

**The investigation of indigenous South African medicinal plants for
activity against *Mycobacterium tuberculosis***



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DEDICATION

This dissertation is dedicated to my parents, Gaerutoe and Phuti Mokgethi for teaching me valuable life lessons. This is also for those who have gone before me, so I could be here.

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List of Abbreviations

Plant extracts:

- | | |
|---------|--|
| 1. Aga | <i>Agapanthus praecox</i> |
| 2. Olea | <i>Olea europaea subspecies Africana</i> |
| 3. SC | <i>Syzgium cordatum</i> |
| 4. ZC | <i>Zanthoxylum capense</i> |

Solvents:

- | | |
|---------|-----------------|
| 5. ace | Acetone |
| 6. aq | Aqueous |
| 7. dcm | Dichloromethane |
| 8. EtAc | Ethyl acetate |
| 9. Hex | Hexane |
| 10. met | Methanol |

General:

- | | |
|--------------------------------|--|
| 11. AIDS | Acquired Immuno Deficiency Syndrome |
| 12. APC | Antigen Presenting Cell |
| 13. BCG | Bacillus Calmette Guérin |
| 14. CD (eg. CD4 ⁺) | Cluster of Differentiation |
| 15. CFU | Colony Forming Unit |
| 16. DMSO | Dimethyl Sulphoxide |
| 17. DOTS | Directly Observed Treatment Short course |
| 18. HIV | Human Immuno Virus |
| 19. HPLC | High Pressure Liquid Chromatography |

20. IC ₅₀	Concentration producing 50% growth inhibition
21. IC ₉₀	Concentration producing 90% growth inhibition)
22. IFN- γ	Interferon-gamma
23. IL (eg. IL-10)	Interleukin
24. iNOS	inducible nitric oxide synthase
25. LAM	Lipoarabinomannan
26. LPS	Lipopolysaccharide
27. MABA	Microplate Alamar Blue Assay
28. MIC	Minimum Inhibitory Concentration
29. MHC	Major Histocompatibility Complex
30. M.tb	<i>Mycobacterium tuberculosis</i>
31. MTT	3-(4,5-dimethylthiazol-2,5- diphenyltetrazoliumbromide)
32. NO	Nitric Oxide
33. OADC	Oleic Albumin Dextrose Catalase
34. TB	Tuberculosis
35. TNF- α	Tumour Necrosis Factor-alpha
36. WHO	World Health Organisation

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Abstract

Tuberculosis is the longest running health catastrophe. It remains an escalating health crisis worldwide and mandates innovative approaches to find more effective therapeutic strategies. The rich plant biodiversity of South Africa has a significant role to play in being able to provide new therapeutic strategies or as a source of novel drug leads for Tuberculosis. There is also a need for validation of the safety and efficacy of medicinal plants used in traditional medicine as a large proportion of the South African population relies on traditional medicine for primary health care. This study investigated four indigenous South African medicinal plants that are commonly used in traditional medicine for their bioactivity against *Mycobacterium tuberculosis*. Crude plant extracts were prepared and characterized using HPLC analysis. The crude rhizome extracts of *Agapanthus praecox*, leaf extracts of *Olea europaea* subsp. *Africana* and bark methanol extracts of *Zanthoxylum capense* showed activity against the virulent strain of *Mycobacterium tuberculosis* H37Rv in a direct susceptibility screening assay *in vitro* using the Microplate Alamar Blue assay, whereas the *Syzigium cordatum* and *Zanthoxylum capense* crude leaf extracts were inactive. The methanol and ethyl acetate crude leaf extracts of *Olea europaea* subsp. *Africana* displayed the most potent anti-mycobacterial activity *in vitro* at 125 and 250µg/ml respectively. Furthermore, the majority of the crude extracts from all four plants were very cytotoxic against murine peritoneal macrophages. In addition, the plant extracts significantly reduced growth of intracellularly growing *Mycobacterium tuberculosis* residing within peritoneal macrophages. None of the extracts tested in *in vivo* using wildtype C57BL/6 murine models showed anti-mycobacterial activity. Treatment with the plant extracts also did not have an effect on cellular infiltration and granuloma formation in the lung and liver tissues of infected mice.

PART 1: TUBERCULOSIS

1. INTRODUCTION

1.1 History

Tuberculosis (TB) has been reported to have origins dating back to pre-historic biblical times (Rom, 1996). Skeletal remains of humans dating back to around 4000 BC have displayed lesions characteristic of TB and tubercular decay has been found in the spines of Egyptian mummies dating from 3000-2400 BC. Reports have been made throughout recorded history that tuberculosis has been the major cause of death and it remains the most prevalent infectious disease in the world. However, due to the variety of its symptoms, it was only recognized as a unified disease in the 1820's and was termed tuberculosis in 1839 by J. L. Schoenlein (Encyclopedia, ; McKinney, 1998; Sakula, 1981). The ancient name for tuberculosis is consumption, derived from the Greek term Phthisis. It has also been referred to as "Captain among these Men of death" (John Bunyon, 1660), white death and white plague.

The spread of the disease was facilitated by migration of Europeans into the West and Eurasia, exposing previously unaffected populations. By 1000 BC, TB had spread throughout the world (Microbiology@Leicester). TB caused the most widespread public alarm in the 19th century as the endemic disease of poor people living in urban areas. Before it was established that the disease was contagious, the morbidity rate in Europe was extremely high; one in four deaths were caused by TB (Dubos, 1952). This outbreak was termed the white plague. Efforts to try and control the spread of the disease included isolation of infected individuals who were housed in sanatoria. Seventy five percent of those who entered the sanatoria were reported dead within five years. A German physician scientist, Robert Koch, later demonstrated the actual causative agent of the disease in 1882 and he identified *Mycobacterium tuberculosis* as the cause of tuberculosis in humans (Koch, 1932).

Furthermore, Koch later discovered around the 1890's that a glycerin extract of the tubercle bacilli could be used as a "remedy" for tuberculosis. This failed to cure the

disease. In 1906, Albert Calmette and Camille Guérin developed a successful vaccine from attenuated bovine tuberculosis strain named *Bacillus of Calmette and Guérin* (BCG). It was first used in humans on the 18th July 1921 and is still the only available TB vaccine today (O'Brien, 2001). There were various other methods of treating the infection in the early 20th century, which were not highly successful. These included the pneumothorax technique, which was a surgical procedure that involved collapsing the infected lung and allowing the lesions to heal. This practice was not very efficient and was discontinued after 1946. A breakthrough and major improvement in reducing TB was the introduction of antibiotics such as streptomycin, the first antibiotic used for TB treatment, during the 20th century (Garvin et al., 1974). This offered the possibility of treating and potentially eliminating the infection rather than the previous strategies that were more focused on prevention. Subsequently, great progress has been made to develop health programs, therapeutic strategies and drugs that aim to eliminate TB completely.

It is therefore evident from literature that TB is an ancient phenomenon that remains prevalent even today, despite the vigorous development of chemotherapies and widespread vaccination efforts. The search for new treatment is now fuelled by the emergence of multi-drug resistant strains of TB amongst other challenges.

2. EPIDEMIOLOGY

2.1 Incidence of Tuberculosis

Tuberculosis remains the leading infectious cause of death worldwide, being responsible for about three million deaths annually (Zumla et al., 2000). Approximately one third (two billion people) of the world's population is infected with TB. Every year, approximately 8 million of the infected populations develop active TB, and an estimated 2 million of these individuals die from the disease (Stewart et al., 2003). The burden and case notifications of TB continue to rise globally, particularly in developing countries (Fig.1). Among the factors that complicate TB control, human immunodeficiency virus (HIV) co-infection is one of the most important and challenging (O'Brien, 2001). HIV promotes progression to active TB in both people with recently acquired TB and increases the risk of reactivating latent TB resulting in TB being the leading cause of

HIV/AIDS related deaths. Moreover, there has been an alarming increase in multi-drug resistant strains of mycobacterium tuberculosis in Africa. Of the 22 high burden countries that constitute 80% of the global burden of TB, nine countries are in Africa (Maher et al., 2005). It is apparent from the rising incidence and prevalence of the disease that it is a long way from being eradicated. Reportedly, there are more cases of TB in the world at present than at any previous time in human history (Zumla et al., 2000). Hence the World Health Organization (WHO) has declared TB a global emergency. In South Africa, rapid increase in TB notification cases together with overwhelming HIV infection rates and the emergence of MDR-TB has led TB control to be priority for the Department of Health. South Africa is currently ranked number eight (8) country in the world with the most number of TB cases (WHO, 2004). TB is the longest enduring health calamity, and despite major efforts to manage the disease, it remains an increasing worldwide health crisis that requires novel and innovative approaches to be prevented or cured.

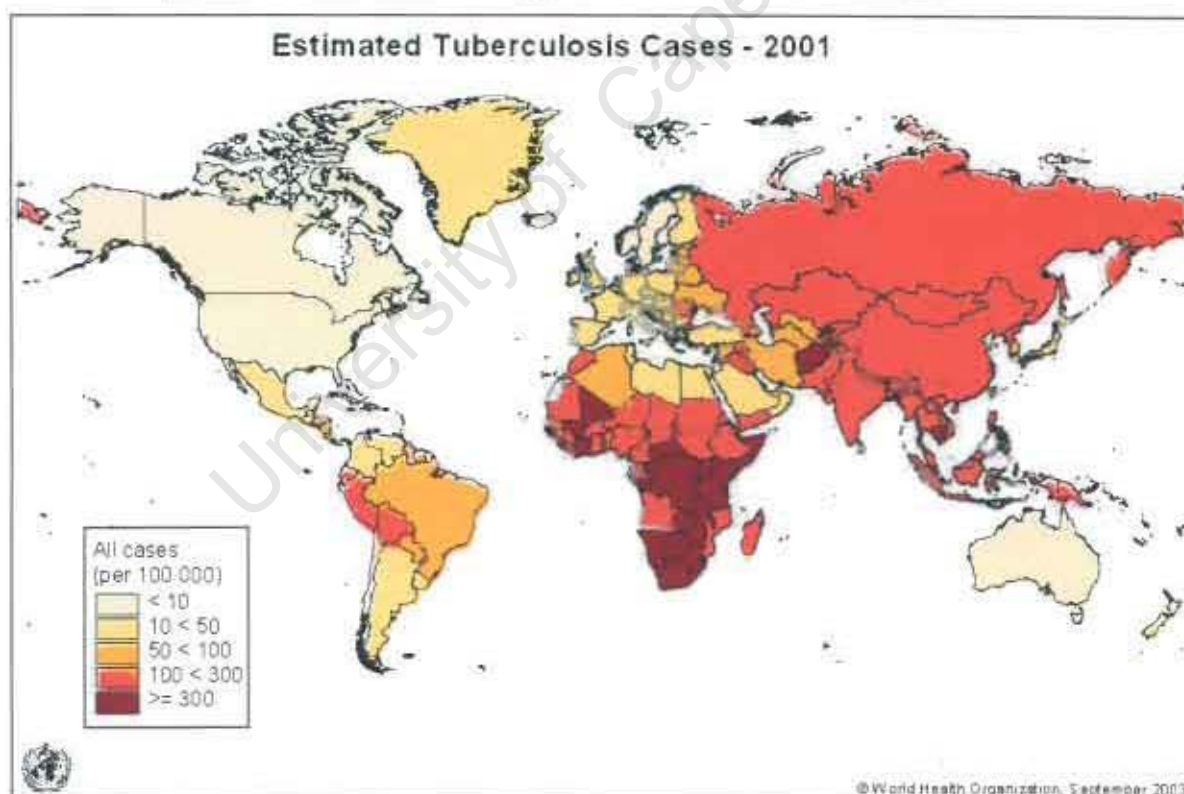


Fig.1 A map demonstrating the estimated global TB incidence in 2001. It is apparent from the diagram that the disease is more prevalent in developing countries as compared to the lower cases in many developed countries including the USA and UK. Sub-Saharan Africa carries the overwhelming share of the global burden (WHO, 2003a).

2.2 Pathogenesis

TB is a bacterial infectious disease caused by the obligate human pathogen, *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is primarily transmitted through the respiratory route via aerosol. Primary TB, the response following the first exposure to *M. tuberculosis*, usually develops in the terminal air spaces of the lung (alveoli). Transmission can also occur through other means such as the gastrointestinal tract; however pulmonary infection is the most common route (Rom, 1996). People with active TB infection, through coughing, talking or sneezing; generate aerosolized droplets containing infectious *M. tuberculosis* and if inhaled by an uninfected person, the viable bacilli lodge in the resting lung alveoli and an infection is established. It takes between 2-3 viable bacteria to establish an infection. People who are infected with TB but have not developed active disease are not infectious (Glickman and Jacobs, 2001). The infecting *M. tuberculosis* replicate at the initial site of infection and the bacteria then erode and escape into the interstitium resulting in spreading of the infection to local lymph nodes and eventually disseminating to remote sites in the body (Orme and Cooper, 1999). This promotes the influx of local macrophages and a series of immune responses. Not everyone who is infected with the tubercle bacillus develops symptomatic or clinically evident disease. Ninety percent (90%) of all infections remain latent, 5% of infected individuals progress rapidly to primary disease, and 5% of those who initially suppressed the infection later re-activate, developing acute disease sometime during their lifetime (Wigginton and Kirschner, 2001).

During latent infection, which can be maintained for the lifetime of the host, the infection is controlled by the immune response but is not eradicated. The only clinical evidence of infection is the delayed type hypersensitivity against mycobacterial antigens, demonstrated by a tuberculin skin test, a TB diagnosis technique. Hence there are cases where people harbour the infection and test positive for TB, but will not exhibit the symptoms of the disease. This usually happens in people who initially control the infection by mounting a strong immune response that prevent disease, but leave a residual population of viable mycobacteria. Reactivation of latent TB can be triggered by a host of events such as aging, malnutrition and largely by immuno-suppression due to diseases

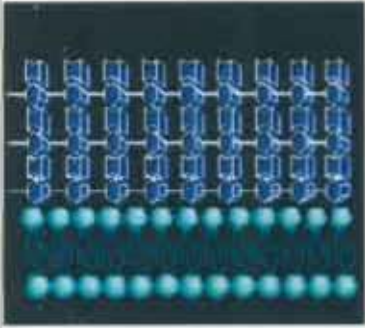
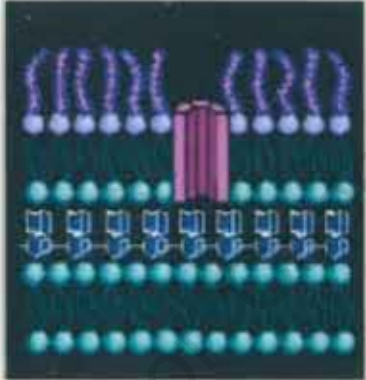
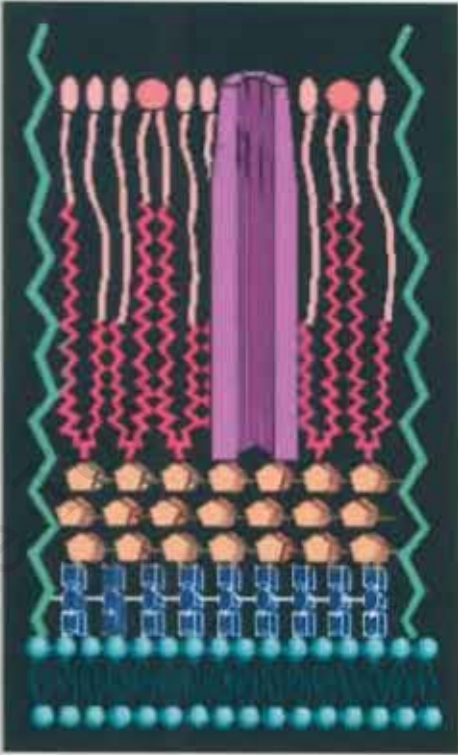
such as AIDS and infection with HIV among other factors (Flynn, 2004a; Stewart et al., 2003). More TB cases in people infected with HIV/AIDS create an increased risk of transmitting TB to the general community, hence elevating the TB incidence.

The course of the disease is heavily influenced by the immune response mounted against *M. tuberculosis*. Primary TB develops one to two years subsequent to initial infection. However, immuno-compromised people have a much higher risk of developing active TB soon following infection. Active TB disease is a chronic wasting illness characterized by fever, loss of appetite and weight loss, nighttime sweating, and constant tiredness. In the case of pulmonary reactivation, there is a persistent cough and often blood in sputum, which may be an indication that the infected portion of the lung has been damaged. This is also a symptom of pulmonary inflammation as it has been reported that tissue destruction and pathology is mediated by the host's immune response rather than the direct toxicity of the bacterium itself (Glickman and Jacobs, 2001).

2.3 Causative agent

M. tuberculosis is an obligate pathogen that can infect a wide variety of animals; however man is the principal host. Other human pathogens belonging to the mycobacterium genus include *Mycobacterium avium*, which causes TB-like disease mainly prevalent in AIDS patients, and *Mycobacterium leprae*, the causative agent of leprosy. *M. tuberculosis* is a fairly large (2-4µm and 0.2-0.5µm in length and width respectively), non-motile, acid-fast, rod-shaped bacillus. Mycobacterial species are classified as acid-fast bacteria due to their impermeability to certain dyes and stains. Acid-fast bacteria will only retain dyes when heated and treated with acidified organic compounds. *M. tuberculosis* is also a non-encapsulated and non-spore forming mycobacterium, although it belongs to the order of actinomycetes (Todar, 2002). It is also an obligate aerobe, which explains a classic case of TB where *M. tuberculosis* complexes are typically found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of alveolar macrophages, which paradoxically characteristically function to eliminate pathogens and other foreign material from the body. This pathogen has a very slow generation time (it divides every 15-28 hours), a physiological characteristic that may contribute to its

virulence; the immune system may not readily recognize its presence or may not be triggered sufficiently to eliminate them (Flynn and Chan, 2001a). In addition, the slow growth of this pathogen forms the basis for the chronic nature of the infection and disease, which complicates microbiological diagnosis and necessitates long-term drug treatment. This pathogen is shielded by a unique and complex cell wall, which possesses several distinctive structures, which may be major determinants of its virulence. The cell envelope differs substantially from the canonical cell wall structure of both gram negative and gram-positive bacteria (Fig.2). Despite such an elaborate array of bioactive chemical entities in the cell envelope, detailed understanding of the role of each of these molecules in *M. tuberculosis* pathogenesis has been limited due to lack of defined mutants defective in the synthesis of the individual molecules. The complexity of the cell wall contributes to the capacity of *M. tuberculosis* to survive in host cells, resist chemical injury and have low permeability to antibiotics (Huang, 2002). The cell wall consists of peptidoglycan, lipoarabinomannan (LAM) and a large hydrophobic layer of mycolic acids, which are unique alpha-branched lipids, and are thought to be a significant determinant of virulence in *M. tuberculosis* (Fig.2). The mycolic acids form a lipid shell around the mycobacterium and are thought to prevent attack of the mycobacterium by cationic proteins, lysozyme and oxygen and nitrogen radicals in the phagocytic granule (Orme, 1995). The cell wall therefore helps the microorganisms to survive the hostile environment within the macrophage by resisting oxidative damage.

Gram-positive organisms	Gram-negative organisms	Mycobacteria cell wall
A porous peptidoglycan layer covers the lipid bilayer cell membrane of most of the Gram-positive bacteria, which is permeable to most antimicrobial agents.	A double membrane encapsulates Gram-negative bacteria. The outer membrane functions as an efficient barrier as it contains lipopolysaccharides (LPS) and porins.	Mycobacteria are shielded by a thick mycolate-rich membrane, which functions as a very efficient barrier.
		
 lipid bilayer	 peptidoglycan	 lipid + LPS
	 porins	 MYCOLATE
		 acyl
		 lipids
		 LAM
		 arabinogalactan

Graphic © Linda M. Stannard, 1996 (Steyn, 1999) (<http://web.uct.ac.za/depts/mmi/steyn/cellwall.html>)

Fig.2 The complex cell wall of *M. tuberculosis* differs substantially to the gram positive and gram-negative bacterial cell walls. It is composed of unique structures, mycolic acids, arabinogalactan and glycolipids that make the cell envelope hydrophobic and impede entry of most antibiotics and polar chemotherapeutic agents and even lysosomal chemicals of phagosomes. It essentially protects the pathogen by acting as an efficient barrier and affords it resistance to most drugs. LAM, which is attached to the mycobacterial cell membrane through phosphatidylinositol moiety, has been directly implicated in the virulence of *M. tuberculosis*.

3. THE IMMUNOLOGY OF *M. tuberculosis*

The lung represents both the main port of entry and the important site of TB infection. There are several possible outcomes following the deposition of the *M. tuberculosis* bacilli in the lungs. The bacilli are ingested primarily by resident alveolar macrophages. Following the recognition of the pathogen by the host's cells, the unique pathogen-associated molecules such as peptidoglycan and mycolic acids, bind to the pattern recognition receptors and Toll-like receptors on the macrophage surfaces (Ernst, 1998). This presentation triggers effector mechanisms such as the production of cytokines, which in turn promote innate immune defences that include inflammation, phagocytosis and activation of the complement pathway. *M. tuberculosis* uses multiple cell surface receptors to gain entry into the macrophage including the mannose receptor, complement receptors and Fc receptors. Following phagocytosis, the bacilli are either i) killed, ii) survive by evading macrophage-killing mechanisms and replicate or iii) erode into the local endothelial cells and into surrounding vessels where they will eventually drain to the lymph node or into the blood to establish a new site of infection in another part of the lung (Orme, 1995). Alveolar macrophages are an ideal target for *M. tuberculosis*, but granulocyte and dendritic cells also represent the first line of defense against *M. tuberculosis* entering the lungs. During *M. tuberculosis* infection, there is activation of macrophages and dendritic cell populations to enhance anti-mycobacterial activity (Gonzalez-Juarrero et al., 2003), an antibody (humoral) mediated response does not aid in the control of TB infection because *M. tuberculosis* is an intracellular pathogen and its high lipid content cell wall (Fig.2) makes it resistant to complement killing.

Much less is known about the activation of anti-mycobacterial activity in human macrophages compared to the murine system. Most of the current knowledge regarding immune mechanisms involved in protection and pathogenesis in TB is derived from studies in murine models (Flynn and Chan, 2001a; Gomez and McKinney, 2004). When phagocytosed by macrophages, bacteria typically enter a specialized phagosome that undergoes progressive acidification (to pH 5.0-5.5) followed by fusion with lysosomes. Lysosomes are complex vacuolar organelles with vacuoles containing potent hydrolytic

enzymes capable of degrading ingested microbes. Hence fusion of the phagosome and lysosomes subject the ingested microorganisms to degradation by intralysosomal acidic hydrolases. In contrast, phagosomes containing viable tubercle bacilli lack fusion with lysosomes (Armstrong and Hart, 1975). The evasion of this mechanism allows *M. tuberculosis* to survive inside macrophages. Early studies have shown that *M. tuberculosis* products, sulfatides, might disrupt phagolysosome fusion (Goren et al., 1976). Activated macrophages are more effective at killing *M. tuberculosis* because they are more efficient at phagosome-lysosome fusion than resting macrophages, and also produce reactive oxygen intermediates (ROI), nitric oxide (NO), and other antimicrobial molecules (Chan et al., 1992; D'Arcy Hart, 1987).

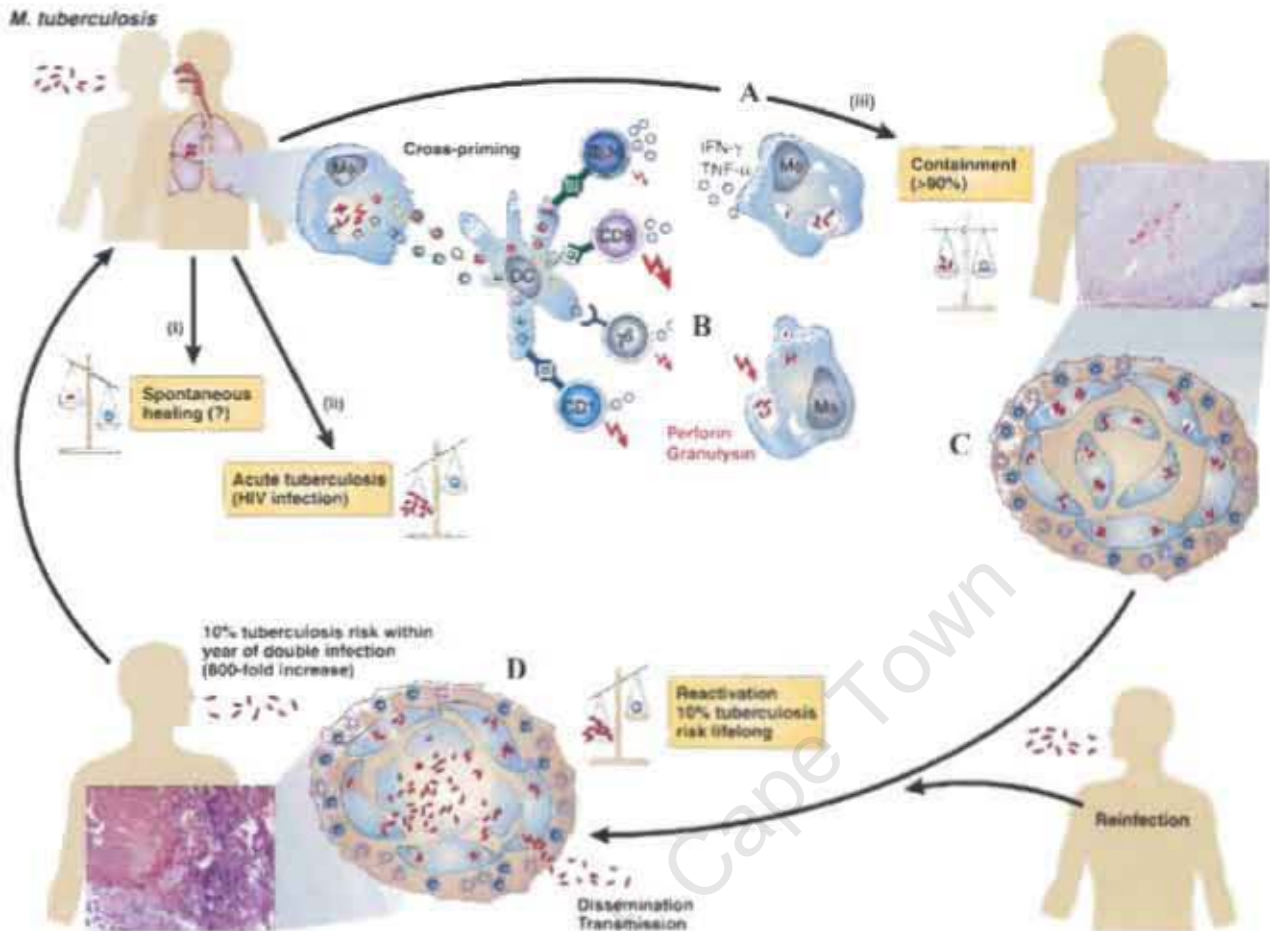
3.1 Granuloma formation

Within 15-20 days post the infection the bacilli will have eroded into the interstitium which causes damage to the tissue, and subsequently results in the production of vasoactive amines and prostaglandins. These chemicals increase the capillary permeability and promote the influx of local macrophages, and also allow the infection to spread. The macrophage influx is followed by activation of the innate immune defense. If the mycobacteria are not eradicated by the innate macrophage response within a short period (1-2 weeks) post-infection, the host will mount a T-cell mediated immune defense (adaptive response). Lymphocytes, macrophages and other cells of the immune system gradually accumulate to the site of infection (inflammation) in response to chemotactic signals generated by infected macrophages to form the beginnings of a granuloma (Eruslanov et al., 2004; Orme and Cooper, 1999). Infection of macrophages by *M. tuberculosis* induces the secretion of tumor necrosis factor- α (TNF- α) as well as other inflammatory cytokines. Dendritic cells are also infected and are a key cellular population at the site of infection because of their unique ability to transport and present pathogen-derived antigens via major histo-compatibility complex (MHC) class II molecules to naïve T cells in the secondary lymphoid organs where they are sensitized or primed (Fig.3). Sensitized CD4⁺ and CD8⁺ T cells secrete interferon (IFN)- γ , which synergizes with TNF- α to induce anti-mycobacterial effects of macrophages (Orme et al., 1993; Serbina and Flynn, 1999), however their main function is target cell killing (Flynn

(Flynn and Chan, 2001b; Kaufmann, 1999). IFN- γ is a key cytokine for control of infection, but it is not sufficient to mediate protection on its own. TNF- α acts on macrophages to induce chemokines (small molecular-mass chemotactic cytokines that mediate recruitment of leukocytes from the blood into tissues) and it is a key cytokine for granuloma formation (Bean et al., 1999). One of the most important effector mechanism responsible for the antimycobacterial activity of IFN- γ and TNF- α is induction of the production of nitric oxide and related reactive nitrogen intermediates (RNI) by macrophages via the action of the inducible form of nitric oxide synthase (iNOS) (Flynn and Chan, 2005). In addition CD8⁺ T cells can induce the death of infected macrophages (apoptosis) and the bacteria within them by producing perforin which forms pores and lyses infected cells and enabling granulysin to access and kill intracellular *M. tuberculosis* (Fig.3b) (Cooper et al., 1997a; Serbina et al., 2000).

Activated T lymphocytes and macrophages and other T helper-1 (Th1) lymphocytes that migrate to the site of infection subsequent to the cytokine/chemokine signals form major cellular components of a granuloma, which generally mediates control of *M. tuberculosis* numbers in the lungs. The formation of a granuloma is an attempt by the host to contain the infection by orchestrating communication between the immune cells and limiting bacterial replication and dissemination by physically containing the bacilli (Algood et al., 2003). The inflammatory cells also produce type 1 cytokine mediators such as IFN- γ and TNF- α which activate macrophages and enhance anti-mycobacterial activity. The tuberculous granuloma is usually found in the lungs and is a hallmark of *M. tuberculosis* infection, but it can be in any organ. The granuloma environment also leads to inhibition of growth or death of *M. tuberculosis* by enhancing macrophage activation and creating an oxygen and nutrient deprived situation (Voskuil et al., 2003). The *M. tuberculosis* is controlled in the lesions, but not completely eradicated, and a dynamic balance between mycobacterial persistence and host defense activation develops. This balance might be life-long and 10% of the infected individuals will develop clinical disease in their lifetime. *M. tuberculosis* possesses several mechanisms for evading elimination by the immune response, and on-going studies are investigating how *M. tuberculosis* survives the strong immune response (Flynn and Chan, 2003).

