Development of novel anti-tuberculosis drugs from African medicinal plants

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DECLARATION

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Date
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Abstract

The need for the development of new antitubercular drugs is essential, especially with the resurgence of tuberculosis (TB). This problem is aggravated by patient in compliance, multi-drug resistant strain development and Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome (HIV/AIDS). In Africa, it is estimated that there are 50 000 terrestrial plant species, of which some 5 000 are believed to have medicinal significance. Although some of these plants are used by traditional healers, no efficacy studies have evaluated their use.

The aim of the study was to exploit the African wild plant diversity for discovering novel TB drugs. High throughput screening of potential drugs against Mycobacterium tuberculosis is hampered by the infectious nature and slow growth of the organism. Mycobacterium smegmatis was employed as a surrogate strain for preliminary screening then tested against M. tuberculosis. In the preliminary screening against M. smegmatis, 110 plant extracts were tested at 1 mg/ml and 36 synthetic compounds were tested at 10 μg/ml using Bioscreen C. Of the 110 plant extracts, two extracts (P02301B and P04495B) showed inhibition (42% and 66%, respectively) against M. smegmatis. Four of the 36 compounds (F1082, F1052, F37, and F952) showed inhibition (38%, 41%, 45%, and 55%, respectively) against M. smegmatis.

The active extracts and compounds were tested against M. tuberculosis using the BACTEC 460 system at the same concentrations as the primary screen. Extract P02301B showed 99% inhibition and P04495B did not inhibit growth against M. tuberculosis. Compound F1082 showed 99% inhibition and others (F952, F37, and F1052) showed growth inhibition of 42%, 41%, and 47%, respectively against M. tuberculosis. Plant extracts are being fractionated to identify the active principle. The MIC of F1082 was determined to be 8 μg/ml. This compound was further tested against selected Gram positive and Gram negative bacteria and showed no inhibition in all six bacteria. This compound appears to have selective activity for mycobacteria. F1082 needs to be further
investigated against multi-drug resistant tuberculosis (MDR-TB), in animal model systems, further more the mode of action against *M. tuberculosis* needs to be determined.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AFB</td>
<td>Acid fast bacilli</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
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<tr>
<td>CFA</td>
<td>Freund's complement adjuvant</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOTS</td>
<td>Directly observed treatment short-course</td>
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<tr>
<td>eCS</td>
<td>Electro-colloidal silver</td>
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<tr>
<td>EMB</td>
<td>Ethambutol</td>
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<tr>
<td>FQ</td>
<td>Fluoroquinolones</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GI</td>
<td>Growth index</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>IL</td>
<td>Interleukins</td>
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<td>INF</td>
<td>Interferon</td>
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<td>INH</td>
<td>Isoniazid</td>
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<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
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MIC  Minimal inhibitory concentration
µ       Micro-
MTB  Mycobacterium tuberculosis
NTM  Nontuberculous mycobacteria
OADC Oleic acid, albumin, dextrose, catalase supplement
OD   Optical density
PMN  Polymorphonucleocytes
PPD  Purified protein derivative
PZA  Pyrazinamide
Rif  Rifampicin
RNA  Ribonucleic acid
SM   Streptomycin
T-h  T-helper lymphocyte
USA  United States of America
WHO  World Health Organization
Z-N  Ziehl Neelsen stain
γ    Gamma
CHAPTER ONE

Literature Review
1.1 Introduction

At the beginning of the 18th century, tuberculosis was a devastating infectious disease in Europe and was known as the “white plague” (Ryan, 1992). At the time, mankind had no effective chemotherapy, and morbidity and mortality were high, especially among the poor. However, as the standard of living of urban poor improved and various preventative measures became widely practiced, the incidence of the disease fell. Better nutrition, less crowded living circumstances, pasteurization of milk, quarantine, use of sanitaria, prohibition of expectoration in public places, periods free from depression and war, all played a useful role (Mitscher and Backer, 1998).

In the middle years of the 20th century, a succession of effective antitubercular chemotherapeutic agents were discovered. The incidence of the disease continued to decrease until it largely faded from the public consciousness as a heath hazard in industrialized nations. Consequently, the research on TB fell to very low levels in industry and academia alike. In recent years this picture has changed significantly as many inner city neighborhoods deteriorated economically and populations almost everywhere continued to increase. Homelessness, poor nutrition, crowded conditions, development of multiple drug resistant strains of \textit{M. tuberculosis}, ease of international travel, immune suppression due to HIV/AIDS and other causes are regarded as largely responsible (Ryan, 1992). Perhaps more disturbingly, the mortality rate (about 50% in the preantibiotic era; falling to about 15% in the antibiotic era with conventional TB therapy) has increased to about 40% in immunocompetent patients with multi drug resistant TB (MDR-TB), and to 80% with MDR-TB in HIV patients. Thus MDR-TB in otherwise healthy persons has a mortality which is nearly equivalent to that in the preantibiotic era and in immunocompromized patients, it is actually greater. There can be no doubt that a reinstitution of preventative measures and discovery of effective new agents are urgently needed in order to deal with this situation.
1.2 Bacteriology

The name "mycobacterium", meaning fungus bacterium, was derived from the way the tubercle bacillus grows, as mould-like pellicles on the surface of liquid media (Ratlege et al., 1989). There are more than 70 species of mycobacteria. Of these, two are major human pathogens, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The remaining mycobacteria are environmental organisms and are known as Nontuberculous mycobacteria (NTM). *M. tuberculosis* is well known and studied species of tuberculosis complex (*M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*) because of its clinical importance. The second and third are rare causes of the disease in immunocompetent people, and *M. microti* is not usually pathogenic, although it is possible that the prevalence of its infections has been underestimated (Nieman et al., 2000).

*M. tuberculosis* is a slow grower and divides every 16 to 20 hours (Cox, 2004). *M. tuberculosis* is a fairly large non-motile rod-like bacillus, which survives under dry conditions for weeks. It is identified microscopically by its staining characteristics: it retains certain stains with acidic decolourisation, and is thus classified as "acid fast bacilli" or AFB (Madison, 2001). In the most common staining technique, the Ziehl Neelsen stain, AFB are stained a bright red which stands out clearly against a blue background. Acid-fast bacilli can also be visualized by fluorescent microscopy, and by an auramine rhodamine stain.

The cell wall structure of *M. tuberculosis* deserves special attention because it is unique amongst prokaryotes and it is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. The lipid fraction consists of three major components: 1) Mycolic acids, are unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium*. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic acids are thought to be a significant determinant of virulence in *M. tuberculosis*. Most likely, they prevent attack of the mycobacteria by cationic proteins, lysozyme and oxygen
1.3 Tuberculosis the disease

TB in humans is an extremely complex disease that primarily affects lungs in discrete consolidated foci known as granulomas. TB is spread by airborne droplet nuclei, which are particles of 1-5 μm in diameter that contain *Mycobacterium tuberculosis* (MTB). Due to their small size, the particles can remain airborne for minutes to hours after expectoration by people with pulmonary or laryngeal tuberculosis during coughing, sneezing, singing or talking (Wells, 1934). MTB is an obligatory intracellular pathogen, which has a predilection for the lung tissue, which is rich in oxygen supply. The bacilli enter the body via the respiratory route. The bacilli spread from the site of infection in the lung through lymphatics or blood to the other parts of the body, with the apex of the lung and the regional lymph nodes being favoured sites. Only 15% of TB patients develop extra-pulmonary TB of the pleura, lymphatic, bone, genito-urinary system, meninges, peritoneum, or skin (Raja, 2004). A TB patient can have active disease, which is characterized by presenting symptoms such as cough, weight-loss and night sweats. The diagnosis is confirmed by isolation of bacteria from the site of infection. Latent infection, which is in the absence of clinical symptoms, and is defined by a delayed-type hypersensitivity reaction when the patient is subcutaneously challenged with purified protein derivative (PPD) (Boshoff et al., 2005).

1.4 Epidemiology

TB is the world’s second most common cause of death from infectious disease, it is second to HIV/AIDS (Frieden et al., 2003). TB has probably killed 100 million people in
the past 100 years (Iseman, 2000). There were an estimated 8-9 million new cases of TB in 2000, less than a half of which were reported, 3-4 million cases were sputum smear positive, the most infectious form of the disease (Corbett et al., 2003). Most cases are in people aged 15-49 years. Sub-Saharan Africa has the highest incidence rate (290 per 100 000 population), but the most populous countries of Asia have the largest number of cases: India, China, Indonesia, Bangladesh and Pakistan together account for more than a half of the global burden.

Case numbers have declined more or less steadily in western and central Europe, North and South America, and the Middle East. In 2003, the World Health Organization (WHO) reported that tuberculosis has increased in the former Soviet Union and Sub Saharan Africa. Worldwide, an estimated 11% of new adult cases in 2000 were infected with HIV with wide variation among regions, 38% in Sub- Saharan Africa, 14% in more developed countries, and 1% in the Western Pacific region. The rate of HIV infection among patients with tuberculosis has so far remained below 1% in Bangladesh, China and Indonesia (Frieden et al., 2003).

The increase in tuberculosis incidence in Africa is strongly associated with the prevalence of HIV infection (Corbett et al., 2002). Rates of HIV infection among TB patients are correspondingly high, exceeding 60% of HIV/AIDS patients in Botswana, South Africa, Zimbabwe and Zambia. Of the 10 countries with the highest incidence of TB, nine are in Africa (Dye et al., 1999). Africa bears the additional high level of poverty and some of the lowest health indicators in the world. In South Africa over three in a thousand people die of TB, the highest rate in the world. One in 200 people suffer from active TB. Parts of South Africa such as the Western Cape have among the highest incidences of TB ever recorded (Van Rie et al., 1999). This is because a large proportion of the population lives under conditions favourable to the development of TB. These conditions include unfavourable socio-economic conditions, over-crowding and lack of adequate health facilities. This is highly prevalent among the population living in informal settlements in the outskirts of the urban cities. South Africa is also witnessing an explosion in the
number of cases of drug-resistant TB. In some parts of South Africa one in ten cases of TB is resistant to the treatment (Lall and Meyer, 1999).

