduction in MIC values respectively. Such a disproportionate reduction in the MICs of both compounds with bactericidal action against *Ps. aeruginosa* often makes the well containing the best synergistic combination concentrations to fall outside (farther away from) the 45° axis previously described. The MICs obtained when methicillin-sensitive *S. aureus* 6571 was challenged with a range of F1 and ciprofloxacin concentrations independently applied were 64 and 32 µg/ml respectively. Both MIC values were however synergistically reduced to 4 and 8 µg/ml respectively when both compounds were applied in combination, yielding a FIC_{index} of 0.313 that indicated these compounds were synergistic in action.

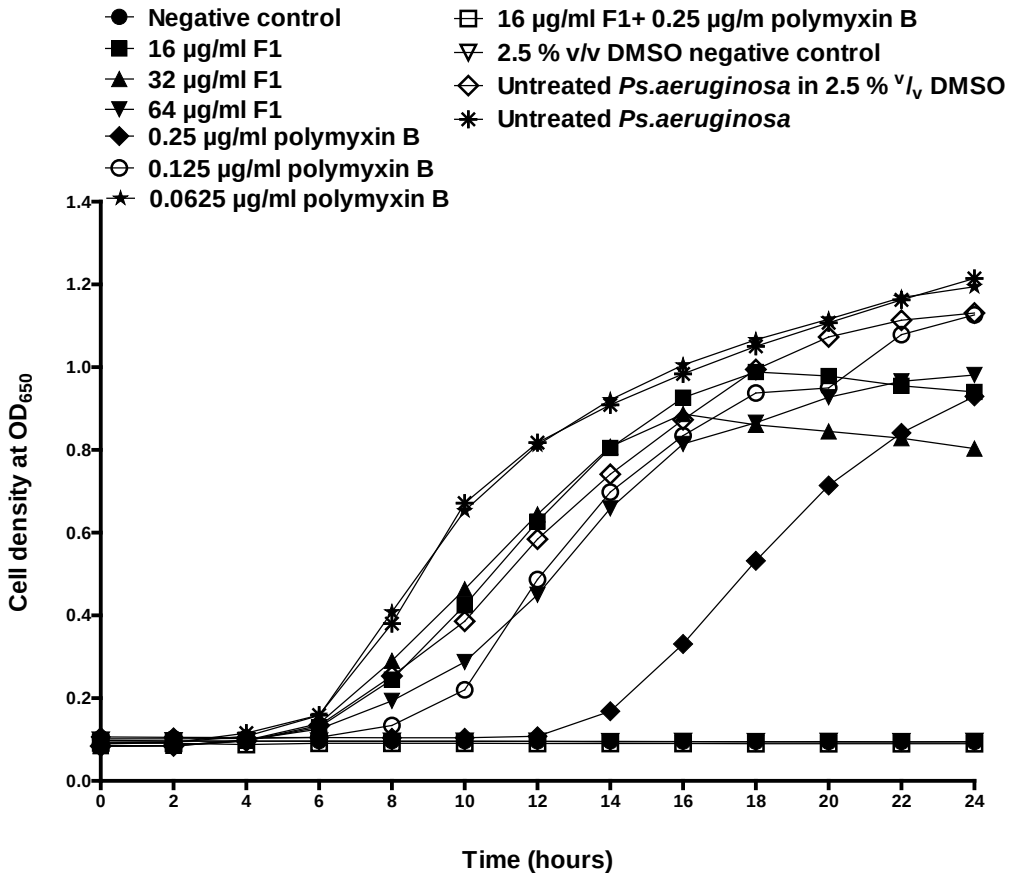


Figure 4.4b: Data from chequerboard assays for 16 µg/ml F1 applied with 0.25 µg/ml polymyxin B against *Ps. aeruginosa*. The mean cell densities at OD_{650} from two experiments repeated on separate days were plotted against time (hours).

4.3.2 The antibacterial action of F23 applied in combination with polymyxin B

The antibacterial effect of F23 applied in combination with polymyxin B against test bacterial species is shown in table 4.2. The data presented in the table demonstrate that in the *in vitro* combination, the MICs of F23 and polymyxin B against MSSA were synergistically reduced from 64 and 1024 (when applied separately) to 16 and 256 µg/ml (when applied in combination) respectively, thereby yielding a FIC_{index} of 0.5. The combination reduced the MICs of both compounds against MRSA from >1024 and 128 to 32 and 64 µg/ml respectively yielding a FIC_{index} of <0.53 that indicated both compounds were additive in action against this bacterial species (figure 4.5). Similarly, both compounds applied in combination were also additive in action against QRSA populations, by having their MICs reduced from 32 and 256 (when applied alone) to 16 and 64 µg/ml (when applied together), thereby yielding a FIC_{index} of 0.75.

Bacterial species	MIC (µg/ml)			MBC (µg/ml) of F23 with polymyxin B	FIC_{index}	Interpretation
	F23	Polymyxin B	F23 with polymyxin B			
MSSA	64	1024	16/256	16/256	0.5	synergism
MRSA	>1024	128	32/64	32/64	<0.53125	additive
QRSA	32	256	16/64	16/64	0.75	additive
E. coli	512	0.03125	32/0.03125	32/0.03125	0.1625	synergism
C. violaceum	>512	32	32/8	64/4	0.3125	synergism
Ps.aeruginosa	256	0.5	32/0.125	32/0.125	0.375	synergism

Table 4.2: The data ($FIC_{indexes}$) obtained from a chequerboard assay for F23 applied in combination with polymyxin B against test bacterial species. The data were at least 2 consistent readings from 3 replicates conducted on separate days. MIC: minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MSSA: *S. aureus* 6571; MRSA: Methicillin-resistant *S. aureus* 11940 and QRSA: quinolone-resistant *S. aureus* 1628. Note that the MIC of polymyxin B applied alone against MSSA had changed from the previously reported 256 to 1024 µg/ml. Inoculum density was 1 x 10^6 cfu/ml.

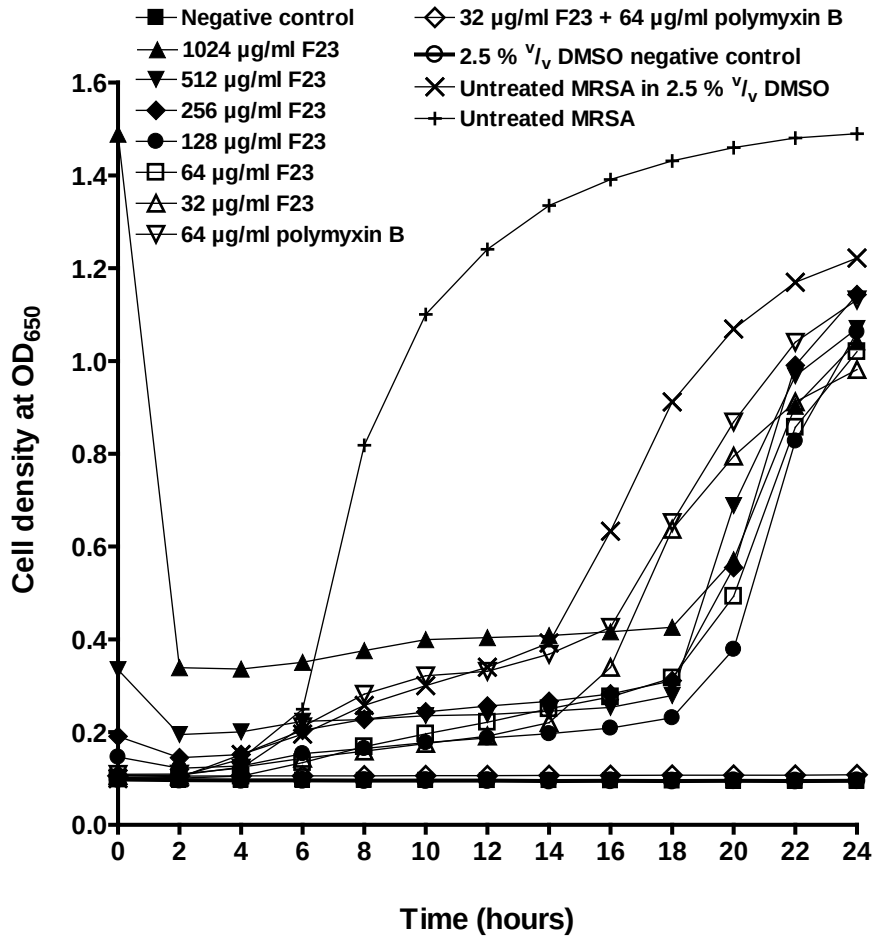


Figure 4.5: Data from chequerboard assays showing the synergistic activity of 32 µg/ml F23 applied with 64 µg/ml polymyxin B against *S. aureus* 11940 (MRSA). The mean ODs are plotted for the outcomes of two experiments repeated on separate days.

The antibacterial activity of F23 applied in combination with polymyxin B against *E. coli* was synergistic but conspicuously outside the imaginary 45^{o} axis, as obtained for F1 applied in combination with polymyxin B against *Ps. aeruginosa*. In this instance, whilst the MIC of F23 was reduced 16-fold from 512 to 32 µg/ml, that of polymyxin B was retained at the initial value of 0.03125 µg/ml. There was another observation in the antibacterial actions of either F1 or F23 applied in combination with polymyxin B against *E. coli*. When F1 was applied in combination with polymyxin B against *E. coli*, the MICs of both F1 and polymyxin B were retained at their initial values of 256 and 0.0313 µg/ml respectively. Whereas with F23 was applied in combination with polymyxin B against *E. coli*, the MIC of F23 was reduced from 512 to 32 µg/ml whilst that of polymyxin B was also retained at the initial value of 0.03125 µg/ml.

The antibacterial activity of F23 applied in combination with polymyxin B against *C. violaceum* is once again noteworthy. The MICs of F23 and polymyxin B were synergistically reduced from 512 and 32 to 32 and 8 µg/ml respectively, yielding a FIC_{index} of 0.313 (figure 4.6).

In this same assay, the combination of either 16 or 32 µg/ml F23 each with 16 µg/ml polymyxin B were equally bactericidal against *C. violaceum*, thereby exposing the various bactericidal flexibilities from different concentration combination of both compounds against this bacterial species that can be further explored.

Finally, in contrast to the additive activity of F1 applied in combination with polymyxin B against *Ps. aeruginosa*, the MICs of F23 and polymyxin B were synergistically reduced from 256 and 0.5 to 32 and 0.125 µg/ml respectively, yielding a FIC_{index} of 0.375.

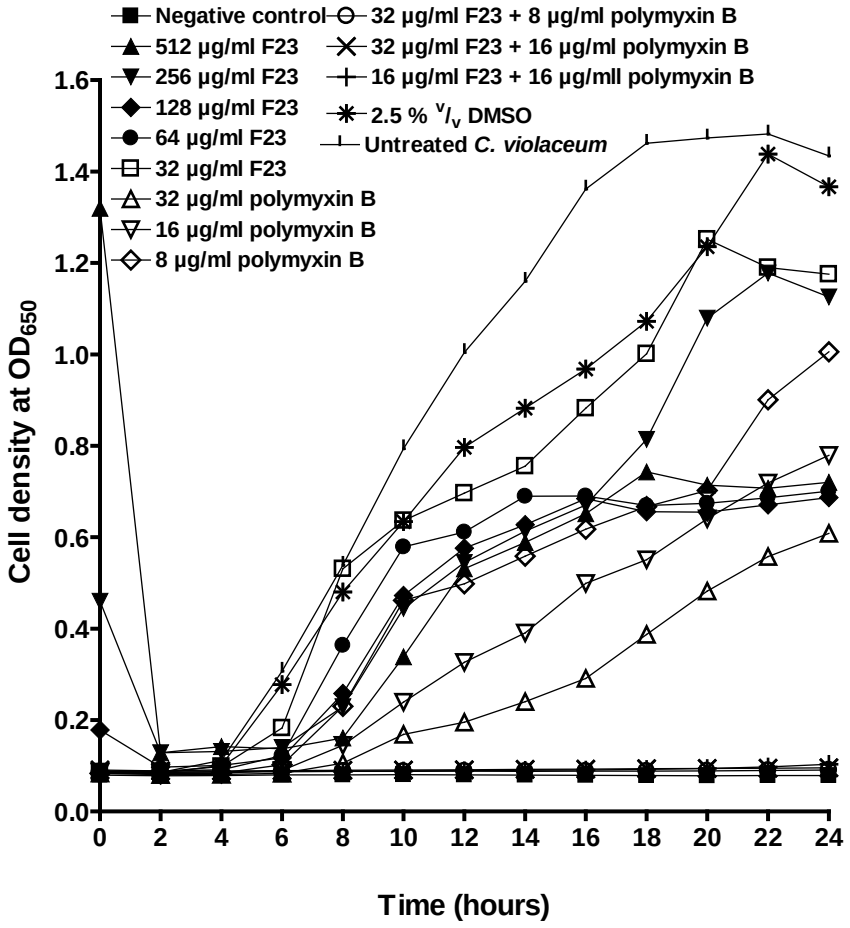


Figure 4.6: Data from chequerboard assays showing the synergistic actions of 32 µg/ml F23 applied in combination with either 8 or 16 µg/ml polymyxin B as well as 16 µg/ml F23 combined with 16 µg/ml polymyxin B against *C. violaceum*. The mean ODs are plotted for the outcomes of two experiments repeated on separate days.

4.4 Discussion

Antibiotics applied in combination in a chequerboard assay are assumed to possess an identical dose response and to contribute equally to the overall outcome of their collective effect upon the challenged bacterial population (Roling et al. 2002a). Such is the case in an ideal situation and if the combined effect of both compounds was synergistic, the most effective concentrations would be contained in wells positioned along the imaginary 45^{o} axis on the micro-titre plate (Lorian 2005). An observation with the chequerboard assay is that although dilutions of concentrations of the chalcone-derivatives applied in combination with the established antibiotics were made on this premise; both compounds could possess dissimilar dose responses at the single growth-inhibitory endpoint (Lewis, Klepser and Pfaller 1999). But a limitation of this *in vitro* assay is that

even if the growth inhibitory end-point concentration wells were located along the 45^{o} line, the information obtained could be misleading if extrapolated and applied presumptuously in an *in vivo* condition, as the ratios of the two compounds may not remain constant upon administration in doses that are proportional to their MICs due to differences in their biological half-lives as well as susceptibility to metabolic degradation (Lorian 2005). This is connected to differences in pharmacodynamic and pharmacokinetic parameters for varying chemical substrates (Owens and Ambrose 2007). It is therefore important to take note of additive and synergistic actions that occur outside the 45^{o} axis as further *in vivo* assays may be required to guarantee the usefulness of the most effective combination concentrations.

The data presented in table 4.1 indicate that the combination of F1 with polymyxin B was synergistic in action against MSSA, MRSA, QRSA, *E. coli* and *C. violaceum* and additive against *Ps. aeruginosa* respectively in the chequerboard assays. Similar data presented in table 4.2 indicate that F23 applied in combination with polymyxin B was synergistic in action against MSSA, *E. coli*, *C. violaceum* and *Ps. aeruginosa*; and additive against MRSA and QRSA respectively. None of the combinations gave indications that were suggestive of the occurrence of antagonism.

The synergisms demonstrated by F1 when applied with polymyxin B against MSSA, MRSA, QRSA and *C. violaceum* were displayed along the midline suggesting both compounds were exhibiting a similar dose-response against these bacterial species. This observation may suggest the cells were equally sensitive to the combined presence of these agents and an inhibition of either a common target site or metabolic pathway in these organisms by both compounds, as seen with the application of trimethoprim with sulphonamides (Grim et al. 2005). Trimethoprim and sulphonamides are individually bacteriostatic agents applied as a combinational antibiotic to achieve a synergistic outcome due to their capacity to inhibit at two separate points simultaneously the folic acid synthetic pathway, a small component of a very broad and complex metabolic process known as the aromatic biosynthesis pathway (Jain 2012). Sulphonamides being derivatives of *p*-aminobenzenesulphonamide are structural analogues and competitive antagonists of *p*-aminobenzoic acid, probably upon the attachment of the sulpha molecule to the benzene ring (Hanson 2006). Sulphonamides are therefore able

to inhibit dihydropteroate synthase, an enzyme responsible for the incorporation of *p*-aminobenzoic acid into dihydropteroic acid which is an immediate precursor of folic acid (Goldstein and Proctor 2008, Lumb et al. 2009).

F1 and polymyxin B applied in combination demonstrated synergism against MSSA, MRSA and QRSA by displaying a ≥4-fold reduction in their MICs. The most profound antibacterial activity reported from the combination assay was that of F1 applied in combination with polymyxin B against *C. violaceum*, which yielded a FIC_{index} of 0.094. This combination demonstrated a 32 and 16-fold reduction in the MICs of both compounds. Considering that *C. violaceum* is a member of the Neisseriaceae family, this response would make the examination of the antibacterial activity of this combination of compounds against antibiotic resistant strains of *Neisseria* species very worthwhile; as recently, a strain of *N. gonorrhoea* that is resistant to all available antimicrobial agents in the clinic was recovered indicating a possibility for the emergence of untreatable gonorrhoea shortly (Bolan, Sparling and Wasserheit 2012).

The activities of F1 and polymyxin B reported against *E. coli* and *Ps. aeruginosa* were indifferent and additive respectively, with both organisms, which incidentally are Gram-negative, showing either little or no further sensitivity to the presence of the combined agents than they would to polymyxin B alone. This may indicate that the degree of bactericidal action of polymyxin B, a Gram-negative specific antibiotic (Zavascki et al. 2008) surpasses that of the combination against both pathogens and that the presence of the chrome-derivatives is not required to facilitate the activity of this polycationic agent against the anionic lipopolysaccharide structure, which is considered the primary target site in the outer membrane bilayer of this class of organisms for this antibiotic (Fernandez et al. 2010, Martti 2010). Although the combination of F1 with polymyxin B was additive in action against *Ps. aeruginosa*, the application of F23 with the latter antibiotic was synergistic in activity, with this organism displaying greater sensitivity to polymyxin B in the presence of this latter chalcone-derivative, as the MIC shifted from 0.5 to 0.125 µg/ml (table 4.2). The data presented show that the additive and synergistic actions of F1 and F23 each in combination with polymyxin B against *Ps. aeruginosa* respectively also required a relatively smaller fraction of both chalcone derivatives. For in table 4.1, the MIC of F1 was reduced from 256 to 16 µg/ml and in table 4.2 that of F23 was reduced from 256 to 32 µg/ml respectively. In the combination,

although F1 and F23 demonstrated a 16 and an 8-fold reduction in MIC, polymyxin B only showed a 2-fold reduction.

It was reported in chapter 3 that both F1 and F23 displayed poor activity against *C. violaceum*, *Ps. aeruginosa* and *E. coli* at concentrations lower than 256 µg/ml and that F23 was inactive against MRSA at concentrations lower than 1024 µg/ml. With the chequerboard assay, F1 applied in combination with sub-inhibitory concentrations of polymyxin B was additive in action against *Ps. aeruginosa*. Generally, tolerance seemed to be expressed to compound F1 by the 3 Gram-negative bacterial species of *E. coli*, *C. violaceum* and *Ps. aeruginosa* by the demonstration of either a one or two steps increment in the MBC values recorded in table 4.1 against these organisms. This observation was indeed not made for polymyxin B, although both agents were applied in combination. This occurrence may be partly due to difficulties associated with both permeability and efflux (Walsh et al. 2003, Cottell et al. 2009). A possibility for the development of tolerance by Gram-negative bacterial species to hydrophobic agents through both above mentioned mechanisms is well established (Hancock 1997, Hancock and Rozek 2002). In fact, structurally unrelated antimicrobial agents, including biocides are sometimes ineffective due to efflux-mediated resistance mechanism (Walsh et al. 2003, Poole 2000).

In table 4.2, F23 applied in combination with sub-inhibitory concentrations of polymyxin B was synergistic against these same Gram-negative organisms. And, the occurrence of tolerance for the action of F23 was also noted when this agent was applied in combination with polymyxin B against C. violaceum by a one-step increment in the MBC value recorded against this organism. Meanwhile, the reported value for polymyxin B reduced by a factor of one-step dilution. On the other hand, all the reported MIC values were retained as the MBC values for the combination of F23 with polymyxin B against all three Gram-positive bacterial species of *S. aureus* (MSSA, MRSA and QRSA respectively). *Ps. aeruginosa* is a Gram-negative bacterium possessing a capacity for both intrinsic and adaptive resistance to antimicrobial peptides (Hocquet et al. 2003, Fernandez et al. 2010) and harbouring numerous virulence factors (Schurek, Breidenstein and Hancock 2012). The intrinsic resistance and virulence of *Ps. aeruginosa* are hugely up-regulated in response to sub-inhibitory concentrations of polymyxin B, colistin and some naturally occurring peptides (Gooderham and Hancock 2009). Hence, it would be worthwhile to examine the mechanism by which these chalcone-

derivatives are able to demonstrate enhanced synergistic activity with sub-inhibitory concentrations of polymyxin B against *Ps. aeruginosa*.

There are on-going attempts to achieve a better understanding of the mechanisms by which antimicrobial peptides induce adaptive resistance responses in *Ps. aeruginosa.* It has been shown that the two-component response regulator proteins PhoP-PhoQ and PmrA-PmrB are up-regulated when treated with polymyxin B and influence the expression of the arnBCADTEF lipopolysaccharide modification operon (Fernandez et al. 2010, Zavascki et al. 2006). In so doing, these regulatory systems activate resistance to polymyxin B and other cationic peptides in this organism (Fernandez et al. 2010, McPhee, Lewenza and Hancock 2003). It would therefore be sensible to examine whether either F1 or F23 down-regulates these resistance genes in *Ps. aeruginosa*, in the presence of sub-inhibitory concentrations of polymyxin B within the course of their combined synergistic activity in the combination assay.

In the presence of polymyxin B, the MICs of both F1 and polymyxin B against *E. coli* were retained at their individual values; an indication that both compounds neither enhanced nor interfered with the activity of each other against this bacterial strain. This may suggest the anionic lipopolysaccharide outer membrane in this organism was a highly effective barrier against the permeation of F1 especially considering the absence of a net (positive) charge on the molecular structure of this compound. However, F23 applied in combination with polymyxin B was synergistic in action against *E. coli* (like this same agent did against *Ps. aeruginosa* and *C. violaceum*, all of which are Gram-negative bacterial species) with the former chalcone compound displaying a 16-fold reduction in MIC whilst the latter retained the previous individual MIC value of 0.0313 µg/ml. This may suggest that polymyxin B promoted the translocation of F23 across the outer membrane bilayer barrier of *E. coli* (these organisms), thereby enhancing the antibacterial action of this chalcone-derivative in their combined applications against this class of bacterial species. This speculation could be explored further to guide the development of polymyxins combinations that would be potentially effective for the treatment of infections mediated by both Gram-positive and Gram-negative bacterial species simultaneously. The lipopolysaccharide (LPS) layer in Gram-negative species accounts to a great degree for their low permeability to hydrophobic compounds, including certain antibiotics (Kumar and Schweizer 2005). The LPS is composed of lipid A (a

polysaccharide) and an *O*-antigen. Fatty acid substituents of the LPS form a thick, impermeable gel because of complete saturation and the *O*-antigen is strongly anionic with cross-bridging of the core region with phosphate groups and divalent cations (Wiese et al. 1999). All these factors enhance the low permeability of the LPS, especially in *E. coli* as well as *Ps. aeruginosa*, wherein the LPS is most highly phosphorylated in comparison to other Gram-negative bacterial species, resulting in a stronger diffusion barrier (Sadovskaya et al. 1998, Sadovskaya et al. 2000) and may explain why combined applications of F1 with polymyxin B were indifferent and additive to both organisms in the data presented in table 4.1.

However, although the outer membrane is an effective barrier against hydrophobic substrates, the fraction of these compounds that could permeate barrier membranes are actually extruded by efflux pumps at a rate faster than that with which such compounds entered (Hancock 1997a, Nikaido and Takatsuka 2009). Molecules of polymyxins (and antibacterial peptides generally) are capable of self-translocation and thereby also promoting the entry of other hydrophobic substrates across bacterial outer and cytoplasmic membranes (Jenssen, Hamill and Hancock 2006a), a process commonly termed self-promoted uptake (Hancock 1997a). Molecules of polymyxins consist of hydrophobic and hydrophilic regions and can aggregate to span the lipid bilayer with micelle-like complexes made up of peptides and lipids. These molecules can also insert themselves at a 90^{o} angle to the plane of the bilayer by the interaction of their hydrophilic regions with the phospholipid head groups of the outer membrane structure whilst their hydrophobic region is associated with the lipid core. The membrane then curves inward allowing more of the polymyxins molecules to line the formed pore to permit the creation of a free entry route for both itself and other molecules (Jenssen, Hamill and Hancock 2006a). This strategy is more pronounced in the “barrel-stave” peptides entry model (Zhang, Rozek and Hancock 2001a, Bradshaw 2003) wherein the polymyxins orientate and insert themselves at an angle of 90^{o} to the bilayer plane forming staves (holes that extend from outward to inward) with their hydrophilic region facing the inner open space of the pore and their hydrophobic region interacting with the lipid bilayer (Jenssen, Hamill and Hancock 2006a). The latter two mechanisms have a capacity to promote the entry of polymyxins and other molecules, even hydrophobic compounds. Such hydrophobic molecules are likely

to begin their transition by interacting with the outer hydrophobic regions of the peptide molecules already in association with the lipid bilayer (Hancock 1997a, Wu and Hancock 1999, Hancock 1997d). This self-promoted uptake can occur in both Gram-positive and negative organisms (Falagas, Kasiakou and Saravolatz 2005, Hancock and Chapple 1999, Hancock and Lehrer 1998a) and is often accompanied by an increased permeability of the bacterial membrane to a variety of compounds, including hydrophobic agents. For Gram-negative cells, the preliminary electrostatic bonding occurs between the cationic peptides and negatively charged phosphate groups within the lipopolysaccharide in the outer membrane (Zhang et al. 2000) whilst lipoteichoic acids occurring on the surfaces of Gram-positive bacteria interacts with these compounds. Therefore, it can be speculated that polymyxin B possibly acts in a manner that promotes the enhanced translocation of both F1 and F23 across both Gram-positive and Gram-negative bacterial membrane barriers leading to a continued accumulation and retention of both compounds (due to a simultaneous down-regulation of efflux mechanisms), within the cytoplasm and periplasmic spaces, for potential targeting of other intracellular sites and enzymes.

In both San Diego and Kingston in Ontario, MPex Pharmaceuticals have been developing some combinational antibacterial compounds (Lomovskaya and Bostian 2006, Kriengkauykiat et al. 2005). One of their strategies is to combine compounds, one of which is sometimes an adjunct that can interfere with the bacterial efflux pump, with an antimicrobial agent (Poole and Lomovskaya 2006). The compound MC-2077,110 and its analogues MC-002595 as well as MC-004124 are dibasic peptide-like agents that are competitive inhibitors of multiple resistance-nodulation-division (RND) transporters in Gram-negative bacterial species, especially in *Ps. aeruginosa* (Lomovskaya and Bostian 2006). These compounds being substrates of their target efflux-pumps compromise the extrusion of fluoroquinolone and macrolide antibiotics when applied in combination by competitively inhibiting the binding of such antibiotics to a common site on the pumps (Pagès, Masi and Barbe 2005). Such a combinational formulation has demonstrated an enhanced antibacterial activity for laevofloxacin against *Ps. aeruginosa*, by preventing the efflux of laevofloxacin from the cytoplasmic space (Kriengkauykiat et al. 2005).

QacA is a member of the small multi-drug resistant family (Paulsen et al. 1996) and NorA belongs to the major facilitator super-family that are multi-drug and

toxic compound extrusion pumps prevalent in Gram-positive bacterial species, especially *S. aureus* (Yoshida et al. 1990, Yamagishi et al. 1996). Whilst QacA is plasmid encoded, NorA is chromosomally encoded and responsible for intermediate fluoroquinolone resistance in *S. aureus* (Poole 2000). Considering that the data presented in this section indicated a synergism for the combined antibacterial activity of F1 with ciprofloxacin, it might be worthwhile to examine whether these extrusion pumps are down-regulated in *S. aureus* by the combination of the chalcone-derivative with a fluoroquinolone. Likewise, it may also be worthwhile examining whether either F1 or F23 in combination with polymyxin B inhibit QacA and NorA in Gram-positive as well as the multiple resistance-nodulation-division pumps in Gram-negative bacterial species in achieving their synergistic action.

Tackling the efflux systems may not be the only potential mechanism by which the combination of the chalcone-derivatives with polymyxins elicited their antimicrobial synergistic action. The loss of the MexAB-OprM efflux system (RND) family in strains of *Ps. aeruginosa* that are resistant to fluoroquinolones and macrolides did not induce susceptibility of this pathogenic organism to these antibiotics (Masuda et al. 2000); an indication that a capacity to overcome the activity of the latter enzymes is a more important factor in determining the potency of a given antibiotics. Although the mechanisms of action of these chalcone-derivatives have not been explored, some similar flavonoid compounds are known to function as adjuncts, modifying the activities of established antibiotics. As mentioned in chapter 3, (-)-epicatechin gallate (ECg) sensitizes MRSA to the actions of β-lactam antibiotics, reducing the oxacillin MICs as much as 512-fold (Cushnie and Lamb 2011) through mechanisms that are neither related to the occurrence of decreased penicillin binding protein nor the binding of the flavonoid to same sites on the peptidoglycan with this class of antibiotic (Stapleton et al. 2004). This flavonoid (ECg) is now thought to potentiate the actions of β-lactams by inflicting structural changes to the cell wall teichoic acid, promoting the release of lipoteichoic acid from the cytoplasmic membrane thereby inducing an enhanced susceptibility of MRSA to the β-lactams (Stapleton et al. 2007). Clavulanic acid acts synergistically as an adjunct with amoxicillin, in a combinational formulation called co-amoxiclav, by rendering strains that are β-lactamase expressing susceptible to amoxicillin through a competitive binding of the former compound to the enzyme (Vree, Dammers and Exler 2003). A

reversible complex is formed initially upon the binding that progresses into an irreversible inactivation of the β-lactamase enzyme and clavulanic acid (Drawz and Bonomo 2010). Both compounds share similar pharmacokinetic properties and a biological half-life of 1 hour illustrating an ideal antimicrobial combination (Vree, Dammers and Exler 2003, Petitpretz et al. 2002). The combination of the chalone-derivatives with polymyxin B may be eliciting their potentiated activity against these bacterial species in a manner comparable to that of co-amoxiclav although it is yet to be ascertained which of the two compounds may be augmenting the antimicrobial activity of the other. Both compounds may collectively potentially inhibit the activities of bacterial metabolic enzymes such as NADH and succinate dehydrogenase that are very significant for bacterial respiratory chain and viability (Wang et al. 2010a).

4.5 Conclusion

The fact that F1 and F23 applied in combination with polymyxin B are synergistic against different species of *S. aureus*, *C. violaceum*, *Ps. aeruginosa* and *E. coli* suggest these combinations should be further evaluated as leads for the development of potentially useful antimicrobial compounds. Combinational therapy targeted at enhancing the antibacterial activity of polymyxin B and other established antibiotics can support their clinical re-evaluation and a re-assessment of their combined spectrum of activity (Owens and Ambrose 2005, Owens and Ambrose 2007). Although the antibacterial activities of F1 and F23 were of low individual potency, the combination of these compounds with polymyxin B clearly demonstrated synergistic bactericidal activities against a wide range of test bacterial species. The synergism demonstrated by the combination of polymyxin B with the chalcone-derivatives, if further evaluated, could lead to smaller doses of polymyxins being required to achieve more effective and extended spectrum of therapeutic goals against pathogenic organisms. This can also lead to a reduction in the potential for polymyxin B to elicit its dose-dependant adverse effect of nephrotoxicity.

Chapter 5

Evaluating the mode of action for polymyxin B chalcone-derivatives combinations

5.1 Introduction

In order to gain some insight to the influence of time upon the nature of the antibacterial activity for polymyxin B applied with the chalcone derivatives, colony counting experiments were undertaken using selected strains of antibiotic-sensitive and resistant bacterial species. The data obtained from chequerboard and MBC analysis for the effective combination concentrations in the latter assay suggesting this polypeptide was either additive or synergistic in action with either F1 or F23 were re-assessed. Such information was further verified by examining either the bacteriostatic or bactericidal concentrations of such combinations in a time-course viability assay. Although the chequerboard technique is very useful, the assay is not as sensitive and discriminatory as a time-course viability analysis to accurately detect synergism (Lewis et al. 2002). The time-course viability assay remains the most reliable technique to verify an antibiotic's mode of action (Lin et al. 2010). This latter assay can elucidate the rate and extent of action of antimicrobial compounds and is therefore still considered most suitable for the characterisation of newly developed agents (McKay et al. 2009b), being able to clearly differentiate bacteriostatic from bactericidal action *in vitro*. Bactericidal effect was defined as $\geq$ 3 log $_{10}$ cfu/ml (99.9 %, equivalent to a 3 log cfu/ml decline in exposed cell density) of the initial bacterial population whilst a bacteriostatic effect was defined as <3 log depletion of the inoculum population within 24 hours (Mangili et al. 2005).

At the moment, most multi-resistant Gram-negative bacterial strains remain susceptible to polymyxin B and colistin (Evans, Feola and Rapp 1999, Li et al. 2005a). Serious concerns have been raised about the potential capability of these Gram-negative pathogens to develop resistance to polymyxins (Vaara and Porro 1996). Clinicians are therefore being discouraged from using polymyxins as a monotherapy, especially when applied as an antibiotic of last resort (Falagas, Kasiakou and Saravolatz 2005) in order to decrease the likelihood for the emergence of resistant bacterial strains due to selective pressure (Vaara and

Vaara 2010). When a given bacterial population is continually exposed to lethal (and especially sub-inhibitory) doses of an antibiotic, a small fraction may evade the killing-effect due to mutation and transfer the resistant gene to the next generation onward (Pond and Frost 2005). There is therefore a strong rationale for continuous development of new antibacterial compounds and more effective synergistic combination of compounds against pathogenic bacterial species (Gunderson et al. 2003, Hogardt et al. 2004). It is thought the development of effective synergistic combinations will permit the use of lower strengths, leading to lower potential for toxicity to the host, an extended spectrum of activity and an extension of the clinical life-span for both agents, as has been demonstrated with (Navarro 2005, Alou et al. 2004) co-amoxiclav (Navarro 2005) and mupirocin with amoxicillin-clavulanate formulation (Alou et al. 2004).

The capacity of some novel synthetic antibacterial peptides to increase the permeability of bacterial membranes and act synergistically with hydrophobic compounds has been demonstrated (Wu and Hancock 1999, Vaara and Porro 1996). Many of the peptides that were developed show some form of resemblance to the polymyxins by having a cyclic structural conformation in common (Vaara and Porro 1996, Reddy, Yedery and Aranha 2004). The capacity of polymyxin B to act synergistically with each of two hydrophobic semi-synthetic chalcone-derivatives (F1 and F23) against selected bacterial species (Gram-positive and Gram-negative) was studied to understand how both classes of compounds promote each other's antimicrobial activity. Polymyxins are ineffective against Gram-positive organisms (MSSA, MRSA and QRSA) probably due to the absence of an outer membrane, which is the primary target of these agents (Hancock 1997b, Jenssen, Hamill and Hancock 2006a, Hancock and Lehrer 1998b). The occurrence of a much thicker peptidoglycan layer in this class of bacterial species may also be facilitating the insusceptibility of this class of bacterial species to these polycationic antimicrobial agents (Lorian 2005). The activity of polymyxin B in combination with the chalcone derivatives against strains of *S. aureus* was investigated in an attempt to overcome the obstacle responsible for the inactivity of polymyxins against Gram-positive bacterial species and potentially extend their spectrum of activity. On the other hand, the activity of both compounds applied together against *C. violaceum* and *Ps. aeruginosa* was evaluated in an attempt towards developing potentially effective formulations with improved antibacterial activity at lower concentrations of

polymyxins against Gram-negative bacterial species in the presence of low amounts of chalcone-derivatives. Considering that polymyxin B is the antibiotic of reserve for ailments like cystic fibrosis mediated by multi-resistant Gram-negative organisms, especially *Ps. aeruginosa* (Kasiakou et al. 2005, Schurek, Breidenstein and Hancock 2012, Bjarnsholt et al. 2009), a reduction in the effective concentrations of polymyxins administered against this group of organisms will lower the potential for the dose-related adverse events of nephrotoxicity associated with the use of these agents and promote their use as antibiotics of choice in the clinics (Vaara and Vaara 2010). The objectives in this chapter were to:

i. verify the data that indicated either an additive or synergistic antibacterial activity for either F1 or F23 applied in combination with polymyxin B against MSSA, MRSA, QRSA, *C. violaceum* and *Ps. aeruginosa*
ii. establish whether the mode of action for polymyxin B in combination with the chalcone derivatives is either bacteriostatic or bactericidal
iii. compare and contrast the antibacterial activity of F1 and F23 applied alone and each in combination with polymyxin B against the test bacterial species.

5.2 Materials and Methods

5.2.1 Time-course viability assay for bacterial suspensions treated with a single test compound

A bacterial suspension containing ~2 x 10^7cfu/ml in 0.9 % $^{w}/_{v}$ saline solution in a conical flask was prepared as described in section 2.4.1 above. A suitable volume of the stock test compound was added to a 20 ml volume of the bacterial suspension in order to obtain the desired final concentration. The flask was incubated in a Clifton shaking water bath, set at 37 ^{o}C and 100 rpm. At time intervals of usually 0, 2, 4, 8 and 24 hours, 100 µl of the suspension was withdrawn for viable count determination in order to ascertain the viable bacterial population in the suspension, as previously described in section 2.3.3 and 2.3.4. Bacteriostatic and bactericidal activities were defined as <3 $\log_{10}$ and ≥ 3 $\log_{10}$ reductions in bacterial population (cfu/ml) respectively, within 24 hours

(CLSI 2009, (Yamagishi et al. 1996). Data reported were obtained from at least 2 independent assays.

5.2.2 Time-course viability assay for bacterial suspensions challenged with polymyxin B applied with the chalcone-derivatives

The data obtained from a chequerboard technique described in chapter 4 that indicated either additive effects or synergism for polymyxin B applied in combination with chalcone-derivatives were verified in a bacterial time-course viability assay. Bacterial suspension containing ~2 x 10^7cfu/ml in 0.9 % $^{w}/_{v}$ sodium chloride solution in a conical flask was prepared as described in section 2.4 above. A suitable volume of the stock concentration of the chalcone-derivative was added to 4 ml of the bacterial suspension in a conical flask, in order to obtain the desired final concentration. The chalcone- derivative was combined with polymyxin B either at MIC proportion or an effective combination concentration previously determined by the chequerboard assay (against the selected bacterial species). Then, the test tube was kept in a suitable glass beaker containing water and incubated in a shaking water bath as described in section 2.6. Also, as described in the same section, at the set intervals, 100 µl samples were withdrawn for colony plate count determination. Graphs of bacterial density ($\log_{10}$ cfu/ml) against time (hours) were plotted. Controls containing the bacterial suspension treated with suitable concentrations of the chalcone-derivative, the antibiotic, untreated bacterial suspension and solvent treated bacterial suspension were included. The antibacterial activities in this assay were further described as either antagonistic, indifferent or synergistic. Antagonism was defined as a $\geq$ 2 $\log_{10}$ cfu/ml decrease in the antibacterial activity of the combination of compounds in comparison to the action of the most potent agent applied alone; indifference was defined as a < $2\log_{10}$ cfu/ml decrease in the action displayed by the combination in comparison to the activity of the most potent single agent (Clinical and Laboratory Standards Institute 2009, Roling et al. 2002a). The activity characterised as synergistic (bactericidal) were those where the combination of compounds demonstrated $\geq$ 2 $\log_{10}$ cfu/ml increase in antibacterial activity in comparison to the activity demonstrated by the most potent single agent (Clinical and Laboratory Standards Institute 2009, Roling et al. 2002a). The data reported were obtained from assays conducted in

duplicate with at least three independent replicates carried out on separate days (n = 6).

5.2.3 Statistical analysis of data

The data obtained from the methods described in section 5.2.1 and 5.2.2 above were subjected to statistical analysis for standard error of mean and standard deviation using a GraphPad Prism 4. The software was used to plot graphs of the bacterial population (cfu/ml) against sampling time (hours). The graphs were interpreted as previously described in sections above. The limit of sensitivity of the assay was also determined.

5.3 Results

5.3.1 Examination of the activity of F1 applied alone and with polymyxin B upon selected strains of *S. aureus* species

5.3.1.1 Bactericidal activity of F1-polymyxin B combination against methicillin-sensitive *S. aureus*

Figure 5.1 represents the data from a time-course viability assay for F1 applied in combination with polymyxin B against methicillin-sensitive *S. aureus* (MSSA). In conducting the time-course viability assays, the MIC value of polymyxin B against MSSA was regarded as 256 µg/ml based on the data previously recorded from a micro-broth dilution assay in chapter 2. In the current technique, this strength of polymyxin B demonstrated about 1 log reduction in the population of the challenged *S. aureus* (MSSA), with reference to the solvent-treated control.

With reference to both the solvent and untreated controls, it appears the presence of F1 seem to have had no effect upon the viability of MSSA in the presence of DMSO, as the bacterial population challenged with only 64 µg/ml F1 had a slightly higher colony count than the solvent after 24 hours suggesting this bacterial species demonstrated a slightly higher sensitivity to the presence of DMSO alone (in the absence of the chalcone-derivatives).

Considering that *S. aureus* 6571 (MSSA) samples challenged with 5 and 10 % $^{v}/_{v}$ DMSO in some preliminary investigations showed appreciable sensitivity to the presence of these strengths of the solvent, the DMSO concentration used for the

dissolution of the test compounds as well as the solvent control were reduced to 1.28 % $^{v}/_{v}$ DMSO. MSSA also demonstrated sensitivity to this strength of DMSO by the display of an approximately 2 log reduction in the challenged inoculum population after 24 hours as shown in figure 5.1.

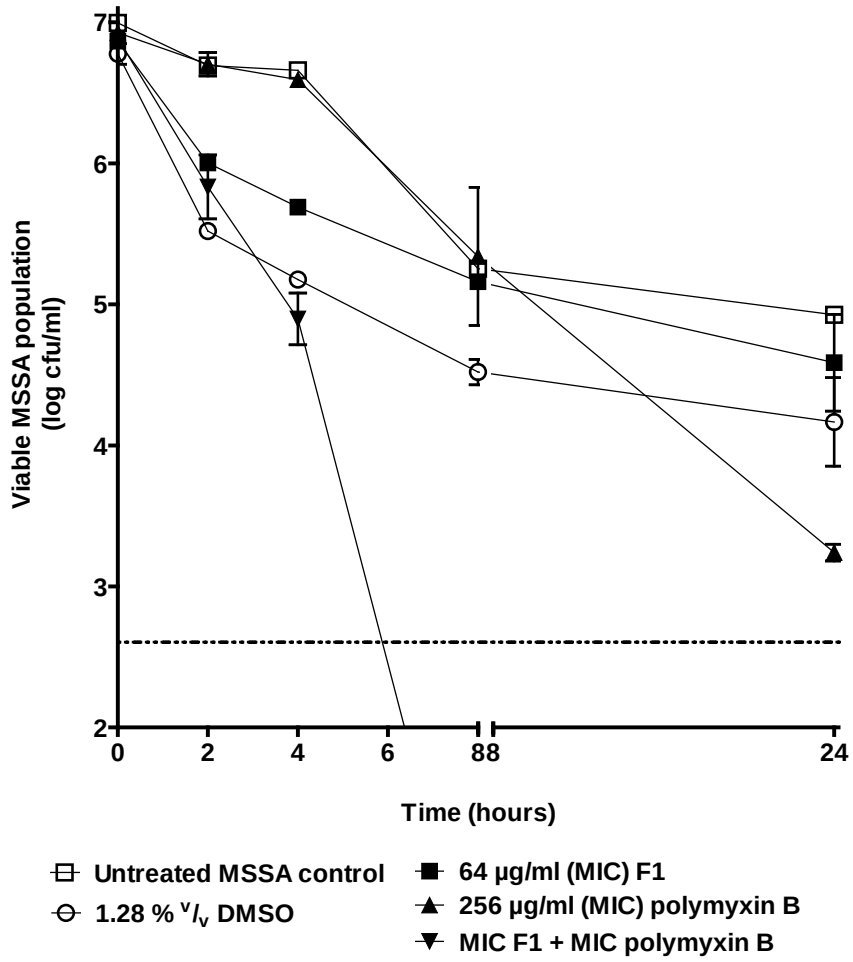


Figure 5.1: Synergistic activity for F1 applied with polymyxin B against *S. aureus* 6571 (MSSA). Inoculum density was ~1 x 10^{7} cfu/ml. The data were mean values obtained from 3 replicates and repeated on separate days.

The untreated positive control demonstrated about 1.5 log cfu/ml reduction in cell density attributable to the poor survival capacity of *S. aureus* in 0.9 % $^{w}/_{v}$ sodium chloride solution over time. Therefore, the effects of DMSO solvent and 0.9 % $^{w}/_{v}$ sodium chloride upon this bacterial species may have been cumulative in the solvent treated sample. The bacterial population challenged with 64 µg/ml (MIC) F1 in combination with 256 µg/ml (MIC) polymyxin B was reduced by more than 4 log cfu/ml to below detectable limit within 8 hours indicating their combined killing-effect surpassed the action of the most potent single compound,

polymyxin B. A profound synergism was therefore displayed over the 24 hours period that greatly surpassed the antibiotic action of the polypeptide and the solvent-effect that were recorded. Given that at least 1 colony forming unit (cfu) can be obtained from 20 µl of a 10-fold dilution, 50 cfu were obtainable from 1 ml of same dilution and 500 cfu from 1 ml of the neat suspension. Therefore, the limit of sensitivity of this assay was 5×10^2 cfu/ml, which is approximately log 2.69. This limit is applicable for all the assays performed in this section using this same technique. A 2 way analysis of variance with the Bonferroni post test sow that a significant difference existed between the activity of the single polymyxin B concentration and the untreated sample at 24 hours at $p < 0.0001$. With the same analysis, there is a significant difference between the activity of polymyxin B-treated and the solvent-treated bacterial suspension from 2 to 24 hours. Although, the synergistic action of the combination of F1 with polymyxin B against the bacterial suspension was significantly different in comparison to the untreated sample at the same conditions, such a difference only occurred between the activity recorded for the synergistic combination and the solvent-only treated samples after 8 and 24 hours respectively affirming that the cumulative negative effect of both solvent and 0.9 % $^{w}/_{v}$ NaCl upon MSSA was enormous.

5.3.1.2 Effect of F1 concentrations upon the viability of methicillin-resistant *S. aureus*

Data for the viability of equivalent densities of MRSA suspensions treated with increasing strengths of F1 in a time-kinetic assay is shown in figure 5.2.

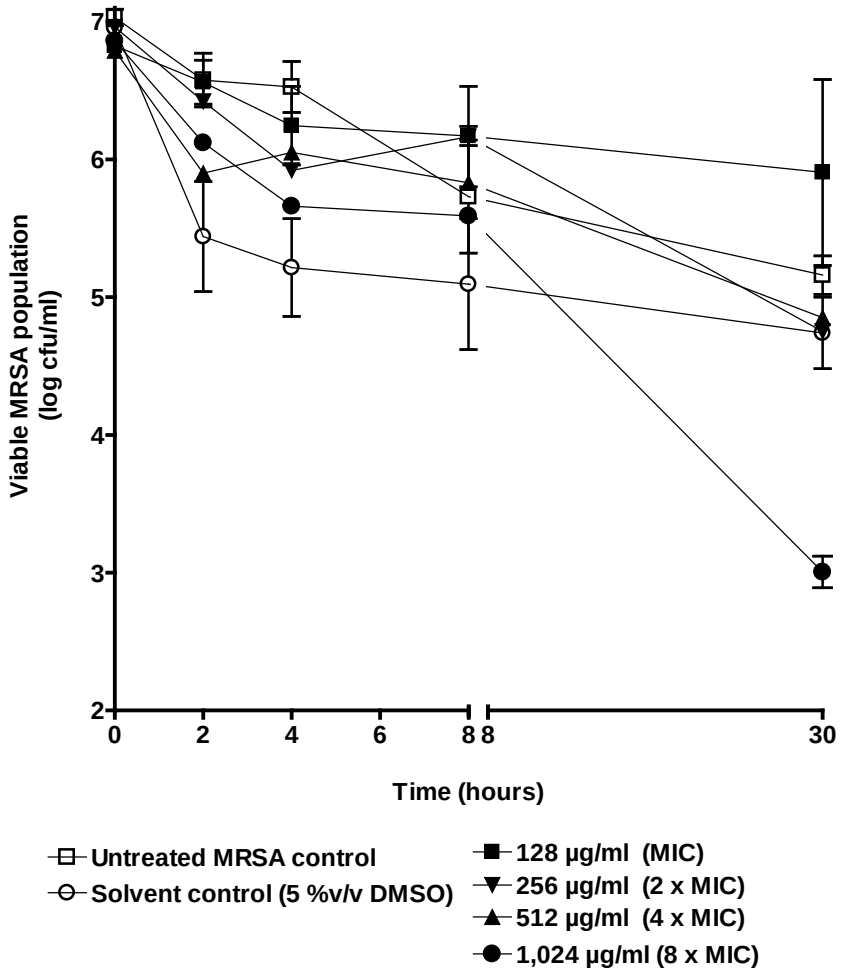


Figure 5.2: The effect of increasing concentrations of F1 applied against *S. aureus* (MRSA) in a time-kinetic assay. Inoculum population was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

Bearing in mind that cell suspension exposed to only 5 % $^{v}/_{v}$ DMSO as a control displayed a 99 % (2 log cfu/ml) depletion in colony count after 24 hours, obviously concentrations within the range of MIC (128 µg/ml) to 4 x MIC (512 µg/ml) F1 demonstrated no apparent antibacterial effect against MRSA, in relation to the untreated and solvent-treated controls, when applied alone.

Relative to the solvent-treated control, 8 x MIC (1024 µg/ml) F1-treated bacterial sample demonstrated a 1.5 log cfu/ml reduction in density within 24 hours; indicating a bacteriostatic action at best with such a high concentration of the chalcone-derivative. As previously explained, the limit of sensitivity for the assay results was log 2.69.

5.3.1.3 Bactericidal activity of F1-polymyxin B combination against methicillin-resistant *S. aureus*

The data presented in figure 5.3 were obtained from a time-course viability assay for methicillin-resistant strain of *S. aureus* challenged with 128 µg/ml (MIC) F1 with 128 µg/ml (MIC) polymyxin B.

