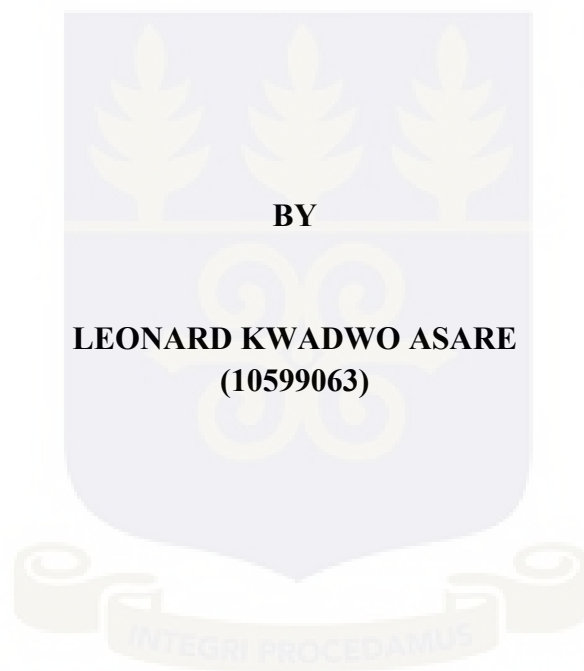


**UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCE**

**INTERACTIONS OF ANTIMICROBIAL COMPOUNDS WITH SELECTED DRUGS
USED IN THE CLINICAL MANAGEMENT OF SICKLE CELL DISEASE (SCD)**



BY

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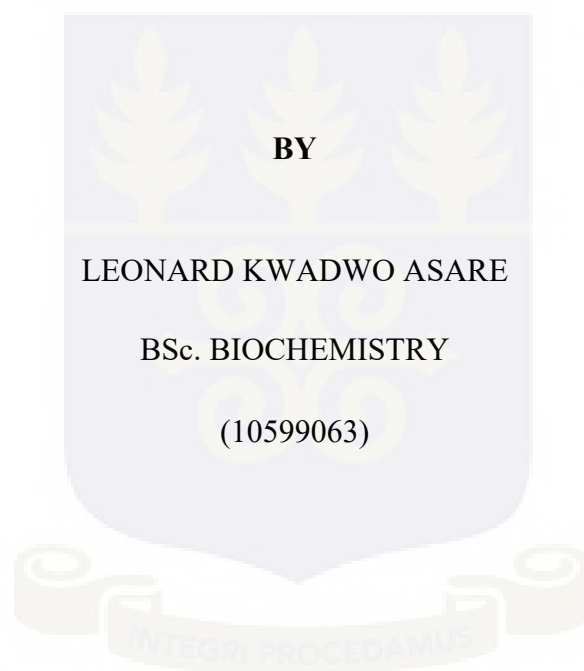
**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF
M. PHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE**

JULY, 2018

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SICKLE CELL DISEASE (SCD)**

A THESIS PRESENTED TO THE WEST AFRICAN CENTRE FOR CELL BIOLOGY OF
INFECTIOUS PATHOGENS (WACCBIP), DEPARTMENT OF BIOCHEMISTRY, CELL
AND MOLECULAR BIOLOGY

UNIVERSITY OF GHANA



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JULY, 2018

DECLARATION

I Leonard Kwadwo Asare (Department of Biochemistry, Cell and Molecular Biology, University of Ghana) hereby declare that this thesis is the result of my own research under the supervision of Dr. Patrick Kobina Arthur and Dr. Elmer Ametefe (Department of Biochemistry, Cell and Molecular Biology, University of Ghana). To the best of my knowledge, this thesis contains no material previously presented for another degree in this University or elsewhere, or any material previously published by another author, except where due acknowledgement has been made in the text of the thesis.

.....
Leonard Kwadwo Asare
(Student)

.....
Dr. Patrick Kobina Arthur
(Principal Supervisor)

.....
Dr. Elmer Ametefe
(Co-Supervisor)



DEDICATION

Glory be to the Father and to the Son, and to the Holy Spirit;

As it was in the beginning, is now and ever shall be, world without end. Amen

This work is dedicated to all scientists in drug discovery research and sickle cell disease individuals.



ACKNOWLEDGEMENT

I sincerely want to express my heartfelt gratitude to my supervisor, Dr. Patrick K. Arthur. He has been very instrumental in my training and this project would not have been positive without him. I appreciate this greatly. I am exceedingly grateful to WACCBIP for this study opportunity to gain relevant research experience and for fully funding my attendance to the EMBL Conference on New Approaches and Concepts in Microbiology in Germany.

I also express my profound thanks to Dr. Elmer Ametefe for her time and input towards this work. My sincere thanks go to Dr. Vincent Amah, Ethel J. S. Blessie, Rebecca Yeboah and Isaac Carilo for all their help, encouragement and support.

I also express my gratitude to the faculty and technical staff of the Department of Biochemistry, Cell and Molecular Biology for their immense contribution towards my training and this project.

Thank you Daddy and Mummy for your continuous provision and prayers for me.

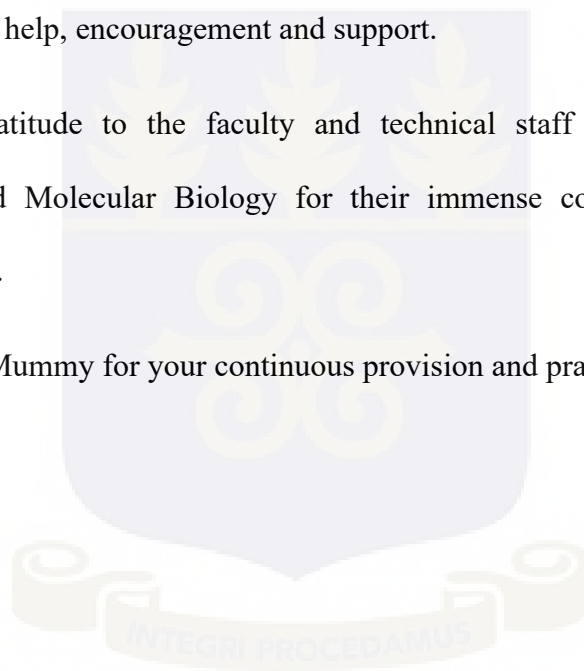


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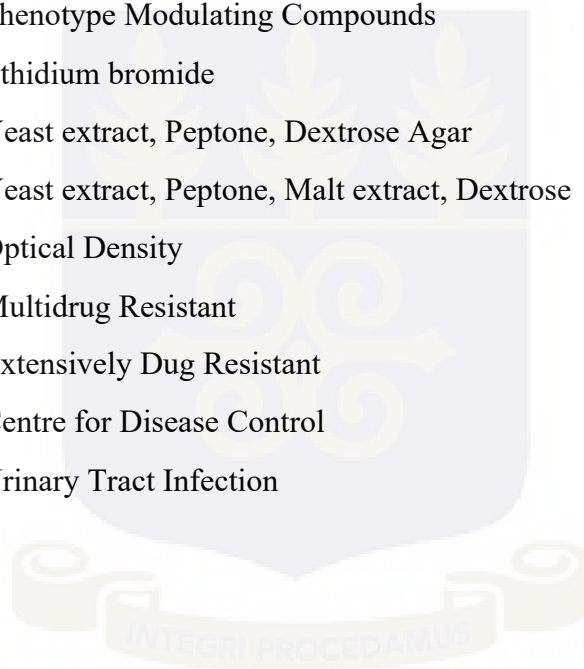
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LIST OF ABBREVIATIONS

MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MHA	Mueller Hinton Agar
SBF	Soil-borne fungi
TEF	Terrestrial Endophytic fungi
4-NQ	4 nitro-quinolone oxide
1,10-PMH	1,10-Phenanthroline monohydrate
SCD	Sickle Cell Disease
GLASS	Global Antimicrobial Surveillance system
WHO	World Health Organization
PMC	Phenotype Modulating Compounds
EtBr	Ethidium bromide
YPDA	Yeast extract, Peptone, Dextrose Agar
YPMD	Yeast extract, Peptone, Malt extract, Dextrose
OD	Optical Density
MDR	Multidrug Resistant
XDR	Extensively Drug Resistant
CDC	Centre for Disease Control
UTI	Urinary Tract Infection





ABSTRACT

Though the promotion of resistant bacteria worldwide has mainly been attributed to the injudicious use of many antibiotics, the role of non-antimicrobial drugs used in the clinical management of pathological conditions of non-infectious diseases in the modulation of microbial drug resistance or susceptibility needs to be assessed. The effects of the antibiotic-non-antibiotic drug-drug interactions against bacteria in SCD patients and in other chronic diseases cannot be overemphasized. The interactions of nine SCD management drugs and fifteen phenotype modulating compounds on the activities of 19 standard antimicrobials against clinical isolates of methicillin-resistant *S. aureus* (MRSA) and *E. coli* were investigated by the agar plate disc diffusion method. We also analyzed the effects of the compounds on ethidium bromide (EtBr) uptake and efflux in the bacterial cells, as well as their biofilm inhibitory and disrupting abilities.

SCD drugs showed mostly resistance-inducing interactions with standard antibiotics particularly the cell wall inhibitors, amoxicillin and ampicillin and some few weak resistance-breaking features mostly with gentamicin. The oral iron chelator, deferasirox and the opioid, morphine emerged as the key resistance-breaking (antibiotic enhancing) SCD drugs. Methotrexate, an anti-inflammatory and antifolate compound exhibited significant resistance-inducing effects in both organisms. In *E. coli*, no SCD drug exceeded the intracellular ethidium bromide (EtBr) accumulation achieved by verapamil, the control for maximum accumulation. Hydroxyurea and paracetamol induced higher uptake of EtBr within MRSA relative to the control. A lower uptake of EtBr and higher rate efflux was associated with *E. coli* whereas MRSA generally showed very high accumulation levels and lower efflux rates. The most effective biofilm inhibiting abilities (approximately greater than 60%) was observed for methotrexate and pethidine against *E. coli* and none (greater than 50%) against MRSA. Deferasirox showed above 50% biofilm disrupting activity against *E. coli* biofilm

and again none against MRSA biofilm. Biofilm formation in MRSA tended to be more robust than in *E. coli*. Our analyses of microbial stress responses to the unique antibiotic-non-antibiotic interactions in further studies would reveal a number of endogenous resistance-promoting genes that represent possible therapeutic targets in countering antimicrobial resistance.



CHAPTER ONE

1.1 INTRODUCTION

The overuse of antimicrobial agents have resulted in the development of antibiotic resistance, which has become a global threat (Brogan and Mossialos, 2016). The pace of new antibiotics production has declined as the number of drug-resistant microbes continue to increase (Zheng *et al.*, 2018). This has profoundly affected current infection rates, morbidity and mortality (Carlet *et al.*, 2012; Freeman *et al.*, 2014; WHO, 2014), much more in developing countries (WHO, 2014).

Antibiotics have for many decades, been helpful in treating bacterial infections (Newman *et al.*, 2011; Collignon *et al.*, 2016). The efficacy of these drugs is reduced by antibiotic resistance increasing the risk of morbidity and mortality in serious diseases. By employing mechanisms such as overexpression of efflux pumps, alteration of drug targets within cells, establishment of biofilms and production of antibiotic-modifying enzymes, many bacterial strains are adapting to the effects of antibiotics (Bhullar, 2012), with genetic mutations being the primary cause (Petrosino *et al.*, 2009). Bacteria also inherit exogenous antibiotic-resistant genes on mobile genetic elements from different bacterial species through horizontal gene transfer (Woods and Read, 2015).

The promotion of resistant bacteria worldwide has usually been attributed to inappropriate antibiotic prescription and misuse, as well as the decline in new antibiotics production over the years (Ventola, 2015). Apart from the injudicious use of many antibiotics, the role of non-antimicrobial drugs used in the clinical management of pathological conditions of non-infectious diseases needs to be assessed. That is, how they may be involved in the modulation of microbial resistance or susceptibility. A large number of people belong to the group of

chronic disease sufferers including sickle cell disease, hypertension, diabetes, arthritis and mental abnormalities for which they depend on lifelong medications (Iqbal *et al.*, 2014).

According to Ramakrishnan *et al.* (2010), Sickle Cell Disease (SCD) patients are highly vulnerable (about 400 times higher) to bacterial infections especially *S. pneumonia* and *H. influenzae*. These infections are common precipitants of pain and mortality particularly in children with SCD due to dysfunctional spleen and reduced immune response (Williams *et al.*, 2009; Cober & Phelps, 2010). Penicillin-resistant pneumococci has been reported to be consistently prevalent in children having the sickle cell phenotype (62% versus 41% in normal children) (Dayie *et al.*, 2015). SCD is generally managed both with preventive (avoiding crises) and symptomatic therapies (Shapiro *et al.*, 1995). The disease modifying agent, hydroxyurea is known to induce formation of fetal haemoglobin which helps reduce the episodes of bone pain crises, acute chest syndrome and mortality in adult patients (Lanzkron *et al.*, 2008). Other therapies include opioid analgesics (codeine, morphine, pethidine, tramadol) (Telfer *et al.*, 2014) and anti-inflammatory drugs such as ibuprofen and celecoxib for the management of severe and mild pains, respectively (Rees *et al.*, 2003). Deferasirox is an oral iron chelator administered to prevent chronic iron overload that may result from long-term blood transfusions (Choudhry and Naithani, 2007).

The effects of the antibiotic-non-antibiotic drug interactions against bacteria in SCD patients as well as other chronic diseases cannot be overemphasized. Studies have revealed the promotion of persistence, tolerance and resistance among bacterial populations upon exposure to chemical and environmental stressors (Poole, 2012; Cohen *et al.*, 2013; Lázár *et al.*, 2013). Bacteria usually have to deal with many chemical entities in their natural environments and this stimulates a variety of stress responses as a means of protecting them from noxious chemicals (Poole, 2012). As a result, alterations in gene expression arrays and cellular functions that influences the organism's antimicrobial susceptibility significantly

occurs (De Kievit *et al.*, 2001). Similarly, within humans, bacteria are constantly exposed to both biological metabolites and other drugs used in managing chronic diseases, some of which may serve as stress sources. Of notable mention is hydroxyurea, which has been shown to specifically inhibit class I ribonucleotide reductase (RNR), stalls the replication fork and further induce stress responses that results in formation of hydroxyl radicals, culminating in *E. coli* cell death (Davies *et al.*, 2009). Oxidative stress and DNA damage are known to promote persistence and resistance to fluoroquinolone antibiotics both in *E. coli* and *P. aeruginosa* (Vega *et al.*, 2012; Wu *et al.*, 2012). The formation of protective biofilm by bacteria can also be induced in the presence of certain routine drugs and other stressors (He *et al.*, 2012). Multi-drug efflux pumps and alteration of membrane proteins are based on the persistence of resistance genes (Piddock, 2006). Akilandeswari and Ruckmani (2015), noted that among the non-antibiotic compounds, neuroleptic and antipsychotic drugs alter the extracellular and intracellular efflux pump systems of multi-drug resistant Gram-positive and Gram-negative microorganisms.

This project was designed to study the interactions between antimicrobials and non-antibiotic agents and their contribution to the selection of resistant strains. We intend to further apply the findings to guide the isolation of bioactive chemical agents from fungal extracts against drug-resistant bacteria.

1.2 RATIONALE

The research concept presented herein results from the worldwide escalation of clinical infections caused by multi-drug resistant microorganisms and the spread of resistance among bacteria to commonly used antibiotics. Also, the contributions of other drugs used in the management of non-infectious diseases to the selection of resistant microbes needs to be unraveled. Some sickle cell disease drug therapies have been shown to possess antimicrobial action from previous studies; particularly, pethidine (Grimmond and Brownridge, 1986; Rota *et al.*, 1997), deferasirox (Ibrahim *et al.*, 2009; Neupane and Kim, 2010) and tramadol (Tamanai-Shacoori *et al.*, 2007) while others have been shown to be immunosuppressive (Wang *et al.*, 2005; Ibrahim *et al.*, 2009; Dublin *et al.*, 2011).

Since antimicrobial therapy still constitutes a major form of treating bacterial infections in general and also in sickle cell patients in Ghana, it is important to investigate the interactions that drug therapies usually given to sickle cell individuals, have, in combination with these antibiotics. These interactions will guide the selection and isolation of novel and effective antimicrobial compounds from fungal species in Ghana. These fungal species have rich potential as source of diverse antimicrobial compounds which needs to be explored for the effective control of drug-resistant infections.

1.3 HYPOTHESES

- Selected SCD drug therapies will exhibit resistance breaking effects and resistance inducing interactions against gram-negative and gram-positive bacteria.
- Phenotype modulating compounds will show unique interactions with standard antimicrobials that will inform the selection and isolation of bioactive molecules effective against resistant bacterial strains.

1.4 AIM AND OBJECTIVES

1.4.1 Aim

To investigate the role of SCD drug therapies and phenotype modulating compounds (PMC) in the modulation of bacterial resistance or susceptibility to antimicrobial compounds *in vitro* and relating the findings to the isolation of bioactive molecules from fungi against drug-resistant bacteria.

1.4.2 Specific Objectives

1. To determine the phenotypic interactions mediated by some selected SCD drugs as well as a panel of PMCs with standard antimicrobial compounds against gram-negative and gram-positive bacteria.
2. Assess the impact and role of SCD drugs and PMCs on efflux and biofilm mediated drug resistance *in vitro*.
3. To isolate bioactive molecules from soil-borne fungal and terrestrial endophytic fungal extracts based on possible interactions of SCD drugs and PMCs with crude extracts from SBF and TEF.
4. Carry out molecular analyses of bacterial stress responses to the uniquely interacting phenotype modulating drugs using quantitative PCR.

1.5 PROJECT CONCEPT OVERVIEW

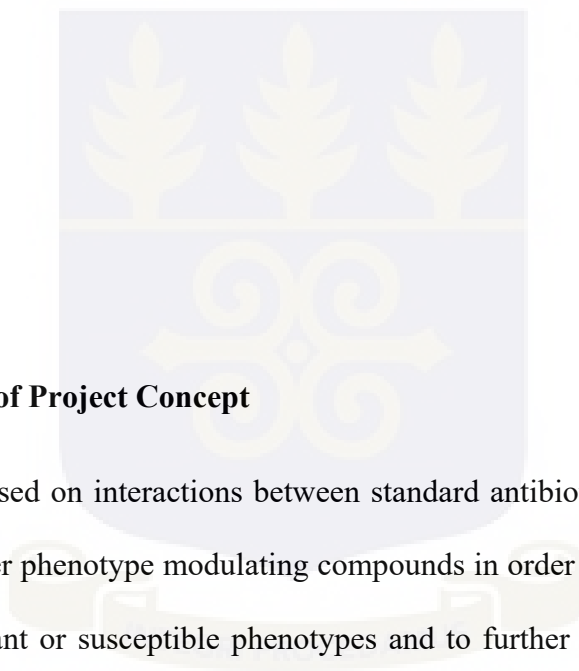


Figure 1.1: Schematic of Project Concept

This study is mainly based on interactions between standard antibiotics and sickle cell drug therapies as well as other phenotype modulating compounds in order to assess their impact in the promotion of resistant or susceptible phenotypes and to further utilize the findings as a guide to isolate antibacterial molecules from fungal extracts; this being a more stringent and effective approach. Phenotypic interaction screening data and stress response data obtained from Figure 1.0 above can be used to appropriately inform the selection of natural products for further product isolation. Data will be obtained on resistance inducing and resistance breaking phenotype modulating compounds (PMCs). Natural products that exhibit resistant breaking interactions with resistant breaking compounds will proceed further to product isolation while those showing indifferent or resistance inducing interactions will be halted. Also, products giving resistance breaking interactions with resistance inducing compounds

are classified specially as diseases relevant antimicrobial sources and will also go further into product isolation and development.



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 ANTIBIOTIC RESISTANCE

The World Health Organization's new Global Antimicrobial Surveillance System (GLASS) has revealed extensive prevalence of resistant bacterial infections among 500,000 individuals across twenty two countries (WHO, 2018). The most commonly reported resistant bacteria included *E. coli*, *K. pneumoniae*, *S. pneumoniae* and *S. aureus*.

Controlling of infectious diseases is made problematic by the increase of drug-resistant microorganisms (Ventola, 2015) since infections they cause often fail to respond to conventional treatment. The primary cause of antibiotic resistance is genetic mutation in bacteria (Petrosino *et al.*, 2009), as well as inappropriate and irrational use of antimicrobial medicines also contributing to emergence, spread and persistence of resistant microorganisms (Odonkor and Addo, 2011). The antibiotics kill drug-sensitive bacteria and resistant bacteria remain and reproduce due to natural selection (Woods and Read, 2015).

Antibiotics have revolutionized the field of medicine right from the discovery of penicillin by Alexander Fleming in the 1920s (Martens and Demain, 2017). Currently, there has been a decline in the discovery of new and effective antibiotics due to economic and regulatory hurdles (Piddock, 2006; Bartlett *et al.*, 2013; Wright, 2014) and also because most antibiotics have a short lifespan. Therefore, most drug discovery firms have left the antibiotic field and rather focused on developing drugs used on a chronic basis (Bartlett *et al.*, 2013; WHO, 2014; Ventola, 2015; Martens and Demain, 2017). The dissemination of resistant bacteria, that makes infections hard to treat, is a critical global threat (Lewis, 2013) and so there is a critical need to produce newer and more effective antibiotics.

2.1.1 Antibiotic-Resistant Bacterial Infections and Resistance Mechanisms

Many antibiotics have lost their effectiveness against even the simplest infections; a crisis which many health organizations consider as a public health threat (CDC, 2013; WHO, 2014). Organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), MDR and XDR *M. tuberculosis*, *S. pneumoniae*, carbapenem-resistant Enterobacteriaceae and the extended spectrum β -lactamase-producing *E. coli* are causes of great concern (CDC, 2013; WHO, 2014).

Infection with *S. aureus* can lead to limited and systemic infections in humans ranging from superficial skin infections to life-threatening pneumonia or sepsis (Gordon & Lowy, 2008; Klevens *et al.*, 2008; Ippolito *et al.*, 2010). *S. aureus* has developed resistance to the penicillins, cephalosporins, and carbapenems, due to synthesis of chromosomal or plasmid encoded β -lactamases within the staphylococcal cassette chromosome mec (SCC mec) (Grundmann *et al.*, 2006; Liu, 2009).

S. pneumoniae is a major cause of bacterial pneumonia and meningitis (CDC, 2013; Gross, 2013). Resistance among *S. pneumoniae* to penicillins and β -lactams results from chromosome-linked mutations to high-molecular-weight penicillin binding proteins (PBPs) (Appelbaum, 2002). Modifications in the PBP enzymes reduces attraction between the PBP and the β -lactam drug leaving them to play their role in cell wall maintenance (Markiewicz & Tomasz, 1989).

Gram-negative bacterial resistance to carbapenem is known to occur through a number of mechanisms; one being loss of, or mutation in outer membrane porins that prevents antibiotic entry. Also, carbapenem-resistant Enterobacteriaceae (CRE) produce enzymes called carbapenemases, a form of β -lactamase (Shin *et al.*, 2012), that cleave the β -lactam ring of β -lactam antibiotics (King *et al.*, 2012).

2.1.2 Influence of Efflux in the development of Multidrug resistant phenotypes

Innate resistance in Gram-negative bacteria to antibiotics is heightened by alteration in membrane permeability (Piddock, 2006), usually the first step in development of resistance (Viveiros *et al.*, 2007). This occurs by the control of the outer membrane permeability and by the efficiency of efflux of noxious chemicals (Nikaido, 2001). Efflux pumps generate intrinsic resistance to antibiotics by extruding the drug from the periplasmic space to the environment allowing the organism to survive in the presence of the toxic agents (Viveiros *et al.*, 2007). According to Piddock (2006), over expressed efflux pumps of Gram-negative bacteria confer a multidrug resistant phenotype with the ability to extrude different classes of compounds. Efflux has been recognized as a resistance mechanism to antimicrobials in *Staphylococcus aureus* leading to fluoroquinolone resistance in clinical isolates. Several multidrug efflux pumps have been identified in *S. aureus*, including NorA, NorB, NorC, MepA and MdeA (Couto *et al.*, 2008; Costa *et al.*, 2013). Couto *et al.* (2008), again noted that most of these pumps are capable of extruding compounds of different chemical classes though they possess different substrate specificities, thus enabling the cell to develop a multi-drug resistance phenotype that allows survival in hostile environments. There is currently increased search for efflux pump inhibiting agents and this work tries to assess the efflux inhibitory activities of twenty-four phenotype modulating compounds which include nine SCD management drugs.

