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# Optimising the efficacy of clofazimine against biofilm-encased *Mycobacterium tuberculosis*

**by**

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**Master of Science**

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**The Department of Immunology**

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**DECLARATION**

I declare that the work contained in this dissertation is my own original work and has not been presented for a degree in any other institution. It is submitted in fulfillment of the requirements for Master of Science degree at the University of Pretoria.

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**PRESENTATIONS**

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**ABSTRACT**

**Background**: The chemotherapy of tuberculosis (TB) patients is administered for a six to nine-month period consisting of an intensive phase during the first two months with four primary anti-TB drugs, rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), followed by a continuation phase during the remaining four to seven months with RMP and INH. During the intensive phase the active-replicating organisms (AR) are effectively and rapidly eliminated (99% killing), while the slow-replicating (SR) / non-replicating (NR) populations are targeted during the continuation phase. These latter bacterial populations respond poorly to treatment and are often associated with disease reactivation and relapse in treated patients, highlighting the necessity of identifying effective antimicrobial agents against these bacteria. Clofazimine (CFZ) has demonstrated high antimycobacterial activities against the AR, SR and NR microbial populations *in vitro*. However, its effects in combination with the primary drugs against these bacteria have not been demonstrated.

**Aim and objectives**: To investigate the antimycobacterial activity of CFZ in combination with primary anti-TB drugs against the AR and SR organisms isolated in planktonic and biofilm-forming cultures respectively, by evaluating their inhibitory and bactericidal activities.

**Methods**: The inhibitory activities of CFZ and three primary anti-TB drugs viz. RMP, INH and EMB were evaluated individually and in combination using minimum inhibitory concentration (MIC) and fractional inhibitory concentration index (FICI) determinations by the microtitre Alamar blue assay (MABA) and biofilm formation/ and crystal violet quantification for planktonic and biofilm cultures respectively. The bactericidal activities of these various combinations of the test agents were evaluated using minimum bactericidal concentration (MBC) and fractional bactericidal concentration index (FBCI) determinations by colony-counting procedures.

**Results**: In planktonic cultures, CFZ demonstrated a high inhibitory (MIC: 0.15 µg/mL), but low bactericidal activity (MBC: 5 µg/mL). In combination with primary anti-TB drugs, CFZ demonstrated synergistic inhibitory activities in combination with RMP and INH individually, as well as when the two antibiotics were used together. With respect to bactericidal activity, CFZ exhibited synergistic activity only in a two-drug combination with RMP. Synergistic activities

were also demonstrated in a two-drug combination of RIF and INH and in a three-drug combination of these two antibiotics with EMB.

However, in biofilm-forming cultures, CFZ demonstrated high inhibitory and bactericidal activities, achieving equal MIC and MBC values of 0.15 µg/mL. All CFZ-containing anti-TB drug combinations exhibited synergistic effects, with high activities being shown in combinations containing RIF and INH.

**Conclusion**: CFZ exhibited synergistic effects in combination with primary anti-TB drugs against both planktonic and biofilm-forming cultures, showing potential benefit in promoting treatment outcome when used in TB chemotherapy.

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### List of abbreviations

| | |
|---|---|
| AG | Arabinogalactan |
| AIM2 | Absent in melanoma 2 |
| AR | Actively-replicating |
| ART | Antiretroviral therapy |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| ATPase | Adenosine triphosphatase |
| BCG | Bacillus Calmette-Guérin |
| BDQ | Bedaquiline |
| C4H*8*$N_2$O3 | Asparagine |
| $C_6H_8O_7$2 | Citric acid |
| CC | Cysteine-cysteine, β chemokine |
| CD | Cluster of differentiation |
| CFP | Culture filtrate protein |
| CFU | Colony-forming unit |
| CFZ | Clofazimine |
| cGAS | Cyclic GMP-AMP synthase |
| CISH | Cytokine-inducible SH2-containing protein |
| CLDIs | Crystal-like drug inclusions |
| $CO_2$ | Carbon dioxide |
| CXC | cysteine-cysteine, α chemokine |
| D0 | Day 0 |
| D6 | Day 6 |
| D7 | Day 7 |
| DC | Dendritic cells |
| $dH_2O$ | Distilled water |
| DIM | Dimycocerosates |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |

| | |
|---|---|
| DS-TB | Drug-susceptible tuberculosis |
| EMB | Ethambutol |
| EPS | Extracellular polymeric substance |
| ESAT-6 | Early secretory antigenic target 6 |
| ESX-1 | ESAT-6 secretion system 1 |
| FBC | Fractional bactericidal concentration |
| FBCI | Fractional bactericidal concentration index |
| $FeH_4N_5O_{12}$ | Ferric ammonium nitrate |
| FIC | Fractional inhibitory concentration |
| FICI | Fractional inhibitory concentration index |
| Gal8 | Galectin 8 |
| GI | Gastrointestinal |
| GTPase | Guanosine triphosphatase |
| HIV | Human immunodeficiency virus |
| IFN | Interferon |
| IGRAs | Interferon gamma release assays |
| IL | Interleukin |
| INH | Isonicotinyl hydrazide (isoniazid) |
| IPT | Isoniazid preventive therapy |
| $K^+$ | Potassium cation |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| LAM | Lipoarabinomannan |
| LRRCR | Leucine-rich repeat-containing receptors |
| LM | Lipomannan |
| M cells | Microfold cells |
| *M. bovis* | *Mycobacterium bovis* |
| *M. tuberculosis* | *Mycobacterium tuberculosis* |
| MABA | Microtitre Alamar Blue Assay |
| Man-LAM | Mannose-capped-lipoarabinomannan |
| MBC | Minimum bactericidal concentrations |
| mDCs | Mononuclear dendritic cells |

| | |
|---|---|
| MDR-TB | Multidrug-resistant tuberculosis |
| MGIT | Mycobacterial growth indicator tube |
| $MgSO_4$ | Magnesium sulphate |
| MHC | Major histocompatibility complex |
| MIC | Minimum inhibitory concentration |
| MIP | Monocyte inflammatory protein |
| MODS | Microscopic observation drug susceptibility |
| MPI | Mannosyl-phosphatidyl-myo-inositol |
| *Mtb* | *Mycobacterium tuberculosis* |
| NAA | Nucleic acid amplification |
| NADH | Nicotinamide adenine dinucleotide |
| NaOH | Sodium hydroxide |
| NHLS | National Health Laboratory Services |
| NICD | National Institute for Communicable Disease |
| NK | Natural killer |
| NLR | Nucleotide-binding oligomerization domain and |
| NR | Non-replicating |
| NRF | National Research Foundation |
| NRLP3 | NLR family pyrin domain containing 3 |
| OADC | Oleic acid, dextrose, catalase |
| OD | Optical density |
| PAS | Para-amino salicylic acid |
| PBS | Phosphate buffered saline |
| PDIM | phthiocerol dimycocerosates |
| pH | Potential of hydrogen |
| PRR | Pattern recognition receptors |
| PTPA | Protein tyrosine phosphatase A |
| PZA | Pyrazinamide |
| Rab GTPase | Ras-related in brain GTPase |
| RMP | Rifampicin |
| RNA | Ribonucleic acid |

| | |
|---|---|
| ROS | Reactive oxygen species |
| SA | South Africa |
| SAMRC | South African Medical Research Council |
| SANAC | South African National AIDS Council |
| SapM | Secreted acid phosphate M |
| SD | Standard deviation |
| SM | Streptomycin |
| STATS SA | Statistics South Africa |
| TACO | Tryptophan aspartate-containing coat protein |
| TB | Tuberculosis |
| TDR-TB | Totally drug-resistant tuberculosis |
| TNF-α | Tumour necrosis factor-alpha |
| TST | Tuberculin skin test |
| USA | United States of America |
| UV | Ultraviolet |
| W0 | Week 0 |
| W5 | Week 5 |
| WHO | World Health Organization |
| XDR-TB | Extensively drug-resistant tuberculosis |
| ZN | Ziehl-Neelsen |
| $ZnS0_4$ | zinc sulphate |

**List of signs**

| | |
|---|---|
| % | Percent |
| µg | Microgram |
| mg | Milligram |
| mL | Millilitre |
| α | Alpha |
| γ | Gamma |

## INTRODUCTION TO THE STUDY

### Problem statement and rationale

Tuberculosis (TB) is an infectious disease that is responsible for high morbidity and mortality globally. The high disease burden is complicated by human immunodeficiency virus (HIV)-TB co-infection and the emergence of drug-resistant (DR)-TB isolates in patients, challenging the management of the disease (WHO, 2018).

Chemotherapy of TB, constituting of four primary anti-TB agents, viz. rifampicin (RMP), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), has been shown to be the most effective control measure of the disease resulting in significant reductions in the number of transmissible cases. However, treatment of TB patients is not always effective, as a result of non-compliance due to the long duration of therapy and this, in turn, resulting in relapse, as well as emergence of drug resistance in isolates, necessitating the search for effective, either new or repurposed, anti-TB agents.

Clofazimine (CFZ), which was originally synthesized as an anti-TB drug in the 1950s, but which is now routinely used for the treatment of leprosy, has recently been repurposed for the treatment of TB patients (Barry, 1957; WHO, 2018; Singh *et al*., 2019). Due to its impressive *in vitro* antimicrobial activity against different strains of *Mycobacterium tuberculosis* (*Mtb*) with varying drug resistance profiles and metabolic status, such as the actively-replicating (AR), slow-replicating (SR) or non-replicating (NR), CFZ has been included in the treatment regimens of DR-TB patients (Mothiba *et al*., 2015; Cholo *et al*., 2017; Berube and Parish, 2018; WHO, 2018). However, despite its application in chemotherapy, information on CFZ antimicrobial activities in combination with other anti-TB agents against these bacterial populations is limited.

The AR and SR/NR microbial populations have been isolated *in vitro* in planktonic and biofilm cultures respectively. This issue represents the focus of the current study in which the antimicrobial effects of CFZ, in combination with primary anti-TB drugs, has been investigated against AR planktonic and SR biofilm-forming organisms.

### Hypothesis

Clofazimine potentiates the antimycobacterial activity of primary anti-TB agents against planktonic and biofilm-forming *Mtb* bacteria.

## Aim and objectives

### Aim

The aim of this study was to determine the antimicrobial potency of CFZ alone and in combination with primary anti-TB chemotherapeutic agents against *Mtb* planktonic and biofilm cultures.

### Objectives

To determine the inhibitory and bactericidal activities of CFZ in combinations with primary anti-TB agents against these cultures by determining their:

i) minimum inhibitory concentrations (MIC) individually
ii) fractional inhibitory concentration index (FICI) values in combinations,
iii) minimum bactericidal concentration (MBC) individually and
iv) fractional bactericidal concentration index (FBCI) values, in combinations

## Scope and limitations

The current study consisted of two phases, which included the evaluation of the activities of the antibiotics in planktonic and biofilm-forming cultures. In planktonic cultures, the MIC of the individual agents, and the fractional inhibitory concentrations indices of the combinations of these agents were determined using the microtitre Alamar blue assay (MABA) procedures while in biofilm-forming cultures these effects were determined by biofilm quantification using visual examination and crystal violet procedures. Bactericidal activities of the antibiotics against both cultures were evaluated by determination of MBC of the individual agents and the fractional bactericidal interaction indices of these agents, using colony-counting procedures. The results showed that the inclusion of CFZ had led to improvement to antimicrobial activities of primary drugs against both planktonic and biofilm-forming cultures, being more effective against the latter bacterial population. These results demonstrated the potential useful information on the impact of CFZ on treatment regimens for TB. However, despite this impact in chemotherapy, the effects of CFZ were not evaluated against PZA, which is also used in TB chemotherapy as well as the NR bacteria, which are also found in TB lesion. Despite this, the research presented in this study, is also original and will be submitted for publication in international, peer-reviewed journals.

**Organisation of the thesis**

This dissertation includes a comprehensive review of past and current literature in Chapter One, illustrating the magnitude of the burden of TB and its control measures, which include treatment strategies. This is followed by a detailed description of the properties of CFZ in Chapter Two. In the three subsequent chapters (Chapters Three to Five) the methodologies used and results on the effects of CFZ and the primary anti-TB agents on planktonic and biofilm *Mtb* cultures are described. Chapter Six provides a final discussion on the results obtained with limitations of the study being discussed in Chapter Seven.

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# CHAPTER ONE: LITERATURE REVIEW

## 1.1. Background of *Mycobacterium tuberculosis*

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis (Mtb)* bacterium. The bacteria are inhaled as aerosols from the TB-infected person and infect the lungs (Esteban and Muñoz-Egea, 2016; Esteban and García-Coca, 2018).

In immunocompetent persons, infection is stopped from spreading, but the bacteria remain viable, resulting in latent disease, which is characterised by absence of development of symptoms. However, in immunocompromised individuals, the bacteria multiply, leading to manifestation of disease resulting in the development of active pulmonary TB (Jilani *et al*., 2019). The symptoms include, but are not limited to, a cough that lasts for more than three months, chest pain, sometimes coughing of blood, tiredness, night sweats, chills and fever, loss of appetite and weight. The disease can also spread to other parts of the body such as the brain and spine causing extra-pulmonary TB (Xu *et al*., 2018).

## 1.2. *Mycobacterium tuberculosis* bacterium

The *Mtb* bacteria belong to the species Mycobacterium, characterized by rod-shaped acid-fast bacilli on Ziehl-Neelsen staining. The bacteria are also weak Gram-positive bacilli (Barksdale and Kim, 1977; Abdelaziz *et al*., 2016; Dzodanu et al., 2019). The organisms grow slowly in aerobic conditions in the presence of minimum levels of carbon dioxide ($CO_2$) (5 - 10%) forming aggregates due to cord formation (Figure 1.1).

The *Mtb* bacterium is approximately 0.2 mm in length and has a cell wall, which is rich in lipids (Cook *et al*., 2009; Pasechnik *et al*., 2017).

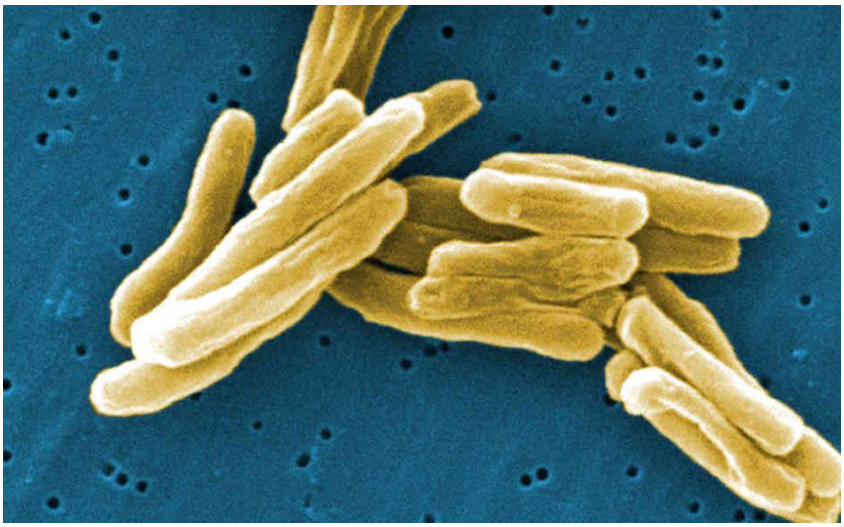

**Figure 1.1:** The cross-sectional structure of *Mycobacterium tuberculosis* (Smith *et al*., 2019, with permission).

### 1.2.1. Cell wall structure of *Mycobacterium tuberculosis*

The cell wall structure of *Mtb* consists of three segments viz. the outermost layer, cell wall core and plasma membrane (Figure 1.2) (Kaur *et al*., 2009; Maitra *et al*., 2019).

The outermost layer consists of lipomannan (LM), mannose-capped lipoarabinomannan (Man-LAM), and glycoproteins with mannan and arabinomannan on the surface (Grzegorzewicz *et al*., 2016). These molecules are anchored to the plasma membrane by mannosyl-phosphatidyl-*myo*-inositol (MPI) (Barry *et al*., 2017).

The cell wall core is made up of mycolic acids, arabinogalactan (AG) and peptidoglycan attached together covalently by phosphoryl-*N*-acetyl-glucosaminosylrhamnosyl linkage units (Vincent *et al*., 2018). The *Mtb* mycolic acids are β-hydroxyl fatty acids with long α-alkyl side chains (Slama *et al*., 2016), providing protection to the cell from oxidative stress (Minnikin, 1982; Shang *et al*., 2018; Barry *et al*., 2017). The AG is a polymeric structure comprised of D-galactofuranose and D-arabinofuranose polysaccharides, providing the cell with integrity (rigidity) and permeability to external factors such as antibiotics (Olmos *et al*., 2017). The peptidoglycan is also a polymeric structure consisting of glycan sugars and alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid amino acids (Barreteau *et al*., 2008; Cava *et al*., 2018). Its function is to provide shape, strength and protection to the cell wall (Radkov *et al*., 2018).

The plasma membrane is the inner structure of a cell wall made up of a phospholipid bilayer consisting of various fatty acids and proteins. Its function is to protect the cell from penetration by external elements (Cholo *et al*., 2017).

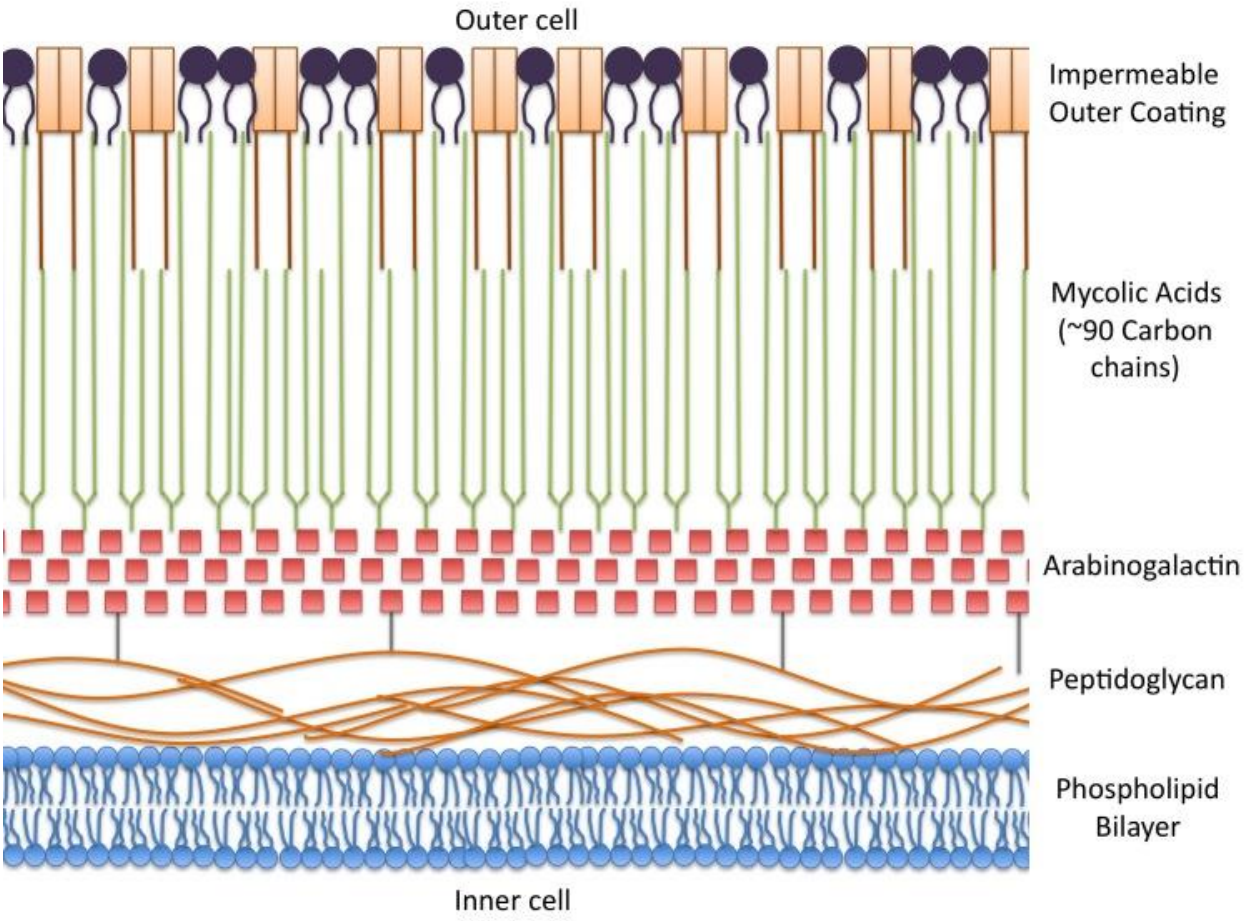


**Figure 1.2:** Basic structure of the *Mycobacterium tuberculosis* cell wall (Smith, 2011, with permission).

### 1.3. History of tuberculosis

Tuberculosis is an ancient disease dating back millions of years (Pezzella *et al*., 2019). The first evidence of human TB was discovered in a 500 000year-old skull in Turkey (Kappelman *et al*., 2008). Additionally, historic presence of the disease in humans had been shown in Europe and Asia with the detection of TB in several burial sites (Donoghue, 2004; Hershkovitz *et al*., 2008; Nicklisch *et al*., 2012; Barberis *et al*., 2017).

In 1882, TB was discovered as an infectious disease by Robert Koch, by the visual detection of the microorganism using his invented microscopy-based staining technique, showing that the microorganisms are rod-shaped entities, appearing as groups within the lesions which failed acid-alcohol decolorisation (acid-fast) (Kapur *et al*., 1994; Murray *et al*., 2016). The staining technique was later modified in 1885 to Ziehl-Neelson (ZN) staining as it is currently known. Robert Koch further isolated the tubercle bacilli from a TB lesion using a solid medium that he developed (Cambau and Drancourt, 2014; Murray *et al*., 2016). He later confirmed that TB is transmissible by reproducing the disease in a non-infected animal, transmitting the disease from patients to rabbits, cow to rabbit and rabbit to rabbit, findings which were demonstrated by the French army doctor, Jean-Antoine Villemin (Villemin, 1865; Villemin, 1868; Koch, 1882; Sakula, 1982; Cambau and Drancourt, 2014; Murray *et al*., 2016). These discoveries led to other milestones in the history of TB, which the development of intervention strategies in reducing the spread of the disease, affording him the award of the Nobel Prize in Physiology or Medicine in 1905 (Philip, 1913; Clayson, 1957; Ehrlich, 1967; Murray *et al*., 2016).

Subsequently, the tuberculin skin test (TST), which involves the development of delayed hypersensitivity reactions in suspected TB-infected persons following injection with a purified protein mixture of TB bacteria was discovered. This procedure has since been adopted for testing exposure to TB (Mantoux, 1910). Currently, in the post-human immunodeficiency virus (HIV) era, the efficacy of the test is being reviewed as its performance and sensitivity is affected by the HIV status of the TB-infected persons (Rangaka *et al*., 2012; Tesfaye *et al*., 2018).

Later, in 1920, a TB vaccine was developed by Leon Charles Albert Calmette and Camille Guerin, who discovered that a subculture of *Mycobacterium bovis* (*M. bovis*) loses virulence after several passages. This culture was then used for the preparation of the Bacillus Calmette-Guerin (BCG) vaccine, named after the discoverers, which has since been used for the immunization of TB-unexposed and exposed individuals for protection from development of TB disease (Calmette and Guerin, 1920; Calmette *et al*., 1924). This vaccine has remained the only available vaccine against TB to date.

Furthermore, in 1943; an antibiotic era in TB began with the discovery of streptomycin (SM) by Albert Schatz and Selman Waksman. This was later followed by the discovery of other anti-TB agents, including isoniazid (INH), rifampicin (RMP) and pyrazinamide (PZA). These antibiotics have since being used for the development of the current treatment regimen for the

chemotherapy of TB and are effective in the treatment of drug-sensitive (DS)-TB patients with achievable success rates of > 95% (Schatz *et al*., 2005; Ahmad, 2011). However, due to the emergence of drug-resistant (DR)-TB isolates, TB has remained a major public health problem (Dye *et al*., 2008; Barberis et al., 2017).

Concurrent to the drug resistance era, was the co-infection of TB in HIV-infected persons. This resulted in an increase in the global burden of TB, undermining previous control measures as patients presented with altered symptoms and disease response to treatment (Gezae *et al*., 2019*)*.

### 1.4. The global tuberculosis burden

TB is a major threat to global public health, leading to high mortality and morbidity especially in low socio-economic countries, despite longstanding intense efforts to control the disease. About 23% of the world's population is estimated to have developed latent TB infection with nine million new cases of active TB and 1.3 million TB-associated deaths being reported in 2016 and 2018 (WHO, 2016; WHO, 2018).

Approximately 29% of the global burden of TB cases and 34% of TB-related deaths are reported in Africa (Figure 1.3) (Rebekah, 2017). In sub-Saharan Africa, which contributes to the majority of global TB cases, South Africa accounts for approximately 60% of these cases (WHO, 2016; WHO, 2018).

