Molecular Characterisation and Antimicrobial Activities of Novel Substituted Ferrocenyl Chalcone Derivatives

Elecia Jaime Henry

BSc (Hons), PgCHPE, PgCRM, MSc

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

March 2019

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

1 Abstract

The increasing issues of drug-resistant bacteria has quickly become a global concern as infections spread from healthcare settings to the wider community. The rapid spread of these infections is partly facilitated by a decrease in the development of new drugs.

Because of their antimicrobial properties, ferrocenyl chalcones, which are organometallic chalcone derivatives, have gained attention from researchers. Early classes of ferrocenyl chalcones were reportedly cytotoxic, which encouraged the need to develop next-generation ferrocenyl chalcone compounds that are less toxic.

One-to-one dilutions of stock ferrocenyl chalcone solution and MHB, resulting in the formation of small, brown particles, was suggested to arise from iron ion chelation of casein hydrolysate, a component of MHB. After solubilisation in DMSO, each of the iodine-containing ferrocenyl chalcone compounds, changed from deep red to dark brown. There was no evidence that iodine was displaced when diethyl ether was used.

Using 2-fold broth microdilution, results demonstrated that five of the ten methylated ferrocenyl chalcones, possessed greater antimicrobial activity against resistant and non-resistant Gram-positive organisms than Gram-negative organisms. These compounds contain increasing alkyl chain lengths from pentyl to decyl on ring 'B' of the compounds.

The results of MTT assays of Gram-positive bacteria indicated that there were no viable cells, where the mean (± SD) percentage of actively respiring cells were observed.

SEM images of bacterial cells, which were treated with an active ferrocenyl chalcone, exhibited significant cell membrane damage as ultrastructurally observed.

Key findings of this research indicate that further investigations into these compounds are required so that the inhibition of biofilm formation and the cytotoxicity towards mammalian cells are clearly defined. Additionally, the results

advance the possibility that these newly developed ferrocenyl chalcone compounds could be important antimicrobial agents in the treatment of infections from clinically resistant bacteria in an environment where current treatments are failing.

Table of Contents

2 Table of Figures

3 Table of Tables

4 List of Abbreviations

ABX	Antibiotic
ANOVA	Analysis of Variance
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BSAC	British Society of Antimicrobial Chemotherapy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
CFU	Colony forming units
Ch	Chalcone
CIP	Ciprofloxacin
CLI	Clindamycin
CLSI	Clinical & Laboratory Standards Institute
CPE	Carbapenemase-producing Enterobacteriaceae
CRH	Chesterfield Royal Hospital
ERY	Erythromycin
EUCAST	European Union Committee on Antimicrobial Susceptibility Testing
HCAI	Healthcare-associated infections
IH	Ian Hopkins (Staffordshire University)
IR	Infrared Spectroscopy
ISA	IsoSensitest agar
ISB	IsoSensitest broth
MBC	Minimum Bactericidal Concentration

MDR	Multidrug-resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MHB	Mueller-Hinton broth
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient agar
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCIMB	National Collection of Industrial Food and Marine Bacteria
NCTC	National Collection of Type Cultures
^{1}H NMR	Proton Nuclear Magnetic Resonance
Org	Organism
OXY	Oxytetracycline
PEN	Penicillin
PBS	Phosphate Buffered Saline
SDH	Succinate dehydrogenase
SEM	Scanning Electron Microscopy
UCLAN	University of Central Lancashire
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organisation

1 Introduction

The prevention and treatment of infectious diseases remain critical goals in maintaining a healthy population. While several approaches, such as health surveillance and health management have been implemented, clinicians are faced with substantial challenges in achieving these targets. One of these challenges is the increasing prevalence of the spread of bacterial pathogens and the consequences of this phenomenon, as discussed by Davies (2011).

1.1 Significance and classification of bacterial pathogens

1.1.1 Importance to humans

Published data from the World Health Organisation (WHO) (2015) indicates that, while non-communicable diseases (e.g., cardiovascular disease and diabetes) are responsible for approximately 41 million deaths annually across the globe, infections from microbial pathogens continues to be a significant concern in world public health and remain as one of the top 10 causes of worldwide deaths. Within the past century, there were over half million deaths annually caused by microbial infections, which was higher combined when compared with deaths resulting from other causes such as malaria, with billions more enduring various pathogenic disorders (Mishra & Agrawal, 2012). As detailed by Hogg (2013), several infectious diseases are caused by micro-organisms, mainly bacteria, some of which are identified in Table 1-1.

Table 1-1 *Common infections caused by bacteria* (Hogg, 2013).

Disease	Bacterial Pathogen
Anthrax	*Bacillus anthracis*
Gonorrhoea	*Neisseria gonorrhoeae*
Typhoid fever	*Samonella typhi*
Tuberculosis	*Mycobacterium tuberculosis*
Cholera	*Vibrio cholerae*
Diphtheria	*Corynebacterium diphtheriae*
Tetanus	*Clostridium tetani*
Bacterial Pneumonia	*Streptococcus pneumonia*
Gas gangrene	*Clostridium perfringens*
Plague	*Yersina pestis*
Botulism	*Clostridium botulinum*
Dysentery	*Shigella dysenteriae*
Whooping cough	*Bordetella pertussis*
Rocky Mountain spotted fever	*Rickettsia rickettsii*

Many of the diseases highlighted in the above table, such as tuberculosis, are defined as healthcare-associated infections (HCAIs). *M. tuberculosis*, which is the causative agent of this disease, is one of many bacterial pathogens that are being increasingly isolated from hospital patients who became infected while under care in health settings (WHO, 2015). With a prevalence of 6.4% (HPA, 2012), the spread of HCAI and their subsequent control has become an important issue in hospitals. The frequency of HCAIs are reported to cause adverse consequences, including patient death (WHO, 2015). The Institute of Medicine (2000) reported that there were 90,000 deaths annually in U.S. hospitals because of care-associated complications as discussed by Baron, Jorgensen, & Landry (2007). Nosocomial infections, which also includes those that result from the presence of drug resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA), have begun to emerge in the community and in non-clinical environments (Hadley, 2014). Subsequently, the WHO (2015) compiled a comprehensive report on the existence of other multidrug-resistant (MDR) bacteria

including carbapenemase-producing Enterobacteriaceae (CPEs), the most common species being *Klebsiella pneumonia* and *Escherichia coli* as identified by Tseng *et al*., (2011). The occurrence of both non-resistant and resistant bacterial pathogens, and their effect on human health, promotes the urgent need for potent antimicrobial compounds as reviewed by Henry *et al*. (2017).

The increasing resistance of microorganisms to antibacterial agents is a global threat to public health with over-prescription and misuse of antibacterial drugs being identified as key factors in the development of bacterial antimicrobial resistance (Davies & Davies, 2010). Resistance in bacteria can be influenced by the minimum inhibitory concentration (MIC) of a particular antimicrobial compound at lethal or, more often, at sub-lethal levels, where treatment with low antimicrobial concentrations indirectly result in selection for drug resistance (Andersson & Hughes, 2012). MIC refers to the lowest concentration of antibacterial agent that inhibits growth. Other key contributors to drug resistance in bacteria are those which involve environmentally-derived mutations in bacterial genes that are responsible for antimicrobial susceptibility, both of which may be insular or transferable, (Gullberg *et al*., 2011), and antimicrobial efflux pumps in bacterial cells (Edwards *et al*., 2014). Another contributing factor of drug resistance involves dosage increases and/or inefficient breakdown of antimicrobial agents in the blood leading to raised levels of active drug to favour selection for resistant strains or physiological changes, which may result from altered bacterial growth conditions, that trigger natural resistance, as reviewed by Normark *et al*. (1977) and Song *et al*. (2009). Mechanisms of antimicrobial resistance will be discussed in Section 1.4. The expression of these mechanisms may be determined by the category of bacteria, which are primarily classified based on morphological identification using the Gram stain technique.

1.1.2 Classification of bacteria based on Gram Stain

In 1884, Hans Christian Gram developed a staining method that enables the differentiation between most bacterial cells, as discussed by several researchers including Sumbali & Mehrotra (2009). This differential staining method facilitates the classification of most bacterial cells into Gram-positive and Gram-negative bacteria (Heritage, Evans, & Killington, 2000), classifications which cover the most frequent

micro-organisms involved in antimicrobial resistance. These organisms differ by the structure of their peptidoglycan layer, which surrounds the bacterial cell membrane. Peptidoglycan, also called murein, is a structural, polymer scaffold within the bacterial cell wall (Salton, 1964; Ghuysen, 1968; Schleifer & Kandler, 1972; Vollmer, Blanot, & Pedro, 2008). This polymer is composed of glycans, N-acetylmuramic acid and N-acetylglucosamine, cross-linked with short peptides (Rogers, Perkins, & Ward, 1980; Williams & Edwards, 1994; Heijenoort, 2001; Desmarais *et al*., 2013). The differential feature of Gram's stain involves the use of crystal violet dye, which is trapped within the peptidoglycan layer, depending on the thickness of the layer (Willey, Sherwood, & Woolverton, 2008).

1.1.3 Gram-positive organisms

A Gram-positive bacterial cell possesses a thick peptidoglycan layer (Figure 1-1), approximately 30-100 nm thick and 60% of cellular mass, and is comprised of many sub-layers (Navarre & Schneewind, 1999; Silhavy, Kahne, & Walker, 2010).

This thick layer, which provides protection against turgor pressure from the cell membrane, contains teichoic acids that are primarily copies of various phosphate polymers (Silhavy, Kahne & Walker, 2010). Wall teichoic acids, which are a sub-group of teichoic acids, are covalently bonded to the peptidoglycan layer, while another sub-group of teichoic acids, which are called lipoteichoic acids, are bonded to the lipid components of the cell membrane. Wall teichoic acids are usually comprised of a disaccharide unit with repeating units of either ribitol phosphate or glycerol phosphate, as discussed by Silhavy, Kahne & Walker (2010). Both groups of polymers, which strengthen the structural integrity of the cell, are interwoven within peptidoglycan (Silhavy, Kahne & Walker, 2010). Although lipoteichoic acids are similar to wall teichoic acids in that they contain glycerol phosphate polymers, the chiral nature of their phosphate polymers differ, and their phosphate polymers are comprised of fewer sub-units.

The thick peptidoglycan layer found with Gram-positive bacteria also contain protein units. These structures may be covalently bonded to peptide components of peptidoglycan or they may be attached via ionic bonds to peptidoglycan and teichioic

acid components as surface proteins. The primary role of these proteins involves the identification of constituents of mammalian cellular matrix (Silhavy, Kahne & Walker, 2010).

The main difference between the cell envelopes of Gram-positive bacteria involved the presence of diverse peptide cross linkages within glycan units, e.g., pentaglycine branches are found in *S. aureus* but not in *Bacillus subtilis*.

Due to their thick peptidoglycan layer retaining the crystal violet dye, Gram-positive bacteria appear purple to violet when viewed under the compound light microscope as reviewed by Madigan *et al*. (2012).

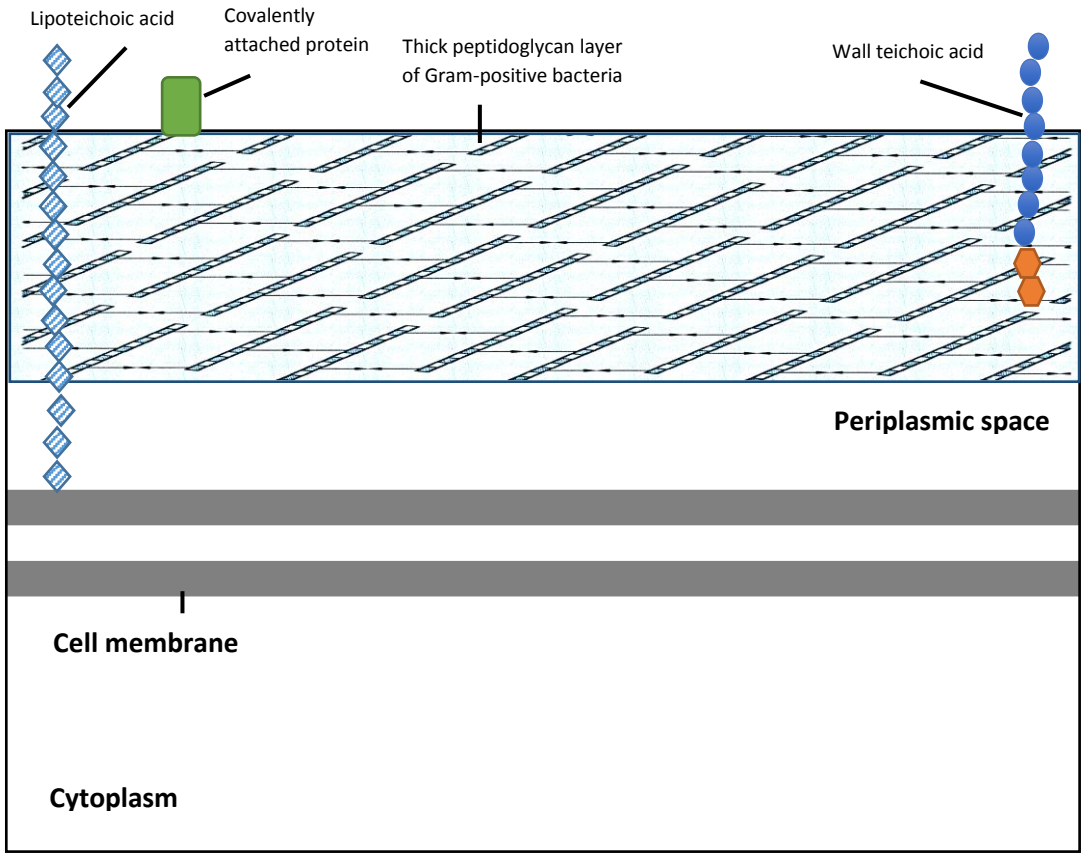


Figure 1-1 *Structure of Gram-positive bacteria with thick peptidoglycan layer (30-100 nm thick), periplasmic space and lipid bilayer of cell membrane (illustration by E. Henry, 2016).*

Bacteria that are classified in this group may be both non-pathogenic and pathogenic. Non-pathogenic Gram-positive bacteria are considered harmless to humans and may be part of the normal microbiota. However, pathogenicity may be induced by physiological and environmental conditions in the host. Pathogenic Gram-positive

bacteria, such as *Staphylococcus aureus*, *Clostridium botulinum*, *Clostridium perfringens* and *Bacillus cereus*, produce enterotoxins and exotoxins that cause foodborne diseases (Jay, Loessner, & Golden, 2005). Common Gram-positive bacteria that cause HCAIs include *Streptococcus mitis*, *Enterococcus spp.*, *Corynebacterium jeikeium* (Zinner, 1999; Gaynes *et al*., 2005).

1.1.4 Gram-negative organisms

The outer membrane, which is not found in Gram-positive bacteria, is a lipid bilayer that is comprised of phospholipids in its inner sub-layer and liposaccharides in its outer sub-layer (Silhavy, Kahne & Walker, 2010). Liposaccharides are endotoxins that contribute to infections in mammalian cells and are comprised of O-antigen and core saccharides. The outer membrane also contains lipoproteins, which possesses lipid groups that are bound to the inner sub-layer of the outer membrane, and β-barrel moieties, which are considered to be transmembrane proteins. While the function of these lipoproteins may not be fully understood, the function of the transmembrane β-barrel porins primarily involve the transport of small molecules such as amino acids (Silhavy, Kahne & Walker, 2010).

Peptidoglycan is also present in Gram-negative bacterial cells. This structure is approximately 1-10 nm thick and also contains sub-layers (Yao, Jericho, & Pink, 1999). Additionally, peptidoglycan is covalently linked to the outer membrane by a lipoprotein, which is commonly known as Braun's lipoprotein, and is primarily found in *E. coli*. In contrast, ionically bonded lipoprotein can be found in *P. aeruginosa*, as discussed by Yao, Jericho, & Pink (1999).

Unlike Gram-positive bacteria, Gram-negative bacteria possess thin peptidoglycan layers (Figure 1-2), which do not trap crystal violet dye (Willey, Sherwood, & Woolverton, 2008). When counterstained with safranin, Gram-negative bacteria appear pink to red when viewed under the compound light microscope (Madigan *et al*., 2012). Gram-negative bacteria can also be both non-pathogenic and pathogenic. Pathogenicity in these microorganisms can also be activated in the host under physiological and environmental conditions. Common Gram-negative bacteria that

cause HCAIs include *E. coli*, *K. pneumonia*, and *Pseudomonas aeruginosa* (Gaynes *et al*., 2005).

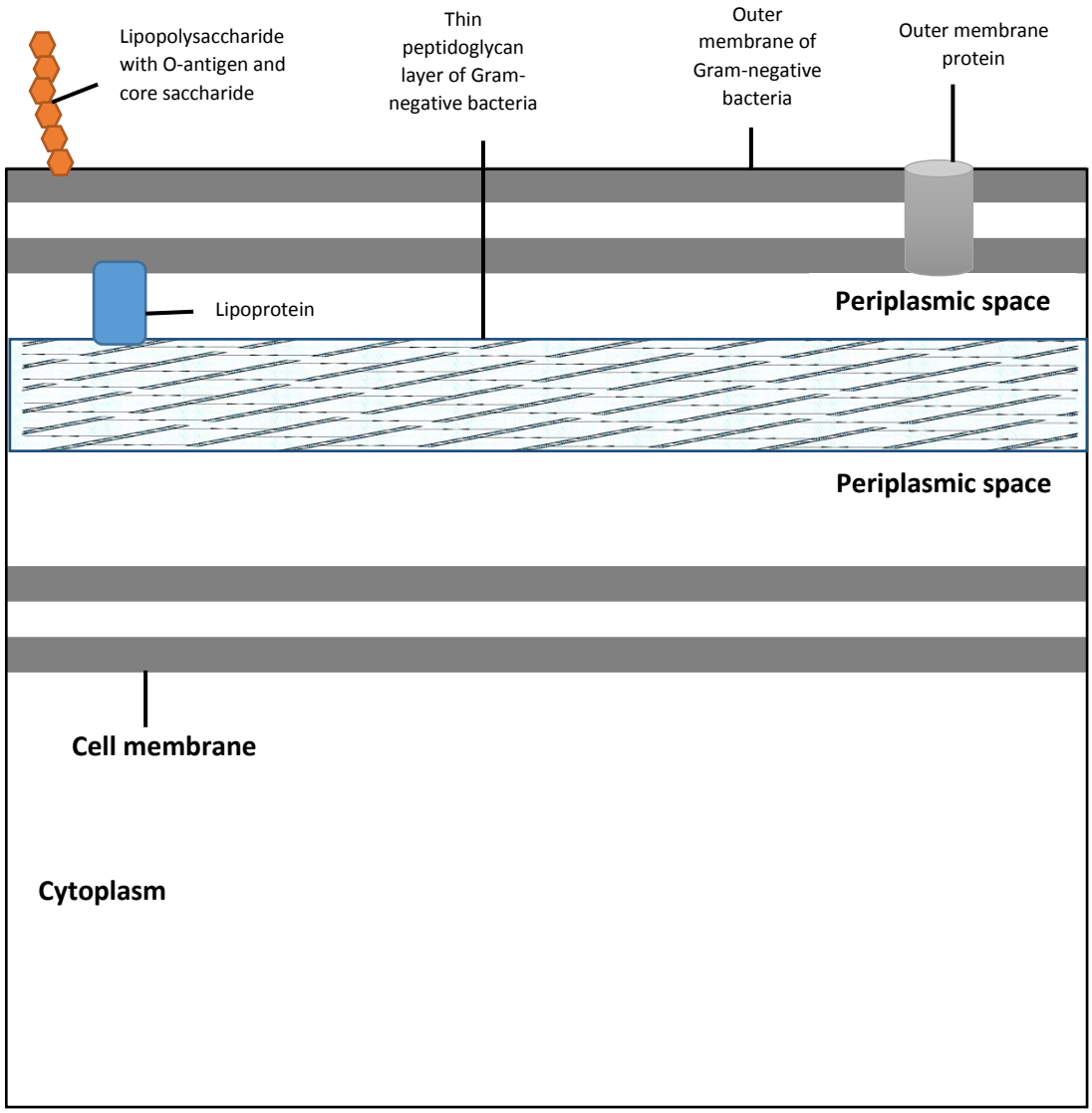


Figure 1-2 *Structure of Gram-negative bacteria with outer membrane, thin peptidoglycan layer (1-10 nm thick), periplasmic space and lipid bilayer of cell membrane (illustration by E. Henry, 2016).*

1.2 Traditional antibiotics and antimicrobial agents used in the treatment of bacterial infections

1.2.1 Classification and sources of antibiotics and antimicrobial compounds

An antibiotic, a term that was first used by Waksman in 1941, is a compound that is used to treat microbial infections by inhibiting microbial growth, as reviewed by Clardy, Fischbach, & Currie (2010). Conventional antibiotics, which are manufactured by micro-organisms such as bacteria and fungi or as synthetic agents, possess either bactericidal (killing) or bacteriostatic (growth inactivity) mechanisms (Madigan *et al*., 2012). The first well-known antibiotic, penicillin (PEN), which was produced by the *Penicillium* fungal species, was discovered by Alexander Fleming in 1928 (Aminov, 2010). This discovery initiated the "Golden Era of Antibiotics", which lasted until the 1970s, and resulted in the development of several classes of solely synthetic compounds known as antimicrobial agents, as reviewed by Phoenix, Harris, & Dennison (2015). Antibacterial agents can be classified as antibiotic compounds or antimicrobial compounds.

Conventional antibiotics and antimicrobial compounds that have exhibited clinical and financial success are classified according to their target bacterial enzymes (Hughes & Karlén, 2014). The major classifications of these therapeutic agents are detailed in Table 1-2, which is based on information taken from Irving *et al.* (2005) and Coates, Halls, & Hu (2011). Discovery of these antimicrobial agents led to the production of analogues of these drugs.

Table 1-2 *Major antibiotic and antimicrobial classifications, mechanisms of action and examples (Irving, Ala'Aldeen, & Boswell, 2005; Coates, Halls, & Hu, 2011).*

Class	Mode of action	Examples of antibiotics
β-lactam: Penicillins	Inhibit synthesis of bacterial cell wall	Penicillin G, Ampicillin, Piperacillin
β-lactam: Cephalosporins	Inhibit synthesis of bacterial cell wall	Cephradine (1st gen.) Cefprozil (2nd gen.) Ceftriazone (3rd gen.) Cefipime (4th gen.) Ceftaroline (5th gen.)
β-lactam: Monobactam	Inhibit synthesis of bacterial cell wall	Aztreonam
β-lactam: Carbapenems	Inhibit synthesis of bacterial cell wall	Imipenem, Meropenem, Ertapenem
Glycopeptides	Inhibit bacterial cell wall formation	Vancomycin, Teicoplanin, Telavancin
Fluoroquinolones	Inhibit bacterial deoxyribonucleic acid (DNA) replication and transcription	Norfloxacin, Ciprofloxacin, Fleroxacin
Tetracyclines	Inhibit bacterial protein synthesis	Tetracycline, Doxacycline, Methacycline
Aminoglycosides	Inhibit bacterial protein synthesis	Gentamycin, Netilmycin, Kanamycin
Macrolides	Inhibit bacterial protein synthesis	Erythromycin, Clarithromycin, Streptomycin
Other: Polymixins	Produce physicochemical effect that disrupts bacterial cell membrane	Polymixin B, Polymixin E (Colistin)
Other: Sulphonamides	Inhibit folate synthesis in bacteria	Sulfamethoxazole, Sulphanilamide, Sulfadiazine
Other: Isoniazid	Inhibits synthesis of bacterial cell wall components	Not applicable
Other: Trimethoprim	Inhibits bacterial DNA synthesis	Not applicable

1.2.2 Common mechanisms of action of antibiotic and antimicrobial agents

The β-lactam class of antibiotics contains a four-ring chemical structure (Figure 1-3), which inhibits cell wall synthesis by irreversibly binding to penicillin-binding proteins (PBP) such as transpeptidase (Madigan *et al*., 2012).

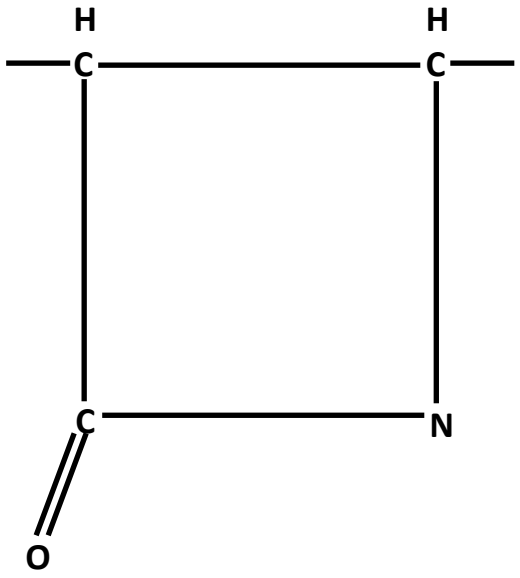


Figure 1-3 *Structure of β-lactam ring (based on a review by Hacker, Messer, & Bachmann, 2009).*

Penicillins, which belong to the β-lactam class of antibiotics, are involved in the inhibition of transpeptidase, which is an enzyme that catalyses the cross-linking of peptidoglycan components (Wise *et al*., 1965; Kong, Schneper, & Mathee, 2011). This class of antibiotics includes PEN-G, ampicillin and methicillin (Willey, Sherwood, & Woolverton, 2008). Cephalosporins, antimicrobial agents that also belong to the β-lactam class of antibiotics, exhibit a mechanism of antimicrobial action that is similar to penicillins but differ in their chemical structure (Madigan *et al*., 2012). This drug is divided into generations from 1st to 5th based on their degree of antimicrobial activity, or antimicrobial spectrum, as detailed by Watkins & Bonomo (2017). Cephalosporins are administered to patients who develop an allergy to penicillins (Campagna *et al*., 2012) and include analogues such as cephalothin (1st generation), cefoxitin (2nd generation) and ceftriaxone (3rd generation) (Willey, Sherwood, & Woolverton, 2008). Fluoroquinolones, also known as quinolones, are broad-spectrum antimicrobial agents that inhibit bacterial DNA gyrase and topoisomerase

IV, which are enzymes that are involved in DNA cleavage, a crucial step in bacterial DNA replication and transcription (Wolfson & Hooper, 1989; Kohanski, Dwyer, & Collins, 2010). Common quinolones include ciprofloxacin (CIP), which is a derivative of an older quionolone known as nalidixic acid, and the next generation quinolone called moxifloxacin, as reviewed by Madigan *et al*. (2012). Tetracyclines block the binding of aminoacyl-transfer ribonucleic acid and the 30S sub-unit in the bacterial ribosome, which results in the inhibition of bacterial protein synthesis (Chopra & Roberts, 2001). Compounds that belong to the tetracycline class of antibiotics include chlortetracycline (1st generation), doxycycline (2nd generation) and tigecycline (3rd generation) (Nguyen *et al*., 2014). The mechanism of action of the aminoglycoside class of antibiotics involves the inhibition of bacterial protein synthesis by binding to bacterial ribosomal ribonucleic acid and transfer ribonucleic acid (Kotra, Haddad, & Mobashery, 2000). These bactericidal agents include neomycin, spectinomycin and streptomycin (Wilson, 2014). Macrolides also possess an affinity for bacterial ribosomes, which results in the inhibition of polypeptides from exiting ribosomes and the synthesis of bacterial proteins (Tenson & Lovmar, 2003; Kannan *et al*., 2014). Antimicrobial agents that belong to this class include ERY, a broad spectrum macrolide, clarithromycin and azithromycin (Madigan *et al*., 2012). Antimicrobials that inhibit bacterial metabolism comprise the classification of other. These antimicrobial agents are structurally similar to bacterial growth factors and metabolites. Examples include sulphonamides, e.g., sulfmethoxazole, which is an analogue of the folic acid precursor called p-aminobenzoic acid, the narrow-spectrum antimicrobial agent known as isoniazid, which is a nicotinamide analogue, and trimethoprim, which inhibits the conversion of dihydrofolic acid to tetrahydrofolic acid by binding to dihydrofolate reductase (Willey, Sherwood, & Woolverton, 2008; Madigan *et al*., 2012).

Antibiotic discovery and antimicrobial development are critical in the treatment of patients with bacterial infections. However, susceptibility of pathogenic bacteria to antimicrobial agents must be assessed prior to therapy, ensuring that health recovery is successful.

1.3 Antimicrobial Susceptibility Testing (AST) of bacteria

AST is the method of categorizing microorganisms as treatable or non-treatable based on the MIC breakpoints, which are the antimicrobial concentrations used to determine susceptibility, of antimicrobial agents used to inhibit growth (Doern, 2011). In terms of antimicrobial activity, results of susceptibility tests classifies bacteria as clinically susceptibility, clinically intermediate and clinically resistant, as detailed by Kahlmeter *et al*. (2003).

European Union Committee on Antimicrobial Susceptibility Testing (EUCAST), which now incorporates the British Society of Antimicrobial Chemotherapy (BSAC), defines a microorganism as susceptible based on the level of antimicrobial susceptibility that leads to a successful therapeutic outcome (MacGowan & Wise, 2005). Intermediate is defined as the level of antimicrobial susceptibility that leads to an indeterminate therapeutic outcome, while resistant is defined as the level that results in antimicrobial susceptibility that leads to the higher possibility of therapeutic failure (MacGowan & Wise, 2005). Several assays can be used to determine the susceptibility of bacteria post-exposure to antimicrobial agents.

The BSAC and the EUCAST have developed standard AST protocols, including the preparation of stock and working antimicrobial solutions, the preparation of bacterial inocula and the steps involved in MIC determination. These standardised methods were detailed by Andrews (2006), Matuschek, Brown, & Kahlmeter (2013) and Wootton *et al*. (2014). Since both committees provide harmonized standardized protocols in AST, the current study, which commenced in 2014, prior to the migration of BSAC susceptibility testing methods to those of the EUCAST in 2016, will primarily refer to BSAC methods (Chapter 2).

1.3.1 Agar well diffusion method

One common assay used to measure the susceptibility of bacteria to antimicrobial drugs is the agar well diffusion method. This method involves the even spreading of an appropriate volume of bacterial inoculum on the entire surface of a sensitivity agar Petri dish, followed by the boring of wells in the sensitivity agar using a sterile cork-borer (Balouiri, Sadiki, & Ibnsouda, 2016). Desired volumes of antimicrobial

solution at various concentrations are pipetted into the wells and the agar plate is incubated at the optimal growth temperature for a period of time, usually overnight (Balouiri, Sadiki, & Ibnsouda, 2016). After incubation, the diameter of the zones of growth inhibition are measured. This method has been used to determine the antimicrobial activity of new drugs, including medicinal plant compounds (Das, Tiwari, & Shrivastava, 2010). Agar well diffusion is a simple assay that requires little to no specialised equipment and the clear zones of growth inhibition can be easily read by clinical technicians. The antimicrobial compound can diffuse directly into the sensitivity agar. This method will be used to determine antimicrobial activity of novel compounds in Section 2.2.4.

1.3.2 Disk diffusion method

Another diffusion method used in antimicrobial sensitivity testing is the disk diffusion method, which is also known as the Kirby-Bauer method as detailed by Engelkirk & Duben-Engelkirk (2008). The standard protocol of this method is similar to that of the agar well diffusion method where the bacterial inoculum is spread on the entire surface of the sensitivity agar. However, unlike the agar well diffusion method, disk diffusion employs paper disks of diameter of 6 mm that are impregnated with a predetermined concentration of antimicrobials (Matuschek, Brown, & Kahlmeter, 2013). These disks are deposited aseptically on the inoculated sensitivity agar, followed by incubation of the plates for the optimal time period and temperature, and the diameter of the zones of growth inhibition are measured (Matuschek, Brown, & Kahlmeter, 2013). While fastidious and slow-growing bacteria are difficult to assess when using this method, the zones of growth inhibition that are produced in disk diffusion are considered to be easy to interpret by technicians in the clinical field, and the method does not require specialised instrumentation (Jorgensen & Ferraro, 2009). This method will be used in antimicrobial sensitivity testing in Section 2.2.3.

1.3.3 Broth dilution methods

Broth dilution methods are used to assess the MIC and the minimum bactericidal concentration (MBC) of antimicrobial compounds against bacteria. MIC refers to the lowest concentration of antimicrobial agent that inhibits bacterial growth, while the

MBC is defined as the lowest concentration at which an antimicrobial agent reduces the bacterial colony count by ≥99.9% as stated by Clinical & Laboratory Standards Institute (CLSI) (1999). These *in vitro* quantitative methods can be performed in tubes (macrodilution) or 96-well microtitre plates (microdilution) as discussed by Varma & Sharma (2017). In both macrodilution and microdilution, appropriate volumes of antimicrobial compound are serially diluted using MHB (Parija, 2012), which is the recommended sensitivity broth used in antimicrobial sensitivity testing (EUCAST, 2003; CLSI, 2012). Equivalent volumes of bacterial inoculum are added to the tube or microtitre plate, which is then incubated at the optimal temperature and time for growth dependent on the bacterial species. After incubation, MIC can be visibly measured as the lowest concentration of an antimicrobial compound at which inoculated solution is clear, i.e. no growth. MIC can also be measured using a spectrophotometer at 620 nm, where absorbance of the solution is obtained. The absorbance of inoculated solution corresponds with the concentration of bacterial cells that are present in the solution, a theory that is based on Beer-Lambert's Law.

While the MIC is a measure of the bacteriostatic (growth inhibition) concentration of an antimicrobial agent, MBC is a measure of the bactericidal (reduction of bacterial concentration by ≥99.9%). MBC can be determined by inoculating growth agar plates with post-treated solutions from tubes or microtitre plates that show no visible bacterial growth, as detailed by Schaechter *et al*. (2012). Although the MBC method may be laborious, the results are important in antimicrobial chemotherapy in terms of dosage of antimicrobials given to infected patients and the efficacy of drugs in therapy. Broth dilution methods will be further explored in Section 2.2.5.

1.3.4 Thin-Layer chromatography-Bioautography (TLC-B)

Bioautography is a method used to screen for the activity and inactivity of antimicrobials and is often combined with thin-layer chromatography. TLC-B detects the mobility of antimicrobial drugs on a thin-layer chromatography plate in a suitable solvent as described by Marston (2011). This method, which can be either contact, immersion or direct, the latter being the most preferred technique because of its use in biological and chemical screening assays, e.g., screening of antimicrobial residues in milk, was reviewed by Choma & Grzelak (2011). In the contact TLC-B technique,

the TLC plate, which contains spots of antimicrobial agent, is placed on the inoculated agar plate for a short time to facilitate diffusion of an antimicrobial agents to the inoculated agar plate. After incubation at optimal temperature for growth, the agar plate is observed for zones of growth inhibition. In immersion TLC-B, also known as agar-overlay, the TLC plate is submerged in growth medium for a long period, inoculated with bacteria and incubated at optimal temperature and time for bacterial growth. After incubation, the agar layer is observed for zones of growth inhibition. The direct technique (TLC-DB) involves the submersion of the TLC plate, which is pre-coated with spots of antimicrobial agent, into a solution of bacteria, followed by incubation. The zones of growth inhibition are visualised using a dehydrogenase-based colorimetric reduction reaction, e.g., the use of a tetrazolium salt. TLC-DB has been used to successfully screen for the antimicrobial activity of natural products, which were extracted from plants, fungi and lichen, against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Valgas *et al*., 2007).

1.3.5 Other methods of AST

With the exception of TLC-DB, the aforementioned tests for antimicrobial susceptibility are the standard methods that are recommended by EUCAST, BSAC and CLSI. However, these methods are usually completed within 24-48 hours in the clinical setting, which may result in delayed antimicrobial treatment of infected patients. Therefore, rapid tests are required so that clinical therapy may begin earlier. Although some of these assays indirectly determine antimicrobial susceptibility, they may be effective ways of providing recommendations to clinicians of antimicrobial use in patient therapy.

One rapid AST method includes the use of polymerase chain reaction (PCR). This method involves the use of restriction endonucleases and ligases in the amplification of nucleic acids from specific genetic sequences (Mullis, Ferre, & Gibbs, 2012). PCR was initially used to identify pathogenic bacteria by amplifying their genomes (Espy *et al*., 2006). However, some PCR methods have been modified to detect bacterial genes that facilitate resistance to antimicrobial drugs (Pulido *et al*., 2013). One successful PCR-based method detects the presence of the *mec A* gene in MRSA that encodes for an altered penicillin-binding protein (PBP) with decreased affinity for β-

lactam drugs (Pournajaf *et al*., 2014). One advantage of this method is that clinicians can quickly eliminate the administration of antimicrobials to which bacteria may be resistant. The disadvantage of this method is that the presence of resistance genes do not always equate to phenotypic manifestations (Pulido *et al*., 2013).

Like PCR-based methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has also been used to identify bacterial pathogens in a rapid manner (Bizzini & Greub, 2010; Emonet *et al*., 2010; Sauer & Kliem, 2010; Sogawa *et al*., 2011; Croxatto, Prod, & Greub, 2012). The MALDI-TOF MS identifies bacteria by comparing their protein fingerprint to a database of known protein structures, as reviewed by Calderaro *et al*. (2014). (Lee *et al*., 2013) have successfully identified bacterial genes, such as single-minded homologue-1 and New Dheli metallo-beta-lactamase-1, which encode for proteins that are resistant to carbapenems. This inexpensive method is high in accuracy when compared to conventional phenotypic techniques such as Gram staining. Prompt identification of bacterial pathogens, and possible presence of resistant genes, allow for the clinical treatment of patients with bacterial infections (Biswas & Rolain, 2013).

Another technique that is used in the identification of bacterial nucleic acids is the microarray. This process relies on the hybridization of complementary DNA strands, which may be substituted with ribonucleic acid (Knudsen, 2011). These complementary nucleic acid sequences, or nucleotides, can be deposited on to membranes, which allows for the identification of thousands of sequences in one assay (Pulido *et al*., 2013). DNA microarrays have been successfully employed in the identification of extended-spectrum β-lactamase genes (Leinberger *et al*., 2010) and carbapenemase genes in Gram-negative bacteria (Naas *et al*., 2011; Peter *et al*., 2012).

Microfluidics, known as “lab on a chip”, are micro-assays used to identify bacterial antimicrobial resistance as reviewed by Pulido *et al*. (2013). This method utilizes minute volumes of sample and reagent that are inserted into polymer-based or glass-based channels, and can be combined with techniques based on nucleic acid identification (Saleh-lakha & Trevors, 2010). Choi *et al*. (2012) reported that this droplet-based method effectively identified bacterial growth in the presence of

antimicrobial agents using a microscope. In addition to the use of small volumes (10 – 100 µl), advantages of microfluidics include no loss of analyte and high-throughput and concurrent assays (Saleh-lakha & Trevors, 2010). Some disadvantages of this method include the possibility of sample contamination and the ability of the system to differentiate target bacteria in polymicrobial samples (Saleh-lakha & Trevors, 2010).

The afore-mentioned alternative approaches to AST reduces the time taken to identify resistant bacterial strains. However, one limitation of these techniques that must be considered is inconsistent concordance of results obtained from each method. While this is an important factor in clinical laboratory identification of bacteria, these methods demonstrate effective and rapid bacterial classification, and resistance genes, when compared to conventional methods.

1.4 Common mechanisms of antimicrobial resistance

Antimicrobial resistance mechanisms are commonly classified into three types: alteration of proteins located in bacterial cell membranes, e.g. efflux pumps, and acquisition of genes that alter the metabolism of key structural target sites found in the bacterial cell wall, and acquisition of antimicrobial-destroying genes (Tenover, 2006).

1.4.1 Role of efflux pumps in antimicrobial resistance

Efflux pumps are membrane-bound proteins that prevent compounds, such as antimicrobial agents, from entering the bacterial cell, and may block the transport of one antimicrobial or multiple antimicrobials (Piddock, 2006). Pumps that block more than one antimicrobial drug are called MDR efflux pumps and are usually classified into 5 groups: ATP-binding cassette, major facilitator superfamily, multi-drug and toxic compound extrusion, resistance nodulation division and small multi-drug resistance, as reviewed by Piddock (2006). These proteins effectively stabilise membrane permeability in both Gram-positive bacteria and Gram-negative bacteria as detailed by Mahamoud *et al*. (2007).

1.4.2 Acquisition of genes involved in metabolism – Plasma-mediated gene transfer

Resistance genes are often located on circular extrachromosomal DNA material called plasmids, which may be transferred from one bacterial cell to another via conjugation (Thomas, 2003). Liu *et al*. (2016) reported that there was increased prevalence of plasma-mediated transfer of the *mcr-1* gene, which is responsible for bacterial resistance against colistin, in Enterobacteriacieae. The presence of such genes that are transferred via plasmids can be detected by the random amplified polymorphic DNA method, which is a PCR-based assay (Nyberg *et al*., 2016).

1.4.3 Acquisition of genes involved in metabolism – Emergence of MRSA

MRSA is a subgroup of *S. aureus* that possesses the *mecA* resistant gene, which encodes a subgroup of PBP called PBP2a and is located in the cell membrane, resulting in reduced susceptibility to β-lactam drugs, as reviewed by researchers

including Fuda *et al*. (2004) and Assis, Nedeljković, & Dessen (2017). Production of this protein results in bacterial resistance to first-line antimicrobials, such as the β-lactams, used to treat patients with *S. aureus* infections (Lindsay, 2014). This resistant microorganism was first reported in 1961 and quickly became one of the most prevalent forms of resistant *S. aureus*, as discussed by Moreira Osório *et al*. (2012). There are two common theories that explains the presence of the *mecA* gene in MRSA. The first theory is that methicillin-sensitive *S. aureus* acquired the gene via horizontal gene transfer from *S. epidermidis* and other bacterial strains (Wielders *et al*., 2002). The second theory is that the *mecA* gene entered the *S. aureus* by horizontal gene transfer at an unknown stage of development resulting in a single MRSA strain that has mutated several times and rapidly spread across the globe (Wielders *et al*., 2002). The expression of mutant PBP in MRSA strains is facilitated by the presence of the sequence of resistant genes that are found in the mobile genetic element known as the Staphylococcal Cassette Chromosome *mec* as reviewed by Assis, Nedeljković, & Dessen (2017).

1.4.4 Acquisition of genes involved in metabolism – Emergence of VRSA

The emergence of *S. aureus* microorganism that exhibit resistance to β-lactam drugs advanced the use of the glycopeptide vancomycin (Smith *et al.*, 1999). However, this evolved into the phenotypic manifestation of heteroresistant vancomycin-intermediate *S. aureus*, which produced multiple genotypic stages of vancomycin resistance from vancomycin-intermediate *S. aureus* to vancomycin-resistant *Staphylococcus aureus* (VRSA), as discussed by Bal *et al*. (2013). Cafiso *et al*. (2012) summarised the complexity of the bacterial cell changes that result in reduced vancomycin susceptibility, which includes an aggregation of D-Ala-D-Ala subunits, a proliferation in the number of non-amidated muropeptides and a reduction in the alanylation of techoic acids. These genotypic alterations produce structural changes, such as thicker cell walls, which leads to a loss of surface binding sites for antimicrobial action (Bal *et al*., 2013).

1.4.5 Acquisition of antimicrobial-destroying genes – Emergence of CPEs

Carbapenem drugs can be effective therapeutic agents against Enterobacteriaceae since these compounds are usually resistant to hydrolysis by either class A, B or D β-lactamases (Oelschlaeger *et al*., 2010). These transpeptidase analogues hydrolyse the β-lactam ring in penicillins and cephalosporins, which inactivates the antimicrobial compounds, as detailed by Oelschlaeger *et al*. (2010). However, increased use of carbapenems against Enterobacteriaceae, which produce serine β-lactamases resulted in the development of resistant Enterobacteriaceae strains. These strains produce four main subclasses of hydrolytic carbapenemases, which are oxacillinase, *K. pneumoniae* carbapenemase, Verona integron-encoded metallo-betalactamase and imipenemase (Oelschlaeger *et al*., 2010). The oxacillinase group of carbapenemases are members of the class D enzymes and have been identified in Gram-negative bacteria such as *Acinetobacter baumannii* (Smith *et al*., 2013) and *Enterobacter cloacae* (Majewski *et al*., 2014). *K. pneumoniae* carbapenemase-producing *K. pneumonia*, which produce class A *K. pneumoniae carbapenemase*, was first reported in 2001 in the United States and its rapid spread has become a major concern, as reviewed by Vaux *et al*. (2011). These resistant strains have been isolated in the United Kingdom in healthcare environments (Virgincar *et al*., 2011). Class B enzymes, which include Verona integron-borne metallo-β-lactamase and inosine monophosphate dehydrogenase, have been isolated from *Pseudomonas aeruginosa* (Ageevets *et al*., 2014) *Citrobacter freundii*, *E. cloacae*, *Escherichia coli*, and *Enterobacter aerogenes* (Li *et al*., 2014).

1.5 Alternative Methods of Combatting Resistant Bacterial Strains

Increased prevalence of infections caused by MDR bacteria has advanced the development of alternative treatment methods. These methods include synergistic therapies, antisense therapy and the use of bacteriophages (Giamarellou, 2006).

1.5.1 Combatting Resistant Bacterial Strains – Synergistic Therapies

One form of treatment against MDR bacteria is to combine two or more antimicrobial drugs, as reviewed by Worthington & Melander (2013). This method has been used in the treatment of cancers (Lane, 2006) and Human Immunodeficiency Virus as discussed by Worthington & Melander (2013). Stepanović *et al*. (2003) reported that propolis, a resinous compound comprised of bee saliva and tree sap and used by bees in the construction of their hives, exhibited antimicrobial activity against MDR bacteria when mixed with oxacillin. A combinatorial approach was also used to investigate the antimicrobial activity of pannarin, a lichen-based organic compound, with five known antimicrobial agents against MRSA (Celenza *et al*., 2012). Although the findings of this study indicated that activity ranged from antagonistic to moderate synergism, pannarin appeared to be a promising scaffold for combination-type antimicrobial treatment. In another synergistic study, essential oils that were extracted from *Myrtus communis leaf*, commonly known as true murtle, were combined with eight antimicrobial agents against several MDR strains of *A. baumannii* (Aleksic *et al*., 2014). Aleksic *et al*. (2014) reported that the combinatorial method significantly reduced growth of the resistant organisms. Combinations of aminoglycosides and 3-benzylchroman derivatives of sappan lignum, a medicinal compound that was extracted from the Chinese plant known as *Caesalpinia sappan* were also assessed by Zuo *et al*. (2014). Findings of the study indicated that at least one aminoglycosides/3-benzylchroman combination exhibited a reduction in MIC values by more than 50% against clinical isolates of MRSA. Despite promising results from the previously mentioned synergistic studies, some combination approaches that utilise only conventional antimicrobial drugs show little success. Máthé *et al*. (2007) have reported that amikacin and imipenem exhibit no significant difference in antimicrobial activity when used individually or in synergism against extended-spectrum β-lactamase-producing *K. pnuemoniae*.

1.5.2 Combatting Resistant Bacterial Strains – Antisense Therapy

Antisense therapy involves the use of synthetic ribonucleic acid sequences that bind to messenger RNA, which results in gene inactivation of genes coding for essential growth proteins and inhibits their production, as detailed by Rasmussen *et al*. (2007). Despite the initial difficulties of utilising this technology in clinical practice, researchers have become increasingly interested in this therapeutic method and have developed novel antisense nucleic acid material such as peptide nucleic acids, as reviewed by Good (2002). In terms of antimicrobial treatment, antisense technology can be used to restore antimicrobial susceptibility in resistant bacteria. One method employed in this process is the production of phosphorothioate oligodeoxynucleotides by substituting one of the oxygen atoms that are present in the phosphodiester bonds of DNA with sulphur (Rasmussen *et al*., 2007). Meng *et al*. (2006) reported that these oligonucleotides were used to successfully re-establish the sensitivity of MRSA to oxacillin by inhibiting the expression of antimicrobial-resistant *mecA* gene. In a similar study, Bai *et al*. (2012) utilised synthetic peptide nucleic acids to inhibit the expression of the resistant *rpoD* gene in MDR Gram-negative bacteria, such as *E. coli* and *K. pneumonia*, which resulted in reduced bacterial growth.

1.5.3 Combatting Resistant Bacterial Strains – Bacteriophages

Bacteriophages are naturally-occurring viruses that specifically target bacterial cells (Kutateladze & Adamia, 2010). These bacteria-targeting microorganisms contain double-stranded DNA and may be lytic or temperate (lysogenic) (Madigan *et al*., 2012). Lytic bacteriophages infect bacterial cells with their dsDNA, which results in viral DNA replication and lysis. Conversely, the injected dsDNA of lysogenic bacteriophages integrates with the host bacterial genome, which produces a dormant state and prevents bacterial cell lysis (Madigan *et al*., 2012). While antimicrobials are usually broad-spectrum, bacteriophages explicitly target narrow ranges of bacterial species. Thus, precise knowledge of the bacterial causative agent of an infection is of significant importance prior to use of bacteriophages (Kutateladze & Adamia, 2010). The use of bacteriophages in clinical therapy possess several advantages including rapid and cost-effective production compared to

antimicrobial development, inhibition of the horizontal transfer of resistant genes and increased potency when synergistically used with antimicrobial drugs (Lu & Koeris, 2011). In terms of antimicrobial therapy, virulent or lytic bacteriophages are more commonly used. Additionally, these microorganisms can be modified to enhance their lytic activity. Clinically isolated MRSA strains exhibited susceptibility to enhanced phage K organisms, which was achieved by multiple successive subculturing of the bacteriophage in *S. aureus* DPC5246 (O'Flaherty *et al*., 2005).

1.6 Novel Compounds as Potential Antimicrobial Agents

Due to the growing unavailability of effective antimicrobial agents, there is an urgent need for the discovery and development of novel compounds that exhibit antimicrobial activity. Research into new sources of potential antimicrobial agents has exponentially increased to rectify this problem. These new sources have contributed to the synthesis of novel antimicrobial compounds with bacteriostatic and bactericidal activity and can be categorised into primary classifications based on their sources.

1.6.1 Classifications and sources of novel antibiotic and antimicrobial compounds

During the "Golden era" of antibiotic drug discovery, the origin of many of the therapeutic agents was natural-based. Many of the novel antibiotic compounds have been extracts from natural sources, while other agents have been synthetically produced.

1.6.2 Antimicrobial agents from plants

One major natural source of antimicrobial compounds are plants, both land plants (Gechev *et al*., 2014; Yong *et al*., 2015; Vandal *et al*., 2015) and marine plants (Kavita *et al*., 2014). In a study by Duric *et al*. (2013), methanolic extracts, both in powdered form and as decoctions, were isolated from *Betula pendula*, which is a species of birch. Methanolic extracts from birch possess antimicrobial active compounds, such as triterpenes, oleanolic acid and betulinic acid, whose mode of action is not yet defined (Cîntă-Pînzaru *et al*., 2012). AST of these compounds demonstrated mixed efficacy against non-resistant bacteria where the compounds inhibited growth of Gram-positive cells, while dimethyl sulfoxide (DMSO) solvent, and not the extracts, contributed to growth inhibition of Gram-negative cells (Duric *et al*., 2013).

Karioti *et al*. (2011) investigated the antimicrobial activity of phenolic compounds that were extracted from the leaves of *Quercus ilex*. The findings of the Karioti study indicated that compounds 1-11 possessed greater antimicrobial activity than compounds 12-17 against Gram-negative and Gram-positive bacteria (Karioti *et al*., 2011).

Another plant-based compound of therapeutic importance is α-mangostin. This xanthone compound is extracted from *Garcinia mangostana* (mangosteen), which is a tropical fruit tree that is a noted source of traditional medicinal compounds used to treat various bacterial infections (Pedraza-Chaverri *et al*., 2008). α-Mangostin exhibited antimicrobial activity against non-resistant Gram-positive bacteria and against three MRSA strains (Koh *et al*., 2013). Koh *et al*. (2013) proposed that the mode of action of α-mangostin is the depolarisation of the bacterial cell membrane resulting in cell lysis.

In a study of 38 methanol extracts from seaweed, antimicrobial activity was demonstrated against two Gram-positive bacteria and two Gram-negative bacteria (Kavita *et al*., 2014). Due to the growth inhibiting activity of these compounds from a renewable marine source, seaweed can provide potent antimicrobial agents (Kavita *et al*., 2014).

1.6.3 Antimicrobial agents from the human body

Natural-based antimicrobial agents can also be isolated from the human body. β-defensins are composed of 36-42 amino acids and have been assessed as possessing antimicrobial activity (Yamaguchi *et al*., 2002). These cationic antimicrobial peptides belong to one of two families of defensins (α or β), which contain six cysteine residues (Yamaguchi *et al*., 2002). Yadava *et al*. (2006) evaluated the antimicrobial activity of three human β-defensins against four *Bacillus spp*. Findings of the study indicated that the compounds inhibited growth of the four isolates (Yadava *et al*., 2006). It was proposed that cell lysis, caused by penetration of the bacterial cell membrane, and inhibition of DNA and/or protein synthesis were possible modes of action of these peptides (Yadava *et al*., 2006).

Another naturally occurring defense mechanism against bacteria are antibodies. These structures, which are found in serum, were first used in the 19th Century against diphtheria and tetanus, and were further administered in serum therapy against pneumonia and meningitis until the introduction of antimicrobials (Casadevall & Scharff, 1994). Antibodies are polypeptide structures produced by B cells and possess active sites that bind with foreign entities known as antigens

(Alberts *et al*., 2002). Antibody-producing B cells can produce cloned cells called hybridomas, which secrete structures, known as monoclonal antibodies that recognise specific antigens (Alberts *et al*., 2002). Monoclonal antibodies were the more common type of antibody used in early therapeutic strategies. Due to the increased occurrence of infections caused by MDR bacteria, there has been a resurgence in the use of monoclonal antibodies against bacterial infections (Oleksiewicz *et al*., 2012). Domanski *et al*. (2005) induced the production of monoclonal antibodies against non-resistant *S. aureus* and MRSA. These proteins successfully opsonized the bacterial cells, which was proposed to be their primary mode of action.

1.6.4 Antibiotic Agents from Bacteria

Bacteria produce defensive mechanisms known as bacteriocins, which are defined as protein molecules that possess bactericidal activity (James *et al*., 1991; Riley & Wertz, 2002). These proteins are found in almost all bacteria species and there are many types that have been identified (James *et al*., 1991; Riley & Wertz, 2002; Cavera *et al*., 2015). Bacteriocins differ from antimicrobial agents in that they possess narrow spectrum of activities and target closely related species (Riley & Wertz, 2002). In a review by Cavera *et al*. (2015), bacteriocins that are produced by Gram-positive bacteria are grouped in to Class I and Class II, while bacteriocins that are produced by Gram-negative bacteria are classified as small peptides, such as microcins, and large peptides, such as colicins. These defensive peptides inhibit the growth of related bacteria species by using modes of action that are similar to those used by antimicrobial agents (Cavera *et al*., 2015). A common type of bacteriocin that has been studied extensively is Nisin A. Studies indicate that many bacteriocins lack N-terminal leaders, making these “leaderless” bacteriocins favoured for their ease of chemical modification and their small size, as reviewed by Ovchinnikov *et al*. (2014). In a recent study by Ovchinnikov *et al*. (2017), a leaderless bacteriocin known as enterocin K1 was evaluated for its antibiotic activity. The findings of the assay indicated that enterocin K1 exhibited antibiotic activity against non-resistant *Enterococcus spp.* and *Streptococcus pneumonia* (Ovchinnikov *et al*., 2017).

1.6.5 Antibiotic Agents from Soil Bacteria

Teixobactin is an antibiotic agent that was isolated and identified from uncultured bacteria in soil using iChip technology (Ling *et al*., 2015). Due to its reported ability to avoid bacterial resistance, this newly discovered antibiotic agent has gained extreme interest by researchers and clinicians (Ling *et al*., 2015). The report by Ling *et al.* (2015) proposed that the mode of antibiotic action of teixobactin was inhibition of bacterial cell wall synthesis, which involved binding of the antibiotic compound to specific compounds in the cell wall (Ling *et al*., 2015). Because of its reported antibiotic activity against non-resistant Gram-positive bacteria and against MRSA, teixobactin could be a potential candidate for antibiotic therapy.

1.6.6 Synthetic Antimicrobial Agents

Another approach to developing novel antimicrobial agents involves the production of laboratory-produced antimicrobial drugs. A solution of silver ion was assayed for its potential antimicrobial action against *S. aureus* and *E. coli* (Jung *et al*., 2008). Due to its inhibition of thiol groups found in enzymes, silver ion exhibited reductions in the growth of both bacterial samples (Jung *et al*., 2008). In a 2013 review, gold was described as an antimicrobial scaffold of interest because of its chemical stability (Khan *et al*., 2013). Gold nanoparticle surfaces have been employed as antimicrobial surfaces against *E. coli* (Ehmann *et al*., 2015). Ehmann *et al.* (2015) attempted to enhance the antimicrobial properties of chitosan, which is an anticoagulant polysaccharide, by coating gold nanoparticles with the polymer (Ehmann *et al*., 2015). This technique proved successful as bacterial growth was reported to have been significantly reduced (Ehmann *et al*., 2015).

1.7 Chalcone compounds

Another compound that has gained attention from researchers are chalcones. Chalcones are plant-based flavonoids (Cushnie & Lamb, 2011; Henry *et al*., 2017) that can be found widely in most plant material, including leaves and stems (Cushnie & Lamb, 2005; Henry *et al*., 2017). These chemicals are responsible for the colour of pollinating flowers and for protection against harmful ultra violet rays from the sun (Harborne & Williams, 2000; Henry *et al*., 2017). Chalcones have been reported to possess many biological activities such as anticancer, antiparasitic, antifungal and antibacterial (Dhar, 1981; Dimmock *et al*., 1999; Ni *et al*., 2004; Go *et al*., 2005; de Carvalho Tavares *et al*., 2011; Rozmer & Perjési, 2016; Henry *et al.*, 2017).

1.7.1 Biosynthesis and Synthetic Production of Chalcones

The biosynthesis of chalcones is catalysed by the enzyme chalcone synthase (Kreuzaler & Hahlbrock, 1972). This enzymatic reaction begins with the consecutive condensation of one p-coumaroyl-coenzyme-A and three malonyl-coenzyme-A moieties and terminates with the Claisen condensation of a tetraketide intermediate into a hydroxylatedaromatic ring system (Kreuzaler & Hahlbrock, 1972; Ferrer *et al*., 1999).

In the laboratory, synthesis of chalcones and their derivatives is achieved by the Claisen-Schmidt condensation reaction, which is also known as aldol condensation (Pavia, 2005). This process involves a reaction between a benzaldehyde and an acetophenone in the presence of a base solution (Vyvyan *et al*., 2002).

1.7.2 Derivatives of Synthetic Chalcones

Because synthetic chalcone compounds can be easily altered, several active derivatives of the organic scaffold have been developed. For example, Liu *et al*. (2008) have synthesised a library of functionalised chalcone derivatives with antibacterial activity against *S. aureus* (Liu *et al*., 2008). These compounds differ by the substitution of Ring A, with organic moieties that possessed molecules such as methoxy groups and phenolic hydroxide (OH) groups, and the addition of R groups on Ring B (Liu *et al*., 2008).

1.7.3 Potential Mode of Action of Chalcones

The mechanism of action of chalcones has not yet been defined. However, the consensus regarding the nature of chalcone activity on cells is that the hydrophobic compounds disrupt the disrupt the integrity of the cell membrane resulting in morphological damage and cell death (Nielsen *et al*., 2005; Sivakumar *et al*., 2009). Sivakumar *et al.* (2009) demonstrated that synthetic chalcone derivatives exhibited bactericidal activity against *S. aureus* (Sivakumar *et al*., 2009). One method of enhancing the antibacterial properties of chalcone compounds is the substitution of either Ring A or Ring B with a ferrocene moiety.

1.8 Antimicrobial Activity of Ferrocenyl Chalcones

Chalcone derivatives, especially those comprised of ferrocenyl groups, are being developed as antimicrobial agents (Attar *et al*., 2011; Pejović *et al*., 2012; Kowalski *et al*., 2013; Prasath *et al*., 2013; Ahmed *et al*., 2015; Henry *et al*., 2017). These combination compounds possess useful benefits including small size, increased lipophilicity for diffusing across lipid membranes, and ease of chemical modification (Attar *et al*., 2011).

Ferrocenyl chalcone compounds are primarily classified into Type I or Type II, where the carbonyl group could be located near the ferrocenyl ring in Type I compounds and where the carbonyl group can be found near the phenyl ring in Type II (Attar *et al*., 2011). One theory of the mode of action of these chalcone derivatives is the interference of respiratory enzymes found in the electron transport chain, which is located on the bacterial cell membrane (Attar *et al*., 2011). Inhibition of bacterial respiration may result in severe morphological damage to the cell membrane followed by cell death.

1.9 Synopsis of research

1.9.1 Description of research

In the current study, Type II ferrocenyl chalcone compounds (Appendix 1, Table 7.1) were created and produced by Robert B Smith (RS), a medicinal/organic chemist and senior lecturer at University of Central Lancashire (UCLAN) with a PhD in design and development of novel metalloporphyrin compounds, using a combined approach involving a Claisen-Schmidt base-catalysed condensation reaction and a nitrogen and pyrimidine methylation. This technique incorporated the substitution of Carbon-5 on Ring A with a nitrogen atom followed by the addition of increasing chains of alkyl iodide, and the substitution of Ring B with a ferrocenyl group (Figure 1-4). Due to their desirable electrochemical characteristics, which may involve inhibition of reproduction as a result of DNA binding, ferrocenyl chalcones have been shown to have anti-nematodal, anti-cancer and anti-plasmodial activities (Attar *et al*., 2011). Thus, derivatives of these compounds may also show antibacterial activity.

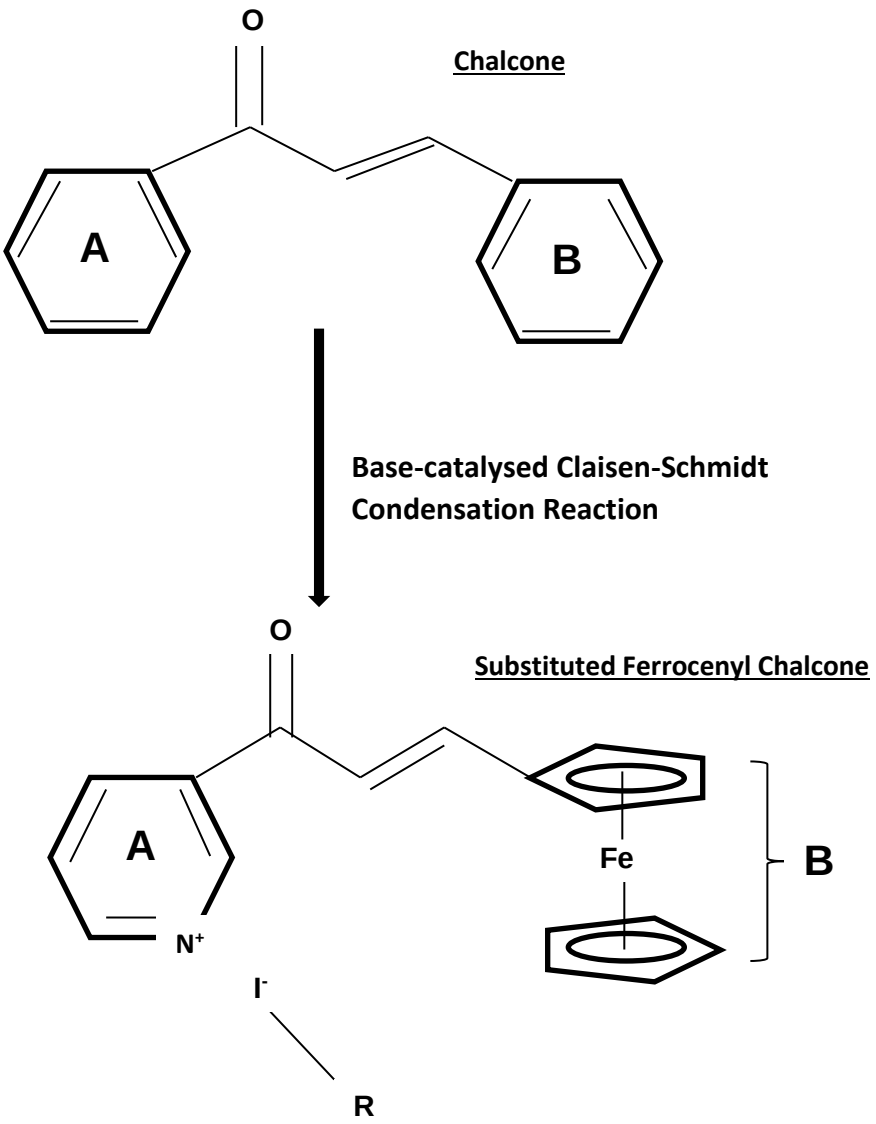


Figure 1-4 Basic structure of a novel ferrocenyl ferrocenyl chalcone compound used in the current study. This compound was derived from the nitrogen substitution and alkyl iodide addition on Ring A, and ferrocenyl group substitution on Ring B of a chalcone scaffold (illustration by Henry, 2014).

1.9.2 Aims of the research

The aim of this project is to investigate the antimicrobial capacity of new ferrocenyl chalcone derivatives with increasing lipophilicity using antimicrobial sensitivity tests to measure MIC.

1.9.3 Objectives of the research

The objectives of this research are:

- To assess the physical properties of the ferrocenyl chalcone compounds using solubility tests and the effect of environmental conditions on these compounds (Chapter 3).

- To investigate the antimicrobial activity of these compounds against non-resistant laboratory-adapted bacteria using disc diffusion and the minimal inhibitory concentrations using 2-fold broth microdilution (Chapter 2).
- To determine the antimicrobial activity of these compounds against non-resistant and resistant clinical isolates, which were donated by Mr. Michael Collins, Service Manager in the Department of Microbiology at Chesterfield Royal Hospital (CRH), using 2-fold broth microdilution (Chapter 2).
- To investigate the molecular profile of these novel compounds using proton nuclear magnetic resonance (^{1}H NMR) and mass spectrometry (MS) as a means of quality control (Appendix 2).
- To determine the possible mechanisms of action used by these compounds against such organisms using and the MTT assay to determine cell viability in terms of cellular respiration (Chapter 4).
- To evaluate the potential external physical changes of bacterial cells after exposure to the ferrocenyl chalcone compounds using scanning electron microscopy (SEM) (Chapter 5).

2 Materials and Methods

2.1 Chemicals and reagents

2.1.1 Standard conditions for sterilisation

All consumables, reagents, deionised water, agar, etc. were autoclaved at 121^{o}C at 15 per square inch for 15 minutes.

2.1.2 Preparation and sterilisation of sterile agar, reagents, tubes and other consumables

IsoSensitest agar (ISA) plates for the diffusion assays were prepared according to the manufacturer's instructions by dissolving 15.7 g ISA powder (Oxoid, Thermo Fisher, UK) in 500 ml deionised water, while nutrient agar (NA) (Lab M Limited, Bury, UK) plates used for broth dilution assays were prepared by dissolving 28 g of agar powder in 1 L of sterile deionised water. These were then autoclaved as described in Section 2.1.1 and cooled to 50^{o}C before pouring into sterile agar plates. Stock phosphate buffered saline (PBS) (Sigma, Dorset, UK), deionised water, Meuller-Hinton Broth (MHB), IsoSensitest broth (ISB), tubes and blank paper disks were also sterilised as detailed in Section 2.1.2. Stock solutions were dispensed into 10 ml aliquot post-sterilisation.

2.1.3 Preparation of stock ferrocenyl chalcone solutions for sensitivity determinations, MTT assays and SEM

Ferrocenyl chalcones of increasing alkyl chain lengths (methyl to decyl) were provided by RS (UCLAN, UK). For broth macrodilution assays, fresh stock of each ferrocenyl chalcone solution were prepared at concentrations of 5 mg/ml in DMSO (Alfa Aesar, Heysham, UK). Fresh stock solutions of each compound were prepared at concentrations of 1 mg/ml in DMSO for diffusion assays and for broth microdilution. The final concentrations of each ferrocenyl chalcone are shown in Table 2-1 and Table 2-2.

Table 2-1 Final concentrations of ferrocenyl chalcones (mg/ml) and DMSO (% $^{v}/_{v}$) for broth macrodilution assays.

Tube #	Concentrations of Chalcone and DMSO	
	Chalcone (mg/ml)	**DMSO (% $^{v}/_{v}$)**
1	2.500	50.000
2	1.250	25.000
3	0.630	12.500
4	0.310	6.250
5	0.150	3.125

Table 2-2 Final concentrations of ferrocenyl chalcones (mg/ml) and DMSO (% $^{v}/_{v}$) for broth microdilution assays.

Well#	Final Concentrations of Chalcone and DMSO	
	Chalcone (mg/ml)	DMSO % $^{v}/_{v}$
1	0.500	50.000
2	0.250	25.000
3	0.125	12.500
4	0.063	6.250
5	0.031	3.125
6	0.016	1.563
7	0.008	0.782
8	0.004	0.391
9	0.002	0.195
10	0.001	0.098

2.1.4 Preparation of antimicrobial solutions for sensitivity determinations and MTT assays

Stock antimicrobial solutions (250 mg/ml) of PEN-G (Sigma, Dorset, UK) and oxytetracycline (OXY) were prepared in sterile deionised water according to the standard method described by Andrews (2001). Each solution was divided into 1 ml aliquots in sterile microcentrifuge tubes and then stored at -20^{o}C.

2.1.5 Preparation of stock MTT solution

Appropriate volumes of stock MTT solution were prepared at a ratio of 5 mg/ml of MTT reagent powder in PBS. The stock solution was stored at -20^{o}C until required.

2.2 Organisms used in each assay and their origins

All organisms that were donated by Mr. Michael Collins of Chesterfield Royal Hospital (CRH) were stored in cryogenic storage vials with beads in nutrient broth (Thermo Fisher, Loughborough, UK) at -80°C. Other organisms were subcultured once per month on NA plates, incubated at 37°C for 18-24 hours and stored at 4°C.

Table 2-3 Organisms used in the current study for the determination of MIC, time-kill assay, MTT assay and SEM.

Organism	Origin
***Klebsiella pneumoniae* (IH)**	Mr. Ian Hopkins (IH)
***Enterococcus aerogenes* NCIMB 10102**	National Collection of Industrial, Food and Marine Bacteria
***Escherichia coli* NCIMB 9483**	National Collection of Industrial, Food and Marine Bacteria
***Pseudomonas aeruginosa* NCTC 10332**	National Collection of Type Cultures
***Staphylococcus aureus* NCIMB 8244**	National Collection of Industrial, Food and Marine Bacteria
***Kocuria kristinae* NCIMB 8884**	National Collection of Industrial, Food and Marine Bacteria
***Enterococcus faecalis* NCTC 12697**	National Collection of Type Cultures
***Staphylococcus aureus* Fully Sensitive (CRH)**	Clinical isolate, CRH
***PEN-resistant Staphylococcus aureus* (CRH)**	Clinical isolate, CRH
PEN/ERY*/CLI**-resistant Staphylococcus aureus (CRH)	Clinical isolate, CRH
MRSA (CRH)	Clinical isolate, CRH
***Salmonella "Manchester"* NCTC 7372**	National Collection of Type Cultures
***Escherichia coli* Fully Sensitive (CRH)**	Clinical isolate, CRH

*Erythromycin

**Clindamycin

2.2.1 Preparation of inocula for sensitivity assays, MTT assays and SEM

Each inoculum was freshly prepared each time by suspending at least 3-4 colonies of the appropriate organism in 10 ml of ISB for broth macrodilution and 10 ml of sterile MHB for broth microdilution, time-kill assays, MTT assays and SEM. The colonies were dispersed until the turbidity of the suspension was equal to, or greater than,

that of 0.5 McFarland Standard and compared against a white background with a contrasting black line, which produced bacterial concentrations of 10^7 cfu/ml to 10^8 cfu/ml. These suspensions were further diluted in sterile ISB or MHB to approximately 10^5 cfu/ml. Each inoculum was used within 30 minutes of preparation as detailed by Andrews (2001).

2.3 Disk Diffusion

Eight plates were divided into 6 sections and labelled for each compound (2 plates per organism). A minimum of 3-4 colonies of pure cultures of *Klebsiella pneumonia* (IH), *Enterococcus aerogenes* NCIMB 10102, *Escherichia coli* NCIMB 9483 and *Pseudomonas aeruginosa* NCTC 10332 were each suspended in 10 ml aliquots of sterile PBS (Sigma, Dorset, UK), derived from stock PBS, and each inoculum was spread onto the appropriately labelled plates. Two types of disk diffusion techniques were used: wet disk diffusion (Sharma *et al.*, 2012) and dry disk diffusion *(*modified method by Sharma *et al.*, 2012). The wet disk diffusion method involves the pipetting of a known volume and concentration of antibiotic solution on a sterile paper disk that has been aseptically placed on an inoculated agar spread plate. The dry disk diffusion method involves the aseptic placement of a dried paper disk, which has been impregnated with a known concentration of antibiotic solution, on an inoculated agar spread plate.

2.4 Preparation of standard curves for broth microdilution and MTT assays

Standard curves of *Staphylococcus aureus* NCIMB 8244, *Streptococcus pyogenes* NCIMB 8884, *Enterococcus faecalis* NCTC 12697, *Salmonella "Manchester"* NCTC 7372, *Pseudomonas aeruginosa* NCTC 10332, *Klebsiella pneumoniae* (IH) and *Escherichia coli* NCIMB 9483 were prepared by making 10-fold serial dilutions of each overnight bacterial sample (0.1 ml broth culture and 0.9 ml sterile deionized water) from 10^{-1} cfu/ml to 10^{-5} cfu/ml. From each dilution, further 10-fold serial dilutions were made in sterile PBS from 10^{-1} cfu/ml to 10^{-8} cfu/ml, which will be used to determine the bacterial concentration. This preparation was also done for overnight

cultures of clinical isolates of fully sensitive *S. aureus* (CRH), PEN-resistant *S. aureus* (CRH), PEN/ERY/CLI-resistant *S. aureus* (CRH) and a MRSA (CRH). Ten NA plates were divided into quadrants and labelled according to the serial dilutions in PBS (2 agar plates per dilution made from sterile deionized water). Three 20 µl drops of each dilution made from PBS were pipetted onto each quadrant of the NA plates and allowed to dry. The NA plates were incubated at 37^0C in air for 18-24 hours. The number of cfu/ml were determined by identifying the dilution that had 3-30 colonies, followed by multiplying the average number of colonies by the appropriate dilution factor and then multiplying by 50. This method was first described by Miles *et al*. (1938). Absorbance values of the dilutions in deionized water were measured at 620 nm on a WPA Biowave II spectrophotometer (Biochrom Limited, Cambourne, UK).

2.5 Initial determination of cfu/ml for sensitivity determinations and MTT assays

Each prepared inoculum was serially diluted 10-fold in sterile PBS from 10^{-1} cfu/ml to 10^{-8} cfu/ml. NA plates were divided into quadrants and labelled 1 to 8 (2 agar plates per inoculum). The plates were then inoculated and incubated followed by the determination of the number of cfu/ml as described in Section 2.4.

2.6 Preparation of 96-well microtitre plates for broth microdilution and MTT assays

Seventy-five microlitres of MHB was pipetted into all wells except those of columns 1, 11 & 12. A further 75 µl of prepared ferrocenyl chalcone solution was added to wells of columns 1 & 2 followed by 2-fold serial dilutions from column 2 – 10. Microdilution plates containing only diluted DMSO were treated in the same manner with MHB diluent. Seventy-five microlitres µl of PEN was added to wells of column 11 and 75 µl of 5 % $^{v}/_{v}$ DMSO was pipetted into wells of column 12. Seventy-five microlitres of MHB was added to wells of rows A & B. This was followed by pipetting of 75 µl of prepared inocula (*S. aureus* NCIMB 8244, *S. pyogenes* NCIMB 8884, *E. faecalis* NCTC 12697, *Salmonella "Manchester"* NCTC 7372, *K. pneumoniae* (IH) and

E. coli NCIMB 9483, fully sensitive *S. aureus* [CRH], PEN-resistant *S. aureus* [CRH], PEN/ERY/CLI-resistant *S. aureus* [CRH] and a MRSA [CRH]) into duplicated rows for each organism. The plates were sealed with a microplate lid and incubated at 37°C for 18-24 hours in air. After incubation, the absorbance values were measured at 620 nm on a Rosys Anthos 2010 microplate reader. The blank wells were subtracted from the sample wells to give the absorbance of cells.

2.7 Time-kill assay procedure

Nine sterile polypropylene tubes were labelled for 1xMIC, 2xMIC and control (3 per condition). Five hundred microlitres of sterile MHB was pipetted into each control tube. Five hundred microlitres of diluted chalcone was pipetted into the MIC tubes according to the required concentration. Five hundred microlitres of each prepared inoculum was added to all 9 sample tubes, which represented hour 0. Three sets of sterile Eppendorf tubes were labelled for each polypropylene sample tube to which were added 900 µl of PBS. One hundred microlitres of sample tube (hour 0) solution (chalcone/inoculum or inoculum only) was added to each corresponding sterile Eppendorf tube containing PBS followed by serial dilution (for colony forming unit determination using Miles & Misra method). The polypropylene sample tubes were then incubated at 37°C in air and 100 µl were removed at consecutive 2-hour intervals (2, 4, 6, 8 and 24 hr) followed by the determination of the number of cfu/ml as described in Section 2.4.

2.8 Preparation of bacterial samples for SEM

Treated and untreated non-resistant bacterial inocula were examined in the SEM. The treated organisms were exposed to decyl ferrocenyl chalcone solution at MIC value and incubated for 18-24 hours at 37°C, whilst untreated cells were incubated under the same conditions in MHB in the absence of chalcone. The total volume of each suspension was 500 µl. After incubation at 37°C, 10 µl of crystal violet stain was added to both treated and untreated suspensions, which were then centrifuged for 5 minutes at 13,000 rpm (11,337 g) in a Microcentrifuge Sigma 1-14 (Sigma Germany,

Osterode am Harz, Germany). After removal of the supernatant, the treated and untreated cells were incubated with 100 µl of 2% glutaraldehyde for 1 hour. This was followed by centrifugation of each solution for 1 minute at 13,000 rpm (11,337 g) and then washing of the pellets with PBS as proposed by Hartmann *et al*. (2010). After the suspensions were centrifuged for 1 minute at 13,000 rpm (11,337 g), and supernatants were removed, the cells were then dehydrated with a graded series of ethanol (20% $^{v}/_{v}$, 40% $^{v}/_{v}$, 60% $^{v}/_{v}$, 80% $^{v}/_{v}$, 95% $^{v}/_{v}$, 100% $^{v}/_{v}$, 100% $^{v}/_{v}$, 100% $^{v}/_{v}$) as detailed by Kaláb *et al*. (2008). After centrifugation for one 1 minute at 13,000 rpm (11,337 g), the cells were re-suspended in sterile deionised water. Ten microlitres of re-suspended cells were pipetted on to 0.2 µm Cyclopore Track Etch polycarbonate membrane filter disks (Whatman International Limited, Maidstone, UK) and sputter-coated with gold for 1.5 minutes. Secondary electron images were taken using the JEOL JSM 6610V SEM (Herts, UK). The method development for the visualisation of bacteria in SEM is further detailed in Section 6.2 of Chapter 6.

3 Determination of MIC

3.1 Introduction

3.1.1 AST

The evidence that chalcones with ferrocenyl moieties effectively exhibit antimicrobial action against Gram-negative and Gram-positive bacteria has increased in the last five years as evidenced by publications from a range of researchers in the field (Kowalski *et al*., 2013; Mishra *et al*., 2015; Gopi, Sastry, & Dhanaraju, 2016; Khan *et al*., 2017). In such studies, antimicrobial action was determined by the observed MIC values of the tested compounds, which reflected the inhibition of bacterial growth and susceptibility of the microorganisms.

The susceptibility of bacteria to antimicrobial drugs can be determined by measuring the zone of inhibition using agar-disk diffusion (Wheat, 2001; Howe & Andrews, 2012) and by using two-fold serial broth dilution (Andrews, 2001; Andrews, 2006; Valgas *et al*., 2007; Wiegand, Hilpert, & Hancock, 2008; CLSI, 2012).

3.1.2 Disk diffusion test

The disk diffusion test involves the measuring of zones of inhibition, which is influenced by the MIC. Conventionally, this approach refers to the inoculation of a pure culture of a bacterial suspension on a plate of Mueller Hinton agar medium or similar sensitivity agar and paper disks impregnated with antimicrobials that are placed on the surface of the medium (Moosdeen, Williams, & Seeker, 1988; Howe & Andrews, 2012; Matuschek, Brown, & Kahlmeter, 2013). After incubation at the optimal temperature for the organism for 18-24 hours, clear zones of growth inhibition, which indicates antimicrobial susceptibility, is measured in millimetres. The larger the zone of growth inhibition, the more susceptible, indicated by reduced bacterial growth, a microorganism is to the antimicrobial compound, as elaborated by several investigators, including Jorgensen & Ferraro (2009). Impregnation and drying of sterile disks can also be done with specific concentrations of novel antimicrobial drugs, as reported by Thompson (1950) and Vineetha *et al*. (2015).

3.1.3 Agar diffusion test

The agar diffusion relies on a similar method whereby sensitivity agar plates are inoculated with test bacteria followed by the use of a sterile cork-borer that punches wells into the agar. This method has been used effectively over a number of years as detailed in various publications (Magaldi *et al*., 2004; Valgas *et al*., 2007; Balouiri, Sadiki, & Ibnsouda, 2016). A specified volume of antimicrobial solution is pipetted into the wells and this is allowed to diffuse into the agar. The agar plate is then incubated at the optimal temperature for bacterial growth for 18-24 hours after which the zone of growth inhibition is measured to give an indication of the efficacy of the antimicrobial agent.

3.1.4 Broth dilution assays

Another method used to show sensitivity of bacteria to antimicrobial agents is the broth macrodilution assay. This is one of the first susceptibility methods (Wiegand, Hilpert, & Hancock, 2008), and involves serially diluting one measured volume of an antimicrobial agent with an equal volume of sensitivity broth in a tube, as detailed by Jorgensen & Ferraro (2009). After inoculation of the diluted solution with test bacteria at 10^5 mg/ml, the suspension is incubated for 18-24 hours and growth is checked visually or by spectrophotometry at approximately 600 nm. The inoculum concentration of 10^5 mg/ml is the standardized quantity since lower quantities may give false positive results, while higher quantities may give false negative results (Wiegand, Hilpert, & Hancock, 2008). Broth microdilution is a modified version of the macrodilution method where the assay is performed in a sterile 96-well microplate instead of tubes, followed by measurement using a 96-well microplate reader, again detailed by Jorgensen & Ferraro (2009). In both methods, the MIC of the antimicrobial compound is therefore determined. Advantages of this method are that many samples can be assayed at one time and a small quantity of sample, broth and antimicrobial drug can be used. The bactericidal activity of an antimicrobial agent can also be determined. This is termed as the MBC and is defined by the CLSI as the lowest concentration at which a drug kills $\geq$ 99.9% of bacteria after incubation with the antimicrobial agent over a specified period (CLSI, 1999). This method has been employed by various researchers in the assessment of the antimicrobial activity of a

drug (Pankey & Sabath, 2004; Andrews, 2006; Hernandes *et al*., 2013). In both assays, PEN will be used as a control antimicrobial drug against PEN-sensitive Gram-positive bacteria, while OXY will be used as a control antimicrobial agent against PEN-resistant Gram-positive bacteria and against Gram-negative bacteria, which possess resistance mechanisms against PEN as detailed by researchers such as Sutherland (1964).

3.1.5 Time-kill assay

The time-kill assay measures the rate at which the bacterial killing activity of an antimicrobial agent occurs and is dependent on concentration (CLSI, 1999). The number of colony forming units of the test sample, which is converted to $\log_{10}$, is estimated over specific time intervals, e.g., 1-2 hours (Messick, Rodvold, & Pendland, 1999) and is plotted against time. The CLSI defined killing activity as the reduction of bacterial colonies by ≥99%, while other studies report killing activity as the reduction of bacterial colonies by ≥3 $\log_{10}$ (Belley *et al*., 2008; Bremmer *et al*., 2017; Cai *et al*., 2017).