2.1.3 Role of biofilms in conferring bacterial resistance

Bacteria residing in biofilms are the cause of many infections in humans and animals (Richter *et al.*, 2017). An exopolysaccharide matrix encloses biofilm-forming bacteria, serving as a blockade against the entry of certain antimicrobial compounds (Ishida *et al.*, 1998). Large molecules such as antimicrobial proteins and sometimes smaller peptide compounds are

prevented from penetrating biofilm cells (Lewis *et al.*, 2001). Bacteria residing in biofilms possess the advantage of a reduced susceptibility to antimicrobial agents. This decreased susceptibility allows for the evolution of antimicrobial resistance that makes biofilm-related infections hard to treat (Richter *et al.*, 2017).

Microorganisms that live in biofilms are also protected against low pH environments and have slower growth rates. Biofilm resistance qualities are partially due their slow growing nature (Costerton *et al.*, 1999), since rapid growth of cells is a requirement for some antibiotics to be effective (Costerton *et al.*, 1999). Non-growing cells are known to be resistant to Penicillin and Ampicillin (Lewis *et al.*, 2001). Their slow penetration through biofilm dwelling bacteria decreases the amount of antibiotic and also allows for the synthesis of β -lactamases to destroy the β -lactam containing antimicrobials. The presence of persistent biofilms on implanted medical devices such as catheters and prosthetic joints have been associated with recurrent infections (Cohen, 2013). A biofilm phenotype can be considered a virulence factor since bacterial biofilms are implicated in many serious chronic infections including pneumonia and *S. aureus* bloodstream infections (Zhao *et al.*, 2012; Chong *et al.*, 2013). Biofilm-forming bacteria are particularly tough to eliminate and so compounds that are able to destroy non-replicating bacterial biofilms are of great importance since most standard antimicrobials are virtually ineffective against this group (Basak *et al.*, 2015).

2.2 SICKLE CELL DISEASE AND INVASIVE BACTERIAL INFECTIONS

Infections are common precipitants of pain and mortality among Sickle cell patients (William *et al.*, 2009). Children with Sickle Cell Disease (SCD) are prone to infections such as *Streptococcus pneumoniae* and *Haemophilus influenzae* due to non-functional spleen and reduced immune response (Cober and Phelps, 2010).

There is increased risk for fatal infection if the sickle cell patient is colonized with antimicrobial drug-resistant bacteria (Miller *et al.*, 2005). Penicillin-resistant pneumococci was detected to be consistently more common in children with sickle cell disease (62% versus 41% in healthy children) (Steele *et al.*, 1996). In a study by Dayie *et al.* (2015), on the susceptibility of 115 pneumococcal isolates recovered from Ghanaian children below age six, 72.2%, 99.1%, 73% and 33.9% of isolates were penicillin (I or R), trimethoprim, tetracycline and sulfamethoxazole-resistant respectively, with 90.4% of isolates showing intermediate resistance to Penicillin. According to Serjeant (2005), sickle cell children in malaria hyper-endemic settings may be at increased risk of death from other pathogens such as invasive salmonellosis and *E. coli* UTI.

2.2.1 Drug Therapies for Management of Sickle Cell Disease

The SCD is generally managed both with preventive (avoiding crises) and symptomatic therapies (Shapiro *et al.*, 1995). These include the disease modifying agent Hydroxyurea, known to induce formation of fetal hemoglobin (HbF), and hence, reduce the frequency of crises, acute chest syndrome and mortality in adult patients (Lanzkron *et al.*, 2008). Other agents include opioid analgesics (codeine, morphine, pethidine, tramadol), as well the oral iron chelator, Deferasirox (Choudhry and Naithani, 2007) for managing pain and preventing chronic iron overload due to blood transfusions respectively. Systemic treatments (red blood transfusion, 5% dextrose, 0.45% sodium chloride) are given as well (WHO, 2011).

Figure 2.1: Chemical Structures of SCD Drug Therapies used the study

(A) Disease modifier (B) Oral iron chelators (C) Anti-inflammatory agents (D) Opioid medications

2.2.2 Reported Antimicrobial Activities of some Sickle Cell Disease Drug Therapies and the need to study their Interactions with Antibiotics

According to Zheng *et al.* (2018), drug repurposing has developed as an alternative option for discovery of effective therapeutics to treat infectious diseases. Ejim *et al.* (2011), indicated that combination of non-antibiotic drugs (Fig. 2.1) with antibiotics provides an opportunity to sample an existing unexploited expanse of bioactive chemicals. Synergistic drug combinations with drugs approved from drug repurposing screens may be useful to overcome the problem of weak activity of individual drugs (Zheng *et al.*, 2018).

The opioid drugs represent a class of non-antibiotic compounds with high potential for repurposing. They include morphine and its derivatives, codeine, pethidine and tramadol.

These drugs act as analgesic by binding to mu-opioid receptors in the brain. Some opioids have been shown to have antibacterial effect *in vitro*. Tramadol, a synthetic analogue of codeine, was experimentally shown to possess antibacterial activity against *E. coli* and *S. epidermidis* compared with controls, at 25 mg/mL for 6 hours and at 12.5 mg/mL for 24 hours (Tamanai-Shacoori *et al.*, 2007). In concentrations commonly used in epidural practice, pethidine inhibited six out of ten common microorganisms *in vitro* by agar dilution method (Neupane and Kim, 2010).

Morphine and its pharmacological derivatives are the most prescribed analgesics for moderate to severe pain management (Banerjee *et al.*, 2016). In spite of the reported *in vitro* antimicrobial activities of certain opioid pain medications, some studies also indicate that their chronic use increases host's susceptibility to bacteria. In one such study by Banerjee *et al.*, (2016), morphine induced the disruption of the gut microbial composition in chronic morphine-treated mice leading to increase of gram-positive bacteria as well as disruption of the mucosal barrier function and metabolic homeostasis. Another work by Wang *et al.* (2015), showed that chronic morphine treatment significantly hindered neutrophil recruitment and increased *S. pneumoniae* bacterial burden in the lung, spleen, and blood in mouse models with a consequent increase in mortality. A case control study undertaken by Dublin *et al.* (2011), indicated that the use of opioids was associated with increased risk of pneumonia in older adults (aged between 64 and 95 years).

The combination of the iron chelator, deferasirox and ciprofloxacin created a novel form of synergism against *Vibrio vulnificus* after twenty-four hours in a study by Neupane and Kim (2010). In another experiment conducted by Ibrahim *et al.* (2009), combination of deferasirox and liposomal amphotericin B synergistically improved survival and reduced lung fungal burden in mice infected with *Aspergillus fumigatus* compared with either drug alone.

The anticancer drug hydroxyurea (El-Said *et al.*, 2009), has also been used as a sickle cell disease (SCD) modifying agent as it is known to reduce the frequency of acute pain crises, episodes of acute chest syndrome and overall, reduce mortality in adult SCD patients (Lanzkron *et al.*, 2008). Davies *et al.* (2009), worked out the sequence of active cellular events that lead to the death of *Escherichia coli* in the presence of the drug hydroxyurea. Experiment showed that hydroxyurea specifically inhibits class I ribonucleotide reductase (RNR) resulting in nucleotide depletion and halting of the replication fork. This further induced stress responses that resulted in formation of hydroxyl radicals that caused *E. coli* cell death. The study presents a strong indication for the repositioning potential of hydroxyurea into an effective antimicrobial compound or as an adjuvant in combination therapy.

2.3 PHENOTYPE MODULATING COMPOUNDS (PMCs)

Compounds such as small molecules, peptides and drugs that can alter the phenotype of a cell or an organism are phenotype modulating compounds. These compounds are used in biological research and drug discovery in phenotypic screening assays, sometimes to mimic a physiological condition (Kotz, 2012). These phenotype modulating compounds may include certain drugs used in management of pathological conditions of non-infectious diseases. Several studies have been done on the antimicrobial properties of non-antibiotic compounds as well as their synergistic effects with standard antibiotics.

Phenothiazines are well-known antipsychotic drugs with efflux pump and bacterial quorum sensing inhibitory properties (Amaral *et al.*, 2014). Examples include thioridazine, and chlorpromazine (Table 2.2). Antimalarial drug artesunate has also been experimentally shown to enhance the antibacterial effect of β -lactam antibiotics against *Escherichia coli* through

inhibition of the multidrug efflux pump system AcrAB-TolC (Li *et al.*, 2011). Non-steroidal anti-inflammatory drug, diclofenac, is reported to have antimicrobial activity against a broad spectrum of clinical species, including *Escherichia coli* (Dutta *et al.*, 2007; Mazumdar *et al.*, 2009), *Klebsiella* sp., *Salmonella* sp., *Shigella* sp. and *Vibrio cholerae* (Dutta *et al.*, 2007). Inhibition of incorporation of thymidine, vital to DNA synthesis, has been reported as one of the likely mechanisms for the bactericidal action of diclofenac in *E. coli* (Dutta *et al.*, 2007). Ibuprofen is also shown to be synergistic in action with cefuroxime and chloramphenicol against multidrug-resistant MRSA (Chan *et al.*, 2017). Verapamil is a well-recognized efflux pump inhibitor (Gupta *et al.*, 2014) and also a well-known modifier of antibiotic resistance (Gunics, 2000). Tables 2.1 and 2.2 show the profiles of a panel of 24 phenotype modulating compounds of which nine are SCD drugs, employed in this work.

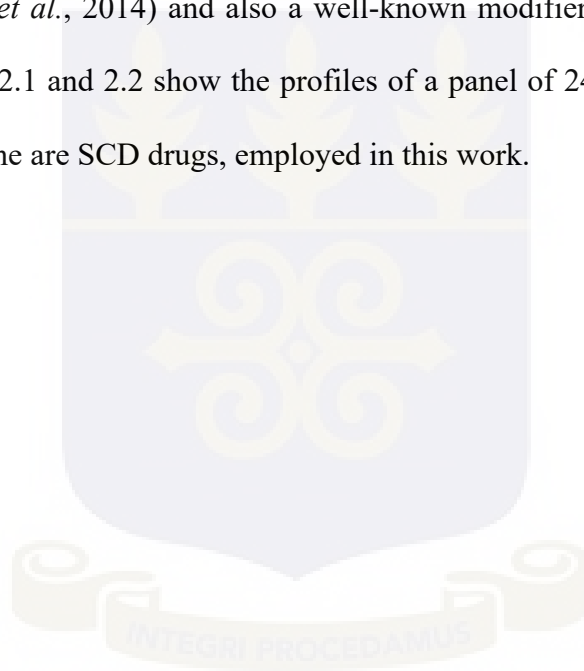


Table 2.1: Systematic review of Phenotype modulating compounds (SCD Drugs)

CODE PC04-	SCD DRUG	INTERACTIONS WITH ANTIMICROBIAL COMPOUNDS	ANTIMICROBIAL ACTIVITY
04	Methotrexate	The highest rate of synergy was obtained upon combination with terbinafine against <i>Aspergillus spp.</i> (Yang <i>et al.</i> , 2009)	Methotrexate is active against <i>Staphylococcus aureus</i> strains (Kruszewska <i>et al.</i> , 2000)
15	Hydroxyurea	No known literature	Induction of OH radical cell death in <i>E. coli</i> (Davies <i>et al.</i> , 2009); Caused reduction in DNA synthesis in <i>Staphylococcus epidermidis</i> and <i>Micrococcus lysodeikticus</i> (Feiner <i>et al.</i> , 1973)
16	Deferasirox	Reported to synergize with ciprofloxacin against <i>Vibrio vulnificus</i> (Neupane and Kim, 2009) and vancomycin against MRSA <i>in vivo, in vitro</i> (Luo <i>et al.</i> , 2015)	Inhibition of growth of <i>Vibrio vulnificus in vitro</i> (Neupane and Kim, 2009)
17	Deferoxamine	Enhanced the activities of cephalothin, gentamicin, vancomycin, and fusidic acid (and in the presence of vitamin C) against <i>S. aureus.</i> (Hartzen <i>et al.</i> , 1991)	Showed bacteriostatic activity against species of <i>Staphylococcus</i> (Hartzen <i>et al.</i> , 2009; Van Asbeck <i>et al.</i> , 1983)
18	Morphine	No known literature	No known activity
19	Tramadol	No known literature	Against <i>S. epidermis</i> and <i>E. coli</i> at 25mg/mL (Tamanai-Shacoori <i>et al.</i> , 2007)
20	Pethidine	No known literature	Known antibacterial activity (Grimmond and Brownridge, 1986)
21	Ibuprofen	Synergistic in action with cefuroxime and chloramphenicol against multidrug-resistant MRSA (Chan <i>et al.</i> , 2017)	3.20 mg/mL MIC against <i>E. coli</i> (Laudy <i>et al.</i> , 2016); 6.25mg/mL MIC against <i>S. aureus</i> (Obad <i>et al.</i> , 2015)
22	Paracetamol	Showed indifferent interactions with Amoxicillin and clavulanic acid and Erythromycin against sensitive pneumococcal strains (Ponte <i>et al.</i> , 2003)	MIC of 2.5mg/mL against <i>E. coli</i> and 1.25mg/mL against <i>S. aureus</i> (Al-Janabi, 2010).

Table 2.2: Systematic review of Phenotype modulating compounds

CODE PC04-	PMC	INTERACTIONS WITH ANTIMICROBIAL COMPOUNDS	ANTIMICROBIAL ACTIVITY
01	Benomyl	No documented interaction	Known to have antifungal activity (Gupta, 2004)
02	Estradiol	Estradiol increases the level or effect of miconazole nitrate by altering drug metabolism	No known antifungal activity
03	4-NQ	No documented interaction	Known to have antifungal activity (Mukhopadhyay, 2004)
05	Sulfometuron methyl	No documented interaction	Known to have antifungal activity (Kingsbury <i>et al.</i> , 2004)
06	1,10-PMH	No documented interaction	Known to have antifungal activity (Mukhopadhyay, 2004)
07	Rifampicin	Rifampin and amphotericin B were found to be synergistic in vitro against <i>Saccharomyces cerevisiae</i> , <i>Histoplasma capsulatum</i> , and several <i>Aspergillus spp.</i> (Medoff, 1983)	Active against <i>H. capsulatum</i> , <i>Blastomyces dermatitidis</i> , and <i>Aspergillus species in vitro</i>
08	Bromperidol	Increased susceptibility of <i>M. tuberculosis</i> in combination with rifampicin (Ramón-García <i>et al.</i> , 2011)	No inherent antimicrobial properties found
09	Thioridazine	Active against extensively drug resistant tuberculosis when added to other second- and third-line antibiotics (de Keijzer <i>et al.</i> , 2016)	Active against intracellular antibiotic-sensitive and drug resistant <i>M. tuberculosis</i>
10	Chlorpromazine	Synergistic with isoniazid, streptomycin, pyrazinamide, rifampin, rifabutin and penicillin against intramacrophage <i>M. tuberculosis</i> (Crowle <i>et al.</i> , 1992)	Bacteriostatic for Gram-negative bacteria and bactericidal for Gram-positive bacteria
11	Trifluoperazine	Not much is known about its effects with other antibiotics	Inhibits ATP synthesis in <i>Mycobacterium leprae</i> (Katoch <i>et al.</i> , 1998)
12	Triflupromazine	Not much is known about its effects with other antibiotics	Active against single and multi-drug resistant tuberculosis
13	Trimeperazine	Trimeperazine is synergistic with trimethoprim against drug resistant bacteria (Dastidar <i>et al.</i> , 2013)	Not much is known about its inherent antimicrobial properties
14	Flupenthixol	Augment its antimicrobial activity when combined with streptomycin, gentamicin, ciprofloxacin, and the non-antibiotic methdilazine against <i>Salmonella sp.</i> (Jeyaseeli <i>et al.</i> , 2011).	Not much is known about its inherent antimicrobial properties
23	Praziquantel	Praziquantel increased the AUC of Albendazole (Pawluk <i>et al.</i> , 2015).	No known activity
24	Albendazole	No documented interaction	No known activity

2.3.1 Possibility of Cross-resistance and Hyper-susceptibility Interactions between Phenotype Modulating Compounds and Antibiotics

Generation of resistance to a specific drug may cause resistance to another drug simultaneously in a phenomenon known as Cross-resistance (Suzuki *et al.*, 2014). Though it is expected that cross resistance may occur between drugs having the same mechanisms of action, cross-resistance between drugs with different mechanisms of action have also been observed (Lázár *et al.*, 2013; Suzuki *et al.*, 2014). For example, the study by Suzuki *et al.* (2014), demonstrated cross resistance networks between the quinolones, enoxacin and ciprofloxacin to β -lactams using antibiotic-resistant *E. coli* strains obtained by laboratory evolution. Another case is described as hyper-susceptibility, in which resistant strains to a drug became more susceptible than the parent strain to another drug, as in the case of the ciprofloxacin-resistant strains showing cross-susceptibility to the aminoglycosides neomycin and amikacin. This was reported by Suzuki *et al.* (2014). Due to the fact that some of these PMCs above possess some level of antibacterial properties, they may also possess the ability to induce resistance or increase susceptibility of bacteria to antibiotics through cross resistance or hyper-susceptibility mechanisms.

2.3.2 Phenotype Modulating Compound-based Screening of Bioactive Molecules

It will, therefore, be necessary to employ phenotype modulating compounds in combinatorial phenotypic screenings in order to take advantage of their resistance inducing or susceptibility inducing effects as well as their physiological mimicking properties during drug discovery from natural products or small molecule libraries. The aim is to obtain antimicrobial products that are more robust in countering the effect of resistance evolution and also with relevance in certain chronic disease conditions (Project concept, Figure 1.1). PMCs and small-molecules as probes aids in the understanding of biological processes and systems, by inducing cellular heterogeneity within biological systems (Schreiber *et al.*, 2015) as compared to drug screens

in stress-free *in vitro* culture environments. A number of small molecules have been shown to induce stress related resistance in bacteria. Processes such as persistence, drug tolerance and virulence are closely linked to stress responses to PMCs. Stress response mediates phenotypic variation and hence, serve as source of potential new drug targets that will lead to more effective therapies against persistent and resistant organisms (Cohen *et al.*, 2013).

2.4 FUNGI AS SOURCES OF NOVEL ANTIMICROBIAL COMPOUNDS

Ghana has an enormous diversity of fungal species that can produce bioactive secondary metabolites. Fungi are the most diverse forms of eukaryotic organisms on earth and produce a wide range of bioactive compounds with important pharmaceutical applications, such as antibiotic penicillins and cholesterol-lowering statins (Strobel, 2003; Nielsen *et al.*, 2017). However, less attention has been paid to fungal secondary metabolites compared to those from bacteria (Nielsen *et al.*, 2017). Due to the high diversity of secondary metabolites they produce, the Actinomycete, *Streptomyces*, has become a main target for screening for bioactive compounds both from terrestrial and marine environments (Seikpe *et al.*, 2012; Traxler and Kolter, 2015). The broad-spectrum polyene macrolide antibiotic, natamycin, is produced industrially by fermentation, using soil-borne fungi, *Streptomyces natalensis* or *Streptomyces gilvosporeus* (Aparicio *et al.*, 2003). Natamycin is used to treat bacterial and fungal keratitis and is especially effective against *Candida*, *Aspergillus*, *Cephalosporium*, *Fusarium* and *Penicillium* (Prajna *et al.*, 2002; Malecha, 2004).

Since 2001, a global scientific effort to study endophytic fungi and their natural products has been ongoing (Strobel, 2003); the result of which may be the production of a large and diverse classes of biologically derived molecules, having a range of bioactivities. Schutz *et al.* (2001), indicated from a comprehensive study, that about 51% of biologically active

substances isolated from endophytic fungi were novel as compared with 38% of novel products from soil microflora (Strobel, 2003).



CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Bacterial Strains

Methicillin-Resistant *Staphylococcus aureus* (MRSA GGP E120) and *Escherichia coli* (*E. coli* GGP E100), both multidrug resistant clinical isolates were obtained from the Bacteriology department of Noguchi Memorial Institute for Medical Research. The bacterial cells were stored and maintained on MHA (Mueller-Hinton Agar) slants at 4 °C and were sub-cultured on MHA plates at 37 °C for 12 h before each experiment.

3.2 Collection of Fungal Isolates (SBF and TEF)

Archived Soil borne fungal isolates (SBF 001 – SBF 201) and Terrestrial endophytic fungal isolates (TEF 001 – TEF 306) were obtained from the Laboratory for Chemical Systems Biology of Infectious Pathogens, Department of Biochemistry, University of Ghana, from the collections of past students.

3.3 Chemicals and Reagents

Media: Growth media for bacterial strains, Mueller Hinton Agar (MHA), Nutrient broth and for fungal isolates, Yeast extract, malt extract, peptone, dextrose and agar (YPDA) were all obtained from Becton, Dickson and company (BD, USA).

Media conditions: Nutrient broth was prepared by adding 50 mL of distilled water to 0.4 g of the powder and the Mueller Hinton Agar made by dissolving 3.8 g of the agar powder in 100 mL of water after which they are autoclaved at 121 °C.

Standard antibiotics: Ampicillin, amoxicillin, vancomycin, isoniazid, ethambutol, pyrazinamide, moxifloxacin, rifampicin, linezolid, tetracycline, chloramphenicol, erythromycin, streptomycin, cycloserine, 5-fluorouracil, gentamicin, paramomycin, rifampicin, metronidazole and clindamycin were obtained from Sigma Aldrich, Germany.

SCD drugs: Morphine, tramadol, pethidine, paracetamol and ibuprofen were purchased from Ernest Chemist Limited, Ghana. Methotrexate, hydroxyurea, deferoxamine and deferasirox were obtained from Sigma Aldrich, Germany.

Phenotype modulating compounds: Benomyl, estradiol, 4-nitroquinoline oxide, sulfometuron methyl, 1, 10-phenanthroline monohydrate, bromperidol, thioridazine, chlorpromazine, trifluoperazine, triflupromazine, trimeperazine tartarate were also purchased from Sigma Aldrich, Germany. Resazurin dye and crystal violet stain was obtained from the Laboratory for Chemical Systems Biology of Infectious Pathogens (LCSBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana.

3.4 Minimum Inhibitory Concentrations (MICs) Determination

The minimum inhibitory concentrations (MICs) of nine selected SCD drug therapies and 15 other phenotype modulating compounds were determined against MRSA GGP E120 and *E. coli* GGP E100. The disc diffusion assay was used to determine the MICs of all compounds used in this study. Experiments were conducted in triplicate.

3.4.1 Inoculum preparation

E. coli GGP E100 and MRSA GGP E120 were streaked on MHA media plates and incubated for 24 h at 37°C. The stock of inoculum was used to inoculate 50 ml nutrient broth. Incubation was done for 24 h at 37°C with shaking (180 rpm) in the dark. The OD_{600 nm} of the overnight cultures of both strains was then readjusted to 0.1 in 50 mL nutrient broth to prepare starter cultures. After 24 h of incubation in an incubator shaker, at 37°C, the OD_{600 nm} was readjusted to give a working OD_{600 nm} of 0.3 for MRSA and 0.2 for *E. coli*. This bacterial suspension was used to spread MHA plates for the bioassay.

3.4.2 Disc diffusion assay

Cells were spread on MHA plates using a sterile swab stick. Whatman No. 1 filter papers were cut into 6 mm diameter discs and autoclaved. The discs were then infused with solutions of the phenotype modulating compounds (Tables 2.1 and 2.2) at concentrations ranging from 0.5 µg to 40 µg per disc for each compound. The infused discs were placed on the MHA plates spread with the organisms and incubated at 37°C for 24 h. Zones of inhibition around each disc in mm were then measured. Experiments were conducted in triplicate.

