INVESTIGATIONS INTO THE MECHANISMS OF ANTI-MYCOBACTERIAL DRUG RESISTANCE USING ANTIPSYCHOTIC COMPOUNDS

A THESIS PRESENTED TO THE WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF THE AWARD OF MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

I Isaac Carilo (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra) hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Patrick Kobina Arthur and with advice from Dr. Jonathan Adjimani (Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra). To the best of my knowledge, this thesis contains neither materials that have been accepted for the award of any degree or any material previously published by another author, except where due reference is made in the text of the thesis.

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DEDICATION

I dedicate this work to the meritorious contributions of all members of the Laboratory for Chemical Systems Biology of Infectious Pathogens (a.k.a PAKAR Lab) especially Ethel Juliet Blessie.

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TABLE OF CONTENTS

Contents **Page**

Contents **Page**

Contents **Page**

Contents **Page**

LIST OF TABLES

Contents **Page**

LIST OF FIGURES

Contents **Page**

Contents **Page**

LIST OF ABBREVIATIONS

M. smeg	*Mycobacterium smegmatis*
Ery M. smeg A	Erythromycin-resistant *M. smegmatis* A
Ery M. smeg B	Erythromycin-resistant *M. smegmatis* B
SBF	Soil borne fungi
TEF	Terrestrial endophytic fungi
YPMD	Yeast extract, Peptone, Malt extract and Dextrose broth
YPDA	Yeast extract, Peptone, Dextrose and Agar
DNA/RNA	Deoxyribonucleic acid/ Ribonucleic acid
OD	Optical density
MIC	Minimum Inhibitory Concentration
MDR	Multidrug-resistant
CDR	Candida drug resistance
XTR	Extensively drug-resistant
MTB	*Mycobacterium tuberculosis*
TB	Tuberculosis
DR-TB	Drug-resistant tuberculosis
qRT-PCR	Reverse transcription quantitative polymerase chain reaction
PBS	Phosphate buffered saline
NADH	Reduced nicotinamide adenine dinucleotide

ABSTRACT

The continual emergence of drug resistant strains of *Mycobacterium tuberculosis* has caused global public health concerns. This project establishes basis for deciphering diverse resistance mechanisms in mycobacteria which would lead to the development of novel therapeutic options. Unique classes of antipsychotic compounds have been found to possess antifungal and anti-mycobacterial activities. The study sought to use antipsychotic compounds and a panel of phenotype modifying compounds to probe for resistance mechanisms in *Mycobacterium smegmatis*. Pairwise drug combinations (480 drug-pair conditions) between antipsychotic compounds/phenotypic compounds and antimicrobial agents produced resistance breaking interactions (37%) and resistance inducing interactions (6%) in *M. smegmatis*. The membrane disrupting antipsychotic compound, thioridazine, generated resistance breaking effects with a number of antibiotics with different antimicrobial profiles suggesting cell envelope homeostasis as a possible mechanism of antimicrobial tolerance and resistance. The DNA damaging agent 4-nitroquinoline oxide produced resistance breaking effects in pairwise combinations with 20 antibiotics tested in the study and disrupted biofilm in *M. smegmatis*, hence supporting the evidence that DNA damage repair is a possible mechanism of antibiotic tolerance. Deferoxamine and sulfometuron which induced biofilm formation in *M. smegmatis* also promoted mycobacterial resistance to a number of antibiotics. All antipsychotics tested promoted ethidium bromide uptake and reduced the rate of extrusion of ethidium bromide in *M. smegmatis* and erythromycin-resistant *M .smegmatis* A. DNA damaging agents inhibited biofilm formation in *M. smegmatis* and multidrug resistant erythromycin resistant *M. smegmatis* A, suggesting DNA disruption as a biofilm breaking strategy. The study highlights the usefulness of unrelated drugs and natural products in TB drug discovery while unveiling other determinants of drug resistance.

CHAPTER ONE

INTRODUCTION

Tuberculosis (TB) continues to be a global challenge due to the high morbidity and mortality rates associated with the disease. The disease poses significant threat to people in low income countries and is further complicated by co-infection with HIV/AIDS. One-third of the world population is infected globally, with new cases affecting 10 million people worldwide, causing an approximated 1.3 million deaths, among HIV-negative people. The disease caused an additional 300, 000 deaths among HIV-positive people in 2017 (WHO, 2018).

Tuberculosis is transmitted by the inhalation of aerosol containing the tubercle nuclei (Wells *et al*., 1948). The introduction of potent anti-mycobacterial agents about fifty years ago apparently controlled the spread and prevalence of tuberculosis (Faustini *et al.*, 2005). Since 1990, drug-resistant tuberculosis (DR-TB) has become a major public health problem (Yadav and Rawal, 2016; Pablos-Méndez *et al.*, 1998). In 2015, an estimated 480, 000 new incidence of multidrug-resistant tuberculosis (MDR-TB) and an additional 100, 000 people were reported to have rifampicin-resistant TB (RR-TB) treatment. The additional 100, 000 cases of rifampicin-resistant TB were eligible for treatment against MDR-TB (WHO, 2016).

The continual emergence of drug resistant *Mycobacterium tuberculosis* strains, co-infection with HIV and lengthy and complex treatment regimen all complicate and limit therapeutic options for TB. The continual emergence of drug resistant strains of *Mycobacterium tuberculosis* drives the urgent need to decipher diverse molecular mechanisms underlying the evolution of resistance. This would ultimately support the development of novel therapeutic agents needed to improve current treatment regimen for tuberculosis (Zhang *et al.*, 2016). Furthermore, due to the slow pace of drug discovery, there is the urgent need for novel

combination therapies and drug 'repurposing' strategies which would encounter no cross-resistance to existing and emerging *Mycobacterium tuberculosis* strains (Ramón-García *et al.*, 2011).

Antipsychotic compounds are taken by special group of people with psychological disorders and have known pharmacological parameters of absorption, distribution, metabolism, excretion and toxicity profiles in the management of psychotic patients. High prevalence of psychological distress and psychosis has been recently associated with tuberculosis especially with patients infected with multi-drug resistant *Mycobacterium tuberculosis* (Lasebikan and Ige, 2015). Therefore, the inclusion of antipsychotic compounds in TB treatment provides opportunities for the effective management of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis using antipsychotic compounds with anti-tuberculosis activities.

According to Gupta *et al.* (2013), verapamil (an FDA approved drug) potentiates bedaquiline activity by inhibiting efflux-mediated bedaquiline resistance. In addition, according to medical Xpress report (2017), a small drug-like molecule called SMARt-420 (Small Molecule Aborting Resistance) in combination with ethionamide in a mouse model has been shown to reverse resistance to ethionamide (Blondiaux *et al.* 2017). These observations support the idea that diverse means of probing for resistance in *Mycobacterium tuberculosis* could unveil resistance mechanisms.

Mycobacterial phenotypic response to unique drug-drug interactions could be used as predictive indicators of resistance or stress response strategies in a number of drug resistant strains of *Mycobacterium tuberculosis.* These diverse means of probing for resistance in *Mycobacterium tuberculosis* would reveal new strategies for TB treatment. This would further expose classical or other resistance mechanisms employed by MTB in drug resistance.

Biologically active products and their derivatives have served as chief source of therapeutic agents for treating infectious diseases since historical times (Cragg and Newman, 2005). According to Cheng *et al.* (2012), bioactive compounds of fungal origin have been shown to be good candidates for tuberculosis treatment. Secondary metabolites produced by the endophytic fungus *Phomopsis stipata* have been shown potent anti-mycobacterial activities (de Prince *et al*., 2012). In a recent study, secondary metabolites from mangrove endophytic fungi exhibit promising activity against *Mycobacterium tuberculosis* (Demers *et al*., 2018). These findings support the strategy of considering bioactive compounds of fungal origin as potential candidates for treating drug-resistant tuberculosis and other bacterial infections.

In earlier studies, sertraline, an antidepressant was shown to inhibit growth of different isolates of *Candida albicans* and was also found to enhance the activity of antibiotics against clinically relevant pathogens (Ayaz *et al.*, 2015; Lass-Flörl *et al.*, 2001). In a large compound screen against *Mycobacterium tuberculosis*, the antipsychotic bromperidol enhanced the activity of rifampicin, streptomycin, clofazimine, and clarithromycin against *Mycobacterium tuberculosis* (Ramón-García *et al.*, 2011). The antipsychotic compounds prochlorperazine and methdilazine have been shown to possess significant antibacterial action (Rani *et al*., 2005). This shows the usefulness of antipsychotics in antimicrobial drug discovery. Further to this earlier finding, antipsychotic compounds from three groups of antipsychotics (butyrophenones, phenothiazines and thioxanthenes), have been found to possess potent anti-mycobacterial effects mainly as bacterial efflux pump inhibitors (Vesenbeckh *et al.*, 2016). Other studies revealed the potential of thioridazine in TB treatment since it alters the cell membrane permeability, inhibits efflux activity, affects respiratory chain components of mycobacteria and increases expression of sigma B regulon (Dutta *et al.*, 2010; Dutta *et al.*, 2011). Chlorpromazine, an antipsychotic has

been found to predominantly inhibit bacterial efflux activity and thus reduce drug resistance (Coelho *et al.*, 2015). Trifluoperazine inhibits the synthesis of proteins, DNA and lipids in mycobacteria by restricting the incorporation of their respective synthetic precursors (Ratnakar and Murthy, 1992). The unique mechanism of action of these antipsychotics make them useful compounds that can be used to uncover new mechanisms of resistance towards the enhancement of their anti-mycobacterial effects and the patterns of expected phenotype.

The study seeks to investigate the mechanisms of anti-mycobacterial drug resistance using antipsychotic compounds and diverse means of using antipsychotics to guide isolation of bioactive metabolites from fungal sources such as Terrestrial Endophytic Fungi (TEF) and Soil Borne Fungi (SBF).

1.1 Problem statement

Drug-resistant tuberculosis still remains a global challenge and has attracted serious public health concern (WHO, 2016). Treatment of *Mycobacterium tuberculosis* infections is lengthy and hindered by the emergence of drug resistance. Considering the slow pace of conventional drug discovery and development process, coupled with the global burden of continual detection of drug resistant *Mycobacterium tuberculosis*, lengthy and complex treatment regimen with MDR tuberculosis, therapeutic options for tuberculosis are limited. These drive the need to further understand resistance and stress response mechanisms in *Mycobacterium tuberculosis* towards the generation of novel therapeutic options.

1.2 Justification

According to Lass-Flörl *et al.* (2001), three patients with premenstrual dysphoric disorder (PMDD) and vulvovaginal candidiasis (VVC) but were undergoing treatment for PMDD only, surprisingly had no recurrent episodes of VVC during sertraline administration. However, a

relapse of VVC occurred when sertraline administration ceased. This serendipitous discovery identified sertraline as a potent antifungal agent in further studies against different species of *Candida albicans* by attenuating virulence (Lass-Flörl *et al.*, 2003; Zhai *et al.*, 2012). In a large compound library screen against *Mycobacterium tuberculosis*, bromperidol (an antipsychotic drug) was accidentally shown to enhance the anti-mycobacterial activities of a number of standard anti-TB drugs and antibiotics. Therefore antipsychotics with structural resemblance to bromperidol may produce similar or peculiar potentiating effects with a number of standard TB drugs and fungal extracts against drug-resistant tuberculosis. Antimicrobial activities have been described for antipsychotic compounds belonging to phenothiazine and thiozanthene classes (Cederlund and Mardh, 1993). Findings by Vesenbeckh *et al.* (2016), show that antipsychotic compounds demonstrate anti-mycobacterial activity by inhibiting efflux activity in MDR and XDR *Mycobacterium tuberculosis*. While there still remains a number of unknown drug resistance strategies in *Mycobacterium tuberculosis* (Palomino and Martin, 2014), further investigations are required to elucidate mycobacterium drug resistant mechanisms/stress response mechanisms in order to stimulate the search for lead anti-mycobacterial agents. Therefore employing diverse means of probing for resistance in mycobacterium tuberculosis can potentially unveil drug resistance strategies in mycobacteria. Biologically active secondary metabolites and their derivatives have been exploited as chief treatment options for infectious diseases (Saga and Yamaguchi, 2009; Tajkarimi *et al*., 2010; Gouda *et al*., 2016; Jin *et al*., 2016). Hence, unique interactions between antipsychotics and fungal extracts would direct isolation of novel compounds from soil borne and terrestrial endophytic fungi.

1.3 Hypotheses

The hypotheses of the study are:

- Antipsychotic drugs would unveil new patterns of drug resistance in mycobacteria
- Interactions of antipsychotic compounds and bioactive fungal extracts (SBF and TEF) will guide/indicate/ provide the basis of selecting extracts of the highest priority (the most efficacious and uncommon mechanism of action).

1.4 Project concept

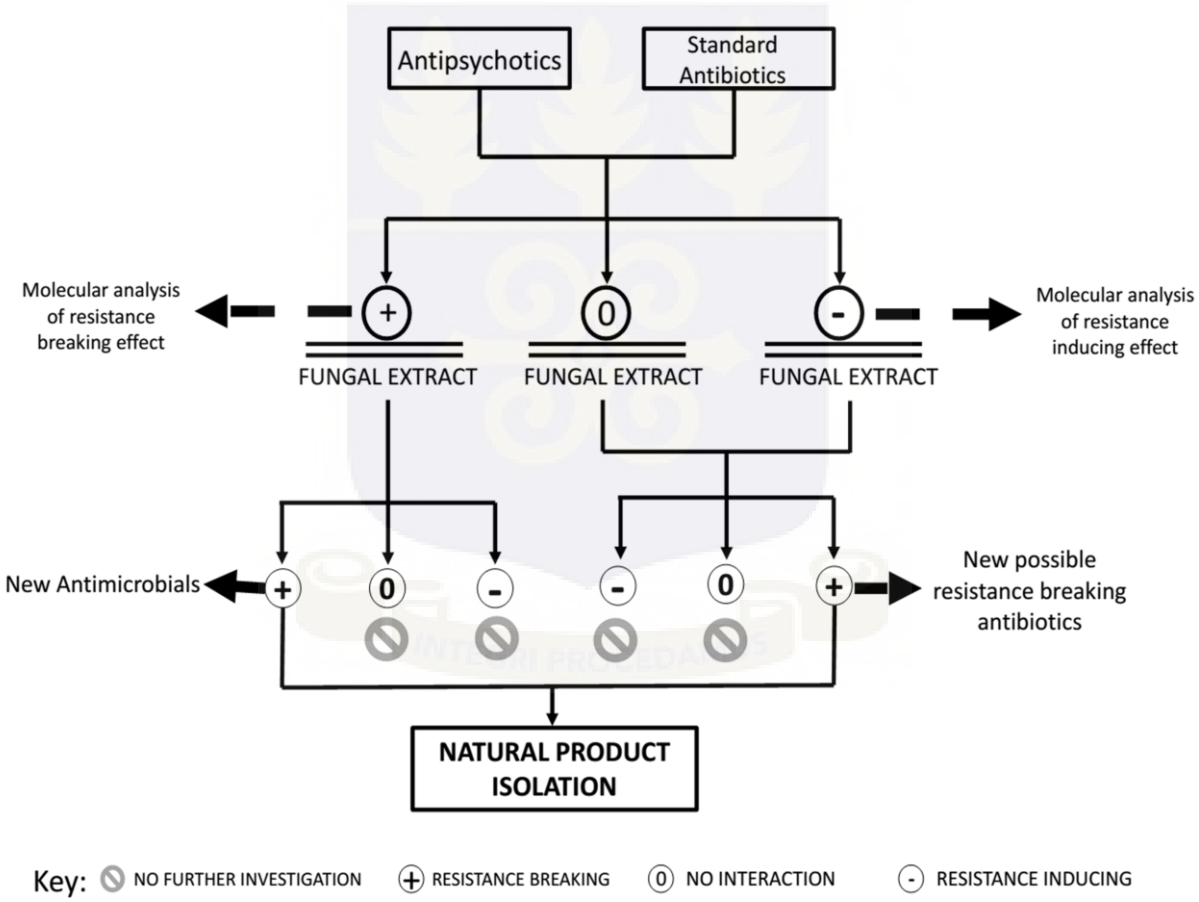

Figure 1.1 Overview of project concept

The project concept primarily highlights the usefulness of antipsychotics in unveiling drug resistance mechanisms. Initial pairwise combinations of antibiotics and antipsychotics at sub-

minimum inhibitory concentrations (sub-MIC) levels would produce resistance breaking or resistance inducing effects or no interactions. Interactions producing resistance breaking effects (+) provides new regimen for therapeutic interventions. Interactions generating resistance inducing (-) or neutral effects (0) provide opportunities for isolating new resistance-breaking antibiotics of fungal origin based on interactions between fungal extracts and antipsychotics. Resistance breaking effects arising from interactions between antipsychotics provide the basis for the isolation of novel bioactive compounds. Molecular analysis of resistance breaking and resistance inducing effects unveils stress-induced antibiotic resistance mechanisms.

1.5 AIM AND OBJECTIVES

1.5.1 Aim

Investigate resistance and susceptibility phenotypes mediated by drug-drug combinations involving antipsychotic compounds, anti-mycobacterials and active extracts from soil borne fungi (SBF) and terrestrial endophytic fungi (TEF).

1.5.2 Objectives

- To determine mycobacterial phenotypic response to specific drug-drug interactions using disc diffusion (standard anti-TB versus antipsychotic) on *M. smegmatis*.
- To determine specific mycobacterial phenotypic response to active fungal extracts and generate priority lists of fungal extracts

CHAPTER TWO

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is a contagious airborne disease caused by inhalation of droplet nuclei of organisms of the *Mycobacterium tuberculosis* complex. Tuberculosis continues to be one of the major causes of morbidity in low-income countries (Figure 2.1), and drug-resistant tuberculosis is a major concern in many clinical settings (Pescarini *et al.*, 2017). Although *M. tuberculosis* is for the most part a pulmonary pathogen (Figure 2.2), disease can progress other parts of the body causing extrapulmonary tuberculosis. Tuberculosis can advance from latent TB infection to active transmissible TB, at which point the patient manifests clinical symptoms such as cough, fever, weight loss and night sweats (Schluger and Burzynski, 2010). Only active pulmonary TB is contagious (Albert *et al.*, 2016).

2.2 Global burden of tuberculosis

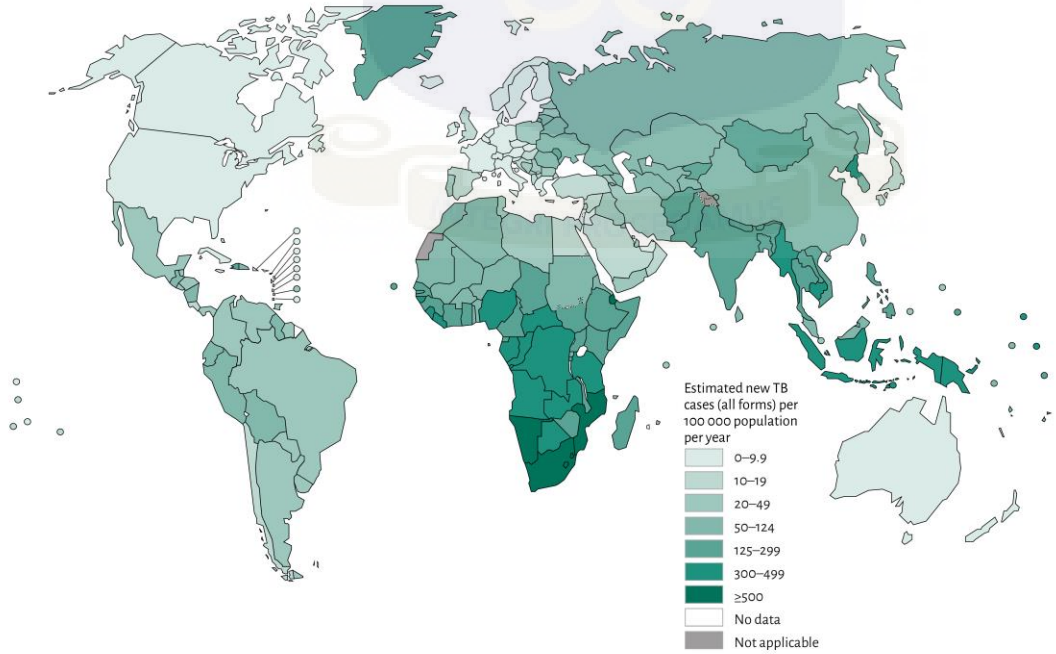

Figure 2.1: Global incidence of active tuberculosis disease (WHO, Tuberculosis report 2015)

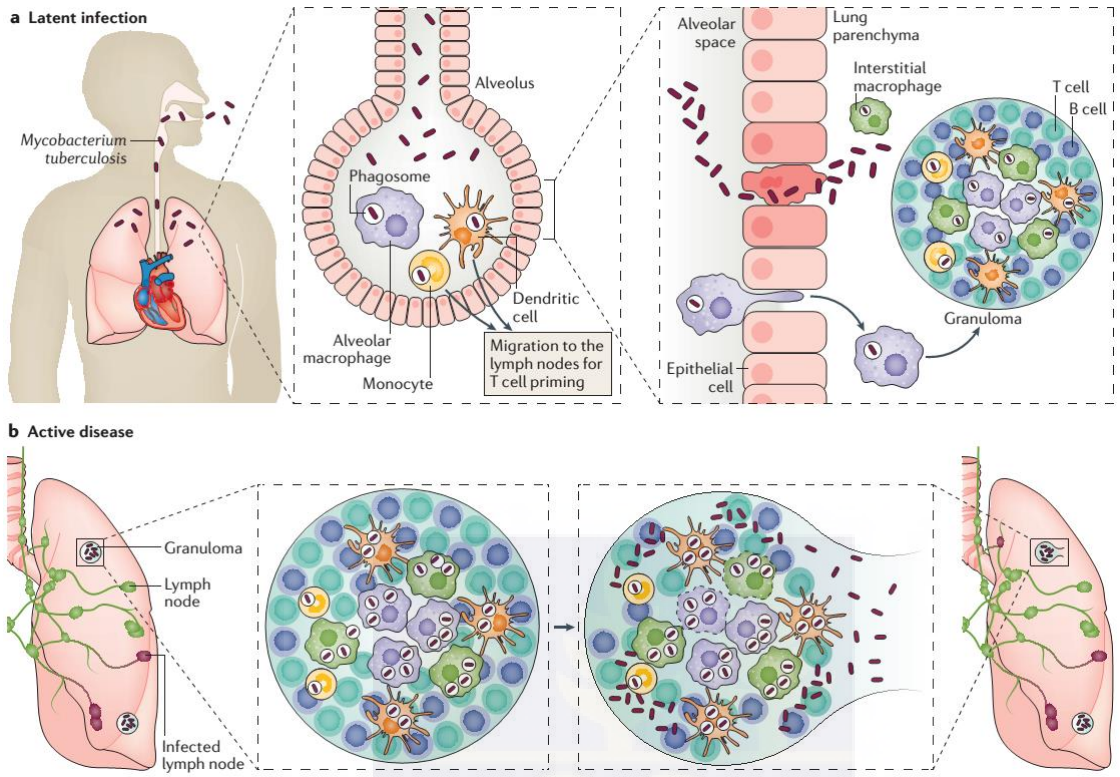

Figure 2.2: *Mycobacterium tuberculosis* infection (Pai *et al*., 2016).

a) As shown in Figure 2.2, infection begins via the inhalation of aerosol containing tubercle nuclei. The tubercle bacilli upon arriving in the lung alveolar space are internalised by resident alveolar macrophages. Failure by macrophages to eliminate the bacteria enables the *M. tuberculosis* to progress to the lung interstitial tissue. Dendritic cells or inflammatory monocytes later move *M. tuberculosis* to pulmonary lymph nodes for prior recognition by T lymphocytes. This finally leads to the attraction of B and T lymphocytes, to the parenchyma of the lung to form granuloma

b) The mycobacteria replicate inside the growing granuloma. In case the mycobacterial load becomes too heavy, the granuloma fails to handle the bacterial load. The bacteria eventually advances towards other organs, a situation called extrapulmonary tuberculosis. The infected person is said to be symptomatic and now has active transmissible tuberculosis.

2.3 Therapeutic options for tuberculosis

For new cases of drug-sensitive tuberculosis, the recently recommended treatment strategy is a four-drug regimen featuring rifampicin, isoniazid, pyrazinamide and ethambutol for two months, followed by rifampicin and isoniazid only for additional four months (WHO, 2010). Treatment for multidrug-resistant tuberculosis (MDR-TB) requires lengthier treatment period

(lasts for as long as twenty months) and requires more expensive and toxic drugs. For most patients with MDR tuberculosis (resistance to isoniazid and rifampicin), the recent prescribed course of treatment recommended by World Health Organization (WHO) has low success rates. At present, an effective vaccine that can be used in preventing tuberculosis remains elusive (Nagelkerke *at al*., 2006).

2.4 Drug resistance in tuberculosis treatment

Though mutations in a number of genes are associated with drug resistance in *Mycobacterium tuberculosis* (MTB) (Table 2.1), there are still a number of cases of unknown mutations and mechanisms conferring resistance. For instance, according to Merker *et al.* (2013), whole genome sequencing reveals that tuberculosis drug resistance is a phenomenon more complex than previously suggested. Therefore more investigations are needed to understand the role of other determinants of drug resistance and their contribution to the occurrence of multidrug-resistant tuberculosis (MDR-TB) - or extensively drug-resistant tuberculosis (XDR-TB). Other studies suggest the involvement of mechanisms other than what is previously known (Fonseca *et al.*, 2015). In *Mycobacterium tuberculosis*, both specific gene mutation and the efflux pump have been shown to play major roles in the resistance to anti-mycobacterial drugs (Machado *et al.*, 2017).

Table 2.1: Anti-mycobacterial drug resistance mechanisms

Clinically relevant antitubercular drugs and their commonly associated resistance mechanisms.

Drug name	Mechanism of action	Common mechanism of resistance
Rifampicin	RNA synthesis inhibition	Mutation of *rpoB* induces a conformational change at β-subunit of RNA polymerase causing a decrease in binding affinity
Isoniazid	Mycolic acid biosynthesis inhibitor and effects on DNA, lipid, carbohydrate, and NAD metabolism	*KatG* suppression causing decreased prodrug activation, and a mutation in the promoter region of *InhA* causing an overexpression of InhA
Pyrazinamide	Not fully resolved, may include membrane potential disruption	Mutations in *pncA* reducing conversion to active acid form
Ethambutol	Arabinogalactan biosynthesis inhibition	Mutations in *embB* at codon *embB306*
Amikacin/Kanamycin	Protein synthesis inhibition	16S rRNA target site modulation (1400 and 1401 *rrs* gene) Increased drug inactivation via overexpression of *eis* aminoglycoside acetyltransferase
Capreomycin	Protein synthesis inhibition	Cross-resistance with aminoglycosides plus mutation of *tlyA* which decreases rRNA methyltransferase activity
Streptomycin	Protein synthesis inhibition	Mutations in *rpsL* and *rrs* confer binding site modulation
Fluoroquinolones	DNA gyrase and topoisomerase IV inhibitor	Mutations in *gyrA and gyrB* causing an alteration to DNA Gyrase A/B binding site (later generations not always cross-resistant with first generation) and increased ABC-type efflux pump expression
Ethionamide	Mycolic acid biosynthesis inhibition	Mutations in *ethA* and *inhA* causing decreased prodrug activation and InhA overexpression (cross-resistance with Isoniazid)
Cycloserine	Peptidoglycan biosynthesis inhibition	Overexpression of *alrA* decreasing drug efficiency
Para-aminosalicyclic acid	Folic acid and iron metabolism inhibition	Mutations in the *thyA* causing a decrease in activated drug concentrations and *folC* mutations which cause binding site mutations
Clofazimine	Release of Reactive Oxygen Species (ROS) and cell membrane disruption	Mutation to Rv0678 causes upregulation of MmpL5, a multisubstrate efflux pump (cross-resistance with Bedaquiline)
Linezolid	Protein synthesis inhibitor (50S subunit)	T460C mutation in *rplC*, encoding the 50S ribosomal L3 protein and possible efflux mechanisms
ß-lactam/ß-lactamase inhibitor: Amoxicillin Meropenem Imipenem	Cell wall disruption via peptidoglycan modulation	Overexpression of β-lactamases, (*BlaC*), point mutations at target site altering deacylation rate and binding affinity, cell permeability (alteration in porins and outer membrane composition), and increased efflux (Rv0194)
Thiacetazone	Inhibits methyltransferases in mycolic acid biosynthesis	*ethA* mutation minimizes prodrug activation and mutations to *hadABC* operon affecting dehydratase activity
Clarithromycin	Protein synthesis inhibition (50S subunit)	Low cell wall permeability and the expression of *emr37*, confers 23S rRNA site modulation
Bedaquiline	Inhibition of mitochondrial ATP synthase	*atpE* mutations introduces binding site modulation. Noted efflux via mmpL5 (cross-resistance with Clofazimine)
Delamanid	Mycolic acid biosynthesis inhibition	Mutation of reductive activating Rv3547 gene

(Hoagland *et al.*, 2016)

2.4.1 Efflux pump mediated anti-mycobacterial resistance

Efflux pumps have become increasingly significant in drug resistance strategies. Bacterial efflux pumps are membrane proteins that are capable of actively pumping out a broad range of substances, including drugs, from the intracellular environment to the exterior of the cell. They are involved in physiological processes, such as division of cell wall, maintenance of hydrogen and ion balance and pumping of intracellular metabolites (Piddock, 2006).

Efflux pumps are increasingly becoming attractive targets for drug discovery programs. The goal of identifying the role of efflux pumps in tuberculosis drug discovery programmes is to

find out whether new anti-mycobacterial drugs will be subject to efflux activity or may be potential efflux pump inhibitors. This is particularly relevant in a time when MDR-TB and XDR-TB continue to worsen therapeutic options for the treatment of serious forms of drug-resistant tuberculosis (Rodrigues *et al.*, 2012).

For example, bedaquiline which acts as an ATP synthase inhibitor of drug sensitive, multi-drug and extensively drug resistant tuberculosis is made ineffective by efflux-mediated activity (Nagabushan and Roopadevi, 2014).

Bacterial efflux pumps are categorized into five distinct super families with different structural architectures, substrate preference and energy requirements (Rossi *et al.*, 2005). These include the major facilitator superfamily (MFS), the resistance–nodulation–cell division (RND) superfamily, the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) superfamily, and the multidrug and toxic compound extrusion (MATE) superfamily. The MFS, RND, ABC, SMR and MATE efflux pumps are chromosomally encoded. Efflux pumps belonging to the ABF family are predominant in mycobacterial drug resistance and drives efflux by using the energy of hydrolysis of adenosine triphosphate (ATP) (Balganesh *et al.*, 2012).

Currently, efflux pump inhibitors have been described as potential synergistic partners in anti-mycobacterial treatment since they have the ability to re-establish sensitivity to current anti-mycobacterial agents. This re-establishment requires the presence of an efflux inhibitor in conjunction with the anti-tuberculosis drugs at sub-minimum inhibitory concentrations (sub-MIC). The anti-mycobacterial drug combination is important as the efflux pump inhibitors alone at their sub-inhibitory concentrations produce little or no mycobacterial growth inhibition

(Viveiros *et al.*, 2012). These findings present opportunities for the use of efflux pump inhibitors as synergistic partners in tuberculosis treatment (Pule *et al.*, 2015).