3.1.6 Approaches to determining the antibacterial activity of newly developed ferrocenyl chalcone compounds

One approach used in this current determination is to measure the MIC values of the newly developed ferrocenyl chalcone compounds against non-resistant laboratory-adapted bacteria, as well as non-resistant and resistant clinical isolates. Another approach is to determine the rate of bactericidal activity of effective compounds over time.

3.2 Materials and Methods

3.2.1 Preparation of sterile agar, reagents, tubes and other consumables

SA plates and NA plates were prepared as described in Section 2.1.1 and Section 2.1.2.

3.2.2 Wet Disk Diffusion (Sharma *et al*., 2012)

1 mg of each chalcone chemical was dissolved in 1 ml of 100% (DMSO) (Alfa Aesar, Heysham, UK) and thoroughly mixed, as detailed in Section 2.1.3. The solutions were then diluted 10X and 100X with sterile, deionized water, which was previously autoclaved at 121^0C for 15 minutes. Sterile blank paper disks (approximately 6 mm in diameter) were aseptically placed in each of the labelled sections of the inoculated ISA plates, which were prepared as described in Section 2.1.1. 30 µl of each diluted chalcone was pipetted onto a sterile blank disk. CIP disks (Oxoid, Thermo Fisher, UK), a broad-spectrum antimicrobial agent (Ball, 2000), were used as positive controls while 30 µl 10X DMSO on sterile blank disks was used as the negative control. The plates were incubated at 37^0C in air for 18-24 hours.

3.2.3 Dry Disk Diffusion (Modified Method by Sharma *et al.,* 2012)

Sterile blank disks were soaked overnight in stock chalcone solution, which were prepared as described in Section 2.1.3 (1 mg chalcone powder in 1 ml DMSO) and 10X dilutions of the solutions. The disks were then air-dried in a Petri-dish in a sterile fume hood. Using a sterile pair of forceps, the dried disks were placed on the appropriate section of labelled inoculated ISA plates, which were prepared as described in Section 2.1.1. CIP disks were used as the positive controls while disks impregnated with 10X DMSO were used as the negative controls. The plates were then incubated at 37^oC in air for 18-24 hours.

3.2.4 Agar well diffusion

Chalcone stock solutions were prepared by dissolving 1 mg of the chalcone powder in 1 ml of DMSO and mixed by vortexing, as detailed in Section 2.1.3. From these stock solutions, 10X working solutions were prepared in sterile deionised water. The inocula were prepared by suspending at least 3-4 colonies of pure cultures of *Klebsiella pneumonia* (IH), *Enterococcus aerogenes* NCIMB 10102, *Escherichia coli*

NCIMB 9483 and *Pseudomonas aeruginosa* NCTC 10332 in 10 ml aliquots of sterile PBS. Each inoculum was spread onto appropriately labelled ISA plates where 2 plates were allocated for the stock solution and 2 plates were allocated for the 10X solutions (4 plates per organism). A sterile cork-borer was used to create 6 holes (diameter = 6 mm) in each agar plate. 30 µl of appropriate chalcone solution (stock and 10X solutions) were pipetted into their respective holes. A 10X DMSO solution was used as the negative control while OXY (Alfa Aesar, Heysham, UK), which was prepared by dissolving 0.128 mg per ml of sterile deionised water, was used as the positive control. The solutions were allowed to diffuse into the agar and then the plates were inverted before incubation at 37^{o}C in air for 18-24 hours.

3.2.5 MIC using Two-fold serial broth macrodilution and microdilution

3.2.5.1 Preparation of stock ferrocenyl chalcone solution

For broth macrodilution assays, fresh stock of each ferrocenyl chalcone solution were prepared as described in Section 2.1.3 at concentrations of 5 mg/ml in DMSO. Fresh stock solutions of each compound were prepared as described in Section 2.1.3 at concentrations of 1 mg/ml in DMSO for each broth microdilution. The final concentrations of each ferrocenyl chalcone are shown in Table 2-1 and Table 2-2.

3.2.5.2 Preparation of stock antimicrobial compound

Stock antimicrobial solutions of PEN-G and OXY were prepared as detailed in Section 2.1.4. Each solution was divided into 1 ml aliquots in sterile microcentrifuge tubes and then stored at -20^{o}C.

3.2.5.3 Preparation of inocula

Each inoculum was freshly prepared each time as detailed in Section 2.2.1.

3.2.5.4 MIC determination using Broth Macrodilution

Four sets of sterile borosilicate glass tubes (one set per organism) were labelled "Org 1-5" (Organisms used = *E. coli* NCIMB 9483*, K. pneumonia* (IH)*, E. faecalis* NCTC 12697*, S. aureus* NCIMB 8244, another 4 sets of glass tubes (1 set per organism) were labelled "Org +DMSO 1-5" and 4 tubes (1 per organism) was labelled "Org + PEN/OXY". 500 µl of ISB was pipetted into all tubes. 500 µl of stock chalcone (5 mg in

1 ml of 100% $^{v}/_{v}$) was pipetted into the tubes labelled "Org 1", which was then followed by 2-fold serial dilutions up to tubes labelled "Org 5". A similar series of steps were performed for the tubes labelled "Org + DMSO 1" using 500 µl of DMSO while 500 µl of PEN (for Gram-positive organisms) and OXY (for Gram-negative organisms) was added to the tubes labeled "Org + PEN/OXY". An equal volume of each prepared inoculum (500 µl) was added to the appropriately labelled tubes and mixed by vigorous shaking. The tubes were then incubated at 37°C in air for 18-24 hours. The absorbance values for each solution were read at 600 nm on the spectrophotometer.

3.2.5.5 Preparation of bacterial standard curves for broth microdilution

Standard curves of *Staphylococcus aureus* NCIMB 8244, *Streptococcus pyogenes* NCIMB 8884, *Enterococcus faecalis* NCTC 12697, *Salmonella "Manchester"* NCTC 7372, *Pseudomonas aeruginosa* NCTC 10332, *Klebsiella pneumoniae* (IH) and *Escherichia coli* NCIMB 9483 were prepared as described in Section 2.4. The number of cfu/ml, which were converted to $\log_{10}$ cfu/ml, were determined for each organism and plotted against absorbance using the graph package 'MS Excel' to produce standard curves (Appendix 3, Figure 7-34 to 7-43).

3.2.5.6 Initial determination of cfu/ml

Each prepared inoculum was serially diluted 10 X in sterile PBS from 10^{-1} mg/ml to 10^{-8} mg/ml as detailed in Section 2.4.

3.2.5.7 MIC determination using Broth Microdilution

The microdilution plates for ferrocenyl chalcones and DMSO only were prepared as detailed in Section 2.6 and as seen in Appendix 2 (Table 7-2 and Table 7-3, respectively). Measurements of $\log_{10}$ cfu/ml were calculated from equations generated from the respective standard curves (Appendix 3, Figure 7-34 to Figure 7-43). Mean (± SD) MIC values of the ferrocenyl chalcone compounds were extrapolated from the calculated $\log_{10}$ cfu/ml values of each bacterial sample that were within 1 SD of the initial $\log_{10}$ cfu/ml. Each assay was performed in triplicate producing a total of 6 measurements.

3.2.5.8 Determination of MBC

After incubation of each inocula and ferrocenyl chalcone as described in Section 3.2.5.7, a loopful of each solution with no visible growth was applied to quadrants of labelled NA plates. The plates were incubated at 37^{o}C in air for 18-24 hours and visually assessed for bacterial growth.

3.2.6 Time-kill assay of Gram-positive organisms

3.2.6.1 Preparation of stock decyl ferrocenyl chalcone solution

The compound with the best overall MIC activity, as estimated in Section 3.2.5.7, was used in this assay. Fresh stock decyl ferrocenyl chalcone solution was prepared as described in Section 2.1.3. The solution was then serially diluted in a 1:1 ratio in sterile MH broth up to the required MIC.

3.2.6.2 Preparation of inocula

Each inoculum was prepared as described in Section 2.2.1 in sterile MHB. The organisms used in this assay were *S. aureus* NCIMB 8244, Fully Sensitive *S. aureus* (CRH), *E. faecalis* NCTC 12697 and *K. kristinae* NCIMB 8884 (previously misidentified as *S. pyogenes*).

3.2.6.3 Time-kill assay procedure

Nine sterile polypropylene tubes were labelled for 1xMIC, 2xMIC and control (3 per condition). The assay was then prepared and completed as described in Section 2.7. Mean (± SD) cfu/ml was plotted against time (hours) in Excel.

Results

3.2.7 Disk Diffusion

3.2.7.1 Wet Disk Diffusion

The results showed zones of growth inhibition from 6 mm to 7 mm for disks containing 10-fold diluted solutions, 6 mm to 9 mm for disks containing 100-fold diluted solutions, and 6 mm-13 mm for disks containing stock solutions (1 mg/ml). Zones of inhibition from 27 mm to 41 mm were seen for disks containing CIP against all bacterial samples.

Table 3-1 Zones of inhibition (mm) of 10 ferrocenyl chalcones 10-fold diluted solutions, DMSO and CIP against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	6	7	7	7
Ethyl	6	6	7	6
Propyl	6	7	6	7
Butyl	7	7	6	7
Pentyl	7	6	6	7
Hexyl	7	6	7	6
Heptyl	6	6	7	7
Octyl	6	7	7	7
Nonyl	7	7	6	6
Decyl	6	7	6	6
DMSO (10% $^{v}/_{v}$)	6	7	6	7
CIP (1 µg)	28	38	33	31

Table 3-2 Zones of inhibition (mm) of 10 ferrocenyl chalcones 100-fold diluted solutions, DMSO and CIP against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	7	7	6	7
Ethyl	6	7	6	6
Propyl	7	7	7	6
Butyl	6	6	8	6
Pentyl	6	6	7	7
Hexyl	6	6	7	7
Heptyl	7	6	7	7
Octyl	7	7	6	7
Nonyl	7	6	6	6
Decyl	6	6	6	7
DMSO (10% $^{v}/_{v}$)	6	9	6	8
CIP (1 µg)	27	41	36	32

Table 3-3 *Zones of inhibition (mm) of 10 ferrocenyl chalcones stock solutions, DMSO and CIP against non-resistant laboratory bacteria.*

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	6	11	6	12
Ethyl	6	11	6	11
Propyl	7	12	8	13
Butyl	8	11	8	10
Pentyl	7	10	7	10
Hexyl	9	10	9	12
Heptyl	10	11	10	12
Octyl	10	11	9	12
Nonyl	10	13	8	13
Decyl	10	11	10	11
DMSO (10% $^{v}/_{v}$)	6	8	6	9
CIP (1 µg)	29	40	36	31

3.2.7.2 Dry Disk Diffusion

The results showed zones of growth inhibition from 6 mm to 8 mm for disks containing 10-fold diluted solutions and for disks containing stock solutions (1 mg/ml). Zones of inhibition from 29 mm to 41 mm were seen for disks containing CIP against all bacterial samples.

Table 3-4 Zones of inhibition (mm) of 10 ferrocenyl chalcones 10-fold diluted solutions, DMSO and CIP against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	6	6	7	6
Ethyl	7	6	7	7
Propyl	7	6	6	7
Butyl	6	7	8	7
Pentyl	8	7	7	6
Hexyl	7	6	8	7
Heptyl	6	6	6	7
Octyl	7	7	7	7
Nonyl	7	7	8	6
Decyl	7	6	6	7
DMSO (10% $^{v}/_{v}$)	6	8	7	6
CIP (1 µg)	30	39	36	31

Table 3-5 Zones of inhibition (mm) of 10 ferrocenyl chalcones neat solutions, DMSO and CIP against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	8	6	8	6
Ethyl	6	7	6	6
Propyl	7	7	8	7
Butyl	7	8	8	7
Pentyl	7	7	7	8
Hexyl	8	7	7	6
Heptyl	7	7	6	8
Octyl	6	6	8	7
Nonyl	7	6	6	7
Decyl	7	7	6	8
DMSO (10% $^{v}/_{v}$)	8	8	7	6
CIP (1 µg)	31	40	35	29

3.2.8 Agar well diffusion

After incubation for 18-24 hours at 37°C, the results showed zones of growth inhibition from 6 mm to 8 mm for wells containing 10-fold diluted solutions and for wells containing stock solutions (1 mg/ml). Zones of inhibition from 30 mm to 41 mm were seen for wells containing OXY against all bacterial samples.

Table 3-6 Zones of inhibition (mm) of 10 ferrocenyl chalcones 10-fold diluted solutions, DMSO and OXY against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	7	6	8	7
Ethyl	6	7	6	6
Propyl	8	7	7	6
Butyl	8	7	7	6
Pentyl	8	6	7	7
Hexyl	8	8	6	7
Heptyl	7	7	6	8
Octyl	7	7	8	8
Nonyl	6	6	7	8
Decyl	6	6	7	7
DMSO (10% $^{v}/_{v}$)	7	8	8	6
OXY	34	40	36	30

Table 3-7 Zones of inhibition (mm) of 10 ferrocenyl chalcones neat solutions, DMSO and OXY against non-resistant laboratory bacteria.

Chalcone/Compound	Zone of inhibition (mm)			
	***K. pneumonia* (IH)**	***E. aerogenes* NCIMB 10102**	***E. coli* NCIMB 9483**	***P. aeruginosa* NCTC 10332**
Methyl	6	6	6	7
Ethyl	7	6	6	6
Propyl	6	6	8	7
Butyl	6	8	8	7
Pentyl	8	8	7	6
Hexyl	7	7	7	6
Heptyl	7	8	7	7
Octyl	6	8	8	7
Nonyl	8	6	8	7
Decyl	7	7	8	7
DMSO (10% $^{v}/_{v}$)	6	8	6	6
OXY	30	41	33	32

It became apparent that the initial diffusion assays were performed using DMSO contaminated with water thus affecting validity of the results presented in Sections 3.2.7 and 3.2.8. Even so, the results gave sufficient indication of the potential MIC and are included here to justify subsequent research. Progress with the following investigations did not allow time to repeat the original diffusion assays.

3.2.9 MIC using Two-fold serial broth microdilution and broth microdilution

3.2.9.1 Broth macrodilution

Table 3-8 *Mean (± SD) MIC values of 10 ferrocenyl chalcone compounds (5 mg/ml) against non-resistant laboratory-adapted bacteria.*

Chalcone	MIC values (mg/ml)			
	S. aureus **NCIMB 8244**	***E. faecalis*** **NCTC 12697**	***K. pneumonia*** **(IH)**	***E. coli*** **NCIMB 9483**
Methyl	2.500	2.500	1.250	1.250
Ethyl	2.500	2.500	2.500	2.500
Propyl	2.500	2.500	2.500	2.500
Butyl	2.500	1.250	2.500	2.500
Pentyl	1.250	0.630	2.500	2.500
Hexyl	0.310	0.310	2.500	2.500
Heptyl	0.310	0.630	2.500	2.500
Octyl	0.310	0.310	2.500	2.500
Nonyl	2.500	1.250	2.500	2.500
Decyl	0.150	0.150	2.500	1.250

The MIC values determined from broth macrodilution ranged from 0.150 mg/ml to 2.500 mg/ml where the percentage of DMSO present ranged from 3.130% $^{v}/_{v}$ to 50.000% $^{v}/_{v}$. Additionally, the concentration (5 mg/ml) used in the broth macrodilution assays was derived from Medu (2013). However, the absorbance values obtained from the current study indicated the difficulty in distinguishing between the dark brown colour observed after incubation and actual bacterial growth. Even so, the results provided enough indication of the potential MIC, which are approximately 5X those obtained from the broth microdilution assays, and are included here to justify subsequent research. Progress with the following investigations did not allow time to repeat the macrodilution assays.

3.2.9.2 Broth microdilution

Methyl to pentyl ferrocenyl chalcones and non-methylated ferrocenyl chalcone showed lower antimicrobial activity than hexyl to decyl ferrocenyl chalcones (Table 3-9). The former group of compounds showed MIC values of 0.125 mg/ml (± 0.000)

for all organisms tested. MIC values at 0.125 mg/ml contained 12.500% $^{v}/_{v}$ DMSO, which was also the threshold at which microbial growth was seen. The MBC assays revealed that bacterial growth in DMSO was only inhibited at greater than, or equal to, 12.500% $^{v}/_{v}$ DMSO. The chalcones with longer alkyl chain lengths (hexyl-decyl) also had lower MICs against Gram-positive bacteria than against Gram-negative bacteria (Table 3-9). MIC values of ferrocenyl chalcones with longer alkyl chains ranged from 0.008 mg/ml (± 0.000) and 0.063 mg/ml (± 0.000) for *S. aureus* NCIMB 8244, *E. faecalis* NCTC 12697, *K. kristinae* NCIMB 8884 and a non-resistant clinical isolate of S. aureus (Table 3-9), while MIC values of the same group of ferrocenyl chalcones against all Gram-negative bacteria were equal to 0.125 mg/ml (± 0.000). MIC values for the same longer alkyl chain ferrocenyl chalcones against resistant clinical isolates range of *S. aureus* from 0.031 mg/ml (± 0.000) to 0.063 mg/ml (± 0.000) (Table 3-9). The MBC values of the hexyl to decyl ferrocenyl chalcones against the laboratory -adapted bacteria were also equal to the MICs.

Table 3-9 *Mean (± SD) MIC values of 11 ferrocenyl chalcone compounds (1 mg/ml) against non-resistant and resistant laboratory-adapted bacteria and clinically isolated bacteria. NM = non-methylated ferrocenyl chalcone. *MBC values.*

Organism	**Mean (±0.000, n=6) MIC (mg/ml)**										
	NM	**Methyl**	**Ethyl**	**Propyl**	**Butyl**	**Pentyl**	**Hexyl**	**Heptyl**	**Octyl**	**Nonyl**	**Decyl**
***S. aureus* NCIMB 8244**	0.125	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.031*
***K. kristinae* NCIMB 8884**	0.125	0.125	0.125	0.125	0.125	0.125	0.031*	0.008*	0.016*	0.016*	0.016*
***E. faecalis* NCTC 12697**	0.125	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.063*
S. aureus* Fully Sens. *(CRH)	0.125	-	-	-	-	-	0.125*	0.063*	0.063*	0.063*	0.063*
PEN-resistant S. aureus (CRH)	0.125	-	-	-	-	-	0.063*	0.031*	0.063*	0.063*	0.063*
PEN/ERY/CLI-resistant (CRH)	0.125	-	-	-	-	-	0.063*	0.031*	0.063*	0.063*	0.063*
MRSA (CRH)	0.125	-	-	-	-	-	0.063*	0.063*	0.063*	0.063*	0.063*
***E. coli* NCIMB 9483**	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
K. pneumoniae (IH)	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
***Salmonella "Manchester"* NCTC 7372**	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
E. coli* Fully Sens. *(CRH)	0.125	-	-	-	-	-	0.125	0.125	0.125	0.125	0.125

3.2.10 Time kill assay

At 1xMIC (0.031 mg/ml), mean (± SD) cfu reduced to 0 (± 0.000) log_{10} cfu/ml from 4 hours, while reduction of mean (± SD) cfu to 0 (± 0.000) log_{10} cfu/ml occurred from 2 hours after the organism was exposed to 2xMIC (0.063 mg/ml) (Figure 3-1). In the same assay, the percentages of DMSO present in solution exhibited no bactericidal activity at 3.100% and 6.300%. Untreated cells showed normal growth trends up to 24 hours. The results of the time kill assay for decyl ferrocenyl chalcone against *S. aureus* NCIMB 8244 confirmed that bactericidal activity occurred.

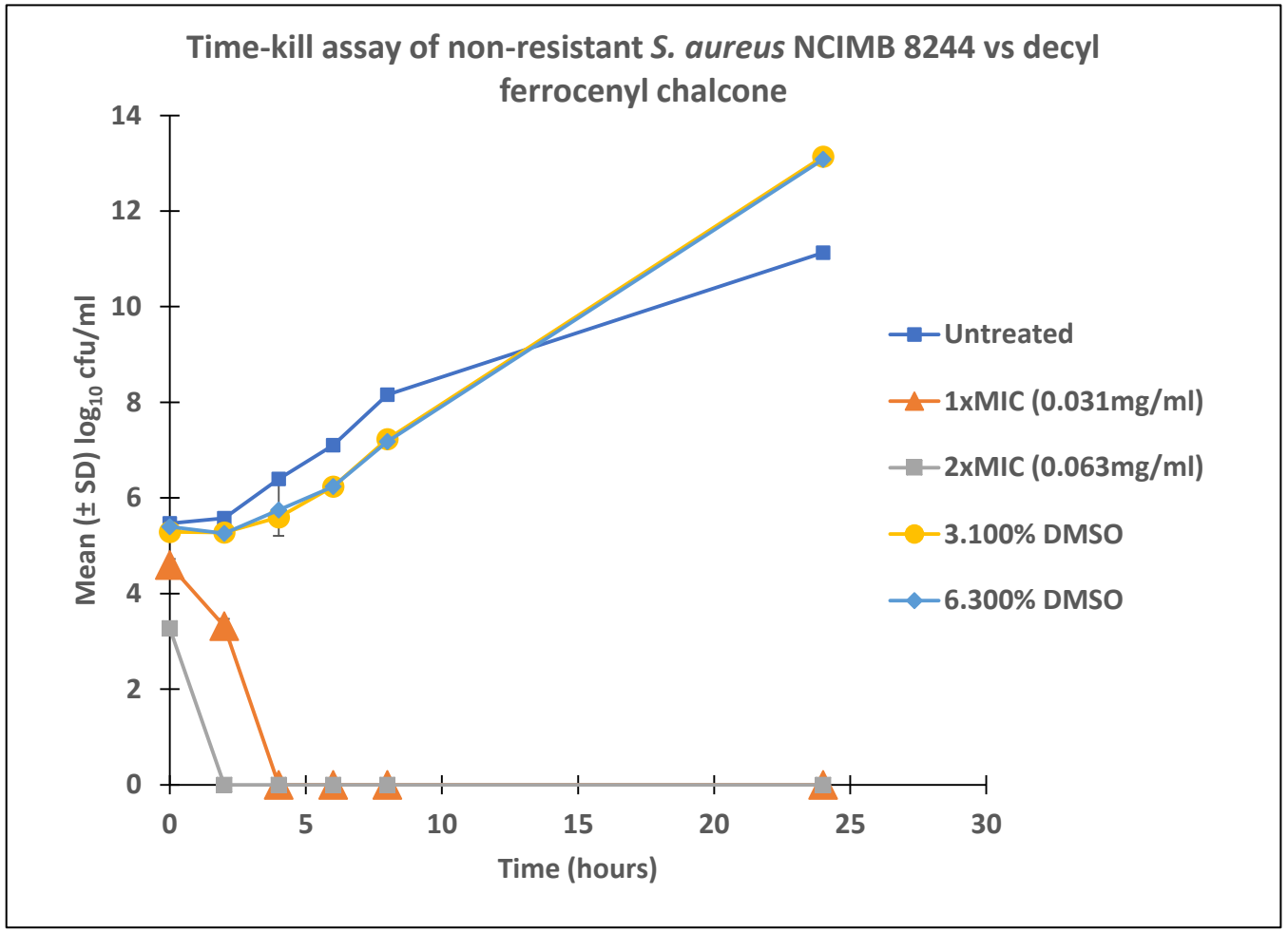


Figure 3-1 *Time-kill curve (n=3) of decyl ferrocenyl chalcone compound and DMSO against S. aureus NCIMB 8244 at 1xMIC and 2xMIC. The curve for 3.1% DMSO (circle) contained similar values to those measured for 6.3% DMSO (diamond).*

Results of the time kill assay for decyl ferrocenyl chalcone also confirmed that the compound possessed bactericidal activity against a MRSA. At 1xMIC (0.063 mg/ml) and 2xMIC (0.125 mg/ml), mean (± SD) cfu reduced to 0 (± 0.000) $\log_{10}$ cfu/ml from 2 hours, (Figure 3-2). In the same assay, the percentages of DMSO present in solution exhibited no bactericidal activity at 6.300% $^{v}/_{v}$ and 12.500% $^{v}/_{v}$. Untreated cells showed normal growth trends up to 24 hours.

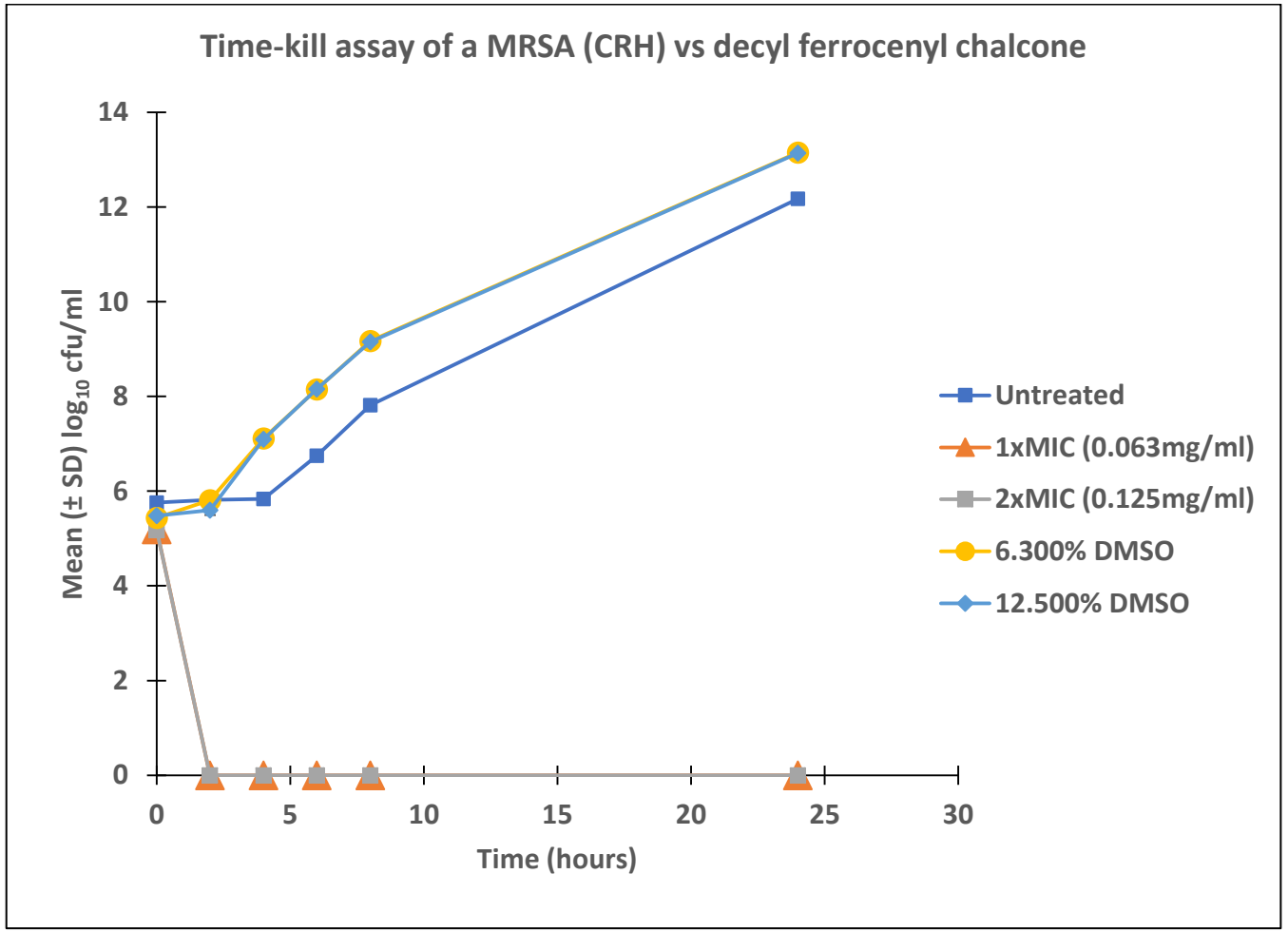


Figure 3-2 *Time-kill curve (n=3) of decyl ferrocenyl chalcone compound and DMSO against a MRSA at 1xMIC and 2xMIC. The curve for 6.300% DMSO (circle) contained similar values to those measured for 12.500% DMSO (diamond), while the curve for 1xMIC (triangle) contained similar values to those measured for 2xMIC (large square).*

Findings of the time kill assay for decyl ferrocenyl chalcone against *E. faecalis* NCTC 12697 revealed the effect of bactericidal activity. At 1xMIC (0.063 mg/ml), mean (± SD) cfu reduced to 0 (± 0.000) $\log_{10}$ cfu/ml from 2 hours, while reduction of mean (± SD) cfu to 0 (± 0.000) $\log_{10}$ cfu/ml occurred from 0 hours after the organism was exposed to 2xMIC (0.125 mg/ml) (Figure 3-3). In the same assay, the percentages of DMSO present in solution exhibited no bactericidal activity at 6.300% $^{v}/_{v}$ and 12.500% $^{v}/_{v}$. Untreated cells showed normal growth trends up to 24 hours.

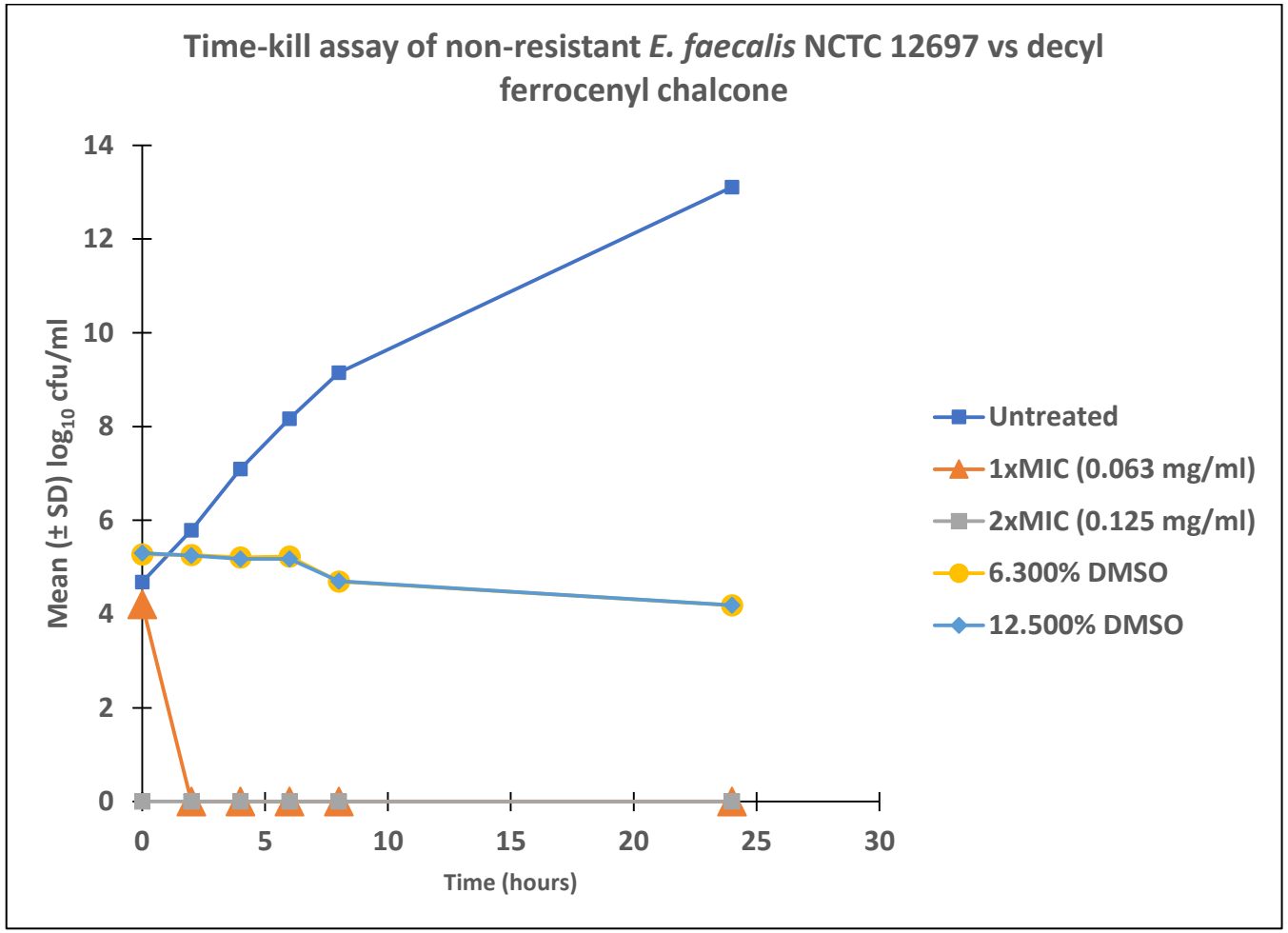


Figure 3-3 *Time-kill curve (n=3) of decyl ferrocenyl chalcone compound and DMSO against E. faecalis NCTC 12697 at 1xMIC and 2xMIC. The curve for 6.300% DMSO (circle) contained similar values to those measured for 12.500% DMSO (diamond).*

Evidence of bactericidal activity was also seen for decyl ferrocenyl chalcone against *K. kristinae* NCIMB 8884. At 1xMIC (0.016 mg/ml) and 2xMIC (0.031 mg/ml), mean (± SD) cfu reduced to 0 (± 0.000) $\log_{10}$ cfu/ml from 0 hours (Figure 3-4). In the same assay, the percentages of DMSO present in solution exhibited bacteriostatic, but not bactericidal activity, at 1.600% $^{v}/_{v}$ and 3.100% $^{v}/_{v}$. Untreated cells showed normal growth trends up to 24 hours.

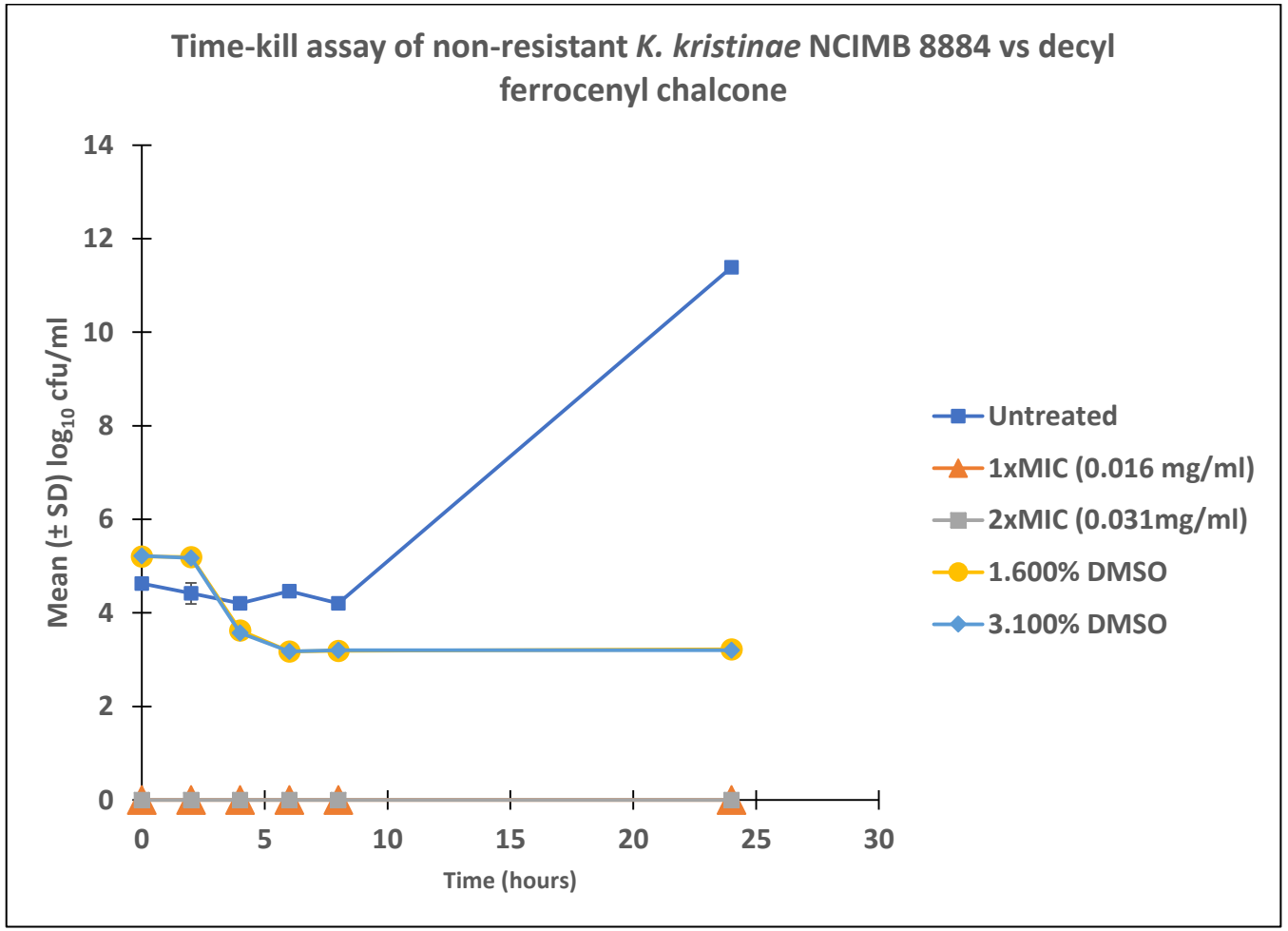


Figure 3-4 *Time-kill curve (n=3) of decyl ferrocenyl chalcone compound and DMSO against K. kristinae NCIMB 8884 at 1xMIC and 2xMIC. The curve for 1.600% DMSO (circle) contained similar values to those measured for 3.100% DMSO (diamond), while the curve for 1xMIC (triangle) contained similar values to those measured for 2xMIC (large square).*

3.3 Discussion

3.3.1 Determination of antimicrobial activity using disc diffusion and agar well diffusion methods

The techniques used in these assays were pilot experiments to determine the appropriateness of using diffusion methods in the current study. While most of the results indicated that the methods were unsuitable, key details about the inhibition of diffusion of the ferrocenyl chalcone compounds into the agar were obtained. Therefore, as stated at the end of Section 3.2.8, the inclusion of the results of the diffusion methods was necessary in order to determine more suitable procedures as the research work progressed.

The results of both the agar well diffusion and disc diffusion showed little to no clear zones of inhibition, with zone diameters from 6 mm to 13 mm, when the organisms were treated with stock chalcone solution of all ferrocenyl chalcone compounds. The presence of no clear zones of growth inhibition, when compared to visible clear zones of bacterial growth with large diameters (29 mm to 40 mm) after exposure to CIP were lower than the reported susceptibility values of 14 mm to 40 mm (Wootton *et al*., 2014). The values were also lower than the reported susceptibility values of ≥22 mm (Enterobacteriaceae) and ≥15 mm (*Enterococcus spp.*) (EUCAST, 2016). This result was unexpected and may be explained by further examination of the solvent DMSO. It is frequently used to solubilise lipophilic compounds used in antimicrobial sensitivity assays but can be a bacteriostatic agent, i.e., an agent that stops bacterial growth but does not kill bacteria as reported by (Basch, Gadebusch, & Brunswick, 1968). Although the chalcones were completely solubilised in DMSO, they began to precipitate after a few minutes. Further investigations have suggested that the DMSO used in these assays appeared to be saturated with water, as DMSO is hygroscopic. This contamination results in characteristics such as lower solubilisation ability and the solvent emits a pungent aroma. As the chalcones were no longer in solution, it is possible that the precipitated particles, which may have been larger than the pore size of the agar lattice (≥3 mμ) (Hewitt, 2005), could not diffuse into the agar, hence resulting in little to no zones of growth inhibition.

3.3.2 Determination of MIC using broth macrodilution

The results of the broth macrodilution showed unusual results with no discernible trend. Results of the broth macrodilution tests revealed that bacterial growth was inhibited at ≥12.500% $^{v}/_{v}$ DMSO, which was the concentration of DMSO in solutions containing 0.630 mg of ferrocenyl chalcone compound. The inhibitory concentration of DMSO that was obtained in the current tests were also reported by Basch *et al*. (1968) and Duric *et al*. (2013). In the same current tests, results showed that alkyl chain influenced antibacterial activity of the ferrocenyl chalcone compounds. Thus, the results would have been expected to show an increase in sensitivity as chalcones alkyl chain lengths increased, particularly with the hexyl to decyl ferrocenyl chalcone compounds. However, *S. aureus* NCIMB 8244 sensitivity was seen against hexyl (0.310 mg/ml), heptyl (0.310 mg/ml), octyl (0.310 mg/ml) and decyl (0.150 mg/ml) but was reduced against nonyl (2.500 mg/ml). The concentration of DMSO contained in solutions with hexyl to octyl and decyl was 3.100% $^{v}/_{v}$ to 6.250% $^{v}/_{v}$, which was lower than the inhibitory concentration of 12.500% $^{v}/_{v}$ and indicated that growth inhibition was caused by the presence of ferrocenyl chalcone. In contrast, nonyl ferrocenyl chalcone did not have an effect on the same organism since the MIC value was 2.500 mg/ml and the DMSO concentration (50.000%) was ≥12.500% $^{v}/_{v}$. Thus, DMSO, and not nonyl ferrocenyl chalcone, inhibited bacterial growth.

A similar trend was observed for nonyl ferrocenyl chalcone against *E. faecalis* NCTC 12697, where the MIC value was 1.250 mg/ml and the inhibitory concentration of DMSO (25.000% $^{v}/_{v}$) was ≥12.500% $^{v}/_{v}$. Conversely, pentyl (0.630 mg/ml), hexyl (0.310 mg/ml), heptyl (0.630 mg/ml), octyl (0.310 mg/ml) and decyl (0.150 mg/ml) showed antibacterial activity against *E. faecalis* NCTC 12697 since the concentrations of DMSO (3.125% $^{v}/_{v}$ - 12.500% $^{v}/_{v}$) were ≤12.500% $^{v}/_{v}$.

The MIC values for all ferrocenyl chalcone compounds against *K. pneumonia* (IH) and *E. coli* NCIMB 9483 were 1.250 mg/ml and 2.500 mg/ml with DMSO concentrations (25.000% $^{v}/_{v}$ – 50.000% $^{v}/_{v}$) that were greater than the inhibitory concentration of 12.500% $^{v}/_{v}$. Therefore, DMSO, and not the ferrocenyl chalcone compounds, exhibited antibacterial activity against the Gram-negative organisms.

The absorbance values that were derived from the experiment were sometimes high at increased chalcone concentrations (Table 3-8), which would indicate bacterial growth. However, no growth was seen when the solutions were inoculated on NA plates. A theory of why no growth was observed is that a quantity of ferrocenyl chalcone remained solubilised in hydrated DMSO. Although hydrated DMSO is reported to lose its power of solubility with organic compounds (Ellson *et al*., 2005), an unknown quantity of solubilised ferrocenyl chalcone may have exhibited bactericidal activity in the assay. This was further proof for the suggestion that the DMSO was hydrated and that the ferrocenyl chalcones were reversibly solubilised. The insoluble chalcones were possibly rendered ineffective in this suspended state since they were unable to cross the lipid bilayer of the bacterial cell membrane. Negative absorbance values (Table 3-8) could also be indicative of incomplete solubilisation in DMSO. This indicated that DMSO and/or broth, and not chalcone, was absorbing more light than the reference solutions.

3.3.3 Determination of MIC using broth microdilution

In the broth microdilution assay, fresh DMSO was used as the solvent for the ferrocenyl chalcones. The results in Table 3-9 showed that MIC values (0.125 mg/ml in 12.500% $^{v}/_{v}$ DMSO) of the non-methylated and methylated ferrocenyl chalcone compounds with shorter alkyl chains (methyl to pentyl) were within the reported values for PEN (0.000015-0.128 mg/ml) (Andrews, 2001; Andrews, 2006) and the reported value of ≤0.125 mg/ml for the same antimicrobial compound (EUCAST, 2016) against *Staphylococci*. In the same assay, the MIC values of all 10 compounds were 0.125 mg/ml in 12.500% $^{v}/_{v}$ DMSO against Enterobacteriaceae (Table 3-9), which were within the values reported by Andrews (2001 & 2006) for tetracycline (0.00025-0.128 mg/ml) against Enterobacteriaceae. Breakpoint values for tetracycline against Enterobacteriaceae were not reported by EUCAST (2016). Reported values by Andrews (2001 & 2006) were used since OXY is an analogue of tetracycline. While the MIC values of the above-mentioned ferrocenyl chalcone compounds were similar to those of the appropriate control antimicrobial agents, growth inhibition, as exhibited by these novel compounds, may also have resulted from exposure of the organisms to DMSO. DMSO has been shown to have an

inhibitory effect at percentages equal to and/or greater than 12.500% $^{v}/_{v}$ (Basch, Gadebusch, & Brunswick, 1968; Duric *et al*., 2013). The chalcone MIC values for *S. aureus* NCIMB 8244 began to decrease as alkyl chain length increased. This was especially seen with hexyl to nonyl (0.063 mg/ml) and decyl (0.031 mg/ml) (Table 3-9). Except for hexyl against clinically isolated *S. aureus* (fully sensitive) (CRH), sensitivity was also seen for hexyl to decyl against PEN-resistant *S. aureus* (CRH), PEN-resistant, ERY-resistant, CLI-resistant *S. aureus* clinical isolates (CRH) (0.031 mg/ml to 0.063 mg/ml) (Table 3-9). Therefore, the presence of long alkyl chains increases antibacterial activity.

The MIC/MBC values reported in Table 3-9 varied between each organism and between each chalcone. When used against *S. pyogenes* NCIMB 8884, which was later confirmed to be *K. kristinae*, all chalcones with longer alkyl chains showed MIC/MBC values of 0.016 mg/ml except for hexyl (0.031 mg/ml) and heptyl (0.008 mg/ml). When used against *E. faecalis* NCTC 12697, hexyl to decyl ferrocenyl chalcones showed MIC/MBC values of 0.063 mg/ml. These values were also within the expected range of known antimicrobials (0.0005-0.128 mg/ml), such as PEN, for *Enterococci* (Andrews, 2001; Andrews, 2006). Although antimicrobial activity was seen with hexyl to decyl against *K. pneumoniae* (IH), *E. coli* (CRH), *E. coli* NCIMB 9483 and *Salmonella "Manchester"* NCTC 7372 (0.125 mg/ml), it may have resulted from sensitivity to 12.500% $^{v}/_{v}$ DMSO.

The overall trend appeared to be that the chalcones had a greater inhibitory effect on Gram-positive bacteria than on Gram-negative bacteria. The difference in MIC values with respect to the Gram-negative and Gram-positive organisms may be as a result of increasing alkyl chain length. One explanation why the Gram reaction may be a factor is that the compounds may pass across the hydrophilic peptidoglycan layer of Gram-positive bacteria because of the amphipathic DMSO (Matthews *et al*., 1975; Hassan, 2014). The long chains may become trapped in the cell membrane allowing the attached ferrocenyl groups, which are relatively smaller than the alkyl chains, to enter the cytoplasm. Since Gram-negative bacteria have outer envelopes with membrane transporter proteins such as porins, followed by thin peptidoglycan layers and cell membranes in their cellular envelopes, entry into these cells may be

more difficult. These porins allow hydrophilic compounds to enter, while hydrophobic compounds may diffuse across the lipid bilayer of the outer envelope, as detailed by various researchers (Nikaido, 2003; Pagès, James, & Winterhalter, 2008; Bolla *et al*., 2011). However, because of the fluidity of the outer lipid bilayer of Gram-negative bacteria (Seltmann & Holts, 2002), the long alkyl chains of the ferrocenyl chalcones may become trapped in the outer envelope and would be unable to cross the peptidoglycan layer and cell membrane into the cells (Henry *et al*., 2017). Another reason why the difference between Gram-positive and Gram-negative bacteria may be important is that organisms, such as *E. coli*, have become used to surviving in enriched media, which promotes vigorous growth (Cooper, Rozen, & Lenski, 2003; Fux *et al*., 2005) and may result in phenotypic changes to the cell wall.

3.3.4 Time-kill assay

In the time-kill assay, decyl ferrocenyl chalcone compound was selected since the compound exhibited the best overall bactericidal activity against the Gram-positive organisms in the current study (Table 3-9). The mean cfu of *S. aureus* NCIMB 8244 was reduced from 4.600 (± 0.130) $\log_{10}$ cfu/ml to 0.000 (± 0.000) $\log_{10}$ cfu/ml after 4 hours of incubation with the compound at 1xMIC (0.031 mg/ml), while the mean cfu decreased from 3.270 (± 0.080) $\log_{10}$ cfu/ml to 0.000 (± 0.000) $\log_{10}$ cfu/ml after 2 hours of incubation at 2xMIC (0.063 mg/ml) (Figure 3-1). Equivalent concentrations of only DMSO solvent (3.100% $^{v}/_{v}$ and 6.300% $^{v}/_{v}$) against the organism did not inhibit bacterial growth, since DMSO exhibits bacteriostatic activity at ≥12.500% $^{v}/_{v}$ (Basch, Gadebusch, & Brunswick, 1968; Duric *et al*., 2013). This meant that in the presence of chalcone, increased bactericidal activity was proportional to decreased MIC of ferrocenyl chalcone compound. This proportional trend was markedly different to the activity seen in the study by Scheerans *et al*. (2015) with linezolid against *S. aureus* ATCC 29213 where the compound exhibited antibacterial activity from 4-6 hours at 0.004 – 0.008 mg/ml of linezolid. At this time period and concentration, activity was determined to be bacteriostatic since mean (± SD) cfu were reduced from approximately 6.00 $\log_{10}$ cfu/ml to 4.50 – 5.50 $\log_{10}$ cfu/ml, which is ≤99.9% of cells.

However, from 24 hours at 0.016 mg/ml (8xMIC), there was evidence of bactericidal activity since mean (± SD) cfu was estimated to be ≤3 $\log_{10}$ cfu/ml.

At 1xMIC (0.063 mg/ml) and 2xMIC (0.125 mg/ml) of decyl ferrocenyl chalcone, mean (± SD) cfu of a MRSA was reduced from 5.190 (± 0.030) $\log_{10}$ mg/ml and 5.180 (± 0.000) $\log_{10}$ mg/ml, respectively, to 0.000 (± 0.000) $\log_{10}$ mg/ml from 2 hours (Figure 3-2). No bactericidal activity was seen at equivalent concentrations of DMSO solvent (6.300% $^{v}/_{v}$ and 12.500% $^{v}/_{v}$), since the chemical inhibits bacterial growth at ≥12.500% $^{v}/_{v}$ as reported by Basch *et al* (1968) and Duric *et al* (2013). Thus, the ferrocenyl chalcone possessed bactericidal activity, since ≤99.9% of cells were killed. The time taken to kill the MRSA cells in the current assay was less than the time taken for ceftobiprole to kill MRSA ATCC 33591 and MRSA clinical isolates reported in a previous study by Barbour *et al*. (2014), and was less than the time taken for telavancin and vancomycin to kill a clinical isolate of a MRSA reported in a later study (Rolston *et al*., 2017). In the former study, the mean (± SD) cfu of MRSA ATCC 33591 was reduced to ≤3 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml after 24 hours at ≥0.002 mg/ml (1xMIC), while the same time was taken to reduce the mean (± SD) cfu of MRSA clinical isolate from approximately 7.00 $\log_{10}$ cfu/ml to ≤3.00 $\log_{10}$ cfu/ml at ≥2xMIC. However, in the study by Rolston *et al*. (2017), the cfu of all 4 MRSA clinical isolates were reduced to ≥3.00 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml after 24 hours at 0.004 mg/ml (4xMIC) of vancomycin, while the cfu/ml of only 1 MRSA clinical isolate was reduced from approximately 6.00 $\log_{10}$ cfu/ml to ≥3 $\log_{10}$ cfu/ml after 24 hours at 0.008 mg/ml (8xMIC) of telavancin.

In the current study, mean (± SD) cfu of *E. faecalis* NCTC 12697 was reduced to 0.000 (± SD) $\log_{10}$ cfu/ml from 4.27 (± 0.030) $\log_{10}$ cfu/ml from 2 hours at 0.063 mg/ml (1xMIC), while bactericidal activity was seen immediately from 0 hour at 0.125 mg/ml (2xMIC) of decyl ferrocenyl chalcone (Figure 3-3). At equivalent concentrations of DMSO solvents (6.300% $^{v}/_{v}$ and 12.500% $^{v}/_{v}$), bacteriostatic activity and not bactericidal activity, was seen, since there was ≤99.9% reduction of bacterial cells. No known previous studies reported DMSO antimicrobial activity against *E. faecalis*. In a previous study (del Valle *et al*., 2016), cfu of *E. faecalis* ATCC 29212 was reduced to 2.23 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml from 10 hours at 0.075

mg/ml to 0.150 mg/ml (1xMIC) of kaempferol, while cfu of *E. faecalis* 1076 clinical isolate was reduced to 2.24 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml from 10 hours at 0.106 mg/ml to 0.175 mg/ml (1xMIC) of the same drug. Del Valle *et al.* (2016) also reported that cfu of *E. faecalis* ATCC 29212 was reduced to 1.38 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml from 10 hours at 0.113 mg/ml to 0.200 mg/ml of resveratrol, while cfu of *E. faecalis* 1076 clinical isolate was reduced to 1.45 $\log_{10}$ cfu/ml from approximately 6.00 $\log_{10}$ cfu/ml from 10 hours at 0.113 mg/ml to 0.163 mg/ml (1xMIC) of the same drug.

While there were no known reported findings from time-kill assays involving *K. kristinae*, the current study proposes that favourable bactericidal activity was achieved, since the mean (± SD) cfu of *K. kristinae* NCIMB was estimated to be 0.000 (± 0.000) $\log_{10}$ cfu/ml at 0.016 mg/ml (1xMIC) and 0.031 mg/ml (2xMIC) from 0 hour (Figure 3-4). Furthermore, equivalent concentrations of DMSO solvent (1.600% $^{v}/_{v}$ and 3.100% $^{v}/_{v}$) showed evidence of bacteriostatic activity. No known previous studies reported DMSO antimicrobial activity against *K. kristinae*.

3.4 Conclusion

Ferrocenyl chalcone compounds have been reported to possess broad-spectrum antibacterial activity (Kowalski *et al*., 2013; Mishra *et al*., 2015; Gopi, Sastry, & Dhanaraju, 2016; Khan *et al*., 2017). This activity, which is characterised by the MIC, can be assessed using agar-disk diffusion (Wheat, 2001; Howe & Andrews, 2012) and broth dilution (Andrews, 2001; Valgas *et al*., 2007; Wiegand, Hilpert, & Hancock, 2008; CLSI, 2012).

Overall findings of the present study indicate that the ferrocenyl chalcone compounds with longer alkyl chains (heptyl to decyl) inhibited bacterial cell growth when compared to a non-methylated ferrocenyl chalcone compound and those with shorter alkyl chains (methyl to pentyl). This means that the addition of alkyl chains and the length of these chains influence antibacterial activity (MIC values) of the ferrocenyl chalcone compounds.

The MIC values, obtained from the broth microdilution assays of the compounds with longer alkyl chains, were lower against Gram-positive bacteria than against Gram-negative bacteria. This means that the compounds with longer alkyl chains were more effective against Gram-positive bacteria than against Gram-negative bacteria. Additionally, their efficacy was evident against both non-resistant laboratory-adapted bacteria, and against non-resistant and resistant clinical isolates. Particularly, activity against resistant clinical isolates used in the current study indicates that the ferrocenyl chalcone compounds should be considered as potential antibacterial agents.

In terms of the time-kill assays, the active compounds also possessed bactericidal activity at 1xMIC and 2xMIC after 0 to 4 hours and this activity was maintained up to 24 hours. This activity was due to the presence of ferrocenyl chalcone compound since bacterial growth was either inhibited or unaffected by DMSO depending on the test organism. The time taken for bactericidal activity to occur was less than that for known compounds in other studies (Scheerans *et al*., 2015; del Valle *et al*., 2016; Rolston *et al*., 2017).

Although these compounds exhibited antibacterial activity, colour change from deep red to dark brown and the presence of brown precipitate were seen. The data presented in Chapter 4, including a review of an MSci study performed by Charles Bennett, highlights the approaches used to determine causes of these issues and whether antibacterial activity is affected.

4 Elucidation of the possible causes and consequence of colour change and particulate formation in diluted ferrocenyl chalcone solutions

4.1 Bioavailability of antibacterial agents

Clinical diagnoses, prognoses and treatment of diseases with the use of appropriate drugs are determined by assays involving human body fluids such as serum, plasma and cerebro-spinal fluid (Hu, Loo, & Wong, 2006; Driscoll *et al*., 2012). As reviewed by Varanda *et al*. (2006), successful activity of antimicrobial agents relies on the solubility of an agent in body fluids, which is a key characteristic of the bioavailability of the active ingredient in antimicrobial drugs. The primary routes of antimicrobial drug absorption, which ensure maximum system circulation of the active ingredient within the body, are intravenous and oral (Levison & Levison, 2009). Oral administration of drugs is preferred because of the availability of less expensive, but more effective, antibacterial compounds, which can be taken home out of the healthcare setting by the patient (Cyriac & James, 2014). Systemic distribution of antimicrobials within the body via either route usually necessitates that most drugs be water-soluble as detailed by Gao *et al.* (2011). However, since orally administrated antimicrobials are absorbed through the intestinal mucosa, these agents must possess hydrophobic properties (Goddard, 1998; Levison & Levison, 2009).

Within the last fifteen to twenty years, newly developed antibacterial drugs have gained the interest of researchers because they are water-insoluble and passively diffuse across the lipid bi-layer of cell membranes (Calvey & Williams, 2007). Unlike hydrophilic therapeutic molecules, such as β-lactam drugs, which easily diffuse into cells through membrane-bound porins, hydrophobic drugs diffuse into bacterial cells across the outer membranes of Gram-negative bacteria, which performs the role of additional protection (Delcour, 2009). For example, lipophilic antibacterial drugs are needed for treatment of bacterial prostatitis, which is enveloped in a hydrophobic membrane (Lipsky, Byren, & Hoey, 2010), and infections of the central nervous system, which is protected by the blood-brain barrier (Nau, Sorgel, & Eiffert, 2010). Due to increased prevalence of infections caused by MDR bacteria, some of which

are Gram-negative, there is the need for effective hydrophobic antimicrobial drugs. Therefore, the ferrocenyl chalcone compounds used in the current study were engineered to be lipophilic chemicals. These chemicals are synthetic derivatives of flavonoids, on which lipophilic data, defined by the octanol-water partition coefficient, is variable, as researched by Rothwell *et al*. (2005).

4.1.1 Solubility of ferrocenyl chalcone compounds

The ferrocenyl chalcone chemicals developed as antibacterial agents, and the focus of this study to determine their effectiveness and mode of action, are highly hydrophobic compounds. The hydrophobicity of the compounds is enhanced due to the addition of increasing lengths of alkyl chains, the addition of ferrocene groups, and the substitution of C5 on the benzene rings with nitrogen atom (Chapter 1, Figure 1-3). The alkyl groups of saturated, unbranched alkanes are water-insoluble and their hydrophobicity increases as chain length increases, as described by Bell (1973) and Stoker (2015). The second group, ferrocene, an aromatic metallocene, is also insoluble in water (Remington, 2006). The third component of the ferrocenyl chalcone compounds used in the current study, a nitrogen substituted benzene ring, is a highly water-insoluble aromatic cyclic structure, as discussed by several papers (May, Wasik, & Freeman, 1978; Buttrick & King, 2017; Taleb *et al*., 2017). The overall structure and chemical group composition of each ferrocenyl chalcone compound, minus the iodide component, was confirmed by Robert B Smith (UCLAN) using ^{1}H NMR (Appendix 2, Figure 9-1 to Figure 9-11), MS (Appendix 2, Figure 9-12 to Figure 9-22) and IR (Appendix 2, Figure 9-23 to Figure 9-33). The hydrophobicity of the ferrocenyl chalcone compounds necessitates the use of an appropriate solvent to enable the preparation of stock solutions to be further diluted in an aqueous broth for AST. In the current study, a number of solvents were used. In spite of completely dissolving the ferrocenyl chalcone compounds, with the exception of one, the stock solutions exhibited precipitation when diluted with bacterial growth solutions. DMSO was the most favourable compound of choice since it is amphipathic and is less inhibitive in terms of bacterial cell growth (Basch, Gadebusch, & Brunswick, 1968; Hassan, 2014) when compared to other assessed compounds such as absolute

ethanol, polyethylene glycol and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox).

4.1.2 Dilution of ferrocenyl chalcone compounds

All of the compounds investigated were found to be completely soluble in 100% DMSO. It was observed that the solutions change colour from deep red to dark brown within 2 hours. At 2X and 4X dilutions in MHB, fine brown particles were also seen when the solutions were diluted with MHB, which contains dehydrated infusion from beef, casein hydrolysate and starch, as detailed by Oxoid (2017). However, the brown particles were observed after incubation. In a study by Kanellopoulos & Wood (1976), as well as a review by Nitin (2009), particle size in suspensions are reportedly 0.4 µm to 12 µm, and are visible to the naked eye. MHB is the preferred medium since the broth efficiently supports the growth of most aerobic non-fastidious bacteria and extensive research information has been gathered from assays involving the medium (CLSI, 2012). This broth is the reference medium for AST (EUCAST, 2003).

4.1.3 Colour change of ferrocenyl chalcone compounds

Once dissolved in DMSO, the stock ferrocenyl chalcone solutions were observed to change from deep red to dark brown in polypropylene tubes at a faster rate than in borosilicate glass tubes. Assuming that the colour change in the current study was due to displaced iodine, diethyl ether was added to the solutions containing colour change. Hildebrand *et al*. (1950) were the first to report the use of diethyl ether to demonstrate the possible presence of displaced iodine, where a top layer comprised of a brown ring of iodine suspension and a clear, colourless bottom layer were observed. This was also observed and reported by Smith *et al*. (2013). This solvent was used since solid iodine readily dissolves in polar solvents such as diethyl ether, as discussed by Kaiho (2015). Concurrent with this assay, Charles Bennett conducted a research project as part of his Master of Science studies at Staffordshire University in 2016 to assess the effect of specific environmental conditions on the compounds used in this study, and the consequent antimicrobial activity of the altered compounds.

4.1.4 Approaches to elucidate causes and consequence of colour change and particulate formation in diluted ferrocenyl chalcone solutions

In the current methods, ferrocenyl chalcone solutions were randomly selected for assessment. One approach of this assay is to evaluate the solubility of ferrocenyl chalcone chemicals pre- and post-dilution with MHB. Another approach is to determine the possible cause of the colour change of these solubilised compounds over time. Finally, the study will review the findings of the Bennett project, which involved assessing the effect of environmental conditions of solubilised ferrocenyl chalcone compounds in terms of their rate of colour change and antibacterial activity after the visible colour change.

4.2 Material and Methods

4.2.1 Preparation of a selected ferrocenyl chalcone for observed precipitate

Fresh stock nonyl ferrocenyl chalcone solutions were prepared by dissolving 1 mg of powder in 1 ml of DMSO in a sterile polypropylene tube (tube 1) and in a sterile borosilicate glass tube (tube 2). Stock MHB and deionised water were autoclaved at 121^{o}C for 15 minutes and dispensed into 10 ml aliquots. Test sterile polypropylene tubes (2 x 75 µl sterile deionised water and 2 x 75 µl MHB) were prepared and 75 µl of dissolved stock ferrocenyl was added to each tube (tubes 3 – 6). A control tube (tube 7) containing 150 µl MHB was also prepared. One tube with MHB diluent (tube 3) and one tube with water diluent (tube 5) were sonicated for approximately two minutes. All tubes were sealed and observed for colour change and precipitate pre- and post-incubation at 37^{0}C for 72 hours. This method was performed in duplicate.

4.2.2 Preparation of sample for displaced iodine assay

Approximately 1 mg of solid decyl ferrocenyl chalcone was dissolved in 1 ml of DMSO in a 1.5 ml Eppendorf microcentrifuge tube made of polypropylene and incubated overnight at room temperature in a cupboard. After observing a colour change from deep red to dark brown in each solution, 0.1 ml of dissolved decyl chalcone was added to an equal volume of diethyl ether in a borosilicate glass tube. The solution was then visually observed for the presence of an upper brown layer.

4.3 Results

4.3.1 Assay for observed precipitate

Solutions in all tubes (tubes 1-6), except control (tube 7), changed colour from deep red to dark brown in the presence of DMSO. 2 of the 6 solutions with MHB diluent exhibited fine brown particles (Figures 4-3 & 4-4) regardless of sonication. Tube 7 exhibited no particle formation.

Figure 4-1 *A 2-fold diluted nonyl ferrocenyl chalcone solution with water as diluent post-incubation displaying no fine brown particles (tube 6) (drop size of 20 µl on white tile).*

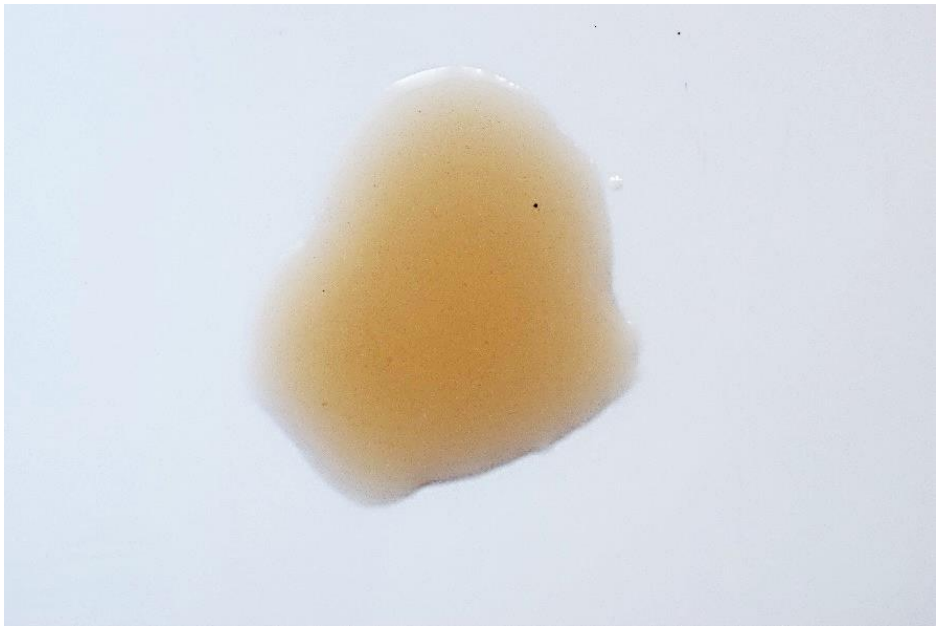


Figure 4-2 *A 2-fold diluted Nonyl ferrocenyl chalcone solution with water diluent after sonication and incubation displaying no fine brown particles (tube 5) (drop size of 20 µl on white tile).*

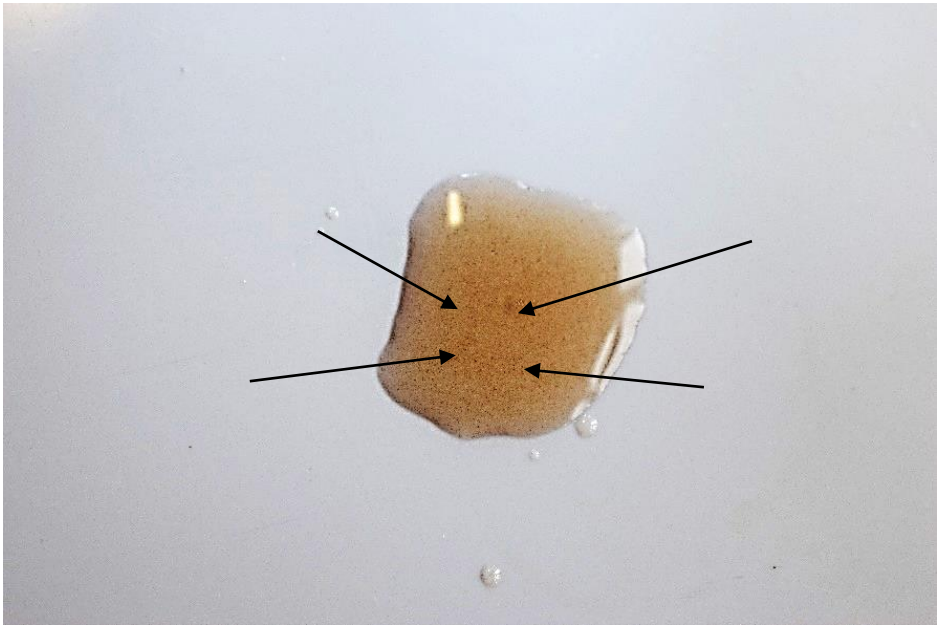


Figure 4-3 *A 2-fold diluted Nonyl ferrocenyl chalcone solution with MHB diluent post-incubation (tube 3) showing aggregates of fine brown particles (black arrows) (drop size of 20 µl on white tile).*

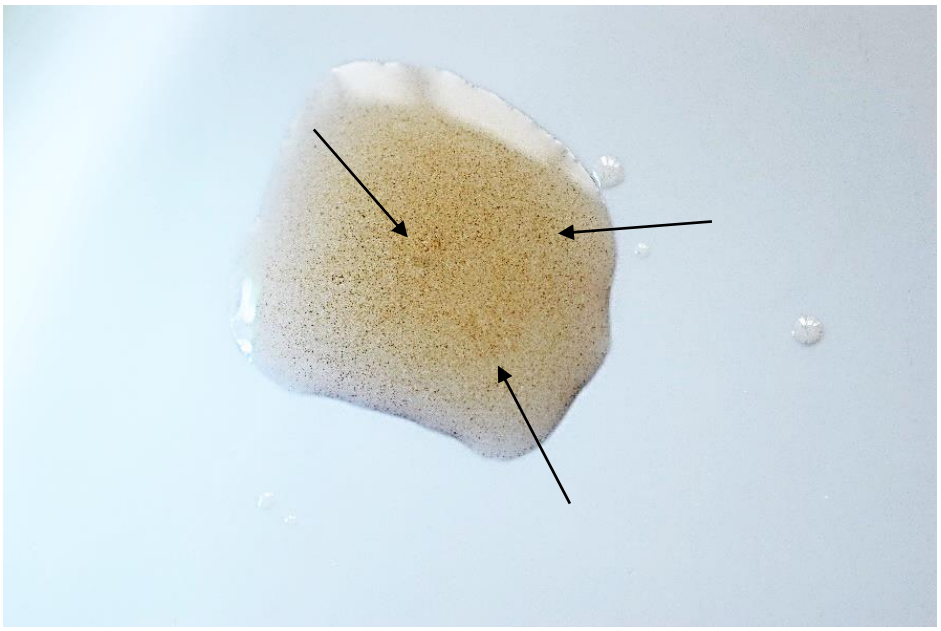


Figure 4-4 *A 2-fold diluted Nonyl ferrocenyl chalcone solution with MHB diluent after sonication and incubation (tube 6) showing aggregates of fine brown particles (black arrows) (drop size of 20 µl on white tile).*

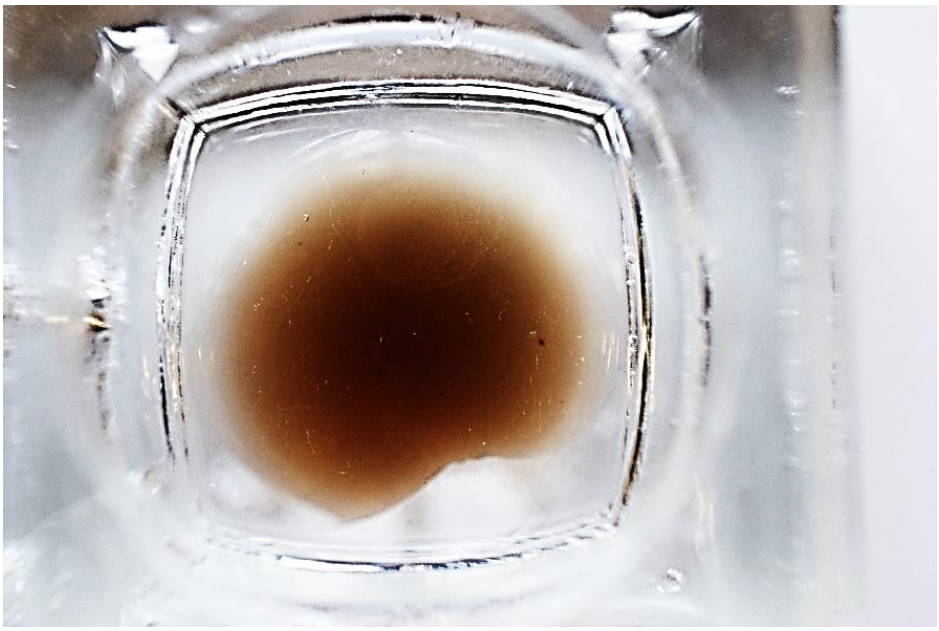


Figure 4-5 *Stock nonyl ferrocenyl chalcone solution prepared in polypropylene tube (tube 1) with no observed fine brown particles and presented here in a glass well.*

Figure 4-6 *Stock nonyl ferrocenyl chalcone solution prepared in borosilicate glass tube (tube 2).*

Tubes 1 and 2 (figures 4-5 and Figures 4-6) were shown to demonstrate the lack of formation of fine brown precipitate when MHB is not added.

4.3.2 Assay for displaced iodine

Brown residue was seen at the bottom of the borosilicate tubes instead of a brown ring at the top of the suspension.

4.4 Discussion

4.4.1 Assay for observed precipitate

After diluting the stock ferrocenyl chalcone solution with MHB, and incubating at 37^{o}C for 72 hours, brown particles, were observed. One theory for the appearance of these brown particles, visible to the naked eye, may be the chelation of Fe^{2+} ions, present in the ferrocene group, by hydrolysate in MHB (Luo *et al*. , 2014). The group have observed that hydrolysis of proteins, such as sodium caseinate, produces casein hydrolysate, a component of MHB, and enhances the metal ion chelating activity of the protein hydrolysate. Gu *et al*. (2010) reported that Maillard Reaction Products (MRP), which contain casein hydrolysate, exhibited Fe^{2+} chelation. This reaction was also reported by Luo, Pan and Zhong (2014), where the group assessed the Fe^{2+} chelation activity of casein hydrolysates. The IUPAC (1997) defines chelation as "the formation or presence of bonds (or other attractive interactions) between two or more separate binding sites within the same ligand and a single central atom". It has been reported that Fe^{2+} chelation activity of hydrolysates can result in hydrophobic protein-metal complexes (Chinedu & Min, 2017), which potentially caused the appearance of macroscopic particles. Therefore, a possible theory on the presence of observed fine brown particles in the current method may be the formation of casein hydrolysate-iron complexes caused by the chelation of iron ions in MHB-diluted ferrocenyl chalcone solution. When observed in the MIC assay (Chapter 3), these complexes were reversible since increased dilution with MHB led to reduced appearance of particles.

4.4.2 Assay for displaced Iodine

As indicated in Section 4.1.3, diethyl ether, an organic solvent that completely solubilises iodine to form a stable solution, would dissolve the precipitate if it is iodine (Hildebrand, Benesi, & Mower, 1950; Smith, Smith, & Nikonov, 2013). The reaction between the ferrocenyl chalcone and diethyl ether produced a brown precipitate at the bottom of the tube. Since a brown precipitate was seen, the change in colour of the ferrocenyl chalcone was not caused by displaced iodine but may be the presence of an equal mix of ferrocene (deep red) and ferrocenium (deep blue), which creates the brown colour. Although ferrocene is predicted to be soluble in

most organic solvents (Yousefinjad, Honarasa, & Solhjoo, 2015), Herrmann (1997) has indicated that ferrocene is sparingly soluble in diethyl ether.

4.5 Review of subsequent research into solution changes and potential antimicrobial effects

The MSci research project at Staffordshire University completed by Bennett (2016), supervised by Dr Pauline Gowland and Mrs Elecia Henry, and further explored the factors and antimicrobial effects of the visible colour change observed in stock ferrocenyl chalcone solutions. This project was influenced by observations from parallel antimicrobial sensitivity tests (as further described in Chapter 3) where the dissolved ferrocenyl chalcones changed from reddish-brown to dark brown after incubation at room temperature (approximately 21^{o}C) and 37^{o}C in 96-well polystyrene plates and disposable 13 ml Sarstedt polypropylene tubes. This occurred during preparation of stock solution and working solutions for the determination of MIC of the compounds against a selected panel of bacteria. It was also observed that when the compounds were dissolved and diluted in borosilicate glass tubes, followed by incubation at 37^{o}C, visible colour change appeared to happen at a slower rate. The visible change in colour of the compounds, which were possible indications of instability after dissolution and whether this potential issue resulted in structural changes and, further to that, affected antimicrobial activity, warranted further investigation.

Due to the absence of literature on the colour change of ferrocenyl chalcone compounds in solution, Bennett (2016) attempted to identify the possible conditions that influenced the visible colour change of the ferrocenyl chalcone compounds with longer chains (hexyl to decyl) (See Appendix 1, Table 9-1). These compounds were determined to be the more effective compounds as described in Chapter 3, in polypropylene and borosilicate glass tubes. Bennett's (2016) project involved incubation of the compounds, dissolved in appropriate volumes of DMSO, under the following conditions: room temperature (21^{o}C ± 1^{o}C) and light, room temperature (21^{o}C ± 1^{o}C) and darkness, and 37^{o}C in an incubator. Another aim of Bennett's (2016) study was to assess whether discoloured solutions post-incubation in the stated conditions affected the antibacterial activity of the compounds against Gram-positive and Gram-negative bacteria were affected post-incubation under the aforementioned conditions. Based on initial observations regarding the occurrence

of colour change in DMSO, Bennett proposed that change in absorbance, indicative of visible colour change, would occur at a faster rate in polypropylene tubes than in borosilicate glass tubes across all the environmental conditions. These observations also led Bennett to propose that the antibacterial activity of the solubilised compounds would be the same, regardless of the type of tube used.

4.5.1 Influence of conditions on colour change

In the first phase of Bennett's study, 1 mg/ml stock solutions of hexyl to decyl ferrocenyl chalcone compounds in DMSO were prepared in 5 polypropylene tubes and 5 borosilicate glass tubes for a total of 10 tubes. Absorbance values were measured at 620 nm using a WPA Biowave II spectrophotometer (Biochrom Limited, Cambourne, UK) at 30-minute intervals up to 5 hours. As reviewed by Lengeler *et al*. (1999), most cells, including bacteria, do absorb light between wavelengths of 500 nm and 660 nm, facilitating measurement of turbidity, which is linearly proportional to concentration.

After 5 hours at room temperature, Bennett observed that the ferrocenyl chalcone solutions appeared to change colour from deep red to a dark brown in both polypropylene and borosilicate glass tubes, and in all three environmental conditions (Figure 4-7). The assay was performed in triplicate.

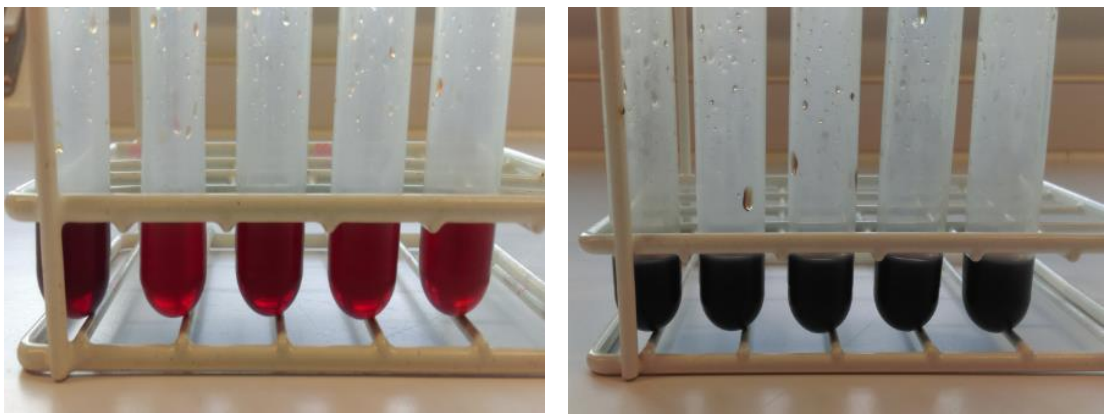


Figure 4-7 *Colour change of the five ferrocenyl chalcones used at 0 minutes (left) and after five hours (right). The same colour change was observed in glass tubes (reproduced with permission from Bennett, 2016).*

Bennett also plotted the mean (± SD) absorbance of each ferrocenyl chalcone after exposure to specified environmental conditions against sampling intervals in hours.

The derived curves were used to estimate the rate of change in absorbance per minute (RA [ΔA/min]) from 0 to 180 minutes (Table 4-1), which he proposed to be the time period of exponential change in colour. Bennett estimated these values by dividing the height of the graph to the measured absorbance value by the width from 0 to 180 minutes (Appendix 5, Figures 9-44 to 9-53) (Bennett, 2016, pers. comm.). Using a two-sample Student's t-test, Bennett determined the significant difference between the RA of ferrocenyl chalcone compounds in polypropylene tubes and borosilicate glass tubes. He also used a single-factor ANOVA test to determine the significant difference between the RA under exposure to the three environmental conditions.

Table 4-1 RA (ΔA/min) of each ferrocenyl chalcone based on type of vessel and environmental condition.

Chalcone	Vessel	Condition	RA (ΔU/min)
Hexyl	Polypropylene tubes	37°C	0.0102 ± 0.0005
		21°C (±1) + light	0.0097 ± 0.0007
		21°C (±1) + dark	0.0051 ± 0.0080
	Borosilicate glass tubes	37°C	0.0078 ± 0.0018
		21°C (±1) + light	0.0056 ± 0.0022
		21°C (±1) + dark	0.0024 ± 0.0007
Heptyl	Polypropylene tubes	37°C	0.0096 ± 0.0007
		21°C (±1) + light	0.0095 ± 0.0008
		21°C (±1) + dark	0.0045 ± 0.0010
	Borosilicate glass tubes	37°C	0.0076 ± 0.0032
		21°C (±1) + light	0.0068 ± 0.0021
		21°C (±1) + dark	0.0023 ± 0.0005
Octyl	Polypropylene tubes	37°C	0.0092 ± 0.0009
		21°C (±1) + light	0.0089 ± 0.0016
		21°C (±1) + dark	0.0044 ± 0.0008
	Borosilicate glass tubes	37°C	0.0060 ± 0.0024
		21°C (±1) + light	0.0068 ± 0.0024
		21°C (±1) + dark	0.0021 ± 0.0003
Nonyl	Polypropylene tubes	37°C	0.0096 ± 0.0007
		21°C (±1) + light	0.0095 ± 0.0006
		21°C (±1) + dark	0.0042 ± 0.0007
	Borosilicate glass tubes	37°C	0.0058 ± 0.0020
		21°C (±1) + light	0.0053 ±0.0017
		21°C (±1) + dark	0.0024 ± 0.0006
Decyl	Polypropylene tubes	37°C	0.0088 ± 0.0007
		21°C (±1) + light	0.0091 ± 0.0012
		21°C (±1) + dark	0.0041 ± 0.0009
	Borosilicate glass tubes	37°C	0.0059 ± 0.0022
		21°C (±1) + light	0.0056 ± 0.0017
		21°C (±1) + dark	0.0023 ± 0.0008

Bennett's findings indicated that at room temperature and in light, the mean (± SD) rates of change in absorbance were 0.0097 ± 0.0007 ΔA/min (hexyl), 0.0095 ± 0.0007 ΔA/min (nonyl) and 0.0091 ± 0.0012 ΔA/min (decyl) in polypropylene tubes. He also discovered that the mean (± SD) rates of change in borosilicate glass tubes were 0.0056 ± 0.0022 ΔA/min (hexyl), 0.0053 ± 0.0017 ΔA/min (nonyl) and 0.0056 ± 0.0017 ΔA/min (decyl). These estimated values were significantly different between polypropylene tubes and borosilicate tubes where p=0.0015 (hexyl), p=0.0001 (nonyl) and 0.0021 (decyl). In the same study, Bennett found that there were no significant differences in the mean (± SD) rates of change in polypropylene and borosilicate glass tubes produced by heptyl (p=0.1894) and nonyl (p=0.1049).