3.5 Determination of Effect of Phenotypic Compounds on Antibiotic Activity against Test Organisms (Antibiotic-Phenotypic Compound Interactions)

Seed and starter cultures of organisms were set up as described previously in section 3.4.1. OD_{600nm} of MRSA GGP E120 and *E. coli* GGP E100 were adjusted to 0.3 and 0.2, respectively. MHA plates were spread with 200 µl of sub-minimum inhibitory concentration of each of the compounds determined below (Table 3.3). For all SCD drugs and phenotypic compounds that showed MIC of above 40 µg in the disc diffusion assay, the working concentration for agar plate modification was calculated using a selected sub disc MIC of 5 µg:

$$\text{Working concentration for agar plate modification} = \frac{5 \mu\text{g}}{4} \times 100 = 125 \mu\text{g}$$

Hence, an amount of 125 µg, equivalent to pipetting 200 µl of 0.625 µg/µl PMC solution, was spread on each plate. Standard antibiotics with working concentrations as listed in Table 3.4 were pipetted onto sterile 6 mm Whatman filter paper discs and were then placed on the PMC modified plates spread with bacterial strains. Plates were incubated for 24 h at 37°C and zones of inhibition measured. All antibiotic-PMC paired interactions were tested in triplicates.

Table 3.3: List of SCD Drug therapies and Phenotypic Compounds used in the study and their selected disc MIC and working concentrations on plate

PC04-	Phenotype Modulating Compounds	MIC (μg)		Selected Disc MIC for Bioassay (μg)		Plate MIC for Bioassay (μg) (based on Selected Disc MIC of 5 μg for all MICs >40)	
		MRSA GGP E120	E. COLI GGP E100	MRSA GGP E120	E. COLI GGP E100	MRSA GGP E120	E. COLI GGP E100
01	Benomyl	> 40	> 40	5	5	0.625	0.625
02	Estradiol	> 40	> 40	5	5	0.625	0.625
03	4-nitroquinoline oxide	10	> 40	5	5	0.625	0.625
04	Methotrexate	> 40	20	5	5	0.625	0.625
05	Sulfometuron methyl	> 40	> 40	5	5	0.625	0.625
06	1,10-PMH	1	1	1	1	0.125	0.125
07	Rifampicin	1	5	1	5	0.125	0.625
08	Bromperidol	> 40	> 40	5	5	0.625	0.625
09	Thioridazine	> 40	> 40	5	5	0.625	0.625
10	Chlorpromazine	> 40	> 40	5	5	0.625	0.625
11	Trifluoperazine	> 40	> 40	5	5	0.625	0.625
12	Triflupromazine	> 40	> 40	5	5	0.625	0.625
13	Trimeperazine	> 40	> 40	5	5	0.625	0.625
14	Flupenthixol	> 40	> 40	5	5	0.625	0.625
15	Hydroxyurea	> 40	> 40	5	5	0.625	0.625
16	Deferasirox	10	5	5	5	0.625	0.625
17	Deferoxamine	> 40	> 40	5	5	0.625	0.625
18	Morphine	> 40	> 40	5	5	0.625	0.625
19	Tramadol	> 40	> 40	5	5	0.625	0.625
20	Pethidine	> 40	> 40	5	5	0.625	0.625
21	Ibuprofen	> 40	> 40	5	5	0.625	0.625
22	Paracetamol	> 40	> 40	5	5	0.625	0.625
23	Praziquantel	> 40	> 40	5	5	0.625	0.625
24	Albendazole	> 40	> 40	5	5	0.625	0.625

Table 3.4: Profile of Standard Antibiotics used in the study and their working amounts in (μg) pipetted unto disc



3.6 Ranking of PMCs Based on Extent of Effect on Antibiotic Activity

PMCs were ranked based on the number of antibiotics they significantly affected (either suppression (resistance-inducing) or enhancement (resistance-breaking) of antibiotic activity and also on the extent of change in antimicrobial zones of inhibition by simple counting and addition. The best three resistance-inducing and resistance-breaking PMCs for each organism was selected for further interaction analyses in liquid cultures.

3.7 Determination and Validation of Unique PMC-Antibiotic Interactions in 96-well Micro-titre Plate (Resazurin liquid culture assay)

The selected compound-antibiotic pairs from the rankings were taken through further validation in broth culture using the 96 well micro-titre plates and Resazurin dye to determine cell viability. The selected sub-minimum inhibitory concentration of the SCD drugs and PMCs of 5 μg was used (Table 3.3). Appropriate or sub-lethal concentrations of standard antibiotics involved were determined by disc diffusion assay with the Mueller Hinton Agar plate modified with 125 μg of the PMC (i.e., 200 μL of a 0.625 $\mu\text{g}/\mu\text{L}$ stock) (Interaction MIC). Tables of antibiotic concentrations used for each organism is shown in Tables B1 and B2 (Appendix).

3.7.1: Resazurin 96 micro-titre well plate assay

Seeds and starter cultures of organisms were set up as previously described. Cells were adjusted to their respective working concentrations using nutrient broth. A total reaction mixture volume of 1 mL comprising of cells, PMC and antibiotic was made for each PMC-antibiotic combination in 2 mL micro-tubes. PMC was added to the mixture to obtain a final concentration of 5 $\mu\text{g}/\text{ml}$ and antibiotic to the concentrations shown in Tables B1 and B2 (Appendix). Control reactions of: **cells only**, **cells + PMC only**, **Cells + antibiotic only** were also set up. A volume of 100 μL of each reaction mixture was aliquoted into three wells of the 96-well plate. Sterile water and fresh media were placed in the remaining wells. The plates were incubated for 4 h after which absorbance $\text{OD}_{600 \text{ nm}}$ was taken. 10 μL of 1% Resazurin dye was added to each well and fluorescence read at time interval of 0 min to 2 h at 595 nm emission and 550 nm excitation ($\text{Em}_{595 \text{ nm}}$, $\text{Ex}_{550 \text{ nm}}$) with the Varioskan Fluorescence plate reader.

3.8 Ethidium Bromide Accumulation and Efflux Assessment

3.8.1 Accumulation assay

The accumulation of ethidium bromide (EtBr) by bacterial strains in the presence of PMCs was carried out as described by Viveiros *et al.* (2008), with slight modifications.

E. coli GGP E100 and MRSA GGP E120 strains were each cultured in 50 mL of nutrient broth at 37°C and 180 rpm in the dark for 12 h until they reached mid-log phase corresponding to an OD_{600 nm} of 0.6. The bacteria were then centrifuged at 15,000 *x g* for 5 min, and the pellet washed twice with the same volume of 0.8% saline. The OD_{600 nm} of the cellular suspension was adjusted to 0.3. 970 µL of cells were aliquoted into each of twenty-seven 2 mL micro-tubes (24 tubes for PMCs and 3 tubes for controls). 3 µL of EtBr was aliquoted into each tube to obtain a final concentration of 3 µg/mL EtBr. Each PMC was added at a final concentration of 1 µg/mL including verapamil, which was used as a control for maximum uptake. Following incubation at room temperature for 1 h, three 100 µL aliquots of each reaction set-up were taken for fluorescence measurement using the Varioskan Fluorescent plate reader.

3.8.2 Efflux assay

The cells (from accumulation assay) loaded with EtBr were centrifuged (15,000 *x g* for 5 min) and washed once with 0.8% saline. Pelleted cells were dissolved in saline containing glucose at 0.4% (v/v) and the PMCs were also added at final concentrations of 1 µg/mL including the verapamil tube, serving as a control for minimum efflux. Aliquots of 100 µL were taken at 15, 30, 60, and 120 min after incubation at 37°C for fluorescence measurement using the Varioskan Fluorescent plate reader (Em_{585 nm}; EX_{530 nm}).

3.9 Establishment of the Bacterial Biofilm Model in 96-well Micro-titre Plates Using a Manual Assay: Biofilm Optimization, Inhibition and Disruption Assessment.

3.9.1 Effect of cellular concentration and growing time

Seed and starter cultures of organisms were set up as described previously in 3.4.1. Two different bacterial concentrations and growing times were tested to determine optimal conditions for biofilm formation. Bacterial suspensions of OD_{600 nm} 0.2 and 0.4 were dispensed onto sterile, flat-bottomed, 96-well polystyrene micro-titre plates (200 µL per well) and incubated for two different times (24 h and 48 h) at 37°C under two different conditions; shaking at 180 rpm or static (Fig. 3.1A).

3.9.2. Crystal violet staining protocol

Crystal violet staining of the biofilms was carried out according to Sandberg *et al.* (2008), with modifications. The planktonic suspension was removed from the wells following incubation and the wells were washed with 150 µL of sterile saline to remove any unbound cells. Wells were air dried for 20 min. Biofilm attached cells were stained by the addition of 25 µL of a 1% crystal violet solution per well and allowed to incubate at room temperature for 15 min. Stain was removed, and wells were washed four times with autoclaved distilled water to remove excess stain. Stained biofilm was solubilized by adding 200 µL of 95% ethanol, and the suspension transferred into a new plate. The biofilm was then quantified by reading absorbance (OD) at 595 nm with the Varioskan plate reader.

3.9.3 Biofilm inhibition and disruption effects of SCD drugs and PMCs

Screening was done of the 24 PMCs for both prevention of biofilm formation (Inhibition) and for destruction of formed biofilm (Disruption). In the biofilm inhibition experiment, the compounds were added simultaneously with the bacterial suspension to a concentration of 1 µg/ml and were incubated in optimal biofilm-forming conditions obtained from 3.7.2 (See Figure 4.7.0). In the biofilm disruption experiment, biofilm was grown normally under the

optimal biofilm-forming conditions and the planktonic suspension was then removed and replaced by fresh nutrient broth along with the compounds at 1 µg/ml and incubated in optimal biofilm-forming conditions for 2 hrs.

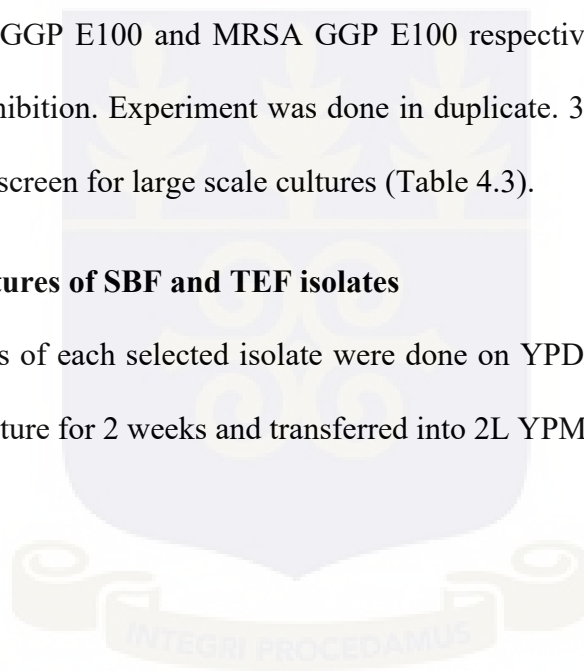
3.9.4 Screening of fungal extracts and Preparation of fungal cultures

3.9.4.1 Screening of SBF and TEF fungal extracts

The antibacterial activities of 201 SBF extracts and 306 TEF extracts were determined by the disc diffusion method against the two bacterial strains. 40 µL of each extract was pipetted onto sterile filter paper discs and placed on MHA plates spread with organisms at OD_{600nm} of 0.2 and 0.3 for *E. coli* GGP E100 and MRSA GGP E100 respectively. The activities were measured as zone of inhibition. Experiment was done in duplicate. 31 high priority fractions were selected from this screen for large scale cultures (Table 4.3).

3.9.4.2 Large scale cultures of SBF and TEF isolates

Expansion plate cultures of each selected isolate were done on YPDA media. Cultures were allowed to grow and mature for 2 weeks and transferred into 2L YPMD broth.



A

24 HRS & 48HRS / STATIC & SHAKING (4 wells)

	1	2	3	4	5	6	7	8	9	10	11	12
A		E coli GGP E100							MRSA GGP E120			
B		0.2 NB	0.2 NB	0.2 NB					0.2 NB	0.2 NB	0.2 NB	
C												
D		0.4 NB	0.4 NB	0.4 NB					0.4 NB	0.4 NB	0.4 NB	
E												
F		ONLY NUTRIENT MEDIA							ONLY NUTRIENT MEDIA			
G		STERILE WATER							STERILE WATER			
H												

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	BENOMYL PC04-01			ESTRADIOL PC04-02								
B												
C	MEDIA + CELLS + COMPOUND (PC04-03 – PC04-24)											
D												
E												
F										ALBENDAZOLE PC04-24		
G	MEDIA + CELLS ONLY						NUTRIENT MEDIA ONLY					
H	STERILE WATER											

Figure 3.1: Micro-plate layouts for Biofilm Assays. (A) Layout for biofilm optimization assays. (B) Layout for biofilm inhibition and disruption assays. 200 μ L of each set-up was pipetted into three wells. Staining was done using 25 μ L of a 1% crystal violet solution and absorbance read at 595nm.

CHAPTER FOUR

4.0 RESULTS

4.1 Drug-drug interactions between phenotype modifying compounds and Standard Antibiotics

The resistance and susceptibility patterns of gram-positive MRSA GGP E120 and gram-negative *E. coli* GGP E100 to standard antibiotics in the presence of SCD drug therapies and other phenotype modulating compounds were determined in a phenotypic based screening assay involving pairwise combinations of PMCs and 19 standard anti-microbial agents by the disc diffusion assay.

Antibiotics whose activities were significantly altered in most interactions included Amoxicillin, Gentamicin, Paramomycin and Rifampicin. Ampicillin, Tetracycline, Erythromycin, Streptomycin, Chloramphenicol and Moxifloxacin activities were affected in a few interactions. Activities of Vancomycin, Metronidazole, Cycloserine and the anti-mycobacterial drugs remained largely unchanged. Resistance breaking and resistance inducing effects were defined as increase and decrease in zones of inhibition by four millimetres or more respectively, compared to the control.

4.1.1 Resistance inducing and Resistance breaking interactions produced by SCD Drugs against *E. coli* GGP E100 (Table 4-1A)

Total resistance of gram-negative *E. coli* GGP E100 to Amoxicillin, a β -lactam cell wall inhibitor, was induced by the sickle cell drugs Deferoxamine, Methotrexate and Paracetamol (13mm to 0mm zone of inhibition). Reduced susceptibility to this antibiotic was also observed with Hydroxyurea (8mm), Pethidine (9mm) and Ibuprofen (9mm). Upon interactions with Morphine, the activities of Ampicillin, also a β -lactam cell wall inhibitor and the fluoroquinolone antibiotic, Moxifloxacin were suppressed as zones of inhibition decreased from 18mm to 13mm and 18mm to 14mm respectively. Morphine however enhanced the activities of the protein synthesis inhibitors Erythromycin, Streptomycin,

Paramomycin and Gentamicin, an aminoglycoside (Kohanski *et al.*, 2008) against the organism. Gentamicin activity was also potentiated by all of the SCD drugs particularly by Morphine (10mm to 17mm) and Paracetamol (10mm to 18mm). Suppression of Tetracycline activity was mediated by Tramadol from 13mm to 9mm and resistance to Streptomycin was induced by the anti-inflammatory agent, Methotrexate (8mm to 0 zone of inhibition).

4.1.2 Resistance inducing and Resistance breaking interactions produced by Phenotype modulating compounds against *E. coli* GGP E100 (Tables 4-1B &4-1C)

From Table 4-1B, the RNA synthesis inhibitor, Rifampicin (PC04-07), induced total resistance to the cell wall inhibiting antibiotics, Ampicillin (18mm to 0mm) and Amoxicillin (13mm to 0mm) but exhibited resistance breaking interactions with the protein synthesis inhibitors Tetracycline (13mm to 18mm), Erythromycin (12mm to 17mm) and Gentamicin (10mm to 21mm). Compound PC04-03 (4-NQ), increased resistance of the organism to Moxifloxacin (18mm to 12mm) and to protein synthesis inhibitor Chloramphenicol (20mm to 14mm) but rather increased susceptibility to Gentamicin, an aminoglycoside antibiotic (10mm to 15mm). 4-NQ, an agent that oxidizes guanines and eventually leads to double-strand breaks (Arima *et al.*, 2006) displayed antagonistic interactions with Erythromycin and Tetracycline (protein synthesis inhibitors). Song *et al.*, (2016) in study observed high synergies between 4-NQ and Gentamicin but no synergies with Tetracycline and Erythromycin against *E. coli*. Compound PC04-05 (Sulfometuron methyl) mediated resistance to Ampicillin (18mm to 13mm) but potentiated Tetracycline activity (13mm to 20mm). Total resistance to Amoxicillin (13mm to 0mm) was observed on interaction with compound PC04-01 (Benomyl) and total resistance to Gentamicin (10mm to 0mm) was mediated by compound PC04-02 (Estradiol).

Other PMCs, PC04-08 (Bromperidol), PC04-11 (Trifluoperazine) and PC04-14 (Flupenthixol) increased the resistance of *E. coli* GGP E100 to Ampicillin and PC04-13

(Trimeperazine) induced total resistance to Amoxicillin. Gentamicin activity was increased by compound PC04-07 (Thioridazine) and PC04-10 (Chlorpromazine), which are well studied phenothiazines with efflux pump inhibiting effects (Amaral *et al.*, 2014). PMCs PC04-23 (Praziquantel), PC04-24 (Albendazole) and PC04-13 (Trimeperazine), also enhanced Gentamicin activity. Results are shown in Table 4-1B and C. Praziquantel was observed to break resistance of *E. coli* GGP E100 to Rifampicin (0 to 7mm) and also enhance Streptomycin activity (8mm to 12mm). Anti-mycobacterial activity of Praziquantel against *M. chelonae* and *M. abscessus* has been reported (Chopra *et al.*, 2011).

4.1.3 Resistance inducing and Resistance breaking interactions produced by SCD Drugs against MRSA GGP E120 (Table 4-1D)

Suppression of Ampicillin activity (20mm in the control) was observed upon interactions with Methotrexate (14mm) and Ibuprofen (16mm). Activity of Amoxicillin was suppressed in the presence of Deferoxamine, an iron chelator (Farmaki *et al.*, 2011), Pethidine and Ibuprofen from 12mm to 7mm, 8mm (partially) and 8mm respectively. Susceptibility of the gram-positive organism to Streptomycin (10mm in the control) was slightly increased in presence of the oral iron chelator, Deferasirox (14mm) and opioid, Morphine (13mm). Resistance to Gentamicin was significantly induced by Methotrexate and Pethidine; reducing Gentamicin activity from 22mm in the control, to 11mm and 13mm respectively. Deferasirox and Paracetamol also slightly induced resistance to Gentamicin (22mm to 17mm each). Partial zones were produced by antibiotics Paramomycin (0 to 22mm) and 5-Fluorouracil (0 to 9mm) in the presence of Deferoxamine and Morphine respectively.

4.1.4 Resistance inducing and Resistance breaking interactions produced by Phenotype modulating compounds against MRSA GGP E120 (Tables 4-1E & F)

We observed induction of resistance to Amoxicillin by Benomyl (12mm to 0mm), Sulfometuron methyl (12mm to 8mm), and Rifampicin (12mm to 8mm). Compound PC04-06 (1,10 Phenanthroline monohydrate [1,10 PMH]) increased resistance of the gram-positive

bacteria to Ampicillin (from 20mm to 15mm). Enhancement of Erythromycin activity was seen upon interaction with 4-NQ (11mm to 16mm) while Rifampicin induced resistance to Streptomycin (10mm to 0mm). PMCs Estradiol and 1,10 PMH induced resistance to Gentamicin reducing the zone of inhibition from 22mm to 14mm and 16mm respectively. Paramomycin, previously inactive, produced inhibition zones of 10mm and 7mm in combinations with compounds 4-NQ and Sulfometuron methyl, respectively. These results are shown in Table 4-1E.

In Table 4-1F, the presence of PC04-12 (Triflupromazine) suppressed the activity the two cell wall inhibitors, Amoxicillin (12mm to 6mm) and Ampicillin (20mm to 16mm). PC04-08 (Bromperidol) and PC04-09 increased resistance of MRSA to Gentamicin. PC04-09 (Thioridazine) enhanced activity of Moxifloxacin (22mm to 26mm) and PC04-11 (Trifluoperazine) enhanced activity of Streptomycin (10mm to 14mm). Paramomycin showed activity of 10mm upon interaction with PC04-10 (Chlorpromazine) and Trifluoperazine.

In a summary, in *E. coli*, most resistance inducing interactions by the SCD drugs were associated with cell wall inhibiting antibiotics Amoxicillin and Ampicillin whereas some of the few resistance breaking effects were linked to the protein synthesis inhibitors and particularly the aminoglycoside, Gentamicin (Table 4-1A). Most of the interactions detected between the SCD drug therapies and the antimicrobial standards were resistance inducing against MRSA (Table 4-1D). The anti-inflammatory drug, Methotrexate exhibited significant resistance-inducing interactions with a broad range of antibiotics) against both organisms. The iron chelating agent, Deferasirox, displayed resistance breaking effects with mostly protein synthesis inhibitors against both organisms.

Figures 4.1 and 4.2 shows agar plate pictures of samples of resistance breaking and resistance inducing scenarios observed from antibiotic-PMC interactions against *E. coli* GGP E100 and MRSA GGP E120 respectively.



Table 4-1A: Resistance inducing and Resistance breaking effects of SCD drugs on the antimicrobial activities of Standard Antibiotics against *E. coli* GGP E100

Table 4-1B: Resistance inducing and Resistance breaking effects of Phenotype Modulating compounds on the antimicrobial activities of Standard Antibiotics against *E. coli* GGP E100



Table 4-1C: Resistance inducing and Resistance breaking effects of Phenotype Modulating compounds on the antimicrobial activities of Standard Antibiotics against *E. coli* GGP E100.

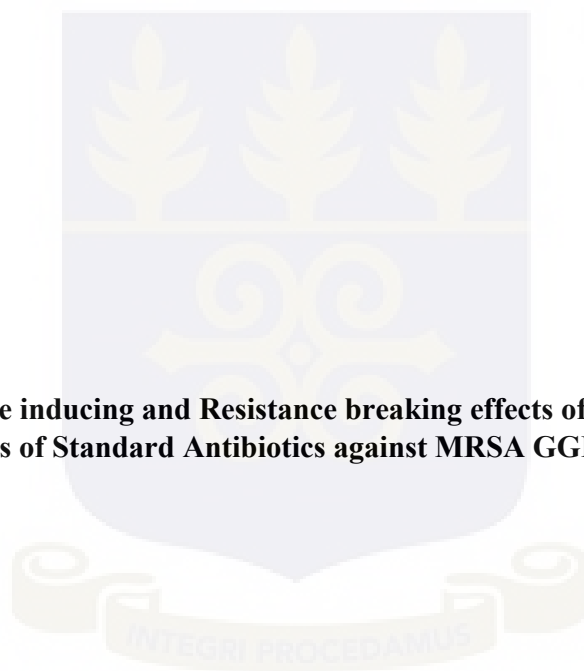
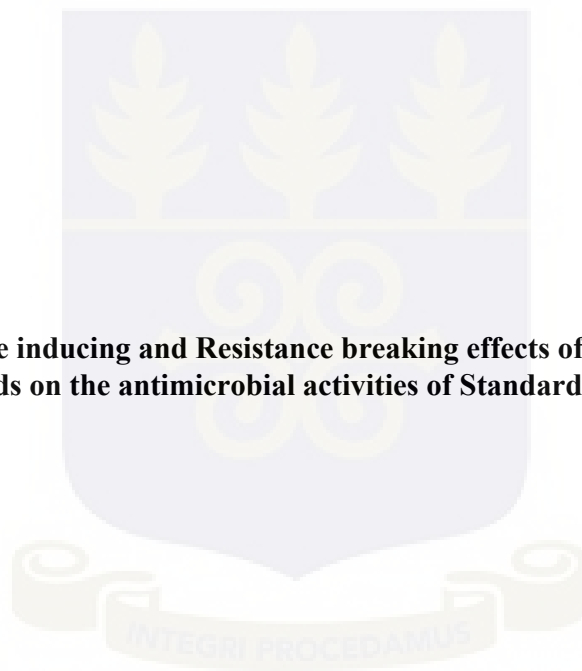


Table 4-1D: Resistance inducing and Resistance breaking effects of SCD drugs on the antimicrobial activities of Standard Antibiotics against MRSA GGP E120.