Fig 1: The distribution of M. tuberculosis in the world in 2003 (WHO, 2006)

1.5 Tuberculosis in HIV infected patients

1.5.1 Background

MTB is still a problem in many countries. According to WHO estimates in 2000, one third of world population (1.8 billion people) is infected with TB, including 8% of subjects co-infected with HIV (Dolin, 2000). The problem of co-infection concerns primarily poor, developing countries, where TB is common. In sub-Saharan Africa, 1/3 of patients with active TB are co-infected with HIV. In the region of west Pacific and south east Asia, the co-incidence of HIV and TB is less frequent than in Africa, but according
to WHO, HIV infection tends to be underdiagnosed there (Dye et al., 1999). The problem of co-infection is also important in countries with low incidence of TB. Coinfection with HIV and MTB is called a fatal coincidence. HIV infection is known to be a serious factor in TB. In HIV infected subjects, 30-40% develop active TB when exposed to mycobacteria, whereas in healthy populations this rate reaches 2-5% only. TB in HIV infected patients is characterized by atypical clinical presentation, severe course and dissemination of the process. Active TB accelerates the progression of asymptomatic HIV infection phase towards AIDS. TB causes 5-160 fold enhancement of HIV replication (Barnes et al., 2002; Havlir and Barnes, 1999).

1.5.2 Pathogenesis of the co-infection

In HIV and MTB co-incidence, the infection exerts mutual negative effect. Immune system deficiency, and especially reduced count and impaired function of T-CD4, or T-helper (Th) lymphocytes, are known to be the main disorder associated with HIV infection. These lymphocytes play the main role in systematic defense against mycobacteria. CD4 lymphocytes are divided into two groups Th1 and Th2 which release different cytokines: Th1---INF-γ and Th2---IL4, IL5, and IL10, respectively. These cytokines play a very significant role in the response to infection with intracellular pathogens by activating alveolar macrophages and synthesis of free oxide radicals, stimulation of class II MHC expression and migration of lymphocytes. Studies by Condos et al (Condos et al., 1997; Condos et al., 1998) demonstrated that patients with advanced TB present a weak Th1 response and low levels of IFN-γ concentrations were observed. Conversely, in mild forms of TB high INF-γ concentrations were observed. Since the CD4 lymphocytes are the main source of IFN-γ, a deficiency of these cells in the course of HIV infection leads to impaired INF-γ synthesis and weaker antimycobacterial response. On the other hand, proinflammatory cytokines, mainly TNF alpha, IL-1 and IL-6, enhance HIV replication. Also mycobacteria and their products enhance HIV replication by stimulating transcription factor NF-κB, a cellular factor stimulating the active HIV replication region (Goletti et al., 1996; Hoshino et al., 2002).
1.6 Treatment of tuberculosis

Currently TB is treated with an initial intensive phase: 2-month regime comprising rifampicin (Rif), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB) or streptomycin (SM)-to ensure that mutants resistant to single drug do not emerge (MMWR, 1993). The next 4 month (continuation phase), only Rif and INH are administered to eliminate any persisting tubercle bacilli. INH and Rif, the two most potent antituberculous drugs, kill more than 99% of tubercule bacilli within two months of initiation of therapy (Mitchison, 1998; Iseman and Madsen, 1989). Along with those two drugs, PZA, with a high sterilizing effect, appears to act on semi-dormant bacilli not affected by any other antitubercular drug (Heifets et al., 1992). Using these drugs in conjunction with each other reduces antitubercular therapy from 18 months to 6 months in susceptible strains. The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission and to prevent the emergence of drug resistance.

Although the duration of treatment of TB has been reduced to as short as 6 months, where rifampicin is used throughout the course of the treatment, this is still very long compared with the treatment of most other bacterial infections. Many countries in Africa, however, tend to use the cheaper continuation phase combination of ethambutol and isoniazid after the first 2 months of rifampicin-based therapy, which lengthens the total treatment period to 8 months. It is an accepted fact that patient adherence is a major determining outcome of treatment. This has resulted in promotion by WHO of directly observed treatment short-course (DOTS) strategy as a means of improving the outcome of treatment. Although the currently available drugs are effective, delay in diagnosis, interruption or non-availability of treatment and drug resistance lessens the likelihood of achieving a high cure rate. It has been argued that, incorrect prescription, inadequate drug supply, poor case holdings and failure to ensure that the patients follow the prescribed regimens drug selection by patients are major contributing factors to treatment failure and the development of drug resistance (Blomberg et al., 2001).
1.7 BCG vaccine

The bacillus Calmette-Guerin (BCG) was derived between 1906 and 1919 by in vitro attenuation of an isolate of Mycobacterium bovis, and was first used as an antitubercular vaccine in humans in 1921. The protective efficacy against TB has been raised for several decades (Soysal et al., 2005). Evidence from several randomized controlled trials and observational studies showed that, in adults, efficacy varied greatly in different populations, from 0 to 80% (Fine, 1995). The most common explanation for the variability in BCG vaccine’s protective efficacy is the difference between populations in terms of exposure to cross reacting environmental mycobacteria, which can either mask or inhibit the protection induced by BCG (Black et al., 2002). In children, however, the protection from BCG appears to be better, especially against most severe disseminated forms of the disease (military TB and meningitis) (Rodrigues et al., 1993). This finding has been justified by the WHO in that infants should be vaccinated as soon after birth as possible with a single intradermal dose of BCG in countries with a high risk of TB infection. The standard test for TB in the absence of significant disease was, until recently, the tuberculin skin test, which measures the reaction to intradermal injection of purified protein derivative (PPD). However, because this test lacks specificity due to the presence of antigen shared among most mycobacteria (including BCG) (Huebner et al., 1993), it has never been clear whether BCG vaccination prevents MTB infection as distinct from clinical disease (Mittal et al., 1996).

1.8 MDR-TB and the Mechanism of Resistance

Multidrug-resistance tuberculosis (MDR-TB) is among the most worrisome element of the pandemic of antibiotic resistance (Anderson, 1999). MDR-TB now has been found on all continents, with especially high rates in countries of the former Soviet Union (Mendez et al., 1998). WHO currently recommends, for all new cases of TB, standardized short course chemotherapy (SCC) based on a regimen of four first line drugs taken for 6-8 months (Pio and Chaulet, 1998). The phrase “MDR” in mycobacteriology refers to
simultaneous resistance to at least rifampicin (Rif) and isoniazid (INH) (Vareldzis et al., 1994) (with or without resistance to other drugs). Genetic and molecular analysis of drug resistance in *M. tuberculosis* suggest that resistance is usually acquired by the bacilli either by alteration of the drug target through mutation (Spratt, 1994) or by titration of the drug through overproduction of the target (Davis, 1994). MDR-TB results primarily from accumulation of mutations in individual drug target genes. The probability of resistance is very high for less effective antitubercular drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin (one in a thousand \(10^{-3}\)); intermediate for drugs such as isoniazid (INH), streptomycin (SM), ethambutol (EMB), kanamycin, and p-aminosalicylic acid \(10^{-6}\) and lowest for rifampicin (Rif) \(10^{-8}\) (Shimao, 1987; Crofton, 1970). Consequently, the ability of a mutation is directly proportional to bacterial load. A bacillary load of \(10^9\) will contain several mutants resistant to any one antitubercular drug (Grange, 1990). As mutation conferring drug resistance are chromosomal, the likelihood of a mutant being simultaneously resistant to two or more drugs is the product of individual probabilities, thus the probability of MDR is multiplicative.

### 1.8.1 Resistance to isoniazid (INH)

INH (isonicotinic acid hydrazide, 4-pyridinecarboxylic acid hydrazide), highly active against the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*), has a very low MICs (0.02μg/ml to 0.06μg/ml) (Youatt, 1969). The mechanisms of action of INH, as well as the mechanism conferring INH resistance, are complex and not completely understood. However, evidence suggests that INH inhibits the biosynthesis of cell wall mycolic acids, thereby making the mycobacteria susceptible to reactive oxygen radicals and other environmental factors. Activation of INH to an unstable electrophilic intermediate requires the enzyme catalase peroxidase (KatG, coded by katG) and an electron sink (hydrogen peroxide) (Shoeb et al., 1985). Although hydrazine formed after INH decomposes may also mediate activation of INH (Maggiozzo and Marcinkeviciene, 1996). Nevertheless, KatG is the only enzyme
 capable of activating INH, and consequently, KatG mutants *M. tuberculosis* strains are invariably INH resistant.

### 1.8.2 Resistance to rifampicin (Rif)

Rifampicin (Rif), first introduced in 1972 as an antitubercular drug is extremely effective against *M. tuberculosis*. It has MICs of 0.1µg to 0.2µg (Mitchison, 1985; Woodley *et al.*, 1972). Due to its high bactericidal action, Rif, along with INH, forms the backbone of the short course chemotherapy (Kochi *et al.*, 1993). Rif has long been believed to target the mycobacterial RNA polymerase and thereby kill the organism by interfering in the transcription process (Ovchinnikov *et al.* 1981). Using purified RNA polymerase from *M. smegmatis*, strain mc² 155, Levin and Hatfull demonstrated that Rif specifically inhibited the elongation of full-length transcripts and had virtually no effect on the initiation of transcription (Levin and Hatfull, 1993). It was demonstrated that Rif specifically interacted with the β subunit of RNA polymerase, thereby hindering transcription, and that mutation in the *rpoB* locus conferred conformational changes leading to defective binding of the drug and consequently resistance (Jin and Gross, 1988). Single amino acid substitution in the 81 base pair core-region of the *rpoB* gene is responsible for conferring Rif’s resistance (in ≥95% of cases).