The granuloma is composed of a variety of cells, including macrophages, CD4 and CD8 T lymphocytes, multi-nucleated giant cells and B-lymphocytes (Gonzalez-Juarrero et al., 2001). In human granuloma lesions, the macrophages are located in the center with the lymphocytes surrounding and infiltrating the macrophage area. In murine models, the macrophages in the granuloma are arranged in sheets adjacent to loose aggregates of lymphocytes. Although they are arranged differently in humans and in mice, the granulomas still perform the same function of limiting bacterial growth and localizing the immune and inflammatory response to the site of infection (Algood et al., 2003). During the course of TB infection, severe tissue damage may occur as a consequence of the immune response. This may happen when the Th1-type cellular response is not well regulated. Naïve CD4 T cells that are first activated into a Th0 state which subsequently differentiate into either Th1 or Th2 cells depending on the cytokine and chemokine signals received. Th1 cells are potent IFN- γ producers, and IFN- γ is central in the activation of anti-mycobacterial activities of macrophages, hence crucial for protection against TB. The immunologic control of *M. tuberculosis* is based primarily on a type 1 T cell response (Wigginton and Kirschner, 2001).



(Kaufmann and McMichael, 2005)

Fig.3 *M. tuberculosis* transmission and pathogenesis: Droplets containing tubercle bacilli are inhaled and there are 3 possible outcomes: (i) Infection is not established, bacilli are eliminated, (ii) 5% of infected people, mainly immuno-compromised individuals, progress rapidly to acute primary disease, (iii) 90% of all infections are contained and the person becomes a carrier. During containment of the pathogen the host's immune responses are activated: bacilli lodge within alveoli macrophages and multiply. Some are killed by macrophage effector mechanisms (A) and later (B) the antigens are presented on the cell surfaces of APC such as dendritic cells, and a cell-mediated response is triggered. T-cells bind to the APC and set off a cascade of cytokine and lymphokine release. There is a dynamic balance of growth and killing of *M. tuberculosis* in the lung, cells are recruited to localize and contain the infection, forming a granuloma (C). In the case where a carrier is re-infected, there's a 10% chance of the infection progressing to disease, with the granuloma lesions disintegrating and disseminating the infection to other parts of the body (D). In this case the individual becomes highly infectious.

3.2 Essential cytokines

When the Th1 response is not well synchronized during chronic latent infection, the effector mechanisms of the robust immune response may result in severe pathology. It has been reported that reactivation of latent infection to active disease and sometimes even death is concomitant with tissue damage due to the immune response rather than the mycobacterium itself (Turner et al., 2002). It is therefore critical to balance the immune response by maintaining equilibrium in cellular activation and deactivation hence controlling cytokine production. IFN- γ , a Th1 cytokine produced by activated macrophages, CD4⁺ and CD8⁺ T-cells is central to the development of an effective cell-mediated response to *M. tuberculosis*. It activates resting macrophages and enhances their ability to effectively eliminate pathogens and produce cytokines (Cooper et al., 1993; Flynn et al., 1993). Interleukin (IL)-12, a Th1 cytokine produced by activated and infected macrophages and dendritic cells, is also a crucial cytokine in controlling *M. tuberculosis* infection (Cooper et al., 1997b). IL-12 regulates the on-going immune response primarily by inducing differentiation of Th0 cells to Th1 cells (Cooper et al., 1995; Manetti et al., 1993). It also promotes IFN- γ secretion by primed CD4⁺ T cells (O'Donnell et al., 1999). TNF- α influences and regulates the expression of chemokine receptors, chemokines and adhesion molecules but its principal function is promoting cell migration and effective granuloma formation (Mohan et al., 2001; Roach et al., 2002; Sedgwick et al., 2000). TNF- α has also been implicated as a major factor in host-mediated destruction of lung tissue; hence it plays a dual role mediating both protection and immunopathology (Rook et al., 1987). IL-10, in contrast to the above-mentioned inflammatory cytokines, deactivates macrophages and inhibits T cell proliferation, MHC class II expression, NO production and other anti-mycobacterial effects activated by IFN- γ , hence down-regulating the immune response to TB (Gazzinelli et al., 1992; Koppelman et al., 1997; Murray et al., 1997). IL-10 is primarily released by macrophages in response to *M. tuberculosis* infection. It is clear that although the Th1-type response plays an important role in control and immunity to *M. tuberculosis* infection, it must also be carefully moderated by IL-10 and other cytokines to prevent severe tissue damage and immunopathology. Other cytokines that are involved during *M. tuberculosis* infection control are lymphotoxin (Th1, involved in maintaining the structural integrity of the

granuloma) and IL-6, IL-4, IL-5 and Transforming Growth Factor (TGF)- β which are classic anti-inflammatory cytokines (Aggarwal, 2003; Bean et al., 1999; Kaufmann, 2001). The immune response to *M. tuberculosis* is evidently very complex and multifaceted, and it is important to elucidate the role of each immune component and understand how this pathogen evades elimination by the vigorous immune response in order to develop effective vaccine and therapeutic strategies.

4. EXPERIMENTAL ANIMAL MODELS

M. tuberculosis infection in humans has distinct phases of replication, dissemination, establishment and maintenance of latency and reactivation. There is no adequate animal model that can mimic this complex setting. The mouse is a useful model for experimental *M. tuberculosis* infection because considerable research has been done on it and its immune system is thoroughly known. The immune response to *M. tuberculosis* in the mouse has also been shown to have direct correlation with the human system (Capuano et al., 2003) although pathology of TB in mice and humans varies significantly (Orme, 2003). Murine models remain attractive because of the reproducibility of the infection, ease of housing in biosafety level 3 facilities, well characterized inbred and genetically altered mouse strains including knockout mice and the commercial availability of immunological reagents (Orme, 2004). Murine models of *M. tuberculosis* infection in knockout or transgenic mice have allowed the roles of some specific cytokines, chemokines and chemokine receptor molecules to be elucidated. Other animal models including guinea pigs, rabbits and even Zebrafish and frogs (Cosma et al., 2004) have contributed immensely to the understanding of TB infection, immune response and vaccine development.

Each model has its inherent advantages and disadvantages. Guinea pigs are more susceptible to *M. tuberculosis* than humans and succumb more readily to the pathological consequences of the infection (McMurray, 2001). However, the necrotic lesions formed in guinea pigs resemble the pathology that develops in humans (Smith et al., 1991). This model is therefore useful for studying both primary and post-primary granulomatous

lesions. Primate models offer the prospects of significant pre-clinical information as they display pathology that closely resemble human infection (Capuano et al., 2003; Walsh et al., 1996). However, cost, time frame and ethical considerations limit the widespread use of the non-human-primate models. The mouse model is used more frequently because it reproduces the basic phenomenon of an infection that is contained, but not eliminated by a natural immune response. Infection of the C57BL/6 mice, a strain relatively resistant to TB, generates a distinctive profile in the lung with an initial acute phase of logarithmic bacterial growth that triggers the host to develop protective immunity and a vigorous cell-mediated bacteriostatic response. The adaptive immune response increases granuloma formation and contains the bacterial load, maintaining a constant titer over many months during which the animal does not exhibit symptomatic disease (Rhoades et al., 1997).

This model is useful in studying the process of bacterial adaptation during persistent/chronic infection, rather than the latent phase observed in humans; and the role of different immunological mechanisms. Some of the shortcomings of the mouse model are that all mice eventually succumb to *M. tuberculosis* infection, as opposed to the 5-10% immuno-competent humans who contain the latent infection indefinitely. Mice also fail to develop caseous necrotic lesions, the precursors of the cavities characteristic of advanced TB in humans. They do develop granulomas however, but they differ in morphology to the human granulomas as they lack caseous cavities concomitant with human pathology (Rhoades et al., 1997; Stewart et al., 2003). The mouse model remains useful because despite the differences to the human infection, there are extensive similarities in terms of the basic immune response (control of chronic infection by immune response and presence of granulomas), and it also provides a cost-effective approach to the issue of screening new drugs and vaccines (Orme, 2003; Tufariello et al., 2003).

5. MANAGEMENT OF TB CONTROL

5.1 TB diagnosis

TB cases that remain undiagnosed contribute to ongoing transmission in communities (Pronyk et al., 2004). Traditionally diagnosis of TB involves detection of acid-fast bacilli in biological specimen. There are various diagnostic tools but microscopic diagnosis

using the Ziehl-Neelsen (ZN) stain procedure is commonly used especially in developing countries where resources are scarce. This stain indicates the presence of acid-fast bacteria, in this case mycobacterium in various clinical specimens such as sputum, biopsy sections of the lung or other organs as well as bronchoalveolar lavage (Zumla et al., 2000). Although rapid, the ZN stain only indicates the presence but not activity of the infection. Another type of diagnosis, the Tuberculin skin test, is used to identify patients with active TB. This involves intra-cutaneously injecting a partially purified derivative or extract of *M. tuberculosis* proteins (PPD) in the patient's forearm and the reaction is left for 48-72 hours. The test is subsequently read by measuring the resulting lesion, which is characterized by erythma (redness) and swelling. The redness and swelling are caused by an inflammatory response, a delayed- type- hypersensitivity (DTH) reaction, which is essentially an influx of macrophages and monocytes from the blood to the site of infection/injection (Adler, 2004; Todar, 2002). A positive tuberculin skin test only indicates presence of infection, and not activity of the infection. Infected individuals with advanced active disease and HIV infected individuals may produce negative test results due to inhibiting antibodies, lack of T- cell recruitment to the skin as they are mostly recruited in the lung lesions or low CD4 cell counts may lead to lack of reaction on the skin in HIV patients (MERCK). Radiography, in particular the traditional chest X-ray, is also used to diagnose TB. A typical chest X-ray pattern of a TB case may show classic upper lobe infiltrates, cavity lesions or fibrosis (Horne, 1996). In cases where multi-drug resistant TB is suspected, samples are taken for laboratory culturing, susceptibility testing and microbiological molecular testing such as the polymerase chain reaction (PCR) to verify and detect drug resistance (Drobniewski and Wilson, 1998). It is important to consider overall presentation, including symptoms and severity of the illness, so that suitable treatment is administered.

5.2 Overview of current TB regimen

5.2.1 Drug therapy

TB control mechanisms that are currently employed include a short course combination chemotherapy regimen involving at least four synthetic antimicrobial drugs. First line chemotherapy usually includes isoniazid, rifampicin, ethambutol and pyrazinamide

during the initial phase of treatment. The duration of treatment for newly diagnosed TB cases is 6 months and treatment for re-treatment cases should be continued for 9 months and includes the fifth drug streptomycin (a second line drug). A new drug, gatifloxacin, which is currently undergoing Phase III clinical trials, has been reported to shorten the treatment regimen from 6 to 4 months when included in place of ethambutol in the drug combination (WHO, 2005). The length of treatment is extensive and includes a combination of drugs to prevent the emergence of multi-drug resistant strains of *M. tuberculosis*. The drugs used kill a large proportion of the actively replicating *M. tuberculosis* (bactericidal), so the extended treatment period is needed to eliminate the persistent *M. tuberculosis* that evade killing. The multiple drugs work through different mechanisms and the lengthy treatment ensures that the disease is treated sufficiently to kill and sterilize the residual organisms within the granulomatous lesions.

i. Isoniazid

Isoniazid is in particular the most useful and least costly drug for TB. It was first synthesized in 1912 but its anti-TB properties were realised only in 1951 (Heym B., 1997). It is a bactericidal drug (kills rapidly growing mycobacteria) in that acts by inhibiting the enzymes involved in synthesis of mycobacterial cell wall components such as mycolic acids and unsaturated fatty acids (Mohamad et al., 2004; Takayama, 1979; Tsukamura and Tsukamura, 1963). Isoniazid requires oxidative activation by the mycobacterial catalase-peroxidase *katG* and the active form of the drug targets the long chain ACP-enoyl fatty acid reductase (Zhang et al., 1992). It is administered orally, is readily absorbed by cells in the body and is highly effective against large populations of extra cellular bacteria. Resistance to isoniazid is largely due to mutations on *katG* (Zhang et al., 1992). The primary side effects of isoniazid are hepatic toxicity and in some cases peripheral neuropathy (Adler, 2004).

ii. Rifampicin

Rifampicin is the second most important drug in the TB therapeutic regimen. It is active against a diverse population of mycobacteria. It is bactericidal and is easily absorbed into cells. It is especially valuable in killing dormant organisms residing within macrophage

or caseous lesions; hence it is central in the TB therapy regimen. Rifampicin inhibits mycobacterial growth by interfering with RNA polymerase; hence prevent mycobacteria from transcribing their DNA (Campbell et al., 2001). Mutants that are resistant to rifampicin have been found to have mutations within the beta sub-subunit of the RNA polymerase gene (*rpoB*) (Drobniewski and Wilson, 1998; Miller et al., 1994). The common side effects of rifampicin include fever or flu-like symptoms, jaundice and renal failure. It can also form unfavourable drug interactions if patients are on other causes of treatments (MERCK).

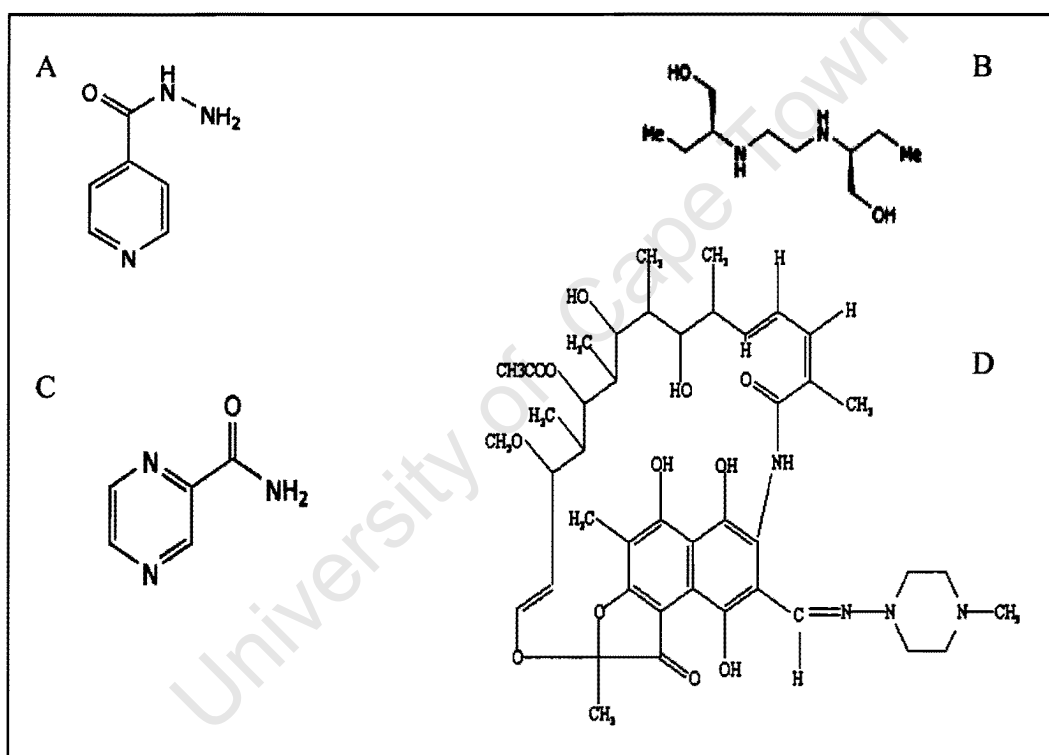
iii. Pyrazinamide

Pyrazinamide is included as a front line drug that is always used simultaneously with isoniazid and rifampicin. Its anti-TB activity was discovered in 1952 (Heym, 1997). It is a bactericidal, orally administered drug, which is used mainly to guard against treatment failure due to isoniazid resistance. Pyrazinamide needs to be activated through deamination by the mycobacterial hydrolytic enzyme (pyrazinamidase) to the active form pyrazinoic acid, which kills the growing populations of mycobacteria. Mutation in the gene encoding the activating enzyme (pyrazinamidase *pncA*), can lead to the mycobacteria being resistant to the drug (Scorpio and Zhang, 1996). The major side effect of pyrazinamide is that it increases uric acid levels in the blood (hyperuricemia), which induces gout and can also interfere with blood glucose management in diabetes patients (MERCK).

iv. Ethambutol

Ethambutol is delivered orally but unlike isoniazid, rifampicin and pyrazinamide, it is primarily a bacteriostatic drug. It has no effect on the viability of non-growing cells, only when administered at high doses (Jindani et al., 1980). Inclusion of ethambutol in the combination therefore ensures that mutants that have developed resistance towards bactericidal drugs are still susceptible to elimination by the drug combination. Ethambutol works in a similar manner as isoniazid, rifampicin and pyrazinamide by interfering with cell wall biosynthesis. The exact mode of action has not been clearly defined yet, but it is known that the drug directly inhibits arabinosyl transferase, an

enzyme that is involved in the polymerization of arabinose sub-units into the arabinogalactan branched structure (Belanger et al., 1996). This leads to decreased incorporation of mycolic acids into the cell envelope and therefore a mal-formed cell wall. Resistance to ethambutol may be due to mutations in the arabinosyl transferase gene, *embA*. (Belanger et al., 1996; Mikusova et al., 1995). Ethambutol toxicity may result in impairment of visual sharpness and problems in distinguishing colours (MERCK).



Graphics: (TAACF, 2003)

Fig. 4 Structural representation of the first front-line drugs of the current TB therapy regimen: a) Isoniazid, targets the long chain ACP-Enoyl fatty acid b) ethambutol, targets the synthesis of Arabinose, Arabinomannan and Lipoarabinomannan c) pyrazinamide, target unknown and d) rifampicin targets RNA polymerase (TAACF, 2003).

v. Fluoroquinolones

The outbreak of MDR-TB (especially resistance to isoniazid and rifampicin, the two most important frontline drugs) has prompted the incorporation of quinolones as second-line TB therapy. This group of compounds has a different target to the first line drugs; their principal target being a DNA gyrase (Heym, 1997). Various fluoroquinolones have demonstrated good activity against *M. tuberculosis in vitro* and have been recommended by many health authorities including the WHO (Bryskier and Lowther, 2002). Ofloxacin and ciprofloxacin have been used clinically, especially in cases where patients developed major side effects to the standard anti-TB drugs. These two drugs and an additional one, levofloxacin have been reported to be as effective as first-line therapy in TB and are being used as alternative or second-line TB drugs (Bryskier and Lowther, 2002; Kennedy, 1996; Tsukamura, 1985). Moxifloxacin has also been shown to be active against *M. tuberculosis in vitro* and *in vivo*, and is currently undergoing clinical trials (Ji, 1998; Miyazaki, 1999). Although they may offer an alternative regimen for managing MDR-TB, fluoroquinolones also need to be administered over a long period of time, give rise to intolerance (side-effects) and also drug resistant strains (Adler, 2004).

5.2.2 Dots

The current TB treatment regimen can be highly effective when followed as prescribed. However poor patient compliance often leads to treatment failure and contributes to the emergence of multi-drug resistant strains of *M. tuberculosis* (MDR-TB) (Petrini and Hoffner, 1999; Smith et al., 2004). The Directly Observed Treatment Short course (DOTS) strategy was developed by WHO to control the overwhelming TB epidemic by facilitating adherence to treatment and therefore maintaining the efficacy of the lengthy regimen. The basic elements of DOTS are that i) patients are to receive quality-assured TB diagnoses ii) access to safe and high-quality chemotherapy under proper case-management, iii) patients taking treatment are to be monitored directly for at least the first two months of the short course regimen, each patient's progress and outcome should be assessed, reported and recorded. In South Africa, where access to drugs is limited and the majority of the affected population is poor, TB treatment is given free of charge. This is in support of one of the DOTS elements that appeal to national governments to commit

to the programme either socially or financially and help make TB control a nation-wide activity and an integral part of the national health system (WHO, 2003a). The social, cultural, economic and poverty issues in poor and less developed countries affect factors such as access to care, diagnosis and delivery of care all of which lack thereof leads to high death rates. Hence TB control programmes such as DOTS are contributing significantly in reducing the burden of disease and infection in less developed countries especially in sub-Saharan Africa (Harries et al., 2001).

5.3 *Bacillus Calmette-Guérin (BCG) vaccine*

Vaccination is another strategy that could combat the TB disease. An effective TB vaccine has not yet being developed, but a vaccine that is still widely in use is BCG. BCG vaccine consists of a live attenuated *Mycobacterium bovis* strain. The efficacy of BCG is high against diseases such as TB meningitis (particularly in children), but it offers little protection (efficacy varies from 0-80%) against adult pulmonary TB (Flynn, 2004a, b). BCG-immunized people can be infected with *M. tuberculosis* and develop disease. Most of the world's population is routinely vaccinated with BCG at birth, followed by a boost revaccination during childhood. Some of the drawbacks of BCG vaccination are that i) it results in positive tuberculin skin tests, hence complicate the reading of the skin test (when treatment is successful in TB patients, they test negative for the skin test, however if they were previously immunized with BCG, the test can still come out as positive, it cannot discriminate between *M. tuberculosis* and BCG); ii) the vaccine does not prevent infection, only progression to disease and iii) BCG may be fatal if given to a person with active TB or immuno-compromised individuals (Flynn, 2004a). It is therefore evident that BCG is not adequately effective as a vaccine hence there is ongoing rigorous research that aims at developing a more effective vaccine against *M. tuberculosis*.

6. Conclusion

Tuberculosis is a complex disease that mandates innovative approaches to treat and control. TB infection can be controlled when treatment is followed thoroughly. However, there are several underlying factors that contribute to the persistence of the disease and even high morbidity rates globally. The emergence of HIV/AIDS has catapulted the TB pandemic into an urgent position (Corbett et al., 2003). A large proportion of people infected with HIV are highly susceptible to TB infection as their immune system is compromised and TB infection is inevitably fatal in immuno-suppressed patients unless a very effective course of therapy is developed. Patient non-compliance and failure to complete the standard short course therapy promote the proliferation of MDR-TB strains and this fuels the pandemic.

Drugs that are currently used in the short course regimen are bactericidal, that is they target the mycobacteria that are actively replicating. Most of the drugs target cell growth and cell division pathways (Gomez and McKinney, 2004). However, during the latent phase of TB infection, there are different populations of *M. tuberculosis*; a proportion of the population is dormant whilst others are in an unknown metabolic state (Wayne and Sohaskey, 2001). Hence some of the mycobacterial populations are recalcitrant to treatment with the current drugs. The striking variation in drug susceptibility of different mycobacteria populations therefore has profound implications for the treatment of TB. Drug interactions are also a predicament in cases where patients are receiving TB and HIV medication simultaneously (Grange et al., 1994). There is therefore a need to develop new anti-mycobacterial agents that will be able to overcome these challenges.

PART 2: TRADITIONAL MEDICINE

7. Introduction

Traditional medicine is described as ‘health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, supplied singularly or in combination to treat, diagnose and prevent illnesses or maintain wellbeing’ (WHO, 2003b). Most of the ingredients used in traditional medicine are prepared from medicinal plants. The relationship between man and plants has been exceptionally close throughout the development of human cultures. Historically, plants have served as drugs used as the result of accumulated knowledge and experience passed on from generation to generation. According to reports by the WHO, 80% of the population in Africa relies on traditional medicine for their everyday healthcare requirements (WHO, 2003b). Various strategies are being pursued to identify potential TB drug candidates that will act more rapidly than the existing short course regimen and investigating natural products and medicinal plants that have been previously used in traditional medicine as potential sources of new classes of therapeutic compounds is one strategy.

In Africa, the belief in traditional medicine and remedies remains firm despite urbanization and westernization. African traditional medicine is thought to be the oldest and possibly the most diverse of all medicine systems (Gurib-Fakim, 2006). The system comprises of both spiritual and physical healing in the form of the diviner (Isangoma) who consults with the spirits and psychological diagnosis to identify the source of the problem, and the herbalist (Inyanga) who prescribes herbal medicine to treat the symptoms (Bye and Dutton, 1991). Some of the information regarding the medicinal plants used in these herbal remedies has been recorded, but most herbal formulations still exist only as oral records. Preservation of traditional knowledge is fundamentally important as a range of medicinal plants that have been implicated as remedies for ailments, such as persistent coughs, fever, chest pains and other symptoms that resemble TB are now being evaluated as potential drugs or drug leads for TB. Furthermore it is

important to investigate and regulate the traditional medicine system and establish consistent and standardized formulations as traditional healers play an influential role in the lives of African people. Traditional medicine has the potential to provide an economical but fundamental component of a comprehensive health care infrastructure.

Drug discovery from medicinal plants has evolved to include many fields of inquiry. Since medicinal plants typically contain an array of chemical compounds such as alkaloids, flavonoids, lignans, fatty acids, polyphenols, triterpenoids and quinones (Cowan, 1999) that may act individually, additively or in synergy to improve health, most studies aim to standardize the herbal remedies and to isolate and characterize the pharmacologically active compounds from the medicinal plants (Balunas and Kinghorn, 2005). Medicinal plants can therefore play an important role as sources of new drugs, drug leads and new chemical compounds (Butler, 2004). The chemical substances derived from medicinal plants can play a role in the treatment of various diseases including cancer, AIDS, malaria and TB (Cowan, 1999; Newman et al., 2000). In developing countries traditional medicine and healers have a crucial contribution to make in building the health system and strengthening and supporting the national response to the devastating HIV epidemic and the closely associated TB epidemic.

7.1 Current state of traditional medicine in SA

Presently there are improved and concerted efforts from government, academic and research institutions around the country to accumulate the current and developing state of knowledge about the indigenous traditional medicines of South Africa. An institute for African traditional medicines has been set up in partnership with the WHO, MRC and CSIR to develop new remedies for chronic diseases and to safeguard indigenous knowledge (Kahn, 2003). The mutual plan is to establish a database of traditional medicines, set of standards that will define the medicine's therapeutic benefits, efficacy, identity, purity and toxicity and safety. Such developments will accelerate the incorporation of traditional medicine into the national health system.

7.2 The need for new TB treatment therapeutics

There are several factors that mandate new development of TB drugs including:

- The emergence of MDR-TB strains
- The need to shorten the duration of the current treatment regimen
- Minimize adverse drug interactions between anti-TB treatment agents and other drugs, especially in patients who are co-infected with HIV and are receiving TB and anti-retro viral therapy simultaneously
- Additional and more efficient drugs that will achieve complete sterility and eradication of the infection

7.3 Existing drugs derived from natural products

Literature indicates that natural products play an important role in the chemotherapy of various diseases. A large number of substances used in modern medicine for the treatment of serious diseases have originated from research on medicinal plants (Lall and Meyer, 1999). Examples of medicinal plant-derived drugs that have been recently introduced in the market are artemisinin, a potent anti-malarial drug derived from a compound isolated from a Chinese medicinal plant, *Artemisia annua* L (van Agtmael et al., 1999); galantamine, which is used for the treatment of Alzheimer's disease, was discovered from a drug lead isolated from the medicinal plant *Galanthus woronowii* Losinsk in Russia (Heinrich, 2004) and the anti-cancer agents Vinblastine and vincristine which were isolated from the *Catharanthus roseus* (L.) and have been in clinical application for more than 40 years for cancer treatment (van Der Heijden et al., 2004). Some of the antibiotics used in TB treatment today were also derived from natural products. Streptomycin, which is used as a second-line drug in the anti-TB regimen, was isolated from *Streptomyces griseus*, and several semi-synthetic derivatives have been prepared from it to serve as drug leads (Copp, 2003). A compound that has shown *in vitro* activity against *M. tuberculosis* (H37Rv) has also been isolated from a Rwandanese medicinal plant *Tetradenia riparia* (Van Puyvelde, 1994). Other medicinal plants that have shown anti-mycobacterial activity include *Hydrocotyle asiaticum* (Grange and Davey, 1990), a Chinese medicinal plant *Dipsacus asperoides* (Zhou, 1994) and *Salvia hypargeia*, from which a new anti-mycobacterial compound, hypargenin, was isolated

from the roots (Ulubelen et al., 1988). The plants that were investigated in our study were selected on the basis of their reported ethno botanical use in South African traditional medicine and on their availability (Hutchings A, 1996; Van Wyk B, 1997).

7.3.1 *Agapanthus praecox* [Agapanthae]

Agapanthus praecox, commonly known as the blue lily, is an evergreen plant that is endemic to Southern Africa. It is widespread in regions that receive high levels of rainfall, particularly the Eastern parts of Southern Africa (Van Wyk B, 1997). In vernacular, it is commonly known as isicathi (Xhosa) and ubani (Zulu) and traditionally has multiple functions. Xhosa women use it as antenatal medicine. In the Zulu culture, this plant is used to treat coughs, chest pain and tightness, colds and even heart disease. It is also combined with other plants in traditional medicine for use in pregnancy to induce labour (Hutchings A, 1996).



(Norten, 2004)

Fig.5 A) *Agapanthus praecox* has strap-like leathery leaves and an umbellate inflorescence on a stalk held above the leaves. We collected whole plant in January 2005 as seen on A. *Agapanthus* species can easily hybridize when grown in close proximity, hence plants growing in their natural habitat (wild) may differ to garden grown species. B). It flowers in summer (December-February).

Pharmacological studies have shown that the *Agapanthus* species contains several saponins and sapogenins that generally have anti-inflammatory, antitussive and immunoregulatory properties (Norten, 2004). The active compounds responsible for these properties are not known yet, however existing scientific evidence indicates that the compounds in *Agapanthus* have an effect on the activity uterus muscles (Kaido et al., 1997).

7.3.2 *Olea europaea* subsp. *Africana* [Oleaceae]

Olea europaea subspecies *Africana*, commonly referred to as wild olive, is an evergreen tree (Fig.6) that is widespread throughout Southern Africa and towards the North through to East Tropical Africa. This plant has been reported to be the most popular and important plant used in traditional medicine in Southern Africa (Dold T, 1999; Somova et al., 2003). The *O. europaea* species is widespread in the Mediterranean Islands, Arabia, India, Spain, Italy, and Greece and its use in folk medicine is well documented in most countries. In vernacular the African subspecies is known as umNquma (Xhosa), isandlulambazo (Zulu) and motlhwane (Setswana). Traditional remedies of this plant are usually teas or herbal infusions prepared by boiling the leaves. This medicinal plant is reportedly used as a diuretic, in lowering blood pressure and as a tonic for sore throat, urinary and bladder infections (Hutchings A, 1996).

There is limited published literature regarding the bioactivity of the South African population of the *O. europaea* subsp. *Africana*. However some information from studies of other *Olea europaea* collections from Europe is available. This plant contains oleanolic and ursolic acids and a range of compounds that are antibacterial, antioxidant, hypotensive, cytotoxic, and some reports have even suggested anti-HIV activity (Serra et al., 1994). *O. europaea* extracts have also been reported to exhibit anti-inflammatory activities by inhibiting TNF- α production (Bitler et al., 2005). Studies on most of the active principles have been extensive. However, limited studies have been done to evaluate the anti-TB activity of *O. europaea* subsp. *Africana* (Grange and Davey, 1990).

A



B



(Joffe, 2002)

Fig 6. A) *Olea europaea* subsp. *Africana* is a neatly shaped tree that ranges from 3-15m in height, and it has small grayish-green leaves. It flowers between October-March, and produces small black/dark purple fruits (B). The leaves are used for medicinal purposes, whilst the wood and fruit are used to make furniture and alcoholic drinks respectively.