Table 4-1E: Resistance inducing and Resistance breaking effects of Phenotype Modulating compounds on the antimicrobial activities of Standard Antibiotics against MRSA GGP E120.

Table 4-1F: Resistance inducing and Resistance breaking effects of Phenotype Modulating compounds on the antimicrobial activities of Standard Antibiotics against MRSA GGP E120.



PMCs were investigated for resistance breaking and resistance inducing effects produced when interacted with standard antimicrobials against gram-positive MRSA. Zones of inhibition represent the average of triplicate experiments. Activities of cell wall antibiotics, Amoxicillin and Ampicillin were mostly suppressed.

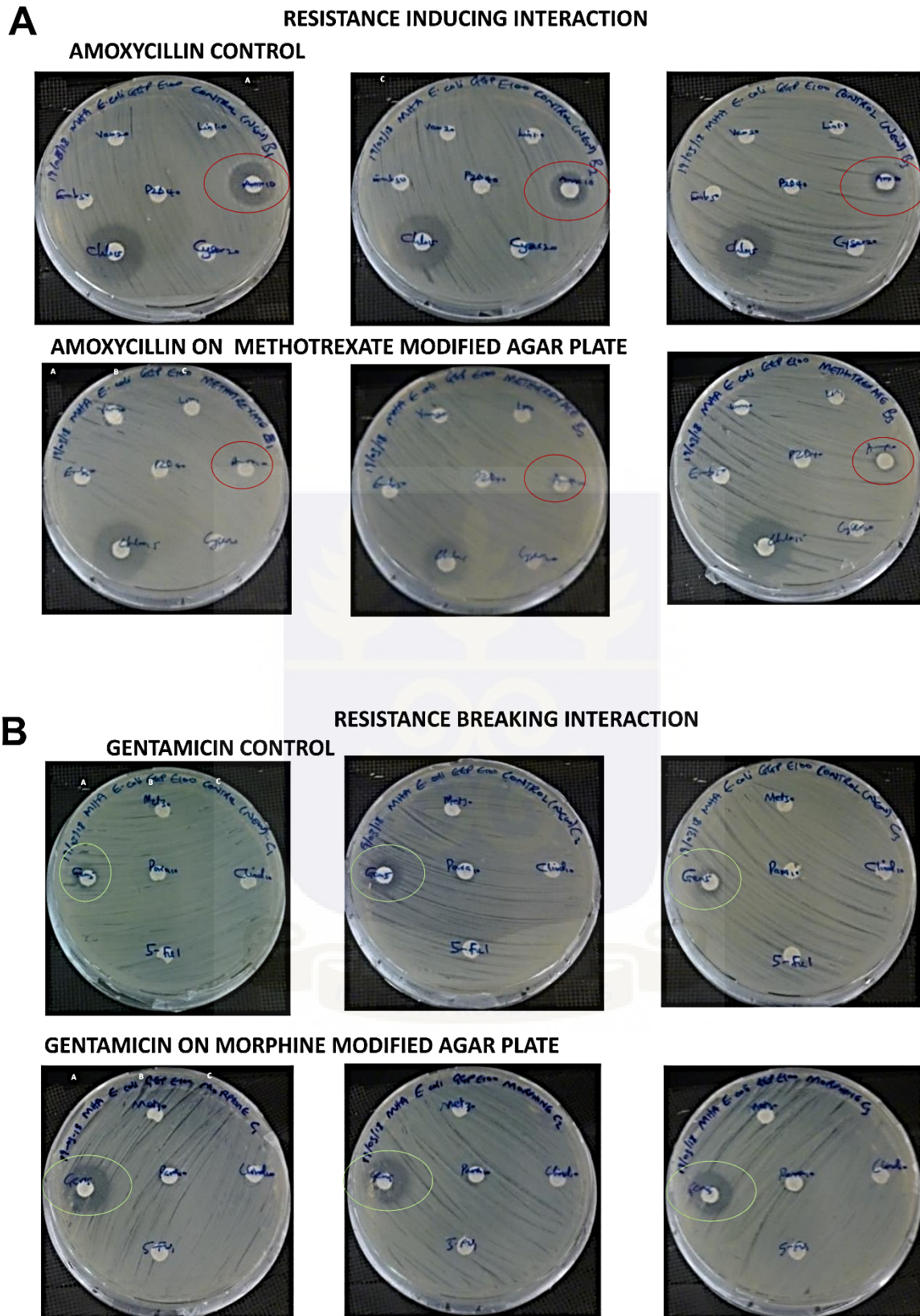


Figure 4.1: Plate pictures of Drug-drug interactions against *E. coli* GGP E100 (A) Resistance inducing interactions produce between Amoxicillin and Methotrexate (red circles) and (B) Resistance breaking interactions between Gentamicin and Morphine (green circles).

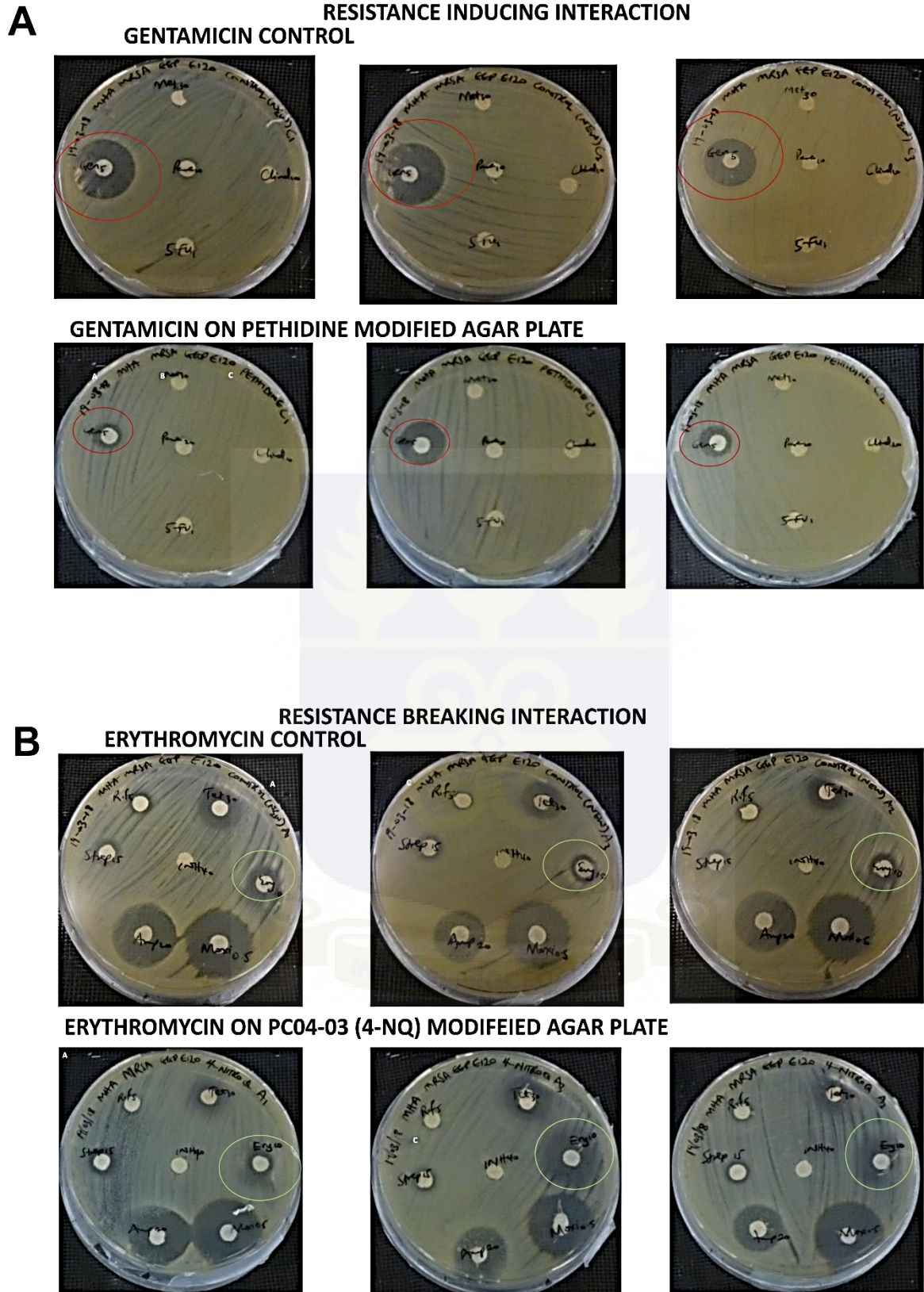


Figure 4.2: Plate pictures of Drug-drug interactions against MRSA GGP E120 (A) Resistance inducing interactions produce between Gentamicin and Pethidine (red circles) and (B) Resistance breaking interactions between Erythromycin and PC04-03 (4-NQ) modified agar plate (green circles). PC04-03 (4-Nitroquinolone oxide) is a chemical mutagen that oxidizes DNA to generate double strand breaks.

4.2 Ranking of Phenotypic Interactions

All resistance inducing interactions and resistance breaking interactions were ranked based on the number of antibiotics affected and also the extent of effect (total zones of inhibition) generated by each PMC. These rankings are shown in Tables 4.2A to 4.2D for *E. coli* GGP E100 and Tables 4.2E to 4.2H for MRSA GGP E100. The highlighted compounds in each table were selected for further validation in liquid cultures.

The opioid drug, Morphine topped the list of resistance breaking interactions for *E. coli* GGP E100. It was selected together with Praziquantel and Deferasirox (iron chelator) (Table 4.2A). For the resistance inducing side, Methotrexate (anti-inflammatory drug), 4-NQ and Flupenthixol were selected for further confirmation (Table 4.2B). In the case of MRSA, Trifluoperazine, Deferasirox and Thioridazine were chosen for resistance breaking effects (Table 4.2G) while Triflupromazine, Methotrexate and Benomyl were selected for resistance inducing effects (Table 4.2F). The antibiotics affected by these selected PMCs are shown in Tables 4.2C, 4.2D and Tables 4.2G, 4.2H for *E. coli* and MRSA respectively. The selected antibiotics for the broth culture validations have been highlighted in each table.

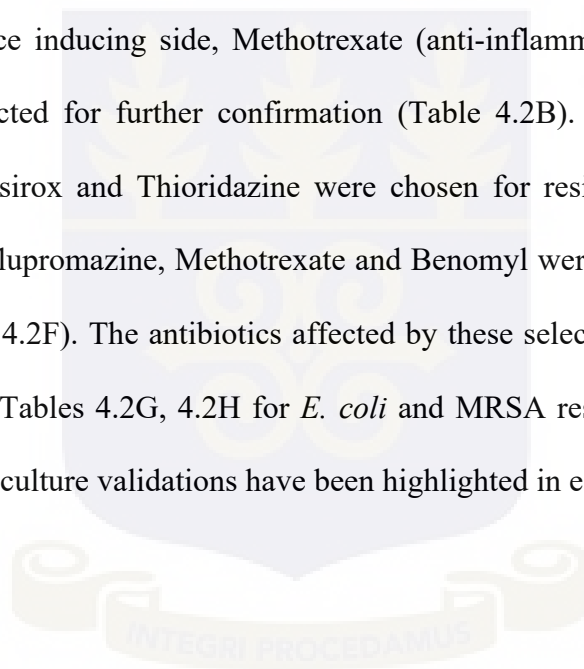


Table 4.2: Ranking of Phenotypic Interactions against *E. coli* GGP E100



Table 4.2C: Phenotype modulating compound-Antibiotic pairs of selected Resistance breaking interactions against *E. coli* GGP E100

Table 4.2D: Phenotype modulating compound-Antibiotic pairs of selected Resistance inducing interactions against *E. coli* GGP E100

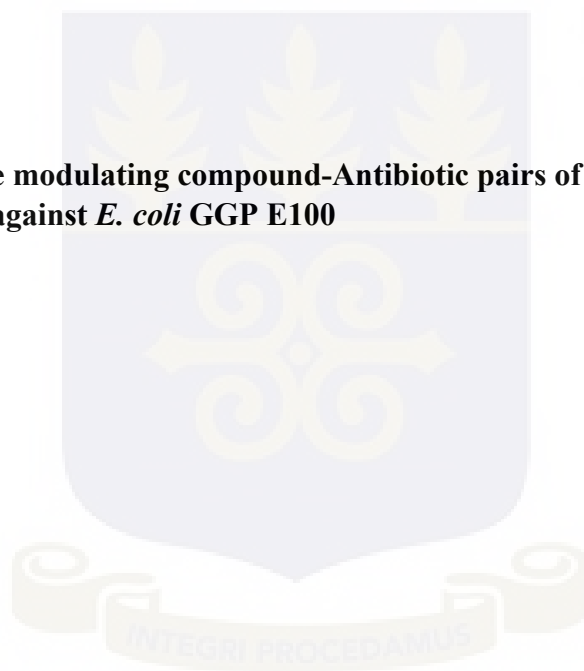


Table 4.2: Ranking of Phenotypic Interactions against MRSA GGP E120



Table 4.2G: Phenotype modulating compound-Antibiotic pairs of selected Resistance breaking interactions against MRSA GGP E120

Table 4.2H: Phenotype modulating compound-Antibiotic pairs of selected Resistance inducing interactions against MRSA GGP E120



4.3 Further validation of PMC-Antibiotic interactions in 96-well micro-titre plate (Resazurin Assay)

The selected compound-antibiotic pairs were taken through further validation in broth culture using the 96 well micro-titer plates and Resazurin dye to determine cell viability. This was also done in order to identify break point concentrations of PMC and antibiotic at which optimum cell viability would be attained for mRNA isolation to be used in further analyses of bacterial stress response to the interactions by quantitative PCR.

***E. coli* GGP E100**

Resistance breaking interaction was confirmed in the Morphine–Gentamicin combination (Fig. 4-3A) as well as in the Praziquantel-Gentamicin combination (Fig 4-3B). *E. coli* cells showed reduced viabilities in the pairwise combinations compared to the single drug reactions. However, in the Deferasirox-Paramomycin combination, cell viability in the pairwise combination was not significantly different from the Deferasirox only reaction (Fig 4-3C).

The Flupenthixol-Gentamicin resistance inducing interaction was confirmed by the liquid medium interaction assay. The combination reaction showed about two-fold increase in cell viability compared to the single drug reactions (Fig 4-3E). The remaining resistance inducing pairs showed quite different patterns compared to the observations from the agar plate assay (Fig 4-3D and Fig 4-3F). This observation may be attributed to the ability of bacterial cells to form protective biofilm when growing on solid surfaces. Cells from disrupted biofilm have been shown to gain increased sensitivity to antibiotics when grown planktonically (Zuroff *et al.*, 2010).

MRSA GGP E120

MRSA exhibited far greater susceptibilities to the selected PMCs in liquid cultures than on agar plates. Viability of the cells was drastically reduced in the presence of Trifluoperazine,

Thioridazine and Triflupromazine (Fig 4-4A, 4-4C and 4-4F). Interestingly, these compounds belong to the class of compounds known as Phenothiazines and are known to exert their primary effect on the plasma membrane of bacteria (Amaral *et al.*, 2004). The Deferasirox-Streptomycin interaction assay confirmed the agar plate observation as cells in the drug combination reaction showed much reduced viabilities compared to the single drug set-ups (Fig 4-4B). Methotrexate, known to be an inflammatory agent with activity against *S. aureus* strains (Kruszewska *et al.*, 2000) showed resistance breaking effect with Gentamicin in the broth cultures (Fig 4-4D) as compared to the agar plate experiment where it decreased Gentamicin inhibition zone from 22mm to 11mm (Table 4-1D). The two other resistance inducing set-ups showed phenotypic compounds Triflupromazine (Fig 4-4E) and Benomyl (Fig 4-4F) only slightly increasing cell viabilities in the drug combinations as compared to the compound only controls.

Further titration and adjustment of PMC and antibiotic concentrations are required to achieve optimum cell viability and corresponding mRNA expression levels for future stress response analyses using primers for the selected genes shown in Tables 4.3 and 4.4 below.



Table 4.3: Selected Genes for Stress response analyses in *E. coli* GGP E100

GENE (<i>E. coli</i>)	COMPONENT
rrsA (16s rRNA)	CONTROL GENE (Rocha <i>et al.</i>, 2015)
recA	SOS induction
tonB	Iron uptake
OxyR	Oxidative stress regulon
ahpC	Oxidative Stress
CpxA	Cell envelope stress response
envZ	Osmotic pressure
csgD	Biofilm (LPS Biosynthesis)
mazE / mazF	Toxin-Antitoxin system
acrA	Efflux pump component

Table 4.4: Selected Genes for Stress response analyses in MRSA GGP E120

GENE (<i>S. aureus</i>)	COMPONENT
gyrB (DNA gyrase B)	CONTROL GENE (Rocha <i>et al.</i>, 2015)
recA	SOS induction
isdB	Iron Homeostasis
MgrA	Oxidative stress regulon
ahpC	Oxidative Stress
sigB	Cell envelope stress response
betA	Osmotic stress
icaD	Biofilm
mazE / mazF	Toxin-Antitoxin system

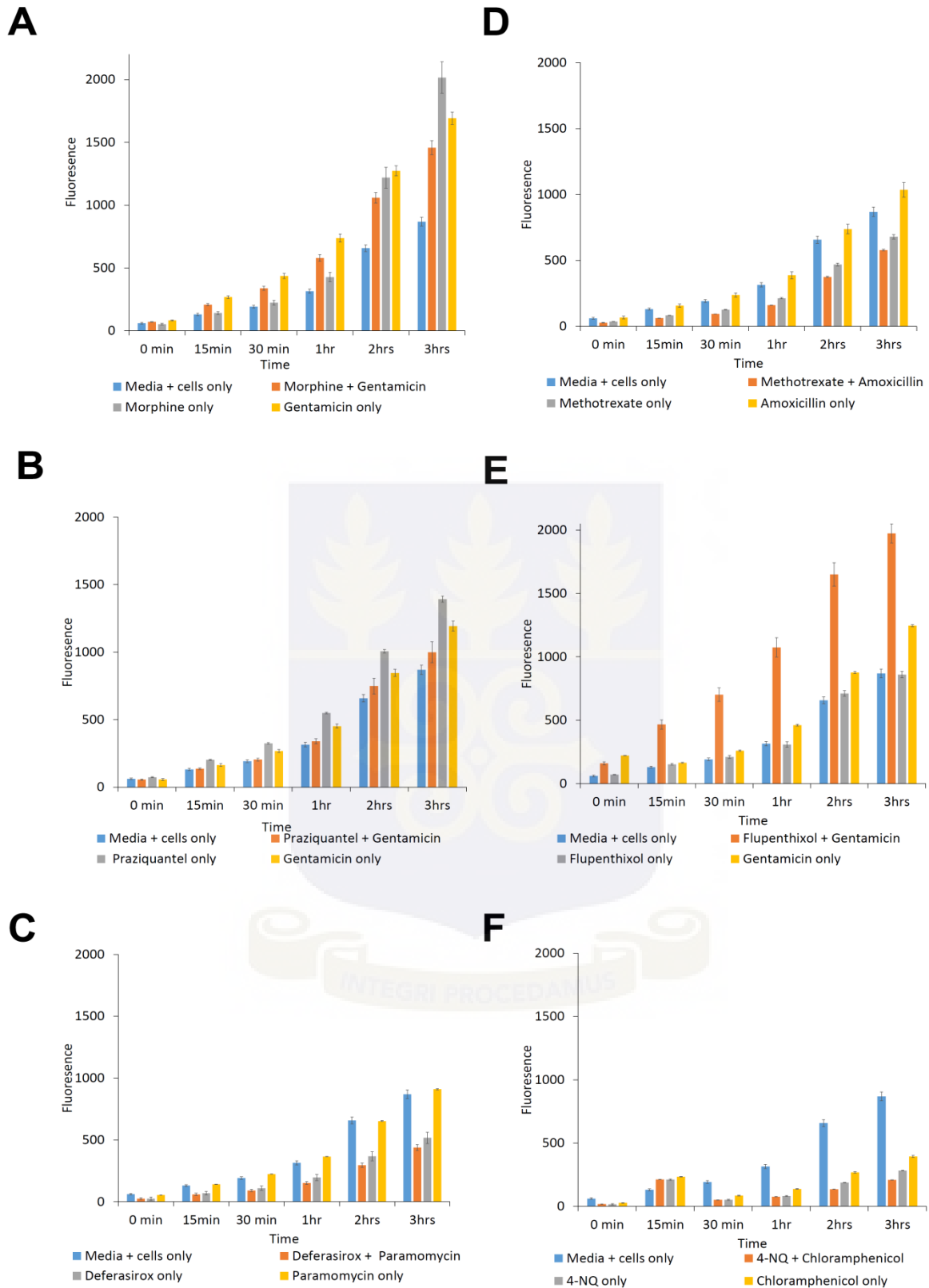


Figure 4-3: Further validation of selected phenotypic interactions against *E. coli* GGP E100 using Resazurin cell viability dye in a 96 well plate. (A – C) Selected Resistance breaking pairs. (D – F) Selected Resistance inducing pairs. To 100 μ L of reaction mixtures, 10 μ L of 1% Alamar blue was added after 4 h incubation. Fluorescence reading was taken periodically over 3 h at 595nm emission and 550nm excitation.

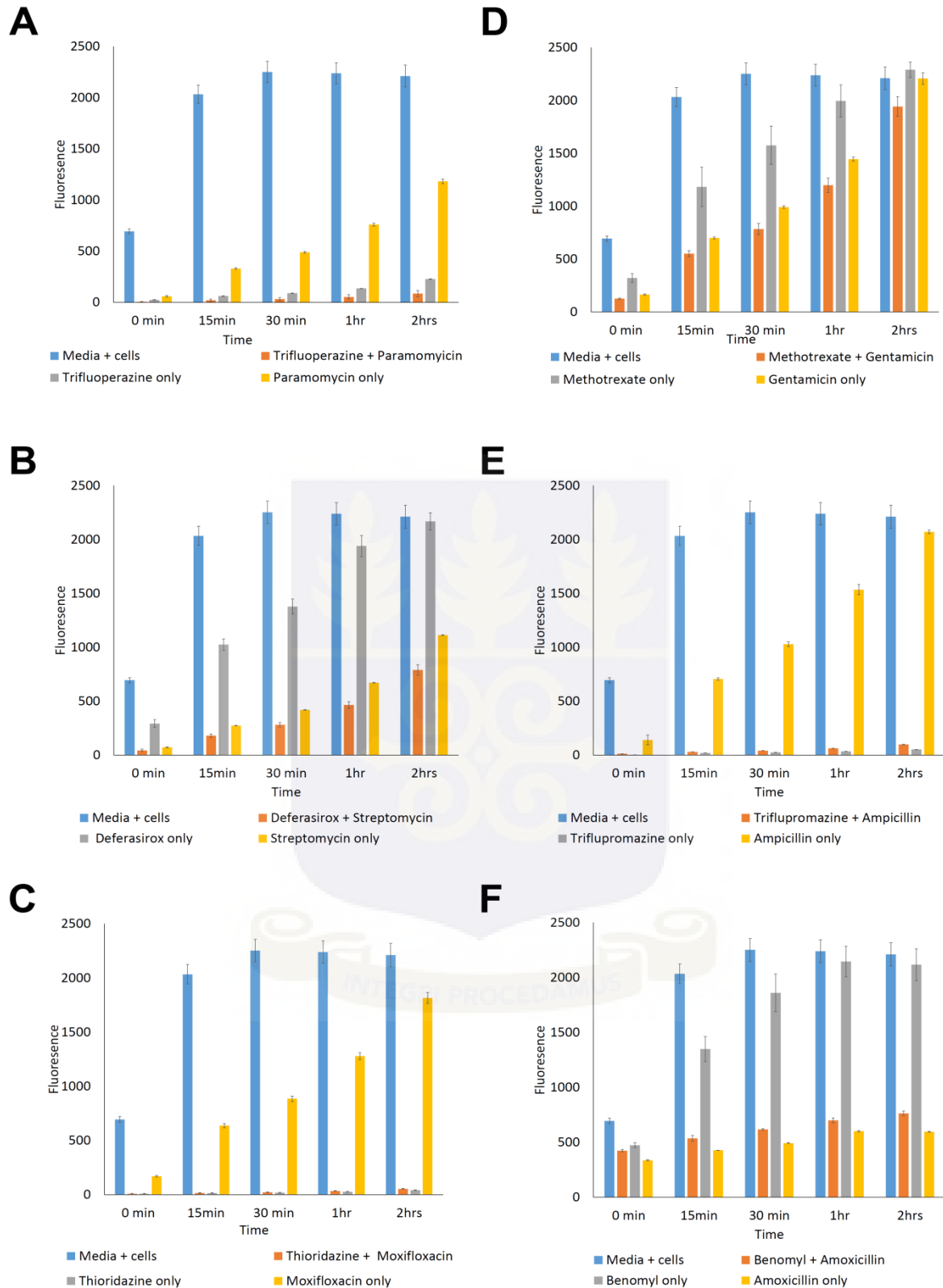


Figure 4-4: Further validation of selected phenotypic interactions against MRSA GGP E120 using Resazurin cell viability dye in a 96 well plate. (A – C) Selected Resistance breaking pairs. (D – F) Selected Resistance inducing pairs. To 100 μ L of reaction mixtures, 10 μ L of 1% Alamar blue was added after 4 h incubation. Fluorescence reading was taken periodically over 2 h at 595nm emission and 550nm excitation.