### 1.8.3 Resistance to ethambutol (EMB)

EMB [dextro-2,2’-(ethylenediimino)-di-1-butanol], synthetic compound with profound antimycobacterial effects (Thomas *et al.*, 1961), is a first line anti-TB drug with a broad spectrum of activity. EMB’s mechanism of action and the genetic basis for resistance were largely obscure. Synergy resulting in co-administration of EMB and other drugs gave evidence for the involvement of EMB in obstructing the formation of cell wall. The synergistic effect was explained as a consequence of increased permeability of the mycobacterial cell wall leading to increased drug uptake (Rastogi *et al.*, 1990). Mycolic acid binds to arabinogalactan and forms mycolyl-arabinogalactan peptidoglycan complex
in the cell wall. Disruption of the arabinogalactan synthesis inhibits the formation of this complex and may lead to increased permeability of the cell wall. Subsequently, it was demonstrated that EMB specifically inhibited arabinogalactan synthesis. It was later shown that EMB specifically inhibited arabinosyl transfer, suggesting that arabinosyl transferase was the primary cellular target for EMB. EMB resistant strains have been shown to have a mutation on the embB gene encoding an arabinosyl transferase which catalysis cell wall synthesis.

1.8.4 Resistance to pyrazinamide (PZA)

PZA, a structural analog of nicotinamide, was shown to have considerable anti-*M. tuberculosis* activity in 1952, but it became an important component of the short course chemotherapy only in the 1980s. PZA, active against semi-dormant bacilli not affected by any other drug, has a strong synergy with INH and Rif (MMWR, 1993). Depending on the assay system and the conditions applied, MICs of PZA vary from 8μg/ml to 60μg/ml. However, even at very high MICs, PZA has no significant bactericidal effect and is primarily considered a “sterilizing drug” (Heifets and Lindohlm-Levy, 1992). The mechanism of action of PZA has not been clearly understood. It is apparent that its activity depends on the bacterial amidase, which converts PZA into pyrazinoic acid (PZOA), an active form (Mitchison, 1996). PZA resistant bacilli lack this amidase activity. The gene encoding this has been identified, and the mutation to this pncA has been associated with resistance to PZA (Scorpio and Zhang 1996).

1.8.5 Resistance to streptomycin (SM) and other inhibitors of protein synthesis

Various drugs exert their antibacterial effect by inhibiting the protein transitional machinery. Among these, aminoglycosides, macrolites, tetracyclines, and basic peptides
like viomycin and capriomycin are active against mycobacteria (Benveinstie and Davies, 1973).

SM, one of the oldest drugs known to be active against *M. tuberculosis*, disrupts the decoding of aminoacyl-tRNA and thus inhibits mRNA translation or causes inefficient translation (Inderlied, 1991). One of the most common mechanisms of acquisition of resistance to SM is acetylation of the drug by aminoglycoside-modifying enzyme (Davies and Wright, 1997) Benveinstie and Davies, 1973). However, this mechanism is not found in *M. tuberculosis*. Instead, resistance to SM is attributed, at least partially, to two distinct classes of mutation including point mutations in the S12 ribosomal protein, encoded by rpsL gene (Douglas and Steyn, 1993) and mutation in the rrs operon encoding the 16S rRNA (Finken et al., 1993).

1.8.6 Resistance to other drugs

Related aminoglycosides such as kanamycin, amikacin, and paramomycin demonstrate no obvious cross resistance to SM and thus are alternative in the SM resistance. Viomycin and capriomycins are bacteriostatic agents that act by binding to the 50S ribosomal subunit and inhibit the translocation reaction (Benveinstie and Davies, 1973). Although cross-resistance between viomycin and capreomycin does occur, the exact mechanism for acquisition of drug resistance is not known.

1.8.7 Resistance to Flouroquinolones (FQ)

FQ as antimycobacterial agents were first described in 1984 and have primarily been used as therapeutic alternatives in MDR-TB cases (Gay et al., 1984). DNA gyrase (Gyr), a member of the type II DNA topoisomerase (Gellart et al., 1976), is the primary target for FQ action. Gyr introduces negative supercoils in closed circular DNA molecule and is heterotetramer (A2B2), coded by gyrA and gyrB respectively (Kirchausen et al., 1978). Quinolones activity is determined by the GyrA protein, which contains the
cleavage/relocation activity, while GyrB contains the intrinsic coumarin-sensitive ATPase activity (Gellart et al., 1976). FQs, synthetic derivatives of nalidixic acid, act by inhibiting the DNA supercoiling and relaxation activity of Gyr without affecting the ATPase activity (Sugino et al., 1977) and enhance the rate of DNA cleavage by Gyr. Mutations clustered in a small region in GyrA conferred resistance to FQs (Takiff et al., 1994).

1.9 Tuberculosis drug development

1.9.1 The value of plants used in traditional medicine for drug discovery

Fossil records date human use of plants as medicine at least to the Middle Paleolithic age, some 60,000 years ago (Solecki and Shanidar, 1975). From that point the development of traditional medical systems incorporating plants as means of therapy can be traced back only as far as recorded documents of their likeness. The value of these systems is as a methodology of medicinal agents which according to WHO, almost 65% of the world's population have incorporated into their primary modality of health care (Fransworth et al., 1985). The goals of using plants as source of therapeutic agents are 1) To isolate bioactive compounds for direct use as drugs e.g., digoxin, digitoxin, morphine, reserpine; 2) To produce bioactive compounds of novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/ or lower toxicity, e.g., metformin, nabilone, oxycodon (and other narcotic analgesic), taxotere, teniposide; 3) To use agents as pharmacologic tools, e.g., lysergic acid diethylamide, mescaline, yohimbin, and 4) To use the whole plant or part of it as a herbal remedy, e.g., cranberry, echinacea, garlic, feverfew. The number of higher plants (angiosperm and gymnosperm) on this planet is estimated at 250,000 (Ayensu and DeFilipps, 1978), with a lower level at 215,000 (Cronquist, 1981) and an upper level as high as 500,000 (Tippo and Stern, 1977). Of these, only about 6% have been screened for biological activity, and a reported
15% have been evaluated phytochemically (Verpoorte, 2000). It is important to note that, chemical diversity of secondary plant metabolites that results from plants evolution may be equal or superior to that found in synthetic combinatorial chemical libraries. One cannot therefore discount the past importance of plants as a source of structurally novel drugs. Ethnomedicine may be defined broadly as the use of plants by humans as medicines (Farnsworth, 1994); but this can be called more accurately ethnobotanic medicine. Traditional medicine is abroad term used to define any non-Western medical practice.

1.9.2 Approaches to drug discovery using higher plants

Several reviews pertaining to approaches for the selecting plants as candidates for drug discovery programs have been published (Phillipson and Anderson, 1989). Here is an outline these approaches briefly before concentrating on the ethnomedical approach, 1) Random selection followed by chemical screening: The so called phytochemical approaches [i.e., for the presence of cardenolites/bufadenolites, alkaloids, triterpenes, flavonoids, isothiocyanates, iridoids, etc (Farnsworth, 1966)], have been used in the past and currently pursued in the developing countries. The tests are simple to perform, but false-positive and false-negative tests often render results difficult to assess (Segelman et al., 1968). More important, it is usually impossible to relate one class of phytochemical to specific biological target; for example, the alkaloids or flavonoids produce a vast array of biological effects that are usually not predictable in advance; 2) Random selection followed by one biological assay: In the past, plant extracts were evaluated in experimental animals, primarily mice and rats. More than 35,000 species were screened in vitro and later in vivo at the NCI (National Cancer Institute) from 1960 to 1981. Using this approach, taxol and camptothecin (Wall and Wani, 1996) were discovered in this program as well as several other plant-derived compounds that were not successful in human studies; 3) Follow-up of biological active reports: These reports showed that the plant extracts had interesting biological activity, but the extracts were not studied for their active principle. The literature from the 1930s through the 1970s contains these types of
Literature Review

reports; 4) Follow-up of ethnomedical (traditional medicine): a) Plants used in organized traditional medicine—Ayurveda, Unani, Kampo, and traditional Chinese medicine have been in use for thousands of years. Even though the Western medical science views such systems as lacking credibility, undeniably they are used widely by most people of this planet. Adverse effects from those widely used plants are not well documented in literature, and efficacy of these plants and plant mixtures is more difficult to assess by Western scientific methods; b) Herbalism, folklore, and shamanism—these center on an apprenticeship systems of information passed to the next generation through a shaman, curandero, traditional healer, or herbalist. The plants that are used are often kept secret by the practitioner, so little information about them is recorded; thus there is less dependence on scientific evidence as in systems of traditional medicine that can be subject to scrutiny. The shaman or herbalist combines the roles of pharmacist and medical doctor with cultural/spiritual/religious beliefs of a region or people, which are often regarded as magic or mysticism. This approach is widely practiced in Africa and South America. Ethnomedical information can be acquired from various sources such as books on medical botany (Lewis and Elvin-Lewis, 1977) and herbals (Cruz et al., 1940), review articles, notes placed on voucher herbarium specimens by the botanist at the time of collection (Von Reis and Arboretum, 1973); 5) Use of database—the NAPRALERT database (Loud et al., 1985) currently contains information of 43,879 species of higher plants covering ethnomedical, chemical, and pharmacologic uses. Of these, 13,599 species contain ethnomedical data distributed among 3,607 genera and 273 plant families.

1.9.3 Interventions and new drugs

The need for new anti-tuberculosis agents is urgent because of the increasing resistance of mycobacteria to the classic anti-tuberculosis drugs. The new or improved effective drug must improve the current treatment through facilitating patient and provider compliance. This would be possible with availability of drugs that result in shorter regimens and regimens that require less supervision. Other requirements would be to improve the treatment of multi-drug resistant tuberculosis (MDR-TB) and the provision of a more effective regimen for the treatment of latent TB infection (O’Brien and Nunn
2001). The process of developing new drugs against TB has been facilitated by the availability of the complete genome sequence of *M. tuberculosis*. In addition, the development of new molecular markers for infection and the response to treatment are likely to have an important role in the development of new drugs for TB. Other new technologies that can be used in the research and development of new drugs include proteomics (analysis of proteins in the cell or pathogen), the use of molecular diversity and combinatorial chemistry, as well as the use of the whole genome to discover drug targets (Mwinga and Fourie, 2004). However, lead compounds based on the application of these technologies are only expected to be available several years from now.

