Development of novel anti-tuberculosis drugs from African medicinal plants

Andile H. Ngwane

Submitted in fulfillment of the requirements for the degree,

Master's of Science (Medicine)

in

Medical Microbiology

at the

Institute of the Infectious Disease and Molecular Medicine,

University of Cape Town

Supervisors

Prof. L.M. Steyn

Co-supervisor

Miss Z. Skepu

November

2006

University of Cape Town

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

DECLARATION

Development of novel anti-tuberculosis drugs from African medicinal plants

I, Andile H. Ngwane, hereby declare that the work on which this dissertation is based is my original work unless stated otherwise and that neither the substance nor any part of this work has been, or is to be submitted for another degree at this or any other university.

University of Cape Town

I grant the University of Cape Town the right to reproduce any portion of this dissertation for the purposes of research.

signature removed

Signed

01/06/07

Date

ACKNOWLEDGEMENTS

Firstly, I thank God, the Almighty, for providing me with this opportunity through prayers. He gave me the power to go forward all the time, with His grace I pulled through.

I would like to thank my supervisor Prof. Lafras. M. Steyn, who gave me the courage, support and empowered me with enthusiasm in this project and taking my career further into the next level of my studies. Working together with my co-supervisor Zoleka Skepu and assisted by Lamese Ahtow, I managed to fulfill my goal, thanks mama.

My many thanks go to the support base I have got in all areas involved in this project: 1) National Drug Development Program; 2) CSIR for providing with plant extracts; 3) Department of chemistry UCT, Kelly Chibale for providing the compounds; 4) Department of Pharmacology UCT, Prof. Peter Smith, Tracy Seamen (BATEC training), Carmen Lategan; 5)Department of Medical Microbiology Groote Schuur, Vanessa Februarie and all the staff and members of Medical Microbiology UCT, you have been great to me.

I would like to give a special thanks to the people who funded this project, Innovation Fund; UCT; MRC; Department of Medical Microbiology. You have made me to see no other obstacles in my work.

Lastly, and by no means the least, my family and friends who were there for me at times of need. Ndiyabulela ko-Rhadebe, oo-Bhungane, ndithi makube chosi kube hele.

University of Cape Town

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 incompliance, 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%, 14%, 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

University of Cape Town

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.

University of Cape Town

TABLE OF CONTENTS

Title page i
Declaration ii
Acknowledgements iii
Abstract iv
Table of Contents vi
List of Figures xi
List of Tables xii
List of Abbreviations xiii

CHAPTER ONE 1
Literature Review 1
1.1 Introduction 2
1.2 Bacteriology 3
1.3 Tuberculosis the disease 4
1.4 Epidemiology 4
1.5 Tuberculosis in HIV infected patients 6
1.5.1 Background 6
1.5.2 Pathogenesis of the co-infection 7
1.6 Treatment of tuberculosis 8
1.7 BCG vaccine 9
1.8 MDR-TB and the Mechanism of Resistance 9
1.8.1 Resistance to isoniazid (INH) 10
1.8.2 Resistance to rifampicin (Rif) 11
1.8.3 Resistance to ethambutol (EMB) 11
1.8.4 Resistance to pyrazinamide (PZA) 12
1.8.5 Resistance to streptomycin (SM) and other inhibitors of protein synthesis 13
1.8.6 Resistance to other drugs 13
1.8.7 Resistance to Flouroquinolones (FQ) 13
1.9 Tuberculosis drug development 14
1.9.1 The value of plants used in traditional medicine for drug discovery 14
1.9.2 Approaches to drug discovery using higher plants 15
1.9.3 Interventions and new drugs 16
1.9.4 Prospective drugs 17

University of Cape Town

1.10 Aim and objectives 19
1.10.1 Aim 19
1.10.2 Objectives 19
CHAPTER TWO 20
Methodology 20
2.1. Introduction 21
2.2. Materials 24
2.2.1 Preparation of the media 24
2.2.2 Preparation of the antimicrobials 24
2.2.3 Bacterial strains, growth conditions and inocula preparation 26
2.3. Methods 28
2.3.1 Primary screen against *M smegmatis* 28
2.3.2 Screening against *Mycobacterium tuberculosis* using the BACTEC 460 TB System 30
2.3.3 Determination of minimal inhibitory concentration (MIC) 30
2.3.4 Testing against selected Gram-positive and Gram-negative bacteria 31
CHAPTER THREE 32
Results 32
3.1. Determination of M. smegmatis growth curve 33
3.2. M. smegmatis growth curve with organic solvents 34
3.2.1. Dose response curve of *M. smegmatis* with DMSO 35
3.3. M. smegmatis with controls 35
3.4. Primary screen of plant extracts, eCS and synthetic compounds against M. tuberculosis using Bioscreen C 37
3.4.1. Plant extracts 37
3.4.2. Colloidal silver 38
3.4.3. Synthetic compounds 39
3.5. Secondary screen of plant extracts, eCS and synthetic compounds against M. tuberculosis using BACTEC 460 system 42
3.5.1 Extracts 42
3.5.2. Colloidal silver 43
3.5.3. Synthetic compounds 45
3.6. Determination of minimal inhibitory concentration (MIC) of F1082 synthetic compound 47
3.7. Testing of F1082 against selected Gram-positive and Gram-negative bacteria 48
3.7.1 Antimicrobial positive controls used 49

University of Cape Town

CHAPTER FOUR 50
Discussion and Conclusion **50**
4.1 Discussion 51
4.2 Conclusion 54
REFERENCES 55

University of Cape Town

List of figures

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

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

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.

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

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 (6.25µg/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.

Fig. 3.4.1.1: Growth curve of *M. smegmatis* with most active crude plant extracts at 1mg/ml. Wells (1-3): P02301c; (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.

Fig. 3.4.1.2: A percentage growth of *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.

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

University of Cape Town

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).

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.

Fig. 3.5.1.1: The 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, 2μg/ml) and 0.111: (*M. tuberculosis* culture diluted in 1:100).

Fig. 3.5.1.2: The percentage growth of *M. tuberculosis* with the two potential inhibitor extracts. The neat (*M. tuberculosis* culture only), 0.111 (*M. tuberculosis* culture diluted 1:100), *M. tubeculosis* culture with Rif (rifampicin, 2μg/ml).

Fig. 3.5.2: The growth curve of *M. tuberculosis* with colloidal silver with results interpreted over ten days. Neat (*M. tuberculosis* culture only), 0.111 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml), *M. tuberculosis* culture with ECS (Electro-colloidal silver).

Fig. 3.5.3: The growth curves of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 0.111 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2 μg/ml).

Fig. 3.5.3.1: The percentage growth of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 0.111 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml).

University of Cape Town

Fig. 3.6: The growth curve of *M. tuberculosis* at various concentrations of F1082 in a period of ten days. Neat (*M. tuberculosis* culture only), 0.111 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2 μg/ml).

University of Cape Town

List of tables

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 2: A summary of percentages 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 3: The effects of F1082 at various concentrations on selected Gram positive and Gram negative bacteria.

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

University of Cape Town

List of abbreviations

AFB	Acid fast bacilli
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guerin
CFA	Freund's complement adjuvant
CFU	Colony forming units
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment short-course
eCS	Electro-colloidal silver
EMB	Ethambutol
FQ	Flouroquinolones
g	Grams
GI	Growth index
HIV	Human immunodeficiency virus
IL	Interleukins
INF	Interferon
INH	Isoniazid
MDR-TB	Multi-drug resistant tuberculosis

University of Cape Town

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

University of Cape Town

CHAPTER ONE

Literature Review

University of Cape Town

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 *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.

University of Cape Town

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 Zielh 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

University of Cape Town

radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum. 2) Cord factor is toxic to mammalian cells and also inhibitor of PMN (Polymorphonucleocytes) migration. Cord factor is most abundantly produced in virulent strains of *M. tuberculosis*. 3) Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA) (Pan *et al.*, 2001)

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 1-5μm 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).

University of Cape Town

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

University of Cape Town

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).

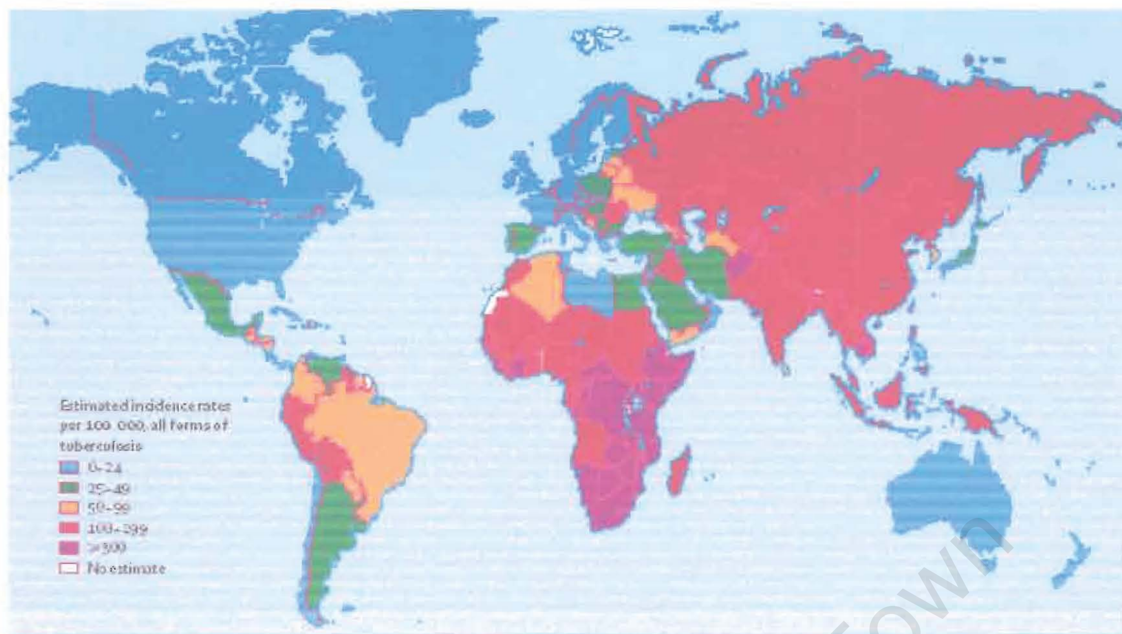


Fig 1: The distribution of *M. tuberculosis* in the world in 2003 (WHO, 2006)

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

University of Cape Town

to WHO, HIV infection tends to be underdiagnosed there (Dye *et al.*, 1999). The problem of coinfection 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-CD_4, or T-helper (T_h) lymphocytes, are known to be the main disorder associated with HIV infection. These lymphocytes play the main role in systematic defense against mycobacteria. CD_4 lymphocytes are divided into two groupsT_h1 and T_h2 which release different cytokines: T_h1---INF-γ and T_h2---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 T_h1 response and low levels of IFN-γ concentrations were observed. Conversely, in mild forms of TB high INF-γ concentrations were observed. Since the CD_4 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, proinflamatory 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-$_{K}$B, a cellular factor stimulating the active HIV replication region (Goletti *et al.*, 1996; Hoshino *et al.*, 2002).