2.4.2 Biofilm mediated drug resistance

Colonies of microbes joined to one another and attached to a surface are described as biofilms (Kumar *et al.*, 2015). Formation of biofilm has been shown to be regulated by quorum sensing (Davies *et al.*, 1998). Biofilm formation in mycobacteria is known to be one of the common factors affecting their pathogenesis (Hall-Stoodley and Stoodley, 2005). Formation of biofilms helps bacteria to become tolerant to antibiotics and persists in chronic infections (Brennan, 2017). Bacterial biofilms affects the antibiotic pattern of a number of antimicrobials (Tabatabaei and Sohrabi, 2017). Catheter-associated pathogenic infections progresses to biofilm development with time and accounts for ineffective antibiotic treatment in *Mycobacterium avium* (Falkinham III, 2007).

2.4.3 Repurposing of antipsychotic compounds in TB drug discovery

One of the effective therapeutic and economic alternatives that have been suggested to combat antimicrobial drug resistance is drug repositioning and synergistic drug combinations (Jin *et al.*, 2014). Antipsychotic compounds are used to treat or manage patients with psychological disorders. However, antipsychotic compounds (butyrophenones, phenothiazines and thioxanthenes) have been shown to possess anti-mycobacterial activities via inhibition of a number of mycobacterial efflux pumps (Vesenbeckh *et al.*, 2016). Efflux mediated resistance have been found to be an alternative mechanism of drug resistance in mycobacteria (da Silva *et al.*, 2011; Kanji *et al.*, 2018). According to Ramón-García *et al.* (2011), bromperidol, which is a butyrophenone, enhances the pairwise synergistic combinations with commonly used antibiotics against *M. tuberculosis*. Thioridazine and chlorpromazine, which are phenothiazines,

have been shown to be effective against multidrug-resistant and extensively drug-resistant tuberculosis, as well as enhancing the activity of some agents used for first line TB treatments (Rodrigues *et al.*, 2008). Phenothiazines have been shown to enhance the activities of rifampicin and streptomycin (Amaral *et al.*, 2001). Chlorpromazine have been shown to be involved in inhibiting components of the electron transport chain involved in ATP oxidative phosphorylation and target membrane proteins (Weinstein *et al.*, 2005).

Research findings by Ratnakar and Suryanarayana Murthy, (1993), show that trifluoperazine possesses a multi-mechanism of action by inhibiting incorporation of synthetic precursors of proteins, lipids and DNA. Trifluoperazine interferes with the activity of a novel calcium binding protein (calmodulin-like protein) in *Mycobacterium tuberculosis* (Koul *et al.*, 2009). These calmodulin- like proteins occur in many mycobacterial species such as *M. smegmatis*, *M. tuberculosis* H37Rv and *M. bovis* (Advani *et al.*, 2014). A number of studies on thioridazine show that this antipsychotic causes permeability of the cell envelope, inhibits efflux pump activity and target NADH:menaquinone oxidoreductase activity, which is involved in aerobic respiration in mycobacteria (de Keijzer *et al.*, 2016). It is also known to target enzymes involved in fatty acid metabolism and membrane proteins in mycobacteria (Dutta *et al.*, 2010). It has been observed that when immune cells are exposed to thioridazine, cytokines responsible for eliciting immune responses against tuberculosis are activated (Ordway *et al.*, 2003). In a follow up study, these cytokines were associated with tuberculosis progression from latent state to active TB state (Ordway *et al.*, 2004). Thioridazine, in combination with other antibiotics, has also been used to treat patients with totally drug resistant tuberculosis (Amaral, 2012). In mouse models, these phenothiazine antipsychotics have been found to possess antibacterial

activity against MDR and XDR TB (Van Soolingen *et al.*, 2010). For these compounds, no resistance mechanisms have been reported yet to their activities in bacteria (Yano *et al.*, 2006). Five of the antipsychotics chlorpromazine, thioridazine, trifluoperazine, triflupromazine and trimeprazine are phenothiazines (Figure 2.3). Flupenthixol is a thioxanthene and bromperidol belongs to the butyrophenone class of antipsychotics. All except bromperidol share structural similarities by possessing three fused ring system (Figure 2.3). Phenothiazines are largely known to be inhibitors of potassium and calcium channels, and to also reverse phenotypes of multidrug resistance in *M. tuberculosis*, *M. smegmatis* and *M. avium* (Amaral *et al.*, 2001). Flupenthixol, a thioxanthene, with similar tricyclic ring system is also known to be a potent efflux pump inhibitor (Machado *et al.*, 2016).

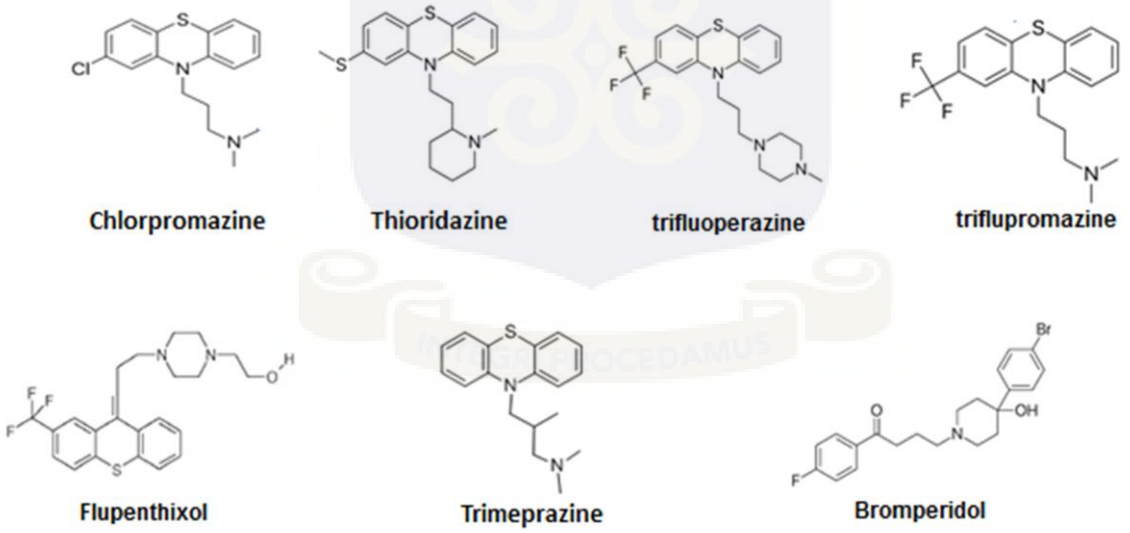

Figure 2.3 Partial structures of antipsychotic compounds

2.4.4 Mycobacterial phenotypic response to anti-TB agents and antipsychotic compounds

A number of strategies have been employed to tackle the occurrence of drug resistance especially in combination therapies. According to Xu *et al.* (2017), verapamil which is an FDA-approved efflux pump inhibitor apparently potentiate clearance of *M. tuberculosis* by bedaquiline. It has been suggested that one inexpensive but effective solution would be to explore new uses for existing drugs ("repurposing"), either alone or in combination therapies (Boguski *et al.*, 2009).

Antipsychotics have been shown to exhibit potent anti-mycobacterial activities with the majority acting as efflux pump inhibitors hence their potential application in TB treatment. An added advantage is that antipsychotics in use have known pharmacological profiles and any redirected use can be more rapidly assessed, thereby decreasing the overall average time for approval (Chong and Sullivan Jr, 2007). Currently, prescribed course of treatment for drug resistant tuberculosis are expensive, lengthy, costly and toxic compared to treatment course for drug-sensitive tuberculosis. These challenges drive the immediate need for the development of novel therapeutic options with little or no side effects. Most importantly, there is the pressing need to develop novel therapeutic treatment courses that are effective against drug-resistant TB and are physiologically tolerant. Phenotypic based assay utilising drug-drug interactions would expand knowledge of heterogeneity of cellular response playing important roles in drug resistance and other cellular behaviour (Slack *et al.*, 2008). From the body of literature, a number of non-antibiotic compounds have been shown to possess antimicrobial properties (Table 2.2). Therefore, drug combinations involving these compounds (Table 2.2) could provide additional strategies in combating MDR and XTR tuberculosis.

Table 2.2: Systematic reviews on the 24 phenotypic compounds

Number	Code name	Compound Names	Interaction with other antibiotics	Antimicrobial activity
1	PC04-01	Benomyl	No documented interaction	Known to have antifungal activity (Gupta 2004)
2	PC04-02	Estradiol	Estradiol increases the level or effect of miconazole nitrate by altering drug metabolism	No known antifungal activity
3	PC04-03	4-nitroquinoline oxide	No documented interaction	Known to have antifungal activity (Mukhopadhyay, 2004)
4	PC04-04	Methotrexate	The highest rate of synergy was obtained for the combination of terbinafine and methotrexate, which exhibited synergy against Aspergillus spp. No interaction was detected for the combinations of methotrexate plus itraconazole or amphotericin B(Yang et al.,2009)	methotrexate is active against staphylococcus aureus strains(Kruszewska et al, 2000)
5	PC04-05	Sulfometuron Methyl	No documented interaction	Known to have antifungal activity (Kingsbury et al., 2004)
6	PC04-06	1,10-phenanthroline	No documented interraction	Known to have antifungal activity (Mukhopadhyay, 2004)
7	PC04-07	Rifampicin	Rifampin and amphotericin B were found to be synergistic in vitro against Saccharomyces cerevisiae, Histoplasma capsulatum, and several species of Aspergillus, The uptake of rifampin by the fungi was increased in the presence of amphotericin B (Medoff, 1983)	Active against H. capsulatum, Blastomyces dermatitidis, and Aspergillus species in vitro
8	PC04-08	Bromperidol	Increases susceptibility of Mtb in combination with rifampicin (Ramón-García et al., 2011)	no inherent antimicrobial properties found
9	PC04-09	Thioridazine	Active against extensively drug resistant tuberculosis when added to other second- and third-line antibiotics (de Keijzer et al., 2016)	Active against intracellular antibiotic-sensitive and drug resistant *M. tuberculosis*
10	PC04-10	Chlorpromazine	synergistic with isoniazid, streptomycin, pyrazinamide, rifampin, rifabutin and penicillin against intramacrophage M. tuberculosis (Crowle et al., 1992)	Bacteriostatic for Gram negative bacteria and bactericidal for Gram positive bacteria
11	PC04-11	Trifluoperazine	Not much is known about its effects with other antibiotics	Inhibits ATP synthesis in *Mycobacterium leprae*
12	PC04-12	Triflupromazine	Not much is known about its effects with other antibiotics	Active against single and multidrug resistant tuberculosis
13	PC04-13	Trimeperazine	Trimeprazine is synergistic with trimethoprim against drug resistant bacteria (Dastidar et al., 2013)	Not much is known about its inherent antimicrobial properties
14	PC04-14	Flupenthixol	Augment its antimicrobial activity when combined with streptomycin, gentamicin, ciprofloxacin, and the non-antibiotic methdilazine against salmonella sp (Jeyaseeli et al., 2011. It is also found to be synergistic with chlorpromazine against drug resistant bacteria.	Not much is known about its inherent antimicrobial properties
15	PC04-15	Hydroxyurea	No known literature	Induction of OH radical cell death in E. coli (Davies *et al.*, 2009); Caused reduction in DNA synthesis in *Staphylococcus epidermidis* and *Micrococcus lysodeikticus (Feiner et al., 1973)*
16	PC04-16	Deferasirox	Reported to synergize with Ciprofloxacin against Vibrio vulnificus (Neupane and Kim, 2009) and vancomycin against MRSA *in vivo*, *in vitro* (Luo *et al.*, 2015)	Inhibition of growth of *Vibrio vulnificus in vitro* (Neupane and Kim, 2009)
17	PC04-17	Deferoxamine	Enhanced the activities of cephalothin, gentamicin, vancomycin, and fusidic acid (and in the presence of vitamin C) against *S. aureus*. (Hartzen *et al.*, 2009)	Showed bacteriostatic activity against species of Staphylococcus (Hartzen *et al.*, 2009; Van Asbeck *et al.*, 1983)
18	PC04-18	Morphine	No known literature	No known literature
19	PC04-19	Tramadol	No known literature	Against S. epidermis and E. coli at 25mg/ml (Tamanai-Shacoori *et al.*, 2007)
20	PC04-20	Pethidine	No known literature	Known antibacterial activity (Grimmond and Brownridge, 1986)
21	PC04-21	Ibuprofen	Synergistic in action with cefuroxime and chloramphenicol against multidrug-resistant MRSA (Chan et al., 2017)	3.20 mg/ml MIC against E. coli (Laudy et al., 2016); 6.25mg/ml MIC against S. aureus (Obad et al., 2015)
22	PC04-22	Paracetamol	Showed indifferent interactions with Amoxicillin and clasvulanic acid and Erythromycin against sensitive pneumococcal strains (Ponte et al., 2003)	MIC of 2.5mg/ml against E. coli and 1.25mg/ml against S. aureus (Al-Janabi, 2010).
23	PC04-23	Praziquantel	praziquantel increased the AUC of albendazole (Pawluk et al., 2015).	No known activity
24	PC04-24	Albendazole	Cimetidine increased the elimination half-life of albendazole and maximum concentration (Cmax) of mebendazole; dexamethasone increased the area under the plasma concentration-time curve (AUC) of albendazole; levamisole decreased the Cmax of albendazole; anticonvulsants (phenytoin, phenobarbital, carbamazepine) decreased the AUC of albendazole, and ritonavir decreased the AUC of both albendazole (Pawluk et al., 2015).	No known activity

2.4.5 Natural products in tuberculosis treatment

Natural products have historically played significant roles in the drug discovery process and have become one of the treatment options for infectious diseases. According to Cragg and Newman, (2005), 80% of the world's population largely depends on natural products for disease treatment and 60% of the conventional drugs currently in use were derived from natural products.

Fungi and plants have been found to be rich sources of secondary metabolites. These secondary metabolites production are linked to biosynthetic gene clusters required for adaptive stress response in their microenvironments (Pusztahelyi *et al*., 2015). According to Cheng *et al*., (2012), secondary metabolites isolated from the endophytic fungus *Biscogniauxia formosana* possess potent antimycobacterial activities. In addition, the compound 3-Nitropropionic acid, isolated from the extracts of endophytic fungi, possesses potent anti-mycobacterial activity (Chomcheon *et al*., 2005). Findings by Wang *at al*., (2013), shows two compounds, 4-deoxybostrycin and nigrosporin, isolated from the fungus *Nigrospora sp.* with better anti-mycobacterial activities than the first line TB drugs used to treat multidrug resistant tuberculosis. Three alkaloid compounds, gliotoxin, helvolic acid and 12,13-dihydroxy-fumitremorgin C extracted from cultures of a deep-sea-derived fungus *Aspergillus* sp. SCSIO Ind09F01, were shown to possess potent anti-mycobacterial activity against *Mycobacterium tuberculosis* (Luo *et al*., 2017).

These findings present the potential for promising anti-mycobacterial agents of fungal origin which can further stimulate the search for new bioactive compounds that would be useful for drug development against tuberculosis.

CHAPTER THREE

MATERIALS AND METHODS

3.1.0 Bacterial strains: *Mycobacterium smegmatis* mc^2 155 (obtained from ETH-Zurich, Switzerland) and erythromycin-derived drug-resistant *M. smegmatis* A *(*Ery *M. smeg* A*)* and erythromycin resistant *M. smegmatis* B (Ery *M. smeg* B) possessing multi-drug resistant phenotype. Erythromycin-resistant *M. smegmatis* A and B are laboratory generated drug-resistant strains (Arthur *et al*., 2019*)*, obtained from the Laboratory for Chemical Systems Biology of Infectious Pathogens at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon.

3.1.1 Standard TB/antibiotic drugs: Ampicillin, Amoxicillin, Vancomycin, Isoniazid, Ethambutol, Ethionamide, Pyrazinamide, Moxifloxacin, Rifampicin, Linezolid, Tetracycline, Chloramphenicol, Erythromycin, Streptomycin, Cycloserine, Metronidazole, Gentamycin, Paramomycin, 5-fluorouracil and Clindamycin were all obtained from Sigma Aldrich.

3.1.2 Phenotypic compounds: Benomyl, estradiol, 4-nitroquinoline oxide, methotrexate, sulfometuron methyl, rifampicin, bromperidol, Chlorpromazine, thioridazine, flupenthixol, trimeprazine, trifluoperazine, triflupromazine, Hydroxyurea, deferrasirox, deferoxamine, Morphine, tramadol, pethidine, ibuprofen, paracetamol, praziquantel, albendazole and 1, 10 phenanthroline were all obtained from Sigma Aldrich.

3.1.3 Media preparation: According to manufacturer's instructions, Middlebrook 7H9 (M 7H9) broth base was supplemented with 0.085% (w/v) NaCl, 0.44% (v/v) glycerol and 0.25% (v/v) Tween 80 to prepare 7H9 liquid broth.

Middlebrook 7H10 (M 7H10) agar base was supplemented with 0.085% (w/v) NaCl and 0.5% (w/v) dextrose to prepare agar media for culturing mycobacterial cells. (See appendix A 1.0 – 1.3 for components of 7H9/ 7H10 and detailed preparation of M 7H9/ M 7H10).

3.1.4 Growth conditions: As described by Arthur *et al*. (2019), *M. smegmatis* and erythromycin resistant *M. smegmatis* A and B were streaked on 7H10 agar media and incubated for 24 hr at room temperature. The stock of inoculum was used to inoculate 50 ml 7H9 broth. After 24 hr of incubation at room temperature in the incubator shaker (140 rpm), the OD_{600nm} of the mycobacterial suspension was readjusted to 0.1 by diluting with 7H9 broth. The OD_{600nm} was readjusted to OD_{600nm} of 0.1 in 50 ml 7H9 broth to prepare starter culture. After 24hrs of incubation, the OD_{600nm} was readjusted to give a working OD_{600nm} of 0.7 for the bioassay. Mycobacteria suspensions at this working OD_{600nm} of 0.7 was used to plate 7H10 media for the bioassay.

3.1.5 Preparation of compounds for bioassay: The phenotypic compounds were prepared as described by Arthur *et al*. (2019) for disc diffusion method. Discs were soaked with the respective concentrations of the compounds in a 96 well microtitre plate (Table 4.0). The plates containing soaked discs were allowed to air dry to allow solvents to evaporate.

3.1.6 Bioassay: Mycobacterial suspensions at the working OD_{600nm} (0.7) were spread on the surface of 7H10 media supplemented with NaCl and dextrose as described above (section 3.1.4). Each antibiotic fused disc was gently pressed on the surface of the inoculated agar plates as described by Arthur *et al*. (2019) for the bioassay. The cultures were kept at room temperature and the zones of inhibition were measured at 48 hr. The sensitivity of the test organisms on the plates was determined by the disc diffusion method and zones of inhibition

were measured as a halo around each disc including the diameter of the disc. The standard anti-mycobacterial agents/antibiotics (Section 3.1.1) were used as controls.

3.1.7 Measurement of Minimum Inhibitory Concentration (MIC) of antipsychotic compounds: Each antipsychotic at the following concentrations from 400µg/µL to 0.5µg/µL was tested for their intrinsic antimicrobial properties. The minimum concentration of each antipsychotic/phenotypic compound producing no zone of inhibition was used as the MIC of each compound as described by Arthur *et al*. (2019). Compounds for which MICs were not obtained over the concentration ranges above were further diluted and tested at lower concentrations from 0.5 to 0.002 µg/µL. The MICs of the compounds are provided (Table 4.0).

3.1.8 Phenotypic assay: The effects of other compounds on the activities of standard anti-mycobacterial drugs were analysed by first spreading 200 µL sub MIC concentrations of phenotype modulating compounds on 7H10 media supplemented with NaCl and dextrose. Discs containing antibiotics were gently laid on the media containing the compounds. The effects of the phenotype modulating compounds on the activity of the standard anti-mycobacterial agents/antibiotics were analysed by measuring the zones of inhibition. Plates which were not modified with phenotypic compounds were used as controls. The lists of antipsychotic compounds and other phenotype modifying compounds used in the study are listed (Table 4.0).

3.1.9 Analyses of resistance breaking and resistance inducing effects

Unique resistance breaking and resistance inducing interactions from the pairwise drug-drug interactions were selected. Resistance breaking interactions were considered as those drug-drug interactions producing increase in zones of inhibition ≥ 4mm compared with the control. Drug-drug interactions producing reduction in zones of inhibition of ≥ 4mm compared with the control were considered as resistance inducing interactions.

3.2.0 Fungal extracts: Archive ethylacetate extracts of 201 SBF and 306 TEF were obtained from the laboratory for Chemical Systems Biology of Infectious Pathogens, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon. As described by Arthur *et al*. (2019), discs were soaked with the ethylacetate extracts 201 SBF and 306 TEF and prepared for preliminary screening of the extracts and extracts against *M. smegmatis* mc^2 155, Erythromycin-resistant M. *smegmatis* A (Ery *M. smeg* A*)*

3.2.1 Collection of Soil Borne Fungi (SBF) and Terrestrial Endophytic Fungi (TEF)

Archival fungal isolates of 201 SBF and 306 TEF were obtained from the Laboratory for Chemical Systems Biology of Infectious Pathogens, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon-Accra. These archival fungal isolates have been kept at + 4°C. Fungal isolates of SBF and TEF whose extracts generated high bioactivities against *M. smegmatis* and erythromycin-resistant *M. smegmatis* A were selected for inoculation. The colony morphology and appearance of each isolate on YPDA plates was rechecked to confirm their identity.

3.2.2 Fermentation of fungal cultures SBF and TEF

Yeast peptone dextrose agar (YPDA) plates were inoculated with 31 archival fungal isolates of SBF and TEF which generated highly active extracts in the preliminary screen (Figure 6.1 and 6.2). The plate cultures were used to prepare expansion cultures of four YPDA plates per isolate to obtain high fungal load for large culture fermentation. The 2L YMPD cultures were incubated at room temperature with daily swirling to allow aeration and distribution of nutrients for 3 months. Fermentation reactions in 2L cultures were quenched with 200 mL ethyl acetate after 3 months (see Table 4.6) to be extracted using ethylacetate.

3.3 Uptake and efflux analysis in *M. smegmatis* mc2 155, erythromycin-resistant *M. smegmatis* A and erythromycin-resistant *M. smegmatis B*

Effects of phenotypic compounds on the accumulation and efflux activity in the test organisms were carried out as described by Rodrigues *et al.* (2008). The procedure is summarised as follows; overnight cultures of mycobacteria were adjusted to an OD of 0.8. The culture was centrifuged at 13 000 rpm for 3 min and the pellet was washed twice in PBS. The OD was finally adjusted to 0.4 and ethidium bromide was added at a final concentration of 3 ug/mL.

To investigate the effects of the phenotypic compounds on the accumulation of ethidium bromide, 990 uL of ethidium bromide (3 ug/mL) loaded cells in PBS (without glucose and verapamil) at an OD 0.4 were set up. The phenotypic compounds at a final concentration of 0.001 ug/uL were added to each reaction tube. Verapamil at this final concentration was also added to one of the tubes as a positive control and compound free culture loaded with ethidium bromide was set up the negative control. From each reaction mixture, 100 uL was pipetted into 96 well microtitre plates and fluorescence (Excitation wavelength 530 nm and emission 585 nm) was measured at 0, 15, 30, 60 and 120 min using a Varioskan plate reader.

To investigate the effects of the phenotypic compounds on efflux, 0.4% glucose was added to each of the reaction tubes to stimulate efflux activity. The fluorescence of the ethidium bromide accumulated cells was measured at excitation and emission wavelengths of 530 nm and 585 nm respectively. Each phenotypic compound was added at a final concentration of 0.001 ug/uL. Tubes that did not receive any phenotypic compounds were used as negative controls whereas tubes containing verapamil served as positive controls. From each reaction mixture, 100 uL was pipetted into 96 well microtitre plates and the fluorescence was measured at 0, 15, 30, 60 and 120 min.

3.4 Biofilm inhibition and disruption assay

Biofilm inhibition and disruption assay was performed as described by Sandberg *et al.* (2008). The conditions for biofilm formation were optimized to mycobacterial starting OD of 0.5 with incubation at room temperature and shaking at 60 rpm. Overnight cultures of *M. smegmatis* mc^2 155, erythromycin-resistant M. *smegmatis* A (Ery *M. smeg* A) and erythromycin-resistant *M. smegmatis* B (Ery M. smeg B) were adjusted to an OD of 0.5.

To investigate the inhibitory effects of the phenotypic compounds on biofilm formation in the test organisms, 990 uL of the mycobacterial suspension (OD 0.5) was aliquoted into 2 mL microfuge tubes. To each mycobacterial suspension, 10 uL (final concentration 0.001 ug/uL) of the phenotypic compound was added. From each reaction mixture, 200 uL was pipetted into a 96 well microtitre plate and the plate was incubated at room temperature with orbital shaking at 60 rpm. After 72 hr incubation with shaking, the cultures in the microtitre plates were washed with PBS and the plate was air dried. To each well, 20 uL crystal violet was added and the plate was incubated at room temperature for 15 min. Each well was washed twice with distilled water to remove unbound cells and 95% ethanol was added to dissolve the crystal violet. The inhibitory effect of each compound on the biofilm formation was determined by measuring absorbance at 595 nm in the Varioaskan plate reader.

The ability of each compound to disrupt already formed biofilm in the test organisms was carried. The procedure is summarised as follows; 200 uL of mycobacterial suspension (OD 0.5) was pippeted into each well in a 96 well microtitre plate. After 72 hr of incubation at room temperature with orbital shaking at 60 rpm, each plate was washed twice and 200 uL of 7H9 broth containing 0.001 ug/uL of each phenotypic compound was added to their respective wells in the microtitre plate. The reaction mixture was incubated for 2 hr with shaking at 60 rpm.

Each well was washed twice with PBS and 20 uL crystal violet was added. The plate was incubated at room temperature for 15 min. Each well was washed twice with distilled water and 95% ethanol was added to solubilise the crystal violet. The inhibitory effect of each compound on the biofilm formation was determined by measuring absorbance at 595 nm using the Varioskan plate reader.

CHAPTER FOUR

RESULTS

4.1.0 Resistance breaking and resistance inducing effects between antipsychotics and anti-TB agents/antibiotics

In a phenotypic array based screening assay involving pairwise combinations of antipsychotics with standard anti-mycobacterial agents, unique interactions producing resistance breaking effects were identified. Resistance breaking effects were defined as increase in zones of inhibition of 4mm or more relative to the control. Conversely resistance inducing effects were defined as reduction in the zones of inhibition of 4 mm or more compared with the corresponding control plate. The minimum inhibitory concentrations (MICs) of antipsychotic compounds and other phenotypic compounds were determined (Table 4.0). Pairwise drug combinations between phenotypic compounds at their sub-MICs (Table 4.0) and standard anti-mycobacterial agents /antibiotics were carried out. The MICs of five modulators of fungal MDR/CDR gene expression, 4-nitroquinoline oxide, benomyl, methotrexate, rifampicin and 1,10-phenanthroline were relatively smaller compared with the other phenotypic compounds (Table 4.0).

Table 4.0: Minimum inhibitory concentration (MIC) table for 24 phenotype modifying compounds

Number	Compound code	Measured disc MIC (ug/uL)		Selected disc MIC for bioassay (ug/uL)		Plate MIC for Bioassay based on Selected Disc MIC of 20 ug for all MICs >40ug/uL		Plate MIC for Bioassay based on Selected Disc MIC of 5 ug for all MICs >5ug/uL	
		M. smeg MIC (ug/uL)	Ery M. smeg MIC (ug/uL)	M. smeg MIC (ug/uL)	Ery M. smeg MIC (ug/uL)	M smeg (ug/uL)	Ery M smeg (ug/uL)	M smeg (ug/uL)	Ery M smeg (ug/uL)
1	PC04-01	0.25	>40	0.25	5	0.03125	2.5	0.0315	0.625
2	PC04-02	>40	>40	5	5	2.5	2.5	0.625	0.625
3	PC04-03	0.5	1	0.5	1	0.0625	0.125	0.0625	0.125
4	PC04-04	0.25	20	0.25	5	0.03125	2.5	0.03125	0.625
5	PC04-05	5	1	5	1	0.625	0.125	0.625	0.125
6	PC04-06	0.125	0.125	0.125	0.125	0.015625	0.015625	0.015625	0.015625
7	PC04-07	0.002	20	0.002	5	0.00025	2.5	0.00025	0.625
8	PC04-08	>40	>40	5	5	2.5	2.5	0.625	0.625
9	PC04-09	20	20	5	5	2.5	2.5	0.625	0.625
10	PC04-10	20	10	5	5	2.5	1.25	0.625	0.625
11	PC04-11	1	1	1	1	0.125	1.25	0.125	0.125
12	PC04-12	1	10	1	5	0.125	1.25	0.125	0.625
13	PC04-13	1	10	1	5	0.125	1.25	0.125	0.625
14	PC04-14	1	10	1	5	0.125	1.25	0.125	0.625
15	PC04-15	>40	>40	5	5	2.5	2.5	0.625	0.625
16	PC04-16	>40	>40	5	5	2.5	2.5	0.625	0.625
17	PC04-17	>40	>40	5	5	2.5	2.5	0.625	0.625
18	PC04-18	>40	>40	5	5	2.5	2.5	0.625	0.625
19	PC04-19	>40	>40	5	5	2.5	2.5	0.625	0.625
20	PC04-20	>40	>40	5	5	2.5	2.5	0.625	0.625
21	PC04-21	5	>40	5	5	0.625	2.5	0.625	0.625
22	PC04-22	>40	>40	5	5	2.5	2.5	0.625	0.625
23	PC04-23	>40	20	5	5	2.5	2.5	0.625	0.625
24	PC04-24	>40	>40	5	5	2.5	2.5	0.625	0.625

All antipsychotics used in the study produced resistance breaking effects upon interacting with rifampicin (RNA polymerase inhibitor) and chloramphenicol (a protein synthesis inhibitor) (Table 4.1). With the exception of chlorpromazine, all other antipsychotics produced resistance breaking effects with the protein synthesis inhibitors tetracycline and paramomycin (Table 4.1). Trifluprомazine, bacterial metabolic energy targeting compound (Kigondu *et al.*, 2014) and flupenthixol, a potent efflux pump inhibitor (Kaatz *et al.*, 2003) enhanced the anti-mycobacterial activity of the DNA replication inhibitor, Moxifloxacin (Table 4.1). Three cell wall synthesis inhibitors, ethionamide, ethambutol and pyrazinamide did not produce any interactions with the antipsychotics (Table 4.1). The thioxanthene based antipsychotic flupenthixol caused resistance breaking effects with moxifloxacin (known to block DNA replication) (Table 4.1). Resistance to 5-fluorouracil, a thymidine synthesis inhibitor was not challenged by any of the antipsychotic compounds. Resistance breaking effects was largely identified with drug-drug interactions between antipsychotics and protein synthesis inhibitor, tetracycline and paramomycin, as well as the RNA synthesis inhibitor, rifampicin. With the exception of chlorpromazine, all antipsychotic compounds generated resistance breaking effects when combined with tetracycline and paramomycin (Table 4.1). Thioridazine generated the highest number of resistance breaking interactions (with 9 antibiotics), followed by flupenthixol (with 8 antibiotics). Trifluoperazine generated resistance breaking effects in combination with chloramphenicol (bacterial protein synthesis inhibitor) against *M. smegmatis* (Figure 4.1).

A number of resistance inducing effects arising from the drug-drug interactions between antipsychotics and standard antimycobacterial agents were identified in the phenotypic based screening. Generally few interactions produced resistance inducing effects (Table 4.1). Two bacterial efflux pump inhibitors chlorpromazine and thioridazine (Ratnakar and Murthy, 1993)

and bromperidol (mechanism not known), each in combination with ampicillin augmented resistance in *M. smegmatis* (Table 4.1). In addition, resistance inducing effects were identified in a pairwise combination involving chlorpromazine, triflupromazine, trimeprazine and the antibiotic erythromycin. Chlorpromazine which targets the NADH:menaquinone oxidoreductase in the electron transport chain of mycobacteria (Weinstein *et al*., 2005) produced resistance inducing effects upon interacting with three inhibitors ampicillin (a cell wall synthesis inhibitor), vancomycin (a cell wall synthesis inhibitor), erythromycin (a protein synthesis inhibitor and the DNA replication inhibitor moxifloxacin as shown in table 4.1. The three ion channel blockers, Chlorpromazine, thioridazine and bromperidol induced resistance in pairwise combinations with ampicillin, a cell wall disrupting antibiotic (Table 4.1). Chlorpromazine in combination with the DNA replication inhibitor moxifloxacin generated resistance inducing effects (Table 4.1).