Another set of findings from Bennett's study indicated that, after exposure to 37^{o}C, the rates of change in absorbance of all five ferrocenyl chalcone compounds were significantly different between polypropylene tubes and borosilicate glass tubes where p=0.0112 (hexyl), p=0.0100 (heptyl), p=0.0123 (octyl), p=0.0017 (nonyl) and p=0.0129 (decyl). The rates of change in the polypropylene tubes were 0.0102 ± 0.0005 ΔA/min for hexyl, 0.0096 ± 0.007 ΔA/min for heptyl, 0.0092 ± 0.009 ΔA/min for octyl, 0.0096 ± 0.0007 ΔA/min for nonyl and 0.0088 ± 0.0007 ΔA/min for decyl. In borosilicate glass tubes, the rates of change were lower for hexyl (0.0078 ± 0.0018 ΔA/min), heptyl (0.0076 ± 0.0032 ΔA/min), octyl (0.0060 ± 0.0024 ΔA/min), nonyl (0.0058 ± 0.0020 ΔA/min) and decyl (0.0059 ± 0 0.0022 ΔA/min). This indicated that higher temperatures affected colour stability of the ferrocenyl chalcone compounds.

Bennett discovered that exposure of all 5 ferrocenyl chalcone compounds to room temperature and darkness resulted in significant differences between the rates of change in absorbance in polypropylene tubes and borosilicate glass tubes where p=0.0042 (hexyl), p=0.0006 (heptyl), p=0.0006 (octyl), p=0.0008 (nonyl), and p=0.0067 (decyl). The rates of absorbance change in the polypropylene tubes were 0.0051 ± 0.0080 ΔA/min for hexyl, 0.0045 ± 0.0010 ΔA/min for heptyl, 0.0044 ± 0.0008 ΔA/min for octyl, 0.0042 ± 0.0007 ΔA/min for nonyl and 0.0041 ± 0.0009 ΔA/min for decyl. In the borosilicate glass tubes, the rates of absorbance change were 0.0024 ± 0.0007 ΔA/min for hexyl, 0.0023 ± 0.0005 ΔA/min for heptyl, 0.0021 ± 0.003 ΔA/min for octyl, 0.0024 ± 0.0006 ΔA/min for nonyl and 0.0023 ± 0.0008 ΔA/min for

decyl. Although absorbance change occurred under dark conditions, the rates of change were lower than those that were observed under light conditions. Another finding of the Bennett study was that the effect of the selected environmental conditions was significantly different. He determined that, for the five ferrocenyl chalcone solutions in polypropylene tubes, there were significant differences in the rates of change in absorbance between the environmental conditions, where p=0.00002 (hexyl), p=0.00005 (heptyl), p=0.000006 (octyl), p=0.0000007 (nonyl) and p=0.000003 (decyl). There were significant differences in the rates of change in absorbance also for the same solutions in borosilicate glass tubes, between the environmental conditions, where p=0.0005 (hexyl), p=0.0032 (heptyl), p=0.0039 (octyl), p=0.0076 (nonyl) and p=0.0069 (decyl).

Based on the results presented, Bennett deduced that visible colour change of the ferrocenyl chalcone solutions was influenced by the composition of the sample vessels, where samples were prepared in polypropylene and borosilicate glass tubes, and that light and heat accelerated this change. The change in the observed colour of the ferrocenyl chalcone solutions may have resulted from the oxidation of Fe^{2+}, found in the ferrocene group, to Fe^{3+}, found in the ferrocenium group (Charette & Sholkovitz, 2002). This may indicate a reaction between the solution and potential pro-oxidant components in polypropylene tubes (Corti *et al.*, 2010). However, iron oxidation in aqueous ferrocene, which is reddish brown in colour, produces aqueous ferrocenium, which is blue in colour. Thus, this oxidation may be reversible and may result in the presence of both ferrocene and ferrocenium (Rao, Kumar, & Ravikanth, 2010), producing a dark brown colour. This is the first known report that specifically addresses the change in colour of ferrocenyl chalcone compounds in relation to the sample vessel and environmental conditions to which the compounds are exposed. Therefore, there is a need for further investigation of this theory, including the identification of the presence of ferrocenium.

4.5.2 Antibacterial activity of the ferrocenyl chalcone compounds after exposure to environmental conditions

The second phase of Bennett's study was the evaluation of the antimicrobial activity of randomly selected compounds (hexyl and heptyl) after visible colour change. The MIC values (mg/ml) of these compounds, indicative of antibacterial activity, were determined using the broth macrodilution method (EUCAST, 2003). The method involved using 2-fold serial broth macrodilution in both polypropylene and borosilicate glass tubes, where samples were done in duplicate. Bennett prepared appropriate volumes of each stock ferrocenyl chalcone solution at concentrations of 2 mg per 1 ml of DMSO, and used MHB, derived from 10 ml aliquots of stock MHB that was autoclaved at 121^{o}C for 15 minutes, as the diluent to produce working concentrations of 1 mg/ml. The positive control was either PEN-G or OXY, an analogue of tetracycline. Each stock antimicrobial solution was prepared to 0.256 mg per 1 ml of sterile deionised water (Andrews, 2001). The negative control was 5% $^{v}/_{v}$ DMSO, which was diluted with MHB.

Bennett used two Gram-positive bacteria, *S. aureus* NCIMB 8244 and *E. faecalis* NCTC 12697, and two Gram-negative bacteria, *Salmonella "Manchester"* NCTC 7832 and *E. coli* NCIMB 9483 in this assay. He prepared each inoculum using the growth method (Andrews, 2001), which involved the suspension of more than 3-4 colonies of each bacterial sample in sterile MHB followed by incubation for 15-20 minutes in air at 37^{o}C until the turbidity was either equal to or greater than that of a 0.5 McFarland standard. This resulted in suspensions that contained approximately 10^{7} and 10^{8} cfu/ml. Bennet then diluted the suspensions to achieve the final inoculum of 10^{5} cfu/ml. After adding equal volumes of diluted ferrocenyl chalcone solution and inocula, Bennett's final chalcone concentration ranged from 0.500 mg/ml to 0.001 mg/ml, and a final antimicrobial concentration of 0.128 mg/ml. After incubating in air for 18-24 hours at 37^{o}C, Bennett measured absorbance values at 620 nm, followed by numeration of viable bacterial colonies using the Miles and Misra method, as described in several studies (Miles, Misra, & Irwin, 1938; Slack & Wheldon, 1978; Codd, Richardson, & Andrews, 1998; Hedges, 2002; Mishra, Taneja, & Sharma, 2012; Nunes, Graça, & Santo, 2017).

Findings of Bennett's assay indicated that heptyl ferrocenyl chalcone compound possessed more efficacy in overall antibacterial activity than hexyl ferrocenyl chalcone compound. In the study, Bennett found that heptyl produced the largest overall MIC value of 0.500 mg/ml against *E. coli* NCIMB 9483 (Table 4-2). The MIC values estimated against Gram-negative bacteria in Bennett's study (0.250 mg/ml to 0.500 mg/ml) were not considered to reflect antibacterial activity, since the percentage of DMSO that was present in the solution were greater than 12.5% $^{v}/_{v}$ DMSO. DMSO has been reported to exhibit antibacterial activity at concentrations ≥12.5% $^{v}/_{v}$ (Basch, Gadebusch, & Brunswick, 1968; Duric *et al*., 2013). Bennet also discovered that hexyl produced the same MIC values in both polypropylene tubes and borosilicate glass tube with 0.063 mg/ml against *S*. *aureus* NCIMB 8244 and 0.125 against *E. faecalis* NCTC 12697 (Table 4-2). The heptyl ferrocenyl chalcone compound produced different MIC values in polypropylene tubes when compared to assays in borosilicate glass tubes. The MIC values were estimated to be 0.016 mg/ml against *S*. *aureus* NCIMB 8244 and 0.031 mg/ml against *E. faecalis* NCTC 12697 in polypropylene tubes, and 0.031 mg/ml against *S*. *aureus* NCIMB 8244 and 0.063 mg/ml against *E. faecalis* NCTC 12697 in borosilicate glass tubes (Table 4-2). At these MIC values, Bennett proposed that antibacterial activity was caused by the presence of ferrocenyl chalcone, since DMSO displays antibacterial activity a ≥12.5% (Basch, Gadebusch, & Brunswick, 1968; Duric *et al*., 2013). The antibacterial activity of DMSO was explored further in Chapter 3. When Bennet exposed the same four organisms to DMSO, which was serially diluted 2-fold with MHB, in both polypropylene and borosilicate glass tubes, this resulted in MIC values from 12.5% $^{v}/_{v}$ DMSO to 25% $^{v}/_{v}$ DMSO (Table 4-2). Overall, Bennett's results indicated that antibacterial activity of the ferrocenyl chalcones remained after exposure to environmental conditions.

Table 4-2 *MIC values of hexyl and heptyl ferrocenyl chalcone compounds and DMSO control against non-resistant laboratory-adapted bacteria (reproduced with permission from Bennett, 2016).*

Organism	MIC values in polypropylene tubes (mg/ml)		MIC values in borosilicate glass tubes (mg/ml)		MIC values of DMSO (% $^{v}/_{v}$)
	Hexyl	Heptyl	Hexyl	Heptyl	DMSO
S. "Manchester" **NCTC 7372**	0.250	0.250	0.250	0.250	12.50
E. coli **NCIMB 9483**	0.250	0.500	0.250	0.500	25.00
S. aureus **NCIMB 8244**	0.063	0.016	0.063	0.031	12.50
E. faecalis **NCTC 12697**	0.125	0.031	0.125	0.063	12.50

4.6 Conclusion

The bioavailability of an antimicrobial drug in the human body relies on the solubility of the compound in hydrophilic body fluids (Varanda *et al*., 2006; Gao *et al*., 2011). The most effective antimicrobial agents are orally administered antimicrobial compounds, which are absorbed in the intestine (Cyriac & James, 2014). Orally administered antimicrobial drugs must be hydrophobic so that they may enter the mucosa by diffusion (Levison & Levison, 2009). Additionally, hydrophobic antimicrobial drugs are preferred since they diffuse across highly protected tissues such as the blood brain barrier and the prostate (Lipsky, Byren, & Hoey, 2010; Nau, Sorgel, & Eiffert, 2010). Due to their hydrophobic nature, such drugs must be dissolved in amphipathic compounds, which allow the drug to diffuse across the hydrophobic barriers but still be capable of targeting hydrophilic targets.

The antimicrobial compounds used in this study contained hydrophobic moieties. DMSO, an amphipathic chemical, was determined to be the best solvent for the ferrocenyl chalcone compounds. After solvation in DMSO, the ferrocenyl chalcone solutions were diluted with MHB, the standard medium used in AST (EUCAST, 2003; CLSI, 2012). However, initial solvation and dilution resulted in a colour change from deep red to dark brown, and the production of brown particles that were visible to the naked eye.

Fe^{2+} chelation by casein hydrolysate, a chelating component of MHB (Oxoid, 2017), was suggested to be the potential cause of particulate formation in the ferrocenyl chalcones in the current study. The cause of the colour change from deep red to dark brown was hypothesised to be the presence of displaced iodine. However, a negative diethyl ether test (Hildebrand, Benesi, & Mower, 1950; Smith, Smith, & Nikonov, 2013), meant that iodine was not displaced after solubilisation. The presence of pro-oxidant compounds in polyethylene vessels (Corti *et al*., 2010) was proposed to be the possible cause of the oxidation of Fe^{2+} in ferrocene to Fe^{3+} in ferrocenium (Rao, Kumar, & Ravikanth, 2010). This reversible colour change may have resulted in an equivalent mix of ferrocene (deep red) and ferrocenium (deep blue), which formed the dark brown colour.

A review of the Bennett (2016) study on the effect of environmental conditions of ferrocenyl chalcone compounds and their consequent antimicrobial activity revealed key findings. These findings were that solubilised ferrocenyl chalcone compounds should be stored at room temperature in the dark. Another finding was that the antimicrobial activity of the compounds remained, despite exposure to specific environmental conditions, so therefore, this is not an issue affecting the ability of the chalcones to exhibit antibacterial activity. In Chapter 3, the antimicrobial activity of freshly prepared ferrocenyl chalcone compounds against non-resistant laboratory-adapted bacteria, as well as resistant and non-resistant clinical isolates, were assessed using a number of reference assays. The work presented in both Chapter 3 and Chapter 4 were performed simultaneously since the colour change and presence of brown particulates were observed during the determination of MIC. The next chapter will be focussed on the potential mode of action of these ferrocenyl chalcone compounds in using the MTT assay.

5 Determination of cell viability using MTT assay in the identification of a potential mode of action of ferrocenyl chalcone compounds

5.1 Introduction

Cell viability refers to the capacity of living cells to perform the same range of functions that are exhibited pre-exposure and post-exposure to a chemical, drug or condition (Pegg, 1989). Pegg (1989) proposed that these functions include the amount of enzyme or product that is released from metabolic reactions, and the amount of oxygen consumption that can be measured. In his attempt to define viability, Pegg (1989) stressed that the term "viability", which has a more limited meaning, differs from "life". Further to this, Pegg has stated that loss of the functions that constitute viability result in expiration. Using Pegg's definition of viability, the manifestations of these functions, such as the concentration of product, can be precisely measured. Techniques used to measure these functions belong to several classifications including physical integrity and metabolic activity. The current report will attempt to rationalise the use of the MTT cell viability assay as a means of identifying the potential mode of action of ferrocenyl chalcone compounds. This justification of the use of this approach became necessary since the functional compounds exhibited antibacterial activity against bacteria that possessed resistance mechanisms such as the production of β-lactamase against PEN, use of enzymes that alter ERY and streptomycin structure or the use of efflux pumps, and alteration of PBPs that facilitate methicillin binding.

5.1.1 Cell viability assays

Traditional cell viability assays involved the use of staining techniques such as those that include the use of proteins labelled with ^{51}Cr or radioactive nucleotides such as ^{3}H Thymidine and ^{125}I Iododeoxyuridine (Mosmann, 1983). The use of tetrazolium salts in a rapid cell viability assay was developed by Mosmann (1983) where washing steps, used in the conventional staining methods, were no longer required. Evidence for the use of these salts in cell viability assays was reported by Slater *et al*. (1963). These compounds, which inhibit different enzymes within the respiratory chain, are

2-p-nitrophenyl-3-p-iodophenyl-5-phenyltetrazolium chloride , triphenyltetrazolium chloride, neotetrazolium chloride, 2,2'-di-pnitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride and MTT (Slater *et al*., 1963). The most common tetrazolium salt used in cell viability assays is MTT, which is used to measure metabolic functions.

5.1.2 MTT assay

MTT is a yellow tetrazolium salt that is reduced by respiratory dehydrogenases in cells to a purple formazan product (Slater *et al*., 1963). This colorimetric assay is performed in microtitre plates and can be quantified by measuring absorbance at 560 nm to 570 nm in a microplate spectrophotometer (Denizot & Lang, 1986). This rapid and cost-effective test produces a proportional relationship between the amount of formazan product and the amount of actively respiring cells, which, in turn, is indicative of cell growth. One limitation of the Mosmann MTT assay (1983), as discovered by Denizot & Lang (1986), was that formazan was an insoluble compound. Due to its insolubility in cells, formazan accumulates as a crystals within the cytoplasm and on the exterior of the cell, which results in rupture of the membrane during exocytosis of the formazan crystals as reported by Lü *et al*. (2012). Adapted MTT methods used organic solvents such as sodium dodecylsulphate-dimethylformamide (Garn *et al*., 1994) and DMSO (Carmichael *et al*., 1987) to solubilise formazan. The Mosmann MTT assay and its derived methods were first used to demonstrate viability with respect to mitochondrial respiration in mammalian cells but has been adapted for use in other cell types such as bacteria, which share respiratory genes with eukaryotes.

5.1.3 The endosymbiotic link between bacteria and eukaryotic cells

Gray *et al*. (1999) proposed that mitochondria in eukaryotic cells contain genetic material that were derived from a proteobacterium ancestor following invasion of the cell by the symbiont (Martin *et al*., 2001). The contentious theory of endosymbiosis, which refers to the formation of new cells after the incorporation of organelles from two unrelated cells, arguably originated from Mereschkowsky (1905), as detailed by Martin & Kowallik (1999). After many years of debates from Wallin (1927) to Margulis (1970), a common thread of all proposed hypotheses is that

host and endosymbiont share similar genes (Dyall, Brown, & Johnson, 2004). Loss of some protein-coding genes that were retained through endosymbiosis resulted in the active cellular organelles chloroplasts in plants (Gabaldón & Huynen, 2003) and mitochondria Gray *et al*. (1999) in eukaryote. Historically, use of the MTT assay relied on the presence of conserved respiratory genes in eukaryotes, which are responsible for essential respiratory enzymes that have been extensively studied.

5.1.4 Respiratory enzymes in eukaryotes

Margulis (1970) successfully argued that endosymbionts developed into mitochondria and chloroplasts, which are functional cellular entities, by gaining metabolites and protection, and producing the energy-rich compound adenosine triphosphate (ATP) derived from the respiratory process(Margulis , 1970; Andersson & Kurland, 1999). A mitochondrion is a complicated structure comprised of an outer membrane and an inner membrane with convolutions known as cristae, as described by Fritiof & Sjorstrand (1953). Although various researchers identified the physical characteristics of the crista membrane, Vogel *et al*. (2006) detailed the system of protein complexes, including succinate dehydrogenase (SDH), located within the structure, and the primary function of this system being respiration and ATP synthesis (Figure 5-1). SDH, which was first observed by Thunberg (1909), is a crucial component of the Krebs Cycle (Krebs *et al*., 1938) where the enzyme catalyses the oxidation of succinate to fumarate, and has been reviewed by several researchers including Hederstedt & Rutberg (1981).

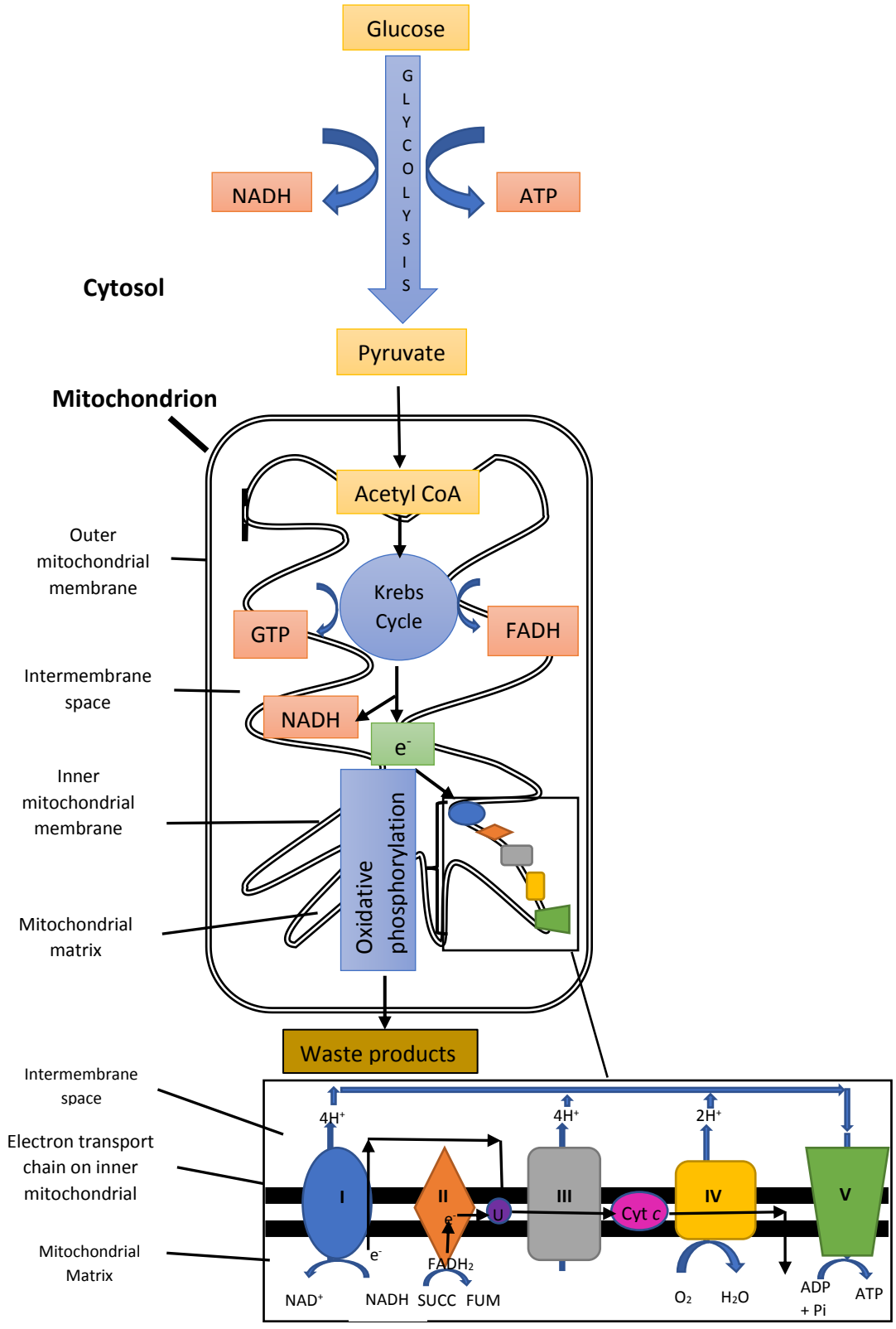


Figure 5-1 A summary of metabolism in eukaryotes indicating the major complexes within the electron transport system. Complex I = NADH dehydrogenase; Complex II = SDH; Complex III = cytochrome b-c_1; Complex IV = cytochrome c oxidase; Complex V = ATP synthase; SUCC = succinate; FUM = fumarate; U = ubiquinone; Cyt c = cytochrome c (adapted from Alberts et al., 2002a; Sazanov, 2015).

5.1.5 Respiration in aerobic bacteria

Like mitochondria, the energy-producing system present in aerobic bacteria (Figure 5-2) is also located on its inner membrane and includes SDH as detailed by (Hederstedt & Rutberg, 1981). In a review by Haddock & Jones (1977), membrane-bound proteins involved in bacterial respiration are orientated to derive full advantage of chemiosmotic reactions resulting in electron transfer and ATP production. Although the constitution of the bacterial respiratory complexes differs according to environmental conditions, such as accessibility of oxygen and enzymes, the configuration is identical to that of mitochondria as discussed by Melo & Teixeira (2015). Oxidase positive bacteria, such as *Pseudomonas spp*., utilise cytochrome c oxidase and employ oxygen as the terminal electron acceptor. In contrast, oxidase negative bacteria, such as *E. coli*, utilise other oxidases and may perform aerobic, anaerobic or facultative respiration. While Gram-positive bacteria, such as *S. aureus* are usually oxidase negative, they may be facultative anaerobes. Bacterial respiration is not only similar to mitochondrial respiration, but the process is also adaptable since a variety of other electron receptors, such as sulphur- and nitrogen-based compounds, are used, as described by Richardson (2000).

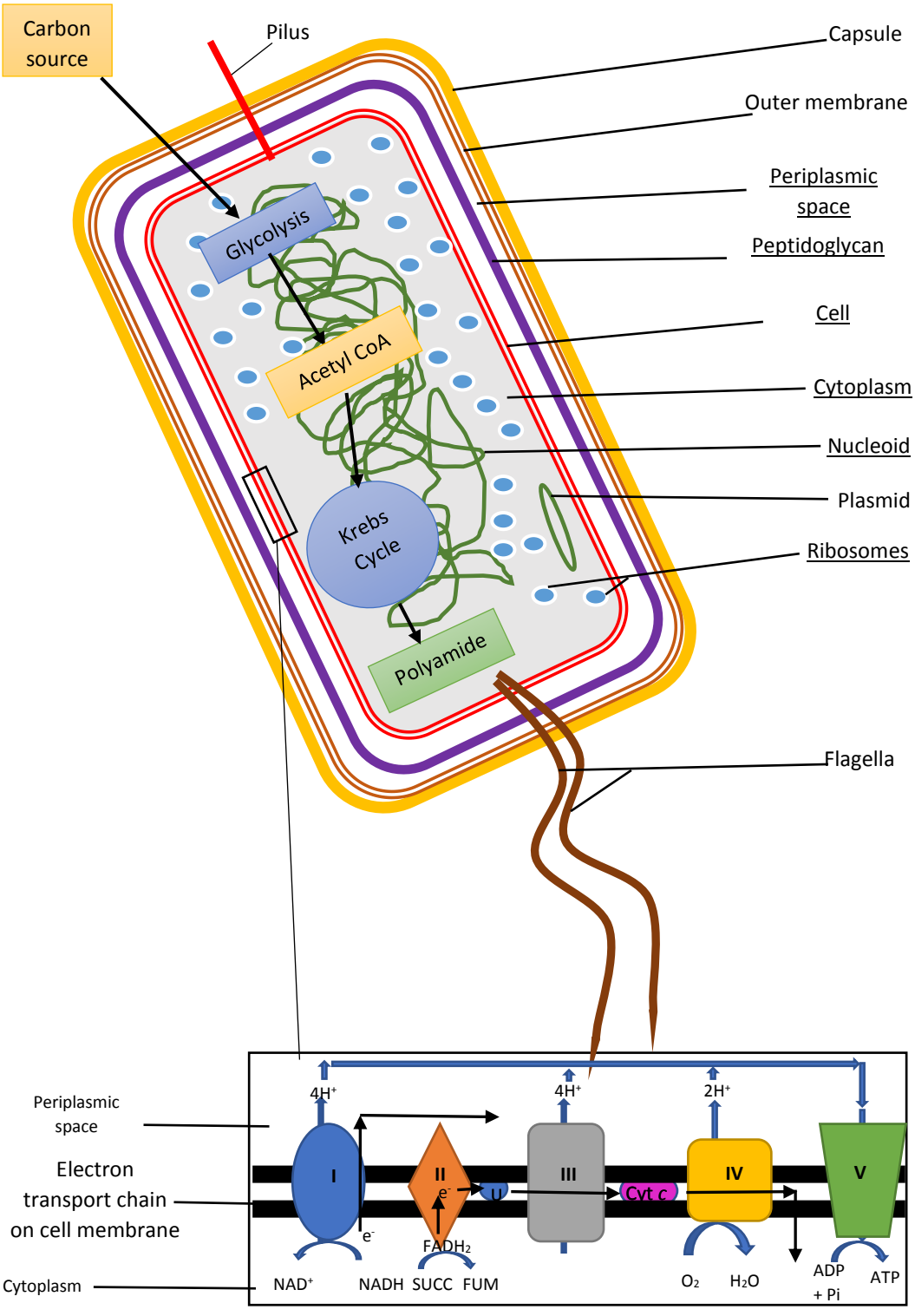


*Figure 5-2 Summary of a classic metabolic pathway in aerobic bacteria indicating the electron transport system. Structures **with underlined labels** are common in all bacterial cells. Complex I = NADH dehydrogenase; Complex II = SDH; Complex III = cytochrome b-c_1; Complex IV = cytochrome c oxidase; Complex V = ATP synthase; U = ubiquinone; Cyt c = cytochrome c; SUCC = succinate; FUM = fumarate (Alberts et al., 2002c; Portnoy et al., 2010)*

5.1.6 Examples of the use of the MTT assay in the determination of bacterial cell viability and alternative methods used to determine bacterial cell viability

Mshana *et al*. (1998) were among the first research groups that attempted to use the MTT assay to assess the viability of bacterial cells. Findings of this study indicated that rifampin-sensitive *Mycobacterium bovis* BCG 390955B, *Mycobacterium tuberculosis* H37Ra strain 35836 and clinical isolates of *Mycobacterium tuberculosis* were unable to reduce MTT to formazan after incubation with rifampin. Concurrently, rifampin-resistant *Mycobacterium tuberculosis* H37Rv strain 35838 did reduce MTT to formazan after exposure to rifampin (Mshana *et al*., 1998). The group concluded that the rapid and inexpensive method could be used to determine the presence of rifampin-resistant *Mycobacterium tuberculosis*. Consideration could also be given to assess the presence of isoniazid-resistant *Mycobacterium tuberculosis* strains using a combined MTT assay for rifampicin and isoniazid since this antibacterial drug combination is the primary approach to treat infections against *Mycobacterium tuberculosis*.

In another study completed by Alenezi *et al*. (2017) explored the use of the MTT assay in measuring cell viability of *E. coli* GC4468 after incubation with Zinc porphyrin-based photosensitizers. Results of the study indicated that the bacterial cells were unable to completely reduce MTT after photoillumination, which was possibly due to bacteriostatic, and not bactericidal, activity (Alenezi *et al*., 2017). The group reported that structural reconfiguration of the compounds could have a greater impact, than possession of high cationic charge and lipophilicity, on cell viability resulting in cell death.

Fluorescent dyes have also been proposed as alternative indicators of the viability of bacterial cells that adhere to and are phagocytised by mammalian cells. SYTO9 and 4',6'-diamidino-2-phenylindole, which is also known as DAPI, are membrane permeable dyes that have been used in determining the viability of total bacterial population of *N. gonorrhoea* used to infect human neutrophils (Johnson & Criss, 2013). In the same study, SYTOX Green and propidium iodide have been used to assess phagocytised *N. gonorrhoea* (Johnson & Criss, 2013).

5.1.7 Aim of assay

The aim of this phase of the research work was to measure the viability of bacterial cells, which exhibited various known mechanisms of action excluding inhibition of bacterial cell respiration, using MTT post-incubation with hexyl to decyl ferrocenyl chalcone compounds, which exhibited greater antimicrobial activity as reported in Table 3-9 of Chapter 3.

5.2 Materials and Methods

5.2.1 Preparation of stock antimicrobial control

Each stock antimicrobial solution was prepared as described in Section 2.1.4. The antimicrobial compounds used were PEN-G and OXY. Stock solution when prepared were stored at -20^{o}C.

5.2.2 Preparation of stock ferrocenyl chalcone

Fresh stock solution of each ferrocenyl chalcone (hexyl to decyl) was prepared as described in Section 2.1.3. The solutions were used immediately after preparation.

5.2.3 Preparation of MTT solution

Appropriate volumes of stock MTT solution were prepared at a ratio of 5 mg/ml of MTT reagent powder in of PBS as detailed in Section 2.1.5. The stock solution was stored at -20^{o}C until required.

5.2.4 Preparation of inocula

Each inoculum of *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884, *E. faecalis* NCTC 12697, fully sensitive *S. aureus* (CRH), PEN-resistant *S. aureus* (CRH), PEN/ERY/CLI-resistant *S. aureus* (CRH) and a MRSA (CRH) was freshly prepared each time as detailed in Section 2.2.1.

5.2.5 Preparation of bacterial standard curves for MTT assay

Standard curves of *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884, *E. faecalis* NCTC 12697, fully sensitive *S. aureus* (CRH), PEN-resistant *S. aureus* (CRH), PEN/ERY/CLI-resistant *S. aureus* (CRH) and a MRSA (CRH) were prepared as described in Section 2.4. The dilutions were converted to percentage of actively respiring cells in terms of formazan product and plotted against the absorbance values in Excel producing standard curves per organism (Appendix 6, Figure 9-54 to 9-60).

5.2.6 Adapted Bacterial MTT assay (Moodley et *al*., 2014)

The 96-well microtitre plates were prepared as described in Section 2.6 for *S. aureus* 8244, *K. kristinae* NCIMB 8884, *E. faecalis* NCTC 12697, fully sensitive *S. aureus* (CRH), PEN-resistant *S. aureus* (CRH), PEN/ERY/CLI-resistant *S. aureus* (CRH) and a MRSA (CRH) where the organisms were treated with ferrocenyl chalcone solution. After incubation of the microplate at 37^{o}C in air for 18-24hrs, 10 µl of MTT solution (5 mg/ml) was added to all wells. The microplates were then incubated at 37^{o}C in air for 3 hours. 50 µl of DMSO was added to all wells and mixed by pipetting. The absorbance values were measured at 570 nm on a microplate reader, where n= 10. The blank wells, where no bacteria were added, were subtracted from the sample wells to give the sample absorbance values. The percentage of actively respiring cells (in terms of formazan product gained) was calculated using the equation derived from the MTT standard curve of each organism. The estimated percentage of each assay was plotted in a Box and Whiskers plot showing the mean, median, upper and lower values.

5.2.7 Statistical analysis

The Kolmogorov-Smirnoff test was used to determine data normality of the MTT assay data. Statistical analysis of the MTT assay data in the study was performed using a one-way analysis of variance (ANOVA) (Appendix 6, Table 9-4 to Table 9-10) to determine whether the mean percentage of actively respiring cells differed between the hexyl to decyl ferrocenyl chalcone treatments.

5.3 Results

The results of the MTT assay of non-resistant Gram-positive laboratory organisms demonstrated that the percentage of actively respiring cells, in terms of formazan product observed (Figure 5-3) decreased after exposure to chalcones at the MIC value. No viable cells (mean estimated percentage of 0%) were seen for *S. aureus* NCIMB 8244 when exposed to hexyl, octyl, nonyl and decyl. For *K. kristinae* NCIMB 8884, no viable cells (mean estimated percentage of 0%) were seen after exposure to heptyl, octyl and nonyl. When exposed to hexyl and heptyl, mean percentage of actively respiring *E. faecalis* NCTC 12697 cells were estimated to be 0%. The highest percentage was measured for *S. aureus* NCIMB 8244 after incubation with heptyl (6.681%). Exposure of all cells to PEN produced mean percentages estimated to be 0%, while the absence of ferrocenyl chalcone or known antimicrobial compound produced mean percentages of actively respiring cells from 14.938% to 74.156%.

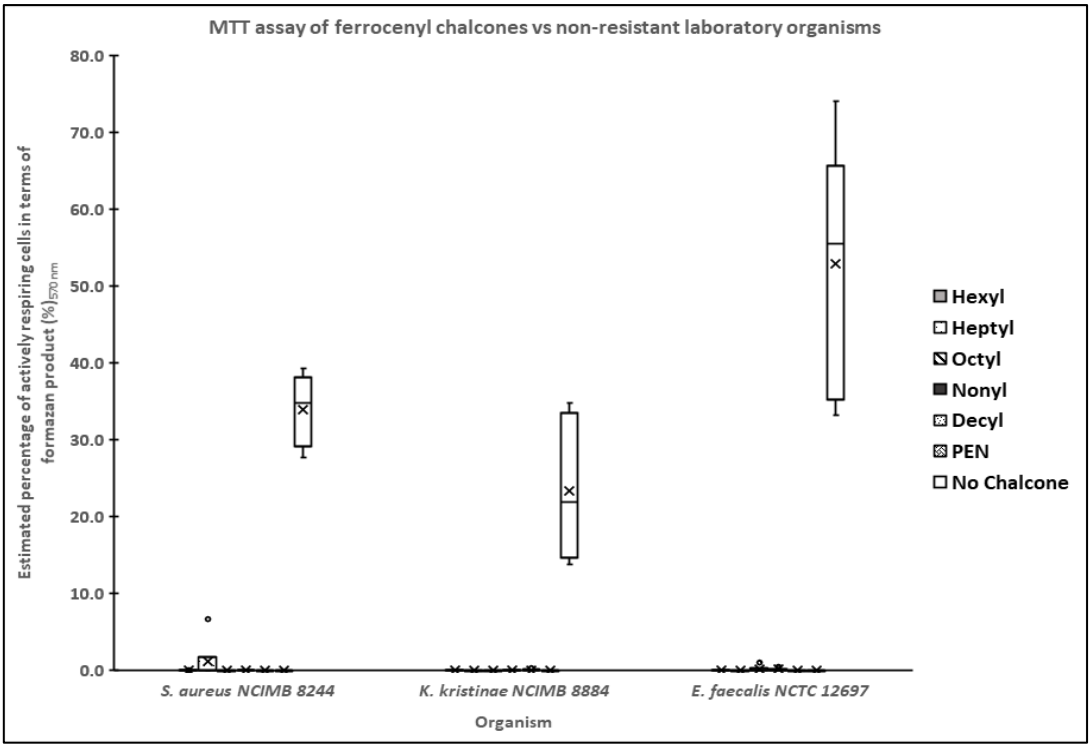


Figure 5-3 Estimated percentage (n=10) of actively respiring non-resistant lab bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlying values.

In the MTT assay of resistant and non-resistant Gram-positive clinical isolates the percentage of actively respiring cells, in terms of formazan product observed (Figure 5-4) also decreased after exposure to chalcones at the MIC value. No viable cells (mean estimated percentage of 0%) were seen for fully sensitive *S. aureus* (CRH) when exposed to heptyl octyl, nonyl and decyl, for *PEN-resistant S. aureus* (CRH) when exposed to hexyl, heptyl, octyl, and decyl, and for *PEN/ERY/CLI-resistant S. aureus* (CRH) when exposed to hexyl and decyl, and for *MRSA* when exposed to decyl. The highest percentage was determined for *PEN-resistant S. aureus* (CRH) after incubation with nonyl (1.342%). Exposure of all cells to PEN produced mean percentages estimated to be 0%, while the absence of ferrocenyl chalcone or known antimicrobial produced mean percentages of actively respiring cells from 4.200% to 14.304%.

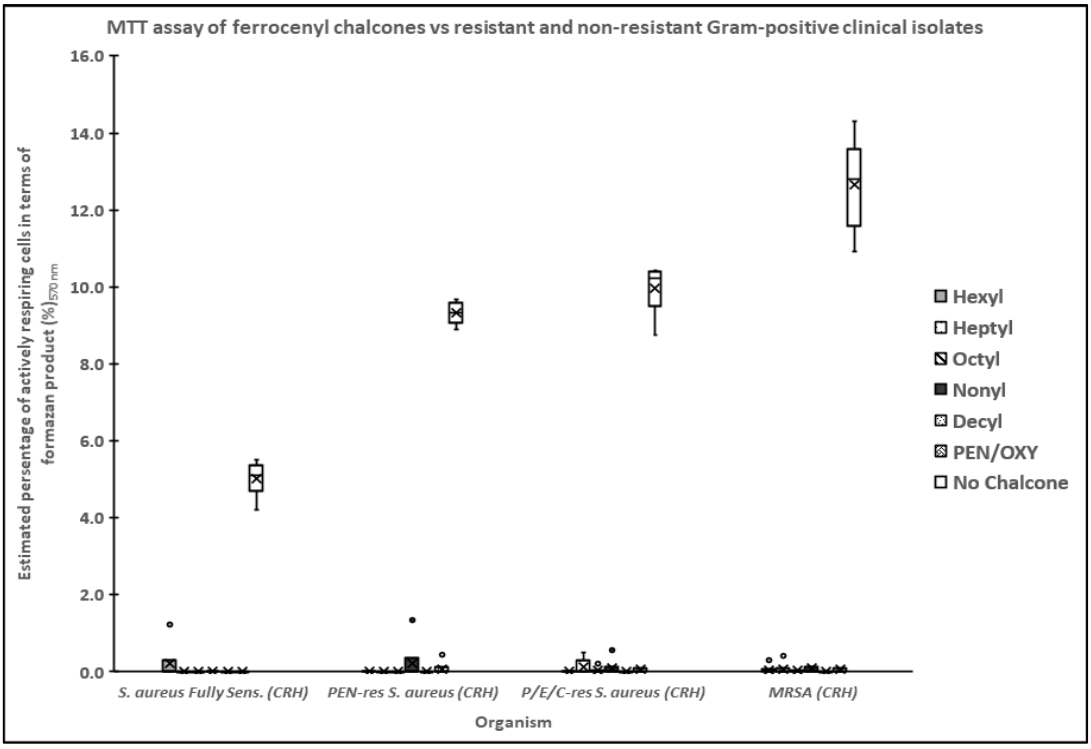


Figure 5-4 Estimated percentage (n=10) of actively respiring resistant and non-resistant clinically isolated bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlying values.

5.4 Discussion

The MIC values of the ferrocenyl chalcones against the organisms used in this study corresponded with the percentage of actively respiring cells in terms of the formazan product seen. This suggests that the metabolic process used to convert MTT was not active at the concentrations of chalcones present in the cells. Therefore, little or no formazan product was detected at 570 nm. In the MTT assay involving Gram-negative bacteria, growth inhibition, which was seen at 0.125 mg/ml, may also have resulted from exposure of the organisms to DMSO. This implies that the percentage of viable cells that were involved in MTT metabolism to formazan were very low at the assessed MIC values in combination with DMSO, making it difficult to define the exact cause of reduced viability off Gram-negative cells in the current study.

When compared to MTT screening of ferrocenyl chalcone antimicrobial activity against *Mycobacterium tuberculosis* H37Rv, the MIC values in this study (Chapter 3, Table 3-9) sat within the reported range (0.016-0.128 mg/ml) (Moodley *et al*., 2014), except for *K. kristinae* NCIMB 8884 where a lower MIC (heptyl chalcone) was achieved. In the microplate assays, the overall trend showed that the ferrocenyl chalcone compounds had a greater inhibitory effect on Gram-positive bacteria than on Gram-negative bacteria. Another observation of the ferrocenyl chalcone activity in this study was that there were minute variations of complete inhibition of cellular respiration across Gram-positive species. This could be due to the diversity of cross-linkage within peptidoglycan and polymer constituents, such as polysaccharides and polypeptides, within the cell wall of different Gram-positive bacterial species (Shockman & Barrett, 1983).

One-way ANOVA tests (Appendix 6, Tables 9-4 to 9-10) were conducted to determine whether there were significant differences ($p<0.05$) between the longer chain chalcones against each Gram-positive organism with respect to their effect on bacterial respiration. Results of these analyses indicated that the differences between hexyl to decyl chalcones in terms of mean (± SD) percentage (n=10) of actively respiring cells present were not significant for *S. aureus* NCIMB 8244 ($p=0.426$), *K. kristinae* NCIMB 8884 ($p=0.442$) and *E. faecalis* NCTC 12697 ($p=0.522$). Thus, the compounds were equally effective at inhibiting respiration in all selected

non-resistant Gram-positive bacteria. Similarly, the hexyl to decyl ferrocenyl chalcones were equally effective at respiration inhibition in fully sensitive *S. aureus* (CRH) (p=0.426), PEN-resistant *S. aureus* (CRH) (p=0.408), PEN/ERY/CLI-resistant *S. aureus* (CRH) (p=0.434) and a MRSA (CRH) (p=0.852).

5.4.1 Possible mode of action

Ferrocene groups have been theorised to be inhibitors of cellular respiration, in which the ferrocene groups act as respiration uncouplers as seen when *Caenorhabditis elegans* are exposed to organic chalcones (Gonzalez & Estevez-Braun, 1997) and ferrocenyl chalcones (Attar *et al*., 2011). The ferrous (Fe^{2+}) centre of ferrocene groups has also been hypothesised to exhibit electrochemical properties. Findings of a study by Jaouen *et al*. (2015) involving ferrocifen demonstrated that Fe^{2+} may be oxidised to Fe^{3+} by oxygen present in cells. This property facilitates anti-cancer activity where ferrocifen binds to oestrogen receptors, essential for cancer growth, causing dimer formation of the proteins (Top *et al*., 1996; 2001; 2003). In the current study, the ferrocenyl chalcone compounds possibly enter the peptidoglycan layer of Gram-positive organisms and the increased chain length may allow the ferrocene groups of the compounds to be trapped in the cell membrane adjacent to SDH in the electron transport chain (Figure 5-6). This is followed by inhibition of the SDH by the ferrocene moiety where succinate reduction to fumarate is blocked and the electron transport machinery is stalled.

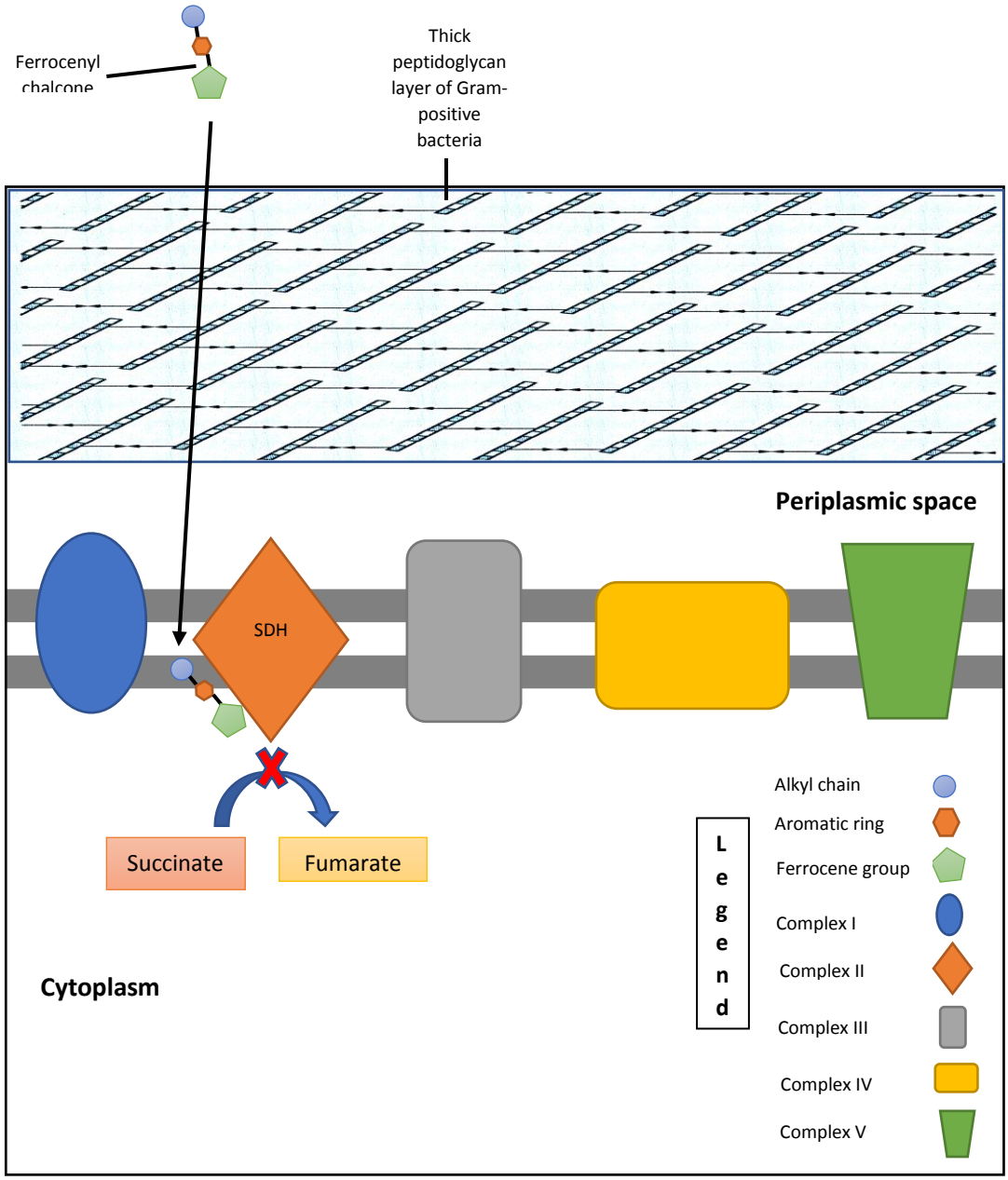


Figure 5-5 Summary of proposed mode of action of longer alkyl chain ferocenyl chalcone compounds showing blockage of succinate reduction in the Krebs Cycle within Gram-positive bacteria (produced by E. Henry, 2017).

Since Gram-negative organisms possess outer envelopes, thin peptidoglycan layers with increased periplasmic space and cell membranes in their cell envelopes, entry into these cells may be more difficult (Figure 5-6). Another possibility is that the cell membrane of Gram-positive bacteria is compromised such that the electron transport chain cannot function (Haddock & Jones, 1977). Cell viability, as indicated by MTT metabolism to formazan, decreased in Gram-positive bacteria when compared to Gram-negative bacteria. Therefore, a possible mechanism of action of the chalcones with longer alkyl chain lengths may be inhibition of cellular respiration.

The physical and external effect of this theorised action of the ferrocenyl chalcone chemicals was further investigated using bacterial SEM.

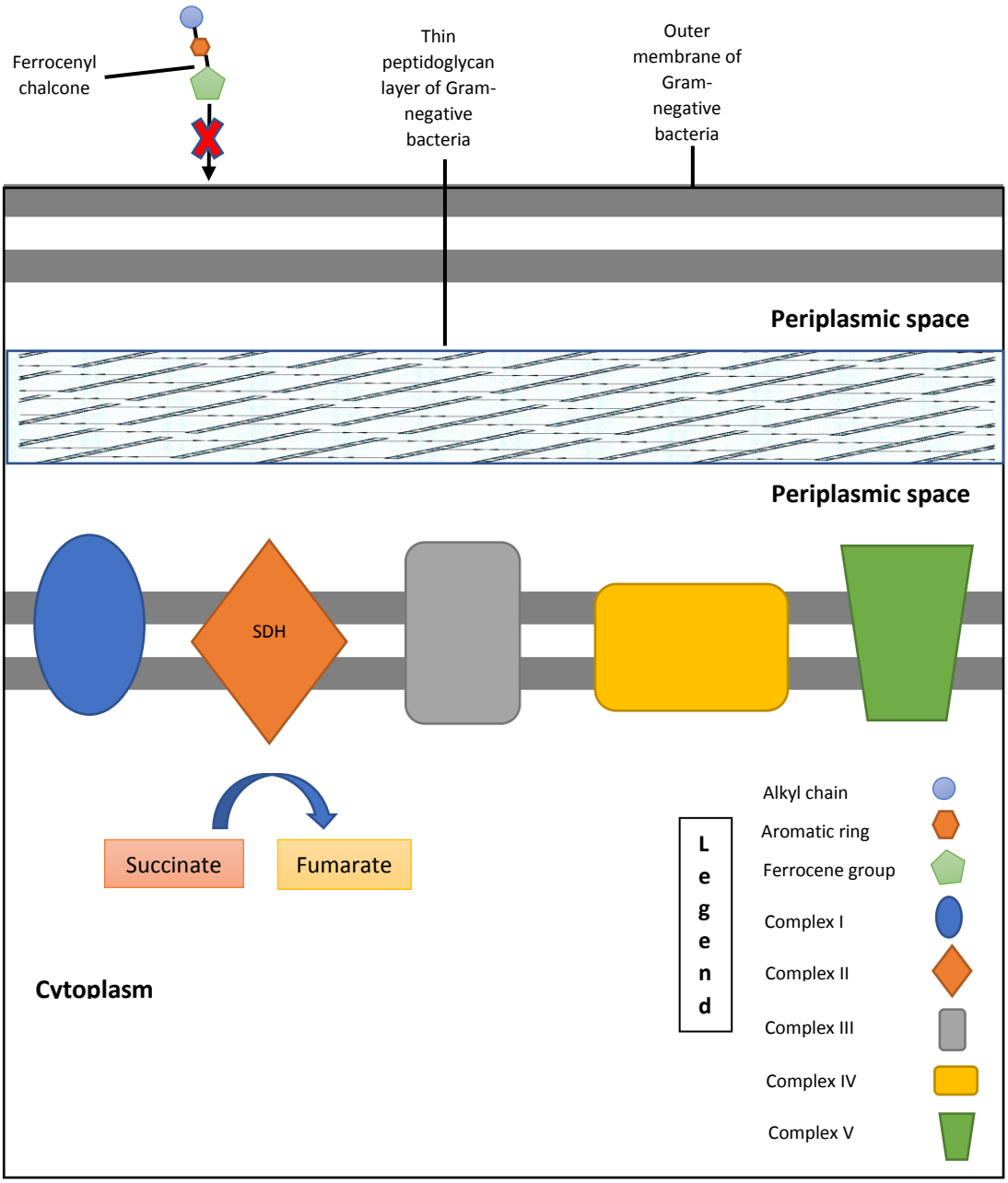


Figure 5-6 Summary of proposed mode of action of longer alkyl chain ferocenyl chalcone compounds showing no blockage of succinate reduction in the Krebs Cycle within Gram-negative bacteria (produced by E. Henry, 2017).

5.5 Conclusion

The MTT colorimetric assay, which is used to affirm the presence of actively respiring cells, involves the reduction of MTT to formazan by SDH and can be used for both eukaryotic and prokaryotic cells. In the present study, percentage of actively respiring non-resistant laboratory-adapted, and non-resistant and resistant clinical isolates of Gram-positive bacteria were significantly reduced post-incubation with ferrocenyl chalcone compounds (hexyl to decyl) comprised of longer alkyl chains. Each compound was equally effective at blocking respiration in all tested cells. A possible reason for this result was that the compounds were able to penetrate the thick peptidoglycan layer of the Gram-positive cells, becoming trapped in the cell membrane near SDH and, consequently, block succinate reduction. Thus, cell respiration and growth were inhibited. In terms of external damage to the cells, micrographic evidence of the effects will be explored in Chapter 6.

6 Determination of Changes to Cellular Morphology using SEM

6.1 Introduction

As described previously, aerobic respiration in bacteria is facilitated by membrane-bound enzymes, including SDH, that comprise the electron transport chain as detailed by several researchers including Hederstedt & Rutberg (1981). Thunberg's (1909) description of SDH as a critical enzyme in electron transfer within the Krebs Cycle was confirmed by many successive researchers, including Vogel *et al*. (2006) who demonstrated the ATP-synthesising and respiratory function of the enzyme. Hederstedt & Rutberg (1981) also reviewed that SDH catalyses the reduction of succinate to fumarate in the Krebs Cycle, an essential function performed in actively respiring eukaryotic and prokaryotic cells that constitutes cell viability (Pegg, 1989).

In Chapter 5, evidence was presented that aerobic bacteria, which were exposed to ferrocenyl chalcone compounds with longer alkyl chains (hexyl to decyl), exhibited reduced viability. This would infer that there was inhibition of the respiratory process, which may indicate damage to the cell membrane structure. SEM provides an opportunity of observing this loss of structure.

6.1.1 SEM

The principle of the SEM, first proposed by Knoll (1935), involves the transmission of a focused electron beam on to a minute area of an object, followed by accumulation of the electron currents, under vacuum, that rebound from the object, and compilation and amplification on to a screen of a cathode-ray tube to produce an image with high resolution and magnification (Smith *et al*., 1955). The first SEM instrument, constructed by McMullan (1953), obtained micrographs of opaque metal specimens. While SEM proved effective in elucidating the topography of metallic samples, it was observed by researchers that the imaging method was in effective in gaining images of biological samples. This was due to the fact that the conservation of biological samples became a major issue, since the maintenance of sample integrity under vacuum conditions was of paramount importance as discussed by Fischer *et al*. (2012). Another key factor in the utilisation of SEM for acquiring images

of biological samples is that such specimens are comprised of non-conductive material from which electrons are not rebounded. These issues resulted in the development of modified SEM preparation techniques for organic samples. In 1975, Malick & Wilson were among the first research groups to adapt this technology for biological samples using air drying preparation. One limitation of this method was that it was only effective for hard biological specimens since vacuum conditions, which were required to obtain high resolution images, destroyed such samples. To overcome this, other preparation techniques such as freeze drying (Boyde & Wood, 1969), critical point drying (Hayat, 1978), and vapour fixation with osmium tetroxide (Quattlebaum & Carner, 1980) have been employed. Another successful sample preparation method, developed by Nation (1983), involved pre-fixing in glutaraldehyde, dehydrating in a graded series of ethanol and post-fixing in hexamethyldisilazane. In a review by Fischer *et al*. (2012), it was noted that SEM methods should be used with caution since chosen techniques should be adapted depending on the type of biological sample under assessment.

6.1.2 Bacterial SEM

Glauert (1980) designed a method in which solid-based bacterial samples were pre-fixed in 2-3% $^{w}/_{v}$ glutaraldehyde and post-fixed in osmium tetroxide or Ruthenium Red for bacterial samples in fatty or polysaccharide sources. Variations of the Nation (1983) method have also been used to prepare bacterial specimens, some of which specifically focus on the preparation of bacterial samples from liquid-based media. Researchers who have developed such techniques for gaining images of bacterial samples in solution discovered that suspension-based bacterial samples require filtration using polycarbonate membrane filters diameters of 13 mm and pore sizes from 0.2 µm to 1 µm, as discussed by Kaláb, Yang, & Chabot (2008). Previous research on antimicrobial agents have used SEM to show changes in the topography of bacterial cells when treated with those agents. Successful studies that employed the use of bacterial SEM include exposure of bacterial cells to antimicrobial peptides (AMP) (Hartmann *et al*., 2010), treatment of food-related bacteria with sugar fatty acid esters (Zhao *et al*., 2015) and the antimicrobial activity of cinnamon essential oil against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Zhang *et al*., 2016). Images

derived from these studies indicated that untreated cells retained their smooth forms and their normal size, while treated cells exhibited blisters on the surface of AMP-treated *E. coli* cells and craters on the surface of AMP-treated *S. aureus* cells (Hartmann *et al*., 2010), uneven surfaces post-exposure to sugar fatty acid esters (Zhao *et al*., 2015) and misshapen morphology *(Zhang et al.*, 2016).

6.1.3 Morphological effects of conventional antimicrobial agents against bacteria

In a study of the morphological effects of conventional antimicrobial agents on clinical isolates of *S. aureus*, Greenwood and O'Grady (1972) obtained SEM images of affected bacterial cells exhibiting various types of external damage. After treatment of clinically isolated *S. aureus* with cloxacillin, which inhibits the synthesis of cell walls, there was evidence of topographical damage where the cell appeared shrunken and amorphous (Greenwood & O'Grady, 1972). In the same study, Greenwood and O'Grady (1972) also obtained morphological evidence of damage to *S. aureus* clinical isolates after exposure of the cells to lincomycin, erythromycin and fusidic acid, which are inhibitors of bacterial protein synthesis. The cells exhibited total structural collapse after exposure to the compounds with "discrete" lesions that were observed on cells affected by lincomycin, and the appearance of "doughnuts" were observed on cells that were treated with erythromycin (Greenwood & O'Grady, 1972).

Evidence of external damage to bacterial cells were also obtained in SEM images of treatment of *S. aureus* ATCC 25923 with common antimicrobial agents (Anam *et al*., 2010). After treatment with PEN-G, *S. aureus* ATCC 25923 cells exhibited incomplete cell wall synthesis with pores that possessed needle-like structures, which resulted from partial transpeptidation (Anam *et al*., 2010). In the same study, the cells exhibited shrinkage and pores after exposure to vancomycin, which is an inhibitor of cross-linkage in bacterial cell walls, resulting in weakened peptidoglycan and cell lysis (Anam *et al*., 2010).

6.1.4 Aim of the assay

If the ferrocenyl chalcone compounds are exerting their effect by disrupting the cell membrane, then this disruption potentially can cause an effect on the physical structure of the organism that can be seen using SEM. The morphological effect(s) of these compounds will possibly differ from those observed in previous studies described in 6.1.2 and 6.1.3, which could provide further evidence that these compounds exhibit a possible alternate mode of action against bacteria. The aim of this assay is to reveal external damage to *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884 and *E. faecalis* NCTC 12697 post-incubation with decyl ferrocenyl chalcone compound at the respective MIC values. Decyl ferrocenyl chalcone compound, which possessed antibacterial activity against all tested bacteria, was randomly selected to address this aim.

6.2 Materials and Methods

6.2.1 Preparation of ferrocenyl chalcone solution

For each bacterial sample, stock solutions of decyl ferrocenyl chalcone were prepared as detailed in Section 2.1.3. Each stock solution was further serially diluted 2-fold in sterile MH broth down to the required MIC values that were measured in Chapter 3 (Table 3-9).

6.2.2 Preparation of inocula

Fresh inocula of *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884 and *E. faecalis* NCTC 12697 were prepared as described in Section 2.2.1.

6.2.3 Preparation of bacterial samples for SEM

Treated and untreated non-resistant bacterial inocula were examined in the SEM. The method of preparation of both treated and untreated organisms were detailed in Section 2.8.

6.3 Results

Low electron energies between 2 keV and 2.5 keV (Hartmann *et al*., 2010) and 4keV were initially used to capture secondary electron images. However, use of these conditions resulted in loss of contrast where cell morphology and possible cell damage could not be determined. The next option used to obtain clear images was the use of back scattered electron imagery, which proved unsuccessful. After productive discussions with other technical staff, it was recommended that the electron energy be incrementally increased, and that the working distance be incrementally decreased until clear images can be obtained (Figures 6-1, 6-2, 6-3, 6-4 and 6-5). This approach helped in the SEM method development of the current study in relation to the SEM conditions that would produce useful images. Additionally, this method development aided in confirming that the preparation technique used in the current study was successful. The most successful conditions involved the use of 15 keV and WD = 11 mm (Figures 6-6, 6-7, 6-8, 6-9, 6-10 and 6-11).

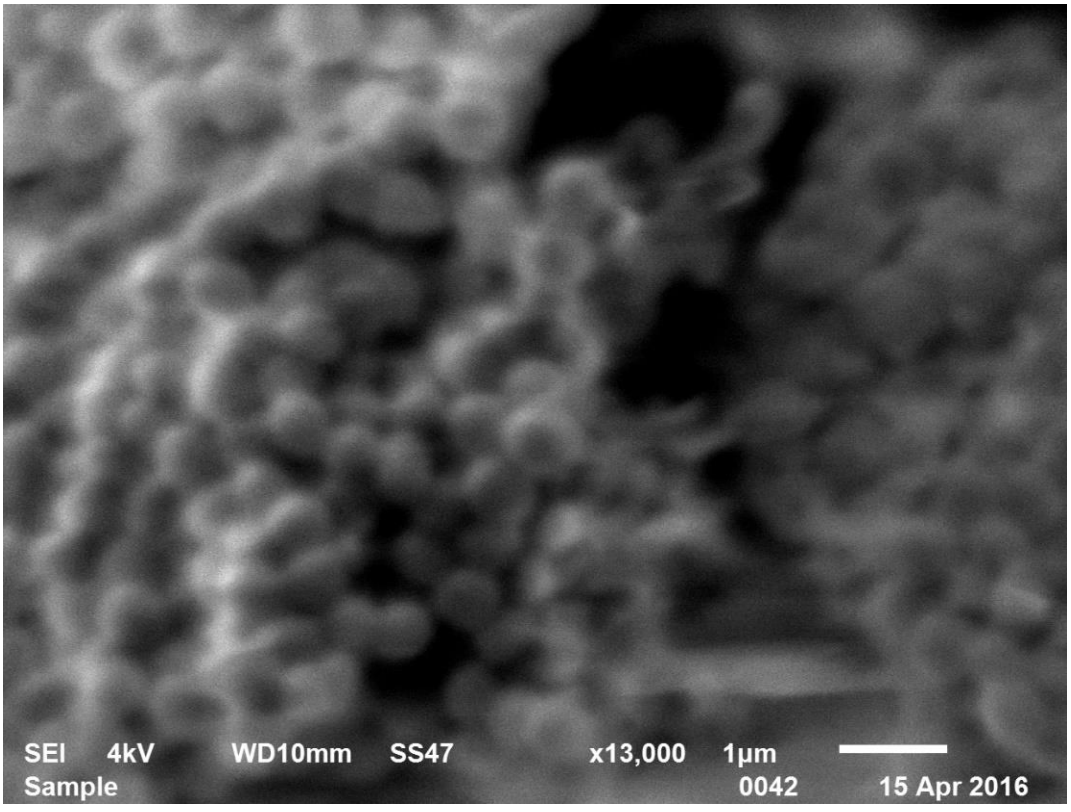


Figure 6-1 SEM image of untreated S. aureus NCIMB 8244 using 4kV. Although the use of similar SEM conditions as described by Hartmann et al. (2010) produced an image with less contrast, sufficient details were obtained that indicated that the untreated cells were undamaged.

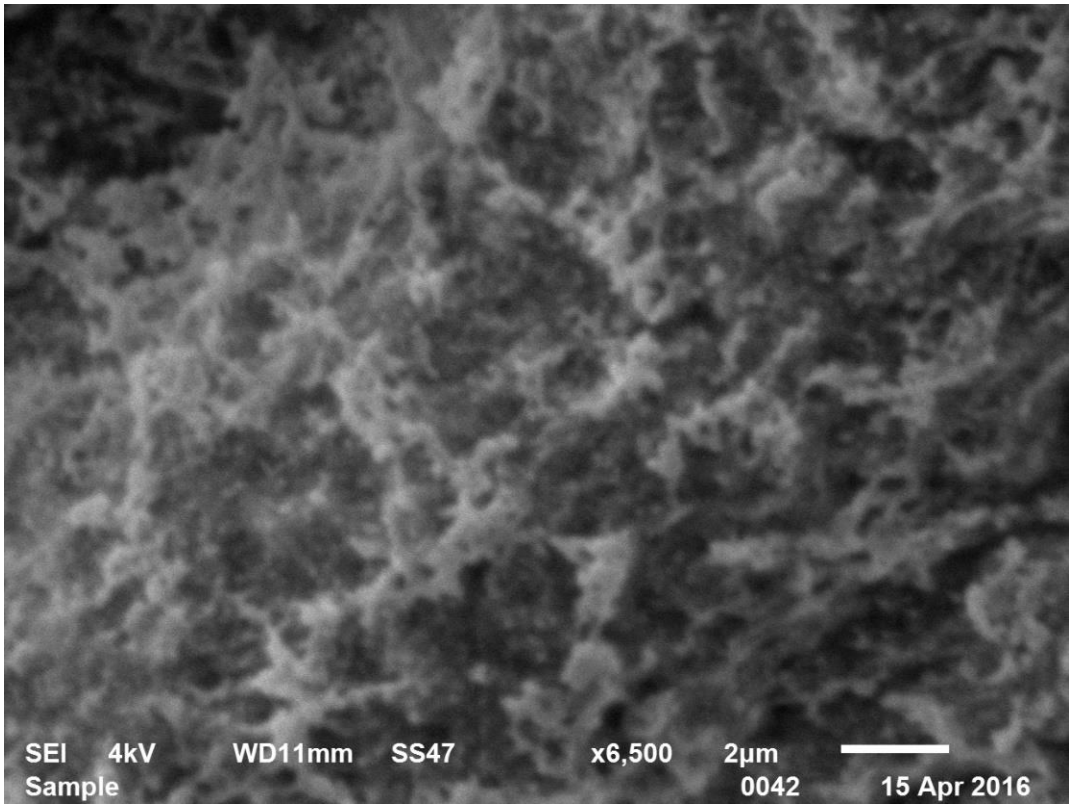


Figure 6-2 SEM image of treated S. aureus NCIMB using 4kV with increased working distance and decreased magnification were considered in an effort to obtain an image with sharper contrast. However, this combination of changes in conditions did not yield clear images since cellular damage could not be determined.

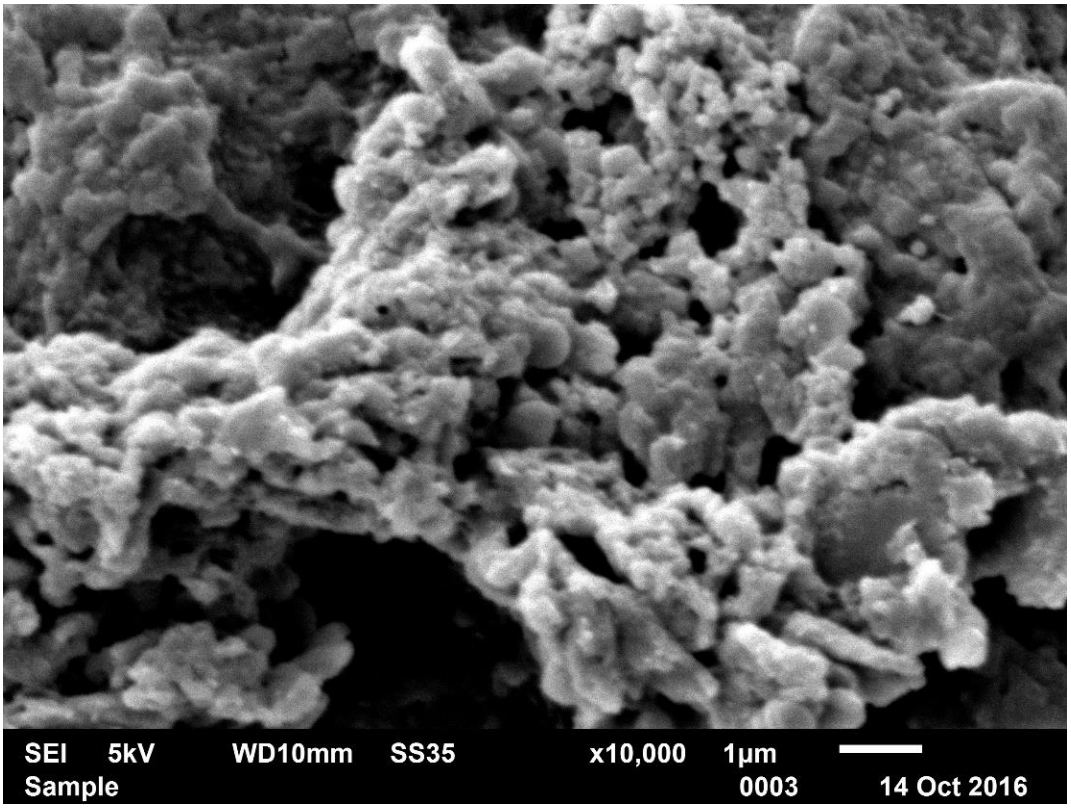


Figure 6-3 SEM image of treated S. aureus NCIMB 8244 using 5kV with decreased working distance and spot size, and increased magnification. Increased electron energy and magnification produced an image with greater contrast showing cells with loss of structural integrity.

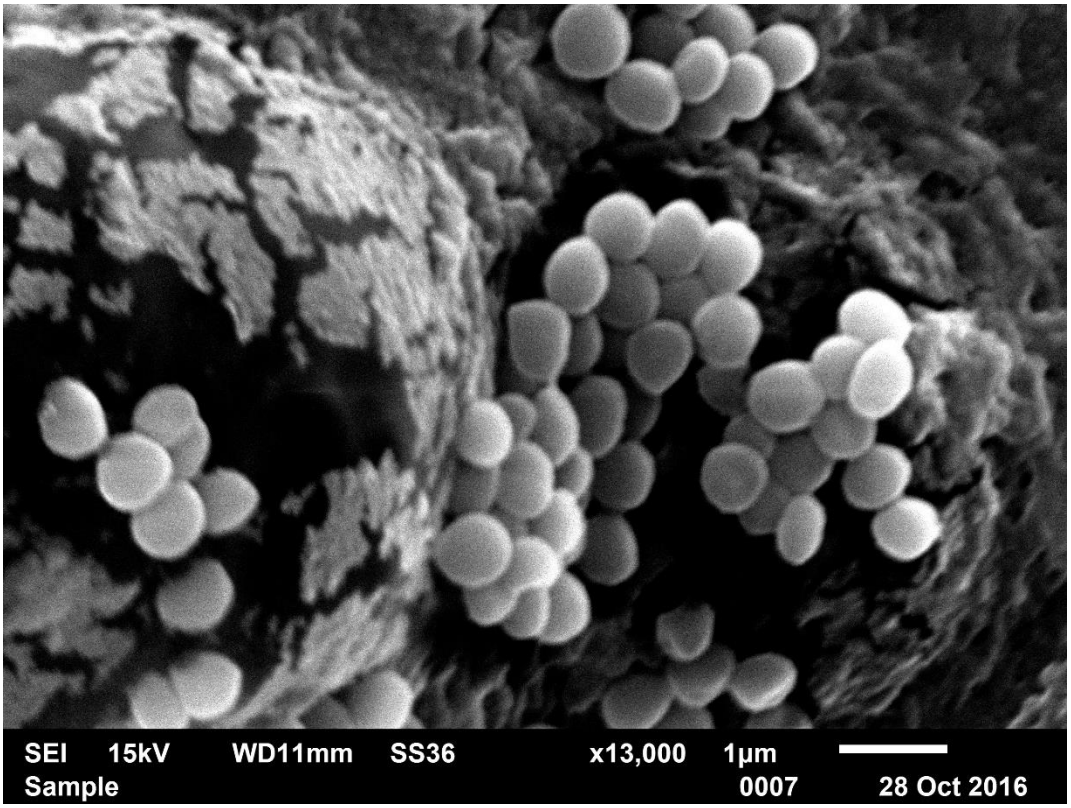


Figure 6-4 SEM image of untreated S. aureus NCMIB obtained after increasing all conditions. Image appears clearer than those in in which lower electron energies were used. Greater cell morphology can be seen but any cellular damage could not be clearly defined.

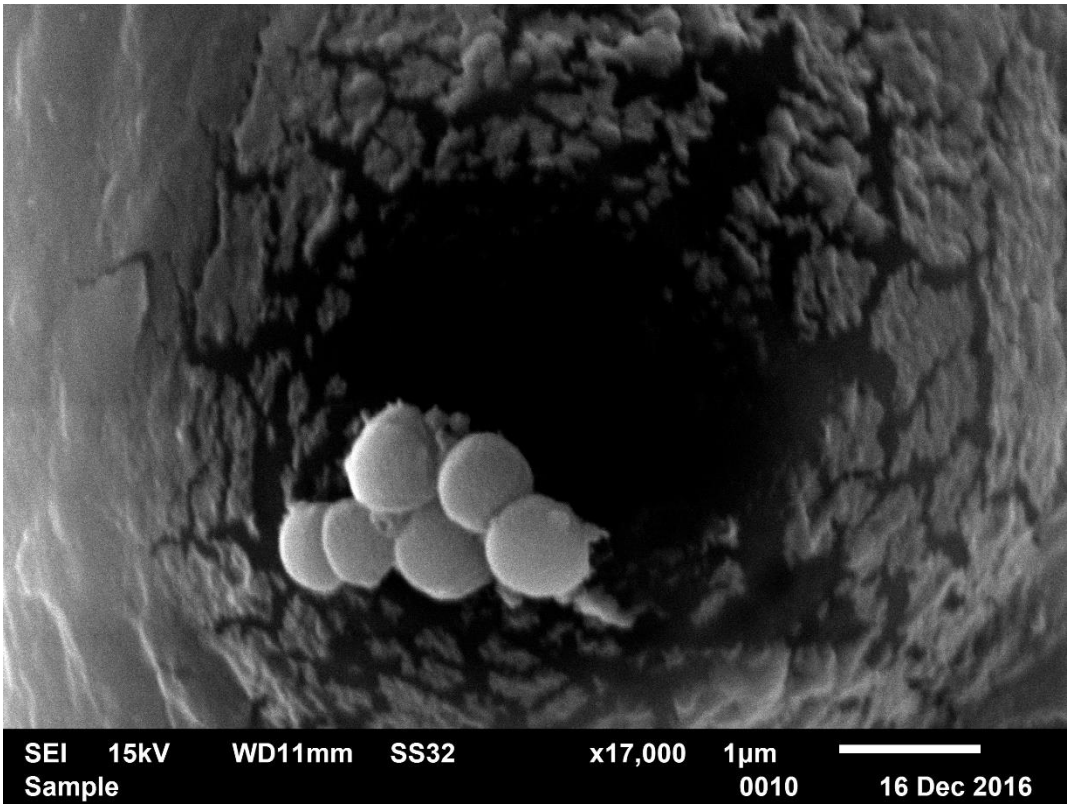


Figure 6-5 SEM image of untreated S. aureus NCIMB 8244 at 15kV with decreased spot size and increased magnification. This combination of changes in SEM conditions resulted in greater contrast where undamaged cellular morphology was observed.

The SEM images of untreated *S. aureus* NCIMB 8244 (Figure 6-6), *K. kristinae* NCIMB 8884 (Figure 6-8) and *E. faecalis* NCTC 12697 (Figure 6-10) revealed bacterial cells with their normal, spherical or spherical-like appearance. However, after exposure to decyl ferrocenyl chalcone compound at respective MIC values, *S. aureus* NCIMB 8244 (Figure 6-7), *K. kristinae* NCIMB 8884 (Figure 6-9) and *E. faecalis* NCTC 12697 (Figure 6-11) exhibited severe external damage. The affected cells appeared partially or fully lysed. Cell lysis indicates that the integrity of the bacterial cell membrane is compromised, which consequently contributes to the inhibition of bacterial respiration.

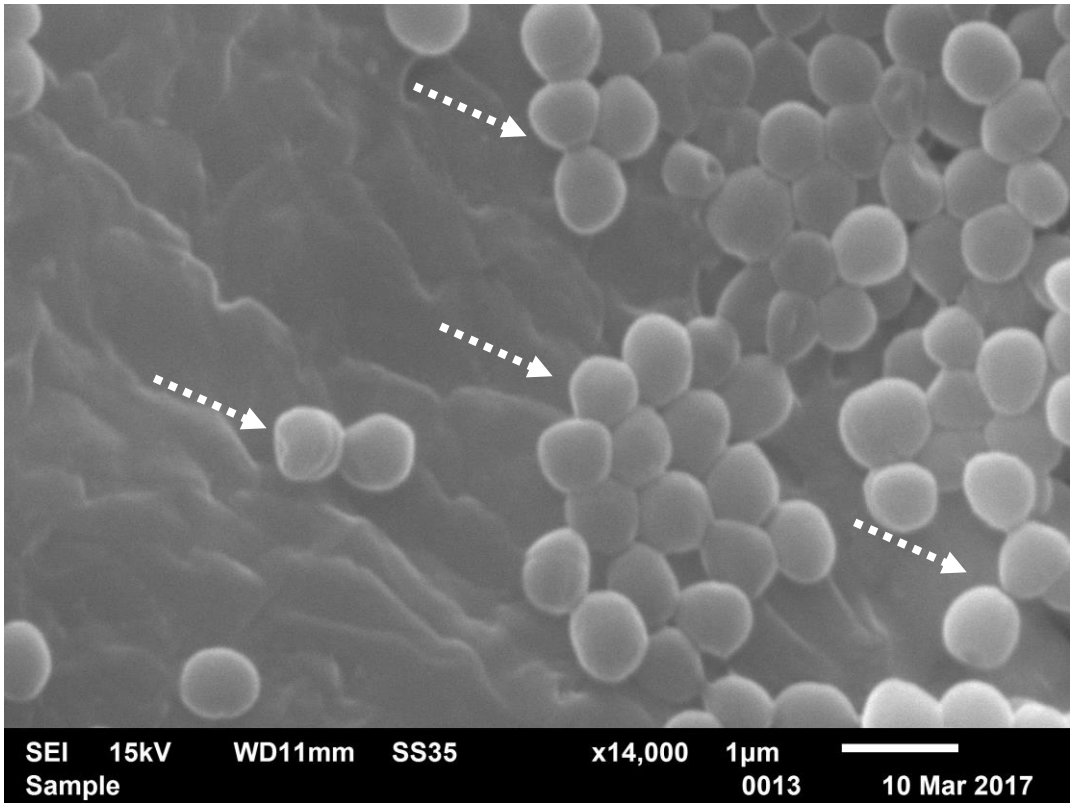


Figure 6-6 SEM image of untreated S. aureus NCIMB 8244 where dotted arrows indicate some of the cells with normal spherical appearance and undamaged cell membranes where bacterial respiration occurs.

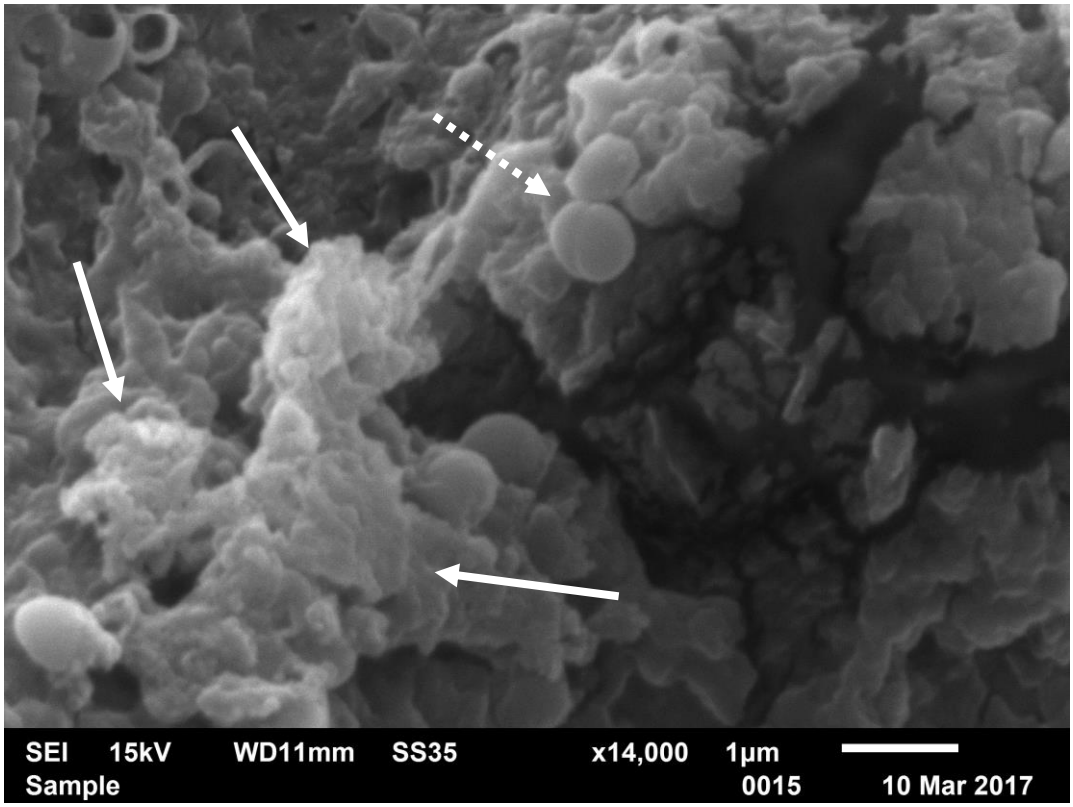


Figure 6-7 SEM image of treated S. aureus NCIMB 8244. Dotted arrow indicates an unaffected cell, while solid arrows indicate some of the fully lysed cells at MIC 0.031 mg/ml of decyl ferrocenyl chalcone. Cell lysis is similar to the damage observed by Li et al. (2014) and Zengin and Baysal (2014), which was caused by the inhibition of bacterial cellular respiration. This also shows evidence that the MIC value is equivalent to the MBC value (Chapter 3).

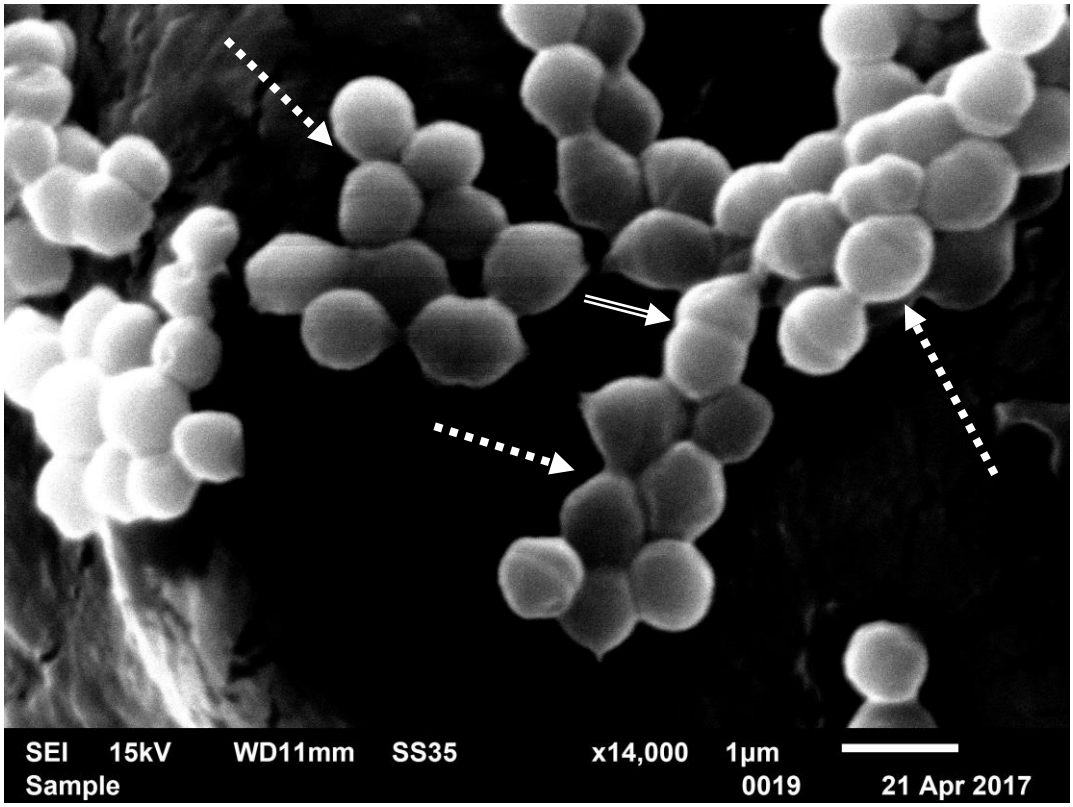


Figure 6-8 SEM image of untreated K. kristinae NCIMB 8884 where dotted arrows indicate some of the cells with normal spherical-like appearance and undamaged cell membranes where bacterial respiration occurs. Double-lined arrow indicates a dividing cell.