University of Cape Town

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).

University of Cape Town

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

University of Cape Town

simultaneous resistance to at least rifampicin (Rif) and isonaizid (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 isonaizid (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.

University of Cape Town

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 (Maggliozzo 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 mc^2 155, 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 ß subunit of RNA polymerase, thereby hindering transcription, and that mutation in the *rpo*B 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 *rpo*B 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 (Rastoggi *et al.*, 1990). Mycolic acid binds to arabinogalactan and forms mycolyl-arabinogalactan peptidoglycan complex

University of Cape Town

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 tranferase 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 *pnc*A 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

University of Cape Town

like viomycin and capriomycin are active against mycobacteria (Benveinste 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) Benveinste 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 *rps*L 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 (Benveinste 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 (A_2B_2), coded by *gyr*A and *gyr*B respectively (Kirchausen *et al.*, 1978). Quinolones activity is determined by the GyrA protein, which contains the

University of Cape Town

cleavage/religation 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

University of Cape Town

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, tritepenes, 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

University of Cape Town

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 apprentiship 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.

University of Cape Town

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).

University of Cape Town

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 antimycobacterial activity. These include flouroquinolone compounds, oxazolidinone compounds, clofazimine. Phenazine, phenathiazine, azoles and peptides, nitroimidazopyrans and long acting rifamycins (rifapentine, rifabutin, rifalazil) (Hudson *et al.*, 2003; Duncan, 2003; O'Brian 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, Emerylville, California) (Elias, 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 Jannssen 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-butan-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).

University of Cape Town

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. tubeculosis.*

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

University of Cape Town

CHAPTER TWO

Methodology

University of Cape Town

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

University of Cape Town

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 (Δ) 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. 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. tuberculosis* was used. The test materials were screened against a species of saprophytic, rapid growing, non-pathogenic mycobacteria, namely *Mycobacterium smegmatis*. This species of mycobacteria is used as test model organisms in the initial screening process while *M. tuberculosis* is usually used at a later stage for confirmation. This organism has been shown to have a similar

University of Cape Town

drug sensitivity profile to that of *M. tuberculosis* (Mitscher and Backer, 1998). Microbiology Biocreen 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 under-go 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.

University of Cape Town

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 5ml of glycerol. It was then autoclaved at 121 °C for 15 minutes. The medium was cooled to 52 °C- 56 °C before 100ml 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, Basingstoke, 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.

University of Cape Town

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.

University of Cape Town

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 mucobromic 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.

2.2.2.4 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 nano-metres 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.

University of Cape Town

2.2.3 Bacterial strains, growth conditions and inocula preparation

Mycobacterium smegmatis (*M. smegmatis*) mc^2 155 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

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 (OD_{600} 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 from10^{-4} to 10^{-6} 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 CO_2 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

University of Cape Town

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 pate as each assay was done in triplicate. A 100 μl aliquot of bacterial culture, diluted to an OD_{600} of 0.05 (3 x 10^6 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

University of Cape Town

bacterial culture at an 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 µg/ml and the volume becomes 200 µl. 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 µg/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. 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 µl of bacterial culture at an OD_{600} of 0.05 was added to 100 µl of eCS and three controls 1) bacterial culture only, 2) medium only and 3) wells containing rifampicin at 6.25 µg/ml were included. A dose response curves was drawn by taking the OD_{600} values at mid-exponential phase of growth for each concentration and MIC_{90} of colloidal silver against *M. smegmatis* tested, was determined. eCS was further tested against *M. 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 OD_{600} measurement intervals was set for every two hours (generation time of *M. smegmatis*), temperature set at 37°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.

University of Cape Town

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

University of Cape Town

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 $^{14}CO_2$ 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.

University of Cape Town

CHAPTER THREE

Results

University of Cape Town

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 $x10^6$ CFU/ml, mid-log phase (4.8 x 10^8 CFU/ml) and stationary phase (3 x 10^9 CFU/ml)

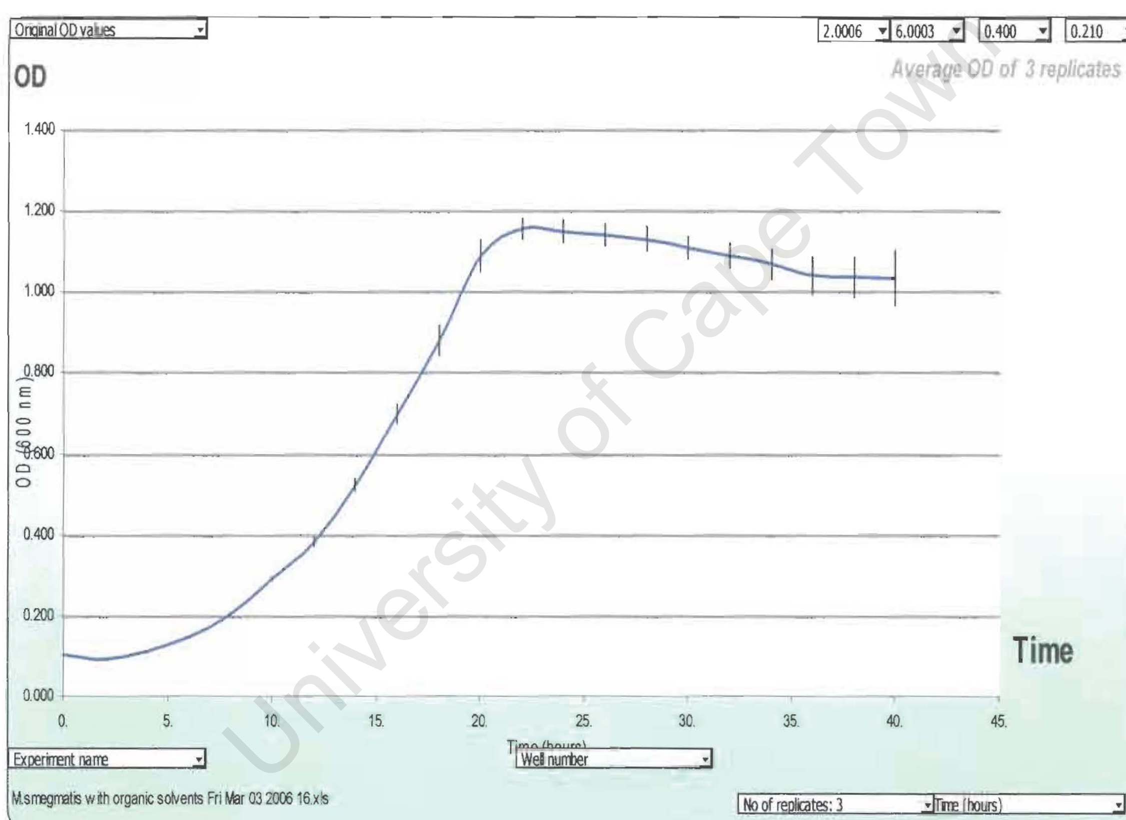
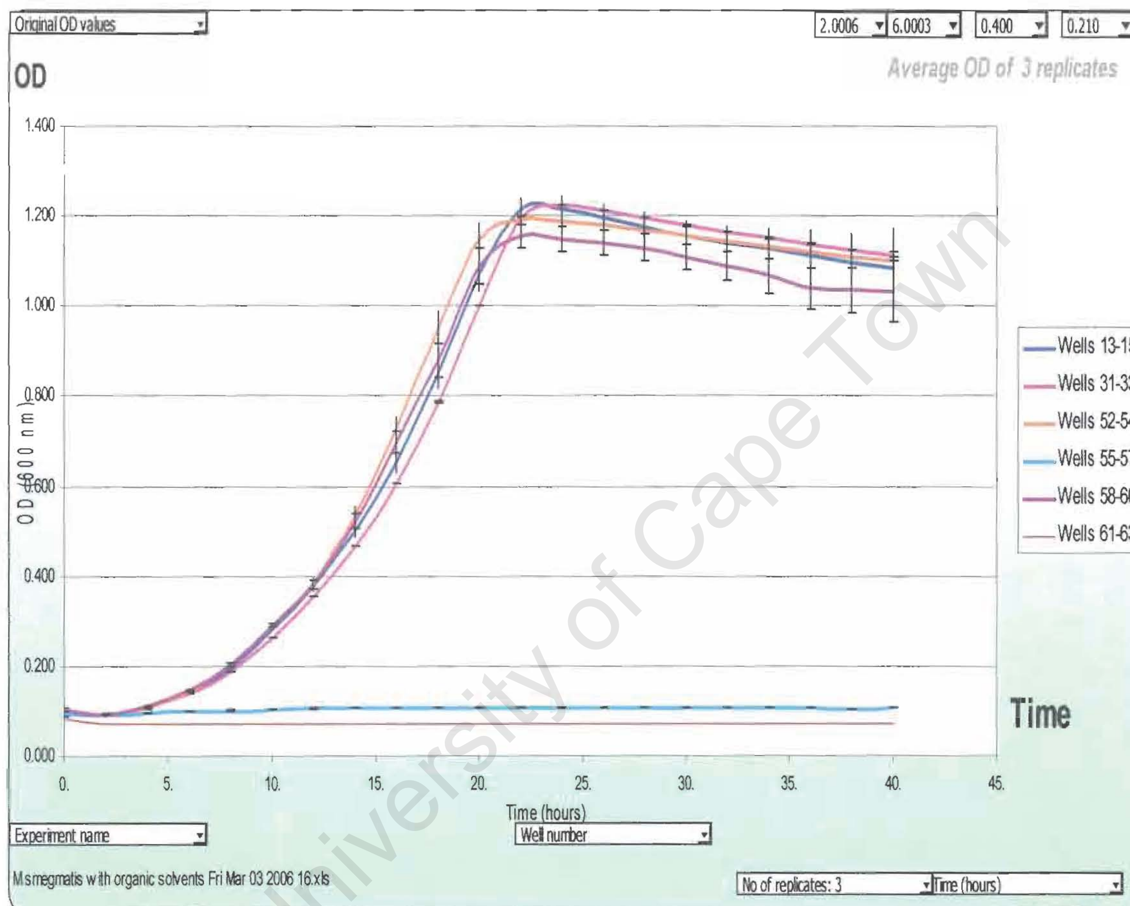


Fig. 3.1: A standard growth curve of *M. smegmatis*

University of Cape Town

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.