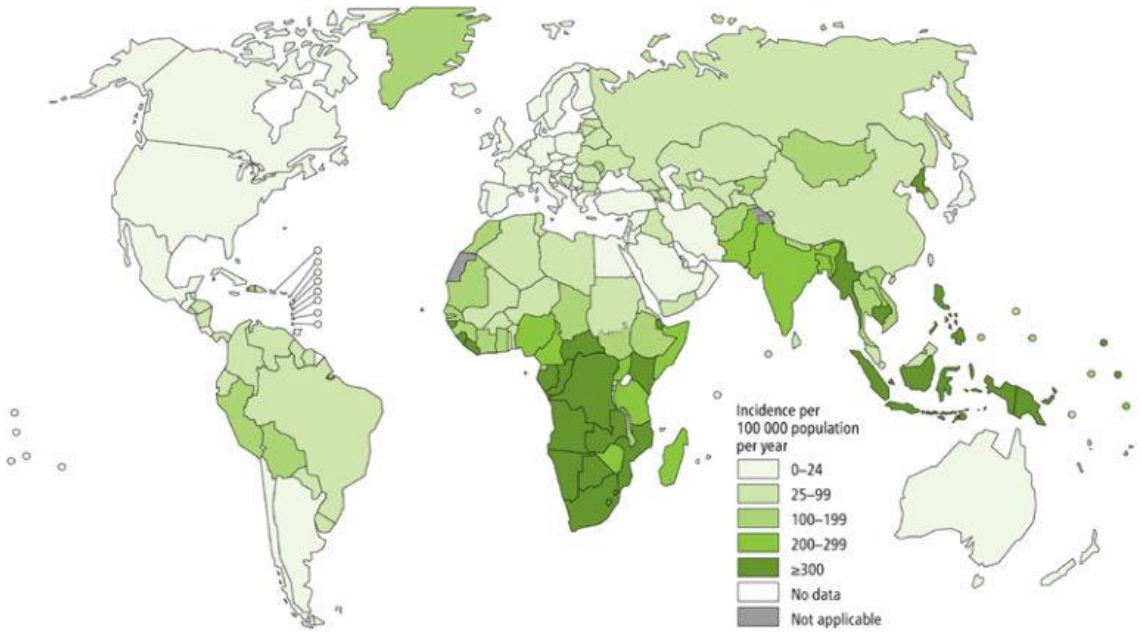


**Figure 1.3:** Estimated tuberculosis incidence rates per 100 000 populations, 2017 (WHO, 2018).

Despite the high burden, the incidence rates of TB have declined since 2016 from 834 to 520 per 100 000 population. This was attributed to the 2020 Sustainable Development Goals efforts (Stats SA, 2018). In South Africa, the number of cases differ in the different provinces with the highest number of cases being reported in the Eastern Cape, KwaZulu-Natal and Western Cape (Figure 1.4) (Loveday *et al*., 2018; WHO, 2018).

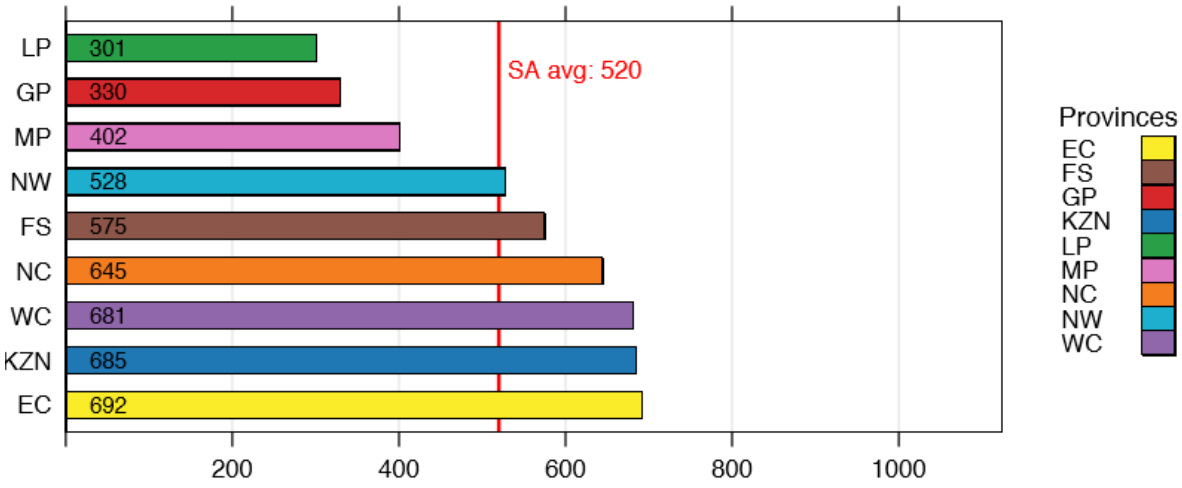


**Figure 1.4:** Incidence of tuberculosis by province in South Africa per 100 000 population, 2016 (Loveday *et al*., 2018, with permission).

Several factors contributing to the burden of TB that have been identified and include long-term persistence of *Mtb*, TB-HIV co-infection, and the emergence of multi- and extensively-drug resistant (M/XDR)-TB strains (Raviglione, 2003; Schumacher *et al*., 2016; NICD, 2017). Other individuals prone to development of TB include immune-compromised people, due to prolonged use of medicines such as tumour necrosis factor-alpha (TNF-α) inhibitors or steroids, and patients with diabetes, renal insufficiency and silicosis (Glaziou *et al*., 2015). Furthermore, TB infection is also influenced by low socio-economic status leading to poverty, which is a risk factor for development of ill health (WHO, 2016). The disease is also more common in men than in women and children (WHO, 2018).

### 1.5. Pathogenesis of *Mycobacterium tuberculosis* infection

TB is spread to susceptible persons via aerosols (Shiloh, 2016). The risk of infection is dependent on several factors such as the infectiousness of the source, bacillary load inhaled, the closeness of contact, and the immune status of the potential host (Mathema et al., 2008; Pienaar et al., 2010; Ahmad, 2018). Upon inhalation, larger aerosol particles are trapped in the upper airway or oropharynx where they can potentially cause TB of the oropharynx, while small particles transit past the nasopharyngeal or tracheobronchial regions and are deposited in the distal airways (Fennelly *et al*., 2015; Hinchey *et al*., 2019).

The primary route of TB infection involves the lungs (Figure 1.5) (Shiloh, 2016). Infection begins when the inhaled small airborne particles (droplet nuclei) are deposited in the terminal alveoli, where they are phagocytosed by the resident alveolar macrophages and/or tissue dendritic cells (Wang *et al*., 2011; Shiloh, 2016; Wang *et al*., 2017). Other infected cells include the microfold cells (M cells), alveolar endothelial and pneumocytes (Hunter *et al*., 2018). During this early phase of infection, the bacteria replicate intracellularly leading to infection of other phagocytic cells. These infected cells migrate to the draining pulmonary lymph nodes and activate adaptive immunity, returning to the site of infection (Russel *et al*., 2010; Shiloh, 2016). An effective cell-mediated immune response develops at the site of infection within 2 to 8 weeks after infection, blocking further replication of the bacilli by forming a granuloma (Shiloh, 2016; Hunter *et al*., 2018).

However, the formation of the granuloma does not completely eliminate the bacteria but allows the bacteria to become dormant or non-replicating forming a solid granuloma. These

bacteria are protected from the immune system and persist in the host for a long time (Kaufmann *et al*., 2015; Lee *et al*., 2016). This type of infection is commonly found in latent disease. Upon TB reactivation, active disease takes place leading to development of necrotic and caseous granulomas (Lee *et al*., 2016; Gengenbacher *et al*., 2017).

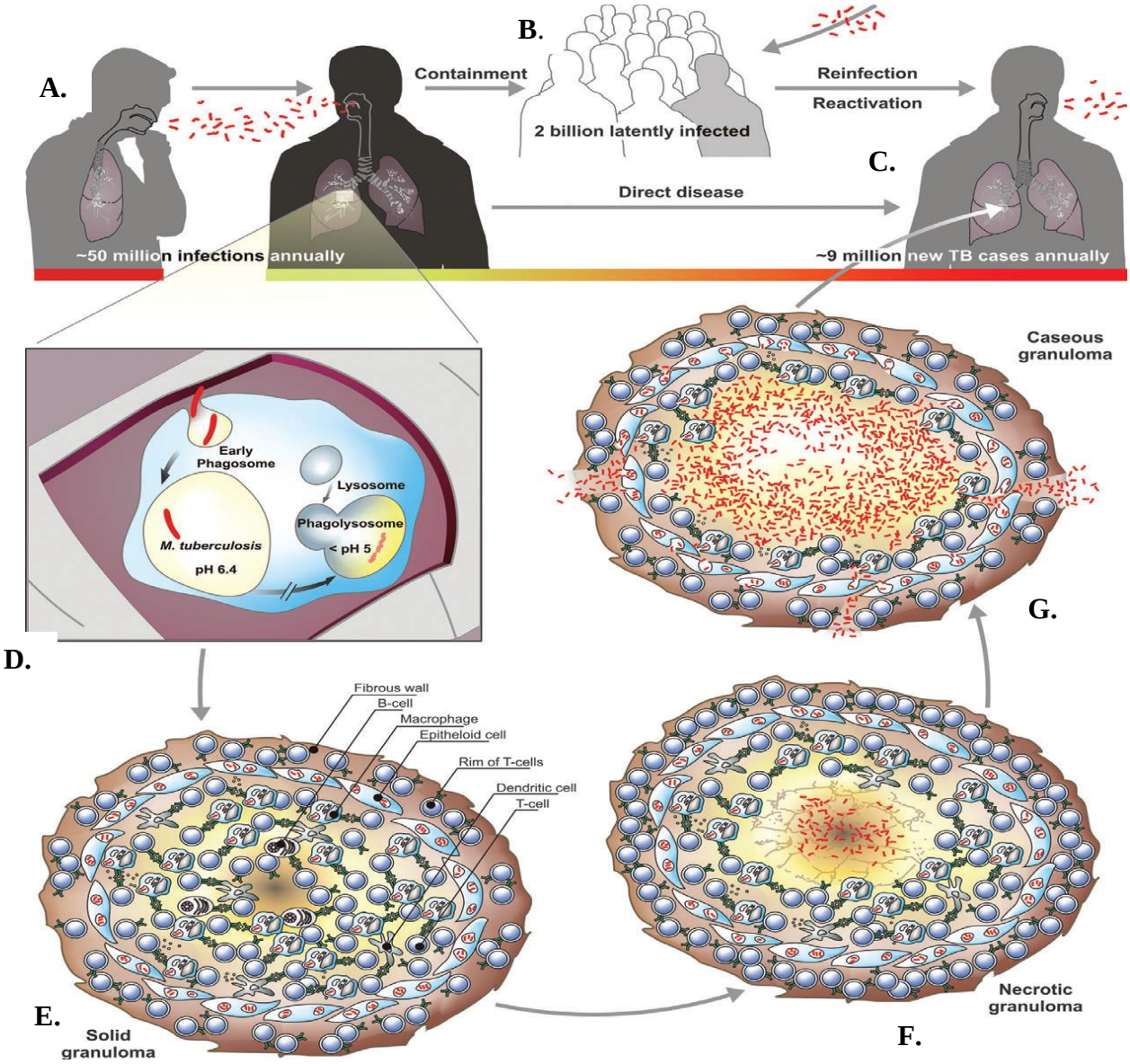


**Figure 1.5:** Transmission and pathology of tuberculosis. A) Transmission of TB between individuals via aerosols. B) Estimated number of people infected due to transmission. C) Number of people who develop the disease. D) Intracellular survival in alveolar macrophages following phagocytosis. E) Activation of adaptive cell-mediated immunity leading to granuloma formation, resulting in solid granuloma in latent TB infection. F) Maturation of granuloma to necrotic lesions at early stage of active disease. F) Maturation of granuloma to caseous lesions found in active TB patients, releasing bacteria into the airways which are coughed out as infectious aerosols (Gengenbacher *et al.*, 2014, with permission).

### 1.6. Human host-tuberculosis pathogen interaction during infection

#### 1.6.1. Infection of macrophages

Macrophages are the first line of defense against the invasion of pathogenic bacteria. The bacteria are phagocytosed by macrophages forming phagosomes which undergo maturation from the early to late stage using the vacuolar proton adenosine triphosphatase (ATPase), mediated by the regulatory activity of the Ras-related in brain, guanosine triphosphatase (Rab GTPase) enzymes (Aguet *et al*., 2016; Prashar *et al*., 2017; Queval *et al*., 2017). This results in acidification of the phagosome leading to fusion of the phagosomal vacuole with the lysosome, forming a phagolysosomal vacuole. The acidic pH of the phagolysosomal vacuole is an optimal condition for the activity of the lysosomal digestive enzymes and for production of reactive oxygen species (ROS), promoting elimination of the intracellular bacteria (Prashar *et al*., 2017).

In the macrophages, bacteria are exposed to additional mechanisms such as cytosolic pattern recognition receptors (PRRs), which lead to activation of antibacterial autophagy (Zheng *et al*., 2017). The *Mtb* phagosome also interacts with numerous organelles such as lipid droplets, mitochondria and the endoplasmic reticulum, which have antibacterial properties (Tobin, 2015; Guiterrez *et al*., 2019).

Additionally, the host utilizes induction of apoptosis, which leads to infected host cell death, resulting in elimination of infection. Apoptosis development leads to cell body shrinkage, nuclear condensation, fragmentation and the formation of apoptotic bodies, which are membrane-bound cell fragments, which are rapidly phagocytosed by neighbouring cells and resident phagocytes (Lamkanfi and Dixit, 2017).

However, *Mtb* has developed a wide range of strategies to counteract macrophage defence mechanisms for its survival and growth. These include failure of phagosomal maturation, allowing the phagosome to express features of an immature endosome (phagosomal vacuole), preventing vacuolar acidification, and inhibiting phagolysosomal fusion (Hu *et al*., 2017).

The factors utilized by bacteria for intracellular survival include production of lipoarabinomannan (LAM), secreted acid phosphate M (SapM) and protein tyrosine phosphatase A (PTPA), all of which lead to the arrest of phagosomal acidification by excluding the vacuolar proton ATPase from the phagosomal membrane (Astarie-Dequeker *et al*., 2009; Augenstreich *et al*., 2017; Burbaud *et al*., 2017). Additionally, the bacteria release dylinositol mannosides (DIM)/ phosphatidylinositol mannosides (PDIM) that allow bacterial access to the cytosol, leading to host

cell death or necrosis. This in turn allow for bacterial escape and spread of infection (Figure 1.6) (Andreu *et al*., 2010; Augenstreich *et al*., 2017; Groschel *et al*., 2017).

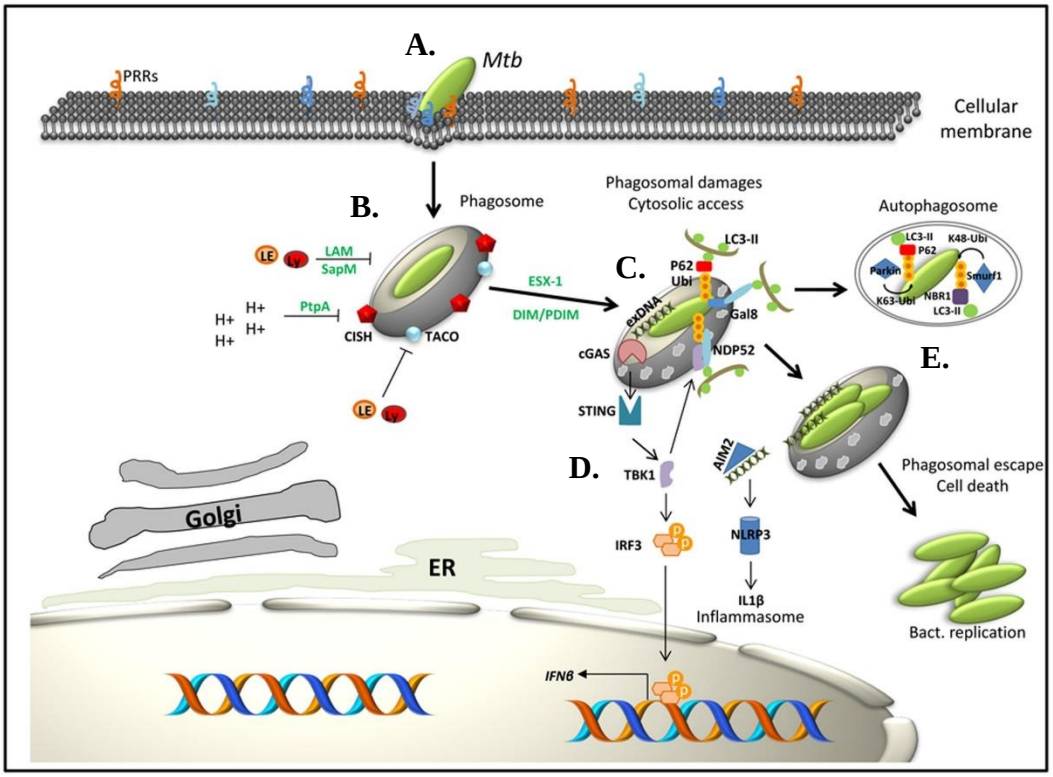


**Figure 1.6:** Macrophage effector and bacterial intracellular survival mechanisms.

In green: bacterial factors; in black: macrophage factors. A) Interaction of *Mtb* with macrophage PRRs, triggering bacteria internalization, B) Inhibition of phagosome maturation and acidification through the expression or secretion of bacterial factors LAM, SapM, PTPA or by subverting host pathways such as tryptophan aspartate-containing coat protein (TACO) or cytokine-inducible SH2-containing protein (CISH) signaling, C) Bacterial cytosolic access through the action of the ESAT-6 secretion system 1 (ESX-1) secretion system and DIM)/ PDIM, D) Bacteria are targeted by ligases (Parkin, Smurf1) and/or activate specific cytosolic recognition pathways (Galectin 8 (Gal8), cyclic GMP-AMP synthase (cGAS), or absent in melanoma-2 (AIM2)), which induces expression of type I interferon (IFN), activation of the nucleotide-binding oligomerization domain and leucine-rich repeat-containing receptors (NLR) family pyrin domain containing 3 (NRLP3) inflammasome and initiation of autophagy and E) *Mtb* induces host cell death programs (apoptosis and necrosis) enhancing bacterial dissemination (Queval *et al*., 2017, with permission).

### 1.6.2. Control of infection in granuloma

The granuloma which creates a microenvironment where the infection can be controlled is the hallmark of TB disease (Qualls *et al*., 2010; Danielle and Heimall, 2017). The formation of the granuloma begins with aggregation of different subsets of cells from the macrophage/monocyte lineage, which include the blood-derived monocytes, epithelioid cells and multinucleated giant cells formed by fused macrophages, surrounded by T lymphocytes (Puissegur *et al*., 2001; Danielle and Heimall, 2017; Schwenkenbecher *et al*., 2018).

Other cells found in the granuloma of TB patients are the monocyte-derived dendritic cells (mDCs), found at the periphery of the granuloma lesion containing fewer bacteria (Pedroza-Gonzalez *et al*., 2004; Gutierrez *et al*., 2019), enabling antigen presentation and expression of major histocompatibility class (MHC)-II and costimulatory molecules (Lussier and Schreiber, 2016). Although the dendritic cells (DCs) are not bactericidal against *Mtb*, they do, however promote bacterial dormancy (Peyron *et al*., 2004; Gutierrez *et al*., 2019). Other cells include neutrophils, which are also involved as a first line of defence against the bacteria, assisting in the killing of the microorganisms by initiating the inflammatory process and induction of oxygen radical production during the early stage of granuloma formation (Zhang *et al*., 1991; Danielle and Heimall, 2017). Others include natural killer (NK) cells (Tsai *et al*., 2016) and B lymphocytes (Maglione *et al*., 2019). Bacterial control in the host is mediated by the activity of the pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and IFN-gamma (γ), which promote the formation and function of the granuloma (Jo *et al*., 2007; Ndlovu and Marakalala, 2016).

The granuloma also provides the mycobacteria with a survival platform. The bacteria survive by modulating the immune response through reduced expression of cysteine-cysteine, β (CC) and cysteine-cysteine, α (CXC) chemokines, preventing the recruitment of macrophages and cluster of differentiation $4^+$ ($CD4^+$) T cells (Adams 1976; Roach *et al*., 2002; Ndlovu and Marakalala, 2016). Infection also leads to the production of anti-inflammatory cytokines such as interleukin (IL)-10, which is a negative regulator of the immune response, leading to the development of necrotic and caseous granuloma lesions (Jo *et al*., 2007; Ndlovu and Marakalala, 2016; Danielle and Heimall, 2017). These lesions result in the release of the bacteria into the extracellular environment, promoting inflammation and tissue damage. The bacteria are also

released into the atmosphere by coughing and exhaling, leading to the spread of infection to susceptible individuals (Wallis *et al*., 2016).

### 1.6.3. Biofilm formation in the host

The formation of biofilm in TB lesions has been reported recently in histological samples from lung cavitary disease (Fennelly *et al*., 2016). The first reports came from cases of TB infection associated with clinical biomaterial (Ha *et al*., 2005; Chakrabory and Kumar, 2019). Biofilms are suggested to play an important role in TB disease by participating in the process of caseous necrosis in cavitary disease in lung tissues (Basaraba and Ojha, 2017; Chakrabory and Kumar, 2019).

*Mtb* develops biofilm *in vitro* (Ojha *et al*., 2008; Mothiba *et al*., 2015; Ojha *et al*., 2015; Chakrabory and Kumar, 2019). The bacteria form pellicles at the air interface of a detergent-free medium. This phenomenon is different from biofilm formation in other bacteria which develop along the material surfaces (Ojha *et al*., 2008; Esteban and García-Coca, 2018). *Mtb* biofilm development requires specific environments such as 5 - 10% $CO_2$ levels, elevated pH of between 7.0 - 8.0, nutrient-limitation and presence of nitric oxide (Kumar *et al*., 2011; Trivedi *et al*., 2012; Basaraba and Ojha, 2017).

The process begins with planktonic bacteria moving to the surface of the growth medium, resulting in the formation of a monolayer of cells on the surface (Chakrabory and Kumar, 2019). The bacteria alter their gene expression from metabolic activities to those responsible for the formation of organized biofilm communities known as sessile bacilli (Basaraba and Ojha, 2017). These organisms release adhesin molecules from the cell wall, which mediate initial attachment of bacteria to the surfaces (Richards and Ojha, 2014; Chakrabory and Kumar, 2019), followed by the synthesis of an extracellular matrix, known as extracellular polymeric substance (EPS), consisting of lipids, predominantly the mycolic acids, glycopeptides, deoxyribonucleic acid (DNA) and other molecules produced by the sessile bacilli. The bacteria aggregate and attach to one another, forming organized communities using intercellular communication via quorum-sensing (Menozzi *et al*., 1996; Zamora *et al*., 2007; Ojha *et al*., 2015; Basaraba and Ojha, 2017).

The *Mtb* bacteria in a mature biofilm are slow replicating (SR) to non-replicating (NR) (Kumar *et al*., 2011; Trivedi *et al*., 2012; Solokhina *et al*., 2017). During TB infection, NR bacteria are found in solid granulomas in latent disease, resulting in a lack of development of signs or

symptoms of the disease. However, these bacteria are activated in cavitation in necrotic and caseous granulomas, causing active TB disease (Ojha *et al*., 2015; Chakrabory and Kumar, 2019).

Biofilm development is an important factor in antimicrobial resistance since these metabolically quiescent cells are drug tolerant (Ciofu *et al*., 2017). Most of the anti-mycobacterial drugs target components of active cell growth. Thus, bacterial drug tolerance leads to treatment failure in TB (Hall-Stoodley *et al*., 2012; Chakrabory *et al*., 2019).

### 1.7. Control of tuberculosis

#### 1.7.1. Diagnosis

Tuberculosis diagnosis involves clinical presentation and laboratory-based procedures. The clinical presentations are detected by procedures such as the tuberculin skin test (TST), chest X-rays and the IFN gamma release assays (IGRA) tests.

However, in the case of laboratory-based techniques, TB diagnosis is performed using the Xpert *Mycobacterium tuberculosis* bacteria (MTB)/Rifampicin, microscopy and molecular techniques.

Most microscopy centers and diagnostic laboratories use the Mycobacterial Growth Indicator Tube (MGIT) liquid culture system for culture and drug susceptibility testing using Xpert MTB/ Rifampicin (Sulis *et al*., 2016). The procedure provides rapid diagnosis and detection of RMP resistance. It is a rapid detection, nucleic acid amplification (NAA) test whereby results can be obtained within two hours and has a high sensitivity of 99% and specificity of 99.2% (Musial *et al*., 2017). Based on these advantages, Xpert technology has been introduced as a replacement for sputum smear microscopy for the diagnosis of pulmonary TB since 2011. About 1.3 million Xpert MTB/Rifampicin tests have been performed in SA between 2011 and 2013, which account for more than half of the global usage of Xpert MTB/Rifampicin (Abdool-Karim *et al*., 2009; WHO, 2016).

The microscopic observation drug susceptibility (MODS) assay is one of the current *Mtb* diagnostic tools and relies on light microscopy to visualize the characteristic cording morphology of *Mtb* in liquid culture. It has a shorter time to culture positivity. However, the effectiveness of MODS in HIV/TB case detection is not yet clearly established (Baya *et al*., 2019).