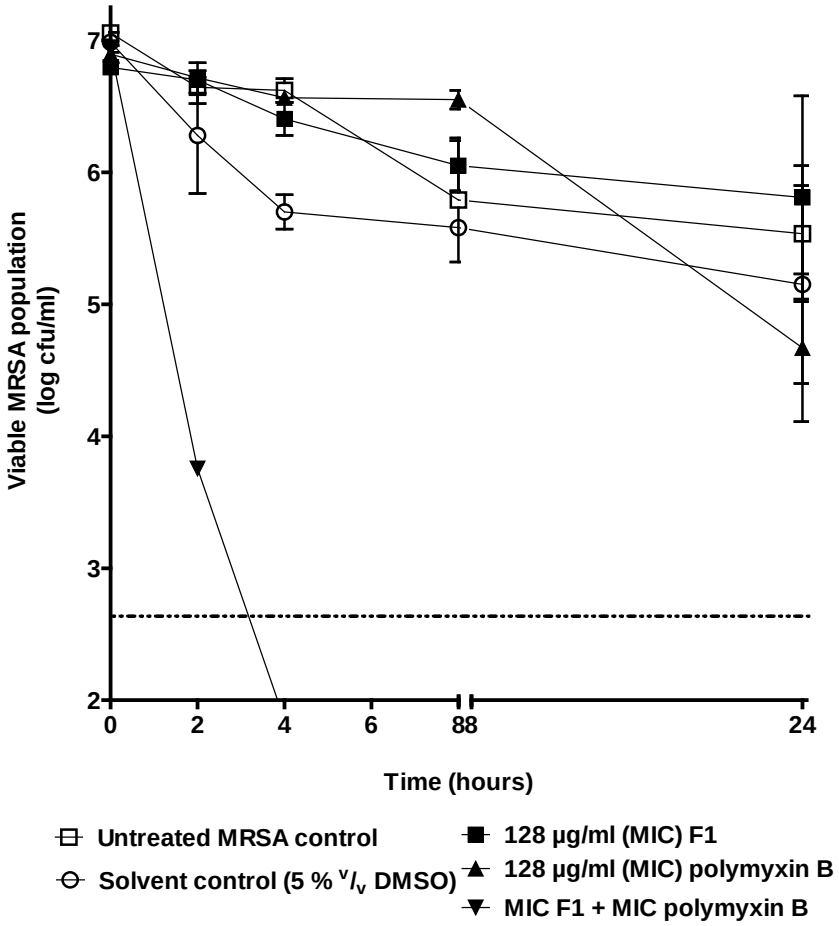


Figure 5.3: Synergistic activity for F1 applied with polymyxin B against MRSA. Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

In comparison to the solvent treated control, MRSA population challenged with 128 µg/ml (MIC) polymyxin B demonstrated less than 1 log cfu/ml reduction in viability after 24 hours. The independently applied 128 µg/ml F1 was ineffective against this bacterial species, in this assay, as had been previously demonstrated in the data presented in figure 5.2. Any appreciable loss in cell viability was not recorded in bacterial suspension treated with this concentration of the chalcone-compound in relation to the solvent-treated cells. The ~1.5 log cfu/ml reduction

in the viability of the solvent-treated MRSA population may be attributable to the weak survival potential of *S. aureus* in 0.9 % $^{w}/_{v}$ sodium chloride coupled with the sensitivity of these cells to DMSO. As before, the loss in the viability in both control samples over 24 hours is greatly surpassed by the rapid and profound synergism demonstrated by F1 with polymyxin B. The application of 128 µg/ml (MIC) F1 with 128 µg/ml (MIC) polymyxin B reduced MRSA population by more than 4 log cfu/ml within 8 hours, although the limit of cell density detection for this technique was 500 cfu/ml. In relation to the activity recorded for the independent application of 128 µg/ml polymyxin B (which is considered the most active single compound), the F1-polypeptide combination displayed a > 4 log cfu/ml depletion to the inoculum population similar to the recorded values against MSSA thereby indicating a profound synergism in both instances.

5.3.1.4 Effect of F1 concentrations upon the viability of quinolone-resistant *S. aureus* under growth conditions

The effect of F1 upon the viability of QRSA suspension in a growth (Mueller-Hinton broth) rather than a buffer medium was undertaken in order to investigate the antibiotic property of this semi-synthetic chalcone compound under an environment that permits cell wall synthesis and cell replication. This assay was also intended to give an insight into the influence of F1's interaction with broth on the antibacterial action of the chalcone-derivative. The choice of QRSA was based on the fact that the relatively low MIC value of 32 µg/ml demonstrated by the chalcone-derivative against this species will enable the examination of the effects of very high multiple-folds of this inhibitory concentration whilst consuming a relatively small cumulative quantity of the compound, since the supply was limited. On this occasion (figure 5.4), the untreated and solvent controls manifested unhindered growth over the time-course suggesting that unlike in previous assays conducted under buffer medium, the deleterious effect of DMSO and 0.9 % $^{w}/_{v}$ sodium chloride on *S. aureus* viability were absent.

The cell sample challenged with 32 µg/ml F1 (equivalent to the MIC) displayed a 1 log cfu/ml growth within 2 hours and this density was maintained for 8 hours. Due to cell replication, the inhibitory effect of this strength of F1 was

overwhelmed with time as the cell population multiplied to equate with those of the untreated and solvent-treated samples after 24 hours. The QRSA population challenged with either 64 (2 x MIC), 128 (4 x MIC 128), 256 (8 x MIC) or 1024 µg/ml (32 x MIC) F1 demonstrated growth inhibitory activity in broth, impeding the capacity of these cells to multiply.

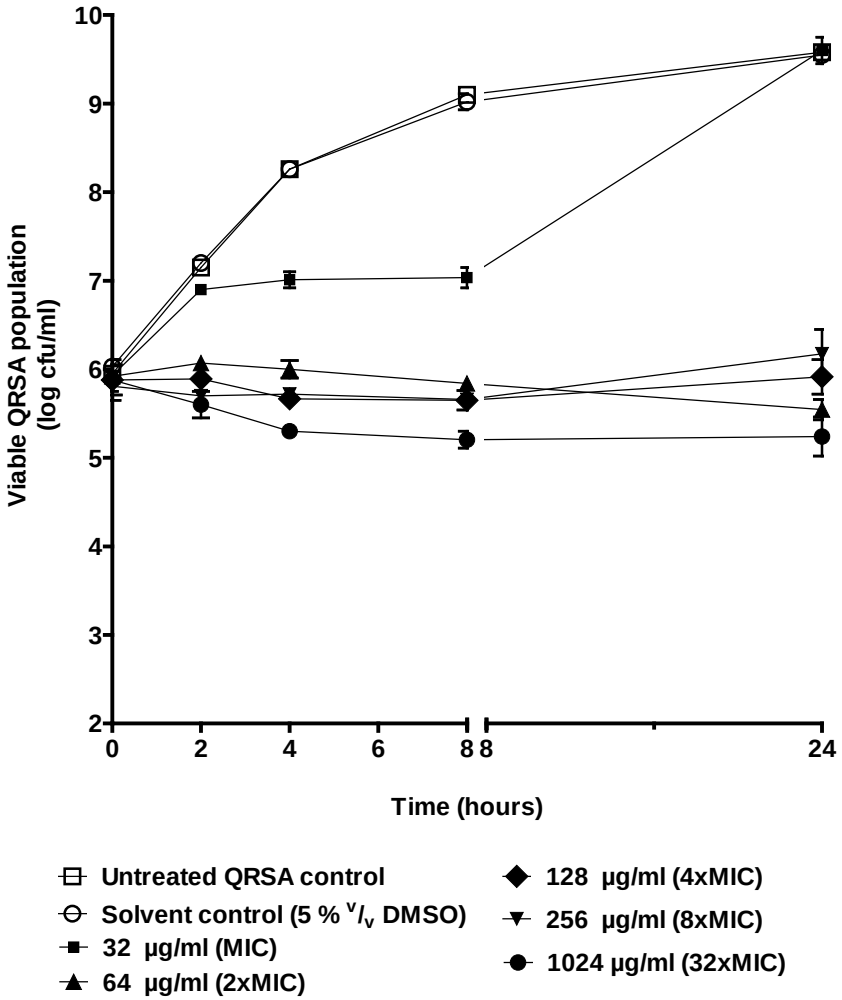


Figure 5.4: Bacteriostatic activity of increasing concentrations of F1 against QRSA in broth. Inoculum density was ~1 x 10^6 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

It is unclear whether either the inoculum populations were being replaced by new cells at an almost equal rate of proliferation or the original cell densities were simply retained within the assay duration. But given that the untreated as well as the solvent treated suspensions exhibited unimpeded cell replication over the same time duration, it can be inferred that the data gleaned from the challenged cell suspensions cannot be attributed to the effect of depletion in nutrient supply

with time. These data suggest that given a high enough concentration (≥2 x MIC), F1 has capacity to inhibit *S. aureus* growth in broth. A comparative view of data presented in this section with that previously recorded in figure 5.2 suggests F1 is only at best bacteriostatic against *S. aureus* at a concentration up to 8 x MIC, in both buffer and growth media. A much higher concentration of 32 x MIC did not show any additional activity in the growth medium. Bearing in mind that the effect of F1 appears to be independent of concentration, subsequent time-kinetic assays were conducted under buffer condition with low concentrations of the chalcone-derivatives in order to verify this compound's lowest synergistic concentrations with polymyxin B against selected bacterial species in combinational assays.

5.3.1.5 Bactericidal activity of F1 with polymyxin B against quinolone-resistant strain of *S. aureus*

Figure 5.5 shows data for a time-course viability assay for a quinolone-resistant strain of *S. aureus* challenged with F1 with polymyxin B. A concentration of 128 µg/ml polymyxin B applied independently did not demonstrate any appreciable activity whilst 32 µg/ml (MIC) F1 was bacteriostatic against QRSA by demonstrating a 1 log cfu/ml reduction to an equivalent population with respect to the solvent-treated sample over 24 hours. As observed with the other strains of *S. aureus* reported earlier, the loss of some degree of viability with the controls is likely due to the combined adverse effects of DMSO and 0.9 $^{w}/_{v}$ sodium chloride.

This loss is however not comparable to the synergistic outcome recorded from the combined application of the same concentrations of both compounds above against an equivalent cell density of the same bacterial species, wherein a rapid and complete loss of viability was reported after only 8 hours of exposure although the reliable limit of detection of this assay was 500 cfu/ml, which approximates to log 2.69 on the y-axis. The three strains of *S. aureus* employed in the investigations discussed were thought to have given adequate representation of the activity of F1 against this Gram-positive bacterial species necessitating the use of other species in subsequent examinations in this assay.

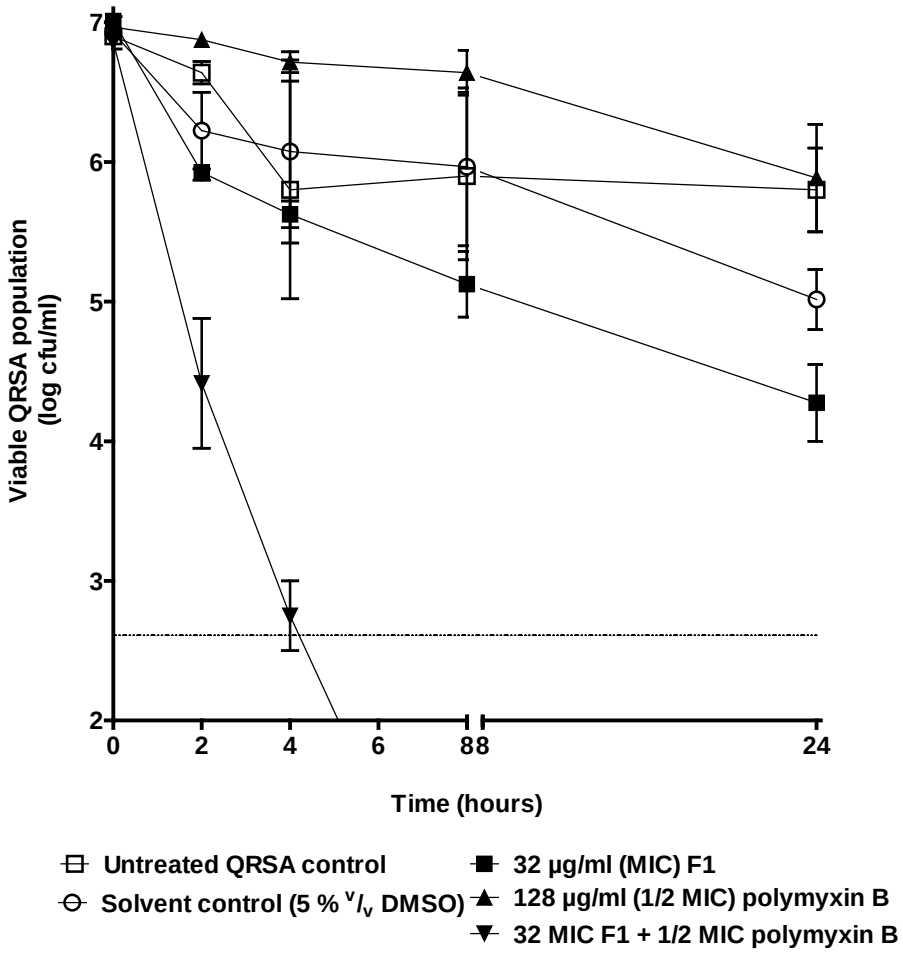


Figure 5.5: Synergistic activity for F1 applied with polymyxin B against QRSA. Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

5.3.2 Examining the antibacterial effect of F1 with polymyxin B against Gram-negative bacterial species

The antibacterial activity of polymyxin B applied with the chalcone-derivative against *E. coli* was not investigated using a time-kinetic colony counting technique because data from prerequisite chequerboard assay suggested the combination of F1 with polymyxin B was indifferent in antibacterial action against this Gram-negative bacterial species (table 4.1). The antibiotic activities from the chequerboard assay that indicated the existence of either additive or synergistic actions were especially considered for re-assessment using the time-course colony counting technique.

5.3.2.1 Bactericidal activity of sub-inhibitory concentrations of F1 with polymyxin B against *C. violaceum*

The data presented in figure 5.6 was obtained from a time-course viability assay for *C. violaceum* suspensions challenged with F1 applied alone and in combination with polymyxin B.

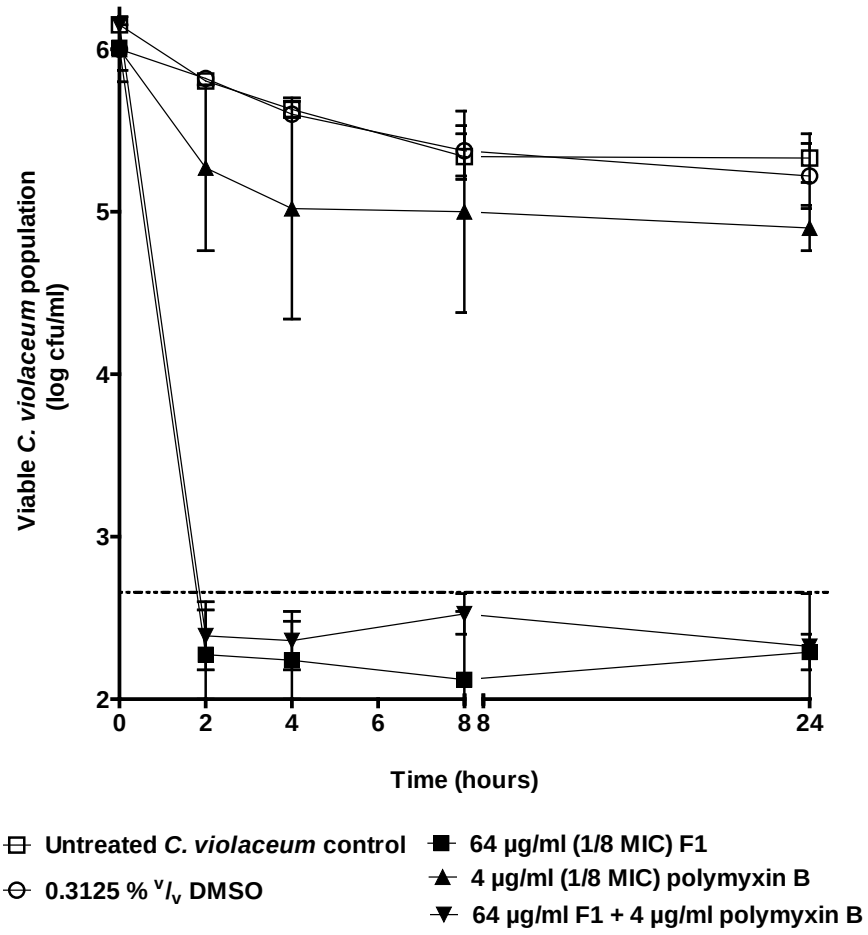


Figure 5.6: Bactericidal activity of sub-inhibitory concentrations of F1 applied alone and indifferent activity of sub-inhibitory concentration of F1 applied with sub-inhibitory concentration of polymyxin B against *C. violaceum*. Inoculum density was ~2 x 10^6 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

The concentrations of both compounds employed was based upon guidance from data obtained from the chequerboard assay previously undertaken (table 4.1). Although 16 µg/ml (1/32nd MIC) F1 applied in combination with 2 µg/ml (1/16th) MIC polymyxin B was synergistic against *C. violaceum* in the previously reported technique, 2 to 4 times the effective chequerboard combination concentrations were used considering that this species is a Gram-negative organism which can

display greater insusceptibility because of the additional drug permeation barrier posed by the presence of an outer membrane structure.

Considering that 5 % $^{v}/_{v}$ DMSO demonstrated appreciable deleterious effect against the viability of *S. aureus* populations, the solvent concentration in this assay was reduced to 0.3125 $^{v}/_{v}$ DMSO which was tolerable to this bacterial species within the 24 hours period. It can also be seen that in comparison to *S. aureus* species, *C. violaceum* showed a lesser sensitivity to the presence of 0.9 % $^{v}/_{v}$ sodium chloride by demonstrating a lower degree of loss in cell viability in the unchallenged control suspension over time. The *C. violaceum* sample challenged with 4 µg/ml (1/8 MIC) of polymyxin B demonstrated only a < 1 log reduction with reference to the unchallenged suspension in DMSO.

Both densities of *C. violaceum* populations challenged with the 64 µg/ml sub-inhibitory concentration of F1 alone and together with polymyxin B were reduced from ~2 x 10^{6} to ~250 cfu/ml within 120 minutes (although the limit of detection of the assay was 500 cfu/ml). Although this 1/8 MIC concentration of F1 displayed a rapid and profound bactericidal action against *C. violaceum*, F1 with polymyxin B demonstrated indifference in their combined antibiotic effect since the addition of the latter agent did not improve the recorded action of the former compound alone within 24 hours. The activity of agents applied together is described as indifferent if their combined antibiotic effect amounts to a < 2 log cfu/ml depletion of the challenged inoculum density.

5.3.2.2 Bactericidal activity of F1-polymyxin B combination against *Ps. aeruginosa*

The result from a time-course viability assay for *Ps. aeruginosa* population challenged with 64 µg/ml (1/4 MIC) F1 applied in combination with 0.25 µg/ml (1/2 MIC) polymyxin B is presented in figure 5.7. As with other instances, the selection of strengths of both compounds to be used was informed by data previously obtained from a chequerboard assay presented in table 4.1. Although 16 µg/ml (1/16 MIC) F1 applied with 0.25 µg/ml (1/2 MIC) polymyxin B had recorded an additive antibacterial action against *Ps. aeruginosa* in the former technique, this combination was eventually not bactericidal in action upon the inoculation of a freshly prepared antibiotic and chalcone-derivative-free agar with

a 1 µl volume obtained from a well containing this sample. The agar plate displayed bacterial growth following incubation at 37 ^{o}C, for 24 hours.

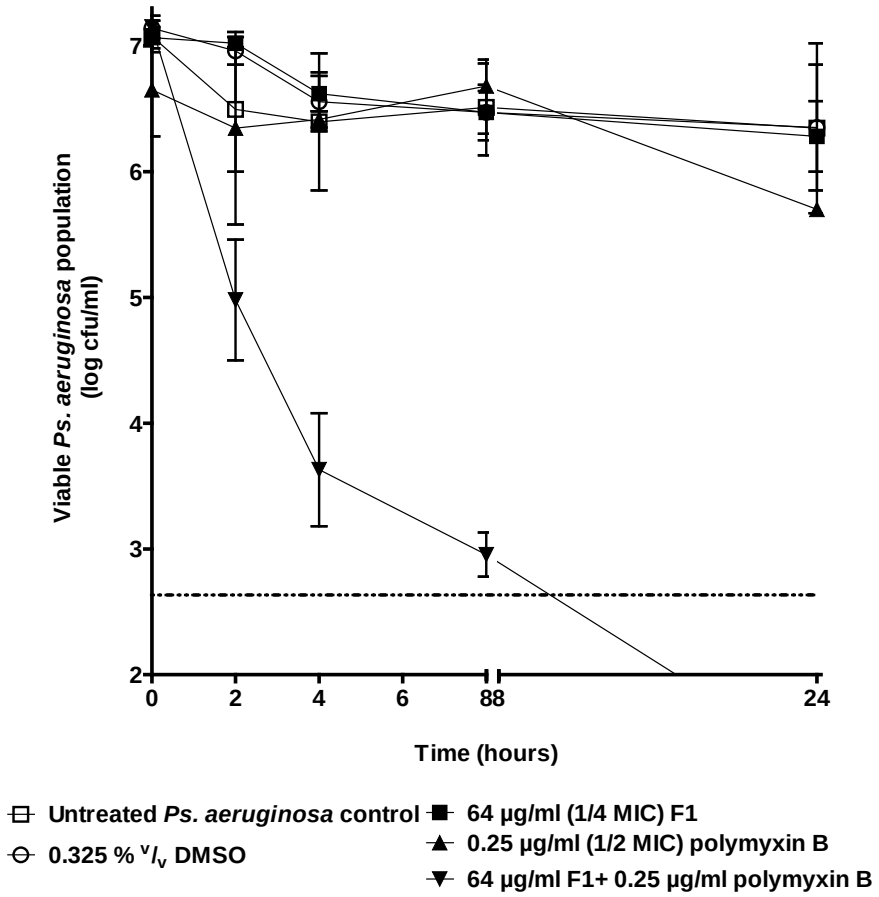


Figure 5.7: Synergistic activity of sub-inhibitory concentrations of F1 applied with sub-inhibitory concentration of polymyxin B against *Ps. aeruginosa.* Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

The data presented in table 4.1 however also shows that the application of 64 µg/ml F1(4 x the former concentration) with 0.25 µg/ml polymyxin B was bactericidal against this bacterial species in both of the previously conducted techniques. Bearing this in mind and especially considering that an increased inoculum density (1 x 10^7 cfu/ml) of this Gram-negative bacterial suspension was to be used for the re-assessment, the latter more effective combination was chosen to be applied for the investigation. This choice is also supported by findings from other researchers as previously published data show that the action of polymyxin B (as well as colistin) against *Ps. aeruginosa* is both concentration-dependent and influenced by inoculum population (Li et al. 2001b, Tam et al. 2005), such that restricting the amount of the chalcone-derivative whilst

increasing the strengths of polymyxin B would be expected to demonstrate an increasing antibiotic effect against this bacterial species.

In comparison to the DMSO-bacterial control, 0.25 µg/ml (1/2 MIC) polymyxin expressed a less than 1 log reduction to population of *Ps. aeruginosa* suspension over 24 hours and 64 µg/ml (1/4 MIC) F1 showed no appreciable activity against this organism. Both compounds applied together at the same strengths against an equivalent bacterial density demonstrated a synergistic outcome by reducing the bacterial density from ~1 x 10^7 to ~1 x 10^3 cfu/ml within the first 8 hours and further down to ~20 cfu/ml (considered to be below the detection limit of 500 cfu/ml for the assay) after 24 hours.

An overview of the results presented so far in this chapter indicate F1 is at best only bacteriostatic against species of *S. aureus* in both buffer and growth media even at concentrations as high as 8 x MIC whilst polymyxin B demonstrated no appreciable antibacterial action against Gram-positive *S. aureus* species, especially methicillin-resistant strains. The combined applications of both compounds demonstrated a profoundly bactericidal action against all tested *S. aureus* species and *Ps. aeruginosa* but were indifferent in their antibacterial effect against *C. violaceum* since the activity demonstrated by F1 alone was not surpassed by that of the combination. Of note however is the immense degree of activity demonstrated by F1 against this organism.

5.3.3 Kinetic colony counting for Gram-positive bacterial species challenged with F23 and polymyxin B

Using the same inoculum densities for the five bacterial species employed in the evaluation of the antibacterial potential of polymyxin B in combination with F1, the antibiotic effect of polymyxin B in the presence of F23 was examined and the findings are presented below. Given that the data presented in tables 4.1 and 4.2 suggest neither of the chalcone-derivatives alone had activity against the examined bacterial species in chequerboard assays and that this was verified when single applications of F1 concentrations were previously tested against QRSA, the action of F23 in combination with polymyxin B was examined against the selected bacterial species based on guidance from the findings recorded in table 4.2.

5.3.3.1 Synergistic activity of F23 with polymyxin B against MSSA

The data presented in figure 5.8 was obtained from a time-course viability assay for MSSA suspensions challenged with 256 µg/ml polymyxin B, 16 µg/ml (1/4 MIC) F23, the latter concentration of F23 with either 64 or 256 µg/ml polymyxin B and twice this F23 strength with 256 µg/ml (MIC) polymyxin B. The selection of concentrations of both compounds examined was based on information previously gleaned from both chequerboard and MBC determination assays (table 4.2) wherein an application of 16 µg/ml F23 with 256 µg/ml polymyxin B was synergistic against MSSA.

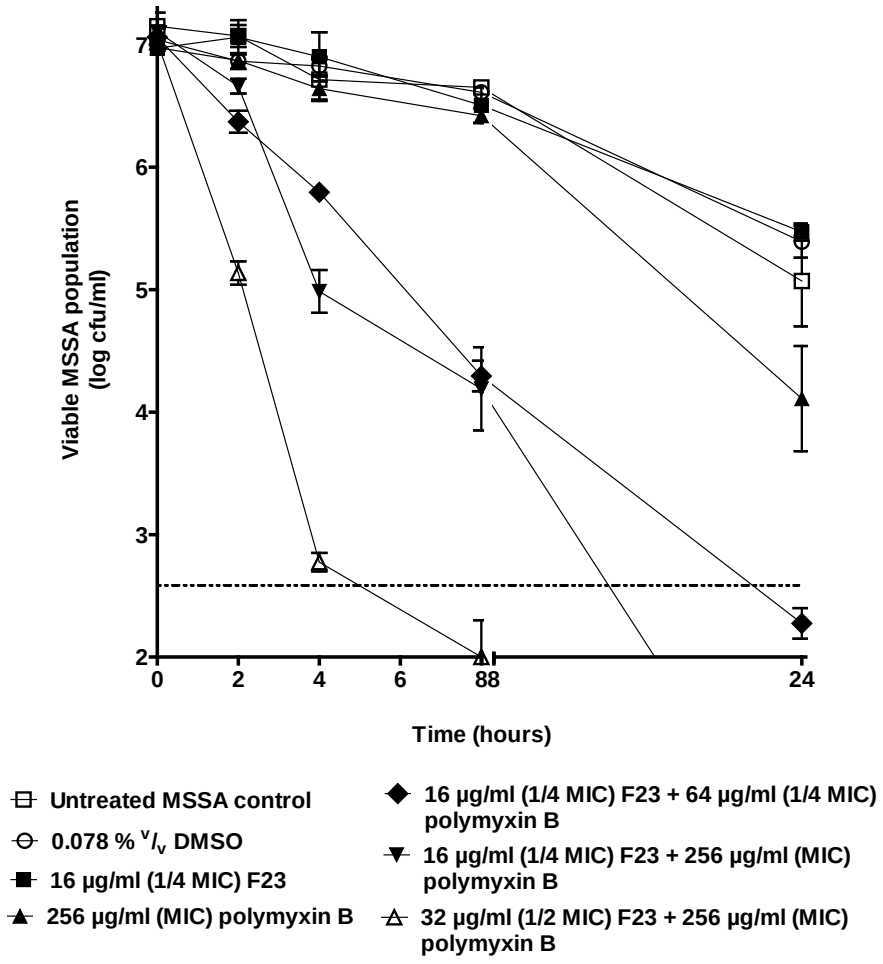


Figure 5.8: Synergistic activity of sub-inhibitory concentrations of F23 applied with polymyxin B concentrations against MSSA. Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

Bactericidal activity was also observed in the well containing 16 µg/ml F23 with 64 µg/ml (1/4 MIC) polymyxin B as well as that containing the cell suspension treated with 32 µg/ml (1/2 MIC) F23 plus 256 µg/ml (MIC) polymyxin B. The

data previously reported in chapter 3 that indicated MIC of polymyxin B against MSSA was 256 µg/ml was used in conducting the time-course viability assay.

Considering that the data recorded in figure 5.1 shows that viability of unchallenged *S. aureus* suspension was negatively affected by the combination of 0.9 % $^{w}/_{v}$ sodium chloride with 1.28 % $^{v}/_{v}$ DMSO over a 24 hours period, a lower strength of 0.078 % $^{v}/_{v}$ final DMSO was used to dissolve F23 and also employed in a control. Although the degree of loss in viability for both controls were lesser in this instance than in the former occasion, the 0.078 % $^{v}/_{v}$ DMSO control still demonstrated a > 1 log reduction in cell density that became more pronounced between 8 to 24 hours. The loss of cell viability observed in the untreated and solvent-treated samples re-emphasized the poor capacity of *S. aureus* to remain viable in 0.9 % $^{w}/_{v}$ sodium chloride over a 24 hours period.

Within 24 hours, 256 µg/ml (MIC) polymyxin B demonstrated bacteriostatic activity against MSSA by causing a ~1.5 log reduction to the inoculum population whilst suspension treated with 16 µg/ml (1/4 MIC) F23 had the same colony count as the solvent control after 24 hours clearly expressing the lack of potency of this semi-synthetic agent against this species at the applied strength.

All the three applications containing sub-inhibitory concentrations of F23 with two varying strengths of polymyxin B were synergistic against MSSA, demonstrating extents of action that were at least 2 log greater than that of polymyxin B alone. Of all the three combinations, the combined application of 16 µg/ml (1/4 MIC) F23 with 64 µg/ml (1/4 MIC) polymyxin B appeared to have shown the lowest degree of antibiotic action by demonstrating a > 3 log reduction to the MSSA suspension within 24 hours. A concentration of 16 µg/ml F23 applied with 64 µg/ml (1/4 MIC) polymyxin B reduced the inoculum population by ~2 log within 2 hours and further down to below detectable limits within 24 hours indicating synergism when viewed with reference to the activity of a higher concentration 256 µg/ml polymyxin B that was singly applied. This recorded level of activity was followed by the action of the combination containing 16 µg/ml F23 plus 256 µg/ml polymyxin B that caused a depletion of the density of an equivalent bacterial population by 2 log cfu/ml within 4 hours and further down to below detectable levels after 24 hours.

As would be expected from the combination containing the highest concentrations of both compounds, the application of 32 µg/ml (1/2 MIC) F23 with 256 µg/ml polymyxin B was the most potent. This latter combination

instituted a 3 log loss in the viability of the challenged cell suspension within 4 hours and a further reduction to below the detectable limit of log 2.69 after 24 hours. This combination therefore demonstrated the most rapid onset and rate of action against MSSA. An increase in the concentration of F23 in the combination therefore translated to a corresponding increase in onset, rate and extent of these compounds antibacterial synergism against MSSA.

5.3.3.2 Synergistic activity of F23 with polymyxin B against MRSA

With an MIC of 1024 µg/ml, F23 was considered inactive against MRSA. However, the chequerboard assay had demonstrated that 32 µg/ml (1/32 MIC) F23 applied with 64 µg/ml (1/2 MIC) polymyxin B was synergistic against MRSA (table 4.2). The technique for MBC determination confirmed the bactericidal property of this combination against MRSA. This combination was therefore applied at the same strength in a time course viability assay whose results are presented in figure 5.9.

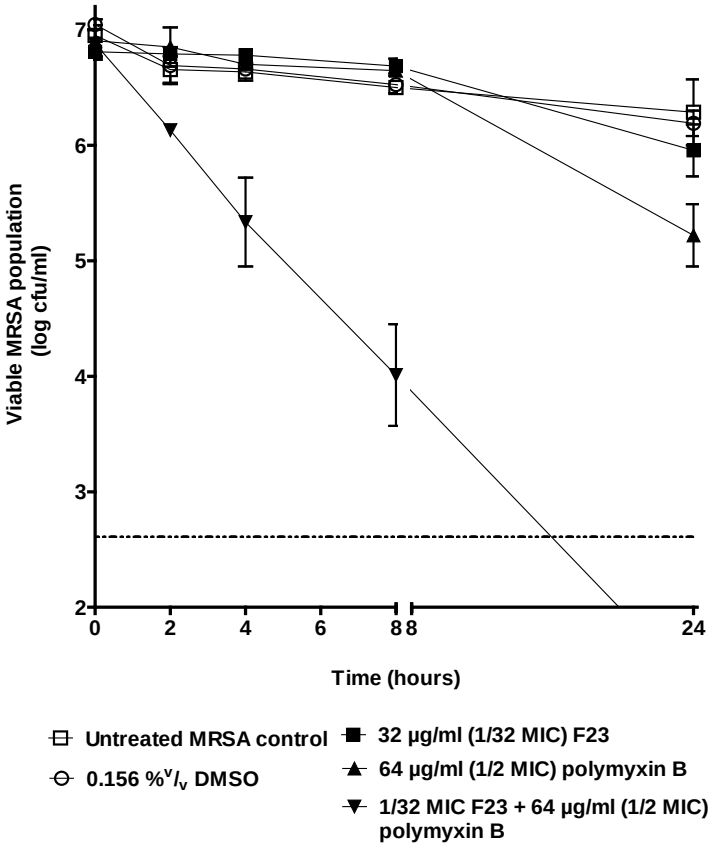


Figure 5.9: Synergistic activity of sub-inhibitory concentrations of F23 applied with polymyxin B against MRSA. Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

Over a 24 hours period, 64 µg/ml (1/2 MIC) polymyxin B was the most active single agent displaying a bacteriostatic action with an approximately 1 log loss in colony count of MRSA that became conspicuous between the 8 and 24 hours with reference to the untreated solvent control. Another aliquot of the bacterial suspension treated with 32 µg/ml (1/32 MIC) of this semi-synthetic agent singly as well as the untreated positive and solvent controls showed no appreciable decline in colony count after 24 hours.

An equivalent density of the bacterial suspension challenged with a combination of the same strengths of both compounds above displayed 2.5 and >4 log losses in viability after 8 and 24 hours respectively, indicating synergism in view of the activity of the most potent compound in the application. The loss in viability recorded for the latter combination was more pronounced and exceeded the detection limit of 500 cfu/ml for the employed technique. The outcome of this assay confirmed the data recorded from the two other techniques that signalled the existence of synergism between both compounds at the examined strengths.

5.3.3.3 Antibacterial action of F23 with polymyxin B against QRSA

The data from a time-course viability assay wherein a QRSA population was challenged with 16 µg/ml (1/2 MIC) F23 with 64 µg/ml (1/4 MIC) of polymyxin B is presented in figure 5.10.

Previous data obtained from chequerboard and MBC determination techniques had shown that this combination of compounds, at the strengths stated above, was bactericidal against QRSA (table 4.2). The polypeptide antibiotic was the most active single agent in the combination since quinolone-resistant *S. aureus* suspension treated with 64 µg/ml (1/4 MIC) polymyxin B demonstrated a 2 log loss in viability within 24 hours, indicating a bacteriostatic action whilst 16 µg/ml F23 alone caused only about 0.5 log depletion to the colony count of an equivalent cell density. When both compounds were applied together against another equivalent QRSA population at the strengths defined above, more than a 4 log reduction in colony count, below the detection limit of log 2.69, was seen after 24 hours with reference to the solvent control whilst only an additional 2 log loss in viability was recorded with reference to the most potent single compound of the combination. This outcome suggests the existence of synergism between polymyxin B and F23 at the evaluated concentrations.

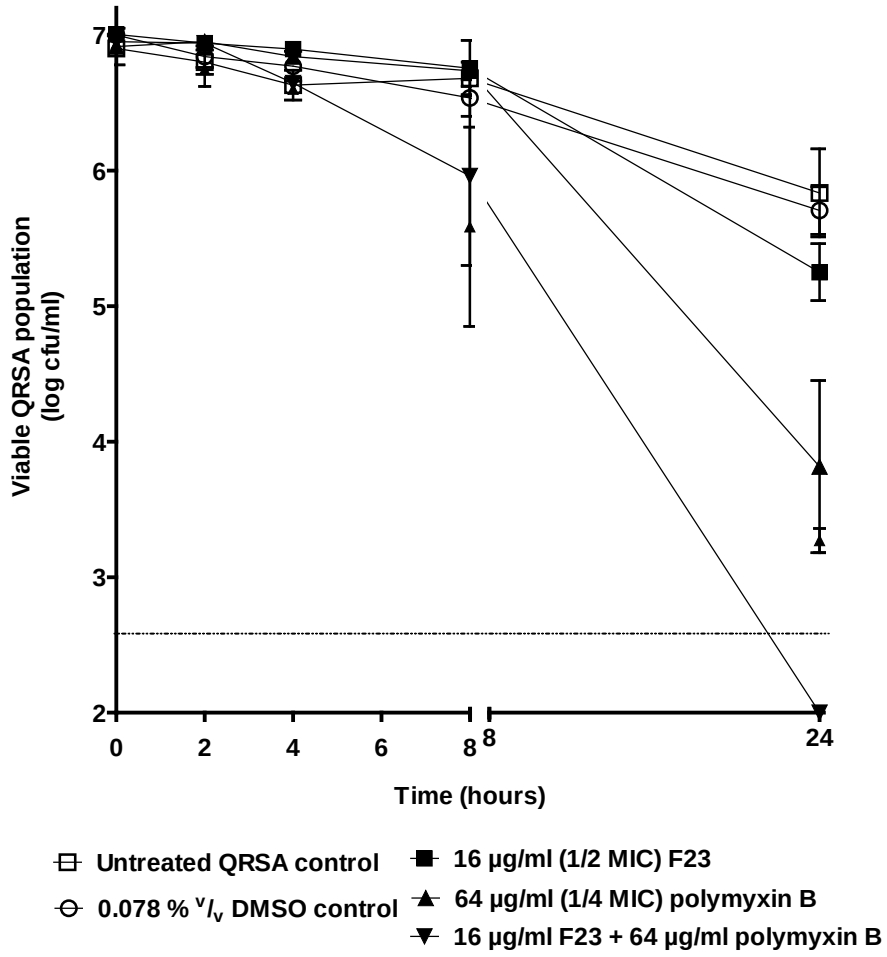


Figure 5.10: Synergistic activity of sub-inhibitory concentrations of F23 applied with sub-inhibitory concentration of polymyxin B against QRSA. Inoculum density was ~2 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

However, the onset of action for this combination was comparatively slow as there was negligible activity within the first 4 hours and only about a 1 log depletion in viability was reported after 8 hours. In a previous time-course assay wherein the activity of F1 applied with polymyxin against QRSA was evaluated in 0.9 % $^v/_v$ sodium chloride (figure 5.5), 5 % $^v/_v$ DMSO final concentration was the solvent for F1 and was also applied as a control. This solvent control sample was seen to have displayed a 2 log loss in the viability of QRSA suspension. The data presented in figure 5.10 however shows a 1 log depletion in viability of the solvent control cells which suggests the organism was less sensitive to the presence of 0.078 % $^v/_v$ DMSO in saline.

5.3.4 Kinetic colony counting for Gram-negative bacterial species challenged with F23 and polymyxin B

5.3.4.1 Antibacterial action of F23 with polymyxin B against *C. violaceum*

The data presented in figure 5.11 were obtained from *C. violaceum* suspensions treated with either 64 µg/ml (1/8th MIC) F23 only or 4 µg/ml (1/8th MIC) polymyxin B for 24 hours.

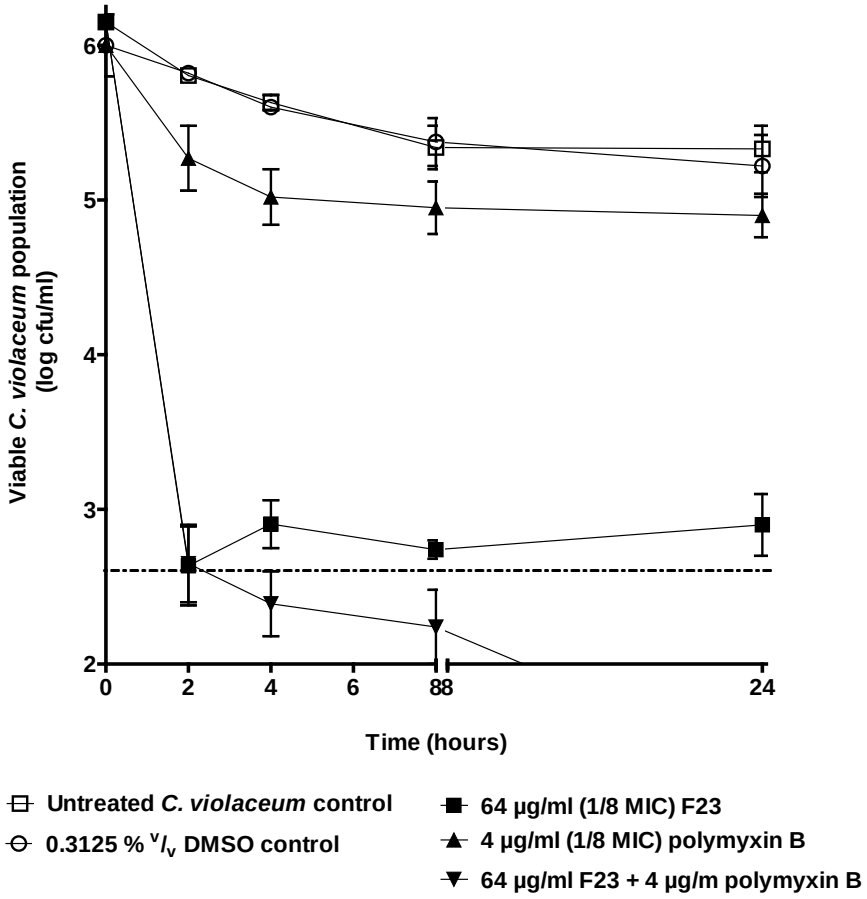


Figure 5.11: Bactericidal activity of sub-inhibitory concentration of F23 and indifferent activity of sub-inhibitory concentration of F23 applied with sub-inhibitory concentration of polymyxin B against *C. violaceum*. Inoculum density was ~2 x 10^6 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

The choice of strengths of both compounds to be evaluated was based on data previously obtained from a chequerboard and MBC determination techniques. The MBC determination assay verified the effectiveness of these combined concentrations of compounds that indicated either an additive or synergistic

outcome in the chequerboard assay in that bacterial re-growth did not occur within 48 hours in treated samples of *C. violaceum* that were sub-cultured onto fresh antibiotic-free agar media (table 4.2).

In the present technique, with respect to the solvent control, 64 µg/ml ($1/8^{th}$ MIC) F23 was on its own bactericidal against *C. violaceum* by demonstrating approximately a 3 log depletion to the density of the treated cell suspension within 2 hours. An equivalent cell density treated with 4 µg/ml (1/8 MIC) polymyxin B was seen to have lost only about 0.5 log in viability within 24 hours. When the chalcone-derivative and polypeptide antibiotic were applied together at the strengths defined above against another standard sample containing an equivalent density of the same bacterial species, the same end-point reduction in colony count previously recorded for the cell suspension treated with 64 µg/ml F23 alone, was obtained. This observation suggested the collective application of both compounds acted indifferently. Therefore, F23 applied singly as well as in combination with polymyxin B demonstrated a rapid onset of antibacterial action by reducing the density of the treated *C. violaceum* from ~2 x 10^6 to ~400 cfu/ml, below the detection limit of the assay, within 2 hours. This action is comparable to the activities of sub-inhibitory concentration of F1 applied alone and with polymyxin B against this bacterial species. Between 2 and 24 hours, the cell suspension challenged with F23 plus polymyxin B showed a slight further drop in colony count suggesting that this combination is relatively more potent with increasing time of exposure than the single application of polymyxin B. Both the solvent and untreated samples demonstrated no appreciable loss in viability, indicating as previously mentioned, that *C. violaceum* is less sensitive to the presence of sodium chloride.

5.3.4.2 Bactericidal action of F23 with polymyxin B against *Ps. aeruginosa*

Figure 5.12 represents the data obtained from a kinetic colony count assay for a *Ps. aeruginosa* suspension challenged with 64 µg/ml (1/4 MIC) F23 with 0.25 µg/ml (1/2 MIC) polymyxin B.

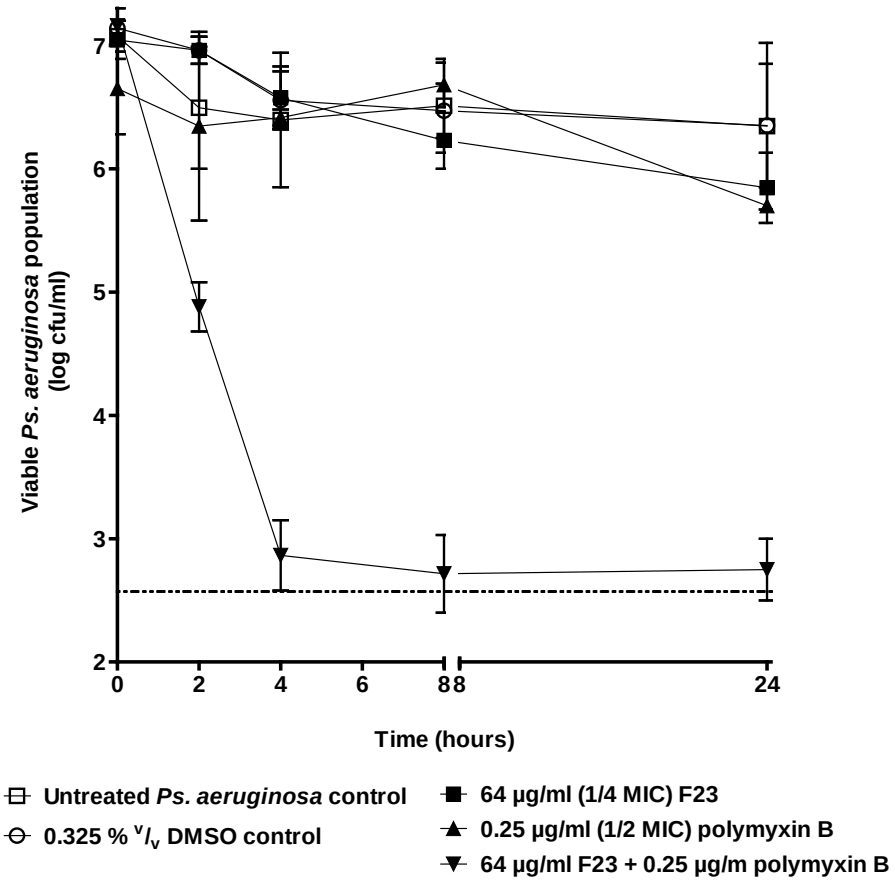


Figure 5.12: Synergistic activity of sub-inhibitory concentration of F23 applied with sub-inhibitory concentration of polymyxin B against *Ps. aeruginosa*. Inoculum density was ~1 x 10^7 cfu/ml. The data were mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

Bearing in mind that this organism is a recalcitrant Gram-negative organism, the applied concentrations were twice the values of bactericidal strengths recorded for these agents during a previously conducted chequerboard assay and verified by an MBC determination technique (table 4.2).

Although the untreated positive and solvent-treated controls showed no appreciable reduction in cell viability within the 24 hours period, cell samples treated with either 0.25 µg/ml (1/2 MIC) polymyxin B or 64 µg/ml (1/4 MIC) F23 demonstrated less than a 1 log reduction in their population.

In contrast, an equivalent cell density challenged with 64 µg/ml (1/4 MIC) F23 and 0.25 µg/ml (1/2 MIC) polymyxin B showed a rapid 4 log depletion in colony count (from ~2 x 10^7 to ~800, which was within the detection sensitivity limit of the technique) within 4 hours. The ~800 cfu/ml *Ps. aeruginosa* density was maintained after 24 hours. The displayed action of both compounds combined

viewed with reference to that of the most potent single agent, polymyxin B, indicates synergism in that the collective action induced > 3 log depletion in the cell viability more than that caused by the former.

5.3.5 Summary of data for the examination of the effects of the chalcone-derivatives in combination with polymyxin B against bacterial species viability

F1 and polymyxin B acted in a synergistic manner against MSSA, MRSA, QRSA and *Ps. aeruginosa*; whilst both agents were indifferent in activity against *C. violaceum* as the application of effective concentrations of both compounds determined by a chequerboard assay expressed no significant additional action to the killing effect of the chalcone-derivative alone. Similarly, F23 concentrations applied with different strengths of polymyxin B were synergistic in action against MSSA, MRSA, QRSA and *Ps. aeruginosa* but also acted indifferently against *C. violaceum*. The results obtained for F1 and F23 each applied with polymyxin B verified the data previously reported from the chequerboard assay (tables 4.1 and 4.2) suggesting such combinations were either additive or synergistic against MSSA, MRSA, QRSA and *Ps. aeruginosa*. Although the chequerboard assay indicated 16 µg/ml F23 applied with 64 µg/ml polymyxin B was additive against QRSA, data recorded from the time-kinetic assay suggested the same combination was synergistic against this species.

After 24 hours of exposure, all the applied combinations (at various concentrations) demonstrated a≥ 2 log reduction to the challenged cell populations with reference to the density of equivalent cell suspensions singly treated with the most potent agent for the various test bacterial species; indicating synergism in all instances.

Finally, figure 5.8 also shows that concentrations of both F23 and polymyxin B in the combination could be adjusted to influence the potency and onset of action for the antibacterial action of the combination against MSSA. Although 1/4 MIC (16 µg/ml) of F23 applied with 1/4 MIC (64 µg/ml) polymyxin B was effectively bactericidal against MSSA, the highest examined strengths of both compounds were half the MIC (32 µg/ml) of F23 applied with the MIC (256 µg/ml) of polymyxin B which as expected demonstrated the most profound and rapid onset of bactericidal action indicating a concentration-dependent potency.

5.4 Discussion

It is an established fact that the outcome and interpretation of a set of data as being either bacteriostatic or bactericidal in a chequerboard assay may not always be consistent with findings from a time-kinetic colony counting assay (Lewis et al. 2002). The latter assay is the most reliable *in vitro* technique for verifying the activity of combination of agents recorded from the former method (Alou et al. 2004). In this chapter, given that 10 to 10^6 –fold dilutions were plated out on agar for incubation and colony counting, the limit of detection for this assay was actually 500 cfu/ml. Although *S. aureus* 6571 (MSSA) demonstrated relatively poor survival capacity in 0.9 % $^{w}/_{v}$ NaCl used as buffer, the viability of *C. violaceum* (figure 5.6) as well as *Ps. aeruginosa* (figure 5.7) in same medium were much better and may not be unconnected with the fact that both are Gram negative organisms with outer membrane barriers. The tenacious ability of *Ps. aeruginosa* to survive in very adverse conditions as well as to persist under antibiotic treatments has been extensively discussed in the literatures (Evans, Adewoye and Poole 2001, Kasiakou et al. 2005, Bjarnsholt et al. 2009, Schurek, Breidenstein and Hancock 2012).