Table 4.1: Drug-drug interactions between antipsychotics and anti-mycobacterial agents against *M. smegmatis*

Standard antibiotics		Antipsychotic compounds						
		Zones of inhibition for drug-drug interactions between anti-psychotic compounds and anti-mycobacterial agents (mm)						
Control Antibiotics	Zones of inhibition for Control (mm)	Chlorpromazine	Trifluoperazine	Thioridazine	Bromperidol	Triflupromazine	Flupenthixol	Trimeprazine
Amp 14	9	0	13	0	0	9	10	9
Amx 14	0	0	0	18	10	0	0	0
Van 14	10	0	40	35	41	13	29	19
INH 10	0	0	0	0	0	0	0	0
Emb 4	0	0	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0	0	0
Moxi 0.2	12	0	14	11	11	14	16	0
Rif 4	15	25	28	26	31	24	24	22
Lin 1	0	0	0	16	0	0	13	0
Tet 8	17	15	28	19	30	25	26	21
Chlo 14	0	14	11	12	18	9	10	11
Ery 14	19	0	19	24	19	14	17	13
Strep 10	0	8	0	18	11	0	14	0
Cyser 20	0	0	0	0	0	0	0	0
Met 10	0	0	0	0	0	0	0	0
Gen 4	10	11	11	10	12	10	11	9
Para 8	8	7	20	19	20	18	14	14
5-Fu 0.4	0	0	0	0	0	0	0	0
Clind 10	0	0	6	13	0	0	0	0

Key : Resistance breaking interactions; Resistance inducing interactions; Neutral interactions

Resistance breaking interactions refer to drug combinations that result in increase in zones of inhibition ≥ 4 mm with respect to the corresponding control plate.
Resistance inducing interactions refer to drug combinations that result in a decrease in zones ≥ 4 mm with respect to the control plate.
Neutral interactions refer to drug combinations where the increase or decrease in zones of inhibition with respect to the control is < 4 mm.
Amp 14 (Ampicillin, 14 µg/µL), Amx 14 (Amoxicillin, 14 µg/µL), INH 10 (Isoniazid, 10 µg/µL), Emb 4 (Ethambutol, 4 µg/µL), Eth 18 (Ethionamide, 18 µg/µL), PZD 40 (Pyrazinamide, 40 µg/µL), Moxi 0.2 (Moxifloxacin, 0.2 µg/µL), Rif 4 (Rifampicin 4 µg/µL), Lin 1 (Linezolid 1 µg/µL), Tet 8 (Tetracycline, 8 µg/µL), Chlo 14 (Chloramphenicol, 14 µg/µL), Ery 14 (Erythromycin, 14 µg/µL), Strep 10 (Streptomycin, 10µg/µL), Cyser 20 (Cycloserine, 20 µg/µL), Met 10 (Metronidazole, 10 µg/µL), Gen 4 (Gentamycin, 4 µg/µL), Para 8 (Paramomycin, 8 µg/µL), 5-Fu 0.4 (5-Fluorouracil, 0.4 µg/µL), Clind 10 (Clindamycin 10 µg/µL).

4.1.1 Resistance breaking and inducing scenarios between selected antipsychotics and anti-TB/antibiotics

Figure 4.1 shows typical resistance breaking and resistance inducing effects from drug-drug combinations involving antipsychotics and selected anti-TB/antibiotics. Trifluoperazine, a calcium binding antipsychotic compound and an inhibitor of bacteria energy metabolism, generated resistance breaking effects when combined with the protein synthesis inhibitor, chloramphenicol (Figure 4.1 A). Resistance breaking interactions similarly occurred between vancomycin, a cell wall synthesis inhibitor, and trifluoperazine (Figure 4.1 A). Neutral interactions were observed especially with interactions involving the antibiotics amoxicillin, linezolid, ethambutol, pyrazinamide and cycloserine, and the antipsychotic, trifluoperazine (Figure 4.1A).

Chlorpromazine, the other phenothiazine counterpart, generated resistance inducing effects when combined with the DNA replication inhibitor, moxifloxacin (Figure 4.1 B). In addition, erythromycin (a protein synthesis inhibitor) and ampicillin (inhibitor of cell wall synthesis) induced resistance effects in drug combination with chlorpromazine (Figure 4.1 B). Rifampicin, the DNA-dependent RNA synthesis inhibitor, generated resistance breaking effects in drug-drug interactions with chlorpromazine (Figure 4.1B). In addition, streptomycin, a protein synthesis inhibitor, generated resistance breaking interactions in pairwise combination with chlorpromazine (Figure 4.1 B). Neutral interactions occurred with drug combinations involving the antipsychotic, chlorpromazine, and the antibiotics linezolid, pyrazinamide, cycloserine and tetracycline (Figure 4.1 B).

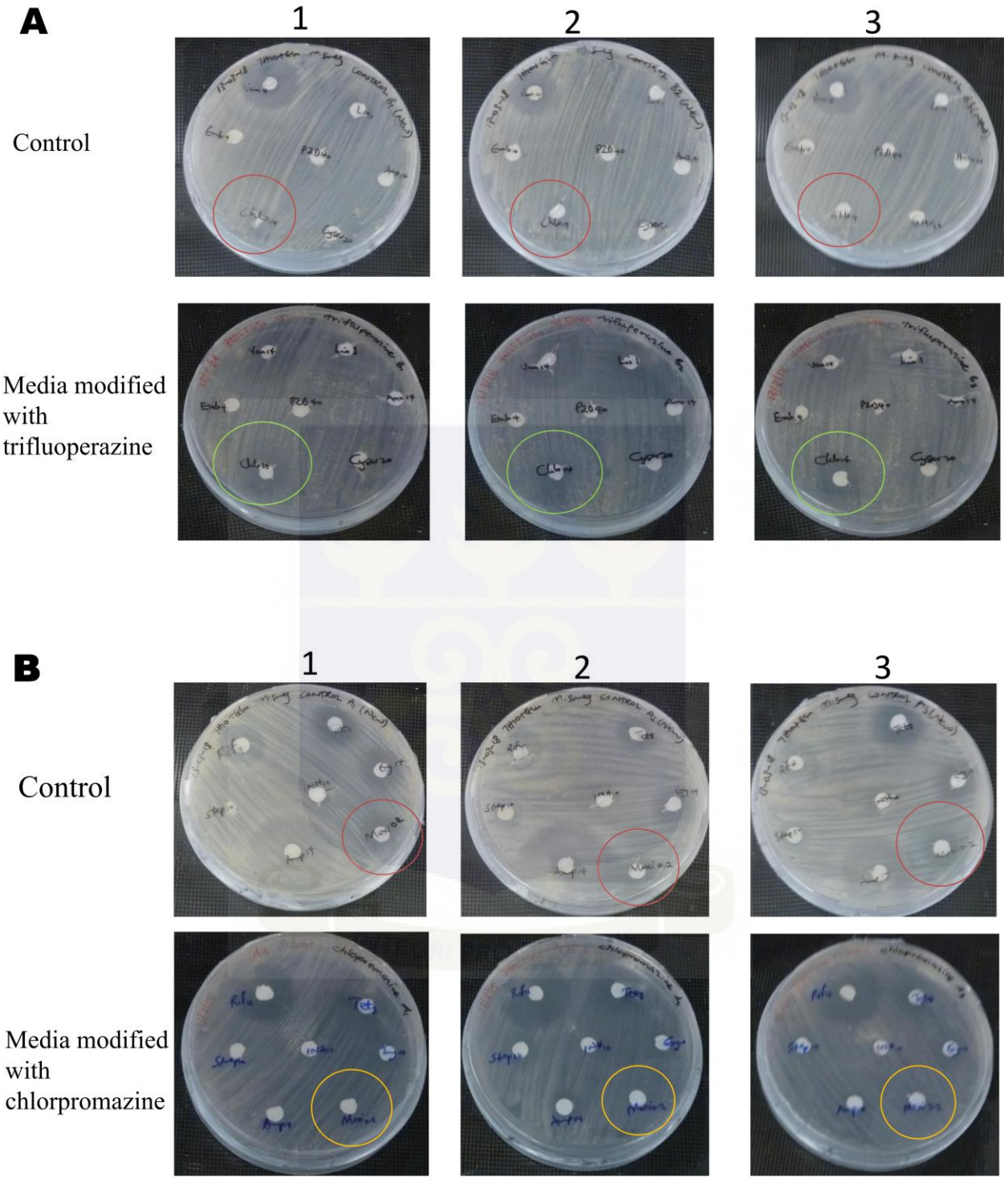

Figure 4.1: Bioassay plates showing (A) Resistance breaking (B) Resistance inducing interactions against *M. smegmatis*. Trifluoperazine in combination with chloramphenicol (chlo circled in green) generated resistance breaking effects and this is evident as increase in zones of inhibition (mm) compared with the control (Figure 4.1A).
Chlorpromazine in combination with moxifloxacin (Moxi circled in yellow) produced resistance inducing effects (reduction in zones of inhibition) (Figure 4.1B). Drug pattern on unmodified agar media were used as control. Each drug combination was carried out in triplicates and the average was used for analysis.

4.1.2 Resistance breaking and resistance inducing effects between modulators of fungal multidrug resistance efflux pump and anti-TB agents/antibiotics

A panel of phenotype modulating compounds was tested for their ability to induce resistance breaking effects in pairwise combination with standard anti-mycobacterial drugs. Notably, compound PC04-03 (4-nitroquinoline oxide) produced resistance breaking effects with cycloserine (cell wall synthesis inhibitor) and metronidazole (a DNA synthesis inhibitor), an effect which was not observed for the antipsychotics-standard TB drug pairwise combinations. To a larger extent, compound 4-nitroquinoline oxide produced resistance breaking effects with all antibiotics (Table 4.2). Outstanding among the resistance breaking effects is drug-drug interactions involving compound PC04-03 (4-nitroquinoline oxide) and pyrazinamide (known to disrupt membrane transport and energetics) (Zhang *et al.*, 2003). Next to 4-nitroquinoline oxide, the RNA synthesis inhibitor, rifampicin, induced resistance breaking effects with 14 antibiotics with different antimicrobial profiles; cell wall synthesis inhibitors, protein synthesis inhibitors and DNA synthesis inhibitors (Table 4.2). A similar effect occurred with the fungal MDR/CDR efflux modulator, sulfometuron, which caused resistance breaking effects with 12 antibiotics possessing diverse mechanisms of antimicrobial action (Table 4.2). Also, 1,10-phenanthroline exhibited resistance breaking effects with 8 antibiotics belonging to cell wall synthesis inhibitors and protein synthesis inhibitors (Table 4.2). However, resistance inducing effects occurred when 1,10-phenanthroline was combined with the DNA synthesis inhibitor, moxifloxacin. Reducing inducing effects also occurred when rifampicin, estradiol and 1, 10-phenathroline were each combined with ampicillin, a cell wall synthesis inhibitor. Three antibiotics, ethambutol, ethionamide and pyrazinamide in combination with the phenotypic compounds, rifampicin, methotrexate, sulfometuron, estradiol and 1, 10-phenanthroline generated resistance inducing effects (Table 4.2).

Table 4.2: Drug-drug interactions between phenotypic compounds and anti-mycobacterial agents against *M. smegmatis*

Standard antibiotics		Phenotypic compounds					
Control Antibiotics	Zones of inhibition for Control (mm)	Zones of inhibition for drug-drug interactions between phenotype modifying compounds and anti-mycobacterial agents (mm)					
		PC04-07	PC04-06	PC04-04	PC04-05	PC04-03	PC04-02
Amp 14	9	0	0	24	27	19	0
Amx 14	0	20	13	0	17	33	12
Van 14	10	40	39	24	40	34	34
INH 10	0	22	0	0	0	21	0
Emb 4	0	0	0	0	0	27	0
Eth 18	0	0	0	0	0	29	0
PZD 40	0	0	0	0	0	31	0
Moxi 0.2	12	27	8	21	0	31	13
Rif 4	15	19	22	24	32	29	25
Lin 1	0	22	13	8	9	27	16
Tet 8	17	40	17	17	31	39	27
Chlo 14	0	29	14	15	15	34	9
Ery 14	19	30	15	17	22	26	16
Strep 10	0	0	0	11	0	35	0
Cyser 20	0	0	0	0	0	32	0
Met 10	0	9	0	0	0	35	0
Gen 4	10	20	20	11	21	30	11
Para 8	8	23	17	8	19	21	15
5-Fu 0.4	0	12	0	0	15	28	0
Clind 10	0	18	17	0	19	34	0

Key : Resistance breaking interactions Resistance inducing interactions Neutral interactions

Resistance breaking interactions refer to drug combinations that result in increase in zones of inhibition ≥ 4 mm with respect to the corresponding control plate.
Resistance inducing interactions were considered for drug combinations that result in a decrease in zones ≥ 4 mm with respect to the control plate.
Neutral interactions refer to drug combinations where the increase or decrease in zones of inhibition with respect to the control is < 4 mm.
Amp 14 (Ampicillin, 14 µg/µL), Amx 14 (Amoxicillin, 14 µg/µL), INH 10 (Isoniazid, 10 µg/µL), Emb 4 (Ethambutol, 4 µg/µL), Eth 18 (Ethionamide, 18 µg/µL), PZD 40 (Pyrazinamide, 40 µg/µL), Moxi 0.2 (Moxifloxacin, 0.2 µg/µL), Rif 4 (Rifampicin 4 µg/µL), Lin 1 (Linezolid 1 µg/µL), Tet 8 (Tetracycline, 8 µg/µL), Chlo 14 (Chloramphenicol, 14 µg/µL), Ery 14 (Erythromycin, 14 µg/µL), Strep 10 (Streptomycin, 10µg/µL), Cyser 20 (Cycloserine, 20 µg/µL), Met 10 (Metronidazole, 10 µg/µL), Gen 4 (Gentamycin, 4 µg/µL), Para 8 (Paramomycin, 8 µg/µL), 5-Fu 0.4 (5-Fluorouracil, 0.4 µg/µL), Clind 10 (Clindamycin 10 µg/µL).

4.1.3 Resistance breaking effects between selected sickle cell management drugs and anti-TB/antibiotics

A number of sickle cell management drugs were tested for their ability to induce resistance breaking or inducing effects in combination with anti-mycobacterial agents and antibiotics (Table 4.3). Pethidine in pairwise drug combinations caused resistance breaking effects with 9 antibiotics whose antimicrobial profiles are different (Table 4.3). Deferasirox generated resistance breaking effects with 8 mechanistically different antibiotics (Table 4.3) whereas its iron chelating counterpart deferoxamine generated resistance breaking effects with 6 antibiotics (Table 4.3). Praziquantel, an antischistosomal drug, produced resistance breaking effects only with 7 antibiotics with different antimicrobial profiles (Table 4.4). The sickle cell management compounds used in the study produced resistance breaking effects when paired with rifampicin (RNA synthesis inhibitor), tetracycline (protein synthesis inhihitor) and paramomycin (protein synthesis inhibitor) (Table 4.3). The compounds deferasirox, deferoxamine, tramadol, pethidine and ibuprofen induced resistance to the protein synthesis inhibitor, erythromycin (Table 4.4). Tramadol and Ibuprofen in combination with ampicillin, vancomycin and erythromycin generated resistance inducing effects (Table 4.3). Deferoxamine, an iron chelator, generated resistance inducing interactions when paired with the antibiotics ampicillin, moxifloxacin and erythromycin. Neutral interactions were occurred when the antibiotics ethambutol, ethionamide pyrazinamide, cycloserine, metronidazole and gentamycin produced neutral were each paired with 5 sickle cell management drugs (Table 4.3).

Table 4.3: Drug-drug interactions between phenotypic compounds and anti-mycobacterial agents against *M. smegmatis*

Standard antibiotics		Phenotypic compounds				
Control Antibiotics	Zones of inhibition for Control (mm)	Zones of inhibition for drug-drug interactions between phenotype modifying compounds and anti-mycobacterial agents (mm)				
		PC04-17	PC04-16	PC04-20	PC04-19	PC04-21
Amp 14	9	0	19	11	0	0
Amx 14	0	0	0	0	0	0
Van 14	10	27	21	21	0	0
INH 10	0	0	0	0	8	0
Emb 4	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0
Moxi 0.2	12	8	18	15	11	16
Rif 4	15	25	25	26	22	22
Lin 1	0	0	14	14	0	0
Tet 8	17	21	33	26	21	25
Chlo 14	0	13	0	14	11	11
Ery 14	19	9	14	13	15	13
Strep 10	0	0	0	0	12	16
Cyser 20	0	0	0	0	0	0
Met 10	0	0	0	0	0	0
Gen 4	10	11	9	12	8	11
Para 8	8	19	17	14	12	17
5-Fu 0.4	0	0	0	11	0	0
Clind 10	0	7	9	10	0	0

Key : Resistance breaking interactions Resistance inducing interactions Neutral interactions

Resistance breaking interactions refer to drug combinations that result in increase in zones of inhibition ≥ 4 mm with respect to the corresponding control plate.
Resistance inducing interactions refer to drug combinations that result in a decrease in zones ≥ 4 mm with respect to the control plate.
Neutral interactions refer to drug combinations where the increase or decrease with respect to the control is < 4 mm.
Amp 14 (Ampicillin, 14 µg/µL), Amx 14 (Amoxicillin, 14 µg/µL), INH 10 (Isoniazid, 10 µg/µL), Emb 4 (Ethambutol, 4 µg/µL), Eth 18 (Ethionamide, 18 µg/µL), PZD 40 (Pyrazinamide, 40 µg/µL), Moxi 0.2 (Moxifloxacin, 0.2 µg/µL), Rif 4 (Rifampicin 4 µg/µL), Lin 1 (Linezolid 1 µg/µL), Tet 8 (Tetracycline, 8 µg/µL), Chlo 14 (Chloramphenicol, 14 µg/µL), Ery 14 (Erythromycin, 14 µg/µL), Strep 10 (Streptomycin, 10µg/µL), Cyser 20 (Cycloserine, 20 µg/µL), Met 10 (Metronidazole, 10 µg/µL), Gen 4 (Gentamycin, 4 µg/µL), Para 8 (Paramomycin, 8 µg/µL), 5-Fu 0.4 (5-Fluorouracil, 0.4 µg/µL), Clind 10 (Clindamycin 10 µg/µL).

4.1.4 Resistance breaking and inducing interactions generated by phenotypic compounds and anti-TB agents/antibiotics

Other phenotypic compounds were tested for their ability to generate resistance breaking or resistance inducing effects in *M. smegmatis* (Table 4.4). on unmodified plates were used as control. The compounds benomyl, paracetamol and albendazole induced resistance when each was paired with the cell wall synthesis inhibitor vancomycin (Table 4.4). Compounds tramadol and ibuprofen induced resistance when each was combined with ampicillin (cell wall synthesis inhibitor), vancomycin (cell wall synthesis inhibitor) and erythromycin (protein synthesis inhibitor). The fungal multidrug resistance (MDR) efflux modulator, benomyl, and two sickle cell management drugs, paracetamol and hydroxyurea, induced resistance when combined with ampicillin. In addition benomyl induced resistance when paired with the ampicillin, vancomycin, tetracycline and erythromycin (Table 4.4). However, benomyl generated few resistance breaking interactions when paired with rifampicin, chloramphenicol and streptomycin. Praziquantel, an antischistosomal drug, generated resistance breaking interactions when paired with 8 antibiotics that are mechanistically diverse (Cell wall synthesis inhibitors, DNA synthesis inhibitors, RNA synthesis inhibitors and protein synthesis inhibitors) (Table 4.4). Whilst praziquantel generated resistance breaking effects with ampicillin and vancomycin, benomyl and paracetamol generated resistance inducing effects when paired with the same antibiotics. The phenotype modifying compounds benomyl, paracetomol, albendazole, praziquantel and hydroxyurea generated resistance breaking effects when combined with the DNA-dependent RNA synthesis inhibitor, rifampicin. Neutral interactions occurred when these compounds paired with isoniazid, ethambutol, ethionamide, pyrazinamide, cycloserine and metronidazole (Table 4.4).

Table 4.4: Drug-drug interactions between phenotypic compounds and anti-mycobacterial agents against *M. smegmatis*

Standard antibiotics		Phenotypic compounds				
Control Antibiotics	Zones of inhibition for Control (mm)	Zones of inhibition for drug-drug interactions between phenotype modifying compounds and anti-mycobacterial agents (mm)				
		PC04-01	PC04-22	PC04-24	PC04-23	PC04-15
Amp 14	9	0	0	8	15	0
Amx 14	0	0	0	0	0	0
Van 14	10	0	0	0	22	32
INH 10	0	0	0	0	0	0
Emb 4	0	0	0	0	0	0
Eth 18	0	0	0	0	0	0
PZD 40	0	0	0	0	0	0
Moxi 0.2	12	12	11	14	18	14
Rif 4	15	25	26	26	23	22
Lin 1	0	0	11	0	0	19
Tet 8	17	12	29	18	22	26
Chlo 14	0	14	11	11	0	13
Ery 14	19	10	27	16	17	14
Strep 10	0	17	0	16	13	0
Cyser 20	0	0	0	0	0	0
Met 10	0	0	0	0	0	0
Gen 4	10	9	10	10	13	23
Para 8	8	8	21	15	16	15
5-Fu 0.4	0	0	0	0	0	0
Clind 10	0	0	8	0	0	10

Key : Resistance breaking interactions Resistance inducing interactions Neutral interactions

Resistance breaking interactions refer to drug combinations that result in increase in zones of inhibition ≥ 4 mm with respect to the corresponding control plate.
Resistance inducing interactions refer to drug combinations that result in a decrease in zones ≥ 4 mm with respect to the control plate.
Neutral interactions refer to drug combinations where the increase or decrease with respect to the control is < 4 mm.
Amp 14 (Ampicillin, 14 µg/µL), Amx 14 (Amoxicillin, 14 µg/µL), INH 10 (Isoniazid, 10 µg/µL), Emb 4 (Ethambutol, 4 µg/µL), Eth 18 (Ethionamide, 18 µg/µL), PZD 40 (Pyrazinamide, 40 µg/µL), Moxi 0.2 (Moxifloxacin, 0.2 µg/µL), Rif 4 (Rifampicin 4 µg/µL), Lin 1 (Linezolid 1 µg/µL), Tet 8 (Tetracycline, 8 µg/µL), Chlo 14 (Chloramphenicol, 14 µg/µL), Ery 14 (Erythromycin, 14 µg/µL), Strep 10 (Streptomycin, 10µg/µL), Cyser 20 (Cycloserine, 20 µg/µL), Met 10 (Metronidazole, 10 µg/µL), Gen 4 (Gentamycin, 4 µg/µL), Para 8 (Paramomycin, 8 µg/µL), 5-Fu 0.4 (5-Fluorouracil, 0.4 µg/µL), Clind 10 (Clindamycin 10 µg/µL).

4.2 Ranking of drug-drug interactions

The phenotypic interactions were categorized into resistance breaking and resistance inducing interactions. The pairwise drug-drug interactions were ranked based on the number of antibiotics affected and their cumulative zones of inhibitions produced by each phenotypic compound (Table 4.5.1 and 4.5.2). Resistance breaking and resistance inducing scenarios from the pairwise interactions studies were ranked based on the number of antibiotics affected by a compound in producing resistance breaking effect (Table 4.5.1 - 4.5.4). Based on the ranking (table 4.5.1), thioridazine (an antipsychotic), Hydroxyurea (PC04-15), and bromperidol (an antipsychotic) were selected for further investigations. The three phenotypic compounds namely thioridazine, hydroxyurea and bromperidol produced resistance breaking effects with 9, 8 and 7 antibiotics respectively (Table 4.5.1). Thioridazine, Hydroxyurea and bromperidol produced cumulative increase in zones of 129 mm, 100 mm and 111 mm respectively. Interactions involving (thioridazine - vancomycin), (Hydroxyurea - chloramphenicol) and (bromperidol - vancomycin) were selected for further investigations (Table 4.5.2). The pairs were selected on the basis that they were mechanistically diverse.

In the same manner, resistance inducing interactions were ranked based on the number antibiotics affected by a compound in inducing resistance (Table 4.5.3). Chlorpromazine (an antipsychotic compound), deferoxamine (sickle cell management compound, and sulfometuron (fungal phenotypic compound) produced cumulative zones of 50 mm, 23 mm and 12 mm respectively. Chlorpromazine, deferoxamine and sulfometuron were selected for further investigations. Resistance inducing interactions involving (chlorpromazine – erythromycin), (deferoxamine – ampicillin) and (sulfometuron – moxifloxacin) were validated in liquid cultures (Figure 4.2).

Table 4.5.1: Rankings of resistance breaking interactions

Phenotype modifying compound	Number of antibiotics affected	Cumulative zones for Resistance breaking interactions
4-NQ	20	495
Rifampicin	14	240
Sulphometh	10	167
Thioridazine	9	129
1,10 phenanthroline	8	112
Hydroxyurea	8	100
Pethidine	8	86
Flupenthixol	8	84
Bromperidol	7	111
Estradiol	7	88
Trifluoperazine	7	87
Methotrexate	7	81
Paracetamol	7	74
Praziquantel	7	68
Deferasirox	6	85
Deferoxamine	6	62
Morphine	6	60
Ibuprofen	6	55
Tramadol	6	46
Trimeprazine	5	37
Albendazole	4	45
Triflupromazine	4	36
Benomyl	3	41
Chlorpromazine	3	32

Table 4.5.2: Selected ranking of resistance breaking interactions

Unique Drug drug interactions	Zone of inhibition (mm) for antibiotic only	Antibiotic plus compound	Change in zone of inhibition (+ mm)
Thioridazine + Vancomycin	10	35	25
Thioridazine + Amoxicillin	0	18	18
Thioridazine + Streptomycin	0	18	18
Thioridazine + Linezolid	0	16	16
Thioridazine + Clindamycin	0	13	13
Thioridazine + Chloramphenicol	0	12	12
Thioridazine + Rifampicin	15	26	11
Thioridazine + Paramomycin	8	19	11
Thioridazine + Erythromycin	19	24	5
Hydroxyurea + Vancomycin	10	32	22
Hydroxyurea + Linezolid	0	19	19
Hydroxyurea + Chloramphenicol	0	13	13
Hydroxyurea + Gentamycin	10	23	13
Hydroxyurea + Clindamycin	0	10	10
Hydroxyurea + Tetracycline	17	26	9
Hydroxyurea + Rifampicin	15	22	7
Hydroxyurea + Paramomycin	8	15	7
Bromperidol + Vancomycin	10	41	31
Bromperidol + Chloramphenicol	0	18	18
Bromperidol + Rifampicin	15	31	16
Bromperidol + Streptomycin	0	11	11
Bromperidol + Paramomycin	8	20	12
Bromperidol + Amoxicillin	0	10	10
Bromperidol + Tetracycline	17	30	13

Table 4.5.3: Rankings of resistance inducing interactions

Compound	Number of antibiotics affected	Cumulative resistance inducing effects
Chlorpromazine	4	50
Deferoxamine	3	23
Trimeprazine	2	18
1,10 phenan	2	8
Ibuprofen	1	6
Thioridazine	1	9
Sulphomethuron	1	12
Benomyl	1	10
Morphine	1	7
Pethidine	1	6
Deferasirox	1	5
Bromperidol	1	9
Triflupromazine	1	14
Tramadol	1	4
Hydroxyurea	1	5

Table 4.5.4: Selected rankings of resistance inducing interactions

Unique Drug drug interactions	Zone of inhibition (mm) for antibiotic only	Antibiotic plus compound	Change in zone of inhibition (- mm)
Chlorpromazine + Erythromycin	19	0	-19
Chlorpromazine + Moxifloxacin	12	0	-12
Chlorpromazine + Vancomycin	10	0	-10
Chlorpromazine + Ampicillin	9	0	-9
Deferoxamine + Erythromycin	19	9	-10
Deferoxamine + Ampicillin	9	0	-9
Deferoxamine + Moxifloxacin	12	8	-4
Sulphomethuron + Moxifloxacin	12	0	-12

4.2.1 Validation of selected resistance breaking and inducing interactions

Selected resistance breaking and resistance inducing interactions were validated in liquid cultures (Figure 4.2 A – F). For resistance breaking effects (Figure 4.2 A-C), drug combinations produced growth inhibitory effects compared with drug free cultures. The growth inhibitory effects contributed by phenotypic compounds only and antibiotics only were also analysed. As expected, the drug combinations had suppressive effects on the growth of mycobacterial cells (Figure 4.2 A-C). Drug combinations involving bromperidol and vancomycin caused suppressive effects on the growth of the cells compared with the drug free media (Figure 4.2 C). Though the phenotypic compound (bromperidol) and the antibiotic (vancomycin) separately caused a decrease in the growth rate of the cells, the drug combinations (bromperidol with vancomycin) further suppressed the growth rate of the cells compared with that generated by the each compound only (Figure 4.2 C). However the other two resistance breaking interactions did not produce a similar pattern (Figure 4.2 A and B), though the drug combinations caused a suppression in the growth rate of the cells. For such interactions (Figure 4.2 A and B), a down titration of vancomycin and chloramphenicol would be required to obtain optimum antibiotic concentration for validation in liquid cultures.

Resistance inducing effects, combinations involving deferoxamine and ampicillin (Figure 4.2 E) validated previous observations with the disc diffusion method. It was observed that cells exposed to the drug combinations grew more than cells in the drug free medium. Ampicillin (cell wall synthesis inhibitor) caused a reduction in the cell growth relative to the drug-free culture and the culture treated with deferoxamine. However, combinations involving ampicillin and deferoxamine boosted the growth of the cells with time (Figure 4.2 E). This validates observations made with the disc diffusion method where combinations involving deferoxamine

and ampicillin produced zones of inhibition higher than the control plates (Table 4.5.4). The other two drug-drug combinations (chlorpromazine – erythromycin) and (sulfometuron – moxifloxacin), did not produce a similar pattern. In these scenarios, the drug combinations suppressed the growth rate of the cells compared with the control. Therefore since these combinations are toxic to the cells, further down titration of the antibiotics would be required to obtain optimum antibiotic concentration for validation in liquid cultures.