4.4 Accumulation and active efflux of Ethidium bromide in bacterial cells

The ability of SCD drugs and PMCs to induce cellular uptake of EtBr and to prevent the efflux of EtBr from the cells was tested. It is a well-established fact that efflux pumps play a significant role in promoting drug resistance and the over expression of efflux pumps confers multidrug resistance phenotypes to bacterial cells (Amaral *et al.*, 2014). Thus compounds that may induce over expression of efflux in bacterial cells may be a contributing factor to the emergence of multidrug resistant phenotypes and compounds that inhibit efflux may serve as important sources of future drug therapies or may be used as adjuvant in combination therapies. Non-lethal concentrations of EtBr and PMCs were used to prevent death of cells. Verapamil was used as a control for maximum uptake and minimal efflux of EtBr as it is known to be an efflux pump inhibitor (Gupta *et al.*, 2014).

4.4.1 Accumulation of EtBr in *E. coli* GGP E100 by PMCs

None of the SCD drugs exceeded the uptake of EtBr beyond that of verapamil. However Pethidine and Deferasirox induced significant uptake relative to the maximum uptake by verapamil. Most of the SCD drugs showed reduced accumulation levels relative to the no treatment control. Interestingly, Tramadol, an opioid pain killer showed the least uptake of EtBr even lower than the no treatment control (Figure 4.5.1A). In Figure 4.5.1B, two compounds belonging to the phenothiazine class, namely Bromperidol and Thioridazine induced significantly high levels of EtBr uptake. Phenothiazines are known to intercalate bacterial DNA and weaken cell walls by down regulation of some selected cell wall proteins (Kristiansen *et al.*, 2003). They are also involved in inhibition of efflux pumps both in human and microbial cells (Amaral *et al.*, 2010). Benomyl, Sulfometuron methyl and Triflupromazine were observed to inhibit accumulation of EtBr in *E. coli* as compared to the no drug treatment control. Previously in the agar plate interaction study Benomyl and

Sulfometuron methyl exhibited some level of resistance induction in *E. coli* to the cell wall inhibitors Amoxicillin and Ampicillin respectively (Table 4-1B).

4.4.2 Accumulation of EtBr in MRSA GGP E120 by PMCs (Fig. 4.5.2)

Among the SCD drug therapies, the ribonucleotide reductase inhibitor, Hydroxyurea and the anti-inflammatory agent, Paracetamol showed higher EtBr accumulation than Verapamil. Hydroxyurea was observed to cause a reduction in DNA synthesis in *Staphylococcus epidermidis* and *Micrococcus lysodeikticus* (Feiner *et al.*, 1973). Deferasirox again induced relatively high uptake close to that of Verapamil followed by Ibuprofen which is known to have some efflux inhibitory effect on bacteria (Al-janabi, 2010). The antipsychotic compound, Flupenthixol showed a two fold increase in EtBr uptake to that of the verapamil control. Other antipsychotic compounds Bromperidol, Chlorpromazine and Triflupromazine had slightly higher accumulation levels than verapamil. Benomyl, Sulfometuron methyl, 1,10-PMH, Trimeperazine and Rifampicin were observed to prevent EtBr accumulation.

Generally, there was higher uptake of EtBr in MRSA GGP E120 than in *E. coli*. GGP E100. Gram negative *E. coli* is known to possess the AcrAB-TolC as its main efflux pump system (Amaral *et al.*, 2014) that confers on the organism the ability to extrude a wide variety of antimicrobial agents resulting in an MDR phenotype.

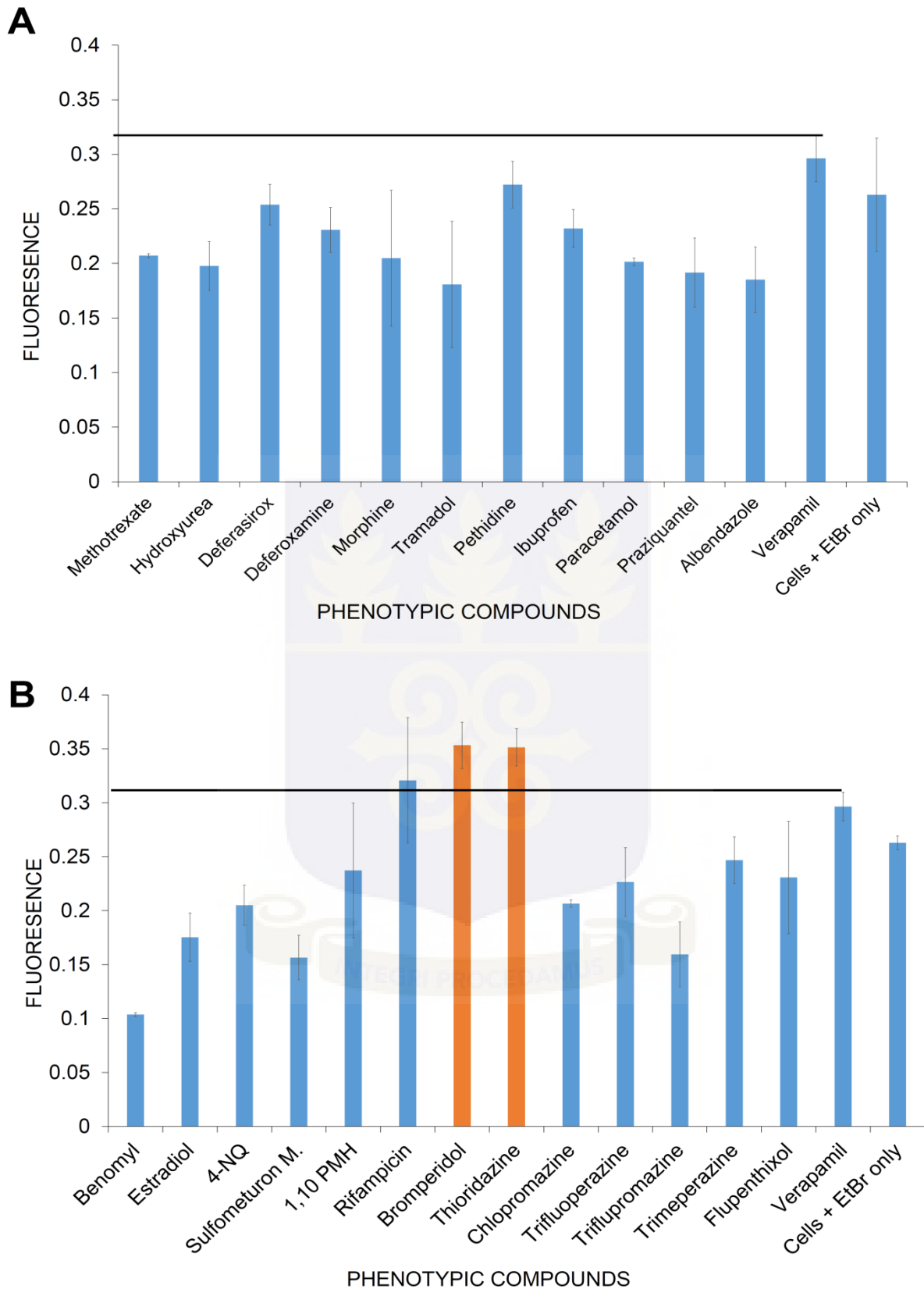


Fig 4.5.1: Effects of PMCs on the accumulation of ethidium bromide in *E. coli* GGP E100. (A) SCD Drugs including Albendazole and Praziquantel (B) other phenotype modulating compounds. Experiment was carried out in triplicates and the graph was constructed using their averages. Fluorescence was measured at excitation and emission wavelengths of 530 nm and 585 nm respectively. Verapamil was used as control for maximal uptake

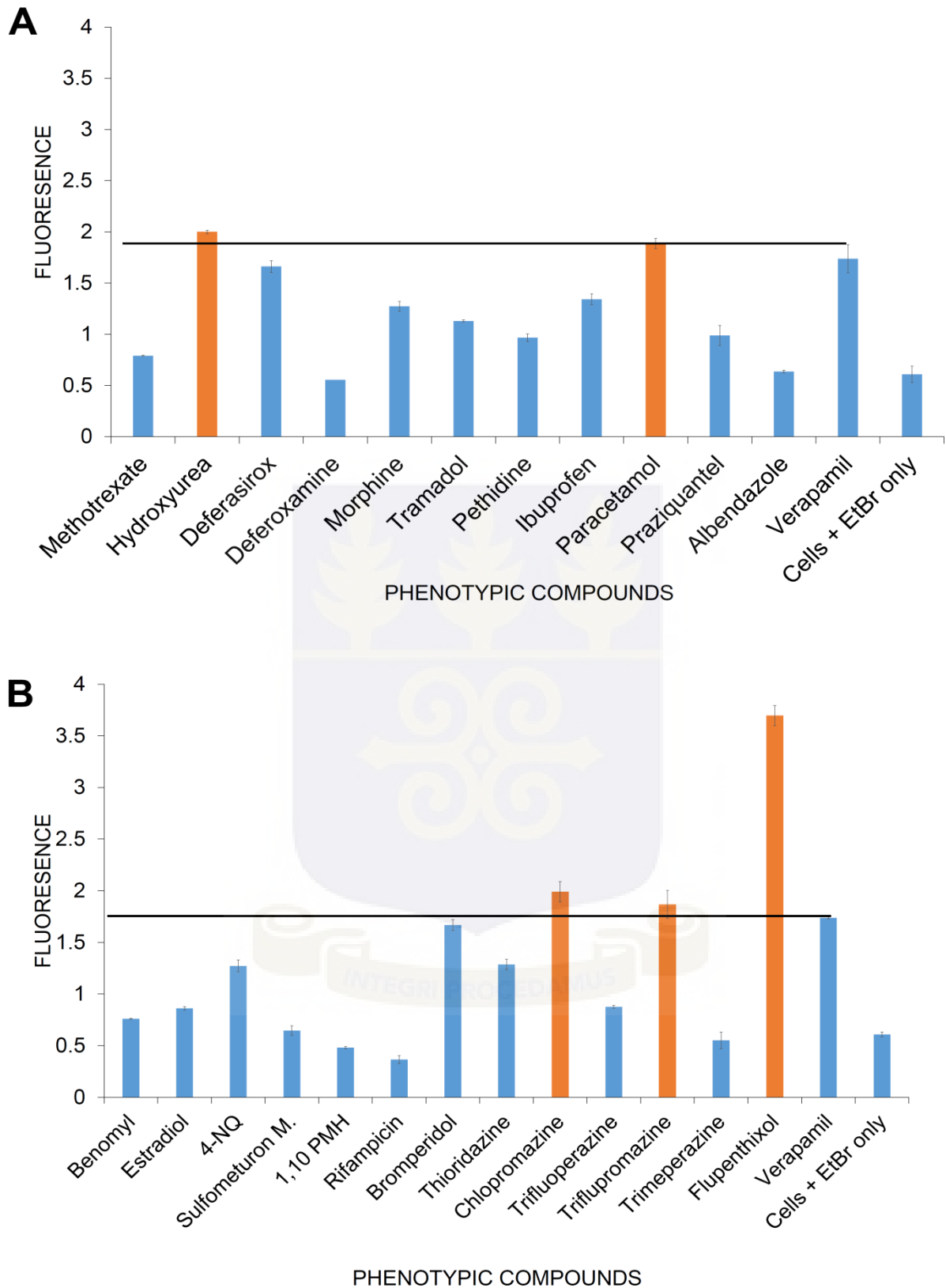


Fig 4.5.2: Effects of PMCs on the accumulation of ethidium bromide in MRSA GGP E120. (A) SCD Drugs including Albendazole and Praziquantel (B) other phenotype modulating compounds. Experiment was carried out in triplicates and the graph was constructed using their averages. Fluorescence was measured at excitation and emission wavelengths of 530 nm and 585 nm respectively. Verapamil was used as control for maximum uptake

4.5 Inhibition of Glucose mediated efflux in bacterial cells by SCD drugs and PMCs

Efflux of accumulated intracellular ethidium bromide from bacterial cells was activated by the addition of 0.4% v/v glucose. After accumulation of ethidium bromide in bacterial cells, efflux was stimulated by the addition of 0.4% glucose. PMC was then added to a concentration of 1 µg/mL to assess efflux inhibitory capability. The rate of EtBr extrusion from the cells was determined by measuring the decline in fluorescence at time intervals between maximum uptake (0 min) and 120 mins.

4.5.1 Rate of Efflux in *E. coli* GGP E100

All SCD drugs showed higher rates of efflux inhibition relative to the no drug treatment control (i.e., below 57% rate of efflux). However, relative to the verapamil control for maximum efflux inhibition rate, the opioids Morphine (18%) and Tramadol (16%), the non-steroidal anti-inflammatory drug Ibuprofen (15%) and Paracetamol (12%) exhibited higher efflux inhibition or lower rate of EtBr extrusion than verapamil (25%)(Fig. 4.6.1B). Closely following the verapamil efflux rate is Hydroxyurea (26%), Deferoxamine (26%) (Fig. 4.6.1A) and Pethidine (28%) (Fig. 4.6.1B).

Rate of EtBr extrusion from *E. coli* GGP E100 among the PMCs was generally lower relative to the minimum efflux verapamil control (25%). Chlorpromazine had the lowest rate of efflux effect (10%) (Fig. 4.6.3C); with Benomyl and Praziquantel both at 18%, Estradiol (19%) and Albendazole (21%) (Fig. 4.6.2A). Rifampicin exhibited the lowest efflux inhibitory activity (62% rate of efflux over time) relative to the no drug treatment control of 57% EtBr extrusion rate (Figure 4.6.2B). Bromperidol showed a higher level of EtBr accumulation than verapamil but exhibited a very poor EtBr efflux inhibitory effect (approximately 40% extrusion rate after 15 mins to 50% after 2 h) but Thioridazine maintained relatively high level of EtBr within the cells over time (23%) (Figure 4.6.3C). Aside Trifluoperazine

showing an efflux rate of 23%, rate of EtBr efflux by Triflupromazine (29%), Trimeperazine (32%) and Flupenthixol (31%) exceeded that of the verapamil control (Fig. 4.6.3D).

4.5.2 Rate of Efflux in MRSA GGP E120

Uptake and maintenance of EtBr within MRSA was generally higher than in *E. coli*. Maintenance of intracellular EtBr by some SCD drugs over time was high as very minimal decrease in fluorescence relative to the no drug treatment control set-up was observed (i.e., below 62% fluorescence decrease). Methotrexate showed the highest level of EtBr maintenance of approximately 2% rate of efflux (Fig 4.6.4A) even compared to the verapamil control for minimal rate of efflux of 7% over 2 h. This was followed by Ibuprofen and Paracetamol (both 13%) and the opioid Pethidine (14%) (Fig. 4.6.4B).

High intracellular EtBr maintenance effect among the PMCs was observed in set ups treated with Albendazole (2%), Estradiol (5%), and Benomyl (15%) (Figure 4.6.5A). In Fig. 4.6.5B, we detected the RNA inhibitor, Rifampicin showing similar maintenance capacity as the verapamil set up (i.e., 7% extrusion rate) followed with the mutagen 4-NQ and 1,10-Phenanthroline monohydrate both at 12% efflux rate. Best performing compounds among the Phenothiazines are Chlorpromazine (5%), Thioridazine (12%) (Fig. 4.6.6C) as well as Trifluoperazine (7%) (Fig 4.6.6D).

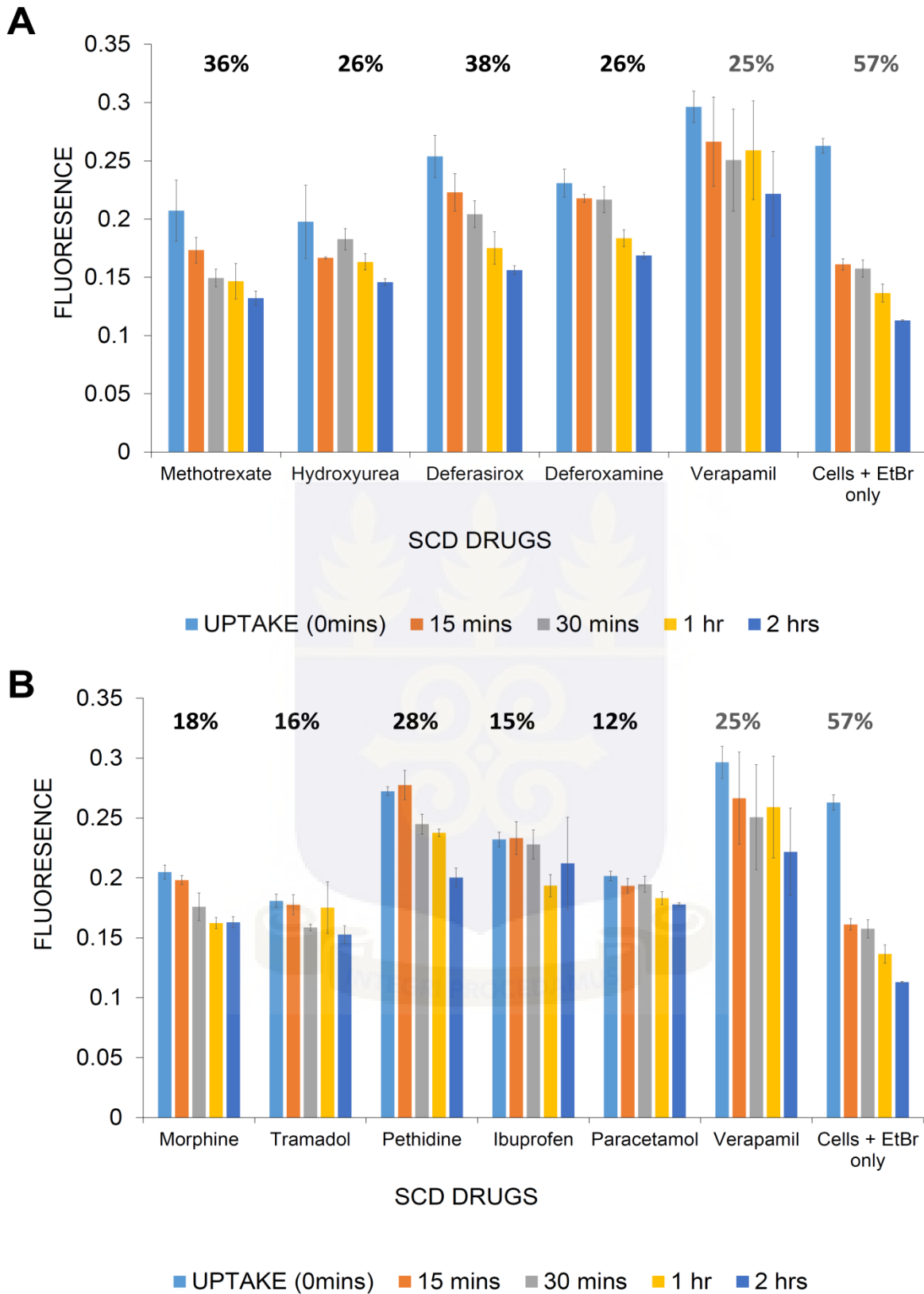


Fig 4.6.1: Effects of SCD drugs on efflux activity in *E coli* GGP E100 (A-B).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

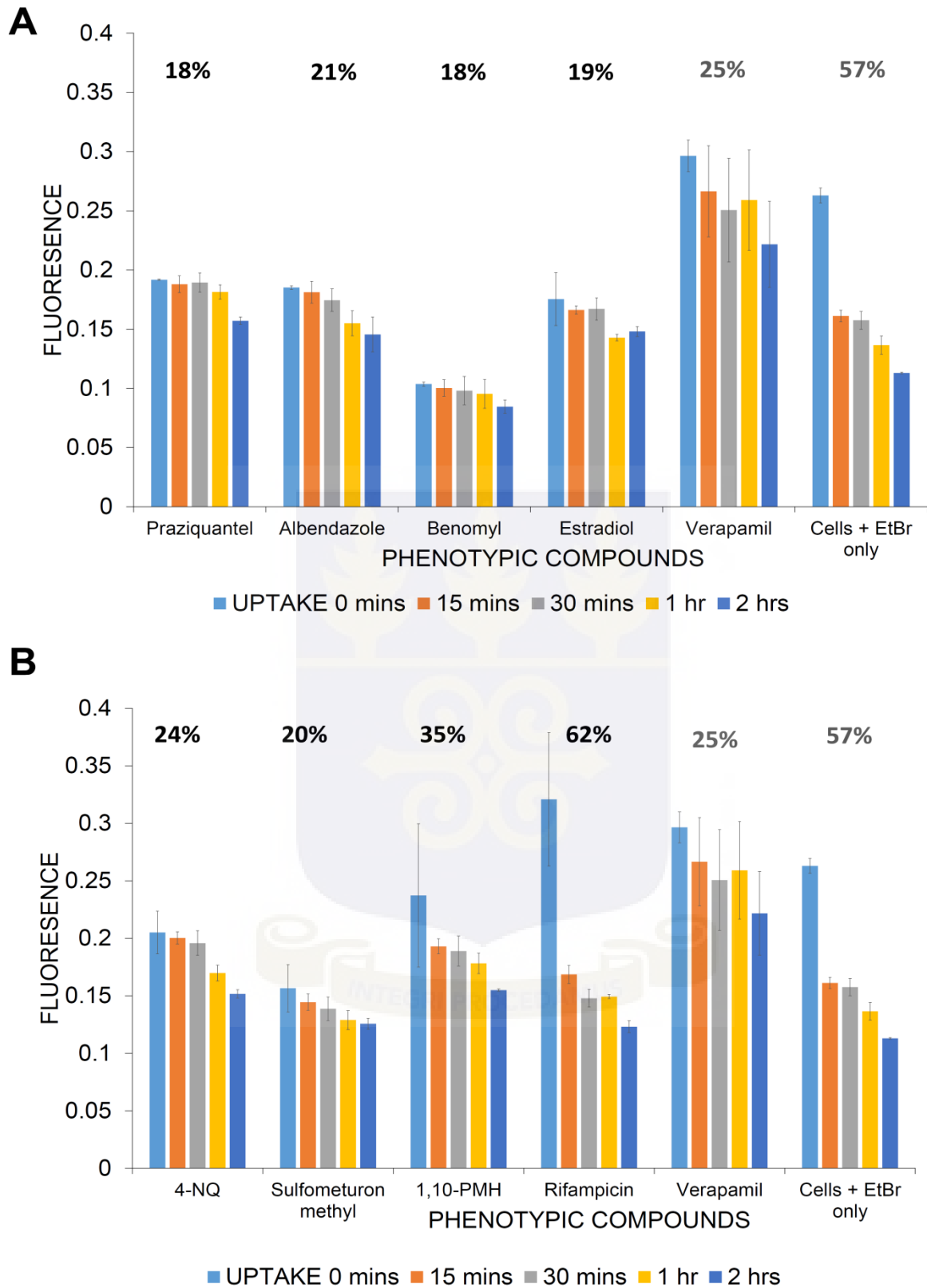


Fig 4.6.2: Effects of PMCs on efflux activity in *E coli* GGP E100 (A-B).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