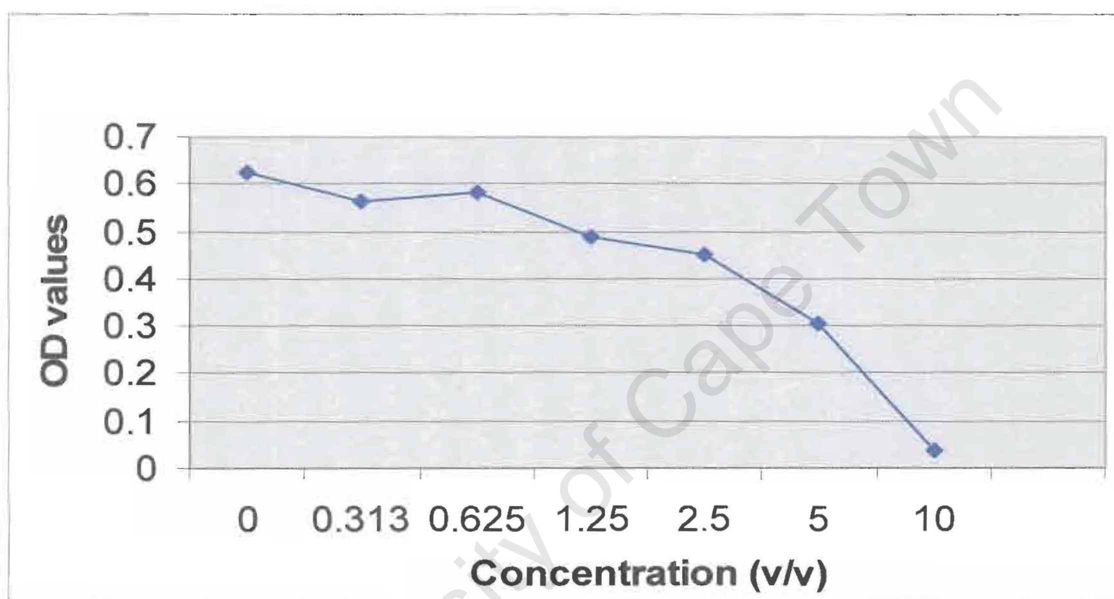


University of Cape Town

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

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 OD_{600} values at 16th hour of *M. smegmatis* growth against each corresponding concentration. The percentage inhibition was calculated by comparing the OD_{600} values of each concentration at the 16th hour of growth relative to the OD_{600} 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*



University of Cape Town

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 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*

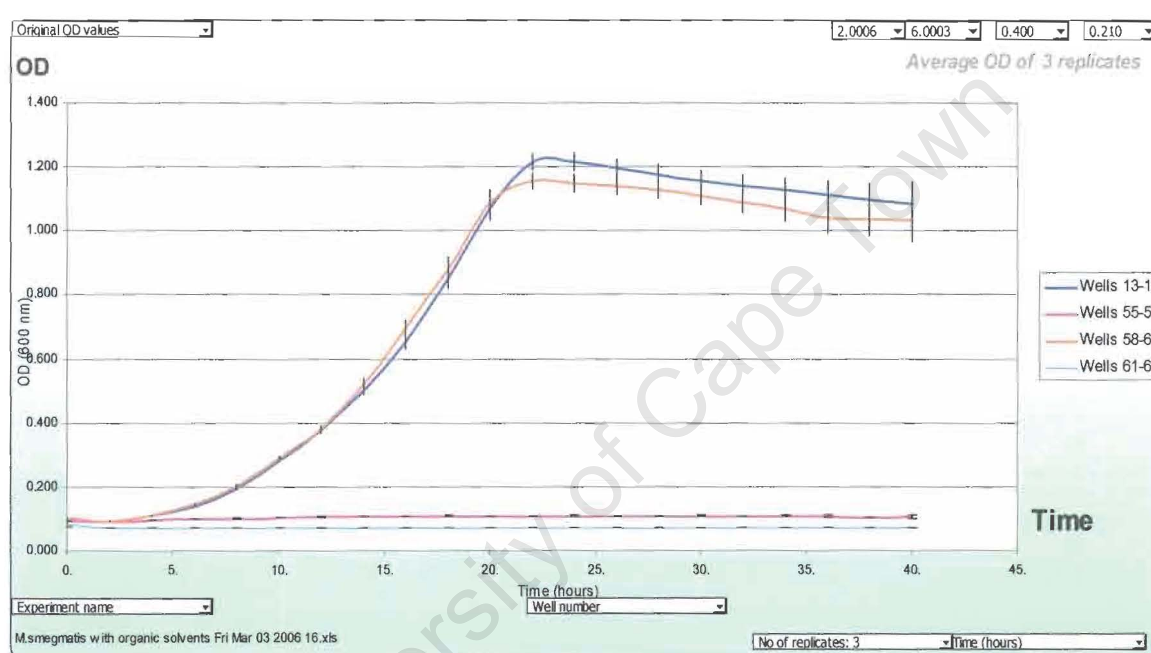


Fig. 3.3: A growth curve of *M. smegmatis* with controls used in the primary screen. Wells 13-15: *M. smegmatis* without any inhibitor, wells 55-57: *M. smegmatis* with rifampicin (6.25 μg/ml), wells 58-60: *M. smegmatis* with 1% DMSO and wells 61-63: medium only. Results are presented as a mean of three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

University of Cape Town

3.4. Primary screen of pant 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.

University of Cape Town

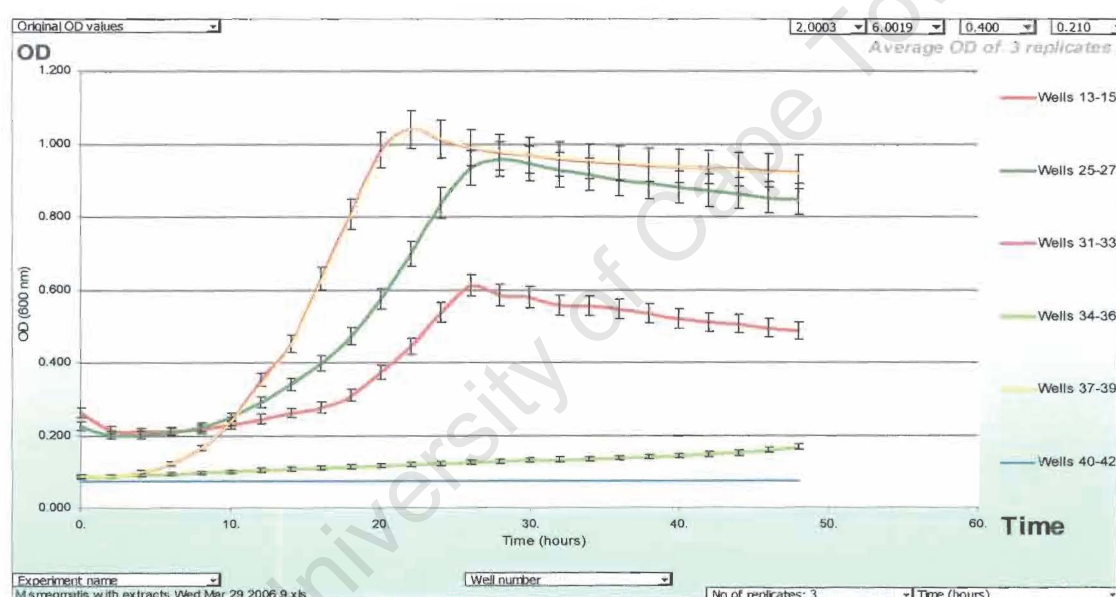


Fig. 3.4.1.1: Growth curve of *M. smegmatis* with most active crude plant extracts at 1mg/ml. Wells: (13-15):P04495b; (25-27): P02301b; (31-33): *M. smegmatis* culture without any inhibitor; (34-36): *M smegmatis* culture with rifampicin (6.25 μg/ml); (37-39): *M. smegmatis* culture with 1% DMSO, (40-42):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

The percentage growth inhibition of *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 *M. smegmatis* culture growth without any inhibitor on the 16th hour of growth.

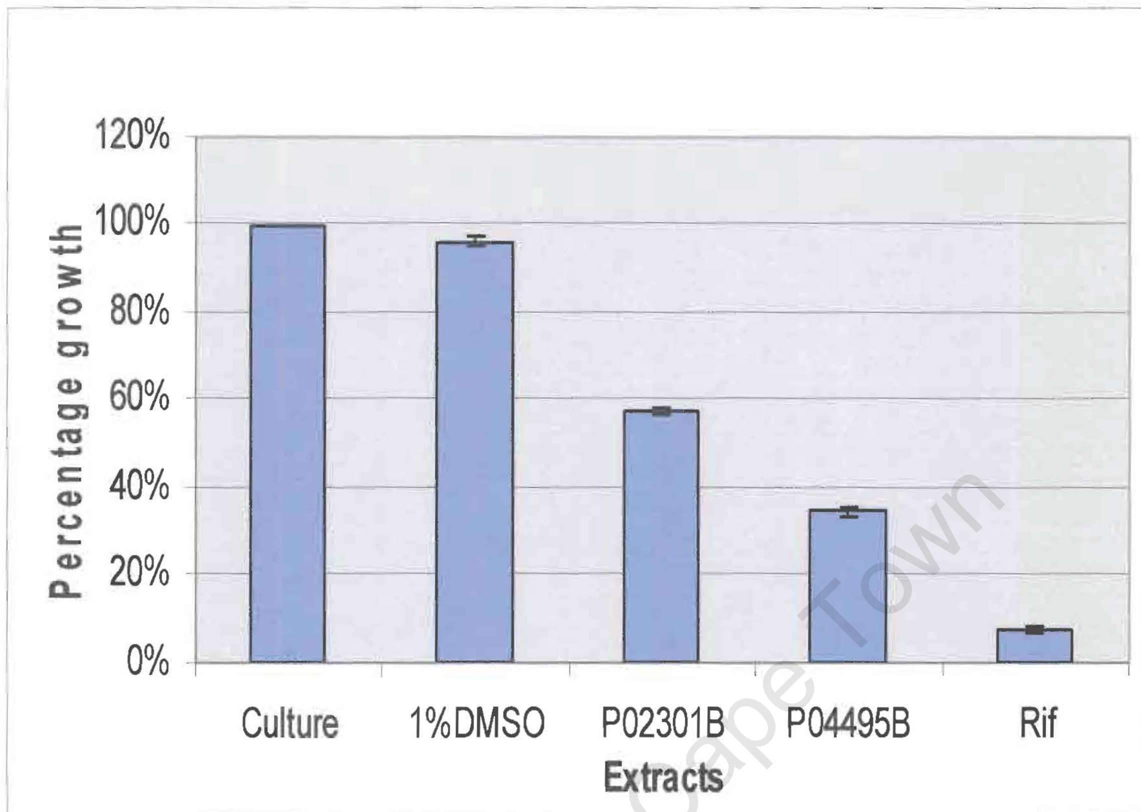


Fig. 3.4.1.2: A percentage growth of *M. smegmatis* on the 16th hour of growth with two extracts that showed inhibition in a Bioscreen C. The results are a mean of three experiments with standard deviation as error bars.