Spoligotyping is also a diagnostic technique which is used for concurrent detection and typing of *Mtb* complex bacteria (Baya *et al*., 2019). This method relies on amplification of a highly

polymorphic direct repeat locus in the *Mtb* genome and results can be obtained within one day (Abadia *et al*., 2011; Baya *et al*., 2019).

### 1.7.2. Prevention

Prevention of TB has been a neglected aspect of TB control. The current TB prevention strategies include treatment of latent TB infection among high-risk persons, case finding for early detection and treatment of infectious TB, which reduces the duration of infectiousness and transmission, and early antiretroviral therapy (ART) for people living with HIV as well as TB vaccine strategies.

To date, BCG is the only vaccine available for the prevention of TB development. It is made up of live bacteria prepared from an attenuated bovine tuberculosis bacillus, *M. bovis* (Olagunju *et al*., 2016). BCG is one of the most widely used vaccines worldwide, but it has variable efficacy against pulmonary TB in adults (Davenne and McShane, 2016; McIlleron *et al*., 2017).

The risk of infection can also be reduced by infection control measures such as effective hygiene measures. These include covering the mouth and nose when coughing or sneezing, which reduces the spread of TB bacteria, as well as ventilation as TB can remain suspended in the air for several hours after individual has coughed or sneezed (Taha *et al*., 2016). In healthcare settings, the spread of TB is reduced through the use of protective clothing, effective ventilation systems, keeping potentially infectious patients separate from other patients and the regular screening of healthcare workers for TB (Taha *et al*., 2016).

Isoniazid preventive therapy (IPT) is another preventive method. It is given for six months to prevent TB in newborn infants and pregnant women, as well as in HIV-TB co-infected patients (Churchyard *et al*., 2014; McIlleron *et al*., 2017). Alternatively, a combination of RMP and INH is given daily for three months (RH preventive therapy) (Spyridis *et al*., 2007; Getahun *et al*., 2015; Mathivha and Vhelaphi, 2017).

### 1.7.3. Treatment

Treatment of TB has been the most effective control measure for the disease resulting in reduction in the number of cases and prevention of the spread of the disease. Tuberculosis chemotherapy is divided into drug susceptible (DS) and drug resistant (DR)-TB patients.

## 1.8. Chemotherapy of tuberculosis

### 1.8.1. Chemotherapy of drug-susceptible tuberculosis

Drug-susceptible tuberculosis (DS-TB) patients are treated with first-line or primary anti-TB drugs which include RMP, INH, EMB and PZA (WHO, 2018). The initial phase of treatment includes these four primary drugs for the first two months followed by the continuation phase for either four months for the smear-converters or for seven months for the non-smear-converters after two months of therapy, with RMP and INH only (Shakya *et al*., 2012; WHO, 2016). This treatment regimen is usually effective resulting in > 95% success rate in treatment outcome. However, its success depends on patient compliance and early disease diagnosis (Singh *et al*., 2019).

#### 1.8.1.1. Primary anti-tuberculosis drugs

##### 1.8.1.1.1. Rifampicin

Rifampicin (RMP) was discovered in 1966 as a very potent anti-TB drug and its structure is given in Figure 1.7. It has high activity against Gram-positive bacteria and *Mtb* with low minimum inhibitory concentrations (MICs) (0.05-0.5 μg/mL), killing both actively-replicating (AR) and NR bacteria. It is a lipid soluble anti-TB agent and is able to penetrate cell membranes. It kills bacteria by inhibiting deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase in bacterial cells, binding to the β-subunit of the enzyme, thus preventing transcription of RNA and subsequent translation of proteins (Aristoff *et al*., 2010; Kora *et al*., 2018). Resistance to RMP is associated mostly with mutations to the *rpoB* gene (Chen *et al*., 2019).

Patients are given a daily regimen of 10 mg/kg (up to 600 mg/day) orally (Buchan *et al*., 2018). Treatment with RMP is not without its side-effects. These include hepatitis with elevation of bile and bilirubin, anaemia, bleeding, fever, eosinophilia, leukopaenia, thrombocytopaenia, purpura, haemolysis and nephrotoxicity (Peters et al., 2019). No serious side-effects have been observed in breastfed infants during RMP therapy (Drobac *et al*., 2005; Peters *et al*., 2019).

##### 1.8.1.1.2. Isoniazid

Isoniazid (INH) was discovered in 1952 (structure given in Figure 1.7). It has shown high inhibitory (bacteriostatic) and bactericidal activities against AR *Mtb* bacteria with MIC and minimum bactericidal concentration (MBC) of 0.003 to 0.01 μg/mL and 0.01 to 0.2 μg/mL, respectively, however it has shown poor anti-mycobacterial activity against SR or NR

mycobacteria. It is used in the treatment of latent TB and more frequently utilized in IPT (Nusrath *et al*., 2016).

Isoniazid is a pro-drug and is activated by the mycobacterial enzyme catalase-peroxidase (KatG), which catalyzes the formation of the isonicotinic acyl-NADH complex. Resistance to isoniazid occurs due to mutations in several genes, which include *katG*, *ahpC*, *inhA*, *kasA* and *ndh* (Shakya *et al*., 2012; Stagg *et al*., 2017).

The recommended daily dose of INH is 8 - 12 mg/kg/day in adults and 5 mg/kg/day in children (McIlleron *et al*., 2009; Francis *et al*., 2019). INH is metabolized in the liver and its metabolites are excreted in the urine. INH toxicity includes hepatotoxicity, peripheral neuropathy and haemolytic anaemia (McIlleron *et al*., 2009; Francis *et al*., 2019).

#### 1.8.1.1.3. Ethambutol

Ethambutol (EMB) was discovered in 1961 (structure given in Figure 1.7). It has demonstrated inhibitory activity against AR *Mtb* bacilli with an MIC range of between 1 and 5 µg/mL. Its mechanism of action involves blocking the formation of the cell wall of *Mtb* by inhibiting the enzyme arabinosyl transferase involved in arabinogalactan synthesis, which is an essential component in the formation of the mycolyl-arabinogalactan-peptidoglycan complex of the *Mtb* cell wall (Aristoff *et al*., 2010; Zimmerman *et al*., 2017). Mutations in the gene *embB* are responsible for resistance to EMB (Tao *et al*., 2006; Fatiguso *et al*., 2016).

Ethambutol is used at concentration of 15 to 25 mg/kg/day for 6 - 8 weeks (Lacroix *et al*., 1989; Zimmerman *et al*., 2017). Fifty percent of the given dose is excreted unchanged in the urine (Zimmerman et al., 2017). Adverse effects of EMB include peripheral neuropathy, red-green color blindness, arthralgia, hyperuricaemia and optic neuritis (Tao *et al*., 2006; Schubert *et al*., 2017).

#### 1.8.1.1.4. Pyrazinamide

Pyrazinamide (PZA) was discovered in 1952 (structure given in Figure 1.7). It is mainly inhibitory but can be bactericidal for AR *Mtb* with a MIC of between 20 and 100 µg/mL. It accelerates the sterilizing effect of INH and RMP when used as part of combination therapy. This has enabled a reduction in the duration of treatment for DS-TB isolates from nine to six months and is therefore used in the first two months of treatment (Heifets and Malenka, 2016).

In acidic conditions, the enzyme pyrazinamidase, converts PZA to the active form, pyrazinoic acid, which subsequently inhibits the enzyme fatty acid synthase (FAS) I, required by the bacterium to synthesize fatty acids. Mutations of the pyrazinamidase gene (*pncA*) are responsible for PZA resistance in *Mtb* (Shakya *et al*., 2012; Stagg *et al*., 2017).

The recommended dose of PZA is between 20 and 25 mg/kg/day or 30 - 40 mg/kg three times a week (Buchan *et al*., 2018). Pyrazinamide is metabolized by the liver and the metabolic products are excreted by the kidneys. Some common side effects of PZA include skin rash, nausea, vomiting, hepatotoxicity, anorexia, hyperuricemia, dysuria, joint pains, urticaria, pruritus, malaise, interstitial nephritis, porphyria and fever (Zimmerman *et al*., 2017).

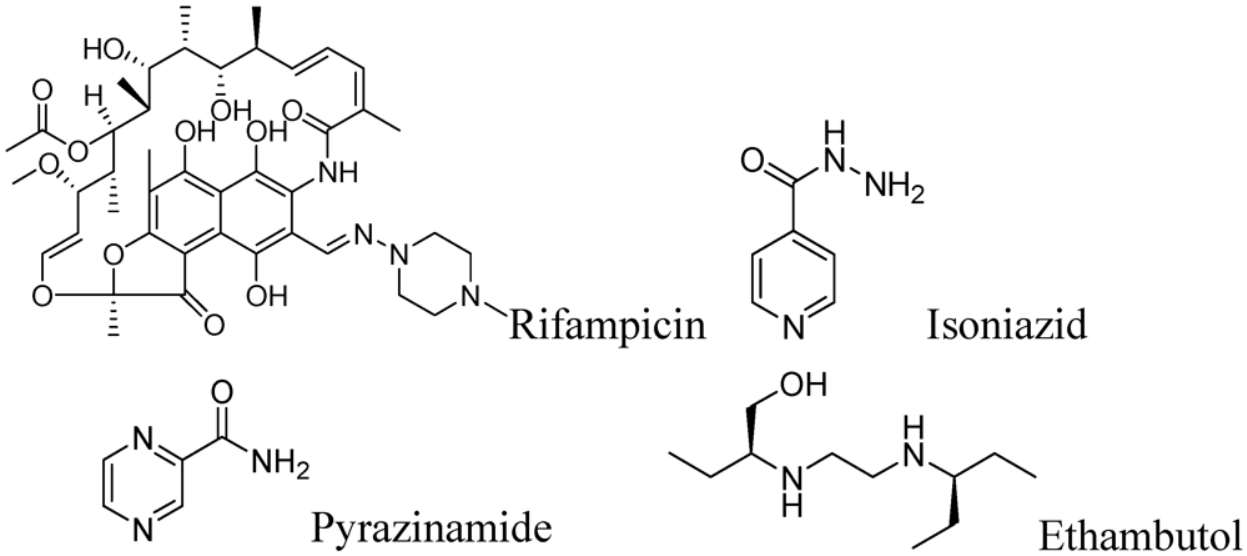


**Figure 1.7:** Molecular structures of first line anti-tuberculosis drugs (Shakya *et al*., 2012, with permission).

### 1.8.2. Chemotherapy of drug-resistant tuberculosis

Drug-resistant TB (DR-TB) develops in situations where there is a late diagnosis, non-compliance, misuse of antimicrobial agents, exposure to DR-TB patient and co-morbidity, leading to the emergence of DR-TB strains such as the multidrug-resistant-TB (MDR-TB: resistant to at least INH and RMP), extensively drug-resistant-TB (XDR-TB: MDR-TB strains resistant to at least second-line agents fluorquinolones and aminoglycoside injectables) and totally drug-resistant-TB (TDR-TB: resistance to all anti-TB drugs tested) (WHO, 2016).

Drug-resistant-TB has seriously complicated the chemotherapy of TB by undermining the effectiveness of available anti-TB agents (Shakya *et al*., 2012; Stagg *et al*., 2017). DR-TB patients are treated with second-line drugs, which often result in high toxicity and side-effects (Phillips *et al*., 2019).

*Mtb* acquires antibiotic resistance through several mechanisms, which include cell wall permeability, clonal selection, efflux pumps, drug degradation and drug tolerance through biofilm formation (WHO, 2016; Singh *et al*., 2019).

The standard chemotherapeutic regimen for DR-TB, which was introduced in 2018, includes a shorter, standardized treatment regimen given for 9 - 12 months. The drugs include members of four groups. These are: Group A: oral fluoroquinolones such as levofloxacin, moxifloxacin and gatifloxacin; Group B: second-line injectables such as aminoglycosides (kanamycin and amikacin), cyclic peptides and aminoglycosides (streptomycin) and Group C: isonicotinic acid derivative (ethionamide), D-cycloserine, terizidone, riminophenazines (clofazimine) and oxazolidinone (linezolid). Treatment regimens consist of at least five antibiotics consisting of PZA and four second line drugs: one from Group A, one from B and at least two from Group C. Treatment includes at least five effective agents during an intensive phase of four months. Bedaquiline (BDQ) may be added to the regimen, either to replace one of the other second-line drugs or to strengthen it (Singh *et al*., 2019). Bedaquiline is currently approved for the treatment of patients with pre-XDR and XDR-TB (Ndjeka *et al*., 2018).

Due to clofazimine forming the basis of the present study, this agent, together with its antimicrobial activity, mechanisms of action, pharmacological properties and clinical presentations and adverse effects will be discussed in greater detail in Chapter Two.

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## CHAPTER TWO: THE ANTITUBERCULOSIS ACTIVITIES OF CLOFAZIMINE

### 2.1. History of clofazimine

Clofazimine (CFZ) is a riminophenazine antibiotic which was synthesized in the 1950s for the treatment of TB (Barry *et al*., 1956; Tyagi *et al*., 2016). It demonstrated high activity against *Mtb in vitro* and therapeutic efficacy in an experimental murine model of TB and was used for the treatment of TB patients. However, it proved ineffective as a monotherapy and also presented with several side-effects such as skin pigmentation, abdominal pain, diarrhea, itchiness, dry skin, and, in some cases, mental disturbances, which include depression, leading to discontinuation of the antibiotic (Tyagi *et al*., 2016; Cholo *et al*., 2017).

CFZ, was, however, subsequently found to be highly effective against multibacillary leprosy and has been used for the treatment of leprosy for over five decades (Pai, 2019). Due to an increasing frequency of drug resistance to other anti-TB drugs in TB isolates, its use in the treatment of TB patients has been revised (Lange *et al*., 2019).

### 2.2. Structure of clofazimine

The structure of CFZ is as shown in Figure 2.1. It has a phenazine nucleus, which is a crucial structural feature of the riminophenazines, with an alkylimino group at position C-2 and phenyl substituents at positions C-3 and N-10 of the phenazine nucleus (Cholo *et al*., 2012; Tan *et al*., 2019). The nitrogen atom of the isopropylimino group located at position C-2 contributes to the cationic amphiphilic properties of the molecule. Cationic amphiphilic drugs contain both hydrophobic and hydrophilic domains in the aromatic ring system and an ionizable amine functional group (Lanoix, 2015; Knight *et al*., 2019).

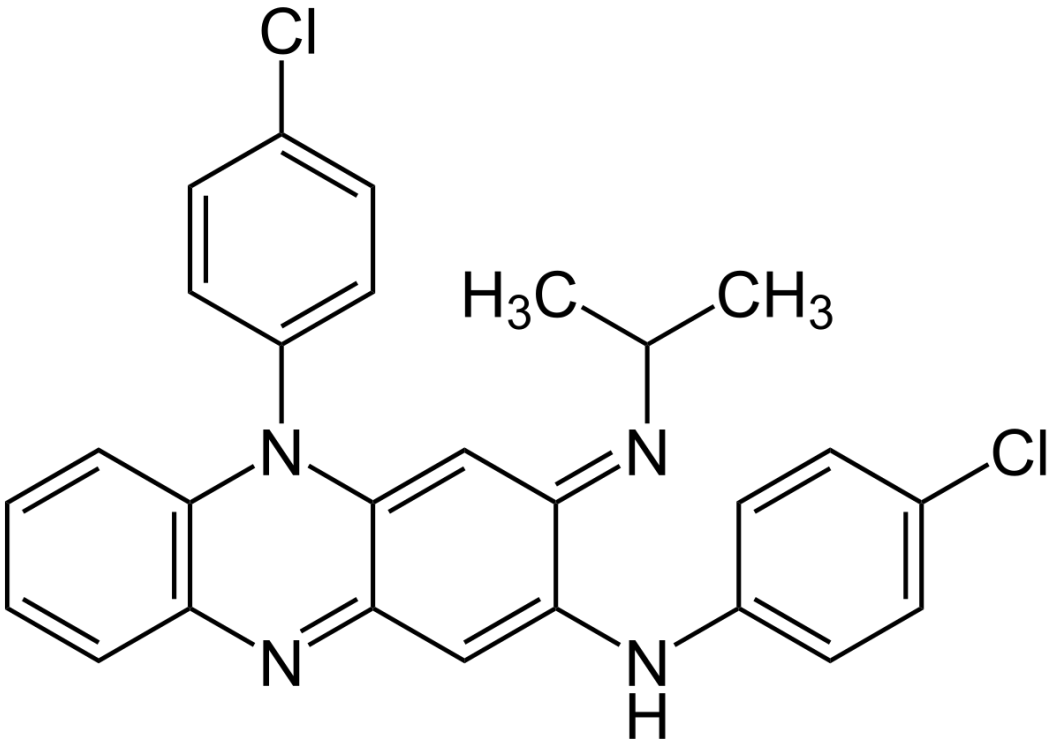


**Figure 2.1:** Molecular structure of clofazimine (Cholo *et al*., 2012, with permission).

### 2.3. Antimicrobial activity of clofazimine

#### 2.3.1. *In vitro* activity

CFZ has demonstrated high antimycobacterial activity against DS- and DR-TB isolates (Aung *et al* 2014; Tang *et al*., 2015; Trébucq *et al*., 2018). It has high inhibitory activity against AR *Mtb*, resulting in low MIC values of between 0.06 and 0.15 µg/mL but has weak bactericidal activity against these organisms. However, it has both high inhibitory and bactericidal activities against SR organisms, resulting in low MIC and MBC values (Mothiba *et al*., 2015). Moreover, it has also demonstrated activity against NR organisms (Cholo *et al*., 2017).

Clofazimine has been shown to exhibit synergistic activity in combinations with second-line drugs such as benzothiazinones, amikacin, tigecycline, clarithromycin, INH, EMB, PZA, linezolid, and BDQ against various species of both rapidly-growing and slow-growing mycobacteria (Van Ingen *et al*., 2012; Pym *et al*., 2016).

### 2.3.2. *In vivo* activity in animal models

*In vivo* studies of experimental TB in mice reported significant therapeutic activity of CFZ (Hwang *et al*., 2014; Ammerman *et al*., 2017), while activity in guinea pigs and monkey models showed poor activity due to low absorption of the drug by the oral route and high accumulation in fatty tissues (Huynh and Marais, 2019).

In a murine model the combination of CFZ with BDQ and PZA showed high bactericidal activity (Tang *et al*., 2015; Diallo *et al*., 2018), while in combination with second-line regimens in mice, led to improvement in treatment outcome leading to shorter culture conversion and relapse prevention (Piubello *et al*., 2014; Diallo *et al*., 2018).

### 2.3.3. Clinical trials of clofazimine alone and in combination with other anti-tuberculosis agents

Patients treated with CFZ-containing regimens, which include the shorter standardized regimen that has been adopted by the World Health Organisation (WHO) for the treatment of MDR-TB patients, showed improvement in treatment success rates ranging from 62% to 87% (Van Deun *et al*., 2010; Sow *et al*., 2016). These treatment success rates were independent of HIV co-infection in patients or drug resistance status of the bacteria (Gupta *et al*., 2010; Nachipo *et al*., 2018). Further cohort studies showing a favourable effect of CFZ-containing regimens have been reported in Benin (Love *et al*., 2017), South Africa (Sow *et al*., 2016) and Ukraine (WHO, 2018) but limited benefits were observed in studies in Brazil (Nachipo *et al*., 2018) and Sri Lanka (Sow *et al*., 2016).

## 2.4. Mechanism of action of clofazimine

The antimicrobial activities of CFZ have been attributed to its high lipophilicity, enabling efficient transmembrane penetration, together with a redox potential of -0.18 V at pH 7 (Barry *et al*., 1957; Lechartier and Cole, 2015; Cholo *et al*., 2017).

The exact mechanisms of antimicrobial action have not been fully elucidated. However, CFZ has been proposed to compete with menaquinone for electrons (Figure 2.2). Menaquinone is the substrate for type 2 NADH: quinone oxidoreductase, which is the initial event in the mycobacterial respiratory chain (Yano *et al*., 2011; Cholo *et al*., 2017). The reduced form of CFZ generated by this mechanism undergoes spontaneous oxidation, resulting in the generation of

antimicrobial ROS such as superoxide and hydrogen peroxide (Grant *et al*., 2010; Yano et al., 2011; Cholo *et al*., 2017).

Another mechanism of action demonstrates that the CFZ stimulates phospholipase A2 activity, resulting in an accumulation of detergent-like lysophospholipids, thus disrupting fundamental cellular functions (Li *et al*., 2014; Feng *et al*., 2015). It has also been hypothesized to inhibit cell replication by binding to the guanine bases of the DNA (Cholo *et al*., 2012; Lechartier and Cole, 2015). It also interferes with potassium ($K^+$) uptake, which is followed by the depletion of adenosine triphosphate (ATP) and inhibition of growth (Cholo *et al*., 2017).

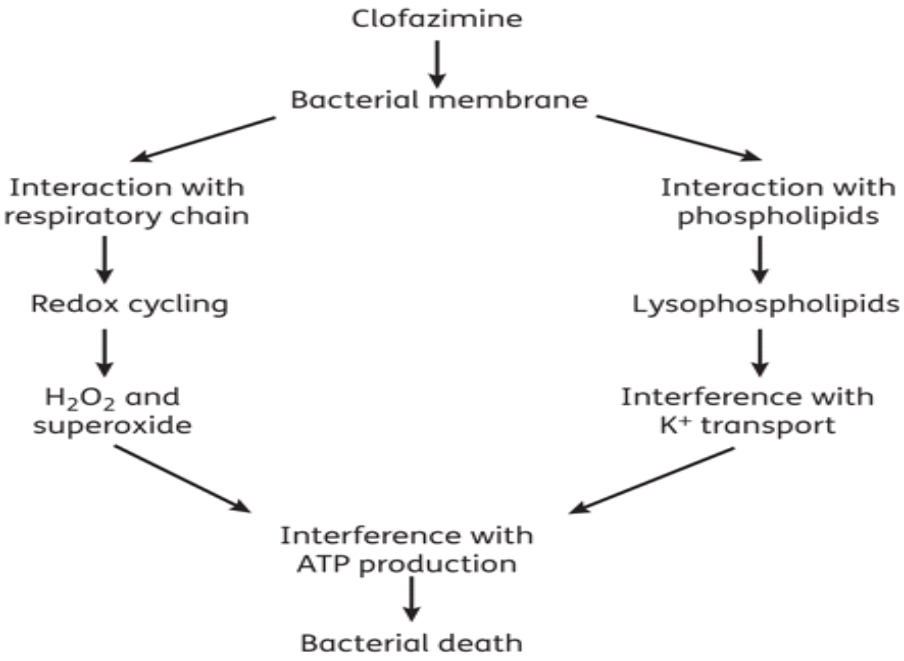


**Figure 2.2:** Membrane-directed mechanisms of the antimicrobial activity of clofazimine (Cholo *et al*., 2012, with permission).

### 2.5. Pharmacological properties of clofazimine

Clofazimine is a highly lipophilic anti-TB drug which accumulates in high concentrations in macrophages of fatty tissues as well as tissues such as the lungs, liver, spleen, brain (Baijnath *et al*., 2015) and bone marrow, but serum concentrations are low (Baik and Rosania, 2012; Srikanth *et al*., 2014; Swanson *et al*., 2015). Its accumulation in macrophages leads to elimination of intracellular bacteria such as *Mtb* and *Mycobacterium leprae* by forming crystal-like drug

inclusions (CLDIs) in the cytoplasm, while it results in discoloration of internal organs and the skin (Yoon *et al*., 2015; Cholo *et al*., 2017; Pai, 2019).

Clofazimine does not cross the blood-brain barrier and the placenta, however it is excreted in breast milk (Arbiser and Moschella, 2014; Cholo *et al*., 2017; Novartis, 2017).

The drug has a long half-life of about 70 days (Nazarov and Lider, 1996; Cholo *et al*., 2017) and is excreted slowly from the body in bile, and only minimally in the urine, sputum, sebum and sweat (Arbiser and Moschella, 1995; Novartis, 2017), taking an average of 6 to 12 months (Pai, 2019). Three metabolites of CFZ have been identified, but their pharmacological roles have not been determined (Baik and Rosania, 2012; McGuffin *et al*., 2017).

Several analogues of CFZ with improved pharmacokinetic properties, which include shorter half-lives, potentially reducing tissue accumulation, have been synthesized and are under investigation (Lu *et al*., 2014; McGuffin *et al*., 2017).

### 2.6. Clinical presentations and adverse effects of clofazimine

As mentioned above, tissue accumulation of CFZ leads to discolouration of the skin, hair, cornea and secretions such as the breast milk, feaces, sweat and urine (McGuffin *et al*., 2017). The discolouration resembles reddish to brownish-black to even orange pink (Winthrop *et al*., 2010; McGuffin *et al*., 2017). However, this pigmentation is reversible upon discontinuation of the antibiotic, although it may take a prolonged time to resolve (Gopal *et al*., 2013; Cholo *et al*., 2017).