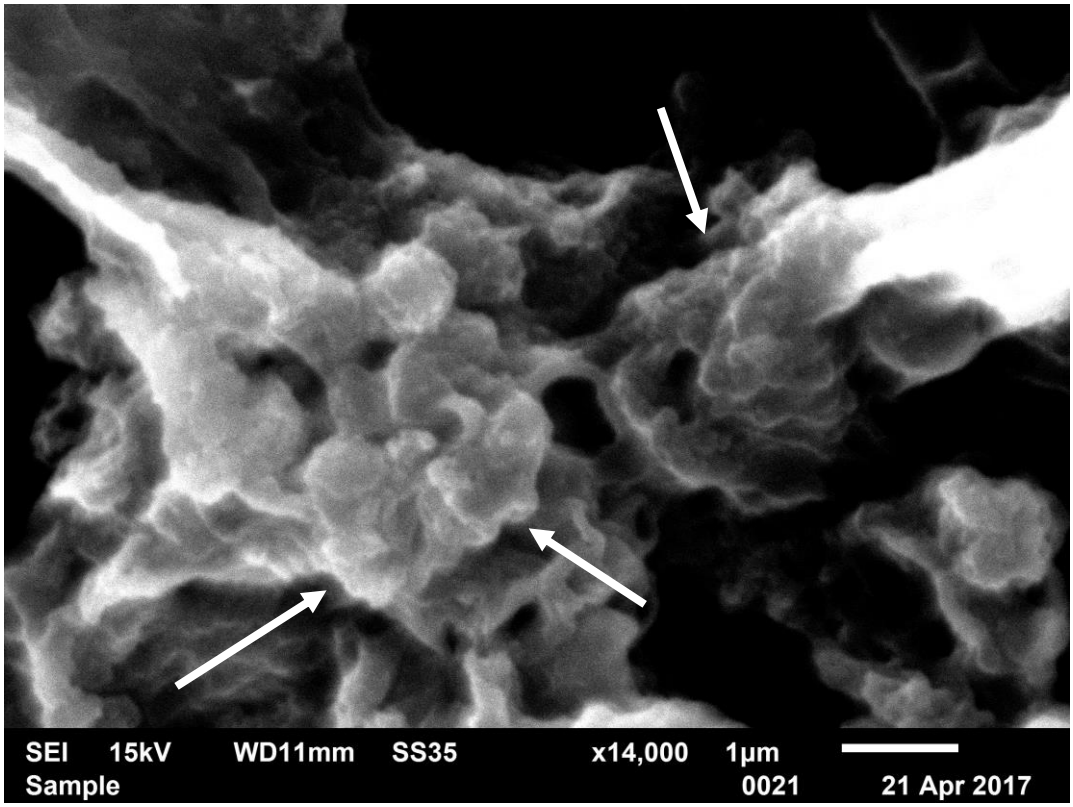


Figure 6-9 SEM image of treated K. kristinae NCIMB 8884 where solid arrows indicate some of the fully lysed cells at MIC 0.016 mg/ml of decyl ferrocenyl chalcone. Cell lysis is similar to the damage observed by Li et al. (2014) and Zengin and Baysal (2014) which was caused by the inhibition of bacterial cellular respiration. This also shows evidence that the MIC value is equivalent to the MBC value (Chapter 3).

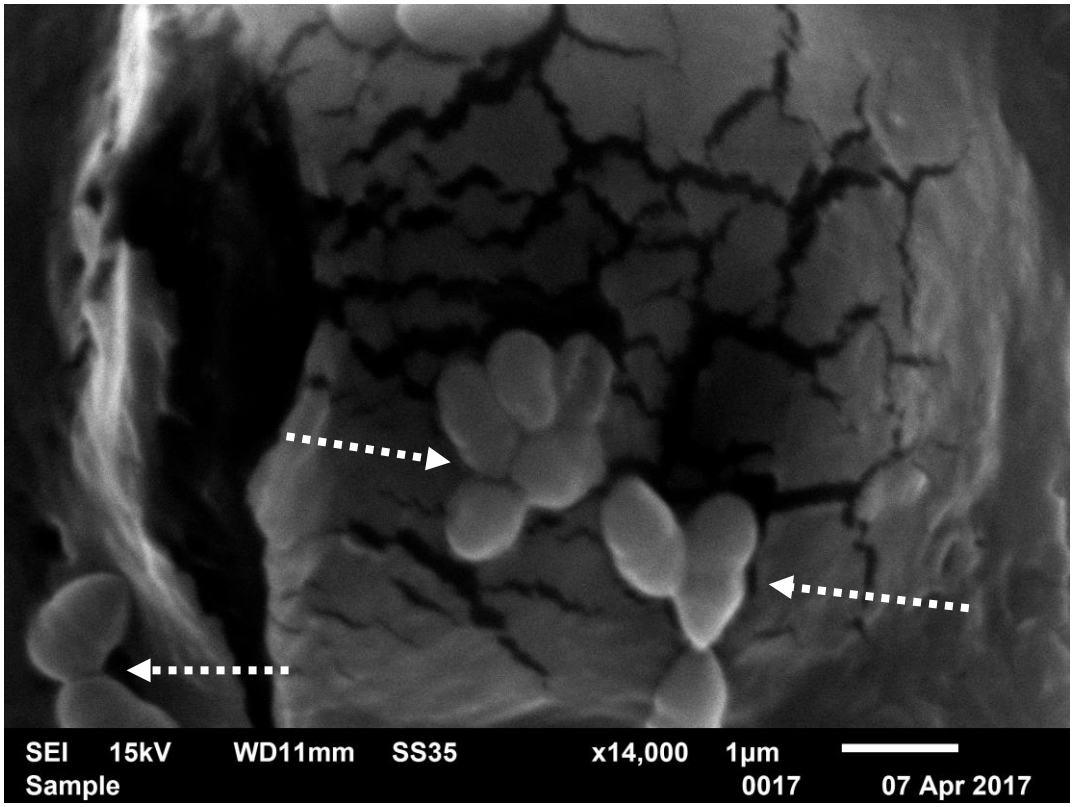


Figure 6-10 SEM image of untreated E. faecalis NCTC 12697 where dotted arrows indicate some of the cells with normal spherical-like (ovoid) appearance and undamaged cell membranes where bacterial respiration occurs..

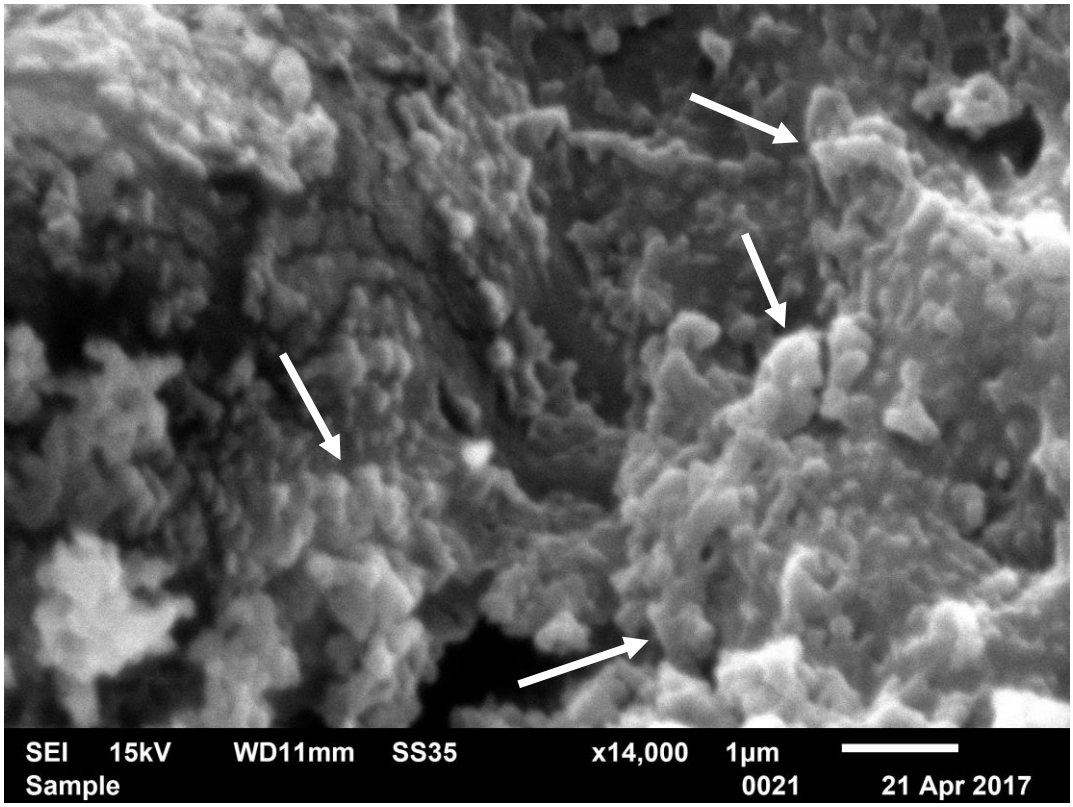


Figure 6-11 SEM image of treated E. faecalis NCTC 12697 where solid arrows indicate some of the fully lysed cells at MIC 0.063 mg/ml of decyl ferrocenyl chalcone. Cell lysis is similar to the damage observed by Li et al. (2014) and Zengin and Baysal (2014) that was caused by the inhibition of bacterial cellular respiration. This also shows evidence that the MIC value is equivalent to the MBC value (Chapter 3).

6.4 Discussion

SEM images revealed fully lysed Gram-positive bacterial cells that may have resulted from the effects of inhibition of cellular respiration in the bacterial cell membranes post-exposure to decyl ferrocenyl chalcone compound at appropriate MIC values (Chapter 3, Table 3-9). Although few unaffected bacterial cells were seen, most of the treated *S. aureus* NCIMB 8244 exhibited extensive external damage post-incubation with decyl ferrocenyl chalcone compound at 0.031 mg/ml MIC value (Figure 6-7). This result corroborated the observations seen in the MTT assay of the same compound against *S. aureus* NCIMB 8244 where the mean percentage of actively respiring cells was 0.782% (Chapter 5, Figure 5-3). In contrast, untreated *S. aureus* NCIMB 8244 cells displayed undamaged spherical shapes (Figure 6-6). After exposure to decyl ferrocenyl chalcone compound at 0.016 mg/ml MIC value, treated *K. kristinae* NCIMB 8884 (Figure 6-9) displayed the same external damage as seen in *S. aureus* NCIMB 8244. Untreated *K. kristinae* NCIMB 8884 cells showed undamaged cells, including some which appear to be dividing, confirming indication of cell viability (Figure 6-8). Treatment of *E. faecalis* NCTC 12697 with decyl ferrocenyl chalcone compound at 0.063 mg/ml MIC value also resulted in external damage to the cells (Figure 6-11), while untreated *E. faecalis* NCTC 12697 exhibited their undamaged, ovoid shape (Figure 6-10).

Similar cell lysis caused by respiration inhibitors was seen when *S. aureus* ATCC 25923 was exposed to graphene films on three types of conductors (Li *et al*., 2014). Li *et al.* (2014) demonstrated that upon microbial cell contact with graphene film on conductor copper (Cu), electrons were transferred from the respiratory system to graphene films and then to conductor Cu, which was the final electron acceptor in the bacterial electron transport system. Exposure of bacterial cells to graphene resulted in the formation of a Schottky barrier, which involved the transfer of electrons from the electron transport system to the semi-metal, and the outcome of cell membrane damage. This damage was reported as a “disruption” of membrane integrity, which resulted in leakage of critical cellular contents and subsequent cell death. Because of the possible removal of electrons from the bacterial respiratory chain that is located in the cell membrane, followed by the interruption of the

membrane structure and cell lysis, which was seen in SEM images obtained by Li *et al.* (2014), the mode of action of the graphene films as proposed by Li *et al* (2014) is comparable with the mechanism of action of the ferrocenyl chalcone compounds used in the current study.

In another study, evidence of cell lysis resulting from respiratory inhibitors against bacterial cells was reported by Zengin & Baysal (2014) when *S. aureus* RSSK01009 cells were exposed to essential oil terpenes. Zengin & Baysal (2014) concluded that monoterpenes, such as eucalyptol, act as inhibitors of bacterial respiration by altering the structural configuration of membrane-bound proteins essential to the electron transport chain followed by the inhibition of oxidative phosphorylation. This inhibition led to extensive damage to the bacterial cell membrane, including the breaking and collapse of the cell membrane, and cell death. The proposed mechanism of action and subsequent SEM images of cell lysis obtained by Zengin and Baysal (2014) can be considered as evidence of a similar mode of action that was displayed after exposure of bacterial cells to ferrocenyl chalcone chemicals in the current study.

As previous research has shown, an association between respiratory inhibition and structural damage can be observed in affected bacterial cells. As discussed in Chapter 5, bacterial cells perform respiration to ensure viability and cell growth. The aerobic version of this process is achieved through the electron transport chain, which is bound to the cell membrane (Hederstedt & Rutberg, 1981). This ATP-producing system includes SDH, an enzyme that reduces succinate to fumarate in the Krebs Cycle (Hederstedt & Rutberg, 1981). Ferrocene, which was present in the ferrocenyl chalcone compounds in this study, has been proposed to inhibit succinate reduction (Gonzalez & Estevez-Braun, 1997; Attar *et al*., 2011). This results in blockage of the Krebs Cycle and eventual cellular respiration and cell damage, which has been observed in SEM micrographs.

6.5 Conclusion

The SEM technique has been shown to be a viable tool to illustrate the topography of bacterial cells. In the current study, images of selected Gram-positive bacteria, post-incubation with decyl ferrocenyl chalcone compound at the appropriate MIC values from Chapter 3, were obtained. The images revealed extensive external cellular damage, which are comparable with previously published research (Li *et al.*, 2014; Zengin and Baysal, 2014). The observed cellular damage may have resulted from inhibition of bacterial respiration. This inhibition was caused by a possible interaction between the ferrocene group of the chalcone compound and SDH in the electron transport chain of the Krebs Cycle. Thus, the SEM images provide further support for the potential mode of action of ferrocenyl chalcones with longer alkyl chains.

7 Conclusions

This study focussed on the characterisation and antimicrobial action of novel ferrocenyl chalcone chemicals, which possess increasing alkyl chains, as well as to determine their possible mode of action. Chalcones are plant-based compounds, which exhibit antibacterial activity and can be easily modified to form ferrocenyl compounds, as reported by several researchers (Attar *et al*., 2011; Pejović *et al*., 2012; Kowalski *et al*., 2013; Medu, 2013; Prasath *et al*., 2013; Ahmed *et al*., 2015; Mishra *et al*., 2015; Smith, 2017 pers. comm.). These ferrocene-type antibacterial agents are potential inhibitors of bacterial respiration.

7.1 Determination of MIC

Key discoveries that were presented in Chapter 3 of this current study are that the ferrocenyl chalcone compounds with longer alkyl chains (hexyl to decyl) exhibited greater antimicrobial activity than those with shorter alkyl chains (methyl to pentyl). Findings also indicated that the compounds, which possessed antimicrobial activity, were more effective against Gram-positive bacteria than against Gram-negative bacteria. Another key finding of the current study was that the ferrocenyl chalcones demonstrated significant antimicrobial activity, with MICs ranging from 0.008 mg/ml to 0.063 mg/ml ± 0.000 mg/ml, against non-resistant laboratory-adapted organisms and resistant and non-resistant clinical isolates. These values were observed using the broth microdilution, as detailed by Andrews (2001), after alternative methods, such as agar well diffusion, disc diffusion and broth macrodilution, yielded minimal practical results. Further assays demonstrated that the MIC values are also equal to the MBC values and that bactericidal activity occurred within 0 to 4 hours as shown by the time-kill assays. The results of the time-kill assays indicate that the time taken to kill bacteria are comparable with other studies (Barbour *et al*., 2014; Scheerans *et al*., 2015; del Valle *et al*., 2016; Rolston *et al*., 2017). These findings indicate that these compounds possess favourable antimicrobial properties.

7.2 Elucidation of the possible causes and consequence of colour change and particulate formation in diluted ferrocenyl chalcone solutions

An issue encountered early in the study was that 2-fold dilution of the solubilised compound in MH broth resulted in a colour change from deep red to dark brown and the formation of brown particles despite the facile solubilisation of the novel ferrocenyl chalcones in DMSO. Initially, disposed iodine was proposed to be the possible cause of the presence of the dark brown solution. Based on the reported findings of Hildebrand *et al*. (1950) and Smith *et al*. (2013), diethy ether was used to indicate the presence of iodine, which is soluble in polar solvents (Kaiho, 2015). However, in the current study, brown residue was seen at the bottom of the borosilicate tubes instead of a brown ring at the top of the suspension, as reported by Smith *et al*. (2013). An alternative explanation was that this reversible colour change was possibly caused by the oxidation of Fe^{2+} ions in ferrocene to Fe^{3+} ions (ferrocenium) as demonstrated in Chapter 4 of this study and reported by Charette & Sholkovitz (2002). This phenomenon resulted in an equivalent mix of ferrocene (reddish orange) and ferrocenium (bluish green), which formed the dark brown colour. The presence of brown particles may have been caused by iron ion chelation due to casein hydrolysate in MH broth. Further investigations involving the reaction between the ferrocene-type compounds and casein hydrolysate are required to verify the cause of this particle formation.

The colour change of these compounds initiated the study by Bennett (2016) to investigate the effect of environmental condition on solubilised compounds and whether the antibacterial activity of these affected compounds were altered. Bennett concluded that solubilised ferrocenyl chalcones were affected by light and heat. However, the study indicated that there was no significant change in antibacterial activity. This means that neither colour change, nor the formation of brown particles, affects the activity of these compounds after solubilisation in DMSO.

7.3 Cell Viability

Cell viability studies implied that the potential mechanism of action of the ferrocenyl chalcone compounds involved the inhibition of bacterial respiration, as reported and discussed in Chapter 5, where there was approximately 0% of actively respiring

bacterial cells present after exposure to the compounds. The absence of actively respiring bacterial cells was represented by the absence of reduced MTT compound, which is a chemical that is reduced from yellow to the purple coloured formazan by membrane-bound respiratory dehydrogenases that are essential for cell viability and production. This absence of the reduced compound may have been achieved by interaction between the ferrocene group and respiratory dehydrogenases in the electron transport chain, which is bound to the bacterial cell membrane as demonstrated by Hederstedt & Rutberg (1981). This mechanism may have resulted in structural alteration of the cell membrane. If this is the mechanism of action, then further work is required to determine the effect of such compounds on mammalian cells, which also utilise respiratory dehydrogenases. This would establish whether mammalian apoptosis occur should these ferrocenyl chalcones be considered as alternative antimicrobial therapeutic agents.

7.4 SEM

After the use of several SEM technical conditions, useful images of bacterial cells that were treated and untreated were obtained. The SEM images highlighted in Chapter 6 demonstrated that exposure to decyl ferrocenyl chalcone, a potent ferrocenyl chalcone, resulted in substantial external damage to bacterial cells at the MIC. The affected cells presented a shrivelled and wrinkled appearance suggesting that the integrity of their cell wall structural had been affected after interaction with the ferrocenyl chalcone compound. Evidence of the external morphological damage was demonstrated in similar studies where bacterial respiration was inhibited (Li *et al*., 2014; Zengin & Baysal, 2014). Thus supporting the view that the inhibition was occurring, the SEM images obtained in the current study further strengthen the findings of the MIC determination using broth microdilution (Chapter 3), time-kill assays (Chapter 3) and the MTT assay (Chapter 5). The overall findings contribute to significant implications in the development of new antimicrobial drugs.

7.5 Overall Implications

The findings from this study indicate that these novel ferrocenyl chalcone compounds possess potential antimicrobial activity against clinical bacterial isolates, specifically fully sensitive *S. aureus*, PEN-resistant *S. aureus*, PEN/ERY/CLI-resistant *S.*

aureus and MRSA. The ferrocenyl chalcone compounds used in the current study may exhibit similar potency to that observed with conventional antimicrobial agents since the observed MICs of the functional ferrocenyl chalcones from hexyl to decyl (0.008 mg/ml-0.063mg/ml) fall within the ranges of PEN (0.000015-0.128 mg/ml) and TET (0.00025-0.128 mg/ml) as reported by Andrews (2001). Additionally, as discussed in Chapter 5, Section 5.3, these ferrocenyl chalcone compounds appear to demonstrate inhibition of respiration in actively respiring bacterial cells, which resulted in no viable cells. This is significant since there was possible evidence of a mode of antimicrobial action that differed from conventional antimicrobial mechanisms but possibly similar to the mode of action of zinc porphyrin-based photosensitisers as proposed by Alenezi *et al*. (2017).

In comparison to reported natural-based antimicrobial compounds, the group of ferrocenyl chalcone compounds used in the current study may be considered to be more effective than methanolic abstracts from birch (Duric *et al*., 2013), similarly effective as α-mangostin (Koh *et al*., 2013) but less effective against Gram-negative bacteria as observed with the activity of methanol extracts from seaweed (Kavita, Kumar, & Jha, 2014). These ferrocenyl compounds, which exhibited antimicrobial activity against a MRSA, PEN-resistant, CLI-resistant and ERY-resistant Gram-positive bacteria, and non-resistant Gram-positive bacteria, also appeared to exhibit mixed efficacy when compared to antimicrobial agents derived from the human body (Yamaguchi *et al*., 2002; Domanski *et al*., 2005; Yadava *et al*., 2006) but can be sufficiently compared to agents derived from pure colonies (Ovchinnikov *et al*., 2017) and from mixed colonies in soil (Ling *et al*., 2015). When compared to other ferrocenyl chalcones, the compounds used in the current study can be considered to be as effective against bacteria. Additionally, an investigation of this family of novel ferrocenyl chalcones, which are semi-synthetic analogues of plant-based compounds, used in the current study reveal that the common structure of these compounds play a vital role in potential antimicrobial therapy. This enhances the increasing interest of researchers into the development of similar chemicals as possible antimicrobial agents.

Some findings of the study failed to support the primary aim of this study, which involves the characterisation and determination of antimicrobial activity of novel ferrocenyl chalcones, but may be useful tools in other directions of research into the development of novel antimicrobial agents. Firstly, despite the fact that the solubility issues and the evidence of colour change of the dissolved compounds that occur under certain conditions require additional scrutiny, the solutions maintained their antimicrobial activity, as explored by Bennett (2016). These characteristics may be used to explore other forms of drug delivery such as topical therapy so as to minimise any adverse effects. Secondly, although the compounds were ineffective against Gram-negative bacteria, evidence of their activity against Gram-positive bacteria may lead to the production of an alternative source in the dwindling supply of antimicrobial therapeutics to which the latter group of bacteria are not resistant. Overall, the antimicrobial characteristics exhibited by the ferrocenyl chalcone compounds used in the current study, such as the favourable MICs and killing times, reduction of viable bacterial cells that is indicative of bactericidal activity, possibly caused by respiration inhibition, and SEM images of severe external damage, positively impacts antimicrobial therapy research by contributing to the exploration and development of similar compounds as potential therapeutic agents.

7.6 Further work

In order to progress the possible use of ferrocenyl chalcones with medium to long alkyl iodide chains as promising alternative antimicrobial drugs, future research into these current chemicals, which includes their effects against biofilms and mammalian cells, as well as chemical modification, should be considered. Further assays involving the efficacy of the compounds against biofilms, such as a comparable study reported by Kunthalert *et al*. (2014), and cytotoxicity against mammalian cells, such as a similar study reported by Kowalski *et al*. (2013) are needed to strengthen the profile of the ferrocenyl chalcone compounds. Another useful investigation would be to expand the current group of ferrocenyl chalcones to include, for example, longer alkyl chains greater than 10 carbons, which may provide a complete profile with respect to the structural relationships among the family of compounds. These modified compounds, along with the ferrocenyl chalcones used the current study, can also be

compared to chalcones that possess a similar structure. Additionally, the determination of the effect of these compounds on spheroblasts, which are cell wall free cells, should be included since this may provide vital information regarding the inefficacy of the compounds against Gram-negative organisms. The outcome would provide additional evidence that these compounds are potential additions to the rapidly decreasing availability of effective antimicrobial agents or that they may be useful as scaffolds in broad spectrum antimicrobial chemotherapy.

7.7 Key Findings of the study

1. Solubilised ferrocenyl chalcone compounds exhibit colour change from deep red to dark brown, which initially was proposed to be caused by the presence of displaced iodine. However, after an assay involving the use of diethyl ether to indicate the presence of iodine resulted in no occurrence of the reported brown ring at the top of the suspension (Hildebrand *et al*., 1950; Smith *et al*., 2013), an alternate cause of the brown colour change was explored. This brown colour change may result from oxidation of Fe^{2+} to Fe^{3+} causing the simultaneous presence of both Fe^{2+} (reddish orange) and Fe^{3+} (bluish green). While the occurrence of Fe^{2+} oxidation has been reported by Charette & Sholkovitz (2002), further work is needed to confirm that this occurred in the current study.
2. Colour change of ferrocenyl chalcone compounds may be influenced by temperature, light and type of reaction vessel. The effect of these environmental conditions on these compounds were further explored by Bennett (2016) who concluded that the observed colour change did not affect the antimicrobial activity of the ferrocenyl chalcone compounds. This is the first known report that focussed on the colour change ferrocenyl chalcones with respect to environmental conditions. Therefore, this theory requires further investigation.
3. Dilution of solubilised compounds in MHB exhibit the reversible formation of particles, which may result from iron ion chelation by casein hydrolysate in MHB as reported by Gu *et al*. (2010) and Luo, Pan & Zhong (2014). The current study is also the first to report this occurrence in

relation to ferrocenyl chalcone chemicals. After exploring the possible effect of the presence of these particles on antimicrobial activity, Bennett (2016) reported that no change in antimicrobial efficacy was observed.

4. Ferrocenyl chalcone compounds with increased alkyl chain lengths demonstrated greater antibacterial activity than those with decreased alkyl chain lengths. This structural feature of the compounds may be a critical factor in the determination of the mode of action. Additionally, the antimicrobial activity of similar compounds with chain lengths that possess more than 10 carbons may be investigated in order to strengthen this theory.
5. Functional compounds exhibited greater antibacterial activity against Gram-positive bacteria than against Gram-negative bacteria. Although antibacterial activity against Gram-negative bacteria was not observed, this result indicates that the compounds used in the current study can be considered as potential additions to the cache of antimicrobial compounds against Gram-positive bacteria. Additionally, spheroblasts may be used to confirm the ineffectiveness of the same compounds against Gram-negative bacteria.
6. Ferrocenyl chalcones compounds exhibited antibacterial activity against non-resistant Gram-positive laboratory-adapted organisms and PEN-resistant, CLI-resistant and ERY-resistant Gram-positive clinical isolates, a MRSA, and non-resistant Gram-positive clinical isolates. The antibacterial activity of these plant-based semi-synthetic compounds against Gram-positive bacteria can be comparable with the antibacterial activity of other plant-based chemicals such as methanolic abstracts (Duric *et al*., 2013) and α-mangostin (Koh *et al*., 2013) but incomparable against Gram-negative bacteria (Kavita *et al*., 2014).
7. Antibacterial activity of these compounds, which is consistent with bactericidal activity, is initiated from 0 to 4 hours after exposure. The results of the time-kill assays, in which MIC values that were obtained against each organism (0.008 mg/ml – 0.063 mg/ml) were used, indicate that the bactericidal activity of the compounds can be comparable to

other reported antimicrobial therapeutic agents such as linezolid (Scheerans *et al*., 2015), telavancin and vancomycin (Barbour *et al*., 2014; Rolston *et al*., 2017) and kaempferol (del Valle *et al*., 2016).

8. Evidence of cellular damage possibly caused by blockage of bacterial respiration was observed in SEM micrographs, which were eventually obtained after several SEM conditions were used. The mode of action may be characterised by chalcone inhibition of membrane-bound respiratory enzymes, which results in interference of bacterial respiration. Morphological damage of the bacterial cells can be comparable to damage observed in cells with inhibited bacterial respiration (Li *et al*., 2014; Zengin & Baysal, 2014).
9. This possible mode of action differs from that of the conventional antimicrobial mechanisms. Further investigations involving the determination of cell death that result from the inhibition of bacterial respiration are required.

8 References

Ageevets, V.A., Partina, I. V, Lisitsyna, E.S., Ilina, E.N., Lobzin, Y. V, Shlyapnikov, S.A. & Sidorenko, S. V, 2014. Emergence of Carbapenemase-Producing Gram-Negative Bacteria in Saint Petersburg, Russia. *International Journal of Antimicrobial Agents*, 44(2), pp.152–5.

Ahmed, N., Konduru, N.K. & Owais, M., 2015. Design, Synthesis and Antimicrobial Activities of Novel Ferrocenyl and Organic Chalcone Based Sulfones and Bis-Sulfones. *Arabian Journal of Chemistry*.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P., 2002a. B Cells and Antibodies. In *Molecular Biology of the Cell*. New York: Garland Science, pp. 476, 1375.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P., 2002b. Chloroplasts and Photosynthesis. In *Molecular Biology of the Cell*. New York: Garland Science, pp. 796–797.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P., 2002c. How Cells Obtain Energy from Food. In *Molecular Biology of the Cell*. New York: Garland Science, pp. 93, 787.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P., 2002d. The Diversity of Genomes and the Tree of Life. In *Molecular Biology of the Cell*. New York: Garland Science, p. 16.

Aleksic, V., Mimica-Dukic, N., Simin, N., Nedeljkovic, N.S. & Knezevic, P., 2014. Synergistic Effect of Myrtus Communis L. Essential Oils and Conventional Antibiotics against Multi-Drug Resistant *Acinetobacter baumannii* Wound Isolates. *Phytomedicine*, 21(12), pp.1666–1674.

Alenezi, K., Tovmasyan, A., Batinic-Haberle, I. & Benov, L.T., 2017. Optimizing Zn Porphyrin-Based Photosensitizers for Efficient Antibacterial Photodynamic Therapy. *Photodiagnosis and Photodynamic Therapy*, 17, pp.154–159.

Allen, J.P. & Williams, J.C., 1998. Photosynthetic Reaction Centers. *FEBS Letters*,

438(1-2), pp.5–9.

Aminov, R.I., 2010. A Brief History of the Antibiotic Era : Lessons Learned and Challenges for the Future. *Frontiers in Microbiology*, 1(134), pp.1–7.

Andersson, D.I. & Hughes, D., 2012. Evolution of Antibiotic Resistance at Non-Lethal Drug Concentrations. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, 15(3), pp.162–72.

Andersson, S.G. & Kurland, C.G., 1999. Origins of Mitochondria and Hydrogenosomes. *Current Opinion in Microbiology*, 2(5), pp.535–541.

Andrews, J.M., 2006. *Determination Of Minimum Inhibitory Concentrations*, Birmingham.

Andrews, J.M., 2001. Determination of Minimum Inhibitory Concentrations. *Journal of Antimicrobial Chemotherapy*, 48(suppl 1), pp.5–16.

Assis, L.M., Nedeljković, M. & Dessen, A., 2017. New Strategies for Targeting and Treatment of Multi-Drug Resistant *Staphylococcus aureus*. *Drug Resistance Updates*, 31, pp.1–14.

Attar, S., O'Brien, Z., Alhaddad, H., Golden, M.L. & Calderón-Urrea, A., 2011. Ferrocenyl Chalcones versus Organic Chalcones: A Comparative Study of Their Nematocidal Activity. *Bioorganic & Medicinal Chemistry*, 19(6), pp.2055–73.

Bai, H. et al., 2012. Antisense Inhibition of Gene Expression and Growth in Gram-Negative Bacteria by Cell-Penetrating Peptide Conjugates of Peptide Nucleic Acids Targeted to *rpoD* Gene. *Biomaterials*, 33(2), pp.659–67.

Bal, A.M., Garau, J., Gould, I.M., Liao, C.H., Mazzei, T., Nimmo, G.R., Soriano, A., Stefani, S. & Tenover, F.C., 2013. Vancomycin in the Treatment of Meticillin-Resistant *Staphylococcus aureus* (MRSA) Infection: End of an Era? *Journal of Global Antimicrobial Resistance*, 1(1), pp.23–30.

Ball, P., 2000. Quinolone Generations: Natural History or Natural Selection? *Journal of Antimicrobial Chemotherapy*, 46(suppl 3), pp.17–24.

Balouiri, M., Sadiki, M. & Ibnsouda, S.K., 2016. Methods for in Vitro Evaluating

Antimicrobial Activity: A Review. *Journal of Pharmaceutical Analysis*, 6(2), pp.71–79.

Barbour, A.M., Schmidt, S., Zhuang, L., Rand, K. & Derendorf, H., 2014. Application of Pharmacokinetic/pharmacodynamic Modelling and Simulation for the Prediction of Target Attainment of Ceftobiprole against Meticillin-Resistant *Staphylococcus aureus* Using Minimum Inhibitory Concentration and Time-Kill Curve Based Approaches. *International Journal of Antimicrobial Agents*, 43(1), pp.60–67.

Baron, E., Jorgensen, J. & Landry, M., 2007. *Manual Of Clinical Microbiology* 9th ed., Washington, DC, USA: ASM Press.

Basch, H., Gadebusch, H.H. & Brunswick, N., 1968. In Vitro Antimicrobial Activity of Dimethylsulfoxide. *Applied Microbiology*, 16(12), pp.1953–1954.

Bell, G.H., 1973. Solubilities of Normal Aliphatic Acids, Alcohols and Alkanes in Water. *Chemistry and Physics of Lipids*, 10, pp.1–10.

Belley, A., Neesham-grenon, E., Arhin, F.F., Mckay, G.A., Parr, T.R. & Moeck, G., 2008. Assessment by Time-Kill Methodology of the Synergistic Effects of Oritavancin in Combination with Other Antimicrobial Agents against *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 52(10), pp.3820–3822.

Bennett, C.T., 2016. *Antibacterial Activity Of Novel Colour-Changing Ferrocenyl Chalcones*. Staffordshire University.

Biswas, S. & Rolain, J., 2013. Use of MALDI-TOF Mass Spectrometry for Identification of Bacteria That Are Difficult to Culture. *Journal of Microbiological Methods*, 92, pp.14–24.

Bizzini, A. & Greub, G., 2010. Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry, a Revolution in Clinical Microbial Identification. *Clinical Microbiology and Infection*, 16(11), pp.1614–1619.

Bolla, J.M., Alibert-Franco, S., Handzlik, J., Chevalier, J., Mahamoud, A., Boyer, G., Kiec-Kononowicz, K. & Pags, J.M., 2011. Strategies for Bypassing the Membrane

Barrier in Multidrug Resistant Gram-Negative Bacteria. *FEBS Letters*, 585(11), pp.1682–1690.

Boyde, A. & Wood, C., 1969. Preparation of Animal Tissues for Surface-Scanning Electron Microscopy. *Journal of Microscopy*, 90(3), pp.221–249.

Bremmer, D.N., Balada-llasat, J., Goff, D.A. & Bauer, K.A., 2017. Ceftriaxone Etest Non-Susceptible Methicillin Susceptible *Staphylococcus aureus* Time-Kill Responses. *Diagnostic Microbiology & Infectious Disease*, 88(2), pp.192–194.

Buttrick, J.C. & King, B.T., 2017. Kekulenes, Cycloarenes, and Heterocycloarenes: Addressing Electronic Structure and Aromaticity through Experiments and Calculations. *Chemical Society Reviews*, 46(7), pp.7–20.

Cafiso, V., Bertuccio, T., Spina, D., Purrello, S., Campanile, F., Di, C., Purrello, M. & Stefani, S., 2012. Modulating Activity of Vancomycin and Daptomycin on the Expression of Autolysis Cell-Wall Turnover and Membrane Charge Genes in hVISA and VISA Strains. *Plos One*, 7(1), pp.1–10.

Cai, Y. *et al*., 2017. Evaluating Polymyxin B-Based Combinations against Carbapenem-Resistant *Escherichia coli* in Time-Kill Studies and in a Hollow-Fiber Infection Model. *Antimicrobial Agents and Chemotherapy*, 61(1), pp.1–11.

Calderaro, A. *et al*., 2014. Matrix-Assisted Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry Applied to Virus Identification. *Nature*, 4(6803), pp.1–10.

Calvey, N. & Williams, N., 2007. Drug Absorption , Distribution and Elimination. In *Principles and Practice of Pharmacology for Anaesthetists*. Massachusetts: Blackwell Publishing, pp. 1–22.

Campagna, J.D., Bond, M.C., Schabelman, E. & Hayes, B.D., 2012. The Use of Cephalosporins in Penicillin-Allergic Patients: A Literature Review. *Journal of Emergency Medicine*, 42(5), pp.612–620.

Carmichael, J., Degraff, W.G., Gazdar, A.F., Minna, J.D. & Mitchell, J.B., 1987. Evaluation of a Tetrazolium-Based Semiautomated Colorimetric Assay:

Assessment of Chemosensitivity Testing Evaluation of a Tetrazolium-Based Semiautomated Colorimetrie Assay : Assessment. *American Association for Cancer Research*, 47, pp.936–942.

de Carvalho Tavares, L. *et al*., 2011. Quinolinyl and Quinolinyl N-Oxide Chalcones: Synthesis, Antifungal and Cytotoxic Activities. *European Journal of Medicinal Chemistry*, 46(9), pp.4448–56.

Casadevalll, A. & Scharff, M.D., 1994. Serum Therapy Revisited: Animal Models of Infection and Development of Passive Antibody Therapy. *Antimicrobial Agents and Chemotherapy*, 38(8), pp.1695–1702.

Cavera, V.L., Arthur, T.D., Kashtanov, D. & Chikindas, M.L., 2015. Bacteriocins and Their Position in the next Wave of Conventional Antibiotics. *International Journal of Antimicrobial Agents*, 46(5), pp.494–501.

Celenza, G. *et al*., 2012. In Vitro Antimicrobial Activity of Pannarin Alone and in Combination with Antibiotics against Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates. *Phytomedicine: International Journal of Phytotherapy and Phytopharmacology*, 19(7), pp.596–602.

Charette, M.A. & Sholkovitz, E.R., 2002. Oxidative Precipitation of Groundwater-Derived Ferrous Iron in the Subterranean Estuary of a Coastal Bay. *Geophysical Research Letters*, 29(10), pp.1–4.

Chinedu, C. & Min, U., 2017. Role of Surface Charge of Hydrolysed Bovine Caseins in Their Iron (II) -Binding Affinity and Antioxidative Capacity in Iron (II) -Facilitated β -Carotene and Glutathione Oxidation. *Journal of Food and Nutrition Research*, 56(2), pp.149–154.

Choi, J., Jung, Y., Kim, J., Kim, S., Jung, Y., Na, H. & Kwon, S., 2012. Rapid Antibiotic Susceptibility Testing by Tracking Single Cell Growth in a Microfluidic Agarose Channel System. *Lab on a Chip*, 13(2), pp.280–287.

Choma, I.M. & Grzelak, E.M., 2011. Bioautography Detection in Thin-Layer Chromatography. *Journal of Chromatography A*, 1218(19), pp.2684–2691.

Chopra, I. & Roberts, M., 2001. Tetracycline Antibiotics : Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews : MMBR*, 65(2), pp.232–260.

Cîntă-Pînzaru, S., Dehelean, C.A., Soica, C., Culea, M. & Borcan, F., 2012. Evaluation and Differentiation of the Betulaceae Birch Bark Species and Their Bioactive Triterpene Content Using Analytical FT-Vibrational Spectroscopy and GC-MS. *Chemistry Central Journal*, 6(67), pp.1–12.

Clardy, J., Fischbach, M. & Currie, C., 2010. The Natural History of Antibiotics. *Current Biology*, 19(11), pp.1–8.

CLSI, 1999. *Methods For Determining Bactericidal Activity Of Antimicrobial Agents; Approved Guideline* 1st ed. A. L. Barry, W. A. Craig, H. Nadler, B. L. Barth Reller, C. C. Sanders, & J. M. Swenson, eds., Pennsylvania: CLSI.

CLSI, 2012. *Methods For Dilution Antimicrobial Susceptibility Tests For Bacteria That Grow Aerobically ; Approved Standard — Ninth Edition*, Pennsylvania.

Coates, A.R.M., Halls, G. & Hu, Y., 2011. Novel Classes of Antibiotics or More of the Same? *British Journal of Pharmacology*, 163, pp.184–194.

Codd, A.A., Richardson, I.R. & Andrews, N., 1998. Lenticules for the Control of Quantitative Methods in Food Microbiology. *Journal of Applied Microbiology*, 85, pp.913–917.

Cooper, T.F., Rozen, D.E. & Lenski, R.E., 2003. Parallel Changes in Gene Expression after 20,000 Generations of Evolution in Escherichia Coli. *Proceedings of the National Academy of Sciences*, 100(3), pp.1072–1077.

Corti, A., Muniyasamy, S., Vitali, M., Imam, S.H. & Chiellini, E., 2010. Oxidation and Biodegradation of Polyethylene Films Containing Pro-Oxidant Additives: Synergistic Effects of Sunlight Exposure, Thermal Aging and Fungal Biodegradation. *Polymer Degradation and Stability*, 95(6), pp.1106–1114.

Croxatto, A., Prod, G. & Greub, G., 2012. Diagnostic Microbiology. *FEMS Microbiological Reviews*, 36, pp.380–407.

Cushnie, T.P.T. & Lamb, A.J., 2005. Antimicrobial Activity of Flavonoids. *International Journal of Antimicrobial Agents*, 26(5), pp.343–356.

Cushnie, T.P.T. & Lamb, A.J., 2011. Recent Advances in Understanding the Antibacterial Properties of Flavonoids. *International Journal of Antimicrobial Agents*, 38(2), pp.99–107.

Cyriac, J.M. & James, E., 2014. Switch over from Intravenous to Oral Therapy : A Concise Overview. *Journal of Pharmacology and Pharmacotherapeutics*, 5(2), pp.83–88.

Das, K., Tiwari, R.K.S. & Shrivastava, D.K., 2010. Techniques for Evaluation of Medicinal Plant Products as Antimicrobial Agent : Current Methods and Future Trends. *Journal of Medicinal Plants Research*, 4(2), pp.104–111.

Davies, J. & Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.*, 74(3), pp.417–433.

Davies, S.C., 2011. *Annual Report Of The Chief Medical Officer, Volume II, 2011, Infections And The Rise Of Antimicrobial Resistance*, London.

Delcour, A.H., 2009. Outer Membrane Permeability and Antibiotic Resistance. *Biochimica et Biophysica Acta*, 1794(5), pp.808–816.

Denizot, F. & Lang, R., 1986. Rapid Colorimetric Assay for Cell Growth and Survival: Modifications to the Tetrozolium Dye Procedure Giving Improved Sensistivity and Reliability. *Journal of Immunological Methods*, 89, pp.271–277.

Desmarais, S.M., Pedro, M.A. De, Cava, F. & Huang, K.C., 2013. Micro Review Peptidoglycan at Its Peaks : How Chromatographic Analyses Can Reveal Bacterial Cell Wall Structure and Assembly. *Molecular Microbiology*, 89(June), pp.1–13.

Dhar, D.N., 1981. *The Chemistry Of Chalcones And Related Compounds* 1st ed., New York: Wiley.

Dimmock, J.R., Elias, D.W., Beazely, M.A. & Kandepu, N., 1999. Bioactivities of Chalcones. *Current Medicinal Chemistry*, 6(12), pp.1125–1149.

Doern, G.V., 2011. Antimicrobial Susceptibility Testing. *Journal of Clinical Microbiology*, 49(9), p.S4.

Domanski, P.J. *et al*., 2005. Characterization of a Humanized Monoclonal Antibody Recognizing Clumping Factor A Expressed by *Staphylococcus aureus*. *Infection and Immunity*, 73(8), pp.5229–5232.

Driscoll, A.J., Bhat, N., Karron, R.A., Brien, K.L.O. & Murdoch, D.R., 2012. Disk Diffusion Bioassays for the Detection of Antibiotic Activity in Body Fluids : Applications for the Pneumonia Etiology Research for Child Health Project. *Clinical Infectious Diseases*, 54(Suppl 2), pp.159–164.

Duric, K., Kovac-besovic, E., Niksic, H. & Sofic, E., 2013. Antibacterial Activity of Methanolic Extracts , Decoction and Isolated Triterpene Products From Different Parts of Birch, *Betula pendula*, Roth. *Journal of Plant Studies*, 2(2), pp.61–70.

Dyall, S.D., Brown, M.T. & Johnson, P.J., 2004. Ancient Invasions : From Endosymbionts to Organelles. *Science*, 304(5668), pp.253–257.

Edwards, B., Andini, R., Esposito, S., Grossi, P., Lew, D., Mazzei, T., Novelli, a., Soriano, a. & Gould, I.M., 2014. Treatment Options for Methicillin-Resistant *Staphylococcus aureus* (MRSA) Infection: Where Are We Now? *Journal of Global Antimicrobial Resistance*, 2(3), pp.133–140.

Ehmann, H.M.A. *et al*., 2015. Gold Nanoparticles in the Engineering of Antibacterial and Anticoagulant Surfaces. *Carbohydrate Polymers*, 117, pp.34–42.

Ellson, R., Stearns, R., Mutz, M., Brown, C., Browning, B., Harris, D., Qureshi, S., Shieh, J. & Wold, D., 2005. In Situ DMSO Hydration Measurements of HTS Compound Libraries. *Combinatorial Chemistry & High Throughput Screening*, 8, pp.489–498.

Emonet, S., Shah, H.N., Cherkaoui, A. & Schrenzel, J., 2010. Application and Use of Various Mass Spectrometry Methods in Clinical Microbiology. *Clinical Microbial Infection*, 16, pp.1604–1613.

Engelkirk, P.G. & Duben-Engelkirk, J.L., 2008. *Laboratory Diagnosis Of Infectious Diseases: Essentials Of Diagnostic Microbiology* 1st ed., Philadelphia: Lippincott Williams and Wilkins.

Espy, M.J. *et al*., 2006. Real-Time PCR in Clinical Microbiology : Applications for Routine Laboratory Testing. *Clinical Microbiology Reviews*, 19(1), pp.165–256.

EUCAST, 2016. Breakpoint Tables for Interpretation of MICs and Zone Diameters. *Version 6.0, 2016.*, pp.35–44. Available at: http://www.eucast.org. [Accessed June 27, 2016].

EUCAST, 2003. *EUCAST DISCUSSION DOCUMENT - Determination Of Minimum Inhibitory Concentrations (MICs) Of Antibacterial Agents By Broth Dilution*, Munich.

Ferrer, J., Jez, J.M., Bowman, M.E., Dixon, R.A. & Noel, J.P., 1999. Structure of Chalcone Synthase and the Molecular Basis of Plant Polyketide Biosynthesis. *Nature Structural Biology*, 6(8), pp.775–784.

Fischer, E.R., Hansen, B.T., Nair, V., Hoyt, F.H. & Dorward, D.W., 2012. Scanning Electron Microscopy. In *Current Protocol Microbiology*. pp. 1–76.

Fritiof, D.R. & Sjorstrand, S., 1953. Electron Microscopy of Mitochondria and Cytoplasmic Double Membranes: Ultra-Structure of Rod-Shaped Mitochondria. *Nature*, 171, pp.30–31.

Fuda, C., Suvorov, M., Vakulenko, S.B. & Mobashery, S., 2004. The Basis for Resistance to β-Lactam Antibiotics by Penicillin- Binding Protein 2a of Methicillin-Resistant *Staphylococcus aureus*. *Journal of Biological Chemistry*, 279(39), pp.40802–40806.

Fux, C.A., Shirtliff, M., Stoodley, P. & Costerton, J.W., 2005. Can Laboratory Reference Strains Mirror “Real-World” Pathogenesis? *Trends in Microbiology*, 13(2), pp.58–63.

Gabaldón, T. & Huynen, M.A., 2003. Reconstruction of the Proto-Mitochondrial Metabolism. *Science*, 301(5633), p.609.

Gao, P., Nie, X., Zou, M., Shi, Y. & Cheng, G., 2011. Recent Advances in Materials for Extended-Release Antibiotic Delivery System. *The Journal of Antibiotics*, 64(9), pp.625–634.

Garn, H., Krause, H., Enzmann, V. & Drößler, K., 1994. An Improved MTT Assay Using the Electron-Coupling Agent Menadione. *Journal of Immunological Methods*, 168(2), pp.253–256.

Gaynes, R., Edwards, J.R., Infections, N. & System, S., 2005. Overview of Nosocomial Infections Caused by Gram-Negative Bacilli. *Clinical Infectious Diseases*, 41, pp.848–854.

Gechev, T.S., Hille, J., Woerdenbag, H.J., Benina, M., Mehterov, N., Toneva, V., Fernie, A.R. & Mueller-roeber, B., 2014. Natural Products from Resurrection Plants: Potential for Medical Applications. *Biotechnology Advances*, 32(6), pp.1091–1101.

Ghuysen, J., 1968. Use of Bacteriolytic Enzymes in Determination of Wall Structure and Their Role in Cell Metabolism. *Bacteriological Reviews*, 32(4), pp.425–464.

Giamarellou, H., 2006. Treatment Options for Multidrug-Resistant Bacteria. *Expert Review of Anti-infective Therapy*, 4(4), pp.601–618.

Glauert, A.M., 1980. *Fixation, Dehydration And Embedding Of Biological Specimens* 3rd ed., Amsterdam: North-Holland Publishing Company.

Go, M.L., Wu, X. & Liu, X.L., 2005. Chalcones: An Update on Cytotoxic and Chemoprotective Properties. *Current Medicinal Chemistry*, 12(117543), pp.481–499.

Goddard, A.F., 1998. Review Article : Factors Influencing Antibiotic Transfer across the Gastric Mucosa. *Alimentary Pharmacology & Therapeutics*, 12, pp.1175–1184.

Gonzalez, J.A. & Estevez-Braun, A., 1997. Phytonematicidal Activity of Aromatic Compounds Related to Shikimate Pathway. *Pesticide Biochemistry and Physiology*, 58(3), pp.193–197.

Good, L., 2002. Antisense Inhibition of Bacterial Gene Expression and Cell Growth. In P. E. Nielsen, ed. *Peptide Nucleic Acids. Methods in Molecular Biology*. New Jersey: Humana Press Inc., pp. 237–248.

Gopi, C., Sastry, V.G. & Dhanaraju, M.D., 2016. Synthesis and Spectroscopic Characterisation of Novel Bioactive Molecule of 3- (2-Substituted) -1H-Indol-3-Yl) - 1- (Thiophen-2yl) Prop-2-En-1-One Chalcone Derivatives as Effective Anti-Oxidant and Anti-Microbial Agents. *Beni-Suef University Journal of Basic and Applied Sciences*, 5(3), pp.236–243.

Gray, M.W., Burger, G. & Lang, B., 1999. Mitochondrial Evolution. *Science*, 283(5407), pp.1476–1481.

Grzelak, E.M. *et al*., 2016. Bioautography with TLC-MS / NMR for Rapid Discovery of Anti-Tuberculosis Lead Compounds from Natural Sources. *ACS Infectious Diseases*, 2(4), pp.294–301.

Gu, F., Kim, J.M., Abbas, S., Zhang, X., Xia, S. & Chen, Z., 2010. Structure and Antioxidant Activity of High Molecular Weight Maillard Reaction Products from Casein – Glucose. *Food Chemistry*, 120(2), pp.505–511.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D. & Andersson, D.I., 2011. Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. *PLoS Pathogens*, 7(7), pp.1–9.

Hacker, M., Messer, W.S. & Bachmann, K.A., 2009. *Pharmacology: Principles And Practice* 1st ed., Oxford: Elsevier Inc.

Haddock, B.A. & Jones, C.W., 1977. Bacterial Respiration. *Bacteriological Reviews*, 41(1), pp.47–99.

Hadley, S., 2014. Resistant Gram-Positive Infections: Where Have We Been, Where Are We Now, and Where Are We Going? *Clinical Therapeutics*, 36(10), pp.1298–1302.

Harborne, J.B. & Williams, C.A., 2000. Advances in FLavonoid Research since 199. *Phytochemistry*, 55(6), pp.481–504.

Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M.F., Gerthsen, D. & Ulrich, A.S., 2010. Damage of the Bacterial Cell Envelope by Antimicrobial Peptides Gramicidin S and PGLa as Revealed by Transmission and Scanning Electron Microscopy. *Antimicrobial Agents and Chemotherapy*, 54(8), pp.3132–3142.

Hassan, A.S., 2014. The Antibacterial Activity of Dimethyl Sulfoxide (DMSO) with and without of Some Ligand Complexes of the Transitional Metal Ions of Ethyl Coumarin against Bacteria Isolate from Burn and Wound Infection. *Journal of Natural Sciences Research*, 4(19), pp.106–111.

Hayat, M.A., 1978. *Introduction To Biological Scanning Electron Microscopy* 1st ed., Baltimore: University Park Press.

Hederstedt, L. & Rutberg, L., 1981. Succinate Dehydrogenase-a Comparative Review. *Microbiological Reviews*, 45(4), pp.542–555.

Hedges, A.J., 2002. Estimating the Precision of Serial Dilutions and Viable Bacterial Counts. *International Journal of Food Microbiology*, 76, pp.207–214.

Heijenoort, J. Van, 2001. Formation of the Glycan Chains in the Synthesis of Bacterial Peptidoglycan 1. *Glycobiology*, 11(3), pp.25–36.

Henry, E.J., Smith, R.B., Collins, M., Bird, S.J., Gowland, P. & Cassella, J.P., 2017. Novel Ferrocenyl Chalcone Compounds as Possible Antimicrobial Agents. In A. Méndez-Vilas, ed. *Antimicrobial Research: Novel Bioknowledge and Educational Programs*. Spain: Formatex Research Center, pp. 140–148.

Heritage, J., Evans, E.V.G. & Killington, R.A., 2000. *Introductory Microbiology* 3rd ed., Cambridge: Cambridge University Press.

Hernandes, C., Coppede, J. da S., Bertoni, B.W., França, S. de C. & Pereira, A.M.S., 2013. Flash Microbiocide: A Rapid and Economic Method for Determination of MBC and MFC. *American Journal of Plant Sciences*, 04(04), pp.850–852.

Herrmann, W.A., 1997. *Synthetic Methods Of Organometallic And Inorganic Chemistry* 1st ed., New York: Georg Thieme Verlag.

Hewitt, W., 2005. *Microbiological Assay For Pharmaceutical Analysis: A Rational*

Approach 1st ed., Florida: CRC Press.

Hildebrand, J.H., Benesi, H.A. & Mower, L.M., 1950. Solubility of Iodine in Ethyl Alcohol, Ethyl Ether, Mesitylene, P-Xylene, 2,2-Dimethylbutane, Cyclohexane and Perfluoro-N-Heptane. *Journal of the American Chemical Society*, 72(2), pp.1017–1020.

Hogg, S., 2013. *Essential Microbiology* 2nd ed., John Wiley & Sons.

Howe, R.A. & Andrews, J.M., 2012. BSAC Standardized Disc Susceptibility Testing Method (Version 11). *Journal of Antimicrobial Chemotherapy*, 67(12), pp.2783–2784.

HPA, 2012. *English National Point Prevalence Survey On Healthcare-associated Infections And Antimicrobial Use, 2011: Preliminary Data*, London.

Hu, S., Loo, J.A. & Wong, D.T., 2006. Human Body Fluid Proteome Analysis. *Proteomics*, 6(23), pp.6326–6353.

Hughes, D. & Karlén, A., 2014. Discovery and Preclinical Development of New Antibiotics. *Upsala Journal of Medical Sciences*, 119(June), pp.162–169.

Irving, W.L., Ala'Aldeen, D.A.A. & Boswell, T., 2005. *Medical Microbiology* 1st ed., New York: Garland Science.

IUPAC, 1997. Chelation. *Compendium of Chemical Terminology*. Available at: http://goldbook.iupac.org/html/C/C01012.html [Accessed July 7, 2017].

James, R., Lazdunski, C. & Pattus, F., 1991. *Bacteriocins, Microcins And Lantibiotics* 1st ed., New York: Springer-Verlag.

Jaouen, G., Vessières, A. & Top, S., 2015. Ferrocifen Type Anti Cancer Drugs. *Chemical Society Reviews*, 44(24), pp.8802–8817.

Jay, J.M., Loessner, M.J. & Golden, D.A., 2005. *Modern Food Microbiology* 7th ed., New York: Springer Science & Business Media.

Jorgensen, J.H. & Ferraro, M.J., 2009. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases:*

An Official Publication of the Infectious Diseases Society of America, 49(11), pp.1749–1755.

Jung, W.K., Koo, H.C., Kim, K.W., Shin, S., Kim, S.H. & Park, Y.H., 2008. Antibacterial Activity and Mechanism of Action of the Silver Ion in *Staphylococcus aureus* and *Escherichia coli*. *Applied and Environmental Microbiology*, 74(7), pp.2171–2178.

Kahlmeter, G. *et al*., 2003. European Harmonization of MIC Breakpoints for Antimicrobial Susceptibility Testing of Bacteria. *Journal of Antimicrobial Chemotherapy*, 52, pp.145–148.

Kaiho, T., 2015. *Iodine Chemistry And Applications* 1st ed., New Jersey: John Wiley & Sons.

Kaláb, M., Yang, A.-F. & Chabot, D., 2008. Conventional Scanning Electron Microscopy of Bacteria. *In Focus*, (10), pp.43–61.

Kanellopoulos, A.G. & Wood, R.J., 1976. Particle Size Analysis Techniques for Suspension Concentrates. *Journal of Pesticide Science*, 7(1976), pp.75–85.

Kannan, K., Kanabar, P., Schryer, D., Florin, T., Oh, E., Bahroos, N. & Tenson, T., 2014. The General Mode of Translation Inhibition by Macrolide Antibiotics. *Proceedings of the National Academy of Sciences*, 111(45), pp.15958–15963.

Karioti, A., Sokovic, M., Ciric, A., Koukoulitsa, C., Bilia, A.R. & Skaltsa, H., 2011. Antimicrobial Properties of *Quercus ilex L.* Proanthocyanidin Dimers and Simple Phenolics: Evaluation of Their Synergistic Activity with Conventional Antimicrobials and Prediction of Their Pharmacokinetic Profile. *Journal of Agricultural and Food Chemistry*, 59(12), pp.6412–6422.

Kavita, K., Kumar, V. & Jha, B., 2014. 24-Branched 5 Sterols from *Laurencia papillosa* Red Seaweed with Antibacterial Activity against Human Pathogenic Bacteria. *Microbiological Research*, 169(4), pp.301–306.

Khan, M.S., Vishakante, G.D. & Siddaramaiah, H., 2013. Gold Nanoparticles : A Paradigm Shift in Biomedical Applications. *Advances in Colloid and Interface Science*, 199-200, pp.44–58.

Khan, S.A., Asiri, A.M., Al-Ghamdi, N.S.M., Zayed, M.E.M., Sharma, K. & Parveen, H., 2017. Optical Properties of Novel Environmentally Benign Biologically Active Ferrocenyl Substituted Chromophores: A Detailed Insight via Experimental and Theoretical Approach. *Journal of Molecular Structure*, 1139, pp.137–148.

Knudsen, S., 2011. *A Biologist's Guide To Analysis Of DNA Microarray Data* 2nd ed., New Jersey: John Wiley & Sons.

Koh, J.-J. et al., 2013. Rapid Bactericidal Action of Alpha-Mangostin against MRSA as an Outcome of Membrane Targeting. *Biochimica et Biophysica Acta*, 1828(2), pp.834–844.

Kohanski, M.A., Dwyer, D.J. & Collins, J.J., 2010. How Antibiotics Kill Bacteria:from Targets to Networks. *Nature Reviews Microbiology*, 8(6), pp.423–435.

Kong, K.-F., Schneper, L. & Mathee, K., 2011. Beta-Lactam Antibiotics: From Antibiosis to Resistance and Bacteriology. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, 118(1), pp.1–36.

Kotra, L.P., Haddad, J. & Mobashery, S., 2000. Aminoglycosides : Perspectives on Mechanisms of Action and Resistance and Strategies to Counter Resistance. *Antimicrobial Agents and Chemotherapy*, 44(12), pp.3249–3256.

Kowalski, K., Koceva-Chy, A., Szczupak, L., Hikisz, P., Bernasińska, J., Rajnisz, A., Solecka, J. & Therrien, B., 2013. Ferrocenylvinyl-Flavones: Synthesis, Structure, Anticancer and Antibacterial Activity Studies. *Journal of Organometallic Chemistry*, 741-742(1), pp.153–161.

Krebs, H.A., Salvin, E. & Johnson, W.A., 1938. The Formation of α-Ketoglutaric Acids in the Mammalian Body. *Biochemical Journal*, 32(1), pp.113–117.

Kreuzaler, F. & Hahlbrock, K., 1972. Enzymatic Synthesis of Aromatic Compounds in Higher Plants: Formation of Naringenin (5,7,4'-Trihydroxyflavanone) from P-Coumaroyl Coenzyme A and Malonyl Coenzyme A. *FEBS Letters*, 28(1), pp.69–72.

Kunthalert, D., Baothong, S., Khetkam, P. & Chokchaisiri, S., 2014. A Chalcone with

Potent Inhibiting Activity against Biofilm Formation by Nontypeable *Haemophilus influenzae*. *Microbiology and Immunology*, 58, pp.581–589.

Kutateladze, M. & Adamia, R., 2010. Bacteriophages as Potential New Therapeutics to Replace or Supplement Antibiotics. *Trends in Biotechnology*, 28(12), pp.591–595.

Lane, D., 2006. Designer Combination Therapy. *Nature Biotechnology*, 24(2), pp.163–164.

Lee, W., Chung, H.-S., Lee, Y., Yong, D., Jeong, S.H., Lee, K. & Chong, Y., 2013. Comparison of Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry Assay with Conventional Methods for Detection of IMP-6, VIM-2, NDM-1, SIM-1, KPC-1, OXA-23, and OXA-51 Carbapenemase-Producing *Acinetobacter Spp*., *Pseudomonas aeruginosa*. *Diagnostic Microbiology and Infectious Disease*, 77(3), pp.227–230.

Leinberger, D.M., Grimm, V., Rubtsova, M., Weile, J., Schro, K., Wichelhaus, T.A., Knabbe, C., Schmid, R.D. & Bachmann, T.T., 2010. Integrated Detection of Extended-Spectrum-Beta-Lactam Resistance by DNA Microarray-Based Genotyping of TEM , SHV , and. *Journal of Clinical Microbiology*, 48(2), pp.460–471.

Lengeler, J.W., Drews, G. & Schlegel, H.G., 1999. *Biology Of The Prokaryotes* 1st ed., Stuttgart: Georg Thieme Verlag.

Levison, M.E. & Levison, J.H., 2009. Pharmacokinetics and Pharmacodynamics of Antibacterial Agents. *Infectious Diseases Clinics of North America*, 23(4), pp.791–819.

Li, H., Zhang, J., Liu, Y., Zheng, R., Chen, H., Wang, X., Wang, Z., Cao, B. & Wang, H., 2014. Molecular Characteristics of Carbapenemase-Producing Enterobacteriaceae in China from 2008 to 2011: Predominance of KPC-2 Enzyme. *Diagnostic Microbiology and Infectious Disease*, 78(1), pp.63–65.

Li, J., Wang, G., Zhu, H., Zhang, M., Zheng, X., Di, Z., Liu, X. & Wang, X., 2014. Antibacterial Activity of Large-Area Monolayer Graphene Film Manipulated by

Charge Transfer. *Sci Rep*, 4(4359), pp.1–8.

Lindsay, J.A., 2014. Evolution of *Staphylococcus Aureus* and MRSA during Outbreaks. *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, 21, pp.548–453.

Ling, L.L. *et al*., 2015. A New Antibiotic Kills Pathogens without Detectable Resistance. *Nature*, 517(7535), pp.455–459.

Lipsky, B.A., Byren, I. & Hoey, C.T., 2010. Treatment of Bacterial Prostatitis. *Clinical Infectious Diseases*, 50(12), pp.1641–1652.

Liu, X.L., Xu, Y.J. & Go, M.L., 2008. Functionalized Chalcones with Basic Functionalities Have Antibacterial Activity against Drug Sensitive *Staphylococcus aureus*. *European Journal of Medicinal Chemistry*, 43, pp.1681–1687.

Liu, Y.Y. *et al*., 2016. Emergence of Plasmid-Mediated Colistin Resistance Mechanism MCR-1 in Animals and Human Beings in China: A Microbiological and Molecular Biological Study. *The Lancet Infectious Diseases*, 16, pp.161–168.

Lü, L., Zhang, L., Sen, M., Wai, M., Tai, D., Yew, W. & Xu, J., 2012. Toxicology in Vitro Exocytosis of MTT Formazan Could Exacerbate Cell Injury. *Toxicology in Vitro*, 26(4), pp.636–644.

Lu, T.K. & Koeris, M.S., 2011. The Next Generation of Bacteriophage Therapy. *Current Opinion in Microbiology*, 14(5), pp.524–531.

Luo, Y., Pan, K. & Zhong, Q., 2014. Physical, Chemical and Biochemical Properties of Casein Hydrolyzed by Three Proteases: Partial Characterizations. *Food Chemistry*, 155, pp.146–154.

MacGowan, A.P. & Wise, R., 2005. *Establishing MIC Breakpoints And The Interpretation Of In Vitro Susceptibility Tests*, Birmingham.

Madigan, M.T., Martinko, J.M., Stahl, D.A. & Clark, David, P., 2012. *Brock Biology Of Microorganisms* 13th ed., San Francisco: Pearson Education Inc.

Magaldi, S., Mata-Essayag, S., Hartung de Capriles, C., Perez, C., Colella, M.., Olaizola, C. & Ontiveros, Y., 2004. Well Diffusion for Antifungal Susceptibility Testing.

International Journal of Infectious Diseases, 8(1), pp.39–45.

Mahamoud, A., Chevalier, J., Alibert-franco, S., Kern, W. V, Page, J. & Moulin, B.J., 2007. Antibiotic Efflux Pumps in Gram-Negative Bacteria: The Inhibitor Response Strategy. *Journal of Antimicrobial Chemotherapy*, 59, pp.1223–1229.

Malick, L. & Wilson, B., 1975. Modified Thiocarbohydrazide Procedure for Scanning Electron Microscopy: Routine Use for Normal, Pathological, or Experimental Tissues. *Stain Technology*, 50(4), pp.265–269.

Margulis, L., 1970. *Origin Of Eukaryotic Cells: Evidence And Research Implications For A Theory Of The Origin And Evolution Of Microbial, Plant, And Animal Cells On The Precambrian Earth* 1st ed., New Haven: Yale University Press.

Marston, A., 2011. Thin-Layer Chromatography with Biological Detection in Phytochemistry. *Journal of Chromatography A*, 1218(19), pp.2676–2683.

Martin, W., Hoffmeister, M., Rotte, C. & Henze, K., 2001. An Overview of Endosymbiotic Models for the Origins of Eukaryotes, Their ATP-Producing Organelles (Mitochondria and Hydrogenosomes), and Their Heterotrophic Lifestyle. *Biological Chemistry*, 382(11), pp.1521–1539.

Martin, W. & Kowallik, K., 1999. Annotated English Translation of Mereschkowsky's 1905 Paper "Über Natur Und Ursprung Der Chromatophoren im Pflanzenreiche." *European Journal of Phycology*, 34(3), pp.287–295.

Máthé, A., Szabó, D., Anderlik, P., Rozgonyi, F. & Nagy, K., 2007. The Effect of Amikacin and Imipenem Alone and in Combination against an Extended-Spectrum Beta-Lactamase-Producing *Klebsiella pneumoniae* Strain. *Diagnostic Microbiology and Infectious Disease*, 58(1), pp.105–10.

Matthews, W.S. *et al*., 1975. Equilibrium Acidities of Carbon Acids. VI. Establishment of an Absolute Scale of Acidities in Dimethyl Sulfoxide Solution. *Journal of the American Chemical Society*, 97, pp.7006–7014.

Matuschek, E., Brown, D.F.J. & Kahlmeter, G., 2013. Development of the EUCAST Disk Diffusion Antimicrobial Susceptibility Testing Method and Its Implementation in

Routine Microbiology Laboratories. *Clinical Microbial Infection*, 20, pp.255–266.

May, W.E., Wasik, S.P. & Freeman, D.H., 1978. Determination of the Solubility Behavior of Some Polycyclic Aromatic Hydrocarbons in Water. *Analytical Chemistry*, 50(7), pp.997–1000.

McMullan, D., 1953. AN IMPROVED SCANNING ELECTRON MICROSCOPE FOR OPAQUE SPECIMENS. *Proceedings of the IEE – Part II: Power Engineering*, 100(75), pp.124–126.

Melo, A.M.P. & Teixeira, M., 2015. Supramolecular Organization of Bacterial Aerobic Respiratory Chains: From Cells and Back. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, pp.1–8.

Meng, J., Hu, B., Liu, J.I.E., Hou, Z., Meng, J.I.A., Jia, M.I.N. & Luo, X., 2006. Restoration of Oxacillin Susceptibility in Methicillin- Resistant *Staphylococcus aureus* by Blocking the MecR1-Mediated Signaling Pathway. *Journal of Chemotherapy*, 18(4), pp.360–365.

Messick, C.R., Rodvold, K.A. & Pendland, S.L., 1999. Modified Time–kill Assay against Multidrug-Resistant *Enterococcus faecium* With Novel Antimicrobial Combinations. *Journal of Antimicrobial Chemotherapy*, 44, pp.831–834.

Miles, A.A., Misra, S.S. & Irwin, J.O., 1938. The Estimation of the Bactericidal Power of the Blood. *The Journal of Hygiene*, 38(6), pp.732–749.

Mishra, A., Taneja, N. & Sharma, M., 2012. Viability Kinetics , Induction , Resuscitation and Quantitative Real-Time Polymerase Chain Reaction Analyses of Viable but Nonculturable *Vibrio cholerae* O1 in Freshwater Microcosm. *Journal of Applied Microbiology*, 112, pp.945–953.

Mishra, S., Tirkey, V., Ghosh, A., Dash, H.R., Das, S., Shukla, M., Saha, S., Mobin, S.M. & Chatterjee, S., 2015. Ferrocenyl–cymantrenyl Hetero-Bimetallic Chalcones: Synthesis, Structure and Biological Properties. *Journal of Molecular Structure*, 1085, pp.162–172.

Mishra, S.K. & Agrawal, D., 2012. *Concise Manual Of Pathogenic Microbiology* 1st ed.,

John Wiley & Sons.

Moodley, S., Koorbanally, N.A., Moodley, T., Ramjugernath, D. & Pillay, M., 2014. The 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay Is a Rapid, Cheap, Screening Test for the in Vitro Anti-Tuberculous Activity of Chalcones. *Journal of Microbiological Methods*, 104, pp.72–78.

Moosdeen, F., Williams, J.D. & Seeker, A., 1988. Standardization of Inoculum Size for Disc Susceptibility Testing : A Preliminary Report of a Spectrophotometric Method. *Journal of Antimicrobial Chemotherapy*, 21, pp.439–443.

Moreira Osório, T. *et al*., 2012. Antibacterial Activity of Chalcones, Hydrazones and Oxadiazoles against Methicillin-Resistant *Staphylococcus aureus*. *Bioorganic & Medicinal Chemistry Letters*, 22(1), pp.225–230.

Mosmann, T., 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assay. *Journal of Immunological Methods*, 65(1-2), pp.55–63.

Mshana, R.N., Tadesse, G. & Abate, G., 1998. Tetrazolium Bromide for Rapid Detection of Rifampin-Resistant *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, 36(5), pp.1214–1219.

Mullis, K.B., Ferre, F. & Gibbs, R.A., 2012. *The Polymerase Chain Reaction* 1st ed., New York: Springer Science & Business Media.

Naas, T., Cuzon, G., Bogaerts, P., Glupczynski, Y., Nordmann, P. & Bacte, S. De, 2011. Evaluation of a DNA Microarray (Check-MDR CT102) for Rapid Detection of TEM, SHV, and CTX-M Extended-Spectrum. *Journal of Microbiological Methods*, 49(4), pp.1608–1613.

Nation, J.L., 1983. A New Method Using Hexamethyldisilazane for Preparation of Soft Insect Tissues for Scanning Electron Microscopy. *Stain Technology*, 58(6), pp.347–351.

Nau, R., Sorgel, F. & Eiffert, H., 2010. Penetration of Drugs through the Blood-Cerebrospinal Fluid / Blood-Brain Barrier for Treatment of Central Nervous

System Infections. *Clinical Microbiology Reviews*, 23(4), pp.858–883.

Navarre, W.W. & Schneewind, O., 1999. Surface Proteins of Gram-Positive Bacteria and Mechanisms of Their Targeting to the Cell Wall Envelope. *Microbiology and Molecular Biology Reviews : MMBR*, 63(1), pp.174–229.

Nguyen, F., Starosta, A.L., Arenz, S., Sohmen, D., Dönhöfer, A. & Wilson, D.N., 2014. Tetracycline Antibiotics and Resistance Mechanisms. *Biological Chemistry*, 395(5), pp.559–575.

Ni, L., Meng, C.Q. & Sikorski, J.A., 2004. Recent Advances in Therapeutic Chalcones. *Expert Opinion on Therapeutic Patents*, 14(February), pp.1669–1691.

Nielsen, S.F., Larsen, M., Boesen, T., Schønning, K. & Kromann, H., 2005. Cationic Chalcone Antibiotics. Design , Synthesis , and Mechanism of Action. *Journal of Medicinal Chemistry*, 48, pp.2667–2677.

Nikaido, H., 2003. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews: MMBR*, 67(4), pp.593–656.

Nitin, K., 2009. *Longman Science* 1st ed., New Delhi: Dorling Kindersley.

Normark, S., Edlund, T., Grundstrom, T., Bergstrom, S. & Wolfwatz, H., 1977. *Escherichia coli* K12 Mutants Hyper Producing Chromosomal Beta Lactamase by Gene Repetitions. *Journal of Bacteriology*, 132(3), pp.912–922.

Nunes, C., Graça, A. & Santo, D., 2017. Growth of *Escherichia coli*, *Salmonella enterica* and *Listeria Spp.*, and Their Inactivation Using Ultraviolet Energy and Electrolyzed Water, on “Rocha” Fresh-Cut Pears. *Food Control*, 77, pp.41–49.

Nyberg, L.K. *et al*., 2016. Rapid Identification of Intact Bacterial Resistance Plasmids via Optical Mapping of Single DNA Molecules. *Nature*, 6(30410), pp.1–10.

O’Flaherty, S., Ross, R.P., Meaney, W., Fitzgerald, G.F., Elbreki, M.F. & Coffey, A., 2005. Potential of the Polyvalent Anti-*Staphylococcus* Bacteriophage K for Control of Antibiotic-Resistant *Staphylococci* from Hospitals. *Applied and Environmental Microbiology*, 71(4), pp.1836–1842.

Oelschlaeger, P., Ai, N., Duprez, K.T., Welsh, W.J. & Toney, J.H., 2010. Evolving Carbapenemases: Can Medicinal Chemists Advance One Step ahead of the Coming Storm? *Journal of Medicinal Chemistry*, 53(8), pp.3013–3027.

Oleksiewicz, M.B., Nagy, G. & Nagy, E., 2012. Anti-Bacterial Monoclonal Antibodies: Back to the Future? *Archives of Biochemistry and Biophysics*, 526(2), pp.124–131.

Ovchinnikov, K. V, Kristiansen, P.E., Straume, D., Jensen, M.S., Aleksandrzak-piekarczyk, T., Nes, I.F., Diep, D.B. & Vederas, J.C., 2017. The Leaderless Bacteriocin Enterocin K1 Is Highly Potent against *Enterococcus faecium*: A Study on Structure , Target Spectrum and Receptor. *Frontiers in Microbiology*, 8(May), pp.1–12.

Ovchinnikov, K. V, Kristiansen, P.E., Uzelac, G., Topisirovic, L., Kojic, M. & Nissen-Meyer, J., 2014. Defining the Structure and Receptor Binding Domain of the Leaderless Bacteriocin LsbB. *The Journal of Biological Chemistry*, 289(34), pp.23838–23845.

Oxoid, 2017. CM0405, Mueller-Hinton Broth _ Oxoid - Product Detail. *Oxoid Microbiology Products*. Available at: http://www.oxoid.com/UK/blue/prod_detail/prod_detail.asp?pr=CM0405 [Accessed May 20, 2017].

Pagès, J.-M., James, C.E. & Winterhalter, M., 2008. The Porin and the Permeating Antibiotic: A Selective Diffusion Barrier in Gram-Negative Bacteria. *Nature Reviews Microbiology*, 6(12), pp.893–903.

Pankey, G.A. & Sabath, L.D., 2004. Clinical Relevance of Bacteriostatic versus Bactericidal Mechanisms of Action in the Treatment of Gram-Positive Bacterial Infections. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 38(6), pp.864–870.

Parija, S.C., 2012. *Textbook Of Microbiology & Immunology* 2nd ed., New Delhi: Elsevier Health Sciences.

Pavia, D.L., 2005. *Introduction To Organic Laboratory Techniques: A Small Scale*

Approach 1st ed., Boston: Cengage Learning.

Pedraza-Chaverri, J., Cárdenas-Rodríguez, N., Orozco-Ibarra, M. & Pérez-Rojas, J.M., 2008. Medicinal Properties of Mangosteen (*Garcinia mangostana*). *Food and Chemical Toxicology*, 46(10), pp.3227–3239.

Pegg, D.E., 1989. Assays for Preserved Cells, Tissues, and Organs. *Cryobiology*, 231, pp.212–231.

Pejović, A., Damljanović, I., Stevanović, D., Vukićević, M., Novaković, S.B., Bogdanović, G. a., Radulović, N. & Vukićević, R.D., 2012. Antimicrobial Ferrocene Containing Quinolinones: Synthesis, Spectral, Electrochemical and Structural Characterization of 2-Ferrocenyl-2,3-Dihydroquinolin-4(1H)-One and Its 6-Chloro and 6-Bromo Derivatives. *Polyhedron*, 31(1), pp.789–795.

Peter, H., Berggrav, K., Thomas, P., Pfeifer, Y., Witte, W., Templeton, K. & Bachmann, T.T., 2012. Direct Detection and Genotyping of *Klebsiella pneumoniae* Carbapenemases from Urine by Use of a New DNA Microarray Test. *Journal of Clinical Microbiology*, 50(12), pp.3990–3998.

Phoenix, D.A., Harris, F. & Dennison, S.R., 2015. History of the Origins, Development, and Use of Conventional Antibiotics. In *Novel Antimicrobial Agents and Strategies*. Weinheim: Wiley-VCH, p. 1.

Piddock, L.J. V, 2006a. Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria. *Clinical Microbiology Reviews*, 19(2), pp.382–402.

Piddock, L.J. V, 2006b. Multidrug-Resistance Efflux Pumps — Not Just for Resistance. *Nature Reviews Microbiology*, 4, pp.629–636.

Portnoy, V.A., Scott, D.A., Lewis, N.E., Tarasova, Y., Osterman, A.L. & Palsson, B.O., 2010. Deletion of Genes Encoding Cytochrome Oxidases and Quinol Monooxygenase Blocks the Aerobic Anaerobic Shift in *Escherichia coli* K-12 MG1655. *Applied and Environmental Microbiology*, 76(19), pp.6529–6540.

Pournajaf, A., Ardebili, A., Goudarzi, L., Khodabandeh, M., Narimani, T. &

Abbaszadeh, H., 2014. PCR-Based Identification of Methicillin-Resistant *Staphylococcus aureus* Strains and Their Antibiotic Resistance Profiles. *Asian Pacific Journal of Tropical Biomedicine*, 4(1067), pp.S293–S297.

Prasath, R., Bhavana, P., Ng, S.W. & Tiekink, E.R.T., 2013. The Facile and Efficient Ultrasound-Assisted Synthesis of New Quinoline-Appended Ferrocenyl Chalcones and Their Properties. *Journal of Organometallic Chemistry*, 726, pp.62–70.

Pulido, M.R., García-Quintanilla, M., Martín-Peña, R., Cisneros, J.M. & McConnell, M.J., 2013. Progress on the Development of Rapid Methods for Antimicrobial Susceptibility Testing. *Journal of Antimicrobial Chemotherapy*, 68, pp.2710–2717.

Quan, D., Nagalingam, G., Payne, R. & Triccas, J.A., 2017. New Tuberculosis Drug Leads from Naturally Occurring Compounds. *International Journal of Infectious Diseases*, 56, pp.212–220.

Quattlebaum, E.C. & Carner, G.R., 1980. A Technique for Preparing *Beauveria Spp*. for Scanning Electron Microscopy. *Can. J. Bot.*, 58(1975), pp.1700–1703.

Rao, M.R., Kumar, K.V.P. & Ravikanth, M., 2010. Synthesis of Boron-Dipyrromethene – Ferrocene Conjugates. *Journal of Organometallic Chemistry*, 695(6), pp.863–869.

Rasmussen, L.C.V., Sperling-Petersen, H.U. & Mortensen, K.K., 2007. Hitting Bacteria at the Heart of the Central Dogma: Sequence-Specific Inhibition. *Microbial Cell Factories*, 26(24), pp.1–26.

Remington, J.P., 2006. *Remington: The Science And Practice Of Pharmacy* 21st ed. D. B. Troy & P. Beringer, eds., Baltimore: Lippincott Williams and Wilkins.

Richardson, D.J., 2000. Bacterial Respiration: A Flexible Process for a Changing Environment. In *Microbiology*. pp. 551–571.

Riley, M.A. & Wertz, J.E., 2002. Bacteriocins: Evolution, Ecology, and Application. *Annual Review of Microbiology*, 56, pp.117–137.

Rogers, H.J., Perkins, H.R. & Ward, J.B., 1980. *Microbial Cell Walls And Membranes* 1st ed., Michigan: Chapman and Hall.

Rolston, K.V., Wang, W., Nesher, L., Smith, J.R., Rybak, M.J. & Prince, R.A., 2017. Time-Kill Determination of the Bactericidal Activity of Telavancin and Vancomycin against Clinical Methicillin-Resistant *Staphylococcus aureus* Isolates from Cancer Patients. *Diagnostic Microbiology and Infectious Disease*, 87(4), pp.338–342.

Rothwell, J.A., Day, A.J. & Morgan, M.R.A., 2005. Experimental Determination of Octanol − Water Partition Coefficients of Quercetin and Related Flavonoids. *Journal of Agricultural and Food Chemistry*, 53(11), pp.4355–4360.

Rozmer, Z. & Perjési, P., 2016. Naturally Occurring Chalcones and Their Biological Activities. *Phytochemistry Reviews*, 15(1), pp.87–120.

Saleh-lakha, S. & Trevors, J.T., 2010. Perspective: Microfluidic Applications in Microbiology. *Journal of Microbiological Methods*, 82, pp.108–111.

Salton, M.R.J., 1964. *The Bacterial Cell Wall* 1st ed., New York: Elsevier Publishing Company.

Sauer, S. & Kliem, M., 2010. Mass Spectrometry Tools for the Classification and Identification of Bacteria. *Nature Reviews Microbiology*, 8, pp.74–82.

Sazanov, L.A., 2015. A Giant Molecular Proton Pump: Structure and Mechanism of Respiratory Complex I. *Nature Reviews Molecular Cell Biology*, 16(6), pp.375–388.

Schaechter, M., Engleberg, N.C., DiRita, V.J. & Dermody, T.S., 2012. *Schaechter's Mechanisms Of Microbial Disease* 5th ed., Philadelphia: Lippincott Williams and Wilkins.

Scheerans, C., Wicha, S.G., Michael, J., Derendorf, H. & Kloft, C., 2015. Concentration-Response Studies and Modelling of the Pharmacodynamics of Linezolid: *Staphylococcus aureus* versus *Enterococcus faecium*. *International Journal of Antimicrobial Agents*, 45(1), pp.54–60.

Schleifer, K.H. & Kandler, O., 1972. Peptidoglycan. Types of Bacterial Cell Walls and Their Taxonomic Implications. *Bacteriological Reviews*, 36(4), pp.407–477.

Seltmann, G. & Holts, O., 2002. *The Bacterial Cell Wall* 1st ed., New York: Springer-Verlag.