7.3.3 *Syzigium cordatum* [Myrtaceae]

Syzigium cordatum is an evergreen, medium-sized tree (Fig.7) that is widely distributed in the Eastern and North-eastern parts of South Africa. Its common names are water berry, umdoni (Zulu and Xhosa) and montlho (Northern Sotho) and they all refer to its water loving nature. Culturally in South African traditional medicine, the plant is used to treat respiratory ailments, tuberculosis, stomach ache and diarrhea (Hutchings A, 1996). The remedies are usually prepared as decoctions using the leaves, bark and roots. In central Africa it is commonly used as a remedy for stomach complaints and diarrhoea (Van Wyk B, 1997).



(Carolus, 2004)

Fig.7 a) The *Syzigium cordatum* tree grows up to 15m in height and it has a rough dark brown bark. b) The broad bluish-green leaves are mostly used in medicinal preparations for various ailments including respiratory complaints. c) Flowers

The *S. cordatum* leaf extracts have been reported to contain compounds that could be effective in diabetes or glucose tolerance impairment (Musabayane et al., 2005); and they have also been investigated as potential anti-cancer agents (Verschaeve et al., 2004). Some of the active constituents of the plant have been identified, which include proanthocyanins, triterpenoids such as arjunolic acids and β -sitosterol that are found in the wood and bark of the tree (Candy HA., 1968.; Van Wyk B, 1997). The pharmacological action of the plant medicine is not known, and the mechanism pertaining to its anti-TB effects remains to be defined.

7.3.4 *Zanthoxylum capense*

Zanthoxylum capense is an indigenous citrus tree that is widely distributed in the Eastern and Northern parts of South Africa. It is small in size (grows up to 4-7m in height) (Fig.8a) and has small citrus scented leaflets. It is also known as knob wood or amabelentombi (Zulu term meaning “breasts of a woman”) due to its characteristic thorny bark (Fig.8b). In traditional medicine, the bark of this plant is known to be excellent for treating long standing chronic coughs and bronchitis (Van Wyk B, 1997). An infusion of

the leaves with other plants is used against colds, gastric and intestinal parasites (Hutchings A, 1996).



Fig.8 A) A *Z. capense* small, branched citrus tree growing at Kirstenbosch National Botanical gardens in Cape Town. The grey bark with thick characteristic thorns (B) is usually used in decoctions to treat bronchitis and TB.

Very little published literature is available on work done on this subspecies. There are two pharmacological studies that have done chemical analyses of the composition of *Z. capense*. Some biologically active compounds such as pellitorine, an insectidal alkalamide, were isolated (Steyn PS., 1998). It also contains sanguinarine, which has anti-inflammatory properties (Van Wyk B, 1997). Other *Zanthoxylum* species that have been investigated are reported to contain various biologically active compounds including sesamin, which is a major lignan in sesame seeds (Fish and Waterman, 1973). Studies with reference to the anti-mycobacterial potential of this plant were not presented.

8. AIMS AND OBJECTIVES

There is a wealth of undiscovered compounds in indigenous South African medicinal plants and this study aims to uncover and also investigate the anti-tuberculosis potential of the medicinal plant extracts used in traditional medicine by:

- Establishing *in vitro* screening bioassays to evaluate the efficacy of crude plant extracts against *M. tuberculosis* H37Rv using the Alamar Blue assay
- Evaluating the potential toxicity of the crude plant extracts against macrophages in culture
- Investigating the efficacy of the crude plant extracts against intracellular *M. tuberculosis* H37Rv *in vitro*
- Establishing an *in vivo* anti-TB screening model using wildtype C57BL/6 mice
- Studying the effects of plant extracts on immuno-histopathology
- Studying the effects of plant extracts on immune response effector mechanisms (NO production by mouse peritoneal macrophages)
- Obtaining and analyzing basic pharmacological profiles of crude plant extracts using High Pressure Liquid Chromatography (HPLC)

9. MATERIALS AND METHODS

9.1 Plant material

Table1 Bioassay Index

Plant Botanical Name	Part used (Days extracted)	Solvent used for extraction	Biological screening evaluated			
			<i>In vitro</i> Anti-TB activity (MIC)	<i>In vitro</i> cell toxicity	<i>In vitro</i> INOS expression	<i>In vivo</i> Anti-TB activity
<i>Sizygium Cordatum</i>	Leaves (5 days)	Hexane	+	+		
		Methanol	+	+		
		Ethyl acetate	+	+		
		Dichloromethane	+	+		
		Water	+	+	+	+
<i>Zanthoxylum capense</i>	Leaves (12 days)	Hexane	+	+		
		Methanol	+	+		+
		Ethyl acetate	+	+		
		Dichloromethane	+	+	+	
	Twigs (8 days)	Water	+	+		
		Methanol	+			
<i>Olea europea subsp. Africana</i>	Leaves (4 days)	Hexane	+	+		
		Ethyl acetate	+	+		+
		Dichloromethane	+	+		
		Methanol	+	+		+
<i>Agapanthus praecox</i>	Rhizome And roots (4 days)	Hexane				
		Ethyl acetate	+	+		
		Dichloromethane	+	+		
		Methanol	+	+		
		Acetone	+	+		

Table 1. Plant parts from the 4 South African medicinal plants were ground and extracted using the indicated solvents for a specific period of time. Crude extracts obtained from the extractions were filtered and dried to obtain dry crude herbal preparations. The extracts were subsequently evaluated for cytotoxic and anti-mycobacterial activity *in vitro* and *in vivo*. This table indicates the plant extracts that were obtained and tested in our study. + indicates the extracts that were tested.

The plants that were investigated in our study were selected on the basis of their reported use in South African traditional medicine and on their availability (Hutchings A., 1996; Van Wyk B., 1997). All plant material was identified and collected from the Kirstenbosch National Botanical Institute in Cape Town, Western Cape Province, South Africa. *S. cordatum* and *Z. capense* leaves and branches (twigs/bark of *Z. capense*) were collected during the summer season (March, 2004); whereas *O. europaea* subsp. *Africana* and *A. praecox* material was collected in January 2005 (See Fig.5- 8, Results chapter). Branches (twigs) and rhizomes were rinsed with water to wash off soil and to prevent potential fungal/microbial contamination. The thick fleshy rhizome tubers of *A. praecox* were cut up into small pieces to accelerate the drying process. The plant material was air-dried for 3-8 weeks at room temperature (25°C) and subsequently ground using a laboratory blender (Waring Commercial, New Hartford, USA) to obtain coarse powdered material.

9.2 Extraction of crude plant extracts

Between 10-20g of finely ground plant material was weighed into conical flasks and extracted with 100-200ml of Millipore water, acetone, dichloromethane, ethyl acetate, hexane or methanol. All organic solvents used for extractions were obtained from Saarchem, MERCK, South Africa. The extractions were shaken continuously at room temperature (~25°C) for 4-10 days. The extractions were subsequently filtered through a sterile funnel fitted with a Whatman filter paper (125mm filter disc, Schleicher & Schuell) and solvents were removed under reduced pressure using a rotary evaporator (BÜCHI Rotavapor R-205, Germany) to concentrate filtrates. The remaining concentrates were subsequently air-dried under a fume hood to remove excess solvent and obtain dry crude extracts. Dried extracts were weighed to determine extract recovery and yield using the following calculation:

$$\text{Percentage yield (\%)} = \frac{\text{mass of recovered crude extract}}{\text{mass of starting plant material}} \times 100$$

The crude extracts were stored in sealed vials in the dark at room temperature (~25°C) to prevent photo-induced degradation until used.

9.2.1 Preparation of extracts for High Pressure Liquid Chromatography analysis (HPLC)

1 mg of each crude extract was dissolved in 500µl of acetonitrile (BDH, HiPerSolv for HPLC). The solution was sonicated (UMC5, Krugersdorp, SA) until completely dissolved and subsequently reconstituted to obtain final 1mg/ml stock samples in 50% acetonitrile (v/v) through addition of Millipore water. Aqueous extracts were re-dissolved in Millipore water instead of 50% acetonitrile. Samples were used immediately for HPLC analysis or stored at 4°C until used. The stock extract solutions were centrifuged at 10 000 rpm (Eppendorf, Centrifuge 5415, Netherlands) for five minutes to exclude any non-solubilized material prior to injecting the sample into the column for analysis. All extracts were analyzed at 1mg/ml.

9.2.2 HPLC protocol

HPLC analyses were conducted using a Shimadzu LC-10 system equipped with an auto sample injector, a LC-10AS pump, Solenoid valve, SPD-M10A diode array detector with a data processor. A Sulpeco flow C18 column (15cm x 4.6mm, 5µm) and a guard column from Discovery® were used in all analyses. Crude extracts were analyzed by injecting a volume of 100µl of sample into the column. An equal volume (100µl) of sample was applied to the column for all extract analysis. The extracts were separated on a linear gradient of water: acetonitrile (10-100%) mobile phase at a flow rate of 1ml/min for 60 minutes. Compounds were detected at a UV wavelength range between 200nm and 300nm. The 210nm spectra were used/captured for analyses.

9.2.3 Preparation of drugs and plant extracts for *in vitro* and *in vivo* biological assays

Defined amounts of crude extracts were dissolved in 10µl of dimethyl sulfoxide (DMSO, BDH AnalaR, England) and vortexed until completely solubilised. 990µl of distilled water (dH₂O) was subsequently added to make up to a stock extract solution in 1% DMSO (v/v). The stock solutions were passed through a 0.45µm filter and working

solutions were prepared accordingly for use in the minimum inhibitory concentration (MIC), cytotoxicity and *in vivo* biological assays. The stock extract solutions were subsequently diluted in Middlebrook 7H9 broth for the Alamar Blue assay, RPMI supplemented (complete) medium for macrophage assays and sterile water for *in vivo* drug treatment. Fresh extract stocks were prepared and used each time for all the experiments. Isoniazid and rifampicin were obtained from Sigma. Stock solutions of isoniazid and rifampicin were prepared in distilled water at 1mg/ml in 1ml aliquots. Rifampicin was prepared in 1% DMSO (v/v) by initially dissolving the drug in 100µl DMSO, vortexing until completely dissolved and making up the volume with 9.9ml dH₂O to a final volume of 10ml. Drugs were stored in 1ml aliquots at -70°C until used for 6 weeks.

9.3 Preparation of mycobacterial culture

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5% glycerol and 10% OADC enrichment medium (BBL, Middlebrook). Cultures were incubated at 37°C with CO₂ supplementation and were grown until mid-log phase. 1ml aliquots were then aseptically prepared from the mid-log phase cultures and were frozen and stored in 2ml screw cap vials (Greiner bio-one, Cryos Cellstar) at -70°C until used. Viable *M. tuberculosis* cell counts (CFU/ml) of the frozen stocks were determined by plating serial dilutions of the stock cultures on Middlebrook 7H10 agar plates containing 10% OADC. The plates were then incubated at 37°C for 3 weeks. Colonies were subsequently enumerated and viable mycobacterium concentration calculated and determined for specific stock batches.

9.3.1 Preparation of inoculum

To prepare well-dispersed clump-free suspensions of *M. tuberculosis* H37Rv for infections, frozen stocks were thawed at room temperature and briefly vortexed to re-suspend. To ensure proper dispersion of mycobacteria, the suspension was drawn 30 times through a 29Gx1/2" 1ml syringe (Omnican B/Braun). The suspended stock culture was subsequently diluted in sterile 0.9% saline solution for *in vivo* infections,

Middlebrook 7H9 broth for anti-mycobacterial screening or RPMI complete medium for *in vitro* macrophage assays to obtain the required inoculum concentration.

9.4 Mice

All experiments in this study were performed using mice bred and maintained under specific pathogen-free (SPF) conditions in the animal housing facility at the University of Cape Town (UCT). Only female C57BL/6 strains were used and all mice were between 6-8 weeks old at the beginning of each experiment. The mice were housed in filter-top cages, and water and food were provided *ad libitum*. Infected mice were housed in the biosafety level 3 facility, whilst pathogen-free mice used for harvesting macrophages were housed in the biosafety level 2 facility (UCT, Cape Town, South Africa).

9.5 Preparation of murine peritoneal macrophages

Macrophages were harvested from 6-8 week old C57BL/6 wild type female mice. 1ml of 3% Brewer thioglycollate (Difco/BBL) was injected intraperitoneally into mice as a stimulant to elicit macrophage recruitment to the peritoneum. The macrophages were collected by peritoneal lavage 3 days after the injection using a 19G needle attached to a 10 ml syringe (Coligan JE., 2001) with 10ml of RPMI 1640 complete medium (Sigma) supplemented with 5% FCS, 2mM L-glutamine and 10% L929 medium. The peritoneal macrophage lavage was subsequently dispensed in a 50ml sterilin tube and kept on ice. The cell number was determined using the trypan-blue exclusion technique. 20µl of the lavage was mixed with 20µl trypan blue dye, and 20µl mixture was counted using a light microscope and a haemocytometer (Clay Adams, Parsippany N.J) to enumerate viable cells. The remainder of the lavage was centrifuged at 1000 rpm, 4°C for 10 minutes. The cell pellet was re-suspended in RPMI complete medium. The cell number was adjusted to have 1×10^6 cells/well for each experiment.

9.6 Drug susceptibility testing of *Mycobacterium tuberculosis*

9.6.1 *In vitro* anti-mycobacterial activity screening (MIC determination) using the Microplate Alamar Blue Assay

To determine the antimycobacterial activity of drugs and plant extracts, the microplate Alamar Blue assay as previously described by Franzblau (Franzblau et al., 1998) was performed with minor modifications. The Microplate Alamar Blue Assay is a colorimetric drug-susceptibility testing method that uses an oxidation-reduction reaction indicator (dye) that changes colour from blue to pink to assess cell proliferation. The blue colour of the reagent is the oxidized form of the dye (resazurin*), whereas the pink colour is the reduced product (resofurin). Viable/ metabolically active cells are able to reduce the dye from blue to pink.

The assay was performed in flat-bottomed 96-well plates (Nunc, Nunc Brand Products, Denmark). The outer perimeter wells were filled with sterile water to prevent evaporation of media in experimental wells. 100 µl of 7H9 broth (Difco, preparation according to manufacturer) was dispensed in the wells in rows B to G in columns 3 to 11. Test drug/plant extract solutions were prepared in 7H9 broth from stocks to obtain 2× highest drug concentrations being tested (16 µg/ml for isoniazid and rifampicin; and 2 mg/ml for all plant extracts). 100 µl of 2× drug concentrations were added to the wells in rows B to G in columns 2 and 3. The contents of wells in column 3 were mixed well and 100 µl transferred to column 4. Identical serial 2-fold dilutions were continued through to the last column containing drugs using a multi-channel pipette (Gilson) and the last 100 µl was discarded. 100 µl of *M. tuberculosis* (H37Rv) inoculum (2×10^5 CFU/ml) was added to all wells excluding those that served as sterility control (contained medium and plant extract only); hence that yielded a total volume of 200 µl per well.

* Resazurin is the original name for Alamar Blue (O'Brien et al., 2000)

Stock plant extracts were initially solubilised in DMSO, hence 0.25% DMSO (v/v) prepared in broth served as solvent control to assess any inhibitory effects of the solvent. Wells containing broth and inoculum served as growth controls (drug-free negative control) and when testing extracts, isoniazid and rifampicin were used as positive controls for growth inhibition. Wells containing broth and plant extract only (no inoculum) were included as a control to monitor possible interference of the extracts with the assay. Plates were sealed with parafilm and aluminum foil to prevent photo-induced reduction of the Alamar Blue reagent™, and incubated at 37°C for five days. Subsequently, 20µl of Alamar Blue reagent™ (Biosource, USA) was added to one well containing inoculum only (growth control) and re-incubated overnight. The well was monitored until the reagent added changed from blue to pink, which indicates mycobacterium growth. If the test well turned pink, 20µl Alamar Blue reagent™ was added to all experimental wells and plates were covered and re-incubated at 37°C. The colours of all wells were monitored and recorded daily for 3 days after all growth control wells had turned pink.

The results (MIC) were interpreted visually by recording blue wells as inhibition of growth and pink wells as mycobacterial growth. Plates with violet/purple wells were re-incubated for a further 24 hours or were recorded as growth 3 days post addition of the reagent. The MIC was defined as the lowest drug/ plant extract concentration that prevented a colour change from blue to pink. Visuals were captured using a digital camera (Canon, Power shot A95).

9.6.2 Macrophage cytotoxicity screening (MTT assay)

Macrophages were plated at 2×10^5 cells/well in flat-bottomed 96-well tissue culture plates (Corning, Costar 3548, NY, USA). Plates were incubated with RPMI complete medium at 37°C overnight to allow cells to adhere to the plate surface. Adherent macrophages were subsequently washed once with 1×phosphate buffered saline (PBS) before incubation with drugs or plant extracts. 200µl RPMI complete medium containing isoniazid, rifampicin and the different plant extracts was added to the macrophage monolayer to determine their cytotoxic potential.

Isoniazid and rifampicin were tested at the following concentrations: 0, 25, 50, 75 and 100µg/ml. A 2-fold serial dilution of DMSO (5%-0%) was tested to determine a non-toxic DMSO concentration to dissolve the plant extracts in. 2-fold serial dilutions of plant extracts ranging from 500µg/ml to 0µg/ml were prepared in complete medium in test tubes. 0.5% DMSO in complete medium was used as solvent control. Cells incubated with drug-free and extract-free medium served as macrophage proliferation control. Each test sample was set up in triplicate wells.

The MTT reagent was purchased from Sigma. A stock solution of 5mg/ml was prepared in 1×PBS and kept in the dark at 4°C. The plates were subsequently incubated with drugs or plant extracts for 8 days at 37°C with 5% carbon dioxide (CO₂) (Forma Scientific water jacketed incubator, model 3121, Ohio). After incubation with drugs/ plant extracts, the medium was removed from the macrophage monolayers. The macrophage monolayers were subsequently incubated with 100µl freshly prepared 2mg/ml MTT solution in 1×PBS for 4 hours at 37°C with 5% CO₂. Subsequently, 100µl of sterile DMSO was added to each well after aspiration of the MTT solution, and the plates were gently shaken to dissolve the converted dye, formazan, which is formed by metabolically viable cells. The formazan solution was then transferred into a new 96-well microplate and absorbances were read immediately thereafter at a wavelength of 570nm with background subtraction at 630nm on a microplate reader (VersaMax, Sunnyvale, CA94089, USA). DMSO was used as a blank when reading the absorbances.

The MTT cell proliferation assay is based on the reduction of the tetrazolium salts (yellow in colour) by metabolically active cells to water-insoluble purple formazan crystals. The amount of formazan product formed is proportional to the metabolic activity and the number of cells present in the sample (Sieuwerts et al., 1995). In our study, the number of viable cells after treatment with drugs and plant extracts was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance}_{\text{reduced test sample}}}{\text{Absorbance}_{\text{reduced untreated control}}} \times 100$$

$$\text{Absorbance}_{570\text{nm}} - \text{Absorbance}_{630\text{nm}} = \text{Absorbance}_{\text{reduced}}$$

9.6.3 Macrophage infection with *M. tuberculosis* and drug treatment of macrophage- ingested mycobacteria

1ml of 1×10^6 cells/ml peritoneal macrophages were plated in 24 or 48-well tissue culture plates in RPMI complete for 24 hours (overnight). The complete medium was removed from the adherent cell monolayers and replaced with 1ml RPMI complete medium containing H37Rv at 1×10^6 CFU/ml concentration. After overnight incubation the plates were washed twice with $1 \times$ PBS to remove the extracellular bacteria that was not phagocytosed. The H37Rv infected adherent macrophages were incubated in complete medium containing isoniazid, rifampicin and plant extract at the indicated concentrations for 8 days. Controls of macrophages incubated with antibiotic-free medium (untreated) and uninfected cells were included. At the end of the incubation period with drugs/plant extracts, the medium was removed and the cells were lysed with 500 μ l of 0.1% saponin (v/v) (CAT 21435 USB, Cleveland, Ohio) dissolved in RPMI complete medium. Lysates were serially diluted (1:10 dilution series) in saline-Tween 80 (0.04%) and 100 μ l was plated on Middlebrook 7H10 agar plates. The plates were subsequently incubated for 3-4 weeks at 37°C, after which colonies of *M. tuberculosis* were enumerated. The results were reported as colony forming units (CFU).

9.7 Macrophage iNOS staining

Peritoneal macrophage cultures were incubated with plant extract-containing RPMI complete medium at 37°C with CO₂ in 8-well Teflon coated slides (Highveld Biological, SA). 0.1 μ g/ml LPS (Sigma Chemical co, St Louis, USA) in RPMI complete medium was used as a positive control for macrophage activation. After incubation period (72 hours), the medium was aspirated off and the cell layer was air dried under sterile conditions. The cells were then fixed and stained for iNOS using the following procedure:

1. Rehydrate slides from Xylol through to water
2. Block the slides using H₂O₂ in methanol for 15 minutes
3. Rinse in water
4. Antigen retrieval in 0.1M Citrate buffer pH 6. Pressure cooker for 2 minutes

5. Rinse in water-cool down for 10 minutes
6. Block with 5% Goat serum for 10 minutes
7. Coat with INOS antibody (1:1000) for 45 minutes
8. Wash with PBS Tween
9. Coat with Envion Rabbit Monoclonal antibody for 30 minutes
10. Wash with PBS Tween
11. DAB Substrate 10 min
12. Rinse in water
13. 1% CuSO₄ – 2 minutes
14. rinse with water for 2 minutes
15. Stain with Haematoxylin for 4 minutes
16. Rinse in water for 5 minutes
17. Dehydrate water to Xylol
18. Coverslip using Entellan

10. *In vivo* anti-mycobacterial screening

10.1 Aerosol infection

Mice were infected by a low dose aerosol in an inhalation exposure system (Glas-Col Inhalation Exposure System Model A4224) in the animal level-3 biosafety facility, UCT. The inoculum was prepared in a total volume of 6ml in 0.9% saline solution containing the well-dispersed *M. tuberculosis* H37Rv stock at a final concentration of 2×10^6 CFU/ml. This concentration allows infection of approximately 100 CFU/lung per mouse. 10-fold serial dilutions of the inoculum were prepared and plated on Middlebrook 7H10 agar plates to confirm the inoculum concentration.

5ml of the inoculum was transferred to the Nebuliser-Venturi unit using a 5ml syringe attached to a 15G' needle, and the instrument was set to operate the aerosol infection procedure with the following parameters:

1. Preheating cycle 900 seconds

2. Nebulising time	2400 seconds
3. Cloud decay time	2400 seconds
4. UV Decontamination	900 seconds
5. Main air flow	60 cubic feet/hour
6. Compressed air flow	10 cubic feet/hour

10.2 Treatment regimen

After infection, the mice were replaced in their cages in their respective treatment groups. Negative control mice did not receive treatment for the duration of the experiment. Treatment was started 25 days post-infection and drugs were administered by oral gavage for 15 consecutive days. Positive control mice were treated with isoniazid (25mg/kg) whilst all plant extracts, *Olea europaea* subsp. *Africana* ethyl acetate and methanol extracts and *Zanthoxylum capense* methanol extract, were administered at 125mg/kg dosage. All drugs/extracts were delivered in a total volume of 200µl using a 1ml syringe (Omnican B/Braun, Switzerland). Treatment efficacy was assessed by first killing 6 mice 1 day post-infection to determine the initial infection dose. 4 untreated mice were killed on day 25 before starting treatment as pre-treatment controls. 4 mice from each treatment group including the untreated control were then killed 7 days after treatment (Day 33) and then finally the treatment was concluded on day 40 where the last groups of mice were killed to enumerate CFUs in the organs and study organ pathology. Mouse body weights were taken at each time point.

Acute infection: Treatment regimen schematic

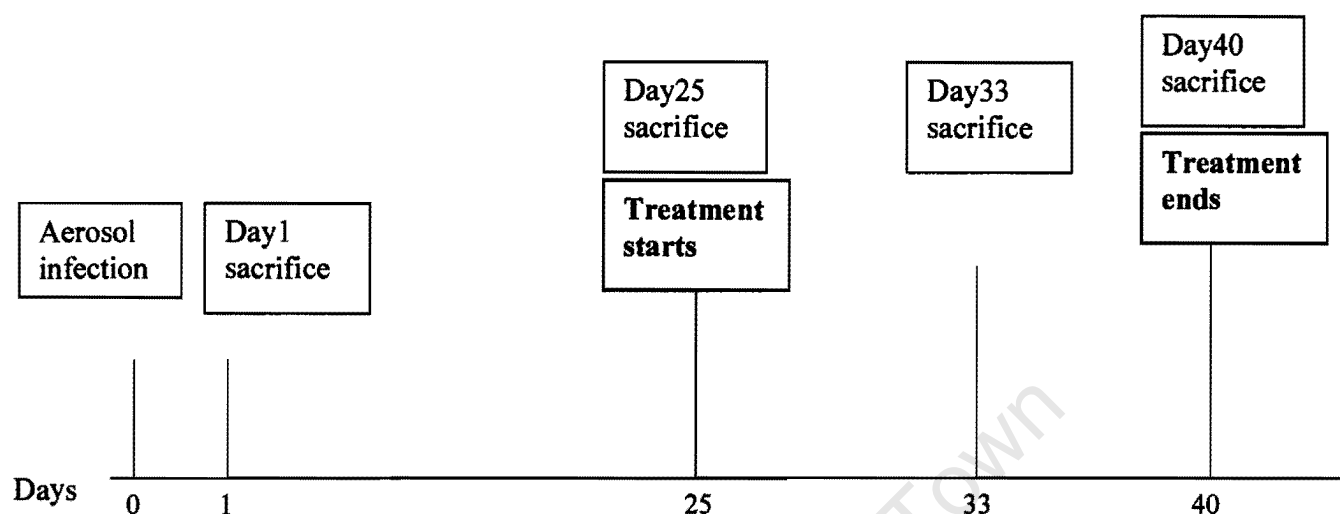


Fig.9 A schematic diagram of the in vivo treatment regimen. 6 mice were sacrifice on day 1, and 4 mice were treated and sacrifice per treatment group/ time point thereafter for the duration of the treatment.

10.3 Organ CFU determinations

Mice were sacrificed by CO₂ and organs (lungs, liver and spleen) were aseptically removed and weighed. The left lobe of the lung and a section of the liver were removed and immersed in 20ml of formalin for histopathological analysis. The remaining lung, liver and whole spleen were weighed and subsequently homogenized in 2ml of 0.9% saline Tween 80 (0.04%) using a sterile Perspex mortar and pestle drill (Black and Decker KD574CRE). 10-fold serial dilutions of the organ tissue homogenates were then plated in duplicates on Middlebrook 7H10 agar plates supplemented with 10% OADC. Plates were semi-sealed in plastic bags and incubated at 37°C with CO₂ for 3 weeks before the colonies were counted. Mean CFU counts were calculated and graphs of *M. tuberculosis* burden in the organs were plotted using the Prism Graph-pad program.

10.4 Histology analysis

10.4.1 Haemotoxylin and Eosin Staining

Organ tissues from all treatment regimens and time points were rehydrated according to the following procedure:

- | | |
|---------------------|---------------|
| 1. Xyol | 3 minutes |
| 2. Xyol | 1 minute (2x) |
| 3. Absolute alcohol | 1 minute (2x) |
| 4. 96% alcohol | 1 minute (2x) |
| 5. 70% | 1 minute |
| 6. Water | 1 minute |

Tissue sections were subsequently stained with Haemotoxylin for 8 minutes and then rinsed with water. Stained sections were then fixed in 1% acid alcohol for 10 seconds and rinsed under running water for 30 minutes. The tissue sections were then counterstained with 1% eosin for 2 minutes then rinsed with water. The stained sections were dehydrated using 70%, 96% alcohol and xyol and then mounted using Canada Balsam.

10.5 Visual imaging Software

The slides of stained tissue sections were analyzed, and images were captured using a Nikon DXM 1200 digital still camera attached to a Nikon Eclipse E400 microscope. The images were subsequently edited using the ACT-1 software application program.

11. Statistical analysis

In vitro drug screens were performed in triplicates, whilst *in vivo* CFU counts were in quadruplicates. The final results are expressed as the mean ($\pm$ standard deviation). The group means were compared using the Student's *t* test and the Dunnett's Multiple Comparison test. *p* values less than 0.05 ($p < 0.05$) were considered significant.

12. Appendix A: Reagents

1. 10×PBS (NaCl, KH₂PO₄, KCl, Na₂HPO₄·2H₂O, pH7.4) stock solution
 - Dissolve 80g NaCl, 2.4g KH₂PO₄, 2g KCl and 14.4g Na₂HPO₄·2H₂O in 900ml distilled water. Adjust pH to 7.4 and make up to 1000ml using distilled water. Autoclave at 121°C for 30 minutes. Store at room temperature. To make working 1×PBS solutions, make 1:10 dilution.
2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) stock
 - Dissolve 50mg of MTT salts (powder) in 10ml 1×PBS solution. Filter sterilize through 0.45µm Millipore filter and store in a bottle covered with aluminium foil to prevent light. Store at 4°C. Prepare working MTT solution by making up a 2mg/ml final concentration solution in 1×PBS and use immediately.
3. 0.9% Saline solution
 - Dissolve 9g NaCl in 900ml distilled water. Adjust volume to 1000ml. Autoclave at 121°C for 10 minutes. Store at room temperature.
4. 0.9% Saline- Tween 80 (0.04%)
 - Add 0.4ml Tween 80 to 900ml distilled water to make 0.04% Tween 80 solution. Dissolve 9g NaCl to the solution. Adjust volume to 1000ml and autoclave at 121°C for 10 minutes. Store at room temperature.
5. Middlebrook 7H10 agar
 - Add 5ml glycerol to 900ml distilled water and dissolve, then add 19mg of Middlebrook 7H10 agar and autoclave at 121°C for 10 minutes. Allow medium to cool down to 55°C and add 100ml of oleic acid albumin catalase (OADC). 7ml of the media was poured in sterile duplicate agar plates per side. Plates were kept at 4°C after they solidified until used.

6. Middlebrook 7H9 broth (Difco)
 - Add 2ml glycerol to 900ml distilled water and dissolve, then add 4.7mg of Middlebrook 7H9 broth and autoclave at 121°C for 10 minutes. Allow broth to cool down to 45°C and add 100ml of oleic acid albumin catalase (OADC). Store at 4°C.
7. RPMI complete medium supplemented for macrophage culturing
 - 84.4ml RPMI 1640 (pH 7.3), 5 ml heat-inactivated Fetal Calf Serum (FCS) (5%), 0.6ml L-glutamine (2mM), 10ml L929 conditioned supernatant (10%). Filter sterilize and store at 4°C.
8. 3% Brewer's thioglycollate
 - Add 0.3g thioglycollate to 10ml distilled water. Heat with frequent agitation for 1 minute. Autoclave at 121°C for 15 minutes. Store at 4°C.
9. LPS
 - Dissolve 1mg of LPS from *Escherichia coli* (*E.coli*) 0127:B8 in 1ml distilled water.
10. Formalin (fixative for histology tissues)
 - Add 10ml formaldehyde (40%w/v formaldehyde solution) to 900ml PBS (pH 7.4). Store in a dark bottle at room temperature.
11. Mayers Haematoxylin stain
 - Dissolve the following reagents in the specified order: 1g Haematoxylin in 800ml distilled water and dissolve. Add 50g Aluminium ammonium sulphate, 0.2g sodium iodate, 1g citric acid and 50g chloral hydrate. Make up to 1000ml, filter through Whatmann filter paper no.1 and store in a dark place at room temperature.
12. Eosin
 - Add 150ml 1% Eosin solution to 75ml 1% Phloxine solution. Add 450ml distilled water, filter through Whatmann filter paper no.1 and store at room temperature.