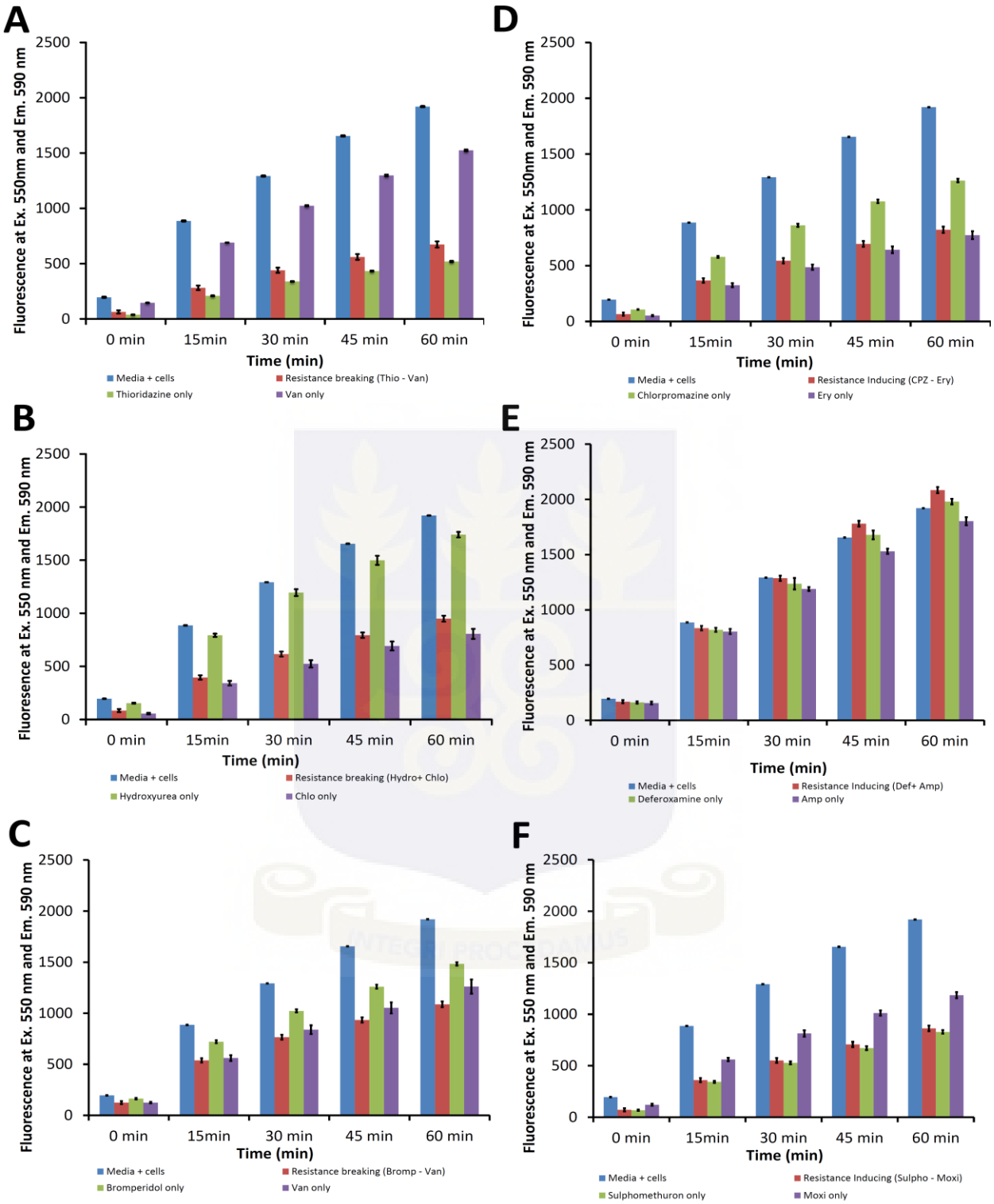

Figure 4.2 Selected resistance breaking interactions (A – C) and resistance inducing interactions (D – F)

To reaction mixtures containing drug combinations, 1% Alamar blue was added and the fluorescence measured at 15 min intervals at excitation and emission wavelengths of 550 and 590 nm respectively. The experiments were carried out in triplicates and averages were used to construct the graphs. Error bars represent standard error of the mean.

(A) Thioridazine and Vancomycin (B) Hydroxyurea and chloramphenicol and (C) Bromperidol and vancomycin against *M. smegmatis.*
Bromp- bromperidol, Van- Vancomycin, Hydro- Hydroxyurea, Chlo- chloramphenicol, Thio- thioridazine

D) chlorpromazine and erythromycin (E) Deferoxamine and ampicillin and (F) Sulfometuron and moxifloxacin against *M. smegmatis*.
CPZ-Chlorpromazine, Ery- erythromycin, Def- deferoxamine, Amp- ampicillin, Sulpho- sulfometuron, Moxi- moxifloxacin

4.3 Uptake of ethidium bromide

The 24 phenotypic compounds were tested for their ability to induce uptake of ethidium bromide in *M. smegmatis*, erythromycin-resistant *M. smegmatis A* (Ery M. smeg A), and erythromycin-resistant *M. smegmatis* B (Ery M. smeg B). Accumulation of ethidium bromide by cells is considered as an indication of efflux disruption (Danquah *et al.*, 2018). All antipsychotics caused increased uptake of ethidium bromide in *M. smegmatis* and erythromycin-resistant *M. smegmatis* B relative to the phenotypic compound free culture containing only ethidium bromide (Figure 4.3.1 A and B, 4.3.2 A and B). Notable among them, bromperidol, chlorpromazine, trifluoperazine, triflupromazine and trimeprazine caused approximately 2 fold increase in the levels of intracellular ethidium bromide compared with verapamil treated cells (Figure 4.3.1 A). The antipsychotic compounds bromperidol, thioridazine and trimeprazine promoted the accumulation of ethidium bromide in *M. smegmatis* and the two multidrug-resistant mutants (erythromycin-resistant *M. smegmatis* A and B) at levels higher than verapamil (standard efflux pump inhibitor) (Figure 4.3.1 A, 4.3.2 A and 4.3.3 A). Five modulators of fungal MDR/CDR gene expression namely; benomyl, estradiol, 4-nitroquinoline, methotrexate and sulfometuron induced accumulation ethidium bromide by approximately 2 fold relative to verapamil induced uptake in *M. smegmatis* (Figure 4.3.1 A). Two anti-schistosomal drugs, albendazole and praziquantel also caused approximately 2 fold increase in the levels of intracellular ethidium bromide compared with verapamil treated culture in *M. smegmatis* (Figure 4.3.1 B). Notably, four sickle cell management drugs hydroxyurea, deferasirox, deferoxamine and ibuprofen caused over 2 fold increase in the intracellular levels of ethidium bromide relative to ethidium bromide uptake in the presence of verapamil (Figure 4.3.1 B).

In erythromycin-resistant *M. smegmatis A* (Ery M. smeg A), all antipsychotic compounds used in the study promoted accumulation of ethidium bromide compared with culture treated with ethidium bromide only (Figure 4.3.2). Notably, the antipsychotic compounds thioridazine, chlorpromazine, bromperidol, flupenthixol and trimeprazine enhanced uptake of ethidium bromide in erythromycin-resistant *M. smegmatis* A compared to verapamil treated culture (Figure 4.3.2 A). The levels of intracellular ethidium bromide mycobacterial culture in the presence of two antipsychotic compounds (thioridazine and flupenthixol) were 1.4 fold relative to verapamil treated erythromycin-resistant *M. smegmatis* A (Figure 4.3.2 A). Benomyl (modulator of MDR/CDR gene expression in *Candida albicans*) induced 1.6 fold increase in the levels intracellular ethidium bromide relative to verapamil treated cells (Figure 4.3.2 A). Compared with verapamil, 20 compounds increased intracellular levels of ethidium bromide in erythromycin-resistant *M. smegmatis* A (Figure 4.3.2 A and B).

In erythromycin-resistant *M. smegmatis* B, bromperidol (an antipsychotic compound) caused 1.6 fold accumulation of ethidium relative verapamil treated culture (Figure 4.3.3 A). In addition, three antipsychotic compounds thioridazine, trifluoperazine and trimeprazine relatively caused approximately 1.2 fold increase in intracellular ethidium bromide relative to verapamil treated culture (Figure 4.3.3A). The DNA dependent RNA polymerase inhibitor (rifampicin), two modulators of fungal MDR/CDR gene expression (sulfometuron and benomyl) and praziquantel (anti-schistosomal drug) caused increased uptake of ethidium bromide in *M. smegmatis*, and erythromycin-resistant *M. smegmatis* A and B relative to verapamil treated culture (Figure 4.3.1 - 4.3.3). In addition, two sickle cell management drugs, deferasirox and hydroxyurea (also known to be a ribonucluotide reductase inhibitor) enhanced uptake of ethidium bromide in all three test organisms (Figure 4.3.1 – 4.3.3).

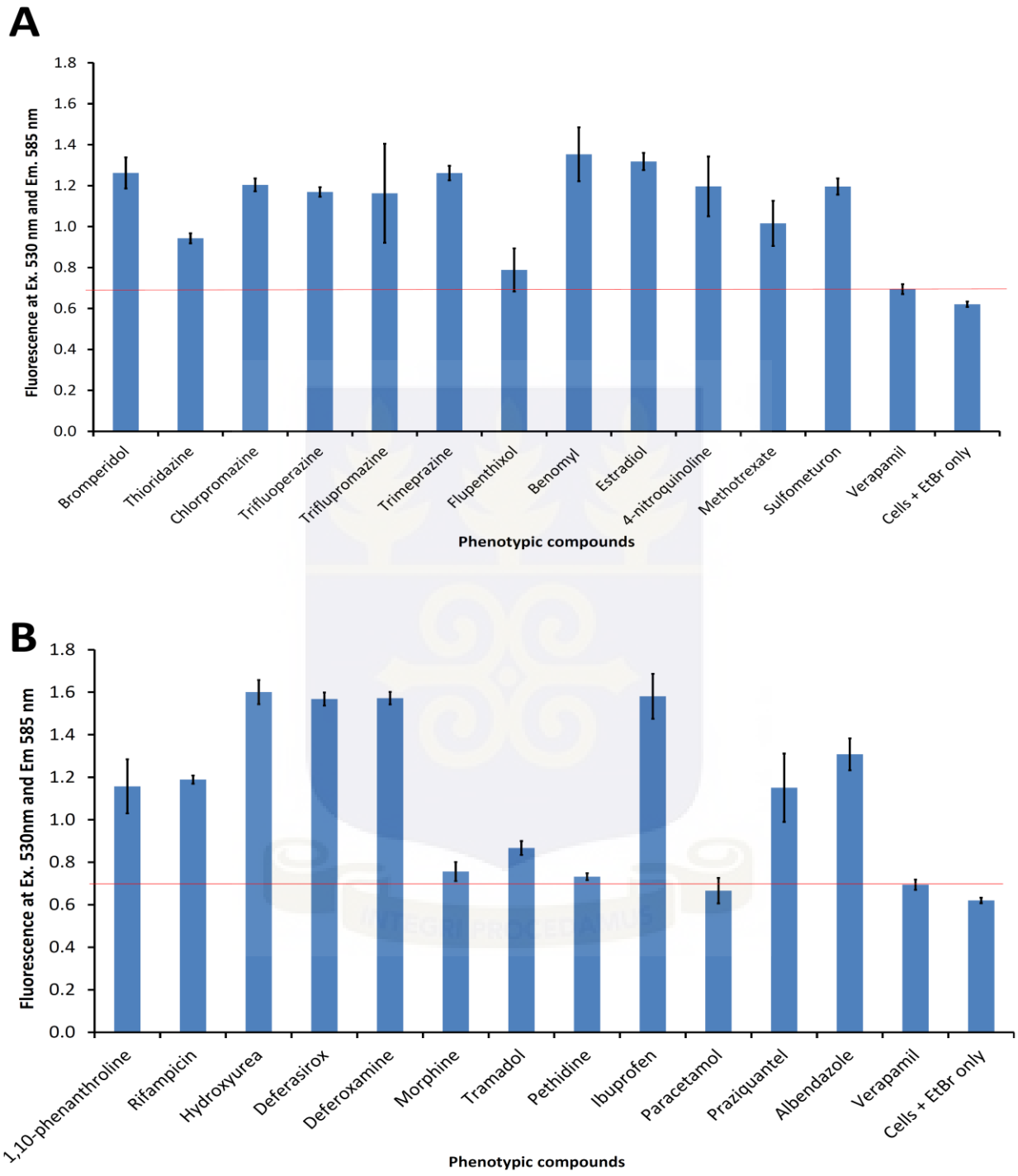

Figure 4.3.1: Effects of antipsychotics and phenotypic compounds on the uptake of ethidium bromide in *M. smegmatis*.
The phenotype modifying compounds were assessed for their ability to induce uptake of ethidium bromide in *M. smegmatis*. Fluorescence over time was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture containing ethidium bromide served as negative control. The experiment was carried out in triplicates and the graph was constructed using their averages. All phenotypic compounds used are distributed across graphs **A** and **B.** Error bars represent the standard error of the mean. The horizontal red line indicates the level of ethidium bromide uptake in verapamil treated cells (positive control) used as a cutoff to assess the effects of other phenotypic compounds.

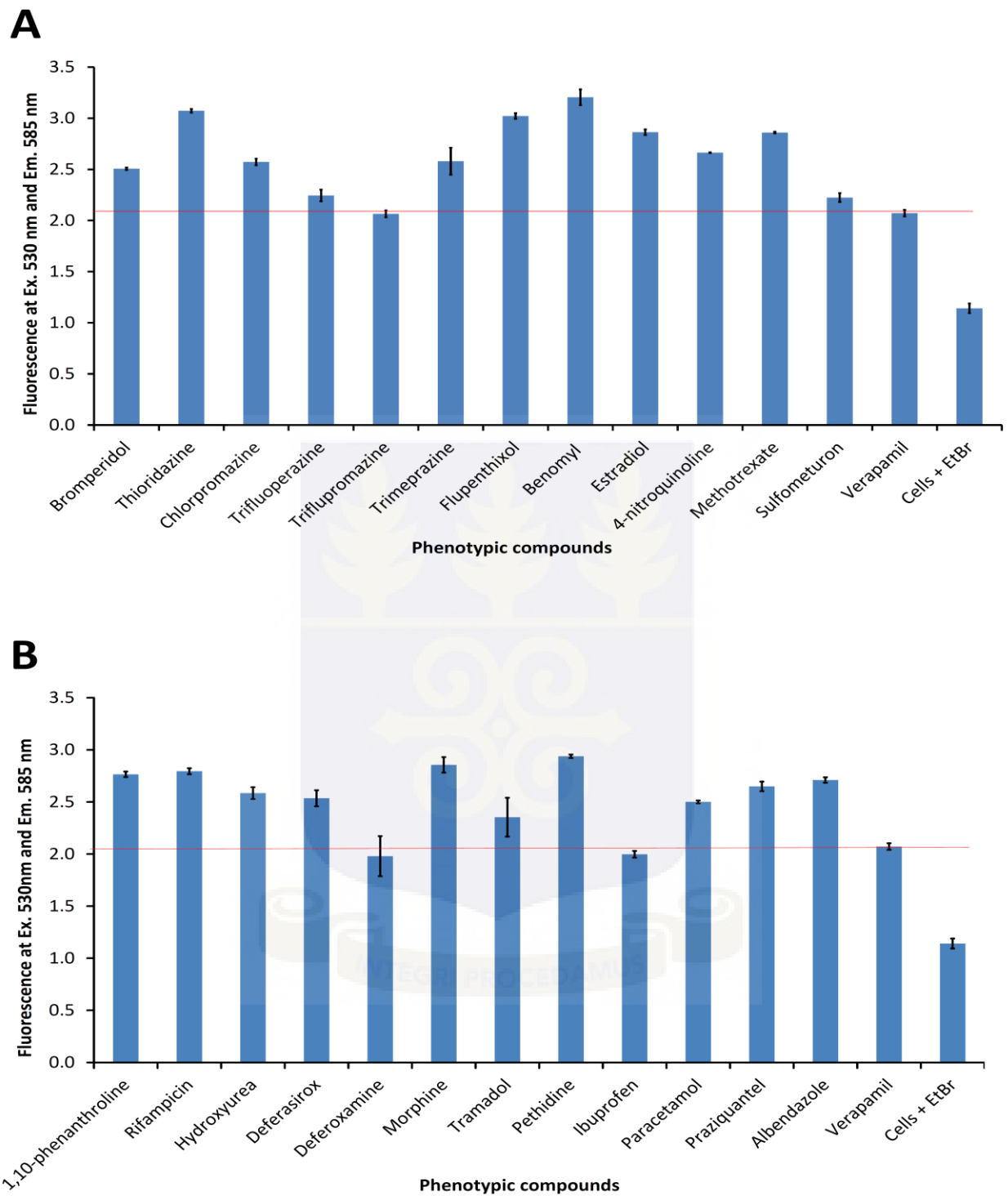

Figure 4.3.2: Effects of antipsychotic compounds and phenotypic compounds on the uptake of ethidium bromide in erythromycin-resistant *M. smegmatis* A

The phenotype modifying compounds were assessed for their ability to induce uptake of ethidium bromide in erythromycin-resistant *M. smegmatis* A. The experiment was carried out in triplicates and the graph was constructed using their averages. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture treated with ethidium bromide were used as negative control. All phenotypic compounds used were distributed across graphs 4.4 **A** and **B.** The error bars represent the standard error of the mean. The horizontal red line indicates the level of ethidium bromide uptake in verapamil treated cells (positive control) used as a cutoff to assess the effects of other phenotypic compounds.

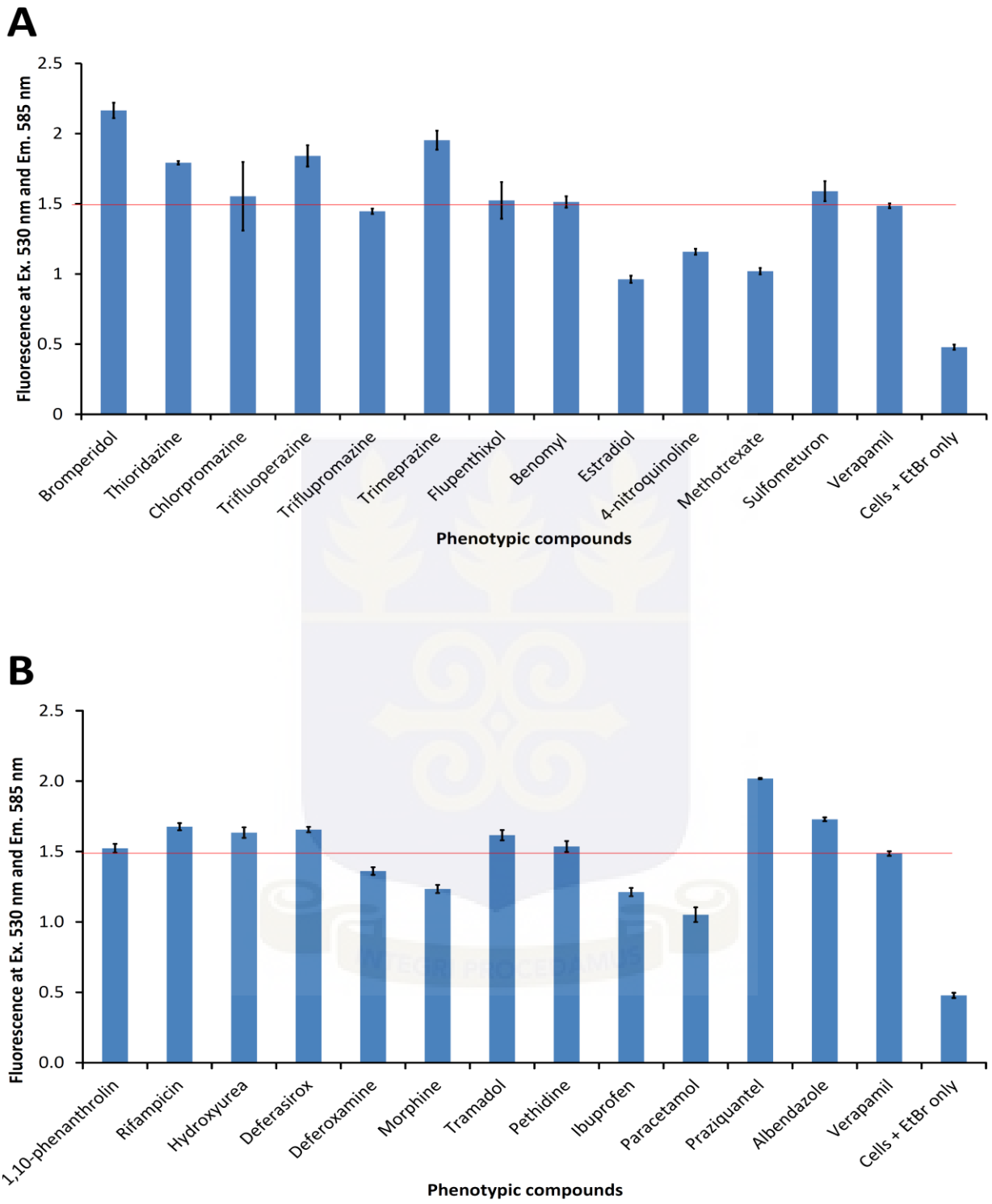

Figure 4.3.3: Effects of antipsychotics and phenotypic compounds on the uptake of ethidium bromide in erythromycin-resistant *M. smegmatis* B

The phenotype modifying compounds were assessed for their ability to induce uptake of ethidium bromide in erythromycin-resistant *M. smegmatis*. The experiment was carried out in triplicates and the graph was constructed using their averages. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture treated with ethidium bromide were used as negative control. All phenotypic compounds used were distributed across graphs **A** and **B.** The error bars represent the standard error of the mean. The horizontal red line indicates the level of ethidium bromide uptake in verapamil treated cells (positive control) used as a cutoff to assess the effects of other phenotypic compounds.

4.4 Effects of antipsychotic compounds on efflux

Three antipsychotic compounds bromperidol, chlorpromazine and thioridazine produced inhibitory effects on the efflux of ethidium bromide in *M. smegmatis* and erythromycin-resistant *M. smegmatis* A (Figure 4.3.4A and 4.3.5A). The percentage drop relative to the accumulated levels of intracellular ethidium bromide in the presence of these three antipsychotics (bromperidol, chlorpromazine and thioridazine) at 120 min were 13%, 11% and 2% respectively in *M. smegmatis* (Figure 4.3.4 A). The amount (percentage change) of ethidium bromide extruded in the presence of bromperidol, chlorpromazine and thioridazine in *M. smegmatis* were relatively smaller than that extruded in the presence of verapamil (Figure 4.3.4 A). In erythromycin-resistant *M. smegmatis* A, the amount (in percentage) of intracellular ethidium bromide effluxed at 120 min were 12%, 17% and 7% respectively (Figure 4.3.5 A), whereas the percentage drop in the presence of verapamil was 22%. Though trifluoperazine, triflupromazine and trimeprazine caused increased uptake levels of ethidium bromide in *M. smegmatis*, efflux activity increased (as evident by the sudden drop in intracellular levels of ethidium bromide) at 15 min after addition of glucose (Figure 4.3.4 A and B), followed by gradual reduction in efflux rate till 120 min. Trifluoperazine induced an uptake of ethidium bromide to levels equal to that of chlorpromazine but upon efflux stimulation, it could not inhibit efflux activity to levels comparable to chlorpromazine (Figure 4.3.4 A). However, in erythromycin-resistant *M. smegmatis* A, trifluoperazine exhibited efflux inhibition profile in a manner closely similar to the standard efflux inhibitor verapamil except that trifluoperazine promoted accumulation of ethidium than verapamil (Figure 4.3.5 A). In *M. smegmatis* the amount of ethidium bromide effluxed in the presence of thioridazine at 120 min was 11% and

whereas in erythromycin resistant *M. smegmatis* A, 17% of accumulated ethidium bromide was effluxed (Figure 4.3.4 and 4.3.5 A).

In erythromycin-resistant *M. smegmatis* A (Ery M. smeg A), the antipsychotics trimeprazine, trifluprom azine and flupenthixol inhibited efflux activity by causing a gradual loss of intracellular ethidium bromide with time (Figure 4.3.5 B) relative to verapamil treated culture (Figure 4.3.5 B). In erythromycin-resistant *M. smegmatis* A, the percentage drop in the intracellular levels of ethidium bromide at 120 min in the presence of trimeprazine and trifluprom azine were 10% and 9% respectively (Figure 4.3.5 B). In the presence of the standard efflux inhibitor verapamil, erythromycin-resistant *M. smegmatis* A effluxed 22% of intracellular ethidium bromide. However, compared with negative control (Ery M. smeg B treated with only ethidium bromide), there were efflux inhibition effects by the antipsychotic compounds against Ery M. smeg B (Figure 4.3.6 A and B). With all antipsychotic compounds, the levels of intracellular ethidium bromide dropped sharply after efflux stimulation in erythromycin-resistant *M. smegmatis* B (Figure 4.3.6). This was similarly observed in *M. smegmatis* in the presence of trifluoperazine, trifluprom azine and trimeprazine (Figure 4.3.4 A). Generally, in erythromycin-resistant *M. smegmatis B* (Ery M. smeg B), none of the antipsychotics produced relatively higher inhibitory effects on the extrusion of ethidium bromide relative to verapamil (Figure 4.3.6). However, all antipsychotics induced accumulation of ethidium bromide in erythromycin-resistant *M. smegmatis* B (Figure 4.3.6 A and B).

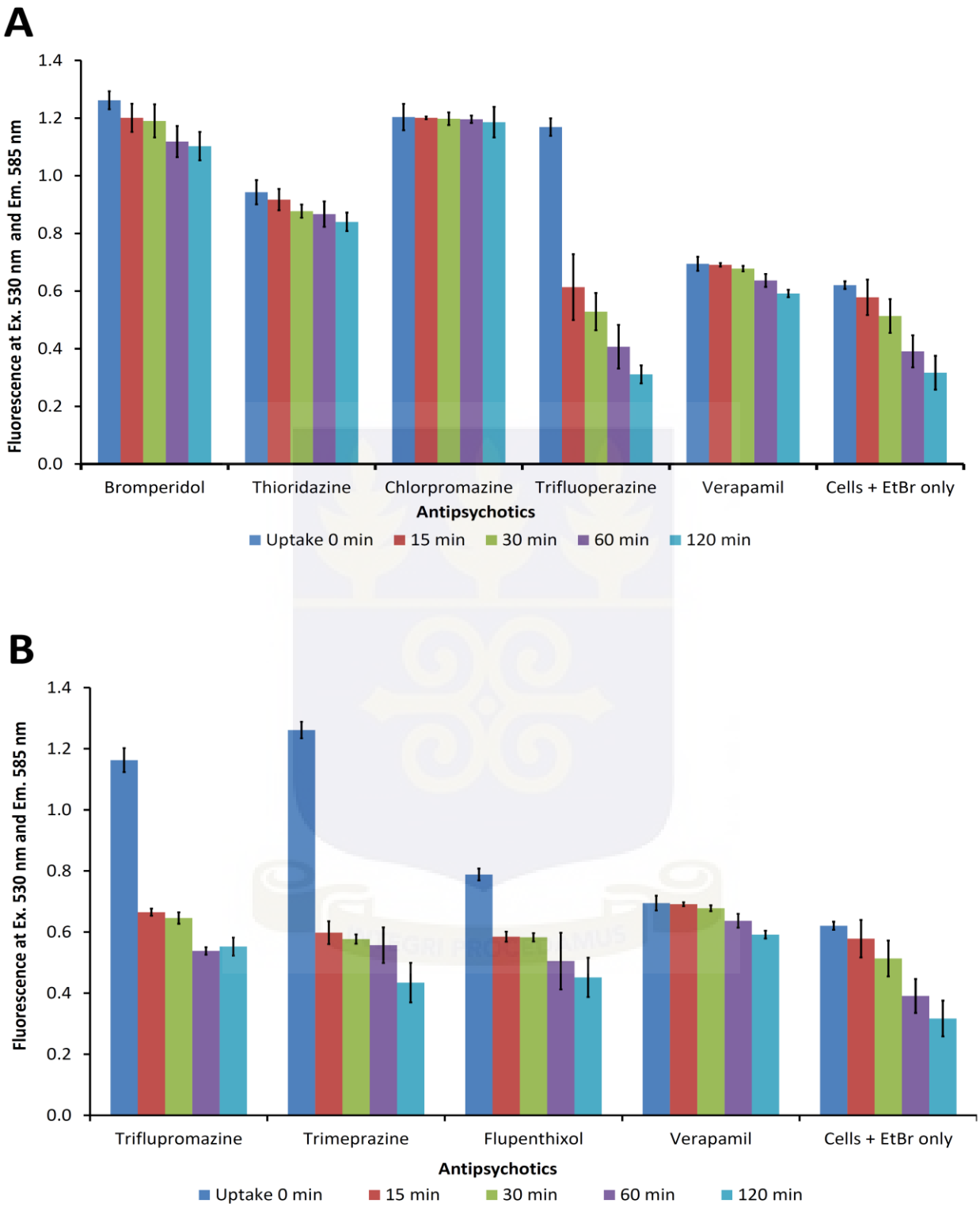

Figure 4.3.4: Effects of antipsychotics on efflux in *M. smegmatis*

Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured with time at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free mycobacterial culture treated with ethidium bromide was used as negative control. The 7 antipsychotics have been distributed across graphs **A** and **B.** The error bars measure the standard error of the mean.

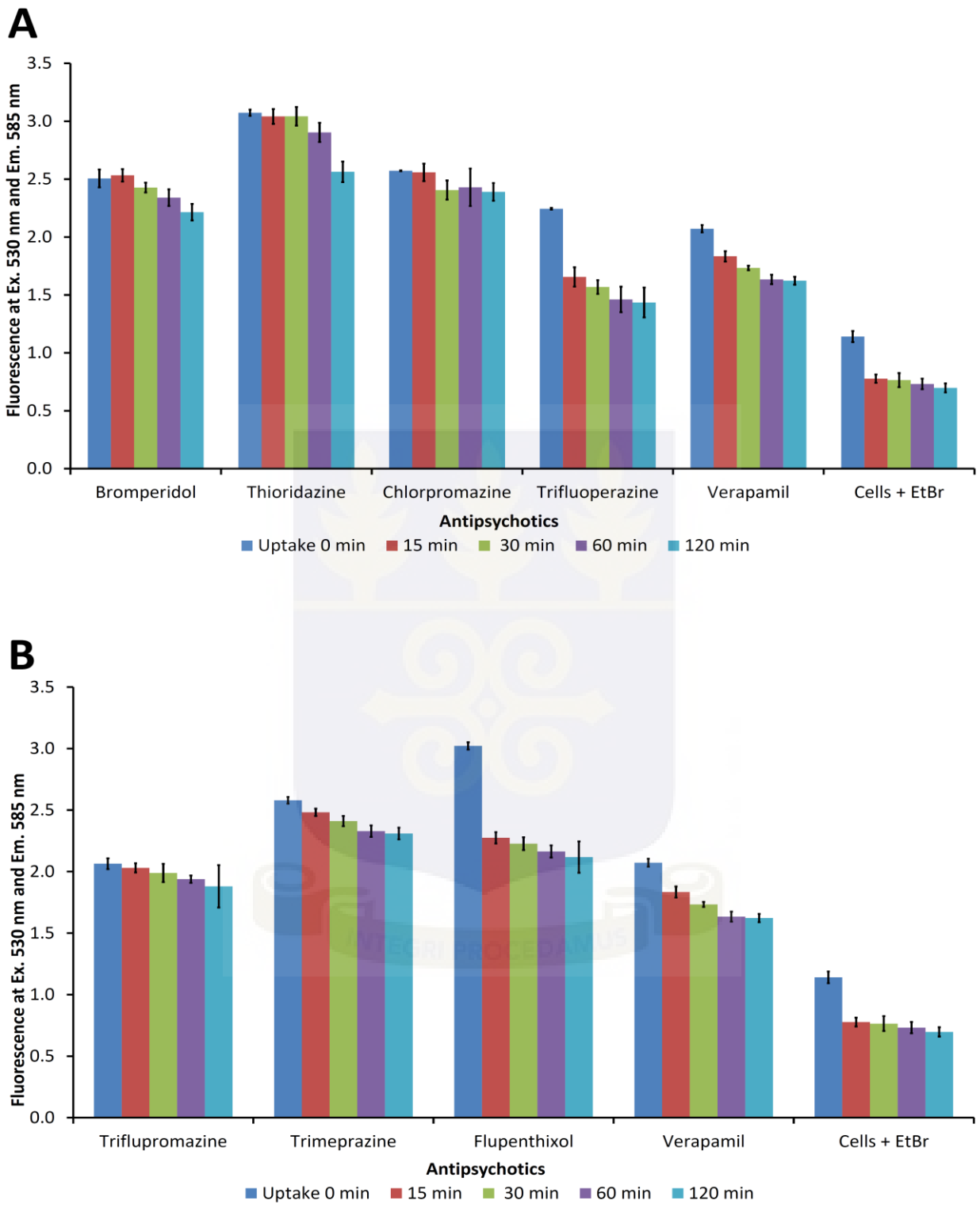

Figure 4.3.5: Effects of antipsychotic compounds on efflux in erythromycin-resistant *M. smegmatis* A.
Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured with time at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free mycobacterial culture treated with ethidium bromide was used as negative control. The 7 antipsychotics have been distributed across graphs **A** and **B.** The error bars indicate the standard error of the mean.