Sharma, V., Singh, G., Kaur, H., Saxena, A.K. & Ishar, M.P.S., 2012. Synthesis of β-Ionone Derived Chalcones as Potent Antimicrobial Agents. *Bioorganic & Medicinal Chemistry Letters*, 22(20), pp.6343–6346.

Shockman, G.D. & Barrett, J.F., 1983. Structure, Function, and Assembly of Cell Walls of Gram-Positive Bacteria. *Annual Review of Microbiology*, 37, pp.501–527.

Silhavy, T.J., Kahne, D. & Walker, S., 2010. The Bacterial Cell Envelope. *Cold Spring Harbour Perspectives in Biology*, 2(a000414), pp.1–17.

Sivakumar, P.M., Priya, S. & Doble, M., 2009. Synthesis, Biological Evaluation, Mechanism of Action and Quantitative Structure – Activity Relationship Studies of Chalcones as Antibacterial Agents. *Chemical Biology & Drug Design*, 73, pp.403–415.

Slack, M.P.E. & Wheldon, D.B., 1978. A Simple and Safe Volumetric Alternative to the Method of Miles, Misra and Irwin for Counting Viable Bacteria. *Journal of Medical Microbiology*, 11(1978), pp.541–545.

Slater, T.F., Sawyer, B. & Strauli, U., 1963. The Determination of the Points of Coupling of Tetrazolium Salts with the Respiratory Chain Has Provoked Several Investigations. *Biochimica et Biophysica Acta*, 77, pp.383–393.

Smith, Theresa L; Pearson, Michele L; Wilcox, Kenneth R; Cruz, Cosme; Lancaster, Michael V; Robinson-Dunn, Barbara; Tenover, Fred C; Zervos, Marcus J; Band, Jeffrey D; White, Elizabeth; Jarvis, W.R., 1999. Emergence of Vancomycin Resistance in *Staphylococcus aureus*. *The New England Journal of Medicine*, 340(7), pp.493–501.

Smith, C.A., Antunes, N.T., Stewart, N.K., Toth, M., Kumarasiri, M., Chang, M., Mobashery, S. & Vakulenko, S.B., 2013. Structural Basis for Carbapenemase

Activity of the OXA-23 β-Lactamase from *Acinetobacter baumannii*. *Chemistry & Biology*, 20(9), pp.1107–1115.

Smith, K.C.A., Oatley, C.W., Zworykin V K, H.J. and S.R.L., D, M., Holdgate H W, M.J.W. and S.M., C, F., A, R., A, H.M.E. and E.P. & S, H., 1955. The Scanning Electron Microscope and Its Fields of Application. *British Journal of Applied Physics*, 6(11), pp.391–399.

Smith, M.B., Smith, M.B. & Nikonov, G., 2013. Iodine. In *e-EROS Encyclopedia of Reagents for Organic Synthesis*. New Jersey: John Wiley & Sons.

Sogawa, K., Watanabe, M., Sato, K. & Segawa, S., 2011. Use of the MALDI BioTyper System with MALDI – TOF Mass Spectrometry for Rapid Identification of Microorganisms. *Analytical and Bioanalytical Chemistry*, 400, pp.1905–1911.

Song, S., Berg, O.G., Roth, J.R. & Andersson, D.I., 2009. Contribution of Gene Amplification to Evolution of Increased Antibiotic Resistance in *Salmonella typhimurium*. *Genetics*, 182(4), pp.1183–1195.

Stepanović, S., Daki, I. & Milena, Š., 2003. In Vitro Antimicrobial Activity of Propolis and Synergism between Propolis and Antimicrobial Drugs. *Microbiological Research*, 158(4), pp.353–357.

Stoker, H.S., 2015. *General, Organic And Biological Chemistry* 7th ed., Boston: Cengage Learning.

Sumbali, G. & Mehrotra, R.S., 2009. *Principles Of Microbiology* 1st ed., New Dehli: Tata McGraw Hill.

Sutherland, R., 1964. The Nature of the Insensitivity of Gram-Negative Bacteria towards Penicillins. *Journal of General Microbiology*, 34, pp.85–98.

Taleb, K., Mohamed-benkada, M., Benhamed, N. & Saidi-besbes, S., 2017. Benzene Ring Containing Cationic Gemini Surfactants: Synthesis, Surface Properties and Antibacterial Activity. *Journal of Molecular Liquids*, 241, pp.81–90.

Tenover, F.C., 2006. Mechanisms of Antimicrobial Resistance in Bacteria. *American Journal of Infection Control*, 34, pp.S3–S10.

Tenson, T. & Lovmar, M., 2003. The Mechanism of Action of Macrolides , Lincosamides and Streptogramin B Reveals the Nascent Peptide Exit Path in the Ribosome. *Journal of Molecular Biology*, 2836(03), pp.1005–1014.

Thomas, C.M., 2003. *Horizontal Gene Pool: Bacterial Plasmids And Gene Spread* 2nd ed., Florida: CRC Press.

Thompson, B.A., 1950. Dried Disc Technique for Determining Sensitivity to the Antibiotics. *Journal of Clinical Pathology*, 3(2), pp.118–121.

Top, S., Tang, J., Vessieres, A., Carrez, D., Provote, C. & Jaouen, G., 1996. Ferrocenyl Hydroxytamoxifen: A Prototype for a New Range of Oestradiol Receptor Site-Directed Cytotoxics. *Chemical Communications*, pp.955–956.

Top, S., Vessières, A., Cabestaing, C., Laios, I., Leclercq, G., Provot, C. & Jaouen, G., 2001. Studies on Organometallic Selective Estrogen Receptor Modulators. (SERMs) Dual Activity in the Hydroxy-Ferrocifen Series. *Journal of Organometallic Chemistry*, 637-639, pp.500–506.

Top, S., Vessières, A., Leclercq, G., Quivy, J., Tang, J., Vaissermann, J., Huché, M. & Jaouen, G., 2003. Synthesis, Biochemical Properties and Molecular Modelling Studies of Organometallic Specific Estrogen Receptor Modulators (SERMs), the Ferrocifens and Hydroxyferrocifens: Evidence for an Antiproliferative Effect of Hydroxyferrocifens on Both Hormone-Depen. *Chemistry - A European Journal*, 9(21), pp.5223–5236.

Tseng, S.-H., Lee, C.-M., Lin, T.-Y., Chang, S.-C. & Chang, F.-Y., 2011. Emergence and Spread of Multi-Drug Resistant Organisms: Think Globally and Act Locally. *Journal of Microbiology, Immunology, and Infection*, 44(3), pp.157–165.

Valgas, C., Souza, S., Smania, E. & Jr, S., 2007. Screening Methods to Determine Antibacterial Activity of Natural Products. *Brazilian Journal of Micrboiology Universidade do Sul de Santa Catarina*, 38, pp.369–380.

del Valle, P., García-Armesto, M.R., de Arriaga, D., González-Donquiles, C., Rodríquez-Fernández, P. & Rúa, J., 2016. Antimicrobial Activity of Kaempferol and Resveratrol in Binary Combinations with Parabens or Propyl Gallate against

Enterococcus faecalis. *Food Control*, 61, pp.213–220.

Vandal, J., Abou-Zaid, M.M., Ferroni, G., Leduc, L.G., Vandal, J., Abou-zaid, M.M., Ferroni, G. & Leduc, L.G., 2015. Antimicrobial Activity of Natural Products from the Flora of Northern Ontario, Canada. *Pharmaceutical Biology*, 53(6), pp.800–806.

Varanda, F., Pratas de Melo, M.J., Cacüo, A.I., Dohrn, R., Makrydaki, F.A., Voutsas, E., Tassios, D. & Marrucho, I.M., 2006. Solubility of Antibiotics in Different Solvents. 1. Hydrochloride Forms of Tetracycline, Moxifloxacin, and Ciprofloxacin. *Industrial & Engineering Chemistry Research*, 45, pp.6368–6374.

Varma, A. & Sharma, A.K., 2017. *Modern Tools And Techniques To Understand Microbes* 1st ed., Cham: Springer International Publishing.

Vaux, S., Carbonne, A., Thiolet, J.M., Jarlier, V. & Coignard, B., 2011. Emergence of Carbapenemase-Producing Enterobacteriaceae in France, 2004 to 2011. *Euro Surveillance*, 16(22), pp.1–7.

Vineetha, N., Vignesh, R.A. & Sridhar, D., 2015. Preparation, Standardization of Antibiotic Discs and Study of Resistance Pattern for First-Line Antibiotics in Isolates from Clinical Samples. *International Journal of Applied Research*, 1(11), pp.624–631.

Virgincar, N., Iyer, S., Stacey, A., Maharjan, S., Pike, R., Perry, C., Wyeth, J. & Woodford, N., 2011. *Klebsiella pneumoniae* Producing KPC Carbapenemase in a District General Hospital in the UK. *The Journal of Hospital Infection*, 78(4), pp.293–296.

Vogel, F., Neupert, W., Reichert, A.S. & Bornhovd, C., 2006. Dynamic of the Subcompartmentalizat Membrane Mitochondrial Inner. *The Journal of Cell Biology*, 175(2), pp.237–247.

Vollmer, W., Blanot, D. & Pedro, M.A. De, 2008. Peptidoglycan Structure and Architecture. *FEMS Microbiological Reviews*, 32, pp.149–167.

Vyvyan, J.R., Pavia, D.L., Lampman, G.M. & Kriz, G.S., 2002. Preparing Students for

Research: Synthesis of Substituted Chalcones as a Comprehensive Guided-Inquiry Experience. *Journal of Chemical Education*, 79(9), pp.1119–1121.

Wallin, I., 1927. *Symbionticism And The Origin Of Species* 1st ed., London: Williams and Wilkins Company.

Watkins, R.R. & Bonomo, R.A., 2017. *Beta-lactam Antibiotics* 4th ed., Amsterdam: Elsevier.

Wheat, P.F., 2001. History and Development of Antimicrobial Susceptibility Testing Methodology. *Journal of Antimicrobial Chemotherapy*, 48 Suppl 1, pp.1–4.

WHO, 2015. *Worldwide Country Situation Analysis: Response To Antimicrobial Resistance*, Geneva.

Wiegand, I., Hilpert, K. & Hancock, R.E.W., 2008. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nature Protocol*, 3(2), pp.163–175.

Wielders, C.L.C., Fluit, A.C., Brisse, S., Verhoef, J. & Schmitz, F.J., 2002. *mecA* Gene Is Widely Disseminated in *Staphylococcus aureus* Population. *Journal of Clinical Microbiology*, 40(11), pp.3970–3975.

Willey, J.M., Sherwood, L.M. & Woolverton, C.J., 2008. *Prescott, Harley And Klein's Microbiology* 7th ed., New York: McGraw Hill.

Williams, A.C. & Edwards, H.G.M., 1994. Fourier Transform Raman Spectroscopy of Bacterial Cell Walls. *Journal of Raman Spectroscopy*, 25, pp.673–677.

Wilson, D.N., 2014. Ribosome-Targeting Antibiotics and Mechanisms of Bacterial Resistance. *Nature Publishing Group*, 12(1), pp.35–48.

Wise, E.M. & Park, J.T., 1965. Penicillin: It's Basic Site of Action as an Inhibitor of a Peptide Cross-Linking Reaction in Cell Wall Mucopeptide Synthesis. In *Proceedings of the National Academy of Sciences*. pp. 75–81.

Wolfson, J.S. & Hooper, D.C., 1989. Fluoroquinolone Antimicrobial Agents. *Clinical Microbiology Reviews*, 2(4), pp.378–424.

Wootton, M., Howe, R., MacGowan, A., Livermore, D., Brown, N., Winstanley, G., Mackenzie, F. & Burns, P., 2014. *BSAC Methods For Antimicrobial Susceptibility Testing*, Cardiff.

Worthington, R.J. & Melander, C., 2013. Combination Approaches to Combat Multidrug-Resistant Bacteria. *Trends in Biotechnology*, 31(3), pp.177–184.

Yadava, P., Zhang, C., Sun, J. & Hughes, J. a, 2006. Antimicrobial Activities of Human Beta-Defensins against *Bacillus* Species. *International Journal of Antimicrobial Agents*, 28(2), pp.132–137.

Yamaguchi, Y., Nagase, T., Makita, R., Fukuhara, S., Tomita, T., Tominaga, T., Kurihara, H. & Ouchi, Y., 2002. Identification of Multiple Novel Epididymis-Specific Beta-Defensin Isoforms in Humans and Mice. *Journal of Immunology*, 169, pp.2516–2523.

Yao, X., Jericho, M. & Pink, D., 1999. Thickness and Elasticity of Gram-Negative Murein Sacculi Measured by Atomic Force Microscopy. *Journal of Bacteriology*, 181(22), pp.6865–6875.

Yong, A., Ooh, K., Ong, H., Chai, T. & Wong, F., 2015. Investigation of Antibacterial Mechanism and Identification of Bacterial Protein Targets Mediated by Antibacterial Medicinal Plant Extracts. *Food Chemistry*, 186, pp.32–36.

Yousefinjad, S., Honarasa, F. & Solhjoo, A., 2015. On the Solubility of Ferrocene in Nonaqueous Solvents. *Journal of Chemical & Engineering Data*, 10, pp.1–8.

Zengin, H. & Baysal, A.H., 2014. Antibacterial and Antioxidant Activity of Essential Oil Terpenes against Pathogenic and Spoilage-Forming Bacteria and Cell Structure-Activity Relationships Evaluated by SEM Microscopy. *Molecules*, 19(11), pp.17773–17798.

Zhang, Y., Liu, X., Wang, Y., Jiang, P. & Quek, S., 2016. Antibacterial Activity and Mechanism of Cinnamon Essential Oil against *Escherichia coli* and Staphylococcus Aureus. *Food Control*, 59, pp.282–289.

Zhao, L., Zhang, H., Hao, T. & Li, S., 2015. In Vitro Antibacterial Activities and

Mechanism of Sugar Fatty Acid Esters against Five Food-Related Bacteria. *Food Chemistry*, 187, pp.370–377.

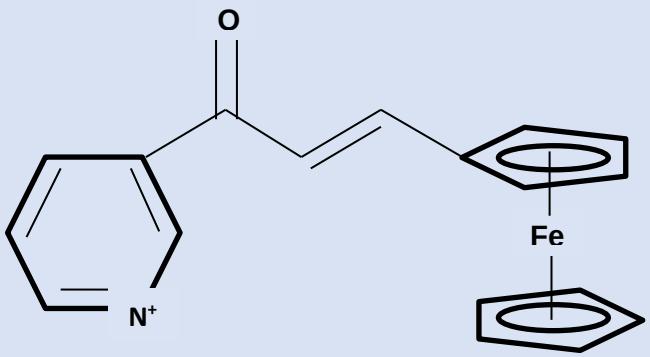
Zinner, S.H., 1999. Changing Epidemiology of Infections from Gram-Positive Bacteria. *Clinical Infectious Diseases*, 29, pp.490–494.

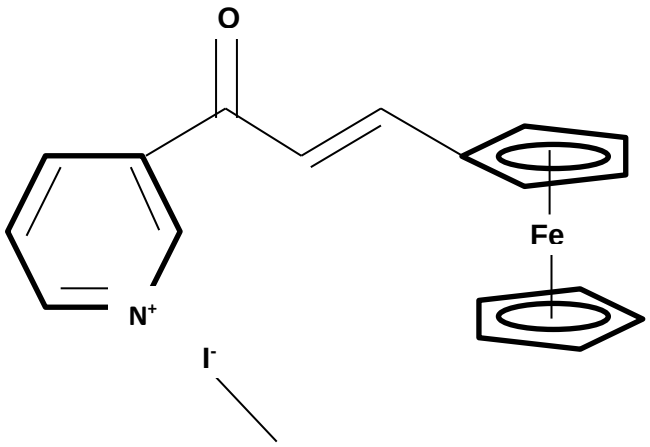
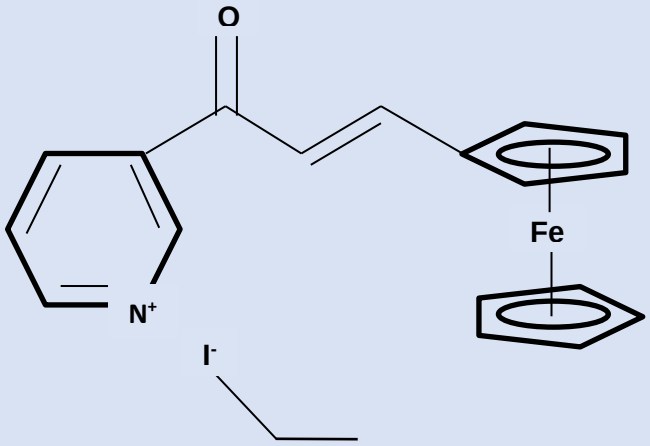
Zuo, G.Y., Han, Z.Q., Hao, X.Y., Han, J., Li, Z.S. & Wang, G.C., 2014. Synergy of Aminoglycoside Antibiotics by 3-Benzylchroman Derivatives from the Chinese Drug Caesalpinia Sappan against Clinical Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology*, 21(7), pp.936–941.

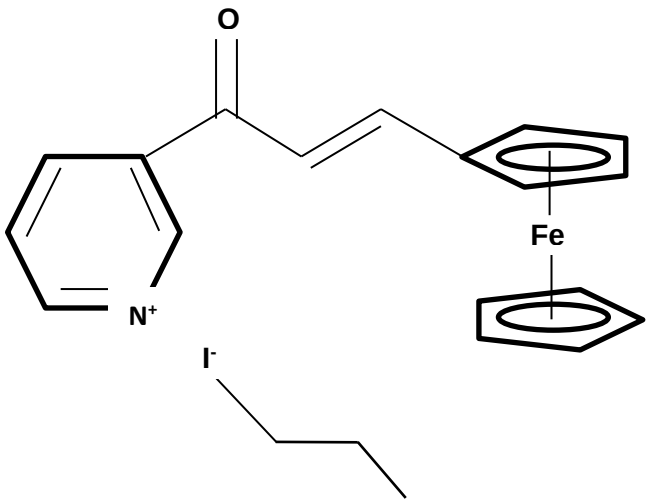
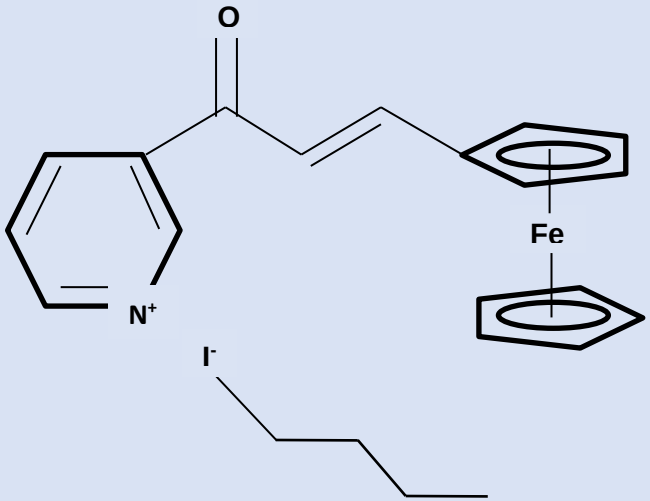
9 Appendices

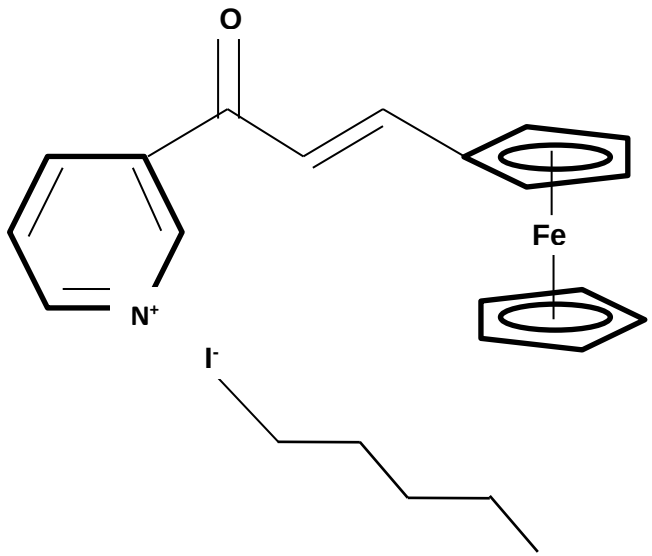
9.1 Appendix 1 – Structures and molecular weights of ferrocenyl chalcone compounds

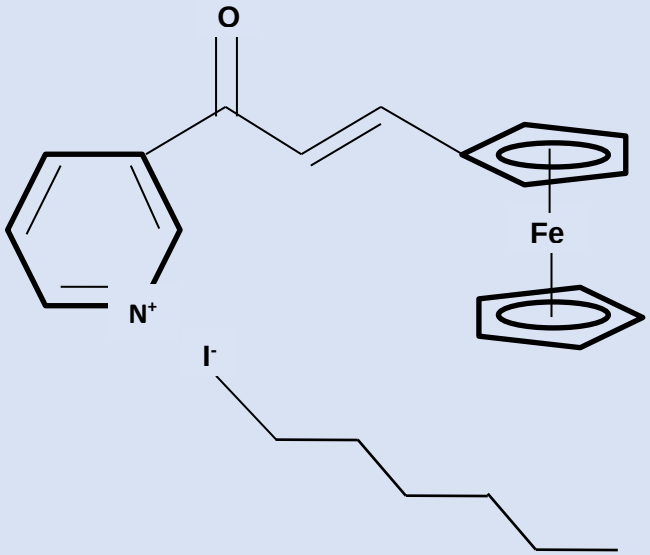
Table 9-1 *Structures and Molecular Weights (g/mol) of 11 ferrocenyl chalcone compounds.*

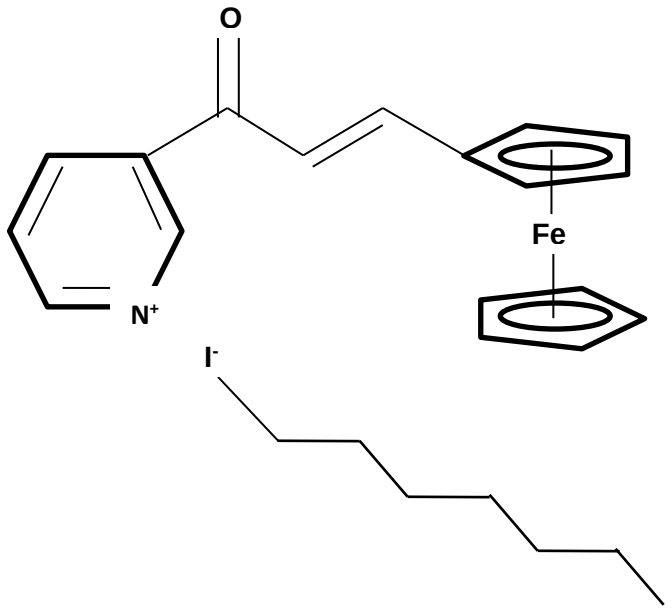
Name of ferrocenyl chalcone	Structure of ferrocenyl chalcone	Molecular weight of ferrocenyl chalcone (g/mol)
Nonmethylated		317.16

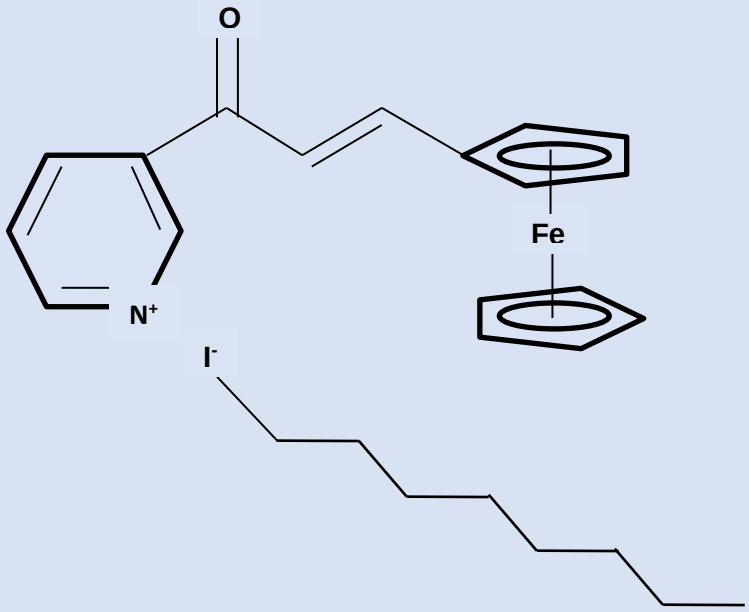
Methyl		459.10
Ethyl		473.13

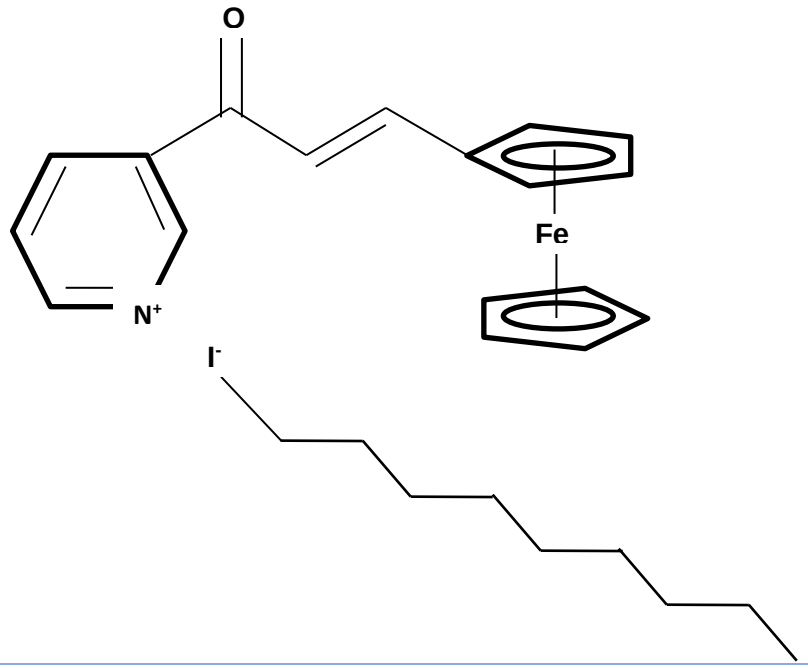
Propyl		487.15
Butyl		501.18

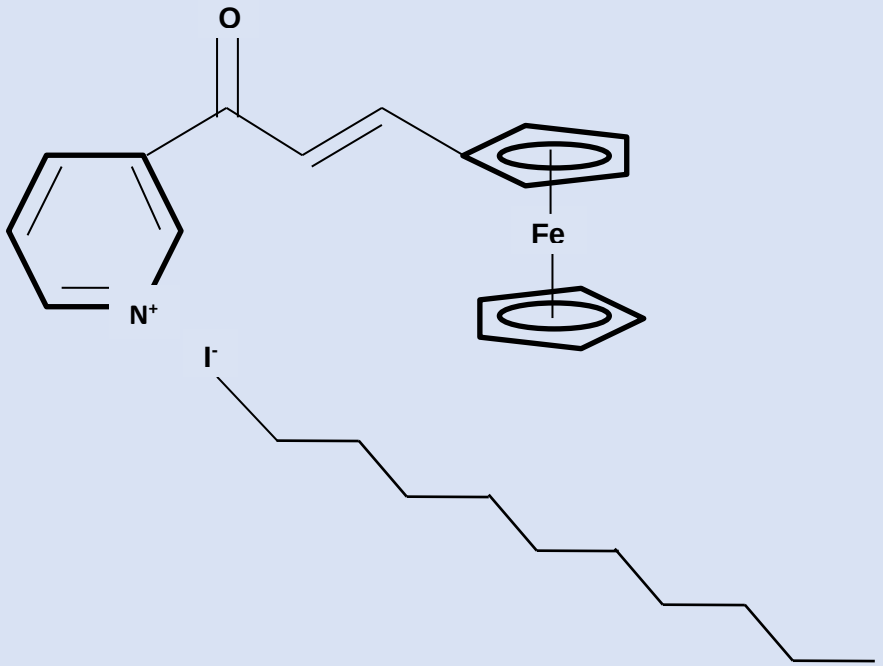
Pentyl		515.21

Hexyl		529.23

Heptyl		543.26

Octyl		557.29

Nonyl		571.31

Decyl		585.34

9.2 Appendix 2 – ^{1}H NMR, MS and IR spectra of ferrocenyl chalcone compounds

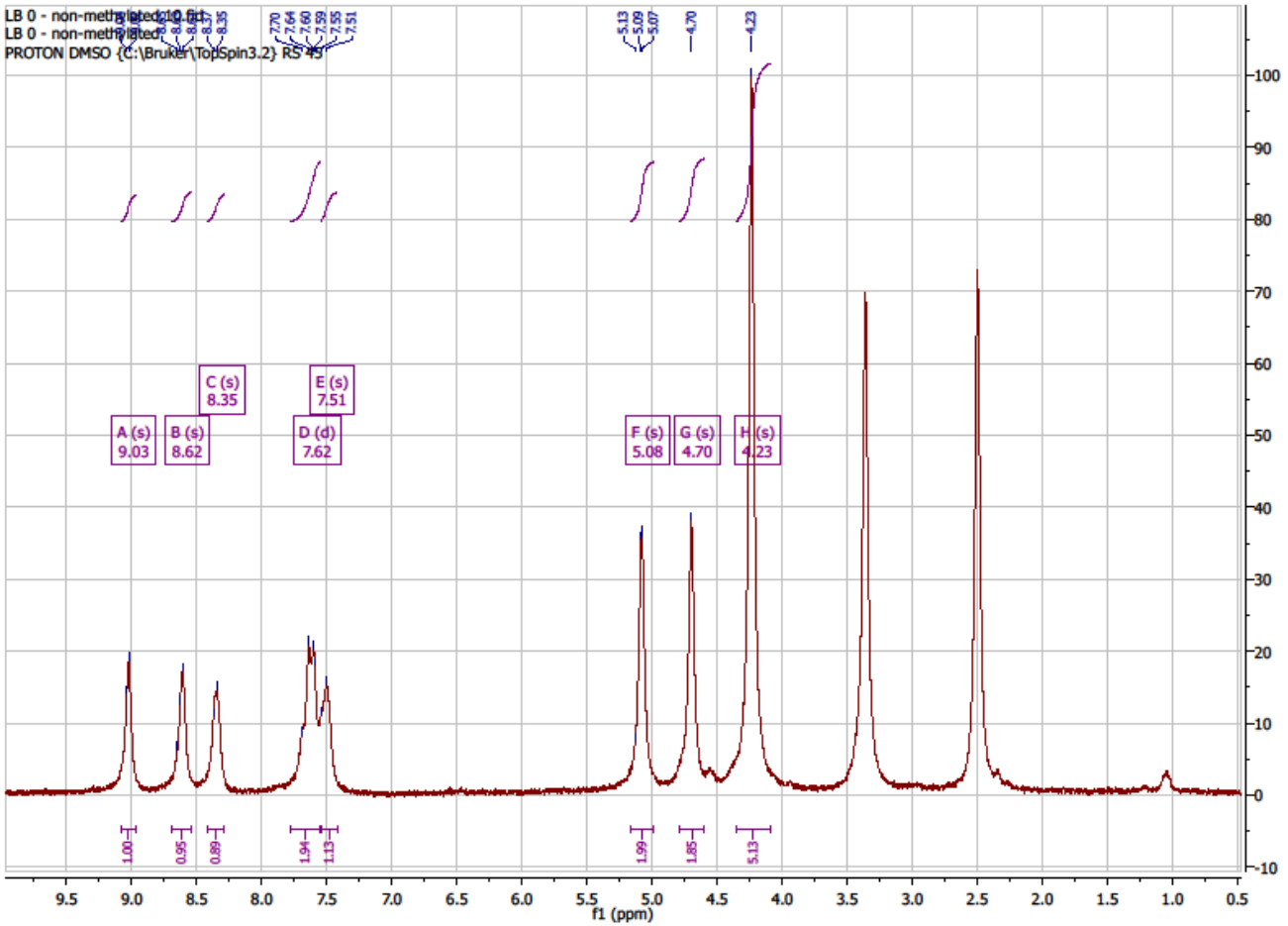


Figure 9-1 *^{1}H NMR spectrum of nonmethylated ferrocenyl chalcone compound without the iodide component.*

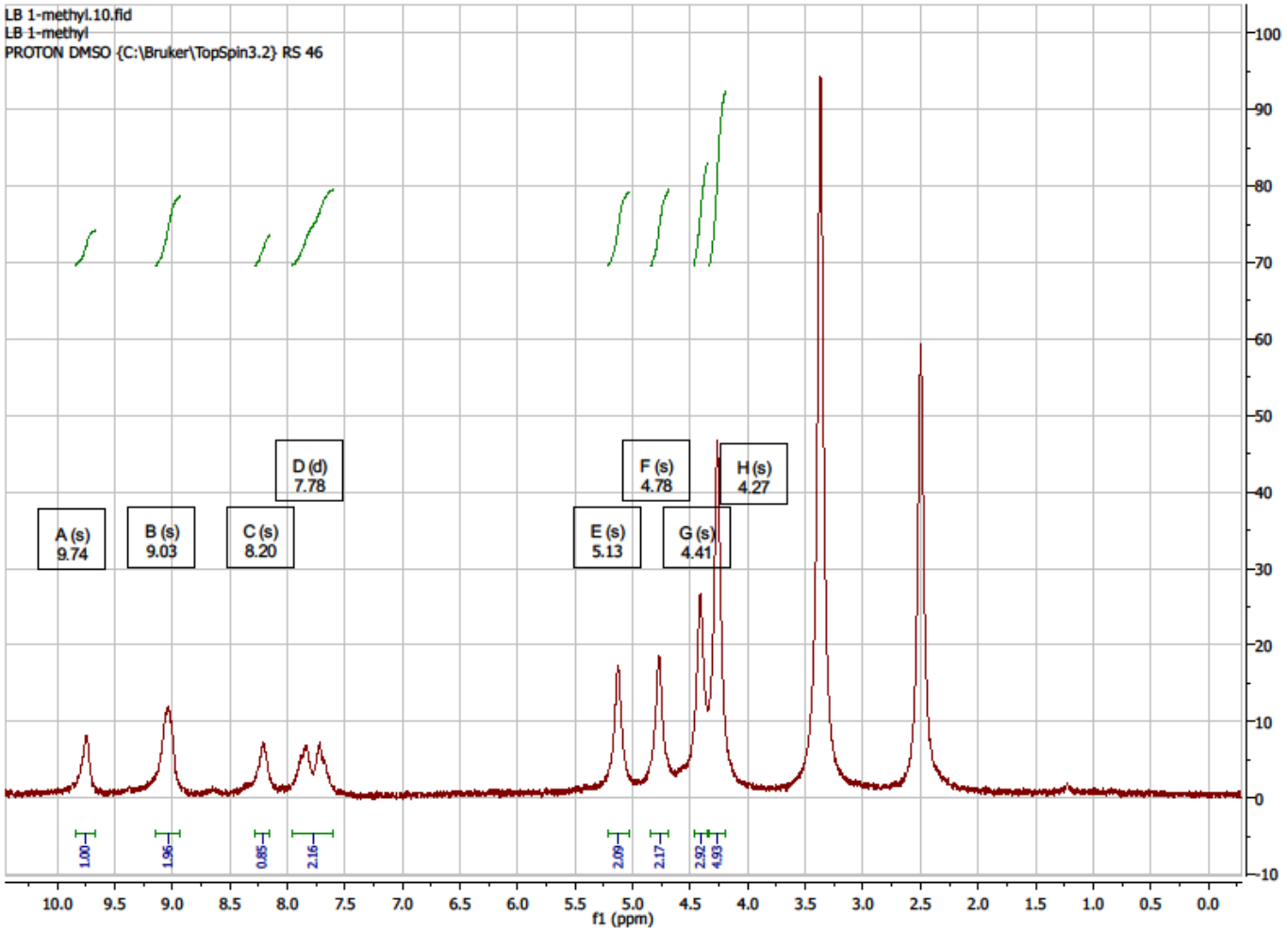


Figure 9-2 *^{1}H NMR spectrum of methyl ferrocenyl chalcone compound without the iodide component.*

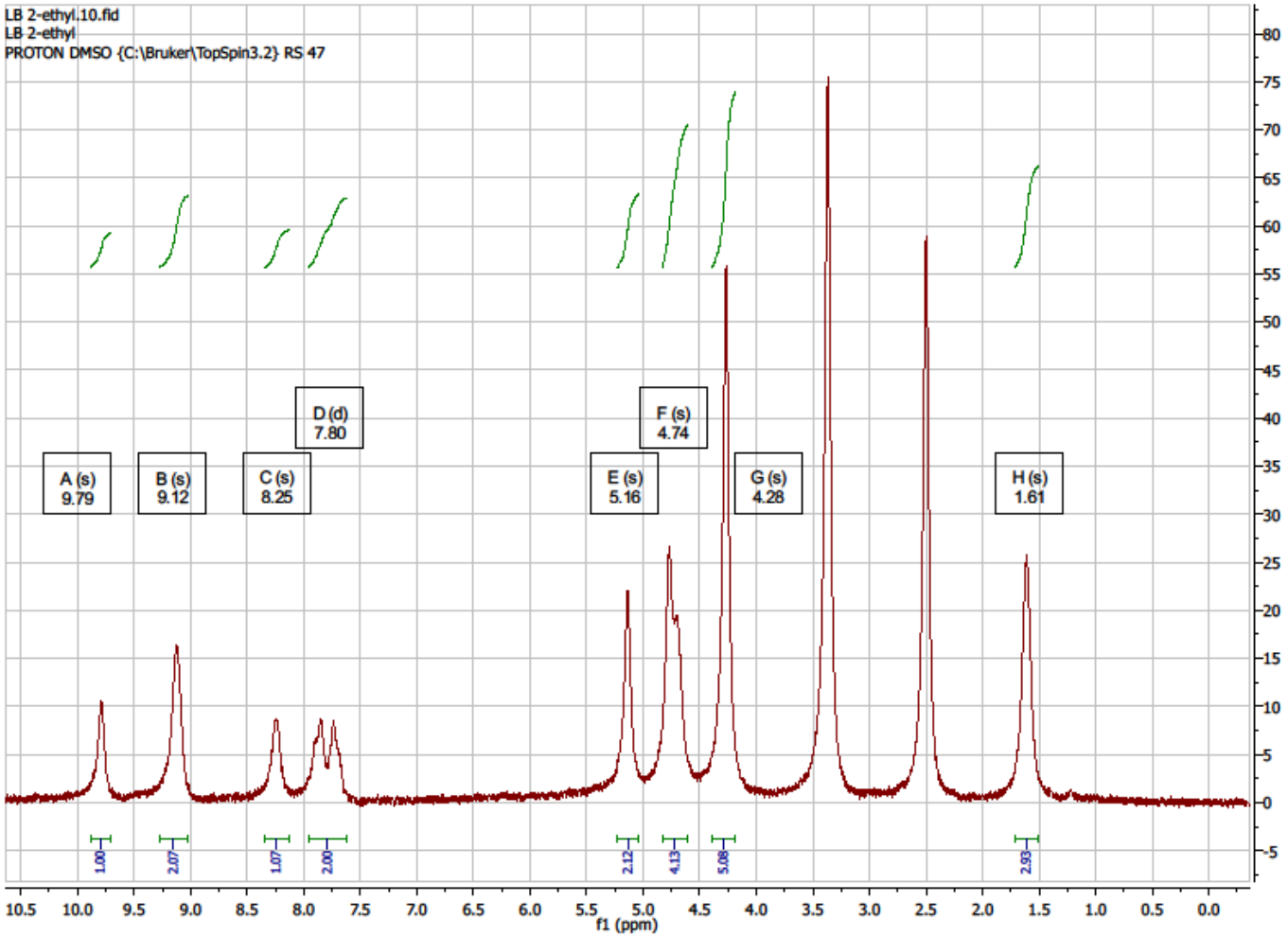


Figure 9-3 *^{1}H NMR spectrum of ethyl ferrocenyl chalcone compound without the iodide component.*

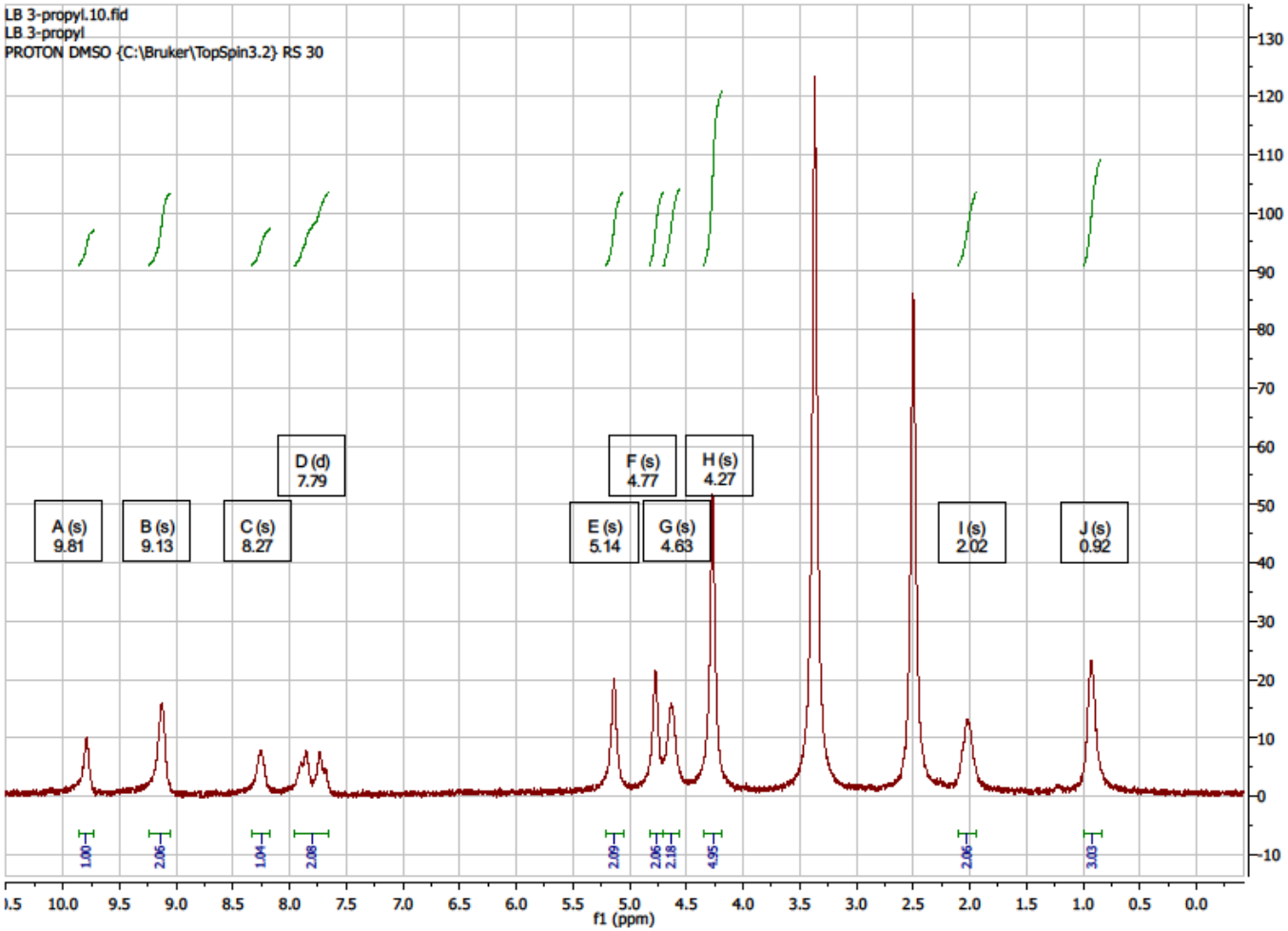


Figure 9-4 *^{1}H NMR spectrum of propyl ferrocenyl chalcone compound without the iodide component.*

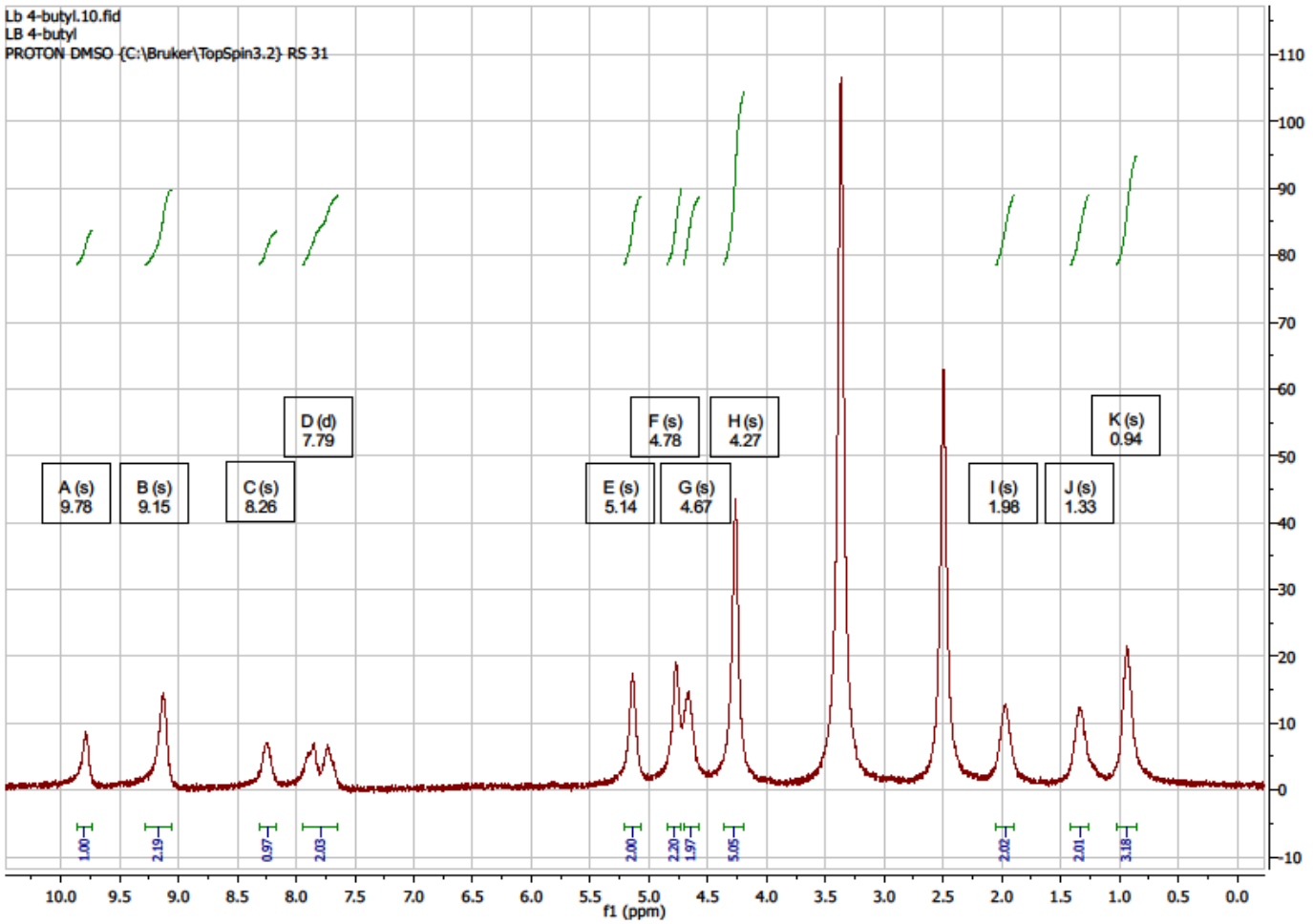


Figure 9-5 *^{1}H NMR spectrum of butyl ferrocenyl chalcone compound without the iodide component.*

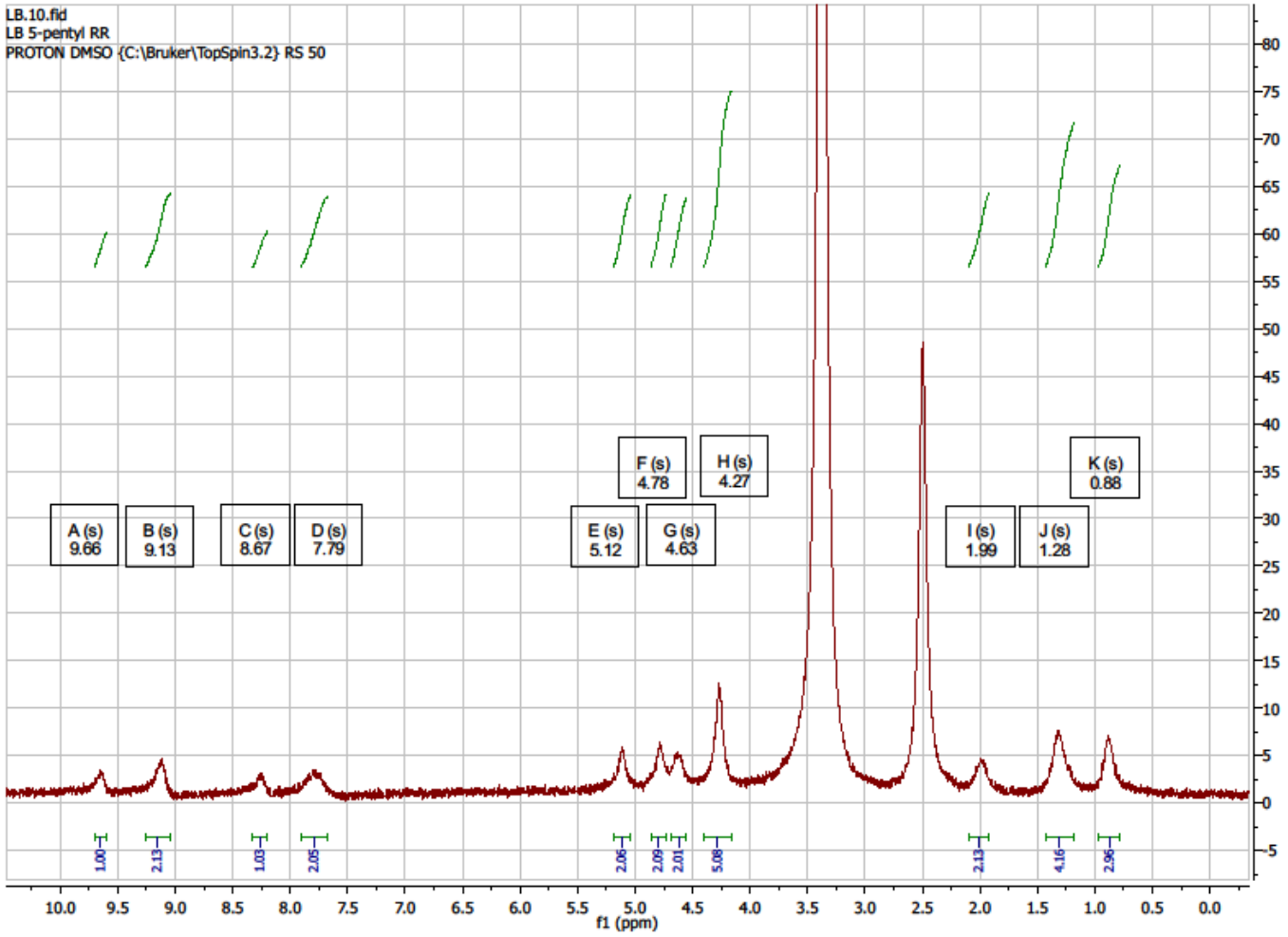


Figure 9-6 *^{1}H NMR spectrum of pentyl ferrocenyl chalcone compound without the iodide component.*

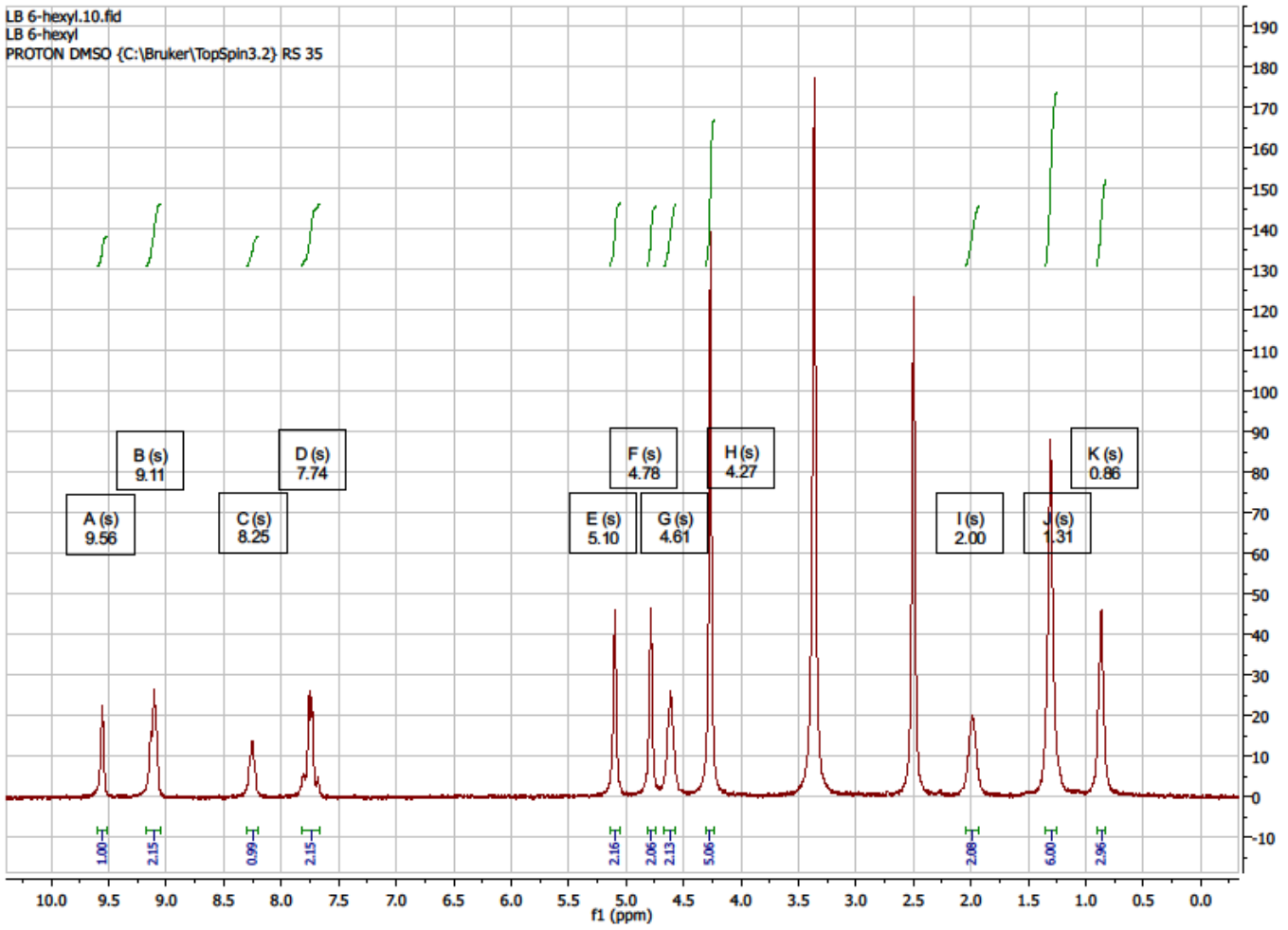


Figure 9-7 *^{1}H NMR spectrum of hexyl ferrocenyl chalcone compound without the iodide component.*

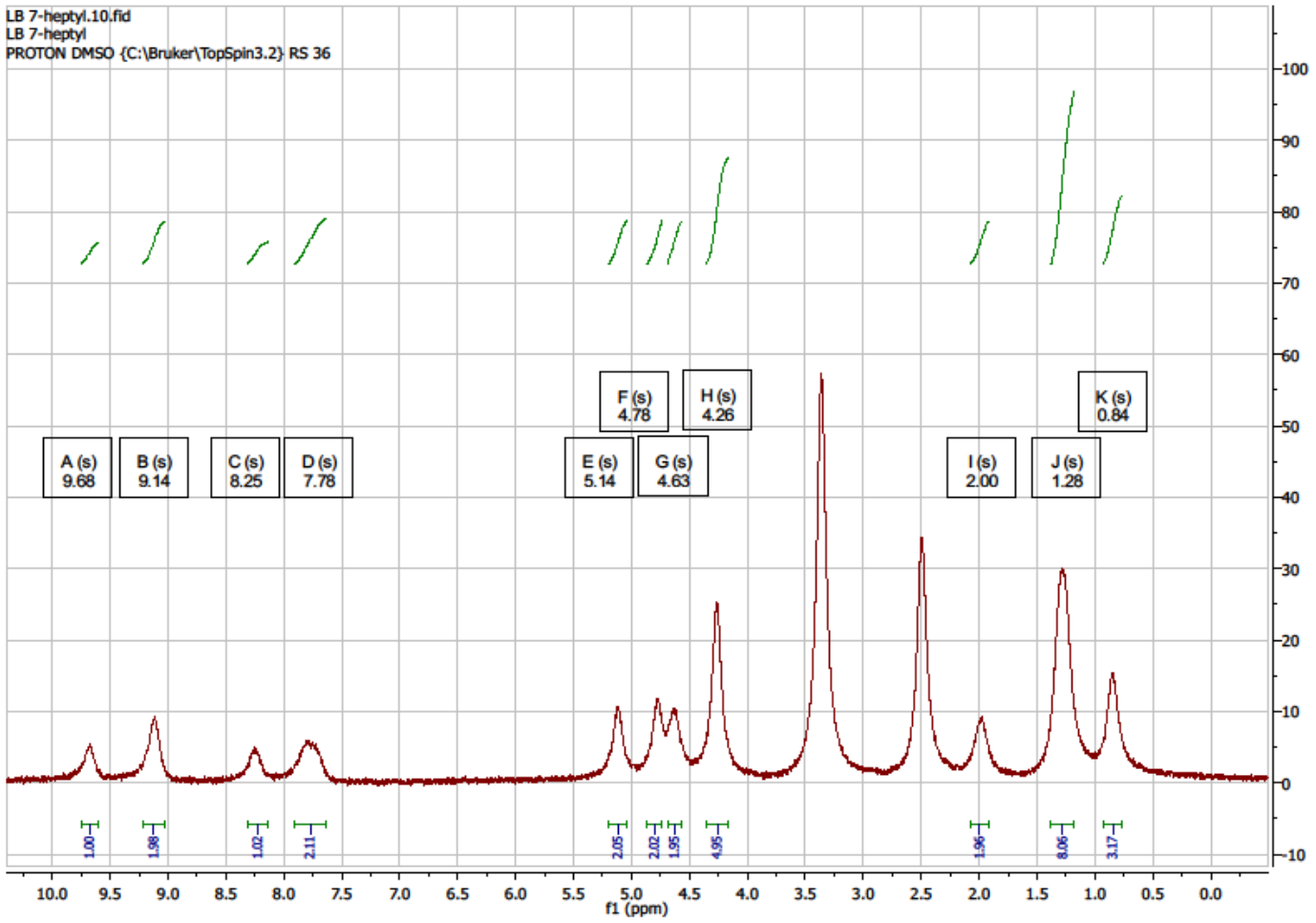


Figure 9-8 *^{1}H NMR spectrum of heptyl ferrocenyl chalcone compound without the iodide component.*

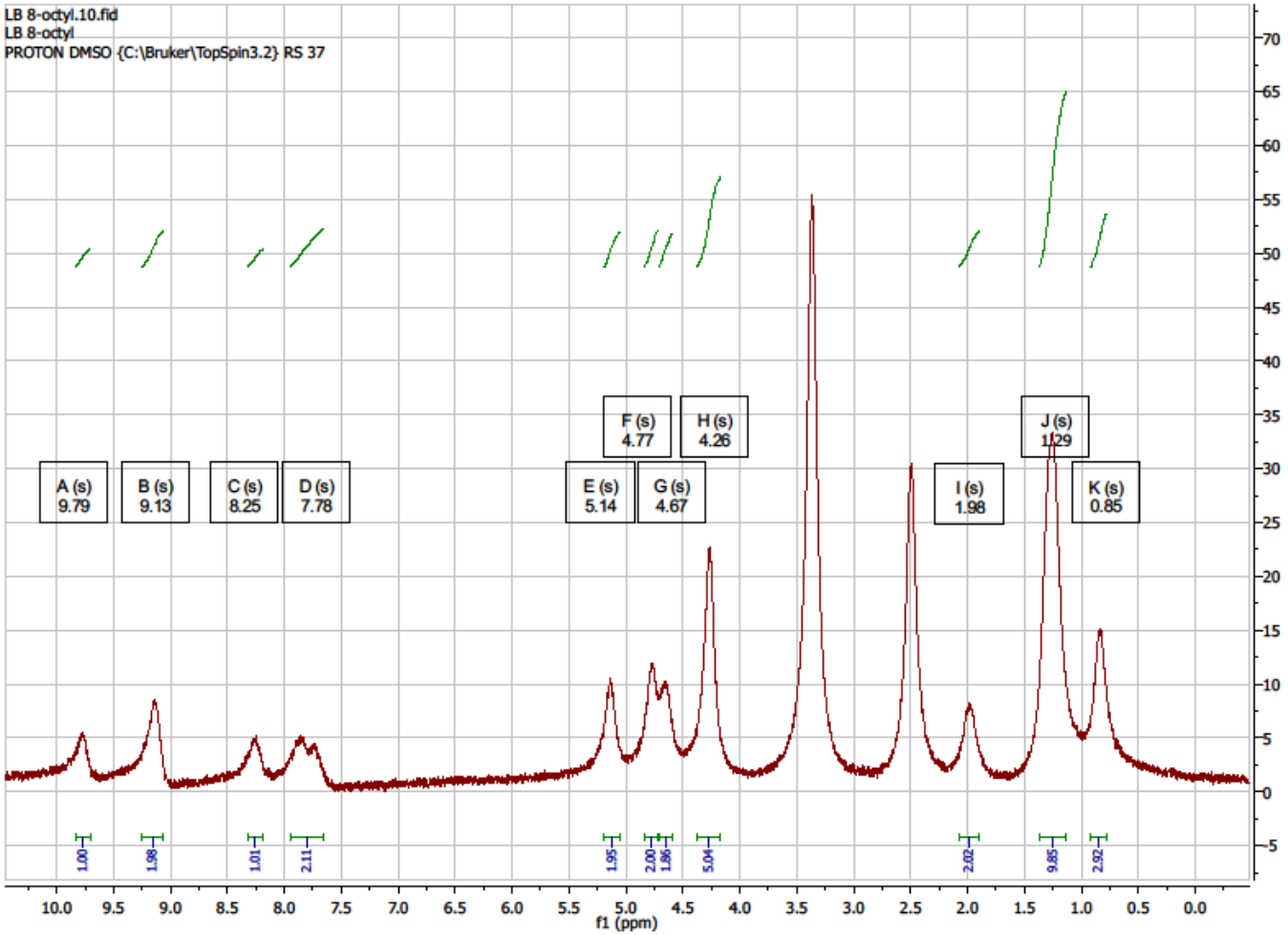


Figure 9-9 *^{1}H NMR spectrum of octyl ferrocenyl chalcone compound without the iodide component.*

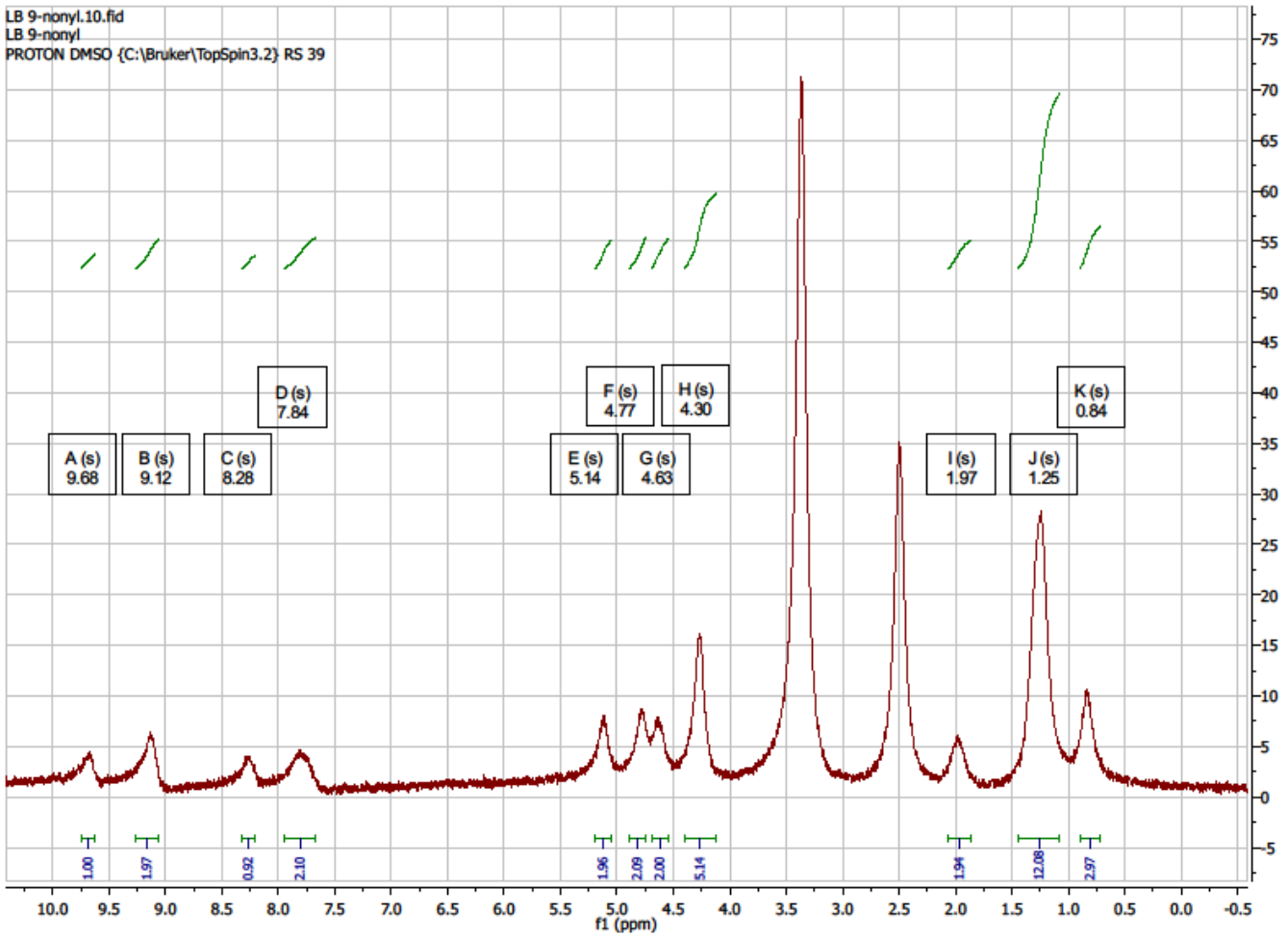


Figure 9-10 *^{1}H NMR spectrum of nonyl ferrocenyl chalcone compound without the iodide component.*

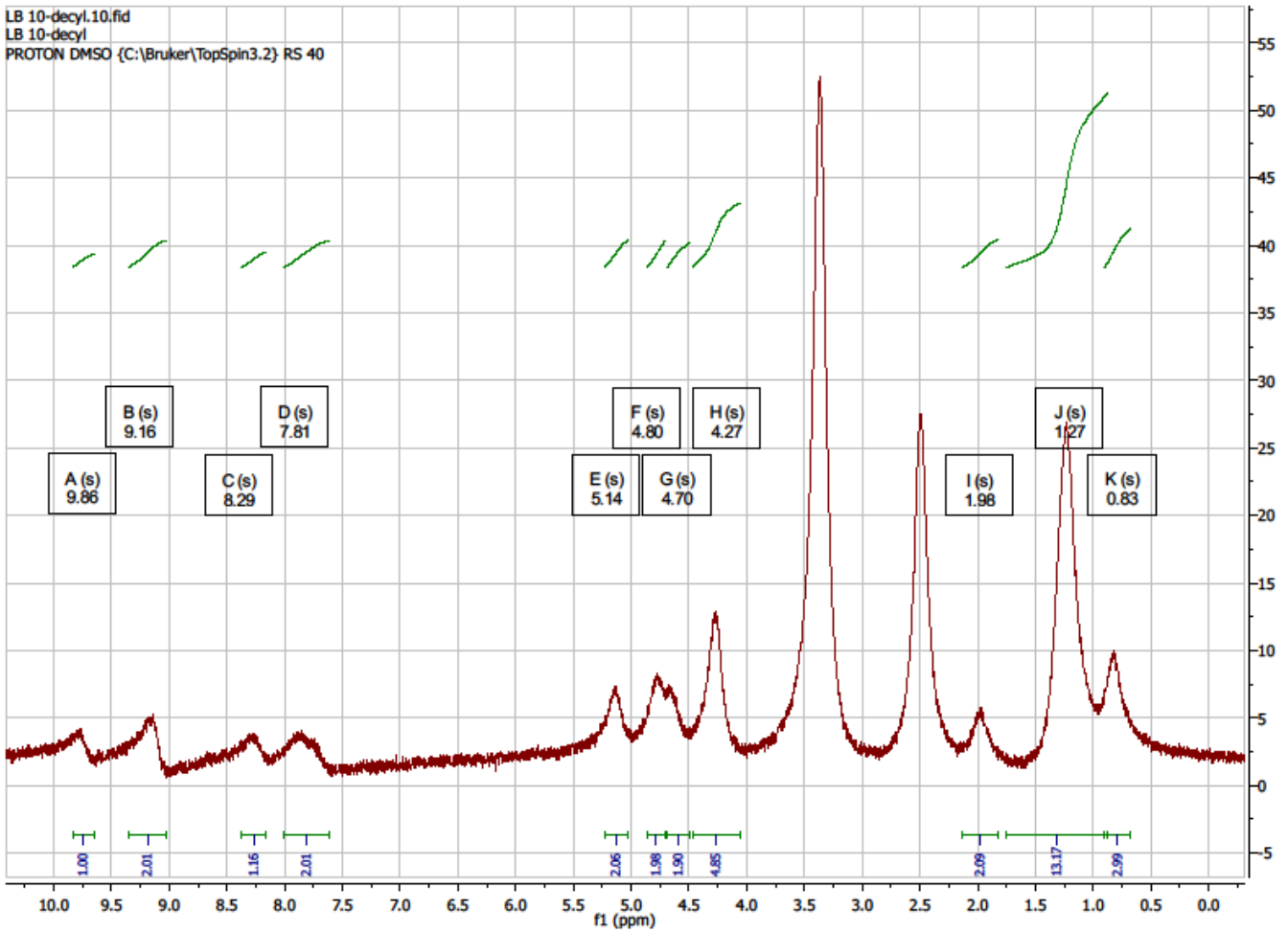


Figure 9-11 *^{1}H NMR spectrum of decyl ferrocenyl chalcone compound without the iodide component.*

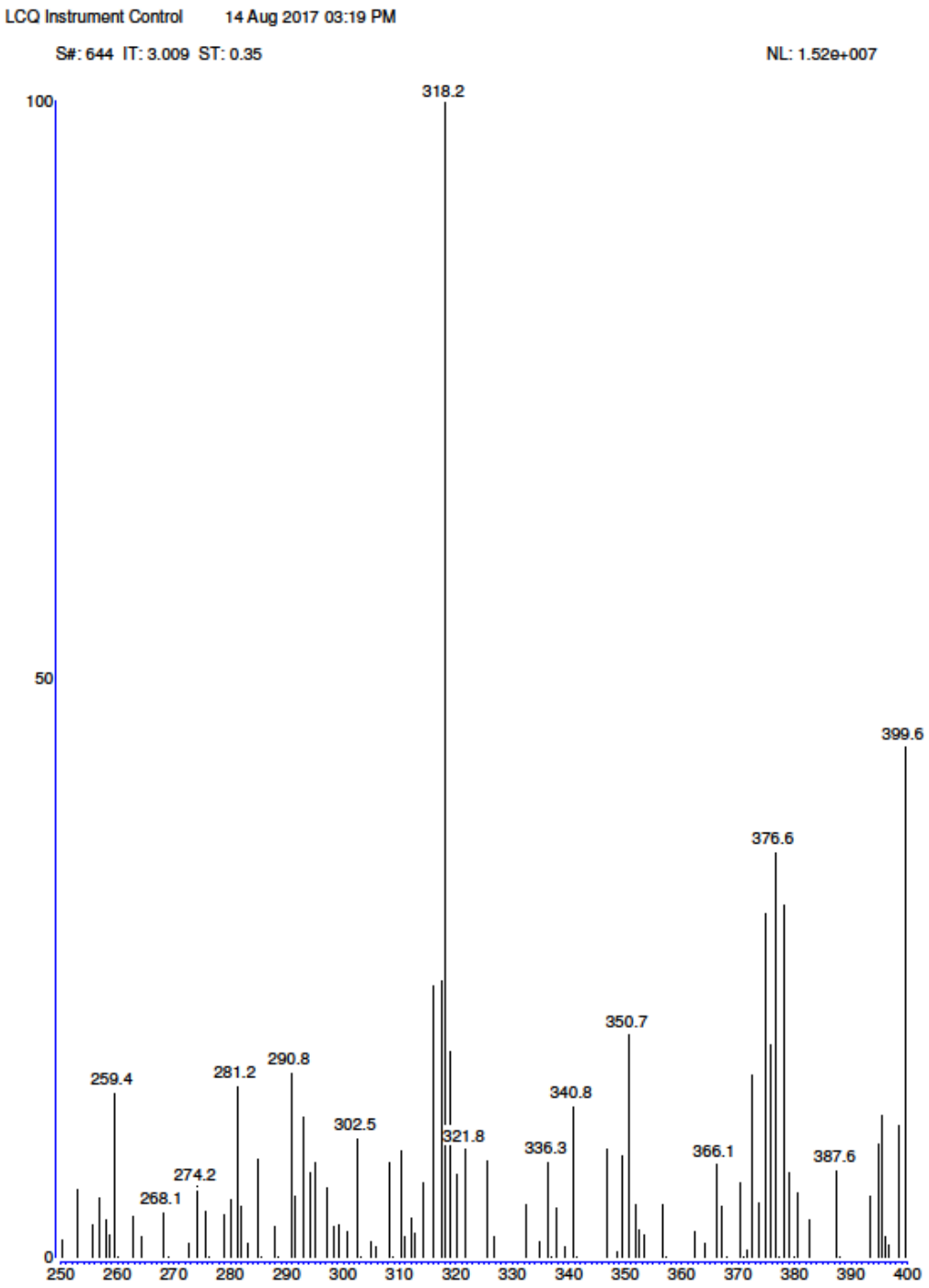


Figure 9-12 *MS spectrum of nonmethylated ferrocenyl chalcone compound without the iodide component.*

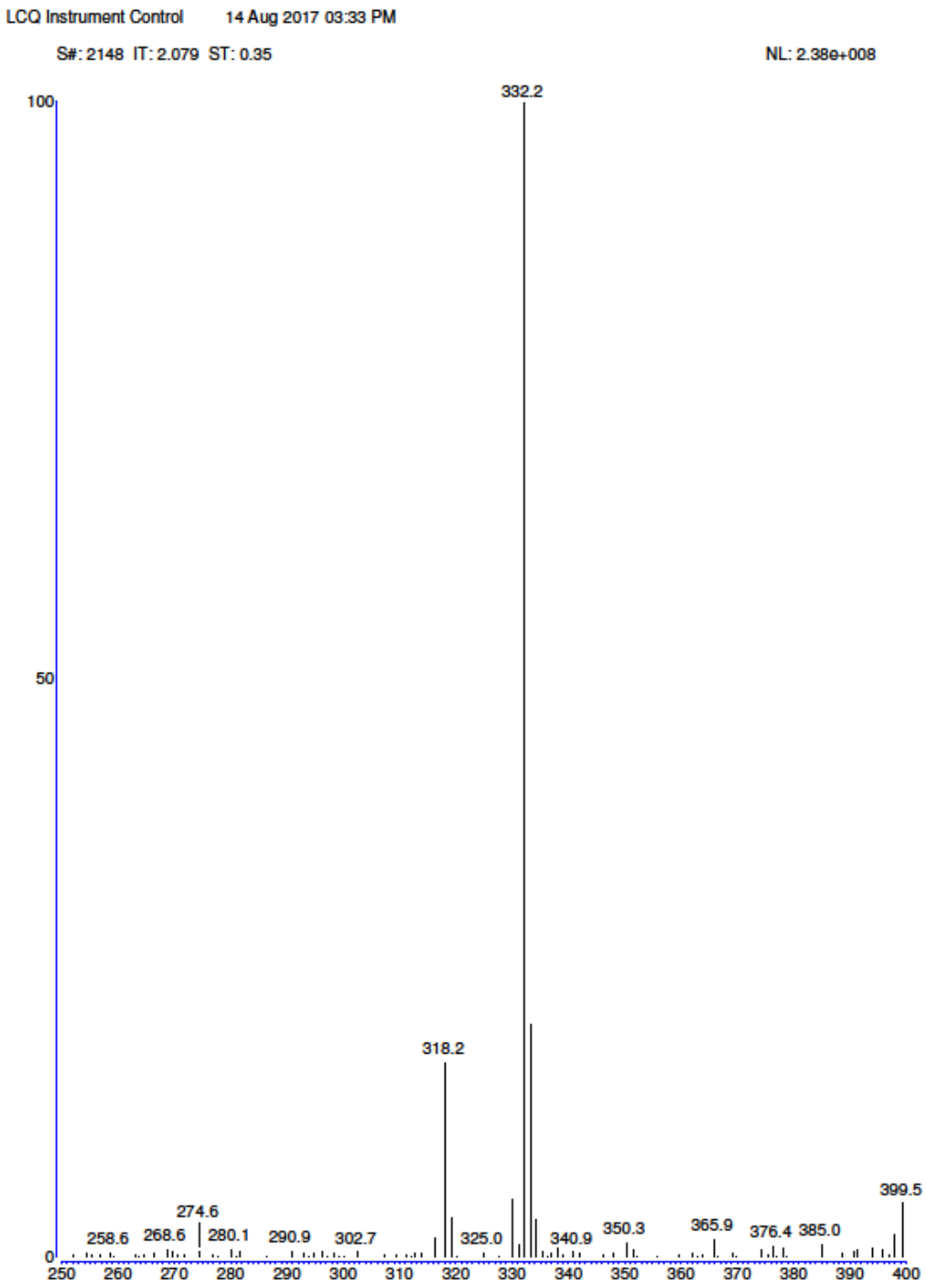


Figure 9-13 *MS spectrum of methyl ferrocenyl chalcone compound without the iodide component.*

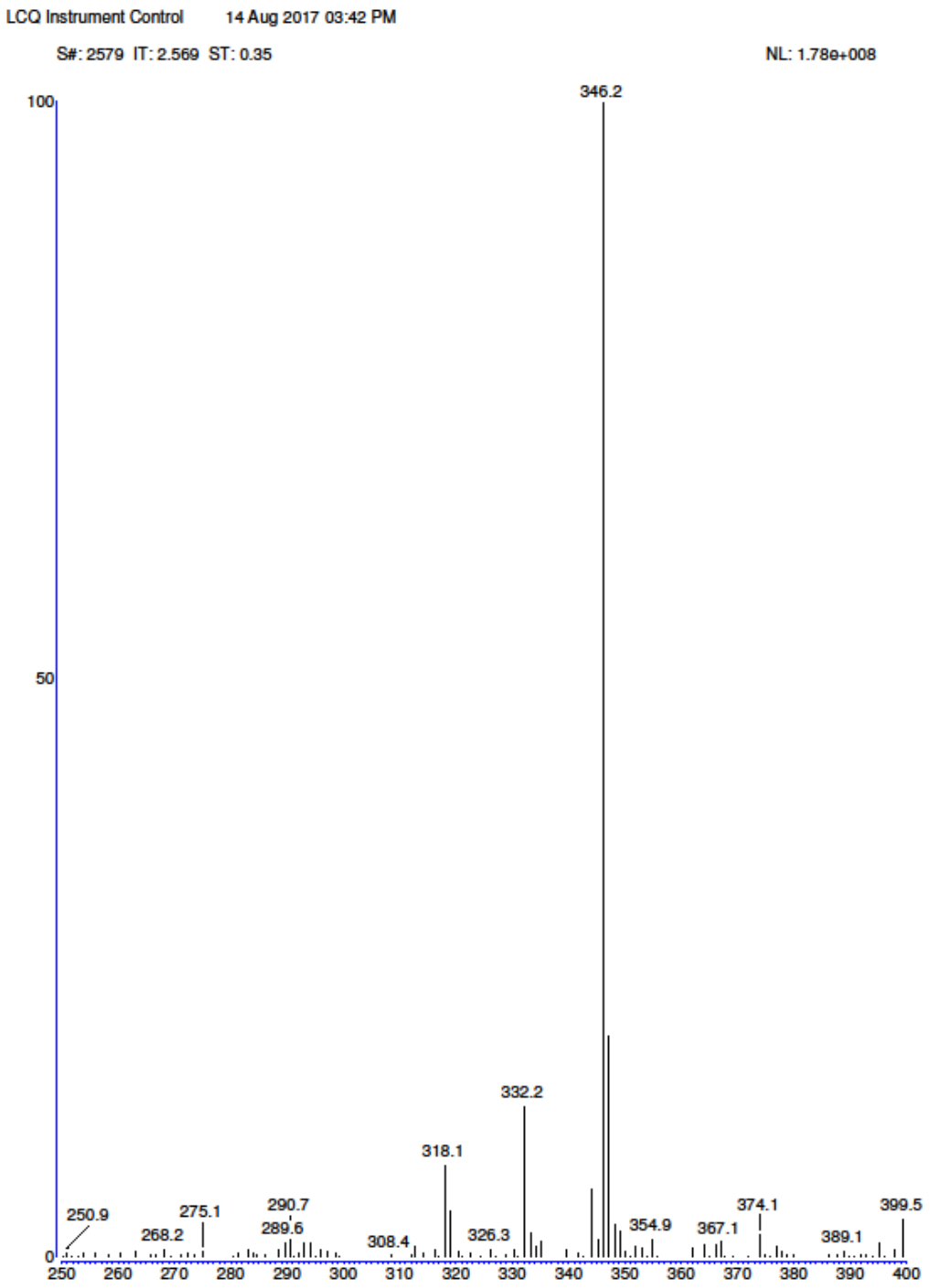


Figure 9-14 *MS spectrum of ethyl ferrocenyl chalcone compound without the iodide component.*