The *in vitro* bactericidal activities of the chalcone-derivatives (F1 and F23) applied with polymyxin B against MSSA, MRSA, QRSA, *C. violaceum* and *Ps. aeruginosa* were significant when compared to the single application of each compound. The combined concentrations of both compounds examined in this assay, which in most cases were found to be synergistic in action, were estimated from previous chequerboard and MBC determination techniques. These results therefore verified the data gleaned from the previous micro broth MIC determination, chequerboard and MBC determination assays. In addition, the time-course viability technique displayed the mode of bacterial killing over time for these agents, showing in some cases, a more extensive action than had been earlier reported. For instance, certain combination concentrations thought to be additive in action in the chequerboard assay were currently reported as synergistic, even in the presence of a higher inoculum population. This is clearly exemplified for *Ps. aeruginosa* suspensions treated with concentrations of F1 in combination with polymyxin B (table 4.1 and figure 5.7). The most likely explanation for these minor discrepancies would be that the time-course assay exposes some of the weaknesses inherent in the other techniques (Pankey and

Ashcraft 2005). The inability to sometimes align findings from time-kinetic colony counting assays with those from a chequerboard and the often irreproducible nature of data from the latter technique has been previously established (Petersen et al. 2006, Lewis et al. 2002). Therefore, data obtained from chequerboard assays often need to be verified by other techniques of which the most recommended is the time-kinetics colony counting experiment (Petersen et al. 2006).However, the data obtained from *in vitro* assays conducted with effective strengths of compounds that were previously determined by either chequerboard or time-course viability assay are some times neither mirrored nor comparable with results from *in vivo* methods due to interference from body fluid. A depletion of the applied strengths of compounds to either below the minimum effective concentrations of both compounds or to an ineffective combination ratio can occur due to the activity of biological enzymes coupled with the existence of a variation in these agents individual half-lives (Reddy 2000, Jacqueline et al. 2005, Pinto et al. 2005, Johnson 2008).

The data presented from assays carried out with an inoculum density of 1×10^6 cfu/ml in the published manuscript of V. H. Tam show that polymyxin B is rapidly bactericidal against various antibiotic sensitive and resistant strains of *Ps. aeruginosa* in growth medium, albeit with extensive re-growth after 2 to 4 hours (Tam et al. 2005). This polypeptide antibiotic is effective against many other Gram-negative bacterial species (Fernandez et al. 2010, Martti 2010, Hogardt et al. 2004, Hermsen, Sullivan and Rotschafer 2003) and notably demonstrates a rapid *in vitro* action against multi-drug resistant *A. baumanii* and *K. pneuomoniae* (Zavascki et al. 2007) yet considered ineffective against most Gram-positive organisms including *S. aureus* (Res 2003). Single applications of polymyxin B were at best only weakly bacteriostatic against species of *S. aureus* in this report.

The findings that F1 applied together with polymyxin B demonstrated an enhanced activity against MSSA, MRSA, QRSA, *C. violaceum* as well as *Ps. aeruginosa* in time-course colony counting assays is consistent with reports presented in the previous chapter and is very remarkable. But a weakness in this study is the fact that it could not be verified whether any of the combinations were antagonist in action as all the assays were carried out in a buffer medium which can not permit the demonstration of increment in population over time due to the inability of organisms to replicate therein.

The data presented in figure 5.2 suggest F1 concentrations up to 512 µg/ml (4 x MIC) were ineffective against MRSA populations and at 1024 µg/ml (8 x MIC) was only bacteriostatic with a 1.5 log cfu/ml reduction to the inoculum population. Meanwhile, under growth conditions (figure 5.4), a concentration of F1 equivalent to the 32 µg/ml (MIC) demonstrated some degree of blockage to the ability of the quinolone-resistant strains of *S. aureus* to multiply in comparison to the untreated and solvent-treated controls. At higher concentrations in the range of (64 to 1024 µg/ml) 2 to 32 x MIC, this compound successfully impeded the ability of the quinolone-resistant cells to replicate within 24 hours. The information gleaned from both assays presented in figures 5.2 and 5.4 appears to strongly suggest that F1 is more active under growth media than in buffer conditions and may have the capacity to cause a blockage to the biosynthesis of peptidoglycan in *S. aureus* species. Within the range of concentrations used in the growth medium above, the capacity of QRSA cells to develop new structures (e.g. cell wall, proteins, DNA, etc.) and to replicate were apparently being impeded. Penicillin inhibits cell wall formation by blocking peptidoglycan biosynthesis in viable cells especially under growth conditions (Lewis 2001). This antibiotic is bactericidal under growth conditions but ineffective against most bacterial species suspended in buffer; except mycoplasma, which is inherently resistant to the activity of penicillin due to the absence of peptidoglycan and conventional cell wall (Normark and Normark 2002).

Figures 5.8 to 5.10 and 5.12 similarly show that sub-inhibitory concentrations of F23 applied alone were devoid of antibacterial activity against the entire test bacterial species (except *C. violaceum* in figure 5.11). These results are comparable to those obtained from bacterial populations challenged with F1 alone in figures 5.1 to 5.7. At best therefore, single applications of F1 were only bacteriostatic against methicillin-sensitive and antibiotic resistant strains of Gram-positive *S. aureus* while F23 was also bacteriostatic against *Ps. aeruginosa*. Both compounds were each bactericidal against *C. violaceum* at the applied concentrations that were seen to be sub-inhibitory and ineffective in the chequerboard technique.

Considering that F1 and F23 demonstrated weak antibacterial action when applied alone, both compounds were examined for potential augmentative effect upon the antibacterial action of established agents like that provided by

clavulanic acid (Navarro 2005). Clavulanic acid is devoid of appreciable antibacterial activity when applied alone (Rolinson 1998, Kim et al. 2009) but can greatly potentiate the antibacterial activity of penicillin and amoxicillin against β-lactamase producing bacterial strains (Therrien and Levesque 2000, Bremner, Ambrus and Samosorn 2007). This compound has demonstrable capacity to competitively inhibit the activity of β-lactamase enzymes, thereby preventing the cleavage of the β-lactam ring and allowing amoxicillin to exert maximum effect upon susceptible pathogens (Mark, Vocadlo and Oliver 2011).

In these last two chapters, F1 and F23 have shown demonstrable capacity to potentiate the antibacterial action of polymyxin B (or vice-versa), leading to enhanced activity against both Gram-positive and negative bacterial species. Just like (-)-epicatechin gallate that reduces the MIC of oxacillin by as much as 512-fold (Cushnie and Lamb 2011) by the enforcement of structural changes upon the cell wall teichoic acid, which is a component of peptidoglycan as well as inducing the release of lipoteichoic acid from the cytoplasmic membrane of MRSA (Stapleton et al. 2007). It is yet to be investigated how the combination of polymyxin B with the chalcone-derivatives act to synchronise and amplify their action against *S. aureus* species but many antibiotics that are effective against Gram-positive cells such as the β-lactams - penicillins, carbapenams, etc. usually primarily block cell replication by inhibiting the capacity of these organisms to expand their peptidoglycan layer as well as develop new cell wall structures (Pinho and Errington 2005). There is a speculation that epicatechin gallate is able to increases the susceptibility of MRSA to β-lactam antibiotics in MRSA either through binding to penicillin binding proteins at target locations distinct from the normal penicillin-binding sites or by intercalation into the cytoplasmic membrane thereby causing the dislodgement of lipoteichoic acid from the phospholipid structure (Stapleton et al. 2007). Newer antibiotic agents that can inhibit the biosynthesis of peptidoglycan by novel mechanisms such as either binding to new targets within penicillin-binding proteins or essential enzymes such as Mur, lipid I and II, in the cell wall biosynthesis pathway, are still being sought after (Hao et al. 2012).

At the moment, one speculation is that the chalcone-derivatives may be having some form of activity against the formation of new peptidoglycan structures which manifested as inhibition of cell replication under growth conditions. Upon single applications, this semi-synthetic agent may have been unable to access its

primary target site at sufficient concentrations leading to the poor antibiotic action. During combined application with polymyxin B, it is speculated that this polypeptide promotes the entry of these semi-synthetic agents and their capacity to reach their active sites thereby facilitating the establishment of their antibacterial action. On the other hand, polymyxins can competitively bind to the anionic lipopolysaccharide, undergo self-translocation and disrupt the outer membrane integrity in Gram-negative bacterial species (Hancock and Sahl 2006, Jenssen, Hamill and Hancock 2006a, Hancock 2001). For this group of bacterial cells, a second speculation is that the synergistic action recorded from the combined application of the chalone-derivatives with polymyxin B is either through the augmentation of this latter mechanism of action or and in addition, the blockage of other intracellular enzyme activity, such as that of the bacterial reducing enzymes, NADH and succinate dehydrogenases. Their combined effect is extended against Gram-positive cells probably due to the action of the chalcone-derivatives against the thicker peptidoglycan layer and cell wall development. The inhibition of certain enzymes activities and defined biosynthetic pathways can culminate in the blockage of cell wall formation. The impairment of transpeptidation and transglycosylation steps, which are the cross-linking stages of cell wall biosynthesis, are known to halt cell wall formation (Kahne et al. 2005). Penicillin can inhibit transpeptidase enzyme by the formation of slowly hydrolysing covalent acyl enzyme intermediates whilst vancomycin causes a complexation of the D-ala-D-ala termini of growing peptidoglycan by making five hydrogen bonds to this dipeptide structure of each uncrosslinked peptidoglycan pentapeptide side chain (Walsh 2000). Further assays will be required to determine whether an application of these chalcone-derivatives either by themselves or in combination with the polypeptide antibiotics blocks essential enzymes required for biosynthetic and metabolic pathways in the examined bacterial species.

Based on the information previously gathered from the chequerboard and MBC determination assays, sub-MIC concentrations of both compounds were some times re-assessed in the current assay, in order to verify, in some instances, the lowest most effective concentrations of the chalcone-derivatives required to potentiate the action of polymyxin B (or vice versa), against the test bacterial species. For instance, table 4.1 and figure 5.7 show that only a relatively small fraction of F1 (1/4 MIC) was needed to augment the antibacterial activity of

polymyxin B against *Ps. aeruginosa*, a recalcitrant Gram-negative bacteria. This observation is comparable to the action of clavulanic acid, as only a small amount of this agent is required to potentiate the antibacterial action of amoxicillin against β-lactamase producing strains of *S. aureus* when applied as co-amoxiclav (Navarro 2005).

The fact that F1 applied with polymyxin B was synergistic in action against both methicillin-sensitive (MSSA) and resistant strains (MRSA) of *S. aureus* may be suggesting a relative reduction in the thickness of the peptidoglycan wall as well as a depletion in the binding capacity of penicillin-binding proteins were having little impact on the combined action of both agents. Mutant strains of MRSA grown in the presence of oxacillin and examined under scanning electron microscope were seen as having enlarged, malformed and rough surface cells (Raju et al. 2007). The increase in cell size was compensated for by a decline in the size of this cell envelope which translates to a reduction in the binding site (capacity) as well as the antibiotic effect of oxacillin on these cells.

Colonies of MRSA used in this project were seen to have a larger morphology on agar plate than their MSSA and QRSA counterparts. This suggests they may well have had a smaller cell envelope and a reduction in the binding site for agents that have the peptidoglycan as primary target. The data presented however showed the combination of polymyxin B with the chalcone-derivative demonstrated comparable antibiotic action against all three strains of *S. aureus* irrespective of their morphology.

Resistance of *S. aureus* to penicillin has in some cases been proven to be due to a reduction in the ability of the binding proteins in the cell wall to interact with penicillin, as well as this compound's derivatives such as the beta-lactams (Enright et al. 2002, Dowson, Coffey and Spratt 1994). Such cells could also be seen to be having thickened cell walls as is observable with clinical strains Mu3 and Mu5 of MRSA with varying degrees of resistance to vancomycin (Hanaki et al. 1998). The demonstration of resistance to vancomycin and teicoplanin by some strains of MRSA is some times due to the inactivation of penicillin-binding-protein (pbp4) (Sieradzki, Pinho and Tomasz 1999) and a thickening of the cell wall, otherwise described as activated peptidoglycan synthesis (Appelbaum 2007). This latter increase in peptidoglycan biosynthesis leads to a decrease in the capacity of certain antibiotics like vancomycin to gain access to required target active sites as the molecules end up entrapped within the wall (Sieradzki,

Pinho and Tomasz 1999). In MRSA that express intermediate resistance to vancomycin (VISA), glycopeptides are unable to gain access to cell wall synthesis sites permitting the continued expansion of existing cell wall structures and the development of new ones even in the presence of this antibiotic unlike in those strains that are sensitive to vancomycin (Appelbaum 2007). As previously speculated, the findings in this study suggest that polymyxin B promotes the translocation of either of these chalcone derivatives across bacterial membrane bilayers thereby providing them unhindered access to the cell wall synthesis sites of both antibiotic sensitive and resistant strains of *S. aureus*. A suitable formulation of both compounds may be a potentially effective alternative against glycopeptide-sensitive and resistant MRSA strains. Further laboratory work involving the use of polymyxin B with either of these semi-synthetic compounds against such MRSA strains, that are CLSI recommended, is however required to verify this speculation. It is known that MRSA grown in the presence of (-)-epicatechin gallate demonstrates a decline in the substitution of wall teichoic acid backbone by D-alanine (D-ala) and ratios of *N*-acetyl glucosamine to D-ala are reduced by as much as about 50 %, which translates to a decrease in the positive charge of the bacterial wall during molecular simulations, and confirmed by an increase in cationized ferritin binding (Bernal, Zloh and Taylor 2009). These observations have given further insight into the mechanisms by which this latter semi-synthetic flavonoid compound increases the susceptibility of MRSA to β-lactam antibiotics.

The findings in this study also suggest that either of the two compounds is capable of promoting the trans membrane up-take of the other or both and thereby facilitating the inhibition of some form of metabolic activities in both Gram-positive and negative bacterial species. This latter speculation is because of the rapidity with which the combination was bactericidal against the challenged bacterial species, despite the high biological load of the inoculum population. Antibiotics that manifest such a high speed at the killing of micro-organisms are thought to be able to inhibit essential metabolic processes, such as the activities of NADH dehydrogenase and ATP synthase in bacterial respiration (Zhang, Rozek and Hancock 2001a, Tam et al. 2005), as well as cause the impairment of protein synthesis to stop cell replication. Bacterial membrane de-energization (Zhang, Rozek and Hancock 2001a), depolarisation and disruption for which polymyxin is known (Hancock and Rozek 2002a) can

initiate processes that lead to impairment of metabolism as well as leakage of intracellular materials. The synergistic activity of both compounds is therefore thought of at the moment as being most probably initiated by the presence of this polypeptide antibiotic both as a bilayer-barrier translocation agent and as the catastrophic element initiating the augmented blockade of yet to be verified processes.

The data presented in figure 5.6 also showed that although F1 applied alone was bactericidal against *C. violaceum*; F1 applied in combination with polymyxin B demonstrated the same rapid and profound quantitative reduction in colony count. This is suggestive of indifference in action for both compounds applied together contrary to the report in table 4.1 from the chequerboard assay in which F1 applied in combination with polymyxin B was outstandingly synergistic with a FIC_{index} of 0.09375. But the more reliable time-course viability assay (Petersen et al. 2006) clearly showed both compounds were indifferent in activity when examined under buffer conditions. Considering that the chequerboard assay was conducted under growth medium, additional time-kinetic assays for *C. violaceum* populations challenged with polymyxin B together with each of the chalcone-derivatives is required to give more insight to the mode of action of both compounds against this organism and enable a more appropriate comparison of the data from the two different assays. The mechanism of action by which either of the chalcone-derivatives alone, as well as polymyxin B, were able to display rapid bactericidal actions against *C. violaceum* should be investigated in future studies. At the moment, the available data seemingly suggest either of the chalcone-derivatives, as well as polymyxin B, were capable of self-translocation across the membrane-barriers of this Gram-negative organism and had unimpaired access to either their primary target site or enzymes.

In the data presented, despite the high starting inoculum densities of approximately $1x10^7$ cfu/ml for all the test species ($1x10^6$ cfu/ml for *C. violaceum*), all combinations examined demonstrated bactericidal activities. Either F1 or F23 applied in combination with polymyxin B demonstrated ≥3 log cfu/ml reduction (equivalent to 99.9 % loss in viability) to the bacterial populations of *C. violaceum* with at least 2 log reduction more than the activity of the most active agent in the combination at 24 hours for challenged populations of *Ps. aeruginosa*, MRSA and QRSA. The combinations usually

demonstrated remarkable activity within the first 4 hours (except with MSSA) and after the 8th hour, bacterial viability for the various species were reduced to below detectable limits for all the *S. aureus* strains that were evaluated. The rapidity of bactericidal action for these combinations is comparable to that published for the combination of two concentrations (48 and 144 x MIC) of colistin each with (1.5 x MIC) ceftazidime against a clinical isolate of *Ps. aeruginosa* (Gunderson et al. 2003). In the published report however, despite the high concentrations of colistin used, all the bacterial populations challenged with both single or a combination of antibiotics expressed re-growth at different times within 24 hours (Gunderson et al. 2003). It is certainly worth verifying whether the killing effect of the chalcone-derivatives applied with polymyxin B against these bacterial species reported in this study will be permanent by repeating the time-course colony counting assays of challenged cell densities in growth media.

Other published reports indicate the killing effect of both colistin and polymyxin B against *Ps. aeruginosa* in kinetic colony counting experiments is concentration-dependent (Gunderson et al. 2003, Tam et al. 2005, Li et al. 2001a) and greatly diminished by a high inoculum load (Tam et al. 2005). Data from this study presented in figure 5.8 showed that higher concentrations of both F1 and polymyxin B applied together displayed increased extents and rates of synergistic effect suggesting their activity was concentration-dependent as well. But despite the high inoculum populations used all through this study, the combinations were effectively bactericidal. For although an increase in inoculum density from log 10^5 to 10^7 cfu/ml of *Ps. aeruginosa* samples causes a reduction in the antibiotic action of polymyxin B when applied alone (Tam et al. 2005) the data presented in this report show that at 8 hours, either F1 or F23 applied in combination with polymyxin B demonstrated a ≥ 3 log cfu/ml reduction to the high biological populations. Their combined action also caused a greater ≥ 2 log cfu/ml reduction to the starting inoculum density of this bacterial species in comparison to the activity of the polypeptide only, which was the most potent individual compound in the assay thereby indicating both agents were synergistic in action (Pillai et al. 2005b).

It appears that *Ps. aeruginosa* was more sensitive to the application of F1 with polymyxin B than to the same strengths of F23 with the polypeptide antibiotic as the challenged populations were reduced down to a lower colony count after 24

hours by the former combination. The antibacterial activity of F23 can be further compared and contrasted with that of F1 using the data presented in tables 4.1 and 4.2, as well as figures 5.8 to 5.12. Despite the high inoculum densities of $1x10^7$ cfu/ml, concentrations of F23 and polymyxin B in combination demonstrated synergistic activity, with ≥4 log cfu/ml reduction to the population of MSSA within the first 4 hours. After 8 hours, concentrations of F23 applied in combination with polymyxin B were bactericidal against MSSA and *Ps. aeruginosa* with approximately 5 and 4 log cfu/ml reductions in population densities of the bacterial species above respectively; and an approximately ≥3 log cfu/ml reduction to the population density of MRSA. At the 24^{th} hour, the combinations had demonstrated ≥4 log reductions to the population densities of MSSA, MRSA, QRSA and *Ps. aeruginosa*. This confirms the existence of synergism following the definition given to this concept by Eliopoulos and Moellering (Pillai et al. 2005b) between either of the chalcone-derivatives applied in combination with polymyxin B against the aforementioned 4 bacterial species.

On the other hand, the chequerboard assay had indicated F1 applied with polymyxin B was synergistic against MSSA, MRSA, QRSA and *C. violaceum*; but additive against *Ps. aeruginosa* respectively. The latter kind of assay had also indicated F23 applied together with polymyxin B was synergistic against MSSA, *C. violaceum* and *Ps. aeruginosa* although additive against MRSA as well as QRSA respectively. But when applied independently at the selected strengths, figures 5.1 to 5.12 suggest polymyxin B had no appreciable activity under buffer conditions against the challenged bacterial populations except QRSA to which this agent appears to be bacteriostatic. This report is mindful that colistin and polymyxin B are reported to be bactericidal against *Ps. aeruginosa* with extensive re-growth under growth conditions (Gunderson et al. 2003, Tam et al. 2005).

The activities of sub-inhibitory concentration of F23 applied alone and with sub-inhibitory concentration of polymyxin B against *C. violaceum* are comparable to those obtained for F1. The application of either F1 or F23 was rapidly and profoundly bactericidal against *C. violaceum*. Considering that the singly applied effective concentrations were lower than the MIC values for these compounds previously recorded from broth micro-dilution and chequerboard assays, it becomes expedient for the time-course colony count for these semi-synthetic agents against *C. violaceum* to be repeated in broth in follow-up experiments. Sub-inhibitory concentrations each of F1 and F23 singly applied in combination

with sub-inhibitory concentrations of polymyxin B were indifferent in action against *C. violaceum*, given the conventional definition for this terminology (Pillai et al. 2005a, Petersen et al. 2006); as neither combinations displayed a significant reduction to the colony count of challenged inoculum populations, at least not to an extent≥ 2 log cfu/ml more than the most active agent (in this instance F1 and F23). However, after 2 hours, F23 applied alone was slightly less active over time than F23 applied together with polymyxin B against *C. violaceum*.

The table 4.2, figures 5.9 and 5.12 also show that with MRSA and *Ps. aeruginosa*, only a relatively small fraction (1/32 and 1/4 MIC respectively) of F23 was required to potentiate the antibacterial action of polymyxin B. Therefore another comparison of F23 to F1 is that both compounds were required in relatively smaller quantities to augment the antibacterial potency of polymyxin B against some of the tested bacterial species. In a given *in vivo* model for the evaluation of the antimicrobial efficacy of cefepime against *E. coli* in mouse, magainin 2 was inactive but only a small fraction was required to potentiate the activity of cefepime against this bacterial strain (Krieger et al. 2007, Darveau et al. 1991). This is also true for the combined action of clavulanic acid with amoxicillin as only a relatively smaller amount of the former compound is required to potentiate the action of the latter antibiotic in different formulations (Navarro 2005, Vree, Dammers and Exler 2003).

5.5 Conclusion

The results from the time-course viability assay presented in this chapter verified the data previously obtained from a chequerboard assay recorded in tables 4.1 and 4.2. The data presented in this chapter verified the existence of synergism for selected concentrations of either F1 or F23 applied in together with polymyxin B against the test bacterial species.

Chapter 6

Evaluating the effect of polymyxins chalcone-derivatives combinations upon bacterial metabolism

6.1 Introduction

Cationic amphiphilic peptides have been studied for a wide range of potential mechanisms that could account for their antibacterial action, in particular membrane depolarisation, disruption and blockade of molecular energization activity (Zhang, Rozek and Hancock 2001a, Vaara et al. 2008, Vaara 2010). Polymyxin B and colistin were investigated for their ability to interfere with the activity of metabolic enzymes essential for normal functioning of the electron transport chain that facilitate adenosine triphosphate (ATP) production. The enzymes nicotinamide adenine dinucleotide (NADH) dehydrogenase and succinate dehydrogenase are involved in bacterial metabolism of carbon sources such as glucose, for the generation of ATP required as the currency for regeneration and synthesis of new proteins (Bernas and Dobrucki 2002). Considering that the inhibition of these fundamental reduction enzyme could lead to the blockage of a cascade of metabolic processes, the assay is often also described as examination of the effect of antibiotics upon bacterial metabolism.

In prokaryotic cells, NADH generated from the ubiquinone cycle attached to the periphery of the bacterial cytoplasmic membrane and the enzymes obtained as a primary product from Krebb's cycle, combine to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt to water-insoluble purple formazan crystals (Berridge, Herst and Tan 2005a). Therefore, the capacity of a given bacterial population to reduce MTT salt through their electron transport chain can be used to assess their metabolic potential (respiratory capacity) (Wang et al. 2010a). The effect of polymyxin B, the chalcone-derivatives and both compounds applied in combination upon the activity of bacterial reducing enzymes, NADH dehydrogenase and succinate dehydrogenase, was assessed. The growth phase to which the bacterial population belongs can influence the rate and reproducibility of the reduction reaction (Wang et al. 2010b). Cells in their exponential growth phase are employed in the assay because of the need for the abundance of nutrients required to support the rapid structural development, cell

proliferation and a high rate of metabolism (Koutny et al. 2006). Considering that the levels of dissolved /available oxygen, nutrient and temperature can influence the biological reduction reaction of MTT salt (Takahashi et al. 2002), the experimental centrifuge tubes should be left open and incubated at 37 ^{o}C to allow for optimum bacterial growth and metabolism. This factor also necessitates the need for a surplus supply of energy reserve, in the form of glycerol and an abundant oxygen supply (Wang et al. 2010a). All these factors should be guaranteed in order to achieve optimum efficiency of the electron transport chain required for the conversion of MTT into formazan crystal by bacteria, catalyzed by several dehydrogenases enzymes most of which are located in the respiratory chain (Berridge, Herst and Tan 2005b, Smith and McFeters 1997, Berridge and Tan 1993b).

For a fixed population of viable cells, until enzyme saturation is achieved, a higher MTT concentration would yield more formazan crystals production over time (Gabrielson et al. 2002, Stentelaire et al. 2001). Therefore, a fixed concentration of MTT solution in the final volume of the reaction mixture should be applied to avoid variability in the rate and quantity of formazan crystal production by a given cell density. Considering that growth media type, volume and strength can influence the rate of the reaction, a fixed volume of Luria Bertani glycerol (LBG) broth should always be inoculated with a known density of the test bacterial species. Background absorbance can be caused by the interaction of incompletely removed culture medium, residual MTT and entrapped bacterial cells before the addition of DMSO (Wang et al. 2010b). To deal with this, the formazan crystals should be immediately washed upon recovery with 0.9 % $^{w}/_{v}$ sodium chloride solution before the introduction of DMSO solvent. To avoid inconsistency in the absorbance readings due to an incomplete dissolution of formazan crystal precipitates as a result of aggregation and formation of complexes with bacterial cells (Wang et al. 2010b), the recovered crystals should be dissolved in DMSO, in the dark, at room temperature overnight. In addition, the solution should be mixed/swirled in a rotary mixer before readings are taken with a spectrophotometer.

Unlike in mammalian culture (Wang et al. 2010b), bacterial cells assayed with the MTT reduction protocol did not form needle-like formazan crystals on cell surfaces and in the broth medium. Formazan crystals instead covered the bacterial cells, forming complexes that led to an entrapment of the substrate.

Each bacterium functions as a unit of dehydrogenase system and the rapid precipitation of formazan crystals upon them leads, as well as the formation of complexes with individual units of these cells causes a decline in the amount of available dehydrogenase enzymes, which is incidentally required to progress the reaction infinitely (Berridge, Herst and Tan 2005a, Wang et al. 2010a). This assay cannot therefore progress infinitely and generally a period of 20 to 30 minutes is required for completion of the reaction (Morgan 1997).

The objectives of this chapter were to:

i. Develop, as well as standardise, an MTT protocol for evaluating the biological reducing enzymes capacity of unchallenged viable bacterial populations by establishing a relationship between fixed density of cell populations and the quantity of formazan crystals produced

ii. Examine the effect of polymyxin B, colistin and combinations of these compounds with the chalcone-derivatives upon the capacity of bacterial species to reduce MTT salt into formazan crystals.

6.2 Materials and Methods

6.2.1 Preparation of Luria Bertani glycerol (LBG) broth

Five grams of tryptone T (Oxoid LP0043), 2.5 g yeast extract (Oxoid LP0021) and 2.5 g sodium chloride (Aldrich) were weighed, the powders suspended in distilled water and the volume made up to 500 ml using a volumetric flask. A 250 ml stock solution of 0.5 % $^{v}/_{v}$ glycerol stock solution was prepared. The bottles containing the media suspension and glycerol solution were autoclaved by moist heat sterilisation as previously described in section 2.1.2.1 When required, the sterile broth and glycerol solutions were combined in a 5:1 ratio.

6.2.2 Preparation of bacterial culture in LBG medium

Using 30 ml of LBG medium in a 100 ml Erlenmeyer conical flask, an 18 hour culture was prepared as previously described in section 2.2.3.2. A 500 µl aliquot of the overnight culture was transferred into fresh LBG and incubated at 37 ^{o}C for 4 - 6 hours in an orbital incubator set at 100 rpm (Sanyo Gallenkamp PLC, Loughborough, UK.).

6.2.3 Establishing a correlation between bacterial density and quantity of formazan crystals produced

The bacterial suspension in LBG was adjusted using a spectrophotometer to obtain 5 samples with densities within the wavelength range of 0.1 to 1.0 units at OD_{600}. Colony plate count was carried out for each density as previously described in sections 2.3.3 and 2.3.4, in order to verify the actual number of viable cells (cfu/ml). Suitable volumes of the defined cell densities and stock MTT solution (5 mg/ml dissolved in 0.9 % $^{w}/_{v}$ sodium chloride solution) were mixed and incubated for 20 minutes, in a static water bath previously set at 37 ^{o}C. Each sample was centrifuged at 14,000 rpm for 2 minutes and the recovered insoluble formazan crystals washed once with 0.9 % $^{w}/_{v}$ sodium chloride solution and re-centrifuged at the conditions described above. After supernatant removal, the crystals were dissolved in 1 ml DMSO and optically quantified with a spectrophotometer (Cecil CE 3021, Cecil instruments Ltd., Milton Technical Centre, Cambridge, UK.) at OD_{550}. The assay was conducted in duplicate with at least three independent experiments. A graph consisting of absorbance (A_{550}) of formazan crystals produced against the optical density (OD_{600}) of the bacterial population that yielded the formazan was plotted and statistically analysed for standard error of mean and standard deviation with GraphPad prism 4 software.

6.2.4 Examining the effects of polymyxins with the chalcone-derivatives upon bacterial metabolism

A defined volume of stock (5 mg/ml) MTT solution and a suitable volume of 0.3 units OD_{600} bacterial suspension in LBG as well as antibiotic solutions required to prepare the desired final concentration were added together and mixed. The mixture was incubated in a static water bath pre-set to 37 ^{o}C. At time intervals of 0, 20, 40, 60 and 80 minutes, samples were collected and centrifuged at 14, 000 rpm for 2 minutes. The recovered pellets were washed once and re-centrifuged at 14,000 rpm. A solution of the recovered formazan crystals was prepared in 1 ml DMSO and the absorbance determined with either a DTX 880 (Molecular Devices, California, USA.) at A_{595} or a with a spectrophotometer (Cecil 7400 UV-Visible), set at A_{550}. The assay was repeated with at least three independent experiments on separate days. A graph of optical density of the broth culture (at

OD_{600}) against absorbance of formazan produced (at A_{550}) was prepared and statistically analysed for standard error of mean as well as standard deviation with GraphPad prism 4 software.

6.3 Results

6.3.1 Calibration curves for formazan quantity produced by bacterial populations

The sensitivity of the assay as a measure of detecting the capacity of untreated bacterial cells to reduce MTT salt into formazan crystals was initially established. The assay was then standardised by determining the relationship between quantity of formazan produced and bacterial density. On this premise, figure 6.1 shows that as the optical density of the viable *E. coli* suspensions were increasing from 0.1 to 1.0 unit at OD_{600}, the quantity of formazan crystals obtained as yield increased proportionately, indicating an increase in enzyme activity.

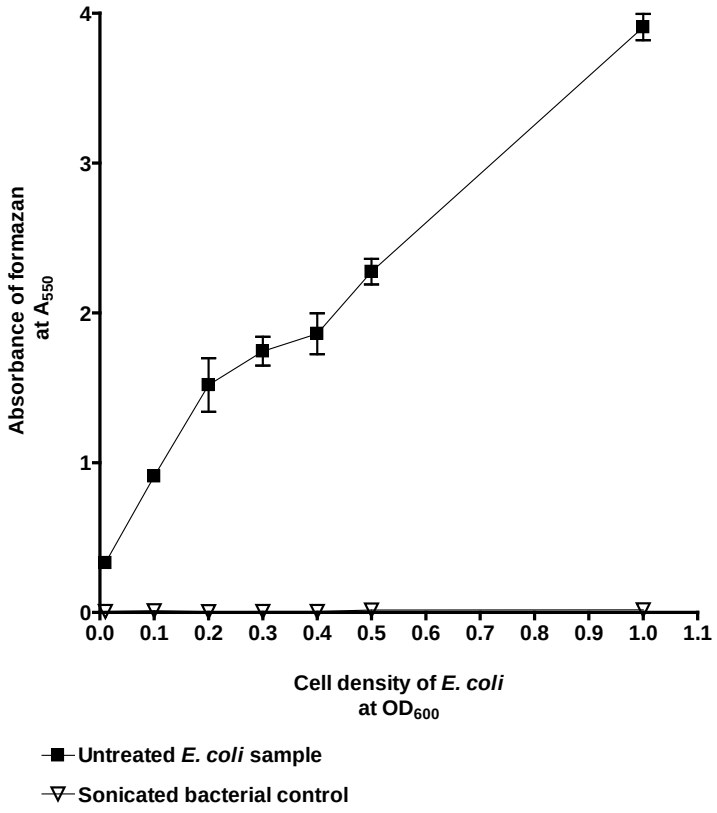


Figure 6.1: Absorbance (at A_{550}) of formazan plotted against the corresponding optical density (at OD_{600}) of *E. coli* that produced the crystals. The data were mean values obtained from experiments conducted in duplicate and repeated thrice on separate days.

Upon establishing the assay on a macro-scale, the substrate concentrations employed in the analysis were reduced to microgram levels and the formazan crystals obtained as yield were quantified in a micro-titre plate using a DTX-880 reader at A_{595}. The assay from which data for figure 6.1 were obtained was designed to standardise the protocol for bacterial MTT metabolic assay. The figure indicates the existence of a direct correlation between the quantity of formazan crystals and bacterial density when *E. coli* population was within the range of 0.1 to 1.0 units at OD_{600}. A fixed cell population selected from this range could therefore be used to assess the effect of antibiotics upon the reducing capacity of bacterial enzymes. Using this as a guide, all further analysis for polymyxins and the chalcone-derivatives were carried out using a fixed cell density of 0.3 unit at OD_{600} which can produce measurable quantities of formazan crystals whilst maintaining a correlation between absorbance of formazan and bacterial density. Having established the sensitivity and reliability of the assay at a macro-scale level, a scaled down technique was employed in most cases such that the reduced/smaller amount of formazan crystals obtained as yield was quantified using a micro-titre plate in a pre-calibrated DTX-880 reader. This explains why subsequent data may have been presented using a different absorbance of 595 nm on the y-axis. The use of varying absorbance for the measurement of formed formazan did not affect the data analysis as the results were normalised in terms of percentage with reference to the control samples.

Figure 6.2 shows data for the calibration curve of absorbance of formazan at A_{595} obtained as yield from the biological reduction of MTT salt against that of the optical density (at OD_{600}) of the corresponding *E. coli* population, using a micro-titre plate incubated in a DTX-880 reader at 37 ^{o}C. A linear correlation exists between the amount of formazan produced and the cell density from which crystals were obtained. As the bacterial population was increasing from 0.1 to 0.2 unit at OD_{600}, the absorbance for the quantities of formazan produced were seen to be increasing in a proportionate manner from 0.5 to 0.85 unit at A_{595}.

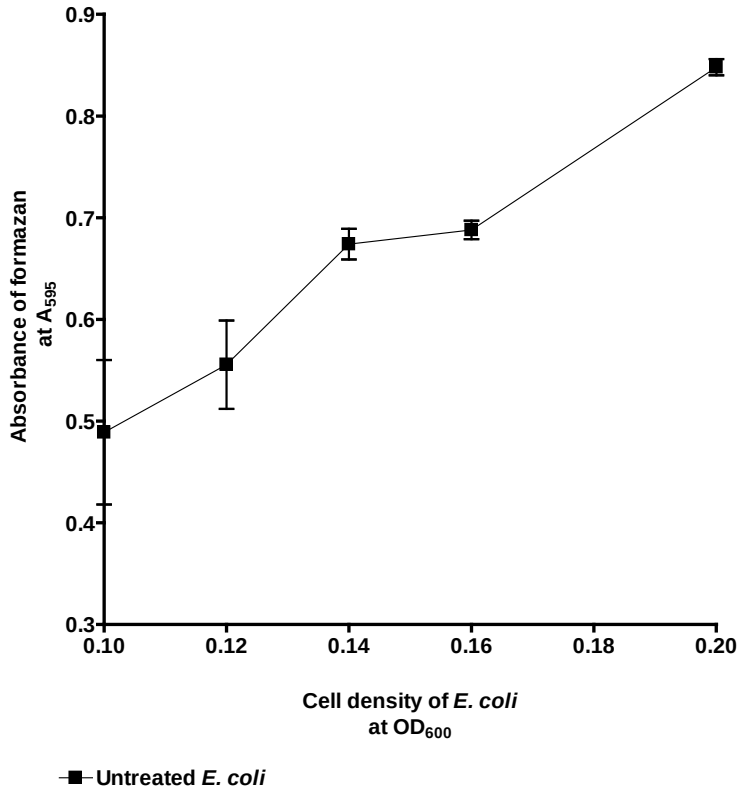


Figure 6.2: Absorbance (A_{595}) of formazan plotted against the corresponding optical density (OD_{600}) of *E. coli* that produced the crystals. The data were mean values obtained from experiments conducted in duplicate and repeated thrice on separate days.

The readings demonstrate a direct correlation between the quantity of formazan produced and the cell density, even within a smaller range of 0.1 to 0.2 units at OD_{600}, indicating the sensitivity of MTT salt to the presence of NADH and succinate dehydrogenase enzymes. This assay can therefore be used to assess the effect of either new or established antimicrobial compounds upon bacterial metabolism.

6.3.2 Evaluating the effect of polymyxin B upon selected bacterial metabolism

6.3.2.1 Impact of polymyxin B upon *E. coli* metabolism

The effect of polymyxin B upon the activity of *E. coli* reducing enzymes was assessed with the bacterial MTT reduction protocol and the data obtained is presented in figure 6.3.

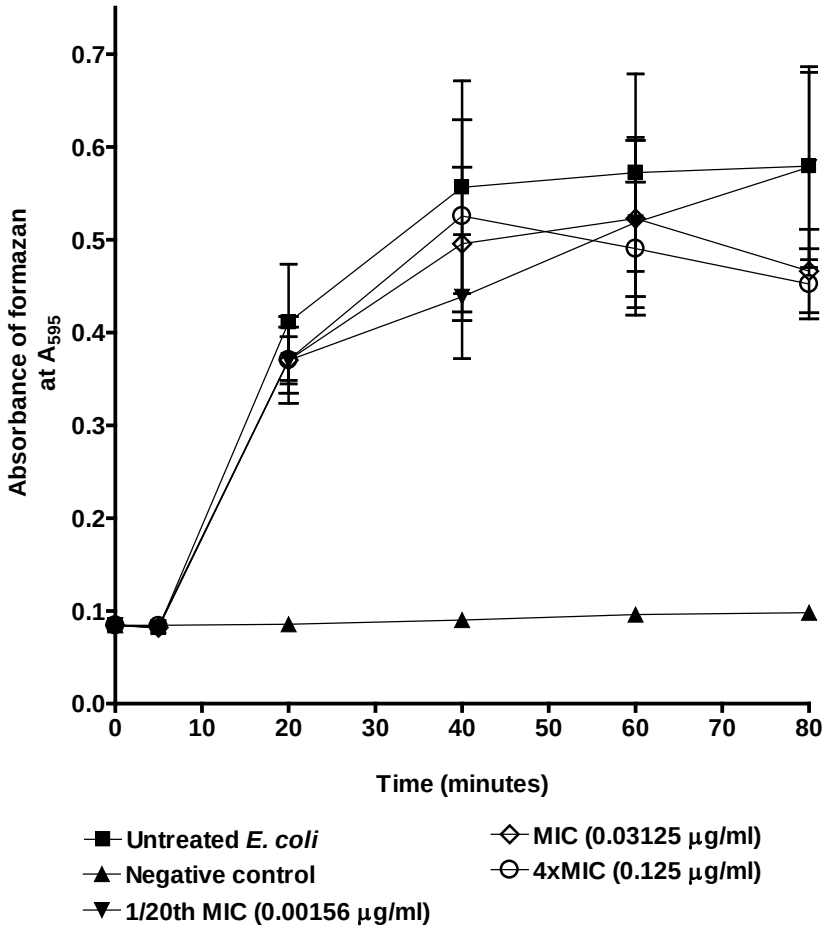


Figure 6.3: The effect of 1/20 x MIC, MIC and 4 x MIC of polymyxin B on reducing enzyme activity of *E. coli.* The data were mean values obtained from experiments conducted in duplicate and repeated three times on separate days.

In comparison to the untreated bacterial sample, *E. coli* challenged with 1/20 MIC of polymyxin B demonstrated no remarkable blockage to the biological production of formazan crystals from MTT salt, whilst cells treated with concentrations of the polypeptide equivalent to MIC and 4 x MIC both displayed about 20 % inhibition. The LBG medium containing an equivalent amount of the MTT salt but devoid of the inoculum bacterial population displayed no optically detectable quantity of formazan crystals thereby giving an indication that the presence of active biological reducing enzymes from viable organisms is required for the salt reduction to occur.

Concentrations of polymyxin B equivalent to 0.0313 µg/ml (MIC) and 0.125 µg/ml (4 x MIC) also had no notable influence upon formazan production from MTT by *E. coli* within 80 minutes. Considering that concentrations of polymyxin B within the range of 1/20 to 4 x MIC only weakly affected the metabolic activity of

E. coli, a higher concentration 0.625 µg/ml (20 x MIC) of this polypeptide antibiotic was subsequently applied in the data presented in figure 6.4.

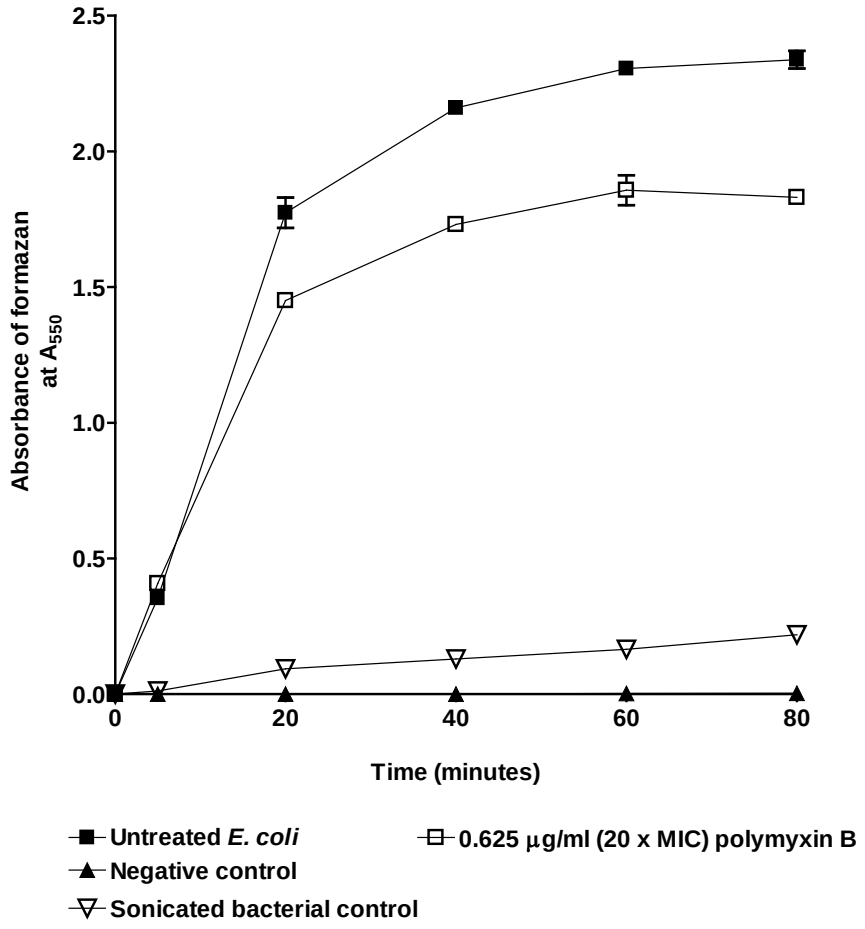


Figure 6.4: Inhibitory effect of 20 x MIC polymyxin B upon the MTT reduction capacity of *E. coli*. The data were mean values obtained from experiments conducted in duplicate and repeated on 3 separate days.

The data presented in figure 6.4 were obtained from MTT reduction analysis of untreated, sonicated and viable *E. coli* suspensions challenged with 20 x MIC polymyxin B. This experiment had been conducted using the macro-protocol as is being reflected on the scale for the absorbance values recorded on the y-axis for formazan production. The production of formazan crystals from MTT salt by *E. coli* was gradually inhibited within 80 minutes. The activity of the biological reducing enzymes was however still partially affected by the presence of a higher concentration of this polypeptide antibiotic. The treated bacterial population demonstrated approximately 25 % reduction in the quantity of formazan produced in comparison to the untreated bacterial population after 80 minutes of incubation. The capacity of sonicated *E. coli* cells to produce formazan crystals

was initially completely inhibited but the cells in this sample eventually produced about 10 % of the amount of crystals yielded by the untreated bacterial control after 80 minutes. This observation indicates that the sonication process probably did not completely destroy 100 % of the reducing enzymes. As expected, there were no formazan crystals in the LBG medium devoid of inoculum populations of *E. coli* over the same time period.

6.3.2.2 Impact of polymyxin B upon the metabolic activity of *S. aureus*

S. aureus 6571 (MSSA) populations were challenged with polymyxin B concentrations within the range of 12.8 µg/ml (1/20 MIC) to 2.56 mg/ml (10 x MIC) in the presence of MTT salt and examined for the rate and quantity of formazan production. The data obtained from the assay is presented in figure 6.5

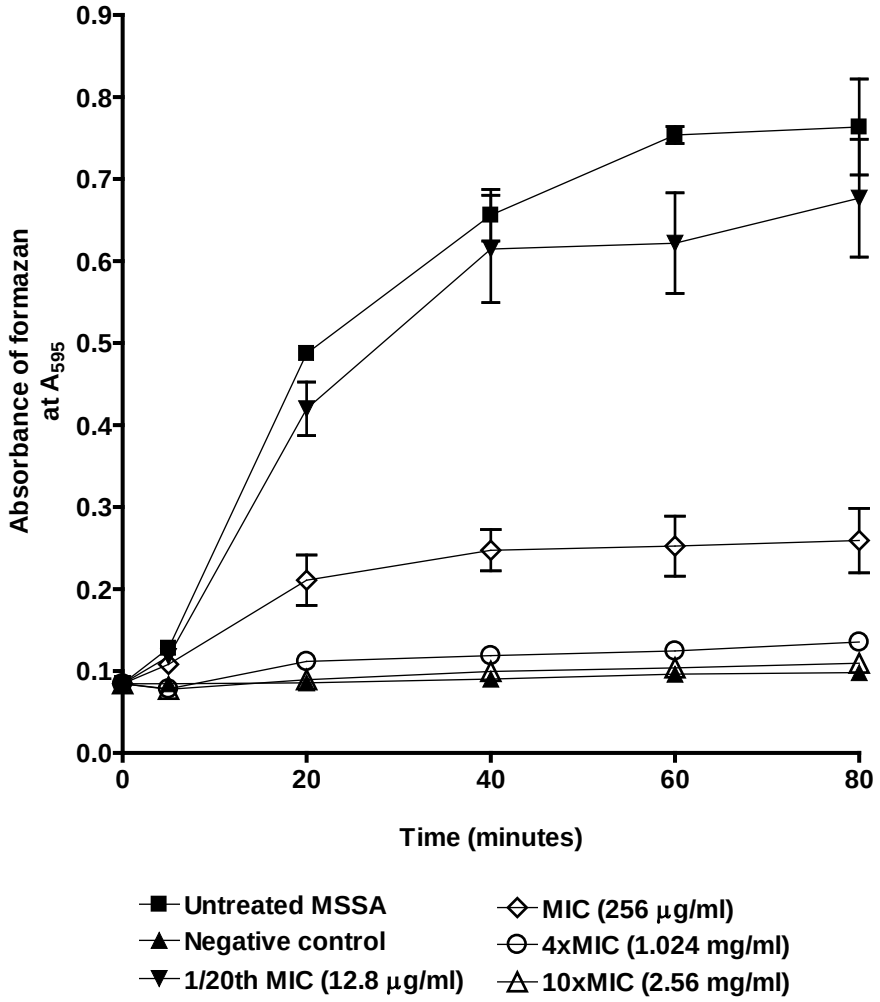


Figure 6.5 Concentration-dependent inhibitory effect of polymyxin B upon the reducing enzymes activity of MSSA. The data were mean values obtained from experiments conducted in duplicate and repeated on 3 separate days.

In comparison to the untreated MSSA suspension, the bacterial samples challenged with 1.024 (4 x MIC) and 2.56 mg/ml (10 x MIC) polymyxin B demonstrated almost 95 and 100 % inhibition of formazan production respectively within 80 minutes. This strongly suggests the presence of both concentrations of this polypeptide antibiotic halted the activities of NADH and succinate dehydrogenase enzymes even in the presence of glycerol, broth and oxygen. As can be seen from the figure, the bacterial population challenged with 256 µg/ml (MIC) polymyxin B demonstrated approximately 75 % reduction in the quantity of formazan produced suggesting the activity of this antibiotic against the biological reducing enzymes in MSSA was concentration-dependent.

To further reinforce this point, the MSSA population challenged with 12.8 µg/ml (1/20 MIC) polymyxin B demonstrated approximately only a 15 % reduction in the quantity of formazan recovered, indicating a reduction in the enzyme inhibitory capacity of this antibiotic with a lesser concentration. The effect of polymyxin B upon the reducing enzymes of Gram-positive *S. aureus* (MSSA) and Gram-negative *E. coli* is therefore concentration-dependent. Although 0.625 µg/ml (20 x MIC) polymyxin B only partially affected (by about 25 %) the metabolising enzymes activity in the latter Gram-negative organism, 1.024 mg/ml (4 x MIC) and 2.56 mg/ml (10 x MIC) polymyxin B completely inhibited the capacity of reducing enzymes to convert MTT salt into formazan in Gram-positive MSSA. But as observed with *E. coli* previously, the negative control devoid of MSSA did not produce formazan crystals within the assay duration.

6.3.2.3 Impact of polymyxin B on the metabolic activity of *Ps. aeruginosa*

The result presented in figure 6.6 was obtained from *Ps. aeruginosa* populations challenged with polymyxin B concentrations and examined for the rate and quantity of formazan production. The *Ps. aeruginosa* population challenged with 0.05 µg/ml (1/20 MIC) of polymyxin B demonstrated a comparable rate and quantity of formazan production with the unchallenged population after 80 minutes. The close proximity of the curves recorded from both samples gives an indication that such a sub-inhibitory concentration of polymyxin B had very little inhibitory effect on the capacity of the biological reduction enzymes, NADH and

succinate dehydrogenases, to convert MTT salt to the reduced form. Meanwhile, *Ps. aeruginosa* samples challenged with the polypeptide concentrations equivalent to 1 µg/ml (MIC), 4 µg/ml (4 x MIC) and 20 µg/ml (20 x MIC) demonstrated a 25, 40 and 50 % reduction in the quantity of formazan crystals produced and thereby manifested a concentration-dependent blockade of the reducing capacity of the biological enzymes in this bacterial species. This systematic quantitative decrease in recoverable formazan shows the biological system is sensitive to the presence of this antibiotic.