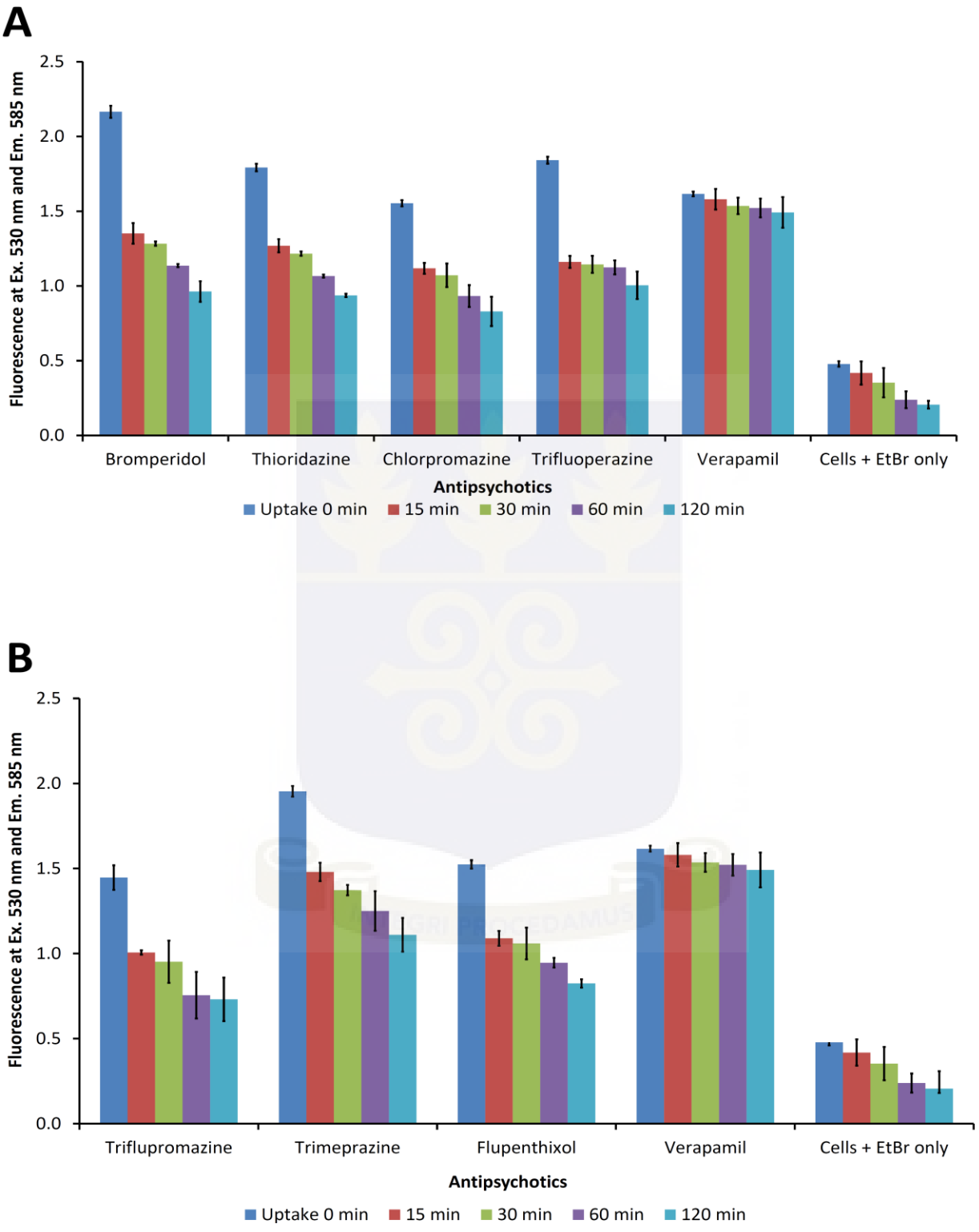

Figure 4.3.6: Effects of antipsychotics compounds on efflux in erythromycin-resistant *M. smegmatis* B
Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture containing ethidium bromide were used as negative control. The error bars represent the standard error of the mean.

4.5 Effects of phenotypic compounds on efflux activity in *M. smegmatis*

The effects of other phenotypic compounds on the rate of efflux in the test organisms were assessed. Benomyl was observed to both stimulate uptake and impede extrusion of ethidium bromide in *M. smegmatis* (Figure 4.3.7 A). In *M. smegmatis* benomyl caused 13% reduction in the intracellular levels of ethidium bromide whilst in the presence of verapamil, *M. smegmatis* extruded 15% ethidium bromide. Apart from benomyl which challenged efflux activity at levels higher than verapamil, the rest of the phenotypic compounds did not challenge extrusion of ethidium bromide after stimulation of efflux in *M. smegmatis* (Figure 4.3.7 and 4.3.8). At 60 min, the levels of intracellular ethidium bromide in the presence of 4-nitroquinoline oxide (DNA damaging agent), morphine, tramadol and pethidine were similar to the negative control (cells treated with ethidium bromide only) (Figure 4.3.7 and 4.3.8). Two DNA disrupting compounds 4-nitroquinoline oxide and methotrexate stimulated uptake of ethidium bromide in *M. smegmatis* but could not inhibit efflux (Figure 4.3.7 A). These two DNA disrupting compounds could not sustain gradual loss of intracellular ethidium bromide which is indicative of their weak propensity to inhibit efflux (Figure 4.3.7 A). Further to this, these two DNA disrupting agents inhibited sudden efflux of ethidium bromide in erythromycin-resistant *M. smegmatis* A after efflux stimulation at 15 min (Figure 5.1 A). Although estradiol is a weak efflux inhibitor in *M. smegmatis* (Figure 4.3.7), its efflux inhibitory effects is higher in erythromycin-resistant *M. smegmatis* A (Figure 5.1). The anti-schistosomal drug albendazole inhibited extrusion of ethidium bromide in erythromycin-resistant *M. smegmatis* A (Figure 5.1). Similarly, sickle cell management drugs hydroxyurea, deferasirox, deferoxamine and ibuprofen promoted accumulation ethidium in *M. smegmatis* but could not inhibit efflux at low rates comparable to standard efflux inhibitor verapamil (15%).

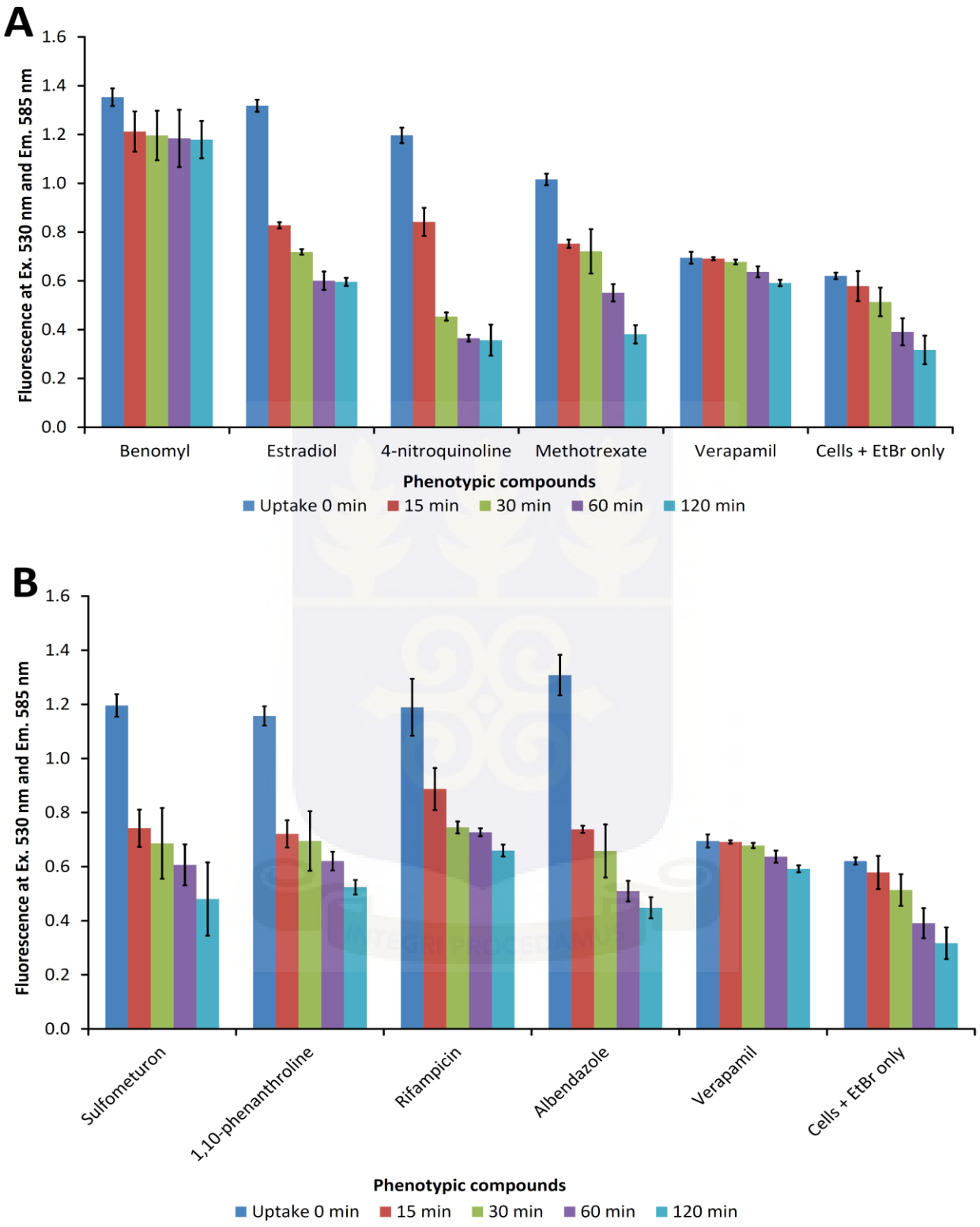

Figure 4.3.7: Effects of phenotypic compounds on efflux in *M. smegmatis*

Efflux activity was stimulated by the addition of 0.4% glucose after ethidium bromide uptake. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence over time was measured at excitation and emission wavelengths of 530 and 585 nm respectively. The phenotypic compounds were distributed across graphs **A and B.** The error bars represent the standard error of the mean.

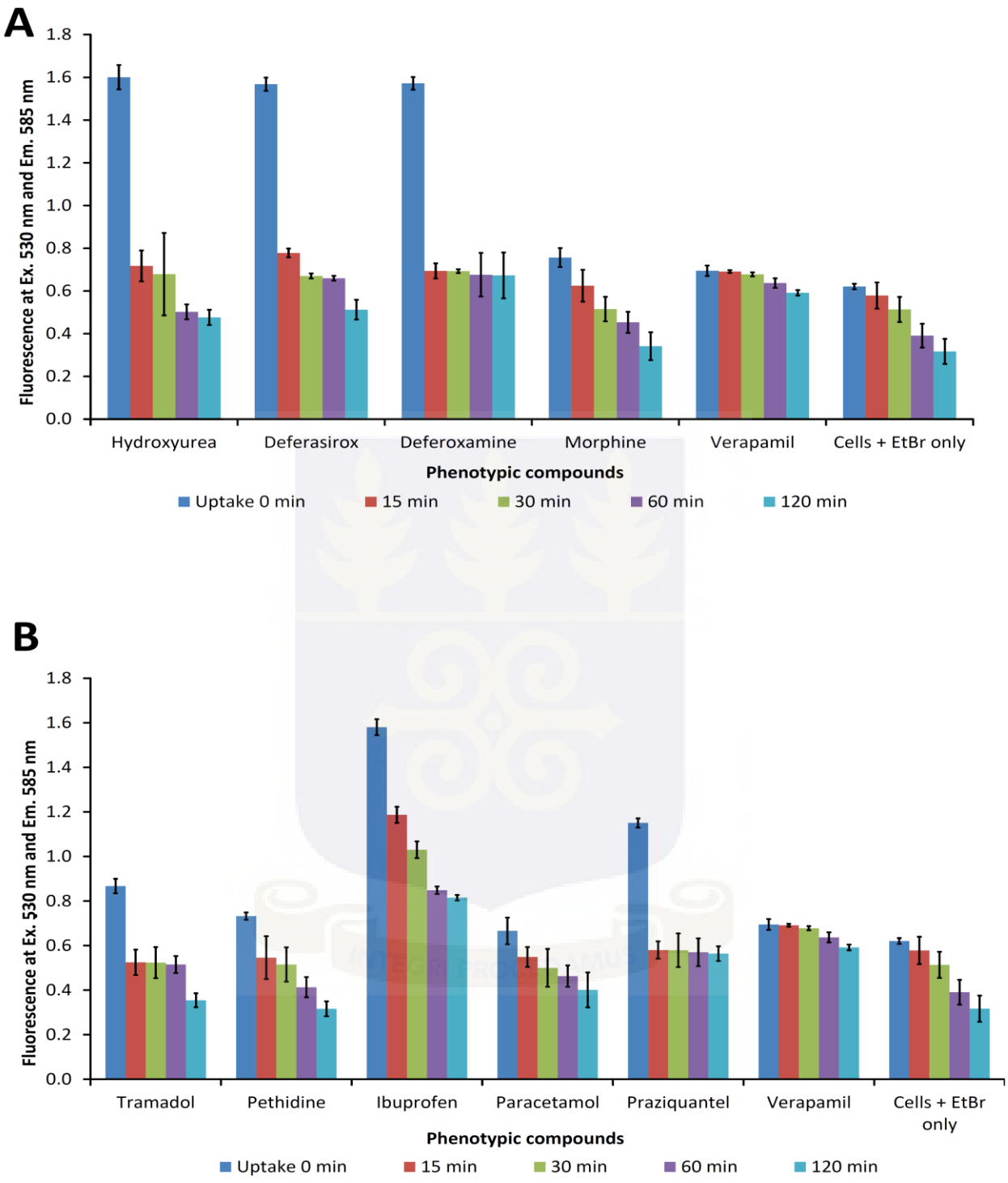

Figure 4.3.8: Effects of phenotypic compounds on efflux in *M. smegmatis*

Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. The phenotypic compounds were distributed across **A and B.** The error bars represent the standard error of the mean.

4.6 Effects of phenotypic compounds on efflux in erythromycin-resistant *M. smegmatis* A

Other phenotypic compounds were tested for their ability to inhibit efflux in erythromycin-resistant *M. smegmatis* A (Figure 4.3.9 and 4.4.0). The percentage decrease in the levels of intracellular ethidium bromide in benomyl treated cells was relatively smaller (10%) compared with that obtained with verapamil treated cells (22%) at 120 min of efflux induction. With deferasirox treated cells, percentage decrease in the intracellular levels of ethidium bromide relatively smaller (14%) compared with verapamil treated cells (22%) at 120 min of efflux stimulation (Figure 4.4.0A). With the exception of benomyl and deferasirox which caused 10% and 14% ethidium bromide respectively to be effluxed, other phenotypic compounds could not induce extrusion of relatively smaller amount of ethidium bromide compared with verapamil treated cells (Figure 4.3.9 and 4.4.0). Generally, all the phenotypic compounds induced accumulation of ethidium bromide in erythromycin-resistant *M. smegmatis* A at levels relatively higher than verapamil treated cells (Figure 4.3.9 and 4.4.0). Apart from benomyl and deferasirox, other phenotypic compounds caused a sharp drop in the intracellular levels of ethidium bromide after 15 min of efflux induction in erythromycin-resistant *M. smegmatis* A (Figure 4.3.9 A and B). It was observed that 4-nitroquinoline oxide and 1, 10 – phenanthroline generated efflux inhibitory effects against erythromycin-resistant *M. smegmatis* A by reducing the intracellular levels of ethidium bromide by 24% and 20% respectively (Figure 4.3.9 A and B). Methotrexate, estradiol, sulfometuron and rifampicin caused relatively higher reduction in intracellular levels of ethidium bromide at 120 min compared with verapamil treated cells (Figure 4.3.9 A and B). Similarly, morphine, pethidine, ibuprofen, praziquantel and paracetamol caused a higher decrease in the intracellular levels of ethidium bromide compared with verapamil treated cells (Figure 4.4.0 A and B)

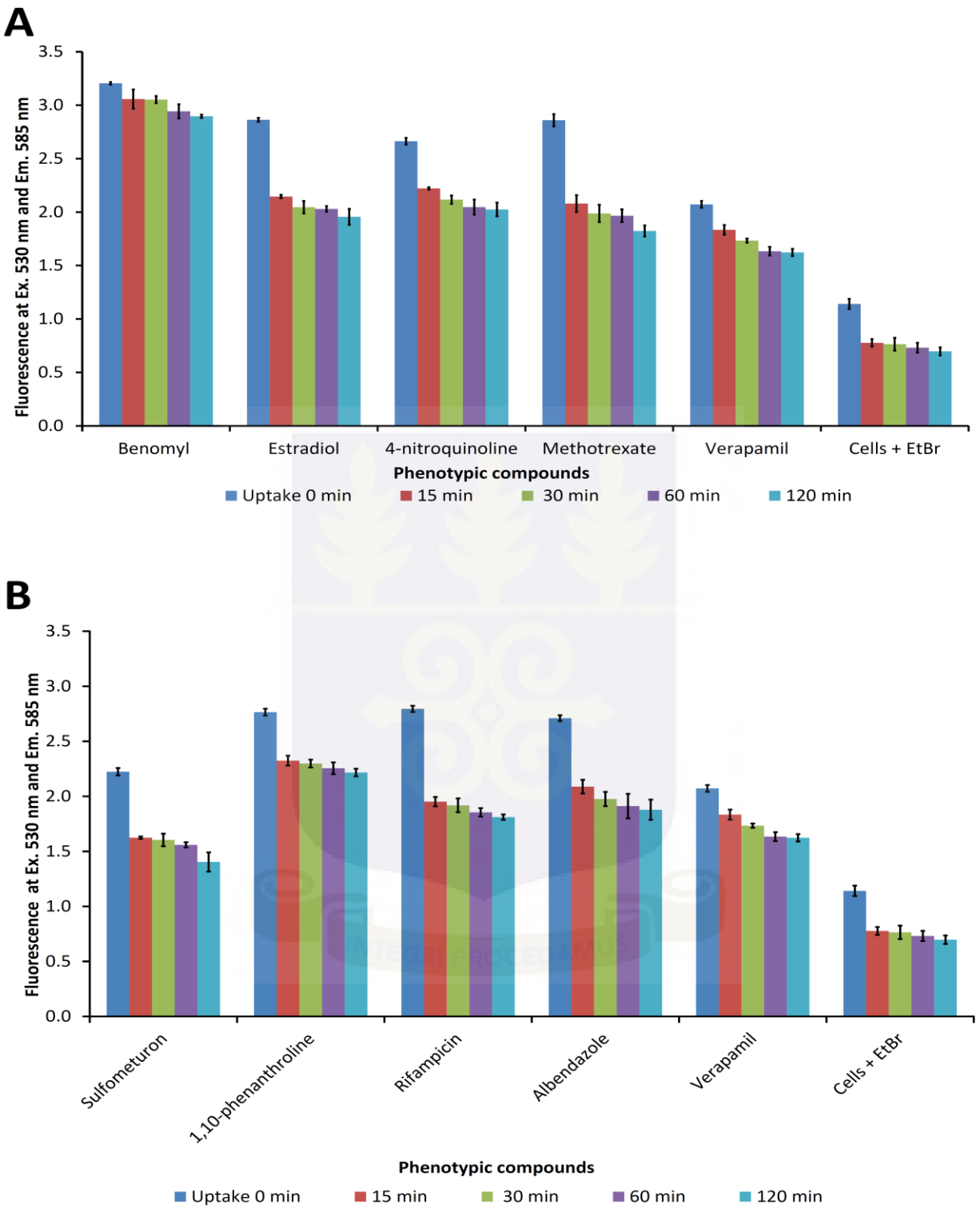

Figure 4.3.9: Effects of phenotypic compounds on efflux in erythromycin –resistant *M. smegmatis* A.
Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free mycobacterial culture containing ethidium bromide was used as negative control. The phenotypic compounds were distributed across **A and B.** The error bars represent the standard error of the mean.

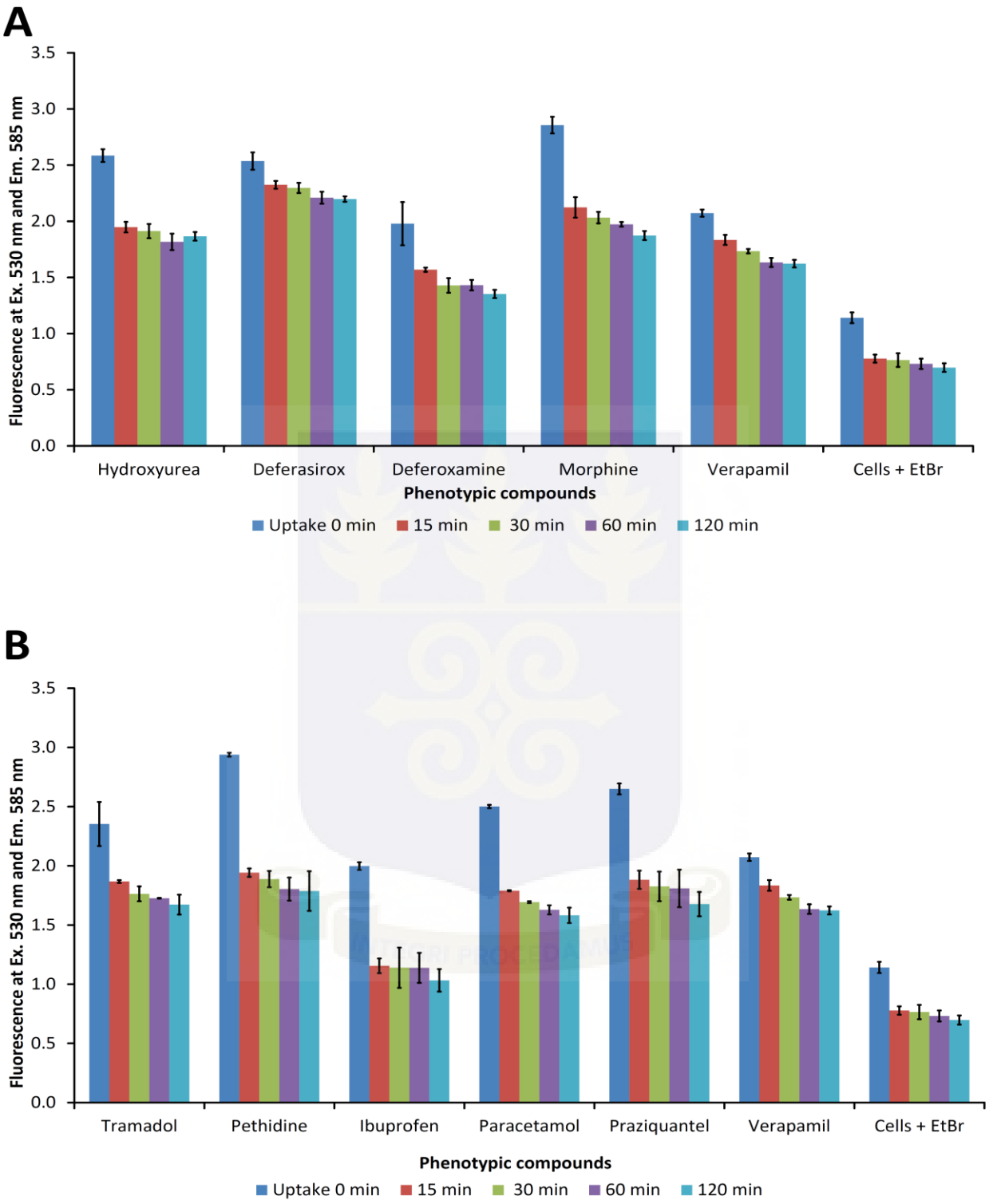

Figure 4.4.0: Effects of phenotypic compounds on efflux in erythromycin-resistant *M. smegmatis* A.
Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free culture containing ethidium bromide was used as negative control. The phenotypic compounds were distributed across **A and B.** The error bars indicate the standard error of the mean.

4.7 Effects of phenotypic compounds on efflux in erythromycin-resistant *M. smegmatis* B

The effects of other phenotypic compounds on efflux activity in erythromycin-resistant *M. smegmatis* B (possesses a relatively robust resistant background compared with erythromycin-resistant *M. smegmatis* A) were analysed (Figure 4.4.1 and 4.4.2). None of the phenotypic compounds could maintain intracellular levels of ethidium bromide after efflux induction compared with verapamil treated cells (Figure 4.4.1 and 4.4.2). Generally all phenotypic compounds induced accumulation of ethidium bromide in erythromycin-resistant *M. smegmatis* B at 0 min. However after 0 min, a sharp drop in the levels of intracellular ethidium bromide occurred till the 120 min of efflux induction (Figure 4.4.1 and 4.4.2). Compared with the negative control (untreated cells), percentage decrease in intracellular levels of ethidium bromide by the phenotypic compounds in erythromycin-resistant *M. smegmatis* B was relatively lower (Figure 4.4.1 and 4.4.2). Notable among the efflux inhibitory effects of the phenotypic compounds was that generated by morphine which caused a percentage drop of 19% in the intracellular levels of ethidium bromide at 120 min in erythromycin-resistant *M. smegmatis* B (Figure 4.4.2 A). Compared with the negative control (untreated cells), the phenotypic compounds induced accumulation of ethidium bromide in erythromycin-resistant *M. smegmatis* B, however, these compounds could not inhibit extrusion of ethidium bromide in a steady fashion (Figure 4.4.1 and 4.4.2). Benomyl, sulfometuron, 1, 10 – phenanthroline, rifampicin, albendazole, praziquantel, hydroxyurea and deferasirox induced accumulation of ethidium to levels matching that generated by verapamil (Figure 4.4.1 and 4.4.2). The percentage drop in the intracellular levels of ethidium bromide at 120 min was highest when erythromycin-resistant *M. smegmatis* B culture was treated with hydroxyurea and deferoxamine (Figure 4.4.1 A).

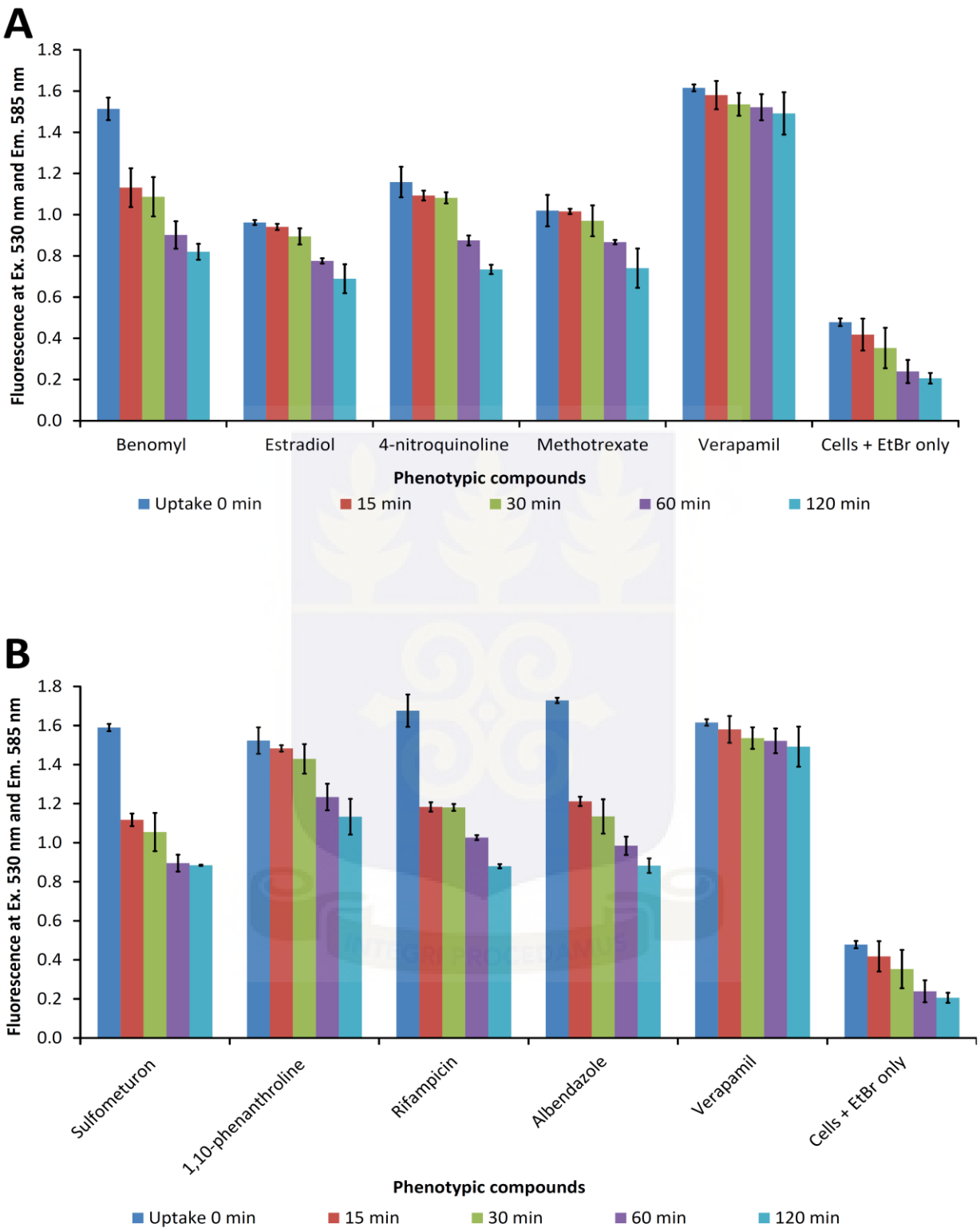

Figure 4.4.1: Effects of phenotypic compounds on efflux in erythromycin-resistant *M. smegmatis* B

Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture containing ethidium bromide were used as negative control. The error bars represent the standard error of the mean.