Other common adverse reactions, which are manageable, have been reported in >10% of patients and include gastrointestinal (GI) disorders (nausea, vomiting, abdominal pain, diarrhea), ichthyosis and dry skin (Hwang *et al*., 2014; Cholo *et al*., 2017; Novartis, 2017). Decreased visual acuity, eye irritation/dryness, rash or pruritus and weight loss occur in 1 - 10% of patients (McGuffin *et al*., 2017). These adverse effects have not been considered severe enough to discontinue the use of CFZ (Gopal *et al*., 2013; Arbiser *et al*., 2014; Tang *et al*., 2015; Cholo *et al*., 2017; WHO, 2017). Less common, but more serious adverse effects that are potentially fatal, due to prolonged use of high doses of CFZ for more than four months, include enteropathy, potentially complicated by intestinal obstruction, gastro intestinal bleeding and splenic infarction (Cholo *et al*., 2012; Arbiser *et al*., 2014; Tang *et al*., 2015; McGuffin *et al*., 2017; Ammerman *et al*., 2017; Cholo *et al*., 2017; Novartis, 2017).In addition, long term use in leprosy often leads to oedema of the lower legs (Pai, 2019).

Mortality rates due to the use of CFZ vary substantially, ranging from 1 to 63%. In the case of pregnancy, CFZ has a low teratogenic effect, while no CFZ-associated deaths in infants receiving breast milk from treated mothers have been reported (Gopal, 2013; McGuffin *et al*., 2017; Ozturk and Tatliparmak, 2017).

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## CHAPTER THREE: GENERAL AIM AND OBJECTIVES

### 3.1. Background

During infection of the host, different populations of *Mtb* are found and include AR, SR or NR. The AR are predominantly found intracellularly in macrophages, where they are exposed to sufficient oxygen concentrations, in nutrient-rich conditions and elevated pH levels, whereas the SR and NR are mostly found in granuloma in nutrient-limiting and hypoxic environments (Dalton *et al*., 2016).

The AR and SR/NR microbial populations have been isolated *in vitro* in planktonic and biofilm cultures respectively. These cultures have been used previously to evaluate the antimycobacterial activity of antibiotics *in vitro* (Ojha *et al*., 2008; Mothiba *et al*., 2015; Dalton *et al*., 2016; Iacobino *et al*., 2017).

In the current study, the anti-mycobacterial activity of CFZ as well as the primary anti-TB agents, which include RMP, INH and EMB, against *Mtb*, were evaluated using planktonic and biofilm-forming cultures.

The current study consists of two phases, which include the evaluation of the activities of the antibiotics in either planktonic or biofilm-forming cultures, and these are discussed separately in Chapters Four and Five respectively.

### 3.2. Aim

The aim of this study was to determine the antimicrobial potency of CFZ alone and in combination with primary anti-TB chemotherapeutic agents against *Mtb* planktonic and biofilm cultures.

### 3.3. Objectives

To determine the inhibitory and bactericidal activities of the antibiotics individually and in combination by determining their MIC/ fractional inhibitory concentration index (FICI) and MBC/ fractional bactericidal concentration index (FBCI) values respectively against the above-mentioned cultures.

### 3.4. References

Dalton JP, Uy B, Phummarin N, Copp BR, Denny WA, Swift S, Wiles S. (2016) Effect of common and experimental anti-tuberculosis treatments on *Mycobacterium tuberculosis* growing as biofilms. *PeerJ*. **4**: e2717. doi 10.7717/peerj.2717.

Iacobino A, Piccaro G, Giannoni F, Mustazzolu A, Fattorini L. (2017). *Mycobacterium tuberculosis* is selectively killed by rifampin and rifapentine in hypoxia at neutral pH. *Antimicrobial Agents and Chemotherapy*. **61(3)**: 2296-2316. doi: 10.1128/AAC.02296-16.

Mothiba TM, Anderson R, Fourie B, Germishuizen WA, Cholo MC. (2015). Effects of clofazimine on planktonic and biofilm growth of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *Journal of Global Antimicrobial Resistance.* **3**: 13-18.

Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel YU, Alahari A, Kremer L, Jacobs WR, Hatfull GF. (2008). Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Molecular Microbiology*. **69**: 164-174.

# CHAPTER FOUR: ANTIMICROBIAL ACTIVITY OF CLOFAZIMINE ALONE AND IN COMBINATION WITH PRIMARY ANTI-TUBERCULOSIS AGENTS AGAINST PLANKTONIC *MYCOBACTERIUM TUBERCULOSIS* CULTURES

## 4.1. Background

Planktonic cultures consist predominantly of AR *Mtb*, which are grown in nutrient-rich broth medium at near-neutral pH of 6.7 - 6.8, in the presence of a detergent under aerobic conditions as isolated cells. During infection, these bacteria are found in macrophages, where they replicate and are associated with active TB disease (Cholo *et al*., 2015).

The antimicrobial activity of CFZ and the three primary anti-TB agents against *Mtb* have been demonstrated previously individually in planktonic cultures, demonstrating high inhibitory and bactericidal activities (Bhirud *et al*., 2017). Furthermore, the interactions of the primary anti-TB drugs in combination against these bacteria have also been reported, demonstrating high synergistic effects (Montelongo-Peralta *et al*., 2019).

The primary drugs are used mainly in the chemotherapy of DS-TB patients with beneficial effects on the elimination of AR bacteria. However, this treatment schedule is administered over six to nine months, leading to noncompliance in patients, which contributes to emergence of drug resistance in isolates. It is noteworthy that, these primary anti-TB antibiotics have high levels of drug resistance (~ $10^{-6}$ and $10^{-8}$ cfu/mL for INH and RMP respectively). However, in the case of CFZ, the drug resistance rate remains low (~ $10^{-26}$ cfu/mL) (Cholo *et al*., 2017).

Despite high antimicrobial activity against planktonic organisms, CFZ is not routinely used in the treatment of DS-TB patients but is used for the treatment of DR-TB patients due to limited availability of effective anti-TB drugs against these bacteria. Its activity in combination with primary anti-TB drugs has been determined *in vivo* in mouse model of experimental TB resulting in a shorter treatment schedule from six to four months (Tyagi *et al*., 2015). However, its interactive effects with the primary anti-TB drugs on the bacteria have not been determined. In the current study, the effects of CFZ, in combination with the primary drugs, on planktonic cultures of AR bacteria were evaluated.

## 4.2. Aim and objectives

### 4.2.1. Aim

As the focus of the current study is on planktonic bacteria, the aim is as follows:

To determine the antimycobacterial activity of CFZ, alone and in combination with primary anti-TB agents against planktonic *Mtb* cultures.

### 4.2.2. Objectives

- To prepare *Mtb* planktonic cultures using 7H9 broth medium.
- To determine:
    - the inhibitory activities of CFZ and primary anti-TB drugs individually against planktonic *Mtb* cultures by measuring their MIC values using microtitre Alamar blue assay (MABA) procedure.
    - the inhibitory activities of CFZ in combination with primary anti-TB drugs against planktonic *Mtb* cultures by determining their FICI values using the MABA procedure.
    - the bactericidal activities of CFZ and primary anti-TB drugs individually against planktonic *Mtb* cultures by measuring their MBC values using colony-counting procedure.
    - The bactericidal activities of CFZ in combination with primary anti-TB drugs against planktonic *Mtb* cultures by determining their FBCI values using colony-counting procedure.

## 4.3. Materials

i. **Strain of *Mycobacterium tuberculosis:*** *Mtb* H37Rv laboratory strain, ATCC 25618, was used.

ii. **Chemicals and reagents:** Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich Chemicals (Merck KGaA, Darmstadt, Germany).

iii. **Growth media:** Middlebrook 7H9 broth (Difco, Michigan, USA) and 7H10 agar (Difco), supplemented with 10% (v/v) oleic acid, dextrose, catalase (OADC) (Becton Dickinson, New Jersey, USA), 0.2/0.5% (v/v) glycerol with/without Tween 80 (preparation in appendix) were used for preparation of planktonic cultures and plate cultures respectively.

iv. **Antibiotics:** The antimycobacterial agents, purchased from Sigma-Aldrich, were CFZ and the primary anti-TB agents, viz. INH, RMP and EMB. CFZ and RMP were prepared in 100% dimethyl sulphoxide (DMSO) to stock concentrations of 2 mg/mL each, while INH and EMB were dissolved in sterile distilled water to stock concentrations of 10 mg/mL each. The water-soluble antibiotics were filter-sterilised using 0.22 µm Millex-GP filters (Merck KGaA, Darmstadt, Germany) and the antibiotic solutions were stored at -20 ºC until use. The final working concentration ranges for each antibiotic, prepared by two-fold serial dilutions with appropriate solvents are as shown in Table 1. The final DMSO concentration used in all the assays was 1% (v/v).

**Table 4.1:** Concentration ranges for individual antibiotics used in planktonic culture experiments.

| Antibiotic | Stock concentration (mg/mL) | Concentration ranges in cultures (µg/L) |
|---|---|---|
| CFZ | 2 | 0.002 - 40 |
| INH | 10 | 0.00009 - 1 |
| RMP | 2 | 0.00003 - 1 |
| EMB | 10 | 0.003 - 40 |

## 4.4. Methods

### 4.4.1. Inoculum preparation

The *Mtb* culture used for inoculum was prepared by inoculating 500 µL of frozen stock cells into 50 mL 7H9 broth. The culture was incubated at 37 °C under stirring conditions until bacterial growth reached mid-logarithmic phase (optical density (OD)) of 0.6 to 1 at 540 nm ($OD_{540\ nm}$ = 0.6 - 1), measured spectrophotometrically using a Spectronic Helios UV-Vis spectrophotometer (Merck, New Jersey, USA)). The bacterial cells were pelleted by centrifugation at 3500 × *g* for 15 min, at 4 ºC followed by washing of the pellet twice with equal volumes of phosphate-buffered saline (PBS: pH 7.4). The pellet was resuspended in PBS and an $OD_{540nm}$ of 0.6 (yielding approximately $10^7$ colony-forming units (cfu)/mL) was determined spectrophotometrically. The suspension was stored at -20 ºC and used as inoculum.

### 4.4.2. Preparation of planktonic cultures

Planktonic cultures were prepared in 7H9 broth in 96-well micro-tissue culture plates to final volumes of 200 µL. An inoculum of $10^5$ cfu/mL bacterial cells was added to the growth medium in each well and plates incubated at 37 °C in 5% $CO_2$ for seven days with frequent mixing to promote bacterial growth. Bacterial growth was determined using the MABA method, which uses the colorimetric indicator Alamar (resazurin), which remains blue in the absence of growth and turns pink during growth (Luna-Herrera *et al*., 2007; Mendoza-Aguilar et al., 2012; Khalifa *et al*., 2013; Torrey *et al*., 2016).

### 4.4.3. Inhibitory activities

***Minimum inhibitory concentration determinations using single drugs***

The inhibitory activity of each antibiotic was evaluated against the planktonic cultures by determining their MIC values. Bacterial cultures were prepared as described above (Section 4.3.2.1). For each antibiotic, an appropriate drug-free, solvent control systems was included (Table 4.1). The contents of each well were mixed, and the cultures incubated at 37 °C for seven days in 5% $CO_2$ for bacterial growth. The growth of the bacteria was determined by the MABA method. On day 6 (D6) 10% (v/v) Alamar blue reagent (blue) was added to the wells, followed by a further incubation for 24 hours for colorimetric detection of bacterial growth as described above (Section 4.3.2.1). The MIC of each antibiotic was taken as the lowest concentration of the antibiotic where no growth was detected.

***Fractional inhibitory concentration determinations using drug combinations***

The inhibitory interactions of combinations of the antibiotics were evaluated by determining their inhibitory interaction indices as a fractional inhibitory concentration index (FICI). The antibiotic mixtures consisting of ratios of 1/2, 1/3, and 1/4 MIC values of each antibiotic in a combination and their two-fold dilutions for two-, three- and four-drug combinations, respectively were added to the bacterial cultures. The cultures were incubated, and bacterial growth was determined by the MABA method (Section 4.3.2.2.1.1). The lowest concentration demonstrating inhibition was used for FICI determinations. The FICI was determined as the sum of the fractional inhibitory concentrations (FICs) of the individual antibiotics as described in the following formula: e.g. for a two-drug combination.

- FICI = FIC of drug A + FIC of drug B
- FIC = $\frac{\text{MIC of the drug in combination}}{\text{MIC of the drug alone}}$

The inhibitory interaction of the antibiotics is interpreted as being synergistic, additive, indifferent or antagonistic when the FICI values are ≤ 0.5, 0.5 - 1.0, 1.0 - 4.0 or > 4.0 respectively (Naghmouchi *et al*., 2011; Andrejak *et al*., 2012; Draper *et al*., 2013; Kitazaki *et al*., 2017).

### 4.4.4. Bactericidal activities

***Minimum bactericidal concentration determinations using single drugs***

The bactericidal activity of each antibiotic was evaluated by determining their MBC values. Bacterial cultures exposed to antibiotics (Table 1) were prepared and incubated as described above (Section 4.3.2.2.1.1). The contents of each well were sampled and 10× serial dilutions were made, followed by plating of the dilutions (100 µL volumes) onto 7H10 agar medium. The plates were incubated at 37 ºC in 5% $CO_2$ for three weeks for the development of colonies. The number of colonies per plate were counted and the numbers of bacteria were determined using the dilution theory as described previously (Mothiba et al., 2015):

- Number of bacteria (cfu/mL) per well = number of colonies on a plate × inverse of dilution plated × 10 (as 100 µL instead of 1 mL was plated).

The number of bacteria was determined at the initial and final time points, which were Day 0 (D0) and Day 7 (D7) respectively. The bactericidal activity of an antibiotic was determined by comparing the number of bacteria in the antibiotic-treated cultures at D7 to those of the D0 control. The lowest concentration of an antibiotic yielding at least a 2-log reduction in bacterial number in comparison to that of D0 was taken as the MBC of that antibiotic.

***Fractional bactericidal concentration determinations using drug combinations***

The bactericidal interaction of antibiotics in a combination was evaluated by determining the bactericidal interaction indices of antibiotics as the fractional bactericidal concentration index (FBCI). The cultures were prepared, and the numbers of bacteria determined as described above (Section 4.3.2.3.1.1). The lowest antibiotic combination that yielded a 2-log reduction in number of bacteria relative to that of the D0 control was used for FBCI determination as described above

(Section 4.3.2.2.2.1) (Naghmouchi *et al*., 2011; Andrejak *et al*., 2012; Draper *et al*., 2013; Kitazaki *et al*., 2017).

### 4.4.5. Statistical analysis

Statistical analyses were performed on all data using the GraphPad Instat 3 Programme and the results are expressed as the mean values ± standard deviations (SDs). The inhibitory and bactericidal activities of the antibiotics individually and in combinations against planktonic cultures were determined by using dose-response plots. Comparisons between drug-free controls and drug-treated systems were performed using the unpaired t test/ Mann Whitney U-test. Each assay was repeated three times, with triplicate determinations for each assay. Statistical significances for the effect of antibiotics on bacterial cultures were taken as a p-value $< 0.05$.

## 4.5. Results

### 4.5.1. Inhibitory activities

***Minimum inhibitory concentration determinations***

The inhibitory activity of the antibiotic was regarded as high or low when the MIC of the antibiotic was lower or higher than 1 µg/mL. CFZ and the primary drugs, RMP and INH, showed high inhibitory activities. However, the inhibitory activities of these primary drugs were significantly greater than those of CFZ (Table 4.2 and Figure 4.1). EMB demonstrated the least inhibitory activity.

**Table 4.2:** The minimum inhibitory concentration values of the individual antibiotics for planktonic *Mycobacterium tuberculosis*.

| **Antibiotics** | **MIC Concentration (µg/mL)** |
|---|---|
| CFZ | 0.15 |
| RMP | 0.002 |
| INH | 0.006 |
| EMB | 2.4 |

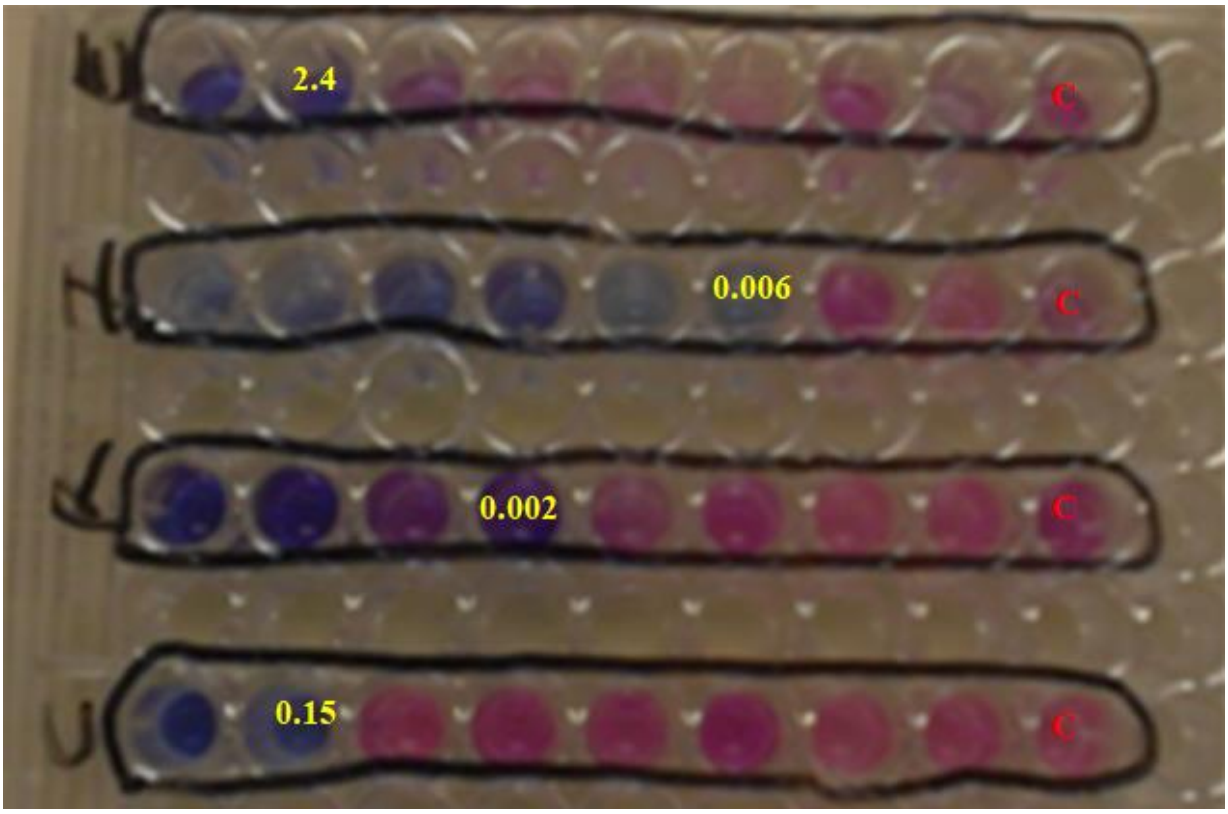



**Figure 4.1:** The minimum inhibitory concentration values of the individual antibiotics against planktonic *Mycobacterium tuberculosis* bacteria using the MABA technique. The symbols C, R, I and E represent clofazimine, rifampicin, isoniazid and ethambutol respectively. The drug concentrations are as described in Table 4.1 ranging from 0.3 - 0.002, 0.016 - 0.00012, 0.2 - 0.0016 and 4.8 - 0.04 µg/mL for CFZ, RMP, INH and EMB respectively, with the lowest concentrations being from right to left. The drug-free control wells (C) and the MIC values are illustrated with red and yellow respectively.

***Fractional inhibitory concentration determinations***

Combinations of two, three and four antibiotics resulted in six, four and one set of combinations respectively. Three of the two- and all three-drug combinations, as well the four-drug combination contained CFZ. The results are as shown in Table 4.3 and Figure 4.2.

Two-drug combinations

CFZ exhibited synergistic anti-mycobacterial effects in combination with RMP and INH and achieved an additive effect in combination with EMB.

However, for the primary anti-TB agents, growth inhibitory synergistic activities were achieved with combinations of RMP with either INH or EMB. These combinations were more inhibitory than those involving CFZ. The combination of EMB and INH were additive.

Three-drug combinations

All three-drug combinations demonstrated synergistic inhibitory activities in the growth of the planktonic bacteria. For the CFZ-containing sets, the greatest synergistic activity was observed in combination with RMP and INH.

However, the combination of the three primary drugs also exhibited high synergistic inhibition of mycobacterial growth, which was comparable to that of CFZ with RMP and INH.

Four drug combinations

The combination of all four antibiotics also resulted in synergistic inhibition of mycobacterial growth with FIC index of 0.0625, which was comparable to that observed with combinations of CFZ or EMB with RMP and INH.

For all the inhibitory combinations investigated, the lowest interactive effect, which was additive, was achieved in two-drug combinations between EMB and either INH or CFZ.

**Table 4.3:** The fractional inhibitory concentration index values of antibiotics against planktonic growth *Mycobacterium tuberculosis*.

| **Antibiotics** | **Antibiotic ratio** | **FIC (µg/mL)** | **FICI** | **Interaction** |
|---|---|---|---|---|
| CONTROL | N/A | N/A | N/A | N/A |
| CFZ + INH | 1/4 + 1/4 | 0.03 + 0.001 | 0.5 | Synergistic |
| CFZ + RMP | 1/8 + 1/8 | 0.01 + 0.0002 | 0.25 | Synergistic |
| CFZ + EMB | 1/2 + 1/2 | 0.07 + 1.2 | 1.0 | Additive |
| EMB + INH | 1/2 + 1/2 | 1.2 + 0.003 | 1.0 | Additive |
| RMP + EMB | 1/16 + 1/16 | 0.0001 + 0.15 | 0.125 | Synergistic |
| RMP + INH | 1/32 + 1/32 | 0.00006 + 0.0002 | 0.0625 | Synergistic |
| CFZ + INH + EMB | 1/24 + 1/24 + 1/24 | 0.006 + 0.0003 + 0.1 | 0.125 | Synergistic |
| CFZ + RMP + EMB | 1/12 + 1/12 + 1/12 | 0.01 + 0.0001 + 0.2 | 0.25 | Synergistic |
| CFZ + RMP + INH | 1/48 + 1/48 + 1/48 | 0.003 + 0.00004 + 0.0001 | 0.0625 | Synergistic |
| RMP + INH + EMB | 1/48 + 1/48 + 1/48 | 0.00004 + 0.0001 + 0.05 | 0.0625 | Synergistic |
| CFZ + RMP + INH + EMB | 1/64 + 1/64 + 1/64 + 1/64 | 0.002 + 0.00003 + 0.00009 + 0.003 | 0.0625 | Synergistic |

**N/A. Not applicable**

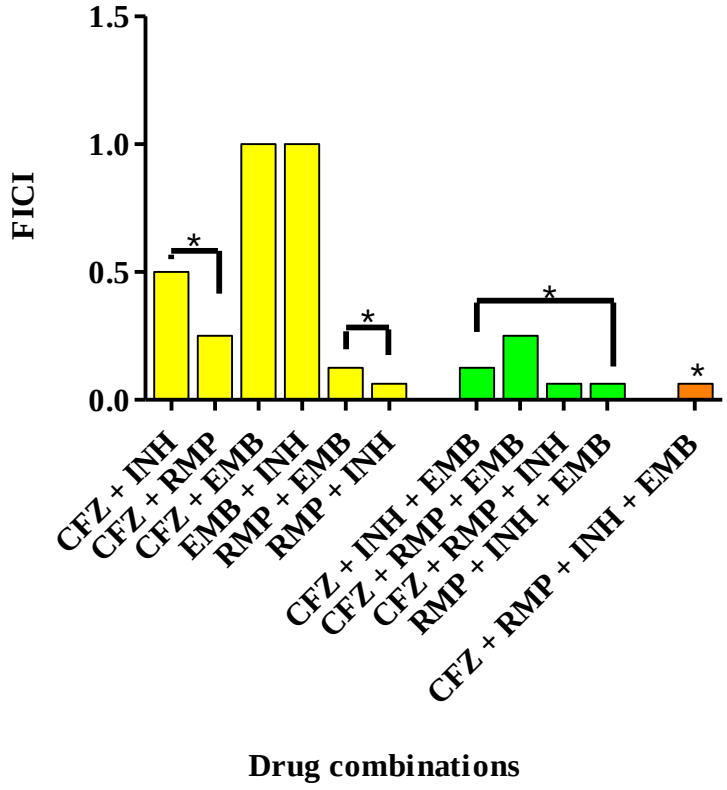



**Figure 4.2:** Inhibitory interactions of clofazimine in combination with the primary anti-tuberculosis drugs on the growth of planktonic *Mycobacterium tuberculosis* using the fractional inhibitory concentration indices. Synergistic inhibitory effect representing FICI value ≤ 0.5 is shown by an asterisk (*).