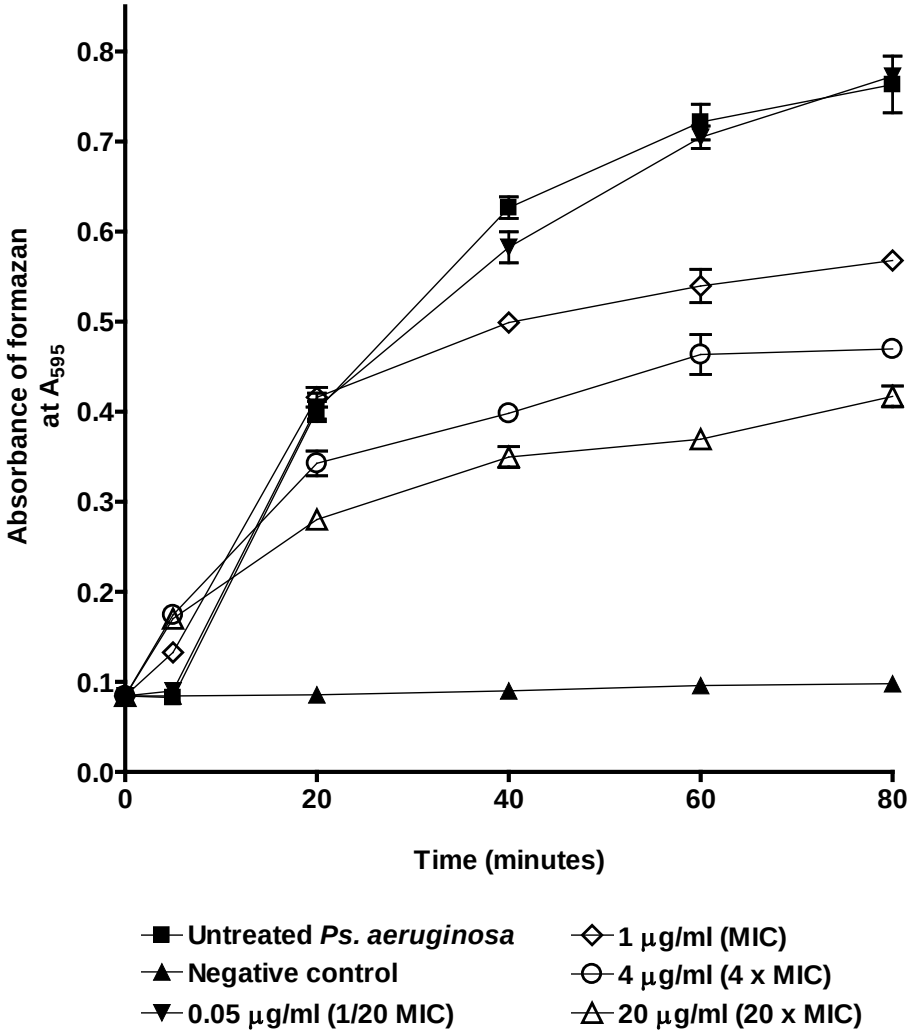


Figure 6.6: Concentration-dependent inhibitory effect of polymyxin B upon the reduction capacity of *Ps. aeruginosa*. The data were mean values obtained from experiments conducted in duplicate and repeated on 3 separate days.

6.3.3 Evaluating the effect of colistin upon the activity of reducing enzymes in selected bacterial species

The ability of colistin to influence the activity of the reducing enzymes in selected bacterial species was examined using the MTT metabolic assay.

6.3.3.1 Effect of colistin upon the activity of reducing enzymes in *E. coli*

Figure 6.7 shows the varying effect of different concentrations of colistin upon the metabolic activity of *E. coli*.

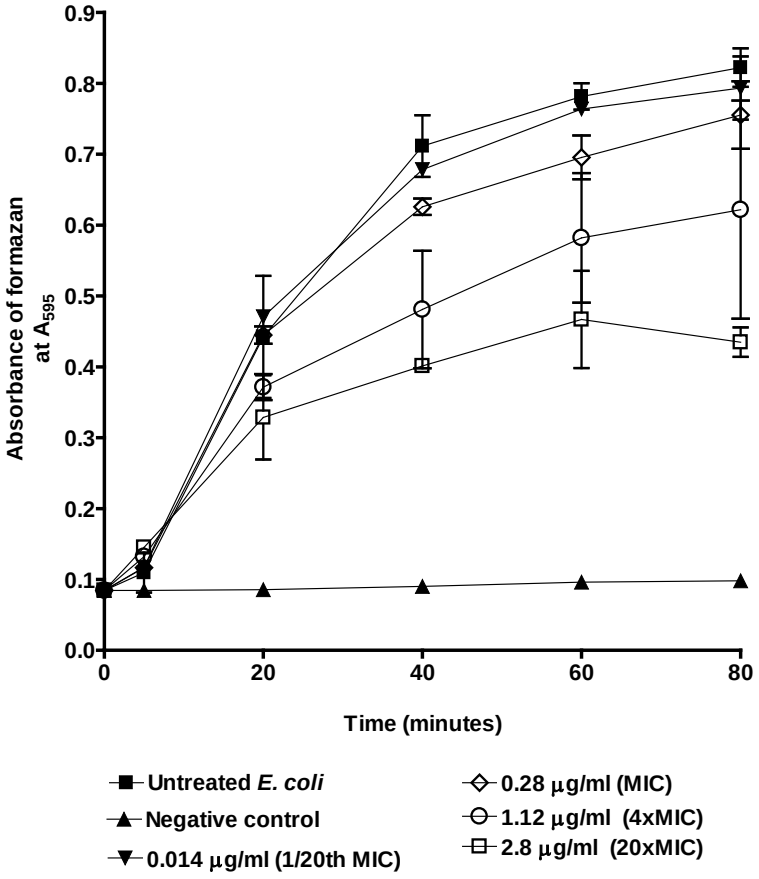


Figure 6.7: Concentration-dependent inhibitory effect of colistin upon the activity of reducing enzymes in *E. coli*. The data were mean values obtained from experiments conducted in duplicate and repeated on three separate days.

There was a demonstration of a concentration-dependent inhibitory effect by colistin upon the capacity of *E. coli* to produce formazan crystals from MTT salt. In comparison to the untreated population, *E. coli* suspensions challenged with 0.014 µg/ml (1/20 MIC), 0.28 µg/ml (MIC), 1.12 µg/ml (4 x MIC) and 2.8 µg/ml (20 x MIC) of colistin demonstrated approximately 2.5, 10, 25 and 50 %

reduction in the quantity of formazan produced respectively. The inhibitory effect demonstrated by 1.12 µg/ml (4 x MIC) colistin was almost equivalent to that shown by 0.625 µg/ml (20 x MIC) polymyxin B against this same species, suggesting colistin was demonstrating more impact upon bacterial reducing enzymes than polymyxin B. As with the inhibitory effect of polymyxin B, 20 x MIC colistin demonstrated the greatest impairment to the activity of the biological reducing enzymes in *E. coli*, with the highest recorded inhibition to the reduction of MTT salt to formazan. As expected, the LBG medium devoid of bacterial inoculum had no formazan crystals.

6.3.3.2 Examining the effect of colistin upon the reducing enzymes activity of MSSA

The data presented in figure 6.8 was obtained from populations of *S. aureus* 6571 (MSSA) that were challenged with different concentrations of colistin in the range of 7 µg/ml (1/20 MIC) to 1.4 mg/ml (10 x MIC).

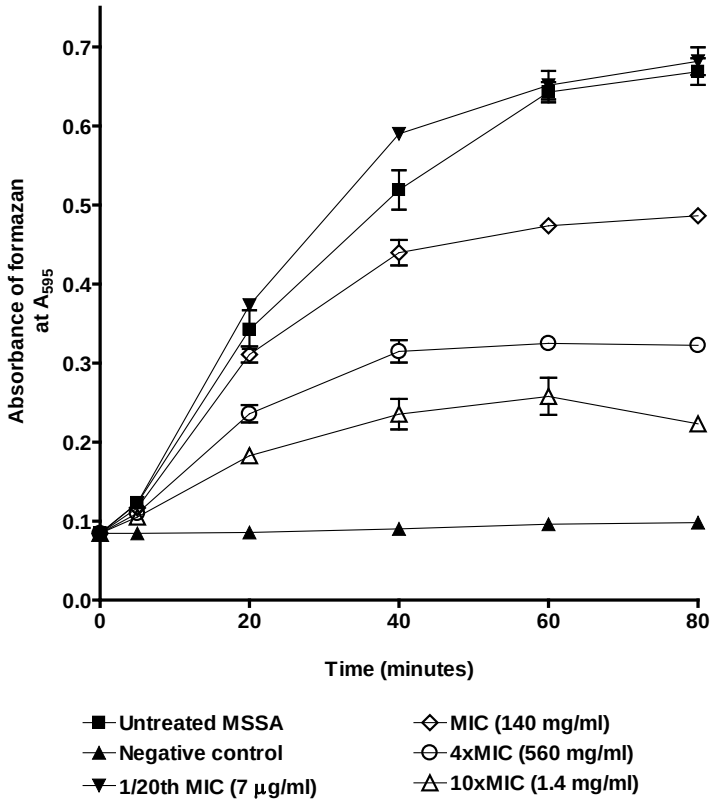


Figure 6.8: Concentration-dependent inhibitory effect of colistin upon the reducing enzymes activity of *S. aureus* 6571 (MSSA). The data were mean values obtained from experiments conducted in duplicate and repeated 3 times on separate days.

The information provided in the figure suggests the occurrence of a concentration-dependent inhibitory effect of colistin upon the metabolising capacity of MSSA in the MTT reduction analysis. In comparison to the untreated population, the bacterial densities challenged with 7 µg/ml (1/20 MIC), 140 µg/ml (MIC), 560 µg/ml (4 x MIC) and 1.4 mg/ml (10 x MIC) of colistin demonstrated a proportionate sensitivity to the presence of this antibiotic.
There was an approximately 2, 30, 55 and 70 % cessation respectively to the quantity of formazan crystals produced after 80 minutes of incubation, suggesting that NADH as well as succinate dehydrogenase enzymes were being inhibited to a varying degree that is dependent upon the concentration of the polypeptide antibiotic. As would be expected, the LBG medium devoid of inoculum bacteria had no formazan crystals.

6.3.3.3 Examination of the effect of colistin upon the MTT-reduction capacity of *Ps. aeruginosa*

The effect of a range of concentrations from 0.075 µg/ml (1/20 MIC) to 15 µg/ml (10 x MIC) of colistin upon the respiratory capacity of *Ps. aeruginosa* was assessed and the information obtained is presented in figure 6.9.
The data obtained demonstrate a concentration-dependent inhibitory effect of colistin upon the capacity of *Ps. aeruginosa* to produce formazan crystals from MTT salt. Compared to the untreated population, the *Ps. aeruginosa* populations challenged with 0.075 µg/ml (1/20), 1.5 µg/ml (MIC), 6 µg/ml (4 x MIC) and 15 µg/ml (10 x MIC) of colistin demonstrated approximately 0, 25, 50 and 68 % impairment to the quantity of formazan produced within 80 minute, again giving the impression that the biological reducing enzymes in these cells were demonstrating a dose-dependent inhibitory response to the presence of colistin.

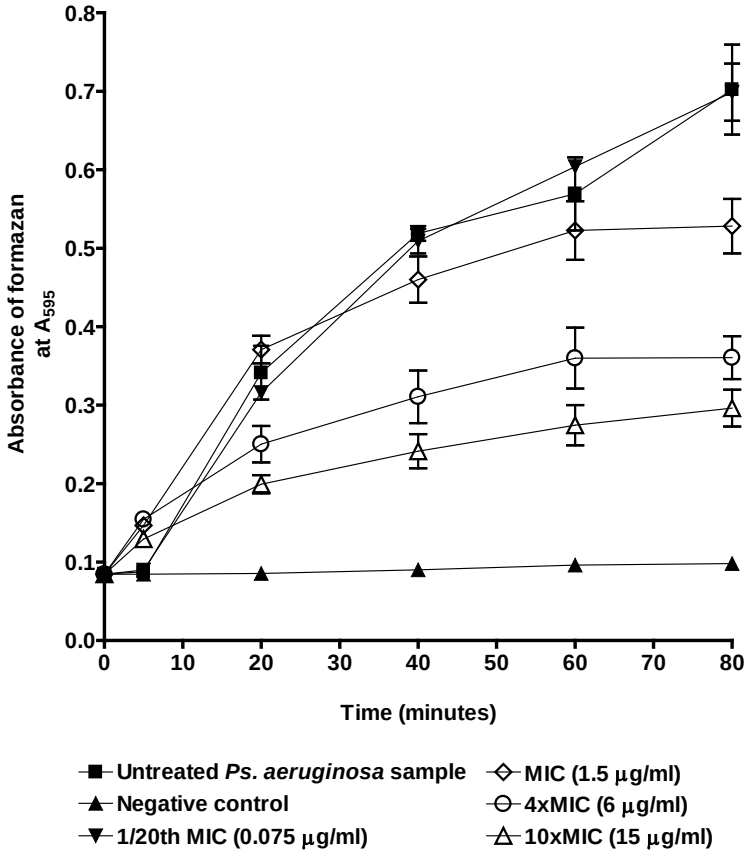


Figure 6.9: Concentration-dependent inhibitory effect of colistin upon the MTT-reduction capacity of *Ps. aeruginosa.* The data were mean values obtained from experiments conducted in duplicate and repeated 3 times on separate days.

6.3.4 Evaluation of the effect of F1 with polymyxin B upon the activity of bacterial reducing enzymes

This analysis should have begun by considering the effect of the combined application of F1 with polymyxin B upon the reducing enzymes activity of *E. coli*. This was however not carried out since the data obtained from the chequerboard assay indicated indifference in the antibacterial action of both compounds combined against this organism. As already stated, the examination of the effect of polymyxin B upon the metabolic activity of *E. coli* reported previously was conducted for the purpose of standardising the protocol using bacterial cells. The effect of polymyxin B with F1 upon bacterial metabolism was investigated for species wherein either an additive or synergistic action had been recorded during a chequerboard and time-kinetic colony counting assay in an attempt to have an insight into these agents potential mechanism of action.

6.3.4.1 Effect of F1 with polymyxin B upon the biological reduction capacity of MSSA

Figure 6.10 shows the effect of F1 applied with polymyxin B upon the activity of reducing enzymes in *S. aureus* 6571 (MSSA).

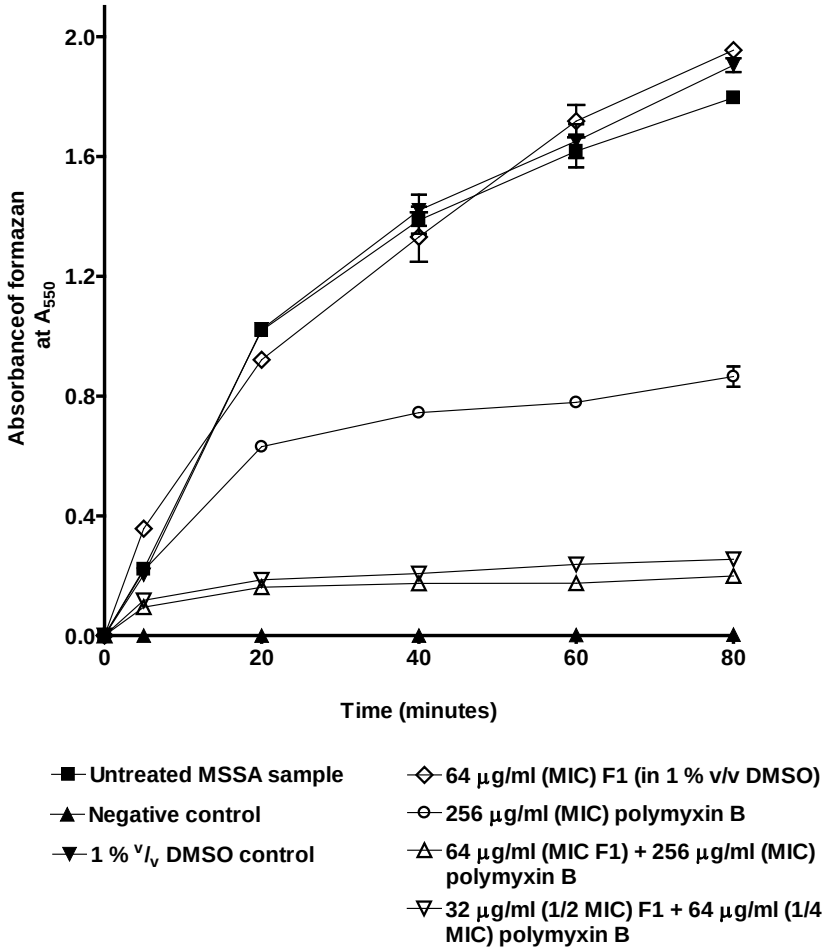


Figure 6.10: Enhanced inhibitory effect of F1 applied with polymyxin B upon the MTT-reduction capacity of MSSA. The data were mean values obtained from experiments conducted in duplicate and repeated three times on separate days.

The concentrations applied in this technique were based on guidance received from the activity of both compounds in the chequerboard (table 4.1) and time-course colony counting (figure 5.1) assays.

The untreated MSSA population, the solvent treated and the sample challenged with 64 µg/ml (MIC) F1 all demonstrated comparable and uninhibited production of formazan. Comparatively, the MSSA density that was challenged with 256 µg/ml (MIC) polymyxin B demonstrated approximately 60 % inhibition to the

quantity of formazan produced. Whilst, the MSSA populations that were challenged with 64 µg/ml (MIC) F1 or 32 µg/ml (1/2 MIC) F1 in combination with 256 µg/ml (MIC) or 64 µg/ml (1/4 MIC) of polymyxin B respectively, demonstrated approximately 90 % decline in the quantity of formazan produced. This reported action suggests a pronounced inhibition of the activity of NADH and succinate dehydrogenase in MSSA by the combined action of these compounds.

This data complements the results recorded from the kinetic colony counting assay wherein a 7 log cfu/ml MSSA inoculum population challenged with 64 µg/ml F1 and 256 µg/ml polymyxin B demonstrated a decline in cell viability to below detectable limits after 8 hours (figure 5.1). It follows therefore that timing for the impairment of cell metabolic activity preceded that of the loss in viability for the challenged bacterial species.

The LBG broth without bacteria to which MTT solution was added did not produce formazan; an indication that the combination of the salt with the media alone cannot reduce the salt to water insoluble purple crystals. As reflected by the results in the figure, the lack of inhibitory activity in the solvent treated control suggests the absence of interference with the assay by the presence of 1 % $^{v}/_{v}$ DMSO (solvent for F1) in the activity recorded for the combinations of F1 with polymyxin B against bacterial NADH and succinate dehydrogenase enzymes.

6.3.4.2 Effect of F1 with polymyxin B upon MTT-reduction capacity of MRSA

Figure 6.11 demonstrates the effect of F1 applied with polymyxin B upon the metabolic enzymes of *S. aureus* 11940 (MRSA). As for *S. aureus* 6571 (MSSA), the concentrations of both compounds applied were chosen based on information already gleaned from a chequerboard (table 4.1) and time-course viability (figure 5.3) assays.

The untreated MRSA population, the solvent treated and the sample challenged with 128 µg/ml (MIC) F1 demonstrated comparable and unhindered production of formazan crystals within 80 minutes.

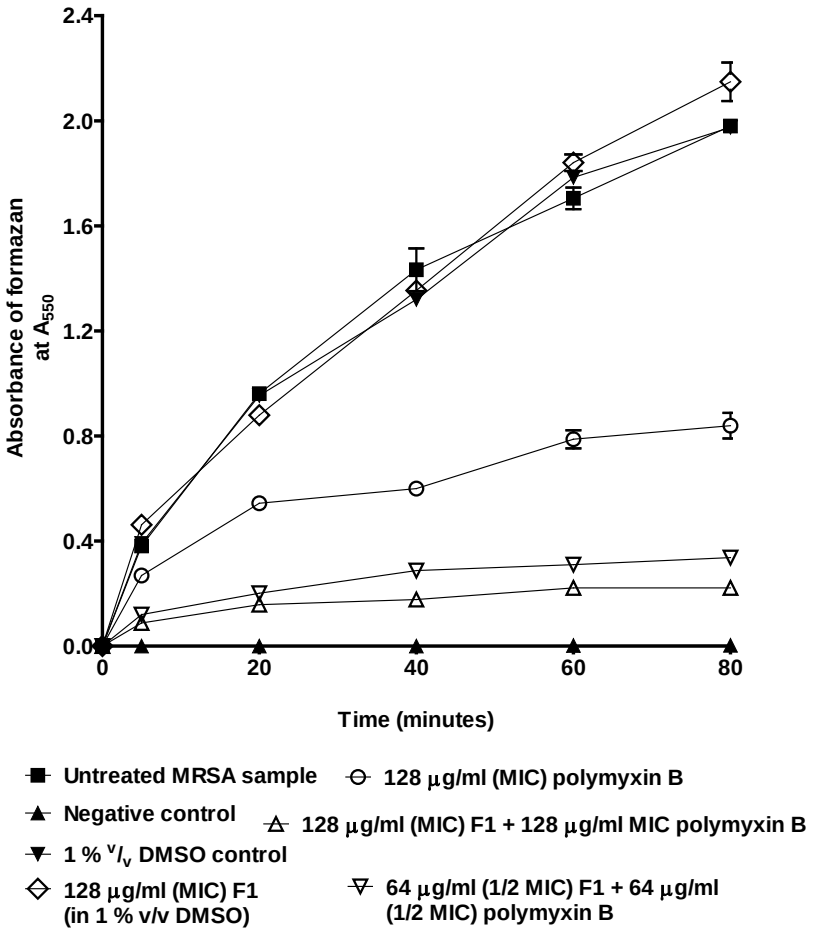


Figure 6.11: Enhanced inhibitory effect of F1 with polymyxin B upon biological MTT-reduction by *S. aureus* 11940 (MRSA). The data were mean values obtained from experiments conducted in duplicate and repeated three times on separate days.

In comparison to the untreated control, the MRSA population challenged with 128 µg/ml (MIC) polymyxin B demonstrated approximately 60 % inhibition to the quantity of formazan crystals produced. The MRSA populations exposed to 64 µg/ml (1/2 MIC) F1 with 64 µg/ml (1/2 MIC) polymyxin B and 128 µg/ml (MIC) F1 with 128 µg/ml (MIC) polymyxin B both demonstrated approximately 85 and 90 % blockage of the biological formation of formazan crystals respectively. The inhibitory activity of the former combination of strengths of both compounds complements the data presented in figure 5.3 from a time-course viability assay, wherein a 7 log cfu/ml inoculum population of MRSA was reduced to below detectable limits after 8 hours, suggesting cessation of metabolic functions preceded the loss of cell viability. The levels of interference of these compounds with the activities of bacterial reducing enzymes (NADH and succinate

dehydrogenase) seem to bear a correlation with their synergistic effects against *S. aureus* species.

6.3.4.3 Effect of F1 with polymyxin B upon the biological reducing capacity of quinolone-resistant *S. aureus*

The figure 6.12 below shows the effect of F1 applied with polymyxin B upon the capacity of quinolone-resistant *S. aureus* (QRSA) to reduce MTT salt into formazan.

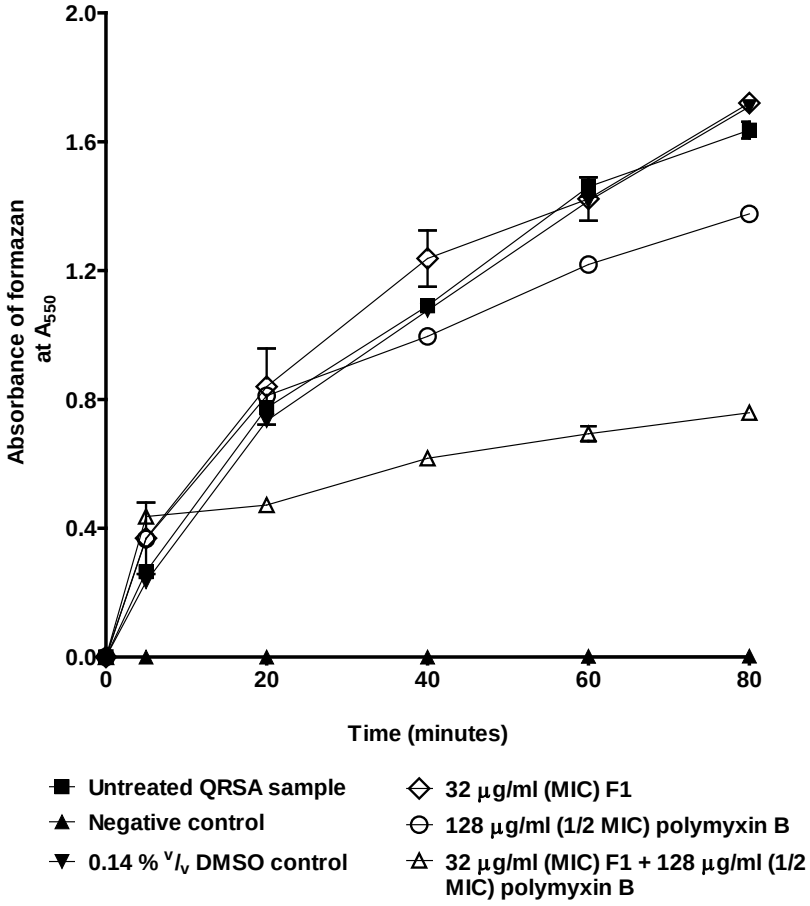


Figure 6.12: Enhanced inhibitory effect of F1 with polymyxin B upon the biological reducing power of QRSA. The data were mean values obtained from 3 experiments conducted in duplicate on separate days.

The choice of concentrations of both compounds applied was based on information obtained from both chequerboard (table 4.1) and time-course viability (figure 5.5) assays.

The untreated, solvent-treated and the QRSA populations challenged with 32 µg/ml F1 demonstrated comparable quantities of formazan crystal production over the 80 minutes duration of the experiment. Given that the untreated QRSA sample displayed a 100 % production of formazan from the salt, then bacterial suspensions challenged with 128 µg/ml (1/2 MIC) polymyxin B alone and in combination with 32 µg/ml (MIC) F1 demonstrated approximately 25 and 60 % reduction in the quantity of recovered formazan, respectively.

The enzyme inhibitory activity of these combined compounds at the same concentrations above also correlates with their synergistic action against this same strain of *S. aureus*, in a time-kinetic colony counting experiment recorded in figure 5.5. Therein, a 7 log cfu/ml population was reduced to below detectable limit after 8 hours, suggesting the establishment of cessation of metabolic activity in advance of eventual cell death, in the combined agents' mechanism of action against *S. aureus*. This observation seems to support the speculation that a correlation existed between the synergistic action of both compounds and their inhibitory effect upon the activities of biological reducing enzymes (such as NADH and succinate dehydrogenase), in *S. aureus* species.

6.3.5 Evaluation of the effect of F23 with polymyxin B upon bacterial metabolism

Following the outcome above for F1, the activity of polymyxin B when applied with the second semi-synthetic agent, F23, against the metabolic action of selected bacterial species in the presence of MTT salt was investigated. The data obtained is subsequently presented.

6.3.5.1 Effect of F23 with polymyxin B upon reducing enzymes activity of MSSA

Figure 6.13 demonstrates the effect of F23 applied in combination with polymyxin B upon the metabolising activity of MSSA.

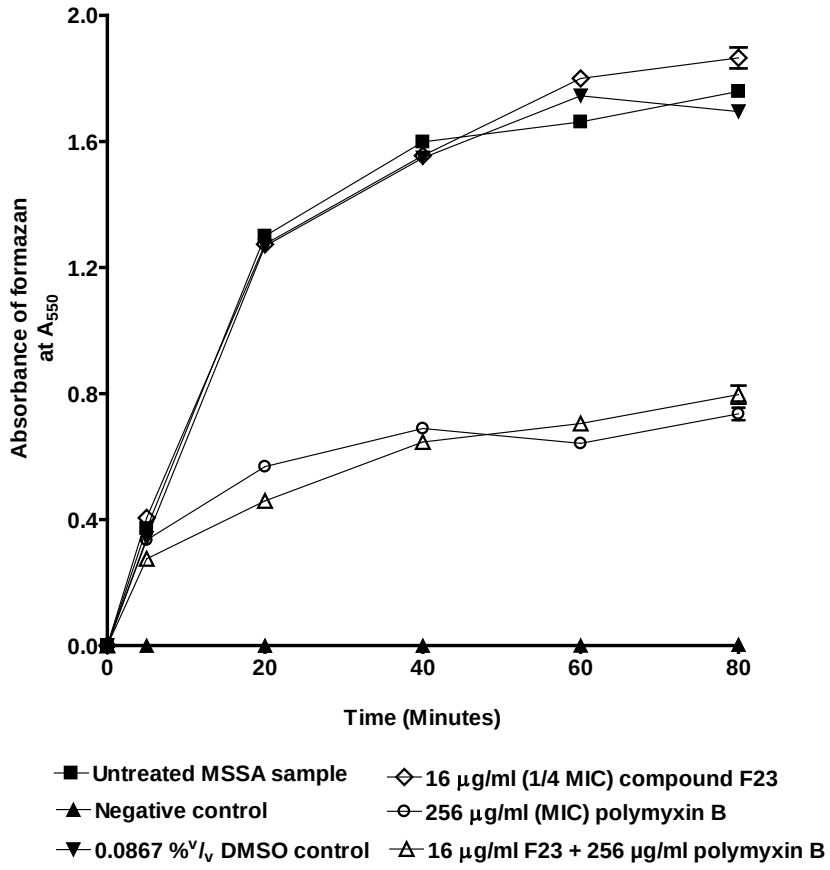


Figure 6.13: Inhibitory effects of polymyxin B alone and in combination with sub-inhibitory concentration of F23 upon the MTT-reducing capacity of *S. aureus* 6571 (MSSA). The data were mean values obtained from 3 experiments conducted in duplicate on separate days.

As in the previous assays with F1 and polymyxin B, the selection of the concentration of both compounds to be used in this protocol was made based on the information collected from chequerboard (table 4.2) and time-kinetic colony counting (figure 5.8) assays. In both assays, as well as in an MBC-determination technique, the combination of 16 µg/ml F23 with 256 µg/ml polymyxin B was synergistic against *S. aureus* 6571. The effect of these same concentrations of both compounds upon this bacterial species metabolism was examined in this protocol.

The untreated, solvent-treated and MSSA populations challenged with 16 µg/ml F23 demonstrated comparable quantities of formazan crystal production over the 80 minutes duration of the experiment. In comparison to the untreated bacterial suspension, *S. aureus* challenged with 256 µg/ml (MIC) polymyxin B alone and in combination with 16 µg/ml (1/4 MIC) F23, both demonstrated approximately 70 % inhibition to the quantity of formazan produced over time. This result verified

the data reported in sections 6.3.2.2 (figure 6.5) and 6.3.4.1 (figure 6.10) wherein 256 µg/ml polymyxin B was seen to have demonstrated about 75 and 60 % inhibition to the activity of the reducing enzymes in MSSA with the application of the same concentration of this polypeptide antibiotic, thereby giving an overall average of 68 %.

Whereas 32 or 64 µg/ml F1 applied with 64 or 256 µg/ml polymyxin B respectively both displayed an enhanced blockade of the activity of reducing enzymes in MSSA (figure 6.10), 16 µg/ml F23 applied with 256 µg/ml polymyxin B demonstrated almost the same degree of impairment with 256 µg/ml of the polypeptide antibiotic used alone against the activity of these enzymes. But clearly, the degree of metabolic enzyme activity impairment by the latter combination is noteworthy whilst F23 had no significant individual impact. Further assays involving the use of higher strengths of F23 in combination with the polypeptide are required before a conclusion can be reached on the possible absence of enhanced impairment of the activity of these reducing enzymes in MSSA by both compounds.

6.3.5.2 Effect of F23 with polymyxin B upon MTT-reducing capacity of MRSA

It was reported in table 4.2 that 32 µg/ml (1/32 MIC) F23 applied with 64 µg/ml (1/2) polymyxin B was additive in antibacterial action against MRSA. These strengths of both compounds applied together were seen to be synergistic in a kinetic colony-counting assay by displaying a >4 log cfu/ml reduction in the viability of the same bacterial species after 24 hours (figure 5.9). In order to gain an insight into the possible mechanism of action for both agents, the same concentration above were used to challenge a population of this bacterial species and examined for the blockage of the cells capacity to reduce MTT salt into formazan crystals. The data obtained is presented in figure 6.14.

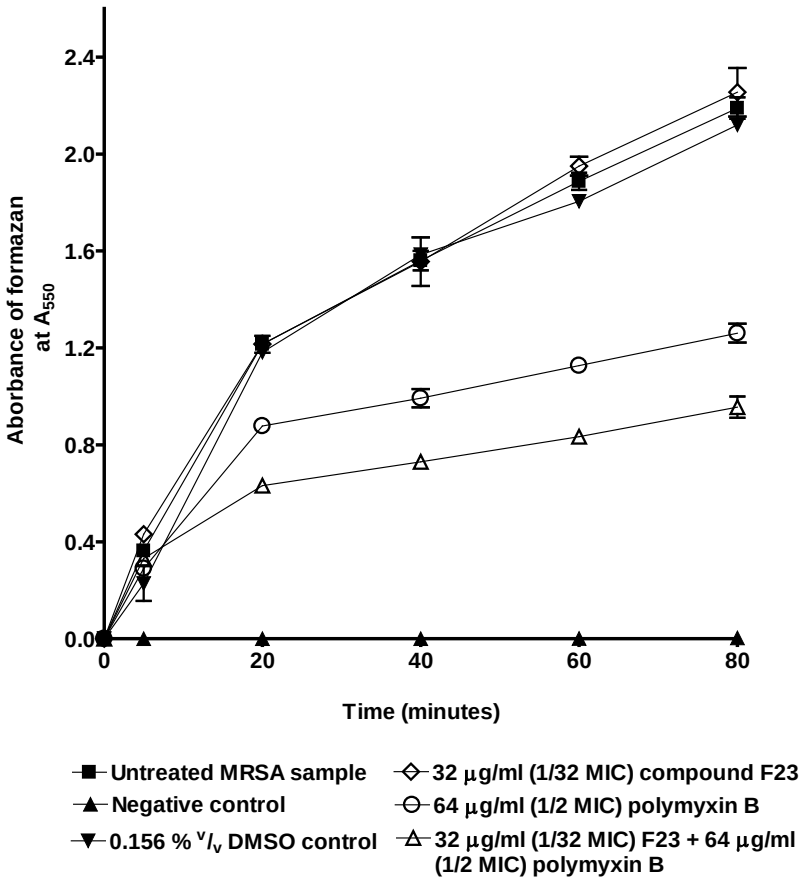


Figure 6.14: The effects of sub-inhibitory concentrations of polymyxin B and F23 applied alone as well as together upon the capacity of *S. aureus* 11940 (MRSA) to produce formazan crystals from MTT salt. The data were mean values obtained from 3 experiments conducted in duplicate on separate days.

Whereas the untreated, solvent-treated and MRSA populations challenged with 32 µg/ml (1/32 MIC) F23 demonstrated comparable quantities of formazan crystal production over 80 minutes, MRSA suspension challenged with 64 µg/ml (1/2 MIC) polymyxin B alone displayed about 45 % blockade to the activity of bacterial reducing enzymes. This recorded activity is lower than the approximately 60 % inhibition produced by twice this concentration of the polypeptide antibiotic in section 6.3.4.2 (figure 6.11). This verified the capacity of polymyxin B to impede the actions of enzymes thought to be responsible for enforcing biological reduction reactions within MRSA. As can also be seen from the results in figure 6.14, an application of 32 µg/ml of this chalcone-derivative with 64 µg/ml (1/2 MIC) polymyxin B demonstrated a 60 % reduction in the quantity of formazan produced. This observation correlates with the results recorded in table 4.2 and figure 5.9 suggesting the impairment of metabolic

activity preceded cell death in the possible mechanism of action for the combination against this bacterial strain.

6.3.5.3 Effect of F23 with polymyxin B upon the formazan crystals production capacity of quinolone-resistant *S. aureus*

Figure 6.15 below shows the effect of a sub-inhibitory concentration of F23 applied alone and with a sub-inhibitory concentration of polymyxin B upon the biological reduction of MTT salt to formazan crystals by QRSA.

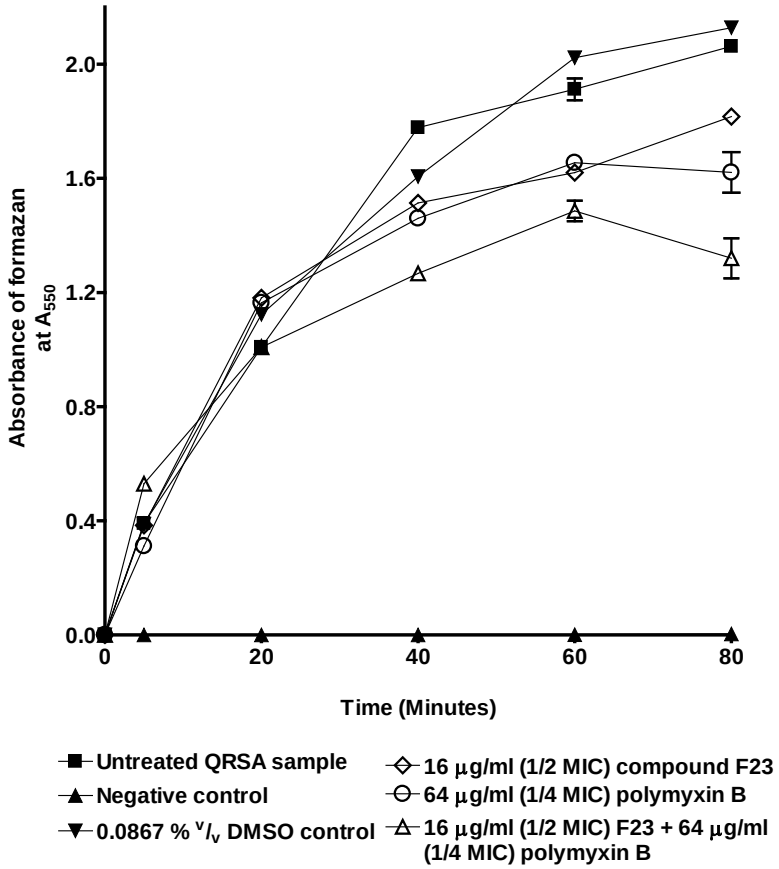


Figure 6.15: Inhibitory effect of a sub-inhibitory concentration of polymyxin B with a sub-inhibitory concentration of F23 applied in combination upon the biological reduction capacity of *S. aureus* 1628 (QRSA) in an MTT metabolic assay. The data were mean values obtained from 3 experiments conducted in duplicate on separate days.

The concentrations of both compounds used in this assay were the same as those reported as synergistic against quinolone-resistant strain of *S. aureus* in table 4.2 and figure 5.10. The 16 µg/ml (1/2 MIC) F23 that was seen to have caused more than 0.5 log cfu/ml loss in the viability of QRSA population within 24 hours is now observed to induce an approximately 12.5 % blockage to the

activity of NADH and succinate dehydrogenase after 80 minutes, with respect to the untreated and solvent-treated QRSA controls.

Similarly, an application of 64 µg/ml (1/2 MIC) polymyxin B that was responsible for a 2 log cfu/ml loss in cell viability is also seen to cause a 25 % impairment to the activity of the biological reducing enzymes; suggesting a cessation of metabolic activity occurred in advance of cell death. A combined application of both compounds at the same strengths previously mentioned demonstrated about 40 % impairment to the activity of these enzymes after 80 minutes.

The fact that the combination of this same 16 µg/ml F23 with 64 µg/ml polymyxin B had been reported to have reduced the colonies of this bacterial species by about 4.5 log cfu/ml after 8 hours suggests again that the cessation of cell metabolism occurred before the killing of these cells.

The curve for the combination of F23 with the polypeptide antibiotic above is also suggestive of a possibility for a continued enhanced impairment of reducing enzymes activity and an increment in the amount of recovered formazan crystals, if the experiment were extendable beyond 80 minutes. But this cannot be achieved because of the deposition and aggregation of the formed crystals with the cells, thereby reducing the accessibility of the metabolic enzymes over time.

As already demonstrated for MSSA (figure 6.5), an increase in the concentration of polymyxin B may have led to an increase in the degree of impairment upon the metabolic enzymes activity in QRSA. A similar speculation is that an increase in concentrations of both F23 and polymyxin B in the combination may also have yielded a corresponding decrease in the rate and quantity of formazan production over time.

In any case, there is a correlation between the data provided and those previously recorded in table 4.2 and figure 5.10. When viewed together, these results strongly suggest the blockage of bacterial metabolism (reducing enzymes activity) in QRSA, is an eminent mechanism of action for the polypeptide combined with F23, which has also been demonstrated for this antibiotic combined with F1 (figure 6.12).

An inconsistency that is noticeable is the use of different wavelengths for the measurements of optical densities of formazan crystals and the estimation of bacterial populations as the presentation of data was progressing. This variation was because the first set of experimentations were conducted using large

quantities of materials and the formazan crystals were measured using 1 ml transparent disposable cuvette in a spectrophotometer. The experimental procedure was later scaled down to permit the use of reduced quantities of substrates and the formazan crystals obtained were quantified in a micro-titre plate wells using a DTX-880 reader (Molecular Devices). The difference in wavelength scales did not interfere with the interpretation of the results data as analysis were done in percentages, relative to the untreated control in each set of experiment.

The absence of either MTT reagent or inoculum bacteria led to lack of production of formazan as mixtures of bacterial-free broth, test-antimicrobial compound and MTT solution incubated at 37 ^{o}C for 80 minutes had no formazan crystals, in comparison to blank DMSO control. This data indicates there was no interference between LBG and MTT in the bacterial metabolic assay as well as that both MTT salt and inoculum bacteria were essentially required for the technique. Similarly, the combination of MTT, broth and glycerol in the presence of oxygen under ideal bacterial growth temperature did not lead to the formation of formazan crystals.

6.4 Discussion

6.4.1 General observations in this protocol

The MIC and minimum bactericidal concentration (MBC) values reported for polypeptides against the different bacterial species were almost always the same (table 3.5), and the use of either value for a given polypeptide antibiotic can be considered almost always interchangeable in this report. The data presented in figures 6.1 and 6.2 suggest that the capacity of the bacterial population to reduce MTT salt to formazan crystals increases proportionately to cell density, as a linear correlation existed between the quantities of formazan produced and cell populations within the range of 0.1 to 1.0 unit at OD_{600}. Therefore, the MTT metabolic assay was justifiably used to assess the inhibitory effect of polymyxins on the reduction capacity of *S. aureus* species, *Ps. aeruginosa* and *E. coli* in relation to untreated cell samples. The untreated cell suspensions and negative controls devoid of either MTT reagent or bacterial inoculum consistently formed the highest and lowest amounts of formazan crystals respectively within the 80 minutes time-course.

Formazan production rate will drop if the assay is continued infinitely after 80 minutes due to enzyme saturation and exhaustion of available units of dehydrogenases. Absorbance readings for formazan production were monitored at 550 nm because formazan crystal-bacterial complex dissolved in DMSO gives broad peak between 510 and 570 nm, with a single absorption maximal at 550 nm (Wang et al. 2010b).

6.4.2 Effect of colistin and polymyxin B upon bacterial reducing enzymes activity

Cationic antimicrobial peptides interact with the polyanionic outer surfaces created by the presence of cell-wall associated teichoic and lipoteichoic acids in Gram-positive as well as the lipopolysaccharide (LPS) in Gram-negative bacterial species (Hale and Hancock 2007). For the latter class of organisms, the initial electrostatic attraction between these agents and the cell surface is followed by a self-promoted translocation (Hancock 1997a, Hancock 1997b). This may explain why colistin and polymyxin B have greater antibacterial potency against most Gram-negative than Gram-positive organisms. These antibiotics appear to display a relatively higher affinity for lipopolysaccharide than for teichoic and lipoteichoic acids (Hancock 1997b). Their affinity for the LPS is so strong that these agents competitively displace native Mg^{2+} and Ca^{2+} ions, causing a local destabilisation that facilitates trans membrane bilayer uptake of more peptides, as well as allied molecules (Hale and Hancock 2007). But in addition to the absence of this outer bilayer in Gram-positive cells, the presence of a thicker peptidoglycan layer may also be limiting the antibacterial action of these polypeptides (Zhang, Rozek and Hancock 2001a, Hancock and Rozek 2002a, Vaara et al. 2008), considering that this latter structure functions as a form of exoskeleton and scaffolding that further strengthens this latter class of organisms (Lorian 2005).

Since both colistin and polymyxin B demonstrated a greater inhibition to the metabolic activity of reducing enzymes in Gram-positive *S. aureus* than those of Gram-negative *Ps. aeruginosa* and *E. coli* in the data recorded from the MTT-reduction assays, these results seem to suggest that the two latter bacterial species can transition between aerobic and anaerobic respiration, unlike the former, thereby enhancing their capacity to better evade this mechanism of

action. For whilst polymyxin B concentrations up to 0.625 µg/ml (20 x MIC) only showed a partial blockade to the metabolic activity of *E. coli* (figures 6.3 and 6.4), strengths of this antibiotic equivalent to the 256 µg/ml (MIC), 1.024 mg/ml (4 x MIC) and 2.56 mg/ml (10 x MIC) were seen to display about 75, 100 and 100 % cessation respectively to the biological MTT-reduction capacity of *S. aureus* in figure 6.5. Since enzymes are proteins that are universal irrespective of the bacterial species, the heavy amounts of this antibiotic employed against the reducing enzymes of *S. aureus* reported in the latter data have undoubtedly contributed to the significant inhibitory action against these proteins activity. But the activity of this polypeptide reported against these same reducing enzymes actions for *Ps. aeruginosa*, another Gram-negative organism was also partial (supporting this species' capacity for respiratory transitioning), despite the use of lesser amounts in comparison to that applied against *S. aureus*. Concentrations equivalent to 1 µg/ml (MIC), 4 µg/ml (4 x MIC) and 20 µg/ml (20 x MIC) applied against this organism were seen to exhibit only partial inhibitions to the activity of NADH and succinate dehydrogenase (figure 6.6). A speculation is that larger quantities of this antibiotic, as those applied against *S. aureus*, may have yielded more impairment (perhaps a complete blockage), given that the actions of this compound against the reducing enzymes were concentration-dependent, as indicated by the curves in figure 6.6. But such an assay would mean the use of concentrations that were grossly disproportionate from the MIC value of polymyxin B against *Ps. aeruginosa*. As a follow on, the action of colistin against the metabolic activity of these bacterial species above may be considered comparable to that of polymyxin B. The degree of inhibitory actions of equivalent multiples of MIC of this latter antibiotic against the activity of bacterial reducing enzymes were also seen to be greater in *S. aureus* than in both *Ps. aeruginosa* and *E. coli*, which may be explained by the fact that the latter two bacterial species can undergo respiratory flexibility (Richardson 2000) by transitioning from aerobic to anaerobic respiration (Hassett et al. 2002), thereby minimising the potential damage upon the cells metabolic capacity by colistin. A number of electron acceptors are utilised, including oxygen, nitrate, nitrite, nitric oxide and fumarate (Hassett et al. 2010).The production of succinate dehydrogenase in *E. coli* exposed to noxious agent and undergoing dual-phase fermentation, has been shown to be dependent upon the time of transitioning from aerobic to anaerobic respiration (Vemuri, Eiteman and Altman 2002).

Concentrations of polymyxin B equivalent to 0.125 µg/ml (4 x MIC) were seen to display a partial blockage of the enzyme-reduction capability of *E. coli* (figure 6.3). A concentration equivalent to 0.625 µg/ml (20 x MIC) polymyxin B also only demonstrated ~ 25 % impairment to the biological reduction capacity of this organism (figure 6.4). Whereas, this antibiotic also demonstrated about 50 % loss to the reduction activity of metabolising enzymes in *Ps. aeruginosa* at strengths equivalent to 20 µg/ml (20 x MIC) and much less at concentrations equivalent to 4 µg/ml (4 x MIC) and 1 µg/ml (MIC). Considering that both compounds are selectively and rapidly bactericidal against Gram-negative bacterial pathogens in kinetic-colony counting assays (Tam et al. 2005, Li et al. 2005b), the data presented in figure 6.3, 6.6, 6.7 and 6.9 wherein both compounds were only partially effective at high concentrations in blocking biological reducing enzymes in these organisms suggest *E. coli* and *Ps. aeruginosa* may be evading metabolic impairment by undergoing a rapid transition from aerobic to anaerobic respiration (Poole and Cook 2000). The cellular physiologies as well as metabolic pathways for both routes of bacterial respiration are vast and diverse (Sawers 1999). With the aerobic growth phase, *E. coli* produces CO_2 as the main extracellular by-product whilst a mixed acid fermentation is undertaken with anaerobic growth leading to the accumulation of formate, lactate, acetate, ethanol as well as relatively small quantities of succinate (Vemuri, Eiteman and Altman 2002). Succinate dehydrogenase enzyme contributes to the biological reduction of MTT salt to formazan crystals (Berridge, Herst and Tan 2005a). And under this circumstance above, certain genetic manipulations can increase succinate yield (Vemuri, Eiteman and Altman 2002). Such genetic engineering manoeuvres include the deletions of *ldhA* (that encodes fermentative lactate dehydrogenase) (Bunch et al. 1997, Jiang, Nikolova and Clark 2001), as well as pyruvate formate lyase (PfL) (Wendisch, Bott and Eikmanns 2006, Leibig et al. 2011); over expression of *ppC* encoding phosphoenolpyruvate (PEP) carboxylase (Millard et al. 1996) as well as the genes that code for malic enzyme and pyruvate carboxylase (*pyC*) (Gokarn, Eiteman and Altman 2000).

Since the anaerobic process for *Ps. aeruginosa* involves the utilisation of alternative electron acceptors such as either NO_3^-, or NO_2^- (Hassett et al. 2002). This organism can replicate under both (aerobic and anaerobic) conditions but robust growth is undertaken via the latter phase respiration through the use of

either of these inorganic terminal electron acceptors above or a very slow growth through substrate level phosphorylation with arginine (Hassett et al. 2002). The NO_3^- is then reduced either to ammonia and subsequently used as a nitrogen source or to nitrogen gas for respiration (Pinzon-Gamez 2009).

Antimicrobial cationic peptides can display a rapid bactericidal activity against susceptible pathogens with greater than 99.999 % reduction to the population of the challenged bacterial inoculum within 5 to 120 minutes at concentrations equivalent to 4 x MIC (Tam et al. 2005, Hermsen, Sullivan and Rotschafer 2003), suggesting the mechanisms of such peptides include inhibition of metabolic enzymes as well as impairment of energization activities (Hancock and Rozek 2002b). This is in addition to their verified capacity to competitively interact with and rapidly disrupt bacterial lipopolysaccharide bilayer (Jenssen, Hamill and Hancock 2006a), which by itself can lead to a substantial loss in metabolism considering that the bilayer accommodates some enzymes such as cytochrome P_{450} reductase (Wadsater et al. 2012). Such membrane anchored-enzymes can catalyse electron transfers from NADPH to cytochrome P_{450}, which is an essential enzyme for substrate metabolism (Malonek et al. 2004).