University of Cape Town

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 *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.

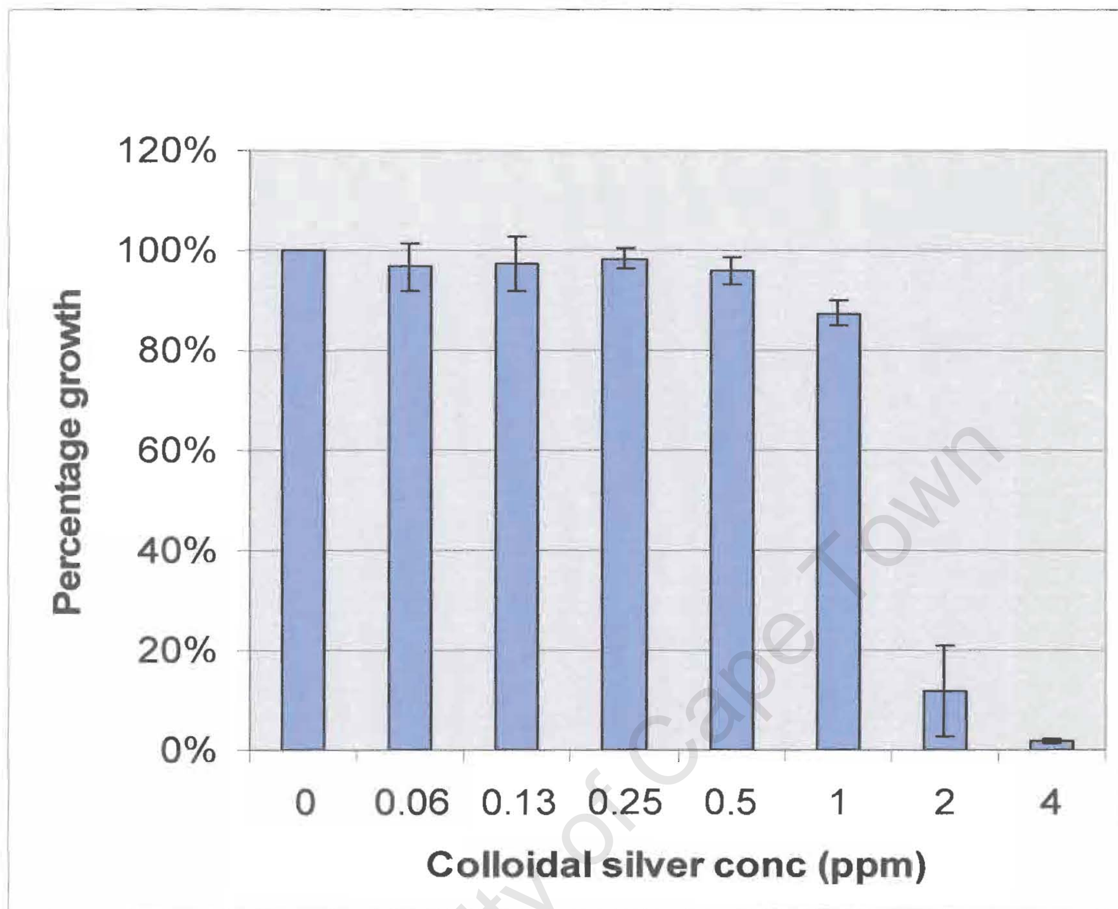


Fig. 3.4.2: The percentage growth of *M. smegmatis* with various concentrations of colloidal silver.

University of Cape Town

3.4.3. Synthetic compounds

All the synthetic compounds were screened at an initial concentration of 10 μg/ml. *M. smegmstis* culture (untreated cells) showed a normal growth pattern, lag, log and stationery 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. smegamtis* as shown in the figure below

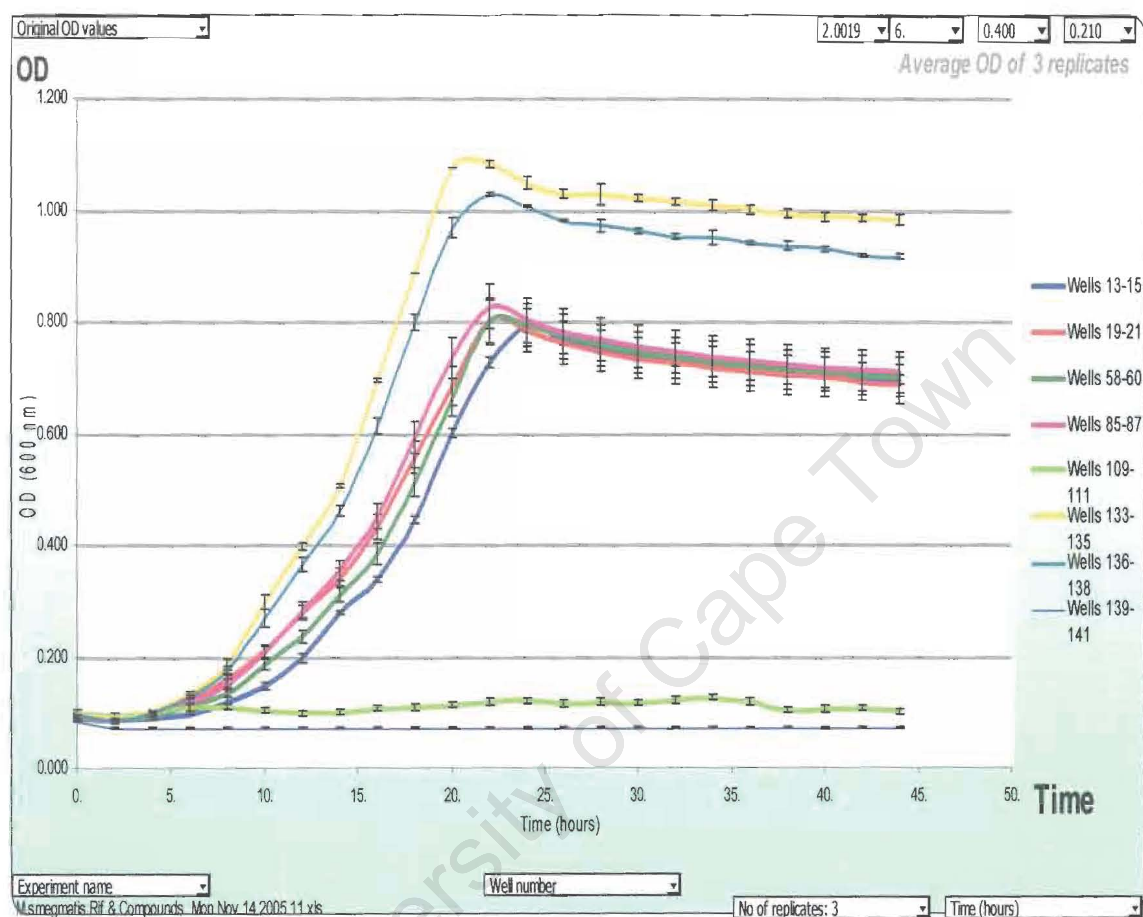


Fig. 3.4.3.1: A sample of 4 synthetic compounds that showed most inhibition screened against *M. smegmatis* and four controls. Wells (13-15): F952; wells (19-21): F1052; wells (58-60): F37; wells (85-87): F1082; wells (109-111): *M. smegmatis* with rifampicin (6.25 µg/ml); wells (133-135): *M. smegmatis*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

University of Cape Town

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).

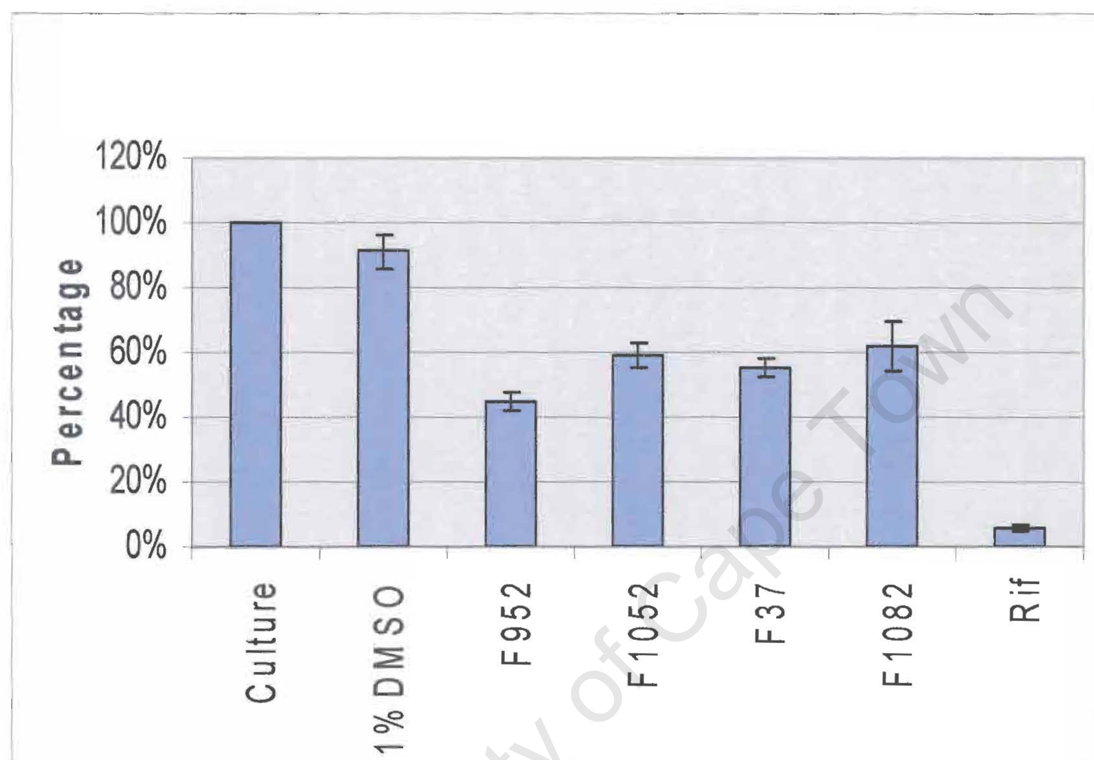


Fig. 3.4.3.2: The percentage growth of *M. smegmatis* with the four "hit" compounds and controls. All these compounds were tested at a concentration of 10 μg/ml. Three different experiments were performed on separate days with standard deviation as error bars of the three experiments.