RESULTS

13. Chemical evaluation of plant extracts

13.1 Plant extractions [Quantitative analyses]

In this study defined amounts of plant material were extracted using the specified solvents to determine and quantify the crude extract yields. Plants contain an array of compounds with different moieties and the solvents used for extractions have different chemical properties, hence will extract varying amounts of compounds from the plant material. The organic solvents used included hexane, methanol, ethyl acetate, dichloromethane, acetone and water. The crude extracts recovered from *A. praecox*, *O. europaea* subsp. *Africana*, *S. cordatum* and *Z. capense* plant parts using these solvents were of variable formulations and quality. Most extractions yielded dry solid, crystalline or powder-like crude extracts with the exception of the *Z. capense* methanol (ZC met), ethyl acetate (ZC EtAc), aqueous (ZC aq) and dichloromethane (ZC dcm) extracts, which were recovered as sticky gel-like products.

The solvents used for extractions have different chemical properties and polarities; thus will dissolve different compounds and chemical entities at varying quantities from the plant material being extracted. The relative polarities of the solvents used are as follows and specified according to increasing polarity: hexane (0.009), ethyl acetate (0.228), dichloromethane (0.309), acetone (0.355), and methanol (0.762) with water being the most polar solvent at 1.00 relative polarity (Reichardt, 1988).

The quantity of recovered material varied according to the plant and solvent used. Methanol and water extractions from all plants yielded relatively high amounts of crude extracts. *S. cordatum* leaves extracted with methanol yielded the highest extract (25.7%), and the second highest extract recovered was the water extract of *S. cordatum* leaves yielding 16.2% plant material (Table 2). Ethyl acetate and dichloromethane recovered comparatively low crude extracts, and hexane yielded the lowest amounts of crude extracts in most of the extractions (Table 2). The most polar solvents (water and methanol), therefore yielded large quantities of crude extracts from the plant material and the percentage recovery decreased with the decreasing solvent polarity.

Table 2. Plant extract recovery

Plant Botanical name	Plant part used	Solvent used for extraction	Starting plant material (g)	Crude extract recovered (g)	Percentage recovery (%)
<i>Syzigium cordatum</i>	Leaves	Hexane	10	0.14	1.4
		Methanol	10	2.57	25.7
		Ethyl acetate	10	0.36	3.6
		Dichloromethane	10	0.42	4.2
		Water	10	1.62	16.2
<i>Zanthoxylum capense</i>	Leaves	Hexane	10	0.33	3.3
		Methanol	10	1.21	12.1
		Ethyl acetate	10	0.44	4.4
		Dichloromethane	10	0.27	2.7
	Twigs (branches)	Water	10	0.62	6.2
		Methanol	10	1.23	12.3
<i>Olea europaea</i> subsp. <i>Africana</i>	Leaves	Hexane	10.6	0.97	9.15
		Ethyl acetate	13.11	0.67	5.11
		Dichloromethane	15.91	0.56	3.52
		Methanol	14.72	0.54	3.67
<i>Agapanthus praecox</i>	Rhizome and roots	Hexane	11.29	0.08	0.71
		Ethyl acetate	12.23	0.11	0.9
		Dichloromethane	16	0.17	1.06
		Methanol	15	0.47	3.13
		Acetone	11.87	0.51	4.29

Table 2. Various plant parts were extracted with organic solvents to recover crude plant extract. Crude extract recovered, represented as a percentage (%), from the plant material was quantified by weighing the starting plant material and the final plant material after extractions.

13.2 Chemical [Qualitative] analysis of crude plant extracts using High Pressure Liquid Chromatography (HPLC)

This study was undertaken to determine and compare the nature of compounds present in the crude plant extracts extracted using different solvents. Furthermore, this study compared the quantities of the compounds present in the different crude plant extracts. Crude plant extracts were separated using HPLC through a hydrophobic (C18) column down an acetonitrile and water concentration gradient (10-100%) over a period of 60 minutes. The relative polarity of acetonitrile is 0.46 (Reichardt, 1988). The constituents of the crude plant extracts were eluted from the column down a concentration gradient of 10-100% acetonitrile; hence the most polar compounds were eluted first, whilst the most non-polar (hydrophobic) constituents were retained longer in the column, and eluted later during the separation.

A. praecox crude rhizome extracts

Analyses of the acetone, dichloromethane, ethyl acetate and methanol crude rhizome extracts of *A. praecox* showed a similar pattern of compound elution in the chromatograph when detecting at a wavelength 210nm. However, the methanol extract (Aga met) spectrum displayed smaller peaks, which suggests that it contained significantly lower concentrations of the chemical constituents compared to the other *A. praecox* extracts (Fig.10d). Analyses of all 4 extracts revealed four major peaks that appeared at comparable times in all 4 analyses/profiles namely, 24 minutes and 3 clustered peaks at 27 minutes (Fig10a-d). Essentially the Aga ace, Aga dcm, Aga EtAc and Aga met eluted the same or very similar constituents which were present at different concentrations in the respective extracts (See Table 3). The *A. praecox* extracts contained relatively polar compounds, as most compounds were eluted with the mostly polar mobile phase early during analysis, and very little to none compounds were eluted when the mobile phase became non-polar towards the end of the run. Acetone, dichloromethane, ethyl acetate and methanol therefore extracted similar constituents from the *A. praecox* rhizome, but in varied amounts.

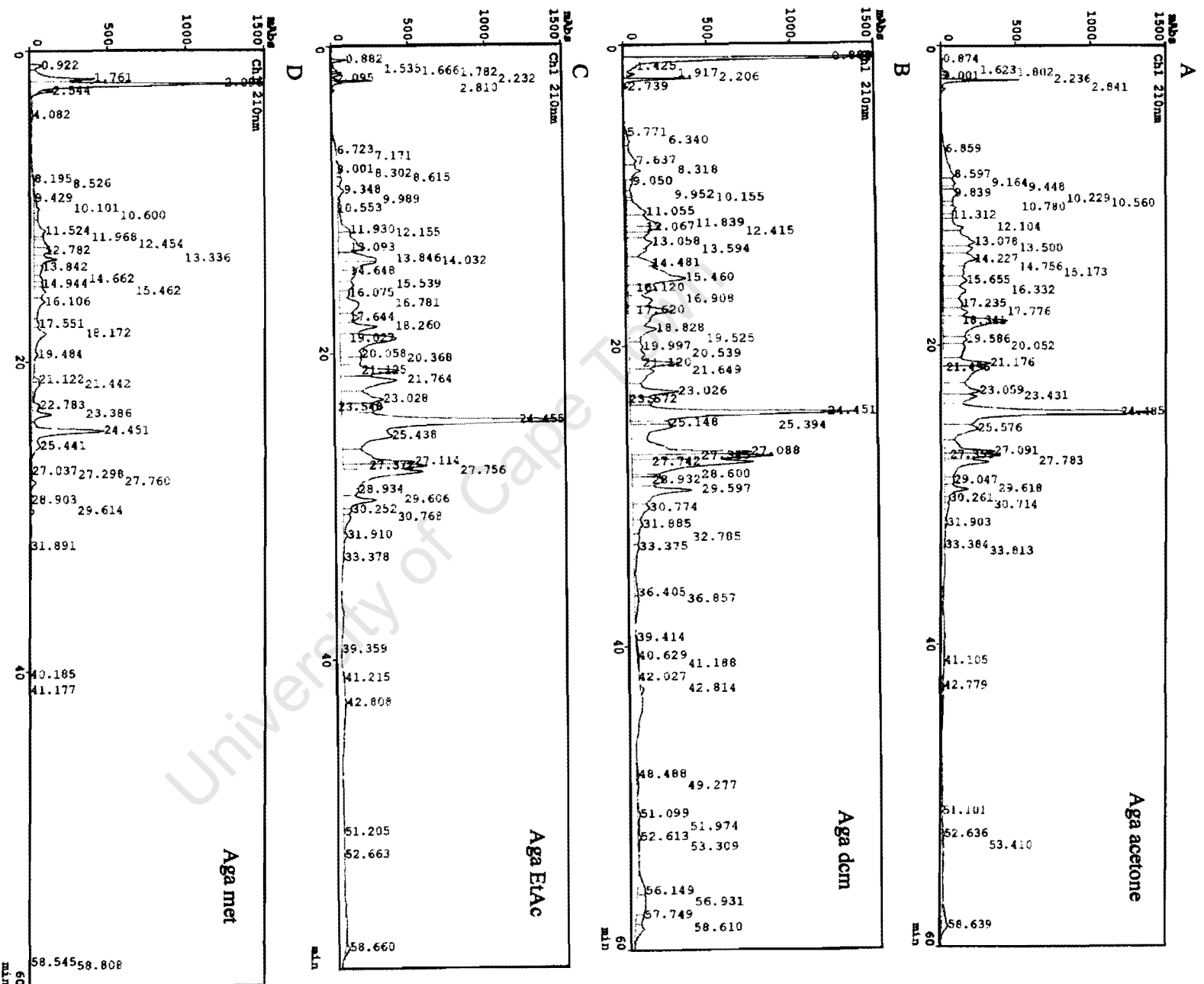


Fig. 10 *A. praecox* crude rhizome a) acetone, b) dichloromethane, c) ethyl acetate, d) methanol extracts separated using HPLC according to the constituents' hydrophobicity. 100µl of 1mg/ml sample was applied to column in all analyses. Hydrophilic compounds were eluted first, whilst hydrophobic compounds were retained longer in the column using a 10-100% gradient acetonitrile mobile phase.

Table 3.Retention times and concentrations of peaks in *A. praecox* extracts HPLC analyses

Plant extract	Peak number	Retention time (min)	Concentration in crude extract (%)
Aga acetone	36	18.34	8.33
	47	24.48	17.85
	49	27.09	3.9
	50	27.35	2.7
	51	27.78	4.14
Aga dcm	24	15.46	5.94
	44	24.45	11.45
	48	27.08	6.03
	49	27.34	5.04
	50	27.74	8.11
Aga EtAc	37	19.02	6.55
	47	24.45	18.77
	50	27.11	5.66
	51	27.37	3.96
	52	27.75	7.81
Aga met	27	18.17	4.06
	34	24.45	13.32
	36	27.03	0.10
	37	27.29	0.10
	38	27.76	0.80

Table 3. The Peak (compound) elution profiles and concentrations of *A. praecox* crude rhizome extracts obtained from the HPLC analyses (Fig.10).

***O. europaea* subsp. *Africana* crude leaf extracts**

The *O. europaea* subsp. *Africana* dichloromethane, ethyl acetate, hexane and methanol crude leaf extracts displayed similar chromatograph spectra when detecting at a wavelength of 210nm. All four extracts eluted four major compounds at 13, 15, 42 and 46 minutes but at varying concentrations (Fig.11, Table 4). The ethyl acetate extract (Olea EtAc) and the methanol extract (Olea met) spectra both had a significant amount of a compound eluted at 15.7 minutes, which was barely detected in the other extracts (Fig.11b, d). Most major peaks appeared early in the first 20 minutes indicating the hydrophilic nature of the compounds, with only a few compounds being eluted at later time points suggesting the *O. europaea* leaves contain very few hydrophobic compounds. The elution pattern was qualitatively consistent in most extract analyses, with the exception of the hexane extract (Fig.11c) that contained polar compounds at very low concentrations and higher amounts of hydrophobic compounds. The methanol and ethyl acetate extracts show a very comparable elution profile, the composition of these extracts may be similar. The *O. europaea* crude leaf and *A. praecox* crude rhizome extracts are all simple extracts as they do not contain a large assortment of compounds. The plant extracts contain a few major constituents at high concentrations. The methanol extract spectrum was also consistent with published *O. europaea* subsp. *Africana* methanol extract spectra (Fig.11i) (MRC).

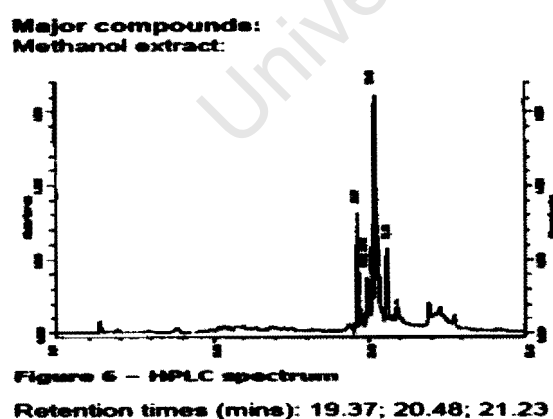


Fig.11i) HPLC spectrum of *O. europaea* subsp. *Africana* methanol extract separated on a C18 column (www.plantzafrica.com/medmonographs/oleaeuropafri.pdf). This spectrum compares to the peaks with retention times: 13.65, 15.00, and 15.72 in our analysis (Fig.11d)

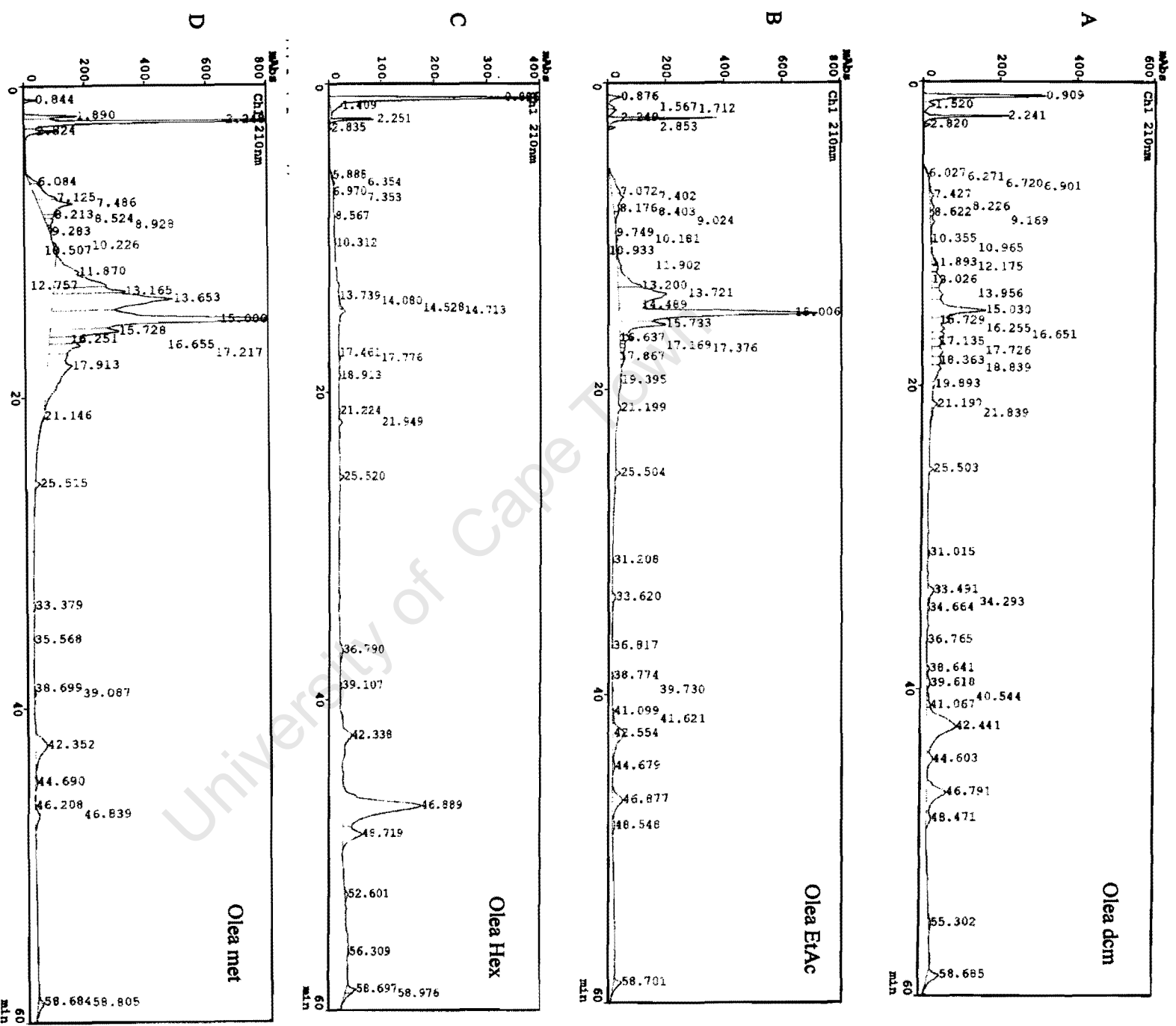


Fig.11 HPLC profiles obtained by analyses of crude extracts of *O. europaea* subsp. *Africana* leaves a) dichloromethane, b) ethyl acetate, c) hexane and d) methanol extracts on a C18 column. 100µl of 1mg/ml samples were applied to column and a mobile phase of acetonitrile (10-100% gradient) was used to elute compounds.

Table 4. Retention times and concentrations of peaks in *O. europaea* extracts

Plant extract	Peak number	Retention time (min)	Concentration in crude extract (%)
Olea dcm	19	13.02	3.96
	21	15.03	12.87
	42	42.44	17.10
	44	46.79	7.69
Olea EtAc	16	13.72	16.16
	18	15.00	32.47
	19	15.73	8.29
	35	42.55	5.08
	37	46.87	3.6
Olea Hex	11	13.73	0.051
	23	42.33	2.92
	24	46.88	46.31
	25	48.71	8.45
Olea met	23	12.75	5.68
	26	13.65	17.44
	27	15.00	24.99
	28	15.72	5.24
	33	17.91	6.15
	41	42.35	2.27
	44	46.83	0.64

Table 4. The Peak (compound) elution profiles and concentrations of *O. europaea* subsp. *Africana* crude leaf extracts obtained from the HPLC analyses. Extracts were separated on a C18 column using a 10-100% gradient acetonitrile mobile phase for 60 minutes (Fig.11).

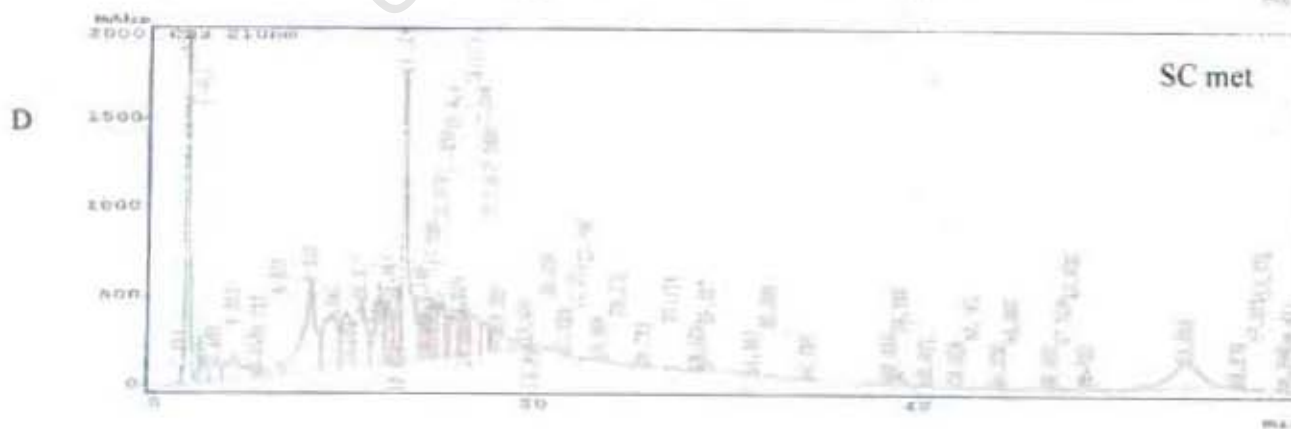
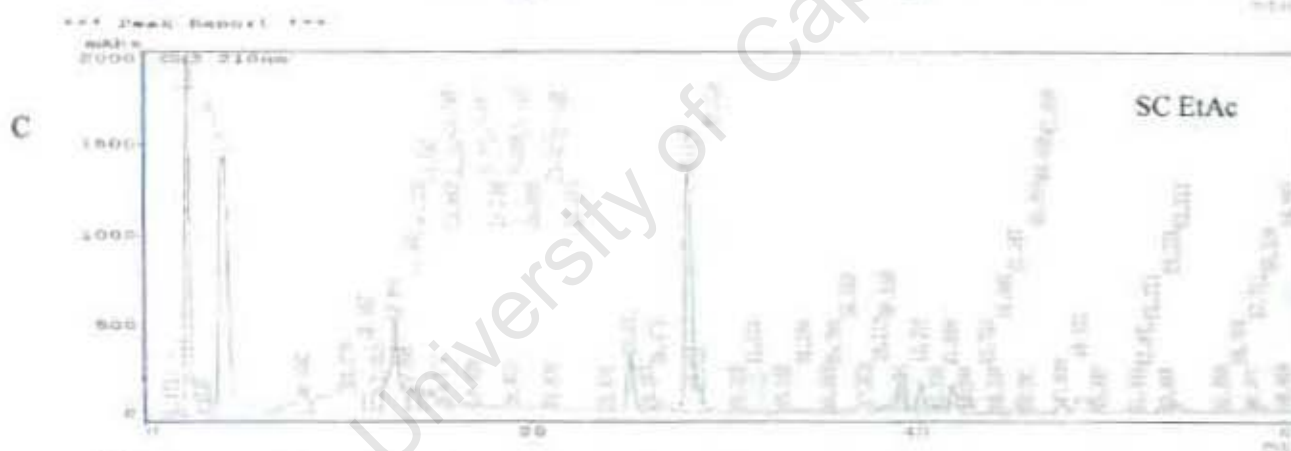
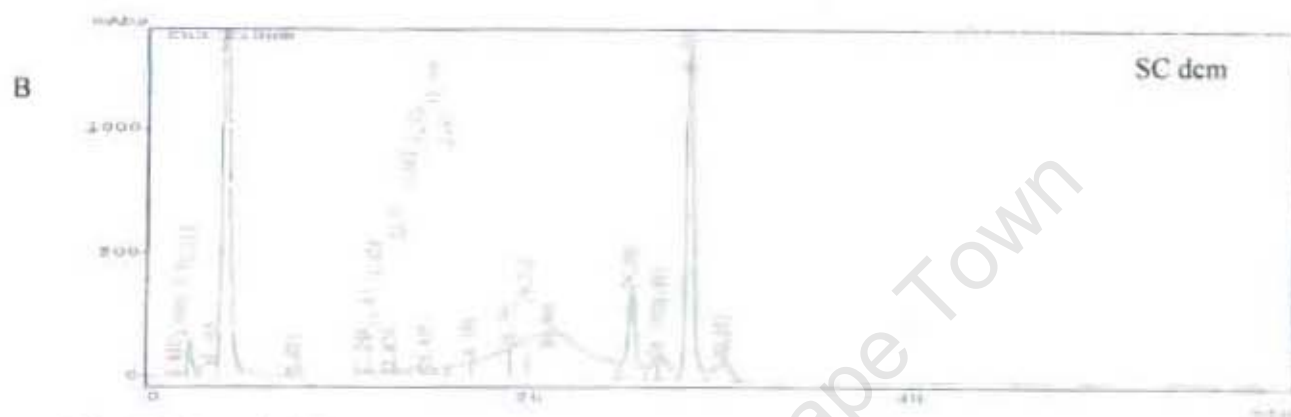
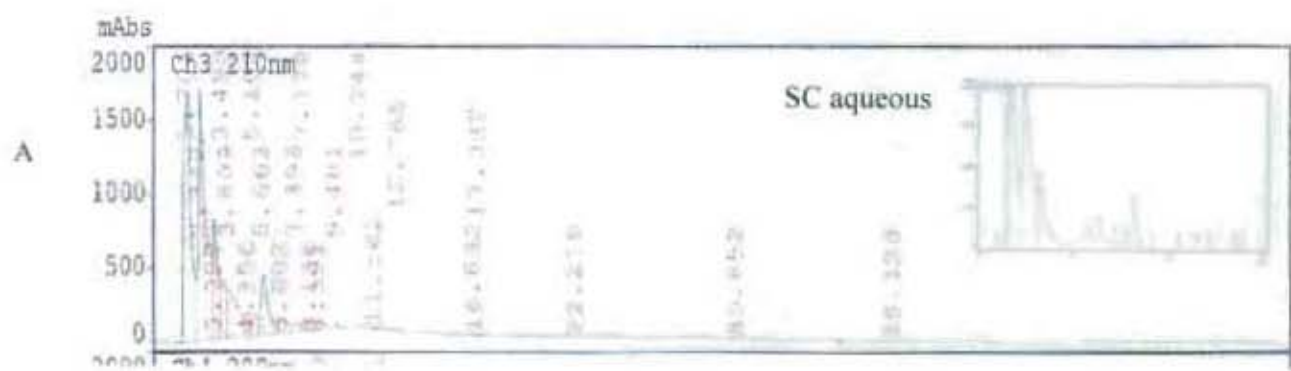
***S. cordatum* crude leaf extracts**

The *S. cordatum* leaf aqueous extract (Scaq) chromatograph revealed 4 major peaks eluted prior to 20 minutes and very few compounds were eluted thereafter (Fig.12a). A repeat analysis of the same extract was performed over a gradient of 20-50% acetonitrile for 15 minutes to separate and simplify visualization of the peaks, and the equivalent 4 compounds were eluted at 1.66, 2.4, 3.2 and 8.08 minutes (Fig.12a insert). This suggests that the aqueous extract contains exceptionally polar compounds and very few non-polar constituents.

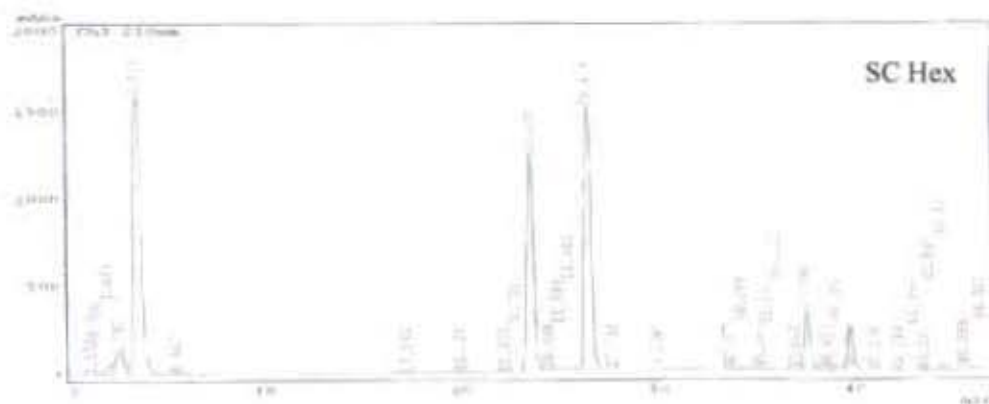
The methanol extract (SC met) was eluted rapidly. A complex combination of compounds was eluted within 20 minutes with major peaks appearing at 8.335, 11.9, 12.83 and a sharp characteristic peak noted at 13.148 minutes (Fig.12d). There were other peaks that were present in relatively significant concentrations eluted between 10-16 minutes of the run (Fig.12d)

Some of the major peaks that appeared on the chromatographs of the dichloromethane (SC dcm), ethyl acetate (SC EtAc) and hexane (SC hex) analyses were common to all three extracts. SC dcm eluted sharp characteristic peaks at 25.26, 26.70, a major peak at 28.358 and the final peak at 30.2 minutes (Fig.12b). No compounds were eluted after 30 minutes.

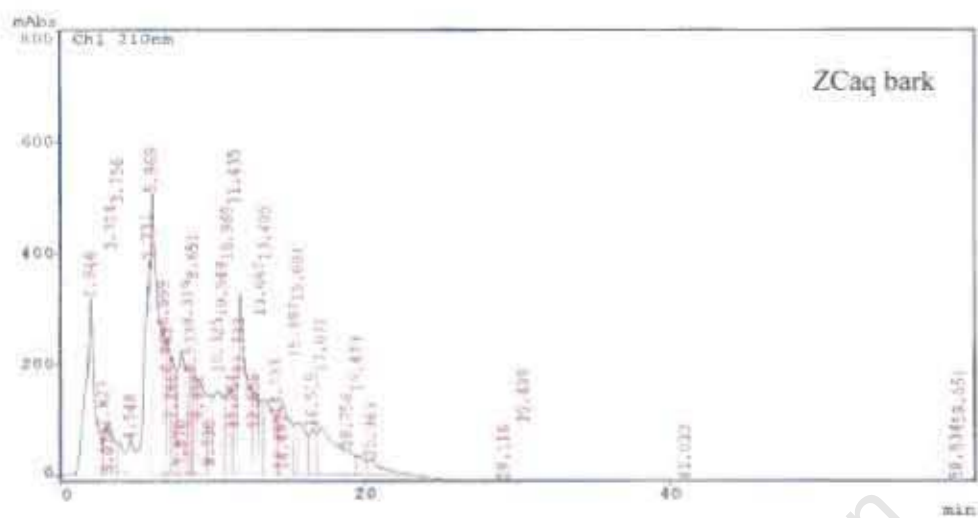
SC hex extract compounds were retained within the column for a longer period of time suggesting that they are fairly hydrophobic. Two major elution peaks were observed at 23.55 and 26.478 minutes respectively. Two further peaks were eluted after 37.59 and 39.78 minutes at lower concentrations (Fig.12e). Additional compounds were detected in the SC EtAc extract compared to the other *S. cordatum* extracts suggesting that ethyl acetate extracted more constituents from the leaves than the other solvents. Major peaks were observed at 12.8, 25.03, a sharp characteristic peak at 28.14 minutes and a cluster of less concentrated but well separated compounds at 39.19, 40.21 and 41.85 minutes (Fig.12c). The major peaks that appeared at 26 and 28 minutes were common in the ethyl acetate, hexane and dichloromethane extracts.