Medicinal plants have also become the focus of research in terms of conservation, and to determine whether the traditional uses are supported by actual pharmacological effects or merely based on folklore (Cunningham 1998; Locher *et al.*, 1995; Williams 1996). The development of new anti-tuberculosis agents, which can be readily and simply produced from some local source (e.g. medicinal plants), would be desirable, especially for developing countries such as South Africa (Lall and Meyer, 1999).

**1.9.4 Prospective drugs**

It is quite interesting that, substantial amount of work has been done and documented on plant derived compounds in search of antmycobacterial activity. These include fluoroquinolone compounds, oxazolidinone compounds, clofazimine. Phenazine, phenathizine, azoles and peptides, nitroimidazopyrans and long acting rifamycins (rifapentine, rifabutin, rifalazil) (Hudson *et al.*, 2003; Duncan, 2003; O'Brien and Nunn, 2001; Tomiaka 2000; Zang and Amzel, 2002). Rifapentine, which was approved for the treatment of TB in USA in 1998, is a rifamycin antibiotic. It acts by inhibiting RNA synthesis. Rifapentine has a half life of 16- 20 hours, compared to rifampicin which has 2 to 3 hours, which makes it possible to reduce the dose to once weekly administration during the continuation phase of treatment. Although it is more active against MTB than rifampicin, it is not effective against drug-resistant MTB as rifampicin resistant stains are
also cross resistant to rifapentine (Jarvis and Lamb, 1998; Tam et al., 1998; Chaulet, 1998)

An application to the Food and Drug Administration (FDA) has been filed by the GATB for the new investigational drug, the nitroimidazole PA-824 (Chiron Corporation, Emeryville, California) (Elías, 2005). Sequella Incorporated (Rockville, Md) are also currently performing preclinical work on the ethambutol analogue, SQ109. A few drug candidates are in clinical trial stage, including pyrrole LL-3858 (Lupin Limited, Mumbai, India), moxifloxacin and gatifloxacin, which are already approved for the treatment of other ailments, and a diarylquinolone (Johnson and Johnson Pharmaceutical Research and Development Division of Janssen Pharmaceutical N.V), R207910, under development as TMC207 (Tibotec Pharmaceuticals Limited, Mechelen, Belgium) (Hampton, 2005). The latter chemical known as 1-(6-bromo-2-methoxy-quinolin-3-yl)-4dimethylamino-2-naphthalen-1-yl-1-phenyl-butyl-2-ol, has MICs of 0.030 to 0.120µg/ml against reference and antibiotic sensitive strains of MTB. Also of equally important, it is potent against MDR-TB isolates, as well as a broad range of non-tuberculous mycobacterial strains. This compound has a novel drug target, namely mycobacterial ATP synthase, and this means that it has no cross-resistance with existing anti-TB drugs (Andries et al., 2005).
1.10 Aim and objectives

1.10.1 Aim

The purpose of this study was to investigate African medicinal plants and synthesized compounds for potential antimicrobial activity by preliminary bioassay screening. The aim of the study was to find novel TB drugs for the treatment of TB.

1.10.2 Objectives

1. To perform high throughput screening of plant extracts and synthesized compounds against *M. smegmatis*.

2. To screen potential inhibitors (plant extracts and compounds) against *M. tuberculosis* H37Rv.

3. To determine the MIC of the most active compound/s against *M. tuberculosis*.

4. To determine the activity of the most active compound against selected Gram positive and Gram negative bacteria.
CHAPTER TWO

Methodology
Methodology

2.1. Introduction

Every wild strain of tubercle bacilli contains some mutants resistant to antibacterial drugs. The difference between the resistant strain and the susceptible strain is that the proportion of resistant bacteria amongst the total number of bacteria making up the strain is much higher in resistant strain than in a susceptible one. In order to deduce the proportion of resistant bacteria in the total viable bacterial population, the first proportional methods were developed in the 1950's (Canetti et al., 1963, Middlebrook and Cohn, 1958) which includes (The Standard Test, Population study test, and Economical test). Conventional antibiotic susceptibility testing protocols were developed from these methods and include testing on both solid and liquid medium. Solid media have included Lowenstein-Jensen (Canetti et al., 1963) and Middlebrook 7H11 (McClatchy, 1978) agars. Several problems are inherent in this type of assay, including drug instability (long incubation periods and protein binding of the drug affect potency), binding of the drug to some medium components, and contamination by extraneous micro-organisms, and because of slow growth of MTB, it may take up to 3 weeks to obtain a result. A microdilution minimum inhibition concentration (MIC) method with Middlebrook 7H9 broth has been developed (Wallace et al., 1986), but it still takes 2 weeks to produce detectable growth. The value of susceptibility testing is obvious for the physician and the patient in terms of shorter delays required for an eventual correction of the chemotherapy regimen. In recent years a rapid semi-automated radiometric drug susceptibility testing (RAD) method has been developed. BACTEC 460 system is a rapid, radiometric, broth susceptibility system which is used to measure drug susceptibilities (Heifets, 1986) and was used in this study.

The BACTEC 460 procedure for susceptibility testing of mycobacteria is based on the same basic principle employed in the conventional method. The only difference is that a liquid medium is used and instead of counting colonies after 3 weeks, the growth is monitored radiometrically and the results are reportable within 4 to 5 days. The principle of the BACTEC radiometric susceptibility assay for mycobacteria is similar to the one utilized in the primary isolation procedure (Siddiqi et al, 1981). When mycobacteria grow
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in 7H12 medium containing \(^{14}\)C-labelled substrate (palmitic acid), they utilize the substrate and \(^{14}\)CO\(_2\) is produced. The amount of \(^{14}\)CO\(_2\) produced reflects the rate and the amount of growth occurring in the vial, and is expressed in terms of the "Growth Index" (GI). When an antituberculous drug is added to the medium, suppression of growth occurs if the test organisms are susceptible. This suppression can be detected by a decline of the daily GI compared to the control. However, if the organisms are resistant, little or no suppression occurs. To determine the 1% proportion of resistance, the control bacterial inoculum used in the vial is one-hundred fold less than that used for the drug containing vial. The drug and the control vials are tested daily at the same time as previous after the inoculation. The rate of increase in the GI, or the amount of change over that of the previous day, called delta (\(\Delta\)) GI, is compared between the control vial and vials containing drugs. If the daily GI increase in a drug vial is equal to or greater than in the control vial, the test organisms are considered resistant to the drug. For a susceptible population, the daily GI increase for the control would be higher than that of the drug vial. For example, if 1% of the mycobacterial population is resistant to isoniazid (INH), then 99% of the organisms would be inhibited by INH and only 1% will grow in the drug vial. The growth rate in the drug vial would be similar to the growth rate in the control vial in which the original bacterial inoculum was only 1/100 of that of the drug vial. Thus, instead of counting colonies in the control and the drug medium, the GI is used to determine susceptibility. Although the results are generally available in 5 days, the test performed on the BACTEC 460 system are costly, require high concentrations of test agents, accumulation of radio-active waste and are not suited to the evaluation of large numbers of compounds.

As a result of \(M.\) \textit{tuberculosis} being a slow growing organism and most importantly, the high infectious nature of the organism, precludes its use in high-throughput screens. In order to circumvent this problem, a surrogate strain to \(M.\) \textit{tuberculosis} was used. The test materials were screened against a species of saprophytic, rapid growing, non-pathogenic mycobacteria, namely \textit{Mycobacterium smegmatis}. This species of mycobacteria is used as test model organisms in the initial screening process while \(M.\) \textit{tuberculosis} is usually used at a later stage for confirmation. This organism has been shown to have a similar
drug sensitivity profile to that of *M. tuberculosis* (Mitscher and Backer, 1998). Microbiology Bioscreen C was used for the primary high-throughput screen in this study.

Microbiology Bioscreen C is a fully automated instrument developed to perform a wide range of microbiological experimentation automatically. The system consists of Bioscreen C reader-analyzer, which is an incubator and measurement unit, integrated with PC, software, printer and consumables. With the software Research Express a Microbiology Workstation Bioscreen C monitors culture growth (turbidity) kinetically in 1-200 wells simultaneously in a liquid medium. Bioscreen C is efficient for a high throughput screen since it can measure the growth of up to 200 wells from each of its honey comb plates of 100 wells. The only limitations are that the Bioscreen uses liquid medium and small volumes of samples. Each well can take up to 400 µl.

Since all cultured microorganisms increase the turbidity of their growth medium, the principle of Bioscreen C is to measure the turbidity versus time (growth curve). All micro-organisms undergo a 4 phase growth curve i.e. lag phase, log phase, stationery phase and death phase. By using this system, it is easy to measure drug susceptibility of an organism against a particular drug. By growing an organism in the presence of a drug or test material, a susceptible organism’s growth curve will be plotted as a straight or decline line from the time or phase at which the drug was introduced. If the organism is resistant to the drug a standard growth curve with all the growth phases will be observed. In the case of a bacterial strain that is partially resistant to the drug, a delayed growth curve will be observed. For susceptibility testing, using this method it is quite important to compare optical density (OD) readings at one time-point in the mid-log phase of growth. At this phase the organism is more susceptible to the drug as the cells are actively dividing. This makes it important to know at what time an uninhibited organism reaches mid log phase. In order to be able to monitor this, it is important to have a control culture with no drug, but grown under the same conditions as the test drug.
2.2. Materials

2.2.1 Preparation of the media

Middlebrook 7H11 agar medium (Difco) was prepared by dissolving 21 g 7H11 in 900 ml distilled water, supplemented by 5 ml of glycerol. It was then autoclaved at 121 °C for 15 minutes. The medium was cooled to 52 °C- 56 °C before 100 ml ADC (albumin fraction V, dextrose, and catalase; Difco) enrichment was added. Middlebrook 7H9 broth medium (Difco) was prepared by dissolving 4.9 g of 7H9 in 900 ml distilled water, supplemented with 0.05 % Tween® 80. It was then autoclaved at 121 °C for 10 minutes; cooled to a temperature less than 50 °C before 100 ml OADC (oleic acid, albumin fraction V, dextrose and catalase; Difco) enrichment was added. Tryptone Soya broth and agar (Oxoid, LTD, Basingstroke, Hampshire, England) were prepared according to manufacturer’s description. Solid and agar media were freshly prepared as required and broth media were sterilized by filtering with 0.22 μm filter.