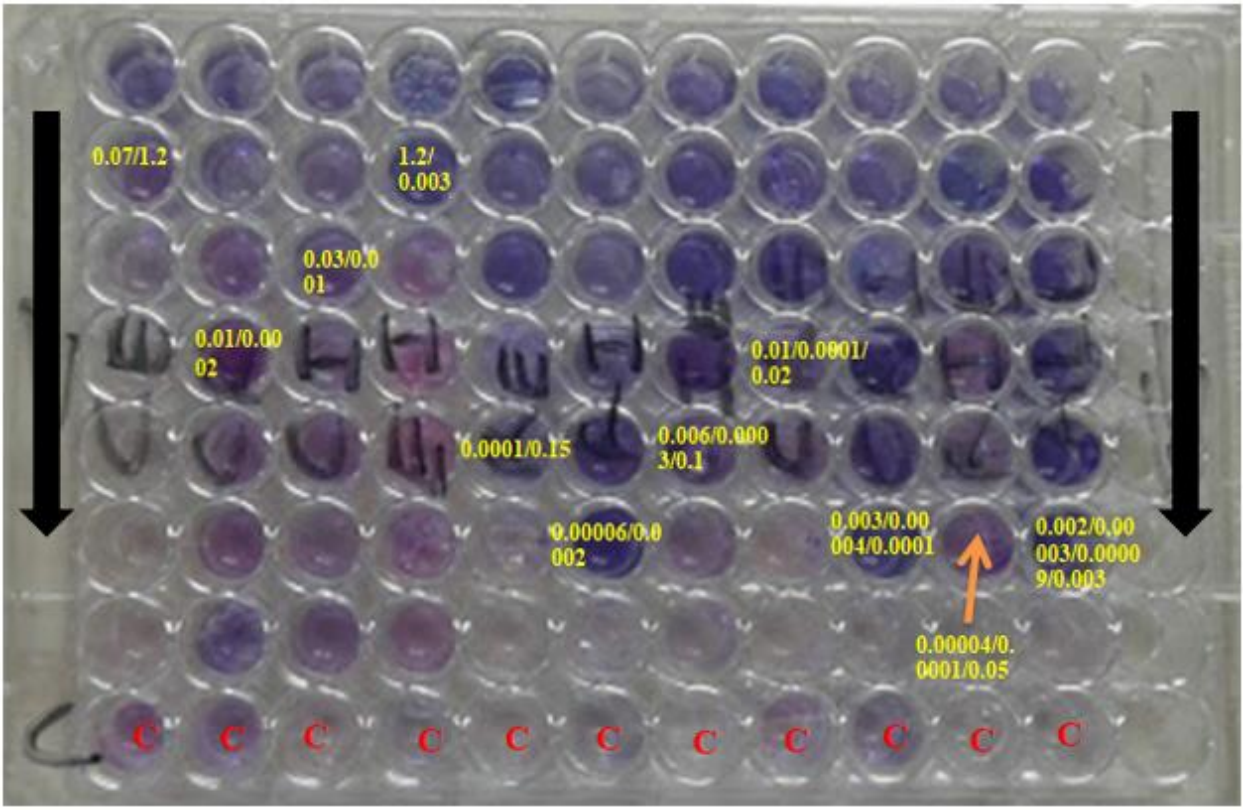



**Figure 4.3:** Inhibitory interactions of the test antibiotics against planktonic *Mycobacterium tuberculosis* in two-, three- and four-antibiotic combinations. The lowest concentrations of the combinations are plated from top to bottom (shown with arrows). The symbols C, R, I and E in black represent clofazimine, rifampicin, isoniazid and ethambutol respectively. The symbol C in red represents the antibiotic-free control. The antibiotic concentrations are as described in Table 5.2 ranging from one concentration above their minimum inhibitory concentrations (MICs) for all the antibiotics. The antibiotic-free, control wells and the MIC values are illustrated in red and yellow respectively.

### 4.5.2. Bactericidal activities

***Minimum bactericidal concentration determinations***

The planktonic bacteria grew rapidly increasing from $2.9 \times 10^4 \pm 1.01 \times 10^4$ to $6.5 \times 10^7 \pm 1.5 \times 10^7$ cfu/mL. Similar to the inhibitory activity determinations, the bactericidal activities of the test antibiotics against planktonic bacteria were regarded as low or high when the MBC value of an antibiotic was higher or lower than 1 µg/mL respectively.

CFZ exhibited low bactericidal activity against planktonic bacteria. The MBC value was 30-fold higher than its MIC value. These results confirm those previously reported by Mothiba et al., (2015). In the case of the three primary drugs, RMP and INH exhibited the highest bactericidal activities, while the activity of EMB was low (Table 4.4, Figure 4.3). Furthermore, the MBC values of these antibiotics were 2-fold higher than their MICs against *Mtb*.

**Table 4.4:** The minimum bactericidal concentration values of the individual antibiotics for planktonic *Mycobacterium tuberculosis*.

| **Antibiotics** | **Concentration (µg/mL)** | **MBC** | |
|---|---|---|---|
| | | **cfu/mL ± SD (Day0)** | **cfu/mL ± SD (Day7)** |
| CONTROL | N/A | $4.8 \times 10^4 \pm 1.6 \times 10^4$ | $9.6 \times 10^6 \pm 1.3 \times 10^7$ |
| CFZ | 5 | $4.7 \times 10^4 \pm 1.6 \times 10^4$ | $3.2 \times 10^2 \pm 19 \times 10^0$ |
| INH | 0.0125 | $4.8 \times 10^4 \pm 1.7 \times 10^4$ | $2.99 \times 10^2 \pm 58 \times 10^0$ |
| RMP | 0.004 | $4.8 \times 10^4 \pm 1.6 \times 10^4$ | $1.17 \times 10^2 \pm 1.01 \times 10^2$ |
| EMB | 4.8 | $4.7 \times 10^4 \pm 1.6 \times 10^4$ | $8.3 \times 10^2 \pm 5.9 \times 10^2$ |

**N/A, Not applicable.**

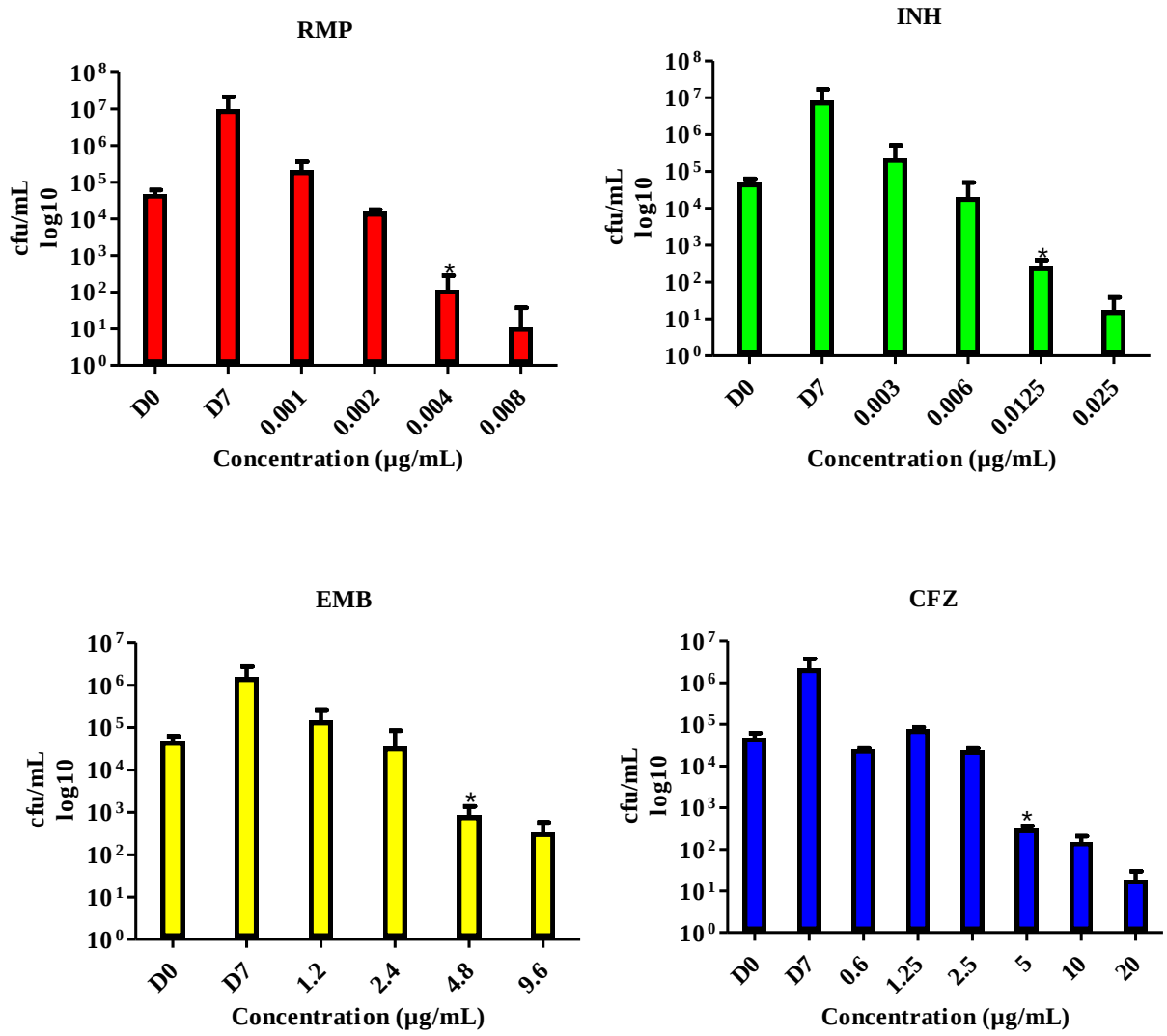



**Figure 4.4:** Minimum bactericidal concentrations of clofazimine and the primary anti-tuberculosis agents against planktonic *Mycobacterium tuberculosis*. The results, shown as log10 graph, are for two separate experiments performed in triplicates for each concentration of clofazimine and are presented as the mean ± standard deviation (SD). The MBC values are shown with an asterisk (*).

***Fractional bactericidal concentration determinations***

The results are as shown in Table 4.5 and Figures 4.5 for the two-, three- and four-antibiotic combinations. In all of the antibiotic combinations tested, CFZ was used at a fixed, final concentration of 0.2 µL.

Two-drug combinations

Synergistic bactericidal activity was only observed in one of the three combination sets containing CFZ, viz., CFZ + RMP and in another consisting of the primary agents, RMP + INH. Both combinations exhibited comparable levels of synergy with similar FICI values (Figure 4.6i).

Three-drug combinations

None of the three CFZ-containing antibiotic combinations demonstrated synergistic inhibitory effects on bacterial growth. However, synergistic inhibition of growth was observed with the combination consisting of the three primary drugs (Figure 4.6ii).

Four-drug combinations

Interaction of CFZ with the three primary drugs in a four-drug combination achieved an additive inhibitory effect on bacterial growth (Figure 4.6iii).

For all the antibiotic combination sets investigated for synergistic bactericidal activity, the lowest bactericidal interactions were observed in the two EMB-containing combinations with CFZ and INH, with EMB being indifferent in combination with RMP and antagonistic with INH or CFZ.

**Table 4.5:** The fractional bactericidal concentration index values of the test antibiotic combinations for planktonic *Mycobacterium tuberculosis*.

| Antibiotics | cfu/mL ± SD (Day0) | cfu/mL ± SD (Day7) | Antibiotic ratio | FBC (µg/mL) | FBCI | Interaction |
|---|---|---|---|---|---|---|
| + CONTROL | $5.6 \times 10^4 \pm 2.5 \times 10^4$ | $2.6 \times 10^6 \pm 4.2 \times 10^5$ | N/A | N/A | N/A | N/A |
| CFZ + INH | $5.6 \times 10^4 \pm 2.5 \times 10^4$ | 10 ± 0 | 1/2 + 1/2 | 2.5 + 0.006 | 1 | Additive |
| CFZ + RMP | | $1.95 \times 10^2 \pm 7$ | 1/4 + 1/4 | 1.25 + 0.001 | 0.5 | Synergistic |
| CFZ + EMB | | $3.5 \times 10^4 \pm 3.9 \times 10^3$ | NA | NA | NA | NA |
| EMB + INH | | $6.1 \times 10^4 \pm 1.5 \times 10^4$ | NA | NA | NA | NA |
| EMB + RMP | | $1.7 \times 10^3 \pm 1.8 \times 10^2$ | 1 + 1 | 2.4 + 0.006 | 2 | Indifferent |
| RMP + INH | | 13 ± 5 | 1/4 + 1/4 | 0.001 + 0.003 | 0.5 | Synergistic |
| CFZ + INH + EMB | $4.1 \times 10^4 \pm 2.4 \times 10^3$ | $3.4 \times 10^2 \pm 8$ | 1/3 + 1/3 + 1/3 | 1.6 + 0.004 + 1.2 | 1 | Additive |
| CFZ + RMP + EMB | | 72 ± 70 | 1/3 + 1/3 + 1/3 | 1.6 + 0.001 + 1.2 | 1 | Additive |
| CFZ + RMP + INH | | 33 ± 0 | 1/3 + 1/3 + 1/3 | 0.1 + 0.001 + 0.004 | 1 | Synergistic |
| RMP + INH + EMB | | $2.9 \times 10^2 \pm 2.1 \times 10^2$ | 1/12 + 1/12 + 1/12 | 0.0004 + 0.001+ 0.5 | 0.25 | Synergistic |
| CFZ + RMP + INH + EMB | | 6.0 ± 8.0 | 1/4 + 1/4 + 1/4 + 1/4 | 1.25 + 0.001 + 0.003 + 1.2 | 1.0 | Additive |

**NA. Not achieved; N/A. Not applicable**

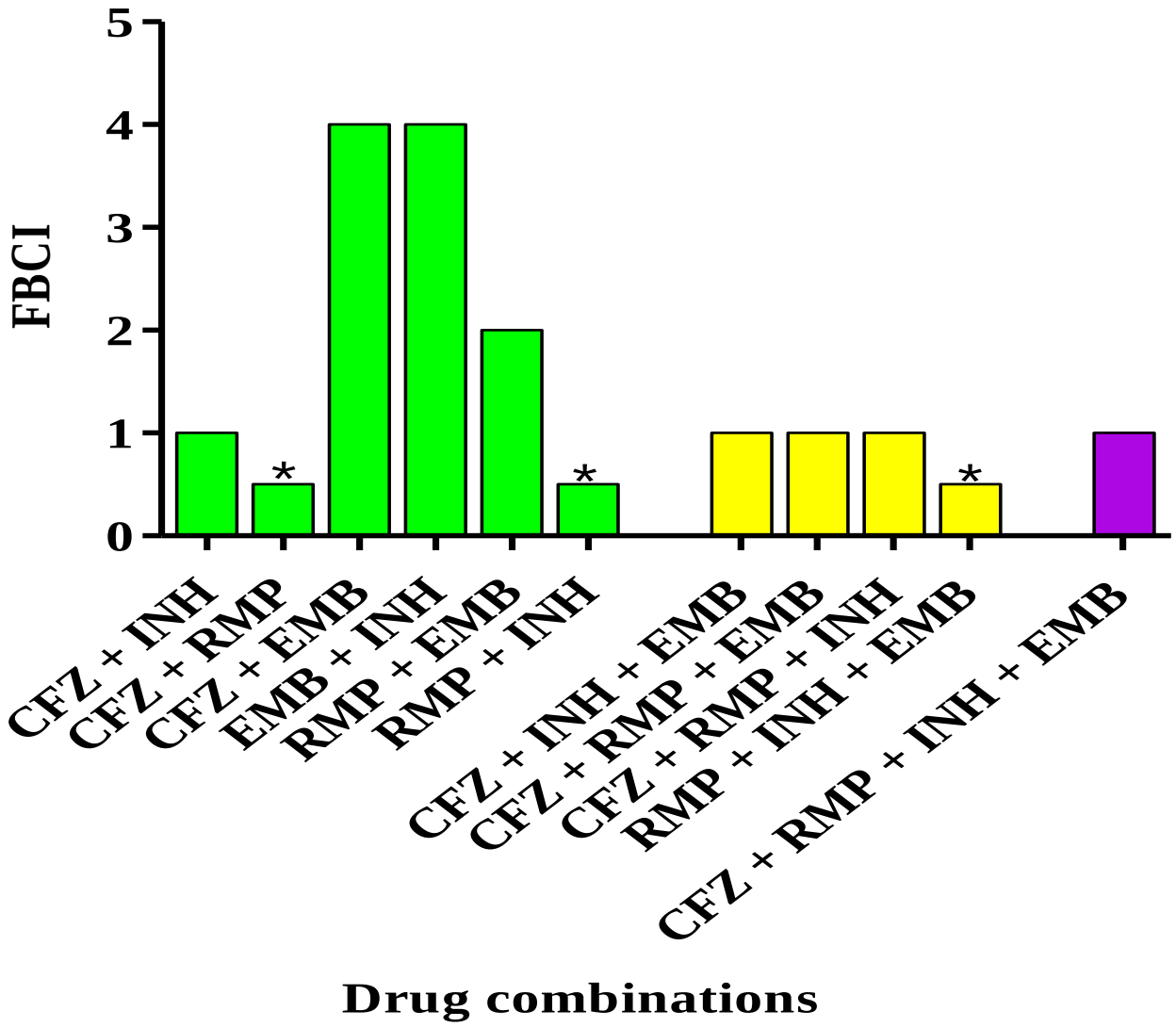
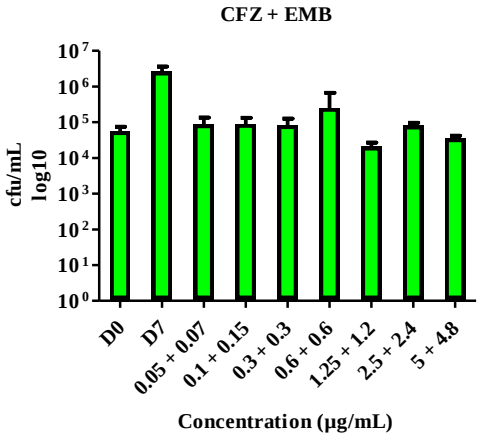
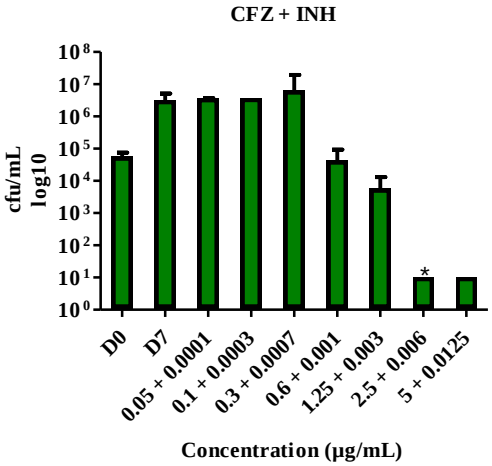
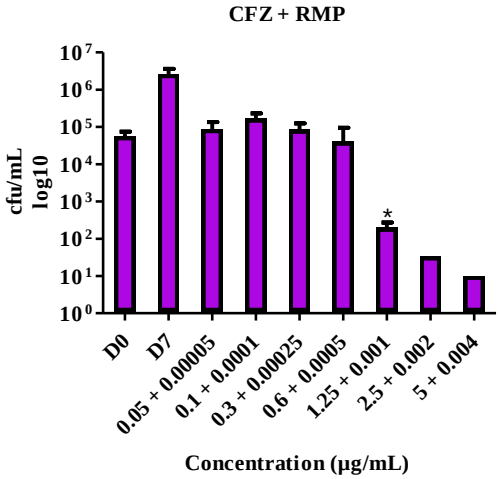
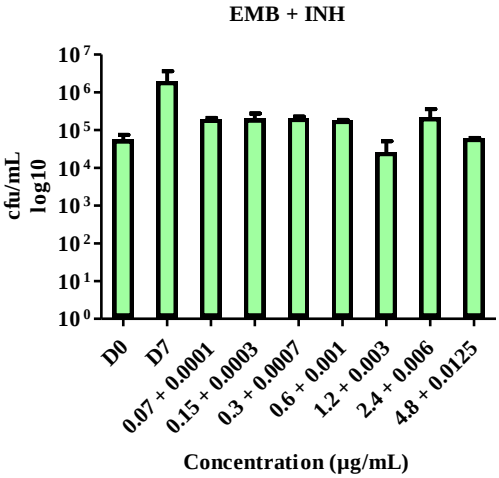


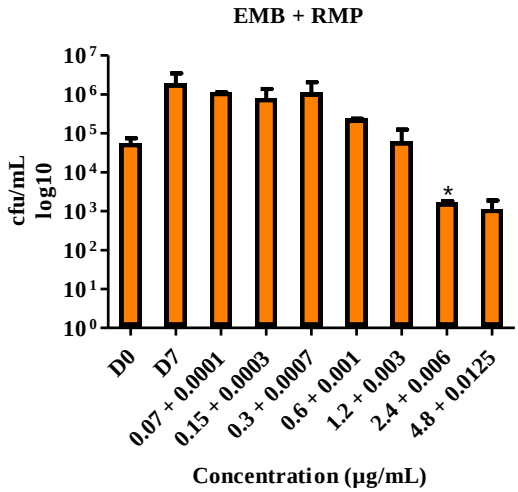

**Figure 4.5:** Bactericidal interactions of clofazimine with the primary antibiotics against planktonic *Mycobacterium tuberculosis* using the fractional inhibitory concentration indices. Synergistic bactericidal effect representing FBCI value ≤ 0.5 is shown by an asterisk (*).

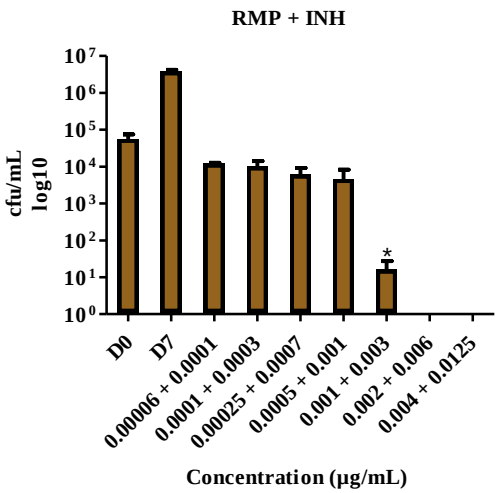
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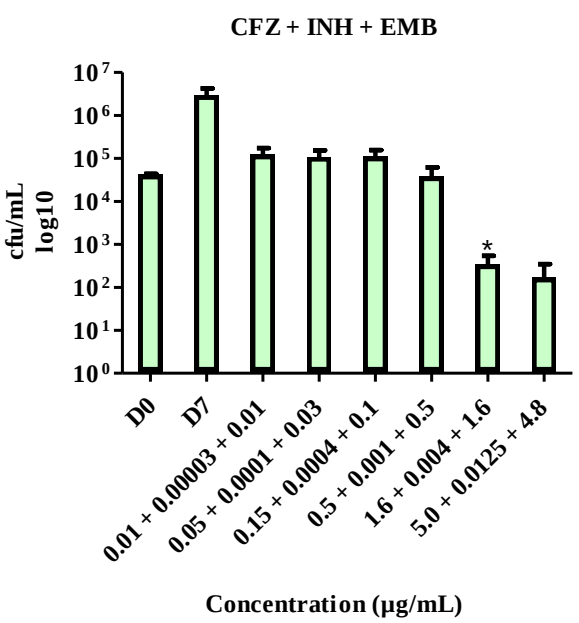