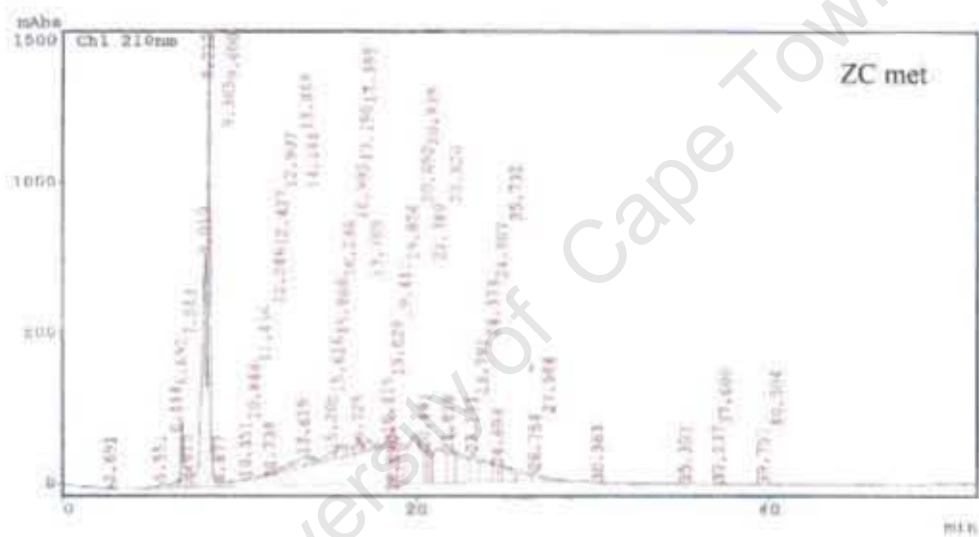
E



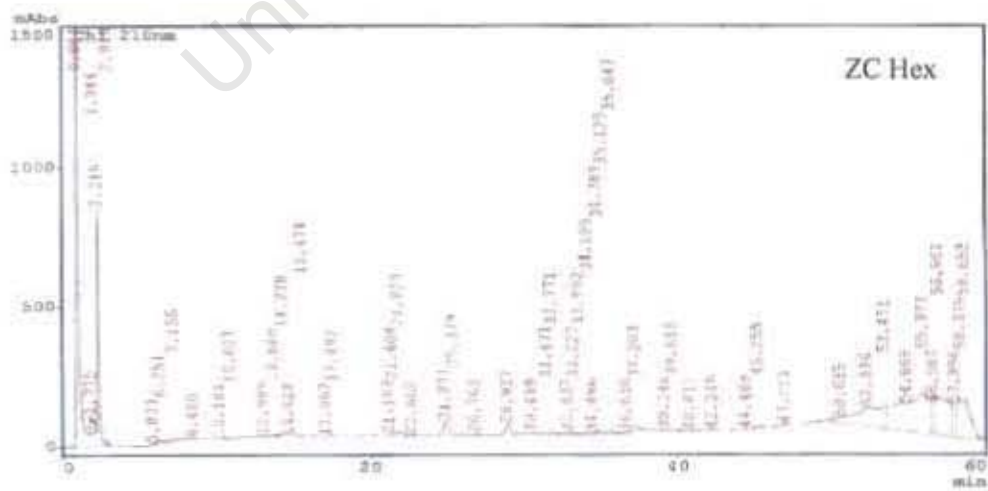
A



B



C



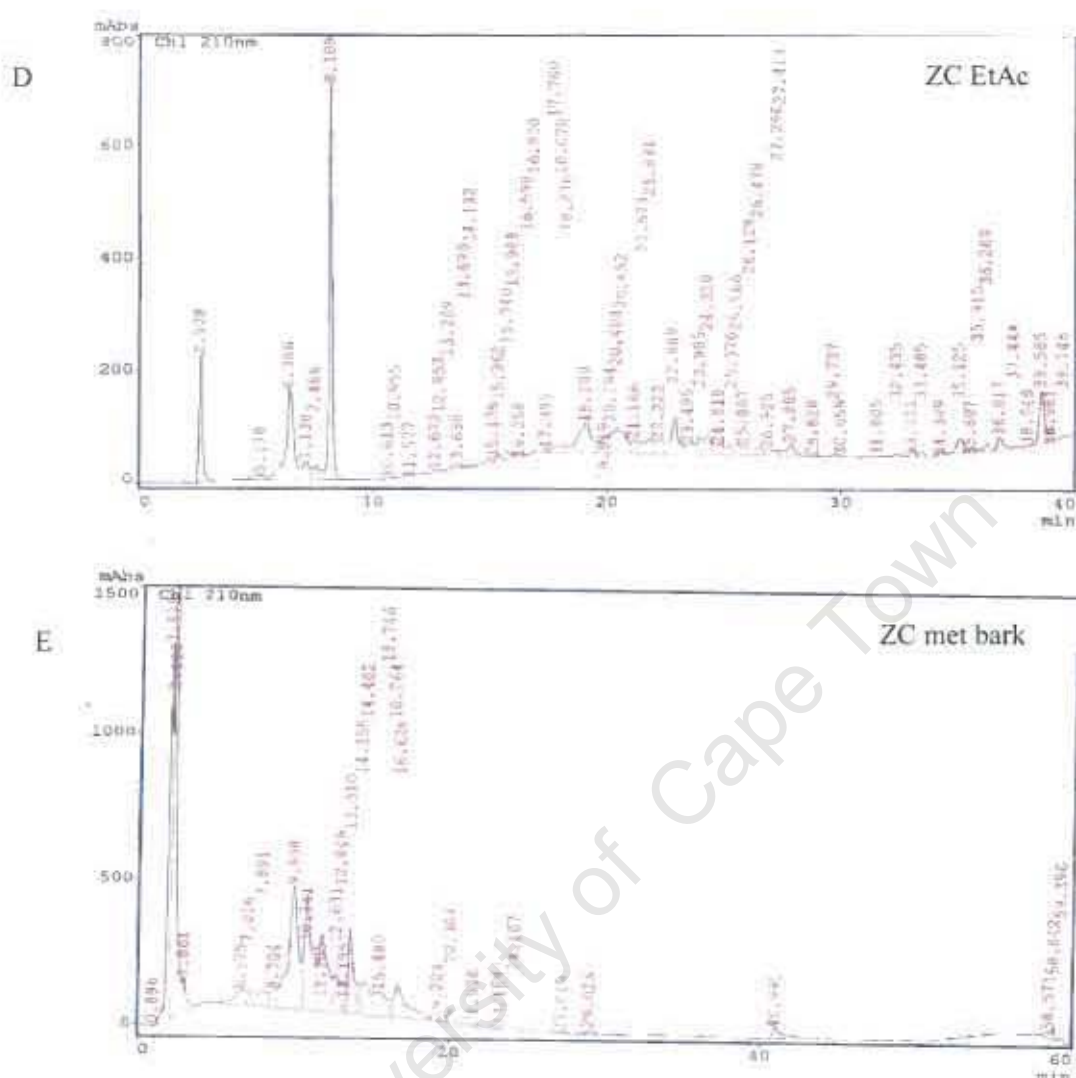


Fig.13 HPLC profiles of *Z. capense* crude extracts. 100 μ l of each sample (1mg/ml extracts) were applied to a C18 hydrophobic column. Compounds were eluted using a 10-100% acetonitrile/water gradient eluting at the flowing rate of 1ml/min for 60 minutes. A-E: *Z. capense* aqueous bark, methanol leaves, hexane, ethyl acetate and methanol bark extracts.

The methanol bark extract is a moderately simple extract as it contains few compounds absorbing at 210nm. There were 3 closely related peaks eluted very early with retention times of 1.879, 2.086 and 2.110 (Fig.13e). The compounds appeared to be a mixture of 3 compounds constituting 12.73% of the crude extract (Table 5). The compounds detected in this extract were very hydrophilic and there were very little non-polar compounds absorbing at 210nm.

Table 5. Retention times and concentration of peaks in *Z. capense* crude extracts

Plant extract	Peak number	Retention time (min)	Concentration in crude extract (%)
ZC _{aq} bark	14	5.73	6.58
	16	5.96	11.01
	39	11.73	8.37
ZC met	4	6.69	1.90
	8	8.01	10.67
	9	8.21	42.94
	50	19.82	5.87
ZC Hex	1	0.88	24.31
	5	2.219	7.69
	53	54.86	9.73
	54	55.97	11.98
	56	56.96	10.15
ZC EtAc	1	2.57	6.82
	3	6.38	11.90
	7	8.10	19.14
	29	19.10	6.37
ZC met bark	*5	2.086	12.73
	7	2.86	24.75
	15	9.90	13.64
	18	10.74	10.70
	20	11.70	7.15

Table 5. Retention times and concentration of compounds obtained from an HPLC analyses. 1mg/ml of crude plant extracts were applied to the column and eluted at 1min/ml flow rate with a 10-100% acetonitrile mobile phase, for 60 minutes (Fig.13). This table shows the peaks that were present in high concentration at 210nm.

* Peak number 5 of the ZC methanol (bark) extract is a mixture of 3 peaks with retention times 1.87, 2.086 and 2.11 minutes.

14. Biological evaluation of plant extracts

14.1 *M. tuberculosis* susceptibility testing: MIC determination

This study assesses the anti-mycobacterial potential of known TB drugs and plant extracts *in vitro* using the visual microplate-based Alamar Blue assay by determining minimum inhibitory concentrations (MIC). To validate the assay isoniazid and rifampicin, as known inhibitors of mycobacterial growth, were tested in a concentration gradient against *M. tuberculosis* H37Rv.

M. tuberculosis was incubated in Middlebrook 7H9 broth containing serial concentrations of drugs or plant extracts ranging from 15µg/ml and 1000µg/ml. 20µl of the Alamar Blue reagent was added to growth control wells containing mycobacteria 5 days after incubation and monitored for a colour change from blue to pink, which indicates mycobacterial growth. The Alamar Blue reagent was subsequently added to the rest of the wells on the microplates when the growth control wells turned pink and the plates were re-incubated at 37°C. Results were captured visually using a digital camera 24, 48 and 72 hours post-reagent addition. The visual MIC's were interpreted as the lowest drug or plant extract concentration that prohibited a colour change from blue to pink for at least 48 hours incubation.

It was established in our study that isoniazid completely inhibited growth at 0.06125µg/ml, whereas inhibition by rifampicin was observed at 0.03125µg/ml after 8 days of incubation (Fig.14). These values differ slightly with published MIC ranges of 0.025-0.05µg/ml and 0.05-0.1µg/ml for isoniazid and rifampicin respectively (Collins and Franzblau, 1997).

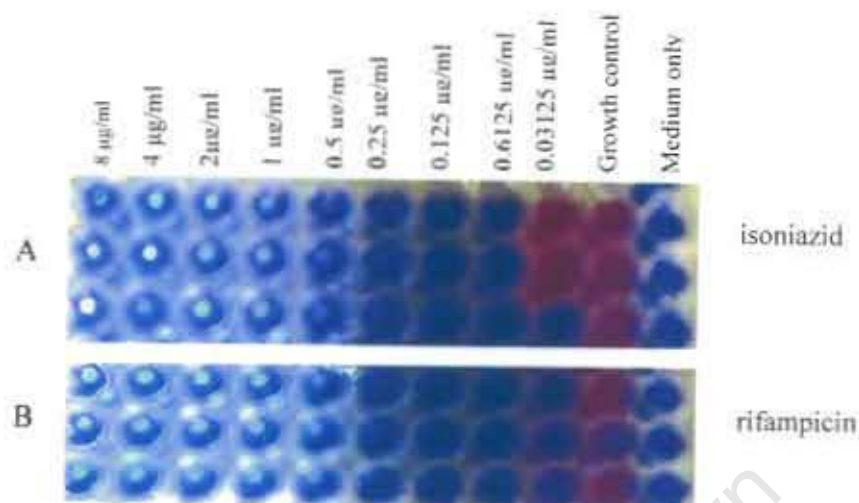


Fig.14 MIC testing of a) isoniazid and b) rifampicin were determined against *M. tuberculosis* H37Rv by the microplate Alamar Blue assay: isoniazid inhibited growth at 0.06125µg/ml and rifampicin at 0.031µg/ml after 8 days incubation ($n=3$). The experiment was done in duplicate.

A. praecox: Day 12-13

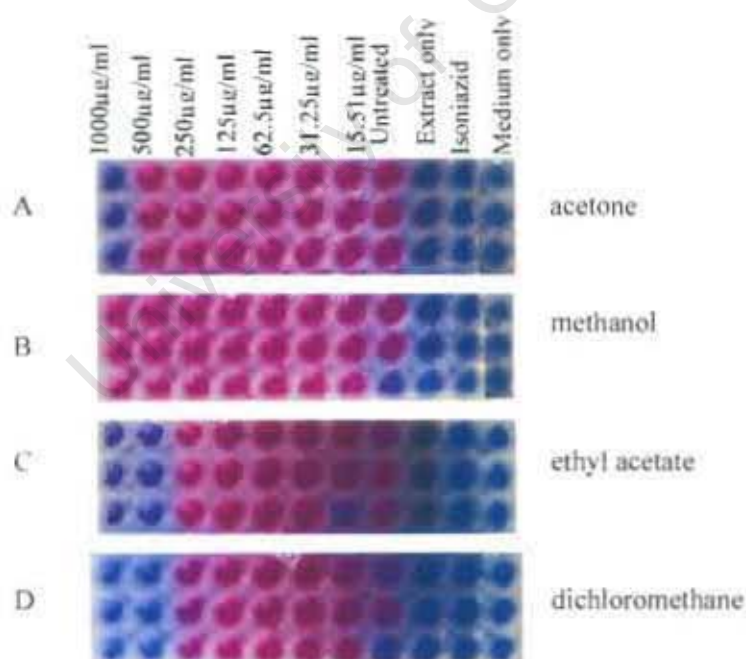


Fig.15 MIC screening of crude *A. praecox* rhizome extracts against *M. tuberculosis* H37Rv using the Alamar Blue assay. Alamar Blue reagent was added to wells containing rapidly growing *M. tuberculosis* 11 days post-incubation. Reduction of the Alamar Blue reagent to a pink colour indicates mycobacterial growth, whereas the blue colour indicates inhibition of growth. ($n=3$)

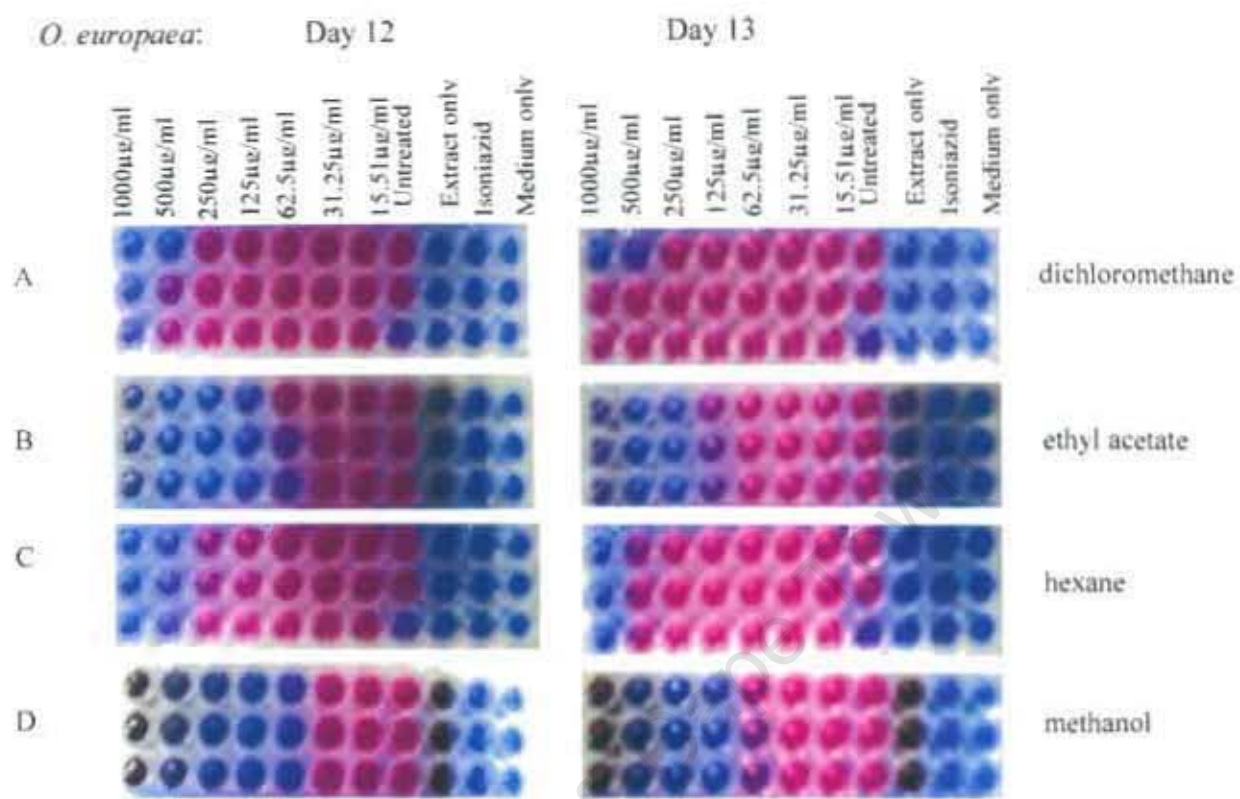


Fig.16 *M. tuberculosis* H37Rv growth inhibitory effect of crude *O. europaea* subsp. *Africana* leaf extracts was assessed using the Alamar Blue assay. Alamar Blue reagent was added 11 days after incubation with extracts and growth inhibition was monitored over 48 hours by capturing the colour change visually. A colour change from blue to pink indicates susceptibility, whereas blue colour indicates mycobacterial growth inhibition by plant extracts ($n=3$).

The efficacy of crude rhizome extracts of *A. praecox* was evaluated against *M. tuberculosis* H37Rv. All *Mycobacterium* cultures treated with the methanol extract (15-1000µg/ml) reduced Alamar Blue to a pink colour 24 hours post addition of the reagent (Fig.15b), indicating that *M. tuberculosis* is not susceptible to the methanol extract of *A. praecox* at any of the tested concentrations. The acetone, dichloromethane and ethyl acetate crude extracts inhibited *M. tuberculosis* growth at 1000µg/ml, 500µg/ml and 500µg/ml respectively (Fig.15a, c and d). The visual MICs of *A. praecox* extracts remained the same 12 and 13 days post incubation, hence figure 15 represents both days. This could also suggest that the extracts have a bacteriostatic inhibitory effect.

Olea dem extract inhibited growth at 1000µg/ml on day 12, but on day 13 growth inhibition had subsided (Fig.16a). Olea Hex inhibited growth at 500µg/ml after 13 days incubation (Fig.16c). *M. tuberculosis* displayed a greater susceptibility toward the Olea met extract with a visual MIC of 125µg/ml (Fig.16d), followed by the Olea EtAc extract which killed mycobacteria at 250µg/ml (Fig.16b, also see Table6) after 13 days incubation. The MIC values reported on Table 6 represent the observations made 48 hours after the addition of the reagent, which was day 13 after the beginning of the experiment or incubation period. The samples treated with *O. europaea* subsp. *Africana* crude extracts continued to change colour indicating the bacteriostatic efficacy of the extracts against *M. tuberculosis* H37Rv.

Z. capense: Day 12-13

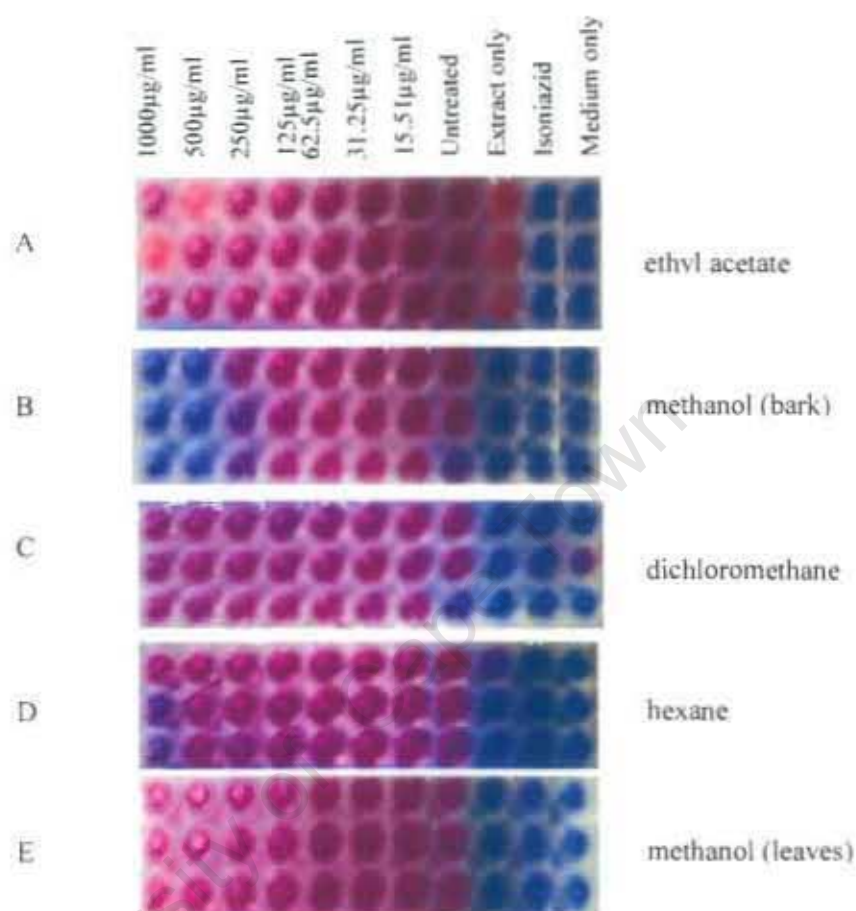


Fig.17 *Z. capense* crude leaf and bark extracts were tested for anti-mycobacterial activity *in vitro* using the Alamar Blue assay. A colour change from blue to pink indicates that all the *Z. capense* leaf extracts failed to kill or inhibit mycobacteria growth at the tested concentrations (a, c, d, e) whereas the blue colour indicates that the methanol bark extract (b) killed mycobacteria at $\geq 500\mu\text{g/ml}$. ($n=3$)

Crude plant extracts of *Z. capense* leaves and bark (branches/twigs) were tested for anti-mycobacterial activity *in vitro*. The dichloromethane, ethyl acetate, methanol, hexane leaf extracts and the aqueous bark extract did not inhibit *M. tuberculosis* growth at the maximum or any of the tested concentrations (Fig.17a, c, d and e). An interesting observation was made; the wells containing medium and ZC EtAc and ZCaq (bark) extract only (control to monitor extract redox capacity), displayed a colour change to pink 24 hours post-addition of the reagent (Fig.17a). This suggests that the ethyl acetate extract of *Z. capense* leaves contains constituents that have the potential to reduce the Alamar blue reagent, thereby interfering with the redox indicator. Hence the colour change in the test wells cannot be entirely attributed to mycobacterial growth. The efficacy of the ZC EtAc extract against *M. tuberculosis* is therefore inconclusive. The methanol extract of *Z. capense* bark inhibited mycobacterial growth at 500µg/ml after 12 and 13 days of incubation.

S. cordatum: Day 12-13

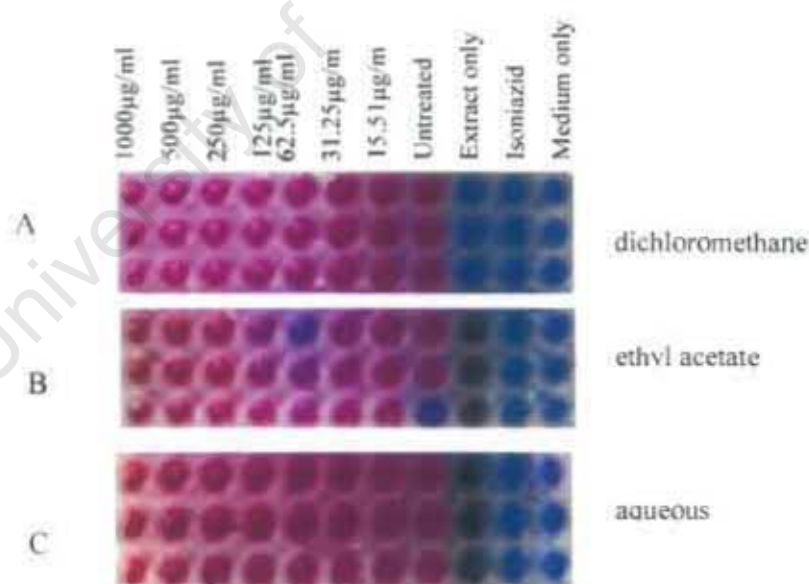


Fig.18 Evaluation of anti-mycobacterial activity of *S. cordatum* crude leaf extracts using the Alamar Blue assay. The colour change of the reagent from blue to pink indicates that the mycobacteria are proliferating in presence of plant extracts. Viable cells are able to metabolize and reduce the blue redox indicator dye to pink. ($n=3$)

There was no inhibition of growth observed when *M. tuberculosis* was exposed to *S. cordatum* dichloromethane, ethyl acetate and aqueous extracts for 12 and 13 days (Fig.18a, b and c). The crude methanol and hexane extracts were also inactive up to 1000µg/ml (picture not shown). This suggests that these *S. cordatum* extracts do not have anti-mycobacterial activity at the highest concentration of 1000µg/ml.

Table 6. A visual minimum inhibitory concentration (MIC) table of crude plant extracts determined using the microplate Alamar Blue assay.

Extraction solvent	MIC of plant extract (µg/ml)			
	<i>A. praecox</i>	<i>O. europaea</i> subsp. <i>Africana</i>	<i>S. cordatum</i>	<i>Z. capense</i>
Acetone	1000			
Dichloromethane	500	>1000	>1000	>1000
Ethyl acetate	500	250	>1000	>1000
Hexane		1000	>1000	>1000
Methanol	>1000	125	>1000	>1000 500*
Water			>1000	>1000*

Table 6. The anti-mycobacterial activity of the crude plant extracts was evaluated using the visual microplate-based Alamar Blue assay. The Alamar Blue oxidation-reduction dye is a general indicator of cellular viability or proliferation. Visual MIC was defined as the lowest concentration of the extract that prevented colour change from blue to pink 48 hours after addition of the reagent (13 days after incubation). See Fig.10, 11 and 12.

* denotes *Z. capense* bark extract

In conclusion, cell viability using the Alamar Blue assay can be assessed spectrophotometrically or fluorometrically by measuring the absorbance/fluorescence of the reduced Alamar Blue redox indicator dye. In our study *M. tuberculosis* proliferation was determined and recorded by simple visual inspection because the pigments of the plant extracts made it complicated to read absorbances at the designated wavelengths (570- 600nm). Plant extracts contain a complex mixture of compounds and pigments which may have common absorption patterns with the absorption spectra of the oxidized (blue) and reduced (pink) form of the reagent hence interfere with the assay. MIC data could therefore not be expressed as percentage (%) of Alamar blue reduced (as an indication of % growth surviving under treatment with drug/ plant extract), but as visual MIC.

The methanol and ethyl acetate extracts of *O. europaea* subsp. *Africana* leaves displayed the most efficient anti-TB activity by inhibiting mycobacterial growth at the lowest concentration, followed by *A. praecox* rhizome extracts. *Z. capense* and *S. cordatum* crude leaf extracts exhibited the least activity against *M. tuberculosis* compared to all the plant extracts screened in our study (Table 6).

15. Cell cytotoxicity testing of crude plant extracts

This study evaluated the toxic effect of the plant extracts on mouse peritoneal macrophages as an indication of their potential toxicity towards human cells. In addition, the assay was also performed as a preliminary test prior to testing the efficacy of the crude plant extracts against intracellular, macrophage ingested *M. tuberculosis* populations. Plant extracts that displayed direct inhibition of *M. tuberculosis* proliferation and those that did not show any anti-mycobacterial activity in the susceptibility screen (MIC determination, Table6) were screened for macrophage cytotoxicity to facilitate further assessment of the extracts against intracellular mycobacteria. Some plant extracts that did not inhibit *M. tuberculosis* growth directly may perform differently within a cellular environment due to factors such as pH variations or possible modifications of the compounds within the extract that ultimately confers activity against *M. tuberculosis* growth.

The cytotoxicity effect of isoniazid, rifampicin and plant extracts was evaluated on mouse peritoneal macrophages using the MTT assay. The MTT assay measures cell growth/growth inhibition by ability of viable cells to reduce the yellow tetrazolium reagent (MTT) to a purple formazan residue which was solubilized in DMSO and quantified spectrophotometrically (570- 630nm). The plant extracts stock preparations were re-dissolved in 1% DMSO (v/v); therefore the highest DMSO concentration in the working extract preparations was less than 1%. DMSO concentrations less than 1% (v/v) displayed minimal killing effects hence the growth inhibition can be principally attributed to the plant extracts (Fig.19a).

Isoniazid and rifampicin were tested as positive controls, and assessed to verify the assay. Isoniazid exhibited no inhibitory effects on cell growth up to the concentration of 100µg/ml (Fig.19b) whereas rifampicin displayed increased toxicity relative to isoniazid. Rifampicin inhibited 50% of cell growth at ~34µg/ml, and total cell growth was inhibited at 75µg/ml (Fig.19c).

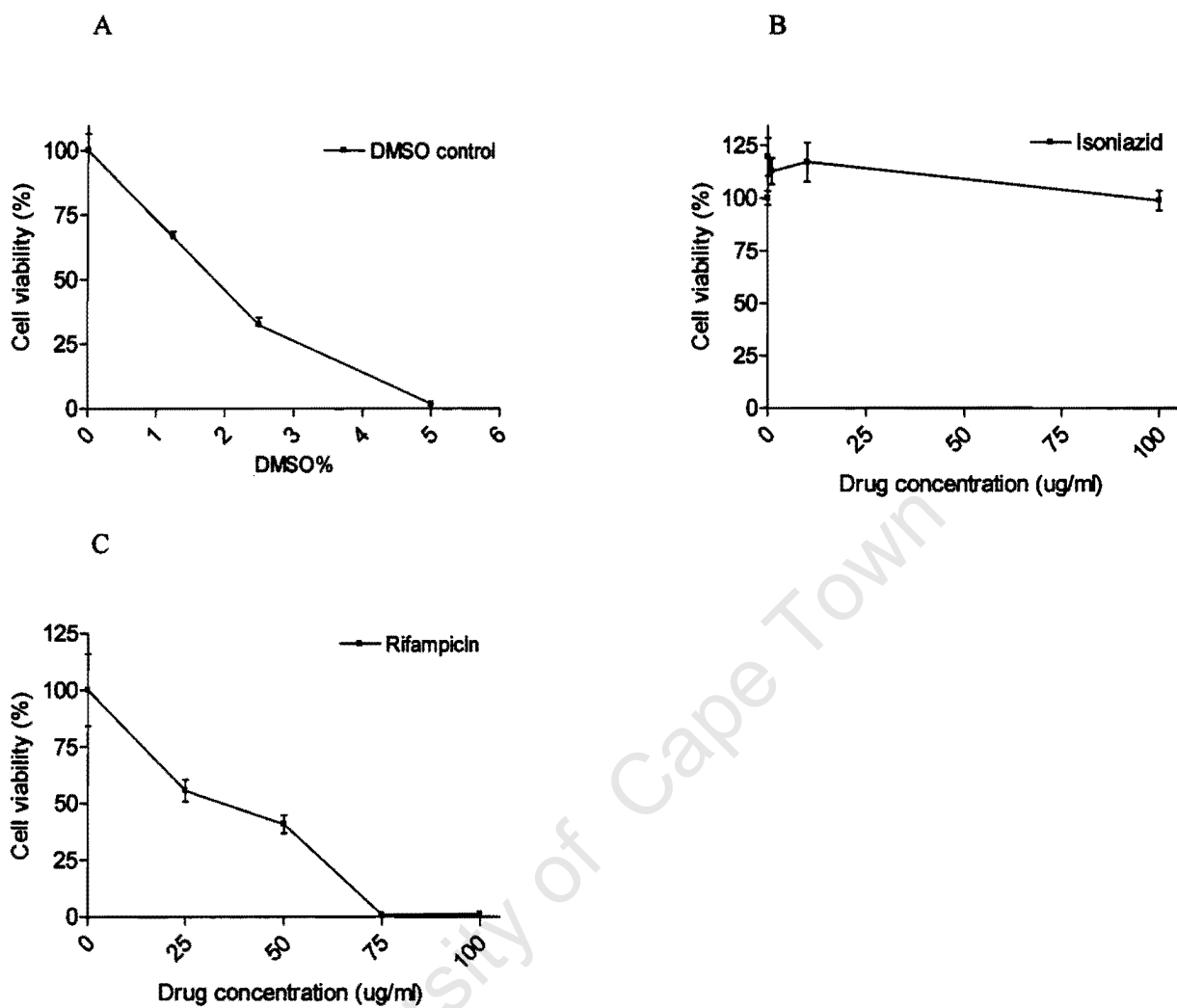


Fig.19 Macrophage cultures were incubated with drugs for 8 days. Extracts were subsequently removed and cells incubated with MTT solution for 4 hours. Cell growth was measured by amount (%) of MTT reduced. Cytotoxicity effects of a) DMSO b) isoniazid and c) rifampicin on mouse peritoneal macrophages were tested over an 8-day incubation period.