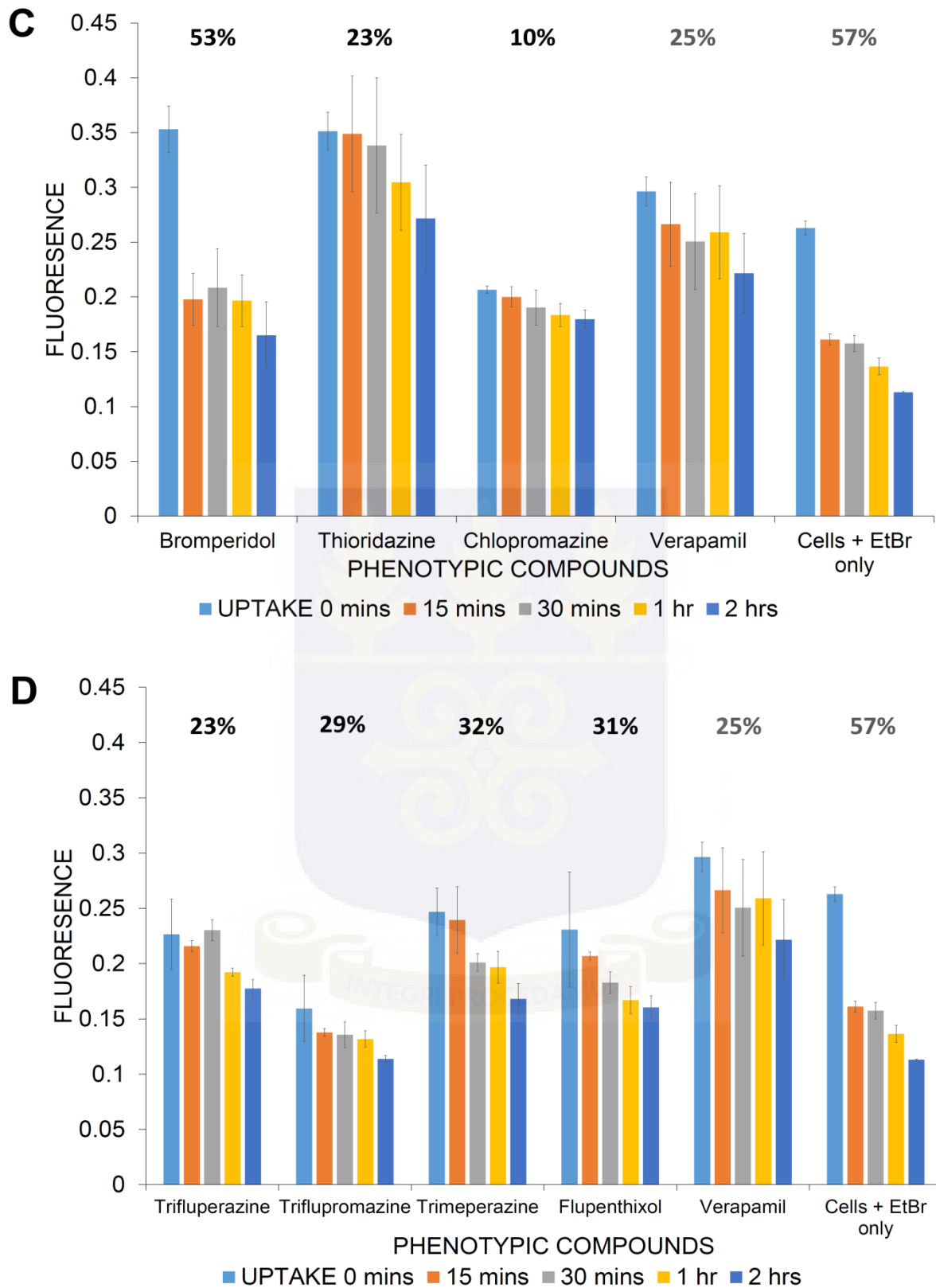


Fig 4.6.3: Effects of PMCs on efflux activity in *E coli* GGP E100 (C-D).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

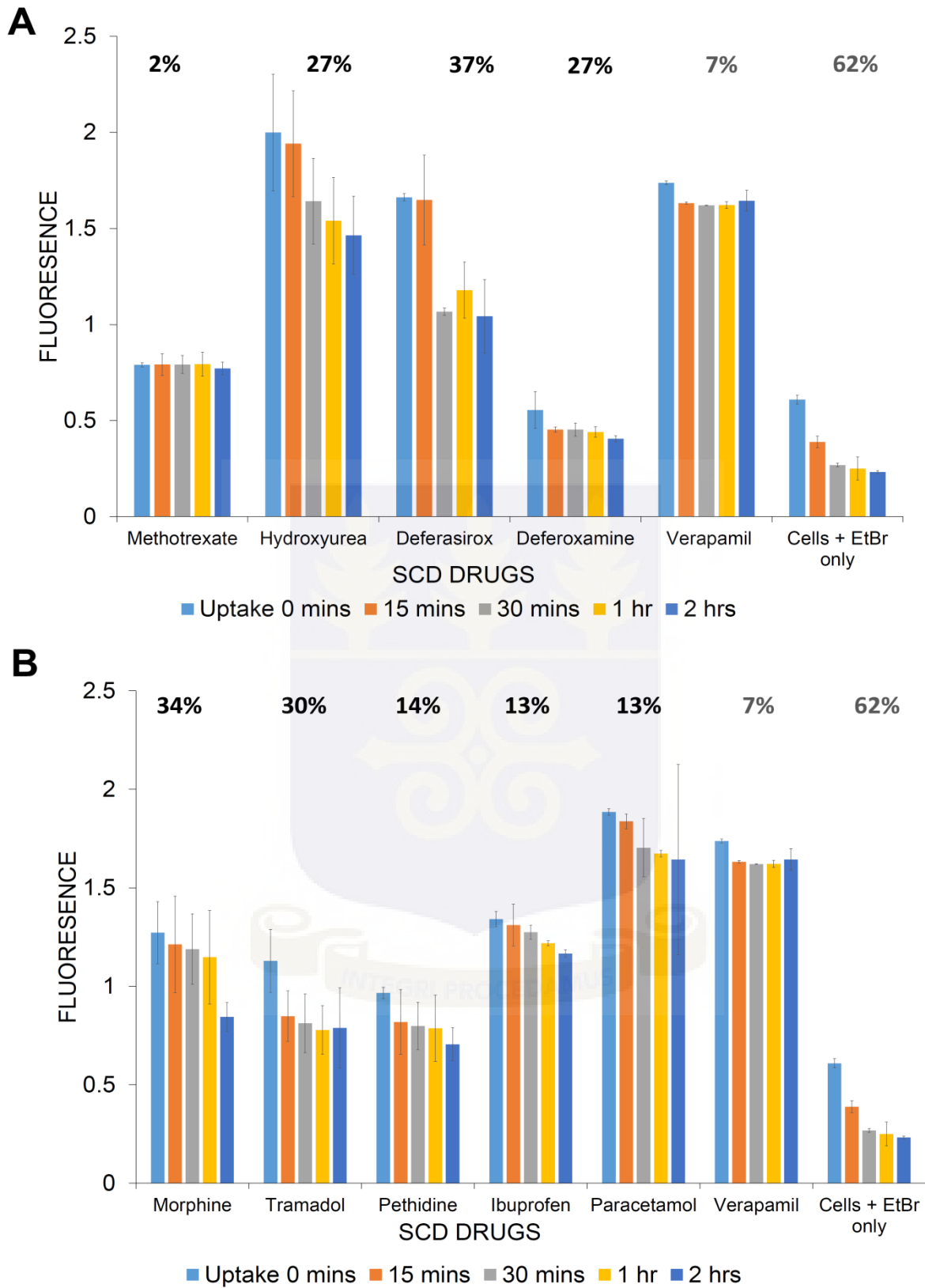


Fig 4.6.4: Effects of SCD drugs on efflux activity in MRSA GGP E120 (A-B).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

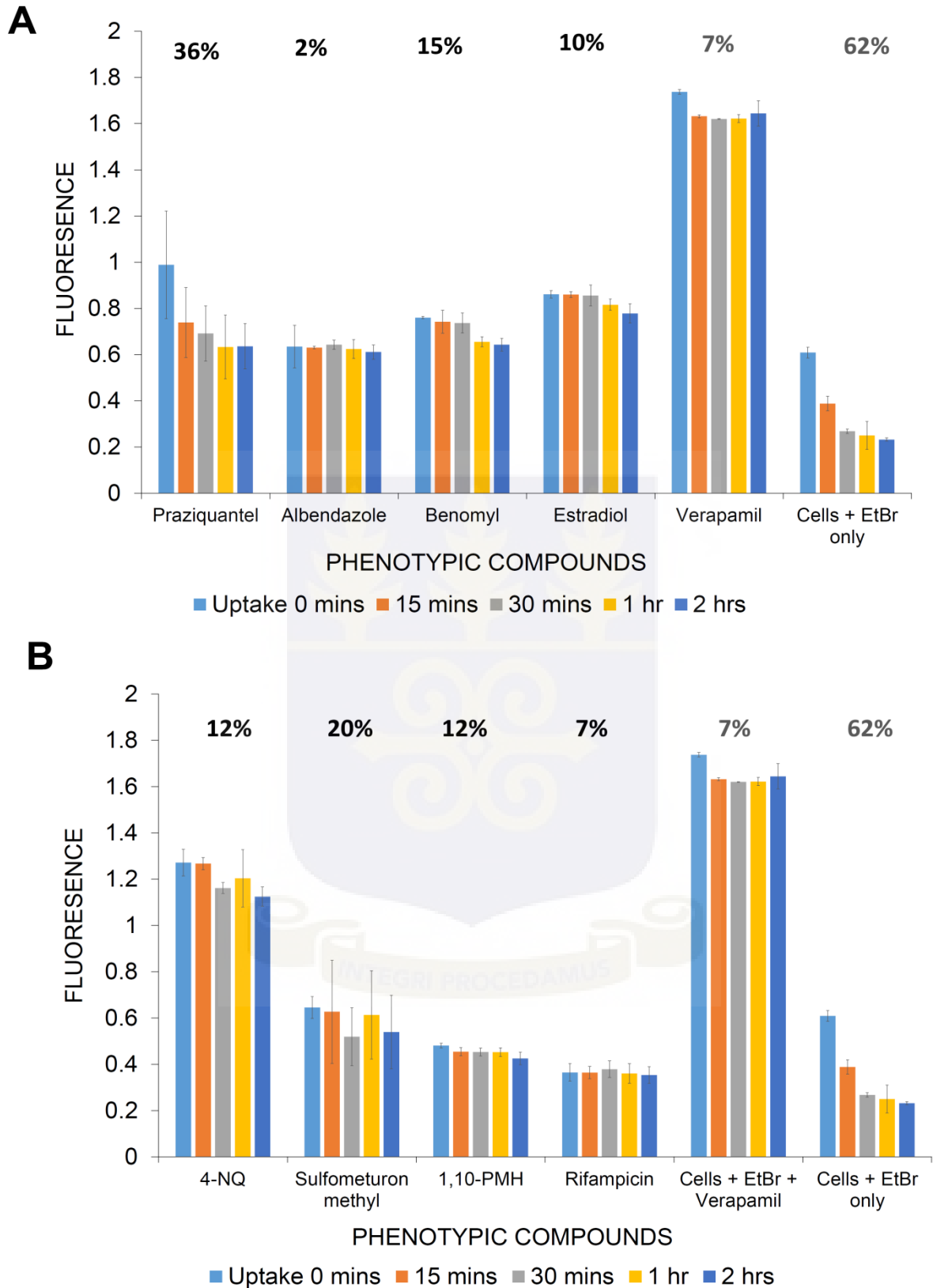


Fig 4.6.5: Effects of PMCs on efflux activity in MRSA GGP E120 (A-B).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

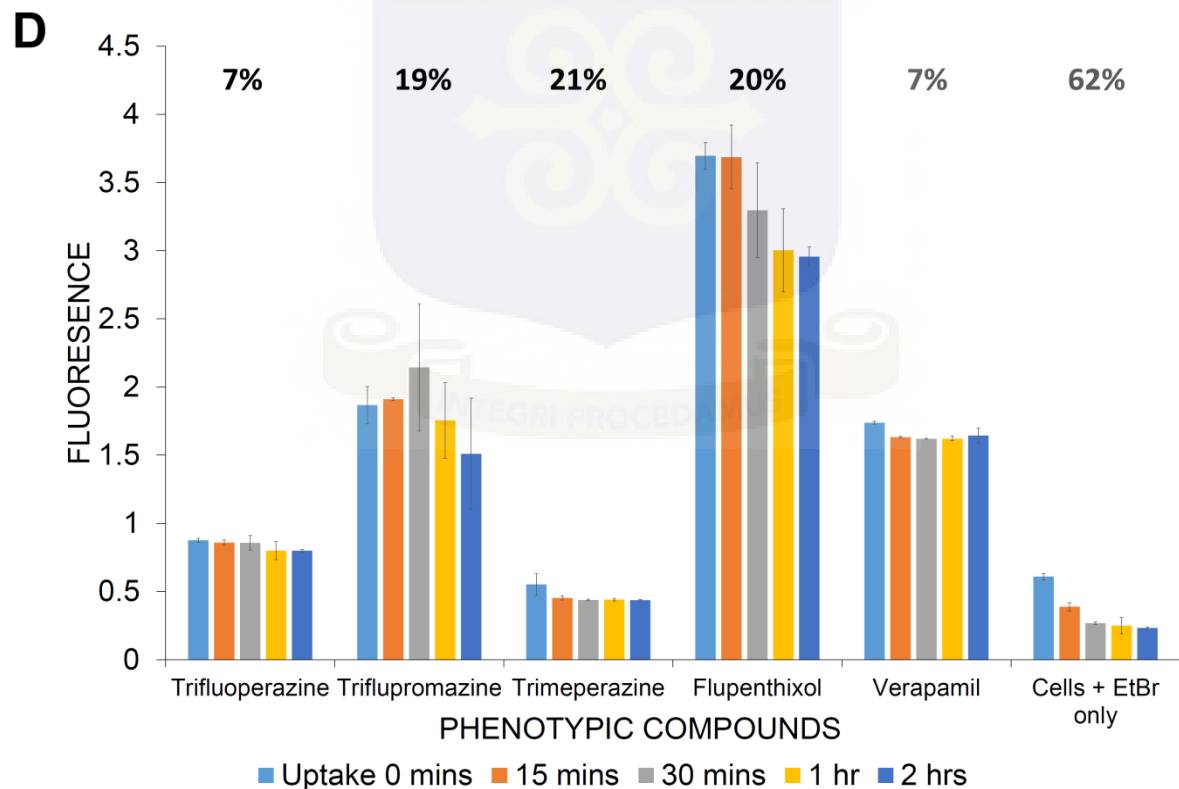
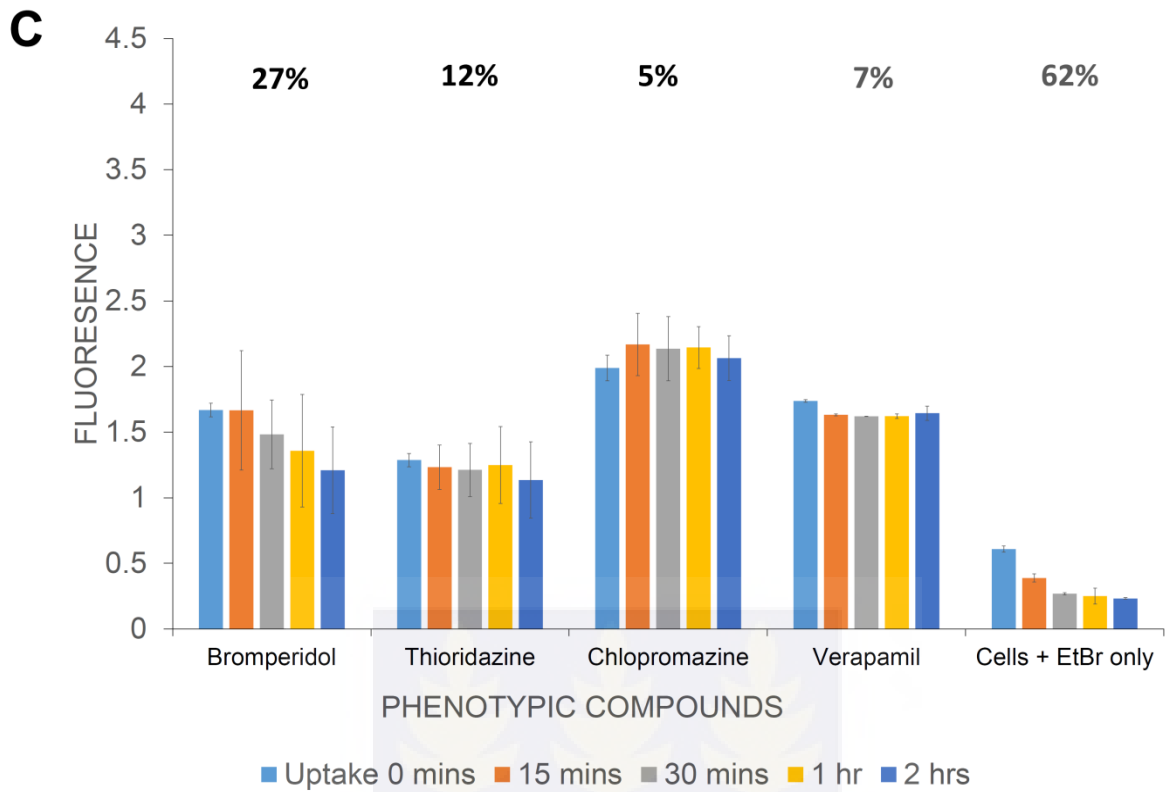


Fig 4.6.6: Effects of PMCs on efflux activity in MRSA GGP E120 (C-D).

Efflux was stimulated by the addition of glucose (0.4% v/v) at time 0 min. Efflux activity with time was monitored by measuring excitation and emission wavelengths at 530 nm and 585 nm respectively. Experiment was carried out in triplicates and the graph was constructed using their averages. Verapamil was used as control for minimal efflux.

4.6 Biofilm Inhibitory and Disruptive effects of SCD drugs and PMCs

Biofilm formation is a major contributing factor to the development of resistance to antimicrobial agents (Mirzaee *et al.*, 2014). As a result agents that can repress the development of biofilm are of high priority and thus the search for non-antibiotics with anti-biofilm activity is ongoing through screening of repurposing libraries (Richter *et al.*, 2017). The ability of the SCD drugs and the PMCs to prevent or induce biofilm formation (inhibition) was assessed as well as their biofilm destroying capabilities (disruption).

4.6.1 *E. coli* GGP E100 biofilm inhibition and disruption by SCD drugs and PMCs

In *E. coli* GGP E100, optimum conditions that produced thick biofilm was obtained by incubating a starter culture of OD₆₀₀ 0.4 for 48 h without shaking (Figure 4.7.0A). These optimum conditions were utilized for the inhibition and disruption assays. All the SCD drugs tested had some biofilm inhibiting activity relative to the no drug treatment control but none completely prevented biofilm formation. Best inhibiting compounds among them were Methotrexate and Pethidine showing greater than 50% inhibitory activity including the anthelmintic drug Albendazole. Hydroxyurea, Deferasirox, Deferoxamine, Tramadol and Praziquantel showed the ability to inhibit biofilm by half. Morphine, Ibuprofen and Paracetamol had less than 50% inhibitory ability (Figure 4.7.1A). Among the Phenothiazine class of compounds, Bromperidol, Thioridazine, Chlorpromazine, Triflupromazine and Trimeperazine had greater than 50% biofilm inhibitory activity excluding Flupenthixol and (Figure 4.7.1B). It is good to note that none of the drugs induced biofilm formation in *E. coli*.

In the biofilm disruption assay, biofilms were initially formed under the optimal conditions and the compounds diluted in fresh media at 1µg/mL were aliquoted unto the formed biofilms and incubated for 2 hrs. The SCD drug that showed significant 50% disrupting ability was the iron chelator, Deferasirox. Pethidine, Deferoxamine and Ibuprofen had approximately 40% disrupting capability. Methotrexate, Hydroxyurea, Morphine and

Tramadol exhibited no significant biofilm disrupting properties (Table 4.7.2A). PMCs with significant greater than 50% disrupting activity included Benomyl and the phenothiazines Bromperidol and Triflupromazine. Sulfometuron methyl, Thioridazine and Trimeperazine showed between 45% to 50% disrupting effect (4.7.2B)

4.6.2 MRSA GGP E120 biofilm inhibition and disruption by SCD drugs and PMCs

Chong *et al.*, (2013), indicates that regardless of prolonged treatment courses, *S. aureus* skin and bloodstream infections continue to recur. *S. aureus* is also highly implicated in biofilm-related infections associated with implanted devices such as catheters (Cohen *et al.*, 2013). Thick MRSA biofilm was obtained by incubating a starting culture of OD_{600nm} 0.2 with 180 rpm orbital shaking at 37°C for 48 h (Fig 4.7.0B). On the whole, MRSA biofilm appeared as tough to intrude as none of the SCD drugs including Albendazole and Praziquantel could significantly inhibit its biofilm development (Figure 4.7.3A). However, Estradiol, Sulfometuron methyl and the phenothiazines Thioridazine and Trimeperazine showed significant inhibitory activity (i.e., greater than 50%) (Figure 4.7.3B).

MRSA biofilm disruption by SCD drugs and PMCs followed a similar trend as observed in inhibition experiment. No compound showed 50% or higher disruption activity relative to the no drug control. Methotrexate and Ibuprofen being the best inhibitory compounds in this case, showed between approximately 35% and 40% disruptive activities (Fig. 4.7.4A). Also, Benomyl, Estradiol, Sulfometuron and Thioridazine exhibited close to 35% biofilm disruption, being the best among the remaining PMCs (Fig 4.7.4B).