2.2.2 Preparation of the antimicrobials

2.2.2.1 Controls

Rifampicin (Rif) was obtained from Sigma Chemicals. A stock solution of Rif was prepared by first dissolving 10 mg in 1 ml of methanol to a final concentration of 10,000 μg/ml then diluted in distilled water to a final concentration of 100 μg/ml before the solution was sterilized by filtration through a 0.22 μm filter. Aliquots of 1 ml of the stock were dispensed in 1.5 ml eppendorf tubes which were then stored at -70°C until use.

2.2.2.2 Plant extracts

In this study we consider the past, present and the future values of employing information from plants used in traditional medical practices (ethnomedicine), and by medical doctors and scientists for the discovery of novel bioactive compounds. The African continent has wide plant diversity in both terrestrial and marine origin. The aim of this study was to
exploit the African wild plant diversity for the discovery of a novel TB drug. A number of plants have been shown to demonstrate significant in vitro antimycobacterial activities and active plant derived compounds belonging to various chemical classes have been isolated (Newton et al, 2000; Cantrell et al, 1999). A database containing more than 13 000 plant taxa of medicinal significance has been established. Plants were selected by the SANBI (South African National Biodiversity Institute) across sub-Saharan Africa on basis that they are of African origin (indigenous plants) and have medicinal value. Various plant parts such as roots, stem, leaves, bark, etc were collected as used by indigenous people.

One hundred and ten (110) plant extracts were provided by the CSIR (Council of Scientific and Industrial Research). Crude plant extraction was prepared in various solvents that included [methanol/dichloromethane (1:1), dichloromethane, water]. These plant extracts were sent to the Division of Pharmacology, Department of Medicine, University of Cape Town (UCT) and were initially dissolved in dimethylsulfoxide (DMSO). Initial concentrations (5 mg/ml) of plant extracts were sent to the Division of Medical Microbiology, UCT for screening for anti-mycobacterial activity. Plant extracts were diluted in de-ionized water to a concentration that is four times higher (4 mg/ml) than the concentration to be used in the primary screening assay. At this concentration the extracts were stored at -20°C as stock drug. This was a blinded study, and the screener did not know anything about the origin of the extracts. Plant extracts were given voucher specimen names for the protection of intellectual property and patenting.

2.2.2.3 Compounds

Thirty six pure (36) compounds were supplied by Prof. K. Chibale, Department of Chemistry, UCT. The compound has been chemically synthesized from the commercially available mucohalic furanones, mucochloric and mucochromic acids. Being furanones these compounds are biologically privileged in the sense that they are found in many biologically active natural products and clinically available drugs (Smith et al, 1981). Furanones are endowed with a broad range of biologically activities including
antibacterial, herbicidal and antitumour activities. These compounds were modified as part of structural activity relationship (SAR) studies aimed at optimizing activity in vitro and ultimately in vivo. SAR studies are an essential component of drug discovery and development. These compounds were given voucher names for the protection of intellectual property and patenting. They were provided in powder (5 mg) form and were initially dissolved in DMSO (5 mg/ml) and then diluted in distilled water to a concentration four times higher (40 μg/ml) than the concentration to be used in the assay. At this concentration the compounds were stored at -20°C until testing.

2.2.4 Colloidal silver

From the early times silver has been known to be an effective treatment for bacterial infection (Bechold H., 1919; Searl A.B., 1919; Custom C.G., 1926). The three most important factors governing the activity of non-toxic properties of silver were found to be; 1) particle concentration, 2) particle size and, 3) particle charge. The ideal particle concentration is between 5 and 10 ppm. The ideal particle size is between 5 and 15 nanometres and the particle must be positively charged. The American Biotec Laboratory (ABL) products contain nano-sized particles of metallic silver dispersed in a matrix of water. One of their products used in this study is Electro Colloidal Silver (eCS) which is 7 ppm and was provided by Dr P.J. Price, Department of Clinical Pathology, University of Stellenbosch, Cape Town. eCS is pure silver in its sub atomic state and consists of $10^8$ of positively charged silver ions suspended in pure distilled water. The eCS was stored at room temperature until testing.

2.2.3 Bacterial strains, growth conditions and inocula preparation

*Mycobacterium smegmatis* (*M. smegmatis*) mc² 155 was obtained from W.R. Jacobs, Albert Einstein College of Medicine, New York, USA. *M. smegmatis* was routinely maintained in Middlebrook 7H9 broth containing 0.05% Tween 80 to prevent clumping and 10% v/v OADC (Oleic acid, Albumin Fraction V, Dextrose and Catalase) enrichment. Glycerol (10%) stocks were prepared from a culture that was grown for 72
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hour at 37°C and were stored in 2 ml screw-cap cryovials with O-ring at -70°C. A stock culture of *M. smegmatis* from -70°C was thawed and 100μl of this was inoculated into 10 ml of 7H9 broth, incubated at 37°C overnight. The purity of the overnight culture (OD₆₀₀ usually ±1), was tested by Ziehl-Neelsen and Gram staining followed by plating out 0.1 ml of bacterial culture on Tryptone Soya Agar. Once the culture was confirmed to be pure Acid Fast Bacilli (AFB’s), the assay was initiated. The culture was diluted to OD of 0.05. An aliquot of 100 μl of the diluted culture at a concentrations ranging from 10⁻⁴ to 10⁶ were spread plated on 7H11 agar for each of the growth phases, four plates per concentration. This was performed for colony counting to determine the number of colony forming units (CFU) per milliliter. By plating out the bacteria there was a further confirmation that the culture was pure.

*Mycobacterium tuberculosis* H37Rv (ATCC 27294) was obtained from Dr Mark. Nicol, Division of Medical Microbiology, University of Cape Town. Ten percent glycerol stock cultures of this strain were prepared in Middlebrook 7H9 supplemented with 0.05% Tween 80 and OADC and were stored at -70°C. A 12B vial was inoculated with *M. tuberculosis* H37Rv from a glycerol stock stored at -70°C. The vial was incubated at 37°C and was read on a daily basis until a GI reading of 500-800 was obtained. Culture purity was checked by Ziehl-Neelsen, Gram staining and plating out on Trypton Soya agar. Prior to any inoculation, BACTEC vials were tested to establish the recommended CO₂ atmosphere. Any vial that showed a GI of 20 or more was not used. The operator has to wear gloves, and work in a biological safety cabinet using a 1 ml syringe with permanent fixed needles.

Stock cultures of Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 13883) and Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778) bacteria were prepared. These bacteria were provided by Tracy Seamen from the Division of Pharmacology, Department of Medicine, UCT. The stock cultures were stored at -70°C. For inoculum preparation the stock cultures were thawed and 100 μl of each organism was inoculated in 10 ml of Tryptone Soya broth and
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incubated over-night. The purity of the culture was determined by first Gram staining and visualization of bacterial morphology under the light microscopy. Further confirmation was performed by plating the organism on a Tryptone Soya Agar (TSA) plate. Once the culture was confirmed to be pure, the assay was performed.

Bacterial cultures were routinely maintained in Trypton Soya Broth (TSB) for assay preparation.

2.3. Methods

2.3.1 Primary screen of plant extracts, synthetic compounds and electro-colloidal silver against *M. smegmatis*

Plant extracts stored at -20°C at a concentration of 4 mg/ml, were thawed. For the assay, extracts were diluted 1:2 in 7H9 broth to a concentration of 2 mg/ml. A 100 μl aliquot of each extract was transferred into each of three wells of the honey-comb plate as each assay was done in triplicate. A 100 μl aliquot of bacterial culture, diluted to an OD₆₀₀ of 0.05 (3 x 10⁶ CFU/ml), was added. The final concentration of the extract was 1000 μg/ml in a final volume of 200 μl. Four controls were included in each experiment. The controls were 1) a bacterial culture only, to monitor uninhibited bacterial growth; 2) bacterial culture with rifampicin at a concentration of 6.25 μg/ml, to serve as a positive control; 3) bacterial culture with 1% DMSO, to determine the effect of 1% DMSO on bacterial growth (extracts contained less than 1% DMSO); and 4) medium only, to see if there is any contamination in the growth medium. The plates were placed in the Bioscreen C and growth was monitored at two hour intervals over two days (48 hours). The extracts were tested in triplicate in three different experiments. Extracts which showed 40% inhibition or more were regarded as “hits” and were tested against *M. tuberculosis*.

Synthetic compounds at a concentration of 40 μg/ml were thawed from -20°C. The compounds were diluted 1:2 in 7H9 broth to 20 μg/ml. Three 100 μl aliquot of each compound were transferred into three wells of the honey-comb plate. A 100 μl aliquot of
bacterial culture at an \( \text{OD}_{600} \) of 0.05 (3 x 10\(^6\) CFU/ml) was added to each well containing the compounds such that the final concentration becomes 10 \( \mu \text{g/ml} \) and the volume becomes 200 \( \mu \text{l} \). Four controls were also prepared 1) wells containing bacterial culture only, 2) wells containing medium only, 3) wells containing bacterial culture with 1\% DMSO and 4) wells containing bacterial culture with rifampicin at 6.25 \( \mu \text{g/ml} \). The honey-comb plate was placed in a Bioscreen C and growth was monitored at two hour intervals over two days (48 hours). Each compound was tested in triplicate in three different experiments. Compounds which showed 40\% or more inhibition were taken as "hits" and were tested against \( M. \text{ tuberculosis} \).