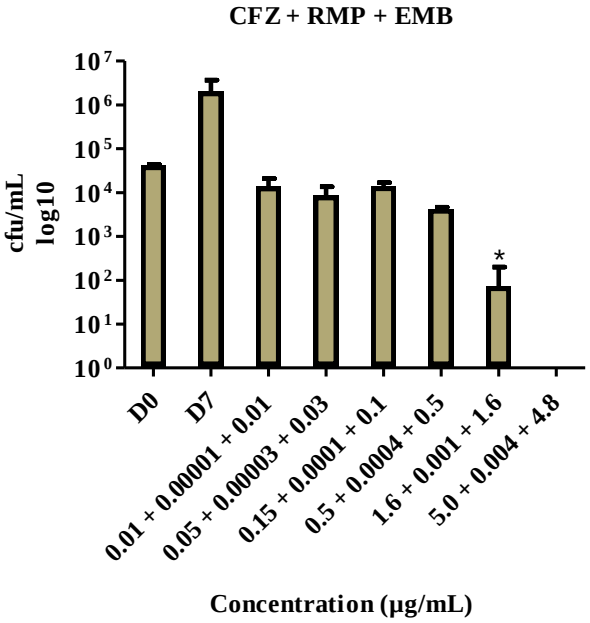


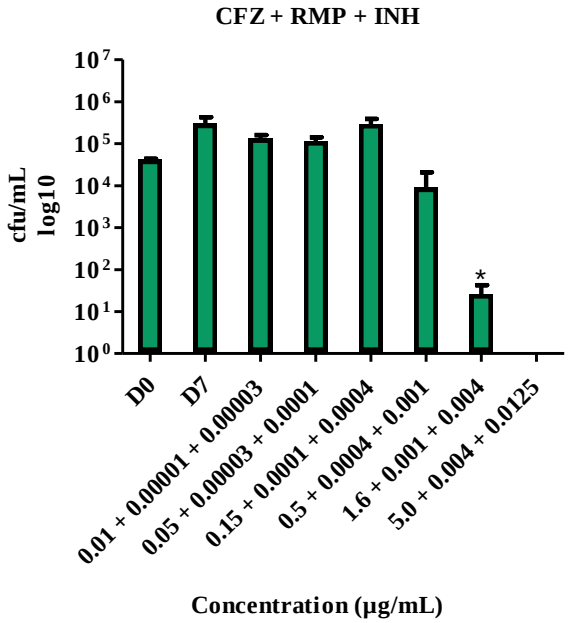


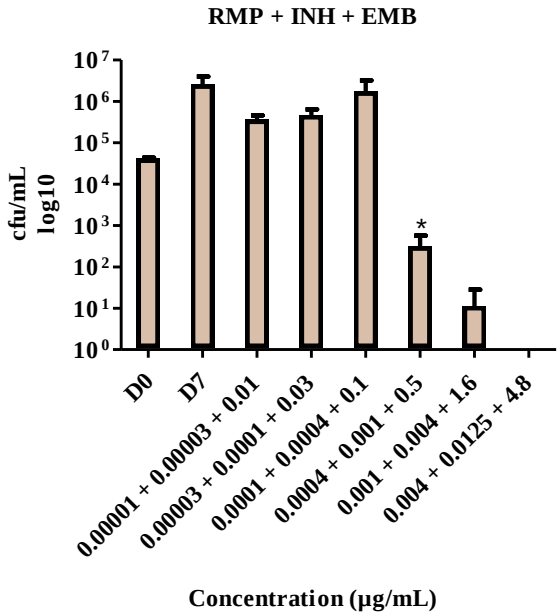


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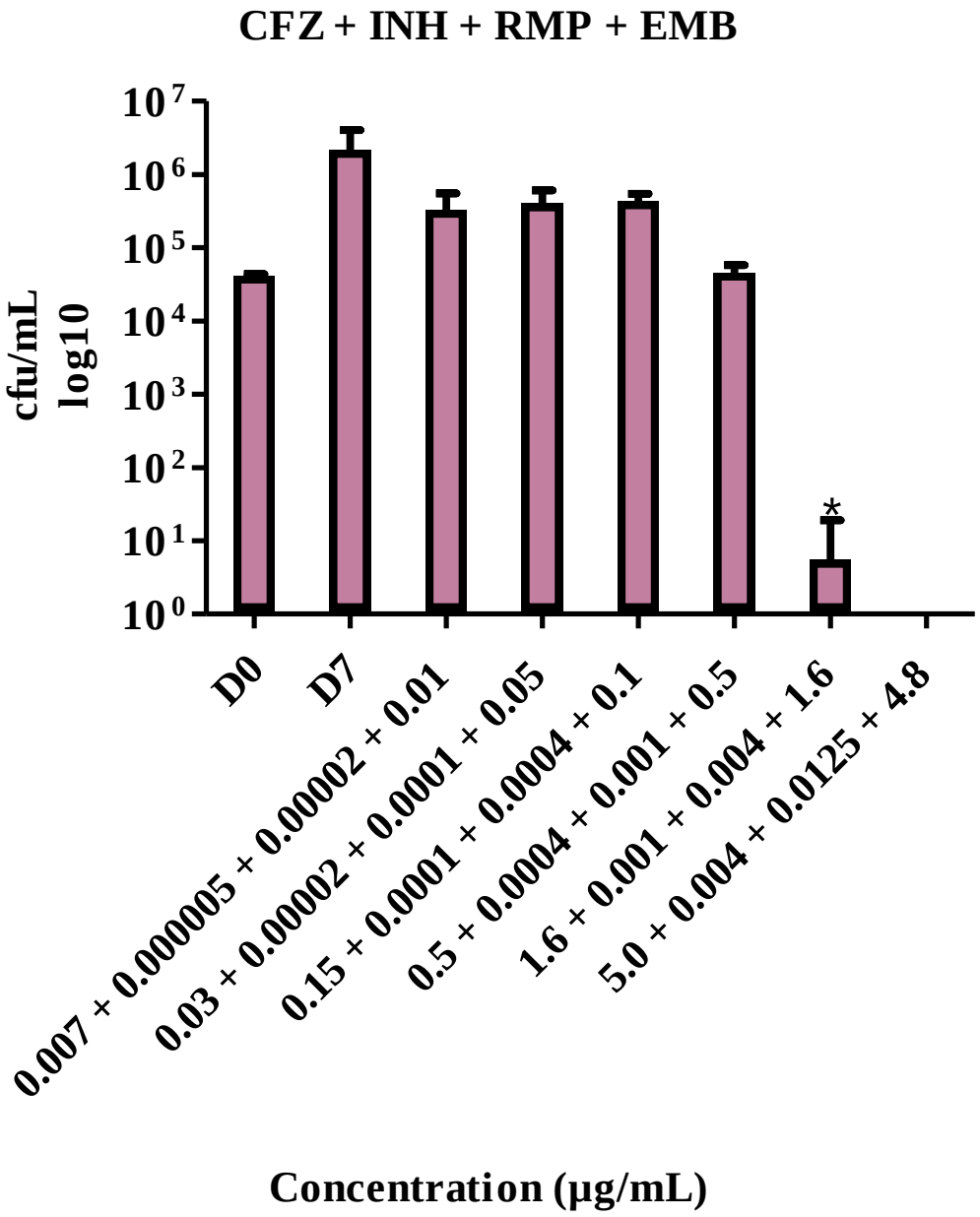


**iii.**

**Figure 4.6:** Fractional bactericidal concentrations of antibiotics against planktonic *Mycobacterium tuberculosis* bacteria at two-, three- and four-drug combinations shown at panel (i), (ii) and (iii) respectively. The results, represented as log10 graph, are for two separate experiments performed in triplicates, presented as mean ± standard deviation (SD). The asterisk (*) denotes FBCI values ≤ 0.5 for each combination set.

### 4.6. Discussion

During infection of the host, the TB lesion consists of heterogenous microbial populations with AR organisms found intracellularly in macrophages, while the SR and NR are located extracellularly in the granuloma (Raviglione and Sulis, 2016).

Treatment of TB is administered for six to nine months consisting of intensive and continuation phases. During the intensive phase patients are treated with the four primary drugs RMP, INH, EMB and PZA which target the AR bacteria. This phase of chemotherapy is usually effective, resulting in elimination of approximately 99% of the AR bacteria, which based on the rapid growth rates, the bacteria are mostly killed within weeks and effectively eliminated within two months (Ojha *et al*., 2008; Basaraba and Ojha, 2017). However, during the continuation phase of antimicrobial chemotherapy, patients are treated with RMP and INH for four to seven months, targeting the SR and NR bacilli.

This treatment schedule is long, often leading to treatment non-compliance, which is a risk factor for the development of drug resistance. In some instances, patients experience relapse despite completion of therapy. In addition, anti-TB agents such as EMB, have high levels of toxicity, presenting with side effects such as peripheral neuropathy and optic neuritis (Schubert *et al*., 2017). There is therefore a need for development of a shorter chemotherapy regimen for TB that will promote compliance in patients.

*In vitro,* the AR are produced in the planktonic cultures, provided with a sufficient supply of nutrients and high levels of oxygen at near-neutral pH (Ojha *et al*., 2008; Cholo *et al*., 2015; Mothiba *et al*., 2015). CFZ has demonstrated inhibitory activity on bacterial growth, but low bactericidal activity against the AR bacteria in planktonic cultures and as shown in the current study, its activity against these bacteria was lower than that of the primary drugs RMP and INH. These findings are in agreement with previous studies evaluating the activities of these drugs against planktonic bacteria individually (Mothiba *et al*., 2015; Dalton *et al*., 2016).

In the current study, the effects of CFZ on the activity of the primary anti-TB drugs against *Mtb* were investigated by determining its inhibitory and bactericidal interaction effects with three primary antibiotics, RMP, INH and EMB, in various combinations, which included two-, three- and four-drug combination sets.

The inclusion of CFZ in combinations with the primary agents resulted, in some cases, in synergistic inhibition of growth of the planktonic bacteria. However, the growth inhibitory

activities of CFZ-containing combinations were less than those achieved by some combinations of the primary agents in the absence of CFZ. In this context, CFZ achieved synergistic inhibition of bacterial growth in two-drug combinations with either RMP or INH, which was lower, however, than that observed with the RMP/INH combination. In the various three-drug combination sets, no CFZ-containing combination achieved higher growth inhibitory activity than that observed with the combination of the three primary drugs. Similar effects were observed when CFZ was used in a four-drug combination.

These results demonstrate that combining CFZ with primary anti-TB agents in the setting of planktonic bacterial growth does not potentiate the antimicrobial activity of the combination of the primary antibiotics, RMP and INH.

Inclusion of CFZ in a four-drug regimen resulted in comparable activity to the three-drug combinations with RMP and INH present. Despite these comparable effects, the inclusion of CFZ in a four-drug combination with the primary drugs may be beneficial in addressing drug dose-related toxicity in patients due to lower concentrations used.

The results of the current study also highlighted RMP and INH as the most effective drugs against the AR bacteria when used alone and in combinations together. However, on the other hand, EMB was shown as the least active drug with low inhibitory and bactericidal activities against these bacteria. Furthermore, in combinations, it demonstrated poor interactive effects with both primary drugs, RMP and INH as well as CFZ.

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# CHAPTER FIVE: ANTIMICROBIAL ACTIVITY OF CLOFAZIMINE ALONE AND IN COMBINATION WITH PRIMARY ANTI-TUBERCULOSIS AGENTS AGAINST BIOFILM-FORMING *MYCOBACTERIUM TUBERCULOSIS* CULTURES

## 5.1. Background

Biofilms are a cluster of heterogenous population of bacteria composed of few AR bacilli and predominantly SR and NR organisms, which are insulated mostly in biofilm-forming and preformed cultures *in vitro* respectively. The *Mtb* biofilm cultures develop in hypoxic and nutrient-limiting conditions in detergent-free medium to promote cellular clustering in a high glycerol concentration as a carbon source in stationary cultures, preferably in high pH conditions of 7.0 - 8.0, forming a layer or pellicle on the surface of the medium (Ojha *et al*., 2008; Brennan, 2017).

During TB infection, the SR and NR are found predominantly in granuloma lesions with the AR in the macrophages that form a rim of these structures. During treatment, these bacteria are targeted at the continuation phase by administration of RMP and INH only. Due to low activity of these antibiotics on these bacteria, treatment is given over four to seven months (Ojha *et al*., 2008; Brennan, 2017). Despite the prolong treatment schedule, these microbial populations have poor response to the primary anti-TB agents, resulting in dormancy development in bacteria. Upon reactivation, these organisms may lead to relapse in patients.

CFZ has demonstrated a high anti-mycobacterial activity on both bacterial populations *in vitro*. However, the effects of CFZ in combination with RMP and INH on these bacteria have not been evaluated.

In the current study, the effects of CFZ on the activities of three primary anti-TB agents (RMP, INH, EMB) against biofilm-forming cultures of SR *Mtb* have been evaluated.

## 5.2. Aim and objectives

### 5.2.1. Aim

As the focus of the current study is on biofilm-forming bacteria, the aim is as follows:

To determine the antimycobacterial activity of CFZ, alone and in combination with primary anti-TB agents against biofilm forming *Mtb* cultures.

### 5.2.2. Objectives

- To prepare *Mtb* biofilm-forming cultures using Sauton synthetic broth medium.
- To determine:
    - the inhibitory activities of CFZ and primary anti-TB drugs individually against biofilm forming *Mtb* cultures by determining their MIC values by means of visual examination.
    - the inhibitory activities of CFZ in combination with primary anti-TB drugs against biofilm forming *Mtb* cultures by determining FICI values by means of visual examination.
    - the effects of CFZ and primary anti-TB agents individually and in combination on *Mtb* biofilm production using the crystal violet procedure.
    - the bactericidal activities of CFZ and primary anti-TB drugs individually against biofilm-forming *Mtb* cultures by measuring their MBC values using colony-counting procedure.
    - the bactericidal activities of CFZ in combinations with primary anti-TB drugs against biofilm-forming *Mtb* cultures by determining their FBCI values using colony-counting procedure.

### 5.3. Materials

i. **Strain of *Mycobacterium tuberculosis:*** Bacterial strain as described in Section 4.3.1.1.

ii. **Chemicals and reagents:** Unless otherwise stated, all other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, Missouri, United States).

iii. **Growth media:** The growth media used included Sauton broth medium (preparation in Appendix 1) for biofilm culture preparation and Middlebrook 7H10 agar for colony development.

iv. **Antibiotics:** The same antibiotics as for planktonic cultures (Chapter Four) were used at concentration ranges as shown in Table 5.1.

**Table 5.1:** Concentration ranges for individual antibiotics in biofilm-forming cultures.

| Antibiotic | Stock concentration (mg/mL) | Final concentration range in assays (µg/mL) |
|---|---|---|
| CFZ | 2 | 0.009 - 1.25 |
| INH | 10 | 0.0007 - 1 |
| RMP | 2 | 0.009 - 1 |
| EMB | 10 | 0.06 - 16 |

## 5.4. Methods

### 5.4.1. Inoculum preparation

The bacterial inoculum preparation was as described in Section 4.4.1.

### 5.4.2. Preparation of biofilm-forming cultures

Biofilm cultures were prepared in Sauton broth medium in 24-well micro-tissue culture plates to final volumes of 2000 µL. An inoculum $10^5$ cfu/mL cells from the inoculum was added to Sauton broth and the contents of the wells were thoroughly mixed. The plates were wrapped in three layers of parafilm, followed by incubation at 37 °C in 5% $CO_2$ in the dark for five weeks with no shaking. Biofilm growth was evaluated by biofilm development, characterised by formation of a dense white irregular layer on the surface of Sauton broth medium (Ojha *et al*., 2008; 2010; Mothiba *et al*., 2015).

### 5.4.3. Inhibitory activities

The inhibitory activities of antibiotics were evaluated by determination of the amount of biofilm formed in the biofilm-forming cultures using visual examination and objective quantification using the crystal violet procedure.

***Minimum inhibitory concentration determinations using single drugs***

Similar to planktonic cultures, the inhibitory activities of the individual antibiotics were determined by measurement of the MIC values. Bacterial cultures prepared as described (Section 5.4.1) were mixed with various concentrations of antibiotics (Table 5.1) followed by incubation for biofilm development. Antibiotic activity was determined by visual examination of biofilm formation. The MIC of each antibiotic on biofilm formation was regarded as the lowest concentration of an antibiotic resulting in the absence of formation of biofilm by visual examination.

***Fractional inhibitory concentration determinations using drug combinations***

The inhibitory drug interactions were determined by FICI determination as described for planktonic cultures in Section 4.4.3.

***Biofilm quantification***

The effects of antibiotics on biofilm formation were also determined by biofilm quantification using crystal violet procedure as described (Mothiba *et al*., 2015). Supernatants from biofilm-forming cultures were removed by aspirating with pipette and the residual biofilm biomass was washed twice with 1 mL distilled water and air-dried. Thereafter 1 mL of 1% (v/v) of crystal violet was added to each well and incubated for 30 min at room temperature. The wells were washed three times with distilled water to remove the unbound dye followed by air drying. The air-dried crystal violet-bound biofilm residues were extracted with 70% (v/v) ethanol, followed by determination of biofilm quantities by OD measurements of the samples at 570 nm using a UV spectrophotometer (Bio-Tek instruments).

The quantities of biofilm were determined at the end of week 5 (W5). The effects of the antibiotics on biofilm formation were determined by comparing the amount of biofilm in the drug-treated system with those of the drug-free controls. Bacteriostatic activity of each antibiotic was determined by significant reduction in biofilm to OD of < 0.5 in drug-treated systems.

### 5.4.4. Bactericidal activities

***Minimum bactericidal concentration determinations using single drugs***

The bactericidal activities of antibiotics against biofilm-forming *Mtb* cultures were determined using the colony-counting procedure. Biofilm-forming cultures were prepared as described (Section 5.2.2). Prior to plating, sterile Tween 80 (0.05% (v/v) final concentration) was added to each well and the cultures were incubated for six hours at 37 ºC with frequent shaking to dissolve the biofilm matrix, releasing the bacteria into the culture medium forming bacterial suspension. Similar to planktonic cultures, the bacterial suspensions in the wells were plated and the numbers of bacteria were determined. The bacterial numbers were determined at initial and final time points which were Week 0 (W0) and W5 respectively. The lowest concentration of each drug that resulted in at least a 2-log reduction in bacterial cells compared to that of W0 was taken as the MBC of that antibiotic.

***Fractional bactericidal concentration determinations using drug combinations***

Bactericidal effects of the antibiotic combinations on biofilm were determined using the FBCI procedure as described for planktonic cultures in section 4.4.2.2.1.

### 5.4.5. Statistical analysis

Statistical analyses were performed on all data as described for planktonic cultures in Section 4.4.5.

## 5.5. Results

### 5.5.1. Inhibitory activities

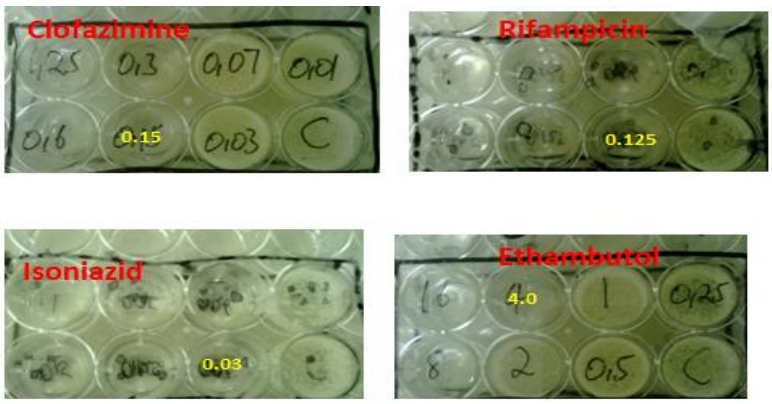
***Minimum inhibitory concentration determinations***

Similar to planktonic cultures, the inhibitory activity of an antibiotic was considered as being high or low when the MIC value was less than, or higher than 1 µg/mL respectively. CFZ demonstrated high inhibitory activity against biofilm-forming cultures (Figure 5.1 and Table 5.2). However, the MIC value was similar to that observed for planktonic bacteria.

In the case of the primary drugs, RMP and INH also exhibited high inhibitory activity against biofilm-forming bacteria, while the activity of EMB was low. However, the MIC values of these antibiotics were higher than those for planktonic bacteria increasing by 100-, 20- and two-fold for RMP, INH and EMB respectively.

**Table 5.2:** The minimum inhibitory concentration values of the individual antibiotics for biofilm-forming *Mycobacterium tuberculosis*.

| **Antibiotics** | | **MIC** |
|---|---|---|
| | **Concentration (µg/mL)** | **OD $_{570}$ nm ± SD** |
| CONTROL | W5 | 2.8 ± 0.05 |
| INH | 0.03 | 0.052 ± 0.001 |
| RMP | 0.125 | 0.03 ± 0.004 |
| EMB | 4.00 | 0.007 ± 0.002 |
| CFZ | 0.15 | 0.026 ± 0 |

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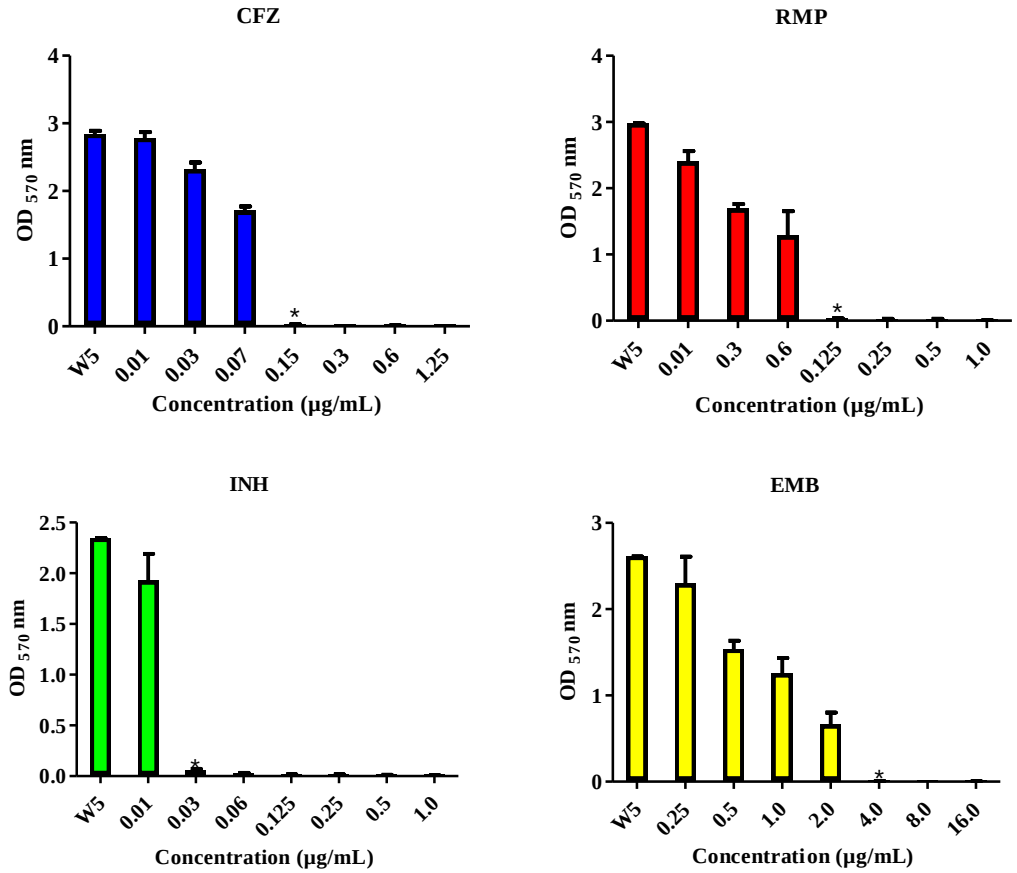



ii.

**Figure 5.1:** Measurement of the minimum inhibitory concentration values of clofazimine and the primary anti-TB drugs against biofilm-forming *Mycobacterium tuberculosis* cultures using (**i**) visual examination and the MIC values are indicated in yellow. The amounts of biofilm were also determined by (**ii**) the crystal violet procedure. The results are for three separate experiments performed in duplicate, presented as the mean ± standard deviation (SD). The (*) denotes the MIC value of each agent.

***Fractional inhibitory concentration determination using drug combinations***

Similar to studies using planktonic cultures, the growth inhibitory activities of combinations of the various test antibiotics against biofilm-forming organisms were determined using the same sets of two-, three- and four-drug combinations. The results are as shown in Table 5.2 and Figure 5.2.

Two-drug combinations

The three CFZ-containing combinations demonstrated synergistic inhibitory effects on the growth of biofilm-forming *Mtb*, with the combination with INH or EMB being least effective (Table 5.3 and Figure 5.2 and 5.3i). In the case of the primary antibiotics, synergistic activity was observed with combinations of RMP with INH or EMB. The combinations of EMB and INH achieved additive effect.

Three-drug combinations

All three-drug combinations showed synergistic effects (Table 5.3, Figure 5.3ii), with two of the three CFZ-containing combinations being most effective. The synergistic inhibitory effect on the growth of biofilm-forming *Mtb* observed with the combination of the three primary drugs r was comparable to that the least effective CFZ-containing combination.

Four-drug combination

Addition of CFZ to the primary antibiotics in a four-drug combination resulted in synergistic inhibitory activity, which demonstrated the highest inhibitory effect of all the combinations tested (Table 5.3 and Figure 5.4).