Subsequently, the crude extracts of *A. praecox* rhizome were tested for potential cytotoxic effects against macrophages. The extracts showed varying effects on the growth of macrophages. The acetone (Fig.20a) and ethyl acetate (Fig.20c) extracts exhibited a concentration dependent cell growth inhibition with 50% of macrophages being killed at 63µg/ml and 65µg/ml respectively. The dichloromethane and methanol extracts inhibited growth at very low concentrations with IC₅₀ values of 31µg/ml and 10µg/ml respectively (Fig.10b and d). The methanol extract (Aga Met) was the most cytotoxic of the group; inhibiting 90% of growth (IC₉₀) at ~20µg/ml. Earlier, our susceptibility studies showed that the *A. praecox* methanol extract does not inhibit *M. tuberculosis* proliferation (Fig15b), whereas the same extract displays potent toxicity towards macrophage cells at very low concentrations. This observation may be due to the following possible rationales:

- i. The extract does not contain active compounds that target pathways that lead to *M. tuberculosis* cell death
- ii. The extract concentration was not sufficient to inhibit *M. tuberculosis* growth
- iii. The extract was not able to penetrate the complex mycobacterium cell wall due to its hydrophilic moieties, hence could not access and interfere with the metabolic pathways that maintain the pathogen proliferation.

The macrophage and *M. tuberculosis* are different cell types; hence the methanol extract of *A. praecox* rhizome will inevitably interact in a different way with cells with different properties. This finding suggests that this extract is potent against other cell types and is potentially poisonous, although it has no evident anti-mycobacterial activity.

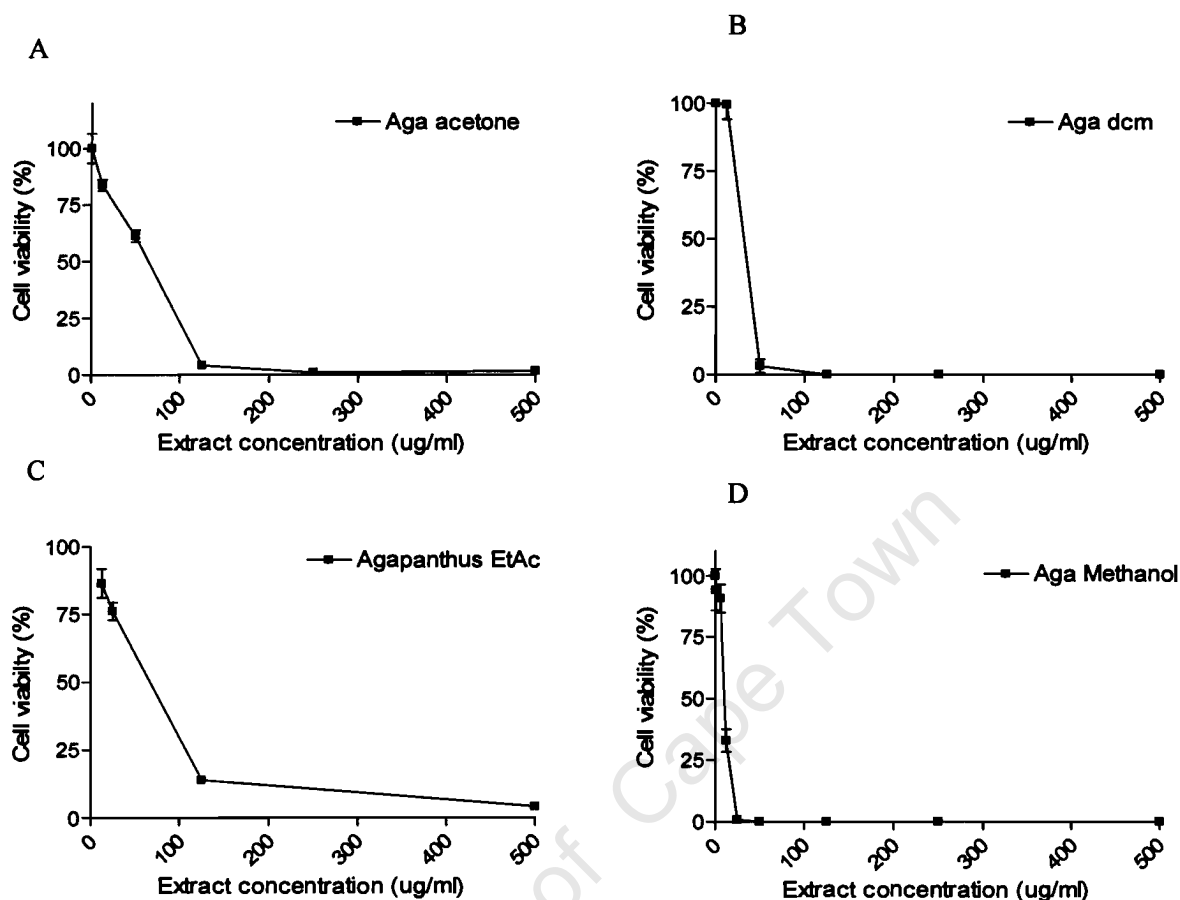


Fig.20 *A. praecox* crude rhizome extracts tested for cytotoxicity against mouse peritoneal macrophages for 8 days using the MTT assay. Total cell killing occurred at concentrations lower than 250 μ g/ml, with the exception of the ethyl acetate (Aga EtAc) extract which allowed some minimal growth at the highest tested concentration, 500 μ g/ml.

The *O. europaea* subsp. *Africana* extracts were tested for cytotoxicity against peritoneal macrophages following the same method as above. *O. europaea* subsp *Africana* crude leaf extracts were generally less toxic towards the proliferating macrophage cultures compared to the *A. praecox* rhizome extracts. Treatment with hexane (Olea Hex) and methanol (Olea met) extracts displayed no significant growth inhibition at high concentrations (Fig.21c and d) and hence they are not cytotoxic at

the tested concentrations. The dichloromethane extract (Olea dcm) did not inhibit macrophage viability at concentrations up to 250 μ g/ml, but gradually declined and displayed 80% inhibition at 500 μ g/ml (Fig.21a). The ethyl acetate extract (Olea EtAc) was the most cytotoxic with cell growth gradually declining with increasing extract concentration (Fig.21b). 50% of the cell population was killed at ~168 μ g/ml and 90% inhibited at ~440 μ g/ml (Table 7).

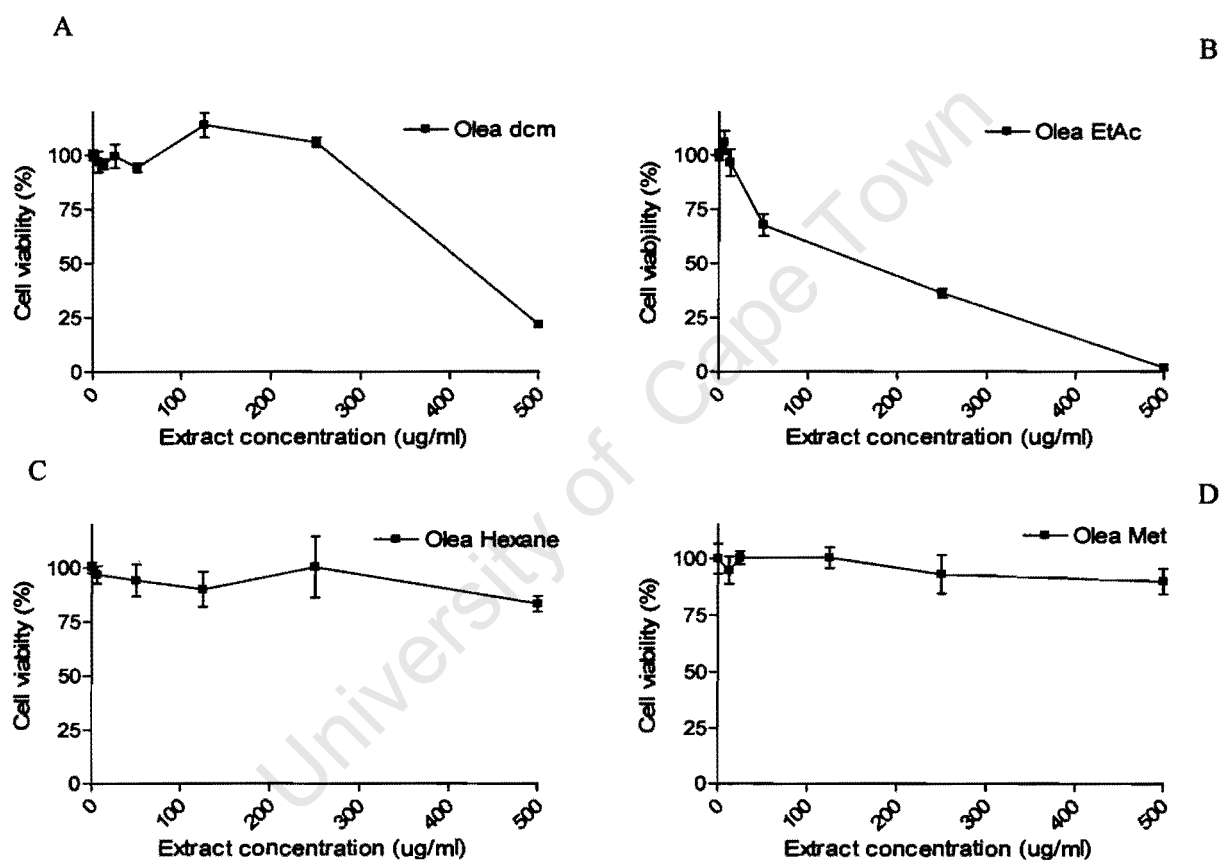
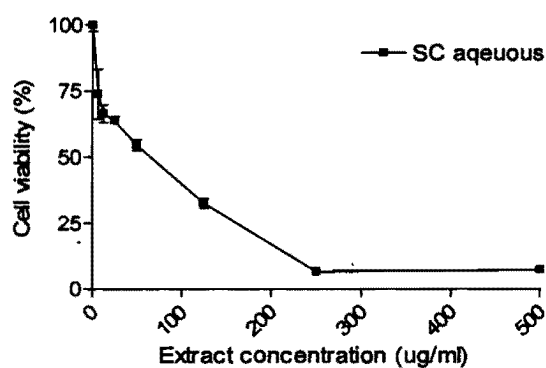


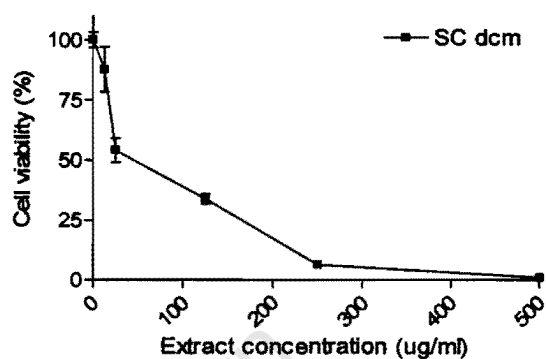
Fig.21 *O. europaea* subsp. *Africana* crude leaf extracts cytotoxicity was evaluated against mouse peritoneal macrophages using the MTT assay. Cell cultures were incubated with the plant extracts for 8 days and growth/ growth inhibitory monitored by a spectrophotometer at the wavelengths 570-630nm

The *S. cordatum* extracts were tested for cytotoxic effects against macrophages. All *S. cordatum* crude leaf extracts had relatively similar capabilities of inhibiting macrophage growth (Fig.22) (also see Table.7 for IC₅₀ and IC₉₀ values). Inhibition of cell viability was concentration dependent in all of the extracts tested. Approximately 90% of cell growth was inhibited at concentration 250µg/ml in leaf extracts of aqueous, dichloromethane, ethyl acetate and methanol solvents (Fig.22a-e). The hexane extract (SC Hex) which had ≥25% cell survival at 250µg/ml was the least toxic whilst the ethyl acetate extract (SC EtAc) was the most toxic with total cell killing occurring at 250µg/ml (Fig.22c and d). All the *S. cordatum* leaf extracts however, did not display any inhibitory effects against *M. tuberculosis* growth in our susceptibility studies.

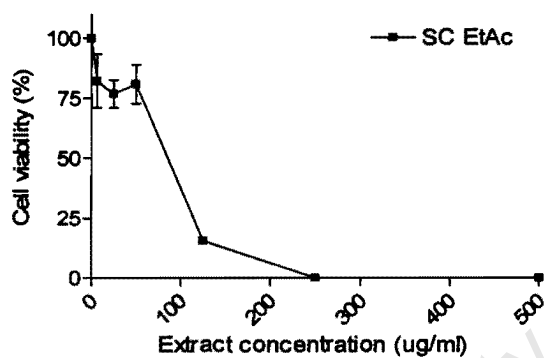
A



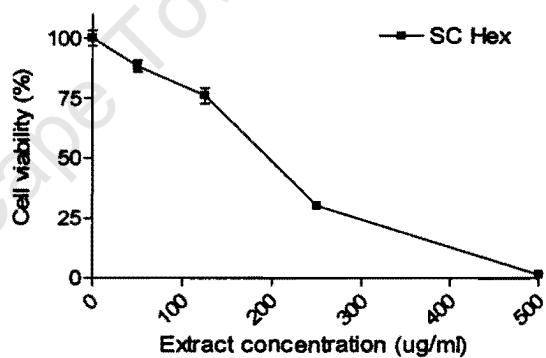
B



C



D



E

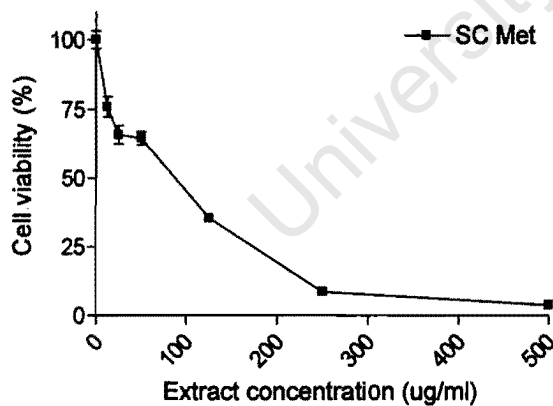


Fig.22 *S. cordatum* crude leaf extracts cytotoxic effects were evaluated on peritoneal macrophage cultures using the MTT assay. All *S. cordatum* extracts show relatively similar inhibition profiles.

Extracts from *Z. capense* leaves and bark were evaluated for toxic effects against proliferating macrophages using the MTT assay. Some *Z. capense* crude leaf extracts had similar inhibitory profiles. The aqueous extract of twigs (bark) had no growth inhibitory effects even at high concentrations (Fig.23a). However, the leaf extracts, ZC dcm, ZC EtAc, ZC Hex and ZC met inhibited cell proliferation at very low concentrations with IC₅₀ values of 31µg/ml, 35µg/ml, 58µg/ml and 25µg/ml respectively (Table 7).

In our susceptibility studies we showed that all *Z. capense* extracts, with the exception of the methanol bark extract, displayed no anti-mycobacterial activity up to 1000µg/ml. These extracts however, show potent inhibitory effects against macrophages. Hence the extracts may not contain active constituents against *M. tuberculosis* H37Rv, but there may be some compounds present that interfere with cellular processes of macrophages, which leads to cell death.

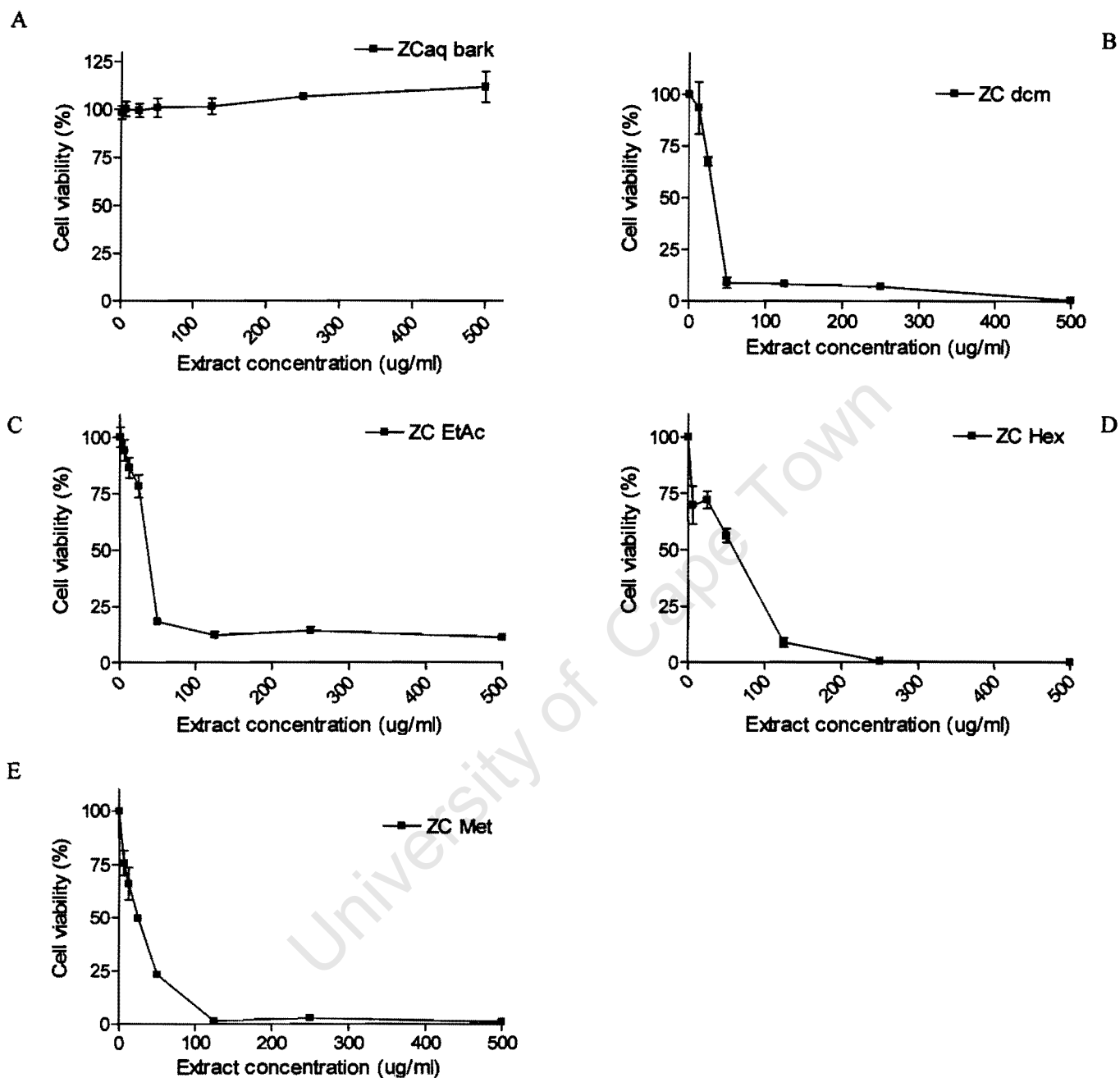


Fig.23 *Z. capense* crude plant extracts cytotoxicity effects screened against mouse peritoneal macrophages using the MTT assay. The leaf extracts showed potent toxicity whereas the aqueous extract of bark/twigs did not inhibit growth at the highest concentration.

Summary Table 7. Inhibitory concentrations of crude plant extracts on macrophage proliferation

Plant name part used	Extraction solvent	IC ₅₀ (µg/ml)	IC ₉₀ (µg/ml)
<i>A. praecox</i> rhizome/roots	acetone	63,3	116
	dichloromethane	31	46.6
	ethyl acetate	65	265
	methanol	10	20
<i>O. europaea</i> leaves	dichloromethane	416	>500
	ethyl acetate	168	440
	methanol	-	-
	hexane	-	-
<i>S. cordatum</i> leaves	dichloromethane	46	230
	ethyl acetate	86	170
	methanol	85	240
	hexane	195	435
	water	65	228
<i>Z. capense</i> leaves twigs (bark)	dichloromethane	31	50
	ethyl acetate	35	>500
	methanol	25	93
	hexane	58	123
	water	-	-

Table 4. Inhibitory concentrations of crude plant extracts on mouse peritoneal macrophage proliferation were obtained using the MTT cell proliferation assay. - indicates that there were no cytotoxic effects

IC₅₀ denotes the concentration at which 50% of the cell growth was inhibited
 IC₉₀ denotes the concentration at which 90% of the cell growth was inhibited

16. Anti-mycobacterial screening of crude plant extracts against intracellular *M. tuberculosis* H37Rv

This study evaluated the efficacy of standard anti-TB drugs and plant extracts against mycobacteria that are ingested and able to survive within macrophages. During TB infection, alveolar macrophages engulf the infecting mycobacteria as the first line of defense. However, when the macrophage is not activated, the mycobacteria are able to evade the macrophage killing mechanisms by various mechanisms (Salyers AA., 1994; Wadee and Clara, 1989) and can survive within the macrophages. Anti-mycobacterial drugs effective in macrophages are therefore needed. This study assessed the potential anti-mycobacterial effects of plant extracts against *M. tuberculosis* residing within the macrophages. The plant extracts that did not show anti-mycobacterial activity against *M. tuberculosis* in the direct susceptibility assay were also evaluated in this study to examine their potential activity within a cellular environment.

Murine peritoneal macrophages were infected with *M. tuberculosis* H37Rv overnight. The inoculum was subsequently removed and the infected macrophage monolayers were incubated with RPMI complete medium containing different concentrations of drugs or crude plant extracts for a defined period of time. Following exposure to drugs/ plant extracts, the macrophage monolayers were lysed. The lysates were serially diluted and plated on Middlebrook 7H10 agar plates. The plates were incubated for 3-4 weeks at 37°C, after which *M. tuberculosis* colonies were counted. The results were reported as percentage (%) growth inhibition of colony forming units (CFU).

Isoniazid and rifampicin were tested as positive controls to validate the assay. Both isoniazid and rifampicin had significant (~60%) anti-mycobacterial effects at 0.1µg/ml (Fig.24). Isoniazid equally reduced mycobacterial growth at 1µg/ml and 10µg/ml by approximately 90%, whilst rifampicin eradicated intracellular mycobacterial growth completely at 10µg/ml.

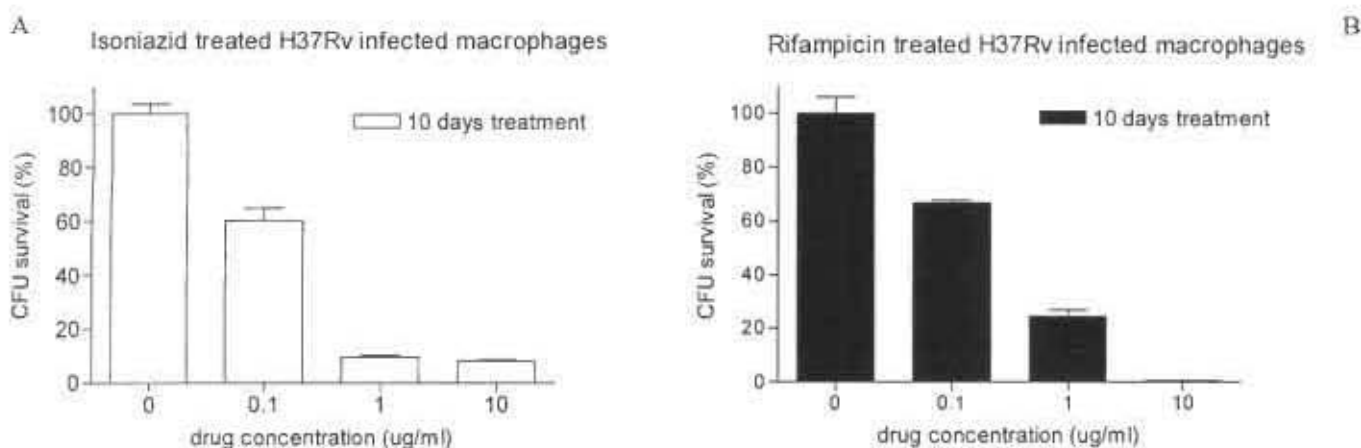


Fig.24 Anti-mycobacterial activity of isoniazid and rifampicin tested against macrophage ingested *M. tuberculosis*. Infected macrophages were incubated with drugs for 10 days, and subsequently lysed and plated on Middlebrook 7H10 agar plates. Growth inhibition was determined by comparing CFU counts of treated and untreated samples.

In other studies rifampicin was shown to be effective against intracellular *M. tuberculosis* H37Rv phagocytosed by human macrophage cultures at 0.5µg/ml and 2.5µg/ml ($p<0.001$) (Duman et al., 2004). Our result is therefore consistent with published data.

All plant extracts were evaluated for anti-mycobacterial activity against intracellular *M. tuberculosis* at one or two concentrations below the macrophage IC_{50} (Table 7) over a fixed period of time, 8 days. Isoniazid was included as a positive control for growth inhibition at a concentration of 10µg/ml, which killed approximately 95% of the intracellular mycobacteria (Fig.25)

Anti-mycobacterial activity of plant extracts against intracellular mycobacteria

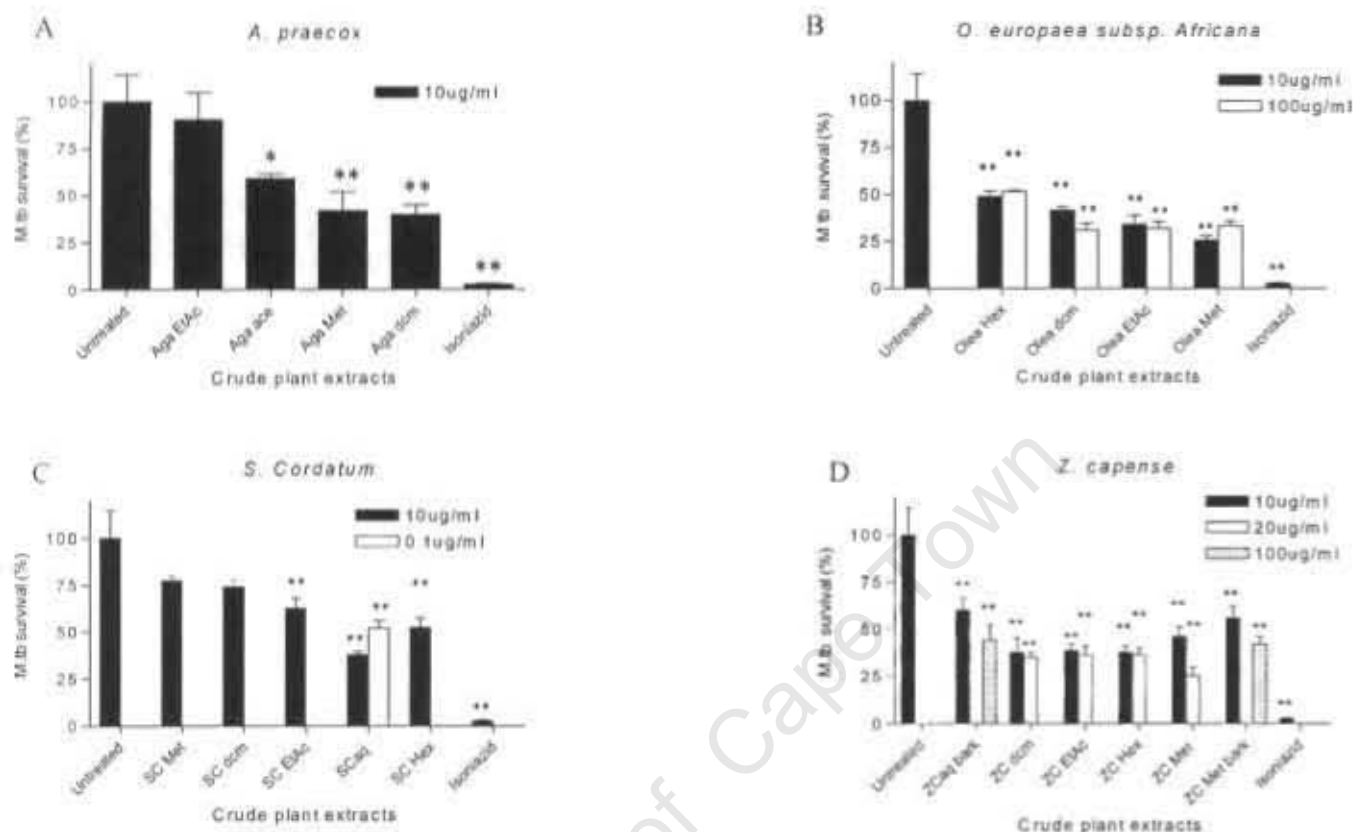


Fig.25 Anti-mycobacterial activity of crude plant extracts against intracellular mycobacteria ingested by mouse peritoneal macrophages. H37Rv infected macrophage cultures were incubated with plant extracts for 8 days prior to lysing and plating on Middlebrook 7H10 agar plates to enumerate CFUs. ($n=3$ per extract screened). *Statistically significant difference from the untreated control, $p<0.05$; ** $p<0.01$ compared to the untreated control.