Electro Colloidal Silver (eCS) was tested at a serial two fold dilution ranging from 4 parts per million (ppm) to 0.125 ppm. Each concentration was tested in triplicate in three different experiments. The same procedure was used as mentioned above i.e. 100 \( \mu \text{l} \) of bacterial culture at an \( \text{OD}_{600} \) of 0.05 was added to 100 \( \mu \text{l} \) of eCS and three controls 1) bacterial culture only, 2) medium only and 3) wells containing rifampicin at 6.25 \( \mu \text{g/ml} \) were included. A dose response curves was drawn by taking the \( \text{OD}_{600} \) values at mid-exponential phase of growth for each concentration and MIC\(_{90} \) of colloidal silver against \( M. \text{ smegmatis} \) tested, was determined. eCS was further tested against \( M. \text{ tuberculosis} \) (section 2.3.2).

The honey-comb plates were placed in the incubation chamber, which was then closed to enable orbital shaking and maintenance of a constant temperature. The machine and the computer were switched on. Setting up of the name and the date of the experiment, experiment location in a suitable file for retrieval and growth parameters were done on the computer. The \( \text{OD}_{600} \) measurement intervals was set for every two hours (generation time of \( M. \text{ smegmatis} \)), temperature set at 37\( ^\circ \text{C} \) (optimal temperature), shaking set at continuous and medium (aeration), an average of three replicates for growth curve if the testing is done in triplicate, the system was set to run for 48.
2.3.2 Secondary screen of plant extracts, eCS and synthetic compounds against *Mycobacterium tuberculosis* using the BACTEC 460 TB System

Stock solutions of plant extracts (100 mg/ml) were diluted (1:2.38) in pure distilled water to a concentration of 42 mg/ml. An aliquot of 100 µl of an extract at a concentration of 42 mg/ml was transferred into a 4 ml BACTEC vial, and 100 µl of *M. tuberculosis* bacterial culture was inoculated to give a final extract concentration of 1 mg/ml in a total volume of 4.2 ml. An aliquot of 100 µl of colloidal silver was transferred into a 4 ml BACTEC vial, and 100 µl of *M. tuberculosis* bacterial culture was inoculated to give a final colloidal silver concentration of 0.7 ppm. All compounds were screened at 10 µg/ml. An aliquot of 100 µl of a compound at a concentration of 420 µg/ml was transferred into 4 ml BACTEC vial, and 100 µl of bacterial culture was inoculated to give a final volume of 4.2 ml. Four controls were also included; 1) *M. tuberculosis* culture only, 2) *M. tuberculosis* culture that has been diluted 1:100 (this was prepared by transferring 0.1 of the suspension into 9.9 ml of special diluting fluid), 3) *M. tuberculosis* culture with 1% DMSO and 4) *M. tuberculosis* with rifampicin at 2 µg/ml. The experiment was done in duplicate and BACTEC vials were swabbed with 3% Virkon before and after inoculation. BACTEC vials were incubated at 37°C and were read on a daily basis at the same time each day. Readings were taken up until the 1:100 vial reached GI of 60. Extracts which showed 99% or more inhibition against *M. tuberculosis* were no further tested and bio-assay guided fractionation was to be performed. Compounds which showed 99% or more inhibition were further tested on a log scale to determine the range of the MIC. Once the range was determined, a serial two fold dilution within the range was prepared to determine where the MIC lies.

2.3.3 Determination of minimal inhibitory concentration (MIC) of F1082

*M. tuberculosis* culture was prepared as described in section 2.2.3 above. 12B vials were tested for the recommended $^{14}$CO$_2$ as above. Antitubercular compounds were prepared
within the concentration range in which the MIC was determined to lie as above. Volumes of 100 µl of the compounds were transferred into 12B vials to give final concentrations ranging from 2 to 10 µg/ml. Rifampicin was used as a positive control at a final concentration of 2 µg/ml. *M. tuberculosis* culture was inoculated into the vials already containing the antitubercular compounds. In each experiment, one drug free vial containing a 1:100 dilution of *M. tuberculosis* culture (1:100, control) and one drug free vial containing *M. tuberculosis* culture only were also included. Vials were incubated at 37°C and the 14CO2 released by metabolically active organisms was measured daily and recorded as growth index on a scale of 0 to 999. The MIC was determined as the lowest concentration of compound in which growth of *M. tuberculosis* was less than that of the 1:100 fold diluted cells.

2.3.4 Testing of F1082 against selected Gram-positive and Gram-negative bacteria

The assay was performed using Microbiology Bioscreen C as described in section 2.1 above. Two fold dilutions of the most active antitubercular compounds were prepared in Tryptone Soya broth. Aliquots (100 µl) of each concentration were transferred into wells of the honey-comb plate, in duplicate. A volume (100 µl) of assay bacterial culture (OD of 0.05) of each of the selected Gram-positive and Gram-positive organisms were added to each well containing compounds, diluting the compounds further such that the final concentrations ranged from 3.125 to 50 µg/ml. Four controls were included in each experiment: wells containing assay bacterial culture only, wells containing assay bacterial culture with ciprofloxacin, wells containing assay bacterial culture with 1% DMSO and wells containing medium only. The plates were placed in the Microbiology Bioscreen C and the growth was monitored every 30 minutes intervals at 37°C with shaking over 36 hours. The experiments were performed in duplicate.
CHAPTER THREE

Results
3.1. Determination of M. smegmatis growth curve

This experiment was done in order to establish mid-log phases of growth of *M. smegmatis* using Bio-screen C. The log phase is important in drug susceptibility testing as the drug is more active against active growing organisms (which are obviously those in the log phase). Fig. 3.1 shows a growth curve of *M. smegmatis* over a period of 40 hours. Three growth phases were observed, a lag phase (0-6 hours); an exponential phase (6-24 hours) and stationary phase (24-36 hours). The mid log phase was observed at 16th hours of growth in all three experiments done on different days. At each phase of growth per time point (0, 16, 30 hours), 100 μl of mycobacterial culture was plated out on 7H11 agar in order to determine CFU/ml. It was found that in the lag phase there are 3 x10⁶ CFU/ml, mid-log phase (4.8 x 10⁸ CFU/ml) and stationary phase (3 x 10⁹ CFU/ml)

Fig. 3.1: A standard growth curve of *M. smegmatis*
Results

3.2. M. smegmatis growth curve with organic solvents

M. smegmatis was grown with three different solvents, namely: DMSO, Methanol and Acetone. This experiment was carried out in order to compare the effects of each solvent on the growth rate of M. smegmatis. All solvents were tested at 1.25% v/v concentration. It was found that all solvents had less than 10% inhibition on the growth of M. smegmatis as shown in fig. 3.2. DMSO was chosen because extracts are readily solubility and its good quality solvent properties.

![Graph showing growth curve of M. smegmatis with different solvents](image)

Fig. 3.2: Determination of the effects of different solvents tested at 1.25% concentration (v/v). Well 13-15: M. smegmatis with DMSO; 31-33: M. smegmatis with methanol; 52-54: M. smegmatis with acetone; 55-57: M. smegmatis with rifampicin; 58-60: M. smegmatis bacterial culture only; 61-63: Medium only
Results

3.2.1. Dose response curve of *M. smegmatis* with DMSO

*M. smegmatis* was grown with varying concentrations of DMSO. A dose response curve was determined by plotting OD₆₀₀ values at 16ᵗʰ hour of *M. smegmatis* growth against each corresponding concentration. The percentage inhibition was calculated by comparing the OD₆₀₀ values of each concentration at the 16ᵗʰ hour of growth relative to the OD₆₀₀ values of the untreated cells. Each concentration was prepared in triplicate and two experiments were performed. Fig. 3.2.1 shows that 1.25% DMSO inhibited less than 10% and 0.625% v/v did not inhibit the growth of *M. smegmatis*.

![Graph showing dose response curve of M. smegmatis with DMSO](image)

Fig. 3.2.1: A dose response curve of *M. smegmatis* with DMSO at different concentrations ranging from 0-10% concentration.

3.3. *M. smegmatis* with controls

In a series of experiments with rifampicin, it was found that rifampicin at a concentration of 6.25 µg/ml has more than 90% growth inhibition against *M. smegmatis*. Based on the
Results

results of section 3.2.1, we decided to use 1% DMSO in subsequent tests. Here we show that 1% DMSO does not significantly affect the growth of *M. smegmatis* (Fig 3.3). Since all extracts were possibly dissolved in ≤1% DMSO, then, 1% DMSO was chosen as a control of the solvent effect on the growth of *M. smegmatis*. In all assays performed, four controls were used. These controls includes: rifampicin (6.25 µg/ml) as a positive control, mycobacterial culture without any inhibitor as a negative control, 1% DMSO as a measure of the effects of DMSO in the extracts/compounds as they contained ≤1% of DMSO and medium only in which the mycobacteria have been grown and diluted, to see if there is any contamination. Fig.3.3 represents three experiments done in different days, each done in triplicate. It is clear from the graph that 1% DMSO has very little effect on the growth of *M. smegmatis*

![Graph showing growth of M. smegmatis with controls](image)

Fig. 3.3: A growth curve of *M. smegmatis* with controls used in the primary screen. Wells 13-15: *M. smegmatis* without any inhibitor, wells 55-57: *M. smegmatis* with rifampicin (6.25 µg/ml), wells 58-60: *M. smegmatis* with 1% DMSO and wells 61-63: medium only. Results are presented as a mean of three readings with standard deviation as the error bars.
Results

3.4. Primary screen of plant extracts, eCS and synthetic compounds against M. smegmatis using Bioscreen C

3.4.1. Plant extracts

All plant extracts containing 1% DMSO solvent were screened at 1 mg/ml. Out of 110 plant extracts, only 2 extracts showed 40% or more inhibition (P02301b and P04495b) and others showed less than 20% inhibition. Plant extracts with 40% or more inhibition were regarded as “hits” and were further screened against M. tuberculosis. Fig. 3.4.1.1 shows only plant extracts with 40% or more inhibition and the one that appeared to stimulated growth of M. smegmatis. The experiment was performed 3 times on separate days, each in triplicate. The results are a mean of the three experiments with standard deviation as a mean of the three experiments.