**Table 5.3:** The fractional inhibitory concentration index values of antibiotic combinations against biofilm-forming *Mycobacterium tuberculosis*.

| Antibiotics | Concentrations ratio | FIC (µg/mL) | FICI | Interaction | $OD_{570}$ nm ± SD |
|---|---|---|---|---|---|
| CONTROL | W 5 | N/A | N/A | N/A | 2.990 ± 0.002 |
| CFZ + INH | 1/4 + 1/4 | 0.03 + 0.01 | 0.5 | Synergistic | 0.332 ± 0.021 |
| CFZ + RMP | 1/8 + 1/8 | 0.015 + 0.015 | 0.25 | Synergistic | 0.306 ± 0.003 |
| CFZ + EMB | 1/4 + 1/4 | 0.03 + 1 | 0.5 | Synergistic | 0.028 ± 0.003 |
| RMP + EMB | 1/4 + 1/4 | 0.03 + 1 | 0.5 | Synergistic | 0.341 ± 0.009 |
| INH + EMB | 1/2 + 1/2 | 0.015 + 2 | 1 | Additive | 0.0.321 ± 0.01 |
| RMP + INH | 1/4 + 1/4 | 0.03 + 0.015 | 0.5 | Synergistic | 0.324 ± 0.032 |
| CFZ + RMP + EMB | 1/12 + 1/12 + 1/12 | 0.01 + 0.01 + 0.3 | 0.25 | Synergistic | 0.355 ± 0.003 |
| CFZ + RMP + INH | 1/24 + 1/24 + 1/24 | 0.006 + 0.005 + 0.001 | 0.125 | Synergistic | 0.186 ± 0.016 |
| CFZ + INH + EMB | 1/6 + 1/6 + 1/6 | 0.02 + 0.005 + 0.66 | 0.5 | Synergistic | 0.056 ± 0.006 |
| RMP + INH + EMB | 1/6 + 1/6 + 1/6 | 0.02 + 0.05 + 0.6 | 0.5 | Synergistic | 0.057 ± 0.005 |
| CFZ + RMP + INH + EMB | 1/64 + 1/64 + 1/64 + 1/64 | 0.002 + 0.001 + 0.0004 + 0.06 | 0.0625 | Synergistic | 0.365 ± 0.003 |

**N/A. Not applicable**

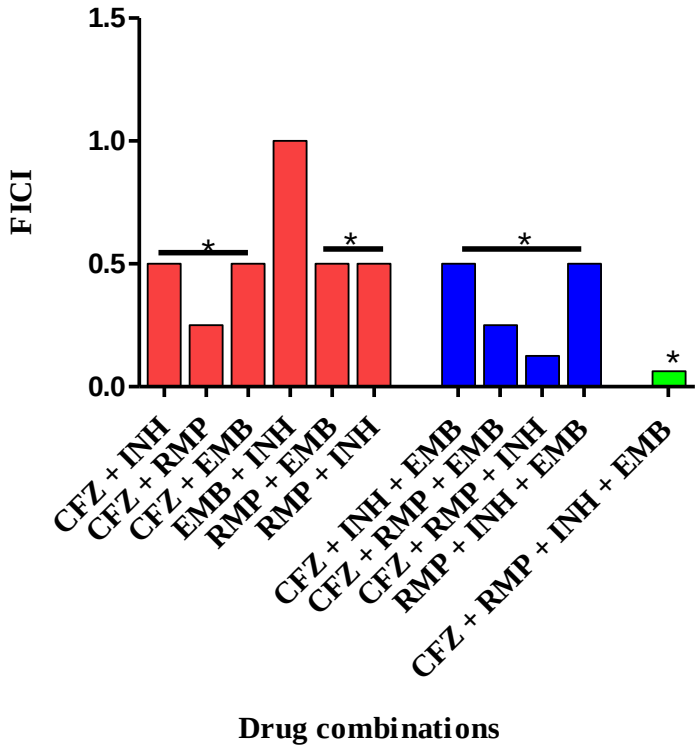
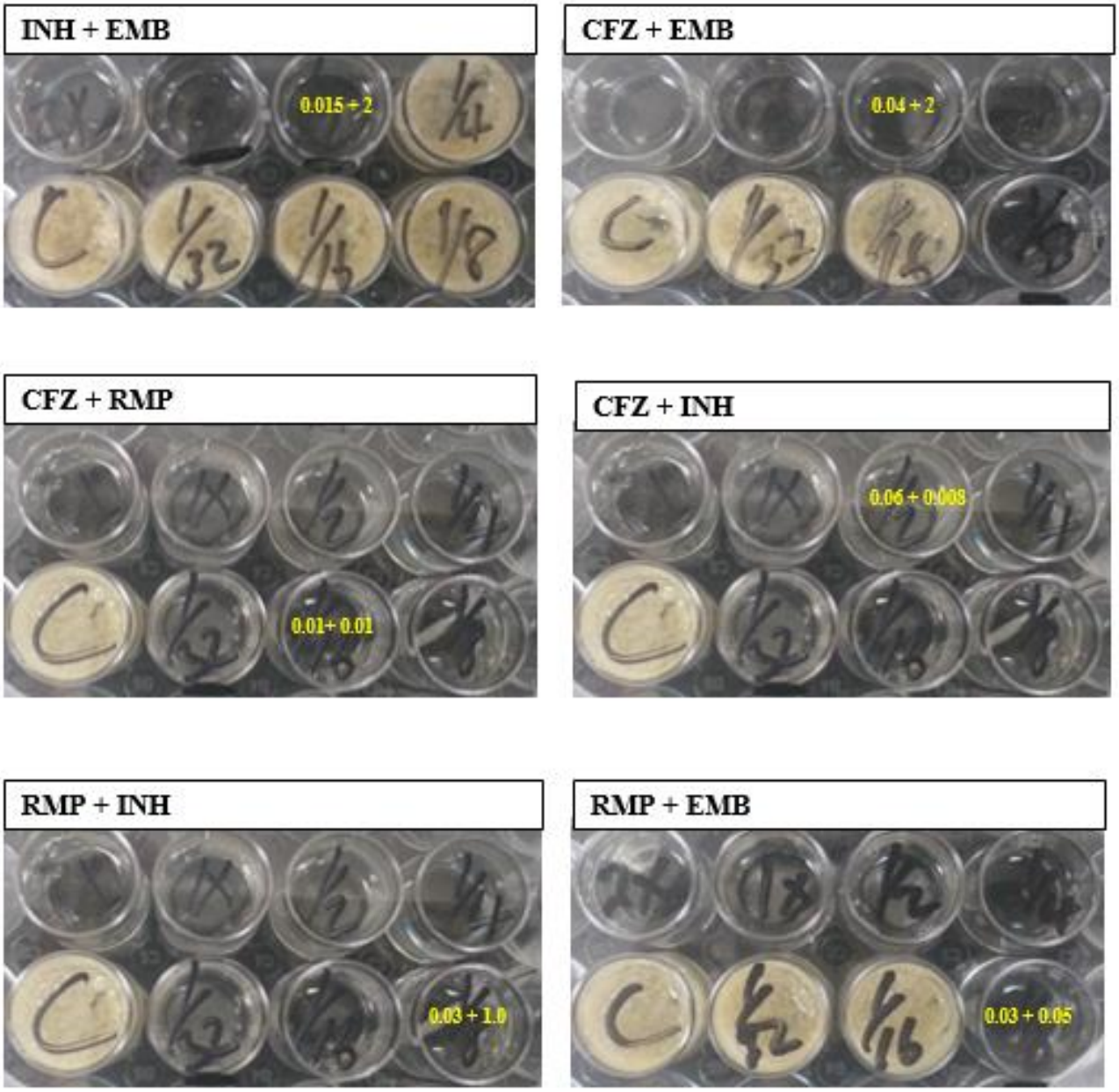



**Figure 5.2**: Inhibitory interactions of clofazimine in combination with the primary drugs on the growth of biofilm-forming *Mycobacterium tuberculosis* using the fractional inhibitory concentration index. Synergistic inhibitory effect representing FICI value ≤ 0.5 is shown by an asterisk (*).

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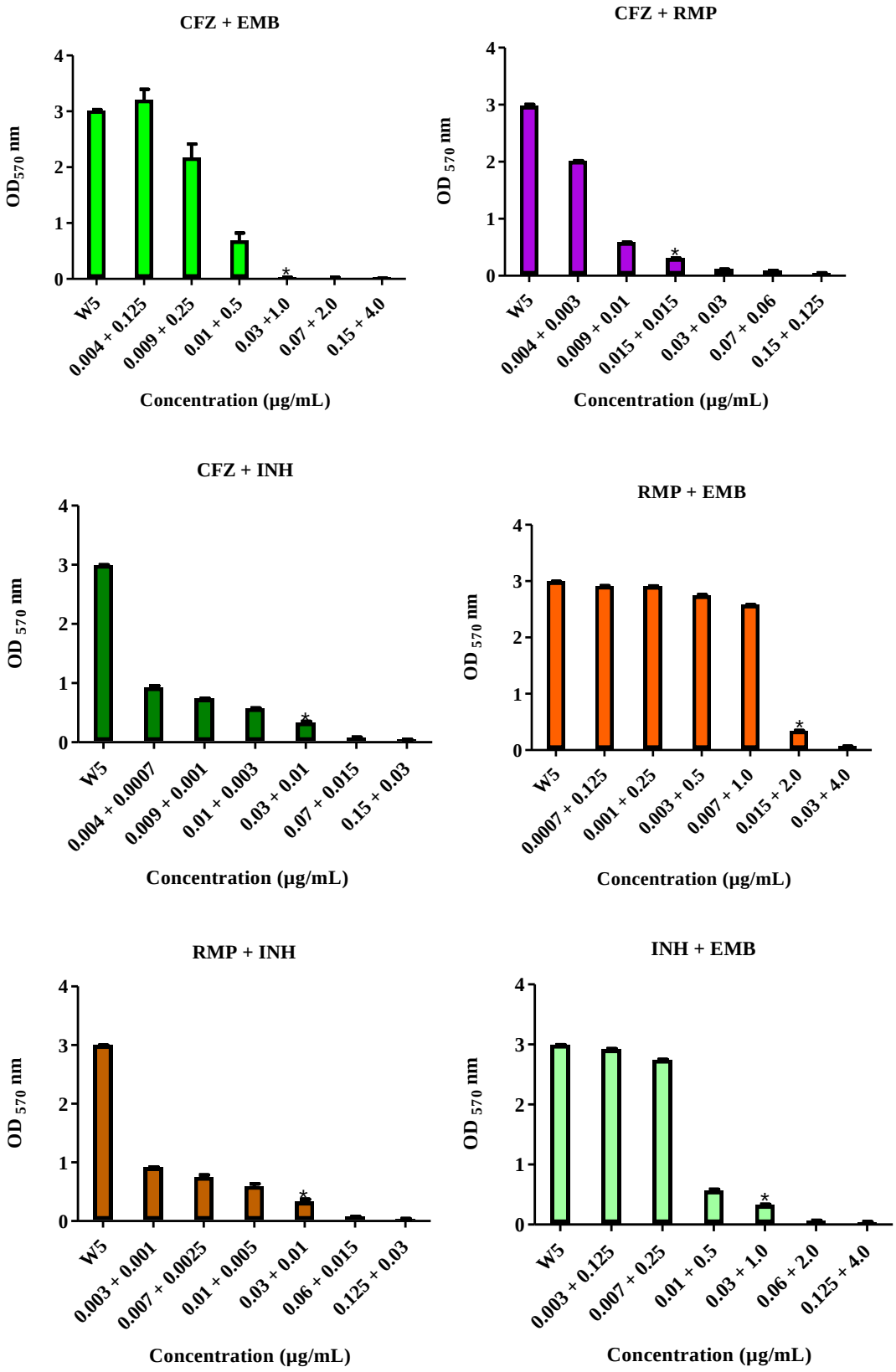


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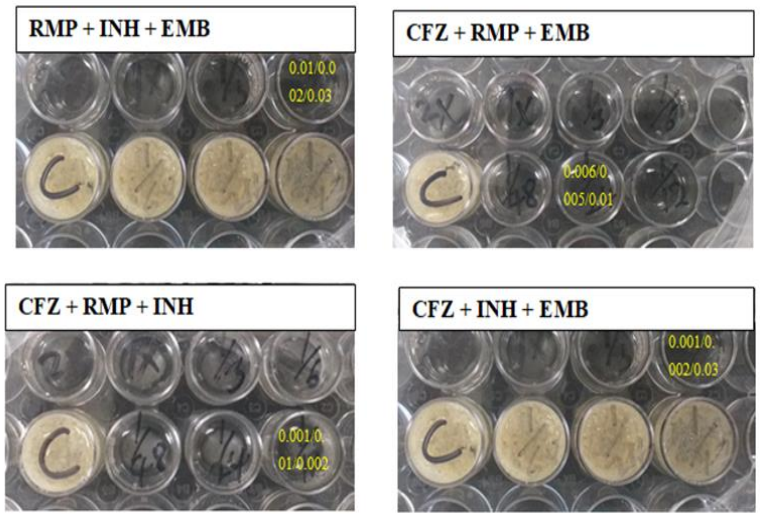


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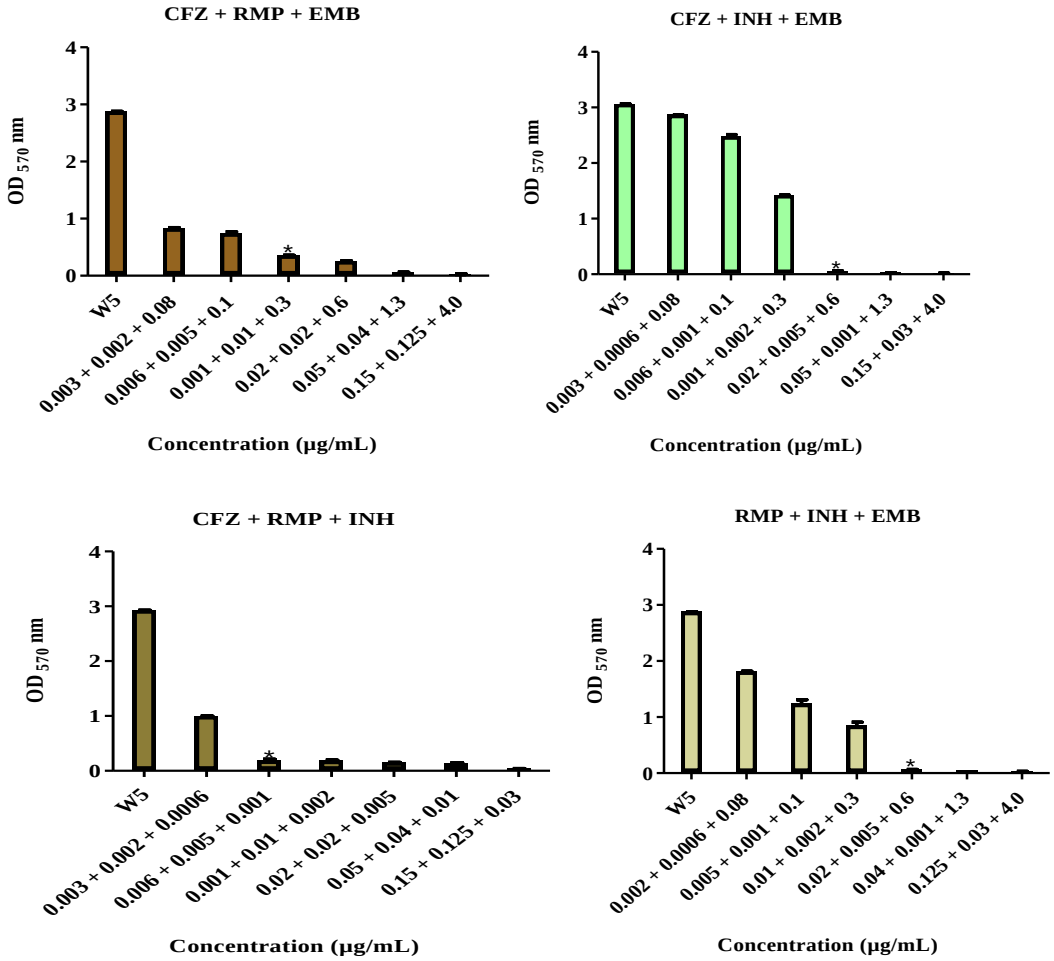


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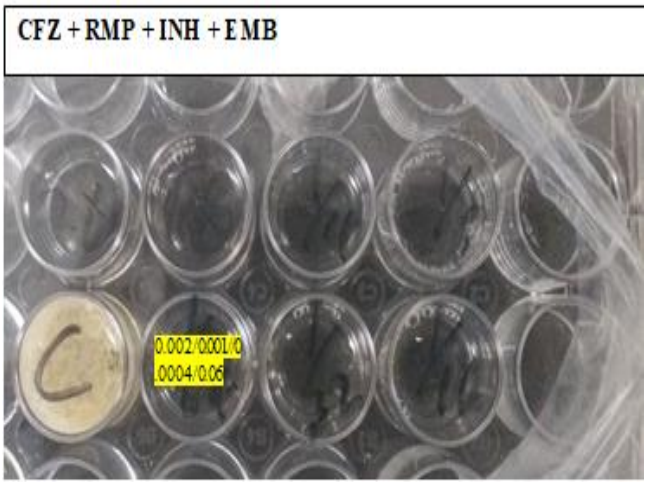


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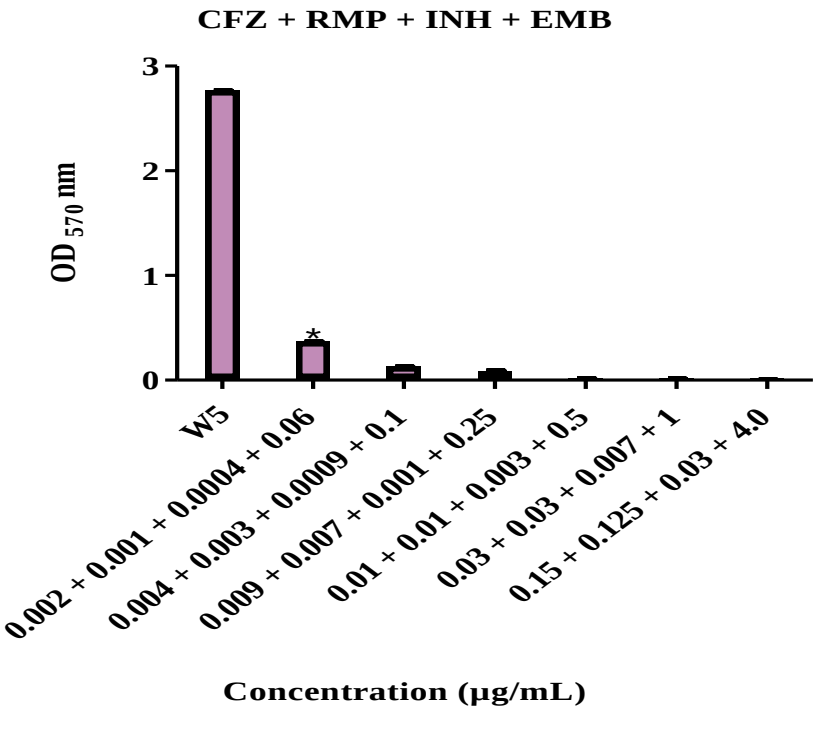



**iii.**

**Figure 5.3:** Inhibitory interactions of the test antibiotics against biofilm-forming *Mycobacterium tuberculosis* in two-, three- and four-antibiotic combinations determined by (**i**) visual biofilm development examination, and (**ii**) quantitated using the crystal violet procedure. The results are for three separate experiments performed in duplicate, presented as the mean ± standard deviation (SD). Synergistic inhibitory effects representing as FICI values ≤ 0.5 is shown by (*).

### 5.5.2. Bactericidal activities

***Minimum bactericidal concentration determinations***

The results of the MBC determination of the antibiotics against biofilm-forming bacteria are as shown in Table 5.3 and Figure 5.5. The biofilm-forming bacteria grew slowly, increasing from 6.9 × $10^5$ ± 6.3 × $10^5$ at W0 to 2.8 × $10^7$ ± 4.6 × $10^7$ cfu/mL at W5. CFZ showed high bactericidal activity against the biofilm-forming bacteria, with an MBC value similar to that of its MIC value against these organisms.

In the case of the primary drugs, RMP and INH demonstrated higher bactericidal activity with MBC values lower than that observed for CFZ. In the case of the primary antibiotics, the primary antibiotics, the MBC values were two-fold higher than the MIC values for INH and EMB, while the MBC and MIC values of RMP were similar.

**Table 5.4:** The minimum bactericidal concentration values of the individual antibiotics for biofilm-forming *Mycobacterium tuberculosis*.

| **Antibiotics** | | **MBC** | |
|---|---|---|---|
| | **Concentration (µg/mL)** | **cfu/mL (W0) ± SD** | **cfu/mL (W5) ± SD** |
| CONTROL | 0.00 | | 2.8 × $10^7$ ± 4.6 × $10^7$ |
| CFZ | 0.15 | | 4.6 × $10^2$ ± 2 × $10^2$ |
| RMP | 0.125 | 6.9 × $10^5$ ± 6.3 × $10^5$ | 1.1 × $10^3$ ± 1.1 × $10^3$ |
| INH | 0.06 | | 3.1 × $10^2$ ± 34 |
| EMB | 8.0 | | 1.2 × $10^3$± 5.1 × $10^2$ |

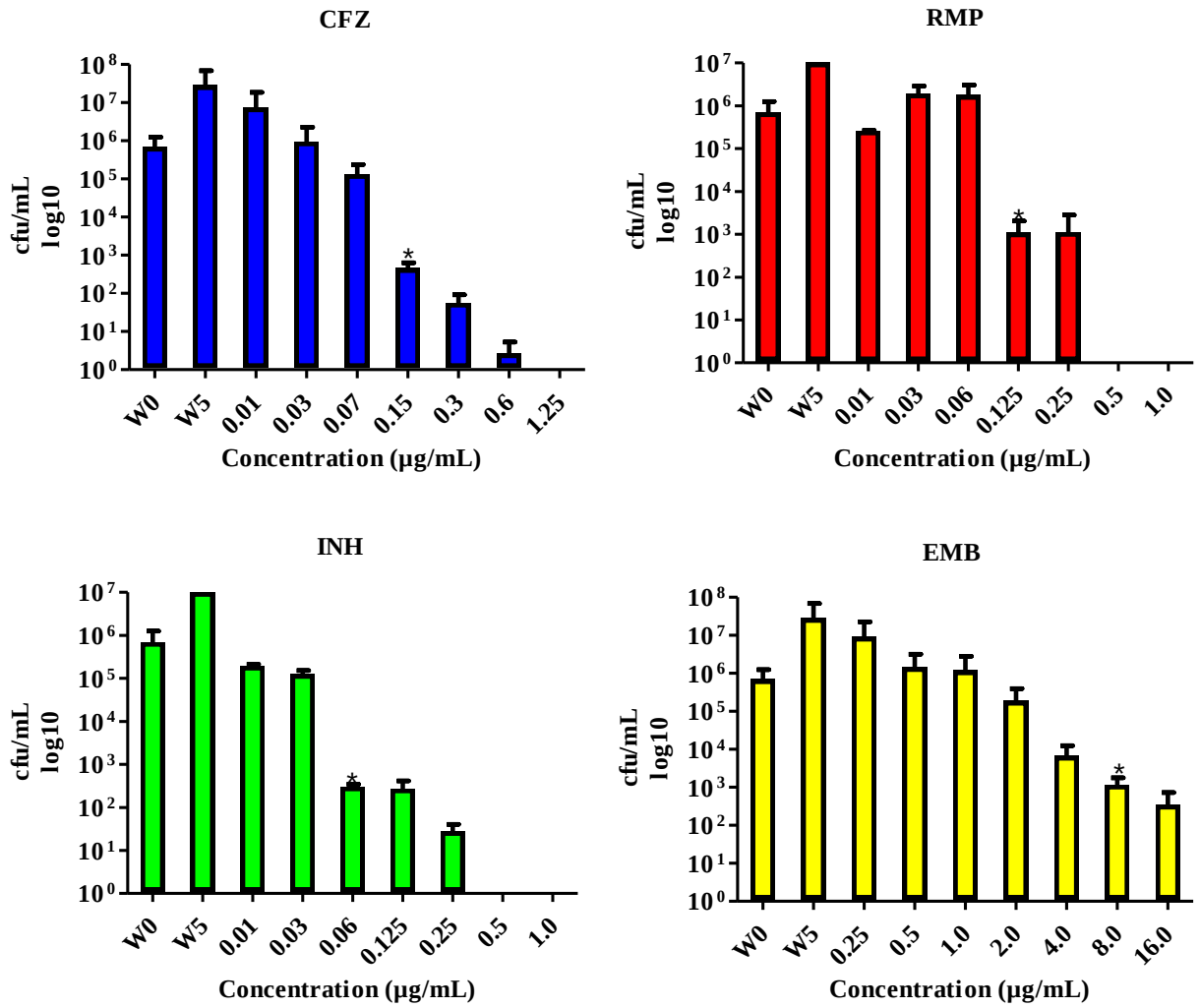



**Figure 5.4:** Determination of the minimum bactericidal concentrations of clofazimine and the primary anti-tuberculosis agents against biofilm-forming *Mycobacterium tuberculosis* organisms. The results, represented as log10 graph, are for three separate experiments performed in duplicate for each concentration of each agent and are presented as the mean ± standard deviation (SD). The MBC values are shown with asterisks (*)

***Fractional bactericidal concentration determinations***

These results are as shown in Table 5.5 and Figures 5.6 - 5.8 for the two-, three- and four-antibiotic combinations.

Two-drug combinations

All the three CFZ-containing two-drug combinations showed synergistic bactericidal effects with the highest synergistic activity shown in combination with RMP. For the primary drugs, synergistic bactericidal activity was exhibited by the combination of RMP and INH (Table 5.5 and figure 5.8i).

Three-drug combinations

All of the four three-antibiotic combinations demonstrated synergistic bactericidal effects against biofilm-forming *Mtb* bacteria. Two of the three CFZ-containing and the three primary antibiotic combination demonstrated the highest, albeit comparable synergistic activities (Table 5.5 and Figure 5.8ii).

Four-drug combination

The combination of CFZ with the three primary drugs resulted in synergistic bactericidal activity against biofilm-forming bacteria. This combination was the most potent of all the combinations tested (Table 5.5, Figure 5.8).