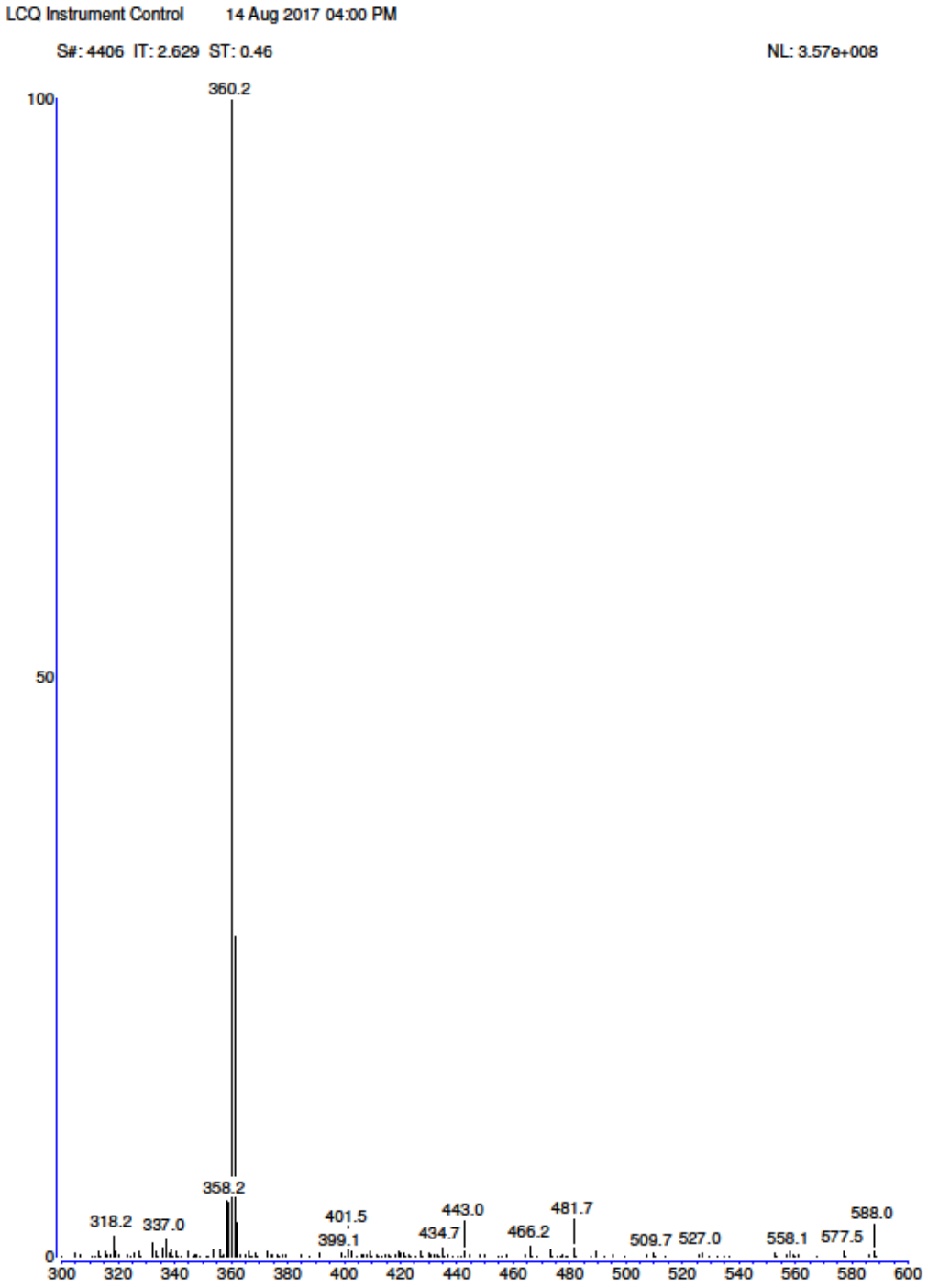


Figure 9-15 *MS spectrum of propyl ferrocenyl chalcone compound without the iodide component.*

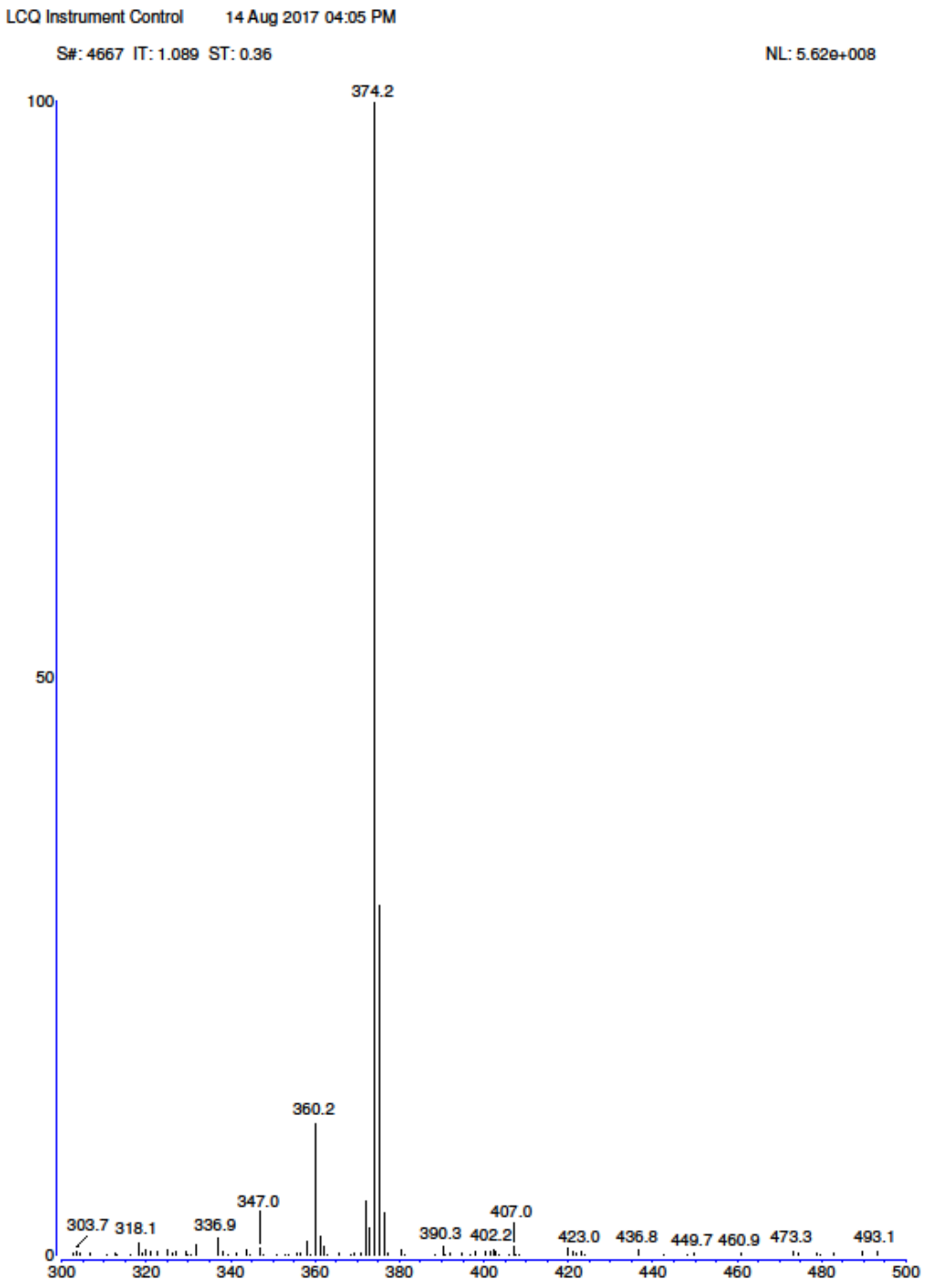


Figure 9-16 *MS spectrum of butyl ferrocenyl chalcone compound without the iodide component.*

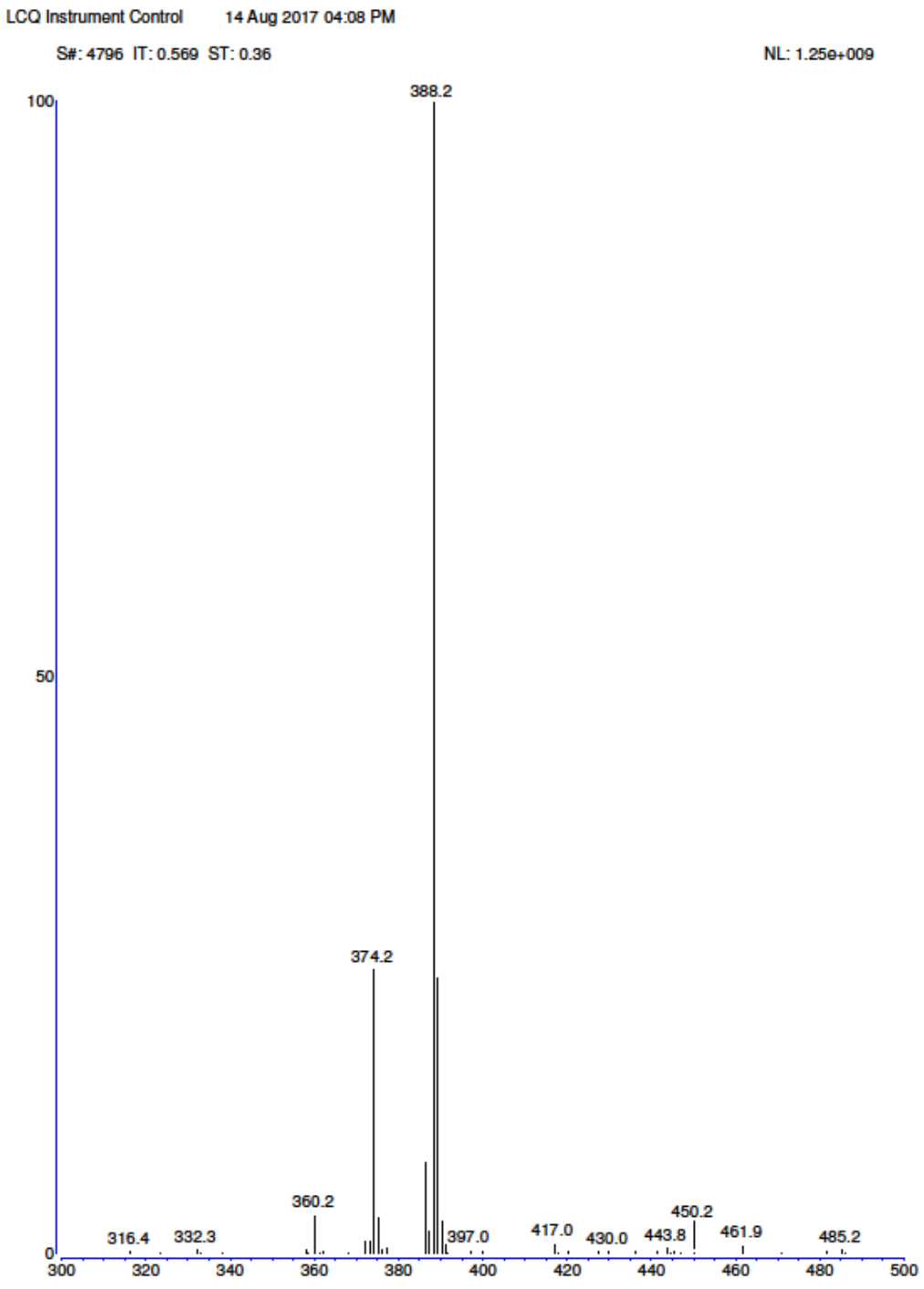


Figure 9-17 *MS spectrum of pentyl ferrocenyl chalcone compound without the iodide component.*

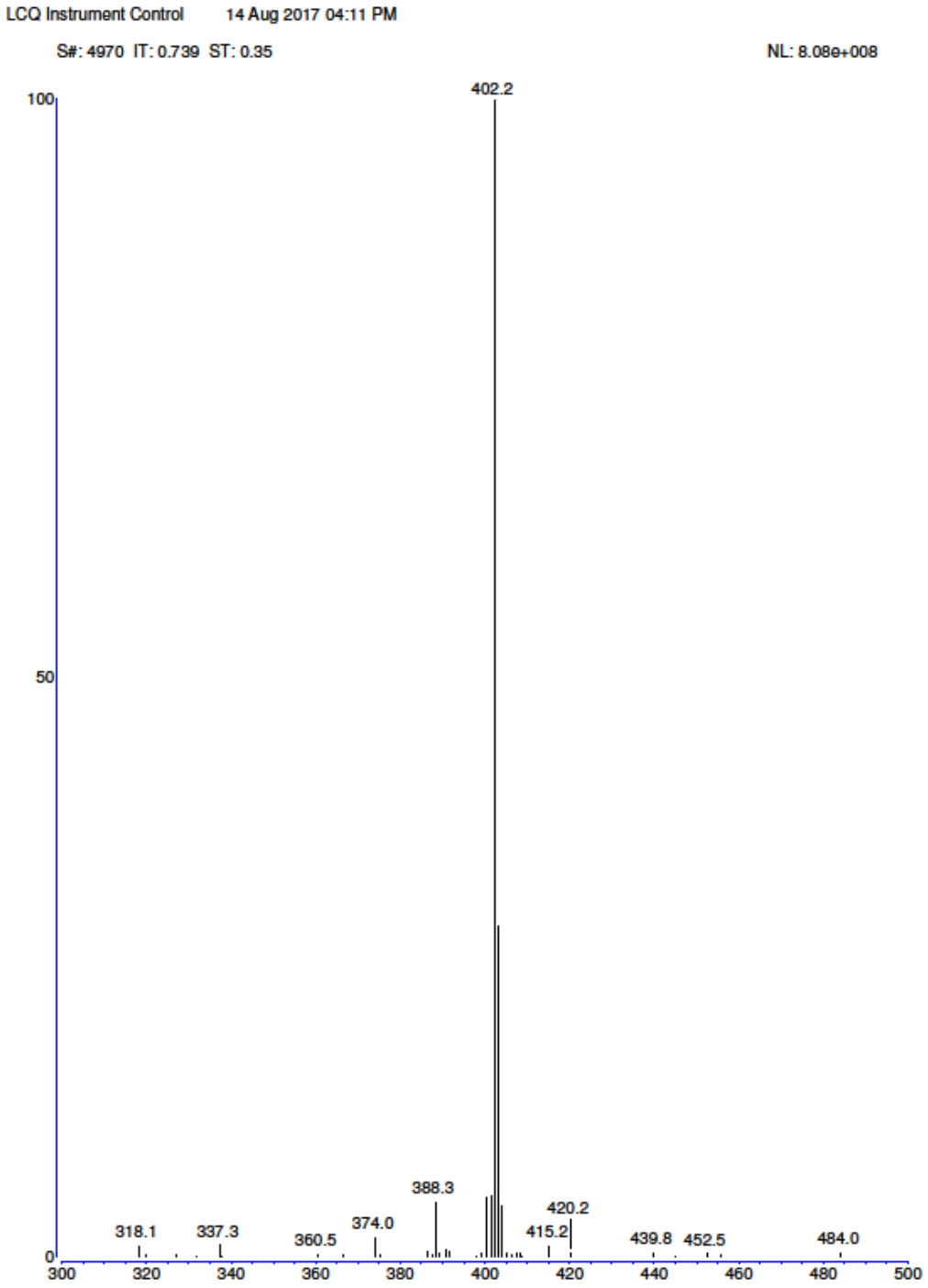


Figure 9-18 *MS spectrum of hexyl ferrocenyl chalcone compound without the iodide component.*

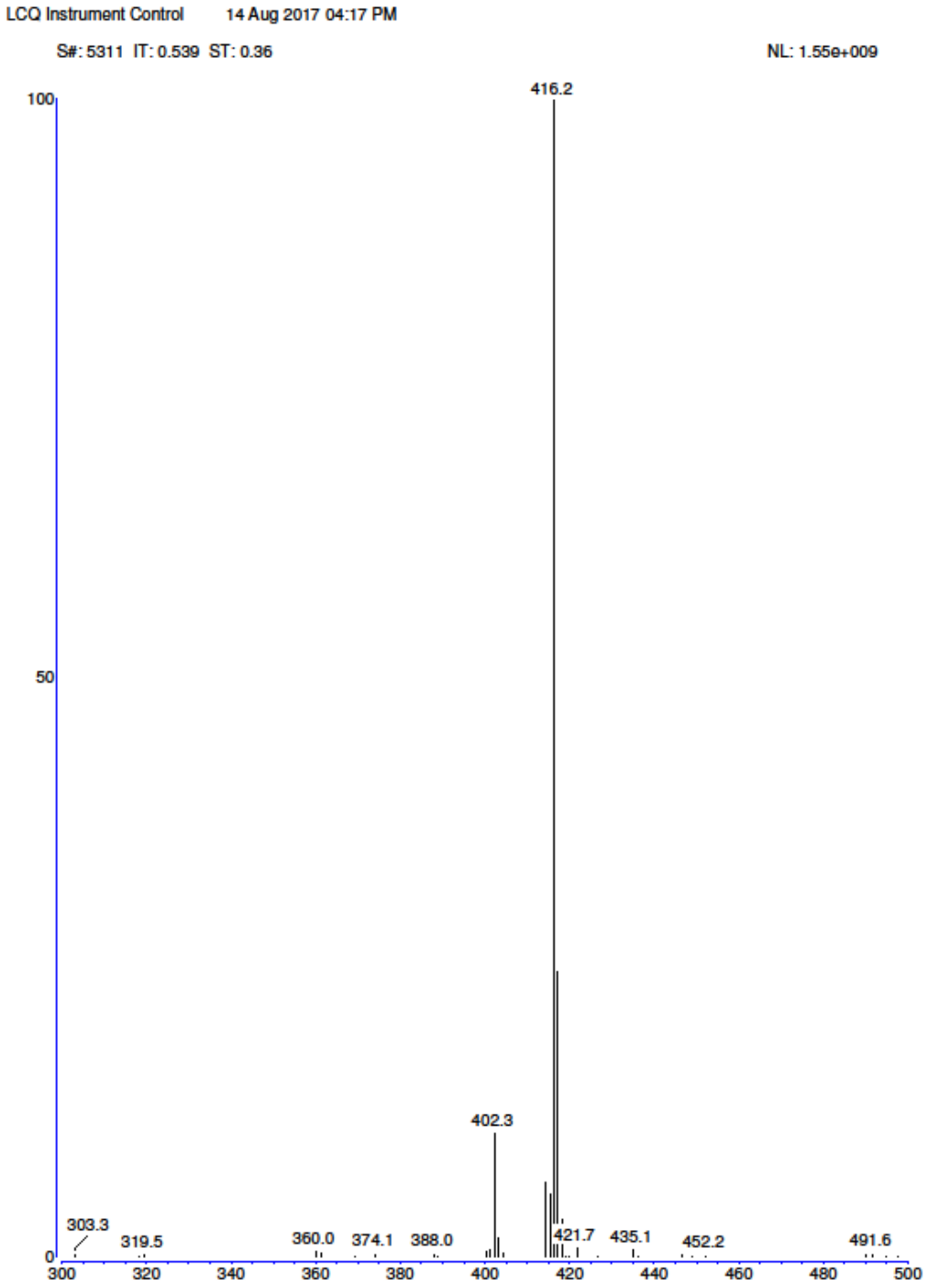


Figure 9-19 *MS spectrum of heptyl ferrocenyl chalcone compound without the iodide component.*

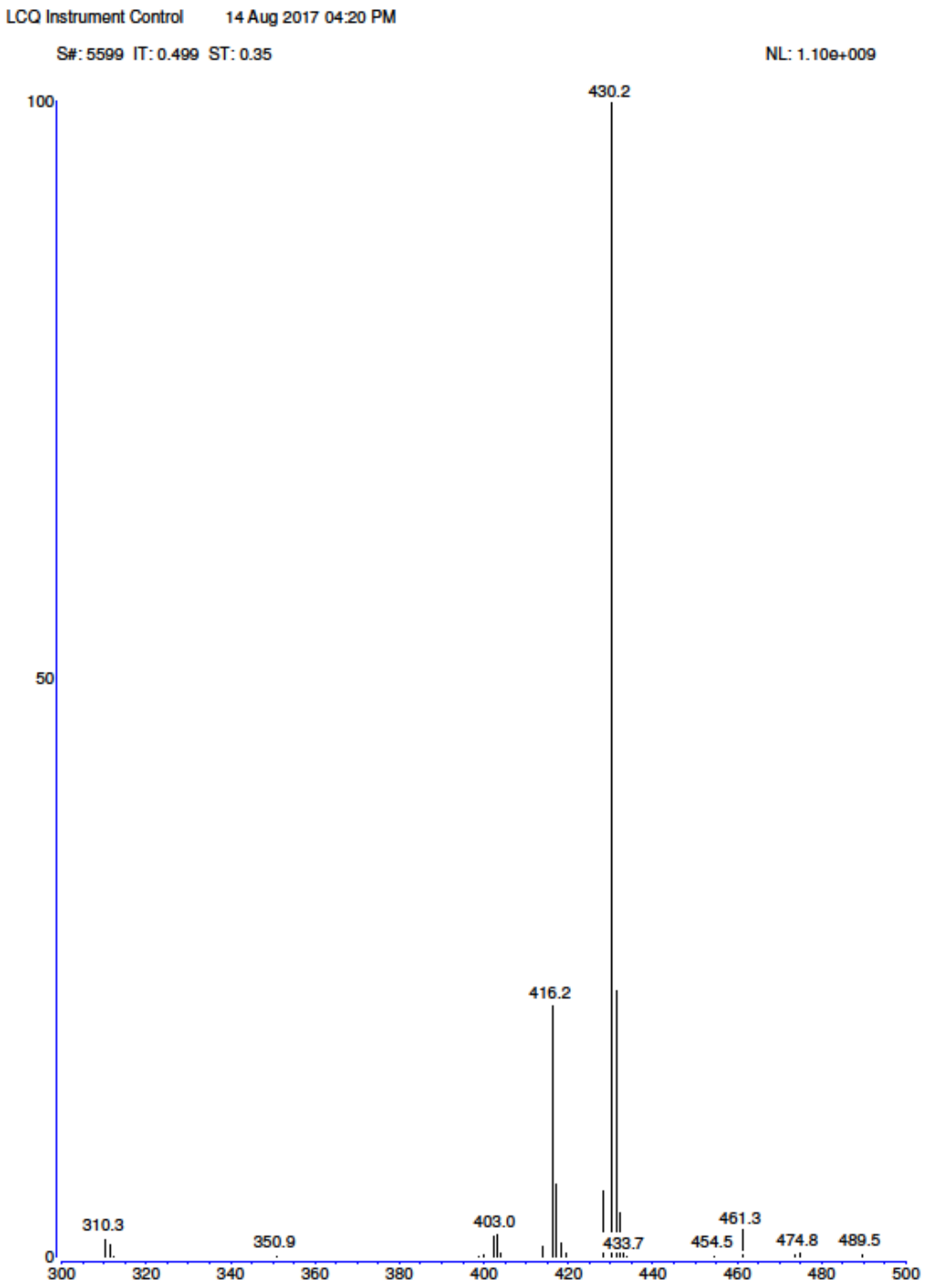


Figure 9-20 *MS spectrum of octyl ferrocenyl chalcone compound without the iodide component.*

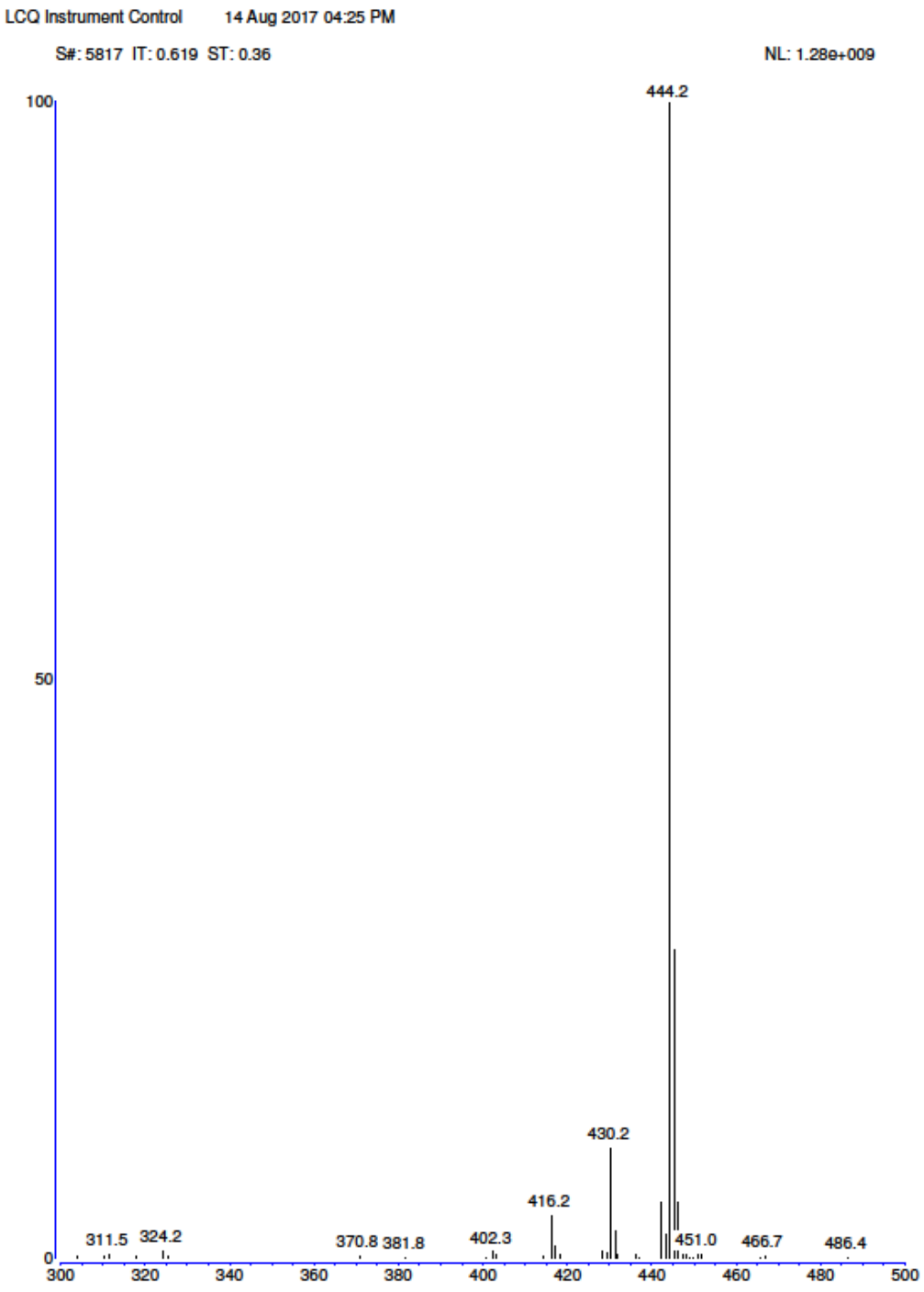


Figure 9-21 *MS spectrum of nonyl ferrocenyl chalcone compound without the iodide component.*

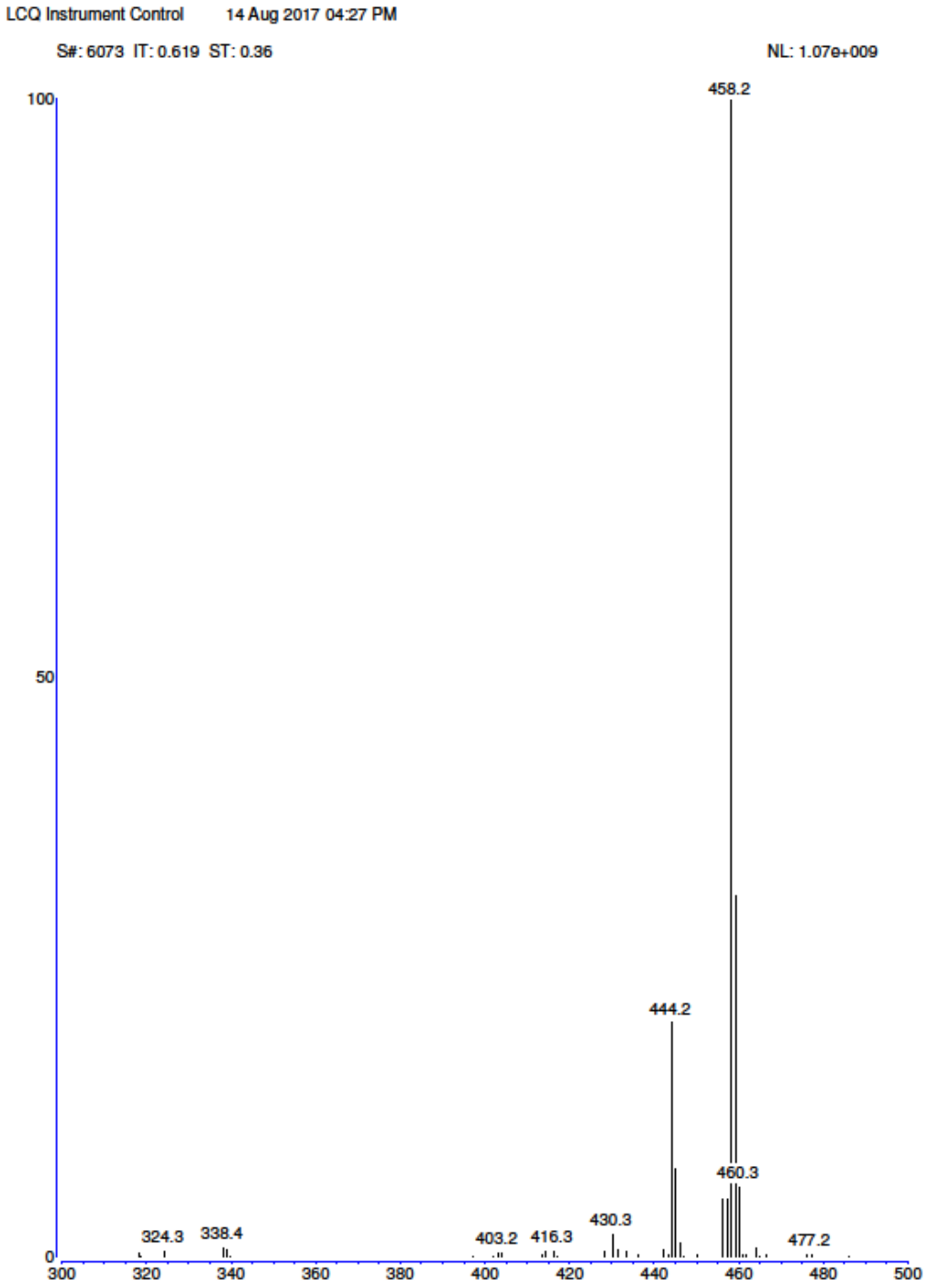


Figure 9-22 *MS spectrum of decyl ferrocenyl chalcone compound without the iodide component.*

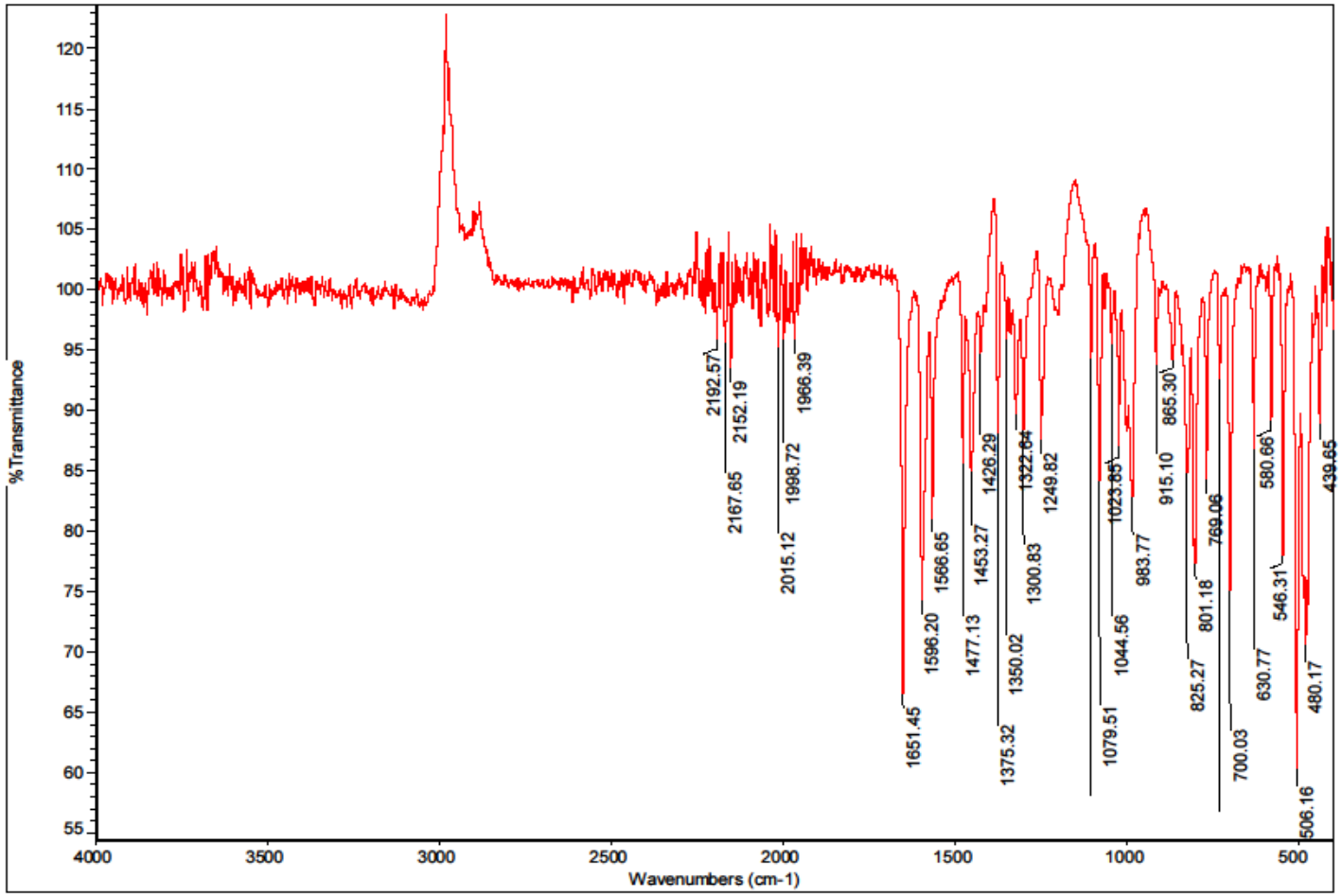


Figure 9-23 *IR spectrum of nonmethylated ferrocenyl chalcone compound with the iodide component.*

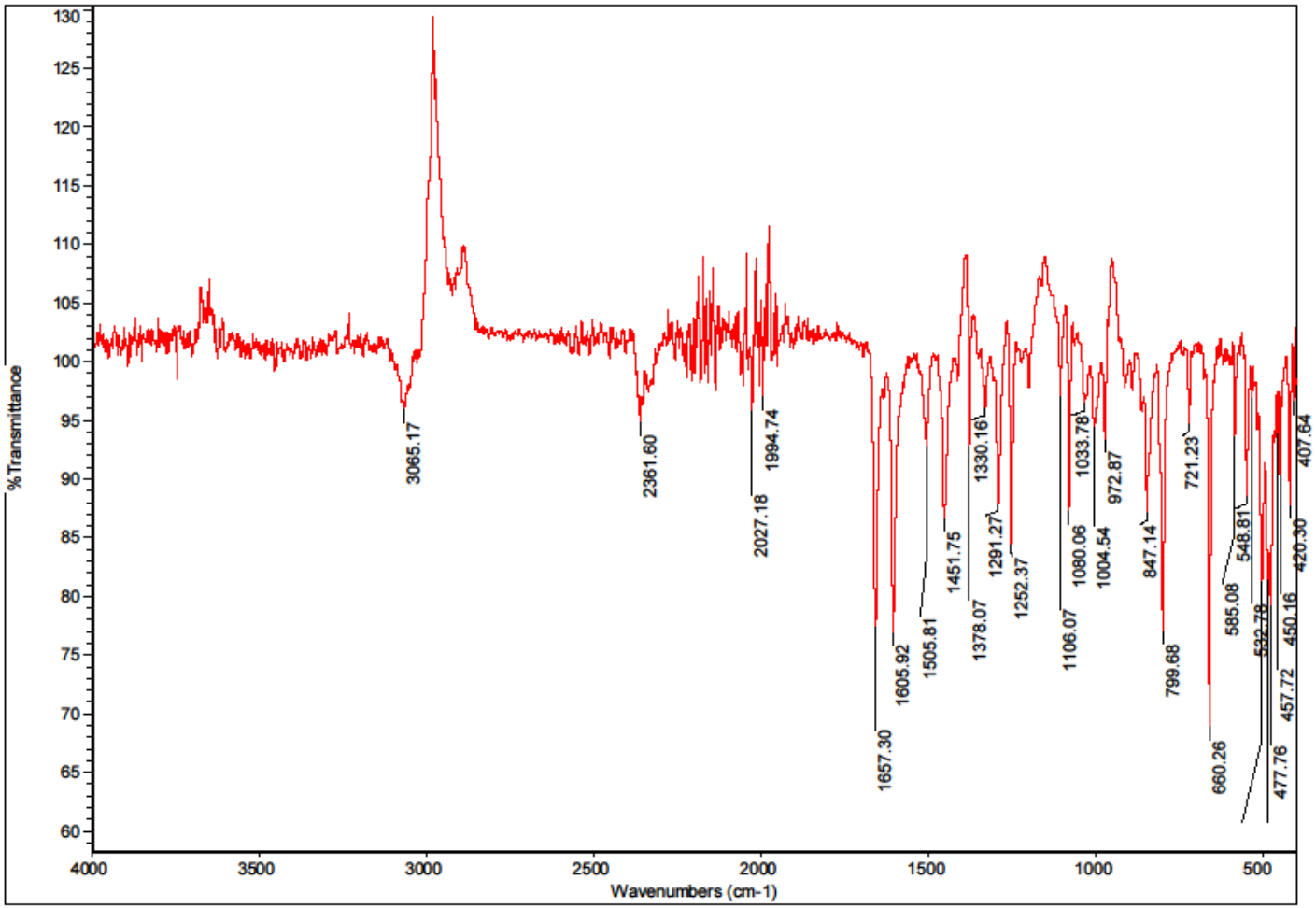


Figure 9-24 *IR spectrum of methyl ferrocenyl chalcone compound with the iodide component.*

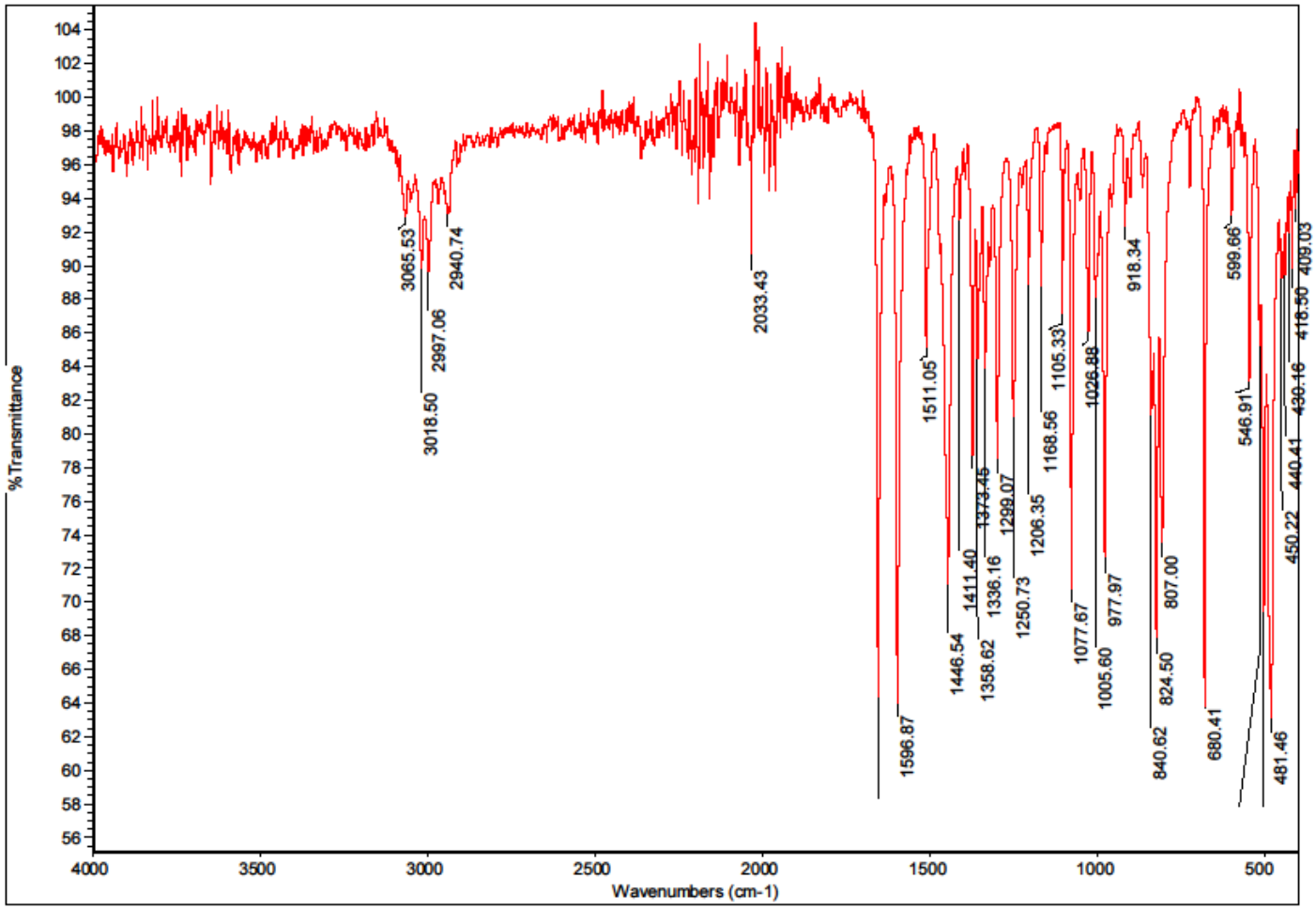


Figure 9-25 *IR spectrum of ethyl ferrocenyl chalcone compound with the iodide component.*

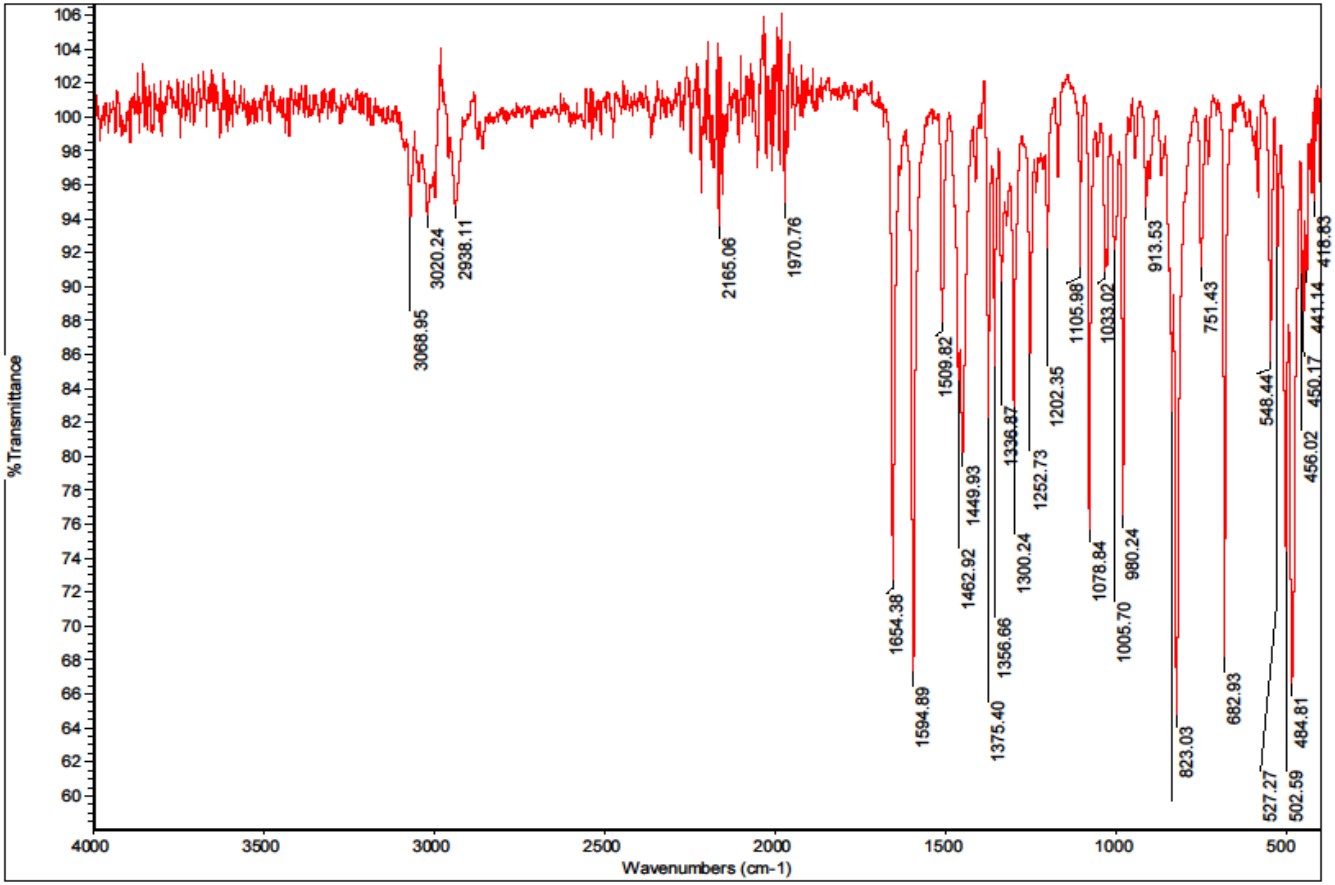


Figure 9-26 *IR spectrum of propyl ferrocenyl chalcone compound with the iodide component.*

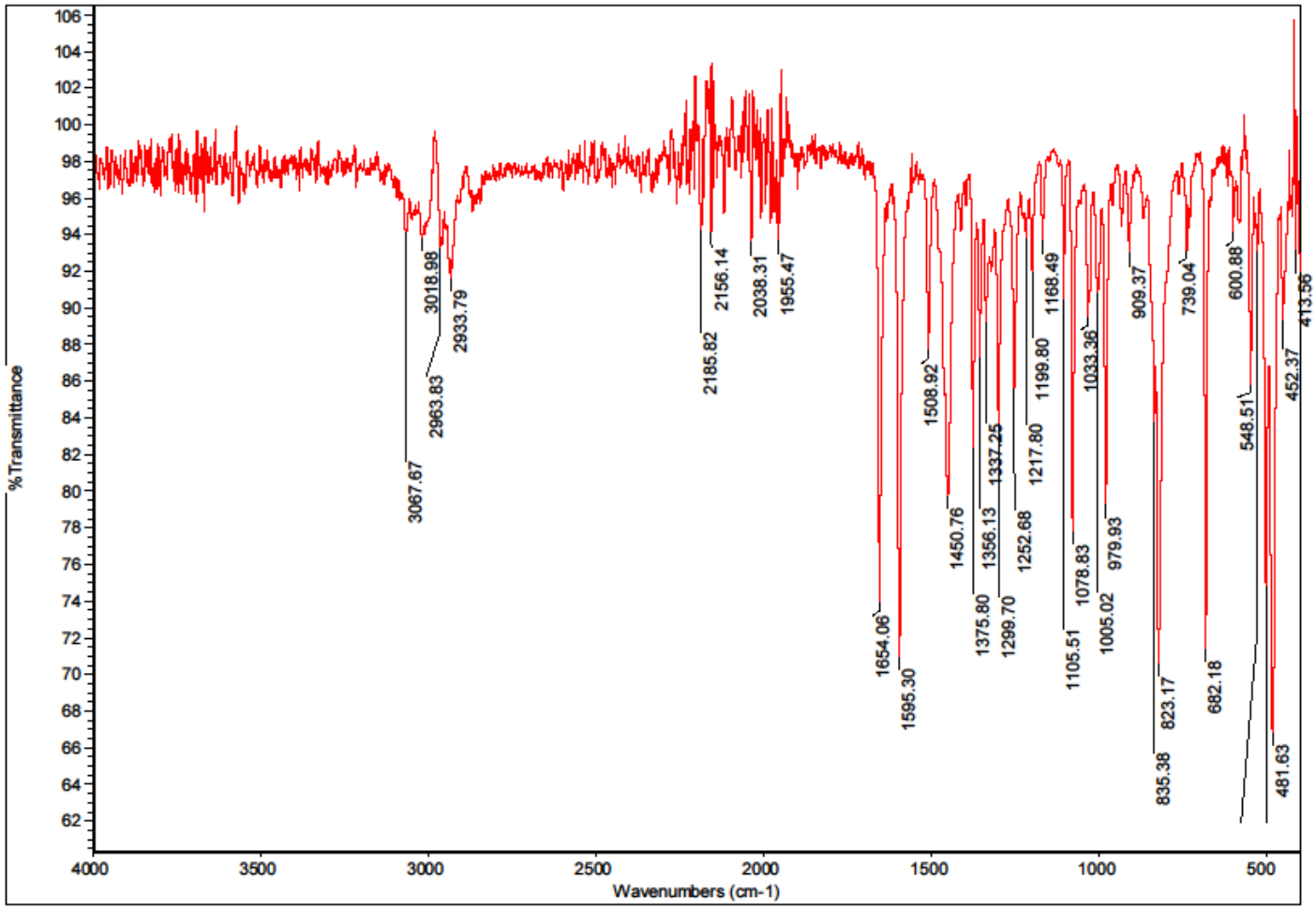


Figure 9-27 *IR spectrum of butyl ferrocenyl chalcone compound with the iodide component.*

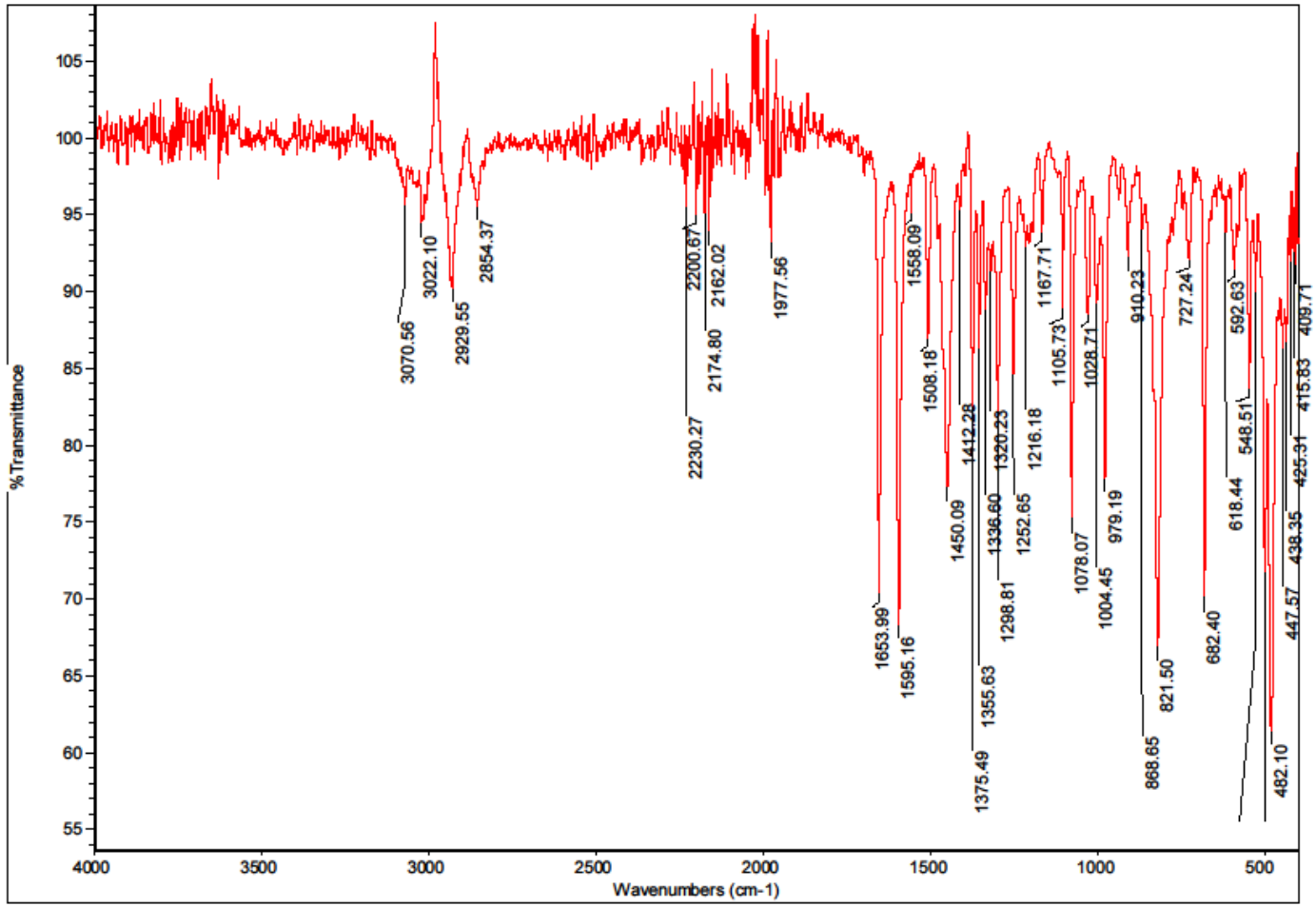


Figure 9-28 *IR spectrum of pentyl ferrocenyl chalcone compound with the iodide component.*

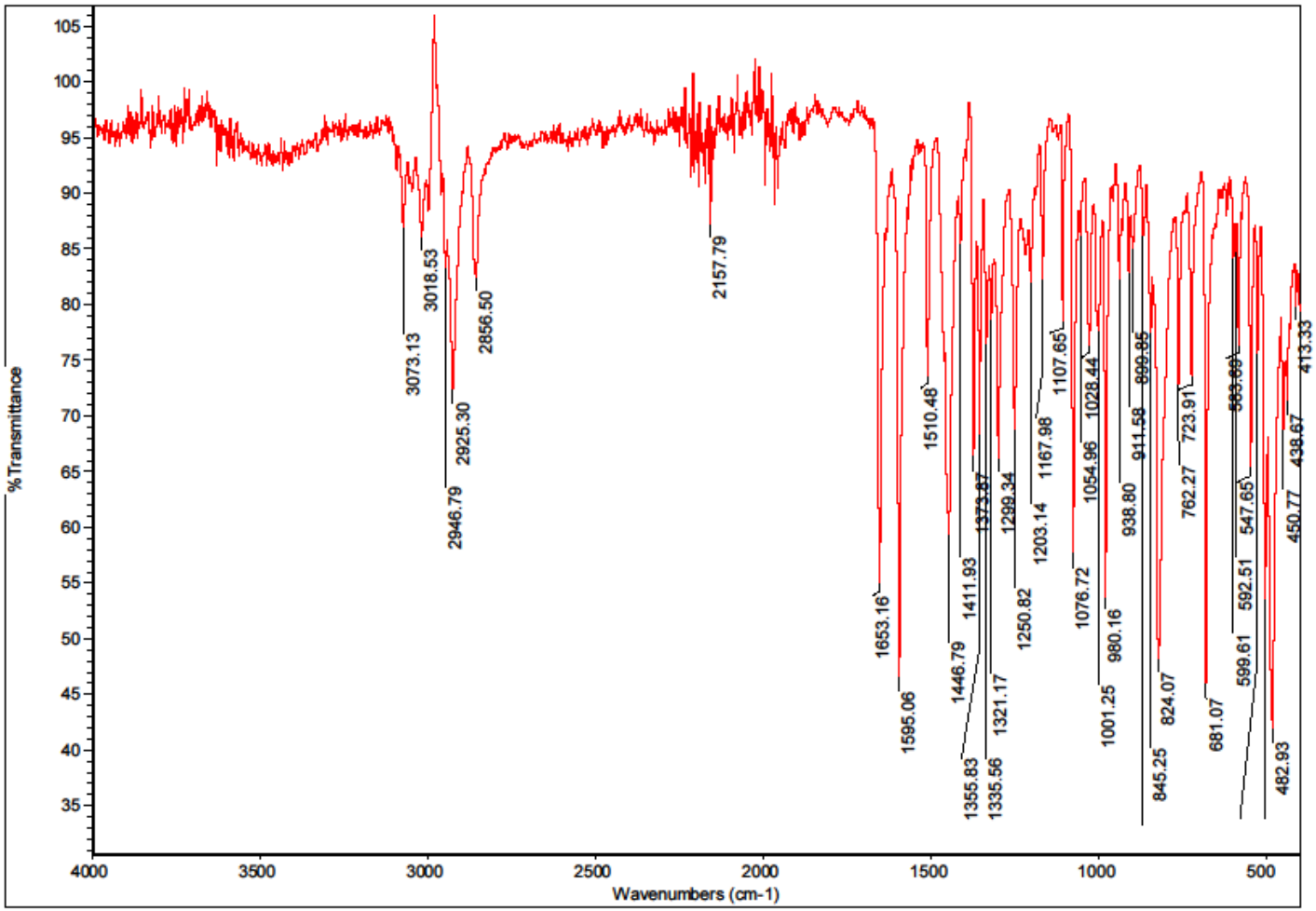


Figure 9-29 *IR spectrum of hexyl ferrocenyl chalcone compound with the iodide component.*

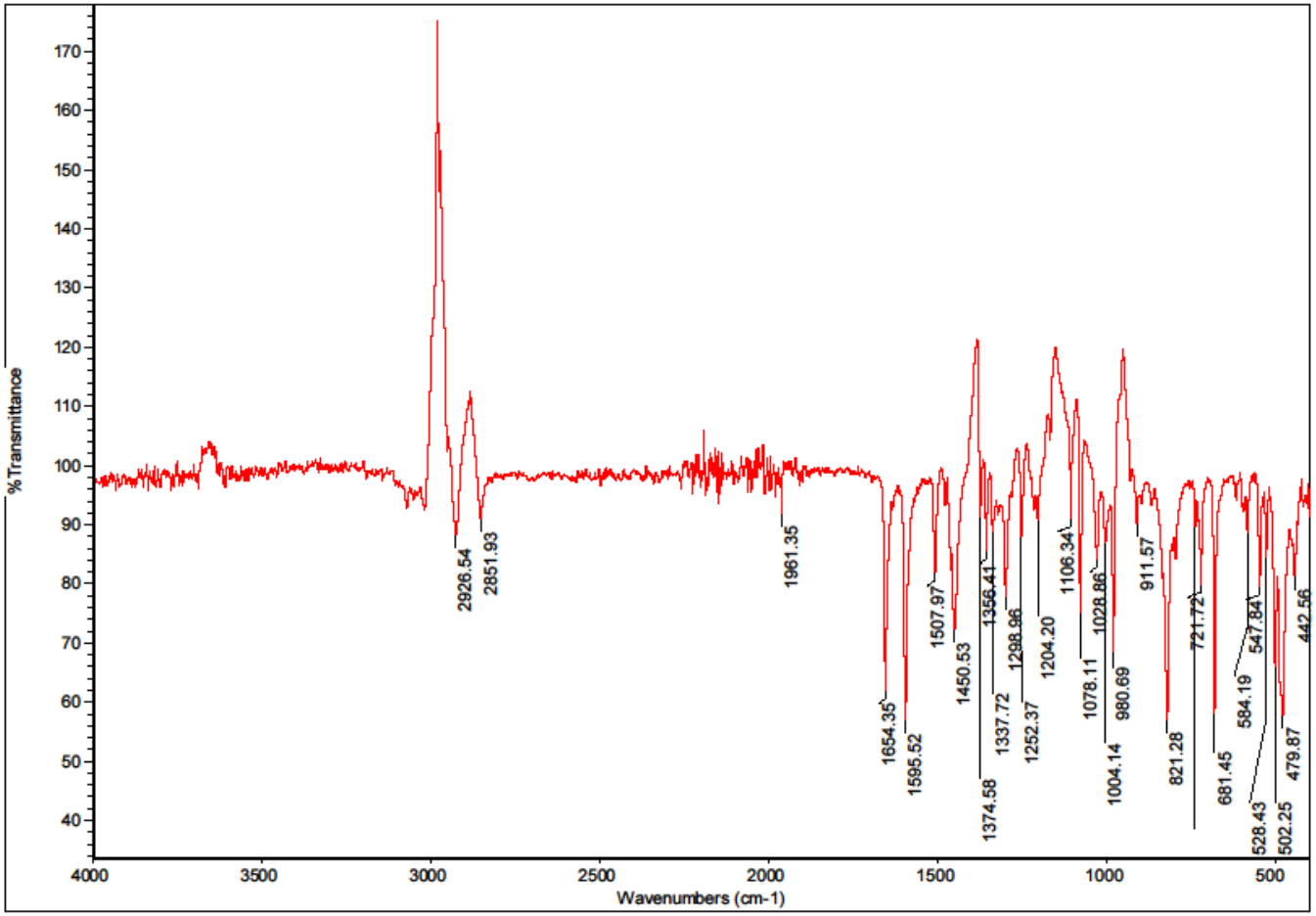


Figure 9-30 *IR spectrum of heptyl ferrocenyl chalcone compound with the iodide component.*

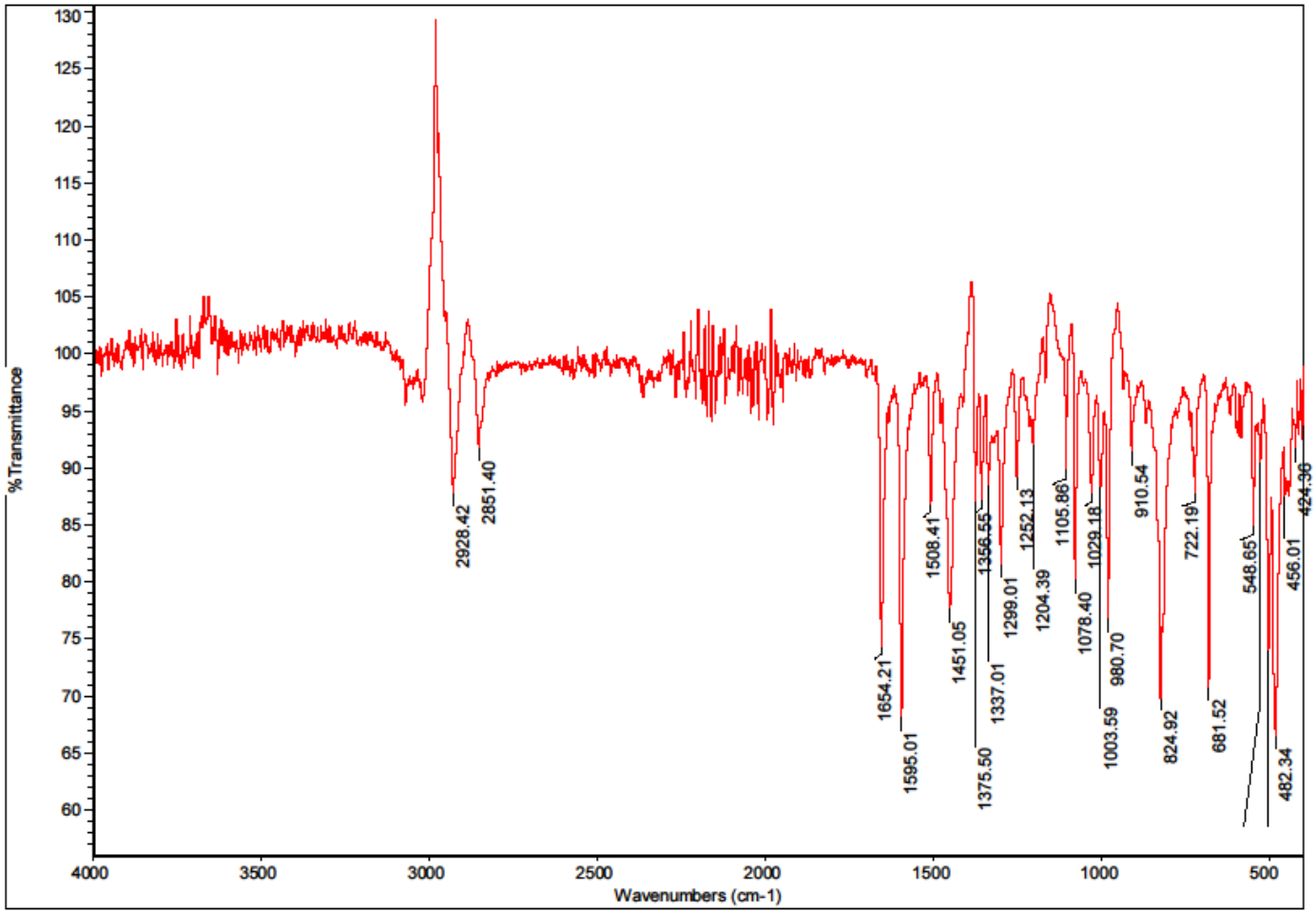


Figure 9-31 *IR spectrum of octyl ferrocenyl chalcone compound with the iodide component.*

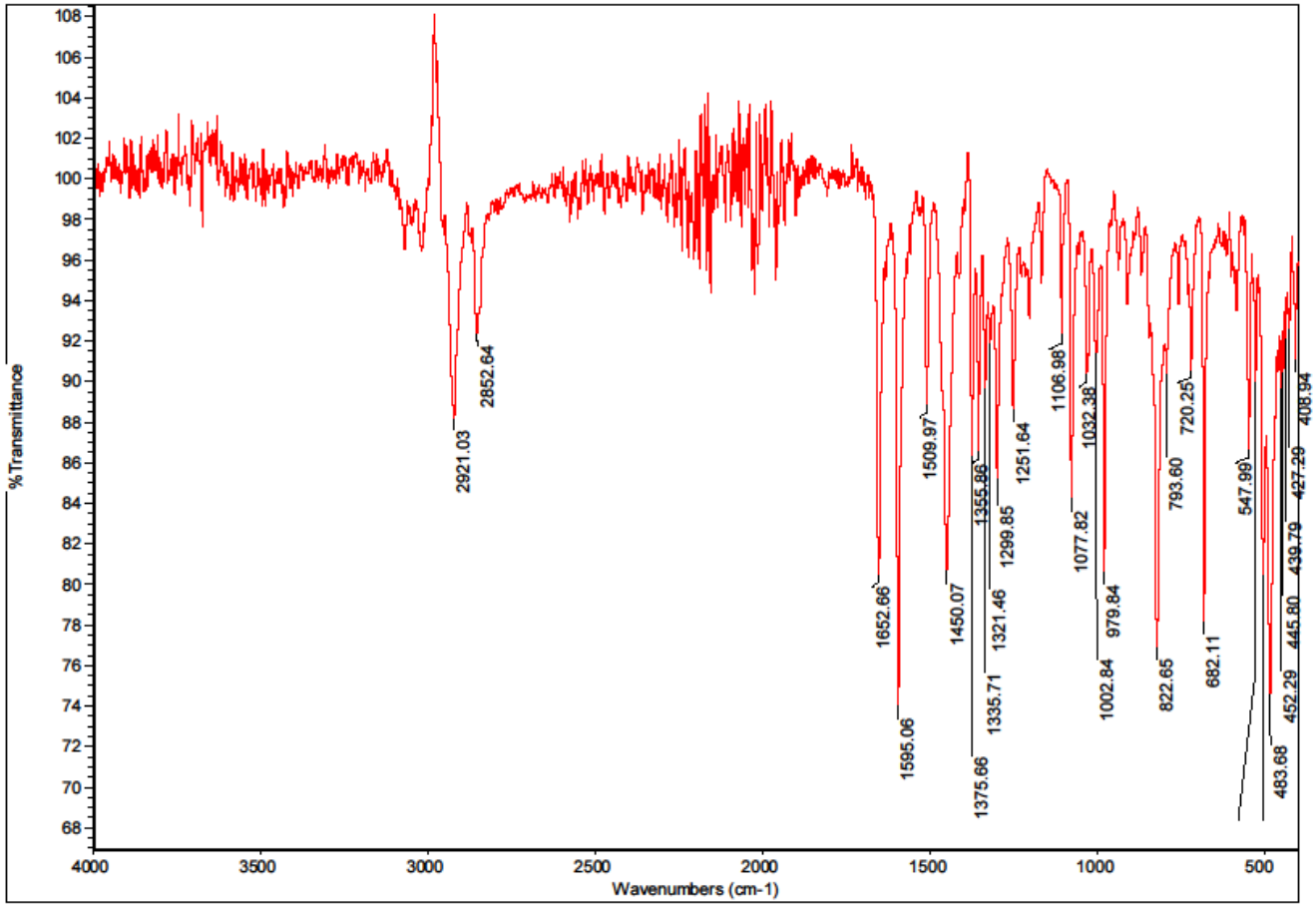


Figure 9-32 *IR spectrum of nonyl ferrocenyl chalcone compound with the iodide component.*

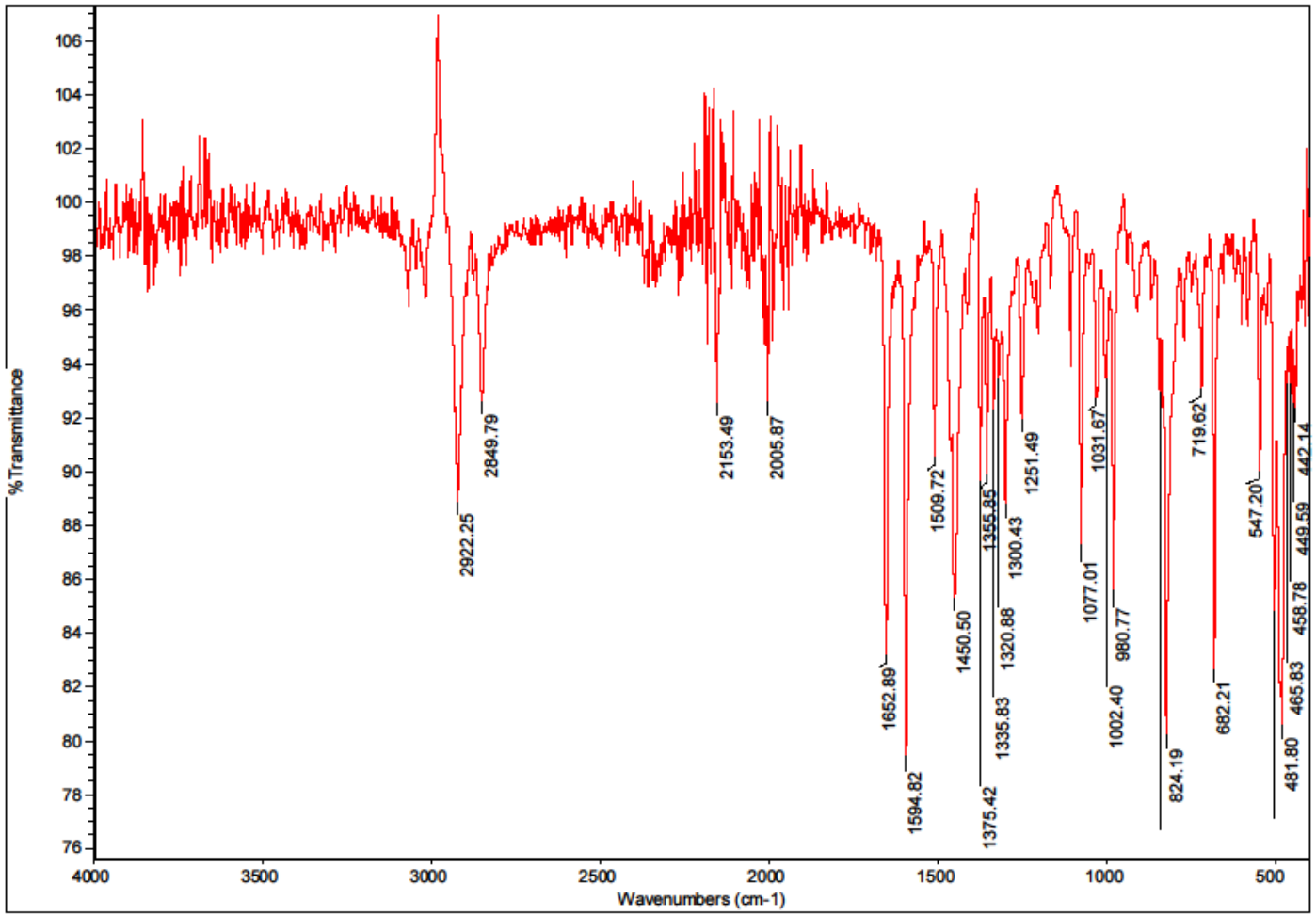


Figure 9-33 *IR spectrum of decyl ferrocenyl chalcone compound with the iodide component.*

9.3 Appendix 3 – Schematics of 2-fold broth microdilution plates

Table 9-2 *Schematics of 2-fold broth microdilution 96-well plate: Ch (n) = initial chalcone dilution (mg/ml).*

	1	2	3	4	5	6	7	8	9	10	11	12
A **Blank**	Ch (1.0) + MHB	Ch (0.5) + MHB	Ch (0.25) + MHB	Ch (0.125) + MHB	Ch (0.063) + MHB	Ch (0.031) + MHB	Ch (0.016) + MHB	Ch (0.008) + MHB	Ch (0.004) + MHB	Ch (0.002) + MHB	ABX + MHB	5% DMSO + MHB
B **Blank**	Ch (1.0) + MHB	Ch (0.5) + MHB	Ch (0.25) + MHB	Ch (0.125) + MHB	Ch (0.063) + MHB	Ch (0.031) + MHB	Ch (0.016) + MHB	Ch (0.008) + MHB	Ch (0.004) + MHB	Ch (0.002) + MHB	ABX + MHB	5% DMSO + MHB
C **Org 1**	Ch (1.0) + Org 1	Ch (0.5) + Org 1	Ch (0.25) + Org 1	Ch (0.125) + Org 1	Ch (0.063) + Org 1	Ch (0.031) + Org 1	Ch (0.016) + Org 1	Ch (0.008) + Org 1	Ch (0.004) + Org 1	Ch (0.002) + Org 1	ABX + Org 1	5% DMSO + Org 1
D **Org 1**	Ch (1.0) + Org 1	Ch (0.5) + Org 1	Ch (0.25) + Org 1	Ch (0.125) + Org 1	Ch (0.063) + Org 1	Ch (0.031) + Org 1	Ch (0.016) + Org 1	Ch (0.008) + Org 1	Ch (0.004) + Org 1	Ch (0.002) + Org 1	ABX + Org 1	5% DMSO + Org 1
E **Org 2**	Ch (1.0) + Org 2	Ch (0.5) + Org 2	Ch (0.25) + Org 2	Ch (0.125) + Org 2	Ch (0.063) + Org 2	Ch (0.031) + Org 2	Ch (0.016) + Org 2	Ch (0.008) + Org 2	Ch (0.004) + Org 2	Ch (0.002) + Org 2	ABX + Org 2	5% DMSO + Org 2
F **Org 2**	Ch (1.0) + Org 2	Ch (0.5) + Org 2	Ch (0.25) + Org 2	Ch (0.125) + Org 2	Ch (0.063) + Org 2	Ch (0.031) + Org 2	Ch (0.016) + Org 2	Ch (0.008) + Org 2	Ch (0.004) + Org 2	Ch (0.002) + Org 2	ABX + Org 2	5% DMSO + Org 2
G **Org 3**	Ch (1.0) + Org 3	Ch (0.5) + Org 3	Ch (0.25) + Org 3	Ch (0.125) + Org 3	Ch (0.063) + Org 3	Ch (0.031) + Org 3	Ch (0.016) + Org 3	Ch (0.008) + Org 3	Ch (0.004) + Org 3	Ch (0.002) + Org 3	ABX + Org 3	5% DMSO + Org 3
H **Org 3**	Ch (1.0) + Org 3	Ch (0.5) + Org 3	Ch (0.25) + Org 3	Ch (0.125) + Org 3	Ch (0.063) + Org 3	Ch (0.031) + Org 3	Ch (0.016) + Org 3	Ch (0.008) + Org 3	Ch (0.004) + Org 3	Ch (0.002) + Org 3	ABX + Org 3	5% DMSO + Org 3

Table 9-3 *Schematics of 2-fold broth microdilution 96-well plate: DMSO (n) = initial DMSO concentration (%).*

	1	2	3	4	5	6	7	8	9	10	11	12
A Blank	DMSO(100) + MHB	DMSO(50) + MHB	DMSO(25) + MHB	DMSO(12.5) + MHB	DMSO(6.3) + MHB	DMSO(3.1) + MHB	DMSO(1.6) + MHB	DMSO(0.8) + MHB	DMSO(0.4) + MHB	DMSO(0.2) + MHB	ABX + MHB	5% DMSO + MHB
B Blank	DMSO(100) + MHB	DMSO(50) + MHB	DMSO(25) + MHB	DMSO(12.5) + MHB	DMSO(6.3) + MHB	DMSO(3.1) + MHB	DMSO(1.6) + MHB	DMSO(0.8) + MHB	DMSO(0.4) + MHB	DMSO(0.2) + MHB	ABX + MHB	5% DMSO + MHB
C Org 1	DMSO(100) + Org 1	DMSO(50) + Org 1	DMSO(25) + Org 1	DMSO(12.5) + Org 1	DMSO(6.3) + Org 1	DMSO(3.1) + Org 1	DMSO(1.6) + Org 1	DMSO(0.8) + Org 1	DMSO(0.4) + Org 1	DMSO(0.2) + Org 1	ABX + Org 1	5% DMSO + Org 1
D Org 1	DMSO(100) + Org 1	DMSO(50) + Org 1	DMSO(25) + Org 1	DMSO(12.5) + Org 1	DMSO(6.3) + Org 1	DMSO(3.1) + Org 1	DMSO(1.6) + Org 1	DMSO(0.8) + Org 1	DMSO(0.4) + Org 1	DMSO(0.2) + Org 1	ABX + Org 1	5% DMSO + Org 1
E Org 2	DMSO(100) + Org 2	DMSO(50) + Org 2	DMSO(25) + Org 2	DMSO(12.5) + Org 2	DMSO(6.3) + Org 2	DMSO(3.1) + Org 2	DMSO(1.6) + Org 2	DMSO(0.8) + Org 2	DMSO(0.4) + Org 2	DMSO(0.2) + Org 2	ABX + Org 2	5% DMSO + Org 2
F Org 2	DMSO(100) + Org 2	DMSO(50) + Org 2	DMSO(25) + Org 2	DMSO(12.5) + Org 2	DMSO(6.3) + Org 2	DMSO(3.1) + Org 2	DMSO(1.6) + Org 2	DMSO(0.8) + Org 2	DMSO(0.4) + Org 2	DMSO(0.2) + Org 2	ABX + Org 2	5% DMSO + Org 2
G Org 3	DMSO(100) + Org 3	DMSO(50) + Org 3	DMSO(25) + Org 3	DMSO(12.5) + Org 3	DMSO(6.3) + Org 3	DMSO(3.1) + Org 3	DMSO(1.6) + Org 3	DMSO(0.8) + Org 3	DMSO(0.4) + Org 3	DMSO(0.2) + Org 3	ABX + Org 3	5% DMSO + Org 3
H Org 3	DMSO(100) + Org 3	DMSO(50) + Org 3	DMSO(25) + Org 3	DMSO(12.5) + Org 3	DMSO(6.3) + Org 3	DMSO(3.1) + Org 3	DMSO(1.6) + Org 3	DMSO(0.8) + Org 3	DMSO(0.4) + Org 3	DMSO(0.2) + Org 3	ABX + Org 3	5% DMSO + Org 3