Working extracts solutions were prepared in RPMI complete medium from stock extracts dissolved in 1% DMSO (v/v). *A. praecox* acetone, ethyl acetate, dichloromethane and methanol extracts (Aga ace, Aga EtAc, Aga dcm and Aga met respectively) were tested at a single concentration of 10µg/ml. Aga EtAc had no significant anti-mycobacterial activity at this concentration (Fig.25a). The acetone extract reduced mycobacterial growth by approximately 40% ($p<0.05$) whilst the methanol and dichloromethane extracts inhibited more than 50% of the mycobacterial growth at 10µg/ml ($p<0.01$) (Fig.25a). These extracts were reported to inhibit *M. tuberculosis* growth directly in our susceptibility studies; hence their activity is maintained against intracellular mycobacteria. The methanol extract was not directly active against *M. tuberculosis* at 1000µg/ml (Fig.25b). The reduced mycobacterial growth in the macrophages may therefore be due to the following reasons:

- i. The methanol extract killed a significant amount of the macrophages as it is very cytotoxic ($IC_{50} = 10\mu\text{g/ml}$), hence the mycobacteria were possibly excluded from the dead cells into the extracellular medium and subsequently removed when the monolayer was washed before lyses. The medium was also not supplemented for mycobacterial extracellular growth; hence the bacteria may have not survived outside the macrophages. The total amount of bacteria plated was therefore low due to loss of inoculum.
- ii. The extract may possibly up-regulate macrophage effector functions such as nitric oxide (NO) and reactive nitrogen (RNI) and oxygen (ROI) intermediates production, which in turn inhibit mycobacterial growth

Treatment of infected macrophages with *S. cordatum* crude extracts also resulted in significant reduction of intracellular *M. tuberculosis* growth. Approximately 75% of intracellular mycobacteria survived when treated with the *S. cordatum* methanol, dichloromethane crude leaf extract (SC met and SC dcm) (Fig.25c). The growth reduction was not significant compared to the untreated control. The ethyl acetate and hexane extracts reduced approximately 35% of intracellular mycobacterial growth ($p<0.01$). The aqueous extract (SCaq) reduced just over 50% of the intracellular infection

at 10µg/ml and mycobacterial growth was slightly higher at 0.1µg/ml (Fig.25c). This indicates that the killing action of the SCaq extract is concentration dependent (bactericidal).

All *O. europaea* subsp. *Africana* crude extracts had significant inhibitory effects on intracellular *M. tuberculosis* ($p<0.01$). The dichloromethane (Olea dcm), ethyl acetate (Olea EtAc), hexane (Olea Hex) and methanol (Olea met) extracts were tested at 10µg/ml and 100µg/ml. Olea Hex reduced approximately 50% of mycobacterial growth at 10 and 100µg/ml extract concentrations. Olea dcm and Olea EtAc reduced mycobacterial growth similarly by approximately 60% at 10µg/ml with Olea dcm killing reducing more growth at 100µg/ml whilst Olea EtAc reduced 50% at both concentrations (Fig.25b). Olea met reduced approximately 75% of the mycobacterial growth at both 10µg/ml and 100µg/ml concentrations with no significant difference.

Z. capense bark (twigs) methanol and aqueous (ZC met bark and ZCaq bark) extracts commonly inhibited approximately 40% of intracellular mycobacterial growth at 10µg/ml and approximately 60% growth at 100µg/ml (Fig.25d). Treatment with dichloromethane (ZC dcm), ethyl acetate (ZC EtAc) and hexane (ZC hex) extracts resulted in approximately 70% mycobacterial growth inhibition at 10µg/ml and 20µg/ml, with no significant difference between the two concentrations (Fig.25d). The methanol leaf extract (ZC met) reduced just approximately 50% of the mycobacterial growth at 10µg/ml and significantly reduced the infection by approximately 75% at 20µg/ml (Fig.25d).

The plant extracts that showed significant direct activity against *M. tuberculosis* such as the ethyl acetate and methanol extracts of *O. europaea* subsp. *Africana*, and the methanol extract of *Z. capense* bark maintained their anti-mycobacterial activity against intracellular *M. tuberculosis*. The extracts that did not inhibit mycobacterial growth directly, but reduced growth of macrophage ingested bacilli (eg. All the *S. cordatum* and *Z. capense* leaf extracts) may be due to interference with macrophage proliferation or damage to the macrophages. The extract constituents may also have undergone

modification that resulted in their being active once inside the macrophage due to changes in pH and enzymatic involvement within the macrophage environment. Another possible explanation is that the extracts may have secondary effects such as activating the macrophages and enhancing their mycobacterial killing capabilities by up-regulating anti-TB responses such as NO, RNI and ROI production.

17. Immune-modulation properties of extracts: iNOS expression in macrophages

This study was performed to evaluate the ability of plant extracts to induce secondary anti-mycobacterial effects in macrophages. NO production is one of the main responses by activated macrophages to *M. tuberculosis* infection (Rojas et al., 1997). Hence this study assessed whether the plant extracts enhanced or reduced macrophage activation by comparing NO accumulation as a marker for activation in non-stimulated, stimulated and plant extract treated macrophage monolayers.

Murine peritoneal macrophage monolayers were cultured in flat-bottomed 8-well culture plates with RPMI complete medium supplemented for macrophage growth in the presence of LPS or plant extracts in the specified concentrations for 48 hours (Fig.26). The medium was subsequently removed and the macrophage monolayers stained for NO.

LPS (0.1 µg/ml) was tested as a positive control for NO production, whereas macrophages cultured with medium without LPS or extracts served as a negative control for macrophage activation. NO presence was not observed in the negative control macrophage cultures (untreated) (Fig.26a). LPS stimulated macrophages indicated accumulation of NO by exhibiting a luminous fluorescence green colour (Fig.26b).

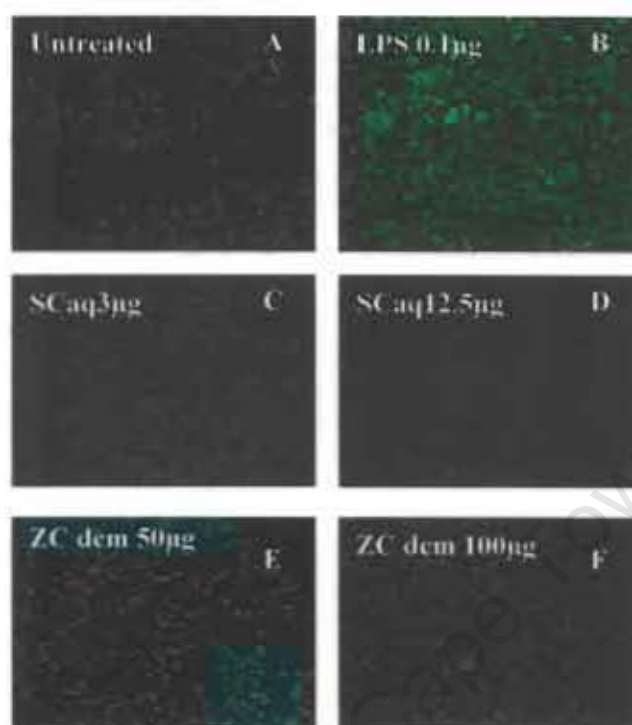


Fig.26 Peritoneal macrophage monolayers were incubated with plant extracts for 48 hours and subsequently evaluated for macrophage activation by staining for NO. Luminous green areas indicate accumulation of nitric oxide. Magnification=40×

The plant extracts evaluated were chosen because they did not show direct inhibition of *M. tuberculosis* growth at high concentrations (Fig.17c and Fig.18c), but reduced growth of *M. tuberculosis* inside macrophages (Fig.25c and d). Therefore, possible induction of macrophage effector mechanisms (iNOS expression) were investigated. *S. cordatum* crude aqueous extract of the leaves did not induce NO production at 3 and 12.5µg/ml (Fig.26c, d). Low levels of NO were detected in macrophages treated with *Z. capense* dichloromethane (ZC dcm) crude extract at 50 and 100µg/ml (Fig.26e, f). The macrophage activation was not significant relative to LPS treated samples. SCaq and ZC dcm extracts therefore do not induce iNOS expression in macrophages or enhance macrophage activation significantly.

18. *In vivo* anti-mycobacterial activity of crude plant extracts

18.1 Physiological effects of plant extract

This study was performed to assess the physiological effects of plant extracts in animal models during acute *M. tuberculosis* infection. The mouse strain used (C57BL/6) has been reported to be relatively resistant and does not show evident signs of disease during acute phase TB infection (Chackerian and Behar, 2003). Our study therefore investigated the effect of treatment with plant extracts on mortality and disease symptoms such as body-weight loss, physical appearance and behavioural patterns.

Mice were infected with a low dose of *M. tuberculosis* H37Rv via aerosol route. Treatment with drugs and crude plant extracts was initiated 3 weeks post-infection and was administered by oral gavage daily, 7 times a week for 2 weeks Isoniazid was administered as a positive control at 25mg/kg to ensure killing of infecting bacilli. The following extracts, *O. europaea* ethyl acetate, methanol (Olea EtAc and Olea met respectively) and *Z. capense* methanol (ZC met) crude leaf extracts were tested at a single concentration of 125mg/kg. The *O. europaea* subsp. *Africana* extracts were selected based on their potent inhibitory effects on *M. tuberculosis* growth *in vitro* (Fig.16b and d). The *Z. capense* methanol extract of the leaves did not show direct inhibition of mycobacterial growth (Fig.17e), but it showed significant activity against intracellular growing mycobacteria (Fig.25d). This extract was therefore evaluated in an *in vivo* model to assess its potential anti-mycobacterial activity in a living system.

All mice in all treatment groups survived the duration of the treatment, there were no mortalities were recorded (Fig.27a). There was no significant difference between the body-weights of the animals treated with isoniazid, plant extracts and untreated mice at all time points. The body weights were also consistent with those of healthy pathogen free mice. The body masses remained relatively constant throughout the treatment (Fig.27b). Mice treated with plant extracts did not exhibit any unusual phenotypes or signs of diseases such as laboured breathing, ruffled fur, loss of appetite, change in colour

of urine, hunched posture, hyperactivity or aggression. Treatment with crude plant extracts therefore did not induce any apparent indication of toxicity *in vivo*.

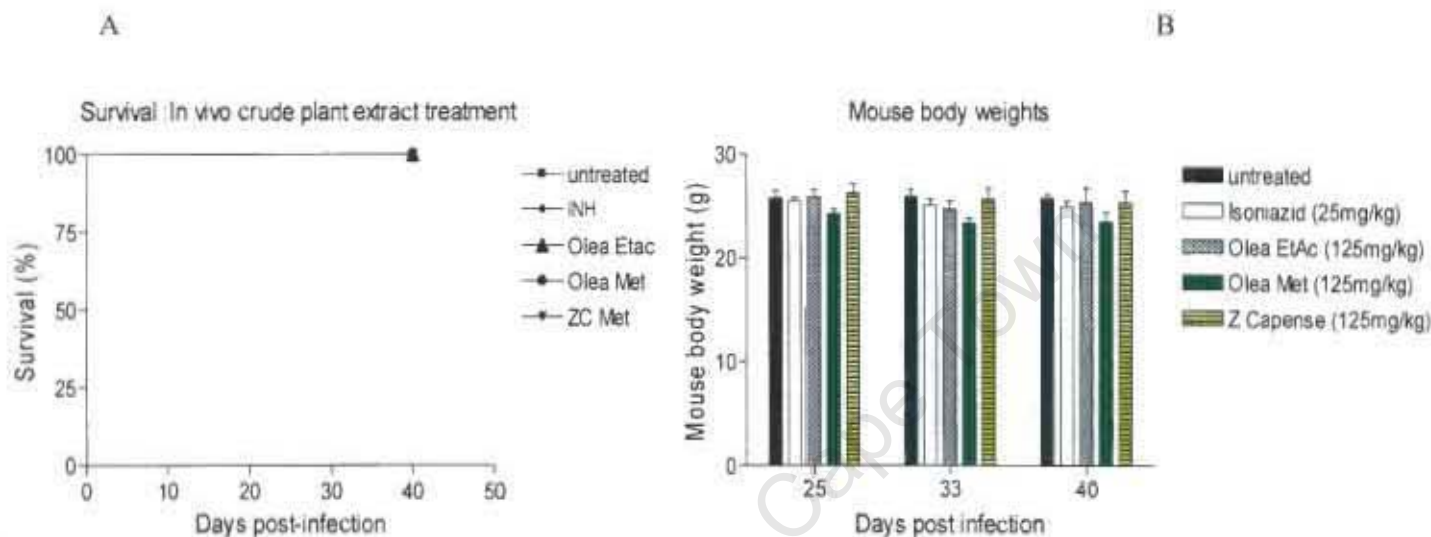


Fig.27a) Survival curve for mice infected with *M. tuberculosis* H37Rv and treated with crude plant extracts at a single concentration of 125mg/kg for two weeks. Treatment was initiated 25 days post-infection and commenced daily for 14 consecutive days. b) Body weights of mice undergoing treatment were taken at the beginning of treatment (day 25), 7 and 14 days after treatment. ($n=4$)

18.2 *In vivo* anti-mycobacterial activity of crude extracts

This study evaluated the anti-mycobacterial activity of the crude plant extracts in animal models. The therapeutic efficacy was defined as the reduction in *M. tuberculosis* burden (CFU) in the lungs, livers and spleens of infected mice.

M. tuberculosis infected mice were treated with isoniazid or crude plant extracts from day 25 post-infection for 14 days. The mice were sacrificed by CO₂ asphyxiation at day 25, 33 and 40 post-infection. Lungs, liver and spleen were aseptically removed and

homogenized in 2ml saline Tween-80 solution. The homogenates were serially diluted in sterile saline and a 100µl plated on Middlebrook 7H10 agar plates. Plates were incubated at 37°C with CO₂ supplementation for 3-4 weeks. The number of viable mycobacteria in the organs was subsequently determined by counting colonies. To evaluate the efficacy of isoniazid and plant extracts, the differences in organ CFUs between treated groups and untreated control were compared.

In untreated mice, mycobacterial growth increased exponentially until around 3 weeks, after which a constant level of *M. tuberculosis* titer was maintained until the end of the experiment (Fig.28a). Treatment with isoniazid reduced mycobacterial proliferation in the lungs by approximately 1log CFU after 7 and 14 days. However, the untreated and isoniazid treated lung CFU means were not statistically significantly different ($P=0.3394$). This finding is consistent with published data, where isoniazid (25mg/kg) was reported to reduce the mycobacterial load in the lungs of wildtype C57BL/6 mice with a reduction of 1 to 1.5log CFU after 30 or 45 days (Lenaerts et al., 2003). There was no significant reduction of *M. tuberculosis* growth in the lungs of mice treated with the Olea EtAc, Olea met and ZC met crude leaf extracts ($P>0.05$) (Fig.28). The CFU burden in these lungs was similar to those of mice receiving no treatment (untreated control). Therefore, treatment with plant extracts did not inhibit *M. tuberculosis* H37Rv proliferation in the lungs of infected mice.

Treatment with isoniazid controlled dissemination and growth of *M. tuberculosis* in secondary organs. CFU loads were reduced by 1log difference in the livers and spleens after 14 days of treatment with isoniazid (Fig28b, c), but the difference was not statistically significant ($P>0.05$). The CFU levels in spleens and livers of Olea EtAc, Olea met and ZC met treated mice were not reduced after 14 days of treatment, in fact, the growth was higher than that of untreated controls. Spleens of Olea EtAc and ZC met treated mice maintained a constantly high CFU load (1×10^4 CFU) throughout the treatment. There was no significant reduction in *M. tuberculosis* infection. Therefore, although the *O. europaea* subsp. *Africana* methanol and ethyl acetate leaf extracts inhibit

mycobacterial growth *in vitro*, they do not show activity in animal models when administered at 125mg/kg.

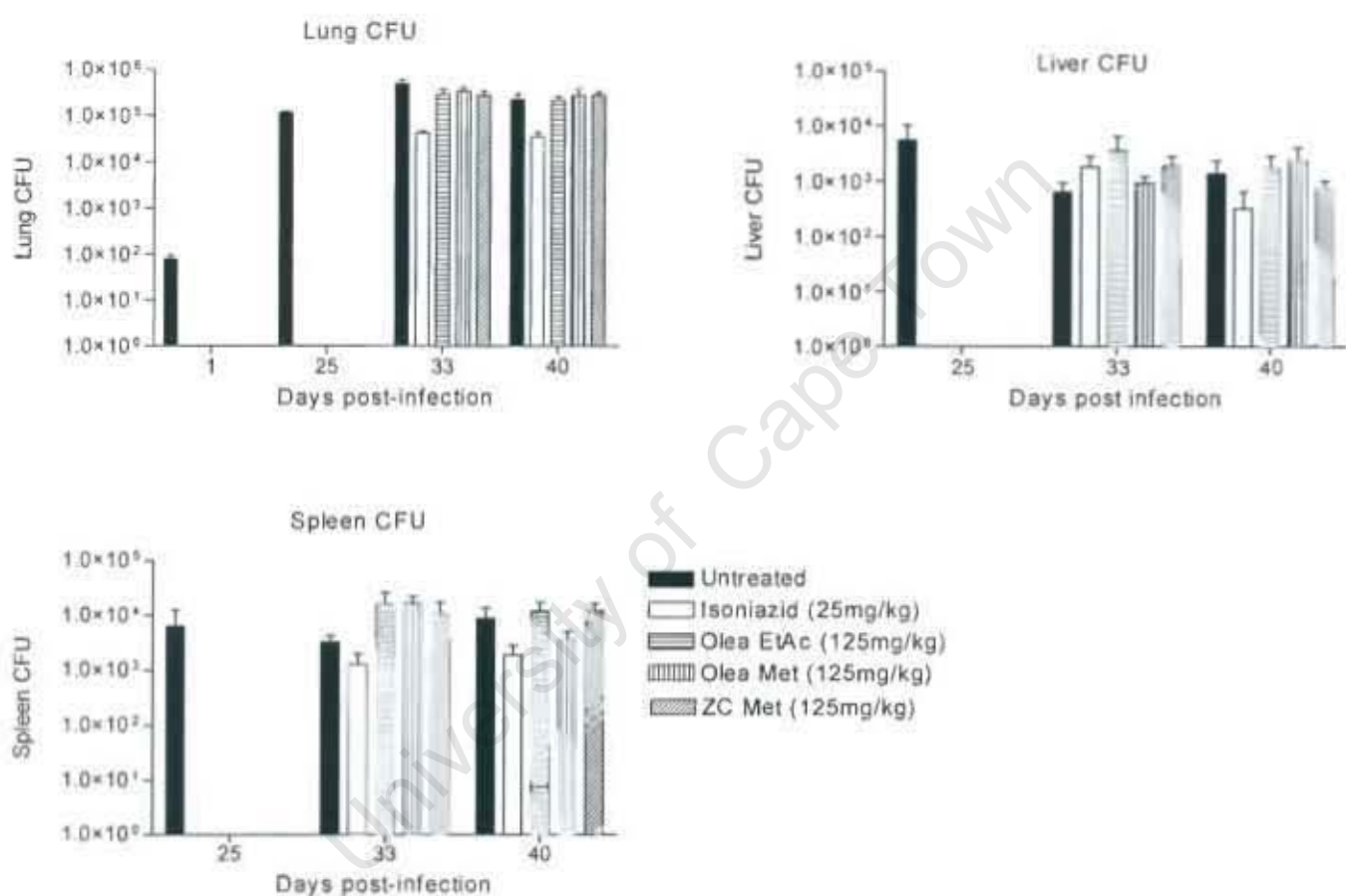


Fig.28 *In vivo* anti-mycobacterial screening of crude plant extracts. Mice were infected with *M. tuberculosis* H37Rv and treatment with isoniazid and plant extracts was started 25 days post infection. Treatment was given orally, daily for 14 consecutive days, and growth (represented as CFU) was compared in the lungs, livers and spleens of untreated mice and mice treated with plant extracts to determine the efficacy of the plant extracts *in vivo*, ($n=4$)

18.3 Histo-pathological analyses

These analyses were done to assess the effect of plant extracts on inflammatory response and pathology of the primary and secondary infection sites. Treatment with plant extracts failed to reduce mycobacterial proliferation in the lungs, livers and spleens of mice during acute phase infection (Fig.28). Granuloma formation was therefore studied as an indication of the effect of the extracts on bacterial containment and cellular recruitment (inflammation) to contain the infection in the lungs and livers. Mice were infected with a low dose of *M. tuberculosis* and infection allowed to progress for 25 days. Treatment with isoniazid, *Z. capense* methanol extract (ZC met), *O. europaea* ethyl acetate and methanol leaf extracts (Olea EtAc and Olea met respectively) was subsequently started on day 25, and continued for 14 consecutive days. The lungs, liver, kidney and hearts of the mice treated with crude plant extracts, isoniazid and a control group of untreated mice were analyzed for immune-regulated responses and pathological effects.

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Acute infection: Lung tissue

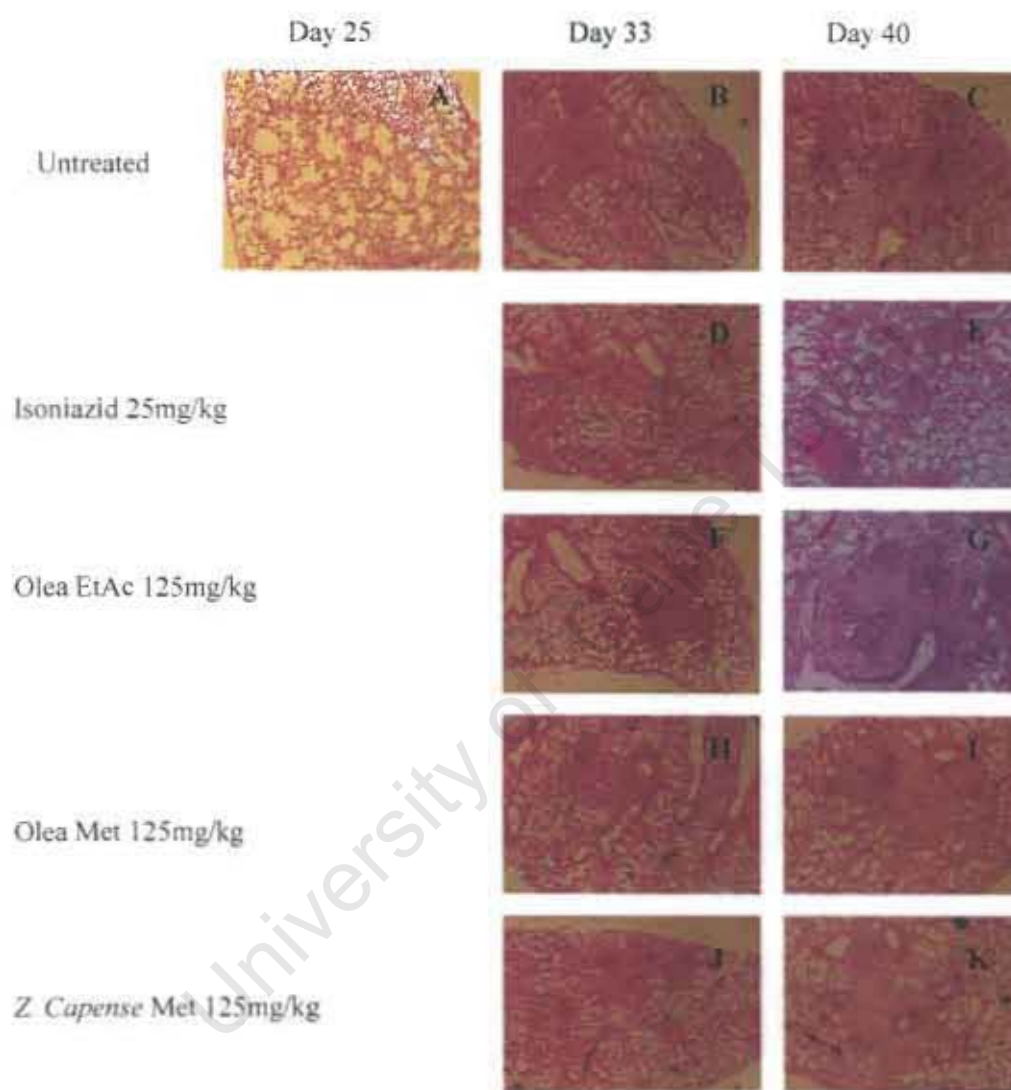


Fig.29 Comparisons of the pathological dynamics in the lungs of mice treated with plant extracts and isoniazid during acute phase of *M. tuberculosis* H37Rv infection. Heamatoxylin-eosin staining, magnification= 40×

Chronic infection: lung tissue

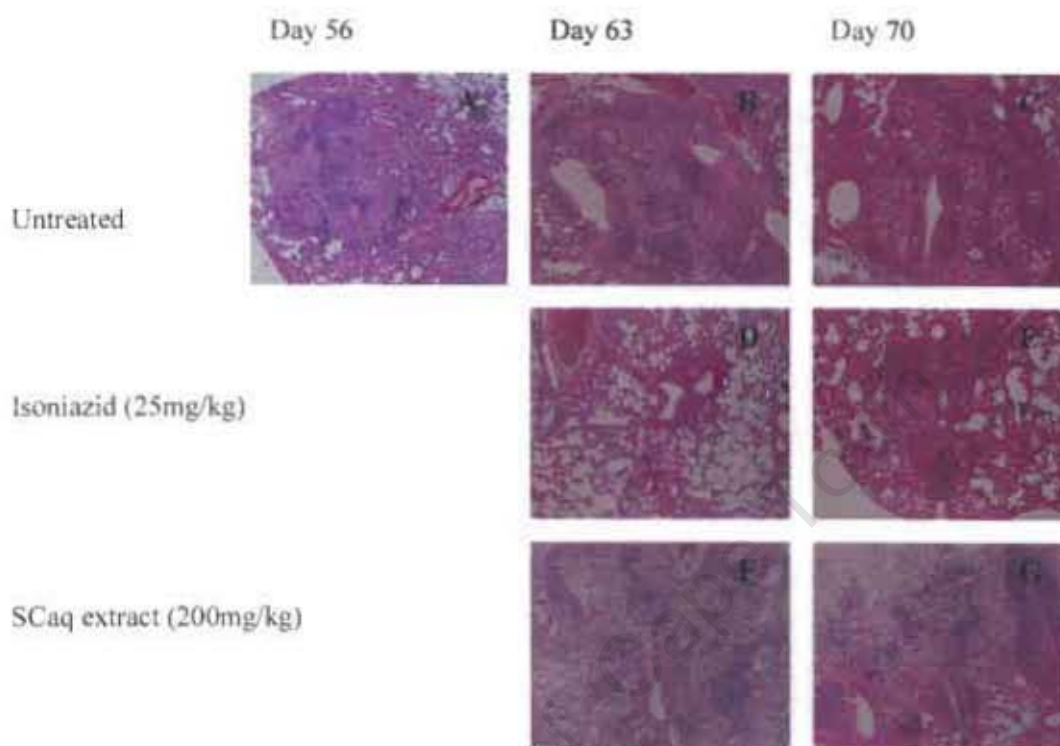


Fig. 30 Histopathology study of lung tissue from chronically infected mice. Mice were infected with *M. tuberculosis* for 56 days before starting treatment with isoniazid (d) and *S. cordatum* methanol extract (e) for 14 days. Heamatoxylin-eosin staining, magnification=40×

Lung tissue from mice infected for 25 days displayed limited inflammation and absence of granuloma lesions (Fig.29a). The formation of medium-size, widespread and loosely arranged granulomas was manifested in the lungs of untreated mice after 33 and 40 days post-infection (Fig.29b, c). There were few air spaces visible in lung tissues of untreated mice 40 days post-infection; suggesting increased inflammatory response. There were few and small, organized granulomas observed in the lung tissue of isoniazid treated mice 7 and 14 days after initiation of treatment (Fig.29d, e). The lung tissue was relatively normal 7 days following drug treatment; alveolar air spaces were relatively large and significant lung pathology was observed 14 days following treatment. There was no significant variation between lung tissue of mice treated with Olea EtAc (125mg/kg),

Olea met (125mg/kg) and ZC met (125mg/kg) crude extracts 33 days post-infection (Fig.29.f, h, j respectively). Small, compact granulomas were observed in the upper lobes of lung tissue from mice treated for 7 days with Olea EtAc (125mg/kg), Olea met (125mg/kg) and ZC met (125mg/kg) crude extracts (Fig.29.f, h, j respectively). At day 40, increased pathology was evident in lungs from mice treated with plant extracts; the granulomas were overlapping and widely spread throughout most of the lung area (Fig.29g, I, k). Lung tissue from untreated mice at day 40 did not differ significantly from that of mice treated with Olea met and Olea EtAc (Fig.29i and k respectively). Lung tissue from mice treated with Olea EtAc (Fig.29g) exhibited increased inflammation and pathology following 14 days of treatment. It is therefore apparent that cellular recruitment and granuloma formation was not disrupted by treatment with plant extracts. The granuloma lesions in untreated and treated mice were well-defined. This suggests that the plant extracts did not have adverse effects on cellular recruitment and immune responses during acute phase infection.

The chronic infection model involved low dose infection of mice with *M. tuberculosis* H37Rv for 56 days before initiation of short course (14 days) drug and plant extract therapy. Cellular infiltration was observed in the lung tissue of untreated mice 56 days post-infection, with well-defined granulomas present at day 56 and 70. (Fig.30a). Lung tissue from mice receiving isoniazid treatment for 7 and 14 days had fewer granulomas compared to the untreated mice (Fig.30d,e), hence there was less damage to the lung tissues. The observation in untreated lungs was consistent with that of the lungs from mice treated with *S. cordatum* aqueous leaf extract (SCaq) following 7 and 14 days treatment where there was severe pathology (Fig.30f, g). In conclusion, it appears that during chronic infection, alleviation of mycobacterial load through isoniazid treatment results in reduced inflammation. There was no significant difference in the pathology of lung tissue from mice treated with ZC met, Olea met, Olea EtAc plant extracts and untreated controls. Hence the plant extracts did not adversely affect the inflammatory response and exacerbate tissue damage.