![Graph](image)

Fig. 3.4.1.1: Growth curve of M. smegmatis with most active crude plant extracts at 1mg/ml. Wells: (13-15): P04495b; (25-27): P02301b; (31-33): M. smegmatis culture without any inhibitor; (34-36): M. smegmatis culture with rifampicin (6.25 μg/ml); (37-39): M. smegmatis culture with 1% DMSO, (40-42): Medium only.
Results

The percentage growth inhibition of \textit{M. smegmatis} in the presence of P02301b and P04495b was 42% and 66%, respectively (Fig 3.4.1.2) as was determined using Bioscreen C method. The percentage growth was calculated relative to the \textit{M. smegmatis} culture growth without any inhibitor on the 16th hour of growth.

![Graph of percentage growth inhibition](image)

Fig. 3.4.1.2: A percentage growth of \textit{M. smegmatis} on the 16th hour of growth with two extracts that showed inhibition in a Bioscreen C. The results are a mean of three experiments with standard deviation as error bars.

3.4.2. Colloidal silver

Colloidal silver was tested at various concentrations ranging from 0-4 ppm. In this study, colloidal silver inhibited more than 90% growth of \textit{M. smegmatis} between 2 and 4 ppm. The percentage growth was calculated for each concentration relative to the uninhibited
organism at one time point (16th hour) of growth. Colloidal silver was further tested against *M. tuberculosis* (see section 3.5.2). Fig. 3.4.2. shows percentage growth of *M. smegmatis* with colloidal silver at different concentrations (ppm). Results show a mean of three experiments with standard deviation as error bars.

![Graph showing percentage growth of M. smegmatis with various concentrations of colloidal silver.](image)

**Fig. 3.4.2:** The percentage growth of *M. smegmatis* with various concentrations of colloidal silver.

### 3.4.3. Synthetic compounds

All the synthetic compounds were screened at an initial concentration of 10 μg/ml. *M. smegmatis* culture (untreated cells) showed a normal growth pattern, lag, log and stationary phases. *M. smegmatis* cells treated with 1% DMSO showed a similar growth
pattern (Fig. 3.4.3.1). As was expected rifampicin (at MIC, 6.25 μg/ml) showed more than 90% inhibition of *M. smegmatis* growth (Fig. 3.4.3.2). Compounds that showed significant inhibition when compared to the untreated cells are presented in (Fig. 3.4.3.1) and those (other 32 compounds) that did not inhibit the *M. smegmatis* growth are not presented. All four compounds showed different degrees of inhibition against *M. smegmatis* as shown in the figure below.

Fig. 3.4.3.1: A sample of 4 synthetic compounds that showed most inhibition screened against *M. smegmatis* and four controls. Wells (13-15): F952; wells (19-21): F1052; wells (58-60): F37; wells (85-87): F1082; wells (109-111): *M. smegmatis* with rifampicin (6.25 μg/ml); wells (133-135): *M. smegmatis* culture; wells (136-138): *M. smegmatis* with 1% DMSO; wells (139-141), 7H9 only. 

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Results

Effects of the different compounds on the growth of *M. smegmatis* were compared on the 16th hour. Percentage of growth was calculated by comparing the OD$_{600}$ of the untreated cells with treated at one time point [16th hour of growth] (Fig. 3.4.3.2). Compounds F952, F1052, F37 and F1082 showed 55%, 40%, 45% and 38% inhibition, respectively when compared to the untreated cells (Fig. 3.4.3.2). These four compounds were regarded as "hits" and were further screened against *M. tuberculosis* (see section 3.5.3).

![Graph showing percentage growth of M. smegmatis with different compounds](image)

Fig. 3.4.3.2: The percentage growth of *M. smegmatis* with the four "hit" compounds and controls. All these compounds were tested at a concentration of 10 μg/ml. Three different experiments were performed on separate days with standard deviation as error bars of the three experiments.
3.5. Secondary screen of plant extracts, eCS and synthetic compounds against \textit{M. tuberculosis} using BACTEC 460

3.5.1 Extracts screened against \textit{M. tuberculosis}

Three plant extracts were screened against \textit{M. tuberculosis} using BACTEC 460 system at the same concentration (1 mg/ml) as in the primary screen. Fig 3.5.1.1 shows the neat (untreated cells) with normal growth curve as opposed to Rif (2 μg/ml) which showed no growth. Extracts P04495B showed little inhibition when compared to the neat, and P02301B showed 99% or more inhibition when compared to the 1:100 controls, as this represents only 1% of bacterial growth.

![Graph showing growth curves of M. tuberculosis with extracts over a period of ten days. The controls used are a neat (M. tuberculosis culture only); M. tuberculosis with Rif (rifampicin, 2 μg/ml) and 1:100: (M. tuberculosis culture diluted in 1:100).]
The effects of the two extracts that showed inhibition were represented as percentage growth in Fig 3.5.1.2. The percentage growth was calculated relative to the neat (untreated cells) and percentages of growth were plotted on the graph below.

![Graph showing percentage growth of M. tuberculosis with two potential inhibitor extracts](image)

Fig. 3.5.1.2: The percentage growth of *M. tuberculosis* with the two potential inhibitor extracts. The neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2µg/ml)

### 3.5.2. Colloidal silver screened against *M. tuberculosis*

The possible high concentration of colloidal silver to be tested was 0.7 ppm, this was because of the high initial concentration requirement for the BACTEC 460 system. Fig 3.5.2 shows that the eCS did not inhibit the growth of *M. tuberculosis* when compared to
the neat. The results are a mean of two experiments, each performed in duplicate. At this concentration there was no inhibition on the growth of *M. tuberculosis*.

**Results**

Fig. 3.5.2: The growth curve of *M. tuberculosis* with colloidal silver with results interpreted over ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml), *M. tuberculosis* culture with eCS (Electro-colloidal silver).

**Table 1:** A summary of percentages growth of *M. tuberculosis* with extracts, colloidal silver and controls over ten days. In each day percentage growth was calculated relative to the neat (*M. tuberculosis* culture only).

<table>
<thead>
<tr>
<th>Days</th>
<th>Neat</th>
<th>1/100</th>
<th>Rif</th>
<th>eCS</th>
<th>P02301B</th>
<th>P04495B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100%</td>
<td>4%</td>
<td>19%</td>
<td>77%</td>
<td>27%</td>
<td>73%</td>
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<tr>
<td>2</td>
<td>100%</td>
<td>1%</td>
<td>2%</td>
<td>78%</td>
<td>2%</td>
<td>76%</td>
</tr>
<tr>
<td>3</td>
<td>100%</td>
<td>1%</td>
<td>1%</td>
<td>82%</td>
<td>1%</td>
<td>77%</td>
</tr>
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<td>4</td>
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<td>0%</td>
<td>89%</td>
<td>0%</td>
<td>83%</td>
</tr>
<tr>
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<td>100%</td>
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<td>0%</td>
<td>95%</td>
<td>0%</td>
<td>84%</td>
</tr>
<tr>
<td>6</td>
<td>100%</td>
<td>1%</td>
<td>0%</td>
<td>105%</td>
<td>0%</td>
<td>90%</td>
</tr>
<tr>
<td>7</td>
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<td>2%</td>
<td>0%</td>
<td>105%</td>
<td>1%</td>
<td>93%</td>
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<tr>
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<td>3%</td>
<td>0%</td>
<td>98%</td>
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<td>91%</td>
</tr>
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<td>7%</td>
<td>0%</td>
<td>120%</td>
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</tr>
<tr>
<td>10</td>
<td>100%</td>
<td>13%</td>
<td>0%</td>
<td>126%</td>
<td>4%</td>
<td>131%</td>
</tr>
</tbody>
</table>
3.5.3. Synthetic compounds screened against *M. tuberculosis*

All “hit” compounds were screened against *M. tuberculosis* at 10 μg/ml using the BACTEC 460 system. The readings were taken on daily basis and the experiment was stopped on the tenth day. Figure 3.5.3.1 illustrates the growth curves of *M. tuberculosis* with the four “hit” compounds. All four compounds showed varying degrees of inhibition on the growth of *M. tuberculosis*. F37, F1052, F952 and F1082 showed (14%, 47%, 48% and 99% inhibition respectively) when the percentages of growth were calculated relative to the neat (Fig 3.5.3.2).

![Graph showing growth curves of M. tuberculosis with synthetic compounds](image)

Fig. 3.5.3.1: The growth curves of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2 μg/ml).
Results

Fig. 3.5.3.2: The percentage growth of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml).

Table 2: A summary of percentages of growth of *M. tuberculosis* with synthetic compounds over ten days period. In each day percentage growth was calculated relative to the neat (bacterial culture only).

<table>
<thead>
<tr>
<th>Days</th>
<th>Neat</th>
<th>1/100</th>
<th>Rif</th>
<th>F952</th>
<th>F1082</th>
<th>F37</th>
<th>F1052</th>
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<td>1%</td>
<td>23%</td>
<td>14%</td>
<td>85%</td>
<td>64%</td>
</tr>
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<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>31%</td>
<td>10%</td>
<td>91%</td>
<td>60%</td>
</tr>
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<td>1%</td>
<td>0%</td>
<td>40%</td>
<td>6%</td>
<td>84%</td>
<td>56%</td>
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<td>0%</td>
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<td>57%</td>
</tr>
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<td>52%</td>
<td>2%</td>
<td>86%</td>
<td>53%</td>
</tr>
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<td>96%</td>
<td>77%</td>
</tr>
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<td>100%</td>
<td>13%</td>
<td>0%</td>
<td>112%</td>
<td>1%</td>
<td>126%</td>
<td>104%</td>
</tr>
</tbody>
</table>
3.6. Determination of minimal inhibitory concentration (MIC) of F1082 synthetic compound.

The MIC was performed from the synthetic compound that showed most inhibition against \textit{M. tuberculosis}. The compound (F1082) was tested at concentrations ranging from 2 to 10 \(\mu\text{g/ml}\) against \textit{M. tuberculosis} using BACTEC 460 system. The results are shown in figure 3.6. The MIC was determined to be 8 \(\mu\text{g/ml}\), which inhibited 99\% growth of \textit{M. tuberculosis}.