The data presented from this study showed that concentrations equivalent to MIC, 4 x MIC as well as 10 X MIC, colistin and polymyxin B demonstrated a rapid onset of suppression of reduction enzyme activity in Gram-positive *S. aureus*, against which both compounds are adjudged to be clinically ineffective (Res 2003) and are not drugs of choices for infections diseases mediated by such pathogens either as primary or opportunistic pathogens (Li et al. 2006). Although both polypeptides caused a cessation to the activity of the bacterial reducing enzymes in *S. aureus* species, this action did not translate to a bactericidal effect under buffer conditions *in vitro* (chapter 5), giving an insight to why these compounds are not effective clinically against *S. aureus* species, bearing in mind the capacity of this organism to transit from aerobic to anaerobic respiration, under biological stress conditions (Martin et al. 1999), producing smaller quantities of succinate (Vemuri, Eiteman and Altman 2002). This is an indication that the rapid inhibition of NADH and succinate dehydrogenases during bacterial aerobic respiration, may not always translate to a rapid loss of cell viability. Time-course viability data presented in figures 5.1 and 5.2 show that 256 and 128 µg/ml polymyxin B, being MICs of this antibiotic against MSSA and MRSA in table 3.5, were only bacteriostatic against these organisms, with an approximate

1 log cfu/ml reduction to the starting inoculum populations within 24 hours. But both concentrations showed a 75 % (figure 6.5) and 60 % (figure 6.11) inhibition to the metabolic enzyme activities in *S. aureus*, within 80 minutes. It is therefore possible that the impairment imposed upon the activities of these reducing enzymes did not translate to cell death because of the capacity of *S. aureus* to transition between aerobic and anaerobic respiration (Martin et al. 1999, Malonek et al. 2004).This may suggest that although both antibiotics were able to block aerobic respiration, these *S. aureus* species probably switched over to anaerobic respiration which generates lower levels of glucose utilisation (Kohler et al. 2003), expressing a decline in the capacity of these organisms to form formazan from MTT salt. As such, despite the demonstrated capacity to inhibit bacterial reduction activity, polymyxin B is not bactericidal against *S. aureus* species even over an extended period of 24 hours as shown in the time-course viability data presented in the previous chapter. But in addition, the inoculum population of 0.3 units at OD_{500} (equivalent to ~1 x 10^8 cfu/ml) used for the assays may be considered as a large bio-load, bearing in mind that the bactericidal efficacy of polymyxin B is significantly diminished in the presence of a high inoculum density (Tam et al. 2005).

Although the time-course viability data presented for polymyxin B against MSSA, MRSA and QRSA in figures 5.1, 5.2 and 5.4 show that strengths of this antibiotic equivalent to MIC were at best only bacteriostatic within 24 hours, these concentrations were seen to have demonstrated a rapid and profound inhibition of formazan production through blockade of the activity of bacterial reduction enzyme. The explanation previously suggested that a lack of correlation between kinetic viability data and inhibition of MTT-reduction capacity existed is also applicable for *Ps. aeruginosa*. Data published for the time course-viability assay of polymyxin B against *Ps. aeruginosa* show that there was a rapid bactericidal action against antibiotic sensitive strains within 2 hours at polymyxin B concentrations of 4, 8 as well 16 µg/ml, given a starting inoculum population of 10^5 to 10^7 cfu/ml with an MIC in the range of 0.5 to 1 µg/ml (Tam et al. 2005). Findings from the MTT reduction assay presented in this section show that concentrations of 1 µg/ml (MIC), 4 µg/ml (4 x MIC) and 20 µg/ml (20 x MIC) demonstrated an incomplete inhibition of biological reduction of MTT salt in a sensitive strain of *Ps. aeruginosa*. This observation may suggest that Gram-negative bacterial species are killed solely by this mechanism. The involvement

of this pathway in the killing effect of polymyxins against these bacterial species may only have been partial. The involvement of outer membrane disruption as the primary mechanism of action of the polymyxins in Gram-negative bacterial species has been extensively discussed (Hancock 1997a, Zhang, Rozek and Hancock 2001b, Hancock and Rozek 2002a, Hancock and Chapple 1999, Zhang et al. 2000, Jenssen, Hamill and Hancock 2006b). The fact that the impairment of bacterial reduction activity remained incomplete even at 20 µg/ml (20 x MIC) seems to fortify the speculation for the occurrence of transitioning between aerobic and anaerobic respiration (with the use of either NO_3^- or NO_2^- in *Ps. aeruginosa* (Hassett et al. 2002), as well as the partial engagement of non-competitive enzyme impairment in the aerobic pathway). The published data (Tam et al. 2005), also show that with the use of broth, the challenged *Ps. aeruginosa* populations began to display extensive re-growth after 4 hours. In fact, even the populations challenged with higher strengths of polymyxin B that had their densities reduced below detectable levels also demonstrated re-growth (Tam et al. 2005). Bearing this in mind whilst viewing data presented in figures 6.6, 6.3, 6.4, 6.9 and 6.7 (in this order) suggest Gram-negative *Ps. aeruginosa* and *E. coli* are not killed primarily through inhibition of their metabolic enzyme activity by either polymyxin B or colistin, especially considering that the organisms can undergo a transition to an alternate respiratory pathway. The inhibition of reduction enzyme activity is therefore only partially effected upon the cells in the entire challenged population. A weakness in the analysis may be attributed to the high inoculum density employed as although this was essential to allow the formation of measurable quantities of formazan, it is uncertain if this factor may be negating the correct interpretation of the data given that the antibacterial activity of polymyxins are greatly influenced by cell density (Hancock and Rozek 2002a, Tam et al. 2005).

The pronounced inhibition of biological MTT salt reduction to formazan crystals by colistin and polymyxin B in MSSA (figures 6.5, 6.8, 6.10, 6.13), MRSA (figures 6.11, 6.14) and QRSA (figures 6.12, 6.15) suggest these organisms are probably able to switch into an alternative means of respiration as well as ATP generation. A *srrAB* (staphylococcal respiratory response) gene, whose homologue *resDE* was previously identified in *B. subtilis*, has been determined in *S. aureus* (Yarwood, McCormick and Schlievert 2001). Both genes code for the two-component regulatory system, ResD-ResE, implicated for the global regulation of both

aerobic and anaerobic respiration (Yarwood, McCormick and Schlievert 2001). The induction of stress signals is known to activate anaerobic respiration in *S. aureus* (Hausladen, Gow and Stamler 1998). It appears these polypeptide antibiotics can activate stress signals that induce transitioning into anaerobic respiration in the organisms examined in this assay. Although both antibiotics demonstrated extensive capacity to halt *S. aureus* conversion of MTT salt to formazan through inhibition of these cells conventional (aerobic) respiratory pathway, colistin and polymyxin B remain Gram-negative specific antibiotics (Falagas, Kasiakou and Saravolatz 2005, Li et al. 2006), and unable to kill Gram-positive *S. aureus* probably because of the absence of the occurrence outer membrane disruption, which is these compounds primary mechanism of action (Vaara 2010).

This rapid onset of action for the pronounced inhibition of the biological reduction process is neither correlated nor reflected in a time-course viability assay for *S. aureus* species. The bacteriostatic effect reported for polymyxin B against some species of *S. aureus* in chapter 5 is unaccountable for by the pronounced inhibition of biological reducing enzymes in this species as these organisms are able to take advantage of alternative routes of respiration (Richardson, Libby and Fang 2008), that by-passes formation of NADH, a biological reducing enzyme. This metabolic adaptation involves the production of either stress-induced (Seidl et al. 2006) or nitric oxide radical (produced by activated phagocytes) inducible L-lactate dehydrogenase, known to have aided *S. aureus* to become resistant to innate antimicrobial defence peptides such as defensins and cathalicidins LL-37 (Richardson, Libby and Fang 2008, Kraus and Peschel 2008). L-Lactate dehydrogenase is thought to catalyse the conversion of pyruvate to lactate when *S. aureus* is grown and stressed/challenged in the presence of rapidly catabolizable carbon source (glycerol in this instance), under anaerobic respiration (Seidl et al. 2006). If the impairment of the activities of the reducing enzymes (NADH and succinate dehydrogenase) by the applied polymyxins made *S. aureus* to transit into anaerobic respiration, the stress induced by the presence of these agents probably led to an induced formation of L-lactate dehydrogenase, an alternate enzyme. This will translate to an unhindered supply of ATP and possibly repair mechanisms that lead to bacterial survival as well as insusceptibility to these antibiotics. The effect of colistin and polymyxin B on stress proteins in the challenged bacterial species may be worth examining in

subsequent studies. Colonies of Gram-positive bacterial species can also exist as biofilms *in vivo*, precluding access of oxygen to cells within the biofilm, which can then switch to alternative mechanisms of sugar metabolism and respiration, thereby evading the lethal effects of cationic antimicrobial peptides, as well as acting as inoculi for cell propagation (Flemming and Wingender 2010, Burmølle et al. 2010). All these factors seem to provide possible explanations for the clinical inefficacy of polymyxins against *S. aureus* species. Considering that Gram-positive cells are bereft of an outer membrane bilayer (Lorian 2005), which is the primary target of these polypeptides (Falagas, Kasiakou and Saravolatz 2005), these organisms are advantageously physiological positioned to evade the killing effects of these agents. In fact, the inability of polymyxins to induce a bactericidal effect upon *S. aureus* species in colony counting assays at concentrations many-fold higher than the MICs (chapter 5) further exposes the ineffectiveness of this polypeptide antibiotic against Gram-positive bacterial species.

On the other hand, polypeptides that are bactericidal against Gram-positive bacterial species (such as indolicidin (Friedrich et al. 2000)) do so by interacting with the phospholipids, teichoic and lipotechoic acids (Scott, Gold and Hancock 1999, Brogden 2005), by depolarisation of the cytoplasmic membrane (Hancock 1997b) and by either the induction of some degree of disturbances or formation of channels across the bilayer (Brogden 2005) (as indeed is applicable in Gram-negative species, upon translocation across the outer membrane (Jenssen, Hamill and Hancock 2006a)). There seems to be an association between the substantial blockade of the reducing enzymes (NADH and succinate dehydrogenases) activity by polymyxins with their infringement upon the integrity of cytoplasmic membrane, as it has been speculated that pore formation across the bilayer alone is insufficient to account for the bactericidal action of antimicrobial peptides against Gram-positive bacterial species, but necessary for these agents to access other intracellular targets (Friedrich et al. 2000, Brogden 2005). Although the permeabilization as well as disruption of the cytoplasmic membrane permits the self-translocation of these peptides, there is another speculation that this action simultaneously leads to an impairment of localised enzyme activities and cell de-energization (Zhang, Rozek and Hancock 2001a). In addition to both latter speculations, is the effect of membrane depolarisation which all collectively culminates into a lethal event (Brogden

2005). Therefore, the capacity of polymyxins to induce membrane depolarisation as well as disruption in *S. aureus* species should be evaluated in subsequent sections of the this study in order to enable a more robust and accurate interpretation of the data gleaned from this chapter. The results obtained from the MTT-reduction assay for the effect of colistin and polymyxin B upon the metabolic reducing enzymes of *S. aureus* species cannot be comprehensively interpreted in isolation as an association is thought to exist between cell membrane depolarisation, channelization, essential enzymes activities inhibition and cell de-energization (Zhanel et al. 2010, Zhang, Rozek and Hancock 2001a).

6.4.3 Partial inhibition of biological reducing enzymes activity in *E. coli* and *Ps. aeruginosa*

The inhibitory action of polymyxin B and colistin upon *E. coli* and *Ps. aeruginosa* reducing enzymes activity were therefore concentration-dependent, as reflected in the data presented in figures 6.3 to 6.9. These observations conform to published reports that demonstrate both antibiotics display concentration-dependent action against *Ps. aeruginosa* in time-course colony counting assays (Gunderson et al. 2003, Tam et al. 2005, Li et al. 2001a), suggesting that outer membrane disruption (Falagas, Kasiakou and Saravolatz 2005), as well as cessation of respiration-associated reducing enzymes can probably collectively account for the bactericidal action of polymyxins against *E. coli* and *Ps. aeruginosa*.

6.4.3.1 Effective drug combinations that impede bacterial enzyme activities

With the assessment of the effect of polymyxin B applied in combination with either of the chalcone-derivative upon bacterial metabolism, the semi-synthetic compounds were on their own devoid of inhibitory action against the activity of the cells reducing enzymes (except about 12.5 % impairment against the reducing enzymes activity in QRSA, at 16 µg/ml in figure 6.15). Yet either these compounds displayed substantial augmentation to the inhibitory action of polymyxin B (or vice versa) against the reducing enzymes activities in species of *S. aureus*. Figures 6.10 to 6.12 show that F1 applied in combination with polymyxin B at sub-inhibitory concentrations demonstrated an enhanced

impairment to the biological reducing enzymes action in MSSA, MRSA and QRSA. The combined application of 32 µg/ml (1/2 MIC) F1 with 64 µg/ml (1/4 MIC) polymyxin B showed a greater inhibitory action against the biological reduction potency of MSSA (figure 6.10) suggesting that the combination causes an enhanced impairment to the activity of NADH and succinate dehydrogenases that results in an increase in the rate as well as extent of the inhibition. Given that data presented in figure 5.1 showed that 64 µg/ml F1 applied in combination with 256 µg/ml polymyxin B also demonstrated a rapid bactericidal activity (unlike polymyxin B applied alone) with greater than 4 log cfu/ml reduction to inoculum population relative to the solvent treated sample within 8 hours, such a rapid activity had been speculated to very likely involve the inhibition of an essential metabolic pathway (Hancock and Rozek 2002b) as is now being shown in figure 6.10. The observation above may also provide an explanation for the rapid synergistic activity of F1 applied in combination with polymyxin B against MRSA (figure 5.2) and QRSA (figure 5.4). In fact, time course viability data in figure 5.2 showed a greater than 3 log cfu/ml reduction in MRSA suspension treated with 128 µg/ml of both F1 and polymyxin B after 2 hours; whilst the same concentrations of both compounds applied separately were clearly ineffective after 24 hours. These observations strongly suggest the application of the chalcone-derivatives with polymyxin B leads to an inhibition of biological MTT-reduction process as well as a killing-effect (unlike the single application of polymyxin B) because the combination seems to possess the capacity to block both aerobic and anaerobic bacterial respiration in these species culminating into a complete cessation of all possible pathways for ATP production as well as speculatively, the blockage of the biosynthesis of essential proteins, such as NADH and succinate dehydrogenases. In such circumstances, it has been demonstrated that in a bid for the cells to generate more NADH by any other pathway, albeit unsuccessfully, the organisms eventually rapidly expend their stock of ATP reserve resulting to a rapid loss of viability (Baker et al. 2004, Seidler 2013). Data presented in figure 6.11 showed that although 128 µg/ml polymyxin B applied alone demonstrated about 60 % inhibition to the activity of the reducing enzyme, 128 µg/ml F1 applied in combination with this same concentration of polymyxin B demonstrated 90 % blockade to the activity of the enzyme, with a faster onset/rate, suggesting an inhibition of both the conventional and alternative metabolic pathways, such as both aerobic and

anaerobic respiration mechanisms. It is possible that the activity of enzymes such as NADH, succinate as well as L-lactate dehydrogenases that are regulated by different pathways have all been impaired. An application of half the aforementioned concentrations for both compounds also demonstrated 85 % impairment to the activity of reducing enzymes at a fast rate, an indication that at lower concentrations, both compounds still enhance each other's activity when applied together.

Other instances exist wherein the inhibition of vital metabolic process leads to and correlates with cell death, sometimes accompanied with rapid onset (Hancock and Rozek 2002a), using combinational drug therapy. The anti-metabolic and synergistic action of trimethoprim applied in combination with sulfamethoxazole is established through their direct blockade of the folic acid synthetic pathway in two independent positions, leading to bactericidal activity in susceptible pathogens (Grim et al. 2005). The development of this effective combination has resulted in the successful treatment of acute urinary tract infections such as cystitis (Kavatha et al. 2003), even with the application of lower synergistic concentrations of both compounds, resulting into a reduced potential for adverse events (Knaapen and Barrera 2007). Folic acid is structurally composed of para-aminobenzoic acid (PABA), pteridine and glutamate (Bermingham and Derrick 2002, Brain et al. 2008). Sulphamethoxazole, like other sulphonamides such as sulfisoxazole is structurally similar to PABA and both are competitive substrates for the biological enzyme dihydropteroate synthetase which is responsible for the addition of PABA to pteridine (Grim et al. 2005, Lumb et al. 2009). On the other hand, trimethoprim like pyrimethamine, is a structural analogue of pteridine and both are competitive substrates for dihydrofolate reductase (Lumb et al. 2009, Basco et al. 2000). Co-trimoxazole combinational antibiotic demonstrates selectivity (preferential interaction) for bacterial dihydropteroate synthetase with a greater than a 1000-fold sensitivity, because unlike mammalian cells, prokaryotic organisms synthesize their folic acid requirements and possess genes encoding for the latter enzyme (Knaapen and Barrera 2007).

The improved activity of F1 in combination with polymyxin B against species of *S. aureus*, even at reduced concentrations, is probably due to the blockage of these organisms' respiratory pathways/enzymes. The antibiotic combination, co-trimoxazole is bactericidal through a mechanism that involves the inhibition of

folic acid synthetic pathway at two separate points (Knaapen and Barrera 2007). Data presented in this section for the chalcone-derivatives alone correlates with those recorded from the time-course viability assays. The chalcone-derivatives were almost always inactive against species of *S. aureus* during individual applications, and these agents were also devoid of inhibitory action against the activity of NADH and succinate dehydrogenases in the MTT reduction analysis. But 16 µg/ml F23 that was bacteriostatic against QRSA with a 2 log cfu/ml loss in viability of the exposed population displayed a 12.5 % inhibition to the activity of this species reducing enzymes within 80 minutes. This observation suggested the antibacterial action of the chalcone-derivatives could be strain related in *S. aureus* as well as the partial inhibition of aerobic and anaerobic respiratory metabolic enzymes.

Another obvious limitation of this study is the inability to make an inference from the data provided so far as to whether or not the inhibition of NADH and succinate dehydrogenases by the combination of F1 with polymyxin is reversible given that the time-course assays were conducted in buffer media and the formation of complexes between bacterial cells and formazan crystals in the biological MTT reduction analysis prohibits the culturing of the organisms for assessment of potential re-growth. Assuming the time-course viability analysis were conducted in broth and if cell samples used for the MTT-reduction test were cultivable (viable), then the occurrence of bacterial re-growth would have verified that the activity of both compounds against these dehydrogenases were either partial or reversible. The activity of polymyxin B applied alone against *Ps. aeruginosa* recorded an eminent bacterial re-growth in all the challenged strains (Tam et al. 2005), supporting this speculation of the occurrence of either a partial or reversible enzyme inhibition. In the presence of polymyxin B concentrations equivalent to MIC, 4 x MIC and 10 or 20 x MIC, *Ps. aeruginosa* and *S. aureus* species probably transited between aerobic and anaerobic respiration thereby enabling an incomplete impairment of these organisms respiration as well as other essential metabolic mechanisms which facilitates cell re-growth in broth.

The viability and virulence of these organisms are determined by cell wall/membrane-associated proteins that are inducible by adverse growth conditions and stress (Seidl et al. 2006). The expression of genes responsible for resistance to specific antibiotic action is driven by specific and sensitive

mechanisms that are often undertaken at the transcriptional level (Bronner, Monteil and Prévost 2006). The regulatory factors are influenced by the two component regulatory genes such as *saeRS* and *srrAB*, that are sensitive to environmental signals. For instance, *srrAB* (*srhSR*) is known to code for certain proteins, including those responsible for energy metabolism and is required for fermentative growth of *S. aureus* wherein the up-regulation of enzymes such as alcohol and L-lactate dehydrogenases that are involved in fermentation are enabled in anaerobic and stressful conditions (Throup et al. 2001). Therefore, a blockage of the activities of NADH and succinate dehydrogenases manifested by an inhibition of MTT-reduction to formazan crystals may be insufficient to always culminate into a bactericidal action in species of *S. aureus* and other bacterial species. For instance, although gentamicin is bactericidal against *E. coli* species, this agent is known to display weak activity against this organism when undergoing anaerobic respiration, especially at reduced pH (Bringer et al. 2007). It may therefore be worthwhile to conduct future time-course viability assays for the chalcone-derivatives applied in combination with polymyxin B against strains of *S. aureus* (and *Ps. aeruginosa*) in growth media in order to facilitate a more elaborate interpretation of the pool of data.

The speculation above is also applicable for the data presented in figures 6.14 and 6.15. Sub-inhibitory concentrations of F23 applied in combination with polymyxin caused a greater impediment to formazan production from MTT salt suggesting the occurrence of a blockade (of both aerobic as well as anaerobic respiratory pathways) to the reducing activity of NADH, succinate, alcohol and L-lactate dehydrogenases, in MRSA and QRSA. This may have contributed substantially to the synergistic activity of both compounds against these bacterial species reported in figures 5.9 and 5.10 respectively.

The concentrations of both compounds applied to challenge the bacterial population were selected based on guidance from data previously obtained from the chequerboard and time-course-viability studies. The combination of 1/32 MIC (32 µg/ml) of F23 with 1/2 MIC (64 µg/ml) polymyxin B demonstrated a remarkable inhibition to the reducing enzymes activity of MRSA (figure 6.12), similar to 64 µg/ml (1/2 MIC) F1 with 64 µg/ml (1/2 MIC) polymyxin B (figure 6.9) and in agreement with the synergistic effects reported for the former concentrations against the growth of this resistant strain of *S. aureus* in both a chequerboard (table 4.2) and time-course viability assays (figure 5.9)

respectively. Considering that these same concentrations of F23 and polymyxin B when applied in combination demonstrated a greater than 4 log cfu/ml reduction to the MRSA inoculum within 24 hours, the data presented in figure 6.2 may be suggesting the mechanisms of action of the combination is not unconnected with the inhibition of the bacterial reducing enzymes activity. At a concentration of 64 µg/ml (1/2 MIC), the polypeptide and Gram-negative specific antibiotic polymyxin B was bacteriostatic against MRSA with a 1 log cfu/ml reduction to the cell population within 24 hours (figure 5.9), and also demonstrated a remarkable cessation of the activity of NADH and succinate dehydrogenase (figure 6.14). The onset of action of between 20 and 80 minutes recorded for the impairment of enzyme activity is not reflected in the previously reported time-course viability bacteriostatic loss in cell count, as no remarkable reduction in colony count is noted until after 8 hours. This may suggest that the impairment of dehydrogenase activity in isolation is inadequate to induce loss of cell viability, in line with a speculation that not all inhibition of biological metabolic processes may lead to cell death in bacterial species (Cushnie et al. 2007). It is therefore possible that other mechanisms still underlie the activity of the chalcone-derivatives applied in combination with polymyxin B against various species of *S. aureus*. For instance, it is thought that an association exists between membrane depolarisation, disruption, cell de-energization and antibiotic bactericidal action (Zhang, Rozek and Hancock 2001a, Hale and Hancock 2007, Baltz 2009). The existence of such a relationship for the activity of the synergistic action of polymyxin B with the chalcone-derivatives is worth examining in subsequent studies.

The data presented in figures 6.13 and 5.8 show that 16 µg/ml F23 applied alone was ineffective at inhibiting the activity of NADH and succinate dehydrogenase in MSSA. This same concentration of F23 applied in combination with 256 µg/ml polymyxin B had demonstrated synergistic activity against viable populations of MSSA with a greater than 4 log cfu/ml reduction to the challenged inoculum population and also demonstrated an approximately 60 % blockade to the activity reducing enzymes in this bacterial species. In addition, 256 µg/ml polymyxin B applied alone that was previously shown to be bacteriostatic in action also showed a 60 % impairment to the activity of the biological reducing enzymes suggesting that the action of polymyxin B may have solely prompted the obstruction to the activity of NADH and succinate dehydrogenases in MSSA

by the combined agents. But considering that polymyxin B single application was not bactericidal against *S. aureus* (MSSA), but that a combined action with F23 was synergistic against this species (as shown in figure 5.8), it is probable that the combination of both agents blocked all alternative respiratory as well as metabolic channels in this organism, unlike the single application of the polypeptide antibiotic.

Data presented in figure 5.10 showed that 64 µg/ml polymyxin B was bacteriostatic against QRSA with about 2 log cfu/ml reduction to the inoculum population in comparison to the solvent-treated sample. This same polypeptide strength showed a 20 % inhibition to the activity of NADH and succinate dehydrogenases after 80 minutes (figure 6.15) in the MTT reduction analysis suggesting the existence of a correlation and a contributory action to the overall mechanism of action for the polypeptide synergism with the chalcone-derivatives against QRSA (figure 5.10). This observation also suggests the synergistic action of both compounds is related to the enhanced blockage of biological reducing enzymes activities of the examined species in the MTT-reduction analysis. Similarly in this same figure, 16 µg/ml F23 that displayed about 0.5 log cfu/ml loss in viability was later seen to induce about 12.5 % inhibition to the activity of biological reducing enzymes.

6.5 Conclusion

Colistin and polymyxin B have demonstrated capacity to obstruct the activity of the essential metabolic pathways required for bacterial respiration and viability. The impairment caused by these agents to the activity of these biological reducing enzymes is evidenced by a reduction in the capacity of these cells to convert MTT-salt into formazan crystals. The previously tested combined synergistic concentrations of chalcone-derivatives and polymyxin B also demonstrated potentiated blockage of bacterial respiratory pathway and metabolic enzyme reduction capacity. The inhibition of bacterial metabolic pathway by polymyxins alone and in combination with chalcone-derivatives possibly occurs simultaneously with membrane potential dissipation and integrity disruption in susceptible Gram-positive as well as Gram-negative species (Falagas, Kasiakou and Saravolatz 2005, Hancock and Chapple 1999, Hancock and Lehrer 1998a). Given the need for target specificity in antibacterial action,

the fact that these metabolic enzymes are also present in eukaryotic cells and that concerns of neuro as well as nephro toxicities in the host were previously raised for polymyxin B when applied clinically, there is a great need for toxicity analysis to be conducted for the synergistic action of both compounds against the selected bacterial species. DMSO-polymyxin B control should be included in future studies on the action of polymyxin B either alone or in combination with the chalcone-derivatives. The capacity of the combination to induce membrane depolarisation and disruption is also to be examined in subsequent sections.

Chapter 7

Examining bacterial membrane potential dissipation and integrity disruption by polymyxin B combined with the chalcone-derivatives

7.1 Introduction

The cytoplasmic membrane depolarisation capacity of polymyxin B in combination with the chalcone-derivatives was examined using fluorimetry as well as fluorescent microscopy in the presence of $DiOC_3(5)$, a membrane potential-sensitive stain, using protocols similar to those employed for the investigation of the demonstration of similar actions by β-lactams (Penyige et al. 2002) and diverse cationic peptides (Friedrich et al. 2000). Under normal polarised conditions, the dye distributes between the cells and medium initially then gains intracellular entrance by permeating the bacterial membrane according to the electrical potential gradient, aggregating within the cytoplasm (Ruissen et al. 2001). Once within the cytoplasm, the stain becomes concentrated and self-quenches its fluorescence due to dye aggregation (Farha and Brown 2010). Depolarisation of the membrane potential by an external agent can cause the disintegration and release of the aggregated dye molecules which is accompanied by measurable fluorescence (Silverman, Perlmutter and Shapiro 2003). The fluorescence intensity can be assessed with a fluorimeter as well as being observed with a suitable fluorescence microscope. The fluorescence of this dye is usually neither a reflection of the degree of membrane perturbation nor destruction (Poole and Cook 2000) but rather gives the extent of depolarisation. Compounds that have capacity to induce bacterial membrane depolarisation include nisin, lantibiotics, daptomycin, oritavancin and some peptide antibiotics (Domenech et al. 2009, Hancock and Rozek 2002a, Silverman, Perlmutter and Shapiro 2003).

The membrane bilayer in *S. aureus* acts as an effective semi-permeable barrier that is porous to water (via osmosis) and uncharged organic molecules up to the size limit of glycerol, with molecular weight of 92 Daltons (Lorian 2005). The mechanisms of substrate uptake through the phospholipid bilayer include either simple diffusion, facilitated diffusion (fluoroquinolones like ciprofloxacin) or active transport (both primary and secondary systems) (Sengupta and Mukherji 2006).

When the integrity of the cell membrane is slightly compromised, small substrates like potassium ions with a molecular weight of 32 g/mol, are readily extruded either through any fractures or by diffusion and the degree of leakage can be quantified with a flame emission spectrophotometer (O'Driscoll 2011, Burt 2004, Cushnie and Lamb 2005b). The flame emission spectrophotometer is a very sensitive device that can identify a metallic substance in the excited state by their atomic weight and the molecules can be quantified with reference to a pre-calibrated curve. The difference in the quantity of potassium ion within the reaction meliu before and after the cell population was challenged over a defined time period can be used to extrapolate the impact of the activity of the compound upon bacterial membrane integrity (and viability) (Cushnie and Lamb 2005b).

Antibacterial peptides are selectively effective against Gram-negative bacterial species due to the presence of an outer membrane bilayer; upon which these peptides' action can cause the formation of protrusions (blebs) following the competitive displacement of Ca^{2+} and Mg^{2+} cations (Zhang, Rozek and Hancock 2001a, Hancock and Sahl 2006, Jenssen, Hamill and Hancock 2006a, Hancock and Rozek 2002b). This outer membrane disruption capacity has provided an explanation for the selective activity of cationic antimicrobial peptides against Gram-negative bacterial species, since these compounds are seen to display an initial electrostatic interaction with the net negative charge on anionic phospholipids and phosphate groups on the lipopolysaccharide layer (Martti 2010, Hancock and Chapple 1999, Hancock and Rozek 2002b). Peptide antibiotics can however also be bound to teichoic acids on the surface of Gram-positive bacterial species (Scott, Yan and Hancock 1999), and may thereby establish a destabilization of the membrane bilayer. Some peptides have demonstrable capacity to bind to both Gram-positive and Gram-negative structures. Artificial chimeric peptides such as the cecropin/melittin hybrid (CEME), produced by recombinant DNA techniques (Piers and Hancock 1994, Subhadra 2011), are bactericidal against *Ps. aeruginosa* by being able to bind with strong affinity to lipopolysaccharide, permeabilizing this same structure and inhibiting the production of lipopolysaccharide-induced tumour necrosis factor (TNF-α) (Piers and Hancock 1994). CEME is likewise able to kill Gram-positive organisms by interacting with lipoteichoic acids (Scott, Gold and Hancock 1999).

The increasing incidence of sepsis and other infections caused by Gram-positive bacterial species provides a strong rationale supporting the need for the development of either new antibiotics or combinations of compounds with broad-spectrum activity against both classes of Gram-positive and Gram-negative bacterial species. It is also pertinent for the mechanisms of action of effective potential agents to be established. New antimicrobial compounds with either novel or multiple mechanisms of action are likely to evade existing antimicrobial resistance strategies of bacterial pathogens and thereby provide a longer clinical lifespan (Yeaman and Yount 2003, Sibanda and Okoh 2010).

When a given bacterial population is exposed to an antimicrobial agent in the presence of cell impermeable dye such as Sytox Green, the stain is able to permeate compromised membranes, interacting with nucleic acids materials to fluoresce (Lebaron, Catala and Parthuisot 1998). The dye is incapable of self-translocation across intact bacterial membrane bilayer and is therefore non-emitting. When the cell membrane is disrupted, Sytox Green undergoes a rapid passage across the damaged bilayer, binding irreversibly to polyanionic nucleic acid materials within the cytoplasm and strongly emitting at a wavelength around 525 nm (Roth et al. 1997), thereby establishing the susceptibility of the challenged pathogen to the applied antibiotic. The change in intensity of emission over time can be used to extrapolate the effect of the applied agent upon bacterial membrane integrity.

The antimicrobial agents applied as controls in this section included the *in vitro* *S. aureus* aggregator galangin (Cushnie et al. 2007), a quaternary ammonium compound benzalkonium chloride (Sütterlin, Alexy and Kümmerer 2008), cell wall synthesis inhibitor penicillin G (Rivera and Boucher 2011) and the DNA gyrase (as well as topoisomerase II) inhibitor ciprofloxacin (Pankey and Ashcraft 2005). Galangin is obtainable from the crude alcoholic extracts of the aerial parts *Helichrysum aureonitens* Sch. Bip. (Asteraceae), used as a topical anti-infective by indigenous South Africans (Afolayan and Meyer 1997). This flavonol is purified and characterised from the acetone extracts of the leaves and within a range of 100 to 200 µg/ml demonstrates bactericidal action against sensitive as well as multi-resistant bacterial species of *S. aureus*, *Enterococcus sp*. and *Ps. aeruginosa* (Pepeljnjak and Kosalec 2004, Cushnie and Lamb 2005c, Cushnie and Lamb 2006). This flavonoid compound was however previously thought to be bereft of activity against Gram-negative organisms, except *Enterobacter cloacae*

(Afolayan and Meyer 1997). Galangin is thought to be bactericidal against *S. aureus* by inducing leakage of intracellular potassium ions (Cushnie and Lamb 2005c) and enforcing cell aggregation (Cushnie et al. 2007).

The objectives in this chapter were to assess the impact of polymyxin B alone, as well as in combination with a chalcone-derivative upon cell membrane potential dissipation and disruption.

7.2 Materials and Methods

7.2.1 Membrane potential depolarisation and cell microscopy assays

A 90 ml aliquot 0.3 unit OD_{500} *S. aureus* 11940 suspension in PBS solution containing 1 % $^{w}/_{v}$ D-glucose at pH 7.2 was prepared from a 24 hours culture (section 2.2.3.2). A 10 ml volume of 1 M KCl was added to the suspension, adjusted to a final concentration of 4 µM (2.186 µg/ml) $DiOC_3(5)$ (Molecular Probes, Life Technologies Ltd, Paisley, UK.) and kept in the dark for 15 minutes, to allow for dye up-take in accordance with the manufacturer's guideline (Haugland, Spence and Johnson 2005, Allison, Brynildsen and Collins 2011). Five 20 ml aliquots were then prepared from this bacterial suspension, three of which were treated with either chalcone-derivative alone, polymyxin B alone or both compounds applied together, using suitable volumes of stock required to obtain the desired effective either single or combination concentration. The fourth and fifth 20 ml aliquot bacterial suspension were used as the untreated stained control and 256 µg/ml (4 x MIC) gramicidin-treated control samples respectively. All samples were incubated in a static water bath pre-set at 37 ^{o}C. At time intervals of 0, 15, 30, 60, 120 and 240 minutes, 1 ml sample was withdrawn into a glass cuvette and fluorescence was read at excitation of 625 nm and emission of 690 nm respectively. The reported data represent the mean result from three independent assays conducted in duplicate on separate days.

For the examination of changes in fluorescence intensity of the cells over time using microscopy, 5 aliquots of 4 ml 0.3 unit OD_{500} *S. aureus* 11940 suspension in PBS solution containing 1 % $^{w}/_{v}$ D-glucose at pH 7.2 was treated as described above and incubated in a Clifton water bath at 37 ^{o}C. A 100 µl volume was withdrawn from each tube and applied onto a 100 µl 0.5 % $^{w}/_{v}$ agarose gel in a hollow glass slide at time intervals of 1, 2, 3 and 4 hours. Observations of

changes in fluorescence intensity were made with a suitable microscope ((Leica DMLB, Leica Microsystems, Wetzlar, Mannheim, Germany) set at a magnification of x1000 and recordings were made using an attached camera (Leica DC 300F, Leica Microsystems, Wetzlar, Mannheim, Germany). The reported data were obtained from three independent assays conducted on separate days.

7.2.2 Quantifying potassium lost from bacterial cytoplasm

7.2.2.1 Preparation of glassware and utensils

All glasswares employed in the flame-emission assay were soaked in deionised ultra-pure water for 24 hours prior to the assay. This was to enable the removal of glass-borne potassium from the surface of the vessels.

7.2.2.2 Calibration of atomic emission spectrophotometer

The calibration of the flame spectrophotometer was made following a procedure similar to a previously published protocol (O'Driscoll 2011). An analysis aimed at quantifying the leakage of intracellular potassium ions was undertaken with a flame atomic absorption spectrophotometer (Model AA3110, Perkin Elmer Las UK., Beaconfield, UK.). The instrument mode was set for standard flame emission for detection of potassium ions at 766.5 nm, slit at 0.2/0.4 nm and was powered by air/acetylene gas mixture (B.O.C., Worsley, Manchester, UK.). A stock solution of 1000 µg/ml KCl in ultra pure de-ionised water was prepared. Five reference standards containing 1.0, 2.0, 3.0, 4.0 and 5.0 µg/ml KCl were made from the stock solution by suitable dilutions. The stock solution and the reference dilution samples were all prepared in propylene volumetric flasks (Azlon; Fisher Scientific). The 5 reference standards were then used to calibrate the flame photometer to obtain a reference graph with a correlation coefficient ≥0.99. The instrument subsequently quantified concentrations of potassium ions in actual samples against this reference calibration graph. Actual samples were a 100-fold dilution of supernatant solutions obtained from bacterial suspensions that were challenged with the test compound(s).

7.2.2.3 Quantifying leakage of potassium ions from antibiotic-treated *S. aureus* 6571

A 200 ml *S. aureus* NCTC 6571 suspension with density of 5 x 10^8 cfu/ml was prepared as previously described in section 2.4. Aliquots of 24 ml were made from the bacterial suspension and treated with suitable volumes of the stock antimicrobial test compounds needed to obtain the desired final concentrations, whose effect on membrane activity were to be examined. All samples were incubated in a Clifton shaking water bath at 37 ^oC and 100 rpm. Then, 1 ml volume was withdrawn from each sample at 0, 10, 20, 30, 60 and 120 minutes respectively and centrifuged at 12,000 rpm for 1.5 minutes. Hundred-fold dilutions of the supernatants were assayed for K^+ concentrations using the calibrated atomic absorption spectrometer (Perkin Elmer, A Analyst 400, UK.). Control samples included negative (untreated) and positive (either 20 µg/ml benzalkonium chloride or 5 % $^v/_v$ chlorhexidine treated) samples. The data obtained were analysed with GraphPad prism software 4 for standard error of mean and standard deviation followed with the plotting of a graph of K^+ lost (µg/µl) against time (minutes).

7.2.3 Quantifying influx of Sytox Green into bacterial cytoplasm

7.2.3.1 Preparation of dye stock solution

A five ml stock solution of 0.5 µM Sytox Green stain (S7020, Invitrogen; Molecular Probes, Life Technologies Ltd, Paisley, UK.) was prepared with distilled water in McCartney bottle wrapped in an aluminium foil. The solution was stored in the dark at -20 ^oC until required; therein, suitable sub-aliquots of the stock required to obtain the desired final concentration were withdrawn.

7.2.3.2 Preparation of experimental sample

A 60 ml volume of stock bacterial suspension containing 1 x 10^8 cfu/ml bacterial density in 0.9 % $^w/_v$ sodium chloride was prepared as previously described in section 2.4. The suspension was adjusted to 5 x 10^7 cfu/ml with same medium and treated with a suitable volume of stock test antibacterial compound solution to obtain a final 20 ml aliquot that was immediately incubated in a Clifton water

bath pre-set at 37 ^{o}C and 100 rpm. One millilitre samples were collected at 0, 15, 30, 60, 120, 180, 240 and 1440 minutes, centrifuged at 12,000 rpm for 2 minutes and the supernatant carefully removed. The recovered bacterial pellets were re-suspended in fresh 1 ml volumes of the same buffer followed by the addition of 100 µl volume of stock 5 µM of Sytox Green stain and kept in the dark for 15 minutes. Untreated control samples and bacterial suspensions treated with established membrane active agents used as controls were included. The fluorescence change for the entire cell suspensions over time were measured using a 1 ml volume glass cuvette to take readings in a Perkin Elmer LS Luminescence Spectrometer, set to take readings at excitation 488 and emission 523 nm. This method was adapted from protocols published by Mark, Vocadlo and Oliver 2011 and Hancock 2001. At the same sampling time intervals indicated above, 100 µl volumes of the bacterial suspension were removed for cell viability determination by colony counting technique previously described in sections 2.3.3, 2.3.4 and 2.4.2. Both assays were conducted in duplicate and repeated on three separate days.

Subsequent fluorescence assays were conducted using a protocol designed for the DTX-880 micro-titre plate reader that was pre-calibrated to take readings at 37 ^{o}C, excitation 488 and emission 535 nm. Wells were prepared containing a final 200 µl volume of 5 x 10^{7} cfu/ml suspensions of treated and untreated bacteria that were stained with a final strength of 0.25 µM of Sytox Green with readings taken every 15 minutes for 24 hours. Assays were conducted in duplicate and repeated on three separate days (n = 6).

7.3 Results

7.3.1 Assessment of bacterial membrane potential depolarisation

7.3.1.1 Fluorimetry analysis of effect of test compounds upon membrane potential

The ability of either of the chalcone-derivatives applied alone and in combination with polymyxin B to induce membrane depolarisation in bacteria was investigated and reported in figures 7.1 and 7.2.

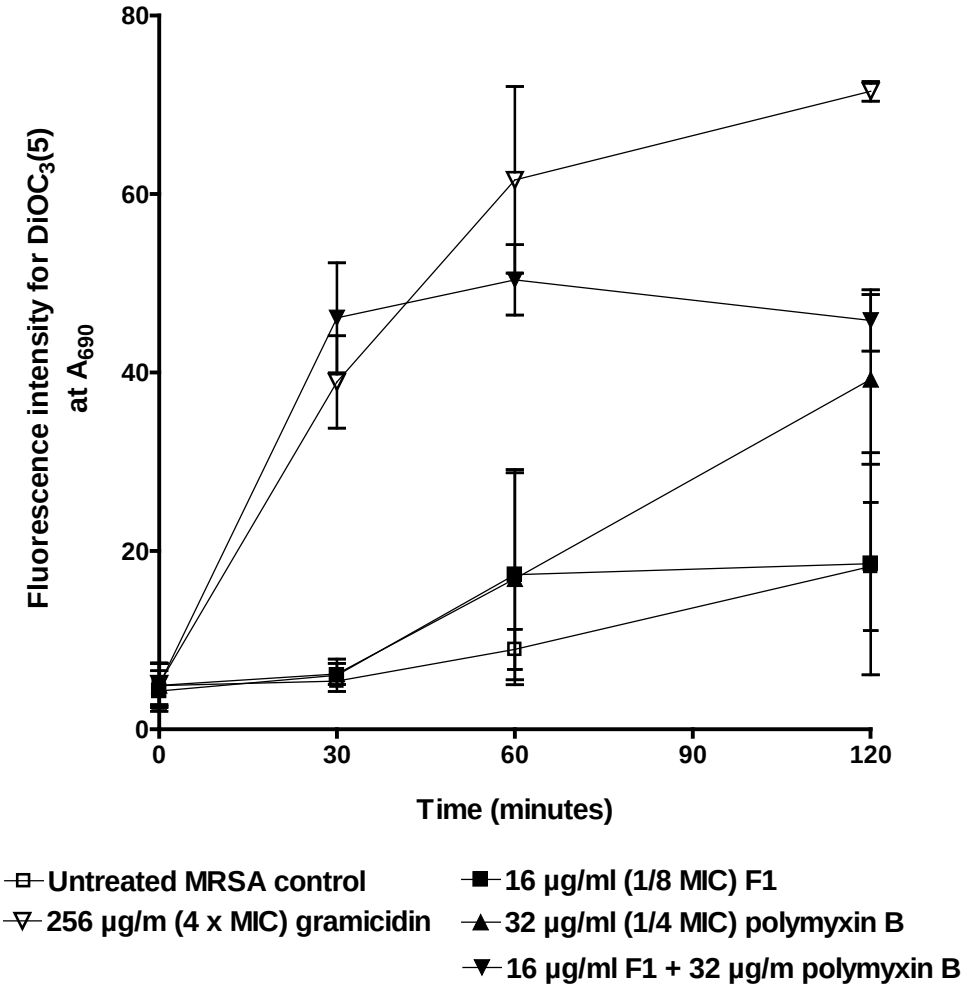


Figure 7.1: Depolarisation of *S. aureus* NCTC 11940 membrane potential by combined sub-inhibitory concentrations of F1 with polymyxin B in the presence of $DiOC_3(5)$. The data presented were obtained from assays conducted in duplicate and repeated on three separate days (n = 6).

The concentrations of the compounds applied in this assay were chosen based on guidance from the previously conducted micro-broth dilution and chequerboard assays (tables 4.1 and 4.2) and were examined for membrane bilayer dissipation. As would be expected, cell suspensions exposed to 4 x MIC gramicidin (section 3.3.4), a membrane active ionophore used as the positive control, displayed the highest extent of staphylococcal membrane depolarisation in both charts. Similarly, the activity recorded in samples treated with polymyxin B were comparable to those of the untreated bacterial suspension within the first 30 minutes but subsequently increased steadily. In both instances, cell suspension exposed to single applications of 32 µg/ml of this polypeptide demonstrated a degree of membrane dissipation equivalent to 50 % after 120 minute, in comparison to the activity recorded for the positive control.

The data recorded in figure 7.1 show that 16 µg/ml (1/8 MIC F1) caused no overall alteration to the membrane potential of the exposed cells, in comparison to the untreated control. But the combination of 16 µg/ml F1 with 32 µg/ml polymyxin B (previously seen to be bactericidal in table 4.1a in both a chequerboard and MBC-determination assays), demonstrated a membrane depolarisation rate that was slightly higher than that effected by gramicidin for the first 30 minutes, although at 120 minutes, this was about 30 % lower in extent. Therefore, although polymyxin B demonstrated a notable extent of the cell membrane bilayer depolarisation, the combination of this polypeptide with F1 displayed a much faster onset and an enhanced extent.

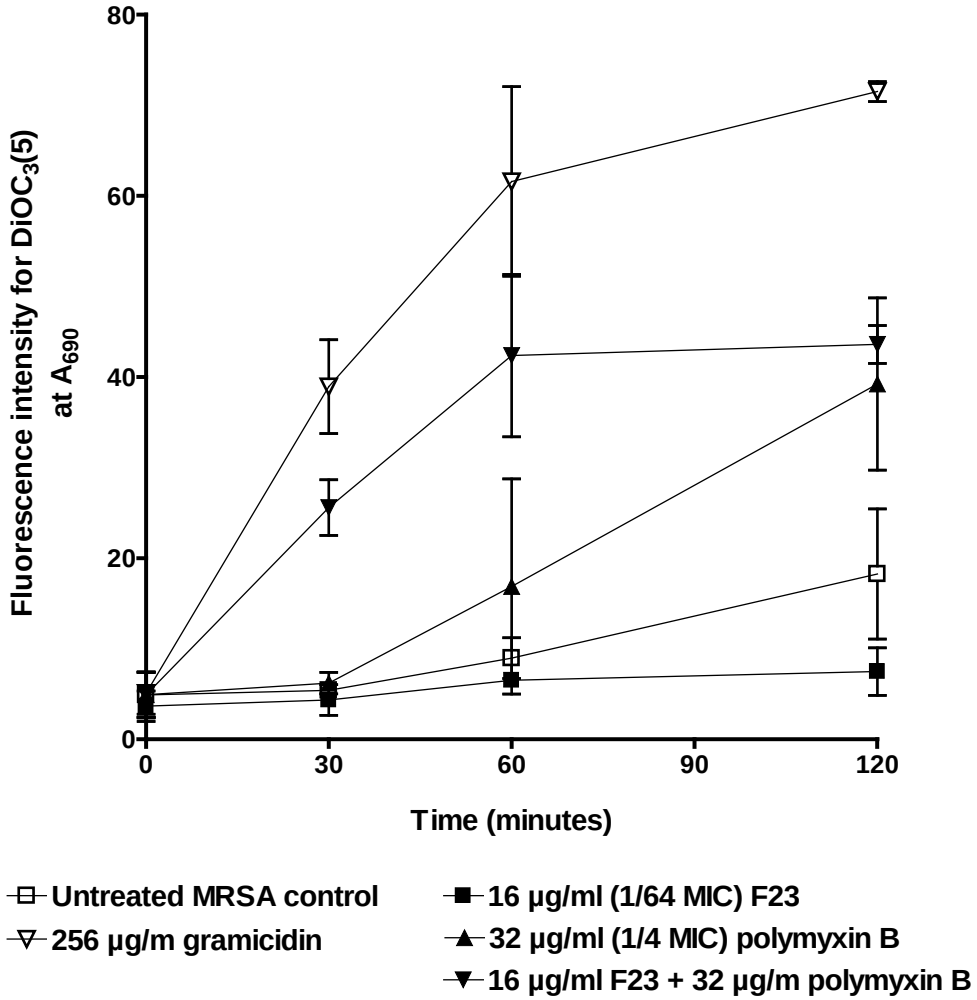


Figure 7.2: Depolarisation of *S. aureus* NCTC 11940 membrane potential by combined sub-inhibitory concentrations of F23 with polymyxin B in the presence of $DiOC_3$ (5). The data presented were obtained from assays conducted in duplicate and repeated on three separate days (n = 3).

The data presented in figure 7.2 also show that 16 µg/ml (1/64 MIC) F23 displayed no impact upon the MRSA membrane potential for the first 30 minutes.

This concentration of the chalcone compound later demonstrated an overall extent of bilayer potential dissipation of about 20 %, in comparison to the positive control. A combined application of both compounds at the same stated concentrations above (equivalent to half their synergistic concentrations recorded in table 4.2), exhibited a much more rapid onset of action than the sub-inhibitory concentration of polymyxin B alone but a lower rate and extent (about 45 %) of action than gramicidin D.

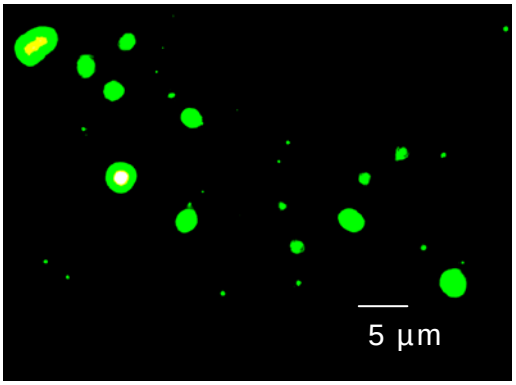
7.3.1.2 Membrane potential examination using microscopy

Morphological assessment using fluorescence microscopy was used to complement the data obtained from the fluorimetry assay above. But to guarantee that dye-uptake occurred during the dark-room incubation period, an initial examination was undertaken.

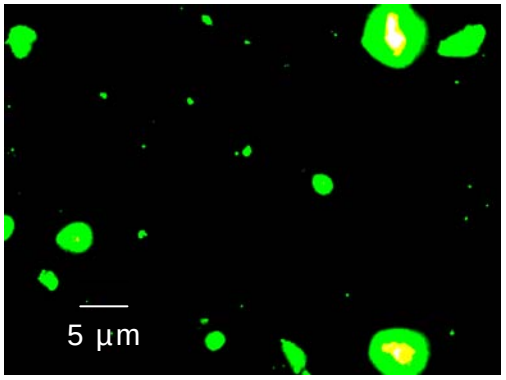
7.3.1.2.1 Verifying dye-uptake by stained cells

In order to ascertain that cells in bacterial suspension treated with $DiOC_3(5)$ experienced dye-uptake and aggregation, a comparative view of *S. aureus* 11940 suspensions stained with this stain, as well as the intact-cell membrane impermeable Sytox Green was undertaken, after the 15 minutes incubation period, using an inverted fluorescence microscope (Leica DMI 4000B, Leica, Wetzlar, Mannheim, Germany) and recordings were taken with an attached camera (Leica DFC 300 FX, Leica, Wetzlar, Mannheim, Germany). The data obtained are presented in figure 7.3.