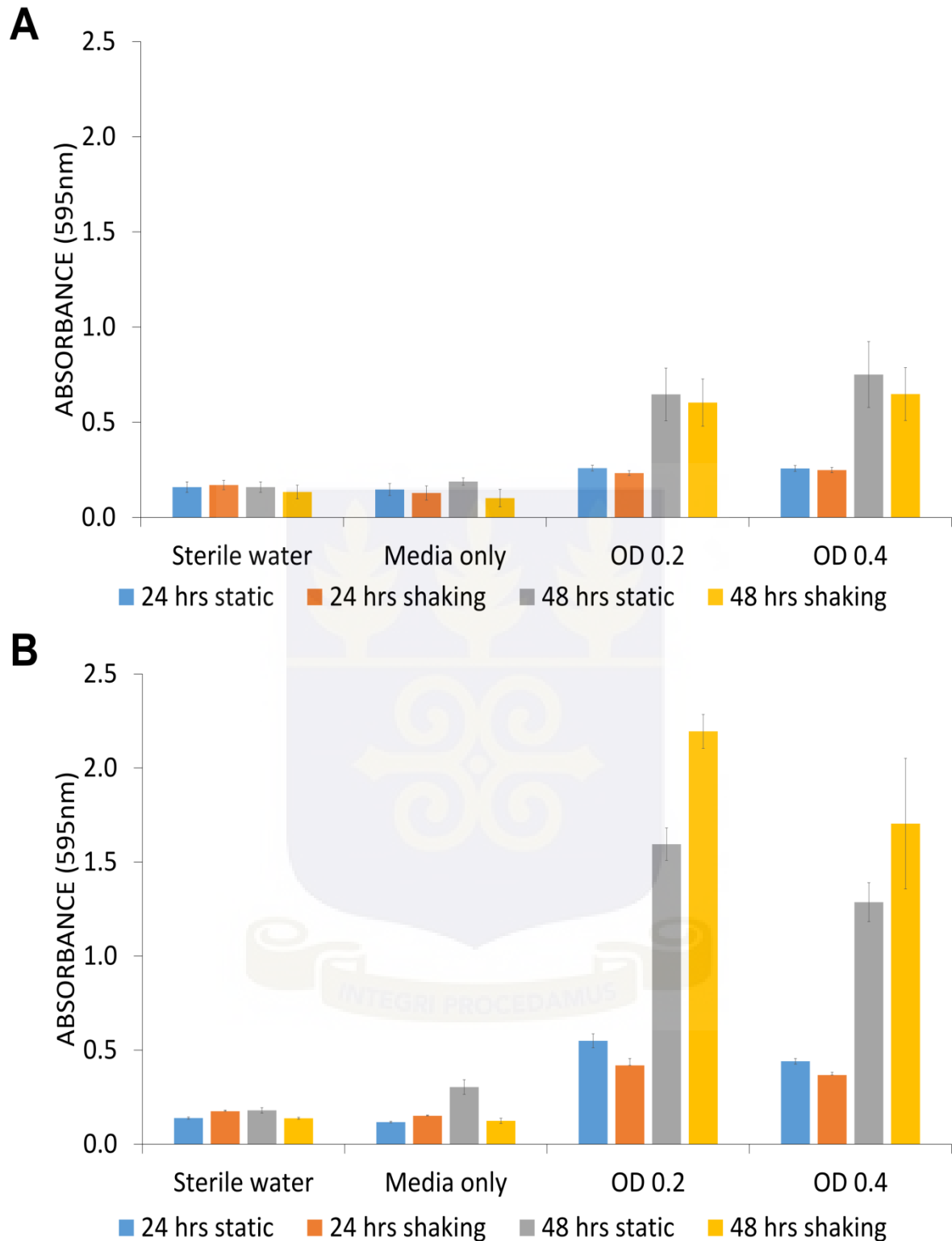


Figure A1: Optimization of culture conditions for thick biofilm formation in (A) *E. coli* GGP E100 (B) MRSA GGP E120. In *E. coli* GGP E100, optimum conditions that produced thick biofilm was obtained by incubating a starter culture of OD_{600nm} 0.4 for 48 h without shaking. Thick MRSA biofilm was obtained by incubating a starting culture of OD_{600nm} 0.2 with 180 rpm orbital shaking at 180 rpm at 37°C for 48 hrs. Biofilm formed was quantified by measuring absorbance at 595 nm after crystal violet staining and solubilization of attached stain with 95% alcohol.

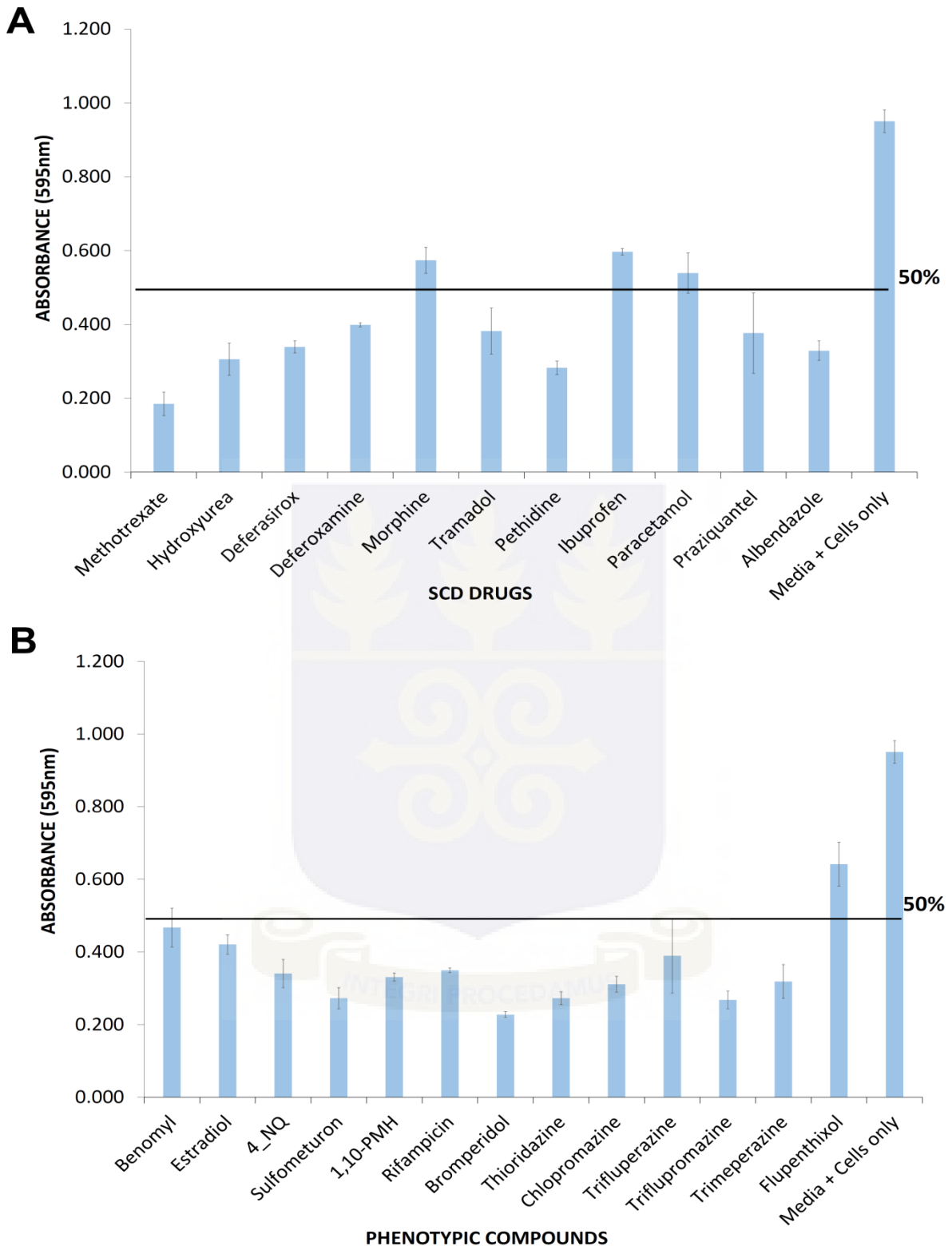


Figure 4.7.1: Inhibitory Effect of Phenotypic compounds on *E. coli* GGP E100 biofilm formation (A) SCD Drugs including Albendazole and Praziquantel (B) Other Phenotype modulating compounds. Each SCD drug and PMC was added to cells at a final concentration of 1 $\mu\text{g}/\text{mL}$, and 100 μL of each compound reaction aliquoted into 3 wells of a polystyrene microtitre 96 well plate. Incubation was done for 48 h static at 37°C. Biofilm formed was quantified by measuring absorbance at 595 nm after crystal violet staining and solubilization of attached stain with 95% alcohol.

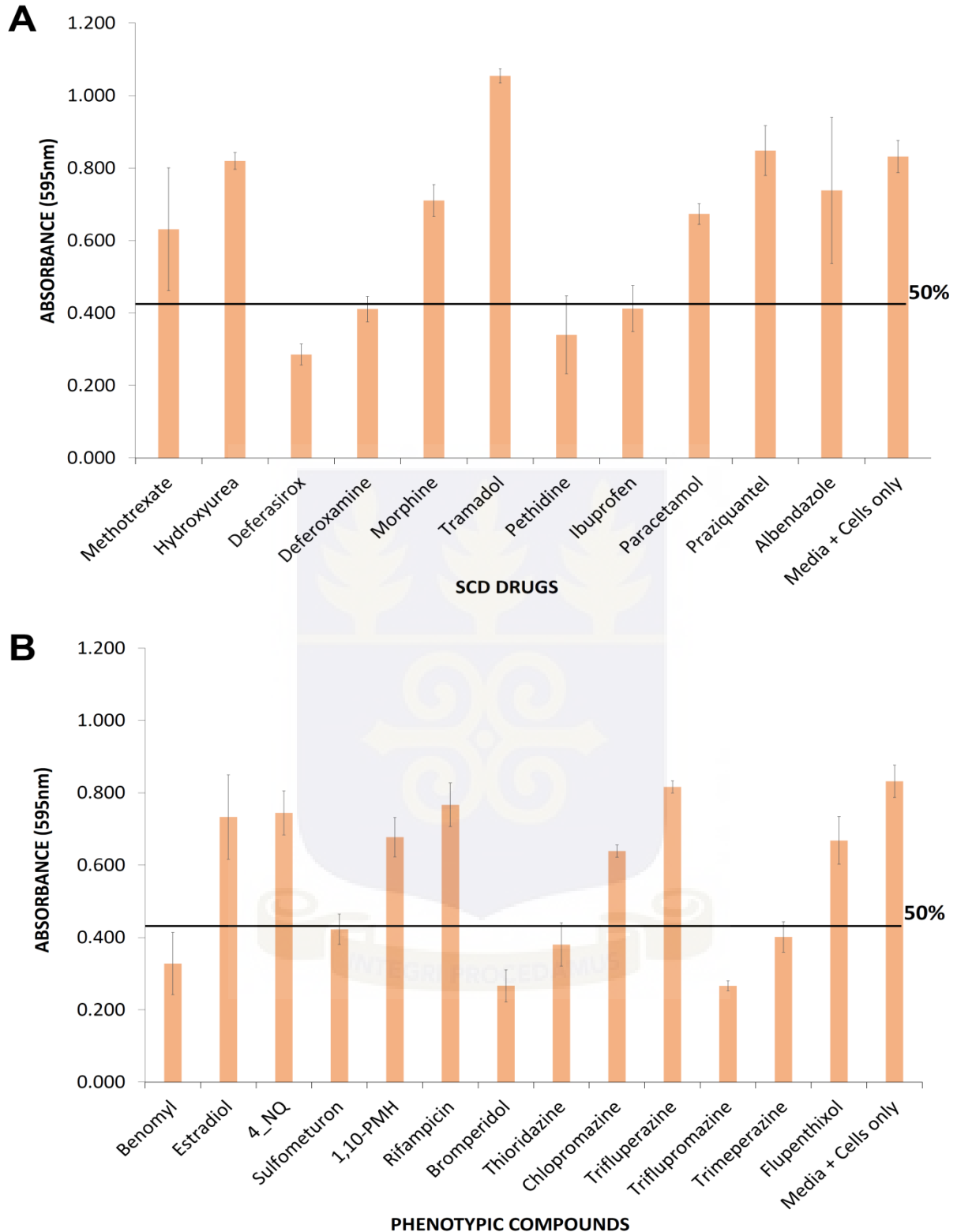


Figure 4.7.2: Disruption of formed biofilm in *E. coli* GGP E100 (A) SCD Drugs including Albendazole and Praziquantel (B) Other Phenotype modulating compounds. Cells without compounds were grown in 96 well micro-plates for 48hours, static at 37°C. Fresh media containing SCD drug or PMC at a final concentration of 1 µg/mL were aliquoted into 3 wells for each compound following washing of planktonic cells. Incubation was done for 2 h at room temperature, followed by washing. Biofilm remaining was quantified by measuring absorbance at 595 nm after crystal violet staining and solubilization of attached stain with 95% alcohol.

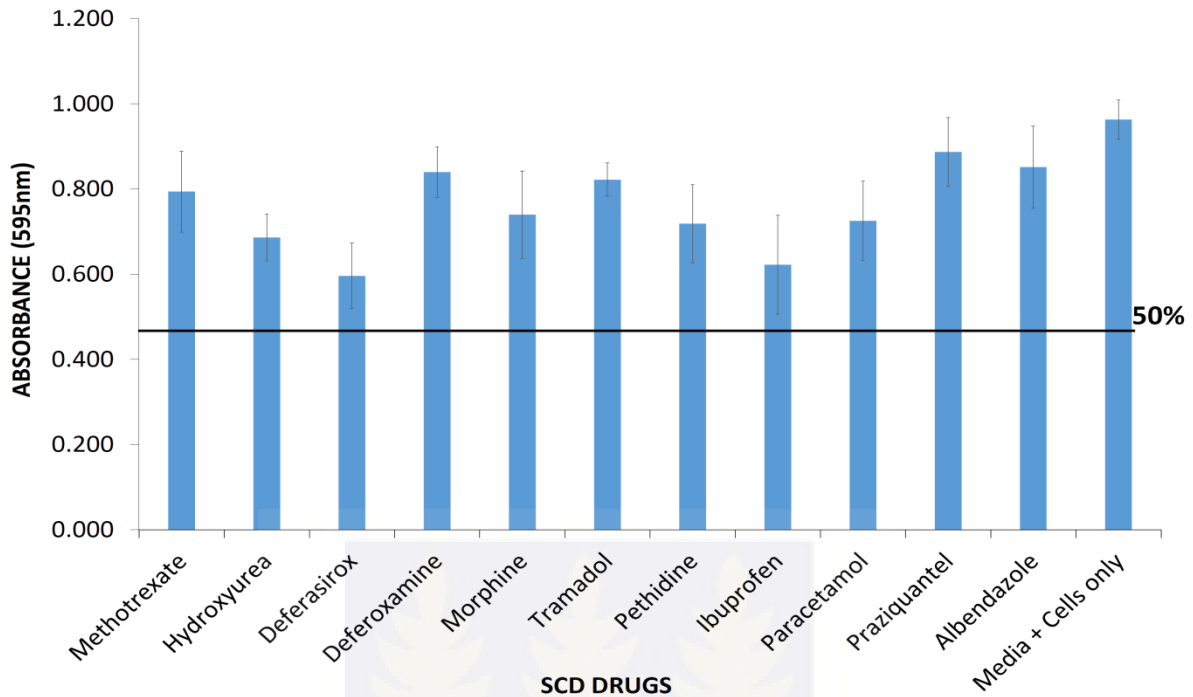
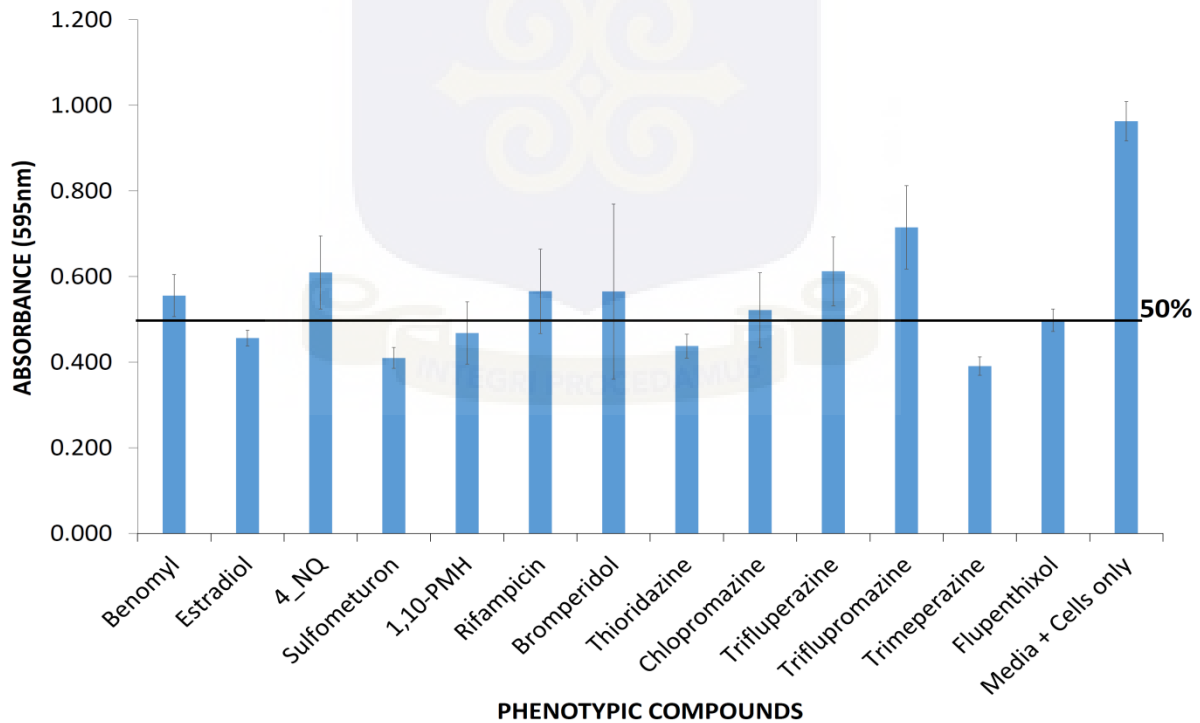
A**B**

Figure 4.7.3: Inhibitory Effect of Phenotypic compounds on MRSA GGP E100 biofilm formation (A) SCD Drugs including Albendazole and Praziquantel (B) Other Phenotype modulating compounds. Each SCD drug and PMC was added to cells at a final concentration of 1 $\mu\text{g}/\text{mL}$, and 100 μL of each compound reaction aliquoted into 3 wells of a polystyrene microtitre 96 well plate. Incubation was done for 48 h with shaking (180rpm) at 37°C. Biofilm formed was quantified by measuring absorbance at 595 nm after crystal violet staining and solubilization of attached stain with 95% alcohol.

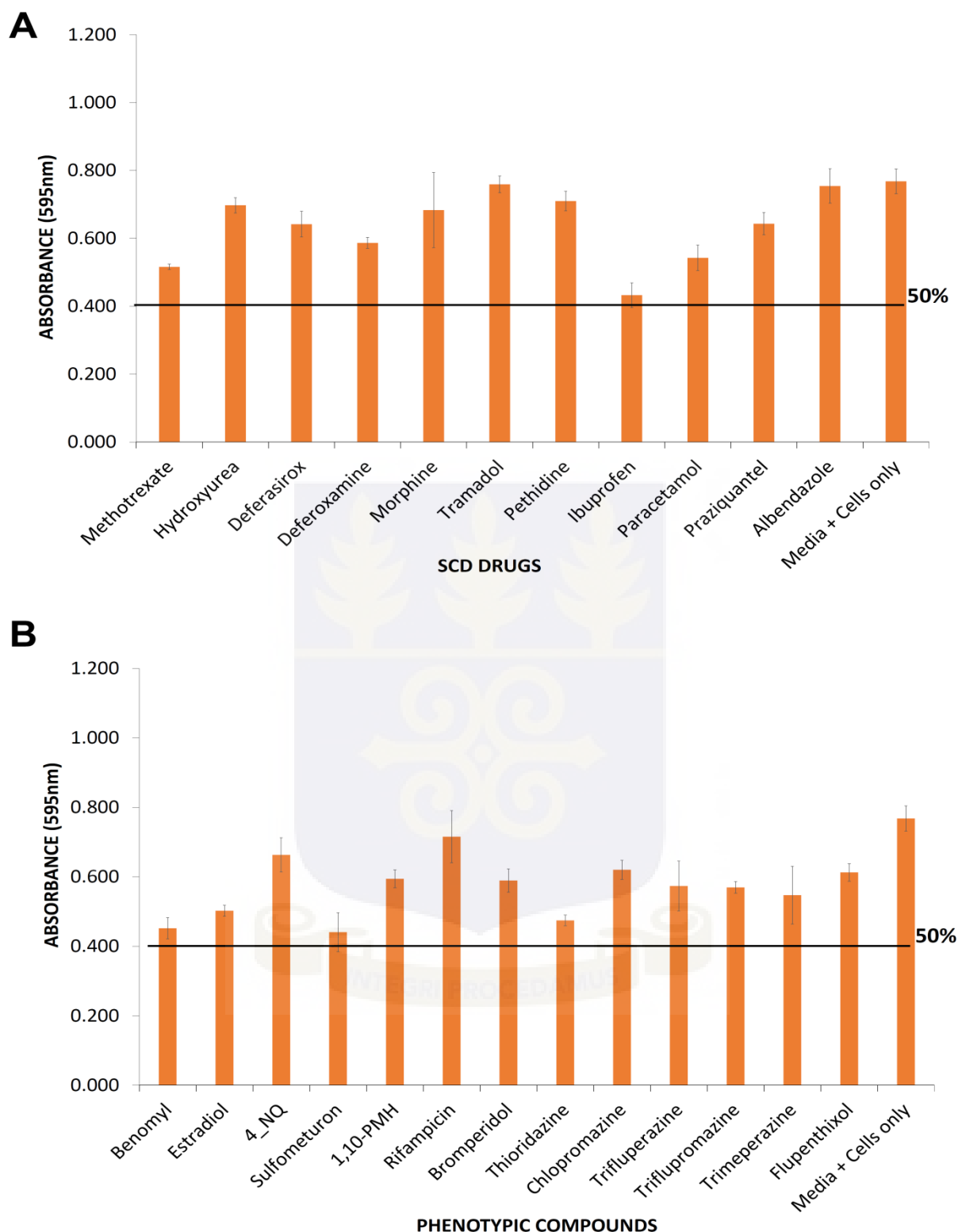
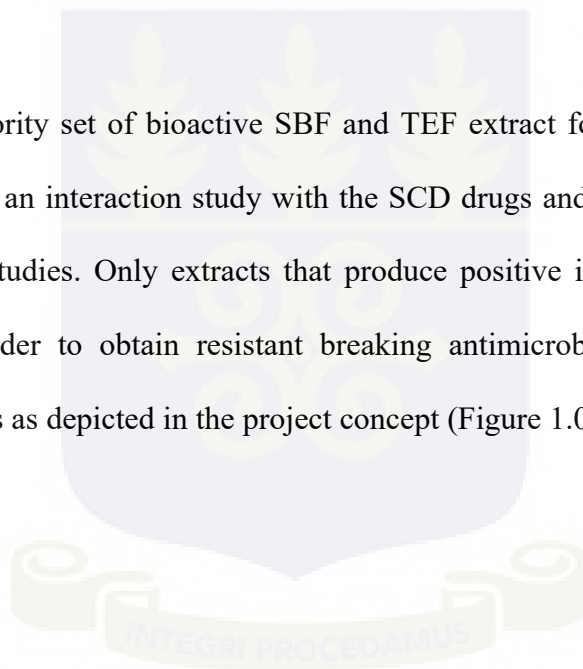


Figure 4.7.4: Disruption of formed biofilm in MRSA GGP E120 (A) SCD Drugs including Albendazole and Praziquantel (B) Other Phenotype modulating compounds. Cells without compounds were grown in 96 well microtitre plate for 48hours, with shaking (180 rpm) at 37°C. Fresh media containing SCD drug or PMC at a final concentration of 1 µg/mL were aliquoted into 3 wells for each compound following washing of planktonic cells. Incubation was done for 2 h at room temperature, followed by washing. Biofilm remaining was quantified by measuring absorbance at 595 nm after crystal violet staining and solubilization of attached stain with 95% alcohol.

4.7 Preliminary Screen of Soil-borne fungal (SBF) and Terrestrial Endophytic Fungal (TEF) isolates towards selection for product isolation

Bioactivities of 201 Soil Borne Fungal extracts and 306 Terrestrial Endophytic Fungal extracts was screened against the two drug resistant organisms. Only two of the SBF extracts, (SBF 052 and SBF 062) was active against both gram positive and gram negative bacteria (Figures 4.8.1 A & B). In addition to these two, five TEF extracts (TEF 246, TEF 248, TEF 249, TEF 266 and TEF 293) have been chosen for further interaction studies and product isolation including 24 other isolates that showed activity against other pathogenic organisms (Table 4.3). 12 TEF extracts was active against *E. coli* GGP E100 and MRSA GGP E120, 16 (Table 4.8.2).

In order to select a priority set of bioactive SBF and TEF extract for product isolation, the extracts will be used in an interaction study with the SCD drugs and Phenotype Modulating Compounds in future studies. Only extracts that produce positive interactions will go into product isolation in order to obtain resistant breaking antimicrobials and SCD relevant antibacterial compounds as depicted in the project concept (Figure 1.0).