![Growth curve of M. tuberculosis at various concentrations of F1082 in a period of ten days. Neat (M. tuberculosis culture only), 1/100 (M. tuberculosis culture diluted 1:100), M. tuberculosis culture with Rif (rifampicin, 2\(\mu\text{g/ml}\)).](image)

Fig. 3.6: The growth curve of \textit{M. tuberculosis} at various concentrations of F1082 in a period of ten days. Neat (\textit{M. tuberculosis} culture only), 1/100 (\textit{M. tuberculosis} culture diluted 1:100), \textit{M. tuberculosis} culture with Rif (rifampicin, 2\(\mu\text{g/ml}\)).
3.7. **Testing of F1082 against selected Gram-positive and Gram-negative bacteria**

Compound F1082 was further tested against selected Gram-positive (*S. aureus, E. faecalis, B. cereus*) and Gram-negative (*E. coli, K. pneumoniae, P. aeruginosa*) bacteria at concentrations ranging from 3.125 to 50 μg/ml. In the presence of F1082, more than 90% growth of all bacterial strains was observed, even at concentrations five times higher than the antitubercular MIC (Table 3). As expected, ciprofloxacin did inhibit the growth of all the selected Gram-positive and Gram-negative bacteria at determined MICs (Table 4). These results show that F1082 is a selective inhibitor of mycobacteria.

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**Table 3: The effects of F1082 at various concentrations on selected Gram-positive and Gram-negative bacteria.**

<table>
<thead>
<tr>
<th>Gram negative bacteria</th>
<th>Gram positive bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage growth</td>
</tr>
<tr>
<td><strong>F1082 concentration</strong></td>
<td><strong>E. coli</strong></td>
</tr>
<tr>
<td>(μg/ml)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>90%</td>
</tr>
<tr>
<td>25</td>
<td>90%</td>
</tr>
<tr>
<td>12.5</td>
<td>90%</td>
</tr>
<tr>
<td>6.25</td>
<td>90%</td>
</tr>
<tr>
<td>3.125</td>
<td>90%</td>
</tr>
</tbody>
</table>
3.7.1 Antimicrobial positive controls used

The antimicrobial agent used against selected Gram positive and Gram negative bacteria was ciprofloxacin. The MICs of ciprofloxacin for each bacterium were determined and are illustrated on table 4.

Table 4: The control drug and the MIC's used for the experiment

<table>
<thead>
<tr>
<th>Organism</th>
<th>Drug</th>
<th>MICs (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Ciprofloxacin</td>
<td>0.5</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

Discussion and Conclusion
Discussion and conclusion

4.1 Discussion

In considering a screening system to find agents which inhibit the growth of *M. tuberculosis*, we used a system which was robust, reproducible, and easy to operate. A key priority, therefore, was to select an organism for use in the screen which would not require specialized containment facilities, which would not have the inherent problems of slow growth, yet which would be a good predictor of activity against pathogenic bacteria. The rapidly growing mycobacteria are generally much less susceptible than slow growing mycobacteria to antibiotics and, hence, would not be expected to select out even the commonly used antituberculosis agents from a pool of test samples. In literature we found out that *M. aurum* and *M. smegmatis* have similar drug sensitivity profiles to *M. tuberculosis* (Mitscher and Baker, 1998) and have been extensively used in high throughput screen of agents against *M. tuberculosis*. Although *M. aurum* has been found to resemble susceptibility of *M. tuberculosis* more than *M. smegmatis*, we employed *M. smegmatis*. In this study, we used an automated system that measures the optical density (OD) of the organism growing in a broth medium and plots the growth curve.

The key for the system in susceptibility testing is to determine the mid-exponential phase of the organism. This is the phase where the organism is actively growing and most susceptible to the drug. We firstly grew the *M. smegmatis* in order to determine all the phases of its growth and we found the mid-exponential phase at 16\textsuperscript{th} hours of growth when an overnight bacterial culture was diluted to an OD\textsubscript{600} of 0.05. The next step was to use a known antitubercular drug and determine its MIC in order to validate our system. Rifampicin was used at an MIC of 6.25 µg/ml. Since our plant extracts and compounds were dissolved in DMSO, we tested the effects of the DMSO on the growth of *M. smegmatis*. After three different experiments, each done in triplicate we found that 1.25% DMSO had less than 10% inhibition against *M. smegmatis* but since extracts contained 1% DMSO then 1% DMSO was used as a control. It is quite important to point out that any percent greater than 2% will affect the growth of *M. smegmatis* creating difficulty in interpreting the results. In all our experiments we used the Rif as a positive control; 1% DMSO to check the effects of DMSO in each experiment; bacterial culture only, as a
Discussion and conclusion

negative control, and medium only, to check if there is any contamination. An assay was
developed in a 200-well microtiter format of the Bioscreen C to suit the requirements of
high-throughput screening. Despite measuring different parameters (metabolism versus
macroscopic growth) there was a good correlation with the MICs.

In this study we screened 110 pant extracts, 36 synthesized compounds and tested
colloidal silver. The plant extracts were chosen on basis that they have some
pharmaceutical value or have been used in traditional medicine; it is interesting to note
that some plants extracts did not show any antimycobacterial activity against the
surrogate strain. It may be that the plants are used to treat the symptoms of the disease
rather than actually cure the disease. In addition some plants may not contain compounds
which inhibit the growth of or kill *M. tuberculosis* but it is possible that they may have
stimulant or modulatory effects on the immune system. Furthermore individual plants
within species may vary according to a number of factors including where the plant is
grown, climate (temperature and rainfall), soil type, season in the plant is collected, the
length of the day (including the quality of light), altitude and the storage conditions
(Evans, 1996). These may have pronounced effects on the nature and the quality of
secondary metabolites within the plant. In addition, factors such as chemical races and
varieties of plant species may also affect the chemical composition of a plant. It must also
be noted that the activities of plant extracts *in vitro* may not parallel those *in vivo*. This
gives rise to the possibility that potentially useful compounds may be missed, as our test
does not mimic the *in vivo* environment. Some drugs for an example may need to be
metabolically activated *in vivo* for example, by a specific enzymes or may have a pH
dependent biological activity (Grange and Snell, 1996; Zhang et al., 1999).

In the results of extracts in primary screen against *M. smegmatis*, only 2 plant extracts
P02301B and P04495B showed (42% and 66% inhibition, respectively). The results in
the secondary screen against *M. tuberculosis* were not as predictive: extract (P02301B)
with least inhibition in primary screen (42%), showed 99% inhibition and (P04495B)
with most inhibition (66%) showed 17% inhibition against *M. tuberculosis* when tested
at the same concentration of 1 mg/ml. This was surprising but the results of the primary screen have shown inhibition.

In this study we were also interested find extracts or compounds that could stimulate the growth of \textit{M. tuberculosis}. This would be a good result for TB diagnosis as the patients and physician would not have to wait long time to know the response of the treatment. One plant extract which appeared to have stimulatory activity was contaminated. Only two experiments were done with extracts and no further test was done because of high initial concentration required in the BACTEC system. The plant extracts were to be further studied using bio-assay guided fractionation. The assay is guided by screening of the fractionations from the crude extract and screened against \textit{M. tuberculosis}, followed by identification of the most active fraction, then using the HPLC in separation and identification of the most active compound.

Of the 36 synthetic compounds only 4 (11\%) showed inhibition in the primary screen against \textit{M. smegmatis}. The 4 potential inhibitors were screened against \textit{M. tuberculosis}, and all of them showed some inhibition. F1082, F37, F1052, F952 showed (99\%, 14\%, 37\%, 48\%, respectively) when tested at the same concentration as the primary screen (10µg/ml) against \textit{M. tuberculosis}. The compound that showed most inhibition (F1082) had its MIC determined, and was found to be 8µg/ml. This compound was further tested against selected Gram-positives and Gram-negatives. In two different experiments, in duplicate, the compound showed no inhibition against all 6 bacteria. This was a remarkable finding, as the compound appears to be selective for mycobacteria.

The results on the colloidal silver in the primary screen showed an MIC\textsubscript{90} of 2 ppm against \textit{M. smegmatis}. Although colloidal silver is claimed to have antimycobacterial activity but was never scientifically proven we sought to justify that and test it against \textit{M. tuberculosis}. Since we only had 30.342 ppm and because of high initial concentration required for the BACTEC system, we were only able to test it at 0.7 ppm. At this concentration there was no inhibition against \textit{M. tuberculosis} and no further tests were performed.
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4.2 Conclusion

In conclusion, with the global impact of TB on up to two billion people, there is a strong case for the development of interventions that would reduce the progression from latent infection to active TB. It is likely that there is a considerable heterogeneity among the infected population, and that multiple pathways can lead from infection to disease. Different combinations of drug-based and immune-based interventions might be appropriate for populations that differ in their rate of exposure to infection with *M. tuberculosis*. The body of the existing ethnomedical knowledge has lead to a great development in the health care. With the rapid industrialization of the planet and the loss of ethical cultures and customs, some of this information will no doubt disappear. An abundance of ethnomedical information on plant uses can be found in the scientific literature but has not yet been compiled into usable form. It is in this study that we seek to investigate such values, and came up with a selective TB drug.

In this study we report a plant extract (P02301B) and a compound (F1082) with 99% inhibition against *M. tuberculosis*. The plant extract will be fractionated by bio-guided fractionation in identifying the active principle. The compound has an MIC of 8μg/ml against *M. tuberculosis*. This compound has shown some selective activity against mycobacteria as it has not shown any activity against selected Gram-negatives and Gram-positives. F1082 needs further investigation against MDR-TB and NTM and be investigated its mode of action. It is interesting to identify a compound that has selectivity for the mycobacteria especially with the alarming, recent outbreak of extremely resistant strains of *M. tuberculosis*. This study has pointed out some plants and use of combinatorial chemistry which may prove to be of value in the continuing struggle to control tuberculosis.
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