University of Cape Town

3.5. Secondary screen of plant extracts, eCS and synthetic compounds against M. tuberculosis *using BACTEC 460*

3.5.1 Extracts screened against *M. tuberculosis*

Three plant extracts were screened against *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.

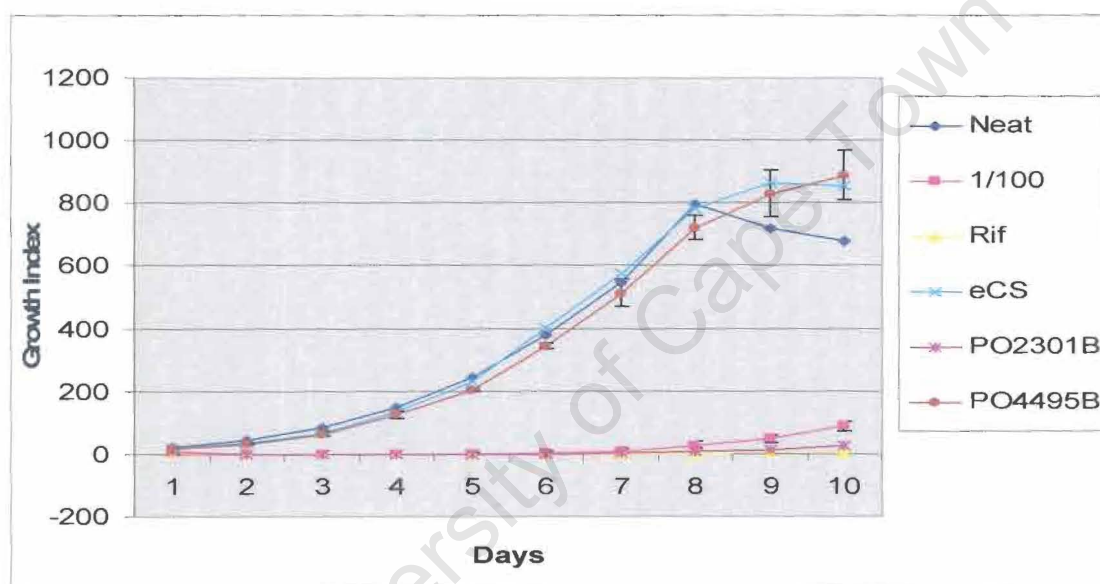


Fig. 3.5.1.1: The growth curves of *M. tuberculosis* with extracts over a period of ten days. The controls used are a neat (*M. tuberculosis* culture only); *M. tuberculosis* with Rif (rifampicin, 2μg/ml) and 1/100: (*M. tuberculosis* culture diluted in 1:100).

University of Cape Town

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.

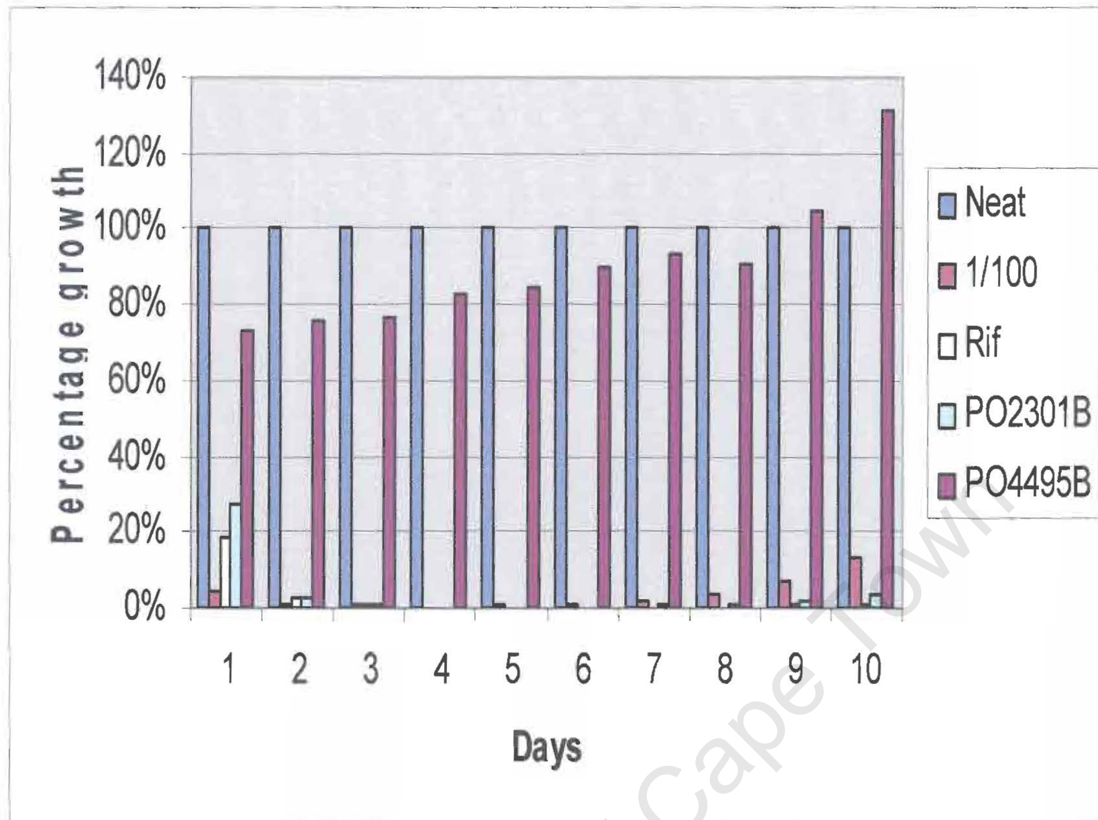


Fig. 3.5.1.2: The percentage growth of *M. tuberculosis* with the two potential inhibitor extracts. The neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tubeculosis* culture with Rif (rifampicin, 2μg/ml)

University of Cape Town

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*.

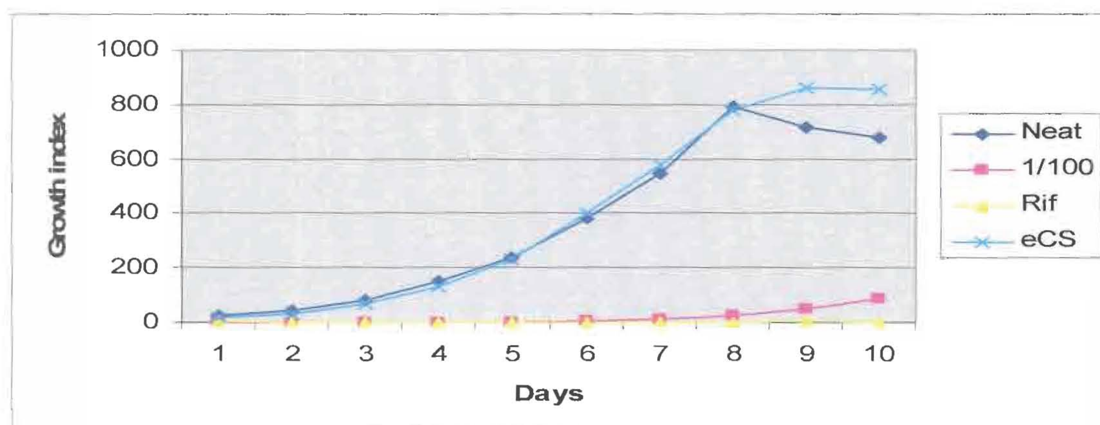


Fig. 3.5.2: The growth curve of *M. tuberculosis* with colloidal silver with results interpreted over ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml), *M. tuberculosis* culture with eCS (Electro-colloidal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

Days	Neat	1/100	Rif	eCS	P02301B	P04495B
1	100%	4%	19%	77%	27%	73%
2	100%	1%	2%	78%	2%	76%
3	100%	1%	1%	82%	1%	77%
4	100%	0%	0%	89%	0%	83%
5	100%	1%	0%	95%	0%	84%
6	100%	1%	0%	105%	0%	90%
7	100%	2%	0%	105%	1%	93%
8	100%	3%	0%	98%	1%	91%
9	100%	7%	0%	120%	2%	104%
10	100%	13%	0%	126%	4%	131%

University of Cape Town

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).