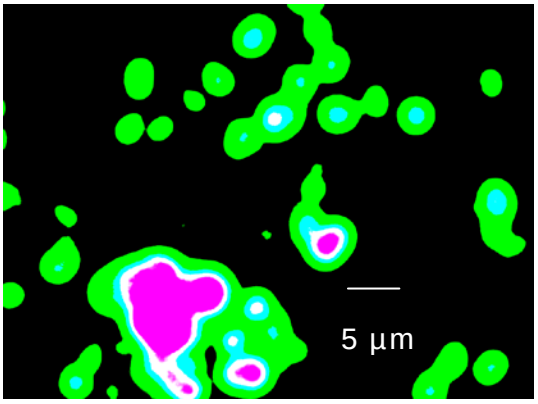
The images in 7.3.1a,b suggests Sytox Green was unable to permeate the MRSA cell membrane whilst those in 7.3.1 c,d suggests apparent intracellular dye-uptake and aggregation for $DiOC_3(5)$ treated cells.



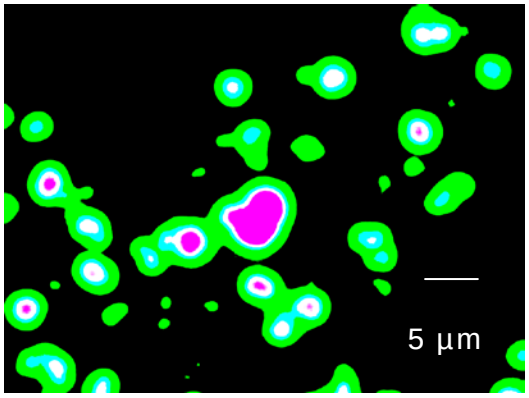
7.3a: MRSA cells stained with Sytox Green



7.3b: MRSA cells stained with Sytox Green



7.3c: Untreated MRSA cells stained with $DiOC_3(5)$



7.3d: MRSA cells stained with $DiOC_3(5)$.

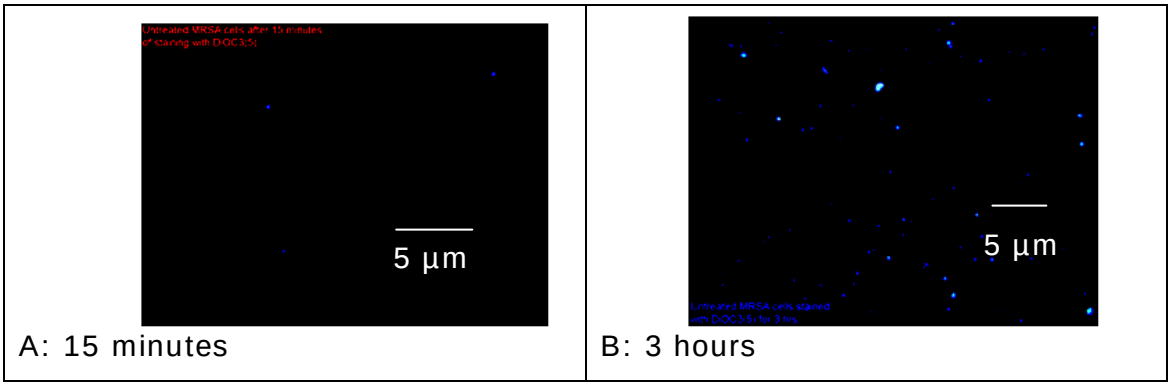
Figure 7.3: Inverted fluorescence microscope images taken at x600 of cells stained with (a,b) Sytox Green (c,d) $DiOC_3(5)$ after 15 minutes dark-room waiting period.

7.3.1.2.2 Examining changes in fluorescence intensity due to the effect of test compounds in bacterial cells

Polymyxin B, each of the chalcone-derivatives applied alone, as well as together with the polypeptide antibiotic and gramicidin D as a control agent, were used to challenge defined *S. aureus* 11940 populations that were subsequently examined for changes in fluorescence intensity using a time-lapse microscopy technique.

7.3.1.2.3 Time-course assessment of changes in fluorescence intensity for stained untreated cells

Stained but unchallenged cells were initially viewed over time for morphological changes with a fluorescence microscope. These control experiments were essential to avoid ascribing morphological and structural changes that may occur due to cell degradation over time as well as defects that were artefactual in origin, to the effect of applied agents. The data obtained when untreated MRSA suspensions were simply exposed to $DiOC_3$ over time is recorded in figure 7.4. Despite the heavy biomass, the stained cells were barely visible under the fluorescent microscope at 15 minute and up to the second hour. As previously stated, the cells were viewed at intervals within 4 hours and the recordings taken at 15 minute and 3 hours are presented in figure 7.4.



A: 15 minutes

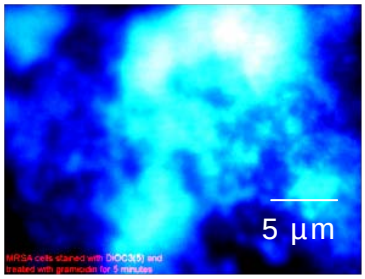
B: 3 hours

Figure 7.4: $DiOC_3(5)$ stained and untreated MRSA cells after (a) 15 minutes (b) 3 hours. Images were taken at x400 using a fluorescence microscope.

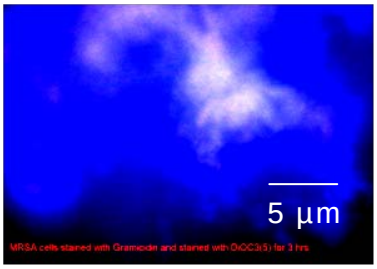
This observation suggests the dye was not emitting due to lack of adequate uptake and dissipation probably due to the existence of a fully functional cytoplasmic membrane bilayer with an intact proton motive force. At the third hour (figure 7.4b), the cells fluoresced slightly as their cells became relatively more visible suggesting the membrane potential of unchallenged MRSA were slowly dissipated over time, enabling the desegregation and fluorescence of previously accumulated stain.

7.3.1.2.4 Changes in fluorescence intensity over time for stained gramicidin-treated cells

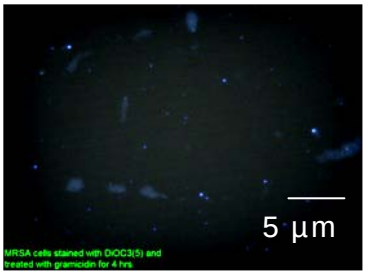
S. aureus stained and challenged with gramicidin were overwhelmingly depolarised after 5 minutes (figure 7.5) leading to the formation of hazy clumps of (colonies) cell.



A: 5 minutes



B: 3 hours



C: 4 hours

Figures 7.5: $DiOC_3(5)$ stained MRSA cells treated with 256 µg/ml gramicidin for (a) 5 minutes (b) 3 hours and (d) 4 hours respectively. Images were taken at x400 using a fluorescence microscope.

The degree of intensity of the stain fluorescence diminished gradually after 2 hours. After 4 hours, the data presented is suggestive of cells displaying a complete collapse of fluorescence, probably due to absolute disintegration of the previously aggregated stain. The accumulated stain disintegrates in response to changes in the cell membrane potential and confirms gramicidin's capacity to effectively dissipate the proton motive force, as well as to be used as a control agent (Penyige et al. 2002).

7.3.1.2.5 Time-Kinetic changes in fluorescence intensity for stained *S. aureus* treated with polymyxin B

The data presented in figure 7.6 is for MRSA stained with $DiOC_3(5)$ and treated with single applications of the polypeptide antibiotic polymyxin B.

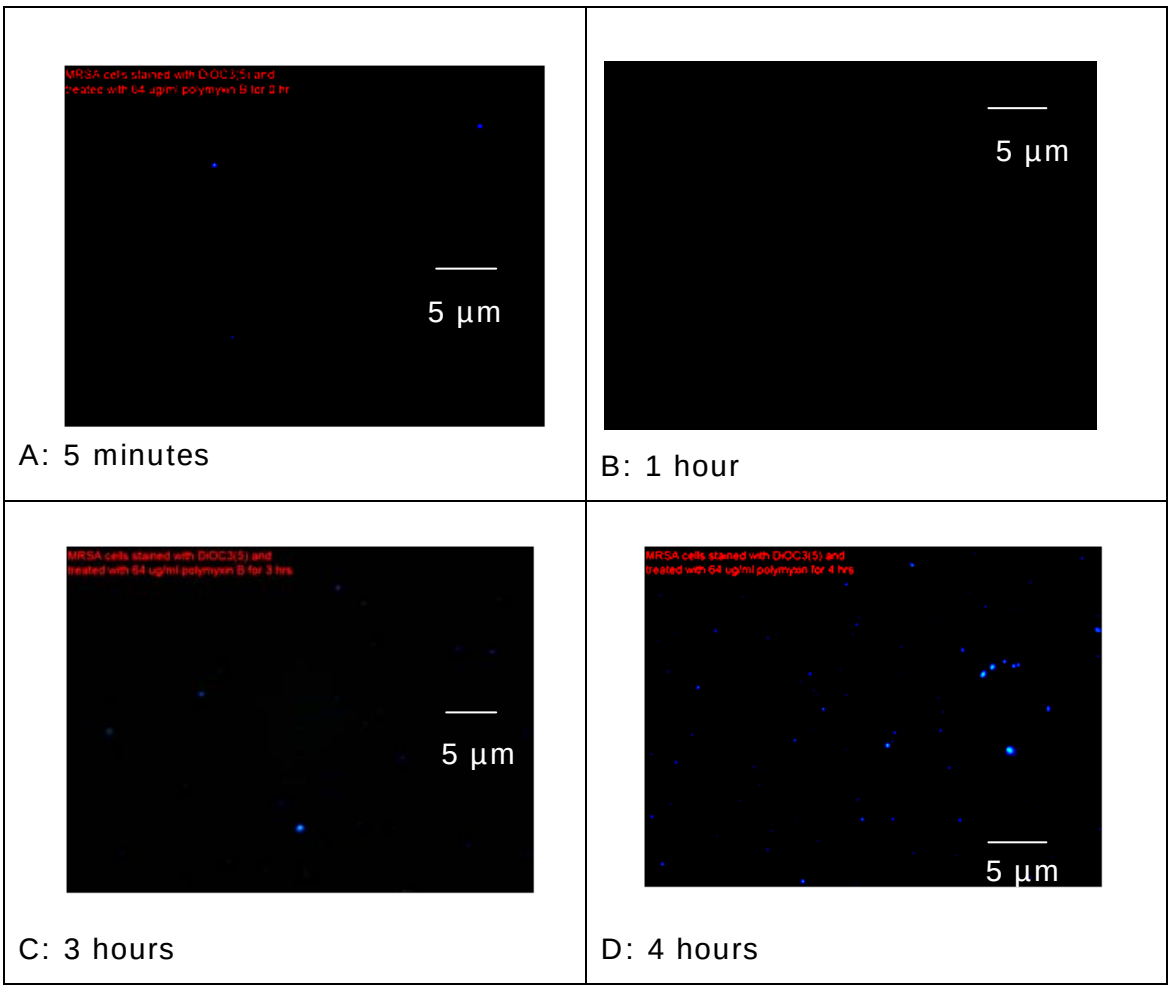


Figure 7.6: $DiOC_3(5)$ stained MRSA cells treated with 64 µg/ml polymyxin B for (a) 5 minutes (b) 1 hour (c) 3 hours and (d) 4 hours respectively. Images were taken at x400 using a fluorescence microscope.

The data suggests the dye accumulated and fluoresced within the treated cells after 1 hour. It was however noticed that the recorded intensity was increasing with time and that the cells appeared depolarised individually after 4 hours. The recorded intensity is however much less than that reported for gramicidin D-treated MRSA, suggesting that the capacity of polymyxin B to dissipate cell membrane potential is lower than that of gramicidin D.

7.3.1.2.6 Time-course changes in fluorescence intensity for bacterial cells stained and challenged with both F1 and F23 chalcone-derivatives

The data shown in figure 7.7 is for MRSA cells that were stained and treated with 32 µg/ml of either F1 or F23 and examined for membrane depolarisation evidenced by dye fluorescence.

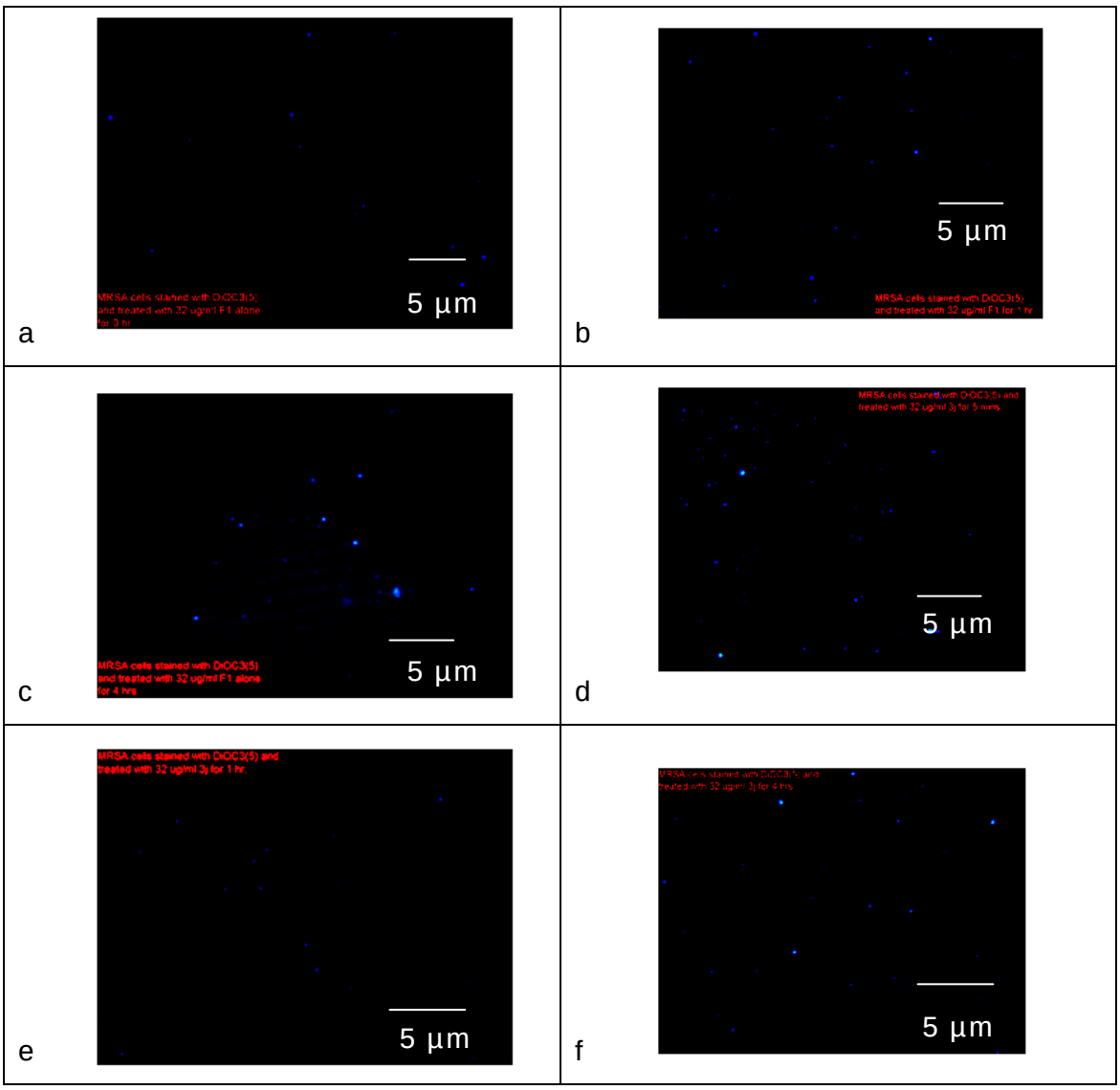


Figure 7.7: $DiOC_3(5)$ stained MRSA cells treated with 32 µg/ml F1 for (a) 5 minutes (b) 1 hour and (c) 4 hours; as well as $DiOC_3(5)$ stained MRSA cells treated with 32 µg/ml F23 for (d) 5 minutes (e) 1 and (f) 4 hours. Images were taken at x400 using a fluorescence microscope.

All the treated samples showed weaker degrees of fluorescence indicating a lower capacity of the semi-synthetic agents to depolarize the bilayer barrier to promote the intracellular disaggregation and fluorescence of the stain. The degrees of observable fluorescence with time appear to be similar to that displayed by the untreated bacterial suspension up to the second hour, indicating the absence of membrane dissipation.

7.3.1.2.7 Monitoring time-course changes in fluorescence intensity for for stained cells challenged with F1 in combination with polymyxin B

The ability polymyxin B when applied with F1 to dissipate the membrane potential of MRSA was re-examined using a fluorescence microscope in the presence of $DiOC_3(5)$ stain. The data obtained is recorded in figure 7.8.

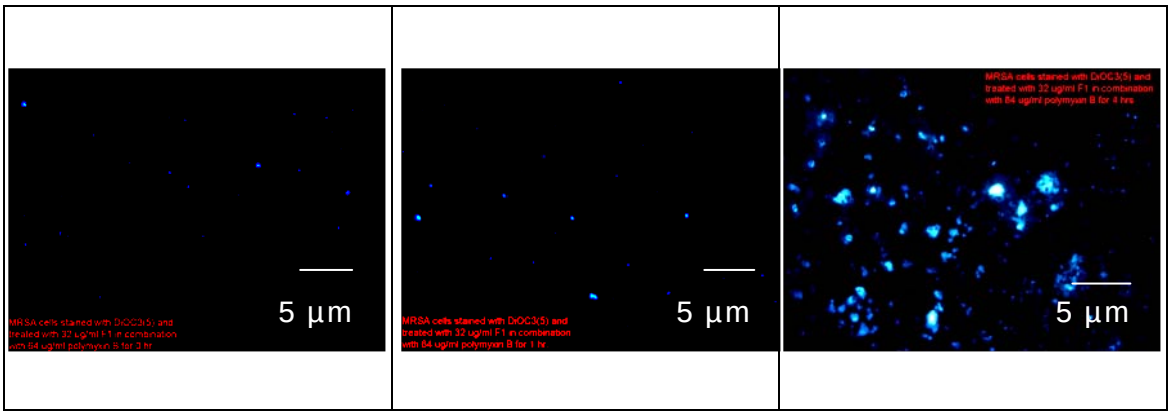


Figure 7.8: $DiOC_3(5)$-stained MRSA cells treated with 32 µg/ml F1 applied with 64 µg/ml polymyxin B for (a) 5 minutes (b) 1 hour and (c) 4 hours. Images were taken at x400 using a fluorescence microscope.

At the applied concentrations, the combined agents induced a significant degree of membrane dissipation and dye fluorescence that became very pronounced over time (figures 7.8), being very obvious at the 4th hour (figure 7.8c). The intensity of fluorescence caused by the combination of both compounds surpassed those seen in samples treated with either polymyxin B (figure 7.6) or any of the chalcone-derivatives when applied alone (figures 7.7). This data therefore show that the application of both agents demonstrated an enhanced capacity to dissipate MRSA membrane potential by the inducement of a degree of stain fluorescence that surpasses those produced by either of the agents' individual applications.

7.3.1.2.8 Morphological time-kinetics for cells stained and treated with both F23 and polymyxin B

MRSA was stained with the same dye and challenged with 64 µg/ml of the polypeptide antibiotic with 32 µg/ml of F23, the second chalcone compound that was examined in this project. These concentrations represent the strengths that were effective synergistically against the same bacterial species in both a chequerboard and MBC-determination assays (as recorded in table 4.2). The

data obtained from the examination of the cells using a fluorescence microscope is presented in figure 7.9.

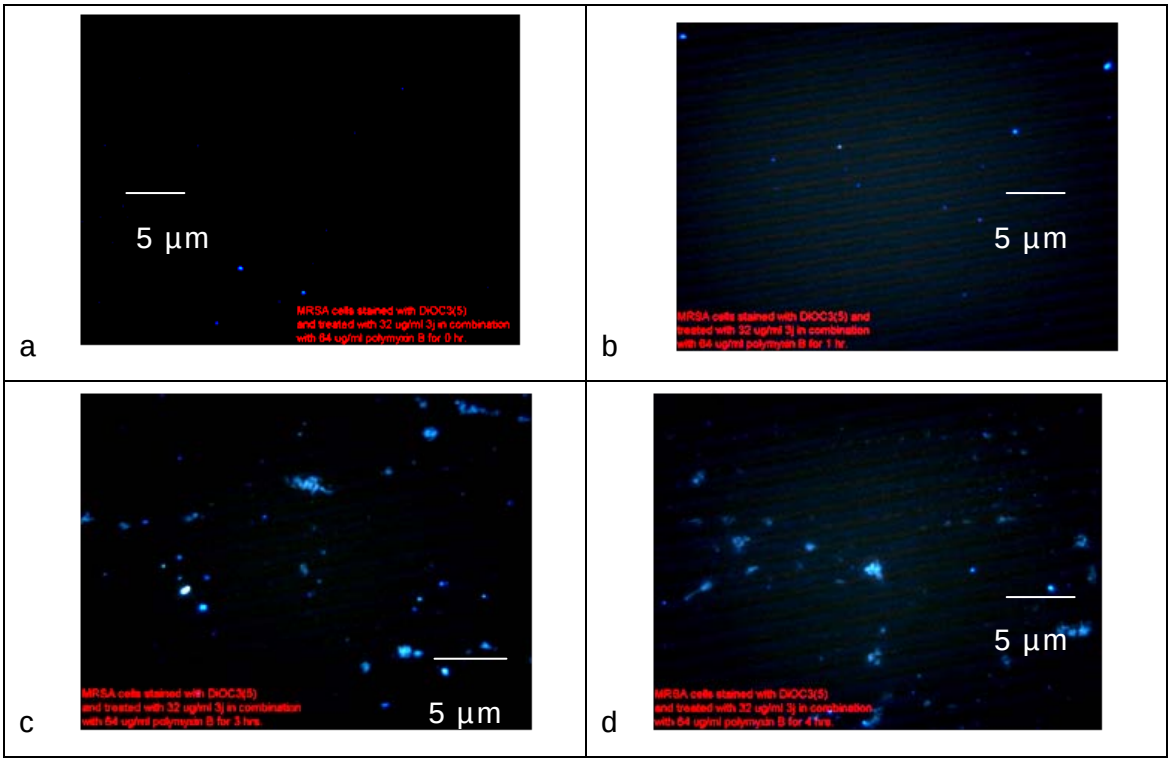


Figure 7.9: $DiOC_3(5)$ stained MRSA cells treated with 32 µg/ml F23 with 64 µg/ml polymyxin B for (a) 5 minutes (b) 1 hour (c) 3 hours and (d) 4 hours. Images were taken at x400 using a fluorescence microscope.

In comparison to the action of the polypeptide applied with F1, this latter combination demonstrates a lower degree of fluorescence. In this case, the induced fluorescence made the cells more visible after 3 (figure 7.9c) and up to after 4 hours (figure 7.9d) respectively. The data presented in both figures is strongly suggestive of cells with depolarised membrane potential and fluorescence especially after 3 and 4 hours.

7.3.1.2.9 Summary of data findings from microscopic changes in cells fluorescence intensity

Another comparative analysis of the data in this section indicates the untreated and polymyxin B–treated cells were seen to undergo degrees of depolarisation over time, producing cells with dissipated membrane potentials that appeared as distinguishable colonies. On the other hand, gramicidin treated cells were recorded as clumped up and hazy colonies, indicating wide-spread dissipation of

their membrane potential; whilst cells treated with combinations of polymyxin B with the chalcone-derivatives were recorded as having both distinct and smaller diameter hazy-clumped, dissipated colonies, indicating a lesser degree of depolarisation than gramicidin-treated control. Cell suspensions treated with F1 and polymyxin B displayed a greater degree of their membrane-bilayer dissipation than those treated with the combination of F23 with polymyxin B. Cell suspensions stained with the dye and treated with either F1 or F23 alone did not show signs of any significant dissipation of their membrane potential after 4 hours as illustrated in the data previously presented in figure 7.7.

7.3.2 An assessment of the effect of selected compounds upon bacterial membrane integrity through measurement of leaked intracellular K^+

7.3.2.1 Effect of benzalkonium chloride upon bacterial membrane integrity

In order to verify the approach using the flame emission spectrophotometer, the rate of potassium ion leakage from a *S. aureus* 6571(MSSA) population challenged with benzalkonium chloride in ultra-pure de-ionised water was assessed and the data obtained is shown in figure 7.10.

The assay results give an indication of the rate and quantity of leakage of intracellular K^+ from the bacterial cells due to the membrane activity of this quaternary ammonium compound. The MSSA samples treated with 4 and 20 µg/ml benzalkonium chloride demonstrated a rapid and profound onset of cell membrane disruption evidenced by the quick release of intracellular potassium ions. The MSSA suspension challenged with 20 µg/ml of this antimicrobial agent was seen to display the highest level of intracellular cation release and was subsequently used as the positive control during the evaluation of the activity of polymyxin B with the chalcone-derivatives.

The untreated bacterial sample released about 0.3 µg/ml intracellular potassium ions after 5 as well as 15 minutes and the amount of released cations increased to about 0.5 µg/ml after 60 minutes.

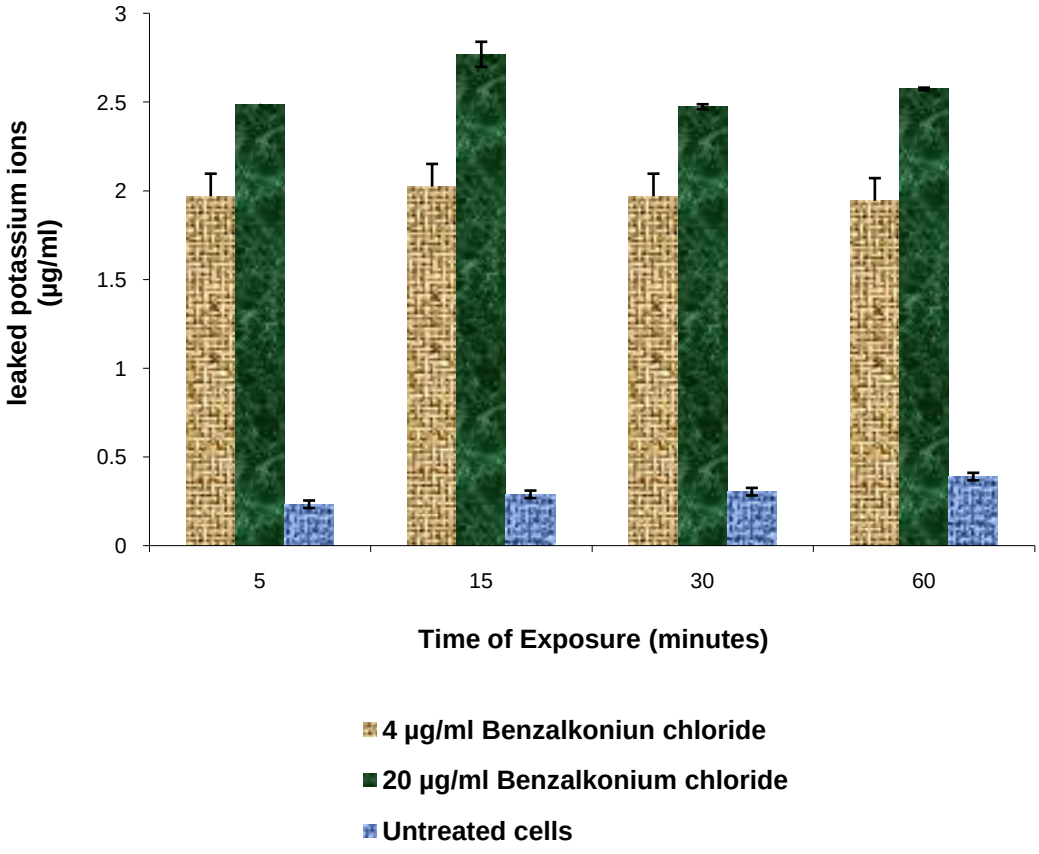


Figure 7.10: Intracellular potassium ion leakage from 1 x 10^8 cfu/ml *S. aureus* 6571 cells treated with benzalkonium chloride and analysed with the flame emission analysis. These data represent the mean result from three independent assays conducted in duplicate.

The release of intracellular potassium ion from bacterial samples that were not challenged is an indication that the cations were either undergoing diffusion from the intracellular space due to concentration gradient or membrane bilayer perturbation since the cells were suspended in de-ionised water rather than a buffer solution.

A complementary plate count determination was undertaken (figure 7.11) and demonstrates a rapid as well as profound bactericidal activity for 20 µg/ml benzalkonium chloride against the inoculum population. After 30 minute, this concentration of quaternary ammonium compound had caused a complete loss of viability to the exposed population. This observation is corroborated by the fact that these samples suspensions displayed a sustained maximum release of potassium after this same period in figure 7.10 as detected by the flame photometer. The bacterial sample challenged with 4 µg/ml benzalkonium chloride demonstrated an approximately 75 % release of intracellular potassium ion

(figure 7.10) and a reduction of their cell colony count from 8 to 6 log cfu/ml (an approximate loss of 10 million colonies) after 60 minutes (figure 7.11).

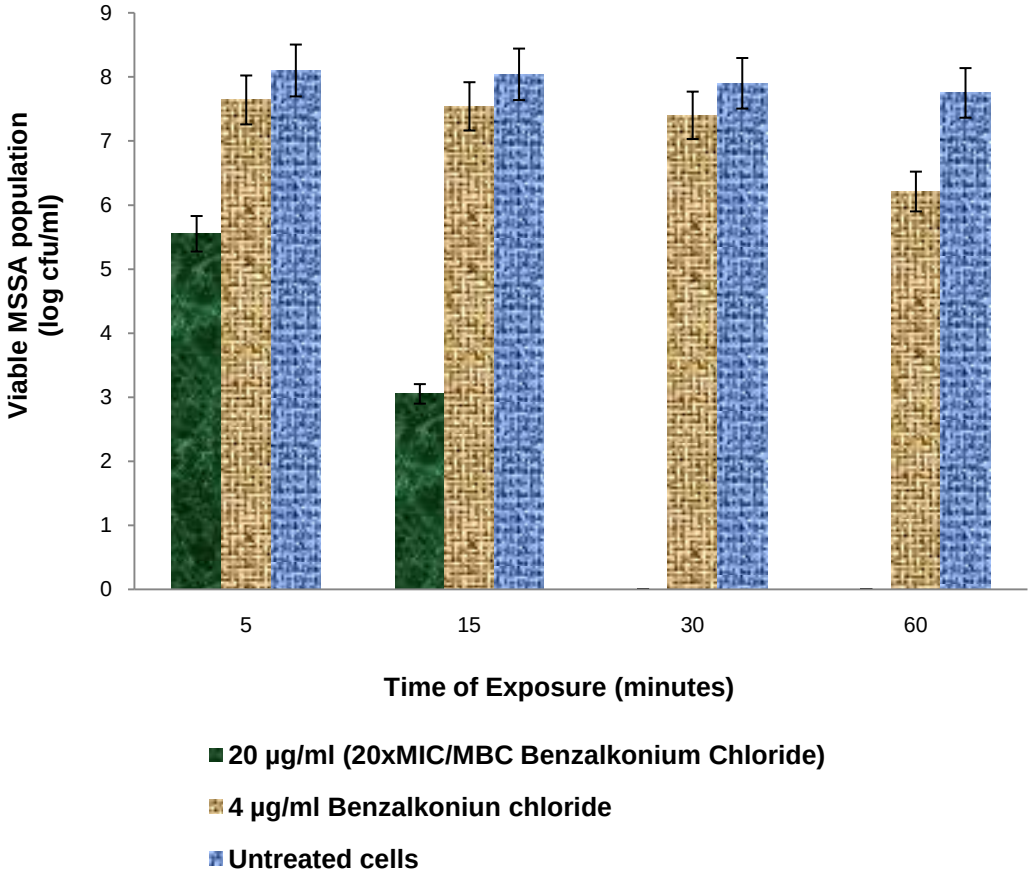


Figure 7.11: Time-kinetic viability assay for the 1 x 10^8 cfu/ml *S. aureus* 6571 suspension exposed to concentrations of the quaternary ammonium compound. Samples for the latter analysis were withdrawn concurrently with those used for the flame emission analysis. These data represent the mean result from three independent assays conducted in duplicate.

Both data appear to suggest that the degree of membrane bilayer disruption induced by this latter concentration of benzalkonium chloride was lesser than that caused by 20 µg/ml of this same agent. The membrane activity of the quaternary compound was therefore concentration-dependent and the degree of cell cytoplasmic bilayer damage was reflected by the percentage of potassium ion loss and mirrored by the extent of loss in cell viability. Future time course viability studies with benzalkonium chloride should be conducted with a neutraliser such as tween 80 in order to avoid potential drug carry-over effect. The untreated cells suspended in de-ionised water did not demonstrate appreciable loss in viability probably due to the short duration of the assay.

7.3.2.2 Effect of polymyxin B with the chalcone-derivatives upon cell membrane integrity

The data presented in figures 7.12 and 7.13 show the rate and extent of leakage of potassium ions leaked from MSSA populations challenged with polymyxin alone and in combination with F1. The release of 4 µg/ml K^+ from the 20 µg/ml benzalkonium chloride-treated control population indicates an increase of 1.5 µg/ml over that previous value obtained from a lower MSSA density of 1 x 10^8 cfu/ml that was treated with the same concentration of this quaternary ammonium compound.

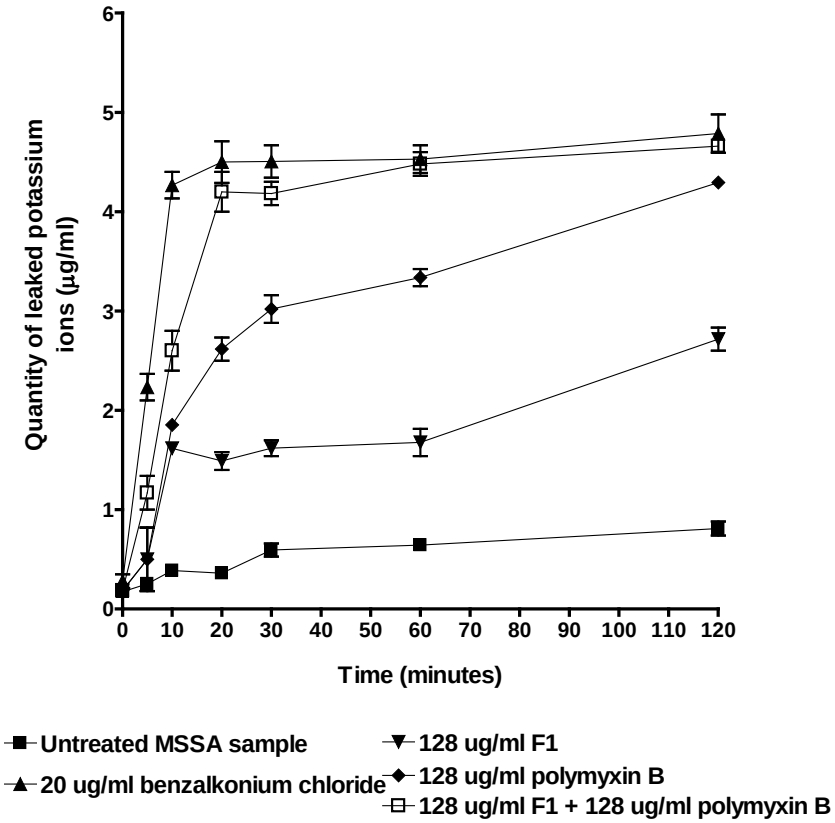


Figure 7.12: Inducement of potassium ion leakage in *S. aureus* 6571 populations challenged with 128 µg/ml of both F1 and polymyxin B. The cell density used was A_{500} 0.50 MSSA that was washed once and re-suspended in ultra pure deionised water. Upon agar plate count, this inoculum population was equivalent to ~5.25x10^8 cfu/ml. The data represent the mean result from three separate experiments that were conducted in duplicate, n = 6.

This observation indicates an increase in the population of the challenged bacterial suspension leads to an increase in the amount of leaked potassium. In comparison to the untreated bacterial suspension, MSSA challenged with only polymyxin B displayed a release of 45, 75 and 82 % K^+ after 10, 20 and 60

minutes respectively whilst another aliquot suspension treated with a single application of F1 released intracellular ions to an extent of 32, 32 and 50 % (figure 7.12). An equivalent bacterial population challenged with F1 and polymyxin B together showed an enhanced leakage of potassium ions. This cell population released about 60, 75 and 100 % of potassium ions at the defined time periods above. As noted previously in the calibration assays with control agents, the MSSA suspension in de-ionised ultra-pure water also released some amount of K^+, probably due to the cation concentration gradient and some degree of membrane perturbation. An analysis from 2 way ANOVA with the Bonferroni post test show that at $p > 0.05$ there was no significant difference between all the treated samples and the untreated control at time 0 but a significant difference existed for both 20 µg/ml benzalkonium chloride and F1-polymyxin B treated samples between 5 and 120 minutes of the assay at $p < 0.0001$. However, both samples treated with polymyxin and F1 separately also demonstrated a non-significant difference in comparison to the untreated control after 5 minutes, with $p > 0.05$.

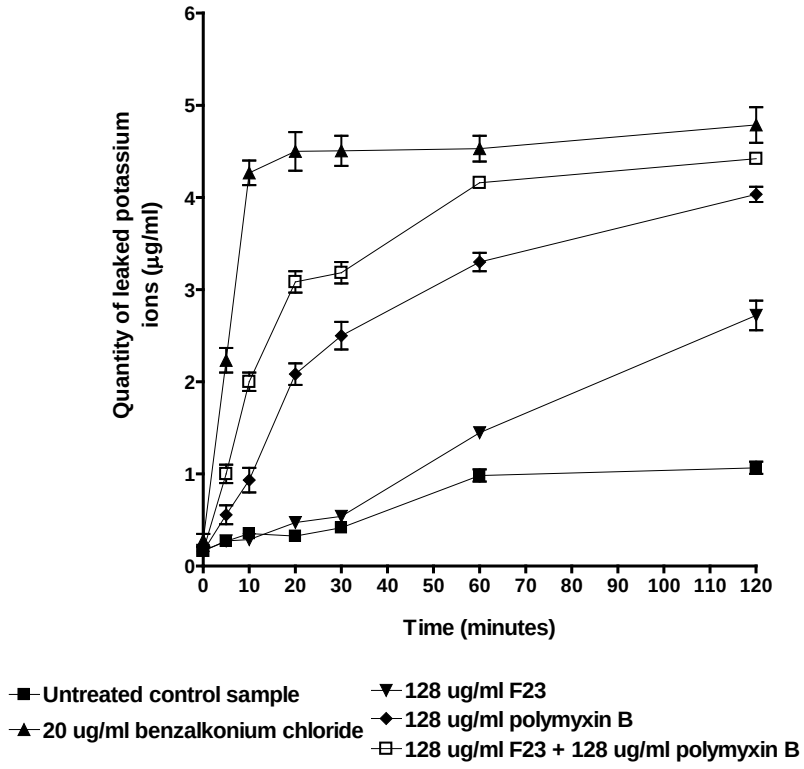


Figure 7.13: Inducement of potassium ion leakage in *S. aureus* 6571 populations challenged with 128 µg/ml of both F23 and polymyxin B. The cell density used was A_{500} 0.50 MSSA that was washed once and re-suspended in ultra pure deionised water. Upon agar plate count, this inoculum population was equivalent to ~5.25×10^8 cfu/ml. The data represent the mean result from three separate experiments that were conducted in duplicate, n = 6.

In figure 7.13, the amount of potassium ions released by bacterial suspension treated with 20 µg/ml benzalkonium chloride was also used as the reference.
MSSA population challenged with a single application of 128 µg/ml F23 displayed a released 8, 12.5 and 37.5 % potassium ions after 10, 30 and 60 minutes respectively. Cells exposed to only 128 µg/ml polymyxin B were seen to have leaked much higher amounts of approximately 45, 75 and 82 % at the same time intervals.
Although cell populations exposed only to polymyxin B, as well as those treated with both compounds were seen to have released about the same amount of the intracellular cation after 20 minutes. The augmentative action of the combined agents induced the release of about 105 % of the cation after 120 minutes whilst cell suspensions treated with only 128 µg/ml polymyxin B leaked only 90 %, but there was a significant difference at $p < 0.0001$ with the use of a 2 way analysis of variance with the Bonferroni post test. The same statistical analysis show that at $p > 0.05$ there was also no significant difference between all the treated samples and the untreated control at time 0 and between F23-treated sample and the latter reference up to after 30 minute with p value >0.05. Menawhile, all the other treated bacterial suspensions expressed a significant difference between 5 and 120 minutes with $p < 0.0001$.
In this assay, bacterial suspensions were prepared in ultra-pure de-ionised water in order to avoid contamination from extraneous particulate materials and dissolved ions as these can interfere with data obtained from flame spectrophotometry. This device is highly sensitive with known capacity to detect microscopic levels of free ions. In comparison to the previous data obtained during the verification of the protocol of the spectrophotometer, after 60 minutes, the untreated sample had no appreciable loss in viability but was seen to have released potassium ion concentration equivalent to 12.5 %. After 120 minutes, this same sample had demonstrated a release of intracellular potassium ion equivalent to 28 %.

7.3.3 Examining cell membrane disruption with fluorimetry analysis

The degree of cell cytoplasmic membrane disruption enforced upon *S. aureus* species challenged with established as well as test antibacterial compounds was assessed with the use of fluorescing dyes in fluorimetry.

7.3.3.1 Calibration of the fluorimeter and testing of the protocol

A calibration of the fluorimetry protocol for assessing bacterial membrane integrity upon the challenge of a given cell population was conducted by evaluating the effect of already established antimicrobial compounds upon MSSA. The data obtained is presented in figure 7.14.

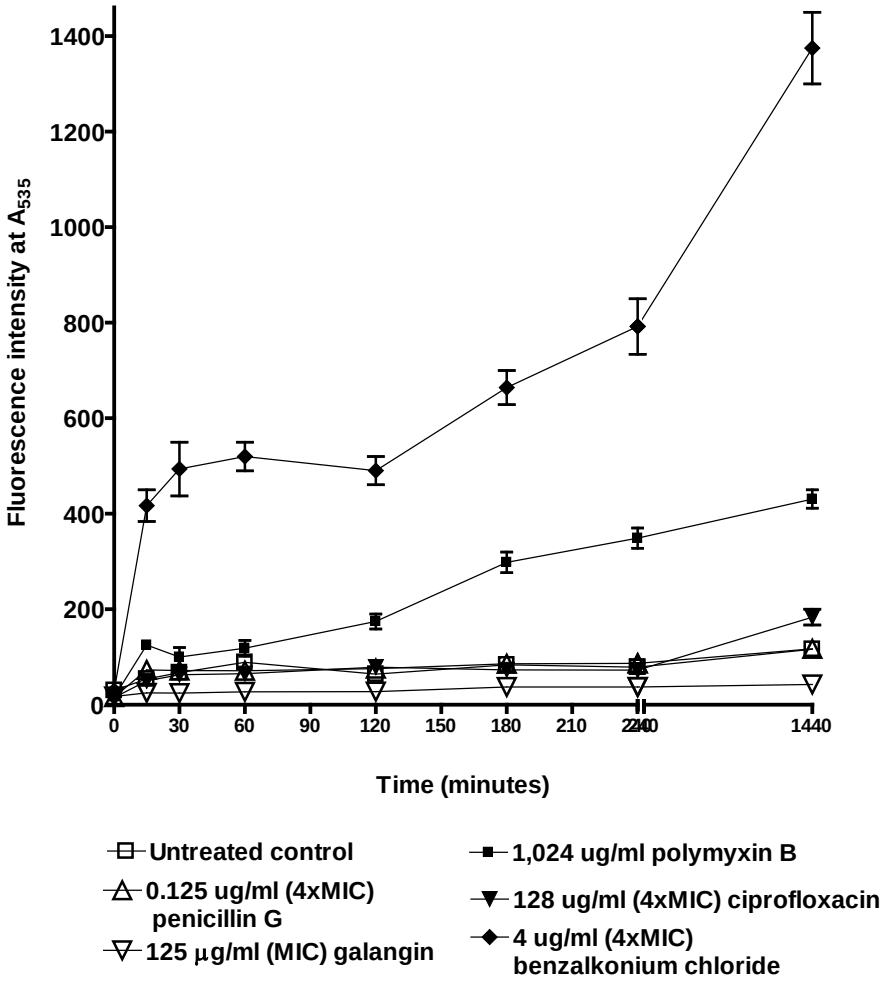


Figure 7.14: An assessment of the impact of representative antimicrobial compounds upon membrane integrity of *S. aureus* 6571 (MSSA) populations. n = 6.

The peak emission demonstrated by MSSA population challenged with benzalkonium chloride after 24 hours was taken to equivalent to a 100 % fluorescence. This quaternary ammonium compound was seen to have displayed a 30, 35 and 55 % emission at 15, 60 and 240 minutes respectively. Polymyxin B displayed 12, 9, 24 and 30 % emission at 15, 30, 60, 240 and 1440 minutes. These data imply both compounds disrupted MSSA cytoplasmic membrane integrity to such an extent that enabled the influx of Sytox Green into the

bacterial cytoplasm for binding to nucleic acid and fluorescing of the dye (Salas-Vidal 2004). On the other hand, MSSA populations challenged with penicillin, ciprofloxacin and galangin displayed emission levels that were below 12 % all through the 24 hours period. In addition, the lowest recorded value of about 1.7 % was displayed by the flavonol compound all through the analysis. This observation suggests the mechanism of action of all of these four compounds is unconnected with disruption of bacterial membrane integrity.

The data presented in figure 7.15 illustrate about a 2.5 log reduction in viability of MSSA populations challenged with the same strengths of galangin, polymyxin B and benzalkonium chloride applied in the examination of membrane integrity, in a complementary assay.

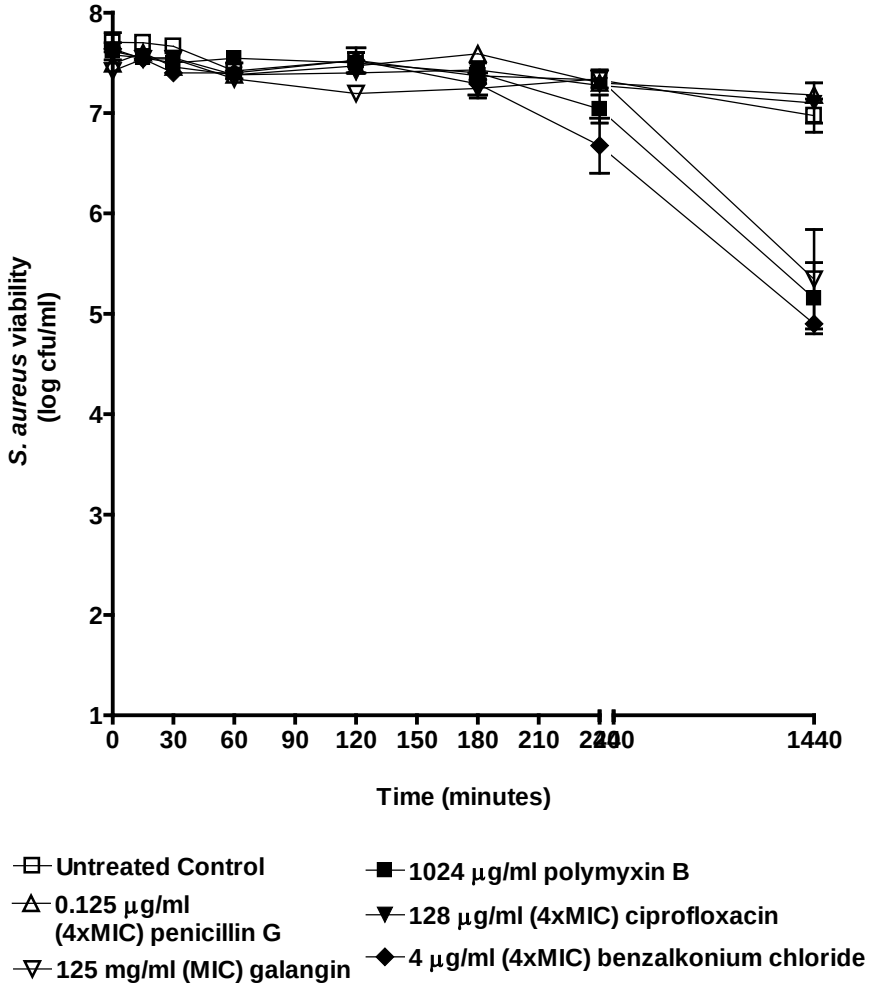


Figure 7.15: Data from a time-course viability assay for the 5 x 10^7 cfu/ml *S. aureus* 6571 (MSSA) populations challenged with 128 µg/ml (4 x MIC) ciprofloxacin, 0.125 µg/ml (4 x MIC) penicillin G, 1.024 mg/ml polymyxin B and 4 µg/ml (4 x MIC) benzalkonium chloride. Samples for viability determinations were collected at the same time as those for the fluorimetry analysis following challenge of the cell sample. The data was mean of values obtained from experiments conducted in duplicate and repeated on three separate days.

This indicates that at the applied concentrations, these three agents were bacteriostatic with about a 500-fold reduction to the high biomass of 5 x 10^7 cfu/ml. The data in figure 7.14 show a rapid onset in the influx of Sytox Green (within 15 minutes) for bacterial populations challenged separately with benzalkonium chloride and polymyxin B. But the data obtained from the time-course viability analysis presented in figure 7.15 suggest that the decline in population of viable cells in these samples were relatively slow in onset and only became notable as from 180 minutes. This observation was despite the occurrence of a prompt as well as steadily increasing membrane disruption, verified by influx of Sytox Green into cells treated with benzalkonium chloride and polymyxin B in figure 7.14, suggesting that membrane disruption may not always translate to an immediate loss in viability. Similar to the data previously presented in figure 7.10 for the leakage of intracellular potassium ions, *S. aureus* suspension exposed to 4 µg/ml (4 x MIC) benzalkonium chloride demonstrated a notable influx and fluorescence of the intact-membrane impermeable stain, as well as a 2.5 log cfu/ml loss in viability after 24 hours. Both information verified the previous data that this concentration of the quaternary ammonium compound disrupted the cytoplasmic membrane bilayer of *S. aureus*, permitting the leakage of potassium ions and in addition gives an insight into the degree of assault upon the membrane bilayer by enabling the intracellular influx, binding and fluorescing of the cationic stain (molecular weight 600 g/mol (Saugar et al. 2006)) to polyanionic nucleic acids. The information also verifies the suitability of the use of this protocol for examination of the membrane activity of the synergistic action of the combined application of polymyxin B with the chalcone-derivatives.