**Table 5.5:** The fractional bactericidal concentration index values of the antibiotic combinations for biofilm-forming *Mycobacterium tuberculosis*.

| Antibiotics | FBC | | | | | |
|---|---|---|---|---|---|---|
| | cfu/mL (W0) ± SD | cfu/mL (W5) ± SD | Concentration ratio | FBC (µg/mL) | FBCI | Interaction |
| CONTROL | $1.4 \times 10^4 \pm 1.5 \times 10^3$ | $1.17 \times 10^7 \pm 1.6 \times 10^6$ | NA | NA | NA | NA |
| CFZ + INH | | 32 ± 4 | 1/4 + 1/4 | 0.03 + 0.01 | 0.5 | Synergistic |
| CFZ + RMP | | $1.4 \times 10^2 \pm 58$ | 1/8 + 1/8 | 0.03 + 0.03 | 0.25 | Synergistic |
| CFZ + EMB | | $5.4 \times 10^2 \pm 5.4 \times 10^2$ | 1/4 + 1/4 | 0.03 + 2.0 | 0.5 | Synergistic |
| INH + EMB | | 45 ± 8 | 1/2 + 1/2 | 0.03 + 4.0 | 1.0 | Additive |
| RMP + EMB | | $3.3 \times 10^2 \pm 0$ | 1/2 + 1/2 | 0.06 + 4 | 1.0 | Additive |
| RMP + INH | | $1.1 \times 10^2 \pm 6$ | 1/4 + 1/4 | 0.03 + 0.015 | 0.5 | Synergistic |
| CFZ + INH + EMB | $1.7 \times 10^4 \pm 9.5 \times 10^2$ | $5 \times 10^2 \pm 1.7 \times 10^2$ | 1/6 + 1/6 + 1/6 | 0.02 + 0.003 + 1.3 | 0.5 | Synergistic |
| CFZ + RMP +EMB | | 33 ± 0 | 1/12 + 1/12 + 1/12 | 0.01 + 0.01 + 0.6 | 0.25 | Synergistic |
| CFZ + RMP + INH | | 32 ± 1.5 | 1/12 + 1/12 + 1/12 | 0.01 + 0.01 + 0.005 | 0.25 | Synergistic |
| RMP + INH + EMB | | 1 ± 0 | 1/12 + 1/12 + 1/12 | 0.01 + 0.006 + 0.6 | 0.25 | Synergistic |
| CFZ + RMP + INH + EMB | | 1 ± 0 | 1/32 + 1/32 + 1/32 + 1/32 | 0.004 + 0.003 + 0.001 + 0.25 | 0.125 | Synergistic |

**NA. Not Achievable, Not Applicable.**

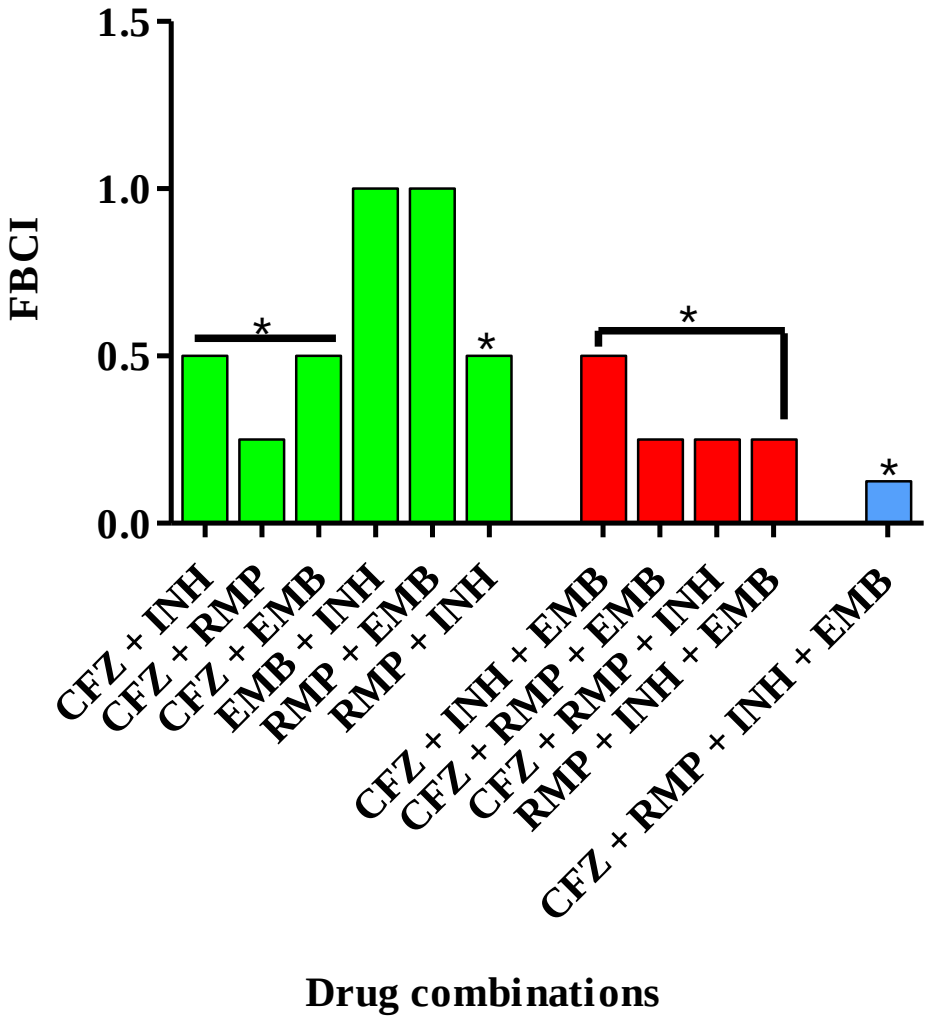
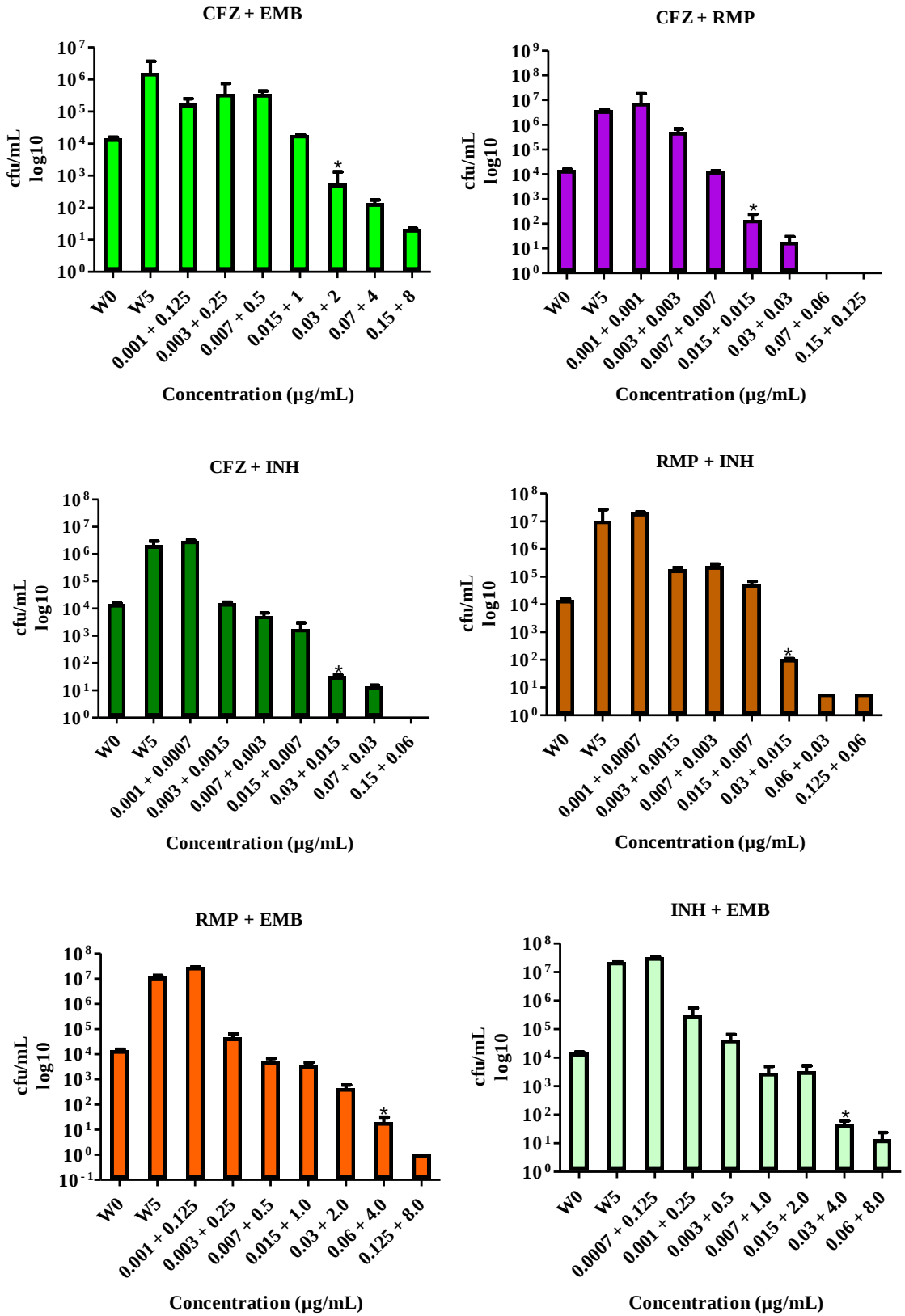
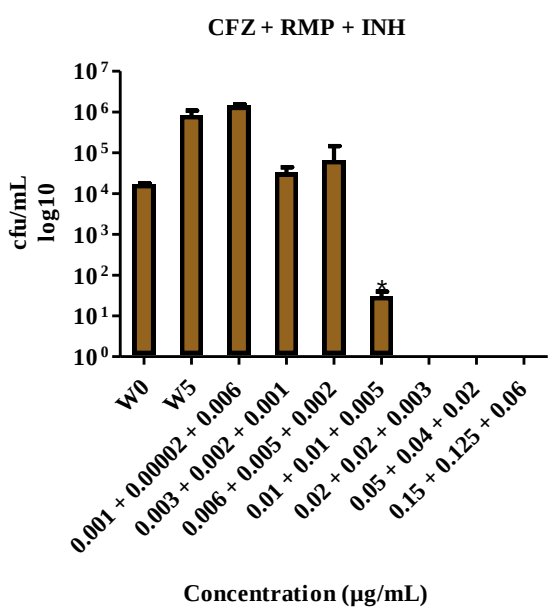
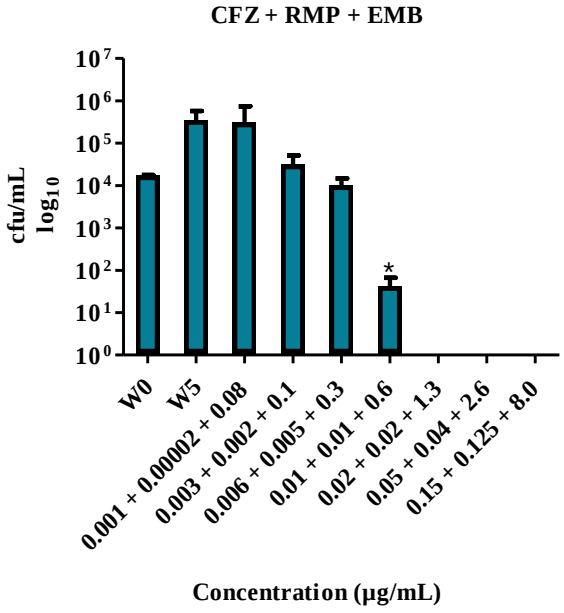


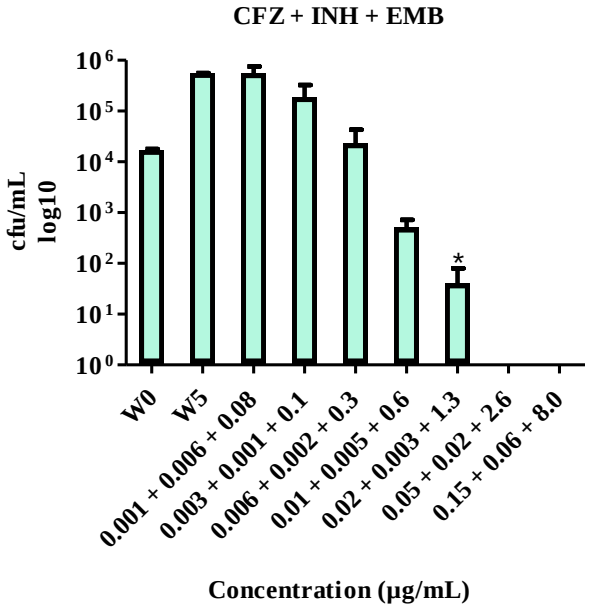

**Figure 5.5**: Inhibitory interactions of clofazimine with the primary antibiotics against biofilm-forming bacteria using the fractional bactericidal concentration indices. Synergistic effect showing FBCI values ≤ 0.5 is represented by an asterisk (*).

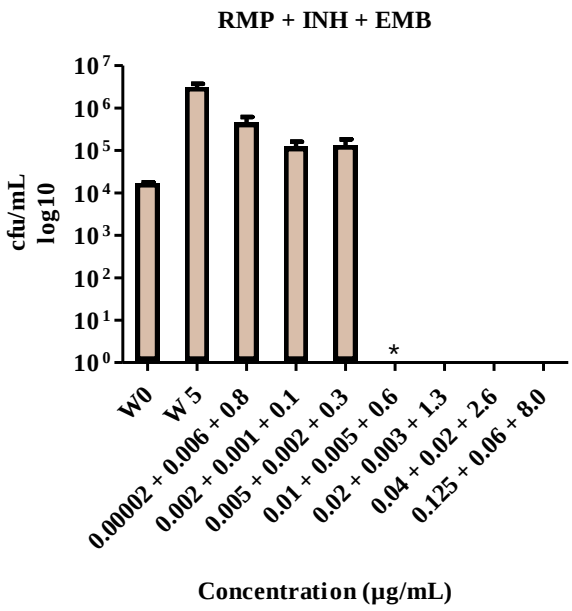



**i.**

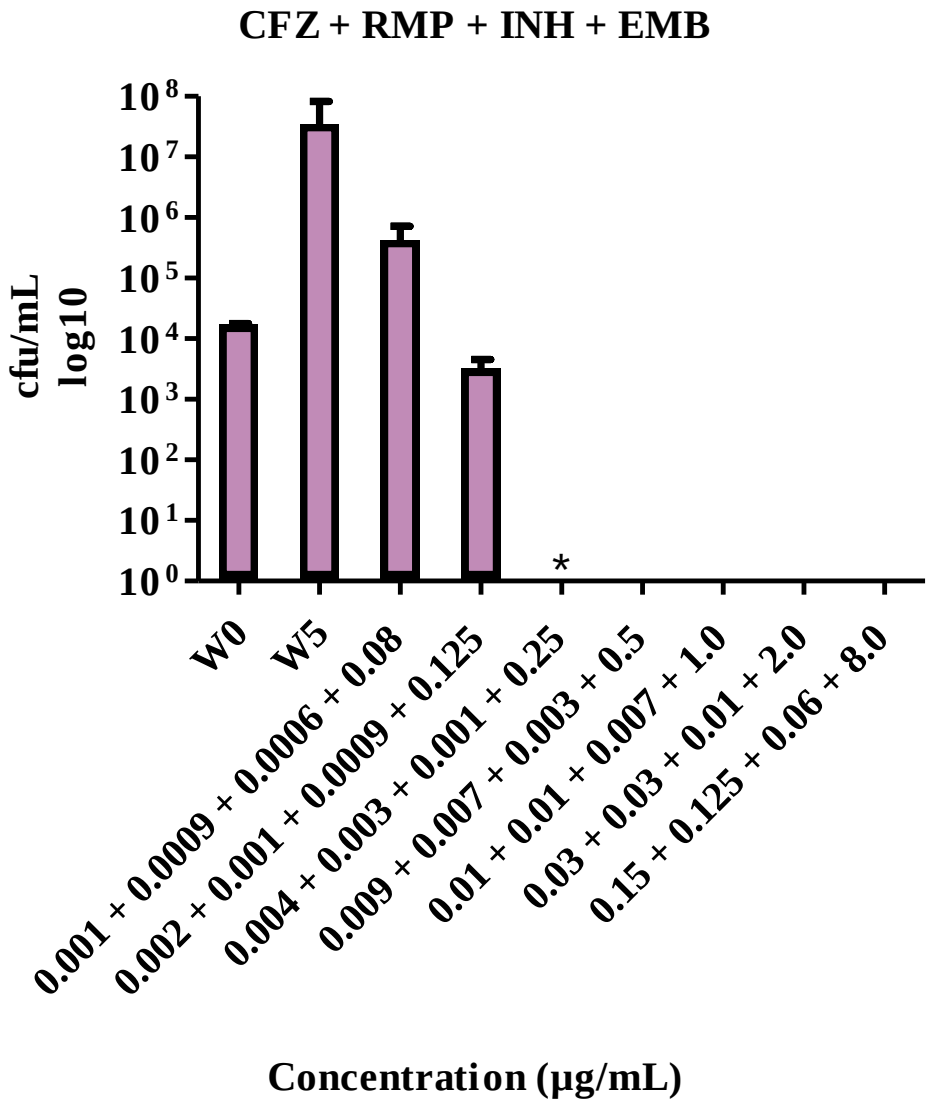



**ii.**




**iii.**

**Figure 5.6:** Fractional bactericidal concentrations of antibiotics against biofilm-forming *Mycobacterium tuberculosis* bacteria with the activities of the two-, three- and four-drug combinations shown in panels (i), (ii) and (iii) respectively. The results, represented as log10 graph, are of two separate experiments performed in triplicate and are presented as the mean ± standard deviation (SD). The asterisk (*) represents the achieved FBC of each antibiotic combination.

## 5.6. Discussion

Biofilms are important pathogenic mechanisms of bacteria, including mycobacteria (Brennan, 2017; Esteban and Garcia-Coca, 2018). In the case of *Mtb,* biofilm formation plays an important role in the process of caseous and necrotic granuloma formation during infection (Ojha *et al*., 2008; 2010; Mothiba *et al*., 2015). These granuloma lesions harbour predominantly persistent, SR and dormant, NR organisms (Esteban and Garcia-Coca, 2018).

During TB treatment, the SR and NR organisms are targeted during the continuation phase with RMP and INH for prolonged periods of four to seven months, due to slow responsiveness to antibiotic therapy. Despite extended treatment, eradication of these organisms is not always achieved, with a number of patients who have completed their therapy relapsing, indicating reactivation of surviving organisms during chemotherapy (Veatch and Kaushal, 2017)

To date, the number of antibiotics that is effective against *Mtb* biofilms is limited (Xu *et al*., 2018). CFZ, as reported in the current study, is one of the antibiotics that possess anti-mycobacterial activity. Importantly, the findings of this study have demonstrated that CFZ, in addition to suppressing the growth of planktonic organisms, also possesses both inhibitory and bactericidal activities against biofilm forming *Mtb,* achieving these effects at concentrations equivalent to those effective against planktonic bacteria. Although the primary anti-TB drugs, RMP and INH, also demonstrated high inhibitory and bactericidal activities against biofilm forming *Mtb*, these effects were only evident at concentrations considerably than those that inhibited the growth of planktonic organisms.

An important additional aim of this study was to evaluate the effects of CFZ on the activities of the primary anti-mycobacterial agents, RMP, INH and EMB against the SR organisms of biofilm-forming cultures. In contrast to the moderate synergistic inhibition of the growth of planktonic organisms when CFZ was used in combination with the primary anti-TB agents, CFZ-containing anti-TB drug combinations were considerably more effective against slow-growing, biofilm-forming organisms. In this context, combining CFZ with the primary anti-TB agents achieved higher levels of growth inhibitory synergism than any of the CFZ-free combinations of the primary anti-TB agents.

Despite high *in vitro* activity, the activity of these antibiotics *in vivo* in granuloma lesions, may be affected by various pharmacokinetic properties such as drug distribution, microenvironmental conditions of the TB lesion and the half-lives of the antibiotic. In the case of

CFZ, this anti-mycobacterial agent has a high level of accumulation in the cellular rim consisting of macrophages. RMP accumulates in high concentrations in necrotic foci and cellular compartments, while INH has poor accumulation in granuloma lesion due to rapid clearance (Prideaux *et al*., 2015; Strydom *et al*., 2019). These differences in drug distribution, suggest that the addition of CFZ to RMP- and INH-containing anti-TB drug regimens may augment therapeutic efficacy Although speculative, it is possible that the high accumulation of CFZ in macrophages will lead to disruption of granuloma membrane, which consists mainly of infected macrophages allowing access of the hydrophilic agent INH to the aqueous central necrotic foci. INH will then act synergistically in combination with RMP.

**5.7. References**

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## CHAPTER SIX: GENERAL DISCUSION

The findings of this study have revealed that combining CFZ with primary anti-TB drugs, especially RMP, results in synergistic suppression of the growth of AR bacteria, albeit less so than that observed with CFZ-free combinations of the primary drugs, specifically RMP and INH. However, CFZ effectively substituted for EMB in drug combinations evaluated for synergistic inhibition of growth of AR *Mtb* organisms. Based on these findings, CFZ may indeed be useful during the initial phase of chemotherapy by replacing EMB.

Of potential importance, CFZ, on the other hand, demonstrated significant augmentative interactions with RMP and INH against SR, organisms. Indeed, combinations of CFZ these primary anti-TB agents resulted in synergistic suppression of both the growth and survival of these slow-growing, biofilm-forming organisms. In this setting, the concentrations of RMP and INH required to target SR bacteria were higher than those which inhibited the growth of planktonic organisms, while the concentrations of CFZ were similar to those active against planktonic bacteria. In the case of RMP and INH these are potentially important findings as both these primary drugs are used during the continuation phase of TB chemotherapy during which they appear to have limited effects on this microbial population, with many patients developing relapse due to reactivation of the disease on completion of treatment. Addition of CFZ, with both high inhibitory and bactericidal activities for the SR *Mtb* population, to this primary two-drug regimen, may enable more effective elimination of these organisms.

This contention is supported by findings originating from a murine model of experimental TB chemotherapy, in which the inclusion of CFZ in the primary-drug regimen, resulted in delayed bacterial regrowth following treatment (Swanson *et al*., 2016).

In conclusion, the findings of the current study are novel and have demonstrated the potential benefit of addition of CFZ to standard anti-TB chemotherapy, possibly shortening treatment and/or preventing disease relapse.

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# CHAPTER SEVEN: LIMITATIONS OF THE STUDY AND FUTURE STUDIES

## 7.1. Limitations of the study

During TB treatment DS-TB patients are treated with four primary drugs including three agents tested in the current study used in combination with PZA during the intensive phase, targeting the AR bacteria. However, in the current study, interaction of PZA with CFZ was not investigated due to absence of an acidic growth medium, which is required for the optimum antimicrobial activity of this agent.

Additionally, during infection, the TB lesions are populated with heterogenous microbial populations which include the AR, SR and NR. In the current study, the antimicrobial activities of CFZ in combinations with primary anti-TB drugs were determined only on the AR and the SR organisms.

## 7.2. Future studies

The anti-mycobacterial activities of CFZ in combination with PZA on different microbial organisms in the absence and presence of the three tested primary anti-TB drugs *in vitro* is required. This information will be valuable in the design of future effective anti-TB treatment regimens.

## APPENDICES

### Appendix one: Preparation of media

### Middlebrook 7H9 broth and 7H10 agar

Media purchased from Beckton Dickinson (USA) were prepared following the manufacturer's instructions. A litre of 7H9 broth medium was prepared by dissolving 4.7 g of 7H9 broth base powder in distilled water, supplemented with 0.2% glycerol and 0.05% Tween 80 while 7H10 agar medium was prepared by dissolving 19 g of agar base powder in water supplemented with 0.5% glycerol. The mixtures were autoclaved at 121°C for 10 minutes (min), and cooled to 55°C, followed by addition of (Beckton Dickinson).

### Sauton broth preparation

A litre of Sauton broth was prepared by dissolving mineral solution mixture consisting of 4 g L-asparagine ($C_4H_8N_2O_3$), 0.5 g magnesium sulphate ($MgSO_4$), 0.5 g potassium dihydrogen phosphate ($KH_2PO_4$), 0.05 g ferric ammonium nitrate ($FeH_4N_5O_{12}$), 2 g citric acid ($C_6H_8O_7$), in distilled water ($dH_2O$), followed by the addition of 0.1 ml of 1% zinc sulphate solution ($ZnS0_4$) and 50 mL glycerol. The pH was adjusted to 7.2 with 10 M sodium hydroxide (NaOH), and the final volume was adjusted to 1L with distilled water. The medium was autoclaved at 121°C for 10 min.

## Appendix two: Ethics approval letter

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

**Institution:** The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001782 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

14 May 2020

**Approval Certificate**
**Annual Renewal**

**Ethics Reference No.: 300/2013**
**Title: Optimising the efficacy of clofazimine against biofilm-encased Mycobacterium tuberculosis**

Dear Mr SA Mashele

The **Annual Renewal** as supported by documents received between 2020-04-22 and 2020-05-13 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-05-13.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2021-05-14.
- Please remember to use your protocol number (300/2013 ) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

**Ethics approval is subject to the following:**
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

**Yours sincerely**

**Dr R Sommers**
MBChB MMed (Int) MPharmMed PhD
**Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

*The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)*

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