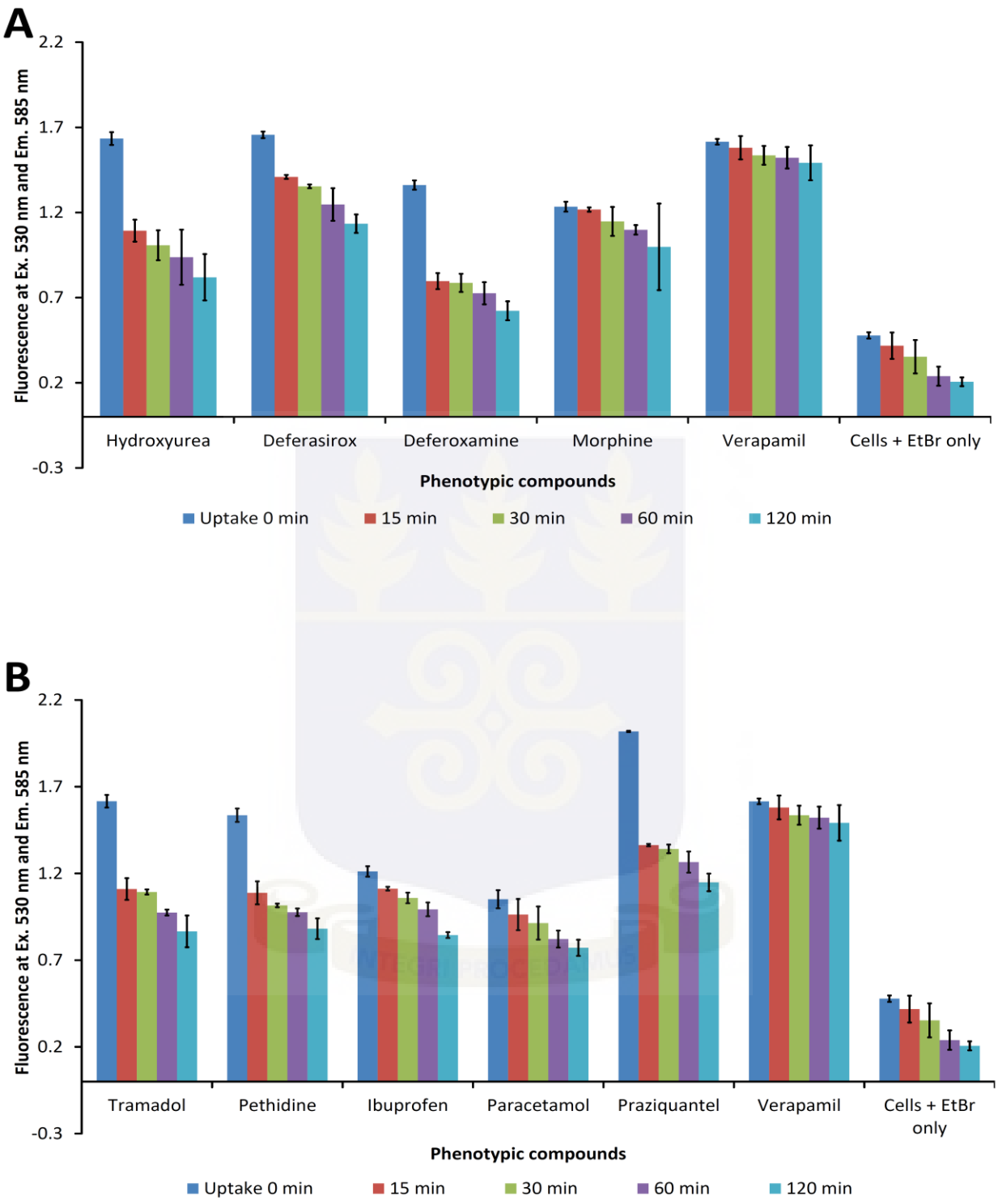

Figure 4.4.2: Effects of phenotypic compounds on efflux in erythromycin-resistant *M. smegmatis* B
Efflux activity was stimulated by the addition of 0.4% glucose after uptake of ethidium bromide. The experiment was carried out in triplicates and the average was used to construct the graphs. Fluorescence was measured at excitation and emission wavelengths of 530 and 585 nm respectively. Reaction mixture containing verapamil only were used as positive control whilst compound free liquid culture containing ethidium bromide were used as negative control. The error bars measure the standard error of the mean.

4.8.0 Biofilm inhibition and disruption by antipsychotic and other phenotypic compounds on *M. smegmatis*

Biofilm formation has been shown to contribute drug resistance in mycobacteria (Bhunu *et al.*, 2017). The effect of the phenotypic compounds on biofilm formation in *M. smegmatis*, erythromycin-resistant *M. smegmatis* A and erythromycin-resistant *M. smegmatis* B was analysed. The conditions for biofilm formation were optimized and used for the biofilm inhibition and disruption experiment (Figure 4.5.0). The optimized conditions obtained were found to be using mycobacterial culture at an OD of 0.5 and incubating them for 72 hr with orbital shaking at 60 rpm for *M. smegmatis*, erythromycin-resistant *M. smegmatis* A and erythromycin-resistant *M. smegmatis* B (Figure 4.5.0).

Biofilm formation in *M. smegmatis* was highly induced by deferoxamine, a sickle cell management drug, to eight-fold compared with compound free medium (Figure 4.5.1 B). Sulfometuron, fungal phenotype modifying compound, increased biofilm formation by two-fold relative to compound-free culture in *M. smegmatis* (Figure 4.5.1 A). However, 4-nitroquinoline oxide, methotrexate, bromperidol (butyrophenone antipsychotic compound), triflupromazine (a phenothiazine antipsychotic compound) and rifampicin inhibited biofilm formation (Figure 4.5.1 A), and partially disrupted already formed biofilm in *M. smegmatis* (4.5.2 A). Generally, with the exception of trimeprazine, none of the antipsychotic compounds induced biofilm formation in *M. smegmatis* (Figure 4.5.1 A). The two anti-schistosomal drugs praziquantel and albendazole inhibited biofilm formation and reduced the levels of already formed biofilm in *M. smegmatis* (Figure 4.5.1 B and 4.5.2 A). Two sickle cell management drugs ibuprofen and pethidine induced two-fold increase in biofilm formation in *M. smegmatis* relative to the compound free culture (Figure 4.5.1 B). Whilst deferoxamine did not disrupt biofilm formation in *M. smegmatis*, its iron chelating counterpart deferasirox disrupted already formed biofilm by

36% in *M. smegmatis* (Figure 4.5.2 B). Two phenothiazine antipsychotic compounds, thioridazine and trimeprazine, could not disrupt already formed biofilm in *M. smegmatis* (Figure 4.5.2 A). In addition the fungal phenotypic compound, sulfometuron, was ineffective at disrupting biofilm formed in *M. smegmatis* (Figure 4.5.2 A).

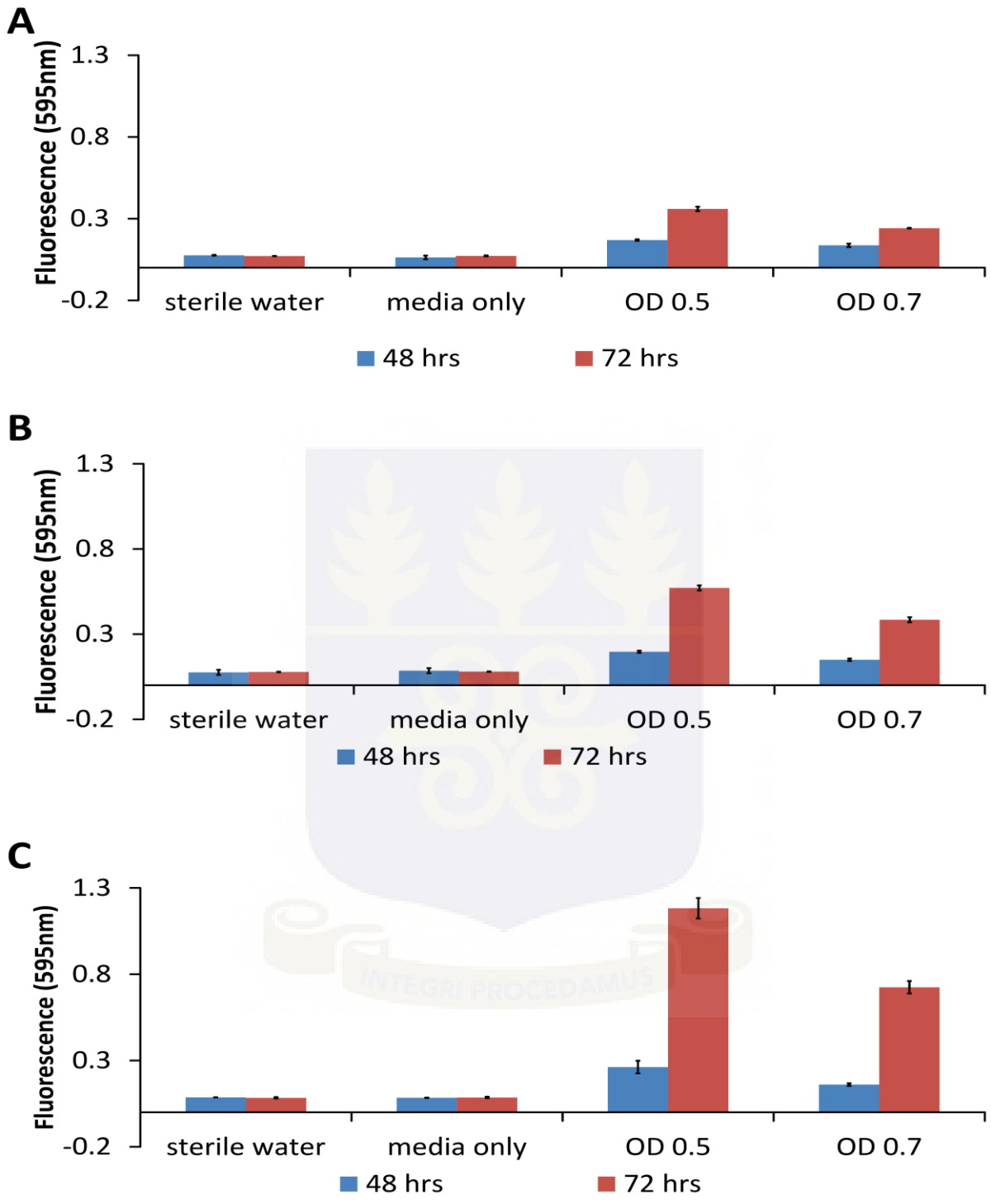

Figure 4.5.0: Biofilm optimisation in (A) *M. smegmatis*, (B) erythromycin-resistant *M. smegmatis* A and (C) erythromycin-resistant *M. smegmatis* B.

Mycobacterial cultures of *M. smegmatis*, erythromycin-resistant *M. smegmatis* A and erythromycin-resistant *M. smegmatis* B at ODs 0.5 and 0.7 were allowed to form biofilms over a period of 48 hr and 72 hr for both ODs. Planktonic cells were then washed and crystal violet added to the wells. The amount of biofilm formed was quantified by measuring absorbance at 595 nm. The experiments were carried out in triplicates and the averages were used to construct the graphs. The error bars represent the standard error of the mean.

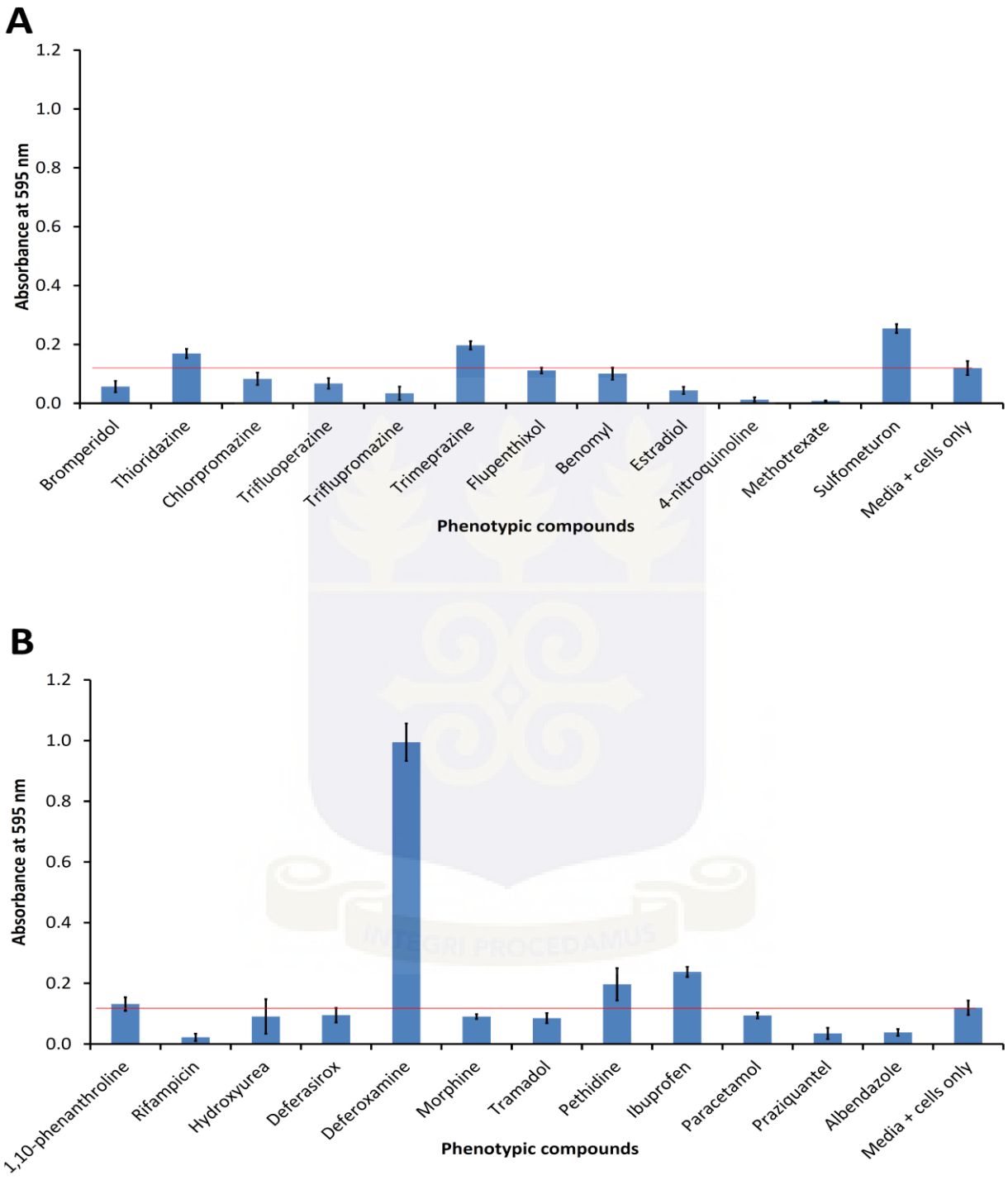

Figure 4.5.1: Effects of antipsychotics and phenotypic compounds on biofilm formation in *M. smegmatis*.
Mycobacteria culture (OD 0.5) was allowed to form biofilm in the presence of the 24 compounds. Following washing of planktonic cells, crystal violet was added to the biofilm formed cells and the absorbance was measured at 595 nm. The experiments were carried out in triplicates and the averages were used for constructing the graphs for analysis. Compound free liquid culture was used as a control. The error bars measure the standard error of the mean. The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the effects of other phenotypic compounds on biofilm formation.

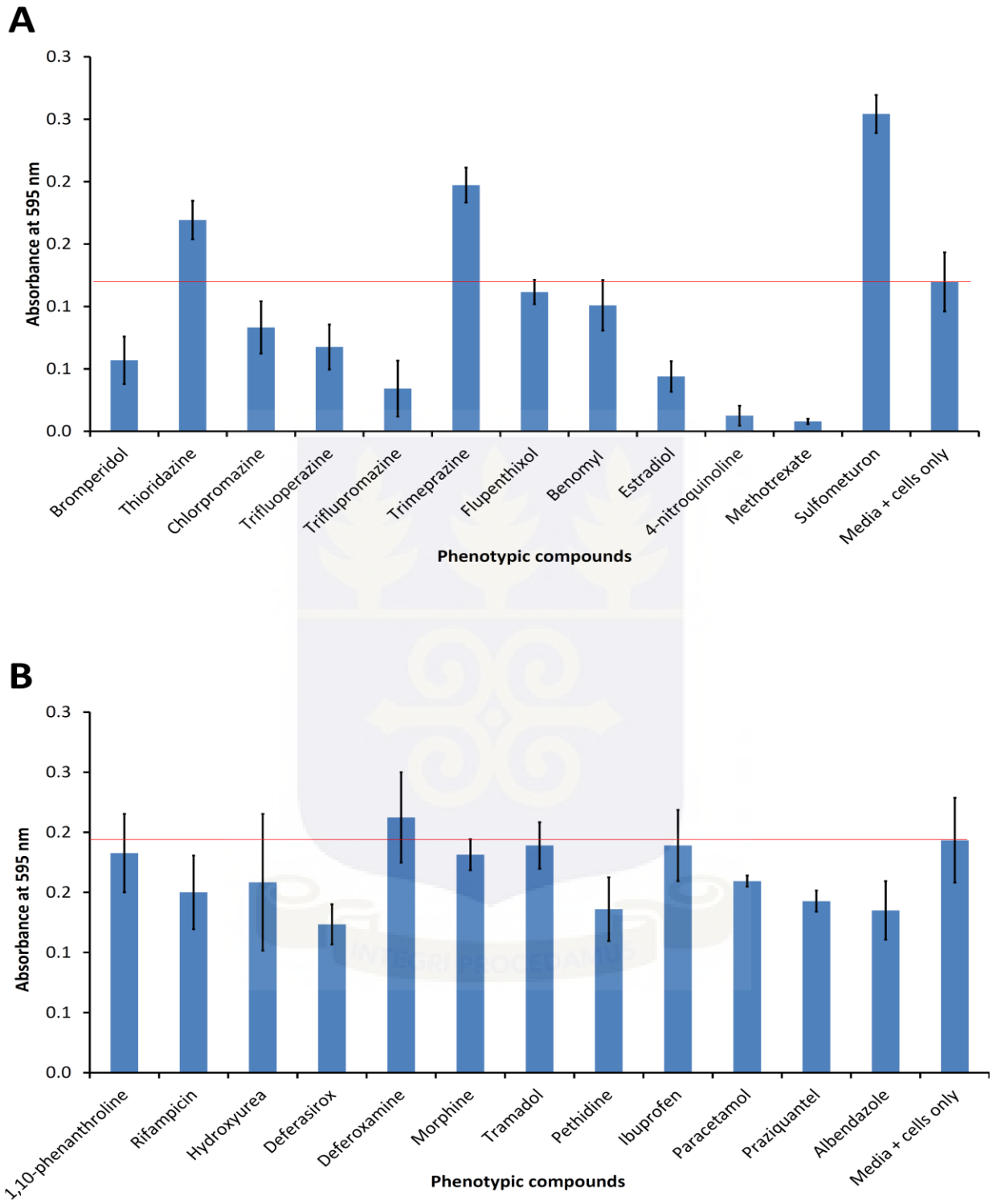

Figure 4.5.2 Biofilm disruption by phenotypic compounds against *M. smegmatis*
Mycobacterial culture (OD 0.5) was allowed to form biofilm under optimum conditions. The biofilm was disrupted by adding phenotypic compounds. Crystal violet was added after washing the planktonic cells and absorbance was read at 595 nm to quantify amount of biofilm formed. The phenotypic compounds were distributed across **A and B.** The error bars represent the standard error of the mean. The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the extent of biofilm disruption by other phenotypic compounds.

4.8.1 Biofilm inhibition and disruption by antipsychotic and other phenotypic compounds on erythromycin-resistant *M. smegmatis* A.

The 7 antipsychotics and other phenotypic compounds were tested for their ability to inhibit biofilm formation and disrupt biofilm in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 and 4.5.4). Bromperidol (a butyrophenone antipsychotic compound) and methotrexate (a sickle cell management drug) inhibited biofilm formation in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 A). Further to this, the antipsychotic compounds thioridazine, trifluoperazine, flupenthixol and trimeprazine induced biofilm formation in erythromycin-resistant *M. smegmatis* A by respective increases of 2-fold, 1.6-fold, 1.5-fold and 2-fold (Figure 4.5.3 A and B). However, chlorpromazine and triflupromazine, could neither induce nor inhibit biofilm formation in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 A). Two sickle cell management drugs, deferoxamine and hydroxyurea, induced biofilm formation by three-fold and two fold respectively in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 B).

The sickle cell management drugs morphine, tramadol, pethidine and paracetamol neither induced nor inhibited biofilm formation in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 B). The fungal phenotypic modifiers benomyl, estradiol and 4-nitroquinoline neither inhibited nor induced formation of biofilm in erythromycin-resistant *M. smegmatis* A (Figure 4.5.3 A). However, benomyl and estradiol disrupted already formed biofilm in erythromycin-resistant *M. smegmatis* A by 28% (Figure 4.5.4 A). The sickle cell management drug, pethidine, and fungal phenotypic compound 1, 10-phenanthroline, disrupted already formed biofilm by 25% and 23% respectively in erythromycin-resistant *M. smegmatis* A (Figure 4.5.4 B). The two iron chelators deferoxamine and deferasirox could not disrupt biofilm formed in erythromycin-resistant *M. smegmatis* A (Figure 4.5.4 B).

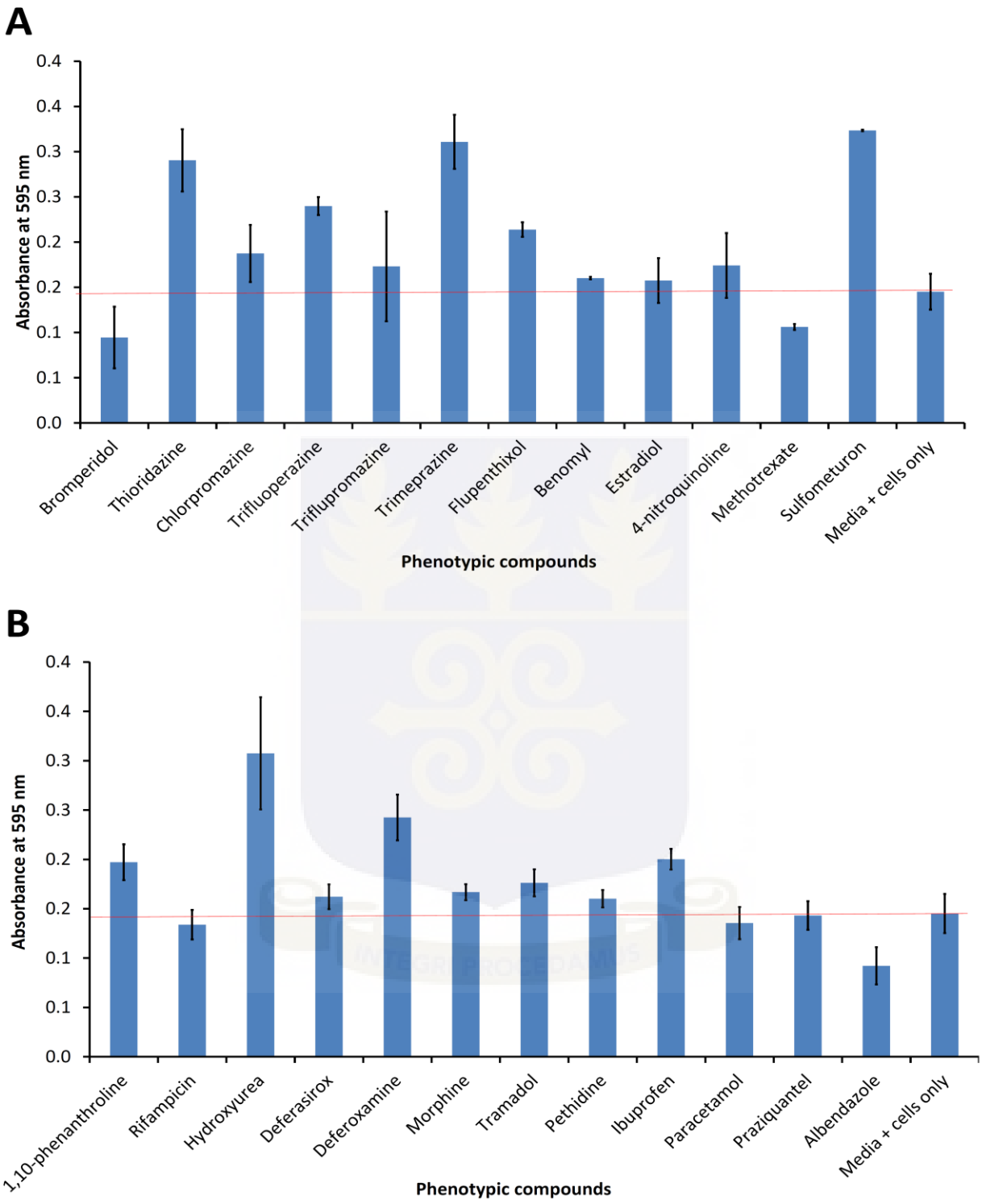

Figure 4.5.3: Effects of antipsychotics and phenotypic compounds on biofilm formation in erythromycin-resistant *M. smegmatis* A.

Mycobacterial culture (OD 0.5) was allowed to form biofilm under optimum conditions. Following washing of the planktonic cells, crystal violet was added to the biofilm formed cells and the absorbance was measured at 595 nm to estimate amount of biofilm formed. The experiments were carried out in triplicates the averages were used for constructing the graphs for analysis. Compound free liquid culture was used as a control. The error bars indicate the standard error of the mean. The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the effects of other phenotypic compounds on biofilm formation.

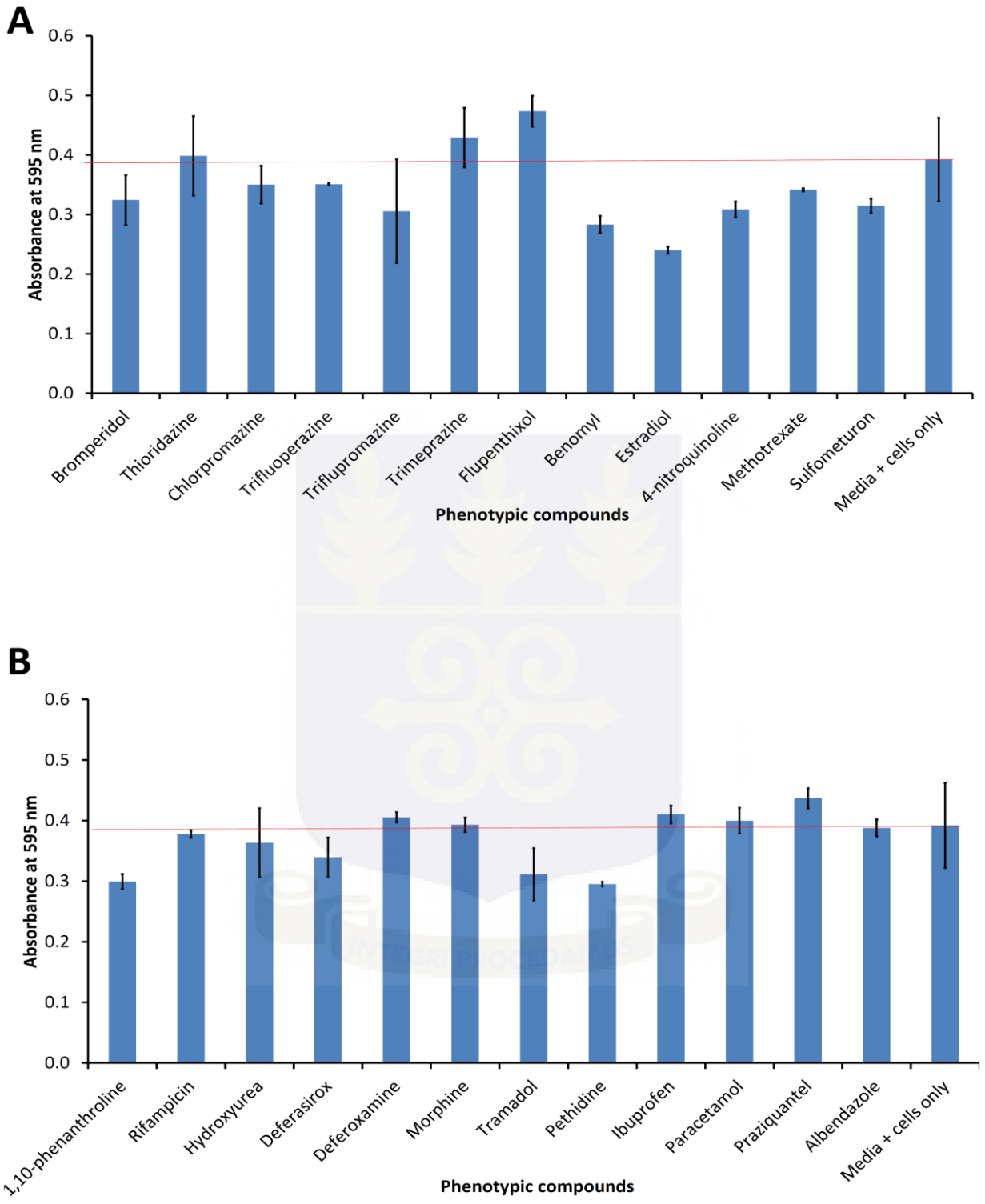

Figure 4.5.4: Biofilm disruption by antipsychotics and phenotypic compounds against erythromycin-resistant *M. smegmatis* A Mycobacterial culture (OD 0.5) was allowed to form biofilm under optimum conditions. The biofilm was disrupted by adding phenotypic compounds. Crystal violet was added after washing the planktonic cells and absorbance was read at 595 nm to quantify amount of biofilm formed. The experiments were carried out in triplicates and the averages were used in constructing the graphs. The error bars represent the standard error of the mean. . The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the extent of biofilm disruption by other phenotypic compounds.

4.8.1 Biofilm inhibition and disruption by antipsychotic and other phenotypic compounds on erythromycin-resistant *M. smegmatis* B

The ability of antipsychotic compounds and other phenotypic compounds to inhibit biofilm formation and disrupt already formed biofilm in erythromycin –resistant *M. smegmatis* B were tested (Figure 4.5.5 and 4.5.6). None of the antipsychotic compounds tested inhibited biofilm formation erythromycin-resistant *M. smegmatis* B (Figure 4.5.5 A). Benomyl, a modulator of fungal efflux pump, caused 0.4-fold increase in biofilm formation in erythromycin-resistant *M. smegmatis* B (Figure 4.5.5 A). However, benomyl could not disrupt biofilm formation in erythromycin-resistant *M. smegmatis* B (Figure 4.5.6 A). Deferasirox, a sickle cell management compound (also an iron chelator) inhibited biofilm formation by 33% relative to compound free culture of erythromycin-resistant *M. smegmatis* B (Figure 4.5.5 B). Notably, apart from deferasirox, the other phenotypic compounds neither induced nor reduced biofilm formation in erythromycin-resistant *M. smegmatis* B (Figure 4.5.5A).

None the 7 antipsychotic compounds tested disrupted biofilm formed by erythromycin-resistant *M. smegmatis* B (Figure 4.5.6 A). Deferasirox, an iron chelating compound which inhibited biofilm formation in erythromycin-resistant *M. smegmatis* B (Figure 4.5.5 B) increased biofilm formation when biofilm disruption assay was carried out in erythromycin-resistant *M. smegmatis* B (Figure 4.5.6 B). In addition, in the biofilm disruption assay, pethidine and estradiol caused 0.3-fold increases in the amount of biofilm formed in erythromycin-resistant *M. smegmatis* B in the biofilm disruption assay (Figure 4.5.6 B). Generally, none of the 24 compounds tested disrupted biofilm formed in erythromycin-resistant *M. smegmatis* B (Figure 4.5.6 B).