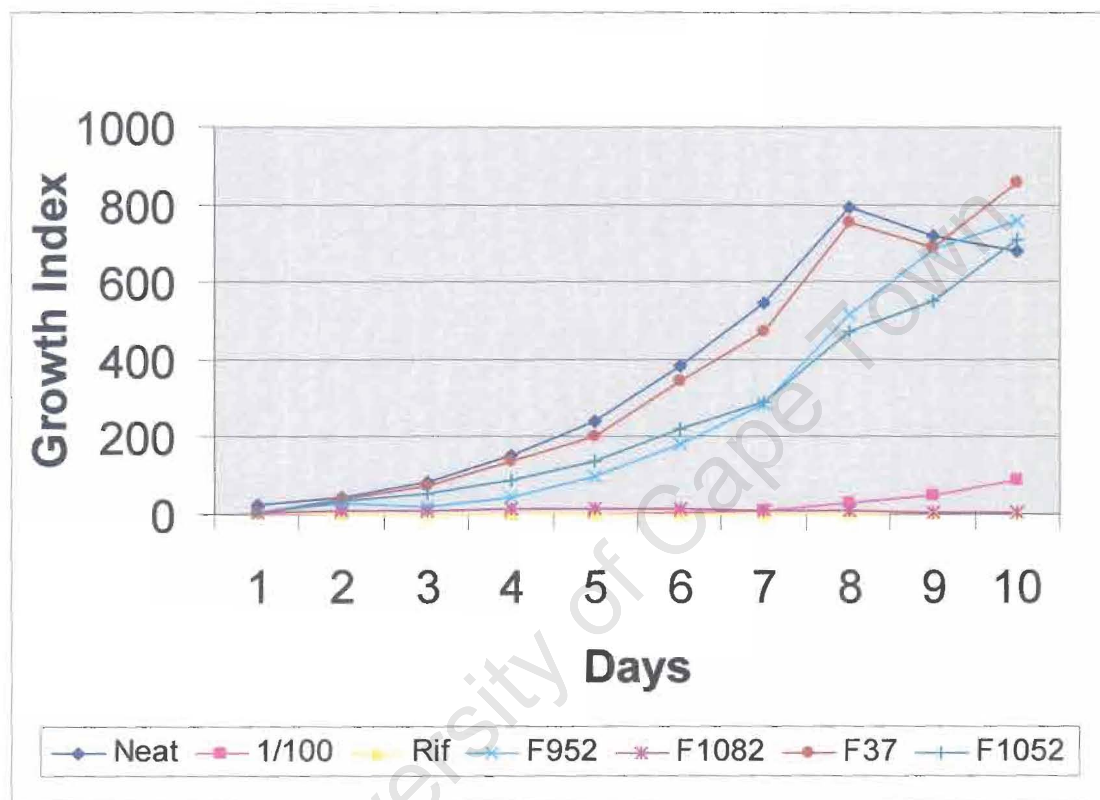


Fig. 3.5.3.1: The growth curves of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2 μg/ml).

University of Cape Town

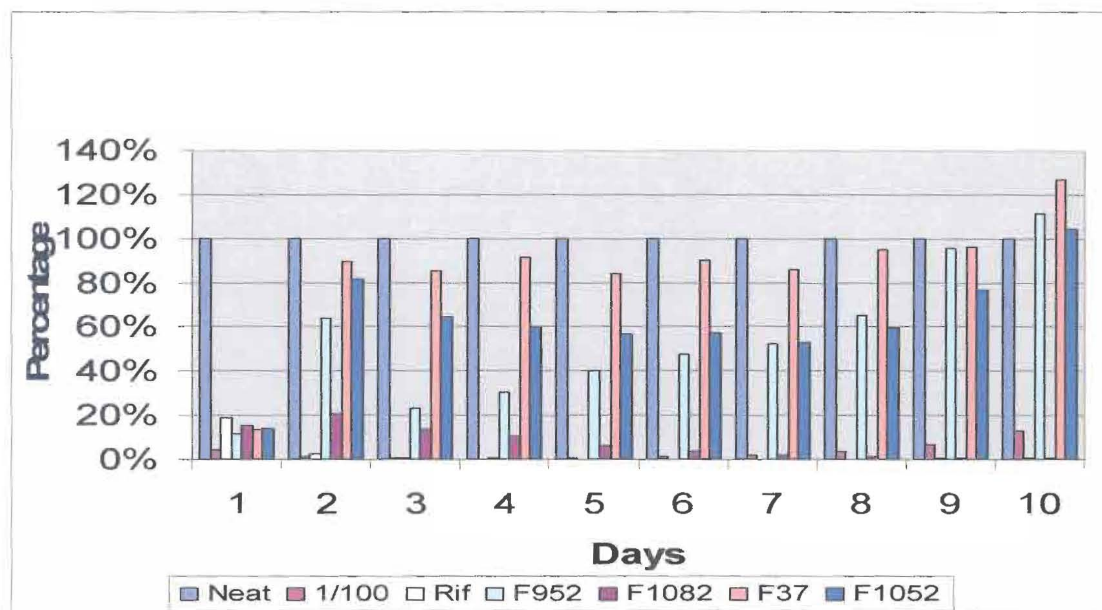


Fig. 3.5.3.2: The percentage growth of *M. tuberculosis* with synthetic compounds in a period of ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2μg/ml).

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)

Days	Neat	1/100	Rif	F952	F1082	F37	F1052
1	100%	4%	19%	11%	15%	13%	14%
2	100%	1%	2%	64%	21%	90%	81%
3	100%	1%	1%	23%	14%	85%	64%
4	100%	0%	0%	31%	10%	91%	60%
5	100%	1%	0%	40%	6%	84%	56%
6	100%	1%	0%	48%	4%	90%	57%
7	100%	2%	0%	52%	2%	86%	53%
8	100%	3%	0%	65%	1%	95%	59%
9	100%	7%	0%	95%	1%	96%	77%
10	100%	13%	0%	112%	1%	126%	104%

University of Cape Town

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 *M. tuberculosis*. The compound (F1082) was tested at concentrations ranging from 2 to 10 µg/ml against *M. tuberculosis* using BACTEC 460 system. The results are shown in figure 3.6. The MIC was determined to be 8 µg/ml, which inhibited 99% growth of *M. tuberculosis*.

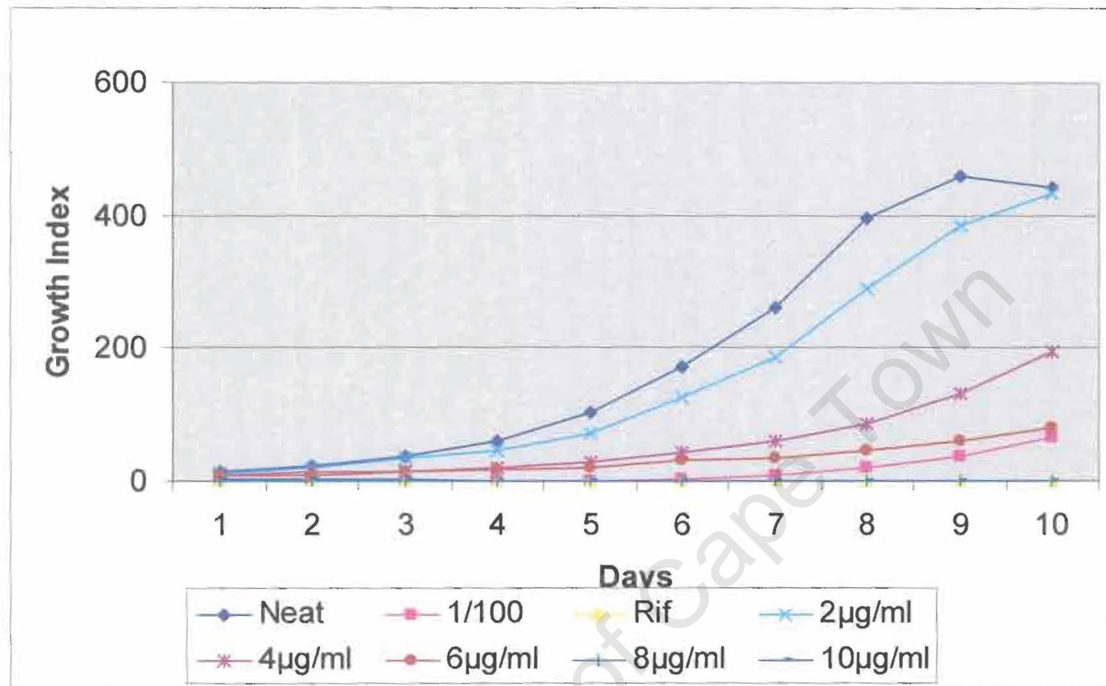


Fig. 3.6: The growth curve of *M. tuberculosis* at various concentrations of F1082 in a period of ten days. Neat (*M. tuberculosis* culture only), 1/100 (*M. tuberculosis* culture diluted 1:100), *M. tuberculosis* culture with Rif (rifampicin, 2µg/ml).

University of Cape Town

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

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

Gram negative bacteria				Gram positive bacteria		
	Percentage growth			Percentage growth		
F1082 concentration (μg/ml)	*E. coli*	*P.aeruginosa*	*K. pneumoniae*	*S.aureus*	*E. faecalis*	*B. cereus*
50	90%	90%	90%	90%	90%	90%
25	90%	90%	90%	90%	90%	90%
12.5	90%	90%	90%	90%	90%	90%
6.25	90%	90%	90%	90%	90%	90%
3.125	90%	90%	90%	90%	90%	90%

University of Cape Town

3.7.1 Antimicrobial positive controls used

The antimicrobacterial 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

Organism	Drug	MICs (µg/ml)
E. coli	Ciprofloxacin	0.5
P. aeruginosa		0.1
K. pneumoniae		0.5
S. aureus		2.0
E. faecalis		1.0
B. cereus		0.2

University of Cape Town

CHAPTER FOUR

Discussion and Conclusion

University of Cape Town

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 16th hours of growth when an overnight bacterial culture was diluted to an OD_{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

University of Cape Town

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).

University of Cape Town

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 *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 *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 *M. smegmatis*. The 4 potential inhibitors were screened against *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 *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_{90} of 2 ppm against *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 *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 *M. tuberculosis* and no further tests were performed.

University of Cape Town

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.

University of Cape Town

REFERENCES

1. Anderson RM (1999). The pandemic of antibiotic resistance. *Nature Medicine* 5:147-149

2. Andries K, Verhasselt P, Guillemont J, Gohlmann HWH, Neefs J-M, Winkler H, Van Gestel J, Timmermann P, Zhu M, Lee E, Williams P, De Chaffoy D, Huitric E, Hofner S, Cambau E, Truffort-Pernot C, Lounis N, Jalier V, (2005). A diarylquinoline drug active on ATP synthase of *Mycobacteium tuberculosis*. *Science* 307: 223-227

3. Ayensu ES, DeFilipps RA (1978). Endangered and Threatened Plants of the United States. Washington, DC: Smithsonian Institution.