9.4 Appendix 4 – Standard curves of test organisms in MIC determination

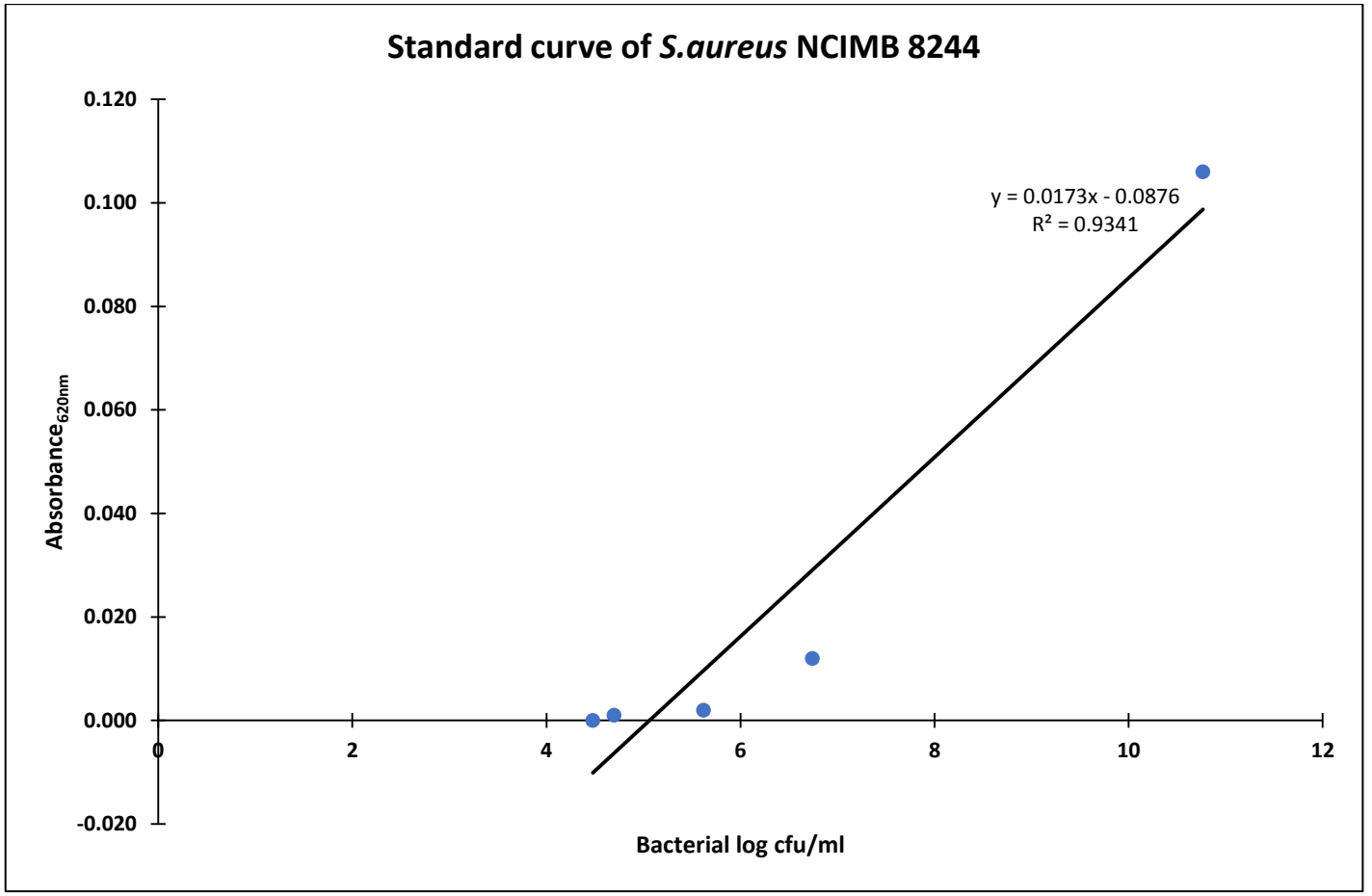


Figure 9-34 *Broth microdilution standard curve of S. aureus NCIMB 8244.*

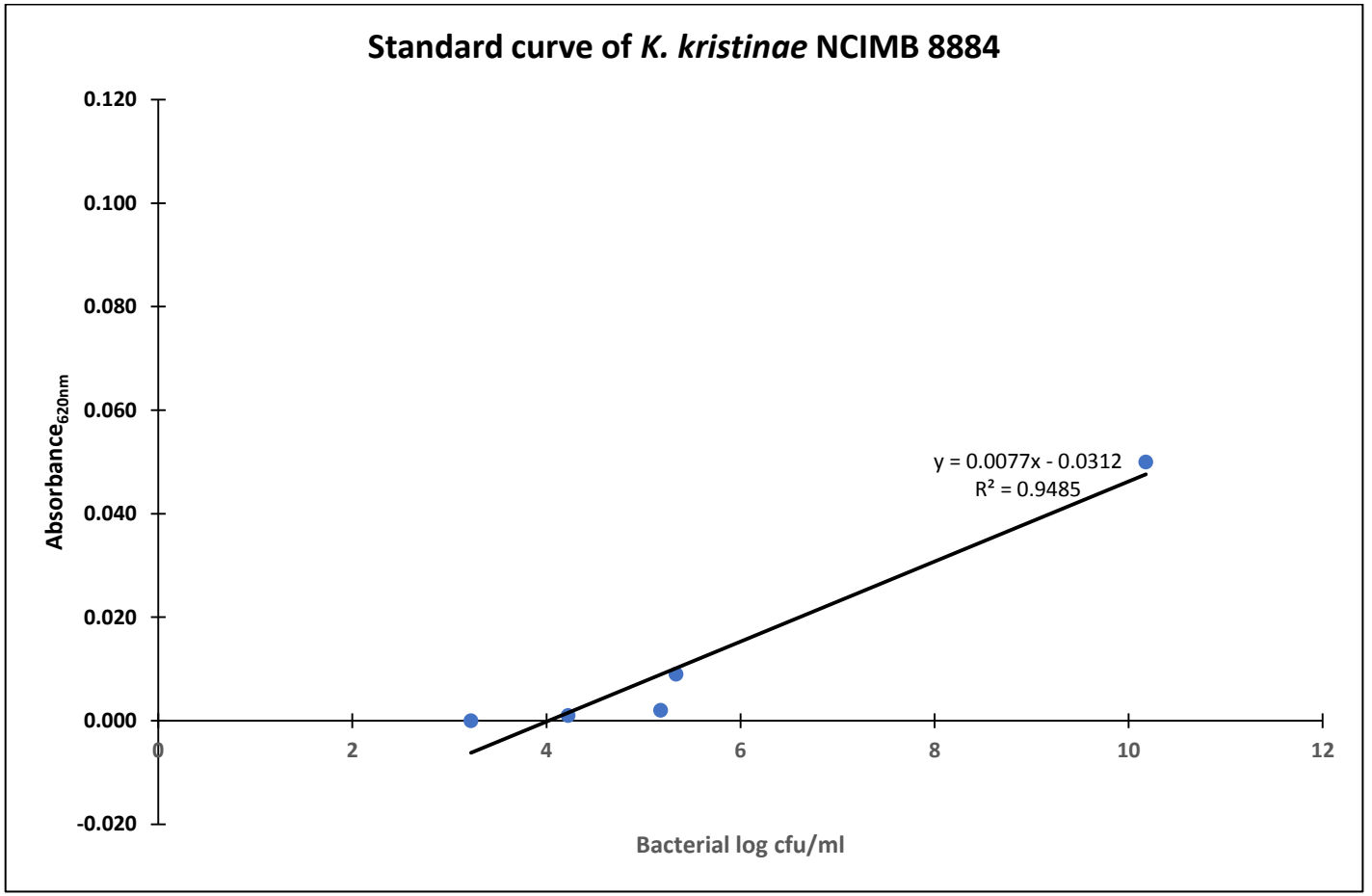


Figure 9-35 *Broth microdilution standard curve of K. kristinae NCMIB 8884.*

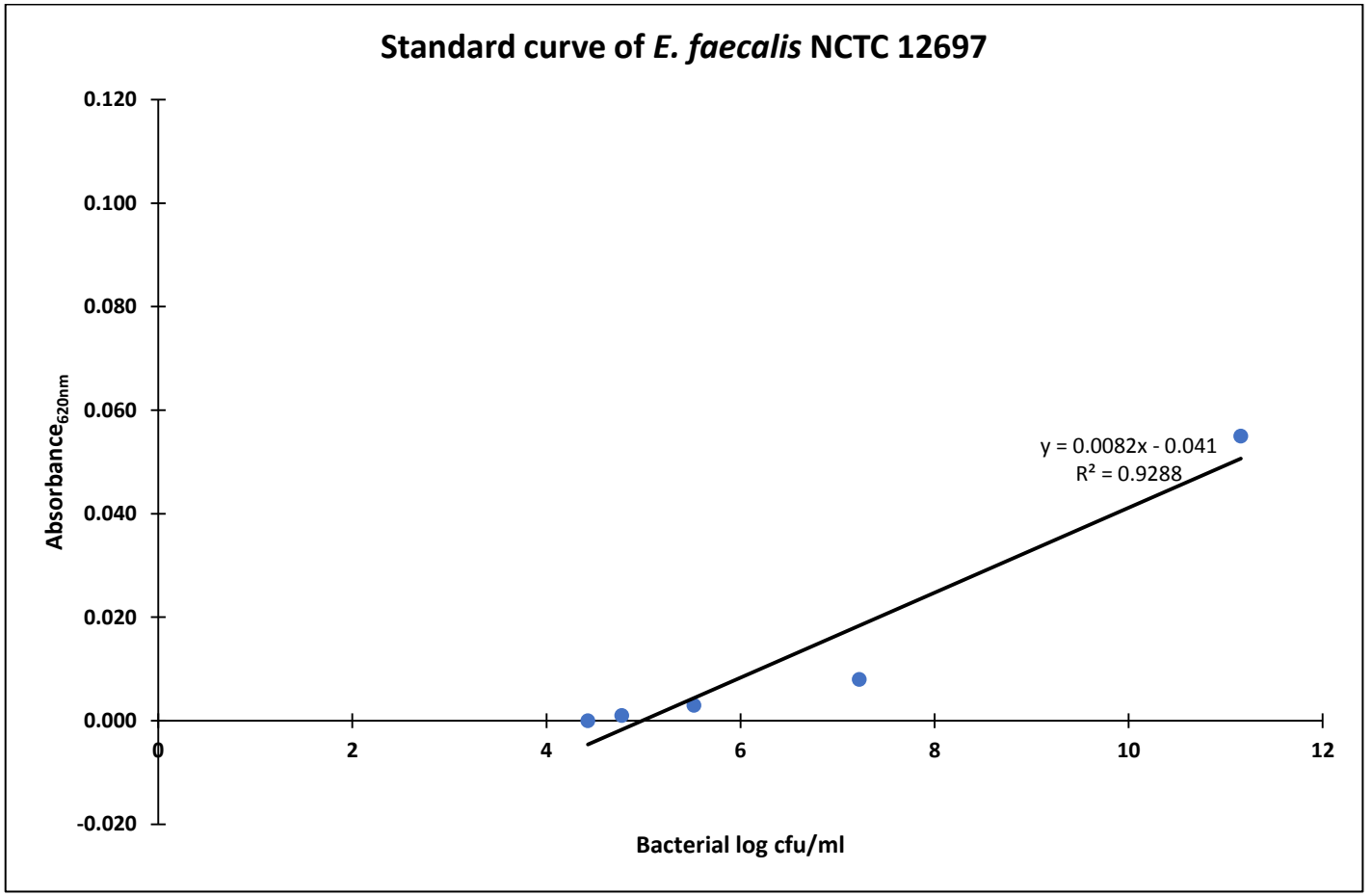


Figure 9-36 *Broth microdilution standard curve E. faecalis NCTC 12697.*

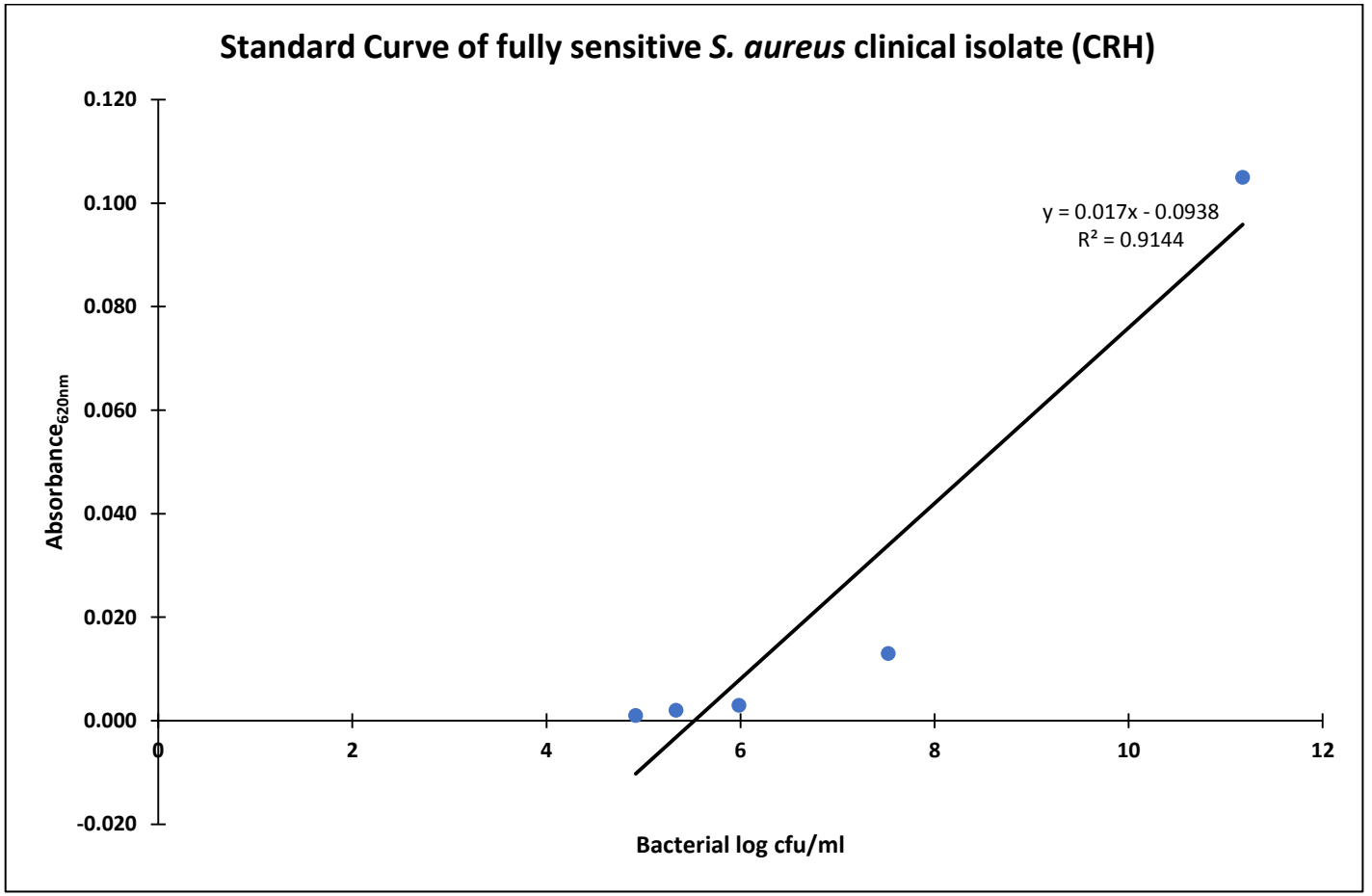


Figure 9-37 *Broth microdilution standard curve of fully sensitive S. aureus (CRH).*

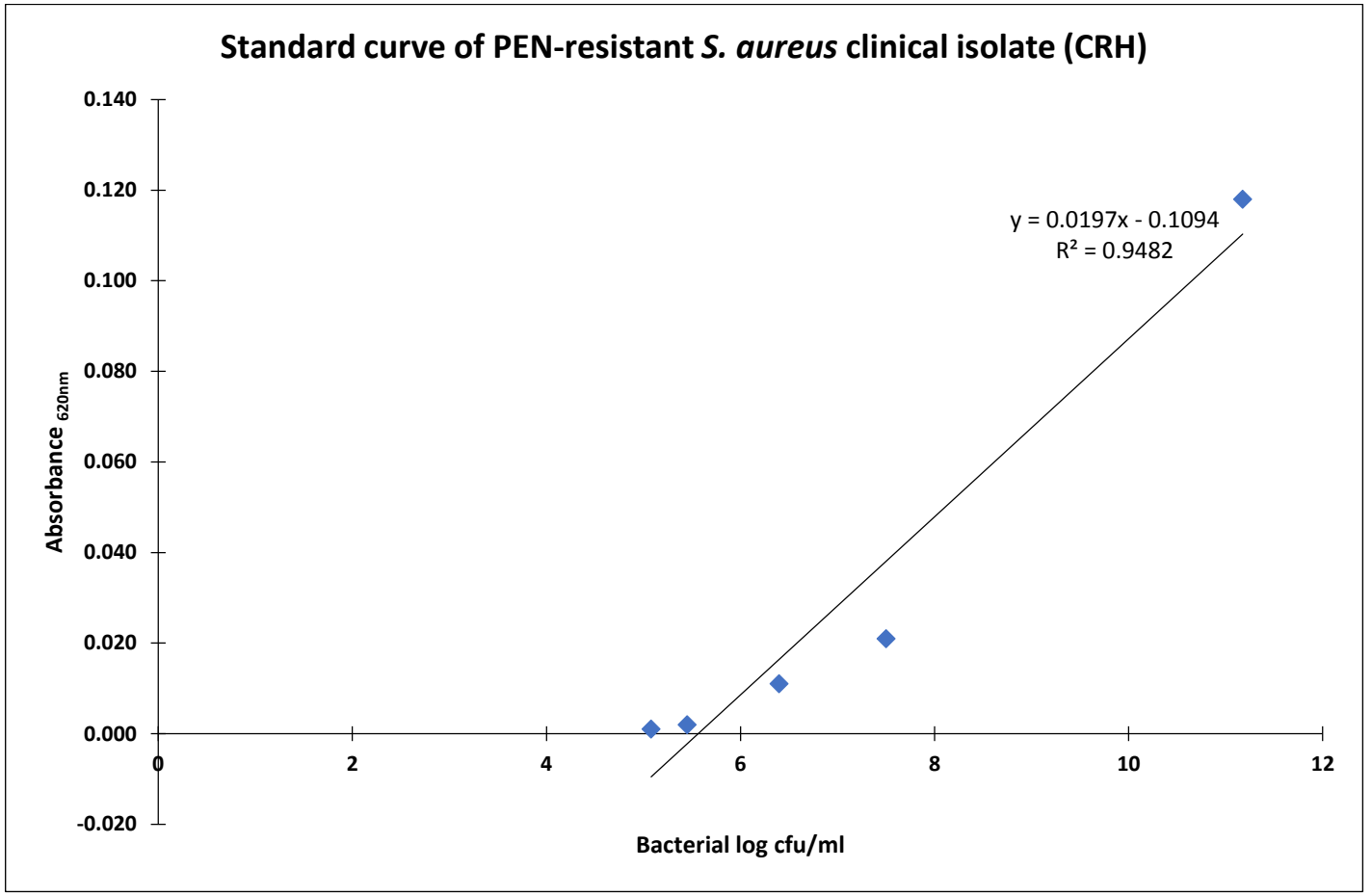


Figure 9-38 *Broth microdilution standard curve of PEN-resistant S. aureus (CRH).*

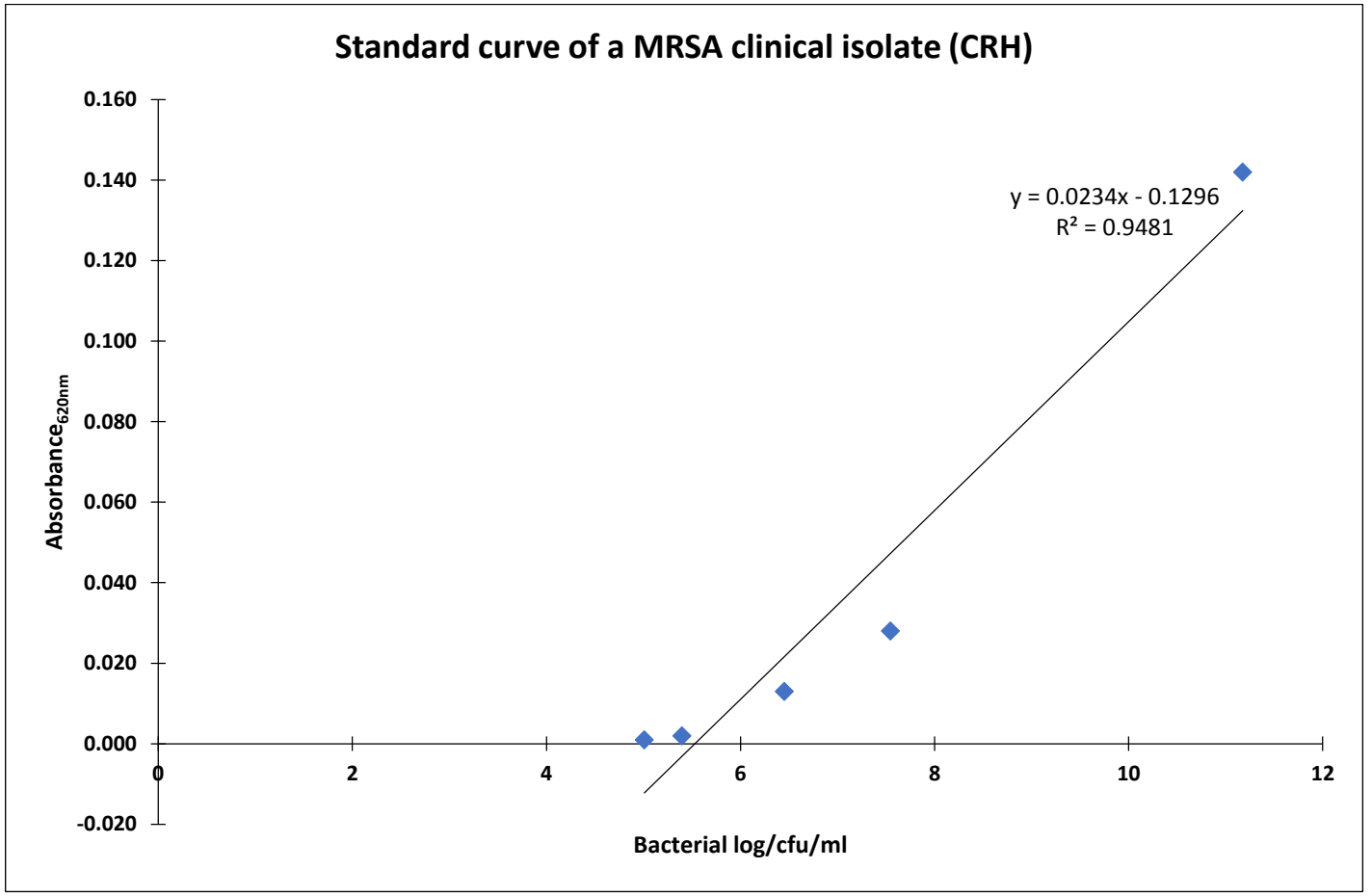


Figure 9-39 *Broth microdilution standard curve of a MRSA (CRH).*

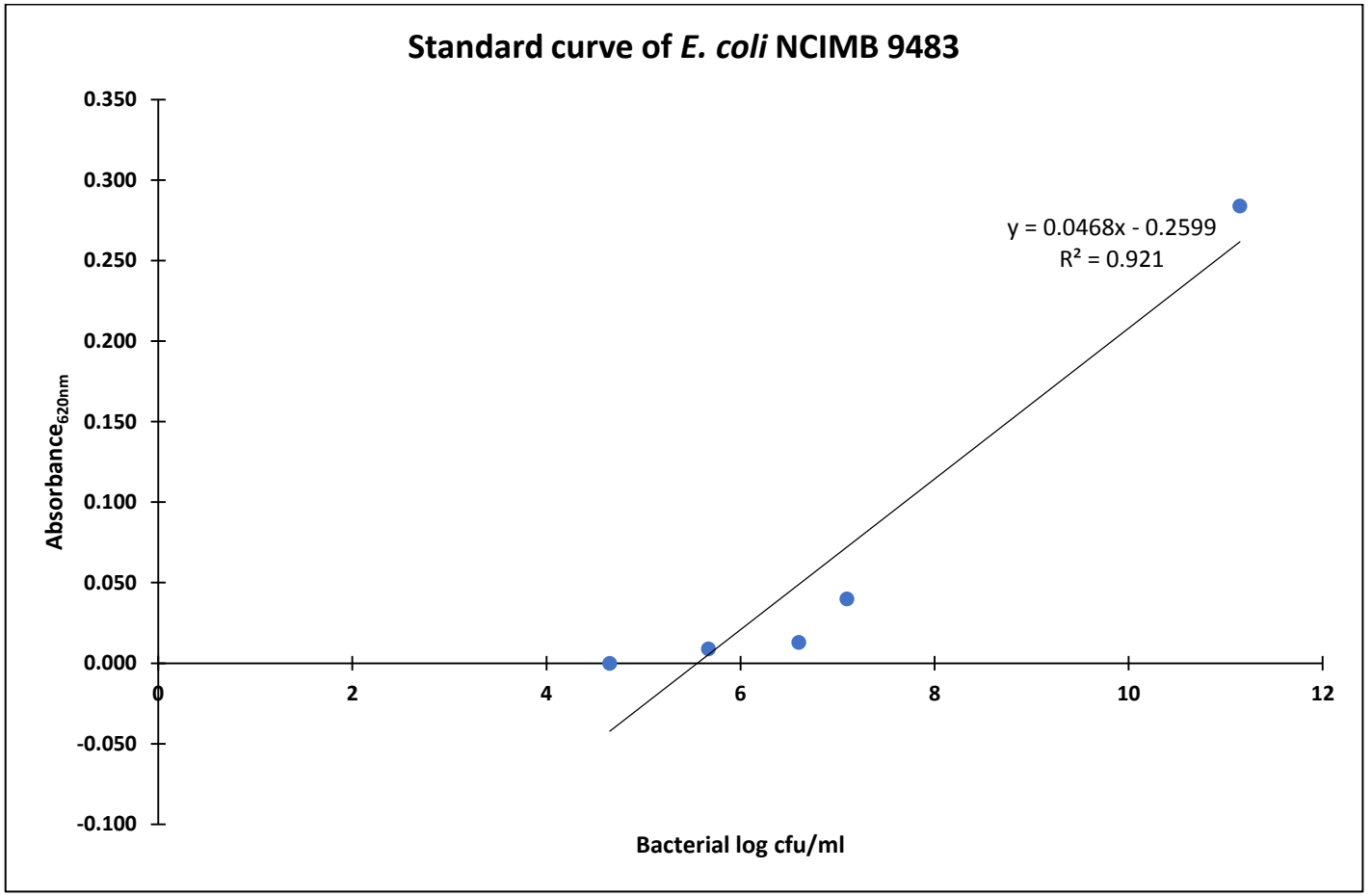


Figure 9-40 *Broth microdilution standard curve of E. coli NCIMB 9483.*

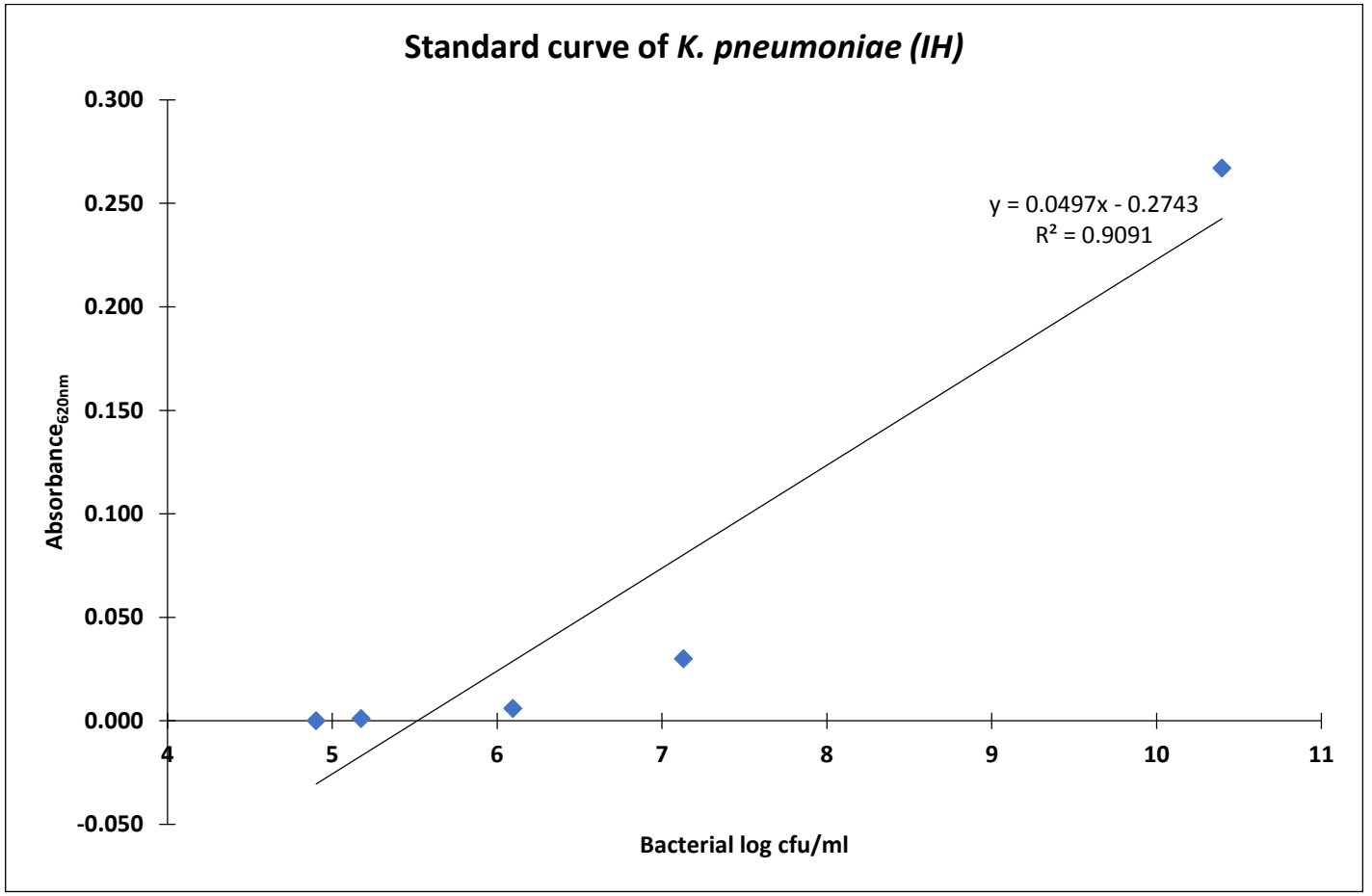


Figure 9-41 *Broth microdilution standard curve of K. pneumonia (IH).*

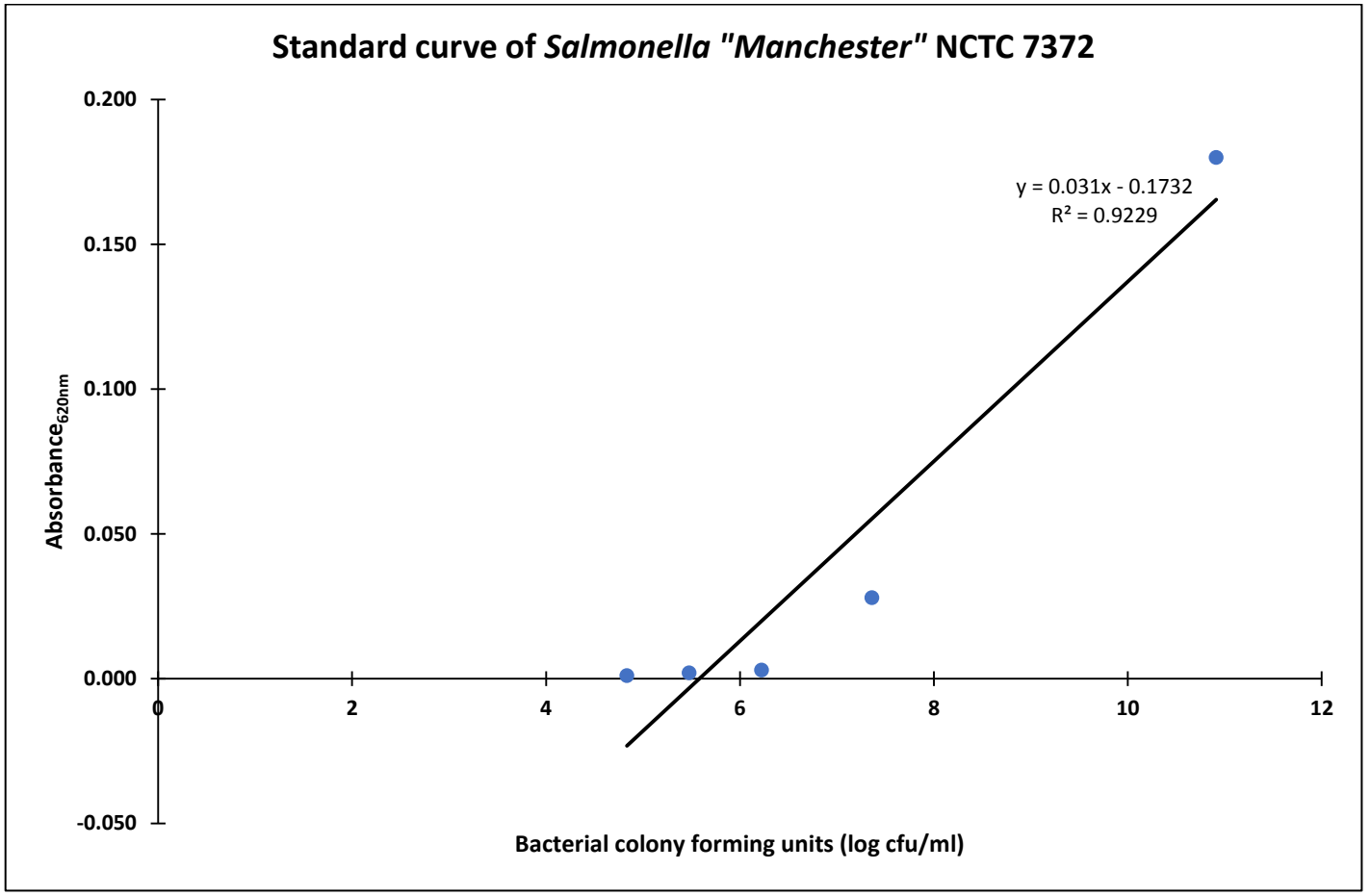


Figure 9-42 *Broth microdilution standard curve of Salmonella "Manchester" NCTC 7372.*

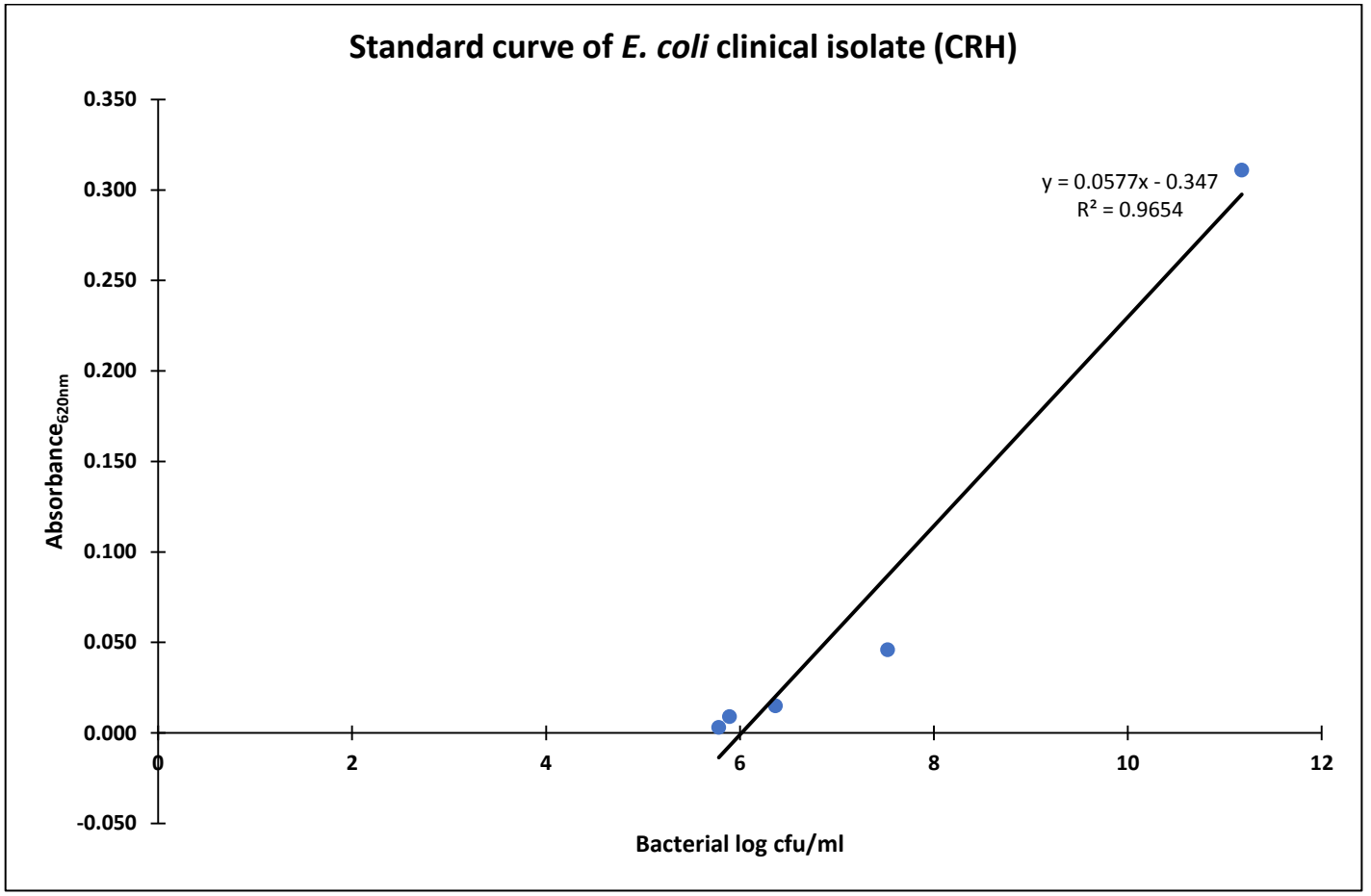


Figure 9-43 *Broth microdilution standard curve of E. coli (CRH).*

9.5 Appendix 5 – Estimations of RA of each ferrocenyl chalcone based on type of vessel and environmental condition

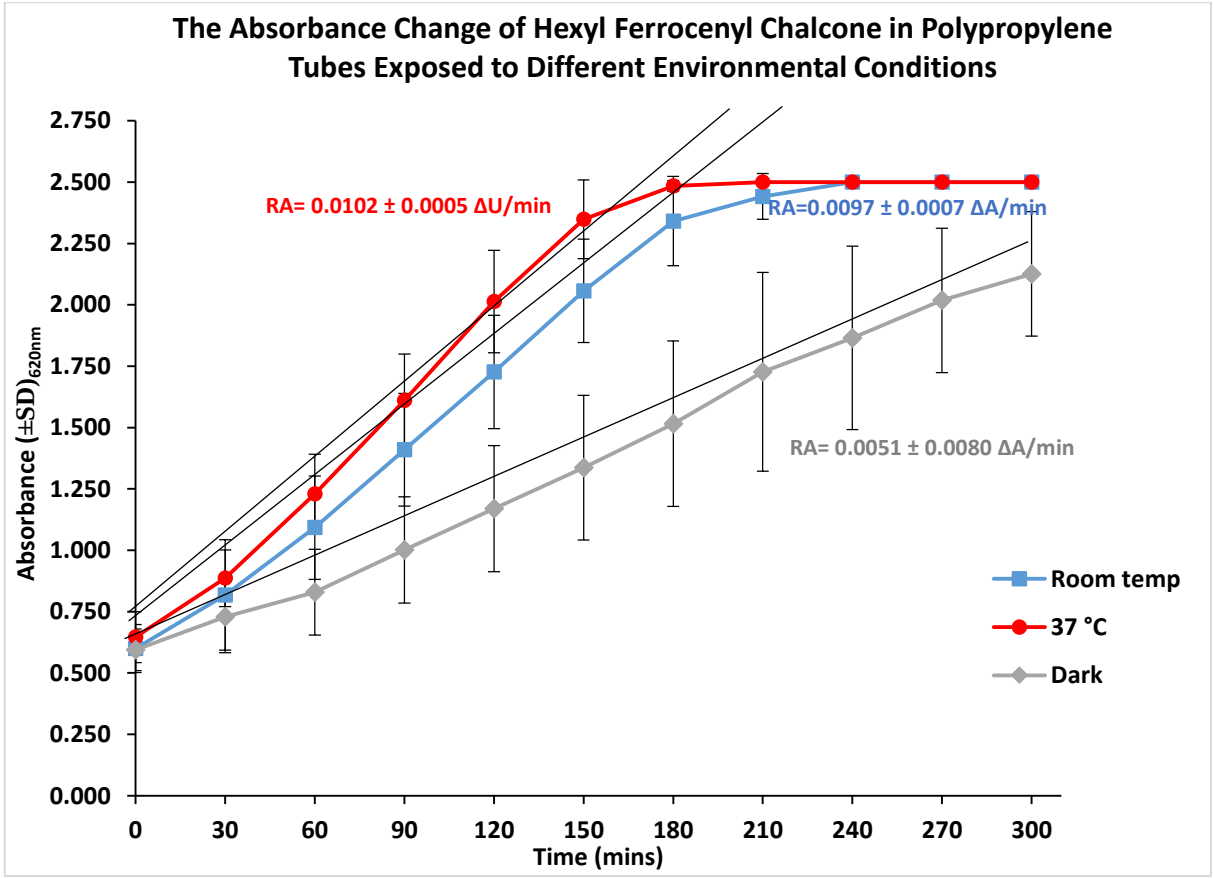


Figure 9-44 *– Mean (± SD) absorbance values of hexyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

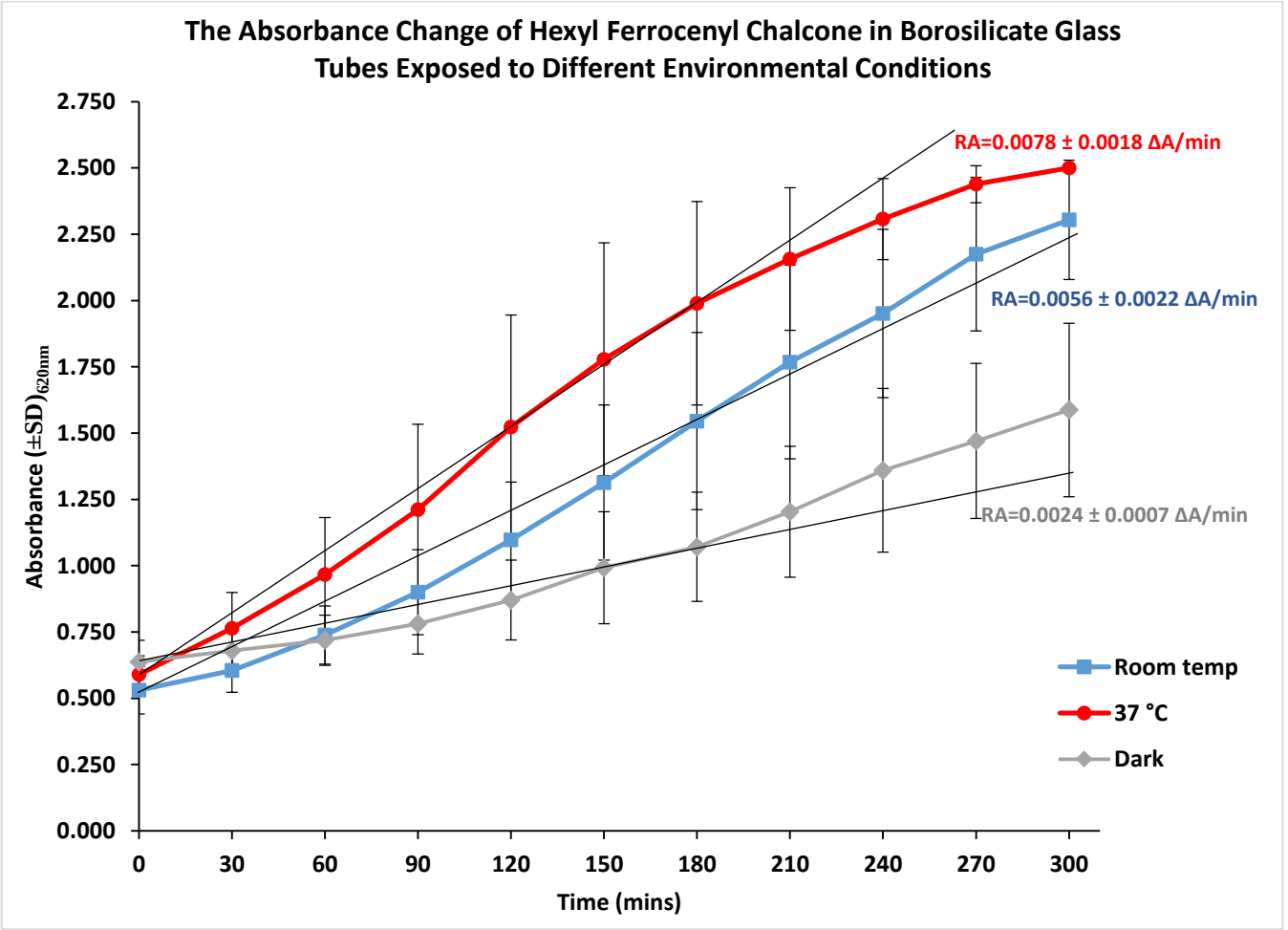


Figure 9-45 *– Mean (± SD) absorbance values of hexyl ferrocenyl chalcone in borosilicate glass tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

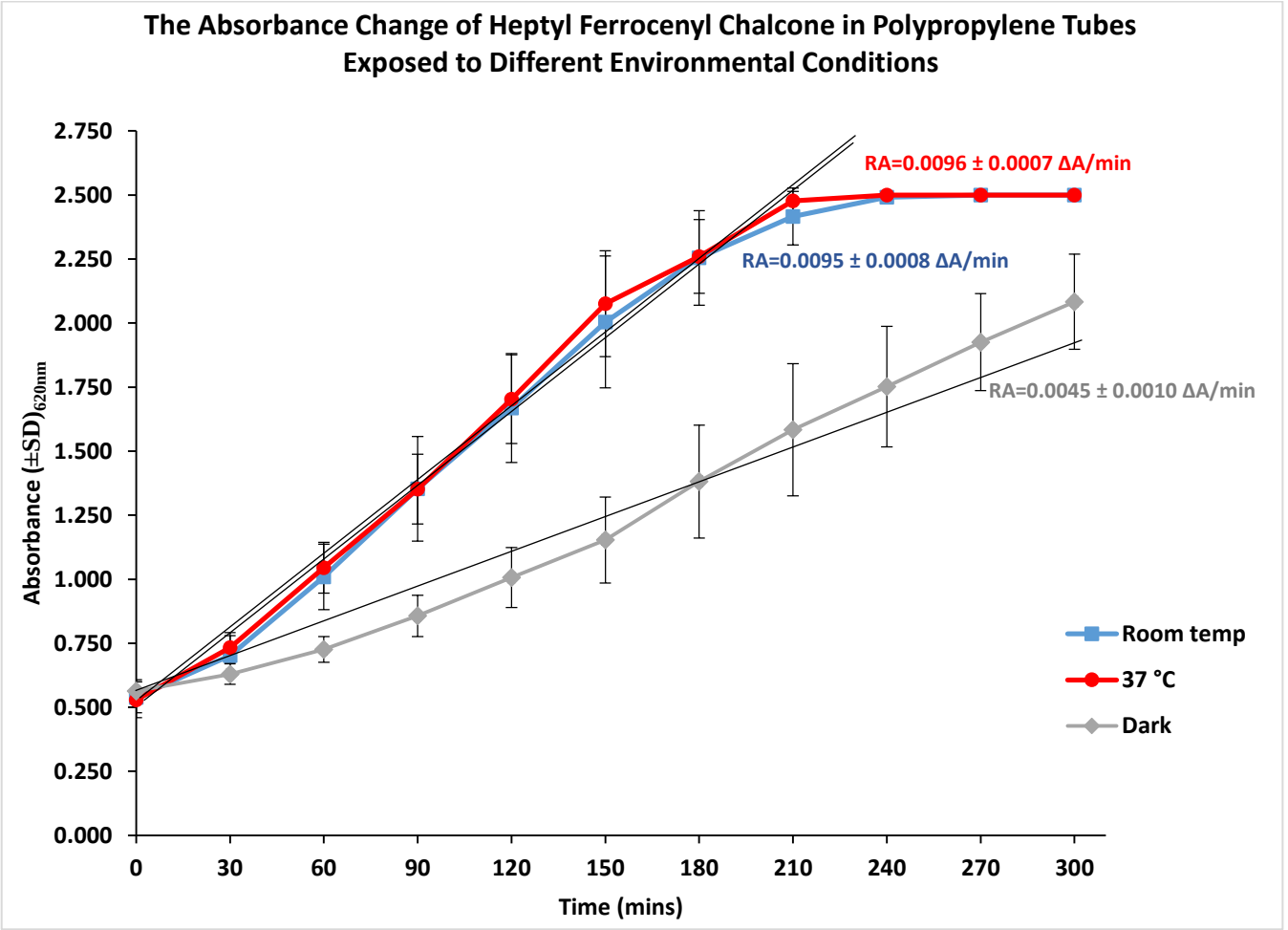


Figure 9-46 *– Mean (± SD) absorbance values of heptyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

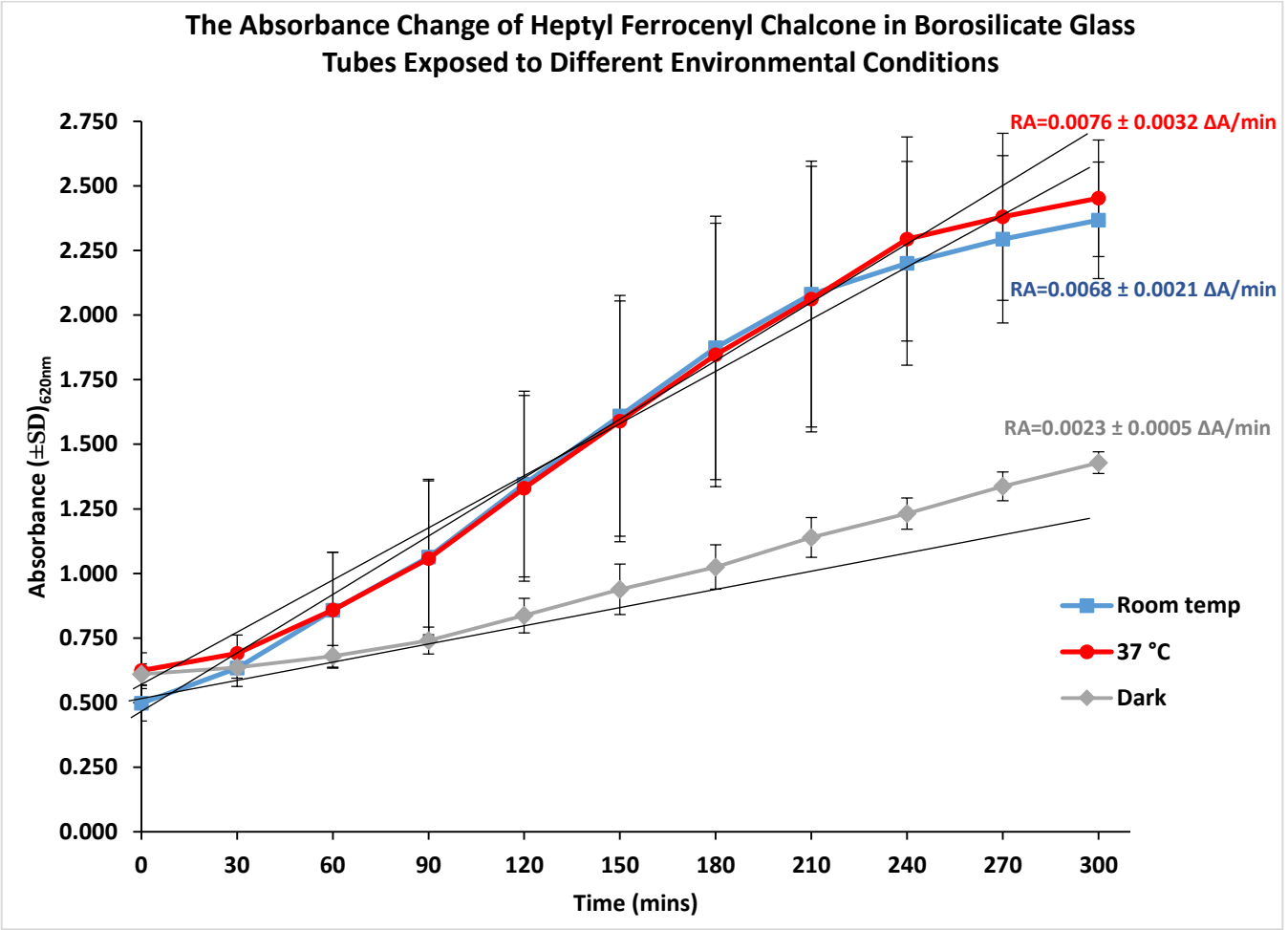


Figure 9-47 *– Mean (± SD) absorbance values of heptyl ferrocenyl chalcone in borosilicate glass tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

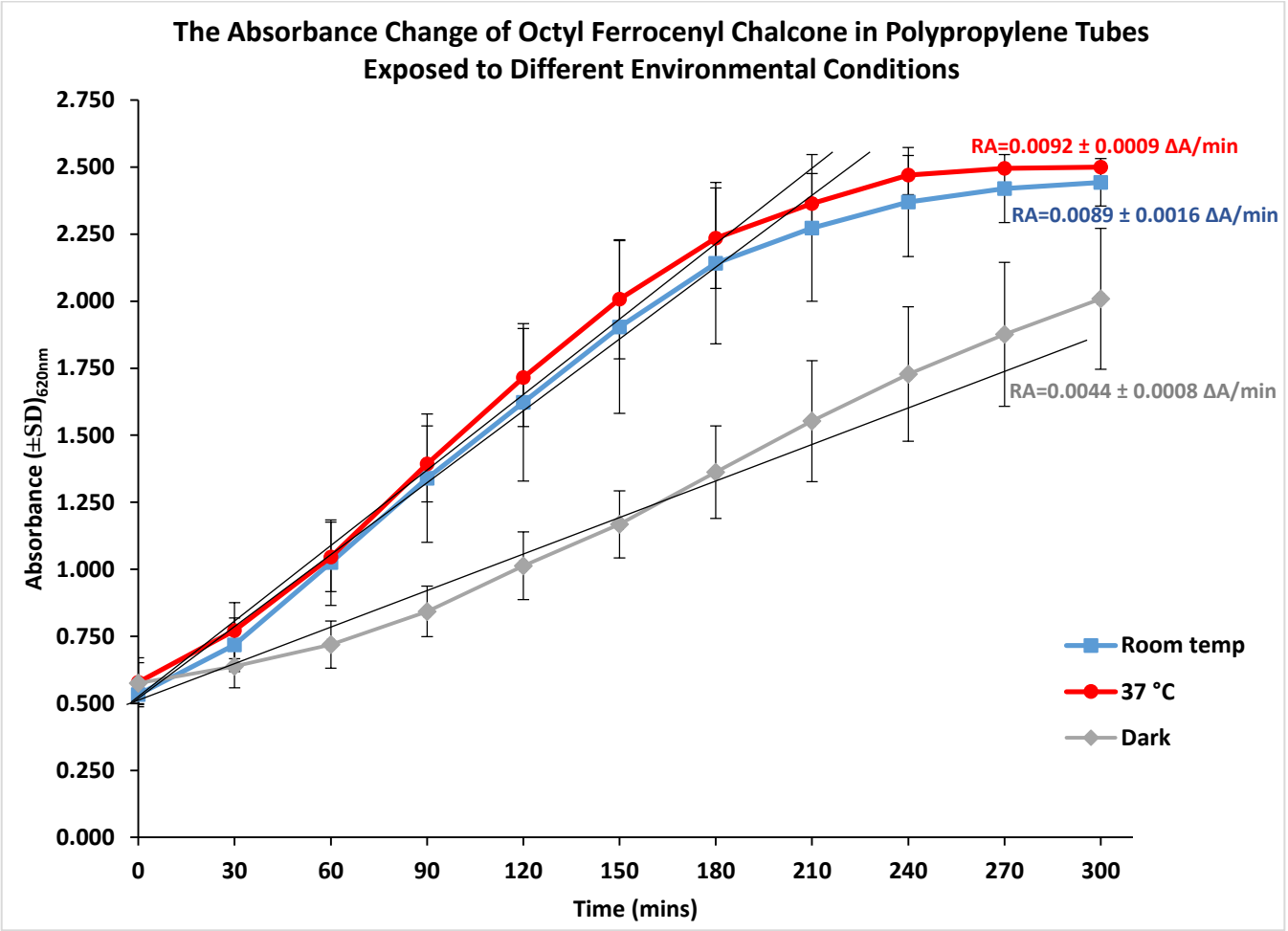


Figure 9-48 *– Mean (± SD) absorbance values of octyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

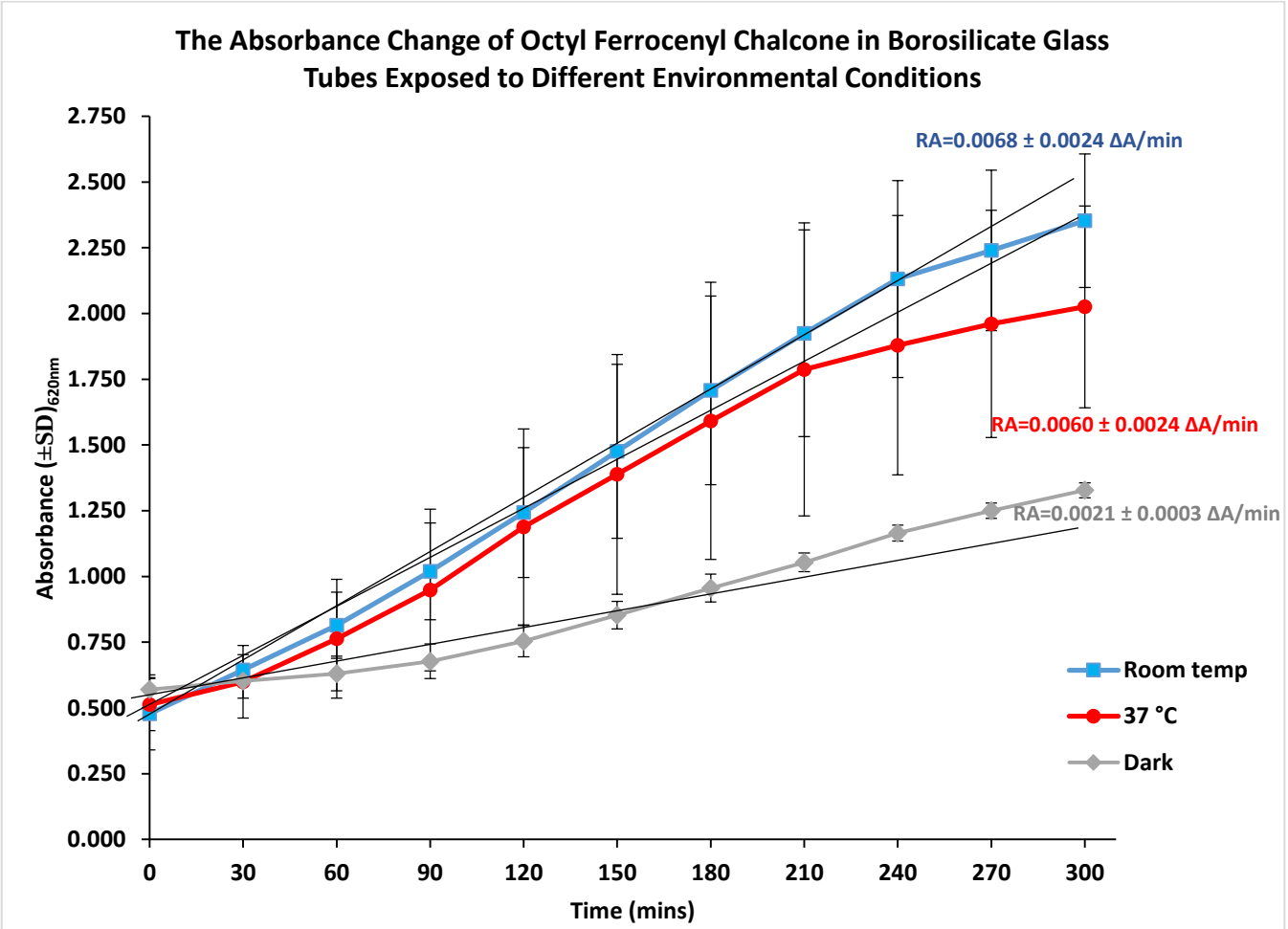


Figure 9-49 *– Mean (± SD) absorbance values of octyl ferrocenyl chalcone in borosilicate glass tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

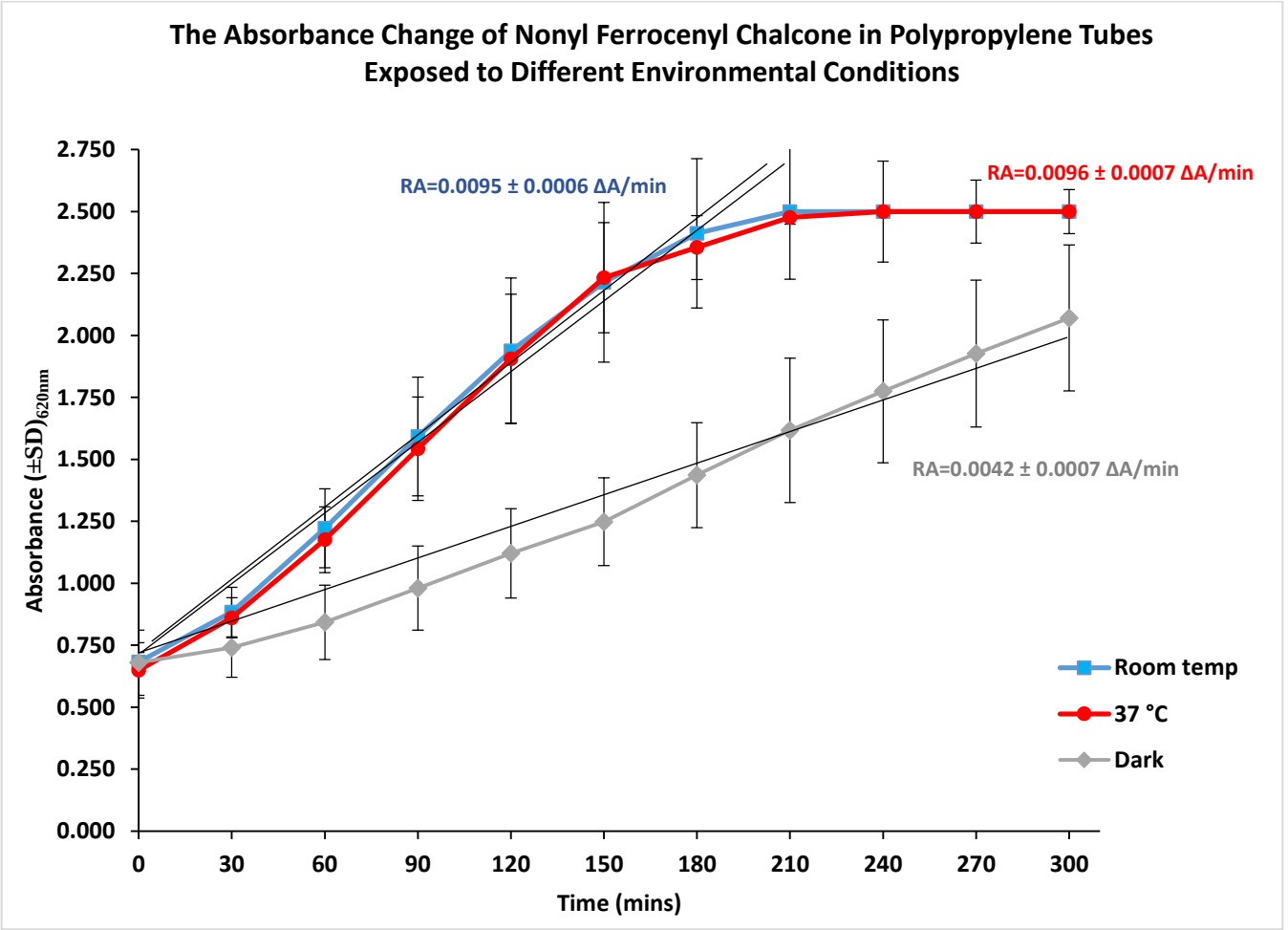


Figure 9-50 *– Mean (± SD) absorbance values of nonyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

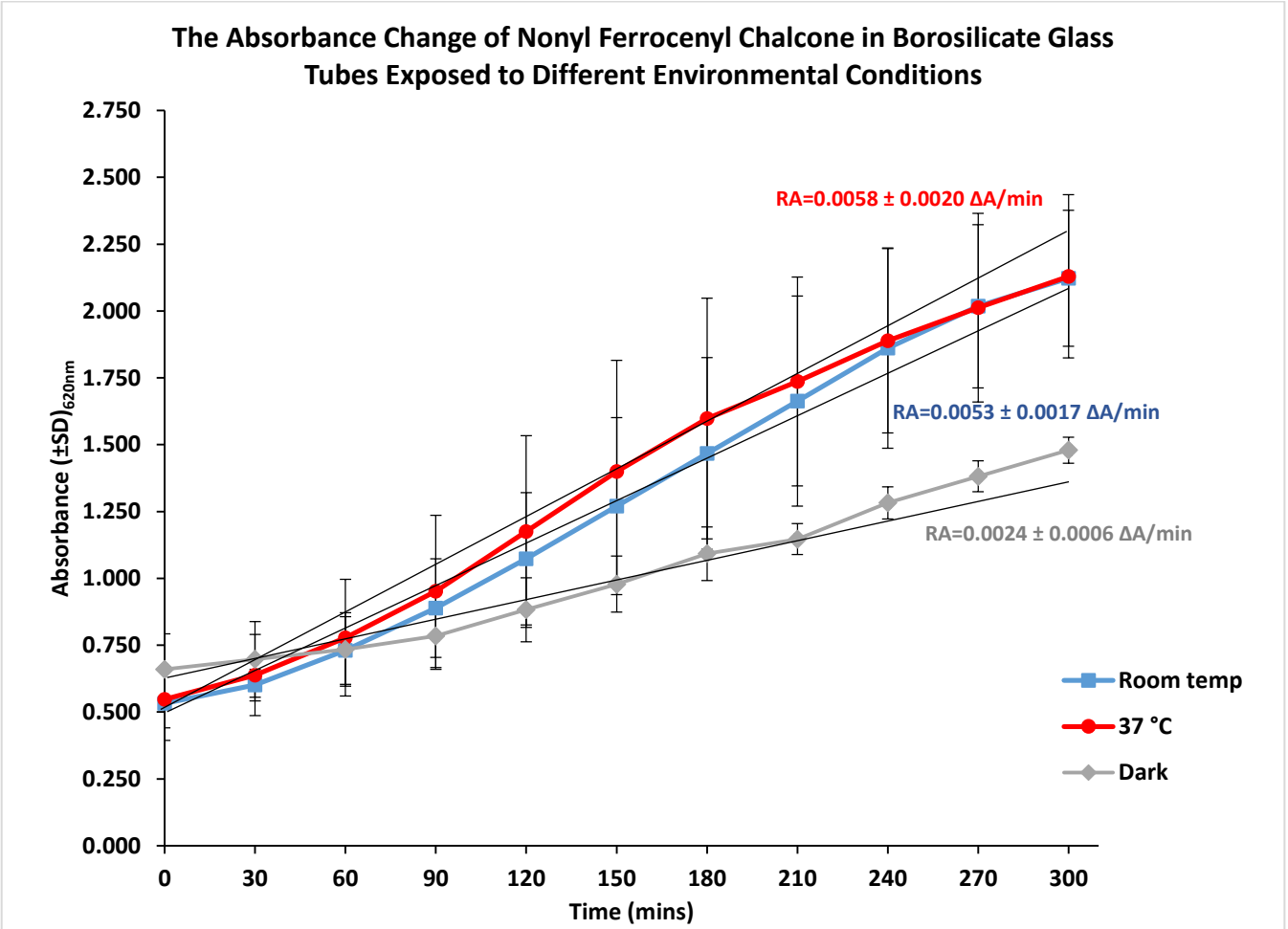


Figure 9-51 *Mean (± SD) absorbance values of nonyl ferrocenyl chalcone in borosilicate glass tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

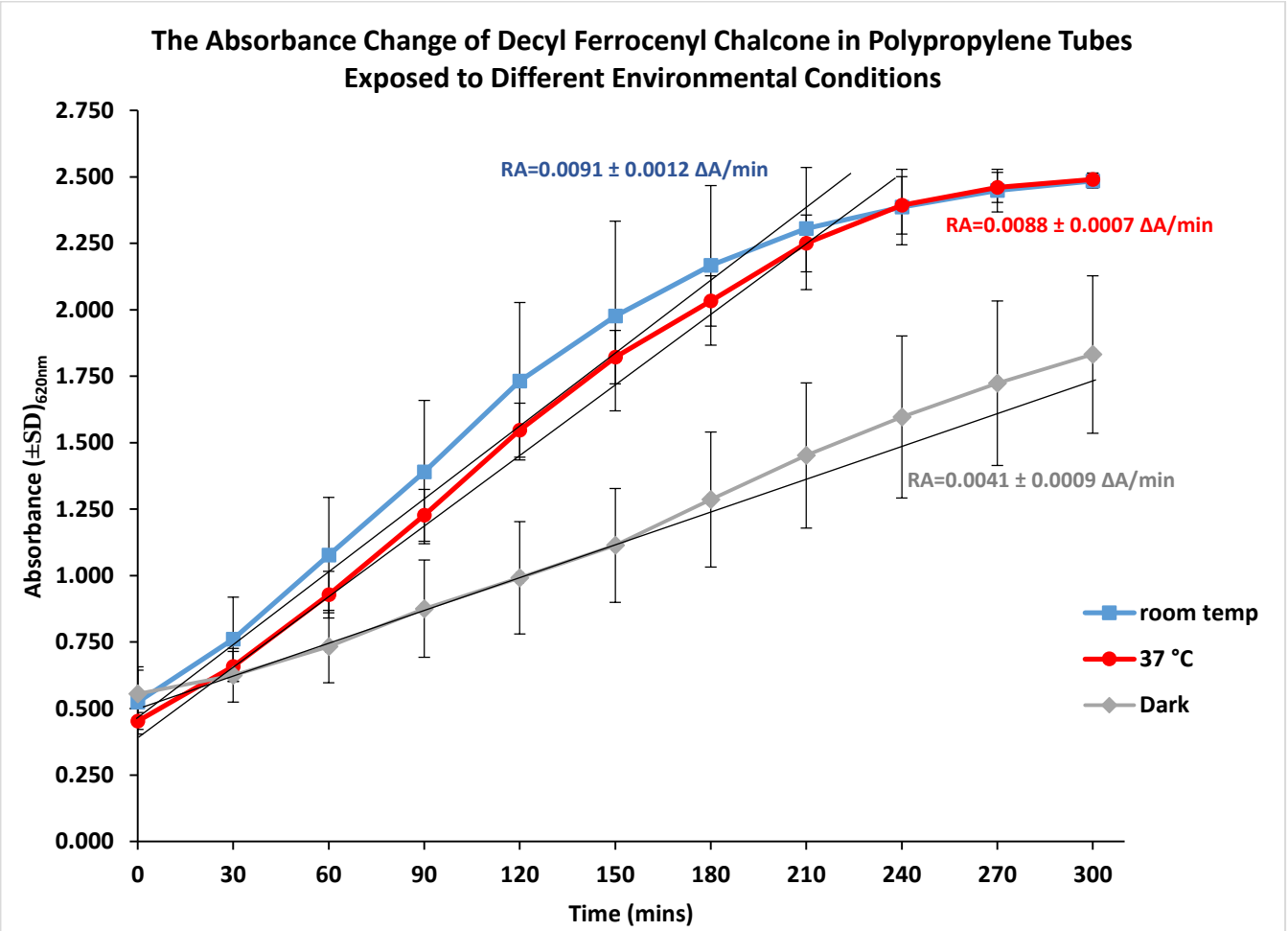


Figure 9-52 *– Mean (± SD) absorbance values of decyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016).*

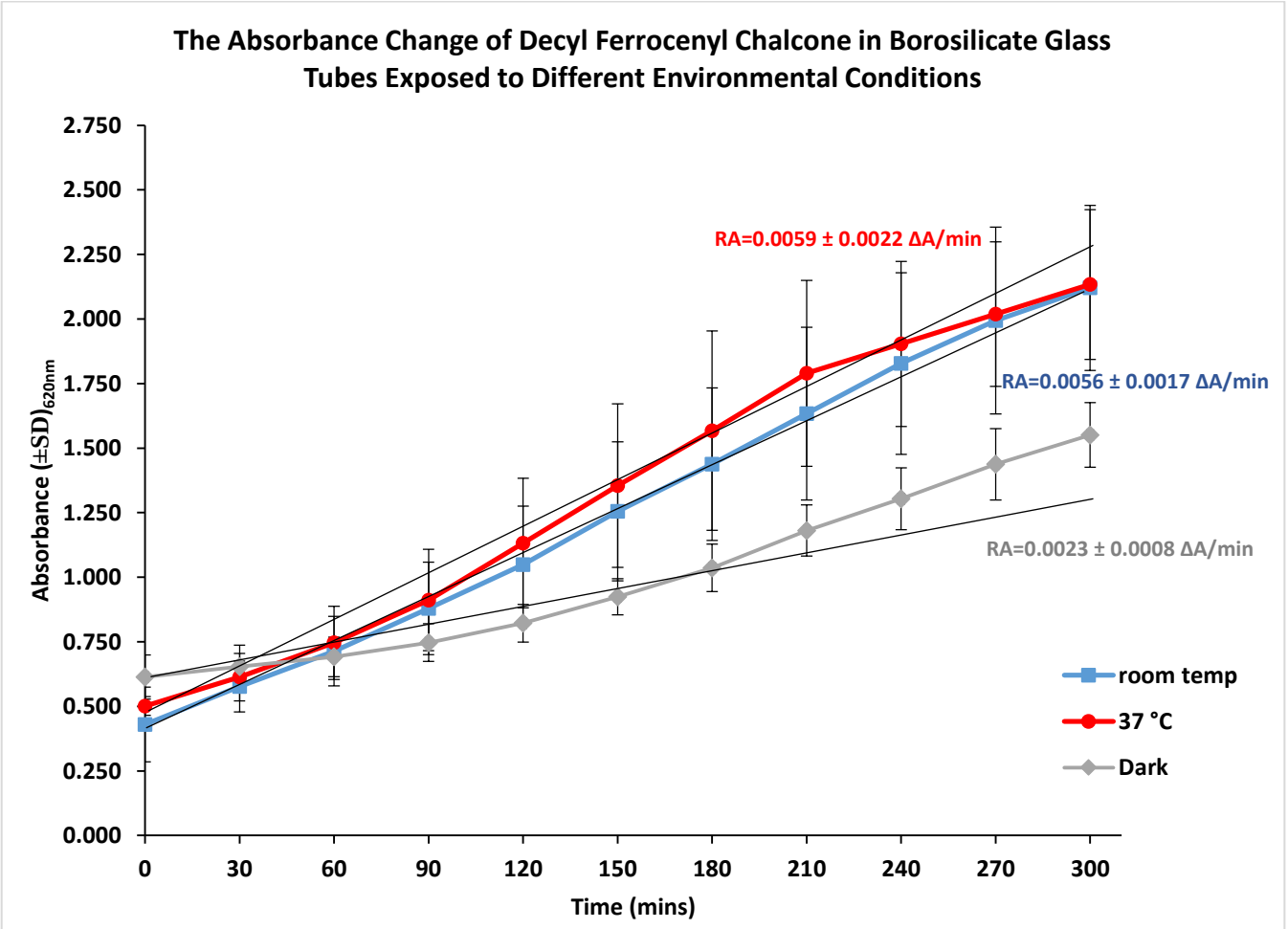


Figure 9-53 *– Mean (± SD) absorbance values of dexyl ferrocenyl chalcone in polypropylene tubes exposed to three different environmental conditions (room temperature and light, 37°C, room temperature in darkness) over time. RA (ΔU/min) from 0 to 180 minutes (reproduced with permission from Bennett, 2016)*