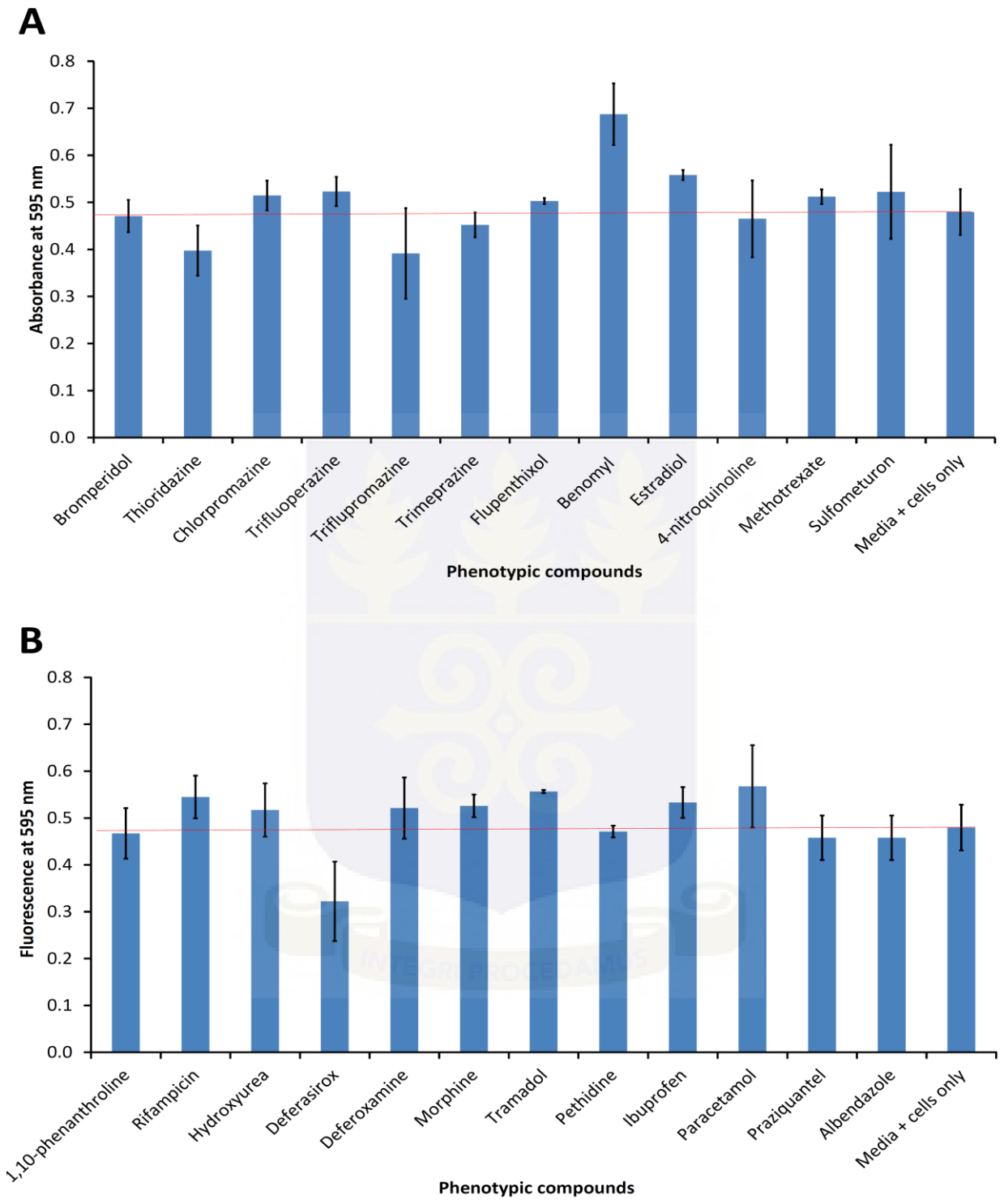

Figure 4.5.5: Effects of antipsychotics and phenotypic compounds on biofilm formation in erythromycin-resistant *M. smegmatis* B.

Mycobacterial culture (OD 0.5) was allowed to form biofilm under optimum conditions. Following washing of the planktonic cells, crystal violet was added to the biofilm formed cells and the absorbance was measured at 595 nm. The experiments were carried out in triplicates and the averages were used for constructing the graphs for analysis. Compound free liquid culture was used as a control. The error bars represent the standard error of the mean. The error bars indicate the standard error of the mean. The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the effects of other phenotypic compounds on biofilm formation.

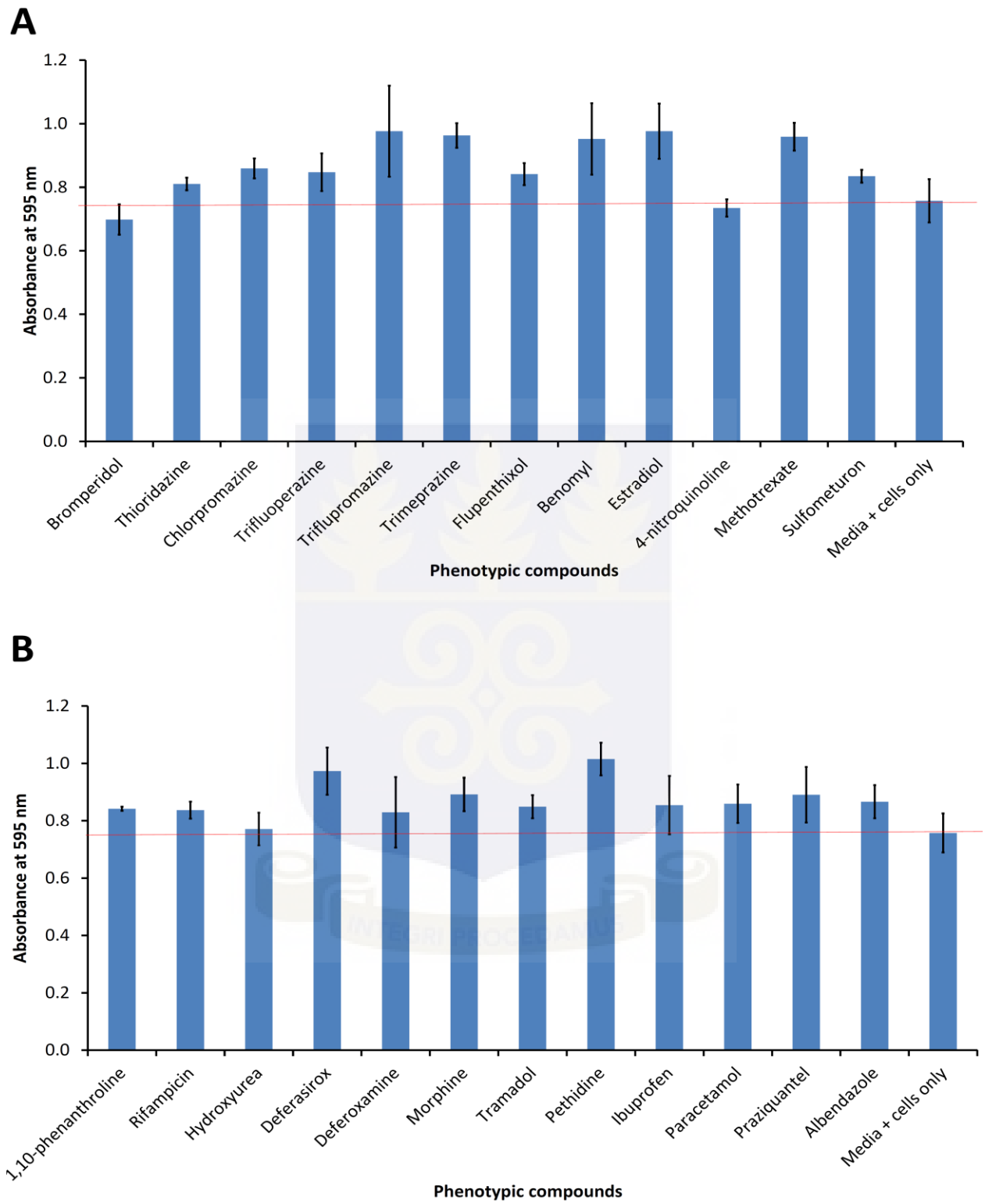

Figure 4.5.6: Biofilm disruption by phenotypic compounds against erythromycin-resistant *M. smegmatis* B. Mycobacterial culture (OD 0.5) was allowed to form biofilm under optimum conditions. The biofilm formed was disrupted by addition of the phenotypic compounds. Crystal violet was added after washing the planktonic cells and absorbance was read at 595 nm to quantify the amount of biofilm formed. The experiments were carried out in triplicates and the averages were used in constructing the graphs. The error bars represent the standard error of the mean. The horizontal red line indicates the level of biofilm formation in compound free medium used as a cutoff to assess the extent of biofilm disruption by other phenotypic compounds.

4.9 Bioactivity of extracts from soil borne fungi (SBF) and terrestrial endophytic fungi (TEF) extracts

Soil borne fungal extracts (201) and terrestrial endophytic fungal extracts (306) were screened against *M. smegmatis* and erythromycin-resistant *M. smegmatis* A (Figure 6.2 and 6.3). Of the 201 SBF extracts screened, 172 SBF (85%) extracts showed activity against *M. smegmatis* and 25 SBF (12%) extracts were active against erythromycin-resistant *M. smegmatis* A (Ery M. smeg A) respectively (Figure 6.2 A and B).

Of the 306 TEF extracts screened, 208 (68%) were active against *M. smegmatis* whilst 57 TEF (19%) extracts were active against erythromycin-resistant *M. smegmatis* A (Ery M. smeg A) (Figure 6.3 A and B). The zones of inhibition of the active extracts were ranked from the highest to the lowest and extracts producing high zones of inhibition (high activity-generating extracts) were considered for further bioactivity guided isolation of active products. From the ranking, 32 highly active SBF and TEF extracts were selected and cultured for large scale secondary metabolites production over a period of three months (Table 4.6).

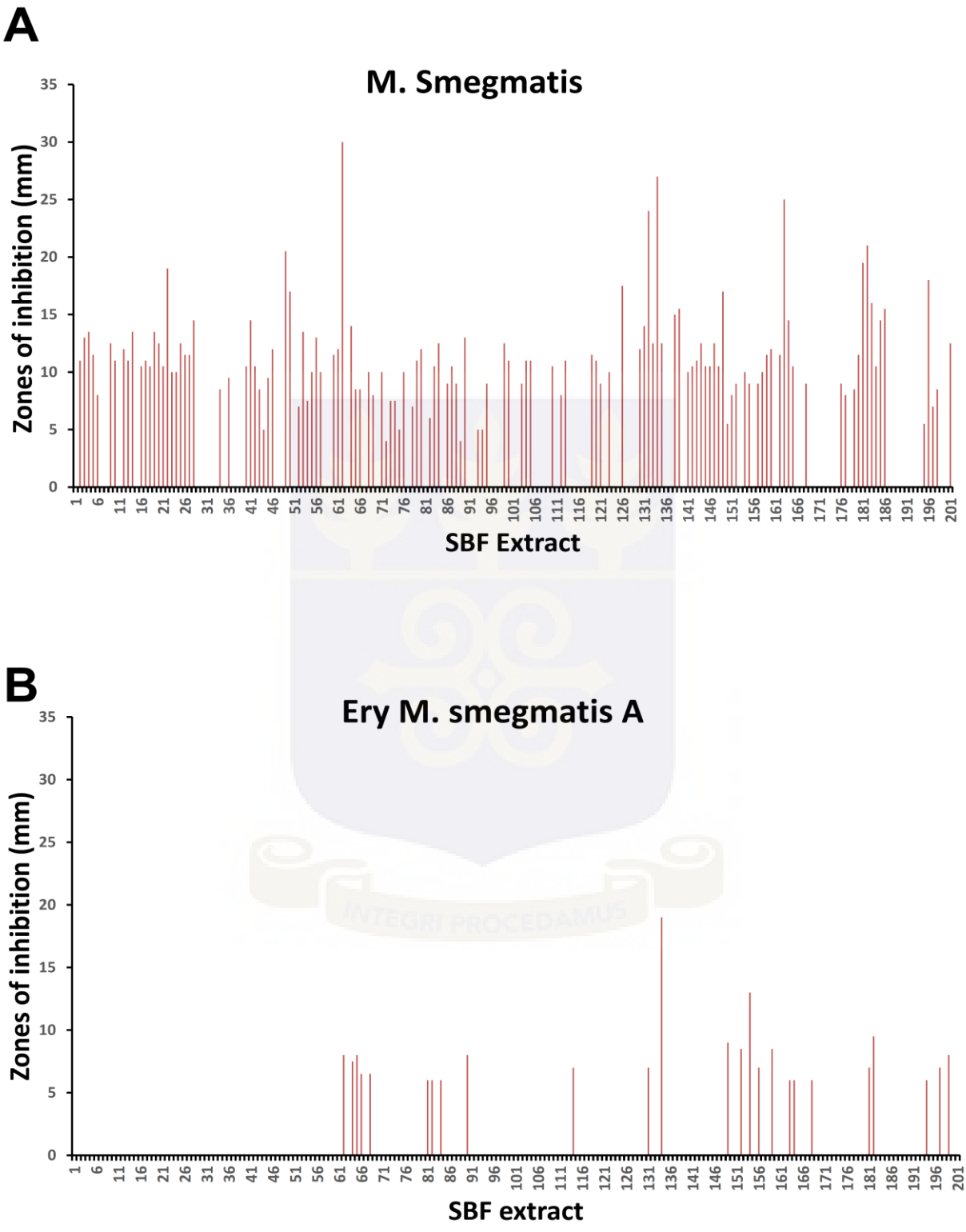

Figure 4.6: Bioactivity of 201 SBF extracts against (A) *M. smegmatis* and (B) erythromycin-resistant M. smegmatis A. Using disc diffusion method, bioactivity of SBF extracts were tested against *M. smegmatis and* erythromycin-resistant *M. smegmatis* A. The experiment was carried out in duplicates and the average zones of inhibition were used to construct the graphs. Fungal isolates producing the most active extracts were selected and cultured for large scale of secondary metabolites.

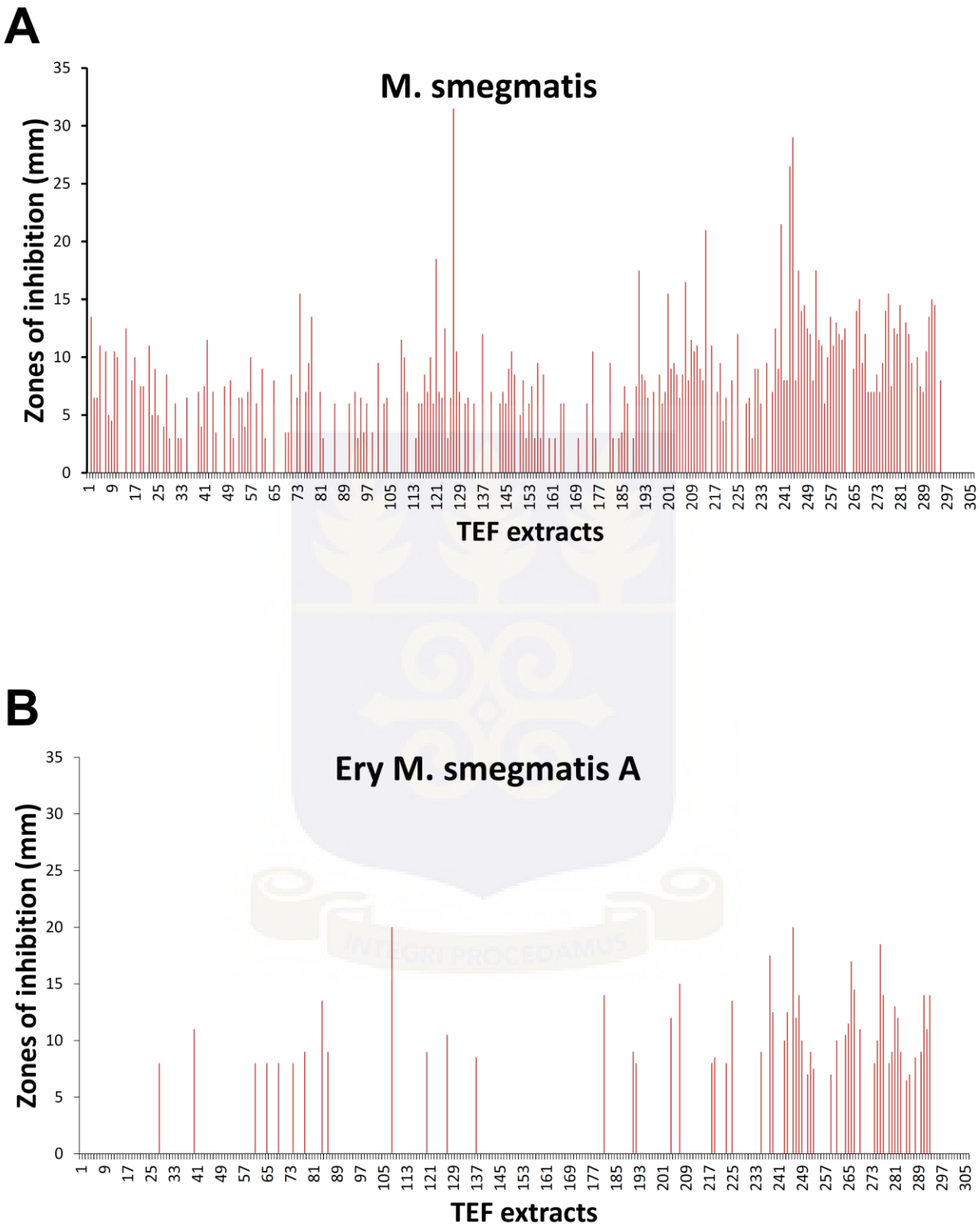

Figure 4.7: Bioactivity of 306 TEF extracts against (A) M. smegmatis and (B) erythromycin-resistant *M. smegmatis* A. The bioactivity of TEF extracts were tested against *M. smegmatis* and erythromycin-resistant *M. smegmatis A* using disc diffusion method. The experiment was carried out in duplicates and the average zones of inhibition were used to construct the graphs. Fungal isolates producing the most active extracts were selected and cultured for large scale of secondary metabolites.

Table 4.6: List of Selected SBFs and TEFs for large culture fermentation

Extract	Grown	Expansion	2L culture
TEF 277	Done	Done	Done
TEF 105	Done	Done	Done
TEF 293	Done	Done	Done
TEF 284	Done	Done	Done
TEF 281	Done	Done	Done
TEF 249	Done	Done	Done
TEF 285	Done	Done	Done
TEF 243	Done	Done	Done
TEF 248	Done	Done	Done
TEF 148	Done	Done	Done
TEF 238	Done	Done	Done
TEF 261	Done	Done	Done
TEF 291	Done	Done	Done
TEF 074	Done	Done	Done
TEF 239	Done	Done	Done
TEF 287	Done	Done	Done
TEF 276	Done	Done	Done
TEF 266	Done	Done	Done
TEF 248	Done	Done	Done
TEF 244	Done	Done	Done
TEF 246	Done	Done	Done
SBF 052	Done	Done	Done
SBF 061	Done	Done	Done
SBF 062	Done	Done	Done
SBF 068	Done	Done	Done
SBF 118	Done	Done	Done
SBF 252	Done	Done	Done
SBF 182	Done	Done	Done
SBF 188	Done	Done	Done
SBF 181	Done	Done	Done
SBF 197	Done	Done	Done
SBF 134	Done	Done	Done

CHAPTER FIVE

DISCUSSIONS

From the body of literature, previous studies pointed out the potential of sertraline (an antidepressant compound) in the treatment of fungal infections (Lass-Flörl *et al.*, 2003; Zhai *et al.*, 2012). In a different study, bromperidol (an antipsychotic compound) was identified to enhance the activity of clarithromycin and rifampicin against *Mycobacterium tuberculosis* (Ramón-García *et al.*, 2011). Further studies suggested the potential of bromperidol derivatives in the treatment of fungal infections (Holbrook *et al.*, 2017). It is against this background that the study was conducted to investigate the mechanisms of resistance in mycobacteria using unrelated compounds including antipsychotics and other phenotype modifying agents.

In a phenotypic drug-drug interaction between 24 phenotypic compounds (including 7 antipsychotic compounds) and anti-mycobacterial agents, 37% (178/480 conditions) resistance breaking and 6% (29/480 conditions) resistance inducing interactions were identified using *M. smegmatis* (Tables 4.1, 4.2, 4.3, 4.4). Of the 7 antipsychotics, thioridazine was found to generate resistance breaking effects with 9 antibiotics (amoxicillin, vancomycin, rifampicin, tetracycline, chloramphenicol, streptomycin, paramomycin, clindamycin and erythromycin) (Table 4.5.1). These antibiotics in resistance breaking scenarios with thioridazine belong to different classes of antibiotics. Thioridazine has been shown to alter membrane permeability of *Mycobacterium tuberculosis* and also enhance the activity of a number of antibiotics irrespective of the resistance profile of the bacteria (Amaral and Molnar, 2014; de Keijzer *et al.*, 2016). Thus better membrane permeability altering agents like thioridazine are potential adjuvants in breaking resistance in mycobacteria (Nasiri *et al.*, 2017). Trifluoperazine, the calmodulin binding antipsychotic, generated resistance breaking effects with 7 antibiotics with

different antimicrobial profiles (Table 4.5.1). Of the five phenothiazines used in the study, thioridazine and trifluoperazine contain piperidine and piperazine respectively as one of the components in their side chains (Figure 2.3). Hence, it is likely these nitrogenous six membered rings (piperidine and piperazine) could play significant roles in enhancing their outstanding resistance breaking scenarios among other phenothiazenes used in the study. As presented in Table 4.5.1, flupenthixol (a thioxanthene antipsychotic compound) produced resistance breaking effects with 8 antibiotics with different antimicrobial profiles. The parent structure (fused tricyclic system) of a thioxanthene is closely related to that of a phenothiazine, except that the nitrogen atom in the parent structure of phenothiazines is replaced by a carbon atom in thioxanthenes (Figure 2.3). Flupenthixol also possesses piperazine as one of the components in its side chains which further supports the earlier speculation that the nitrogenous six membered ring could play significant roles in their resistance breaking interactions. Previous findings showed that flupenthixol is a potent efflux pump inhibitor (ion channel inhibitor) in bacterial systems (Kaatz *et al.*, 2003; Machado *et al.*, 2016). These observations confirm one of the major determinants of drug resistance in mycobacteria as possessing very active efflux systems (Balganesh *et al.*, 2012). In sync with previous findings in the drug-drug interactions study, the efflux assays revealed thioridazine and bromperidol as efflux inhibitors in *M. smegmatis* and erythromycin-resistant *M. smegmatis* A (Figure 4.3.4 and 4.3.5). Notably, 4-nitroquinoline oxide induced resistance breaking effects with all 20 standard antimicrobial agents used in the study. From the body of literature, 4-nitroquinoline oxide is a DNA-damaging agent in bacterial systems (Downes *et al.*, 2014; Fronza *et al.*, 1992), hence DNA-damaging agents will find potential in combination therapy for TB treatment. In addition hydroxyurea produced resistance breaking scenarios with 7 antibiotics with unique mechanisms of action (Table

4.5.1). Hydroxyurea is known to be an inhibitor of ribonucleotide reductase responsible for synthesis of deoxyribonucleotides for the synthesis and repair of DNA (Mowa *et al.*, 2009). This confirms DNA damage repair mechanisms (bacterial SOS response mechanisms) as one of the essential factors promoting mycobacterial persistence in the host (Naran, 2015; Singh, 2017; Žgur-Bertok, 2013). All antipsychotics used in the study produced resistance breaking effects with rifampicin, a bacterial RNA polymerase inhibitor (Wehrli, 1983). DNA-dependent RNA transcription is essential for most bacteria since it drives synthesis of proteins for life processes (Crick, 1970). Phenothiazine and thioxanthene antipsychotics generally targets efflux pumps in bacterial systems (Kaatz *et al.*, 2003; Rodrigues *et al.*, 2011), therefore, it is possible bacterial systems that are secondarily challenged with RNA synthesis inhibitors will be weakened and made susceptible to the primary compounds/antibiotics. Though the mechanism of bromperidol is not yet known, its butyrophenone structurally similar counterpart haloperidol has been found to be a potent ion channel blocker in bacterial cells (Machado *et al.*, 2016), suggesting its likelihood to act as an ion channel blocker. This may explain why bromperidol generated resistance breaking effects with 7 antibiotics with different antimicrobial activity backgrounds (Table 4.5.1). In addition, bromperidol also possesses piperidine (six membered nitrogenous ring) as similarly found in the phenothiazines (thioridazine and trifluoperazine). This further supports the suggestion that six membered nitrogenous rings (piperdidine or piperazine) could play significant roles in resistance breaking interactions. The effects of bromperidol-vancomycin drug pair on the growth of *M. smegmatis* were further validated in liquid cultures (Figure 4.2 C). Bromperidol-vancomycin drug pair combination was found to suppress the growth rate of *M. smegmatis* relative to mycobacterial culture containing either compound (Figure 4.2 C). For phenotypic compound – antibiotic combinations that did not produce

expected resistance breaking interactions, lower concentrations of the antibiotics should be used to generate expected suppressive growth effects by the antibiotic-compound pair.

A number of resistance inducing interactions from the pairwise drug-drug combinations were identified (Tables 4.1, 4.2, 4.3, 4.4). The two ion channel blockers, chlorpromazine, thioridazine and the proposed ion channel blocker bromperidol induced resistance in pairwise combinations with ampicillin, a cell wall disrupting antibiotic (Table 4.1). Since efflux pumps are mostly linked to cell membrane (Szumowski *et al.*, 2012), it is possible using two agents that affect the permeability of the cell membrane will induce remarkable remodeling of the cell envelope as a stress response mechanism (Black *et al.*, 2014). This could explain the resistance inducing effects observed with interactions between the three ion channel blockers (including proposed ion channel blocker bromperidol) and ampicillin. Notably, the iron chelator deferoxamine induced resistance effects in *M. smegmatis* when paired with erythromycin, ampicillin and moxifloxacin (Table 4.5.3 and 4.5.4). Resistance inducing effects on *M. smegmatis* in the presence of deferoxamine and ampicillin was further validated in liquid cultures (Figure 4.2 E). It was observed that in the presence of deferoxamine only the growth rate of *M. smegmatis* relative to drug free culture was enhanced. The drug-drug pair (deferoxamine-ampicillin) further enhanced the growth of *M. smegmatis* (Figure 4.2 E). Iron chelation has been found to modulate growth of mycobacteria (Cronjé *et al.*, 2005). Iron deprivation has been shown to produce persistence in *Mycobacterium tuberculosis*, since under such conditions, the organism depend on the exclusive control of endogenous iron leading to the activation of a number of determinants of antibiotic resistance genes (Kurthkoti *et al.*, 2017). This observation is consistent with the ability of *M. smegmatis* to significantly form biofilm in the presence of deferoxamine (Figure 4.5.1 B). This suggests that iron deprivation

boosts the formation of biofilm in *M. smegmatis* as a mechanism of persistence and antibiotic resistance in mycobacteria. In addition sulfometuron (mechanism of action unknown in mycobacteria) which promoted biofilm formation in *M. smegmatis* (Figure 4.5.1 A) and erythromycin-resistant *M. smegmatis* A (Figure 5.8 A) produced resistance phenotypes when paired with the DNA replication inhibitor moxifloxacin in the interaction studies (Table 4.5.3 and 4.5.4). Therefore, biofilm-induced tolerance contributes to drug resistance in mycobacteria.

Mechanism of resistance by efflux is a common mechanism in most mycobacterial species (da Silva *et al.*, 2011; Smith *et al.*, 2012). Ethidium bromide (EtBr) transport assays were carried out to quantify efflux activity in the presence of the phenotypic compounds since its fluorescence is detectable within bacilli but loses fluorescence in an aqueous environment (Machado *et al.*, 2016). Bromperidol, thioridazine and chlorpromazine enhanced accumulation of ethidium bromide and prevented abrupt efflux of ethidium bromide in *M. smegmatis* and erythromycin-resistant *M. smegmatis* A with time (Figure 4.3.4 A). In a similar fashion, bromperidol, thioridazine and chlorpromazine caused increased intracellular levels of ethidium bromide and inhibited sudden extrusion of ethidium bromide in erythromycin-resistant *M. smegmatis* A (Figure 4.3.5 A). Bromperidol and chlorpromazine were found to exhibit similar efflux inhibition pattern in *M. smegmatis* and erythromycin-resistant *M. smegmatis* A (Figure 4.3.4 A and 4.3.5 A). Therefore it is possible a similar efflux modulating mechanism is exhibited by these two compounds in mycobacteria though they belong to different classes of antipsychotic compounds. As described earlier, haloperidol, an ion channel inhibitor (Machado *et al.*, 2016), shares structural similarities with bromperidol, therefore, bromperidol is likely to act as an ion channel inhibitor. Thioridazine and chlorpromazine are phenothiazines which are mainly bacterial efflux pump inhibitors (Amaral *et al.*, 2001; Black *et al.*, 2014; Kaatz *et al.*,

2003; Pule *et al.*, 2015). Thioridazine has been shown to alter the permeability of mycobacterial cell membrane, thereby enhancing uptake of compounds (de Keijzer *et al.*, 2016). Hence cellular strategies enhancing membrane homeostasis can possibly contribute to antibiotic tolerance in mycobacteria (Fonseca *et al.*, 2015). From these observations, it is possible mechanisms of efflux in mycobacteria can be perturbed by cell envelope (membrane homeostasis) disrupting agents. Two other phenothiazine based antipsychotics trimeprazine and trifluoperazine and the thioxanthene flupenthixol, produced stronger efflux inhibitory effects in erythromycin-resistant *M. smegmatis* A than verapamil treated cells. These observations are consistent with their mode of action as efflux pump inhibitors (Kaatz *et al.*, 2003). Trifluoperazine is also known to be a novel calcium binding protein in mycobacteria thereby affecting all calcium dependent cellular processes (Ratnakar *et al.*, 1995). In a macrophage infection model, thioridazine, chlorpromazine and trifluoperazine are known to concentrate within the macrophage and kill phagocytosed mycobacteria by multi-mechanisms of action (van Ingen, 2011). However, in erythromycin-resistant *Mycobacterium smegmatis* B, none of the antipsychotics and other phenotypic compounds maintained a gradual loss of ethidium bromide in a manner similar to verapamil. This shows that the erythromycin-resistant *M. smegmatis* B has a stronger efflux system or highly resistant background compared with *M. smegmatis* and erythromycin-resistant *M. smegmatis* A or is not very sensitive to binding by the antipsychotics. Although, estradiol produced uptake and efflux profile similar to verapamil in *M. smegmatis*, its uptake and extrusion pattern is higher and well controlled in erythromycin respectively resistant *M. smegmatis* A (Figure 4.4.0 A and B). Drug resistance in mycobacteria is known to impart metabolic and fitness cost (Salvatore *et al.*, 2015), therefore it is possible the multidrug resistance phenotype observed in erythromycin-resistant *M. smegmatis* A imposes

metabolic remodelling consequences thereby weakening the organisms efflux activity rate towards estradiol.

M. smegmatis formed biofilm in the presence of the phenothiazine trifluoperazine. Trifluoperazine, a calmodulin binding compound has also been shown to possess other mechanisms of action in mycobacteria. Trifluoperazine has been shown to inhibit synthesis of ATP *in vitro* in *Mycobacterium leprae* (Katoch *et al.*, 1998), and also possess multiple mechanisms of action on the biosynthesis of macromolecules (Ratnakar and Suryanarayana Murthy, 1993). Hence in the presence of trifluoperazine, its multifactorial mechanisms may induce cellular stress response that culminates in the formation of biofilm as was observed. The DNA damaging agent 4-nitroquinoline oxide (Fronza *et al.*, 1992), RNA synthesis inhibitor rifampicin (White *et al.*, 1971) and DNA synthesis inhibitor methotrexate (Stone *et al.*, 1984) all inhibited biofilm formation in *M. smegmatis* in addition to the antipsychotic bromperidol (Figure 4.5.1 A). Hence it is very likely DNA and RNA disrupting agents are potential inhibitors of biofilm in mycobacteria. Hence DNA repair mechanisms are crucial in the formation of biofilms for bacterial persistence (Debbia *et al.*, 2001). In addition rifampicin caused universal resistance breaking effects with all phenotypic compounds used in the study (Table 4.1 – 4.4). Since rifampicin is an inhibitor of DNA directed RNA synthesis, it causes extensive non-specific reduction in mRNA transcripts therefore in combination with a number for phenotypic compounds induces enhancement of activity. This reveals mechanisms that promote bacterial homeostasis of mRNA transcripts as essential for its survival and drug tolerance

To mirror previous drug-drug interactions between phenotypic compounds and anti-mycobacterial agents per the project concept (Figure 1.1), extracts obtained from soil borne

fungi and terrestrial endophytic fungi were tested against *M. smegmatis* and erythromycin-resistant *M. smegmatis* A. Natural products have been suggested to be one of the dependable sources that can provide lead compounds for the development of anti-mycobacterial agents against the emergence of drug resistant tuberculosis (Salomon and Schmidt, 2012). From the preliminary screening (Figure 6.2 and 6.3), 12% of SBF and 19% of the TEF extracts respectively showed activity against the multidrug resistant *M. smegmatis* (A and B). This observation throws more light on the fact that natural products are potential sources for the development of novel TB drugs.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The proportion of resistance breaking interactions and resistance inducing interactions generated from the drug-drug combinations were (37%; 178/480 conditions) and (6%; 29/480 conditions) respectively against *M. smegmatis*. Drug combinations involving antipsychotics generated 9% (44/480 conditions) resistance breaking interactions and 2% (9/480 conditions) resistance inducing interactions.