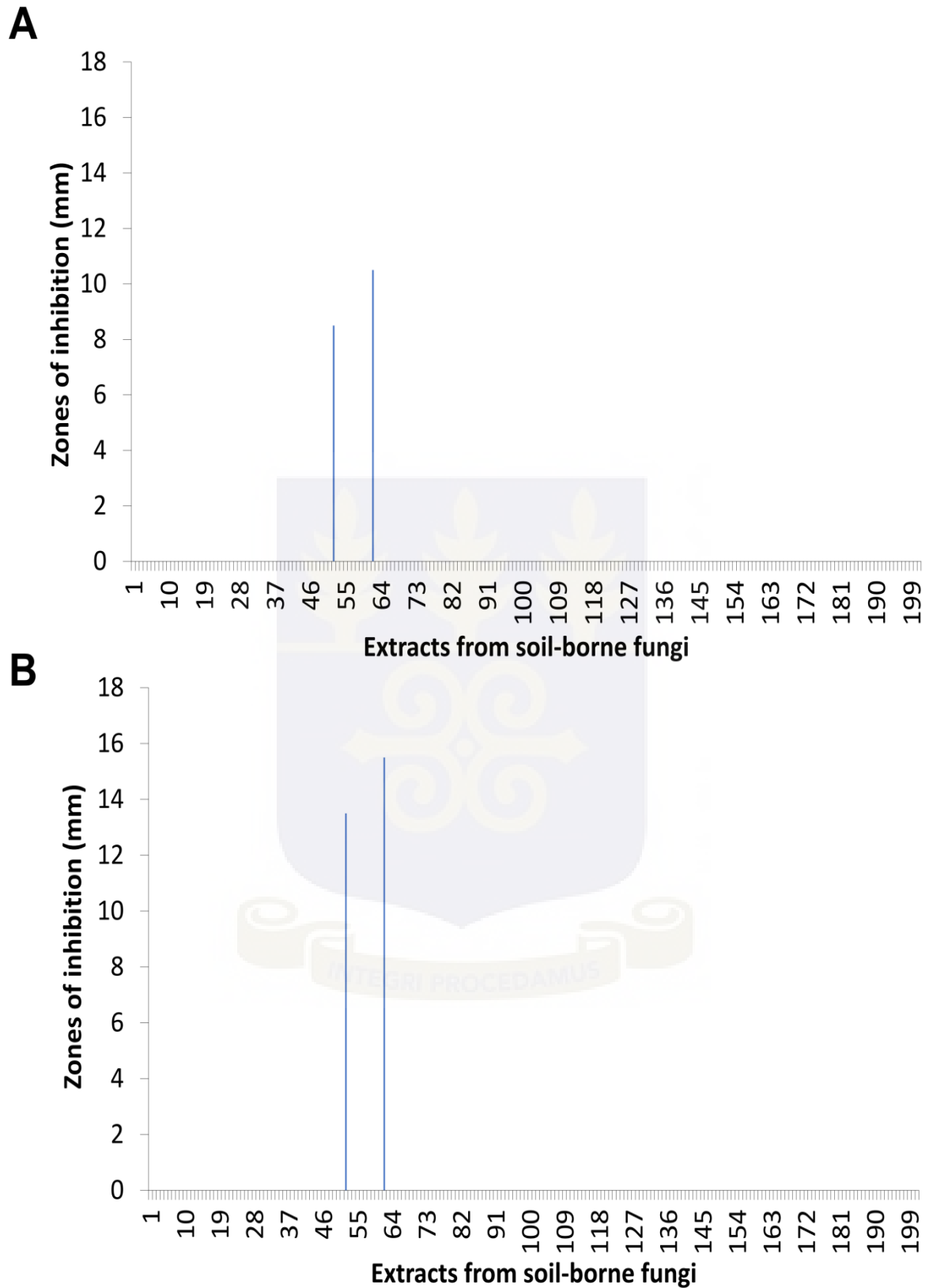


Figure 4.8.1: Preliminary Screen of 201 soil-borne fungal extracts against (A) Antibacterial activities of 201 soil borne fungal extracts against *E. coli* GGP E100 (B) Antibacterial activities of 201 soil borne fungal extracts against MRSA GGP E120. Screening was done using the disc diffusion method. Each extract was tested in duplicate

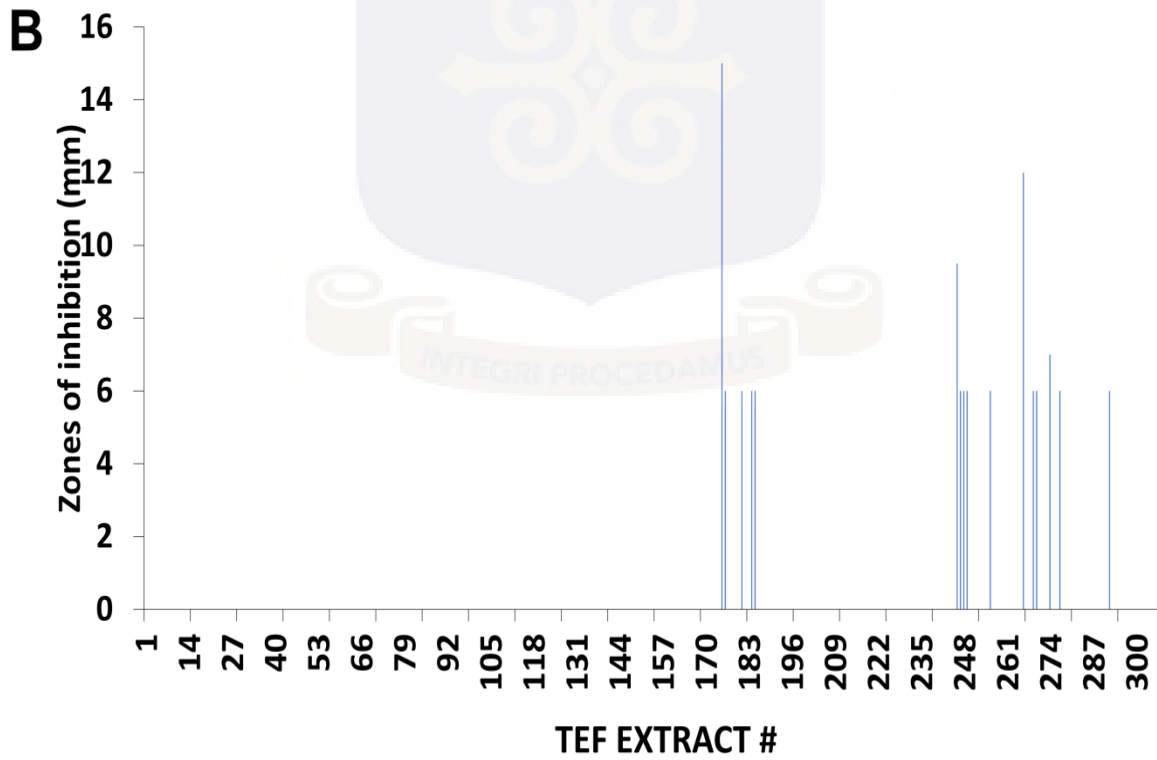
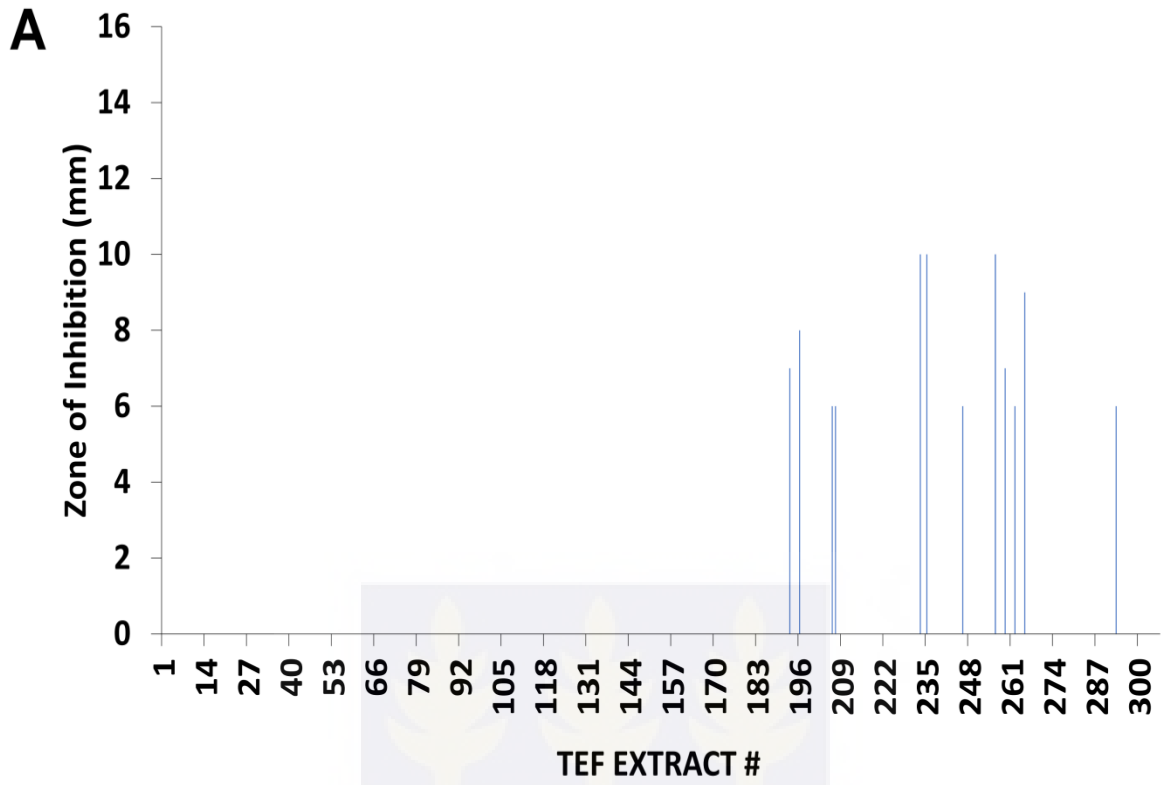


Table 4.5 Selected 31 Fungal Isolates for product isolation

SBF 052	TEF 074
SBF 061	TEF105
SBF 062	TEF 148
SBF 068	TEF 238
SBF 118	TEF 239
SBF 134	TEF 243
SBF 154	TEF 244
SBF 181	TEF 246
SBF 182	TEF 248
SBF 188	TEF 249
SBF 197	TEF 261
	TEF 266
	TEF 276
	TEF 277
	TEF 281
	TEF 284
	TEF 285
	TEF 287
	TEF 291
	TEF 293

CHAPTER FIVE

5.0 DISCUSSION

Antimicrobial compounds by themselves prevent growth and survival of bacteria and they often induce defense responses in bacteria leading to their own resistance-promoting responses (Fajardo & Martínez, 2008; Poole 2012; Poole, 2014). Additionally, environmental stressors such as exposure to nutrient starvation, reactive oxygen and nitrogen species, membrane damage, elevated temperature and ribosome disruption all influence bacterial resistance and susceptibility to a variety of antimicrobials, usually by acquisition of resistance determinants or promotion of physiological changes that compromise antimicrobial activity (Poole, 2012).

Bacteria, in their natural environments, are challenged by a variety of many chemical entities leading to stimulation of a variety of stress responses as a protective mechanism (Poole, 2012). Similarly, within humans, bacteria are constantly exposed to both biological metabolites and other drugs used in managing chronic diseases. Some of these drugs possess inherent bactericidal abilities and can therefore stimulate stress-response pathways, which can promote horizontal gene transfer in bacteria leading to acquisition of cross resistance to standard antimicrobials. For example, induction of the SOS response by the quinolone class of antibiotics is known to increase the rate of horizontal gene transfer in *E. coli* and *Vibrio cholerae* (Beaber *et al.*, 2004), and specifically promotes the sharing of mobile genetic elements that result in resistance to aminoglycosides, lincosamides, and anti-folate antibiotics (Van Hoek *et al.*, 2011).

It is in view of this that this study was undertaken to investigate the roles that SCD management therapies and other non-antibiotics or phenotype modulating compounds (PMCs) may play in modulating resistance or susceptible phenotypes in bacterial strains. We

also further assessed the contributions of these PMCs to efflux mediated drug resistance and antibiotic resistance due to formed biofilms.

The combinatorial effect of two drugs that differs from each drug alone expected for a given phenotype describes a drug interaction (Greco *et al.*, 1995). In the case of antimicrobial drugs and non-antibiotic compounds combinations, drug interaction may be labeled as resistance-breaking or positive (enhanced antimicrobial activity), resistance inducing or negative (decreased antibiotic activity) or indifferent (antibiotic activity unchanged) (Greco *et al.*, 1995; Keith *et al.*, 2005; Weinstein and Zaman, 2017).

The opioid, morphine, exhibited the best antibiotic potentiation effect against *E. coli* mainly with the protein synthesis inhibitors but suppressed the activities of Ampicillin (Cell wall inhibitor) and Moxifloxacin (inhibitor of DNA gyrase) (Table 4-1A). Similar trend was seen for Rifampicin, a known RNA inhibitor, as it enhanced the activities of Tetracycline, Erythromycin and Gentamicin but showed resistance inducing interactions with the anti-cell wall agents Ampicillin and Amoxicillin (Table 4-1B). A study by Dryselius *et al.*, (2005), showed synergistic interactions between mRNA inhibitors and protein level inhibitors having similar genetic targets. Rifampicin acts more or less like a protein synthesis inhibiting agent by inhibiting RNA polymerase (Calvori *et al.*, 1965), through a physical blocking of RNA elongation thus preventing synthesis of RNA and hence bacterial protein synthesis (Campbell *et al.*, 2001). Further studies could determine whether Morphine has similar action in due to the similar interaction fingerprints observed as well as producing a synergistic activity with Rifampicin in *E. coli* (0mm to 7mm) (Table 4-1B).

The suppressive interactions produced between the DNA mutagen 4-nitroquinolone oxide (4-NQ) (PC04-03) and Moxifloxacin against *E. coli* could be attributed to a site specific competitive inhibition or probably cross resistance mechanisms between two DNA disrupting

quinolones (Lázár *et al.*, 2013; Suzuki *et al.*, 2014). Moxifloxacin is known to be active against both gram-positive and gram-negative bacteria by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV (Drlica and Zhao, 1997). The mode of action of fluoroquinolone is the formation of DNA-fluoroquinolone-topoisomerase complexes and the subsequent generation of detrimental double-stranded DNA breaks (Drlica *et al.*, 2008). 4-NQ also oxidizes guanines in DNA to generate double strands break (Arima *et al.*, 2006). However resistance inducing effect between 4-NQ and Chloramphenicol, an inhibitor of protein chain elongation (Table 4-1B) agrees with reports from other experiments suggesting that the pairing of DNA synthesis and translation (protein synthesis) inhibitors show suppressive drug interaction (Yeh *et al.*, 2006; Bollenbach *et al.*, 2009). Bollenbach *et al.*, (2009), explains that in the presence of DNA stress, there is non-optimal regulation of ribosomal genes thus enhancing survival of bacteria in the presence of a protein synthesis inhibitor. A different scenario however, is observed in the gram-positive MRSA GGP E120 as 4-NQ produced resistance breaking interactions with the protein inhibitors, Erythromycin and Paramomycin (Table 4-1E), and in *E. coli* with Gentamicin (Table 4-1B).

Methotrexate, an anti-inflammatory compound (Li *et al.*, 2017), is known to be a first line medication for the treatment of Rheumatoid arthritis (Kremer & Phelps, 1992; Nayak *et al.*, 2016). Rheumatoid arthritis is among the complications of Sickle cell disease and it causes inflammation and irreversible damage in certain organs and joints (Bunn, 1997; Marino & McDonald, 1990; Nistala & Murray, 2001). Methotrexate exhibited significant resistance-inducing effects in both organisms, rendering *E. coli* GGP E100 resistant to Amoxicillin (13mm to 0) (Fig. 4.1 A) and Streptomycin (8mm to 0). It suppressed the activities of Ampicillin (20mm to 14mm) and Gentamicin (22mm to 11mm) against MRSA GGP E120, even though it has been reported to show strong antimicrobial activity against only *Staphylococcus aureus* strains and not *E. coli*, *P. aeruginosa* and *C. albicans* in a study by

Kruszewska *et al.*, (2000). Methotrexate as an antibacterial (Nayak *et al.*, 2016), binds and inhibits dihydrofolate reductase (DHFR) activity (Miller, 1972) which leads to loss of reduced folates, essential for purines, thymidylate and amino acid biosynthesis (Baccanari *et al.*, 1981; Schweitzer *et al.*, 1990). Thus, there is subsequent obstruction of DNA synthesis resulting ultimately in cell death. However, Kopytek *et al.*, (2000), has observed the resistance of many laboratory strains of *E. coli* to Methotrexate. Further tests by inactivating either *tolC* or *acrA* of the efflux pump system restored Methotrexate sensitivity (Kopytek *et al.*, 2000). It is possible then, that the resistance inducing interactions produced by Methotrexate to a variety of antibiotics in this work resulted from cross resistance due to activation of one or more efflux pump systems (Lewis, 1994; Nikaido, 2001). Similar reason could be attributed to the resistance inducing interactions observed with strong efflux inhibitor, Flupentixol (Jeyaseeli *et al.*, 2012) against *E. coli* GGP E100 (Table 4.2B) as sub-lethal concentrations may have elicited stress leading to overexpression of efflux pump genes.

The Resazurin assay was used to validate the interactions observed on agar plates in liquid medium. Most of the resistance breaking interactions involving *E. coli* were confirmed in the validation. However, two of the resistance inducing compounds, Methotrexate and 4-NQ on agar plates were observed to enhance antibiotic activity in broth cultures. With MRSA GGP E120, susceptibilities to compounds were highly increased in broth cultures than on agar plates. This observation highly depicts the ability of *Staphylococcus aureus* to form protective biofilms on solid surfaces (Grinholc *et al.*, 2007; Götz, 2002). Also, most surface associated recurrent infections have been linked to *S. aureus* biofilms (Chong *et al.*, 2013; Cohen *et al.*, 2013; Reffuveille *et al.*, 2017). Interaction between Flupentixol, an antipsychotic compound (Jeyaseeli *et al.*, 2012) and aminoglycoside Gentamicin was highly resistance inducing in *E. coli* GGP E100 both in the agar plate and liquid culture assays.

Flupenthixol is known to have strong efflux pump inhibiting properties (Jeyaseeli *et al.*, 2012; Machado *et al.*, 2016) and thus, sub-inhibitory concentrations of it may have sensitized *E. coli* cells to up-regulate its main AcrAB-TolC efflux pump system (Amaral *et al.*, 2014; Li *et al.*, 2011) rendering it resistant to Gentamicin.

Efflux has been recognized as a resistance mechanism to antimicrobials (Costa *et al.*, 2013). *S. aureus* is one of the most common human pathogens responsible for several infections. Besides this, it is known to show many different mechanisms of resistance to antibiotics. However, efflux related antibiotic resistance for *S. aureus* is less well characterized (Couto *et al.*, 2008). Uptake of Ethidium bromide (EtBr) by MRSA GGP E120 was generally far higher than in *E. coli* depicting a weak efflux activity or system in MRSA. Despite this, Deferoxamine significantly inhibited EtBr accumulation in MRSA, followed by Methotrexate and Albendazole. Deferoxamine is extensively used for iron chelation in iron-overloaded conditions (Moreau-Marquis *et al.*, 2009). Though it decreases susceptibility to infections, some microorganisms are able to use Deferoxamine as a siderophore or iron sequestering agent for their own use, thus increasing virulence (vanAsbeck *et al.*, 1983). Conversely, the counterpart iron chelator, Deferasirox, induced a higher uptake of EtBr. According to Chan *et al.*, (2009), such an unfavorable situation is not found in Deferasirox.

The Sickle cell disease modifier, Hydroxyurea (Green and Barral, 2011; Lanzkron *et al.*, 2008; Thornburg *et al.*, 2011) and Paracetamol showed higher EtBr accumulation capabilities than the verapamil control in MRSA GGP E120. Hydroxyurea is an inhibitor of class I ribonucleotide reductase, whose inhibition depletes nucleotide pools and results in replication fork arrest (Rosenkranz *et al.*, 1967). Antibacterial activities of Paracetamol and Ibuprofen have been reported in most studies (Al-Janabi, 2010; Obad *et al.*, 2015, Laudy *et al.*, 2016). Flupenthixol had the greatest uptake effect on MRSA with Chlorpromazine and

Triflupromazine following. These antipsychotic compounds are well known for their efflux inhibiting effects (Amaral *et al.*, 2014).

Many infectious diseases are associated with multidrug resistant bacteria residing in biofilms (Richter *et al.*, 2017; Chong *et al.*, 2013). Thus, agents that possess anti-biofilm properties are of high priority and those that may induce biofilm formation in bacteria are a cause for concern. *E. coli* biofilm formation was easily inhibited by the SCD drugs and other PMCs unlike the MRSA biofilms which tended to be very robust. Pethidine, an intravenous pain medication, appeared as both a good inhibitor and disruptor of *E. coli* biofilms and can be further explored for its anti-biofilm property. The antipsychotic compounds Bromperidol, Chlorpromazine, Thioridazine and Triflupromazine also showed high anti-biofilm effects.

Recently, there have been several reports of abuse of the pain killer, Tramadol. Tramadol is a mild opioid analgesic relative to Morphine and Codeine and have been reported to have some antimicrobial properties (Tamanai-Shacoori *et al.*, 2007). In our study, Tramadol slightly suppressed Tetracycline activity but boosted Gentamicin activity against *E. coli* GGP E100. In the ethidium bromide accumulation assay, uptake by Tramadol treated cells was the lowest, about 30% lower than the no drug treatment control in *E. coli*. Apart from a slight inhibition of *E. coli* biofilm, Tramadol showed no capability to inhibit or destroy biofilms.

Currently several studies are ongoing to develop methods for the analyses of drug interactions through drug interaction fingerprinting as a potent tool for discovery of effective combination therapies, identification of active pharmaceutical ingredients and to predict mechanisms of drug action as well as interrelations between cellular components (Yeh *et al.*, 2006; Ankomah & Levin, 2012; Suzuki *et al.*, 2014; Bollenbach, 2015; Song *et al.*, 2016; Weinstein and Zaman, 2017). In addition, these drugs and small molecules aid in exploring the effects of stress on cellular systems aside genetic mutations (Schreiber, 2003).

Stress responses are important determinants of antimicrobial resistance in bacteria and are representative of possible therapeutic targets in countering antimicrobial resistance (Poole, 2014). Determining bacterial stress response to a variety of small molecules and phenotype modulating compounds will reveal unique cellular targets for drug discovery (Bredel & Jacoby, 2004; Poole, 2012; Ziegler *et al.*, 2013). For example, in a report by Lee *et al.*, (2009), the mutation of the 2-component regulator of *Pseudomonas aeruginosa*, AmgRS, enhanced aminoglycoside action against bacteria grown planktonically and in antibiotic tolerant biofilms, and also against genetically resistant clinical isolates, as well as in lethal infections of mice. Hence, drugs that target AmgRS would be expected to increase the clinical efficiency of aminoglycosides. It has been suggested that AmgRS responds to envelope stress, which can be caused by aminoglycoside-induced translational misreading (Kohanski *et al.*, 2008).

Going forward, we intend to determine bacterial stress response to the unique interactions generated by some of these SCD drugs and Phenotype Modulating compounds to reveal endogenous bacterial genes that may serve as a valuable source of therapeutic targets for further discovery.



CHAPTER SIX

6.0 CONCLUSION AND FUTURE WORK

In all, SCD drugs showed unique interactions with antimicrobial compounds, most being resistance inducing, particularly to the cell wall inhibitors Amoxicillin and Ampicillin. Very few interactions enhanced activities of protein synthesis inhibiting antibiotics – Erythromycin, Streptomycin, Paramomycin and Gentamicin, an aminoglycoside. The oral iron chelator, Deferasirox and the opioid, Morphine emerged as the key resistance breaking (antibiotic enhancing) SCD drugs. Methotrexate, an anti-inflammatory compound exhibited significant resistance-inducing effects in both organisms.

Deferasirox, Pethidine and Morphine have high potential for repurposing into adjuvants for antibiotic compounds as they showed beneficial anti-biofilm, EtBr accumulation and efflux prevention effects.

31 large scale cultures of SBF and TEF isolates have been set up. The extracts obtained will be phenotypically screened using the panel of 24 PMCs to select a set of high priority resistance breaking extracts that will proceed for product isolation.

Analyses of bacterial stress response to certain unique interactions observed between PMCs and standard antibiotics as well as PMCs and isolated bioactive products from crude extracts will be carried out in order to identify genes that may be considered as therapeutic targets in future drug research.

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APPENDIX