4. Barnes PF, Lakey DL, Burman WJ (2002). Tuberculosis in patients with HIV infection. *Infectious Disease Clinic of North America* 16:107-126

5. Bechold H, Van Nostrand D, (1919). Colloids in Biology and Medicine. New York: 364-376

6. Benveinste R, Davies J (1973). Mechanism of antibiotic resistance in bacteria. *Annual Review in Biochemistry* 42:471-506

7. Black GF, Weir RE, Floyd S, Bliss L, Warndorff DK, Crampin AC, Ngwira B, Sichali L, Nazareth B, Blackwell JM, Branson K, Chaguluka SD, Donovan L, Jarman E, King E, Fine PE, Dockrell HM (2002). BCG-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. *Lancet* 359(9315):1393-401

8. Blomberg B, Spinaci S, Fourie B Laing R (2001). The rationale for recommending fixed-dose combination tablets for the treatment of tuberculosis. *Bulletin of the World Heath Organization* 79(1): 61-68

9. Boshoff HIM Barry 3rd CE (2005) Tuberculosis-Metabolism and Respiration in the absence of growth. *Nature Reviews/Microbiology* 3: 70-80.

10. Canetti G, Froman J. Grosset M, Haunduroy M, Lngerova HT, Mahler O, Meissner DA, Michison DA, Sula L (1963). Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bulletin of the world Health Organization* 29:565-578

11. Cantrell C L, Abate L, Fronczek F R, Franzblau S G, Quijano L, Fischer N H (1999). Antimycobacterial eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa*. *Planta Medica* 65:351-355

University of Cape Town

12. Chaulet P (1998). Rifapentine, a reviewpoint. *Drugs* 56(4): 617

13. Condos R, Rom NW, Liu MY, Schluger NW (1998). Local immune responses correlated with presentation and outcome in tuberculosis. *American Journal of Respiratory and Critical Care Medicine* 157:729-735

14. Condos R, Rom WN, Schluger NW (1997). Treatment of multidrug-resistant pulmonary tuberculosis with interferon-γ via aerosol. *Lancet* 349:1513-1515

15. Corbett EL, Steketee RW, ter Kuile FO, Latif AS, Kamali A Hayes RJ (2002) HIV-1/AIDS and the control of other infectious diseases in Africa. *Lancet* 359(9324), 2177-87

16. Corbett EL, Watt CJ, Walker N (2003). The growing burden of tuberculosis: global trends and interaction with the HIV epidemic. *Archives of International Medicine* 163(9), 1009-1021

17. Cox RA (2004). Quantitative relationships for specific growth rates and macromolecular compositions of Mycobacterium tuberculosis, Streptomyces coelicolor A3(2) and Escherichia coli B/r: an integrative theoretical approach. Microbiology. 150(Pt 5):1413-26

18. Crofton J (1970). The assessment and treatment of drug-resistance problems in tuberculosis. *Journal of the Irish Medical Association* 63:75-8

19. Cronquist A (1981). An Integrated System of Classification of Flowering Plants. New York:Columbia University Press

20. Cruz M, Badiano J, Trueblood EWE (1940). The Badianus Manuscript, Codex Barberini, Latin 241, Vatican Library: An Aztec Herbal of 1552. Baltimore, MD:Johns Hopkins Press

21. Cunnigham AB (1998). An investigation of herbal medicine trade in Natal/Kwa-Zulu. Investigation report No.29. Institute of Natural Resources, Scottsvile, South Africa

22. Custom CG, Knoff AA, (1926). History of Medicine. New York: 216

23. Davies J, Wright JD (1997). Bacterial resistance to aminoglycoside antibiotics. *Trends in Microbiology* 5:234-9

24. Davis J (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* 264:375-82

25. Dolin PJ (2000). Global tuberculosis incidence and mortality during 1990-2000. *Bulletin of the World Health Organization* 72:213-220

26. Douglass J, Steyn LM (1993). A ribosomal gene mutation in streptomycin-resistant *Mycobacterium tuberculosis* isolates. *Journal of Infectious Diseases* 167:1505-6

University of Cape Town

27. Duncan K (2003). Progress in TB drug development and what is still needed. *Tuberculosis* 83: 201-207

28. Dye C, Scheele S, Dolin P, Pathania V Raviglione MC (1999) Global burden of tuberculosis: estimated incidence, prevalence and mortality by country. WHO Global Surveillance and Monitoring Project. *Journal of American Medical Association* 282(7), 677-86

29. Elias M (2005). Development of new drugs for TB chemotherapy (analysis of the current). www.accessmed-msf.org/documents

30. Evans WC (1996). Trease and Evans' Phamarmagnosy. W.B. Saunders Company Ltd., London.

31. Farnsworth NR (1994). Ethnopharmacology and drug development. *Ciba Foundation Symposium* 185:42–51

32. Farnsworth NR, Akerele O, Bingel AS, Soejarto DD, Guo Z (1985).Medicinal plants in therapy. *Bulletin f the World Health Organization* 63:965–981.

33. Farnsworth NR, Henry LK, Svoboda GH, Blomster RN, Yates MJ, Euler KL (1966). Biological and phytochemical evaluation of plantsI: Biological test procedures and results from 200 accessions. Lloydia 29:101–122

34. Fine PEM (1995). Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* 346:1339-1345

35. Finken M, Kirschner P, Meier, A, Wrede A, Bottger EC (1993). Molecular basis of streptomycin-resistance in *Mycobacterium tuberculosis*: alteration of the ribosomal protein S12 gene and point mutations within a functional 16S rRNA pseudoknot. *Molecular Microbiology* 9:1239-46

36. Frieden TR, Sterling TR, Munsiff SS, Watt CJ Dye C (2003). Tuberculosis. *Lancet* 362(9387): 887-99

37. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C (2003) Tuberculosis. *Lancet* 362(9387), 887-99

38. Gay JD, de Young DR, Roberts GD (1984). In vitro activities of norfloxacin and ciprofloxacin against *Mycobacterium tuberculosis, M. avium* complex, *M chelonei, M. forfuitre, and M. kansasii. Antimicrobial Agents and Chemotherapy* 26:94-6

39. Gellert M, Mizuuchi K, O'Dea MH, Nash HA (1976). DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proceedings of the Natural Academy of Sciences USA* 73:3872-5

University of Cape Town

40. Gellert M, O'Dea MH, Itoh T, Tomizava J (976). Novobiocin and coumeromycin inhibit DNA supercoiling catalyzed by DNA gyrase. *Proceedings of the Natural Academy of Sciences U S A* 73:4474-8

41. Goletti D, Weissman D, Jackson RW, Graham NM, Vlahov D, Klein RS, Munsiff SS, Ortona L, Cauda R, Fauci AS (1996). Effect of Mycobacterium tuberculosis on HIV-replication. Role of activation. *Journal of Immunology* 157:1271-1278

42. Grange J M, Snell N J C (1996). Activity of bromhexine and ambroxol, semi-synthetic derivatives of vasicine from Indian shrub *Adhatoda vasica*, against *Mycobacterium tuberculosis in vitro*. *Journal of Ethnopharmacology* 50:49-53

43. Grange JM (1990). Drug-resistance and tuberculosis elimination. Bulletin International Union Against Tuberculosis and Lung Diseases 65:57.

44. Hampton T (2005). Tb drug research picks up the pace. *Journal of American Medical Association* 293(22): 27052707

45. Havlir DV, Barnes PF (1999). Tuberculosis in patients with human immunodeficiency virus infection. *New England Journal of Medicine* 340: 367-373

46. Heifets L.B. and Lindohlm-Levy P.J. (1992) Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* populations. *American Review of Respiratory Diseases* 145:1223-5

47. Heifets LB (1986). Rapid automated methods (BACTEC System) in clinical mycobacteriology. Seminars in Respiratory Infections. ;1(4):242–249

48. Heifets LB, Lindohlm-Levy PJ (1992). Pyrazinamide sterilizing activity in vitro against semidormant *Mycobacterium tuberculosis* populations. *American Review of Respiratory Diseases* 145:1223-5

49. Hoshino Y, Nakata K, Hoshino S, Honda Y, Tse BD, Shioda T, Rom NW, Weiden M (2002). Maximal HIV-1 replication in alveolar macrophages during tuberculosis requires both lymphocyte contact and cytokines. *Journal of Experimental Medicine* 18:495-505

50. Hudson A, Imamura T, GutteridgeW, Kanyok T, Nunn P (2003). The current anti-tuberculosis drug research and development pipeline. Special program for Research and training in Tropical Diseases (TDR). TDR/PRD/TB/03.1W

51. Huebner RE, Schein MF, Bass JB (1993). The tuberculin skin test. *Clinical Infectious Diseases* 17:968-975

52. Iseman M.D. and Madsen L.A. (1989) Drug-resistant tuberculosis. *Clinical Chemistry Medicine* 1989;10:341-53

University of Cape Town

53. Iseman MD (2000) A clinicians guide to tuberculosis. Philadelphia: Lippincott, Williams and Wilkins.

54. Jarvis B, Lamb HM (1998). Rifapentin. *Drugs* 56(4): 607-616

55. Jin D, Gross C (1988). Mapping and sequencing of mutations in the *Escherichia coli rpo*B gene that leads to rifampicin resistance. *Journal of Molecular Biology* 202:45-58

56. Kirchausen T, Wang JC, Harrison SC (1978). Purification of the subunits of *Escherichia coli* DNA gyrase and reconstitution of enzyme activity. *Proceedings of the National Academy of Sciences USA* 75:1773-7

57. Kochi A, Vareldzis B, Styblo K (1993). Multi-Drug resistant tuberculosis and control. *Research in Microbiology* 144:104-10

58. Lall N, Meyer JJM (1999) In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *Journal of Ethnopharmacology* 66. 347-354

59. Levin ME, Hatfull GF (1993). *Mycobacterium smegmatis* RNA polymerase: DNA supercoiling, action of rifampicin and mechanism of rifampicin resistance. *Molecular Microbiology* 8:277-85

60. Lewis WH, Elvin-Lewis MPF (1977). Medical Botany: Plants Affecting Man's Health. New York:Wiley

61. Locher CP, Burch MT, Mower HF, Berestecky J, Davis H, van Poel B, Lasure A, Vanden Berghe DA Vlietinck AJ (1995). Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaiian medicinal plants. *Journal of Ethnopharmacology* 49(1): 23-32

62. Loud W D, Farnsworth N R, Soejarto D D, Quinn M L (1985). NAPRALERT; computer handling of natural products research data. *Journal of Chemical. Information and Computer Science* 25:99-103