Two antipsychotic compounds, thioridazine and bromperidol induced uptake and inhibited extrusion of ethidium bromide in *M. smegmatis* and the multidrug-resistant *M. smegmatis* (erythromycin-resistant *M. smegmatis* A*)*. Thioridazine, bromperidol and flupenthixol generated resistance breaking effects when combined with a number of antibiotics with different antimicrobial profiles, thus suggesting efflux activity and cell envelope homeostasis in mycobacteria as possible mechanisms of antimicrobial tolerance. It is possible that cellular factors driving influx of ethidium bromide are different from factors modulating efflux since most compounds could not inhibit efflux at levels where they stimulated uptake.

The DNA disrupting agent, 4–nitroquinoline oxide induced resistance breaking effects with all antibiotics tested. Two DNA disrupting phenotypic compounds, methotrexate and 4-nitroquniline oxide, and the RNA synthesis inhibitor rifampicin inhibited biofilm formations in *M. smegmatis*, therefore implying mechanisms of DNA repair and mRNA homeostasis to be relevant to biofilm formation and antibiotic tolerance.

Two biofilm inducing compounds deferoxamine and sulfometuron also induced resistance with a number of antibiotics in *M. smegmatis* during the drug interactions studies. Therefore, biofilm induction is one of the possible mechanisms mediating anti-mycobacterial tolerance and resistance.

The activity profile of extracts from soil borne and terrestrial endophytic fungi shows secondary metabolites of fungal origin to gain promising potential in anti-mycobacterial drug discovery.

The impacts of the study highlight the usefulness of antipsychotics and other non-antibiotics in tuberculosis drug discovery and the potential management of drug-resistant tuberculosis. The study further unveils the bioprospecting potential of fungi in tuberculosis drug discovery.

6.2 Future directions

1. Molecular analysis of antibiotic associated stress response and efflux pump to assess their role in the observed resistance and susceptibility phenotypes.
2. Active fungal extracts from soil borne fungi (SBF) and terrestrial endophytic fungi (TEF) would be further fractionated using bioactivity guided procedure to obtain novel antimicrobials.

6.3 Recommendation

1. Drugs used in the management of other chronic diseases should be included to expand the usefulness of non-TB drugs in repurposing.

REFERENCES

Advani, M. J., Rajagopalan, M., & Reddy, P. H. (2014). Calmodulin-like protein from M. tuberculosis H37Rv is required during infection. *Scientific reports*, *4*, 6861.

Albert, H., Nathavitharana, R. R., Isaacs, C., Pai, M., Denkinger, C. M., & Boehme, C. C. (2016). Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better? *European Respiratory Journal*, ERJ-00543-02016.

Amaral, L. (2012). Totally Drug resistant tuberculosis can be treated with thioridazine in combination with antibiotics to which the patient was initially resistant. *Biochem. Pharmacol*, *39*(5), 376-380.

Amaral, L., Kristiansen, J. E., Viveiros, M., & Atouguia, J. (2001). Activity of phenothiazines against antibiotic-resistant Mycobacterium tuberculosis: a review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. *Journal of Antimicrobial Chemotherapy*, *47*(5), 505-511.

Amaral, L., & Molnar, J. (2014). Mechanisms by which thioridazine in combination with antibiotics cures extensively drug-resistant infections of pulmonary tuberculosis. *In Vivo*, *28*(2), 267-271.

Arthur, P. K., Armaah, V., Cramer, P., Arkaifie, G. B., Blessie, E. J. S., Fuseini, M., Carilo, I., Yeboah, R., Asare, L., & Robertson, B.D (2019). Characterization of Two New Multidrug-ResistantStrains of *Mycobacterium smegmatis*: Tools for Routine In Vitro Screening of Novel Anti-Mycobacterial Agents. *Antibiotics MDPI* 8 (4).

Ayaz, M., Subhan, F., Ahmed, J., Khan, A.-u., Ullah, F., Ullah, I., & Hussain, S. (2015). Sertraline enhances the activity of antimicrobial agents against pathogens of clinical relevance. *Journal of Biological Research-Thessaloniki*, *22*(1), 4.

Balganesh, M., Dinesh, N., Sharma, S., Kuruppath, S., Nair, A. V., & Sharma, U. (2012). Efflux Pumps of Mycobacterium tuberculosis play a significant role in anti-tuberculosis activity of potential drug candidates. *Antimicrobial agents and chemotherapy*, AAC. 06003-06011.

Bhunu, B., Mautsa, R., & Mukanganyama, S. (2017). Inhibition of biofilm formation in Mycobacterium smegmatis by Parinari curatellifolia leaf extracts. *BMC complementary and alternative medicine*, *17*(1), 285.

Black, P. A., Warren, R. M., Louw, G. E., van Helden, P. D., Victor, T. C., & Kana, B. D. (2014). Energy metabolism and drug efflux in Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, AAC. 02293-02213.

Blondiaux, N., Moune, M., Desroses, M., Frita, R., Flipo, M., Mathys, V., & Djaout, K. (2017). Reversion of antibiotic resistance in Mycobacterium tuberculosis by spiroisoxazoline SMARt-420. *Science*, *355*(6330), 1206-1211.

Boguski, M. S., Mandl, K. D., & Sukhatme, V. P. (2009). Repurposing with a difference. *Science*, *324*(5933), 1394-1395.

Brennan, M. J. (2017). Biofilms and Mycobacterium tuberculosis. *Infection and immunity*, *85*(10), e00411-00417.

Cederlund, H., & Mardh, P. A (1993). Antibacterial activity of non-antibiotic drugs. *J Antimicrob Chemother*, 32: 355-365.

Cheng, M.-J., Wu, M.-D., Yanai, H., Su, Y.-S., Chen, I.-S., Yuan, G.-F., & Chen, J.-J. (2012). Secondary metabolites from the endophytic fungus Biscogniauxia formosana and their antimycobacterial activity. *Phytochemistry Letters*, *5*(3), 467-472.

Chong, C. R., & Sullivan Jr, D. J. (2007). New uses for old drugs. *Nature*, *448*(7154), 645.

Chomcheon, P., Wiyakrutta, S., Sriubolmas, N., Ngamrojanavanich, N., Isarangkul, D., and Kittakoop, P (2005). 3-Nitropropionic acid (3-NPA), a potent antimycobacterial agent from endophytic fungi: is 3-NPA in some plants produced by endophytes? J Nat Prod. 2005 68(7):1103-5.

Coelho, T., Machado, D., Couto, I., Maschmann, R., Ramos, D., von Groll, A., & Viveiros, M. (2015). Enhancement of antibiotic activity by efflux inhibitors against multidrug resistant Mycobacterium tuberculosis clinical isolates from Brazil. *Frontiers in microbiology*, *6*, 330.

Cragg, G. M., & Newman, D. J. (2005). Biodiversity: A continuing source of novel drug leads. *Pure and applied chemistry*, *77*(1), 7-24.

Crick, F. (1970). Central dogma of molecular biology. *Nature*, *227*(5258), 561.

Cronjé, L., Edmondson, N., Eisenach, K. D., & Bornman, L. (2005). Iron and iron chelating agents modulate Mycobacterium tuberculosis growth and monocyte-macrophage viability and effector functions. *FEMS Immunology & Medical Microbiology*, *45*(2), 103-112.

da Silva, P. E. A., Von Groll, A., Martin, A., & Palomino, J. C. (2011). Efflux as a mechanism for drug resistance in Mycobacterium tuberculosis. *FEMS Immunology & Medical Microbiology*, *63*(1), 1-9.

Danquah, C. A., Kakagianni, E., Khondkar, P., Maitra, A., Rahman, M., Evangelopoulos, D., & Bhakta, S. (2018). Analogues of Disulfides from Allium stipitatum demonstrate potent

anti-tubercular activities through drug efflux pump and Biofilm inhibition. *Scientific reports*, *8*(1), 1150.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science*, *280*(5361), 295-298.

de Keijzer, J., Mulder, A., de Haas, P. E., de Ru, A. H., Heerkens, E. M., Amaral, L., & van Veelen, P. A. (2016). Thioridazine alters the cell-envelope permeability of Mycobacterium tuberculosis. *Journal of proteome research*, *15*(6), 1776-1786.

Debbia, E., Roveta, S., Schito, A., Gualco, L., & Marchese, A. (2001). Antibiotic persistence: the role of spontaneous DNA repair response. *Microbial drug resistance*, *7*(4), 335-342.

Demers, D. H., Knestrick, M. A., Fleeman, R., Tawfik, R., Azhari, A., Souza, A., Vesely, B., Netherton, M., Gupta, R., Colon, B. L., Rice, C. A., Rodriguez-Perez, M.A., Rhode, K. H., Kyle, D. E., Shaw, L. N., Baker, B. J (2018) Exploitation of mangrove endophytic fungi for infectious disease drug discovery. *Mar drugs*, 16(10):376.

de Prince, K. A., Sordi, R., Pavan, F. R., Santos, A. C. B., Araujo, A. R., Leite, S. R. A., & Leite, C. Q. F (2012). Anti-mycobacterium tuberculosis activity of the fungus *Phomopsis stipata*. *Braz J. Micribiol*. 43 (1): 224 - 229.

Downes, D. J., Chonofsky, M., Tan, K., Pfannenstiel, B. T., Reck-Peterson, S. L., & Todd, R. B. (2014). Characterization of the mutagenic spectrum of 4-nitroquinoline 1-oxide (4-NQO) in Aspergillus nidulans by whole genome sequencing. *G3: Genes, Genomes, Genetics*, g3. 114.014712.

Dutta, N. K., Mehra, S., & Kaushal, D. (2010). A Mycobacterium tuberculosis sigma factor network responds to cell-envelope damage by the promising anti-mycobacterial thioridazine. *PloS one*, *5*(4), e10069.

E Salomon, C., & E Schmidt, L. (2012). Natural products as leads for tuberculosis drug development. *Current topics in medicinal chemistry*, *12*(7), 735-765.

Falkinham III, J. O. (2007). Growth in catheter biofilms and antibiotic resistance of Mycobacterium avium. *Journal of medical microbiology*, *56*(2), 250-254.

Faustini, A., Hall, A., & Perucci, C. (2005). Tuberculosis treatment outcomes in Europe: a systematic review. *European Respiratory Journal*, *26*(3), 503-510.

Fonseca, J., Knight, G., & McHugh, T. (2015). The complex evolution of antibiotic resistance in Mycobacterium tuberculosis. *International journal of infectious diseases*, *32*, 94-100.

Fronza, G., Campomenosi, P., lannone, R., & Abbondandolo, A. (1992). The 4-nitroquinoline 1-oxide mutational spectrum in single stranded DNA is characterized by guanine to pyrimidine transversions. *Nucleic acids research, 20*(6), 1283-1287.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016) Endophytes: A treasure house of bioactive compounds of medicinal importance. Front Microbiol. 7:1538.

Gupta, S., Cohen, K. A., Winglee, K., Maiga, M., Diarra, B., & Bishai, W. R. (2013). Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, AAC. 01462-01413.

Hall-Stoodley, L., & Stoodley, P. (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends in microbiology, 13*(1), 7-10.

Hoagland, D. T., Liu, J., Lee, R. B., & Lee, R. E. (2016). New agents for the treatment of drug-resistant Mycobacterium tuberculosis. *Advanced drug delivery reviews, 102*, 55-72.

Holbrook, S. Y., Garzan, A., Dennis, E. K., Shrestha, S. K., & Garneau-Tsodikova, S. (2017). Repurposing antipsychotic drugs into antifungal agents: Synergistic combinations of azoles and bromperidol derivatives in the treatment of various fungal infections. *European journal of medicinal chemistry, 139*, 12-21.

Jin, L., Tu, J., Jia, J., An, W., Tan, H., Cui, Q., & Li, Z. (2014). Drug-repurposing identified the combination of Trolox C and Cytisine for the treatment of type 2 diabetes. *Journal of translational medicine, 12*(1), 153.

Jin, L., Quan, C., Hou, X., Fan, S (2016). Potential Pharmacological Resources: Natural Bioactive Compounds from Marine-Derived Fungi. Mar Drugs. 14(4):76.

Dutta, N. K., Mazumdar, K., Dastidar, G. S., Karakousis, C. P., & Amaral, L. (2011). New patentable use of an old neuroleptic compound thioridazine to combat tuberculosis: a gene regulation perspective. *Recent patents on anti-infective drug discovery, 6*(2), 128-138.

Kaatz, G. W., Moudgal, V. V., Seo, S. M., & Kristiansen, J. E. (2003). Phenothiazines and thioxanthenes inhibit multidrug efflux pump activity in Staphylococcus aureus. *Antimicrobial agents and chemotherapy, 47*(2), 719-726.

Kanji, A., Hasan, R., & Hasan, Z. (2018). Efflux pump as alternate mechanism for drug resistance in Mycobacterium tuberculosis. *Indian Journal of Tuberculosis*.

Katoch, V., Saxena, N., Shivannavar, C., Sharma, V., Katoch, K., Sharma, R., & Murthy, P. S. (1998). Effect of trifluoperazine on in vitro ATP synthesis by Mycobacterium leprae. *FEMS Immunology & Medical Microbiology, 20*(2), 99-102.

Kigondu, E. M., Wasuna, A., Warner, D. F., & Chibale, K. (2014). Pharmacologically active metabolites, combination screening and target identification-driven drug repositioning in antituberculosis drug discovery. *Bioorganic & medicinal chemistry*, *22*(16), 4453-4461.

Koul, S., Somayajulu, A., Advani, M. J., & Reddy, H. (2009). A novel calcium binding protein in Mycobacterium tuberculosis—potential target for trifluoperazine.

Kumar, V., Sachan, T. K., Sharma, P., & Rawat, K. D. (2015). Ultrastructural morphologic changes in mycobacterial biofilm in different extreme condition. *Ultrastructural pathology*, *39*(1), 38-48.

Kurthkoti, K., Amin, H., Marakalala, M. J., Ghanny, S., Subbian, S., Sakatos, A., & Rodriguez, G. M. (2017). The capacity of Mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. *MBio*, *8*(4), e01092-01017.

Lasebikan, V., & Ige, O. (2015). Prevalence of psychosis in tuberculosis patients and their nontuberculosis family contacts in a multidrug treatment-resistant treatment center in Nigeria. *General hospital psychiatry*, *37*(6), 542-547.

Lass-Flörl, C., Dierich, M. P., Fuchs, D., Semenitz, E., & Ledochowski, M. (2001). Antifungal activity against Candida species of the selective serotonin-reuptake inhibitor, sertraline. *Clinical Infectious Diseases*, *33*(12), e135-e136.

Lass-Flörl, C., Ledochowski, M., Fuchs, D., Speth, C., Kacani, L., Dierich, M. P., & Würzner, R. (2003). Interaction of sertraline with Candida species selectively attenuates fungal virulence in vitro. *FEMS Immunology & Medical Microbiology*, *35*(1), 11-15.

Luo, X., Zhou, X., Lin, X., Qin, X., Zhang, T., Wang, J., Tu, Z., Yang, B., Liao, X., Tian, Y., Pang, X., Kaliyaperumal, K., Li, J. L., Tao, H., & Liu, Y (2017). Antituberculosis compounds from a deep-sea-derived fungus *Aspergillus* sp. SCSIO Ind09F01. *Natural Products Research* 31 (16): 1958-1962.

Machado, D., Pires, D., Perdigão, J., Couto, I., Portugal, I., Martins, M., & Viveiros, M. (2016). Ion channel blockers as antimicrobial agents, efflux inhibitors, and enhancers of macrophage killing activity against drug resistant Mycobacterium tuberculosis. *PloS one*, *11*(2), e0149326.

Machado, D., Coelho, T. S., Perdigão, J., Pereira, C., Couto, I., Portugal, I., & Rossetti, M. L. (2017). Interplay between mutations and efflux in drug resistant clinical isolates of Mycobacterium tuberculosis. *Frontiers in microbiology*, *8*, 711.

Medical Xpress Report, 2017.

Merker, M., Kohl, T. A., Roetzer, A., Truebe, L., Richter, E., Rüsch-Gerdes, S., & Varaine, F. (2013). Whole genome sequencing reveals complex evolution patterns of multidrug-resistant Mycobacterium tuberculosis Beijing strains in patients. *PloS one, 8*(12), e82551.

Mowa, M. B., Warner, D. F., Kaplan, G., Kana, B. D., & Mizrahi, V. (2009). Function and regulation of class I ribonucleotide reductase-encoding genes in mycobacteria. *Journal of bacteriology, 191*(3), 985-995.

Nagelkerke, N. J. D., de Vlas, S. J., Mahendradhata, Y., Ottenhoff, T, H. F., & Borgdorff, M. (2006). The search for a tuberculosis vaccine: an elusive quest? *Science direct Tuberculosis.* 86 (1): 41-46.

Nagabushan, H., & Roopadevi, H. (2014). Bedaquiline: a novel antitubercular drug for multidrug-resistant tuberculosis. *Journal of postgraduate medicine, 60*(3), 300.

Naran, K. (2015). Elucidation of mechanisms of antibiotic subversion in mycobacteria. University of Cape Town.

Nasiri, M. J., Haeili, M., Ghazi, M., Goudarzi, H., Pormohammad, A., Imani Fooladi, A. A., & Feizabadi, M. M. (2017). New insights in to the intrinsic and acquired drug resistance mechanisms in mycobacteria. *Frontiers in microbiology, 8*, 681.

Ordway, D., Viveiros, M., Leandro, C., Bettencourt, R., Almeida, J., Martins, M., & Amaral, L. (2003). Clinical concentrations of thioridazine kill intracellular multidrug-resistant Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy, 47*(3), 917-922.

Ordway, D. J., Costa, L., Martins, M., Silveira, H., Amaral, L., Arroz, M. J., & Dockrell, H. M. (2004). Increased Interleukin-4 Production by CD8 and γδl T cells in health-care workers is associated with the subsequent development of active tuberculosis. *Journal of Infectious Diseases, 190*(4), 756-766.

Pablos-Méndez, A., Raviglione, M. C., Laszlo, A., Binkin, N., Rieder, H. L., Bustreo, F., & Chaulet, P. (1998). Global surveillance for antituberculosis-drug resistance, 1994–1997. *New England Journal of Medicine, 338*(23), 1641-1649.

Pai, M., Behr, M.A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C.C., Ginsberg, A., Swaminathan, S., Spigelman, M., Getahun, H., Menzie, D and Raviglione, M (2016). Tuberculosis. *Nature primer vol 2*

Palomino, J. C., & Martin, A. (2014). Drug resistance mechanisms in Mycobacterium tuberculosis. *Antibiotics, 3*(3), 317-340.

Pescarini, J. M., Rodrigues, L. C., Gomes, M. G. M., & Waldman, E. A. (2017). Migration to middle-income countries and tuberculosis—global policies for global economies. *Globalization and health, 13*(1), 15.

Piddock, L. J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical microbiology reviews*, *19*(2), 382-402.

Pule, C. M., Sampson, S. L., Warren, R. M., Black, P. A., van Helden, P. D., Victor, T. C., & Louw, G. E. (2015). Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *Journal of Antimicrobial Chemotherapy*, *71*(1), 17-26.

Pusztahelyi, T., Holb, I. J., & Pocsi, L. (2015). Secondary metabolites in fungus-plant interactions. *Front plant Sci* 6: 573.

Ramón-García, S., Ng, C., Anderson, H., Chao, J. D., Zheng, X., Pfeifer, T., & Thompson, C. J. (2011). Synergistic drug combinations for tuberculosis therapy identified by a novel high throughput screen. *Antimicrobial agents and chemotherapy*, AAC. 00474-00411.

Rani, B. L., Mazumdar, K., Dutta, N. K., & Dastida, N. G (2005). Antibacterial property of the antipsychotic agent prochlorperazine, and its synergism with methdilazine. *Microbiol Res*. 160 (1): 95-100.

Ratnakar, P., & Murthy, S. P. (1992). Antitubercular activity of trifluoperazine, a calmodulin antagonist. *FEMS microbiology letters*, *97*(1-2), 73-76.

Ratnakar, P., Rao, S., Sriramarao, P., & Murthy, P. (1995). Structure-antitubercular activity relationship of phenothiazine-type calmodulin antagonists. *International clinical psychopharmacology*.

Ratnakar, P., & Suryanarayana Murthy, P. (1993). Trifluoperazine inhibits the incorporation of labelled precursors into lipids, proteins and DNA of Mycobacterium tuberculosis H37Rv. *FEMS microbiology letters*, *110*(3), 291-294.

Rodrigues, L., A Ainsa, J., Amaral, L., & Viveiros, M. (2011). Inhibition of drug efflux in mycobacteria with phenothiazines and other putative efflux inhibitors. *Recent patents on anti-infective drug discovery*, *6*(2), 118-127.

Rodrigues, L., Villellas, C., Bailo, R., Viveiros, M., & Aínsa, J. A. (2012). Role of the Mmr efflux pump in drug resistance in M. tuberculosis. *Antimicrobial agents and chemotherapy*, AAC. 01482-01412.

Rodrigues, L., Wagner, D., Viveiros, M., Sampaio, D., Couto, I., Vavra, M., & Amaral, L. (2008). Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in Mycobacterium avium and Mycobacterium smegmatis. *Journal of Antimicrobial Chemotherapy (JAC)*, *61*(5).

Rossi, E. D., Aínsa, J. A., & Riccardi, G. (2005). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS microbiology reviews*, *30*(1), 36-52.

Saga, T., Yamaguchi, K. (2009). History of antimicrobial agents and resistant bacteria. JMAJ.52(2):103-8.

Salvatore, P. P., Becerra, M. C., Abel zur Wiesch, P., Hinkley, T., Kaur, D., Sloutsky, A., & Cohen, T. (2015). Fitness costs of drug resistance mutations in multidrug-resistant Mycobacterium tuberculosis: a household-based case-control study. *The Journal of infectious diseases, 213*(1), 149-155.

Sandberg, M., Määttänen, A., Peltonen, J., Vuorela, P. M., & Fallarero, A. (2008). Automating a 96-well microtitre plate model for Staphylococcus aureus biofilms: an approach to screening of natural antimicrobial compounds. *International journal of antimicrobial agents, 32*(3), 233-240.

Schluger, N. W., & Burzynski, J. (2010). Recent advances in testing for latent TB. *Chest, 138*(6), 1456-1463.

Singh, A. (2017). Guardians of the mycobacterial genome: A review on DNA repair systems in Mycobacterium tuberculosis. *Microbiology, 163*(12), 1740-1758.

Slack, M. D., Martinez, E. D., Wu, L. F., & Altschuler, S. J. (2008). Characterizing heterogeneous cellular responses to perturbations. *Proceedings of the National Academy of Sciences, 105*(49), 19306-19311.

Smith, T., Wolff, K. A., & Nguyen, L. (2012). Molecular biology of drug resistance in Mycobacterium tuberculosis *Pathogenesis of Mycobacterium tuberculosis and its Interaction with the Host Organism* (pp. 53-80): Springer.

Stone, S. R., Montgomery, J., & Morrison, J. F. (1984). Inhibition of dihydrofolate reductase from bacterial and vertebrate sources by folate, aminopterin, methotrexate and their 5-deaza analogues. *Biochemical pharmacology, 33*(2), 175-179.

Szumowski, J. D., Adams, K. N., Edelstein, P. H., & Ramakrishnan, L. (2012). Antimicrobial efflux pumps and Mycobacterium tuberculosis drug tolerance: evolutionary considerations *Pathogenesis of Mycobacterium tuberculosis and its Interaction with the Host Organism* (pp. 81-108): Springer.

Tabatabaei, M., & Sohrabi, N. (2017). Comparison of biofilm formation and antibiotic resistance pattern of Pseudomonas aeruginosa in human and environmental isolates. *Microbial pathogenesis, 109*, 94-98.

Tajkarimi, M., Ibrahim. S., & Cliver, D (2010). Antimicrobial herb and spice compounds in food. Food Control. 2010;21(9):1199-218

van Ingen, J. (2011). The broad-spectrum antimycobacterial activities of phenothiazines, in vitro: somewhere in all of this there may be patentable potentials. *Recent patents on anti-infective drug discovery, 6*(2), 104-109.

Van Soolingen, D., Hernandez-Pando, R., Orozco, H., Aguilar, D., Magis-Escurra, C., Amaral, L., & Boeree, M. J. (2010). The antipsychotic thioridazine shows promising therapeutic activity in a mouse model of multidrug-resistant tuberculosis. *PloS one, 5*(9), e12640.

Vesenbeckh, S., Krieger, D., Bettermann, G., Schönfeld, N., Bauer, T. T., Rüssmann, H., & Mauch, H. (2016). Neuroleptic drugs in the treatment of tuberculosis: Minimal inhibitory concentrations of different phenothiazines against Mycobacterium tuberculosis. *Tuberculosis, 98*, 27-29.

Viveiros, M., Martins, M., Rodrigues, L., Machado, D., Couto, I., Ainsa, J., & Amaral, L. (2012). Inhibitors of mycobacterial efflux pumps as potential boosters for anti-tubercular drugs. *Expert review of anti-infective therapy, 10*(9), 983-998.

Wang, C., Wang, J., Huang, Y., Chen, H., Lee, Y., Zhong, L., Chen, Y., Chen, S., Wang, J., Kang, J., Peng, Y., Yang, B., Lin, Y., She, Z., & Lai, X (2013). Antimycobacterial activity of marine fungus-derived 4-deoxybostrycin and nigrosporin. *Molecules*. 18(2): 1728–1740.

Wells, W. F., Ratcliffe, H. L., & Grumb, C. (1948). On the mechanics of droplet nuclei infection; quantitative experimental air-borne tuberculosis in rabbits.*Am J Hyg*. 47 (1): 11-28.

Wehrli, W. (1983). Rifampin: mechanisms of action and resistance. *Reviews of infectious diseases, 5*(Supplement_3), S407-S411.

Weinstein, E. A., Yano, T., Li, L.-S., Avarbock, D., Avarbock, A., Helm, D., & Rubin, H. (2005). Inhibitors of type II NADH: menaquinone oxidoreductase represent a class of antitubercular drugs. *Proceedings of the National Academy of Sciences, 102*(12), 4548-4553.

White, R., Lancini, G., & Silvestri, L. (1971). Mechanism of action of rifampin on Mycobacterium smegmatis. *Journal of bacteriology, 108*(2), 737-741.

The treatment of tuberculosis guidelines (2010). *World Health Organization*. WHO/HTM/TB/2009.420 WHO, Geneva. 4th edition. pp 30-33.

Global Tuberculosis report (2015). *World Health Organisation*. WHO/ HTM/ TB/ 2015. 22 WHO, Geneva. 20th edition. pp 18.

Global Tuberculosis report 2016. *World Health Organisation*. WHO/ HTM/ TB/ 2016.13 WHO, Geneva. pp 1.

Global Tuberculosis report 2018. *World Health Organisation*. WHO/ CDS/ TB/ 2018. 20 WHO, Geneva. pp 1.

Xu, J., Tasneen, R., Peloquin, C. A., Almeida, D. V., Li, S.-Y., Barnes-Boyle, K., & Nuermberger, E. (2017). Verapamil increases the bioavailability and efficacy of bedaquiline but not clofazimine in a murine model of tuberculosis. *Antimicrobial agents and chemotherapy*, AAC. 01692-01617.

Yadav, S. (2015). A new concept in tuberculosis awareness in the low income countries. *Edorium J Tuberc, 5*, 1-4.

Yadav, S., & Rawal, G. (2016). Primary extrapulmonary multidrug-resistant tuberculosis of the sternum without HIV infection. *Journal of clinical and diagnostic research: JCDR, 10*(1), RD01.

Yano, T., Li, L.-S., Weinstein, E., Teh, J.-S., & Rubin, H. (2006). Steady-state kinetics and inhibitory action of antitubercular phenothiazines on Mycobacterium tuberculosis type-II NADH-menaquinone oxidoreductase (NDH-2). *Journal of Biological Chemistry, 281*(17), 11456-11463.

Žgur-Bertok, D. (2013). DNA damage repair and bacterial pathogens. *PLoS pathogens, 9*(11), e1003711.

Zhai, B., Wu, C., Wang, L., Sachs, M. S., & Lin, X. (2012). The antidepressant sertraline provides a promising therapeutic option for neurotropic cryptococcal infections. *Antimicrobial agents and chemotherapy*, AAC. 00212-00212.

Zhang, Q., Wu, Z., Zhang, Z., Sha, W., Shen, X., & Xiao, H. (2016). Efficacy and effect of free treatment on multidrug-resistant tuberculosis. *Experimental and therapeutic medicine, 11*(3), 777-782.

Zhang, Y., Wade, M. M., Scorpio, A., Zhang, H., & Sun, Z. (2003). Mode of action of pyrazinamide: disruption of Mycobacterium tuberculosis membrane transport and energetics by pyrazinoic acid. *Journal of Antimicrobial Chemotherapy, 52*(5), 790-795.

APPENDIX

A 1.0: Components of Middlebrook 7H9 broth base (Sigma Aldrich)

Ammonium sulfate (0.50 g/L), Disodium phosphate (2.50 g/L), Monopotassium phosphate (1.00 g/L), Sodium citrate (0.10 g/L), Magnesium sulfate (0.05 g/L), Calcium chloride (0.0005 g/L), Zinc sulfate (0.001 g/L), Copper sulfate (0.001 g/L), Ferric ammonium citrate (0.04 g/L), L-Glutamic acid (0.50 g/L), Pyridoxine (0.001 g/L) and Biotin (0.0005 g/L)

A 1.1 Preparation of 7H9 broth for culturing mycobacteria

According to manufacturer's instructions, Middlebrook 7H9 broth was prepared by adding 50 ml of distilled water to 0.261 g (0.522%) 7H9 in a conical flask. The resulting suspension was supplemented with 0.0425 g (0.085%) NaCl, 125 µL (0.25%) of 20% Tween 80 and 220 µL (0.44%) glycerol.

A 1.2 Components of Middlebrook 7H10 agar base (Sigma Aldrich)

Ammonium sulfate (0.50 g/L), L-Glutamic acid (0.50 g/L), Monopotassium phosphate (1.50 g/L), Disodium phosphate (1.50 g/L), Sodium citrate (0.40 g/L), Ferric ammonium citrate (0.04 g/L), Magnesium sulfate (0.025 g/L), Calcium chloride (0.0005 g/L), Zinc sulfate (0.001 g/L), Copper sulfate (0.001 g/L), Pyridoxine hydrochloride (0.001 g/L), Biotin (0.0005 g/L), Malachite green (0.00025 g/L) and Agar (15.00 g/L)

A 1.3 Preparation of 7H10 agar medium for culturing mycobacteria

According to manufacturer's instructions, Middlebrook 7H10 agar media was prepared by adding 1.9g 7H10 to 100 mL distilled water to make 1.9% 7H10. The resulting suspension was supplemented with 0.5 g (0.5%) dextrose and 0.085 g (0.085%) NaCl.