Acute infection: liver tissue

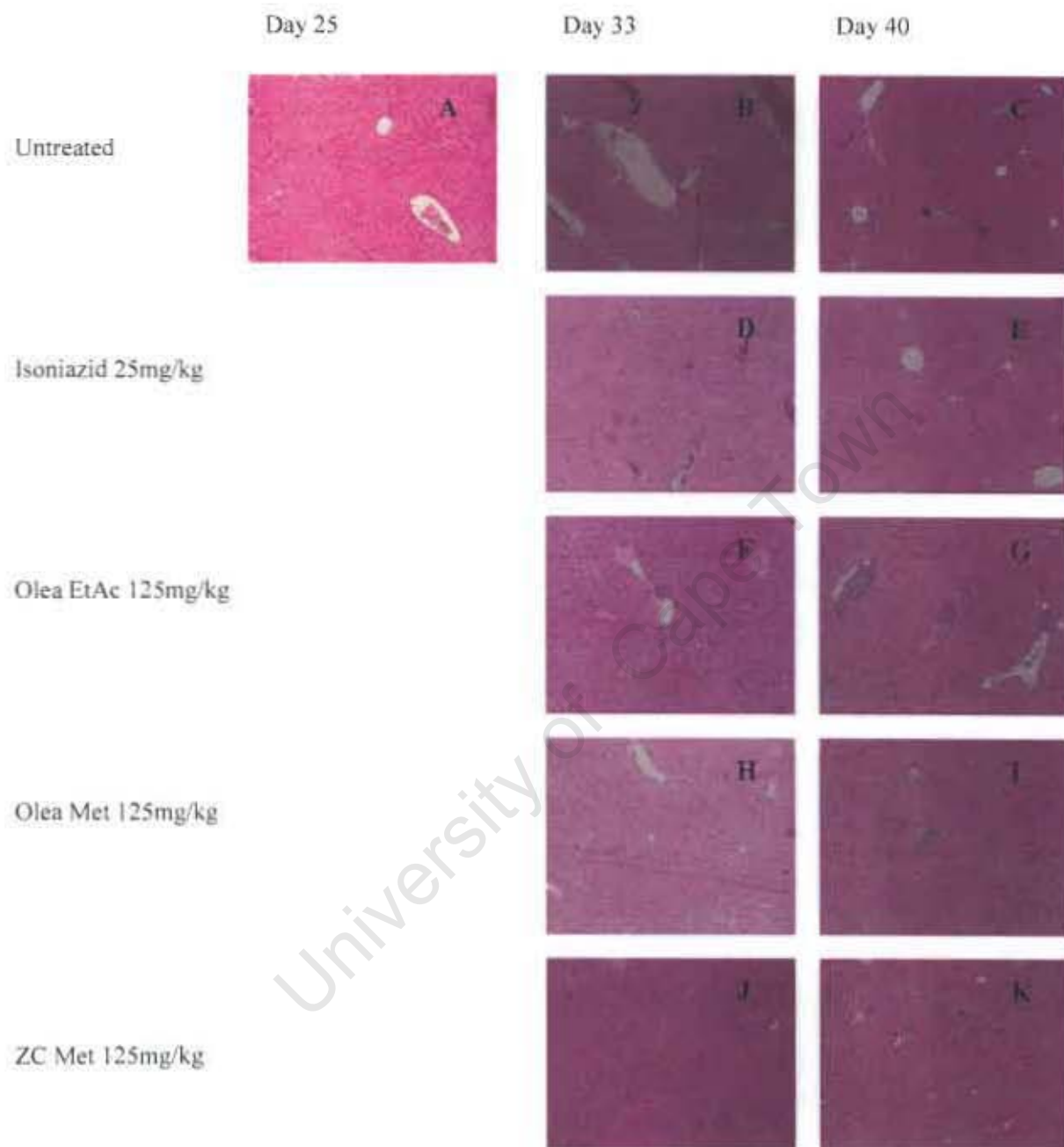


Fig.31 Histopathology comparison between liver tissues from mice treated with isoniazid, Olea EtAc, Olea Met, ZC Met and untreated mice. Treatment was started on day 25 and continued for 14 consecutive days. Mice were sacrificed on days 33 and 40 to evaluate the efficacy of the treatment. Tissues from all treatment groups show several small compact granuloma lesions. Haematoxylin-eosin staining, magnification=40×

Chronic infection: liver tissue

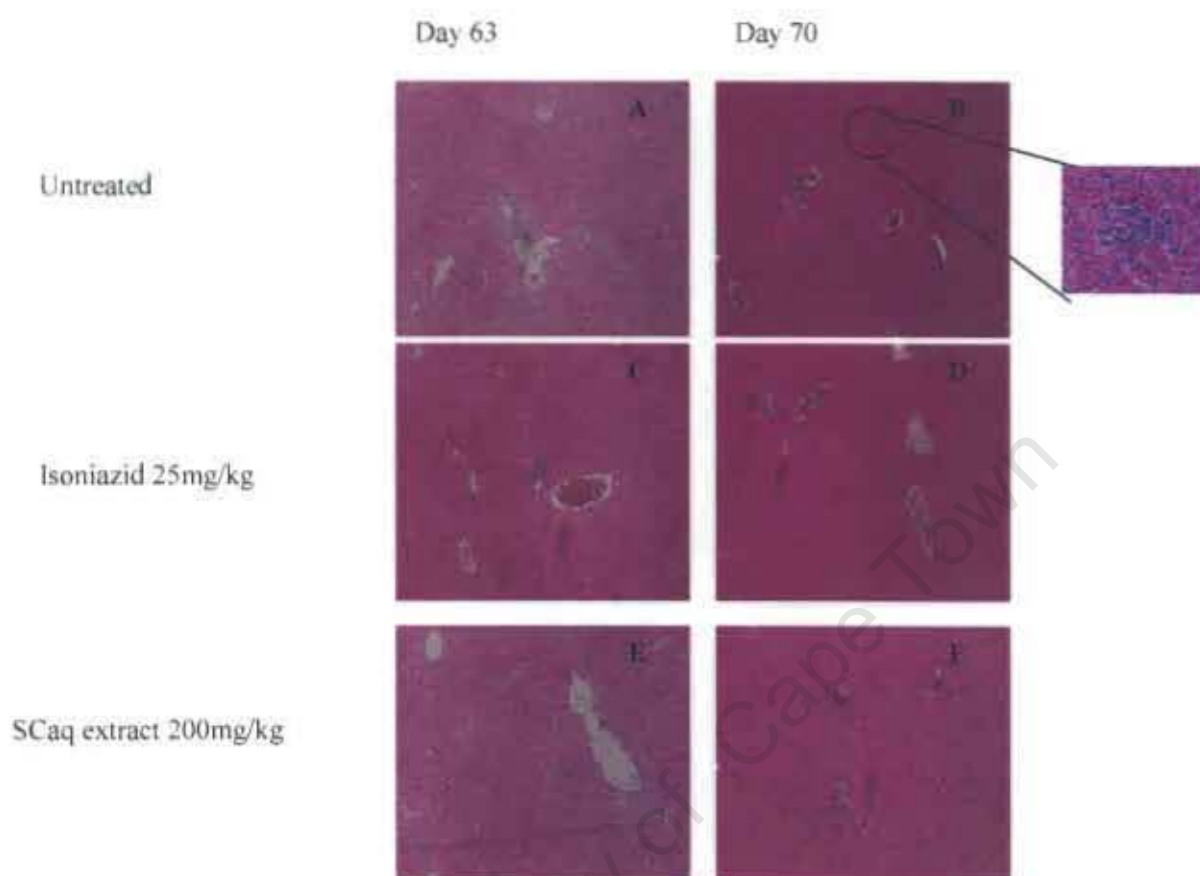


Fig.32 Comparison of pathological dynamics in the liver tissues of chronically infected mice treated with isoniazid and crude plant extract, SC aqueous extract. Haematoxylin-eosin staining. Magnification=40 $\times$, insert showing compact liver granuloma (magnification=100 $\times$)

Presence of granuloma lesions in secondary organs such as the liver indicated dissemination of the infection from the primary site of infection. There were no lesions in liver tissue of mice infected for 25 days (Fig.31a). Granuloma formation was evident 33 days post-infection in livers of mice receiving no treatment (Fig.31b). There was evidence of cellular infiltration in liver tissue of isoniazid (Fig.31d) as well as mice receiving treatment with Olea EtAc (Fig.31f) and ZC met extracts (Fig.31j). Olea met extract treated mice had livers with compact granuloma lesions observed 7 days post treatment (Fig.31h). At day 40, liver tissue from all treatment groups displayed small compact lesions, with the majority granulomas being observed in the untreated and Olea

EtAc (Fig.31c, g) treated tissues. Granulomatous reaction in the liver tissue of isoniazid treated mice had subsided following 14 days of treatment (Fig.31e). Olea met and ZC met extract treated liver tissue also had a granulomatous response similar to day 7 following 14 days of treatment (Fig.31i, k).

Advanced dissemination of the infection to the liver was observed in chronically infected mice. Additional granulomas were observed in liver tissues of all groups (Fig.32), but the lesions remained small, compact and structured as in acute infection. There was no significant difference observed in the pathology of the liver tissues of mice receiving chemotherapy, phytotherapy and untreated mice. The plant extracts did not appear to cause any hepatic injury at the concentrations they were tested, as there was no hepatic damage observed (Fig.31-32). The plant extracts therefore did not reduce dissemination of the infection to secondary organs, interfere with cellular recruitment or exacerbate destructive inflammation in the liver.

19. Discussion

The purpose of this study was to investigate four indigenous South African medicinal plants for anti-mycobacterial activity *in vitro* and *in vivo*. The search for new anti-TB drugs is driven by escalating incidence of multi-drug resistant *M. tuberculosis* strains (MDR-TB), increasing rates of HIV and *M. tuberculosis* co-infection and the long duration of the current therapy regimen. The current therapeutic regimen which includes the following front-line drugs, isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin is taken for the duration of 6-12 months. These drugs are very effective when treatment is adhered to, however the length of the treatment duration often results in patient non-compliance, hence compromises the efficiency of the treatment (East African/British Medical Research councils, 1972). The elevating demand for developing new anti-mycobacterial compounds mandate unique routes and strategies to discovering novel drug leads. According to (Balganesh TS, 2004), the most successful anti-microbial 'active principles' are natural products with potent *in vitro* activity against the microbe of interest.

Our approach to discovering novel therapeutics for TB was based on studying the activity of crude plant extracts from South African medicinal plants. The plants that were investigated in our study were selected based on their reported use as anti-TB or related TB symptoms treatment in South African traditional medicine (Hutchings A, 1996; Van Wyk B, 1997). Our study is therefore valuable as an evaluation and discovery process for potential drug leads and also as an investigation of safety and efficacy for the continued use of medicinal plants. This study therefore adds to the current state of knowledge of some relatively undefined herbal remedies.

We investigated the following indigenous South African medicinal plants for anti-mycobacterial activity, *O. europaea* subsp. *Africana*, *A. praecox*, *Z. capense* and *S. cordatum*. The initial stage of screening included the determination of the MIC of the plant extracts against *M. tuberculosis* using the Alamar Blue colometric assay, and then the extracts were evaluated for cytotoxic effects in murine macrophages to determine the

IC₅₀ and IC₉₀ concentrations. Some extracts were evaluated for iNOS expression as an indication of their potential as immuno-regulatory agents. The next stage of screening was the murine peritoneal macrophage infection assay to determine if the extracts can inhibit growth of *M. tuberculosis* in its intracellular environment, and lastly the extracts were screened in a mouse model with a low-dose aerosol infection with *M. tuberculosis* H37Rv.

The ideal TB drug or combination therapy should target the bacteria in its replicating and non-replicating state and ideally completely eliminate the infection; it should be able to penetrate and be active within the macrophage environment and extracellularly; should not have toxic side-effects towards the host and should have a relatively fast mode of action to avoid extensive treatment.

O. europaea subsp. *Africana* methanol, ethyl acetate and hexane crude leaf extracts inhibited *M. tuberculosis* growth significantly at 1000, 250 and 125µg/ml. Our finding corresponds with reports in literature that show anti-mycobacterial (H37RvTMC102 strain) activity of ethanolic extracts of *O. europaea* subsp. *Africana* using the broth dilution method (Grange and Davey, 1990). Activity of methanol extracts against *Mycobacterium aurum* A⁺ has also been reported at 200µg/ml using the firefly luciferase bioluminescence assay (Ntutela, 2002).

Our study also showed that the methanol, ethyl acetate, hexane and dichloromethane crude extracts of *O. europaea* subsp. *Africana* leaves had significant activity against intracellular *M. tuberculosis* ingested by murine peritoneal macrophages. The methanol and hexane extracts are not cytotoxic at 500µg/ml, whereas the dichloromethane and ethyl acetate extracts exhibit cytotoxic effects. These findings have not been reported anywhere else. This finding is novel, and may have numerous implications, as the active principles appear to be able to access and target the pathogen extracellularly and intracellularly. Many candidate drugs fail due to the poor absorption of the molecules into cells. This finding also indicates that the active principles are able to permeate the macrophage and retain their anti-mycobacterial activity in the macrophage cytoplasm

where the local pH and environment differs from a broth culture assay. Methanol and ethyl acetate extracts were found to be inactive against *M. tuberculosis in vivo* at a concentration of 125 mg/kg, and had no effect on the inflammatory response in the lung and liver tissue of wildtype C57BL/6 mice. The lack of *in vivo* activity may have been due to the low testing concentrations as the active principles within the crude extracts may have been in very low amounts. Studies by Bitler et al., 2005 have shown that the aqueous extract of *O. europaea* has potent anti-oxidant and anti-inflammatory activity. The simple phenol compound, hydroxytyrosol, found in the extract reduces serum TNF- α in BALB/c mice and decreases iNOS production in macrophage cultures *in vitro* (Bitler et al., 2005). It is therefore possible that the methanol and ethyl acetate extracts tested in our study do not reduce *M. tuberculosis* growth *in vivo* because they contain this phenol compound and other polyphenols that down-regulate anti-mycobacterial functions such as TNF- α expression and inhibit ROI and RNI from combating the infection. The anti-oxidant and anti-inflammatory properties of this plant may also not be attractive as anti-TB therapy particularly during chronic infection where TNF- α is pivotal for granuloma formation and maintenance.

Other effects of the *O. europaea* subsp. *Africana* leaves include anti-hypertensive, anti-hypotensive, cardiotonic or coronary dilating and antioxidant activities (Somova et al., 2004; Somova et al., 2003). It is therefore evident why it has been recently reported that “umquma” as this plant is traditionally known, was designated “the most important plant” in use in traditional medicine (Dold T, 1999), it has a wide range of therapeutic properties.

We found that the active *O. europaea* subsp. *Africana* extracts (methanol and ethyl acetate extracts) contain 3 major peaks/compounds of relatively high polarity (retention times: 13.7, 15 and 15.7 minutes). The major compounds were present at very high concentrations in the leaves (see Table 4). This result is highly consistent with the HPLC analysis reported in another published study where the retention times of the methanol extract analysis were 19.37, 20.48 and 21.23 (MRC). The difference in the retention times may be due to differences in the method and mobile phase used to elute the

compounds. The compound identities were not elucidated in our study, hence further analysis needs to be done to identify these compounds and their structures. There are limited pharmacological studies in published literature regarding the chemical composition of the *O. europaea Africana* subspecies. Clinical studies on the *O. europaea* collections from Europe have been well documented. Anti-viral including anti-HIV (Kashiwada et al., 2000; Micol et al., 2005; Serra et al., 1994), anti-inflammatory (Bitler et al., 2005), anti-bacterial (Braca et al., 2000), anti-diabetic (Taniguchi et al., 2002), anti-ulcer activity (Farina et al., 1998) and hepato-protective effects have been attributed to compounds isolated from *O. europaea* plants. The active principles/constituents that have been isolated from the European species include the two secoiridoids, oleacein and oleuropein which has been reported as the major component of olive leaf extracts (Lasserre et al., 1983; Micol et al., 2005), various alkaloids and triterpenes such as β -amyryn and olenic acid (Bitler et al., 2005; MRC, ; Somova et al., 2003). The chemical components of the *Africana* subspecies might differ from that of the European subspecies due to geographic variations of climate, soil, environment and seasonality.

We also found in our study that the *A. praecox* ethyl acetate, dichloromethane and acetone rhizome extracts were active against *M. tuberculosis in vitro*, and *A. praecox* extracts were very cytotoxic. The acetone, methanol and dichloromethane extracts also showed significant inhibitory effects against intracellular growing *M. tuberculosis*. There have been no published reports on the anti-mycobacterial activity of this plant thus far, hence our finding is new. Furthermore, our finding of the cytotoxic effects of *A. praecox* may substantiate and justify the toxic effects observed in humans whereby ingestion of the plant and/or plant sap causes haemolytic poisoning and severe ulceration of the mouth (Norten, 2004).

The HPLC extract spectrum analysis of *A. praecox* crude ethyl acetate, dichloromethane and acetone rhizome extracts revealed very similar profiles suggesting that these 3 extracts contained very similar polar constituents. It is highly likely that the constituents extracted by these solvents are the same due to their very similar relative polarities. Low concentrations of the triplet peaks (retention times: 27.03, 27.29 and 27.76) in the

methanol extract, which may represent structurally related compounds, could be the reason for non-activity against *M. tuberculosis* of this extract. This observation therefore suggests that the activity of the *A. praecox* extracts may be dependent on the compounds associated with the triplet peak; which could potentially be the active principles. Fractionation of the crude extract and determination of the active fraction is necessary to determine the exact identity of the active component of the extract.

A. praecox is one of the 10 *Agapanthus* species that are widely grown and used in traditional medicine in South Africa. Published literature on the biological activity of *A. praecox* was not found. However, various properties of other *Agapanthus* subspecies have been reported. Anti-depressant activity of extracts from *A. campanulatus* has been investigated (Nielsen et al., 2004). Anti-hypertensive (Duncan et al., 1999) and uterotonic (uterus) activity (Veale et al., 1999) of the *A. africanus* subspecies have been reported. The latter property of the *Agapanthus* species explains the plants' common use as an antenatal medicine in South African traditional medicine (Kaido et al., 1997). Isolation and characterization of possible biologically active compounds from the roots of *A. africanus* showed the presence of a known yellow phenolic flavonoid, isoliquiritigenin and a novel polyphenol, dimeric dihydrochalcone (Kamara et al., 2005). The *A. praecox* rhizome crude extracts in our study may contain some of these flavonoids (chalcones) as they all had a yellow pigment, which is characteristic of chalcones (Ali, 1974; Roux, 1974).

Our study showed that *Z. capense* leaf extracts were inactive against *M. tuberculosis* *in vitro* and *in vivo*. However, the methanol extract of the bark showed anti-mycobacterial activity *in vitro*. It is interesting to note that in traditional medicine, the bark was specifically used in treating tuberculosis by the Zulu people, whilst the other plant parts (roots and leaves) were used for other ailments such as acne, sores, toothache etc (Steyn, 1998). Based on the claims of traditional medicine practice, it is not surprising that the bark extract was active, whereas leaf extracts did not exhibit anti-mycobacterial activity. Our finding may therefore validate the use of *Z. capense* for treating pulmonary ailments

and TB in South African traditional medicine, and justify the particular use of bark as opposed to any other plant part for treating TB.

The leaf extracts were highly cytotoxic, whereas the aqueous bark extract had no toxic effects towards murine macrophages. It has been reported that the *Zanthoxylum* genus is well known for the production of certain alkaloids that are well known cytotoxins (Luis, 1998). Aerial parts of *Z. capense* may therefore possibly contain these alkaloids as its extracts exhibit potent cytotoxic effects. An interesting observation was made with the aqueous bark and the ethyl acetate leaf extracts. These extracts reduced the Alamar Blue reagent when incubated with medium, in the absence of *M. tuberculosis* inoculum. This may suggest that these two extracts contain compounds that have charged groups that interfere with the redox reaction that occurs when the Alamar Blue dye is reduced from a blue substrate to the pink reduced form. The Alamar Blue assay which is oxidation-reduction indicator based assay would therefore not be suitable to evaluate the efficacy of these extracts if they have reducing capacity (power) as it will not provide reliable results. Alternative bio-assays that can be used to assess extracts of this nature include the radiometric BACTEC 460 assay (Collins and Franzblau, 1997) or the agar proportion method (Hall, 2002). Dried crude herbal extracts have been reported to be prone to contamination with fungal spores and bacteria which are always present in the air. Although our extracts were prepared under sterile conditions; it is possible that the deterioration and de-composition of the herbal extracts during storage can lead to growth of fungi, bacteria or even mites (Mukherjee, 2002). Although highly unlikely, this possibility may be considered as an alternative explanation for the observation of reduction in control samples. The extracts were screened twice in independent experiments with the same outcome each time.

The peak analysis of the *Z. capense* extracts revealed that the extract composition was complex. Several small peaks were observed in all extracts signifying compounds present at low concentration. We found that the active methanol bark extract eluted 3 closely related hydrophilic compounds with retention times 1.87, 2.08 and 2.11 minutes; and another major compound eluting at 2.86 minutes which constituted 24.7% of the crude

extract. HPLC analyses of *Z. capense* crude extract (twigs and leaves combined) in a study by Steyn (Steyn, 1998) isolated the active constituents from the plant and identified them as β -sitosterol, sitosterol- β -D-glucoside which have anti-ulcer bioactivity; xanthoxylol- γ,γ -dimethylallyl ether with unknown biological activity and pellitorine whose bioactivity is well documented. Pellitorine occurred as a mixture of 3 isomers with retention times of 2.4, 3.1 and 3.9 minutes in a HPLC separation (Steyn, 1998). Our HPLC analysis of the methanol bark extract revealed a similar elution pattern; hence there is a possibility that the 3 peaks/ compounds observed in our extract analysis might be the pellitorine isomers. This compound is also reportedly found in several other *Zanthoxylum* species (Steyn, 1998). It has established bioactivity against a range of economically important agricultural insects and pests, hence its regular inclusion in pesticides (Steyn, 1998). Another study reported the presence of sesamin in the root and stem barks of *Z. capense* (Fish and Waterman, 1973). Neither of the studies reported on the anti-mycobacterial activity of the *Z. capense* crude extracts nor the activity of the isolated and identified compounds. Further studies to confirm the identity of the compounds obtained in our extract analysis are required.

Other *Zanthoxylum* species have been investigated for various biological activities. *Z. budrunga* bark extracts have shown antibacterial and anti-fungal properties. Similar to our findings, the methanol extracts of this species also demonstrated potent cytotoxic effects (Islam et al., 2001). *Z. culantrillo* ethanol extracts of bark and leaves were found to lack anti-bacterial activities (Luis, 1998). This particular species contains some of the same compounds that are found in *Z. capense* such as sesamin and sitosterol (Luis, 1998). Methanol extracts and an isolated compound from *Z. bungeanum* showed inhibitory effects on NO production in macrophage cultures (Tezuka et al., 2001). In our studies, we also found that there was no iNOS expression when murine peritoneal macrophage cultures were treated with the *Z. capense* dichloromethane leaf extract (Fig.26). Macrophage iNOS is not expressed in resting (non-activated) macrophages; maximal iNOS expression in murine macrophages is obtained by co-stimulation with IFN- γ and bacterial LPS (Xie et al., 1992). The *Z. capense* dichloromethane extract therefore does not activate macrophages. Our result however, shows that iNOS

expression was not induced rather than inhibited by the extract because the macrophages in our experiments were not activated at the outset. This potential anti-inflammatory property may be a beneficial therapeutic strategy for chronic TB as it will reduce organ pathology caused by excessive inflammatory responses such as excessive NO production.

Furthermore all the *Z. capense* extracts appeared to reduce intracellular *M. tuberculosis* growth in our study. This may have been due to metabolic activation of the active principles within the macrophage. By and large our study and the reviewed published literature show that the *Zanthoxylum* genus does not exhibit potent direct anti-microbial properties at least at the low concentrations evaluated. The general lack of activity may also be due to the variation in metabolism and composition of secondary metabolites in the plant during different seasons. The active principles may have not been expressed in the plant when we harvested the material. Hence depending on the season of collection, the chemical composition of the plant may be different as established with *Z. capense* secondary metabolites in a study by Steyn et al., 1998. Thus the time of collection of raw plant material may influence the efficacy of the plant.

Furthermore, our study showed that all the *S. cordatum* extracts did not have inhibitory effects against *M. tuberculosis* *in vitro* using the Alamar Blue assay and intracellular growth inhibition was also not significant in most extracts of this plant. Possible reasons for the lack of bioactivity of the *S. cordatum* leaf extracts include those forwarded for *Z. capense*. A further possible explanation for the lack of activity in our biological assay as opposed to the reported efficacy in traditional use of the plant may be the deterioration of the active secondary metabolites in the extract during storage, as the stability of the metabolites is not known. Therefore factors such as light, humidity, temperature etc. may compromise the chemical stability of the active principles and the quality of the crude extract (Mukherjee, 2002). Furthermore, *S. cordatum* may be used to treat the symptoms of TB in traditional medicine, rather than cure the disease. Therefore it is possible that we did not detect anti-mycobacterial activity in our assays because this plant may not contain active compounds which directly target and kill *M. tuberculosis*, but stimulates secondary and effector mechanisms that alleviate the disease symptoms.

The *S. cordatum* extracts had cytotoxic effects on murine macrophages, suggesting that the plant may be poisonous for consumption at elevated concentrations. Basic pharmacological analysis by HPLC revealed that the *S. cordatum* extracted with polar solvents contained very few constituents absorbing at 210nm. The aqueous leaf extract eluted two major compounds at 1.70 and 2.34 minutes when separated by HPLC using a 10-100% acetonitrile mobile phase. The methanol leaf extract showed presence of 2 major compounds eluting at 1.91 and 13.14 minutes. Both these extracts were very hydrophilic. The hexane, ethyl acetate and dichloromethane leaf extracts contained compounds with very similar retention times, a possible indication that the extracts have similar or even the same constituents. The dichloromethane and ethyl acetate extracts had 3 major common peaks with retention times of 3.9, 25 and 28 minutes. Similar peaks were observed in the hexane extract analysis at 3.5, 23.5 and 26.4 minutes. The slight difference in the retention times may be due to the differences in the relative polarities of the extraction solvent, and the overall charge that they give to the compounds in the extracts. The hexane extracts also displayed prominent peaks at 37.7 and 39.7 minutes, which were present in very low concentration in the ethyl acetate extract, and not detected at all in the dichloromethane extract. The hexane and ethyl acetate extract may therefore contain similar chemical components. There were no chemical analysis spectra of the *Syzygium* genus found in published literature to compare with our analysis.

Previous studies have shown that the majority of compounds found in *S. cordatum* are proanthocyanidins such as delphinidin, cyaniding and pentacyclic triterpenoids such as friedelin, epifriedelinol and β -sitosterol (Candy HA., 1968.). The latter 3 compounds have been shown to be the major components of hexane extracts of the bark (Candy HA., 1968.). Hence it is possible that some of the major peaks observed in our pharmacological analysis of the hexane extract represent some of the mentioned compounds. Other compounds that have been isolated from this plant include arjunolic, gallic and ellagic acids. The bioactivities of some these triterpenoids have been established. Betulinic acid, a triterpenoid isolated from the *S. claviflorum* species, was found to have potent anti-HIV activity (Fujioka et al., 1994), and has even been further modified to form a novel compound that has higher anti-HIV efficacy (Kanamoto et al.,

2001). Previous work on *S. cordatum* has also revealed its potential therapeutic use for mild diabetes (Musabayane et al., 2005) and it has been reported to have potential anti-mutagenic properties (Verschaeve et al., 2004) and therefore may be used in protection against chemical mutagens that may lead to cancer. Activity against *M. tuberculosis* H37Rv has not been reported thus far. Our data is therefore novel and adds valuable information to the database of this species as a potential source for identifying lead compounds.

The extracts that were tested *in vivo*, *Z. capense* leaves methanol extract, *O. europaea* subsp. *Africana* methanol and ethyl acetate leaf extracts did not demonstrate any anti-mycobacterial activity at 125mg/kg. This may have been due to the concentration tested, as the active principles in the crude extracts may have been present in low amounts. Hence the bioavailability of the active compounds may have been low, and the final concentration of active compound reaching the site of action (alveolar macrophages) was not sufficient to target the infection. Another possible explanation may be that the active compounds were metabolized and structurally altered *in vivo*, resulting in loss of activity.

Our *in vivo* model tested the efficacy of the plant extracts against bacterial populations that were either in log phase (rapidly replicating) and /or early stationary phase where they were either in a dormant or non-growing state. This was important as *M. tuberculosis* has the ability to survive in different physiological states in the human host; hence it is important that the drugs being evaluated are screened against the different populations of the pathogen to obtain reliable indication of clinical activity. The *in vitro* biological assays prior to *in vivo* screening should be reliable and sensitive. The assays employed should ideally be simple, inexpensive, rapid and reproducible in order to cope with the large number of crude plant extracts and their fractions. The assays set up in our study satisfied most of these criteria.

20. Conclusion

This study has highlighted some plants/ extracts which are worthwhile of further analysis for their anti-mycobacterial activities. *O. europaea* subsp. *Africana* leaf extracts demonstrated the most potent anti-mycobacterial activity *in vitro*, followed by *A. praecox* rhizome extracts and lastly *Z. capense* bark extract. The *S. cordatum* extracts did not show anti-mycobacterial activity *in vitro*. An alternative assay not involving oxidation-reduction indicators can also be used to confirm the extracts that reduced the indicator dye in the Alamar blue assay. This will ensure that potentially active extracts/compounds are not missed due to false negative results. The extracts tested *in vivo* (Olea met, Olea EtAc and ZC met) did not show any activity against *M. tuberculosis* H37Rv. Future experiments should involve increasing the dose of the plant extracts being tested *in vivo*. The period of drug treatment with the active extracts in our study can also be extended in future to evaluate effects of extensive therapy. In the case where there is not enough plant extract for an extensive/prolonged treatment regimen, the Cornell model can be used, where extracts can be tested against IFN- γ deficient mice which cannot control the infection (Lenaerts et al., 2003). This model is rapid; it requires a short duration of treatment the differences are more pronounced between untreated and treated groups (Lenaerts et al., 2003).

Mycobacterial persistence within the macrophage is central to its recalcitrance to standard anti-TB chemotherapy. The *A. praecox*, *O. europaea* subsp. *Africana*, *Z. capense* and *S. cordatum* crude extracts in our study seemed to have to some extent reduced intracellular *M. tuberculosis* proliferation in murine macrophages. Hence this feature implies that the extracts are able to access and inhibit intracellular mycobacterial growth. Further chemical investigation of these extracts needs to be done to elucidate the active compounds responsible for the antimycobacterial activity. In depth analysis macrophage secondary responses such as cytokine production can be done in future experiments to investigate whether the plant extracts that reduce intracellular growth target the mycobacterium directly, or if they have secondary effects such as inducing the production of cytokines that activate various signaling events. For example, production of

TNF- α and IFN- γ may induce NO release, which in turn directly kills *M. tuberculosis* or induces macrophage apoptosis (death).

While these results are encouraging, more work still needs to be done. The active extracts (Olea met, Olea EtAc, Olea hex, Aga acetone, Aga EtAc, Aga dcm) need further pharmacological analysis. Further investigation will involve fractionation of the crude extracts using Solid Phase Extraction (SPE) column chromatography; identifying the active fractions and isolating the active compound. The identity and chemical structures of the compounds can subsequently be elucidated using Nuclear Magnetic Resonance (NMR) analysis. Finally the pharmacological mode of action of the pure active compounds can be studied. *In vivo* activity of the active and the inactive extracts and isolated active compounds if available should also be assessed in future.

In conclusion, this study has established suitable, concise and affordable bioassays that can be used to screen libraries of South African medicinal plants that have been previously implicated in TB treatment in traditional medicine.

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