63. Maggliozzo RS, Marcinkeviciene JA (1996). Evidence for isoniazid oxidation by oxypressors mycobaterial catalase-oxidase. *Journal American Chemical Society* 118:11303-4

64. McClatchy JK (1978). Susceptibility testing of mycobacteria. *Laboratory Medicine* 9:47-52

65. Mendez P, Raviglione A, Laszo MC, Binkin A, Rieder N, Busteo HL, Cohn F, Kim CSB, Chaulet SJ, Nunn P (1998). Global surveillance for antituberculosis-drug resistance. *New English Journal of Medicine* 338:1641-1649

66. Middlebrook G, Cohn ML (1958). Bacteriology of tuberculosis: laboratory methods. *American Journal. Public Health* 48:944-853

University of Cape Town

67. Mitchison D.A. (1985) Mechanism of drug action in short-course chemotherapy. Bulletin International Union Against Tuberculosis 65:30-7

68. Mitchison DA (1996). Pyrazinamide: on the antituberculosis frontline. *Nature Medicine* 2(6): 635-636

69. Mitscher LA, Baker WR. (1998). A search for novel chemotherapy against tuberculosis among natural products. *Pure Applied Chemistry* 70,365-371

70. Mittal SK, Aggarwa, V, Rastogi A, Saini N (1996). Does BCG vaccination prevent or postpone the occurrence of tuberculous meningitis? *Indian Journal of Pediatrics* 63: 659-664

71. MMWR (Morbidity and Mortality weekly Report) (1993) Initial therapy for tuberculosis in the era of multi-drug resistance: recommendations of the advisory council for the elimination of tuberculosis.;42 (RR-7)

72. Mwinga A Fourie BP (2004). Prospects for new tuberculosis treatment in Africa. *Tropical Medicine and International Health* 9 (7): 827-832

73. Newton SM, Lau C, Wright CW, (2000). A review of antimycobacterial natural products. *Phytotherapy Research* 14:302-322

74. Niemann S, Richter E, Dalugge-Tamm H, Schlesinger H, Graupner D, Konigstein B, Gurath G, Greinert U, Rusch-Gerdes S, (2000). "Two cases of Mycobacterium microti derived tuberculosis in HIV immunocompetent patients." Emerging Infectious Diseases.

75. O'Brien RJ Nunn P (2001). The need for new drugs against tuberculosis-obstacles, opportunities and new steps. *American Journal of Respiratory and Critical Care Medicine* 163(5): 1055-1058

76. Ovchinnikov YA, Monastyrskaya GS, Gubanov VV, Lipkin VM, Sverdlov ED, Kiver IF, et al (1981). Primary structure of *Escherichia coli* RNA polymerase nucleotide substitution in the ß-subunit gene of rifampicin resistant *rpo*B255 mutant. *General Molecular Genetics* 84:536-8

77. Pan F, Jackson M, Ma Y, McNeil MR (2001). Determination that cell wall galactofuran synthesis is essential for growth of mycobacteria. *Journal of Bacteriology* 183:3991-3998

78. Phillipson JD, Anderson LA (1989). Ethnopharmacology and Western medicine. *Journal of Ethnopharmacology* 25:61–72

79. Pio A, Chaulet P (1998). Tuberculosis handbook. Geneva:World Health Organization

80. Raja A (2004). Immunology of tuberculosis. *Indian Journal of Medical Research* 120. 213-232

University of Cape Town

81. Rastoggi N, Goh KS (1990). Action of 1-isonicotinyl-2-palmitoyl hydrazine against the *Mycobacterium avium* complex and enhancement of its activity by m-flurophenyl alanine. *Antimicrobial Agents and Chemotherapy* 34:2061-4

82. Ratledege C, Stanford J, Grange JM (1989). The biology of mycobacteria. Vol. 3, London Academic Press

83. Rodrigues LC, Diwan VK, Wheeler JG (1993). Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. *International Journal of Epidemiology* 22: 1154-1158

84. Ryan F (1992). The forgotten plague: How the battle against tuberculosis was won and lost, Little, Brown and Co., Boston

85. Scorpio A, Zhang Y (1996). Mutation in pncA, a gene encoding pyrazinamide/ nicotinamidase cause resistance to the antituberculosis drug PZA in tubercle bacillus. *Nature Medicine* 2(6):662-667

86. Searle AB, Dutton EP (1919). The use of Colloids and Disease. New York: 83

87. Segelman AB, Farnsworth NR, Quimby MW (1968). Biological and phytochemical evaluation of plants. III: False-negative saponin test results induced by the presence of tannins. Lloydia 32:52–58

88. Shimao T (1987). Drug-resistance in tuberculosis control. Tubercle; 68(suppl):5-15

89. Shoeb HA, Bowman BU Jr, Ottolenghi AC, Merola AJ (1985). Peroxidase-mediated oxidation of isoniazid. *Antimicrobial Agents and Chemotherapy* 27:399-403

90. Siddiqi SH, Libonati JP, Middlebrook G (1981). Evaluation of rapid radiometric method for susceptibility testing of Mycobacterium tuberculosis. *Journal of Clinical Microbiology* 13:908-912

91. Smith AB 3rd, Levnberg PA, Jerris PJ, Scarborough RM., Wovkulich PM (1981). *Journal of American Chemistry* 103:1501-1513

92. Solecki R (1975). Shanidar IV, a Neanderthal flower burial in northern Iraq. *Science* 190:880–881

93. Soysal A Millington AK Bakki M (2005). Effects of BCG vaccination on risk of Mycobacterium tuberculosis infection in children with house hold tuberculosis contact: a prospective community-based study. *Lancet* 366:1443-1451

94. Spratt BG (1994). Resistance to antibiotics mediated by target alterations. *Science* 264:388-93

University of Cape Town

95. Sugino A, Peebles CL, Kreuzer KN, Cozzarelli NR (1977). Mechanism of action of Nalidixic acid: purification of *Escherichia coli nal*A gene product and its relationship to DNA gyrase and a novel nicking closing enzyme. *Proceedings of the National Academy of Sciences U S A* 74:4667-71

96. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreisworth B (1994). Cloning and nucleotide sequencing of *Mycobacterium tuberculosis gyr*A and *gyr*B genes and detection of quinolone resistant mutations. *Antimicrobial Agents and Chemotherapy* 38:773-80

97. Tam CM, Chan SL, Lam CW, Leung CC, Kam KM, Morris JS, Mitchison DA (1998). Rifapentine and isoniazid in the continuation phase of treating pulmonary tuberculosis. *American Journal of Respiratory Care Medicine* 157: 1726-1733

98. Thomas JP, Baughan CO, Wilkinson RG, Shephard RG (1961). A new synthetic compound with anti-tuberculous activity in mice: ethambutol (dextro-2,2'-[ethylenediimino]-di-1-butonol). *American Review of Respiratory Diseases* 83:891-893

99. Tippo O, Stern WL (1977). Humanistic Botany. New York:W.W. Norton.

100. Tomiaka H (200). Prospects for development of new antimycobial drugs. *Journal of Infection and Chemotherapy* 6:8-20

101. Van Rie A, Warren RM, Beyers N, Gie RP, Clasen CN, Richardson M, Sampson SL, Victor TC van Helden PD (1999) Transmission of multidrug-resistant Mycobacterium tuberculosis strain among non-institutionalized, human immunodeficiency virus-seronegative patients. *Journal of Infectious Diseases* 180: 1608-1615

102. Vareldzis B.P., Grosset J., de Kantor I., Crofton J., Laszlo A., Felten M (1994). Drug-resistant tuberculosis: laboratory issues. World Health Organization recommendations. *Tubercle and Lung Diseases* 75:1-7

103. Verpoorte R (2000). Pharmacognosy in the new millennium: leadfinding and biotechnology. *Journal of Pharmacology* 52:253–262

104. Von Reis S (1973). Arnold Arboretum, Harvard University, Gray Herbarium. Drugs and foods from little-known plants; notes in Harvard University herbaria. Cambridge, MA: Harvard University Press

105. Wall ME, Wani MC (2000). Camptothecin and taxol: from discovery to clinic. *Journal of Ethnopharmacology* 51:239–254

106. Wallace RJ, Nash Junior DR, Steel LC, Steingrube V (1986). Susceptibility testing of slow mycobacteria by microdilution MIC method with 7H9 broth. *Journal of Clinical Microbiology* 24:976-981

University of Cape Town

107. Wells WF (1934). On air-borne infection: study 11, droplets and droplet nuclei. *Journal of American Hygiene* 20:435-442

108. WHO (2006). Global tuberculosis control: surveillance, planning, financing. Geneva: World Health Organization 242.

109. Williams VL (1996). The Witwatersrand muti trade. *Veld and Flora* 82: (12)1-14

110. Woodley CL, Kilburn JO, David HL, Silcox VA (1972). Susceptibility of mycobacteria to rifampin. *Antimicrobial Agents and Chemotherapy* 2:245-249

111. Youatt J (1969). A review of the action of isoniazid. *American Review of Respiratory Diseases* 99:729-749

112. Zhang Y, Amzel M (2002). Tuberculosis drug targets. *Current Drug Targets* 3: 131-154

113. Zhang Y, Scorpio A, Nikaido H, Sun Z (1999). The role of acid pH and efficient efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *Journal of Bacteriology* 181:2044-2049

University of Cape Town

106. Wells WF (1934). On air-borne infection: study 11, droplets and droplet nuclei. *Journal of American Hygiene* 20:435-442

107. WHO (2006). Global tuberculosis control: surveillance, planning, financing. Geneva: World Health Organization 242.

108. Williams VL (1996). The Witwatersrand muti trade. *Veld and Flora* 82: (12)1-14

109. Woodley CL, Kilburn JO, David HL, Silcox VA (1972). Susceptibility of mycobacteria to rifampin. *Antimicrobial Agents and Chemotherapy* 2:245-249

110. Youatt J (1969). A review of the action of isoniazid. *American Review of Respiratory Diseases* 99:729-749

111. Zhang Y, Amzel M (2002). Tuberculosis drug targets. *Current Drug Targets* 3: 131-154

112. Zhang Y, Scorpio A, Nikaido H, Sun Z (1999). The role of acid pH and efficient efflux of pyrazinoic acid in unique susceptibility of *Mycobacterium tuberculosis* to pyrazinamide. *Journal of Bacteriology* 181:2044-2049

113. Elias M (2005). Development of new drugs for TB chemotherapy (analysis of the current). www.accessmed-msf.org/documents

University of Cape Town