9.6 Appendix 6 – Standard curves of test organisms and One-way ANOVA data tables in MTT assay

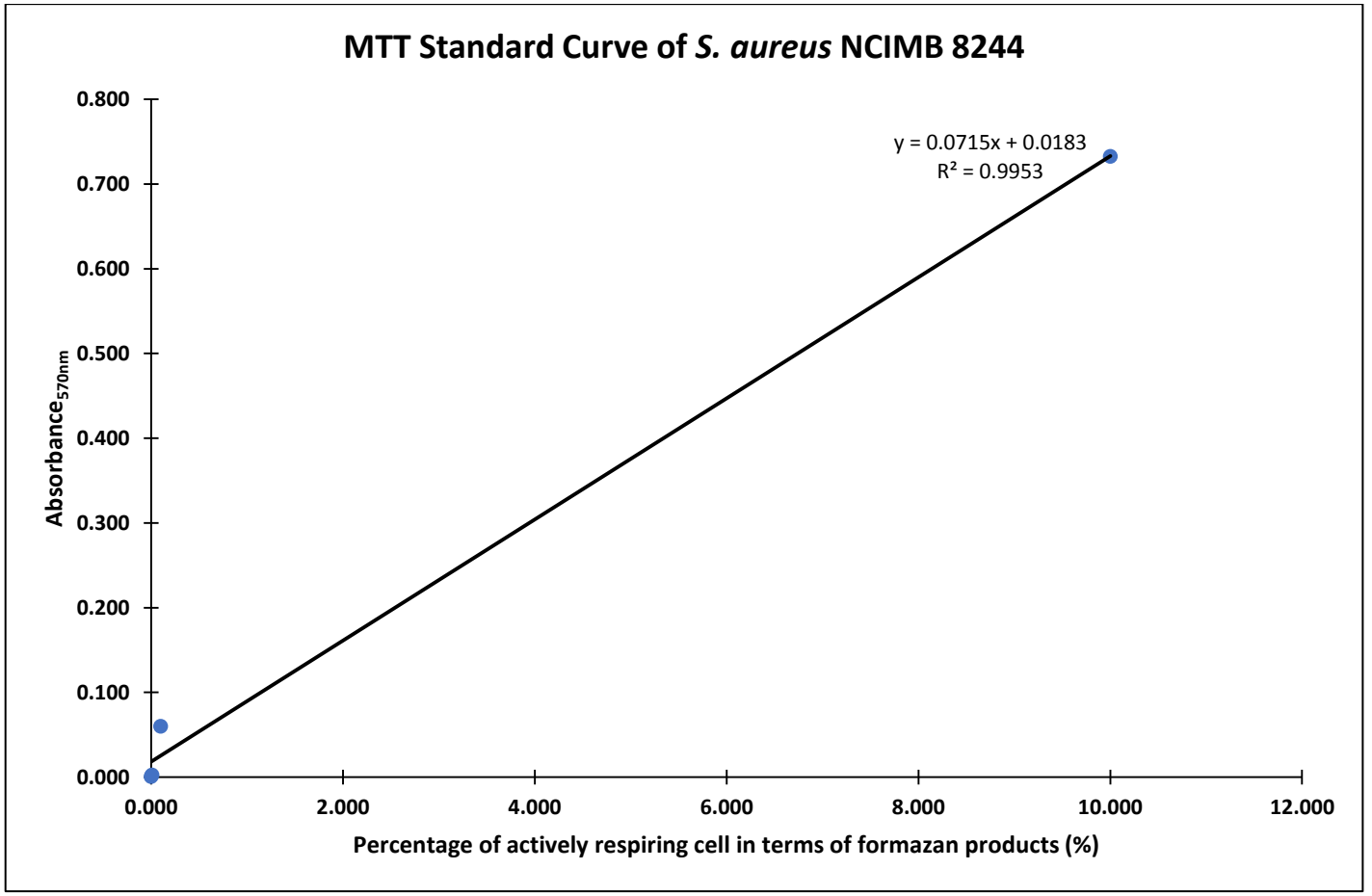


Figure 9-54 *MTT standard curve of S. aureus NCIMB 8244.*

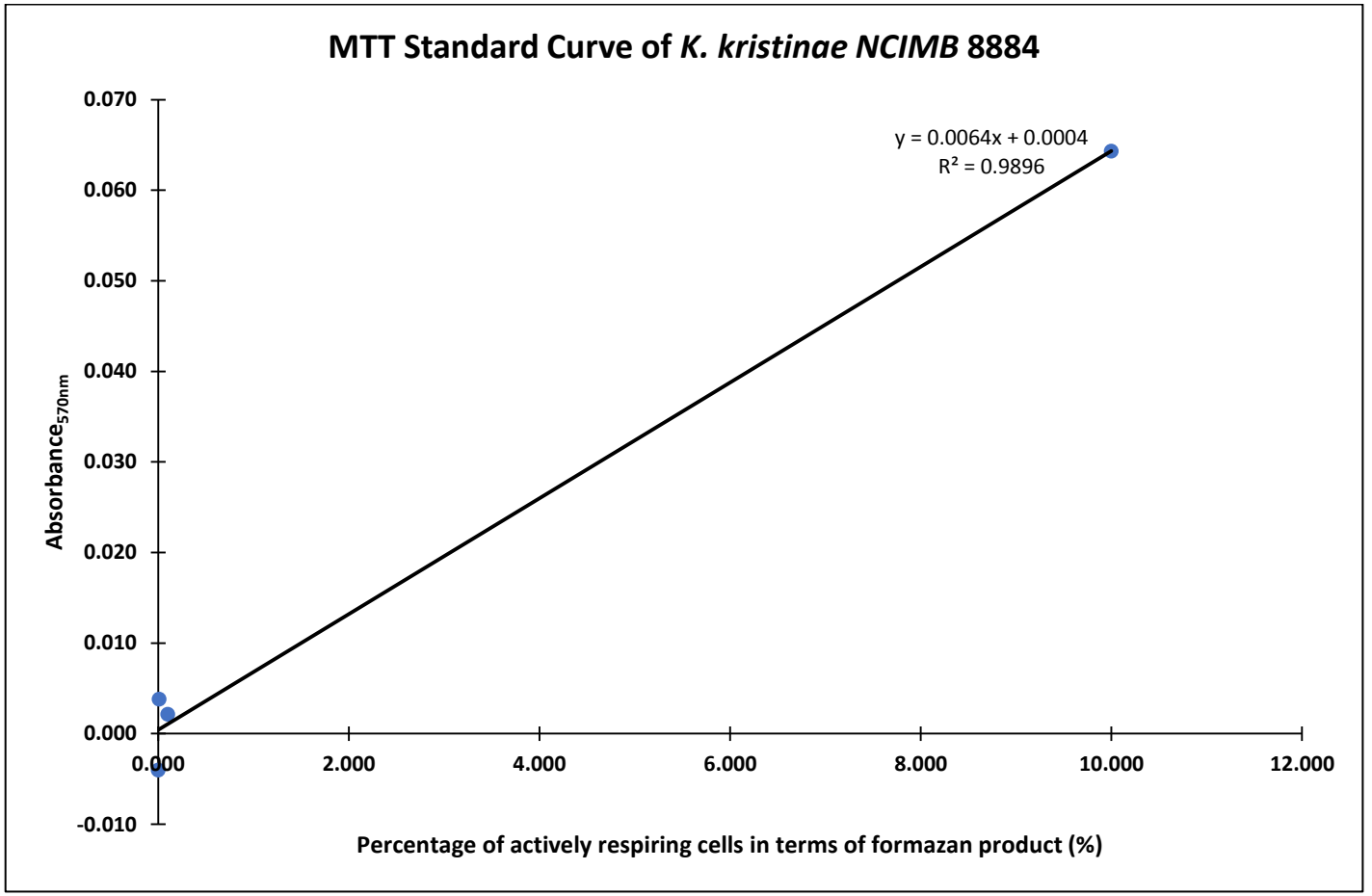


Figure 9-55 *MTT standard curve of K. kristinae NCIMB 8884.*

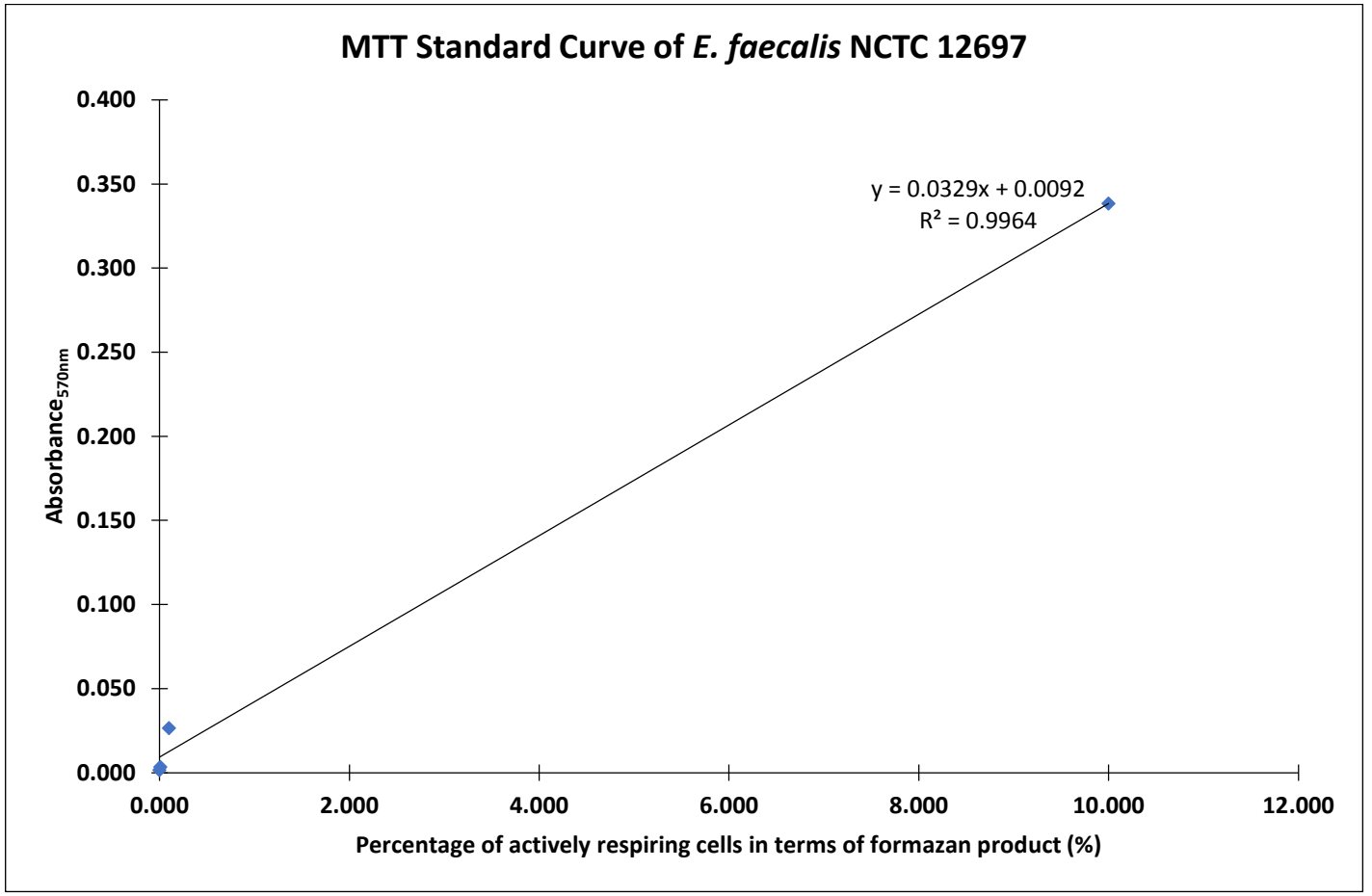


Figure 9-56 *MTT standard curve of E. faecalis NCTC 12697.*

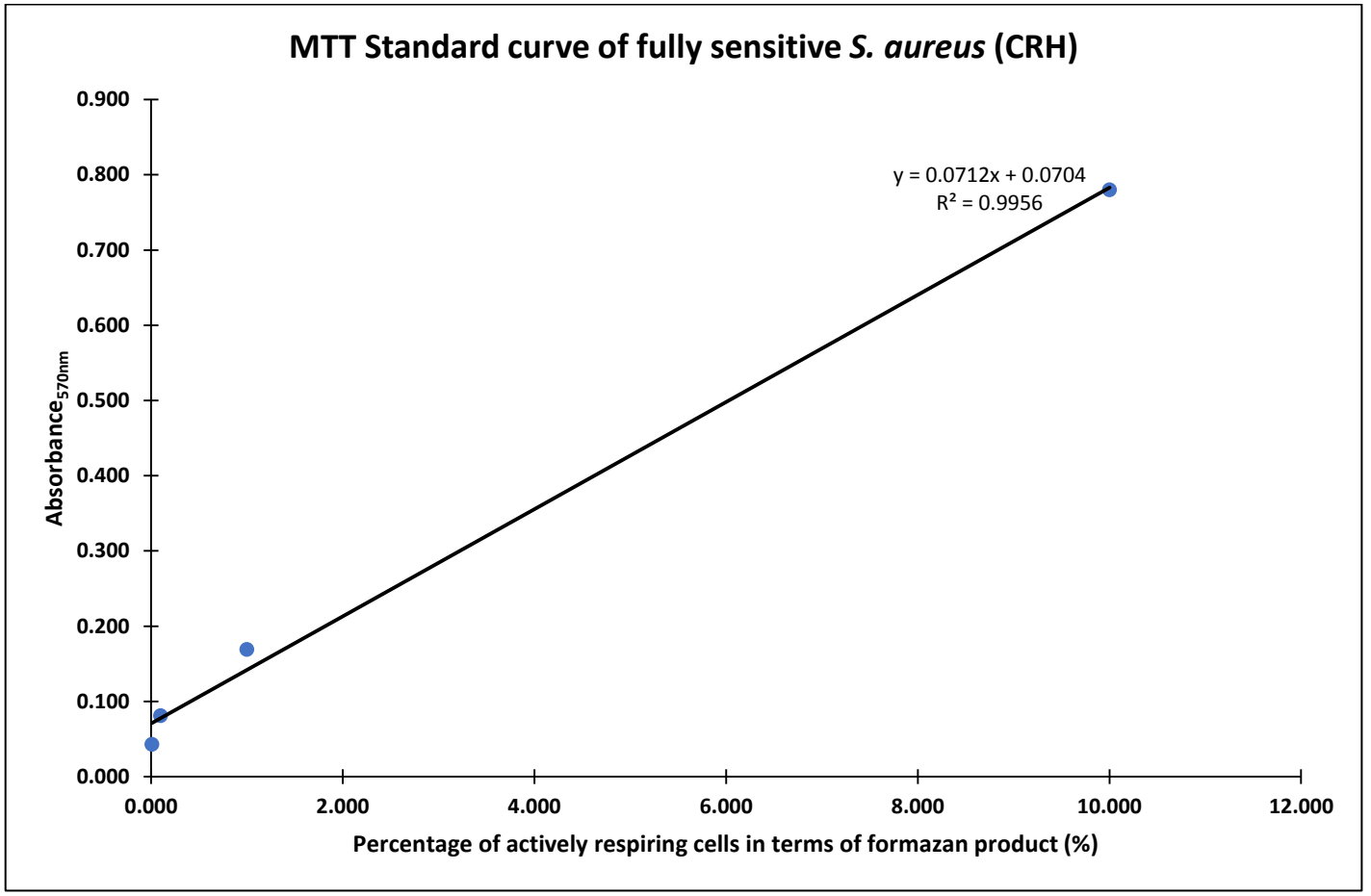


Figure 9-57 *MTT standard curve of fully sensitive S. aureus (CRH).*

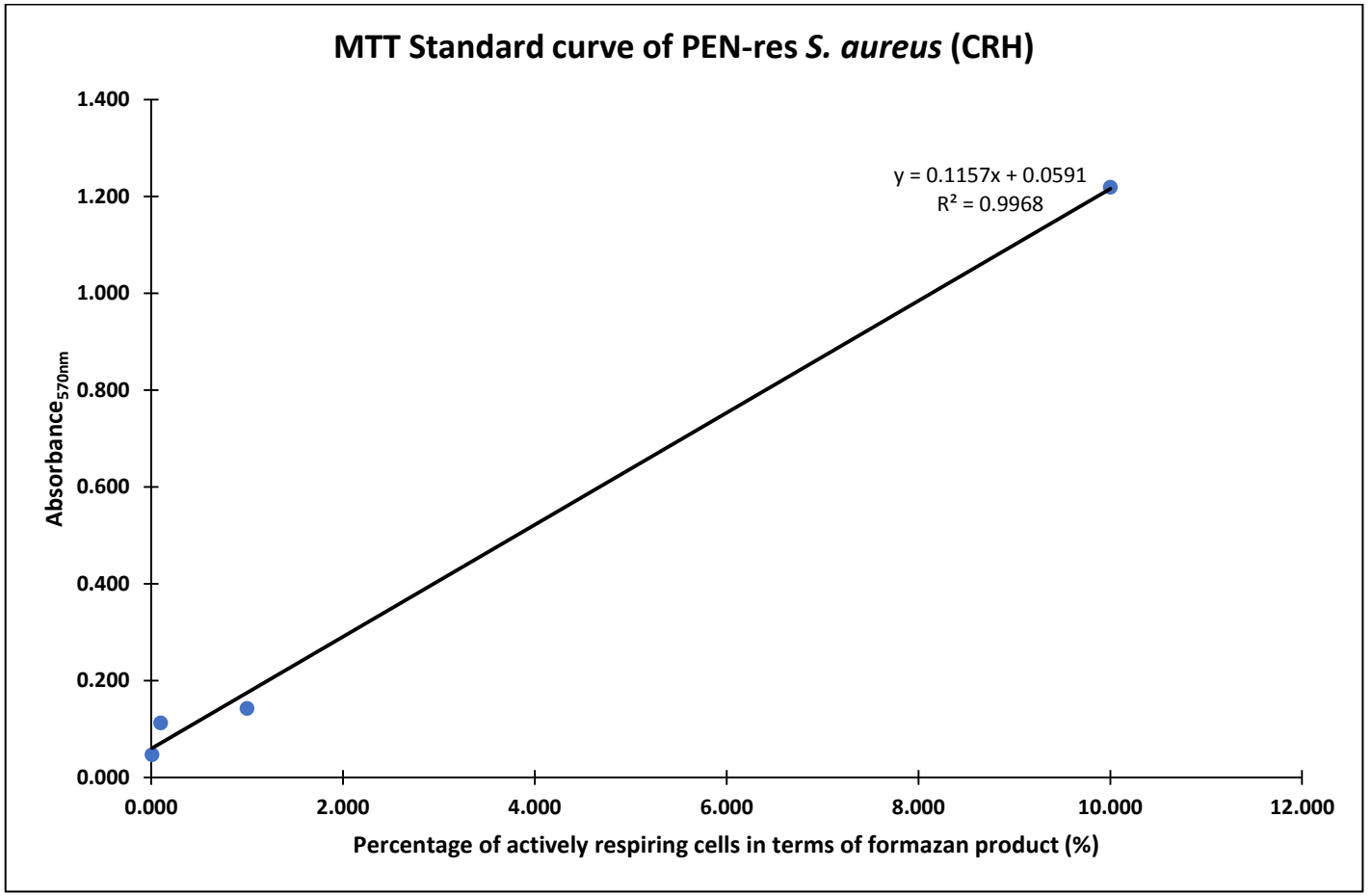


Figure 9-58 *MTT Standard curve of PEN-resistant S. aureus (CRH).*

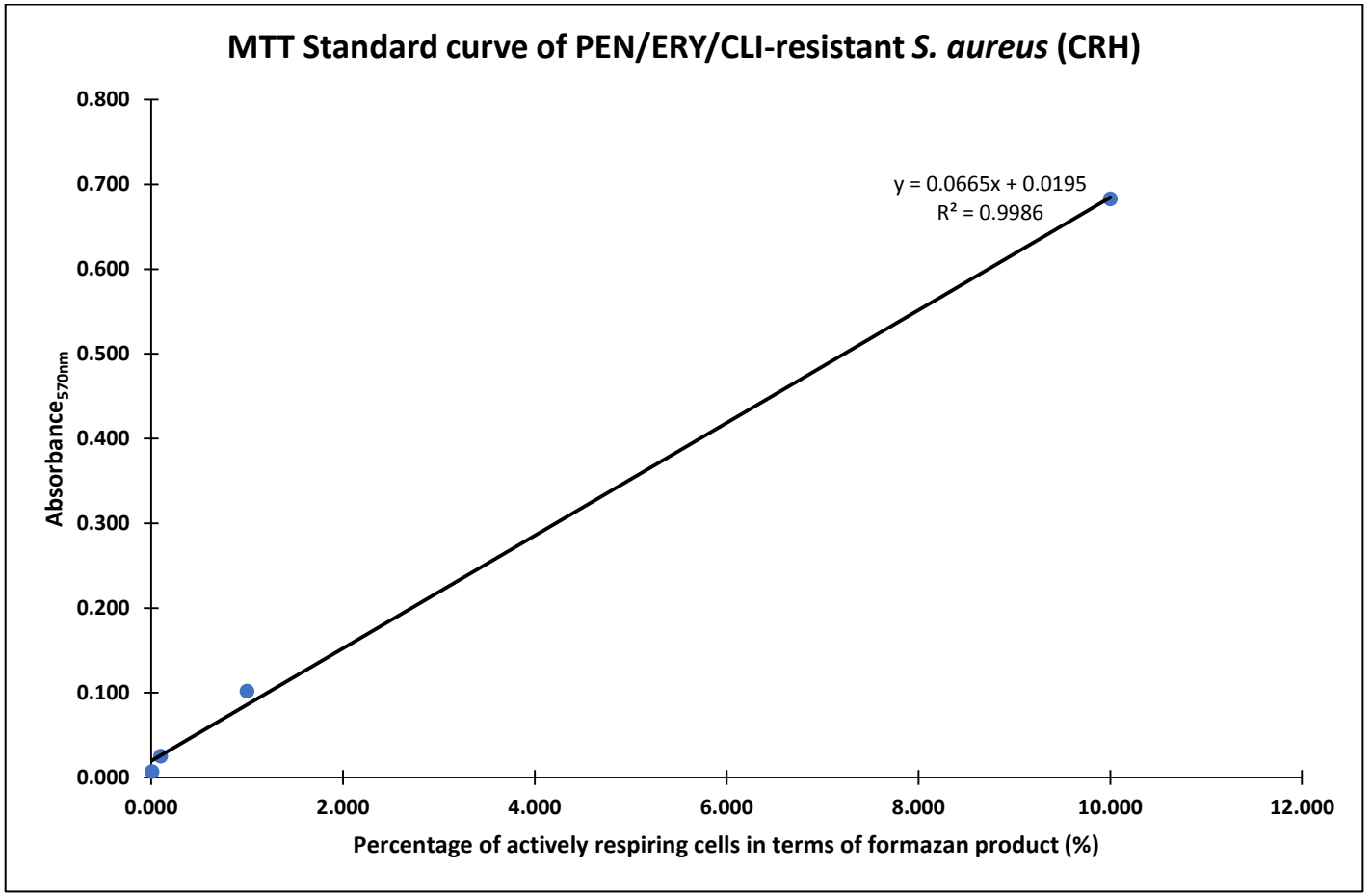


Figure 9-59 *MTT Standard curve of PEN/ERY/CLI-resistant S. aureus (CRH).*

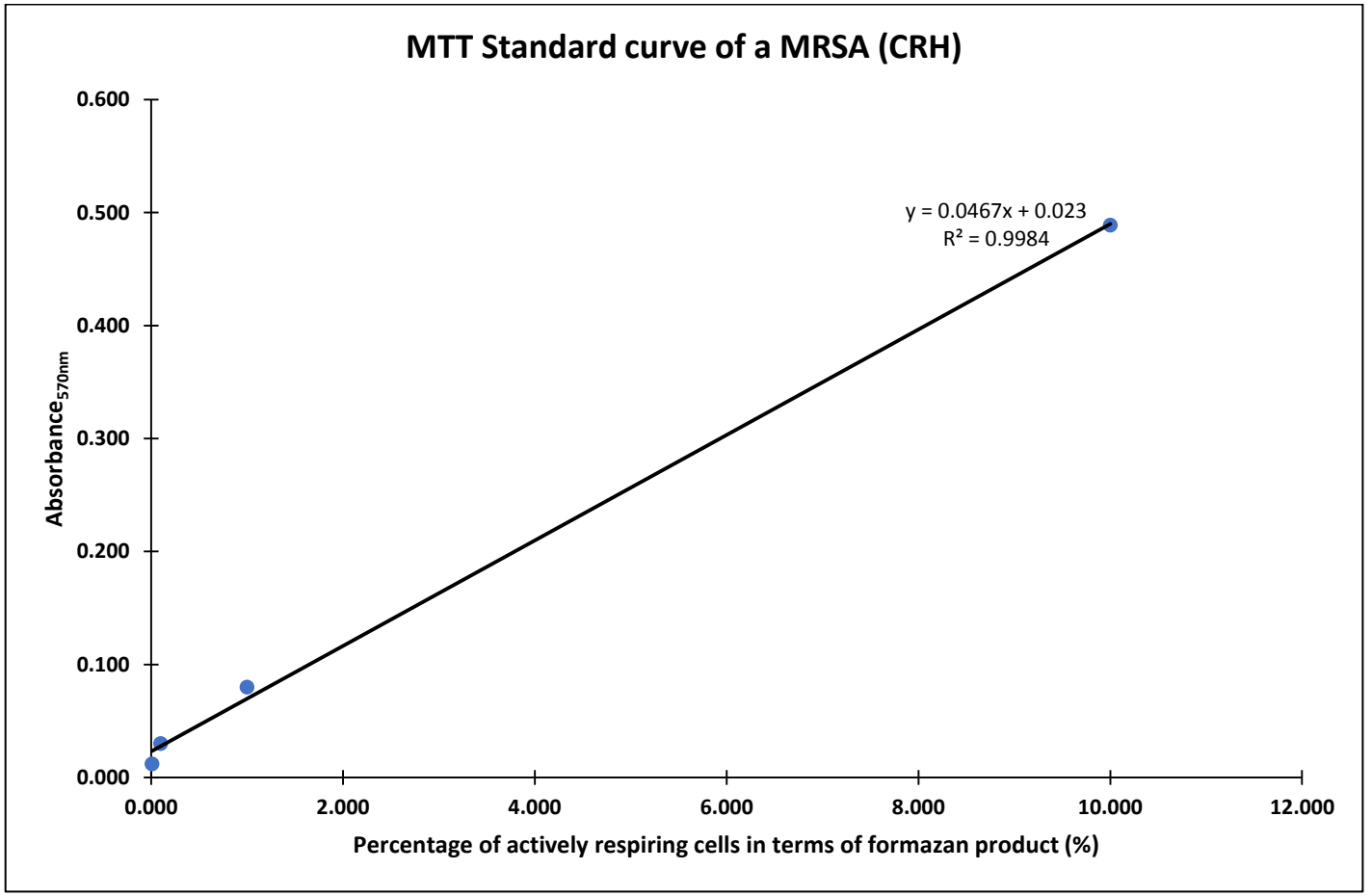


Figure 9-60 *MTT Standard curve of a MRSA (CRH).*

Table 9-4 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against S. aureus NCIMB 8244 in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	5.951647	4	1.487912	1	0.426093	2.75871
Within Groups	37.19779	25	1.487912			
Total	43.14944	29				

Table 9-5 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against K.kristinae NCIMB 8884 in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.008105	4	0.002026	0.968872	0.441968	2.75871
Within Groups	0.052287	25	0.002091			
Total	0.060392	29				

Table 9-6 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against E. faecalis NCTC 12697 in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.107456	4	0.026864	0.824965	0.521732	2.75871
Within Groups	0.814094	25	0.032564			
Total	0.92155	29				

Table 9-7 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against fully sensitive S. aureus (CRH) in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.197154	4	0.049289	1	0.426093	2.75871
Within Groups	1.232213	25	0.049289			
Total	1.429367	29				

Table 9-8 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against PEN-resistant S. aureus (CRH) in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.247876	4	0.061969	1.03753	0.407599	2.75871
Within Groups	1.493188	25	0.059728			
Total	1.741065	29				

Table 9-9 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against PEN/ERY/CLI-resistant S. aureus (CRH) in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.080136	4	0.020034	0.984489	0.433942	2.75871
Within Groups	0.508744	25	0.02035			
Total	0.58888	29				

Table 9-10 *Results of the One-way ANOVA of hexyl to decyl ferrocenyl chalcones against a MRSA (CRH) in the MTT assay.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.026656	4	0.006664	0.335127	0.851667	2.75871
Within Groups	0.497121	25	0.019885			
Total	0.523777	29				

Appendix 7 – Details and copies of publications

- Henry, E.J., Smith, R.B., Collins, M., Bird, S.J., Gowland, P. & Cassella, J.P., 2017. Novel Ferrocenyl Chalcone Compounds as Possible Antimicrobial Agents. In A. Méndez Vilas, ed. *Antimicrobial Research: Novel Bioknowledge and Educational Programs*. Spain: Formatex Research Centre, pp. 140–148.
- Henry, E., Smith, R.B., Collins, M., Bird, S.J., Gowland, P. & Cassella, J.P., 2017. Infection Control in the UK : An Antimicrobial Resistance Perspective. *International Journal of Infection Control*, 13(i2), pp.1–7.

Novel Ferrocenyl Chalcone Compounds as Possible Antimicrobial Agents

Elecia J Henry[1a], Robert B Smith[2], Michael Collins[3], Susan J Bird[1a], Pauline Gowland[1a], John P Cassella[1b]

[1a] School of Life Sciences and Education, Staffordshire University, Stoke-on-Trent, Staffordshire, ST4 2DF, United Kingdom
[1b] School of Law, Policing and Forensics, Staffordshire University, Stoke-on-Trent, Staffordshire, ST4 2DF, United Kingdom
[2] Chemistry, School of Physical Sciences and Computing, Preston UCLan, PR1 2HE, United Kingdom
[3] Chesterfield Royal Hospital NHS Foundation Trust, Chesterfield Road, Calow, Chesterfield, Derbyshire, S44 5BL, United Kingdom

The increased presence of drug-resistant bacteria has quickly become a worldwide concern as infections spread from healthcare settings to the wider community. The swift spread of infections caused by bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) is influenced by factors such as misuse and abuse of traditional antimicrobial treatments and inferior drugs. Ferrocenyl chalcones, which are derivatives of plant-based flavonoids, have gained further attention from researchers because of their antimicrobial activity. Using 2-fold broth microdilution, results demonstrated that 5 of the 10 newly developed ferrocenyl chalcones, which contain increasing alkyl chains from 5-10 carbons on ring B, possessed greater antimicrobial activity against Gram-positive organisms than Gram-negative organisms. These novel compounds were active against 3 types of drug-resistant *S. aureus*, including a MRSA, and other non-resistant Gram-positive bacteria. The same compounds inhibited growth by potentially obstructing cellular respiration in Gram-positive bacteria. Images obtained through scanning electron microscopy revealed bacterial cells with severe external damage once exposed to a selected compound that showed activity. Findings indicate that these newly developed compounds could be important antimicrobial agents in the treatment of infections from clinically resistant bacteria.

Keywords Antimicrobial resistance; antimicrobial activity; ferrocenyl chalcones; lipophilicity; mechanism of action; cellular respiration; scanning electron microscopy

1. Introduction

Because of the dwindling supply of antibiotics, and the rise in multi-drug resistant (MDR) bacteria, there is a need for new antimicrobial agents (1). This drug resistance in microorganisms has resulted in the emergence of MDR bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA) and carbapenemase-producing *Enterobacteriaceae* (CPE) leading to a greater prevalence in healthcare-acquired infections (HCAI) (2). Infections arising from these organisms have quickly become an issue of grave importance worldwide (3,4).

1.1 Emergence of colistin-resistant bacteria

Infections resulting from CPEs have driven greater use of colistin (polymixin E). This drug is a critical antimicrobial agent against some common MDR Gram-negative aerobic bacilli, including CPEs and has been considered as the last form of protection. The mode of action of colistin is to damage the integrity of the outer envelope of Gram-negative bacilli by causing instability of membrane-bound lipopolysaccharides (LPS) (5). This damage allows leakage of cellular matter, resulting in cell death, but the lethal effects on the human kidney prevented its use in routine antimicrobial therapy (6). Overuse of colistin has now resulted in infections caused by colistin-resistant CPEs. Initially, resistance was thought to result from chromosomal mutations (7) but recent studies have revealed that resistance can be facilitated by the transfer of plasmids containing the colistin-resistant gene known as MCR-1 (7). In a 2016 report by the European Centre for Disease Prevention and Control (ECDC), infections caused by this type of colistin resistance are described as a critical public health issue (ECDC, 2016).

1.2 Natural-based antimicrobial agents

Increasing prevalence of MDR bacteria has intensified research into natural-based compounds as a possible solution to this global concern (8,9). Potential sources of natural antimicrobial agents include soil and plants (10). In particular, researchers have discovered teixobactin, which is a compound derived from soil-based microbes (11). This chemical exhibits antimicrobial activity against *Mycobacterium tuberculosis* (12,13) and Gram-positive bacteria, including drug-resistant *S. aureus* (14) and *E. faecium* (15). Teixobactin inhibits bacterial cell wall synthesis by binding to lipids that are essential for cell wall integrity (16,17).

Another group of compounds that has gained interest are the chalcones, which are plant-based flavonoids (18). These biosynthetic intermediates are found widely in most plant material, including leaves and stems (19). These chemicals

are responsible for the colour of pollinating flowers and for protection against harmful ultra violet rays from the sun (20). Chalcones possess many biological activities such as anti-cancer, anti-parasitic, anti-fungal and antibacterial (21–26). From these chalcone compounds, medicinal chemists have synthesised derivatives that contain a ferrocene moiety (27,28).

Ferrocenyl chalcones have useful benefits including small size, increased lipophilicity for crossing cell membranes, they can be easy modified (29), and they show biological activities similar to those that were previously mentioned, especially their antimicrobial activity (30–33). In this study, ferrocenyl chalcone derivatives with increasing alkyl iodide chains were prepared [RS $^{(2)}$] to determine their antimicrobial activity.

2. Antimicrobial activity of ferrocenyl chalcones derivatives

Due to their hydrophobic nature, ferrocenyl chalcones are usually dissolved in organic solvents such as ethanol, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) (28). The ferrocenyl chalcone compounds that were provided by RS (UCLAN, UK) were highly water-insoluble because they contained alkyl iodide chains (methyl to decyl). Therefore, DMSO was the most favourable solvent used in this study because of its amphipathic feature, which allows for the delivery of lipophilic antibacterial agents, and because it is less inhibitive in terms of bacterial growth (34). For all assays, we prepared fresh stock solutions of each compound at 1 mg/ml in DMSO.

We assessed antimicrobial activity of each ferrocenyl chalcone solution in terms of their minimum inhibitory concentration (MIC) values against a panel of non-resistant and resistant bacterial clinical isolates as well as non-resistant laboratory-adapted organisms. *Staphylococcus aureus* NCIMB 8244, *Enterococcus faecalis* NCTC 12697, *Kocuria kristinae* NCIMB 8884, *Escherichia coli* NCIMB 9483, *Klebsiella pneumoniae* and *Salmonella serotype Manchester* NCTC 7832 were prepared by suspending at least 3-4 colonies of each organism in individual sterile 10 ml aliquots of sterile Oxoid Mueller-Hinton (MH) broth (Fisher Scientific, Loughborough, UK) and incubated for 15-20 minutes at 37^{0}C in air while stirring. Each inocula was compared to 0.5 MacFarland standard. Suspensions were diluted 1:100 in sterile MH broth to gain starting inocula of 10^5 per BSAC standards. Clinical isolates of non-resistant *E. coli*, fully sensitive *S. aureus*, resistant *S. aureus* (penicillin; erythromycin/penicillin/clindamycin) and a highly resistant MRSA were prepared as previously described. *K. kristinae* NCIMB 8884 was prepared 1:10 also according to British Society of Antimicrobial Chemotherapy standards (35).

2.1 Minimum inhibitory concentration assay

Antimicrobial activity of newly developed compounds is commonly determined using 2-fold serial broth microdilution (36–38). This method is used to measure MIC values, the lowest concentration of antimicrobial agent that inhibits growth of organism (39). Each ferrocenyl chalcone compound was diluted with sterile MH broth. 75 µl of each prepared inocula was added to an equal value of diluted ferrocenyl chalcone solution in Nunc 0.2 ml flat bottom 96-well 12-column microtitre plates (Fisher Scientific, Loughborough, UK). Column 11 was treated with either penicillin or oxytetracycline and column 12 was left untreated. Plates were then incubated at 37^{0}C for 18-24 hours. Absorbance values were measured using a Rosys Anthos 2010 microplate reader (Salzberg, Austria) at 620 nm adapted from Medu (2013). Results from this assay indicated that the ferrocenyl chalcone compounds with shorter alkyl chains (from methyl to pentyl) were less effective than those compounds with longer alkyl chains (from hexyl to decyl). The mean (± SD) MIC values of the methyl to pentyl ferrocenyl chalcones were determined to be 0.125 mg/ml (± SD), while the mean (± SD) MIC values of the hexyl to decyl ferrocenyl chalcones ranged from 0.008 mg/ml (± SD) to 0.063mg/ml (± SD) (Table 1). The MIC values of the ferrocenyl chalcone compounds were within the reported values for penicillin (0.000015-0.128 mg/ml) against *S. aureus* and *E. faecalis*, and for oxytetracyline, an analogue antibiotic of tetracycline, against *Enterobacteriaceae* (0.00025-0.128 mg/ml) (35). However, growth inhibition of Gram-negative bacteria may also have resulted from exposure of the organisms to DMSO. DMSO has been shown to have an inhibitory effect at percentages equal to or above 12.5% $^v/_v$ (34). The latter group of compounds also showed greater activity against Gram-negative bacteria than Gram-positive bacteria (Table 1). The MIC values of the hexyl to decyl ferrocenyl chalcone compounds are also equal to the minimum bactericidal concentration (MBC) of the same group of compounds for non-resistant *S. aureus* NCIMB 8244, *E. faecalis* NCTC 12697 and, *K. kristinae* NCIMB 8884 (Table 1). MBC is defined as the lowest concentration at which an antibiotic either completely inhibits bacterial growth or facilitates the reduction of the bacterial population to $\leq$ 99.9% (40).

Table 1 Mean (± SD) MIC values of 10 ferrocenyl chalcone compounds against non-resistant and resistant laboratory-adapted bacteria and clinically isolated bacteria. CRH = Chesterfield Royal Hospital. *Minimum Bactericidal Concentration of the compounds.

Organism	Mean (± 0.000, n=6) MIC (mg/ml)									
	Methyl	Ethyl	Propyl	Butyl	Pentyl	Hexyl	Heptyl	Octyl	Nonyl	Decyl
***S. aureus* NCIMB 8244**	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.031*
***K. kristinae* NCIMB 8884**	0.125	0.125	0.125	0.125	0.125	0.031*	0.008*	0.016*	0.016*	0.016*
***E. faecalis* NCTC 12697**	0.125	0.125	0.125	0.125	0.125	0.063*	0.063*	0.063*	0.063*	0.063*
S. aureus* Fully Sens. *(CRH)	-	-	-	-	-	0.125	0.063	0.063	0.063	0.063
PEN-resistant S. aureus (CRH)	-	-	-	-	-	0.063	0.031	0.063	0.063	0.063
PEN/ ERY/ CLI-resistant (CRH)	-	-	-	-	-	0.063	0.031	0.063	0.063	0.063
MRSA (CRH)	-	-	-	-	-	0.063	0.063	0.063	0.063	0.063
***E. coli* NCIMB 9483**	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
K. pneumoniae (1H)	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
***Salmonella "Manchester"* NCTC 7372**	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
E. coli* Fully Sens. *(CRH)	-	-	-	-	-	0.125	0.125	0.125	0.125	0.125

2.2 Inhibitory activity of ferrocenyl chalcone chemicals

The difference in MIC values with respect to the Gram-negative and Gram-positive organisms may be because of increasing alkyl chain length of the ferrocenyl chalcone chemicals. One possible reason that contributed to the difference in activity is that the compounds may pass across the thick hydrophilic peptidoglycan layer of the Gram-positive bacteria because of the amphipathic DMSO. The long chains may become trapped in the cell membrane allowing the attached ferrocenyl groups, which are relatively smaller than the alkyl chains, to enter the cytoplasm. Since Gram-negative bacteria have outer envelopes with membrane transporter proteins such as porins, followed by thin peptidoglycan layers and cell membranes in their cellular envelopes, entry into these cells may be more difficult (41).

These porins allow hydrophilic compounds to enter, while hydrophobic compounds may diffuse across the lipid bi-layer of the outer envelope (42). However, because of the fluidity of the outer lipid bi-layer (43), the long alkyl chains of the ferrocenyl chalcones may become trapped in the outer envelope and would be unable to cross the peptidoglycan layer and cell membrane into the cells. Another possible reason is that the organisms such as E. coli have become used to living in enriched media, which promotes vigorous growth (44). The ferrocenyl chalcone compounds exhibited antimicrobial activity against bacteria that were resistant to penicillin (PEN), clindamycin (CLI), erythromycin (ERY), and a MRSA that was resistant to penicillin, flucoxacillin, trimethoprim, ciprofloxacin and a cephalosporin. The modes of action of these antibiotics include cell wall synthesis inhibition (45), protein synthesis inhibition (46), blockage of essential bacterial reductases (47) and the inhibition of DNA replication (48). The possible antimicrobial mechanism of the ferrocenyl chalcone compounds was further explored using a bacterial cell viability assay.

3. Bacterial cell viability assay

In order to determine a potential mode of action of the ferrocenyl chalcones, cell viability, in terms of respiration, can be used. The assay involved using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a positively charged compound that can easily diffuse into cells (49). The yellow MTT compound is reported to be reduced to the purple formazan product by a dehydrogenase system of enzymes within the cell (50). Once formed, the formazan product, which is insoluble in water, can be dissolved in an organic solvent such as dimethyl sulfoxide and then measured by spectrophotometry at 570 nm (50). Although the exact mechanism is not known, the process occurs in actively respiring cells (51). The amount of formazan present is proportional to cell viability. Bacterial cell viability of resistant and non-resistant bacteria at MIC was determined by inoculating 96-well microplates as described above, followed by the addition of 10 µl of MTT solution (5 mg/ml) (Sigma-Aldrich, Dorset, UK). Plates were incubated at room temperature for 3 hours followed by the addition of 50 µl of DMSO. Absorbance values were measured at 570 nm.

The results of the assay of non-resistant Gram-positive laboratory organisms demonstrated that the percentage of actively respiring cells, in terms of formazan product observed (Figure 3-1) decreased after exposure to ferrocenyl chalcone compounds at the MIC value. A mean percentage of 0% was observed for *S. aureus* NCIMB 8244 when exposed to hexyl, octyl and nonyl. Similar percentage were seen for *E. faecalis* NCTC 12697 when exposed to hexyl, heptyl and octyl. For *K. kristinae* NCIMB 8884, mean percentage of 0% were seen for hexyl and heptyl. The highest percentage was measured for *S. aureus* NCIMB 8244 after incubation with decyl (4.241%). In the MTT assay of resistant and non-resistant Gram-positive clinical isolates the percentage of actively respiring cells, in terms of formazan product observed (Figure 3-2) also decreased after exposure to chalcones at the MIC value. Mean percentage of 0% were seen for fully sensitive *S. aureus* (CRH) when exposed to hexyl, heptyl and octyl, for PEN-resistant *S. aureus* (CRH) when exposed to heptyl, octyl, nonyl and decyl, for PEN/ERY/CLI-resistant *S. aureus* (CRH) and MRSA when exposed to hexyl, heptyl, nonyl and decyl ferrocenyl chalcones. The highest percentage was determined for fully sensitive *S. aureus* (CRH) after incubation with nonyl (2.242%).

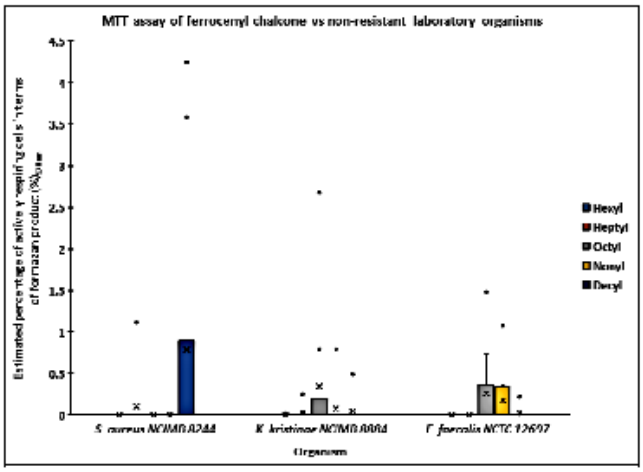


Figure 3-1 Estimated percentage of actively respiring non-resistant laboratory bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlier values.

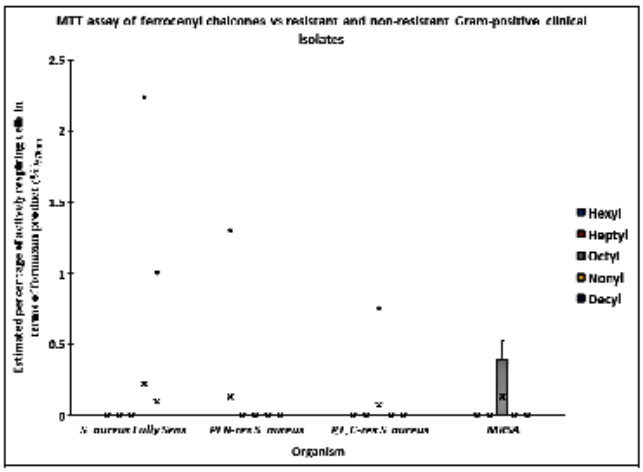


Figure 3-2 Estimated percentage of actively respiring resistant and non-resistant clinically isolated bacterial cells when treated with ferrocenyl chalcone at MIC. Box plots represent the lower and upper quartiles with the medians shown as black lines. Whiskers represent the minimum and maximum percentages and each X represents the mean values. Dots represent outlier values.

3.1 Statistical analysis

Statistical analysis of the MTT assay data in the study was performed using a One-Way ANOVA to determine if the mean percentage of actively respiring cells differed between the hexyl to decyl ferrocenyl chalcones treatments. The Kolmogorov-Smirnoff test was used to determine data normality of the MTT assay data.

In the microplate assays, the overall trend demonstrated that the chalcones had a greater inhibitory effect on Gram-positive organisms than on Gram-negative organisms. The trend also indicated that the differences between hexyl to decyl chalcones in terms of mean percentage (± SD) of actively respiring cells present were not significant for *S. aureus* NCIMB 8244 (p=0.107), *K. kristinae* NCIMB 8884 (p=0.326) and *E. faecalis* NCTC 12697 (p=0.118). Thus, the compounds were equally effective at inhibiting respiration in these bacterial cells. Similarly, the hexyl to decyl

ferrocenyl chalcones were equally effective at respiration inhibition for fully sensitive S. aureus (p=0.523), penicillin-resistant *S. aureus* (p=0.418), PEN/ERY/CLI-resistant *S. aureus* (p=0.418) and a MRSA (p=0.418).

3.2 Possible mechanism of action

When compared to MTT screening of ferrocenyl chalcone antimicrobial activity against *Mycobacterium tuberculosis*, the MIC values in this study lay within the reported range (0.016-0.128 mg/ml) (49), except for *K. kristinae* NCIMB 8884 where a lower MIC (heptyl chalcone) was used. Increased chain length may allow the ferrocene group to enter the cytoplasm of Gram-positive organisms. Ferrocene groups have been theorised to be inhibitors of cellular respiration, in which the ferrocene groups act as uncouplers (29). Since Gram-negative organisms have outer envelopes, thin peptidoglycan layers with increased periplasmic space and cell membranes in their cell envelopes, entry into these cells may be more difficult. Another possibility (SB) is that the cell membrane of Gram-positive bacteria is compromised such that the electron transport chain cannot function (52). Cell viability, as indicated by MTT metabolism to formazan, decreased in Gram-positive organisms when compared to Gram-negative organisms. Therefore, a possible mechanism of action of the chalcones with longer alkyl chain lengths may be inhibition of cellular respiration. This effect causes physical damage to the bacterial cells and was observed in images obtained from scanning electron microscopy.

4. Bacterial Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy is an imaging technique used to investigate the surface topography of biological specimens at the nanometre level (53). These samples usually possess a higher water content, which decreases their conductivity under high vacuum conditions in the electron microscope and results in distortion and destruction of the material (54). Biological samples, such as bacterial cells, must be chemically fixed, dehydrated, dried and sputter coated with a metal such as gold or using carbon under vacuum (55). Fixed bacterial samples must also be washed with an appropriate buffer such as phosphate buffered saline (PBS). For the purpose of SEM, bacterial samples should be captured on membrane filter with a diameter 13 mm and with a pore size that ranges between 0.2 µm to 1 µm (56).

SEM has been used to examine the external morphological effects of sugar fatty acid esters on selected Gram-positive and Gram-negative bacteria (57), and to observe the morphological changes to *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 after exposure to cinnamon essential oil (58).

4.1 SEM study of treated and untreated bacteria

In the current study, treated and untreated non-resistant bacteria were assessed. *S. aureus* NCIMB 8244, *K. kristinae* NCIMB 8884 and *E. faecalis* NCTC 12697 cells, which were examined in the SEM. The treated organisms were exposed to decyl ferrocenyl chalcone solution at MIC values and incubated for 18-24 hours at 37^0C, whilst untreated cells were incubated under the same conditions in the absence of chalcone. Treated and untreated cells were incubated with 2% glutaraldehyde (prepared with sterile deionised water) for 1 hour then washed with sterile phosphate buffer saline (PBS) (59). The cells were then dehydrated with a graded series of sterile ethanol and re-suspended in sterile deionised water. At the end of each step, the solutions were centrifuged. 10 µl of re-suspended cells were pipetted on to 0.2 µm Cyclopore Track Etch polycarbonate membrane filter discs (Whatman International Limited, Maidstone, UK) and sputter-coated with gold. Secondary electron images were taken using the JEOL JSM 6610V SEM (Herts, UK).

4.2 Morphological damage to bacterial cells

The SEM images revealed that exposure to decyl ferrocenyl chalcone resulted in severe external damage to bacterial cells at MIC and supra-MIC. The affected cells were shrivelled and wrinkled in appearance as if their cell wall structural integrity had been affected (Figure 3-3).

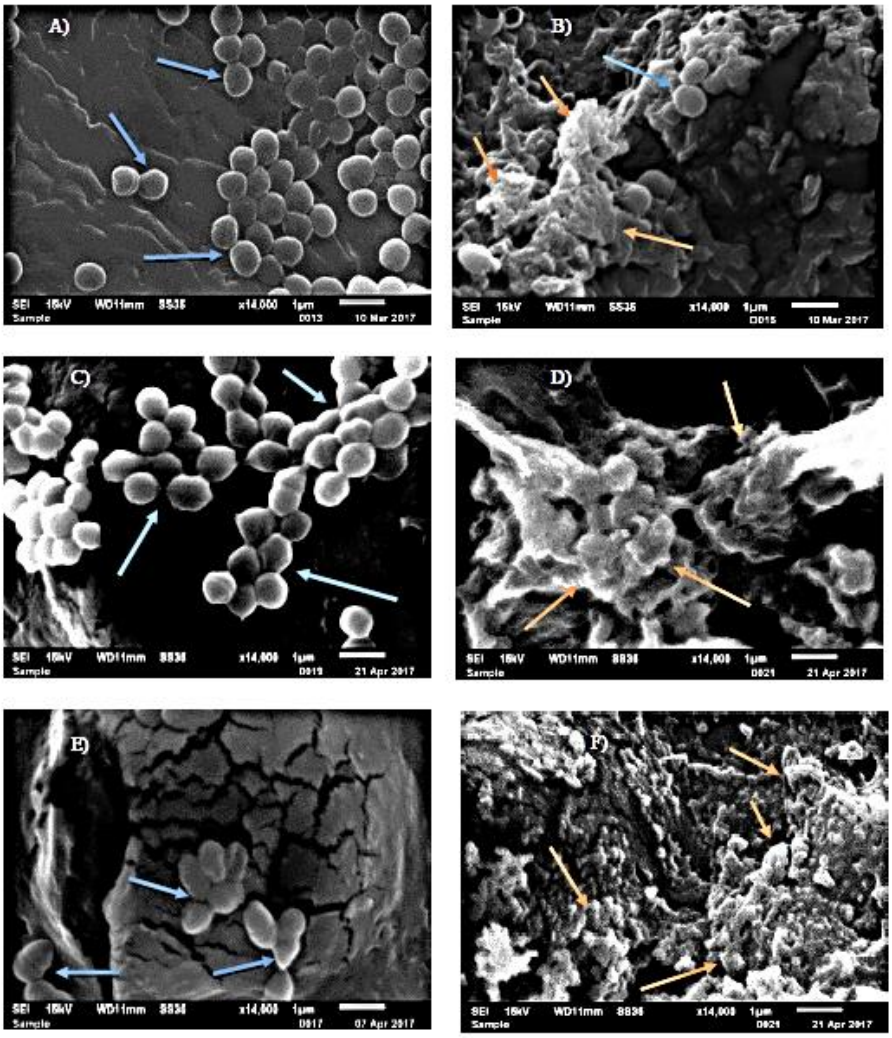


Figure 3-3 SEM images of untreated bacterial cells and cells that were treated with decyl ferrocenyl chalcone compound where blue arrow indicates unaffected cell and orange arrows indicate severely damaged cells. A) Untreated S. aureus NCIMB 8244. B) Treated S. aureus NCIMB 8244. C) Untreated K. kristinae NCIMB 8884. D) Treated K. kristinae NCIMB 8884. E) Untreated E. faecalis NCTC 12697. F) Treated E. faecalis NCTC 12697.

5. Conclusion

Infections caused by the spread of drug-resistant bacteria must be addressed as quickly as possible. This spread is in part caused by the misuse of antibiotics and the unavailability of new antimicrobial agents. Key discoveries of the

research are that ferrocenyl chalcone compounds with longer alkyl chains (hexyl to decyl) exhibit greater antimicrobial activity than those with shorter alkyl chains (methyl to pentyl) and that compounds with antimicrobial activity are more effective against Gram-positive bacteria than against Gram-positive bacteria. This is the first report to also demonstrate that ferrocenyl chalcones, which can be structurally altered by synthetic methods, possess significant antimicrobial activity against non-resistant laboratory organisms and resistant and non-resistant clinical isolates. Another important result of this study is that chalcone activity may be characterised by blocking bacterial respiration. Findings from this study reveal that these novel ferrocenyl chalcone compounds are potential antimicrobial agents against clinical bacterial isolates requiring further investigation involving efficacy of the compounds against biofilms and cytotoxicity against mammalian cells.

Acknowledgements The support of Susan Avery, Alison Davidson, Denise Figgins, Ian Hopkins and Rob Manning at Staffordshire University are gratefully acknowledged.

References

[1] Shlaes DM, Spellberg B. Overcoming the challenges to developing new antibiotics. *Curr Opin Pharmacol*. Elsevier Ltd; 2012 Oct;12(5):522–6.
[2] Hadley S. Resistant Gram-Positive Infections: Where Have We Been, Where Are We Now, and Where Are We Going? *Clin Ther*. Elsevier; 2014 Oct 3;36(10):1298–302.
[3] Tseng S-H, Lee C-M, Lin T-Y, Chang S-C, Chang F-Y. Emergence and spread of multi-drug resistant organisms: think globally and act locally. *J Microbiol Immunol Infect*. Elsevier Taiwan LLC; 2011 Jun;44(3):157–65.
[4] WHO. Worldwide country situation analysis: response to antimicrobial resistance. Geneva; 2015.
[5] Newton BA. The properties and mode of action of the polymixins. *Bact Rev*. 1956;20:14–27.
[6] Falagas ME, Kasiakou SK. Colistin: The Revival of Polymyxins for the Management of Multidrug-Resistant Gram-Negative Bacterial Infections. *Clin Infect Dis*. 2005;40:1333–41.
[7] Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect Dis*. 2016;16:161–8.
[8] Laxminarayan R, Duse A, Wattal C, Zaidi AKM, Wertheim HFL, Sumpradit N, et al. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis*. 2013 Dec;13(12):1057–98.
[9] Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, et al. The global threat of antimicrobial resistance: Science for intervention. *New Microbes New Infect*. Elsevier Ltd; 2015 Apr;6(April 2015):22–9.
[10] Moloney MG. Natural Products as a Source for Novel Antibiotics. *Trends Pharmacol Sci*. Elsevier Ltd; 2016;37(8):689–701.
[11] Ling LL, Schneider T, Peoples AJ, Spoering AL, Engels I, Conlon BP, et al. A new antibiotic kills pathogens without detectable resistance. *Nature*. 2015;517(7535):455–9.
[12] Dong M, Pfeiffer B, Altmann K-H. Recent developments in natural product-based drug discovery for tuberculosis. *Drug Discov Today*. Elsevier Ltd; 2016;22(3):585–91.
[13] Quan D, Nagalingam G, Payne R, Triccas JA. New tuberculosis drug leads from naturally occurring compounds. *Int J Infect Dis*. International Society for Infectious Diseases; 2017;56:212–20.
[14] Assis LM, Nedeljković M, Dessen A. New strategies for targeting and treatment of multi-drug resistant Staphylococcus aureus. *Drug Resist Updat*. 2017;31:1–14.
[15] Paganelli FL, Van de Kamer T, Brouwer EC, Leavis HL, Woodford N, Bonten MJM, et al. Lipoteichoic acid synthesis inhibition in combination with antibiotics abrogates growth of multi-drug resistant Enterococcus faecium. *Int J Antimicrob Agents*. Elsevier B.V.; 2017;49(3):355–63.
[16] Hampton T. Novel Programs and Discoveries Aim to Combat Antibiotic Resistance. Journal of the American Medical Association. United States; 2015.
[17] Piddock LJ V. Teixobactin, the first of a new class of antibiotics discovered by ichip technology? *J Antimicrob Chemother*. 2015;70(10):2679–80.
[18] Cushnie TPT, Lamb AJ. Recent advances in understanding the antibacterial properties of flavonoids. *Int J Antimicrob Agents*. 2011 Aug;38(2):99–107.
[19] Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents*. 2005;26(5):343–56.
[20] Harborne, J.B. und Williams CA. Advances in flavonoid research since 1992. *Phytochemistry*. 2000;55(6):481–504.
[21] Dhar DN. The chemistry of chalcones and related compounds. 1st ed. New York: Wiley; 1981. 285 p.
[22] Dimmock JR, Elias DW, Beazely MA, Kandepu N. Bioactivities of chalcones. *Curr Med Chem*. 1999;6(12):1125–49.
[23] Ni L, Meng CQ, Sikorski J a. Recent advances in therapeutic chalcones. *Expert Opin Ther Pat*. 2004;14(February):1669–91.
[24] Go ML, Wu X, Liu XL. Chalcones: an update on cytotoxic and chemoprotective properties. *Curr Med Chem*. 2005;12(117543):481–99.
[25] de Carvalho Tavares L, Johann S, Maria de Almeida Alves T, Guerra JC, Maria de Souza-Fagundes E, Cisalpino PS, et al. Quinolinyl and quinolinyl N-oxide chalcones: synthesis, antifungal and cytotoxic activities. *Eur J Med Chem*. Elsevier Masson SAS; 2011 Sep;46(9):4448–56.
[26] Rozmer Z, Perjés P. Naturally occurring chalcones and their biological activities. *Phytochem Rev*. 2016;15(1):87–120.
[27] Mishra S, Tirkey V, Ghosh A, Dash HR, Das S, Shukla M, et al. Ferrocenyl–cymantrenyl hetero-bimetallic chalcones: Synthesis, structure and biological properties. *J Mol Struct*. Elsevier B.V.; 2015;1085:162–72.

[28] Gopi C, Sastry VG, Dhanaraju MD. Synthesis and spectroscopic characterisation of novel bioactive molecule of 3- (2-substituted) -1H-indol-3-yl) - 1- (thiophen-2yl) prop-2-en-1-one chalcone derivatives as effective anti-oxidant and anti-microbial agents. *Beni-Suef Univ J Basic Appl Sci*. Elsevier B.V.; 2016;5(3):236–43.
[29] Attar S, O'Brien Z, Alhaddad H, Golden ML, Calderón-Urrea A. Ferrocenyl chalcones versus organic chalcones: a comparative study of their nematocidal activity. *Bioorg Med Chem*. Elsevier Ltd; 2011 Mar 15;19(6):2055–73.
[30] Pejović A, Damljanović I, Stevanović D, Vukićević M, Novaković SB, Bogdanović G a., et al. Antimicrobial ferrocene containing quinolinones: Synthesis, spectral, electrochemical and structural characterization of 2-ferrocenyl-2,3-dihydroquinolin-4(1H)-one and its 6-chloro and 6-bromo derivatives. *Polyhedron*. 2012 Jan;31(1):789–95.
[31] Kowalski K, Koceva-Chy A, Szczupak L, Hikisz P, Bernasińska J, Rajnisz A, et al. Ferrocenylvinyl-flavones: Synthesis, structure, anticancer and antibacterial activity studies. *J Organomet Chem*. 2013;741–742(1):153–61.
[32] Prasath R, Bhavana P, Ng SW, Tiekink ERT. The facile and efficient ultrasound-assisted synthesis of new quinoline-appended ferrocenyl chalcones and their properties. *J Organomet Chem*. Elsevier B.V; 2013 Feb;726:62–70.
[33] Ahmed N, Konduru NK, Owais M. Design, synthesis and antimicrobial activities of novel ferrocenyl and organic chalcone based sulfones and bis-sulfones. *Arab J Chem*. King Saud University, 2015; http://dx.doi.org/10.1016/j.arabjc.2014.12.008.
[34] Basch H, Gadebusch HH, Brunswick N. In Vitro Antimicrobial Activity of Dimethylsulfoxide. *Appl Microbiol*. 1968;16(12):1953–4.
[35] Andrews JM. Determination of minimum inhibitory concentrations. *J Antimicrob Chemother*. 2001 Jul 1;48(suppl 1):5–16.
[36] Stubbings WJ, Bostock JM, Ingham E, Chopra I. Assessment of a microplate method for determining the post-antibiotic effect in Staphylococcus aureus and Escherichia coli. *J Antimicrob Chemother*. 2004;54(1):139–43.
[37] Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis*. 2009 Dec 1;49(11):1749–55.
[38] Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. *J Pharm Anal*. Elsevier; 2016;1–9.
[39] Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc*. 2008;3(2):163–75.
[40] Pankey G a, Sabath LD. Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis*. 2004;38(6):864–70.
[41] Galdiero S, Falanga A, Cantisani M, Tarallo R, Della Pepa ME, D'Oriano V, et al. Microbe-host interactions: structure and role of Gram-negative bacterial porins. *Curr Protein Pept Sci*. 2012;13:843–54.
[42] Bolla JM, Alibert-Franco S, Handzlik J, Chevalier J, Mahamoud A, Boyer G, et al. Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria. *FEBS Lett*. 2011;585(11):1682–90.
[43] Seltmann G, Holts O. The Bacterial Cell Wall. 1st ed. New York: Springer-Verlag; 2002. 98-99 p.
[44] Fux C a., Shirtliff M, Stoodley P, Costerton JW. Can laboratory reference strains mirror "real-world" pathogenesis? *Trends Microbiol*. 2005;13(2):58–63.
[45] Wise EM, Park JT. Penicillin: Its Basic Site of Action as an Inhibitor of a Peptide Cross-linking Reaction in Cell Wall Mucopeptide Synthesis. In: Proceedings of the National Academy of Sciences. 1965. p. 75–81.
[46] Menninger JR, Otto DP. Erythromycin, carbomycin, and spiramycin inhibit protein synthesis by stimulating the dissocation of peptidyl-tRNA from ribosomes. *Antimicrob Agents Chemother*. 1982;21(5):811–8.
[47] Hitchings GH. Mechanism of Action of Trimethoprim-Sulfamethoxazole-I. *J Infect Dis*. 1973;128(Suppl. 3):S433–6.
[48] Chalkley LJ, Koornhof HJ. Antimicrobial activity of ciprofloxacin against Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus determined by the killing curve method: Antibiotic comparisons and synergistic interactions. *Antimicrob Agents Chemother*. 1985;28(2):331–42.
[49] Moodley S, Koorbanally N a, Moodley T, Ramjugernath D, Pillay M. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a rapid, cheap, screening test for the in vitro anti-tuberculous activity of chalcones. *J Microbiol Methods*. Elsevier B.V.; 2014 Sep;104:72–8.
[50] Wang H, Cheng H, Wang F, Wei D, Wang X. An improved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for evaluating the viability of Escherichia coli cells. *J Microbiol Methods*. Elsevier B.V.; 2010;82(3):330–3.
[51] Riss TL, Moravec R, Niles AL, Benink H, Worzella TJ, Minor L. Cell Viability Assays Assay Guidance Manual. Assay Guidance Manual. 2013.
[52] Haddock BA, Jones CW. Bacterial Respiration. *Bact Rev*. 1977;41(1):47–99.
[53] Fischer ER, Hansen BT, Nair V, Hoyt FH, Dorward DW. Scanning Electron Microscopy. In: Current Protocol Microbiology. 2012. p. 1–76.
[54] Piroeva I, Dimowa L, Sbirkova H. A simple and rapid scanning electron microscope preparative technique for observation of biological samples : application on bacteria and DNA samples. *Bulg Chem Commun*. 2013;45(4):510–5.
[55] Mestres P, Pütz N, Garcia Gómez de Las Heras S, García Poblete E, Morguet A, Laue M. The surface topography of the choroid plexus. Environmental, low and high vacuum scanning electron microscopy. *Ann Anat*. Elsevier GmbH.; 2011;193(3):197–204.
[56] Kalàb M, Yang A-F, Chabot D. Conventional Scanning Electron Microscopy of Bacteria.pdf. In Focus. 2008;(10):43–61.
[57] Zhao L, Zhang H, Hao T, Li S. In vitro antibacterial activities and mechanism of sugar fatty acid esters against five food-related bacteria. *Food Chem*. Elsevier Ltd; 2015;187(2015):370–7.
[58] Zhang Y, Liu X, Wang Y, Jiang P, Quek S. Antibacterial activity and mechanism of cinnamon essential oil against Escherichia coli and Staphylococcus aureus. *Food Control*. Elsevier Ltd; 2016;59:282–9.
[59] Hartmann M, Berditsch M, Hawecker J, Ardakani MF, Gerthsen D, Ulrich AS. Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrob Agents Chemother*. 2010;54(8):3132–42.

International Journal of Infection Control
www.ijic.info ISSN 1996-9783

REVIEW

Infection control in the UK: an antimicrobial resistance perspective

Elecia Henry[1], Robert B Smith[2], Michael Collins[3], Susan J Bird[1], Pauline Gowland[1] and John P Cassella[4]
[1]School of Life Sciences and Education, Staffordshire University, Stoke-on-Trent, Staffordshire, ST4 2DF, United Kingdom
[2] Chemistry, School of Physical Sciences and Computing, Preston UCLan, PR1 2HE, United Kingdom
[3] Chesterfield Royal Hospital NHS Foundation Trust, Chesterfield Road, Calow, Chesterfield, Derbyshire, S44 5BL, United Kingdom
[4] School of Law, Policing and Forensics, Staffordshire University, Stoke-on-Trent, Staffordshire, ST4 2DF, United Kingdom

doi: 10.3396/IJIC.v13i2.011.17

Abstract
The spread of healthcare-associated infections has become a matter of global concern. These infections, which were once solely limited to hospital settings, have emerged in the community. Reduced infection control practices strengthen this rapid spread of infections, which has led to the increase in the occurrence of multi-drug resistant organisms. The state of infection control in the United Kingdom is of extreme importance because of several reported cases of infections caused by these organisms. In addition to antimicrobial usage in human medicine, antimicrobial agents used in agriculture must be considered as major factors in the prevalence of resistant organisms and the implications to the UK. However, this increased occurrence of antimicrobial resistant bacteria and their resulting infections could be reduced with the application of effective policies for antibiotic use in agricultural environments, stringent decontamination and sterilisation techniques and better regulations that encourage the search for, and development of, new and novel drugs.

Keywords: Infection control, healthcare associated infections, antimicrobial drug resistance, prophylaxis, health policy.

Corresponding Author
Elecia Henry
School of Life Sciences and Education, Staffordshire University, Stoke-on-Trent, Staffordshire, ST4 2DF, United Kingdom.
Email: Elecia.Henry@research.staffs.ac.uk

Introduction

With a prevalence of 6.4%,[1] the spread of healthcare-associated infections (HCAIs) and their subsequent control has become an important issue in hospitals.[2] Historically, the hospital setting was subjected to daily rigorous sanitisation by dedicated cleaning staff, and all medical students and staff had to observe a stringent hygiene regimen.[3] Over time, there was a decrease in following these strict rules of hospital hygiene resulting in an increase in HCAIs. As a result, infection control practices, although not the primary cause of disease transmission, suffered great setbacks leading to the unintended spread of infectious diseases.[3]

Reduction in global infection control practices has led indirectly to the development of antimicrobial resistance (AMR), which is the evolution of pathogenic microorganisms to become resistant to anti-infective agents. Occurrence of these resistant microorganisms has increased worldwide,[4] spreading to the UK and increasing the risks of both patients and healthcare workers acquiring these infections in a healthcare setting as reported by the National Institute for Health and Care Excellence (NICE).[5] These infections, which were once mainly hospital-acquired, are now seen in the community and the issue of increasing resistance, as reported by WHO,[6] to antimicrobials has also become an issue beyond infection control in the hospital environment.

This paper focuses on the current state of infection control in the UK in terms of the major issues regarding anti microbial resistance. Consideration of antimicrobial usage in agriculture and human medicine will be reviewed, as well as their consequences relating to the major causes of AMR, the prevalence of global threat from this resistance and the implications to the UK. The need to develop new antibiotics as a consequence of this issue will be considered.

Methods

The methodology used for data collection was an internet search using keywords and phrases related to infection control in the UK and antibiotic use on UK farms and in UK human medicine. Other phrases used in the search were those involving causes and occurrences of AMR including the importance of new antimicrobial agents. The internet search engines used included ScienceDirect, Google Scholar, the Antimicrobial Resistance and Healthcare-associated Infections (AMRHAI) reference unit, the Department of Public Health (UK), Public Health England and the World Health Organisation (WHO).

Antibiotic use on UK farms

In a 2014 Review on Antimicrobial Resistance that was announced by the UK Prime Minister, several measures were identified to deal with the growing problem of AMR. One of these measures was a global commitment to monitor the use of antibiotic drugs by humans, in agriculture and in the environment.[7]

Although overlooked because of the restrictions placed on the class of antibiotics used, antimicrobial agents in livestock farming do contribute to AMR.[8] These drugs are used for various reasons such as for medicinal purposes, whether by mass medication (metaphylaxis) or preventative medication (prophylaxis), and to promote growth of the animals. Although the use of antibiotics as growth aids have been banned in Europe, they are still being used as prophylactics.[9]

In the United Kingdom, De Briyne *et al.*[10] reported that penicillins and tetracylines were the most commonly used antibiotics in cattle (32% and 10% respectively) and horses (24% and 11% respectively) to treat disorders such as mastitis and respiratory infections. In another study involving 88 UK farms, heifer calves with respiratory diseases, e.g., pneumonia, were treated with antibiotics (11.3% ±15.8%).[11] Despite the availability of guidelines on antibiotic use in agriculture, 50% of farmers appear to be unfamiliar with these guidelines leading to possible inefficient farming practices.[12]

Commercially available antimicrobial agents, such as ivermectin, are also used on goat farms in the UK to treat ectoparasites such as lice and mange.[13] Extensive use of this macrocyclic lactone and other unnamed antibiotics by many goat farmers may have been ineffectively regulated and could have contributed to the increase in antibiotic resistance of infectious ectoparasites.[13]

A survey of UK broiler farms conducted by Hughes *et al.*[14] showed that 32% of poultry farmers used prescribed antibiotics such as amoxicillin for metaphylactic

purposes. On some participating farms, other antibiotics such as trimethoprim/sulfadiazine (16.8%) and tylosin tartrate (11.7%) were used as prophylactics.[14] Results of the 2008 study indicated that antibiotics were mainly used to prevent diseases in broilers rather than to treat them. Antibiotic management on broiler farms needs further investigation since antibiotic misuse is a potential factor in AMR.

United Kingdom pig farmers, like some European pig farmers,[15] seemed to focus more on their economic issues rather than infections in their animals[16] leading to overuse of antibiotics. As a consequence, it has been shown that there is a clear connection between the poor regulation of antibiotic use in UK farms and AMR in humans.[8]

Antibiotic use in UK human medicine

It is without doubt that antimicrobial agents have been effective against many human infectious diseases. The incidence of infectious diseases in the UK has not notably increased but the total usage of these agents have reportedly increased by 4.1% across the NHS Trusts in a five-year period (2008-2012).[17] While there has been a reduction in the use of fluoroquinolones, such as ciprofloxacin (-23.8%), within the same period, the 2014 study showed that there was an increase in the use of other drugs such as meropenem of the carbapenem class (94.8%), and penicillins such as piperacillin/tazobactam (142.3%). While the report provides expected and observed data on point-of-care antimicrobial usage, it does not show progressive usage, which is crucial in antimicrobial management.

Consequential spread of infectious diarrhoea caused by *Clostridium difficile* is reported to be a major concern in hospitals. In a 2015 study,[18] the mean number of patients suspected to be infected with these organisms was estimated to be 149.6 ± 173.6 in small hospitals, 75.0 ± 27.8 in medium hospitals and 43.78 ± 37.7 in large hospitals. In order to treat patients with diarrhoea that resulted from *Clostridum difficile* infection, metronidazole and fidaxomicin were the primary antimicrobial agents used while vancomycin was the secondary drug of choice.[18] One concern that emerged from the study was that with the increasing incidence there was the possible spread of infections due to poor infection control practices. Without adequate infection control, the risk of the emergence of antimicrobial resistant *Clostridium difficile* increases. Since more first-line and second-line antibiotics are being used to treat infectious diarrhoea and a number of patients (mean = 10 ± 0.0) are re-admitted after an uncompleted course of treatment of the same drugs, there is the concern that AMR in *Clostridium difficile* could develop.

The concern of the spread of HCAIs was also noted in a 2009 study by Burnett *et al.*[19] Findings from the report that focused on patients with and without *Staphylococcus aureus* septicaemia identified critical issues of infection control that needed attention, such as communication, patient confidence in the NHS, and health and safety practices. Antibiotics are understandably used against HCAIs but there is the need to monitor the possible spread of resistant organisms with increased use in a closed environment found in healthcare settings. Another important concern is the use of antibiotics by persons outside of the hospital environment. Antimicrobial agents are also prescribed by general practitioners in the community. A review of databases such as the Clinical Practice Research Datalink (CPRD) by Shallcross and Davies[20] indicates that 80-90% of antibiotic prescriptions are given in primary care facilities. However, these programmes do not record actual use of antibiotics by patients. The possibility for the spread of resistant organisms between hospitals and community environments in conditions promoting the maintenance of resistance can be inferred.

The prevalence of antimicrobial resistance

Antimicrobial resistance is the development of reduced sensitivity to antimicrobial agents in microorganisms. Major antimicrobial resistant organisms as listed by Tang *et al.*[21] include meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), extended spectrum β-lactamase-producing (ESBL) *Enterobacteriaceae*, carbapenemase-producing *Enterobacteriaceae* (CPE) and carbapenem-resistant *Acinetobacter baumannii* (CRA). The main mechanisms of resistance in such organisms usually involve either the transmission of resistant genetic material from one bacterial cell to another or mutation/s in new genes. Antimicrobial resistance has become a global epidemic resulting in the generation of AMR policies

in many regions, including Europe. The European Centre for Disease Prevention and Control (ECDC) has acknowledged the threat of AMR and has identified the major causes of the rapid spread of AMR across Europe with a particular source being the use of antibiotics in agriculture.[22] A report from the B-Debate meeting with public health authorities across Europe stated that the unchecked use of antibiotics in farming practices led to selective pressure for resistant organisms, which could spread to humans.[22] These resistant organisms are spread through human consumption of inadequately prepared meat products[23] and dairy milk,[24] and human contact with animal waste.[25,26] Snow *et al.*[25] determined that 17 of 65 sampled dairy farms received animals with various strains of CTX-M *E. coli*, which is an ESBL-producing microorganism. Findings of the study outlined insufficient infection control practices, such as slurry containment, open herding and inadequate cleaning of feeding equipment used for calves.[25]

Mismanaged antimicrobial chemotherapy in relation to ineffective infection control strategies is also a source of the development of AMR in bacteria as well as the spread of AMR bacteria. The prevalence of CTX-M *Klebsiella pneumoniae*,[27] *Salmonella typhi* and *Salmonella paratyphi*,[28] and MRSA[29] has been documented in UK hospitals. Each study has identified poor infection control measures as a contributing factor to the spread of antimicrobial resistant microorganisms. While studies have found isolates of non-resistant *Pseudomonas aeruginosa* from water systems in UK hospital wards[30] as well as isolates of non-resistant *Staphylococcus spp* from various clinical instruments,[31] the risk of AMR evolution still exists. High standards in infection control are crucial, especially when staff are charged with the care of critically ill patients who are most susceptible to infections from antimicrobial resistant organisms.[32]

In the community setting, unchecked prescribing of antimicrobial agents has been identified as a contributing factor to the spread of antimicrobial resistant bacteria.[33] Due to misuse and abuse of antibiotics, the ECDC has recommended that antibiotic usage should be recorded as defined daily doses (DDD) over time.[22] Supplementary data such as the number of packages used per day and details about the package content enhances knowledge regarding the antibiotic consumption.[22] Support for this recommendation was evidenced by a European study which showed that the average DDD per package increased between 0.04 and 0.31 per year.[34] This marked increase in antibiotic use is an indication of the potential risk of antimicrobial resistant bacteria transmission.[35]

Another aspect of inadequate infection control is the disposal of waste containing antibiotics. Samples of waste water and surface water from the UK environment were shown to contain multiple antibiotics such as amoxicillin, oxytetracycline and trimethoprim.[36] Emerging contaminants (EC) found in water sources at sites in Oxford along the Thames river and Boxford along the River Lambourn included pharmaceuticals.[37] ECs were also found in the Chalk aquifer, which provides potable water to parts of the UK.[38] The presence of antibiotics in water sources is an important factor in AMR since these compounds tend to be water-soluble and remain in the environment.[39,40] Improper waste disposal from farms and communities could contribute to the presence of antibiotics in UK water sources.[41] The constant exposure of non-resistant bacteria to antibiotics in water sources may encourage the evolution of antimicrobial resistant bacteria.

Another important consideration is the exposure of humans to water sources that are contaminated with antimicrobial resistant bacteria. A recent study showed that samples taken from coastal waters of England and Wales had a prevalence of 1.2% of 3rd generation cephalosporin-resistant *E. coli*.[42] This value may be low since only one strain of resistant *E. coli* was investigated. Persons taking part in recreational activities in these areas, such as water sports, swimming and diving, had an increased risk of infection from antimicrobial resistant bacteria due to ingestion of contaminated water.

Antimicrobial resistance to colistin

Colistin, also known as polymixin E, has been considered as a critical agent against some common multidrug-resistant Gram-negative aerobic bacilli, including CPEs. The mechanism of action of colistin is to damage the integrity of the outer envelope of Gram-negative bacilli by causing instability of membrane-bound lipopolysaccharides.[43] This damage allows cellular contents to escape, which results in

apoptosis. Because of its toxic effects on the human kidney, colistin use in antimicrobial therapy was halted.[44] However, the rapid emergence of CPEs had prompted the return of the usage of this drug. Overuse of colistin has now resulted in infections caused by colistin-resistant CPEs. Initially, resistance was thought to result from chromosomal mutations. Recent reports on colistin resistance in Europe, including a study published by the ECDC,[45] revealed that resistance can now be mediated by the transfer of plasmids containing the colistin-resistant gene known as *MCR-1*.[46-50]

Infection control strategies to curb the spread of antimicrobial resistant bacteria

Effective infection control strategies are important in reducing the spread of antimicrobial resistant bacteria. One strategy is the implementation of policies and guidelines. The Department of Health has issued a 5-year plan that involves policies to lessen the impact of AMR on both humans and animals. The paper identifies seven important actions needed to achieve this goal, which includes the revision of infection control practices in agriculture and human settings.[51]

Adherence to proper and effective animal husbandry was recommended as a way of protecting animals from infectious antimicrobial resistant bacteria.[9,51] In terms of antimicrobial therapy, farmers and agricultural authorities should maintain accurate information regarding health and safety practices and antibiotic use.[9] Other recommendations include a total ban of antibiotic use in prophylaxis, a ban on unregulated antibiotics in animals and discontinuation of cephalosporins and fluoroquinolones in animal antimicrobial chemotherapy.[8]

The 2013 edition of the NICE clinical guidelines has defined clear rules that govern infection control practices in both hospital and non-hospital environments. These include proper training of all staff, availability of items needed to carry out their duties (gloves, antiseptic agents, etc.), effective sterilisation and disinfection techniques and proper waste disposal management.[52] These measures should be at the forefront of infection control since there has been an increase in public interest in CPEs in the UK.[53] Decontamination, disinfection and sterilisation have been identified as excellent methods of the elimination and extermination of infectious agents.[54] Another method of reducing the spread of infectious organisms is the proper use of gloves.[55]

A key action of the UK five-year plan is to encourage research and development in new antibiotic drug therapies.[51] Due to the overuse of antibiotics in humans and animals, which was first reported by the Swann Committee (1969) and the increase of the "major public health threat" of AMR as highlighted in House of Lords' report (1998), there is an immediate need for new antibiotic drugs.[56] The progress of novel drug discovery is limited by factors such as financial gain and strict regulations regarding drug trials and manufacture.[57] Authorities have altered such regulations, which has revitalised the interest of large pharmaceutical companies into resuming the search for new and more effective drugs against antimicrobial resistant bacteria.[58] This endeavour could also be strengthened with synergistic studies that show the mechanism(s) of combinatorial therapies.[59] Researchers are also encouraged to investigate natural-based and semi-synthetic compounds as viable alternatives. Some medicinal scientists have identified chalcones,[60] which are plant-based intermediates of flavonoids, and their ferrocenyl derivatives as potential drugs since these exhibit potent antimicrobial activity.[61-64]

Conclusion

Infection control practices and appropriate prescribing of antibiotics are essential tools against AMR. With the overuse of antibiotics on farms and in human medicine across the UK, it is evident that AMR continues to spread. However, this increase in the prevalence of antimicrobial resistant bacteria and their resulting infections could be slowed with the implementation of effective policies for antibiotic use in animals and farms, strict decontamination and sterilisation techniques and better regulations that encourage the search for new and novel drugs.

Conflicts of Interest

The authors whose names are listed in this article certify that they have NO affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript. No sources of funding were required for this review.

References

1 HPA. English National Point Prevalence Survey on Healthcare-associated Infections and Antimicrobial Use, 2011: Preliminary data. Health Protection Agency, London; 2012.
2 Mitchell BG, Hall L, MacBeth D, Gardner A, Halton K. Hospital infection control units: Staffing, costs, and priorities. *Am J Infect Control* 2015; **43(6):** 612–616. https://doi.org/10.1016/j.ajic.2015.02.016
3 Warren P. The UK's National Health Service does not meet its control standards. *J Hosp Infect* 2005; **59(1):** 1–3. https://doi.org/10.1016/j.jhin.2004.07.017
4 Budd E. ARHAI's 5th annual report 2013 to 2014. London; 2014.
5 NICE. Infection - Prevention and control of healthcare-associated infections in primary and community care. National Institute for Health and Care Excellence, London; 2012.
6 WHO. Worldwide country situation analysis: response to antimicrobial resistance. World Health Organisation, Geneva; 2015.
7 O'Neill J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. The Review on Antimicrobial Resistance, London; 2014.
8 ASOA. Antimicrobial resistance - why the irresponsible use of antibiotics in agriculture must stop. The Alliance to Save Our Antibiotics, Bristol; 2014.
9 Elliott K. Antibiotics on the Farm : Agriculture's Role in Drug Resistance. CGD Policy Paper 059. Washington, DC, USA; 2015.
10 De Briyne N, Atkinson J, Borriello SP, Pokludova L. Antibiotics used most commonly to treat animals in Europe. *Vet Rec* 2014; **175:** 325. https://doi.org/10.1136/vr.102462
11 Boulton AC, Rushton J, Wathes DC. A Study of dairy heifer rearing practices from birth to weaning and their associated costs on UK dairy farms. *Open J Anim Sci* 2015; **5:** 185–197. https://doi.org/10.4236/ojas.2015.52021
12 Jones PJ, Marier E, Tranter RB, Wu G, Watson E, Teale CJ. Factors affecting dairy farmers' attitudes towards antimicrobial medicine usage in cattle in England and Wales. *Prev Vet Med* 2015; **121(1-2):** 30–40. https://doi.org/10.1016/j.prevetmed.2015.05.010
13 Cornall K, Wall R. Ectoparasites of goats in the UK. *Vet Parasitol* 2015; **207(1-2):** 176–179. https://doi.org/10.1016/j.vetpar.2014.11.005
14 Hughes L, Hermans P, Morgan K. Risk factors for the use of prescription antibiotics on UK broiler farms. *J Antimicrob Chemother* 2008; **61(4):** 947–952. https://doi.org/10.1093/jac/dkn017
15 Visschers VHM, Backhans A, Collineau L, *et al.* Perceptions of antimicrobial usage, antimicrobial resistance and policy measures to reduce antimicrobial usage in convenient samples of Belgian, French, German, Swedish and Swiss pig farmers. *Prev Vet Med* 2015; **119(1-2):** 10–20. https://doi.org/10.1016/j.prevetmed.2015.01.018
16 Alarcon P, Wieland B, Mateus ALP, Dewberry C. Pig farmers' perceptions, attitudes, influences and management of information in the decision-making process for disease control. *Prev Vet Med* 2014; **116(3):** 223–242. https://doi.org/10.1016/j.prevetmed.2013.08.004
17 Cooke J, Stephens P, Ashiru-Oredope D, Johnson AP, Livermore DM, Sharland M. Antibacterial usage in English NHS hospitals as part of a national Antimicrobial Stewardship Programme. *Public Health* 2014; **128(8):** 693–697. https://doi.org/10.1016/j.puhe.2014.06.023
18 Buchanan J, Wordsworth S, O'Connor L, *et al.* Management of patients with suspected infectious diarrhoea in hospitals in England. *J Hosp Infect* 2015; **90(3):** 199–207. https://doi.org/10.1016/j.jhin.2014.12.021
19 Burnett E, Lee K, Rushmer R, Ellis M, Noble M, Davey P. Healthcare-associated infection and the patient experience: a qualitative study using patient interviews. *J Hosp Infect* 2010; **74(1):** 42–47. https://doi.org/10.1016/j.jhin.2009.07.027
20 Shallcross LJ, Davies SC. GP antibiotic overuse and microbial resistance. *Br J Gen Pract* 2015; **65(631):** 61–62. https://doi.org/10.3399/bjgp15X683413
21 Tang SS, Apisarnthanarak A, Hsu LY. Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. *Adv Drug Deliv Rev* 2014; **78:** 3–13. https://doi.org/10.1016/j.addr.2014.08.003
22 Roca I, Akova M, Baquero F, *et al.* The global threat of antimicrobial resistance: Science for intervention. *New Microbes New Infect* 2015; **6:** 22–29. https://doi.org/10.1016/j.nmni.2015.02.007
23 Woolhouse M, Ward M, van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. *Philos Trans R Soc Lond B Biol Sci* 2015; **370(1670):** 20140083. https://doi.org/10.1098/rstb.2014.0083
24 Randall L, Heinrich K, Horton R, *et al.* Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011. *Res Vet Sci* 2014; **96(1):** 15–24. https://doi.org/10.1016/j.rvsc.2013.10.009
25 Snow LC, Warner RG, Cheney T, *et al.* Risk factors associated with extended spectrum beta-lactamase Escherichia coli (CTX-M) on dairy farms in North West England and North Wales. *Prev Vet Med* 2012; **106(3-4):** 225–234. https://doi.org/10.1016/j.prevetmed.2012.03.009
26 Moore JE, Huang J, Yu P, *et al.* High diversity of bacterial pathogens and antibiotic resistance in salmonid fish farm pond water as determined by molecular identification employing 16S rDNA PCR, gene sequencing and total antibiotic susceptibility techniques. *Ecotoxicol Environ Saf* 2014; **108:** 281–286. https://doi.org/10.1016/j.ecoenv.2014.05.022
27 Lob SH, Biedenbach DJ, Badal RE, Kazmierczak KM, Sahm DF. Antimicrobial resistance and resistance mechanisms of Enterobacteriaceae in ICU and non-ICU wards in Europe and North America: SMART 2011–2013. *J Glob Antimicrob Resist* 2015; **3(3):** 190-197. https://doi.org/10.1016/j.jgar.2015.05.005
28 Dave J, Sefton A, de Pinna E, *et al.* Trends in antibiotic susceptibility of enteric fever isolates in East London. *Travel Med Infect Dis* 2015; **13(3):** 230–234. https://doi.org/10.1016/j.tmaid.2015.04.001
29 Newitt S, Myles PR, Birkin JA, *et al.* Impact of infection control interventions on rates of *Staphylococcus aureus* bacteraemia in National Health Service acute hospitals, East Midlands, UK, using interrupted time-series analysis. *J Hosp Infect* 2015; **90(1):** 28–37. https://doi.org/10.1016/j.jhin.2014.12.016
30 Loveday H, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J. Association between healthcare water systems and Pseudomonas aeruginosa infections: a rapid systematic review. *J Hosp Infect* 2014; **86(1):** 7–15. https://doi.org/10.1016/j.jhin.2013.09.010
31 Mahida N, Levi K, Kearns A, Snape S, Moppett I. Investigating the impact of clinical anaesthetic practice on bacterial contamination of intravenous fluids and drugs. *J Hosp Infect* 2015; **90(1):** 70–74. https://doi.org/10.1016/j.jhin.2014.11.024
32 Bauchmuller K, Faulds MC. Care of the critically ill patient. *Surg* 2015; **33(4):** 165–171. https://doi.org/10.1016/j.mpsur.2015.01.009
33 Spellberg B, Guidos R, Gilbert D, *et al.* The epidemic

of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. *Clin Infect Dis* 2008; **46(2):** 155–164. https://doi.org/10.1086/524891

34 Bruyndonckx R, Hens N, Aerts M, Goossens H, Molenberghs G, Coenen S. Measuring trends of outpatient antibiotic use in Europe: jointly modelling longitudinal data in defined daily doses and packages. *J Antimicrob Chemother* 2014; **69(7):** 1981–1986. https://doi.org/10.1093/jac/dku063

35 Busfield J. Assessing the overuse of medicines. *Soc Sci Med* 2015; **131:** 199–206. https://doi.org/10.1016/j.socscimed.2014.10.061

36 Petrie B, Barden R, Kasprzyk-Hordern B. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Res* 2014; **72(0):** 3–27.

37 Stuart ME, Lapworth DJ, Thomas J, Edwards L. Fingerprinting groundwater pollution in catchments with contrasting contaminant sources using microorganic compounds. *Sci Total Environ* 2014; **468-469:** 564–577. https://doi.org/10.1016/j.scitotenv.2013.08.042

38 Lapworth DJ, Baran N, Stuart ME, Manamsa K, Talbot J. Persistent and emerging micro-organic contaminants in Chalk groundwater of England and France. *Environ Pollut* 2015; **203:** 214–225. https://doi.org/10.1016/j.envpol.2015.02.030

39 Zhang D, Gersberg RM, Ng WJ, Tan SK. Removal of pharmaceuticals and personal care products in aquatic plant-based systems: A review. *Environ Pollut* 2014; **184(2014):** 620–639. https://doi.org/10.1016/j.envpol.2013.09.009

40 Fernandes JP, Almeida CMR, Pereira AC, *et al.* Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms. *Bioresour Technol* 2015; **182:** 26–33. https://doi.org/10.1016/j.biortech.2015.01.096

41 Stuart M, Lapworth D, Crane E, Hart A. Review of risk from potential emerging contaminants in UK groundwater. *Sci Total Environ* 2012; **416:** 1–21. https://doi.org/10.1016/j.scitotenv.2011.11.072

42 Leonard AFC, Zhang L, Balfour AJ, Garside R, Gaze WH. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. *Environ Int* 2015; **82:** 92–100. https://doi.org/10.1016/j.envint.2015.02.013

43 Newton BA. The properties and mode of action of the polymixins. *Bact Rev* 1956; **20:** 14–27.

44 Falagas ME, Kasiakou SK. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. *Crit Care* 2006; **10(1):** 1–13. https://doi.org/10.1186/cc3910

45 European Centre for Disease Prevention and Control (ECDC). Plasmid-mediated colistin resistance in Enterobacteriaceae. Stockholm; 2016.

46 Liu YY, Wang Y, Walsh TR, *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect Dis* 2016; **16:** 161–168. https://doi.org/10.1016/S1473-3099(15)00424-7

47 Doumith M, Godbole G, Ashton P, *et al.* Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J Antimicrob Chemother* 2016; **71(8):** 2300–2305. https://doi.org/10.1093/jac/dkw093

48 Schwarz S, Johnson AP. Transferable resistance to colistin: A new but old threat. *J Antimicrob Chemother* 2016; **71(8):** 2066–2070. https://doi.org/10.1093/jac/dkw274

49 Day M, Doumith M, Jenkins C, *et al.* Antimicrobial resistance in Shiga toxin-producing *Escherichia coli* serogroups O157 and O26 isolated from human cases of diarrhoeal disease in England, 2015. *J Antimicrob Chemother* 2016; **72:** 145-152. https://doi.org/10.1093/jac/dkw371

50 Findlay J, Hopkins KL, Alvarez-buylla A, *et al.* Characterization of carbapenemase-producing Enterobacteriaceae in the West Midlands region of England: 2007–14. *J Antimicrob Chemother* 2017; **10:** 1–9. https://doi.org/10.1093/jac/dkw560

51 DOH. UK Five Year Antimicrobial Resistance Strategy 2013 to 2018. Department of Health, London; 2013.

52 Loveday HP, Wilson JA, Pratt RJ, *et al.* epic3: national evidence-based guidelines for preventing healthcare-associated infections in NHS hospitals in England. *The Journal of Hospital Infection* 2014; **86(1):** S1–S70. https://doi.org/10.1016/S0195-6701(13)60012-2

53 Otter JA. What's trending in the infection prevention and control literature? From HIS 2012 to HIS 2014, and beyond. *J Hosp Infect* 2015; **89(4):** 229–236. https://doi.org/10.1016/j.jhin.2015.02.004

54 de Zoysa H, Morecroft E. Cleaning, disinfection and sterilization of equipment. *Anaesth Intensive Care Med* 2007; **8(11):** 453–456. https://doi.org/10.1016/j.mpaic.2007.09.001

55 Loveday HP, Lynam S, Singleton J, Wilson J. Clinical glove use: Healthcare workers' actions and perceptions. *J Hosp Infect* 2014; **86(2):** 110–116. https://doi.org/10.1016/j.jhin.2013.11.003

56 Wise R. The urgent need for new antibacterial agents. *J Antimicrob Chemother* 2011; **66(9):** 1939–1940. https://doi.org/10.1093/jac/dkr261

57 Livermore DM. Discovery research: the scientific challenge of finding new antibiotics. *J Antimicrob Chemother* 2011; **66(9):** 1941–1944. https://doi.org/10.1093/jac/dkr262

58 Bax R, Green S. Antibiotics: the changing regulatory and pharmaceutical industry paradigm. *J Antimicrob Chemother* 2015; **70(5):** 1281–1284. https://doi.org/10.1093/jac/dku572

59 Bollenbach T. Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. *Curr Opin Microbiol* 2015; **27:** 1–9. https://doi.org/10.1016/j.mib.2015.05.008

60 Harborne JB, Williams CA. Advances in flavonoid research since 1992. *Phytochemistry* 2000; **55(6):** 481–504. https://doi.org/10.1016/S0031-9422(00)00235-1

61 Pejović A, Damljanović I, Stevanović D, *et al.* Antimicrobial ferrocene containing quinolinones: Synthesis, spectral, electrochemical and structural characterization of 2-ferrocenyl-2,3-dihydroquinolin-4(1H)-one and its 6-chloro and 6-bromo derivatives. *Polyhedron* 2012; **31(1):** 789–795. https://doi.org/10.1016/j.poly.2011.11.006

62 Kowalski K, Koceva-Chy A, Szczupak L, *et al.* Ferrocenylvinyl-flavones: Synthesis, structure, anticancer and antibacterial activity studies. *J Organomet Chem* 2013; **741-742(1):** 153–161. https://doi.org/10.1016/j.jorganchem.2013.05.009

63 Prasath R, Bhavana P, Ng SW, Tiekink ERT. The facile and efficient ultrasound-assisted synthesis of new quinoline-appended ferrocenyl chalcones and their properties. *J Organomet Chem* 2013; **726:** 62–70. https://doi.org/10.1016/j.jorganchem.2012.12.022

64 Ahmed N, Konduru NK, Owais M. Design, synthesis and antimicrobial activities of novel ferrocenyl and organic chalcone based sulfones and bis-sulfones. *Arab J Chem* 2015. https://doi.org/10.1016/j.arabjc.2014.12.008