7.3.3.2 Assessing the degree of membrane disruption caused by polymyxin B with the chalcone-derivatives

The fact that an application of polymyxin B with the chalcone-derivatives caused cytoplasmic membrane disruption in *S. aureus* has been evidenced by the leakage of intracellular potassium ions. But the degree of this damage still needs to be verified since this cation is considered to be small in size, having a molecular weight of 32 g/mol. The effect of the combined application of the

polypeptide antibiotic with either of the chalcone derivatives upon bacterial membrane integrity was determined by quantifying the intra-cellular influx of Sytox Green over time, after challenging *S. aureus* suspensions with defined strengths of both compounds. As stated previously, this stain with a molecular weight of about 600 g/mol is unable to permeate intact cytoplasmic membranes and would fluoresce only upon binding to nucleic acid materials (Vives-Rego, Lebaron and Nebe-von Caron 2000). The data presented in figures 7.16 to 7.18 show the effect of various concentrations of control as well as single and combined applications of test compounds upon the membrane integrity of equivalent MSSA populations, measured as a reflection of the intensity of Sytox Green emission.

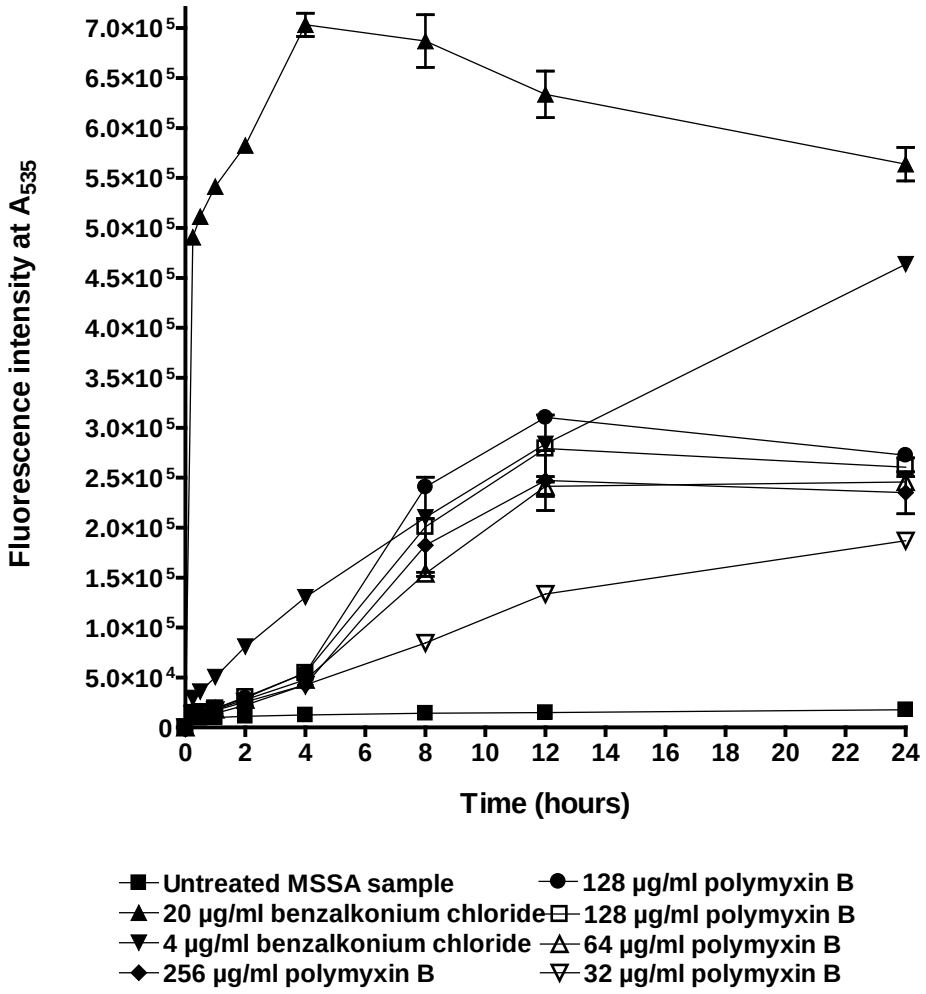


Figure 7.16: Determination of the membrane integrity of *S. aureus* 6571 (MSSA) challenged with concentrations of benzalkonium chloride, polymyxin B, F1 alone and F1 applied in combination with polymyxin B at varying strengths. All data are means of duplicate experiments that were repeated three times on separate days. Statistical evaluations were by 2-way ANOVA and post hoc investigations were by Bonferonnis test of multiple comparisons where all the samples were analysed relative to the untreated control at $p < 0.001$.

The data presented from all three figures suggest Sytox Green was unable to permeate the membrane of untreated MSSA reflected by the near-zero level emission perpetually recorded from this sample all through the assay period. In figure 7.16, the 4 µg/ml benzalkonium chloride treated bacterial suspension demonstrated a gradual incremental uptake of the dye whilst cell suspension treated with 20 µg/ml of this cationic surface active agent shows a pronounced and rapid onset of emission within 5 minutes indicating a very substantial disruption of the cell membrane by this cationic surface active agent, thereby permitting the rapid intracellular influx of the stain. The data from subsequent assays were interpreted based on the premise that cells exposed to 20 µg/ml benzalkonium chloride displayed a 100 % membrane disruption and Sytox Green emission. Further standardisation of the protocol was carried out by examining the influx of the stain into cells treated with various strengths of polymyxin B alone. The data presented in figure 7.16 also contains curves for cell suspensions exposed to varying concentrations of this polypeptide showing that although this agent demonstrated membrane action, membrane disruption that was accompanied by the influx of the dye became pronounced approximately after 4 hours for all the examined strengths within the range of 32 to 256 µg/ml. The degree of disruption appears to be time and concentration dependent in character. The membrane activity of the highest concentration of this polypeptide antibiotic peaked at about 48 % at 10 hours whilst that of the lowest concentration tested was 32 % after 24 hours of exposure.

The data presented in figure 7.17 show that bacterial suspensions treated with single applications of the chalcone-derivative F1 produced curves with lower degrees of fluorescence in comparison to that from cells exposed to the control surface active agent, thereby indicating a relatively lesser degree of membrane activity reflected by the lower onset as well as extent of dye emission. The membrane disruption activities of the semi-synthetic agents were also in a concentration-dependent order. MSSA cells treated with 128 µg/ml F1 demonstrated the highest emission followed by those challenged with 64 and 32 µg/ml (figure 7.17).

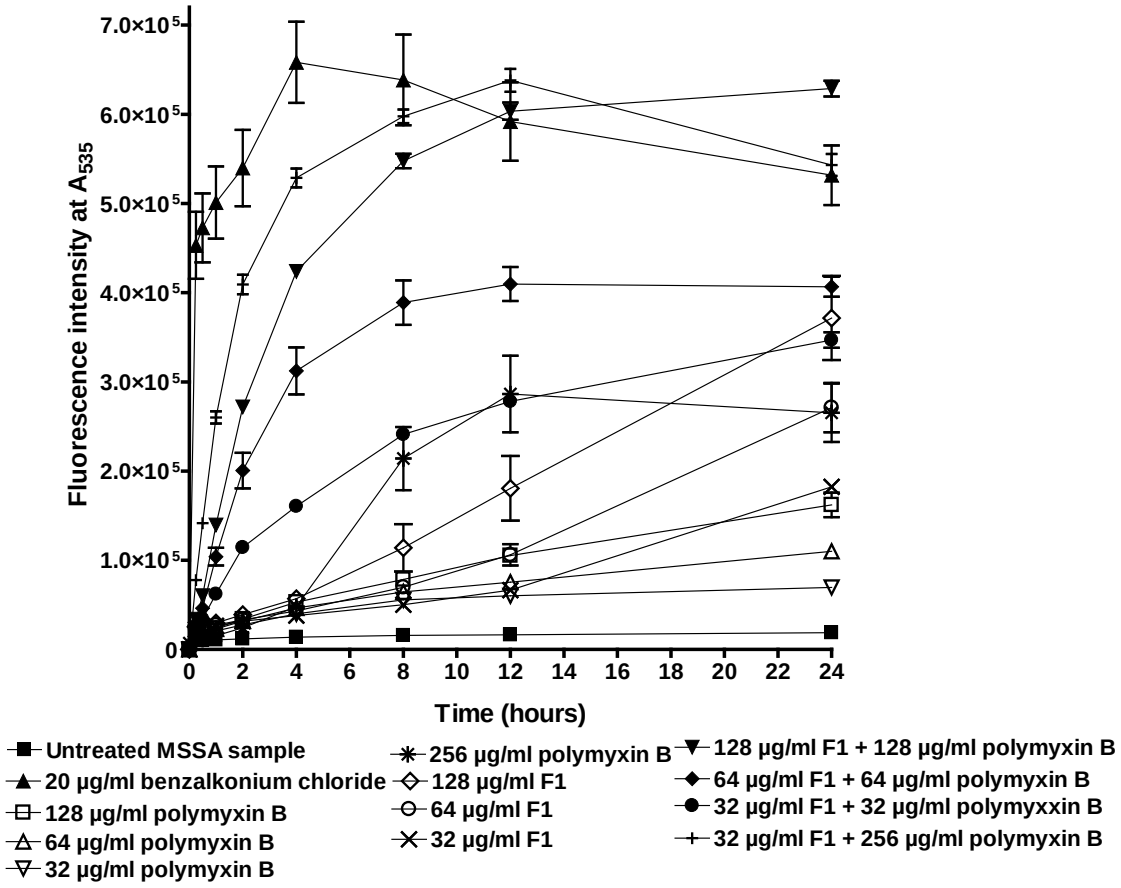


Figure 7.17: Determination of the membrane integrity of *S. aureus* 6571 (MSSA) challenged with concentrations of benzalkonium chloride, polymyxin B, F1 alone and F1 applied in combination with polymyxin B at varying strengths. All data are means of duplicate experiments that were repeated three times on separate days.

Similarly, cells exposed to 128 F23 displayed the highest degree of emission followed by 64 and 32 µg/ml (figure 7.18) respectively. The data presented in figure 7.17 also clearly show a potentiated membrane disruption activity between various concentrations of F1 with polymyxin B as the curves express higher degrees of fluorescence that are comparable to that caused by 20 µg/ml benzalkonium chloride. This latter concentration of the control agent produced a membrane bilayer disruption action that remarkably surpassed (in both rate and extent) the bilayer assault caused by 4 µg/ml of this same compound (figure 7.16). With reference to the 20 µg/ml benzalkonium chloride control, cell suspensions exposed to 32 µg/ml of both compounds applied together showed the lowest degree of activity that was followed by 64 and 128 µg/ml of both compounds. However, a combination of 32 µg/ml F1 with 256 µg/ml polymyxin B demonstrated the highest activity of 105 % amongst all the combinations.

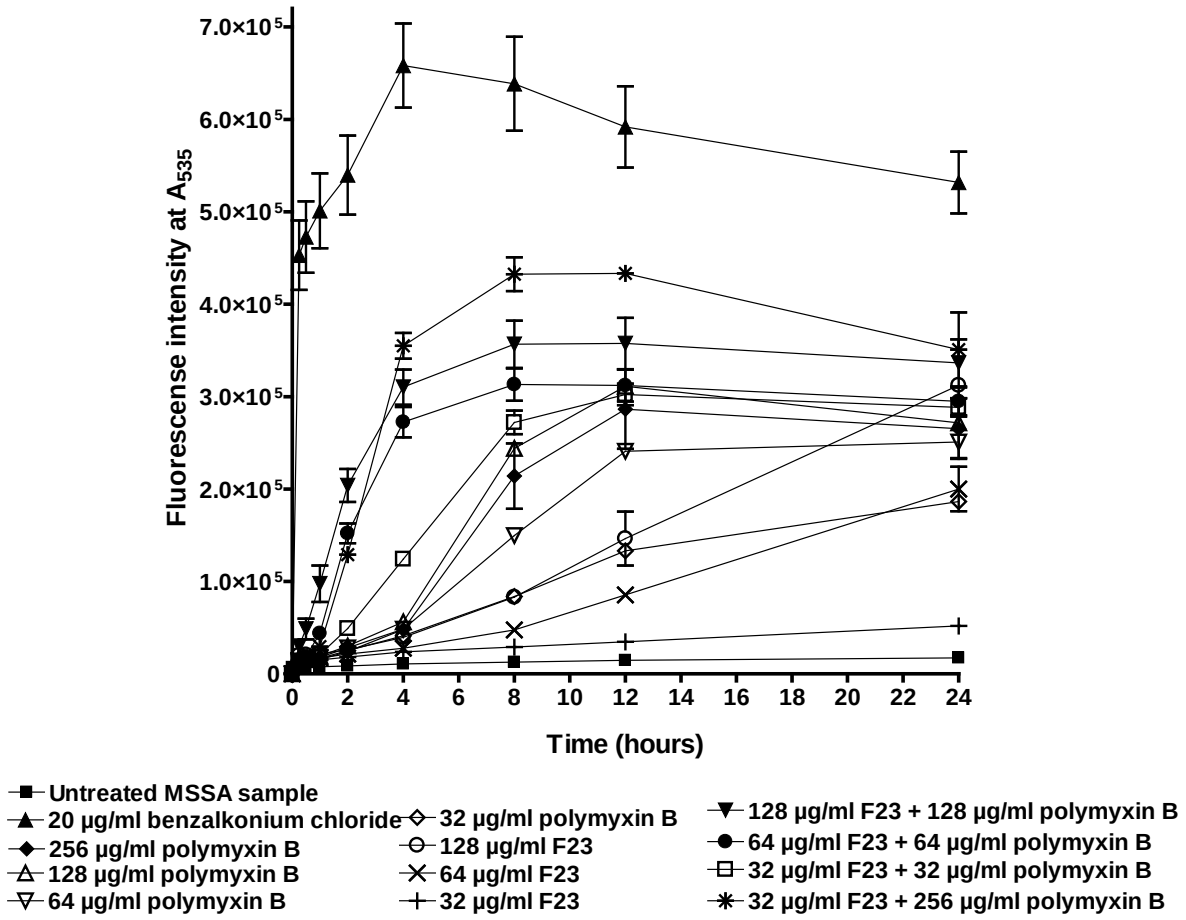


Figure 7.18: Determination of the membrane integrity of *S. aureus* 6571 (MSSA) challenged with concentrations of benzalkonium chloride, polymyxin B, F23 alone and F23 applied in combination with polymyxin B at varying strengths. All data are means of duplicate experiments that were repeated three times on separate days.

Although the activity of benzalkonium chloride peaked after about 4 hours, that of this latter combination was at optimum after 10 hours, in addition to demonstrating the most rapid onset of action which was only closely followed by that of the combination of 128 µg/ml of both compounds. The activity of this latter combination of both agents was seen to be at optimum after about 20 hours. Therefore a combination of 32 µg/ml F1 with 256 µg/ml displayed the best overall onset of action amongst all the strengths and combinations examined fluorimetrically with an extent of action that surpassed that displayed by the surface active agent used as control. This was closely followed by the activity of 128 µg/ml F1 combined with 128 µg/ml polymyxin B that displayed an extent of action which also exceeded that of benzalkonium chloride. This latter combination demonstrated a remarkable potentiated membrane disruption, exceeding that displayed by the individual compounds. Colony counting assay

data presented in chapter 5 showed this same combination displayed tremendous synergistic actions by reducing 1 x 10^7 cfu/ml populations of both MSSA and MRSA to below detectable levels after 8 hours clearly indicating the existence of a correlation between membrane activity and inducement of loss in viability by the combined application of both agents.

The data presented in figure 7.18 suggest the application of 32 µg/ml F23 with 256 µg/ml polymyxin B was superior in extent of activity over other strengths of both compounds examined in combination against MSSA. It is again interesting to note that the extent of membrane disruption displayed by this combination was better than that reported for 128 µg/ml F23 applied with 128 µg/ml polymyxin B; emphasizing that with a small fraction of this semi-synthetic agent, the antibiotic action of polymyxin B against Gram-positive organism is augmented partly through the enhanced disruption of the cytoplasmic membrane integrity. Another important observation is that 32 µg/ml F23 applied in combination with 256 µg/ml polymyxin B that induced the highest rate and extent of loss in *S. aureus* viability (figure 5.8) also demonstrated the highest rate and extent of influx of the fluorescing dye after 24 hours. This again emphasizes the possibility that cytoplasmic membrane bilayer disruption is amongst the core mechanisms of action of polymyxin B combined with the chalcone-derivatives against Gram-positive *S. aureus*.

In figure 7.18, in comparison to the action of 20 µg/ml benzalkonium chloride, MSSA challenged with 128 µg/ml of both polymyxin B and F23 that were singly applied displayed about 48 % stain emission that peaked after about 10 and 24 hours respectively. A concurrent view of figures 7.16, 7.17 and 7.18 suggest that applications containing only polymyxin B gave emissions with a faster rate and onset of action than those singly treated with either F1 or F23. The cells challenged with a combination of 128 µg/ml F23 and 128 µg/ml polymyxin B demonstrated about 55 % membrane disruption with an action that was slightly more rapid in onset, which peaked after 4 hours like cells challenged with only polymyxin B but exhibited a peak that is lower than was obtained with the combined application of 32 µg/ml F23 with 256 µg/ml polymyxin B as well as that of the cationic surface active agent control. As previously mentioned, 32 µg/ml (1/2 MIC) F23 applied with 256 µg/ml (MIC) polymyxin B demonstrated the fastest onset and extent of bactericidal action in the kinetic-colony count presented in figure 5.8. The fact that the bactericidal activity demonstrated by

this combined concentrations of both compounds is higher than that shown by the combined application of 128 µg/ml of both agents (as is indeed also shown for F1 in figure 7.17), verifies that only a small fraction of either of the chalcone-derivatives was required to be applied with polymyxin B in order to elicit augmented activity against strains of Gram-positive *S. aureus*. Overall, these data give an insight into a potential mechanism for the bactericidal action of combinations of both agents against *S. aureus* as previously reported from a chequerboard and time-kinetic assays.

7.4 Discussion

7.4.1 Membrane depolarisation and the release of *S. aureus* intracellular K^+ by the combination of polymyxin B with the chalcone-derivatives

There are variations in the definitions and descriptions of bacterial membrane damage as well as arguments on the correct order of events that lead to cell death caused by antimicrobial agents. But certainly, these events are all likely related in a cascade. The dissipation of trans membrane potential, formation of ion channels, trans membrane pores and membrane rupture should be viewed as a chain of events rather than as completely different mechanisms (Dathe and Wieprecht 1999). In terms of sequence of events, there is a strong rationale behind the opinion that the distortion of membrane-potential as well as the pH-gradient by agents such as gramicidin, valinomycin, nisin, etc., certainly precedes all other events and are likely to be accompanied by impairment of osmotic regulation, formation of trans membrane pores and inhibition of bacterial respiration (Matsuzaki et al. 1997, Dathe et al. 2002). The data presented in figure 7.1 and 7.2 show induced depolarisation of MRSA membrane potential by the combinations of sub-inhibitory concentrations of the chalcone derivatives (F1, F23) with polymyxin B within 30 to 60 minutes. This dissipation is likely to have occurred before the extensive loss of the membrane bilayer integrity in cells exposed to the combinations, evidenced by the massive influx of Sytox Green within the first 75 minutes, as recorded in figures 7.16 to 7.18.

The formation of peptide-induced ultra structural lesions has been noted to lag behind loss in cell viability (Brogden 2005). The reported data suggest whereas substantial loss in viability by *S. aureus* species challenged with the combinations

of polymyxin B with the chalcone-derivatives in time-kinetic colony counting assays were recorded between 2 and 8 hours (chapter 5), membrane depolarisation (figures 7.1 and 7.2), leakage of intracellular K^+ (figures 7.12 and 7.13), blockade of essential metabolic enzyme function (chapter 6) and extensive membrane disruption (figures 7.16 to 7.18) were seen to occur much earlier. Although the treatment of *E. coli* with megainin 2 leads to immediate release of intracellular potassium ion that is accompanied by prompt cell death (Matsuzaki et al. 1997), this was not the case for the combined application of polymyxin B with the chalcone-derivatives against *S. aureus* as a time-lag existed between the occurrence of both events.

It is notable that lower concentrations of these compound combinations (than those used for time-kinetic colony counting assays) demonstrated these agents capacity to depolarise *S. aureus* membrane potential. The antimicrobial peptide cecropin A has been shown to dissipate the lipid ion gradients in lipid vesicles at concentrations much lower than is required to cause extensive membrane damage following the release of encapsulated calcein (Silvestro et al. 1997). The antibacterial action of lower strengths of this antimicrobial peptide is therefore attributed to the mechanistic dissipation of trans membrane electrochemical ion gradients.

The dissipation of trans membrane electrochemical potential in MRSA

by as low as 16 µg/ml of either of F1 applied with 32 µg/ml of polymyxin B complemented the synergistic activity of this same combination recorded in table 4.1 and in addition suggests membrane depolarisation occurred in advance of actual cell death, being initiated at less than 30 minutes in figures 7.1 and 7.2. The rapid onset of bacterial membrane potential depolarisation in *S. aureus* species membrane is responsible for triggering a cascade of sub-cellular events that may eventually lead to bactericidal effects (Penyige et al. 2002, Mirzoeva, Grishanin and Calder 1997).

The release of intracellular potassium ions has largely been described as the first sign of impairment to the cytoplasmic membrane integrity as evidenced by the immediate release of this cation upon the application of certain antimicrobial agents such as the ionophore valinomycin, as well as daptomycin (Silverman, Perlmutter and Shapiro 2003). The leakage of intracellular cations may be accompanied by the release of inorganic phosphates, a pool of amino acids as well as materials absorbing at 260 nm (nucleic acids and proteins) (Maillard

2002). If quickly halted, these destructive actions may lead to only a bacteriostatic effect if the bacterial cell is able to initiate and effect the repair of the membrane bilayer as well as other measures that counteract the action of the noxious agent (Maillard 2002). The data presented in figures 7.12 and 7.13 showing that polymyxin B caused an inducement of the leakage of intracellular potassium ions from exposed *S. aureus* cells does not therefore always automatically translate to a bactericidal action (> 3 log cfu/ml loss in cell viability (Petersen et al. 2006)). This assertion was corroborated by data presented in figures 5.1 to 5.10 wherein varying concentrations, including 128 µg/ml, of polymyxin B were at best only bacteriostatic against species of *S. aureus* but demonstrated the capacity to induce the intracellular leakage of this cation (figures 7.12 and 7.13) and enabled the influx of the intact-membrane impermeable dye (figures 7.16 to 7.18). The molecular weight and size of potassium ion is so small that any minor infringement on the intactness of the bilayer membrane results into leakage of this cation (Lambert and Hammond 1973). Bacterial suspensions in de-ionised water may also lose viability over time because of a perturbation of the membrane bilayer due to the difference in osmotic pressure between the extracellular and intracellular fluid.

However, a massive and unobstructed leakage of this cation followed by the cascade of events previously described, often leads to a bactericidal effect. Data presented in figures 7.12 and 7.13 showing the inducement of enhanced leakage of cytoplasmic cation by the action of either F1 or F23 applied with polymyxin B against *S. aureus* correlates with the synergistic action of both compounds reported in figures 5.1 to 5.10. This observation also corroborated the enhanced anti-metabolic action of the application of both compounds against various strains of this bacterial species reported in figures 6.10 to 6.15. Although massive induced leakage of the cation was seen to have occurred from the initial time of exposure up to 120 minute of the assay, substantial cell deaths for these bacterial species were only recorded between 2 and 8 hours in the colony-counting assay. This is in order with published opinions that sometimes the timing for the occurrence of ultra-structural lesions caused by certain harmful agents, including antimicrobial peptides, may be seen to lag behind the period wherein loss of cell viability was noted (Brogden 2005).

Upon the application of sufficient negative potential to an artificial planar lipid bilayer, the introduction of the antimicrobial peptides HNP-1 and rabbit NP-1 to

the opposite side causes formation of trans membrane pores (Kagan, Ganz and Lehrer 1994). It is therefore thought that the interaction of polymyxin B-chalcone derivatives with lipoteichoic acid of Gram-positive *S. aureus* possibly caused a depolarisation of the cells that triggered the formation of trans membrane pores that is accompanied by the massive outflow of cytoplasmic materials as well as the blockage of essential metabolic enzyme activities (chapter 5) and loss of cell viability.

The combination of polymyxin B with the chalcone-derivatives demonstrated a rapid blockage of the activity of biological metabolic enzymes (chapter 6), which may have eventually lead to an impairment of ATP production (Dinning et al. 1998, Zhang et al. 2003). It may be worthwhile to verify the inhibition of the activity of this essential adenosine-derived nucleotide in future studies. Nisin has been reported to cause a time and concentration-dependent decline in internal ATP levels in treated *M. bovis* strain bacillus Carmette Guerin treated without the detection of ATP efflux (Chung, Montville and Chikindas 2000), suggesting the inhibition of ATP production mechanisms occurs almost simultaneously with the onset of pore-formation and the utilisation of energy reserve in unsuccessful attempts to generate essential metabolites. But *M. bovis* is also seen to demonstrate the maintenance of a fairly constant level of ATP during the initial treatment period despite a decline in both components of the proton motive force (membrane potential,$\Delta\psi$ and ΔpH) (Chung, Montville and Chikindas 2000). Therefore, despite the dissipation of membrane potential, blockage of bacterial metabolic action in the MTT-reduction assay and membrane disruption evidenced by the fluorescence of nucleic acid bound stain, additional assays are required to ascertain whether the combination of polymyxin B with the chalcone-derivatives alters ATP/ATPase levels.

It is also pertinent to investigate whether the polypeptide combined with these semi-synthetic agents inhibits the activity of genes encoding for efflux mechanisms in *S. aureus* as this would amount to a more rapid accumulation of both compounds to facilitate their potential destructive effects within the cell (Kumar and Schweizer 2005).

7.4.2 *S. aureus* membrane disruption and influx of Sytox Green stain

The assay involving the interaction and binding of unsymmetrical and polycationic intact-membrane impermeable Sytox Green stain (Roth et al. 1997) to polyanionic nucleic acid, gave an insight into the possible degree of membrane disturbances caused by the assault of the combined agents application. The degree of membrane disturbance/disruption may be estimated to be minor if it only permitted the leakage of K^+ (MW. 32 g/mol), but prevented the outflow of the pool of amino acids as well as nucleic acids and disallowed the influx of the stain. On the other hand, the degree of membrane disturbance/disruption enforced upon cell membrane bilayer would be estimated to be major if the disturbance permitted either the influx of the dye into the cells or an unhindered outflow of nucleic acids demonstrated by the binding and fluorescing of the stain (bearing in mind that the molecular weight of Sytox Green is 600 g/mol (Saugar et al. 2006)). The antimicrobial peptide alamethicin forms trans membrane pores using the barrel-stave model wherein the hydrophobic regions of the compound align with the lipid core regions of the bilayer whilst the hydrophilic regions of the agent form the interior region of the pore which can contain 3 – 11 parallel helical molecules (He et al. 1995, Ludtke et al. 1996). The inside and outside diameters of the pore were found to be ~1.8 and 4.0 nm (Spaar, Münster and Salditt 2004). The sizes of toroidal trans membrane pores induced by magainin are observed to be bigger with more variable sizes than those formed by alamethicin (Yang et al. 2001). These pores measure 3.0 – 5.0 and ~7.0 – 8.4 nm in inner and outer diameter respectively and each can accommodate either 4-7 molecules of magainin or approximately 90 lipid molecules (Matsuzaki et al. 1998). The polycationic Sytox Green stain with a larger molecular weight (600 g/mol) than the compounds mentioned above (Saugar et al. 2006), demonstrates great affinity for RNA as well as DNA but an inability to permeate intact bacterial membranes, whilst this stain can flow freely across compromised cytoplasmic bilayers (Lebaron, Catala and Parthuisot 1998, Joux and Lebaron 2000). The data presented in figure 7.16 suggests that various concentrations of polymyxin B were able to disrupt *S. aureus* membrane integrity to a degree that allows either the outflow of the intracellular contents or influx of Sytox Green stain only after about 300 minutes. The combinations of the chalcone-derivatives with this same polypeptide were however seen to cause an immediate

impairment of membrane integrity to allow for the occurrence of the same process reflected by the prompt fluorescence of the stain (figure 7.17 and 7.18), sometimes to an extent that exceeded the emission induced by benzalkonium chloride.

The data presented in this assay shows that the release of intracellular potassium ions is not a guarantee for the stain to fluoresce as the extent of membrane disturbance caused by the action of an antimicrobial agent may not be sufficient to either permit the outflow of DNA and RNA or the influx of the stain. The formation of pores with smaller diameters has been noted to be able to prevent the release of the pool of intracellular amino acids (Daniel R. Marshak 1996). Considering the high inoculum population of 5×10^7 cfu/ml, galangin at 125 µg/ml demonstrated as much as 2.5 log cfu/ml reduction in the cell viability after 24 hours (figure 7.15) with only 1.7 % fluorescence intensity of the dye over the same period (figure 7.14). But this flavonol causes damage to the cytoplasmic membrane of *S. aureus*, evidenced by the leakage of intracellular potassium ions (Cushnie and Lamb 2005b). It can therefore by speculated, going by the findings in this report that the damage to the cytoplasmic bilayer of *S. aureus* instituted by the action of galangin was not sufficient to either permit the influx of Sytox Green or enable the outflow of nucleic acid containing materials. There may be a desire in the future, to determine the actual size/width of the trans membrane infringements that are instituted by galangin's action. It is a well established fact the primary mechanism of action of the other control antibiotics penicillin G and ciprofloxacin involves the blockade of the synthesis of peptidoglycan (Anderl, Franklin and Stewart 2000) and DNA replication (Pankey and Ashcraft 2005) respectively. Both compounds function via a membrane non-lytic mechanism of action.

The antibacterial activities of β-lactams and fluoroquinolones are diminished in non-growing cells and by high inoculum densities (Mizunaga et al. 2005). Both conditions were factored into the experiment for control purposes. The data show penicillin G and ciprofloxacin at four times and twenty times their MICs demonstrated less than 0.5 log cfu/ml reduction to the inoculum population of MSSA. The data also show the combinations of the chalcone-derivatives with polymyxin B demonstrated enhanced entry and binding to nucleic acid for Sytox Green indicating a potentiated membrane permeabilization and influx for both compounds.

It has been suggested that the use of Sytox Green for membrane integrity analysis should be considered with caution and restricted to the evaluation of antibiotic susceptibility of bacteria with undamaged nucleic acids (Lebaron, Catala and Parthuisot 1998). Considering that the organisms employed in this project were not in starvation mode, neither were they obtained from the natural habitat, these bacterial species may be assumed to have undamaged as well as the normal proportion of nucleic acids, unlike cell samples collected from the Mediterranean Sea (Roth et al. 1997). There is also another assumption that the combination of polymyxin B with these chalcone-derivatives does not destroy nucleic acids. If both compounds were later found to kill bacteria by mechanisms that include destruction of RNA and DNA, then the reported degree of fluorescence by the stain upon the combined application of both compounds may need to be reviewed. The use of Sytox Green for determining the fraction of dead cells amongst marine oligotrophic bacterial species has been restricted by the fact that the degree of nucleic acids containing cells may be some times lower than 50 % (Karner and Fuhrman 1997). For the same reason, the use of this stain in the determination of the fraction of dead cells in natural habitats is limited. In the presence of a background stain for live cells, Sytox Green is unable to effectively differentiate live from dead cells when the sample is obtained from the Mediterranean Sea because of an inadequate supply of RNA and DNA (Lebaron, Catala and Parthuisot 1998). In this project however, the use of background stain was avoided, preferring colony counting assay that is a more reliable means of ascertaining cell viability (Moriarty, Elborn and Tunney 2005, Tunney et al. 2004). The adoption of the latter technique also made the interpretation of the data from the stain unambiguous since determination of the intactness of the bacterial membrane bilayer and viability were simultaneously ascertained by two different mechanisms.

7.4.3 Monitoring changes in fluorescence intensity in *S. aureus* using microscopy

The data presented in figures 7.4 and 7.5 show clearly that the controls put in place for the fluorescence microscopy assay were reliable. The $DiOC_3(5)$ stained but untreated *S. aureus* cells did not fluoresce in for the first three hours, indicating the absence of membrane depolarisation within this period. The

stained cells fluoresced with greater intensity for gramicidin-treated bacterial suspensions indicating dye disaggregation within the intracellular space, after 15 minutes of exposure. This ionophore antibiotic has demonstrable capacity to dissipate bacterial cytoplasmic membrane potential thereby enabling dye disaggregation that caused the recorded intense fluorescence (Suller, Stark and Lloyd 1997). The use of such stains has been adapted in flow cytometry analysis for the prompt assessment of antibiotic susceptibility through determination of membrane depolarisation, integrity impairment and loss in bacterial viability (Kirk et al. 1998, Shapiro 2001). Data presented in figure 7.7 suggests the cells exposed to either of the chalcone-derivatives alone demonstrated higher degrees of changes in fluorescence intensity over time in comparison to the untreated cells but that was higher in intensity when viewed in relation to samples treated with the latter agents in combination with polymyxin B. The data presented in figures 7.3 to 7.8 likewise suggests firmly that the application of polymyxin B with either of the chalcone-derivatives caused a depolarisation of the *S. aureus* bilayer that enabled the disaggregation and notable fluorescence of the stain.

With the use of a similar stain, and likewise gramicidin as a control, valinomycin has been shown to demonstrate complete membrane depolarisation in *S. aureus* (MSSA) as well as MRSA evidenced by intense fluorescence. Understandably, methicillin is unable to induce an emission in stained MRSA cells (Suller, Stark and Lloyd 1997). Effective β-lactam antibiotics are thought to be able to induce depolarisation of membrane potential, inhibition of cell wall biosynthesis and an induction of cell wall autolysis that are both dose and bacterial-growth phase related (Penyige et al. 2002).

Sometimes, alterations and damage to cell structures are expected to occur at the same time with loss of viability during mechanistic studies for antibacterial compounds, in order to correlate cause and effect. But data from some studies have shown that this is not always the case. But the timing for the occurrence of cellular damage caused by antimicrobial peptides may lag substantially behind that for recorded cell death; one study concluded a reported induced ultra-structural damage may have been artefactual (Brogden 2005). In other instances, the morphological and structural defects caused by the application of such agents continue to increase even after the establishment of a killing action. *E. coli* cells treated with the polypeptide, HNP, are seen to increase the formation of membranous blebs even after a substantial decrease in colony count is noted

(Lehrer et al. 1989). The interpretation of data from that publication was that cell death was not caused by bleb formation but rather an event that occurred afterwards (Hancock and Lehrer 1998a). Similarly, although a complete loss in viability was recorded for *E. coli* cells exposed to another peptide DEFB118 after 15 minutes of exposure, morphological and structural damage are seen to continue 30 to 120 minutes later (Yenugu et al. 2004). Data reported in figures 7.5 to 7.13 show that although gramicidin D induced prompt MRSA membrane depolarisation that was evidenced by an immediate and intense fluorescence of the stain, this was not the case with the combination of polymyxin B with either of the semi-synthetic agents. This combination required 3 to 4 hours for notable fluorescence which tallied with the timing needed for initiation of substantial loss in viability against various strains of this organism (figures 5.1, 5.3, 5.5, 5.8, 5.9 and 5.10). The occurrence of the reported bactericidal effects for both compounds lagged behind the timing for the induced leakage of intracellular potassium ions, which was very rapid in onset (figures 7.12 and 7.13).

7.4.4 Combined discussion of the data in this chapter

Published data show that sub-inhibitory concentrations of the cAMP myeline could dissipate the membrane potential of the cytoplasmic membranes of *Ps. aeruginosa* and *S. aureus* species, as well as disrupt the outer membrane of the former organism (Rasul et al. 2010), following an initial electrostatic interaction with both of these cells surfaces (Zhang et al. 2000). It was observed however that the major cell damaging/killing effect for both bacterial species was the impact of the agent's destructive effect upon their cytoplasmic membranes. For whilst a rapid dissipation of cytoplasmic membrane potential demonstrated by a quicker release of $DiSC_3(5)$ stain (previously aggregated within the cytoplasm) correlates with the timing of the loss of cell viability in *Ps. aeruginosa* (Rasul et al. 2010), all the concentrations used to challenge *S. aureus*, including the non-killing concentrations in this report demonstrated a comparable extent and rate of intracellular release of this stain. This suggests that membrane depolarisation and release of the dye in *S. aureus* may not always result into an immediate loss of cell viability. The data presented in this section although show existence of a correlation between the disaggregation of this stain, membrane depolarisation, leakage of intracellular cations and loss viability in MRSA suspension challenged

with the combination of polymyxin B with the chalcone-derivative; however the timing of the former events preceded that of the latter (chapter 5). Moreover, the timing for the rapid loss in intracellular potassium ions tallied with that recorded earlier for the blockage of bacterial metabolic activity in chapter 6.

Previously published data from an agar dilution technique demonstrated galangin had MICs in the range of 25 – 50 µg/ml against various strains of *S. aureus* including MSSA (*S. aureus* NCTC 6571) (Cushnie and Lamb 2005c, Cushnie, Hamilton and Lamb 2003) and that this flavonol was bactericidal in action by causing a 100,000-fold decrease in inoculum population within 2 hours in time-course viability studies through rapid cell aggregation (Cushnie et al. 2007) as revealed by light microscopy. Although it was thought that the cell aggregation effected by galangin was due to the compounds action upon the cytoplasmic membrane (Cushnie et al. 2007), data presented in figures 7.12 and 7.13 show that Sytox Green is unable to permeate MSSA cytoplasmic barrier when challenged with 125 µg/ml of this agent for 24 hours strongly suggesting that the integrity of the treated cells were intact during this period despite a≥ 2.5 log cfu/ml reduction in inoculum population. Time-kinetic data not shown from this study demonstrated 125 µg/ml was the concentration of galangin required for optimum activity against MSSA.

The inability of Sytox Green to permeate *S. aureus* cytoplasmic membrane upon treatment with galangin, that indicated cell membrane intactness, did not however dismiss the possibility for the occurrence of leakage of potassium ions due to either infringed minor disturbances upon the bilayer or induced activation and/or formation trans membrane channels (Cushnie and Lamb 2005b). It is therefore possible for a given agent to induce pore formation and leakage of intracellular cations, without actually causing a killing effect through membrane disruption and cell lysis. Indeed, daptomycin causes the leakage of potassium ions from *S. aureus* upon the insertion of the compounds lipophilic acyl tail into the cytoplasmic membrane (Rasul et al. 2010). The bleeding of cytoplasmic potassium ion could lead to an imbalance in the ionic concentration gradient that causes membrane depolarisation, inhibition of protein, DNA, RNA synthesis and eventually cell death (Silverman, Perlmutter and Shapiro 2003). Although galangin has been shown to cause bacterial death through cell aggregation (Cushnie et al. 2007), it may be worth investigating whether the loss of intracellular potassium ion induced by the flavonol (Cushnie and Lamb 2005b) is

caused by an imbalance in ionic concentration, membrane depolarisation and the sub-cellular cascade of events that results to cell death, alongside the induced aggregation.

The data above also therefore suggests that the inability of Sytox Green to pass through cytoplasmic membrane may not always imply absolute intactness of the bilayer structure as the molecular weight of potassium ion makes this element small enough to permit its leakage across such barriers that are effective against the polycationic dye. Understandably, the loss of potassium ion is the first sign and primary indication of bacterial membrane damage, leading to a chain of events that could terminate in cell death (Johnston et al. 2003, Castillo et al. 2006), even in the absence of cell lysis (Cotroneo et al. 2008). Daptomycin is rapidly bactericidal *in vitro* against *S. aureus* (Thorne and Alder 2002) through a cascade of sub-cellular events that is initiated with the insertion of the compounds lipophilic region that seems to initiate membrane depolarisation and the leakage of cytoplasmic potassium ions (Silverman, Perlmutter and Shapiro 2003, Benvenuto et al. 2006) but without the occurrence of cell lysis (Cotroneo et al. 2008).

At various concentrations, polymyxin B demonstrated the capacity to permeate *S. aureus* cytoplasmic membrane and permit the influx of Sytox Green stain. This agent has a reputation for self-promoted uptake across bacterial membrane bilayer (Hancock 1997b). Antimicrobial peptides with such characteristics are also known to promote the uptake of hydrophobic antibiotics across bacterial bilayer barriers and act synergistically with these agents (Vaara and Porro 1996, Sánchez-Gómez et al. 2011). Therefore, it is being speculated that polymyxin B probably promoted the entry of the chalcone derivatives, through which both compounds demonstrated augmented bactericidal effect against several Gram-positive as well as Gram-negative species.

Cationic antimicrobial peptides (cAMPs) have been known to function as anti-infective agents through permeabilization of bacterial membranes (Zhang, Rozek and Hancock 2001a) and have now been overwhelmingly suggested to also function through alternative mechanisms and as such via multiple routes (Jenssen, Hamill and Hancock 2006a). The renewed clinical interest in some cAMPs such as colistin and polymyxin B, apart from the shortfall in supply of new effective agents, is due to their efficacy against multi-resistant Gram-negative bacterial species, rapid onset of action and low potential for the emergence of

mutant-resistant strains during their applications (Gordon, Romanowski and McDermott 2005). Data obtained from this study suggest the combination of the chalcone derivatives with polymyxin B can extend the latter's spectrum of activity to include Gram-positive species such as *S. aureus* through enhanced depolarisation and permeabilization of cytoplasmic membrane, increased rate and extent of leakage of intracellular potassium ions, blockage of respiratory metabolic enzymes (chapter 6) and cell death (chapter 5).

7.5 Conclusion

The data presented in this chapter strongly suggest the combination of polymyxin B with the chalone-derivatives elicits multiple mechanisms of action against *S. aureus*.

Chapter 8

Erythrocyte lysis assay for polymyxin B combined with either of the chalcone derivatives

8.1 Introduction

The sensitivity of erythrocytes to the polymyxin B applied alone and in combination with either of the chalcone-derivatives were also assessed, as a measure of haemolysis. This is thought to be able to give an insight into the outcome of possible interactions between potential formulations delivering synergistic concentrations of both compounds with these mammalian cells (Plank et al. 1994, Miwa et al. 2002). Given the available literature information concerning the dose-related nephrotoxicity of polymyxin B alone and the aqueous solubility challenges affiliated with the use of the chalcone-derivatives, potential future formulations will be likely inclined towards external formulations such as ointments, creams, powders for skin ulcers, as well as nebulised inhalations that can be applied directly into the lungs in ailments such as cystic fibrosis which is normally mediated by multi-resistant Gram-negative organisms such as *Ps. aeruginosa* (Schurek, Breidenstein and Hancock 2012).
The aims of this section are to examine the sensitivity of mammalian red blood cells to the presence of various concentrations of polymyxin B alone and in combination with various synergistic strengths of either F1 or F23.

8.2 Materials and Methods

8.2.1 Erythrocyte lysis assay

Erythrocytes were recovered from defribinated horse blood by centrifuging six 1 ml volumes at 10,000 rpm for 2 minutes. The recovered pellets were washed in phosphate buffer solution three times and all were combined in a conical flask. The pellets were re-suspended and diluted 20-fold in the same buffer. Following on, 4 ml of the suspension, in a non-adherent disposable plastic 20 ml vial (Fisher Scientific), was treated with suitable volumes of the stock solutions of chalcone-derivative and polymyxin B separately and together, in order to obtain the desired final concentrations. All the vials were incubated for 1 hour in a water

bath previously set at 37 ^{o}C, following which haemolysis was assessed by measuring the optical densities of the suspensions at OD_{540}. A four ml aliquot of erythrocyte suspension challenged with 5 % $^{w}/_{v}$ sodium dodecyl sulphate (SDS) and an equivalent untreated volume were used as controls, representing 0 and 100 % haemolysis, respectively. This assay was developed by adaptations from procedures described by (Fernandez-Lopez et al. 2001) and (Lee and Oh 2000).

8.3 Results

8.3.1 Effect of the test compounds on erythrocyte membrane integrity

The haemolytic effect of the chalcone-derivatives applied alone and in combination with polymyxin B upon the membrane integrity of erythrocytes was examined and the data obtained are presented in figures 8.1 and 8.2.

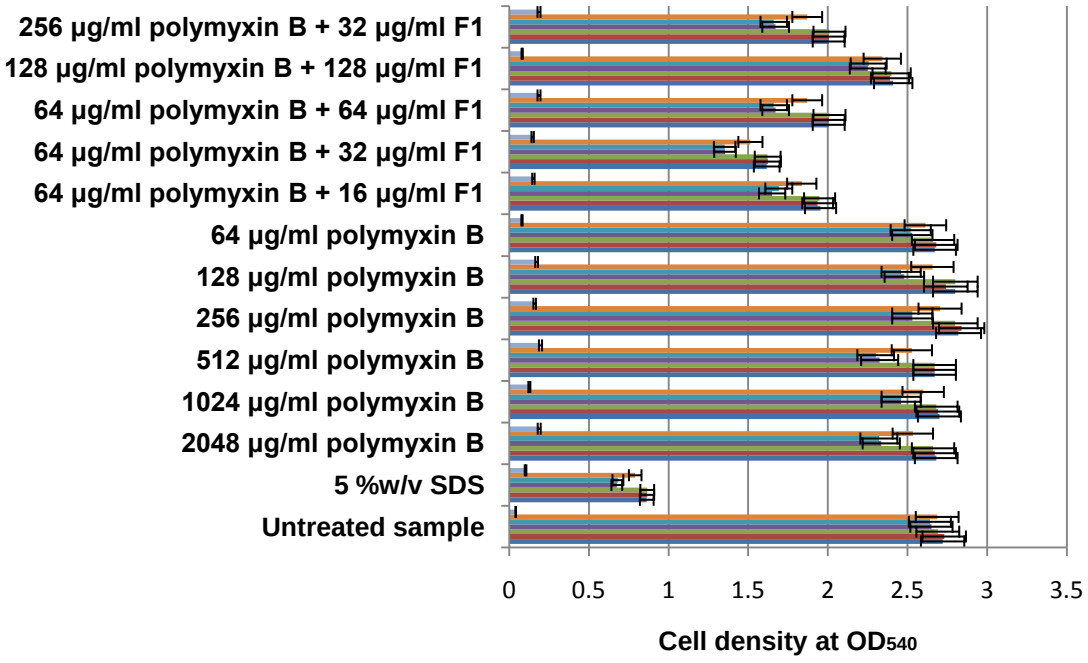


Figure 8.1**:** Data for erythocyte cells treated with various concentrations polymyxin B alone and in combination with F1. SDS represents sodium dodecyl sulphate. Results are from experiments conducted in duplicated and repeated twice.

The data expresses the effect of different concentrations of single polymyxin B concentrations and treatments made in conjunction with varying concentrations of either of the chalcone-derivatives against erythrocytes and showed that this polypeptide did not induce membrane lysis upon mammalian cells, even at extremely high concentrations of up to 2048 µg/ml, in comparison to

unchallenged cells. Although 5 % $^{w}/_{v}$ SDS used as the positive control displayed a 100 % haemolysis, the applications of different synergistic combination concentrations of polymyxin B with either of the chalcone-derivatives demonstrated varying degrees of haemolysis.

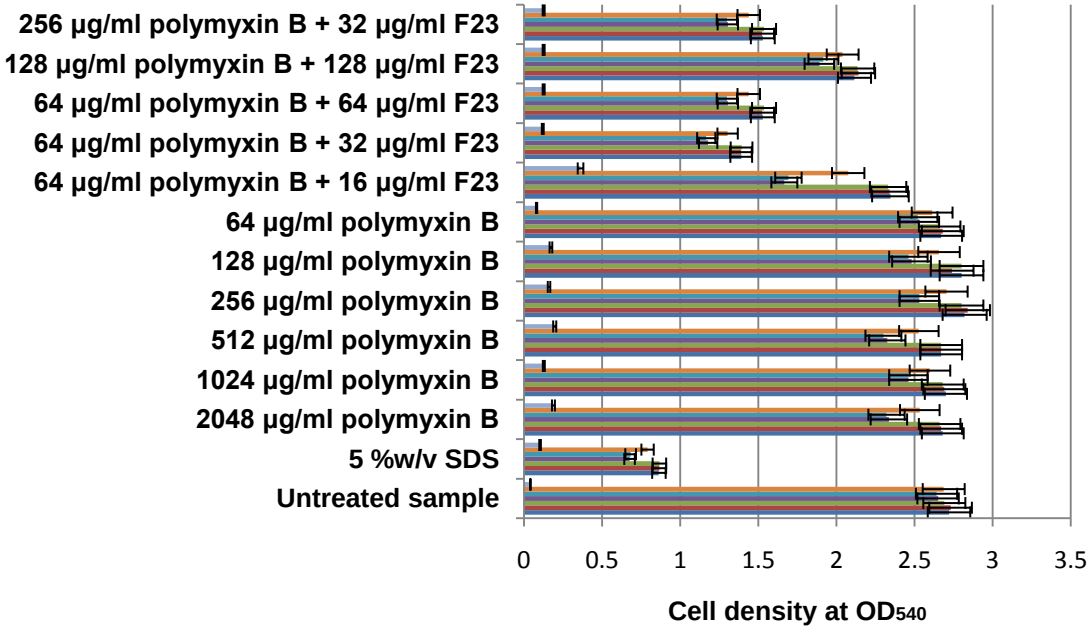


Figure 8.2**:** Data for erythocyte cells treated with various concentrations polymyxin B alone and in combination with F23. SDS represents sodium dodecyl sulphate. Results are from experiments conducted in duplicated and repeated twice.

In comparison to the action of SDS, the combination of 128 µg/ml of either of the chalcone-derivatives with the same concentration of polymyxin B displayed 80.4 % of unlysed cells (the combination with lowest toxicity), whilst being notably synergistic (figure 5.1, 5.3), inducing profuse and rapid leakage of intracellular potassium ions (figures 7.12 and 7.13), haven't ruptured *S. aureus* cytoplasmic membrane to an extent that permitted the inflow of Sytox Green stain (figures 7.16 to 7.18) and caused the blockage of the activities of metabolic enzymes (figure 6.9). The degree of haemolysis caused by the latter combination concentrations was followed by a 28 % haemolysis effected by 32 µg/ml of either of the chalcone-derivatives applied with 256 µg/ml polymyxin B. This combination had demonstrated the most rapid onset as well as extent of cytoplasmic membrane disruption (figure 7.17 and 7.18) and was the most synergistic concentration combination against *S. aureus* species (figure 5.8). It was noted that the combination of 64 µg/ml polymyxin B with 64 µg/ml F1 as

well as that of 64 µg/ml polymyxin B with 32 µg/ml F1 demonstrated more deleterious effects against the exposed cells in the data presented in figure 8.1 by leaving behind 72 % unruptured cells after the treatment period and that this was also consistent with the findings recorded in figure 8.2 for the combined action of equivalent concentrations of both F23 with this same antibiotic. Therefore, the combination of 128 µg/ml of polymyxin B with either of the chalcone compounds appears to have the optimum activity if considerations between efficacy and safety was undertaken on the basis of the information obtained so far. At the moment, this finding seems ambiguous and would require further analysis as lower strengths of antibiotics are expected to display lesser adverse effects as has been noted with the dose-related nephrotoxicity syndrome of polymyxins (Schurek, Breidenstein and Hancock 2012).

Combinations containing these concentrations demonstrated a notable bactericidal effect against Gram-positive *S. aureus* and displayed the lowest lysis of red blood cells. The combination of 64 µg/ml of both F1 and polymyxin B that demonstrated an enhanced inhibition of the activity of metabolic enzymes activity in *S. aureus* (figure 6.9), also displayed a 28 % haemolytic effect. Also, the combination of 32 µg/ml F1 with 64 µg/ml polymyxin B displayed as high as approximately 40 % haemolysis and was the highest degree of haemolysis recorded in this assay. This latter combination concentration instituted the blockage of the activity of *S. aureus* metabolic enzymes (figure 6.8) and induced membrane bilayer depolarisation (figure 7.8). The data obtained with the combination of polymyxin B with F23 were comparable at some points to those from the combination of this same antibiotic with F1. But the combination of 16 µg/ml F23 with 64 µg/ml polymyxin B induced only about 17 % haemolysis (unlike with same concentration of F1 which was 30 %). This combination of F23 with polymyxin B was synergistic against *S. aureus* species in the chequerboard (table 4.2) as well as in the time-kinetic colony counting (figures 5.8 and 5.9) assays. The combinations of either 32 or 64 µg/ml F23 with 64 µg/ml polymyxin B induced the same degree of haemolysis (36 %). The combined application of 32 µg/ml F23 with 64 µg/ml polymyxin B was bactericidal in table 4.2 and figure 5.9 and induced a notable degree of cell depolarisation in figure 7.9 in *S. aureus* species. The haemolysis action effected by the 5 % $^{w}/_{v}$ SDS positive control was approximately twice the value instituted by the most haemolytic combination concentration of polymyxin B with either of the semi-synthetic compounds. It

appears that red blood cells displayed the lowest level of sensitivity to the presence the combination of 128 µg/ml polymyxin B with 128 µg/ml of either of both chalcone-derivatives, which had also incidentally demonstrated very notable antibacterial actions. If further investigations are warranted in the future, controls samples examining the haemolytic activities of F1, F23, strengths of DMSO used as solvent in their single applications as well as DMSO-polymyxin B should be included in the analysis.

8.4 Discussion

8.4.1 Haemolytic effect of polymyxin B with the chalcone-derivatives

The enhanced haemolytic activity of the application of the chalone-derivatives with polymyxin B *in vitro* may make it necessary to investigate whether this combination may show preference for binding with either sodium dodecyl sulphate (SDS) or 3-[(3-cholamidoprpyl) dimethylammonio]-1-propanesulphonic acid (CHAPS). Cationic antimicrobial peptide antibiotics which prefer interacting with SDS over CHAPS are expected to exhibit low haemolytic activity (Rasul et al. 2010). Upon independent application, polymyxins have preferential binding for Gram-negative bacterial membranes over mammalian cell bilayers (Giuliani, Pirri and Nicoletto 2007, Ding et al. 2004, Gregory and Mello 2005) and also display an overall preference for prokaryotic membranes, especially at lower doses (Giuliani, Pirri and Nicoletto 2007), a desired characteristic of an ideal antibiotic. The data in figures 8.1 and 8.2 also suggest mammalian red blood cells displayed a very high degree of insensitivity to the presence of polymyxin B, even at extremely high concentrations, emphasizing the selective toxicity attribute of this agent when applied alone. When applied with varying concentrations of the chalcone-derivatives, erythrocytes demonstrated increased sensitivity to certain combinations that had shown notable antibacterial actions in other sessions. The data presented suggest the combination of 128 µg/ml of polymyxin B with either of the chalcone-derivatives had both notable synergistic action and the lowest lysis of red blood cells.

In the past, the concentration-dependent adverse effects of nephrotoxicity caused by polymyxins have necessitated studies aimed at improving antimicrobial activity whilst enhancing selectivity by varying the number of

positive charges (Vaara et al. 2008), hydrophobic interactions with membrane bilayers (Dathe et al. 1996) and helicity (Dathe et al. 1997) of the molecule. But most of the investigations produced compounds that demonstrated enhanced activity with reduced selectivity (Dathe et al. 1997), just like it became clearer that helicity and planar orientation at membrane surfaces are also crucial for bilayer translocation and antibacterial action (Giuliani, Pirri and Nicoletto 2007, Vaara et al. 2008, Vaara 2010, Brogden 2005). A strong correlation however also exists between the helicity of cationic amphiphatic model peptides and haemolytic activity which seems to verify the hypothesis that helicity significantly contributes to haemolysis (Dathe et al. 2002, Dathe et al. 1997). It may be worth investigating whether the combined application of polymyxin B with lower concentrations of these chalcone-derivatives causes an increase in haemolytic tendency through alteration to the hydrophobic moment and planar orientation of both compounds when in contact with mammalian (erythrocyte) cell membranes; and whether a further increase in the amount of the semi-synthetic agents leads to helical movements that effect lower degrees of disruption against these bilayers.

8.5 Conclusion

Certain combinations of polymyxin B with the chalone-derivatives, which were previously reported to be synergistic in action against the various bacterial species, displayed lower degrees of lysis of mammalian red blood cells.

Chapter 9

General discussion and future works

9.1 The need for new agents and combinations of antibiotics with either novel or multiple mechanisms of action

In view of the prevailing universal crises and continually emerging patterns of microbial resistance to antibiotics (Zhang et al. 2006, DiazGranados, Cardo and McGowan 2008, Kumarasamy et al. 2010, Johnson et al. 2012), there remains a strong rational need for the discovery and development of new effective antimicrobial agents with either uncommon or multiple mechanisms of action (Fischbach and Walsh 2009). Newly introduced agents that do not belong to the existing classes of antibiotics, possessing either novel or multiple mechanisms of action will ensure a prolonged delay in the time required by susceptible organisms to develop resistance strategies (Gould 2007). Research efforts must therefore be aimed at the search for either unfamiliar molecular structures with antibiotic activities or new combinational approaches that yield synergistic outcomes, entailing the applications of established antimicrobial compounds with novel agents, such as semi-synthetic flavonoids (Cushnie and Lamb 2011, Anderson et al. 2005).

The methods described in this thesis were used to evaluate the antibacterial activity of the synthetic hydrophobic compounds, F1 and F23. The chequerboard method used for preliminary investigations aided the detection and evaluation of their synergistic action with polymyxin B. Although the use of this technique is considered adequate for the examination of synergistic action amongst cationic antimicrobial peptides (cAMPs) (Yan and Hancock 2001), the data obtained showing synergism between the applied agents, were verified using more reliable time-course viability assays (Lewis et al. 2002). The modified bacterial MTT-reduction protocol also gave demonstrable insight into possible mechanisms of action by polymyxins against selected bacterial species through the inhibition of the cells respiratory metabolic pathway. The timing of the latter event shows it occurred in advance of the loss of cell viability, suggesting metabolic enzyme inhibition is amongst the initial mechanisms of action for the synergistic

combinations of polymyxin B with the chalcone-derivatives against Gram-positive *S. aureus* species.

Although the chalcone-derivatives were ineffective as antimicrobial agents alone, a combination of either F1 or F23 with polymyxin B induced a notable antibacterial effect. Similarly, although polymyxin B alone is known to be ineffective against Gram-positive bacterial species, the combination of this polypeptide antibiotic with either of the semi-synthetic agents elicited an extended spectrum of activity, resulting in antibacterial actions against both Gram-positive and negative bacterial species.

The predominant reason for using antibiotic combinational therapy, especially during an empirical treatment, includes the need for a wider spectrum of coverage (Viscoli and Castagnola 2002), improved action against multiple-drug resistant strains (Nicas, Zeckel and Braun 1997), a reduction in the potential for dose-related adverse events due to the use of lower doses of the combined drugs (Pal and Mitra 2006) and an improvement in the antibiotic pharmacokinetic profile (Roling et al. 2002a). Although a combined application of different antibiotics can result in competitive antagonism if the agents share either a common mechanism of action or are acting on the same target site (Roling et al. 2002b), the use of polymyxin B with the chalcone-derivatives (either F1 or F23), elicited an enhanced bactericidal effect. Considering that the molecular structures presented in figure 1.5 show the absence of a net-charge in the chalcone-derivatives and that these compounds are hydrophobic, these agents may on their own be possessing low membrane activity and were also to have been antagonistic in action with the polymyxins, whose primary target is the disruption of the cell membrane (Vaara 2010).

Previous studies have shown that the antibacterial activity of aminoglycosides and daptomycin are affected by media composition (König, Schwank and Blaser 2001), including the addition of Ca^{2+} (Barry, Fuchs and Brown 2001). The extent of the interference caused by these factors varies with the class of organism such that Gram-negative species have been shown to be more sensitive to both antibiotics *in vivo,* in the presence of externally added Ca^{2+} (Benvenuto et al. 2006, da Cunha Camargo et al. 2008,Baltz 2009, Kotra, Haddad and Mobashery 2000). This observation is thought to be associated with the preferential binding of these cationic agents to the lipopolysaccharide layer and competitive displacement of native Ca^{2+} as well as Mg^{2+} from the membrane bilayer surface

(Zhang, Rozek and Hancock 2001a, Hancock and Rozek 2002a). Variation in media content has been shown to significantly influence the degree of action of some antibiotics, especially those with weak basic molecular structures such as daptomycin, which has a pKa value of approximately 7.5 as well as about 50 % ionisable functional side-chains at pH 7 (Chopra 2007). The addition of 20 % albumin and 50 mg/ml Ca^{2+} significantly improves the antibacterial action of daptomycin (Thorne and Alder 2002) through mechanisms thought to involve the reduction of the quantity of free divalent cations in the test medium, thereby promoting competitive binding of daptomycin to the anionic lipopolysaccharide which in turn results in depolarisation and disruption of the outer membrane structure (Baltz 2009). One lapse in the present study was that the effect of growth media on the activity of the semi-synthetic chalcone-derivatives was conducted with only Mueller-Hinton broth, such that the effect of media-content variation on the antibacterial action of these chalcone derivatives with polymyxin B remains to be determined. So the effect of pH fluctuation, broth content variation and presence of cations like Ca^{2+}, Mg^{2+} and Zn^{2+}. on the antibacterial activity of effective combination concentrations of polymyxin B with the chalcone-derivatives would be worth investigating in future. Furthermore, the effect of nutrients present in Mueller-Hinton broth on the activity of chalcone-derivatives was considered only against Gram-positive *S. aureus*. Such an analysis should be extended to cover Gram-negative organisms in order to give a balanced view and a more reliable interpretation of the entire data.

Polysorbate 80 enhances the concentration-dependent antibacterial activity of oritavancin when assessed by *in vitro* time-kinetic assays (Belley et al. 2009, McKay et al. 2009a) by functioning as a surfactant that promotes solubilisation of this oligopeptide antibiotic whilst minimising the adherence of this drug to experimental vessel walls. The effect of this pharmaceutical excipient (Gourley et al. 2007) on the antibacterial activity of both chalcone-derivatives alone and their effective combinations with other agents should be considered in the future, since DMSO was used as a solvent for the analysis of these semi-synthetic compounds.

A systematic review of PubMed and Scopus databases on the studies conducted so far to determine the comparative effectiveness of polymyxins either as a monotherapy or as a combinational treatment with other antibiotics such as rifampicin and carbapenems was undertaken (Petrosillo, Ioannidou and Falagas

2008). This review found 16 studies that had previously evaluated the effectiveness of these agents. All the studies were carried out using Gram-negative bacterial species giving an impression that their objectives included a view towards improving the action of this polypeptide agent against resistant strains of this class of organisms, with a particular emphasis on *Ps. aeruginosa*, *Acinetobacter baumannii* and *E. coli* (Petrosillo, Ioannidou and Falagas 2008), whilst preserving the clinical lifespan of this antibiotic class (Falagas, Kasiakou and Saravolatz 2005). On the other hand, the data presented in this report primarily focused on extending the antibacterial coverage of polymyxin B to include Gram-positive organisms through a combined application with chalcone-derivatives whilst also improving the efficacy of polymyxin B against Gram-negative organisms. The finding that F1 applied in combination with polymyxin B demonstrated an augmented activity against MSSA, MRSA, QRSA, *C. violaceum*, *E. coli* and *Ps. aeruginosa* in both a chequerboard and time-course colony counting assays is therefore noteworthy.

The antibacterial action of the combination of the polypeptide antibiotic with either of the chalcone-derivatives may not be attributable to one specific mechanism, as there are several possible targets which include dissipation of membrane potential, disruption of membrane integrity, blockage of cell wall synthesis, interaction with nucleic acids as well as impairment of the activity of metabolic and essential enzymes, (Carson, Mee and Riley 2002). Although these represent the most likely targets, some of these potential sites may not be affected in isolation due to the triggering of a cascade of events. The locations within bacterial cells thought to be the sites of action upon which the combination of polymyxin B with the chalcone compounds act include membrane depolarisation, especially in Gram-positive organisms, as has been demonstrated in published data for *S. aureus* challenged with daptomycin (Silverman, Perlmutter and Shapiro 2003). The blockage of cell wall synthesis by the combined agents, especially under bacterial growth conditions, may also be a possibility considering that these compounds have demonstrated the capacity to prevent the multiplication of *S. aureus* in broth under suitable growth conditions (figure 5.4). Similarly, the impairment of cell wall synthesis is a proven mechanism of action with β-lactams, glycopeptides and others (Rivera and Boucher 2011, Pinho and Errington 2005).

Leakage of intracellular potassium ions is one of the first signs of a damaged bacterial membrane (Johnston et al. 2003, Ioannou, Hanlon and Denyer 2007). Data provided in this study show that the application of polymyxin B with the chalcone-derivatives demonstrated an enhanced leakage of intracellular potassium ions. The occurrence of this event, like the cessation of respiratory metabolic activity, preceded the loss of cell viability in *S. aureus* species. The simultaneous occurrence of membrane depolarisation, leakage of intracellular cations and either interference with cell wall expansion and synthesis or an outright damaging of existing cell wall structures have been noted with vancomycin, teicoplanin, daptomycin and oritavancin (Howden et al. 2010, Kim et al. 2008, Domenech et al. 2009, Baltz 2009). But an area that was clearly not investigated in this study is the capacity of the combined agents (polymyxin B with chalcone-derivatives) to induce damage to membrane proteins. Damage to membrane proteins especially by reactive oxygen species and oxidative stress are recognised mechanisms of bacterial destruction by antibiotics (Cabiscol, Tamarit and Ros 2010). There is little possibility that the combination of polymyxin B with either of the chalcone-derivatives can impede the activities cell proteins within the cytoplasmic membrane as seen with other antimicrobial peptides, which are able to impair septum formation in bacterial binary fission (Kois et al. 2009), but there is a strong speculation that the induction of pore formation in the cytoplasmic bilayer (Brogden 2005) may be involved. The data provided from bacterial metabolic assays showing a cessation of the activity of reducing enzymes in *S. aureus* by the combination of polymyxin B with either F1 or F23 may also be supporting another speculation for the existence of a simultaneous impairment of the activity of other enzymes such as ATPase, located within the cytoplasmic membrane (Fekkes and Driessen 1999, Qu and Sharom 2002). This needs to be further investigated.

The combination of polymyxin B with the chalcone-derivatives may have led to the production of a partly lipophylic entity that can be attached/accumulated in the lipid bilayer, causing a distortion of either the lipid-lipid or lipid-protein interaction. Because the newly assembled moiety may have an amphiphilic character, the lipophilic component of this structure could have exercised a direct engagement with the hydrophobic areas of the bacterial bilayer proteins (Denich et al. 2003), potentially causing a depletion of the proton-motive force. The depletion of the proton-motive force by the activity of certain antibiotics [such as

lactosporins, bacteriocins, megainin, etc., (Riazi, Dover and Chikindas 2012)], and by nisin in species of mycobacterium (Chung, Montville and Chikindas 2000) is well established. Nisin causes a time and concentration dependent reduction of cytoplasmic ATP levels without any evidence of ATP efflux in *M. bovis*-BCG by decreasing both components of the proton motive force (membrane potential, Δψ and ΔpH) (Chung, Montville and Chikindas 2000). The depletion of ATP can lead to catastrophic events that culminate in cell death (Nicholls and Budd 1998). Similarly depletion of NAD+, an important co-factor in bacterial metabolism, is known to occur through hyper-activation of PARP-1 following DNA damage through the activity of alkylating agents (Grahnert et al. 2011). This leads to the reduction in the amount of NAD+ within the cytoplasm, a cessation of glycolysis as well as depletion of the intracellular ATP pool and eventually cell death (Blank and Shiloh 2007). This form of induced cell apoptosis occurs because, in a bid to re-synthesize more NAD+, the bacterial species consume the reserve stock of ATP, thereby exacerbating the energy crisis that leads to cell death (Pieper et al. 1999).

The data provided in this study clearly shows polymyxin B (and colistin) exhibited capacities to initiate sub-cellular events that led to the impairment of the activities of essential metabolic respiratory enzymes in species of *S. aureus*, *Ps. aeruginosa* and *E. coli*. The combination of polymyxin B with the chalcone-derivatives demonstrated an extension of the activity of polymyxin B to cover Gram-positive *S. aureus* through an enhanced membrane depolarisation, increased leakage of intracellular content as well as an augmented impairment of the activity of biological reducing enzymes.

9.2 Enhanced uptake may be a vital step for the synergistic actions of polymyxin B with the chalcone-derivatives

The possible means by which polymyxin B undergoes self-promoted uptake across bacterial membranes have been extensively studied (Hancock 1997b, Brogden 2005). The barrel-stave method appears most suitable for explaining the uptake of polymyxin B with the chalcone-derivatives since the trans membrane pore formation would more likely facilitate a continuous translocation of both compounds. It can be speculated that polymyxin B was being synergistic

with these semi-synthetic hydrophobic compounds through their promoted uptake across the outer (in Gram-negative bacterial cells) and cytoplasmic membrane barriers. The antimicrobial activity of hydrophobic compounds is greatly impeded because of a reduction of access to their intracellular target sites due to the barrier instituted by the presence of the lipopolysaccharide layer in Gram-negative bacterial species, as well as by highly effective efflux pumps mechanisms (Hancock 1997c, Hancock and Chapple 1999). It is possible that their poor aqueous solubility makes it more difficult for these semi-synthetic agents to permeate bacterial outer and cytoplasmic membranes despite their relatively modest molecular weights (figure 1.5) and in addition, a great fraction of hydrophobic compounds are extruded by the activity of efflux pumps (Fernández, Breidenstein and Hancock 2011, Hancock 1997d). More so, both F1 as well as F23 appear to bear no net surface charge and may consequently not be exhibiting enough physical and ionic interactions with either the polyanionic lipopolysaccharide outer bilayer in Gram negative bacterial species or teichoic and lipoteichoic acids in Gram-positive species, limiting their trans-location capacities. Such physical and chemical ionic interactions are prerequisites for enhanced antibiotic uptake and actions which can ultimately result in cell death, especially those initiated through membrane depolarisation and disruption (Falagas, Kasiakou and Saravolatz 2005, Hancock and Sahl 2006).

Unlike chalcone-derivatives, polymyxins are amphipatic compounds with hydrophobic fatty acyl regions attached to their amino terminus (Tsubery et al. 2000, Giacometti et al. 2000). Polymyxin-like peptide, ranalexin, enhances the entry of several hydrophobic substrates into both Gram-positive and Gram-negative bacterial cells (Giacometti et al. 2000). Hydrophilic cAMPs applied together with hydrophobic fluorescent compounds increase the diameter of the outer membrane porins and inadvertently enhance the influx of hydrophobic fluorescent probes (Delcour 2009). Therefore both ranalexin and polymyxins can act in a manner that potentiates the antibacterial action of lipophilic and amphiphilic agents such as rifampicin, erythromycin, fusidic acid and novobiocin against *Ps. aeruginosa* (Hancock 1997c, Giacometti et al. 2000, Vaara and Porro 1996). In addition, the application of polycationic peptides (ranalexin) in combination with polymyxins yields synergistic antimicrobial action (Knoetze 2006). This is understandable considering that both compounds have similar mechanisms of interacting with the phospholipids of cell membranes, increasing

bilayer permeability and disrupting osmotic integrity (Giacometti et al. 2000, Giacometti et al. 1999).

Compared to Gram-positive cells, Gram-negative species possess a layer of peptidoglycan with a reduced thickness and an outer membrane in addition to the cytoplasmic membrane that exists in both classes of bacteria (Pagès, James and Winterhalter 2008). The outer-membrane functions as a selective sieve and a permeability barrier, bearing additional efflux mechanisms as well as a periplasmic space that can accommodate β-lactamase enzymes (Hancock and Rozek 2002b). All these factors collectively function to make Gram-negative bacteria intrinsically more resistant to most antibiotics (Li, Zhang and Poole 2000). But, Gram-negative bacterial species are more susceptible to colistin and polymyxin B because these agents competitively interact with the anionic lipopolysaccharide, underging a self-promoted uptake (Hancock 1997b), and causing disruption of this polyanionic outer membrane bilayer that is completely absent in Gram-positive organisms (Falagas, Kasiakou and Saravolatz 2005). Therefore, the observation that the combination of polymyxin B with the chalcone-derivatives was bactericidal against Gram-positive *S. aureus* species became very crucial in this study.

The thicker peptidoglycan layer in Gram-positive bacteria may also be partly responsible for making species of *S. aureus* particularly insusceptible to the activities of some antibiotics, including the polymyxins (Lorian 2005, Russell 1998, Epand et al. 2006). Vancomycin-intermediate resistant *S. aureus* (VISA) isolated from patients with previous exposure to vancomycin have been noted for their exceptionally thicker cell wall (Appelbaum 2007) developed in response to an altered composition of the peptidoglycan layer (Sieradzki, Pinho and Tomasz 1999). This altered morphology creates an environment enabling the continuation of cell wall synthesis even in the presence of glycopeptides due to the inability of these agents to gain sufficient access to these target sites, due to entrapment of a large fraction of the administered dose within the enlarged wall structure (Sieradzki, Pinho and Tomasz 1999). Findings in this study suggest that although the chalcone-derivatives were weak in antibacterial actions on their own, their effectiveness may be greatly improved with increased translocation across bacterial membrane bilayer barrier.

9.3 Inhibition of efflux mechanisms as a potential mechanism of polymyxin B combined with the chalcone-derivatives

Although the inhibition of bacterial efflux mechanisms, such as the blockage of the activities of the ABC transporters involved in the extrusion of tetracyclines by reserpine and verapamil (Mahamoud et al. 2007), has not been previously ascribed to the polymyxins, it may be worthwhile investigating the possibility of this combination of this latter agent with the chalcone-derivatives to act through this mechanism. The combination of MC-207,110 with laevofloxacin against *Ps. aeruginosa* and *E. coli* caused a reduction of intrinsic resistance of these organisms to fluoroquinolone (Lomovskaya et al. 2001b). An 8-fold reduction in the intrinsic resistance of both organisms was induced by the drug combination through the potentiating action of MC-207,110, a broad spectrum efflux pump inhibitor effective against all three Mex efflux pumps in *Ps. aeruginosa* and their homologues AcrAB-TolC in *E. coli* (Lomovskaya et al. 2001b). It may be worth investigating whether the potentiated action of polymyxin B with the chalcone-derivatives against Gram-negative species of *Ps. aeruginosa* and *C. violaceum* is due to an inhibitory effect upon mechanisms responsible for an early efflux of the combined agents. Regardless of how the synergistic action is imparted, the antibacterial activity of polymyxin B singly applied against *S. aureus* (MSSA, MRSA and QRSA), *C. violaceum* as well as *Ps. aeruginosa* was potentiated from being bacteriostatic to bactericidal with the addition of small fractions of either of the semi-synthetic agents. The activity of ciprofloxacin when combined with the semi-synthetic agents was also augmented against *S. aureus* in a chequerboard assay.

But apart from efflux, bacterial resistance to fluoroquinolone antibiotics can also occur by target mutations, occurring mainly in quinolone-resistant determining regions (QRDRS) (Piddock 1995, Hartmann et al. 1998), in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). It would be important to investigate in future whether the chalcone derivatives are able to block the activity of efflux pumps through the inactivation of QRDR in *S. aureus* when applied with polymyxin B. In this regard, reserpine is a known MDR pump inhibitor for Gram-positive bacteria, mammalian cells and p-glycoproteins (Markham 1999), with an additional capacity to suppress the emergence of

ciprofloxacin-resistant mutants in *S. aureus* and *S. pneumonia* (Markham 1999, Markham and Neyfakh 1996). It may be worthwhile to examine whether the chalcone derivatives are able to inhibit NorA MDR efflux pump activity in *S. aureus*. Such an investigation may be extended to cover whether the activity of the chalcone-derivatives, when combined with polymyxin B involves the inhibition of MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux mechanisms in *Ps. aeruginosa*. Such an action is known to be able to significantly improve the antibiotic activity of complement compounds via a decrease in intrinsic resistance, reversal of acquired resistance as well as a reduction in the potential for the emergence of mutant strains (Lomovskaya et al. 2001b).

9.4 The semi-synthetic flavonoids (F1 and F23) demonstrated a potential to synergistically extend the spectrum and improve the efficacy of polymyxin B

Although polymyxins are ineffective against Gram-positive species, an extension of their antibacterial spectrum through the complementary activity of the chalcone-derivatives would be a development with clinical advantage. But on their own, the polymyxins are effective agents against Gram-negative bacterial species and are reserved for multi-resistant strains (Falagas, Kasiakou and Saravolatz 2005). Published data show that colistin was rapidly bactericidal with as much as a 5 log cfu/ml reduction to strains of *Ps. aeruginosa* within 2 hours (Gunderson et al. 2003, Li et al. 2001a). However, cell re-growth occurs after about 8 hours. The data presented from this study (chapters 4 and 5) show that the combination of polymyxin B with the chalcone-derivatives demonstrated improved/synergistic activity against *Ps. aeruginosa* under buffer environment. Such studies should be repeated with polymyxin B applied with either of the chalcone-derivatives in broth. In the event of an absence of re-growth, this would verify that it could be advantageous to use polymyxin B with these semi-synthetic agents. It is worth mentioning at this point that these chalcone-derivatives were synthesized through a one-step microwave assisted heterocyclisation pathway (Abdel Ghani et al. 2008), making it relatively simple for these compounds to be produced. Hence if the combined application with

polymyxin B is considered to be clinically useful, a continuous supply of the chalcone-derivatives at a reasonable cost is guaranteed.

Cationic antimicrobial peptides (cAMPs) that are bactericidal with kill rates as high as 99.99 % within a short duration are thought to cause depolarisation of membrane potential (Baltz 2009, Cotroneo et al. 2008), disruption of the bilayer structure (Hancock and Rozek 2002b), as well as cell de-energization (Hancock and Rozek 2002a, Tam et al. 2005, Steinberg et al. 1997). The data presented from this study strongly suggests that these polycationic agents are able to block the activity of enzymes responsible for essential metabolic processes. The capacity of these agents to dissipate the membrane bilayer was previously exemplified with the accumulation of triphenymethylphosphonium and safranine *O* by *E. coli* membrane vesicles in the presence of suitable electron donors, which is regarded as an indication of membrane depolarisation (Sakai, Houdebert and Matile 2003), as this process occurs only in response to an induced intracellular potassium ion gradient. But the ability of the combination of polymyxin B with the chalcone-derivatives to induce the leakage of intracellular potassium ions from *S. aureus* demonstrated in this study (figures 7.12 and 7.13), may have been preceded by membrane depolarisation through the dissipation of trans membrane ΔpH and Δψ across the bilayer.

With *Ps. aeruginosa*, the suppression of ATP generation and utilisation might have been temporary or else the organism developed a by-pass mechanism enabling the transitioning from aerobic to anaerobic respiration (Hassett et al. 2002) and thereby overcoming the effect of the inhibition of essential metabolic pathways. This speculation may provide an explanation for the re-growth recorded with higher cell densities challenged with single applications of colistin (Gunderson et al. 2003) and polymyxin B (Tam et al. 2005) for assays conducted in broth. The synergistic action recorded for the combination of 128 µg/ml of polymyxin B with either of the chalcone-derivative that demonstrated an excellent reduction of the *S. aureus* colonies should be re-evaluated under bacterial growth conditions. This combination concentration that displayed an excellent antibacterial action alongside a weak haemolytic effect should also be noted for further analysis. Like the commencement of antibiotic action of daptomycin (Castanheira et al. 2008, Cotroneo et al. 2008), it is possible that the combined application of both compounds stated above interacted with the acyl portion of the bacterial membrane bilayer to institute the depolarisation as

well as cause an alteration of the surface membrane structure in a manner that enables the inward permeation of more of both drug entities, similar to the barrel-stave analogy (Bradshaw 2003, Brogden 2005). This speculation is based on the knowledge that antimicrobial peptides always associate with membrane lipid head groups (Brogden 2005). There is no evidence at the moment as to whether or not pore formation ensues, but the data provided strongly suggest the occurrence of cytoplasmic membrane depolarisation (figures 7.1 and 7.2), as well as disruption, which is evidenced by the leakage of intracellular K^+ (figures 7.12 and 7.13), influx and emission of the intact membrane bilayer-impermeable Sytox Green stain (figures 7.16 to 7.18), followed by cell death (chapter 5). This is almost the same sequence of events for daptomycin, albeit with emphasis on the occurrence of membrane depolarisation as a prerequisite (Silverman et al. 2005, Silverman, Perlmutter and Shapiro 2003).

The disruption of the trans membrane electrochemical gradient by daptomycin in *B. megaterium* leads to the blockage of active transport of cell wall amino acids, formation of sugar-peptide precursors and peptidoglycan synthesis (Wagenlehner and Naber 2004). Although Jung and colleagues have shown evidence that membrane depolarisation can occur after cell death upon bacterial exposure to daptomycin (Jung et al. 2004b), the data provided in this study for the membrane activity of polymyxin B with the chalcone-derivatives suggest depolarisation preceded cell death for challenged *S. aureus* species. For whilst membrane depolarisation and inhibition of metabolic activity were seen to occur within 60 minutes (figures 7.1 and 7.2; chapter 6), significant loss in viability were often recorded beyond 120 minutes (chapter 5). The bactericidal action of daptomycin and vancomycin is optimal when cells are challenged in the logarithmic rather than stationary growth phase (Snydman et al. 2000), unlike with oritavancin (Mercier, Stumpo and Rybak 2002). It is yet to be ascertained as to whether or not the combined action of the chalcone-derivative with polymyxin B is growth-phase specific. However, an equivalent of 8 x MIC F1 only application was seen to demonstrate bacteriostatic action against species of *S. aureus* in both broth and buffer. The data provided in this study also suggest that, like the action of daptomycin against *S. aureus* (Sauermann et al. 2008), the antibiotic effect of the combined application of the semi-synthetic agents with polymyxin B was concentration-dependent. Therefore, should *in vivo* studies be warranted in future, the efficacy of the combinations would best be correlated

with either the peak concentration (C_{max}) or a 24 hours area under the concentration versus time curve (AUC), in relation to the MIC demonstrated against the given bacterial strain (Thorne and Alder 2002, Dandekar et al. 2004, Safdar, Andes and Craig 2004), since this will give a clearer indication of the bioavailability profiles for both agents in relation to their activity over time.

Also in the future, further toxicity studies will be required given that the combination of either of the chalcone derivatives with polymyxin B has been suggested to cause a blockage of the activity of reducing enzymes such as NADH and succinate dehydrogenase, which are present in both prokaryotic and mammalian host cells. But a more specific toxicity study should focus on the potential of these polymyxin B chalcone-derivatives combinations to aggravate the neuro and nephro toxicities that were previously associated with the former antibiotic. Structural studies to improve the aqueous solubility of these semi-synthetic flavonoids may include the addition of either gallate or citrate sub-group to the chromone-ring of these compounds. This sort of structural modification improved solubility, hydrolytic stability and enhanced the antibacterial action of both (-)-epicatechin (as the gallate) (Stapleton et al. 2007). This might speculatively lower their MICs to sub-microgram values and encourage the undertaking of other molecular biology experimentations as well as formulation studies. Considerations may latter be given to potential formulations containing polymyxin B with either of the chalcone-derivatives in the form of external applications such as ointments, lotions, nebulised powders (for cystic fibrosis), dusting powders for skin ulcers and as either sanitizers or disinfectants.

9.5 Conclusion

In addition to membrane bilayer depolarisation and disruption, another plausible mechanism for the antibacterial action of polymyxins demonstrated in this study is the inhibition of essential bacterial enzymes activity, displayed by their capacity to block cell respiratory metabolism. The combination of polymyxin B with either of the chalcone-derivatives (F1 and F23), demonstrated a potential extended spectrum to include Gram-positive species of *S. aureus*, as well as an enhanced efficacy against *Ps. aeruginosa* and *C. violaceum*, by this decapeptide antibiotic. The combination of both agents dissipated cell membrane potential,

induced leakage of intracellular cations as well as enabled the influx of Sytox Green thereby indicating the infliction of major membrane disruption. Furthermore, the combination of either of both compounds caused the cessation of metabolic activity and eventually led to cell death. Future optimisation through structural activity modifications as well as formulation studies are needed to develop suitable and effective therapeutic combinations of the chalcone-derivatives with polymyxin B against clinical strains of Gram-positive and negative bacterial species.

10

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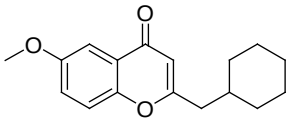
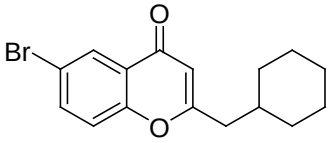
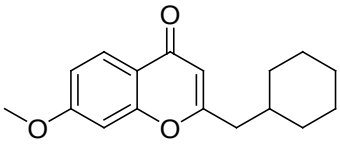
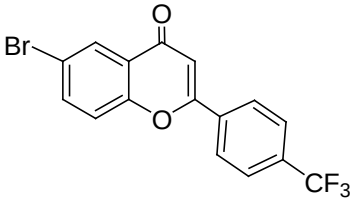
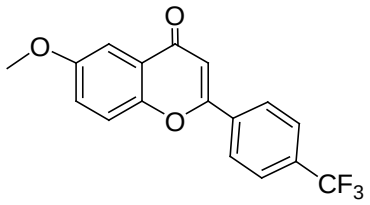
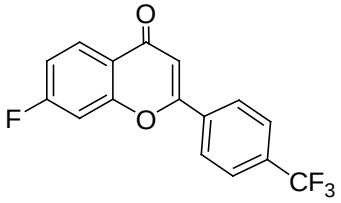
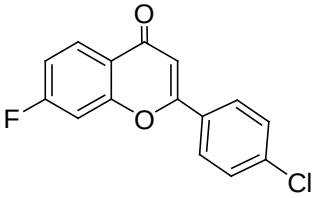
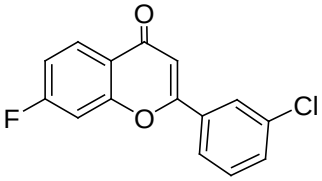
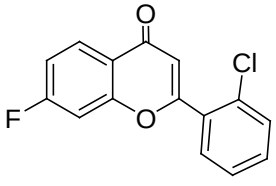
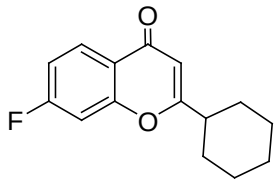
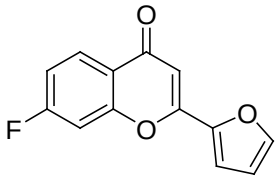
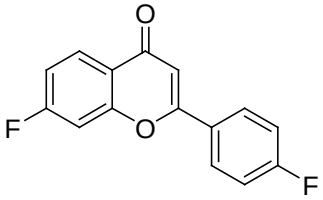
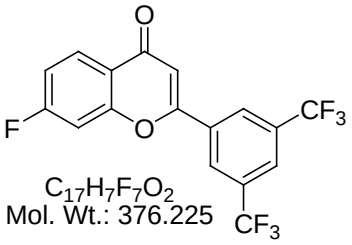
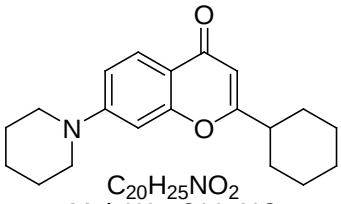
11

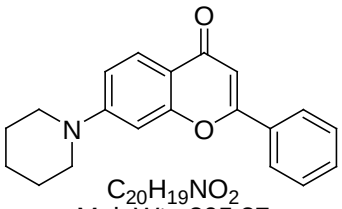
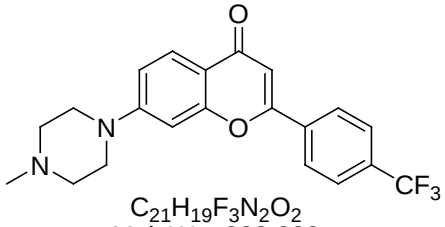
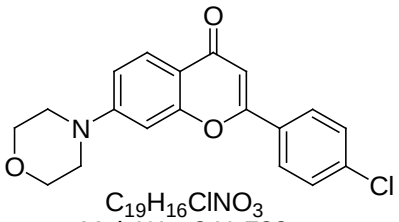
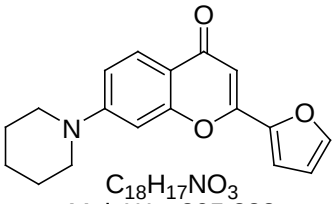
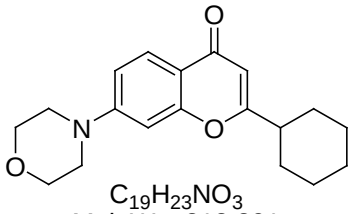
Appendices

11.1 List of the examined chalcone-derivative compounds

Code	Chemical Structure
F1	 $C_{17}H_{20}O_3$ Mol. Wt.: 272.339 2-(cyclohexylmethyl)-6-methoxy-4*H*-chromen-4-one
F2	 $C_{16}H_{17}BrO_2$ Mol. Wt.: 321.209 6-bromo-2-(cyclohexylmethyl)-4*H*-chromen-4-one
F3	 $C_{17}H_{20}O_3$ Mol. Wt.: 272.339 2-(cyclohexylmethyl)-7-methoxy-4*H*-chromen-4-one
F4	 $C_{16}H_8BrF_3O_2$ Mol. Wt.: 369.133 6-bromo-2-(4-(trifluoromethyl)phenyl)-4*H*-chromen-4-one

F5	 $C_{17}H_{11}F_3O_3$ Mol. Wt.: 320.263 2-(4-(trifluoromethyl)phenyl)-6-methoxy-4*H*-chromen-4-one
F6	 $C_{16}H_8F_4O_2$ Mol. Wt.: 308.227 7-fluoro-2-(4-(trifluoromethyl)phenyl)-4*H*-chromen-4-one
F7	 $C_{15}H_8ClFO_2$ Mol. Wt.: 274.674 2-(4-chlorophenyl)-7-fluoro-4*H*-chromen-4-one
F8	 $C_{15}H_8ClFO_2$ Mol. Wt.: 274.674 2-(3-chlorophenyl)-7-fluoro-4*H*-chromen-4-one
F9	 $C_{15}H_8ClFO_2$ Mol. Wt.: 274.674 2-(2-chlorophenyl)-7-fluoro-4*H*-chromen-4-one

F10	 $C_{15}H_{15}FO_2$ Mol. Wt.: 246.277 2-cyclohexyl-7-fluoro-4*H*-chromen-4-one
F11	 $C_{13}H_7FO_3$ Mol. Wt.: 230.191 7-fluoro-2-(furan-2-yl)-4*H*-chromen-4-one
F12	 $C_{15}H_8F_2O_2$ Mol. Wt.: 258.22 7-fluoro-2-(4-fluorophenyl)-4*H*-chromen-4-one
F13	 $C_{17}H_7F_7O_2$ Mol. Wt.: 376.225 2-(3,5-bis(trifluoromethyl)phenyl)-7-fluoro-4*H*-chromen-4-one
F14	 $C_{20}H_{25}NO_2$ Mol. Wt.: 311.418 2-cyclohexyl-7-(piperidin-1-yl)-4*H*-chromen-4-one

F15	 $C_{20}H_{19}NO_2$ Mol. Wt.: 305.37 2-phenyl-7-(piperidin-1-yl)-4*H*-chromen-4-one
F16	 $C_{21}H_{19}F_3N_2O_2$ Mol. Wt.: 388.383 2-(4-(trifluoromethyl)phenyl)-7-(4-methylpiperazin-1-yl)-4*H*-chromen-4-one
F17	 $C_{19}H_{16}ClNO_3$ Mol. Wt.: 341.788 2-(4-chlorophenyl)-7-morpholino-4*H*-chromen-4-one
F18	 $C_{18}H_{17}NO_3$ Mol. Wt.: 295.332 2-(furan-2-yl)-7-(piperidin-1-yl)-4*H*-chromen-4-one
F19	 $C_{19}H_{23}NO_3$ Mol. Wt.: 313.391 2-cyclohexyl-7-morpholino-4*H*-chromen-4-one

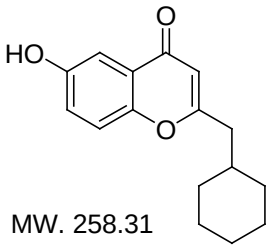
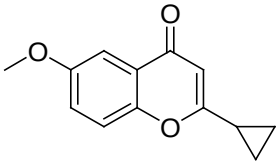
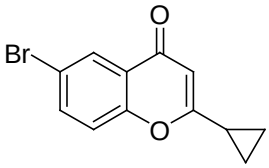
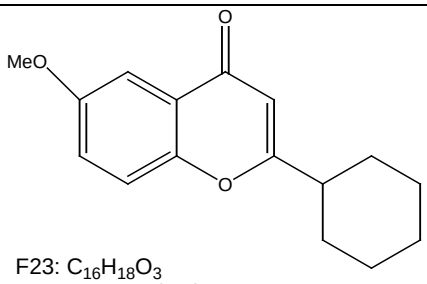
F20	 MW. 258.31 2-(cyclohexylmethyl)-6-hydroxy-4*H*-chromen-4-one
F21	 $C_{13}H_{12}O_3$ Mol. Wt.: 216.2326 2-cyclopropyl-6-methoxy-4*H*-chromen-4-one
F22	 $C_{12}H_9BrO_2$ Mol. Wt.: 265.1027 6-bromo-2-cyclopropyl-4*H*-chromen-4-one
F23	 F23: $C_{16}H_{18}O_3$ MW. 258.31 g/mol 2-cyclohexyl-6-methoxy-4*H*-chromen-4-one

Table 11.1: Chemical structure, molecular weight and scientific names of the examined chalcone derivatives

11.2 Calibration curves for *S.aureus* strains

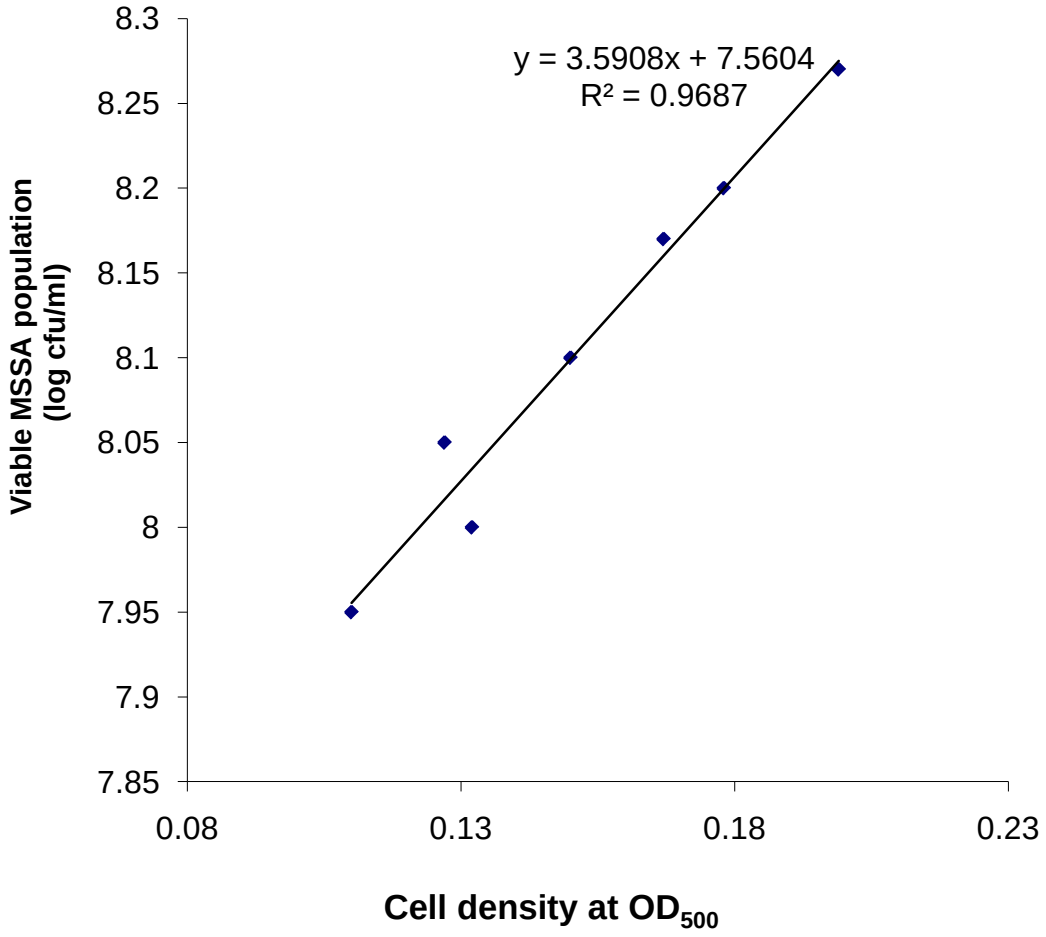


Figure 12.1: Calibration curves for *S. aureus* 6571 (MSSA)

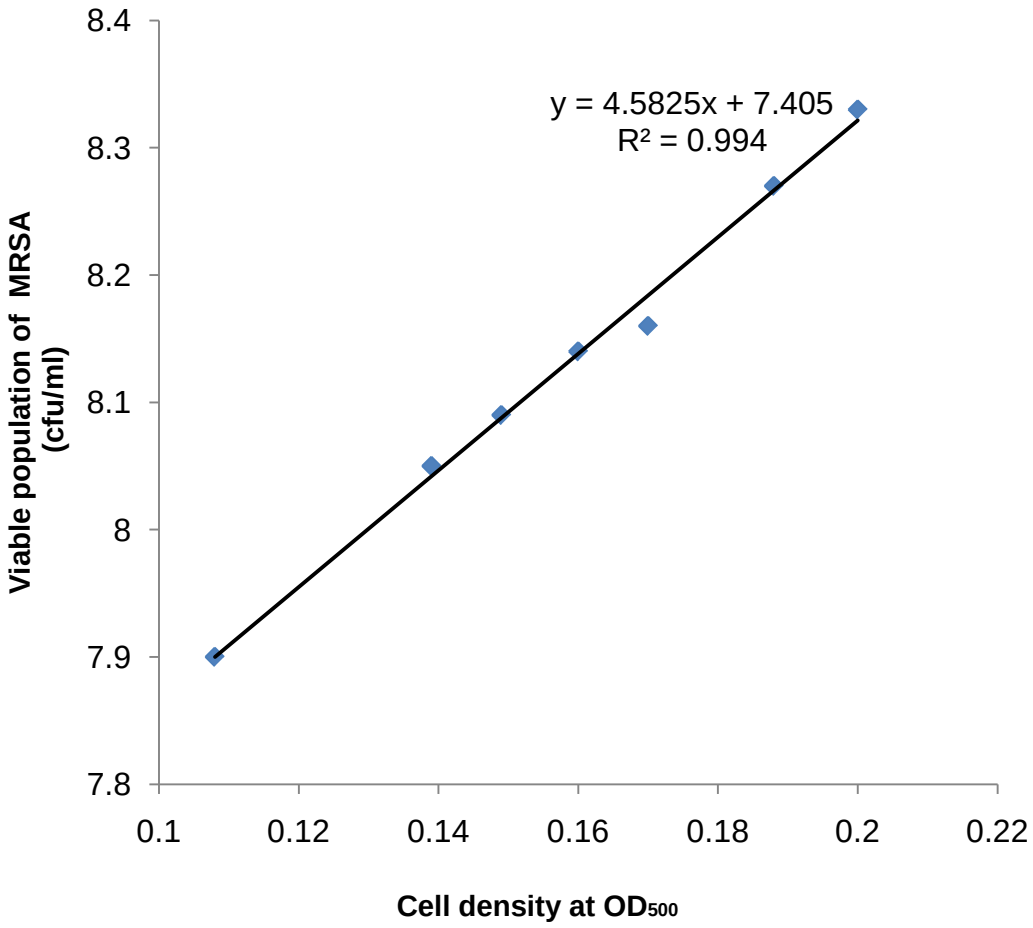


Figure 12.2: Calibration curves for *S. aureus* 11940 (MRSA)

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List of Publications:

i. Convenient One-Pot Synthesis of Chromone-Derivatives and their Antifungal and Antibacterial Evaluation – Paper publication, synthetic communications June 2012.

Complete List of Authors: Abdel Ghani, Sherif; Ain Shams University, Faculty of Agriculture
Mugisha, Patrick; University of Southampton, Chemistry
Wilcox, Juliet; University of Southampton, Chemistry
Gado, Emad; Ain Shams University, Faculty of Agriculture
Medu, Erere; RGU, School of Pharmacy & Life Sciences
Lamb, Andrew; RGU, School of Pharmacy & Life Sciences
Brown, Richard; University of Southampton
Keywords: Flavonoids, Chrom ones, Heterocyclic, Synthesis
Web link: http://www.tandfonline.com/eprint/Xv23YTybNhNJGAe6GxrY/full

ii. Examination of the effect of colistin and polymyxin B upon the metabolic activity of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* in an MTT reduction assay – Abstract and Poster presentation at the research showcase event of the Institute for Social Research and Welfare, RGU, May 2012.

Author: Erere O. Medu.
School of Pharmacy and Life Sciences, RGU, Schoolhill, Aberdeen. AB10 1FR.

iii. Antibacterial evaluation of some semi-synthetic flavone compounds against quinolone- and methicillin-resistant *Staphylococcus aureus* – Abstract and podium presentation in a symposium at the Institute for Social Research and Welfare, RGU, May 2011.

Erere Medu[1], Kerr Matthews[1], Juliet Wilcox[2], Patrick Mugisha[2], Richard Brown[2] and Andrew Lamb[1]

[1]School of Pharmacy and Life Sciences, RGU, Schoolhill, Aberdeen. AB10 1FR.

[2]School of Chemistry, University of Southampton, Highfield, Southampton. SO17 1BJ.

iv. *In vitro* antibacterial evaluation of synthetic flavone compounds against quinolone- and methicillin-resistant *Staphylococcus aureus.*

Abstract and poster presentations at a conference on antibacterial agents supported by the Society for Applied Microbiology and hosted at RGU, Aberdeen. Jun 2010

Erere Medu[1], Richard Brown[2] and Andrew Lamb[1]

[1]School of Pharmacy and Life Sciences, RGU, Schoolhill, Aberdeen. AB10 1FR.

[2]School of Chemistry, University of Southampton, Highfield, Southampton. SO17 1BJ.

v. Structural confirmation by NMR of 3-Octanoyl-epicatechin, a novel antibacterial compound

Poster presentation at a conference on antibacterial agents supported by the Society for Applied Microbiology and hosted at RGU, Aberdeen. Jun 2010

A. Di Salvo[1], **E. Medu**[1], A. Lamb[1]

[1]School of Pharmacy and Life Sciences, RGU, Schoolhill, Aberdeen. AB10 1FR. Scotland.

vi. Msc. Thesis: Evaluation of the antibacterial and antifungal activities of eleagnine from *Chrysophyllum albidum*, G.Don-Holl (Sapotaceae).